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WILD MAMMALIAN BIOMONITORS FOR ASSESSING IMPACTS OF ENVIRONMENTAL CONTAMINATION ON POPULATION AND COMMUNITY ECOLOGY.

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The second year (01 Jun 92- 31 May 93) of this research project was devoted primarily to in situ biomonitoring to compare relative sensitivities of selected measures of metabolic, immunologic, genetic, and histopathologic toxicity in small-mammalian residents of terrestrial ecosystems contaminated with complex mixtures of petrochemicals. Results of these measures will be compared to common laboratory bioassay tests (fathead minnow survival, rice seed germination test, etc.), relative to their ability to predict ecotoxicity risks (as indexed by demographic changes in the small mammal community). Our principal in situ biomonitor has been the cotton rat (*Sigmodon hispidus*), which is the dominant member of the small mammal community on 3 uncontaminated reference and 3 heavy metal-petrochemical contaminated study sites located on the Refinery Waste Site on Cyril, Oklahoma. Chemical analyses of soil and soil extracts has revealed a variety of heavy metal and organic contaminants on the 3 suspected toxic study sites, which was reflected in common laboratory bioassay results using fathead minnow, microtox, rice seed germination, and *Ceriodaphnia* assays. At the ecosystem level, in situ small mammal total biomass, sex ratios, reproduction, recruitment, survival, and density were measured as indices of population integrity;

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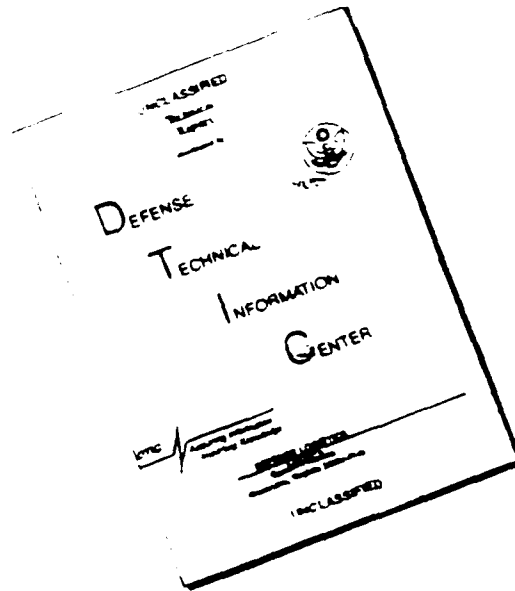
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community profiles included measurements of diversity, richness, and similarity. Both population and community indices demonstrated sensitivity as ecosystem endpoint markers of contaminant exposure, with density and survival estimates differing significantly between toxic and reference study sites. Altered immune function resulting in increased susceptibility to infection and disease could be responsible for increased mortality on toxic sites. Resident cotton rats returned the laboratory have been subjected to detailed postmortem examinations to include gross and histological examinations and profiles of immune system, cytogenetic, and metabolic status. Cotton rats collected from suspected toxic sites possess teeth that lack pigmentation, which is attributed to degenerative and necrotic changes in the ameloblasts. Immune system lesions

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ANNUAL TECHNICAL REPORT

Wild Mammalian Biomonitors for Assessing Impacts of Environmental Contamination on Population and Community Ecology.

PROJECT SUMMARY

The second year (01 Jun 92- 31 May 93) of this research project was devoted primarily to in situ biomonitoring to compare relative sensitivities of selected measures of metabolic, immunologic, genetic, and histopathologic toxicity in small-mammalian residents of terrestrial ecosystems contaminated with complex mixtures of petrochemicals. Results of these measures will be compared to common laboratory bioassay tests (fathead minnow survival, rice seed germination test, etc.), relative to their ability to predict ecotoxicity risks (as indexed by demographic changes in the small mammal community). Our principal in situ biomonitor has been the cotton rat (*Sigmodon hispidus*), which is the dominant member of the small mammal community on 3 uncontaminated reference and 3 heavy metal-petrochemical contaminated study sites located on the Refinery Waste Site in Cyril, Oklahoma. Chemical analyses of soil and soil extracts has revealed a variety of heavy metal and organic contaminants on the 3 suspected toxic study sites, which was reflected in common laboratory bioassay results using fathead minnow, microtox, rice seed germination, and *Ceriodaphnia* assays. At the ecosystem level, in situ small mammal total biomass, sex ratios, reproduction, recruitment, survival, and density were measured as indices of population integrity; community profiles included measurements of diversity, richness, and similarity. Both population and community indices demonstrated sensitivity as ecosystem endpoint markers of contaminant exposure, with density and survival estimates differing significantly between toxic and reference study sites. Altered immune function resulting in increased susceptibility to infection and disease could be responsible for increased mortality on toxic sites. Resident cotton rats returned the laboratory have been subjected to detailed postmortem examinations to include gross and histological examinations and profiles of immune system, cytogenetic, and metabolic status. Cotton rats collected from suspected toxic sites possess teeth that lack pigmentation, which is attributed to degenerative and necrotic changes in the ameloblasts. Immune system lesions were indicated by significant differences in in vitro measures of cell-mediated immunity, circulating levels of immune cells, and immune organ development. Analysis of methoxyresorufin (MROD) and ethoxyresorufin (EROD) O-dealkylase activity of cytochrome P-450 enzyme systems also indicated the presence of contaminant-induced lesions. Initial chromosome aberration and flow cytometric analyses suggest that animals are suffering induced chromosome lesions on toxic study sites. Seasonal effects in the degree and type of physiologic lesions documented in resident small mammals were observed for metabolic, cytogenetic, and immunologic end-points, suggesting seasonal changes may have occurred in the volatility of organic contaminants on toxic study sites.

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Dr. Charles A. Qualls
Dr. S. L. (Bud) Burks

OTHER PROJECT PERSONNEL

Several graduate students and laboratory technicians are both directly and indirectly supported or provide assistance to this project. The following list of individuals have contributed to the project during the second year of funding.

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Jing Ren Zhang (Ph.D. candidate)
Timothy Propst (M.S. candidate)
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Kathleen Thies (Research Technician)
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RESEARCH OBJECTIVES

Objective I. To determine the ecological effects and sensitivity of complex mixtures of environmental toxicants at contaminated petrochemical waste sites on the structure and composition of resident populations and communities of small mammals by quantitating:

1. age structure, sex ratio, recruitment, survival of populations.
2. species richness, diversity, similarity of communities.

Objective II. To determine the immunotoxicity, genotoxicity, and metabolic toxicity (physiological response-profiles) of complex mixtures of environmental toxicants in resident small mammals inhabiting contaminated petrochemical waste sites through evaluation of:

1. immune organ development, lymphocyte subpopulations, lymphoproliferation, humoral and cell-mediated immunity.
2. chromosome aberrations, variation in nuclear DNA content, DNA strandbreaks.
3. total and isoenzyme levels of hepatic cytochrome P-450, ultrastructural pathology, free radical concentration.

Objective III. To evaluate the use of enclosed terrestrial mesocosms for conducting both subacute and chronic in situ exposures of cotton rats to complex mixtures of environmental toxicants at contaminated petrochemical waste sites by measuring:

1. reproductive and survival response.
2. physiological response-profiles.

Objective IV. To examine correlations between in situ physiological response-profiles (immunologic, genetic, metabolic endpoints) of a mammalian biomonitor (cotton rat and white-footed mouse) and standard laboratory-derived biological assays for soil leachates from contaminated petrochemical waste sites by using:

1. *Ceriodaphnia* short-term chronic bioassay.
2. fathead minnow larval survival bioassay.
3. rice seed germination toxicity assay.

STATUS OF RESEARCH

Study Site

Site Selection

Our research team for this project is currently composed of four tenure-track faculty, two technicians, three doctoral and four masters candidates. Tremendous progress was made over the second year monitoring populations of small mammals in situ on six study sites (3 reference, 3 suspected petrochemical-contaminated study sites), conducting controlled in situ mesocosm exposures, and profiling metabolic, genotoxic, immunotoxic, and histopathologic responses of cotton rats, both resident and mesocosm-introduced animals, on the Oklahoma Oil Refinery Superfund Site, Cyril, Oklahoma. The three contaminated sites include a sludge land-farming area containing heavy metal and petroleum contaminants, a spoil-site adjacent to a series of API-separator ponds containing heavy metal and petroleum contaminants, and a site adjacent to asphalt disposal pits containing a variety of petroleum contaminants (Fig. 1). The six mesocosms on the study site include 3 on the contaminated study sites described above and 3 reference mesocosms off-site.

Contaminants and Bioassay Results

A primary objective of this project was to evaluate correlation between laboratory derived toxicity response factors of aquatic organisms and plants with community response factors of resident small mammals living in ecosystems contaminated with petrochemical hazardous wastes. As a secondary objective, we compared chemical contaminant concentration data from the proposed on-site study areas with EPA recommended Chronic Toxicity Reference Levels specified in the Toxics Characteristics (TC) rules (EPA, March 29, 1990, Fed. Reg., 55(61)11796-11877). EPA established the chronic toxicity reference levels on the basis of potential human health effects from consuming groundwater contaminated with concentrations greater than the CTR levels. Therefore the question of ecosystem effects was not considered in establishing the TC regulatory levels. We used the EPA Toxic Characteristics Leachate Procedure (TCLP) (EPA Method 1311) to prepare extracts of soil samples for chemical analyses and toxicity assays. This procedure was primarily designed to determine the potential mobility of specific toxic organics and metals from combined municipal and industrial sanitary landfills, by performing chemical analyses of the leachates for specific compounds (Fig. 2). We evaluated the potential use of TCLP extract for toxicity tests by performing acute toxicity tests with the test organisms microbes (*Photobacterium phosphoreum*), water flea (*Ceriodaphnia dubia*), and fathead minnow (*Pimephales promelas*) using extracts from inert silica sand, the blank extraction media, and soils from both contaminated and uncontaminated control sites. The modified Neubauer seed germination phytoassay using rice seeds (*Oryza sativa*) was also used to test soil samples.

The TCLP procedure has not provided a good protocol for obtaining samples for toxicity tests with aquatic organisms, since the blank routinely causes too much toxicity to be acceptable. We have returned to using reconstituted water as the extracting fluid. The

aqueous extraction procedure imparts no solvent blank toxicity, but it may not simulate "worst-case" conditions that could occur in the field situations.

The concentration of toxic metals in samples collected from the area in December, 1991 indicated relatively high concentrations of cadmium, zinc, manganese, copper, lead, sodium, chloride, and sulfates from various toxic grids (Figs. 3 and 4). The most contaminated soil sample analyzed to date, was collected from enclosure 3. This sample had a physical appearance and odor of "oily" materials. Aqueous extracts of this sample were analyzed by a combination of solid phase adsorbent-methanol elution-high performance liquid chromatography, but failed to detect any peaks. The soil sample was directly extracted with methylene chloride, concentrated to 1 ml, and injected onto a gas chromatograph. The chromatogram indicated a cluster of organic compounds which were chromatographically similar to alkyl-aromatics. The aqueous extracts from Enclosure 3 exhibit the following physical-chemical characteristics; dark brown color, color not removed by centrifugation at 8000 G, color not removed by filtration with 8 um pore glass fiber filters, not digested in boiling concentrated nitric acid, and not adsorbed on non-polar solid phase adsorbent (C18). The aqueous extract had an extremely high level of organic contaminants, COD of 8,000 mg/l, compared to most of the other areas of less than 200 mg/l. The extract was then passed through C18 solid adsorbent to determine if the contaminants would be removed by a non-polar adsorbent. The single pass through C18 was approximately 5000 ppm and the double pass through C18 was 3800 ppm. Thus some of the organic contaminants were nonpolar, but a relatively high percentage was not adsorbed and must be semipolar to polar in nature. The HPLC chromatographic characteristics of the extracts are similar to naphthalene and phenanthrene, however the extract appears to a complex mixture of semi-polar to polar organic contaminants. The concentrations of metals and anions may also interact to create some level of toxicity. Additional studies will be performed in this area to narrow the cause of this toxicity.

The only location to consistently exhibit extreme toxicity using bioassay tests was Enclosure 3 (toxic site). The biological and chemical analyses indicated acutely lethal reactions to contaminants within this location. The location of Enclosure 3 over old leaded gasoline tanks may be a contributing factor to its toxicity. Some areas may be more concentrated with toxins at one location compared to a location just a few meters away. The reasons for this toxicity may be due to several factors. There are sufficient non-polar organics here to induce toxic results, however, there is evidence that some toxins may be more polar and water soluble in nature. The concentrations of metals and anions may also interact to create some level of toxicity. Additional studies will be performed in this area to narrow the cause of this toxicity. Grid trap 5 (reference site) did elicit noticeable toxicity with the *Ceriodaphnia* tests but the reasons for this have not been explored at this time.

Monitoring of Wild Populations

Mammalian Population Dynamics

Field monitoring of resident small mammal populations and community dynamics at Cyril has provided evidence of petrochemical-induced toxicity at the population level; suggesting that demographic indices are useful measures of ecotoxicity in terrestrial environments. Small mammal populations on the 6 study sites have been monitored since January 1991 (through August 1992) at 8 week intervals except in summer and winter where a 3 week interval was followed for 4 consecutive trapping periods (total 17 trapping bouts). Total biomass, sex ratios, reproduction, recruitment, survival, and density were measured as indices of population integrity. Community profiles included measurements of diversity, richness, and similarity.

During the course of the study, a total of 11 species of small mammals was captured, representing 9 rodents and 2 insectivores. House mice (*Mus musculus*), white-footed mice (*Peromyscus leucopus*), deer mice (*P. maniculatus*), and fulvous harvest mice

(*Reithrodontomys fulvescens*) were seasonally abundant on most areas sampled. However, cotton rats (*Sigmodon hispidus*) represented the most abundant small mammal collected on all areas during the entire course of the study. Cotton rat density was seasonally variable and was significantly different between toxic and reference sites (Fig. 5). Greatest differences in density between toxic and reference sites was observed in 1991, especially in July and August when reference study sites supported greater densities than suspected toxic study sites. Differences were less obvious in 1992 although August results showed similar trends as in 1991.

Reproductive status of adult female cotton rats do not appear to indicate any differences between toxic and reference sites (Fig. 6). However, recruitment (as indicated by percentage of juvenile cotton rats in the trapable population, Fig. 6) trends closely resembled responses seen for density in this species. The lack of obvious differences in female reproductive activity coupled with lower recruitment patterns on toxic sites indicated possible differences in juvenile survival. Analysis of survival of all trapable cotton rats collected in the study do not appear to indicate any differences between toxic and reference sites (Fig. 7). However, these results represent a very cursory examination of survival. It is likely that differences in survival do exist within discrete age-classes. For example, differential survival of juvenile (both in utero and neonatal mortality) or aged animals may be possible among study sites. Further analysis of the existing data set will aid in determine if differential survival existed among specific age classes within populations.

Diversity of small mammal communities on toxic and reference sites showed a relatively high degree of divergence from May 1991 through February 1992 (Fig. 8). During this time, diversity was consistently higher on toxic sites compared to references due to the presence of a high number of house mice on toxic sites. It is difficult at this time to determine the significance of this difference. Increased numbers of house mice could represent a remnant population previously associated with the refinery. Another more interesting possibility is that house mice could be more resistant to contaminant exposure than their native counterparts and thus outcompeting them for resources. Similarity indices were included as a measure of the composition and relative abundance of species within communities (Fig. 9). Similarity indices indicated differences in the structure of small mammal communities existed between toxic and reference sites in summer samples; differentiation between sites was less obvious for winter samples.

Both population and community indices demonstrate sensitivity as ecosystem endpoint markers of contaminant exposure. The primary "lesion" at the population level appears to be differential survival rates between toxic and reference sites, although at this time we are uncertain exactly which component of the population is most susceptible. Differences in density of cotton rat populations coincide nicely with the majority of immunological lesions observed in the same populations. Altered immune function resulting in increased susceptibility to infection and disease could be responsible for increased mortality on toxic sites.

Four collections of resident cotton rats have been conducted (January and September 1991, March and September 1992), resulting in a total collection of 217 cotton rats from 6 areas (3 toxic and 3 reference sites) for physiological response profiling. Animals were returned to the laboratory where pathological, immunological, metabolic, and cytogenetic endpoints measured. The preliminary results of these findings are described below:

Pathologic Examinations

Cotton rats returned to the laboratory from wild caught populations were subjected to detailed postmortem examination, to include gross and histologic evaluations. The most prominent morphological alterations were in dental morphology. The normal color of cotton rat incisors is deep yellow-orange, which is imparted by a pigment produced by ameloblasts. Sixty-one of 107 from the contaminated site and one of 102 from the

reference sites had grossly abnormal teeth. The abnormal incisors were pale and mottled. Microscopic examination of the undecalcified normal and abnormal incisors revealed that the enamel of the abnormal teeth was irregular, and lacked pigment, focally or diffusely unlike the regular, smooth, normally pigmented enamel in the teeth that were grossly normal. We have determined that this lack of pigmentation is attributed to the degenerative and necrotic changes in the ameloblasts of abnormal incisors. Possible causes of these lesions will be discussed.

Our initial results provide strong evidence in support of using both gross and histologic examinations in assessing toxicity in situ using small mammalian residents. These results are only preliminary and further study will be needed to validate their usefulness.

Immunotoxicity Evaluations

Unlike other studies which used controlled dosing protocols to examine the immunotoxicity of known compounds in wild rodents maintained in the laboratory, our results demonstrate the usefulness and response of immunological endpoints to contaminant exposure in a wild population of rodents simultaneously subjected to a plethora of environmental stressors routinely encountered under natural conditions.

As expected, a considerable amount of variation in immune responses were observed among and within populations over time. Several immune parameters, including complement levels, phagocytic activity of macrophages, and proliferative response of lymphocytes to the B and T cell mitogens, PWM and IL-2, have not been sensitive indicators of contaminant exposure (Table 1). Additionally, no single immune response endpoint showed significant differences between toxic and reference sites during every seasonal collection period. However, several trends are apparent from our initial results in this study. The majority of the differences were observed in the September collections compared to winter, possibly indicating a seasonal dependence in response linked to other extrinsic or intrinsic stressors. Also, all indices of leukocyte cellularity were typically lower on toxic compared to reference sites. T lymphocytes, as indicated by mitogenic response to Con A and Con A-based subtyping, appeared to be the most sensitive immune lesion. B lymphocytes, as indicated by mitogenic response to PWM and IgG-based subtyping, appeared to be relatively insensitive to contaminant exposure.

Immune organ indices of general condition showed significant variation between toxic and reference sites and among collection grids during September 1991 and 1992 collection periods (Table 2). Relative spleen mass of cotton rats was heavier and paired adrenals and popliteal nodes were lighter in cotton rats collected on toxic sites in September 1991 and September 1992. Overall, kidney mass in September 1991 and kidney mass and liver mass in September 1992 were not significantly ($P > 0.100$) different between toxic and reference sites although significant variation among the six collection grids was evident (Table 2).

A number of different leukocyte and erythrocyte indices showed significant variation between toxic and reference sites and among toxic and reference collection grids, typically reflecting the highest degree of sensitivity in September collection periods (Table 3). Total leukocyte, lymphocyte, and neutrophil counts were significantly lower in cotton rats collected from toxic sites compared to reference sites in January 1991. Total number of splenocytes was higher in cotton rats collected from toxic sites compared to references in September 1992, whereas cellularity of paired popliteal nodes (total cells and cells per mg of node) was lower on toxic sites during the same period. Lymphocyte subtyping showed Con A positive T-lymphocytes to be significantly depressed in cotton rats collected from toxic sites compared to reference sites. Erythrocyte indices showed significant variation in September 1991 and September 1992 collections (Table 3). Erythrocyte counts and packed cell volume were significantly higher in September 1991 and erythrocytes lower in September 1992 in cotton rats collected from toxic sites compared to references.

Depressed erythrocyte levels on toxic sites in September 1992 were accompanied by a significant increase in corpuscular volume and hemoglobin. Platelet counts were significantly ($P < 0.040$) higher in cotton rats collected from toxic sites compared to reference sites in September 1992.

In addition to the above indices, a number of different functional measures of immunity were also assessed. In general, proliferative capacity of splenocytes, both innate (unstimulated) and Con A-stimulated, was the most consistent discriminator of immune dysfunction between cotton rats from toxic and reference sites (Fig. 10 and 11). In vitro proliferation of unstimulated splenocytes was significantly greater on toxic sites in September 1991 and September 1992 compared to reference sites (Fig. 10). T lymphocyte proliferation following stimulation with Con A at 5ug/ml culture in January 1991 and September 1992 and 40ug/ml culture in September 1991 was significantly higher for cotton rats collected from toxic sites compared to reference sites (Fig. 11).

In vivo cell mediated immune response as measured by a 24-hr hypersensitivity response to an intradermal injection of phytohemagglutinin was significantly higher in cotton rats collected from reference sites than toxic sites in March 1992 (Fig. 12). Metabolic activity of peritoneal macrophage populations showed a significant degree of variation among collection grids in January 1991 and September 1992 (Fig. 13). There appeared to be a definite seasonal trend in the degree of toxicity. Late summer and autumn represents a stressful period for most small mammals due to reproduction demands, high densities, and nutritional stress. Possible interactions between xenobiotic stressors and other seasonally dependant natural stressors (e.g., reproduction) could act synergistically and enhance an otherwise essentially benign toxic effect. Similar types of interactions have been shown by other investigators, where mice were subjected to several combinations of food, water, pathogens, and immunotoxicants.

Increased in vitro splenocyte proliferation in unstimulated and stimulated (T cell mitogen, Con A) cultures were the most consistent indicators of immune dysfunction in cotton rats collected from toxic sites. It is unclear what mechanism is promoting the increase in blastogenesis, especially in the unstimulated cultures, although several possibilities exist. Regulatory activity of the immune system may be impaired with possible selective depletion and/or functional impairment of suppressor T cells. Lymphocytes may be stimulated to secrete factors which promote cell proliferation. Contaminants may alter cell surface markers, thereby rendering them antigenic in nature and eliciting an in vivo immune response against the transformed cells. At this point it is difficult to determine the exact mechanism which would require more elaborate immunological assays. T suppressor cell generation is known to be sensitive to mercury exposure. Also, depletion of peripheral cells can lead to enhanced proliferation and differentiation of the pluripotent stem cell pool as a mechanism to replenish the depleted populations. Other studies have demonstrated the stimulatory effects of lead on lymphocyte blastogenesis, although most studies concur that lead suppresses proliferation. It is apparent that in situ monitoring can detect a variety of immune system lesions; the possible interactions between the numerous toxicants present on the sites and the impacts of, and interactions with, other extrinsic and intrinsic stressors which resident rodents are undoubtedly exposed to may be contributing factors.

Hepatic Cytochrome P450 Induction Studies

Analysis of methoxyresorufin (MROD) and ethoxyresorufin (EROD) O-dealkylase activity was performed on hepatic microsomes from wild caught hispid cotton rats (*Sigmodon hispidus*). Results comparing pooled activity of contaminated to reference sites revealed a significant increase in both MROD and EROD activity in the Sept. collections (MROD- Sept. 91 $P \mu 0.0003$, Sept. 92 $P \mu 0.0012$; EROD- Sept 91 $P \mu 0.0138$, Sept. 92 $P \mu 0.0339$). No significant increase in MROD OR EROD activity was seen at the same sites for collections carried out in Jan. 91 and Mar. 92. This difference in activity in summer

versus winter seasons may be associated with higher volatility of chemicals in the warmer months resulting in higher levels of exposure.

Genotoxicity Evaluations

For each animal returned the laboratory, chromosomes were extracted from bone marrow for analysis of mitotic metaphase chromosome aberrations, spleens were disaggregated and fixed for flow cytometric analysis, and liver biopsies and thigh skeletal muscle tissues were frozen in liquid nitrogen for subsequent analysis of DNA single strand lesions using the alkaline unwinding assay. Analysis of 100 metaphase spreads in each of 53 individuals from the first two sampling periods has been completed. Because all samples in the genetic analyses are number coded and analyzed in a "blind" manner (i.e. the person conducted the analysis does not know from which grid an animal was collected), we will not uncode these data until all animals have been analyzed. It would be premature to draw any conclusions from the chromosome aberration analyses at this point. However, aberrant cells per individual range from 0 to 11%; background levels of aberrant cells per individuals in reference populations from previous studies have been approximately 2% so we are encountering significantly elevated levels of chromosome damage in some individuals. Slightly less than 90% of observed damage is attributable to chromatid breaks. No complex rearrangement figures, rings, or dicentrics have been found. Although these data are not yet completely analyzed, they do indicate that animals are suffering induced chromosome lesions.

Flow cytometry (FCM) has been successfully used with wild vertebrates as a biomarker of exposure to environmental genetic toxicants, where FCM can indicate greater variation in DNA content and possible aneuploidy in spleen cells of wild animals. Flow cytometric analysis of all individuals from the September 1991 and the March 1992 sampling period has been completed. This includes generation of over 60 internal standard DNA distribution histograms and over 300 DNA distribution histograms from test animals for each collection period. Number-coded samples were analyzed using a Partec Pas-II fluorescent flow cytometer calibrated with a commercial standard preparation of calf thymocyte nuclei. Aliquots of the calf thymocyte standard were run at the beginning of analysis and after every fifth rat sample to ensure instrument stability. For each rat, data from five replicates of 20,000 cells each were collected and averaged. Variables derived from the DNA histograms for each of these runs were statistically compared between Superfund and reference grids using Mann-Whitney U.

Mann-Whitney U indicated statistical differences among grids for 4 of 8 variables for the 1991 sample. Grids 1 and 6 were significantly different in the percent of cells in synthesis. Grids 2 and 4 and 3 and 4 (all toxic sites) were significantly different for the percent of debris. All remaining statistically significant differences were for the coefficient of variation of the G1 peak (CV G1) or for the mean channel for the G1 peak (Mean G1), a relative measure of DNA content, and are summarized in Fig. 14. Mean G1 peak channel (relative DNA content) values for each grid in are given in Fig. 15a. Pooled values were 63.00 and 64.18 for toxic grid animals and reference grid animals, respectively. Mean CV G1 values for each grid are given in Fig. 17c. Pooled values were 1.78 and 1.70 for Superfund grid animals and reference grid animals, respectively. Mann-Whitney U indicated no statistical differences among grids for the 8 variables in 1992. Mean G1 peak channel (relative DNA content) values for each grid in are given in Fig. 15b. Pooled values were 61.11 and 60.73 for toxic grid and reference grid animals, respectively. Mean CV G1 values for each grid are given in Fig. 15d. Pooled values were 2.48 and 1.94 for toxic grid and reference grid animals, respectively. The 1991 and 1992 samples were significantly different for mean G1 and CV G1 among all grids.

Mean CV G1 values for each animal were also analyzed by calculating the 95% confidence range around the mean CV G1 for pooled reference sites animals in each year. For pooled sites in 1991, 14% of reference site animals and 40% of toxic site animals

exceeded the upper confidence limits. For pooled sites in 1992, 48% of reference site animals and 55% of Superfund site animals exceeded the upper confidence limits. These data show that animals from the toxic grids in September 1991 had significantly ($P=0.05$) increased CVs for mean DNA content indicating the presence of genetic lesions and significantly ($P=0.05$) decreased relative G1 DNA content suggesting that genetic lesions may be resulting in net loss of DNA. During March 1992, animals from toxic grids were not significantly different from reference grid animals for either CVs or DNA content; although patterns for CVs were similar to those from 1991, and animals from the toxic grids consistently had higher CVs than animals from reference grids. Possible reasons for seasonal differences may include increased volatility of clastogenic organics during hotter summer months resulting in increased exposure for resident rodents.

Tissues for the Alkaline Unwinding Assay are being stored at -80°C . The method of DNA extraction and purification originally planned for use on this project yields large quantities of DNA but it is too sheared to use in accurately assessing the ratio of single to double stranded DNA. Because of this problem, we have investigated several different methods for extraction and purification and found an alternative method that yields consistently high quality DNA from small quantities of tissue. As indicated in the proposal, analysis for this portion of the project will continue during 1993/1994.

Mesocosm Experimental Trials

General

We have successfully completed 2 eight-week in situ exposure trials, one in the winter of 1992 and one in the summer of 1992. Six mesocosms, 3 suspected contaminated and 3 reference locations, were utilized in both trials. The use of mesocosms to monitor or assess ecotoxicity has proved to be quite useful due to the greater experimental control that they afford. Animals of known age and exposure history can be placed upon a site of known contaminant composition for a specified period of time. This ability to control many of the variables affecting a terrestrial mammalian system was probably instrumental in increasing the sensitivity of the assays we used in this study. Furthermore, site specific toxic effects were able to be delineated between reference and treatment mesocosms. As a result, mesocosms have proved to be effective tools for monitoring site-specific, physiological responses of feral rodents.

In our trials, equal numbers of juvenile male and female cotton rats were introduced to each mesocosm and provided with food and water ad libitum over the duration of exposure. At the end of the exposure period, animals were collected for necropsy and physiological response assessments.

Immunotoxicity Assessment

Our laboratory utilized a battery of assays to profile immunocompetence of animals. The controlled in situ environments provided by the mesocosms enabled us to conduct a variety of in vivo assessments of humoral and cell-mediated immunity, as animals could be periodically recaptured and handled as needed. Immune organ size and cellularity, basic hematological screening, and general health screens were performed on all individuals returned to the laboratory. Additionally, in vitro assays of immune system function included lymphoproliferative mitogenic responsiveness, lymphocyte subtyping, natural killer cell tumoricidal activity, and macrophage metabolic and phagocytic activity. In vivo assays included 24-hr phytohemagglutinin (PHA) hypersensitivity and delayed-type hypersensitivity to a recall-antigen (oxazolone) to assess cell-mediated immune function, and keyhole limpet hemocyanin challenge (T-dependent protein antigen) was used to assess humoral immunity.

..... One of the most revealing indicators of immunotoxicity was the in vivo assessment of cell-mediated immune function. Hypersensitivity responses of animals to PHA (Fig. 16) on the three reference sites were approximately 60%

greater than those housed on suspected sites of toxicity. Interestingly, the reverse trend was observed for the delayed-type hypersensitivity reaction to the recall-antigen oxazalone (Fig. 17). Although both assays measure in vivo cell-mediated immune function, they differ greatly in their mode of action. The PHA assay was a measure of the degree to which the animal could respond to a known mitogenic substance where memory cells are not involved. In comparison, the oxazalone reaction measures memory cell function as well. An important component of the immune system is the self regulatory mechanism, whereby cells modulate or down-regulate immune response. The observed in vivo cell-mediated immune responses have lead us to hypothesize that the mixture of petrochemical contaminants at the study site have induced a lesion within the suppressive branch of the immune system. It is important to remember that any deviation from the normal response level, enhancement or reduction, can detrimentally affect survival of the host animal. Ongoing analysis of additional immune parameters will allow us to monitor these changes in greater detail.

Laboratory Sensitivity Assays

Pathologic examination of data from a study of lead, received via water consumption were performed. Thorough necropsy examinations were performed and recorded in all the cases. A control rat (female) from a 7-week study and a second female rat from a 13-week study receiving 100 ppm of lead acetate died before the termination of the experiment. All tissues were routinely processed. Histological sections of adrenals, bone (femur), brain, epididymis, heart, kidney, liver, lung, lymph node, muscle, ovary, spleen and testicle were examined. Special stains included Ziehl-Neelsen's acid fast stain and Fite's ferraco on all sections of liver and kidney. In addition, a complete immunocompetence profile was described for each animal.

Preliminary Results

Gross changes at necropsy were generally unremarkable in treatment and control rats. On light microscopic examination of H & E stained sections altered renal proximal tubular epithelium was found in all the rats dosed with 1000 ppm of lead acetate for either 7 or 13 weeks. The changes in the tubules were quite uniform in all the specimens. Most epithelial cells were enlarged and had irregular apical borders encroaching upon the lumina of the tubules. Occasionally necrosis of tubular epithelium and sloughing of cells into the lumina of tubules was observed. Intra-nuclear inclusions were present in the kidneys of all the 24 rats receiving 1000 ppm of lead acetate for either 7 or 13 weeks. Inclusion were not present in the liver. The inclusions were usually solitary, spherical and varied from 2 to 7 microns in diameter. They were pink to red in H & E stained sections and were usually found in enlarged nuclei with marginated chromatin. Most inclusions were in the straight segments of proximal tubules. The inclusions were not acid fast with Ziehl-Neelsen's method which is routinely used to demonstrate lead inclusions. However the inclusions were acid fast with Fite's ferraco method. The glomeruli in these kidneys were normal. Rats which received 100 ppm of lead acetate for 7 or 13 weeks had the tubular changes but failed to demonstrate the lead inclusions in the kidney or liver. Sections of the kidney from the control animals did not contain the inclusions and were entirely free from the tubular changes. Testicles of 3 rats on 1000 ppm of lead for 13 weeks had fewer sperms and in one rat (7 weeks) there was virtually no spermatogenesis. Three rats on 100 ppm of lead for 13 weeks had fewer sperm with no sperm in one rat. Ovaries of 4 rats on 1000 ppm of lead (3 of 13 week and 1 of 7 week) consisted of corpora lutea with few developing follicles. Only 2 rats (7 week) receiving 100 ppm of lead had the ovarian lesion. Testicles and ovaries from the control rats were normal. Hepatic fatty change was observed in both control and treatment rats.

Immune system function was also sensitive to lead exposure as indicated by depressed splenocyte proliferation, smaller popliteal lymph nodes, heavier spleens, and altered circulating levels of immune cells. No differences in the in vivo cell-mediated immune response or macrophage activity were observed. These data are presently being prepared for publication.

Discussion

The gross findings were unremarkable in rats supplemented with lead acetate. Eosinophilic, acid fast, intra nuclear inclusions considered pathognomonic for lead intoxication were present in rats receiving 1000 ppm of lead acetate. The presence of inclusion bodies reflect the amount of lead that can be found in body tissues. Inclusions are not readily formed in the liver even when the lead content is high. Histologic sections of decalcified femur from control and treatment rats were normal. Metaphyseal sclerosis which is usually observed in young animals in lead poisoning were not present since mature rats were used in the experiment. Pathological changes associated with lead encephalopathy were not observed in the brain of treatment rats. Large doses of lead are required to produce central nervous system effects and also pathological lesions appeared to differ greatly among different animals.

A variety of controlled laboratory toxicity trials will be conducted during the next funding year using several known contaminants occurring on the Cyril Refinery study site. These toxicity assays will allow us to pin-point which contaminants are most influential in inducing abnormal physiological responses among wild-caught and mesocosm subjects.

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FIGURE LEGENDS

Fig. 1. Site location map illustrating position of trap grids and enclosures.

Fig. 2. Flow chart illustrating sample preparation and extraction procedures.

Fig. 3. Comparison of trace metal concentration in TCLP extracts from selected locations at the Cyril refinery, Dec. 1991, April 1992, May 1992 and Sept 1992..

Fig. 4. Comparison of anion concentration in TCLP extracts from selected locations at the Cyril refinery, May 1992, April 1992, and second analyses of April/May 1992 samples.

Fig. 5. Differences in mean ($\pm$ SE) mark-recapture density estimates of cotton rat populations on replicate reference and toxic sites at an abandoned oil refinery in Oklahoma from January 1991 to August 1992.

Fig. 6. Differences in mean percent reproductively active (pregnant or lactating) adult female cotton rats and percent juvenile ($\leq$ 60g) cotton rats captured on replicate reference and toxic sites at an abandoned oil refinery in Oklahoma from January 1991 to August 1992.

Fig. 7. Differences in mean finite survival rates of all trapable cotton rats on replicate reference and toxic sites at an abandoned oil refinery in Oklahoma from January 1991 to July 1992.

Fig. 8. Differences in mean diversity indices of small mammal communities on replicate reference and toxic sites at an abandoned oil refinery in Oklahoma from January 1991 to August 1992.

Fig. 9. Similarity in species composition of small mammal communities on replicate reference and toxic sites at an abandoned oil refinery in Oklahoma during winter 1991 (January and March pooled) and summer 1991 (June, July, and August pooled). Similarity based on the species present and their relative proportion to each other within each community.

Fig. 10. Differences in unstimulated proliferative response ($\text{MEAN} \pm \text{SE}$) of splenocytes (72 hr culture) from cotton rats collected on replicate reference and toxic sites at an abandoned oil refinery in Oklahoma during September 1991 and 1992. Values represent absorbance of solubilized formazan granules produced by splenocyte reduction of tetrazolium salt. Values above bars represent sample size (n) and common letters denote no statistical difference between grids ($P \leq 0.10$).

Fig. 11. Differences in elicited proliferative response ($\text{MEAN} \pm \text{SE}$) of splenocytes (72 hr culture) from cotton rats collected on replicate reference and toxic sites at an abandoned oil refinery in Oklahoma during January and September 1991 and September 1992. Cells were stimulated in vitro with either 5 or 40 ug/ml of culture Concanavalin A lectin. Values represent corrected absorbances (stimulated minus unstimulated cultures) of solubilized formazan granules produced by splenocyte reduction of tetrazolium salt. Values above bars represent sample size (n) and common letters denote no statistical difference between grids ($P \leq 0.10$).

Fig. 12. Differences in mean ($\pm$ SE) hypersensitivity response as reflected by the absolute increase (postimmune minus preimmune thickness) in skin fold thickness (inches) 24 hr after an intradermal injection of phytohemagglutinin (250 ug protein) in cotton rats.

Animals were collected from replicate reference and toxic sites at an abandoned oil refinery in March 1992. Values above bars represent sample size (n) and common letters denote no statistical difference between grids ($P \leq 0.10$).

Fig. 13. Differences in mean ($\pm$ SE) metabolic activity of peritoneal macrophages shown as absorbance values of cell cultures following reduction of nitroblue tetrazolium dye to formazan after 15 min incubation. Cells were recovered from cotton rats collected from replicate reference and toxic sites at an abandoned oil refinery in Oklahoma during January 1991 and September 1992. Values above bars represent sample size (n) and common letters denote no statistical difference between grids ($P \leq 0.10$).

Fig. 14. Pair-wise comparisons among all grids for coefficient of variation for mean DNA content in the G1 peak (above the diagonal) and mean channel of the G1 peak (relative DNA content) (below the diagonal). * indicates values significantly different at the $P=0.05$ level with Mann Whitney test.

Fig. 15. a. Mean G1 peak for each grid in 1991; b. mean G1 peak for each grid in 1992; c. mean CV peak for each grid in 1991; d. mean CV G1 peak for each grid in 1992.

Fig. 16. Differences in in vivo cell-mediated immunity as measured by the 24-hr hypersensitivity response to an intradermal injection of phytohemagglutinin. Response was measured as percent change in skin fold thickness of animals returned from wild populations on reference (A-C sites) and toxic (D-F sites) study sites.

Fig. 17. Differences in in vivo cell-mediated immunity as measured by the delayed-type hypersensitivity response to a percutaneous application of oxazolone to previously sensitized cotton rats. Response was measured as percent change in ear thickness of animals returned from wild populations on reference (A-C sites) and toxic (D-F sites) study sites.

FIGURE 1.

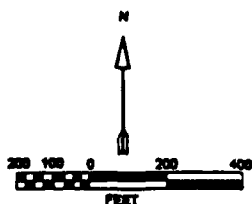
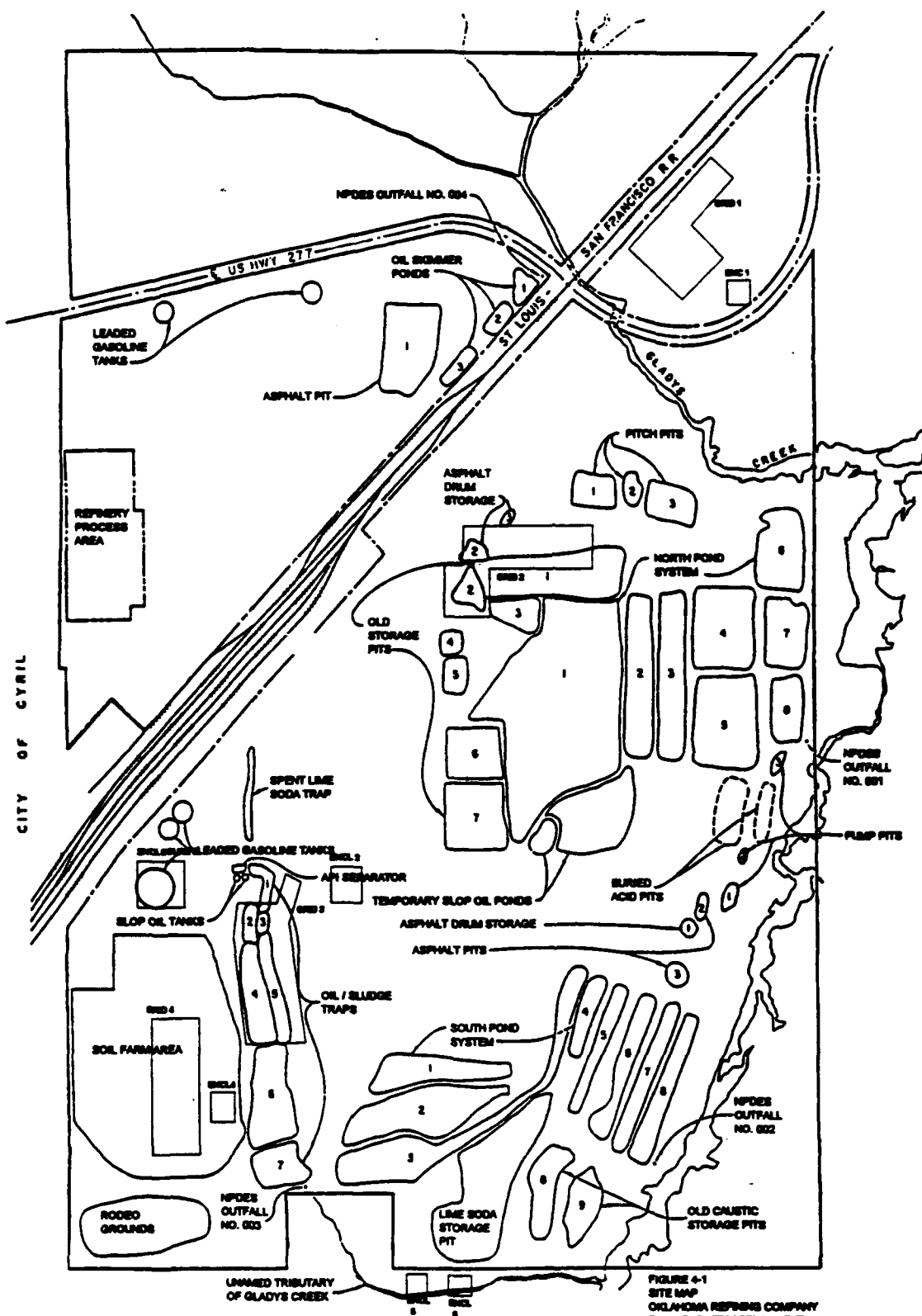


FIGURE 2.

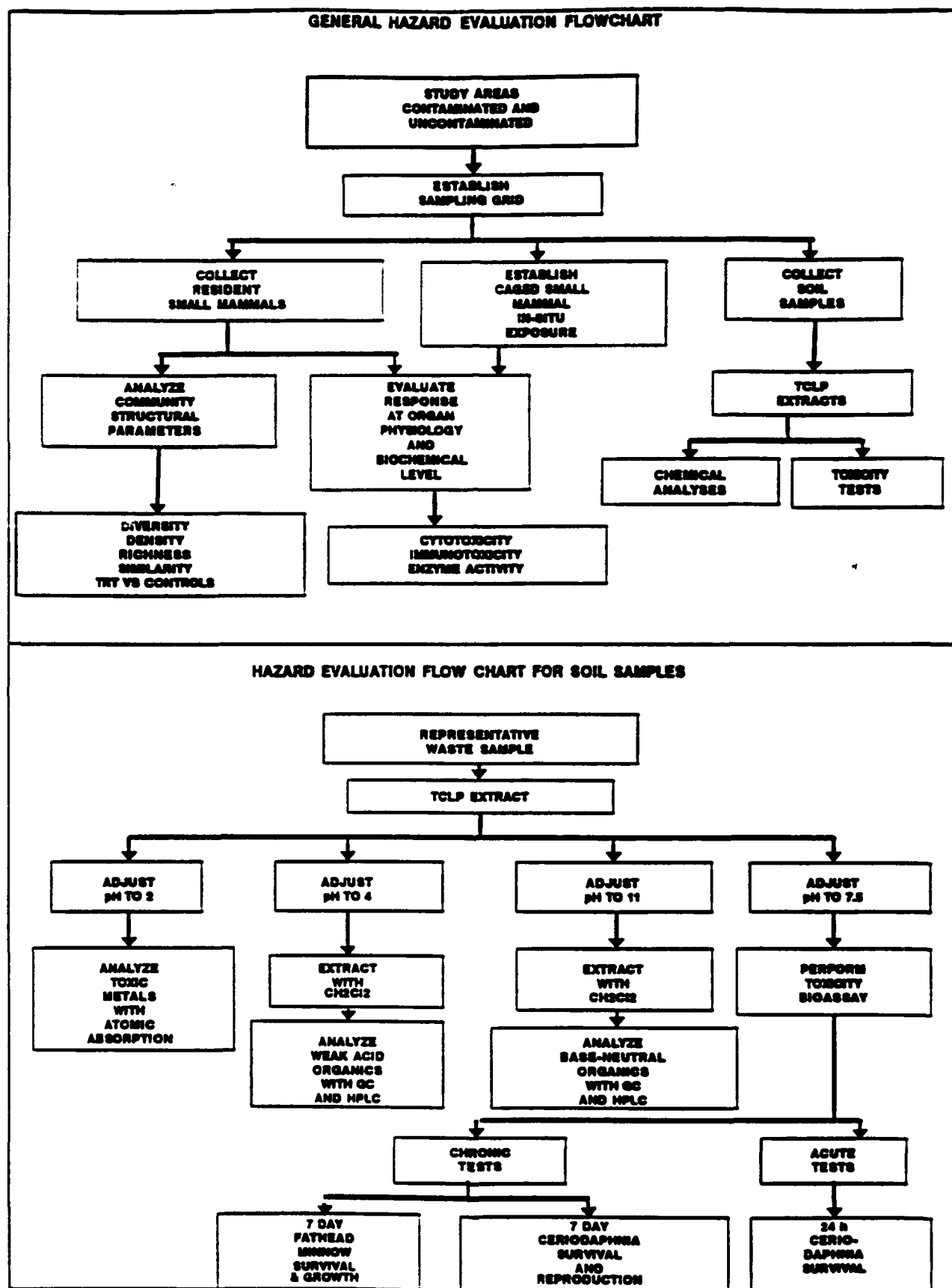


FIGURE 3.

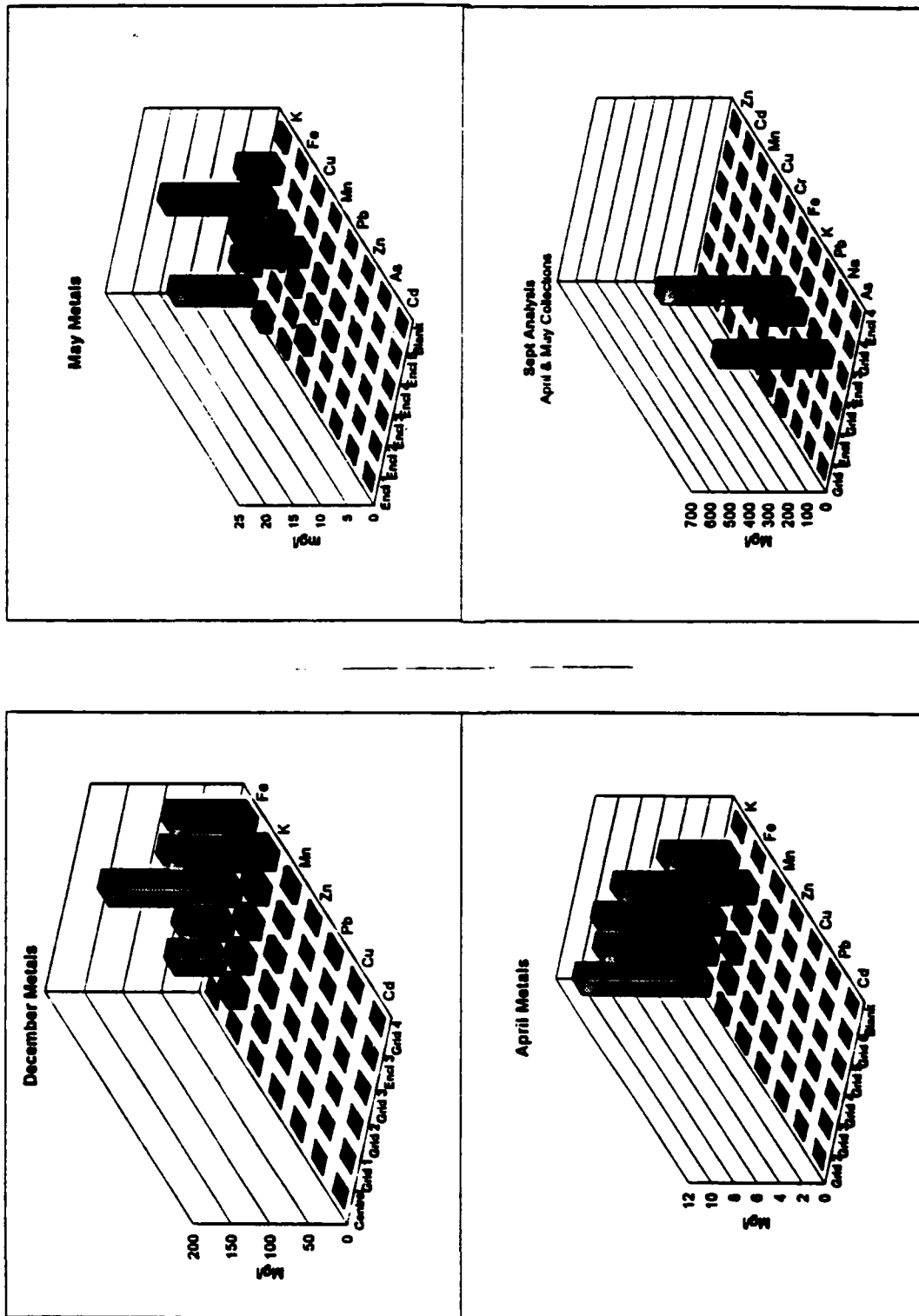


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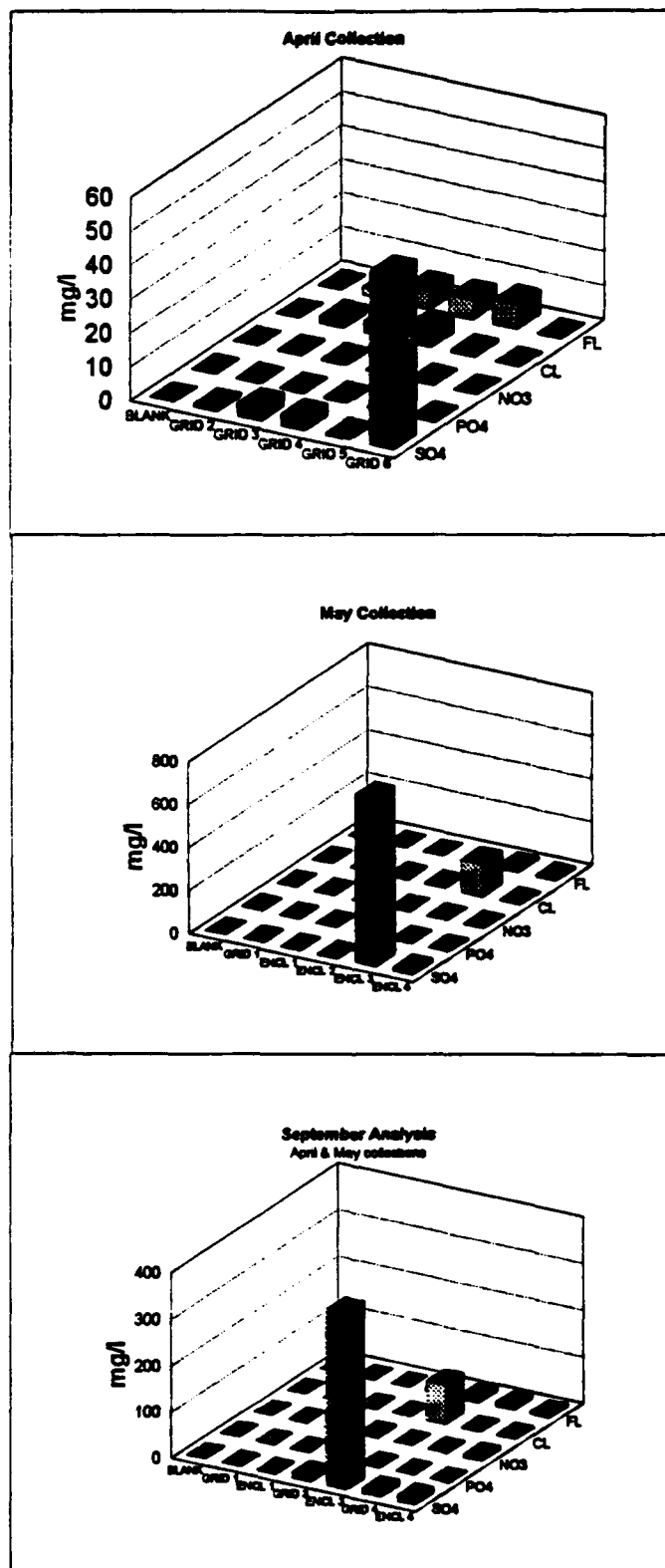


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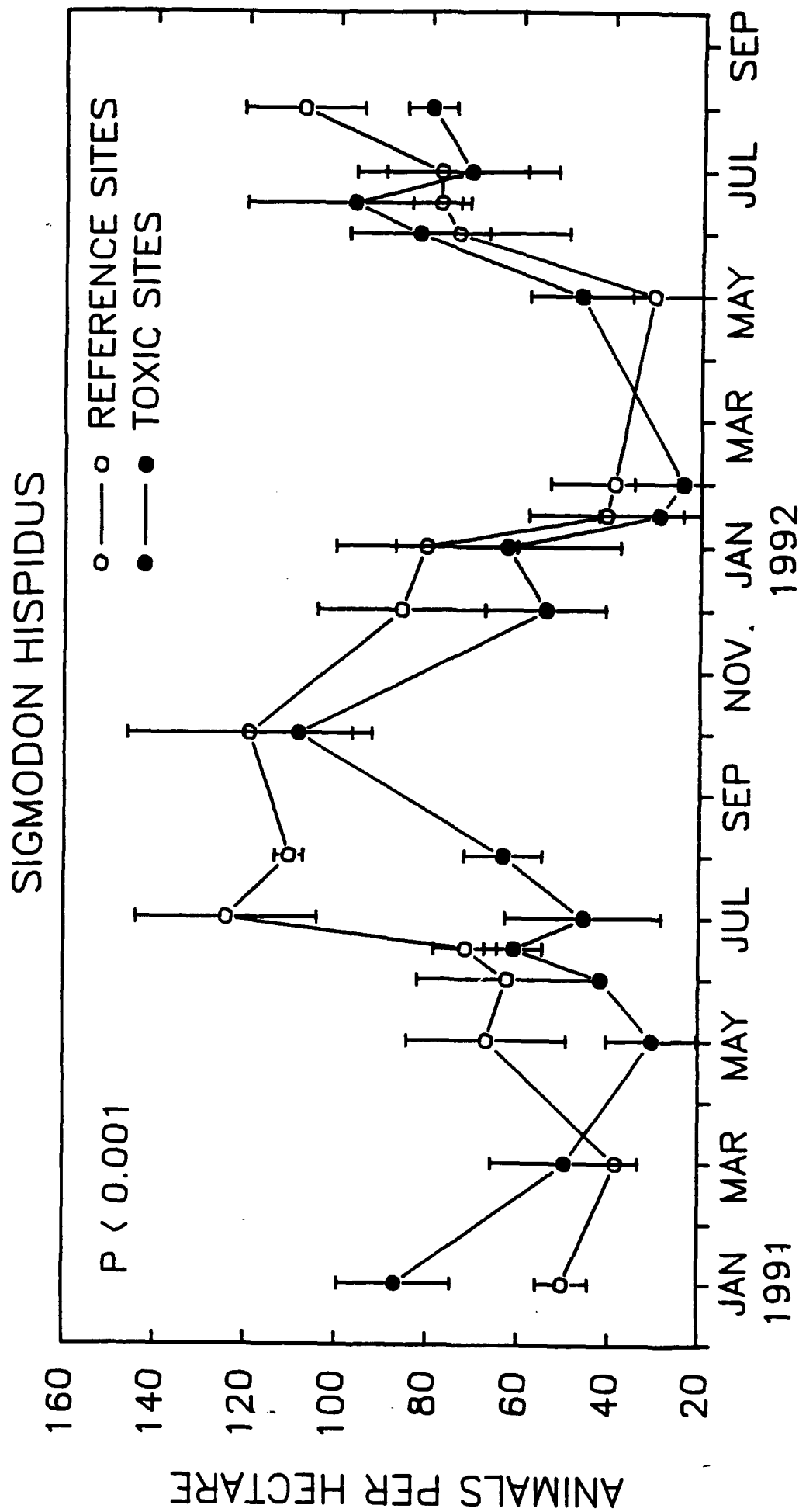


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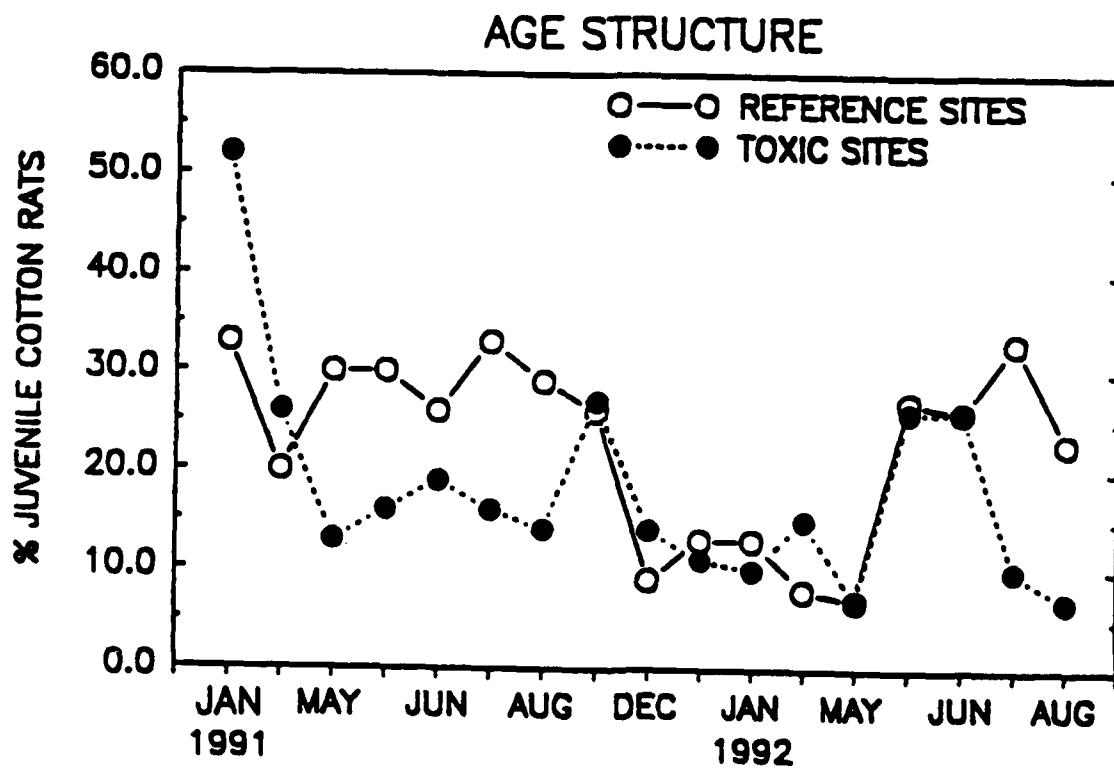
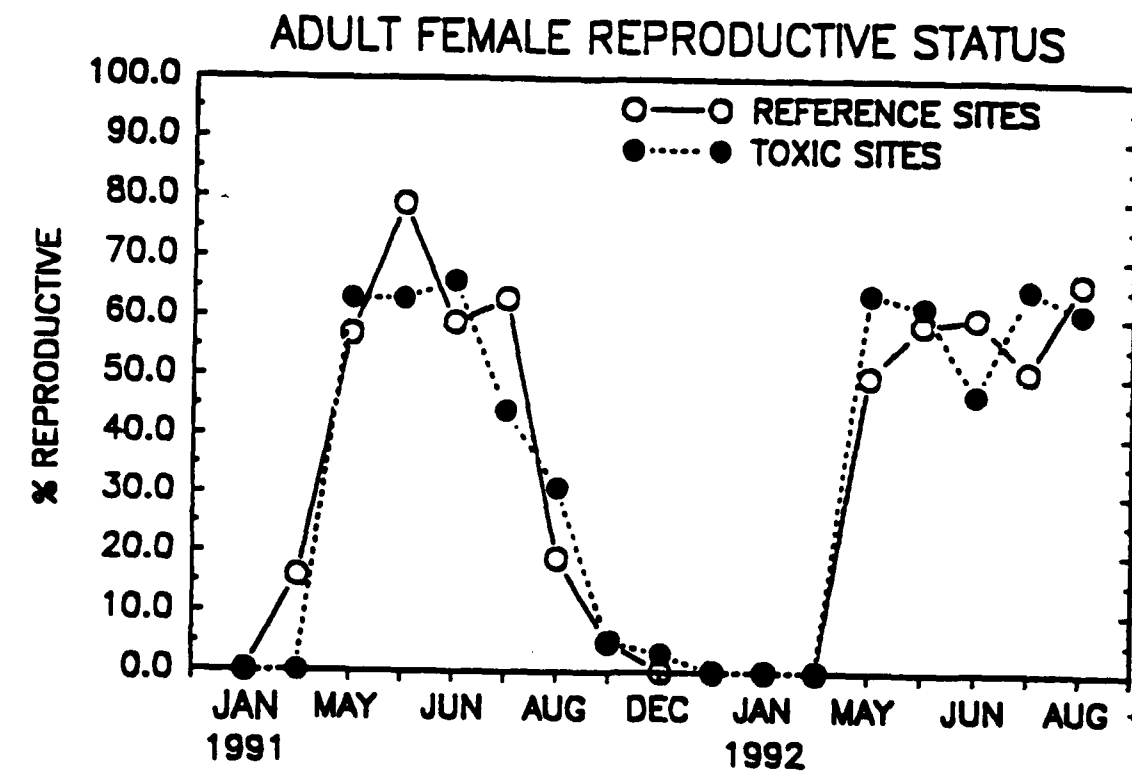


FIGURE 7.

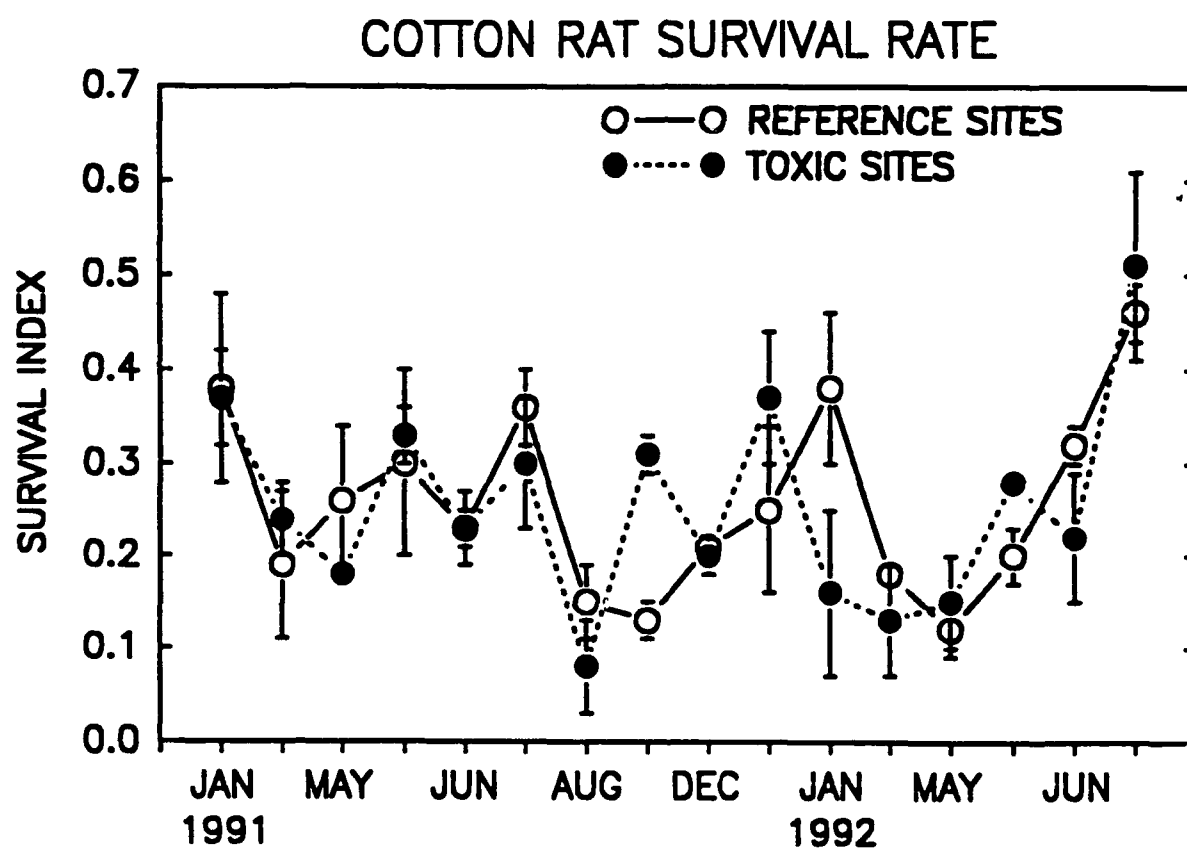


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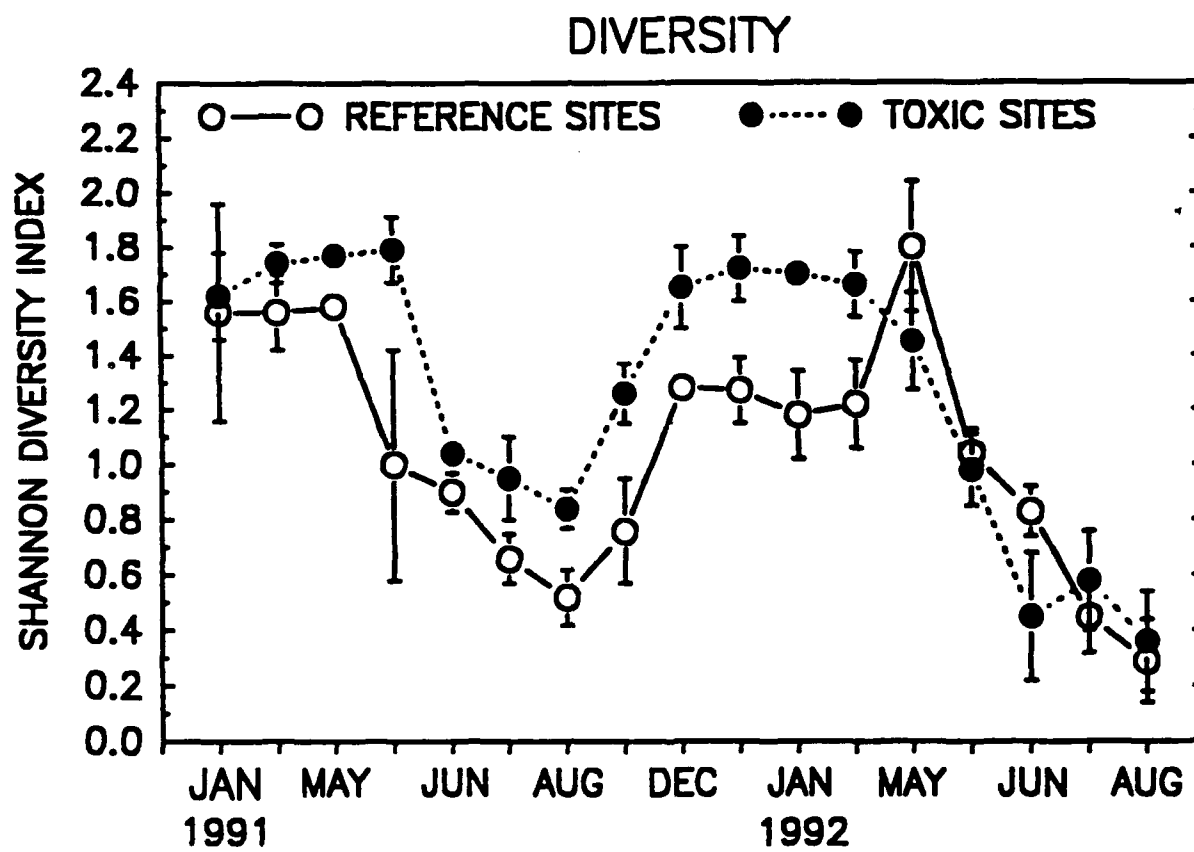


FIGURE 9.

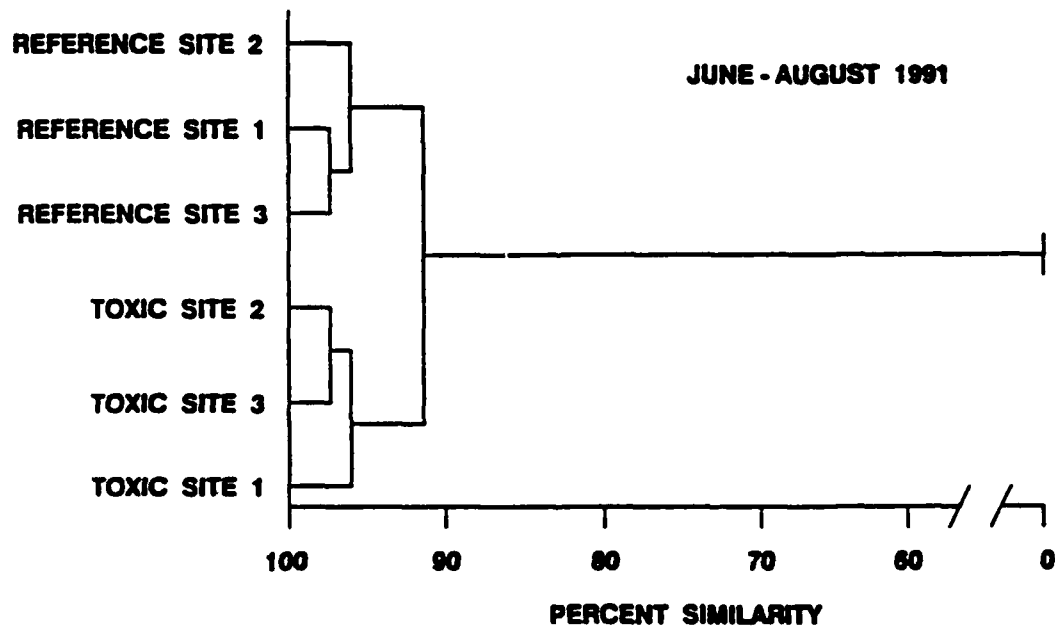
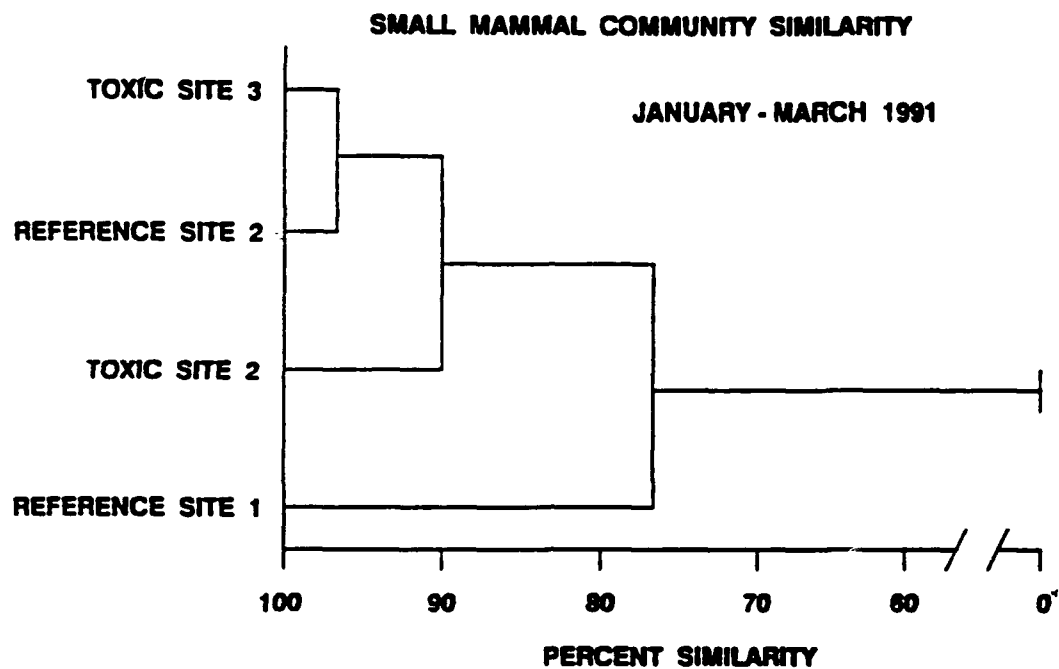


FIGURE 10.

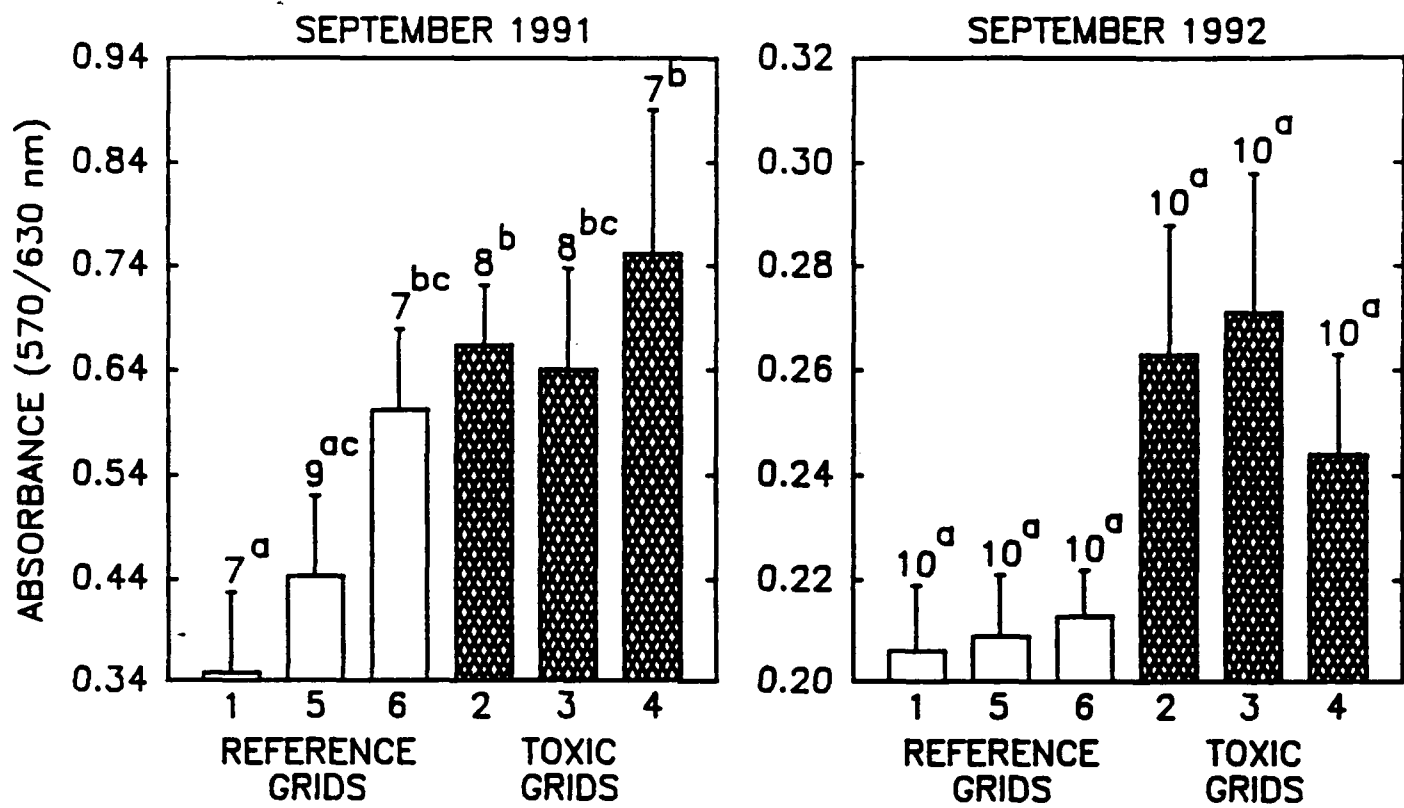


FIGURE 11.

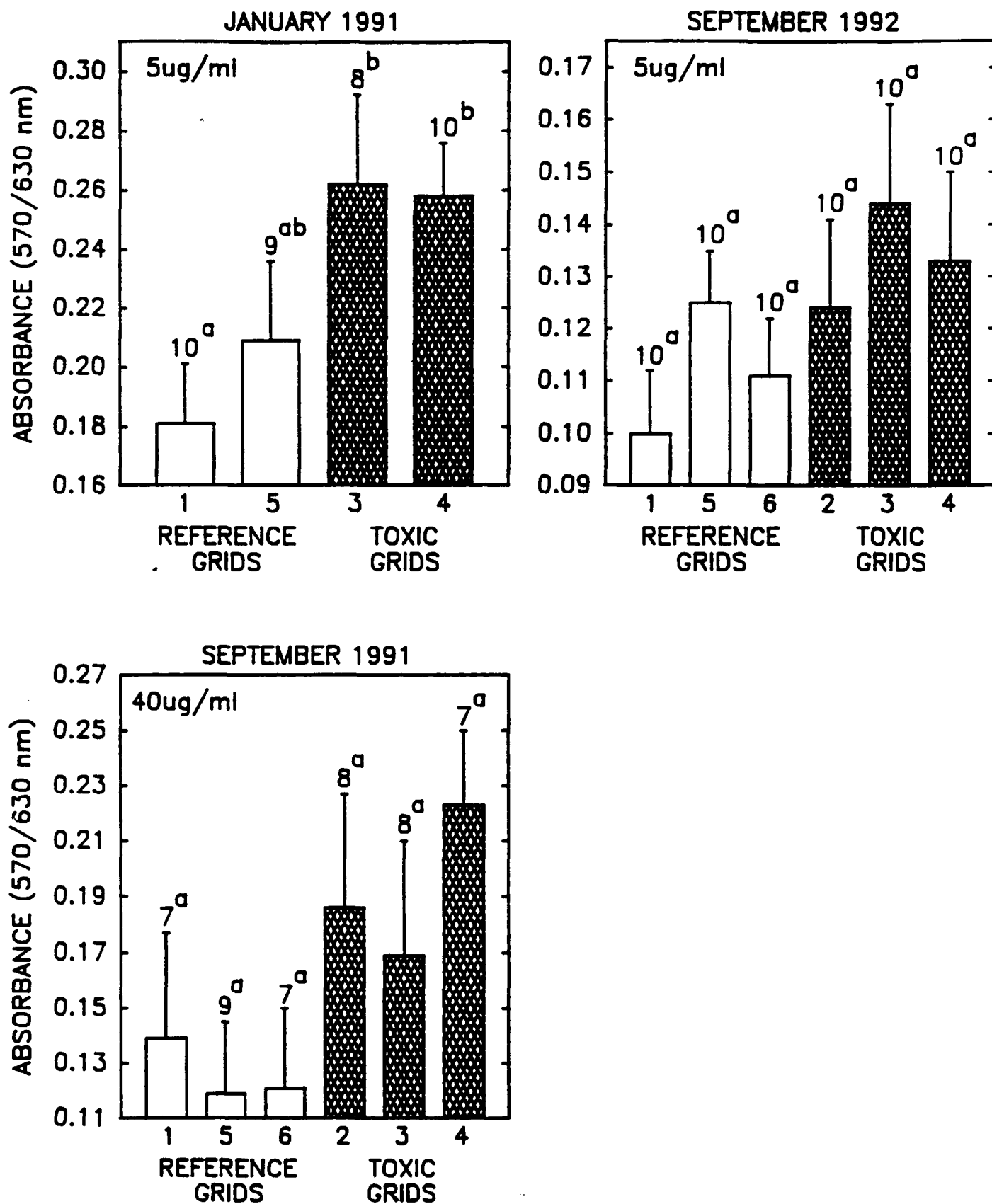


FIGURE 12.

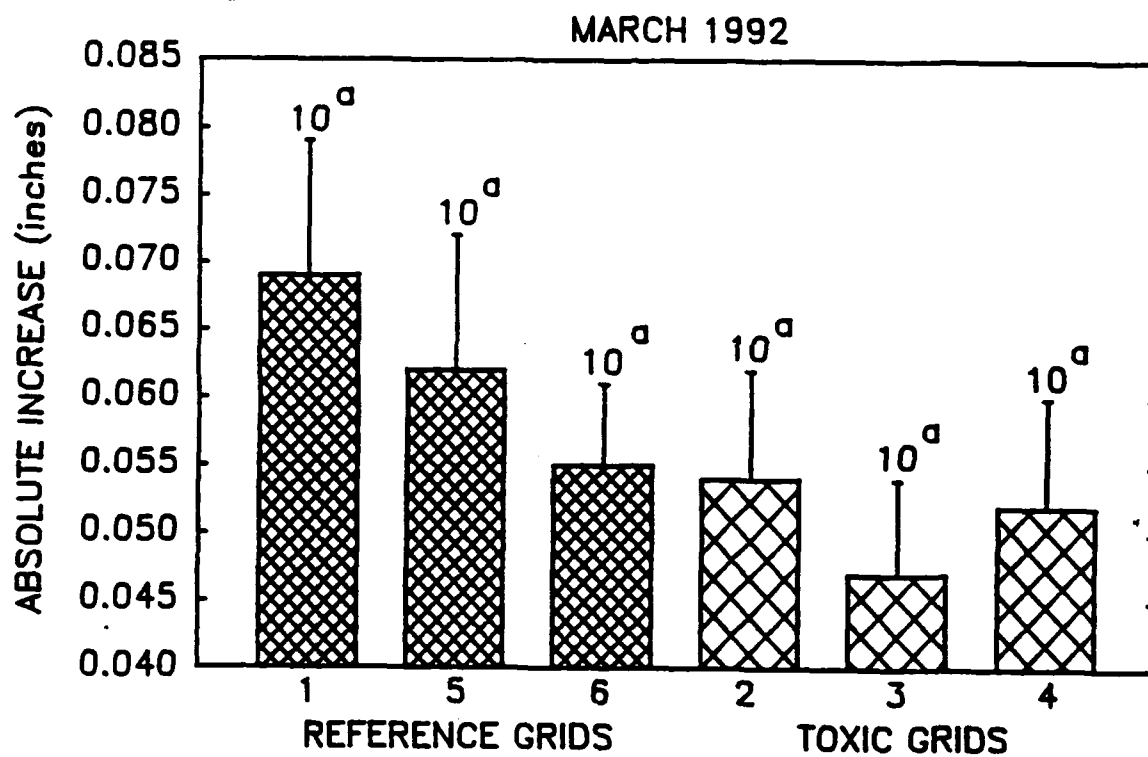


FIGURE 13.

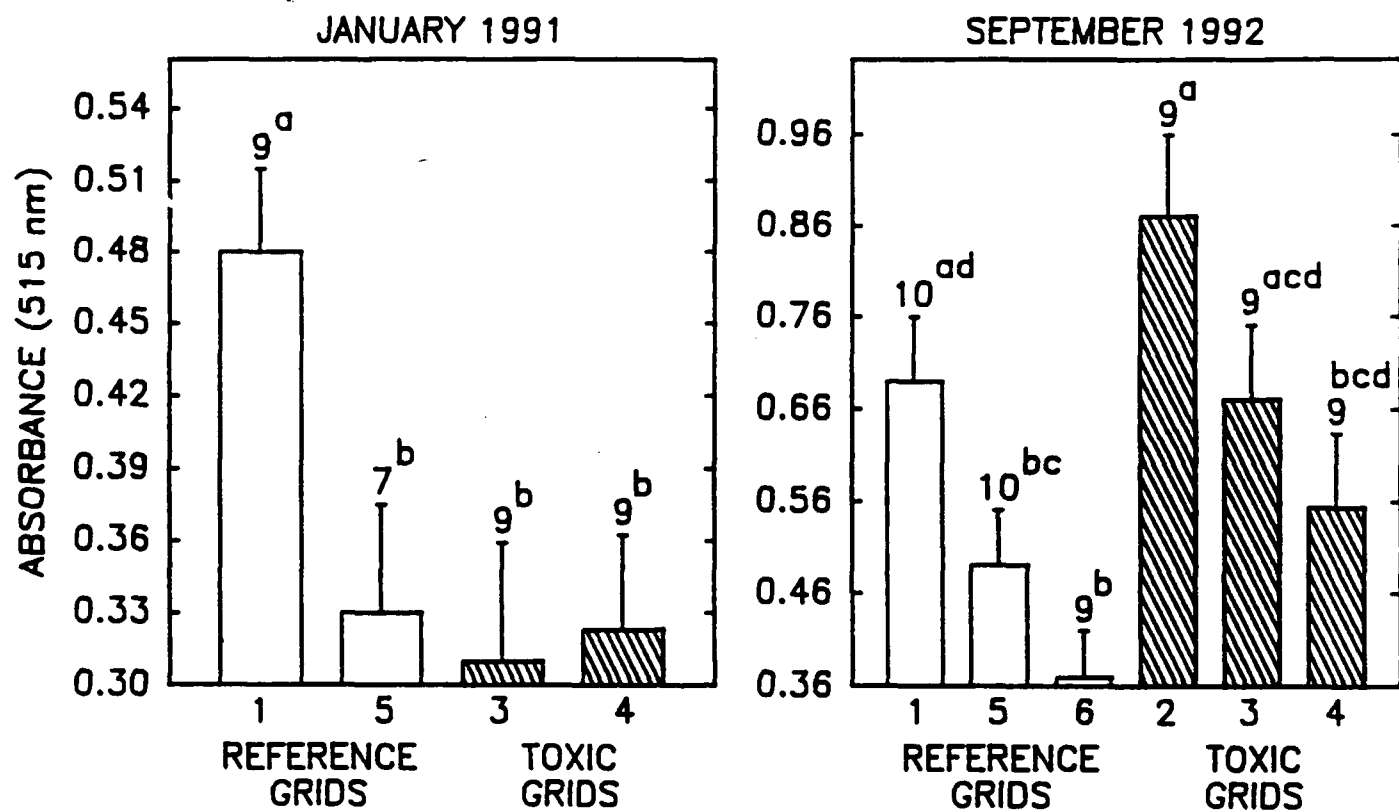


FIGURE 14.

		Coefficient of Variation - G1					
		1	2	3	4	5	6
Mean Channel - G1	1	—	*	NS	NS	NS	NS
	2	NS	—	NS	NS	*	NS
	3	NS	NS	—	NS	NS	NS
	4	NS	NS	NS	—	*	NS
	5	NS	*	NS	*	—	NS
	6	NS	*	*	*	NS	—

FIGURE 15.

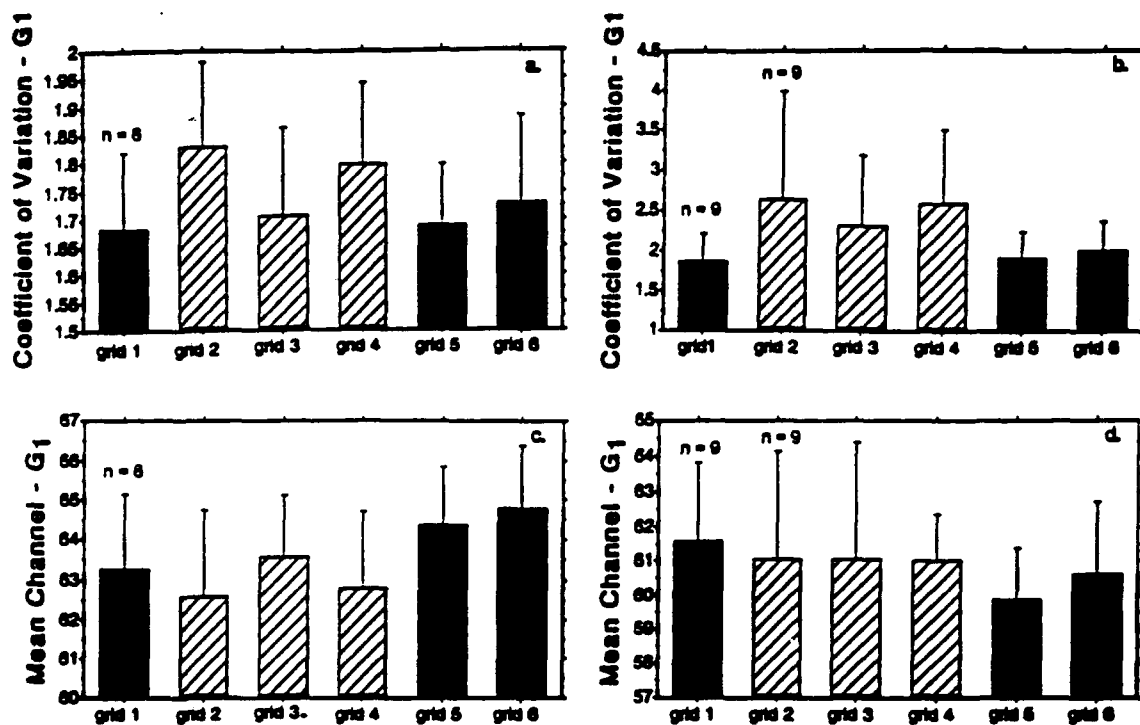


FIGURE 16.

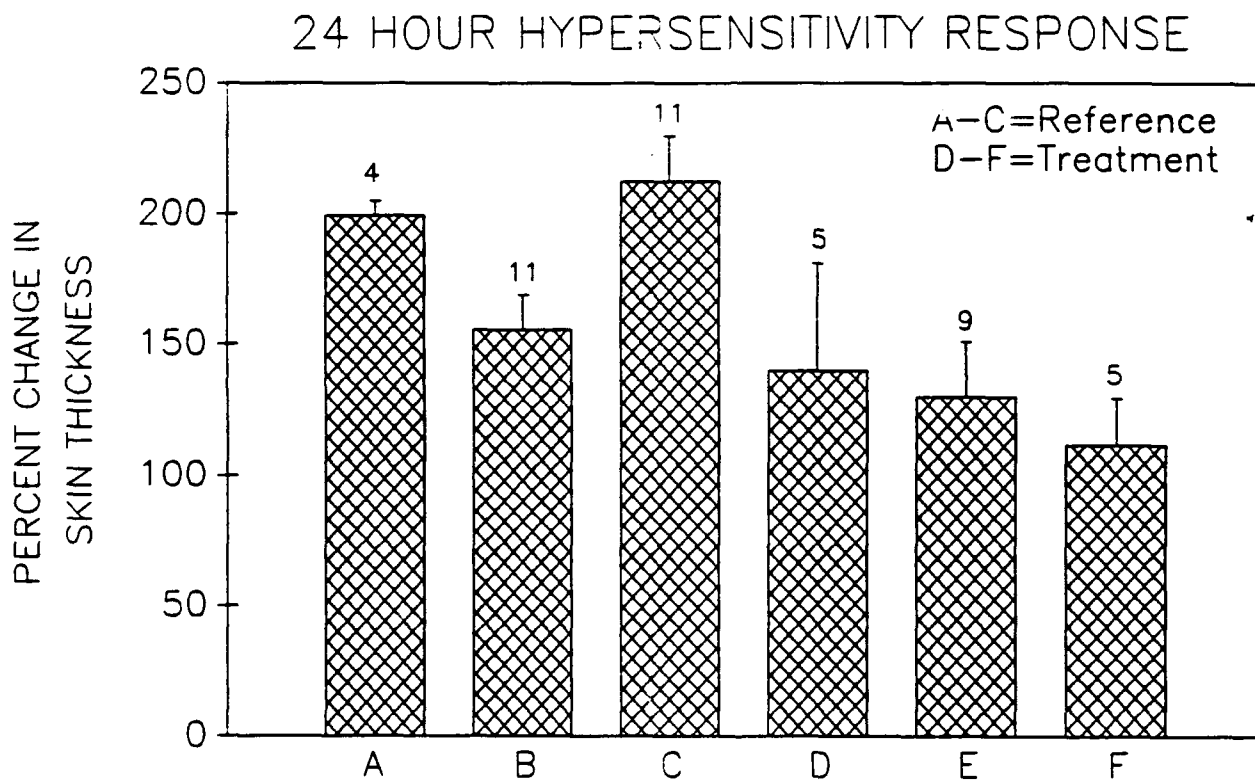


FIGURE 17.

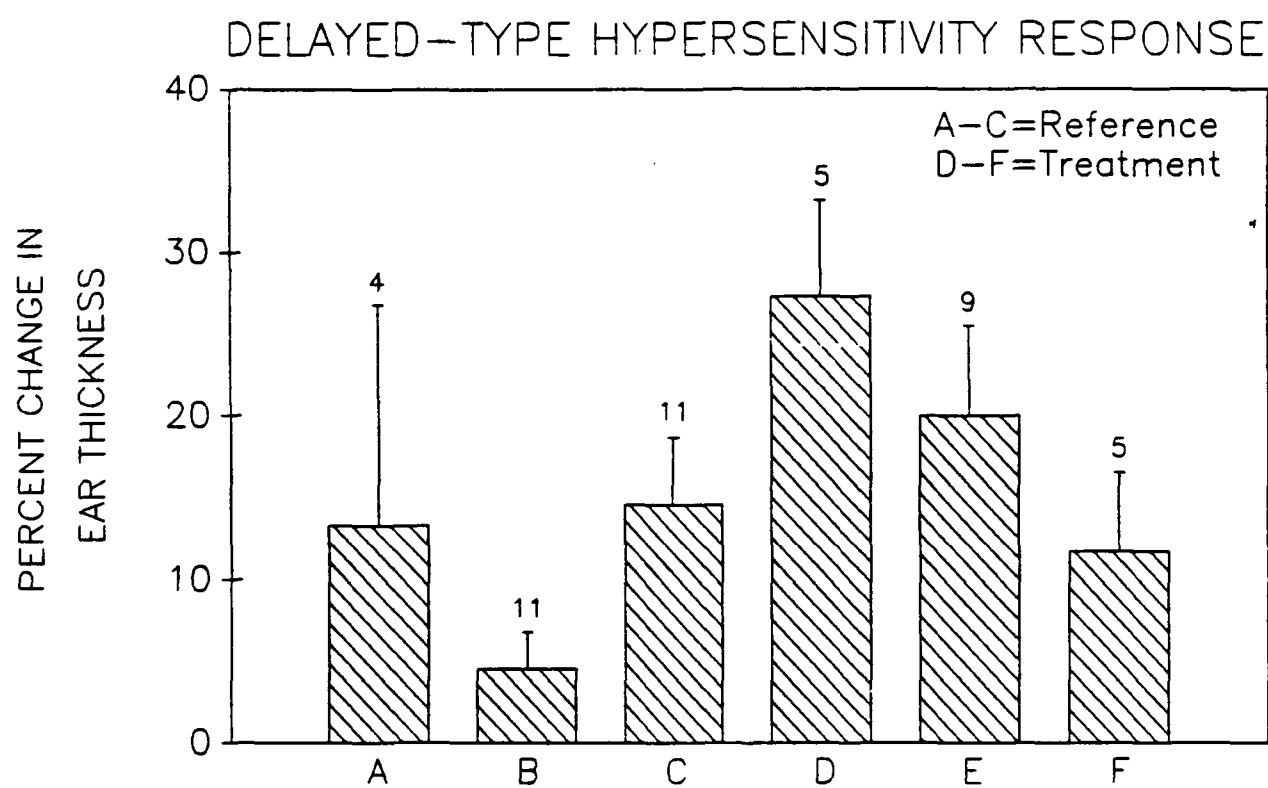


Table 1. Mean ($\pm$ SE) values for immunological and condition variables from cotton rats collected from a Superfund Hazardous Waste facility which were not significantly ($P > 0.05$) different between matched reference grids and toxic grids

	n		Toxic grids		Reference grids	
Cellularity						
Eosinophils (per ul)	48		905(174)		781(115)	
Basophils (per ul)	48		23(9)		9(4)	
Monocytes (per ul)	48		356(43)		324(56)	
Lymphocytes (%)	48		51.0(2.3)		48.8(2.3)	
Neutrophils (%)	48		36.4(1.1)		39.8(1.9)	
Eosinophils (%)	48		8.3(0.1)		7.9(0.9)	
Basophils (%)	48		0.3(0.4)		0.1(0.04)	
Monocytes (%)	48		4.1(0.1)		3.4(0.5)	
Splenocytes/mg spleen $\times 10^3$	98		349(18)		373(16)	
IgM positive splenocytes (%)	47		58.7(1.0)		57.8(1.0)	
Hemoglobin (g/dl)	60		13.7(0.2)		13.6(0.2)	
Mean corpuscular hemoglobin concentration (%)	60		31.7(0.2)		31.7(0.2)	
Barological immunity						
Complement activity (CH50)	108		3955(259)		3819(243)	
Non-specific immunity						
Number of latex beads engulfed/ phagocytically active macrophage	30		1.96(0.12)		1.96(0.12)	
Percentage of total macrophages phagocytizing latex beads	30		21.0(2.6)		21.2(2.9)	
Percent natural killer cell tumoricidal activity	60		51.9(2.7)		52.9(2.6)	
Nitrogen-induced splenocyte proliferation (AR 570/430 nm)						
Pokeweed mitogen (0.625 ug/ml culture)	101		0.141(0.016)		0.138(0.015)	
Pokeweed mitogen (1.25 ug/ml culture)	100		0.128(0.017)		0.119(0.014)	
Interleukin-2 (40 U/ml culture)	83		0.145(0.015)		0.172(0.017)	
In vivo cellular immunity						
24-hr hypersensitivity (%)	90		122(10)		129(9)	

Table 2. Mean ($\pm$ SE) relative mass[§] of selected organs from cotton rats collected from replicate toxic and reference grids on an abandoned oil refinery in Oklahoma in September 1991 and September 1992

Collection date	Toxic grids				Reference grids			Overall means	
	Grid 2	Grid 3	Grid 4	Grid 1	Grid 5	Grid 6	Toxic	Reference	
<u>September 1991</u>									
Spleen ^a	2.65(0.20) ^{ab}	2.94(0.29) ^a	2.29(0.15) ^b	1.65(0.16) ^c	2.21(0.13) ^b	2.24(0.19) ^b	2.62(0.13) [*]	2.05(0.10)	
Adrenals	0.27(0.01) ^a	0.29(0.02) ^{ac}	0.31(0.03) ^{abc}	0.31(0.02) ^{abc}	0.34(0.02) ^{bc}	0.36(0.03) ^b	0.29(0.01) [*]	0.34(0.01)	
Kidneys	7.20(0.20) ^a	7.20(0.20) ^a	6.50(0.20) ^c	7.40(0.10) ^{ab}	7.80(0.20) ^b	7.40(0.20) ^{ab}	7.00(0.10) ^{**}	7.50(0.10)	
<u>September 1992</u>									
Spleen	2.50(0.33) ^a	2.44(0.15) ^a	2.32(0.19) ^a	2.32(0.23) ^a	1.98(0.16) ^a	1.90(0.34) ^a	2.42(0.13) [*]	2.07(0.15)	
Popliteal nodes	0.18(0.03) ^a	0.14(0.03) ^a	0.13(0.02) ^a	0.17(0.03) ^a	0.22(0.05) ^a	0.20(0.03) ^a	0.15(0.01) [*]	0.20(0.02)	
Kidneys	7.70(0.20) ^a	7.30(0.30) ^{ab}	6.80(0.30) ^b	7.50(0.20) ^{ab}	7.80(0.20) ^a	7.70(0.20) ^a	7.30(0.20) ^{**}	7.70(0.10)	
Liver	37.7(0.9) ^a	33.4(1.1) ^b	32.3(1.1) ^b	34.1(1.3) ^b	33.2(0.9) ^b	35.3(1.3) ^{ab}	34.5(0.7) ^{**}	34.2(0.7)	

[§]mg/g of body mass

^aMeans among grids with the same letter are not significantly different at $P \leq 0.05$

^{*}Overall mean of toxic grids significantly different from reference grids at $P \leq 0.09$

^{**}Not different from reference grids at $P \geq 0.10$

Table 3. Mean ($\pm$ SE) cellularity values for blood and selected immune organs of adult cotton rats collected from replicate toxic and reference grids on an abandoned oil refinery in Oklahoma in January and September 1991 and September 1992.

Collection date	Toxic grids			Reference grids			Overall means	
	Grid 2	Grid 3	Grid 4	Grid 1	Grid 5	Grid 6	Toxic	Reference
JANUARY 1991								
Leukocytes ($\times 10^3/\mu\text{l}$) ^a	ND	4.43(0.31) ^a	4.84(0.50) ^a	6.68(0.48) ^b	7.98(1.10) ^b	ND	4.66(0.31) ^a	7.30(0.58)
Lymphocytes ($\times 10^3/\mu\text{l}$)	ND	2.43(0.37) ^a	2.87(0.43) ^{ac}	3.63(0.39) ^{bc}	4.35(0.61) ^b	ND	2.68(0.29) ^a	3.97(0.36)
Neutrophils ($\times 10^3/\mu\text{l}$)	ND	1.57(0.30) ^{ac}	1.47(0.23) ^a	2.60(0.19) ^b	2.93(0.67) ^{bc}	ND	1.51(0.18) ^a	2.76(0.32)
September 1991								
Erythrocytes ($\times 10^6/\mu\text{l}$)	5.63(0.30) ^a	5.25(0.25) ^a	5.42(0.32) ^a	4.67(0.22) ^a	4.91(0.32) ^a	4.88(0.24) ^a	5.43(0.16) ^a	4.82(0.15)
Packed cell volume (%)	42.8(1.5) ^{abc}	44.6(0.7) ^{bc}	45.3(1.0) ^b	41.1(1.7) ^{ac}	39.3(1.0) ^a	41.6(1.4) ^{ac}	44.2(0.7) ^a	40.6(0.8)
FlTC-Concanavalin A positive splenocytes (%)	71.8(2.3) ^a	74.4(1.9) ^a	75.0(2.1) ^a	77.3(2.6) ^a	76.1(2.8) ^a	77.7(2.1) ^a	73.7(1.2) ^a	77.0(1.4)
September 1992								
Erythrocytes ($\times 10^6/\mu\text{l}$)	6.61(0.28) ^a	6.35(0.25) ^a	6.55(0.18) ^a	6.61(0.22) ^a	7.04(0.16) ^a	6.97(0.21) ^a	6.50(0.13) ^{aa}	6.87(0.12)
Mean corpuscular volume (μm^3)	64.3(0.8) ^{ab}	67.0(1.0) ^a	67.4(1.3) ^a	66.2(0.8) ^{ab}	64.1(1.1) ^b	63.8(0.9) ^b	66.9(0.6) ^{aa}	64.7(0.6)
Mean corpuscular hemoglobin (pg)	21.2(0.3) ^{ab}	21.6(0.4) ^a	21.4(0.5) ^a	21.0(0.3) ^{ab}	20.3(0.4) ^b	20.2(0.3) ^b	21.4(0.2) ^a	20.5(0.2)
Platelets ($\times 10^3$)	618(77) ^a	676(73) ^a	607(64) ^a	549(59) ^a	569(49) ^a	485(30) ^a	633(41) ^a	534(27)
Spleen cellularity ($\times 10^6$)	80.5(14.8) ^a	76.8(13.5) ^a	95.8(12.0) ^a	70.2(7.4) ^a	70.6(10.0) ^a	57.9(12.3) ^a	84.4(7.7) ^{aa}	66.2(5.7)
Paired popliteal node cellularity ($\times 10^6$)	21.3(4.4) ^a	16.9(4.5) ^a	19.8(4.9) ^a	25.4(6.2) ^a	26.6(6.6) ^a	26.7(6.6) ^a	19.4(2.6) ^a	26.2(3.6)
Paired popliteal node cellularity ($\times 10^6/\text{mg nodes}$)	1.04(0.10) ^{ac}	0.92(0.08) ^a	1.02(0.09) ^a	1.36(0.16) ^{bc}	1.22(0.12) ^{abc}	1.39(0.13) ^b	0.99(0.05) ^a	1.32(0.08)

n = 10 for all means except: leukocytes, lymphocytes, neutrophils for grids 3 and 5 where n = 8 and 9, respectively; erythrocytes in September 1991 for grid 1 where n = 9; packed cell volume for grids 1-3 and grid 6 where n = 9 and 8, respectively

^aMeans among grids with the same letter are not significantly different at $P \leq 0.05$

^{aa}Overall mean of toxic sites significantly different from reference sites at $P \leq 0.05$

^{ac}Overall mean of toxic sites significantly different from reference sites at $P \leq 0.05$