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13. ABSTRACT *(Maximum 200 words)*

Technical and Scientific Support Services

This report covers research efforts conducted in Government laboratories through a series of Task Order assignments. The research encompassed environmental quality, occupational health, and research methods development. Much of the effort was related to toxicological research using non-mammalian species, specifically medaka, bluegill, and Xenopus frogs. Assays were developed and tested to determine the potential hazards to human and ecological health that may result from complex mixtures of chemical contaminants in water, soil, sediment, and air. Most of the research took place on-site at the U.S. Army Biomedical Research and Development Laboratory at Fort Detrick, Maryland. This organization was renamed the U.S. Army Center for Environmental Health Research (USACEHR). Other sites where research took place included Edgewood Research, Development, and Engineering Center, National Institute of Environmental Health Sciences, Rocky Mountain Arsenal, Colorado State University, and Wright Patterson Air Force Base. During the period of this research the concept of Deployment Toxicology was developed and the Master Plan was completed. This effort uses the concepts developed in the laboratory research to help prepare soldiers for exposure to complex environmental contamination while deployed or to detect the potential hazards from exposure to mixtures of contaminants.

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FOREWORD

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NA In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (National Academy Press, 1996)

NA For the protection of human subjects, the investigator(s) have adhered to policies of applicable Federal Law 45 CFR 46.

In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

Project Manager's Signature

30 SEPTEMBER 1998
Date

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EXECUTIVE SUMMARY

This is the Final Report for Contract DAMD17-93-C-3006, dated 14 January 1993. This contract is a Task Order contract for Technical and Scientific Support Services for the Health Effects Research Division (HERD) at the U. S. Army Biomedical Research and Development Laboratory (USABRDL), Fort Detrick, Maryland. During the course of the five-year Period of Performance, USABRDL was reorganized and renamed the U. S. Army Center for Environmental Health Research (USACEHR). For convention, the Task Orders were numbered in accordance with the originating Branch of HERD at USABRDL: EQ for Environmental Quality Research Branch (EQRB), OH for Occupational Health Research Branch (OHRB), and RM for Research Methods Branch (RMB). Initial Task Orders were issued for work commencing 8 March 1993. During the course of this contract, Task Orders were issued as follows: EQ-1 though EQ-7, OH-1 through OH-5 (absent OH-3, which was rescinded), and RM-1 through RM-4. This report is organized by Task Order.

Deliverables throughout the course of the contract consisted of both periodic technical progress reports and supplemental reports in special cases. In addition, many publications, presentations, and abstracts were completed for peer review of the scientific effort. All these were made public with the review and approval of the Contracting Officer's Representative (COR) and GEO-CENTERS' management. Supplemental reports consisted of the following two types: interim reports (drafted as documentation of progress to date upon the departure of a scientist from the research project) and Army Technical Reports (publications retained within the Defense Technical Information Center, DTIC, as opposed to publication in peer-reviewed journals). Interim reports of progress are included herein; however, the volume of published documents is far too great to include in this report. A complete Bibliography is contained in Appendix A, listing the 119 publications, abstracts, presentations, and posters resulting from this contract. Appendix B contains interim reports on portions of the work completed on EQ-1 written at the time of the departure of the researchers responsible for those separate efforts (Dr. Robert Steighner and Ms. Susan Burns). Appendix C contains a final task report on EQ-6 written when a researcher permanently shifted, with the approval of the Army, from EQ-6 to RM-1. Appendix D contains a list of documents delivered by our subcontractor, Life Systems, Inc. as a Final Report of their subcontract in support of this effort. Other final task reports and other deliverables that were submitted at the conclusion of task order period of performance and accepted by the Government have not been included in this report.

Initially, technical progress reports were required monthly. During the second year of the contract, the frequency of reports was changed to quarterly. During each reporting period, progress was reported for each Task Order. In this way, the ongoing progress made for the most recent period was evident. Although this information is comprehensive, reviewing over five years of progress using this format is cumbersome. Accordingly, for this report we have recompiled these periodic reports chronologically within each Task Order. In this way, the progress reported within each period is evident along with the overall progress toward research goals. To retain the concepts employed at the time, as well as the mood and technical content



of the effort, other than re-organizing by Task Order, little editing has taken place for this report. By the nature of the efforts, in some cases, this approach results in some repetition; however, overall it is an effective presentation style.

Following the first year of the contract, GEO-CENTERS requested and was granted an opportunity to present orally the first year's effort. This lengthy briefing for both contract and technical personnel contained a comprehensive technical review of research results as well as contractual and financial status reports. The report was well received and resulted in modification to all Task Orders to reduce the cost estimates for all years remaining. In addition, there was a better understanding by all concerned regarding the priorities for tracking data and reporting of financial and contractual information. Following the second year of the contract, a similar annual briefing was held.

The Request for Proposal (RFP) which resulted in competitive award of this contract was based on the level of effort being provided by GEO-CENTERS through previous contracts at the time of the drafting of that portion of the RFP. By the time of award of the first Task Orders, eleven months after submittal of the proposal, it was recognized that the man-hour and dollar ceiling on the contract would be insufficient to maintain the required level of effort for five years. To reduce the administrative burden inherent in frequently exercising option years to keep up with contract requirements, the contract was awarded as a five-year contract, rather than one year with four option years. In addition, it was estimated that the overall ceiling of the contract would be reached within three years at the level of effort required at contract award. Accordingly, for those efforts requiring continuing research, Task Orders were issued with a three-year Period of Performance.

As the end of the third year approached, the predictions proved accurate, and the contract required recompete and renewal or extension and expansion. The Army expanded the contract ceiling to a level that was estimated to be required for a total of five years from the original award date. While awaiting award of the follow-on contract so the work could continue, the Period of Performance was extended in increments from 14 January 1998 and finally ended 31 August 1998.

The remainder of this report contains the chronological progress for each Task Order, as reported contemporaneously while the research was being conducted.

Task Order EQ-1



GEO-CENTERS, INC.

TECHNICAL AND SCIENTIFIC SUPPORT SERVICES
FINAL REPORT

The following is the report of progress for each task under Contract Number DAMD17-93-C-3006 (GC-P-93-2533):

Title: Bioeffects and Biomarkers of Pollution
Task Number: EQ-1 (2533-001)

This task requires technical scientific support for basic and applied research on the effects of nitroaromatic munitions compounds on mammalian cell lines.

MARCH 1-30, 1993

The development of methods to quantify the type and spectra of mutations induced by munitions in a human cell line is continued by the commencement of this task. The N-methyl-N-Nitrosoguanidine (MNNG) mutagenesis pilot experiment begun last month was discontinued due to incubator CO_2 malfunction. The data obtained prior to termination indicated a cloning efficiency of 26.4% for the control cells and 6.7% for the treatment group; *i.e.,* survival of 25% at 12.5 ng/ml MNNG. One problem encountered with the procedure was the difficulty in carrying a large enough number of cells throughout the experiment to prevent loss of induced mutants. The polyacrylamide gel electrophoresis (PAGE) system for purification of polymerase chain reaction (PCR) products was refined. PAGE purification of PCR-amplified genomic DNA went extremely well; no further modifications to the technique are necessary. Computer spreadsheet programs to calculate the cloning efficiencies and mutation frequencies (with 95% confidence levels) were designed and are now being employed to analyze the data.

The cytotoxicity and mutagenicity of the munitions, 2,4- dinitrotoluene and 2,6-dinitrotoluene, were monitored *in vitro* in the TK6 human lymphoblast cell line. Two genes were used in the munition analysis: the TK gene and the HGPRT gene. TK+ and HGPRT+ mutants were treated with different concentrations of 2,4-dinitrotoluene or 2,6-dinitrotoluene, and growth curves generated. Doses causing 50% or greater cytotoxicity were used to experimentally derive plating efficiency and mutation frequency. From this point, the mutants will be picked from the plates, cultured, and frozen for future testing of the actual molecular mutation within the TK or HGPRT gene.

APRIL 1-30, 1993

The development of methods to quantify the type and spectra of mutations induced by munitions in a human cell line continued. The cell culture segment of the second N-methyl-N-Nitrosoguanidine (MNNG) mutagenesis pilot experiment was completed. Survival and mutation frequency data were in agreement with data from other laboratories and validate the cell culture segment of the project. The cloning efficiency of the cells improved from 26% to 67% by using



horse serum obtained from a different vendor. Cells from both the treated and untreated populations were frozen for later use. Aliquots were processed for DNA amplification through polymerase chain reaction techniques. The molecular nature of the mutations will then be elucidated after first isolating those molecules containing base substitution mutations through denaturing gradient gel electrophoresis (DGGE). Subsequent "Sanger" DNA sequencing will elucidate the exact nature of the base substitutions. Additionally, the annual meeting of the Environmental Mutagenesis Society was attended. One full day of the conference was devoted to mutational spectrometry and specifically the DGGE technique. Information presented at the meeting was particularly worthwhile regarding this project.

The cytotoxicity and mutagenicity of the munitions, 2,4- dinitrotoluene and 2,6-dinitrotoluene, were monitored *in vitro* in the TK6 human lymphoblast cell line. A mutation assay, which generates a growth curve, plating efficiency and mutation frequency, was performed using TK6 cells treated with MNNG, 2,4-DNT, 2,6-DNT, and 2,4,6-TNT. This experiment generated 15 mutants, four of which were frozen to be used in further molecular testing. Testing with 2,4-DNT and 2,6-DNT showed no significant toxicity response. These drugs will be retested in the near future. Testing with 2,4,6-TNT shows a toxic response at 200 mg/mL, 100 mg/mL, and 50 mg/mL concentrations. The mutation assay has not quite been perfected with this compound and is in its third trial. Thus, no mutants have been harvested. Testing of this compound and its monoamine derivatives will continue using the mutation assay coupled with metabolic activation of the cytochrome C system in order to enhance the mutagenic properties of 2,4,6-TNT.

MAY 1-31, 1993

The development of methods to quantify the type and spectra of mutations induced by munitions in a human cell line continued. DNA prepared from 6-thioguanine resistant cells treated with N-methyl-N'-nitro-N-nitrosoguanidine, or from untreated control cells, was amplified by the polymerase chain reaction. The 224 base pair fragment produced was then subjected to two further PCR amplifications to isolate the high and low temperature melting domains. While production of the 204 bp fragment was successful the 180 bp fragment amplification proved problematic with the "vent" (and "deep vent") polymerases. Success was achieved using "Taq" polymerase, but the poor fidelity of this enzyme precludes its routine adoption in the procedure. Manipulation of the experimental variables involved in PCR amplification with the "vent" polymerases have so far proven ineffectual. Initial indications are that the "vent" (and "deep-vent") polymerases are unable to bind to the GC clamp containing primer. Efforts to resolve the problem through further analysis and manipulation of reaction conditions, or replacement with another heat stable, high fidelity polymerase are under consideration.

The cytotoxicity and mutagenicity testing of 2,4,6-trinitrotoluene (TNT) continued using the TK6 human lymphoblast cell line. Testing with 2,4,6-TNT demonstrated significant cytotoxicity and mutagenicity at various exposure levels. This experiment was expanded to troubleshoot for certain inconsistencies such as a slow recovery of the control group as well as an extremely high doubling rate once the cells reached their log phase. Experiments are planned for

next month to evaluate the effect of metabolic activation of 2,4,6-TNT on its mutagenicity.

JUNE 1-30, 1993

The development of methods to quantify the type and spectra of mutations induced by munitions in a human cell line continued. The focus this month was on screening six compounds for toxicity to TK-6 lymphoblasts. The purpose was to establish the concentration range in which the compounds cause significant toxicity to TK-6 cells. These data will then be used for designing mutagenicity studies. Toxicity was determined by the percent survival of treated cell cultures compared to control cell cultures, measured by taking the ratio of cloning efficiencies (after 2 weeks growth) or total cell number in bulk cultures (24 hours post-treatment).

The following compounds were significantly toxic to TK- cells; 1,3 dinitrobenzene, 2,4-dinitrotoluene, and 2,6-dinitrotoluene. 4-Methyl-3-nitroaniline, 2-methyl-3-nitroaniline, 2-methyl-5-nitroaniline were relatively nontoxic to the cells. Replication of the experiment with a shorter exposure period (cell growth during a 24-hour exposure period poses technical difficulties) is now underway, as is quantitation to determine the lethal dose causing 50% toxicity (LD50).

The cytotoxicity and mutagenicity testing of 2,4,6-trinitrotoluene (TNT) continued using the TK6 human lymphoblast cell line. After several trials of exposing 2,4,6-TNT to the TK6 cells, mutants were produced and frozen for further analysis. Metabolic activation utilizing the cytochrome P450 system is being used to test for increased cytotoxicity and mutagenicity. These tests are currently being conducted at the highest concentration that produced mutants. However, this concentration is expected to be too toxic to quantitate so the concentration will probably be lowered. Work was initiated this month to begin a new project to determine whether superoxide generated by exposure of munitions to the pBr322 plasmid will cause single strand or double strand mutations.

JULY 1-31, 1993

The development of methods to quantify the type and spectra of mutations induced by munitions in a human cell line continued. The focus this month was on calculating the induced resistance to 6-thioguanine of TK-6 lymphoblast cells treated with the six chemicals screened for toxicity last month. The purpose was to identify which chemical would induce a significant increase in resistance to 6-thioguanine to warrant molecular analysis. The chemicals tested were 1,3 dinitrobenzene, 4-methyl-3-nitroaniline, 2-methyl-3-nitroaniline, 2-methyl-5-nitroaniline, 2,4-dinitrotoluene, and 2,6-dinitrotoluene. Only 4-methyl-3-nitroaniline appears to produce a significant increase in the frequency of cells resistant to 6-thioguanine. The result, which is still tentative, indicates an induced 6-thioguanine resistance frequency of 12 cells out of a million compared to a background frequency of 3 cells in a million, or a four fold increase. In addition to the 4-methyl-3-nitroaniline work, experiments with the parent compound, 2,4-dinitrotoluene, are also underway.

The cytotoxicity and mutagenicity testing of 2,4,6-trinitrotoluene (TNT) continued using the TK6 human lymphoblast cell line. Exposure of various munitions causes chromosomal mutations that have been theorized to either be single stranded breaks, double stranded breaks, or single stranded breaks that occur at the same point on each of the DNA helices. To test this hypothesis the plasmid pBr322 was grown up in the *E. Coli* HB101 system, extracted using a cesium chloride gradient, exposed to the munition, and analyzed on an agarose gel. RTe first trial of this experiment produced a very low concentration of plasmid DNA and was unable to be analyzed further. Another trial is planned but with the addition of chloramphenicol to the growing bacterial culture. This chemical halts genomic DNA and protein synthesis but allows for the mass replication of the plasmid. With this alteration a usable concentration of plasmid DNA is expected that can be carried on to further experimental steps.

AUGUST 1-31, 1993

The development of methods to quantify the type and spectra of munitions induced by munitions in a human cell line continued. The focus this month was determining the optimal concentration of serum to use in cloning efficiency experiments, and replicating the 4-amino-2-nitrotoluene induced increase in resistance to 6-thioguanine seen in Tk-6 cells last month. Results of the serum experiment indicated 10% serum as the optimal concentration. The results of the 4-amino-2-nitrotoluene experiment were somewhat disappointing. In this experiment three doses of 4-amino-2-nitrotoluene were used, but only the highest dose resulted in significant cytotoxicity. The fact that the lone cytotoxic treatment resulted in an increase in 6-thioguanine resistance correlates very well with the data generated in the first screening experiment. The quantitatively lower value is reflected by a similar decrease in the mutation frequency. The fact that the cloning efficiencies were once again out-of-range for accurate calculation of cytotoxicity using the Poisson distribution was also disappointing. A new approach to cell quantitation prior to plating has been devised and should resolve this problem. A third experiment with 4-amino-2-nitrotoluene has now been completed with the cells in the expression stage, and a fourth experiment is scheduled for next week.

The cytotoxicity and mutagenicity testing of 2,4,6-trinitrotoluene (TNT) continued using the TK6 human lymphoblast cell line. Prior experiments testing the hypothesis that exposure to various munitions causes single stranded, double stranded, or single stranded mutations at the same loci on each DNA helix were evaluated. Plasmid preparation was previously done through a Cesium Chloride gradient which was very time consuming. The alternative method chosen for this procedure is alkaline lysis. This method reduces the preparation time for one week to about 4-5 hours. This would make it possible to produce more plasmid to expose to the various munitions.

SEPTEMBER 1-30, 1993

The development of methods to quantify the type and spectra of mutagenicity induced by munitions in a human cell line continued. This month results from the third 4-amino-2-nitrotoluene experiment were obtained. The cloning efficiency plates indicate a decrease in

survival (33%) of cells treated with 0.2% dimethyl sulfoxide (DMSO) for a 24-hour period. This result was subsequently confirmed in a separate study in which cells were exposed to varying concentrations of DMSO for 24 hours (toxicity was seen at 0.2 % DMSO but not 0.1 % or 0.05 %). DMSO is the solvent of choice for non-water-soluble chemicals in mutagenesis screening, but is generally used at 1 % in a four-hour exposure period. We originally decided to use a 24-hour exposure with 0.2 % DMSO due to the lack of toxicity seen with the chemicals to be screened in shorter exposure periods. Toxicity at 0.2 % DMSO was not anticipated. Although 0.1 and 0.05 % DMSO over 24 hours are non-toxic, it is difficult to dissolve the chemicals in this small a volume at concentrations which will result in cytotoxicity and still remain in solution. It now appears we will be forced to stick to a four-hour exposure period with 1.0 % DMSO, although this may limit toxicity of the chemicals to be screened. Concomitant with the decision to limit the exposure period, a new cytotoxicity experiment with 4-amino-2-nitrotoluene and 2,4-dinitrotoluene was performed. It is anticipated this experiment will indicate the optimal dose for a mutagenicity experiment within this exposure period.

The cytotoxicity and mutagenicity testing of 2,4,6-trinitrotoluene (TNT) continued using the TK6 human lymphoblast cell line. Metabolic activation of 2,4,6-TNT in the cytochrome C P450 system proved to be toxic in both the treated cells and in the controls at 50 mg/L for 24 hours. To correct this problem we exposed the cells for 3- and 6-hour exposure periods at various concentration levels. There was no significant toxicity at 3 hours; however, at 6 hours, 200 mg/L was approximately 30-40% more toxic than the lower concentrations. Tests are now in progress to generate a growth curve of both metabolically activated cells and regular exposure cells to determine the degree that the metabolic activation system increases toxicity.

After having little success with the *E. Coli* strain HB101 pBr322 in the experiment examining the mutational effects of munition exposure to DNA, we chose to continue this experiment using a more versatile plasmid, *E. Coli* HB101 puc10/SOD1.

OCTOBER 1-31, 1993

The development of methods to quantify the type and spectra of munitions induced by munitions in a human cell line continued. A fresh TK-6 lymphoblast culture was begun this month to be used in future experiments. The new line was started to guard against the selection of atypical cells which have been known to emerge and dominate long-term tissue cultures. A new mutagenesis experiment with trinitrotoluene was begun. Cells are to be treated with trinitrotoluene (TNT) this week at doses of 250, 200 and 150 mg/L. Prior to treating the cells solubility of TNT at the higher dose will be ascertained. Survival data for the 4-Amino-2-Nitrotoluene and 2,4-Dinitrotoluene study performed last month are being calculated. The cells from these experiments were frozen down until the survival data are gathered. Provided sufficient toxicity is attained, these cells will then be plated for mutagenicity.

Note: It was shown that the metabolic activation of the munition 2,4,6-TNT, utilizing the cytochrome C P-450 system for a 6-hour exposure period and at a 200 mg/L concentration level, caused a less toxic response than if the munition is solely exposed to the cells.

The mutational effects of munition exposure to DNA has been the latest project. After working out the "bugs" in the purification process of the *E. Coli* strains HB101 puc18/sod and pBr322, we began to analyze the plasmids through gel electrophoresis. Each of the strains, as well as molecular weight standard, was run on a gel at 3.33 V/cm at 3.75 hours. The gel was stained with ethidium bromide, and a positive/negative picture was taken under transmitted UV light. The negative was then analyzed spectrophotometrically, and a graph was produced which corresponds to the band positioning and concentration of DNA per band. Cutting the plasmid with restriction enzymes, exposing them to the munition, and reannealing the DNA strands are the next steps in this experiment. Certain munitions are suspected frameshift mutagens, and, through the exposure to the nicked plasmid, they should intercalate into the plasmid DNA structure causing a different structure and different electrophoretic properties, thus different positioning of the band in the gel.

NOVEMBER 1-30, 1993

The development of methods to quantify the type and spectra of munitions induced by munitions in a human cell line was discontinued this month.

Work continues on the mutational effects of munition exposure to DNA. Through many trials of varying enzymatic concentrations it soon will be determined which concentration of DNase causes 50% nicking of the plasmid pBr322. Once this is established, the plasmid will be exposed to various munitions to determine mutagenicity in reference to known frameshift mutagens. Various concentrations of DNase were tested on the plasmid along with the restriction enzyme PstI (to determine where double-strands were in the gel in reference to the supercoiled plasmid). The gel was run at 5 V/cm for 2.5 hours in 1% agarose. Efforts are being made to run a gel overnight in a 1.4% agarose system. Resolution has been poor in the gels that have been run overnight thus far and the parameters, such as time and voltage, will be varied to possibly correct this problem.

DECEMBER 1-31, 1993

Work continues on the mutational effects of munitions exposure to DNA. The parameters for the electrophoresis gel to be run overnight were established as 3 V/cm for 16 hours in a 1.4% agarose system. Resolution was greatly increased in this system over the one run at 5 V/cm for 4 hours in 1% agarose. This will prove valuable as research progresses and definition of closely placed bands is essential.

It was also discovered that the previous plasmid preparations contained a great deal of double stranded DNA caused by handling of DNA during the preparation. The expected results should contain double strands generated by the possible frameshift mutagenicity of munitions compounds. The contaminating double strands caused in the preparation had to be removed to accurately define the mutagenicity of these compounds relative to known frameshift mutagens. This problem was alleviated by running the preparations through a Cesium Chloride gradient at 45,000 rpm for 48 hours.

The development of methods to quantify the type and spectra of munitions induced by munitions in a human cell line was discontinued last month. The interim report for this portion of Task EQ-1 is included as Appendix B.

JANUARY 1-31, 1994

The development of methods to quantify the type and spectra of munitions induced by munitions in a human cell line continued. Previous concentration levels of the pBr322 plasmid extracted from *E. Coli* were very low. Several different purification techniques were tried to increase this yield including, an alkaline lysis with both a continuous cesium chloride gradient and polyethylene glycol methods. A purification kit from Promega was also used to purify the plasmid. There was no significant increase in plasmid yield; however, the kit produced a greater number of double stranded DNA which would interfere with the expected generated results when exposed to munitions compounds. An additional cesium chloride gradient would allow the supercoiled DNA to be liberated from the contaminated samples. This procedure was very time consuming and the yield of the preparations were extremely low. The plasmids puc18/SOD and pCAT are to be tested for increased yield and compatibility with pBr322 in respect to molecular weight, copy number, and electrophoretic properties.

FEBRUARY 1-28, 1994

The development of methods to quantify the type and spectra of mutations induced by munitions in a human cell line continued. Preparations of the plasmid pCAT provided yields in excess of 1 mg. These yields were 5 to 10 greater than previous preparations of pBr322. Additional tests were preformed to nick 50% of the plasmid generating double strand DNA. The plasmid sample was then exposed to two concentrations of 9-aminoacridine, a known frameshift mutagen, +/- 5% DMSO. The exposed DNA sample was then relegatedyzed electrophoretically. One of three trials of this experiment clearly showed a mutagenic effect. To test for possible denaturing of the ligase used in the two trials which did not show a mutagenic effect, DNA samples from the other two trials were relegated lot of DNA ligase. New ligase enzyme was ordered and this experiment will be repeated.

MARCH 1-31, 1994

The development of methods to quantify the type and spectra of mutations induced by munitions in a human cell line continued. Work with double and single stranded super helical DNA forms from various cell lines were fractionated by extraction with phenol under acidic conditions at pH 4.1. The purified super helical form was exposed to munitions in the presence of microsomes to determine whether strand breaks occur or adducts were formed as a result of the reduction process.

APRIL 1-30, 1994

Horizontal and vertical type polyacrylamide gels were done using molecular weight standards to practice the procedures. However, the major work effort was on organizing the laboratory in preparation for the upcoming realignment of personnel.

MAY 1-31, 1994

An Annual Report on year 1 (March, 1993 - March, 1994) progress for the contract was prepared and presented to the client May 31, 1994. The presentations consisted of a 2-hour "Technical Progress Summary" and a 2-hour "Contract Administration/Finance Summary."

No work was performed on this task due to resignation of employee assigned to task. Search for replacement is in progress.

JUNE 1-30, 1994

Work continued on this task with training of a new laboratory assistant.

JULY 1- SEPTEMBER 30, 1994 (Government approved Quarterly Progress Reports in lieu of Monthly Progress Reports)

Work continued on this task with investigation of stress proteins as biomarkers of toxicity in TK6 human lymphoblast cells. It was observed that the loading buffer used to resuspend the cells interfered with the Pierce□ method of protein determination. For this reason, it is necessary to resuspend pelleted cells in water for protein analysis. An heat shock experiment was conducted using TK6 cells exposed to 44° C for the following times: 0, 2, 5, 16, and 24 hours. The cells were pelleted, resuspended in loading buffer and separated on polyacrylamide gels (discontinuous Laemmli). It was empirically determined that the total amount of protein added to each well would be standardized at 20□g.

Visualization was difficult with this analytical system, and three alternative systems were tested. First, the quantification of the gels by scanning transparencies was attempted. It was observed that peak area did increase with increased concentration. However, the responses were not linear. Secondly, a two-dimensional gel separation method which used a horizontal gel under various conditions to maximize protein separation was coupled with iso-electric focusing to bind the principle constituents. It was postulated that this technique would permit observation of new proteins. But, the method was abandoned due to difficulty in casting and handling of the gels. Lastly, an immunological detection system for 72/73 KD heat shock proteins in TK6 lymphoblast cells was attempted. Proteins from normal and heat treated cells separated on SDS-PAGE gels were successfully transferred to nitrocellulose membranes. However, it was determined through reconstruction experiments that the presence of SDS interferes with 72/73 kd heat shock protein immune detection. SDS interference was

minimized by the addition of a washing step into the immune blot procedure. It has also been determined that blotting proteins to PVDF membranes produces sharper bands than blotting with nitrocellulose.

Using the improved electrophoresis/western blotting system, it has been observed that there is an increase in the amount of 72/73 kd HSP present after cell exposure to 44^{0}C for 5 and 22 hours. Currently, experiments are being performed to find run conditions for one dimensional gels which will facilitate separation of the 72 and 73 HSPs. Also, attempts are being made to lower the background levels on the X-Ray film so that quantification could be made by directly scanning the films. Also, an exposure study of TNT on TK6 cells is currently being performed. The effect of various concentrations of TNT at various times on the production of HSPs will be examined.

OCTOBER 1 - DECEMBER 31,1994

Attempts to separate the 72/73kd Heat Shock Proteins using a one-dimensional electrophoresis system were unsuccessful. As a result, it will be necessary to study the combined 72/73 HSP increases in response to munitions. The effect of various concentrations of TNT in DMSO on the production of 72/73kd HSP's was examined. It was determined that DMSO does not enhance the 72/73kd HSP response. Preliminary results support a response in cells exposed to 5µg/mL TNT for five hours. Also, there may be a slight increase in the cells exposed to 24µg/mL TNT for five hours. Additional experiments are being conducted in an attempt to verify the response at 5µg/mL TNT after five hours and to determine the effect of a recovery period on the production of 72/73kd HSP's.

Replicate experiments were conducted in an attempt to confirm increased levels of 72/73kd HSP's in TK6 cells exposed to TNT. Although a definitive conclusion could not be reached from these investigations, it does not appear at this time that 72/73kd HSP's are reproducibly stimulated by exposure to TNT. To further support this working hypothesis, experiments are being conducted to determine additional positive controls for 72/73kd HSP induction. It was determined that ethanol at concentrations of 1 %, 2 %, and 4 % does induce the 72/73kd HSP's after a variety of times. In addition, ZnC12 induced the 72/73kd HSP's at concentrations of 3, 12.5, and 25 µg/mL after various exposure times. Therefore, additional positive controls for later comparison with munitions have been obtained. While conducting the prior experiments it was determined that improved contrast between positive and negative controls in the colorimetric detection system is obtained by loading less protein to the wells of the gel. Using this improved technique in conjunction with the additional positive controls, tests are currently being conducted with an extensive battery of TNT-exposed samples to determine if there is induction of the 72/73kd HSP's.

JANUARY 1 - MARCH 31, 1995

Experiments investigating the possible induction of the 72/73 kd HSP in TK-6 cells due to munitions exposure have begun. Cells were exposed to TNT at concentrations of 2, 5, 10,

and 25 μg/mL at various timed intervals. Screening studies were performed to compare the known induction effects from $ZnCl_2$, ethanol (EtOH), and heat shock with that of TNT exposure using western blots. The results of the experiments determined that TNT exposure of 10 μg/mL for 10 hours appears to induce 72/73 kd HSPs at a comparable rate to $ZnCl_2$, EtOH, and heat shocking.

Work has also been done in the area of quantitation of western blot membranes with a colorimetric detection system. This method would be advantageous because it is possible to obtain low background levels and good contrast between positive and negative controls. Initial investigations seem to indicate that screening transparencies in a gel scanner may provide a means of quantitating induction. This observation was tested further by conducting several experiments to correlate induction of 72/73kd HSPs with cytotoxicity.

These experiments compared 72/73kd HSPs induction in samples exposed for 10 hours to 10μg/mL TNT or heat. A battery of protein concentrations were electrophoresed and blotted from samples serially diluted. These experiments provided a sample concentration and the sensitivity limits of the colorimetric detection system. Results from these experiments indicated that the induction effects from the exposure to TNT is below the sensitivity limit of the colorimetric detection system. Therefore, the induction of 72/73kd HSPs by TNT can not be quantitated by scanning transparencies of blotted membranes using current methods.

APRIL 1 – JUNE 30, 1995

Multiple experiments have been conducted which compare induction of the 72/73kd HSP after a 10-hour time period using samples exposed to heat and to 10 µg/mL TNT. Results indicate that the 72/73kd HSP is not induced by TNT under these conditions at levels comparable to positive heat shock controls. This experiment is being repeated to confirm the results.

In addition, preparation for two dimensional electrophoresis has begun. Currently, work is being done to refine the techniques of casting and running first dimension gels including sample preparation for iso-electric focusing. Using two-dimensional electrophoresis will provide a means to separate and visualize new proteins which are induced in cells exposed to TNT. Methods of sample preparation for two-dimensional gel electrophoresis have been compared. It has been determined that optimal results are obtained by placing samples in equal volumes of iso-urea solution E (Bio-Rad) after being heated at 95^{0}C for five minutes. The samples are then centrifuged at 14,000xG for ten minutes to remove any insoluble proteins. A final concentration of 25-30µg of protein is loaded onto each tube gel in the first dimension (iso-electric focusing).

Experiments are also being conducted which compare cellular protein in control cells with those exposed to 10µg/mL TNT for 24 hours. Two-dimensional electrophoresis coupled with silver staining will provide a means to separate and identify proteins which are up or down regulated in exposed cells. Experiments which compare cellular proteins from control

TK-6 cells with those exposed to 10μg/ML TNT for 24 hours have been conducted. These proteins were successfully separated by two-dimensional electrophoresis and silver stained. The resulting gels are being compared.

Following two-dimensional electrophoresis, 72/73kd heat shock proteins were isolated and identified through a western blot onto a PVDF membrane. The results of this experiment in conjunction with those obtained through previous studies indicate that there appears to be enhancement of the 72/73kd HSP following exposure to TNT. However, the amount of stress protein found in treated cells is not at levels comparable to the positive heat shock control.

In addition, mRNA from control TK-6 cells and from cells exposed to TNT has been purified and quantitated. It will be used in an *in vitro* translation experiment which will search for new or different transcripts.

JULY 1 - SEPT 30, 1995

An analysis of purified mRNA from control TK-6 cells and cells exposed to 10μg/mL TNT by *in vitro* translation was conducted. In order to proceed with these experiments, it was first necessary to develop a working mini-gel electrophoresis and electroblot procedure. It was determined empirically that optimal separation was obtained by running a discontinuous gel system (6% stacking gel/10% separating gel; 0.75mm thick) at 100V for 75 minutes. It was concluded that additional wash steps must be incorporated into the previously used electroblot protocol in order to reduce background levels during chemiluminescent detection. Initially, the positive and negative controls provided by the manufacturer did not give results consistent with what was expected from the kit. New materials were ordered and the experiment was repeated. The positive and negative controls did react to give results consistent with what was expected from the kit. However, RNA extracted from the cell did not appear to translate any proteins. Work on this project has been discontinued.

Experiments have been performed to investigate thermotolerance in TK-6 cells after exposure to a range of concentrations of TNT. This was accomplished by comparing growth curves of cells at 37°C and 44°C after TNT exposure. It was observed that the cells were unable to recover from heat treatment at 44°C, and therefore determined that growth following heat exposure can not be used as a measure of themotolerance in TK-6 cells.

Currently, an experiment is being conducted to determine the relative plating efficiency on TK-6 cells following exposure to a battery of concentrations of TNT for 16 hours. This investigation should provide additional information on the effect of TNT on TK-6 cell growth.

OCTOBER 1 - DECEMBER 31, 1995

An experiment was conducted which determined the relative plating efficiencies of TK-6 cells following exposure to a battery of concentrations of TNT for 16 hours. It was observed that the cells were able to recover from TNT exposure at 10*u*g/mL and below. At 25*u*g/mL,



the TK-6 cells were unable to recover completely. This provided additional information on the effect of TNT on the growth of this cell line.

In addition, work has begun on customizing a protocol to conduct reverse transcriptase-polymerase chain reaction (RT-PCR) experiments. This technique will initially be used to screen for the production of new or enhanced, randomly primed sequences, relative to controls, following TNT exposure. Twenty different random 10-mers (Operon) will be utilized in the original screening process.

The results from the screening experiments of the random 10-mers (Operon) are in the process of being evaluated. It has initially been concluded that primers OPA-3, OPA-4, OPA-7, OPA-8, OPA-9, OPA-11, OPA-13, and OPA-18 are potentially viable primers and may be examined further. The evaluation process will continue into the next quarter.

Currently, we are involved in the extraction and purification of RNA which will be used throughout the evaluation process.

JANUARY 1 - MARCH 31, 1996

Total RNA was extracted from TK-6 cells which had been exposed to various TNT concentrations. Recent applications of the single step purification method developed by Chomcyznski and Sacchi produced impure RNA as determined by an absorbance ratio 260/280. This value was consistently below that given for pure RNA (1.6-1.8). Multiple extractions were performed in an unsuccessful attempt to determine the nature of the problem. Total RNA was then extracted and purified using TRIzol reagent and protocol obtained from Life Technologies™. Since both protocols involve essentially the same chemicals, it was concluded that one of the reagents used was not responding as expected. A micro extraction protocol for total RNA is being developed. It may be implemented in PCR experiments in which a small amount of RNA is required.

Additional screening experiments of random I 0-mer primers (OPA 1-20) are being conducted. Initially, twenty reverse transcriptase-polymerase chain reactions (RT-PCRs) will be performed using purified RNA from cells exposed to l0pg/mL TNT for 16 hours and each primer. Primers are being evaluated on the presence of bands (transcripts) on silver stained gels between 200-1000 bps and the darkness or strength of these bands. The primers which satisfy these criteria will then be evaluated further in tests which will eliminate transcripts resulting from contamination and false positives.

APRIL 1 - JUNE 30, 1996

Additional screening experiments were performed to evaluate primers on the basis of reproducibility, uniqueness, and strongly amplifiable transcripts not due to DNA contamination. Primer OPA-20 was determined to have the overall best characteristics for further investigation. Results from a RT-PCR experiment using Primer OPA-20 and RNA

from cells exposed to a battery of concentrations of TNT appear to show a new transcript at approximately 500 bp. This band was then excised from the gel, reamplified, and cloned into a plasmid using a "TA" vector system. Clones were selected through ampicillin resistance and alpha-complementation. Eight clones were randomly chosen for use in "mini-preps" to confirm the size of the insert. Following confirmation, the inserts of two of these clones were non-radioactively labeled in order to serve as a probe against total and mRNA in future experiments.

The heat shock protein research which was performed last year was accepted for publication. "The Effects of 2,4,6-Trinitrotoluene and Associated Munitions on HSP72/73 Production in a Human Lymphoblast Cell Line" by Rajnik and Mitchell will appear in *In Vitro Toxicology*, Volume 9, #2.

JULY 1 - SEPTEMBER 30, 1996

Work on this project has been discontinued.

Task Order EQ-2



GEO-CENTERS, INC.

Title: Toxicity and Metabolism of Munition Compounds
Task Number: EQ-2 (2533-002)

This task requires technical scientific support for basic and applied research on the toxicity and metabolism of nitroaromatic munitions compounds *in vivo* and *in vitro*.

MARCH 1-30, 1993

Work on the effects of trinitrobenzene (TNB) in the rat continued with the commencement of this task. The epoxide hydrolase assay for microsomes was continued, and efforts were initiated to write the protocol for a new project to investigate the role, if any, of cytochrome P450 in superoxide anion production. The Society of Toxicology annual meeting in New Orleans, LA, was attended. The following posters were presented at this year's meeting: "Toxicokinetics of ^{14}C-1,3,5-Trinitrobenzene (TNB) in F344 rats after oral administration" and "Hematologic effects of orally administered 1,3,5-trinitrobenzene (TNB) in Fischer 344 Rats." Both posters were well received.

APRIL 1-30, 1993

Work on the effects of trinitrobenzene (TNB) in the rat continued. The scope of the project has been expanded to include another test compound, Tetryl, as well as to continue the project initiated last summer with visiting faculty. Continued epoxide hydrolase assay for microsomes frozen last summer. Enzymatic activity and effects in the blood will also be investigated. Initiated writing of the safety protocol and animal use amendment. Collaborative effort to investigate the role, if any, of superoxide anion generation by cytochrome P450 has been discontinued.

MAY 1-31, 1993

Work on the effects of trinitrobenzene (TNB) in the rat continued. The epoxide hydrolase assay was continued on microsomal samples collected last summer. Also glutathione-s-transferase assays on all cytosol from this past summer were continued. The protocol for *in vitro* metabolism by erythrocytes and formation of methemoglobin was completed. Training was completed at RIID on procedures for the barrier suite work. All animal work will be performed in barrier suites this summer due to an outbreak of murine hepatitis in the RIID animal facility.

JUNE 1-30, 1993

Work on the effects of trinitrobenzene (TNB) in the rat continued. Blood samples were obtained from untreated rats in order to practice the methemoglobin assay and re-calibrate the spectrophotometer for this assay. A pilot *in vivo* test with TNB and Tetryl was performed. One animal was dosed (by oral gavage) with 71mg/kg TNB and another with corn oil. After 2.5 hrs these rats were sacrificed and the liver and testes were removed and made into microsomes and cytosol. One animal was dosed, again by oral gavage, with 2ml of Tetryl



(100mg/ml - 1000mg/kg) and the other rat was untreated. The liver was removed and frozen for future practice of microsome prep. The blood from all four animals was collected and methemoglobin concentrations were determined. Blood smears were also practiced. The blood smears will be for heinz body detection. The frozen livers were used for microsome and cytosol prep practice.

JULY 1-31, 1993

Work on the effects of trinitrobenzene (TNB) in the rat continued. This month 48 rats were dosed with either TNB or Tetryl and then sacrificed after 10, 4, or 1 day(s). The livers and testes were taken and both microsomes and cytosol were made from each. Blood was taken from the animals. Blood chemistry, complete blood count, and reticulocyte counts, along with methemoglobin assay and heinz body slides, were done on each animal.

AUGUST 1-31, 1993

Work on the effects of trinitrobenzene (TNB) continued. The rat exposure protocol was amended and a five-hour TNB exposure experiment was repeated with ten additional rats; only blood was collected for analysis from this experiment. Preliminary data, including blood chemistry, complete blood count, reticulocyte counts, gross pathology weights along with the results from methemoglobin assay and individual animal weights and doses were compiled and sent to Oklahoma State University Department of Pathology. The microsomes have had a preliminary protein assay done on them and Glutathione-s-transferase assays have been begun.

SEPTEMBER 1-30, 1993

Work on the effects of trinitrobenzene (TNB) continued. Glutathione-s-transferase assays have been done on TNB 10- and 4-day and Tetryl experiments. The preliminary results show little to no effect on liver and testis cytosol of Tetryl treated rats, however the TNB treated rats showed significant increase in glutathione-s-transferase content at the higher dose levels in both liver and testis cytosol.

Preliminary cytochrome P450 and cytochrome b5 experiments have been conducted on the Tetryl treated rat microsomes. The liver microsomes showed a decrease in the quantity of P450 and an increase in b5. The testis microsomes had undetectable cytochrome b5 and the P450 showed a decrease.

Cytosolic reduced and oxidized glutathione content assay has begun on the Tetryl treated rats.

Three abstracts for the Society of Toxicology (SOT) have been written and sent to SOT for the annual meeting in March.

OCTOBER 1-31, 1993

Work on the effects of trinitrobenzene (TNB) continued. Glutathione-S-transferase assays have been done on TNB (10- and 4-day) and Tetryl experiments. The final results show little to no effect on liver and testis cytosol of Tetryl treated rats; however, the TNB treated rats showed significant increase in glutathione-S-transferase content at the higher dose levels in both liver and testis cytosol.

Preliminary cytochrome P-450 and cytochrome b5 experiments have been conducted on the 10-day TNB-treated rat microsomes. The testis microsomes were not measured for cytochrome b5.

Cytosolic reduced and oxidized glutathione content assay has continued on the Tetryl treated rats.

Pentoxy- and Ethoxy- resorufin assays have been started on the Tetryl treated rats.

NOVEMBER 1-30, 1993

Work on the effects of trinitrobenzene (TNB) continued. Pentoxy- and ethoxy- resorufin assays were continued on the Tetryl treated rats. Assessment of cytosolic reduced and oxidized glutathione concentrations has been postponed due to a delay in delivery of new software for the fluorometer.

DECEMBER 1-31, 1993

Work on the effects of trinitrobenzene (TNB) continued. Still awaiting delivery of new software for the fluorometer. Assessment of cytosolic reduced and oxidized glutathione concentrations were initiated without the new software for the fluorometer. An assay was developed using the existing software and accurate results were obtained.

JANUARY 1-31, 1994

Work on the effects of trinitrobenzene (TNB) continued. Several assays were performed on multiple sample groups including; glutathione-s-transferase activity, determination of reduced and oxidized glutathione concentration, and cytochromes P450 and b5 activities using resorufin, ethoxy and pentoxy as substrates.

The samples in the Tetryl experiment were finished. The poster on the Tetryl experiments for presentation at the 1994 Annual Meeting of the Society of Toxicology (March 13-18, Dallas, Texas) is in preparation.

FEBRUARY 1-28, 1994

Work on the effects of trinitrobenzene (TNB) continued. Multiple assays were done on different sample groups: glutathione-s-transferase, reduced and oxidized glutathione, cytochromes P450 and b5, resorufin, ethoxy and pentoxy. The graphical display of these data (to be presented at the 1994 Annual Meeting of the Society of Toxicology) is in progress.

MARCH 1-31, 1994

Work on the effects of trinitrobenzene (TNB) continued. Microsomal, cytosol, and blood samples from TNB and Tetryl-treated rats were processed for Glutathione-s-transferase activity, reduced and oxidized glutathione concentrations and cytochromes P450 and b5, activity using resorufin, ethoxy and pentoxy. A manuscript was prepared for presentation at a conference scheduled in Florida.

APRIL 1-30, 1994

Work on the effects of trinitrobenzene (TNB) continued. Experimental data from the study on acute and chronic exposure from TNB and Tetryl on microsomal and cytosolic enzyme activities, blood chemistry and methemoglobin were analyzed using the statistical program SAS. The TNB experiment assessing reduced and oxidized glutathione concentrations was finished and results were calculated. Cytochrome P450 activity assays (ethoxy- and pentoxy-resorufin) were continued on all the TNB samples. Efforts have been made to perform all statistical analysis using SAS. This will improve the interpretation of the data. New data sets will also use other applications of this software.

MAY 1-31, 1994

Experimental data from the study on acute and chronic exposure from TNB and Tetryl on microsomal, cytosol, blood chemistry and methemoglobin was analyzed using the statistical program SAS. The TNB experiment using reduced and oxidized glutathione test was finished and results were calculated. Ethoxy- and Pentoxy-resorufin assays were continued on all the TNB samples. Completion of these tests will conclude the data collection for this study.

JUNE 1-30, 1994

Work on the effects of trinitrobenzene (TNB) continued. Ethoxy and Pentoxy resorufin assays were completed and data tabulated on all the TNB samples. Preparation began to repeat experiments to study the effects of Tetryl at the 1000 mg/kg dose. The experiment will augment the hematology data from previous experiments which was lost due to coagulation of blood samples. The hypercoagulation of the blood can be attributed to the exposure to Tetryl. To retard the coagulation the next experiment will be conducted using tubes containing heparin. Standing Operating Procedures (SOPs) were written for new equipment.



JULY 1- SEPTEMBER 30, 1994 (Government approved Quarterly Progress Reports in lieu of Monthly Progress Reports)

Work on the effects of Tetryl on blood cells continued. Rats were dosed with Tetryl and euthanized after 24 hours. Blood and tissue samples were collected. Blood samples were sent to the clinical lab for chemical and hematologic analysis. Methemoglobin assays were preformed on the remaining blood samples. Hypercoaggulation did not occur in this experiment as in previous experiments, therefore the use of heparinized test tubes appears to have eliminated this problem. Tissues were weighed and preserved with formalin for shipment to another laboratory for pathological analysis.

Microsomes and cytosol cell fractions were extracted from liver and testes for enzymatic assays. Total Cytochrome P450 and Cytochrome b_5 activities were assessed in the new microsomes. Ethoxy and Pentoxy resorufin assays were performed on remaining samples. Cytosol samples were assayed for reduced glutathione, oxidized glutathione and Glutathione-*S*-transferase (GST). Data is currently being reviewed and organized for manuscript preparation.

A literature review was conducted to prepare protocols and SOP's for *in vitro* experiments on the inhibition of GST by TNB and identification of metabolites in the urine of ^{14}C-TNB treated rats. Preliminary inhibition study was done measuring GST inhibition by TNB. Non-treated rat urine was collected to begin preliminary metabolite identification.

OCTOBER 1 - DECEMBER 31,1994

Work on the acute effects of Tetryl on blood chemistry and methemoglobin and xenobiotic metabolizing enzyme systems continued. Experiments for this phase of the investigation are complete. Data are currently being reviewed and organized for manuscript preparation.

Work continues on *in vitro* investigations of munitions metabolism. A literature review was conducted to prepare protocols and SOP's for in *vitro* experiments on the inhibition of glutathione transferase activity by trinitrobenzene (TNB) and identification of TNB metabolites in the urine of ^{14}C-TNB treated rats. Microsomal metabolism of TNB was assessed and the results analyzed using HPLC. The TNB peak completely disappeared within 5 minutes, with concurrent appearance of putative metabolites. Metabolite identification has begun with this preliminary information. Extraction of organic soluble metabolites from the water-soluble metabolites was performed, and the majority of the radioactivity was in the aqueous phase, indicative of water-soluble metabolites. Preparation of an SOP for the analysis of aqueous phase enzyme release, acid release and water soluble metabolites generated is in progress. Also an SOP for assessment of fl-glucuronidase and sulfatase activities and glutathione conjugate concentration is in the process of being written.

JANUARY 1 - MARCH 31, 1995

Work with *in vitro* metabolism of munitions by microsomes and cytosol by Phase I and Phase II enzymes was done. Also, extraction of organic soluble metabolites from water-soluble metabolites was repeated at different time points. This data was analyzed with high performance liquid chromatography (HPLC). Standing operating procedures (SOP) for enzyme or acid released and water soluble metabolites has been written to aid the study of residues present in the aqueous phase. Also a SOP for □-glucuronidase, sulfatase and glutathione conjugates was written. Further, reviewed collected data to determine possible effects of munitions on the Phase I and Phase II enzymes and future direction for research.

Data from the TNB and Tetryl experiments has been consolidated into presentable charts for publications. Writing of a manuscript titled "Biochemical and Pathological Effects of N-methyl-N,2,4,6-tetranitroaniline (Tetryl) in Male rats" for submission to *Bulletin of Environmental Contamination and Toxicology* is in progress.

APRIL 1 – JUNE 30, 1995

Plans were made to continue previous *in vitro* metabolism studies to verify metabolites and determine V_{max} and K_m of the microsomal and cytosolic enzymes. Planning was also initiated to repeat *in vivo* metabolism of ^{14}C-Trintrobenzene and ^{14}C-Tetryl during the next quarter.

Data from the TNB and Tetryl experiments have been consolidated into presentable charts for publications. The first draft of the manuscript titled "Biochemical and Pathological Effects of 2,4,6-Trinitrophenyl-N-methylnitramine (Tetryl) in Male rats" was completed. The manuscript will be submitted to "Bulletin of Environmental Contamination and Toxicology" for publication. An abstract for 5th International Symposium on Analysis and Detection of Explosives titled "Metabolism of ^{14}C-Trintrobenzene (TNB) by F344 Male Rat Liver Microsomes" was also completed.

Additional efforts were spent on writing an Acute Toxicity Evaluation for the following chemicals: Brass Powder, Diesel Fuel/Brass Powder Mix, Diesel Fuel, Fog Oil/ Brass Powder Mix, HC Smoke, Copper-Zinc Coated Powder, Copper-Zinc Powder, CI Solvent Yellow 33, CI Solvent Green 3/ CI Solvent Yellow 33, and 1-acetyl-,3,5,7-trinitro-1,3,5,7-octahydrotetrazocine.

JULY 1 - SEPT 30, 1995

Ethoxy and Pentoxy assay of previous TNB and Tetryl experiments was found to be miscalculated. Review of the raw data concluded that it was appropriate to repeat these assays. The Ethoxy and Pentoxy assays were performed on the TNB microsomes. The data corresponded to previously reported data.

Five- and thirty-minute *in vitro* metabolism of TNB was completed. Evaportation is currently underway in order for the resulting organic phase concentrate to be analyzed by HPLC.

Lyophalizing samples from collaberation of metabolism of TNB by skin was begun.

Abstracts

- submitted to 1996 Annual Meeting of the Society of Toxicology
  - ✓ G. Reddy, A.E.G. Hampton, J. Amos, and M. Major. Metabolism of ^{14}C-1,3,5-Trintrobenzene (TNB) *In Vitro*

Manuscripts

- to be submitted to the *Bulletin of Environmental Contamination and Toxicology*

  ✓ Reddy, C.W. Qualls Jr., A.E.G. Hampton, A. Yelamanchili, S. Kim. Biochemical and Pathological Effects of 2,4,6-Trinitrophenyl-N-methylnitramine (Tetryl) in Male Rats.

- submitted to *Acute Toxicity Data, Journal of the American College of Toxicology*, part B

  ✓ Gunda Reddy, Dale A. Mayhew, and Anne E.G. Hampton.
  Acute Toxicity Evaluation of Diesel Fuel
  Acute Toxicity Evaluation of Fog Oil
  Acute Toxicity Evaluation of Diesel Fuel and Brass Powder
  Acute Toxicity Evaluation of Fog Oil and Brass Powder

- Assisted in the writing of the following, also submitted to *Acute Toxicity Data, Journal of the American College of Toxicology*, part B:

  ✓ Gunda Reddy, Indu A. Muni, Elliot B. Gordon, Jane B. Goodband, Dale A. Mayhew, and Howard T. Bausum. Acute Toxicity Evaluation of Brass Powder

  ✓ Gunda Reddy, Indu A. Muni, Elliot B. Gordon, Jane B. Goodband, and Howard T. Bausum
  Acute Toxicity Evaluation of Brass Coated Powder
  Acute Toxicity Evaluation of CI Solvent Green 3/ CI Solvent Yellow 33 Mix
  Acute Toxicity Evaluation of 2-(2-quinolyl)-1,3-inandione
  Acute Toxicity Evaluation of 1-acetyl-3,5,7-trinitro-1,3,5,7-octahydrotetrazocine

- Assisted in the writing of the following, to be submitted to *Acute Toxicity Data, Journal of the American College of Toxicology*, part B:

  ✓ Gunda Reddy, Dale A. Mayhew, and Indu A. Muni
  Acute Toxicity Evaluation of Simulated Hexachloroethane (HC) Smoke

  ✓ Gunda Reddy and Dale A. Mayhew

Acute Toxicity Evaluation of Disperse Red 11
Acute Toxicity Evaluation of Disperse Blue 3
Acute Toxicity Evaluation of Solvent Red 1
Acute Toxicity Evaluation of Disperse Red 11 and Solvent Red 1 (Red Mix)
Acute Toxicity Evaluation of Disperse Red 11 and Disperse Blue 3 (Violet Mix)

✓ Don. W. Korte, Jr., Earl W. Morgan, Larry D. Brown, and Gunda Reddy
14-Day Toxicity Studies with Nitroguanidine in Sprague-Dawley Rats

OCTOBER 1 - DECEMBER 31, 1995

Lyophalizing of samples from collaboration of metabolism of TNB by skin was completed, and efforts on this task were suspended. Funding was re-directed to higher priority programs following the decision that this task needed to take a different tact. Effort may resume in the future, perhaps with different skills required.

Manuscripts

submitted to "Bulletin of Environmental Contamination and Toxicology"

G. Reddy, C.W. Qualls Jr., A.E.G. Hampton, A. Yelamanchili, S. Kim. Biochemical and Pathological Effects of 2,3,6-Trinitrolphenyl-N-methylnitramine (Tetryl) in Male Rats.

JANUARY 1 - MARCH 31, 1996

No work was performed on this task.

APRIL 1 - JUNE 30, 1996

No work was performed on this task.

JULY 1 - SEPTEMBER 30, 1996

Work on this project has been discontinued.

Task Order EQ-3



GEO-CENTERS, INC.

Title: Environmental Chemistry
Task Number: EQ-3 (2533-003)

This task requires technical support in chemistry to investigate the environmental and health effects associated with the firing of moderate to high concentration explosives.

MARCH 1-30, 1993

Methods development for detection of munitions residue in soil continued with the initiation of this task. Ten preliminary soil samples are waiting to be analyzed using the sample oxidizer for the protocol titled "Bioavailability, metabolism, and pulmonary toxicity of inhaled particulates from composts of trinitrotoluene (TNT)-contaminated soils." The oxidizer is being repaired. An experiment was initiated to ascertain the changes in solubility of HMX, RDX, TNT, and TNB in the presence of various environmental compounds. The use of humic acid and fatty acids resulted in little change in solubility of HMX, RDX, TNT, and TNB at concentrations $\leq$ 10 ppm. The concentration of TNB and TNT did increase with the addition 100 ppm of humic acid, and HMX and RDX remained the same. The concentration of HMX and RDX increased with the addition of 1000 ppm of Curpic Sulfate and 100 ppm of humic acid. Carbohydrates will be used next to determine its effects on solubility of the tested compounds. This study will be repeated to validate the results. Twenty samples were analyzed in support of the TNB metabolism studies (Task 2533-002).

APRIL 1-30, 1993

Methods development for detection of munitions residue in soil continue. Over 200 samples are awaiting analysis using the sample oxidizer for the protocol titled "Bioavailability, metabolism, and pulmonary toxicity of inhaled particulates from composts of trinitrotoluene (TNT)-contaminated soils." Currently, the oxidizer is being repaired by the Packard technical representative and the samples are being stored in our refrigerator. Methods development continues to analyze samples for the bioavailability study using HPLC and a radioisotope detector to detect metabolites of TNT. The method will also use a UV detector to aide in the identification of metabolites. Experiments to ascertain the changes in solubility of HMX, RDX, TNT, and TNB due to the concentration of various environmental compounds were continued. The humic acid portion of the solubility study was repeated and results were confirmed. The data have been faxed to a colleague to prepare for publication. Planning for upcoming projects was initiated. These projects will include, (1) a collaboration with Edgewood Research, Development and Engineering Center (formerly CRDEC) to test a new smoke for potential hazards, (2) analysis of tissue samples for Tetryl, and (3) preparation of a five-year plan to study the application of bioremediation in TNT contaminated soils.

MAY 1-31, 1993

Methods development for detection of munitions residue in soil continue. Work is in progress to ascertain the changes in solubility of HMX, RDX, TNT, and TNB due to the

concentration of various environmental compounds. The fatty acid portion of the solubility study was repeated; an increase in solubility with concentrations above 10ppm of linolenic acid was observed. A Bioremediation Symposium in Dallas, Texas was attended.

JUNE 1-30, 1993

Methods development for detection of munitions residue in soil continue. Work is in progress to ascertain the changes in solubility of HMX, RDX, TNT, and TNB due to the concentration of various environmental compounds. The fatty acid portion of the solubility study was repeated a second time to confirm an increase in solubility with concentrations above 10ppm of linolenic acid. Also completed the portion using syringic acid as a co-solute. Five samples were analyzed for trinitrobenzene for in-house investigations. Initiated discussions with Dr. Bollag at Penn State on experiments to study the degradation of TNT in soil.

JULY 1-31, 1993

Methods development for detection of munitions residue in soil continue. Completed experiments with syringic acid and linolenic acid and found a significant correlation with syringic acid and the solubility of TNT and TNB. The presence of fatty acids does not effect the concentration of any of the studied compounds. Analyzed 32 plant extracts for Dr. R.S. Wentsel of ERDEC to investigate if interference exists between plant pigments and the detection of RDX and TNT. The samples were obtained from radishes, lettuce, green beans, and tomatoes.

AUGUST 1-31, 1993

Methods development for detection of munitions residue in soil continue. Initiated data analysis and manuscript write-up for the syringic acid study. Synthesized Nitrogen-15 labeled TNT and commenced reducing it to 4-amino 2,6-dinitrotoluene. The compounds will be used in experiments that will be conducted from August 30th to September 2nd at Penn State in Dr. Bollag's lab. Processed samples for in-house compost study.

SEPTEMBER 1-30, 1993

Methods development for detection of munitions residue in soil continue. Worked at Penn State in Dr. Bollag's laboratory and performed experiments with anilines and TNT using peroxidase from horseradish to form a complex between aniline and humic acid. These experiments were continued with 4-amino- 2,6-dinitrotoluene at USABRDL.

Continuing to work on the manuscript titled "The effects of cosolutes on the solubility of HMX, RDX, TNB and TNT in water."

OCTOBER 1-31, 1993

Methods development for detection of munitions residue in soil continue. Performed experiments with 4-amino- 2,6-dinitrotoluene using horseradish peroxidase. The reaction of horseradish peroxidase with syringic acid caused the 4-amino-2,6-dinitrotoluene to disappear from UV detection at 244 nm within 20 minutes. The next step of the experiment is to use GC-MS at Penn State to determine the compounds created.

Work continued on the manuscript titled "The effects of cosolutes on the solubility of HMX, RDX, TNB and TNT in water." Now waiting for data from Ron Checkai, Ph.D. at Chemical Biological Defense Agency.

Preliminary analysis has been performed on alfalfa, corn, green beans, and radish samples for a Cornhusker Army Ammunition Plant Project for Chemical Biological Defense Agency to determine if any plant constituents will interfere with the analysis of HMX and RDX.

Urine samples were analyzed for TNT metabolites for the in-house composting study in progress [Task EQ-6 (2533-012)].

NOVEMBER 1-30, 1993

Methods development for detection of munitions residue in soil continue. Performed experiments with 4-amino- 2,6-dinitrotoluene using horseradish peroxidase. Horseradish peroxidase was reacted with syringic acid in the presence of 4-amino-2,6-dinitrotoluene. This reaction resulted in the disappearance of 4-amino-2,6-dinitrotoluene from UV detection at 244 nm within 20 minutes. The samples were sent to NATICK R&D Center for analysis. Additionally, urine samples for TNT metabolites for the composting study in support of task 012 were analyzed.

DECEMBER 1-31, 1993

Methods development for detection of munitions residue in soil continue. Performed experiments with 4-amino- 2,6-dinitrotoluene (4-Am) using horseradish peroxidase. Horseradish peroxidase was reacted with syringic acid in the presence of 4-amino-2,6-dinitrotoluene. This reaction resulted in the disappearance of 4-amino-2,6-dinitrotoluene from UV detection at 244 nm within 20 minutes. Experiments were also conducted using equal amounts of syringic acid to 4-Am. The results demonstrated that the syringic acid is utilized at a greater rate then the 4-Am. The experiments to determine pH effects on horseradish peroxidase activity demonstrated that the enzyme is still active at pH 3.2.

JANUARY 1-31, 1994

Work on methods development for detection of munitions residue in soil continued. Performed experiments with 4-amino- 2,6-dinitrotoluene (4-Am) using horseradish peroxidase.

Reaction of horseradish peroxidase with syringic acid caused the 4-Am to disappear from UV detection at 244 nm within 20 minutes. A similar experiment was performed using TNT and syringic acid with the horseradish peroxidase. However, unlike 4-Am, TNT was not removed from the mixture.

Performed hydrolysis on the horseradish peroxidase product from the pH 3.2 experiment and can only retrieve 10 to 15% of the 4-Am. The products from the pH 3.2 and 6.0 experiments have been sent to Penn State and U.S. Natick labs for analysis.

Continued to work on the manuscript titled "The effects of cosolutes on the solubility of HMX, RDX, TNB, and TNT in water" and plan to have the manuscript ready for review in February.

FEBRUARY 1-28, 1994

Work on methods development for detection of munitions residue in soil continued. Completed the manuscript titled: "The effects of cosolutes on the solubility of HMX, RDX, TNB and TNT in water" by John C. Amos, Michael A. Major, Michael Simini, and Ronald T. Checkai. The manuscript is under review by authors and will be submitted to the *Journal of Contaminant Hydrology* for peer reviewed publication. The Technical Report titled: "Bioremediation Methods for the treatment of TNT contaminated soils" by Michael A. Major, Jean-Marc Bollag and John C. Amos was completed and sent for printing this month. Work was initiated this month on a manuscript detailing the results from the horseradish peroxidase experiments.

MARCH 1-31, 1994

Work on methods development for detection of munitions residue in soil continued. Two manuscripts were submitted for peer review: (1) "Bioremediation method for treatment of TNT contaminated soils" Michael A. Major, Jean-Marc Bollag and John C. Amos, submitted to *The Journal of Bioremediation,* and (2) "Toxicity Testing of Soils from Joliet Army Ammunition Plant," Michael Simini, Randall S. Wentsel, Ronald T. Checkai, Carlton T. Phillips, Nancy A. Chester, Michael A. Major and John C. Amos, submitted to *Environmental Toxicology and Chemistry.* Technical Report 9305, "Bioremediation Methods for the treatment of TNT contaminated soils" by Michael A. Major, Jean-Marc Bollag and John C. Amos, U.S. Army Biomedical Research & Development Laboratory, Ft. Detrick, Frederick, MD was released. The manuscript "Development of an Effective Bioremediation Method for TNT-contaminated sites: Phase I Studies" by Michael A. Major, Jean-Marc Bollag, and John C. Amos is currently under review by authors.

TNT contaminated compost was hydrolyzed to determine the stability of the bonds linking the TNT. The results yielded diamino-nitrotoluene product which indicates an unstable bond. Current research efforts were presented to U.S. Army Environmental and Hygiene Agency at Edgewood Arsenal, Aberdeen Proving Ground, MD.

APRIL 1-30, 1994

Methods development for detection of munitions residue in soil continue. The product of the horseradish peroxidase experiments was analyzed by GC-mass spectrometry analysis at the Pennsylvania State University the results identified a 379 amu molecule which is the size of the product predicted in the theoretical model. Method development for separation of metabolic products of TNT by HPLC continued. Future efforts will analyze urine from rats exposed to TNT contaminated compost. Investigation of the binding properties of TNT, HMX, RDX, and TNB with soil continue.

The paper, "Bioremediation method for treatment of TNT contaminated soils" by Michael A. Major, Jean-Marc Bollag and John C. Amos was accepted for publication in *The Journal of Bioremediation.*

Abstracts on "The effects of cosolutes on the solubility of TNT, TNB, RDX, and HMX in water" by John C. Amos, Michael A. Major, Ronald T. Checkai, and Michael Simini were submitted for presentation at the Army Environmental Research and Development Symposium and the Society of Environmental Toxicology and Chemistry meetings.

MAY 1-31, 1994

"Bioremediation method for treatment of TNT contaminated soils" Michael A. Major, Jean-Marc Bollag and John C. Amos is scheduled for publication in the June issue of *The Journal of Bioremediation.*

Urine samples were analyzed using an UV-absorbance and radiolabeled detectors attached to a HPLC system. The results demonstrated that TNT-composted soil administrated to rats is excreted in urine as nonpolar molecule that elutes from a C-8 reverse phase HPLC column approximately at 5.5 minutes. This peak exhibited all of the radiolabeled C-14. A similar peak was noted in the TNT-composted soil samples. Beta-glucuronidase has been used to cleave the linkage to ascertain the compound; but efforts have not yielded satisfactory results. Trinitrobenzaldhyde standard was measured by HPLC to compare the elution time with the unknown compound. The trinitrobenzaldhyde standard eluted at 9.7 minutes, which is considerably later than the unknown peak.

JUNE 1-30, 1994

Methods development for detection of munitions residue in soil continue. The laboratory work performed this month focused on determining the fate of C-14 radiolabeled TNT after composting. The experiment used 59.8 mg of compost (containing ^{14}C-TNT) which was sonicated for 12 hours in acetonitrile. The mixture was filtered and the filtrate was evaporated under nitrogen to 5 mL. A 500 µL sample of the filtered mixture was counted in a scintillation counter. The compost remaining on the filter was hydrolyzed with a mixture of

methanol and hydrogen chloride for 2 hours. The resulting hydrosylate was once again filtered and evaporated as described previously. The remaining compost was burn in an oxidizer and counted. The theoretical recovery was 85%. This recovery was derived by comparing the results from oxidizing three representative samples. Experimental results demonstrated that 63% remained bound to the compost, 22% was removed after hydrolysis and only 0.36% could be extracted by acetonitrile. These results are similar to the first experiment which yielded 79% bound to the compost, 19% present in the hydrosylate and 1% in acetonitrile sample.

Laboratory work to produce N-15 labeled 4-amino-2,6-dinitrotoluene (4-AM) from N-15 label TNT yielded an impure mixture of 4-AM and 2-amino-4,6-dinitrotoluene. The synthesis will be continued in July. The product is needed to continue soil-binding studies with horseradish peroxidase.

The technical report titled "*Changes in the Solubility of Military Explosives in Relation to Alteration of Environmental Condition and Cosolutes*" by John C. Amos, Michael A Major, Ronald Checkai, and Michael Simini is in review and will be submitted to AEHA for approval in July, 1994.

The paper, "Bioremediation method for treatment of TNT contaminated soils" by Michael A. Major, Jean-Marc Bollag and John C. Amos is scheduled for publication in the September, 1994 issue of *The Journal of Bioremediation.*

JULY 1- SEPTEMBER 30, 1994 (Government approved Quarterly Progress Reports in lieu of Monthly Progress Reports)

Methods development for detection of munitions residue in soil continue. Work continued on the cosolute and cosolvent effects of humic acid on the solubility of TNT, TNB, RDX, and HMX in water. Different soil types were selected from Anniston Army Depot, Milan Army Ammunition Plant, Pueblo Army Depot and Radford Army Ammunition Plant. Humic acid was extracted by dissolving 5 grams of soil into 30 mL of distilled water, followed by the addition of 10 mL of 10 M NaOH. Lastly, 3 ml of 12 N HCL was added to the mixture and sonicated for 12 hours the mixture was dried under nitrogen. Currently, humic acid extracted from Hagerstown Loam is in use to determine its effects on solubility of TNT, TNB, RDX, and HMX. Additional, work is also continuing on the reduction of N-15 labeled TNT.

The chosen method may not yield a product of 4-amino-2,6-dinitrotoluene pure enough for the present experiments. A pure reduction product is needed to study the binding properties of TNT degradation products to soil components.

The manuscript titled "Evaluation of soil toxicity at Joliet Army ammunition Plant" is in press for *Environmental Toxicology and Chemistry Journal.* The galleys for the manuscript "Bioremediation method for the treatment of TNT contaminated soil" were sent to the *Journal of Bioremediation.* A poster titled "Peroxidase-Catalyzed Linkage of TNT Metabolites to Soil

Humus" was prepared and presented (by a co-author) at the Mutual Weapons Development Master Data Exchange Agreement USA/Germany Annual Meeting held at Bonn, Germany.

OCTOBER 1 - DECEMBER 31,1994

Work was completed on the cosolute and cosolvent effects of humic acid on the solubility of TNT, TNB, RDX, and HMX in water with soils from Anniston Army Depot, Milan Army Ammunition Plant, Pueblo Army Depot, and Radford Army Ammunition Plant. Data are being analyzed and prepared for publication.

Research has begun on the metabolic pathway of TNB by microsomal degradation. Eighty-two samples were analyzed for this study. Research efforts on the Compost Exposure Study focused on determining the metabolic product using mass spectrometry, which revealed that the product is a diphenol. Also, new separation procedures are being implemented to further elute the degradation products present in the compost and in the rat specimens.

JANUARY 1 - MARCH 31, 1995

Efforts performed on this task included work with C-14 radiolabeled TNT and non-radiolabeled TNT in experiments to determine the degradative characteristics of this compound under alkaline conditions. The experiment consisted of titrating a mixture of TNT and water with a concentrated solution of NaOH while changes in pH and the concentration of TNT was measured. The results showed a decrease in the concentration of TNT and an increase of unknown degradative compound. The chromatographs were compared to 2,6-dinitro-*p*-cresol and 4,6-*o*-dinitro-cresol and the elution of these peaks did not correspond with the unknown peak. Further analysis of the solution by ion chromatography revealed three possible degradative products. This was suspected considering the meta arrangement of the nitro groups on TNT. Therefore it is highly plausible that the reduction of one or two of the nitro-groups would yield a suite of compounds.

The alkaline derived degradative compound was characterized further by reacting it with pyridine and acetic anhydride. This reaction forms esters and increases the retention time. This effort yielded the desired results with this compound. The product was further characterized by comparing the octanol/water partition coefficient (k_{ow}) of the compound with the reacted product. The results showed that the k_{ow} changed significantly. These experiments were repeated on urine from rats exposed to compost and the results indicated that the metabolic products are different from the alkaline compound.

Successful efforts were achieved in the synthesis of 4-amino-2,6-dinitrotoluene (4amdnt) by the reduction of 2,4,6-Trinitrotoluene (TNT). The methods used started with TNT dissolved in 1,4 dioxane and catalyzed by ammonium hydroxide while bubbling hydrogen sulfide gas. The first attempt used ammonium sulfide and hydrogen sulfide and yield 98% product. The synthesis was repeated without the ammonium sulfide and achieved similar results. This is the final synthesis procedure that was required to make 4amdnt that is C-14

radiolabeled and N-15 labeled. This double-labeled compound will be used to determine the mechanism(s) that bind nitroaromatic compounds to soils.

Work was completed on the cosolute and cosolvent effects of humic acid on the solubility of TNT, TNB, RDX, and HMX in water with soils from Anniston Army Depot, Milan Army Ammunition Plant, Pueblo Army Depot, and Radford Army Ammunition Plant. Data indicate that all energetic compounds are removed from solution. The statistical analysis of the data has commenced in preparation for publication.

Research continued on the metabolic pathways of TNB. Currently, the results have demonstrated removal of TNB below present detection limits in fijve minutes by microsomes from rat livers. An enzyme kinetics curve was plotted to determine the K_m, but the data points were not consistent. The experiment was repeated, yielding more consistent data; however, a K_m has not been determined because the presence of different enzymes has complicated the efforts of producing a reliable Michaelis-Menten plot. Possible metabolites were observed which correspond with the degradation of TNB. The experimental data do not resemble any standards that were tested. The closest match is with 3,5 dinitroaniline. Further efforts to determine the metabolites of TNB were conducted by measuring the concentration of C-14 in aqueous phase compared to organic phase. The results yielded C-14 in the ethanol with no detectable levels in the aqueous phase.

APRIL 1 – JUNE 30, 1995

Studies were conducted to determine the effects of Triton X-100 and humic acid on the maximum concentration of explosives in water. In initial experiments, explosives were added to water in excess of the amount that could be dissolved, and equilibrium achieved by shaking in 25°C water bath for four days. The explosives used were 2,4,6-trinitrotoluene (TNT) and 1,3,5-trinitrobenzene (TNB), hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) and ocatahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX). No cosolubility effects were observed when these explosives were added together. In subsequent experiments, Triton X-100 and various humic acids were added to saturated solutions of this mixture and maximum concentrations of individual explosives were determined. The results showed linear increases in the concentration for TNT (r^2=.800), TNB ($r^{2=}$=.789), RDX (r^2=.912), and HMX (r^2=.946) in relation to the amount of Triton X-100 added. However, similar experiments with various humic acids produce an apparent increase in solubility only with HMX.

In experiments in which Triton X-100 and humic acid were used together, the increases in solution concentrations of nitramines were lower then when Triton X-100 was used alone. This effect was not observed with nitroaromatic concentrations. A manuscript entitled "Effects of Humic Acid and Triton X-100 on the Aqueous Solubility of TNT, TNB, RDX, and HMX" is being prepared.

Research continued on the metabolic pathways of TNB. Currently, the results have demonstrated removal of TNB below present detection limits within 5 minutes by microsomes from rat livers. An enzyme kinetics curve was plotted to determine the km, but the data points

were not consistent. The experiment was repeated and yielded more consistent data. However, a km has not been determined because the presence of different enzymes has complicated the efforts of producing a reliable Michaelis-Menten plot. Metabolites (including 3,5 dinitroaniline) which may correspond with the degradation of TNB were observed. Further efforts to determine the metabolites of TNB were made by measuring the concentration of C-14 in aqueous phase compared to organic phase. The results yielded C-14 in the ethanol with no detectable levels in the aqueous phase. An abstract for the 5th International Symposium on Analysis and Detection of Explosives entitled "Metabolism of ^{14}C-Trinitrobenzene (TNB) by F344 Male Rat Liver Microsomes" was prepared for submission.

The Department of Defense Environmental Technology Workshop was attended during this quarter. The following manuscripts were published or completed during this reporting period:

- Simini, M., R.S. Wentsel, R.T. Checkai, C.T. Phillips, N.A. Chester, M.A. Majors and J.C. Amos. Evaluation of soil toxicity at Joliet Army Ammunition Plant. Environmental Toxicology and Chemistry, Vol. 14, No. 4 pp. 623-630. 1995.

- Major, M.A., W.H. Griest, J.C. Amos, and W.G. Palmer. The Chemistry of TNT in Composting of TNT-Contaminated Soils. Army Environmental Health Agency Technical Report: (in review).

- Major, M.A., J.C. Amos, and Jean-Marc Bollag. Enzyme Catalyzed Linkage of TNT Metabolites to Soil Humus. Army Environment Health Agency Technical Report: (in press).

- Checkai, R.T., M.A. Major, R.O. Nwanguma, J.C. Amos, C.T. Phillips, and R.S. Wentsel and M.C. Sadusky. Transport and fate on Nitroaromatic and Nitramine Explosives in soils from open burning open detonation operations at Pueblo Army Depot. ERDEC Technical Report (in press).

- Checkai, R.T., M.A. Major, R.O. Nwanguma, J.C. Amos, C.T. Phillips, and R.S. Wentsel and M.C. Sadusky. Transport and Fate of Nitroaromatic and Nitramine Explosives: I. RDX, TNT, 2,4-DNT, and 2,6-DNT in Clarksville-Fullerton Stony/Cherty Loam. Environmental Toxicology and Chemistry (in press).

- M.A. Major, J. Bollag, and J.C. Amos. Bioremediation methods for the treatment of TNT contaminated soils. Journal of Bioremediation (in press).

JULY 1 - SEPT 30, 1995

During the past quarter, work was completed on the cosolute and cosolvent effects of Triton X-100 on the solubility of TNT, TNB, RDX, and HMX in water and with humic acid derived from Anniston Army Depot, Milan Army Ammunition Plant, Pueblo Army Depot and Radford Army Ammunition Plant. Data indicate that humic acid minimizes the solubilizing

effects of Triton X-100. The statistical analysis of the data has begun, and a manuscript is being prepared for publication.

A poster titled, "The Chemistry of TNT in Composting TNT-Contaminated Soils," Major, M.A., W.H. Griest, J.C. Amos, and W.G. Palmer was completed and will be presented at the U.S./German Mutual Weapons Development Master Data Exchange Agreement Annual Meeting, Denver, Colorado, October 9-13, 1995. A technical report with the same title also was prepared and is currently being reviewed for publication.

OCTOBER 1 - DECEMBER 31, 1995

A manuscript on the cosolute and cosolvent effects of Triton X-100 on the solubility of TNT, TNB, RDX and HMX is being prepared for publication.

Research has begun on the solubility of degradative products of TNT. This study will determine the aqueous solubility of 2,4 Diamino-6 Nitrotoluene; 2-Amino-4,6 Dinitrotoluene; 1,3-Dinitrobenzene, 2,6-DNT; 2,4-DNT, 4-Amino-2,6-DNT; 4-4 Azoxytoluene; 2,2 Azoxytoluene; 3,5 Dinitroaniline; 4,6 Dinitro-O-Cresol; 2,6 Dinitro-*P*-cresol; 2-Amino 4-Nitrotoluene; 4-Amino-2-Nitrotoluene; 2,4 Dinitroaniline; 3,4 Dinitrotoluene; 2,4-Diaminotoluene and 2,6-Diaminotoluene. The aqueous solubility will be estimated by Regression equations using the Kow, TmB and the Regression Koc, and theoretical equations using estimated activity coefficients based on the structure, Hf, TmB and octanol/water. The octanol/water coefficient will be estimated using the Leo's fragment constants method. The theoretical estimates will be compared to the experimental data for analysis.

Samples were also analyzed for the degradation of TNB from samples taken from guinea pig, rat, and human skin studies. Statistical analysis is now being performed in preparation for peer review publication.

JANUARY 1 - MARCH 31, 1996

During this quarter, work was completed on the cosolute and cosolvent effects of Triton X-100 on the solubility of TNT, TNB, RDX, and HMX in water and with humic acid derived from soils located at Anniston Army Depot, Milan Army Ammunition Plant, Pueblo Army Depot and Radford Army Ammunition Plant. The manuscript titled "Effects of Humic Acid and Nonionic Surfactant on the Aqueous Solubility of TNT, TNB, RDX AND HMX" is being reviewed by U.S. Army Center for Health Promotion and Preventive Medicine (USACHPPM) for outside publication. The manuscript will be sent to *Environmental Science and Technology* (ES&T) journal for consideration for publication after internal review. Revisions were started on the manuscript titled "Chemistry of TNT in Composting of TNT-Contaminated Soils." This manuscript will also be sent to ES&T for publication.

Work was also done on a poster for the Society of Toxicology which was presented at the Anaheim, California meeting in March. The poster was titled, "Metabolism of

"C-1,3,5Trinitrobenzene (TNB) In Vitro." G. Reddy, A.E.G. Hampton, J. Amos, M. Major. USACHPPM, Fort Detrick, Frederick, MD. Fundamental and Applied Toxicology. Supplement. The Toxicologist 30, (1) part 2, 125, 1996. A slide presentation was prepared for the Bioremediation of Surface and Subsurface Contamination Conference held in February. A response for Badger Army Ammunition Plant on monitoring procedures for 2,4,6-Trinitrotoluene metabolites and a proposal for SERDP funds titled "Application of Coupled Chemical Oxidative Denitration and Microbial Mineralization For Destruction of Nitroaromatic and Nitramine Explosives in Soil and Water" were edited.

Laboratory work was dedicated to the synthesis of Nitrogen-15 labeled 4-amino-2,6-DNT and the completion of samples on the degradation of TNB by guinea pig, rat, and human skin studies. A meeting with collaborators at Pennsylvania State University was held to discuss research goals and funding requirements.

APRIL 1 - JUNE 30, 1996

During the past quarter, the manuscript titled "Effects of Humic Acid and Nonionic Surfactant on the Aqueous Solubility of TNT, TNB, RDX and HMX" was reviewed and approved by U.S. Army Center for Health Promotion and Preventive Medicine (USACHPPM) for outside publication. The manuscript is now being reviewed for publication in *Environmental Science and Technology* (ES&T) journal. The manuscript titled "Chemistry of TNT in Composting of TNT-Contaminated Soils" was revised and currently being reviewed for publication by ES&T under the title "Evidence for the Chemical Reduction and Binding of TNT during the Composting of Contaminated Soils." The manuscript "Studies on the Stability of the Binding of TNT Residues to Organic Fractions of Soil/Compost during Composting of TNT-Contaminated Soil", Michael A. Major, John C. Amos and Winifred G. Palmer is under reviewed for release as a technical report.

Laboratory work was dedicated to the development of a synthesis methods for 4-amino-2,6-DNT and TNT. Nitrogen-15 labeled TNT synthesized in this laboratory was sent to collaborators at Pennsylvania State University and University of Stuttgart Institute for Microbiology. The following manuscripts and proposals were reviewed:

1. "Biological Remediation of TNT-Contaminated Soils by Sequential Reductive Metabolism and Humification Methodologies" by Michael A. Major, Jean-Marc Bollag and Timothy E. Saylor.

2. "Bioavailability of TNT Residues in Composts of TNT-Contaminated Soil" by Winifred G. Palmer, Joseph R. Beaman, Dianne M. Walters, Michael A. Major, and Donald A. Creasia.

3. "Assessment of the Toxicity of Enzyme Linked Products Generated as Chemical Models for the Bound Metabolites of TNT formed During Composting Proposal", Michael A. Major, Jean-Marc Bollag and Glenn Leach.

4. "Working Plan for the Characterization of the Irreversible Bound Metabolites of TNT to Soil Components During the Anaerobic/Aerobic Treatment of TNT-Contaminated Soil" by H.-J Kanckmuss.

A research plan was developed for the study of TNT and RDX reduction in soils by the enhancement of humification process.

JULY 1 - SEPTEMBER 30, 1996

During the past quarter, the manuscript titled "Effects of Humic Acid and Nonionic Surfactant on the Aqueous Solubility of TNT, TNB, RDX and HMX" was rejected for publication in *Environmental Science and Technology* (ES&T) journal. The manuscript has been corrected and has been sent to the *Journal of Environmental Science and Health.*

Laboratory work was dedicated to the development of a synthesis method for TNT that will increase the yield but lower the amounts of toluene needed. An improved synthesis method will decrease the cost of making dual labeled TNT, which is required for planned research efforts. The remainder of the quarter was devoted to packing and moving the laboratory from Fort Detrick, Maryland to Edgewood Proving Ground.

OCTOBER 1 - DECEMBER 31, 1996

During the past quarter, the manuscript entitled "Effects of Humic Acid and Nonionic Surfactant on the Aqueous Solubility of TNT, TNB, RDX and HMX" was reviewed and approved by U.S. Army Center for Health Promotion and Preventive Medicine (USACHPPM) for outside publication. This manuscript is now being released as a USACHPPM technical report. The manuscript entitled "Chemistry of TNT in Composting of TNT-Contaminated Soils" was revised and is being released as a USACHPPM technical report.

Laboratory work was dedicated to the development of a high performance liquid chromatography (HPLC) method for 2,2'-thiodiethanol. The lambda max was determined to be 215 nm, but good absorption was also seen at 234 nm. Therefore 234 nm may be a better selection then 215 nm due to less interference from other compounds and extraction solvents. The mobile phase is distilled water pumped at 1 mL min^{-1} through a C-18 column. The retention time was approximately 10 minutes. An experiment to determine the degradation of HMX and RDX in an alkaline solution was conducted and degradation was determined in both compounds. This work will be repeated as a time study to determine how rapidly these compounds are degraded under alkaline conditions.

A radiation safety standing operating procedure (SOP) was completed and is under review for the use of C^{14} and H^{3} in future research efforts.

JANUARY 1 - MARCH 31, 1997

This research project, which was being conducted at USACHPPM, was shifted to another contract, and the effort under this task order was terminated.

Task Order EQ-4



GEO-CENTERS, INC.

Title: Environmental Monitoring
Task Number: EQ-4 (2533-004)

This task requires technical support to characterize and examine the environmental and health effects associated with toxic substances generated from various military operations.

MARCH 1-30, 1993

Work continued on developing gas monitors at the commencement of this task. Assisted in the preparation and execution of a field trip to Aberdeen Proving Grounds. The purpose of this trip was to characterize the smoke produced in the "Super Box". A real time HCl monitor was used for these analyses.

The majority of this month was spent working toward updating the Ion Chromatograph with the latest technology for running cations. DIONEX has a new column and a new suppressor that alleviates the need for regenerant. Methods were developed for running Li^+, Na^+, NH_4^+, K^+, Mg^{2+}, and Ca^{2+}. These methods were used to begin characterizing "raw" and "processed" water used by various labs at USABRDL.

APRIL 1-30, 1993

Work continued on developing gas monitors. Assisted in the preparation and execution of a field trip to characterize the exhaust from a rocket firing at Aberdeen Proving Grounds. The real time toxic gas monitor developed at USABRDL was used in this test. The data collected during these tests will be presented next month at the annual ACGIH meeting. Ran impinger samples on the ion chromatograph to validate the data collected in the field by the toxic gas monitor.

Work continued to enable detection of Li^+, Na^+, NH_4^+, K^+, Mg^{2+}, and Ca^{2+} on the ion chromatograph using the new columns and new suppressor developed by DIONEX. Used this method to continue characterizing "raw" and "processed" water used by various labs at USABRDL. Analysis of these ion concentration will be used to help define the cause of water associated problems experienced in those same labs. Began developing method to extract magnesium off filter samples taken by the Air Force during a rocket firing at Thiokol.

MAY 1-31, 1993

Work continued on developing gas monitors by performing a series of experiments to determine the efficiency of the HCl monitor developed in house by Dr. Hoke. Samples were taken of HCl aerosols and HCl gases by both the monitor and impingers and the data compared. Initiated work to prepare dataloggers for next field test, tentatively scheduled for the middle of June.

Chemical analysis and quantitation of anions in tap water from room 122 was performed this month in order to determine if carbon filtered tap water will be adequate for medaka culture in the immunotoxicology laboratory. Additionally, initiated set up of methods for detecting anions on the ion chromatograph using the new columns and new suppressor developed by DIONEX.



JUNE 1-30, 1993

Work continued on developing gas monitors. Provided support for a Combat Systems Test Activity (CSTA) in a halon degradation study. Impinger and real time toxic gas monitor samples were collected for analysis. New dataloggers were tested under field conditions. Analyzed impinger samples for fluoride once back in the laboratory. Retrieved data from dataloggers; dataloggers did not perform up to desired standards and will have to be modified. Support was provided to the Institute of Chemical Defense (Edgewood Arsenal, Aberdeen Proving Ground, MD) to aid in the set up an experiment involving CN. Water analysis support was provided to the in-house immunotoxicology group. Analysis consisted of quantitation of anions (Cl, NO_2) and cations (NH_3, Ca, Mg) from water samples in order to characterize the water to be used in that particular laboratory. The technical report "MR 1-93: *Atmospheric Analysis Of Exhaust From The Solid Propulsion Integration And Verification Motor No. 1"* was completed and distributed.

JULY 1-31, 1993

Work continued on developing gas monitors. Worked toward developing methods on the ion chromatograph for methylphosphonate (MPA), ethyl methylphosphonate (EMPA), isopropyl methylphosphonate (IMPA), and pinacolyl methylphosphonate (PMPA). A method is being developed which allows all four compounds and chloride concentrations to be determined in one run. A calibration curve was determined for MPA, EMPA, and IMPA. MPA can be detected down to 0.25 ppm; EMPA can be detected down to 0.1 ppm; and IMPA can be detected down to 0.5 ppm. A calibration curve for PMPA has been developed from 1 to 10 ppm. Work continues to determine the lowest limit of detection for PMPA. Once method development is complete, samples will be run.

AUGUST 1-31, 1993

Work continued on developing toxic gas monitors. Ran several experiments to determine the efficiency of the newest impinger for the monitor. These experiments were designed to compare the monitor's impinger with standard impingers in their response to 10,000 ppm Cl^- aerosol, 5000 ppm Cl^- aerosol, 10,000 ppm HCl aerosol, 40 ppm HCl gas, and 10 ppm HCl gas. It was difficult to design a sampling chamber for the aerosol portion of the experiment. Once this was achieved, the aerosol experiments were successful in showing that the monitor impinger was efficient in sampling for the forms of aerosol that were tested. Difficulty also arose in determining the efficiency of fritted impingers verses regular standard impingers while sampling aerosols. Several experiments were run to determine which was more efficient. The monitor's impinger and the standard impingers also showed good agreement when sampling for HCl gases.

Work also continued on developing a method for SDS (sodium dodecyl sulfate). It was shown that SDS can be detected and quantitated on the ion chromatograph. A calibration curve was determined from 0.5 ppm to 10 ppm SDS with an r^2 of 0.99971. A stability study and precision and accuracy data need to be obtained before samples can be run.

SEPTEMBER 1-30, 1993

Work continued on developing toxic gas monitors. Also ran anion and cation scans on various water samples from within the laboratory.

Supported field study of JAVELIN firings at Redstone Arsenal in Huntsville, AL. Personal samplers were used to collect airborne lead samples representative of the air the soldiers were exposed to in various firing scenarios. These results will be compared with blood-lead samples of the gunner and the assistant gunners. Samples were taken for two firings. Due to problems experienced by the JAVELIN crew, samples from a third firing were not taken. Once these problems are corrected, samples will be taken.

OCTOBER 1-31, 1993

Work continued on development of a multigas analyzer. Initiated work with LabVIEW2. It is a combination of hardware and software designed to collect data from the multigas analyzer and report that data in various forms. Also installed the hardware and went through the six-hour tutoring program that came with the software. The next task is to figure out how the LabVIEW2 software and hardware are connected to the multigas analyzer and then to write a program to run the multigas analyzer with LabView2.

Continued developing a method on the ion chromatograph for isomethyl phosphonate (IMPA) and methylphosphonate (MPA).

NOVEMBER 1-30, 1993

Work continued on development of a multigas analyzer. The multigas analyzer was tested using bromide gas and bromide aerosols. This was done by pulling duplicate impinger samples at each level of gas. The impinger samples were run on the ion chromatograph. The calibration curve ranged from 0.5-10.0 ppm Br^- and the r^2=0.998804. The monitor and impingers agreed well at each level of gas sampled. The 1,000 and 10,000 ppm Br^- aerosol impinger samples were more difficult; the impinger samples were consistently lower than the monitor readings of the same concentration aerosol. It was decided to use a fritted impinger to collect the samples. Once this was done, the impingers and monitor agreed fairly well. Work continued with LabView2. Some progress was made in developing a program to read data from the multigas analyzer; however, it will not store the data. Work continues on developing the program.

DECEMBER 1-31, 1993

Work continued on development of a multigas analyzer. Preparations were completed for gathering data from the firing of a Sidewinder missile at the Naval Surface Weapons Center in Dahlgren, VA. This involved getting pumps, impingers, and the sequential sampler ready for the field. After returning to the laboratory, the samples were run on the ion chromatograph.

JANUARY 1-31, 1994

Work continued on development of a multigas analyzer. Assisted in condensing the size of the multigas analyzer. Also prepared new ion chromatograph to run routine anions (fluoride, chloride, bromide, nitrate, phosphate, and sulfate).

Compared commercial sequential sampler with the sequential sampler developed in-house and provided a list of significant differences to be given to the patent lawyers at Ft. Detrick. Also continued to troubleshoot the program that operates the sequential sampler.

FEBRUARY 1-28, 1994

Work continued on development of a multigas analyzer. Assisted in condensing the size of the multigas analyzer. Also prepared new ion chromatograph to run routine anions (fluoride, chloride, bromide, nitrate, phosphate, and sulfate). Continued to troubleshoot the program that operates the sequential sampler developed in-house. Work continued on methods development for ion chromatograph detection of EMPA (ethylmethylphosphonate), a nerve agent degradation product. Detection methods development for PMPA is scheduled for next month.

MARCH 1-31, 1994

Work continued on development of a multigas analyzer. Work on development of a phosphonate method with ion chromatography (IC) was done; attempts to duplicate an existing method with the present column did not achieve satisfactory results. Another type of column for the IC is on order. The work will continue upon the arrival of the new IC column. The primary focus for next month will be the multigas analyzer.

APRIL 1-30, 1994

Work continued on development of a multigas analyzer. A field study at APG Airbase 3 was conducted to test the multigas analyzer to determine it's ability to detect HBr, HCl, HCN, HF, and changes in pH. The test condition was a simulated vehicle fire extinguished with CO_2. No data was obtained during the first run; the tubing from the vent tube melted and collapsed. This problem was corrected and data was obtained when the test was repeated. The multigas analyzer operated well. The next effort will be to link the multigas analyzer to a PC computer for direct input into the Labview program. Several logistical problems were encountered with this field exercise. However, these concerns are being addressed and will be corrected before the next field trip.

MAY 1-31, 1994

The multigas analyzer was field tested at Eglin Air Force Base in Florida. The device was used to measure the amount of harmful impurities produced by new military smokes. Field tests were conducted at Redstone Arsenal during the last week of the month.



JUNE 1-30, 1994

Work continued on development of a multigas analyzer. Field tests at APG continued. Several problems were encountered during field operations. These problems are in the process of being identified and corrected. One problem was maintaining the proper airflow, which was caused by mist being pulled from the trap into the filters. This has been corrected by modifying the caps to the trap. Secondly, the fluoride and bromide electrodes did not respond properly. Additional tests in the laboratory demonstrated that the fluoride and bromide electrodes did not work. Subsequently, the electrodes were sent back to Microelectrodes for repair. The multigas analyzer's critical orifice also did not function as expected. Experiments to troubleshoot the device will continue in July.

One success of the field test was to get a signal from the multigas analyzer into a computer through Labview. The program is still in the development stage, but progress is continuing as scheduled. Another success is the new case design which will make it faster and easier to calibrate the instrument before and after burns.

Three HCl monitors were prepared and delivered to AEHA for use at a large rocket firing test in Hawaii.

JULY 1- SEPTEMBER 30, 1994 (Government approved Quarterly Progress Reports in lieu of Monthly Progress Reports)

Work continued on development of a multigas analyzer. Several fluoride and bromide electrodes were tested but did not work properly. Researchers from Aberdeen Proving Ground (APG) tested their equipment with a known concentration of HCl gas in this laboratory. The HCl monitor, the multigas analyzer, and impingers were tested as well. Four of the impinger samples taken were analyzed by ion chromatography and half were sent to APG to be analyzed. Testing of a ion chromatography method for iodide has begun. Iodide electrodes for the multigas analyzer were received and will be tested.

Several trips were made to Airbase 3, APG to test the multigas analyzer and support Live Fire Test Program. The field test was simulated engine fires that were extinguished by a prototype system. The position of the multigas analyzer was moved to the front of the vehicle in order to be closer to the fires which minimized the interference of the smoke reacting with the vent tube. The fumes were analyzed for fluoride and bromide levels by the analyzer.

A field study was conducted at Ft. Huachuca for HCl from a RATO bottle propelled UAV (Unmanned Aerial Vehicle). A report is being prepared on the results.

OCTOBER 1 - DECEMBER 31,1994

Work continued on development of a Multigas Analyzer. Sorbent tube samples from the trip to Ft. Huachuca, AZ, have been run. Data from monitor, multigas analyzer, and sorbent tubes were compiled into a report reflecting findings from the Ft. Huachuca launching of Hunter Unmanned Air Vehicle (UAV).

A series of cold temperature studies were performed on the Multigas Analyzer. Each channel was tested to determine any temperature effects on the electrodes as well as the solenoid pump. The data from these tests have been translated into graphic form and are currently being evaluated. A problem with the 0.1 M sodium hydroxide (NaOH) trapping solution for cyanide was noticed. The Tween used as a surfactant in the 0.1 M NaOH completely breaks down over a period of six (6) hours. This is unacceptable. A substitute for the Tween is needed. Nonidet is also a nonionic surfactant and is currently being studied as a possible substitution for the Tween. Sodium dodecyl sulfate (SDS) is also being looked at, but it is an ionic surfactant and therefore is not an ideal substitute.

The *LabVIEW* program for collecting data from the Multigas Analyzer was written by an outside contractor. Writing of an instruction manual on how to operate the program, which collects data from samplings of toxic gases with the Multigas Analyzer, was completed this quarter.

A method was set up and an SDS analysis was run for the USABRDL Killifish project. Stock solutions were correct; however, there was a problem with the final samples pulled after SDS exposure to the fish. The SDS peak had disappeared over the exposure time and an additional peak appeared on the tail of the chloride peak. Additional study will be necessary to determine what is happening to the SDS during testing.

A study of the stability of iodide in solution was conducted using the ion chromatograph (IC). Iodide aerosol samples were collected in midget impingers and compared with data from the Multigas Analyzer using the iodide channel. The results were recorded in the notebook. Next, bromide (Br) aerosol samples were collected and run on the IC; these results were also placed in the notebook. The ASRS in the IC was regenerated and put in the DX100 IC. A new ASRS was installed in the primary IC. A method for running creosol on the IC was investigated.

JANUARY 1 - MARCH 31, 1995

Nonidet was tested as an alternative surfactant to replace Tween 20 in the 0.1 M sodium hydroxide (0.1 M NaOH) trapping solution. Initial experiments indicated that Nonidet does not degrade after three weeks of testing. Next, various trapping solutions were prepared with Nonidet for the Multigas Analyzer. The data from testing four solenoid pumps used in the Multigas Analyzer were graphed. The test studied flow rate of the solenoid verses temperature. Test results with the fluoride, chloride, pH, and bromide systems within the Multigas Analyzer performed properly with Nonidet. The cyanide system is scheduled to be tested.

Tests were performed on ten new blue boxes used in the Multigas Analyzer. The blue boxes work in conjunction with the signal amplifier to enhance the noise-to-signal ratio. Only two of the blue boxes performed properly on all of the channels in the Multigas Analyzer during the first test. The remaining eight only worked on channel 6. Two days later, the same test was repeated, and all of the blue boxes now worked with all channels. More tests were conducted at Aberdeen Proving Grounds (APG) with a new signal amplifier in the Multigas Analyzer. The

amplifier and the blue boxes were incompatible. The signal from the Multigas Analyzer needs to be amplified for the LabVIEW program to perform properly. This problem will be addressed during the next quarter.

A method was developed to determine TNT reduction products in an alkaline solution (pH 12). The method was developed using a Dionex ion chromatograph with a PAX-500 column. A conductivity detector and a UV detector (wavelengths 244 and 490) were used to detect the constituents when basic eluants were used. Only the UV detector was used with the acidic eluants. The eluants used ranged from approximately a pH of 2 up to 13. Three peaks were resolved but were not identified. None of the three peaks was 2,4 dinitrobenzene and 2,6 dinitrobenzene.

APRIL 1 – JUNE 30, 1995

Work began on developing a system to detect lower levels of HCl in the environment. Efforts are underway using peristaltic pumps in order to decrease the liquid flow rate by a factor of ten. The desired result should then be obtained.

Attempts were made to get equipment operational for an absorption study. Several acid gases will be used to test a number of different types of tubing. The tests will determine which types of tubing, if any, are best for environmental sampling of toxic acid gases (i.e. HCl, HF, HBr and HI). HCl data has been gathered.

HF monitors were prepared and used for a field trip to Chesapeake Beach.

JULY 1 - SEPT 30, 1995

Work continued on developing a system to detect low levels of hydrogen chloride (HCl) gas and aerosol real time in the atmosphere. A peristallic pump was used to decrease the liquid flow rate by ten-fold. The goal to lower the detection limit by a factor of 10 was successful. A VICI calibrator was used to produce low levels of HCl in order to validate the new low level system. Impinger results were compared with low level HCl Multigas Analyzer apparatus. The results showed excellent agreement between the two sampling methods. More work is needed to reduce the response time of the new system.

Two field studies were conducted at Airbase 3 at APG (Aberdeen Proving Grounds) in order to compete work on the LabVIEW□ program used in the Live Fire program. The first study was designed to address and correct signal problems. During the second study, all six channels (chloride, pH, bromide, iodide, fluoride, and cyanide) of the Multigas Analyzer were configured and calibrated; all of the data were taken into the new LabVIEW□ program. Once this was completed, a burn was initiated. The fluoride channel specifically was used since Hydrogen Fluoride (HF) was the only expected gas to be detected. The data from the burn were collected through LabVIEW. The system worked, but noise levels were unacceptable. This problem will be addressed.

The last part of the quarter was spent preparing for a field test to take place on the *Ex-USS Shadwell*, Mobile, AL. HF readings will be taken during the study. The monitors were prepared and shipped to the site. Dataloggers were prepared for recording during the burns. The field test will take place September 20 through October 7, 1995.

OCTOBER 1 - DECEMBER 31, 1995

Efforts during the first month of the quarter were primarily focused on field work. HF (hydrogen fluoride) measurements were taken aboard the Ex-*USS Shadwell* in Mobile, AL. Engine compartment fires were set and extinguished using a halon alternative. The tests were conducted over the period 20 Sep 95 through 14 Oct 95. Six real-time HF monitors developed in house by USACHPPM were used to gather the data. New dataloggers were used to gather data from the monitors for the first time in the field. Work during the remainder of the month was devoted to clean up of instruments and preparation for tests conducted in the lab.

All seven of the HF monitors used aboard the Ex-*USS Shadwell* were evaluated in laboratory during the following month. All seven operated properly. FM200, a fire suppression agent, was obtained from Aberdeen to conduct tests studying the effect of the compound on the fluoride electrode. The FM200 did not effect the fluoride electrode. A manual for the HF monitor is being written and is near completion. Once completed, it will be submitted to the Army as a technical report.

Work also began to determine a method for capturing aerosol from a smoke generator.

A trip was made to Aberdeen Proving Ground, Edgewood area, in an attempt to collect aerosolized fog oil from an Army smoke generator. The method of sample collection was not efficient and a new way of sampling needs to be determined. Once determined, another attempt will be made to collect the aerosolized fog oil.

JANUARY 1 - MARCH 31, 1996

Toxic Gas Monitors were prepared for testing aboard the Ex-*USS Shadwell*. The monitors were set up to test for HF and HBr. A new laptop computer was configured to support data collection on board the ship. Data loggers from the previous trip to the ship were modified to collect data from two channels and were then tested in the lab. Several sets of instructions were prepared to explain the various tasks to be performed in the field. A procedure for operating the DX-100 ion chromatograph was also prepared.

APRIL 1 - JUNE 30, 1996

Funding for this project has ended. No further work will be performed on this task.

Task Order EQ-5



GEO-CENTERS, INC.

Title: Plant Uptake of RDX and TNT
Task Number: EQ-5 (2533-011)

This task requires technical scientific support for basic and applied research on the development of analytical methods for extraction and qualification of RDX and TNT from plant tissues.

AUGUST 1-31, 1993

Work was initiated on a study to investigate the bioconcentration of RDX and TNT in commercial and home garden crops. This study is simulating irrigation of crop plants indigenous to Cornhusker Army Ammunition Plant (CAAP) with contaminated water. Methods development of plant extraction procedures were begun to determine interferences by plant compounds. Extracts were analyzed from untreated crop plants for background matrices that potentially may interfere with HPLC analyses. A solvent peak was found to interfere with the TNT peak and a plant pigment peak was found to interfere with the RDX peak. Solvent ratios will be adjusted to try to correct this problem. A meeting was held the beginning of August to discuss collection of soils from CAAP. Soil was collected per instructions that were left with CAAP personnel during a previous site visit. A 20ft. X 30ft. X 12in. deep area (600 cubic feet) was collected from an active alfalfa field at CAAP. The soil was placed in eighty 55-gallon drums, sealed and shipped to APG, building E5641. The soil arrived in two shipments (30 drums and 50 drums, respectively).

SEPTEMBER 1-30, 1993

Work continued on a study to investigate the bioconcentration of RDX and TNT in commercial and home garden crops. This study is simulating irrigation of crop plants indigenous to Cornhusker Army Ammunition Plant (CAAP) with contaminated water. Methods development of plant extraction procedures were continued to determine interferences by plant compounds. Solvent and plant pigment peaks had previously been shown to interfere with RDX and TNT peaks in HPLC analyses. Plant extracts were dried and reconstituted. HPLC analyses are currently being performed using different solvent ratios in an effort to get better separation of peaks. Soil previously collected and transported to APG for this study was dried and sieved. Paperwork was processed to obtain clearances to receive RDX and TNT for the study. The first experiment will begin when all the paperwork is cleared and RDX and TNT is shipped to building E5641.

OCTOBER 1-31, 1993

Work continued on acquiring the components and processing the paperwork.

NOVEMBER 1-30, 1993

Work continued on acquiring the components and processing the paperwork.

DECEMBER 1-31, 1993

Work was diverted to higher priority tasks this month in coordination with the Government. Work is expected to resume next month.

JANUARY 1-31, 1994

Work was initiated on a study to investigate the bioconcentration of RDX and TNT in commercial and home garden crops. This study is simulating irrigation of crop plants indigenous to Cornhusker Army Ammunition Plant (CAAP) with contaminated water. Methods development of plant extraction procedures were begun to determine interferences by plant compounds. Extracts were analyzed from untreated crop plants for background matrices that potentially may interfere with HPLC analyses. A solvent peak was found to interfere with the TNT peak and a plant pigment peak was found to interfere with the RDX peak. Solvent ratios will be adjusted to try to correct this problem. A meeting was held the beginning of August to discuss collection of soils from CAAP. Soil was collected per instructions that were left during our site visit in June. A 20ft. X 30ft. X 12in. deep area (600 cubic feet) was collected from an active alfalfa field at CAAP. The soil was placed in 80, 55-gallon drums, sealed and shipped to APG, building E5641. The soil arrived in two shipments (30 drums and 50 drums, respectively) on 18 August.

FEBRUARY 1-28, 1994

Work continued on a study to investigate the bioconcentration of RDX and TNT in commercial and home garden crops. This study is simulating irrigation of crop plants indigenous to Cornhusker Army Ammunition Plant (CAAP) with contaminated water. Preliminary methods development of plant extraction procedures have been completed. Soil previously collected and transported to APG for this study was dried and sieved for phase I of the experiment. RDX and TNT to be used for analytical standards were ordered. The safety SOP for receiving small amounts of explosives was revised and submitted for review. An experimental procedure and statistical design were prepared. Greenhouse facilities and necessary equipment were also being prepared for the experiment.

MARCH 1-31, 1994

Work continued on a study to investigate the bioconcentration of RDX and TNT in commercial and home garden crops. This study is simulating irrigation of crop plants indigenous to Cornhusker Army Ammunition Plant (CAAP) with contaminated water. Preliminary methods development of plant extraction procedures have been completed. Soil previously collected and transported to APG for this study was dried and sieved for phase I of the experiment. RDX and TNT to be used for analytical standards were ordered. The safety SOP for receiving small amounts of explosives was received from review, edited, and re-submitted to the Safety Office. Crystalline standards of RDX, HMX, TNT, 2,4-DNT, 2,6-DNT were received and dissolved in solvent. These standard materials will be used to verify concentrations of the chemicals in plant tissues during HPLC analyses. The experimental procedure and statistical design were revised. Greenhouse facilities and necessary equipment were also prepared for the experiment.

APRIL 1-30, 1994

Work continued on a study to investigate the bioconcentration of RDX and TNT in commercial and home garden crops. This study is simulating irrigation of crop plants indigenous to Cornhusker Army Ammunition Plant (CAAP) with contaminated water. Soil previously collected and transported to APG for this study was dried and sieved. A sub-sample was taken for determination of dry fraction content and water-holding capacity. The analysis is currently in progress. Revisions of the safety SOP for receiving small amounts of explosives was approved by the Safety Office. The quantity of alfalfa plants needed per pot to simulate field planting rate was determined.

MAY 1-31, 1994

Work continued on a study to investigate the bioconcentration of RDX and TNT in commercial and home garden crops.

JUNE 1-30, 1994

Work continued on a study to investigate the bioconcentration of RDX and TNT in commercial and home garden crops. This study is simulating irrigation of crop plants indigenous to Cornhusker Army Ammunition Plant (CAMP) with water contaminated with RDX and TNT. Soil previously collected at CAMP and transported to APG were sieved and dried. Containers were filled with soil and prepared for experimentation with four crop species. Calculations were made to determine the watering regime to simulate irrigation. Pots will be watered to the water-holding capacity (WAC) which is 16% according to previous calculations. Plot plans were designed and drawn and stock solutions were prepared. Corn, soybean, radish, and tomato seeds were planted. Treatment levels will include a control (distilled water),2 µg/L RDX, 20 µg/L RDX, 100 µg/L RDX, 2 µg/L TNT, 100 µg/L TNT, 800µg/L TNT, and 100 µg/L RDX + 800µg/L TNT.

JULY 1- SEPTEMBER 30, 1994 (Government approved Quarterly Progress Reports in lieu of Monthly Progress Reports)

Work continued on a study to investigate the bioconcentration of RDX and TNT in commercial and home garden crops. This study is simulating irrigation of crop plants indigenous to Cornhusker Army Ammunition Plant (CAMP) with contaminated water. Corn, soybean, radish, and tomato seeds were planted July 1, in previously prepared containers. Containers were watered to the water-holding capacity (WAC) which is 16% according to previous calculations. Treatment levels included a control (distilled water), 2µg/L RDX, 20 µg/L RDX, 100 µg/L RDX, 2 µg/L TNT, 100 µg/L TNT, 800µgg/L TNT, and 100 µg/L RDX + 800µg/L TNT. Plants were thinned after two weeks to two per pot. Pots were watered two to three times per week to maintain moisture near WHC. Seedlings have been fertilized twice since planting. A preliminary study with radishes was terminated. Thirty days after planting, radish roots were harvested, rinsed, weighted, sectioned and lyophilized for 48 hrs. At least one sample per treatment was harvested. Samples were reweighed following lyophilization and stored in a

desiccator for one day prior to shipping. The tissues were shipped to an analytical lab for HPLC analysis of explosive compounds. These analyses will be used to test the methodology and to determine traces of explosive residue in edible tissues. Irrigation solutions as well as purified standard solutions were also shipped for analysis. A full experimental set of radishes, planted August 1, were harvested in the beginning of September. Tissues, irrigation solutions and soils will be analyzed for RDX and TNT.

OCTOBER 1 - DECEMBER 31,1994

Due to priority efforts on related tasks, no work was conducted in October, November, or December 1994 on this task.

JANUARY 1 - MARCH 31, 1995

Work continued on a study to investigate the bioconcentration of RDX and TNT in commercial and home garden crops. This study is simulating irrigation of crop plants indigenous to Cornhusker Army Ammunition Plant (CAAP) with contaminated water. Corn stover, soybean seed, radish root, tomato fruit, bush bean fruit, lettuce leaves, and alfalfa shoots have been harvested from plants grown in soil irrigated with water containing distilled water, 2 μg/L RDX, 20 μg/L RDX, 100 μg/L RDX, 2 μg/L TNT, 100 μg/L TNT, 800 μg/L TNT, or 100 μg/L RDX + 800 μg/L TNT.

Previously lyophilized tissues of radish, soybean, and tomato are being analyzed for RDX and TNT concentration. Lyophilized corn seeds and stover (leaves and stems), bush bean fruit (seeds and pods), and lettuce leaves are presently being stored in a freezer in the dark until analysis for RDX and TNT.

Alfalfa plants were cut to approximately 5 mm above the soil line. Shoot tissue was immediately weighed, stored on dry ice, and frozen in preparation for lyophilization. The 5 mm of alfalfa plant remaining in each previously-harvested pot is being allowed to grow back for a second harvest and continues to be irrigated with treatment solutions. The second harvest will occur in early April 1995.

Yield and biomass (dry weight) of tomato fruit, bush bean fruit, corn stover, soybean seeds, lettuce leaves, and radish roots were calculated. Analysis of variance (ANOVA) showed significantly ($p=0.05$) reduced yield and biomass of tomato, bush bean, corn, and soybean tissues from plants irrigated with the 100 μg/L RDX + 800 μg/L TNT treatment compared to control plants. Lettuce and radish were unaffected by treatment level.

Total soil loading of RDX and TNT (mg contaminant/kg soil) was calculated for each pot per treatment per species. Soil loading was greatest for tomato, bush bean, corn, and soybean, and least for lettuce and radish.

APRIL 1 – JUNE 30, 1995

Work continued on a study to investigate the bioconcentration of RDX and TNT in commercial and home garden crops. This study involves simulating irrigation of crop plants indigenous to Cornhusker Army Ammunition Plant (CAAP) with contaminated water. Corn stover (leaves and stems), soybean seed, radish root, tomato fruit, bush bean seeds and pods, lettuce leaves, and alfalfa shoots (twice @ 60 and 30 days) have been harvested from plants grown in soil irrigated with distilled water, 2 µg/L RDX, 20 µg/L RDX,100 µg/L RDX, 2 µg/L TNT, 100 µg/L TNT, 800 µg/L TNT, or 100 µg/L RDX + 800 µg/L TNT.

Previously lyophilized tissues of radish, soybean, tomato, and corn have been analyzed for RDX and TNT by HPLC. Data are currently undergoing statistical and quality assurance analyses. Lettuce tissue is currently being analyzed for RDX and TNT. Bush bean and alfalfa tissues have been lyophilized and are being stored frozen awaiting analysis. Yield and biomass (dry weight) of all tissues have been statistically analyzed. Analysis of variance (ANOVA) showed significantly ($p=0.05$) reduced yield and biomass of tomato, bush bean, corn, and soybean tissues from plants irrigated with 100 µg/L RDX + 800 µg/L TNT compared to control plants. Lettuce, radish, and alfalfa yield and biomass were unaffected by treatment level.

Total soil loading (mg RDX and TNT/kg soil) was calculated for each pot per treatment per species. Soil loading, presented as greatest to least for all species was tomato > corn > alfalfa > soybean > bush bean > lettuce > radish. Loading was a function of the duration of the life cycle (planting to harvest) for each species.

JULY 1 - SEPT 30, 1995

Due to other government priorities, no work was performed on this task during this reporting period.

OCTOBER 1 - DECEMBER 31, 1995

Work continued on a study to investigate the bioconcentration of RDX and TNT in commercial and home garden crops. This study involves simulated irrigation of crop plants indigenous to Cornhusker Army Ammunition Plant (CAAP) with contaminated water. HPLC analytical data of uptake of RDX and TNT in radish root, soybean seed, tomato fruit, bush bean fruit, and corn stover (leaves and stems) were quality assured and analyzed statistically. Data indicate that RDX was taken up into corn stover and lettuce leaves. However, concentrations found in plant tissues were not significantly greater than concentrations loaded onto the soil. Tomato, radish, soybean, and bush bean data did not indicate significant uptake of RDX into the plant tissue from the soil. Results from analyses of TNT in plant tissues have shown no significant uptake of TNT in corn, tomato, radish, lettuce, bush bean, or soybean. Data of the crop species were charted in histograms showing the relationship between irrigation treatment, and yield and biomass of edible plant parts. These charts were incorporated into a poster presented at the national meeting of the American Phytopathological Society (APS).

Chemical analysis of irrigation water used in the study was conducted. Samples of water from test wells at CAAP were used as reference materials for the irrigation water analysis. There was concern when little or no TNT was found in irrigation water analyzed via HPLC. It was hypothesized that either the TNT in the irrigation solutions degraded over time, since it had been at least eight months since the solutions were prepared and refrigerated in the dark, or the nutrient solution added to the water was binding the TNT and rendering it insoluble. Fresh solutions of TNT were prepared, one with 800 ppb TNT with nutrient solution and one with 800 ppb TNT without nutrient solution. Analyses were performed and results showed that TNT peaks were found in HPLC chromatograms from both treatments. The peak from the water with nutrient solution appears to be smaller than the peak from water without nutrients. These results indicate that TNT may be partially bound by the nutrient solution, but enough remained in solution for satisfactory treatment. Peak areas are being integrated. When concentrations have been calculated, the estimated soil loading will be adjusted accordingly.

Chemical analysis of freshly-made irrigation water at 800 ppb TNT and 100 ppb RDX was also conducted. TNT and RDX were found in the water at approximately 20% less than original ppb levels. However, the solutions in the study were made from field-grade munitions so they were not expected to be pure. Original stock solutions of 30,000 ppm TNT and 10,000 ppm RDX will be diluted with purified water to the appropriate treatment levels, with and without nutrient solution, and analyzed by HPLC. These analyses will confirm the precise concentrations of explosives at Day 1 and Day 7, with and without nutrient solution.

A methodology was developed to analyze soil used in irrigation studies for TNT and RDX concentrations via HPLC. Analysis results are forthcoming. The program manager of CAAP and the Army Environmental Center (AEC) were briefed at appropriate times during the study. A draft interim report was submitted to AEC and the final report is nearly complete.

JANUARY 1 - MARCH 31, 1996

Work continued on a study to investigate the bioconcentration of RDX and TNT in commercial and home garden crops. This study involves simulating irrigation of crop plants indigenous to Cornhusker Army Ammunition Plant (CAAP) with contaminated water. The draft final report of the study is currently under review by the Army Environmental Center (AEC). A meeting will be held with AEC personnel to discuss and revise the report. In the interim, the draft is at the editing stage and abstracts have been presented to the CAAP study at the Triservice Environmental Technology Workshop (May 1996) and the American Phytopathological Society annual meeting (July 1996).

APRIL 1 - JUNE 30, 1996

A final report on methodology developed to analyze soil used in irrigation studies for TNT and RDX concentrations via HPLC was submitted to the Army Environmental Center (AEC).

JULY 1 - SEPTEMBER 30, 1996

Due to priority in other areas, no work was performed on this task during this reporting period.

OCTOBER 1 - DECEMBER 31, 1996

Due to priority in other areas, no work was performed on this task during this reporting period.

JANUARY 1 - MARCH 31, 1997

Due to priority in other areas, no work was performed on this task during this reporting period.

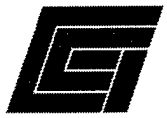
APRIL 1 - JUNE 30, 1997

Due to priority in other areas, no work was performed on this task during this reporting period.

JULY 1- SEPTEMBER 30, 1997

Due to priority in other areas, no work was performed on this task during this reporting period. No other work is expected to be undertaken under this Task Order.

Task Order EQ-6



GEO-CENTERS, INC.

Title: Environmental Chemistry, Composting
Task Number: EQ-6 (2533-012)

AUGUST 1-31, 1993

Repeated the female C-14/TNT portion of the study due to observations concerning significantly higher rates of excretion of the radiolabel in the female urine. Have completed sample oxidation of tissues for first female run, and in process of oxidizing tissue samples for the second female run.

Beginning b-glucoronidase extraction of metabolites in urine samples from selected 14C-TNT articles. We are interested in differentiating metabolic products in the 14C-TNT and 14C-TNT/compost test articles.

Have begun long-term acute study with male rats and the 14C-TNT test article. Will begin the long term acute study with the 14C-TNT/Compost test article in the beginning of September.

SEPTEMBER 1-30, 1993

Work continued on long-term acute study with the male Fisher F- 344/VAF Plus rats and the 14C-TNT/Compost test article. Currently on day 28 of the 100-day proposed study.

Completed the sampling portion of the long-term acute study with the 14C-TNT test article. We are in the process of oxidizing samples.

Submitted an abstract for a manuscript entitled "Bioavailability of TNT in composts of TNT-contaminated soils" to be presented in poster format at the Society of Toxicology 1994 annual meeting.

Began to write the Materials and Methods section for manuscript which will be submitted to an appropriate journal in the field.

OCTOBER 1-31, 1993

Work continued on long-term acute study with the male Fisher F- 344/VAF Plus rats and the 14C-TNT/Compost test article. Samples through day 50 (both sampling and analysis by tissue oxidation) were completed for the long-term acute study involving the TNT/compost test article. Oxidation of the 14C-TNT test article tissue samples was completed this month. Will begin data analysis in the near future. Work continues on developing a standard protocol for column chromatography and subsequent HPLC of rat urine samples containing radiolabeled TNT metabolites. We are using the nonionic resins XAD-2 and XAD-4 (Sigma) for this procedure. Work continues on writing "Materials and Methods" section for manuscript.

NOVEMBER 1-30, 1993

Work continued on long-term acute study with the male Fisher F- 344/VAF Plus rats and the ^{14}C-TNT/Compost test article. Samples through day 75 (both sampling and analysis by tissue oxidation) were completed for the long-term acute study involving the TNT/compost test article. Work continues on developing a standard protocol for column chromatography and subsequent HPLC of rat urine samples containing radiolabeled TNT metabolites. Abstract submitted October 1, 1993 was accepted for 1994 Society of Toxicology meeting. Presentation will be in poster format. First draft of materials and methods section for manuscript and poster was completed.

DECEMBER 1-31, 1993

Work continued on long-term acute study with the male Fisher F- 344/VAF Plus rats and the ^{14}C-TNT/Compost test article. Due to notification of task termination at the end of this month, attempted to complete all remaining lab work on task to include all necropsies, sample oxidation, and analysis by liquid scintillation counting. Will require approximately 4 hours additional time to complete work. Will also require approximately 8 additional hours to complete dosing of rats and urine collection from TNT and compost treated rats. Need clean urine for metabolite determination, which is the last experiment necessary for work to be submitted for publication. Manuscript write-up will be handled by Government PI, with technical support available to clarify procedural questions on an as-needed basis. Methods section of paper has been completed and submitted to PI for review.

JANUARY 1-31, 1994

This task has been completed.

Task Order EQ-7



GEO-CENTERS, INC.

Title: The Environmental Fate of Smoke/Obscurants
Task Number: EQ-7 (2533-013)

This task requires technical scientific support for basic and applied research on the environmental fate and effects of candidate smokes/obscurants in terrestrial ecosystems.

OCTOBER 1-31, 1993

Work was performed to determine the effect of terephthalic acid (TPA) smoke on field planted tree species. TPA smoke is a candidate for replacement of HC smoke. Foliar injury data from a previous field study were tabulated and statistically analyzed by analysis of variance. Injury was not statistically greater on treated vs. untreated seedlings of black locust, sweetgum, black cherry, and white pine following two exposures, although slight injury occurred on black cherry leaves. Black cherry and black locust seedlings were much less sensitive to TPA smoke than to HC smoke exposures in previous studies.

NOVEMBER 1-30, 1993

Work continued on the analysis of data to determine the effect of terephthalic acid (TPA) smoke on field planted tree species. Data were sorted by date and analyzed by analysis of variance to determine if injury developed and/or decreased over time. Results showed that slight injury occurred immediately after exposure in one of the plots and decreased with time. However, it was concluded that this injury was caused by the burn of a malfunctioning canister and not directly by TPA smoke. TPA smoke injury was not statistically different among treatments when sorted by date.

DECEMBER 1-31, 1993

Work continued on the analysis of data to determine the effect of terephthalic acid (TPA) smoke, a potential replacement for HC smoke, on field planted tree species. An interim report of research results was prepared, reviewed, and edited. Work will now begin on developing a peer-reviewed journal article that evaluates the phytotoxicity of TPA smoke. Results will be compared to those compiled for HC smoke in previous tests.

JANUARY 1-31, 1994

Work continued on the analysis of data to determine the effect of terephthalic acid (TPA) smoke, a potential replacement for HC smoke, on field planted tree species. An interim report of research results was completed. Results showed that TPA smoke is much less toxic than HC smoke to the same forest tree species. Review of the interim report was begun to develop a peer-reviewed journal article that compares the phytotoxicity of TPA smoke to that of HC smoke determined in previous tests.

FEBRUARY 1-28, 1994

Work continued on the analysis of data to determine the effect of terephthalic acid (TPA) smoke, a potential replacement for HC smoke, on field planted tree species. An interim report of research results was completed. Results showed that TPA smoke is much less toxic than HC smoke to the same forest tree species. Review of the interim report was completed. Preparation of a final report will begin. A peer-reviewed journal article will be developed from this report that compares the phytotoxicity of TPA smoke to that of HC smoke determined in previous tests.

MARCH 1-31, 1994

Work continued on the effect of terephthalic acid (TPA) smoke, a potential replacement for HC smoke, on field planted tree species. A research protocol was written which described the background and methods for the study of phytotoxicity of TPA smoke. Preparation of a final report will begin. A peer-reviewed journal article will be developed from this report that compares the phytotoxicity of TPA smoke to that of HC smoke determined in previous tests.

APRIL 1-30, 1994

Work continued on the effect of terephthalic acid (TPA) smoke, a potential replacement for HC smoke, on field planted tree species. Preparation of an interim report has begun. The open-top field chamber site was visited and assessed for potential work needed to prepare the site for FY94 field experiments. Replacement tree seedlings for this work were received.

MAY 1-31, 1994

Work continued on the effect of terephthalic acid (TPA) smoke, a potential replacement for HC smoke, on field planted tree species.

JUNE 1-30, 1994

Works continued on the effect of terrephthallic acid (TPA) smoke, a potential replacement for HC smoke, on field planted tree species. The open-top field chamber site was visited. Black cherry seedlings were replaced and the site was inspected. An abstract was submitted to the Smoke/Obscurants Symposium XVIII. This presentation will compare and contrast results of the TPA smoke experiments with those of the previously completed HC smoke experiments.

JULY 1- SEPTEMBER 30, 1994 (Government approved Quarterly Progress Reports in lieu of Monthly Progress Reports)

Works continued on the effect of terrephthallic acid (TPA) smoke, a potential replacement for HC smoke, on field planted tree species. The open-top field chamber site was visited. Black cherry seedlings were replaced and the site was inspected. Supplies were moved from storage building to the site. An oral presentation entitled "*Phytotoxicity of Hexachloroethane Smoke and Terephthalic Acid Smoke*" by M. Sadusky, M. Simini, J.M. Skelly,



R.T. Checkai, and R.S. Wentsel was presented by M. Sadusky at the Smoke/Obscurants Symposium XVIII, August 23-26, Eglin Air Force Base, Ft. Walton Beach, FL. This paper presented the potential phytotoxicity of TPA smoke compared to HC smoke based on previous work.

OCTOBER 1 - DECEMBER 31,1994

Works continued on the effect of terephthalic acid (TPA) smoke, a potential replacement for HC smoke, on field planted tree species. An on-line search was performed for chemical and toxicological information on terephthalic acid (TPA) smoke. This information was compiled to serve as a database for any future work that may be conducted using TPA. The presentation given by M. Sadusky at the Smoke Symposium XVIII entitled "Phytotoxicity of Hexachloroethane Smoke and Terephthalic Acid Smoke" by M.C. Sadusky, M. Simini, J.M. Skelly, R.T. Checkai, and R.S. Wentsel, will be published in the proceedings. This paper presented the observed non-toxic effects of TPA smoke to tree seedlings compared to phytotoxicity of hexachloroethane smoke seen in the investigators' previous work.

On 29 November 1994, a briefing by ERDEC's Product Manager for Smoke/Obscurants (PM Smoke) was attended. The briefing summarized all the smoke program developments and production involving PM Smoke. It was beneficial to learn about the larger systems for which smokes are developed in addition to developing points-of-contact for the smokes that are used in our toxicological testing.

JANUARY 1 - MARCH 31, 1995

No work was performed on this task during this reporting period.

APRIL 1 – JUNE 30, 1995

No work was performed on this task during this reporting period.

JULY 1 - SEPT 30, 1995

No work was performed on this task during this reporting period.

OCTOBER 1 - DECEMBER 31, 1995

Due to priority assigned to other task areas, no work was performed on this task during this reporting period.

JANUARY 1 - MARCH 31, 1996

Previous studies on the fate and effects of U.S. Army smokes were reviewed and a paper is being written on the relative phytotoxicity of terephthalic acid smoke and hexachloroethane smoke. An abstract written about the comparative studies was submitted for poster presentation at the Smoke/Obscurant Symposium XIX (June 1996).

APRIL 1 - JUNE 30, 1996

Due to priority assigned to other task areas, no work was performed on this task during this reporting period.

JULY 1 - SEPTEMBER 30, 1996

Work continued on the effect of terephthalic acid (TPA) smoke, a potential replacement for hexachloroethane (HC) smoke, on field-grown tree species. A repeated measured analysis of variance was performed on the data to determine the effect of foliar injury caused by TPA smoke or HC smoke (from previous studies) over time. Results indicated that HC is chronically toxic to black locust and acutely toxic to black cherry seedlings when exposed to high levels of smoke over an eight week period during the summer months. TA is virtually non-phytotoxic to the same tree species during approximately the same time period. Results from this study were incorporated into a draft manuscript that will be submitted to a peer-reviewed journal for publication.

OCTOBER 1 - DECEMBER 31, 1996

Due to priority in other areas, no work was performed on this task during this reporting period.

JANUARY 1 - MARCH 31, 1997

Due to priority in other areas, no work was performed on this task during this reporting period.

APRIL 1 - JUNE 30, 1997

Due to priority in other areas, no work was performed on this task during this reporting period.

JULY 1- SEPTEMBER 30, 1997

Due to priority in other areas, no work was performed on this task during this reporting period. It is expected that no additional work will be undertaken in execution of this Task Order.

Task Order OH-1



GEO-CENTERS, INC.

Title: Evaluation of Diagnostic Media for Testing Water Quality
Task Number: OH-1 (2533-005)

This task requires technical scientific support for basic and applied research to develop a rapid field test kit for water quality determination.

MARCH 1-30, 1993

Awaiting personnel assignment and technical coordination.

APRIL 1-30, 1993

This position is unfilled at this time.

MAY 1-31, 1993

This position is unfilled at this time.

JUNE 1-30, 1993

This position is unfilled at this time.

JULY 1-31, 1993

This position is unfilled at this time.

AUGUST 1-31, 1993

This position is unfilled at this time. It is anticipated that this task will be cancelled as a result of a shift in emphasis by the Army.

SEPTEMBER 1-30, 1993

Due to re-programming of funds by USABRDL, this task has been terminated. No funds have been expended. Future reports will not include reference to this task.

Task Order OH-2



GEO-CENTERS, INC.

Title: Occupational Health Effects of Army Chemicals
Task Number: OH-2 (2533-006)

This task involves providing technical review of data and documents which include, but are not limited to, final reports produced outside USABRDL and in-house reports produced by USABRDL and/or GEO-CENTERS, INC. personnel.

MARCH 1-30, 1993

Review of draft USABRDL technical report entitled "Evaluation of Test Strips for Determining Inorganic contaminants in Field Water" was completed.

Work on investigation of the effects on soldiers of exposure to metals via gun and rocket exhaust continued with the commencement of this task. This project involves both in-house and extramural investigators. The objective of this project is to examine the exposures and review the health effects data base to determine if there is a need for specific military exposure standards for any of the metals identified: Al, At, Ba, Cu, Pb, Sn, and Zn. An extensive data base on toxicity of the selected metals has been compiled and reviewed. A report is being prepared for aluminum, antimony, barium, cadmium, tin, and zinc, documenting the exposure data and reviewing the toxicity data.

Work continued on development of a physiologically-based pharmacokinetics (PB-PK) model for the presence of lead in humans and animals. The goal of this project is to apply a PB-PK model to the Army's air and blood lead data collected during artillery exercises to be able to predict the crewmen's responses in future exercises and to avoid hazardous blood lead concentrations. Work to date has shown that a modified version of a published model by J. Bert et al. [*Environ. Res.* **48**:117-27 (1989)] fits well with blood lead (PbB) and air lead (PbA) data in the recent literature. However, it tends to over-predict the PbB levels resulting from Howitzer firing exercises.

Three Planavin manuscripts were reviewed and final revisions made; they have been returned to the Editor of *J. Amer. Coll. Toxicol.* for publication.

A draft manuscript received from Dr. Bucci, the Principal Investigator for contract DAMD17-91-C-1088, titled "A 90-Day Oral Toxicity of Diisopropyl Methylphosphonate (DIMP) in Mink", has been reviewed and returned to Dr. Bucci for final review prior to BRDL in-house manuscript review.

Commenced work on tasking entitled "White Phosphorus LOEL;" which was combined into the existing task order and will be reported accordingly. Literature searches were initiated; review of papers obtained to date is in progress.

Continued with literature acquisition for the review of Agent GA (Tabun).

APRIL 1-30, 1993

Reviews of the following technical/final reports and journal articles were completed: (1) contractor final report, "Experimental Design and Instrumentation for a Field Experiment" (PI: Beningus/USEPA), (2) draft technical report, "Reverse Osmosis Water Purification Unit: Efficacy of Cartridge Filters for Removal of Bacteria and Protozoan Custs when RO Elements are Bypassed" (PI: Schaub/USABRDL), and (3) paper submitted for the Halon Alternatives Conference, "Development of a Multigas Analyzer" (Authors: S.H. Hoke/USABRDL and C. Herud/USACSTA).

Work continued on development of physiologically-based pharmacokinetic (PB-PK) model of blood lead concentrations after exposure to airborne lead. Applied data collected from firing of JAVELIN missiles to the modified Bert model (*Environ. Res.* 48(1):117-28) and the Bernard model (*Health Phys.* 32:44-6) which is the accepted regulatory model for lead absorption. Both models predicted similar elevation of blood lead levels after one firing episode, but this level would not approach the blood lead concentration that calls for any regulatory action under current or proposed Federal standards for lead.

Assessment of the data base on health effects of zinc chloride smoke produced by HC smoke munitions continued. The bibliography on health effects of zinc chloride inhalation has been updated. The author of a NIOSH study of zinc chloride smoke used in fire training has provided information useful in documenting effects of exposure to this smoke.

Review of short-term high-level exposure to select metals continued. Recent additions to the literature on effects of inhaling the selected metals have been reviewed and added to the data base.

A report on the status of the two manuscripts being prepared under MRDC Contract DAMD17-91-C-1088 titled "A 90-Day Oral Toxicity Study and a 5-Day Metabolic Study of Diisopropyl Methylphosphonate (DIMP) in Mink" was submitted on 14 April 1993. Continued with the literature acquisition for the review of Agent GA (Tabun).

The task order to evaluate data for potential establishment of a White Phosphorous LOEL was completed 28 April 1993.

MAY 1-31, 1993

Work continued on development of physiologically-based pharmacokinetic (PB-PK) model of blood lead concentrations after exposure to airborne lead. A recently-published model of the kinetics of lead disposition in humans (O'Flaherty, E.J. Toxicol. Appl. Pharmacol. 118:16-29 (1993)) has been retrieved. It will be compared to the model currently being used for the project Physiologically based Pharmacokinetic (PB-PK) Model of Blood Lead Concentrations after Exposure to Airborne Lead.

Assessment of the data base on health effects of zinc chloride smoke produced by HC smoke munitions continued with updating of the bibliographies and retrieval and review of

relevant documents.

A memo proposing assistance in preparing environmental assessments and environmental impact statements for the proposed move of the smoke training mission from Fort McClellan to Fort Leonard Wood was completed and delivered to the client.

Two manuscripts titled "A 90-Day Oral Toxicity Study of Diisopropyl Methylphosphonate (DIMP) in Mink" and "A Pharmacokinetic Study of Diisopropyl Methylphosphonate (DIMP) in Mink and Rats" were completed and approved for publication. They were submitted to the journals *Fundamental and Applied Toxicology* and *Xenobiotica* respectively.

An abstract titled "*Hazard Evaluation of Army Munitions Compounds in the Environment*" was prepared for the Fifth European ISSX Meeting in Tours, France.

Literature searching continued for the review of Agent GA (Tabun).

JUNE 1-30, 1993

This task involves providing technical review of data and documents which include, but are not limited to, final reports produced outside USABRDL and in-house reports produced by USABRDL and/or GEO-CENTERS, Inc. personnel.

Work continued on development of physiologically-based pharmacokinetic (PB-PK) model of blood lead concentrations after exposure to airborne lead. The results of modeling the physiological response (blood lead concentration, PbB) to the air lead concentrations (PbA) measured during testing of the JAVELIN missile were discussed with LTC Langford, Chief of the Occupational Health Research Branch of USABRDL. He indicated that more data from testing of this weapon would soon be available to apply to the model. Current model runs indicate that, although PbA levels may exceed the regulatory limits, PbB would not approach the action level that indicates the danger of long-term ill effects from exposure to lead.

USABRDL Technical Report No 9303, "Review of Short-term High-level Exposure to Metals: Antimony, Barium, and Cadmium," has been prepared and is going through the USABRDL technical review process.

Work on the assessment of the data base on Health Effects of Zinc Chloride Smoke has been resumed.

Work was initiated on preparing the paper "Hazard Evaluation of Army Munitions Compounds in the Environment" for presentation at the Fifth Environmental ISSX Meeting in France.

A final revision of the manuscript titled "Genotoxicity of the Phosphoramidate Agent Tabun (GA)" was completed.

JULY 1-31, 1993

USABRDL Technical Report No 9303, "Review of Short-term High-level Exposure to Metals: Antimony, Barium, and Cadmium," has been through the USABRDL technical review process. Several sections were extensively re-written in response to reviewers' comments. The report has been approved by all technical reviewers and has been authorized for printing by the Commander, BRDL.

Work on the assessment of the database on health effects of zinc chloride smoke produced by HC smoke munitions is continuing. Several new references have been reviewed and will be assimilated into the final document.

Work continues on preparing the paper "Hazard Evaluation of Army Munitions Compounds in the Environment" for presentation at the Fifth Environmental ISSX Meeting in France.

The manuscript "A 90-Day Oral Toxicity Study of Diisopropyl Methylphosphonate (DIMP) in Mink and Rats" has been accepted for publication in the journal *Fundamentals of Applied Toxicology*.

AUGUST 1-31, 1993

The report "Lead Exposures and Biological Responses in Military Weapons Systems: Effects of Long-term Exposure among U.S. Army Artillerymen" for Army Project Order No. 86PP8621 (M. H Bhattacharyya/Argonne National Laboratory) was received and reviewed during August. The recommended changes were submitted to Dr. W. D. Burrows for transmittal to the author for preparation of the distribution copies of the report.

The final report for Project Order 87PP7808, Toxicity of Red and Violet Dyes in M18 Grenade (D. L. Costa/USEPA) is overdue. This report will be reviewed upon receipt.

The modified Bert *et al.* model was applied to airborne lead exposures measured during firing of the JAVELIN missile during tests held in 1992 and 1993. A briefing package of projected blood lead elevations from the measured exposures was prepared for presentation at a briefing of the Army Infantry School concerning the need for further biological monitoring of soldiers exposed during JAVELIN exercises. Documentation of the basis for the original model and the modifications introduced in order to accommodate the characteristics of the present-day soldier population was prepared and submitted to Chief, Occupational Health Research Branch, USABRDL, along with a disk containing the computer algorithm to run the model, per request.

USABRDL Technical Report No. 9303, "Review of Short-term High-level Exposure to Metals: Antimony, Barium, and Cadmium," has been submitted for printing and distribution.

Work on the project to assess the health effects of zinc chloride is continuing. Several errors have been found and corrected in the document, some of which originated in the primary references.

Work continues on preparing the paper "Hazard Evaluation of Army Munitions Compounds in the Environment" for presentation at the Fifth Environmental ISSX Meeting in France.

The manuscript titled "A Pharmacokinetics Study of Diisopropyl Methlyphosphonate (DIMP) in Mink and Rats" is undergoing revision in conjunction with the contractor P.I. Dr. T. Bucci.

SEPTEMBER 1-30, 1993

A manuscript by W. Palmer et al., "Mutagenicity of Emissions from the M16 Rifle," was reviewed.

Work continued on the task Assessment of the Data Base n Health Effects of Zinc Chloride Smoke Produced by HC Smoke Munitions. The sections of the report concerned with chemistry of the smoke and of the mixture which produces it and with human exposure to the smoke and the smoke mix have been thoroughly revised with new tables and incorporating new references. Work is continuing on the sections dealing with pharmacokinetics, toxicology, and risk assessment.

A draft chapter, "Toxicology and Environmental Hazards," from the forthcoming book, "Organic Energetic Materials," was sent to LTC D. Caldwell, Occupational Health Research Detachment, USABRDL, Dayton, OH.

In response to a request from the Chief, Environmental Quality Research Branch, the review of exposure and toxicity of colored dyes used in smoke grenades was updated and forwarded to Dr. Maurice Weeks, Toxicology Division, USAEHA, for his use in a submission to the Committee on Toxicology, National Research Council, requesting a review of the health risks associated with those materials.

Work continued on the preparation of a manuscript from the paper from the Tours Meeting which will be published in the meeting proceedings journal *Drug Metabolism Reviews*. This paper was presented during the Fifth Environmental ISSX Meeting held in Tours, France.

OCTOBER 1-31, 1993

A meeting with LTC W. C. Roberts, Health Hazard Assessment Officer, Office of the Command Surgeon, Army Materiel Command, was held 12 October 1993 at Fort Detrick. We discussed application of the model to his project to develop a military standard for short-term, high-level exposure of soldiers to lead resulting from artillery fire. We also exchanged ideas

concerning the whole area of military lead exposures, including reproductive effects and animal and *in-vitro* tests to examine the effects of short-term, high-level exposures to lead. I provided him with a copy of the model, some runs using Army data, and supporting documentation.

Sections on pharmacokinetics and toxicity of the smoke to humans and animals were completed in October. Work is continuing on the sections of the report dealing with toxicology of the components, carcinogenicity, and risk assessment. The risk assessment section is being completely revised and greatly amplified, with new tables, new references, and a discussion of acute versus chronic hazards of exposure to HC smoke and its components.

Work continues on preparation of a manuscript from the paper presented at the Fifth Environmental ISSX Meeting held in Tours, France, last month. The paper will be published in the meeting journal *Drug Metabolism Reviews*.

Numerous requests for BRDL reports and publications have been received and sent to requestors from overseas.

The following papers were published, and reprints received:

a. *p-Chlorophenyl Methyl Sulfide, p-Chlorophenyl Methyl Sulfoxide, and p-Chlorophenyl Methyl Sulfone. I. Acute Toxicity and Bacterial Mutagenicity Studies.* A. Philip Leber, Jack C. Dacre, Daryl C. Thake and Dennis C. Mays. *J. Amer. Coll. Toxicol.*, **12**, 369-376, (1993).

b. *II. Subchronic Effects in Rodents and Rhesus Monkeys.* Dayrl C. Thake, Dennis C. Mays, A. Philip Leber and Jack C. Dacre. *J. Amer. Coll. Toxicol.*, **12**, 377-395, (1993).

c. *III. Toxicokinetics and Metabolism in Rats and Rhesus Monkeys.* Jack C. Dacre, A. Philip Leber and Dennis C. Mays. *J. Amer. Toxicol.*, **12**, 397-407, (1993).

d. *Dominant Lethal Study of Sulfur Mustard in Male and Female Rats.* L.B. Sasser, J.A. Cushing and J. C. Dacre. *J. Appl. Toxicol.*, **13(5)**, 359-368, (1993).

NOVEMBER 1-30, 1993

Technical review of the report "Bioremediation of TNT-Contaminated Soils" was completed. Documentation and references concerning the PB-PK model was provided to LTC Langford, Occupational Health Research Detachment (OHRD), USABRDL, at Wright-Patterson Air Force Base (WPAFB), Dayton, OH. Revision of the text of the report on "Assessment of the Data Base on Health Effects of Zinc Chloride Smoke Produced by HC Smoke Munitions" was completed. A draft copy of the completed manuscript has been submitted for initial in-house review. Material on the composition, degradation products, and toxicity studies of the dyes used in current and developmental colored smoke munitions was provided to CPT D. Lundy of

ORHD, WPAFB, OH.

Work continues on the manuscript from the paper presented at the Fifth Environmental ISSX Meeting. Proofs for the paper titled "Genotoxicity of the Phosphoramidate Agent Tabun (GA)" were received, corrected and returned to the publisher. The paper will be published in the journal *Toxicology*, Vol. 74. The proofs of the paper "Subchronic Oral Toxicity Study of Diisopropyl Methyl-phosphonic Acid in Mink" were received, corrected and returned to the publishers, Academic Press, and will be published in the journal *Fundamentals and Applied Toxicology*. Visited the National Center for Toxicological Research at Jefferson, AR to confer with Dr. Bucci and finalize two outstanding chemical agent reports. A manuscript titled "A Comparative Metabolism Study of DIMP in Mink and Rats" was also completed.

DECEMBER 1-31, 1993

Applied the Physiological-based Pharmacokinetic Model of Blood Lead Concentrations after Exposure to Airborne Lead to data from PALADIN exercises supplied by CPT D. Lundy, Occupational Health Research Detachment (OHRD), USABRDL, at Wright Paterson Air Force Base (WPAFB), Dayton, OH. Reported the results to CPT Lundy at OHRD/WPAFB. Revision of the text of the report on "Assessment of the Data Base on Health Effects of Zinc Chloride Smoke Produced by HC Smoke Munitions" was completed. The draft will next be submitted to OHRD/WPAFB for the formal review process. (Dr. Palmer will assume responsibility for publication of the report.)

A revised proof of the paper "Subchronic oral toxicity study of diisopropyl methylphosphonic acid in mink" was received from the editor of *Fundamental and Applied Toxicology*. It was reviewed, corrected and returned to the publishers, Academic Press. A final revised manuscript titled "A comparative metabolism study of diisopropyl methylphosphonate (DIMP) in mink and rats" (MRDC contract No. DAMD17-91-C-1088) was finalized and submitted for publication in the journal *Archives of Environmental Contamination and Toxicology*.

JANUARY 1-31, 1994

This task has been completed.

Task Order OH-4



GEO-CENTERS, INC.

Title: Toxicity of Microbial Metabolites of TNT
Task Number: OH-4 (2533-009)

APRIL 1-30, 1993

Work commenced on the task order "Toxicity of Microbial Metabolites of TNT." Literature searches on TNT were initiated and appropriate papers and reports are being obtained for review and evaluation of the toxicological data.

MAY 1-31, 1993

This task involves compilation and critical technical review of all data and documents relating to the toxicity of microbial metabolites of 2,4,6-trinitrotoluene (TNT). Insofar as the data permit, estimate a NOAEL for each metabolite. Identify data gaps and propose essential research to establish appropriate remediation levels for the TNT metabolites in soil to protect human health and the environment.

Literature searching continues and all appropriate papers and reports are being obtained through the library for subsequent review and evaluation of the toxicity data.

JUNE 1-30, 1993

Literature searching continues. Papers already obtained are being reviewed and the toxicological data is being evaluated.

JULY 1-31, 1993

Literature searches have been completed. Papers already obtained are being reviewed and the toxicological data are being evaluated. A draft report is in preparation.

AUGUST 1-31, 1993

Literature searches have been completed. Papers already obtained are being reviewed and the toxicological data are being evaluated. A draft report is nearing completion.

SEPTEMBER 1-30, 1993

Work was completed on this project. The final report was submitted .

Task Order OH-5



GEO-CENTERS, INC.

Title: Toxicity of Chemical Warfare Agents - Manuscript Preparation
Task Number: OH-5 (2533-014)

OCTOBER 1 - DECEMBER 31,1994

This task requires technical support for preparation and publication of USABRDL-funded chemical agent toxicological project reports.

The sulfur mustard manuscript, "Subchronic toxicity evaluation of sulfur mustard in rats," by L.B. Sasser, R.A. Miller, D.R. Kalkwarf, J.A. Cushing and J.C. Dacre, was revised as requested by journal editors and accepted for publication by the *Journal of Applied Toxicology* as of 12/13/94.

No progress has been made on submission of Agent Sarin report due to the fact that senior author (an extramural investigator) has not submitted a draft manuscript despite repeated requests. Information received the last week of December indicated that the draft should be received in early January.

Review of the USABRDL Technical Report on sulfur mustard is in progress. Preparation of a manuscript for peer review publication of the same data is scheduled to begin in January.

The draft report on agent lewisite "Dominant lethal study of lewisite in male rats" has been reviewed. The final report, which incorporated the recommended corrections, was submitted for approval and processing to USABRDL on 12/19/94.

JANUARY 1 - MARCH 31, 1995

Agent Sarin draft manuscript for publication in *Teratology Journal* was received; in-house technical review was completed January 17, 1995. Manuscript was sent back to the first author for correction and submission to the journal. We are currently awaiting a copy of the final version of the manuscript from the first author (James B. LaBorde) as submitted for publication.

In-house review of the USABRDL Technical Report on sulfur mustard continued this quarter. Preparation of a manuscript for peer review publication of the same data was initiated this quarter and is still in progress. It is anticipated that both papers will be completed early next quarter.

APRIL 1 – JUNE 30, 1995

This task was completed this quarter with the submission of the manuscript on sulfur mustard to the journal *Drug Metabolism.* The task will be closed out upon final acceptance of the Technical Report and other deliverables submitted to the government.



JULY 1 - SEPT 30, 1995

Work on this task has been completed.

Task Order RM-1



GEO-CENTERS, INC.

Title: Integrated Biological Assessment
Task Number: RM-1 (2533-007)

This task requires technical support for basic and applied research to develop new approaches for assessing the nature and extent of chemical contamination in the environment.

MARCH 1-30, 1993

This task commenced with the continuation of the development of an immunotoxicology program using Teleosts as the test animal. Several plating efficiency assays have been performed to determine the serum concentration (medaka serum) required to optimize macrophage adherence to tissue culture vessels. Once this optimal concentration is determined, phagocytic cell function assays will be repeated and adherent cells characterized by staining properties (myeloperoxidase, esterase,etc.). Setting up the new immunotoxicology laboratory was continued.

Work began to refurbish and outfit the laboratory trailer in front of Building 568 for field use.

Work continues on investigation of methods available for identifying biomarkers of mammalian heptocarcinogenesis for adaptation to fish models. Once these methods have been developed in the Japanese medaka fish (*Oryzias latipes*) in the laboratory, they will be applied to fish being exposed to on-site environmental contaminants in the USABRDL Aquatic Biomonitoring Mobile Laboratory. This will provide a method for *in vivo* detection of carcinogens in a water source prior to the actual development of hepatic neoplasia in the test fish. Well-defined methods for using BrdU as such a biomarker have been adapted to the medaka in the laboratory. Pilot experiments to use this technique in the field were initiated this month on medaka currently being exposed to potentially hazardous groundwater in the biomonitoring trailer at Aberdeen Proving Grounds. Quantification of the BrdU-staining results with the Image Analyis system on slides received back from the completed laboratory BrdU study (Test EE1) were initiated. Different types of bacteria associated with the Medaka used for the EE1 BrdU test and the possible involvement of that bacteria with the unexpected disappearance of BrdU after 72 hours were identified. Five separate colonies of bacteria with a known amount of BrdU dissolved in the culturing media were cultured. The rate at which the bacteria metabolized the BrdU over a 72-hour period was measured. The results were interesting, and further studies are being designed to investigate BrdU metabolism by endogenous bacterial flora in medaka.

Studies of chemical carcinogenesis in medaka were continued with the initiation of this task. Six tanks of DEN exposed fish (Test CC) were sacrificed and prepared for Dr. van Beneden (Duke University Marine Lab). Livers were removed, halved and either frozen in liquid nitrogen or processed for histologic evaluation. Both sets of liver samples have been delivered to respective investigators.

Analytical chemistry methods development continued from previous efforts with the



initiation of this task, as well. Analysis of water samples for trace metals was performed by ICAP. Ground water samples used to expose medaka at Aberdeen Proving Ground for 5-bromodeoxyuridine were analyzed. Development of methodology for the analysis of metabolites of trinitrobenzene by high performance liquid chromatography (HPLC) proceeded. The preliminary results, based on retention times, were that trinitrobenzene metabolizes into 1,4-nitroaniline, 3,5-dinitroaniline, trinitrobenzene, and another unidentified compound.

Aquatic laboratories were maintained in immaculate condition the entire month for the American Association for the Accreditation of Laboratory Animal Care (AAALAC) recertification inspection and for several other laboratory tours which occurred throughout the month. The AAALAC recertification inspection took place March 2, as scheduled, and resulted in three non-mandatory recommendations for improvement in the areas of animal use protocol review and worker safety and efficacy (showers for female employees and desk space outside of laboratory for several technicians).

APRIL 1-30, 1993

Work continues on investigation of methods available for identifying biomarkers of mammalian heptocarcinogenesis for adaptation to fish models. Pilot experiments to use BrdU as a biomarker in the field were completed this month on medaka currently being exposed to potentially hazardous groundwater in the biomonitoring trailer at Aberdeen Proving Grounds. Continued quantifying BrdU-staining results with the Image Analysis system on slides received back from the completed laboratory BrdU study (Test EE1). A five-day training session in SPSS (a statistical analysis program adapted to IBM PC) was attended. This program will be used for statistical analysis of the image analysis data currently being collected from Test EE1 investigating the role of early cellular proliferation in chemically-induced carcinogenesis in medaka. However, this SPSS training did reduce the amount of time devoted to data collection; image analysis data collection for Test EE1 will resume next month.

Studies of chemical carcinogenesis in medaka continued. Received final histology reports from EPL, the laboratory that provides us histology support. Tests Y2, U, P, S, a mini-study comparing two kinds of fixatives, and two medaka health assessments were summarized. Results were as expected, and the reports were filed with the test study data. Preliminary data from the histological analysis of Test Z indicated that an intermediate MNNG dose had more neoplasms than the higher dosed tanks. The exposure records were reviewed and were in order. As this was a nominal study, there are no chemical data to review. In light of this, the exposure part of this study was repeated (with no animals) and analyzed by HPLC. Results are due from chemistry next month, however, there seems to be at least one breakdown product in the samples analyzed.

Continued converting SETAC poster presentation on comparison of rapid toxicity tests into manuscript form for submission for publication. The first draft was completed and sent to co-authors for review.

Work continued on the development of an immunotoxicology program using Teleosts at the test animal. Comparisons of plating efficiencies using both fetal bovine serum and medaka serum continued. Preliminary results indicated that low levels of medaka serum tend to bind cells more efficiently after a 24-hour period. Pilot experiments were initiated to assay protein concentration directly in microtiter plates. Most assays for assessment of macrophage function will be normalized to mg protein. Studies to correlate protein concentration to cell numbers in medaka will be initiated next month. Work continued on organizing the workshop "Modulators of Fish Immune Responses" to be held this September in Breckenridge, Colorado. USABRDL is one of the meeting co-sponsors. A mailing list for potential participants was put together, and meeting announcements will be mailed next month.

Work continued on analytical chemistry methods development in support of task 2533-007. Performed the analysis of water samples for trace metals by ICAP. Analyzed ground water samples used to expose medaka at Aberdeen proving ground for 5-bromodeoxyuridine. Developed methods and analyzed for munitions (HMX, RDX, TNT, TNB,Tetryl, 2,4-DNT, and 2,6-DNT) in well water by high performance liquid chromatography (HPLC). Developed methodology and analyzed well water samples for MNNG.

Work continued to design a new fish chamber to be used in the ventilatory tests. The chamber design was completed and building of a prototype chamber and tank is currently in progress. Once the prototype is complete it will be run alongside the existing tanks to assess its functional ability in ventilatory data collection. Work continued to refurbish and outfit the trailer in front of Building 568 for field use.

Work continues to optimize aquatic laboratory facilities. Testing of new tubing for incoming water was initiated. Before retiring the medaka from Breeding Tank 2 room 5, opaque silicon tubing replaced the silicon tubing normally used for the incoming water supply line. After one week, eggs were collected (~200) and reared. These fish will have the new tubing as the water supply line through out their life. If no detrimental effects are observed, the opaque tubing will be used throughout the fish culture laboratory. This would greatly reduce clean-up effort prior to inspections. Aquaculture personnel received training on accessing the EPA database ACQUIRE. This database is a compilation of aquatic toxicity effects published in scientific journals. Access at USABRDL is through DIALOGLINK software and is password driven. The most direct search is by chemical CAS number and organism scientific name. General fish culture included collection of 2000 eggs from the medaka colony for culture renewal on April 27. These eggs will hatch in early May. Tanks 1 and 2 were retired, and the fish hatched on February 7, 1993, were rotated into the breeding colony.

MAY 1-31, 1993

Work continues on investigation of methods available for identifying biomarkers of mammalian heptocarcinogenesis for adaptation to fish models. Data collection continued to quantify BrdU-staining results with the image analysis system on processed slides from the completed laboratory BrdU study (Test EE1). Work also continued on developing a worksheet

format in SPSS for statistical analysis of data. Image analysis data collection for Test EE1 will continue next month.

Studies of chemical carcinogenesis in medaka continued. No new studies were initiated this month; project work consisted of maintenance of ongoing studies.

Work continued on the development of an immunotoxicology program using Teleosts at the test animal. Pilot experiments continued to assay cellular protein concentration directly in microtiter plates. Modification of both the Lowry and Bradford protein assays were used to correlate protein concentration to cell numbers in medaka. The Lowry modification worked well, and work on the Bradford modification is in progress. Background reading of articles dealing with the comparison of interspecies immune responses was initiated this month. Design and initiation of a study to compare macrophage biochemistry between species (human, rodent and teleost) is planned for next month. Approximately 180 medaka were shipped to Dr. Judith Zelikoff at New York Medical Center for immunology research. A visit to BRDL by Dr. Zelikoff is planned for next month to observe and learn medaka-related laboratory techniques. Work continued on organizing the workshop "Modulators of Fish Immune Responses" to be held this September in Breckenridge, Colorado. USABRDL is one of the meeting co-sponsors. The first group of meeting announcements was mailed to potential participants this month, and another group is being assembled for mailing next month.

Work continued on analytical chemistry methods development. Analytical methods for detection of MS-222, Phenol, and Pentachlorophenol are being prepared for introduction into the standard book of methods for HPLC. Also in preparation is precision and accuracy data for the pesticide 2,4-D and the munitions TNT, TNB, 2,4-DNT, 2,6,-DNT, HMX, RDX, and Tetryl for analysis by HPLC.

Work continued on the set-up of the ventilatory test system for tests to be run the summer. This included providing check lists for technicians to follow during test operation and putting together a packet of data forms for the test.

Work continues to optimize aquatic laboratory facilities. The quarterly medaka colony renewal occurred in this month. Two tanks of old breeders were retired, and three month old fish were sexed and rotated into the colony. New fry hatched on May 5 and were placed in the 4 x 55 gallon wall tanks for three-month grow out. Inventory of exhaustible supplies was conducted for budgetary planning purposes. Needs were projected on a quarterly basis through January 1, 1994.

Three binders of final draft SOPs were reviewed for EPL. This included the equipment SOPs, the trailer SOPs, and a binder with miscellaneous items such as record keeping and facilities use. Comments were reviewed with a BRDL co-worker prior to the binders returning to EPL.

JUNE 1-30, 1993

Work continues on investigation of methods available for identifying biomarkers of mammalian heptocarcinogenesis for adaptation to fish models. Data collection continued to quantify BrdU-staining results with the image analysis system on processed slides from the completed laboratory BrdU study (Test EE1). Work also continued on developing and programming a worksheet format in SPSS for statistical analysis of data. This SPSS program is almost complete. Image analysis data collection for Test EE1 will continue next month.

Studies of chemical carcinogenesis in medaka continued. Test CC, a DEN high level exposure, was completed. The last animals (4 tanks of ~55 medaka each) were shipped to Dr. Bunton at Johns Hopkins on 30 June.

Work continued on the development of an immunotoxicology program using Teleosts at the test animal. The first week of June was spent preparing for and hosting a 2 day visit from collaborators from New York University Medical Center, Dr. Judith Zelikoff and her technician Wei hua Wong. The purpose of the visit was to demonstrate laboratory procedures using medaka and discuss overall project goals with our research director. Procedures demonstrated included organ collection, isolation and separation of monocytes into single cell suspensions, plating cells into primary culture and running superoxide anion generation assays (cytochrome *c* and NBT). Discussions on project goals and integration with in-house work went well. Plans have been made to ship more retired breeders to Dr. Zelikoff in early July. In light of her projected need of fish for research, 1400 medaka eggs will be pulled for her during the July egg culture. The first run of a time course for generation of superoxide anion following stimulation was conducted this month. This experiment will need to be repeated several times before the optimal time for assessment of macrophage function in culture can be reliably determined. In preparation for future 96 hr LC50 studies, experiments to determine optimal loading densities (number of fish per tank) were initiated. Water qualities (dissolved oxygen, hardness, ammonia and nitrite concentrations, etc.) were performed for all tanks daily either in the immunotoxicology lab itself, or by the in-house chemistry group. Background reading of articles dealing with the comparison of interspecies immune responses continued this month. Design and initiation of the study to compare macrophage biochemistry between species (human, rodent and teleost) is in progress. Work continued on organizing the workshop "Modulators of Fish Immune Responses" to be held this September in Breckenridge, Colorado. USABRDL is one of the meeting co-sponsors. The second mailing of meeting announcements to potential participants was completed this month. A manuscript for presentation at the meeting was received and will be reviewed next month.

Work continued on analytical chemistry methods development in support of task 2533-007. Filter samples were prepared for trace metal analysis by ICAP. The explosive RDX was purified by recrystalization so that it may be used in analysis with HMX. Continued preparation of precision and accuracy data the munitions TNT, TNB, 2,4-DNT, 2,6,-DNT, HMX, RDX, and Tetryl for analysis by high performance liquid chromatography. Provided schedule of maintenance for analytical instrumentation used for analytical chemistry.

Submission of the manuscript comparing rapid toxicity tests (data presented in poster form at the November, 1992 SETAC meeting) to the SETAC journal has been delayed. One of the co-authors is rewriting one of the sections. After co-author agreement, the article will be circulated at BRDL for internal peer review.

Work continues to optimize aquatic laboratory facilities. An experiment to investigate the wavy fry problem of May 1991 was conducted. The culture practices that were in use then were abandoned for a process that did not bend the spines of the medaka. By varying the components of the prepared Rearing Solution, the old chemicals were checked to see if they were the cause of the abnormalities. There were seven treatments and three replicates, yielding a total of 21 flasks of 35 eggs each. One treatment was grown in darkness to simulate poor lighting. One treatment was the current practice of using well water to raise the eggs. The hatch rate from the well water flasks averaged 70%, while the hatch from the rearing solution averaged 80%. The darkness treatment produced no adverse effect on the eggs. There were no wavy fish seen. Since there have been so many changes to the culturing process (examples: diet change, lighting change, fish density change), it was determined that this investigation would not be continued at this time. The egg culturing will continue in well water.

JULY 1-31, 1993

Work continues on investigation of methods available for identifying biomarkers of mammalian hepatocarcinogenesis for adaptation to fish models. Data collection continued to quantify BrdU-staining results with the image analysis system on processed slides from the completed laboratory BrdU study (Test EE1). SPSS program for statistical analysis of image analysis data was completed, although future modifications may be required. Image analysis data collection for Test EE1 will continue next month. Work on a procedural paper for *in vivo* labelling of medaka cells with BrdU was initiated.

Studies of chemical carcinogenesis in medaka continued. No new tests were initiated; fish currently on test were maintained.

Work continued on the development of an immunotoxicology program using Teleosts at the test animal. The time course for macrophage generation of superoxide anion following stimulation was repeated several times this month; however, the data were not as clean as could be hoped due to several mishaps in the lab (contamination, equipment failure, labor intensive nature of collecting cells). This experiment will still need to be repeated before the optimal time for assessment of macrophage function in culture can be reliably determined, although all data to date indicate that a 24 - 48 hour time point post-isolation should work well. Experiments are planned for next month to test macrophage activation 6 to 12 days post-isolation; this will be the age of the primary rodent cells we will be receiving from Johns Hopkins for our species comparison studies. Background reading of articles dealing with the comparison of interspecies immune responses continued this month. Design and initiation of the study to compare macrophage biochemistry between species (human, rodent and teleost) is in progress. A meeting with our collaborators at Johns Hopkins has been set for August 5. Work continued on

organizing the workshop "Modulators of Fish Immune Responses" to be held this September in Breckenridge, Colorado. The manuscript from W.E. Hawkins for presentation at the meeting was reviewed and returned (with comments) to the authors with instructions for abstract and paper preparation. Work was initiated with medical illustrations to make a "Laboratory Techniques" video for presentation at the meeting. We are one of four labs asked to provide video tapes for these sessions. Three trips to the U.S. Fish and Wildlife Laboratories in Kearneysville, WV, were undertaken this month to discuss and set up a USABRDL and USFWS collaboration in fish immunology. Two of these trips involved swapping fish (medaka for trout) and learning bleeding techniques. A two-day trip is planned for next month to learn fish bacterial culture techniques.

Work continued on analytical chemistry methods development. Water samples were analyzed for lead, copper, zinc, and cadmium by ICAP in support of range finding tests on killifish. Continued preparation of precision and accuracy data of TNT, TNB, 2,4-DNT, 2,6,-DNT, HMX, RDX, and Tetryl for analysis by high performance liquid chromatography.

Work continues to optimize aquatic laboratory facilities. Eggs were pulled for colony renewal and various research projects. The cultures in Room 5 produced more eggs than the Room 18 cultures. Lighting is the biggest difference between the two rooms. Will inquire why the Room 18 lights have not been upgraded. Cross-training was initiated with the immunotoxicology laboratory; culture personnel learned how to remove head kidneys from medaka and helped isolate cells for a large immunotoxicology experiment. Quarterly light measurements over every tank and quarterly notebook and binder review were performed.

AUGUST 1-31, 1993

Work continues on investigation of methods available for identifying biomarkers of mammalian hepatocarcinogenesis for adaptation to fish models. Data collection continued to quantify BrdU-staining results with the image analysis system on processed slides from the completed laboratory BrdU study (Test EE1). Work on a procedural paper for *in vivo* labelling of medaka cells with BrdU continued. Data from Group B slides of Test EE1 were summarized for use in the paper.

Studies of chemical carcinogenesis in medaka continued. Interim sacrifice was performed for Test 401-001R, the Beach Point Groundwater Study. At test day 180, 20 fish from each of the 12 test tanks in the biomonitoring trailer were returned to Fort Detrick. After a day of rinsing, the animals were sacrificed and prepared for histology. Twenty fish from the four control tanks maintained at USABRDL were also sacrificed and prepared for histology. The prepared fish will be sent to Experimental Pathology Laboratories for histology/pathology evaluation by Dr. Wolfe.

Work continued on the development of an immunotoxicology program using Teleosts at the test animal. The time course for medaka macrophage generation of superoxide anion following stimulation was repeated twice this month, and results indicated that 24-hour post-isolation is the optimal time for macrophage stimulation and assessment of superoxide anion

generation. Forty-eight hours post-isolation also gave acceptable results. Macrophages activated 6 to 12 days post-isolation did not produce measurable amounts of superoxide anion. These results led to a modification of species comparison protocol; both principle investigators agreed to a change from 12 days to 24-48 hour post-isolation as the assay time for the species comparison studies. Additional progress on this project included a trip to Johns Hopkins University to observe the techniques used to isolate bone marrow stromal cells from rat and mouse and initiation of pilot experiments with mouse, rat and trout bone marrow/head kidney macrophages. Work continued on organizing the workshop "Modulators of Fish Immune Responses" to be held this September in Breckenridge, Colorado. The abstract for the paper to be presented was submitted August 15 following in-house review. Work was completed with medical illustrations to make a "Laboratory Techniques" video for presentation at the meeting. The tape is currently being edited. Two trips to the U.S. Fish and Wildlife Laboratories in Kearneysville, WV, were undertaken this month to learn fish bacterial culture and identification techniques. The first pathogen to test in LC50 tests with medaka was obtained, *Aeromonas salmonicida*, and studies are scheduled to begin within the next few months.

Work continued on analytical chemistry methods development. Water samples were analyzed for phenol, pentachlorophenol, and 2,4-dichlorophenoxy acetic acid (2,4-D) by high performance liquid chromatography (HPLC) in support of ventilatory test on bluegills and range finding tests on killifish. Continued preparation of precision and accuracy data for the munitions TNT, TNB, 2,4-DNT, 2,6,-DNT, HMX, RDX, and Tetryl for analysis by HPLC. Training was initiated for the operation of the Leeman 3000 PS series ICAP.

The article, "A Comparison of the Sensitivity of Rapid Toxicity Screening Tests" to be submitted for publication in *Environmental Toxicology and Chemistry*, was submitted for in-house review.

Work continues to optimize aquatic laboratory facilities. Eggs were pulled for colony renewal and various research projects.

Work commenced with the addition of an analytic chemist at Rocky Mountain Arsenal. Commenced familiarization with the laboratory and conducted initial sample analysis on GC. Identified a senior chemist to commence work in September.

SEPTEMBER 1-30, 1993

Work continues on investigation of methods available for identifying biomarkers of mammalian hepatocarcinogenesis for adaptation to fish models. Data collection continued to quantify BrdU-staining results with the image analysis system on processed slides from the completed laboratory BrdU study (Test EE1). The SPSS syntax program needed to run the first group of fish was completed this month. Work will continue with both the Bioquant and SPSS data analysis next month. Journal literature on cell proliferation/BrdU/apoptosis was updated; retrieved references were entered into branch database.

Studies of chemical carcinogenesis in medaka continued. The first six months of records from the biomonitoring trailer at Beach Point (Test 401-001R) were reviewed and entered into the project notebook. Five scientists from this facility attended the Aberdeen Proving Ground Installation Restoration Meeting held in Lancaster, PA. Deployment of the newest set of mobile laboratory facilities developed by USABRDL to Aberdeen Proving Ground and/or adjacent sites is anticipated within the near future.

Work continued on the development of an immunotoxicology program using teleosts as the test animal. The time course for medaka macrophage generation of superoxide anion following stimulation was repeated twice this month with 12-month-old fish. In contrast to results from the younger fish obtained last month, considerable activity was demonstrated by freshly isolated cells in older fish. These results agreed nicely with results obtained independently by USABRDL's extramural contractor, Dr. Judy Zelikoff of NYU Medical Center.

Work continued on trouble-shooting optimization of the hydrogen peroxide assay technique. Work continued on the species comparison studies. A new technician was added to the staff for this project and laboratory training is going well. Techniques demonstrated and practiced included medaka anterior kidney cell isolation, slide preparation and staining, superoxide anion generation assays and experiments to correlate protein content to cell number. The workshop "Modulators of Fish Immune Responses" was held September 17-21 in Breckenridge, Colorado. Four scientists attended from this facility, and the meeting went well. There is potential for useful information exchange/collaboration with eminent fish immunologists as a results of the contacts made at this meeting. The paper and "Laboratory Techniques" video presented were well received. A manuscript based on the presented paper was completed and reviewed by all authors. The manuscript, "Development and use of medaka as a model for immunotoxicity," is currently in in-house review; submission of the final version to the meeting chairman is anticipated by the middle of next month. Two abstracts entitled "Immunotoxicological methods development and validation in the medaka (*oryzias latipes*) for potential hazard assessment applications" and "Phylogenetic conservation of the nonspecific immune response: A species comparison," were written, reviewed and submitted to the Program Committee of the Society of Toxicology for platform/poster presentation at the annual meeting next March in Dallas, TX.

Work continued on analytical chemistry methods development. Initiated method development and method validation for the analysis of trace metals in waste waters using the Leeman 3000 PS series ICAP in the analytical chemistry trailer. Methods development for the analysis of 2,4-D was completed. Digested and analyzed filter disks for lead by ICAP. Work continued on the disposal of waste chemicals. A study on the stability of munitions in well water was initiated.

The manuscript, "A comparison of the sensitivity of rapid toxicity screening tests," was received by *Environmental Toxicology and Chemistry* for peer review.

Two shipments of retired breeders went to Dr. Zelikoff of NYU, and one shipment of



retired breeders went to Dr. Bunton of Johns Hopkins University.

Work continues to optimize aquatic laboratory facilities. Began Temp Scribe calibration for the laboratory. The design of operation was changed from the tear-off mode to the continuous rewind mode. All Temp Scribes not currently in operation were calibrated at three temperatures. On October 1, the Temp Scribes will be changed out so that all on-going measurements will be made with a calibrated instrument. The "retired" Temp Scribes will be calibrated in October and switched to the continuous mode of operation. As part of the culture maintenance, the baths were cleaned. The heater coils were acid cleaned. The circulators were taken apart and scrubbed out. The bath surfaces were scrubbed and siphoned. The tank drains to the bath were all replaced and repaired. With the receipt of the signed and dated SOPs, new forms were copied to be put into use in October. Some pages that do not fill up monthly will be changed out whenever they fill up, to eliminate extra paperwork.

OCTOBER 1-31, 1993

Work continues on investigation of methods available for identifying biomarkers of mammalian hepatocarcinogenesis for adaptation to fish models. Data collection continued to quantify BrdU-staining results with the image analysis system on processed slides from the completed laboratory BrdU study (Test EE1).

Studies of chemical carcinogenesis in medaka continued. Prepared submission form for EPL histology analysis of the 6-month interim sacrifice of Test 401-001R (Beach Point). Three reports on Beach Point Groundwater Analysis by an outside contractor were collated for in-house review.

Work continued on the development of an immunotoxicology program using teleosts at the test animal. Work continued on trouble-shooting optimization of the hydrogen peroxide assay technique. Work continued on the species comparison studies; to date, superoxide anion production and protein determination assays have been performed with cells from all of the species involved in order to obtain optimal conditions. Training of the new project technician continued; laboratory techniques were studied and improved. Two protein assays using Medaka were performed. Isolation of trout head kidney was a technique learned and a protein assay using trout was also done. Reading/studying of immunology reference material continued. Five papers were reviewed for publication in the meeting proceedings from the workshop "Modulators of Fish Immune Responses" held September 17-21 in Breckenridge, Colorado. In-house review of the paper presented at this meeting, "Development and use of medaka as a model for immunotoxicity," was completed and the manuscript was submitted to the meeting chairman on approximately 10/18/93. The reviewed manuscript was returned for technical and editorial corrections at the end of the month; corrections and final review are in progress and submission of the final manuscript is expected early next month.

Work continued on analytical chemistry methods development. Method development and validation continued for the analysis of trace metals in waste waters using the Leeman 3000 PS

series ICAP in the analytical chemistry trailer. Standing Operating Procedure (SOP) for the analysis of munitions by HPLC continued. Work continued on the disposal of waste chemicals. The study on the stability of munitions in well water initiated last month continued; water samples were analyzed for zinc and munitions. Method development for analysis of rubidium by ICAP was initiated this month.

Writing of animal use protocol(s) for mummichogs (*Fundulus heteroclitus*) was initiated this month. Tank 18-C-2 will be the designated salt water tank for the holding of the mummichogs. The mummichog project will be in collaboration with NIEHS. Feral fish purchased from a commercial supplier will be used to design the study. Once the study design is set, transgenic mummichogs (supplied by NIEHS) will be exposed to a carcinogen, and then returned to NIEHS for evaluation. In preparation for this project, salt water culturing of fish was researched. The proper filtration system was ordered, and water quality parameters were established for mummichogs.

Work continues to optimize aquatic laboratory facilities. Extensive laboratory cleaning was done for the tour after the RMB workshop of October 26-27. Everyone in the laboratory was able to attend some or all of the RMB Research Review held October 26 and 27th at Ceresville Mansion.

NOVEMBER 1-30, 1993

Work continues on investigation of methods available for identifying biomarkers of mammalian hepatocarcinogenesis for adaptation to fish models. Data collection continued to quantify BrdU-staining results with the image analysis system on processed slides from the completed laboratory BrdU study (Test EE1). SPSS program for data analysis was updated. BrdU/cell proliferation/apoptosis literature was updated.

Studies of chemical carcinogenesis in medaka continued. The final sacrifice of Test 401-001R (Beach Point) began at the end of the month and will continue into the first day of December.

Work continued on teleost immunotoxicology methods development. Optimization of the hydrogen peroxide assay technique continued; reduction of the concentration of phenol red improved results. Assays with trout cells are now consistent and reproducible; trout can now serve as the positive control in further troubleshooting with medaka. Work continued on the species comparison studies. Macrophage protein determination for 0-hr, 90-min and 12-hr attachment time points for both the medaka and the rainbow trout were completed. All species comparison data generated to date is being analyzed in preparation for planning remaining experiments for project completion. A 2-day site visit to New York Medical University Institute of Environmental Medicine was taken November 8th and 9th. The research goal for this trip was to enhance the interlaboratory methods standardization efforts by working cooperatively with extramural contractors on the medaka project. Four different assays were performed side-by-side by NYU and in-house technicians at the NYU laboratory. SOP-required documentation is being



integrated as part of laboratory procedures for the immunotoxicology laboratory. Final revision of the manuscript "Development and use of medaka as a model for immunotoxicity," was completed. The manuscript was submitted for publication in the meeting proceedings from the September workshop "Modulators of Fish Immune Responses." Publication of the proceedings is expected by early 1994.

Work continued on analytical chemistry methods development. Method development and method validation was initiated for the analysis of N-Nitroso-N-ethylurea (ENU) by high performance liquid chromatography in salt water. Gradient elutions from 0 to 100 percent methanol provided a range in which ENU could be eluted in a short period of time (under 10 minutes). The final conditions used during the study was 20 percent methanol with a 1.5 Ml/minute flow rate. Soon after method development began, it was determined that ENU is not a very stable compound. Various diluents for stock were used to determine the most stable medium for standards. One hundred percent acetonitrile provided the most stability of solvents that did not absorb UV in the same range as ENU. Using acetonitrile limits the injection volume to 25 ml due to the strong elution strength in a weak mobile phase. Stability studies were then performed on ENU in salt water, well water, DI water and salt water in the absence of light. The results of these tests were summarized in a report "The Stability of ENU in Water" provided to aquaculture personnel. Disposal of waste chemicals continues. Additionally, 15 filter samples were digested and analyzed for lead. Sand flies samples were analyzed by ICAP for rubidium but no rubidium was found.

The animal use protocol for mummichogs (*Fundulus heteroclitus*) was completed and submitted for approval by the animal use committee. Mummichogs (*Fundulus heteroclitus*) were received in early November. Fish appear to be doing well in the salt water culture system set up last month. The mummichogs survived the quarantine period. Three fish of each size (1" and 3") were placed in square glass containers (one side is mesh) and suspended from the edge of the tank. These fish were held this way for three days, with no obvious ill effects. This was a pilot study to test the isolation procedure planned for post-exposure holding of exposed fish. Nitrite levels continue to exceed recommended amounts, but overall, they are declining to acceptable levels. We are replacing 40 liters of tank water daily until the nitrite levels stabilize.

Work continues to optimize the aquatic laboratory facilities. Animal use records for all fish for FY 93 were compiled. This included all fish reared, shipped, or sacrificed for intramural and extramural work.

DECEMBER 1-31, 1993

Work continues on investigation of methods available for identifying biomarkers of mammalian hepatocarcinogenesis for adaptation to fish models. Analysis of fish sacrifice Group B, tanks 1 - 14 on the Bioquant Image Analysis System was completed this month. Since it was decided to evaluate additional slide sections after the analysis of Group B had begun, missing data points from tanks 3 - 9 will be filled in before proceeding to Group D, the next sacrifice scheduled to be analyzed. SPSS program for data analysis was updated. The final version of the

SPSS macro should be complete by the end of the month. Work was initiated on the "quality control" portion of the cell proliferation project (randomly selected slides which have already been analyzed will be read by another technician and results compared for reproducibility). BrdU/cell proliferation/apoptosis literature was updated.

Studies of chemical carcinogenesis in medaka continued. Final sacrifice data from Test 401-001R was compiled and summarized. The mean lengths and weights for 16 tanks of fish were calculated. Began preliminary planning for the next trailer test at Aberdeen Proving Grounds at the West Branch of Canal Creek site. When all of the details have been sorted out, this study will be submitted as an addendum to the animal use protocol at the first site (i.e., Test 401-001R).

Work continued on teleost immunotoxicology methods development. During the month of December, time was devoted to cleaning out laboratories on the basement and first floor levels to accommodate the painting of building 568. New storage space was obtained and many supplies were relocated to this area. Personnel offices were also relocated. Work on the species comparison studies was suspended this month due to laboratory and building painting. Optimization of the hydrogen peroxide assay technique continued. A hydrogen peroxide assay was run to test current protocol and compare reactivity of freshly obtained kidney cells to kidney cells stored overnight. Results from one assay are not definitive, but storage of organs overnight appeared to decrease the reactivity of the cells. Work continued on interlaboratory methods standardization between USABRDL and NYU. Detailed and definitive procedural protocols incorporating all of the information gathered from the site-visit to NYU in November were received from our NYU collaborators. Upon review, it was decided that the protocols require extensive correction, rewriting and clarification. These revisions are in progress and should be completed by the first or second week of January. Definitive studies for the methods standardization are scheduled to begin the third week of January. SOP-required documentation is being integrated as part of laboratory procedures for the immunotoxicology laboratory. The final manuscript was received for review for publication in the Proceedings from the September workshop "Modulators of Fish Immune Responses"; manuscript will be reviewed and returned to the publisher by early January. Publication of the Proceedings by early 1994 is still on schedule.

Work continued on analytical chemistry methods development. Method development and method validation was completed for the analysis of N-Nitroso-N-ethylurea (ENU) by high performance liquid chromatography in salt water. Plots of standard curves, stability data, and chromatogram were prepared for use in this method. Maintenance was performed on the TJA 61E ICAP. A difficulty with the nebulizer pressure controller was found and traced the difficulties to a bad remote on the regulator which adjusts the argon pressure. It was necessary to replumb the argon flow to compensate for the increased pressure. Disposal of waste chemicals continues. Completed the removal of all chemicals from building 1058 and retained the chemicals that were retained for future use were placed on the chemical inventory for building 568.

The animal use protocol for mummichogs (*Fundulus heteroclitus*) was approved, pending certain modifications, by the animal use committee. Protocol corrections have been made and



review of the changes is underway.

Work continues to optimize the aquatic laboratory facilities. The fish labs were painted during the month of December. Preparation for this event included stripping the lab of everything on the counters and walls, and moving the 100-gallon fish tanks away from the wall. The fish tanks and baths were draped with dropcloths and sealed off from the paint fumes. Aeration to the tanks was continued, so that the tanks were positively pressurized. The ceilings were spray painted and the walls were roller and brush painted with latex paint. On the day of painting, the 100-gallon tanks had to be drained and moved back to their wall position after the wall behind them was painted. At this time the water was also shut off to the tanks because the drain lines could not be on the floor during the floor painting. Epoxy paint was used to seal the floors, and fumes from the paint were irritating to the extent that the building was evacuated by the Commander. The water was restored to the tanks 6 hours later when the floor was dry enough to walk on. There was no mortality within the fish population as a result of painting. Of special note was the attention to detail given by Ron Miller. He came in early to begin the draping, and he was one of the people who came back at 9 pm to turn the water back on and check on the fish.

JANUARY 1-31, 1994

Work continues on investigation of methods available for identifying biomarkers of mammalian hepatocarcinogenesis for adaptation to fish models. Completed missing data points on fish livers from Group B, tanks 3 - 9 on the Bioquant. Started marking livers to be analyzed from Group D. SPSS program for data analysis was updated. The final version of the SPSS macro needed to compute the results obtained from sacrifice B was completed. Several graphs depicting the results were made.

No work on chemical carcinogenesis in medaka was conducted this month.

Work continued on teleost immunotoxicology methods development. Work on the species comparison project continued with superoxide anion assays on trout, medaka, rat and mouse cells. Results from these tests are now in the process of being tabulated. Optimization of the hydrogen peroxide assay technique continued. Work continued on interlaboratory methods standardization between USABRDL and NYU. During the month of January, the major goal for this project was to finish refining and optimizing assay conditions and finalize protocols. Five of eight protocols (Cell Isolation, Slide Preparation and Routine Staining, Serum Collection, Hydrogen Peroxide Assay, and Cytochrome *c* Assay), were finalized, and the remaining three (5' Nucleotidase Activity, NBT Assay and Protein Determination) will be finalized in early February. Three Hydrogen Peroxide assays, three Cytochrome *c* assays, three 5' Nucleotidase assays and two NBT assays were performed. In consultation with collaborators at NYU, it was decided to add four more parameters for comparison studies; fish weight, anterior kidney weight, hematocrit and leukocrit. The five definitive experiments (incorporating all of the assays listed above) for the interlaboratory standardization are scheduled to be run February 7-25. SOP-required documentation continues to be integrated as part of laboratory procedures for the immunotoxicology laboratory. Publication of the meeting proceedings from the September

workshop "Modulators of Fish Immune Responses" is still on schedule, copies of completed publication are expected next month. A new member of the group was introduced to the various laboratory duties, assays and methods used in immunotoxicology.

Work continued on analytical chemistry methods development. Continued method development and method validation for the analysis of N-Nitroso-N-ethylurea (ENU) by high performance liquid chromatography in salt water. Due to the unstable nature of the compound, it was requested that the stability of ENU be examined at each of the concentrations used to expose Fundulus (6, 12.5, 25, 50, and 100 mg/L. One hundred mL of salt water was placed in a sealed, jacketed flask to simulate the conditions of the exposure. A stock of 1000 mg/L was prepared in DI water. Proportional amounts of the stock were added to the salt water to give the concentrations used during the exposures. Samples were pulled immediately after the mixing and at 10 minute intervals until the ENU had completely degraded. The samples were injected onto a Supelco C-18 DB column with a 1.5 mL/minute flow rate of 20 percent methanol. The injection volume was reduced to 15 from 25 µL to eliminate a problem with double peaking of the standards which was caused by the strong elution strength of acetonitrile in a relatively weak mobile phase. Fifteen samples of ENU were analyzed under test number 103-001. Work on the disposal of waste chemicals continued. Received training on the removal and installation of the optics on the Leeman PS-3000 in the mobile lab and prepared the instrument for transport to Aberdeen Proving Grounds.

Analytical chemistry support for Rocky Mountain Arsenal included the following:

- DIMP Analysis:

The present method involving liquid/liquid extraction has been taken through the certification process to document that a lower reporting limit can be used. A reporting limit of 2µg/L was required, and a reporting limit of just over 1µg/L was achieved. Since an acceptable reporting limit has been documented, additional samples (5 to 6 or more per week) can be brought back in-house. Solid phase extraction procedures are being investigated for possible method improvement. At present, the most favorable results have been obtained with a C-8 cartridge. A limited number of solid phase extraction disks have been received, and these are in the process of being evaluated.

- GC/MS Volatiles:

Changes have been made in the standards, calibration files, QC acceptance criteria, and analyte list used in the procedure. The changes were made to the procedure in order for it to conform to EPA Method 8260.

- Organosulfur Compounds:

Solid phase extraction techniques are also being investigated as a possible improvement for this procedure.

- Lewisite Analyses:

Available documentation on laboratory procedures for the analysis of Lewisite has been reviewed. One of the procedures utilizing ethanedithiol was investigated in the laboratory for possible use as a confirmatory procedure. Unacceptable results were obtained with this procedure. Another procedure utilizing HPLC appears much more promising. the laboratory's HPLC equipment was brought back into the building from storage and has been set up. The solvent delivery system, built-in diode array detector (DAD), and computerized data acquisition system all function properly. However, the flow cell for the DAD had been cracked, and a new cell is on order. The stand-alone refractive index detector will be installed and utilized (if possible) for method development.

- Back-up Headspace Instrument for Lewisite Analysis:

The laboratory has a Headspace GC/FID which was intended for use as a back-up for the primary instrument. The laboratory has been unable to use the instrument for this purpose due to a lack of sensitivity. Work is underway to improve the sensitivity of this instrument.

Work on the mummichogs (*Fundulus heteroclitus*) project continues. The mummichogs were exposed to ethylnitrosourea (ENU) on Tuesday, January 11. There were 3 fish per exposure vessel, with replicates run of each treatment. Concentrations tested were 6, 12, 25, 50, and 100 mg/L ENU and control fish (no exposure). Each vessel was exposed for one hour at staggered intervals throughout the day to allow for chemical analysis of the solutions during the exposure. No mortality was observed during the exposure, and the fish were transferred to grow-out containers within our 55-gallon salt water tank. This test is labelled Test 103-001. Two modifications to Test 103-001 were necessary. Glass tops were siliconed to the top of the grow-out containers because the fish were unusually active post-exposure (three fish jumped out of exposure beakers during the test). Several fish were injured by this "hyperactive" behavior, so the fish were visually shielded by placing a strip of paper around the outside of the tank. This shielding seems to have soothed the fish and reduced injuries.

Work continues to optimize the aquatic laboratory facilities. The quarterly light intensity measurements were made. Approximately 2000 eggs were collected for immunology research.

FEBRUARY 1-28, 1994

Work continues on investigation of methods available for identifying biomarkers of hepatocarcinogenesis for adaptation to fish models. Bioquant image analysis of liver areas of fish from tanks 1,2 (control) and tanks 5,6 (100mg/l DEN) from sacrifice point Group D, Test EE1 was completed. "Materials & Methods" section for analysis of liver areas using the Bioquant was written up in anticipation of manuscript preparation. Bimonthly update on cell proliferation references was conducted this month. References were subsequently entered into Ref-Man. Starting March 7, effort on this project is expected to increase.

No work on chemical carcinogenesis in medaka was conducted this month.

Work continued on teleost immunotoxicology methods development. Work on the species comparison project continued with superoxide anion assays on trout, medaka, rat and mouse cells. All pilot work for the species comparison was completed. The definitive N3 studies for rat, mouse, trout and medaka were completed. The definitive experiments using ML-1 human cells are scheduled for March 3, which will complete the laboratory portion of this phase of the species comparison studies. Data analysis is in progress for presentation of the results at the Society of Toxicology meeting next month.

Work continued on interlaboratory methods standardization between USABRDL and NYU. The final three protocols (5' Nucleotidase Activity, NBT Assay and Protein Determination) were finalized. The five definitive experiments for the interlaboratory methods standardization were completed February 28. NYU data was received the last week of February. Data analysis for presentation as a poster at Society of Toxicology meeting next month is in progress. SOP-required documentation continues to be integrated as part of laboratory procedures for the immunotoxicology laboratory. The meeting proceedings from the fish immunology workshop (September, 1993, Brechenridge, CO) were published this month, as scheduled; "Modulators of Fish Immune Responses, Volume One, Models for Environmental Toxicology, Biomarkers, Immunostimulators," J.S. Stolen and T.C. Fletcher (eds), SOS Publications, Fair Haven, NJ, 1994. Compilation of video presentations from the same meeting are in progress.

Work continued on analytical chemistry methods development. The stability study of N-nitroso-N-ethylurea (ENU) by high performance liquid chromatography in salt water at 6, 12.5, 25, 50, and 100 mg/l was completed. All of the concentrations exhibited similar logarithmic degradations in salt water. A stock of 1000 mg/l ENU was prepared in DI water and was injected periodically over a 24-hour period at ambient temperatures. The injection volume of the sample was reduced from 15 to 1.5 µL to prevent overloading of the column. This stock sample showed degradation of less than 10% in DI water. Procedure titled "Method for the Determination of Trace Metals in Brine Shrimp" was written this month. Completed the digestion and analysis of a sample of Brine Shrimp. Transferred methods for a replacement computer for the TJA 61E ICAP and wrote the method "The Determination of Trace Metals in Water by ICAP" for the instrument. Received four water samples, two salt samples and a sample of sand for analysis of trace metals from a contract facility having difficulty with killifish mortality. Continued tying down equipment in the mobile lab for trailer transport to Aberdeen Proving Ground early next month.

Analytical chemistry support for Rocky Mountain Arsenal included the following:

- DIMP Analysis:

Samples from the Peoria Street Treatment Plant have successfully been brought back in-

house. Solid phase extraction procedures are continuing to be investigated for possible method improvement. Several automated systems for sample extraction utilizing solid-phase techniques are being evaluated for potential use.

- Organosulfur Compounds:

Solid phase extraction techniques are also being investigated as a possible improvement for this procedure. In addition, alternate liquid-liquid extraction procedures are being evaluated to allow certification of this method at a lower reporting limit.

- Back-up Headspace Instrument for Lewisite Analysis:

The laboratory has a Headspace GC/FID which was intended for use as a back-up for the primary instrument. The laboratory has been unable to use the instrument for this purpose due to a lack of sensitivity. Modifications were made to the instrument, and sensitivity equivalent to the primary instrument was achieved. The timing of this modification was very auspicious. The primary instrument went down, and the back-up instrument is now being used for all Lewisite analyses.

- Support of Soil Washing Project:

Harding Lawson and Associates (HLA) is conducting a study of soil washing as a potential remediation technique for use at Rocky Mountain Arsenal. The technology was developed by Resources Conservation Company (RCC), and utilizes triethylamine as a solvent to remove organochlorine pesticides (OCP's) from contaminated soil. Support was provided to develop appropriate sample preparation procedures for GC/MS and GC/ECD OCP's analyses. In addition, a procedure based on RCC methodology was developed for residual triethylamine in soil. Samples are presently being analyzed for residual triethylamine.

- Miscellaneous:

The dual FID gas chromatograph was modified to allow the use of the packed column required for triethylamine analysis. The ECD, which was installed on the primary headspace instrument, was installed in another gas chromatograph to allow its use as back-up for the primary GC/ECD.

Work on the mummichogs (*Fundulus heteroclitus*) project continues. The interim sacrifice for Test 103-001 (*Fundulus heteroclitus*) was postponed pending histological analysis by EPL of non-test fish.

Work continues to optimize the aquatic laboratory facilities. Eggs were collected, reared and hatched for medaka colony renewal.

MARCH 1-31, 1994

Work continues on investigation of methods available for identifying biomarkers of hepatocarcinogenesis for adaptation to fish models. Bioquant Image Analysis of liver areas of fish from tanks 1,2 (control), 3,4 (10mg/L DEN), tanks 5,6 (100mg/L DEN), and tanks 13,14 (10mg/L DEN, 1mg/L TCE) from sacrifice point Group D are completed. Work also continued on the statistical portion of the carcinogenicity study. However, software problems have hampered the progress; these problems should be rectified within a couple of weeks. Retrieval of library cell proliferation articles into Ref-Man continues. Slides from Test EE1 from histopathology were archived and slide sections for Image Analysis marked.

No work on chemical carcinogenesis in medaka was conducted this month. The exposure room is still in the process of being remodeled.

The article "A Comparison of the Toxicity of the Sensitivity of Rapid Toxicity Screening Tests" submitted for publication in *Environmental Toxicology and Chemistry* was resubmitted after addressing reviewers' comments and recommendations.

Work continued on teleost immunotoxicology methods development. Work on the species comparison and interlaboratory methods standardization projects continued with presentation of data at the 33rd annual meeting of the Society of Toxicology in Dallas, TX. Both poster presentations were well received, stimulating discussions and interest in our work. In preparation for future work the laboratory was rearranged to accommodate an isolated area for bacteriological studies. Necessary S.O.P record logs have been coalesced and record keeping instituted. Method comparisons of NBT assays were undertaken as well as media optimization. The fish population for the methods standardization testing was changed from retired breeders to naive (untreated; not culture fish), 8-month-old fish (hatch date 6/14/93 - 6/16/93). Naive group of fish was not as "healthy" as the retired breeders. This observation was based on appearance, kidney harvests, and cell yields. Naive fish yielded only 2.4×10^7 and 7.2×10^6 cells/ml per 60 fish taken. Data from past experiments with retired breeders yielded the same numbers of cells with only 40 fish of comparable weight. Necropsy of fish from several age groups, and bacterial culture and isolation from major organs (kidney, liver, abdominal wall, etc.) are currently underway to determine if a health problem is developing in the medaka in culture for future immunotoxicological studies. SOP-required documentation continues to be integrated as part of laboratory procedures for the immunotoxicology laboratory. Planning was initiated for the 2nd Annual "Modulators of Fish Immune Responses"; meeting is scheduled, for the second week of July 1995, in Breckenridge CO. Members of the organizing committee for the next meeting are Joanne Stolen, Christopher Bayne, Doug Anderson, Steve Kaattari, Judy Zelikoff and Lorraine Twerdok. Compilation of video presentations from the 1993 meeting is on hold awaiting the final ELISA video from Dr. Steve Kaattari at VIMS.

Work continued on analytical chemistry methods development. Preparations for a technical report on the analysis of N-Nitroso-N-ethylurea (ENU) by high performance liquid

chromatography and its stability in salt water were begun. Experiments with ENU showed good stability in an acidic buffer while a neutral and basic buffer showed rapid degradation.

The move of the Analytical Chemistry and Support Trailers to Aberdeen Proving Grounds was completed; the move went well. Preliminary testing of equipment is complete and more extensive testing will be undertaken at the time the trailer is put into full operation. Preliminary planning for West Branch Canal Creek Biomonitoring Trailer Study was initiated this month.

Analytical chemistry support for Rocky Mountain Arsenal included the following:

- Perkin-Elmer GC/PID-HALL:

Installation of this instrument has been completed. The purge and trap system has been connected to the GC, and all remote ready/start outputs have been properly configured. In order to fully automate the system, it was necessary to remove the data processing board from the GC. The on-board system caused bus errors when the instrument was used in a multiple run configuration. In addition, the board was redundant, as all data processing is accomplished via the OMEGA software. A new nickel reaction tube was installed in the Hall detector. The solvent pump for the detector had "seized" after being idle for such an extended period. The pump was dismantled and repaired, and is now operational. The process to certify this instrument for the analysis of benzene, toluene, ethylbenzene, and xylenes has been initiated. Plans are to certify this instrument for the analysis of methylene chloride and the trihalomethanes as well. Certifying this instrument for these analyses will allow additional routine analyses to be brought back in-house.

- Organosulfur Compounds:

Certification to allow the use of standards prepared directly rather than standards which have been extracted with the sample batch has been initiated. Experimentation with solid phase extraction techniques continues.

Work on the mummichogs (*Fundulus heteroclitus*) project continued. Test 103-001 was terminated early due to incidence of parasitic disease discovered during histologic examination of non-test fish. Decontamination and cleaning measures were taken. Histology samples and feed samples will be sent to testing laboratories.

Work continues to optimize the aquatic laboratory facilities. Maintenance and quality assurance continues on all culture and test fish. Construction on room #9 required removal of equipment and supplies from that area; these supplies are being temporarily stored in the tank room. Medaka were shipped to Dr. Judy Zelikoff at New York University, Dr. Malins at Pacific Northwest Research Foundation and Dr. Winn at Duke Marine Laboratory.

APRIL 1-30, 1994

Work continues on investigation of methods available for identifying biomarkers of mammalian hepatocarcinogenesis for adaptation to fish models. Bioquant Image Analysis of liver areas of fish from Tanks 7 & 8 (0.1 mg/L TCE) and Tank 9 (10mg/L DEN, 0.1 mg/L TCE) was completed from sacrifice point Group D. Bioquant system was down for 2 weeks this month due to software installation problems as well as optical drive inaccessibility. The problem was finally resolved by removing the computer memory board and reconfiguring the optical disk. Test EE1 and now Test 401-001R (Beach Point trailer BRDU study) slides were received back from histopathology lab labeled and archived. Materials and supplies were ordered and received for Apoptosis Detection Kit. The feasibility of staining archived tissue from Tests EE1 and 401-001R using this kit is being investigated. Cell proliferation articles were entered into Ref-Man.

Studies of chemical carcinogenesis in medaka are still suspended due to remodeling of exposure facilities in rm 10.

Work on teleost immunotoxicology methods development has been temporarily suspended in order to institute health screening for medaka cultures. Due to low cell yields of medaka anterior kidney cells, representative samples of fish across all age groups were necropsied and preserved for histopathology. The existing contract with EPL is being revised to include health screening histopathology to be performed every three months. Necropsy specimens of liver, spleen, kidney, fin, skin scrape and gill showed indications of septicemia. Tissue samples were plated for bacterial growth and sent to Maryland Medical Labs for analysis. Final report will be prepared upon receipt of all data. Preliminary observations suggest the phenomena may be caused by stress-induced chronic infection of *Pseudomonas spp.* and/or *Aeromonas spp.* Overall fish mortality has not changed significantly. Rapid diagnostic approaches are being studied for use in our own laboratory at the quarterly health screen planned for medaka. Differential and selective agars have been prepared and the formula for another selective agar has been obtained from Dr. Jeff Teska, U.S. Biological Service, Kearneysville, WV. A training program for technicians was also discussed with Dr. Teska. A cytochrome-c assay was attempted using fish from August 1993 hatch but significantly lower cell yields aborted the assay. Gram stain slides were prepared from these kidney cells for assessment.

Two shipments of medaka went to Dr. Zelikoff (immunotoxicology) at N.Y. University Medical Center this month. Each shipment contained 75 fish.

Work continued the preparation of a technical report on the analysis of N-Nitroso-N-ethylurea (ENU) by high performance liquid chromatography and ENU stability in salt water. The development of a method to analyze low levels of Aldicarb, Aldicarb sulfoxide, Aldicarb sulfone, Atrazine and Simazine in water by HPLC was begun. These compounds are representative of some of those included in the California pesticide/fertilizer mix being tested using Fetax.



Analytical chemistry support for Rocky Mountain Arsenal included the following:

- Perkin-Elmer GC/PID-HALL:

Certification for BTEX analysis on this instrument has been put on hold. A contractor working for Jacobs Engineering is using the instrument to provide support for a soil-gas sampling project.

- HPLC Method for Lewisite as 2-Chlorovinylarsenous Acid (CVAA):

The flow cell for the DAD was received and installed. The installation and operational check of the HPLC was completed. A procedure for Lewisite as CVAA developed by Dr. Paul C. Bossle (Aberdeen Proving Ground) was modified for use in this laboratory. Modifications were made to the mobile phase to eliminate the use of acetonitrile. Modifications are also planned for the soil extraction procedure. Results to date look very promising. The next step is to use this procedure on samples which the laboratory has been unable to clear for Lewisite due to possible interferences with the Headspace-GC procedure. Once it has been determined whether the HPLC procedure is free from these interferences, the potential exists for many areas of the Arsenal which show possible Lewisite contamination to be cleared.

A saltwater flow through system for the transgenic Fundulus holding post-exposure was built. Several design modifications to our freshwater apparatus were necessary to ensure success. Two 80-gallon reservoirs were purchased for saltwater preparation. A teflon air float was attached to each aeration line because the air pressure was not sufficient to aerate more than ~18" from the surface. A "sinker" was attached to the pump line to consistently pull feed water from the bottom of the tank. (The sinker was an extra airstone clamped to the line with a teflon clamp). Timely mixing of the salt with the water was not achieved by either aeration or overhead stirring. A circulator was found to work well, and the black rubber cord was covered with silicon tubing. The planned flow of one tank turnover a day was not sufficient, and we have now gone to two tank turnovers per day. Transgenic Fundulus and medaka arrived on 4/21/94. In addition to creating the special flow-through system for the Fundulus, a separate tank was prepared for the transgenic medaka. This is the first of several inter-agency projects (NIEHS and BRDL).

Work continues to maintain and optimize the aquatic laboratory facilities. Medaka eggs were reared in Prepared Synthetic Medium this month on the advice of the immunotoxicology group. All egg culturing seemed routine, until fry hatch. The fry were bent (or "wavy") and were all discarded. Several mini-experiments will be run to determine if the bending is pH related. The tanks for the April fry will be left in place for May fry. Laboratory maintenance was performed on all culture (medaka, guppy, and killifish) and test fish (medaka). Daily activities included three times a day feeding of fish and associated record keeping, tank cleaning, siphoning, and maintenance of live foods (brine shrimp and microworms). Weekly



activities included water quality analysis.

In support of the Environmental Engineering Division at Rocky Mountain Arsenal (RMA), the following is reported:

Tasks Performed:

- Analytical Review and support pertaining to waste water encountered by a contractor during demolition of the acetylene (C_2H_2) gas above ground storage tanks.
- Health and safety procedures development.
- Attended an OSHA 500 course.
- Research locations for a hazardous and non-hazardous debris storage area.

Tasks Continuing:

- Waste management policy review and development.
- Review monitoring well locations and perform a cross comparison to RMA site history such as storage areas, burial sites, landfills, reported spills, production areas, and ground water flow.
- Assistance with developing of statements of work and health and safety programs.
- Revise RMA's standard operating procedure for Remediation Field Inspectors.
- Asbestos containing material (ACM) disposal options for the tank demolition projects.
- Become physically familiar with Rocky Mountain Arsenal (RMA), since the Bald Eagle Management Areas (BEMA) locations are open.
- Review and aid in the development of an asbestos disposal program.

Tasks Delayed:

- Confirmation for debris storage area.
- Continuing with monitoring well cross reference.
- Collecting data pertaining to Basin F.
- Review the effectiveness and improve current systems for drum and/or hazardous materials containers auditing system.
- Collect data regarding solvent extraction system for soils.
- Collect data regarding the test burn of the mustard pits.
- Collection of data from the North Plants remediation efforts.

As of 13 April 1994, Mr. Muehlmann and Mr. Baughman lost their office privileges. This means as of the specified date the two GEO-CENTERS, INC. personnel have no or limited access to computers, telephones, and everyday office furnishings. Pertinent portions of the contractual requirements for Government Furnished Support have been sent to RMA, and



local government assistance has been sought.

Risk Assessment work at Rocky Mountain Arsenal (RMA) continued with familiarization with the history of chemical contamination at the various sites. This history is key to helping complete the Endangerment Assessment (EA), which is expected to be completed in late fall of this year. To complete the EA, a Supplemental Field Study (SFS) will be conducted to collect additional samples. The SFS is to be conducted by Enserch Environmental; however, the Army has requested that GEO-CENTERS observe this effort to ensure valid data collection and to advise the Army on methods employed and improvement recommendations. Risk assessor attended a Superfund workshop in Boston on short notice; OSHA training is scheduled for mid-May to enable participation in the SFS in the contaminated areas.

MAY 1-31, 1994

Work continues on investigation of methods available for identifying biomarkers of mammalian hepatocarcinogenesis for adaptation to fish models. Bioquant Image Analysis of liver areas from Group D sacrifice was completed. A brief summary report of materials & methods and data from groups B & D was written. After review of data, it was decided to evaluate areas from sacrifice point J next. The final statistical analysis for sacrifice B and D for the carcinogenicity project (EE1) was completed. Current efforts will be continuing on the EE2 study. All data analysis was done with the SPSS program. Samples of Test EE1 (unstained slides) were shipped to GCRL for PCNA staining. All materials for apoptosis staining were received with a Ref-Man line search done on apoptosis. References and information gathered was given to a summer student who will be working on this project. The first draft of a techniques paper on using BrdU as a biomarker of cell proliferation in Japanese medaka was completed. The BrdU portion of West Branch Canal Creek Carcinogenicity Study for coordination of fish sacrifices with FCRF histopathology lab is currently being reviewed. The paperwork and supplies needed for this study are being evaluated. Work continued on maintaining literature database on "Cell Proliferation"; articles continue to be entered into Ref-Man.

Work on chemical carcinogenesis in medaka resumed this month. An Animal Use Protocol for the proposed Canal Creek Study at Aberdeen Proving Ground was written and submitted. This groundwater study with medaka (Test 401-002R) will be run on-site in our biomonitoring trailer. There will be two studies running concurrently, each with a different diluent water. Preliminary range finds for the 32-tank test will begin in early June. A 96-hour LC50 with medaka is also part of the study design. The chronic carcinogen study should begin in July and will include the use of the cell proliferation marker, bromodeoxyuridine (BrdU). The protocol was approved at the May 24, 1994 meeting of the BRDL LACUC.

Work on teleost immunotoxicology methods development continues. Experiments are still temporarily suspended due to low cell yields of medaka anterior kidney cells. Fish mortality has still not been observed; however cell yields are declining in fish from the 8/93

and 11/93 hatches. All necropsy work and bacterial sampling in culture and immunotox fish was completed for our initial health screen. In a continuing effort to investigate the problems with the medaka, water samples were collected from different sites in the flow-through intake water system and plated on selective and non-selective media. Overabundance of bacteria in the culture water was suspected as a possible factor in degenerating health conditions. Positive growth plates were sent to a reference laboratory identification and drug sensitivities; we are still awaiting results. Bottom water samples were also drawn from random tanks to be tested for nitrites. Results were negative on all samples ruling out high nitrite concentrations as a toxic contaminant. Pertinent literature is being read to gain further information. One possible course of action would be treatment with potentiated sufonamide (Romet 30), a drug most bacteria isolated from the medaka were killed by in the sensitivity screening performed by Maryland Medical Laboratories. Drug-treated fish food has been ordered for a trial treatment using "Immunotox fish" from the 8/93 and 11/93 hatch dates. Thirty fish were fixed and will be sent to EPL for histopathology evaluation once the "Fish Health Screening" task order and purchase requests are approved and processed. A report will be prepared upon receipt of histopathology data. Once this report is prepared, an appointment with Dr. Vicki Blazer, U.S. Biological Survey, Leetown, VA, will be make to aid in evaluation of fish overall health status. The transfer of equipment and supplies from laboratory room 7 to laboratory room 111 was completed. The allocation of room 111 as a immunotoxicology laboratory will allow much more efficient flow of work effort between the new lab and immunotoxicology laboratory room 122. A schedule for the immunotoxicology species comparison project detailing a realistic amount of time needed to complete the project was prepared. The project should be finished by August 1994. Three posters ("Conservation of the Nonspecific Immune Response: A Species comparison"; "Medaka Fish as a Model for Immunotoxicological Field Studies: Methods Development and Standardization"; and "A Fish Model for Predicting the Immunotoxicity of Aquatic Metal Pollution") were presented at the "Alternatives in the Assessment of Toxicity: Theory and Practice" at Aberdeen Proving Ground, MD on May 25, 1994. Work was initiated on the first draft of a manuscript on "Methods Standardization" from present research efforts for the *Journal of Applied Toxicology*.

Two shipments of medaka went to Dr. Zelikoff (immunotoxicology) at N.Y. University Medical Center this month. The shipments contained 370 (2/24/94 HD) and 50 (6/15/93 HD) fish, respectively.

The analysis by HPLC of 11 salt water samples for an exposure of N-Nitroso-N-ethylurea (ENU) was performed. A method was developed to analyze the compounds included in the California pesticide/fertilizer mix; this method will detect low levels of Aldicarb, Aldicarb sulfoxide, and Aldicarb sulfone in water. The Atrazine and Simazine compounds also included in the test mixture and originally planned for HPLC analysis will be analyzed by gas chromatography to improve the detection limits. The concentrations tested did not produce the expected endpoint, therefore, higher concentrations will be tested in the future. Precision and accuracy data will be performed for this method when an appropriate range for the test mixture is found. A new diode array detector (DAD) and Chemstation for the HPLC was received. Training on the tutorial provided has begun on this instrument. The Chemstation will provide

automated data analysis and allow for remote control of pumps, autosampler and other accessories on the HPLC.

Test 103-002 was begun. This test used transgenic *Fundulus heteroclitus* from NIEHS that were approximately 2 inches long. Two sets of six fish were exposed to 100 mg/L ethylnitrosourea (ENU) for one hour. A third set of six fish were handled as the treated fish without ENU exposure. The fish are being held in Room 22 in the flow through salt water system described in last month's report.

Work continues to maintain and optimize the aquatic laboratory facilities. Six thousand (6000) medaka eggs were cultured this month in well water. Approximately 5000 fry hatched from this batch; this is an unusually high hatch rate of 85%. Fry from this hatch are designated for the breeding colony, immunotoxicology research, range finding for Test 401-002R, and the 96 hr. LC50 for Test 401-002R. The lab was cleaned for the MRDC general's tour in mid-May. Office space for was allocated on the second floor and relocation took place in mid-May.

JUNE 1-30, 1994

Work continues on investigation of methods available for identifying biomarkers of mammalian hepatocarcinogenesis for adaptation to fish models. Analysis of data from the in-house BrdU study continued; all raw data for each fish was prepared for statistical analysis by the Naval Post Graduate School in Monterey, CA. Also an investigation of past experiments involving TCE was conducted to identify other possible water parameters that could produce hepatic neoplasms. Consulted with a representative from EPL to sex fish from sacrifice D. Revisions were made on BrdU Materials & Methods paper. A copy was given to the senior author for review. Supplies were ordered and paperwork started for the BrdU portion of West Branch Canal Creek Carcinogenicity Study coordinating paperwork and sacrifice timepoints with FCRF histopathology lab. Slides continued to be archived that were received for Test EE1. Cell proliferation articles were entered into Ref-Man.

Studies of chemical carcinogenesis in medaka continued. Supplies were prepared and shipped for the biomonitoring trailer at Aberdeen Proving Ground. This included items related to fish care for thirty-two tanks of fish: food, nets, siphon hoses, drains, nets, scrub brushes, and related items. Due to a change in Test 401-002R, a survival study was necessary. Eighty fry were placed in an exposure pail setup and monitored for survival after 48 hours. No mortality was observed. This allows us to combine the BrdU fish with the chronic assay fish. The new number of fish in each exposure vessel will be eighty. Seven thousand eggs were pulled from the medaka breeding colony for Test 401-002R, the West Branch Canal Creek Groundwater Study with Medaka. These eggs were cultured in well water for eight days. After hatching, the fry were randomized to the 36 test vessels.

Work on teleost immunotoxicology methods development contnues. Due to the health problems in our medaka, most work performed this month did not include animal use.

Progress on the species comparison project included developing two protocols to measure the levels of alpha-naphthyl acetate esterase and myeloperoxidase (cellular enzymes) in cells. These protocols still need to be tested. Arrangements to obtain rainbow trout from NBS, Leetown, WV, to finish the species comparison project were completed. Efforts to compile all of the species comparison data sets has begun. This work will aid in identifying the best way to express data and determine the need for future projects. Work began on determining the LC_{50}'s of *Aeromonas salmonicida* in medaka. Prepared bacterial medias for ID and quantification of *A. salmonicida* other bacteria endogenous to medaka. Pilot LC_{50} studies will begin next month, providing that plumbing of well water into rm 122 is completed on time. Articles pertaining to Immunotoxicology were added to a reference manager information system (Refman) to facilitate easy access for investigators. A one-day seminar conducted by National Capital Area Chapter of the Society Of Toxicology in Baltimore, MD on aquatic models in toxicology and ecotoxicology was attended. The morning session was organized and chaired by Dr. Lorraine Twerdok. Personnel training was conducted to orientate and in-process a new student hire. The training consist of general laboratory skills to enhance performance on various projects and to conduct the weekly fish health screen. Assistance was given to the aquaculture labs to aid in the randomization process for a large-scale study in the mobil laboratories at Edgewood Arsenal, Edgewood, MD.

Continuing efforts were made to understand the health problems currently affecting the medaka fish population. Met with Drs. Anderson, Blazer and Teska from NBS, Kearneysville, WV, to discuss data and obtain advice on determining the possible causes and solutions to the fish health problem. NBS scientists suggested that the health problems we are observing are most likely stress-related. Possible causes of the problem could be inbreeding, vitamin and/or other nutritional deficiencies, or stress from culture room traffic, noise, and observation. Recommendations on ways to treat the problem from will be discussed at a meeting of all aquaculture personnel on July 6, 1994. New parameters were added to the health screen being conducted on a weekly basis with the 111093 hatch fish to provide a database for comparison in future assessment. Information on the effects of stress on fish has been reviewed and new recommendations for improved fish husbandry will be discussed at the July 6th meeting with aquaculturists. Two cytochrome-*c* assays to determine superoxide anion generation were aborted due to failure to obtain an adequate number of cells from the anterior kidney to proceed. Slides were made using the cytospin method and stained for esterase, myeloperoxidase, and as reference slides. A task with EPL for health screening histopathology on a quarterly basis was initiated; fixed samples from the April health screen were sent to EPL in mid-June for processing and evaluation. The next quarterly health screen for all medaka in the culture facility is scheduled for mid-July. This screening will include: histopathology, necropsy and bacterial sampling and blood work.

The development of a method to analyze low levels of Aldicarb, Aldicarb sulfoxide, Aldicarb sulfone, Atrazine and Simazine in water by HPLC was completed and approximately 60 samples in FETAX were analyzed. Atrazine and Simazine were compounds included in the California test mixture being examined by FETAX. In order to analyze for these compounds a method to improve the detection limits of Atrazine and Simazine must be developed. The

concentrations tested did produce some malformations, but higher concentrations will be tested in the future. When an appropriate range for the test mixture is found, precision and accuracy data will be performed for this method.

Method development for the chemical analysis of the Iowa test mixture in FETAX began this month. This mixture contains Alachlor, Atrazine, Cyanazine, Metolachlor, and Metribuzin. The laboratory was able to obtain standards for all of the chemicals with the exception of Metribuzin, therefore, no analysis will be performed on this chemical. Although these compounds are similar in structure, adequate separation was obtained for the compounds of interest. The concentration range being tested has not been determined and detection limits may be a complication.

Full time work on the project area "Rapid Toxicity Assessment in Killifish," began this month. New hatching methods for killifish (*Nothobranchius guentheri*) were developed to alleviate previous control mortalities associated with prior test methods. An addendum to the existing protocol was drafted to correct for control mortalities. Routine laboratory maintenance and experimental preparation were performed to prepare for killifish testing. Preliminary testing of new hatching methods will begin the first week in July, contingent upon receipt of eggs from Dr. Hull. Water quality was also performed to verify the suitability of the modified soft water for testing. Using last summer's data (1993) as a guide, new concentration levels were set for the killifish tests to allow for more precise measurements of the LC_{50} and EC_{50} for each toxin tested. Concentration levels also had to be adjusted and calculated to compensate for concentration fluctuations associated with new hatching and handling methods. Verification of correct concentrations will be reviewed by in-house chemists and project PI, Tom Shedd, prior to initiation of tests. Additionally, laboratory assistance was given in support of the Japanese medaka (*Oryzias latipes*) test for Edgewood Arsenal. Egg collection and randomization procedures were performed.

Work continued on the "Lettuce Rapid Toxicity Test." Lettuce seeds were exposed to sodium dodecyl sulfate (SDS) in the laboratory for four days; root elongation in the 24-hour period between day three and four was measured. Several parameters were changed to adapt this assay to our biomonitoring trailer, and preliminary results indicate that this will not affect the sensitivity of the test. Statistical analysis of the data has not been performed but the results are similar to earlier results.

Three shipments of medaka were shipped to NYU in June and one shipment of fundulus was sent out to NIEHS. This shipment of fundulus consisted of all fundulus at BRDL. The salt-water culture area will be temporarily vacant until receipt of more test animals from NIEHS.

A shipment of eleven tanks of transgenic medaka was received from NIEHS. As there was no bath space available, a benchtop was pressed into service as the fish holding area. Flow-through tanks were set up for each separate batch of fish. This brings the total of transgenic medaka tanks to twelve including the shipment received in April.

Work continues to maintain and optimize the aquatic laboratory facilities. A well water sample was prepared and shipped for analysis by an outside contractor. An identical sample was taken for in-house analysis of trace metals; in-house results will be compared to results from the contracted analysis. Four kinds of fish food were prepared and shipped for a semi-annual analysis (food certification) by an outside contractor. Laboratory maintenance was performed on all culture (medaka, guppy, and killifish) and test fish (medaka). Daily activities included feeding fish three times daily plus associated record keeping, tank cleaning, siphoning, and maintenance of live foods (brine shrimp and microworms). Weekly activities included water quality analysis. Document registers were set up and maintained for all purchasing within the aquaculture labs. Maintenance of fundulus ended with the shipping out of fish to NIEHS. Maintenance of the transgenic medaka, and preparation for the Edgewood Arsenal test is ongoing.

JULY 1- SEPTEMBER 30, 1994 (Government approved Quarterly Progress Reports in lieu of Monthly Progress Reports)

Work continues on investigation of methods available for identifying biomarkers of mammalian hepatocarcinogenesis for adaptation to fish models. The work on the carcinogenicity project consisted of completion of a data collection notebook for the EE1 study containing raw data, graphs and statistical analysis. Analysis of Group J livers was started for Test EE1. Work on the Aberdeen Beach Point Study included data collection from 4 tanks (test 400-001R); data collection from sacrifice point A (BrdU study), and the tabulation of all water chemistry and mutagenicity data from the project. Slides received from Tests EE1 & 401-001R were archived. A 72-hr BrdU exposure on extra fish for gut controls for histopathology was performed. Paperwork and planning and gathering of supplies for BrdU portion of West Branch Canal Creek Carcinogenicity Study was undertaken. The paperwork was completed for Test 401-002R and vials and cassettes for final BrdU trailer exposure were labelled. The fourth and final 72-hour BrdU exposure at the biomonitoring trailer at Aberdeen Proving Grounds (West Branch Canal Creek) was also completed. The glassware from BrdU trailer exposure was cleaned and a binder notebook for the BrdU portion of Test 401-002R was put together. Remaining slides were marked from Test 401-001R for subsequent Bioquant Analysis. An updated version of Bioquant was installed on the image analysis computer at USABRDL. However, installation of the new Bioquant system at the Fort Collins, CO, location was postponed because the furniture for the image analysis system has not, as yet, arrived. Additionally, assistance was given to the Hood student, Tomoko Nii, with apoptosis project when needed, such as providing slides, literature, and ordering supplies.

Studies of chemical carcinogenesis in medaka continued. Work continues on Test 401-002R, the West Branch Canal Creek Groundwater Study with medaka. Due to water chemistry, doses for the test have been changed to 1%, 5%, and 25% groundwater. We will also be using water from Well 27 B instead of 27 A. Eggs were pulled on 7/18-19 and cultured until hatch on 7/27. Five thousand fry were compiled in one 10-gallon tank for overnight. The tank was flow through and in a 25°C water bath. Randomization occurred on



7/28 with help from laboratory personnel in all technical areas. DEN exposure was August 9-11, and the on-site exposure to test water began on August 12.

Work on teleost immunotoxicology methods development continues. Due to the health problems in our medaka, project related definitive studies were not conducted during this quarter. Progress on the species comparison project included preliminary assays to measure the levels of myeloperoxidase in cells. Efforts to compile all of the species comparison data sets was completed. This work will aid in identifying the best way to express data and determine the need for future projects. Work continued on determining the LC_{50} of *Aeromonas salmonicida* in medaka. SOP for procedures and techniques to be followed when working with fish pathogens was written, reviewed, approved and circulated to all technical laboratory personnel. A large amount of effort was spent setting up the aquaculture system in 122b. Water quality data was collected on acclimation tanks for 16 days consecutive days to verify stabilization of the parameters; the laboratory SOP schedule for water quality analysis was then put into affect for aquaria in rm 122b by immunotoxicology personnel. *Aeromonas salmonicida* stock acquired from NBS on 8/93 was biochemically characterized. Stocks had maintained infective characteristics as assessed by pigment characteristics on TSA and TSA+BCC, TSI agar and mobility assays. Stock was amplified and frozen down in 1 ml aliquots in T-S broth from 24, 48, 72, and 96 hr growth points for use in future studies. Results from the first *Aeromonas salmonicida* LC_{50} pilot experiment was disappointing; no mortality was observed. Possible reasons under investigation include extended freezing time of bacteria, loss of infectivity in bacterial cultures at room temperature (infectivity can be lost in as little as 48 hrs), or excessive population passage at room temperature. The pilot study will be repeated using fresh *Aeromonas salmonicida* stocks (from NBS, Kearneysville, WV) which have demonstrated virulence in brook trout.

Esterase and Myeloperoxidase immune organ stains were performed this month on three separate groups of fish kidneys. Slides will be characterized for positive or negative activity. Results from these studies will be included in the poster currently in preparation for presentation at SETAC meeting, October 31 - November 3, in Denver, CO. Three weekly health screens were done in July and August on 11/93 and 02/94 hatchdate lots of medaka which indicated an inability to stimulate the cells with PMA. Testing has begun on 5/94 fish that have been held in 20 degree water temperatures and fed brine shrimp are scheduled for experimental use. Optimization of 5' Nucleotidase assay using a microtiter plate method continued. We would like to incorporate this assay into our battery of inflammatory cell function assays planned for next month (n=4) with naive fish 6-8 months of age (*i.e.* not treated or used for breeding). Assistance was given to the aquaculture labs to aid in the randomization process for a large-scale study in the mobile laboratories at Edgewood Arsenal, Edgewood, MD. Articles pertaining to immunotoxicology were added to a reference manager information system (Refman) to facilitate easy access for investigators. Two abstracts (one first author, one co-author) were written, reviewed, approved and submitted for presentation at the Society of Toxicology Annual Meeting scheduled for March 5-9, 1995, in Baltimore, MD.

Continuing efforts were made this quarter to understand the health problems currently affecting the medaka fish population. Recommendations on ways to improve overall culture health status were discussed at a meeting of all aquaculture personnel on July 6, 1994. New culture procedures designed to minimize stress in our culture animals were drafted into a memo [memorandum SGRD-UBS (70)] by the Associate Director for Research and distributed for immediate implementation in mid-July. One additional change was made to medaka culture for immunotoxicology study fish only: brine shrimp feeding was increased from 3X weekly to daily beginning in □mid-August. Weekly health screening of fish from 8/93, 11/93, 1/94 and 2/94 hatchdate lots of fish continued through September. Results from assays performed in June, July and August (anterior kidney cell yields and immunotoxicological endpoints) were discouraging; which lead to the decision not to use any fish from the 11/93 hatch for definitive immunotoxicological studies. However, there was a remarkable improvement in fish of all ages examined in September, so we hope to get back on track with immunotoxicological methods development studies soon. The third quarterly health screen for all medaka in the culture facility was conducted in the third week of July. The next quarterly health screen is scheduled for early November. This screening will include: histopathology, necropsy and bacterial sampling and blood work. Data analysis for all health screen data is in progress, including assembly of SPSS databases. We are working with Dr. Don Gaver of the Naval Post-Graduate School in database design and data analysis. Details on routine health screening in the medaka were presented at the Sixth Annual Research Review. Preliminary discussions were initiated between Drs. Robert Finch, L. Twerdok and Paul Bowser of Cornell University, to investigate the feasibility of collaborating on work to characterize and evaluate the viral health status of medaka. Discussions in the same vein, with respect to bacterial health status of medaka, were also initiated with Dr. Jeff Teska of the National Fisheries Academy (NBS, Kearneysville, WV). Additionally, a holding area was set up in room 111 for incoming fish for health screen/status comparison studies. Fifty 6-8 month old medaka were received from Gulf Coast Research Labs (Ocean Springs, Mississippi) and assayed immediately (n=3; 16, 16, and 18 fish, respectively) using our weekly health screen protocol. GCRL fish were apparently healthy; weight, length, anterior kidney weight, hematocrit and leukocrit were within medaka normal ranges established to date. Anterior kidney cells isolated from GCRL fish exhibited vigorous extracellular superoxide anion production upon *in vitro* stimulation with phorbol ester. Future projects include acclimation studies with GCRL fish, as well as similar projects with medaka from different labs in the USA, so as to establish species "norms" for health screening and immunological parameters of interest.

Analytical method development and analysis of chemicals used in carcinogenicity and mobil laboratory studies continued. Samples of tap, creek and ground water that will be used in the BrdU exposures at Aberdeen Proving Grounds were received. The exposure concentrations of 0, 1, 5 and 25 percent ground water in tap and creek water were prepared. The test concentrations were then spiked to contain 75 mg/L BrdU to examine the stability of the compound in the test matrices. The samples were analyzed at O and 72 hours and it was found that the concentration of BrdU decreased in creek water but remained fairly constant in the tap water. The pH of these samples was also examined and a very slight decrease in pH was found over the 72-hour period. The analysis of approximately 200 samples of BrdU by



HPLC was completed in support of the exposure of medaka at APG. Training was received on the Hewlett Packard 7686 Prep Station for the HPLC. Difficulties with the interface between the 1050 HPLC autosampler and the Prep station continued and the technical service department of Hewlett Packard is working on a solution and will return to repair the interface and provide more software training.

Method development for the chemical analysis of the Iowa test mixture in FETAX continued. A method was developed for the analysis of Atrazine and Simazine, compounds which were included in the California test mixture being examined by FETAX. Controls and stocks from the first range find were analyzed by this method. Due to dilution of FETAX when stocks are prepared in DI water the test protocol was modified to use FETAX to prepare the stocks in future pesticide/fertilizer mix tests. With higher test concentrations of Atrazine and Simazine it may be possible to analyze all of the pesticides in the California mix using one method. When an appropriate range for the test mixture is found, precision and accuracy data will be performed for this method. Approximately 50 samples for the Iowa test mixture were analyzed, which contains Alachlor, Atrazine, Cyanazine, Metolachlor. The tested range for this test mixture was too high and lower concentrations (<25X) will be used for the next range find. The concentrations that will be tested are below the detection limits of the method, but stocks and blanks can still be analyzed. Blanks and stocks for the Iowa test mixture being used in the FETAX assay are currently being analyzed.

Methods development for the chemical analysis for compounds scheduled for killifish (*Nothobranchius guentheri*) testing were initiated this quarter. A series of compounds (trinitrotoluene, phenol, pentachlorophenol, zinc, cadmium, and copper) being used in the exposure of killifish were analyzed. Each test includes a set of 38 samples. The method for the analysis of pentachlorophenol had to be altered due to difficulties with a column. The column was changed from a Waters C18 10 X .8 cm, 4 □m column to a Supelco C18 25 X .46 cm, 5 □m particle size. Due to the length and internal diameter of the new column, the flow rate was reduced from 2 to 1.5 mL/minute to reduce column pressure to an acceptable limit. Eluent composition, injection volume and wavelength used for detection remained constant.

Analytical chemistry support for Rocky Mountain Arsenal included the following:

Routine Analyses (Changes)

Approximately 170 additional samples were analyzed for volatile organics in conjunction with the Western Tier investigation and the water treatment POPS pilot study. Additional samples were received for DIMP analysis. We have resumed all DIMP analyses due to government personnel going on leave.

Method Improvement/Development

1. Extraction/Direct Injection Procedure for High Level Benzene in Water:
   - Approximately 50 samples were analyzed for the Enserch *in-situ* biotreatability study.



Other analyses are planned for the future. All parties seemed pleased with the analytical results obtained during this round. Results for a sampling duplicate were 4.3% different, while results for a method duplicate were 0.4% different.

2. Organosulfurs Analysis:
   - We are still awaiting QA/QC review of the data packet and approval for use. However, experimentation has continued to attempt to improve the extraction efficiencies and lower the method detection limit.

3. GC/MS Volatile Organics Analysis:
   - New standard materials and sorbent traps specified in EPA Method 8260 were received. New identification files were created and new calibration curves were prepared. The new traps appear to give superior performance. Development of an SOP is presently underway. This will allow future transition of the analysis to government personnel.

4. DIMP Analysis:
   - A column splitter was received and installed in one of the dual FPD gas chromatographs. This will allow simultaneous analysis and confirmation of DIMP from a single injection. The method with the above changes was taken through the certification process and a revised SOP was prepared.

Miscellaneous

Training is continuing to be provided to government personnel to transition the DIMP analysis. In addition, training is being provided to government personnel on the analysis of volatile organics via GC/MS. Work continues on data forms, checklists, and SOP's needed to bring the in-house laboratory into compliance with CQAP.

A week was spent in training at Oak Ridge National Laboratories. ORNL has developed a direct sampling ion-trap mass spectrometer for use in a mobile-lab setting. The instrument appears to work quite well. The unit was shipped to Rocky Mountain Arsenal in September.

Toxicology support for Armstrong Laboratory included the following:

Work in the development and use of the microdialysis probes continued as various potential concentrations of trichloroethylene (TCE) have been analyzed for future use. The required animal training protocol was approved in July, and initial *in vitro* use of the microdialysis probes has begun; results were encouraging. The animal use protocol for the dose route/matrix study of TCE was approved; training in mouse gavage techniques began in July and was completed in August. Additionally, the temperature telemetry transmitters were calibrated in preparation for training which commenced in August. Also, work began for the gas uptake system to be used for this project. Loss of the system due to a hardware failure

(leak) delayed data production. The new parts arrived at the end of September, and gas uptake data, along with mouse body temperatures, should be ready shortly.

The "1994 Workshop on Physiologically Based Pharmacokinetic/Pharmacodynamic Modeling and Risk Assessment" meeting was attended in August in Fort Collins, Colorado. This very intense and educational course on PBPK computer modeling not only served as an enhancement educationally in the field of Pharmacokinetics, but also provided an enriching perspective of the entire field. As a result of this experience, an abstract was submitted to present a poster at the Society of Toxicology 1995 Annual Meeting in Baltimore, MD. The models produced should show that there is a relationship between stratum corneum diffusion coefficients and temperature for halogenated hydrocarbons. A copy of the abstract is attached.

The PE Nelson system, which controls the GC used for this project, became disabled when a computer board failed due to a power outage. Repair is now complete, and the entire system is functional again. Some *in vitro* experiments with the probe were recently completed with unexpected results; experiments will continue in October.

Work continues with the investigation of various isoforms of cytochrome P450 in mouse and rat liver microsomes. Experiments have been conducted on the effects of TCE on nine classes of isoforms to date. Research is currently centering on the use of Paranitrophenol (PNP) as a substrate for the determination of P450 2E1 isoforms. PNP could replace the more hazardous Dimethylnitrosamine which has historically been used to determine 2E1 activity. The formation of trichloroethanol (TCOH) from CH, which is the next step in the metabolism of TCE, is also being studied. Quantitation of TCOH from rodent liver cytosol is achieved by electron-capture gas chromatography, with the HP 5890 GC. An additional portion of this study involves incubation of rodent hepatic cytosol with limiting concentrations of chloral hydrate at 37 degrees C for five minutes. TCOH is then extracted in ethyl acetate and analyzed from headspace by electron capture GC vs. an external TCOH standard curve. Michaelis-Menten parameters, K_m and V_{max}, were determined from the results. Data obtained from these experiments will be presented in a poster session at the 34th annual Society of Toxicology Meeting. An abstract has been submitted and is attached.

Work also continues on protocol writing/revision/editing for each of the cytochrome P450 assays. Based on the preliminary results obtained by another member of the team, experiments have been initiated to investigate the possible involvement of glutathione in DCA degradation. Work continues on training and development of animal handling skills by assisting and occasionally performing intestinal perfusion surgery. This is a relatively complex surgery and will require continued effort in order to perfect the technique. The purpose of the surgery is to ascertain the uptake of TCE into the blood stream when injected directly into the lumen of the small intestine of the test animal (Fischer 344 rat). A new Bechman spectrophotometer was purchased for the lab and training is underway to enable it to be used in these P450 assays.

Work on the project area "Rapid Toxicity Assessment in Killifish," continued this quarter. Due to an insufficient supply of killifish *(Nothobranchius guentheri)* embryos from Dr. Eugene Hull the majority of the month of July was devoted to multi-task laboratory functions. Support efforts were performed to aid in the re-outfitting and reorganization of rooms 9 and 10. Room 1 (the end basement closet) was reorganized and outfitted with all laboratory tools formerly housed in rooms 5 and 18. All laboratory tubing was relocated from the tank room and also organized in room 1. Support efforts were also performed to facilitate efficient functioning of all related *Oryzias latipes* activities in conjunction with the ongoing studies at Aberdeen. Various glassware cleaning activities were performed in support of research efforts. Water quality assistance was also given in support of laboratory objectives. *N. guentheri* laboratory facilities were relocated from room 22 to room 7 to comply with memorandum SGRD-UBS (70) relating to animal health in the laboratory. Room 7 was cleaned to upgrade laboratory hygiene and outfitted as necessary.

N. guentheri embryos were received from Dr. Eugene Hull on 19 July. Embryos were used to practice handling techniques as outlined in addendum TRS-1A2 to *N. guentheri* protocol. New handling techniques proved to be successful. Attempts were made to activate diapause II eggs into diapause III. Results are still pending. PCP and 1-octanol test assays were performed the week of September 12. Both tests displayed a positive dose response with low control mortality. An un-ionized ammonia test assay was performed the week of September 19, with a positive dose response and no control mortality. Chemical analysis was also performed on all ammonia test samples. Obligatory pretest chemical procedures associated with the complex acid-base chemistry of ammonia and ammonium chloride were also performed to facilitate the proper functioning of the test assay. Due to an unfortunate error by Airborne Express the malathion test assay scheduled for the week of September 26 was postponed one week due to shipping difficulties. The TNT test assay was performed as scheduled. Aid was given in the efforts of medaka embryo culturing due to down time that was created from the canceled malathion assay. Data compilation and analysis for all previously performed killifish assays were also initiated. Results are very positive with good dose response and low control mortality in all tests performed to date. Efforts to improve embryo shipment and holding have been ineffective to date but new methodologies with promising future successes will be explored in the upcoming month in conjunction with on-going inter-laboratory efforts with Orbis Scientific and Dr. Eugene Hull.

Comments from *Journal of Environmental Toxicology and Chemistry* were received on the article "A comparison of the sensitivity of rapid toxicity screening tests" (Toussaint [GEO-CENTERS, INC.] *et al.)* submitted in March 1994. The comments are currently being addressed and reviewed by co-authors.

The 24-foot aquatic biomonitoring trailer located on the north east side of building 568 was cleaned and upgraded to make it suitable for laboratory purposes. Reconstruction and recalibration of the ventilatory distribution system was performed to replace outdated technologies. All electrical circuitry of the 32 ventilatory chambers were reconditioned, repaired, or replaced as needed.

Work continues to maintain and optimize the aquatic laboratory facilities. Laboratory maintenance on all culture (medaka, guppy, bluegill, white sucker, and killifish) and test fish (medaka) located in rooms 5 and 18 was performed. Daily activities include three times a day feeding of culture and test organisms. Associated record keeping was performed on all in-house test organisms. Associated tank cleaning, siphoning, and maintenance of live foods (brine shrimp and microworms) was performed to facilitate ongoing laboratory efforts. Weekly activities also include the following water quality analyses; dissolved oxygen, pH, conductivity, water flow measurements, ammonia. Transgenic medaka were maintained. In July, 10,000 medaka eggs were pulled and cleaned, and fry were sorted and randomized for Test 401-002R (Aberdeen/Edgewood Arsenal - Canal Creek area mobile laboratory test). The diluter for the Aberdeen test was also set up. Assistance was given to aid in ongoing killifish acute toxicity assays; randomization and initial testing procedures were initiated for Ammonia and TNT test assays. In September, 3600 medaka eggs were pulled for new breeding stock. Document registers were set up and maintained for all purchasing within the Division. The preliminary responses to three Quality Assurance audits of Test 401-002R was drafted.

Fish shipped/received to/from other research facilities:

Number of medaka	Institution	Date	Method
200	NYU	7/18/94	shipped
150	NYU	7/27/94	shipped
114 + 50 (2 age groups)	NYU	9/6/94	shipped
50	GCRL	9/94	received
200	U of ME	9/94	shipped

The following meetings were attended by most, if not all, of the technical staff associated with USABRDL: in-house animal care/husbandry (fish and frogs) optimization meetings, July and September; laboratory-wide safety meeting, August 17, presented by Post Safety Office; Animal Use Seminar, 30 Aug 1994, presented by the Post LACUC Office (Col. Powell); and the Sixth Annual Research Review meeting at Ceresville Mansion, Frederick, MD, September 20-21. Additional meetings attended by individuals from relevant project areas included: informal animal care/husbandry optimization meetings at the National Fisheries Academy (NBS), Kearneysville, WV; the Foundation for Immunotoxicology Ninth Annual Meeting on Mechanisms in Immunotoxicology, Virginia Beach, VA, August 31 - September 2; and SERDP Semiannual Progress and Planning Meeting, USABRDL, Frederick, MD, September 22, 1994.

In support of the Environmental Engineering Division at Rocky Mountain Arsenal (RMA), the following is reported:

Tasks Performed:

- Collected and presented disposal option for RMA; collected information regarding disposal of non-hazardous and hazardous debris. Also, collected information for asbestos containing materials (ACM) disposal. The following information will detail various disposal options currently proposed to RMA:

  Debris non-hazardous/hazardous:
  - Fuel recovery of materials such as wood, road oil, etc.
  - Debris BDAT - researching various methods to treat different types of debris in order to exempt from subtitle C regulation. Collect and distribute information pertaining to a CO_2 pelletizer (DI-250). The instrument is used in lieu of sandblasting; however, the DI-250 uses CO_2 pellets instead of Al_2O_3 and does not generate extra waste during decontamination procedures.
  - Hazardous waste landfill (subtitle C).

  Asbestos:
  - In-state disposal versus out-of-state disposal.

- Regulatory review meeting: Wrote a position paper and established a regulatory review meeting with RMA personnel. The first meeting did not meet expectations. The attendance did not review the position paper (summary of the Debris Rule) prior to the meeting, which meant nobody was prepared for discussions. The purpose of the meeting was to review and share interpretations and agree upon a common interpretation of the regulation being reviewed.

- Presented options for oil and water separating. Remediation contractors have to remove heavier hydrocarbons (oils) from their used decontamination water prior to transporting to CERCLA Waste Water Treatment Plant for treatment.

- Telephones installed at GEO-CENTERS' field office located at building number 311.

  - Observed remediation contractors performing remediation and demolition activities.

  Contractors' activities included the following:

- Jacobs Engineering: worked on the pilot demolition project, work activities include: removal of the ethylene generators (EG) which were used to manufacture ethylene ($H_2C:CH_2$). Ethylene is a precursor of chemical agent Dichlorodiethylsulfide (mustard



agent). Jacobs dismantled the EG by means of hot cutting. The EGs are being salvaged for carbon steel and refractory brick. The non-salvageable materials such as asbestos gaskets are being disposed of at a TSCA landfill (This landfill is one of the in-state landfills recommended by GEO-CENTERS personnel for non-RCRA material disposal). Observed Jacobs performing pipeline characterization. The pipeline was used during the production of dichlorodiethylenefulfide. Jacobs did encounter some non-agent hot spots within the pipeline, which were determined to be mercury vapors. Note: mercury was used in the manometer which metered steam that supplied heat to various buildings in South Plants.

- Gonzales Construction Company: worked on the demolition of the acetylene (C_2H_2) above ground storage tanks (ASTs). Gonzales removed the concrete wall using a 9,000 lb hydraulic hammer and cut up the inner steel liner using a hydraulic shear. The carbon steel is being recycled and non-salvageable materials such as concrete is being disposed of at a subtitle D landfill. Also observed Gonzales decontaminate an AST which contained road oil. In characterizing the steam line in South Plants, Gonzales used a mercury vapor analyzer (Jerome) to verify Jacobs preliminary findings that mercury was present in the line. Demolition and excavation was then commenced.

- Weston: worked on demolition of diesel fuel ASTs. The number 6 fuel oil is considered off-specification due to trace amounts of dichloropentadiene (DCPD). The estimated volume of the material is 500,000 gallons. The oil is being placed into railroad cars, then transported to a recycler. Weston's first task was to remove sources of potential explosive atmosphere by decontaminating the interior of the ASTs, then used a hydraulic shear to cut up the ASTs. The approximate 300 tons of scrap steel was then loaded for transport to a recycler.

- Enserch Environmental: worked on boiler plant equipment removal; work activities consisted of: identifying PCB equipment, removal of PCB equipment to RMA's PCB accumulation area, lockout and tagout of all electrical wiring and equipment, setup of a temporary electrical station, collection of criticals (establishment of a background ambient for asbestos fibers), removal of friable asbestos (cryolite) from bulding number 321, and staging of work area for day-to-day operations.

- Tennessee Valley Authority (TVA): worked on removing mustard storage tanks out of building 742-A. TVA removed reactors from building 422. These reactors were used to make the raw dichlorodiethyl sulfide (mustard agent). TVA performed area monitoring within suspected agent buildings.

Tasks Continuing:

- Working on trying to obtain a computer with link-up to the network. The present lap-top has limited capabilities.

- Trying to acquire telephones at the main office in Building 111.

- Contractors Evaluation Form: coordinating with Mr. Terry Grush (RMA Contracts) revisions and edits to the form. Mr. Grush likes the draft version and wants to implement a remediation contractor's evaluation system.

- Monitoring well review: review monitoring well locations and compare the locations of the wells to RMA site history such as: storage areas, burial sites, landfills, reported spills, production areas, and ground water flow.

- Assist RMA personnel with developing should-cost estimates on an as needed basis; with implementing friable asbestos, asbestos containing material (ACM), and small quantities of hazardous debris disposition policies; assisted with development and implementation of debris and hazardous waste characterization policies; assisted the asbestos inspectors with development of a field oversight program; assisted with developing and implementing a program for characterizing PCBs; assisted the Safety and Health Office in setting up an asbestos awareness course.

Tasks Delayed:

- Collecting data pertaining to Basin F remediation clean-up techniques and remaining waste to be disposed.

- Collecting data regarding solvent extraction system for soils.

- Collecting data regarding the test burn of the mustard pits in buildings number 536, 537, and 538.

- Collecting data regrading North Plants remediation efforts.

- Presentation of DI-250 which is a CO2 pellet blaster used in decontamination operations.

- Collecting videos and overheads for training courses.

- Collecting data regarding solvent extraction system for soils.

The above tasks have been delayed due to priorities and will be accomplished on a not-to-interfere basis.

The Risk Assessment support at Rocky Mountain Arsenal has accomplished the following:

Endangerment Assessment Status

The Pre-White version of this report was delivered to the Organizations and State

(OAS) on 1 July 1994. The OAS met on 11 July to discuss this report. The Army received a letter from Shell stating they had no problems worth further delaying of this document. They and U.S. Fish and Wildlife Service (USFWS) also had no problems with the document being finalized as is. No letters were received from the State. However, EPA sent a letter asking that the document be changed due to the Army's added comment. The Army's comment was a response to the EPA's comment on the document. The Army decided to place EPA's letter in the document after their comment. EPA also requested that their letter be listed in the main Table of Contents. Errata will be out in October. This should close out the Final IEA/RC process.

Supplemental Field Study

The contractor's collection of Deermice was thought to be complete but had to be repeated due to a sampling error. They have completed all stages of the SFS except they are four grams short of grasshoppers in one of the plots and they are still working on the collection of beetles.

Problems

- The contractor was supposed to wash the Deermice traps between each site, as outlined in the work plan; however, this was not done, and sampling was complete before it was noticed. The contractor pointed this out to us, and it was agreed that, due to the sensitivity of this study, the sampling should be repeated. Not sanitizing the traps between sites could result in cross contamination. The objective is to collect animals that have been solely exposed to a certain area. Erroneous results could cause the study to have to proceed to Phase II. The Army would not like to incorrectly proceed into Phase II, if possible, since that could cost the Army hundreds of thousands of dollars per year. Since the contractor is repeating the work, those involved already know the strategy behind all the sites, so this work should only take a few weeks. Our advice has prepared the Army for negotiation with that contractor should it become necessary.

- Grasshopper collection went slowly because the nets had to be washed between sites. More nets were ordered to speed up the process.

- The Starling breeding season has ended and, due to other studies and heat, the number of starlings available for our study was only 15. The goal was 50. This information was presented to the OAS along with a list of five alternatives. As of right now the EPA and State are pushing to extend the study to 1995. No word was received from Shell. The Army is sending out a letter stating the outcome of the analysis with the current collected species will determine if the study will be continued into next summer.

- A laboratory has still not been chosen to do the analysis for this study. The Laboratory Support Division (LSD) expects one will be appointed in the next four weeks. This problem stems from the AEHA lab not accepting deermice due to the Hantivirus threat. A

meeting was held on August 31st to discuss what precaustions our LSD should take when sending deermice samples and what literature should accompany them. The literature explains to the receiving laboratory any precautions, as well as handling techniques, taht they shold be aware of. The lack of a lab is still a problem, however, we have obtained a freezer to handle the increase in specimens.

- During the latest committee meeting it was decided that the field study document does not need to be signed by the Parties; however, EPA had a concern with the proxy value chosen for the analysis in the Final SFS document. An errata is due to go out in October stating the proxy value as □CRL.

National Resource Conservation Committee (NRCC)

On 12 July 1994, we attended the quarterly NRCC meeting. These meetings contain information on what the USFWS is doing at the Arsenal to establish the area as a wildlife refuge habitat. We observe these meetings and report to the Army what the USFWS is doing and if that follows the outlined procedure as written in their Annual Management Plan. As a result, USFWS has been asked to use us as the technical point of contact for their activities at the Arsenal. Thus far, this function has worked well, with the USFWS personnel being very helpful in bringing us up to date.

Natural Resource Damage Assessment (NRDA)

We have been attending meetings with the NRDA committee. They need to initiate a preliminary assessment of the economic impact of the natural resource damage that has occurred. This report would indicate the damage caused to the economy of this portion of Colorado due solely to the contamination caused by the Army and Shell Chemical at this site. A mid-project meeting was held on September 8th to make sure the contractor was on the right track. The contractor is preparing the report for the Army's use only.

OCTOBER 1 - DECEMBER 31,1994

Work continues on investigation of methods available for identifying biomarkers of mammalian hepatocarcinogenesis for adaptation to fish models:

Bioquant slide analysis was completed for Test 401-001R.

Amendments were written for the BrdU trailer exposure for test 401-002R in response to EPL's quality assurance audit. Setup and paperwork for a small-scale (30 fish) BrdU exposure was completed. This study will provide histology controls and methods development information for BrdU-staining of formalin-fixed fish tissue.

Work continues on the analysis of Group J medaka livers from Test EE1 on the Bioquant. Data were collected from sacrifice J and transformed into SPSS format. Statistical

analysis of these data is scheduled to begin the first week of January 1995. Arrangements for histopathological. evaluation of H&E slides for this study by EPL *(i.e.,* the slides adjacent to slides stained and scored for proliferation with BrdU) were initiated.

Slides and materials were prepared for presentation of the paper "Assessment of the carcinogenic potential of contaminated waters" at the Society of Toxicology and Environmental Chemistry, Denver, CO, Oct. 31 - Nov. 4. The paper was presented at the symposium "Integrating Molecular Toxicology with Field Studies" and was well received.

Support in the way of providing materials, slides, literature, and training on the Bioquant system was given to graduate student Tomoko Nii for the apoptosis project. A literature search was reviewed for pertinent papers on cell proliferation for the TGF/apoptosis project and articles of interest were entered into Refman for later reference.

A one-day training session on microscopic photography given by Bunton Instruments was attended in November.

The new Bioquant image analysis system was installed at the BRDL laboratory on the campus of Colorado State University at Fort Collins, CO. The installation required housing design and purchase, purchase of image analysis software (CHRIS), writing of usage procedure draft, and physical setup of the instrument itself in Colorado. Two scientists are scheduled to attend a two-day workshop on the CHRIS software in the middle of January.

Studies of chemical carcinogenesis in medaka continued:

Work continues on Test 401-002R, the West Branch Canal Creek Groundwater Study with medaka consisting of routine care, maintenance and exposure of the animals by field personnel. The six-month interim sacrifice of animals in this study is scheduled for February 1995.

Quality Assurance (QA) Audit responses were finalized for five QA audits of Test 401-002R and returned to our contract QA unit. Critical events from this test audited to date are: Randomization/distribution of medaka fry to test chambers; preparation and transfer of medaka from Ft. Detrick to APG-Edgewood Area; biomonitoring trailer facility inspection; termination of DEN exposure to medaka; and medaka removed from BrdU and sacrificed. Inadequacies noted were minimal.

Progress on work in support of rodent and medaka physiologically based pharmacokinetic modeling (PBPK) conducted at Armstrong Laboratory, Wright-Patterson AFB follows:

This work includes investigation and modeling of rodent metabolism and disposition of trichloroethylene and perflurorhexyl iodide, as well as characterization of medaka liver cytochrome p-450 activities and isoform profiles.

Initial work began on the Microdialysis project. The focus of this project is to develop a methodology to determine an *in vivo* partition coefficient for a physiologically based pharmacokinetic model using microdialysis sampling techniques. The model compound for this experiment was trichloroethylene (TCE). Microdialysis sampling is a new technique for the study of *in vivo* pharmacokinetics and the metabolism of chemicals. In this approach, a microdialysis probe, tipped with a very small semipermeable membrane, is inserted into a blood vessel or tissue. Chemicals and their metabolites diffuse across a semipermeable membrane due to a concentration gradient and are carried away by the constantly pumping perfusion medium for on-line or off-line analysis. During this quarter, studies were conducted in the rat to determine the recovery efficiency of the microdialysis probes in a Ringers Solution containing TCE and the time to reach the steady state level. Efforts were also made to improve the recovery of TCE. An *in vitro* station was configured to allow for microdialysis of excised kidney in the Ringers Solution containing TCE for determination of *in vitro* recovery efficiency. TCE was analyzed using a gas chromatographic method with electron capture detection. Preliminary *in vivo* microdialysis experiments were also initiated. This involved insertion of the probe in the kidney and the blood vessels of anesthetized rats. The rats were exposed to TCE. The TCE concentrations were determined by collecting the microdialysis perfusion Ringers solutions. The TCE partition coefficient was determined following an inhalation exposure to TCE from the tissue-to-blood ratio at the steady state. Work progressed with the *in vivo* microdialysis probe experiments to prove that the probes are consistent from day to day at various exposure concentrations of TCE. To date the results are encouraging, and the anesthetized rat's blood and tissue TCE concentrations meet expectations.

Work began and continues on the rodent intestinal metabolism of TCE. The major objectives of the project include: (1) Determination of the capacity of intestinal 700g supernatant, microsomes, cytosol and mitochondria to metabolize trichloroethylene; (2) identification of cofactor requirements; (3) determination of the ability of gut contents to anaerobically metabolize trichloroethylene as well as its metabolites (CH, TCA, TCOH, and DCA); and (4) set up an isolated perfused rat intestine model. Experiments were conducted to determine the effects of various compounds on the cytosolic metabolism of DCA *in vitro*. Work was done to assess the effects of small molecules, and preliminary experiments have indicated the involvement of the glutathione system. Based on results, testing began to assess the exact nature of the involvement of the glutathione system in the cytosolic degradation of DCA. Experiments performed have demonstrated that the ultrafiltration of cytosol, which removes very small molecules such as glutathione, diminishes DCA degradation and that the treatment of cytosol with chemicals known to deplete glutathione results in an inhibition of DCA degradation. The replenishment of glutathione from exogenous sources seems to restore DCA degradation. Further testing was done to assess the involvement of the glutathione S-transferase enzyme. Experiments indicated that a cytosolic protein is responsible, and we have postulated that it is glutathione S-transferase. This enzyme was removed from cytosol by affinity chromatography and was verified by conducting glutathione S-transferase assays on the different proteins eluted from the column. The results of this experiment indicated that the glutathione S-transferase enzyme is not involved in DCA degradation. It has been previously

demonstrated that gut microflora can form DCA from TCA, and several bacteria are known to express another enzyme, DDT-dehydrochlorinase, involved in this metabolism. In future experiments, we may assess the distribution of this enzyme to the liver cytosol. The publication "Dichloracetic Acid: Metabolism in Cytosol from Mice, Rats and Humans" was co-authored and has been submitted for internal review and clearance. Submission for review by BRDL will follow.

A project using a closed chamber gas uptake experiment of perfluorohexyl iodide was performed to determine the uptake and distribution kinetics using a physiologically based pharmacokinetic (PBPK) model. Uptake data were obtained from five six-hour close chamber gas uptake studies for perfluorohexyl iodide concentrations ranging from 5,000 to 10,000 ppm with male F-344 rats. Loss rates were determined at each concentration level. A PBPK model was used to mathematically describe the disposition and metabolism of perfluorohexyl iodide employing specific parameters and apparent whole-body constants determined from the gas uptake experiments. The results indicated that perfluorohexyl iodide absorbs to the sodium hydroxide (C02 scrubber) and the skin and fur of the rat. Following an investigation, lower loss rates were obtained with barium hydroxide instead of sodium hydroxide. Uptake and disposition of perfluorohexyl iodide appeared to be first-order.

Work began and continues on testing of microsomes from the medaka liver. Recent experiments have assessed the distribution of a number of cytochrome P-450 activities to microsomes prepared from medaka liver. The results of these experiments have yielded preliminary data which suggest that medaka microsomes have a significant amount of activity toward ethoxyresorufin, indicative of P450 lAl/lA2. Data suggest that very little other P450 activity is present, which is in agreement with data published for other fish species. Continued effort should be able to confirm the presence of P450 lAl/lA2 through the use of gel electrophoresis and immunostaining with specific antibodies. This technique will be applied to assess the presence of other major P-450 isoforms.

Work on teleost immunotoxicology methods development continued as follows:

A complete battery of tests indicative of superoxide anion production were run in October on the January (1/17/94) hatch of medaka as a comparison with previous data gathered. Extracellular superoxide stimulation was enhanced as shown in the cytochrome-*c* assay with high values of 10.1 nanomoles of cytochrome-*c* reduced per $2x10^5$ cells which corresponded with values obtained in recent assays of Gulf Coast Research Lab medaka tested. Results from this assay were encouraging as an indicator of renewed health of BRDL medaka. Methods development continue for assessment of 5'-nucleotidase activity (activity decreases as monocytes mature into fully functional macrophages) and the quantitation of intracellular superoxide anion using NitroBlue Tetrazolium (NBT) to simplify and optimize these assays for field use. Data and graphs were compiled for the SETAC poster. A notebook was prepared with necessary forms for documentation of calibration and use for laboratory equipment to meet SOP requirements. Abnormal medaka culled from the culture facility were fixed for histology and will be sent to EPL along with the next Health Screen fish.

Work resumed in December on the species comparison project. Following review of existing data, the decision was made to repeat some of the medaka, trout, and ML-1 assays before these data should be considered for publication; the rodent data are acceptable. This work is scheduled for the middle of next quarter.

Medaka were received from Gulf Coast Research Lab to repeat health screen and functional assays in our efforts to collect information on health parameters of fish from other laboratories using medaka for toxicological studies. Testing began the day after receipt and the following two days in order to assess possible shipping stress that may affect test results. Cytochrome-*c* results for these assays showed suppression of ability to stimulate anterior kidney cells with phorbol ester with a high value of 2.8 nanomoles for the first two tests and 5.2 nanomoles for the third test, indicating the beginning of a return to a stabilized state. GCRL medaka held at our facility will be tested again after an 8-week holding period. As a comparison, high values of 9.4 were observed in GCRL fish tested in September after five days acclimation at our facility. These preliminary data suggest that there is a temporary suppression inflammatory cell function in medaka associated with the stress of shipping and handling, with activity returning to normal in a relatively short period of time (3-5 days).

A collaboration was formed with Dr. Jeff Teska from the Eastern Fish Disease Lab in Kearneysville, WV, to provide a better profile of the organisms inherent in our fish and those possibly acting as pathogens. All bacteriology plates from fish tissues and water samples will be sent to him for identification. After discussions with Dr. Teska, it was decided that Brain Heart Infusion agar would replace the Trypticase Soy agar for the bacterial plate media in order to culture a wider range of organisms. Arrangements for delivery of samples was also worked out for subsequent health screens.

Health Screen # 3 was completed the second week of November. Data from in-house assays are currently being analyzed, and we are waiting on analysis of fish tissues from EPL. Results from bacterial samples characterized by Dr. Teska were received mid-December. Data analysis for all health screen data is in progress, including assembly of SPSS databases. A database in SPSS was designed for bacteriology results. We are working with Drs. Don Gaver and Pat Jacobs of the Naval Post-Graduate School in database design and data analysis. Health screen # 4 is scheduled for the beginning of January, with data from all health screens to be presented at the Society of Toxicology meeting in March.

Temperature comparison studies were begun with May hatch (5/18/94) fish held at 20°C and 25°C to see if one condition was more favorable than the other. Fish initially tested were from the 25°C group and gave a poor cell yield per fish in the first n = 4, consequently, only two assays were conducted. Suzanne Jacobson, a graduate student from the University of Maryland at Baltimore, came to observe the assays for possible inclusion in their program using koi, talipia, and goldfish as experimental models. A set of three batteries was done using the 20°C fish with better results. Cell yields were in the range usually seen. This study is scheduled for completion next quarter.

The study to repeat *Aeromonas salmonicida* LC_{50} pilot experiment using fresh *Aeromonas salmonicida* stocks (from NBS, Keameysville, WV), which have demonstrated virulence in brook trout, was not conducted this quarter. Continuation of this study is scheduled for next quarter.

Two posters, "Methods development and standardization in medaka for immunotoxicological field studies" by L.E. Twerdok, M.W. Curry, J.R. Beaman, R.A. Finch, H.S. Gardner, and J.T. Zelikoff and "Species comparison of inflammatory cell reactivity" by E. M. Boncavage-Hennessey, L.E. Twerdok, R.A. Finch, and H.S. Gardner were presented at the 15th Annual Meeting of the Society of Environmental Toxicology and Chemistry, Denver, CO, October 31 - November 3, 1994. Both posters were extremely well received, with many potentially productive interactions with US and foreign investigators. Follow-up for the meeting included mailing of copies of the posters and a copy of our "Small Fish Techniques" video, made for the fish immunology meeting in Breckenridge (September 1993).

Work with transgenic medaka (in collaboration with Dr. James Burkhart at NIEHS) being held at the USABRDL facility was as follows:

Three pairs of transgenic medaka were cultured with fry resulting from each breeding pair. Fin clipping done on the transgenic fish in October revealed 2 out of 15 offspring were transgenic for tank 5-C-18 (both males). Two wild type females were kept with the transgenic medaka and the remainder were euthanized. Fin clipped in December from two tanks of fish were digested and frozen at -70 degrees C and shipped on dry ice to Dr. Burkhart at NIEHS. One tank of fish was then euthanized and the other held for a retry when the fins grow back. Two batches of eggs were cultured from transgenic parents. Fifty-eight fry resulted from the cross *04 line* and approximately 40 from the *267 line.*

Work on methods development for rapid toxicity assessment continued as follows:

Four lettuce tests *(Lactuca sativa)* were conducted in November. Germination of new seeds was found to be acceptable. Tests with groundwater samples from West Branch Canal Creek were conducted with no significant reduction of growth in Lactuca sativa when compared to the controls. Copper sulfate was used as the positive control. Test 403-001R used 5 concentrations of groundwater from the West Branch of Canal Creek ranging from 100% to 6.25% (0.5 dilution factor). A slight inhibition was observed at 100%, but EC_{50} results have not been calculated yet. Test 104-001 used 5 concentrations of copper sulfate ranging from 0.34 mg/L as copper to 5.5 mg/L as copper. Greater than 50% reduction in growth was observed for 1.38, 2.75, and 5.5 mg/L copper. Statistical analysis of the data is pending.

The article "A comparison of the sensitivity of rapid toxicity screening tests" (Toussaint *et al.)* submitted to *Journal of Environmental Toxicology and Chemistry* in March 1994, was accepted for publication. Publication is scheduled for Vol. 14, No. 5, which is due out 4/18/95.

2,4-D killifish acute toxicity experiments performed in October showed a positive dose response, while subsequent tests showed no toxicity at the same concentrations. The pH of the stock concentrations of 2,4-D was raised to determine if pH was responsible for the dose response initially observed; no toxicity was observed at the adjusted pH. The sensitivity of newly hatched embryos was compared to the sensitivity of 24-hour old fry using zinc sulfate as a test substance. Results of LC50 and EC50 data were found to be similar for both groups. The final toxicant for the SDS killifish test system was completed in October again testing the newlyhatched fish and the 24-hour old fish with very similar values. Data compilation for all killifish test data was completed. Analysis of all zinc comparative data was also completed. Embryo viability from batch to batch proved to be negligible. A zinc test utilizing 90-day old embryos was also performed to see if shelf life had any effect on embryonic sensitivity with little difference noted. Overall study results suggest that the killifish test system is comparable to other standard tests employed for rapid toxicity assessment for the toxins tested.

Killifish diapause holding studies continued comparing embryos held in saline well water and saline synthetic soft water. The well water embryos underwent massive hatching mortality while the survival rate with the soft water was 80%. Embryos stored out of water in a semi-arid environment maintained a similar survivability.

Efforts were made to induce fertilized Japanese medaka (*Oryzias latipes*) eggs into a state of diapause by incubating them in saline, well, or softened water. Ionic flux across the egg membrane may be critical in maintaining a steady embryonic resting stage. Saline water with very high ionic strength proved less successful in maintaining viable embryos in medaka or killifish trials. Medaka eggs were also plated on filter paper one week after fertilization to test whether they could withstand desiccation. One week after drying, 9 of 10 embryos remained viable. After one week post drying, 8 of 9 fry hatched within a few hours time. Future attempts to hold dry medaka eggs (embryos) are planned for the beginning of January, concurrent with the development of appropriate experimental protocols.

Attempts to culture killifish were also made by collecting sixty eggs from one breeding pair. No embryonic development has been noted. Further culturing methods development are being planned upon arrival of necessary supplies. Maintenance and record keeping were performed on all killifish aquaria held in room V. Approximately 100 killifish fry were reared from embryos received in November and transferred to the fish culture lab to be held with 140 other killifish from previous hatches. Dr. Eugene Hull visited BRDL in December to observe our hatching and test methods and to collaborate on future efforts for shipping and handling of embryos. Ideas on holding methods were exchanged.

A prototype chamber was developed using state-of- the-art waterproof connectors and a new wiring system designed for the ventilatory chamber. The chamber was constructed and tested for signal quality compatibility with the current ventilatory computer system and proved to produce signals as good if not better than past chambers used with the added benefit of reduced maintenance time and cost. After final revisions, materials were ordered to produce 20

new chambers to retrofit all current biomonitoring trailer systems with the new technology. Technical difficulties with the biomonitoring trailer system were also addressed. Signal quality was a major focus of this R&D phase. Related electrical and fluid dynamic difficulties were investigated to try to alleviate problematic signal interference. Diagnostic testing was performed using a variety of alterations to the biomonitoring system. Bonnie Wemer from Cumberland Electronics and John Hayden, a Fluke application engineer, were contacted to provide a demonstration of the Fluke 99 series scopemeter to determine its usefulness as a diagnostic tool for the trailer system. The meter was determined to be an invaluable diagnostic tool to replace out current out-dated storage oscilloscope.

Analytical method development and analysis of chemicals used in carcinogenicity and mobile laboratory studies continued as follows:

Training was received on the Hewlett Packard 7686 Prep Station for the HPLC to perform various sample handling tasks including dilution, internal standard addition, liquid/liquid extraction, and solid phase extraction. In support of the killifish project, 43 samples of 2,4-D and zinc were analyzed. Eight samples of copper were analyzed for the lettuce seed testing. FETAX chemistry support required 170 sample analyses of Aldicarb, Aldicarb sulfoxide, Aldicarb Sulfone, Atrazine and Simazine (California mix 11A). Modification of methods was necessary to detect lower levels of the first four of these compounds used in the assays. Solid phase extraction and increased injection volumes to 300 µm, along with monitoring effluent at 205 nm rather than 210 nm made lower concentration detection possible. Sensitivity was increased to 0.015-0.150 mg/L. Data manipulations were simplified by using an Excel spreadsheet for faster input of data and automated calculations while reducing transcription errors. Precision and accuracy data were generated, and results were acceptable. Solid phase extraction was examined as a means to detect lower levels of Atrazine and Simazine, but instrumental difficulties with the Prepstation did not allow the analysis to be performed. Hewlett Packard representatives are currently working on correcting the problem. The solubilities of β-estradiol and Nonylphenol are being examined in support of a new series of experiments involving FETAX. Preliminary methods have been developed using HPLC. Standards for these compounds were prepared in methanol. β-estradiol, at 100 mg/L, was placed in FETAX at 25°C and stirred for three days, then filtered through a 0.2 µm nylon filter and injected. No β-estradiol was detected by the instrument; an estimated detection limit for this method is 0.05 ng/L. It will be necessary to use an intermediate solvent for this test.

Analytical chemistry support for Rocky Mountain Arsenal (RMA) included the following:

Routine analyses at RMA: Several samples were analyzed for benzene in connection with the Enserch In Situ Biotreatability Study. The method used for these analyses included the High-Level Benzene in Water method, developed with the cooperation of *GEO-CENTERS* and RMA chemists.

Method improvement/development at RMA: (1) Work on detection of methylene chloride in air continues. Twelve air samples in Tedlar bags were received from Enserch for methylene chloride analysis. Air samples from the southern boundary of the Arsenal have contained variable levels of methylene chloride, and there are some indications that the analyte may be coming from off-post. The Enserch samples were analyzed using a GC/ECD and the ion trap MS (ITMS). Levels of 5μL methylene chloride in air were readily detectable by the ITMS as well as the GC/ECD. Future plans are to install the ITMS in one of the mobile labs and utilize this technique in the field in an attempt to determine where the methylene chloride might be coming from. (2) Nitrosamines analysis work is underway to bring a low-level method developed by Oak Ridge National Laboratories in-house. The method involves the use of a short-path thermal desorber and a chemiluminescent nitrogen detector. The necessary instrumentation needs to be acquired, and assistance is being provided to obtain the information required. (3) Refinement of the Organosulfurs Analysis method continued. It is expected to be certified for quantitation by QA/QC in the near future. Once approved, this method will be used to determine levels of the Organosulfurs oxathiane, dithiane, chlorophenylmethyl sulfide, chlorophenylmethyl sulfone, and chlorophenylmethyl sulfoxide, on a regular basis. (4) Work continued in the development of a standard method for the determination of volatiles using Purge-and-Trap GC/MS in conjunction with government personnel. This method will not however, be suitable for determination of high levels of benzene and other volatiles. (5) Sample preparation was also conducted in cooperation with government personnel.

Work continues to maintain and optimize USABRDL aquatic laboratory facilities:

Essential laboratory maintenance continues on all culture (medaka, guppy, bluegill, white sucker, and killifish) and experimental medaka located in rooms 5 and 18. Daily activities entail three feedings of all organisms and maintenance of live food systems. Associated record keeping was performed to maintain necessary test records to meet SOP regulatory review requirements. Water analyses and light measurements were performed weekly. Transgenic medaka being held at the facility in collaboration with Dr. Jim Burkhart of NIEHS were also maintained. Assistance was given in the killifish acute toxicity assays and egg culturing effort. Paperwork and water quality readings were also documented for *Nothobranchius guentheri* species held in rooms 7 and 18. A new feeding regime in room 10 was coordinated with the government. Seven tanks (60 fish per tank) will be receiving the regular feedings and seven tanks will get frozen brine shrimp in place of flake food. Essential laboratory supplies were documented and ordered as needed for the quarter.

Brood stock was renewed with fry from GCRL. The December medaka hatch was successful and designations to various usages were made. 1200 fish will be used for colony renewal, 720 for immunotoxicological studies, 50 for BrdU research, 840 for food study, and 500 for trailer LC_{50} studies. The total number of medaka maintained in the aquatic toxicology lab increased from 9300/month in FY93 to approximately 10,000/month. Natural deaths for the year were summarized and unusually high death rates were observed for November 1993 and June 1994. The 1994 fiscal year summary of animal use was provided to the head of the Animal Use Committee.

Fish food was sent to Johnston Labs for contaminant analysis.

A quarterly temperature scribe calibration was performed, and several instruments were found to need repair or to be replaced.

The annual SOP review was begun this quarter. SOP's were reviewed for fish culture and glassware cleaning.

Fish shipped/received to/from other research facilities this quarter:

No. of medaka	Institution	Location	shipped/received
650	NYU	Tuxedo, NY	shipped
100	GCRL	Ocean Springs, MI	received
650	WV	Morgantown, WV	shipped
500	U of MD	APG, MD	shipped
200	U of MD	Baltimore, MD	shipped
500	U of MD	Edgewood Area, APG, MD (Canal Creek)	shipped

In support of the Environmental Engineering Division at Rocky Mountain Arsenal (RMA), the following is reported:

To better understand the technology needs of the Army during cleanup of contaminated sites, our technicians observed contractors performing remediation and demolition activities at Rocky Mountain Arsenal. Contractors activities included the following:

- Jacobs Engineering: Preparing for the pilot building demolition project. Jacobs performed the following remediation tests on several buildings:
    - Solvent Extraction (using acidic/caustic as solvents)
    - Pressure Washing (using water)
    - Sweeping (shoveling/brooms)
    - The analytical confirmations results for the remediation tests have not been compiled at this time. Removed piping and ancillary equipment from pilot demolition buildings. The piping was decontaminated and sent to a recycler for reclamation.

- Gonzales Construction Company: Worked on dismantling the South Plants steam line. The metal is being reclaimed by a recycler and the wooden stanchions have been disposed of as a characteristic waste (DO08) to a subtitle C landfill due to the lead base paint. Completed the acetylene tank demolition project. The tasks include loading up concrete debris and tank residue which consisted of deteriorating wood and

ferrous oxide. GEO-CENTERS provided RMA personnel with technical support in order to develop an appropriate disposition plan for the debris. Originally RMA personnel were going to treat the debris as a hazardous waste and send it to a Subtitle C landfill. After review of our recommendations, the debris was sent to a Subtitle D landfill for disposal.

- Weston: Worked on demolition of the number 6 fuel oil tanks. The scrap steel from these tanks was sent to a recycler. Weston is also removing ancillary equipment and piping from the eastern side of building number 515, a pesticide and agent production area. Worked on decontaminating the number 6 fuel oil tanks.

- Enserch Environmental: Worked on the Boiler Plant Demolition Project. Enserch is removing boilers from building number 325 and steel removal in building number 321. Enserch received final clearance on building number 321, 19 December 94. This means Enserch personnel can downgrade personnel protection to OSHA level D. Observed Enserch packaging brick from the boilers that is contaminated with asbestos into IAI, 55-gallon drums. Each one of the boilers will generate approximately 600 drums of waste. Following review, Enserch is now using one cubic yard pallet boxes in lieu of the 1Al drums (1 pallet box = ~5 drums).

- Tennessee Valley Authority (TVA): Worked on removing piping and ancillary equipment from building numbers 511 and 537. These buildings were part of the mustard (HD) production and demilitarizing. TVA removed all identified above ground agent lines from South Plants.

- Arthur D. Little: Worked on installing two (2) horizontal monitoring wells. The horizontal wells are a pilot test to see how effective product recovery will be at RMA. The product being recovered from these wells is benzene.

Reviewed and edited RFI-SOP. The RFI-SOP is in the final draft form. Worked on developing and implementing a government remediation field inspectors training program. This program includes on-the-job-training of RMA's Field Inspectors and regulatory compliance interpretation.

Assisted and advised regarding various regulatory issues, such as:

- Day-to-day procedures pertaining to waste characterization, transportation, handling, and disposal.

- Provided technical assistance regarding the clarification of double permitted disposal facilities, e.g. SCA/RCRA disposal facilities.

- Provided technical assistance regarding identification of deficiencies in RMA's on-site Hazard Communications Program, OSHA CFR 29 Part 1910.1200.

- Packaging of materials contaminated with asbestos. The Department of Transportation (DoT) recognizes and approves of various types of containers for transportation of hazardous materials.

Coordinated identification of health and safety deficiencies with Management Assistance Corporate of America (MACA) and RMA's Safety Health and Environmental Office (SH&E). MACA was recently contracted by RMA SH&E Office to implement and conduct health and safety training programs and site audits.

Helped to develop alternative method for water disposition at RMA. Note: the CERCLA plant is backed up until approximately middle of January 1995. The current estimated backlog of water that is awaiting treatment at CERCLA is over 250,000 gallons.

The Risk Assessment support at Rocky Mountain Arsenal has accomplished the following:

Endangerment Assessment: The errata containing the Cancer Risk map correction and the Table of Contents correction has been changed. The final erratum to this document will only contain the Table of Contents correction. The final errata to the Integrated Risk Assessment/Risk Characterization was mailed to the EPA, Shell, USFWS, and Colorado State on December 6, 1994. All but one of the existing reports has been updated.

The revised maps will come out as an Addendum to the IEA/RC report. *GEO-CENTERS* is working with Foster Wheeler, (formerly Enserch Environmental) to make the necessary revisions on these maps so they more accurately reflect the risks at Rocky Mountain Arsenal. Internal review of several maps that went out in the final document is currently in progress. Monitoring of the progress of this Addendum will continue into next quarter.

Supplemental Field Study: The field effort of biota tissue collection has been completed by Foster Wheeler. The total number of species was not acquired. Only 16% of the beetles, 36% of the starlings, and 96% of the grasshoppers were collected. One hundred percent of the prairie dogs, rabbits, and deer mice were attained. A summary of the sampling field effort has been prepared by Foster Wheeler. The Standard Analytical Reference Material for Oxychlordane has been produced. Oxychlordane will now be a part of the analysis performed. This summary of the field effort was sent out to the Parties (EPA, State, Shell, and FWS) on December 7, 1994. A meeting was held on December 15, 1994, to make the necessary decisions to proceed with the analysis. The decisions will be formalized as a written summary and sent to the parties in January. Identification of a laboratory to perform the analysis on the biota tissue is a recurrent problem. A laboratory is expected to be identified in mid-January.

EPA is pushing for Phase-II planning to be initiated. The Army's position on the planning of Phase-H is to hold off until the Phase-II sampling results are back. *GEO-CENTERS*

is supporting the Army in their position and providing technical advice and support for the completion of Phase-I before Phase-II is planned.

The FWS gave representatives from the EPA a tour of the Area of Dispute. *GEO-CENTERS* personnel accompanied the tour and answered questions about the field study events that took place this summer.

DIMP: A contract has been awarded by the Army to initiate a DIMP study on female lactating mink. *GEO-CENTERS* will be reviewing all reports and progress on this study and providing biological support and expertise. The Army now owns the 220 mink that will be used for the two-year study.

Risk Expert: *GEO-CENTERS* is providing technical advice on risk support to the Army for a Scope of Work for contracting a Risk Expert on the Endangerment Assessment (On-post and Off-post). The Statement of Work is being prepared to award this contract. In keeping with alleviation of conflict of interest, *GEO-CENTERS* cannot propose on this effort.

Relative Risk: Most of the work effort carried out in the month of October was in support of the "Relative Risk Site Evaluation" project. This project, sent from the Army Environmental Center, requested an evaluation of each of the 209 sites at RMA based on the highest levels of contamination in the soil, ground water, surface water, and sediment. This was assigned to all the Army facilities throughout the nation. AEC plans to use this information to score each site to determine clean-up priority. This will be done for each site in the country. This work effort consisted of searching through Contamination Assessment Reports (CAR's and SAR's) for the necessary information to complete each form. A final review was performed on the Relative Risk Project handed down by AEC and sent out on December 16, 1994.

Special Projects:

Biota Remedial Investigation reports and the Comprehensive Monitoring Program reports were searched to tally the number of biota tissue samples the Army has attained for analysis in preparation for the 15th Annual Society of Environmental Toxicology and Chemistry Meeting (October 31 - November 3). *GEO-CENTERS* provided technical advice on the ten presentations Foster Wheeler made at this meeting.

A meeting was held between the Army and FWS to review upcoming projects in the Biomonitoring Program. *GEO-CENTERS* was present to provide biological advice to the Army for the FWS requests.

GEO-CENTERS attended the 4th quarter National Resource Conservation Committee meeting and provided a summary to the Army.

JANUARY 1 - MARCH 31, 1995

Investigation of methods available for identifying biomarkers of mammalian hepatocarcinogenesis for adaptation to fish models:

Evaluation of the slides from Test EE1, Group J on the Bioquant Image Analysis system was finished. Hematoxylin and Eosin slides from Groups B, D, and J of this test were prepared for shipment to EPL for liver pathology evaluation. Pictures of the BrdU-stained livers were also taken of these slides. All data for sacrifices B, D and J in the EE1 and EE2 study were compiled and analyzed using SPSS. An Executive Summary was prepared on *BrdU as a marker of hepatocellular proliferation in an initiation promotion study with the Japanese medaka*. Training on a new image analysis software (C.H.R.I.S.), the Bioquant image analysis system, and cc:Mail class were completed. The instruction sheets for the Bioquant were edited and one was prepared for photomicroscopy for use with the Orthomat E and Orthoplan 2 scope. Necessary slides and paperwork were organized for transfer to Maxine Hennessey and Alex Constan at CSU for TGF and apoptosis staining. Cell proliferation papers were entered into REFMAN.

Training was concluded with Tomoko Nii, a Hood College Master's candidate, on the Bioquant Image Analysis System. Ms. Nii's project report, *The Examination of Apoptosis at Early Stages in Chemical Carcinogenesis in Japanese Medaka,* was reviewed and edited; all changes were made by Ms. Nii. The final report was submitted by Ms. Nii to Hood College to document satisfactory completion of her independent study project on apoptosis.

Thirty medaka fry were exposed to 75 mg/L BrdU for 72 hours (protocol addendum 94-002A-02). This fish tissue was then submitted to FCRF-histopathology laboratory to be used for development of a BrdU-staining technique for formalin-fixed fish so that fish from the West Branch Canal Creek study (401-002R) can be processed for BrdU-staining. Preliminary slides were evaluated for quality of BRDU-staining and the possible use in the Bioquant and C.H.R.I.S. analysis systems. The binder and notebook for this study were also updated.

The manuscript entitled *The Use of Non-Traditional Assays in an Integrated Environmental Assessment of Contaminated Groundwater*, by Lorraine E. Twerdok, Dennis T. Burton, Henry S. Gardner, Tommy R. Shedd, and Marilyn J. Wolfe, was written for submission to *Environmental Toxicology and Chemistry* as part of an accelerated publication of papers from the symposia "Integrating Molecular Toxicology with Field Studies" presented at the SETAC annual meeting in Denver last November. Co-author review was completed March 13, in-house technical review was completed March 15, and manuscript was submitted to session chair, Tom Abbott, on March 21.

Studies of chemical carcinogenesis in medaka:

Work continues on Test 401-002R, the West Branch Canal Creek Groundwater Study with medaka consisting of routine care, maintenance and exposure of the animals by field

personnel. Test 401-002R records for data audit were prepared. This included filing all completed data forms generated both at BRDL and the biomonitoring trailer. Also filed were the chemical analysis from two water sampling intervals that were done by a contract laboratory. The six-month interim sacrifice of animals in this study was conducted in February, 1995. The fish were prepared for transport to USABRDL by *GEO-CENTERS* personnel (Toussaint and Miller), and the procedure was audited by EPL of Herndon, VA. The fish were held in tanks in Diluters Two and Three, with four sets of fish housed in Diluter One. Eighteen tanks of animals were sacrificed on 2/9/95 (360 animals), and eighteen tanks were sacrificed on 2/10/95 (360 animals). The fish were processed through Bouins Solution, two 70% ethanol rinses, stored in formalin and were then shipped to EPL for histology on 2/21/95. During the sacrifice, the data collected were audited by EPL. The QA report was received with responses and problems being addressed.

Procedures to have Diluters Two and Three in Room 10 operational for the rinsing portion of Test 401-002R sacrifice were begun. Diluter Three will be fitted with a flow splitter tube for water delivery. The PVC contaminated waste lines from Diluter One will be fitted on Diluter Three, so that all waste from Diluters Two and Three are carbon filtered. Supplies were inventoried and ordered where needed to get this project operational.

Planning was initiated for two medaka studies with methylene chloride scheduled to begin with a range-find in mid-April and the definitive study proceeding in July or August. Another medaka study in the planning stages is a high level DEN exposure projected to begin in May 1995.

Rodent and medaka physiologically based pharmacokinetic modeling (PBPK) conducted at Armstrong Laboratory, Wright-Patterson AFB:

This work includes investigation and modeling of rodent metabolism and disposition of trichloroethylene and perflurorhexyl iodide, as well as characterization of medaka liver cytochrome p-450 activities and isoform profiles.

Work continued on the method development for the Microdialysis Probes. The goal of this project is to develop a method in which an *in vivo* tissue:blood partition coefficient can be determined using microdialysis sampling techniques. This partition coefficient is necessary for a functional physiologically based pharmacokinetic (PBPK) model of a particular chemical. The model compound for this experiment was Trichloroethylene (TCE). *In vitro* calibration of the probes was completed, and the *in vivo* method development began. A microdialysis probe is constructed from two concentric tubes tipped with a semipermeable membrane one mm in diameter and 10 mm in length. For the *in vivo* method, one probe is implanted into each tissue area, the liver, fat, jugular vein, and/or kidney of an anesthetized male F-344 rat. Multiple nose-only exposures were completed at 2500, 5000 or 7500 ppm TCE, each ranging from four to eight hours in length. A perfusate, Lactated Ringers, is flushed at a rate of 5 □L/min through the probe and accumulated in a collection vial. Over time, the test chemical in the blood or tissue will be driven into this perfusate due to a concentration gradient. The TCE is

then extracted from the Lactated Ringers into methyl *t*-butyl ether and analyzed on a gas chromatograph. The tissue:blood partition coefficient can be calculated from this information. The completion of this project is planned for the next quarter with the submission of a technical report.

Work continues on the Trichloroethylene Biologically Based Health Risk Modeling project. The purpose of this project is to study pharmacokinetics of trichloroethylene and its major metabolites in mice, then develop and validate a biologically based pharmacokinetic model for trichloroethylene and it major metabolites. The investigation of the pharmacokinetics and metabolic disposition of trichloroethylene (TCE) and its metabolites after 60-day repeated oral gavage dosing of TCE in B6C3F1 mouse protocol has been approved. The results of this study will be linked with the biological effects observed in the 60 days repeated gavage dosing study by the pharmacodynamics team. From this, a biologically based health risk model for trichloroethylene will be developed. To support this project, pharmacokinetic investigations of chloral hydrate and trichloroethanol levels after intravenous administration in mice are in progress. A physiologically based pharmacokinetic model was developed to describe the disposition of chloral hydrate and trichloroethanol in mice. Initial disposition and metabolic parameters were determined with the model using the preliminary results of these studies. This model will be an initial step toward the development of the comprehensive model at the end of this project.

The Species Differences in Skin Penetration project work has been completed. The project goal was to determine the relationship between stratum corneum diffusion coefficients and temperature for halogenated hydrocarbons. The chemicals used in this study were Dibromomethane (DBM), Tetrachloroethylene (PERC), and Chloropentafluorobenzene (CPFB). A PBPK model was utilized to determine the diffusion coefficients for temperatures ranging from 27°C - 40°C. Stratum corneum samples studied were from rat and human back skin. According to the Stokes-Einstein Equation, diffusion was shown to be proportional to temperature for the chemicals studied. The diffusion coefficient is commonly used to determine the dermal permeability coefficient, an important parameter of most PBPK models. This work resulted in a presentation at the international 1995 Society of Toxicology Annual Meeting entitled "*The Relationship Between Stratum Corneum Diffusion Coefficients and Temperature for Halogenated Hydrocarbons.*" A technical report titled the same is in draft.

Work began under the Halon 1301 Replacement project. The blood:air partition coefficient for Bromotrifluormethane (CF3Br) using microdialysis probes is in progress. This is the first utilization of the microdialysis probes since their introduction to the Armstrong Laboratory. Whole blood is exposed to 10,000 ppm of CF3Br *in vitro* and is sampled by the microdialysis probes at a rate of five µL/min. The perfusate obtained from the probe is then sampled on a gas chromatograph using an electron capture detector. Previously, the blood:air partition coefficient was not determinable for this chemical, as it was below the permissible range of the former method (vial equilibration). The initial results from using the probes however, suggest that a value is obtainable.



Work progresses in the study of metabolism of medaka microsomes to evaluate sex differences in CYP 450 1A enzyme activity. Collaboration with Dr. Michael Miller, Dept of Biochemistry West Virginia University School of Medicine, provided the female, male and mixed male and female Medaka microsomal fractions to our laboratory. EROD assays were conducted on these microsomes to measure 1A1 and 1A2 activities. Preliminary results indicate that microsomes from male medaka exhibit higher EROD activity per unit protein than do female or mixed microsomes. However, the study also showed that microsomes from male medaka contain more P450 per unit protein than do microsomes from female medaka, which accounts for the observed difference. Future collaboration to study medaka microsomal metabolism involving substrates specific for other P450 isoforms are being planned.

Work continues on the project to determine the intestinal metabolism of trichloroethylene (TRI) using the isolated perfused rat intestine model. A protocol has been approved for mastery of surgical techniques using Fisher 344 rats. A technical report is in draft detailing the surgical technique, optimization of sampling procedure and apparatus, and preliminary intestinal uptake results.

Work continues on the project Determination of Rate of Formation of Tricholoroethanol from Chloral Hydrate in Hepatic Cytosolic Fractions from Rodents. It was found that mice exhibit a higher K_m and V_{max} for TCOH formation than rats. The study also indicates that formation of TCOH and TCA from CH is accomplished through the exchange of an oxidized/reduced cofactor. Results revealed that NAD+ was the cofactor preferred in the cytosolic formation of TCA and that NADH stimulated the formation of TCOH much more than did NADPH. While aldehyde reductase is reported to have a preference for NADPH in the conversion of acetaldehyde to ethanol, our results with NADH indicate that alcohol dehydrogenase may be largely responsible for the formation of TCOH. This information was presented at the 1995 Society of Toxicology Annual Meeting. A written technical report has been submitted under the same title, "A Species Comparison of the Metabolism of Chloral Hydrate to Trichloroethanol *in Vitro*." A second technical report is in draft entitled "The *In Vitro* Metabolism of Chloral Hydrate I. Kinetics of Trichloroacetic Acid and Trichloroethanol Formation in Rat and Mouse Liver."

Teleost immunotoxicology methods development:

The fourth medaka health screen was initiated during the first week of January beginning with the fixation of specimens for histopathology examination by EPL. Thirty-six samples were sent out for histopathologic evaluation this quarter. Qualitative and quantitative bacteriology assessment plates were prepared the following week on representative fish. These plates along with tank water bacterial samples from corresponding tanks and in-coming feeder lines were delivered to Dr. Jeff Teska at the National Fish Health Research Laboratory in Kearneyville, WV for bacterial assessment. Extracellular superoxide anion production assays were run on all fish groups large enough to obtain adequate anterior kidney samples.

Data from this and the previous three health screens were compiled and comparisons were made between age groups, culture and immunotoxicology test fish. Over 1150 individual data records were entered and QA'd in the SPSS individual database. In addition, the bacteriology from health screens 2-4 were entered and statistically analyzed and incidence tables were constructed from this data. Four additional assays for superoxide anion generation and 16 plasma Ig assays were run to provide more information on age group tendencies. This information was assembled into the poster presentation for the Society of Toxicology Meeting held in Baltimore, Maryland, the second week of March.

Work continues with Drs. Don Gaver and Pat Jacobs of the Naval Postgraduate School in Health Screening database design and data analysis. Health Screen # 5 is scheduled for mid-April. Following completion of Health Screen #5, all data will be evaluated, and if appropriate, a recommendation made to alter the frequency for conducting routine health screens.

Immunotoxicology laboratory SOP review and update is in progress as part of the laboratory-wide SOP update. A comparison study is underway on medaka held at temperatures of 20°C versus 25°C, which is currently the standard. A large series of Hydrogen Peroxide production and Nitro Blue Tetrazolium reduction assays were conducted to test the reproducibility/variability of simplified versions of these two assays for potential use as part of our "field" immunotoxicology battery. If reproducibility is good, they may become part of the standard battery of inflammatory cell functional assays along with the Cytochrome "c" reduction assay. Results and status of these assays will be forthcoming in the next quarterly report.

Work continued on the species comparison project. A protocol to measure the amount of myeloperoxidase in cells colorimetrically was written. Preliminary assays utilizing the myeloperoxidase colorimetric procedure yielded promising results, but more refinement of the assay system is still needed. Cytochrome *c*, NBT and hydrogen peroxide tests were repeated using trout cells from Leetown, West Virginia. Also, protocols to be used for medaka and ML-1 (human cell line) definitive studies were finalized. The laboratory portion and the data will be collected next quarter.

Data analysis and a summary report of health data collected from medaka received from Gulf Coast Research Laboratory is in progress.

The first attempt for isolation of mycobacterium from in-house medaka by Dr. Jeff Teska of the National Biological Service (NBS), Kearneysville, WV, took place in January of this quarter. Dr. Teska, working at his laboratory in West Virginia, kept the plates as long as possible, but did not observe any "presumptive-positive" colonies. Dr. Teska was not satisfied with the selective media he used in these experiments. Dr. Teska is scheduled to receive a second batch of fish in Mid-April to continue these experiments.

The study to repeat Aeromonas *salmonicida* LC_{50} pilot experiment using fresh *Aeromonas salmonicida* stocks (from NBS, Kearneysville, WV), which have demonstrated virulence in brook trout, was not conducted this quarter. Continuation of this study is scheduled for next quarter.

Two posters, "Development of routine health monitoring methods for use with an aquatic model (*Oryzias latipes)* used in immunotoxicological testing" by L.E. Twerdok, J.R. Beaman, M.W. Curry, and J.T. Zelikoff (NYU), and "Immunological biomarkers in fish: Influence of host age" by W. Wang (NYU), N. Islam (NYU), L.E. Twerdok and J.T. Zelikoff (NYU), were presented at the 34th Annual Meeting of the Society of Toxicology in Baltimore, MD, March 5-9, 1995. Both posters were well received.

Transgenic medaka research at USABRDL [in collaboration with Dr. James Burkhart at the National Institute of Environmental Health Sciences (NIEHS)]:

Eggs were collected from transgenic medaka from three breeding pairs. These eggs were cultured for the required time and the resulting fry are being raised in Bath Three. Transgenic breeding pairs were transferred to a 10-gallon tank that was divided into quarters. Each section has a separate water supply and drain. A prototype top is being worked up by the shop for permanent housing of these animals; an interim flow splitting device has been installed as a temporary measure. Another group of transgenic medaka were fin clipped. F1 generations of three different sets of transgenic parents were used. A total of 60 animals were screened, and none were found to be positive. This set of medaka had been raised from birth at USABRDL. The negative animals were euthanized, and the egg culturing/colony renewal for the transgenic medaka was put on hold. At this time, two sets of F1 fry have been cultured and reared. There are no F2 animals. Investigations are being made into the equipment used and scaling down the reagents involved in the DNA extraction.

Transgenic animal research conducted at NIEHS, Research Triangle Park, NC:

In this portion of the task, *GEO-CENTERS* is providing support for a project to develop *in vivo* mutagenicity testing in fish and mammals (medaka and mice). Transgenic animals containing exogenous bacteriophage DNA are the model systems in development.

Training of a new employee was initiated by learning and practicing techniques for DNA extraction, restriction enzyme digestion, gel elctrophoresis, and bacteriophage recovery. DNA was extracted using three different protocols; (1) Quiagen, (2) the mouse tail DNA extraction, and (3) the small scale sucrose DNA extraction protocols. The tissue digests differ by the method in which the DNA is removed from solution. The Quiagen protocol removes DNA from solution via ion exchange. The others are non-ionic exchange methods. Restriction enzyme digests were based on the Quiagen protocol. No other methods of restriction enzyme digests were performed. In association with the restriction enzyme digests, gel preparation was also performed. The gel was photographed, transferred to nitrocellulose paper, and prehybridized. Probes were produced. Autoradiography was used to detect the

probe on the gel; however, no probes were detected. The process will be repeated when time allows. Problems with phage recovery are under investigation. One experiment tests the ligation buffer that was used. The lilgation buffer is made in-house; the buffer that comes with the kit is not used. The concern is that the locally-prepared buffer could have been prepared improperly, leading to low phage recovery. Another experiment will test the concentration of DNA that should be used. The suspicion is that there was too much DNA used. Other duties were associated with general laboratory set up while moving into a new laboratory. Some time was spent assembling and installing laboratory equipment along with preparing stock solutions for the new laboratory.

Methods development for rapid toxicity assessment:

Proofs were returned to *Environmental Toxicology and Chemistry* for the article: "A Comparison of Standard Acute Toxicity Tests with Rapid-Screening Toxicity Tests." The article will appear in April 1995, Vol. 14, No. 5.

A second lettuce root (*Lactuca sativa*) elongation study with Canal Creed groundwater was completed. Statistical analysis on this study has been delayed.

Daily maintenance and record keeping was performed on all killifish aquaria being held in room #7. Weekly water changes were made for fresh and saline aquaria. Bi-weekly water changes for water incubated embryos were also done routinely. It was noted that laboratory cultured killifish embryos are beginning to develop. Collaboration with Dr. Eugene Hull was initiated to facilitate further ideas on holding and shipping techniques of killifish embryos. New shipment and holding methods are planned for the upcoming months.

Bluegill ventilatory monitoring project:

Dismantling of old ventilatory equipment was accomplished. Personnel at USAMA collaborated to convert current ventilatory chamber housings into compatible housings for the new ventilatory chamber constructs.

A complete technical and physical construction of the new biomonitoring test chamber system was initiated in January. Amplifier systems were reconstructed and rewired to accommodate new hardware upgrades. New ventilatory chambers were assembled with all necessary electronic hardware. Plumbing of the diluter system was re-engineered to produce an improved test system as well as to accommodate new ventilatory chamber constructs. Time was spent to become familiar with the functioning and operation of the new Fluke 99 series scopemeter. The meter was then used to perform diagnostic evaluations of all newly engineered systems.

During February, assistance was provided during the construction of the permanent ventilatory laboratory facility at O-field on Aberdeen Proving Ground. Due to technical difficulties and poor signal quality from the ventilatory chambers the new ventilatory chambers

were installed at the O-field facility during mid-construction. The new ventilatory chambers and refurbished amplifier system were installed and received initial field testing at Aberdeen. The highly improved signal responses gained with the new chambers cured the technical difficulties encountered with the old chamber design.

All construction of the ventilatory system in the trailer adjacent to USABRDL was completed. Plans to outfit the new trailer system located in the bullpen area of Building 1054 were also completed.

Tasks for the month of March were divided between ventilatory applications at O-Field and construction and assembly of the aquatic biomonitoring system in the new biomonitoring trailer located in the bullpen area of Building 1054. Construction efforts in the new trailer included plumbing of the ventilatory diluter, construction and assembly of the ventilatory chambers, housings, and drainage system, construction and assembly of the ISCO automated water sampling system, along with various other duties that were performed to prepare the biomonitoring trailer for the"Command in the Spotlight" to be held on April 3.

Tasking related to O-Field included the composition of an Operation and Maintenance manual of the Biomonitoring Facility at O-Field. This was delivered along with a data forms packet for the ventilatory test. A copy of USABRDL SOPs will be generated for this project and housed at the test site.

In collaboration with USABRDL employees, ventilatory pre-test procedures were performed and real-time ventilatory monitoring was initiated at the Biomonitoring Facility at O-Field. All systems are functioning as designed and fish response to the process water is being monitored as it is discharged into the Gunpowder River.

Analytical method development and analysis of chemicals used in carcinogenicity and mobile laboratory studies:

A method for the analysis of Aldicarb, Aldicarb Sulfoxide and Aldicarb by HPLC was completed. The Prepstation was repaired and a method to analyze Atrazine and Simazine by solid phase extraction was attempted. The samples were concentrated by a factor of 10 but this did not provide sufficient precision at the lower levels of the FETAX exposure. By increasing the injection volume from 100 to 200□L, detection limits were reached. The precision and accuracy of the method were within acceptable limits. The entire analysis of Atrazine and Simazine was automated using the HP Prepstation. A limiting factor in the extraction of samples will be the availability of only five sample inlets. A 24-hour stability study was performed on the California mixture and the compounds being analyzed by HPLC were found to be stable. Two methods for the analysis of the California mix IIA by HPLC were completed and put into the format specified by the laboratory SOP's.

The testing of the solubilities of Nonylphenol and B-estradiol was completed. Both compounds were insoluble in water, and it will be necessary to use an intermediate solvent if

they are to be used in a FETAX assay.

Fourteen samples of BrdU were analyzed in support of Medaka exposures. Solid phase extraction of the Iowa III mixture is beginning. It will be necessary to concentrate the samples by a factor of 100. Interference has been found in FETAX blanks; therefore, either the extraction procedure or the chromatography will have to be changed.

Two samples of water from APG were analyzed for iron by ICAP to test the efficiency of an iron removal system.

A week was spent in Omaha, Nebraska at a training session on the ICPMS instrument sponsored by Hewlett Packard.

Analyzed approximately 120 samples in support of a FETAX study involving the California Mix IIA. There was a failure in the HP Prepstation and the unit was replaced. Received samples for munitions testing from APG. Attended a class on the operation of the Inductively Coupled Plasma Mass Spectrometer (ICP/MS). The instrumentation is capable of reaching sub ppb levels of detection for trace metals. Assisted on the installation of a Leeman 3000 ICP in the analytical chemistry trailer and ran instrumental checks. A problem with the system stability is being caused by temperature fluctuation in the trailer and it may be necessary to compensate by using internal standards.

Analytical chemistry support for Rocky Mountain Arsenal (RMA):

Routine analyses at RMA: Several samples were analyzed for benzene in connection with the Foster Wheeler (formally Ebasco) *In Situ* Biotreatability Study. The method used for these analyses included the High-Level Benzene in Water method, developed with the cooperation of *GEO-CENTERS* and RMA chemists. This method has not yet been certified for use under the RMA Laboratory Support Division (LSD) Quality Control Plan. A preliminary copy of the method has been resubmitted for review. The QC data should be finalized by next quarter.

GEO-CENTERS has taken over the routine analysis of DIMP, a byproduct of Nerve Agent which was produced at RMA in the 1950's. As part of the "lab within a lab" concept, this is the first step in converting RMA LSD into a Civilian Contractor laboratory.

Method improvement/development at RMA: The Organosulfurs Analysis method, submitted to the LSD QC Chemist for certification, has been temporarily delayed. Questions arose regarding the future application of the method and whether the number of regularly scheduled analyses would be sufficiently small that a more involved method, to achieve lower detection limits, would better meet the needs of RMA LSD.

Work on the development of a standard method for the determination of volatiles using Purge-and Trap GC/MS in conjunction with *GEO-CENTERS* has ceased for the present, due to

priority conflicts. This method, because it is not suitable for determination of high levels of benzene, does not satisfy government requirements regarding the biotreatability study.

Volatile Organic Analysis sample preparation continues to be conducted in cooperation with government personnel.

GEO-CENTERS personnel will be involved with the development of a method for the analysis of NDMA. The *GEO-CENTERS* LSD staff has recently expanded. *GEO-CENTERS* will now be involved in GC/MS and IT/MS work, provide chemical expertise, perform routine analysis, assist in method development, perform QA, and develop a QC plan for *GEO-CENTERS*.

Maintenance and optimization of USABRDL aquatic laboratory facilities:

Essential laboratory maintenance continues on all culture (medaka, guppy, bluegill, and killifish) and experimental medaka located in rooms 5 and 18. Daily activities entail three feedings of all organisms and maintenance of live food systems. Associated record keeping was performed to maintain necessary test records to meet SOP regulatory review requirements. Water analyses and light measurements were performed weekly. Transgenic medaka being held at the facility in collaboration with NIEHS were also maintained. Assistance was given in the killifish acute toxicity assays and egg culturing effort. Paperwork and water quality readings were also documented for *Nothobranchius guentheri* species held in rooms 7 and 5. Maintenance and culturing of all live food (brine shrimp and microworms) systems was performed to facilitate ongoing laboratory efforts. Weekly activities also included the following water quality analyses: dissolved oxygen, ph, conductivity, water flow measurements, ammonia, and light measurements.

A new frozen brine shrimp feeding study was coordinated with USABRDL in room 10 Diluter One. Seven tanks received the regular feeding schedule, and seven tanks received frozen brine shrimp in place of flake food. Loading rate is 60 medaka per tank. The first three sacrifices for the food study were completed at the designated intervals. Control tanks and treated tanks were sacrificed; data indicated that the treated fish consistently weighed more and were larger than the control fish.

A live brine shrimp feeding study was begun (28 FEB 1995) to determine if the amount of brine shrimp currently being fed can be increased without significant change in flake food consumption. Consumption of brine shrimp by all fish was at least double the amount currently being fed according to the standard operating procedure in place.

Quarterly light measurements, ammonia testing, and ground fault testing were performed. Preparations were made for February sacrifice of test fish from APG. All FETAX waste was disposed.

Cleanup of first APG sacrifice was initiated which included cleaning of numerous 5-

gallon tanks and assorted glassware. Techniques for the collection of medaka blood and anterior kidney were practiced.

Preparations were begun for the range find study for upcoming tests scheduled for this summer. All three of the diluters will be used; therefore, they need to be outfitted and operational by mid-April.

Collection of eggs for the medaka colony renewal and immunotoxicology research was initiated. Egg collection in late January was difficult. Egg production in the medaka colony was reduced from approximately 1000 eggs per tank to about 200 eggs per tank. With six tanks being used for collection, four days of collecting were required to get enough eggs. There have been no procedural changes in the fish maintenance regime. Medaka egg culture was successful; the hatch occurred 2/3/95. Fry were released from their beakers about one week posthatch. In March, eggs were pulled for colony rotation with one bath scheduled for immunotoxicology and one for Test 100-002. Fry will hatch on or about April 8, 1995.

The first stage of data automation that includes a multiprobe capable of logging water quality readings was implemented.

Fish shipped/received to/from other research facilities this quarter:

Total No. of medaka	Institution	Location	Shipped/Received
745	NYU	Tuxedo,NY	five shipments
560	U of WV	Morgantown, WV	two shipments
300	U of MD	APG, MD	one shipment
30	Nat'l Biol. Survey	Kearneysville, WV	one shipment
720	EPL (fixed fish)	Herndon, VA	one shipment
15	EPL (live fish)	Herndon, VA	one shipment

Mummichog slides from Test 103-001 were sent to Dr. Robin Overstreet of Gulf Coast Research Lab in Ocean Springs, Mississippi. This was done to aid in the diagnosis of parasites in these animals.

Support of the Environmental Engineering Division at Rocky Mountain Arsenal (RMA):

To better understand the technology needs of the Army during cleanup of contaminated sites, our technicians observed contractors performing remediation and demolition activities at the Rocky Mountain Arsenal. Contractors activities included the following:

- **Jacobs Engineering:** Removed sediments from building number 412. Approximately 72 drums of material were collected. The material was analyzed and characterized as polychlorinated biphenyls (PCB) contaminated and negative for agent (mustard).

- Prepared for the pilot building demolition project. Jacob's preparation tasks include the following:

  - Glass window removal from the buildings
  - Electrical hardware removal from the buildings
  - Asbestos abatement of the buildings
  - Stabilize ground surface immediately adjacent to the buildings

- Performed actual building demolitions as follows:

  - Building number 412 (raw mustard storage/reactor area)
  - Building number 433 (ethylene dehydrator &compressor/storage)

- **Gonzales Construction Company:** Worked on dismantling the South Plants steam line. The metal is being reclaimed by a recycler and water decanted from the steam line is being reclaimed by RMA-CERCLA Waste Water Treatment Plant.

- **Weston:** Worked on removing ancillary equipment and piping from the eastern side of building number 515, a pesticide and agent production area. Weston is also working on excavating, purging, decontaminating, and removing the underground pipe line from South Plants Tank Farm. The scrap metal is being decontaminated and sent to a recycler. Items that cannot be certifiably decontaminated are being consolidated into a roll-off for disposal at a Subtitle C landfill. Decanted materials (Dichloropentadiene) from the pipe line are being relinquished to Shell Chemical Company for storage.

- **Enserch Environmental:** Worked on the Boiler Plant Demolition Project. Enserch has received final clearance for building number 325 and has commenced boiler removal. Ancillary equipment removal is completed in building numbers 321 and 325. This project task was completed 17 March 95. Enserch disposed of approximately 2.8 million pounds of asbestos-containing materials, and approximately 1.8 million pound of steel was recycled.

- **Tennessee Valley Authority (TVA):** Worked on removing piping and ancillary equipment from building number 537. These buildings were part of the mustard (HD) and other agent component production and demilitarizing.

  Assisted in clarifying various regulatory issues, such as:

  - Day-to-day procedures pertaining to waste characterization, transportation, handling, and disposal.

  - Provided technical assistance regarding the clarification of double-permitted

disposal facilities, e.g. TSCA/RCRA disposal facilities.

- Hazardous waste characterization and identification.

- Department of Transportation motor carrier regulations pertaining to hazardous materials transportation.

Worked on developing and implementing a government remediation field inspectors training program. This program includes on-the-job training and regulatory compliance, including, but not limited to: developing technical training programs relating to issues such as OSHA (General Industry and Construction Standards), EPA (Clean water, RCRA, CERCLA standards) developing IGCE, Health and Safety Plans.

Performed regulatory research and provided technical support for disposal options regarding equipment contaminated with PCB and agent.

Assisted with waste profiling and characterization for the Underground Storage Tank Removal Project. Worked on developing an outline for the "Project Manager's Project Handbook." Assisted with identifying an unknown material encountered during Weston's South Plants Tank Farm excavation work. The material was deteriorated pipe insulation made of expanded glass foam.

The Risk Assessment support at Rocky Mountain Arsenal:

Integrated Endangerment Assessment/Risk Characterization (IEA/RC): *GEO-CENTERS* has been providing the Army with any ecological risk presentation material to be presented at the Monthly Restoration Advisory Board (RAB) meetings. *GEO-CENTERS* also attends these evening meetings, where risk is the issue, to provide technical support and expertise to the RAB panel.

GEO-CENTERS has been appointed as the Technical Point-of-Contact on the IEA/RC task, including the Supplemental Field Study. *GEO-CENTERS* is providing the Army with advice on how much effort is needed to close out this task.

Supplemental Field Study (SFS) --Further starling collection will take place in the spring of 1995 due to the inability to obtain a complete data set for starlings last summer. *GEO-CENTERS* has provided expertise in the decision of the starling box locations and box structure. A laboratory has been chosen to perform the analyses for the SFS. The Method Proficiency Demonstration is complete, and it is expected that samples will be sent by the first week in April.

Detailed Analysis of Alternatives (DAA) - The DAA is a result of the Feasibility Study. *GEO-CENTERS* will become involved in the DAA to provide a link between the IEA/RC Report and the DAA Report. This link is necessary to assist those involved with the DAA in

understanding how a "risk management" decision (part of the Feasibility Study) was made based on the information provided in the IEA/RC report. A meeting is scheduled in April for *GEO-CENTERS* to address the Organizations and States concerns over the biota risk maps used in the DAA.

Risk Expert - The Risk Expert contract has been tied into the IEA/RC task. This modification is expected to be awarded by April 15th.

APRIL 1 – JUNE 30, 1995

Investigation of methods available for identifying biomarkers of mammalian hepatocarcinogenesis for adaptation to fish models:

Preparations Test 100-002, a Methylene chloride range find, were begun. The literature was checked for toxicity information on MeCl. Paperwork and set-up for the BrdU portion of the test was completed. Four 72-hour exposures were done and animal sacrifice made. Sixty additional fish were sacrificed for histopathology. These slides were examined and catagorized for shipment to Colorado State University GEO-CENTERS personnel for further analysis and development of staining techniques, such as apoptotic and TGF staining.

A collection of cell proliferation papers was entered in REFMAN. Paperwork, glassware, and coordinating histology work for the cell proliferation aspects of studies 100-003 and 100-004 were prepared. The first exposure of this series for test 100-003, Medaka Carcinogenicity Test with Two Concentrations of Diethylnitrosamine (DEN) began June 30, 1995. These fish will be held for grow-out in Room 18 for the nine-month study.

A one-day training session at Oncor in Gaithersburg was attended to become familiar with the use of their Apoptag kit for apoptosis staining. Budget estimates and timelines for cell proliferation studies were prepared. An abstract was also written titled "The usefulness of 5-bromo-2'deoxyuridine (BrdU) as a marker of hepatocellular proliferation in the Japanese medaka (*Oryzias latipes*)" for *Aquatic Toxicology*.

Studies of chemical carcinogenesis in medaka:

A trial run was set up using the diluter with methylene chloride on April 6 to 7. Samples were collected from the tank feed lines and from the tanks themselves after 24 hours of flow. Measured analysis showed that the amounts lost through vaporization paralleled our TCE (trichloroethylene) results from a previous study. Several new engineering features for this system were developed. These include a self-starting siphon for the effluent overflow chambers, viton tubing used as the delivery lines between the tanks and diluter, hinged glass tops, a new type of overhead mixer used for super stock mixing, and liquid level controllers added to the waste pump. Measured analysis showed that the carbon filtration was not completely removing the toxicant, so an aeration sump was added.

Fry for Test 100-002, Medaka with methylene chloride study, were hatched with the culture fry. The fish underwent toxicant induction on April 20th. Test doses were 200, 100, 50,

25, and 12.5 mg/L of methylene chloride. Fry were released from beakers after seven days in the toxicant. This test was scheduled to run six weeks, but, due to the small size of fish and the absence of significant mortality, the sacrifice date was postponed until July 12th.

A response to a six-month audit of Test 401-002R was completed and returned. The final sacrifice was performed on the Canal Creek fish (Test 401-002R). Approximately 1000 medaka were weighed, measured, observed, and placed in labeled cassettes in the span of 48 hours. The fish were processed according to protocol before being delivered for histopathology. Fish from tanks 1-16, the creek water diluent tanks, were not sent out at this time. All remaining study records that had not been previously audited were prepared for this process. A total of 15 study binders were submitted for final inspection.

A response was received from the animal use committee chair regarding the protocol amendment for Test 100-003, medaka carcinogen test with two doses of DEN. The study design of 12 tanks allows four tanks at each treatment level: control, 10 mg/L DEN and 100 mg/L DEN. There will be multiple test endpoints with several researchers using the exposed medaka. The type of protocol submitted had been terminated, therefore a new one needed to be written. The chair gave permission to begin the test and the new protocol was submitted to the committee. A protocol was also written for Test 100-004, medaka carcinogen test with methylene chloride. This protocol is currently under committee review.

Supplies for the definitive methylene chloride study were ordered. Three times as many pumps, air sumps, and associated equipment will be needed since all three diluters will be used concurrently. A review of waste minimization efforts for the range find test showed that three aeration sumps would be sufficient to reduce the amount of methylene chloride going to the drain to below 5 mg/L.

Rodent and medaka physiologically based pharmacokinetic modeling (PBPK) conducted at Armstrong Laboratory, Wright-Patterson AFB:

The development of Microdialysis Probe project work concluded during this reporting period. An internal technical report for this work was completed on May 18, 1995 entitled "Development of Microdialysis Probe Method for Partition Coefficient Determination for Pharmacokinetic Modeling."

Currently, partition coefficients for volatile chemicals are determined by an *in vitro* vial-equilibration method. In this *in vitro* method, an animal must be sacrificed and the tissue and blood harvested to complete the procedure. Thus certain physiological aspects of a living system are compromised. In the *in vivo* method described in this report, these compromises could be reduced or eliminated.

In this method, one probe is implanted into the jugular vein and another probe is implanted into the tissue area of interest (liver, fat, and/or kidney) of an anesthetized male F-344 rat. Multiple nose-only exposures are completed each ranging from four to eight hours in length. Over time, the test chemical in the blood or tissue will be driven into this perfusate due to a concentration gradient. The chemical is then extracted from the Lactated Ringers into methyl

t-butyl ether and analyzed on a gas chromatograph. From this information, the tissue:blood partition coefficient could be calculated.

Unfortunately, the tissue:blood partition coefficients for the test chemical as determined by the microdialysis probe method was never equal to those found in published journal articles. There were multiple reasons for this disparity, including anesthetizing problems, chemical affinity to water (or lack thereof), and a low relative recovery. Therefore, with the completion of the technical report, this project was put aside until new ideas surface.

Work progressed on the Trichloroethylene Biologically Based Health Risk Modeling project with the pharmacokinetic investigation of trichloroethylene (TCE) and its metabolites in mice after oral gavage dosing. Experiments were conducted continuously in mice with 1200 mg/kg and 600 mg/kg TCE in corn oil to explore the dose dependent kinetic disposition of TCE and its metabolites. In order to eliminate an artifact involved in analysis of trichloroacetic acid (TCA) and dichloroacetic acid (DCA) in fresh blood, plasma and red blood cells were collected from mice in addition to the blood and analyzed for TCA and DCA concentrations. Pharmacokinetic parameters were determined by compartmental analysis (PCNONLIN computer program) of the blood concentration-time profiles of TCE in mice.

Development of a physiologically based pharmacokinetic model for TCE and its primary metabolite chloral hydrate (CH) in B6C3F1 mice was initiated. The model structure consisted of liver, kidney, lung, richly perfused tissues, slowly perfused tissues and fat that were interconnected by arterial and venous blood pools. The physiological parameters were obtained from the literature.

The partition coefficients (PCs) of chloral hydrate were determined using non-volatile methods. The TCE partition coefficient were modeled initially using data from the rat. Experiments were initiated to determine TCE partition coefficients in mouse blood and tissues.

Pharmacokinetic studies of chloral hydrate and trichloroethanol after intravenous administration in mice continued, and determinations were completed at two dose levels (100 mg/kg and 10 mg/kg) for each compound. Pharmacokinetic analysis and parameter determination were made for chloral hydrate and trichloroethanol (TCOH) using PCNONLIN. The decline of the concentration-time profiles of CH and TCOH were biexponential, and the dispositions were best described by a two compartment model. The results of the studies were used to validate physiologically based pharmacokinetic models for chloral hydrate and trichloroethanol in mice. This work will be presented at the upcoming International Congress of Toxicology-VII, Seattle, Washington, July 2-6, 1995.

As part of the TCE project, human TCE inhalation exposure was contracted to the Research Triangle Institute (RTI) facility. The protocol for blood and urine sample collection, sample treatment, shipment and preparation for analysis of TCE and its metabolites in humans to determine the pharmacokinetics of TCE and its metabolites was submitted to RTI. These experiments were designed and carried out at Wright-Patterson AFB to determine the stability of TCE and its metabolites, CH, TCA, DCA, TCOH (trichloroethanol), TCOHG (trichloroethanol glucuronide) in blood, urine under storage conditions, urine containers, and during shipment

(from RTI).

These studies indicated that there was neither a significant amount of TCE partition through the catheter wall nor residual TCE left in the tube to effect the kinetic study. TCE and its metabolites were stable in human blood and in urine during freezer storage, dry ice shipment, and in urine containers.

The Species Differences in Skin Penetration project work was completed during the previous reporting period. The resulting technical report entitled □The relationship between stratum corneum diffusion coefficients and temperature for halogenated hydrocarbons□ was completed on April 15, 1995.

Work for the Halon 1301 Replacement project has concentrated in two areas:

Continuing from last quarter was an attempt to determine the blood:air partition coefficient for Bromotrifluoromethane (CF3Br) by using microdialysis probes. The blood:air partition coefficient is one of the many variables which is required for the Physiologically Based Pharmacokinetic (PBPK) model.

Previously, this value was reported as 0.1 B1 0.2, obviously not a preferable response. It is predicted that the blood:air partition coefficient for CF3Br should be approximately 0.01. The use of the microdialysis probes for determining the partition coefficient has been non-profitable to date. However, by directly comparing the blood and air concentrations of CF3Br, a partition coefficient of 0.01 B1 0.001 has been determined. The chemical of interest was then changed to Dibromomethane (DBM) for experimental design purposes, as it has a well-established blood:air partition coefficient of 74.1.

When this method of determining blood:air partition coefficients was applied to DBM a value of 38 B1 4 resulted. Clearly, the new method of determining blood:air partition coefficients is not valid as of yet. Although most of the current ideas have been exhausted, this investigation will continue as time allows.

A physiologically based model for closed chamber gas uptake in rats was developed by modifying the existing model to predict pharmacokinetics of 1-Iodoheptafluoropropane (C3F7I). Metabolic constants were determined for C3F7I in rats by the model. Combined with the results of the previous modeling and simulation of CF3I and C6F13I, a paper titled "Inhalation Uptake and Metabolism of Iodohalogenated Compounds, CF3I, C6F13I and C3F7I" was submitted as a government technical report.

Work was started and completed on the second area of interest covering the gas uptake investigation of 1,1,1,3,3,3-hexafluoropropane (HFC-236fa) for the Halon 1301 Replacement project, as described above. Currently we are working on a technical report titled "Gas Uptake Kinetics of 1,1,1,3,3,3-Hexafluoropropane (HFC-236fa) and Identification of Its Potential Metabolites" to summarize this data.

Work began on Hydrocarbon Remediation Issues project (TPH). Initially for this project, the usual series of gas uptake analysis was planned to determine the metabolic constants for the PBPK model. However, it was soon discovered that the chemical of interest, Nonane, has an unusual affinity for adhering to glass and stainless steel -- the primary components of the gas uptake chamber system. Since these metabolic constants are still needed for the PBPK model, another method of determining them, which has yet to be identified, will be devised in the near future.

Cross-specied comparison of the TCE metabolism project continues. Last quarter experiments focused on the several P-450 forms believed to be involved in Trichloroethylene metabolism: 2E1, 2B1/2, 2C 6/11, and 1A 1/2. Two particular forms of cytochrome P-450, 2E1 (DMN) and 1A 1/2 (EROD) were studied.

Medaka liver microsomes were assayed for activity toward these two particular forms. It was determined that medaka demonstrate high levels of EROD and activity of the 1A-1 and 1A-2 forms. From *in vitro* and *in vivo* studies of other species, it is known that the forms 1A 1/2 are active when a biological system having these forms is exposed to trichloroethylene at high concentrations. Hepatocytes will be used as the ultimate model for medaka metabolism of trichloroethylene. Due to a lack in quantity of medaka hepatocytes, initial studies have involved trichloroethylene (TCE) metabolism by rat and human hepatocytes. This quarter, three more rat and a total of four human *in vitro* experiments were completed at TCE concentrations ranging from 50 PPM to 5000 PPM. A nearly linear relationship was observed between human hepatocyte uptake of TCE and an increasing TCE concentration range of 50 - 1000 PPM. The search is continuing for the saturation concentration of TCE for 3 mls (6 million) cells. At 5000 PPM a sharp decline in TCE uptake was seen, and our AST, ALT, LDH analysis indicated a level of toxicity may have been reached. The analysis of the concentrations between 1000 and 5000 PPM to confirm these results in human hepatocytes is underway. A technical report describing these experiments is being prepared currently and will be released pending approval.

Work continues on the "Determination of Rates of Formation of Trichloroethylene (TRI) from Metabolites in Human Hepatic Cytosolic and Microsomal Fractions."

It was determined that while NAD stimulates trichloroacetic acid (TCA) production from chloral hydrate (CH) most efficiently in all three species tested, trichloroethanol (TCOH) production was stimulated best by NADH in mice and rats but by NADPH in humans. Also it was found that at under identical conditions of protein, cofactor and substrate concentration, humans form much more TCA and TCOH than do rodents. TRI uptake by microsomes was found to be dependent on NADPH and was observed to be highest in humans followed by mice, and rats. Characterization of cytochrome P450 distribution and activity within the human hepatic microsomes has also begun. Preliminary assays for 2E1 (Paranitrophenol hydroxylase), 2A (Benzoxyresorufin O-deethylase), 3A (Pentoxyresorufin O-deethylase, and Erythromycin N-deethylase) were completed.

Results indicate that no human sample tested was devoid of any activity examined and the range of variability between individuals was less than ten-fold. Our results support that the highly conserved P450 2E1 that is responsible for TRI metabolism in the rodent functions in the

same capacity in the human. Another important isoform in the human is P450 3A, which accounts for approximately 29% of the total P450 protein in the human liver. While the Erythromycin assay showed no evidence of inhibition of 3A activity by TRI, the BROD assay did indicate possible inhibition. A more selective test for 3A such as nifedipine activity should be conducted to obtain conclusive results. A technical report entitled, "The Effect of Trichloroethylene on Form-S elective Cytochrome P450 Activities in the Rat, Mouse, and Human," detailing this information was released in May. Testing is also underway to assess TRI uptake and metabolite formation in human hepatocytes.

Work has begun on the study of the effect of the organ procurement process on *in vitro* analysis. Data derived from human organ preparations has often been used to recommend standards for safe exposures of humans to potentially toxic compounds. The process by which the donor tissue is prepared for shipment may alter its ability to metabolize xenobiotic compounds and standard *in vitro* substrates. The potential effect(s) of organ procurement processing methods on relevant drug metabolizing activity has not been characterized. This study proposes to assess the effect of the tissue removal process and subsequent cold time on cytochrome P450 activity. This major family of drug metabolizing enzymes is comprised of several tens of individual substrate-specific isoforms. Standard enzyme assays will be employed to determine the activity of P450 2E1, 1A1, 1A2, 2B, 2C, and 3A.

A Technical Report entitled "Isolated Perfused Small Intestine--Profile of Absorption and Metabolism of Trichloroethylene in the Fischer 344 Rat" was completed and approved for public release in April. An additional technical report was co-authored entitled, "Dichloroacetic Acid Metabolism In Vitro: II. Kinetics in Hepatic Cytosol." A presentation entitled "Chloral Hydrate Metabolism in Three Mammalian Species" was also co-authored and will be delivered by Captain John Lipscomb at the ISSX Conference on August 27th.

Abstracts submitted:

1) R. Abbas, J. W. Fisher, R. K. Black, T. J. Janicki and B. L. Garrity.
"Physiologically Based Pharmacokinetic (Pbpk) Model For Trichloroethylene And Its Metabolites in B6C3F1 Mice." Submitted to the Tenth Annual Meeting and Exposition of American Association of Pharmaceutical Scientists, November 5-9, 1995.

2) R. Abbas, R. K. Black, T. J. Janicki , B. L. Garrity, J. D. McCafferty and J. W. Fisher. "Disposition of Trichloroethylene And Its Metabolites in B6c3f1 Mice After Oral Administration." Submitted to the Tenth Annual Meeting and Exposition of American Association of Pharmaceutical Scientists, November 5-9, 1995.

Abstracts presented:

1) C.M. Garrett, J.C. Lipscomb, D.K. Hoover, and D.A. Mahle. "A Species Comparison of The Metabolism of Chloral Hydrate to Trichloroethanol *In Vitro*" Presented at the Annual Society of Toxicology Meeting, Baltimore, MD, 1995 and Conference of Risk Assessment Issues for Sensitive Human Populations, Dayton, OH, 1995.



2) J.E. Snawder, C.M. Garrett, and J.C. Lipscomb. "The Effect of Trichloroethyle on Cytochrome P450 Form-specific Activities of Mouse, Rat and Human Microsomes." Presented at the Annual Society of Toxicology Meeting, Baltimore, MD, 1995 and Conference of Risk Assessment Issues for Sensitive Human Populations, Dayton, OH, 1995.

3) J.C. Lipscomb, C.M. Garrett and D.A. Mahle. "Kinetics and Inhibition of Dichloroacetic Acid Metabolism *In Vitro."* Presented at the Annual Society of Toxicology Meeting, Baltimore, MD, 1995.

4) J.C.Lipscomb, D.A. Mahle, C.M Garrett. "Chloral Hydrate Metabolism in Three Mammalian Species."

Papers submitted for publication:

1) J.C. Lipscomb, D.A. Mahle, C.M. Garrett, and H.A. Barton. "Dichloracetic Acid: Metabolism in Cytosol From Mice, Rats And Humans." *Drug Metabolism and Disposition.*

2) J.C. Lipscomb, D.K. Hoover, C.T. Bishop, D.A. Mahle, W.T. Brashear, G.W. Butler, C.M. Garrett. "The in Vitro Metabolism of Chloral Hydrate I. Kinetics of Trichloroacetic Acid And Trichloroethanol Formation in Rat and Mouse Liver." *Toxicology and Applied Pharmacology.*

Technical Reports:

1) Creech, J. R., Black, R. K., Garrity, B. L., Abbas, R., Dong, L., Williams, R. J., McDougal, J. N., Vinegar, A. and Jepson, G. W. "Inhalation Uptake And Metabolism of Iodohalogenated Compounds, CF3I, C6F13I and C3F7I Iodohalogenated Compounds, CF3I, C6F13I and C3F7I." Submitted as a government technical report.

2) M.C. Caracci, R. Geary, C. Wall, G.W. Jepson. "Development of Microdialysis Probe Method for Partition Coefficient Determination for Pharmacokinetic Modeling."

3) M.C. Caracci, Z. Liron, J. D. McCafferty, G.W. Jepson. "The Relationship Between Stratum Corneum Diffusion Coefficients And Temperature For Halogenated Hydrocarbons."

4) J.C. Lipscomb, C.M. Garrett, D.A. Mahle, and G.W. Butler. "Dichloracetic Acid Metabolism: II Kinetics in Hepatic Cytosol."

5) J.C. Lipscomb, C.M. Garrett, D.A. Mahle, J.E. Snawder. "The Effect of Trichloroethylene on Form-selective Cytochrome P450 Activities in the Rat, Mouse, and Human."

6) C.M. Garrett, D.A. Mahle, S.G. Stavrou, J.C. Lipscomb. "Isolated Perfused Small Intestine-application for Absorption and Metabolism of Trichloroethylene in the Fisher 344 Rat."

7) C.M. Garrett, J.C. Lipscomb, D.K. Hoover, and D.A. Mahle. "A Species Comparison

of the Metabolism of Chloral Hydrate to Trichloroethanol *In Vitro*."

8) A. Vinegar, G.W. Butler, M.C. Caracci, J.D. McCafferty. "Gas Uptake Kinetics of 1,1,1,3,3,3-hexafluoropropane (Hfc-236fa) and Identification of its Potential Metabolities."

Teleost immunotoxicology methods development:

The fifth medaka health screen was completed during the second half of April. This screen consisted of histopathology, bacteriology, wet mounts, length, weight, hematocrit, leukocrit, plasma immunoglobulin, and functional assays to characterize health norms in test and culture medaka. The data obtained from this health screen will be evaluated along with data from the other four health screens performed to date. The results of this analysis will be presented in poster format at the SETAC meeting in Vancouver in November 1995. Presentations will also be made at Modulators of the Immune Response Meeting held in July in Breckenridge, CO, and at an American Fisheries Society Meeting in Syracuse, NY.

In the beginning of April, experimental and data analysis portions of NBT (Nitroblue Tetrazolium) and Hydrogen Peroxide assays Standard and Tube methods were assessed. The data indicated that the NBT Tube method resulted in better detection of superoxide anion production. The Hydrogen Peroxide Standard method yeilded more consistent results using the 90- and 120-minute timepoints rather than earlier timepoints previously used. More work was needed on the Hydrogen Peroxide Tube method to determine the usefulness of this type of assay. In early May trials continued. It was observed that reaction times of 210 to 250 minutes using two different concentrations of cells gave consistent results if assays were run on the same day. Time of peak activity was approximately 125 minutes. Variations occurred when assays were run on different dates, possibly due to changing stress levels in the fish, but still showed less variation than the standard method. Further efforts to reduce fluctuating stress levels in medaka were made by eliminating any fish tank cleaning for two days prior to scheduled use in upcoming assays.

Data from Health Screen 5 was entered into the SPSS database for analysis. This information will be incorporated into poster format for scientific meetings in July (Modulators of Immune Response and AFS fish Health Meeting) and November (SETAC).

A bacterial LC50 trial was performed using *Aeromonas salmonicida.* No mortality was observed even in the highest bacterial concentration of 2×10^7 CFU/ml. The results were discussed with Dr. Jeff Teska of the National Fish Health Lab. It was decided to try another aquatic pathogen, *Yersinisa ruckeri,* in future bacterial LC_{50} experiments.

Dr. Teska successfully isolated a pathogen, *Mycobacterium,* from 17-18 month-old medaka originating from the USABRDL aquaculture facility. Different age groups of both test fish and breeders will be sent for analysis to see if this occurrence is found predominately in older medaka or throughout age groups. Presently, Dr. Teska is attempting to characterize the isolates.

Immunotoxicology provided experimental support for medaka feeding studies conducted by the aquaculture facility. Age-related differences to an augmented live brine shrimp diet were studied as well as a freeze-dried brine shrimp diet as a sole source of nutrition. The controls for both groups were fish fed according to the SOP diet. Both studies showed an increase in length and weight over controls. The fish fed the Frozen Brine Shrimp (FBS) diet also had higher cell yield, greater cell viability and better *in vitro* response to an immunostimulant. A meeting was convened to discuss results and decisions to change diet was delayed until GCRL has results of the NTP diet standardization study. Histopathology results have not yet been received on fish fed higher doses of brine shrimp. An increase in heptocellular vacuolation is a possible negative side effect.

Immunotoxicology technology transfer trip to NYU resulted in the acquisition of two new bioassays which quantify the Plaque-Forming Cell potential using medaka kidney cells and the Leukocyte Proliferation potential of medaka spleen cells after stimulation with a mitogen. These assays will be performed and optimized for use in the field at USABRDL in the near future. Biomarkers in Toxicology Symposium was attended in Baltimore on June 1st.

The summary report concerning comparison data generated between medaka from the two laboratories was sent to Gulf Coast Research Lab.

Using medaka as a test species, a three-part chemical exposure assay using Zinc Chloride has been initiated. A static renewal, 96-hour LC_{50} range-find using published values from the literature is the first assay underway. Using data generated from this range-find, a fourteen-day flowthrough system observing the effect of time on immunotoxicity will be performed in a USABRDL field trailer. Preparations for this assay have included diluter reburbishing, cleaning and calibration as well as tank and toxicant delivery set-up. A third part of the program will combine dose and time exposures selected from part II of the study. A battery of immunotoxicity assays will be performed on these fish to assess immune system reaction. Two additional organic chemicals, malathion and pentachlorophenol (PCP) are planned for repeat studies. Data obtained will be used as a comparative tool for assessing immunotoxic effects of groundwaters and surface waters at Aberdeen Proving Grounds in the Fall.

Species Comparison studies were run on two different occasions. Medaka kidney cells were plated for 24 hours then tested for intracellular and extracellular superoxide anion production. Hydrogen Peroxide production was also assayed to complete the testing. Results were sent to GEO-CENTERS-CSU for analysis and work-up as part of a larger study initiated last year.

Training was given to aquaculture personnel in the collection and quantification of medaka kidney cells for use in functional assays. This cross-training enables technicians to pool resources during high work load periods, if necessary, and provides information for the expansion of parameters examined to assess fish health within the aquaculture facility.

Transgenic medaka research at USABRDL [in collaboration with Dr. James Burkhart at the National Institute of Environmental Health Sciences (NIEHS)]:

Transgenic medaka test organisms were maintained in conjunction with ongoing inter-laboratory functions with Dr. Jim Burkhart. In addition, efforts were made to culture transgenic medaka. Further investigations with the transgenic medaka are scheduled for the upcoming month. Essential laboratory supplies were documented and ordered as needed for the quarter.

Transgenic animal research conducted at NIEHS, Research Triangle Park, NC:

In this portion of the task, *GEO-CENTERS* is providing support for a project to develop *in vivo* mutagenicity testing in fish and mammals (medaka and mice). Transgenic animals containing exogenous bacteriophage DNA are the model systems in development.

The new laboratory has been made fully functional. Most, if not all, operations can be performed now without working at more than one campus.

Results from the work with *in vivo* mutagenicity testing concerning fish have been produced. Liver and dorsal muscle tissues of *Fundulus heteroclitus* were examined for mutagenicity. The controls for mutagenicity in the liver tissue showed that 1.99×10^{8} phage were recovered per tissue sample. Thirty-nine mutants were recovered, leading to a mutation frequency of 1.96×10^{-7}. The ENU-treated (*N*-ethyl-*N*-nitrosourea) fish had 5.44×10^{8} phage recovered from their livers. The number of mutants were 964 with a mutation frequency of 17.72×10^{-7}.

As for the dorsal muscle tissues, the number of phage recovered for the control samples were 3.46×10^{7}. Mutants numbered 85. Surprisingly, 61 mutants from the total of 85 came from one repetition of a single sample. The mutation frequency was 24.56×10^{-7}. For the ENU-treated samples, phage recovery from dorsal muscle tissues were 2.36×10^{7}. Three hundred thirty-one mutants were counted. The resulting mutation frequency was 140.25×10^{-7}.

The determination one can make from these results is that induced somatic mutations can be recovered from laboratory animals.

Methods development for rapid toxicity assessment:

Daily maintenance and record keeping was performed on all killifish aquaria being held in room #7. Weekly water changes were performed. An addendum to killifish protocol BRDL-92-017-02 was drafted to test new techniques of killifish embryo storage. Current embryo holding methods have proven unsatisfactory at preserving healthy embryos for long term storage and subsequent testing.

An embryo storage plan was sent to Dr. Eugene Hull for his review. Ongoing efforts to produce an environment or embryo capable of yielding long term storage of a stable population

of embryos will continue in the upcoming months.

An article entitiled" A Comparison of Standard Acute Toxicity Tests with Rapid-Screening Toxicity Tests" was published in May 1995 in *Environmental Toxicology and Chemistry*, Vol 14, no.5, pp. 907-915.

Bluegill ventilatory monitoring project:

Assistance was given in the set up of the Aquatic Biomonitoring trailer for the □Command in the Spotlight□ held at the beginning of April. Walk-through tours of the Biomonitoring trailer were also given during this function. Final electrical wiring of the ISCO automated water sampler and the Hydrolab automated water analyzer was completed on the new trailer. The liquid level detector for water holding tanks was also installed.

Efforts were made to develop an improved ventilatory amplifier for the ventilatory system in the new trailer and to replace the amplifier system previously used in the Canal Creek trailer. A new system needed to be developed due to the discontinuation of our current amplifier system manufactured by Intelligent Instrumentation. Because of the customized nature of our amplifier system several engineers from different specialty electronics corporations were contacted to determine if an appropriate replacement amplifier/signal conditioner could be commercially obtained or if special components would need to be manufactured to meet our specialized needs. A commercial amplifier manufactured by Intelligent Instrumentation was tested and found to be an unsuitable replacement for our current system. The amplifier was not sensitive enough to measure voltages in the microvolt range. An auxiliary plan of action is being pursued with applications engineer Tim Knowlton of Dataforth Corporation to produce a customized amplifier system. A prototype will be available for testing by mid-June.

Additions and revisions to the Operation and Maintenance manual for the ventilatory biomonitoring system at Aberdeen Proving Ground were made to incorporate changes to the Aquatic Biomonitoring Program in its function as a continuous online monitor of bluegill ventilatory response. A stand-alone single fish amplifier unit was constructed to be used as a demonstrational instrument at Aberdeen Proving Ground for upcoming tours by public interest groups and the general public.

Field collection of bluegill species was performed to support ventilatory applications at Old O-Field and for upcoming ventilatory sensitivity studies at BRDL.

Efforts were made to prepare the 24' trailer located in front of building 568 for upcoming ventilatory sensitivity tests. In preparation for these events information given by Jeff Leach helped solve problems associated with the program configuration of the Aquatic Biomonitoring Program. Upon completion of this work, final diagnosis of the system was made and problems with hardware and signal transmission corrected. Collaboration with Jeff Leach also resulted in additional information regarding possible upgrades and modifications to the Aquatic Biomonitoring Program that would make it more compatible and user friendly for our applications at Old O-field in Aberdeen.

Assistance was given at Old O-Field. Operations were performed concomitant with all time-based scheduled events. An "out of control" response light was installed and tested. The light is wired into the main control panel of the process plant to notify plant operators when ventilatory responses are going out of control. A remote monitor system was also installed and tested to allow plant operators to monitor all ventilatory parameters.

Analytical method development and analysis of chemicals used in carcinogenicity and mobile laboratory studies:

A variety of samples were analyzed by High Performance Liquid Chromatography (HPLC). Included in this were approximately 60 samples in support of a FETAX study involving the California Mix IIA, 135 samples of BrdU, and several munitions samples for Aberdeen Proving Grounds. A class was attended on the development of methods by HPLC. This course emphasized the use of a systematic approach to the development of methods. It provided a review of separation basics, new column developments, and detailed explanations of the various types of liquid chromatography. The course proved to be very useful in the development of a method to analyze Iowa Mix III in FETAX. The mixture of Cyanazine, Metribuzin, Atrazine, Metolachlor, and Alachlor was very difficult to separate under normal chromatographic conditions. Most columns would not resolve Metolachlor and Alachlor. It was necessary to combine an isocratic and gradient elution to provide resulotion of the compounds. A Supelco C-18 DB (25X.46cm, 5 µm particle size) was used for the separation. The solvent delivery system was programmed to deliver 45% Acetonitrile for 20 minutes to 100% Acetonitrile at 30 minutes at a flow rate of one mL/minute. The injection volume was increased to 200 uL and the amount of FETAX extracted was reduced from 100 to 50 mL. Three days of precision and accuracy data were generated, and the method provided acceptable results. Approximately 60 samples were analyzed for the FETAX assay.

Aberdeen Proving Grounds will provide soil and water samples for analysis by EPA method 8330. This method analyzes trace levels of explosives residue in water and soil by HPLC. A substantial amount of sample preparation is involved and it will be necessary to demonstrate the capability to produce acceptable results utilizing this method.

Installation of the Hewlett Packard 5600 Inductively Coupled Plasma - Mass Spectrometer (ICP-MS) was completed. The instrumentation was tested and performed according to the manufacturer's specifications. A problem was noted with sample introduction using the new ultrasonic nebulizer and a cause has not been identified.

Analytical chemistry support for Rocky Mountain Arsenal (RMA):

Routine analyses at RMA: A major portion of the routine analysis for this quarter involved diisopropylmethyl phosphanate (DIMP). The average sample load has been 60 samples per week requiring a two day turnaround time. To date, all MK (Morrison Knudsen) DIMP samples have been analyzed and reported within the short reporting time.

Steady benzene analysis continues at approximately ten samples per week. Benzene analysis is occasionally being done by purge and trap GC/MS when the DIMP sample load is

heavy. The two methods have given similar results. The results that had been produced by RMA differed from the results reported by an outside lab. To find out which lab was in error, blind QC samples were sent with actual field samples. Results from the QC samples showed that the RMA laboratory was reporting correct concentrations, while the outside lab was only reporting about 30% of the actual concentration.

Sample screening continues to be done using the Ion Trap Mass Spectrometer (ITMS). Routine air samples now arrive on a weekly basis. These samples are used in identifying air scrubbers that have become non-functional and need to be replaced. The ITMS has also been used for the identification of several unknown samples.

Purge and Trap GC/MS analysis for VOA compounds is being done by GEO-CENTERS. The weekly average sample load is ten samples per week. The number is expected to increase in the next couple of weeks. One set is expected to start in July. By doing this in-house anaylsis, GEO-CENTERS is helping the Army to save 86,000 dollars. The GC/MS instrumentation also has new software which should increase the capabilities of the instrumentation.

All data produced by GEO-CENTERS is being reviewed, entered in data deliverable packages, and transmitted to customers by GEO-CENTERS.

Method Improvement/Development: Method development continues for several analyses. The method for DIMP analysis has been modified and those modifications have been sent for review. The Benzene method is still in the process of certification. The organo sulfur method was close to certification when two additional compounds were requested by the management of the Army's laboratory. These two additional compounds are expected to add several weeks to the certification of the method.

GEO-CENTERS performed a QA laboratory walk through and found several areas that were deficient. Recommendations were made and corrective actions are being taken by the laboratory director. GEO-CENTERS is writing a laboratory QA plan, which will help to ensure that the laboratory is operating with good laboratory practices. There are also several standard operating procedures (SOP's) that have been written in conjunction with the laboratory QA plan.

Maintenance and optimization of USABRDL aquatic laboratory facilities:

Essential laboratory maintenance was performed on all culture (medaka, guppy, bluegill, white sucker, and killifish) and test fish (medaka) located in rooms 5 and 18. Daily activities include three daily feedings of all culture and test organisms. Associated record keeping was performed to maintain necessary test records to meet SOP and regulatory review requirements. Associated tank cleaning and siphoning was performed to maintain overall tank hygiene to accommodate healthy test organisms. Maintenance and culturing of all live food (brine shrimp and microworms) systems was performed to facilitate ongoing laboratory efforts. Weekly activities also include the following water quality analyses: dissolved oxygen, pH, conductivity, water flow measurements, ammonia, and light measurements.

Eggs were collected for medaka colony renewal, immunotox research and carcinogen



tests. Due to the genetic contribution of new broodstock, the hatch time for eggs has been lengthened. This factor will be taken into account in future egg cultures. The medaka colony was renewed and Bath 4 was filled with 720 animal for the immunotoxicology research group. Training was received in the following immunotoxicology procedures: hematocrit, leukocrit, superoxide production, kidney cell collection and cell number determination.

The Shedd feeding study was continued in April with the last sacrifice occuring in June. Fish that were fed frozen brine shrimp were consistently larger in length and weight. Medaka were also studied after being fed a free consumption of live brine shrimp in addition to their normal flake food diet. Brine shrimp intake approximately doubled from the amount specified in the SOP. A Fish Feeding meeting was coordinated and held on June 7. The topic of the meeting was assessment of the brine shrimp feeding studies. The consenses was that live brine shrimp would be fed to all medaka at current SOP amounts on a daily basis. Decisions on dietary changes will be withheld until histology results are back from the two recent food studies.

Fish Shipped to other research facilities this quarter:

No. of Medaka	Institution	Location	No. of shipments
1180	NYU	Tuxedo, NY	four
860	U of WA	Morgantown, WV	two
300	U of MD	Wye Research	one
699	EPL (fixed fish)	Herndon, VA	one

Support of the Environmental Engineering Division at Rocky Mountain Arsenal (RMA):

To better understand the technology needs of the Army during cleanup of contaminated sites, our technicians observed contractors performing remediation and demolition activities at the Rocky Mountain Arsenal. Contractors activities included the following:

- **JacobsEngineering**: Pilot building demolition project
- **Gonzales Construction Company:** South Plants steam line demolition project.
- **Roy F. Weston:** Above ground storage tank demolition project. PCB sampling, equipment removal, and remediation project.
- **Foster Wheeler Environmental Corp:** Non-process equipment demolition project (amended to incorporate remediation of an overhead crane contaminated with PCBs).
- **Tennessee Valley Authorities(TVA):** Process equipment removal project.
- **R&R:** Rifle Range restoration (bullet removal).

- **Harding Lawson Associates:** Hydro conductivity study of section 25.
- **Arthur D. Little:** Horizonal well pump tests.
- **ETCS:** Vinyl chloride ton container sampling and product transfer.
- **Gus Anderson:** Underground storage tank removal project.

Assisted in clarifying issues, such as:

- Day-to-day procedures pertaining to waste characterization, transportation, handling, and disposal.
- Hazardous waste characterization and identification.
- Department of Transportation motor carrier regulations pertaining to hazardous materials transportation and agent contaminated materials.

The implementation of a field inspectors training program that would be specific to the needs of the RMA is underway. This program includes but is not limited to; on the job training, regulatory compliance issues such as OSHA (General Industry & Construction Standards), RCRA, and developing an IGCE, SOW, and Health & Safety Plans. The training program has been evaluated and approved by the Army. Future training requirements are being planned for the inspectors.

Other tasks have been the development of an independent Government Cost Estimate (IGCE) for demolition of 12 RMA non-process related buildings. Assistance was given with waste profiling and characterization for the Underground Storage Tank Removal Project. Work was done on helping develop a "Project Manager's Project Handbook" for government personnel. Per the request of RMA personnel, the Program Manager for Non-Stockpile Chemical Material at Aberdeen Proving Grounds was provided with technical information regarding alternative methods for decontamination of ton containers.

The Risk Assessment support at the Rocky Mountain Arsenal (RMA) has accomplished the following:

Integrated Endangerment Assessment/Risk Characterization (IEA/RC): GEO-CENTERS has been providing the Army with any ecological risk presentation material to be presented at the Monthly Restoration Advisory Board (RAB) meetings. This material is written by GEO-CENTERS and is included in the RAB packet for their reference. GEO-CENTERS also attends these evening meetings, where risk is on the agenda, to provide technical support and expertise to the RAB panel.

The IEA/RC is a document that defines potential risk. Since GEO-CENTERS was involved in finalizing this document, they have also been asked to assist in the finalization of the Feasibility Study Document, Detailed Analysis of Alternatives Report in providing technical expertise and advise on ecological risk management decisions. Due to Settlement being reached by the parties for the clean-up of RMA, this report is due to go final on October 16, 1995.

To provide a link between the Public Affairs Office (PAO) and the Environmental Engineering Division, GEO-CENTERS has also been asked to assist PAO with producing the Proposed Plan document. GEO-CENTERS is to provide technical advice to the Public Affairs group to ensure their document reflects the agreed upon clean-up settlement. This plan is due to go final by December 7, 1995.

Supplemental Field Study (SFS) - Collection of the remainder of starlings needed for the SFS-Phase I has been completed by the contractor, Foster Wheeler. GEO-CENTERS has orchestrated this task to insure proper collection, necropsy, and paperwork procedures were carried out. GEO-CENTERS accompanied the contractor and USGS when the Global Positioning System was used to pinpoint these starling collection locations as well as ten collection sites left over from last year□s sampling effort.

A laboratory has been chosen to perform the analyses for the SFS. The Method Proficiency Demonstration is complete, but Target Reporting Limits (TRL's) do not meet the SFS criteria. The Committee is still deciding on whether or not to send these samples to the designated lab. Samples will not be sent until there is agreement. GEO-CENTERS is coordinating all necessary meetings to arrive at a decision.

Risk Expert - The Risk Expect contract has been tied into the IEA/RC task. This modification was awarded on April 14th.

JULY 1 - SEPT 30, 1995

Investigation of methods available for identifying biomarkers of mammalian hepatocarcinogenesis for adaptation to fish models:

Histopathology slides from Test 100-002 Methlylene chloride range find study continued to be examined and categorized. Selected slides were shipped to GEO-CENTERS at Colorado State University at Fort Collins for further analysis and development of apoptosis and TGF staining techniques. The first sacrifice point has been marked for analysis at Colorado State. Work on reading this study has been delayed due to necessary upgrades in computer image analysis hardware on the Bioquant System. Slides continue to be archived at BRDL for future reference. Pathology consultation was requested concerning the gut of medaka from test and control animals involved in this test. A possible parasite infestation was suspected. It was determined by the pathologists consulted that the histopathology slides showed no abnormalities in this organ. A one-day course to be held at BRDL has been scheduled for October 11 with Dr. Marilyn Wolfe of Experimental Pathology Laboratories, Inc. on the evaluation of histopathology slides and pathology in medaka in order to improve assessment of results from



tests conducted. A Bioquant training workshop was given by Mr. Lloyd Kinzer of R&M Biometrics, Inc. on September 18-21 at CSU.

Three exposures of 5-bromo-2'deoxyuirdine (BrdU) and associated sacrifices were completed for Test 100-003 carcinogenicity test with two concentrations of Diethylnitrosamine (DEN). The binder for this test was also completed.

Livers were removed from medaka that had been shipped frozen from the Pacific Northwest Research Foundation. The fish were thawed and livers were excised. The extracted livers were then refrozen and will be held at USABRDL until the six-month sacrifice for Test 100-003.

The optimizations of TGF-alpha and TGF-beta staining procedures are being conducted at Colorado State as well as a new GST-p staining procedure. The results for the GST-p method were promising, therefore more supplies were ordered to continue the trials.

Studies of chemical carcinogenesis in medaka:

A quality assurance debriefing for Test 400-002R, the West Branch Canal Creek Medaka Study was attended during the beginning of the quarter. During this meeting, the decision was made to have two separate protocols for all future studies. One protocol would meet the Animal Use Committee guidelines while the other would serve as a study protocol which would include all tests performed at the test site. Two Animal Use protocols for Test 100-003 and Test 100-004 were revised after review by the Animal Use Committee. A section was added to each protocol concerning levels of pain. These protocols were then reviewed by a statistician and specific methods of data analysis were selected. The two protocols were then submitted for quality assurance auditing.

Test conditions were summarized for Test S, a TCE recovery test, for the Pacific Northwest Research Foundation. Water flow, water quality, fish length, fish weight, feeding, light intensities, and toxicant measured values for the test duration were compiled.

Cleanup from the methylene chloride ($MeCl_2$) range find (Test 100-002) was completed. All remaining medaka from the $MeCl_2$ range find were sacrificed. Test animals were weighed, measured, and disected for removal of liver and gills. Half of each tissue removed was frozen in liquid nitrogen while the other half was perserved in Bouin=s solution. The frozen tissue samples will be held at -70C until shipment to the University of Maine is made.

The preparation of the diluters for the $MeCl_2$ Test 100-004 was completed. The proportional diluter will deliver $MeCl_2$ in cycles of 4 minutes and 10 seconds " 5 seconds. Daily checks will be made to insure accuracy. Fresh stock will be prepared daily and tank samples will be taken weekly to verify that the $MeCl_2$ is within test limits. The diluter lighting system was upgraded to comply with light intensities required for the test. A waste

system was developed to remove toxicant from the waste stream. Aeration sumps were configured in triplicate and vented to the diluter. Waste air sumps were sealed with silicone to eliminate leakage of test chemical. The waste will be monitored weekly through analysis by the chemistry department. 2,500 eggs were collected and test binder prepared. Randomization of the fish took place on September 8 and thinned to 70 fish per tank on September 18. DEN exposure occurred September 19-21. $MeCl_2$ exposure began on September 26. Two apoptosis sacrifices (24 hr post DEN exposure and 48 hr post $MeCl_2$ initiation) used a total of ten medaka per tank. For the chronic exposure, six control and six exposure tanks will each contain 60 fish with sacrifices scheduled at six and nine months.

An interim sacrifice of ten medaka per test tank was made for Test 100-003 in September. Two methods were used to prepare the fish for analysis. The first five were euthanized, weighed, measured and preserved in Bouins solution. The second five were euthanized, weighed , measured, and livers removed for histopathology. The livers were frozen in liquid nitrogen and stored at -70EC. The fish carcasses were preserved in Bouins solution to be sexed at a later date and destroyed. The frozen livers will be mailed to Pacific Northwest Research Foundation. The next sacrifice for this test is scheduled for December 1995.

Rodent and medaka physiologically based pharmacokinetic modeling (PBPK) conducted at Armstrong Laboratory, Wright-Patterson AFB:

Work continues on the Determination of Rates of Formation of Trichloroethylene (TCE) Metabolites in Human Hepatic Microsomal Fractions. It was determined that TCE uptake by microsomes was highest in humans followed by mice and rats. However, to date, epidemiologists have been unable to demonstrate a link between known TCE exposure and toxicity in humans. Therefore, as part of an ongoing investigation into the toxicity associated with TCE exposure, the kinetics of TCE metabolism in the hepatic microsomes of three species -- rat, mouse, and human were determined. CH and TCOH formation from TCE at pH 7.4 was evaluated and Km and Vmax values were determined. Results from this study have been submitted in abstract form for internal clearance and will be presented at the annual Society of Toxicology Meeting in March of 1996. Testing is also underway to assess TCE uptake and metabolite formation in human hepatocytes. Results from the human hepatocyte work will be presented at SOT in March 1996, followed by publication as a technical report.

Work continues on the study of the Effect of the Organ Procurement Process on *in Vitro* Analysis. This study is to determine whether the process by which human organs are processed into the samples used for *in vitro* analyses adversely affect the liver=s ability to metabolize chemicals. To date, approximately 20 rat livers have been perfused with either KCl/Tris buffer or University of Wisconsin media and prepared into microsomes after 0-, 6-, 12- or 24-hour cold times. Protein content has been determined for each lot of microsomes produced in order to determine enzymatic activity as nmol product formed per milligram protein. Initial enzymatic assays for 1A1, 1A2 (EROD), 3A (PROD), and 2B (BROD) have been completed and submitted for statistical analysis. Using a paired t-test, we were able to



demonstrate differences in enzymatic activity between control and test groups. Assays for 2A (Coumarin) and 2E1 (PNP, DMN) activity still need to be performed, as well as determining the activities of each isoform at 6- and 24-hr cold times. In addition, total P450 content will be determined for each lot of microsomes prepared and glucose-6-phosphatase assays will be conducted on each lot of liver homogenate and microsomes in order to determine microsomal protein recovery. Results of this study will be organized into a technical report and submitted for internal publication as soon as all data have been gathered.

Work began on the Sidsplus of Ammonium Perchlorate project. The purpose of the project is to evaluate the toxicity of ammonium perchlorate (AP) administered in the drinking water of Sprague-Dawley rats. The project is relevant to the DoD because the production and storage of AP, which is used in solid rocket and missile engine propellant mixtures, has resulted in contamination of soil and water. During the past quarter, the pilot study was conducted. Evaluation of the AP dosing solutions to determine the quantity and stability of AP in the solutions at room temperature for 28-days was performed using HPLC coupled with a conductivity detector. The dosing solutions were determined to be stable. In addition, radioimmunoassay (RIA) to quantitatively determine the measurements of thyroglobulin (Tg), reverse triiodothyronine (rT3) and triiodothyronine (T3) in serum samples obtained from control and AP-exposed rats were completed. Ammonium perchlorate-exposed rats showed increased Tg levels, increased rT3 levels and decreased T3 levels when compared to control rats.

Work is underway to prepare a protocol for the Establishment of Isolated Hepatocyte Cultures from Medaka (Oryzias latipes) and the Assessment of Trichloroethylene Metabolism by Medaka Liver Preparations *In Vitro*. An extensive literature search on medaka has been conducted.

The Halon 1301 Replacement Toxicity project has concentrated in two areas. Continuing from last quarter was the determination of blood:air partition coefficients for Bromotrifluoromethane (Halon 1301) by using microdialysis probes. The blood:air partition coefficient is one of the many variables which is required for the Physiologically Based Pharmacokinetic (PBPK) model. It is predicted that the blood:air partition coefficient for Halon 1301 should be approximately 0.13. During this quarter it was determined that the original study chemical, Dibromomethane (DBM), will not work for experimental design purposes of this new method. DBM has a boiling point of 970EC. In order to determine the concentration of chemical in the blood, an aliquot of exposed blood is placed in a vial and heated at 600EC. Theoretically, all of the chemical should be driven into the headspace prior to sampling the headspace. Since DBM has a boiling point greater than the maximum temperature we can heat a vial (600EC), not all of the chemical was driven into the headspace: Therefore, the study chemical was changed to Dichloromethane (DCM). DCM has a boiling point of 400EC and a published blood:air partition coefficient of 19.40.8. Preliminary data using the new microdialysis probe method yields a blood:air partition coefficient of 16.49 1.5 for DCM. Encouraged by these results, an attempt to determine Bromotrifluoromethane blood:air partition coefficient yielded a result of 0.101- 0.012. Based upon these initial results, it is

believed that the new method of determining blood:air partition coefficients may be valid. Further studies during the next quarter should confirm these results. In addition, a third chemical, which has not yet been determined, will be examined for method confirmation purposes.

Gas uptake investigation of 1,1,1,3,3,3-hexafluoropropane (HFC-236fa) for the Halon 1301 Replacement project was completed during this quarter. A technical report entitled Gas Uptake Kinetics of 1,1,1,3,3,3-Hexafluoropropane (HFC-236fa) and Identification of its Potential Metabolites was written; U.S. Air Force clearance is pending.

During this quarter, a collaboration was formed with researchers associated with the University of North Carolina (UNC), the USEPA, and ICF Kaiser Inc. Currently, their work involves the Physiologically Based Pharmacokinetic (PBPK) model development of Bromodichloromethane (BDCM). Data collected from a blood time course study completed at UNC did not agree with the data collected from their gas uptake analysis of metabolic constants. Our colleagues were fairly confident in the blood time course data, and asked us to complete a gas uptake study of BDCM for them. Mr. Patrick Lily of UNC visited Tri-Service Toxicology to observe our gas uptake chamber and procedures. Three exposure levels were completed, 200, 800, and 3200 ppm BDCM, along with the corresponding loss runs at each level. Our gas uptake data confirmed their previous gas uptake data, and their proposed PBPK model.

Work on the Hydrocarbon Remediation Issues project (or Total Petroleum Hydrocarbons project) continues. As noted last quarter, gas uptake analysis of metabolic constants is not feasible for the study chemical, Nonane. Therefore a series of oral gavage and intravenous dosing experiments was initiated. Following dosing, the animals are sacrificed at appropriate times via CO_2 asphyxiation, and tissues containing Nonane (blood, liver, fat, and brain) are harvested. Currently, it is believed that abdominal fat and brain are two of the major deposit sites. In addition, it was determined that up to 48 hours following a Nonane oral gavage, a notable amount of Nonane is found in the feces. Therefore, a method for periodic collection and sampling of rat feces was developed.

Work progressed under the Trichloroethylene (TCE) Project with the pharmacokinetic investigation of TCE and its metabolites in mice after oral gavage dosing. Experiments were conducted continuously in mice with 1200, 600 and 300 mg/kg TCE in corn oil to explore the dose dependent, metabolism, kinetic and disposition of TCE and its metabolites. After analyzing the data from 1200, 600 and 300 mg/kg TCE gavage dosing, it appeared that TCE disposition and metabolism were linear (dose independent) at 300 and 600 mg/kg TCE dose levels. Saturation of the TCE metabolism occurred at 1,200 mg/kg TCE dose. Therefore, 2,000 mg/kg dose of TCE was included in the oral gavage dosing study to determine non-linear kinetic parameters of TCE.

The initial analysis of the oral gavage study data was performed by non-compartmental analysis techniques. Development of PBPK models for TCE and its major metabolites were

continued along with the experimental data collection in mice. An initial model was proposed for TCE and major metabolites. It consisted of a detailed main model for TCE parent and relatively simple sub-models for the metabolites, CH, TCOH, TCOG, TCA and DCA. Partition coefficients were determined for TCE in blood, liver, kidney, lung, fat and muscle from B6C3F1 mice, and human blood for use in the models.

Pharmacokinetic and metabolic parameters were determined by compartmental analysis of the chloral hydrate (CH) and trichloroethanol (TCOH) IV dosing studies data. An initial physiologically based pharmacokinetic model was developed for chloral hydrate and its metabolites in B6C3F1 mice. The model structure for CH consisted of liver, kidney, lung, richly perfused tissues and slowly perfused tissues that were interconnected by arterial and venous blood pools. The physiological parameters were obtained from the literature. The partition coefficients (PCs) of chloral hydrate were determined using non-volatile methods. The metabolites were modeled as simple one or two compartments. The results of the chloral hydrate IV study were used for validation of the physiologically based pharmacokinetic models for chloral hydrate in mice. This work was presented at the International Congress of Toxicology-VII, Seattle, Washington, July 2-6, 1995.

The long delayed human TCE inhalation exposure was started in mid-July at Research Triangle Institute (RTI), Research Triangle Park, North Carolina. Travel to RTI was accomplished to observe the human exposure during the first 24 hours of the study. Validation of sample collection, preparation and handling was performed for experiment quality assurance. Human TCE exposure studies were underway at a rate of two subject exposures per week. The human samples were divided into several parts; samples analyzed for P-450 pathway metabolism such as TCE, CH, TCOH, TCOG, TCA and DCA were sent to WPAFB, glutathion path related analysis samples were sent to Wayne State University, and samples for biological effect analysis were sent to Creighton University. At WPAFB, samples from RTI are organized for analysis to include: GC analysis of each metabolite and its concentration. Three to four hundred samples/week of blood and urine are analyzed for TCE, CH, TCOH, TCOG, TCA and DCA. Data analysis is ongoing to estimate pharmacokinetic and metabolic parameters. Efforts are also being made to develop an initial PBPK model for human TCE inhalation exposure. Ongoing communication continues with RTI for organization of each week=s exposure, sample shipment and related details.

During this reporting period, a kinetic study and animal use protocol titled Metabolic Disposition and Kinetics of Trichloroethylene and its Metabolites was prepared, submitted and approved thru AL/OEVM Animal Care and Use Committee.

Publications, Abstracts, Posters, etc.

Abstracts/Presentations:

Lipscomb, JC, Silvers, LA, Mahle, DA, Garrett, CM and Confer, PD. Trichloroethylene Metabolism by Human Hepatocytes. Hepatocyte Users Group Meeting.

Host: University of Glasgow. Glasgow, Scotland. September 10 - 11, 1995.

Publications:

A. Vinegar, G. W. Buttler, M. C. Caracci, J. D. McCafferty, Gas Uptake Kinetics of 1,1,1,3,3,3-Hexafluoropropane (HFC-236fa) and Identification of its Potential Metabolites, U. S. Air Force Technical Report, submitted July 1995.

Abbas R., Seckel C S, Creech J R, Garrity B L, Black R K, Bzcykowski J Z and Fisher J. A Physiologically Based Pharmacokinetic (PBPK) Model to Describe Disposition of Chloral Hydrate and Trichloroethanol in Mice. *The International Toxicologist*, July 2-6, 1995, 33-P-20

Hayton W L and Abbas R. Kinetics of Paraoxon in Rainbow Trout: PBPK-PD Model of Paraoxon and Acetylcholinesterase. *The International Toxicologist*, July 2-6, 1995, 89-P-17

Hayton W L, Yu Z, Abbas R, Vick A and Doddapaneni S. Pharmacokinetics of Acriflavine in Rainbow Trout *The International Toxicologist*, July 2-6, 1995, 89-P-16

Abbas R, Schultz I R., Doddapaneni S and Hayton W L. Toxicokinetics of Parathion and Paraoxon in Rainbow Trout after Intravascular Administration and Water Exposure *Toxicol. Appl. Pharmacol.* In press, 1995.

Abbas R, and Hayton W L. Gas Chromatographic Determination of Parathion and Paraoxon in Fish Plasma and Tissues. *Journal of Analytical Toxicology*, In press, 1995.

Teleost immunotoxicology methods development:

Two posters were presented at the Modulators of the Immune Response Meeting held in Breckenridge, CO, July 8-14. Species comparison work was presented in the poster discussion session. The composite work of data collected from five immunotoxicological health screens entitled "Health Status Determination and Monitoring in an Aquatic Model (*Oryzias latipes*) Used in Immunotoxicological Testing" by L.E. Twerdok, J.R. Beaman, M.W. Curry, J.D. Teska, and J.T. Zelikoff was presented in poster session format. The meeting outlined a phylogenetic approach for identification, quantification, and utilization of immune system models of both vertebrates and invertebrates. An overview of the species comparison work has been written and submitted for publication in the Modulators of Immune Response Proceedings. Another poster recapitulating findings from the health screen data entitled "Health Status Determination and Monitoring in an Aquatic Model (*Oryzias latipes*) Used in Toxicological Testing" by L.E. Twerdok, J.R. Beaman, and M.W. Curry was presented at the Joint Meeting of the Fish Health Section of the American Fisheries Society and the Eastern Fish Disease Workshop held in Syracuse, NY, July 19-22. Both basic and applied research were presented to answer questions concerning fish health and disease as it relates to gamefish stocks in this country and in Canada.

A static 96 hour acute LC50 pilot study was performed using zinc chloride ($ZnCl_2$). No mortality was observed. Analytical chemistry results showed that desired concentrations were not acheived due to a miscalculation in stock preparation. The hygroscopic properties of $ZnCl_2$ led to further variations in test concentrations. Zinc sulfate was determined to be a more appropriate source of zinc for future testing.

A test was conducted in the biomonitoring trailer toward the goal of refining assays suitable for field use that quantify the effects of contaminants on subsystems of the immune system *in situ*. The test material used was zinc sulfate at two low level concentrations of 70 and 280 ppb Zn_2+ with sacrifices occurring at day seven and fourteen. Length, weight, hematocrit, leukocrit, organ weight, viability, and functional assays were assessed. The method of calibration and operation of proportional diluters had to be learned with instruction and assistance given by other departments. Results from the study showed that seven-day exposures at the high dose promoted significantly higher reduction of cytochrome-*c* than at the lower dose or control. The cell proliferation assay indicated proliferation was most adversely effected at the low dose after seven days of exposure. Small sample size prevented statistical analysis from being conducted on this parameter. Cell yields from the spleens taken for this test were lower than previously conducted trial assays. As the exposure progressed to day fourteen, cell yields from the spleen decreased. Further assay results are being investigated.

Medaka health screen six has been completed sampling across age categories of four culture and three test fish groups. Water samples were taken for microbial analysis from associated fish tanks as well as samples from three tanks of frogs used in the FETAX program. Histopathology results from health screen five have been received and are being integrated into data previously gathered for a poster presentation at the SETAC World Congress in Vancouver, BC, in November. Live fish from seven age groups of medaka were delivered to Dr. Jeff Teska at the National Fish Health Research Laboratory in Kearneyville, WV. This was done as part of a mycobacterium surveillance project. Difficulties in culturing techniques for mycobacterium precluded prior identification of this bacteria in fish tank samples. Preliminary reports show that mycobacteria exist in culture and test tanks of the aquaculture facility. The final report on Dr. Teska=s findings should be available early in the next quarter.

A session is scheduled on the use of the Bioquant system for reading fluorescent beads as part of a phagocytic index assay. This assay will help determine the effect of phagocytic capablilty on macrophages when exposed to environmental contaminants. The use of fluorescent beads together with the Bioquant system is expected to greatly enhance the accuracy of scoring this assay.

Work continues on the Species Comparison Study. Procedures and protocols have been started to add two amphibian species to the comparison. Pilot medaka studies carried out in Colorado were unsuccessful and will be repeated. All equipment has arrived to complete the project. Research accomplished thus far will be presented at the Quantitative and

Computational Biological Research (QCBR) meeting September 28. Meetings of the toxicology colloquium seminars and QCBR have been attended regularly. Two abstracts have been written for poster presentations at the Mountain West Society of Toxicology Meeting (MWSOT). A superfund meeting at CSU was attended September 6.

Transgenic medaka research at USABRDL [in collaboration with Dr. James Burkhart at the National Institute of Environmental Health Sciences (NIEHS)]:

In this portion of the task, GEO-CENTERS is providing support for a project to develop *in vivo* mutagenicity testing in fish and mammals (medaka and mice). Transgenic animals containing exogenous bacteriophage DNA are the model systems in development.

The N-ethyl-N-nitrosoures-induced mutagenesis work with *Fundulus* is under review. Work with 7, 12-dimethylbenz[a]anthracene (DMBA) will begin after fish husbandry problems are addressed. Currently, fish have been unsuccessful at breeding and mortality is being observed. An efficient method of electroporating fish eggs and sperm with a viral insert will also have to be determined.

Production of competent cells has been successful. The percentage competency has increased by a factor of ten. This result is possibly due to a faulty viral DNA standard. Additional experimentation will soon verify results. A paper will be written on this work if results are as expected.

Cell lines that were transfected with phi x inserts are still under propagation. Confirmation that the inserts have incorporated successfully into the genomes of these cell lines has still not been estabilished.

Methods development for rapid toxicity assessment:

A second generation of killifish (*Nothobranchius guentheri*) was cultured from the KF-7 generation. A population of 245 killifish were successfully hatched from embryos that had been held in the laboratory for a period of two months. Further efforts were pursued in developing a method of long term killifish embryo storage. The goals are as follows: to hold embryos for three months or longer, achieve less than 50% embryo storage mortality , to exceed a 50% hatch rate for embryos that survive the incubation period, and maintain less than 10% mortality over a 48-hour period for hatched embryos held in reconstituted soft water. The process involves storing embryos in refined peat moss in which the embryos can later be extricated from the peat and utilized for hatching in an acute toxicity test. The first extrication was performed at 31 days post peat moss incubation. An embryo recovery rate of 97% was recorded with a greater than 90% hatch rate, and less than 10% mortality over 48 hours. Extrication and hatch events are scheduled for two- and three-month incubation periods in the upcoming months.

A letter was written to the Polybac Corporation in response to questions concerning the publication in *Environmental Toxicology and Chemistry* entitled AA Comparison of Standad Acute Toxicity Tests with Rapid-Screening Toxicity Tests.@ Forty-four reprints of this article have been mailed in response to requests received from around the world.

Bluegill ventilatory monitoring project:

Routine and emergency procedures were carried out at Old O-Field. Bi-weekly scheduled bluegill transfers and configuring of the Aquatic Biomonitoring Program were performed during the month of July. Assistance was given to Jeffery Leach of USAG (MCHD-IM) to install a biomonitoring computer system in USAG facilities. This system will be used by Mr. Leach to create remote system software that will allow remote access to the Aquatic Biomonitoring Program via computer networking. A complete inventory and evaluation of Hydrolab water quality transmitters was performed. All probes were reconditioned and calibrated. The version number and computer compatibility of each unit was verified. Four versions of Hydrolab exist at BRDL. Two versions of the Hydrolab were found to be incompatible with the Aquatic Biomontoring Program configuration. Configuration files will need to be altered to make these versions compatible with the Aquatic Biomonitoring Program.

The new amplifier system being developed in conjunction with Dataforth Corporation was re-evaluated after the filtering bandwidth was expanded to meet requirements of the current ventilatory amplifier. The bandwidth was expanded from 4 Hz to 30 Hz to capture cough frequencies that were attenuated by a previously evaluated amplifier system. Assessment of the new amplifier showed that cough events were now discernable at the higher bandwidth. Events other than coughs were also transmitted; therefore, a computer comparison of the old and new amplifier signal outputs was made indicating that notable differences in output values were produced from the Aquatic Biomonitoring Program. Further evaluations of the two amplifiers by Chris Stivers of Dataforth and Mark Kady of KD Associates determined that matching the exact bandwidth of our old amplifier system was necessary to replicate similar response signals. An evaluation board was given to Chris Stivers to evaluate the bandwidth filtering capacity of our old amplifier. To aid in this process a diagnostic comparison of all amplifier channels using the same input ventilatory signal was performed at USABRDL. All amplifier boards tested showed similar results proving that no significant variances were present among existing amplifier boards. Our system was then compared with the amplifier system designed by Dataforth and a difference was noted between the two. It was determined that a match of bandwidth and response time was necessary. The new system was constructed by Tim Knowlton of Dataforth. The new amplifier has been received and will be evaluated in the upcoming quarter.

A sample of the current ventilatory amplifier system used in the Ventilatory Biomonitoring System of USABRDL was sent to Mr. Ron Shuey, an applications engineer at American Products Incorporated to quote an estimate for customization of Intelligent Instrumentation amplifier boards PCI-20044T-1 and PCI-20045T-1. After consulting with Mr.



Shuey it was verified that duplicate boards of our old ventilatory amplifier boards could be replicated. The system is being engineered to outfit the second Ventilatory Biomonitoring Trailer.

A ventilatory time-to-response study was carried out according to USABRDL protocol "Validation of Response and Sensitivity of the Ventilatory Biomonitoring System to Selected Test Articles." Ethyl 3-Aminobenzoate Methanesulfonate (MS-222) was used as the test substance. A sublethal ventilatory response was recorded for 50% of the test organisms (*Lepomis macrochirus*) within the first hour of testing at a concentration just below the 96-hour LC50. The ventilatory system accurately validated its ability to detect lethal levels of toxicant well in advance of a mortality response.

The Operation and Maintenance Manual for the Ventilatory Biomonitoring Facility at Old O-Field, Aberdeen Proving Ground, Edgewood Arsenal was completed and submitted for review by the Army Corps of Engineers at Aberdeen Proving Ground. Operating procedures for the Biomonitoring Facility in conjunction with the Ground Water Treatment Facility at Old O-Field were discussed with the Corps and with the Roy F. Weston Company.

A final systems check was performed on the new biomonitoring trailer located at site 1058. The trailer is slated for transport to a new site in the early spring. All internal and external plumbing was evaluated and tested. System checks were performed on all ventilatory and proportional diluter systems. All equipment and electrical systems were evaluated and re-engineered, if needed, to ensure proper functioning of all biomonitoring systems.

Analytical method development and analysis of chemicals used in carcinogenicity and mobile laboratory studies:

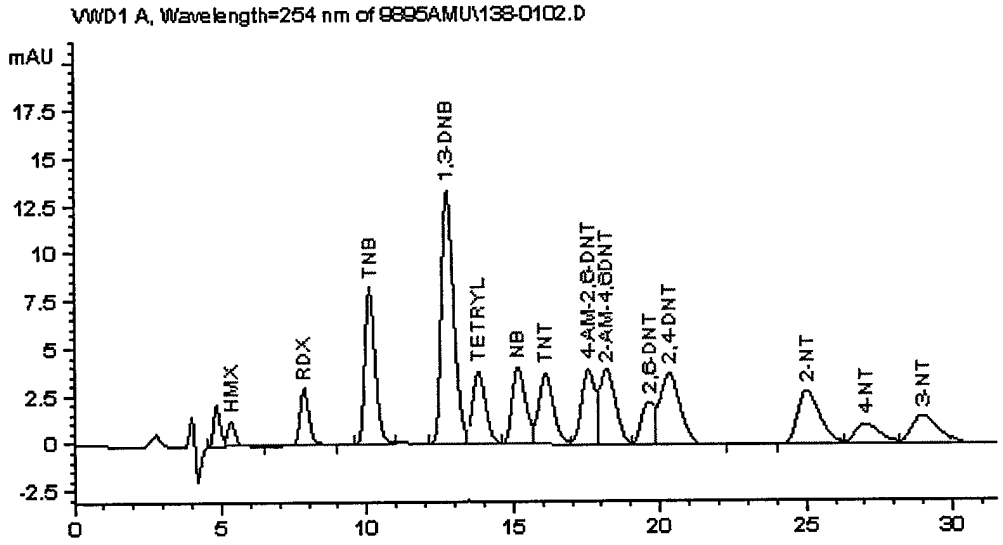
A variety of samples was analyzed by High Performance Liquid Chromatography (HPLC). This work included approximately 60 samples in support of a FETAX study involving the Iowa Mix III. This completed the series of experiments involving the mixtures of pesticides and herbicides for the FETAX project. Thirty-five samples of MS-222 were analyzed in support of experiments being conducted in the ventilatory trailer. Fifty samples of BrdU were analyzed in support of medaka research.

Samples from the analytical chemistry trailer at APG and 130 samples of Zinc were analyzed by ICP-MS. The O-Field effluent samples that were tested proved to be a useful series of tests to determine the capabilities of the ICP-MS in analyzing samples. The use of multiple internal standards gives good reproducibility for low and high mass elements. Through the ICP-MS software, an analytical chemistry report template was created which automatically fills out the report. This will eliminate possible transcription errors for samples that may contain over 20 analytes.

Work on the analysis of 14 munitions by EPA method 8330 has continued. The columns suggested by the EPA method do not provide resolution of all 14 compounds. The



separation can be improved by using a Rainin Microsorb C18 25X.46 cm column as shown in Figure 1. The conditions of the separation were fine tuned to produce the best resolution by varying the percentage of organic solvent and temperature. The final conditions were 49% Methanol: water with a column temperature of 35EC. Very small changes in these variables seem to have a large effect in the resolution of compounds and care must be taken to ensure adequate resolution of the compounds. Several secondary columns for conformation of any munitions found in a sample were tested. None of the secondary columns tested (HP Hypersil BDS, Supelco C8, and Supelco Cyano) provided resolution of all of the compounds being tested. A Supelco C8 column using a 20 to 50% Methanol gradient provided the best resolution and will be used as the secondary Column. If HMX and RDX are suspected to be present in the sample, the Supelco Cyano column will be used, the stationary phase on this column is more polar than a C-18 and reverses the order of elution. Spikes of well water containing 10 ug/L of each munition were extracted by the EPA method and approximately 75 percent of the munitions were recovered. The salting out procedure will be examined for a



method to increase the recovery.

Analytical chemistry support for Rocky Mountain Arsenal (RMA):

In order to meet the increasing sample load anticipated for RMA, the focus for this quarter was to upgrade the Gas Chromatograph/Mass Spectrometer (GC/MS). This upgrade was necessary in order to decrease the analysis time and improve chromatography.

Water and soil samples are analyzed for volatiles using an EPA standard purge and trap method. In order to upgrade the GC/MS, the effort involved removing the jet separator from the instrument. The split was done by routing the purge and trap line through the injection port, and replacing the mega-bore column with a capillary column. The idea for the switch came from a technical paper provided by Hewlett-Packard. Using the old setup, a volatile

analysis took approximately 50 minutes, while the new setup takes approximately 30 minutes. Overall, more samples can be analyzed before the instrument needs to be recalibrated, thus lowering the cost.

A standard operating procedure (SOP) was produced for the analysis of water samples using the GC/MS with purge and trap. The method, currently under review, uses EPA method 8260A where possible. It is expected that this method will be finalized next month. The written SOP for the analysis of soil samples, using GC/MS with purge and trap, will be initiated as soon as the water SOP is approved. To address the air sampling program, GEO-CENTERS is aiding in the production of a SOP for the operation of Finnigan Ion Trap Mass Spectrometer.

Method certification was started for the GC/MS using the new instrument conditions as outlined above. The method certification involved analyzing a set of calibration solutions, and a set of spike solutions. The calibration spike solutions were remade and reanalyzed within seven days from the first set. The data generated will be analyzed and practical quantitation limits determined.

Routine Analysis at RMA:

Routine analyses for volatiles continue. Approximately 15 water samples per week were analyzed using the purge and trap GC/MS. Ten samples per week were analyzed on the ITMS for volatile compound contamination in air samples.

The ITMS was featured in the yearly conference held at the RMA on air programs on post. A demonstration and a short discussion was given to the 14 attendees on the uses and potential of the ITMS.

Routine DIMP analysis continues with a weekly load of approximately 30 samples. A project with over 100 samples is scheduled the week of September 25. GEO-CENTERS personnel have taken on full responsibility for the completion of all DIMP analyses. Each quarter, Tri-County Health Department submits DIMP samples for analyses, as part of the off post private well monitoring program. Samples were also analyzed for DIMP in support of the Lillard & Clark Groundwater Intercept and Treatment System project and the Jacobs Engineering RMA Wastewater Lagoon monitoring program. The Morrison Knudsen A-Neck and North Boundary Containment System Plant Sampling Programs included a semi-weekly monitoring program, necessitating two-day results returns. This however, has been extended to seven days for the end of the program. In addition to routine DIMP analysis, an effort to lower the current method reporting limit is underway.

Method Development at RMA:

In order to meet the future needs of the Army and requirements from the state and EPA, method development for the determination of N-nitrosodimethylamine (NDMA) in

waters is underway. This will be done with the GC/thermal desorber/chemiluminescent nitrogen detector. *GEO-CENTERS* personnel are being sent to Oakridge National Laboratory for training on this method. A status date of 15 October 1995, has been set for the NDMA method certification in order to process the initial 200 incoming samples scheduled. In addition, method development for the determination of dibromochloropropane (DBCP) in water and soils has begun.

Certification of the method UL10, to determine organosulfurs in water, was completed in August. Samples are being analyzed on a routine basis. Results are typically reported within 6 days of the sample receipt.

Maintenance and optimization of USABRDL aquatic laboratory facilities:

Essential laboratory maintenance was performed on all culture (medaka, guppy, bluegill, fathead minnows, and killifish) and test fish (medaka) located in rooms 5 and 18. Daily activities include three daily feedings of all culture and test organisms. Associated record keeping was performed to maintain necessary test records to meet SOP and regulatory review requirements. Associated tank cleaning and siphoning was performed to maintain overall tank hygiene necessary to accommodate healthy test organisms. Maintenance and culturing of all live food (brine shrimp and microworms) systems was performed to facilitate ongoing laboratory efforts. Weekly activities also include the following water quality analysis; dissolved oxygen, ph, conductivity, water flow measurements, ammonia, and light measurements. Quarterly GFI testing and light intensity measurements were finished.

Transgenic medaka test organisms were maintained in conjunction with ongoing inter-laboratory research with NIEHS. Further investigations with the transgenic medaka are scheduled for October. Essential laboratory supplies were documented and ordered as needed for the month. 2,500 eggs from the medaka culture tanks were pulled to restock the breeding colony. Monthly well samples were drawn to be tested by the analytical chemistry department for the presence of TCE. Supplies were ordered to last through the beginning of next year. Fat head minnows were successfully hatched in the laboratory.

Intermittant power outages in the culture facility required an investigation as to the cause. Two baths which had independently lost power were found to have cool water flow from the water splitter. The incoming water was changed to tempered water. A water circulator was replaced with a newer model and the ground fault intensity was checked and found to be operational. Repair was necessary for the incubator located in room 5. A service plan is may be implemented for this peice of equipment due to the frequent recharging required.

Fish shipping to other research facilities this quarter:

Total No. of Medaka	Institution	Location	Shipments
442	NYU	Tuxedo, NY	four
500	U of WV	Morgantown, WV	one
8	NBS	Kearneysville, WV	one
60	PNWRF	Seattle, WA	one
100	ORNL	Oak Ridge, TN	one

A two-day conference in Toronto, Ontario, on the Care and Use of Fish, Amphibians, and Reptiles in Research was attended. Topics of interest to aquaculture process included responsible fish management, guidelines on biotechnology using fish, stress management, stress and disease prevention, and design and operation of fish and research facilities.

Support of the Environmental Engineering Division at Rocky Mountain Arsenal (RMA):

Due to the enormous contractor effort in the clean-up of RMA, GEO-CENTERS provides their observations and technical support of remediation and demolition activities performed by onsight contractors carrying out the clean-up. These projects include: the pilot demolition task, South Plants steam line demolition, above ground storage tank demolition, PCB sampling and equipment removal as part of the PCB Interim Response Action (IRA), Agent related and process equipment removal as part of the Agent IRA, horizontal well pump tests and water treatment system, underground storage tank removal project, and above ground storage tank characterization and ancillary removal task.

To address problems as they arise and to keep the clean-up effort constant, GEO-CENTERS is onsight assess and provid technical expertise related to the technology available to characterize and deal with the complex chemical contamination at the Arsenal and to clarify various regulatory issues such as, waste characterization and transportation, waste handling and disposal, hazardous waste characterization and safe handling procedures, and researching innovative methods for reclamation and disposition of waste materials.

GEO-CENTERS continues to participate in and improve on the Army approved field inspectors training program. This program includes but is not limited to; on the job training, regulatory compliance issues (OSHA-General Industry and Construction Standards and RCRA), developing and an IGCE, SOW, and Health and Safety Plan. Currently all 12 of the RMA government inspectors have performed their 90-day training session/evaluation period. Work on developing the "Project Manager's Handbook" for government personnel continues.

GEO-CENTERS also assists with providing technical information pertaining to waste profiling and characterization for contents contained within underground storage tanks scheduled for removal. As a request from RMA personnel, GEO-CENTERS provided the Program Manager for Non-Stockpile Chemical Material at Aberdeen Proving Grounds with technical information regarding alternative methods for decontamination of ton containers.

The Risk Assessment support at the Rocky Mountain Arsenal (RMA) has accomplished the following:

With the signing of the Agreement for a Conceptual Remedy for the cleanup of RMA, the Environmental Engineering division at RMA has restructured. To ensure all components of the agreement are being acted upon, a planning branch, to oversee this effort, has been established. GEO-CENTERS risk support has been moved into the planning branch.

Since an agreement has been reached to address the cleanup of RMA, it is essential to complete the Detailed Analysis of Alternatives document (DAA), part of the Feasibility Study. It is scheduled to go final on 16 October 1995. GEO-CENTERS personnel have focused on the completion of this document, by attending all dispute meetings in order to aid the Army in resolving these issues raised by the EPA, State, Shell and the US Fish and Wildlife Service (the Service). The DAA outlines the preferred alternative chosen by all the parties involved with the cleanup of the RMA.

As a result of the DAA going final, it will be presented to the public for comment in a synopsis document called the Proposed Plan. GEO-CENTERS personnel have worked with the Public Affairs Office to ensure the DAA is technically correct and accurately reflected in the Proposed Plan, before it goes to the public. Status date for the distribution of the Proposed Plan to the public is 16 October 1995.

As part of the new planning office responsibilities, GEO-CENTERS is aiding the Army in developing workshops for the public. These workshops are held to help address the public's questions and concerns about the cleanup of RMA. These workshops are held onsight and offsight, during work hours, evenings or weekends.

One of the components of the Conceptual Remedy is to initiate Medical Monitoring. GEO-CENTERS is involved in the design and implementation of this plan. The goal of this program is to address the health concerns of the public.

Along with the additional responsibilities, the GEO-CENTERS risk personnel are also monitoring the closing out of the Endangerment Assessment task. The remaining portion of this task still ongoing is the Supplemental Field Study (SFS). After the unacceptable Proficiency Demonstration ruled out the last laboratory, a new lab has been chosen to do the tissue analyses. Samples are being sent now, and a final report is anticipated by the first of the year.

The Supplemental Field Study was initiated to resolve the Biomagnification Factor dispute. Since this time, additional information on tissue levels in animals has been provided to the Army. GEO-CENTERS has been involved in the plotting of this information on maps that show the estimated soil concentrations. Using the GIS system, GEO-CENTERS, along with Foster Wheeler, have been able to show the Army how the estimated soil concentration correlates with the predicted tissue concentration in the animals. Through this mapping technique and actual data, the risk team for the Army can now demonstrate the accuracy of the risk assessment model developed for the Army. This information is being used in presentations and seminars nationally.

Abstracts Published:

Applehans, Fred M., D.J. Tate, M.L. Jones, and L.DiNorcia. An Ecological Risk Assessment for the American Kestrel at a CERCLA Site: Part I, the Model. Foster Wheeler Environmental Corporation, Lakewood, O 80228 (FMA,DJT,MLJ). Rocky Mountain Arsenal, Commerce City, CO 80022 (LD).

Roy, Richard r., F.M. Applehans, M. Sorsby, D.J. Tate, M.L. Jones and L. DiNorcia. An Ecological Risk Assessment for the American Kestrel at a CERCLA Site: Part II, Use of Site-specific Data. U.S. Fish and Wildlife Service, Commerce City, CO 80022 (RRR, MS), Foster Wheeler Environmental Corporation, Lakewood, CO 80228 (FMA, DJT, MLJ). Rocky Mountain Arsenal, Commerce City, CO 80022 (LD).

Quality Assurance at Rocky Mountain Arsenal (RMA):

GEO-CENTERS personnel at the RMA Analytical Laboratory assisted in the coordination of the Lockheed Martin Energy Systems audit on July 11, 1995. As a result, the GEO-CENTERS Quality Assurance Coordinator has worked towards implementing corrections to address the deficiencies pointed out by the auditors. These include writing SOP's covering Corrective Actions, Data Package Generation, Data Review, Control Chart Review, and Balance Calibration. A system has been implemented to accurately document and trace standards and solutions used during analysis. A system has been established for the tracking and storing of data packages. Work continues on the finalization of the QA Plan.

GEO-CENTERS has also taken on the task of reviewing all data generated by the RMA Laboratory Support Division. To aid in the production of litigation quality data packages, and to initiate new QA systems, a semi-monthly training program has been implemented.

OCTOBER 1 - DECEMBER 31, 1995

Investigation of methods available for identifying biomarkers of mammalian hepatocarcinogenesis for adaptation to fish models:

A seminar on the histopathology of medaka was given by Dr. Marilyn Wolfe of Experimental Pathology Laboratories at USABRDL and attended by several GEO-CENTERS employees involved in this task. Normal and abnormal tissue types were identified and compared. Following the seminar, a training session was held utilizing the microscope and fish histopathology slides. Distinctions were made between tumor types and specific information was given on sexing medaka through tissue analysis.

Work continued on compilation of background literature for a cell proliferation paper. The introduction for the paper has been drafted and revised. Budget summaries and estimates for FY96 histopathology were also prepared for cell proliferation studies. Cross-training in the use of the Bioquant was provided to other GEO-CENTERS personnel at USABRDL in order to augment their program with image analysis capabilities and to establish trained technicians qualified to read test slides. A formal training session was also held at Colorado State University (CSU) in the use of Bioquant's fluoroscopy procedures. New protocols have been written for use of this instrumentation by other researchers. Problems associated with the Bioquant Image Analysis System at CSU continue to be investigated. R&M Biometrics has been continuously involved in resolution of the problems. New software is being tested to produce quality slides and viewgraphs for future presentations.

Work continues on statistical analysis for EE1/EE2 carcinogenicity study. All of the data will be normalized to test several hypotheses on trends and models. Assistance in statistics and statistical modeling for this project is being provided by a CSU graduate student. Slides from the methylene chloride range find study continue to be evaluated using the Bioquant Image Analysis System. The first sacrifice point should be completed by mid-January.

Studies of chemical carcinogenesis in medaka:

The histopathology data were reviewed from Test 401-002R: West Branch Canal Creek Carcinogenicity Study With Medaka. The purpose of this review was to determine if statistical analysis of graded incidences would be beneficial. Master summary sheets were compiled by sex for the six-month interim sacrifice of creek water tanks and laboratory controls. Incidence was compared to lab controls, test controls, absence or presence of DEN, and by treatment level. Patterns were noted and compared to the pathology report. In all cases, the report contained a notation of the findings of this independent analysis. It is doubtful that statistical analysis of incidence would reveal new information regarding this test.

A presentation of the Fish Carcinogen Project was given at the November 27th USABRDL Program Review. The chronic exposure process was presented with slides, an

overview of aquaculture capabilities was described and eleven of the recent tests were highlighted. Future tests will include field work and collaborations with nationally recognized scientists. A meeting was also attended at CSU where Dr. Chris Portier, of the National Toxicology Program (NTP) from the National Institute of Environmental Health Sciences (NIEHS), presented information on mathematical modeling schemes used in carcinogenesis and PBPK/PD models (Physiologically Based Pharmacokinetics/Pharmacodynamics).

Stock delivery problems for Test 100-004 continued to be investigated. The pump head was changed to a rapid-load type head and the in-use stock bottle was elevated 16 inches. The vitron tubing's short lifespan necessitates replacement after an 8-week period. To prolong the use of the expensive tubing, the delivery parameters were altered to delay tubing failure. The original design was to deliver 37 mL superstock per cycle to the mixing chamber in forty seconds at pump speed 48 during the 1:44 minutes of the empty cycle. The design was changed to pump speed 24 for eighty seconds, still delivering 37 mL per cycle. This results in less strain on the pump and the tubing but shortens the mixing time in the chamber to 20 seconds. The chemistry sample submission record fomat was also altered at the request of USABRDL chemistry.

Preliminary planning for an upcoming chloroform test with medaka was initiated. The literature was searched for relevant information and to assure no duplication of effort. The range find concentrations were determined to be 6.25, 12.5, 25, 50, and 100 mg/L. Two protocols are being written for this test. Pending approval by the Animal Use committee, this study would begin in late January 1996. An annual summary of information regarding fish use in FY95 was prepared and submitted.

A proposal was outlined for a parallel research project between NASA and USABRDL scientists. The project involves the study of the effects of zero-gravity and cosmic radiation on chemical carcinogenesis of medaka. Control and exposed medaka (50 mg/L methylazolxymethanol acetate) would be reared for approximately 90 days. Statistical analysis of histopathology, including several biomarkers, would determine the effect level. This proposal was submitted to the director of USABRDL. Milestones for the program were planned and possible known human carcinogens were reviewed for future medaka carcinogenicity tests and validation of assays.

Rodent and medaka physiologically based pharmacokinetic modeling (PBPK) conducted at Armstrong Laboratory, Wright-Patterson AFB:

Work continues on the characterization of the kinetics of trichloroethylene (TCE) in human hepatic microsomal fractions. Determination of human Km and Vmax values for TCE metabolism *in vitro* revealed three distinct groupings of individuals with high, mid-range and low Km and Vmax. This led investigators to hypothesize that three separate P450 isoforms are responsible for the metabolism of trichloroethylene in the human. The isoforms thought to be involved are P450 1A, 2E1, and 3A4; therefore, enzyme inhibition experiments are being performed using inhibitors specific to the isoforms in question to attempt to verify the



involvement of these P450 isoforms in human metabolism of TCE.

Work continues on the study of the effect of the organ procurement process on *in vitro* analysis. This study is to determine whether the process by which human organs are processed into the samples used for *in vitro* analyses adversely affect the liver□s ability to metabolize chemicals. Cytochrome P450 assays for all isoforms in all lots of prepared microsomes have been completed and submitted for statistical analysis. Assays for glucose-6-phosphatase activity and total P450 contents are awaiting completion. Results of this study will be organized into a technical report and submitted for internal publication as soon as all data have been gathered.

Work continues on the species dependent metabolism and toxicity of TCE project. The underlying objectives were (1) to develop low-volume hepatocyte culture and validate viability of cells following short-term incubation, (2) to evaluate the metabolism and toxicity of TCE in isolated hepatocytes of the mouse, rat and human, (3) to extrapolate metabolism data derived from

hepatocytes to yield an accurate prediction of metabolism by the intact animal, and (4) to compare mammalian metabolic pathways to hepatic metabolism of TCE and specific marker pathways expressed in the medaka minnow.

Experiments were conducted on isolated human hepatocytes *in vitro* to assess (1) cell viability using trypan blue exclusion, (2) TCE uptake using gas chromatography headspace analysis, (3) toxicity of TCE by measuring aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH) leakage and by analyzing potassium content, and (4) metabolism of TCE by assaying for trichloroethanol (TCOH, free and total), dichloroacetic acid (DCA), trichloroacetic acid (TCA) and chloral hydrate (CH) using two different methods of analysis. Additionally, samples of these human hepatocytes were prepared and sent to Dr. Lawrence H. Lash, Wayne State University School of Medicine, for S-(1,-2-dichlorovinyl)-L-cysteine (DCVC) and S-(1,2-dichlorovinyl) glutathione (DCVG) analysis. Samples were also prepared and sent to Dr. Neil R. Pumford, Division of Interdisciplinary Toxicology, University of Arkansas for Medical Sciences, Little Rock, AR, for protein adduct analysis. Through interaction with the laboratory of Dr. Pumford we will determine whether human hepatocytes display the same tendency to develop trichloro-adducted liver proteins (an indicator of toxicity) as mice. Additional aliquots of the same samples were also sent to Dr. Frank Witzmann in the Molecular Anatomy Laboratory, Indiana University - Purdue University at Columbus, for two-dimensional gel electrophoresis analysis. This analytical procedure examines the distribution of hepatic proteins and can identify specific changes in individual proteins following exposure to toxic chemicals.

During the quarter several important and preliminary accomplishments have been achieved: (1) low-volume hepatocyte culture was successfully established using human cells, (2) analytical training in metabolite identification and quantification was accomplished, and (3) baseline data on metabolism of TCE by human hepatocytes was demonstrated. We expect results from Dr. Pumford's laboratory to be forthcoming. These data will demonstrate

whether the human is at risk to the same protein adducts as the toxicologically-susceptible mouse. These data can be used in human health risk assessments for TCE. Data forthcoming from the laboratory of Dr. Witzmann may reveal indicator proteins, whose distribution parallels TCE exposure. Additionally, should protein adducts be identified by the immunological methods of Dr. Pumford, the antibody will be incubated with two-dimensional gel patterns and a high probability exists that the adducted proteins can be identified. Additional work could determine whether the same proteins are adducted in mice as in humans.

Work began on the Metabolism of Trichloroethylene by the Japanese Medaka Minnow project. The objective of this project is to determine whether the Japanese medaka minnow metabolizes TCE to chloral hydrate (CH), trichloralethanol (TCOH) and trichloral acetic acid (TCA) in a manner similar to rats, mice and humans. Although the gas chromatographic method did not adequately separate TCE from CH, preliminary data indicate that this metabolic step may be catalyzed by the medaka. This is novel information and has stimulated further interest in this species as a potential marker species in the realm of environmental monitoring. An additional goal is to determine the distribution of microsomal protein within medaka liver.

To accomplish these goals during this quarter, whole fish homogenate was prepared from 4, 7 and 12 day-old and adult medaka. Livers were harvested from 49 male and 49 female retired adult breeders, homogenized and centrifuged to yield suspensions containing cytosolic, mitochondrial and microsomal proteins. The microsomal protein content of both male and female adult medaka livers, as well as hepatic S9 were spectrophotometrically determined using the Pierce BCA Protein Assay Reagent. Results indicate that there is a gender-specific distribution of total liver mass, total liver protein and S9 (metabolically active) protein. Although the body mass of the adult female is approximately 71% that of the male, the female fish has more liver mass per fish than the male (18.93 versus 12.44 mg), more S9 protein (1.54 versus 0.88 mg per gram body mass), more cytochrome P-450 (0.38 versus 0.27 nmoles P-450 per gram body mass) and more activity toward an enzyme-marker substrate (ethoxyresorufin o-deethylase: 4.734 pmoles versus 3.674 pmoles/ minute/gram liver). These data may indicate that the female medaka may be at a slightly higher risk to chemicals requiring metabolic activation in the production of toxicity (e.g., TCE). Although this implication is beyond the scope of work underway and planned, the hypothesis should be evaluated in the future.

Current efforts are centered on developing a gas chromatographic method for the evaluation of CH formation following the exposure of hepatic microsomes to TCE. In addition, samples of adult, medaka (male and female) hepatic microsomes were shipped to a collaborator, Dr. Stelvio Bandiera, University of British Columbia, for analysis of individual cytochrome P-450 isoforms. It is hypothesized that TCE is metabolized in the rodent by isoform 2E1, a form which may not be expressed to appreciable degrees in fish. Future work will compare TCE metabolism in fish with the distribution of P-450 isoforms to determine whether forms other than 2E1 may metabolize TCE to more toxic compounds.



Work on the Halon 1301 Replacement Toxicity project during the past quarter has concentrated on the development of a method to determine blood:air partition coefficients for poorly soluble chemicals. Bromotrifluoromethane (Halon 1301) is poorly soluble in blood, as is 1,1,1,3,3,3-Hexafluoropropane (HFC-236fa), and Dichloromethane (DCM). The blood:air partition coefficient for DCM has been established by the vial equilibration method previously. Over the past months, the method developed has been shown to accurately determine blood:air partition coefficients for Halon 1301 and DCM. The vial equilibration method is currently the only reliable method to determine partition coefficients. Presently, work is underway to examine HFC-236fa by this newly developed method. In this method, approximately six milliliters of heparinized rat blood is placed into an exposure flask and exposed to vapor of the chemical of interest at a rate of 30 milliliters per minute. Microdialysis probes are used to determine when the system reaches equilibrium. Every 30 minutes, the exposure concentration is analyzed three times by gas chromatography. Once the system has attained equilibrium, nine 50 ml blood samples are drawn and placed into 2 mL headspace autosampler vials. The vials are heated at 55□C for at least 30 minutes before the headspace is analyzed by hand injections on a gas chromatograph. All of the chemicals studied have a boiling point less than 50□C to ensure that all of the chemical is driven into the headspace. The blood:air partition coefficients are calculated from the blood headspace concentration and the exposure concentration data. Results indicate that this modified version of the vial equilibration technique yields suitable blood:air partition coefficients for chemicals that are poorly soluble in blood. At this time, it is planned to present the information gained by this project at the 1996 annual Society of Toxicology Conference in Anaheim, California, in March of 1996.

Work on the Hydrocarbon Remediation Issues project (or Total Petroleum Hydrocarbons project) continues. The main study chemical, Nonane, has proven to be a difficult chemical to work with, as it has a tendency to adhere to glass, stainless steel, and teflon, among other things. In addition, once Nonane adheres to one of these substances, it can only be removed by heating the item to temperatures greater than 100□C. Despite this, many attempts have been made to experimentally determine all of the routes of excretion and all of the primary deposit sites. Currently blood, liver, fat, brain, feces, and exhaled breath have been examined for Nonane levels.

Work continues on the Trichloroethylene Biologically-Based Health Risk Modeling project. The study's goal is to evaluate the pharmacokinetics of trichloroethylene and its metabolites in animals and humans, explore their toxicities, develop and validate a biologically-based pharmacokinetic model for them.

The human TCE exposure studies progressed; TCE exposure and sample analysis for TCE, CH, TCOH, TCOG, TCA and DCA in blood and urine were completed. The work continued on data analysis to estimate pharmacokinetic and metabolic parameters. Efforts are also being made to develop an initial PBPK model for human TCE inhalation exposure.

Work progressed with the pharmacokinetic investigation of TCE and its metabolites in mice after oral gavage dosing. Experiments were conducted continuously in mice with 2000 mg/kg TCE in corn oil to explore the dose dependent, metabolism, kinetics and disposition of TCE and its metabolites. After analyzing the data from 1200, 600 and 300 mg/kg TCE gavage dosing, it appeared that TCE disposition and metabolism were linear (dose independent) at 300 and 600 mg/kg TCE dose levels. Saturation of the TCE metabolism occurred at 1200 mg/kg TCE dose. Therefore, 2000 mg/kg dose of TCE was included in the oral gavage dosing study to determine non-linear kinetic parameters of TCE. Experiments were also conducted continuously in mice with 1200 and 600 mg/kg of TCE in corn oil to fill in data gaps and also explore TCE kinetics in lung and its dose dependence.

The mice data analysis was continued with Excel by non-compartmental analysis techniques. Development of physiologically based pharmacokinetic models for TCE and its major metabolites were continued along with the experimental data collection in mice. An initial model was proposed for TCE and its major metabolites. It consisted of a detailed main model for TCE parent and relatively simple sub-models for the metabolites; CH, TCOH, TCOG, TCA and DCA. Currently, the initial TCE model is under a process of simulation and comparison with experimental data for the TCE parent. TCE Partition coefficient was determined in human liver, kidney, lung, fat, muscle and blood.

Continuous efforts were being made to analyze CH and its metabolite data after IV administration of CH in mice. Pharmacokinetic and metabolic parameters were determined by non-compartmental and compartmental analysis of the chloral hydrate (CH) IV dosing data. A literature review of TCA pharmacokinetics, followed by design of a pharmacokinetic and metabolism study protocol for TCA after intravenous administration in mice was performed. Initial results of the 100 mg/kg TCA IV administration in mice confirmed that the hypothesis of DCA is produced from the metabolism of TCA in mice.

Late last quarter it came to the attention of Tri-Service Toxicology that the Hazardous Material and Hazardous Waste inventory and storage system was not in compliance with the Wright-Patterson Air Force Base regulations. To ensure uninterrupted continuation of the research, many hours have been applied to cleaning out storage facilities, improving chemical safety practices, updating Hazardous Materials records, and re-organizing the chemical inventory.

Publications and Presentations:

Two posters were prepared and presented at the Tenth Annual Meeting and Exposition of the American Association of Pharmaceutical Scientists.

1) Abbas, R., Black, R. K., Janicki, T. J., Garrity, B. L., McCafferty, J. D. and Fisher, J. W. Disposition of Trichloroethylene and Its Metabolites in B6C3F1 Mice after Oral Administration. Pharmaceutical Research. Vol. 12, No. 9, pp. s-338. 1995.

2) Abbas, R., Fisher, J. W., Black, R. K., Janicki, T. J. and Garrity, B. L. Physiologically Based Pharmacokinetic (PBPK) Model for Trichloroethylene and Its Metabolites in B6C3F1 Mice. Pharmaceutical Research. Vol. 12, No. 9, pp. s-338. 1995.

3) Caldwell, D.J., Kinkead, E.R., Wolfe, R.E., King, J.H., Narayanan, L., Confer, P.D. and Mattie, D.R. Changes in Thyroid Hormone Levels after Fourteen Day Oral-Dosing with Ammonium Perchlorate. Society of Toxicology, 1996 Annual Meeting. Anaheim, CA. March 10-14, 1996. (Abstract Submitted for Presentation Consideration).

Teleost immunotoxicology methods development:

Preparation for a poster presented at Society of Toxicology and Chemistry (SETAC) '95 involved statistical analysis of six quarterly health screens (4/95 - Present). Parameters quantified included standard health aspects, microbiology, histopathology, and immunologic endpoints. These data were summarized and presented in graphic and tabular format. Presentation of the poster was made by Dr. Lorraine Twerdok in Vancouver, WA. In October, the Mountain West chapter of the Society of Toxicology at CSU held a meeting where a poster was presented on species comparison work. This work serves to illustrate cross species conservation of several immune and regulatory mechanisms.

Training of aquaculture personnel in immunotoxicology techniques was initiated to increase research capabilities. Training to date has included collection of blood for hematocrit/leukocrit determination, pooling blood for serum collection, collection of anterior kidneys for use in assays, homogenization of cells, and method of cell counting. Immunotoxicology personnel received training in the use of the Bioquant to perform area counts and pixel counts in fluorescent mode image analysis. The goal is to develop an efficient method of quantifying a phagocytic index assay using fluorescent latex microspheres. This assay may become part of the immunotox assay battery for use in field studies.

Work began on the phagocytic bactericidal assay, which quantifies the ability of macrophages to kill phagocytized bacteria. Up to a 33% reduction in bacterial growth for some dilutions at five hours have been observed. More work needs to be done in estimation and quantification of bacteria. An experiment testing the protein determination used in conjuction with all assays was performed. Templates were designed in the Kineticalc computer program to allow protein determination comparisons between the number of cells used and the actual protein concentrations of cells. Finally, two range-find LC50 experiments were performed with interperitoneal inoculation of *Yersinia ruckeri* in medaka.

Preliminary research was performed in an attempt to establish a route of administration of a bacterial pathogen whereby mortality could be observed in medaka. Several attempts using bath exposures to both *Aeromonas salmonicida* and *Yersinia ruckeri* have failed to produce any mortality. The present approach is examination of an intraperitoneal inoculation of the bacteria. The results are promising. Using *Aeromonas salmonicida,* one out of twelve fish showed mortality (8.3%). *Yersinia ruckeri* resulted in twelve out of twelve mortalities (100%)

while the control showed no mortality (0%).

An internal research review was conducted during the last week of November. The presentation consisted of the present and future status of the research program, accomplishments, and an evaluation of the programs. Immunotoxicology presented a two-part research overview of past, current, and future work. Several meetings were held by GEO-CENTERS staff to critically assess time schedules required for completion of our respective research programs. The meetings included the development of a timeline for research projects, planning by goals or milestones, and program status and directional recommendations. This exercise brought the total research program into prespective and set real-time goals for successful completion.

Transgenic medaka research at USABRDL [in collaboration with Dr. James Burkhart at the National Institute of Environmental Health Sciences (NIEHS)]:

No transgenic medaka research was performed at USABRDL this quarter.

Transgenic animal research conducted at NIEHS, Research Triangle Park, NC:

In this portion of the task, GEO-CENTERS is providing support for a project to develop *in vivo* mutagenicity testing in fish and mammals (medaka and mice). Transgenic animals containing exogenous bacteriophage DNA are the model systems in development.

An age study testing mutation frequency found in the tissues of aged mice is currently being conducted. Husbandry of Fundulus fish requires much attention. Work with the fish entails collecting eggs, feeding, and cleaning the tanks when necessary. A study will be performed to measure metabolism and DNA adducts in conjunction with mutation. Production of new transgenic lines will also begin in the new year. Tail biopsies were performed on 43 OX174-transgenic mice. These mice were the result of a cross between two heterozygous mice for the OX174 allele. A dot blot was made to identify the allele and to help segregate the homozygous, heterozygous, and non-transgenic mice. The homozygotes and heterozygous mice were quantified by the process of phospho-imaging. Matings were set up between one homozygous male and two homozygous females. Other matings were set up between one homozygous male and six heterozygous females, producing a new line.

On November 13, a visiting scientist from the National Center for Toxicology Research began one week of training to learn the techniques used to determine mutation frequency in fish and new transgenic rats.

Methods development for rapid toxicity assessment:

The second and third phases of the refined peat moss embryo holding validation were performed during the quarter. Embryos were extracted from refined peat moss at 60 and 90 days post peat moss incubation. The two-month holding study resulted in recovery of thirty-

four of 37 embryos from the peat yielding a recovery rate of 92%. Hatch initiation occurred within the first 30 minutes of introduction into the hatching tubes. The overall hatch rate was 82%. All embryos were alive at 48 hours post hatch. The three-month holding study yielded 31 of 37 embryos recovered for a recovery rate of 84%. Hatch initiation occurred within the first 30 minutes of introduction into the hatching tubes. The overall hatch rate was 82% with 0% mortality at 48 hours post hatch. Three of the 31 hatched embryos showed signs of stress at 48 hours post hatch. Test goals of <50% holding mortality, >50% hatch rate, and <10% 48-hour mortality were achieved. In-house killifish embryos were stored in refined peat moss. Approximately 400 embryos are in storage. Six-month-old embryos held in un-refined peat moss were placed into soft water to initiate hatch. Twenty-one fry hatched within a 24-hour period. The number of embryos held in the peat moss initially was unknown due to the collection process.

An abstract on Rapid Toxicity Assessment Using the Annual Killifish *Nothobranchius guentheri* was drafted and submitted for a poster presentation at the ASTM Sixth Symposium on Environmental Toxicology and Risk Assessment in Orlando, Florida, on April 14-18, 1996.

Bluegill ventilatory monitoring project:

Regularly scheduled trips were taken to Old O-Field, Edgewood Arsenal, Aberdeen Proving Ground to perform weekly operations and program configuration of the Aquatic Biomonitoring Program at the Aquatic Biomonitoring Facility. An emergency trip was taken to Old O-Field to perform full sanitization of all test and culture systems. This procedure was necessary to control an infestation of an *Ichthyopthirus* species at the Biomonitoring Facility. Emergency trips were also taken to resolve diluter malfunctions. An electrical timer/relay was designed and installed at Old O-Field. This timer is used in conjunction with the conductivity control devices in place at Old O-Field. An animal use protocol entitled Continuous Acute Toxicity Response Monitoring of Treated Effluent at Old O-Field by the Ventilatory Biomonitoring System was drafted.

All data collected at the Aquatic Biomonitoring Facility between July and September was compiled and statistically analyzed. A summary report of events and critical data was drafted and submitted to the Roy F. Weston Company and the Army Corps of Engineers. Continued data archiving and statistical analysis of all data generated at Old O-Field was performed for the quarter.

Diagnostic evaluation of the new ventilatory amplifier system was undertaken to confirm that the new system functions in the same manner as the previous ventilatory amplifier system. Preliminary evaluations indicate this to be true; however, more tests will be necessary due to interference complications between to two systems.

Analytical method development and analysis of chemicals used in carcinogenicity and mobile laboratory studies:

Work on the analysis of 14 munitions by EPA method 8330 has continued. The recoveries found when performing the salting out extraction were found to be unacceptable. The extractions were performed in an identical fashion but the results were not reproducible. An internal standard was added to compensate for the variability of the salting out extractions. Concentrations for the munitions in water samples were based on the amount of 1,4-Dinitrobenzene (1,4-DNB) recovered during an extraction. 1,4-DNB was chosen because it has similar physical and chemical properties to the compounds being studied. Five water samples were analyzed and the reproducibility was compared. The average RSD of the set of samples was improved from 11.8% when not using the internal standard to 6.9%. Several water samples were analyzed producing good results. Matrix interferences make the accurate quantition of HMX very difficult in soil and water. A gradient elution was used to improve the resolution of HMX from interferences that occur in these matrices.

Five soil samples were received for munitions analysis. The samples were dried at room temperature, extracted, and analyzed. No munitions were found in the samples. The only significant interferences found were for the analysis of 2,4-Nitrotoluene (2-NT). A peak with a similar retention time to 2-NT was noted and identified by retention time as 2-NT. Several methods were used to prove that the peak was not 2-NT. The sample was spiked and the chromatograms were overlaid. The unknown peak was resolved from 2-NT. The UV spectra of the unknown peak and a 2-NT standard were compared and the unknown peak lacked a strong absorbance at 280 nm. A cyano column used for the separation and a peak with the same retention time as 2-NT was not found.

A method for the analysis of munitions in hexane was requested. The hexane samples are being produced by the extraction of semipermeable membranes. Due to the incompatibility of hexane with the mobile phase being used the samples must be transferred into another solvent. The samples evaporated to dryness using the HP Prepstation, but it was found that the more volatile munitions (Nitrobenzene, 2-Nitrotoluene, 3-Nitrotoluene, and 4-Nitrotoluene) were being lost. The addition of 100 uL of water to 1 mL of Hexane prior to extraction improved the recoveries of the analytes.

Several sets of water samples from APG were received and analyzed. A discrepancy between the results for Iron was found. This was tracked to an interferant on the Fe 57 line. A new method was created which analyzes several lines for a metal whenever possible. This will allow a comparison of the results for different mass/charge ratios. It is suggested that random water samples be taken to establish a baseline for water samples coming from APG. Currently, samples are only being drawn when the animals in the ventilatory chambers are out of control.

A new software upgrade for the HP4500 ICP-MS was received. The upgrade allows for the automated tuning of the mass spectrometer.

Analytical chemistry support for Rocky Mountain Arsenal (RMA):

Analysis for ppt levels of N-nitrosodimethylamine (NDMA) in samples collected both on and off RMA has been underway since October. Prior to receiving samples, two methods were developed and certified to handle trace and low-level concentrations of NDMA. Approximately 100 samples have been extracted and analyzed to assess the levels of NDMA in the groundwater resulting from past storage of hydrazine fuel on RMA.

Sample screening continues using the Ion Trap Mass Spectrometer(ITMS). Approximately ten air samples are analyzed on a weekly basis to identify when an air scrubber has become non-functional and needs replacement. The ITMS has also been used for the identification of several unknown samples. Purge and trap GC/MS analysis for VOA compounds continues to be done by GEO-CENTERS personnel. The weekly average is approximately five samples. Samples from the on-site treatment plants and from groundwater monitoring wells continue to be analyzed for diisopropylmethyl phosphonate (DIMP). Approximately 30 samples a week are processed by GEO-CENTERS' chemists.

Work on the ITMS was done to lower the detection limits by an order of magnitude. By changing several of the instrument conditions, the lower detection limits were achieved. Work was also done to determine if the samples could be analyzed using an 80-ft heated sample line instead of Tedlar bags. The experiment showed that the heated line could be used, but the detection limits would be slightly higher. The purpose of the test was to determine if the ITMS can ultimately be installed in a mobile laboratory for field sampling. A second ITMS from Teledyne instruments was installed in the laboratory. The new ITMS has several features that were not available on the older ion traps. Because the new ITMS allows MS/MS work to be done on the instrument, identification of interferences in the samples will be possible. Currently only water standards have been analyzed on the instrument. Teledyne, in cooperation with Oak Ridge National Laboratory, is working on a new air sampler that should be available in early 1996. The new air sampler will be sent to RMA for testing and evaluation.

Several new analyses will be brought on-line in the next couple of months to expand the capabilities of the laboratory. Methods to prepare and analyze water and soil samples for dibromochloropropane (DBCP) and organochlorine pesticides are currently underway. Plans to start up the inorganic lab once again will begin with anion analyses.

Data generated by the entire laboratory is now reviewed and reported by GEO-CENTERS. Previously, only data generated by GEO-CENTERS personnel was handled by GEO-CENTERS QA. In addition, standard operating procedures have largely been written for all activities in the laboratory. Lockheed-Martin personnel provided a follow-up audit on October 25 and were pleased with improvements made in the laboratory since July. Because recommendations for improvements in the agent lab were made by the auditors, GEO-CENTERS was asked to work with the Army chemists to improve the quality of their data. GEO-CENTERS personnel also were consulted by Rock Island Arsenal to help establish data

quality for their agent simulant work.

Maintenance and optimization of USABRDL aquatic laboratory facilities:

Essential laboratory maintenance was performed on all culture (medaka, guppy, bluegill, fathead minnows, and killifish) and test fish (medaka) located in rooms 5 and 18. Daily activities include three daily feedings of all culture and test organisms. Associated record keeping was performed to maintain necessary test records to meet SOP and regulatory review requirements. Associated tank cleaning and siphoning was performed to maintain overall tank hygiene necessary to accommodate healthy test organisms. Maintenance and culturing of all live food (brine shrimp and microworms) systems was performed to facilitate ongoing laboratory efforts. Weekly activities also include the following water quality analysis; dissolved oxygen, pH, conductivity, water flow measurements, ammonia, and light measurements. Monthly TCE samples from the well were taken to be analylized by BRDL chemistry lab.

A field trip to was taken to assist with the collection of bluegills for future use at Aberdeen Proving Ground. Three thousand medaka fry were hatched. Of these, 1200 are to be used as new breeding stock, while the remaining fry were separated into 24 tanks to be used by the Immuntoxicology lab. Preparations were made for egg collection from the four Shedd study tanks in November, but adequate viable eggs were not obtained. A later attempt produced the necessary number of eggs for the second generation study fish. Eggs were pulled for culture fish renewal stock the second week of December.

Transgenic medaka test organisms were maintained in conjunction with ongoing inter-laboratory functions with NIEHS. Further investigations with the transgenic medaka will be scheduled. Essential laboratory supplies were documented and ordered as needed for the quarter.

Support of the Environmental Engineering Division at Rocky Mountain Arsenal (RMA):

GEO-CENTERS continues to observe remediation/demolition activities and provide technical support to the Contracting Officer's Representatives for these projects. These projects are carried out by the contractors performing the clean-up of RMA and include:

- Morrison Knudsen Corporation (MK): Above ground storage tank (AST) characterization and deactivation project.
- Gonzales Construction Company: MK's AST demolition project.
- Resources Environmental Group Services, LTD. (REGS): MK's AST interior tank decontamination project.

- Weston: PCB sampling, equipment removal, and remediation project.
- Tennessee Valley Authorities (TVA): Agent related and process equipment removal and waste management project.
- AD Little: Horizonal well pump tests and water treatment system.
- Gus Anderson: Underground storage tank (UST) waste management.
- Foster Wheeler: UST waste management and site characterization.

Assistance was given to the Army in clarifying various regulatory issues, such as waste characterization, transportation, handling, and disposal, hazardous waste characterization and safe handling procedures. Researching innovated methods for reclamation and disposition of waste materials was also a service performed. Technical information was provided pertaining to waste profiling and characterization for contents contained within underground storage tank (UST) scheduled for removal. Assistance was given to the Safety/Health and Environmental Office (SH&E) at RMA developing and implementing health and safety policies for all government employees at RMA.

An in-house OSHA 8-hour Refresher training program for RMA Government Field Inspectors was developed and implemented. Currently, efforts are being coordinated with RMA's SH&E Office to expand the in-house training program to all RMA government employees.

Training and assisting RMA employees with special waste management activities continues. For example, three drums of asbestos materials and debris contaminated with extracts (methyl chloride, chloroform, and isopropyl alcohol) required disposal. GEO-CENTERS found a Toxic Storage Disposal Facility (TSDF) that would dispose of the material. The waste required repackaging, serving as an excellent training exercise.

Two drums of mercury contaminated floor tile with ancillary decontamination equipment and supplies required disposal. GEO-CENTERS found a TSDF that would dispose of the waste.

In this situation, training for government employees in the acquisition of hands-on experience regarding proper waste classification, segregation and sample collection was provided.

Approximately 2,600 gallons of product concentrated Sulfuric Acid (98%) was found on site. RMA first wanted to research methods of disposition. It was suggested by GEO-CENTERS that the material should be reclaimed or recycled as an off spec-product. GEO-CENTERS found a facility to use the sulfuric acid as an off spec-product coordinating all removal and transportation efforts. This task required the involvment of three government



entities (TVA, RMA, and Lockhead Martin) and was successfully completed. A Letter of Appreciation was received from the Program Manager for Rocky Mountain Arsenal, Colonel Bishop, for this effort.

The Risk Assessment/Management support at the Rocky Mountain Arsenal (RMA) has accomplished the following:

The Feasibility Study: To complete the Feasibility Study process in moving towards a Record of Decision (ROD), the Final Detailed Analysis of Alternatives (DAA) was made public in October. GEO-CENTERS assisted the Army in reviewing the risk management decisions presented in the DAA. The next step in working towards the ROD, is making the Proposed Plan for the Rocky Mountain Arsenal On-post Operable Unit public for the 90-day comment period. The Proposed Plan is an abbreviated summary of each alternative outlined in the DAA, although less technical wording is used to assist the public in making appropriate comments on the preferred clean-up scenario. GEO-CENTERS was tasked to assist the Army with producing this document. GEO-CENTERS worked closely with the Army in producing the DAA and Proposed Plan and participated in the public meeting (part of the ROD process) to assist the Army in answering ecological risk questions that the public may have. GEO-CENTERS will also be involved in producing the biological text appearing in the ROD.

To comply with an agreement signed by EPA, Army, Shell, USFWS and the State, a biological sub-committee was established in November to review all biological studies at the Arsenal. The goal of this sub-committee is to serve as a technical resource to decision makers. By using expertise in analyzing and potentially collecting data, recommendations can be made suggesting remedies for surficial soil areas and aquatic resources that will break unacceptable exposure pathways in consideration of minimum habitat disturbances. Monitoring the efficacy of these remedies in breaking unacceptable exposure pathways to biota will also be performed. This sub-committee is made up of technical experts such as ecotoxicologists, biologists, and range/reclamation specialist. GEO-CENTERS has been asked to participate in this committee and advise the government. It is anticipated that this committee will be a key participant in determining the effects of remediation, and, therefore, will be working as a group for at least five years.

GEO-CENTERS is still monitoring the progress on the Supplemental Field Study. The final report on this study is due on March 30, 1996.

GEO-CENTERS received a Memorandum of Appreciation from the Program Manager for Rocky Mountain Arsenal, Colonel Bishop, citing the efforts put forth in helping establish an acceptable plan and achieve a resolution of differences between the agencies.

JANUARY 1 - MARCH 31, 1996

Investigation of methods available for identifying biomarkers of mammalian hepatocarcinogenesis for adaptation to fish models:

Work continued on the revisions for a cell proliferation paper. These revisions included expanding the introduction, editing the materials and methods, and further referencing for the bibliography. Bioquant evaluation of slides from two additional sacrifice points from Test EEI were completed and the data was sent to CSU for statistical evaluation and incorporation into the paper. Cross-training and follow-up in the use of the Bioquant was continued; currently a DEN dose response study is being read for verification of the BRDU cell-labeling method in the medaka after exposure to a genotoxic carcinogen. Approximately 720 slides from the remaining sacrifice points from this group are being analyzed. The data is then transferred from the image analysis computer to a SPSS statistical spreadsheet and intermittent analyses have been performed. Once all of the sacrifice points have been read a thorough analysis will be conducted.

Approximately 150 preserved medaka from Test 100-003 (medaka and DEN) were sexed according to age groups and then disposed. The livers of these fish had previously been excised and frozen for Dr. Malins of the Pacific Northwest Research Foundation. The three age classes of fish were six-week, three-month, and six-month. The sex of the older fish was most easily determined. At three months determination was slightly more difficult due to the range of sexual maturity present. The sex organs of the six-week-old fish were not mature or had been lost during the fixative rinsing process making sexing very difficult. For future tests, a recommendation will be made that sexing medaka will be done on fish that are three months or older. The final sacrifice took place in March with a euthanization of approximately 370 fish. Livers were removed and carcasses retained for sexing in five fish from each of the 12 tanks. The excised livers were frozen in liquid nitrogen and held at -70°C. The remaining 310 fish were prepared for histopathology.

Budget summaries and estimates for possible future applications of cell proliferation experiments in water by-product disinfection protocols were drafted and submitted.

Studies of chemical carcinogenesis in medaka:

The animal use protocol for medaka and chloroform (Tests 100-005 & 100-006) was defended at the 1/30/96 meeting of the Animal Use Committee. This protocol was the first to be submitted using the DOD format and required changes after committee review. The protocol was changed as directed, resubmitted on 1/31/96 and approved. The initiation of this project has been delayed by funding evaluation.

A third unscheduled interim sacrifice was performed on 16 medaka from Test 100-004 (methylene chloride). These fish were submitted for histopathology to EPL. No neoplastic or pre-neoplastic changes were found in the fish. Fish examined were found to be emaciated.



Methylene chloride acts as a central nervous system depressant as well as an anesthetic in fish. It is known that calorie deprivation may be protective against induction of neoplasia in some cases. The surviving fishes' feeding behavior was observed and was found to be normal, therefore, it is suspected that some aspect of the exposure acts to block fish metabolism. It was decided to terminate the flow-though exposure at six months with an interim sacrifice, while continuing the test in the grow-out phase for six additional months. Fifteen fish per tank were sacrificed at six months. The remaining fish have been moved to a culture area for a grow-out period. Glass tops for aquaria were constructed and materials were ordered for waste sumps in preparation for planned testing in the future.

Rodent and medaka physiologically based pharmacokinetic modeling (PBPK) conducted at Armstrong Laboratory, Wright-Patterson AFB:

Work on the Biological Effects of TCE project continues. The study continues to evaluate the pharmacokinetics of trichloroethylene and its metabolites in animals and humans. Their toxicities are being explored and a biologically based pharmacokinetic model will be developed and validated.

In support of the Human TCE Exposure and Modeling study, 100 and 50 pprn trichloroethylene (TCE) human exposures and sample analysis were completed. A summary of the TCE and its metabolites, chloral hydrate (CH), trichloroethanol (TCOH), trichoroethanol glucuronide (TCOG), trichloroacetic acid (TCA) and dichloroacetic acid (DCA) concentration time-profiles in blood and urine, and the calculated accumulative amount of the metabolites in urine were submitted to the TCE project director. Based on the results of the mice and human pharmacokinetic study and physiologically based pharmacokinetic modeling of TCE and metabolites, the TCE project director generated an annual progress report and briefed the Strategic Environmental Research and Development Program (SERDP, sponsor of the TCE project). As a result of the good progress and achievement of the TCE project, SERDP has extended funding for FY1997.

Work continued in the evaluation of the metabolism of TCE in primary hepatocytes obtained from humans, rats and mice. Human health risk assessments for TCE are currently based on rodent data. The toxicity of TCE appears to be contingent upon the production of cytochrome P450-dependent metabolites which include chloral hydrate (CH), trichloroethanol (TCOH, free and total), dichloroacetic acid (DCA) and trichloroacetic acid (TCA). TCE is also metabolized through a glutathione-S-transferase (GST)-dependent pathway to S-(1,2-dichlorovinyl) glutathione (DCVG). It is thought that the DCVG is further processed in the kidney to yield the nephrotoxic S-(1,2-dichlorovinyl)-L-cysteine (DCVC). The purpose of this study was to determine whether human hepatocytes, procured from organ donor agencies, metabolize TCE in a manner similar to rats and mice. To meet this objective, experiments were conducted in which isolated human hepatocytes were assessed in vitro for 1) cell viability using trypan blue exclusion, 2) toxicity of TCE by measuring aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH) leakage, and 3) metabolism of TCE by assaying for TCOH (free and total), DCA, TCA and CH using two



different methods of analysis. Steps taken were: receiving and preparing liver cells, incubation and gas chromatography, extraction and quantification of metabolites and final data analysis. Support for AST, ALT and LDH was provided by Clinical Laboratory Services within AL/OET. Additionally, samples of these human hepatocytes were prepared and sent to Dr. Lawrence H. Lash, Wayne State University School of Medicine, for DCVC and DCVG analysis. Samples were also prepared and sent to Dr. Neil R. Pumford, Division of Interdisciplinary Toxicology, University of Arkansas for Medical Sciences, Little Rock, AR, for protein adduct analysis. Through interaction with the laboratory of Dr. Pumford, it will be determined whether human hepatocytes display the same tendency to develop trichloro-adducted liver proteins (an indicator of toxicity) as mice. Additional aliquots of the same samples were also sent to Dr. Frank Witzmann in the Molecular Anatomy Laboratory, Indiana University -Purdue University at Columbus, IN, for two-dimensional gel electrophoresis analysis. This analytical procedure examines the distribution of hepatic proteins and can identify specific changes in individual proteins following exposure to toxic chemicals.

Work continued on the metabolism of Trichloroethylene by the Japanese medaka project. A gas chromatographic method was developed for the evaluation of CH and TCOH formation following the exposure of hepatic microsomes and S9 to TCE. TCE was incubated with medaka microsomal protein and metabolites were extracted with ethyl acetate. The extracts were analyzed using gas chromatography (liquid injection) with an electron capture detector and separately using gas chromatography-mass spectrometry. Observations showed microsome-mediated metabolism of TCE to CH, a precursor of toxic metabolites. Linear relationships between the formation of CH and both exposure time and protein concentration were demonstrated. In addition, CH was incubated with medaka S9 protein containing cytochrome P450 and soluble enzymes. Incubations were subjected to ethyl acetate extraction and a second method involving acidification and derivitization with dimethylsulfate followed by hexane extraction. Ethyl acetate and hexane extracts were analyzed by gas chromatography (liquid injection) with an electron capture detector. Both methods demonstrated the metabolism of CH to TCOH. Additionally, the hexane extraction method demonstrated TCA formation.

The toxicity of TCE appears to be contingent upon the production of cytochrome P450-dependent metabolites. Cytochrome P-450 2E1 metabolizes TCE in mammals; however, this isoform of cytochrome P450 has not been reported to be expressed in the fish species examined to date. In a second series of experiments, samples of male and female microsomes were analyzed by a collaborator, Dr. Stelvio Bandiera, University of British Columbia, for individual cytochrome P450 forms via polyacrylamide gel electrophoresis and immunoblotting with antibodies selective for individual cytochrome P450 forms. These experiments confirmed expression of the cytochrome P450 1A isoforms in the medaka, while cytochromes P450 of the 3A and 2E families were not detected. Together, these results are the first to indicate that medaka are capable of metabolizing TCE to CH and CH to TCOH and TCA. Metabolism of TCE in a species with no detectable activity towards P450 2E1 substrates and devoid of immunologically detectable 2E1 protein suggests that TCE is metabolized by P450 forms other than 2EL These data further support medaka's use in environmental and conventional risk assessments for this particular groundwater contaminant.



Work completed on the kinetics of trichloroethylene (TCE) in three species of hepatic microsomal fractions was presented in a poster format at the Annual Society of Toxicology Meeting in Anaheim, California. Data concerning the comparisons of metabolic rates in rat, mouse and human microsomes were displayed. This work represents the first time a direct comparison of rodent to human data from identical experimental systems has been accomplished. Results showed that humans exhibit only about one-third the metabolic rate of rodents for TCE. Determination of human Kin and Vmax values for TCE metabolism in vitro revealed three distinct groupings of individuals with high, mid-range and low Kin and Vmax. This led investigators to hypothesize that three separate P450 isoforms are responsible for the metabolism of trichloroethylene in humans. The isoforms thought to be involved are P450 1A, 2E1, and 3A4. Experimental evidence shows that P450 2E1 accounts for 80-85% of the total P450 dependent metabolism of TCE. Further experimentation involving inhibitors specific to these isoforms is currently being conducted to elucidate their involvement in human metabolism of TCE.

Work progressed in support of the Rodent IV and Oral Exposure and Modeling study with the pharmacokinetic investigation of TCE and its metabolites in mice after oral gavage dosing. Due to the variability of the TCE concentrations in mice, experiments are conducted continuously in mice with 2000, 1200 and 600 mg/kg TCE in corn oil to increase sample size and accurately explore the dose dependent metabolism, kinetics and disposition of TCE and its metabolites.

Urinary excretion studies of TCE metabolites were designed and conducted. Mice were oral gavaged with 2000, 1200, 600 and 300 mg/kg TCE in corn oil (5 mouse/dose), urine was collected at 24 hour intervals for 8 days and analyzed for CH, TCOH, TCOG, TCA and DCA concentrations. Accumulative amounts of each metabolite in the percent of dose were calculated from the concentration-time profiles, and dose dependent excretion of the metabolites were examined. This information will be applied to model development. Development of a physiologically based pharmacokinetic model for TCE and five metabolites in mice continues. This model is the most comprehensive model ever developed for TCE and metabolites. It consists of a parent model for TCE and five sub-models for the metabolites plus validation using a blood and tissues concentration-time profile following a wide range of TCE doses. Validation of the model involves comparison of the predicted TCE concentration-time profile in blood, liver, kidney, fat and lung following four dose levels, and five corresponding metabolites in blood, liver, kidney, fat and urine with experimentally determined values. During this reporting period, major efforts have been made to validate the TCE parent model with the experimentally determined concentration-time profiles of TCE in blood, liver, kidney and fat following 300, 600, 1200 and 2000 mg/kg oral gavage doses of TCE. In vivo and in vitro metabolic parameters were used in the model. The results from this work were presented at the 35th Annual Meeting of Society of Toxicology, March 10-14, 1996 in Anaheim, CA.

Continuous efforts are being made to analyze data of TCA and its metabolite, DCA, following iv administration of TCA in mice. Pharmacokinetic and metabolic parameters were



determined by non-compartmental and compartmental analysis of the trichloroacetic acid IV dosing data. A literature review on DCA pharmacokinetics was performed and a pharmacokinetic metabolism study for DCA after intravenous administration in mice was designed. Initial results of the 100 mg/kg DCA iv administration in mice indicated that the DCA is rapidly metabolized following iv administration. The concentration of DCA dropped below detection limit of the GC-ECD method. To better characterize the pharmacokinetics of DCA, a more sensitive GC-MS assay is being used. Great effort has been put into literature reviews, study design, personnel supervision, study group organization as well as extensive laboratory work, data analysis, model development and risk assessment in support of the Biological Effects of TCE project. As a result, more responsibilities of the direction of the project have been assigned.

The development of a method to determine blood-to-air partition coefficients for poorly soluble chemicals for the Halon 1301 Replacement Toxicity project concluded. The method, which was outlined in the previous quarterly report, was shown to accurately determine the blood:air partition coefficient for Bromotrifluoromethane (Halon 1301),1,1,1,3,3,3-Hexafiuoropropane (HFC-236fa), Dichloromethane (DCM) and Diethyl Ether (Ether). This work was presented as a poster at the 1996 Annual Society of Toxicology Conference in Anaheim, California. Due to the availability of this new method, rat and human blood:air partition coefficients are currently being determined for Iodotrifluoromethane (CF31) in an effort to support the PBPK Modeling of Egress Times for Halocarbon Fire Agents aspect of this project.

The previous problems with the study chemical (Nonane) for the Hydrocarbon Remediation Issues project (or Total Petroleum Hydrocarbon project) have been resolved. During the past quarter, a series of twelve nose-only inhalation exposures were completed with this chemical. For a single nose-only exposure, five female F344 rats were individually placed into cone shaped tubes. These tubes were attached to the exposure apparatus, which would blow the chemical (in this case, Nonane vapor) towards the animal's nose. Twenty-four hours prior to the exposure, the animals were surgically implanted with a jugular cannula. During the four-hour exposure, blood from each rat was periodically sampled through the cannula and was analyzed for Nonane. At the end of the exposure, the animals were either sacrificed and tissues were collected, or the animals were allowed to recover for four hours. During the four hour recovery period, the animals blood 'was monitored as before via the jugular cannula. At the end of the four-hour recovery period, the animals were sacrificed and tissues were collected. This procedure was conducted at three different concentrations and included a total of 60 rats. Currently PBPK modeling is being used with these data. Future project work is currently being planned. It will include the gavaging of rats with a mixture of Nonane and aged-soil plus a time course analysis of tissues and blood post-dose.

Work continues on the Quantitative Approaches to Measure and Model Dermal Penetration and Subsequent Chemical Damage project. One of the objectives of this project is to measure the pen-neability coefficients of 3 chemicals; chloropentafluorobenzene, 1,2-dichlorobenzene, and perfluorohexyl iodide in hairless guinea pigs, Hartley guinea pigs

and rats. This involves analyzing blood concentrations during in vivo exposures to the chemical as a pure liquid. The chemical is administered through the septum of a glass exposure cell which is attached to the test animal's skin of the back. Blood is drawn via a jugular cannula that was surgically implanted 24 hours before exposure. Before beginning actual in vivo exposures, however, a new method needed to be developed. Previous efforts on this project involved analyzing blood samples by extracting them in an appropriate solvent and quantitating by gas chromatography. This method was inconsistent and somewhat insensitive to low blood levels of chemical. A new method using headspace analysis GC has been optimized and yields much greater sensitivity and reproducibility. Surgical implantation of cannulas and subsequent in vivo exposures are scheduled to be conducted over the next few weeks.

Work continues on the study of the effect of the organ procurement process on in vitro analysis. This study is to determine whether the process by which human organs are processed into the samples used for in vitro analyses adversely affects the livers ability to metabolize chemicals. Cytochrome P450 assays for all isoforms in all lots of prepared microsomes have been completed and submitted for statistical analysis. Assays for glucose-6-phosphatase activity and total P450 contents are awaiting completion. Results of this study will be organized into a technical report and submitted for internal publication as soon as all data have been gathered.

Work began on the Defense Women's Health Research Program project. The Defense Women's Health Research Program was developed to increase research efforts on health issues that directly affect military women. The objectives of the studies include the establishment of a colony of hydra and the evaluation of various test compounds for developmental toxicity. During this quarter, training was completed on maintenance and feeding of the hydra colony. During the establishment of the hydra colony, it is necessary to feed brine shrimp on a daily basis in an attempt to increase budding rates and eventually increase the population size by asexual reproduction. Assays will begin once the colony size has reached a level capable of providing a continuous supply of hydra to maintain a viable colony, as well as providing enough hydra to perform developmental toxicity assays. During this quarter, maintenance of the Hazardous Material and Waste Inventory continued. Many hours have been used to update Hazardous Materials records and to re-organize the chemical inventory.

Presentations and Publications:

Abbas, R., Fisher, J. W., Black, R. K., Janicki, T. J, and MacMahon, K. L., Development of a Physiologically Based Pharmacokinetic Model for Trichloroethylene and its Metabolites in B6C3F I Mice. *The Toxicologist*, Vol 30, No. 1: 1267. March 13, 1996.

Caracci, M.C., Vinegar, A., Jepson, G.W., Determination of Blood:Air Partition Coefficients for Poorly Soluble Chemicals. *The Toxicologist*, Vol 30, No. 1:256. March 13, 1996

Garrett, C.M., Mahle, D.A., Lipscomb, J.C., Comparison of the In Vitro Metabolism of Trichloroethylene in Three Species: Rat, Mouse, and Human. Triservice Toxicology,

Wright-Patterson AFB, OH. *The Toxicologist*, Vol 30, No. 1: 1634. March 13, 1996

Lipscomb, J.C., Silvers, L., Mahle, D., Garrett, C.M., and Confer, P.D., Trichloroethylene Metabolism by Human Hepatocytes in Primary Culture. *The Toxicologist*, Vol 30, No. 1: 1633. March 13, 1996.

Caldwell, D.J., Kinkead, E.R., Wolfe, R.E., King, J.H., Narayanan, L., Confer, P.D. and D.R. Mattie. Changes in Thyroid Hormone Levels after Fourteen Day Oral-Dosing with Ammonium Perchlorate. *The Toxicologist*, Vol. 30, No. 1: 345. March 13, 1996.

Confer, P.D., Buttler, G.W., Bandiera, SM. and J.C. Lipscomb. Trichloroethylene Metabolism in Hepatic Microsomal and S9 Protein of the Japanese Medaka. Conference on Advances in Toxicology and Applications to Risk Assessment, Wright-Patterson Air Force Base, Ohio. April 23-25, 1996. (Accepted for presentation)

Lipscomb, J.C., Lash, L.H., Silvers, L., Mahle, D.A., Garrett, C.M. and P.D. Confer. Cytochrome P-450 and Glutathione-S-Transferase Dependent Trichloroethylene Metabolism in Human Hepatocytes. Conference on Advances in Toxicology and Applications to Risk Assessment, Wright-Patterson Air Force Base, Ohio. April 23-25, 1996. (Accepted for presentation)

Confer, P.D., Buttler, G.W., and J.C. Lipscomb. Metabolism of Trichloroethylene by the Japanese Medaka Minnow. American Chemical Society Mid-West Regional Meeting, Dayton, OH. June 9-12, 1996. (Accepted for presentation)

Teleost immunotoxicology methods development:

Medaka Health Screen #7 has been completed and encompassed fish sample groups from 2 to 10 months of age. Three age groups of culture fish and 4 age groups of test fish were sampled. Experimental observations were made for all of the standard health parameters. Data from this and the other six health screens are currently being formatted into a journal article which will be submitted to the *Fundamentals of Applied Toxicology Journal.*

The mycobacteriurn surveillance project, a collaborative effort with Dr. Jeff Teska of the National Fish Health Research Laboratory in Kearnyville, WV was completed. Data collected will be submitted for a poster presentation at the 1996 SETAC meeting held in Washington D.C. and also submitted for publication in the *Journal of Aquatic Animal Health.*

Progress has been made on the optimization of several immune-function assays. These assays are candidates for an immunotoxicological assay battery which will analyze effects of toxicants both *in vitro* and *in vivo.* The plaque forming cell (PFC) assay has been modified slightly, in order to increase resolution of plaques and facilitate enumeration of plaques by the technician. Analysis of these two modifications is currently being performed. If successful, these changes will allow this assay to be reproducible and logistically simpler than the

previously used method.

The macrophage bactericidal assay and the lymphocyte proliferation assays continue to be developed. A recent experimental run of the lymphocyte proliferation assay produced results similar to collaborators at NYU, although further analysis is necessary. Bacterial standardization for the macrophage bactericidal assay is only possible at the three highest concentrations using the microtiter plate technique with the spectrophotometer. Work will continue on bacterial standardization methods as this will simplify the assay. It has also been found that a lower concentration of detergent is necessary. Cold double distilled H20 and 10% Tween 20 has been used unsuccessfully in past experiments.

Preliminary investigations into the suitability of the bluegill sunfish *(Lepomis macrochirus)* as a lab/field teleost model are underway. Test runs of five fish each have been completed and data analysis is underway. Multiple assays are possible using the same fish due to the high yield of pronephric tissue. Greater variability between samples is expected with the use of individual animals. Medaka require a combined cell suspension fi7om multiple fish thereby decreasing variability observed. It is also expected that modifications to existing assay methods designed for medaka may be necessary to optimize immune function response in the bluegill.

The final draft of the species comparison work presented at the Immunotoxicology meeting in Breckenridge, CO was submitted and accepted for publication, and should appear in the next edition of *Modulators of Fish Immune Responses.*

Publications:

Zelikoff, Judith T., Weihua Wang, N. Islam, Lorraine E. Twerdok, Marianne Curry, Joseph Beaman, and Eliezer Flescher. 1996. Assays of Reactive Oxygen Intermediates and Antioxidant Enzymes in Medaka (Oryzias latipes): Potential Biomarkers for Predicting the Effects of Environmental Pollution. In "Methods in Aquatic Toxicology," edited by Gary Ostrander, CRC Press. In Press (Publication Date: August 1996)

Transgenic medaka research at USABRDL [in collaboration with Dr. James Burkhart at the National Institute of Environmental Health Sciences (NIEHS)]:

No transgenic medaka research was performed at USABRDL this quarter.

Transgenic animal research conducted at NIEHS, Research Triangle Park, NC:

In this portion of the task, GEO-CENTERS is providing support for a project to develop *in* vivo mutagenicity testing in fish and mammals (medaka and mice). Transgenic animals containing exogenous bacteriophage DNA are the model systems in development.

Work is focusing on the production of double-stranded (DX am 3 DNA for the purpose of producing additional transgenic animals. Improvements in fish husbandry have resulted in successful breeding and many fry available to use in the di-methyl-benz(a)anthracene (DMBA) study. This experiment is scheduled to begin soon.

A dot blot has been performed from fin clips of our *Fundulus heteroclitus* F1 and F2 fry. Results have determined that a number of these fry do contain the ΦX 174 insert. The majority of the animals contain between 15 and 20 copies of the ΦX insert. Two of the specimens, however, contained 40 and 70 copies of ΦX. More fin clips will be performed, but it has now been established that the fish have successfully transmitted the insert to their offspring.

Human fibroblasts were electroporated with ΦX DNA. A portion of these fibroblasts will be tested to confirm the intercalation of the viral DNA into the genome of the human fibroblast to see if there is any stability of the intercalation after continuous passages of the fibroblasts.

Age studies are also being conducted to visualize different rates of mutations between geriatric and pediatric mice. Problems associated with these experiments have resulted in inconclusive data. Media used to culture E. coli was at an incorrect pH. The system used to collect the DNA from the target tissues was overloaded and the number of units of ligase used was too large. This resulted in a toxic effect on the E. coli used to propagate the ΦX as well as preventing the plasmid DNA of the ΦX from properly circularizing.

Work with a new assay has begun. In order to measure the metabolism of 7-ethoxyresorufin using microsomal subcellular fractions, the EROD (ethoxyresorufin optical density) assay was used. The EROD assay measures relative metabolism of 7-ethoxyresorufin to ethoxyresorufin which is mediated by cytochrome P450 and can be helpful in determining induction of mixed function oxidase activity by polycyclic hydrocarbons.

Methods development for rapid toxicity assessment:

Collection and storage of in-house killifish embryos in refined peat moss was completed. A total of 1651 embryos were collected from KF-7 and 3045 embryos were collected from KF- 12.

A poster presentation, Rapid Toxicity Assessment Using the Annual Killifish *Nothobranchius guentheri,* T.R. Shedd, U.S. Army Biomedical Research and Development Laboratory (USABRDL), Building 568, FT. Detrick, Frederick, MD, M.W. Widder, Geo-Centers Inc., Building 568, Ft. Detrick, MD, M.C. Sunkel, USABRDL, Budding 568, Ft. Detrick, Frederick, MD, E. Hull, Orbis Scientific Inc., P.O. Box 3890, Milton, FL, and W.H. van der Schalie, U.S. Environmental Protection Agency, (8501), 401 M. Street SW, Washington, D.C. was prepared for the ASTM Sixth Symposium on Environmental Toxicology and Risk Assessment in Orlando, Florida on April 14-18, 1996. Work is

continuing on producing display posters for the biomonitoring trailer which will be on exhibit for the Department of Energy in Cincinnati in April.

Bluegill ventilatory monitoring project:

Scheduled trips were taken to Old O-Field, Edgewood Arsenal, Aberdeen Proving Ground to assist with biomonitoring operations and program configuration of the Aquatic Biomonitoring Program at the Aquatic Biomonitoring Facility. Two Aquatic Biomonitoring file transfer systems (version 2.41) were constructed and tested. One system was installed at Old O-Field, Edgewood Arsenal, Aberdeen Proving Ground. The other system was installed in the new biomonitoring trailer being outfitted for the Department of Energy demonstration at the Fernald Plant in Cincinnati, Ohio. Data was transferred daily from Old O-Field to Ft. Detrick during the quarter. All statistical analysis and data archiving was performed for thirteen data collection periods spanning from Dec. 1995 through March 1996 at Old O-field.

Efforts were made to resolve difficulties encountered with the Hydrolab water quality analyzers at Old O-Field. It was determined that the continuous monitoring process has a deleterious effect on the dissolved oxygen probes. Efforts were pursued to recondition the probes to extend their life. A damaged pH probe was replaced in the Hydrolab H20 (version 1.03). A recirculation loop and redesigned water distribution system was installed at Old O-Field to alleviate temperature fluctuations that were occurring.

The installation and finalization of all system processes for the DOE demonstration in Cincinnati was completed. Ventilatory amplifiers were assembled and installed in the biomonitoring trailer. Temperature controllers and water heating systems were assembled and installed. A sump water removal system was designed and tested for the DOE demonstration. All other relevant equipment was installed and tested.

Work continues on performance of ventilatory tests in order to determine "time to response." Concentrations of chemicals chosen are calculated, the diluter system in the biomonitoring trailer is calibrated, chemical analysis is coordinated, the peristaltic pumps are calibrated, paper work is prepared and other miscellaneous tasks are completed before testing may begin. Testing is scheduled to begin early next quarter. The initiation of this project requires considerable time spent reading SOP's and historic ventilatory test data.

Analytical method development and analysis of chemicals used in carcinogenicity and mobile laboratory studies:

Preparation for upcoming analyses of Phenol in support of ventilatory testing has begun. 'Phenol analyses will be performed by IHPLC (High Performance Liquid Chromatography). The flow of the separation will be reduced from I mL/min to 0.7 mL/min in order to reduce backpressure on the column. The viscosity of the mobile phase (50% Methanol:water) is very high and causes pressures that exceed 250 bar. The precision and recovery data for the analysis of munitions in soil by BPLC has been completed. The

recoveries for the munitions were within the acceptable limits with the exception of FRAX. The recoveries and relative standard deviations were very high for this explosive. The reason for this is unknown. No interfering peaks were observed in soil blanks and a gradient elution was introduced to separate HMX from interferences found at the beginning of the chromatogram.

SPMD (Semipermeable Membrane Device) extracts were analyzed for munitions, PAH's (polynuclear aromatic hydrocarbons) and trace metals. Samples were analyzed by direct injection following concentration with the PrepStation. A fluorescence detector was used to determine that PAH's were present. Unfortunately specific compounds could not be identified by IHPLC due to the inability to resolve the numerous peaks found in the sample. Concentrations could not be determined but relative concentrations between samples and controls were established. Besides PAH's several trace metals were found but only copper was found in significant concentrations.

Several sets of water samples from APG were received and analyzed for trace metals. The suggestion that random water samples be taken to establish a baseline for water samples coming from APG has been initiated. Several sets of standards and blanks were analyzed to establish quality control criteria for trace metal analysis.

The analytical chemistry trailer has been prepared for an on site demonstration at the U.S. Atomic Energy Commission Fernald Plant. A method has been created to analyze Ag, As, Ba, Cd, Cr, Pb and Se using the Leeman PS-3000 ICP between the levels of 0.05 and I mg/L. The trailers will be transported and displayed at the Fernald site during the month of April.

Analytical chemistry support for Rocky Mountain Arsenal (RMA):

Development was done on the Finnigan Direct Sampling Ion Trap Mass Spectrometer (DSITMS) setup and operation in the Real Time Analytical Platform (RTAP). The RTAP is a mobile laboratory used for field analyses and quick analytical response. Having the DSITMS in the RTAP results in truer sample identification and faster analysis time in the field. Successful operation of the DSITMS using RTAP generators under rough transport conditions and successful sample collection using an 80 ft heated transfer line demonstrated that the DSITMS will work well in the RTAP under the environmental conditions associated with mobile analysis.

A trip to the Pueblo An-ny Depot is currently being planned using the DSITMS on the RTAP to sample wells with known types of contamination. Tests have shown that the DSITMS can detect down to the levels that are needed for this project. The work is expected to start sometime in April or May and is expected to last several months.

Methods for analyzing 1,4-dibromochloropropane (DBCP) in water and soil have been developed and certified. In addition, the certification process for organochlorine pesticides in

waters and soils using solid phase extraction and sonication has begun. Other analytical methods in the developmental stages include: chloride by Ion Selective Electrode, diisopropyl methyl phosphonate (DIMP) in soils, dimethyl methylphosphonate (DMMP) in waters and soils, metals in water and soil by Inductively Coupled Plasma Atomic Emission Spectroscopy, anions by Flow Injection Analysis, and volatile compounds in water by Gas Chromatography/Mass Spectrometry (GC/MS).

Production type analysis continued for volatiles analyses. Ten samples per week were analyzed on the DSITMS for volatile compound contamination in air samples, while approximately five water samples per week were analyzed by purge and trap GC/MS. Work was also done on the identification of an unknown substance from the inside of a pipe. The DSITMS was used to ensure that the sample was not giving off toxic fumes before it was brought into the laboratory and for identification of the unknown. In addition, approximately 40 samples are analyzed weekly by GEO-CENTERS personnel for low-level DIMP. Up to 15 samples weekly were analyzed for methanol in water with a 24-hour turnaround for the results.

Maintenance and optimization of USABRDL aquatic laboratory facilities:

Essential laboratory maintenance was performed on all culture (medaka, guppy, bluegill, fathead minnows, and killifish) and test fish (medaka) located in rooms 5 and 18. Daily activities include three daily feedings of all culture and test organisms. Associated record keeping was performed to maintain necessary test records to meet SOP and regulatory review requirements. Associated tank cleaning and siphoning was performed to maintain overall tank hygiene necessary to accommodate healthy test organisms. Maintenance and culturing of all live food (brine shrimp and microworms) systems was performed to facilitate ongoing laboratory efforts. Weekly activities also included the following water quality analyses; dissolved oxygen, pH, conductivity, water flow measurements, ammonia, and light measurements.

Transgenic medaka test organisms were maintained in conjunction with inter-laboratory investigations with Dr. Jim Burkhart, NIEHS. Further studies with the transgenic medaka are scheduled for April. Essential laboratory supplies were documented and ordered as needed for the month. Waste samples monitored each week in January from the methylene chloride filtration system continued to be free of methylene chloride contamination entering the waste drain.

FY95 aquaculture information was complied and provided to the associate director of the laboratory. A month-by-month analysis provided data on the number of medaka held at the aquaculture facility, medaka mortality by age group, the number of medaka used for immunotoxicology or carcinogenicity research, and yearly information on all other fish species maintained. In response to a new requirement that all culture animals be covered under test protocols, an aquaculture protocol was written and submitted to the USABRDL Animal Use Committee. This protocol directs the care of all species of fish cultured at USABRDL, including medaka, killifish, bluegills, and fathead minnows. Approval was received from the



committee on 2/26/96. Animal Use Protocol Numbers were placed on each tank of fish in the laboratory.

The laboratory was prepared for the AAALAC inspection of 2/28/96. All records were reviewed since the previous AAALAC site visit in 1993. Backlogged chemical analysis of well water and feeds were added to the notebook binder system. The laboratory successfully passed the AAALAC inspection. 4000 medaka eggs were cultured for renewing broodstock and for immunotoxicology research. The fry hatched were assigned as follows: 1200 to broodstock colony (2/20/96 hatch) and 720 to Bath 2 for immunotox research (2/21/96 hatch).

The histopathology budgetary projection for FY96 was submitted with options for partial histopathology on test fish. A draft of a short communication on parasites in *Fundulus heteroclitus* was sent to co-authors. This article will be submitted to Journal of Aquatic Animal Health. A review of laboratory Standing Operating Procedures (SOP's) for the aquaculture facility has been undertaken. This process will continue into the next quarter.

Fish shipments for this quarter were as follows:

Total No. of Medaka	Institution	Location	Shipments
420	NYU	Tuxedo, NY	two
16	EPL	Herndon, VA	one
1000	WVU	Morgantown, WV	two

Support of the Environmental Engineering Division at Rocky Mountain Arsenal (RMA):

GEO-CENTERS continues to observe remediation/demolition activities and provide technical support to the Contracting Officer's Representatives for these projects. These projects are carried out by the contractors performing the clean up of RMA.

Assistance was given in clarifying various regulatory issues, such as waste characterization, transportation, handling, and disposal, hazardous waste characterization and safe handling procedures, research of innovative methods for reclamation and disposition of waste materials. Technical information was provided pertaining to waste profiling and characterization for contents contained within underground storage tank (UST) and contaminated soils. By providing technical guidance for excavating a hydrocarbon-contaminated site, RMA saved approximately $150,000.00. The expense of this project was greatly reduced when excavation was done only on material required under regulatory requirements.

To meet State requirements for the Army, an "Initial Site Assessment and Abatement Report" was drafted to the State of Colorado regarding a hydrocarbon release from an underground storage tank. This report circumvented notice of violation (NOV) from the Environmental Protection Agency (EPA).

Assistance was given to the Safety/Health and Environmental Office (SH&E) at RMA in the development and implementation of health and safety policies for all government employees at the Arsenal, as well as development and implementation of an in-house OSHA eight-hour refresher training program for RMA Government Field Inspectors. Currently, efforts are being coordinated with RMA's SH&E Office to expand the in-house training program to all RMA government employees. Assistance and training continued for RMA employees with special waste management activities. RMA has recently requested a need to perform a South Plants chemical sweep to remove all remaining chemical items from the South Plants production area. RMA personnel will receive training regarding; safe chemical handling (neutralizing and stabilizing), classification, segregation, sampling, Hazcatting, profiling, packaging, and selecting a Toxic Storage Disposal Facility (TSDF).

The Risk Assessment/Management support at the Rocky Mountain Arsenal (RMA) has accomplished the following:

Involvement with producing the Endangerment Assessment, Feasibility Study, and the Proposed Plan documents has prompted the Army to ask for assistance in writing the risk section of the Record of Decision (ROD). The coordination of all human health or ecological risk meetings has been an ongoing responsibility. These meetings are necessary to work on settling possible disputes issues to avoid delaying the Final ROD. Part of the ROD includes a Responsiveness Summary which addresses public comments on the Proposed Plan. The public meeting was attended and help was given in writing the Proposed Plan. Assistance was also requested from the Army in addressing these comments.

Biological Assessment: To meet the requirements of Section 7 of the Endangered Species Act, the lead has been taken, at the Army's request, on producing the Biological Assessment. This document outlines how the Army intends to perform remedial activities while preventing disturbances to endangered or threatened species, and their habitats. Work has been done with the U.S. Fish and Wildlife Service in attaining updated listed species.

Biological Advisory Subcommittee: Representation for the Army was continued by serving on the Biological Advisory Subcommittee (BAS). Recent obstacles the BAS has been addressing are the State's Dioxin study in which they identify Dioxin is a contaminant present on RMA. Problematic is the fact that there are no Dioxin background studies in this area or on the species that the State chose to sample. Another project is the development of a database to incorporate any pertinent data which may assist the BAS in assessing remediation effects on biota.

Supplemental Field Study: Due to Army laboratory delays, the final report due date has been extended to the end of April.

APRIL 1 - JUNE 30, 1996

Investigation of methods available for identifying biomarkers of mammalian hepatocarcinogenesis for adaptation to fish models:

A training session for proliferating cell nuclear antigen, transforming growth factor b, and bromodeoxyuridine staining technique was attended at Colorado State University. Western blots were also performed on transforming growth factor b, and c-jun proteins. Efforts are also being made to include a molecular toxicity component to a battery of environmental assessment assays currently in use. The human hepatoma cell line, HepG2, will initially be used in the CAT-Tox/liver and CAT-Tox/DNA assays. HepG2 has been genetically altered in order to express damage-specific and chemical class-specific gene induction following chemical exposure. Of the fourteen target proteins these assays measure c-fos, p53, GADD45, GADD153, GST-Ya, HSP70, and cytochrome P450 are of particular interest due to their cellular functions of cell cycle regulation, DNA and protein repair, and tumor suppression.

Additional expansions and revisions were done on a cell proliferation paper. It was decided to include data from the TCE portion of the experiment along with the DEN data, which required additional slides to be evaluated on the Bioquant. These data were sent to CSU for statistical evaluation. Modified methods for doing BRDU staining in-house were also studied. An updated version of Refman was installed for cataloging references for the database for these studies. A student contractor was trained to operate this program in order to review and enter a backlog of papers into the cell proliferation database.

At CSU, slides were read from the EE1 test and statistics were run for all of the data available. After analysis, graphs, charts and tables were constructed to reflect significant dose response relationships in the data, as well as changes in the data over time. Two Bioquant demonstrations were given and training on the system began for a new user. Experiments were performed on archived fish slides using immunohistochemical staining in an effort to determine which stains and fixatives work successfully together. Efforts concentrated on staining techniques with propidium iodide. These results will be used in future experiments.

Contact has been made with Candace Matthew of USARIEM, who is interested in evaluating the Bioquant as a tool for a current research projects. The project would involve using the Bioquant to read fluorescent microspheres in the tissues of rodents who have undergone hyperthermia stress. A standard curve will be constructed for this project when appropriate materials arrive.

Studies of chemical carcinogenesis in medaka:

Quality assurance was performed on length and weight statistics from the six and nine month sacrifices of Test 401-002R, The West Branch Canal Creek Carcinogenicity Study with Medaka. The sex of each animal was included in the data before the package was returned for

further statistical analysis to the USABRDL extramural contractor.

The nine-month sacrifice fish (60 medaka) which had livers excised for Dr. Malins were sexed. The testes and ovaries were well shaped and easily distinguished from one another.

It was determined that the foam biopsy pads in the tissue culture cassettes of preserved fish were disintegrating. In order to prevent artifacts during microscopic examination, the pads were removed from all stored fish at USABRDL. In some cases, bits of foam adhered to the fish and could not be removed. Tests which used these pads are: 100-003 (DEN) and 100-004 (MeCl2). Alternative methods of keeping the organs in the fish during fixation are being investigated.

Rodent and medaka physiologically based pharmacokinetic modeling (PBPK) conducted at Armstrong Laboratory, Wright-Patterson AFB:

Future work on this project will be performed through another contract.

Teleost immunotoxicology methods development:

Progress has been made on delineating the 96 hr LC50 for bacterial exposure in medaka. A 48-hr culture of *Yersinia ruckeri* is being used as an opportunistic aquatic bacterial pathogen of freshwater fish for these assays. The results of three assays show that the LC50 for medaka is. 3 X 10^8 CFU/ ml with mortality normally occurring within 24-30 hrs. Growth time for bacterial culture has been an important factor in this experiment. The bacterial culture stock must be allowed to grow at least 48 hrs. Exceeding this time frame may alter results of test. Nine strains of *Aeromonas hydrophila* and *Aeromonas schubertii* from the National Aquarium in Baltimore, MD were received and characterized. Stocks of these bacteria were prepared for use in LC_{50} studies.

Data from the health screens is currently being formatted into a journal article which will be submitted to the Fundamentals of Applied Toxicology journal. The mycobacterium surveillance project, a collaborative effort with Dr. Jeff Teska of the National Fish Health Research Laboratory in Kearnyville, WV was completed. Data collected will be submitted for a poster presentation at the 1996 SETAC meeting held in Washington D.C., and also submitted for publication in the Journal of Aquatic Animal Health. The finalized version of the paper is now going through the review process.

The antibody forming cell (AFC/PFC) assay has been modified slightly, in order to increase resolution of plaques and facilitate enumeration of plaques by the technician. The change consists of using 3.5 ml of sheep red blood cells (SRBC's) diluted in 8 ml phosphate-buffered saline (PBS) rather than 2 ml SRBC's (C_f= 1.75X). Another logistical modification calls for the SRBC's preparation and medaka kidney collection to be performed late afternoon

the day prior to the assay. The analysis of the two modifications has been made. Slides scored into quadrants also facilitate standardized quantification. Using these slides, results can be expressed in terms of # plaques/unit area. It has been decided that these changes will be adopted for use in upcoming studies. This assay will be attempted in bluegill studies by the end of June.

Preliminary investigations into the suitability of the bluegill sunfish (*Lepomis macrochirus*) as a lab/field/feral teleost model has been completed. Four sampling points numbering 5, 5, 5, and 8 fish have been completed. The conclusions from these preliminary tests are: 1) high cell yields from pronephric tissue allow multiple assays from one individual fish; 2) greater variability is likely to be observed since readings will be from individual animals, as opposed to cell suspensions from multiple fish needed with medaka; 3) these advantages may make the bluegill a suitable feral candidate species for immunotoxicological surveillance. Routinely manipulated environmental conditions that test bluegill currently undergo may present a stress factor in the fish. Values may change if fish are kept at constant environmental conditions with minimal handling stress over extended periods of time. Modifications to original medaka assays may be necessary to optimize for immune function response in the bluegill. A poster on this work entitled "Treated Effluent Biomonitoring: Development of the Bluegill (Lepomis macrochirus) as a Model for Assessment of Immunotoxicological Hazard" was presented at the Alternatives in the Assesssment of Toxicity: Issues, Progress and Opportunities - 4th Biennial International Symposium 12-14 June 1996 held at Aberdeen Proving Grounds, MD.

Publications:

Zelikoff, Judith T., Weihua Wang, N. Islam, Lorraine E. Twerdok, Marianne Curry, Joseph Beaman, and Eliezer Flescher. 1996. Assays of Reactive Oxygen Intermediates and Antioxidant Enzymes in Medaka (*Oryzias latipes*): Potential Biomarkers for Predicting the Effects of Environmental Pollution. In "Methods in Aquatic Toxicology", edited by Gary Ostrander, CRC Press. In Press (Publish Date: August 1996)

Transgenic medaka research at USABRDL [in collaboration with Dr. James Burkhart at the National Institute of Environmental Health Sciences (NIEHS)]:

In April, an experiment was performed in an attempt to generate transgenic medaka containing OX 174 DNA using the electroporation method rather than the previously used method of microinjection. The light photoperiod on the day of the experiment was delayed to 10 am in room 5. Tests were run with different parameters to determine the best conditions for electroporating the sesitive embryos. In collaboration with NIEHS scientist, Dr. Jim Burkhart, 500 medaka eggs with soft chorions were electroporated with DNA at 500, 250, and 60 volts. The eggs were then rinsed and cultured according to protocol. Six fry hatched and survived. Confounding factors were the frailty of the eggs, the adhesion of the eggs to each other, the 2mm gap in the cuvettes, and the differing voltages.

These problems were addressed in May=s electroporation experiments. A 4mm gap cuvette, different resistivity and pulse settings, and 60 volts were used. Changing the resistivity did not significantly alter survival, but increasing pulse decreased survival. In each of the four experiments, a minimum of 44 fry hatched. Twelve days after hatch, 25 fry from each treatment were sacrificed and sent to Dr. Burkhart for DNA analysis. June electroporation experiments used settings similar to the May experiments, with 3 potentially transgenic populations (a total of 400 fry) resulting.

Transgenic animal research conducted at NIEHS, Research Triangle Park, NC:

Continual improvements in fish husbandry at the National Institute of Environmental Health Sciences increased the number of the test specimen Fundulus heteroclitus (killifish). Fin clips of the killifish were continually made as fry grew to an acceptable size for the procedure. Probing the DNA resulted in very low numbers of F1 fish containing the OX 174 insert while the F2 have resulted in a 1:2:1 ratio. Micosome preparations of liver tissue from both fish and mice were made to conduct future EROD induction assays.

Methods development for rapid toxicity assessment:

The killifish culture was maintained on a daily basis. Eggs were collected weekly from two of the killifish tanks and placed in peat moss for long term storage. After a minimum incubation period of 30 days, some eggs were hatched in an effort to increase the current breeding colony.

Two bags of 20 eggs each were sent to Portland State University at their request. The university personnel will attempt to verify the recovery rate of Killifish embryos after being stored in peat moss for an extended time period. No results of the recovery rate experiments have been reported.

The peat moss used to store the embryos is kept slightly moist at all times. Because of this moisture, the peat has a tendency to produce fungal growth. Peat moss used at USABRDL is autoclaved before use, as opposed to Dr. Hull's laboratory in Florida, where it is boiled. Dr. Hull has not experienced the fungal problems encountered at USABRDL. An experiment was initiated to determine if autoclaved peat moss develops fungus faster than boiled peat moss. This study is in currently still in progress.

Bluegill ventilatory monitoring project:

A manuscript draft copy of materials and methods was written for: "Rapid Toxicity Assessment Using the Annual Killifish *Nothobranchius guentheri,*" T.R. Shedd, U.S. Army Biomedical Research and Development Laboratory (USABRDL), Building 568, FT. Detrick, Frederick, MD, M.W. Widder, *GEO-CENTERS INC.*, Building 568, Ft. Detrick, MD, M.C. Sunkel, USABRDL, Building 568, Ft. Detrick, Frederick, MD, E. Hull, Orbis Scientific Inc., P.O. Box 3890, Milton, FL, and W.H. van der Schalie, U.S. Environmental Protection

Agency, (8501), 401 M. Street SW, Washington, D.C.

A demonstration of the biomonitoring trailer system was given for the Department of Energy at the Fernald Plant in Cincinnati, Ohio. A number of toxicity tests using multitrophic levels of organization and various modes of toxicity were demonstrated in the aquatic biomonitoring trailer. Test systems on display were as follows: the medaka carcinogenicity assay, fish immune organ toxicity, FETAX, Microtox, lettuce root elongation, the rapid fish acute toxicity test, and the automated ventilatory biomonitoring system. The ventilatory file transfer system was tested and operational for the demonstration. A report of activity was generated for project managers of the Fernald Environmental Management Project.

All statistical analysis and data archiving was performed on data collected from Old O-field over ten collection periods from March 25 through June 21. Reports on Continuous Biomonitoring from five operational periods from April 5 through June 14 at Old O-field were generated for submission to APG project managers. The annual report of Continuous Biomonitoring at Old O-field was reviewed and edited. The report covered operational periods from June 23, 1995 to March 31, 1996. A table of all chemical analyses of Old O-field effluent water samples was submitted for inclusion in the annual report. Training was provided to part-time contract students to assist in data evaluation of ventilatory data generated at Old O-field.

A ventilatory validation of response to new ABP software was performed in the 24' trailer located at building 568. The validation covered a two-week period. Set-up and preparation of the ventilatory time-to-response studies was also performed. Review of scientific and animal use protocols was completed. A number of on-site ventilatory trailer demonstrations were also performed. Technical training and guidance was provided to individuals currently working with ventilatory and killifish test systems. The animal use and scientific protocols for both the in-house and Old O-field ventilatory systems were revised using the updated format. Three protocols underwent and passed a review process.

A three-week ventilatory test was initiated in an effort to validate the new computer software being used in the mobile biomonitoring facilities. This test has been completed during this quarter. New software installed at Old O-field test site was suspected to be more sensitive to the responses of the fish than the old version of the software. Results of the experiment showed that both programs yield similar results. Preparation for ventilatory tests scheduled to begin next month continued. A test was initiated during the first week of June. This test was terminated due to faulty wiring. The mobile biomonitoring laboratory and equipment is now ready to proceed with testing.

Dr. Burton submitted an annual report for the work performed at Old O-field. At the request of APG, USABRDL has been asked to explain "out of control" responses for which there are no obvious explanations. Much time has been spent reviewing fish responses, water quality data, and chemistry analyses results for each of the two week monitoring periods from the last year. Data analyses is now being performed at the termination of each two-week

monitoring period in an effort to stay ahead of problems noted over the last year.

Analytical method development and analysis of chemicals used in carcinogenicity and mobile laboratory studies:

As part of the mobile laboratory on-site demonstration at the U.S. Atomic Energy Commission Fernald Plant, fourteen samples of water were digested and analyzed for Ag, As, Ba, Cd, Cr, Pb and Se by EPA methodologies. A report of the data was provided to FERMCO and ECO. All of the data met the required quality control criteria. Several problems associated with running the backup generator were noted. The generator did not supply clean power to the ICP, causing the plasma to flicker and creating instability in the intensity of light being emitted from the plasma. The power supply to the ICP was corrected by installing a heat sink in the ICP. The backup generator overall did not supply a reliable source of power. Inconsistent power, power failure, and power fluctuations were observed. For future testing, it was recommended that hard power be supplied to the trailers when on site for an extended period of time.

Pentachlorophenol (PCP) was analyzed in support of a FETAX assay. Lower detection limits were required, so the injection volume was increased from 50 to 100 uL, while all other instrumental conditions remained the same. Approximately 200 samples were analyzed in support of the assay. During the exposure it was found that the concentration of PCP was decreasing after exposure to embryos for 24 hours. A 24-hour stability study was performed in FETAX and PCP was showed no decrease in concentration due to the FETAX matrix.

Preparation for upcoming analyses of Phenol in support of ventilatory testing has begun. Phenol analyses will be performed by HPLC (High Performance Liquid Chromatography). The flow rate of the separation will be reduced from 1 mL/min to 0.6 mL/min in order to reduce backpressure on the column. The viscosity of the mobile phase (50% Methanol:water) is very high and causes pressures that exceed 250 bar. The concentration of Phenol was determined to be stable for a 24-hour period in well water. Stocks for the ventilatory study will be freshly prepared each day of the exposure.

Approximately twenty samples were analyzed in support of ongoing ventilatory studies at Abredeen Proving Grounds for trace metals by ICP-MS. The data for this analysis were transferred to a database maintained on all samples received from APG. The method was modified to include several more masses for Barium.

Analytical chemistry support for Rocky Mountain Arsenal (RMA):

Assistance was given in the coordination of the Lockheed Martin Energy Systems audit of the Environmental Analytical Laboratory on May 22. Certified methods for the determination of 1,2-dibromo-3-chloropropane have been approved for use. A certified method for the determination of DIMP in soil has been submitted for final review and

approval. A method for the determination of metals by ICP is also in the certification process. Additionally, methods development is underway for organosulfurs in soil, organochlorine pesticides in water and soil, and dimethylmethylphosphonate (DMMP) in water and soil.

Regular production work continues, including DIMP under method UK16, GC/MS volatiles under SW-846 method 8260A, ITMS volatiles, GC/MS semi-volatiles under SW-846 method 8270, and metals work under SW-846 method 6010B. A visit was made to the Pueblo Army Depot to analyze well samples for dichloroethene and trichloroethene. The samples were analyzed onsite using a Finnigan Magnum ITMS equipped on the real time analytical platform (RTAP). Duplicate samples were also collected for analysis at the EAL using a Teledyne 3DQ ITMS as a comparison.

Maintenance and optimization of USABRDL aquatic laboratory facilities:

Essential laboratory maintenance was performed on all culture (medaka, bluegill, fathead minnows, and killifish) and test fish (medaka) located in rooms 5 and 18. Daily activities include three daily feedings of all culture and test organisms. Associated record keeping was performed to maintain necessary test records to meet SOP and regulatory review requirements. Associated tank cleaning and siphoning was performed to maintain overall tank hygiene necessary to accommodate healthy test organisms. Maintenance and culturing of all live food (brine shrimp and microworms) systems was performed to facilitate ongoing laboratory efforts. Weekly activities also include the following water quality analysis: dissolved oxygen, ph, conductivity, water flow measurements, ammonia, and light measurements. Transgenic medaka test organisms were maintained in conjunction with ongoing inter-laboratory functions with Dr. Jim Burkhart. Essential laboratory supplies were documented and ordered as needed for the quarter.

The aquatic laboratories at USABRDL were utilized this quarter in transgenic medaka culturing and testing. Twelve tanks were dedicated for the fry after the experiments. Fourteen days after hatching, half of each aquaria's population were sacrificed. The remaining fish will be grown out for three months. The diluter tests scheduled for April are still on hold until further notice. Fish selected for the methylene chloride test continue to be maintained. Preparations were made for the bluegill harvest but the field trip for collection met with little success. The number of available fish at the netting site were minimal. Further attempts will be made later this summer and alternate sources for bluegill are being investigated. The medaka broodstock colony was renewed in April and June. Twelve tanks of immunotoxicolgy fry wre also cultured successfully in June.

Yearly water and food samples were collected to be sent to Gascoyne labs for evaluation. Supplies were ordered to restock lab through December, 1996. Preparations began for upcoming sacrifice on the second generation Shedd study fish. The possibility of pulling additional eggs for this study is being discussed. This would make three generations of fish data available if eggs are pulled.

It has been determined that the cost of ordering certified flake food is no greater than the cost of buying flake food requiring analysis by an outside laboratory. The lab will be switching from Tetramin flake food to Zeigler Brothers Aquatox Diet during this quarter. To further regulate freshness and amount fed, certain other measures are being taken. After receipt of the food, sieves of 2mm pore size will be used to grade the ground flake and the prepared food will be packaged in weekly lots. The bags will be sealed, and kept in the freezer until use (expiration date of one year after receipt). Zeigler Brothers Diet has the advantage of being fresher since it is not made until the order is placed.

A seminar on Zoonotic Diseases of Fish and Amphibians given by Major David M. Rubble was attended by all personnel involved in animal use. The possibility of receiving infections from bacterial, viral, and fungal agents in the laboratory was discussed. The risks of infection appear to be very low to lab workers who have no skin breaks, do not eat raw seafood, have a current tetnus booster, and are not immunocompromised.

Free swimming flatworms (Turbellaria) were found in one of the 10 gallon tanks. After observations, it was determined that the worms were of the order Rhabdocoela, either *Microstomum spp.* or *Stentostomum spp.* To further differentiate the worms was not possible at 50X magnification, because the details of the eye cups could not be determined. The worms were not consuming living eggs, but scavenging dead material. The worms were white in color, transparent, with length estimated to be less than 1 mm long. They were composed of chains of zoids, and reproduced by fission. No external cilia were visible.

Fish Shipping for the quarter:

Total No. of Medaka	Institution	Location	Shipments
600	NYU	Tuxedo, NY	four
650	WVU	Morgantown, WV	two
120	PNRF*	Seattle, WA	one
35	USABRDL@DOE	Cincincatti, OH	one
50	EPL	Herndon, VA	one
100	NIEHS	Research Triangle Park, NC	one
10	USABRDL@CSU	Ft. Collins, CO	one
300	UMe**	Orono, ME	one
35	USABRDL@DOE	Cincincatti, OH	one

*PNRF = Pacific Northwest Research Foundation

**University of Maine

Support of the Environmental Engineering Division at Rocky Mountain Arsenal (RMA):

Remediation/demolition activities were observed and technical support provided to the COR for above and below ground storage tanks. Assistance was also given in clarifying various regulatory issues, such as, waste characterization, transportation, handling, and disposal; hazardous waste characterization and safe handling procedures; and researching innovated methods for reclamation and disposition of waste materials.

Training was provided for personnel involved in a chemical sweep of the entire Arsenal to remove all remaining chemical item substances from the various structures. RMA Engineering Technicians are receiving training for safe chemical handling (including neutralizing and stabilizing), chemical classification, chemical segregation, SW-846 sampling methods, chemical hazcatting, chemical profiling, chemical packaging, and selecting a Toxic Storage Disposal Facility (TSDF) for chemical disposition.

RMA Engineering Technicians were provided with technical support for day to day field operations regarding chemical classification, chemical segregation, representative sampling of chemicals, packaging chemicals per DOT specifications, profiling per EPA specifications, and arranging for disposition per EPA specifications. This training program will continue into the next quarter. Future training requirements are in the planning stages for Rocky Mountain Arsenal employees.

The Risk Assessment/Management support at the Rocky Mountain Arsenal (RMA) has accomplished the following:

Record of Decision: All human health or ecological risk meetings continued to be coordinated while providing technical support to the Army to ensure language appearing in the ROD was consistent with the Risk Assessment and the Feasibility Study. Ongoing support was also provided to the Army in writing the Responsiveness Summary of the ROD. The ROD was signed on June 11, 1996. It was ensured that all the Engineering Division aspects of the document were completed before the Ceremony.

Biological Assessment: This document was finalized and received concurrence from the U.S. Fish and Wildlife Service before the signing of the ROD.

Biological Advisory Subcommittee: Representation of the Army was provided by serving on the Biological Advisory Subcommittee (BAS). The State's Dioxin study and answering public concerns continue to be issues for the committee. The development of a database is still in the preliminary stages.

Supplemental Field Study: The draft document was distributed to the BA and comments were incorporated. The Final document is due in July.

JULY 1 - SEPTEMBER 30, 1996

Investigation of methods available for identifying biomarkers of mammalian hepatocarcinogenesis for adaptation to fish models:

A literature summary on fish liver biochemistry was written to include additional background information for the cell proliferation study. Updating and cataloging of related references on the Ref-Man database continued. A new Windows version of the Bioquant Image Analysis System was installed and a rough draft of the SOP's for this system was written. SOP's were also updated and written for the BRDU portions of experiments. The old Bioquant system was labelled, disassembled, and boxed for shipment to CSU.

Research Conducted at Colorado State University:

Work on tests EE1 and EE2 carcinogenicity projects continued. This included reading slides, analyzing statistics and preparing slides and pictures for presentation. A two-week lecture series was attended on physiologically-based pharmacokinetic/pharmacodynamic modelling and risk assessment at CSU. Investigations were made toward developing a faster, more efficient way to count fluorescent microspheres for the USARIEM project on hypothermia. This project should be completed in the next quarter.

Two laboratory rooms are being prepared for the new Bioquant and tissue culture facility. Equipment has been ordered to outfit the rooms. Meetings have been held with Dr. Greg Cosma about possible collaborative studies with his molecular biology group with the goal of illustrating mechanistic relationships as they relate to whole animal observations in the field.

Studies of chemical carcinogenesis in medaka:

The final sacrifice of Test 100-004, Medaka Carcinogenicity Test With Methylene Chloride was performed. Tissue was excised from the gill and liver, then frozen in liquid nitrogen for Dr. van Beneden of the University of Maine. The remainder of the fish carcass was preserved in Bouins for 48 hours, followed by two 24 hour 70% ethanol rinses, then placed in formalin. The preserved fish will be stored on-site until funding is available for histopathology.

Plans for the drinking water disinfection by-products are being implemented. Of the three chemicals to be studied (chloroform, sodium chlorate, and dibromoacetic acid), chloroform was the only chemical to have an approved animal use protocol. The animal use protocols for the remaining two chemicals, as well as the scientific protocols for all three chemicals, were written and are undergoing peer review. The chloroform animal use protocol was amended to reflect the change from one sub-chronic study to two 96 hour tests. Supplies for the project were ordered. Two stock carts were designed and assembled to safely store the large glass stock bottles that will be used for this test. The angle iron inside all three diluters is

being replaced.

Two flow-through range find studies were performed using chloroform and sodium chlorate. Nominal concentrations for chloroform were 1000, 100, 10, 1, and 0.1 mg/L. Measured analysis of chloroform showed that the stock concentration of 8000 mg/L was actually 50% less than anticipated, therefore, the highest test concentration was approximately 400 mg/L. Mortality was observed only in the high concentration, with the dissolved oxygen measurement of -60% saturation in those test tanks. The 96 hour LC50 for this chemical is planned for October. Nominal concentrations for sodium chlorate were 1000, 100, 10, 1, and 0.1 mg/L. No mortality was observed at any test concentrations. The range find will be repeated at a later date.

A quality assurance audit was performed of length and weight data from Canal Creek test fish for a University of Maryland report. Additionally, the fish were sexed, and the lengths and weights were separated based on sex. The possibility of using the Xenometrix assay for a supportive field test was investigated and price quotes were requested from the company.

Teleost immunotoxicology methods development:

A trip to NYU Medical Center Institute of Environmental Medicine was taken to learn techniques for conducting Mixed Lymphocyte Reaction (MLR) and Lymphocyte Proliferation Assay (LPA) assays. An antibody forming cell assay was conducted in bluegill, but was unsuccessful. Optimization of antigenization time may need to be adjusted from the 11 days post-inoculation currently used in medaka assays. Another possible modification necessary may be the adjustment of the serum concentration or the serum source used in the buffer. The use of a dual antigen, such as, SRBC's and *Yersinia ruckeri* bacteria is also being considered. Assays quantifying the cell-mediated immune response continue to be investigated. Optimization of experimental conditions for the MLR and LPA assays was continued. Parameters being optimized included incubation temperature, cell concentration, and mitogen concentration.

A 96 hr static malathion range-find LC50 was run using medaka. The first trial used toxicant concentrations up to 0.4 ppm. No mortality was observed in any of the concentrations. Concentrations were increased significantly (1 - 32 ppm) for the second trial. 100 % mortality was observed in 32, 16, and 8 ppm. 4 ppm resulted in 30 % mortality; 2 ppm - 0%; 1ppm - 10 % with the control showing no mortality. The LC50 was calculated to be approximately 4.8 ppm. A degradation curve was run for malathion under static conditions in well water. It was estimated that a 50% loss occurs over 96 hrs.

A 7 day subchronic flowthrough exposure of medaka to the organophosphate pesticide malathion was completed in the biomonitoring trailer adjacent to building 568 at Ft. Detrick. The goal of the project was to observe possible immunotoxic effects on Japanese medaka caused by exposure to sublethal concentrations of malathion. The concentrations of malathion



to be used, 0.8 and 0.2 ppm (nominal), were based on the range-find static 96 hr LC50. Since exposures occurred under flowthrough conditions (negligible degradation) for 168 hrs, the above doses were deemed to be appropriate. 720 fish (3 concentrations; 4 tanks/concentration; 60 fish/tank) were randomized and allowed to acclimate to trailer conditions for 7 days prior to the toxicant exposure. The mean concentration of stock toxicant was 121.5 " 20.59 ppm.

General (length, weight, hematocrit, leukocrit) , specific (reactive oxygen intermediates), cell-mediated (lymphocyte proliferation), and humoral (Antibody Forming Cell Assay) aspects of immunotoxicity were examined. 19.2 % mortality was oberved in the high dose. Interestingly, 19.5 % of dead fish and 40 % of the experimental fish had a hemorrhagic "spot" or aneurysm along the spine which was lateral in aspect (near the midline) and posterior to the gut region. No mortality was observed in the low dose or controls. Medaka exposed to the high concentration of malathion displayed aberrant behavior (e.g. abnormal swimming, gills and pectoral fins flared out away from body, and lack of avoidance behavior during feeding or cleaning). Trends were seen toward cell viability and cell yield/fish (cell number) decreasing as the toxicant concentration increased. Assays quantifying reactive oxygen intermediate species (ROI's) showed a decline in production as the toxicant concentration increased, though the decrease was not statistically significant. Since the production of ROI's causes cellular damage and destruction, this may be attributed to a downregulation over time to prevent cellular damage that could be injurious or lethal to the fish. To observe optimal differences (increases or decreases), this parameter may have to be evaluated earlier in the exposure (*i.e.*, day 3 - 5). The lymphocyte proliferation assay showed the same general trends of decline as malathion concentration increased, however, T-cell proliferation by Con A was higher in the low dose than in the control. The antibody forming cell assay showed a statistically significant ($p < .01$ by One-Way ANOVA), dose-dependent decrease in AFC numbers as the toxicant concentration increases. Lower concentrations of malathion in future tests would eliminate excess mortality, permitting a refined assessment of immunotoxic effect.

A manuscript entitled "Treated Effluent biomonitoring: Development of the Bluegill (*Lepomis macrochirus*) as a model for assessment of immunotoxicological hazard" was prepared for submission as a government technical report and also as a textbook chapter. This request came as a result of a poster presentation made by the immunotoxicology group at an alternative toxicity assessment methods meeting at Aberdeen Proving Ground. The suitability of submission of this research to such a limited audience was discussed and a decision was made to submit the research at a later date in a journal with wider distribution, thereby disseminating the information more effectively.

Bluegill from Olney and Middletown, Maryland were utilized in studies characterizing the immune function under normal aquaculture conditions. Endpoints included hematological and non-specific immune function parameters. This data will be presented at the SETAC meeting in November, 1996. Animal use protocols were completed and submitted to the IACUC chairman for final approval. A scientific protocol was also written for toxicant exposures to teleost fish conducted in the biomonitoring trailer.

Transgenic medaka research at USABRDL [in collaboration with Dr. James Burkhart at the National Institute of Environmental Health Sciences (NIEHS)]:

Dr. Burkhart of NIEHS visited the lab to continue experiments with transgenic medaka. Electroporated medaka eggs were cultured and hatched to yield 14 new transgenic fry for Dr. Burkhart of NIEHS. Preliminary screening of the previous electroporation experiments showed that the procedure was working. However, due to DNA contamination, the results were not what had been anticipated. Transgenic fry from April and May experiments were euthanized. Screening is underway for the June electroporation fry.

Transgenic animal research conducted at NIEHS, Research Triangle Park, NC:

In this portion of the task, GEO-CENTERS is providing support for a project to develop *in vivo* mutagenicity testing in fish and mammals (medaka and mice). Transgenic animals containing exogenous bacteriophage DNA are the model systems in development.

In August a new experiment was begun using microsomes made from liver tissues of mice and fish treated with dimethylbenzanthracene (DMBA) . The study is an *in vitro* assay to determine the effects of metabolites on genome stability. Fish husbandry continues. Technical setbacks resulted in several deaths of the transgenic fundulus. Air lines were added to the fish room as replacements for the air pumps that normally are used for oxygenating water. Dot blots made from DNA rescued from tissue of medaka fry revealed no transgenic medaka after electroporation with FX 174 DNA as embryos.

Currently, work is concentrating on the development of a new phage as well as a new cell line containing an insert of the newly developed phage. In July, mismatched breeding pairs of mice were discovered. All breeding pairs were screened for the number of copies of the am54 gene by molecular analysis and the problem was corrected. In addition, contamination problems still exist and have proven difficult to remedy.

Bluegill ventilatory monitoring project:

A meeting was attended to assist in the presentation of the Ventilatory Biomonitoring System to the Army Corps of Engineers. This meeting was held to initiate interest in developing a workshop for technology transfer of the Ventilatory Biomonitoring System. A demonstration/training meeting was attended to brief new plant operators (ICF) at Old O-field on the functioning the Ventilatory Biomonitoring System. In preparation for this meeting, the Operation and Maintenance manual for the Ventilatory Biomonitoring Facility was revised to include current working operations.

Data collected at the Ventilatory Biomonitoring Facility during the quarter were analyzed and archived in the working database and on permanent disk. Refinements were made to the Aquatic Biomonitoring System to evaluate responses or to remove responses

caused by changes in water quality (*i.e.*, temperature, dissolved oxygen, pH, conductivity) that are not directly related to acutely toxic events. A collaboration was formed with Mr. Jeff Leach to assist in writing a program that would aid in analyzing ventilatory response information. A meeting was also held to install new ABP programming in the developmental trailer located in front of building 568. Resume mode and abbreviated menu programming was installed. Upon Mr. Leach's request the program was tested for operational compatibility. Several unusual operational conditions were tested. During testing it was noted that the resume function was unable to recover from power outages and Hydrolab initiation malfunctions. The problems were relayed to Mr. Leach and corrected. Instruction was also received on the proper use of the new program that creates raw data files of fish ventilatory parameters. Assistance was provided during Mr. Leach's re-installation of the new Aquatic Biomonitoring software version 1.5 after corrections were made.

A review of chemical analyses was performed for all samples pulled at Old O-field. Also a review was performed of the annual report "Continuous Acute Toxicity Biomonitoring of Aberdeen Proving Ground-Edgewood Area Old O-field Groundwater Treatment Facility Effluent" by Darlene Tiemann and Dennis Burton, University of Maryland. Content was checked for accuracy and scientific relevancy. A draft of the quarterly report (1 April through 28 June 1996) of the ventilatory data from Old O-field was completed and submitted to Mr. Tommy Shedd for comments and review. In addition to drafting the quarterly report, bi-weekly reports of data from Old O-field were generated for the periods 31 May-14June, 14June-28June, 28June-12July, 12July-26July, 26July-9August.

Two ventilatory time-to-response tests were performed during the month. Test compounds used were Phenol and Malathion. An estimate and order form was drafted and submitted to purchase the new design of the ventilatory amplifier system developed in conjunction with Dataforth Corporation. A cost estimate and order form was also submitted for ventilatory amplifier system display for Old O-field.

Setup and initiation of a ventilatory time-to-response study was begun. The test was terminated due to a ground fault problem in the developmental trailer. Pre-test procedures were also performed in preparation for an un-ionized ammonia time-to-response study. Calibration curves, set-up and verification of concentrations and use of the Specific Ion Analyzer were also completed. Historic data were reviewed to determine concentrations. A new plumbing design was installed in the developmental trailer to monitor flow through the Hydrolab unit and to verify Hydrolab readings with manual readings.

In conjunction with on-going research at Aberdeen Proving Ground, a meeting with researchers from the Johns Hopkins Applied Physics Lab was attended. New probe technologies to be deployed at Canal Creek, Beach Point, and Old O-field were discussed. Researchers were taken on-site to discuss design and implementation. Assistance was given in the drafting of a preliminary outline for a ventilatory workshop entitled, "Continuous Freshwater Biomonitoring: Perspectives and Application." Topic areas and guest speakers were discussed. The workshop is being planned in conjunction with the Army Corps of

Engineers Technology Transfer.

Collaborations with researchers from the Midwest Science Center were pursued at APG during the quarter. In preparation for these research efforts, the biomonitoring trailer at Canal Creek was re-outfitted to perform ventilatory and proportional diluter tests. A ventilatory amplifier system was installed. All plumbing and wiring associated with full ventilatory operation was installed and tested. A new ISCO water sampling chamber was developed and installed at Canal Creek. Similar systems are scheduled to be installed with all existing ISCO systems. A ventilatory test using dilutions of Canal Creek ground water was initiated for the purpose of behavioral response data in collaboration with Ed Little of the USGS (formerly NBS) Midwest Science Center. Bluegills were also provided for a parallel behavioral video study at the Canal Creek trailer. Test initiation and randomization procedures were performed. Brain tissues will be collected from both test systems at their termination. Jim Petty of the USGS was contacted to set up and implement procedures for installation of semi-permeable membrane devices (SPMD's) at Canal Creek. The devices were designed to pick up and concentrate various organic constituents that are resident in the water source tested.

Safety straps for the light fixtures in rooms 18 and 5 were constructed. Time delay relays were wired and installed on the diluters in room 10 for the medaka chloroform tests. Killifish embryo extractions were performed. Killifish embryos were sent to Dick Pratt at Portland State to perform extraction and embryo hatching procedures. The 1996 APG Installation Restoration Conference was attended at the Willow Valley Conference Center, Lancaster, Pennsylvania. All clean-up efforts were reviewed and future scientific applications at APG were discussed.

Analytical method development and analysis of chemicals used in carcinogenicity and mobile laboratory studies:

An analytical method for the analysis of honey bees and their pollen for trace metals was developed. The variables examined during the development of this method were, drying temperature, sample weight, digestion pressures and time of digestion. Analysis was performed on approximately 30 samples. Spike recoveries suggest a loss of Selenium during microwave digestion.

An analytical method for the analysis of Thalidomide was developed and an investigation into the solubility and stability of the compound in FETAX was performed. 200 mg of Thalidomide was placed in 100 mL DI water in a jacketed flask maintained at 20° C. After 1 hour of stiring the concentration of Thalidomide reached 30 mg/L. After 24 hours of constant mixing approximately 35 mg/L of Thalidomide was found in solution. This experiment was repeated using 2.5 mg of Thalidomide in 200 mL of FETAX. After 1 hour the concentration reached 8.5 mg/L and after 24 hours the concentration was 2.1 mg/L. The initial concentration was less than expected. DMSO was used as a carrier solvent to insure that all of the compound went into solution and a 24 hour stability study was performed. A stock of 1160 mg/L was prepared in DMSO and diluted 1:100 in FETAX. The initial concentration



was determined to be 11.46 mg/L. Approximately half of the Thalidomide degraded after 6 hours.

A more elaborate determination of the solubility of Thalidomide in FETAX was requested in order to determine if Dimethylformamide (DMF) was a better carrier solvent than Dimethylsulfoxide (DMSO). The solubility of Thalidomide in FETAX, 1% DMF and 1% DMSO was determined. A saturated solution of Thalidomide was prepared by placing 40 ug of Thalidomide into 200 mL of FETAX maintained at 24° C. This provided a solution with a concentration of 200 mg/L. Samples were taken and filtered through a 0.45 um membrane and analyzed by HPLC. An equilibrium was established at approximately 43 mg/L. As some Thalidomide degraded to the soluble product, more Thalidomide went into solution. The concentration of the degradation product is estimated by using the response factor of Thalidomide.

A stock of 20,240 mg/L Thalidomide was prepared in DMSO. This stock was diluted to 202.4 mg/L using FETAX. A sample was drawn immediately, filtered through a 0.45 um membrane and analyzed. Precipitation was observed several minutes after mixing. The concentration of the unknown was estimated using the response factor of Thalidomide. The plot of the solubility shows that when the stock is added Thalidomide goes into solution but soon precipitates and forms an equilibrium with the soluble degradation product.

A stock of 20,300 mg/L Thalidomide was also prepared in DMF. This stock was diluted to 203.0 mg/L using FETAX. A sample was drawn immediately, filtered through a 0.45 um membrane and analyzed. Precipitation was observed a few minutes after mixing. The concentration of the unknown was estimated using the response factor of Thalidomide. The difference in solubility between 1% DMSO and 1% DMF did not seem to be significant.

Forty samples of Phenol were analyzed in support of ventilatory testing. Eleven samples were analyzed in support of ongoing ventilatory studies at Abredeen Proving Grounds for trace metals by ICP-MS. The data for this analysis was transferred to a data base being maintained on all samples received from APG. Approximately 350 samples of Pentachlorophenol were analyzed in support of FETAX assays. Fifteen samples of water were analyzed for TNT in support of an algae assay performed at APG.

Analytical chemistry support for Rocky Mountain Arsenal (RMA):

A method for the determination of metals by ICP is currently being reviewed. The semiannual meeting in June for the Technology Reinvestment Program was attended. A presentation was given on the work that has been done at RMA and Pueblo Depot Activity using the ITMS. Also attended was the World Air conference in August. At this meeting a demonstrattion was given on the capabilities of the Finnegan ITMS installed on the Real Time Analytical Platform (RTAP), also known as a mobile laboratory. The presentation and instrumentation demonstration were well received.

Preliminary work in the agent lab continues. Work with the organosulfurs in soil has moved from method development to the certification process. Method development continues for DMMP. Newly certified methods for organochlorine pesticides in water and volatiles in water by GC/MS have been put into active use. Routine work continues utilizing methods in place during the past year and a half with the exception of semi-volatiles, which are now being analyzed by the recently certified method UM48.

Maintenance and optimization of USABRDL aquatic laboratory facilities:

Essential laboratory maintenance was performed on all culture (medaka, bluegill, fathead minnows, and killifish) and test fish located in rooms 5 and 18. Daily activities include three daily feedings of all culture and test organisms. Associated record keeping was performed to maintain necessary test records to meet SOP and regulatory review requirements. Associated tank cleaning and siphoning was performed to maintain overall tank hygiene necessary to accommodate healthy test organisms. Maintenance and culturing of all live food (brine shrimp and microworms) systems was continued to facilitate ongoing laboratory efforts. Weekly activities also include the following water quality analysis: dissolved oxygen, pH, conductivity, water flow measurements, ammonia, and light measurements. Monthly activities include TCE sampling of well water.

Transgenic medaka test organisms were maintained in conjunction with ongoing inter-laboratory functions with Dr. Jim Burkhart. Further studies with the transgenic medaka are scheduled for October. Essential laboratory supplies were documented and ordered as needed for the month. Medaka eggs were pulled in August and September. An entire bath was set up for immunotoxicology studies. Logistic preparations began for upcoming egg culturing in October. Eggs will be pulled to fill 1 1/2 baths for immunotoxicology work, four 55 gallon tanks for culture restock as well as fish for Dr. Burkhart and upcoming diluter tests at the end of the month. Two October breeding tanks will be held at 27 °C for the range find study with chloroform. Culture stock was replaced and rotated according to age. Bluegill cultures for Aberdeen field work, immunotoxicology, and ventilatory studies also continue to be rotated.

Diluter and waste disposal improvements required extensive ordering of new supplies and equipment. Plans are being made to work with Dr.Burkhart on fin clipping transgenic fish during the beginning of the next quarter. Ten years of well water analyses were summarized. Alkalinity, hardness, pH, and conductivity were averaged in one year increments. The standard deviation, the maximum and the minimum value for each parameter were also reported.

Fish Shipping for the quarter:

300 live medaka to NYU
40 frozen transgenic fry to NIEHS
500 live medaka to WVU
20 live medaka to Johns Hopkins University

Support of the Environmental Engineering Division at Rocky Mountain Arsenal (RMA):

Remediation and demolition activities continue to be observed, and technical support provided for the above ground storage tank (AST) characterization and deactivation project, the agent related and process equipment removal and waste management project, and the exploratory drilling task in Section 36. The latter activity consists of soil characterization to determine soil types in order to design and install slurry trenches around Shell's and Army's Burial Trenches.

Assistance continues in clarifying various regulatory issues, such as waste characterization, transportation, handling, and disposal, hazardous waste and agent waste characterization, and methods for reclamation and disposition of waste materials. RMA is currenly conducting a chemical sweep of the entire Arsenal to remove all remaining chemical items and chemical substances from various structures and process facilities. RMA Engineering Technicians received training and technical support regarding safe chemical handling (including neutralizing and stabilizing), chemical classification, chemical segregation, SW-846 sampling methods (standard methods), chemical hazcatting, chemical profiling, chemical packaging, and selecting a Toxic Storage Disposal Facility (TSDF) for chemical disposition.

As TVA completes its mission of managing RMA's chemical agent waste streams in December 96, technical guidance for managing RMA's chemical agent waste streams will be provided. Assistance is being given to RMA's Safety Health and Environmental Office to design and implement an internal unexploded ordnance (UXO) assessment team. The UXO team mission is to provide support for RMA's intrusive field activities and assess designated UXO areas that are scheduled for clean-up under the Record of Decision (ROD). Representation for RMA will be provided in an Environmental Protection Agency (EPA) Resources Conservation Recovery Act (RCRA) inspection.

Risk Assessment/Management Support at the Rocky Mountain Arsenal:

Assitance continues to the Army in the Record of Decision (ROD) implementation, by participation in the following groups:

The Biological Advisory Subcommittee (BAS) continues to look at the effects of remediation on the biota, as well as review proposed biota studies and check them for scientific soundness. The State=s Dioxin study has consumed much of the BAS's efforts. A recommendation has been made by the BAS to the RMA Committee to do additional analyses of biota on and off post to answer the question, AAre Dioxin=s a RMA contaminant?@. Approval has been given by the committee, and the BAS is moving ahead with refining the details of the study.

The Supplemental Field Study's Phase I report was finalized in July. The decision was made that any further studies, originally slotted for Phase II, would fall under the Biological Advisory Subcommittee (BAS). Two years have passed since the study was originally planned, and more information has been gathered. Therefore, the BAS is reworking the models predicting residual risk by using GIS maps. The BAS is working the maps by removing designated soil borrow areas, and looking at ways to reduce further remediation due to a possible residual risk.

A Biomonitoring Technical Review Group was formed to design a biomonitoring plan for the Army to assist in the decisions whether applied remedies had proven successful. The intention is to utilize the USFWS Biomonitoring Program already in place, but to identify gaps where further analyses is needed. Funding received from Army by the USFWS could be used to do recommended additional studies to determine success of remediation. This recommendation was approved by the Army, and the first meeting was held September 10, 1996.

The Medical Monitoring Advisory Group continues to meet once a month to review the work products of the subgroups. Assistance is given to the Army by attending the Baseline Health and the Human Health subgroups. Ground rules are currently being set. Policy for Old Samples discussions continue with Army lawyers to look at the possibility of disposing old biota samples. Hurdles include attaining EPA policy on the viability of samples and determining how long they are to be maintained, methods of disposal for samples with low levels of contamination, and preventing the State from taking custody of discarded samples.

Environmental Testing for Biological Threat Agents at U.S. Army Medical Research Institute of Infectious Disease:

Experiments were conducted at the Armed Forces Institute of Pathology (AFIP) for the validation of an *in vitro* viability staining procedure for use with the bacterium *Brucella suis.* Experiments conducted with the assistance of LTC Ted Hadfield, Microbiology Division Chief, utilized a two fluorescent dye system that resulted in red staining of non-viable bacteria, while live bacteria stained green when viewed with the epifluorescence. This rapid assay was successfully used to screen liquid fill contents of M114 bomblets for viable *Brucella* cells.

Prior to on-site testing of recovered liquid fill M114 bomblets at Wright-Patterson Air Force Base (WPAFB), a field manual of Standing Operating Procedures (SOP's) was prepared. The manual outlined procedures for the collection, sampling, shipping and testing of M114 submunition samples. Assays for the detection of target agents included slide agglutination, immunofluorescent antibody test, viablity staining, detection by PCR, and culture. This manual was useful for on-site tesing, as well as later tesing at USAMRIID.

A statistical model was developed to aid in determining how many M114 submunitions recovered at WPAFB would have to be tested. Sample sizes were modified several times during the actual recovery of bomblets, but the original model was useful in providing initial

guidance. Modifications to the sampling plan were made as the number of full and empty bomblets became known during the recovery operation.

A deployment was made to WPAFB (Dayton, OH) by a joint USAMRIID-FIP team to provide on-site tesing of recovered M114 bomblets. Previous research indicated that the bomblets contained only *Brucella suis* which had been heat-killed before burial forty years ago. Laboratory supplies and equipment were set up in a secured facility near the bomblet burial site. The team was able to test liquid M114 samples by slide agglutination, viability staining, Immunofluorescent antibody testing and PCR. On-site expert consultation was provided by the team during the week of September 18-23, 1996. Bomblet samples not available during the on-site visit were shipped to USAMRIID for further testing. A total of 372 samples were processed and tested by the Special Pathogens Department of USAMRIID. Samples were also prepared and transported to AFIP for testing. Test results for the first 220 samples tested by USAMRIID and AFIP were compiled and summarized in a preliminary report. The report was submitted to authorities at WPAFB. A final report will be prepared next quarter following the completion of animal studies conducted at AFIP.

Activities related to the College of American Pathologists (CAP) accreditation of a newly remodeled microbiology laboratory were initiated this quarter. Planning and preparation for an upcoming CAP inspection (March 1997) are being coordinated with the supervisor of the Clinical Laboratory. Participation in the special immunization program (SIP) was also begun this quarter. Participation in this program is essential for obtaining clearance to work in a BL3 biocontainment laboratory. Training was received in chemical hygiene, BL4 biocontainment, and medical defense against biolgoical warfare agents. A briefing was also made to LTC Estep, Duty Director, Biological Arms Control Treaty Office, on in-house departmental procedures for sample chain of custody.

The writing, editing, and organizing of SOP's to be followed by Special Pathogens Department personnel has been initiated. These SOP's include sample receipt, handling, isolation and identification procedures.

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Investigation of methods available for identifying biomarkers of mammalian hepatocarcinogenesis for adaptation to fish models:

Preparation for the BRDU portion of the water disinfection study to assess hepatocellular proliferation was initiated, including coordination of chemistry and histopathology efforts.

Research Conducted at Colorado State University:

Work continued on the development of a faster, more accurate means of evaluating fluorescent microspheres. The microsphere work did not lend itself easily or quickly for

Bioquant analysis, as was hoped. This information was relayed to Dr. Candy Matthews at USARIEM. Bioquant training for several university students and staff was provided over several days. A time for a possible two-day training session for interested parties is being arranged. Furniture for the Bioquant room has not arrived which has delayed the set-up of new equipment purchased for the instrument. Shipping of the Maryland Bioquant has also been put on hold due to space restraints.

Equipment for the tissue culture room arrived. Orders were then placed for calibration and certification of the biological hood. Orientation was provided to a new employee, Ms. Sandra Gunselman. The reading of slides and computing data for tests EE1 and EE2 was completed. All final graphs, tables and statistics were given to Dr. Hank Gardner for incorporation into future publication(s). Method development for DNA protein extraction for use in future species comparison work is in the preliminary stages and will continue to be developed over the next few months. Much time was spent obtaining research articles on nuclear factor - kappa B; heat shock proteins, and metallothionein expression in fish and/or amphibians. A search was also conducted by Al Reynolds of WRAIR. This research was required to identify known information in this area using fish as a model. An informal meeting was held with Hank Gardner and Greg Cosma to focus the scope of their collaborative efforts. The work will commence shortly after the first of the year on determining the phylogenetic conservation of the mechanisms of the proinflammatory response using established cell lines from fish, rodents, humans and possibly amphibians. This work may manifest itself as a study of nf kappa B, stress proteins, or both. A preliminary proposal will be written in the next quarter. This will neccessitate further library research and discussions with Dr. Cosma regarding the results in the published literature and how to interpret those results.

Work on establishing a library of scanned images that can be used in presentations, posters, etc. commenced this month. The library will be stored on disk and as hard copy to make it easily assessable to all of USACEHR. Two hard copies of the first edition will be made available after the first of the year. Efforts were made toward gaining proficiency in Microsoft Powerpoint, a software package used to create presentation slides in order to prepare slides and overheads for upcoming briefings.

A new laboratory was set up at CSU. Supplies were ordered and available instrumentation was obtained. Meetings with CSU faculty were attended to raise interest in collaborative work with Fourier transform infrared spectroscopy (FT-IR) and gas chromatography-mass spectrometry (GC-MS). A lecture was given to the undergraduate Enviromental Health class on recently published work involving oxidation DNA modifications and carcinogenesis. Working with Dr. Don Malins, a maunuscript was completed and submitted to *Cancer Research.* A manuscript was also submitted to *Proceedings of the National Academy of Sciences.*

Tissue culture techniques were learned to facilitate future work. Library research was performed for possible projects involving redox chemistry of DNA exposed to environmental

carcinogens. DNA extractions have been performed for the following projects: The Pacific Northwest Research Foundation in Seattle's split tissure samples for a reproducibility investigation as part of contractual agreement through USACEHR, as well as medaka liver DNA for FT-IR analysis; rat liver DNA from Dr. Yang's laboratory that has been exposed to pentachlorobenzene over a 12, 24, and 48 hour, four-day time course; and rabbit prostate, liver and testes that has been exposed to DDT and DDE gestationally from Dr. Cosma's lab. A non-phenolic DNA extraction procedure that was developed as a modification of the protocol used at Pacific Northwest Research Foundation was used. The use of phenol has raised questions of possible oxidation injury to DNA during the extraction procedure. The new protocol appears to be an acceptable improvement as it yields high quality DNA (A260/280 ration = 1.8) and high quantities. An outline of work to be pursued at CSU is being constructed. The focus will be oxidative changes caused by enviromental exposures on human breast MCF cells and the effect on transcription factors. This work will be synchronized with mice embryo fibroblasts (10T1/2) in order to look at the cell cycle dependence of DNA damage. Results of the *in vitro* work will be combined with the *in vivo* work from the rabbit and medaka studies.

Information Transfer:

Malins, DC, Polissar, NL, Gunselman, SJ. Models of DNA structure achieve almost perfect discrimination between normal prostate, benign prostatic hyperplasia (BPH) and adenocarcinoma and have a high potential for predicting BPH and prostate cancer. *Proc.Natl.Acad.Sci., USA* 1997, **94**(1): 259-264.

Malins, DC, Polissar, NL, Garner, MM, Gunselman, SJ. Mutagenic DNA base modifications are correlated with lesions in nonneoplastic hepatic tissue of the English sole carcinogenesis model. *Cancer Research*, 1996, **56**(24): 5563-5565.

Malins, DC, Polissar, NL, Gunselman, SJ. Tumor progression to the metastatic state involves structural modifications in DNA markedly different from those associated with primary tumor formation. *Proc.Natl. Acad.Sci., USA*, **93**(24): 14047-14052.

Studies of chemical carcinogenesis in medaka:

Test 100-005 LC_{50} chloroform study was begun. Nominal concentrations were 250, 150, 90, 54, 32, and 0 mg/L. Within one hour after test initiation all the fry in the high concentration were dead. By day 3 there was a reduced number in the 150 mg/L concentration tanks, and the surviving fry were impaired. The chloroform LC_{50} for medaka is 136 mg/L. A repeat of this experiment was terminated early due to heavy control mortality. Unexplained mortality was also observed in test tanks. A white residue was discovered in the toxicant mixing chamber which was determined to be a fungal contamination after microscopic examination. Samples were taken from several sites of the diluter test system and the effected tanks in order to determine a possible source of the contamination. Sample results indicated fungal infection at two sites in the diluter system. The diluter was acid washed, disassembled



and retested before subsequent testing was resumed. A second set of samples tested negative for fungal growth, therefore, the cholorform LC_{50} of the water disinfection study was restarted. Samples will be taken again while the testing is in progress to monitor for further contamination. The diluter calibration was changed to deliver 1.5, 0.15, and 0.015 mg/L chloroform for the chronic carcinogen test which will begin in January. A new chemical, bromodichloromethane (BDCM) was added to the drinking water disinfection by-product study protocols. Since the chloroform medaka test closely followed literature results, the range find for this chemical was omitted and the 96-hour LC_{50} test performed under flow through conditions. Concentrations tested were 251, 146, 82, 51, 29, and 0 mg/L BDCM. The BDCM LC50 for medaka is 79 mg/L. The chronic BDCM carcinogen test is scheduled to begin during the second quarter of 1997.

A meeting was held to discuss the upgrades to exposure room #10. Representatives from Engineering Computer Optecnomics, Inc (ECO) were in attendance to provide cost estimates on possible upgrades. Equipment to be modified are the diluter boards, the exposure cabinets, and the multiport glove box. A quality assurance check was performed on the water quality data obtained from the West Branch Canal Creek trailer study #401-002R. This data was entered onto Lotus spreadsheets for statistical analysis and checked for transcribing errors. A quality assurance training workshop for Good Laboratory Practices held by the Society of Quality Assurance was attended. The importance of having good data collection practices supported by valid and up-to-date SOPs and test protocols was stressed. Reference materials were collected for general laboratory use.

Teleost immunotoxicology methods development:

Research utilizing the bluegill model continued this quarter. Accomplishments include the optimization of assays involving the nonspecific and specific immune response. Production of reactive oxygen intermediates (ROI's) and plasma circulating antibody (Ig) production assays were completed. Progress was made on the optimization of the lymphocyte proliferation assay. Initial tests were performed which analyze the killing ability of macrophages and selected proteins (complement, lysozyme) in the plasma. Bacterial LC50 trials were also performed which ascertained the levels of bacteria to which the bluegill are susceptible. The species *Aeromonas hydrophila, salmonicida and Yersinia ruckeri* were selected because they are opportunistic aquatic pathogens which may cause disease in immunocompromised fish. They are easy to culture and isolate, and pose little threat to human health. Initial studies demonstrate that bluegill are susceptible to all three species of bacteria. Progress is being made towards defining a bacterial concentration range for each bacterial species which will serve as the LC50 in future studies.

Two research posters were prepared and presented at the Society of Environmental Toxicology and Chemistry's 17th Annual Meeting held in Washington D.C. The posters were entitled "Immunotoxicology Methods Development in the Bluegill (*Lepomis macrochirus*) for use in Laboratory and Field Studies" and "Methods Development for Isolation of *Mycobacterium chelonae* subsp. *abcessus* from the Japanese medaka". Inquiries for copies of

the bluegill research poster were received from approximately 20 scientists from the USA and abroad. A progress briefing was made to representatives of the Army Corps of Engineers at the annual Fate & Effects Research Review conducted 13-14 November at the Waterways Experiment Station in Vicksburg, Mississippi. A program overview highlighting accomplishments of FY96, and goals set for FY 97-98 was given. A seminar was given at the National Cancer Institute's (NCI) Frederick Cancer Research and Development Center at Ft. Detrick on December 4th. It consisted of an overview of the teleost fish immunotoxicology program and was presented as part of the continuing education program for the animal caretakers and lab technicians at NCI. Approximately 50 people attended.

The animal use protocol entitled "Immunotoxicological Methods in Teleosts - assessment of Specific and Non-Specific Immune Function in Medaka and Bluegill" was approved by the IACUC committee. In addition two additional animal use protocols describing medaka health screening and host-resistance/bacterial challenges in medaka and bluegill were prepared for the January 1997 IACUC meeting. A scientific protocol entitled "Immunotoxic Effects of Low-Level Exposure to Insecticides - Effects of Malathion on Japanese medaka host resistance" was also written.

Transgenic medaka research at USACEHR [in collaboration with Dr. James Burkhart at the National Institute of Environmental Health Sciences (NIEHS)]:

Medaka fry from the June electroporation were euthanized. Three medaka from the August electroporation tested postitive as transgenic and are being held separately in 10 gallon tank to await future breeding. Mortality was observed in one of these fish in December. Approximately 100 fry resulted from the October electroporation. Screening of these fish for bacteriophage DNA is scheduled to begin in January.

Transgenic animal research conducted at NIEHS, Research Triangle Park, NC:

In this portion of the task, GEO-CENTERS is providing support for a project to develop *in vivo* mutagenicity testing in fish and mammals (medaka and mice). Transgenic animals containing exogenous bacteriophage DNA are the model systems in development.

Work concentrated on collecting data from liver tissues of mice dosed with 19.0 mg/kg 7, 12-dimethylbenz[a]anthracene (DMBA) exposed for 2, 4 and 30 days. Data Collection continued with corresponding spleen samples. Fish husbandry efforts have resulted in steady egg production with good hatching and survival rates for fry. An additional DMBA study with mice was initiated. Mice were exposed for 72 hours at three different concentrations of DMBA to determine EROD induction. Previous experimentation yielded inconsistent results.

An abstract has been submitted to the Environmental Mutagen Society for a paper to be titled "A Comparative Approach to 7, 12-Dimethylbenz[a]anthracene Effects: Metabolism and Mutagenesis in Mice and Fish." Preparations are underway to move the laboratory to a new

location. Reorganization and setup will require a major portion of the work effort until the lab is assembled into a new work unit.

Methods development for rapid toxicity assessment:

An experiment was conducted during this quarter to investigate the chorion strength of killifish eggs deposited in aquaria containing Instant Ocean□. It has been hypothesized by Dr. Eugene Hull that the Instant Ocean□ used in maintaining the Killifish decreases embryo chorion strength. It is also thought that NaCl does not affect the chorion strength. In the experiment, killifish eggs were subjected to as much as 150 grams of Instant Ocean□ and still exhibited a rate of hatchability equal to that of the control group.

The culture of the killifish continued this quarter. Culturing included daily feedings and temperature recording as well as weekly aquaria cleaning. Killifish eggs were collected weekly. After collection, the eggs were water incubated in Petri dishes for a minimum of 10 days. Incubation water was changed at least twice during the 10-day period. At the end of the incubation period, the eggs were transferred to moist, sterile peat moss for long term storage.

Bluegill ventilatory monitoring project:

A number of developmental refinements of the Aquatic Biomonitoring Program were pursued during the quarter. Ventilatory data was analyzed using new movement categories to determine the suitability of using fish for testing. New parameters were tested and found to be more applicable to conditions in a field operating environment. In collaboration with Mr. Leach, the new parameters were incorporated into the ventilatory BLPGM program. It was also decided to incorporate programming that would automatically determine a fish's suitability for testing based on these new parameters. The new BLPGM and Aquatic Biomonitoring Program automatically select fish that are not suitable for testing and electronically remove them from the test group. These changes simplify the programming procedures of the ventilatory operator but also allow the operator the flexibility to select their own settings if unusual circumstances should occur. All pre-testing and trouble-shooting was performed and new programming was installed at Old O-Field. A Quattro Pro program was created to determine relative weights of bluegills based upon their standard length and wet weight. The program was created to automate the process of determining the relative health of all test fish used at Old O-Field. Determining these values manually is very time consuming and susceptible to human error.

Data collected from Old O-Field during the quarter was analyzed and archived in the working database and on permanent disk. All response and water quality graphs were generated. Bi-weekly reports were generated and submitted for internal review and transmittal to Aberdeen Proving Ground. Reports were generated for six reporting periods spanning from 6 September through 29 November. Reports for the periods of 29 November through 13 December and 13 December through 27 December were submitted for internal review at USACEHR. O-field data collected over the first 15 months of operation were summarized for



inclusion in a presentation at the COE Fate and Effects R&D Research Review at WES in Vicksburg, MS. A number of graphics slides were prepared with ventilatory system operational statistics. Killifish informational slides were also provided for the presentation.

The dissolved oxygen probes of Hydrolab water quality analyzers versions 1.02 and 2.02 were reconditioned with an ammonia hydroxide 10% solution. The reconditioning process was

successful for version 1.02. The process was unsuccessful for version 2.02 and the probe will be replaced upon arrival of a replacement. A new oxygen sensor was installed in the Hydrolab Scout water quality analyzer. Dissolved oxygen sensors were received and installed in the version 2.02 Hydrolab H_20 water quality analyzer. Assistance was given to Mr. Jeff Leach to correct a Hydrolab transmission difficulty at DOIM. A new pH probe was installed into version 1.03 Hydrolab H_20 water quality analyzer. All other probes were reconditioned and calibrated. Research was begun to locate new water quality probe technologies to replace the Hydrolab water quality analyzers. Discussions were held with Dataforth Corporation to finalize the design and construction of the new ventilatory amplifiers. The final production module was received and tested with the ventilatory chambers. The ventilatory display amplifier was re-engineered with an internal power supply making the display module more compact, more portable, and easier to use.

Efforts were directed in converting room #2 into a diagnostic testing facility for the assembly, testing, and trouble-shooting of laboratory equipment and ventilatory amplifier and computer systems. The new ventilatory amplifier systems were received from Dataforth. The systems were assembled in room #2 for evaluation. The system is currently being evaluated for system compatibility and accuracy. A new programmable DC voltage source diagnostic instrument was acquired. The desired capability was to program the test instrument to simulate a fish ventilatory signal. This was successfully accomplished. The instrument will be used to validate and diagnose hardware problems. The instrument has also been helpful in understanding the dynamics of the ventilatory test system. A new method of liquid level detection was researched and developed to replace current liquid level sensors. Instrumentation now being used has been susceptible to fouling and inadvertent displacement. The new sensors utilize a non-intrusive capacitive sensor to detect liquid levels. The new sensors and controllers were ordered and will be installed upon arrival.

The following animal use protocols for Old O-Field and in-house ventilatory studies were revised and submitted for review at the January Animal Use Committee review: Ventilatory

Biomonitoring of Treated Effluent at the Old O-Field Ground Water Treatment Facility and Establishment of the Sensitivity and Response Time of the Ventilatory Biomonitoring System to Chemicals Having Varying Modes of Toxic Action on the bluegill *Lepomis macrochirus.*

Analytical method development and analysis of chemicals used in carcinogenicity and mobile laboratory studies:

Approximately 50 samples of bees and pollen were digested and analyzed for trace metals by ICP-MS. Samples contained metals typically found in environmental samples. Approximately 100 samples were analyzed for thalidomide in FETAX. The concentrations used did not produce malformations in the embryos and will be increased for the next series of tests. The method for the analysis of Thalidomide was changed in order to compensate for the increase in concentrations used to expose embryos in FETAX. The injection volume was decreased from 10 microliters to 1 microliter. Immediate precipitation was observed in concentrations of thalidomide that exceeded 250 mg/L and it was suspected that the metabolic activation used during the exposure was not working properly. Reducing the concentration range may be indicated. Several instrumental difficulties have appeared with the HP 7686 PrepStation that are associated with a software upgrade. These problems will be solved by upgrading the computer hardware used to support the latest revision of the software. Final revisions are being completed on an application note being published with Hewlett Packard on the automated extraction of Atrazine and Simazine in FETAX.

Maintenance and optimization of USACHER aquatic laboratory facilities:

Essential laboratory maintenance was performed on all culture (medaka, bluegill, fathead minnows, and killifish) and test fish (medaka) located in rooms 5 and 18. Daily activities include three daily feedings of all culture and test organisms. Associated record keeping was performed to maintain necessary test records to meet SOP and regulatory review requirements. Associated tank cleaning and siphoning was performed to maintain overa

ll tank hygiene necessary to accommodate healthy test organisms. Maintenance and culturing of all live food (brine shrimp and microworms) systems was performed to facilitate ongoing laboratory efforts. Weekly activities also include the following water quality analysis; dissolved oxygen, pH, conductivity, water flow measurements, ammonia, and light measurements. Monthly activities include TCE sampling of well water.

Transgenic medaka test organisms were maintained in conjunction with ongoing inter-laboratory functions with Dr. Jim Burkhart. Continuing investigations with the transgenic medaka are scheduled throughout the year. Essential laboratory supplies were documented and ordered as needed for the month. Efforts were made to raise the culture tanks to breeding level on December 2nd with medaka eggs culture taking place on the 16th of December for the upcoming chronic chloroform study. Culturing was also performed to restock fish according to the breeder rotation plan. Approximately 10,000 medaka eggs were cultured during this quarter for colony renewal, immunotox, and medaka tests.

The FY 96 fish portion of the Annual Report to Congress was completed. Numbers of medaka, bluegill, killifish, fathead minnows, and guppies cultured and used from October 1995 to September 1996 were summarized.

Fish shipping for the quarter:

710 live medaka to NYU
425 live medaka to WVU
10 live medaka to EPL
55 live medaka to University of Maine
Frozen livers and gills from methylene chloride tests to U of ME

Support of the Environmental Engineering Division at Rocky Mountain Arsenal (RMA):

GEO-Centers continues to observe remediation and demolition activities providing technical support for the ancillary equipment deactivation at tank farm number 105 project, chemical sewer closure project, exploratory drilling task in Section 36, and agent related/process equipment removal/ waste management project. Assistance continues in clarifying various regulatory issues, such as waste characterization, transportation, handling, and disposal, hazardous waste and agent waste characterization and safe handling procedures, researching innovated methods for reclamation and disposition of waste materials, and providing emergency response during an on-site spill of sulfuric acid.

RMA is continuing a chemical sweep of the entire Arsenal to remove all remaining chemical items or substances from various structures and process facilities. RMA engineering technicians are receiving training on safe chemical handling (including neutralizing and stabilizing), chemical classification, chemical segregation, SW-846 sampling methods (standard methods), chemical hazcatting, chemical profiling, chemical packaging, and selecting a Toxic Storage Disposal Facility (TSDF) for chemical disposition. RMA engineering technicians are also receiving technical support regarding day-to-day field operations pertaining to chemical classification, chemical segregation, collecting representative samples, packaging chemicals per DOT specifications, chemical profiling, and arrangments for disposition.

Technical review of TVA's chemical agent waste management operations continues. TVA is currently managing RMA's chemical agent waste streams, however TVA's mission at RMA will be completed as of December 96. RMA has requested technical guidance for managing their chemical agent waste streams. Technical support is provided for an internal unexploded ordnance (UXO) assessment team that assesses potential UXO areas and identifies various types of UXO's that may be present. Support is also given for the development of the RMA Contingency Plan.

Risk Assessment/Management Support at the Rocky Mountain Arsenal:

Assistance continues to the Army in the Record of Decision (ROD) implementation, by participation in the following groups:

The Biological Advisory Subcommittee (BAS) tasked with looking at the effects of

remediation on the biota, as well as reviewing proposed biota studies for scientific soundness. The State□s Dioxin study continues to consume much of the BAS effort. Upon committee's approval of the BAS recommendation to do additional analyses of biota on and off post, the BAS initiated the Dioxin/Furan Study. Tissue samples are being sent to Clemson Laboratory for the H4IIe assay. A select group of samples will be sent to another lab for the PCDD/PCDF analyses.

The Supplemental Field Study's Phase I continues to be evaluated by the BAS. The BAS is reworking the models predicting residual risk by using GIS mapping. They are also working with the RMA Borrow Area Group to incorporate areas of high risk into areas needed for borrow. The BAS is working towards reducing all residual risk areas with a minimal cost to the Army and Shell.

The Biomonitoring Technical Review Group continues to identify gaps or areas where the USFWS exceeds the needs of the Army for a Biomonitoring Program. This group is also looking at the proposed borrow areas to ensure the Army avoids recommending an area be used for borrow that they have revegetated for the USFWS. This is possible NRDA expense.

The Medical Monitoring Advisory Group continues to meet once a month to review the work products of the subgroups. Assistance is given to the Army by attendance of the Baseline Health and the Human Health subgroups. This group is currently working on a document to outline the communities baseline health, and an action plan in the event of exposure to the community during remediation.

Policy for Old Samples continues to work with Army lawyers examining the possibility of disposing old biota samples.

Environmental Testing for Biological Threat Agents at U.S. Army Medical Research Institute of Infectious Disease:

Soil samples from Saudi retrograde ammunition were received by the Bio-Treaty Laboratory. Subsamples were removed and irradiated for chemical analysis. The remaining samples were analyzed for biological threat agents. The Bio-Treaty Lab received 300 surface and subsurfac soil samples from verification sites in Iraq. Samples are currently being assessed for threat agents.

An ILIR proposal was prepared for Department of Defense funding of a project entitled "Development of specialized media of the selective isolation of *Bacillus anthracis* and *Yersinia pestis* from medical and other complex matrices". A manuscript entitled "Rapid viability assessment of biological submunition" was also prepared for publication in *Letters in Applied Microbiology* and is awaiting signature release by the USAMRIID commander before submission to the journal. A cost estimate analysis (personnel, expandable materials, reagents and media) was prepared and a reimbursement schedule for *in vitro* bacterial isolation and identification of submitted test material to the Bio-Treaty Laboratory. Assistance was given in

the production of a 20 minute VHS video of anthrax vegetative cells and spores as requested by the United Naiton, New York. Manuscript review was completed on Terbium Dipicolinate Photoluminescence (TDP) and comments were presented to the Chief of the Diagnostic Systems Divsion. A Personnel Training File was established for each member of the Special Pathogens Department in order to meet GLP compliance. Numerous Standard Operating Procedures (SOPs) regarding administrative and scientific functions of the laboratory were prepared, reviewed and edited. Medical clearance was received to enter BL3 and BL4 containment laboratories. Preparations are currently underway for the transfer of personnel and equipment to a newly renovated BL3 laboratory. Moving activities are expected to continue through January 1997.

Plate/probe based PCR-EIA (Enzyme Immuno Assay) for *Yersinia pestis* was begun this quarter. The goal is the development of a portable plate/probe based assay for use in the field to quickly determine whether a sample is positive for plague. The DIG-labeled PCR reactions required optimization which required much of the work effort this quarter. Primers from the genetic locus plasmidogen activating factor (pla) were used which allowed specific bands for only *Y. pestis* even when testing with other closely associated organisms. Optimization experimentation included length of annealing and extension times, temperatures, number of cycles, different primers and concentrations, $MgCl_2$ concentration, and twelve templates and their concentrations. The next step involves the optimization of the EIA which has currently begun. Various biotinylated probes and concentrations are being used for standardizing colormetric signal.

Training was received in Export and Import of Etiologic Agents, Introduction to Good Laboratory Practices (GLP), Safety Operations for BL3 and BL4 Laboratories, GLP for the Analytical Laboratory, GLP Study Director Training, GLP Workshop for SOP Preparation, Clinical Applicaitons of the Microscan (Automated Bacterial Identificaiton System), automated DNA sequencing, oligo synthesis and purification methods, Restriction Fragment Length Polymorphism (RFLP), and Subversion and Espionage Directed Against the US Army and Deliberate Security Violations.

Information Transfer:

Teska,JD, L.Twerdok, J.Beaman, M.Curry and R.Finch. Prevalence of *Mycobacterium* in cultured Japanese medaka (*Oryzias latipes*) used for aquatic toxicity testing. SETAC 17th Annual Meeting, Washington, D.C., 17-21 November 1996.

Teska,JD, L.Twerdok, J.Beaman, M.Curry and R.Finch. Isolation of *Mycobacterium chelonae* subsp. *abscessus* from Japanese madaka *(Oryzias latipes)*. *Journal of Aquatic Animal Health.* (Submitted)

Teska,JD, T. Hadfield, J.Ezzell, E.Hilyard, and E.Henchal. Rapid viability assessment of biological submunitions. *Letters in Applied Microbiology.* (In Review)

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Investigation of methods available for identifying biomarkers of mammalian hepatocarcinogenesis for adaptation to fish models:

The majority of the work effort for the month of January was spent on Test 100-006, Chloroform Exposure to Medaka. Four 5-Bromo-2' deoxyuridine (BRDU) exposures were done during this time period to assess hepatocellular proliferation in the medaka after 1, 4, 6, and 20 days of chloroform exposure. Tasks included test preparation, actual exposures, fish sacrifices, clean-up, and test-associated binder preparation.

Research Conducted at Colorado State University:

Preparations were made for a meeting concerning the future use of FT-IR technology at CSU university. At the meeting, a presentation was given to attendees. Among those at the meeting were: Drs. Loichi Nishikida and Dave Tracy of the Perkin-Elmer Corporation, Environmental Health Dept. Chairman, Dr. Reif, Dr. Tessari, Dr. Cosma, Dr. Gardner and Dr. Frank. As a result of this effort, Perkin-Elmer Corporation reconfirmed their agreement to consign the FT-IR to CSU. Additional meetings were held on various topics concerning the FT-IR. Protocol procedures were discussed with Dr. Malins of Northwest Pacific Research Foundation, use of the instrumentation with Dr. Tessari, future research projects with Dr. Cosma, and overall program application with Dr. Gardner. A timeline was prepared for Dr. Gardner's approval.

DNA extraction methods were taught to a CSU student for Dr. Ray Yang at CETT. This was considered a priority effort to ensure that free time would be available in the future for upcoming work. A lecture given by Dr. Don Malins was attended at Pepperdine University. The NIEHS-sponsored Superfund Basic Research Conference in Chapel Hill, NC, was also attended where a poster presentation was made on the application of DNA biomarkers to Superfund sites.

Several problems were encountered that have hindered progress in the laboratory. Extraction of Dr. Yang's samples was producing high quality (UV 1.6-1.9) and quantity DNA but the DNA was sheared. The procedure was repeated several times with the same result. Each solvent and chemical used in the procedure was eliminated as a factor. The continual freeze-thaw of the samples was suspected as a possible factor. At Dr. Cosmas's suggestion, liver samples were acquired that were not flash-frozen or thawed. The extraction of these samples produced "stranded" DNA. New work began on an unthawed set of samples for Dr. Yang but results have varied. Shearing of the DNA continued to be observed. In depth discussions with Dr. Cosma indicate that the chemical sample preparation must be contributing to the problem.

The preparation of standards for the GC-MS has begun in order to be ready for samples the end of March. One of the standards custom-synthesized for this work by Cambridge



Isotopes was discovered to have turned a bright orange color. The sample was scanned on the GC-MS and results were sent to Cambridge Isotopes chemists. At their request, the compound was returned for further evaluation by their laboratory. More samples of other compounds will also be run on the GC-MS in the following weeks.

The Bioquant image analysis system at the CETT location was disassembled and moved to an alternate location to accomodate a second Bioquant system's arrival. A new computer was received and configured to adapt to research needs. 486 computers were also reformatted for compatibility with government equipment located on the main campus. Graphics were scanned for use in the image album being assembled for presentation purposes. Overload of data caused a computer crash and information was lost. A new hard drive was ordered and installed. Programs and network connections were revamped but problems continue with proper operation. Graphics for presentations were rescanned into the USACEHR shared drive for access by all users.

A potential medaka holding facility on the CSU campus was surveyed. Information on flow rates, water chemistry and water quality are being compared between the Fort Detrick facility and the CO facility. A possible research project for the species comparison work was outlined. Using established macrophage cell lines from trout, goldfish, rodent and humans, a reactive oxygen species (ROS) using lippopolysaccharide would be generated. NF kappa B and/or AP-1 complex, products of the inflammatory response, will be probed and the presence of cytokines such as IL-1 and TNF-a will be determined. After cellular activation, assays for the presence of cellular defense mechanisms (i.e., IL-6) and the initiation of stress proteins will be performed. An Animal Use Protocol addendum was written for a pilot study of the NF kappa B. All laboratory equipment for future cell culture work has been moved into designated space. Calibration and set up of this equipment was also performed. Statistical analysis was performed on the DEN study read by Experimental Pathology Laboratory (EPL) for neoplastic endpoints and at CEHR for cell proliferation endpoints.

Studies of chemical carcinogenesis in medaka:

Two drinking water disinfection by-products tests began in January. The 96 hour LC50 for medaka with chlorate was 2917 mg/L. The 9 month chronic carcinogen study using medaka and three levels of chloroform (1.5, 0.15, and 0.015 mg/L) was also started. Four cell proliferation tests were run with chloroform exposed medaka. A range-find experiment was performed with dibromoacetic acid (DBAA) and medaka. Concentrations tested included 1000, 100, 10, and 1 mg/L DBAA. 100% mortality occurred at 1000 mg/L, linked to the low pH. Subsequent water testing revealed that 400 mg/L is the highest DBAA concentration with an acceptable pH (pH = 6.7), which will be the high dose in the LC50 test. The LC50 test for DBAA is planned for late April.

Investigation of intermittant mortality of test organisms in room 10 continued. A meeting was held with laboratory personnel, attending veterinarian, and a microbiologist to decide on the proper course of action to be taken. An investigational study was performed

with fish and frogs using several variables to determine a causitive factor in unexplained mortality. Results of this test suggest that fish and frog embryos may not be compatible as test organisms and should not be tested together. Frogs showed a greater sensitivity when held in the same test aquaria as the fish and may be succumbing to bacterial pathogens. The possibility of disinfecting the water pipes with a peroxide solution was discussed. A feasibility study on well room upgrades was begun. Water samples for bacterial and fungal analysis were collected, processed and cultured. Media were prepared, data summaries written, and the first draft of an SOP was completed. Results of this testing indicate that the PVC water lines to room 10 have a higher fungal and bacterial load than the other aquaculture rooms. Bacterial and fungal sampling the last month of the quarter showed that the UV unit 2 is doing an adequate job of disinfecting incoming water, allowing #5 bacterial colonies/100mL to pass into the water system. UV unit 1 allows 5-10 bacterial colonies/100mL sample to pass into the lines, therefore, its use was discontinued. The replacement of RO filters with nanofilters has been discussed with a contractor. This upgrade would lower the operating pressures on the system, waste less water, and require fewer manhours to operate. A presentation on the subject will be made to the laboratory in the near future.

A quality assurance check was done on the water chemistry data from West Branch Canal Creek trailer study #401-002R. This data had been transcribed by Dr. Dennis Burton (University of Maryland) from Johnston Spectra Laboratories reports and was checked for transcribing errors as well as laboratory report inconsistencies. Corrections and a summary table of the corrections were sent to Dennis Burton and Tommy Shedd (USACEHR).

Teleost immunotoxicology methods development:

Research using the Japanese medaka model continued this quarter. Accomplishments include the assessment of host resistance/susceptibility to pathogenic bacterial challenge following

chronic exposure to a potentially immunotoxic environmental chemical, malathion. This completes the first chemical in a series which will be assessed in both the medaka and bluegill model. The study included setup and calibration of the proportional diluter system in the biomonitoring trailer, selection, randomization, and acclimation of - 480 adult (9-11 month) medaka, and a post-acclimation 21-day exposure to sublethal levels of malathion, with assessment of host resistance at day 7, 14, and 21. The host resistance assay involved the interperitoneal inoculation of 20 ml of an LD_{40} dose of *Yersinia ruckeri*, a gram negative opportunistic aquatic pathogen in control, low and high toxicant concentrations.The results are as follows:

Test 303-004: Host Susceptibility to Pathogenic Challenge with *Yersinia ruckeri* Following Exposure to Subacute Concentrations of Malathion

A. Percent Mortality By Exposure Duration and Total Mortality by Toxicant Exposure Group

	7 Days	14 Days	21 Days	Total
High [1]	54.6 " 25.7 %	74.8 " 15.2 %	72.6 " 7.3 %	67.3 " 18.6 %
Low [2]	51.4 " 19.5 %	60.7 " 18.2 %	59.1 " 11.1 %	57.1 " 15.7 %
Control [3]	45.5 " 7.4 %	39.6 " 4.3 %	38.6 " 7.9 %	41.2 " 6.9 %

1 High Toxicant Concentration = 279.6 " 123.3 ppb
2 Low Toxicant Concentration = 87.2 " 44.2 ppb
3 Control = 0.0 ppb

Toxicant stock equaled 125,885 " 24,327 ppb. Stock and aquaria water samples were analyzed 7 times during the study. A model I (fixed-effects) two way analysis of variance (ANOVA) was used to test for effects. Comparisons among means were made using the Tukey-Kramer multiple comparison procedure and point estimation was accomplished using regression analysis. Analysis of variance indicates a good fit to the model, however the only significance observed was that caused by toxicant concentration. Analysis by concentration level yielded no exposure time effect for any concentration. Analysis by exposure time indicates a significant concentration effect for days 14 and 21 (Table 1). Comparisons in mean percent mortality among the concentration levels by exposure time, show that there were no significant differences among any of the concentrations at day 7. At days 14 and 21, mean % mortality of both toxicant concentration levels was different from the control (Table 2).

Table 1: Concentration Effect By ExposureTime

	Statistic	
Sampling Day	F	*p*
7	0.52	0.4891
14	14.05	0.0038*
21	31.27	0.0002*

* = Statistically significant effect.

Table 2: Tukey - Kramer Multiple Comparison Procedure: Comparisons Among Concentration, By Day

Day	Concentration (ppb)	Observed Mean % X100	Predicted Mean % X100	95% C. L. (U + L)	Cat*
7	0	0.4545	0.4539	0.2850, 0.6497	A
	87.164	0.5136	0.5159	0.3939, 0.6378	A
	279.636	0.5455	0.5439	0.3749, 0.7129	A
14	0	0.3955	0.3893	0.2456, 0.5329	A
	87.164	0.6068	0.6266	0.5372, 0.7160	B
	279.636	0.7477	0.7341	0.6102, 0.8580	B
21	0	0.3860	0.3803	0.2874, 0.4732	A
	87.164	0.5909	0.6902	0.5514, 0.6670	B
	279.636	0.7254	0.7128	0.6327, 0.7929	B

* Means with different letters are significantly different from each other.

Preparations for immunotoxic hazard assessment of malathion in bluegill have begun during this quarter. A definitive bacterial LD_{50} in bluegill was performed using *Yersinia ruckeri*. Three replicates of 5 dilutions plus controls were run, each dilution containing 10 fish. Also, a static renewal LC_{50} was run in bluegill, and a No-Observed-Effects-Level (NOEL) was established. The diluter system was disassembled and cleaned along with all test glassware (aquaria, animal jars, etc). The water bath/diluter system is currently being reset/recalibrated and prepared for the bluegill test.

Two animal use protocols were prepared and submitted to the IACUC in January, and are presently undergoing final review and approval. The two protocols are 1) "Routine Health Screening in Cultured Medaka" and 2) "Immunotoxicological Methods Development in Teleosts - Bacterial LC_{50} in Medaka and Bluegill". Seven Standard Operating Procedures (SOP's) have been finalized and are ready for inclusion in the laboratory's SOP collection.

Transgenic medaka research at USACEHR [in collaboration with Dr. James Burkhart at the National Institute of Environmental Health Sciences (NIEHS)]:

Tissue was taken from forty-nine medaka's fins to determine whether or not genes had been transmitted through the vector virus. Each fish was isolated into an individual chamber to await the screening results. The fin tissues were sent to Dr. Burkhart in NC.

Transgenic animal research conducted at NIEHS, Research Triangle Park, NC:

In this portion of the task, GEO-CENTERS is providing support for a project to develop *in vivo* mutagenicity testing in fish and mammals (medaka and mice). Transgenic animals containing exogenous bacteriophage DNA are the model systems in development.

Work concentrated on obtaining data from 7,12-dimethylbenz[a]anthracene (DMBA)-treated murine spleen tissue. Most of the work has been completed with the exception of the control animals. Work is currently in progress on these controls. More analyses is also required. A notable change in the physical appearance of the assay occurred with high background apparent causing speculation on the validity of the results. It was found that the bacterial host for the vector had undergone genetic drift. The problem was rectified and low backgrounds have once again been established in the assay, as well as the ability to reproduce results.

Fish husbandry continues with successful breeding of *Fundulus heteroclitus.* Good numbers of eggs and young fry are being obtained. Fin clips continue to be performed to determine the transmission of the vector through different generations. Problems have been encountered with the dCTP (^{32}P) that has been received to prepare probes. New dCTP has been ordered from another company to see if the incorporation into probes can be more effectively accomplished.

Bluegill ventilatory monitoring project:

Bi-weekly reports were written and submitted for the ventilatory data collected at Old O-Field for five reporting periods. The installation and testing of a new injection diluter system was completed during the quarter. The DDS-2XFDA Dosing Diluter System constructed by Engineering Computer Optecnomics, Inc. (ECO) was designed to inject a volume of desired liquid into a dilution water source. The system was tested with the DVD-8X1A Ventilatory Diluter to ensure that it was properly injecting and delivering the appropriate mix of materials. The system was tested and installed at Old O-Field to be used as a conductivity adjustment device. Testing performed at USACEHR validated that the system was an effective method for achieving and maintaining the conductivity levels necessary for testing purposes at Old O-Field. Summary information comparing year 1 and year 2 operational periods and response information from Old O-Field was compiled and incorporated into summary slides for a presentation to the U.S. Army Corps of Engineers at Vicksburg, MS, and Las Vegas, NV. A computer system was constructed and tested in collaboration with Mr. Tommy Shedd, Mr. Jeff Leach, and Ms. Florence Hoffmann. The system was installed at the Canal Creek Trailer site for use by Ms. Darlene Tiemann, who was given training to generate the biweekly reports of the Old O-Field data. Revisions were made to the Old O-Field Operation and Maintenance Manual to incorporate recent changes to the system. The Old O-field Animal Use protocol was revised in response to comments by the Animal Use committee and the Response Flow Chart was reworked after receiving comments from Aberdeen Proving Ground and Dr. William van der Schalie. The new response chart incorporates a more conventional flow chart

format.

Intellectual property rights and patent disclosure information regarding the ventilatory biomonitoring system and the killifish test system were discussed with Blake Sajonia and Tim Wittig. On their advisement, both the killifish system and the ventilatory system will be submitted for patent applications. The time-to-response/sensitivity Animal Use protocol was revised and submitted to the Animal Use committee. Training on the ventilatory biomonitoring system was given to a new technician. The design forthe converstion of the insectory into a ventilatory exposure facility was completed. This plan for the conversion of the insectory was submitted by the government for construction by post engineering. Further actions are awaiting approval and subsequent interaction from the post engineers.

Efforts were pursued in preparing for the U.S. Army Corps of Engineers Combined Innovative Technology Transfer and Chemistry Workshop on 17-21 March 1997 at the Alexis Park Resort in Las Vegas, NV. Preparations for the half-day workshop on ventilatory biomonitoring included constructing and preparing the ventilatory computer system for transport and subsequent interactive live demonstration of the ventilatory biomonitoring system at the workshop. The constructed system included the final version of the new ventilatory amplifiers which were successfully integrated with the Aquatic Biomonitoring System. All procedures to be performed at the workshop were tested at USACEHR. The live demonstration and a 30 minute presentation on the Ventilatory Biomonitoring System requirements were presented at the Corps of Engineers workshop in Las Vegas.

Analytical method development and analysis of chemicals used in carcinogenicity and mobile laboratory studies:

A Dionex 500 Ion Chromatograph (IC) was received by the laboratory. This new instrument will allow separation and detection of compounds having ionic characteristics. A method developed at Dionex was verified for analyzing chlorate in well water. During testing, it was discovered that careful degassing the mobile phase is necessary. An incompletely degassed mobile phase will cause bubbles to form in the pump head and producing unstable back pressure. Precision and accuracy data was completed at 10 and 100 mg/L. Approximately 200 samples were analyzed for chlorate in support of toxicology testing by ion chromatography. Approximately 100 samples of Bromodeoxyuridine and 300 samples of Thalidomide were and analyzed by HPLC.

Maintenance and optimization of USACEHR aquatic laboratory facilities:

Essential laboratory maintenance was performed on all culture (medaka, bluegill, fathead minnows, and killifish) and test fish located in rooms 5 and 18. Daily activities include three daily feedings of all culture and test organisms. Associated record keeping was performed to maintain necessary test records to meet SOP and regulatory review requirements. Associated tank cleaning and siphoning was performed to maintain overall tank hygiene necessary to accommodate healthy test organisms. Maintenance and culturing of all live food

(brine shrimp and microworms) systems was performed. Weekly activities also include the following water quality analysis: dissolved oxygen, ph, conductivity, water flow measurements, ammonia, and light measurements. Monthly activities include TCE sampling of well water.

Transgenic medaka test organisms were maintained in conjunction with inter-laboratory research with Dr. Jim Burkhart. Essential laboratory supplies were documented and ordered as needed for the quarter. Maintenance of the ongoing chloroform test continued during the quarter including weekly water sample collection. Temperatures and brine shrimp feeding levels were increased on all medaka culture breeding tanks to prepare for a scheduled egg pull. Medaka fry were hatched on March 3rd and 4th with a 75% hatch rate. Two water baths were set up for immunotox fish and to house fish for culture stocks. One of the two baths of retired medaka breeder fish was shipped to Dr. Miller at West Virginia University.

Fish shipping for the quarter:

NYU	952 live medaka
WVU	530 live medaka
EPL	600 preserved medaka

Support of the Environmental Engineering Division at Rocky Mountain Arsenal (RMA):

Health and safety support for Tennessee Valley Authority (TVA) at RMA has been provided. TVA is currently performing chemical agent deactivation tasks for RMA. In cooperation with the Department of Army (DA) and Chemical Agent Safety and Health Policy Action Committee (CASHPAC) discussions have been held regarding the approval of chemical protective clothing (CPC) to be used at CBDCOM facilities. The CPC will save CBDCOM facilities a considerable amount of money each year while meeting the intent of EO12196 and OSH the Act.

Assistance was given in clarifying regulatory issues such as waste characterization, transportation, handling, and disposal, hazardous waste and agent waste characterization and safe handling procedures. Researching innovated methods for reclamation and disposition of waste materials, UXO site assessment requirements, and chemical agent waste management practices and policies are also areas of interest. Technical guidance and training was provided to RMA employees for managing special waste. Assistance continues to be given to the Army in conducting the chemical sweep of the Arsenal. Technical support regarding day-to-day field operations was provided to RMA Engineering Technicians. A review of TVA's chemical agent waste management operations was made. TVA is currently managing RMA's chemical agent waste streams, however TVA's mission at RMA has been down scaled as of December 96. RMA has requested GEO-CENTERS to be the Technical Manager for RMA chemical agent waste streams. Technical support was also provided for an internal unexploded ordnance

(UXO) assessment team which assess all potential UXO areas, and identifies various types of UXOs that may be present at RMA. The 60% design of the slurry wall around the Army complex trenches will require the following support: ambient monitoring for chemical agent, health and safety oversight, UXO pre-site assessment, and emergency response support for chemical agent and UXO incidents during site activities.

The coordination and arrangement of the on-site treatment for 20,000 gallons of sodium hydroxide (NaOH) at RMA Waste Water Treatment Facility was undertaken. The NaOH was generated from agent decontamination operations and surplus NaOH from a tank farm demolition project located in the North Plants area. RMA treating the NaOH on-site is a good management in two aspects; there is no future liability with off-site disposition and a cost saving of approximately $40,000.

Assessment/Management Support at the Rocky Mountain Arsenal:

Assistance continues to be provided to the Army in the Record of Decision (ROD) implementation, by participating and supporting the Army=s position in the groups such as the Biological Advisory Subcommittee (BAS), the Supplemental Field Study, the Biomonitoring Technical Review Group, the Medical Monitoring Advisory Group, and Policy for Old Samples committee.

Environmental Testing for Biological Threat Agents at U.S. Army Medical Research Institute of Infectious Disease:

Plate/probe based PCR-EIA (Enzyme Immunoassay) for *Yersinia pestis* identification continued. Conditions for the plate/probe assay were optimized. Variables considered were biotin-labeled probes (MSI001 @ MSI002) both which targeted the genetic locus plasmidogen activating factor, concentration of DIG-labeled PCR product, length of hybridization, conjugation and substrate incubations. A positive control was cloned from a strain of *Yersinia pestis* known as K25, with optimum concentration determined. This project is in the final stages until unknown samples arrive. Upcoming project will be a plate/probe based PCR-EIA for identification of *Brucella.*

Experiments were conducted to optimize the polymerase chain reaction conditions for use with inner and outer primer sets (BS5/BS3-1 and BS5/BS3-2) specific for *Bacillus subtilis* var. *niger* (*Bacillus globigii*). During this process, various $MgCl_2$ and primer concentrations were examined as well as multiple annealing temperatures. In addition, nested PCR sensitivity studies have been performed using these primer sets. Specificity experiments are being planned. Testing of domestic biotreaty samples obtained from Dugway Proving Grounds, Utah, has begun. Three isolates extracted from these soil samples have been confirmed as *Bacillus subtilis* var. niger through PCR to date. Experiments are also being done to determine the sensitivity of various soil extraction procedures. The aspect which is currently being examined is the effect of lysostaphin on *Bacillus anthracis* spores and vegetative cells during the soil extraction procedure.

Training was received in Class II Type A BioSafety cabinet use. The special immunization program was also completed clearing entry into BL-3 units. The Bio-Treaty laboratory was prepared for an external program audit conducted by LTC Roy. This audit was conducted over the course of two weeks and consisted of a complete review of the Bio-Treaty program; including administrative, personnel, scientific, and quality control issues. The Bio-Treaty continues to evolve toward a quality system and has adopted ISO-9001 guidelines as the standard under which it will operate. A senior military briefing was prepared on the status of the Bio-Treaty lab. The briefing was presented to COL Jaax of the Bio-Treaty Office. Col Jaax replaces LTC Estep as the new Bio-Treaty Office administrator. Additional components were added to the ISO-9001 quality system under which the laboratory will operate. These necessary components include a chemical inventory system, guidelines for preparing assay validation plans, guidelines for preparing test plans, and a system for conducting and recording equipment use and calibration. Over thirty Standard Operating Procedures have been prepared and submitted to the Branch Chief for review. Bio-Treaty Lab SOPs are divided into functional categories, including administrative SOPs, sample preparation SOPs, sample analysis SOPs and quality control SOPs.

Meetings were attended to bring new technologies to the Special Pathogens Branch and ultimately, the Bio-Treaty Lab. Exchanges occurred with John Hopkins University's Applied Physics Laboratory (APL) and with Argonne Laboratories. The joint venture with APL will involve the latest mass spectrometry technology. Argonne will utilize the MicroChip technology. Both technologies have application to threat agents detection and directly impact the mission of the Bio-Treaty lab.

A policy statement was prepared for the dual use of the Clinical Microbiology Laboratory (Clinical Pathology Branch, DSD). This shared laboratory will be used, in part, to help fulfill the mission. This policy statement was critical for outlining primary and secondary functions of Room 410 (Building 1425) in preparation for the upcoming accreditation inspection by the College of American Pathologists (CAP). A report was also prepared for International Technologies, Inc. (IT) addressing final disposition of evidence tags and samples from collected submunitions at Wright-Patterson Air Force Base. This final report was coordinated with LTC Hadfield, Microbiology Division Chief, Armed Forces Institute of Pathology (AFIP). The Wright-Patterson Air Force Base Burial Site-1 remediation project is now completed.

The Special Pathogens Branch was represented at a meeting concerning the renovations to the new BL-3 facilities in building 1412. Numerous maintenance and logistic issues have delayed opening the Bio-Treaty Lab in room 212, building 1412 until June 1, 1997. In the interim, the new facilities will be set up as a functional BL-2 laboratory for Bio-Treaty activities. The manuscript, "Isolation of *Mycobacterium abscessus* from Japanese medaka (*Oryzias latipes)*" by Teska, JD, L.Twedok, J. Beaman, M. Curry and R. Finch submitted to the Journal of Aquatic Animal Health is currently in revision. The poster "Analysis of M114 biologic submunitions unearthed at Wright-Patterson AFB" by Hadfield, T., E. Hilyard, M.E.

D'Nicuola, J. Ezzell and J. Teska was presented at the 21st Annual Meeting, Society of Armed Forces Medical Laboratory Scientists, Spokane, WA March 10-14, 1997 and also in Malaysia, March 1997.

APRIL 1 - JUNE 30, 1997

Investigation of methods available for identifying biomarkers of mammalian hepatocarcinogenesis for adaptation to fish models:

Approximately one-fourth of the work effort for this quarter was spent on Test 600-004 - Medaka Chronic Test with Bromodichloromethane (BrdU). Four BrdU exposures were done during this time period to assess hepatocellular proliferation in the medaka after receiving 1 day, 4 days, 6 days, and 20 days of chloroform. Tasks included test preparation, actual exposures, fish sacrifices, clean-up, scheduling with the histopathology lab (EPL), and test-associated record keeping.

Another portion of the work effort was to provide support for the investigation of microbial contamination of the laboratory water supply. Tasks included collecting, processing, and culturing of water samples as well as setting up test notebooks and binders, and writing the final draft of the SOP. A draft was written of the paper "An *In Vivo* Model for Using 5-bromo-2'deoxyuridine (BRDU) as a Marker of Chemically-Induced Hepatocellular Proliferation in the Japanese Medaka (*Oryzias latipes*)" for publication in *Fundamental & Applied Toxicology*. The paper is currently being reviewed by Maxine Boncavage-Hennessey and Dr. Hank Gardner (both co-authors).

Efforts continued in ordering supplies and setting up a lab in Room 112 for immunohistochemical staining. An abstract was written for the "Chemical Mixtures" meeting at CSU in August. A one-day training session for Handling of Hazardous Chemicals was attended and an external review being conducted at SAIC/NIH was participated in as a representative user of the services of the histopathology lab.

Research Conducted at Colorado State University:

The black and white Bioquant system sent to CSU was set up, calibrated, and is currently in use for macroscopic measurements. Several CSU students were trained in its correct use over a 2- day period and, thus far, no problems have been experienced. A rough draft of an SOP for the windows version of Bioquant was written and sent out for review and revisions. An SOP for the black and white system will be prepared in the next quarter. The scientific protocol written for the preliminary NF-Kβ was approved. The first exposure and nuclear protein extraction was performed at USACEHR, Fort Detrick, in June. Success or failure of the exposure will be determined in mid-July, when the electrophoretic mobility shift assay will be run at the CSU campus under the direction of Dr. Greg Cosma. Revision of the animal husbandry protocol was also done in order to include bleeding grass carp once a month to obtain serum. The serum will be used as a necessary supplement for the goldfish



macrophage line that is being sent from the University of Alberta, Canada. The remainder of the quarter was spent working with Dave Ufferfilge learning nuclear protein extraction and electrophoresis techniques.

Time was spent in literature research for GC-MS methodologies to detect melatonin and melatonin metabolites. Some of the samples for this study were run by the chemist at Fort Detrick. Time was also spent with Dr. Zang learning ESR/EPR techniques and providing him with training on the GC-MS. In cooperation with Dr. Cosma=s laboratory, an experiment was conducted exposing human lung cells to grain dust extract and melatonin to determine if melatonin has any effect on DNA damage measured by GC-MS and FT-IR. FT-IR analysis revealed a significant difference between control vs. grain dust exposure and grain dust exposure vs. grain dust + melatonin exposure. Control and melatonin control samples were similar. There were insufficient amounts of DNA to make GC-MS analysis possible, therefore cells were grown to repeat the experiment. When enough cells had grown for the experiment, they were harvested and DNA extracted only to find that the extraction procedure had produced poor quality DNA and could not be used. Assuming that the enzymes used in the extraction procedure were bad, new enzymes were ordered and the experiment will again be repeated.

Several discussions were held regarding the upcoming deadline for proposal submissions to the US Army Breast Cancer Research Program. Contacts were made with Dr. Jan Hess in Seattle, Washington, who has available a store of 600 breast aspirate samples for research purposes. It was discussed that these samples could be used for DNA analysis, estrogen metabolites analysis and genotyping. Dr. Hess also referred to Dr. Ben Anderson of the Fred Hutchinson Cancer Research Center in Seattle as a contact. The initiative was taken to assist Dr. Cosma in gathering background materials for the grant proposal. A decision was made to also submit a grant proposal on the analysis of human breast milk in conjunction with the aspirate samples. Literature searches were conducted and a contact was established with the laboratory of Dr. Kadlubar of the National Center for Toxicological Research. Contacts here proved very useful and a method for the extraction of DNA from human breast milk was found. It was also discovered that Dr. Kadlubar=s laboratory was planning to submit a proposal to the Army Breast Cancer Program on adduct analysis of human breast milk. This presented a very competitive nature to proposal plans and after much discussion, it was decided to abandon the human breast milk aspect of the research proposal. By early June, however, the decision had been made to postpone submission of a grant until some preliminary laboratory data could be generated. The idea of analyzing some aspirate samples with Dr. Anderson was pursued and six samples were shipped to the laboratory. Upon further scrutiny of the samples, it was discovered that the samples had been centrifuged and the cell pellet discarded. The end result being that the samples had insufficient quantities of cells to make a DNA extraction possible. After so many set-backs with this project, the parties involved at CSU have lost most of their enthusiasm and the focus of current work has shifted to the melatonin project until further notice.



Studies of chemical carcinogenesis in medaka:

A medaka 96-hour flow-through LC50 test with dibromoacetic acid (DBAA) was planned and implemented. Doses tested included 52, 86, 144, 240, and 400 mg/L. The highest dose of 400 mg/L was determined during range finding experiments in March. This was the highest dose of DBAA with a pH above 6, the lowest acceptable pH for medaka. No treatment related mortality was observed. The length and weight measurements will be analyzed to determine if a no observed effects level (NOEL) can be determined. The new diluter board was delivered and installed in Diluter 3. Two minor leaks were identified and fixed by the manufacturer. Familiarization with the new electronic timer was begun. Preparations were made and initiation began of the chronic test with Bromodichloromethane and medaka (Test 600-0040). Waste lines were hard-plumbed in Diluter 3. A new support system for the delivery lines was made with PVC pipe, which removed the angle iron from over the tanks. Approximately 1500 medaka were reared for this experiment and held in room 5 until randomization. Several modifications to the new diluter board were necessary to achieve a 3-minute cycle time, including the use of larger stainless steel lines as transfer lines.

A meeting was held with two representatives of US Filter to discuss the well room upgrade project. A tour was given of the facility and a quotes for various upgrades is expected. An abstract was submitted as a poster presentation for the meeting entitled "Current Issues on Chemical Mixtures," at Colorado State University, August 11-13, 1997. The title of the poster is "Integrated Toxicity Evaluation of a Contaminated Groundwater." Work continued on patent application for the aquarium drain assembly. Sections of SOPs needed for Best Practices Manual were collected and are being transitioned into a patentable generic format.

Teleost immunotoxicology methods development:

Range-Find LC50 for malathion in bluegill was established. LC50 and NOEL - based on NOEL toxicant exposures, will be set at 100 ppb (high concentration) and 25 ppb (low concentration) for malathion exposures. The *in vitro* immune function battery in bluegill was begun, however, during the acclimation period it was noted that dominance was established by the largest bluegill in each aquaria. The dominant bluegill showed aggression toward the subordinate fish in the tank, causing severe stress and physical damage. Overcrowding or the breeding season may have contributed to the behavior. Makeshift dividers were constructed out of heavy plastic screen and stainless steel wire and placed in each tank but proved inadequate to segregate each of the four bluegill. Since the fish had already suffered considerable stress, it was decided to discontinue the test until more permanent structures could be built in the tanks. The fish were euthanized with MS-222 and serum was collected. Dividers for the next test will be constructed of frosted plexiglass so that visual stress between fish will be minimized. Sufficient holes will be drilled in the plexiglass so that diluent water can pass unimpeded, keeping toxicant concentrations within the segregated chambers as equal as possible. Chambers will have diffusion holes cut in three of the partitions but not in the fourth to create a counterclockwise directional flow. The construction of these tanks is in



progress.

Work on the host resistance portion of the malathion assessment in bluegill began. A successful fish collecting trip to a nearby firepond in Middletown, MD, yielded 1000-1500 juvenile bluegill. Of these, 420 were selected and randomized into the 12 exposure aquaria at a stocking density of 35 fish/tank. Since caught recently, the acclimation period was extended to 10 days prior to the exposure, which will begin on Sunday, June 29, 1997. The exposure will last 21 days, and sampling will take place on day 7, 14, and 21; or day 10 and 21, depending on toxicant-induced mortality. All fish will be exposed to an - LD50 of *Yersinia ruckeri* as the challenge agent.

Methods development and standardization continued this quarter. An *in vitro* panel was run with the bluegill using assays methods optimized for medaka. The hydrogen peroxide assay was unsuccessful possibly due to outdated reagents or adjustment to the method may be needed. The lymphocyte proliferation assay's results were also inconclusive indicating the need for optimization for this particular species. The test showed that logistically, it is possible to run the panel successfully with bluegill. Optimization all assays in the test panel to bluegill will take place next quarter.

Health Screen #9 was completed for medaka. Support was given for initial Kappa B experiments in medaka consisting of exposure setup, randomization of - 225 medaka, and maintenance of fish for 1 week. The experiment was run at 1, 4, and 24 hr exposure times and nuclear material was extracted from head kidney lymphocytes following tissue collection. Fish were collected to obtain bluegill sera for experiments. Approximately 20 mature adult bluegilll were euthanized and bled from caudal peduncle. 23 mL of blood yielded 12 mL of sera. A course in advanced statistics entitled "Research Design and Data Analysis" was attended. Two abstracts were prepared for research to be presented at scientific meetings to be held in 1997. The first will be a poster for a conference on Complex Chemical Mixtures to be held at Fort Collins, CO, in August. The second is for the Society of Environmental Toxicology and Chemistry International Conference to be held in San Francisco in November 1997. In addition, a manuscript was prepared for publication in a scientific journal and is in the final editing process prior to submission. Work unit reports for immunotoxicology were prepared for Army Corps of Engineers in preparation for a user's group meeting. The reports detailed plans for basic and applied research in immunotoxicology, highlighting milestones and products expected.

Transgenic medaka research at USACEHR [in collaboration with Dr. James Burkhart at the National Institute of Environmental Health Sciences (NIEHS)]:

Tissue was taken from forty-nine medakas' fins to determine whether genes had been transmitted through the vector virus. Each fish was isolated into an individual chamber to await the screening results. The fin tissues were sent to Dr. Burkhart in NC. Dr. Burkhart electroporated approximately 100 medaka eggs in May which yielded approximately 70 fry. These new fish will be screened when they are about 3 months old.

Fifty of the 10/96 transgenic medaka were fin clipped in May. Of these, 2 fish screened positive, and the remainder were euthanized. Both of these fish were females, and they were placed in the same tank as the positive male from the 8/96 electroporation. Eggs were to be cultured from these animals to start a transgenic colony but the attempt was not successful. Of the approximately 110 eggs collected, none survived to hatch. The increased temperature and feeding regime for egg collection was discontinued, however, the eggs succumbed to fungal infection. An investigation is underway to screen a salt treatment for the eggs. The remaining 18 of the 10/96 medaka were fin clipped in June. Analysis of these clips is pending.

Transgenic animal research conducted at NIEHS, Research Triangle Park, NC:

During the quarter research focused on *in vivo* approaches correlating the use of alternative species with laboratory animal and cell culture models to assess mutagenic hazards from exposure to chemical or physical agents in the environment. Transgenic technology was applied to the study of induced somatic mutation directly at the DNA level using bacteriophage as the transgenic marker in rodents, fish and cultured cells where dose, adduction, DNA repair, and mutation can be compared. Experiments concluded that spontaneous mutation frequencies in a non-transcribed bacteriophage transgene is similar in mice, fish and cultured cells. Mutation in the target sequence can be induced in somatic tissues of fish and mice by treatment to an alkylating agent. Exposure effects of 7, 12-dimethylbenz[a]anthracene (DMBA) were examined through the analysis of cytochrome P4501A induction and DMBA metabolite levels after intraperitoneal injection with doses of 0.26, 1.9, 10 and 19 mg/kg in corn oil. Fish presented an increasing trend of ethoxyresorufin optical density assay (EROD) induction with increasing DMBA dose. There was no significant increase in the mouse CYP1A activity. The same metabolites were detected in fish upon exposure to DMBA, demonstrating the various effects of DMBA in two divergent study organisms. These results are indicative of the importance of examining the differences in response between sentinel species when evaluating the effects of polycyclic aromatic hydrocarbons. *In vivo* induced mutation in the transgene was also examined in mice and fish at 1.9 and 19.0 mg/kg doses of DMBA. There was a respective 2 and 11-fold increase in the mutation frequency over controls in fish; however, the effect of DMBA on FX174 in mice was not as significant. The 10 mg/kg data is incomplete at this time.

These studies represent the first evidence that identical gene indicators, detecting specific classes of chemically induced mutation, combined with analysis of biotransformation in various species may provide a mechanistic basis for comprehending correlations between laboratory species that may serve as environmental sentinels in polluted ecosystems. Experiments studying complex mixtures from contaminated ecosystems are beginning.

Methods development for rapid toxicity assessment:

The killifish were fed twice daily and the water quality parameters of hardness and alkalinity were obtained weekly. Two large populations of killifish were successfully hatched and are now developing into adult fish. Approximately 50% of the recovered embryos developed into adult fish in the most recent hatch attempt. Previous hatch attempts yielded only a few fry, which did not survive more than a week. The length of time in storage is most likely the most significant factor in determining a successful hatch. A collection of embryos was made from one of the new hatches with over 99% of the embryos appearing viable.

An information poster was redesigned and updated for two events at Fort Detrick and used at other information briefings.

Bluegill ventilatory monitoring project:

Seven bi-weekly reports were written and submitted for the ventilatory data collected at Old O-Field. All data was analyzed and archived for these reporting periods. The reporting period from 21 March through 18 April included critical response events that where characterized and discussed with Army Corps of Engineers and DSHE personnel in effort to better understand the events that occurred. A final report was written and submitted to DSHE. Revisions were made to Animal Use protocols for Old O-Field and in-house ventilatory studies and were submitted for approval. Titles of the protocols are" Ventilatory Biomonitoring of Treated Effluent at the Old O-Field Ground Water Treatment Facility" and " Establish the Sensitivity and Response Time of the Ventilatory Biomonitoring System to Chemicals Having Varying Modes of Toxic Action on the bluegill *Lepomis macrochirus*.

A number of trips were made to Old O-Field to work on the injection diluter system. Collaboration with Engineering Computer Optecnomics (ECO) was enlisted. After consultation, ECO will be redesigning the injection system to properly deliver the appropriate dosing of solutions. Old O-Field's problem with the effluent discharge light in the ground water treatment facility control room was investigated. It was determined that the flow switch was malfunctioning. The information was conveyed to the Ground Water Treatment Facility Operators and the problem was corrected. A number of trips were made to Aberdeen to provide training to the new part-time Biomonitoring Facility Operator at Old O-Field. Training in all ventilatory operations was provided. Assistance was given in the evaluation of an intermittent problem with the proportional diluters in room 10. Loose electrical connections were tightened in an effort to remedy the problem.

The ventilatory carbon filter was removed from the well room. The carbon is being replaced for upcoming ventilatory studies. Training on all aspects of performing a ventilatory test, including a validation study were covered with a new technician. A chamber was designed and built for use as a demonstration model. A "display" ventilatory signal amplifier was also built. The larger chamber will accommodate a larger fish than is normally used for a test to allow better viewing for presentations.

Preparations were made for the upcoming validation ventilatory biomonitoring tests. This included rebuilding the amplifier units and other associated equipment. Diagnostics were performed using the electronic fish simulator to verify that all ventilatory hardware was functioning properly. The scientific protocol for these studies was also revised. The first in the series of tests, Zinc Sulfate Sensitivity, was initiated on June 20. Toxicant was introduced the morning of June 27, 1997 and a response was observed in less than two hours. Additional data acquisition computers were connected to the ventilatory system in the small biomonitoring trailer for research being performed by Johns Hopkins University Applied Physics Laboratory. The collaborative efforts with the Applied Physics Lab will attempt to analyze whole ventilatory signal information for changes or variations that may be missed by the current ventilatory biomonitoring system, parameters that may be available for evaluation other than ventilatory rate, *i.e.* average depth, cough rate, and percent movement.

Analytical method development and analysis of chemicals used in carcinogenicity and mobile laboratory studies:

A method for the analysis of Dibromoacetic acid was developed using HPLC. Direct injection provided the detection limits required for a FETAX test and an exposure to medaka. This method eliminated extensive sample preparation required by gas chromatography to perform the analysis. Precision and accuracy data was completed and a stability study is planned. A method for the analysis of Melatonin and the metabolite (AMK) was developed for analyses to be performed at Colorado State University. Approximately 120 samples of Dibromoacetic acid and 100 samples of Bromodeoxyuridine were analyzed in support of toxicology testing by HPLC. Approximately 200 Thalidomide samples were analyzed by HPLC in support of an ongoing FETAX test. Several waste water samples from Aberdeen proving grounds were received due to a response in the bluegills. These samples were analyzed by HPLC, IC, and ICP-MS in order to determine if the response was chemically induced. The results were inconclusive as to what the causative factor may have been. Four water and two soil samples from Aberdeen Proving Grounds were analyzed for munitions.

Maintenance and optimization of USACEHR aquatic laboratory facilities:

Essential laboratory maintenance was performed on all medaka, bluegill, grass carp, and killifish located in rooms 5 and 18. Daily activities include three daily feedings of all culture and test organisms. Associated record keeping was performed to maintain necessary test records to meet SOP and regulatory review requirements. Associated tank cleaning and siphoning was performed to maintain overall tank hygiene necessary to accommodate healthy test organisms. Maintenance and culturing of all live food (brine shrimp and microworms) systems continued. Weekly activities include the following water quality analysis; dissolved oxygen, pH, conductivity, water flow measurements, ammonia, and light measurements. Monthly samples of well water were taken for TCE analysis.

Transgenic medaka test organisms were maintained in conjunction with ongoing inter-laboratory research with Dr. Jim Burkhart. Essential laboratory supplies were documented and ordered as needed for the quarter. The contaminant analysis of a new lot of frozen brine shrimp was reviewed. All parameters were below detection limits or within acceptable ranges. The medaka colony (1104 medaka) was renewed and eggs were cultured for one bath of fry for immunotox research. One batch of retired breeders showed a higher than normal mortality pattern. These fish were euthanized and not offered to extramural researchers.

A new water level sensor device for use in the water purification room, was put together and will soon be tested. These sensors are non-intrusive to the water reservoir, replacing the current system of using direct contact to the water. The new sensors should provide longer and more reliable service.

Fish Shipping Summary:

No. of Medaka	Institution	Location
1190	NYU	Tuxedo, NY
15	EPL	Herndon, VA
250	WVU	Morgantown, WV
2[a]	Cornell U	Ithaca, NY
18[b]	NIEHS	Research Triangle Park, NC

[a] tissue analysis of aged fish for possible lymphosarcomas
[b] frozen tail fin clips, live fish held at USACEHR

Support of the Environmental Engineering Division at Rocky Mountain Arsenal (RMA):

Health and safety support was provided for Tennessee Valley Authority (TVA) at RMA. TVA is currently performing chemical agent deactivation tasks for RMA. Assistance was given to the Department of Army (DA) and Chemical Agent Safety and Health Policy Action Committee (CASHPAC) regarding the approval of chemical protective clothing (CPC) to be used at CBDCOM facilities. The CPC will save CBDCOM facilities a considerable amount of money each year while meeting the intent of EO12196 and OSH the Act. Work continued in clarifying regulatory issues, such as, waste characterization, transportation, handling and disposal, hazardous waste and agent waste characterization and safe handling procedures. Innovated methods were researched for reclamation and disposition of waste materials, UXO site assessment requirements, and chemical agent waste management practices and policies. Technical guidance and training was provided to RMA employees for managing special waste. Assistance was given to the Army in conducting a chemical sweep of the Arsenal to remove all remaining chemical items and chemical substances from various

structures and process facilities. RMA Engineering Technicians are receiving training regarding safe chemical handling (including neutralizing and stabilizing), chemical classification, chemical segregation, SW-846 sampling methods (standard methods), chemical hazcatting, chemical, chemical packaging, and selecting a Toxic Storage Disposal Facility (TSDF) for chemical disposition.

Technical support was provided to RMA engineering technicians regarding day-to-day field operations pertaining to chemical classification, chemical segregation, collecting representative samples, packaging chemicals per DOT specifications, chemical profiling, and arranging for disposition. Technical review of TVA's chemical agent waste management operations was provided. Technical support was provided for the 60% design of the slurry wall around the Army complex trenches. On-site technical assistance will include; ambient monitoring for chemical agent, health and safety oversight, and emergency response support for chemical agent incidents during site activities. Coordination and arrangements were made for the on-site treatment for 20,000 gallons of sodium hydroxide (NaOH) at RMA Waste Water Treatment Facility.

Assessment/Management support at the Rocky Mountain Arsenal:

Assistance is given the Army in the Record of Decision (ROD) implementation, by participating and supporting the Army=s position in the Biological Advisory Subcommittee (BAS) which is tasked with looking at the effects of remediation on the biota, identifying areas of additional surficial soil contamination, as well as reviewing proposed biota studies and checking them for scientific soundness. The State=s Dioxin study continues to consume much of the BAS efforts. Upon committee's approval of the BAS recommendation to do additional analyses of biota on and off post, the BAS initiated the Dioxin/Furan Study. Tissue samples are being sent to Clemson Laboratory for the H4IIe assay. A select group of samples will be sent to another lab for the PCDD/PCDF analyses. The Supplemental Field Study Phase I continues to be evaluated by the BAS. The BAS has reworked the models predicting residual risk by using GIS mapping. The BAS also completed the effort of working with the RMA Borrow Area Group to incorporate areas of high risk into areas needed for borrow. The Biomonitoring Technical Review Group continues to identify gaps or areas where the USFWS exceeds the needs of the Army for a Biomonitoring Program. This group will also be evaluating future studies proposed by the USFWS. The Medical Monitoring Advisory Group now meets bi-monthly to go over the work products of the subgroups. Assistance is given to the Army by sitting on the Baseline Health and the Human Health subgroups, which meet every Monday evening. This group is currently working on a document to outline the communities baseline health, and what to do in case of exposure to the community during remediation. Work product currently produced by the group are the Birth Defects Registry Plan and the Physician Referral Plan. Work with the Policy for Old Samples group involves interaction with Army lawyers looking at the possibility of disposing old biota samples.



Environmental testing for biological threat agents at U.S. Army Medical Research Institute of Infectious Disease:

The Bio-Treaty Laboratory had several opportunities to test its capabilities with actual environmental samples. One such exercise consisted of isolating surrogates of threat agents from domestic soil samples. Personnel from the Bio-Treaty Laboratory gained first hand experience through the analysis of these samples. Reports of all findings were prepared and submitted to the Biological Arms Control Treat Office (BACTO, Ft. Detrick). The Bio-Treaty Laboratory analyzed Iraqi soil samples as part of an UNSCOM verification mission. Soils were analyzed for aerobic and anaerobic spore-forming bacilli. Data was compiled and presented to the United Nations in June 1997. These samples provided an opportunity to test the administrative and technical aspects of the Bio-Treaty Laboratory. Experience was gained in maintaining chain-of-custody, sample storage and archiving, sample distribution, data recording and reporting, and the technical aspects of sample extraction and analysis.

Maintaining and operating a BL2 clinical microbiology laboratory was incorporated into the overall strategic plan for Bio-Treaty Laboratory readiness. Consequently, experience was gained through actual clinical specimen workup in support of the clinical laboratory. Routine cultures and sensitivities were processed according to physician request. The processing of clinical specimens broadens the scope of the Bio-Treaty Laboratory and provides an excellent opportunity to maintain competency in clinical microbiology. A written policy statement was prepared on the use and priorities of the clinical microbiology laboratory. The laboratory supports patient care, but also provides BL2 space for the processing and analysis of Bio-Treaty samples. The policy clearly defines both missions. Three standard operating procedures (SOP) were prepared and approved in support of the clinical microbiology laboratory (Clinical Pathology Branch, DSD). The laboratory operates under the College of American Pathologists (CAP) certification, and helps support the mission of the Bio-Treaty Laboratory.

A written document for the 121st General Army Hospital (Seoul, Korea) was prepared on *Bacillus anthracis* and the clinical aspects of anthrax. Information will be used as a guideline for establishing the clinical capability of isolating and identifying *B. anthracis* from patients in Korea.

Respirator Training was received in April and blood-borne Pathogens Training in May.

Experiments have been conducted to determine non-specific binding of a variety of *Bacillus anthracis* specific primers with *Bacillus subtilis var. niger (Bacillus globigii)* samples. In addition, analysis of isolates obtained from international samples has begun. The identity of a threat agent has been confirmed in multiple samples through PCR. Additional analysis is underway. An investigation of the effect of the Molecular Probes, Inc. BacLight Viability Stain is also being undertaken.

Plate/probe based PCR-EIA (Enzyme Immuno Assay) for *Brucella* identification was begun. Optimized conditions for both PCR and EIA are complete. A positive control for *Brucella* was cloned from a *melitensis* strain and detection limits for this assay determined. Lacking strains of *Brucella* to test this system, DNA was acquired and extracted from 26 different *Brucella* strains that are currently being examined. Robotics were applied to the EIA portion of the detection where a comparison of automation and manual labor to the sensitivity of the positive control was made. Two robotic systems were also compared to one another for reliability and to determine which would be the better suited to the needs of the laboratory. The *Yersinia pestis* project is on hold until unknown samples analysis is completed.

Access to the BL-3 lab was granted, and introductory training was completed. Respiratory clearance was granted upon completion of fitting and use of a personal respirator. Training for use of bloodborne pathogens and a powerpoint course was completed. A presentation was made on Nucleic Acid Isolation and Separation to the DSD division. A summer student was given in-processing, training on procedures in the lab and guidance through his projects. A seminar was attended given by Perkin Elmer/Applied Biosystems entitled ANew Developments in Genetic Research: PCR-Based Fluorescent Discovery, Detection, and Quantitation Technologies@ at the University of Maryland. An abstract was submitted to the American Society of Tropical Medicine and Hygiene entitled ADetection of *Yersinia pestis* by Polymerase Chain Reaction Enzyme Immuno Assay (PCR-EIA)".

A project has been initiated aimed at developing an assay for the quick detection of *Staphlococcus aureus* enterotoxin A and B (SEA, SEB) and *Chlostridium botulinum* toxins A, B and E. A working assay for SEA and SEB has been developed and is awaiting final testing of experimental samples. This test is based on the PCR, followed by ELISA of the amplified product. It has not been possible to test the assay on Cbot because there is no amplifiable material in-house. Paperwork has been initiated for the transference of this material. Work is expected to be completed on this project in the next quarter. The following project planned is designed to quickly identify the various strains of Orthopox, using PCR and ATaqman=, a technique developed by Perkin Elmer to diagnostically identify large pieces of amplified DNA. This assay is already up and running in-house and little trouble is perceived in adapting it to this project.

JULY 1- SEPTEMBER 30, 1997

Investigation of methods available for identifying biomarkers of mammalian hepatocarcinogenesis for adaptation to fish models:

Approximately one-fourth of the work effort for this quarter was dedicated to the development of an apoptotic staining procedure adaptable to formalin-fixed medaka tissue. This was accomplished by modifying the procedures used in an apoptotic kit designed for mammalian tissue. The work is based on previous modifications of this procedure that had been performed by a graduate student working in the lab in 1995. The stain will be used as a biomarker to look for apoptotic activity on medaka slides received from the water disinfection

by-product studies. Another quarter of the work effort was spent on preparing data and slides for the" Environmental Complex Mixture Toxicity Assessment" presentation given at the Current Issues on Chemical Mixtures symposium held at CSU at Fort Collins, Co., Aug 11 - 13, 1997. A rough draft of the presentation was begun for publication in the Environmental Health Perspectives journal. Efforts continued in sampling and culturing of laboratory water for microbial monitoring (Test 105-001). Finalizing of Bioquant hardware installation and imaging procedures was accomplished in preparation of BrdU and apoptotic biomarkers evaluation for water disinfection by-product slides (Tests 100-006 and 600-004). These slides will also be sorted to select out unstained tissues from these same studies received back from histopathology for BrdU and apoptotic staining.

Research Conducted at Colorado State University:

Much time has been spent attempting to obtain a viable fish macrophage line for use in future experiments since live animal facilities in Colorado are not a viable option at this time. Because the amount of nuclear protein sample brought back from the LPS exposure experiment performed at Ft. Detrick is diminishing quickly, efforts are underway to bring in two established fish lines from other laboratories: a carp monocyte line from Dr. Ahne in Munich, Germany, and a monocytic line derived from a marine species from Dr. Faisal=s lab at Virginia Institute of Marine Science (VIMS). Carp being housed at Fort Detrick for serum to use with goldfish macrophages were sacrificed. At the time of sacrifice, the fish were bled and the serum spun down, frozen and sent to the Colorado laboratory for future use.

The first electrophoretic mobility shift assay (EMSA) was run using the fish extracts that were acquired at Fort Detrick in June. The data from the EMSA, which was run using a mammalian probe for NF-Kb, suggest that NF-Kb is present in the medaka with the most remarkable time point being one hour post-exposure. The density difference was measurable between the control and the exposed groups at this timepoint. A super-shift assay was expected to confirm that the band appearing in the lanes with the fish extracts is definitely NF-Kb rather than non-specific binding of fish proteins. The results of the supershift were inconclusive, however. The banding pattern seen on the supershift was somewhat indicative of NF-Kb, but diffuseness of the banding pattern from suspected protease activity made it was difficult to determine exactly where the bands were occurring. Several additional attempts to perform the assay were made to validate the preliminary results seen with the electrophoretic mobility shift assay. Due to unsatisfactory probe labeling and a problem with protein concentrations, both attempts were canceled and rescheduled. Methods in the correct preparation and pouring of polyacrylamide gels were acquired. This work was initiated to determine if fish do indeed have NF-Kb and whether this transcription factor is as involved in fish immune responses as it is in mammalian immune responses. Though no thoroughly conclusive results were obtained, the preliminary studies do demonstrate the presence of a nuclear protein in the fish that appears very similar to the NF-Kb protein found in mammals.

Components for the Bioquant continue to arrive and a reorganization is underway in the Bioquant room in order to accommodate all equipment while maintaining efficient work space.

A problem still to be resolved with the bioquant is the electronic malfunction of the fluorescent lamp. An outline was devised for research describing several possible experiments that could be done to look at NF-Kb, AP-1, heme-oxygenase and heat shock protein interactions in fish macrophages after environmental stimulation. Comparison methods with cells from mammalian species were also investigated.

On July 16th and 17th, the Deployment Toxicology Science Workgroup was attended at CSU to discuss science that is currently being pursued in the area of deployment toxicology. The meeting was composed of scientists from academia as well as from all branches of the service. The Chemical Mixture Meeting was attended in August. This meeting was very informative and touched on several aspects of the ramification of results obtained when working with chemical mixtures as opposed to single chemical exposures. The discussions were very applicable to the work being done by GEO-CENTERS and the Army and provided several ideas for future projects. Computer problems required attention when the floppy drive malfunctioned taking information from the windows file that prevented access to any windows programs without a complete reinstallation. After receiving two replacement drives, one also being defective, the computer was nearly restored. The main bioquant computer with the TCW (color) system also developed major problems when the imaging chip died and the entire MFG board had to be sent to R&M Biometrics for chip replacement. This left only the black and white system operational. Other accomplishment for the quarter were the collection and preliminary tabulation of the methylene chloride data for the first three of four time points.

The Colorado State University=s Module 0 Radiation Training course was attended. A student was trained in the DNA extraction protocol using THP lung cells. Dr. Zang also provided several samples from his melatonin studies. Unfortunately, the samples were unable to be analyzed due to major software configuration problems with the GC-MS. Hewlett-Packard was contacted and a site visit was made, at which time the technician determined that all software had to be reloaded. After reloading the software the system worked correctly. Two weeks of training were provided to Dr. Zang and a studen including the the three-day preparation and analysis processes that are required for these samples. Repeated runs of the samples were also made to insure accuracy.

A presentation was made at a meeting at Lake Chelan, Washington. A visit was also made to the Pacific Northwest Research Foundation in Seattle, Washington. Standards were lost from the -80°C freezer in the Physiology Building. To avoid a recurrence of this kind, all standards kept at low temperatures were moved to Dr. Cosma=s freezer that had recently been cleaned and reorganized. A major effort was expended toward writing text, preparing figures and preparing a poster presentation for the NATO Meeting in Turkey. Several editorial problems with Dr. Malins and Dr. Polissar (co-authors) were resolved. An oral presentation was also requested at the meeting which required preparation of seven slides for the presentation. The injector tower on the GC-MS malfunctioned and several days were spent trouble-shooting the problem. An extra tower from another instrument was borrowed to avoid interruption of analyses. The problem has been narrowed down to the plunger belt or the motor sensor. Many hours have been spent in literature searches netting over 60 separate

manuscripts for study

Studies of chemical carcinogenesis in medaka:

The six month interim sacrifice was performed for Test 100-006, medaka carcinogen test with chloroform. Twenty fish each from sixteen tanks were euthanized and fixed in Bouin's solution for histopathology. An additional five fish per tank were analyzed for hematocrit, leucocrit, cell viability, and cell count. Results of the blood and cell analyses show that treated groups are very similar to control groups. Preparations were made for the nine month sacrifice. Diluters for the chloroform and BDCM test continued to function without any outages or major malfunctions. 1280 fry were randomization for the BDCM test which will be on-line for nine months. Weekly samples from each tank were pulled and submitted to the chemistry lab with good results. The investigation into nanofiltration for the well room was completed. The findings indicated that the improvements to the system of increasing usable water volume did not outweigh the potential detriments of differing line pressures, possible new pump requirements, different mix of raw and processed water, and potential retrofitting obstacles. The softener was still needed for optimal membrane life, so the labor/dollar savings from phasing out the softener would not be realized by changing to nanofiltration. In order to handle increased water volume, the UV Sterilization Unit would require an upgrade to a larger unit. The decision was made to replace the reverse osmosis membranes with more of the same type membranes. As well water use has increased, the UV disinfection system has been pushed to its upper limit. A work order has been recommended to unite the two UV units in series. This should improve bacterial killing capacity and reduce the bacterial load to the well water users.

Two posters were prepared and presented on research performed by USACEHR at the conference entitled "Current Issues on Chemical Mixtures" held at Colorado State University, August 11-13, 1997. Titles of the posters were: "Integrated Toxicity Evaluation of a Contaminated Groundwater" and "Assessment of the Teratogenic Potential of two Simulated Water Samples using the Frog Embryo Teratogenesis Assay." The integrated toxicology poster presented results of a multi-test study performed on contaminated groundwater at the West Branch Canal Creek site at Aberdeen Proving Grounds, Edgewood, MD. The FETAX poster presented results from tests run on simulated groundwater from Iowa and California.

Supplies were ordered for the two remaining chronic carcinogen tests. The waste drainage system for diluter two was rebuilt using fixed PVC lines within the diluter enclosure. The support for the tank supply lines was changed to PVC in a continuing effort to remove angle iron from inside of the diluter enclosures. A quality assurance audit was performed on data from J-Field, Aberdeen Proving Grounds. The raw data was compared to a set of generated data tables. A summary report of findings was issued to the COR for the project.

Teleost immunotoxicology methods development:

Range-Find LC_{50} experiments were performed using the fungicide pentachlorophenol in the bluegill sunfish and Japanese medaka. Concentration ranges were set for the exposures from historical published data. The LC50 and NOEL (No Observed Effects Level) for pentachlorophenol were mathematically established using probit analysis (SAS) and other appropriate statistical procedures. Toxicant exposure concentration is based on NOEL calculations, exposure duration, and historical relevant environmental data. The calculated NOEL range for medaka was calculated to be 0.68 -0.71 mg/L; for bluegill, 0.11 -0.14 mg/L. Host resistance studies were undertaken using juvenile bluegill exposed to malathion. The bluegill were exposed to malathion at 0.20 and 0.050 mg/L for up to 21 days. Concentrations proved to be too high for the survival rates required for immunotoxicity testing. The study was repeated using 0.060 and 0.015 mg/L malathion for the same exposure duration (samples @ day 10 and 21). Minimal mortality occurred in the high dose (1/120 - 0.8%), and no mortality was observed in low or control fish. Although cumulative mortality at day 10 was higher in low and high doses than in control fish, the difference was not significant ($p = 0.07$). Statistically significant differences were observed for cumulative mortality rates between control, low and high treatment groups at day 21. Based on the observed results, the data indicates that malathion suppresses the host's (bluegill) ability to resist pathogenicity caused by experimental infection of *Yersinia ruckeri.* Future functional assays will further define mechanisms of immunomodulation.

Host resistance studies were undertaken using adult medaka (10 months) exposed to pentachlorophenol. Medaka were exposed to pentachlorophenol at 0.215 and 0.050 mg/L for up to 21 days. Fish were sampled at days 10 and 21. Mortality was below 1.5% for all groups, exposed and control. A statistically significant difference between control and treatment groups was observed on both the day 10 and day 21 sampling. Based on the observed results, the data indicates that pentachlorophenol suppresses the medaka's ability to resist pathogenicity caused by experimental infection of *Yersinia ruckeri.*

The chemical mixture meeting at CSU was attended and a poster was presented detailing the use of the bluegill as a feral model for immunotoxicological research aimed at hazard and risk assessment of complex chemical mixtures.

Transgenic medaka research at USACEHR [in collaboration with Dr. James Burkhart at the National Institute of Environmental Health Sciences (NIEHS)]:

Bath #3 in culture room 5 was prepared for fin clipping of transgenic fish for Dr. Burkhart. The remainder of the 10/96 hatch of transgenic medaka were screened and found to be negative. These fish were euthanized. Current inventory of "transgenic" medaka is two confirmed positives that are being used as breeders, approximately 70 fry from eggs electroporated in 5/97, and two sets of potential F1 generation hatched from the positive parents: approximately 40 fry of 8/97 hatch and approximately 50 fry of 9/97 hatch. Out of six electroporations of 2827 medaka eggs, 865 fry have resulted, of which five fish were



positively transgenic.

Transgenic animal research conducted at NIEHS, Research Triangle Park, NC:

Research has focused on *in vivo* approaches correlating the use of alternative/natural environmental species with laboratory animals and cell culture models to assess mutagenic hazards from exposure to chemical or physical agents in the environment. Transgenic technology was applied to the study of induced mutation directly at the DNA level using fX174 bacteriophage as the transgenic marker in rodents, fish and cultured cells where dose, adduction, DNA repair, and mutation can be compared. The latest work has been concentrated on a few control samples to understand the natural mutation rate. Along with each sample, three digests were performed. From each digest, three to four platings were made to determine mutation frequency. Experiments concluded that spontaneous mutation frequencies in a non-transcribed bacteriophage transgene is similar in mice, fish and cultured cells. Mutation in the target sequence can be induced in somatic tissues of fish and mice by treatment to an alkylating agent. Exposure effects of 7, 12-dimethylbenz[a]anthrocene (DMBA) were examined through the analysis of cytochrome P4501A induction and DMBA metabolite levels after intraperitoneal injection with doses of 0.26, 1.9, 10 and 19 mg/kg in corn oil. Fish presented an increasing trend of ethoxyresorufin optical density assay (EROD) induction with increasing DMBA dose. There was no significant increase in the mouse CYP1A activity. The same metabolites were detected in fish upon exposure to DMBA, demonstrating the various effects of DMBA in two divergent study organisms. These results are indicative of the importance of examining the differences in responses between sentinel species when evaluating the effects of polycyclic aromatic hydrocarbons. *In vivo* induced mutation in the transgene was also examined in mice and fish at 1.9 and 10.9 mg/kg doses of DMBA. There was a respective 2 and 11-fold increase in the mutation frequency over controls in fish; however, the effect of DMBA on fX174 DNA in mice was not as significant. 10 mg/kg evaluation will be completed during the next quarter.

These studies represent the first evidence that identical gene indicators, detecting specific classes of chemically induced mutation, combined with analysis of biotransformation in various species may provide a mechanistic basis for comprehending correlations between laboratory species that may serve as environmental sentinels in polluted ecosystems. Experiments studying complex mixtures from contaminated ecosystems are beginning.

Methods development for rapid toxicity assessment:

Research and culturing continues with killifish. Attempts to improve holding methods to produce the maximum number of viable embryos has been the primary effort. Other parameter being studied are length of embryo holding time and moisture content of the storage media. This information will help to improve recovery and hatch rates of the Killifish and determine an Aexpiration date@ for the Killifish Rapid Toxicity Assessment test kit. The *Triops longicaudatus* (Triops) is being developed as a potential organism for rapid toxicity assessment. Triops eggs are collected daily and a record is kept of the number of eggs



collected from each aquarium. Much literature review has been performed to obtain information relevant to the *T. longicaudatus*. Currently research is targeted at developing the culturing of the organism from laboratory collected eggs. Several hatch attempts have been initiated using laboratory collected eggs. Each hatch produced live organisms, none of which lived longer than 1 week. The most recent hatch yielded over sixty live Triops. This hatch has been divided into groups of five *T. longicaudatus* per group. Each group is receiving a different assortment of algae, flake food, micro worms, and protozoa. These foods were selected based on information found in the literature. Groups are fed and receive a water change twice daily. No mortality was noted in the first 24 hours after hatch. Less than 10% mortality was noted after 48 hours. A total of 25 triops were still living after one week. An additional 12 organisms are still alive that were hatched on the second day after water initiation. Literature searches have also begun to yield information on other organisms suitable for addition to the rapid toxicity test battery. Two information posters have been converted to electronic format. One has been printed and is currently in place at Aberdeen Proving Grounds.

Bluegill ventilatory monitoring project:

Bi-weekly reports were written and submitted for the ventilatory data collected at Old O-Field for four reporting periods. All data was analyzed and archived. More sensitive analyses were also performed on the data set from 27 June through 11 July due to a report of mustard breakdown agent that was detected in the bi-weekly chemical analyses. No distinguishable response was noted with the lower sensitivity analyses when compared to the control fish. It was later determined that the chemical detection was a laboratory error and that no mustard breakdown agent was present in the effluent water. Bi-weekly changing of fish at Old O-Field was accomplished on 25 July. Ventilatory programming and bi-weekly test maintenance were also performed. Efforts were made to replace a Hydrolab dissolved oxygen probe for use at Old O-Field.

Remote monitoring of Old O-field ventilatory response information continued. Mortality was noted in three and possibly four fish in effluent on the morning of 16 September. These fish had red-lined on 15 September but did not remain red-lined because recorded (false) ventilation rates rose above 9 ventilations per minute. Discharge continued to occur during this event since ground water treatment facility operators did not effectively diagnose the fish as dead. Operators were immediately notified of the possible mortalities and discharge was stopped. Upon consultation with Mr. Tommy Shedd, Mr. Dennis Fisher and Mr. Dennis Powers of the Army Corps of Engineers it was decided that the mortality events were possibly related to the calibration of the treatment facility dissolved oxygen probe. A trip to Old O-Field that same day verified that fish were actually dead and the decided course of action was to place the control fish on effluent water to assess their level of response to the effluent water. Samples taken by the automated water sampler were also collected and returned to USACEHR for immediate metals analysis. As a result of this event, the Aquatic Biomonitoring Program is under alteration to maintain a redline for fish recording a ventilation rate less than 9 for the duration of the test period.

Three ventilatory validation studies were completed during the quarter. Zinc and MS-222 Sensitivity Studies were performed with both indicating unusually high levels of control response. A third ventilatory validation study was performed with Phenol. The study was prematurely terminated due to a well system shut down. No control response issues had occurred with the Phenol study at the time of termination. A fourth ventilatory study was performed using control water. Previous tests indicated an excessive number of fish deviating from their baseline patterns, especially the control fish. In addition to this study, trend analysis of ventilatory length and weight was compared to level of response for all bluegills used as control test organisms. No noticeable correlation was made between the size of bluegill and group response information. It was initially hypothesized that smaller fish created more of a control group response. More definitive analyses are planned for the next quarter. Collaboration with Dr. Charles Sarabun and Mr. Robert Chaulmers of the Johns Hopkins Applied Physics lab was formed. Information pertaining to ventilatory test specifics were sent to Dr. Sarabun via E-mail. Further collaboration will be facilitated with APL on the test information in the upcoming months. Training was also provided on the procedures for data analysis and graphical preparation of ventilatory response and water quality information. A problem with the APL data acquisition system was diagnosed as a bad data acquisition board which was corrupting signal transmission of raw signals to the ventilatory data acquisition system. The problem was reported to Dr. Sarabun. The APL system was disconnected from the ABP system until APL can correct the problem.

Report drafting and review of the annual report of Old O-Field ventilatory response data for April 1996 through March 1997 was finalized with the assistance of summer student, Mr. Robert Mitchell. The first draft report was submitted for government review. Statistical analysis and graphical summaries of the ventilatory response information were generated in conjunction with the bluegill behavioral studies performed on the Canal Creek groundwater by Ed Little of USGS Midwest Science Center. New ventilatory capacitors were tested using the electronic fish signal generator and also to ensure that the capacitors performed the same as previously existing ventilatory capacitors before installation onto the new ventilatory amplifier system. A cost savings of $730 per test system was expected using the new capacitors. The new capacitive liquid level system was installed on the water storage tank in the well room. This sensor system utilizes non-intrusive sensors to control water levels. They will eliminate corrosion and calcium carbonate electrode plating problems that were encountered with the old electrode system. Similar systems will be installed at the Old O-Field Biomonitoring Facility and in the Biomonitoring Trailer. New ventilatory amplifiers were prepared for shipment to Dr. Robin Hough of the Great Lakes Institute of Systems Research in Rochester, Michigan. A trip was taken to the Great Lakes Institute to transfer fish ventilatory biomonitor technology. The current USACEHR ventilatory biomonitoring system was effectively set up and demonstrated there. This exercise allowed Dr. Hough and his staff to understand how the ventilatory biomonitor works and to assist technical staff in understanding the configurational complexities that exist in integrating the ventilatory biomonitor with other test systems that will be utilized for monitoring efforts at Lake St. Clair. Ideas were exchanged about the possibility of converting the Aquatic Biomonitoring Program to a windows-based environment (Windows NT or UNIX). This would allow a more functional incorporation of all test information into

an operable real-time data format. Computer specialist John Harney was very optimistic that the system could be converted to a suitable windows environment. A ventilatory data acquisition system was also assembled with the assistance of Mr. Jeff Leach for use at the Great Lakes Institute of Systems Research.

A press release of the report and recommendations from the Macomb County Blue Ribbon Commission on Lake St. Clair was attended. The report and release outlined the current status of Lake St. Clair and future efforts that have been recommended. Included in the report's drinking water action plan was the utilization of continuous biological monitoring by local, state, national, and international agencies of government. The report also included the use of continuous biomonitoring of industrial discharges in the state of Michigan. The Commission recommended the creation of a Water Quality Board in the Macomb County Health Department to take the lead in protecting the waters of Lake St. Clair through the recommendations they have outlined in their report. The ventilatory biomonitoring system was also demonstrated for Jay Dosenberry of TARDAC. Possible collaborative research efforts were also discussed in relation to the Lake St. Clair project.

A new ventilatory dosing diluter was developed by ECO. A trip was taken to Annapolis, Maryland for training and to transport the new diluter system to USACEHR for further validation studies. Laboratory studies validated the system's ability to be used for conductivity adjustment of a water source and to provide a 50/50 mix of different two water sources. A major limitation of the system is the need to maintain a constant water level in the two feed tanks of incoming water lines. The system is pressure regulated and any change in hydraulic head pressure has a variable effect on the dilution characteristics of the dosing system. Given a constant head the dosing system appears to provide a consistent level of dosing.

Analytical method development and analysis of chemicals used in carcinogenicity and mobile laboratory studies:

A method for the analysis of Dibromoacetic acid was developed using HPLC. Direct injection provided the detection limits required for a FETAX test and an exposure to medaka. This method eliminated extensive sample preparation required by gas chromatography to perform the analysis. Direct injection by HPLC will not provide the necessary detection limits (10 Fg/L) required for a nine month study involving the diluter tests at USACEHR. It is necessary to use the EPA method 552.2, Determination of haloacetic acids and dalapon in drinking water by liquid-liquid extraction, derivatization and gas chromatography with electron capture detector. Two trail runs with a set of standards plus high and low spikes and blanks were analyzed. Several revisions to the method were made including preparation of standards and the concentrations of internal standards and surrogates. The method is very labor intensive with two days of sample preparation for each set of samples estimated.

A method for the analysis of Melatonin and the metabolite (AMK) by HPLC was developed for analyses to be performed at Colorado State University. Ten dust samples were

digested and analyzed for trace metals by ICP-MS for CSU. Approximately 120 samples of Dibromoacetic acid, 40 samples of Phenol, 75 samples of MS-222 and 100 samples of Pentachlorophenol were analyzed by HPLC in support of toxicology testing at USACEHR. Forty waste water samples from Aberdeen proving grounds were received for analysis due to a response in the bluegills.

Maintenance and optimization of USACEHR aquatic laboratory facilities:

Essential laboratory maintenance was performed on all medaka, bluegill, grass carp, fat-head minnow and killifish located in rooms 5 and 18. Daily activities include three daily feedings of all culture and test organisms. Associated record keeping was performed to maintain necessary test records to meet SOP and regulatory review requirements. Associated tank cleaning and siphoning was performed to maintain overall tank hygiene necessary to accommodate healthy test organisms. Maintenance and culturing of all live food (brine shrimp and microworms) systems continued. Weekly activities include the following water quality analysis; dissolved oxygen, pH, conductivity, water flow measurements, ammonia, and light measurements. Monthly samples of well water were taken for TCE analysis. 3,500 eggs were pulled for culture renewal and 1,200 fry for Immunotoxicological studies.

Fish Shipping Summary:

No.of Medaka	Institution	Location
580	NYU	Tuxedo, NY
1014	WVU	Morgantown, WV
48	UME	Orono, ME
363[a]	EPL	Herndon, VA
65[b]	NIEHS	Research Triangle Park, NC

[a] 320 preserved in Bouins and 30 live fish

[b] 65 fin clips from potential transgenic medaka

Support of the Environmental Engineering Division at Rocky Mountain Arsenal (RMA):

The provision of health and safety support for Tennessee Valley Authority (TVA) at RMA continued. These tasks consisted of sampling and characterizing 1,200 drums of miscellaneous agent contaminated materials and sizing agent related process equipment for smelting at Rock Island. GEO-Centers was involved in verifying decontamination tags regarding the 1,200 drums of miscellaneous agent contaminated medias providing TVA with best management practices for handling the agent related waste and general health and safety support. Work continues with The Department of Army (DA) and Chemical Agent Safety and Health Policy Action Committee (CASHPAC) regarding the approval of chemical protective clothing (CPC) to be used at CBDCOM facilities. Assistance in clarifying regulatory issues continues, such as: waste characterization, transportation, handling, and disposal; hazardous



waste and agent waste characterization and safe handling procedures; researching innovated methods for reclamation and disposition of waste materials; chemical agent waste management practices and policies.

Technical guidance and training continues to be provided to RMA employees for managing special waste. Continuing technical support and management of the chemical agent contaminated waste storage was provided. The chemical agent contaminated waste storage contains over 8,000 drums. Technical insight inclusive of safe handling procedures, site assessment, ambient monitoring, and characterization was provided regarding unknown drums found in remote sections (section number 7 and 24) of RMA. Assistance was given regarding the design of Phase I remediation projects at RMA involving borrow areas, burial trenches/pits (landfills) excavations, and south plants demolition. Historic research, site assessments, and health & safety insight has also been provided for these projects. Chemical agent monitoring was requested for the Foster Wheeler's soil gas survey project phase I and II. Site control was requested in the event of agent detection consisting of: ensuring on-site personnel immediately proceed through gross decontamination and also personnel decontamination; securing chemical agent point source or fugitive emission sources; collecting soil samples for laboratory analysis; site decontamination if required; and notification of RMA officials of the incidents and actions taken.

Health & safety and technical support was provided for the Chemical Weapons Convention Treaty inspection conducted at RMA regarding their four declared sites (GB production and fill facilities, HD distillation facilities, HD fill facilities, and methyl phosphonic dichloride production facility). Emergency site characterization was done of an unknown system found in section number 9. The system consists of; three underground manholes with abandoned ancillary equipment and receptacles contained within. The 3 manholes make an equilateral triangle with the legs measuring approximately 300 feet in length. The Army requested ambient monitoring of the interior of the manholes, entrance of the manholes and removal of the receptacles, photo documentation of the interior, and performance of a hazard characterization on the contents of the receptacles. Training for RMA Engineer Technicians was conducted for polychlorinated biphenyls (PCB) and hazardous waste containing receptacles inspection. The training program covers weekly and monthly inspection criteria, record keeping, notification, and how to respond to an incident.

Support of the Program Controls Office at the Rocky Mountain Arsenal:

GEO-Centers continues to assist the Army in the Record of Decision (ROD) implementation, by participating, and supporting the Army=s position in the following groups:

The Biological Advisory Subcommittee (BAS) is tasked with looking at the effects of remediation on the biota, identifying areas of additional surficial soil contamination, as well as reviewing proposed biota studies and checking them for scientific soundness. Upon Committee's approval of the BAS recommendation to do additional analyses of biota On and Off post, the BAS initiated the Dioxin/Furan Study. Tissue samples, originally to be analyzed

by Clemson Laboratory for the H4IIe assay, have been returned to the Army due to a series of events making it impossible for Clemson to perform the analyses. At this time, the study is on hold until additional information is obtained from other laboratories able to do the H4IIe assay. The Supplemental Field Study Phase I continues to be evaluated by the BAS. The BAS has reworked the models predicting residual risk by using GIS mapping. The BAS also completed the effort of working with the RMA Borrow Area Group to incorporate areas of high risk into areas needed for borrow, and submitted the final recommendation in June 1997. The Biomonitoring of the USFWS requested performance of weekly field audits on the USFWS field activities. This program will begin in the beginning of the next quarter. Fish and Wildlife Funding has requested assistance in tracking of the USFWS expenditures. Policy for Old Samples work continues with the lawyers to form an agreement as to which tissue samples are ready for safe disposal.

Land Transfer of 815 Acres on the western tier of RMA is to be deleted using the EPA guidance on deletion of real property. The Remedial Action Report and carrying this process through the Notice of Deletion procedure in underway. The report is due to be finalized in the last quarter of 1997.

Environmental testing for biological threat agents at U.S. Army Medical Research Institute of Infectious Disease:

Plate/probe based PCR-EIA (Enzyme Immuno Assay) for the identification of *Brucella* was optimized. Optimization was not smooth and another set of primers for PCR will probably be chosen. The testing of primer sets against 7 genes within the pox sequence has begun. An abstract was submitted and accepted by the American Society of Tropical Medicine and Hygiene entitled ADetection of *Yersinia pestis* by Polymerase Chain Reaction Enzyme Immuno Assay (PCR-EIA). Work has begun on preparing for this poster presentation. A PerSeptive Biosystems seminar and user=s meeting was attended at the National Institute of Health in Bethesda, MD for information on their recently received PNA kit. A presentation was given comparing the sensitivity of 96-well PCR plates to the short topic forum. Reorganization of the laboratories continues in order to provide usable areas of research. A high school intern received training during the 3 hours a day/5 days a week she interns at the laboratory.

Work concentrated on the development and testing of an assay to detect the presence of DNA encoding *S. aureus* enterotoxin A (SEA) and B (SEB). The work has been quite successful. A collaboration was formed with Dr. B. Stiles of Toxinology Division to independently assay, by immunological ELISA, a group of 27 *S. aureus* clinical samples for the presence of SEA and SEB. Preliminary evidence suggests that the two assays correlate quite nicely and confirm the efficacy of the original assay. Expansion on this work will be continued to determine the limits of sensitivity and selectivity. Enough data has been accumulated to write a manuscript for publication during the next quarter. Assistance has also been given in developing diagnostic tests to identify and distinguish the presence of small amounts of Orthopoxvirus in blood, saliva etc. as part of the Biotreaty mandate. One

component of this effort involves sequencing several genes common to poxviruses and identifying areas containing enough polymorphic sequence to devise PCR assays capable of detecting and identifying the species(s) present in clinical specimens. Seven separate cones of variola open reading frames were made and assistance was given in sequencing them. This task is over 90% complete. The development of PCR-ELISA, PCR-TaqMan and Long PCR RFLP assays is now in progress. Identification is underway of primer sets which function well in these assays. Some useful primer sets have already been identified. The deadlines imposed by the Biotreaty contract are expected to be met

Stock suspensions of known concentrations of *Bacillus anthracis* spores and vegetative cells were prepared, dispensed in aliquots, and frozen for use in end-point detection experiments. Using a soil extraction procedure obtained from the Navy, experiments have been conducted to determine the limit of detection of *B. anthracis* spores and vegetative cells. The experiment was then repeated with the addition of a one-hour germination incubation which improved detection of spores by approximately one log. Further experiments were performed using a soil extraction procedure obtained from Batelle laboratories. Detection limits for spores and vegetative cells were determined using the complete procedure. Each step in the procedure was then systematically omitted to determine which were critical to the limit of detection. Finally the complete experiment was repeated with the addition of the one hour germination incubation.

Several environmental samples were received this quarter for aerobic and anaerobic microbiological analyses. Sample workup primarily included identification of *Bacillus* and *Clostridium* species. Work continues toward increasing expertise in anaerobic culture techniques and to validate current anaerobic methodologies. A state-of-the-art anaerobic chamber, the latest laboratory acquisition, will greatly supplement our ability to culture anaerobes. The Special Pathogens Branch continues to expand with regards to physical facilities and personnel. Renovations were completed this quarter on a limited-access sample archiving and distribution laboratory. Improvements included additional refrigeration and ultra-low temperature storage, sample archiving areas, and increased security. This laboratory will play a central role in our ability to maintain chain-of-custody procedures required for each sample. A training course was attended entitled AISO 9000 Implementation@ on July 9, 1997 at the PDA Headquarters in Baltimore, MD. Course contents were directly applicable to instituting a quality standard for the Bio-Treaty Laboratory. Work continues to develop a quality system encompassing administrative and technical aspects of the Bio-Treaty Laboratory. A successful College of American Pathologist (CAP) inspection was made of the Clinical Facilities, including the Microbiology Laboratory. Accreditation of the Clinical Microbiology Laboratory by CAP acknowledges that the necessary quality systems are in place to help ensure quality products (i.e., results) being delivered to customers. Preparation and participation in the CAP inspection provided experience that will prove invaluable to future certifying inspections of the Bio-Treaty Laboratory (i.e., ISO 9001).

OCTOBER 1- DECEMBER 31, 1997

Investigation of methods available for identifying biomarkers of mammalian hepatocarcinogenesis for adaptation to fish models:

A quarter of the work effort was spent on preparing the manuscript "Environmental Complex Mixture Toxicity Assessment" for publication in the Environmental Health Perspectives journal. Submission was made to the journal in December. Time was also spent on preparing an abstract,"5-Bromo-2'-deoxyuridine (BrdU) as a Biomarker of Chemically-Induced Hepatocellular Proliferation in the Japanese Medaka *(Oryzias latipes)"* for poster presentation at the 41st Annual Conference on Great Lakes Research in May, 1998.

Approximately one-fourth of the work effort for this quarter was spent monitoring the microbial content of the water in the well room (Test 105-001). A series of water samples was taken from ports in the well room before and after UV quartz sleeves were cleaned to test the bacteriocidal capabilities of the UV lights, and to determine whether or not the amount of viable bacteria present after passing through the UV filters was related to the percent transmittance of the UV lights. Other factors that could possibly affect the quality of water in the well room were also examined, such as frequency of filter changing and water-processing equipment failures. Data from this test will be submitted to the lab in January 98.

Work effort also involved processing the medaka slides generated from water disinfection by-product studies (Tests 100-006 and 600-004). Unstained slides were examined and selected for BrdU staining by FCRF, and BrdU-stained slides received back were marked and randomized for Bioquant evaluation. Staining, marking, and randomization has been completed for all BrdU slides from the chloroform study (Test 100-006). These slides are ready for evaluation on the Bioquant. One sacrifice point will be read at CSU; the other at USACEHR. The first of 4 sets of slides from the BDCM study (Test 600-004) are currently at FCRF for BrdU staining. Three days of time was spent entering references into the Refman system.

Research Conducted at Colorado State University:

A NATO meeting in Antalya, Turkey was attended and a poster presented entitled AUse of infrared spectral models in cancer research and their potential clinical applications. A platform talk was also presented during the meeting which was very well received. Approval was given from the CSU Department of Environmental Health to move all of the equipment and supplies currently used into one laboratory. This constitutes a major work flow improvement by cutting down on shifts between laboratories on different floors for each and every procedure. All equipment is now in Room 114 and space has been designated for the DNA work. Improved supply inventory will now be possible.

Work is presently being done on the assay of DNA damage by GC-MS techniques. Growth-related projects with EPR-Spin trapping techniques are also being performed. By



early next year, publishable data should be ready to be submitted. Further training was given to Dr. Cosma's student and post-doctoral researcher in the DNA extraction, set-up of calibration curves and basic GC-MS operation. The GC-MS protocols were repeated several times and ran smoothly. Independent running of samples has been performed successfully. Training in the data analysis aspect of the process will be given next quarter. A request to present at the IAGLR symposium entitled, "Biomonitors and Biomarkers as Indicators of Environmental Change," has been accepted. The symposium will be held May 18-22, 1998, at McMaster University in Hamilton, Ontario. Three to four days were spent on preparation of the abstract for the presentation.

Studies of antioxidant properties of melatonin have been performed. The neurohormone melatonin has been suggested to act as an antioxidant, however, its direct effects on reactive oxygen species (ROS) are not well understood. Using electron paramagnetic resonance (EPR)-spin trapping technique with 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) as a spin trap, quantitative relationships of melatonin reactivity with specific ROS have been identified. Melatonin was found to inhibit O^2.- formation in a dose-dependent manner. At the level of 1.7 mM, melatonin caused 50% inhibition of EPR signal intensity of DMPO-O^2-, generated by the reaction of xanthine and xanthine oxidase. The reaction rate constant of melatonin with O^2.- was found to be 1.25 x 10^3 M-1s-1. However, melatonin (up to 1.2 mM) did not exhibit a scavenging effect toward the .OH radical produced by Fenton reaction (Fe^2+ +H^2O^2 Fe^3+ + .OH + OH-). In addition, no evidence has been found for the formation of the melatonin indolyl cation radical that presumably precedes conversion of melatonin to its stable N1-acetyl-N2-formyl-5-methoxykynuramine (AMK) metabolite following sequential reactions of melatonin with O^2.- and .OH. On the other hand, melatonin is capable of scavenging H^2O^2 in a dose-dependent manner with an IC50 = 0.5 mM. The reaction rate constant of melatonin with H^2O^2 was found to be 2.52 x 10^5 M-1s-1. Furthermore, melatonin was also found to inhibit $1O^2$-dependent 2,2,6,6-tetramethylpiperidine oxide (TEMPO) radical formation during rose bengal photodynamic reaction, which may be due to the formation of an indolyl anionic species during reaction of melatonin with $1O^2$. The results obtained from the present study demonstrate that melatonin's antioxidant properties, in part, may involve a direct effect on scavenging of ROS. A manuscript has been written and submitted to the preview journal, *Free Radical Biology & Medicine* entitled "Scavenging of Reactive Oxygen Species by Melatonin". Two world experts, Drs. Lester Packer and Barry Halliwell have read this manuscript and gave very positive comments. At present, the assay of DNA damage by GC-MS techniques and antioxidants-related projects with EPR-Spin trapping techniques are being investigated. Publishable data is expected in the next quarter.

Equipment was received and set up to run gel electrophoresis. Laboratory space at CETT and the labs occupied by GEO-CENTERS/USACEHR were reorganized and received surplus cabinets and lab benches. The microtiter plate reader located on the main campus was reported to be taking errant readings. Equipment operation was assessed using troubleshooting methods recommended by Biotek and the machine was found to be in working order; however, a recommendation was made that if the problem occurred again, the machine should be inspected by the Biotek technician. Quality assurance slide reading continued on the methylene



chloride slides. Date of completion along with statistical summaries is expected by spring. A paper was reviewed on the performance of nuclear extracts on rodent bone marrow tissue in an effort to optimize treatment of the fish tissue for good, clean nuclear protein preparations. An outline of the work to be done for the NF-kB studies was orally presented to the Quantitative and Computational Toxicology group (QCT) at the Center for Environmental Toxicology and Technology at CSU on Monday the 17th of November. The presentation was well received and many of the researchers in the group offered advice and ideas for future for NF-kB studies. Progress on the NF-kB work was stalled due to problems in obtaining a fish cell line from Dr. Faisal and/or from the German University. Two slide show presentations were completed. The software needed to make 2x2 slides arrived and was installed. Although the pictures turned out exceptionally well, the slide maker had a defect in the alignment of the cathode tube causing the pictures to print out at a slight angle. The slide maker was sent back for repair. The ability to take slides on-site should result in savings in film processing. Regular maintenance of the Bioquant equipment was also scheduled and should be completed in the next quarter. The New Bioquant computer was received and will be installed next month to ensure that the slides received from the chloroform test are read using the format already established on the present instrument.

Studies of chemical carcinogenesis in medaka:

Medaka Test 100-006 with chloroform has been terminated as scheduled at nine months. Length and weight measurements were taken of the euthanized fish prior to their fixation in Bouin's solution. The fish were cleared with two rinses of 70% ethanol and stored in formalin prior to being sent for histopathology. Histopath results are expected in the first quarter of 1998. As part of the final sacrifice, 5 fish per tank were used for internal dose study. Livers were excised, weighed, and stored at -70EC until analysis. The animal use protocol was amended to allow the use of additional fish to be exposed to chloroform, and then used for tissue methods development on the GC-MS. Analysis of these non-test fish was successful allowing further tissue analysis of the actual test fish. A small fraction of chloroform was seen in exposed livers. Additionally, two metabolites were recorded in the samples. Quantitation of these metabolites was not possible due to the detergent base of the digested samples. NIEHS chemists have been contacted for suggestions on how to estimate the quantities of these materials from the GC-MS traces.

The drinking water disinfection by-products (DWDB) study was summarized by work unit, providing technical and budgetary information for FY97 and FY98. A work unit summary was prepared by individual of USACEHR research personnel for use in budget analysis. Three separate projects have been initiated with regard to facility upgrades. The fiberglass tanks used to house broodstock medaka in room 18 are being rehabilitated on post at a rate of one tank per quarter. This improvement will save us the expense of purchasing replacement tanks. The second project centers on UV disinfection of the processed well water. Current water usage is at the upper limit of our current UV disinfection capability. The backup UV has been cleaned with acid internally and placed online in parallel with the current UV unit. This will reduce the water to each unit by 50%, so that each unit will process

approximately 5 gallons per minute continuously. The final rehabilitation project is the upgrading of the waste collection system from diluter 1 in room 10. Upon completion of this project, all diluters in room 10 will have hard-plumbed collection systems. A project of data analysis and summarization of the medaka/methylene chloride carcinogen test was designed for an intern student. A manuscript entitled "Environmental Complex Mixture Toxicity Assessment" was reviewed which is to be submitted in December to Environmental Health Perspectives for publication. A poster abstract was prepared and submitted for the IAGLR meeting in May, 1998. The abstract title is "Integrated Environmental Assessment of Chemical Contamination", and it compares pentachlorophenol toxicity to frog embryos (FETAX) and fish immune function and respiration. The poster will be presented by a CSU researcher, who will be in attendance at the meeting.

The six month interim sacrifice was performed for Test 600-004, Medaka Carcinogen Test with Bromodichloromethane (BDCM). Twenty fish from each of the sixteen tanks were euthanized and prepared for histopathology. A summary was compiled of the fish length and weights by treatment. The summary of measurements of control and exposed fish has been submitted to the statistician for analysis, however, no significant differences are predicted. The preserved fish have been stored in the flammable cabinet until funding is acquired for their histopathology. Preparations were begun for briefing slides to be used at a NIEHS meeting in February 1998 regarding the progress made on the drinking water disinfection by-products project. Contacted co-investigators at USACEHR and requested summary information from their portions of the project to include in the briefing.

Teleost immunotoxicology methods development:

Range-Find LC_{50} experiments were performed using the heavy metal cadmium (Cd) in the bluegill. Concentration ranges were set for the exposures from historical published data. The LC50 and NOEL (No Observed Effects Level) for Cd will be mathematically established using probit analysis (SAS) and other appropriate statistical procedures. Toxicant exposure concentrations for the host resistance assays will be based on NOEL calculations, exposure duration, and historically relevant environmental data. The NOEL range for medaka was calculated to be 0.68 -0.71 mg/L; the NOEL range for bluegill was calculated to be 0.11 - 0.14 mg/L.

Host resistance studies were undertaken using juvenile bluegill (~6 months) and adult (~10 month old) medaka exposed to pentachlorophenol. Bluegill and medaka (360) were exposed to pentachlorophenol (bluegill - 0.105 mg/L [high], 0.022 mg/L [low]; medaka - 0.210 mg/L [high], 0.050 mg/L [low]) for up to 21 days. Fish were sampled at days 10 and 21. Fish were challenged with the bacterial pathogen Yersinia ruckeri at a dose approximating the LD_{50}. There were statistically significant differences between control and treatment groups for both fish species exposed to pentachlorophenol. Statistically significant differences were observed for cumulative mortality rates between control, low, and high treatment groups.



Fish Species	Exposure Duration	F ratio	p value
Bluegill	10 days	25.29	0.0005
"	21 days	12.81	0.0050
Medaka	10 days	35.82	0.0001
"	21 days	11.15	0.0053

Based on the observed results, the data indicates that pentachlorophenol suppresses some aspect of the host (bluegill & medaka) ability to resist pathogenicity caused by experimental infection of *Yersinia ruckeri.*

The 18th annual SETAC meeting in San Francisco, CA was attended and a poster was presented detailing the use of the host resistance assay in bluegill and medaka as an assessment tool to observe the immunotoxicological effects of pure compounds and possibly complex chemical mixtures. Twenty scientists expressed interest in poster. A manuscript was completed to be submitted for publication in Journal of Toxicology and Environmental Health entitled "Effects of exposure to malathion on host resistance, nonspecific, and specific immunity in a teleost fish model". Submission will be made to the journal in early January.

Transgenic medaka research at USACEHR [in collaboration with Dr. James Burkhart at the National Institute of Environmental Health Sciences (NIEHS)]:

Preliminary results of the transgenic medaka fin clips (May 1997 hatch) do not indicate any strongly positive fish. Several fin clip analyses were questionable, and these will be repeated for clarity. The remaining clipped fish were euthanized. Fifty more fish were clipped in December, including the three questionable fish from the last procedure, approximately 40 fish from the 8/97 hatch (F1 of positive parents), and approximately 7 fish from the 9/97 hatch (also F1 from the same parents). An attempt at hatching more transgenic medaka in November was unsuccessful. During this period, there were many changes in water quality due to the RO membrane change and it is believed that this contributed to the failure of the egg culture. More eggs will be collected in January 98 from the two positive founders.

Transgenic animal research conducted at NIEHS, Research Triangle Park, NC:

Research has focused on *in vivo* approaches correlating the use of alternative/natural environmental species with laboratory animal and cell culture models to assess mutagenic hazards from exposure to chemical or physical agents in the environment. Transgenic technology was applied to the study of induced somatic mutation directly at the DNA level using FX 174 bacteriophage as the transgenic marker in rodents, fish, and cultured cells where dose, adduction, DNA repair, and mutation can be compared. The latest work has been concentrated on a few control samples to understand the natural mutation rate. Each sample

had three digest performed with it. From each digest, three to four platings were made to determine mutation frequency.

Experiments concluded that spontaneous mutation frequencies in a non-transcribed bacteriophage transgene is similar in mice, fish, and cultured cells. Mutation in the target sequence can be induced in somatic tissues of fish and mice by treatment to an alkylating agent. Exposure effects of 7, 12-dimethylbenz{a}anthracene (DMBA) were examined through the analysis of cytochrome P4501A induction and DMBA metabolite levels after intraperitoneal injection with doses of 0.26, 1.9, and 10 and 19 mg/kg in corn oil. Fish presented an increasing trend of ethoxyresorufin optical density assay (EROD) induction with increasing DMBA dose. There was no significant increase in the mouse CYP1A activity. The same metabolites were detected in fish upon exposure to DMBA, demonstrating the various effects of DMBA in two divergent study organisms. These results are indicative of the importance of examining the differences in response between sentinel species when evaluating the effects of polycyclic aromatic hydrocarbons. *In vivo* induced mutation in the transgene was also examined in mice and fishes at 1.9 and 19.0 mg/kg doses of DMBA. There was a respective 2 and 11-fold increase in the mutation frequency over controls in fish; however, the effect of DMBA on FX DNA in mice was not as significant. Analysis of 10 mg/kg DMBA dose is underway. These studies represent the first evidence that identical gene indicators, detecting specific classes of chemically induced mutation, combined with analysis of biotransformation in various species may provide a mechanistic basis for comprehending correlations between laboratory species that may serve as environmental sentinels in polluted ecosystems. Experiments studying complex mixtures from contaminated ecosystems are beginning.

Methods development for rapid toxicity assessment:

Studies are currently underway to find the most rapid methods to hatch Triops. Two killifish hatching tubes and one well on two different well plates were filled with 10 Triops each. One hatching tube and well plate were placed on the laboratory bench top, the remaining well plate and hatch tube were placed in an incubator. The laboratory bench top received 14 hours of light per day while the incubator received 24 hours of light per day. At 20 hours after initiation of hatch, more Triops hatched in the incubator than the well on the bench top. At 48 hours post initiation, the number of Triops hatched that were held on the laboratory bench top surpassed the cysts that had been incubated. The killifish hatch tube hatched no Triops. This study was repeated using 100 cysts in each container, and produced similar results. Similar studies will continue to be performed to evaluate the hatching methods. Hatch temperature is also believed to affect hatch rate. Various diets have been under experimentation with the Triops. They include combinations of algae, live brine shrimp, flake food, frozen brine shrimp, microworms and protozoan. Combinations of brine shrimp as a part of the diet seem to provide the best results. Currently, the feeding program consists of live brine shrimp and frozen brine shrimp early morning, live brine shrimp and flake food and noon, live brine shrimp in the evening. Quantity of food is an important factor in Triops diet due to their rapid metabolism. Cannibalism has been observed when food becomes scarce. A significant amount of waste is produce by the Triops making good water quality difficult to maintain. Debris is

siphoned from the tanks daily and filters have been placed in the tanks. A power filter proved to create too much turbulence in the water and appeared to filter the food from the water before it could be consumed. A baffle was placed in the tank to control the water flow without improvement. An air powered filter was substituted which eliminated turbulence and removed very little food from the water. Experiments with the substrates used for animal culture were also undertaken. Sand used for both the Triops and the killifish was sorted by particle size, then used to determine the effect on the culturing and cyst collection. Fine sand was placed in a killifish embryo collection dish and a Triops cyst collection dish. The cysts were easier to remove from the fine sand but it was also more easily displaced from the dish by the Triops. When collecting killifish embryos, fine sand was found to aspirate more easily into the pipette which placed more debris in the embryo storage dish.

The daily records were maintained on the killifish and Triops. Hatches are generated as needed to maintain population size for Triops. Each group of Triops lasts 5 to 6 weeks before the last organism dies. A plan is being developed to raise killifish in a large bath stainless steel bath. This is in attempt to increase embryo production. Enough embryos should be collected in one week to run a killifish toxicity test. Support was given for an outline and draft research proposals characterizing diapause in the killifish for submission to DARPA for research funding purposes.

Bluegill ventilatory monitoring project:

Seven bi-weekly reports were written and submitted for the ventilatory data collected at Old O-field. All data was analyzed and archived for these reporting periods. Additional analyses and graphics were provided during periods of effluent response. A number of trips were taken to Old O-Field to perform regular test procedures, to evaluate operational concerns, and to perform diagnostic evaluation of system hardware with the electronic fish signal generator. During one of the trips a diluter malfunction of a diaphragm was noted. Materials were researched and a new diaphragm was constructed, which is currently under evaluation. Remote monitoring of Old O-field ventilatory response information was also improved. A new graphics format was developed for presenting bi-weekly ventilatory information which utilizes time and date axial information rather than print intervals, and also incorporates control and effluent water quality information onto the same graphs. The new dosing diluter system was installed at Old O-Field to facilitate conductivity adjustment of control water.

Collaboration continued with Dr. Charles Sarabun of the Johns Hopkins Applied Physics lab. Ventilatory information collected by the APL data acquisition was formatted into a form compatible with the Aquatic Biomonitoring System analysis. Analyses will be performed to ensure that data is compatible and accurate. An eight-channel diagnostic tool was designed to be used with the electronic fish generator. The device will allow for more accurate evaluation of differences between ventilatory chambers. New electrolytic capacitors were installed in the ventilatory biomonitoring trailer. The system was tested with the electronic fish signal generator after installation. A signal multiplexer was designed and constructed to permit the use of the old ventilatory amplifiers with new data acquisition computer hardware.



Existing eight-channel cabling was multiplexed into a 16-channel cable design to make the old amplifiers compatible. The multiplexing system was tested using the electronic fish signal generator. The biomonitoring trailer was prepared for ventilatory demonstrations on November 6th.

A Phenol sensitivity study and an Ammonia time-to-response study was performed. Chemical analysis for ammonia was done with an Orion specific ion analyzer. Reconstruction and calibration of the specific ion analyzer was performed in preparation for the studies. The probe was also used for additional testing to determine the exact concentrations needed for assays due to the unique speciation of ammonia in aqueous environments. Medaka and fathead minnow species were evaluated for ventilatory application during the quarter. Medaka signals were not measurable with existing chamber designs. Construction of smaller chambers yielded measurable yet inconsistent ventilatory signals that were largely superimposed with whole body movement. Fathead minnows yielded small but measurable ventilatory signals with extremely high ventilation rates (>190 respirations per minute). Assistance was given in the outlining and drafting of research proposals for development of a salt water ventilatory probe, and ecological probe technologies. The proposals were submitted to DARPA for research funding purposes. A salt water probe design was constructed utilizing existing electrode technology with a slight modification. The modification arises due to the fact that under increasing conductivity (salinity), signal transmittance is attenuated by the increasing conductivity. This phenomenon is believed to be caused by a short circuiting of the positive and negative electrodes. To avoid this short circuiting condition, a chamber design will be developed to electrically separate the positive and negative electrodes. This will be accomplished by encompassing the electrodes in a less conductive media which is separated from, but in direct electrical contact with the higher conductivity saline water in which the fish resides. This design would regain the capacitive nature of the cell that was lost by the increasing conductivity caused by salt water. A meeting was held with Conrad Carpenter to discuss marketing opportunities for the ventilatory biomonitoring system. A tour of the biomonitoring system at Old O-Field was also provided.

Analytical method development and analysis of chemicals used in carcinogenicity and mobile laboratory studies:

140 samples of bees were digested and analyzed for trace metals by ICP-MS. Data for quality control was completed and reported. An additional 35 bee samples were received. The analysis was performed and the data reported. 75 samples of Thalidomide, 100 samples of Pentachlorophenol and 50 samples of Phenol were analyzed by HPLC in support of ongoing toxicology testing at USACEHR. Approximately 150 samples of Dibromoacetic acid were analyzed in support of FETAX testing. The injection volume of the method was reduced from 20 to 2 uL to compensate for the high levels of DBAA found in the samples (>1000 mg/L). High relative standard deviations were observed and the original injection volume of 20 uL was used to analyze diluted samples. Precision and accuracy data for analyzing low levels of DBAA in well water has been initiated. Good precision and accuracy could not be obtained at the 100 ug/L level. Samples below the 1 mg/L level will be analyzed by the extraction /



derivitization method by gas chromatography. Concentrations for a nine month study are between 10 and 1000 ug/L. Colorado State University sent 24 samples of frog ovaries containing Cadmium. These samples were digested for analysis by ICP-MS. Following digestion a waxy undissolved substance was observed. This substance was analyzed by GC-MS and determined to be a fatty residue. Samples were filtered prior to analysis. Approximately 20 filter samples were also received from CSU for trace metal analysis. The samples were digested and are ready for analysis. A method for the analysis of Dibromoacetic acid was developed using HPLC. Direct injection provided the detection limits required for a FETAX test and an exposure to medaka. This method eliminated extensive sample preparation required by gas chromatography to perform the analysis. Precision and accuracy data was completed and a stability study will be completed as soon as time permits. Approximately 90 samples were analyzed for Dibromoacetic acid in support of toxicology testing by HPLC. Approximately 100 Thalidomide samples were and analyzed by HPLC in support of an ongoing FETAX test. Several samples from Aberdeen proving grounds were received due to a response in the blue gills. These samples were analyzed by HPLC, IC, and ICP-MS in order to determine if the response was due to some unknown chemical. The analytical chemistry department has analyzed over 4500 samples in 1997 more than doubling the output of the previous years.

Maintenance and optimization of USACEHR aquatic laboratory facilities:

Essential laboratory maintenance was performed on all medaka, bluegill, grass carp, fat-head minnow and killifish located in rooms 5 and 18. Daily activities include three daily feedings of all culture and test organisms. Associated record keeping was performed to maintain necessary test records to meet SOP and regulatory review requirements. Associated tank cleaning and siphoning was performed to maintain overall tank hygiene necessary to accommodate healthy test organisms. Maintenance and culturing of all live food (brine shrimp and microworms) systems continued. Weekly activities include the following water quality analysis; dissolved oxygen, pH, conductivity, water flow measurements, ammonia, and light measurements. Monthly samples of well water were taken for TCE analysis.

Summary information was compiled for use in the report to AAALAC on Animal Use for year 1997. Approximately 14,000 fish were added for use in 1997, 14,000 fish were used, and our inventory remains at about 11,000 fish. A cost proposal was prepared in regard to culturing zebra fish in FY 98 at USACEHR for another organization on post. Included in the proposal were maintenance labor and costs, supplies, health screens (labor and histopathology), and facility services (processed well water, analyzed food).

Fish Shipping Summary:

No. of Medaka	Institution	Location
1655	NYU	Tuxedo, NY
510	WVU	Morgantown, WV
439[a]	EPL	Herndon, VA
50[b]	NIEHS	Research Triangle Park, NC

[a] fixed fish from final sacrifice of Test 100-006

[b] frozen tail fin clips from possibly transgenic medaka, fish remain at USACEHR

Support of the Environmental Engineering Division at Rocky Mountain Arsenal (RMA):

In support of the Health and Safety office, efforts were coordinated with the Department of Army (DA) and Chemical Agent Safety and Health Policy Action Committee (CASHPAC) regarding the approval of chemical protective level "A" fully encapsulated suits for the use at CBDCOM facilities. The level "A" suits offer the highest degree of protection at a reasonable cost. Implementation of these NIOSH approved chemical protective suits will help the Department of Army meet the requirements of EO12196 and OSH the Act. Research continues on innovated technologies regarding chemical agent monitoring methods for field applications. The technologies currently being used at RMA; RTAP, minicams, and CAM2 are not reliable because of the wide range of chemical contamination at RMA which causes false positive detections. Technical insight continued to be provided regarding regulatory issues, such as waste characterization, transportation, handling, and disposal, hazardous waste and agent waste characterization and safe handling procedures. Research of innovated methods for reclamation and disposition of waste materials, and chemical agent waste management practices and policies also continued. Technical guidance for project coordination, management and implementation was given to RMA's internal special projects by working directly with RMA's engineering technician group (7 individuals). Training was provided for hazard assessment, hazard abatement, and the safe handling of special waste. Involvement in designing and implementing a chemical sweep program at RMA continues. The chemical sweep program consist of the removal of all remaining chemical related items and chemical substances from various structures and chemical process facilities. RMA engineering technicians are receiving training regarding safe chemical handling (including neutralizing, stabilizing, and decontamination), chemical classification, chemical segregation, SW-846 and standard sampling methods, chemical hazcatting, monitoring, chemical profiling, chemical packaging, and selection of a Toxic Storage Disposal Facility (TSDF) for disposition. RMA Engineering Technicians are provided with technical support for the day-to-day field operations regarding the above topics and general field operations protocol pertaining to project set-up, coordination, and management.

Service was provided as the technical point of contact for the management of RMA's chemical agent contaminated waste storage areas. The chemical agent contaminated waste

management includes management of over 8,000 drums and receptacles, safe handling requirements of new waste streams, and provision of technical insight regarding items encountered in the field. Further technical advice is provided in the support for the Chemical Weapons Convention Treaty at RMA. Field related issues include maintaining accountability of personnel access into declared areas and facilities, inspections of declared equipment, inspections of treaty tagged/locked out specialty equipment, and safety inspections of declared facilities. Point of contact responsibilities also were performed for polychlorinated biphenyls (PCB), debris pile, and hazardous waste containing receptacles inspection program. The program covers weekly and monthly inspection criteria, record keeping, notification, and procedures for responding to an incident (an incident consist of a leaking container or some other abnormality requiring immediate attention in order to protect human health and the environment).

In support of the Program Controls Office, assistance continues with Record of Decision (ROD) implementation, by participating in the Biological Advisory Subcommittee (BAS). The Dioxin/Furan Study tissue samples, originally to be analyzed by Clemson Laboratory for the H4IIe assay, have been returned to the Army due to a series of events making it impossible for Clemson to perform the analyses. Spikes and blanks will be sent directly to MRI to perform the congener specific analyses so that the BAS can better assess the integrity of the samples that Clemson had homogenized. Another collection of kestrel eggs is being planned for this field season to increase the sample size. A subset of samples will be analyzed for the H4IIe assay in order to identify the Ah receptor. The Supplemental Field Study Phase I continues to be evaluated by the BAS. The BAS has reworked the models predicting residual risk by using GIS mapping. This will enable the BAS to pinpoint areas of concern which may require additional remediation. In coordination with the Biomonitoring of the USFWS, the Army has requested weekly field audits on the USFWS field activities. This program began on October 1, 1997. Due to lack of field sampling efforts to monitor, planning of next years field activities has commenced. Assistance was also given in tracking the USFWS expenditures. A procedure has been finalized for disposing of old biota samples that are of no future use. A list of samples has been given to the BAS for their review and approval for disposing of approximately 250 samples. A Remedial Action Report was written and is in review by the regulators on a land transfer of 815 acres on the western tier of RMA which are to be deleted using the EPA guidance on deletion of real property. Representation was provided for the Army on risk issues in Section 20 where contaminated soil may exist. This area was not identified in the ROD, but was discovered by the USFWS while they were preparing the soil for revegetation. Red areas appeared in land that had been mowed. Characterization of the soil is being done to determine any human health or ecological risk numbers.

Environmental testing for biological threat agents at U.S. Army Medical Research Institute of Infectious Disease:

Stock suspensions of known concentrations of *Bacillus anthracis* vegetative cells were prepared and dispensed into aliquots. Sets were frozen at -70°C and -20°C and enumerated

weekly to determine which storage conditions are better for maintaining viability. Experiments were then performed using a soil extraction procedure obtained from Los Alamos National Labs. Detection limits for spores and vegetative cells were determined using the complete procedure. Each step in the procedure was then systematically omitted to determine which were critical to the limit of detection. Preliminary results indicate that the procedure obtained from Batelle is the most sensitive in detecting non-germinated spores and vegetative cells in buffer. Currently, experiments are being performed to determine the detection limit of the procedure using soil samples. PCR analysis of a set of Special Pathogens isolates was performed using a group of primers for the *Bacillus* genus. A one-day training seminar was attended on the MIDI gas-chromatograph which will be used in the containment lab to develop a library based on fatty acid composition of bacteria.

The testing of approximately 100 primer sets against 7 genes within variola for PCR continued on the pox project. Numerous plasmid extractions were performed of cloned variola DNA for template for PCR. The TAQMAN assay was learned while experimenting with VEE samples and efforts are now being combined with a coworker on the *Brucella* project. Overseeing the lab work of a student has continued. Training in extractions, culture handling and gel electrophoresis was given to technicians from the Pathology department. Work was performed on a poster entitled ADetection of *Yersinia pestis* by PCR-Enzyme Immuno Assay@ and presented in Florida at the American Society of Tropical Medicine and Hygiene. Currently, experimentation for *Yersinia pestis* work is being completed in order to begin writing the publication.

A working assay has been developed that will specifically identify *S. aureus* samples that express either Toxins A or B. This assay was tested against 25 *S. aureus* samples and was greater than 95% specific when compared against traditional ELISA procedures. Induction of false positive results were judged to be nil based on testing of 27 common bacterial culture samples. This work is being written for publication in the *Journal of Clinical Microbiology*. Seven variola open reading frame sequences have been cloned, which will be used for developing diagnostic assays offering specificity and sensitivity in identifying this pathogen in clinical or environmental samples. A request to analyze a culture suspected of being *C. botulinum* was made. Although no assay had been developed for the *C. botulinum* toxins, reagents for some of them were available and an attempt to identify the sample was made. Amplification was achieved in one sample and this subsequently was sequenced. The amplified fragment turned out to be a cellular gene of *S. aureus*. One problem with this assay was that a positive control was not available to work out the optimal assay conditions. The negative results were duly recorded.

The Special Agent Laboratory (SAL) of the Special Pathogens Branch became operational this quarter. Equipment acquisitions included the MIDI (Microbial Identification Instrument) and the BIOLOG System. Both will be used for rapid biochemical characterization and identification of threat agents. These instruments, along with other core capabilities of the SAL, will be invaluable to meeting the Branch=s counter-terrorism and counter-proliferation missions. In addition to the CAP and ISO9001 experience gained last quarter, further



regulatory experience was gained by participating in two QA/FDA clinical trial audits. Documentation, record keeping, preparation of SOPs, and conducting clinical trials were addressed in these audits. Developing, conducting and reporting results of experimentation under the watchful eye of a regulatory body offered many learning opportunities. Knowledge gained will serve the Special Pathogens Branch in continuing to offer our customers a quality service. Representation for the Chief, Diagnostic Systems Division, was provided as a voting member of the USAMRIID Scientific Review Committee. The committee functions as a reviewing body to evaluate the scientific soundness of human experimental protocols. Service was also rendered as clinical microbiologist on a clinical trial entitled AExpanded Phase I Outpatient Safety and Immunogenicity Study of a Live, Oral, Attenuated *Shigella flexneri* 2a Vaccine SC602.@ This bacterium, the cause of shigellosis, can be a significant hindrance to U.S. troop readiness during foreign conflicts. It is also considered a threat agent, and first hand experience gained through completion of this project has increased the Special Pathogens Branch=s ability to rapidly and efficiently deal with this threat.

Abstracts written for the quarter were: Teska, J.D., S.R. Coyne, E.A. Henchal, J.W. Ezzell, T.L. Hadfield and E.J Hilyard. 1998. "Rapid Viability Assessment of Biological Threat Agents." ASM Meeting, Atlanta, GA and Teska, J.D., T. Coster, J.R. Colbert, Q. Rance, M. Venkatesan and T.L. Hale. 1998. "Excretion of Live, Oral Shigella flexneri 2a Vaccine SC602 During a Community-Based Phase I Trial." ASM Meeting, Atlanta, GA.

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Investigation of methods available for identifying biomarkers of mammalian hepatocarcinogenesis for adaptation to fish models:

The majority of the work effort this quarter was spent evaluating the cellular proliferation indices on slides of medaka livers generated from the water disinfection by-product studies, tests 100-006 and 600-004. Unstained slides from these studies were examined and selected for BrdU staining by Frederick Cancer Research Facility (FCRF); BrdU-stained slides received back were marked and randomized for Bioquant evaluation. Slide reading has been completed on the Bioquant for all BrdU slides from the two sacrifice time-points from the chloroform study, test 100-006. One of these sacrifice points was read at CSU, the other at USACEHR. Statistical evaluations of the effects of chloroform on hepatocellular proliferation are currently being evaluated at CSU and should be completed by the end of the quarter. Bioquant evaluation of the first two of four sacrifice points from the bromodichloromethane study, test 600-004 should also be completed by the end of this month. The reading of these sacrifice points was also split between CSU, and USACEHR. The two remaining sets of slides from test 600-004 at FCRF are still undergoing BrdU staining. The staining should be completed by the end of April. Projected completion for the Bioquant analysis and statistical evaluation of these slides is the end of May. In addition to evaluating slides, time was also spent preparing viewgraphs for presentation to USACEHR and EPA of the data generated thus far from the water disinfection by-product studies.

Monitoring of the microbial content of our laboratory water supply, test 105-001, continued with bi-monthly sampling of water from the well room and the laboratories. A meeting was arranged with Final Filtration representatives, who met with laboratory personnel to discuss upgrades for our well room ultraviolet light (UV) filters, as well as other components of the water-processing system, such as the in-line pre-UV filters. Price quotes and recommendations were given. The recommendation was made to insure routine maintenance was performed including changing of the pre-UV in-line filters (monthly) and replacing the cotton filters, which may encourage bacterial growth, with synthetic filters. A price quote of $2244.00 was given to replace our current UV filters with an upgraded system. Time was also spent continuing to enter references into the Refman database, training technicians in utilizing the Bioquant image analysis system for reproductive toxicity work, and installing software in the Bioquant.

Research Conducted at Colorado State University:

The first quarter of 1998 has involved the study of oxidative damage and genotoxicity in mammalian cells using *in vitro* models. Initially, time was spent learning tissue culture techniques under the supervision of Russ Drabek, a post-doctoral student in Dr. Charles Waldren's laboratory, Department of Radiation Medicine. Toxicity (survival) curves and single-cell plating for two cell lines (AND6 and 179-1) were established for hydrogen peroxide (H_2O_2) exposures of 1 and 16 hours. Mass cultures (10^6 cells) for the same exposures have been initiated and should be complete within two weeks. A UV-sensitive cell line has also been added to the test group at this point. Concurrently, DNA samples have been extracted from the cell lines and DNA damage will be assessed via GC-MS and FT-IR. H_2O_2 is known to produce the hydroxyl radical, a reactive oxygen species known to cause DNA damage. The long term effects of radical-induced DNA damage upon a cell are unknown and are the basis of intense research efforts world wide. Correlations between killing, mutagenicity and DNA damage will allow for a comprehensive assessment of the effects of H_2O_2 and will be the basis for future studies of radical-induced DNA damage. Upon completion of H_2O_2 exposures, arsenic and cadmium will be evaluated for their mutagenicity/DNA damaging effects using the same techniques. Training has begun in fluorescent *in situ* hybridization (FISH) using human Hela cells provided by Dr. Waldren's lab. Ten chromosomal slides have been made which will be used for the FISH technique to probe for chromosome 11. Future work will involve training in the specialized techniques of Southern, Western and Northern blots and PCR analysis. April and May will involve several hours of training two individuals on the GC-MS as well as preparation for the IAGLR symposium presentation scheduled for May 19 at McMaster University in Hamilton, Ontario.

Data analysis was performed on the methylene chloride study. The report will be finalized when the 4th sacrifice reading is completed by the CSU technician. Data transformation and analysis was also begun on the chloroform study. Results were submitted at the end of the quarter. The data for one time point in the BDCM study will arrive on the 27th with anticipated analysis completed by the 1st of April. Data for the other three BDCM sacrifice points will be ready during the first quarter and the analyzed prior to 1 May. QA/QC

work on the slides continues. SOP's for the equipment at the Colorado State University location are being written with an expected completion by the end of the second quarter. Nuclear protein extraction procedures are being performed on the hematopoietic tissue from 3 different species - medaka, bluegill and the Sprague-Dawley rat. The extracts will be frozen and their NF-kappa B values analyzed during April. The procedure for analysis will use a non-radioisotopic EMSA (electrophoretic mobility shift assay) procedure.

Studies of antioxidant properties of chaparral have been completed. Chaparral is a herbal preparation which is made by grinding leaves of an evergreen desert shrub known as the creosote bush or "greased". The leaves can be brewed for tea or made into capsules or tablets. Chaparral has been recommended as a free radical scavenger to retard aging, maintain proper skin conditions, and protect against various other health disorders. However, its direct effects on reactive oxygen species (ROS) are not well understood. To address whether chaparral can act as a direct scavenger of free radicals, investigations have been undertaken into the effects of chaparral on ROS by spin trapping in combination with electron paramagnetic resonance (EPR) techniques. If chaparral scavenges free radicals, the characteristic EPR signals of trapped free radical species generated in the given reaction systems should decrease with increasing amounts of added chaparral. New experimental evidence has been found that chaparral is capable of scavenging the superoxide anion radical, $O_2^{\cdot}$-. Findings indicate that chaparral scavenges superoxide anion radical ($O_2^{\cdot}$-) in a dose-dependent manner. 5,5-dimethy-1-pyrroline-N-oxide (DMPO) was used as a spin trapping agent and the reaction of xanthine and xanthine oxidase as a source of $O_2^{\cdot}$-. The kinetic parameters, IC_{50} and V_{max}, for chaparral scavenging of $O_2^{\cdot}$- were found to be 0.899 μg/mL and 8.4 ng/mL/sec, respectively. The rate constant for chaparral scavenging $O_2^{\cdot}$- was found to be 1.22×10^6 $g^{-1}s^{-1}$. These studies suggest that the antioxidant properties of chaparral may involve a direct scavenging effect of the primary oxygen radical, $O_2^{\cdot}$-. A manuscript will be submitted for publication with the above results entitled "Scavenging of Superoxide Anion Radical by Chaparral." Authors are Lun-Yi Zang, Greg Cosma, Henry Gardner, Xianlin Shi and Val Vallyathan. Completion of the kinetic studies on DNA damage by hydroxyl radical is planned. Work will also continue on the assay of DNA damage by GC-MS techniques when a new GS-MS column is received.

Studies of chemical carcinogenesis in medaka:

A preliminary draft of a research proposal to be submitted to EPA was prepared in response to an EPA memo entitled, "Call for FY98 Proposals for NCEA Intramural Grants Program". This new program will support two years of research in the area of drinking water contamination. The topic of the preliminary draft was an evaluation of nontraditional vs traditional assays with regard to drinking water contaminants studied at USACEHR. Fish and frog embryo tests will be compared to rodent toxicological results if the proposal is accepted. One of the conditions of the program is that EPA investigators play a lead role in the research. A tentative collaboration has been established. The EPA collaborator declined to submit the proposal due to insufficient time to prepare the document, but EPA's strong interest in the work proposed led to a search for alternate contracting vehicles. In preparation for an upcoming meeting with EPA and other drinking water professionals, a handout was prepared

that outlined the USACEHR research approach to drinking water disinfection byproducts which included selected results. The first draft was prepared of an article for submission to *Toxicologic Pathology*, "Histology of Medaka Chronically Exposed to a Complex Environmental Mixture". Co-authors are Dr. Marilyn Wolfe of EPL, Dr. Dennis Burton of University of MD, Tom Shedd and Dr. Henry Gardner of USACEHR. This article will detail the unusual histological findings in medaka from the West Branch Canal Creek Study at Aberdeen Proving Grounds, Aberdeen, MD. Copies of the first draft have been sent to co-authors Wolfe and Burton for comment.

Selected histopathology findings were submitted to the USACEHR statistician from the USACEHR Test 100-006, Medaka with Chloroform Study. Liver tumors rarely occurred in this study, and findings seemingly treatment related were abnormalities of the gallbladder and the liver biliary system. The exposure phase of USACEHR Test 600-004, Medaka with bromodichloromethane, has been completed. Internal dose analysis of livers from 5 fish per exposure aquaria was completed in February. Metabolites of BDCM were not seen on the GC trace; however, one clear BDCM peak was seen in the exposed fish. The internal dose (0.041 mg/L) of the low dose fish was roughly 2x the external dose of 0.020 mg/L; the mid dose fish had roughly the same internal dose (0.121 mg/L) as external dose (0.136 mg/L); and the high dose fish had a lower internal dose (0.872 mg/L) than external dose (1.38 mg/L). Males had higher internal doses in the low and mid dose concentrations, and lower internal doses in the high dose tanks. The hematology analysis was completed in February. Hematocrit, leukocrit, cell viability, and cell count were not affected by chloroform treatment, although hematocrit was approaching statistical significance at $\alpha = 0.05$ level ($p = 0.0702$). Approximately 460 fish were sacrificed in March at exposure termination. The fish were preserved per USACEHR SOP and are being held at USACEHR until funding is received for histopathology.

Powerpoint slides were prepared for a Drinking Water Disinfection Byproduct study progress meeting in North Carolina. These slides summarized the results to date from medaka and frog embryo testing for chloroform, bromodichloromethane, dibromoacetic acid, and chlorate. Laboratory photographs of the work being done was incorporated into the presentation. Photo micrographs of stained fish tissue was included to show unusual effects of toxicant exposure. Tables, charts, or figures were prepared of chemical analyses, cell proliferation, FETAX testing, acute and chronic fish testing, and histopathology findings. Summary slides were produced for each chemical. A time line showing completed work, test in progress, and projected work completed the presentation. Assistance was given to the USACEHR budgeting process. Previously, man hours had been collected for all the scientific staff, government and contractor. The labor categories in use by the government and contract technical staff differed from the APC codes used to track spending. These man hours were reassigned by APC code in an Excel spreadsheet to categories that had funding designations, or in some cases, to unfunded designations. From this information, others were able to add the cost of labor to have a clearer picture of actual operating costs. A draft was made of an informational brochure for USACEHR. Two versions were circulated for comments among USACEHR personnel. The objective is to have promotional materials available for visitors and interested colleagues at professional meetings. A draft was prepared of the poster entitled

"Integrated Environmental Assessment of Chemical Contamination" to be presented at the International Association for Great Lakes Research (IAGLR) meeting in May. Co-authors include Widder, Beaman, Grossnickle, Gaudet-Hull, Gunselman, Shedd, Finch, and Gardner.

Teleost immunotoxicology methods development:

Health Screen # 10 was performed during this quarter. Six fish groups (3 test groups and 3 culture groups) were monitored in histopathology (4 fish/group), and bacteriology (5 fish/group and a water sample from each tank from which fish were taken). Drop counts were performed to enumerate bacteria in each fish/water sample. All colonies were picked for ID at last readable concentration. The following tests/isolate were performed to delineate biochemical profile: gram Stain, O/F (Oxidative/Fermentative) Glucose, TSI (Triple Sugar Iron) Agar Slant, Catalase Production, Cytochrome Oxidase Production. General/immunological health matrices (15 fish/group of length, weight, hematocrit, leukocrit, cell yield/fish, cell viability, extracellular superoxide anion production by cytochrome *c* reduction test were tested. Setup and start of Test # 304-005, Sublethal exposure of bluegill sunfish to pentachlorophenol: *In vitro* immunological studies, was initiated. The test will consist of a 31 day (10 day acclimation and 21 day exposure) housing of 48 adult (> 1 year old) bluegill (4 fish/5 gal tank) using specially designed tanks fitted with a partition system constructed of frosted plexiglass which segregates the fish, yet allows for equal exposure of fish to toxicant. Forty-eight bluegill were individually randomized into 12 partitioned aquaria and will be allowed to acclimate for 10 days. The bluegill will then be exposed to pentachlorophenol at 2 dose levels (high = 0.140 mg/L; low = 0.035 mg/L) and a no toxicant control for 21 days. Fish will be sampled at days 10 and 21 and immune function assays performed to determine the effect(s) of pentachlorophenol exposure on the immune response of adult bluegill.

Two range-find LC_{50}'s were run in bluegill and medaka with cadmium. Mortality occurred in bluegill at concentrations tested, but sufficient mortality did not occur in enough groups to accurately calculate an LC_{50} using probit analysis. This assay will be performed again using concentrations based on those where mortality was observed so that an accurate calculation of LC_{50} and NOEL may be performed. The medaka range-find was unsuccessful, causing no mortality even at concentrations predicted to cause 100% mortality. After consulting with analytical chemistry advisors, the problem may have occurred as a result of diminished availability of free cadmium due to precipitation or complexing with organic matter. Manipulation of the chemical along with analyses will be performed prior to retesting with medaka.

An in-house IACUC committee meeting was attended in January. An addendum was submitted to an immunotox animal use protocol to include the fathead minnow, *Pimephales promelas*, to be included as a model for host resistance studies. IACUC inspection, with a follow-up safety inspection was conducted in preparation of the upcoming AALAC inspection in 1999. The final editing for a manuscript to be submitted for publication in Journal of Toxicology and Environmental Health entitled "Effects of exposure to malathion on host



resistance, nonspecific, and specific immunity in a teleost fish model". Submission to Journal is expected as soon as draft is received back from editing coauthor, Judy Zelikoff.

Transgenic medaka research at USACEHR [in collaboration with Dr. James Burkhart at the National Institute of Environmental Health Sciences (NIEHS)]:

Results from the first screening of fry from positive transgenic medaka were all negative. When two culturing attempts failed during December, concerns were raised that the positive mating pair may be too old to breed successfully; however, two egg collections in January proved otherwise. December egg failure may have been the result of changes in water quality caused by the reverse osmosis water treatment process fluctuations. The eggs from the positive transgenics appear to be more fragile and more susceptible to failure than regular brood stock medaka eggs. Remaining transgenic fry were fin clipped. DNA analysis results were negative.

Transgenic animal research conducted at NIEHS, Research Triangle Park, NC:

Research has focused on *in vivo* approaches correlating the use of alternative/natural environmental species with laboratory animal and cell culture models to assess mutagenic hazards from exposure to chemical or physical agents in the environment. Transgenic technology was applied to the study of induced somatic mutation directly at the DNA level using ΦX174 bacteriophage as the transgenic marker in rodents, fish and cultured cells where dose, adduction, DNA repair, and mutation can be compared. The latest work has been concentrated on a few control samples to understand the natural mutation rate. Each sample had three digests performed with it. From each digest, three to four platings were made to determine mutation frequency.

Experiments concluded that spontaneous mutation frequencies in a non-transcribed bacteriophage transgene is similar in mice, fish, and cultured cells. Mutation in the target sequence can be induced in somatic tissues of fish and mice by treatment to an alkylating agent. Exposure effects of 7,12-dimethylbenz[a]anthracene (DMBA) were examined through the analysis of cytochrome P4501A induction and DMBA metabolite levels after intraperitoneal injection with doses of 0.26, 1.9, and 10 and 19 mg/kg in corn oil. Fish presented an increasing trend of ethoxyresorufin optical density assay (EROD) induction with increasing DMBA dose. There was no significant increase in the mouse CYP1A activity. The same metabolites were detected in fish upon exposure to DMBA, demonstrating the various effects of DMBA in two divergent study organisms. These results are indicative of the importance of examining the differences in response between sentinel species when evaluating the effects of polycyclic aromatic hydrocarbons. *In vivo* induced mutation in the transgene was also examined in mice and fish at 1.9 and 19.0 mg/kg doses of DMBA. There was a respective 2 and 11-fold increase in the mutation frequency over controls in fish; however, the effect of DMBA on ΦX DNA in mice was not as significant. Analysis of 10 mg/kg DMBA dose is underway. These studies represent the first evidence that identical gene indicators detecting specific classes of chemically induced mutation combined with analysis of biotransformation in



various species may provide a mechanistic basis for comprehending correlations between laboratory species that may serve as environmental sentinels in polluted ecosystems. Experiments studying complex mixtures from contaminated ecosystems are beginning.

Currently, work on genotyping F_1 generation of medaka (Oryzias latipes) is continuing. All offspring (F_1) of medaka appear negative for ΦX174 marker. Scheduling of new transformation experiments for the medaka is in process. Genotyping for killifish (Fundulus heteroclitus) is going well. At this point, all F_3 generation are positive, having high copy numbers. For other genotyping experiments, the use of polymerase chain reaction has been essential for amplification of specific genes. Over the past few weeks, work has centered on defining the constraints for the reaction as well as relative problem solving to prepare for the upcoming workload. Plans for the near future include designing another plasmid with a site-directed point mutation to detect G:C adduct formation induced by xenobiotic chemical exposure. Western blot techniques have been exercised over the past few months to correlate the actual amount of CYP1A to induced CYP1A activity by DMBA. Fish husbandry has required considerable attention until a replacement technician is found for this vacated position.

Methods development for rapid toxicity assessment:

A small feeding study was performed with the Triops to determine the amount of food optimal for a study. Of the replicates tried, 3 μL of the live brine shrimp solution appeared to work very well. There was also very little mortality after 24 hours in the control. The control was not fed, indicating the potential to have a successful test without feeding the organisms.

Bluegill ventilatory monitoring project:

Seven bi-weekly reports were written and submitted for the ventilatory data collected at Old O-Field from 12 December through 19 March. All data was analyzed and archived for these reporting periods. A number of trips were taken to Old O-Field for maintenance activities. These included rewiring and installation of a new injection pump for the diaphragm metering system and replacement of the Hydrolab water quality analyzer, as well as routine fish changes and data archiving. Remote monitoring and data transfer of Old O-field ventilatory response information was also facilitated. Ventilatory graphics were updated due to the corruption experienced from installation of the upgraded version of Excel for Windows NT. A macro program was written that compiles analyzed data files into acceptable graphics format, saving considerable time in generating graphics for the bi-weekly reports. A prototype silicon diaphragm for the diaphragm metering system at Old O-Field was constructed and tested. The system worked successfully and additional diaphragms will be constructed to replace existing diaphragms which are nearing their life expectancy for normal use. A standard curve of signal loss compared to increasing conductivity was generated for the ventilatory electrode and amplifier system. The curve was generated to determine the percentage of signal loss in the ventilatory system that could be directly attributable to increasing conductivities. The graphics were used to help explain recent data measuring difficulties encountered at Old O-Field, which were directly linked to the increasing



conductivity of the effluent water. To aid in this process, whole year conductivity graphs of the effluent water at Old O-Field were generated for all 3 years of data collected form the site. From this, measurement difficulties were easily correlated with the recent conductivity increase. Additional testing was also performed in an attempt to reduce signal noise caused by the water pulses of the diaphragm metering system. Flow reduction and baffle systems were tested and found to have a positive effect. Neither method was totally able to eliminate the water noise. The water pulses appear to be problematic only when signal amplitude is reduced by high conductivity.

A review of the most current year of Old O-Field biomonitoring data (1 April 1997 through March 1998) was compiled for submission to APG which included operating status, mortality and response information, system upgrades, and future cost reduction possibilities.

The Aquatic Biomonitoring System Operation and Maintenance manual was also updated to include current operating procedures and deletions. Completion of un-ionized ammonia validation studies were run during the quarter. Both time-to-response and sensitivity studies were completed. The sensitivity study had to be repeated due to premature mortality caused by water delivery problems from the well system. Stock concentrations were made for the ammonia studies and analyses were performed using the Orion specific ion analyzer. Training was also provided on the proper calibration and measurement techniques needed to operate the specific ion analyzer. Preparations were made for the upcoming ventilatory tests using the insecticide Malathion. Malathion tends to adhere to surfaces with which it comes in contact. This causes the concentration delivered to the chambers, where fish exposure occurs, to be reduced. It was noted in previous tests that the loss decreased over time. For ventilatory tests, it is important that the chemical concentration stays consistent. A study was run using Malathion in the diluter system using a stock concentration of 120 mg/L. The stock was delivered to the dilution chamber at a rate calculated to give 3.0 mg/L in the high and 0.3 mg/L in the low. Samples were taken from the chamber at 1, 2, 12, 24, 36 hours and analyzed for Malathion. The results were then plotted to develop a concentration curve. The curve will help determine what volume of stock should be delivered at a given time interval in the test to obtain the desired concentration. The delivery volume of the peristaltic pumps were then recorded at various pump speed settings to predetermine the delivery volumes. This will allow the flow to be logically reduced throughout the test. The chemical properties of Malathion necessitated the need to run preliminary chemical dose curves for the ventilatory water delivery system.

Collaboration continued with Dr. Charles Sarabun and other researchers form the Johns Hopkins Applied Physics lab. Information pertaining to ventilatory validation test specifics were sent to Dr. Sarabun via E-mail. Two trips were facilitated to APL to discuss results and findings of the ventilatory waveform analysis and to discuss new probe technologies; specifically a saline ventilatory probe and whole-ecosystem monitoring possibilities. A carbon electrode was constructed by APL to be tested with the ventilatory system. Initial testing using the electric fish has proven to be very promising. At low conductivities the carbon electrode yields output similar to current stainless steel electrodes, while at higher conductivities the

carbon electrode provides a more stable signal and eliminates signal drift that is present when using stainless steel. Measurable signals were picked up by the Aquatic Biomonitoring system with additional amplification of existing system. Additional amplifications of 10X and 100X were tested with positive results up to 35 g/L of [NaCl] (salinity of ocean water). A measured conductivity of 40,900 micro S/cm was recorded for this concentration. Additional testing will be performed with the carbon electrode system.

Time was spent preparing the ventilatory invention disclosure information. Initial hand drawings were drafted and submitted to Blake Sajonia to accompany the written text of the disclosure. Technical information on all aspects of the ventilatory system and necessary forms were compiled and submitted to both Mr. Sajonia and Mr. Chuck Harris, the government patent attorney. Review and comments by authors were also made on draft versions of the invention disclosure information. Discussions were held with Stan Finger and Ken Bayer on the construction of ventilatory chambers. Strengths, weaknesses, essential characteristics, and possible improvements of the chambers were discussed. A tour was taken of the laboratory facilities and trailer systems. Ventilatory graphics and information was compiled for inclusion in the IAGLR poster on integrated environmental assessment. The draft poster was reviewed and comments were submitted. A meeting to discuss killifish culturing and holding methods was held to form a plan of continued activity. A presentation of the ventilatory biomonitoring system and the killifish test system was provided to a visiting guest from the Command at the request of Colonel Danley. The Working Integrated Product Team on US Army Medical Research and Materiel Command Support for *Pfiesteria sp.* Research was also attended.

Analytical method development and analysis of chemicals used in carcinogenicity and laboratory studies:

Several samples of treated water from Aberdeen Proving Grounds were analyzed in support of ventilatory biomonitoring efforts. The samples were analyzed for sodium and chloride to determine if sea water was causing an increase in observed conductivity. No previous data was available to compare the relative concentrations of samples prior to the increase in conductivity. The conductivity continued to increase in subsequent samples and an increase in the concentrations of sodium and chloride were observed. Precision and accuracy data for the analysis of anions is currently being performed. There is a difficulty in analyzing real samples due to high levels of chloride that exist in typical samples. Chloride is present at concentrations exceeding 100 mg/L and interferes with the analysis of low levels of nitrite. A spiked sample of well water is shown in Figure 1. High levels of chloride and other halides can be eliminated by solid phase extraction by the formation of insoluble silver salts.

Approximately 40 samples of Dibromoacetic acid were analyzed by high performance liquid chromatography and approximately 90 samples of chlorate were analyzed by ion chromatography in support of FETAX testing. The ICP-MS lab was relocated from room 3 to room 125. Following the transfer of equipment, 60 samples of cadmium were analyzed by ICP-MS. No noticeable effect on the stability of the instrument was observed. A HPLC method was developed for the separation of 2-Hydroxyestradiol and 4-Hydroxyestradiol. This



method may be used to analyze liver samples from CSU.

Maintenance and optimization of USACEHR aquatic laboratory facilities:

A general lab cleanup was undertaken for semi-annual IACUC Facility Inspection. Animal husbandry areas were mopped, refrigerator records were attached to refrigerators, and doors were washed. The plexiglass plates covering the air conditioner ends of baths 5 & 6 were cleaned with 70% ethanol. The inside of the food refrigerator in Room 18 was washed. Essential laboratory maintenance was performed on all medaka, bluegill, fathead minnows and killifish located in rooms 5 and 18. Daily activities include three daily feedings of all culture and test organisms. Associated record keeping was performed to maintain necessary test records to meet SOP and regulatory review requirements. Associated tank cleaning and siphoning was performed to maintain overall tank hygiene necessary to accommodate healthy test organisms. Maintenance and culturing of all live food (brine shrimp and microworms) systems continued. Weekly activities include the following water quality analysis: dissolved oxygen, pH, conductivity, water flow measurements, ammonia, and light measurements. Monthly samples of well water were taken for TCE analysis. Transgenic medaka test organisms were maintained in conjunction with interlaboratory studies with Dr. Jim Burkhart. Essential laboratory supplies were documented and ordered on an as need basis. Work continued with killifish eggs on developing a better hatching method. A culture bath is planned to be set up in the coming quarter. Preparations were begun in earnest for upcoming transgenic fry hatch. TCE samples were submitted to the chemistry lab for each month of this quarter.

Fish Shipping Summary:

No. of Medaka	Institution	Location
1680	NYU	Tuxedo, NY
750	WVU	Morgantown, WV
50	[a]NIEHS	Research Triangle Park, NC

[a]frozen fin clips of live fish housed at USACEHR

Environmental testing for biological threat agents at U.S. Army Medical Research Institute of Infectious Disease:

Experiments have been performed using the soil extraction procedure obtained from Los Alamos National Labs. An additional wash step was included to determine its effect on the detection limit for spores and vegetative cells. A comparison of the effects of the Sephadex G-200 spin column and the Gibco BRL Glass Max purification system on removing inhibitors in soil extracts has been made. Results indicate that the Glass Max procedure is a better purification method for soil extracts. In mid-February, the Special Pathogens Department received approximately 200 samples for analysis. Of these, 142 are soil samples. Systematic extractions of these soil samples and analysis for threat agents by PCR has begun. To date, 38 samples have been extracted and analyzed.

The Special Pathogens Branch continues work toward implementation of the ISO 25 quality system. This system has met with approval from the Branch's primary customers, and a process action team (PAT) has been organized to oversee implementation of the system. The PAT will help ensure successful certification through external audits. Completion of ISO 25, as well as the positive reputation the Branch has been gaining, will assist greatly in maintaining the customers the Branch currently serves. The Branch also continues to play a key role in the analysis of environmental samples for the presence of threat agents. A recent example was the analysis of criminal evidence during a recent FBI investigation. High priority samples continue to be analyzed using both classical and molecular techniques. These samples continue to test the administrative and scientific measures currently established and implemented by the Branch (i.e., sample receipt, chain-of-custody, archiving, distribution and analysis).

The PCR-EIA optimization for *Brucella* has been completed and a collaboration on a publication is currently underway. *Yersinia pestis* PCR-EIA assay has also been completed. A manuscript on this work is now being written. Improvements on these methods are anticipated at a later date. The TAQman optimization has been finished for *Bacillus anthracis*. Supervision continues with students working on a DNA extraction and PCR testing project for use as a specificity tool for all of the biological warfare agents.

APRIL 1 - JUNE 30, 1998

Investigation of methods available for identifying biomarkers of mammalian hepatocarcinogenesis for adaptation to fish models:

Work effort this quarter was spent evaluating the cellular proliferation indices on slides of medaka livers generated from the final sacrifice point, number 4, of the bromodichloromethane study (Test 600-004) using the Bioquant image analysis system. The raw data was sent to CSU for statistical evaluation. Time was spent preparing and finalizing the poster "5-bromo-2'-deoxyuridine (BrdU) as a biomarker of chemically-induced hepatocellular proliferation in the Japanese medaka (*Oryzias latipes)*" for presentation at the 1998 Conference on Great Lakes Research, May 18-22. Final editorial changes were made to the "Environmental Complex Toxicity Assessment" paper, which was sent back to the *Environmental Health Perspectives* journal. This was accepted for publication in the Nov98 issue. Investigations were made into the feasibility of using the proliferating cell nuclear antigen (PCNA) stain as a biomarker of proliferation in the medaka liver. A protocol for the PCNA stain that was developed for rodent livers at CSU is being modified here at USACEHR for use in the medaka. Archived medaka slides from water disinfection by-product studies, and the diethylnitrosamine carcinogen studies are being used for development of this staining technique. Guidance was given to the summer student currently working on this project. The monitoring of the microbial water quality of the well room continued with monthly samples and cultures. Maintenance and updating of the Refman database, and making budget projections for the biomarker components of the upcoming water disinfection by-product studies was also accomplished.

Research Conducted at Colorado State University:

Early in the quarter, Dr. Faisal of Virginia Institute of Marine Science was contacted to request the use of cell lines he has available. Two AML (Atlantic Menhaven - Liver) cell lines were received in the last third of the quarter for use in research. These cells will be used to perform *in vitro* exposures and subsequent NF-kB studies. To prepare a holding environment for the cells, the incubator needed to be repaired and equipped with carbon dioxide as stated in Dr. Faisal"s protocol. The cells appear fairly hardy and have undergone two passages very well. Several cryovials were frozen in liquid nitrogen for future use and characterization.

Five electrophoretic mobility shift assays (EMSA's) were run in order to attain information on reproducibility of the assay as well as to optimize running conditions using the chemiluminescent kit from Boehringer Mannheim. The results from the controls were promising, but the results for the proteins that were designated as NF-kB were not as expected. Unfortunately, to date, the assay has not produced reproducible results even when using controls that were sent with the kit. Further work will be done to assess the feasibility of using this assay in the future. Should the assay fail to work properly over the next few months, efforts will be directed toward the use of nuclear vectors that will provide a means of NF-kB detection. The first of these vectors has been requested and sent from the NCI laboratory located at Ft. Detrick. A scientific protocol addendum was written for the *in vivo* NF-kB analysis at Ft. Detrick during the month of July. The addendum was approved and the work will commence during the week of July 14th. The remainder of the month was spent in quality assurance (QA) of the chloroform slides. Currently, one day a week is being set aside to perform the QA work, allowing only a total of 6-8 fish to be read each week. This schedule may need to be reevaluated. Bioquant training was provided involving the measuring of FETAX embryos and some statistical analysis of the water disinfection by-product BrdU slides. Windows95 was installed on several of the computers at CSU as well as other software upgrades. All computers were checked for Y2K compliance and a report written and given to Al Rosencrance on the status of the CSU computers. Inventory of all hand receipt items was also accomplished and a master list of all equipment and location was created to aid in tracking equipment at CSU housed in several different buildings. During the next quarter, incorporation of a reporter gene construct will be attempted. Should a reporter gene be able to be integrated into the DNA of the AML cell line, EMSA's will no longer be pursued.

Progress has been made in learning new techniques to further independent research. These techniques include tissue culture, fluorescent *in situ* hybridization (F.I.S.H.) procedures, use of a Zeiss microscope for viewing DNA protein cytogenetic techniques, and new, nonphenolic DNA extraction methods. Progress resulting in useful data has been slow due to other commitments (i.e., GC-MS training) that limit time in the laboratory. Several critical experimental errors also resulted in the need to repeat experiments. Mentoring activities have focused on the training of four individuals in DNA extraction methods and the use of the GC-MS located in the Physiology building. A meeting was attended and an oral presentation given entitled, "Clinical applications of Fourier Transform Infrared spectroscopy" at the 41st Conference on Great Lakes Research, McMaster University, Hamilton, Ontario, Canada.

Two posters from research done at USACEHR, Fort Detrick, were also presented. Plans for the next quarter include experimentation with H_2O_2 survival curves of UVL , AND6, and 179-1 cell lines and adduct analysis using GC-MS and FT-IR. In the final quarter, $Balb_c$ and C57 mice cell lines will be used to investigate H_2O_2 mutagenicity and DNA adduct formation.

Studies of chemical carcinogenesis in medaka:

In order to identify the metabolite peaks seen in gas chromatographs of test fish (Test 100-006), an animal use protocol amendment was written, and two static chloroform exposures were performed. In both cases, chloroform was seen in the liver extracts, but no metabolite was identifiable by mass spectroscopy. Six fish remain to be used on the amendment. A static renewal chloroform exposure for six medaka was planned and carried out in a continued effort to elucidate the identity of chloroform metabolites in medaka. This experiment continued for two weeks. Three medaka were sacrificed after one week of exposure, and the excised livers were pooled and frozen. The remaining fish received the same treatment at two weeks. Liver samples were flash frozen and stored at -70°C. GC/MS analysis is pending. A poster was prepared, approved, and sent to the May 18-22, 1998 IAGLR meeting in Ontario, Canada. The poster compiled information that cut across several projects to showcase our current research. Entitled "Integrated Environmental Assessment of Chemical Contamination," the poster examined results of testing pentachlorophenol with FETAX, immune host resistance assays, killifish acute toxicity tests, and bluegill ventilatory tests.

An abstract was submitted entitled "Toxicological Assessment of Chloroform Using Medaka Fish" to be presented at the SETAC meeting in Charlotte, NC, this coming November. This abstract highlights the work done at USACEHR with internal dose, immune hematocrit, and medaka carcinogenicity from chloroform exposure. SETAC abstract,"5-bromo-2-deoxyuridine Labeling of Hepatocytes as an Indicator of Cellular Proliferation" and IAGLR poster, "5-bromo-2'-deoxyuridine (BrdU) as a Biomarker of Chemically-Induced Hepatocellular Proliferation in the Japanese Medaka (*Oryzias latipes*)" was written collaboratively. A report was begun on the chloroform project for the study sponsor, NIEHS. This report will present methods and results for all chloroform related work (FETAX, cell proliferation, medaka acute toxicity, medaka carcinogenicity, fish internal dose, fish immune hematocrit assays, and chemical analysis of samples during test) performed at USACEHR. An archive was established in Room 216 for strip charts from temperature recorders. Rolls of continuous monitored temperature in the laboratory must be stored in a non-burnable container. Subsequent rolls from each location (i.e., bath 1) are stored together. Test rolls are stored separately from culture rolls. Additionally, suitable vendors were determined for the Ames Test component of the upcoming water disinfection by-product studies.

The 1997 annual report for an extramural contractor was reviewed entitled "Evaluation of Biomonitoring Systems for Assessment of Contaminated Water and Sediments at U. S. Army Installations - Aquatic Toxicity Evaluation of Selected Sites During High Surficial Flow at J-Field, Aberdeen Proving Ground," authored by Dennis T. Burton. The COR was notified of the minor report inconsistencies. All fixed medaka from the six and nine month sacrifice of

Test 600-004, the medaka chronic carcinogen exposure to bromodichloromethane were sent to Experimental Pathology Laboratories, Inc. for histopathology in May 1998. A research proposal was drafted for Physiologically Based Pharmacokinetic (PBPK) Models using medaka fish and bromodichloromethane. In order to have enough sample to be analyzed, medaka from each exposure level would have to be pooled. Approximately 1200 fish would be needed for the proposed study.

Teleost immunotoxicology methods development:

Setup, start and completion of Test # 304-005, Sublethal exposure of bluegill sunfish to pentachlorophenol: *In vitro* Immunological Studies occurred. This test consisted of a 31 day (10 day acclimation and 21 day exposure) housing of 48 adult bluegill using specially designed tanks fitted with a partition system constructed of frosted plexiglass which segregated the fish, yet allowed for equal exposure of toxicant. Fifty percent of the bluegill were inoculated intraperitoneally with formalin-inactivated *Yersinia ruckeri* on the day prior to exposure initiation. The bluegill were then exposed to pentachlorophenol at 2 dose levels (high = 0.140 mg/L; low = 0.035 mg/L) and a no toxicant control for 21 days. Fish were sampled at days 10 and 21 and an immune function assay battery (FISTAB) was performed to determine the immunotoxic effect(s) of sublethal pentachlorophenol exposure. Pentachlorophenol exposure resulted in statistically significant alterations in general endpoints (leukocrit), nonspecific immune function (ROI production), and specific immune function (antibody-forming cell assay and serum agglutination assay). Earlier tests showed that sublethal exposure of juvenile bluegill to PCP altered the ability of the fish to resist bacterial infection from *Yersinia ruckeri.*

A manuscript is in preparation to be submitted for publication in *Environmental Toxicology and Chemistry* entitled "Effects of exposure to pentachlorophenol on host resistance, nonspecific, and specific immunity in the bluegill."

Transgenic medaka research at USACEHR [in collaboration with Dr. James Burkhart at the National Institute of Environmental Health Sciences (NIEHS)]:

One transgenic fish remains at the USACEHR facility of the original brood fish. The latest hatch of sixty-five fry will not be tested since no transfer of the fX174 marker has occurred in subsequent generations tested. Injection of bacteriophage into medaka eggs has been proposed. Dr. Burkhart has been contacted and further work awaits his availability.

Transgenic animal research conducted at NIEHS, Research Triangle Park, NC:

Work continues on genotyping F_1 generation of medaka (*Oryzias latipes*). All offspring (F_1) of medaka appear negative for fX174 marker. Scheduling of new transformation experiments for the medaka is in progress. Genotyping for killifish (*Fundulus hetereoclitus*) has shown all F3 generation are positive, having high copy numbers. In another genotyping experiment, the use of polymerase chain reaction has been essential for amplification of specific genes. This project focused on two specific constructs. One construct was a housekeeping gene that was used as a linker to the second construct, a marker for a neurotox

study. The marker was determined to be lethal in mice homozygous for the marker; therefore, the marker was linked to the housekeeping gene, producing a 616 bp PCR product in heterozygous mice (hemizygous for the marker).

In conjunction with the frog metamorphosis study from the Minnesota sampling, a chloramphenical acetyltransferase assay (CAT) is currently in progress to develop data from the sites sampled to determine whether affected retinoic acid receptors observed have resulted in the malformation of limb buds observed. A plasmid preparation is underway to produce more plasmid to continue the assay. Environmental samples will be assayed early next quarter. Plans for the near future include designing another plasmid with a site-directed point mutation to detect G:C adduct formation induced by xenobiotic chemical exposure. Western blot techniques have been exercised over the past few months to correlate the actual amount of CYP1A1 activity to induced CYP1A1 activity by DMBA.

Methods development for rapid toxicity assessment:

The Killifish culturing continues which includes daily feedings and temperature records as well as weekly tank cleaning, water quality analyzes and embryo collection. A large stock of Killifish embryos stored in peat moss has been accumulating in the incubator. The collection was catalogued and all the packages stored over 30 days were hatched. The rate of recovery was recorded for each package and the embryos were then placed in the hatch tubes. Over 150 killifish fry hatched. The fry were placed in 6 well tissue culture dishes overnight and transferred to 10 gallon aquarium the following day.

Bluegill ventilatory monitoring project:

A new poster for the ventilatory system was finalized this quarter. Two copies of the poster were printed. One copy is on display at Aberdeen Proving Grounds, Old B O field treatment facility; the other poster was presented at the Fort Detrick Earth Day celebration and is now displayed in the laboratory for use during tours. The ventilatory system was set up for a Malathion test but well pump failure caused mortality in many of the test organisms. To prevents tests from crashing, an aeration system was installed in the fish chambers to aerate the chambers in the event of a well pump failure. The aeration system was attached to an auxiliary power source to provides power to the aeration pump when electrical power to the well pump is interrupted. Proposed tests with malathion and pentachlorophenol (PCP) were put on hold to investigate marine biotoxins. Marine neurotoxins were researched to determine their efficacy for use in validating the sensitivity of the ventilatory biomonitoring system to neurotoxic agents. After studying the topic and collaborating with Dr. Mark Poli of USAMRIID, development of test methods and testing was initiated with Brevetoxin and Saxitoxin. Range-find studies were performed for both substances using the ventilatory system. The scarcity of the test substance necessitated the need for either a short flow-through exposure duration or a static exposure. Additional uptake and stability studies were performed for Brevetoxin to determine if static exposures would yield appropriate toxin uptake. Results from this study favored a flow through exposure to insure adequate toxin exposure. A full-scale ventilatory

study was performed with a one-hour flow-through Brevetoxin exposure. An excellent dose response was elicited during the exposure. Data for the range find and full-scale exposure studies were analyzed and graphics generated for all ventilatory exposures performed. Results from the Brevetoxin study clearly indicate the effectiveness of the ventilatory system to detect fish responses to both lethal and sub-lethal concentrations of Brevetoxin. The system was then cleaned and recalibrated to prepare for the PCP test. Maintenance on the ventilatory tests continued daily through the duration of each test and range find. The daily maintenance includes water quality analyses, cycle time checks and data acquisition checks.

Seven bi-weekly reports were written and submitted for the ventilatory data collected at Old O-Field for the periods of 19 March through 25 June. All data was analyzed and archived for these reporting periods. Testing and validation of the efficacy of the new version (2.0) of the Aquatic Biomonitoring System was completed. The system was tested and validated at USACEHR using the electronic fish before implementation at Old O-Field. After adequate validation the new Aquatic Biomonitoring System was installed at Old O-Field. A subsequent trip was necessary to diagnose configurational incompatibilities existing between the new Aquatic Biomonitoring System and the ventilatory amplifier system. When the new system was initiated, the test data were compared to baseline data for the wrong fish due to wiring identification discrepancies; thus, a large number of out of control responses occurred. This problem was diagnosed and corrected. Data collected during this monitoring period was re-analyzed with the correct baseline information for each fish at the end of the test period. Historic Old O-Field ventilatory data from 1995-1997 was permanently archived on Jazz cartridge. The Jazz cartridge as well as the 80 diskettes containing archived data were stored in the computer room vault.

Continuous ventilatory data was also recorded using the Applied Physics Lab data acquisition system. This data was prodvided to Dr. Charles Sarabun for analysis. Research was also begun to determine the efficacy of Trimethylolpropane phosphate (TMPP) as possible test substance for validation with ventilatory system. A 30-minute presentation was given about On-Site Integrated Environmental Assessment Using Mobile Laboratory Facilities at the Technical Cooperative Program Technical Workshop on Environmental Aspects of Energetic Materials. The workshop brought together government professionals from the United States, Canada, United Kingdom, and invited government contractors to discuss a wide variety of environmental issues related to the military use of energetic materials. The workshop was held at the Defence Research Establishment Valcartier (DREV), located just north of Quebec City. The research and development facility=s emphasis is one of protecting Canadian Forces from hazards encountered in the field. The workshop was broken down into two concurrent sessions; Human Health and Environmental Risk Assessment of EM; and Site Characterization and Bioremediation. Most of the interest from the presentation seemed to be focused around the ventilatory biomonitoring system, which was mentioned only briefly to avoid biased toward this subject. The majority of the talks focused mainly on soil contamination with TNT and RDX and the their degradation by-products. Canadian and U.K. representatives focused on soil toxicity only. Canada is still in the beginning stages of characterizing and eliminating sources of soil contamination and has yet to the look at groundwater fate at the sites being

tested. A lack of funding was apparent. Testing and evaluation using Microtox and earthworm studies were presented. All were in agreement that toxicity testing is a necessary process in determining the relative hazards of contaminated sites. A CD ROM copy of all of the presenters' papers will be sent to all attendees. The earthworm studies presented seemed to be quite advanced (for testing TNT and RDX) and may have some merit if soil toxicity test methods were to be developed at USACEHR. Researchers from DREV and the Biotechnology Research Institute in Montreal have been collaborating together using the earthworm model. They have found that the degradation by-products of TNT are more toxic to earthworms than TNT. Researchers at DREV seemed very open to collaborative research with other TTCP countries and have participated in joint studies on a number of occasions in the past at various U.S. and Canadian military sites.

Analytical method development and analysis of chemicals used in carcinogenicity and laboratory studies:

Approximately 50 samples of Xenopus ovaries and embryos were analyzed for cadmium by ICP-MS. High levels of cadmium were found in some of the controls. The source of contamination was unknown, but the sample containers and method of preservation are suspect. The samples arrived in formalin in glass containers. Glass contains a variety of trace metals and should be avoided if possible. A second batch of samples was properly preserved in nitric acid in plastic containers. Low recoveries of the spikes on the first set of samples suggested a matrix interference effect on cadmium. 20 % nitric acid was used to prepare the standards used for the analysis of the digested Xenopus samples. This significantly improved the recoveries. The microwave method was also altered to decrease the time required for the samples to reach the assigned pressure. The vessels were not completing the digestion in the time required by the digestion vessels. Several effluent and samples from Aberdeen Proving Grounds and a new batch of brine shrimp were analyzed for the usual suite of trace metals. Approximately 150 samples of pentachlorophenol were analyzed by high performance liquid chromatography in support of immunotoxicology testing. Approximately 40 samples of chlorate were analyzed by ion chromatography in support of FETAX testing. Several samples of water and soil were received from Aberdeen Proving Grounds for munitions testing. No munitions were found in the samples.

Methods for the analysis of Boron and Dibromoacetic acid in various Xenopus tissues are being evaluated. The method for Boron will be similar to the method used for the analysis of Cadmium. The extraction of dibromoacetic acid from the tissue samples will not be as simple. Several methods are being evaluated. The likely method will consist of saponification of the blood or tissue samples, followed by a clean-up step using solid phase extraction and analysis by HPLC.

Maintenance and optimization of USACEHR aquatic laboratory facilities:

Essential laboratory maintenance was performed on all culture (medaka, bluegill, fathead minnows, and killifish) and test fish (medaka) located in rooms 5 and 18. Daily

activities include three daily feedings of all culture and test organisms. Associated record keeping was performed to maintain necessary test records to meet SOP and regulatory review requirements. Associated tank cleaning and siphoning was performed to maintain overall tank hygiene necessary to accommodate healthy test organisms. Maintenance and culturing of all live food (brine shrimp and microworms) systems was performed to facilitate ongoing laboratory efforts. Weekly activities also include the following water quality analysis; dissolved oxygen, ph, conductivity, water flow measurements, ammonia, and light measurements. Monthly activities include TCE sampling of well water. Essential laboratory supplies were documented and ordered as needed for the month.

The medaka colony renewal that was scheduled for early April had a hatch rate of 77%. The fish lab reorganization and reordering of supplies was completed. The three week process of medaka culture renewal for July began on June 8, with fry hatching out in early July. The results of a 102 day light bulb trial in the aquaculture facility were summarized. Of the two bulbs compared, Optima Choice, currently used, remains the bulb of choice. Although higher light intensities and higher egg production were seen in the Power Twist tank, the percent hatch in the Power Twist tanks were lower. Additionally, the Power Twist tank had a slightly higher mortality rate than the Optima Choice tank. The trial continues with a second set of same age fish. Several changes in food analyses were noted during this quarter. Since a sizeable data base has been built for both frozen brine shrimp and brine shrimp cysts, the analysis procedure for these foods has been amended to include metals only for repeat purchases from the same vendor. For the frozen brine shrimp, each shipment will be analyzed for metals, with at least one sample per year being run by an outside lab. For the brine shrimp cysts, the manufacturer analyses records are being requested for each lot received. USACEHR chemists will analyze each lot of brine shrimp cysts for metals to confirm manufacturer results. Due to lower animal numbers and decreased demand for medaka, an excess of flake food from Lot 2 remains in our -70EC freezer. This food was given an extended expiration date (one year added to shelf life) to make use of the current supply.

Fish Shipping Summary:

No. of Medaka	Institution	Location
1190	NYU	Tuxedo, NY
300	WVU	Morgantown, WV
771(fixed)	EPL	Herndon, VA
No. of Bluegills		
5	NYU	Tuxedo, NY

An estimate was made for the material needed to increase the culturing capacity for the *Xenopus*. A large pan was drawn and submitted to the machine shop on post for a cost estimate. A material list was also made and cost estimate developed for expanding the plumbing system to deliver water to the additional culturing tanks.

Environmental testing for biological threat agents at U.S. Army Medical Research Institute of Infectious Disease:

Systematic extraction and analysis has continued on the 142 soil samples received by special pathogens last quarter. To date, 132 samples have been extracted. 106 have been tested for inhibition and analyzed. The sensitivity and specificity determination for the PCR-EIA assay for *Clostridium botulinum A, Clostridium botulinum B, Staphylococcus enterobacter A, Staphylococcus enterobacter B,* and *Francisella tularensis* is halfway completed. Taqman assays for the special pathogens samples were started for the detection of several organisms until the task was assigned to newly hired personnel. Training was provided to the new personnel, as well. Several students were trained in molecular biological techniques and assistance was given to them on their individual projects. The Yersinia pestis PCR-EIA manuscript is undergoing revisions. This work was presented at the Spring Research Festival poster session in May. Priority work now is to obtain results from PCR-EIA for the CAP and PA genes of anthrax in under two hours. Documentation is also being kept compiling optimum conditions, sequence information and sensitivity limits for detection of organisms for PCR-EIA and taqman assays.

The Special pathogens Branch continued to play a key role in the analysis of environmental samples for the presence of threat agents. High priority samples continue to be processed and analyzed using both classical and molecular techniques. The assays currently used by the branch were tested when an outside laboratory submitted spiked control samples for analysis. Final interpretation of the data is currently not complete. Work also continues toward implementation of the ISO Guide 25 quality system. The Office of Product Development and Regulatory Affairs at USAMRIID has been providing guidance and consultation regarding implementation of the quality system. The branch acquired additional laboratory space this quarter and has included this space under the same ISO Guide 25 system. A manual was prepared outlining the administrative and technical activities of the Special Pathogens Sample Testing laboratory (SPSTL). Portions of the manual will be used for a Senate briefing. Proficiency in fatty acid analysis using MIDI instrumentation continued this quarter. Currently, a stock culture collection of quality control organisms is being characterized by the new method. Clinical microbiology consultation was provided to the Medical Division regarding an upcoming protocol on enterotoxigenic *Escherichia coli* (ETEC). Two presentations were made at the 98th Annual Meeting of American Society for Microbiology in Atlanta, GA, May 17-22 entitiled "Rapid viability assessment of biological threat agents" by Teska, J.D., S.R. Coyne, E.A. Henchal, T.L. Hadfield, E.J Hilyard, and J.W. Ezzell, 1998 and "Excretion of live, oral *Shigella flexneri* 2a vaccine SC602 during a comminity-based phase 1 trial" by Teska, J.D., T. Coster, J.R. Colber, Q.Rance, M. Venkatesan and T.L. Hale, 1998. A poster comparing three different extraction procedures for *B.anthracis* from soil was also written and presented at the meeting and at the Spring Research Festival at Fort Detrick. The following manuscripts were written during the quarter:



Teska, J.D., T.Coster, J.R. Colbert, W.R. Byrne, T.L. Hale and M. Venkatesan. 1998. Novel self-sampling culture method to monitor excretion of live, oral Shigella flexneri 2a vaccine SC602 during a community-based phase 1 trail. *J. Lab. Clin. Med.* **(In Review)**

Teska, J.D., S.R.Coyne, E.A. Henchal, T.L. Hadfield, E.J. Hilyard and J.W. Ezzell. 1998. Rapid viability assessment of biological threat agents. *Biomed. Lett.* **(In Review)**

Teska, J.D., E.A. Henchal and J.W. Ezzell. 1998. Establishing an analytical laboratory in support of biological weapon counter proliferation and terrorism. **(In Preparation)**

Task Order RM-2



GEO-CENTERS, INC.

Title: FETAX Method Validation
Task Number: RM-2 (2533-008)

This task requires technical support for basic and applied research to develop new non-mammalian toxicity test models.

MARCH 1-30, 1993

Experiments were initiated on Phase III of the Frog Embryo Teratogenesis Assay - *Xenopus* (FETAX) Validation Project. This phase of the project involves testing unknown test compounds. The first assay (Chemical A) failed due to high mortality among the control embryos. The latter part of the month was spent preparing for the Mid-Atlantic Water Pollution Biology Workshop. A one-day workshop on FETAX was offered as part of the meeting. Nine registrants participated in the workshop.

APRIL 1-30, 1993

Work continued on the Interlaboratory Validation Study of FETAX. Testing of the coded chemical labelled P3C was completed. Screening tests of test compounds P3A and P3B were also completed. Testing in the FETAX assay for compounds P3A and P3B is scheduled for next month.

MAY 1-31, 1993

Work continued on the Interlaboratory Validation Study of FETAX. During the month of May, testing was completed on one coded sample as part of phase three of the Interlaboratory Validation Study.

JUNE 1-30, 1993

Work continued on the Interlaboratory Validation Study of FETAX. During the month of June, testing was completed on the second definitive assay for the coded sample P3A. This test took longer than anticipated due to bacterial contamination of samples, which resulted having to repeat experiments. Screening assays for two coded samples and three definitive assays for coded sample P3D were also completed for phase three of the Interlaboratory Validation Study. Initiation of experiments to investigate incorporation of metabolic activation into the FETAX assays is planned for July.

JULY 1-31, 1993

Work continued on the Interlaboratory Validation Study of FETAX. During the month of July, testing was completed for the first five Phase III coded samples. Length measurements for these tests were completed during the last week of July. The Metabolic Activation Study which incorporates a mammalian metabolizing system into the FETAX assay will begin in August.

AUGUST 1-31, 1993

Work continued on the Interlaboratory Validation Study of FETAX. Work was initiated to incorporate a mammalian metabolic activation system into the FETAX assay. This part of the Interlaboratory Validation Study will serve to correlate the FETAX results with those typically found using a mammalian test model to evaluate the teratogenicity of similar chemicals.

SEPTEMBER 1-30, 1993

Work continued on the Interlaboratory Validation Study of FETAX. During the month of September, work continued on coded chemical P3M1 using the metabolic activation system designed to work in conjunction with the FETAX assay. The progress has been slow due to a number of problems involving the microsomes, including the pH of the generator system and the lack of mortality in the positive controls. Also, OSU has been slow to provide both coded chemicals and microsomes which further impedes progress.

OCTOBER 1-31, 1993

Work continued on the Interlaboratory Validation Study of FETAX. During the month of October, our work on P3M1 was stalled while waiting for supplies from OSU. This "down" time was used for general housekeeping, paperwork, and a massive chemical inventory reduction. During the last week of the month, one assay was run using P3M1 and a doubled volume (2X) of the microsomal preparation. The results showed some microsomal activity. We observed a dose response, but our negative controls exhibited 100% mortality. This microsomal preparation was tested for protein concentration using the Pearson Protein Determination Method. Different spectra were also used to demonstrate the peak due to the presence of cytochrome P-450. The next assay will involve a series of controls, which should be helpful in isolating problems. Some of the parameters to be tested include: vortexing the microsomes, heat inactivation of the microsomes, and changing the order of addition of the metabolic activation system components.

NOVEMBER 1-30, 1993

Work continued on the Interlaboratory Validation Study of FETAX. During the month of November, a screening test on the coded compound P3F was performed. After determining the crucial concentrations, one definitive test was completed and two more definitive tests on this compound are in progress. In addition, a test using the Metabolic Activation System control compounds was completed. This test was instrumental in perfecting the procedure and technique for the proper utilization of the Metabolic Activation System with FETAX.

DECEMBER 1-31, 1993

Work continued on the Interlaboratory Validation Study of FETAX. During the month of December, three definitive tests were performed on the coded compound P3F. Only one of these tests was acceptable for use in the interlaboratory study; the criteria for test acceptance were not met in the other two tests. Work in the laboratory this month was interrupted for a considerable period of time due to painting.



JANUARY 1-31, 1994

Work continued on the Interlaboratory Validation Study of FETAX. During the month of January, we completed testing of coded sample P3F. Length measurements and data analyses of P3F will be performed during February.

Testing of coded sample P3M2 began this month, and work continues on the coded sample P3M1. We are still experiencing problems related to the metabolic activation system. Toxicity related to the microsomes is one problem. This is beyond our control, since OSU provides all participating laboratories with microsomes. We intend to test microsomes for toxicity before using them in an assay. The failure of the negative controls to survive at a satisfactory level is another recurring problem. We have been instructed by Jim Rayburn to "reuse" CO gassed microsomes throughout the week during a single assay. We suggest the use of freshly thawed CO gassed microsomes daily after observing coagulation in the "reused" microsomes. This matter should be resolved next month. We also attempted to use sodium dithionite in conjunction with CO gassing for our negative controls. We were instructed by Dr. Fort as to the amount to be used. This amount caused 100% mortality in the negative controls. We have successfully used sodium dithionite in smaller quantities in a test of controls performed in November. Much work is still necessary in order to obtain the proper results using the metabolic activation system with FETAX.

FEBRUARY 1-28, 1994

Work continued on the Interlaboratory Validation Study of FETAX. Length measurements and data analyses of P3F, P3M1 and two assays of P3M2 were completed this month. Troubleshooting for incorporation of a metabolic activation system into FETAX continued. Work this month concentrated on obtaining the required results in the negative control; controls were tested using sodium dithionite with CO-gassed microsomes. This combination gave the required results. In an attempt to get approximately 50% mortality and higher malformation rate, the generator system was excluded from the negative controls. This did not work at all, however, we still intend to try leaving out the enzyme stock in the next experiment. Cyclophosphamide, in the specified concentration for the positive and negative controls, had no effect on the embryos in the absence of the metabolic activation system. Sodium dithionite alone slightly enhanced the growth of the embryos.

MARCH 1-31, 1994

Work continued on the Interlaboratory Validation Study of FETAX. Failure to obtain viable eggs from 19 pairs of frogs over a two-week period slowed testing of sample material. One viable clutch of eggs was obtained to perform the final test of coded sample P3M1. Determinations for a precise amount of sodium dithionite added to microsome to produce a negative control were also done. Continued to examine various parameters for potential use in monitoring/predicting breeding success (eg. weighing egg clutches).



APRIL 1-30, 1994

Work continued on the Interlaboratory Validation Study of FETAX. Assays are currently being performed on coded samples from the National Toxicology Program (NTP). These chemicals are coded NA, NB and NC. Screening tests for NA will be complete this week, while NB and NC will require additional testing.

One experiment using sodium dithionite was also performed. The purpose of this experiment was to determine the amount of sodium dithionite necessary to obtain the required results in the negative controls.

Pilot experiments for use of volatile chemicals in FETAX were begun this month. Two experiments were conducted to determine the amount of solution required to enable embryos to survive in air tight containers. The experiments to use volatile chemicals will be performed using two water samples from NTP (Iowa mix and California mix). These experiments are scheduled to begin early next month.

MAY 1-31, 1994

Work continued on the Interlaboratory Validation Study of FETAX. Screening tests of the three coded samples from NTP (NA, NB and NC) were completed this month. Definitive testing of these compounds will begin after we have received a spreadsheet from OSU to determine the relevance of using the Metabolic Activation System on each compound.

Testing of the California water sample from NIEHS was initiated this month. Since the sample contains volatile chemicals, wide mouth jars with lids are being used in the place of petri dishes. Dose-independent, excess morality was observed in the first pilot study. This may have caused by low dissolved oxygen. Testing is currently being conducted to determine the maximum number of embryos which may be supported by the dissolved oxygen levels maintained in the jars.

JUNE 1-30, 1994

Work continued on the Interlaboratory Validation Study of FETAX. Three separate tests were completed this month with the California Mix IIA testing series. The first test (209-02) was the second range find for the mix. A dose response was seen for malformations but not mortality. The question was raised as to whether the malformations were due to the chemical itself or the test conditions. The second and third tests performed attempted to answer this question. Preliminary data from a Dissolved Oxygen test (209-04) and a test for Osmolarity (209-05) indicate that the malformations recorded in the second range find are a result of the California Mix IIA solution. Testing of this mixture is expected to continue next month.

For the past several months, the FETAX laboratory has been having a problem obtaining enough "good" eggs to run an assay. The problem is currently being carefully investigated. New breeders have been ordered from *Xenopus I;* in the meantime, individual records are being reviewed to determine if particular animals are no longer reproductively successful.



Euthanization of animals has already begun, and the laboratory expects to reduce the current colony by approximately 35 animals which will then be replaced with the new breeders when they arrive. Dr. Bantle's laboratory has also suggested that the injection regimen be changed by using smaller doses and only one injection rather than two. This new regimen will begin with the new breeders.

The latest NTP protocol was received this month. Work will begin next month using this new protocol.

JULY 1- SEPTEMBER 30, 1994 (Government approved Quarterly Progress Reports in lieu of Monthly Progress Reports)

Work continued on the Interlaboratory Validation Study of FETAX. Training on the use of SPSS, a PC-based statistical software package, has been ongoing throughout the current reporting period. No assay was performed during the first week of July due to the holiday. The week was spent performing miscellaneous tasks to maintain the laboratory and to prepare for future assays.

During the second week of July, the first assay on the Iowa Mix (210-01) was performed. This test was the initial screening for the mixture, however, the concentrations chosen were too high and resulted in 100% mortality for all replicates. A high rate of malformations in the controls (34%) was also experienced.

After attending the in-house aquaculture meeting, new methods were implemented to control stress on the frogs. All extraneous equipment was removed from the frog culture room (room 22), the Killifish experiments were moved to room 7; room 22 will now be locked after working hours. This implementation coincided with the arrival of the new frogs, colony VI. Colony VI consists of 25 females and 10 males.

For the third week of July, the sixth assay (206-06) was performed on coded sample, NA using the latest protocol. This protocol requires running duplicate metabolically activated and inactivated concentration ranges. Metabolic activation appears to increase the toxicity of NA, but the current protocol does not allow for quantification of the differences between the two systems. The current protocol is designed to yield an EC50 and LC50 only for the inactivated concentrations. The FETAX-AB controls exhibited a malformation rate of 18% which is unacceptable, however, the controls for the metabolic activation system were within limits. All the other controls for this assay yielded the expected results.

An assay was scheduled for the fourth week of July, but due to poor quality eggs, a test could not be performed. All six pairs of frogs produced clutches consisting of a large percentage of unfertilized eggs. The frogs used for injection were from the new group, Colony VI. A different injection schedule is being used in which the frogs are only injected once on Sunday with a lower dose of HCG. It has been suggested that the frogs build a tolerance to the HCG and continuously need larger doses until they are no longer sensitive to the HCG. Since the dose of HCG has been decreased, no necrotic clutches of eggs have been observed, which has been common in the past. It has also been noted that one frog from each tank appears to

produce viable eggs more consistently than the others from the same tank. Further investigation is needed on this subject.

Fresh *Xenopus* breeding stock, designated Colony VII and consisting of 25 females and 10 males, was received in-house in late July. Therefore, most of the older frogs were euthanized to make room for the new stock. The most successful breeders were kept to continue mating for comparison purposes. Several of the frogs in the new shipment had skin lesions. The frogs with possible nematode infections were examined by Colonel Powell, the post veterinarian (LACUC). Two of the worst cases were euthanized to give to RIID for a complete necropsy. During the final week of September, a pathology report was received from RIID on the two frogs euthanized for Colonel Powell. RIID reported low numbers of a protozoa-like structures in association with the lesions in the epidermis of the animals. The structures were not identified but consultation is still in progress. Colonel Powell verbally reported that he was expecting more information in the near future and that he would let us know as soon as he received an update.

Definitive test (206-07) of coded sample NA was conducted in August-September. Four pairs of frogs were injected (last day of the full moon), but only one pair produced a usable clutch of eggs. This incidence is in keeping with our moon phase hypothesis. The test fell within acceptable parameters for malformations and mortality, but 100% mortality was not achieved in the positive control. Further, high rates of mortality occurred in lower concentrations than what was expected from previous runs of this chemical. Plans are to run NA again soon in an effort to determine its true teratogenicity. Also, remaining frogs from Colonies III and IV were euthanized due to decreased sensitivity to HCG and subsequent drop in embryo production.

During the second week of September no assay was performed due to the Labor Day holiday. This week was used for cleaning the laboratory, scrubbing the frog baths, paper work, and general lab duties. Time was also spent preparing for our first field study with FETAX at Aberdeen Proving Grounds in the biomonitoring trailer.

The first field study was performed in the biomonitoring trailer in the later part of September. Six pairs of frogs were injected (last day of a new moon); one viable clutch of eggs was produced and used for test 401-003R. The assay was a flow-through system to test the ground water on the West Branch of Canal Creek in Aberdeen. The assay consisted of two distinct parts, one which used creek water as the diluent and one which used tap water as the diluent. Results from this test suggest that the ground water is not as toxic or teratogenic as either of the two diluents. The creek water controls obtained 47% mortality and decreased with the addition of ground water. The tap water controls obtained 31% malformations which also decreased with the addition of ground water. Other problems experienced involved the possible escape of some embryos into the surrounding medaka tanks and the intrusion of medaka into the frog chambers. Also the pump in the trailer stopped working for approximately 18 hours causing a static condition which increased ammonia concentrations and decreased dissolved oxygen levels. Future considerations involve performing static assays on the creek and tap water using the FETAX solution as a diluent and repeating the initial study to determine if the results seen are an accurate depiction of developmental toxicity.

During the final week of September six pairs of frogs were injected (full moon period); one viable clutch of eggs was produced . This clutch was used to perform a definitive Iowa Mix study. Currently, the test appears to be doing well but will not be completed until tomorrow morning.

OCTOBER 1 - DECEMBER 31,1994

Testing of the coded samples designated by the National Toxicology Program was continued throughout the quarter. Nine NTP assays were attempted. Three of these attempts failed due to an unacceptable percentage of mortality and or malformation in the controls. Four attempts failed due to insufficient viable embryos available to initiate an assay. One acceptable definitive test of coded sample NA was completed. The third definitive test remains to be completed before moving on to the next compound.

A significant test of the California water sample from NIEHS was performed during the current reporting period. The test compared the preparation of the stock solution using deionized water versus FETAX solution. The use of different stock solutions had no effect on the embryos. The next assay performed on the California water sample will be designed as a definitive study.

The SETAC conference in Denver was well attended. Valuable information about FETAX and the Interlaboratory Validation Study, as well as information about other biomonitoring assays, was gathered both during the conference and in a meeting of FETAX researchers from around the country who were attending SETAC.

One week of the quarter was spent in the mobile biomonitoring facilities at Aberdeen Proving Ground. The FETAX assay was employed in testing water from the west branch of Canal Creek, ground water from this area, and dechlorinated tap water. The FETAX assay was performed using the normal static renewal procedure as well as under flow-through conditions. Controls in FETAX solution were also included in this study. The results of this study closely paralleled that of the first flow-through assay in that 4 1 % of the embryos treated with tap water were malformed. The percentage of malformed embryos dropped to 10. 5% when I% ground water was added to the tap water. Overall, the malformation rates were much lower in the static renewal assay in which FETAX solution was used as the diluent. The absence of essential salts from the test waters may cause the abnormal development of the *Xenopus* embryos. As a preliminary test of this hypothesis, embryos were treated with well water. The mortality rate was not significantly higher than that of the controls; however, all of the embryos were severely malformed, probably due to the insufficient salt content of the well water.

Native African *Xenopus Laevis* adults were received from Xenopus Express in December. These animals are believed to be more fecund as well as more sensitive to HCG than the laboratory bred animals that are currently being used.

An additional technician began work on the FETAX project during the first week of December. Training began during this quarter and will continue into the next quarter.

During the first week of December, an in-house data seminar on the Interlaboratory Validation Study was presented. The presentation raised some interesting questions about both FETAX and the Interlaboratory Validation Study. These questions are presently being addressed and should be answered in the next quarterly report.

Other work carried out throughout the quarter included the length measurement of embryos using the Bioquant Morphometry System. Further investigation into the moon phase theory using SPSS is currently in progress. Also, light/dark cycle is currently being considered as a more controllable factor which may influence the breeding cycles of Xenopus laevis. Efforts continue in finding a solution to unreliable embryo production.

JANUARY 1 - MARCH 31, 1995

Testing of the coded samples designated by the National Toxicology Program (NTP) was continued throughout the quarter. Ten NTP assays were attempted. Nine of these attempts were successful. One assay failed due to a malformation rate of 20% among the control embryos. During this quarter, the definitive testing of the coded chemicals NB, NC, and ND was completed.

Two definitive tests of the NIEHS water sample, California Mix IIA, were successfully completed during the quarter. Another test of this water sample is scheduled for the second week of April.

Two weeks during the quarter were used for field testing at the mobile biomonitoring facilities at Edgewood Area, Aberdeen Proving Ground, MD. Testing took place in both January and March. During each week, a static test and a flow-through test were performed. The assays performed in January (402-004R and 402-005R) tested the water from the west branch of Canal Creek, ground water from this area, and dechlorinated tap water. Mortality was greater in the flow-through test using water from Canal Creek. Malformations decreased as greater amounts of ground water were added to the creek water in the flow-through test. Malformations and mortality remained insignificant in all flow-through concentrations of tap water and ground water. The static test exhibited the highest mortality (21%) in 100% creek water. All malformations found in the static test were below 10%. The assays performed in March (402-006R and 402-007R) tested the ground water from the area of the west branch of Canal Creek and dechlorinated tap water. The creek water testing was completed in February. There were no significant findings to report from these assays.

Due to unreliable production of fertilized embryos, a telephone conference was arranged with Drs. Bantle and Fort. During this conference several changes were suggested and implemented in our culture facilities and in our injection regimen. Both culture and breeding are maintained at 21 degrees Celsius. The colony is being fed on a daily basis. The injection schedule found to be most effective consists of 150 IU's of HCG administered to all frogs at approximately 1500 hours (3 p.m.) followed by 500 IU's administered to males and 750 IU's administered to females at 1600 hours (4 p.m.). These changes were slightly more successful than our previous format. A fourth change was implemented one week later. This change involved separating the pairs of mating frogs so that each pair cannot be disturbed by the



presence of other frogs. After the implementation of this final change, the mating of six pairs of frogs yielded five viable clutches. This occurred during the last week of February while the barometric pressure remained at a steady high throughout the breeding period. This success could also be associated with the approach of spring, although this kind of breeding success has not been experienced again. Since the breeding temperature has been lowered from 25°C to 21° C, the embryos are often at stages too early to begin sorting. In an effort to eliminate this problem, the preliminary injection is given at 1200 hours (noon) followed by the final injection at 1300 hours (1 p.m.).

Other work carried out during the quarter included length measurement of treated embryos, using a two tailed T-test to find the MCIG, analysis of raw data using Quattro Pro software, as well as general laboratory maintenance and husbandry tasks.

APRIL 1 – JUNE 30, 1995

Testing of the coded samples designated by the National Toxicology Program was continued throughout the quarter. Nineteen NTP assays were attempted during the quarter. Four of these assays failed due to malformation rates among the control embryos that were beyond the acceptable limits. During this quarter, the definitive testing of the coded chemicals NE, NF and NG was completed. The data analysis of the coded chemicals NC, ND, NE and NF using Quattro Pro software was also completed. Thirteen definitive tests remain to be performed in order to complete the NTP chemical testing. That testing is projected to be completed by the end of the next quarter.

One definitive test of the NIEHS water sample, California Mix IIA was successfully completed during this quarter. One definitive test of the NIEHS water sample, Iowa Mix III is currently in progress. The continuation of work on these chemicals is dependent upon the schedule of the chemists.

One week during the quarter was utilized for field testing at the mobile biomonitoring facilities at Aberdeen Proving Ground. Testing took place during the second week in May. A flow-through assay was used in testing dechlorinated tap water and ground water from the immediate area. Also, a static test of the water from Canal Creek, dechlorinated tap water and ground water using FETAX solution as the diluent was conducted concurrently. Both of these tests exhibited high mortality rates and high malformation rates among the control embryos. In comparing these tests to the previous seven tests of this series, no trend was found among the data. The data collected at Aberdeen Proving Ground has been analyzed using Trimmed Spearman-Karber to determine EC50's and LC50's and is currently being summarized to accompany the Aberdeen Proving Ground multi-species test system data in the quarterly report from the University of Maryland.

The third week in April was the only breeding during the quarter that did not produce adequate embryos for testing. Six tests failed due to high mortality and/or high malformation rates among the control embryos. Three of these tests were performed in the same week and all embryos used were taken from the same breeding pair of adult *Xenopus laevis*. Mindi Hull of OSU has reported experiencing the same difficulties with high malformation rates among the



control embryos.

Other work carried out during the quarter included the writing of a protocol for the calibration of micro-pipettes, length measurements of test embryos, using a two tailed T-test to find the MCIG, analyses of raw data using Quattro Pro software, as well as routine laboratory maintenance and husbandry tasks.

JULY 1 - SEPT 30, 1995

Testing of the coded samples designated by the National Toxicology Program continued throughout the quarter. Sixteen NTP assays were attempted. Eleven of these attempts were successful while two assays failed due to a malformation rate that was above the acceptable limits. A power outage that resulted in a temperature drop from 25□C to 15□C was the suspected cause of one of the failed tests. During this quarter, the definitive testing of the coded sample NI was completed. The data analysis of the coded chemicals NG and NI using Quattro Pro software was also completed. Four definitive tests remain to be performed in order to complete the NTP chemical testing. The projected completion date is November 1995.

Three definitive tests of the NIEHS water sample, Iowa Mix III, were successfully completed during this quarter. This finalizes the testing phase of the NIEHS water samples. Length measurements of test embryos and data analysis via Trimmed Spearman-Karber to determine LC50 and EC50 values were also completed and a written summary is in progress.

Difficulties in obtaining consistent embryo yields this quarter have been experienced. Several theories have been postulated to account for this phenomenon. Record high temperatures followed by a sharp temperature decline during a cold front in the mating periods may have had an effect. Investigations are being conducted to alleviate this set back in productivity. One new approach has been to establish proven and reliable breeders within the *Xenopus* colony through the tracking of breeding records. However, this season has historically shown little breeding success. During this quarter, four breeding attempts resulted in nonviable oocytes that were necrotic, unfertilized, or insufficient in number to be used for testing.

Upon completion of the field testing at the mobile biomonitoring facilities at Aberdeen Proving Ground, a summary report is being written to be incorporated into the University of Maryland's quarterly report reviewing the APG multispecies toxicity data.

Other work carried out during the quarter includes length measurements of treated embryos using a two-tailed T-test to determine the MCIG, analysis of raw data using Quattro Pro software, and general laboratory maintenance and husbandry tasks.

OCTOBER 1 - DECEMBER 31, 1995

As part of the Interlaboratory Validation Study, the FETAX laboratory performed several tests including the following: NJ 219-004, NJ 219-005, NM 222-003, NK 220-002, NK 220-003, NJ 219-006, NG 216-004, NJ 219-007, NM 222-004, NJ 219-008, NJ 219-009, NG 216-005, and NG 216-006. Length measurements using a computerized biomorphometry system

were initiated for all the assays yielding acceptable results, and statistical analysis was then performed using a spreadsheet customized for the FETAX assay.

Using information obtained from a professional conference, a new method of euthanizing frogs was attempted with success. Two unhealthy frogs were injected with a lethal dose of MS-222 rather than being immersed in an MS-222 solution. The initial trial required three injections to euthanize the frogs, therefore, further experimentation on proper dose levels for *Xenopus* is needed. However, the investigation still demonstrated that the new method requires less MS-222 and results in a quicker response in the frogs.

Extensive research on the subjects of Neurotoxicity and Reproductive Toxicity has also been initiated in an attempt to prepare for a new project after the completion of the ILS. The laboratory intends to expand its research with *Xenopus* into these two areas. The literature review has revealed that both of these topics are relatively undeveloped with *Xenopus* and that there is a need for an assay which can adequately assess the potential of a substance to effect neurotoxicity and reproductive behavior.

The FETAX laboratory continues to maintain a colony of over 100 South African clawed frogs. Personnel performed general maintenance as well as quarterly chores such as light intensity measurements, water quality analysis, and GFI testing. All laboratory procedures adhere to USABRDL SOP's and follow GLP as much as possible.

JANUARY 1 - MARCH 31, 1996

Testing of the coded samples designated by the National Toxicology Program was continued throughout the quarter. Six NTP assays were attempted. Three of these attempts were successful. Three assays failed due to a malformation rate that was above the acceptable limits. During this quarter, the definitive testing of the coded samples NE and NJ were completed. The data analysis of the coded chemicals NE and NJ using Quattro Pro software was also completed. This finalizes the definitive testing of the NTP designated chemicals.

Other work carried out during the quarter includes length measurements of treated embryos, using a two-tailed T-test to determine the MCIG, analysis of raw data using Quattro Pro software, and general laboratory maintenance and husbandry tasks. Additional effort was made both in the laboratory and animal room to ensure compliance for the AAALAC site visit in February, 1996. The FETAX ILS animal use protocol was updated into the latest format and an animal use protocol was created for culturing adult *Xenopus laevis.*

A cost estimate for the water disinfection by-product study using FETAX was generated. The manuscript for Phase 111, part I of the ILS was proofread and suggestions for revisions were offered. The revision of the ASTM Standard Guide for Conducting the Frog Embryo Teratogenesis *Assay-Xenopus (FETAX)* and associated SOP's will continue into the next quarter.

As part of the biomonitoring trailer demonstration for the Department of Energy scheduled for April in Ohio, frog embryos were allowed to develop in order to determine their culturing needs. The embryos were shipped from BRDL to CSU and back again as part of an



experiment to determine the effects of shipping procedures. Upon their return, the embryos were still viable. Water temperatures had dropped to 12°C, well outside of the 18 to 26°C recommended. The emerging larvae were fed brine shrimp daily as well as ground shrimp pellets and an amphibian/reptile food manufactured by Tetra. At the end of three weeks, survival rates were very low (< 20%). The larvae were then transferred to the biomonitoring trailer where the remaining embryos died. Excessive temperature fluctuation from air-handling system failure within the laboratory and from incoming water supply to the bio-monitoring trailer were contributing factors to the failure of the larva's continuing metamorphosis. More experimentation will be performed the last week of the quarter. To resolve some of the problems encountered, the embryo shipping container will be transported along with medaka and bluegill transportation containers inside an insulated box to help maintain a stable shipping temperature. Frog embryos will be shipped to the biomonitoring mobile laboratories for use in the DOE demonstration if water quality problems (excessively high pH and chlorination) have been corrected.

APRIL 1 - JUNE 30, 1996

Testing of the coded samples designated by the National Toxicology Program was completed this quarter. Data analyses were finalized and sent to Dr. Jack Bantle at Oklahoma State University.

A field demonstration of the FETAX assay in the mobile biomonitoring facilities for the Department of Energy at Fernald Remediation Site in Cincinnati, Ohio was conducted in April, 1996. The Alternatives in the Assessment of Toxicity meeting held at Aberdeen Proving Ground was attended. Standing Operating Procedures for conducting FETAX were revised. After a final review, these revisions will be complete. Animal use and scientific protocols were revised into the updated format and distributed. The procedures for BRDU staining of *Xenopus laevis* embryos and sectioning for histological examination are currently being investigated. Range finding studies were initiated to provide comparative FETAX data to that of the ventilatory in-house validation study conducted with the chemicals pentachlorophenol, phenol, unionized ammonia, malathion and 3-aminobenzoic acid ethyl ester (MS222).

Embryo production has been adequate during the past 18 months. However, many of the most recent assays have exhibited malformation rates among the control embryos beyond the acceptable limit for the standard FETAX assay. Due to recurring high percentages of malformed control embryos, it was determined that the adult *Xenopus laevis* breeding colony should be replaced. The colony was euthanized and replacements frogs have been ordered. Embryos are currently being cultured beyond the standard 96 hours outlined in the FETAX assay in preparation for the extended test period which will be employed in the testing of thalidomide. Twenty one days is the estimated time frame for hind limb development. The current testing will be used to define feeding parameters, normal mortality rates and initiation of hind limb formation. Also, the possibility of testing thalidomide in a flow-through system is being explored. Work is continuing on this Project.

JULY 1 - SEPTEMBER 30, 1996

It has been determined that repetitive exposure to human chorionic gonadotropin causes the embryos of *Xenopus laevis* to become malformed in higher than normal percentages, thus rendering them unusable in the FETAX assay. This finding will make it necessary to renew the *Xenopus* colony on a more routine basis. In the past, the colony was renewed due to declining health or failure to produce viable embryos. During this quarter, a new colony of *Xenopus laevis* was acquired from Xenopus I. Many of the frogs were discolored on the ventral side of their bodies upon arrival. Over a period of ten days, treatment with NaCl was initiated on the advice of Xenopus I. Major Ruble was also consulted during the treatment of the new colony. It was his opinion that the frogs were suffering from shipping stress. After a quarantine and observation period of three weeks, Major Ruble released the new colony for use. Since the release of the colony for use, the newly acquired females have been paired with males that have been in the laboratory since February of 1995. Six injection periods have produced viable embryos with one exception. The period that did not produce viable embryos coincided with a power outage which caused the temperature in the laboratory to rise to 24°C.

In preparation for studying the effects of thalidomide on the formation of limbs in *Xenopus laevis* embryos, embryos are currently being maintained until they develop both forelimbs and hind limbs. In addition to developing husbandry methods for the *Xenopus* embryos, the results of feeding live foods (microworms and *artemia* nauplii) were compared to feeding food marketed by Tetramin□ for fry. The study is still in progress, but preliminary results indicate that the live foods enable the embryos to develop faster and cause less mortality. The mortality noted during this study may be attributed to improper handling of the embryos during cleaning, increased ammonia levels due to the presence of uneaten food and wastes, and possibly to overcrowding. The handling problem has been addressed by carefully scrubbing the bottom of the jars with a brush to dislodge material adhering to the jars and adapting the end of a 50mL pipet in order to avoid injuring embryos accidentally caught in the pipet. Using this technique, the embryos may also be removed for thorough cleaning. The increased ammonia problem may be partially resolved by feeding little or no prepared food as well as renewing the FETAX solution on a daily basis. This study was initiated with ten embryos per jar. Decreasing the number of embryos per jar should also aid in reducing the mortality among the embryos and may allow them to develop at a faster rate by decreasing competition for food.

An IACUC meeting was attended during this quarter. It was determined during the meeting that the FETAX assay does not require an animal use protocol. The embryos used in the assay are not recognized as vertebrates by the U.S. Army since they do not developed vertebrae during the 96 hour test period. However, an animal use protocol must exist for the husbandry of the adult *Xenopus laevis* colony. That protocol was revised during this quarter and is currently being reviewed by the principal investigator.

All Standing Operating Procedures pertaining to FETAX were revised during this quarter. The revised SOP's are currently being reviewed by the principal investigator. The Milli-Q□ water purification system was removed and replaced with an updated model which is equipped with UV sterilization and is more easily maintained. Several attempts were made during the quarter to demonstrate some of the FETAX procedures for Major Ruble. All attempts



were unsuccessful due to schedule conflicts.

The testing of pentachlorophenol was initiated and completed during this quarter. The lipophilic nature of the chemical prevented the use of the metabolic activation system. The microsomes adsorbed the pentachlorophenol from the solution preventing the embryos from becoming malformed or experiencing mortality.

OCTOBER 1 - DECEMBER 31, 1996

The first FETAX assay testing Thalidomide using lot m2 microsomes produced no metabolic activation of Thalidomide and 0% mortality among the positive control embryos. Thalidomide and lot m2 microsomes were tested against lot m18 microsomes. Lot m18 microsomes were used in the Interlaboratory Validation Study. Lot m2 microsomes did not activate the thalidomide and the test was inconclusive. Lot m2 microsomes yielded 85% mortality among the positive control embryos. One hundred percent mortality was obtained in the positive

control containing lot m18 microsomes. Lot m4 microsomes were received from Oklahoma State University and were tested for activation. The preliminary testing of metabolic activation controls yielded 0% mortality among the positive control embryos indicating little or no metabolic activation. Lot m5 microsomes have been received from Oklahoma State University and are scheduled to be tested for activation in FETAX during the first week in January.

New flow-through chambers were designed and fabricated to contain medaka and *Xenopus laevis* in the 96-hour LC50 flow-through test of chloroform. The chambers functioned very well in the first chloroform test. Additional flow-through chambers were fabricated for both medaka and *Xenopus laevis* for testing bromodichloromethane. Some minor improvements were made to the chambers used in testing chloroform.

FETAX was also included in three flow-through 96-hour LC50 tests. The first chloroform test indicated that the 96-hour LC50 and EC50 are both between 97.3 mg/L and 113.0 mg/L. The second chloroform test was terminated due to 75% mortality among the control embryos. High mortality also occurred in the low concentrations. The mortality in the first flow-through 96-hour LC50 test of bromodichloromethane appeared to be adversely affected by the accidental feeding of the *Xenopus laevis* chambers. Taking this into account, the LC50 appears to fall between 145.0 mg/L and 247.7 mg/L. The EC50 appeared to be unaffected by the addition of brine shrimp and microworms into the *Xenopus laevis* chambers and a malformation dose response curve was obtained from the data. The EC50 appears to fall between 28.7 mg/L and 50.0 mg/L. The LC50 and EC50 values for these flow-through tests are estimations. The length measurements of the test and control embryos and the statistical analyses will be completed next quarter.

The report of the California and Iowa Mix FETAX studies was completed during this quarter. The results of the FETAX assays were compared to the standardized mammalian test results obtained by NIEHS. General laboratory maintenance and husbandry of the adult *Xenopus laevis* were also performed during the month. Scientific protocols for thalidomide, water

disinfection by-products (flow-through) and water disinfection by-products (static renewal) were completed. A seminar was given at the National Cancer Institute's (NCI) Frederick Cancer Research and Development Center at Ft. Detrick on December 4th describing the FETAX program.

JANUARY 1 - MARCH 31, 1997

Arochlor 1254 induced microsomes lot number b12 were tested in a FETAX assay in order to assess their ability to bioactivate cyclophosphamide. Oklahoma State University sent two rather than four 4 mL vials of lot b12 microsomes for testing. It has been general practice to thaw a vial of microsomes daily during the four days of testing. The shortage of vials made it necessary to freeze the microsomes a second time in order to use them an additional day. Freezing temperature was -20EC . By the morning of the third day of testing, the cyclophosphamide positive controls appeared to be close to mortality, as expected. On the third and fourth day of testing, refrozen microsomes were used in the static renewal. The cyclophosphamide positive controls recovered and the results were 0% mortality and 0% malformed at the end of the 96-hour test period. It has been determined from this experience that microsomes requiring additional use must be frozen in liquid nitrogen until they are needed again for static renewal.

Sodium chlorate was tested under flow-through conditions. The test failed due to excessive mortality and malformations among the control embryos. The lowest test concentration was similarly affected. The same compound was also tested in two static FETAX assays during the quarter. The first assay yielded no significant information due to the fact that the concentrations were too low. The second assay is currently in progress and 100% mortality has been obtained in the three highest concentrations.

FETAX and *Xenopus laevis* husbandry animal use protocols were reviewed for accurracy and utility. It was determined that maintaining animal use protocols specifically for FETAX is unnecessary. The protocol for the husbandry of *Xenopus laevis* will be maintained without any modifications; the protocol for using the metabolic activation system (MAS) kit was edited to reflect current procedures. The MAS kit was also tested in three of the thalidomide assays performed during the quarter. The kit decreased the amount of time spent preparing for a FETAX assay which include the MAS. Explicit instructions for the use of the metabolic activation system and for the use of the Bioquant7 morphometry system with FETAX were written to aid in the training of new personnel.

The laboratories were cleaned in preparation for the IACUC site visit. Instruction on measuring embryos was given using the Bioquant7 morphometry system. All length measurements of the embryos in chloroform flow-through test number 229-001 were performed by the newly trained technician. Some of the more critical aspects of the assay were also taught, such as injecting the adult *Xenopus laevis*, culling embryos for use in FETAX assays, assessing mortality at 24-hour time intervals during a FETAX assay, and assessing malformations at the end of the 96-hour test period. Training records were updated to reflect new knowledge in these areas.

New test chambers were fabricated for use in the control tests of the well water flow-through system in room 10. Two consecutive flow-through FETAX assays were run in room 10. The first assay included a standard static renewal FETAX assay. The first flow-through assay was assessed for mortality and malformations with negligibleresults, therefore, it was continued until 192 hours. After 192 hours, the aquaria remaining static with only partial water changes daily averaged 75% mortality. The aquaria maintained according to the guidelines described in the Standing Operating Procedures averaged 18% mortality. Remaining test aquaria exhibited negligible mortality. In the static renewal portion of the test performed in the FETAX lab using water from the flow-through tanks in room 10, 24% mortality and 100% malformed embryos were found in one Petri dish which contained well water from one of the aquaria treated following the SOP guidelines. A Petri dish which contained well water taken from one of the two-chambered aquaria containing frog embryos and free-swimming fish exhibited 96% mortality. The remaining Petri dishes exhibited 0% mortality and negligible malformations. The second control flow-through assay was allowed to run for 72 hours with results mirroring the first assay. This data may indicate mortality increases when microorganisms thrive on excess food, then also attack the frog embryos.

Three FETAX assays were performed on thalidomide during the quarter. The same lot of microsomes were used in all three assays. The microsomes used were Mix 7 which was a mixture of microsomes induced with Arochlor 1254 and microsomes induced with isoniazid. The volume of the microsomes used in testing was the only variable that was modified in the three assays. The volumes used in the first two assays failed to bioactivate thalidomide. The third assay was successful in that all of the controls yielded the desired results. Both mortality and malformation dose responses were obtained from the thalidomide concentrations containing the MAS.

APRIL 1 - JUNE 30, 1997

Thalidomide testing was conducted during two weeks of April. The first assay was successful, however, the second assay failed due to high malformations among the control embryos. The most probable reason for the failure was a refractory condition in the male adult *Xenopus laevis* to the human chorionic gonadotropin with which they have been repeatedly injected to induce mating. To complete the testing of thalidomide, one additional assay must be performed.

Dibromoacetic acid was tested in FETAX under continuous flow conditions in room 10. The trial concentrations were designed to produce results with medaka fry but proved insufficient to obtain significant results in Xenopus. The concentrations will be increased after this chemical is tested in standard FETAX. Replicate number 17 in tank 7 (240 mg/L) was affected by either bacterial or fungal contamination. This replicate exhibited both high mortality and high malformation rates.

Thalidomide test number 228-007 failed due to high malformations among the control embryos, also suspected to be from a refractory condition of male *Xenopus laevis.* These males were used because delays were experienced in obtaining new stock. Thalidomide test number 228-008 was attempted, but terminated prior to initiation. The percentage of viable embryos was

too low to be used in a FETAX assay and any viable embryos present in the clutch were past stage 11. Two other attempts at this test were aborted since viable embryos were not obtained. Eggs were unfertilized or contained too many necrotic eggs to be used in a FETAX assay. The fourth attempt at initiating thalidomide test number 228-008 was successful. The injections were administered two hours later than in previous tests. Seasonal changes require modifications in the injection schedule in order to obtain viable embryos. However, inadequate bioactivation in the isoniazid induced microsomes was observed. The embryos developed normally in all negative controls. The cyclophosphamide positive controls exhibited the expected response of 100% mortality, indicating that the arochlor 1254 induced microsomes in the mixture were able to bioactivate the cyclophosphamide. The microsomes induced with isoniazid may have been weaker due to the reduced response obtained in the acetic hydrazide positive controls as well as that of the high concentrations of thalidomide. The acetic hydrazide positive controls were more severely malformed than the acetic hydrazide negative controls. This indicates that some bioactivation was present, but not enough to cause the desired 50% percent mortality in the positive controls. The highest concentration of thalidomide including the metabolic activation system exhibited 0% malformed, but were slightly stunted. When attempting to run this test again, viable eggs were not obtained. The same problem prevented initiation of the next dibromoacetic acid test number 231-008.

Forty eight new adult male *Xenopus laevis* were received from Xenopus Express. All animals appeared to be in good health. The arrival of these new animals necessitated the reorganization of the culture room for *Xenopus laevis*. The animals were quarantined and observed for two weeks prior to their use in experimentation. A dibromoacetic acid assay was successfully initiated but by 36 hours there was 25% malformation and 12% mortality in the controls. The test was continued for range finding purposes and concentrations were determined for the definitive assay. Viable eggs were again unavailable for continuing this test series. An abstract was submitted as poster presentations for the meeting entitled "Current Issues on Chemical Mixtures," at Colorado State University, August 11-13, 1997. The title of the poster is "Assessment of the Teratogenic Potential of Two Simulated Water Samples Using the Frog Embryo Teratogenesis Assay - Xenopus (FETAX)"

Reproductive Toxicology:

Supplies and equipment were ordered for the reproductive toxicology laboratory recently initiated with the assistance of Dr. Eric Clegg of the Environmental Protection Agency. Much time was expended to make the laboratory functional. Information was gathered from the scientific literature, *Xenopus* vendors, and Dr. Bantle, OSU, as a base of knowledge from which to expand. An Animal Use protocol was written for preliminary research and development using *Xenopus laevis* as a model. The protocol was approved in April. Four adult male *Xenopus laevis* were euthanized and testes taken for histological examination. One testis from each frog was fixed in 20 mL 10% formalin while the remaining testis was fixed in 20 mL Bouin's solution to compare the efficacy of each preparation. The Bouin's-fixed testes will be embedded in paraffin and the formalin-fixed testes will be embedded in glycol methacrylate. Sectioning will be done at three locations along the length of the organ in order to view tubule cross-sections. Staining will be performed with either periodic acid Schiff (PAS) - hematoxylin, or hematoxylin - eosin (H&E) depending on results. These samples will provide Experimental Pathology Labs (EPL)

with tissue samples to develop expertise in preparation and optimal orientation for slide study. A memo was submitted to obtain a cost estimate for histological preparation by EPL.

Dissections were performed to learn the anatomy of the frog and to obtain testes for use in sperm head counts. Significant variation has been observed between the size of testes within individual frogs. Maceration of the testes and examination under the microscope revealed motile sperm in the sample. This may be of significance since it is a indication that *Xenopus laevis* does not store mature sperm in a structure equivalent to the epididymis of mammals. In euthanizing adult males for dissestion, the current method using 10g/L MS222 at a dose of 1cc per 50 g body weight was found to be inadequate. A alternate method was obtained from a paper on spermiation supplied by Dr. Bantle. This method, describing a 10% solution of MS222 at a dose of 0.1 cc per 50 g body weight was also found to be inadequate. Increasing the dosage to 0.55 cc per 50 g body weight still failed to produce complete euthanasia. More information is being sought on an efficient way to sacrifice the frogs. Testicular sperm head counts were initiated in order to optimize and perfect the assay for use with *Xenopus*. Definitive counts will begin next quarter. The induction of spermiation has been another area of study. Gonadatropin-Releasing Hormone (GnRH) has been injected in male frogs and periodic cloacal washes performed to obtain live sperm. These attempts have not been successful to date. Concentrations of hormone necessary to produce spermiation are still under investigation. Morphology and motility of these sperm may be used as indicators of fertility. Videotaping capability has been integrated with the Bioquant system for this purpose.

JULY 1- SEPTEMBER 30, 1997

The FETAX assay using dibromoacetic acid, test number 231-009, was attempted but terminated prior to initiation because of the lack of viable eggs. One pair of *Xenopus laevis* was injected with GnRH, the hormone used by Reproductive Toxicology, but produced no eggs nor did it cause amplexus. A second attempt at initiating a dibromoacetic acid assay was successful, however, high malformations across the board (14 - 18%) disallowed the test. It does not appear that dibromoacetic acid is a teratogen as the surviving, normally formed embryos in all the concentrations had attained their hind limb buds by 96 hours. Dibromoacetic acid test number 231-010 was initiated but a 9% mortality rate in the controls disallowed the test. The Dibromoacetic acid test was again attempted but the high concentration (3200 mg/l) only achieved forty percent mortality causing the test to be invalid.

Nineteen adult male frogs from Xenopus Express were received for use in Reproductive Toxicity methods development. Major David Ruble made an inspection the frogs due to the unexplainable increase in male mortality. His assessment was that the males looked healthy and appeared normal and that the next frog that died would be preserved for pathology. Due to the lack of viable eggs, adult *Xenopus laevis* mating pairs were injected with hCG two consecutive weekends. The dosages, the time of injection and the temperature were all varied with no success in obtaining viable embryos for use in the FETAX assay. It is possible that these failures may be attributable to male animals purchased from a new supplier (Xenopus Express). There has been considerable mortality among the males purchased from Xenopus Express. Future injections will include at least two males from Xenopus One in an effort to determine whether the male animals purchased from Xenopus Express are in optimal health for these studies. Fifty pounds of liver

were ordered and received from Hemp's Meat Market in Jefferson, MD. To investigate more efficient feeding methods, three of the fifty pounds were pre-ground and made into liver-burgers on arrival. Observations were made to see if the frogs were better able to eat the liver in this form and if it provided an easier, less time consuming feeding regimen for the technicians. Diced or ground was consumed equally well by the frogs but food preparation and cleanup time was halved making the ground liver preferable. Discussions were held on increasing the holding capacity for the frog colony. A plan to increase capacity by six to twelve tanks was formulated, but will not be initiated until the direction Reproductive Toxicology is determined.

An overview of the FETAX assay and the work that has been done utilizing the assay was presented at the 1997 National Capital Area Branch/American Association for Laboratory Animal Science Seminar.

Reproductive Toxicology:

During the final month of the quarter, two male frogs purchased from Xenopus Express were euthanized due to a muscular injury and to white spots present on the dorsal surface of the other frog. The latter was preserved in 10% formalin for examination by a pathologist. In addition to the two males that were euthanized, one male was found which had died shortly before it was discovered. This animal was also preserved in 10% formalin for pathology. Along with the two animals mentioned above, a third male frog was euthanized and sent to the pathology laboratory at USAMRIID for examination.

Reproductive toxicity data was entered into a Microsoft7 Excel spreadsheet. The spreadsheet was modified to automatically make calculations as data is entered. Six adult male frogs were ordered and received from Xenopus One for use in Reproductive Toxicity methods development. A literature search for articles to support the reproductive toxicity program was submitted to the necessary personnel. Some additional articles were retrieved from the NCI library. Supplies for the use of flow cytometry to study spermatogenesis in *Xenopus laevis* were ordered. Necessary catalogs have been also been requested.

OCTOBER 1- DECEMBER 31, 1997

Dibromoacetic acid test number 231-012 was attempted once but terminated due to the lack of viable embryos. On the second initiation, controls were within acceptable limits for malformations but mortality throughout the treated concentrations disallowed the test. The third attempt was successfully completed using FETAX-AB (FETAX and a penicillin/streptomycin solution) in order to combat the bacterial contamination caused by the dibromoacetic acid's disintegration of the embryos. A second successful dibromoacetic acid test was run using FETAX-AB. Thalidomide test number 228-009 was successfully completed. Length measurements were completed on the three valid thalidomide tests and data was submitted to the statistician for analysis. Length measurements continued on the completed water disinfection by-product chemical tests. Twelve male and twelve female adult *Xenopus laevis* were received from Xenopus One for use in both FETAX and Reproductive Toxicity methods development.

Reproductive Toxicology:

A considerable amount of effort has gone into perfecting the digestion of testes from *Xenopus laevis*. The first attempt diluted the spermatogenic cells excessively. In trial number two, the volume of the media was decreased from 100 mL for two testes to 10 mL per testis. Also, two different media were evaluated; one containing collagenase and one containing trypsin. The volume was assessed to be adequate. The media containing collagenase was preferred over the trypsin because it was not as harsh to the spermatogenic cells and also resulted in better dissociation of the tissue. The spermatogenic cells obtained during the digestion process were videotaped for future reference. The cells were fixed in 70% ethanol at the end of the procedure for possible analysis by flow cytometry. The fixed cells were later placed in a cytotek centrifuge to spin on to slides for staining. After the spinning procedure was complete, examination of the slides determined that the cells were adhering. Another digestion was performed in order to obtain cells for staining. Upon the completion of the digestion, both live and fixed cells were spun onto slides for staining with toluidine blue or periodic acid Schiff - hematoxylin (PAS-hematoxylin). The fixed cells exhibited the same problems as encountered previously. However, it appeared that the staining procedure had removed enough of the cells in order for individual cells to be observed. The toluidine blue stain yielded better results, in that the cells absorbed more of the stain. Also, the cells seemed to become damaged by the PAS - hematoxylin stain. The purpose of the staining was to better identify the early spermatocytes and study morphology. Two more digestions were performed in order to fix cells in 70% ethanol for flow cytometry analysis without adherence. To date, all attempts at preserving cells in various volumes of 70% ethanol have not yielded individual free cells. The most successful attempt consisted of adding 5 mL of 70% ethanol to a 2 mL cell suspension yielding a final concentration of 50% ethanol. Every attempt to increase the final concentration to 70% ethanol resulted in cell adherence. It has been suggested that the addition of the ethanol to the cell suspension denatured the protein in the bovine serum albumin causing the sticking. Cells will be fixed in phosphate buffered solution after the next digestion. Once the preparation is ready for flow cytometry analysis, exploration of some possible reference samples will begin that may be used in conjunction with the analysis of the spermatogenic cell samples. Two possibilities are lymphocyte or red blood cells. The details of the preparation of the reference samples must be researched and refined before proceeding with the initial flow cytometry analysis.

The testes from two adult Xenopus laevis were fixed in 5% glutaraldehyde in cacodylate buffer. Each testis was fixed in 10 mL of the fixative in glass screw top scintillation vials and shipped overnight express to Dr. Robert Sprando at the FDA laboratory in Laurel, Maryland. The testes will be sectioned and examined by Dr. Sprando. *Xenopus laevis* embryos are also being cultured through metamorphosis in order to determine the earliest point at which spermatogenic cells may be harvested from young frogs.

JANUARY 1 - MARCH 31, 1998

The dibromoacetic acid test, number 231-015 was completed using new test stock after four failed attempts caused either by lack of viable embryos or malformed controls. Failure rates were attributed to the poor performance of frogs obtained from Xenopus Express. Two sodium chlorate tests, numbers 230-007 and 008 were successfully completed using new frogs



from Xenopus One. While waiting for Xenopus One frogs to meet the injection schedule rest period requirements, two sodium chlorate tests, number 230-009, were unsuccessfully attempted using Xenopus Express frogs. All remaining frogs from this vendor were euthanized or designated for alternate uses. Another sodium chlorate test attempt was aborted due to lack of viable embryos. T-tests were performed for FETAX studies of dibromoacetic acid, bromodichloromethane and chloroform. EC_{50}'s, LC_{50}'s and teratogenic indices were also generated for the FETAX data on DWBP's. A FETAX assay of sodium chlorate was initiated. Photographic equipment was installed for use with a dissecting microscope and pictures were taken for a presentation of the FETAX study of Water Disinfection By-Products. The FETAX data from testing thalidomide was sent to Oklahoma State University for analysis. Two cost estimates were prepared for testing water samples from Minnesota using the FETAX assay and for participation in an interlaboratory validation of an exogenous metabolic activation system for FETAX. A FETAX demonstration was conducted for Ms. Kelly Lippenholz and two of her colleagues from CHPPM at Aberdeen Proving Ground. Ms. Lippenholz returned to the laboratory a second time in order to learn the selection procedure for choosing embryos for use in the FETAX assay. A meeting with Dr. John Tessari was attended to discuss the initiation of the FETAX assay at Colorado State University Dr. Tessari is interested in using FETAX to investigate DWBP's, specifically dibromoacetic acid.

Excess chemicals from FETAX testing were removed from room 7 and transferred to the safety officer for proper disposal. A new gasket for the autoclave in room 7 was ordered and replaced. The frog husbandry laboratory was prepared for the arrival of replacement animals for FETAX studies and the reproductive toxicology program. Several frogs were euthanized due to contact with powdered gloves. The gloves have been discarded. Natural rubber gloves were ordered and used in an attempt to control a technician's allergic reaction to latex. Cotton glove liners have been ordered to be used by this technician.

Reproductive Toxicology:

During the quarter, work continued to develop a procedure that could be used to obtain spermatogenic cells for flow cytometry analysis. Many variations in the preparation of samples were employed in order to prevent the cells from adhering to one another. All attempts were unsuccessful. It is possible that the adhesion may be occurring mainly among the mature spermatozoa and would therefore bias the data if the larger particles (groups of cells) were filtered from the samples. This portion of the project has been temporarily put on hold. Three male frogs purchased from Xenopus Express were sent to Dr. Gary Zaucha, a veterinary pathologist at USAMRIID, for examination. One frog had a large tumor on the ventral outer surface of its mouth. The cause of the tumor was determined to be infection by *Mycobacterium* species. Two other frogs were sent to Dr. Zaucha for examination. One of these animals was also found to be infected with a *Mycobacterium* species. This problem was discussed with Major David Ruble. His conclusion on this issue was that the infection and resultant tumor were due to poor husbandry by Xenopus Express. *Xenopus laevis* embryos were raised beyond 96 hours in order to begin to define the optimum husbandry conditions for offspring. This work will continue until the mortality among the offspring is within acceptable limits.

Testes were removed from four frogs and fixed via immersion in 5% glutaraldehyde in 1M cacodylate buffer. The tissue was sent to Dr. Robert Sprando of the FDA for histological examination. Through the examination of the immersion fixed tissue, it was determined that perfusion fixation would yield better results. Dr. Sprando visited the laboratory on two occasions during the quarter to perfuse several frogs and to teach the technique to the technical support of the project. Three pairs of adult frogs were injected with hCG and the males were observed for nuptial pad development. The males began to respond to the hormone approximately two hours after the injections were administered. Two spermiation experiments were conducted during the quarter. In the first experiment, five male frogs were injected with hCG. Injections of 1 ml sterile deionized water were administered to each frog following the darkening of the forelimb pads. The cloaca of each animal was flushed with physiological saline 30 minutes after the deionized water was administered in order to obtain samples of spermatozoa. No spermatozoa were obtained. The frogs were then euthanized via dorsal lymph sac injections of MS-222 and fixed in Bouin's solution for pathological examination by Dr. Gary Zaucha. The second experiment consisted of four frogs injected with hCG. Cloacal washes were attempted at hourly intervals throughout the day and the nuptial pad development was monitored for three days. Spermatozoa collection was again unsuccessful. These frogs were euthanized and perfused with glutaraldehyde for histological examination of the testes. Three frogs were perfused with fixative prior to beginning a □spermiation - time after hCG experiment□, in which the hCG injected frogs will be perfused with glutaraldehyde at the completion of the experiment. All three perfusion trials were successful. The third perfusion also included the ligation of arterial vessels leading to one testis prior to excising the same testis for spermatid counting. The perfusion fixed testis was then removed and immersed in the same fixative. Three of four planned □spermiation - time after hCG experiments□ were completed during this quarter. Four frogs were injected with hCG, each frog was euthanized and perfused with glutaraldehyde at four different time intervals. One frog was perfused at each of the following time intervals: 30 minutes, 1 hour, 2 hours, and 4 hours after the hCG injections. One control frog was also perfused that had not received hCG. Prior to perfusion, one testis from each animal was ligated, excised and frozen for spermatid counting. The fourth day of the experiment will be completed early next quarter as will the spermatid counting. All of the fixed tissue from these experiments will be sent to Dr. Sprando for examination. Dr. Sprando will examine the tissue in order to determine the time frame in which the hCG causes the release of spermatozoa from the testis.

Xenopus laevis embryos were examined for excess spermatozoa that were unable to penetrate the eggs. Some spermatozoa were found adhered to the outer membrane of fertilized eggs. One possible future use of this exercise could be to observe unfertilized eggs for the presence of spermatozoa which may have been rendered incapable of fertilization by exposure to a toxicant. A cooperative agreement with the electron microscopy personnel from USAMRIID was initiated during the quarter. A meeting was arranged with Dr. David Fritz, a veterinary pathologist employed by USAMRIID, in order to discuss the project details and to outline the official documentation necessary to begin the work. The protocol for counting spermatids was revised and faxed to Dr. John Bantle and Dr. Douglas Fort. Literature searches for articles to support the reproductive toxicology program were requested from Allen Reynolds. Some articles were retrieved from the NCI library on post. A meeting with command representative, Joan Porter was attended in order to discuss the technical work performed in the laboratory.

Meeting attendees included Colonel David Danley, Dr. Robert Finch, Angela Gaudet-Hull, Margaret Toussaint and Mark Widder. An inventory of all microscope equipment was conducted. Considerable knowledge of the operation and use of the existing equipment was gained over the course of the quarter, including the use of phase contrast and Hoffman Modulation contrast. Also, photomicroscopy using existing equipment was accomplished on both an inverted microscope using Hoffman Modulation contrast and on a dissecting microscope.

APRIL 1 - JUNE 30, 1998

Sodium chlorate test number 230-009 was initiated, but terminated due to high mortality in the controls. Bromodichloromethane test number 232-007 was completed, but again, due to high malformations in the controls, the test was disallowed. Testing was put on hold for four weeks the first two months of the quarter while awaiting funding. Chloroform test number 229-007 was attempted four times with obtaining viable embryos in any of the tests. A good test was finally completed on this chemical. Sodium chlorate test 230-010 was successfully completed. Length measurements were done on all of the sodium chlorate tests and the data was given to the statistician for analysis. Twenty-five pounds of ground liver was received and packaged into one pound increments for easier dissemination. Twelve female and six male frogs that were sick were euthanized. Six of these females were sent to USAMRIID for pathological testing. Results are pending. Twelve albino male frogs were received from Xenopus One. The albinos were chosen because of their breeding efficiency to help allay the recent problems associated with obtaining embryos. The temperature was also decreased from 23° C to 17°C in the frog culture tanks since cooler temperature is more desirable for the female *Xenopus laevis* reproductive system and should reduce the number of necrotic embryos.

A study design was drafted for the pilot FETAX work to be done in collaboration with Oklahoma State University testing bromodichloromethane, dibromoacetic acid and treated water. Cost information on this study was gathered for the laboratory director. Supply demands were coordinated and scheduling with OSU personnel.

Reproductive Toxicology:

Collaboration on electron microscopy with USAMRIID was initiated. Dr. David Fritz assisted Dr. Robert Sprando of the FDA in photographing the stages of spermiation in *Xenopus laevis*. A protocol for counting *Xenopus laevis* spermatids using a hemacytometer was streamlined. The procedure for use in other species was adapted for *Xenopus laevis*. Also, the technical support of the project required training and familiarity with the procedure. Considerable time was spent outlining the criteria for counting and acquiring photographs cataloging the criteria. An experiment entitled Spermiation: Induction of Spermiation by Injection of HCG was completed during the quarter. The purpose of the experiment was to study the release of sperm from the testis following injection of HCG and the timing of that sperm release. The experiment consisted of five repetitions in order to demonstrate reproducibility. The experimental animals were treated with 500□l of HCG. One animal was assigned to each of the following time endpoints: Control, 30 minutes, 1 hour, 2 hours, and 4 hours. One testis from each animal was ligated, excised and reserved for spermatid counting. The remaining testis and excurrent ducts were removed from the animal following whole body perfusion with fixative.

The fixed tissue was shipped to Dr. Robert Sprando of the FDA for histological examination. Training in tissue embedding and sectioning was received from Dr. Sprando at the FDA laboratory in Laurel, MD.

Chemical inventory for the Reproductive Toxicology laboratory, room 117, was updated.

Animal use protocols for Reproductive Toxicology and FETAX were also updated. A scientific protocol for the spermiation experiment was created. Literature searches in MEDLINE were also conducted to support these protocols. A possible flow cytometry collaboration with USAMRIID investigators was initiated. The revised Atlas of Abnormalities for FETAX was proofread.

Husbandry of juvenile frogs was conducted during the quarter.

Task Order RM-3



GEO-CENTERS, INC.

Title: Integrated Biological Assessment Technology Transfer
Task Number: RM-3 (2533-010)

AUGUST 1-31, 1993

Work commenced by our subcontractor TRI to arrange/coordinate the annual Research Review Conference to take place in October. Location of site, confirmation of dates, and notification of speakers were completed. Invitations, handout packages, and travel arrangements will be the bulk of the effort during September.

SEPTEMBER 1-30, 1993

Coordination has begun with Technical Resources, Inc. (TRI) for the Fifth Annual Research Methods Branch Research Review. The conference is scheduled for 26 and 27 October. The invitation list numbering approximately 150, including the speakers, has been completed. The tentative agenda has been completed as well as the registration form (both are to be included in the initial invitation letter).

The invitation letter along with enclosures were mailed out on 29 September.

OCTOBER 1-31, 1993

Final preparations and last minute details for the Fifth Annual Research Methods Branch Research Review have been completed and the Research Review was held on 26 and 27 October with 76 speakers and invited guests attending. All audio/visual equipment was in place on 25 October, provided through TRI, and the meeting rooms prepared the morning of the 26th.

All guests were registered and given hand-out information in a smooth and efficient manner. The scheduled speakers were kept within their time limit. The breaks and lunch were as planned and done in a timely fashion, both days. The bus transportation was on time and very adequate. A tour of the USABRDL facilities was provided for six visitors.

The proceedings of the meeting will be completed by TRI with some coordination through *GEO-CENTERS* if needed. It was felt by all that the Review was successful.

Follow-up questions and information will be provided to TRI via telephone if necessary. TRI has been provided telephone and FAX numbers of each presenter for completion of the proceedings.

NOVEMBER 1-30, 1993

Follow up on the Fifth Annual Research Methods Branch Research Review with TRI is continuing. The initial transcription has been completed by TRI, and technical writers are being assigned to each report for completion. The writers will then edit the initial report into a summary which will be screened/reviewed in-house (Twerdok) prior to being sent to authors for approval. The summarized reports are expected to be sent to USABRDL by mid-December. Workshop



speakers are being contacted with requests for hard copies of slides used in their presentations for inclusion into the publication where appropriate.

The final revised draft of the 1990-92 RMB Workshop Proceedings has been returned to TRI for type-setting. The proceedings will go to press once TRI has received a "Forward" from the Chief of RMB and galley proofs have been reviewed and approved.

Compilation of information from current extramural contractors/grantees on student support provided by USABRDL funding is in progress. This report will include student name, type of degree (B.S., M.S., Ph.D., etc.) and thesis title, if applicable. The report should be completed by the first week of December.

DECEMBER 1-31, 1993

Writing of summary manuscripts for the proceedings of the 1993 workshop is in progress. Six of the 15 summarized reports will be delivered to USABRDL on or about January 10, 1994, to begin review.

A meeting was held on December 14, 1993, with representatives of USABRDL and Aberdeen Proving Ground to establish SOPs for utilizing the mobile biomonitoring laboratory complexes at Aberdeen Proving Ground. Minutes from this meeting are in the process of being transcribed and will be sent to USABRDL for approval the first week of January. When approval is obtained, these notes will be structured into SOPs.

JANUARY 1-31, 1994

The first six summarized reports from the 1993 workshop, prepared by TRI, have been reviewed. Some modifications are needed. Audio-visual support has been obtained to prepare hard copies of slides to be used in the 1993 Workshop Proceedings. Follow-up on meeting proceedings will continue.

Minutes from December 14 meeting for use of the Mobile Biomonitoring Facility have been reviewed and approved. The approved version of the minutes will be sent to the meeting attendees meeting next month. These minutes will also be utilized in-house for writing Standard Operating Procedures for use of the Mobile Laboratories at Aberdeen Proving Grounds.

FEBRUARY 1-28, 1994

Summarized reports from the 1993 workshop prepared by TRI were mailed to presentors along with copies of slides, if provided to TRI. Follow-up on meeting proceedings will continue.

Minutes from the December 14 meeting for use of the Mobile Biomonitoring Facility have been reviewed and approved by the meeting coordinator and will be mailed to attendees following review/approval from Branch Chief and Laboratory Director.

MARCH 1-31, 1994

Completed 1990-1992 workshop proceedings were received back from TRI; once Forward has been written and added to text, proceedings will be ready to send for printing at the post printing facility. Follow-up on 1993 workshop meeting proceedings will continue; edited/corrected manuscripts from presenters are due back to TRI April 15, 1994.

Minutes from December 14 meeting for use of the Mobile Biomonitoring Facility have been reviewed and approved by the meeting coordinator and Branch Chief/Laboratory Director. Addresses of meeting attendees were obtained; packages for mailing to attendees will be assembled and delivered to meeting coordinator early next month for mailing.

APRIL 1-30, 1994

Compilation for the Proceedings for the Fifth Annual Research Methods Branch Research Review continues. Revised manuscripts from presenters were due to TRI April 15, 1994; as of April 30, about half of the revised manuscripts have been received by TRI.

The galley proofs of the 1990-92 RMB Workshop Proceedings will be returned to TRI for final corrections in early May. The proceedings will go to press once a "Forward" from the Chief of RMB and has been incorporated into the compendium.

MAY 1-31, 1994

No work was performed on this task during the current reporting period.

JUNE 1-30, 1994

Compilation for the Proceedings for the Fifth Annual Research Methods Branch Research Review (1993 meeting) continues. Revised manuscripts from presenters were due to TRI April 15, 1994; as of June 30, all but three manuscripts have been received by TRI.

The camera-ready copy of the 1990-92 RMB Workshop Proceedings was received June 30, 1994. The proceedings will go to press once a "Forward" from the Director of USABRDL is received and incorporated into the compendium. Incorporation of the "Forward" into the Proceedings will be done in-house, *i.e.*, it will not be sent back to TRI.

JULY 1- SEPTEMBER 30, 1994 (Government approved Quarterly Progress Reports in lieu of Monthly Progress Reports)

The Sixth Annual Research Review was held on September 20-21, 1994, at Ceresville Mansion, Frederick, MD. Last minute details completed/finalized on September 19, 1994, for the meeting included: compilation of a list of the registrants (by the subcontractor, Technical Resources International, TRI) to be included as a hand-out for attendees; finalization and copying of the meeting agenda; compilation of meeting packets for distribution to attendees; arrangements for audio/visual equipment; and final check on the meals and refreshments. A copy of the attendee packet was made available to TRI.



Speakers and guests were registered in a timely manner and given hand-out folders with a copy of the agenda and registrants enclosed. Copies of the *Proceedings of the 1990-1992 Annual Research Reviews* were available at the meeting. Speakers were kept within the time constraints of the schedule. Breaks and lunch were as planned and satisfactory. The seating arrangements (auditorium style) were felt to be inadequate by the laboratory director, therefore, changes were made by the beginning of the second day's schedule. A tour of USABRDL facilities was provided for two guests at the close of the conference.

A list of attendees will be compiled and mailed to each person in attendance and to USABRDL by TRI. Manuscripts and camera-ready slides from the conference will be sent to USABRDL Associate Director for Research, Dr. Robert Finch, by November 1, 1994, for review. Coordination with TRI and speakers for compilation of the 1994 meeting proceedings will continue.

Compilation for the Proceedings for the Fifth Annual Research Methods Branch Research Review (1993 meeting) continues. All reviewed/revised manuscripts from presenters have been received by TRI, with the possible exception of one paper.

Notes from the Strategic Environmental Research and Development Program (SERDP) meeting, held September 22, 1994, were recorded, and work is in progress on the meeting minutes. A list of attendees with addresses, phone and FAX numbers was obtained and a copy was given to Dr. Lorraine Twerdok as the *GEO-CENTERS* point of contact (POC). Hard copies of meeting presenters' slides and written reports will be sent to USABRDL Associate Director for Administration and Logistics, Ms. Karen Fritz, for review and forwarding to *GEO-CENTERS'* Operations Manager, Mr. David Lovelady. This information will be consolidated into a "Discussion, Recommendation, Action" format and copies will be sent to those in attendance. Meeting attendees will be contacted for any additional information required to finalize the meeting minutes.

OCTOBER 1 - DECEMBER 31,1994

Approximately half of the manuscripts from the speakers at the 1994 Annual Research Review (September 20-21, 1994, at Ceresville Mansion, Frederick, MD) have been received. Most manuscripts were received in hard copy only, without an electronic copy, which had been requested. Work is scheduled to begin next quarter to obtain the outstanding manuscripts and obtain electronic copies of all manuscripts. Work will then proceed with manuscript review and compendium compilation.

Compilation for the Proceedings for the Fifth Annual Research Methods Branch Research Review (1993 meeting) continues. All reviewed/revised manuscripts from presenters have been received by TRI.

Work is still in progress on the meeting minutes from the Strategic Environmental Research and Development Program (SERDP) meeting, held September 22, 1994. This information will be consolidated into a "Discussion, Recommendation, Action" format, and copies will be sent to those in attendance. Delay in the compilation of the meeting minutes has

been due to difficulties in getting hard and/or electronic copies of information from meeting attendees.

JANUARY 1 - MARCH 31, 1995

No work was performed this quarter on the Compilations for the Proceedings for the Fifth (1993 meeting) Annual Research Methods Branch Research Review. All reviewed/revised manuscripts from presenters have been received by TRI for the 1993 Compendium . The government has decided to apply the necessary incremental funding to this task to enable the Compendium to be completed by TRI. TRI has been informed that the government intends to complete the 1994 Compendium in-house, as well as any future research review coordination.

Progress on the Compilations for the Proceedings for the Sixth (1994 meeting) Annual Research Methods Branch Research Review is as follows; three electronic copies of manuscripts have been received and three hard copies of manuscripts have been received. This represents approximately half of the manuscripts due for inclusion of the 1994 proceedings. A completed hard copy of one of the manuscripts from the 1994 Research Review will be available for review and approval in April. If approved, work will begin on completion of the manuscripts received to date, and follow-up calls, requesting electronic and hard copies of manuscripts, will be made to those presenters whose manuscripts are still outstanding.

Work is still in progress on the meeting minutes from the Strategic Environmental Research and Development Program (SERDP) meeting, held September 22, 1994. A draft of the SERDP meeting held on 9/22/94 has been forwarded to USABRDL for review. Information from two presenters is required before theses minutes can be finalized. This information will be obtained from Karen Fritz or from the presenters during the week of April 10, 1995. This information will then be consolidated into a "Discussion, Recommendation, Action" format, and copies will be sent to those in attendance. Delay in the compilation of the meeting minutes has been due to difficulties in getting hard and/or electronic copies of information from meeting attendees.

APRIL 1 – JUNE 30, 1995

A SERDP technical review meeting was held April 19-20, 1995, in Columbia, MO, hosted by the National Biological Service, Midwest Science Center. Action items from the meeting were drafted and sent to all attendees. Minutes of the meeting were drafted as well.

A presentation was drafted and finalized for the Director of USABRDL to present to the Executive Director and Staff of SERDP in May. Subsequently, the TTAWG for the Cleanup pillar recommended to the Scientific Advisory Board that BRDL's SERDP program not be funded for FY96. The progress report and FY96 plan scheduled to be presented in late June to the SAB was cancelled. Plans are underway to minimize the impact of the loss of funding.

During May and June, GEO-CENTERS SERDP Coordinator visited extramural research partners in response to action items generated at the April meeting. Visits were conducted to Oak Ridge National Laboratory to conduct discussions with Dr. Lee Shugart and staff, to the



National Institute of Environmental Health Sciences to conduct discussions with Dr. Jim Burkhart, to the Environmental Protection Agency's Environmental Research Lab at Gulf Breeze, FL, to conduct discussions with Dr. Bill Fisher, Dr. Jack Fournie, and Dr. Leroy Folgar, to Aberdeen Proving Ground to conduct discussions with Dr. Dennis Burton of the University of Maryland, Ms. Michelle Lorah of USGS, and Dr. Jim Petty and Dr. Chris Ingersoll of NBS, and to the U.S. Naval Postgraduate School at Monterey, CA, to conduct discussions with Dr. Don Gaver and Dr. Pat Jacobs. These coordination meetings were very productive, resulting in a better understanding of how to consolidate differing data into meaningful forms. A proposed concept was presented to the Director of BRDL for consideration.

The completed hard copy of a manuscript from the 1994 Research Review was submitted to Dr. Lorraine Twerdok for approval. Follow-up telephone calls were made with TRI, Inc. regarding the status of the proceedings from the 1993 Research Review. TRI was asked to provide a final estimate for delivery of the 1993 Research Review Proceedings by June 30th.

Two additional manuscripts from the 1994 review were completed with a third one nearing completion. A letter was sent to the COR to be approved for circulation to all presenters that have not yet submitted their manuscripts. It requested that hard copies of the manuscripts and diskettes be submitted in order to complete the proceedings. A list of the presenters in this category was also included.

Work continues on the 1994 Proceedings.

JULY 1 - SEPT 30, 1995

Work continures on the Compilations for the Proceedings for the Sixth (1994 meeting) Annual Research Methods Branch Research Review. Presenters that have not submitted manuscripts have been contacted in writing and by telephone. Three manuscripts remain outstanding as of September 23. Dr. Vanabeneden's manuscript has been finalized and Dr. Burkhart's hard copy and electronic copy have been received after review by Dr. Robert Finch.

In addition, support has continued to the Strategic Environmental Research and Development Program (SERDP) through coordination of interagency activities, drafting and submission of monthly and quarterly reports, and assistance in preparation of presentations.

An in-depth review of the laboratory□s capabilities and mission resulted in a draft "Business Plan" requested by USAMRMC. Upon emerging from BRAC and re-aligning within USAMRMC and USARIEM, this review and analysis of future directions was appropriate and timely. Additionally, inputs were submitted regarding briefings given to CG TECOM by the Director of USABRDL.

Preparations are underway for a comprehensive review of the status of all technical and contractual activities on the contract to be presented 13-14 November. It is expected that options will be offered regarding ensuring the entire capacity of the contract and the individual Task Orders is used in the most effective manner, either through re-estimates of completion on each task or through no-cost extensions to the periods of performance.



OCTOBER 1 - DECEMBER 31, 1995

The final monthly progress report and the final monthly financial report on the Strategic Environmental Research and Development Program (SERDP) were completed and submitted to the SERDP Executive Committee. All funds were expended on time with the exception of approximately $8,000 which was not obligated or expended by the EPA□s Environmental Research Laboratory at Gulf Breeze, FL. Cancellation of in internal SERDP conference originally scheduled for September and the disruption caused by two hurricane evacuations caused the funds not to be used within the allotted time.

Work continues on the compliation of the Proceedings for the Sixth (1994 meeting) Annual Research Methods Branch Research Review. It has been determined that efforts will be undertaken to prepare all Proceedings to the point that they are ready for publication. Budget priorities will then determine when publication and distribution actually take place.

A comprehensive Annual Research Review was conducted with detailed presentations by all researchers on their projects. Additionally, detailed adminstrative and contractual status reports were presented. The principal thrust of the contract status review was that the manhour and funding ceilings would be reached within a few months, although the Period of Performance of the contract extended to January of 1998. Options were presented with advantages/ disadvantages for government action in resolving the issue. In addition, it was noted that all Task Orders' Periods of Performance end in March 1996.

As a result of the Annual Research Review, coordination meetings have been held with all GEO-CENTERS researchers at USABRDL to facilitate recommendations on future direction of the efforts. Considerable progress has been made, particularly in standardizing approaches to planning and prioritizing elements in accomplishing the mission of the laboratory. Recommendations will be presented to the Army as they are formulated.

Minutes of the Annual Research Review are in the process of being composed. They will be distributed for management and government review during the next quarter.

JANUARY 1 - MARCH 31, 1996

Cover letters from the Associate Director of USABRDL have been prepared and are ready to be sent with completed manuscripts from the Proceedings for the Sixth Annual Research Methods Branch Research Review. Manuscripts that have been sent to researchers for review will be finalized within a limited time period allowing for comments and editing. At present, four of the manuscripts have been completed.

APRIL 1 - JUNE 30, 1996

Work is continuing on the 1994 Research Review. Five reports have been completed and are ready for authors' approval. Six reports have been received and remain to be completed. Names of contributors whose manuscripts are outstanding have been submitted to the Associate

Director of the laboratory.

JULY 1 - SEPTEMBER 30, 1996

Manuscripts from the 1994 In-House Research Review have been forwarded to Drs. Twerdok, Ostrander, Hinton and Wolfe for approval. Final edits will be made after author's review to complete this portion of the compendium. Work is still in progress on Dr. Gaver's and Burton's submissions due to the length and complexity of the reports. Five manuscripts have been prepared for mailing but will be held until new computer equipment has been received and installed in order to make back-up copies of the reports. Three manuscripts remain outstanding.

Work continues toward completion of reports to be used as the basis for revised IRIS database inputs and the first and second phases of technical support services on chemical agents guidance documents. Peer reviews of the TNT and TNB documents have been completed. Peer reviews have also been completed of draft Edgewood Research, Development and Engineering Center (ERDEC) documents for mustard gas and nerve agent airborne exposure. A workgroup meeting consisting of group members, invited military participants, and observers was held at Aberdeen, MD in September. A summary report including a document outline was submitted. Another workgroup was formed on the Military Effects of Chemical Agents. A statement of work, staffing assignments schedule, and a milestone schedule for mustard agents document preparation and review were written and revised. Initial meetings with combat and doctrine developers were also held.

OCTOBER 1 - DECEMBER 31, 1996

Manuscripts from the 1994 In-House Research Review for Dr. Ostrander, Dr. Wolfe, and Dr. Hinton have been completed and approved by the authors. Dr.Twerdok's approved manuscript remains to be received. Work continues on Dr. Burton and Dr. Gaver's reports. These manuscripts are scheduled to be sent to the authors for final approval within the next two weeks. This will complete the submissions expected for the 1994 compendium.

A report on reverse osmosis membrane chemical rejection performance entitled "Develop Recommended Field Water Quality Standards and Assess Water Quality Characterization Capabilities" was completed. A preliminary draft document on Mustard Agents Performance Degradation was prepared for discussion by the Chemical Agent Workgroup. The second Chemical Agent Workgroup meeting was held at Aberdeen, MD, on December 10, 1996. Workgroup members, invited military participants and observers were in attendance. A third meeting of the workgroup was coordinated and scheduled for January 23, 1997 at APG. Meetings were held at Fort Collins, CO and Fort Detrick, MD, toward the development of a Toxic Hazards Evaluation Tools Master Plan. Information gathering and analysis was conducted supporting the preparation of the CEHR Master and Program Plan.

JANUARY 1 - MARCH 31, 1997

Research Review manuscripts continue to be edited. Dr. Ostrander's, Hinton's and Wolfe's have been approved by the author and are completed. The majority of the others have



been sent to the author for final approvals with the exception of Dr. Gaver's which is still in the editing process due to the complexity of the material. Computer translation problems have held up work on Dr. Burkhart's manuscript. A translation will be attempted at CEHR with programs designed for this purpose. Three manuscripts have not been received.

Efforts were continued in support of CHPPM regarding a panel of experts reviewing the standards for maximum exposure of troops to chemical agents. Follow-on work is anticipated to include reviews for G-Agents and VX. This work is being accomplished by Life Systems, Inc., under a subcontract.

GEO-CENTERS conducted a comprehensive inventory of technologies and intellectual property rights at USACEHR. Included was a one-day course for all GEO-CENTERS and government employees in intellectual property and the importance of protecting rights. A detailed report was submitted to Dr. Gardner and Dr. Finch. It included recommendations for submittal of documents to protect rights in jeopardy due either to time limitations since disclosure or pending public disclosure of new technology applications. Subsequently, the proper documents were submitted by the Government to protect the intellectual property. In addition, this inventory will be used as a comprehensive source of accomplishments for the past several years of research in promotion and marketing of USACEHR capabilities.

Both GEO-CENTERS and our subcontractor, Life Systems, participated in drafting an Joint Environmental Toxicology Research and Development Master Plan (subsequently renamed the Deployment Toxicology Research and Development Master Plan) for Joint Service efforts being led by USACEHR. This plan, in draft form, was presented in San Antonio by LCDR Knechtges, USN, and Dr. Gardner to the TARA on 24 February 1997 to favorable review.

On 13-14 March 1997, the first Deployment Toxicology Users Working Group met at USACEHR. It was well attended and resulted in significant enthusiastic support of the concept being pursued in the Master Plan. The Draft Master Plan was presented and made available for review by the attendees. Reports of all activities leading up to and including the meeting of the DTUWG were submitted to USACEHR as they occurred. In addition, several coordination meetings were held with Government officials to prepare the Master Plan and the presentation materials both for TARA and for DTUWG.

Planning commenced for the first Science Advisory Working Group to be held near the end of May, as well as the Second DTUWG to be held just prior to the SAWG in May. The first SAWG will be an overview of USACEHR activities. A second SAWG meeting will be held in Fort Collins, CO, in late July for review of Deployment Toxicology user needs and how USACEHR can be employ its research programs to meet those needs. It is likely that a Third DTUWG will be held late in the calendar year.

APRIL 1 - JUNE 30, 1997

The Deployment Toxicology Science Advisory Work Group held its first meeting at Fort Detrick May 28, 1997. The panel of experts were briefed on the capabilities and concepts to be employed in the Deployment Toxicology program. The Second Deployment Toxicology Users'

Work Group Meeting was held at USACEHR, Fort Detrick May 29-30. The purpose of the meeting was to provide the users' perspectives and advice to the Joint Deployment Toxicology Research and Development programming and planning efforts. A Master Plan has been developed by Army and Navy medical toxicology research laboratories, with input from the Air Force, to coordinate and integrate research to protect deployed defense personnel from toxic chemical environmental hazards. A meeting synopsis has been prepared and distributed to all attendees. Plans are underway for a second Science Advisory Work Group meeting to be held in Fort Collins, CO, July 16-17. A third Deployment Toxicology Users Work Group will be held later in the year.

JULY 1- SEPTEMBER 30, 1997

During this quarter, research management support continued by finalizing the Joint Deployment Toxicology Research and Development Master Plan for publication. Meetings with USACEHR management resulted in first drafts of the next stage in the three-tiered process, the Program Plans. Upon completion and approval of the Program Plans, work will commence, in collaboration with USACEHR, on the Research Plans.

GEO-CENTERS arranged a Sentinel Species Workshop in September, which was jointly sponsored by USACEHR, USEPA, and ATSDR. The workshop was held at Ceresville Mansion and was well-attended by government agencies and other invitees. A consolidated report of the work accomplished by the breakout groups will be submitted in October. One of the principal action items is the completion of an article during the next quarter detailing the issues addressed in the workshop for publication. This article may bring the attention of the appropriate communities to both the complexity of the undertaking and the value of using species that are exposed to environmental contaminants as sentinels for hazards to human health. All reports for the 1994 Research Review compendium have been finalized and submitted to USACEHR. Assistance was given in the preparation of presentation materials for three briefings given by USACEHR personnel.

OCTOBER 1- DECEMBER 31, 1997

Presentation overheads for various meetings were produced for the Deployment Toxicology Research and Development program. Overheads for a brief on USACEHR program plans to be given to COL Takafuji were also prepared. Preparations were made for a meeting scheduled with Col Danley and staff for dry-run presentation of USACEHR's technology. Three different templates have been produced for evaluation. The presentation is part of the marketing program's transfer technology of USACEHR to DOD and commercial markets. Research and investigations were performed in the following areas: brochure lay-outs for handouts at trade shows, presentations, and for mail inquires; available and suitable trade shows for our product; trade magazines suitable that will reach the market place; and possible sources for production of a video presentation.

A chemical agents effects workgroup meeting was held at Edgewood, MD, on November 13, attended by workgroup members, invited military participants, and observers. A third Work Group was held at the USACEHR Fort Detrick, MD. Printed copies of the final version and

Program Plan material was submitted. A neurobehavioral toxicology workshop was facilitated and a workshop meeting summary was prepared. The sentinel species workshop meeting summary was prepared and the outline for a journal article on the workshop was drafted. Three final documents were delivered on the performance degrading effects of exposure to mustard agent, VX and G-nerve agents.

JANUARY 1 - MARCH 31, 1998

Arrangements were made with the laboratory's computer personnel to have the electronic copies of the posters, created by visual information center, placed on USACEHR's computer system. This allows the posters to be corrected and rearranged "in house" decreasing the lead time needed to create a poster for a given event. A poster on the ventilatory biomonitoring system is near completion for the Fort Detrick Earth Day celebration in April. Another poster currently being revised, is the Integrated Environmental Assessment poster. This poster will be used for the Workshop on Environmental Aspects of Energetic Materials in Canada, to be held the 26th and 27th of May, 1998.

A graphic color layout was completed of a brochure for USACEHR. Mass production will be underway on this brochure next quarter. Graphic presentation of the "Drinking Water Disinfection Byproducts Brief" was completed. As research continues, this presentation will be expanded. A PowerPoint picture layout incorporating slide photos of medaka was completed. Graphics were altered and created for a biomonitoring poster to be used in up coming conferences. Additionally, five drawings and paperwork were completed for the ventilatory biomonitoring patents. Amphibian photos were scanned for the Reproductive Toxicology project. Photos were arranged and altered for a joint Navy project. A ventilatory biomonitoring slide was created for CDR Knechtges's briefings. SOP's were updated and edited. Assistance was given with memos and copying and research test numbers were assigned. 35mm slides/photos scanning was learned on the color scanner to enable USACEHR to become more autonomous in producing graphic presentations which will help meet deadlines in time constrained projects.

A draft technology assessment was prepared as part of the research planning for all technologies considered as either critical or supporting to deployment toxicology research requirements designated to address the user identified needs. A draft white paper was prepared for deployment toxicology medical R&D. Technical assistance for TARA biomedical review was provided. Assistance and preparation went into the development of STO's, MNS's, FOC's, DTO's and other materiel requirements documents. Support was given on the preparation of a Sentinel Species journal article. Review and comment was collected from the Deployment Toxicology Science Work Group on the draft strategic research plan prepared in response to Presidential Review Directive #5. The DTUWG III meeting summary was prepared and distributed. Two IPR's held at Fort Detrick were attended. All meeting actions on the neurobehavioural toxicology work shop were completed. Preparations were made for meetings on disinfectants/disinfectant by-products research project design.



APRIL 1 - JUNE 30, 1998

The Cooperative Research and Development Agreement (CRDA) for the biomonitoring system has been in legal review. Upon completion of this review, it should be signed and put into place. Patent license agreement final draft has been completed and is ready for review. Contact has been made with Pacific Northwest Laboratory regarding the use of USACEHR's technologies and the mobile laboratories for use in Orange County, California, water disinfection program. A cost estimate and options related to this effort are being prepared.

A collaborative effort resulted in a multipurpose informational brochure about USACEHR to be disseminated at professional meetings and to guests. The brochure used state-of-the-art graphics and relevant photographs to describe current research at USACEHR, along with providing the mission statement and a point of contact. This brochure was distributed at the IAGLR meeting in Ontario, Canada and was well received. A presentation was prepared entitled "Study Design for FETAX Studies," visual equipment was set up and arrangements made for the EPA meting April 1st. A Deployment Toxicology brief presented to EPA on April 2nd and a presentation titled "Deployment Toxicology Medical Research and Development" presented to the Office of Technology Assessment, National Academy of Scientists were prepared for Dr. Gardner. A force protection brief entitled "Deployment Toxicology Update" and the "Deployment Toxicology and Environmental Health Surveillance" presentation was prepared for COL Danley. Test number assignments were issued for all experiments. Reports were prepared for T. Shedd. CDR Knechtges's "Deployment Toxicology Medical Research and Development" brief and Dr. Clegg's presentation titled: "The Challenges of Conducting State-of-the-Art Epidemiologic Studies" were edited and polished. Work was done on USACEHR's Research Program presentation. Efforts are also being made to create a database/program for USACEHR to more effectively keep track of costs. USACEHR work unit presentations for the Fate and Effects Research Review were completed. The SO4 Funded Work Unit presentation are as follows: "Reproductive Toxicity Test Procedure Using *Xenopus* Frogs," "Methods to Identify Hazards to Human Reproduction Using Sentinel Species," "Mechanisms of Fish Immune Responses for Interspecies Extrapolation Modeling," "Teleost Fish Models for Immunotoxicological Hazard Assessment," "Measurement of Induced Mutation in Transgenic Fish." The SO4 Unfunded Work Unit presentation was titled "Development Neurotoxicity Model for Environmental Toxicants." The 835 Funded Work Unit presentations were; "Sensitivity and Response Time of the Automated Fish Ventilatory Biomonitoring System" and "Software Development for an Automated Fish Ventilatory Biomonitoring System." The 835 Unfunded Work Unit presentation was titled "A Comparison of Semipermeable Membrane Devices (SPMD's) and Sentinel Species as Indicators of Chemical Contamination."

A revised Tri-Service Research and Development Plan for Deployment Toxicology and a report entitled "Improved Microbiological Testing of Field Water - An Evaluation of Capabilities, Technologies and Research and Development" was submitted. A presentation was given at the Conference on Issues and Applications in Toxicology and Risk Assessment entitled "Deployment Toxicology Medical Research and Development." The conference proceedings will be published in *Drug and Chemical Toxicology*. The Sentinel Species Workshop summary documentation for publication in *Environmental Health Perspectives* was completed. CEHR environmental quality (EQ) R&D strategy meeting at Fort Detrick was attended and continuing

follow-on support has been given in presenting the CEHR EQ R&D program for review and coordination. Planning was done for an independent peer review meeting of the neurobehavioral toxicology research plan prepared by the Naval Medical Research Institute Detachment - Toxicology and Division of Neuroscience, Walter Reed Army Institute of Research.

Task Order RM-4



GEO-CENTERS, INC.

Title: Mixed Exposures and Neurobehavioral Toxicology
Task Number: RM-4 (2533-016)

Life Systems provided support to two projects under this task order. A white paper on Mixed Exposures as part of the National Occupatinal Research Agenda was prepared. A second project, for the Naval Medical Research and Development Command, in volved the peer review and revision of the Neurobehavioral Toxicology Research Plan. Deliverables were Mixed Exposures White Paper (TR-1605-14, September 1998) and Deployment Toxicology Assessment Program (DTAP) Strategic Plan: Neurobehavioral Toxicology Thrust Area (TR-1605-15, September 1998).

Appendix A



GEO-CENTERS, INC.

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Appendix B

INTERIM REPORT

EQ-1

The development of methods to quantify the type and spectra of munitions induced by munitions in a human cell line (This portion of Task EQ-1 was discontinued on November 19, 1993)

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GOALS OF PROJECT

This goals of this project were to screen the mutagenicity of chemicals of interest to the U.S. Army in human cells, and to identify the specific type and spectra of mutations induced in deoxyribonucleic acid (DNA) by those chemicals found to be positive mutagens.

EXPERIMENTAL DESIGN

In this section a brief overview of the experimental design is given. The laboratory procedures followed may be found in the appendixes sumarizing methods developed by many investigators (Dlouhy et al., 1991; Bangham C.R.M. 1991; Farr C. J. 1991; Myers R.M. et al 1987; Forth E. et al., 1981; Fisher S.G. and L.S. Lerman 1983; Thilly W.G. 1990; Keohavong P. and Thilly W.G. 1989).

The experimental approach utilized an initial screening of suspected mutagens by treating TK-6 lymphoblasts with the suspected mutagen. Large numbers of K-6 cells were treated with compounds identified as mutagenic in our initial screening, and cells expressing a mutation "selected" with 6-thioguanine. 6-Thioguanine is a purine analog which is metabolized by cells containing a functional Hypoxanthine-Guanine Phosphoribosyl Transferase protein (HPRT) into a 6-thioguanine nucleotide capable of acting as a substrate in the synthesis of deoxyribonucleic acid (DNA). Incorporation of the 6-thioguanine nucleotide into DNA terminates DNA synthesis resulting in cell death. In contrast, cells not producing a functional HPRT protein are resistant to 6-thioguanine cytotoxicity since no cytotoxic metabolite is produced. Therefore any cell containing a mutation in the HPRT gene that results in the production of a non-functional HPRT protein, or no protein, will survive treatment with 6-thioguanine. The exon 3 region of the HPRT gene within the resistant cells is then analyzed on the molecular level to determine what type of mutation occurred.

DNA analysis of the induced mutations utilized an initial amplification of the entire 224 base pair (bp) HPRT exon 3 region by using polymerase chain reaction (PCR). The product of this amplification was purified on non-denaturing polyacrylamide gels (PAGE), and used in two additional PCR amplifications. The second amplification step was designed to produce two separate species, a 184 bp fragment containing the high temperature melting domain (melting is defined here as the dissociation of duplex DNA into single strands), and a second 204 base pair fragment containing the low temperature melting domain of the exon. The melting characteristics of small DNA fragments are uniquely dependent on the sequence of base pairs the DNA is composed of. Base substitution mutations occurring anywhere within the exon 3 region are capable of being resolved from one another on denaturing gradient gels by manipulating the unique melting characteristics of these two species. The products from the second amplification reaction were run on denaturing gradient gels (after first being purified on non-denaturing PAGE gels) the gradient of which had been independently optimized to separate fragments containing base substitution mutations within the individual melting domains. The final step was to isolate the independent bands and to use DNA sequence techniques to determine the type and spectra of mutation induced. Work was terminated on the project before the final series of experiments were initiated.

RESULTS AND PROGRESS

Please consult laboratory notebooks for specific data from the experiments summarized below. Methodological differences in data acquisition, as well as the preliminary nature of the data preclude compilation in comprehensive tables and charts. The work is summarized below and all procedures employed may be found in the appendixes.

The first year of this project was devoted to developing a non-radioactive technique for analyzing mutations at the molecular level using polyacrylamide gel electrophoresis, the polymerase chain reaction, and denaturing gradient gel electrophoresis (please see notebook #1191). The techniques developed for doing this work may be found in the appendixes. Success of the methods were verified by using DNA from cells treated with the positive control mutagen, N-methyl-N'-nitro-nitrosoguanidine (MNNG). However, the high temperature domain proved difficult to amplify using PCR and the high fidelity, temperature stable, polymerases necessary in mutational studies. Neither the Vent, nor Deep-vent polymerases, were able to amplify the high temperature fragment, although Taq polymerase was effective (the low fidelity of the Taq polymerase precludes it's use in mutagenesis studies). The reason the Vent and Deep-vent polymerases were unable to amplify the high temperature fragment is most likely due to an inability to bind and polymerize from the PCR primer containing the GC clamp (this is a guanine and cytosine rich segment of the primer added to alter the melting behavior of the product). Therefore, no gradient gels designed to separate those mutations occurring in the high temperature domain were run. Since these experiments were conducted, additional high fidelity, temperature stable, polymerases have been discovered, and may prove effective in overcoming this limitation. PCR amplification of the low domain was successful with all the polymerases, as was the production of digitoxigenin-11-UTP (uracil triphosphate) labelled DNA probes through a modification of the standard PCR technique. The techniques for the non-radioactive localization of DNA on gels utilized a modified southern blotting procedure. This process is laborious and requires multiple steps, but is capable of substituting for the standard radioactive method of localizing bands on gels.

The second part of the project focused on screening chemicals for mutagenesis. The project was terminated four months after the work was begun, and prior to the generation of publishable data. This period of time was primarily spent optimizing and verifying the conditions including the exposure time, optimal chemical concentrations and solubility, background mutation frequencies, and solvent toxicity and mutagenicity. Verification of the method was performed with the positive mutagen, MNNG. The mutation frequency derived is consistent with that found in the literature. Problems encountered in this part of the project included solvent toxicity (the purest DMSO commercially available should be used for these experiments; however, it should be noted that no significant DMSO toxicity was observed in similar systems by co-workers), chemical solubility (the compounds chosen to screen were generally quite hydrophobic), and statistical fluctuations in cloning efficiencies on the cytotoxicity plates, presumably due to the difficulty in getting accurate data from small populations (in the cloning efficiency technique used one cell per well is plated in each well of a 96 well plate, although this is the standard technique used in the field it proved problematic in these experiments). This latter problem resulted in large fluctuations in cloning efficiencies making determination of cytotoxicity difficult in some experiments. Although not ideal, estimations of cytotoxicity in those experiments were taken from the number of cells surviving treatment. A separate technique for determining cytotoxicity called "grow back extrapolation" (see Furth et al., (1981)) may result in more consistent data. Although this problem posed

challenges toward gathering consistent cytotoxicity data, the experimental design and mathematical calculation of mutation frequency results in a number unaffected by this problem (any aberrant cloning efficiency cancels out when the ratio of cloning efficiencies in the absence and presence of 6-thioguanine are taken, provided all cells originate from the same dilution calculations). Data sufficient to make observations regarding 4-amino-2-nitrotoluene and 2,4 dinitrotoluene were obtained, with only 4-amino-2-nitrotoluene showing sufficient mutagenicity to be classified as a weak mutagen (highest mutation frequencies obtained were only 3-4 fold over the background frequency for the individual experiment). The lack of mutagenicity of 2,4-dinitrotoluene is consistent with previously published reports in the literature. Of the other five compounds tested, 1,3-dinitrobenzene, 2,6-dinitrotoluene, 2-methyl-3-aniline, trinitrotoluene, and 2-amino-4-nitrotoluene (in which a limited number of experiments were completed, or the experiments were only partially successful), initial indications of mutagenicity were negative (notebook #1198).

SUMMARY AND RECOMMENDATIONS

With the exception of the MNNG experiment, molecular analysis was not performed with any of the chemicals tested for mutagenicity. Although 4-amino-2-nitrotoluene displayed weak mutagenicity, the 3-4 fold increase was not large enough to warrant full-scale molecular analysis (a mutation frequency this low may be difficult to publish). Although laborious, the non-radioactive technique developed to localize DNA fragments in this project for use with PAGE and denaturing gradient gel electrophoresis is a viable one. However considerable time and money would be saved using the standard radioactive techniques.

In general the nitro compounds screened within this period were at best weakly mutagenic at doses bordering on their individual solubility limits (millimolar range). The technique employed is capable of revealing mutagens inducing base substitutions, frameshifts and small deletions. However, this does not preclude their possible mutagenicity in other assays designed to distinguish different classes of mutations (large deletions, recombination, etc.). Detection of these other classes of mutation would require different methods than those discussed above and in the appendix.

Taking into consideration the weak mutagenicity of the nitro compounds tested by this method, these methods do not appear suitable for identifying the type and spectra of mutations caused by nitro-aromatic compounds. However, experiments to increase the mutagenicity of the test compounds were not exhaustive. For example, incorporating metabolic activation systems, using mixtures of the the nitro compounds or using actual environmental samples containing these munition products. In this way, the test compounds would exhibit higher mutation frequencies in the HPRT test system, for subsequent use in identification of genetic biomarkers of munitions toxicity. Additionally, if identification of genetic biomarkers of these same nitro aromatic compounds is pursued without modifications to increase HPRT mutagenicity, it is recommended that an alternate experimental approach, *i.e.* not the HPRT system, be considered.

APPENDIX I

GROWTH OF TK-6 CELLS FOR DGGE EXPERIMENTS

(Furth E. et al., 1981)

1. Tk-6 cells are grown in RPMI 1640 media, 10% horse serum (heat-inactivated at 56°C for 30 minutes), 1% Penicillin/Streptomycin (100 units penicillin and 0.1 mg strptomycin). Cells are incubated 5% CO_2 in 500 ml spinner/roller cultures. Dilute daily to 3 x 10^5 cells/ml. All codes should should consist of two independent cultures.

2. Prior to an experiment cells (2 X10^7 total cells to start with) are grown in anti-selective media (CHAT) to reduce background frequencies for two days.

3. Expand cell cultures to a minimum 3 X 10^8 total cells, per individual treatment at densities between 1 X 10^5 and 1 X 10^6 cells/ml (weaker mutagens will require the treatment of a larger number of cells). Plan on having two independently treated cultures per mutagen (as well as controls) at these densities. Ideally, 10,000 induced mutants per treatment group are necessary to permit identification of mutants making up 1% of the total mutant population.

4. Treat independent cultures with mutagen at a cell density of 5 X 10^5 cells/ml. Remove aliquots of each culture, centrifuge, wash once with 1X PBS, resuspend in dilution media, and count. Dilute and plate cells in 96 well microtiter plates at 2 and 4 cells/well (2 plates per condition) to determine survivals. Centrifuge bulk treated cultures (to remove mutagen), wash once with 1 X PBS, resuspend in dilution media, and count. Then resuspend in fresh media at 3 x 10^5 cells/ml. Grow bulk cultures (cells should reach densities of 1 X 10^6 cells/ml in about 2 days depending on survival) and split by a factor of 3 regularly. Continue subculturing a total number of cells equal to the number that was treated (at least 3 X 10^8 total cells). Count survival plates in 12-14 days. Dilutions are performed in RPMI with 2% serum. Final plating is in regular media (RPMI, 10% horse serum, 1% pen/strep).

5. Continue to grow cells in bulk culture (keeping different treatment groups separate) by counting and/or diluting each day. This is known as the 'expression period" during which the concentration of pre-existing HPRT protein is diminished through natural degradative turnover and dilution consequent to cell growth. Cells suffering a selectable mutation in the HPRT gene will not produce viable enzyme during this period and will be resistant to selection with 6-Thioguanine. It is **CRUCIAL** that the cells are counted often and not allowed to reach confluence. Record all cell densities. If they reach confluence it will be impossible to determine survival by "grow back extrapolation" (please see Forth et al., 1981) .

6. After expression period determine mutation frequencies by plating aliquots of bulk cultures at 2 and 4 cells per well for cloning efficiencies, and 40,000 cells/well to determine mutation frequencies. Ten μM 6-Thioguanine is added to the mutation frequency plate media (**NOT** to those plates used for the cloning efficiencies). Count plates after two weeks. This will result in a mutation frequency which may be compared to that obtained by "grow-back extrapolation".

Perform dilutions in RPMI with 2% serum, cloning efficiencies in regular media, and mutation frequencies in regular media supplemented with 10 μM 6-thioguanine (final concentration).

7. Treat remaining bulk cultures with 1μM thioguanine and grow cells for three days undisturbed. After three days replace two-thirds of the media with fresh media to decrease cell debris. Continue to culture cells until mutant fraction of culture is unity (until all the cells are 6-Thioguanine resistant; non-resistant cells will be attrited during this period). The expression period should be about two weeks. Freeze cells and prepare cells for PCR.

6-Thioguanine:
Weigh out 16.72 mg of 6-Thioguanine and completely dissolve in 2 ml of 1 N NaOH. Add 98 ml of 3 X dH_2O. Filter Sterilize and partition into 10 ml aliquots before freezing. Use at 1:100.

CHAT:
Weight out and mix compounds together into a 20 ml container. Add 2ml 1 N NaOH dropwise and stir until completely dissolved. Add 8ml 3X dH_2O and recheck to make sure it's dissolved before taking up into a total of 100 ml. Filter Sterilize and partition into 10 ml aliquots before freezing. After CHAT treatment resuspend cells in HCT media (hypoxanthine, cytidine, thymidine at the same concentrations used for CHAT media) and grow for two more days. Use at 1:100

	MOLECULAR WEIGHT (grams/mole)	GRAMS/100 ml (100 X conc.)	FINAL MEDIA CONC. (1 X μM)
CHAT			
Cytidine	243.2	0.0243	10
Hypoxanthine	136.1	0.136	100
Aminopterin	440.4	0.0009	0.2
Thymidine	242.2	0.041	17
6-Thioguanine			
2-Amino-6-Mercaptopurine	167.2	0.01672	10

MUTAGENS:
MNNG (N-methyl-N'-nitro-N-nitrosoguanidine) used at 10-15 ng/ml (survival = 85-11% in this range (Thilly, 1990), ideally shoot for about 30 % survival) and dissolved in PBS at pH 7.2 (Thilly 1980). MNNG is useful as a positive control for mutagenesis. The serum bottles of MNNG which came from Sigma contain 10 mg. Add 30 ml anhydrous DMSO for a 0.33 mg/ml (2.2 mM) solution, then dilute 1/100 for a 3.3 μg/ml (22 μM) solution. Discard after use. Use 4.5 ml/1000 ml for a 15 ng/ml solution (approx. 100 nM) and 3.0 ml/1000 ml for a 10 ng/ml (68 nM) solution. MNNG is a potent mutagen and must be inactivated prior to disposal. Inactivation of MNNG is simply done by acidifying the solution to pH=3.0 and letting it stand in a hood overnight.

If using a chemical dissolved in DMSO, do not use DMSO at a concentration greater then 0.5% of total culture volume. With munitions compounds solubility experiments must be done prior to the experiment.

CALCULATIONS-Poisson Distribution:
$P(0) = e^{-x}$ (x = average number of colony forming units/well)
P(0) = probability of any well having no colony (negative wells/total number of wells).
Cloning Efficiency = -Ln P(0) / Number of cells per well (-LN (negative number of wells/total

number of wells))/number of cells plated per well.
Mutation Frequency = Cloning Efficiency in selective media/Cloning Efficiency in non-selective media

ISOLATION OF DNA FROM CELLS PRIOR TO PCR
(Maniatis T. et al., 1982)

1. Pellet 10^5 cells by centrifugation at 1500 g at 4°C in small microfuge tubes.
2. Wash once with 1 X PCR buffer (either TAQ , Vent or whatever buffer is supplied with the polymersae to be used for PCR, no other additions such as dNTP's etc..) and remove as much remaining liquid as possible. Wash with 1 x PBS. Repeat.
3. Store pellet at -20°C or use immediately.
4. Resuspend pellet in 20 μl of lysis buffer. Add proteinase K at a final concentration of 100 μg/ml and mix.
5. Incubate at 50-60 °C for one hour (longer incubations (3 hrs) are recommended in other procedures).
6. Heat to 95°C for 10 minutes to inactivate remaining proteinase K.
7. Pellet cell debris in microfuge. Store at -20°C.

1 X pre-Vent Buffer (pH 8.8 at 25°C) - 10 mM KCl; 20 mM Tris-HCl; 10 mM $(NH_4)SO_4$; 2 mM $MgSO_4$
0.1% Triton X-100.

1 X PBS (Thilly, Mut.Res., 1991) - 15 mM Na_2HPO_4; 1.5mM KH_2PO_4; 137 mM NaCl; 2.7mM KCl; 3 X dH_2O to 1 liter (aliquot, sterilize and store at 4°C):

For 10 X PBS - 150 mM Na_2HPO_4; 15.0 mM KH_2PO_4; 1.37 M NaCl; 27mM KCl; 3 X dH_2O to 1 liter

Lysis buffer - 1 ml of 10 X PCR buffer (do not use Taq lysis buffer with Vent); 0.5% (50μl) of Tween-20
0.5% (100μl) of NP-40 0.5% Nonidet P-40 from Sigma); 3 X dH_2O to 10 ml, store at 4°C

Proteinase k is made up according to Maniatis. Store at -20°C.

6 X 10^6 cells will yield 25μg of DNA. For PCR step use 12.5 μL of the lysis mix or 500 ng of DNA. 2.5 x 10^5 cells yields 1μg DNA; 6 x 10^6 cells yields 25μg of final product (8pg DNA/diploid cell (4pg for haploid cell) assume a 50 % recovery)

APPENDIX II

GROWTH OF PHAGE LAMBDA AND ISOLATION OF DNA FOR PROBE PRODUCTION

(Maniatis T. et al., 1982)

For heteroduplex analysis on denaturing gradient gels and for the production of digioxigenin-11-UTP labelled probes of the HPRT exon 3 region lambda phage clone (Huλ3;American type culture collection # 57238) containing the human exon 3 region was used. This clone was orignially produced by Thomas Caskey and contains the entire proximal portion gene (exon one through intron three). The phage were grown, the DNA isolated, and the exon three region PCR amplified to produce both digioxigenin-11-UTP labelled and unlabelled probes.

GROWTH OF PHAGE LAMBDA AND ISOLATION OF DNA:

Preparation of lambda phage from plate lysates:
Mix 10^5 pfu of bacteriophage in 0.1 ml with 0.1 ml plating bacteria (at O.D. of 2.0=1.6 X 10+9 cells/ml). Incubate 20-30 minutes at 37°C. Add 3 ml of molten top agar (0.7 % agar at 47°C), mix and pour immediately. Incubate overnight at 37°C. The next day check for confluent lysis. If lysis occurred add 5 ml SM to the top of the plate and store at room temperature for several hours with gentle shaking. Collect SM media containing phage. Add 1 ml SM to plate, tilt plate and collect SM after it drains to bottom of slant, combine. Centrifuge at 4000 g for 10 minutes at 4°C to remove debris and remove an aliquot to use to determine titers. Recover supernatant, add 1 drop chloroform and store at 4°C.

Purification of DNA with Qiagen (from Qiagen manual):
1. Add 30 μl of buffer L1, Rnase A to a final concentration of 60μg/ml and Dnase I to a final concentration of 18 μg/ml (these final concentrations are those recommended by Qiagen, Maniatis recommends a final concentration for Dnase I and Rnase A of 1μg/ml). If using 10 mg/ml stock solution of Rnase A add 60 μl/10 ml phage suspension. For Dnase I add 13μl/10 ml phage suspension from the 14,000 μg/ml Sigma stock. Incubate at 37°C for 30 minutes.

2. Add 2ml of ice cold L2, mix gently, and incubate on ice for 60 minutes.

3. Centrifuge at >10,000 X g for 10 min (about 10,000 RPM's if using the SS34 rotor), and discard the supernatent. Then place tubes upside down for one minute to drain remaining supernatent.

4. Resuspend the pellet in 1 ml of buffer L3 using a pipet (pipet up and down a few times and wash tube walls also).

5. Add 1ml of buffer L4 (make sure the SDS has not precipitated), mix gently, incubate at 70°C for 10 minutes,then cool on ice.

6. Add 1 ml of buffer L5, mix immediately but gently (invert tube 4-6 times), incubate 5-10 minutes on ice, and centrifuge at 4°C for 30 minutes (>15,000 X g). Carefully collect the clear supernatent (a layer of potassium dodecylsulfate (PDS) may be on top of it so be careful not to mix the two).

7. Centrifuge the supernatent again at 4°C for 10 minutes (>15,000 X g) to obtain a particle-free clear lysate (non-turbid). Make sure there is no particulate or suspended material in the supernatent (it will clog the column).

8. Equilibrate a Qiagen-tip 20 with 1 ml of buffer QBT.

9. Apply the supernatent from step 7 onto the Qiagen-tip and allow it to enter the resin by gravity flow.

10. Wash the Qiagen-tip 20 with 2 X 2 ml of buffer qc.

11. Elute the DNA with 1.5 Ml of buffer qf.

12. Precipitate the DNA in at least 0.7 volumes of isopropanol (30 minutes on ice) and microfuge for 30 min at high speed. Use isopropenol with Qiagen purified DNA. Normal nucleic acid precipitation uses 2 volumes of Ethanol instead.

13. Carefully remove isopropanol and wash DNA with 1 volume of ice cold 70% ethanol. Re-centrifuge in microfuge for 5-10 minutes at the same speed used above. Carefully remove ethanol and allow the pellet of nucleic acid to dry in the air for 5 minutes. Do not use a speed vac for evaporation. Resuspend in 50 μl of TE buffer, pH=8.0 (until concentration is known). If < 1μg of DNA is present glycogen should be added as a carrier for precipitation.

Qiagen Buffers:
L1 20 mg/ml RNase A, 6 mg/ml DNase I, 0.2 mg/ml BSA, 100 mM Tris•HCL, 300 mM NaCl, 10mM EDTA, pH=7.5, 4°C
L2 30 % Polyethylene Glycol (PEG 6000), 3 M NaCl, 4°C
L3 100 mM Tris•HCL, 100 mM NaCl, 25mM EDTA, pH=7.5, 25°C
L4 4% sodium dodecylsulfate (SDS), 25°C
L5 2.55 M potassium Acetate, pH=4.8, 25 °C
QBT 750 mM NaCl, 50 mM MOPS, 15% ethanol, pH 7.0, 0.15% Triton X-100, 25°C
QC 1.0 M NaCl, 50 mM MOPS, 15% ethanol, pH=7.0, 25°C
QF 1.25 M NaCl, 50 mM MOPS, 15% ethanol, pH=8.2, 25°C

Preparation of lambda phage from large-scale liquid cultures:
This method employs infection at high multiplicity to generate large numbers of phage. Inoculate 10 ml of LB or NZCYM media in a 50 ml flask with a single colony of bacteria host. Incubate at 37°C overnight with vigorous shaking (300 cycles/minute). The next day inoculate 500 ml of LB or NZCYM media (37°C) in each of four 2 liter flasks with 1 ml of the overnight culture and incubate while shaking vigorously until OD600=0.5 (3-4 hours). Infect bacteria with 10^{10} pfu of phage lambda and incubate at 37°C with vigorous shaking until lysis of the culture occurs (3-5 hours). Add 10 ml of chloroform to each flask and continue incubation for 10 minutes. Cool the culture to room temperature and add pancreatic DNAase I and RNAase (both at 1μg/ml). Incubate at room temperature for 30 minutes. Add solid NaCl to a final concentration of 1 M (29.2 grams/500ml) and dissolve by swirling. Let stand for 1 hour on ice. Centrifuge at 11,000 g for 10 minutes at 4°C. Pool all supernatents. Precipitate phage with polyethylene glycol (PEG 8000) by slowly adding to concentration of 10 % (50g/500ml). Cool in ice cold H_2O and place on ice for 1 hour. Recover precipitated phage by centrifugation at 11,000 g for 10 minutes at 4°C.

Discard supernatent and drain remaining liquid (be careful to remove all supernatent). Resuspend phage in SM (8 ml/500 original ml) by pipeting up and down with a wide-bore pipet. Wash walls of flask thoroughly. Add an equal volume of chloroform and vortex. Centrifuge (3000 g for 15 minutes) and recover aqueous layer.

Centrifuge phage at 25,000 rpm for 2 hours at 4°C in a Beckman SW28 rotor. Pour off supernatent and add 1-2 ml of SM, mix and leave overnight.

EXTRACTION OF LAMBDA DNA:

This procedure may be employed, or use the procedure outlined in the Qiagen manual. Add proteinase K to a final concentration of 50 μg/ml and SDS from a 10 % (w/v) stock solution to a final concentration of 0.5 % to the phage preparation. Incubate for one hour at 56°C. Cool to room temperature and add an equal volume of phenol equilibrated with 50 mM Tris (ph=8.0). Mix by inversion and separate phases by centrifugation at 3000 g for 5 minutes. Transfer aqueous phase to a new tube and extract with a 50:50 mixture of phenol/chloroform. Recover aqueous phase and extract with chloroform (equal volume). Dialysis overnight at 4°C against three changes of a 1000 fold volume of TE (ph=8.0).

TITERING PHAGE:
Suspend E-coli LE 392 cells at OD_{600} = 2.0 in SM. Do three successive dilutions of 10 μl of the phage preparation into 2.16 ml of SM (216 fold dilution each for a total of 1 to 10^7). Add 100μl of diluted phage to 100μl of plating bacteria, mix and incubate at 37°C for 30 minutes to allow phage absorption. Add 100μl of phage/bacteria mix to 3 ml of LB top Agar and pour onto plate. The next day count plaques and calculate titers.

MAINTENANCE OF STOCK CULTURES:
To rehydrate freeze-dried bacteria cultures from American Type Culture Collection add 0.3-0.4 ml Lb media with a sterile pasteur pipet. Dissolve pellet and transfer to a 5-6 ml LB culture. Aliquot 0.2 ml into 10 X 6 ml LB cultures and streak onto Agar plates. Grow cultures and re-inoculate into larger volumes of 50 ml. Cells should be harvested in their early stationary phase of growth. To determine growth kinetics inoculate a LB culture at a density of OD600=0.01. Monitor time dependence of bacteria growth every hour at OD600. Don't forget blanks.

Freezing cells:
Centrifuge and resuspend into 2.5 ml of LB media, add 20 % sterile glycerol (autoclavable) and dispense aliquots into sterile vials. Freeze in ethanol-dry ice or liquid nitrogen, store at -70°C. Bacteriophage may be stored in SM with 0.3 % chloroform at 4°C for several years. A second storage method is to add 7 % DMSO, freeze, and then freeze in liquid nitrogen, store at -70°.

Strains and genotypes (check on these-word processor did not convert all properly):
E-coli strain DP50 supF58: (atcc # 39061) F^- dapD8 lacY (galuvrB) thyA $nalA^r$ hsds suII suIII
E-coli strain LE 392: Obtained from the NCI (Don Court)
Lambda phage Huλ3: (atcc # 57238)

Media:
Lb media (adjust to pH 7.0 with 5N NaOH) - 10 g Bacto-tryptone; 5 g bacto-yeast extract; 10 g NaCl
H_2O to 1 liter. Sterilize at 121°C for at least 15 minutes and then add - 8 ml of $MgSO_4 \bullet 7H_2O$ (1M) or $MgSO_4$ (1M); 5ml 1M $CaCl_2$; 10 ml of a 20% maltose Solution (1ml maltose/100 ml culture media)

For LB Plates add 15 grams of agar (or agarose)/ liter, and for top agar/agarose add 7 grams/liter of agar or agarose prior to autoclaving.

LB bottom agar/agarose (should give higher yields then above) as above but add - 0.3% glucose; 0.075 mM $CaCl_2$ (already present at 5 mM); 0.004 mM $FeCl_3$; 2 mM $MgSO_4$ (already present at 8 mM)

SM Buffer - 5.8 g NaCl; 8 ml of $MgSO_4 \bullet 7H_2O$ (1M) or $MgSO_4$ (1M); 1 M tris•Cl (pH=7.5); 2% gelatin solution add 5ml (final % = .01 %); H_2O to 1 liter

NZCYM media - 10 g NZ amine (enzymatic casein hydrolysate); 5 g NaCl; 5 g bacto-yeast; 1 g casamino acids; 2 g of $MgSO_4 \bullet 7H_2O$; H_2O to 950 ml and then adjust ph to 7.0 with 5 N NaOH (about 0.2ml).

Ribonuclease A (10mg/ml; pH7.5): store at -20°C. Maniatis uses RNase A at af i n a l concentration of 1μg/ml - 10 mM Tris•HCL; 15 mM NaCL

Deoxyribonuclease I, If not made up by supplier make up at least 1mg/ml if possible (Sigma's is at 14 mg/ml) in the following buffer - 20 mM Tris pH=7.6; 50 mM NaCl; 1 mM Dithiothreitol (mol. wt. = 154.2g/mole) 100μg/ml BSA; 50 % glycerol

TE - 10 mM Tris•HCL ph=8.0 (121.14 grams/mole);1mM EDTA

Proteinase K (store at 4°C) - Add 200 mg to 10 ml H_2O for a 20mg/ml solution
SDS - 10 % (w/v)

$MgSO_4 \bullet 7H_2O$ - Add 24.65 grams to 100ml for a 1M solution.

$MgSO_4$ - Add 12.04 grams to 100 ml for a 1M solution.

$CaCl_2$ - Add 14.7 grams to 100 ml for a 1 M solution.

50 X TAE is - 242 g Tris base; 57.1 ml glacial acetic acid; 100 ml of 0.5M EDTA (ph=8.0) per 1000 ml.

10 x TBE (actually only 5 X but used as 10 X) is - 54 g Tris base; 27.5 g Boric acid; 20 ml of 0.5M EDTA; ph=8.0

10 mg/ml Ethidium Bromide (wear gloves) - 1g Ethidium Bromide dissolved in 100ml of dH_2O

10 X PBS (aliquot and sterilize) - 150 mM Na_2HPO_4;15.0 mM KH_2PO_4;1.37 M NaCl 27mM KCl; H_2O to 1 liter

APPENDIX III

POLYMERASE CHAIN REACTION
(Mullis K.B., and Faloona F.A., (1987); Keohavong P, and Thilly W.G. (1989); Cariello N.F., et al. (1990))

Primer 1 (reverse primer) is complementary to the sense (plus) strand (3'-end of exon), primer 2 (forward primer) is complementary to the antisense (minus) strand (5'end of exon 3). Primer 3 is located within exon 3, immediately 3' of the 5' splice site, and is complimentary to the antisense strand. Primer 4 is complementary to the sense strand and is located 79 nucleotides (5' direction) from the 3' splice site. Primer 2-GC-clamp, is the same as two, but contains an additional GC clamp sequence. Solutions of oligonucleotides should be made up in 3 X H_2O and kept frozen.

LENGTH OF EXPECTED AMPLIFICATION PRODUCTS:
primers 1/2 = 224 bp entire exon 3 region
primers 1/3 = 204 bp Designed for DGGE of 104 bp low temp region
primers 4/5 = 184 bp Designed for DGGE of 80 bp high temp region

PRIMER NUMBER (MOL.WT.)
Primer 1 (6600) is 3'-CACTCATATAAATTATATAC-5'
Primer 2 (6600) is 5'-TCCTGATTTTATTTCTGTAG-3'
Primer 3 (6600) is 5'-GACTGAACGTCTTGCTCGAG-3'
Primer 4 (5610) is 5'-AAACGACTGGACGACCT-3'
Primer 5 (primer # 2 with a GC-clamp) (24420) is
5'-GCCGCCTGCAGCCCGCGCCCCCCGTGCCCCCGCCCCGCCGCCGGCCCGGGCGCC
TCCTG...
.....ATTTTATTTCTGTAG-3'
The extinction coefficients per nucleotide, and those per primer in ml/μmole are listed below. Coefficients per primer are found by summing up the contributions of each nucleotide.

EXTINCTION COEFFICIENTS FOR PRIMERS:

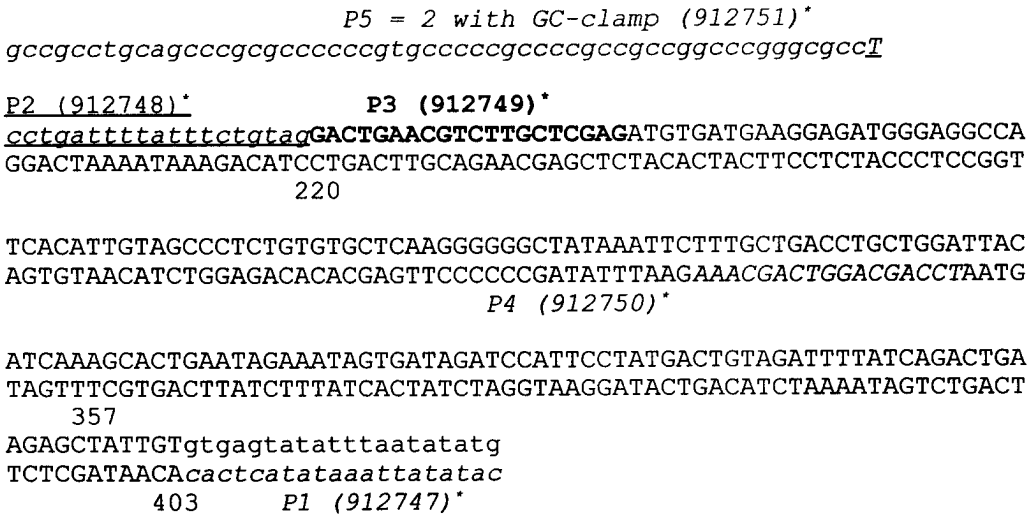
NUCLEOTIDE	PRIMERS				
	1	2	3	4	2-GC
GUANINE	0	35.1	70.2	46.8	234
ADENINE	138.1	46.2	61.6	92.4	61.6
THYMINE	61.6	96.8	44	17.6	114
CYTOSINE	29.2	21.9	36.5	36.5	270
EXT.COEF. (ml/μmole)	229.4	200	212.3	193.3	680
MOL. WT.	6600	6600	6600	5610	24410

APPROXIMATE MOLECULAR WEIGHTS OF PCR AMPLIFIED DOUBLE-STRANDED FRAGMENTS:
(not labelled with Digoxigenin-11-dUTP)

Probe 1 = 224 bp fragment = 1.48 X 10^5 g/mole
Probe 2 = 204 bp (low temp domain) fragment = 1.35 x 10^5 g/mole

Probe 3 = 184 bp (high temp domain) fragment = 1.2×10^5 g/mole

HPRT EXON 3



* Number in parentheses is the Stratagene "part" number for ordering the oligonucleotide.

PCR PRIMERS:

LOW TEMPERATURE PRIMERS (b.p.300-403)

5' 3'
1. CATATATTAAATATACTCAC
2. TCCTGATTTTATTTCTGTAG

HIGH TEMPERATURE PRIMERS (b.p.220-299)

5' 3'
5. GCCGCCTGCAGCCCGCGCCCCCCGTGCCCCCGCCCCGC
CGCCGGCCCGGGCGCCTCCTGATTTTATTTCTGTAG
4. TCCAGCAGGTCAGCAAA
3. GACTGAACGTCTTGCTCGAG

The low temperature melting domain is from positions 300-403 and that of the high temperature domain is 220-299.

PCR FROM HUMAN CELLS:

The PCR machine contains three pre-programmed files which together constitute a specific pcr method. Hotstart PCR is employed in our initial method when amplifying DNA from cells. This procedure is designed to increase specificity of amplification. All the components of the reaction mixture (except the polymerase) are mixed together (it's best to add in the following order; buffer, H_2O, $MgSO_4$ (not used in our protocol), dNTP's, DNA template, primers) and heated for 5 minutes at 95°C. The temperature is lowered to 85°C for 10 minutes during which the enzyme is added. Primer annealing is then carried out at 42°C followed by an extension time of 72°C for

30 seconds. Program #10 carries out all these steps automatically by running the preamplification step at 95°C, the 10 minute 85°C step, and then the first cycle. At the conclusion of the first cycle program #10 calls up program #7 which is the main cycling program. This file is set to cycle through the following parameters for 29 cycles.

1 minute at 94°C (melt)
Ramp down to 42°C
1 minute at 42°C (anneal)
Ramp up to 72°C
30 seconds at 72°C (extension)
Repeat 28 times (30 cycles total)

File 7 is started automatically upon completion of file # 10. Upon it's completion, file # 7 calls up file # 8, which is a holding file designed to keep the samples at 4°C until the machine is physically shut off. The holding file allows for overnight, and unattended runs.

PCR FROM PRIOR AMPLIFICATIONS:

To analyse exon 3 in the Hypoxanthine-Guanine phosphoribosyl transferase gene the entire 224 base pair exon is initially amplified. Primer 1 and Primer 2 which flank the exon and are used in the initial amplification. Exon 3 contains both high and low melting regions which are then amplified independently from the product of the primer 1/2 amplification. Primers P1+P3 are used for the low temperature domain and primers P4+P2-GC-clamp are used for the high temperature domain). The initial pcr reaction is run for 20 cycles (less is best since we do not want any polymerase introduced base pair changes to occur at this stage). 5 μl from the initial amplification is then reamplified to obtain the low temp and high temperature domains. Run the second reactions for 30-40 cycles.

Primer annealing temperatures may be approximated from the equation below. However, a more exact calculation requires base stacking interactions to be taken into consideration, this may be performed on the "Primer" analysis software program available at BSC. It may be necessary to alter the annealing temperature for amplifications of the high and low domains.

$$T_m\ ^\circ C = 2\ X\ (A + T) + 4\ X\ (G + C)$$

PREPARATION OF HPRT-EXON 3 BY PCR

To produce double stranded Exon 3 for heteroduplex formation and for direct amplification of genomic DNA Deep-Vent polymerase is used. Heteroduplex formation produces double stranded DNA useful for forming heteroduplexes with mutants prior to DGGE analysis. Exon 3 sequence for both high (184 bp) and low (224 bp) melting domains are needed. The Deep-Vent polymerase has a higher fidelity then the Ampli-Taq polymerase enabling the production of more pristine DNA. For this reason the Deep-Vent polymerase is used to produce DNA to be used in heteroduplex formation and in the direct amplification of DNA from cells. For production of HPRT probes labelled with digoxigenin-11-dUTP, ampli-taq polymerase is employed (fidelity is less of an issue in this application). Probes for both the high (184 bp) and low (224 bp) melting domains are needed.

VENT AND DEEP-VENT PCR:

Add in the following order; 10X buffer (New England Biolabs), H_2O, $MgSO_4$ (not used in our protocol but may improve yield in some reactions), dNTP's at 200-400 μM (Vent needs a higher concentration of dNTP's to inhibit the 3'-5'exonuclease action which occurs if dNTP's run low, so 300μM is generally employed, 200μM is okay for Deep-Vent and Taq), DNA template (at a concentration of 500 ng - 1 μg which is approximately 10^5 cells), 0.2-1.0 μM Primer A and Primer B (copies of Exon 3 amplified with primers 1 and 2 are re-amplified with using primers P1+P3 for the low temperature domain or primers P4+P2-GC-clamp for the high temperature domain), and 1-2 units of VENT polymerase/100 μl volume).

Run initial reaction for 20-40 cycles (less is best) using primers 1 + 2. Then repeat using 5 μl of the product from the first round for the low temp and high temperature domains. Purify by gel electrophoresis and quantitate by absorbance spectrophotometry (OD=260/OD=280 ratio gives purity which should be about 1.8). OD of 1.0 at OD=260 is approximately equal to a concentration of 50μg/ml for double stranded DNA (20 μg/ml for oligonucleotides and 33 μg/ml for single-stranded DNA).

VENT POLYMERASE REACTION:

ADDITION (STOCK CONC.)	VOLUMES (μL)	FINAL CONCENTRATIONS
VENT-BUFFER (10X)	10	1X
3 X dH_2O	54.5	*
NTP's	12 (3μl each)	300μM
Primer A (10μM)	5	500nM
Primer B (10μM)	5	500nM
DNA Lysate	12.5	$<$1μg/ml
VENT POLYMERASE	1	20
Total	100	

DEEP-VENT POLYMERASE REACTION:

ADDITIONS (STOCK CONC.)	VOLUMES (μL)	FINAL CONCENTRATIONS
VENT-BUFFER (10X)	10	1X
3 X dH_2O	58.5	*
NTP's	8 (2μl each)	200μM
Primer A (10μM)	5	500nM
Primer B (10μM)	5	500nM
DNA Lysate	12.5	$<$1μg/ml
Deep-VENT POLYMERASE	1	20
Total	100	

Notes:

* Vent and Deep-Vent polymerase stock concentration = 2000 units/ml
* Vent-Buffer is -10mM KCL; 10mM $(NH_4)_2SO_4$; 20 mM Tris•HCl (pH 8.8, at 24°C); 2mM $MgSO_4$; 0.1% Triton X-100
* Deep vent buffer is the same as Vent buffer
* Non-acetylated BSA (NOT acetylated) may also be added at 100μg/ml (this is optional but it

sometimes helps with amplification from genomic samples by neutralizing enzyme inhibitors and decreasing non-specific adsorption of reagents).

* 5×10^{10} copies of Exon 3 amplified with Vent may be used as template for further amplification using primers P1 +P3 or primers P4 + P2-GC-clamp.

PCR WITH DIGOXIGENIN-11-dUTP:

Primers P1 and P2 are for initial amplification of entire fragment, P1 and P3 for the low temp domain, and P4 and P2-GC (primer #5) for the high temp domain. Probes are needed for 1/3 and 4/GC-clamp. Digoxigenin labelled probe is produced by pcr. Add 130 μM dTTP and 70 μM digoxigenin-11-dUTP to the reaction mixture (or use 20 μl of Boerhringer-Mannheim's (vial 6) dNTP mix). Probe will migrate 10 % slower then non-digoxigenin labelled probe on agarose gels.

Mix all reaction components (enzyme last), spin down in microfuge, and overlay with 70 μl of mineral oil. Following reactions mineral oil is easily removed by the addition of chloroform (100 μl). The oil/chloroform will partition to the bottom of the sample and the PCR reaction components are easily removed from the top.

AMPLI-TAQ POLYMERASE REACTION:

ADDITION (STOCK CONC.)	VOLUMES (μL)	FINAL CONCENTRATIONS
10 X PCR buffer II	10	1X
3 X dH_2O*	41.5	*
dNTP Boeh/Mann vial 6	20	200μM
(or dNTP Perkin-Elmer)	(2 each)	200μM
Primer A (10μM)	5	500nM
Primer B (10μM)	5	500nM
Ampli-Taq Polymerase	0.5	0.025Units
25 mM $MgCl_2$	8	2.0mM
DNA template	10	$<1\mu g/100\mu l$
Total	100	

Ampli-Taq polymerase stock concentration = 5 units/ml

* If preparing dNTP mix separately use the following (don't forget to change the H_2O volume).

Boerhringer Mannheim dNTP's with digoxigenin-11-dUTP contain 0.65mM dTTP + 0.35mM dig-dUTP as well as the other dNTP's at 1mM. This results in a final concentration of 130μM dTTP, 70μM dig-11-dUTP, and 200μM of the other dNTP's (vial 6 in kit number 1).

Digoxigenin-11-dUTP/dTTP mixture (130 μM dTTP + 70 μM dig-dUTP) - 65 μl of 10 mM dTTP 35 μl of 10 mM dig-11-dUTP

AmpliTaq buffer - 500 mM KCl; 100 mM Tris-HCl (pH 8.0)

IDENTIFICATION OF AMPLIFIED EXON 3 IN MUTAGEN-TREATED CELLS:

3 % Nusieve gels (in 1X TBE with 0.5μg/ml ethidium bromide) are run to identify the presence of amplified Exon 3 product in mutagen-treated cells (this is prior to running PAGE/DGGE gels and is done to assure that exon 3 is present and hasn't been deleted). Pour Nusieve agarose gels

and insert combs. Add gel loading solution (1μl) to PCR product (5μl) and load into wells. Run gels at 70 volts and check migration after 3-4 hours. Photograph.

Loading Dye (store at 4°C) - 0.25% Bromophenol Blue; 0.25% Xylene Cyanol FF; 40% Sucrose in H_2O

10 x TBE (actually only 5 X but used as 10 X); 54 g Tris base; 27.5 g Boric acid; 20 ml of 0.5M EDTA ; ph=8.0

10 mg/ml Ethidium Bromide (wear gloves) - 1g Ethidium Bromide dissolve in 100ml of dH_2O

APPENDIX IV

DENATURING GRADIENT GEL ELECTROPHORESIS
(Cariello N.F., et al (1990); Keohavung P., et al., (1989); Thilly W., (1985); Dlouhy S.R., et al. 1991; Myers R.M., et. al. 1987; Fisher S.G. and L.S. Lerman (1983))

GENERAL INFORMATION:

The electrophoresis apparatus (central core from the Protean II xi unit is used without outer casing) is submerged in 1X TAE buffer (30 liters) in an aquarium. The bottom chamber is represented by the aquarium while the upper chamber is formed when the plates are attached to the Protean II xi unit's central core. Electrodes are attached to each chamber (through terminals on the top of the core) creating an electrical current through the gel. Buffer in the aquarium should reach to about one inch below the top of the plates. It is crucial that the buffers of these two chambers are kept separate to maintain the electrical current through the gel. The same buffer is used in the upper buffer chamber as in the lower chamber and it is necessary for it to be recirculated. A peristalic head pumps buffer from the top chamber to the bottom chamber. The buffer is replaced with one of the four tubes connected to the aquarium, being fed by the large tube coming from the bottom chamber attached to the second head of the pump. The aquarium is equilibrated to 60°C prior to beginning a run and gels are run for 14 hours or more (partially denatured strands only move at around 1 cm per week) at 150 Volts (constant voltage).

Plates must be very clean before gels are poured. Wash plates in warm soapy H_2O and then with ethanol. Silanize (using sigmacoat) the smaller (inner) plate which will be removed for the hybridization step.
Heteroduplex Formation (from Dlouhy S.R. et. al. 1991):

HETERODUPLEX FORMATION:

To prepare heteroduplex DNA using Deep-Vent polymerase. Mix DNA fragments in high salt buffer (300 mM NaCl, 1mM EDTA, 30 mM Tris-HCl, pH = 8.0), heat to 98°C for 10 minutes and allow to anneal at 65°C for 3-4 hours. Following incubation, precitate DNA and resuspend in DGGE buffer.

POURING GRADIENTS:

1. When casting a DGGE gel the gradient-maker chambers are loaded with equal volumes (15 ml if using the 1mm, 16 X 20 cm plates) of low (upstream chamber), and high (downstream chamber), denaturing polyacrylamide solutions. The gels are made at 12.5 % polyacrylamide, 38-53 % denaturant concentration for the high temperature melting domain and 20-35% for the low melting domain (100 % denaturant is 7 M urea/40 % foramide). For the 20-35% denaturant gels, the upstream chamber is filled with 20%, and downstream chamber with 35% denaturant solution (38% in upstream and 53% in downstream chambers for the 38-53% denaturant gels). It is important to note that the acrylamide concentration (at a ratio of 37.5:1 acrylamide to bis-acrylamide) is kept constant at 12.5 % in the gel while the denaturant (urea and foramide) forms the gradient. Keep both solutions ice cold prior to pouring and add a small stir bar to downstream chamber.

2. Add catalyst (1/200 fresh Ammonium persulfate (10 %) and 1/2000 TEMED) to both acrylamide solutions (50 % of total in each). Pour high denaturant acrylamide solution into downstream chamber and begin stirring with a small stirbar. Open stopcock connecting chambers and allow chamber connecting tunnel to fill with high denaturant acrylamide.

3. Close stopcock and remove excess acrylamide from upstream chamber prior to filling with low denaturant acrylamide solution. Add catalyst to low denaturant solution and pour into upstream chamber.

4. Open valve connecting chambers and begin pouring gel. Time it takes to pour should be about 1 minute. Terminate tubing leading to plates with a needle inserted between the plates (this allows for a quick, easy and non-messy casting of the gel). Immediately after pouring gel insert 10-20 tooth comb (the 15 well comb is suppose to hold 147 μl per well, however, 50 μl is more realistic) into gel and rinse tubing thouroughly with H_2O. Gels may be stored at 4°C overnight in plastic wrap.

5. Rinse wells with buffer prior to loading samples. Load DNA into wells with gel loading pipet tips. Run submerged at 60°C for 14 hours at 150 Volts in 1 X TAE (pH= 8.3) buffer (or 120 volts for 16-18 hours).
Hybridization for a probe 95-100 % homologous should be done at 42 °C in 50% formamide.

NONDENATURING POLYACRYLAMIDE GELS (PAGE GELS):
Recirculate buffer through ice water when running non-denaturing PAGE gels (heat is generated by these gels which will separate the DNA strands if not dissipated). Percent acrylamide, run times and voltage need to be determined empirically. The initial 9% acrylamide gel was run for 4 hours but had trailing bands visible. Lower voltage (70-80 volts), 6% acrylamide and longer run times will be tried in the future.

PAGE GELS (pH=8.3)

Acrylamide

30 ml of 30% acrylamide for a 6% acrylamide gel
*45 ml of 30% acrylamide for a 9% acrylamide gel
*60 ml of 30% acrylamide for a 12% acrylamide gel

15 ml 10 X TBE buffer
3 X dH_2O to 150 ml

Prior to pouring add and stir the following:
1.5 ml of 10% Ammonium persulfate (make fresh monthly):
0.1 gram Ammonium persulfate in 1.0 mL 3 X dH_2O

TEMED (N,N,N',N'-Tetramethylethylenediamine-supplied by Bio-Rad) is used as supplied at 70 μl.

* 30% Stock Acrylamide (30:1 acrylamide to bis-acrylamide)

If using solid reagents:
145 grams electrophoresis grade acrylamide
5 grams bisacrylamide

3 X H_2O to 500 mL

If using Amresco liquid Acrylamide reagents:

ml of 40% acrylamide= (MC)(**FINAL** total acrylamide gel conc.)(total volume)
(40%)

MC=29/30 =.966667 for a 1:30 bis/acrylamide ratio
MC=monomer content

ml of 2% Bis-acrylamide= (CC)(**FINAL** total acrylamide gel conc.)(total volume)
(2%)

CC=1/30 =.03333 for a 1:30 bis/acrylamide ratio
CC=cross=linker content

Example. To make up 150 ml of a 9% acrylamide/bis-acrylamide solution add the following (should be enough for 4 PAGE gels):

32.6 ml 40% acrylamide
22.5 ml of Bis-acrylamide
15 ml 10 X TBE
H_2O to 150 ml

Prior to pouring add appropriate amounts of 10% Ammonium persulfate (make fresh monthly and generally used at 1/200) and TEMED (N,N,N',N'-Tetramethylethylenediamine-supplied by Bio-Rad) is used used as supplied at 1/2000.

CRUSH AND SOAK METHOD OF EXTRACTING DNA FROM POLYACRYLAMIDE GELS:
Cut out and dice bands of DNA from gel (match up photocopied transparency-may have to cut out a rectangular area of the transpaency cooresponding to the DNA of interest) and transfer to test tube containing 2 X volumes of elution buffer.

Elution Buffer is:

0.5 M ammonium acetate-(5 ml of 10 M stock)
10 mm magnesium acetate-(1 ml of 1 M stock, m.w.= 214.5 g/mole)
1 mm EDTA (pH=8.0)-(0.2 ml of 0.5 M stock)
0.1% SDS-(1 ml of 10% stock)

Incubate tubes at 37°C with gentle shaking for 4 hours. Centrifuge for 12,000 g for 1 minute at 4°C in microfuge. Remove supernatent and place in second tube. Add 2 volumes of ice cold ethanol and store on ice for 30 minutes. Recover DNA by centrifugation at 12,000g for 10 minutes at 4°C. Redissolve DNA in 200 μl TE (pH=7.6), add 25 μl of 3 M sodium acetate (pH=5.2, m.w.=82.03 g/mole), and reprecipitate with 2 volumes of ethanol. Rinse pellet with 70 % ethanol and dissolve in TE (10 μl).

12.5 % DENATURING GRADIENT GELS:

90% denaturant stock solution:

500 ml

62.5 g of 37.5:1 acrylamide-bisacrylamide
180 ml formamide = (36%)
210 grams urea (6.3M)
10 ml of 50 X TAE (pH=
3 X H_2O to 500 ml

0% denaturant-free stock solution:

500 ml

156.25 ml of 40% acrylamide *
10 ml 50 X TAE
3 X H_2O to 500 ml

40 % Acrylamide is 37.5:1 acrylamide to bis-acrylamide
If using Amresco acrylamide add 30 ml of the 40% solution of acrylamide to 0.8 ml of the bis-acrylamide solution.

10% Ammonium persulfate (must be made fresh monthly):
0.1 gram Ammonium persulfate in 1.0 mL 3 X dH_2O

TEMED (N,N,N',N'-Tetramethylethylenediamine-supplied by Bio-Rad) is used as supplied at 35 μl per 100 ml gel.

50 X Stock TAE (pH=7.4) - 242 grams of Tris base (2M); 57.1 ml of glacial acetic acid (1 M); 100ml of 0.5 M EDTA; pH = 8.3 for DGGE gels.

10 x Stock TBE (actually only 5 X but used as 10 X):
54 g Tris base
27.5 g Boric acid
20 ml of 0.5M EDTA
pH = 8.0 for Agarose gels
pH = 8.3 for PAGE gels
pH = 8.7 for Capillary transfer of DNA onto membranes.

10 mg/ml Ethidium Bromide (wear gloves):
1g Ethidium Bromide dissolve in 100 ml of dH_2O

Sample loading solutions:

DGGE gels (Doury et. al. 1992)-80% glcerol; 10 mM Tris-HCL (pH=7.4); 25 mM EDTA; 0.25% Bromophenol blue

Sequencing gels (Maniatis)-98% Deionized Formamide; 10 mM EDTA (pH=8.0); 0.025% Xylene Cyanol FF; 0.025% Bromophenol Blue

Agarose/PAGE gels-0.25% Bromophenol Blue; 0.25% Xylene Cyanol FF; 30% Glycerol in H_2O

USING THE MILLIPORE-APPARATUS TO TRANSFER AND CROSSLINK DNA FROM DGGE OR PAGE GELS ONTO BOEHRINGER-MANNHEIM MEMBRANES

This outline was extracted and modified from the MILLIPORE Protocols guide for multiplex sequencing and is useful for both PAGE and DGGE gels.

1. Saturate the "wicking" platform with ethanol (for first time use or after drying), rinse a couple of times with deionized H_2O, and fill base with 2.7 liters (not the 2.8-2.9 liters the Millipore manual recommends) of 1 X TBE buffer (pH=8.3). Make sure the buffer level is not above the surface of the platform.

2. Place a frame of parafilm around the perimeter of the "wicking" surface so that the 'wicking" surface is slightly smaller then the surface of the gel. This will prevent "short circuiting" of the buffer.

3. Cut a piece of Whatman 3MM filter paper and Boehringer-Mannheim (BM) nylon membrane slightly larger then the gel. Do not moisten gel or filter paper with buffer at this stage. Pry gel apart and place the filter paper directly onto the gel. Peel off gel/filter paper from glass plate. Gel will stick to filter paper. Place on flat surface.

4. Attach the BM membrane to the gel being careful not to create any bubbles or wrinkles. Don't forget to mark which side of the BM membrane the DNA was transferred to.

5. Trim the membrane/gel/filter paper sandwich making sure edges are even and loading wells are removed. Notch one corner of the sandwich to indicate orientation.

6. Center the sandwich onto the "wicking" platform, slightly overlapping the parafilm applied in step 2. Filter paper should be in direct contact with the 'wicking" surface. Seal the edges of the sandwich with parafilm to avoid "short circuiting" of the buffer.

7. Place a piece of 3MM filter paper directly on top of the second parafilm frame (BM membrane). Place a "pack" of absorbant paper on top of the 3MM paper. Place the blotter lid onto the absorbant paper.

8. Allow the transfer to proceed for 15-30 minutes. Disassemble the apparatus and carefully remove the membrane from the gel. Dry membrane in oven at 120°C for 15-30 minutes (do not exceed 30 minutes) Keep track of which side of the membrane the DNA is on.

9. Meanwhile wrap the PAGE or DGGE gel in plastic wrap and place in refrigerator until membrane is developed. Bands will be excised once location is determined.

COLORIMETRIC DETECTION OF DNA IN PAGE OR DGGE GELS USING DIGOXIGENIN-11-UTP:

1. Transfer BM membrane to a sealable hybridization bag (make sure it doesn't leak) for detection. Hybridization bags must be kept free of air during all steps. To remove air bubbles, lay bag flat, push out air bubbles with a cylindrical object (pipet), then seal bag.

2. For the prehybridization step add 0.1 ml/cm^2 or about 20 ml per 12 X 16 cm gel probe free formamide-hybridization solution. Seal bag and incubate for one hour at 42°C with gentle mixing. Open and drain bag.

3. Denature digioxigenin labelled probe DNA by heating to 68°C for 10 minutes. Add 10 to 100 ng/ml of diluted probe to the hybridization solution. Replace pre-hybridization solution with the hybridization solution (2.5 ml/100 cm^2 or about 5 ml per 12 X 16 cm gel). Incubate overnight at 42°C.

4. Recover hybridization/probe solution and store at -20°C for reuse. Remove membrane and wash 2 X 5 min in 50 ml/100 cm^2 2X SSC with 0.1% SDS (100 ml for 12 X 16 cm gel) at room temp.

5. Wash 2 X 15 min in 50 ml/100 cm^2 0.1X SSC with 0.1 % SDS at 68°C. Filter may be stored air-dried prior to detection, but to prevent DNA migration from occuring in the refrigerated gel it should be processed as soon as possible.

6. Wash filter for 1 minute with detection buffer 1 (Genius solution 1).

7. Incubate filter at room temperature in detection buffer 2 (genius buffer number 2) at 1 ml/cm^2 for 30 minutes with the addition of 50 μg/ml Salmon or Herring sperm DNA (750 μl of 10 mg/ml Salmon sperm per 150 ml equals 50 μg/ml). Agitate

8. Dilute antiDigioxigenin -alkaline phosphatase (DIG-AP, vial number 8) 1:5,000 (final conc. = 150 mU/ml) with detection buffer 2 (no DNA). This is only stable for 12 hours. Incubate filter with DIG-AP at 20 ml/100 cm^2 (40 ml for 12 X 16 cm gel) for 30 minutes with agitation. The diluted antibody solution must cover the entire membrane

9. Wash the membrane 2 X 15 minutes with 100 ml/100 cm^2 (200 ml for 12 X 16 cm gel) detection buffer

10. Equilibrate with detection buffer 3 (no additions) for 2 minutes at 20 ml/100 cm^2 (40 ml for 12 X 16 cm gel). This activates the alkaline phosphatase conjugated to the antibody.

11. Pour off the Genius buffer 3 and add the color substrate solution (0.1 ml/cm^2 (20 ml for a 12 X 16 cm gel). Make up colored substrate solution fresh by mixing 45 μl of NBT solution and 35 μl of X-phosphate solution in 10 mL of detbuf 3 (Genius buffer 3). Store on ice. Colored substrate solution is only good for 12 hours.

Allow color development to take place in a sealed plastic bag or box for 30-60 minutes in the dark at room temperature (do not mix). Incubate for longer time periods to increase sensitivity.

11. When spots are detectable wash filter with TE8 buffer for 5 minutes to stop the reaction. Photocopy results onto transparencies and match up to gel. Cut out bands and elute DNA by the crush and soak method.

12. Compare spot intensities of control verus unknown to estimate concentration of unknown. Gives ballpark estimation only. Probe concentration from a digioxigenin spiked PCR reaction

should be at least 25 μg/ml. Membranes may be dried, stored, and revitilized by soaking in TE8.

DETERMINATION OF LABELLED PROBE CONCENTRATION:

The following procedure is taken from Wayne Mitchells's procedure and contains modifications not in Boehringer Mannheim's ("The Genius System User's Guide for Filter Hybridization") documentation.

1. Make up colored substrate solution fresh. Mix 45 μl of NBT solution and 35 μl of X-phosphate solution in 10 mL of detbuf 3 (Genius buffer 3). Store at 4°C.

2. Make serial dilutions of labelled control DNA in Boerhringer Mannheim's DNA dilution buffer (vial number 3). Check to see if standards have already been made.

Labelled Control DNA	(μl)	Dilution	Final (pg/ml)	Spot 1 μl for:(μg/ml)
A.5000	2/8	(1:5)	1000	100 pg
B.1000	2/18	(1:50)	100	10 pg
C.100	2/18	(1:500)	10	1 pg
D.10	2/18	(1:5000)	1	0.1 pg
E.1	2/18	(1:50,000)	0.1	0.01 pg

3. Spot 1 μl of dilutions B through E onto a positively charged nylon membrane. Allow to air dry and mark membrane lightly with a pencil to identify each dilution.

4. Make 5 serial dilutions (1:10,100,1000,10^4,10^5) of newly labelled experimental DNA probe (unknown concentration) in DNA buffer (vial number 3) according to step 2.

5. Spot 1 μl of each unknown serial dilutions (B through E) made in step 4 onto nylon membrane. Allow to air dry and mark membrane lightly with a pencil to identify each dilution.

6. Bake filters at 120°C for 15-30 minutes to fix DNA onto membrane.

7. Wash filter for 1 minute with detbuf 1 (Genius solution 1).

8. Incubate filter at room temperature in detbuf 2 (genius buffer number 2) at 1mL/cm^2 for 30 minutes with the addition of 50 μg/ml Salmon or Herring sperm DNA.

9. Dilute anti-DIG-alkaline (DIG-AP, vial number 8) phosphatase 1:5,000 with detbuf 3. Incubate filter with DIG-AP at 20 mL/100cm^2 for 30 minutes. The diluted antibody solution must cover the entire membrane

10. Wash the membrane 3 X 10 minutes with 100 ml/100 cm^2 detbuf 1.

11. Equilibrate with detbuf 3 (no additions) for 2 minutes at 20 mL/100 cm^2. This activates the alkaline phosphatase conjugated to the antibody.

12. Pour off the Genius buffer 3 and add the Color Substrate Solution made up in step 1 at 10 mL/cm^2. Allow color development to take place in a plastic bag or box for 30-60 minutes in the dark (do not mix). The 1:5,000 dilution (1pg) of the the DIG-labelled control should be visible in 30 minutes. Incubate for longer time periods to increase sensitivity.

13. When spots are detectable wash filter with 50 ml/cm^2 TE8 buffer for 5 minutes.

14. Compare spot intensities of control verus unknown to estimate concentration of unknown. Gives ballpark estimation only. Probe concentration from a digioxigenin spiked PCR reaction should be at least 25 $\mu g/ml$.
Hybridization solutions may be reused for up to one year. Store in a capped plastic vessel at -20°C. To reuse, thaw and denature at +95°C for 10 minutes. If hybridization solution contains formamide denature at +68°C.

1 M Tris (pH 7.2) - 12.11 g Tris base; adjust pH to 7.2 with conc. HCl (about 6 ml); 3X dH_2O to 100 ml

5 M NaCl - 29.22 g NaCl; 3X dH_2O to 100 ml

1 M $MgCl_2 \bullet 6\ H_2O$ - 10.17 g $MgCl_2\ 6H_2O$; 3X dH_2O to 100 ml

4 M LiCl - 16.96 g LiCl; 3X dH_2O to 100 ml

20X SSC (AUTOCLAVE) - 175 g NaCl (3M final); 88.23 NaCitrate (0.3 M final); 3X dH_2O to 1 liter at pH=7.0

10% SDS - 10 grams SDS; 3X dH_2O to 100 ml

Formamide-hybridization solution

To make up these volumes add the indicated number of milliters of each:

10 ml	100ml	200ml	
2.5	25	50	20X SSC (5x final)
0.1	1	2	10% N-lauryl sarcosine stock (0.1% final)
0.02	0.2	0.4	10% SDS stock (0.02% final)
2 ml	20	40	Blocking stock (2% final)
0.38	3.8	7.6	3X dH_2O
5 ml	50	100	100 % formamide (50% final)

Note : If foramide is yellow deionize with Dowex mixed bed XG8 or equivalent by stirring for 1 hour and passing twice through filter paper).

Detection Buffer 1 (AUTOCLAVE) - 100 mM maleic acid (11.5 g/l Maleic); 150 mM NaCl (8.7 g/l NaCl); pH=7.5; H_2O to 1000 ml

Detection Buffer 2 - 1 part blocking solution/9 parts Detection Buffer 1

Detection Buffer 3 - 100 mM Tris (10 ml of 1 M Tris pH=9.5); 100 mM NaCl (2 ml of 5 M NaCl); 50 mM $MgCl_2$(5 ml of 1 M $MgCl_2$); H_2O to 100 ml

Blocking Solution - 10% Vial 11 blocking reagent in Detection Buffer 1; Dissolve with heating/shaking; Autoclave and freeze at -20°C; Add 50 μg/ml denatured salmon or herring sperm DNA.

TE8 - 10 mM Tris (1 ml of 1 M Tris, ph=8.0); 0.2 ml of 0.5 M EDTA to 100 ml; H_2O to 100 ml

Diluted Digoxigenin-Alkaline phosphatase (Dig-AP) - 150 mU/ml or a 1:5000 dilution of vial 8 in Detection Buffer 2. Unstable for longer then 12 hours.

Color Substrate (prepared that day) - 45 μl NBT solution (vial 9); 35 μl X-phosphate solution (vial 10) in 10 ml Detection Buffer 3

Glycogen in TE - 20 mg/ml glycogen in TE

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Susan Burns
Final Technical Task Report

BACKGROUND

United States Army pollutant compounds were analyzed for mutagenicity via the monitoring of the HGPRT (hypoxanthine-guanine phosphoribosyl transferase) gene and the TK (thymidine kinase) gene. Both genes function in the biochemical salvage pathway of nucleotide metabolism. Growth of TK6 cells in CHAT medium inhibits de novo synthesis of nucleotides due to aminopterin and provides the basic constituents of nucleotides for exogenous nucleoside synthesis- thymidine, cytidine, and hypoxanthine. The TK gene is autosomal and it is essential for cellular survival under these growth conditions that the cell be heterozygous. TK^+ cells can grow because they phosphorylate the exogenous thymidine. Conversely, TK^- cells cannot and subsequently die. The HGPRT gene is located on the X chromosome. Since one allele is located on the inactive Barr Body in females, both males and females are hemizygous for this gene. If the cells are $HGPRT^+$, they can process the purine precursor, hypoxanthine; $HGPRT^-$ cells are unable to do this and die.

TK^+ and/or $HGPRT^+$ cells were exposed to various concentrations of the munition, then treated with a nucleoside analogue selective agent (6-thioguanine for the HGPRT gene and trifluorothymidine for the TK gene). If the salvage pathway is utilized, the non-mutated cells will incorporate the selective agent into their DNA and die. Mutants, however, are unable to utilize the salvage pathway and are unaffected by the selective agents. Cytotoxic levels of 50% or greater were further evaluated via through plating efficiencies and mutation frequencies.

METHODS AND RESULTS

The TK6 cells were grown in CHAT medium (10^{-5} M cytidine, $2x10^{-4}$ M hypoxanthine, $2x10^{-7}$ M aminopterin, $1.75x10^{-5}$ M thymidine in RPMI 1640 with 10% heated horse serum and 100 U/mL penicillin-streptomycin) for 48 hours in a 37°C, humidified, 5% CO_2 incubator. To aid in recovery the cells were grown in THC medium (CHAT medium without aminopertin) for 48 hours, diluting once with completed RPMI 1640.

The munition, 2,4,6-Trinitrotoluene, and two of its monoamine derivatives, 2,4-Dinitrotoluene and 2,6-Dinitrotoluene, were added at concentrations ranging from 0 mg/L to 200 mg/L for 24 hours. The cells were resuspended in completed medium and counted daily for 6

days for the HGPRT gene expression. Those concentration showing a 50% cytotoxic response were plated in microtiter plates. Plating efficiencies and mutation frequencies according to Furth, *et al.* (1981) were established to determine the mutagenicity of these compounds.

CONCLUSIONS

It was shown that the three compounds, 2,4,6-TNT, 2,4-DNT, and 2,6-DNT, were not extremely mutagenic. These compounds were further evaluated via metabolic activation using rat liver S9 extracts (Liber and Thilly, 1982). These extracts proved to be toxic to the cell. To facilitate metabolic activation of the munition compounds, rat liver microsomes along with the generating system enzymes should be added to the cells. This should reduce the toxicity of the cytochrome c system on the TK6 cells.

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FRAMESHIFT MUTAGENICITY OF 2,4,6-TNT

BACKGROUND

Mutations can occur as frameshifts, intercalation into the DNA structure, or through adduct formation. Statistical analysis can quantitatively determine the degree of mutagenicity of various compounds via spectrophotometric scans. Writhe formation of the varying degrees of mutagenicity is reflected in differences in migratory rates in an agarose gel. Since each band within the gel represents a degree of mutagenicity single strands generated during the technical process were removed through an acidified phenol extraction (Zasloff, 1978). The *E. Coli* plasmid, pBr322, was nicked, exposed to trinitrobenzene and 9-aminoacridine, and religated. Positive/negative pictures of an agarose gel allowed for the determination of mutagenicity.

METHODS AND RESULTS

Purified pBr322 was nicked with 6.25×10^{-4} units/mL RQ DNAse I for 15 minutes at 37°C. Trinitrobenzene and 9-aminoacridine were exposed to 0.5 ug of the plasmid. The DNA was then ligated with 0.2 Weiss units of T4 DNA Ligase. The samples were run on a 1% agarose gel at 3.3 V/cm for 3.75 hours. Positive/negative pictures were taken and the negatives were read in the Beckman 7500 spectrophotometer at 260 nm.

Since 9-aminoacridine is a known frameshift mutagen,it was used as the positive control. The trinitrobenzene showed mutagenicity; however, the results were not reproducible.

CONCLUSIONS

It is possible that this experimental design is valid. However, work on this project was directed into another direction to analyze whether mutations occurred on a single strand, on double strands, or at the same site on the complimentary base pair location on both plasmid strands.

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Appendix C

GEO-CENTERS, INC.

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APPENDIX A - Publications in Press

ENVIRONMENTAL CHEMISTRY, COMPOSTING

This task requires technical scientific support for basic research to investigate the pulmonary effects of composting material using an *in vivo* mammalian model.

BACKGROUND

Many of the chemical exposures experienced by military and quasi-military personnel can be attributed to respirable dusts bearing hazardous materials. It is difficult to predict the health hazards associated with hazardous materials adsorbed to respirable particulates, since it is not known whether organic compounds adsorbed to particulate matrices will be released in biological systems. If toxic chemicals remain tightly bound to the particle once inside the lung, the toxicity should be minimal since it is available for interactions with tissues. These chemicals could be toxic to the organism if the compound became disassociated from it's particulate matrix by some physicochemical action; *e.g.* release could be effected by interaction with pulmonary macrophages.

This project is part of a larger study on the bioavailability of TNT in composts of TNT-contaminated soils. A study by Griest *et al.* (1991) determined that during the composting process, a large percentage of the TNT in the experimental sample becomes tightly bound to compost particles and is refractory to extraction with organic solvents. This project examined the distribution, metabolism, and release of TNT or its biotransformation products (metabolites) in male and female Fisher F344/VAF Plus rats following intratracheal installation of various TNT-contaminated test articles. Intratracheal installation is the preferred method of exposure because similar doses can be delivered to the lungs and experimental conditions are easier to control.

METHODS AND RESULTS

Ring-labeled [1-^{14}C]Trinitrotoluene was obtained from New England Nuclear for this experiment. Two grams of soil from Umatilla Army Depot was radiolabeled by addition of 20 mCi of ^{14}C-TNT in tetrahydrofuran, and was then shipped to Roy F. Weston, Inc. near Philadelphia, PA. This company used the radiolabeled soil to prepare a 90-day compost containing approximately 7000 ppm TNT. The compost was allowed to air-dry, coarsely ground using a Braun coffee grinder, and stored dark at 4°C until needed. A TNT control dose was prepared by preparing a solution in phosphate-buffered saline (PBS) followed by filtration to remove particles which did not go into solution.

Male and female F344 VAF/Plus rats weighing between 180-200 g and being approximately six weeks old were obtained from Charles River for the experiment. They were

quarantined for one week and housed in Bio-Clean cage enclosures prior to any laboratory manipulations. Before each treatment, the animals were anesthetized with Halothane gas anesthesia in a carrier gas mixture of O_2 and NO. The animal was then administered a dose of the appropriate test article using intratracheal instillation. Samples of each dose were taken to confirm the amount of radiolabel delivered. Animals were marked, weighed, and placed individually into Nalgene^R metabolic cages and held until sacrificed for sample collection. Animals were observed daily for signs of morbidity and mortality. The intratracheal installation technique was chosen over exposure by inhalation of aerosols because this technique allows more precise control of the delivered dose, thereby allowing the researcher to use less animals in the study. Urine and feces were collected at four-hour post-installation and subsequently at 24-hour intervals thereafter (24, 48, 72, day 7, 14, 21, 28, 50, 75, 100 and 215 for one particular group of compost-treated animals). These were analyzed and data used for urinary radiolabel output calculations. Feces were also collected and radiolabel excretion was quantified. Also, for each time period, a group of three or four animals were sacrificed, and blood and tissue samples were obtained to quantify the distribution of the TNT and/or its metabolites in the body. All biological samples were oxidized with a Packard Model 204 oxidizer and radiolabel quantified in a Beckman scintillation counter.

Based on radiolabel quantification in urine, the free-form TNT administered was released from the lung rapidly, 21.5% being excreted within the first 4-6 hours. By day 28, there was no free TNT radiolabel detectable in the urine. The free TNT was distributed throughout body tissues but was cleared rapidly based on results from sequential temporal necropsies. In contrast, the TNT-compost was released slowly, with 2.23% coming off by 72 hours and about 35% being excreted in the urine by day 100. Also, a buildup of radiolabel in the kidney tissue over time was observed in both male and female rats. The radiolabel dissipated in the male rats by day 200, but no decrease was observed in the females, the last group of which was sacrificed on day 120. We may have observed a decrease if a later time point was possible. The goal of the research was to show that the composting process transformed hazardous munitions wastes so that they were not bioavailable. The data illustrates that some percentage of the tightly-bound TNT was bioavailable. Radiolabel was recovered from every tissue type collected and at every time point out to day 214. Only about 10-20% of the original dose was left in the lungs at this point.

CONCLUSIONS

Further research is needed to confirm possible sex differences in metabolite accumulation in tissues. Also needed is elucidation of the metabolites formed during the composting process and during normal metabolism. We began this work but were unable to complete it because of funding restraints. Research has shown that certain microorganisms, particularly *Phanaerochete chrysosporium* has been able to degrade TNT and similar chemicals under suitable conditions. A two-stage process of composting followed by biodegradation using special microbial cohorts may decrease the bioavailability of TNT to a level whereby this technique could be implemented

as a reliable bioremediation method for hazardous munitions chemicals.

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PUBLICATIONS RESULTING FROM THIS TASK

Palmer, W.G., Beaman, J.R., Walters, D.M. and Creasia, D.A. 1994. Bioavailability of TNT in Composts of TNT-Contaminated Soil. *The Toxicologists*, Vol. 13, *in press.*

Palmer, W.G., Beaman, J.R., Walters, D.M. and Creasia, D.A. 1994. Bioavailability of TNT in Composts of TNT-Contaminated Soil. *Toxicology and Applied Pharmacology*, manuscript in preparation.

APPENDIX A

PUBLICATIONS IN PRESS

Palmer, W.G., Beaman, J.R., Walters, D.M. and Creasia, D.A. 1994. Bioavailability of TNT in Composts of TNT-Contaminated Soil. *The Toxicologists*, Vol. 13, *in press.*

BIOAVAILABILITY OF TNT IN COMPOSTS OF TNT-CONTAMINATED SOIL. WG Palmer[1], JR Beaman[2], DM Walters[3], DA Creasia[3]. USABRDL[1], GEO-CENTERS [2]and USAMRIID[3], Fort Detrick, Frederick, MD. (*Manuscript in preparation*)

ABSTRACT

TNT residues in composts of TNT-contaminated soils cannot be extracted with aqueous or organic solutions. Based on this, composting has been considered for remediating TNT-contaminated soils. To examine health risks associated with exposure to airborne dusts from compost piles, we examined the bioavailability of composted TNT in the lungs. Single doses of ^{14}C-TNT, or suspensions of dusts from compost prepared with ^{14}C-TNT-labeled soils, were administered to rats by intratracheal instillation. The appearance of ^{14}C in urine was taken as an indication of the release of TNT from the compost matrix in the lungs. In rats instilled with ^{14}C-TNT, about 25% of the ^{14}C dose appeared in urine within 3-days, decreased rapidly thereafter, and was undetectable by 4-weeks after treatment. In contrast, less than 2% of the total dose appeared in urine during the first 3-days after treatment with ^{14}C-labeled compost. However, ^{14}C continued to appear in the urine for more than 6-months and the total amount in urine was comparable to that in TNT-treated animals. These results indicate that TNT residues become bioavailable in the lungs. ^{14}C did not accumulate in any of the 10 tissues examined in ^{14}C-TNT-treated animals. However, there was a buildup of ^{14}C in kidney, heart, and liver of rats treated with labeled compost.

Appendix D



GEO-CENTERS, INC.

SUBCONTRACT FINAL REPORT
Subcontract GC-2533-93-001
Contract DAMD 17-93-C-3006

Under Task Order GC-95-001, Life Systems conducted two projects. Army field water criteria were examined and a database developed, and water characterization technologies evaluated for a project for LTC Roland Langford, MRMC-WPAFB Detachment. A second project was completed for Dr. Dick Burrows, USACEHR, examining reverse osmosis membrane performance and database development options. Three major deliverables were prepared:

Field Water Quality Database Development, TR-1664-2-1A, January 24, 1996.

Water Characterization Evaluation Report, TR-1664-2-2A, January 24, 1996.

Reverse Osmosis Membrane Chemical Rejection Performance Database Development, TR-1605-5, December 2, 1996.

Under Purchase Order No. 22267, Life Systems conducted two projects. A panel of toxicologist reviewed CHPPM's IRIS submissions on trinitrotoluene and trinitrobenzene for Dr. Glenn Leach. A second project were performed for CHPPM, under the direction of Mr. Jesse Barkley, which examined information provided to the Army's medical and operational communities on the criteria to be used for chemical warfare agents. Major deliverables prepared included:

Peer Review - Trinitrotoluene, TR-1605-1, July 24, 1996.

Peer Review - Trinitrobenzene, TR-1605-2, July 24, 1996.

Chemical Agent Airborne Exposure - Initial Review and Comment, TR-1605-3, August 8, 1996.

Under Task Order GC-96-002, Life Systems convened a panel of experts and examined the performance degrading effects of chemical warfare agents for CHPPM, under the direction of Mr. Stephen Kistner. Through a series of meetings, a plan of action was developed and individual documents were prepared for the different classes of chemical warfare agents. The major deliverables included:

Information of Combat Developers on Performance Degrading Effects from Exposure to Mustard Agents, TR-1605-6C, December, 1997.

Information for Combat Developers on Performance Degrading Effects from Exposure to G-Agents, TR-1605-10B, December, 1997.

Information for Combat Developers on Performance Degrading Effects from Exposure to VX, TR-1605-11B, December, 1997.

Under Task Order GC-96-003, Life Systems provided technical support services to CEHR, under the direction of Dr. Hank Gardner, in developing the Deployment Toxicology program. Under this task order, Life Systems assisted in the formation of the Deployment Toxicology Users' and Science Work Groups, facilitation and documentation of three meetings of each group, development of the Deployment Toxicology Master Plan, Program Plan, and Research Plan, facilitation, documentation and drafting an article of a workshop on Sentinel Species, preparation of materiel requirement documents, facilitation of neurobehavioral toxicology plan development and review, examination and evaluation of field water microbiological testing capabilities and emerging technologies, and participation in development of a disinfection/disinfectant by-product pilot study. Major deliverables submitted include:

Deployment Toxicology Research and Development Master Plan, TR-1605-7, September, 1997.

Program Plan for Joint Deployment Toxicology Research and Development, TR-1605-8, October 8, 1997.

Tri-Service Deployment Toxicology Research Plan, TR-1605-9C, June 24, 1998.

Improved Microbiological Testing of Field Water - An Evaluation of Capabilities, Technologies and Research and Development, TR-1605-12A, July 29, 1998.

Animals as Sentinels of Human Health Hazards of Environmental Chemicals: A Workshop Summary, TR-1605-13, August, 1998

Under Task Order GC-98-004, Life Systems provided support to two projects. A white paper on Mixed Exposures as part of the National Occupational Research Agenda was prepared. A second project, for the Naval Medical Research Command involved the peer review and revision of the Neurobehavioral Toxicology Research Plan. Major deliverables are

Mixed Exposures White Paper, TR-1605-14, September, 1998.

Deployment Toxicology Assessment Program (DTAP) Strategic Plan: Neurobehavioral Toxicology Thrust Area, TR-1605-14, September, 1998.