USACEHR Test Report

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October 15, 1999

USACEHR TEST REPORT

TEST TITLE: USACEHR DRINKING WATER DISINFECTION BY-PRODUCT TESTING WITH FETAX: BROMODICHLOROMETHANE, DIBROMOACETIC ACID, AND CHLORINATED SURFACE WATER

DATA REQUIREMENT: Tests were conducted according to USACEHR Standing Operating Procedures and ASTM Guidelines for FETAX Testing

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13. ABSTRACT *(Maximum 200 words)*

Real-world environmental contamination of native frog species has demonstrated how fragile aquatic ecosystems can be. The South African clawed frog, Xenopus laevis, dwells in water its entire life span and is easily cultured in the laboratory, rendering it a suitable test species for aquatic toxins. Frog embryo growth malformations, and toxicity can easily be assessed with the 96-hour FETAX assay. Drinking water disinfction by-products of different disinfection processes have varying degrees of toxicity. At the request of the Study Sponsor, two bromination by-products and chlorinated surface water (SW) were tested in the FETAX assay with and without the exogenous metabolic activation system (MAS). To further assure reproducibility, the FETAX testing was carried out in two independent laboratories (USACEHR) in Maryland and Oklahoma State University in Oklahoma. The chlorinated surface water was selected by representatives of the American Water Works Association. Surface water was collected from Manatee County, Florida, and shipped to USACEHR. Bromine was added to the surface water prior to chlorination. Tests were performed on free chlorine water and two lots of the same water that had been chlorinated. The test results are contained in this report.

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Bromodichloromethane; chlorinated surface water; dibromoacetic acid; disinfection by-products; FETAX; metabolic activation system; mutagenicity; trihalomethanes; Xenopus laevis

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GOOD LABORATORY PRACTICE STATEMENT:

These tests were not conducted to meet the requirements of 40 CFR 160 (EPA-FIFRA) or 40 CFR 792 (EPA-TSCA). To the best of my knowledge, these tests were conducted in accordance with USACEHR Standing Operating Procedures and ASTM Guidelines for FETAX Testing in our AAALAC approved facility with the following exception:

Test chambers and methodology to test volatiles with the metabolic activation system were developed during the course of testing for this Study Sponsor.

15 OCT 1999

Dr. Henry S. Gardner, Jr., Study Director — Date

QUALITY ASSURANCE STATEMENT:

There is not an active quality assurance unit at USACEHR, however all data from USACEHR tests were recorded in USACEHR notebooks and are available for review.

15 OCT 1999

Dr. Henry S. Gardner, Jr., Study Director — Date

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SUMMARY

Two individual drinking water disinfection by-product (DWDB) chemicals, bromodichloromethane (BDCM) and dibromoacetic acid (DBAA), as well as two lots of chlorinated surface water were tested for toxicity and teratogenicity in the Frog Embryo Teratogenesis Assay - *Xenopus* (FETAX) with and without metabolic activation (MAS). Three chlorinated surface water samples were tested for mutagenicity in the *Salmonella* Plate Incorporation Mutagenicity Assay.

The metabolic activation system is used when the FETAX assay is used as a predictor of human health hazards. MAS is composed of induced rat liver microsomes and an NADPH generator system which simulates mammalian metabolism. When running MAS with FETAX, additional controls are used to check for toxicity of the activation system components. We did not see any evidence of toxicity from the MAS.

The estimated LC50 for data pooled across the three BDCM tests without metabolic activation (MAS) was 421.6 mg/L. The estimated EC50 for pooled data without MAS was 64.9 mg/L. The teratogenic index (TI) for pooled tests without MAS was 6.5 (a TI >1.5 suggests greater teratogenic potential). The estimated LC50 for pooled BDCM data with MAS was 64.5 mg/L, while the estimated EC50 for pooled BDCM data with MAS was 55.8. The TI for the pooled BDCM tests with MAS was 1.2 (not teratogenic).

In the BDCM test groups without MAS, spinal abnormalities were seen at 50-75 mg/L BDCM, while concentrations higher than 75 mg/L had multiple severe malformations. In the tests with MAS, spinal deformities appeared to occur at lower prevalence rates at both 50 and 75 mg/L (4.6% and 18.4%, respectively) than in the tests without MAS (11.1% and 33.3%, respectively).

BDCM was teratogenic to frog embryos without MAS. Adding metabolic activation to the FETAX assay appeared to increase BDCM embryo toxicity, however, fewer malformations were noted.

At DBAA concentrations of up to 12,800 mg/L, neither fifty percent mortality nor fifty percent malformations were achieved in two of the three tests without the added metabolic activation, therefore the LC50 and the EC50 could not be estimated for those two tests. In Test 231-019, the LC50 without MAS was 7,354 mg/L and the EC50 was 11,723 mg/L. The TI for DBAA without MAS was 0.6. LC50s estimated for the DBAA tests with MAS were 6,244, 69, and 3,787 mg/L. A pooled LC50 for these tests was not estimated. Only one EC50 was estimated: 879 mg/L. The TI for DBAA test, 231-019 with MAS was 0.1. The TI for the other two tests could not be calculated.

DBAA malformations did not appear to increase in severity or prevalence with increasing

concentration in tests with or without metabolic activation.

DBAA was not teratogenic with or without MAS and was toxic only at very high levels that are not likely to be found in disinfected water.

In the 2 lots of chlorinated surface water (SW) used for the three SW tests, there were no LC50s or EC50s estimated from the tests without MAS. No TIs could be calculated for the SW tests without MAS. In the tests with MAS, the LC50s were 1.0, 0.8 and 1.1 mg/L total trihalomethanes. No EC50s with MAS could be estimated for surface water. The only TI that could be calculated for the SW tests with MAS suggested that SW was not teratogenic.

Similar types of malformations were seen in the SW tests without MAS (7.3% prevalence) and the SW with MAS tests (15.9% prevalence).

Total trihalomethanes (chloroform, BDCM, dibromochloromethane, and bromoform) of 3 mg/L in chlorinated surface water were neither toxic nor teratogenic to frog embryos without MAS. Upon addition of MAS to the chlorinated surface water samples, toxicity and malformations in frog embryos appeared to increase above background levels.

None of the three chlorinated surface water samples tested in the Ames Test were mutagenic in two tester strains (TA 98 and TA 100) of *Salmonella typhimurium* with and without S-9 activation.

In conclusion, addition of MAS to the FETAX test system did not result in an increase in the teratogenicity index of BDCM, DBAA, or chlorinated surface water. For BDCM, the addition of MAS appeared to increase toxicity to frog embryos without substantially changing the prevalence of embryo malformations, in turn lowering the TI. For DBAA, the addition of MAS did not appear to increase teratogenicity or toxicity to frog embryos. Chlorinated surface water appeared to increase in toxicity with the addition of metabolic activation to the FETAX system, but fewer malformations were noted.

BACKGROUND

The frog embryo teratogenesis assay - *Xenopus* (FETAX) is a 96 hour toxicity test with endpoints of lethality for estimating the LC50, malformation for estimating the EC50, and length measurement for estimating the no observed effect concentration (NOEL) and minimum concentration to inhibit growth (MCIG). The teratogenicity index (TI) is a ratio of the LC50 to the EC50. The American Society of Testing and Materials (ASTM) approved method for FETAX is supplemented by the Atlas of Abnormalities to ensure reproducibility among technicians evaluating *Xenopus laevis* embryos. Validation studies using known mammalian or human developmental toxicants indicate that the predictive accuracy for FETAX will approach or exceed 85% (Dawson and Bantle, 1987). Since frog embryos do not have a metabolic activation system, addition of the exogenous metabolic activation system (MAS) to the test chambers will expose the embryos to metabolites of test article, which may or may not be more toxic than the test article.

PROJECT DESCRIPTION

The project was designed to use the FETAX assay to test two single chemical drinking water disinfection by-products (DWDB), bromodichloromethane (BDCM) and dibromoacetic acid (DBAA), along with an actual mixture of trihalomethanes, obtained by chlorinating and brominating surface water. Each FETAX assay was run in triplicate with and without MAS in each of two laboratories (USACEHR at Ft. Detrick, MD, and Oklahoma State University, Stillwater, OK). This report summarizes the testing done at the USACEHR facility as well as Ames Test results run by a contract laboratory. The report summarizing the Oklahoma State University (OSU) work will follow.

INTRODUCTION

Real-world environmental contamination of native frog species has demonstrated how fragile aquatic ecosystems can be. The South African clawed frog, *Xenopus laevis*, dwells in water its entire life span and is easily cultured in the laboratory, rendering it a suitable test species for aquatic toxins. Frog embryo growth, malformations, and toxicity can easily be assessed with the 96-hour FETAX assay.

Drinking water disinfection by-products of different disinfection processes have varying degrees of toxicity. The question has been raised, which is more toxic, chlorination or bromination by-products? Previous USACEHR testing for another sponsor indicated that BDCM was teratogenic to frog embryos at low levels, while chloroform was not.

At the request of the Study Sponsor, two bromination by-products and chlorinated surface water (SW) were tested in the FETAX assay with and without MAS. To further assure reproducibility, the FETAX testing was carried out in two independent laboratories (USACEHR

in MD and OSU in OK).

The chlorinated surface water was selected by representatives of the American Water Works Association. Surface water was collected from Manatee County, FL, and shipped to USACEHR. This water had a high organic content. To produce a better distribution of trihalomethanes, bromine was added to the surface water prior to chlorination. The free chlorine was measured after 24 hours to insure that it was at a concentration that would not have a significant effect on the development of the embryos (malformations and mortality less than 10%). Batches of the same lot of water were chlorinated daily during each 96-hour FETAX test. Two lots of water were used for the tests performed. Lot 1 was used for Test 234-003 and lot 2 was used for Tests 234-004 and 234-005. Two water samples from Lot 1 and one water sample from Lot 2 were submitted for Ames mutagenicity testing.

Research began on this project in July 1998. This work was the first of its kind for testing volatile chemicals with MAS. Test chambers previously used for volatiles testing (without MAS) were not used due to the prohibitive cost of microsomes that would be needed for such a large volume. Through process development, the chambers were scaled down to be economical and meet testing needs. The final phase of laboratory testing at USACEHR ended in March 1999.

MATERIALS AND METHODS

Test Materials

FETAX solution is composed of 625 mg NaCl, 96 mg $NaHCO_3$, 30 mg KCl, 15 mg $CaCl_2$, 60 mg $CaSO_4 \cdot 2H_2O$, and 75 mg MgSO4 per liter of Type I ASTM water. The pH of the final solution was adjusted to be between 7.6 to 7.9. All chemicals were reagent grade or better. FETAX solution with antibiotics (FETAX-AB) was prepared daily by adding 10 mL of penicillin-streptomycin solution (Sigma Chemical Company, St. Louis, MO) per 1000 mL of FETAX, and used for all the assays. This was done to discourage bacterial growth in the test chambers during the assays, which has historically been problematic when the MAS system was being used.

Oxygenated FETAX-AB was used for the BDCM and surface water tests since these were conducted in closed test jars due to the volatile nature of the test chemicals. In initial trials, the embryos were unable to survive in the test jars containing MAS without the addition of oxygen. FETAX-AB was not oxygenated for the DBAA tests since the chemical was not volatile and the tests were conducted in petri dishes. Therefore, there was no container-induced oxygen deficiency in the DBAA tests. The procedure for oxygenating the FETAX is as follows: for the BDCM tests, the FETAX solution was oxygenated to 12 mg/L dissolved oxygen on day 1 and to 16 mg/L on days 2, 3, and 4. The antibiotic was added, and this FETAX-AB was used as the solvent for all test solutions, including controls. For the surface water tests, the FETAX was

oxygenated to 12-15 mg/L dissolved oxygen each day. The oxygen level was determined by the oxygen concentration of the stock solution of the surface water, which had to be oxygenated separately prior to chlorination and bromination. A YSI Model 58 Dissolved Oxygen Meter (Yellow Springs Instrument Co., Inc., Yellow Springs, OH) was used to measure the oxygen levels for all the test systems.

The metabolic activation system is used when the FETAX assay is used as a predictor of human health hazards. MAS is composed of induced rat liver microsomes and an NADPH generator system which simulates mammalian metabolism. When running MAS with FETAX, additional controls are used to check for toxicity of the activation system components. We did not see any evidence of toxicity from the MAS.

Bromodichloromethane (CAS # 75-27-4) (lot number 10807kr) of 98% purity was obtained from Aldrich Chemical Company, Milwaukee, WI. A stock solution of 2000 mg/L BDCM was prepared by adding 2.04 g of BDCM to oxygenated FETAX-AB solution in a 1 L volumetric flask. A fresh stock was prepared daily. All test concentrations were prepared by diluting this stock with oxygenated FETAX-AB solution.

The analysis of BDCM was performed using a flame ionization detector interfaced to a model 6890 capillary gas chromatograph (both from Hewlett-Packard, Wilmington, DE) equipped with a model 7694 automatic static headspace sampler and fused silica capillary column (30 m x 0.25 mm inner diameter) coated with cross-linked 1% methylsilicone gum phase, film thickness, 0.33 um (Hewlett-Packard). The analysis of chlorinated Manatee County surface water for trihalomethanes was performed using an electron capture detector with the same capillary column and gas chromatograph. The injection port was maintained at 250°C for all runs with the run times varying from 6 minutes for BDCM analyses with an oven temperature at 40°C to 22.75 minutes when all four trihalomethane compounds were analyzed at 35°C to 150°C. Static headspace sampling was used for the speed of the analysis time which was required to analyze a large number of samples in short period with no sample preparation required. All stocks and standard solutions were prepared fresh daily.

Dibromoacetic acid (CAS # 631-64-1) (lot number 01625N) of 97% purity was obtained from Aldrich Chemical Company, Milwaukee, WI. A stock solution of 16,000 mg/L DBAA was prepared by adding 8.247 g of DBAA to oxygenated FETAX-AB solution in a 500 mL volumetric flask. 400 mL of FETAX-AB solution was added, and the pH was adjusted to approximately 7.7 with 10 N NaOH (CAS # 1310-73-2, Fisher Scientific, Pittsburgh, PA). Following the pH adjustment the flask was filled to 500 mL with FETAX-AB solution. This stock was prepared fresh at the beginning of each test. All test concentrations were prepared fresh daily from this stock by diluting it with FETAX-AB solution. Samples were analyzed by a Hewlett Packard 1050 series Liquid Chromatograph (Hewlett Packard, Avondale, PA) with UV detection at 200 nm. The separation of the analyte was performed on a Supelco C-18 column (25 x 0.46 cm, 5 um particle size (Supelco, Bellefonte, PA). The mobile phase consisted of 2.5%

acetonitrile (CAS # 75-05-8, J.T. Baker, Phillipsburg, PA): 97.5% 0.2% phosphoric acid (CAS # 7664-38-2, Fisher Scientific, Pittsburgh, PA) at a flow rate of 1.5 mL/minute. The injection volume was 20 µl.

Chlorination Procedure

The FETAX assay was performed on surface water received from Manatee County's water treatment plant. All reagents were prepared in chlorine demand free water. Chlorine demand free water was prepared as described in Standard Methods for the Examination of Water and Wastewater section 4500.3.m. The pH of the surface water was adjusted to 7 with 1% sodium hydroxide (CAS # 1310-73-2, Sigma Chemical Co., St. Louis, MO). The water was buffered by adding 0.01 M phosphate buffer prepared from potassium dihydrogen phosphate (CAS # 7778-77-0, Fisher Scientific, Pittsburgh, PA) and sodium hydroxide (CAS # 1310-73-2, Sigma Chemical Co., St. Louis, MO). Oxygen was bubbled through the water to increase the dissolved oxygen to the desired level (16-19 mg/L dissolved oxygen). This was necessary to insure that the oxygen demand of the *Xenopus* embryos was met. Sodium bromide (CAS # 7647-15-6, J.T. Baker, Phillipsburg, NJ) was added to produce a more even distribution of trihalomethanes. The sample was chlorinated by adding sodium hypochlorite solution (CAS # 7681-52-9, Aldrich Chemical Co., Milwaukee, WI) and sealed in an amber glass bottle with no head space. The sample was allowed to stand overnight at room temperature. After 24 hours, a sample was taken for trihalomethane analysis. The free and total chlorine were determined by ampeometric titration. (Fisher Chlorine Titrimeter Model 397, Fisher Scientific, Pittsburgh, PA). Table 1 shows the average values of trihalomethanes formed after 24 hours.

The amount of chlorine and bromide added to the water was determined by preparing a quantity of water, adjusting the pH, then buffering and oxygenating the sample as described. The water was divided into several aliquots and spiked with various levels of chlorine and bromide. It was necessary to determine the volume of chlorine that would produce water with a free available chlorine less than 0.1 mg/L Cl_2 after 24 hours. The surface water was spiked with bromide over a range of 1 to 5 mg/L. Initial chlorination studies showed that the surface water would contain mainly chloroform. The bromide concentration was increased to produce a greater proportion of the brominated disinfection by-products.

TABLE 1. TRIHALOMETHANES FORMED 24 HOURS AFTER CHLORINATION

Test Number	Free Available Chlorine	Total Chlorine	Bromide Added	Chloroform	BDCM	DBCM	Bromoform
	(mg/L)	(mg/L)	(mg/L)	(mg/L)	(mg/L)	(mg/L)	(mg/L)
234-003	0.03	0.44	4	0.185	0.380	0.541	0.160
234-004	0.04	1.54	4	2.139	1.029	0.278	0.021
234-005	0.05	1.30	5	1.885	1.138	0.408	0.035

Test Organisms

The South African clawed frog (*Xenopus laevis*) embryos were supplied from USACEHR in-house cultures. The USACEHR adult frog colony was housed in flow-through (100 ±15 mL/min) ten gallon aquaria (49.5 cm L x 25 cm W) with a water depth of 7-14 cm. Each aquaria contained a maximum of 3 same sex frogs. USACEHR processed well water held at 20 ± 3°C served as the adult frog culture medium. All frogs were fed 2 times per week with USDA approved commercial beef liver supplemented with liquid vitamins (PolyViSol™; Mead-Johnson Nutritionals, Evansville, IN). The colony was held under a photo period of 12 h light and 12 h dark.

Mating was induced by injecting 500 and 750 I.U. of human chorionic gonadotropin (hCG) (CAS # 9002-61-3, Sigma Chemical Co., St. Louis, MO, lot number 68H1100) in the dorsal lymph sac of the males and females, respectively. Amplexus typically occurred 4-6 h after injecting hCG; egg deposition occurred 9-12 h following hCG injection. Mating pairs were bred in the dark at 24 ± 2°C in FETAX solution.

Embryos between normal stage 8 blastulae and normal stage 11 gastrulae were obtained from *X. laevis* breeding pairs (embryo staging as per Nieuwkoop and Faber). The embryos were de-jellied in a 2% L-cysteine solution (2 g of L-cysteine per 98 mL of FETAX solution) that had been pH adjusted to 8.1. Once de-jellied, the embryos were rinsed and resuspended in FETAX solution. Embryos were assigned randomly to test vessels using the USACEHR randomization program. Frog embryos did not require food during a 96 h test.

Static renewal tests of DBAA were conducted in glass Petri dishes as per ASTM Standard Guidelines for FETAX. For the static renewal tests of BDCM and chlorinated SW, the embryos were tested in glass screw top jars, with teflon-lined jar lids, containing 110 mL of solution (10 mL head space). After daily solution renewal, all jars containing MAS were placed in an oxygen tent. The tent was sealed and pure oxygen was pumped into the enclosure for several minutes. This was done to insure embryo survival in MAS jars which previously had experienced problems with oxygen depletion resulting in embryo mortality. Special screen inserts and springs were designed to ease renewal of solutions with minimal disruption to the test organisms. Two replicates of 15 embryos per replicate were used for each test treatment. Four replicates of 25 embryos per replicate were used for the FETAX-AB control jars within a test. MAS controls had 15 embryos per chamber for each of two chambers. The static renewal tests were conducted at 24 ± 2°C in the dark in a constant temperature environment. Daily solution renewal occurred during the 96 h test.

Test Design, Methodology, and Statistical Analysis

General Information. FETAX-AB solution was prepared as per USACEHR SOP. For all tests, appropriate controls of FETAX-AB solution, FETAX-AB solution with MAS,

microsomes, generator, enzyme, and cyclophosphamide were run concurrently with the test article. There were four replicates of FETAX-AB-only controls, while all other treatments of controls or test article had two replicates. Mortality was noted daily, and all dead embryos were removed from the test chamber. Malformations were observed and recorded in the 96 h survivors according to ASTM guidelines. Length measurements were taken on formalin fixed embryos after the test concluded. Oxygen measurements were taken (YSI Model 58 Dissolved Oxygen Meter, Yellow Springs Instrument Co., Inc., Yellow Springs, OH) and recorded on all jars just prior to solution renewal (T_{24}). Oxygen measurements were not taken on Petri dish tests.

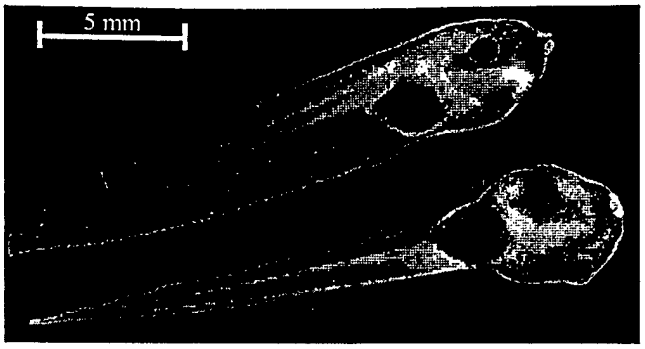
Bromodichloromethane. The same dilution series was used for all three BDCM tests. Nominal concentrations of BDCM tested in the groups without MAS were 25, 50, 75, 150, 250, 350, 450, and 550 mg/L BDCM. Nominal concentrations for the groups with MAS included 25, 50, 75, 150, 250, and 350 mg/L BDCM. Daily samples were collected for analytical chemistry at solution renewal and on the spent solution (T_0 and T_{24}) of all stocks and test chambers. Samples were collected in 40 mL glass headspace vials with teflon caps.

Dibromoacetic Acid. The same dilution series was used for all three DBAA tests. Nominal concentrations of DBAA tested in the groups without MAS included 25, 50, 100, 200, 400, 800, 1600, 3200, 6400, and 12800 mg/L DBAA. The groups with MAS used an identical dilution scheme as the groups without MAS . Daily samples were collected for analytical chemistry of fresh solutions (T_0 only, since this chemical was not volatile).

Chlorinated Surface Water. Range finding tests with the first lot of water did not indicate any toxicity or teratogenicity at 100%, so the first SW test (234-003) did not run dilutions of SW in the part of the test without MAS . The following dilutions of SW with MAS were tested: 1%, 5%, 33%, 66%, and 100%. The two remaining SW tests (234-004 and 234-005) were conducted with a second lot of surface water. Therefore dilutions (1%, 5%, 33%, and 66%) of the SW, with and without MAS, as well as 100% SW, were tested. Daily samples were collected for analytical chemistry at solution renewal and on the spent solution (T_0 and T_{24}) from all stocks and test chambers in glass headspace vials as described above.

Statistical Analysis. Probit analysis was used to estimate the LC50s, the EC50s and their associated estimated 95% fiducial limits. Pearson's chi square was used to test for fit to the model. Abbott's formula was applied to optimize the estimation of the natural threshold response rate. Wald's chi square was used to indicate effects. For length measurements, analysis of variance (ANOVA) was used to test for fit to the model and for effects. Comparisons among or between replicates at each mean measured concentration were made using the Tukey-Kramer multiple comparison procedure. Regression analysis was used for point estimation to provide estimated NOELs, MCIGs, and their associated estimated 95% confidence limits. Length data for the combined tests for each group with and without MAS were analyzed to test for heterogeneity among slopes, which tests whether or not the regression coefficients are constant over the three tests. Type I sums of squares was used to provide the appropriate statistics for

estimating the various regressions of length on concentration. Mean measured concentrations of BDCM, DBAA, and total trihalomethanes (SW) were used for statistical analyses. SAS statistical computer software was used for these analyses (SAS Institute, Inc., Cary, NC).



Controls (no malformations)

5% SW with MAS
Eye, Face, Notochord,
& Gut Malformations

66% SW without MAS
Severe Malformations

Ames Testing. Three water samples (99-020-1, 99-028-6, and 99-050-6) were submitted for Ames testing. Samples 99-020-1 and 99-028-6 were from the same mixture of water; sample 99-028-6 corresponded to water used in Test 234-003. Sample 99-050-6 came from the second lot of surface water received at USACEHR and corresponds to water used in Test 234-004. The tester strains of *S. typhimurium* included histidine auxotrophs TA 98 and TA 100. The water samples were tested at a minimum of five dose levels (2 mL, 1.5 mL, 1.0 mL, 0.2 mL, and 0.1 mL) along with appropriate vehicle and positive controls with and without S9 activation in both tester strains via plate incorporation methodology. One water sample (99-050-6) deviated from this protocol with the addition of a pre-incubation step to see if sample volatility was adequately being taken into consideration. The test protocols and test reports for the mutagenicity testing are included as appendices to this report.

Archiving Requirements. Surviving frog embryos were preserved in formalin and will be held at USACEHR for five years. Chemistry samples were spent during analysis and were not archived. All raw data, data summaries, and test reports will be maintained by USACEHR for a minimum of 10 years after completion of the test report.

RESULTS AND DISCUSSION

Bromodichloromethane. A summary of BDCM testing results is shown in Table 2. Similar trends were suggested in all three tests with BDCM. BDCM was teratogenic without MAS; however BDCM was much more toxic with MAS, while EC50 malformations did not appear to be changed by the addition of MAS. The estimated LC50 for data pooled across three BDCM tests without MAS was 421.6 mg/L. The estimated EC50 for pooled BDCM data without MAS was 64.9 mg/L. The estimated LC50 for pooled BDCM data with MAS was 64.5, while the estimated EC50 for pooled BDCM data with MAS was 55.8 mg/L.

NOELs and MCIGs were calculated from embryo length measurements. The NOELs and MCIGs for BDCM tests without MAS could not be pooled, but the NOEL and MCIG for pooled BDCM data with MAS were 12.6 and 18.5 mg/L, respectively.

BDCM without MAS malformations seen at lower concentrations (50 and 75 mg/L BDCM) were spinal (notochord) deformities (10/90 (11.1%) and 30/90 (33.3%), respectively). Representative photomicrographs of embryos with these types of malformations are shown in Figure 1. Above 75 mg/L, the malformations were edema (12/89 (13.5%) at 150 mg/L), cardiac edema (21/89 (23.6%) at 150 mg/L), or severe multiple malformations(52/89 (58.4%) at 150 mg/L, 89/89 (100%) at 250 mg/L, 90/90 (100%) at 350 mg/L, and 11/11 (100%) at 450 mg/L). Spinal deformities in the BDCM with MAS treatments appeared to occur less frequently with 4/87 (4.6%) at 50 mg/L and 7/38 (18.4%) at 75 mg/L than in the comparable treatments without MAS (10/90 (11.1%) at 50 mg/L and 30/90 (33.3%) at 75 mg/L). Gut coiling rose to 3/38

(7.9%) at the 75 mg/L BDCM treatment with MAS . Severe multiple malformations in the groups with MAS followed a similar trend (6/38 (15.8%) at 75mg/L, no malformations in either the 4 survivors at 150 mg/L or the 3 survivors at 250 mg/L, but 5/5 (100%) at 350 mg/L) as the groups without MAS, although the overall prevalence for this finding was not as frequent.

TABLE 2. STATISTICAL ANALYSES OF BDCM IN FETAX

96 h ENDPOINT	USACEHR TEST NUMBER			POOLED TEST DATA
	232-012	232-013	232-015	
WITHOUT MAS				
LC50 (mg/L) 95% fiducial limits	401.4 (325.0, 545.1)	444.8 *	393.1 (349.6, 417.7)	421.6 (412.2, 432.3)
EC50 (mg/L) 95% fiducial limits	61.7 (54.8, 87.3)	63.5 (49.9, 83.8)	57.0 (50.5, 65.0)	64.9 (60.3, 73.1)
TI (TI >1.5 = teratogenic)	6.5 Teratogenic	7.0 Teratogenic	6.9 Teratogenic	6.5 Teratogenic
NOEL (mg/L)[a] 95% confidence limits	15.0 (1.7-27.7)	0.0 (0.0, 10.0)	0.0 (0.0, 6.1)	**
MCIG (mg/L)[a] 95% confidence limits	36.2 (25.0, 47.0)	20.5 (11.2, 29.8)	20.5 (14.9, 26.1)	**
WITH MAS				
LC50 (mg/L) 95% fiducial limits	62.8 (18.8, 138.0)	54.9 (15.6, 98.0)	61.5 (24.5, 123.2)	64.5 (43.0, 90.3)
EC50 (mg/L) 95% fiducial limits	52.7 *	63.0 *	49.8 (45.4, 53.4)	55.8 (51.7, 65.4)
TI	1.2	0.9	1.2	1.2
NOEL (mg/L)[a] 95% confidence limits	12.6 (2.1, 23.1)	0.0 (0.0, 8.4)	18.6 (9.1, 28.1)	12.6 (6.5, 18.8)
MCIG (mg/L)[a] 95% confidence limits	33.2 (23.2, 43.2)	18.6 (12.2, 25.2)	41.8 (32.4, 51.2)	18.5 (12.9, 24.4)

* Could not be estimated. **Could not be pooled.

[a] NOEL and MCIG are based on length measurements

Selected malformations that occurred (1-4.4% prevalence) with no apparent relationship to treatment in the groups without MAS were gut coiling, multiple edema, facial edema, tail, eye, hemorrhage, and cardiac malformations. Malformations of no major consequence (1-3.4%

prevalence)in the tests with MAS included edema, multiple edema, cardiac edema, facial edema, tail, eye, hemorrhage, and cardiac malformations.

Dibromoacetic Acid. A summary of DBAA testing results is shown in Table 3. DBAA was not teratogenic with or without MAS. Only one LC50, 7354 mg/L, was estimated without MAS. The LC50s for DBAA with MAS were highly variable (69 , 3787, and 6244 mg/L). The only EC50 that could be estimated was 879 mg/L.

TABLE 3. STATISTICAL ANALYSES OF DBAA IN FETAX

96 h ENDPOINT	USACEHR TEST NUMBER			COMMENT
	231-018	231-019	231-020	
WITHOUT MAS				
LC50 (mg/L) 95% fiducial limits	*	7,354 (4,570, 14,001)	*	NOT TERATOGENIC
EC50 (mg/L) 95% fiducial limits	*	11,723 *	*	
TI	**	0.6	**	
NOEL (mg/L)[a] 95% confidence limits	* *	1,539 (84, -2231)	6,153 (0, 8289)	
MCIG (mg/L)[a] 95% confidence limits	* *	3,094 (2,199, 3,989)	12,124 (10,082, 13,140)	
WITH MAS				
LC50 (mg/L) 95% fiducial limits	6,244 *	69 (1, 244)	3,787 (2,658, 5,649)	NOT TERATOGENIC
EC50 (mg/L) 95% fiducial limits	* *	879 *	* *	
TI	**	0.1	**	
NOEL (mg/L)[a] 95% confidence limits	3,137 (0, 4022)	196 (89, 303)	2,959 (469, 3,542)	
MCIG (mg/L)[a] 95% confidence limits	5,726 (5014, 6290)	393 (260, 526)	6,153 (5,359, 6,825)	

* Could not be estimated. ** Could not be calculated.

[a] NOEL and MCIG are based on length measurements

NOELs and MCIGs for DBAA tests with MAS or without MAS could not be pooled and were highly variable. One DBAA test, 231-019 with MAS, had a NOEL of 109 mg/L and a

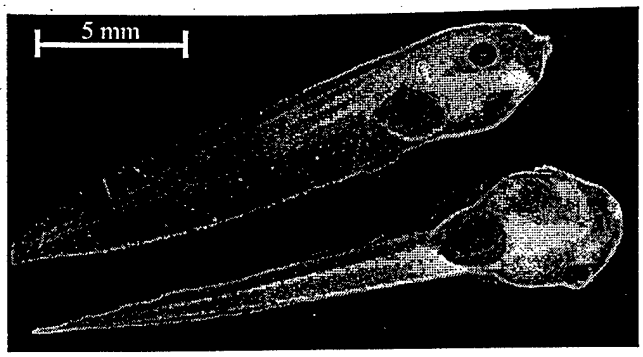
MCIG of 393 mg/L. These results were an order of magnitude lower than results in either test 231-018 or 231-020.

In the tests where DBAA concentrations were high enough to malform at least half of the embryos, malformations did not increase with concentration in the test with MAS, Test 231-019 (χ^2 = 3.061E-8; p = 0.9999), or the same test without MAS, (χ^2 = 1.218E-7; p = 0.9997). The two most commonly seen malformations, severe and gut, occurred in prevalences under 5% in all tests (severe: 16/1285 (1%) without MAS and 37/846 (4%) with MAS; gut: 33/1285 (3%) without MAS and 35/846 (4%) with MAS). Similar malformations were seen in both tests with and without MAS (1-5% prevalences): cardiac edema, facial edema, optic edema, tail, notochord, fin, face, eye, hemorrhage, and cardiac malformations.

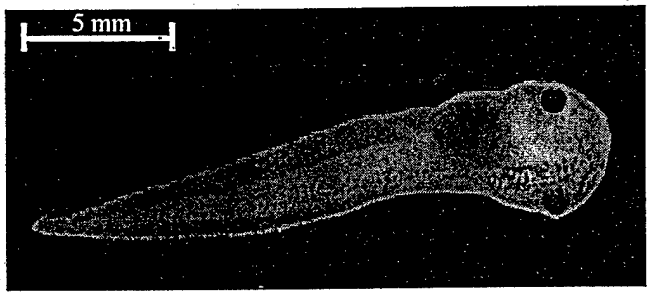
Chlorinated Surface Water. A summary of the SW testing results is shown in Table 4. All SW results were reported in total trihalomethanes ($CHCL_3$, BDCM, DBCM, and $CHBr_3$). Chlorinated SW was not teratogenic in the tests without MAS. No LC50s for SW without MAS could be estimated. The SW LC50s with MAS were 1.0, 0.8, and 1.1 mg/L trihalomethanes. Only one EC50 could be estimated for SW with MAS, 1.9 mg/L.

For SW Test 234-002 without MAS, the NOEL and MCIG could not be estimated. The NOEL for the other two SW tests without MAS was 0.2 mg/L. Adding MAS to these tests lowered the NOEL to 0.1 mg/L. The SW MCIG in the other two tests without MAS was 0.9 mg/L. With the addition of MAS to these tests, the MCIG varied from 0.3 to 0.8 and 0.9 mg/L.

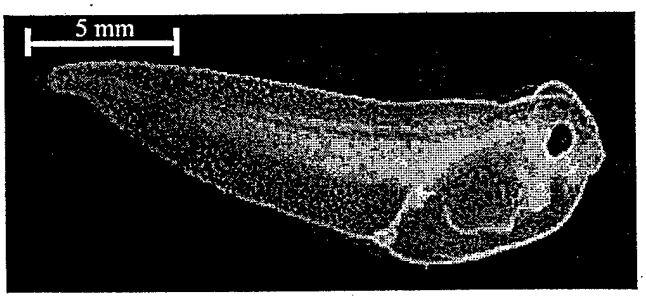
Representative photomicrographs of embryos from SW tests are shown in Figure 2. Malformations seen in the samples without MAS occurred in 22/303 (7.3%) prevalence, and in the samples with MAS, at a prevalence of 34/214 (15.9%). In samples with MAS, mortality and malformations were evident at 33% and 66% dilutions. Only one malformation, gut coiling, appeared to increase in prevalence with increasing SW concentration in MAS tests (at 1% SW, 1/72 with coiled gut; at 5% SW, 3/60; at 33% SW, 1/47; at 66% SW, 7/35; at 100% SW, 2/14). Other malformations that occurred in 1-4% prevalence in all SW tests included: severe multiple malformations, notochord, face, eye, and cardiac malformations. Malformations that occurred at prevalences of 1-2% in the tests without MAS only included tail, multiple edema, and cardiac edema. No malformations occurred in the tests with MAS that had not already been seen in the tests without MAS.



Controls (no malformations)



50 mg/L BDCM without MAS
Notochord Malformation



150 mg/L BDCM without MAS
Severe Malformations

Ames Test Results. All three water samples were not mutagenic at any concentration tested in either tester strain. A complete summary of all Ames Test procedures and results is in Appendix III of this report.

CONCLUSIONS

- BDCM is toxic and teratogenic without MAS; adding MAS appears to increase embryo toxicity with no apparent effect on teratogenicity, thereby altering the Teratogenicity Index.
- DBAA toxicity is not likely to be encountered in disinfected water; DBAA is not a teratogen.
- Composition of chlorinated surface water samples varied.

TABLE 4. STATISTICAL ANALYSES OF CHLORINATED SURFACE WATER IN FETAX, results in total trihalomethanes (mg/L)

96 h ENDPOINT	USACEHR TEST NUMBER			COMMENT
	234-003	234-004	234-005	
WITHOUT MAS				
LC50 (mg/L) 95% fiducial limits	mortality [b] ≤10%	mortality [b] ≤10%	mortality [b] ≤10%	NOT TERATOGENIC
EC50 (mg/L) 95% fiducial limits	malformations [b] ≤10%	malformations [b] ≤10%	malformations [b] ≤10%	
TI	**	**	**	
NOEL (mg/L)[a] 95% confidence limits	* *	0.2 (0.0, 0.4)	0.2 (0.0, 0.5)	
MCIG (mg/L)[a] 95% confidence limits	* *	0.9 (0.7, 1.2)	0.9 (0.6, 1.2)	
WITH MAS				
LC50 (mg/L) 95% fiducial limits	1.0 *	0.8 *	1.1 *	NOT TERATOGENIC
EC50 (mg/L) 95% fiducial limits	* *	* *	1.9 *	
TI	**	**	0.6	
NOEL (mg/L)[a] 95% confidence limits	0.1 (0.0, 0.1)	0.1 (0.0, 0.3)	0.1 (0.0, 0.4)	
MCIG (mg/L)[a] 95% confidence limits	0.3 (0.2, 0.3)	0.8 (0.6, 1.1)	0.9 (0.6, 1.2)	

* Could not be estimated or was an extrapolation beyond the data. ** Could not be calculated.

[a] NOEL and MCIG are based on length measurements

[b] At all dilutions tested, there were no apparent dose responses and no striking abnormalities.

- Addition of MAS appeared to make the chlorinated surface water more toxic and suggested an increase in prevalence of malformations.
- Chlorinated surface water was not mutagenic.
- Further generalizations about chlorinated surface water samples can not be made. It is recommended that other water samples of interest be tested.

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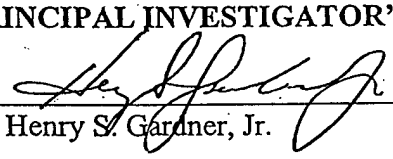
PRINCIPAL INVESTIGATOR'S SIGNATURE



15 OCT 1999

Dr. Henry S. Gardner, Jr. — Date

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APPENDIX I

FETAX Raw Data

BROMODICHLOROMETHANE (BDCM) IN FETAX

NOMINAL BDCM (mg/L)	PERCENT MORTALITY			PERCENT MALFORMED		
	232-012[a]	232-013[a]	232-015[a]	232-012[a]	232-013[a]	232-015[a]
25	0	0	0	0	10	0
50	0	0	0	7	3	30
75	0	0	0	37	33	60
150	0	3	0	100	100	100
250	0	3	0	100	100	100
350	0	0	0	100	100	100
450	10	3	63	100	100	100
550	97	100	100	100	X[b]	X[b]
25 + MAS	0	7	0	0	4	7
50 + MAS	3	3	3	7	7	17
75 + MAS	53	57	73	43	39	88
150 + MAS	100	87	100	X[b]	100	X[b]
250 + MAS	100	90	90	X[b]	100	100
350 + MAS	83	100	100	100	X[b]	X[b]

[a] All FETAX controls within ASTM limits of <10% mortality and malformations.

[b] There were no survivors, hence no malformations in survivors.

BDCM IN FETAX (continued)

NOMINAL BDCM (mg/L)	MEAN BDCM MEASURED VALUES (mg/L)			MEAN LENGTH MEASUREMENTS (% of control)		
	232-012	232-013	232-015	232-012	232-013	232-015
0	BDL[a]	BDL[a]	BDL[a]	100.0	100.0	100.0
25	14.6	20.5	20.5	95.5	92.2	97.2
50	36.2	45.5	45.5	92.8	92.9	91.2
75	56.0	63.3	63.3	92.4	100.2	95.4
150	129.7	131.6	131.6	89.5	84.2	82.3
250	217.6	220.9	221.0	79.5	68.1	68.1
350	301.5	308.4	308.4	70.5	57.2	54.0
450	400.7	408.9	403.8	51.9	50.5	39.7
550	484.1	482.9	482.9	53.9	X[b]	X[b]
0 + MAS	BDL[a]	BDL[a]	BDL[a]	100.0	100.0	100.0
25 + MAS	12.6	18.5	18.5	99.4	98.7	102.6
50 + MAS	22.9	42.2	42.2	96.1	93.7	96.4
75 + MAS	49.7	57.9	57.8	91.1	87.9	86.9
150 + MAS	120.3	122.8	122.8	X[b]	74.1	X[b]
250 + MAS	194.9	206.7	206.7	X[b]	73.9	67.4
350 + MAS	292.9	293.3	293.3	67.3	X[b]	X[b]

[a] BDL = Below detection limits of 5 ug/L.

[b] No survivors, no embryos to be measured.

DIBROMOACETIC ACID (DBAA) IN FETAX

DBAA CONCENTRATION (mg/L)	PERCENT MORTALITY			PERCENT MALFORMED		
	231-018[a]	231-019[a]	231-020[a]	231-018[a]	231-019[a]	231-020[a]
25	0	0	0	0	4	2
50	0	0	2	2	2	4
100	0	4	4	2	6	10
200	2	2	0	4	2	8
400	12	6	2	19	15	6
800	30	18	14	12	5	5
1600	42	2	2	10	4	6
3200	18	22	8	0	6	0
6400	36	56	12	3	0	7
12800	26	72	34	0	64	12
25 + MAS	0	46	2	2	37	6
50 + MAS	8	52	0	22	21	4
100 + MAS	22	46	0	15	11	2
200 + MAS	34	76	6	27	25	11
400 + MAS	36	62	2	22	5	12
800 + MAS	40	60	20	13	25	10
1600 + MAS	14	96	12	7	100	0
3200 + MAS	28	100	14	0	X[b]	2
6400 + MAS	50	100	86	4	X[b]	14
12800 + MAS	100	100	100	X[b]	X[b]	X[b]

[a] All FETAX controls within ASTM limits of <10% mortality and malformations.

[b] There were no survivors, hence no malformations in survivors.

DBAA IN FETAX (continued)

NOMINAL DBAA (mg/L)	MEAN DBAA MEASURED VALUES (mg/L)			MEAN LENGTH MEASUREMENTS (% of control)		
	231-018	231-019	231-020	231-018	231-019	231-020
0	BDL[a]	BDL[a]	BDL[a]	100.0	100.0	100.0
25	27	25	28	98.8	98.7	104.3
50	51	49	51	99.8	100.2	104.1
100	100	98	101	103.2	98.7	103.3
200	196	196	197	98.9	99.4	102.9
400	393	393	396	99.3	94.9	102.7
800	724	783	801	97.1	97.5	102.1
1600	1531	1539	1489	94.6	99.8	104.5
3200	3137	3094	2959	101.0	98.7	104.9
6400	5726	6090	6153	100.9	98.3	102.5
12800	12254	11991	12124	99.7	84.8	97.6
0	BDL[a]	BDL[a]	BDL[a]	100.0	100.0	100.0
25 + MAS	27	25	28	98.1	94.0	101.8
50 + MAS	51	49	51	96.2	96.1	101.7
100 + MAS	100	98	101	92.9	99.1	101.7
200 + MAS	196	196	197	93.1	8.63	97.8
400 + MAS	393	393	396	93.7	97.2	99.9
800 + MAS	724	783	801	96.8	87.8	100.5
1600 + MAS	1531	1539	1489	95.9	79.8	102.5
3200 + MAS	3137	3115	2959	95.0	X[b]	99.3
6400 + MAS	5726	6029	6153	104.1	X[b]	82.9

[a] BDL = Below detection limits of 4 mg/L.

[b] There were no survivors, hence no malformations.

CHLORINATED SURFACE WATER (SW) IN FETAX

SW CONCENTRATION (PERCENT)	PERCENT MORTALITY			PERCENT MALFORMED		
	234-003[a]	234-004[a]	234-005[a]	234-003[a]	234-004[a]	234-005[a]
1%	–[b]	0	0	–[b]	0	10
5%	–[b]	0	3	–[b]	3	10
33%	–[b]	0	0	–[b]	7	0
66%	–[b]	0	0	–[b]	7	7
100%	0	0	3	3	3	3
1% + MAS	0[c]	20	7	5[d]	4	7
5% + MAS	0	0	0	0	7	7
33% + MAS	0	93	50	3	50	0
66% + MAS	30	53	100	24	14	X[e]
100% + MAS	57	100	97	15	X[e]	100

[a] All FETAX controls within ASTM limits of <10% mortality and malformations.

[b] This concentration not tested in Test 234-003.

[c] One jar was aberrant, with 67% mortality; the replicate jar had 0% mortality. If the aberrant data are not used, the mortality for 1% SW falls to 0%, which is in line with the concentration response shown by the other dilutions tested.

[d] There were no malformed embryos in the aberrant jar, so this number would not change if the aberrant jar data were not used.

[e] No survivors to check for malformations.

CHLORINATED SURFACE WATER (SW) IN FETAX (continued)

NOMINAL SW-THM[a] (mg/L)	MEAN SW MEASURED VALUES [a] (mg/L)			MEAN LENGTH MEASUREMENTS (% of control)		
	234-003	234-004	234-005	234-003	234-004	234-005
0	BDL[b]	BDL[b]	BDL[b]	100.0	100.0	100.0
1%	–[c]	0.049	0.035	–[c]	101.1	104.3
5%	–[c]	0.159	0.153	–[c]	97.4	96.3
33%	–[c]	0.936	0.907	–[c]	90.7	96.8
66%	–[c]	1.871	1.810	–[c]	93.5	X[d]
100%	1.135	3.107	3.124	96.2	90.6	81.7
0	BDL[b]	BDL[b]	BDL[b]	100.0	100.0	100.0
1% + MAS	0.026	0.032	0.033	94.9	99.6	97.7
5% + MAS	0.057	0.136	0.136	100.9	96.6	100.0
33% + MAS	0.279	0.821	0.838	94.7	88.4	98.2
66% + MAS	0.585	1.697	1.628	84.0	87.8	94.4
100% + MAS	1.015	2.718	2.792	85.3	X[d]	90.9

[a] Measured values are reported as total trihalomethanes (chloroform, bromodichloromethane, dibromocloromethane, and bromoform).
[b] Below detection limits of 0.003 mg/L.
[c] This concentration not tested in this series.
[d] No survivors to be measured for length.

APPENDIX II

Statistical Reports

March 8, 1999

MEMORANDUM FOR: Dave Kumsher/Maggie Toussaint

FROM: Florence J. Hoffmann
Mathematical Statistician

SUBJECT: Estimates of the LC50 and EC50 for BDCM Exposures, Without MAS, Using FETAX, For Test 232-012

STATISTICAL METHODS

Probit analysis was used to estimate the LC50, the EC50, and their associated estimated 95 percent fiducial limits. Abbott's formula was applied to optimize the estimation of the natural threshold response rate. Wald's chi square was used to test for effects. SAS PROC PROBIT statistical computer software was used for these analyses.

STATISTICAL RESULTS

The best-fitting model for estimating the LC50 and its associated 95 percent fiducial limits was one using BDCM concentrations log10 transformed for calculations. However, estimates with BDCM concentrations on the arithmetic scale provided a model which fit the data and produced good estimates. The best-fitting model for estimating the EC50 and associated 95 percent fiducial limits was one using BDCM concentrations on the arithmetic scale. The model with log10 transformed BDCM concentrations also provided good estimates for the EC50 and associated 95 percent fiducial limits. There was a BDCM effect for the mortality for both models (X^2=11.5057; p=0.0007) for the log10 transformed BDCM concentrations and (X^2=17.8907; p=0.0001) for the model with BDCM concentrations on the arithmetic scale. There also was a BDCM concentration effect for malformations (X^2=8.1900; p=0.0042) for the model with BDCM concentrations on the arithmetic scale (Tables 2a and 2b). The LC50 was estimated to be 384.5 (326.8, 448.3) mg/l BDCM for the estimates based on log10 transformed BDCM concentrations and (401.4 (325.0, 545.1) mg/l BDCM for estimates made with BDCM concentrations on the arithmetic scale. The EC50 was estimated to be 61.7 (54.8, 87.3) mg/l BDCM for estimates made with BDCM on the arithmetic scale (Tables 3a and 3b).

Table 1. Fit to the Model for BDCM Exposures Without MAS, Using FETAX for Estimated LC50, EC50, and Their Associated Estimated 95 Percent Fiducial Limits: Test 232-012

Model	Statistic	
	X^{2a}	p
LC50		
Arithmetic Scale	47.0649	0.0000
Log_{10} Transformed	36.6153	0.0005
EC50		
Arithmetic Scale	8.1163	0.7760
Log_{10} Transformed	8.2026	0.7691

$\alpha = 0.10$

[a] = Pearson's Chi Square

Table 2a. Effects for BDCM Exposures, Without MAS, for LC50 and EC50 Estimates Using FETAX: Test 232-012

Parameter	Effect					
	Intercept			BDCM Concentration		
	Estimate	X^{2a}	p	Estimate	X^{2a}	p
LC50	-2.6083563	24.9198	0.0001	0.00649855	17.8907	0.0001
EC50[b]	-3.9642651	11.5193	0.0007	0.06419929	8.1900	0.0042

$\alpha = 0.05$

[a] Wald's Chi Square

[b] Best Fitting Model

Table 2b. Effects for BDCM Exposures, Without MAS, for LC50 and EC50 Estimates Using FETAX, with BDCM Concentrations Log10 Transformed: Test 232-012

Test	Effect					
	Intercept			BDCM		
	Estimate	X^{2a}	p	Estimate	X^{2a}	p
LC50[(b)]	-27.514375	11.4685	0.0007	10.6442622	11.5057	0.0007
EC50	-14.075749	16.9409	0.0001	7.87819093	16.1700	0.0001

$\alpha = 0.05$

[a] Wald's Chi Square

[b] Best Fitting Model

Table 3a. Estimated LC50 and EC50 and Their Associated Estimated 95 Percent Fiducial Limits, for BDCM Exposures, Without MAS, Using FETAX, With Parameter Estimates using BDCM Concentrations on Arithmetic Scale: Test 232-012

Parameter	Estimate (mg/L)	Estimated 95 Percent Fiducial Limits	
		Lower	Upper
LC50	401.4	325.0	545.1
EC50[a]	61.7	54.8	87.3

[a] Best Fitting Model

Table 3b. Estimated LC50 and EC50 and Their Associated Estimated 95 Percent Fiducial Limits, for BDCM Exposures, Without MAS Using FETAX, With Parameter Estimates using BDCM Concentrations on Arithmetic Scale: Test 232-012

Parameter	Estimate (mg/L)	Estimated 95 Percent Fiducial Limits	
		Lower	Upper
LC50[a]	384.5	326.8	448.3
EC50	61.2	54.0	73.9

[a] Best Fitting Model

February 25, 1999

MEMORANDUM FOR: Dave Kumsher/Maggie Toussaint

FROM: Florence J. Hoffmann
Mathematical Statistician

SUBJECT: Estimates of the LC50 and EC50 for BDCM Exposures, Without MAS, Using FETAX, For Test 232-013

STATISTICAL METHODS

Probit analysis was used to estimate the LC50, the EC50, and their associated estimated 95 percent fiducial limits. Abbott's formula was applied to optimize the estimation of the natural threshold response rate. Wald's chi square was used to test for effects. SAS PROC PROBIT statistical computer software was used for these analyses.

STATISTICAL RESULTS

The LC50 and the EC50 and associated estimated 95 percent fiducial limits, without MAS, were estimated with BDCM concentrations on the arithmetic scale. There was no BDCM effect for the mortality (X^2=6.129 E-6; p=0.9980). There was a significant BDCM effect for malformations (X^2=31.9520; p=0.0001) (Table 2). The LC50 was estimated to be 444.8 mg/l BDCM. The 95 percent fiducial limits could not be estimated for the LC50. The EC50 was estimated to be 63.5 (49.9, 83.8) mg/l BDCM (Table 3).

Table 1. Fit to the Model for BDCM Exposures Without MAS, Using FETAX for Estimated LC50, EC50, and Their Associated Estimated 95 Percent Fiducial Limits: Test 232-013

Model	Statistic	
	X^{2a}	p
LC50		
Arithmetic Scale	9.9966	0.7624
Log_{10} Transformed	9.9966	0.7624
EC50		
Arithmetic Scale	19.7856	0.0713
Log_{10} Transformed	36.0930	0.0003

$\alpha = 0.10$

[a] = Pearson's Chi Square

Table 2. Effects for BDCM Exposures, Without MAS, for LC50 and EC50 Estimates Using FETAX: Tests 232-013

Parameter	Effect					
	Intercept			BDCM Concentration		
	Estimate	$X^{2(a)}$	p	Estimate	X^{2a}	p
LC50	-75.511845	6.339E-6	0.9980	0.16976235	6.129E-6	0.9980
EC50	-1.9343748	40.10062	0.0001	0.03044762	31.9510	0.0001

$\alpha = 0.05$

[a] Wald's Chi Square

Table 3. Estimated LC50 and EC50 and Their Associated Estimated 95 Percent Fiducial Limits, for BDCM Exposures, Without MAS, Using FETAX: Test 232-013

Parameter	Estimate (mg/L)	Estimated 95 Percent Fiducial Limits	
		Lower	Upper
LC50	444.8	***	***
EC50[a]	63.5	49.9	83.8

[a] Wald's Chi Square

*** Could not be estimated

April 2, 1999

MEMORANDUM FOR: Dave Kumsher/Maggie Toussaint

FROM: Florence J. Hoffmann
Mathematical Statistician

SUBJECT: Estimates of the LC50 and EC50 for BDCM Exposures, Without MAS, Using FETAX, For Test 232-015

STATISTICAL METHODS

Probit analysis was used to estimate the LC50, the EC50, and their associated estimated 95 percent fiducial limits. Abbott's formula was applied to optimize the estimation of the natural threshold response rate. Wald's chi square was used to test for effects. SAS PROC PROBIT statistical computer software was used for these analyses.

STATISTICAL RESULTS

The LC50 and associated estimated 95 percent fiducial limits, without MAS, were estimated with BDCM concentrations on the arithmetic scale. The EC50 and associated 95 percent fiducial limits, without MAS, were estimated with BDCM concentrations log10 transformed. There was a significant BDCM concentration effect for the mortality (X^2=8.6789; p=0.0032) and a significant BDCM concentration effect for malformations (X^2= 18.4762; p=0.0001) (Table 2). The LC50 was estimated to be 393.1 (349.6, 417.7) mg/l BDCM. The EC50 was estimated to be 57.0 (50.5, 65.0) mg/l BDCM (Table 3).

Table 1. Fit to the Model for BDCM Exposures Without MAS, Using FETAX for Estimated LC50, EC50, and Their Associated Estimated 95 Percent Fiducial Limits: Test 232-015

Model	Statistic	
	X^{2a}	p
LC50		
Arithmetic Scale	25.7795	0.0276
Log_{10} Transformed	25.9266	0.0264
EC50		
Arithmetic Scale	3.9924	0.9701
Log_{10} Transformed	3.6985	0.9779

$\alpha = 0.10$

[a] = Pearson's Chi Square

Table 2. Effects for BDCM Exposures, Without MAS, for LC50 and EC50 Estimates Using FETAX: Test 232-015

Parameter	Effect					
	Intercept			BDCM Concentration		
	Estimate	X^{2a}	p	Estimate	X^{2a}	p
LC50	-11.583229	8.2739	0.0040	0.0294639	8.6789	0.0032
EC50	-12.45516	18.5792	0.0001	7.0937723	18.4762	0.0001

$\alpha = 0.05$

[a] Wald's Chi Square

Table 3. Estimated LC50 and EC50 and Their Associated Estimated 95 Percent Fiducial Limits, for BDCM Exposures, Without MAS, Using FETAX: Test 232-015

Parameter	Estimate (mg/L)	Estimated 95 Percent Fiducial Limits	
		Lower	Upper
LC50	393.1	349.6	417.7
EC50[a]	57.0	50.5	65.0

[a] Wald's Chi Square

March 8, 1999

MEMORANDUM FOR: Dave Kumsher/Maggie Toussaint

FROM: Florence J. Hoffmann
Mathematical Statistician

SUBJECT: Estimates of the LC50 and EC50 for BDCM Exposures, With MAS, Using FETAX, For Test 232-012

STATISTICAL METHODS

Probit analysis was used to estimate the LC50, the EC50, and their associated estimated 95 percent fiducial limits. Abbott's formula was applied to optimize the estimation of the natural threshold response rate. Wald's chi square was used to test for effects. SAS PROC PROBIT statistical computer software was used for these analyses.

STATISTICAL RESULTS

The LC50 and the EC50 and associated estimated 95 percent fiducial limits, without MAS, were estimated with BDCM concentrations log10 transformed. There was a BDCM effect for the mortality (X^2=9.0758; p=0.0026). There was no significant BDCM effect for malformations (X^2= 0.9816; p=0.3218) (Table 2). The LC50 was estimated to be 62.8 (18.8, 138.0) mg/l BDCM. The EC50 was estimated to be 52.7 mg/l BDCM. The 95 percent fiducial limits could not be estimated for the EC50 (Table 3).

Table 1. Fit to the Model for BDCM Exposures, With MAS, Using FETAX for Estimated LC50, EC50, and Their Associated Estimated 95 Percent Fiducial Limits: Test 232-012

Model	Statistic	
	X^{2a}	p
LC50		
Arithmetic Scale	105.0583	0.0000
Log_{10} Transformed	73.1762	0.0000
EC50		
Arithmetic Scale	3.6378	0.6026
Log_{10} Transformed	3.6971	0.5938

$\alpha = 0.10$

[a] = Pearson's Chi Square

Table 2. Effects for BDCM Exposures, With MAS, for LC50 and EC50 Estimates Using FETAX: Test 232-012

Parameter	Effect					
	Intercept			BDCM Concentration		
	Estimate	X^{2a}	p	Estimate	X^{2a}	p
LC50	-5.54541	8.0918	0.0044	3.08370733	9.0758	0.0026
EC50	-19.447461	1.0110	0.3147	11.296003	0.9816	0.3218

$\alpha = 0.05$

[a] Wald's Chi Square

Table 3. Estimated LC50 and EC50 and Their Associated Estimated 95 Percent Fiducial Limits, for BDCM Exposures, With MAS, Using FETAX: Test 232-012

Parameter	Estimate (mg/L)	Estimated 95 Percent Fiducial Limits	
		Lower	Upper
LC50	62.8	18.8	138.0
EC50[a]	52.7	***	***

[a] Wald's Chi Square

*** Could not be estimated

February 26, 1999

MEMORANDUM FOR: Dave Kumsher/Maggie Toussaint

FROM: Florence J. Hoffmann
Mathematical Statistician

SUBJECT: Estimates of the LC50 and EC50 for BDCM Exposures, With MAS, Using FETAX, For Test 232-013

STATISTICAL METHODS

Probit analysis was used to estimate the LC50, the EC50, and their associated estimated 95 percent fiducial limits. Abbott's formula was applied to optimize the estimation of the natural threshold response rate. Wald's chi square was used to test for effects. SAS PROC PROBIT statistical computer software was used for these analyses.

STATISTICAL RESULTS

The LC50 and associated estimated 95 percent fiducial limits, with MAS, were estimated with BDCM concentrations log10 transformed. The best-fitting model for estimating the EC50 and associated estimated 95 percent fiducial limits, with MAS, were estimated with BDCM concentrations on the arithmetic scale. However, estimates with BDCM concentrations log10 transformed provided a model which fit the data and produced good estimates. There was a significant BDCM effect for the mortality for both models (X^2=9.9147; p=0.0016) for the model with BDCM concentrations on the arithmetic scale and (X^2=10.6915; p=0.0011) for the model with BDCM concentrations log10 transformed. There was a marginally non-significant BDCM effect for malformations for both models, BDCM on the arithmetic scale and log10 transformed, (X^2=3.0173; p=0.0824) and (X^2=3.3187; p=0.685), respectively (Tables 2a and 2b). The LC50 was estimated to be 54.9 (15.6, 98.0) mg/l BDCM, for the model with BDCM concentrations log10 transformed. The EC50 was estimated to be 62.3 mg/l BDCM for the estimate made with BDCM concentrations on the arithmetic scale and 63.0 mg/l BDCM for the estimate made with BDCM concentrations log10 transformed. The 95 percent fiducial limits could not be estimated for the E50 for either model (Tables 3a and 3b).

Table 1. Fit to the Model for BDCM Exposures, With MAS, Using FETAX for Estimated LC50, EC50, and Their Associated Estimated 95 Percent Fiducial Limits: Test 232-013

Model	Statistic	
	X^{2a}	p
LC50		
Arithmetic Scale	59.5558	0.0000
Log_{10} Transformed	46.7836	0.0000
EC50		
Arithmetic Scale	3.4104	0.7559
Log_{10} Transformed	3.4799	0.7466

$\alpha = 0.10$

[a] = Pearson's Chi Square

Table 2a. Effects for BDCM Exposures, With MAS, for LC50 and EC50 Estimates Using FETAX, with BDCM Concentrations Log_{10} Transformed: Test 232-013

Parameter	Effect					
	Intercept			BDCM Concentration		
	Estimate	X^{2a}	p	Estimate	X^{2a}	p
LC50	-1.1860519	5.9483	0.0147	0.01526987	9.9147	0.0016
EC50[b]	-5.6500575	3.7705	0.0522	0.09071255	3.0173	0.0824

$\alpha = 0.05$

[a] Wald's Chi Square

[b] Best Fitting Model

Table 2b. Effects for BDCM Exposures, With MAS, for LC50 and EC50 Estimates Using FETAX, with BDCM Concentrations Log_{10} Transformed: Test 232-013

Parameter	Effect					
	Intercept			BDCM Concentration		
	Estimate	X^{2a}	p	Estimate	X^{2a}	p
LC50[b]	-4.871715	8.5596	0.0034	2.8001602	10.6915	0.0001
EC50	-19.065151	3.5280	0.0603	10.5950162	3.3187	0.0685

$\alpha = 0.05$

[a] Wald's Chi Square

[b] Best Fitting Model

Table 3a. Estimated LC50 and EC50 and Their Associated Estimated 95 Percent Fiducial Limits, for BDCM Exposures, With MAS, Using FETAX, with Parameter Estimates Made Using BDCM Concentrations on the Arithmetic Scale: Tests 232-013

Parameter	Estimate (mg/L)	Estimated 95 Percent Fiducial Limits	
		Lower	Upper
LC50	77.7	12.1	165.0
EC50[a]	62.3	***	***

*** Could not be estimated

[a] Best Fitting Model

Table 3b. Estimated LC50 and EC50 and Their Associated Estimated 95 Percent Fiducial Limits, for BDCM Exposures, With MAS, Using FETAX, with Parameter Estimates Made Using BDCM Concentrations on the Arithmetic Scale: Test 232-013

Parameter	Estimate (mg/L)	Estimated 95 Percent Fiducial Limits	
		Lower	Upper
LC50[a]	54.9	15.6	98.0
EC50	63.0	***	***

*** Could not be estimated

[a] Best Fitting Model

April 2, 1999

MEMORANDUM FOR: Dave Kumsher/Maggie Toussaint

FROM: Florence J. Hoffmann
Mathematical Statistician

SUBJECT: Estimates of the LC50 and EC50 for BDCM Exposures, With MAS, Using FETAX, For Test 232-015

STATISTICAL METHODS

Probit analysis was used to estimate the LC50, the EC50, and their associated estimated 95 percent fiducial limits. Abbott's formula was applied to optimize the estimation of the natural threshold response rate. Wald's chi square was used to test for effects. SAS PROC PROBIT statistical computer software was used for these analyses.

STATISTICAL RESULTS

The LC50 and EC50 and associated estimated 95 percent fiducial limits, with MAS, were estimated with BDCM concentrations log10 transformed. There was a significant BDCM effect for the mortality (X^2=7.7577; p=0.0053) and a significant BDCM effect for malformations (X^2= 11.0394; p=0.0009) (Table 2). The LC50 was estimated to be 61.5 (24.5, 123.2) mg/l BDCM. The EC50 was estimated to be 49.8 (45.4, 53.4) mg/l BDCM (Table 3).

Table 1. Fit to the Model for BDCM Exposures, With MAS, Using FETAX for Estimated LC50, EC50, and Their Associated Estimated 95 Percent Fiducial Limits: Test 232-015

Model	Statistic	
	X^{2a}	p
LC50		
Arithmetic Scale	77.5223	0.0000
Log_{10} Transformed	76.2816	0.0000
EC50		
Arithmetic Scale	4.1428	0.3870
Log_{10} Transformed	4.0747	0.3960

$\alpha = 0.10$

[a] Pearson's Chi Square

Table 2. Effects for BDCM Exposures, With MAS, for LC50 and EC50 Estimates Using FETAX: Test 232- 015

Parameter	Effect					
	Intercept			BDCM Concentration		
	Estimate	X^{2a}	p	Estimate	X^{2a}	p
LC50	-7.7293459	7.3740	0.0006	4.32001417	7.7577	0.0053
EC50[b]	-28.458021	11.3714	0.0007	16.76499713	11.0394	0.0009

$\alpha = 0.05$

[a] Wald's Chi Square

[b] Best Fitting Model

Table 3. Estimated LC50 and EC50 and Their Associated Estimated 95 Percent Fiducial Limits, for BDCM Exposures, With MAS, Using FETAX: Test 232-015

Parameter	Estimate (mg/L)	Estimated 95 Percent Fiducial Limits	
		Lower	Upper
LC50	61.5	24.5	123.2
EC50	49.8	45.4	53.4

March 10, 1999

MEMORANDUM FOR: Dave Kumsher/Maggie Toussaint

FROM: Florence J. Hoffmann
Mathematical Statistician

SUBJECT: Data Analyses for Length Measurements for BDCM Exposures, Using FETAX, Without MAS, for Test 232-012

STATISTICAL METHODS

Analysis of variance (ANOVA) was used to test for fit to the model and for effects. Regression analysis was used for point estimation. SAS statistical software was used for these analyses.

STATISTICAL RESULTS

Analysis of variance indicated that the best-fitting model was a quadratic model (Tables 1 and 2). The equation for providing the estimated values is:

$$y = 8.642515621 - 0.005532826x - 0.000010347x^2$$

There was a significant BDCM effect ($F=47.42$; $p=0.0001$) and a significant quadratic term ($F=23.25$; $p=0.0001$) (Table 2). The NOEL was estimated to be 15.0 (1.7, 27.7) mg/l BDCM. The MCIG was estimated to be 36.2 (25.0, 47.0) mg/l BDCM (Table 4).

Table 1. Fit to the Model for BDCM Exposures, Without MAS, Using FETAX, for Length: Test 232-012

Model	Statistic			
	F	p	R^2	cv
Linear	1559.85	0.0001	0.8360	7.20
Quadratic	848.56	0.0001	0.8477	6.95
Loglinear	388.33	0.0001	0.5593	11.80

$\alpha = 0.05$

Table 2. Effects for BDCM Exposures, Without MAS, Using FETAX, for Length: Test 232-012

Effect	Statistic	
	F	p
Linear		
Concentration	1559.85	0.0001
Quadratic		
Concentration	47.42	0.0001
Concentration^2	23.35	0.0001
Loglinear		
Concentration	388.33	0.0001

$\alpha = 0.05$

Table 3. Observed Means, Estimated Mean and Their Associated Estimated 95 Percent Confidence Limits, for BDCM Exposures, Without MAS, Using FETAX, for Length: Test 232-012

BDCM Concentration[a] (mg/L)	Observed Mean (mm)	n	Estimated Mean (mm)	Estimated 95 Percent Confidence Limits	
				Lower	Upper
0.0	8.78	100	8.64	8.56	8.73 AA
14.2	8.31	15	8.56	8.49	8.64 AB
15.0	8.46	15	8.56	8.48	8.63 AB
36.2	8.15	30	8.43	8.36	8.50 BB
55.7	8.16	15	8.30	8.23	8.37 BB
56.3	8.05	15	8.30	8.23	8.37 BB
128.9	7.31	15	7.76	7.65	7.86 CC
130.5	8.41	15	7.74	7.64	7.85 CC
216.4	7.02	15	6.96	6.84	7.08 DD
218.8	6.93	15	6.94	6.82	7.06 DD
299.3	6.27	15	6.06	5.95	6.71 EE
303.7	6.11	15	6.01	5.90	6.12 EE
398.2	4.85	15	4.80	4.62	4.97 FF
403.3	4.26	12	4.73	4.55	4.91 FF
486.7	4.73	1	3.50	3.15	3.84 GG

[a] Mean measured BDCM Concentrations

Table 4. Estimated NOEL and MCIG and Their Associated Estimated 95 Percent Confidence Limits for BDCM Exposures, Without MAS, Using FETAX, for Length: Tests 232-012

Parameter	Estimate		
	Estimate (mg/L)	95 Percent Confidence Limits	
		Lower	Upper
NOEL	15.0	1.7	27.7
MCIG	36.2	25.0	47.0

$\hat{y} = 8.642515621 - 0.005532826x - 0.000010347x^2$

March 8, 1999

MEMORANDUM FOR: Dave Kumsher/Maggie Toussaint

FROM: Florence J. Hoffmann
Mathematical Statistician

SUBJECT: Data Analyses for Length Measurements for BDCM Exposures, Using FETAX, Without MAS, for Test 232-013

STATISTICAL METHODS

Analysis of variance (ANOVA) was used to test for fit to the model and for effects. Regression analysis was used for point estimation. SAS statistical software was used for these analyses.

STATISTICAL RESULTS

Analysis of variance indicated that the best-fitting model was a linear model (Tables 1 and 2). The equation for providing the estimated values is:

$$y = 8.965635820 - 0.011578403x$$

There was a significant BDCM effect ($F=1247.14$; $p=0.0001$) (Table 2). The NOEL was estimated to be at the control level, 0.0 (<0.0, 10.0) mg/l BDCM. The MCIG was estimated to be 20.5 (11.2, 29.8) mg/l BDCM.

Table 1. Fit to the Model for BDCM Exposures, Without MAS, Using FETAX, for Length: Test 232-013

Model	Statistic			
	F	p	R^2	cv
Linear	1247.13	0.0001	0.8056	10.21
Quadratic	622.45	0.0001	0.8058	10.22
Loglinear	330.58	0.0001	0.5234	15.99

$\alpha = 0.05$

Table 2. Effects for BDCM Exposures, Without MAS, Using FETAX, for Length: Test 232-013

Effect	Statistic	
	F	p
Linear		
Concentration	1247.13	0.0001
Quadratic		
Concentration	103.85	0.0001
Concentration^2	0.37	0.5430
Loglinear		
Concentration	330.58	0.0001

$\alpha = 0.05$

Table 3. Observed Means, Estimated Mean and Their Associated Estimated 95 Percent Confidence Limits, for BDCM Exposures, Without MAS, Using FETAX, for Length: Test 232-013

BDCM Concentration[a] (mg/L)	Observed Mean (mm)	n	Estimated Mean (mm)	Estimated 95 Percent Confidence Limits	
				Lower	Upper
0.0	8.97	96	8.97	8.85	9.08 AA
20.5	7.68	15	8.73	8.62	8.84 BB
20.6	8.85	15	8.73	8.62	8.83 BB
45.1	8.28	15	8.44	8.34	8.54 CC
45.9	8.37	15	8.43	8.34	8.53 CC
62.5	9.54	15	8.24	8.15	8.34 CD
64.1	8.44	15	8.22	8.13	8.32 DD
130.7	7.47	14	7.45	7.36	7.54 EE
132.4	7.63	15	7.43	7.34	7.52 EE
220.3	6.58	15	6.41	6.30	6.53 FF
221.6	5.65	14	6.40	6.29	6.51 FF
307.0	5.01	15	5.41	5.26	5.56 GG
309.8	5.25	15	5.38	5.23	5.53 GG
399.8	4.60	14	4.34	4.13	4.54 HH
407.8	4.46	15	4.24	4.04	4.45 HH

[a] Mean measured BDCM Concentrations

Table 4. Estimated NOEL and MCIG and Their Associated Estimated 95 Percent Confidence Limits for BDCM Exposures, Without MAS, Using FETAX, for Length: Test 232-013

Parameter	Estimate		
	Estimate (mg/L)	95 Percent Confidence Limits	
		Lower	Upper
NOEL	0.0	< 0.0	10.0
MCIG	20.5	11.2	29.8

$\hat{y} = 8.965635820 - 0.011578403x$

April 5, 1999

MEMORANDUM FOR: Dave Kumsher/Maggie Toussaint

FROM: Florence J. Hoffmann
Mathematical Statistician

SUBJECT: Data Analyses for Length Measurements for BDCM Exposures, Using FETAX, Without MAS, for Test 232-015

STATISTICAL METHODS

Analysis of variance (ANOVA) was used to test for fit to the model and for effects. Regression analysis was used for point estimation. SAS statistical software was used for these analyses.

STATISTICAL RESULTS

Analysis of variance indicated that the best-fitting model was a linear model (Tables 1 and 2). The equation for providing the estimated values is:

$$y = 10.14279474 - 0.01489973x$$

There was a significant BDCM effect ($F=2487.35$; $p=0.0001$) (Table 2). The NOEL was estimated to be at the control level, 0.0 (<0.0, 6.1) mg/l BDCM. The MCIG was estimated to be 20.5 (14.9, 26.1) mg/l BDCM.

Table 1. Fit to the Model for BDCM Exposures, Without MAS, Using FETAX, for Length: Test 232-015

Model	Statistic			
	F	p	R^2	cy
Full Model				
Linear	2487.35	0.0001	0.8969	6.97
Quadratic	1254.34	0.0001	0.8980	6.94
Loglinear	362.16	0.0001	0.5587	14.41

$\alpha = 0.05$

Table 2. Effects for BDCM Exposures, Without MAS, Using FETAX, for Length: Test 232-015

Effect	Statistic	
	F	p
Linear		
Concentration	2487.35	0.0001
Quadratic		
Concentration	177.70	0.0001
Concentration^2	3.10	0.0796
Loglinear		
Concentration	362.16	0.0001

$\alpha = 0.05$

Table 3. Observed Means, Estimated Mean and Their Associated Estimated 95 Percent Confidence Limits, for BDCM Exposures, Without MAS, Using FETAX, for Length: Test 232-015

BDCM Concentration[a] (mg/L)	Observed Mean (mm)	n	Estimated Mean (mm)	Estimated 95 Percent Confidence Limits	
				Lower	Upper
0.0	10.09	97	10.14	10.05	10.23 A
20.5	9.81	15	9.84	9.75	9.92 B
20.6	9.82	15	9.84	9.75	9.92 B
45.1	9.39	15	9.47	9.39	9.55 C
45.9	9.01	15	9.46	9.38	9.54 C
62.5	9.54	15	9.21	9.14	9.28 D
64.1	9.71	15	9.19	9.11	9.26 D
130.8	8.28	15	8.19	8.12	8.27 E
132.4	8.34	15	8.17	8.10	8.24 E
220.3	6.90	15	6.86	6.76	6.96 F
221.6	6.84	15	6.84	6.74	6.94 F
307.0	5.34	15	5.57	5.43	5.71 G
309.0	5.57	15	5.53	5.38	5.67 G
407.8	4.01	11	4.07	3.87	4.26 H

[a] Mean measured BDCM Concentrations

Table 4. Estimated NOEL and MCIG and Their Associated Estimated 95 Percent Confidence Limits for BDCM Exposures, Without MAS, Using FETAX, for Length: Test 232-015

Parameter	Estimate		
	Estimate (mg/L)	95 Percent Confidence Limits	
		Lower	Upper
NOEL	0.0	< 0.0	6.1
MCIG	20.5	14.9	26.1

$\hat{y} = 10.14279474 - 0.01489973x$

March 10, 1999

MEMORANDUM FOR: Dave Kumsher/Maggie Toussaint

FROM: Florence J. Hoffmann
Mathematical Statistician

SUBJECT: Data Analyses for Length Measurements for BDCM Exposures, Using FETAX, With MAS, for Test 232-012

STATISTICAL METHODS

Analysis of variance (ANOVA) was used to test for fit to the model and for effects. Regression analysis was used for point estimation. SAS statistical software was used for these analyses.

STATISTICAL RESULTS

Analysis of variance indicated that the best-fitting model was a linear model (Tables 1 and 2). The equation for providing the estimated values is:

$$y = 7.780693654 - 0.009349290x$$

There was a significant BDCM effect (F=140.43; p=0.0001) (Table 2). The NOEL was estimated to be 12.6 (2.1, 23.1) mg/l BDCM. The MCIG was estimated to be 33.2 (23.2, 43.2) mg/l BDCM (Table 4).

Table 1. Fit to the Model for BDCM Exposures, With MAS, Using FETAX, for Length: Test 232-012

Model	Statistic			
	F	p	R^2	cv
Linear	140.43	0.0001	0.5722	6.51
Quadratic	72.16	0.0001	0.5812	6.48
Loglinear	39.30	0.0001	0.2723	8.50

$\alpha = 0.05$

Table 2. Effects for BDCM Exposures, With MAS, Using FETAX, for Length: Test 232-012

Effect	Statistic	
	F	p
Linear		
Concentration	140.43	0.0001
Quadratic		
Concentration	18.95	0.0001
Concentration^2	2.24	0.1378
Loglinear		
Concentration	39.30	0.0001

$\alpha = 0.05$

Table 3. Observed Means, Estimated Mean and Their Associated Estimated 95 Percent Confidence Limits, for BDCM Exposures, With MAS, Using FETAX, for Length: Test 232-012

BDCM Concentration[a] (mg/L)	Observed Mean (mm)	n	Estimated Mean (mm)	Estimated 95 Percent Confidence Limits	
				Lower	Upper
0.0	7.79	29	7.78	7.67	7.89 AA
12.6	7.74	30	7.66	7.56	7.76 AB
33.2	7.43	14	7.47	7.38	7.56 BB
33.9	7.55	15	7.46	7.37	7.56 BB
49.1	7.24	4	7.32	7.22	7.42 BB
50.4	6.96	10	7.31	7.21	7.41 BB
289.7	5.09	4	5.07	4.66	5.48 CC
296.1	5.40	1	5.01	4.59	5.43 CC

[a] Mean measured BDCM Concentrations

Table 4. Estimated NOEL and MCIG and Their Associated Estimated 95 Percent Confidence Limits for BDCM Exposures, With MAS, Using FETAX, for Length: Test 232-012

Parameter	Estimate		
	Estimate (mg/L)	95 Percent Confidence Limits	
		Lower	Upper
NOEL	12.6	2.1	23.1
MCIG	33.2	23.2	43.2

$\hat{y} = 7.780693654 - 0.009349290x$

March 8, 1999

MEMORANDUM FOR: Dave Kumsher/Maggie Toussaint

FROM: Florence J. Hoffmann
Mathematical Statistician

SUBJECT: Data Analyses for Length Measurements for BDCM Exposures, Using FETAX, With MAS, for Test 232-013

STATISTICAL METHODS

Analysis of variance (ANOVA) was used to test for fit to the model and for effects. Regression analysis was used for point estimation. SAS statistical software was used for these analyses.

STATISTICAL RESULTS

Analysis of variance indicated that the best-fitting model was a quadratic model (Tables 1 and 2). The equation for providing the estimated values is:

$$y = 8.272607564 - 0.018266851x + 0.000035155x^2$$

There was a significant BDCM effect ($F=37.58$; $p=0.0001$) and a significant quadratic term ($F=4.39$; $p=0.0386$) (Table 2). The NOEL was estimated to be at the control level, 0.0 (<0.0, 8.4) mg/l BDCM. The MCIG was estimated to be 18.6 (12.2, 25.2) mg/l BDCM (Table 4).

Table 1. Fit to the Model for BDCM Exposures, With MAS, Using FETAX, for Length: Test 232-013

Model	Statistic			
	F	p	R^2	cv
Linear	95.75	0.0001	0.4770	7.12
Quadratic	51.61	0.0001	0.4981	7.01
Loglinear	51.78	0.0001	0.3303	8.05

$\alpha = 0.05$

Table 2. Effects for BDCM Exposures, With MAS, Using FETAX, for Length: Test 232-013

Effect	Statistic	
	F	p
Linear		
Concentration	95.75	0.0001
Quadratic		
Concentration	37.58	0.0001
Concentration^2	4.39	0.0386
Loglinear		
Concentration	51.78	0.0001

$\alpha = 0.05$

Table 3. Observed Means, Estimated Mean and Their Associated Estimated 95 Percent Confidence Limits, for BDCM Exposures, With MAS, Using FETAX, for Length: Test 232-013

BDCM Concentration[a] (mg/L)	Observed Mean (mm)	n	Estimated Mean (mm)	Estimated 95 Percent Confidence Limits	
				Lower	Upper
0.0	8.21	45	8.27	8.12	8.42 AA
18.6	8.10	13	7.95	7.84	8.05 BB
41.8	7.76	14	7.57	7.43	7.71 CC
42.6	7.62	15	7.56	7.42	7.70 CC
58.3	7.22	13	7.33	7.15	7.50 CC
123.7	6.08	4	6.55	6.28	6.82 DD
205.5	6.48	2	6.00	5.41	6.60 DD
208.0	5.67	1	5.99	5.38	6.60 DD

[a] Mean measured BDCM Concentrations

Table 4. Estimated NOEL and MCIG and Their Associated Estimated 95 Percent Confidence Limits for BDCM Exposures, With MAS, Using FETAX, for Length: Tests 232-013

Parameter	Estimate		
	Estimate (mg/L)	95 Percent Confidence Limits	
		Lower	Upper
NOEL	0.0	< 0.0	8.4
MCIG	18.6	12.2	25.2

$\hat{y} = 8.272607564 - 0.018266851x + 0.000035155x^2$

April 5, 1999

MEMORANDUM FOR: Dave Kumsher/Maggie Toussaint

FROM: Florence J. Hoffmann
Mathematical Statistician

SUBJECT: Data Analyses for Length Measurements for BDCM Exposures, Using FETAX, With MAS, for Test 232-015

STATISTICAL METHODS

Analysis of variance (ANOVA) was used to test for fit to the model and for effects. Regression analysis was used for point estimation. SAS statistical software was used for these analyses.

STATISTICAL RESULTS

Analysis of variance indicated that the best-fitting model was a linear model (Tables 1 and 2). The equation for providing the estimated values is:

$$y = 9.038361661 - 0.010934056x$$

There was a significant BDCM effect ($F=118.24$; $p=0.0001$)(Table 2). The NOEL was estimated to be 18.6 (9.1, 28.1) mg/l BDCM. The MCIG was estimated to be 41.8 (32.4, 51.2) mg/l BDCM (Table 4).

Table 1. Fit to the Model for BDCM Exposures, With MAS, Using FETAX, for Length: Test 232-015

Model	Statistic			
	F	p	R^2	cy
Full Model				
Linear	118.24	0.0001	0.5545	5.77
Quadratic	60.38	0.0001	0.5623	5.75
Loglinear	20.65	0.0001	0.1785	7.83

$\alpha = 0.05$

Table 2. Effects for BDCM Exposures, With MAS, Using FETAX, for Length: Test 232-015

Effect	Statistic	
	F	p
Linear		
Concentration	118.24	0.0001
Quadratic		
Concentration	22.08	0.0001
Concentration^2	1.68	0.1978
Loglinear		
Concentration	20.65	0.0001

$\alpha = 0.05$

Table 3. Observed Means, Estimated Mean and Their Associated Estimated 95 Percent Confidence Limits, for BDCM Exposures, With MAS, Using FETAX, for Length: Test 232-015

BDCM Concentration[a] (mg/L)	Observed Mean (mm)	n	Estimated Mean (mm)	Estimated 95 Percent Confidence Limits	
				Lower	Upper
0.0	8.90	29	9.04	8.92	9.16 A
18.5	8.93	15	8.84	8.73	8.94 A
18.6	9.33	15	8.83	8.73	8.94 A
41.8	8.75	13	8.58	8.48	8.68 B
42.6	8.41	14	8.57	8.47	8.68 B
58.3	7.73	8	8.40	8.29	8.52 B
293.7	6.00	3	5.83	5.29	6.36 C

[a] Mean measured BDCM Concentrations

Table 4. Estimated NOEL and MCIG and Their Associated Estimated 95 Percent Confidence Limits for BDCM Exposures, With MAS, Using FETAX, for Length: Tests 232-015

Parameter	Estimate		
	Estimate (mg/L)	95 Percent Confidence Limits	
		Lower	Upper
NOEL	18.6	9.1	28.1
MCIG	41.8	32.4	51.2

$\hat{y} = 9.038361661 - 0.010934056x$

January 25, 1999

MEMORANDUM FOR: Angela Gaudet-Hull/Dave Kumsher

FROM: Florence J. Hoffmann
Mathematical Statistician

SUBJECT: Estimates of the LC50 and EC50 for DBAA Exposures, Without MAS, Using FETAX, For Test 231-018

STATISTICAL METHODS

Probit analysis was used to estimate the LC50, the EC50, and their associated estimated 95 percent fiducial limits. Abbott's formula was applied to optimize the estimation of the natural threshold response rate. SAS PROC PROBIT statistical computer software was used for these analyses.

STATISTICAL RESULTS

DBAA concentrations without MAS were not high enough to kill half of the embryos or elicit a malformation response in half of the embryos, therefore, the LC50 and the EC50 and their associated estimated 95 percent fiducial limits could not be estimated (Table 2).

Table 1. Fit to the Model for DBAA Exposures Without MAS, Using FETAX for Estimated LC50, EC50, and Their Associated Estimated 95 Percent Fiducial Limits: Test 231-018

Model	LC50		EC50	
	X^{2a}	p	X^{2a}	p
Arithmetic Scale	105.0783	0.0000	24.8427	0.0032
Log_{10}	39.9774	0.0000	19.6816	0.0200

$\alpha = 0.10$

[a] = Pearson's Chi Square

Table 2. Estimated LC50, EC50, and Their Associated Estimated 95 Percent Fiducial Limits, for DBAA Exposures Without MAS Using FETAX: Test 231-018

Parameter	Estimate (mg/L)	Estimated 95 Percent Fiducial Limits	
		Lower	Upper
LC50	*	*	*
EC50	*	*	*

* Extrapolation beyond data. (DBAA, without MAS) not high enough to kill or malform half of the embryos

January 25, 1999

MEMORANDUM FOR: Angela Gaudet-Hull/Dave Kumsher

FROM: Florence J. Hoffmann
Mathematical Statistician

SUBJECT: Estimates of the LC50 and EC50 for DBAA Exposures, Without MAS, Using FETAX, For Test 231-019

STATISTICAL METHODS

Probit analysis was used to estimate the LC50, the EC50, and their associated estimated 95 percent fiducial limits. Abbott's formula was applied to optimize the estimation of the natural threshold response rate. SAS PROC PROBIT statistical computer software was used for these analyses.

STATISTICAL RESULTS

The LC50 and the EC50 and associated estimated 95 percent fiducial limits, without MAS, were estimated with DBAA concentrations on the arithmetic scale. The associated estimated 95 percent fiducial limits could not be estimated for the estimated EC50. The LC50 was estimated to be 7374 mg/l (4570, 14001) mg/l. The EC50 was estimated to be 11723 mg/l (Table 2).

Table 1. Fit to the Model for DBAA Exposures Without MAS, Using FETAX for Estimated LC50, EC50, and Their Associated Estimated 95 Percent Fiducial Limits: Test 231-019

Model	LC50		EC50	
	X^{2a}	p	X^{2a}	p
Arithmetic Scale	45.6115	0.0000	18.4940	0.0178
Log_{10}	47.9632	0.0000	35.3406	0.0000

$\alpha = 0.10$

[a] = Pearson's Chi Square

Table 2. Estimated LC50, EC50, and Their Associated Estimated 95 Percent Fiducial Limits, for DBAA Exposures Without MAS Using FETAX: Test 231-019

Parameter	Estimate (mg/L)	Estimated 95 Percent Fiducial Limits	
		Lower	Upper
LC50	7374	4570	14001
EC50	11723	***	***

*** Could not be estimated

January 25, 1999

MEMORANDUM FOR: Angela Gaudet-Hull/Dave Kumsher

FROM: Florence J. Hoffmann
Mathematical Statistician

SUBJECT: Estimates of the LC50 and EC50 for DBAA Exposures, Without MAS, Using FETAX, For Test 231-020

STATISTICAL METHODS

Probit analysis was used to estimate the LC50, the EC50, and their associated estimated 95 percent fiducial limits. Abbott's formula was applied to optimize the estimation of the natural threshold response rate. SAS PROC PROBIT statistical computer software was used for these analyses.

STATISTICAL RESULTS

DBAA concentrations without MAS were not high enough to kill half of the embryos or elicit a malformation response in half of the embryos, therefore, the LC50 and the EC50 and their associated estimated 95 percent fiducial limits could not be estimated (Table 2).

Table 1. Fit to the Model for DBAA Exposures Without MAS, Using FETAX for Estimated LC50, EC50, and Their Associated Estimated 95 Percent Fiducial Limits: Test 231-020

Model	LC50		EC50	
	X^{2a}	p	X^{2a}	p
Arithmetic Scale	26.1721	0.0019	7.7901	0.4542
Log_{10}	18.4685	0.0301	8.8992	0.3509

$\alpha = 0.10$

[a] = Pearson's Chi Square

Table 2. Estimated LC50, EC50, and Their Associated Estimated 95 Percent Fiducial Limits, for DBAA Exposures Without MAS Using FETAX: Test 231-020

Parameter	Estimate (mg/L)	Estimated 95 Percent Fiducial Limits	
		Lower	Upper
LC50	*	*	*
EC50	*	*	*

* DBAA concentrations not high enough to elicit malformation response in half of the embryos.

January 25, 1999

MEMORANDUM FOR: Angela Gaudet-Hull/Dave Kumsher

FROM: Florence J. Hoffmann
Mathematical Statistician

SUBJECT: Estimates of the LC50 and EC50 for DBAA Exposures, With MAS, Using FETAX, For Test 231-018

STATISTICAL METHODS

Probit analysis was used to estimate the LC50, the EC50, and their associated estimated 95 percent fiducial limits. Abbott's formula was applied to optimize the estimation of the natural threshold response rate. SAS PROC PROBIT statistical computer software was used for these analyses.

STATISTICAL RESULTS

The LC 50 with MAS was estimated with DBAA concentrations on the arithmetic scale. Its associated estimated 95 percent fiducial limits could not be estimated. DBAA concentrations with MAS were not high enough to elicit a malformation response in half of the embryos, therefore the EC50 and its associated estimated 95 percent fiducial limits could not be estimated (Table 2).

Table 1. Fit to the Model for DBAA Exposures With MAS, Using FETAX for Estimated LC50, EC50, and Their Associated Estimated 95 Percent Fiducial Limits: Test 231-018

Model	LC50		EC50	
	X^{2a}	p	X^{2a}	p
Arithmetic Scale	56.2294	0.0000	25.3253	0.0007
Log_{10}	69.2449	0.0000	30.9484	0.0001

$\alpha = 0.10$

[a] = Pearson's Chi Square

Table 2. Estimated LC50, EC50, and Their Associated Estimated 95 Percent Fiducial Limits, for DBAA Exposures With MAS Using FETAX: Test 231-018

Parameter	Estimate (mg/L)	Estimated 95 Percent Fiducial Limits	
		Lower	Upper
LC50	6244	***	***
EC50	*	*	*

* DBAA concentrations not high enough to elicit malformation response in half of the embryos

*** Could not be estimated

January 25, 1999

MEMORANDUM FOR: Angela Gaudet-Hull/Dave Kumsher

FROM: Florence J. Hoffmann
Mathematical Statistician

SUBJECT: Estimates of the LC50 and EC50 for DBAA Exposures, With MAS, Using FETAX, For Test 231-019

STATISTICAL METHODS

Probit analysis was used to estimate the LC50, the EC50, and their associated estimated 95 percent fiducial limits. Abbott's formula was applied to optimize the estimation of the natural threshold response rate. SAS PROC PROBIT statistical computer software was used for these analyses.

STATISTICAL RESULTS

The LC50 and associated estimated 95 percent fiducial limits, with MAS, were estimated with DBAA concentrations log10 transformed. The EC50, with MAS, was estimated with DBAA concentrations on the arithmetic scale. The associated estimated 95 percent fiducial limits could not be estimated for the estimated EC50. The LC50 was estimated to be 69 mg/l (1, 244) mg/l. The EC50 was estimated to be 879 mg/l (Table 2).

Table 1. Fit to the Model for DBAA Exposures With MAS, Using FETAX for Estimated LC50, EC50, and Their Associated Estimated 95 Percent Fiducial Limits: Test 231-019

Model	LC50		EC50	
	X^{2a}	p	X^{2a}	p
Arithmetic Scale	51.6040	0.0000	17.7290	0.0033
Log_{10}	42.5870	0.0000	19.8286	0.0013

$\alpha = 0.10$

[a] = Pearson's Chi Square

Table 2. Estimated LC50, EC50, and Their Associated Estimated 95 Percent Fiducial Limits, for DBAA Exposures With MAS Using FETAX: Test 231-019

Parameter	Estimate (mg/L)	Estimated 95 Percent Fiducial Limits	
		Lower	Upper
LC50	69	1	244
EC50	879	***	***

*** Could not be estimated

January 25, 1999

MEMORANDUM FOR: Angela Gaudet-Hull/Dave Kumsher

FROM: Florence J. Hoffmann
Mathematical Statistician

SUBJECT: Estimates of the LC50 and EC50 for DBAA Exposures, With MAS, Using FETAX, For Test 231-020

STATISTICAL METHODS

Probit analysis was used to estimate the LC50, the EC50, and their associated estimated 95 percent fiducial limits. Abbott's formula was applied to optimize the estimation of the natural threshold response rate. SAS PROC PROBIT statistical computer software was used for these analyses.

STATISTICAL RESULTS

The LC50 and its associated estimated 95 percent fiducial limits, with MAS, were estimated with DBAA concentrations on the arithmetic scale. The estimated LC50 was 3787 mg/l (2658, 5649) mg/l. DBAA concentrations with MAS were not high enough to elicit a malformation response in half of the embryos, therefore, the EC50 and its associated estimated 95 percent fiducial limits could not be estimated (Table 2).

Table 1. Fit to the Model for DBAA Exposures With MAS, Using FETAX for Estimated LC50, EC50, and Their Associated Estimated 95 Percent Fiducial Limits: Test 231-020

Model	LC50		EC50	
	X^{2a}	p	X^{2a}	p
Arithmetic Scale	30.4063	0.0002	13.1844	0.0677
Log_{10}	36.9680	0.0000	12.3219	0.0905

$\alpha = 0.10$

[a] = Pearson's Chi Square

Table 2. Estimated LC50, EC50, and Their Associated Estimated 95 Percent Fiducial Limits, for DBAA Exposures With MAS Using FETAX: Test 231-020

Parameter	Estimate (mg/L)	Estimated 95 Percent Fiducial Limits	
		Lower	Upper
LC50	3787	2658	5649
EC50	*	*	*

* DBAA concentrations not high enough to elicit malformation response in half of the embryos.

January 28, 1999

MEMORANDUM FOR: Angela Gaudet-Hull/Dave Kumsher/Maggie Toussaint

FROM: Florence J. Hoffmann
Mathematical Statistician

SUBJECT: Data Analyses for Length Measurements for DBAA Exposures, Without MAS, Using FETAX, for Test 231-018

STATISTICAL METHODS

Analysis of variance (ANOVA) was used to test for fit to the model and for effects. Regression analysis was used for point estimation if appropriate. SAS statistical software was used for these analyses.

STATISTICAL RESULTS

Analysis of variance indicated that none of the models tested linear, quadratic, or loglinear, in either the full or reduced form fit (Table 1). There were no concentration effects and no replicate effects for any of the models (Table 2). Table 3 shows the observed mean lengths for each mean measured DBAA concentration level.

Table 1. Fit to the Model for DBAA Exposures, Without MAS, Using FETAX, for Length: Test 231-018

Model	Statistic			
	F	p	R^2	cy
Full model				
Linear	1.76	0.1355	0.0136	6.94
Quadratic	1.41	0.2194	0.0136	6.95
Loglinear	1.80	0.1270	0.0139	6.94
Reduced model				
Linear	0.10	0.7529	0.0002	6.97
Quadratic	0.05	0.9500	0.0002	6.97
Loglinear	0.54	0.4639	0.0010	6.96

$\alpha = 0.05$

Table 2. Effects, by Model, for DBAA Exposures, Without MAS, Using FETAX, for Length: Test 231-018

Model Effect	Statistic	
	F[a]	p
Full model		
Linear		
Concentration	0.21	0.6473
Replicate	2.31	0.0751
Quadratic		
Concentration	0.00	0.9995
Concentration^2	0.02	0.8969
Replicate	2.31	0.0751
Loglinear		
Concentration	0.38	0.5401
Replicate	2.22	0.0846
Reduced Model		
Linear		
Concentration	0.10	0.7529
Quadratic		
Concentration	0.00	0.9771
Concentration^2	0.00	0.9518
Loglinear		
Concentration	0.54	0.4639

$\alpha = 0.05$

[a] ANOVA (Fisher's "F")

Table 3. Observed Means, Estimated Mean and Their Associated Estimated 95 Percent Confidence Limits, for DBAA Exposures, Without MAS, Using FETAX, for Length: Test 231-018

DBAA Concentration[a] (mg/L)	Observed Mean (mm)	n	Standard Error (mm)
0	9.15	100	0.0489
27	9.04	50	0.0635
51	9.13	50	0.0666
100	9.44	50	0.0554
196	9.05	49	0.1341
393	9.09	44	0.1265
724	8.89	35	0.1509
1531	8.66	29	0.1400
3137	9.24	41	0.0644
5726	9.23	31	0.0704
12254	9.12	37	0.1012

[a] Mean Measured DBAA Concentrations

January 29, 1999

MEMORANDUM FOR: Angela Gaudet-Hull/Dave Kumsher/Maggie Toussaint

FROM: Florence J. Hoffmann
Mathematical Statistician

SUBJECT: Data Analyses for Length Measurements for DBAA Exposures, Without MAS, Using FETAX, for Test 231-019

STATISTICAL METHODS

Analysis of variance (ANOVA) was used to test for fit to the model and for effects. The Tukey-Kramer multiple comparison procedure was used to make comparisons among/between replicates at each mean measured DBAA concentration level. Regression analysis was used for point estimation. SAS statistical software was used for these analyses.

STATISTICAL RESULTS

Analysis of variance indicated that the best-fitting model was a linear model (Tables 1 and 2). However, there was a significant replicate effect ($F=5.69$; $p=0.0008$) (Table 2). Replicate comparisons at each mean measured DBAA concentration group indicated that these difference were not patterned in any way and were found in the control group with replicate 4 having a smaller observed mean value than the other 3 replicates; in the 393 mg/l group with replicate 1 having a smaller value than replicate 2; and in the 783 mg/l group with replicate 1 having a smaller value than replicate 2 (Table 3). The equation for providing the estimated values is:

$$y = 8.880275604 - 0.000073746x$$

The NOEL was estimated to be 1539 (847, 2231) mg/l. The MCIG was estimated to be 3094 (2199, 3989) mg/l (Tables 4 and 5).

Table 1. Fit to the Model for DBAA Exposures, Without MAS, Using FETAX, for Length: Test 231-019

Model	Statistic			
	F	p	R^2	cy
Full model				
Linear	15.77	0.0001	0.1118	6.45
Quadratic	16.33	0.0001	0.1404	6.35
Loglinear	10.23	0.0001	0.0755	6.58
Reduced model				
Linear	44.77	0.0001	0.0816	6.53
Quadratic	31.74	0.0001	0.1121	6.43
Loglinear	18.56	0.0001	0.0355	6.70

$\alpha = 0.05$

Table 2. Effects, by Model, for DBAA Exposures, Without MAS, Using FETAX, for Length: Test 231-019

Model Effect	Statistic	
	F[a]	p
Full model		
Linear		
Concentration	42.04	0.0001
Replicate	5.69	0.0008
Quadratic		
Concentration	2.31	0.1451
Concentration^2	16.58	0.0001
Replicate	5.49	0.0010
Loglinear		
Concentration	20.70	0.0001
Replicate	7.23	0.0001
Reduced Model		
Linear		
Concentration	44.77	0.0001
Quadratic		
Concentration	2.06	0.1517
Concentration^2	17.26	0.0001
Loglinear		
Concentration	18.56	0.0001

$\alpha = 0.05$

[a] ANOVA (Fisher's "F")

Table 3. Observed Means, for DBAA Exposures, Without MAS, Using FETAX, for Length: Test 231-019

DBAA Concentration[a] (mg/L) Replicate	Observed Mean (mm)	n	Tukey Kramer Grouping[b]
0			
1	8.99	25	A
2	9.01	25	A
3	9.15	25	A
4	8.61	25	B
25			
1	8.76	24	A
2	8.88	25	A
49			
1	8.92	25	A
2	8.99	25	A
98			
1	8.89	25	A
2	8.75	23	A
196			
1	8.94	25	A
2	8.83	24	A
393			
1	8.13	22	B
2	8.77	25	A
783			
1	8.21	16	B
2	9.05	25	A
1539			
1	8.93	23	A
2	8.90	24	A
3094			
1	8.94	14	A
2	8.76	25	A
6090			
1	8.74	13	A
2	8.85	9	A
11991			
1	7.62	11	A
2	7.44	3	A

[a] DBAA MEAN MEASURED CONCENTRATIONS

[b] Tukey Kramer groupings with different letters are different from each other for the same mean measured DBAA concentration groups

Table 4. Observed Means, Estimated Mean and Their Associated Estimated 95 Percent Confidence Limits, for DBAA Exposures, Without MAS, Using FETAX, for Length: Test 231-019

DBAA Concentration (mg/L)	Observed Mean (mm)	n	Estimated Mean (mm)	Estimated 95 Percent Confidence Limits	
				Lower	Upper
0	8.94	100	8.88	8.82	8.94 AA
25	8.82	49	8.88	8.82	8.93 AA
49	8.96	50	8.88	8.82	8.93 AA
98	8.82	48	8.87	8.82	8.93 AA
196	8.89	49	8.87	8.81	8.92 AA
393	8.48	47	8.85	8.80	8.90 AA
783	8.72	41	8.82	8.77	8.87 AA
1539	8.92	47	8.77	8.716	8.82 AB
3094	8.82	39	8.65	8.59	8.718 BB
6090	8.79	22	8.43	8.31	8.55 CC
11991	7.58	14	8.00	7.76	8.24 DD

Table 5. Estimated NOEL and MCIG and Their Associated Estimated 95 Percent Confidence Limits, for DBAA Exposures, Without MAS Using FETAX, for Length: Test 231-019

Parameter	Estimate (mg/L)	Estimated 95 Percent Fiducial Limits	
		Lower	Upper
NOEL	1539	847	2231
MCIG	3094	2199	3989

$\hat{y} = 8.880275604 - 0.000073746x$

January 29, 1999

MEMORANDUM FOR: Angela Gaudet-Hull/Dave Kumsher/Maggie Toussaint

FROM: Florence J. Hoffmann
Mathematical Statistician

SUBJECT: Data Analyses for Length Measurements for DBAA Exposures, Without MAS, Using FETAX, for Test 231-020

STATISTICAL METHODS

Analysis of variance (ANOVA) was used to test for fit to the model and for effects. The Tukey-Kramer multiple comparison procedure was used to make comparisons among/between replicates at each mean measured DBAA concentration level. Regression analysis was used for point estimation. SAS statistical software was used for these analyses.

STATISTICAL RESULTS

Analysis of variance indicated that the best-fitting model was a quadratic model (Tables 1 and 2). However, there was a significant replicate effect ($F=3.52$; $p=0.0150$) (Table 2). Replicate comparisons at each mean measured DBAA concentration group indicated that these difference were not patterned in any way and were found in the 51 mg/l group with replicate 2 having a smaller observed mean value than replicate 1, and in the 1489 mg/l group with replicate 2 having a smaller value than replicate 1 (Table 3). The equation for providing the estimated values is:

$$y = 9.420562844 + 0.0000777769x - 0.000000010x^2$$

The NOEL was estimated to be 6152 (<0, 8289) mg/l. The MCIG was estimated to be 12,125 (10,082, 13,140) mg/l (Tables 4 and 5).

Table 1. Fit to the Model for DBAA Exposures, Without MAS, Using FETAX, for Length: Test 231-020

Model	Statistic			
	F	p	R^2	cy
Full model				
Linear	5.05	0.0005	0.0351	7.40
Quadratic	6.03	0.0001	0.0515	7.35
Loglinear	2.68	0.0310	0.0189	7.47
Reduced model				
Linear	7.02	0.0083	0.0124	7.47
Quadratic	9.65	0.0001	0.0334	7.40
Loglinear	2.56	0.1102	0.0046	7.50

$\alpha = 0.05$

Table 2. Effects, by Model, for DBAA Exposures, Without MAS, Using FETAX, for Length: Test 231-020

Model Effect	Statistic	
	F[a]	p
Full model		
Linear		
Concentration	9.54	0.0021
Replicate	4.35	0.0048
Quadratic		
Concentration	4.06	0.0444
Concentration^2	9.61	0.0020
Replicate	3.52	0.0150
Loglinear		
Concentration	0.22	0.6406
Replicate	2.71	0.0444
Reduced Model		
Linear		
Concentration	7.02	0.0083
Quadratic		
Concentration	6.27	0.0125
Concentration^2	12.15	0.0005
Loglinear		
Concentration	2.56	0.1102

$\alpha = 0.05$

[a] ANOVA (Fisher's "F")

Table 3. Observed Means, for DBAA Exposures, Without MAS, Using FETAX, for Length: Test 231-020

DBAA Concentration[a] (mg/L) Replicate	Observed Mean (mm)	n	Tukey Kramer Grouping [b]
0			
1	9.21	25	A
2	9.14	25	A
3	9.40	25	A
4	9.03	25	A
28			
1	9.59	25	A
2	9.60	25	A
51			
1	9.71	24	A
2	9.45	25	B
101			
1	9.49	23	A
2	9.51	25	A
197			
1	9.50	25	A
2	9.43	25	A
396			
1	9.34	24	A
2	9.55	25	A
801			
1	9.39	19	A
2	9.39	24	A
1489			
1	9.77	25	A
2	9.44	24	B
2959			
1	9.60	24	A
2	9.71	22	A
6153			
1	9.58	19	A
2	9.32	25	A
12124			
1	8.83	16	A
2	9.12	17	A

[a] DBAA Mean Measured Concentrations

[b] Tukey Kramer groupings with different letters are different from each other for the same mean measured DBAA concentration groups

Table 4. Observed Means, Estimated Mean and Their Associated Estimated 95 Percent Confidence Limits, for DBAA Exposures, Without MAS, Using FETAX, for Length: Test 231-020

DBAA Concentration (mg/L)	Observed Mean (mm)	n	Estimated Mean (mm)	Estimated 95 Percent Confidence Limits	
				Lower	Upper
0	9.20	100	9.42	9.35	9.49 A
28	9.60	50	9.42	9.35	9.49 A
51	9.58	49	9.42	9.35	9.50 A
101	9.50	48	9.43	9.36	9.50 A
197	9.47	50	9.44	9.37	9.50 A
396	9.45	49	9.45	9.37	9.51 A
801	9.39	43	9.48	9.42	9.54 A
1489	9.61	49	9.52	9.44	9.59 A
2959	9.65	46	9.57	9.45	9.68 A
6153	9.43	44	9.54	9.38	9.69 A
12124	8.98	33	8.95	8.71	9.19 B

Table 5. Estimated NOEL and MCIG and Their Associated Estimated 95 Percent Confidence Limits, for DBAA Exposures, Without MAS Using FETAX, for Length: Test 231-020

Parameter	Estimate (mg/L)	Estimated 95 Percent Fiducial Limits	
		Lower	Upper
NOEL	6153	< 0	8289
MCIG	12124	10082	13140

$\hat{y} = 9.420562844 + 0.0000777769x - 0.000000010x^2$

January 28, 1999

MEMORANDUM FOR: Angela Gaudet-Hull/Dave Kumsher/Maggie Toussaint

FROM: Florence J. Hoffmann
Mathematical Statistician

SUBJECT: Data Analyses for Length Measurements for DBAA Exposures, With MAS, Using FETAX, for Test 231-018

STATISTICAL METHODS

Analysis of variance (ANOVA) was used to test for fit to the model and for effects. Regression analysis was used for point estimation if appropriate. SAS statistical software was used for these analyses.

STATISTICAL RESULTS

Analysis of variance indicated that the best-fitting model was a quadratic model (Tables 1 and 2). There was no replicate effect for this model (Table 2). The equation for providing the estimated values is:

$$y = 8.456215285 - 0.000205506x + 0.000000056x^2$$

The NOEL was estimated to be 3137-(<0, 4022) mg/l DBAA and the MCIG was estimated to be 5726 (5014, 6290) mg/l DBAA (Table 4).

Table 1. Fit to the Model for DBAA Exposures, With MAS, Using FETAX, for Length: Test 231-018

Model	Statistic			
	F	p	R^2	cy
Full model				
Linear	7.82	0.0005	0.0404	8.45
Quadratic	9.68	0.0001	0.0726	8.32
Loglinear	5.37	0.0050	0.0281	8.50
Reduced model				
Linear	10.37	0.0014	0.0270	8.50
Quadratic	12.89	0.0001	0.0648	8.34
Loglinear	1.83	0.1774	0.0049	8.59

$\alpha = 0.05$

Table 2. Effects, by Model, for DBAA Exposures, With MAS, Using FETAX, for Length: Test 231-018

Model Effect	Statistic	
	F[a]	p
Full model		
Linear		
Concentration	7.47	0.0066
Replicate	5.16	0.0237
Quadratic		
Concentration	6.57	0.0108
Concentration^2	12.90	0.0004
Replicate	3.12	0.0783
Loglinear		
Concentration	2.67	0.1030
Replicate	8.88	0.0031
Reduced Model		
Linear		
Concentration	10.37	0.0014
Quadratic		
Concentration	7.29	0.0072
Concentration^2	15.02	0.0001
Loglinear		
Concentration	1.83	0.1774

$\alpha = 0.05$

[a] ANOVA (Fisher's "F")

Table 3. Observed Means, Estimated Mean and Their Associated Estimated 95 Percent Confidence Limits, for DBAA Exposures, With MAS, Using FETAX, for Length: Test 231-018

DBAA Concentration[a] (mg/L)	Observed Mean (mm)	n	Estimated Mean (mm)	Estimated 95 Percent Confidence Limits	
				Lower	Upper
0	8.75	49	8.46	8.36	8.55 A
27	8.58	50	8.45	8.36	8.55 A
51	8.42	46	8.45	8.36	8.54 A
100	8.13	39	8.44	8.35	8.52 A
196	8.15	33	8.42	8.34	8.50 A
393	8.20	31	8.38	8.31	8.46 A
724	8.47	30	8.34	8.25	8.42 A
1531	8.39	36	8.27	8.14	8.41 A
3137	8.31	36	8.36	8.18	8.54 A
5726	9.11	25	9.11	8.83	9.38 B

[a] DBAA Mean Measured Concentrations

Table 4. Estimated NOEL and MCIG and Their Associated Estimated 95 Percent Confidence Limits, for DBAA Exposures, With MAS Using FETAX, for Length: Test 231-018

Parameter	Estimate (mg/L)	Estimated 95 Percent Fiducial Limits	
		Lower	Upper
NOEL	3137	< 0	4022
MCIG	5726	5014	6290

$\hat{y} = 8.456215285 - 0.000205506x + 0.000000056x^2$

January 29, 1999

MEMORANDUM FOR: Angela Gaudet-Hull/Dave Kumsher/Maggie Toussaint

FROM: Florence J. Hoffmann
Mathematical Statistician

SUBJECT: Data Analyses for Length Measurements for DBAA Exposures, With MAS, Using FETAX, for Test 231-019

STATISTICAL METHODS

Analysis of variance (ANOVA) was used to test for fit to the model and for effects. Regression analysis was used for point estimation. SAS statistical software was used for these analyses.

STATISTICAL RESULTS

Analysis of variance indicated that the best-fitting model was a linear model (Tables 1 and 2). The equation for providing the estimated values is:

$$y = 7.274960358 - 0.000875474x$$

The NOEL was estimated to be 196 (89, 303) mg/l. The MCIG was estimated to be 393 (260, 526) mg/l (Table 4).

Table 1. Fit to the Model for DBAA Exposures, With MAS, Using FETAX, for Length: Test 231-019

Model	Statistic			
	F	p	R^2	cy
Full model				
Linear	14.11	0.0001	0.1381	8.95
Quadratic	9.35	0.0001	0.1382	8.98
Loglinear	12.29	0.0001	0.1226	9.03
Reduced model				
Linear	27.70	0.0001	0.1353	8.94
Quadratic	13.77	0.0001	0.1353	8.97
Loglinear	24.23	0.0001	0.1204	9.02

$\alpha = 0.05$

Table 2. Effects, by Model, for DBAA Exposures, With MAS, Using FETAX, for Length: Test 231-019

Model Effect	Statistic	
	F	p
Full model		
Linear		
Concentration	28.14	0.0001
Replicate	0.58	0.4474
Quadratic		
Concentration	4.42	0.0369
Concentration^2	0.00	0.9570
Replicate	0.58	0.4477
Loglinear		
Concentration	24.52	0.0001
Replicate	0.43	0.5119
Reduced Model		
Linear		
Concentration	27.70	0.0001
Quadratic		
Concentration	4.45	0.0362
Concentration^2	0.00	0.9871
Loglinear		
Concentration	24.23	0.0001

$\alpha = 0.05$

Table 3. Observed Means, Estimated Mean and Their Associated Estimated 95 Percent Confidence Limits, for DBAA Exposures, With MAS, Using FETAX, for Length: Test 231-019

DBAA Concentration (mg/L)	Observed Mean (mm)	n	Estimated Mean (mm)	Estimated 95 Percent Confidence Limits	
				Lower	Upper
0	7.44	48	7.27	7.16	7.39 AA
25	6.99	27	7.25	7.15	7.36 AA
49	7.15	24	7.23	7.13	7.34 AA
98	7.37	27	7.19	7.09	7.29 AA
196	6.42	12	7.10	7.01	7.20 AB
393	7.23	19	6.93	6.8148	7.05 BB
783	6.53	20	6.59	6.37	6.807 CC
1539	5.94	2	5.93	5.47	6.38 DD

Table 4. Estimated NOEL and MCIG and Their Associated Estimated 95 Percent Confidence Limits, for DBAA Exposures, With MAS Using FETAX, for Length: Test 231-019

Parameter	Estimate (mg/L)	Estimated 95 Percent Fiducial Limits	
		Lower	Upper
NOEL	196	89	303
MCIG	393	260	526

$\hat{y} = 7.274960358 - 0.000875474x$

January 29, 1999

MEMORANDUM FOR: Angela Gaudet-Hull/Dave Kumsher/Maggie Toussaint

FROM: Florence J. Hoffmann
Mathematical Statistician

SUBJECT: Data Analyses for Length Measurements for DBAA Exposures, With MAS, Using FETAX, for Test 231-020

STATISTICAL METHODS

Analysis of variance (ANOVA) was used to test for fit to the model and for effects. Regression analysis was used for point estimation. SAS statistical software was used for these analyses.

STATISTICAL RESULTS

Analysis of variance indicated that the best-fitting model was a quadratic model (Tables 1 and 2). The equation for providing the estimated values is:

$$y = 9.119243981 + 0.000171940x - 0.000000069x^2$$

The NOEL was estimated to be 2959 (469, 3542) mg/l. The MCIG was estimated to be 6153 (5359, 6825) mg/l (Table 4).

Table 1. Fit to the Model for DBAA Exposures, With MAS, Using FETAX, for Length: Test 231-020

Model	Statistic			
	F	p	R^2	cy
Full model				
Linear	8.27	0.0003	0.0374	7.66
Quadratic	13.09	0.0001	0.0846	7.48
Loglinear	1.12	0.3258	0.0053	7.79
Reduced model				
Linear	16.43	0.0001	0.0370	7.66
Quadratic	19.61	0.0001	0.0843	7.48
Loglinear	2.03	0.1550	0.0047	7.78

$\alpha = 0.05$

Table 2. Effects, by Model, for DBAA Exposures, With MAS, Using FETAX, for Length: Test 231-020

Model Effect	Statistic	
	F[a]	p
Full model		
Linear		
Concentration	16.29	0.0001
Replicate	0.15	0.7021
Quadratic		
Concentration	6.29	0.0125
Concentration^2	21.92	0.0001
Replicate	0.14	0.7052
Loglinear		
Concentration	2.00	0.1578
Replicate	0.22	0.6371
Reduced Model		
Linear		
Concentration	16.43	0.0001
Quadratic		
Concentration	6.28	0.0126
Concentration^2	21.97	0.0001
Loglinear		
Concentration	2.03	0.1550

$\alpha = 0.05$

[a] ANOVA (Fisher's "F")

Table 3. Observed Means, Estimated Mean and Their Associated Estimated 95 Percent Confidence Limits, for DBAA Exposures, With MAS, Using FETAX, for Length: Test 231-020

DBAA Concentration[a] (mg/L)	Observed Mean (mm)	n	Estimated Mean (mm)	Estimated 95 Percent Confidence Limits	
				Lower	Upper
0	9.09	50	9.12	9.04	9.20 A
28	9.25	49	9.12	9.04	9.21 A
51	9.24	50	9.13	9.05	9.21 A
101	9.24	50	9.14	9.06	9.21 A
197	8.89	47	9.15	9.08	9.22 A
396	9.08	49	9.18	9.11	9.24 A
801	9.14	40	9.21	9.13	9.29 A
1489	9.32	44	9.22	9.11	9.34 A
2959	9.03	43	9.03	8.86	9.19 A
6153	7.54	7	7.57	7.08	8.06 B

[a] DBAA Mean Measured Concentrations

Table 4. Estimated NOEL and MCIG and Their Associated Estimated 95 Percent Confidence Limits, for DBAA Exposures, With MAS Using FETAX, for Length: Test 231-020

Parameter	Estimate (mg/L)	Estimated 95 Percent Fiducial Limits	
		Lower	Upper
NOEL	2959	469	3542
MCIG	6153	5359	6825

$\hat{y} = 9.119243981 + 0.000171940x - 0.000000069x^2$

March 11, 1999

MEMORANDUM FOR: Dave Kumsher/Maggie Toussaint

FROM: Florence J. Hoffmann
Mathematical Statistician

SUBJECT: Data Analyses for Estimating the LC50 and the EC50 for Drinking Water (Total Trihalomethanes in Chlorinated Surface Water) Exposures Using FETAX, Without MAS: Test 234-004

STATISTICAL METHODS

Probit analysis was used to estimate the LC50 and the EC50 and associated estimated 95 percent fiducial limits. Pearson's chi square was used to test for fit to the model. Abbott's formula was applied to optimize the estimation of the natural threshold response rate. Wald's chi square was used to indicate effects. SAS PROC PROBIT statistical computer software was used for these analyses.

STATISTICAL RESULTS

The LC50 and it's associated estimated 95 percent fiducial limits could not be estimated since there was no response for mortality. The best fitting model for estimating the EC50 used drinking water concentrations on the arithmetic scale (Table 1). The EC50 was estimated to be 54.432 mg/l drinking water (total trihalomethanes in chlorinated surface water). The 95 percent fiducial limits could not be estimated for the EC50 (Table 3). There was no drinking water effect for malformations (X^2=0.0.605; p=0.8057 (Table 2).

Table 1. Fit to the Model for Drinking Water (Chlorinated Surface Water) Exposures Using FETAX, Without MAS, for Estimated LC50 and EC50 and Their Associated Estimated 95 Percent Fiducial Limits: Test 234-004

Model	Statistic	
	X^{2a}	p
LC50		
Arithmetic Scale	254.000	0.0000
Log_{10} transformed	254.000	0.0000
EC50		
Arithmetic Scale	8.7123	0.3671
Log_{10} transformed	9.7672	0.2817

$\alpha = 0.10$

[a] Pearson's Chi Square

Table 2. Effects for Drinking Water (Chlorinated Surface Water) Exposures, Without MAS Using FETAX for Estimates: Tests 234-004

Test	Effect					
	Intercept			Drinking Water Concentration		
	Estimate	X^{2a}	p	Estimate	X^{2a}	p
LC50	***	***	***	***	***	***
EC50	-1.6455979	22.1109	0.0001	0.03023203	0.0605	0.8057

$\alpha = 0.05$

[a] Wald's Chi Square

*** Could not be estimated (No mortality reported)

Table 3. Estimated LC50 and EC50 and Their Associated Estimated 95 Percent Fiducial Limits for Drinking Water (Chlorinated Surface Water) Exposures using FETAX, Without MAS: Test 234-004

Parameter	Estimate	Estimate 95 Percent Fiducial Limits	
		Lower	Upper
LC50[a]	***	***	***
EC50	54.432	***	***

*** Could not be estimated

[a] No mortality reported

March 12, 1999

MEMORANDUM FOR: Dave Kumsher/Maggie Toussaint

FROM: Florence J. Hoffmann
Mathematical Statistician

SUBJECT: Data Analyses for Estimating the LC50 and the EC50 for Drinking Water (Total Trihalomethanes in Chlorinated Surface Water) Exposures Using FETAX, Without MAS: Test 234-005

STATISTICAL METHODS

Probit analysis was used to estimate the LC50 and the EC50 and associated estimated 95 percent fiducial limits. Pearson's chi square was used to test for fit to the model. Abbott's formula was applied to optimize the estimation of the natural threshold response rate. Wald's chi square was used to indicate effects. SAS PROC PROBIT statistical computer software was used for these analyses.

STATISTICAL RESULTS

Neither the LC50 nor the EC50 and their associated estimated 95 percent fiducial limits could be estimated since concentrations of total trihalomethanes in chlorinated surface water were not high enough to kill half of the embryos or elicit a malformation response in half of the embryos.

February 12, 1999

MEMORANDUM FOR: Dave Kumsher/Maggie Toussaint

FROM: Florence J. Hoffmann
Mathematical Statistician

SUBJECT: Data Analyses for Estimating the LC50 and the EC50 for Drinking Water (Total Trihalomethanes in Chlorinated Surface Water) Exposures Using FETAX, With MAS: Test 234-003

STATISTICAL METHODS

Probit analysis was used to estimate the LC50 and the EC50 and associated estimated 95 percent fiducial limits. Pearson's chi square was used to test for fit to the model. Abbott's formula was applied to optimize the estimation of the natural threshold response rate. Wald's chi square was used to indicate effects. SAS PROC PROBIT statistical computer software was used for these analyses.

STATISTICAL RESULTS

The best-fitting model for estimating the LC50 and the EC50 used drinking water concentrations on the arithmetic scale (Table 1). The LC50 was estimated to be 0.9722 mg/l drinking water (total trihalomethanes in chlorinated surface water). The 95 percent fiducial limits could not be estimated for the LC50. There was no drinking water effect for mortality (X^2=1.3404; p=0.2470). The EC50 was not estimated because the drinking water concentrations were not high enough to elicit a malformation response in half of the embryos.

Table 1. Fit to the Model, for Drinking Water (Chlorinated Surface Water) Exposures, Using FETAX, With MAS, for Estimated LC50 and EC50 and Their Associated Estimated 95 Percent Fiducial Limits: Test 234-003

Model	Statistic	
	X^{2a}	p
LC50		
Arithmetic Scale	49.9992	0.0000
Log_{10}	64.7920	0.0000
EC50		
Arithmetic Scale	12.6540	0.0489
Log_{10}	23.4094	0.0007

$\alpha = 0.10$

[a] Pearson's Chi Square

Table 2. Estimated LC50 and EC50 and Their Associated Estimated 95 Percent Fiducial Limits, for Drinking Water (Chlorinated Surface Water) Exposures, With MAS Using FETAX: Test 234-003

Parameter	Estimate (mg/L)	Estimated 95 Percent Fiducial Limits	
		Lower	Upper
LC50	0.9722	***	***
EC50	*	*	*

* Extrapolation beyond data. Drinking water concentrations not high enough to elicit malformations in half of the embryos

*** Could not be estimated

March 11, 1999

MEMORANDUM FOR: Dave Kumsher/Maggie Toussaint

FROM: Florence J. Hoffmann
Mathematical Statistician

SUBJECT: Data Analyses for Estimating the LC50 and the EC50 for Drinking Water (Total Trihalomethanes in Chlorinated Surface Water) Exposures Using FETAX, With MAS: Test 234-004

STATISTICAL METHODS

Probit analysis was used to estimate the LC50 and the EC50 and associated estimated 95 percent fiducial limits. Pearson's chi square was used to test for fit to the model. Abbott's formula was applied to optimize the estimation of the natural threshold response rate. Wald's chi square was used to indicate effects. SAS PROC PROBIT statistical computer software was used for these analyses.

STATISTICAL RESULTS

The best-fitting model for estimating the LC50 and the EC50 used drinking water concentrations on the arithmetic scale (Table 1). The LC50 was estimated to be 0.784 mg/l drinking water (total trihalomethanes in chlorinated surface water). The 95 percent fiducial limits for the LC50 could not be estimated. The EC50 was estimated to be 3.338 mg/l drinking water (total trihalomethanes in chlorinated surface water). The 95 percent fiducial limits could not be estimated for the EC50 (Table 3). The effect for mortality was marginally non-significant (X^2=3.5558; p=0.0593). There was no drinking water effect for malformations (X^2=0.9462; p=0.3307 (Table 2).

Table 1. Fit to the Model for Drinking Water (Chlorinated Surface Water) Exposures Using FETAX, With MAS, for Estimated LC50 and EC50 and Their Associated Estimated 95 Percent Fiducial Limits: Test 234-004

Model	Statistic	
	X^{2a}	p
LC50		
Arithmetic Scale	52.1555	0.0000
Log_{10} transformed	70.5734	0.0000
EC50		
Arithmetic Scale	5.6310	0.0599
Log_{10} transformed	8.7878	0.0124

$\alpha = 0.10$

[a] Pearson's Chi Square

Table 2. Effects for Drinking Water (Chlorinated Surface Water) Exposures, With MAS Using FETAX for LC50 and EC50 Estimates: Tests 234-004

Test	Effect					
	Intercept			Drinking Water Concentration		
	Estimate	X^{2a}	p	Estimate	X^{2a}	p
LC50	-0.7320668	1.0816	0.2983	0.93352009	3.5558	0.0593
EC50	-1.4506576	8.2282	0.0041	0.43465551	0.9462	0.3307

$\alpha = 0.05$

[a] Wald's Chi Square

Table 3. Estimated LC50 and EC50 and Their Associated Estimated 95 Percent Fiducial Limits for Drinking Water (Chlorinated Surface Water) Exposures using FETAX, With MAS: Test 234-004

Parameter	Estimate	Estimate 95 Percent Fiducial Limits	
		Lower	Upper
LC50	0.784	***	***
EC50	3.338	***	***

*** Could not be estimated

March 12, 1999

MEMORANDUM FOR: Dave Kumsher/Maggie Toussaint

FROM: Florence J. Hoffmann
Mathematical Statistician

SUBJECT: Data Analyses for Estimating the LC50 and the EC50 for Drinking Water (Total Trihalomethanes in Chlorinated Surface Water) Exposures Using FETAX, With MAS: Test 234-005

STATISTICAL METHODS

Probit analysis was used to estimate the LC50 and the EC50 and associated estimated 95 percent fiducial limits. Pearson's chi square was used to test for fit to the model. Abbott's formula was applied to optimize the estimation of the natural threshold response rate. Wald's chi square was used to indicate effects. SAS PROC PROBIT statistical computer software was used for these analyses.

STATISTICAL RESULTS

The best-fitting model for estimating the LC50 used a log10 transformation of the total trihalomethanes in chlorinated surface water. The best-fitting model for estimating the EC50 used total trihalomethanes in chlorinated surface water on the arithmetic scale (Table 1). There was a significant effect for total trihalomethanes in chlorinated surface water for mortality but not for malformations (Table 2). The LC50 was estimated to be 1.113 mg/l total trihalomethanes in chlorinated surface water. The EC50 was estimated to be 1.865 mg/l total trihalomethanes in chlorinated surface water. The 95 percent fiducial limits could not be estimated for either the LC50 or the EC50 (Table 3).

Table 1. Fit to the Model for Drinking Water (Chlorinated Surface Water) Exposures With MAS, Using FETAX for Estimated LC50, EC50, and Their Associated Estimated 95 Percent Fiducial Limits: Test 234-005

Model	Statistic	
	X^{2a}	p
LC50		
Arithmetic Scale	83.0327	0.0000
Log_{10} transformed	45.7889	0.0000
EC50		
Arithmetic Scale	3.7581	0.4397
Log_{10} transformed	7.9698	0.0927

$\alpha = 0.10$

[a] = Pearson's Chi Square

Table 2. Effects for Drinking Water (Chlorinated Surface Water) Exposures Using FETAX, With MAS, For Estimated LC50 and EC50: Test 234-005

Parameter	Effect					
	Intercept			Drinking Water Concentration		
	Estimate	X^{2a}	p	Estimate	X^{2a}	p
LC50	-0.2753338	0.3989	0.5277	5.9208553	4.9180	0.0266
EC50	-12.565326	8.807E-8	0.9998	6.73911452	4.525E-8	0.9998

$\alpha = 0.05$

[a] Wald's Chi Square

Table 3. Estimated LC50, EC50, and Their Associated Estimated 95 Percent Fiducial Limits, for Drinking Water (Chlorinated Surface Water) Exposures, With MAS Using FETAX: Test 234-005

Parameter	Estimate (mg/L)	Estimated 95 Percent Fiducial Limits	
		Lower	Upper
LC50	1.113	***	***
EC50	1.865	***	***

*** Could not be estimated

February 16, 1999

MEMORANDUM FOR: Dave Kumsher/Maggie Toussaint

FROM: Florence J. Hoffmann
Mathematical Statistician

SUBJECT: Data Analyses for Length Measurements for Drinking Water (Total Trihalomethanes in Chlorinated Surface Water) Exposures, Using FETAX, Without MAS, for Test 234-003

STATISTICAL METHODS

Analysis of variance (ANOVA) was used to test for fit to the model and for effects. Regression analysis was used for point estimation. SAS statistical software was used for these analyses.

STATISTICAL RESULTS

Analysis of variance indicated that the best-fitting model was a linear model (Tables 1 and 2). The equation for providing the estimated values is:

$$y = 9.714469051 - 0.323995257x$$

While there was a significant drinking water concentration/response effect ($F=6.35$; $p=0.0130$), differences between concentration groups and control could not be detected (Tables 2 and 3). Therefore, neither a NOEL nor a MCIG could be estimated.

Table 1. Fit to the Model for Drinking Water (Chlorinated Surface Water) Exposures, Without MAS, Using FETAX, for Length: Test 234-003

Model	Statistic			
	F	p	R^2	cy
Linear	6.35	0.0130	0.0480	7.26
Quadratic	3.17	0.0453	0.0483	7.29
Loglinear	6.33	0.0131	0.0478	7.26

$\alpha = 0.05$

Table 2. Effects, by Model, for Drinking Water (Chlorinated Surface Water) Exposures, Without MAS, Using FETAX, for Length: Test 234-003

Model Effect	Statistic	
	F	p
Linear		
Concentration	6.35	0.0130
Quadratic		
Concentration	0.04	0.8358
Concentration^2	0.04	0.8417
Loglinear		
Concentration	6.33	0.0131

$\alpha = 0.05$

Table 3. Observed Means, Estimated Mean and Their Associated Estimated 95 Percent Confidence Limits, for Drinking Water (Chlorinated Surface Water) Exposures, Without MAS, Using FETAX, for Length: Test 234-003

Drinking Water Concentration[a] (mg/L)	Observed Mean (mm)	n	Estimated Mean (mm)	Estimated 95 Percent Confidence Limits	
				Lower	Upper
0.000	9.71	98	9.71	9.57	9.85
1.132	9.32	15	9.35	9.10	9.60
1.138	9.37	15	9.36	9.09	9.60

[a] Mean Measured Drinking Water Concentrations

$\hat{y} = 9.714469051 - 0.323995257x$

March 12, 1999

MEMORANDUM FOR: Dave Kumsher/Maggie Toussaint

FROM: Florence J. Hoffmann
Mathematical Statistician

SUBJECT: Data Analyses for Length Measurements for Drinking Water (Total Trihalomethanes in Chlorinated Surface Water) Exposures, Using FETAX, Without MAS, for Test 234-004

STATISTICAL METHODS

Analysis of variance (ANOVA) was used to test for fit to the model and for effects. Regression analysis was used for point estimation. SAS statistical software was used for these analyses.

STATISTICAL RESULTS

Analysis of variance indicated that the best-fitting model was a linear model (Tables 1 and 2). The equation for providing the estimated values is:

$$y = 9.321126247 - 0.286862585x$$

There was a significant drinking water concentration/response effect ($F=84.17$; $p=0.0001$) (Table 2). The NOEL was estimated to be 0.162 (<0, 0.421) mg/l total trihalomethanes in chlorinated surface water. The MCIG was estimated to be 0.931 (0.698, 1.164) mg/l total trihalomethanes in chlorinated surface water (Table 4).

Table 1. Fit to the Model for Drinking Water (Chlorinated Surface Water) Exposures, Without MAS, Using FETAX, for Length: Test 234-004

Model	Statistic			
	F	p	R^2	cy
Linear	84.17	0.0001	0.2504	5.82
Quadratic	42.14	0.0001	0.2514	5.83
Loglinear	25.57	0.0001	0.0921	6.41

$\alpha = 0.05$

Table 2. Effects for Drinking Water (Chlorinated Surface Water) Exposures, Without MAS, Using FETAX, for Length: Test 234-004

Model Effect	Statistic	
	F	p
Linear		
Concentration	84.17	0.0001
Quadratic		
Concentration	9.12	0.0028
Concentration^2	0.33	0.5678
Loglinear		
Concentration	25.57	0.0001

$\alpha = 0.05$

Table 3. Observed Means, Estimated Mean and Their Associated Estimated 95 Percent Confidence Limits, for Drinking Water (Chlorinated Surface Water) Exposures, Without MAS, Using FETAX, for Length: Test 234-004

Drinking Water Concentration[a] (mg/L)	Observed Mean (mm)	n	Estimated Mean (mm)	Estimated 95 Percent Confidence Limits	
				Lower	Upper
0.000	9.34	99	9.32	9.24	9.40 A
0.048	9.48	15	9.31	9.23	9.39 A
0.049	9.40	15	9.31	9.23	9.38 A
0.157	9.11	20	9.28	9.20	9.35 A
0.162	9.12	15	9.27	9.20	9.35 A
0.931	8.98	15	9.05	8.99	9.12 B
0.940	9.17	15	9.05	8.98	9.12 B
1.866	8.78	15	8.79	8.69	8.88 C
1.876	8.69	15	8.78	8.69	8.88 C
3.102	8.62	15	8.43	8.27	8.59 D
3.112	8.30	15	8.43	8.27	8.59 D

[a] Mean Measured Drinking Water Concentrations

Table 4. Estimated NOEL and MCIG and Their Associated Estimated 95 Percent Confidence Limits, for Drinking Water (Chlorinated Surface Water) Exposures, Without MAS Using FETAX, for Length: Test 234-004

Parameter	Estimate (mg/L)	Estimated 95 Percent Fiducial Limits	
		Lower	Upper
NOEL	0.162	< 0	0.421
MCIG	0.931	0.698	1.164

$\hat{y} = 9.321126247 - 0.286862585x$

March 12, 1999

MEMORANDUM FOR: Dave Kumsher/Maggie Toussaint

FROM: Florence J. Hoffmann
Mathematical Statistician

SUBJECT: Data Analyses for Estimating the LC50 and the EC50 for Drinking Water (Total Trihalomethanes in Chlorinated Surface Water) Exposures Using FETAX, With MAS: Test 234-005

STATISTICAL METHODS

Probit analysis was used to estimate the LC50 and the EC50 and associated estimated 95 percent fiducial limits. Pearson's chi square was used to test for fit to the model. Abbott's formula was applied to optimize the estimation of the natural threshold response rate. Wald's chi square was used to indicate effects. SAS PROC PROBIT statistical computer software was used for these analyses.

STATISTICAL RESULTS

The best-fitting model for estimating the LC50 used a log10 transformation of the total trihalomethanes in chlorinated surface water. The best-fitting model for estimating the EC50 used total trihalomethanes in chlorinated surface water on the arithmetic scale (Table 1). There was a significant effect for total trihalomethanes in chlorinated surface water for mortality but not for malformations (Table 2). The LC50 was estimated to be 1.113 mg/l total trihalomethanes in chlorinated surface water. The EC50 was estimated to be 1.865 mg/l total trihalomethanes in chlorinated surface water. The 95 percent fiducial limits could not be estimated for either the LC50 or the EC50 (Table 3).

Table 1. Fit to the Model for Drinking Water (Chlorinated Surface Water) Exposures With MAS, Using FETAX for Estimated LC50, EC50, and Their Associated Estimated 95 Percent Fiducial Limits: Test 234-005

Model	Statistic	
	X^{2a}	p
LC50		
Arithmetic Scale	83.0327	0.0000
Log_{10} transformed	45.7889	0.0000
EC50		
Arithmetic Scale	3.7581	0.4397
Log_{10} transformed	7.9698	0.0927

$\alpha = 0.10$

[a] = Pearson's Chi Square

Table 2. Effects for Drinking Water (Chlorinated Surface Water) Exposures Using FETAX, With MAS, For Estimated LC50 and EC50: Test 234-005

Parameter	Effect					
	Intercept			Drinking Water Concentration		
	Estimate	X^{2a}	p	Estimate	X^{2a}	p
LC50	-0.2753338	0.3989	0.5277	5.9208553	4.9180	0.0266
EC50	-12.565326	8.807E-8	0.9998	6.73911452	4.525E-8	0.9998

$\alpha = 0.05$

[a] Wald's Chi Square

Table 3. Estimated LC50, EC50, and Their Associated Estimated 95 Percent Fiducial Limits, for Drinking Water (Chlorinated Surface Water) Exposures, With MAS Using FETAX: Test 234-005

Parameter	Estimate (mg/L)	Estimated 95 Percent Fiducial Limits	
		Lower	Upper
LC50	1.113	***	***
EC50	1.865	***	***

*** Could not be estimated.

February 17, 1999

MEMORANDUM FOR: Dave Kumsher/Maggie Toussaint

FROM: Florence J. Hoffmann
Mathematical Statistician

SUBJECT: Data Analyses for Length Measurements for Drinking Water (Total Trihalomethanes in Chlorinated Surface Water) Exposures, Using FETAX, With MAS, for Test 234-003

STATISTICAL METHODS

Analysis of variance (ANOVA) was used to test for fit to the model and for effects. Regression analysis was used for point estimation. SAS statistical software was used for these analyses.

STATISTICAL RESULTS

Analysis of variance indicated that the best-fitting model was a quadratic model (Tables 1 and 2). The equation for providing the estimated values is:

$$y = 9.334580134 - 3.277014981x + 2.007632475x^2$$

The estimated NOEL was 0.057 (0.023, 0.093) mg/l and the estimated MCIG was 0.278 (0.219, 0.345) mg/l drinking water (total trihalomethanes in chlorinated surface water) (Tables 3 and 4).

Table 1. Fit to the Model for Drinking Water (Chlorinated Surface Water) Exposures, With MAS, Using FETAX, for Length: Test 234-003

Model	Statistic			
	F	p	R^2	cy
Linear	89.71	0.0001	0.3888	6.46
Quadratic	59.16	0.0001	0.4580	6.10
Loglinear	9.09	0.0031	0.0606	8.01

$\alpha = 0.05$

Table 2. Effects, by Model, for Drinking Water (Chlorinated Surface) Exposures, With MAS, Using FETAX, for Length: Test 234-003

Model Effect	Statistic	
	F	p
Linear		
Concentration	89.71	0.0001
Quadratic		
Concentration	51.26	0.0001
Concentration^2	17.88	0.0001
Loglinear		
Concentration	9.09	0.0031

$\alpha = 0.05$

Table 3. Observed Means, Estimated Mean and Their Associated Estimated 95 Percent Confidence Limits, for Drinking Water (Chlorinated Surface Water) Exposures, With MAS, Using FETAX, for Length: Test 234-003

Drinking Water Concentration[a] (mg/L)	Observed Mean (mm)	n	Estimated Mean (mm)	Estimated 95 Percent Confidence Limits	
				Lower	Upper
0.000	9.30	30	9.33	9.20	9.47 A
0.026	8.83	19	9.25	9.13	9.37 A
0.057	9.38	30	9.15	9.05	9.26 A
0.278	9.00	15	8.58	8.44	8.71 B
0.280	8.61	15	8.57	8.44	8.71 B
0.580	7.86	10	8.11	7.93	8.28 C
0.589	7.77	11	8.10	7.93	8.28 C
1.007	7.61	1	8.07	7.79	8.34 C
1.024	8.24	12	8.08	7.79	8.37 C

[a] Mean Measured Drinking Water Concentrations

Table 4. Estimated NOEL and MCIG and Their Associated Estimated 95 Percent Confidence Limits for Drinking Water (Chlorinated Surface Water) Exposures, With MAS, Using FETAX, for Length: Tests 234-003

Parameter	Estimate		
	Estimate (mg/L)	95 Percent Confidence Limits	
		Lower	Upper
NOEL	0.057	0.023	0.093
MCIG	0.278	0.219	0.345

$\hat{y} = 9.334580134 - 3.277014981x + 2.007632475x^2$

March 12, 1999

MEMORANDUM FOR: Dave Kumsher/Maggie Toussaint

FROM: Florence J. Hoffmann
Mathematical Statistician

SUBJECT: Data Analyses for Length Measurements for Drinking Water (Total Trihalomethanes in Chlorinated Surface Water) Exposures, Using FETAX, With MAS, for Test 234-004

STATISTICAL METHODS

Analysis of variance (ANOVA) was used to test for fit to the model and for effects. Regression analysis was used for point estimation. SAS statistical software was used for these analyses.

STATISTICAL RESULTS

Analysis of variance indicated that the best-fitting model was a linear model (Tables 1 and 2). The equation for providing the estimated values is:

$$y = 8.131928013 - 0.570911970x$$

There was a significant drinking water concentration/response effect ($F=33.46$; $p=0.0001$) (Table 2). The NOEL was estimated to be 0.136 (<0, 0.343) mg/l total trihalomethanes in chlorinated surface water. The MCIG was estimated to be 0.821 (0.556, 1.086) mg/l total trihalomethanes in chlorinated surface water (Table 4).

Table 1. Fit to the Model for Drinking Water (Chlorinated Surface Water) Exposures, With MAS, Using FETAX, for Length: Test 234-004

Model	Statistic			
	F	p	R^2	cy
Linear	33.46	0.0001	0.2585	7.12
Quadratic	18.70	0.0001	0.2824	7.04
Loglinear	5.40	0.0223	0.0532	8.04

$\alpha = 0.05$

Table 2. Effects, by Model, for Drinking Water (Chlorinated Surface Water) Exposures, With MAS, Using FETAX, for Length: Test 234-004

Model Effect	Statistic	
	F	p
Linear		
Concentration	33.46	0.0001
Quadratic		
Concentration	6.30	0.0137
Concentration^2	3.17	0.0780
Loglinear		
Concentration	5.40	0.0223

$\alpha = 0.05$

Table 3. Observed Means, Estimated Mean and Their Associated Estimated 95 Percent Confidence Limits, for Drinking Water (Chlorinated Surface Water) Exposures, With MAS, Using FETAX, for Length: Test 234-004

Drinking Water Concentration[a] (mg/L)	Observed Mean (mm)	n	Estimated Mean (mm)	Estimated 95 Percent Confidence Limits	
				Lower	Upper
0.000	8.21	28	8.13	8.00	8.26 AA
0.032	8.18	24	8.11	7.99	8.24 AA
0.136	7.93	30	8.05	7.94	8.17 AA
0.821	7.26	2	7.66	7.51	7.81 BB
1.697	7.21	14	7.16	6.87	7.46 CC

[a] Mean Measured Drinking Water Concentrations

Table 4. Estimated NOEL and MCIG and Their Associated Estimated 95 Percent Confidence Limits, for Drinking Water (Chlorinated Surface Water) Exposures, With MAS Using FETAX, for Length: Test 234-004

Parameter	Estimate (mg/L)	Estimated 95 Percent Fiducial Limits	
		Lower	Upper
NOEL	0.136	< 0	0.343
MCIG	0.821	0.556	1.086

$\hat{y}$ = 8.131928013 – 0.570911970x

March 24, 1999

MEMORANDUM FOR: Dave Kumsher/Maggie Toussaint

FROM: Florence J. Hoffmann
Mathematical Statistician

SUBJECT: Data Analyses for Length Measurements for Drinking Water (Total Trihalomethanes in Chlorinated Surface Water) Exposures, Using FETAX, With MAS, for Test 234-005

STATISTICAL METHODS

Analysis of variance (ANOVA) was used to test for fit to the model and for effects. Regression analysis was used for point estimation. SAS statistical software was used for these analyses.

STATISTICAL RESULTS

Analysis of variance indicated that the best-fitting model was a linear model (Tables 1 and 2). The equation for providing the estimated values is:

$$y = 8.844722047 - 0.531981945x$$

There was a significant drinking water concentration/response effect ($F=10.87$; $p=0.0013$) (Table 2). The NOEL was estimated to be 0.137 (<0, 0.372) mg/l total trihalomethanes in chlorinated surface water. The MCIG was estimated to be 0.838 (0.389, 1.287) mg/l total trihalomethanes in chlorinated surface water (Table 4).

Table 1. Fit to the Model for Drinking Water (Chlorinated Surface Water) Exposures, With MAS, Using FETAX, for Length: Test 234-005

Model	Statistic			
	F	p	R^2	cy
Linear	10.87	0.0013	0.0972	7.22
Quadratic	5.38	0.0060	0.0972	7.26
Loglinear	7.62	0.0069	0.0702	7.33

$\alpha = 0.05$

Table 2. Effects, by Model, for Drinking Water (Chlorinated Surface) Exposures, With MAS, Using FETAX, for Length: Test 234-005

Model Effect	Statistic	
	F	p
Linear		
Concentration	10.87	0.0013
Quadratic		
Concentration	2.50	0.1172
Concentration^2	0.00	0.9501
Loglinear		
Concentration	7.62	0.0069

$\alpha = 0.05$

Table 3. Observed Means, Estimated Mean and Their Associated Estimated 95 Percent Confidence Limits, for Drinking Water (Chlorinated Surface Water) Exposures, With MAS, Using FETAX, for Length: Test 234-005

Drinking Water Concentration[a] (mg/L)	Observed Mean (mm)	n	Estimated Mean (mm)	Estimated 95 Percent Confidence Limits	
				Lower	Upper
0.000	8.79	29	8.84	8.71	8.98 A
0.032	9.22	13	8.83	8.69	8.96 A
0.033	9.12	15	8.83	8.69	8.96 A
0.136	8.59	15	8.77	8.65	8.90 A
0.137	8.34	15	8.77	8.65	8.90 A
0.838	8.51	15	8.40	8.16	8.64 B
2.869	7.18	1	7.32	6.45	8.18 B

[a] Mean Measured Drinking Water Concentrations

Table 3. Observed Means, Estimated Mean and Their Associated Estimated 95 Percent Confidence Limits, for Drinking Water (Chlorinated Surface Water) Exposures, With MAS, Using FETAX, for Length: Test 234-005

Drinking Water Concentration[a] (mg/L)	Observed Mean (mm)	n	Estimated Mean (mm)	Estimated 95 Percent Confidence Limits	
				Lower	Upper
0.000	8.79	29	8.84	8.71	8.98 A
0.032	9.22	13	8.83	8.69	8.96 A
0.033	9.12	15	8.83	8.69	8.96 A
0.136	8.59	15	8.77	8.65	8.90 A
0.137	8.34	15	8.77	8.65	8.90 A
0.838	8.51	15	8.40	8.16	8.64 B
2.869	7.18	1	7.32	6.45	8.18 B

[a] Mean Measured Drinking Water Concentrations

Table 4. Estimated NOEL and MCIG and Their Associated Estimated 95 Percent Confidence Limits, for Drinking Water (Chlorinated Surface Water) Exposures, With MAS Using FETAX, for Length: Test 234-005

Parameter	Estimate (mg/L)	Estimated 95 Percent Fiducial Limits	
		Lower	Upper
NOEL	0.137	< 0	0.372
MCIG	0.838	0.389	1.287

$\hat{y}$ = 8.844722047 – 0.531981945x

APPENDIX III

Ames Test Reports

FINAL REPORT

Study Title

***Salmonella* Plate Incorporation Mutagenicity Assay Using Water Samples**

Test Article

Water Sample 99-020-1

Authors

Valentine O. Wagner, III, M.S.
Sean M. Caruthers, B.S.

Study Completion Date

March 26, 1999

Performing Laboratory

BioReliance
9630 Medical Center Drive
Rockville, MD 20850

Laboratory Study Number

AA12BR.501006.BTL

Sponsor

US Army
Center for Enviromental Health Research
568 Doughten Drive
Fort Detrick, MD 21702-5010

STATEMENT OF COMPLIANCE

Study No. AA12BR.501006.BTL was conducted in compliance with the U.S. FDA Good Laboratory Practice Regulations as published in 21 CFR 58, the U.S. EPA GLP Standards 40 CFR 792 and 40 CFR 160, the UK GLP Compliance Programme, the Japanese GLP Standard and the OECD Principles of Good Laboratory Practice in all material aspects with the following exceptions:

> The identity, strength, purity and composition or other characteristics to define the test or control article have not been determined by the testing facility.
>
> Analyses to determine the uniformity, concentration, or stability of the test or control mixtures were not performed by the testing facility.
>
> The stability of the test or control article under the test conditions has not been determined by the testing facility.

Valentine O. Wagner, III
Valentine O. Wagner, III, M.S.
Study Director

26-Mar-1999
Date

QUALITY ASSURANCE STATEMENT

Study Title: *SALMONELLA* PLATE INCQRPORATION MUTAGENICITY ASSAY USING WATER SAMPLES

Study Number: AA12BR.501006.BTL

Study Director: Valentine O. Wagner, III, M.S.

This study has been divided into a series of in-process phases. Using a random sampling approach, Quality Assurance monitors each of these phases over a series of studies. Procedures, documentation, equipment records, etc., are examined in order to assure that the study is performed in accordance with the U.S. FDA Good Laboratory Practice Regulations (21 CFR 58), the U.S. EPA GLPs (40 CFR 792 and 40 CFR 160), the UK GLP Regulations, the Japanese GLP Standard, and the OECD Principles of Good Laboratory Practice and to assure that the study is conducted according to the protocol and relevant Standard Operating Procedures.

The following are the inspection dates, phases inspected, and report dates of QA inspections of this study.

INSPECT ON 25 JAN 99, TO STUDY DIR 25 JAN 99, TO MGMT 25 JAN 99
PHASE: Protocol Review

INSPECT ON 27 JAN 99, TO STUDY DIR 27 JAN 99, TO MGMT 27 JAN 99
PHASE: Preparation of S9 mixture

INSPECT ON 05 MAR 99, TO STUDY DIR 05 MAR 99, TO MGMT 10 MAR 99
PHASE: Draft Report

INSPECT ON 29 MAR 99, TO STUDY DIR 29 MAR 99, TO MGMT 29 MAR 99
PHASE: Draft to Final Report

This report describes the methods and procedures used in the study and the reported results accurately reflect the raw data of the study.

Jennifer Klopsis — 3/29/99

Jennifer Klopsis
QUALITY ASSURANCE — DATE

Salmonella Plate Incorporation Mutagenicity Assay Using Water Samples

FINAL REPORT

Sponsor:	**US Army** **Center for Environmental Health Research** **568 Doughten Drive** **Fort Detrick, MD 21702-5010**
Authorized Representative:	**Maggie Toussaint** **Geo Centers**
Performing Laboratory:	**BioReliance** **9630 Medical Center Drive** **Rockville, Maryland 20850**
Test Article I.D.:	**Water Sample 99-020-1**
BioReliance Study No.:	**AA12BR.501006.BTL**
Test Article Description:	**clear, colorless water**
Storage Conditions:	**room temperature; protected from exposure to light**
Test Article Receipt:	**January 20, 1999**
Study Initiation:	**January 22, 1999**
Study Director:	Valentine O. Wagner, III 26-Mar-1999 Valentine O. Wagner, III, M.S. Date

TABLE OF CONTENTS

SUMMARY

The test article, Water Sample 99-020-1, was tested in the *Salmonella* Plate Incorporation Mutagenicity Assay Using Water Samples with tester strains TA98 and TA100 in the presence and absence of Aroclor-induced rat liver S9. The assay was performed using the plate incorporation method. The mutagenicity assay was used to evaluate the mutagenic potential of the test article.

Water was selected as the solvent of choice based on the Sponsor's request and compatibility with the target cells.

The maximum dose level tested in the mutagenicity assay was 2.0 mL of undiluted test article per plate. Subsequent dose levels were prepared by diluting the test article in deionized water. These dilutions were soluble in water at 0.75 mL/mL, the most concentrated dilution prepared.

In the mutagenicity assay no positive response was observed. Neither precipitate nor appreciable toxicity was observed. However, a reduced revertant count was present at the top doses of tester strain TA100 in the presence of S9 activation due to the presence of contaminating colonies on the plates. The overall evaluation and dose ranges tested are as follows:

S9 Activation	Overall Evaluation[a] and Dose Range Tested (mL/plate)			
	TA98		TA100	
	Low	High	Low	High
None	-		-	
	0.1	2.0	0.1	2.0
Rat	-		-	
	0.1	2.0	0.1	2.0

[a]-=negative, +=positive (maximum fold increase)

In conclusion, the results indicate that under the conditions of this study, test article **Water Sample 99-020-1** was concluded to be negative the *Salmonella* Plate Incorporation Mutagenicity Assay Using Water Samples.

PURPOSE

The purpose of this study was to evaluate the mutagenic potential of the test article (or its metabolites) by measuring its ability to induce reverse mutations at selected loci of two strains of *Salmonella typhimurium* in the presence and absence of S9 activation.

CHARACTERIZATION OF TEST AND CONTROL ARTICLES

The test article, Water Sample 99-020-1, was received by BioReliance on 01/20/99 and was assigned the code number AA12BR. The test article was characterized by the Sponsor as chlorinated drinking water. An expiration date of 1/27/99 was provided. Upon receipt, the test article was described as clear, colorless water and was stored at room temperature, protected from exposure to light.

The vehicle used to deliver the test article to the test system was sterile distilled water, (CAS# 7732-18-5), obtained from Life Technologies, Inc.

Positive controls plated concurrently with the mutagenicity assay are listed below:

Strain	S9 Activation	Positive Control	Concentration (µg/plate)
TA98, TA100	+	2-aminoanthracene (Sigma Chemical Co.)	1.0
TA98	-	2-nitrofluorene (Aldrich Chemical Co., Inc.)	2.0[a]
TA100	-	sodium azide (Sigma Chemical Co.)	2.0[a]

[a] In a deviation from the protocol, the concentration used in the assay was 1.0 µg/plate. Since an acceptable positive response was obtained, which indicated that the test system was capable of detecting a known mutagen, the Study Director has accepted these data.

To determine the sterility of the test articles, the highest test article dose level used in the mutagenicity assay was plated on selective agar with an aliquot volume equal to that used in the assay.

MATERIALS AND METHODS

Test System

The tester strains used were the *Salmonella typhimurium* histidine auxotrophs TA98 and TA100 as described by Ames *et al.* (1975). *Salmonella* tester strains were received on 08/11/98 and 11/10/98 directly from Dr. Bruce Ames, University of California, Berkeley.

Tester strain TA98 is reverted from histidine dependence (auxotrophy) to histidine independence (prototrophy) by frameshift mutagens. Tester strain TA100 is reverted by mutagens that cause both frameshift and basepair substitution mutations.

Overnight cultures were prepared by inoculating from the appropriate master plate or from the appropriate frozen permanent stock into a vessel containing ~50 mL of culture medium. To assure that cultures were harvested in late log phase, the length of incubation was controlled and monitored. Following inoculation, each flask was placed in a resting shaker/incubator at room temperature. The shaker/incubator was programmed to begin shaking at approximately 125 rpm at 37±2°C approximately 12 hours before the anticipated time of harvest. Each culture was monitored spectrophotometrically for turbidity and was harvested at a percent transmittance yielding a titer of approximately 10^9 cells per milliliter. The actual titers were determined by viable count assays on nutrient agar plates.

Metabolic Activation System

Aroclor 1254-induced rat liver S9 was used as the metabolic activation system. The S9 was prepared from male Sprague-Dawley rats induced with a single intraperitoneal injection of Aroclor 1254, 500 mg/kg, five days prior to sacrifice. The S9 was batch prepared on 12/16/98 and stored at ≤-70°C until used. Each bulk preparation of S9 was assayed for its ability to metabolize 2-aminoanthracene and 7,12-dimethylbenz(a)anthracene to forms mutagenic to *Salmonella typhimurium* TA100.

The S9 mix was prepared immediately before its use and contained 10% S9, 5 mM glucose-6-phosphate, 4 mM β-nicotinamide-adenine dinucleotide phosphate, 8 mM $MgCl_2$ and 33 mM KCl in a 100 mM phosphate buffer at pH 7.4. The Sham S9 mixture (Sham mix), containing 100 mM phosphate buffer at pH 7.4, was prepared immediately before its use. To confirm the sterility of the S9 and Sham mixes, a 0.5 mL aliquot of each was plated on selective agar.

Mutagenicity Assay

The mutagenicity assay was used to evaluate the mutagenic potential of the test article. A minimum of six dose levels of test article along with appropriate vehicle and positive controls were plated with tester strains TA98 and TA100 in the presence and absence of rat liver S9 activation. All dose levels of test article, vehicle controls and positive controls were plated in duplicate.

Plating and Scoring Procedures

The test system was exposed to the test article via the plate incorporation methodology originally described by Ames *et al.* (1975) and updated Maron and Ames (1983).

In a deviation from the protocol, the sample was not filter sterilized prior to use. However, in consultation with the Sponsor, it was determined that filtration was inappropriate because of volatile components diluted in the water. Any contaminant colonies observed on the assay plates were excluded from the revertant count. Therefore, the Study Director has accepted these data.

On the day of its use, minimal top agar, containing 3.4% agar (W/V) and 5.4% NaCl (W/V), was melted and supplemented with L-histidine, D-biotin and L-tryptophan solution to a final concentration of 179 μM each. Bottom agar was Vogel-Bonner minimal medium E (Vogel and Bonner, 1956) containing 1.5 % (W/V) agar. Nutrient bottom agar was Vogel-Bonner minimal medium E containing 1.5 % (W/V) agar and supplemented with 2.5 % (W/V) Oxoid Nutrient Broth No. 2 (dry powder). Nutrient Broth was Vogel-Bonner salt solution supplemented with 2.5 % (W/V) Oxoid Nutrient Broth No. 2 (dry powder).

Each plate was labeled with a code system that identified the test article, test phase, dose level, tester strain, and activation, as described in detail in BioReliance's Standard Operating Procedures.

Test article dilutions were prepared immediately before use. One-half milliliter (0.5 mL) of S9 mix or Sham mix, 100 μL of tester strain and 2.0 mL of vehicle or test article were added to 0.5 mL of top agar at 60$\pm$2°C. After vortexing, the mixture was overlaid onto the surface of 25 mL of minimal bottom agar. When plating the positive controls, the test article aliquot was replaced by a 2 mL aliquot of distilled water and a 50 μL aliquot of appropriate positive control. After the overlay had solidified, the plates were inverted and incubated for approximately 48 to 72 hours at 37$\pm$2°C. Plates that were not counted immediately following the incubation period were stored at 2-8°C until colony counting could be conducted.

The condition of the bacterial background lawn was evaluated for evidence of test article toxicity and precipitate by using a dissecting microscope. Toxicity and degree of precipitation were scored relative to the vehicle control plate using the codes shown on the following page.

Code	Description	Characteristics
1	Normal	Distinguished by a healthy microcolony lawn.
2	Slightly Reduced	Distinguished by a noticeable thinning of the microcolony lawn and possibly a slight increase in the size of the microcolonies compared to the vehicle control plate.
3	Moderately Reduced	Distinguished by a marked thinning of the microcolony lawn resulting in a pronounced increase in the size of the microcolonies compared to the vehicle control plate.
4	Severely Reduced	Distinguished by an extreme thinning of the microcolony lawn resulting in an increase in the size of the microcolonies compared to the vehicle control plate such that the microcolony lawn is visible to the unaided eye as isolated colonies.
5	Absent	Distinguished by a complete lack of any microcolony lawn over ≥90% of the plate.
6	Obscured by Precipitate	The background bacterial lawn cannot be accurately evaluated due to microscopic test article precipitate.
NP	Non-Interfering Precipitate	Distinguished by precipitate on the plate that is visible to the naked eye but any precipitate particles detected by the automated colony counter total less than 10% of the revertant colony count (e.g., ≤3 particles on a plate with 30 revertants.)
IP	Interfering Precipitate	Distinguished by precipitate on the plate that is visible to the naked eye and any precipitate particles detected by the automated colony counter exceed 10% of the revertant colony count (e.g., >3 particles on a plate with 30 revertants.)

Revertant colonies for a given tester strain and activation condition, except for positive controls, were counted either entirely by automated colony counter or entirely by hand unless the plate exhibited toxicity. Plates with sufficient test article precipitate to interfere with automated colony counting were counted manually.

Evaluation of Results

For each replicate plating, the mean and standard deviation of the number of revertants per plate were calculated and are reported.

For a test article to be evaluated positive, it must cause a dose-related increase in the mean revertants per plate of at least one tester strain with a minimum of two increasing concentrations of test article. Data sets for strains TA98 and TA100 were judged positive if the increase in mean revertants at the peak of the dose response is equal to or greater than two times the mean vehicle control value.

Criteria for a Valid Test

The following criteria must be met for the mutagenicity assay to be considered valid. All tester strain cultures must demonstrate the presence of the deep rough mutation (*rfa*), the deletion in the *uvr*B gene and the presence of the pKM101 plasmid R-factor. All cultures must demonstrate the characteristic mean number of spontaneous revertants

in the vehicle controls as follows (inclusive): TA98, 10 - 50; TA100, 80 - 240. To ensure that appropriate numbers of bacteria are plated, tester strain culture titers must be greater than or equal to $0.3x10^9$ cells/ml. The mean of each positive control must exhibit at least a three-fold increase in the number of revertants over the mean value of the respective vehicle control. A minimum of three non-toxic dose levels are required to evaluate assay data. A dose level is considered toxic if one or both of the following criteria are met: (1) A >50 % reduction in the mean number of revertants per plate as compared to the mean vehicle control value. This reduction must be accompanied by an abrupt dose-dependent drop in the revertant count. (2) A reduction in the background lawn.

Archives

All raw data, the protocol and all reports will be maintained according to Standard Operating Procedure OPQP3040 by the BioReliance RAQA unit headquartered at: BioReliance, 14920 Broschart Road, Rockville, MD 20850.

RESULTS AND DISCUSSION

Solubility

Water was selected as the solvent of choice based on the Sponsor's request and compatibility with the target cells.

Mutagenicity Assay

The results of the mutagenicity assay are presented in Tables 1 through 4 and summarized in Table 5. These data were generated in Experiment B1. The maximum dose level tested in the mutagenicity assay was 2.0 mL of undiluted test article per plate. Subsequent dose levels were prepared by diluting the test article in water. These dilutions were soluble in water at 0.75 mL/mL, the most concentrated dilution prepared. Neither precipitate nor appreciable toxicity was observed. However, a reduced revertant count was present at the top doses of tester strain TA100 in the presence of S9 activation due to the presence of contaminating colonies on the plates.

In Experiment B1, no positive responses were observed with any of the tester strains in the presence and absence of S9 activation.

CONCLUSION

All criteria for a valid study were met as described in the protocol. The results of the *Salmonella* Plate Incorporation Mutagenicity Assay Using Water Samples indicate that, under the conditions of this study, **Water Sample 99-020-1** did not cause a positive response with any of the tester strains in the presence and absence of Aroclor-induced rat liver S9.

REFERENCES

Ames, B.N., J. McCann and E. Yamasaki (1975) Methods for Detecting Carcinogens and Mutagens with the *Salmonella*/Mammalian Microsome Mutagenicity Test, Mutation Research, 31:347-364.

Maron, D.M. and B.N. Ames (1983) Revised Methods for the *Salmonella* Mutagenicity Test, Mutation Research, 113:173-215.

McCann, J. and B.N. Ames (1976) Detection of Carcinogens as Mutagens in the *Salmonella*/Microsome Test: Assay of 300 Chemicals: Discussion, Proc. Natl. Acad. Sci. USA, 73:950-954.

McCann, J., E. Choi, E. Yamasaki and B.N. Ames (1975) Detection of Carcinogens as Mutagens in the *Salmonella*/Microsome Test: Assay of 300 Chemicals, Proc. Natl. Acad. Sci. USA, 72:5135-5139.

Vogel, H.J. and D.M. Bonner (1956) Acetylornithinase of *E. coli*: Partial Purification and Some Properties, J. Biol. Chem., 218:97-106.

Salmonella Mutagenicity Assay

Table 1

Test Article Id : Water Sample 99-020-1
Study Number : AA12BR.501006.BTL
Strain : TA98
Liver Microsomes : None
Vehicle : water
Plating Aliquot : 2.0mL

Experiment No : B1
Cells Seeded : 1.4 X 10^8
Date Plated : 01/27/99
Counted by : hand

Concentration mL per plate	Plate Number	Revertants per plate	Background Code[a]	Average Revertants	Standard Deviation
Vehicle	01	12	1		
	02	16	1	14	3
0.1	01	14	1		
	02	10	1	12	3
0.2	01	14	1		
	02	16	1	15	1
0.5	01	10	1		
	02	19	1	15	6
1.0	01	12	1		
	02	10	1	11	1
1.5	01	20	1		
	02	20	1	20	0
2.0	01	16	1		
	02	24	1	20	6
Positive Control 2-nitrofluorene 1.0 µg per plate[b]					
	01	409	1		
	02	301	1	355	76

[a]Background bacterial evaluation code
1=Normal 2=Slightly reduced 3=Moderately reduced
4=Extremely reduced 5=Absent 6=Obscured by precipitate
NP=Non-Interfering Precipitate IP=Interfering Precipitate

[b]Positive control plates were machine counted

Salmonella Mutagenicity Assay

Table 2

Test Article Id : Water Sample 99-020-1
Study Number : AA12BR.501006.BTL Experiment No : B1
Strain : TA98 Cells Seeded : 1.4 X 10^8
Liver Microsomes : Rat liver S9 Date Plated : 01/27/99
Vehicle : water
Plating Aliquot : 2.0mL Counted by : hand

Concentration mL per plate	Plate Number	Revertants per plate	Background Code[a]	Average Revertants	Standard Deviation
Vehicle	01	9	1		
	02	13	1	11	3
0.1	01	18	1		
	02	12	1	15	4
0.2	01	13	1		
	02	10	1	12	2
0.5	01	14	1		
	02	17	1	16	2
1.0	01	19	1		
	02	8	1	14	8
1.5	01	12	1		
	02	12	1	12	0
2.0	01	6	1		
	02	13	1	10	5
Positive Control 2-aminoanthracene 1.0 μg per plate[b]					
	01	1526	1		
	02	1699	1	1613	122

[a]Background bacterial evaluation code
1=Normal 2=Slightly reduced 3=Moderately reduced
4=Extremely reduced 5=Absent 6=Obscured by precipitate
NP=Non-Interfering Precipitate IP=Interfering Precipitate
[b]Positive control plates were machine counted

Salmonella Mutagenicity Assay

Table 3

Test Article Id : Water Sample 99-020-1
Study Number : AA12BR.501006.BTL
Strain : TA100
Liver Microsomes : None
Vehicle : water
Plating Aliquot : 2.0mL

Experiment No : B1
Cells Seeded : 1.1 X 10^8
Date Plated : 01/27/99
Counted by : machine

Concentration mL per plate	Plate Number	Revertants per plate	Background Code[a]	Average Revertants	Standard Deviation
Vehicle	01	109	1		
	02	123	1	116	10
0.1	01	116	1		
	02	129	1	123	9
0.2	01	126	1		
	02	122	1	124	3
0.5	01	140	1		
	02	145	1	143	4
1.0	01	131	1		
	02	125	1	128	4
1.5	01	128	1		
	02	137	1	133	6
2.0	01	174	1		
	02	146	1	160	20
Positive Control sodium azide 1.0 µg per plate[b]					
	01	466	1		
	02	388	1	427	55

[a]Background bacterial evaluation code
1=Normal 2=Slightly reduced 3=Moderately reduced
4=Extremely reduced 5=Absent 6=Obscured by precipitate
NP=Non-Interfering Precipitate IP=Interfering Precipitate

[b]Positive control plates were hand counted

Salmonella Mutagenicity Assay

Table 4

Test Article Id : Water Sample 99-020-1
Study Number : AA12BR.501006.BTL Experiment No : B1
Strain : TA100 Cells Seeded : 1.1 X 10^8
Liver Microsomes : Rat liver S9 Date Plated : 01/27/99
Vehicle : water
Plating Aliquot : 2.0mL Counted by : hand

Concentration mL per plate	Plate Number	Revertants per plate	Background Code[a]	Average Revertants	Standard Deviation
Vehicle	01	148	1		
	02	141	1	145	5
0.1	01	100	1		
	02	112	1	106	8
0.2	01	146	1		
	02	127	1	137	13
0.5	01	138	1		
	02	128	1	133	7
1.0	01	40	1		
	02	62	1	51	16
1.5	01	149	1		
	02	75	1	112	52
2.0	01	90	1		
	02	69	1	80	15
Positive Control 2-aminoanthracene 1.0 µg per plate[b]					
	01	1891	1		
	02	1451	1	1671	311

[a]Background bacterial evaluation code
1=Normal 2=Slightly reduced 3=Moderately reduced
4=Extremely reduced 5=Absent 6=Obscured by precipitate
NP=Non-Interfering Precipitate IP=Interfering Precipitate
[b]Positive control plates were machine counted

Salmonella Mutagenicity Assay
Summary of Results

Table 5

Test Article Id : Water Sample 99-020-1
Study Number : AA12BR.501006.BTL Experiment No : B1

Average Revertants Per Plate ± Standard Deviation

Liver Microsomes: None

Dose (mL)	TA98	TA100
0.0	14 ± 3	116 ± 10
0.1	12 ± 3	123 ± 9
0.2	15 ± 1	124 ± 3
0.5	15 ± 6	143 ± 4
1.0	11 ± 1	128 ± 4
1.5	20 ± 0	133 ± 6
2.0	20 ± 6	160 ± 20
Pos	355 ± 76	427 ± 55

Liver Microsomes: Rat liver S9

Dose (mL)	TA98	TA100
0.0	11 ± 3	145 ± 5
0.1	15 ± 4	106 ± 8
0.2	12 ± 2	137 ± 13
0.5	16 ± 2	133 ± 7
1.0	14 ± 8	51 ± 16
1.5	12 ± 0	112 ± 52
2.0	10 ± 5	80 ± 15
Pos	1613 ± 122	1671 ± 311

0.0 = Vehicle plating aliquot of 2.0mL
Pos = Positive Control concentrations as specified in Materials and Methods section.

APPENDIX I

Historical Control Data

Historical Negative and Positive Control Values
1995 - 1997

revertants per plate

Strain	Control	Activation							
		None				Rat Liver			
		Mean	SD	Min	Max	Mean	SD	Min	Max
TA98	Neg	17	7	3	52	23	8	4	65
	Pos	267	189	53	1416	815	426	69	2769
TA100	Neg	120	21	65	262	138	24	62	323
	Pos	570	161	104	2054	904	415	106	2813

SD=standard deviation; Min=minimum value; Max=maximum value; Neg=negative control (including but not limited to deionized water, dimethyl sulfoxide, ethanol and acetone); Pos=positive control

APPENDIX II

Study Protocol

QA 1-25-99
APPROVED

Received by RA/QA 1/22/99

MA Study Number: AA12BR.501006.BTL

Salmonella Plate Incorporation Mutagenicity Assay Using Water Samples

1.0 PURPOSE

The purpose of this study is to evaluate the mutagenic potential of the test article by measuring its ability to induce reverse mutations at selected loci of two strains of *Salmonella typhimurium* in the presence and absence of S9 activation.

2.0 SPONSOR

2.1 Name: **US Army**
Center for Environmental Health Research

2.2 Address: **568 Doughten Drive**
Fort Detrick, MD 21702-5010

2.3 Representative: **Maggie Toussaint**
Geo Centers

3.0 IDENTIFICATION OF TEST AND CONTROL SUBSTANCES

3.1 Test Article: **Water Sample 99-020-1**

3.2 Controls: Negative: Test article vehicle (deionized water)

Positive: 2-aminoanthracene
2-nitrofluorene
sodium azide

3.3 Determination of Strength, Purity, etc.

Unless alternate arrangements are made, the testing facility at MA will not perform analysis of the dosing solutions. The Sponsor will be directly responsible for determination and documentation of the analytical purity and composition of the test article, and the stability and strength of the test article in the solvent (or vehicle).

3.4 Test Article Retention Sample

The retention of a reserve sample of each batch of the test article will be the responsibility of the Sponsor.

4.0 TESTING FACILITY AND KEY PERSONNEL

4.1 Name: Toxicology Testing Facility
MA BioServices, Inc.

4.2 Address: 9630 Medical Center Drive
Rockville, MD 20850

4.3 Study Director: Valentine O. Wagner III, M.S.

5.0 TEST SCHEDULE

5.1 Proposed Experimental Initiation Date: 01/27/99

5.2 Proposed Experimental Completion Date: 02/24/99

5.3 Proposed Report Date: 03/10/99

6.0 TEST SYSTEM

The tester strains will include the *S. typhimurium* histidine auxotrophs TA98 and TA100 as described by Ames *et al.* (1975).

Genotype of the *S. typhimurium* Strains Used for Mutagen Testing

Histidine Mutation		Additional Mutations		
Reversion Specificity		LPS	Repair	UR Factor
TA100	TA98	*rfa*	Δ*uvr*B	+R

Each tester strain contains, in addition to a mutation in the histidine operon, two additional mutations that enhance sensitivity to some mutagens. The *rfa* mutation results in a cell wall deficiency that increases the permeability of the cell to certain classes of chemicals such as those containing large ring systems that would otherwise be excluded. The second mutation is a deletion in the *uvr*B gene resulting in a deficient DNA excision-repair system.

Tester strains TA98 and TA100 also contain the pKM101 plasmid (carrying the R-factor). It has been suggested that the plasmid increases sensitivity to mutagens by modifying an existing bacterial DNA repair polymerase complex involved with the mismatch-repair process.

TA98 is reverted from histidine dependence (auxotrophy) to histidine independence (prototrophy) by frameshift mutagens. TA100 is reverted by both frameshift and base substitution mutagens.

The *S. typhimurium* tester strains were received directly from Dr. Bruce Ames, Department of Biochemistry, University of California, Berkeley.

7.0 EXPERIMENTAL DESIGN AND METHODOLOGY

The test article will be tested neat at a minimum of five dose levels along with appropriate vehicle and positive controls with tester strains TA98 and TA100 with and without S9

activation. All dose levels of test article, vehicle controls and positive controls will be plated in duplicate.

7.1 Selection of Dose Levels

Unless indicated otherwise by the Sponsor, the dose levels for the initial assay will be 2.0, 1.5, 1.0, 0.5, 0.2 and 0.1 mL per plate.

7.2 Frequency and Route of Administration

The test system will be exposed to the test article via the plate incorporation methodology originally described by Ames *et al.* (1975) and updated by Maron and Ames (1983). This test system has been shown to detect a wide range of classes of chemical mutagens (McCann *et al.*, 1975; McCann and Ames, 1976).

7.3 Controls

7.3.1 Positive Controls

All combinations of positive controls and tester strains plated concurrently with the assay are listed below:

Tester Strain	S9 Activation	Positive Control	Concentration (µg/plate)
TA98, TA100	Rat	2-aminoanthracene	1.0
TA98	None	2-nitrofluorene	2.0
TA100		sodium azide	2.0

7.3.2 Vehicle Controls

Vehicle controls will be plated for each tester strain with and without S9 activation.

7.3.3 Sterility Controls

The most concentrated test article dilution and the Sham and S9 mixes will be checked for sterility.

7.4 Exogenous Metabolic Activation

Aroclor 1254-induced rat liver S9 will be used as the metabolic activation system. The S9 homogenate will be prepared from male Sprague-Dawley rats induced with a single intraperitoneal injection of Aroclor 1254, 500 mg/kg, five days prior to sacrifice. The S9 will be batch prepared and stored frozen at approximately -70°C until used. Each batch of S9 homogenate will be assayed for its ability to metabolize 2-aminoanthracene and 7,12-dimethylbenzanthracene to forms mutagenic to *S. typhimurium* TA100.

Immediately prior to use, the S9 will be thawed and mixed with a cofactor pool to contain 10% S9 homogenate, 5 mM glucose-6-phosphate, 4 mM β-nicotinamide-adenine dinucleotide phosphate, 8 mM $MgCl_2$ and 33 mM KCl in a 100 mM phosphate buffer at pH 7.4. This mixture is referred to as S9 mix. Sham mix will be 100 mM phosphate buffer at pH 7.4.

7.5 Preparation of Tester Strain

Overnight cultures will be inoculated from the appropriate master plate or from the appropriate frozen stock. To ensure that cultures are harvested in late log phase, the length of incubation will be controlled and monitored. At the end of the working day, each inoculated flask will be placed in a resting shaker/incubator at room temperature. The shaker/incubator will be programmed to begin shaking at approximately 125 rpm at 37±2°C approximately 12 hours before the anticipated time of harvest.

All cultures will be harvested by spectrophotometric monitoring of culture turbidity rather than by duration of incubation since overgrowth of cultures can cause loss of sensitivity to some mutagens. Cultures will be removed from incubation at a density of approximately 10^9 cells/mL.

7.6 Test System Identification

Each plate will be labeled with a code system that identifies the test article, test phase, dose level, tester strain and activation type as described in MA BioServices, Inc.'s Standard Operating Procedures.

7.7 Test Article Preparation

Unless specified otherwise, test article dilutions will be prepared immediately prior to use. All test article dosing will be at room temperature under yellow light. The water sample will be filter sterilized prior to use.

7.8 Treatment of Test System

One-half milliliter (0.5 mL) of S9 mix or Sham mix, 100 µL of tester strain and 2.0 mL of vehicle or test article dilution will be added to 0.5 mL of molten selective top agar (54.4 g/L NaCl and 33.8 g/L agar supplemented with 56 mL of normal amino acid supplement per 100 mL) at 60±2°C. When plating the positive controls, the test article aliquot will be replaced with 2.0 mL of distilled water and a 50 µL aliquot of appropriate positive control. The mixture will be vortex mixed and overlaid onto the surface of 25 mL of minimal bottom agar. After the overlay has solidified, the plates will be inverted and incubated for approximately 48 to 72 hours at 37±2°C. Plates that are not counted immediately following the incubation period will be stored at 2-8°C.

7.9 Colony Counting

The condition of the bacterial background lawn will be evaluated for evidence of test article toxicity and precipitate. Evidence of toxicity will be scored relative to the vehicle control plate and recorded along with the revertant count for that plate. Toxicity will be evaluated as a decrease in the number of revertant colonies per plate and/or a thinning or disappearance of the bacterial background lawn. Precipitation will be evaluated after the incubation period by visual examination without magnification.

7.10 Tester Strain Verification

On the day of use in the mutagenicity assay, all *S. typhimurium* tester strain cultures will be checked for the following genetic markers:

The presence of the *rfa* wall mutation will be confirmed by demonstrating sensitivity to crystal violet. The presence of the *uvr*B mutation will be confirmed by demonstrating sensitivity to ultraviolet light. The presence of the pKM101 plasmid will be confirmed for tester strains TA98 and TA100 by demonstrating resistance to ampicillin.

8.0 CRITERIA FOR DETERMINATION OF A VALID TEST

The following criteria must be met for the mutagenicity assay to be considered valid:

8.1 Tester Strain Integrity

To demonstrate the presence of the *rfa* mutation, all tester strain cultures must exhibit sensitivity to crystal violet. To demonstrate the presence of the *uvr*B mutation, all tester strain cultures must exhibit sensitivity to ultraviolet light. To demonstrate the presence of the pKM101 plasmid R-factor, tester strain cultures of TA98 and TA100 must exhibit resistance to ampicillin.

8.2 Spontaneous Revertant Background Frequency

Based on historical control data, all tester strain cultures must exhibit a characteristic number of spontaneous revertants per plate in the vehicle controls. The mean revertants per plate must be within the following ranges (inclusive): TA98, 10 - 50; TA100, 80 - 240.

8.3 Tester Strain Titers

To ensure that appropriate numbers of bacteria are plated, all tester strain culture titers must be equal to or greater than 0.3×10^9 cells per milliliter.

8.4 Positive Control Values

Each mean positive control value must exhibit at least a three-fold increase over the respective mean vehicle control value for each tester strain.

8.5 Toxicity

A minimum of three non-toxic dose levels will be required to evaluate assay data. A dose level is considered toxic if it causes a >50% reduction in the mean number of revertants per plate relative to the mean vehicle control value (this reduction must be accompanied by an abrupt dose-dependent drop in the revertant count) or a reduction in the background lawn. In the event that less than three non-toxic dose levels are achieved, the affected portion of the assay will be repeated with an appropriate change in dose levels.

9.0 EVALUATION OF TEST RESULTS

For a test article to be evaluated positive, it must cause a dose-related increase in the mean revertants per plate of at least one tester strain over a minimum of two increasing concentrations of test article. Data sets will be judged positive if the increase in mean revertants at the peak of the dose response is equal to or greater than two times the mean vehicle control value.

10.0 REPORT

A report of the results of this study will be prepared by the Testing Laboratory and will accurately describe all methods used for generation and analysis of the data. The report will include:

- Test substance: identification and CAS no., if known; physical nature and purity, if known; physicochemical properties relevant to the conduct of the study, if known; stability of test article, if known.
- Solvent/Vehicle: justification for choice of vehicle; solubility and stability of test article in solvent/vehicle, if known.
- Strains: strains used; number of cells/mL per culture; strain characteristics.
- Test conditions: amount of test substance per plate with rationale for dose selection and number of plates per concentration; media used; type and composition of metabolic activation system, including acceptability criteria; treatment procedures.
- Results: signs of toxicity; signs of precipitation; individual plate counts; the mean number of revertant colonies per plate and standard deviation; dose-response relationship, where possible; statistical analysis, if any; concurrent negative and positive control data means and standard deviations; historical negative and positive control data with ranges, means and standard deviation.

- Discussion of results.
- Conclusion.

11.0 RECORDS AND ARCHIVES

Upon completion of the final report, all raw data and reports will be maintained in the archives of MA BioServices, Inc., Rockville, MD in accordance with the relevant Good Laboratory Practices Regulations.

12.0 REGULATORY REQUIREMENTS/GOOD LABORATORY PRACTICE

This study will be performed in compliance with the provisions of the Good Laboratory Practice Regulations for Nonclinical Laboratory Studies.

Will this study be submitted to a regulatory agency? YES

If so, to which agency or agencies? EPA - office of water (Research Proj.)

Unless arrangements are made to the contrary, unused dosing solutions will be disposed of following administration to the test system and all residual test article will be disposed of following finalization of the report.

13.0 REFERENCES

Ames, B.N., McCann, J. and Yamasaki, E. (1975). Methods for detecting carcinogens and mutagens with the *Salmonella*/mammalian-microsome mutagenicity test. Mutation Research *31*:347-364.

McCann, J. and Ames, B.N. (1976). Detection of carcinogens as mutagens in the *Salmonella*/microsome test: assay of 300 chemicals: discussion. Proc. Natl. Acad. Sci. USA 73:950-954.

McCann, J., Choi, E., Yamasaki, E. and Ames, B.N. (1975). Detection of carcinogens as mutagens in the *Salmonella*/microsome test: assay of 300 chemicals. Proc. Natl. Acad. Sci. USA 72:5135-5139.

Maron, D.M. and Ames, B.N. (1983). Revised Methods for the *Salmonella* Mutagenicity Test. Mutation Research 113:173-215.

14.0 APPROVAL

Robert A. Finch for HSG 1-19-99

SPONSOR REPRESENTATIVE DATE

Robert A. Finch

(Print or Type Name)

(Henry S. Gardner)

STUDY DIRECTOR DATE

If submission to Japanese Regulatory Agency is indicated in section 12.0, MA management will sign.

MA STUDY MANAGEMENT DATE

Valentine O. Wagner, III
MA Study Director

22-Jan-1999
Date

4.2 Address: 9630 Medical Center Drive
Rockville, MD 20850

4.3 Study Director: Valentine O. Wagner III, M.S.

5.0 TEST SCHEDULE

5.1 Proposed Experimental Initiation Date: 01/27/99

5.2 Proposed Experimental Completion Date: 02/24/99

5.3 Proposed Report Date: 03/10/99

6.0 TEST SYSTEM

The tester strains will include the *S. typhimurium* histidine auxotrophs TA98 and TA100 as described by Ames *et al.* (1975).

Genotype of the *S. typhimurium* Strains Used for Mutagen Testing

Histidine Mutation		Additional Mutations		
*hisG*46	*hisD*3052	LPS	Repair	R-factor
TA100	TA98	*rfa*	Δ*uvr*B	+R

Each tester strain contains, in addition to a mutation in the histidine operon, two additional mutations that enhance sensitivity to some mutagens. The *rfa* mutation results in a cell wall deficiency that increases the permeability of the cell to certain classes of chemicals such as those containing large ring systems that would otherwise be excluded. The second mutation is a deletion in the *uvr*B gene resulting in a deficient DNA excision-repair system.

Tester strains TA98 and TA100 also contain the pKM101 plasmid (carrying the R-factor). It has been suggested that the plasmid increases sensitivity to mutagens by modifying an existing bacterial DNA repair polymerase complex involved with the mismatch-repair process.

TA98 is reverted from histidine dependence (auxotrophy) to histidine independence (prototrophy) by frameshift mutagens. TA100 is reverted by both frameshift and base substitution mutagens.

The *S. typhimurium* tester strains were received directly from Dr. Bruce Ames, Department of Biochemistry, University of California, Berkeley.

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The test article will be tested neat at a minimum of five dose levels along with appropriate vehicle and positive controls with tester strains TA98 and TA100 with and without S9

activation. All dose levels of test article, vehicle controls and positive controls will be plated in duplicate.

7.1 Selection of Dose Levels

Unless indicated otherwise by the Sponsor, the dose levels for the initial assay will be 2.0, 1.5, 1.0, 0.5, 0.2 and 0.1 mL per plate.

7.2 Frequency and Route of Administration

The test system will be exposed to the test article via the plate incorporation methodology originally described by Ames *et al.* (1975) and updated by Maron and Ames (1983). This test system has been shown to detect a wide range of classes of chemical mutagens (McCann *et al.*, 1975; McCann and Ames, 1976).

7.3 Controls

7.3.1 Positive Controls

All combinations of positive controls and tester strains plated concurrently with the assay are listed below:

Strain	S9 Activation	Positive Control	Concentration (µg/plate)
TA98, TA100	Rat	2-aminoanthracene	1.0
TA98	None	2-nitrofluorene	2.0
TA100		sodium azide	2.0

7.3.2 Vehicle Controls

Vehicle controls will be plated for each tester strain with and without S9 activation.

7.3.3 Sterility Controls

The most concentrated test article dilution and the Sham and S9 mixes will be checked for sterility.

7.4 Exogenous Metabolic Activation

Aroclor 1254-induced rat liver S9 will be used as the metabolic activation system. The S9 homogenate will be prepared from male Sprague-Dawley rats induced with a single intraperitoneal injection of Aroclor 1254, 500 mg/kg, five days prior to sacrifice. The S9 will be batch prepared and stored frozen at approximately -70°C until used. Each batch of S9 homogenate will be assayed for its ability to metabolize 2-aminoanthracene and 7,12-dimethylbenzanthracene to forms mutagenic to *S. typhimurium* TA100.

Immediately prior to use, the S9 will be thawed and mixed with a cofactor pool to contain 10% S9 homogenate, 5 mM glucose-6-phosphate, 4 mM β-nicotinamide-adenine dinucleotide phosphate, 8 mM $MgCl_2$ and 33 mM KCl in a 100 mM phosphate buffer at pH 7.4. This mixture is referred to as S9 mix. Sham mix will be 100 mM phosphate buffer at pH 7.4.

7.5 Preparation of Tester Strain

Overnight cultures will be inoculated from the appropriate master plate or from the appropriate frozen stock. To ensure that cultures are harvested in late log phase, the length of incubation will be controlled and monitored. At the end of the working day, each inoculated flask will be placed in a resting shaker/incubator at room temperature. The shaker/incubator will be programmed to begin shaking at approximately 125 rpm at 37±2°C approximately 12 hours before the anticipated time of harvest.

All cultures will be harvested by spectrophotometric monitoring of culture turbidity rather than by duration of incubation since overgrowth of cultures can cause loss of sensitivity to some mutagens. Cultures will be removed from incubation at a density of approximately 10^9 cells/mL.

7.6 Test System Identification

Each plate will be labeled with a code system that identifies the test article, test phase, dose level, tester strain and activation type as described in MA BioServices, Inc.'s Standard Operating Procedures.

7.7 Test Article Preparation

Unless specified otherwise, test article dilutions will be prepared immediately prior to use. All test article dosing will be at room temperature under yellow light. The water sample will be filter sterilized prior to use.

7.8 Treatment of Test System

One-half milliliter (0.5 mL) of S9 mix or Sham mix, 100 µL of tester strain and 2.0 mL of vehicle or test article dilution will be added to 0.5 mL of molten selective top agar (54.4 g/L NaCl and 33.8 g/L agar supplemented with 56 mL of normal amino acid supplement per 100 mL) at 60±2°C. When plating the positive controls, the test article aliquot will be replaced with 2.0 mL of distilled water and a 50 µL aliquot of appropriate positive control. The mixture will be vortex mixed and overlaid onto the surface of 25 mL of minimal bottom agar. After the overlay has solidified, the plates will be inverted and incubated for approximately 48 to 72 hours at 37±2°C. Plates that are not counted immediately following the incubation period will be stored at 2-8°C.

7.9 Colony Counting

The condition of the bacterial background lawn will be evaluated for evidence of test article toxicity and precipitate. Evidence of toxicity will be scored relative to the vehicle control plate and recorded along with the revertant count for that plate. Toxicity will be evaluated as a decrease in the number of revertant colonies per plate and/or a thinning or disappearance of the bacterial background lawn. Precipitation will be evaluated after the incubation period by visual examination without magnification.

7.10 Tester Strain Verification

On the day of use in the mutagenicity assay, all *S. typhimurium* tester strain cultures will be checked for the following genetic markers:

The presence of the *rfa* wall mutation will be confirmed by demonstrating sensitivity to crystal violet. The presence of the *uvr*B mutation will be confirmed by demonstrating sensitivity to ultraviolet light. The presence of the pKM101 plasmid will be confirmed for tester strains TA98 and TA100 by demonstrating resistance to ampicillin.

8.0 CRITERIA FOR DETERMINATION OF A VALID TEST

The following criteria must be met for the mutagenicity assay to be considered valid:

8.1 Tester Strain Integrity

To demonstrate the presence of the *rfa* mutation, all tester strain cultures must exhibit sensitivity to crystal violet. To demonstrate the presence of the *uvr*B mutation, all tester strain cultures must exhibit sensitivity to ultraviolet light. To demonstrate the presence of the pKM101 plasmid R-factor, tester strain cultures of TA98 and TA100 must exhibit resistance to ampicillin.

8.2 Spontaneous Revertant Background Frequency

Based on historical control data, all tester strain cultures must exhibit a characteristic number of spontaneous revertants per plate in the vehicle controls. The mean revertants per plate must be within the following ranges (inclusive): TA98, 10 - 50; TA100, 80 - 240.

8.3 Tester Strain Titers

To ensure that appropriate numbers of bacteria are plated, all tester strain culture titers must be equal to or greater than 0.3×10^9 cells per milliliter.

8.4 Positive Control Values

Each mean positive control value must exhibit at least a three-fold increase over the respective mean vehicle control value for each tester strain.

8.5 Toxicity

A minimum of three non-toxic dose levels will be required to evaluate assay data. A dose level is considered toxic if it causes a >50% reduction in the mean number of revertants per plate relative to the mean vehicle control value (this reduction must be accompanied by an abrupt dose-dependent drop in the revertant count) or a reduction in the background lawn. In the event that less than three non-toxic dose levels are achieved, the affected portion of the assay will be repeated with an appropriate change in dose levels.

9.0 EVALUATION OF TEST RESULTS

For a test article to be evaluated positive, it must cause a dose-related increase in the mean revertants per plate of at least one tester strain over a minimum of two increasing concentrations of test article. Data sets will be judged positive if the increase in mean revertants at the peak of the dose response is equal to or greater than two times the mean vehicle control value.

10.0 REPORT

A report of the results of this study will be prepared by the Testing Laboratory and will accurately describe all methods used for generation and analysis of the data. The report will include:

- Test substance: identification and CAS no., if known; physical nature and purity, if known; physicochemical properties relevant to the conduct of the study, if known; stability of test article, if known.
- Solvent/Vehicle: justification for choice of vehicle; solubility and stability of test article in solvent/vehicle, if known.
- Strains: strains used; number of cells/mL per culture; strain characteristics.
- Test conditions: amount of test substance per plate with rationale for dose selection and number of plates per concentration; media used; type and composition of metabolic activation system, including acceptability criteria; treatment procedures.
- Results: signs of toxicity; signs of precipitation; individual plate counts; the mean number of revertant colonies per plate and standard deviation; dose-response relationship, where possible; statistical analysis, if any; concurrent negative and positive control data means and standard deviations; historical negative and positive control data with ranges, means and standard deviation.

- Discussion of results.
- Conclusion.

11.0 RECORDS AND ARCHIVES

Upon completion of the final report, all raw data and reports will be maintained in the archives of MA BioServices, Inc., Rockville, MD in accordance with the relevant Good Laboratory Practices Regulations.

12.0 REGULATORY REQUIREMENTS/GOOD LABORATORY PRACTICE

This study will be performed in compliance with the provisions of the Good Laboratory Practice Regulations for Nonclinical Laboratory Studies.

Will this study be submitted to a regulatory agency? YES

If so, to which agency or agencies? EPA - office of water (Research Proj.)

Unless arrangements are made to the contrary, unused dosing solutions will be disposed of following administration to the test system and all residual test article will be disposed of following finalization of the report.

13.0 REFERENCES

Ames, B.N., McCann, J. and Yamasaki, E. (1975). Methods for detecting carcinogens and mutagens with the *Salmonella*/mammalian-microsome mutagenicity test. Mutation Research *31*:347-364.

McCann, J. and Ames, B.N. (1976). Detection of carcinogens as mutagens in the *Salmonella*/microsome test: assay of 300 chemicals: discussion. Proc. Natl. Acad. Sci. USA 73:950-954.

McCann, J., Choi, E., Yamasaki, E. and Ames, B.N. (1975). Detection of carcinogens as mutagens in the *Salmonella*/microsome test: assay of 300 chemicals. Proc. Natl. Acad. Sci. USA 72:5135-5139.

Maron, D.M. and Ames, B.N. (1983). Revised Methods for the *Salmonella* Mutagenicity Test. Mutation Research 113:173-215.

14.0 APPROVAL

Robert A. Finch for HSG 1-19-99
SPONSOR REPRESENTATIVE DATE

Robert A. Finch
(Print or Type Name)

(Henry S. Gardner)
STUDY DIRECTOR DATE

If submission to Japanese Regulatory Agency is indicated in section 12.0, MA management will sign.

MA STUDY MANAGEMENT DATE

Valentine O. Wagner, III
MA Study Director

22-Jan-1999
Date

FINAL REPORT

Study Title

SALMONELLA PLATE INCORPORATION MUTAGENICITY ASSAY USING WATER SAMPLES

Test Article

Water Sample 99-028-6

Authors

Valentine O. Wagner, III, M.S.
Susan C. Twardzik, B.A.

Study Completion Date

July 20, 1999

Performing Laboratory

BioReliance
9630 Medical Center Drive
Rockville, MD 20850

Laboratory Study Number

AA12JC.501006.BTL

Sponsor

US Army
Center for Enviromental Health Research
568 Doughten Drive
Fort Detrick, MD 21702-5010

STATEMENT OF COMPLIANCE

Study No. AA12JC.501006.BTL was conducted in compliance with the U.S. FDA Good Laboratory Practice Regulations as published in 21 CFR 58, the U.S. EPA GLP Standards 40 CFR 792 and 40 CFR 160, the UK GLP Compliance Programme, the Japanese GLP Standard and the OECD Principles of Good Laboratory Practice in all material aspects with the following exceptions:

> The identity, strength, purity and composition or other characteristics to define the test or control article have not been determined by the testing facility.
>
> Analyses to determine the uniformity, concentration, or stability of the test or control mixtures were not performed by the testing facility.
>
> The stability of the test or control article under the test conditions has not been determined by the testing facility.

Valentine O. Wagner, III
Valentine O. Wagner, III, M.S.
Study Director

20-Jul-1999
Date

QUALITY ASSURANCE STATEMENT

Study Title: *SALMONELLA* PLATE INCORPORATION MUTAGENICITY ASSAY USING WATER SAMPLES

Study Number: AA12JC.501006.BTL

Study Director: Valentine O. Wagner, III, M.S.

This study has been divided into a series of in-process phases. Using a random sampling approach, Quality Assurance monitors each of these phases over a series of studies. Procedures, documentation, equipment records, etc., are examined in order to assure that the study is performed in accordance with the U.S. FDA Good Laboratory Practice Regulations (21 CFR 58), the U.S. EPA GLPs (40 CFR 792 and 40 CFR 160), the UK GLP Regulations, the Japanese GLP Standard, and the OECD Principles of Good Laboratory Practice and to assure that the study is conducted according to the protocol and relevant Standard Operating Procedures.

The following are the inspection dates, phases inspected, and report dates of QA inspections of this study.

INSPECT ON 02 FEB 99, TO STUDY DIR 02 FEB 99, TO MGMT 02 FEB 99
PHASE: Protocol Review

INSPECT ON 09 FEB 99, TO STUDY DIR 09 FEB 99, TO MGMT 11 FEB 99
PHASE: Scoring the plates

INSPECT ON 15 MAR 99, TO STUDY DIR 15 MAR 99, TO MGMT 17 MAR 99
PHASE: Draft Report

INSPECT ON 21 JUL 99, TO STUDY DIR 21 JUL 99, TO MGMT 21 JUL 99
PHASE: Draft to Final Report

This report describes the methods and procedures used in the study and the reported results accurately reflect the raw data of the study.

Jennifer Klopsis 7-21-99

Jennifer Klopsis
QUALITY ASSURANCE

DATE

Salmonella Plate Incorporation Mutagenicity Assay Using Water Samples

FINAL REPORT

Sponsor:	**US Army** **Center for Environmental Health Research** **568 Doughten Drive** **Fort Detrick, MD 21702-5010**
Authorized Representative:	**Maggie Toussaint** **Geo Centers**
Performing Laboratory:	**BioReliance** **9630 Medical Center Drive** **Rockville, Maryland 20850**
Test Article I.D.:	**Water Sample 99-028-6**
BioReliance Study No.:	**AA12JC.501006.BTL**
Test Article Description:	**clear, colorless water**
Storage Conditions:	**room temperature; protected from exposure to light**
Test Article Receipt:	**January 28, 1999**
Study Initiation:	**February 02, 1999**
Study Director:	Valentine O. Wagner, III 20-Jul-1999 Valentine O. Wagner, III, M.S. Date

TABLE OF CONTENTS

SUMMARY

The test article, Water Sample 99-028-6, was tested in the *Salmonella* Plate Incorporation Mutagenicity Assay Using Water Samples with tester strains TA98 and TA100 in the presence and absence of Aroclor-induced rat liver S9. The assay was performed using the plate incorporation method. The mutagenicity assay was used to evaluate the mutagenic potential of the test article.

Water was selected as the solvent of choice based on the Sponsor's request and compatibility with the target cells.

The maximum dose level plated in the mutagenicity assay was 2.0 mL of undiluted test article per plate. Subsequent dose levels were prepared by diluting the test article in water. These dilutions were soluble in water at 0.75 mL/mL, the most concentrated dilution prepared.

In the mutagenicity assay, no positive response was observed. Neither precipitate nor appreciable toxicity was observed. The overall evaluation and dose range tested are as follows:

S9 Activation	Overall Evaluation[a] and Dose Range Tested (mL/plate)			
	TA98		TA100	
	Low	High	Low	High
None	-		-	
	0.10	2.0	0.10	2.0
Rat	-		-	
	0.10	2.0	0.10	2.0

[a] - = negative, + = positive (maximum fold increase)

In conclusion, the results indicate that under the conditions of this study, test article **Water Sample 99-028-6** did not cause a positive response in the *Salmonella* Plate Incorporation Mutagenicity Assay Using Water Samples.

PURPOSE

The purpose of this study was to evaluate the mutagenic potential of the test article (or its metabolites) by measuring the ability to induce reverse mutations at selected loci of two strains of *Salmonella typhimurium* in the presence and absence of S9 activation.

CHARACTERIZATION OF TEST AND CONTROL ARTICLES

The test article, Water Sample 99-028-6, was received by BioReliance on January 28, 1999 and was assigned the code number AA12JC. The test article was characterized by the Sponsor as chlorinated drinking water. An expiration date of February 05, 1999 was provided. Upon receipt, the test article was described as clear, colorless water and was stored at room temperature, protected from exposure to light.

The vehicle used to deliver the test article to the test system was sterile distilled water, (CAS# 7732-18-5), obtained from Life Technologies, Inc.

Positive controls plated concurrently with the mutagenicity assay are listed below:

Strain	S9 Activation	Positive Control	Concentration (μg/plate)
TA98, TA100	+	2-aminoanthracene (Sigma Chemical Co.)	1.0
TA98	-	2-nitrofluorene (Aldrich Chemical Co., Inc.)	2.0[a]
TA100	-	sodium azide (Sigma Chemical Co.)	2.0[a]

[a]In a deviation from the protocol, the concentration used in the assay was 1.0 μg/plate. Since an acceptable positive response was obtained, which indicated that the test system was capable of detecting a known mutagen, the Study Director has accepted these data.

To determine the sterility of the test articles, the highest test article dose level used in the mutagenicity assay was plated on selective agar with an aliquot volume equal to that used in the assay.

MATERIALS AND METHODS

Test System

The tester strains used were the *Salmonella typhimurium* histidine auxotrophs TA98 and TA100 as described by Ames *et al.* (1975). *Salmonella* tester strains were received on 08/11/98 and 11/10/98 directly from Dr. Bruce Ames, University of California, Berkeley.

Tester strain TA98 is reverted from histidine dependence (auxotrophy) to histidine independence (prototrophy) by frameshift mutagens. Tester strain TA100 is reverted by mutagens that cause both frameshift and basepair substitution mutations.

Overnight cultures were prepared by inoculating from the appropriate master plate or from the appropriate frozen permanent stock into a vessel containing ~50 mL of culture medium. To assure that cultures were harvested in late log phase, the length of incubation was controlled and monitored. Following inoculation, each flask was placed in a resting shaker/incubator at room temperature. The shaker/incubator was programmed to begin shaking at approximately 125 rpm at 37±2°C approximately 12 hours before the anticipated time of harvest. Each culture was monitored spectrophotometrically for turbidity and was harvested at a percent transmittance yielding a titer of approximately 10^9 cells per milliliter. The actual titers were determined by viable count assays on nutrient agar plates.

Metabolic Activation System

Aroclor 1254-induced rat liver S9 was used as the metabolic activation system. The S9 was prepared from male Sprague-Dawley rats induced with a single intraperitoneal injection of Aroclor 1254, 500 mg/kg, five days prior to sacrifice. The S9 was batch prepared on 12/16/98 and stored at ≤-70°C until used. Each bulk preparation of S9 was assayed for its ability to metabolize 2-aminoanthracene and 7,12-dimethylbenz(a)anthracene to forms mutagenic to *Salmonella typhimurium* TA100.

The S9 mix was prepared immediately before its use and contained 10% S9, 5 mM glucose-6-phosphate, 4 mM β-nicotinamide-adenine dinucleotide phosphate, 8 mM $MgCl_2$ and 33 mM KCl in a 100 mM phosphate buffer at pH 7.4. The Sham S9 mixture (Sham mix), containing 100 mM phosphate buffer at pH 7.4, was prepared immediately before its use. To confirm the sterility of the S9 and Sham mixes, a 0.5 mL aliquot of each was plated on selective agar.

Mutagenicity Assay

The mutagenicity assay was used to evaluate the mutagenic potential of the test article. A minimum of six dose levels of test article along with appropriate vehicle and positive controls were plated with tester strains TA98 and TA100 in the presence and absence of rat liver S9 activation. All dose levels of test article, vehicle controls and positive controls were plated in duplicate.

Plating and Scoring Procedures

The test system was exposed to the test articles via the plate incorporation methodology originally described by Ames *et al.* (1975) and updated Maron and Ames (1983).

In a deviation from the protocol, the sample was not filter sterilized prior to use. However, in consultation with the Sponsor, it was determined that filtration was inappropriate because of volatile components diluted in the water. Any contaminant colonies observed on the assay plates were excluded from the revertant count. Therefore, the Study Director has accepted these data.

On the day of its use, minimal top agar, containing 3.4% agar (W/V) and 5.4% NaCl (W/V), was melted and supplemented with L-histidine, D-biotin and L-tryptophan solution to a final concentration of 179 μM each. Bottom agar was Vogel-Bonner minimal medium E (Vogel and Bonner, 1956) containing 1.5 % (W/V) agar. Nutrient bottom agar was Vogel-Bonner minimal medium E containing 1.5 % (W/V) agar and supplemented with 2.5 % (W/V) Oxoid Nutrient Broth No. 2 (dry powder). Nutrient Broth was Vogel-Bonner salt solution supplemented with 2.5 % (W/V) Oxoid Nutrient Broth No. 2 (dry powder).

Each plate was labeled with a code system that identified the test article, test phase, dose level, tester strain, and activation, as described in detail in BioReliance's Standard Operating Procedures.

Test article dilutions were prepared immediately before use. One-half milliliter (0.5 mL) of S9 mix or Sham mix, 100 μL of tester strain and 2.0 mL of vehicle or test article were added to 0.5 mL of top agar at 60$\pm$2°C. After vortexing, the mixture was overlaid onto the surface of 25 mL of minimal bottom agar. When plating the positive controls, the test article aliquot was replaced by a 2 mL aliquot of distilled water and a 50 μL aliquot of appropriate positive control. After the overlay had solidified, the plates were inverted and incubated for approximately 48 to 72 hours at 37$\pm$2°C. Plates that were not counted immediately following the incubation period were stored at 2-8°C until colony counting could be conducted.

The condition of the bacterial background lawn was evaluated for evidence of test article toxicity and precipitate by using a dissecting microscope. Toxicity and degree of precipitation were scored relative to the vehicle control plate using the codes shown on the following page.

Code	Description	Characteristics
1	Normal	Distinguished by a healthy microcolony lawn.
2	Slightly Reduced	Distinguished by a noticeable thinning of the microcolony lawn and possibly a slight increase in the size of the microcolonies compared to the vehicle control plate.
3	Moderately Reduced	Distinguished by a marked thinning of the microcolony lawn resulting in a pronounced increase in the size of the microcolonies compared to the vehicle control plate.
4	Severely Reduced	Distinguished by an extreme thinning of the microcolony lawn resulting in an increase in the size of the microcolonies compared to the vehicle control plate such that the microcolony lawn is visible to the unaided eye as isolated colonies.
5	Absent	Distinguished by a complete lack of any microcolony lawn over ≥90% of the plate.
6	Obscured by Precipitate	The background bacterial lawn cannot be accurately evaluated due to microscopic test article precipitate.
NP	Non-Interfering Precipitate	Distinguished by precipitate on the plate that is visible to the naked eye but any precipitate particles detected by the automated colony counter total less than 10% of the revertant colony count (e.g., ≤3 particles on a plate with 30 revertants.)
IP	Interfering Precipitate	Distinguished by precipitate on the plate that is visible to the naked eye and any precipitate particles detected by the automated colony counter exceed 10% of the revertant colony count (e.g., >3 particles on a plate with 30 revertants.)

Revertant colonies for a given tester strain and activation condition were counted either entirely by automated colony counter or entirely by hand unless the plate exhibited toxicity.

Evaluation of Results

For a test article to be evaluated positive, it must cause a dose-related increase in the mean revertants per plate of at least one tester strain with a minimum of two increasing concentrations of test article. Data sets for strains TA98 and TA100 were judged positive if the increase in mean revertants at the peak of the dose response is equal to or greater than two times the mean vehicle control value.

Criteria for a Valid Test

For each replicate plating, the mean and standard deviation of the number of revertants per plate were calculated and are reported.

The following criteria must be met for the mutagenicity assay to be considered valid. All tester strain cultures must demonstrate the presence of the deep rough mutation (*rfa*), the deletion in the *uvr*B gene and the presence of the pKM101 plasmid R-factor. All cultures must demonstrate the characteristic mean number of spontaneous revertants

in the vehicle controls as follows (inclusive): TA98, 10 - 50; TA100, 80 - 240. To ensure that appropriate numbers of bacteria are plated, tester strain culture titers must be greater than or equal to $0.3x10^9$ cells/ml. The mean of each positive control must exhibit at least a three-fold increase in the number of revertants over the mean value of the respective vehicle control. A minimum of three non-toxic dose levels are required to evaluate assay data. A dose level is considered toxic if one or both of the following criteria are met: (1) A >50 % reduction in the mean number of revertants per plate as compared to the mean vehicle control value. This reduction must be accompanied by an abrupt dose-dependent drop in the revertant count. (2) A reduction in the background lawn.

Archives

All raw data, the protocol and all report(s) will be maintained according to Standard Operating Procedure OPQP3040 by the BioReliance RAQA unit headquartered at: BioReliance, 14920 Broschart Road, Rockville, MD 20850.

RESULTS AND DISCUSSION

Solubility

Water was selected as the solvent of choice based on the Sponsor's request and compatibility with the target cells.

Mutagenicity Assay

The results of the mutagenicity assay are presented in Tables 1 through 4 and summarized in Table 5. These data were generated in Experiment B1. The maximum dose level plated in the mutagenicity assay was 2.0 mL of undiluted test article per plate. Subsequent dose levels were prepared by diluting the test article in water. These dilutions were soluble in water at 0.75 mL/mL, the most concentrated dilution prepared. Neither precipitate nor appreciable toxicity was observed.

In Experiment B1, no positive responses were observed with any of the tester strains in the presence and absence of S9 activation.

CONCLUSION

All criteria for a valid study were met as described in the protocol. The results of the *Salmonella* Plate Incorporation Mutagenicity Assay Using Water Samples indicate that under the conditions of this study, **Water Sample 99-028-6** did not cause a positive response with any of the tester strains in the presence and absence of Aroclor-induced rat liver S9.

REFERENCES

Ames, B.N., J. McCann and E. Yamasaki (1975) Methods for Detecting Carcinogens and Mutagens with the *Salmonella*/Mammalian Microsome Mutagenicity Test, Mutation Research, 31:347-364.

Maron, D.M. and B.N. Ames (1983) Revised Methods for the *Salmonella* Mutagenicity Test, Mutation Research, 113:173-215.

McCann, J. and B.N. Ames (1976) Detection of Carcinogens as Mutagens in the *Salmonella*/Microsome Test: Assay of 300 Chemicals: Discussion, Proc. Natl. Acad. Sci. USA, 73:950-954.

McCann, J., E. Choi, E. Yamasaki and B.N. Ames (1975) Detection of Carcinogens as Mutagens in the *Salmonella*/Microsome Test: Assay of 300 Chemicals, Proc. Natl. Acad. Sci. USA, 72:5135-5139.

Salmonella Mutagenicity Assay

Table 1

Test Article Id : Water Sample 99-028-6
Study Number : AA12JC.501006.BTL Experiment No : B1
Strain : TA98 Cells Seeded : 1.7×10^8
Liver Microsomes : None Date Plated : 02/03/99
Vehicle : water
Plating Aliquot : 2.0 mL Counted by : hand

Concentration mL per plate	Plate Number	Revertants per plate	Background Code[a]	Average Revertants	Standard Deviation
Vehicle	01	7	1		
	02	14	1	11	5
0.10	01	14	1		
	02	11	1	13	2
0.20	01	11	1		
	02	12	1	12	1
0.50	01	7	1		
	02	9	1	8	1
1.0	01	5	1		
	02	5	1	5	0
1.5	01	10	1		
	02	15	1	13	4
2.0	01	9	1		
	02	10	1	10	1
Positive Control 2-nitrofluorene 1.0 µg per plate[b]					
	01	367	1		
	02	559	1	463	136

[a]Background bacterial evaluation code
1=Normal 2=Slightly reduced 3=Moderately reduced
4=Extremely reduced 5=Absent 6=Obscured by precipitate
NP=Non-Interfering Precipitate IP=Interfering Precipitate
[b]Positive control plates were machine counted

Salmonella Mutagenicity Assay

Table 2

Test Article Id : Water Sample 99-028-6
Study Number : AA12JC.501006.BTL Experiment No : B1
Strain : TA98 Cells Seeded : 1.7 X 10^8
Liver Microsomes : Rat liver S9 Date Plated : 02/03/99
Vehicle : water
Plating Aliquot : 2.0 mL Counted by : hand

Concentration mL per plate	Plate Number	Revertants per plate	Background Code[a]	Average Revertants	Standard Deviation
Vehicle	01	12	1		
	02	10	1	11	1
0.10	01	20	1		
	02	20	1	20	0
0.20	01	19	1		
	02	6	1	13	9
0.50	01	18	1		
	02	14	1	16	3
1.0	01	14	1		
	02	13	1	14	1
1.5	01	15	1		
	02	9	1	12	4
2.0	01	13	1		
	02	13	1	13	0
Positive Control 2-aminoanthracene 1.0 μg per plate[b]					
	01	292	1		
	02	366	1	329	52

[a]Background bacterial evaluation code
1=Normal 2=Slightly reduced 3=Moderately reduced
4=Extremely reduced 5=Absent 6=Obscured by precipitate
NP=Non-Interfering Precipitate IP=Interfering Precipitate

[b]Positive control plates were machine counted

Salmonella Mutagenicity Assay

Table 3

Test Article Id	: Water Sample 99-028-6		
Study Number	: AA12JC.501006.BTL	Experiment No	: B1
Strain	: TA100	Cells Seeded	: 1.7 X 10^8
Liver Microsomes	: None	Date Plated	: 02/03/99
Vehicle	: water		
Plating Aliquot	: 2.0 mL	Counted by	: hand

Concentration mL per plate	Plate Number	Revertants per plate	Background Code[a]	Average Revertants	Standard Deviation
Vehicle	01	90	1		
	02	84	1	87	4
0.10	01	107	1		
	02	91	1	99	11
0.20	01	82	1		
	02	86	1	84	3
0.50	01	74	1		
	02	77	1	76	2
1.0	01	77	1		
	02	77	1	77	0
1.5	01	76	1		
	02	78	1	77	1
2.0	01	103	1		
	02	105	1	104	1
Positive Control sodium azide 1.0 μg per plate[b]					
	01	291	1		
	02	357	1	324	47

[a]Background bacterial evaluation code
1=Normal 2=Slightly reduced 3=Moderately reduced
4=Extremely reduced 5=Absent 6=Obscured by precipitate
NP=Non-Interfering Precipitate IP=Interfering Precipitate

[b]Positive control plates were machine counted

Salmonella Mutagenicity Assay

Table 4

Test Article Id	: Water Sample 99-028-6		
Study Number	: AA12JC.501006.BTL	Experiment No	: B1
Strain	: TA100	Cells Seeded	: 1.7 X 10^8
Liver Microsomes	: Rat liver S9	Date Plated	: 02/03/99
Vehicle	: water		
Plating Aliquot	: 2.0 mL	Counted by	: hand

Concentration mL per plate	Plate Number	Revertants per plate	Background Code[a]	Average Revertants	Standard Deviation
Vehicle	01	112	1		
	02	113	1	113	1
0.10	01	102	1		
	02	93	1	98	6
0.20	01	93	1		
	02	100	1	97	5
0.50	01	101	1		
	02	91	1	96	7
1.0	01	77	1		
	02	86	1	82	6
1.5	01	63	1		
	02	71	1	67	6
2.0	01	68	1		
	02	55	1	62	9
Positive Control 2-aminoanthracene 1.0 µg per plate[b]					
	01	399	1		
	02	332	1	366	47

[a]Background bacterial evaluation code

1=Normal 2=Slightly reduced 3=Moderately reduced
4=Extremely reduced 5=Absent 6=Obscured by precipitate
NP=Non-Interfering Precipitate IP=Interfering Precipitate

[b]Positive control plates were machine counted

Salmonella Mutagenicity Assay
Summary of Results

Table 5

Test Article Id : Water Sample 99-028-6
Study Number : AA12JC.501006.BTL Experiment No : B1

Average Revertants Per Plate ± Standard Deviation

Liver Microsomes: None

Dose (mL)	TA98	TA100
0.0	11 ± 5	87 ± 4
0.10	13 ± 2	99 ± 11
0.20	12 ± 1	84 ± 3
0.50	8 ± 1	76 ± 2
1.0	5 ± 0	77 ± 0
1.5	13 ± 4	77 ± 1
2.0	10 ± 1	104 ± 1
Pos	463 ± 136	324 ± 47

Liver Microsomes: Rat liver S9

Dose (mL)	TA98	TA100
0.0	11 ± 1	113 ± 1
0.10	20 ± 0	98 ± 6
0.20	13 ± 9	97 ± 5
0.50	16 ± 3	96 ± 7
1.0	14 ± 1	82 ± 6
1.5	12 ± 4	67 ± 6
2.0	13 ± 0	62 ± 9
Pos	329 ± 52	366 ± 47

0.0 = Vehicle plating aliquot of 2.0 mL
Pos = Positive Control concentrations as specified in Materials and Methods section.

APPENDIX I

Historical Control Data

Historical Negative and Positive Control Values
1995 - 1997

revertants per plate

Strain	Control	Activation							
		None				Rat Liver			
		Mean	SD	Min	Max	Mean	SD	Min	Max
TA98	Neg	17	7	3	52	23	8	4	65
	Pos	267	189	53	1416	815	426	69	2769
TA100	Neg	120	21	65	262	138	24	62	323
	Pos	570	161	104	2054	904	415	106	2813

SD = standard deviation; Min = minimum value; Max = maximum value; Neg = negative control (including but not limited to deionized water, dimethyl sulfoxide, ethanol and acetone); Pos = positive control

APPENDIX II

Study Protocol

APPROVED

Salmonella Plate Incorporation Mutagenicity Assay Using Water Samples

1.0 PURPOSE

The purpose of this study is to evaluate the mutagenic potential of the test article by measuring its ability to induce reverse mutations at selected loci of two strains of *Salmonella typhimurium* in the presence and absence of S9 activation.

2.0 SPONSOR

2.1 Name: **US Army**
Center for Environmental Health Research

2.2 Address: To be provided

2.3 Representative: **Maggie Toussaint**
Geo Centers

3.0 IDENTIFICATION OF TEST AND CONTROL SUBSTANCES

3.1 Test Article: Water Sample 99-028-6

3.2 Controls: Negative: Test article vehicle (deionized water)

Positive: 2-aminoanthracene
2-nitrofluorene
sodium azide

3.3 Determination of Strength, Purity, etc.

Unless alternate arrangements are made, the testing facility at MA will not perform analysis of the dosing solutions. The Sponsor will be directly responsible for determination and documentation of the analytical purity and composition of the test article, and the stability and strength of the test article in the solvent (or vehicle).

3.4 Test Article Retention Sample

The retention of a reserve sample of each batch of the test article will be the responsibility of the Sponsor.

4.0 TESTING FACILITY AND KEY PERSONNEL

4.1 Name: Toxicology Testing Facility
MA BioServices, Inc.

4.2 Address: 9630 Medical Center Drive
Rockville, MD 20850

4.3 Study Director: Valentine O. Wagner III, M.S.

5.0 TEST SCHEDULE

5.1 Proposed Experimental Initiation Date: 02/02/99

5.2 Proposed Experimental Completion Date: 03/02/99

5.3 Proposed Report Date: 03/17/99

6.0 TEST SYSTEM

The tester strains will include the *S. typhimurium* histidine auxotrophs TA98 and TA100 as described by Ames *et al.* (1975).

Genotype of the *S. typhimurium* Strains Used for Mutagen Testing

TA100	TA98	*rfa*	Δ*uvr*B	+R

Each tester strain contains, in addition to a mutation in the histidine operon, two additional mutations that enhance sensitivity to some mutagens. The *rfa* mutation results in a cell wall deficiency that increases the permeability of the cell to certain classes of chemicals such as those containing large ring systems that would otherwise be excluded. The second mutation is a deletion in the *uvr*B gene resulting in a deficient DNA excision-repair system.

Tester strains TA98 and TA100 also contain the pKM101 plasmid (carrying the R-factor). It has been suggested that the plasmid increases sensitivity to mutagens by modifying an existing bacterial DNA repair polymerase complex involved with the mismatch-repair process.

TA98 is reverted from histidine dependence (auxotrophy) to histidine independence (prototrophy) by frameshift mutagens. TA100 is reverted by both frameshift and base substitution mutagens.

The *S. typhimurium* tester strains were received directly from Dr. Bruce Ames, Department of Biochemistry, University of California, Berkeley.

7.0 EXPERIMENTAL DESIGN AND METHODOLOGY

The test article will be tested neat at a minimum of five dose levels along with appropriate vehicle and positive controls with tester strains TA98 and TA100 with and without S9

plated in duplicate.

7.1 Selection of Dose Levels

Unless indicated otherwise by the Sponsor, the dose levels for the initial assay will be 2.0, 1.5, 1.0, 0.5, 0.2 and 0.1 mL per plate.

7.2 Frequency and Route of Administration

The test system will be exposed to the test article via the plate incorporation methodology originally described by Ames *et al.* (1975) and updated by Maron and Ames (1983). This test system has been shown to detect a wide range of classes of chemical mutagens (McCann *et al.*, 1975; McCann and Ames, 1976).

7.3 Controls

7.3.1 Positive Controls

All combinations of positive controls and tester strains plated concurrently with the assay are listed below:

TA98, TA100	Rat	2-aminoanthracene	1.0
TA98	None	2-nitrofluorene	2.0
TA100		sodium azide	2.0

7.3.2 Vehicle Controls

Vehicle controls will be plated for each tester strain with and without S9 activation.

7.3.3 Sterility Controls

The most concentrated test article dilution and the Sham and S9 mixes will be checked for sterility.

7.4 Exogenous Metabolic Activation

Aroclor 1254-induced rat liver S9 will be used as the metabolic activation system. The S9 homogenate will be prepared from male Sprague-Dawley rats induced with a single intraperitoneal injection of Aroclor 1254, 500 mg/kg, five days prior to sacrifice. The S9 will be batch prepared and stored frozen at approximately -70°C until used. Each batch of S9 homogenate will be assayed for its ability to metabolize 2-aminoanthracene and 7,12-dimethylbenzanthracene to forms mutagenic to *S typhimurium* TA100.

Immediately prior to use, the S9 will be thawed and mixed with a cofactor pool to contain 10% S9 homogenate, 5 mM glucose-6-phosphate, 4 mM β-nicotinamide-adenine dinucleotide phosphate, 8 mM $MgCl_2$ and 33 mM KCl in a 100 mM phosphate buffer at pH 7.4. This mixture is referred to as S9 mix. Sham mix will be 100 mM phosphate buffer at pH 7.4.

7.5 Preparation of Tester Strain

Overnight cultures will be inoculated from the appropriate master plate or from the appropriate frozen stock. To ensure that cultures are harvested in late log phase, the length of incubation will be controlled and monitored. At the end of the working day, each inoculated flask will be placed in a resting shaker/incubator at room temperature. The shaker/incubator will be programmed to begin shaking at approximately 125 rpm at 37±2°C approximately 12 hours before the anticipated time of harvest.

All cultures will be harvested by spectrophotometric monitoring of culture turbidity rather than by duration of incubation since overgrowth of cultures can cause loss of sensitivity to some mutagens. Cultures will be removed from incubation at a density of approximately 10^9 cells/mL.

7.6 Test System Identification

Each plate will be labeled with a code system that identifies the test article, test phase, dose level, tester strain and activation type as described in MA BioServices, Inc.'s Standard Operating Procedures.

7.7 Test Article Preparation

Unless specified otherwise, test article dilutions will be prepared immediately prior to use. All test article dosing will be at room temperature under yellow light. The water sample will be filter sterilized prior to use.

7.8 Treatment of Test System

One-half milliliter (0.5 mL) of S9 mix or Sham mix, 100 µL of tester strain and 2.0 mL of vehicle or test article dilution will be added to 0.5 mL of molten selective top agar (54.4 g/L NaCl and 33.8 g/L agar supplemented with 56 mL of normal amino acid supplement per 100 mL) at 60±2°C. When plating the positive controls, the test article aliquot will be replaced with 2.0 mL of distilled water and a 50 µL aliquot of appropriate positive control. The mixture will be vortex mixed and overlaid onto the surface of 25 mL of minimal bottom agar. After the overlay has solidified, the plates will be inverted and incubated for approximately 48 to 72 hours at 37±2°C. Plates that are not counted immediately following the incubation period will be stored at 2-8°C.

The condition of the bacterial background lawn will be evaluated for evidence of test article toxicity and precipitate. Evidence of toxicity will be scored relative to the vehicle control plate and recorded along with the revertant count for that plate. Toxicity will be evaluated as a decrease in the number of revertant colonies per plate and/or a thinning or disappearance of the bacterial background lawn. Precipitation will be evaluated after the incubation period by visual examination without magnification.

7.10 Tester Strain Verification

On the day of use in the mutagenicity assay, all *S. typhimurium* tester strain cultures will be checked for the following genetic markers:

The presence of the *rfa* wall mutation will be confirmed by demonstrating sensitivity to crystal violet. The presence of the *uvrB* mutation will be confirmed by demonstrating sensitivity to ultraviolet light. The presence of the pKM101 plasmid will be confirmed for tester strains TA98 and TA100 by demonstrating resistance to ampicillin.

8.0 CRITERIA FOR DETERMINATION OF A VALID TEST

The following criteria must be met for the mutagenicity assay to be considered valid:

8.1 Tester Strain Integrity

To demonstrate the presence of the *rfa* mutation, all tester strain cultures must exhibit sensitivity to crystal violet. To demonstrate the presence of the *uvrB* mutation, all tester strain cultures must exhibit sensitivity to ultraviolet light. To demonstrate the presence of the pKM101 plasmid R-factor, tester strain cultures of TA98 and TA100 must exhibit resistance to ampicillin.

8.2 Spontaneous Revertant Background Frequency

Based on historical control data, all tester strain cultures must exhibit a characteristic number of spontaneous revertants per plate in the vehicle controls. The mean revertants per plate must be within the following ranges (inclusive): TA98, 10 - 50; TA100, 80 - 240.

8.3 Tester Strain Titers

To ensure that appropriate numbers of bacteria are plated, all tester strain culture titers must be equal to or greater than 0.3×10^9 cells per milliliter.

Each mean positive control value must exhibit at least a three-fold increase over the respective mean vehicle control value for each tester strain.

8.5 Toxicity

A minimum of three non-toxic dose levels will be required to evaluate assay data. A dose level is considered toxic if it causes a >50% reduction in the mean number of revertants per plate relative to the mean vehicle control value (this reduction must be accompanied by an abrupt dose-dependent drop in the revertant count) or a reduction in the background lawn. In the event that less than three non-toxic dose levels are achieved, the affected portion of the assay will be repeated with an appropriate change in dose levels.

9.0 EVALUATION OF TEST RESULTS

For a test article to be evaluated positive, it must cause a dose-related increase in the mean revertants per plate of at least one tester strain over a minimum of two increasing concentrations of test article. Data sets will be judged positive if the increase in mean revertants at the peak of the dose response is equal to or greater than two times the mean vehicle control value.

10.0 REPORT

A report of the results of this study will be prepared by the Testing Laboratory and will accurately describe all methods used for generation and analysis of the data. The report will include:

- Test substance: identification and CAS no., if known; physical nature and purity, if known; physicochemical properties relevant to the conduct of the study, if known; stability of test article, if known.
- Solvent/Vehicle: justification for choice of vehicle; solubility and stability of test article in solvent/vehicle, if known.
- Strains: strains used; number of cells/mL per culture; strain characteristics.
- Test conditions: amount of test substance per plate with rationale for dose selection and number of plates per concentration; media used; type and composition of metabolic activation system, including acceptability criteria; treatment procedures.
- Results: signs of toxicity; signs of precipitation; individual plate counts; the mean number of revertant colonies per plate and standard deviation; dose-response relationship, where possible; statistical analysis, if any; concurrent negative and positive control data means and standard deviations; historical negative and positive control data with ranges, means and standard deviation.

- Conclusion.

11.0 RECORDS AND ARCHIVES

Upon completion of the final report, all raw data and reports will be maintained in the archives of MA BioServices, Inc., Rockville, MD in accordance with the relevant Good Laboratory Practices Regulations.

12.0 REGULATORY REQUIREMENTS/GOOD LABORATORY PRACTICE

This study will be performed in compliance with the provisions of the Good Laboratory Practice Regulations for Nonclinical Laboratory Studies.

Will this study be submitted to a regulatory agency? YES

If so, to which agency or agencies? EPA - office of water (Research Pr

Unless arrangements are made to the contrary, unused dosing solutions will be disposed of following administration to the test system and all residual test article will be disposed of following finalization of the report.

13.0 REFERENCES

Ames, B.N., McCann, J. and Yamasaki, E. (1975). Methods for detecting carcinogens and mutagens with the *Salmonella*/mammalian-microsome mutagenicity test. Mutation Research *31*:347-364.

McCann, J. and Ames, B.N. (1976). Detection of carcinogens as mutagens in the *Salmonella*/microsome test: assay of 300 chemicals: discussion. Proc. Natl. Acad. Sci. USA 73:950-954.

McCann, J., Choi, E., Yamasaki, E. and Ames, B.N. (1975). Detection of carcinogens as mutagens in the *Salmonella*/microsome test: assay of 300 chemicals. Proc. Natl. Acad. Sci. USA 72:5135-5139.

Maron, D.M. and Ames, B.N. (1983). Revised Methods for the *Salmonella* Mutagenicity Test. Mutation Research 113:173-215.

Robert A. Finch for HSG 1-19-99

SPONSOR REPRESENTATIVE DATE

Robert A. Finch

(Print or Type Name)

(Henry S. Gardner)

STUDY DIRECTOR DATE

If submission to Japanese Regulatory Agency is indicated in section 12.0, MA management will sign.

Valentine O. Wagner, III 2-Feb-1999

MA STUDY MANAGEMENT DATE

FINAL REPORT

Study Title

***Salmonella* Preincubation Mutagenicity Assay Using Water Samples**

Test Article

Water Sample 99-050-6

Authors

Valentine O. Wagner, III, M.S.
Susan C. Twardzik, B.A.

Study Completion Date

April 26, 1999

Performing Laboratory

BioReliance
9630 Medical Center Drive
Rockville, MD 20850

Laboratory Study Number

AA13FK.501006.BTL

Sponsor

US Army
Center for Environmental Health Research
568 Doughten Drive
Fort Detrick, MD 21702-5010

STATEMENT OF COMPLIANCE

Study No. AA13FK.501006.BTL was conducted in compliance with the U.S. FDA Good Laboratory Practice Regulations as published in 21 CFR 58, the U.S. EPA GLP Standards 40 CFR 160 and 40 CFR 792, the UK GLP Compliance Programme, the Japanese GLP Standard and the OECD Principles of Good Laboratory Practice in all material aspects with the following exceptions:

The identity, strength, purity and composition or other characteristics to define the test or control article have not been determined by the testing facility.

Analyses to determine the uniformity, concentration, or stability of the test or control mixtures were not performed by the testing facility.

The stability of the test or control article under the test conditions has not been determined by the testing facility.

Valentine O. Wagner, III
Valentine O. Wagner, III, M.S.
Study Director

26-Apr-1999
Date

QUALITY ASSURANCE STATEMENT

Study Title: *SALMONELLA* PREINCUBATION MUTAGENICITY ASSAY USING WATER SAMPLES

Study Number: AA13FK.501006.BTL

Study Director: Valentine O. Wagner, III, M.S.

This study has been divided into a series of in-process phases. Using a random sampling approach, Quality Assurance monitors each of these phases over a series of studies. Procedures, documentation, equipment records, etc., are examined in order to assure that the study is performed in accordance with the U.S. FDA Good Laboratory Practice Regulations (21 CFR 58), the U.S. EPA GLPs (40 CFR 792 and 40 CFR 160), the UK GLP Regulations, the Japanese GLP Standard, and the OECD Principles of Good Laboratory Practice and to assure that the study is conducted according to the protocol and relevant Standard Operating Procedures.

The following are the inspection dates, phases inspected, and report dates of QA inspections of this study.

INSPECT ON 24 FEB 99, TO STUDY DIR 24 FEB 99, TO MGMT 24 FEB 99
PHASE: Protocol Review

INSPECT ON 25 FEB 99, TO STUDY DIR 26 FEB 99, TO MGMT 26 FEB 99
PHASE: Test and/or control material administration

INSPECT ON 05 APR 99, TO STUDY DIR 05 APR 99, TO MGMT 09 APR 99
PHASE: Draft Report

INSPECT ON 28 APR 99, TO STUDY DIR 28 APR 99, TO MGMT 28 APR 99
PHASE: Draft to Final Report

This report describes the methods and procedures used in the study and the reported results accurately reflect the raw data of the study.

Becky D. Schreckengost 4/28/99

Becky D. Schreckengost, B.S.
QUALITY ASSURANCE DATE

Salmonella Preincubation Mutagenicity Assay Using Water Samples

FINAL REPORT

Sponsor:	**US Army** **Center for Environmental Health Research** **568 Doughten Drive** **Fort Detrick, MD 21702-5010**
Authorized Representative:	**Maggie Toussaint** **Geo Centers**
Performing Laboratory:	**BioReliance** **9630 Medical Center Drive** **Rockville, Maryland 20850**
Test Article I.D.:	**Water Sample 99-050-6**
BioReliance Study No.:	**AA13FK.501006.BTL**
Test Article Description:	**clear, colorless liquid**
Storage Conditions:	**room temperature; protected from exposure to light**
Test Article Receipt:	**February 19, 1999**
Study Initiation:	**February 22, 1999**
Study Director:	Valentine O. Wagner, III 26-Apr-1999 Valentine O. Wagner, III, M.S. Date

TABLE OF CONTENTS

SUMMARY

The test article, Water Sample 99-050-6, was tested in the *Salmonella* Preincubation Mutagenicity Assay with tester strains TA98 and TA100 in the presence and absence of Aroclor-induced rat liver S9. The assay was performed using the preincubation method. The mutagenicity assay was used to evaluate the mutagenic potential of the test article.

Deionized water was selected as the solvent of choice based on Sponsor's request and compatibility with the target cells.

The maximum dose level plated in the mutagenicity assay was 2.0 mL of undiluted test article per plate. Subsequent dose levels were prepared by diluting the test article in deionized water. These dilutions were soluble in deionized water at 0.75 mL/mL, the most concentrated dilution prepared.

In the mutagenicity assay, no positive response was observed. Neither precipitate nor appreciable toxicity was observed. The overall evaluation and dose ranges tested are as follows:

S9 Activation	Overall Evaluation[a] and Dose Range Tested (mL/plate)			
	TA98		TA100	
	Low	High	Low	High
None	-		-	
	0.10	2.0	0.10	2.0
Rat	-		-	
	0.10	2.0	0.10	2.0

[a] - = negative, + = positive (maximum fold increase)

Under the conditions of this study, test article **Water Sample 99-050-6** was concluded to be negative in the *Salmonella* Preincubation Mutagenicity Assay.

PURPOSE

The purpose of this study was to evaluate the mutagenic potential of the test article by measuring its ability to induce reverse mutations at selected loci of two strains of *Salmonella typhimurium* in the presence and absence of S9 activation.

CHARACTERIZATION OF TEST AND CONTROL ARTICLES

The test article, Water Sample 99-050-6, was received by BioReliance on February 19, 1999 and was assigned the code number AA13FK. The test article was characterized by the Sponsor as chlorinated drinking water that should be stored at ambient temperature. An expiration date of February 26, 1999 was provided. Upon receipt, the test article was described as a clear, colorless liquid and was stored at room temperature, protected from exposure to light.

The vehicle used to deliver Water Sample 99-050-6 to the test system was sterile deionized distilled water, (CAS# 7732-18-5), obtained from Life Technologies, Inc.

Positive controls plated concurrently with the mutagenicity assay are listed below:

Strain	S9 Activation	Positive Control	Concentration (µg/plate)
All *Salmonella* Strains	+	2-aminoanthracene (Sigma Chemical Co.)	1.0
TA98	-	2-nitrofluorene (Aldrich Chemical Co., Inc.)	2.0
TA100	-	sodium azide (Sigma Chemical Co.)	2.0

To determine the sterility of the test article, the highest test article dose level used in the mutagenicity assay was plated on selective agar with an aliquot volume equal to that used in the assay.

MATERIALS AND METHODS

Test System

The tester strains used were the *Salmonella typhimurium* histidine auxotrophs TA98 and TA100 as described by Ames *et al.* (1975). *Salmonella* tester strains were received on 08/11/98 and 11/10/98 directly from Dr. Bruce Ames, University of California, Berkeley.

Tester strain TA98 is reverted from histidine dependence (auxotrophy) to histidine independence (prototrophy) by frameshift mutagens. Tester strain TA100 is reverted by mutagens that cause both frameshift and basepair substitution mutations.

Overnight cultures were prepared by inoculating from the appropriate master plate or from the appropriate frozen permanent stock into a vessel containing ~50 mL of culture medium. To assure that cultures were harvested in late log phase, the length of incubation was controlled and monitored. Following inoculation, each flask was placed in a resting shaker/incubator at room temperature. The shaker/incubator was programmed to begin shaking at approximately 125 rpm at 37±2°C approximately 12 hours before the anticipated time of harvest. Each culture was monitored spectrophotometrically for turbidity and was harvested at a percent transmittance yielding a titer of approximately 10^9 cells per milliliter. The actual titers were determined by viable count assays on nutrient agar plates.

Metabolic Activation System

Aroclor 1254-induced rat liver S9 was used as the metabolic activation system. The S9 was prepared from male Sprague-Dawley rats induced with a single intraperitoneal injection of Aroclor 1254, 500 mg/kg, five days prior to sacrifice. The S9 was batch prepared on 02/04/99 and stored at ≤-70°C until used. Each bulk preparation of S9 was assayed for its ability to metabolize 2-aminoanthracene and 7,12-dimethylbenz(a)anthracene to forms mutagenic to *Salmonella typhimurium* TA100.

The S9 mix was prepared immediately before its use and contained 10% S9, 5 mM glucose-6-phosphate, 4 mM β-nicotinamide-adenine dinucleotide phosphate, 8 mM $MgCl_2$ and 33 mM KCl in a 100 mM phosphate buffer at pH 7.4. The Sham S9 mixture (Sham mix), containing 100 mM phosphate buffer at pH 7.4, was prepared immediately before its use. To confirm the sterility of the S9 and Sham mixes, a 0.5 mL aliquot of each was plated on selective agar.

Mutagenicity Assay

The mutagenicity assay was used to evaluate the mutagenic potential of the test article. A minimum of five dose levels of test article along with appropriate vehicle and positive controls were plated with tester strains TA98 and TA100 in the presence and absence of rat liver S9 activation. All dose levels of test article, vehicle controls and positive controls were plated in duplicate.

Plating and Scoring Procedures

The test system was exposed to the test article via the preincubation methodology described by Yahagi *et* al. (1977).

On the day of its use, minimal top agar, containing 0.8 % agar (W/V) and 0.5 % NaCl (W/V), was melted and supplemented with L-histidine, D-biotin and L-tryptophan solution to a final concentration of 50 μM each. Top agar not used with S9 or Sham mix was supplemented with 25 mL of water for each 100 mL of minimal top agar. For the preparation of media and reagents, all references to water imply sterile, deionized water produced by the Milli-Q Reagent Water System. Bottom agar was Vogel-Bonner minimal medium E (Vogel and Bonner, 1956) containing 1.5 % (W/V) agar. Nutrient bottom agar was Vogel-Bonner minimal medium E containing 1.5 % (W/V) agar and supplemented with 2.5 % (W/V) Oxoid Nutrient Broth No. 2 (dry powder). Nutrient Broth was Vogel-Bonner salt solution supplemented with 2.5 % (W/V) Oxoid Nutrient Broth No. 2 (dry powder).

Each plate was labeled with a code system that identified the test article, test phase, dose level, tester strain, and activation, as described in detail in BioReliance's Standard Operating Procedures.

Test article dilutions were prepared immediately before use. One-half (0.5) milliliter of S9 or sham mix, 100 μL of tester strain and 2.0 mL of vehicle or test article were added to 13 X 100 mm glass culture tubes pre-heated to 37$\pm$2°C. After vortexing, these mixtures were incubated with shaking for 60$\pm$2 minutes at 37$\pm$2°C. Following the preincubation, 2.0 mL of selective top agar was added to each tube and the mixture was vortexed and overlaid onto the surface of 25 mL of minimal bottom agar. When plating the positive controls, the test article aliquot was replaced by a 50 or 100 μL aliquot of appropriate positive control. After the overlay had solidified, the plates were inverted and incubated for approximately 48 to 72 hours at 37$\pm$2°C. Plates that were not counted immediately following the incubation period were stored at 2-8°C until colony counting could be conducted.

The condition of the bacterial background lawn was evaluated for evidence of test article toxicity by using a dissecting microscope. Precipitate was evaluated by visual examination without magnification. Toxicity and degree of precipitation were scored relative to the vehicle control plate using the codes shown below.

Code	Description	Characteristics
1	Normal	Distinguished by a healthy microcolony lawn.
2	Slightly Reduced	Distinguished by a noticeable thinning of the microcolony lawn and possibly a slight increase in the size of the microcolonies compared to the vehicle control plate.
3	Moderately Reduced	Distinguished by a marked thinning of the microcolony lawn resulting in a pronounced increase in the size of the microcolonies compared to the vehicle control plate.
4	Severely Reduced	Distinguished by an extreme thinning of the microcolony lawn resulting in an increase in the size of the microcolonies compared to the vehicle control plate such that the microcolony lawn is visible to the unaided eye as isolated colonies.
5	Absent	Distinguished by a complete lack of any microcolony lawn over ≥90% of the plate.
6	Obscured by Precipitate	The background bacterial lawn cannot be accurately evaluated due to microscopic test article precipitate.
NP	Non-Interfering Precipitate	Distinguished by precipitate on the plate that is visible to the naked eye but any precipitate particles detected by the automated colony counter total less than 10% of the revertant colony count (e.g., ≤3 particles on a plate with 30 revertants.)
IP	Interfering Precipitate	Distinguished by precipitate on the plate that is visible to the naked eye and any precipitate particles detected by the automated colony counter exceed 10% of the revertant colony count (e.g., >3 particles on a plate with 30 revertants.)

Revertant colonies for a given tester strain and activation condition, except for positive controls, were counted either entirely by automated colony counter or entirely by hand unless the assay was the preliminary toxicity assay or the plate exhibited toxicity. Plates with sufficient test article precipitate to interfere with automated colony counting were counted manually.

Evaluation of Results

For each replicate plating, the mean and standard deviation of the number of revertants per plate were calculated and are reported.

For the test article to be evaluated positive, it must cause a dose-related increase in the mean revertants per plate of at least one tester strain with a minimum of two increasing concentrations of test article. Data sets for strains TA98 and TA100 were judged positive if the increase in mean revertants at the peak of the dose response is equal to or greater than two times the mean vehicle control value.

Criteria for a Valid Test

The following criteria must be met for the mutagenicity assay to be considered valid. All *Salmonella* tester strain cultures must demonstrate the presence of the deep rough mutation (*rfa*) and the deletion in the *uvr*B gene. Cultures of tester strains TA98 and

TA100 must demonstrate the presence of the pKM101 plasmid R-factor. All cultures must demonstrate the characteristic mean number of spontaneous revertants in the vehicle controls as follows (inclusive): TA98, 10 - 50; TA100, 80 - 240. To ensure that appropriate numbers of bacteria are plated, tester strain culture titers must be greater than or equal to $0.3x10^9$ cells/mL. The mean of each positive control must exhibit at least a three-fold increase in the number of revertants over the mean value of the respective vehicle control. A minimum of three non-toxic dose levels are required to evaluate assay data. A dose level is considered toxic if one or both of the following criteria are met: (1) A >50 % reduction in the mean number of revertants per plate as compared to the mean vehicle control value. This reduction must be accompanied by an abrupt dose-dependent drop in the revertant count. (2) A reduction in the background lawn.

Archives

All raw data, the protocol and all reports will be maintained according to Standard Operating Procedure OPQP3040 by the BioReliance RAQA unit headquartered at: BioReliance, 14920 Broschart Road, Rockville, MD 20850.

RESULTS AND DISCUSSION

Solubility

Deionized water was selected as the solvent of choice based on the Sponsor's request and compatibility with the target cells.

Mutagenicity Assay

The maximum dose level plated in the mutagenicity assay was 2.0 mL of undiluted test article per plate. Subsequent dose levels were prepared by diluting the test article in deionized water. These dilutions were soluble in deionized water at 0.75 mL/mL, the most concentrated dilution prepared.

The results of the mutagenicity assay are presented in Tables 1 through 4 and summarized in Table 5. These data were generated in Experiment B1. Neither precipitate nor appreciable toxicity was observed.

In Experiment B1, no positive responses were observed with any of the tester strains in the presence and absence of S9 activation.

CONCLUSION

All criteria for a valid study were met as described in the protocol. The results of the *Salmonella* Preincubation Mutagenicity Assay Using Water Samples indicate that, under the conditions of this study, **Water Sample 99-050-6** did not cause a positive response with any of the tester strains in the presence and absence of Aroclor-induced rat liver S9.

REFERENCES

Ames, B.N., J. McCann and E. Yamasaki (1975) Methods for Detecting Carcinogens and Mutagens with the *Salmonella*/Mammalian Microsome Mutagenicity Test, Mutation Research, 31:347-364.

Yahagi, M., Nagao, Y., Seino, T., Sugimura, T. and Okada, M. (1977). Mutagenicities of N-nitrosamines on *Salmonella*. Mutation Research 48:121-130.

Salmonella Mutagenicity Assay

Table 1

Test Article Id : Water Sample 99-050-6
Study Number : AA13FK.501006.BTL
Strain : TA98
Liver Microsomes : None
Vehicle : water
Plating Aliquot : 2.0 mL
Experiment No : B1
Cells Seeded : 7.6×10^8
Date Plated : 02/25/99
Counted by : hand

Concentration mL per plate	Plate Number	Revertants per plate	Background Code[a]	Average Revertants	Standard Deviation
Vehicle	01	13	1		
	02	16	1	15	2
0.10	01	12	1		
	02	18	1	15	4
0.20	01	12	1		
	02	5	1	9	5
0.50	01	9	1		
	02	13	1	11	3
1.0	01	10	1		
	02	12	1	11	1
1.5	01	13	1		
	02	27	1	20	10
2.0	01	24	1		
	02	18	1	21	4
Positive Control 2-nitrofluorene 2.0 μg per plate[b]					
	01	1021	1		
	02	927	1	974	66

[a]Background bacterial evaluation code
1=Normal 2=Slightly reduced 3=Moderately reduced
4=Extremely reduced 5=Absent 6=Obscured by precipitate
NP=Non-Interfering Precipitate IP=Interfering Precipitate

[b]Positive control plates were machine counted

Salmonella Mutagenicity Assay

Table 2

Test Article Id : Water Sample 99-050-6
Study Number : AA13FK.501006.BTL
Strain : TA98
Liver Microsomes : Rat liver S9
Vehicle : water
Plating Aliquot : 2.0 mL

Experiment No : B1
Cells Seeded : 7.6 X 10^8
Date Plated : 02/25/99
Counted by : hand

Concentration mL per plate	Plate Number	Revertants per plate	Background Code[a]	Average Revertants	Standard Deviation
Vehicle	01	18	1		
	02	14	1	16	3
0.10	01	17	1		
	02	24	1	21	5
0.20	01	20	1		
	02	17	1	19	2
0.50	01	13	1		
	02	18	1	16	4
1.0	01	16	1		
	02	20	1	18	3
1.5	01	20	1		
	02	17	1	19	2
2.0	01	17	1		
	02	27	1	22	7
Positive Control 2-aminoanthracene 1.0 µg per plate[b]					
	01	748	1		
	02	542	1	645	146

[a]Background bacterial evaluation code
1=Normal 2=Slightly reduced 3=Moderately reduced
4=Extremely reduced 5=Absent 6=Obscured by precipitate
NP=Non-Interfering Precipitate IP=Interfering Precipitate

[b]Positive control plates were machine counted

Salmonella Mutagenicity Assay

Table 3

Test Article Id : Water Sample 99-050-6
Study Number : AA13FK.501006.BTL Experiment No : B1
Strain : TA100 Cells Seeded : 1.9 X 10^8
Liver Microsomes : None Date Plated : 02/25/99
Vehicle : water
Plating Aliquot : 2.0 mL Counted by : machine

Concentration mL per plate	Plate Number	Revertants per plate	Background Code[a]	Average Revertants	Standard Deviation
Vehicle	01	189	1		
	02	162	1	176	19
0.10	01	195	1		
	02	200	1	198	4
0.20	01	211	1		
	02	182	1	197	21
0.50	01	169	1		
	02	213	1	191	31
1.0	01	227	1		
	02	189	1	208	27
1.5	01	226	1		
	02	197	1	212	21
2.0	01	224	1		
	02	215	1	220	6
Positive Control sodium azide 2.0 μg per plate					
	01	1158	1		
	02	1196	1	1177	27

[a]Background bacterial evaluation code
1=Normal 2=Slightly reduced 3=Moderately reduced
4=Extremely reduced 5=Absent 6=Obscured by precipitate
NP=Non-Interfering Precipitate IP=Interfering Precipitate

Salmonella Mutagenicity Assay

Table 4

Test Article Id : Water Sample 99-050-6
Study Number : AA13FK.501006.BTL
Experiment No : B1
Strain : TA100
Cells Seeded : 1.9 X 10^8
Liver Microsomes : Rat liver S9
Date Plated : 02/25/99
Vehicle : water
Plating Aliquot : 2.0 mL
Counted by : machine

Concentration mL per plate	Plate Number	Revertants per plate	Background Code[a]	Average Revertants	Standard Deviation
Vehicle	01	213	1		
	02	191	1	202	16
0.10	01	162	1		
	02	200	1	181	27
0.20	01	195	1		
	02	210	1	203	11
0.50	01	205	1		
	02	218	1	212	9
1.0	01	218	1		
	02	202	1	210	11
1.5	01	217	1		
	02	201	1	209	11
2.0	01	196	1		
	02	230	1	213	24
Positive Control 2-aminoanthracene 1.0 µg per plate					
	01	644	1		
	02	587	1	616	40

[a]Background bacterial evaluation code
1=Normal 2=Slightly reduced 3=Moderately reduced
4=Extremely reduced 5=Absent 6=Obscured by precipitate
NP=Non-Interfering Precipitate IP=Interfering Precipitate

Salmonella Mutagenicity Assay
Summary of Results

Table 5

Test Article Id : Water Sample 99-050-6
Study Number : AA13FK.501006.BTL Experiment No : B1

Average Revertants Per Plate ± Standard Deviation

Liver Microsomes: None

Dose (mL)	TA98	TA100
0.0	15 ± 2	176 ± 19
0.10	15 ± 4	198 ± 4
0.20	9 ± 5	197 ± 21
0.50	11 ± 3	191 ± 31
1.0	11 ± 1	208 ± 27
1.5	20 ± 10	212 ± 21
2.0	21 ± 4	220 ± 6
Pos	974 ± 66	1177 ± 27

Liver Microsomes: Rat liver S9

Dose (mL)	TA98	TA100
0.0	16 ± 3	202 ± 16
0.10	21 ± 5	181 ± 27
0.20	19 ± 2	203 ± 11
0.50	16 ± 4	212 ± 9
1.0	18 ± 3	210 ± 11
1.5	19 ± 2	209 ± 11
2.0	22 ± 7	213 ± 24
Pos	645 ± 146	616 ± 40

0.0 = Vehicle plating aliquot of 2.0 mL
Pos = Positive Control concentrations as specified below in the Materials and Methods.

APPENDIX I

Historical Control Data

Historical Negative and Positive Control Values
1995 - 1997

revertants per plate

Strain	Control	Activation							
		None				Rat Liver			
		Mean	SD	Min	Max	Mean	SD	Min	Max
TA98	Neg	17	7	3	52	23	8	4	65
	Pos	267	189	53	1416	815	426	69	2769
TA100	Neg	120	21	65	262	138	24	62	323
	Pos	570	161	104	2054	904	415	106	2813

SD = standard deviation; Min = minimum value; Max = maximum value; Neg = negative control (including but not limited to deionized water, dimethyl sulfoxide, ethanol and acetone); Pos = positive control

APPENDIX II

Study Protocol

2-24-99
APPROVED

PROTOCOL AMENDMENT 1

Sponsor: **US Army Center for Environmental Health Research**

Test Article I.D.: **Water Sample 99-050-6**

BioReliance Study No.: **AA13FK.501006.BTL**

Protocol Title: ***Salmonella* Preincubation Mutagenicity Assay Using Water Samples**

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1. **LOCATION:** Page 1; Title

   AMENDMENT: Replace "Plate Incorporation " with "Preincubation".

   REASON FOR THE AMENDMENT: Sponsor's request to enable better detection of volatile components in the sample.

2. **LOCATION:** Page 3, §7.2; Frequency and Route of Administration

   AMENDMENT: The test system will be exposed to the test article via the preincubation modification of the Ames test described by Yahagi *et al.* (1977). This test system has been shown to detect a wide range of classes of chemical mutagens (McCann *et al.*, 1975; McCann and Ames, 1976).

   One-half milliliter (0.5 mL) of S9 mix or sham mix will be added to pre-heated 13 x 100 mm glass culture tubes (screw-cap with a teflon-lined cap). To these tubes will be added 100 µL of tester strain and 2 mL of vehicle or test article dilution or 50 µL of positive control. After vortexing, the mixture will be allowed to incubate for 60±2 minutes at 37±2°C. Two milliliters of selective top agar (normal concentration) will then be added to each tube and the mixture will be overlaid onto the surface of 25 mL of minimal bottom agar. After the overlay has solidified, the plates will be inverted and incubated for approximately 48 to 72 hours at 37±2°C. Plates that are not counted immediately following the incubation period will be stored at 2-8°C.

   Yahagi, T., Nagao, M., Seino, Y., Matsushima, T., Sugimura, T. and Okada, M. (1977). Mutagenicities of N-nitrosamines on *Salmonella*, Mutation Research 48:121-130.

   REASON FOR THE AMENDMENT: Sponsor's request to enable better detection of volatile components in the sample.

BIORELIANCE
Formerly Microbiotest

3. **LOCATION:** Page 4, §7.7; Test Article Preparation

AMENDMENT: The water sample will not be filter sterilized prior to use.

REASON FOR THE AMENDMENT: Sponsor's request to enable better detection of volatile components in the sample.

APPROVALS:

Valentine O. Wagner, III — STUDY DIRECTOR — 24-Feb-1999 — DATE

Robert A. Hirsch — SPONSOR REPRESENTATIVE — 3-3-99 — DATE

MA Study Number: AA13FK.501006.BTL

Salmonella Plate Incorporation Mutagenicity Assay Using Water Samples

1.0 PURPOSE

The purpose of this study is to evaluate the mutagenic potential of the test article by measuring its ability to induce reverse mutations at selected loci of two strains of *Salmonella typhimurium* in the presence and absence of S9 activation.

2.0 SPONSOR

2.1 Name: **US Army**
Center for Environmental Health Research

2.2 Address: **568 Doughten Drive**
Fort Detrick, MD 21702-5010

2.3 Representative: **Maggie Toussaint**
Geo Centers

3.0 IDENTIFICATION OF TEST AND CONTROL SUBSTANCES

3.1 Test Article: **Water Sample 99-050-6**

3.2 Controls: Negative: Test article vehicle (deionized water)

Positive: 2-aminoanthracene
2-nitrofluorene
sodium azide

3.3 Determination of Strength, Purity, etc.

Unless alternate arrangements are made, the testing facility at MA will not perform analysis of the dosing solutions. The Sponsor will be directly responsible for determination and documentation of the analytical purity and composition of the test article, and the stability and strength of the test article in the solvent (or vehicle).

3.4 Test Article Retention Sample

The retention of a reserve sample of each batch of the test article will be the responsibility of the Sponsor.

4.0 TESTING FACILITY AND KEY PERSONNEL

4.1 Name: Toxicology Testing Facility
MA BioServices, Inc.

4.2 Address: 9630 Medical Center Drive
Rockville, MD 20850

4.3 Study Director: Valentine O. Wagner III, M.S.

5.0 TEST SCHEDULE

5.1 Proposed Experimental Initiation Date: 03/25/99

5.2 Proposed Experimental Completion Date: 03/25/99

5.3 Proposed Report Date: 04/08/99

6.0 TEST SYSTEM

The tester strains will include the *S. typhimurium* histidine auxotrophs TA98 and TA100 as described by Ames *et al.* (1975).

Genotype of the *S. typhimurium* Strains Used for Mutagen Testing

Histidine Mutation		Additional Mutations		
Frameshift Reversion		LPS	Repair	R-factor
TA100	TA98	*rfa*	Δ*uvr*B	+R

Each tester strain contains, in addition to a mutation in the histidine operon, two additional mutations that enhance sensitivity to some mutagens. The *rfa* mutation results in a cell wall deficiency that increases the permeability of the cell to certain classes of chemicals such as those containing large ring systems that would otherwise be excluded. The second mutation is a deletion in the *uvr*B gene resulting in a deficient DNA excision-repair system.

Tester strains TA98 and TA100 also contain the pKM101 plasmid (carrying the R-factor). It has been suggested that the plasmid increases sensitivity to mutagens by modifying an existing bacterial DNA repair polymerase complex involved with the mismatch-repair process.

TA98 is reverted from histidine dependence (auxotrophy) to histidine independence (prototrophy) by frameshift mutagens. TA100 is reverted by both frameshift and base substitution mutagens.

The *S. typhimurium* tester strains were received directly from Dr. Bruce Ames, Department of Biochemistry, University of California, Berkeley.

7.0 EXPERIMENTAL DESIGN AND METHODOLOGY

The test article will be tested neat at a minimum of five dose levels along with appropriate vehicle and positive controls with tester strains TA98 and TA100 with and without S9

activation. All dose levels of test article, vehicle controls and positive controls will be plated in duplicate.

7.1 Selection of Dose Levels

Unless indicated otherwise by the Sponsor, the dose levels for the initial assay will be 2.0, 1.5, 1.0, 0.5, 0.2 and 0.1 mL per plate.

7.2 Frequency and Route of Administration

The test system will be exposed to the test article via the plate incorporation methodology originally described by Ames *et al.* (1975) and updated by Maron and Ames (1983). This test system has been shown to detect a wide range of classes of chemical mutagens (McCann *et al.*, 1975; McCann and Ames, 1976).

7.3 Controls

7.3.1 Positive Controls

All combinations of positive controls and tester strains plated concurrently with the assay are listed below:

[illegible]	Activation	[illegible]	Concentration (µg/plate)
TA98, TA100	Rat	2-aminoanthracene	1.0
TA98	None	2-nitrofluorene	2.0
TA100		sodium azide	2.0

7.3.2 Vehicle Controls

Vehicle controls will be plated for each tester strain with and without S9 activation.

7.3.3 Sterility Controls

The most concentrated test article dilution and the Sham and S9 mixes will be checked for sterility.

7.4 Exogenous Metabolic Activation

Aroclor 1254-induced rat liver S9 will be used as the metabolic activation system. The S9 homogenate will be prepared from male Sprague-Dawley rats induced with a single intraperitoneal injection of Aroclor 1254, 500 mg/kg, five days prior to sacrifice. The S9 will be batch prepared and stored frozen at approximately -70°C until used. Each batch of S9 homogenate will be assayed for its ability to metabolize 2-aminoanthracene and 7,12-dimethylbenzanthracene to forms mutagenic to *S. typhimurium* TA100.

Immediately prior to use, the S9 will be thawed and mixed with a cofactor pool to contain 10% S9 homogenate, 5 mM glucose-6-phosphate, 4 mM β-nicotinamide-adenine dinucleotide phosphate, 8 mM $MgCl_2$ and 33 mM KCl in a 100 mM phosphate buffer at pH 7.4. This mixture is referred to as S9 mix. Sham mix will be 100 mM phosphate buffer at pH 7.4.

7.5 Preparation of Tester Strain

Overnight cultures will be inoculated from the appropriate master plate or from the appropriate frozen stock. To ensure that cultures are harvested in late log phase, the length of incubation will be controlled and monitored. At the end of the working day, each inoculated flask will be placed in a resting shaker/incubator at room temperature. The shaker/incubator will be programmed to begin shaking at approximately 125 rpm at 37±2°C approximately 12 hours before the anticipated time of harvest.

All cultures will be harvested by spectrophotometric monitoring of culture turbidity rather than by duration of incubation since overgrowth of cultures can cause loss of sensitivity to some mutagens. Cultures will be removed from incubation at a density of approximately 10^9 cells/mL.

7.6 Test System Identification

Each plate will be labeled with a code system that identifies the test article, test phase, dose level, tester strain and activation type as described in MA BioServices, Inc.'s Standard Operating Procedures.

7.7 Test Article Preparation

Unless specified otherwise, test article dilutions will be prepared immediately prior to use. All test article dosing will be at room temperature under yellow light. The water sample will be filter sterilized prior to use.

7.8 Treatment of Test System

One-half milliliter (0.5 mL) of S9 mix or Sham mix, 100 µL of tester strain and 2.0 mL of vehicle or test article dilution will be added to 0.5 mL of molten selective top agar (54.4 g/L NaCl and 33.8 g/L agar supplemented with 56 mL of normal amino acid supplement per 100 mL) at 60±2°C. When plating the positive controls, the test article aliquot will be replaced with 2.0 mL of distilled water and a 50 µL aliquot of appropriate positive control. The mixture will be vortex mixed and overlaid onto the surface of 25 mL of minimal bottom agar. After the overlay has solidified, the plates will be inverted and incubated for approximately 48 to 72 hours at 37±2°C. Plates that are not counted immediately following the incubation period will be stored at 2-8°C.

7.9 Colony Counting

The condition of the bacterial background lawn will be evaluated for evidence of test article toxicity and precipitate. Evidence of toxicity will be scored relative to the vehicle control plate and recorded along with the revertant count for that plate. Toxicity will be evaluated as a decrease in the number of revertant colonies per plate and/or a thinning or disappearance of the bacterial background lawn. Precipitation will be evaluated after the incubation period by visual examination without magnification.

7.10 Tester Strain Verification

On the day of use in the mutagenicity assay, all *S. typhimurium* tester strain cultures will be checked for the following genetic markers:

The presence of the *rfa* wall mutation will be confirmed by demonstrating sensitivity to crystal violet. The presence of the *uvr*B mutation will be confirmed by demonstrating sensitivity to ultraviolet light. The presence of the pKM101 plasmid will be confirmed for tester strains TA98 and TA100 by demonstrating resistance to ampicillin.

8.0 CRITERIA FOR DETERMINATION OF A VALID TEST

The following criteria must be met for the mutagenicity assay to be considered valid:

8.1 Tester Strain Integrity

To demonstrate the presence of the *rfa* mutation, all tester strain cultures must exhibit sensitivity to crystal violet. To demonstrate the presence of the *uvr*B mutation, all tester strain cultures must exhibit sensitivity to ultraviolet light. To demonstrate the presence of the pKM101 plasmid R-factor, tester strain cultures of TA98 and TA100 must exhibit resistance to ampicillin.

8.2 Spontaneous Revertant Background Frequency

Based on historical control data, all tester strain cultures must exhibit a characteristic number of spontaneous revertants per plate in the vehicle controls. The mean revertants per plate must be within the following ranges (inclusive): TA98, 10 - 50; TA100, 80 - 240.

8.3 Tester Strain Titers

To ensure that appropriate numbers of bacteria are plated, all tester strain culture titers must be equal to or greater than 0.3×10^9 cells per milliliter.

8.4 Positive Control Values

Each mean positive control value must exhibit at least a three-fold increase over the respective mean vehicle control value for each tester strain.

8.5 Toxicity

A minimum of three non-toxic dose levels will be required to evaluate assay data. A dose level is considered toxic if it causes a >50% reduction in the mean number of revertants per plate relative to the mean vehicle control value (this reduction must be accompanied by an abrupt dose-dependent drop in the revertant count) or a reduction in the background lawn. In the event that less than three non-toxic dose levels are achieved, the affected portion of the assay will be repeated with an appropriate change in dose levels.

9.0 EVALUATION OF TEST RESULTS

For a test article to be evaluated positive, it must cause a dose-related increase in the mean revertants per plate of at least one tester strain over a minimum of two increasing concentrations of test article. Data sets will be judged positive if the increase in mean revertants at the peak of the dose response is equal to or greater than two times the mean vehicle control value.

10.0 REPORT

A report of the results of this study will be prepared by the Testing Laboratory and will accurately describe all methods used for generation and analysis of the data. The report will include:

- Test substance: identification and CAS no., if known; physical nature and purity, if known; physicochemical properties relevant to the conduct of the study, if known; stability of test article, if known.
- Solvent/Vehicle: justification for choice of vehicle; solubility and stability of test article in solvent/vehicle, if known.
- Strains: strains used; number of cells/mL per culture; strain characteristics.
- Test conditions: amount of test substance per plate with rationale for dose selection and number of plates per concentration; media used; type and composition of metabolic activation system, including acceptability criteria; treatment procedures.
- Results: signs of toxicity; signs of precipitation; individual plate counts; the mean number of revertant colonies per plate and standard deviation; dose-response relationship, where possible; statistical analysis, if any; concurrent negative and positive control data means and standard deviations; historical negative and positive control data with ranges, means and standard deviation.

- Discussion of results.
- Conclusion.

11.0 RECORDS AND ARCHIVES

Upon completion of the final report, all raw data and reports will be maintained in the archives of MA BioServices, Inc., Rockville, MD in accordance with the relevant Good Laboratory Practices Regulations.

12.0 REGULATORY REQUIREMENTS/GOOD LABORATORY PRACTICE

This study will be performed in compliance with the provisions of the Good Laboratory Practice Regulations for Nonclinical Laboratory Studies.

Will this study be submitted to a regulatory agency? YES

If so, to which agency or agencies? EPA - office of water (Research Proj

Unless arrangements are made to the contrary, unused dosing solutions will be disposed of following administration to the test system and all residual test article will be disposed of following finalization of the report.

13.0 REFERENCES

Ames, B.N., McCann, J. and Yamasaki, E. (1975). Methods for detecting carcinogens and mutagens with the *Salmonella*/mammalian-microsome mutagenicity test. Mutation Research *31*:347-364.

McCann, J. and Ames, B.N. (1976). Detection of carcinogens as mutagens in the *Salmonella*/microsome test: assay of 300 chemicals: discussion. Proc. Natl. Acad. Sci. USA 73:950-954.

McCann, J., Choi, E., Yamasaki, E. and Ames, B.N. (1975). Detection of carcinogens as mutagens in the *Salmonella*/microsome test: assay of 300 chemicals. Proc. Natl. Acad. Sci. USA 72:5135-5139.

Maron, D.M. and Ames, B.N. (1983). Revised Methods for the *Salmonella* Mutagenicity Test. Mutation Research 113:173-215.

14.0 APPROVAL

Robert A. Finch for HSG 1-19-99

SPONSOR REPRESENTATIVE DATE

Robert A. Finch

(Print or Type Name)

(Henry S. Gardner)

STUDY DIRECTOR DATE

If submission to Japanese Regulatory Agency is indicated in section 12.0, MA management will sign.

Valentine O. Wagner, III 22-Feb-1999

MA STUDY MANAGEMENT DATE