TOXICITY RISKS TO RESIDENT SMALL MAMMALS INHABITING FORMER LAND-TREATMENT FACILITIES FOR PETROCHEMICAL WASTES

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PREFACE

Environmental contamination is a persistent problem throughout the world. Scientists, land managers, and public concerns over past and current practices of waste disposal has lead to a search for valid methods to evaluate the effects of pollution on human and ecological health. Small mammals as resident biomonitors of environmental contamination have potential to be a good measure of ecological health. To this end, we evaluated the immune response and other physiological parameters of cotton rats (*Sigmodon hispidus*) living on land-treated petroleum waste sites to determine risks of exposure. This thesis includes three manuscripts formatted for submission to *Environmental Pollution* (Chapter I), *Archives of Environmental Contamination and Toxicolgy* (Chapter II), and *Journal of Wildlife Diseases* (Chapter III). The manuscripts are complete as written and need no supporting material.

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ABSTRACT:

Land-treatment of petrochemical wastes is a widely used method to dispose of hazardous and non-hazardous waste by biodegradation. However, no comprehensive assessment of the impact of such disposal techniques on terrestrial ecosystems has been conducted. Because wild rodents frequently reside on these waste sites after closure or abandonment, despite the presence of suspected immunotoxicants in the soil, we explored the seasonal sensitivity of the immune system of the hispid cotton rat (*Sigmodon hispidus*) to *in situ* exposures. Animals were monitored on five contaminated land-treatment sites and five ecologically matched-reference sites in Oklahoma, USA over two seasons (summer and winter). Most hematological parameters were not adversely affected by land-treatment, however, platelet counts were 26% greater in cotton rats from land-treatment sites compared to reference sites in winter. Significant treatment-related differences were observed in total serum protein concentrations, organ mass and organ cellularity, but these differences were not consistent across the five land-treatment units. Lymphoproliferative responses of cotton rat splenocytes stimulated *in vitro* were elevated for a T-cell mitogen and

depressed for a B-cell mitogen in animals from land-treatment compared to reference sites. The ability of splenocytes to proliferate in response to interleukin-2 receptor-binding was not influenced by treatment. Total yields of peritoneal cells, yield of peritoneal macrophages, and yield of peritoneal lymphocytes were influenced to varying degrees by land-treatment. Functionally, *in vitro* metabolic activity of peritoneal macrophages was 114% greater in cotton rats from land-treatment sites compared to reference sites during summer. These results indicate that petrochemical wastes applied to soils on these five land-treatment sites had variable immunomodulatory effects in resident cotton rats. Immune alterations for some assays were indicative of enhancement on some land-treatment sites while suppressive on other land-treatment sites, which could have been a function of type and concentration of immunotoxicants present on each site and highlights the uniqueness of each land-treatment site.

Key words: Immunotoxicity, land-treatment, petrochemicals, wildlife toxicology, cotton rats

INTRODUCTION

Land-treatment is a waste management technology that has received increasing attention in recent decades, primarily because of its low cost and versatility for handling both industrial and municipal wastes. This managed disposal technology involves the controlled application of waste onto or into soil for the purpose of biodegradation of organic wastes, immobilization of inorganics, and avoiding bioaccumulation of hazardous compounds (Loehr & Malina, 1986).

Over 50% of hazardous wastes generated by the petroleum industry have been disposed of through land-treatment operations (American Petroleum Institute, 1984). There are an estimated 200 land-treatment sites in the United States that are designed for disposal of hazardous industrial wastes, over half of which are operated by the petroleum industry (American Petroleum Institute, 1984; LaGrega *et al.*, 1994). This number does not include the myriad land-treatment sites designed for disposal of petrochemical wastes that are classified as non-hazardous. Land-treatment facilities that were used for disposal of petroleum refinery wastes are widespread in their distribution and include both active and inactive sites, yet no comprehensive assessment of their impact on terrestrial ecosystems has been completed. Possibly because of accumulation of nutrients in soil, many formerly active land-treatment sites have become heavily vegetated after abandonment and eventually support diverse assemblages of small mammals and other vertebrates (McMurry, 1993).

As with most industrial waste sites contaminated with petrochemicals, a variety of chemical mixtures exists in the soil, including organic hydrocarbons and inorganic compounds. Toxicity of these highly complex chemical mixtures to mammalian systems remains largely unknown (Coppock *et al.*, 1995). Limited analyses of soils from land-treatment waste sites suggests that such sites possess an array of potentially immunotoxic compounds, including heavy metals such as lead and cadmium, and organic hydrocarbons such as benzene, toulene, and benzo(a)pyrene (Yates, 1994; Rafferty, 1998). Complex chemical mixtures such as these can greatly influence the overall phsyiology of an organism (Khan

et al., 1989; Coppock *et al.*, 1995; Khan *et al.*, 1996), including altering the normal state of host immunocompetence (Rocke *et al.*, 1984; Briggs *et al.*, 1996). Potential consequences from *in situ* exposure to a variety of immunotoxicants in wild animal populations have been most evident among marine mammals, which may ultimately be reflected in increased rates of morbidity and mortality within exposed populations (Duffy *et al.*, 1993; Ross *et al.*, 1996; Zentebo-Savin *et al.*, 1997). Comparable studies on immunotoxic risks to terrestrial small mammal assemblages from exposure to complex mixtures of petrochemicals in the environment have not been conducted, although McMurry (1993) documented a higher incidence of immune system alterations in a population of cotton rats residing on an abandoned oil refinery complex.

We hypothesized that habitats where disposal of petrochemical wastes was facilitated by land-treatment techniques posed immunotoxicity risks to resident small mammal populations. To explore this hypothesis, we monitored seasonal differences in various physiological indices of immune response of hispid cotton rats (*Sigmodon hispidus*) from five land-treatment disposal and reference sites in Oklahoma, USA.

MATERIALS AND METHODS

Experimental Design and Study Sites

We selected five land-treatment units located throughout Oklahoma. Each land-treatment unit consisted of a land-treatment waste site and a matched-reference site in similar habitat located from 1 to 16 km from the land-treatment

site. All land-treatment sites were characteristic of a disturbed prairie ecosystem, with early successional species dominating the vegetation community. Reference sites were selected based on a visual assessment of similarity of vegetation structure and composition with their paired land-treatment site; sites also were similar with respect to topography.

Land-treatment units (herein referred to as units 1 through 5) were chosen based on accessibility to the site, historical use for disposal of oil refinery wastes, and presence of suitable vegetation to support a resident small mammal population. One of five land-treatment units was a designated Superfund Waste Site (land-treatment unit 1, Oklahoma Refining Company, Cyril, OK, USA). Virtually no historical information existed on what levels and mixtures of contaminants were disposed of on these sites.

All five land-treatment units were sampled during two seasons (winter [February-March] and summer [September-October]) to evaluate whether season influenced susceptibility to immunotoxicants. Six males and 6 females from each land-treatment and matched-reference site were collected within each season. All animals were reproductively mature and weighed more than 50 g. Each pair of reference and land-treatment sites were evaluated at the same time to minimize sampling error due to interassay variation; only one pair of sites was sampled per day. Sherman live-catch traps were placed in each pair of sites and animals were removed from traps the following morning and returned to an approved laboratory animal facility for further processing. Cotton rats were placed individually in polycarbonate cages (47 x 27 x 20 cm) with wire-lid tops,

wood-chip bedding, and food (Purina 5001 laboratory rodent chow; Purina Mills, St. Louis, MO, USA) and tap water provided *ad libitum*. Animals were housed under a 16L:8D light-dark illumination cycle provided by fluorescent lighting at 23-24 °C, 50% relative humidity, and 15 fresh-air changes per hour. All animals were processed and immunocompetence evaluated within 48 hours of capture.

Hematology and Morphology

Animals were anesthetized by Metofane (Methoxyflurane, Pitman-Moore, Mundelein, IL, USA) inhalation and final body mass was recorded to the nearest 0.1 g. Whole blood was collected from the retro-orbital sinus plexus into a Vacutainer serum separation tube (Becton Dickinson, Rutherford, NJ, USA), centrifuged, and serum collected and stored at -80 °C for later analysis. A heparinized blood sample (40 µl) was obtained for automated hematology analysis and differential whole blood cell counts. Blood samples were analyzed on a Serono-Baker System 9000 automated hematology analyzer (Serono-Baker Diagnostics, Allentown, PA, USA) that was previously calibrated for cotton rats to measure white blood cell count (WBC), red blood cell count (RBC), platelet count, hematocrit, hemoglobin, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC). Total serum protein was measured by the biuret method (Kingsley, 1942) against a human serum standard (Serachem Clinical Chemistry Control, Fisher Scientific, Orangeburg, NY, USA).

Anesthetized animals were killed by cervical dislocation and immediately processed aseptically for collection of peritoneal exudate cells and splenocytes.

Subsequently, mass of liver, kidneys, adrenal glands, spleen, and popliteal lymph nodes were recorded to the nearest 0.1 mg after blotting tissues dry and removing any adherent fat tissue from the organ. Mononuclear lymphocytes were enumerated in paired popliteal lymph nodes as an index of immune organ cellularity. Lymph nodes were disrupted with a glass homogenizer in 3 ml of phosphate buffered saline (PBS) containing 5% fetal calf serum (FCS), and cellularity measured on a previously calibrated automated cell-counter (Serono-Baker System 9000). Cellularity was expressed as total mononuclear cell yield and relative mononuclear cell yield (cells mg^{-1}).

Phytohemagglutinin Hypersensitivity Response

In vivo cell-mediated immune responsiveness was measured using an intradermal injection (rump) of 100 µl of phytohemagglutinin (PHA; Sigma, St. Louis, MO, USA; 2.5 mg ml^{-1} of PBS) 24 hours prior to death (Williams *et al.*, 1979); the opposite rump was challenged with 100 µl of PBS only. Double skin-fold thickness was measured to the nearest 0.001 inches with a pressure-sensitive micrometer (24 hours after injection). PHA-hypersensitivity response was calculated as percent increase in skin-fold thickness of the PHA-injected site corrected for the PBS-injected site.

Lymphoproliferative Responsiveness of Splenocytes

The ability of lymphocytes harvested from the spleen to undergo clonal expansion when stimulated with mitogenic lectins (T-cell polyclonal stimulator, concanavalin A [Con-A], *Canavalia ensiformis*; B- & T- cell activator, pokeweed mitogen [PWM], *Phytolacca americana*; Sigma, St. Louis, MO, USA;) or

stimulated with lymphokines (interleukin-2, IL-2, recombinant human IL-2; Boehringer Mannheim, Germany) was assessed *in vitro* by measuring incorporation of radio-labeled [^{3}H]-thymidine into DNA of proliferating cells. The spleen was removed aseptically and a single-cell suspension prepared according to methods of McMurry *et al.* (1995). Lymphoproliferative responsiveness of cultured splenocytes was assessed *in vitro* following stimulation with Con-A (5 µg ml^{-1} culture), PWM (1.25 µg ml^{-1} culture), and IL-2 (40 U ml^{-1} culture), as described by Dabbert & Lochmiller (1995). Briefly, mitogens or IL-2 (10 µl) were added to 90-µl splenocyte suspensions (final concentration of 500,000 cells per well in a supplemented medium RPMI-S) in 96-well microtiter plates in triplicate; 10 µl RPMI-S was substituted for mitogen or IL-2 in unstimulated control wells. The RPMI-S medium was prepared by addition of 1.0 % sodium pyruvate (100 mM solution, Sigma), 1.0 % penicillin-streptomycin solution (Sigma P-0781), 100 µl 2-mercaptoethanol (50 µM solution, Sigma), and 10 % horse serum to Roswell Park Memorial Institute, RPMI-1640 medium.

After 54 hrs incubation at 37 °C, ^{3} H-thymidine (1 µCi per well) was added to each well and incubated for an additional 18 hrs; cells were then harvested on a semiautomated PhD Cell Harvester (Cambridge Tech Inc., Watertown, PA, USA). Amount of radioactivity incorporated into DNA of proliferating cells in triplicate cultures was measured in a liquid scintillation counter (Packard Instruments, Meriden, CT, USA) as disintegrations per min (dpm). Lymphoproliferative responsiveness of cultured splenocytes was calculated as

the difference in average dpm for three stimulated wells and average dpm for three unstimulated control wells to correct for spontaneous proliferation.

Macrophage Function

Resident peritoneal macrophages were aseptically removed by injecting 20 ml of ice-cold MEM-H medium (500 ml Dulbecco's Modified Eagle's Medium supplemented with 1.85 g of $NaHCO_3$, and 10 ml of heparin 1,000 U / ml, Sigma H-0880) into the peritoneum, followed by vigorous palpation of the abdomen for 1 min. Exudate was removed and transferred into culture tubes where it was centrifuged for 8 min at 1,200 rpm at 4 °C to isolate cells. The cell pellet was resuspended in 5 ml of tris-buffered ammonium chloride (0.83 %, pH 7.2) to lyse erythrocytes and underlayed with 1 ml of sterile FCS, before centrifuging again. After washing cells twice in 5 ml of cold MEM-H-F (MEM-H with 10% FCS), viable cell counts were determined by trypan blue (Sigma T-8154) exclusion using a hemacytometer. Percentage of macrophages was determined by non-specific esterase staining (Koski *et al.*, 1976; McMurry, 1993) and cell numbers adjusted to $1X10^6$ macrophages ml^{-1}. We recorded total number of exudate cells, and numbers of macrophages, mast cells, lymphocytes, and neutrophils harvested from each animal.

Integrity of the respiratory burst of harvested peritoneal (resident) macrophages was assessed by measuring reduction of nitroblue tetrazolium dye (NBT) to formazan by the superoxide anion using a slight modification (see McMurry *et al.*, 1995) of the procedure described by Southwick & Stossel (1986). Macrophages (500,000 cells in 500 µl MEM-H-F) were incubated without

(unstimulated) or with (stimulated) latex beads (Fluorsbite latex beads, 1 μm dia.; Polysciences, Warrington, PA, USA) and NBT (1mg ml^{-1}; Sigma N-6876) as described by Shousha & Kamel (1972). The NBT-formazan that is formed by macrophages during metabolism was extracted with 1 ml pyridine in a dry bath at 110 °C and absorbance measured directly on a spectrophotometer at 515 nm against a pyridine blank (unstimulated) or pyridine with latex beads (stimulated). A stimulation index was calculated as (stimulated - unstimulated)/unstimulated.

Statistical Analysis

All data were tested for normality (Proc Normal; SAS, 1994) and homogeneity of variances (Levines test; Steel & Torrie, 1980). Data not meeting these assumptions were transformed (square-root or arcsine) prior to further statistical analyses. We used square-root (platelet count, lymphoproliferation responsiveness, PHA-hypersensitivity response) and arcsine of square-root (macrophage metabolic activity, total macrophage cell yield) transformations for statistical tests, but we report untransformed means in the text.

We used a randomized complete block design in a 2 X 2 X 5 factorial format with two treatments (land-treatment and reference), and two seasons (summer and winter) and five land-treatment units (1-5) to evaluate whether the land-treatment of petrochemical wastes influenced resident cotton rat populations. Comparisons were made using PROC MIXED (SAS, 1994) with sources of variation distributed among main factor effects and interaction terms (land-treatment unit by treatment interaction, season by treatment interaction, and land-treatment unit by season interaction); we used treatment by land-

treatment unit by season interaction as the error term in our analysis. If there were no significant interaction terms, main effects were compared with the PDIFF option for the LSMEANS statement. Significant interaction term effects were compared using the SLICE option for the LSMEANS statement. Satterthwait's approximation was used in calculating degrees of freedom for the error term. Statistical significance for all hypothesis tests was set a priori at $P \leq 0.05$ and means (±SE) are reported.

RESULTS

All morphologic, hematologic, and immunologic parameters that we measured showed strong seasonal effects ($P < 0.05$). Body mass of cotton rats showed no treatment main effect, but a significant land-treatment unit by treatment interaction was indicated ($P < 0.007$). A closer inspection of this interaction showed the difference to be attributed to land-treatment unit 1 where mean body mass was significantly greater on the land-treatment site (134 ± 7 g) compared to its matched-reference (108 ± 5 g) site (Fig. 1). Mean body masses of animals from other land-treatment units were similar during both seasons.

Hematology and Morphology

Treatment had no appreciable impact on hematology of resident cotton rats, with the exception of platelet counts and total serum protein (Table 1). Platelet counts showed a significant season by treatment interaction ($P < 0.012$), where concentrations in winter were about 26% greater in animals from land-treatment sites than from reference sites (Fig. 2). There were no differences in

platelet counts between land-treatment and reference populations during summer; two land-treatment units in summer had missing values because of logistical problems with the hematology analyzer. Concentration of total serum proteins showed a significant land-treatment unit by treatment interaction ($P < 0.005$; Fig. 3). Least square means analysis showed concentration of total serum proteins were significantly greater ($P < 0.05$) on land-treatment unit 4, where levels were about 8% higher in animals from the land-treatment than reference site. Concentrations of protein in animals on land-treatment and reference sites were similar for the other four land-treatment units.

No treatment differences were observed for organ masses of adrenal glands, spleen, and popliteal lymph nodes (Table 2). Both absolute and relative mass of kidney and liver showed treatment differences, but differences were not always consistent across land-treatment units (Fig. 4). A significant land-treatment unit by treatment ($P < 0.003$) and season by treatment ($P < 0.02$) interaction was demonstrated for absolute kidney mass. Mean kidney mass differed significantly ($P < 0.05$) in summer but not winter, and averaged 8% more in mass for animals from reference sites compared to land-treatment sites. A significant land-treatment unit by treatment interaction ($P < 0.001$) was indicated for relative kidney mass, where differences between land-treatment and reference sites were apparent for land-treatment units 1, 3, and 4 (Fig. 4). Relative kidney mass was greater on reference than land-treatment sites for units 1 and 3, but the reverse occurred on land-treatment unit 4. Significant land-treatment unit by treatment interactions were also observed for absolute ($P <$

0.004) and relative ($P < 0.001$) liver mass. However, differences between land-treatment and reference populations for both absolute and relative liver mass showed no consistent pattern, and differences ranged from 10 to 26% (Fig. 4). The only clear pattern was for land-treatment unit 3, where both kidney and liver were smaller on the land-treatment site.

Yield of mononuclear lymphocytes from popliteal lymph nodes showed no significant relationship with treatment (Table 2); overall mean yield was 14.6 ± 3.5 cells x 10^6. Relative cellularity of spleen (splenocytes mg^{-1}), however, was associated with a significant land-treatment unit by treatment interaction ($P < 0.003$; Fig 5). Least square means indicated differences were attributable to land-treatment units 3 (29% greater on land-treatment), 4 (33% greater on reference), and 5 (27% greater on land-treatment site). Total yield of splenocytes from cotton rats showed a tendency for a treatment effect as suggested by the land-treatment by treatment interaction ($P < 0.07$; Fig. 5).

PHA-hypersensitivity Response

Overall, cotton rats experienced nearly a two-fold (2.1 ± 0.05 units) increase in skin-fold thickness during the 24 h *in vivo* PHA-hypersensitivity test. Cotton rats injected with an intradermal injection of PHA tended to show a treatment difference ($P < 0.10$), with slightly lower responses in animals from land-treatment sites (2.17 ± 0.07 units) compared to reference sites (2.03 ± 0.07 units); other interaction effects were not significant ($P > 0.10$).

Lymphoproliferative Responsiveness

All animals showed a remarkable proliferative response *in vitro* to stimulation with plant lectins and the lymphokine IL-2. Cotton rat splenocytes stimulated with the mitogen Con-A showed a strong season by treatment interaction ($P < 0.013$; Fig. 6), with a 20% greater lymphoproliferative response for animals from land-treatment sites compared to reference sites in winter. Lymphoproliferative responses to Con-A stimulation were similar ($P > 0.10$) between land-treatment and reference sites in summer. Splenocytes stimulated with the mitogen PWM tended ($P < 0.06$) to have lower responses for those from land-treatment sites compared to reference sites (Fig. 6); interaction effects were not significant ($P > 0.10$). The ability of lymphocytes to proliferate in response to the lymphokine IL-2 showed no relationship to treatment ($P > 0.10$).

Macrophage Function

Significant land-treatment unit by treatment interactions were observed for total yield (cells x 10^6) of peritoneal cells ($P < 0.008$), yield of peritoneal macrophages ($P < 0.014$), and yield of peritoneal lymphocytes ($P < 0.04$) in cotton rats (Fig. 7). Least squared means for yield of peritoneal macrophages indicated that differences were attributed to land-treatment units 1 (32% greater on land-treatment) and 3 (82% greater on land-treatment site). Total yield of peritoneal cells also showed differences occurring on land-treatment units 1 and 3 for macrophages. When cell yield from the peritoneal cavity was corrected for body mass (cells x 10^6 g^{-1}), significant differences for total peritoneal cells ($P < 0.003$; 92% greater on land-treatment site) and macrophages ($P < 0.003$; 80%

greater on land-treatment site) were only apparent for land-treatment unit 3. Differences in yield of lymphocytes were attributed to land-treatment units 2 and 3, but these differences were small and inconsistent (Fig. 7). Total yield of peritoneal mast cells in cotton rats tended to show a season by treatment interaction ($P < 0.07$; Fig. 7), where animals from land-treatment sites yielded about three-fold fewer mast cells in summer.

The metabolic activity of peritoneal macrophages as measured by the mitochondrial, NBT-reduction assay (stimulation index) showed a significant ($P < 0.05$) season by treatment interaction (Fig. 7). Least squared means revealed that this difference was attributable to summer ($P < 0.007$), when adult cotton rats from land-treatment sites had stimulation indices that were 114% greater than animals from the five reference sites.

DISCUSSION

Elangbam *et al.* (1989) suggested that the cotton rat was an ideal *in situ* model for evaluating toxicity risks to wildlife populations because of its ubiquitous distribution, large body size, and life history characteristics. Additionally, this animal lives in close association with the soil, which it either purposely or inadvertently ingests (Garten, 1980). Investigators have reported a high frequency of chromosomal aberrations in cotton rat populations inhabiting a variety of waste sites associated with the petrochemical industry, indicating their susceptibility to genotoxicants in the environment (McBee *et al.*, 1987; Thompson *et al.*, 1988; McBee & Bickham, 1988). Significant cytochrome P-450 induction in

cotton rats has been observed at some petrochemical waste sites (Elangbam *et al.*, 1989), whereas moderately lower levels of induction have been observed at other waste sites (Rattner *et al.*, 1993). The question of whether these contaminant-induced alterations ultimately influence recruitment and survival of natural populations residing on waste sites remains equivocal. Flickinger & Nichols (1990) concluded that demographic endpoints for populations of cotton rats were too insensitive to the levels of toxicants they observed on petrochemical waste sites in Texas. A survey of these same sites previously had shown a high incidence of cytogenetic lesions in these populations, which was attributed to mixtures of contaminants in soil (Thompson *et al.*, 1988). In comparison, Elangbam *et al.* (1989) and McMurry (1993) have reported significant differences in demography of cotton rat populations residing on petrochemical waste sites compared to those from ecologically-similar reference sites. In both of these studies, demographic alterations correlated with differences in other physiologic endpoints, supporting a link between mixtures of contaminants in the environment and demographic responses.

Clearly, these studies demonstrate that not all cotton rat populations behave similarly on all petrochemical waste sites. Although sampling error may be partly responsible for these apparent discrepancies, a more likely explanation is that waste sites vary in the mixtures of contaminants or assemblages of small mammals in the environment. With respect to sampling error, selection of an appropriate reference population to make comparisons with populations on land-treatment sites is of paramount importance. Structure and botanical composition

of vegetation in the habitat should be closely matched between sites to minimize potential confounding effects from differing nutritional, microclimatic, and predation stressors. These environmental factors are known to have a remarkable influence on immunity, liver function, neuroendocrine physiology, reproduction, and other potential endpoints of toxicity (Good & Lorenz, 1992; Lochmiller & Dabbert, 1993; Lochmiller, 1996). Lower levels of hepatic cytochrome P-450 observed on at least one waste site studied by Rattner *et al.* (1993) could have been the result of nutritional differences among cotton rats, as vegetation on the waste site differed considerably from the reference site.

Several controlled laboratory studies have demonstrated that the immune system of cotton rats can be altered from exposure to known immunotoxicants such as lead (McMurry *et al.*, 1995), benzene (McMurry *et al.*, 1991; 1994a; 1994b), and cyclophosphamide (McMurry *et al.*, 1994b), as well as multiple nutritional-immunotoxicant stressors (McMurry *et al.*, 1994b). Preliminary studies of McMurry (1993) provided evidence that resident populations of cotton rats on a land-treatment site located on the Oklahoma Refining Company Superfund waste site in southwestern Oklahoma, USA were exposed to immunotoxicants. This was suggested by elevated indices of lymphoproliferative responsiveness of cultured splenocytes stimulated with the mitogen Con-A and percentage of T-cells staining positive for Con-A receptors (McMurry, 1993). McMurry (1993) hypothesized that the complex mixtures of petrochemicals present in soil of land-treatment waste sites are probably immunotoxic to resident small mammal populations. Results from our study of five different land-treatment waste sites

distributed throughout Oklahoma, USA generally supports this hypothesis. However, it was also clear from these results that considerable variation exists among land-treatment facilities with regards to their degree of immunotoxicity or susceptibility of resident host population of cotton rats to these immunotoxicants.

The five contaminated land-treatment units from this study showed elevated levels of numerous heavy metals and organics (Rafferty, 1998) that are known to be immunotoxic (IPCS, 1996; Silkworth *et al.*, 1995), including lead, chromium, vanadium, benzo (a) pyrene, benzo (b and k) fluoranthene, chrysene, dibenz (a,h) anthracene, and benzo (a) anthracene. Concentrations of immunotoxic heavy metals in the soils of these land-treatment facilities ranged from 2- to 20-fold above those found in reference soils (Rafferty, 1998). Total petroleum hydrocarbons in the soil were observed to range 300-800 mg/kg on land-treatment sites; PAH's such as chrysene, ranged from 13-61 ug/kg (Rafferty, 1998).

Alterations in several hematological indices of small mammals such as the white-footed mouse (*Peromyscus leucopus*) and the cotton rat previously have been demonstrated in the field, with alterations correlating with soil levels for lead (Stansley & Roscoe, 1996; McMurry, 1993). Clinically, white-footed mice collected from lead-contaminated sites frequently show reductions in concentrations of blood hemoglobin and ALAD (delta aminolevulinic acid) activity (Stansley & Roscoe, 1996). McMurry (1993) observed elevated platelet counts in cotton rats collected from petrochemical-contaminated waste sites in Oklahoma. This mild form of thrombocytosis was one of the most consistent

indicators of exposure in cotton rats from the land-treatment facilities we studied, and these clinical alterations were most apparent in winter. Silkworth *et al.* (1984) observed increases in splenic megakaryocytes in laboratory mice exposed to soil from the Love Canal waste site. Causative factors contributing to observed thrombocytosis at these various study sites are not known. However, toxic mode of action for several metal ions is known to involve the vascular system and integrity of membranes (Kiss & Osipenko, 1994; Alonso *et al.*, 1990). Myriad factors can contribute to thrombocytosis, including infections, malignancies, hemorrhage, and mineral deficiencies, as well as anything that might disrupt normal dynamic exchange between spleen and blood pools or production of hematopoietic growth factors such as thrombopoietin (Schalm *et al.*, 1975). Some metals such as beryllium are capable of inducing platelet hyperactivity and concurrently increasing thrombopoietin production (Togna *et al.*,1997).

Organ masses, particularly the liver and kidney, frequently have been used as indicators of exposure to environmental contaminants in small mammals. Increases in relative liver mass have been reported in laboratory mice exposed to petrochemical-contaminated soil (Silkworth *et al.*, 1984). Rattner *et al.* (1993) observed elevated relative liver mass in cotton rats from the MOTCO Inc. waste site in Texas, USA that was consistent with exposure to petroleum hydrocarbons. However, at an arsenic-contaminated site, Rattner *et al.* (1993) observed reductions in relative mass of liver in cotton rats. We observed that relative liver mass was elevated on two land-treatment sites yet reduced on

another, suggesting the plausibility of different contaminants being responsible for these disparate results.

Kidney mass was lower in cotton rats from land-treatment sites in summer, whereas relative kidney mass was both reduced (two sites) and increased (one site). This inconsistency also suggests the possibility of differing forms of contamination and toxicity across land-treatment units. Previous studies with cotton rats exposed to lead acetate in drinking water for up to 14 weeks failed to elicit a change in relative kidney mass (McMurry *et al.*, 1995). However, wild bank voles (*Clethrionomys glarolus*) collected from lead-contaminated soils (Ma, 1989) or laboratory mice exposed to Love Canal soils (Silkworth *et al.*, 1984) have been shown to experience an increase in relative kidney mass. Trends in relative liver and kidney masses were similar in our study, suggesting that contaminants in the soil affected these two organ systems in a similar fashion.

Cotton rats collected from land-treatment sites in this study showed an enhanced lymphoproliferative response following stimulation with the plant-lectin Con-A, which was similar to observations reported by McMurry (1993). This assay is useful for assessing the ability of mature and immature T-cells to undergo blastogenesis following antigenic stimulation. Benzo (a) pyrene at low concentrations (10^{-5}M – 10^{-8}M) is capable of enhancing the proliferative response of mouse splenocytes following *in vitro* stimulation with Con-A and PHA, however higher concentrations of this compound can have a suppressive effect on cells (Tomar *et al.*,1991). Constan *et al.* (1995) noted a significant increase in

hepatocyte proliferation *in vivo* for F344 rats following long-term exposures to low levels of a complex petrochemical mixture containing arsenic, benzene, chloroform, chromium, lead, phenol, and trichloroethylene. In contrast, McMurry *et al.* (1995) observed suppressed lymphoproliferative responses to Con-A and PWM in cotton rats exposed to lead acetate in their drinking water for 7 or 14 weeks.

The macrophage arm of the nonspecific immune system consistently has been shown to be responsive to many forms of immunotoxicants under laboratory exposure conditions (Descotes, 1988; Talmage, 1992). We observed both quantitative and qualitative differences in indices of nonspecific immunity in the cotton rat. Total cell yields from the peritoneal cavity, including numbers of recovered macrophages, were frequently elevated in animals from land-treatment sites. Measurements of integrity of the respiratory burst via mitochondrial reduction of NBT showed a trend comparable to that for total cell yield in cotton rats, suggesting exposure caused some up-regulation of macrophage activity. Exposure to metals such as chromium, copper, and manganese can be associated with similar numerical responses in macrophages of laboratory animals models (Johansson *et al.*, 1986; Chukhlovin *et al.*, 1996). Wojdani & Alfred (1984) observed that several PAHs were capable of inducing substantial elevations in macrophage yields in a dose-dependent fashion. Elevated phagocytic activity and H_2O_2 production by mouse macrophages have been observed following exposures to low concentrations of lead, cadmium, (Cd,

3.0 mg kg^{-1} food; Pb 1.5 mg kg^{-1} food; Baykov *et al.*, 1996) and vanadium compounds (Cohen *et al.*, 1996).

Cotton rats collected from land-treatment sites experienced a marginal depression in hypersensitivity responsiveness to an intradermal challenge of PHA, suggesting that some functional suppression of cell-mediated immunity may have resulted from exposure to the complex mixtures of contaminants on these sites. McMurry (1993) showed a similar depression for *in vivo* response to antigenic challenge with PHA for cotton rats collected from petrochemical-contaminated sites. Similarly, Propst *et al.* (1995) showed a 60% lower PHA-hypersensitivity response in cotton rats housed in small *in situ* mesocosms on petrochemical-contaminated waste sites. This type of hypersensitivity reaction is mediated by macrophages and involves T-cells that produce lymphokines in response to PHA (Zelikoff *et al.*, 1994). Laboratory studies have documented dysregulation of skin immune function through loss of Langerhan cells when mice were exposed to 7,12 dimethylbenz[a]anthracene (Halliday *et al.*, 1988). However, elevated yields and metabolic activity of macrophages observed in cotton rats from land-treatment sites suggest that reduced response to PHA challenge may be more T-cell dependent.

Results of this study indicate that petrochemical wastes applied to soils on these five study sites have no uniform immunomodulatory effect on cotton rats as indicated by the significant land-treatment unit by treatment interactions observed for several tests used to evaluate immunotoxicity. In addition, immune alterations sometimes indicated enhancement while at other sites these same

assays indicated suppression of immune response. The frequency of alterations in immune response parameters did not appear equally distributed among land-treatment sites. Land-treatment unit 3 had the most alterations among the parameters measured while land-treatment unit 2 had the fewest. These observations appear reasonable given the considerable diversity of contaminants present in soils of the five different land-treatment facilities we investigated (Rafferty, 1998). Many contaminants such as metals are well known for their differing abilities to either enhance or suppress immune responses (Hong *et al.*, 1992; Chukhlovin *et al.*, 1996). In addition multiple contaminants can have additive, synergistic, or antagonistic effects on animal immune responses. Waste products disposed of through land application technologies such as these vary from one industrial site to another. For example, land-treatment unit 4 was used almost exclusively for disposal of tank-bottom wastes, while land-treatment unit 1 was used for disposal of waste sludges from sedimentation ponds as well as tank-bottom wastes. An additional factor contributing to observed differences in response variables is length of time wastes was actually applied to soils. Most of these sites lacked historical records on what was applied and how long the land-treatment facilities were in operation. Of the assays we employed in this study, assessing cell-mediated immunity in a lymphoproliferation assay, enumerating platelets, and assessing macrophage function appeared to be the most sensitive indicators of exposure for cotton rats from land-treatment sites.

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FIGURE LEGENDS

Figure 1. Body mass (means ± SE) of adult cotton rats collected from five land-treatment units in Oklahoma, USA during summer and winter. (n = 12 for all means).

Figure 2. Seasonal blood platelet (means ± SE) of adult cotton rats collected from land-treatment sites (n = 5) and matched-reference sites (n = 5) in Oklahoma, USA. Difference between values is indicated with an asterisk ($P < 0.05$) for that season.

Figure 3. Concentration of total serum protein (means ± SE) in adult cotton rats collected from five land-treatment units in Oklahoma, USA. Values were pooled across seasons (summer and winter) and difference between land-treatment and matched-reference site within a land-treatment unit is indicated by an asterisk ($P < 0.05$).

Figure 4. Kidney and liver organ mass (means ± SE) in adult cotton rats collected from five land-treatment units in Oklahoma, USA. (A) Kidney mass of adult cotton rats collected from land-treatment sites (n = 5) and matched-reference sites (n = 5). Difference between values is indicated with an asterisk ($P < 0.05$) for that season. (B) Relative kidney mass, (C) liver mass, and (D) relative liver mass, values were pooled across seasons (summer and winter) and difference between land-treatment and matched-reference site within a land-treatment unit is indicated by an asterisk ($P < 0.05$).

Figure 5. (A) Splenocyte relative and (B) total cell yield (means ± SE) in adult cotton rats collected from five land-treatment units in Oklahoma, USA. Values were pooled across seasons (summer and winter) and difference between land-treatment and matched-reference site within a land-treatment unit is indicated by an asterisk ($P < 0.05$).

Figure 6. Lymphoproliferative response (means ± SE) of adult cotton rats collected from five land-treatment units in Oklahoma, USA. (Con A) Seasonal lymphoproliferative response of cotton rat splenocytes to challenge with Con-A of adult cotton rats collected from land-treatment sites ($n = 5$) and matched-reference sites ($n = 5$). Difference between values is indicated with an asterisk ($P < 0.05$) for that season. (PWM) Lymphoproliferative response of cotton rat splenocytes stimulated with PWM of adult cotton rats collected from land-treatment sites ($n = 5$) and matched-reference sites ($n = 5$) with land-treatment sites and matched-reference sites combined over seasons (winter and summer).

Figure 7. Peritoneal cavity cell yields and macrophage metabolic activity (means ± SE) in adult cotton rats collected from five land-treatment units in Oklahoma, USA. (A) Total peritoneal cavity cell yield, (B) total macrophage yield, and (D) total lymphocyte yield, values were pooled across seasons (summer and winter) and difference between land-treatment and matched-reference site within a land-treatment unit is indicated by an asterisk ($P < 0.05$). (C) Total mast cell yield and (E) macrophage stimulation index of adult cotton rats collected from land-

treatment sites (n = 5) and matched-reference sites (n = 5). Difference between values is indicated with an asterisk ($P < 0.05$) for that season.

Table 1. Hematological parameters (mean ± SE) of adult cotton rats collected from five land-treatment units in Oklahoma, USA during summer and winter and land treatment units consisted of a contaminated population residing on the waste site and an off-site reference population. (n = 12 except where superscripted).

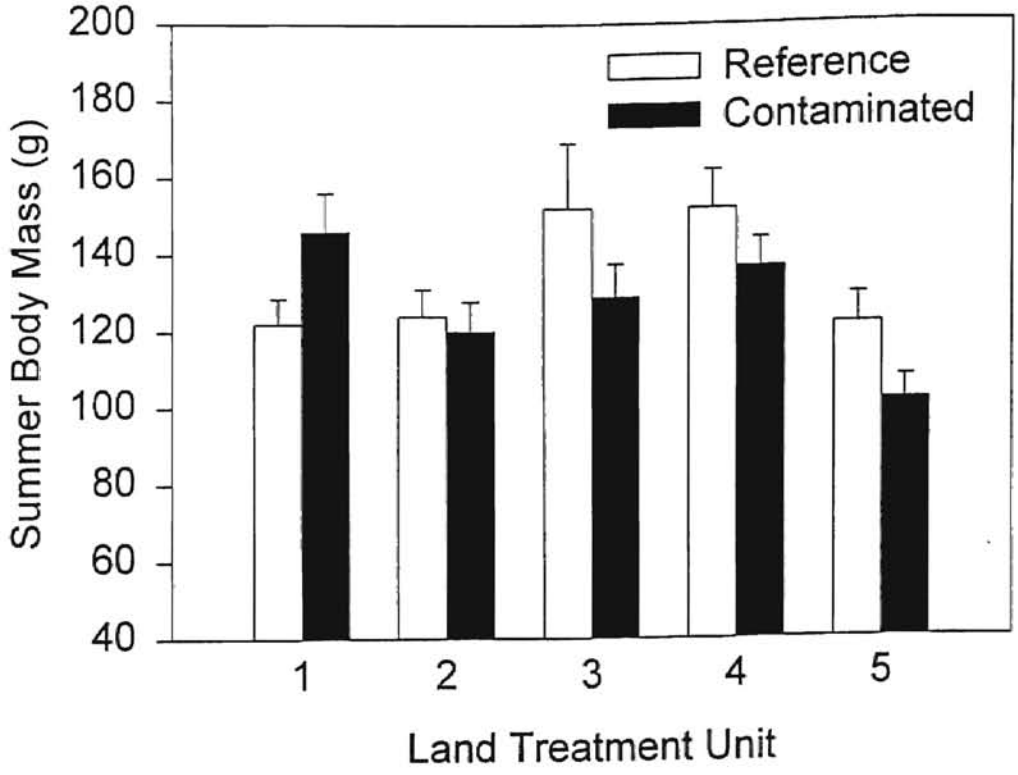
	Land-treatment unit									
	Unit 1		Unit 2		Unit 3		Unit 4		Unit 5	
Season/parameter	Reference	Land-treatment	Reference	Land-treatment	Reference	Land-treatment	Reference	Land-treatment	Reference	Land-treatment
Summer										
White blood cells ($x10^3/\mu l$)	22.68 ± 2.99[c]	20.06 ± 1.83[b]	23.69 ± 1.31[c]	27.15 ± 3.79[c]	20.19 ± 2.89[b]	16.59 ± 1.47	20.61 ± 2.33[c]	19.18 ± 1.41[e]	22.28 ± 4.60[d]	21.87 ± 2.78[d]
Red blood cells ($x10^6/\mu l$)	6.37 ± 0.15[b]	6.21 ± 0.18	6.61 ± 0.17	6.55 ± 0.16	5.47 ± 0.25	5.51 ± 0.19	5.65 ± 0.10	5.47 ± 0.29	6.13 ± 0.11	6.61 ± 0.12
Hemoglobin (g/dl)	13.95 ± 0.26[b]	14.18 ± 0.40	13.63 ± 0.42	13.3 ± 0.23	11.98 ± 0.48	11.94 ± 0.38[b]	12.17 ± 0.24[b]	11.64 ± 0.81[d]	13.22 ± 0.22[b]	13.03 ± 0.29
Hematocrit (%)	41.6 ± 0.8[b]	42.1 ± 1.3	41.3 ± 1.1	39.9 ± 0.8	36.2 ± 1.5	36.6 ± 1.3	37.2 ± 0.7	35.9 ± 2.0	39.9 ± 0.7	39.5 ± 0.8
Mean corpuscular volume (fl)	65.5 ± 0.8[b]	67.9 ± 1.0	62.5 ± 0.6	61.0 ± 0.7	66.6 ± 1.5	66.5 ± 0.7	65.9 ± 1.1	65.5 ± 1.1	65.2 ± 0.7	59.8 ± 0.6
Mean corpuscular hemoglobin (pg)	21.95 ± 0.32[b]	22.88 ± 0.41	20.62 ± 0.32	20.38 ± 0.32	22.08 ± 0.59	21.89 ± 0.30[b]	21.59 ± 0.44[b]	21.79 ± 0.55[d]	21.47 ± 0.31[b]	19.70 ± 0.21
Mean corpuscular hemoglobin concentration (%)	33.53 ± 0.22[b]	33.68 ± 0.23	32.98 ± 0.25	33.36 ± 0.25	33.12 ± 0.18	33.03 ± 0.24[b]	32.85 ± 0.29[b]	33.04 ± 0.42[d]	32.95 ± 0.21[b]	32.94 ± 0.15
Winter										
White blood cells ($x10^3/\mu l$)	14.38 ± 1.85[b]	14.71 ± 1.36	22.52 ± 3.60[c]	25.25 ± 4.22[e]	18.11 ± 2.21[a]	18.49 ± 2.14[a]	33.36 ± 4.94[e]	35.86 ± 5.44[e]	62.58 ± 9.15[g]	54.45 ± 8.14[d]
Red blood cells ($x10^6/\mu l$)	5.69 ± 0.25	5.95 ± 0.11	6.03 ± 0.09	6.42 ± 0.14	6.48 ± 0.16[a]	6.47 ± 0.14[a]	6.29 ± 0.13	5.81 ± 0.29	6.15 ± 0.13	6.35 ± 0.08
Hemoglobin (g/dl)	12.22 ± 0.46	13.02 ± 0.23	12.5 ± 0.16	13.08 ± 0.30	13.38 ± 0.20[a]	13.82 ± 0.23[a]	13.89 ± 0.26	13.38 ± 0.38	12.9 ± 0.34[g]	13.02 ± 0.26[d]
Hematocrit (%)	36.2 ± 1.4	38.5 ± 0.7	37.5 ± 0.4	39.1 ± 0.9	40.1 ± 0.7[a]	40.9 ± 0.7[a]	38.2 ± 0.8	36.4 ± 1.4	38.3 ± 0.4	38.9 ± 0.6
Mean corpuscular volume (fl)	64.0 ± 0.8	64.8 ± 0.5	62.3 ± 0.4	61.0 ± 0.7	62.1 ± 0.8[a]	63.5 ± 1.1[a]	60.7 ± 0.5	63.0 ± 1.0	62.3 ± 0.8	61.3 ± 0.6
Mean corpuscular hemoglobin (pg)	21.62 ± 0.42	21.90 ± 0.18	20.75 ± 0.14	20.40 ± 0.25	21.13 ± 0.31[a]	21.46 ± 0.43[a]	22.13 ± 0.39	23.42 ± 0.82	24.11 ± 2.03[f]	20.62 ± 0.34[d]
Mean corpuscular hemoglobin concentration (%)	33.78 ± 0.33	33.78 ± 0.14	33.33 ± 0.12	33.39 ± 0.18	33.87 ± 0.22[a]	33.78 ± 0.21[a]	36.45 ± 0.61	37.08 ± 1.00	34.62 ± 0.21[g]	33.62 ± 0.21[d]

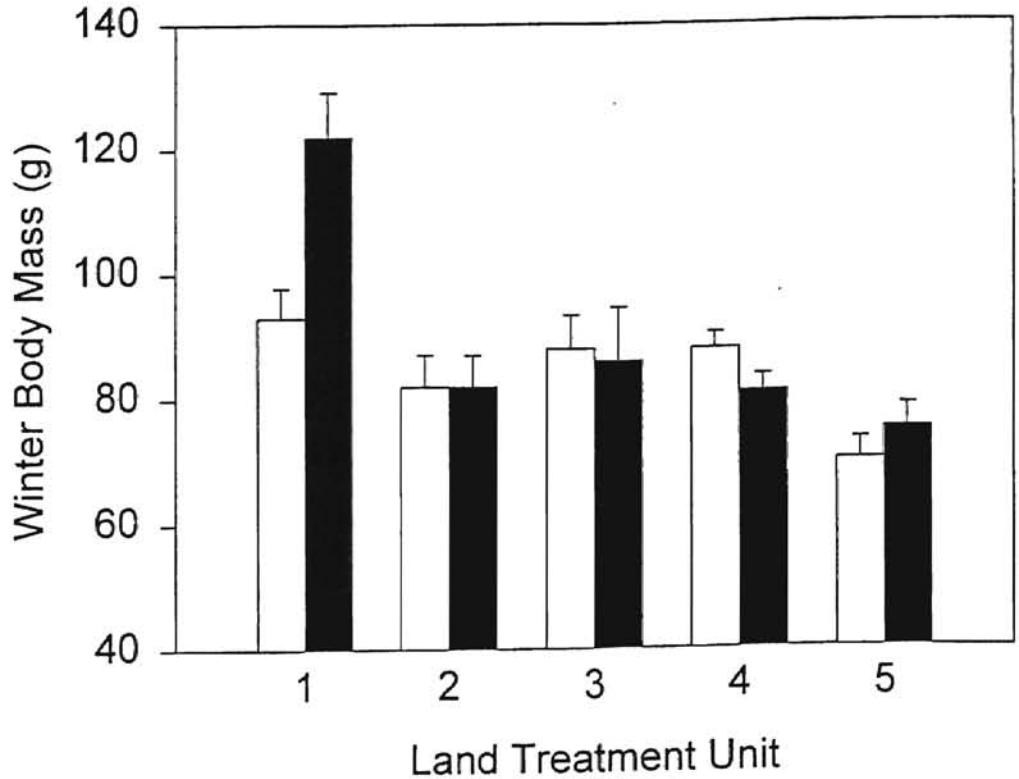
[a] n = 13; [b] n = 11; [c] n = 10; [d] n = 9; [e] n = 8; [f] n = 7; [g] n = 5

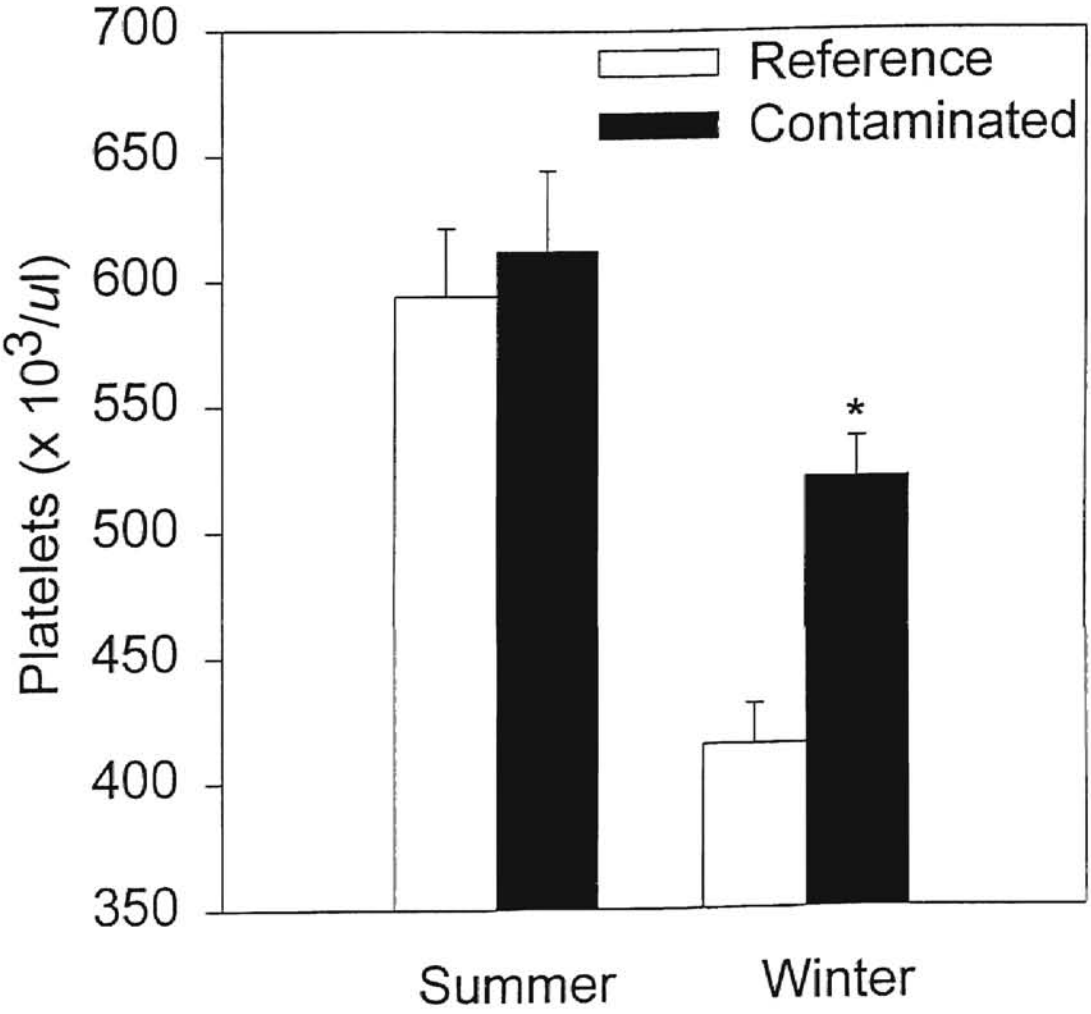
Table 2. Organ mass and cellularity (mean ± SE) of adult cotton rats collected from five land-treatment units in Oklahoma, USA during summer and winter and land treatment units consisted of a contaminated population residing on the waste site and an off-site reference population. (n = 12 except where superscripted).

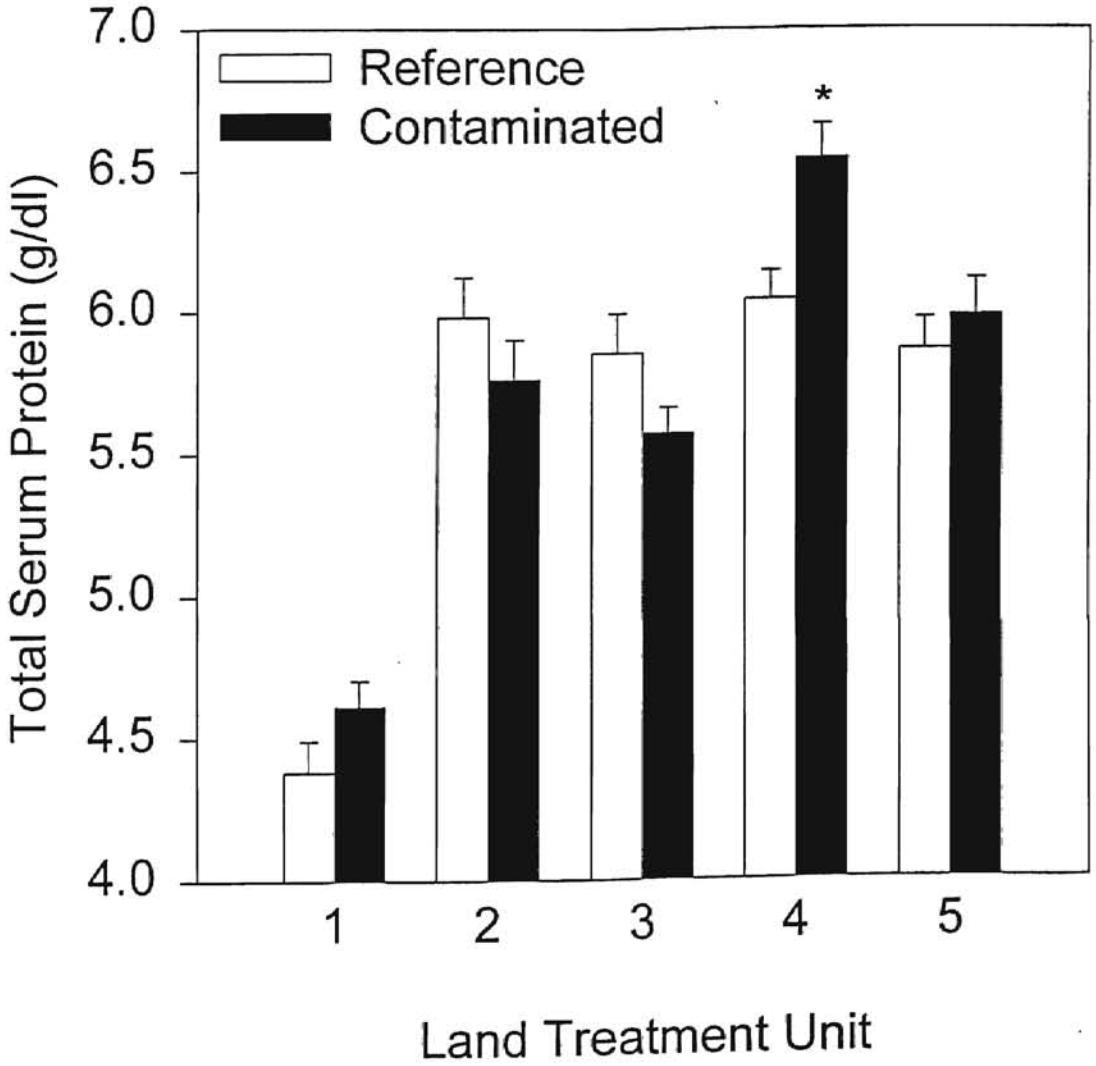
	Land treatment unit									
	Unit 1		Unit 2		Unit 3		Unit 4		Unit 5	
Season/parameter	Reference	Land-Treatment	Reference	Land-Treatment	Reference	Land-Treatment	Reference	Land-Treatment	Reference	Land-Treatment
Summer										
Popliteal lymph nodes										
Mass (mg)	18 ± 4[b]	22 ± 3	16 ± 5	20 ± 4	18 ± 3	9 ± 2	32 ± 8	23 ± 7	20 ± 3	15 ± 2
Relative mass (mg/g)	0.153 ± 0.033[b]	0.154 ± 0.027	0.135 ± 0.040	0.159 ± 0.027	0.132 ± 0.024	0.072 ± 0.017	0.229 ± 0.063	0.164 ± 0.044	0.170 ± 0.036	0.153 ± 0.018
Total cell yield(x 10^6 cells)	11.9 ± 1.7[b]	19.8 ± 3.8	20.3 ± 6.0	26.7 ± 6.5	18.5 ± 5.8	7.0 ± 2.3	24.1 ± 6.0	20.1 ± 7.9	19.7 ± 3.6	17.7 ± 2.9
Relative cell yield (x 10^6 cells/mg)	0.72 ± 0.07[b]	0.89 ± 0.06	1.22 ± 0.13	1.33 ± 0.16	0.90 ± 0.11	0.68 ± 0.05	0.80 ± 0.11	0.81 ± 0.17	1.02 ± 0.07	1.12 ± 0.11
Adrenal gland										
Mass (mg)	54 ± 4[b]	62 ± 6	60 ± 6	58 ± 7	55 ± 7	53 ± 4	43 ± 6	45 ± 6	48 ± 4	47 ± 4
Relative mass (mg/g)	0.434 ± 0.036[b]	0.415 ± 0.029	0.480 ± 0.036	0.477 ± 0.043	0.366 ± 0.031	0.426 ± 0.034	0.293 ± 0.045	0.319 ± 0.037	0.404 ± 0.038	0.459 ± 0.031
Spleen										
Mass (mg)	332 ± 46[b]	418 ± 42	227 ± 22	228 ± 10	315 ± 49	249 ± 31	297 ± 36	283 ± 23	216 ± 21	171 ± 17
Relative mass (mg/g)	2.55 ± 0.21[b]	2.86 ± 0.22	1.83 ± 0.15	1.95 ± 0.11	2.08 ± 0.22	2.02 ± 0.33	1.95 ± 0.18	2.08 ± 0.16	1.77 ± 0.13	1.68 ± 0.14
Winter										
Popliteal lymph nodes										
Mass (mg)	11 ± 3	11 ± 2	6 ± 1	8 ± 2	5 ± 1[b]	6 ± 1[a]	11 ± 4	9 ± 2	9 ± 2	8 ± 2
Relative mass (mg/g)	0.114 ± 0.028	0.090 ± 0.016	0.079 ± 0.008	0.106 ± 0.026	0.067 ± 0.012[b]	0.073 ± 0.011[a]	0.144 ± 0.044	0.107 ± 0.024	0.120 ± 0.024	0.099 ± 0.029
Total cell yield (x 10^6 cells)	10.0 ± 2.0	13.0 ± 2.9	9.2 ± 1.0	11.0 ± 2.5	8.7 ± 2.4[b]	6.4 ± 1.2[a]	9.9 ± 1.3	13.3 ± 3.5	11.8 ± 3.4	12.1 ± 4.4
Relative cell yield (x 10^6 cells/mg)	1.04 ± 0.08	1.12 ± 0.07	1.47 ± 0.10	1.57 ± 0.11	1.30 ± 0.12[b]	1.01 ± 0.10[a]	1.28 ± 0.13	1.42 ± 0.07	1.11 ± 0.13	1.15 ± 0.17
Adrenal gland										
Mass (mg)	39 ± 3	49 ± 4	29 ± 2	28 ± 2	30 ± 3[b]	31 ± 5[a]	31 ± 2	37 ± 9	28 ± 1	24 ± 1
Relative mass (mg/g)	0.419 ± 0.023	0.408 ± 0.038	0.353 ± 0.011	0.345 ± 0.014	0.339 ± 0.035[b]	0.376 ± 0.047[a]	0.398 ± 0.020	0.448 ± 0.111	0.400 ± 0.022	0.332 ± 0.066
Spleen										
Mass (mg)	171 ± 13	194 ± 17	133 ± 17	114 ± 13	124 ± 14[b]	131 ± 21[a]	116 ± 12	118 ± 15	88 ± 11	105 ± 6
Relative mass (mg/g)	1.84 ± 0.08	1.57 ± 0.08	1.62 ± 0.21	1.37 ± 0.09	1.38 ± 0.06[b]	1.45 ± 0.11[a]	1.50 ± 0.14	1.44 ± 0.16	1.22 ± 0.11	1.39 ± 0.08

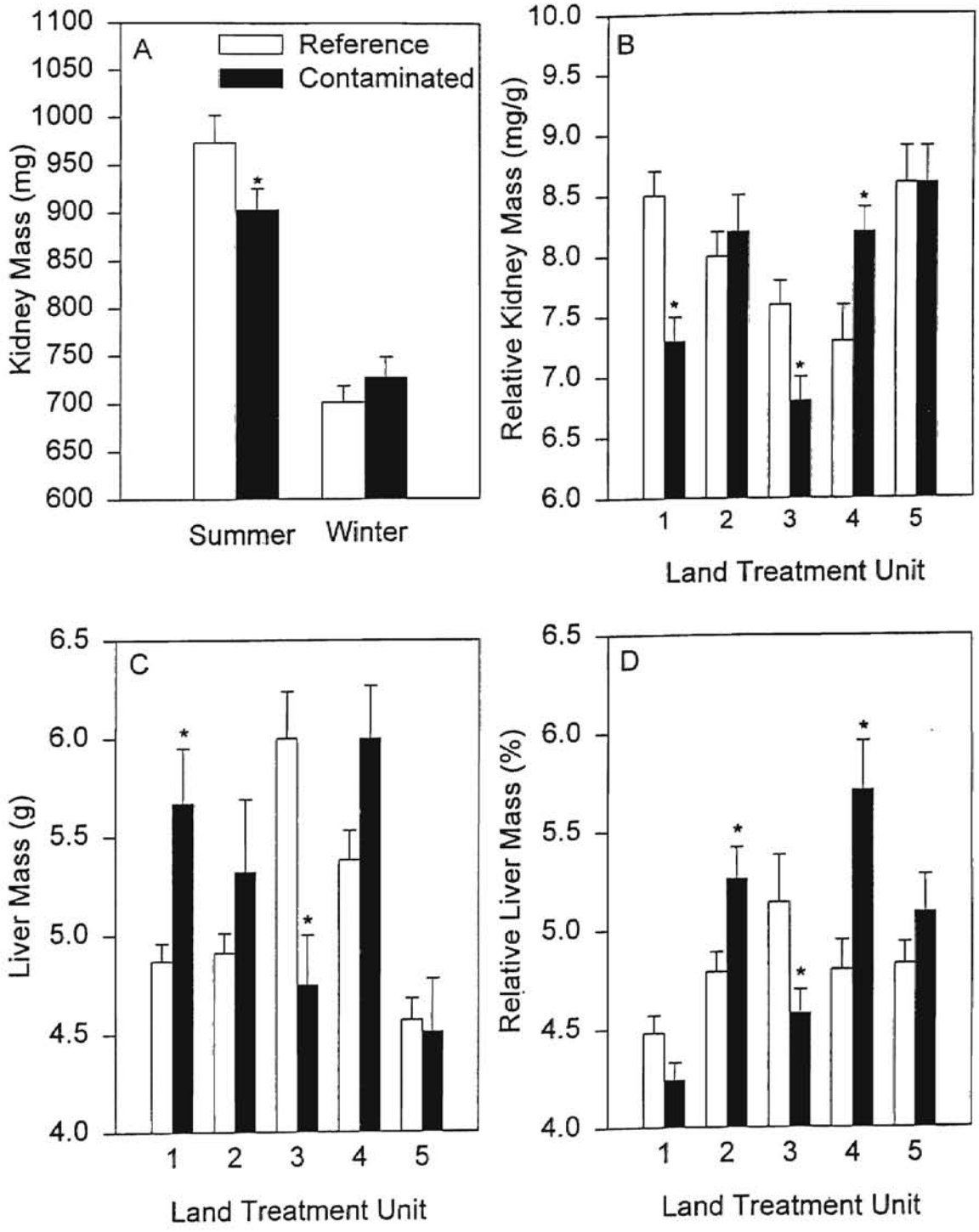
[a] n = 13; [b] n = 11

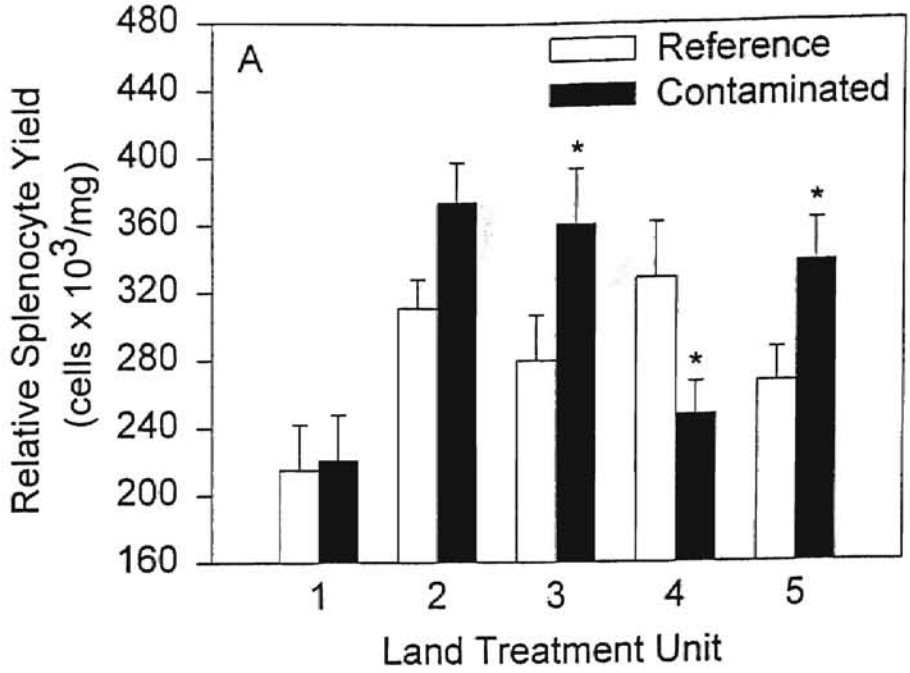

Reference
Contaminated
Summer Body Mass (g)
200
180
160
140
120
100
80
60
40
1
2
3
4
5
Land Treatment Unit

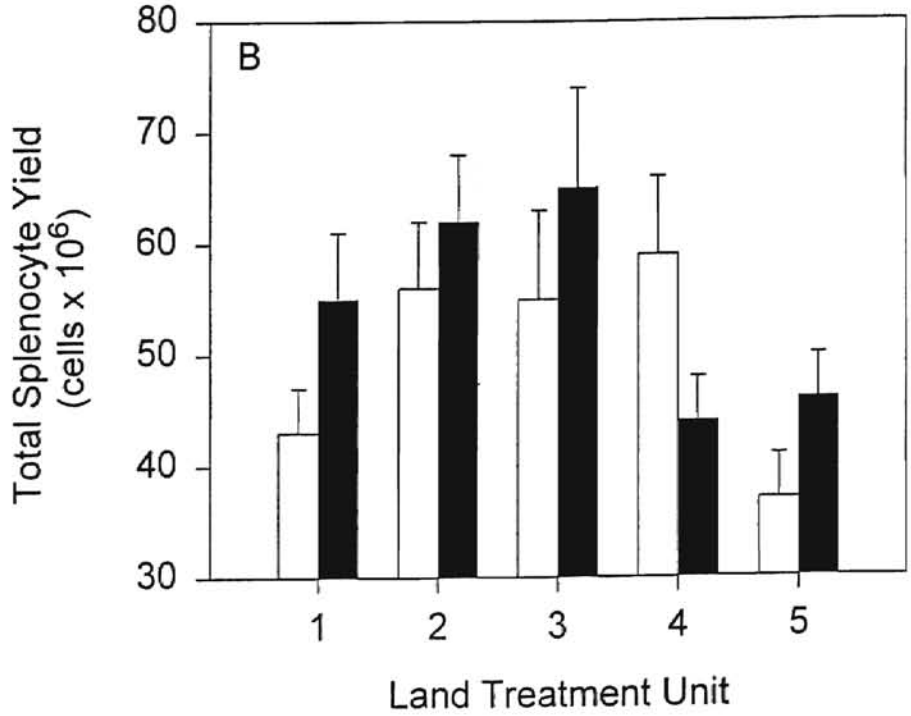

Winter Body Mass (g)
140
120
100
80
60
40
1
2
3
4
5
Land Treatment Unit


700
650
600
550
500
450
400
350
Platelets (x 10^3/ul)
Reference
Contaminated
*
Summer
Winter

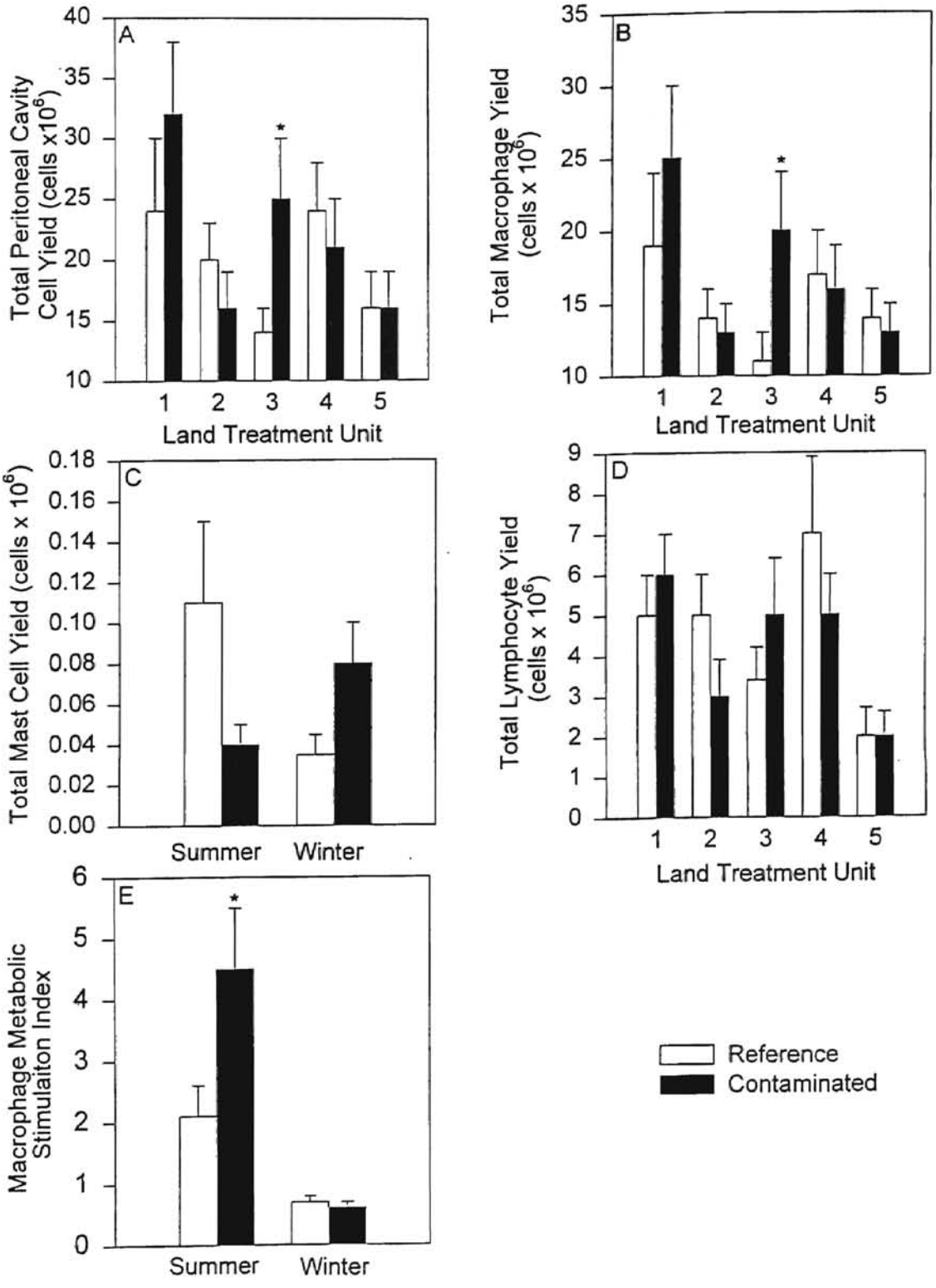

7.0
6.5
6.0
5.5
5.0
4.5
4.0
Total Serum Protein (g/dl)
Reference
Contaminated
*
1
2
3
4
5
Land Treatment Unit


A
Reference
Contaminated
Kidney Mass (mg)
1100
1050
1000
950
900
850
800
750
700
650
600
Summer
Winter
B
Relative Kidney Mass (mg/g)
10.0
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
1
2
3
4
5
Land Treatment Unit
C
Liver Mass (g)
6.5
6.0
5.5
5.0
4.5
4.0
1
2
3
4
5
Land Treatment Unit
D
Relative Liver Mass (%)
6.5
6.0
5.5
5.0
4.5
4.0
1
2
3
4
5
Land Treatment Unit


A
Reference
Contaminated
Relative Splenocyte Yield
(cells x 10^3/mg)
480
440
400
360
320
280
240
200
160
*
*
*
1
2
3
4
5
Land Treatment Unit


B
Total Splenocyte Yield
(cells x 10^6)
80
70
60
50
40
30
1
2
3
4
5
Land Treatment Unit

Stimulation Response (dpm x 1000)
140
120
100
80
60
40
Con A
Reference
Contaminated
*
Summer
Winter
Stimulation Response (dpm x 1000)
250
200
150
PWM


A
Total Peritoneal Cavity Cell Yield (cells x10^6)
40
35
30
25
20
15
10
1 2 3 4 5
Land Treatment Unit
*
B
Total Macrophage Yield (cells x 10^6)
35
30
25
20
15
10
1 2 3 4 5
Land Treatment Unit
*
C
Total Mast Cell Yield (cells x 10^6)
0.18
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
Summer Winter
D
Total Lymphocyte Yield (cells x 10^6)
9
8
7
6
5
4
3
2
1
0
1 2 3 4 5
Land Treatment Unit
E
Macrophage Metabolic Stimulaiton Index
6
5
4
3
2
1
0
Summer Winter
*
Reference
Contaminated

CHAPTER II

Metals, Polycyclic Aromatic Hydrocarbons, and Total Petroleum Hydrocarbons in Surface Soils of Petrochemical Waste Land-Treatment Sites

Abstract. Land-treatment of petroleum wastes is a widely used industrial practice, yet there has been no comprehensive evaluation of the long-term risks to human health or terrestrial ecosystems from such practices. Only limited data exist on heavy metals and polycyclic aromatic hydrocarbon (PAH) concentrations in soils that have been land-treated. We evaluated soils from five sites that historically had been land-treated with a variety of petroleum wastes. Concentrations of heavy metals (evaluated for 5 sites) and PAHs (evaluated for 3 sites) in the soil generally far exceeded background levels on matched-reference sites. Surface soils (0-3 cm) of land-treatment sites contained 100- to 1000-fold higher concentrations of PAHs compared to reference sites. Multivariate analysis revealed that each land-treatment site is unique in the concentration profile of heavy metals and PAHs in the soil, which may be an important consideration in evaluating human and ecological risk.

Introduction

Land-treatment is a waste management technology that has received increasing attention in recent decades primarily because of its low cost and versatility for handling both industrial and municipal wastes. Land-treatment is a managed disposal technology that involves controlled application of waste onto or into soil for the purpose of biodegradation of organic wastes, immobilization of inorganics, and avoiding bioaccumulation of hazardous compounds (Huesemann 1994, Loehr and Malina 1986). This technology has been so successful that over 50% of hazardous wastes generated by the petroleum industry have been disposed of through land-treatment operations (American Petroleum Institute 1984). In 1991 the petroleum industry generated 270 billion pounds of hazardous waste [Resource Conservation and Recovery Act qualified wastes] (Bass et al. 1995). There are an estimated 200 land-treatment sites in the United States that are designed for disposal of hazardous industrial wastes, and over half are operated by the petroleum industry (American Petroleum Inst. 1984, LaGrega et al. 1994). This number does not include myriad land-treatment sites that are designed for disposal of petrochemical wastes classified as non-hazardous. Land-treatment facilities that were used for disposal of petroleum refinery wastes are widespread in their distribution and include both active and inactive sites, yet no comprehensive assessment of their impact on terrestrial ecosystems has been completed. Total petroleum hydrocarbon (TPH) levels have been used as criteria for cleanup and closure of petroleum waste land-treatment sites, but

several authors believe such limited criteria may be insufficient for risk assessment (Heath et al. 1993, Koblis et al. 1993, Michelsen and Boyce 1993). On older, weathered petroleum-waste sites, polycyclic aromatic hydrocarbons (PAHs) may be more relevant than appraising TPHs for evaluating human risk whereas bioassays may be more appropriate for ecological risk assessment (Michelsen and Boyce 1993).

Recently, the U.S. EPA (1993) listed seven PAHs that are commonly found in petroleum-related wastes as probable human carcinogens: benzo(a)anthracene, benzo(a)pyrene, benzo(b)fluoranthene, benzo(k)fluoranthene, chrysene, dibenz(a,h)anthracene, and indeno(1, 2, 3, -c, d)pyrene. Similarly, several compounds in petroleum-related wastes possess immunotoxic properties (IPCS 1986, Silkworth et al.1995), including lead, chromium, vanadium, benzo(a)pyrene, benzo(b and k)fluoranthene, chrysene, indeno(1, 2, 3, -c, d)pyrene, dibenz(a, h)anthracene, benzo(a)anthracene, and benzo(g, h, I)perylene. All of these contaminants are known or suspected to be present in petrochemical wastes that are land-treated (API 1984).

Because of an accumulation of nutrients in the soil, many formerly active land-treatment sites become heavily vegetated after abandonment and eventually support diverse assemblages of small mammals and other vertebrates (McMurry 1993). Thus, these sites pose a potential risk to resident fauna. The purpose of the present work was to survey specific petrochemically land-treated waste sites for concentrations of heavy metals, PAHs, and TPHs in surface soil.

This study focused on the top 3 cm of surface soil only because this was deemed the zone-of-exposure most relevant to the vertebrate fauna residing on the sites.

Materials and Methods

Site Selection

We selected five land-treatment sites (herein referred to as units 1 through 5) for study. These sites were chosen based on the site having been used historically for land-treating oil refinery wastes, no longer being actively cultivated, having vegetation adequate to provide suitable cover to support a resident small mammal population, and because we were able access to the site. All sites were previously or currently owned by corporations and only one of five of these land-treatment sites was a designated Superfund Waste Site (land-treatment unit 1, Oklahoma Refining Company, Cyril, OK) on the U.S. Environmental Protection Agency's National Priorities List. Treatment of petroleum wastes was discontinued on all five sites during the early to mid 1980's. Little or no prior information existed as to duration, loading rates, and types of refinery waste products incorporated into soils on these sites. Interviews with former operators of these land-treatment facilities revealed that land-treatment units 1, 3, and 5 began operation in the early to mid 1970's. These sites were used for disposal of a variety of wastes from most refinery processes and no particular schedule or rate of application was followed during their operation. Land-treatment unit 2

was unique in that treated soil that originated elsewhere was used to cap a multipurpose waste pit; land-treatment unit 4 was used exclusively for disposal of tank-bottom sludges over a two-year period only during the mid 1970's. For each land-treatment unit, an ecologically similar reference site was selected for comparison to the actual land-treatment site. Reference sites were selected based on a visual assessment of similarity of vegetation structure and composition with their paired land-treatment site; sites were also similar with respect to topography, soil types, and located < 16 km from their paired land-treatment site.

Soil Sampling

Soils were sampled within a 70 m by 70 m grid that was established in the center of each land-treatment and matched-reference site. Land-treatment sites were sub-divided into six sample stations and matched-reference sites were sub-divided into two sample stations. At each sample station, a composite sample of soil was made from three random surface (0 to 3 cm) samples of soil obtained from within a 1-m radius of the sample station. Composite soil samples were mixed and sealed in acid-washed glass jars and transported to the laboratory for future analysis.

Composite soil samples (n = 6 for each land-treatment and n = 2 for each reference site) were air-dried in a greenhouse over a 2-day period. For heavy metal analysis (EPA Method 3050) a subsample of each composite was wet

digested with HNO_3. Heavy metals were quantified by inductively coupled plasma emission spectroscopy (ICP; American Resources Laboratory, Fisons Maxim, Boston, MA.); samples were analyzed in duplicate for Ba, Cd, Co, Cr, Cu, Ni, Pb, Sr, Ti, V, and Zn.

Semivolatile organic compounds that included U.S. EPA priority pollutants and total petroleum hydrocarbons (TPH) were analyzed in composite soil samples for land-treatment units 1, 2, and 5. A subsample (10g) of each air-dried composite sample of soil was extracted with 50/50 mixture of methylene chloride and acetone using an automated solvent extraction system (Dionex ASE 200, Dionex Corporation, Houston, TX). Extracts were analyzed for 20 semivolatile PAH compounds using gas chromatography with mass spectroscopic detection (GC-MS; Hewlett-Packard 5890 Series II with 5971 MSD, Hewlett-Packard, San Fernando, CA.) according to EPA method 8270, and TPHs were measured using the Wisconsin method (Wisconsin DNR, 1993; C10-C28).

Data Validation

Analytical data were reviewed for completeness, holding times, GC-MS tuning and system performance, initial and continuing calibrations, laboratory method blank analysis, surrogate recoveries, matrix spike, and matrix spike duplicate analysis, filed duplication precision and compound quantification, and detection limits.

Data Analysis

All data were tested for normality (Proc Normal; SAS 1994) and homogeneity of variances prior to further statistical analysis (Levines test; Steel and Torrie 1980); data not meeting these assumptions were transformed (arcsine or log normal) prior to further statistical analysis. Concentrations of all metals required data transformation, however, only untransformed means are reported in the text.

A randomized complete block design was used in a 2 X 5 factorial format with two treatments (land-treatment and reference) and five land-treatment units (units 1-5) to evaluate differences in concentrations between treatments and among land-treatment units. Comparisons were made using PROC MIXED (SAS 1994) with sources of variation distributed among the main factor effects and the interaction term (land-treatment unit by treatment interaction). If there was no significant interaction term, main effects were compared with the PDIFF option for the LSMEANS statement. Significant interaction term effects were compared using the SLICE option for the LSMEANS statement. Satterthwait's approximation was used in calculating the degrees of freedom for the error term.

Data were subjected to multivariate statistical analysis using principal component analysis (PROC PRINCOMP; SAS 1994). The underlying principle is that data in multidimensional space are projected into a space of fewer dimensions preserving as much as possible of the systematic variation in the data set. This method was used to determine what characteristics make land-treatment sites similar or different from each other and reference sites. It was

also used to identify patterns in the data that otherwise may go unnoticed. Statistical significance for all hypothesis tests was set a priori at $P \leq 0.05$ and means ±SE are reported. Total PAHs were calculated as the sum of the 20 measured PAHs and total immunotoxic PAHs were calculated as the sum of benzo(a)pyrene, benzo(b and k)fluoranthene, chrysene, indeno(1, 2, 3, -c, d)pyrene, dibenz(a, h)anthracene, benzo(a)anthracene, and benzo(g, h, i) perylene.

Results

Metals

Soils from both reference and land-treatment sites in all five land-treatment units had detectable levels of the 11 metals analyzed. Significant treatment main effects indicated concentrations of Pb ($P < 0.0075$), Cd ($P < 0.0042$), and Zn ($P < 0.0001$) in soil differed between land-treatment and reference sites (Table 1). Overall mean concentrations of Pb (83 ± 25 vs 10 ± 1), Cd (0.54 ± 0.05 vs o.37 ± 0.06), and Zn (152 ± 17 vs 35 ± 2 mg/kg) in the five land-treatment soils far exceeded observed mean levels in the five matched-reference soils. Vanadium concentrations in land-treatment soils (overall mean = 42 ± 11) tended to be greater than in reference soils (21 ± 3 mg/kg), but the difference was not significant ($P < 0.10$).

A significant ($P < 0.05$) land-treatment unit by treatment interaction was evident for concentrations of Ba, Co, Cr, Cu, Sr, and Ti in soils (Table 1). Least square means analysis revealed that most of the differences between land-treatment and reference soils, which were responsible for the significant interactive effect, were associated with land-treatment units 1, 3, and 5. Concentrations of Ba were 1.5- to 4-fold greater in land-treatment than reference soil for land-treatment units 1, 2, 3, and 5. Concentration of Co in reference soil was slightly greater on unit 5, but slightly lower in reference soil on units 1 and 3, compared to land-treatment soils. Land-treatment units 1, 3, and 5 had concentrations of Cr that were 3- to 14-fold greater in land-treatment than reference soil. Only land-treatment units 1 (4-fold greater) and 3 (23-fold greater in land-treatment soil) demonstrated differences in Cu concentration. Concentrations of Sr and Ti only differed on land-treatment unit 1; concentrations of Ni were similar across all soils. Overall, concentrations of metals in land-treatment soil differed most from their matched-reference soil for land-treatment units 1, 3, and 5.

Principal component analysis of metal profiles of soil revealed that each land-treatment unit contained a unique suite of contaminants. Principal component 1 accounted for 35.4% of the variation and principal component 2 accounted for an additional 19.9% of the variation among soils. The first 5 principal components together accounted for 89% of the total variation in the data set. A two-dimensional plot of scores for the first two principal components showed a clear separation of soils among land-treatment sites and between land-

treatment and reference soils (Fig. 1). Land-treatment soil samples varied to a larger degree in type of and total metal contamination than the reference soils, which clustered together and were neither negatively nor positively chosen by the variables deemed important by the principal components. The loading plot (Fig. 2) indicated that soil concentrations of Cr, Sr, Ti, Zn, and Ba were the most important variables in describing the variance of principal component 1, which explained most of the variance in the data set. Pb and Cd were the variables most important in explaining the variance associated with principal component 2.

PAHs and TPHs

Polycyclic aromatic hydrocarbons and TPHs were only determined in soils for land-treatment units 1, 2, and 5. Mean TPHs (Table 2) for land-treatment soils ranged from 275 to 765 mg/kg with a mean of 551 ± 61 mg/kg for the 3 land-treatment sites above. Concentrations of TPHs in reference soils ranged from undetected to 31 mg/kg with a mean of 12 ± 8 for the 3 reference sites above. Individual PAHs measured in soils ranged from below detection limits to 1,119 ug/kg (2-methylnapthylene; Table 2). Total PAHs measured ranged from 431 to 3221 ug/kg in land-treatment soils (mean = 1,999 ± 314 ug/kg) and 5 to 385 ug/kg in reference soils (mean = 195 ± 86 ug/kg). Total concentration of immunotoxic PAHs ranged from 160 to 610 ug/kg in land-treatment soils (mean = 440 ± 68 ug/kg) and below detection limits to 66 ug/kg (mean = 24 ± 14 ug/kg) in reference soils (Table 2).

Principal component analysis of PAH profiles in soil also revealed the uniqueness of each land-treatment site. Principal component 1 accounted for 55.3% of the variation and principal component 2 accounted for an additional 17.0% of the variation among soil samples. The first 5 principal components accounted for 89% of the total variation in the data set. The plot of the first two principal components in two-dimensional space showed a clear separation of soils among land-treatment sites and between land-treatment vs. reference sites (Fig. 3). Profiles of PAHs were similar among all reference sites and there was not much separation between the reference sites and land-treatment from land-treatment unit 1 (C1; Fig. 3). Land-treatment soils from land-treatment units 2 (C2) and 5 (C5) were spatially quite disparate from reference soil and land-treatment soils from land-treatment unit 1. The area of each polygon reflected variability in soil concentration of contaminants among subsamples; land-treatment soil samples from land-treatment unit 5 were the most variable. A loading plot (Fig. 4) showing the influence of each variable revealed that most PAHs tested were equally discriminating among principal component 1. Benzo(b and k)fluoranthene, benzo(a)anthracene, benzo(a)pyrene, chrysene, and pyrene were positively selected for in both principle components, indicating that these compounds were the best discriminators among land-treatment and reference sites. Land-treatment soils from land-treatment units 2 (C2) and 5 (C5) were spatially separated due to negative selection of compounds such as acenapthene and fluorene by principal component 2; these compounds were undetected on C2 and present on C5. The heavy loading of dibenz(a,

h)anthracene along principal component 2 appeared to be related to the uniqueness of the reference soil on land-treatment unit 2.

Discussion

Previous surveys of contaminant burdens in soils from land-treatment facilities such as those described in this paper are extremely limited. Genouw et al. (1994) reported that biodegradation of petroleum-related hydrocarbons that were land-treated followed first-order kinetic dynamics during biodegradation for soil residual concentrations. Measurement of the rate of degradation across several different waste application rates revealed that it would take 17-25 years to reach a residual hydrocarbon concentration of 50 to 500 mg/kg of soil (Genouw et al 1994). Although, first order kinetics appears to predict hydrocarbon loss, they are probably not realistic since they assume eventually a complete degradation of the constituent (Loehr et al. 1992). Our observations were similar to those of Loehr et al. (1993) who reported finding elevated levels of both heavy metals and PAHs in the zone of incorporation of former land-treatment facilities. However, PAH concentrations in 15-cm core samples of land-treatment facilities surveyed by Loehr et al. (1993) and Loehr and Webster (1997) were100- to1000-fold greater than the levels reported in this study. Since types of petroleum wastes applied to soils were basically the same in this and other studies, differences in PAH concentrations are probably the result of soil depth sampled. We took surface scrapes (0-3 cm) where there may be enhanced microclimatic and

biodegradative processes occurring that accelerated the rate of degradation of PAHs in soil compared to environments > 3 cm zone of incorporation. A variety of bioassays to assess the toxicity of aqueous extracts of land-treatment soils that had previously received petroleum wastes have largely reflected only minimal toxicity (Genouw et. al. 1994, Ramanathan 1994).

It is apparent from this and other studies that land-treatment of petroleum wastes alters the long-term metal and PAH profiles of soil relative to reference soils. Multivariate statistical analysis revealed that soils are considerably more variable in their metal and PAH composition among than within land-treatment sites. In other words, each site possesses a unique contaminate profile, which should be considered when evaluating human or ecological risks.

It is unclear what the human and ecological risk of exposure to these levels of contaminants is. Currently there are no uniform acceptable levels of heavy metals and PAHs for soil contamination. The potential for additive, synergistic, and antagonistic effects on animal and human biology remains uncertain. Initial evidence indicates that the level of contamination and the exposure scenarios present on former land-treatment sites may be immunotoxic to resident mammalian biomonitors (Rafferty 1998, McMurry, 1993). The complex nature of the contaminants and exposure scenarios remain to be further elucidated.

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Figure Legends

Figure 1. Plot of the first two principal components describing the relationship between concentrations of heavy metals in soil of five land-treatment waste units; (C1-C5) land-treatment sites and pooled reference sites (R). Each solid-lined polygon represents 6 composite soil samples for each land-treatment site and the dashed polygon represents 5 matched-reference sites combined (2 soil samples each).

Figure 2. Principal component analysis loading plot describing the relationship between metal variables used in deriving principle components 1 and 2.

Figure 3. Plot of the first two principal components describing the relationship between concentrations of PAHs in soil of three land-treatment waste units; (C1, C2, and C5) land-treatment sites and pooled reference sites (R). Each solid-lined polygon represents 6 composite soil samples for each land-treatment site and the dashed polygon represents 3 matched reference sites combined (2 soil samples each).

Figure 4. Principal component analysis loading plot describing the relationship between concentrations of PAH compounds, used in deriving principle components 1 and 2: (B) napthalene, (C) acenapthylene, (D) acenapthene, (E) fluorene, (F) phenanthrene, (G) anthracene, (H) fluoranthene, (I) pyrene, (J) benzo(a)anthracene, (K) chrysene, (L) benzo(b and k)fluoranthene, (M) benzo(a)pyrene, (N) indeno(1,2,3,-c,d)pyrene, (O) dibenz(a,h)anthracene, (P) benzo(g,h,i)perylene, (Q)

bis(2-ethylhexyl)phthalate, (R) di-n-butylphthalate, (S) diethylphthalate, (T) butylbenzyphthalate, and (U) 2-methylnapthylene.

Table 1 Concentrations (X ± SE) of metals in composite samples of soil from land-treatment (n=6) and reference (n=2) sites for each of 5 land treatment units surveyed in Oklahoma.

	Land treatment unit (mg/kg)									
	Unit 1		Unit 2		Unit 3		Unit 4		Unit 5	
Metal	Reference	Land-treatment	Reference	Land-treatment	Reference	Land-treatment	Reference	Land-treatment	Reference	Land-treatment
Ba	49 ± 33	206 ± 7*	88 ± 21	193 ± 8*	83 ± 3	141 ± 12*	72 ± 1	86 ± 6	107 ± 11	160 ± 7*
Cd[a]	0.42 ± 0.23	0.46 ± 0.03	0.14 ± 0.04	0.32 ± 0.04	0.54 ± 0.14	0.66 ± 0.08	0.43 ± 0.00	0.88 ± 0.14	0.31 ± 0.13	0.38 ± 0.03
Co	5 ± 1	7 ± 1*	15 ± 1	18 ± 1	4 ± 1	8 ± 1*	4 ± 1	4 ± 1	16 ± 2	11 ± 1*
Cr	16 ± 0	233 ± 31*	39 ± 13	53 ± 8	23 ± 5	80 ± 25*	19 ± 1	40 ± 17	13 ± 1	105 ± 10*
Cu	10 ± 2	37 ± 5*	12 ± 2	19 ± 3	5 ± 1	132 ± 39*	5 ± 1	22 ± 7	12 ± 1	25 ± 3
Ni	11 ± 1	51 ± 26	35 ± 7	31 ± 6	13 ± 2	20 ± 2	9 ± 1	17 ± 4	23 ± 2	20 ± 1
Pb[a]	11 ± 2	61 ± 10	10 ± 2	21 ± 3	4 ± 1	83 ± 16	12 ± 1	221 ± 115	13 ± 2	29 ± 2
Sr	19 ± 7	192 ± 19*	13 ± 3	19 ± 3	10 ± 1	36 ± 7*	8 ± 1	13 ± 2	16 ± 1	24 ± 1
Ti	56 ± 11	163 ± 18*	13 ± 3	19 ± 3	93 ± 24	115 ± 17	36 ± 4	26 ± 3	16 ± 1	24 ± 1
V[b]	14 ± 1	92 ± 52	13 ± 3	23 ± 1	31 ± 3	47 ± 2	32 ± 2	24 ± 1	16 ± 1	23 ± 1
Zn[a]	34 ± 2	173 ± 11	40 ± 8	91 ± 14	30 ± 1	168 ± 48	27 ± 4	69 ± 20	42 ± 2	259 ± 22

[a] Land-treatment sites significantly different from reference sites ($P < 0.05$).

[b] Land-treatment sites marginally different from reference sites ($P < 0.06$).

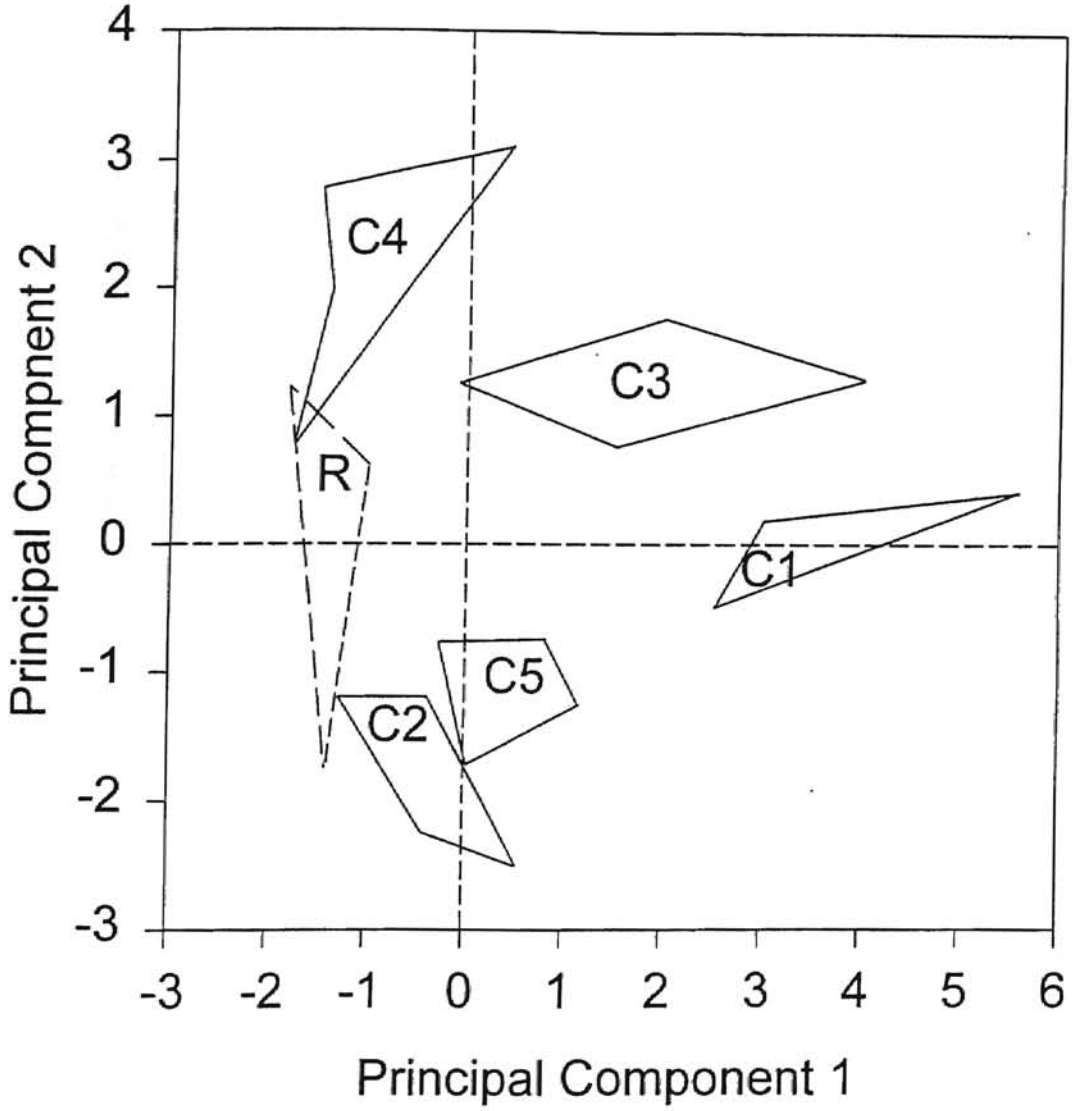
* Land-treatment sites significantly different from reference site within a land treatment unit; least squares mean.

Table 2: Concentrations (X ± SE) of selective organic compounds in composite samples of soil from land-treatment (n=6) and reference (n=2) sites for each of 5 land-treatment units surveyed in Oklahoma.

	Land treatment unit					
	Unit 1		Unit 2		Unit5	
Organic compound	Reference	Land-treatment	Reference	Land-treatment	Reference	Land-treatment
Total petroleum hydrocarbons (mg/kg)	31 ± 22	275 ± 46	bdl	769 ± 41	6.0 ± 6	610 ± 94
Polycyclic aromatic hydrocarbons(ug/kg						
Napthalene	bdl	d	bdl	92 ± 6	bdl	290 ± 65
Acenaphthylene	bdl	bdl	bdl	bdl	bdl	54 ± 30
Acenaphtene	bdl	bdl	bdl	bdl	bdl	20 ± 2
Fluorene	bdl	bdl	bdl	d	bdl	28 ±5
Phenanthrene	bdl	26 ± 5	bdl	131 ± 4	bdl	217 ± 34
Anthracene	bdl	15 ± 4	bdl	25 ± 2	bdl	79 ± 14
Fluoranthene	bdl	bdl	bdl	d	bdl	d
Pyrene	bdl	d	bdl	42 ± 3	bdl	36 ± 3
Benzo (a) anthracene	bdl	2 ± 2	bdl	31 ± 3	bdl	19 ± 4
Chrysene	bdl	13 ± 5	bdl	61 ± 5	bdl	44 ± 3
Benzo (b and k) fluoranthene	d	11 ± 3	bdl	48 ± 7	bdl	33 ± 5
Benzo (a) pyrene	bdl	d	bdl	48 ± 5	bdl	33 ± 9
Indeno (1,2,3,-cd) pyrene	bdl	bdl	bdl	29 ± 2	bdl	63 ± 17
Dibenz (a,h) anthracene	bdl	21 ± 6	66 ± 19	51 ± 5	bdl	bdl
Benzo (g,h,i) perylene	bdl	111 ± 19	bdl	280 ± 12	bdl	418 ± 114
Bis (2-ethylhexyl)phthalate	bdl	17 ± 17	bdl	105 ± 23	bdl	75 ± 24
Di-n-butylphthalate	bdl	157 ± 37	319± 25	870 ± 168	195 ± 195	568 ± 133
Diethylphthalate	bdl	35 ± 35	bdl	bdl	bdl	125 ± 125
Butylbenzylphthalate	bdl	17 ± 17	bdl	bdl	bdl	bdl
2-methylnapthylene	bdl	bdl	bdl	504 ± 23	bdl	1119 ± 124
Σ PAHs	d	424 ± 108	385 ± 43	2320 ±132	195 ± 194	3220 ± 406
Σ Immunotoxic PAHs	d	158 ± 31	66 ± 19	551 ±28	bdl	610 ± 144

bdl = Below detection limit of 10 ug/kg.

d = Compound detected 0 to 10 ug/kg.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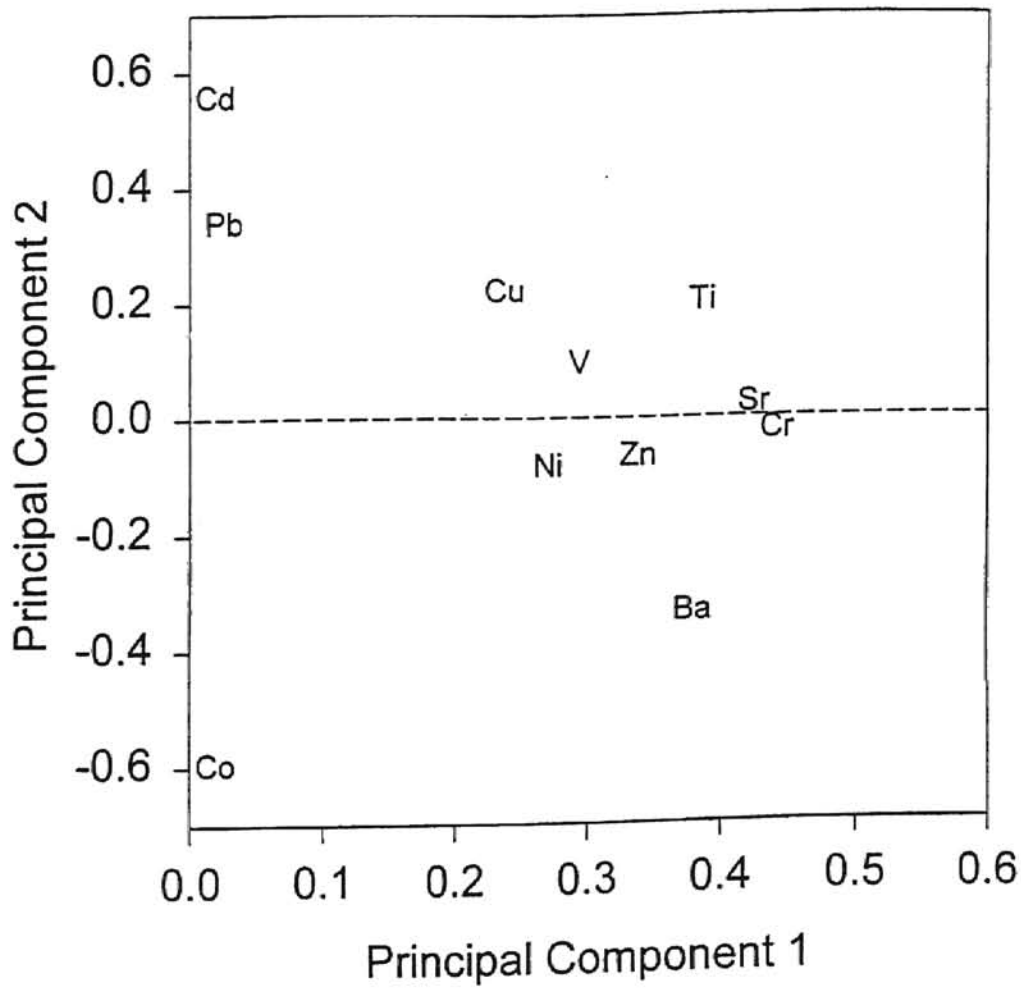
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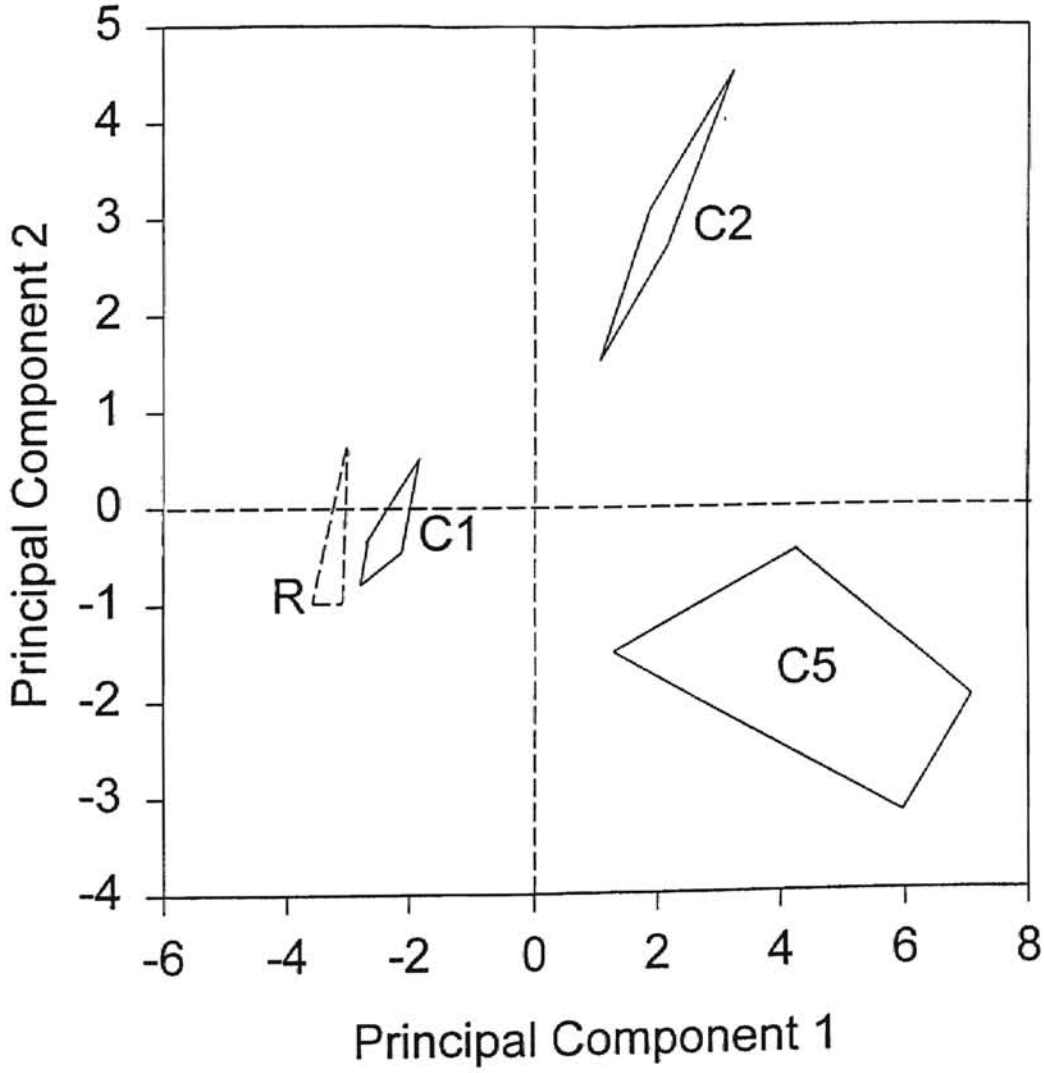
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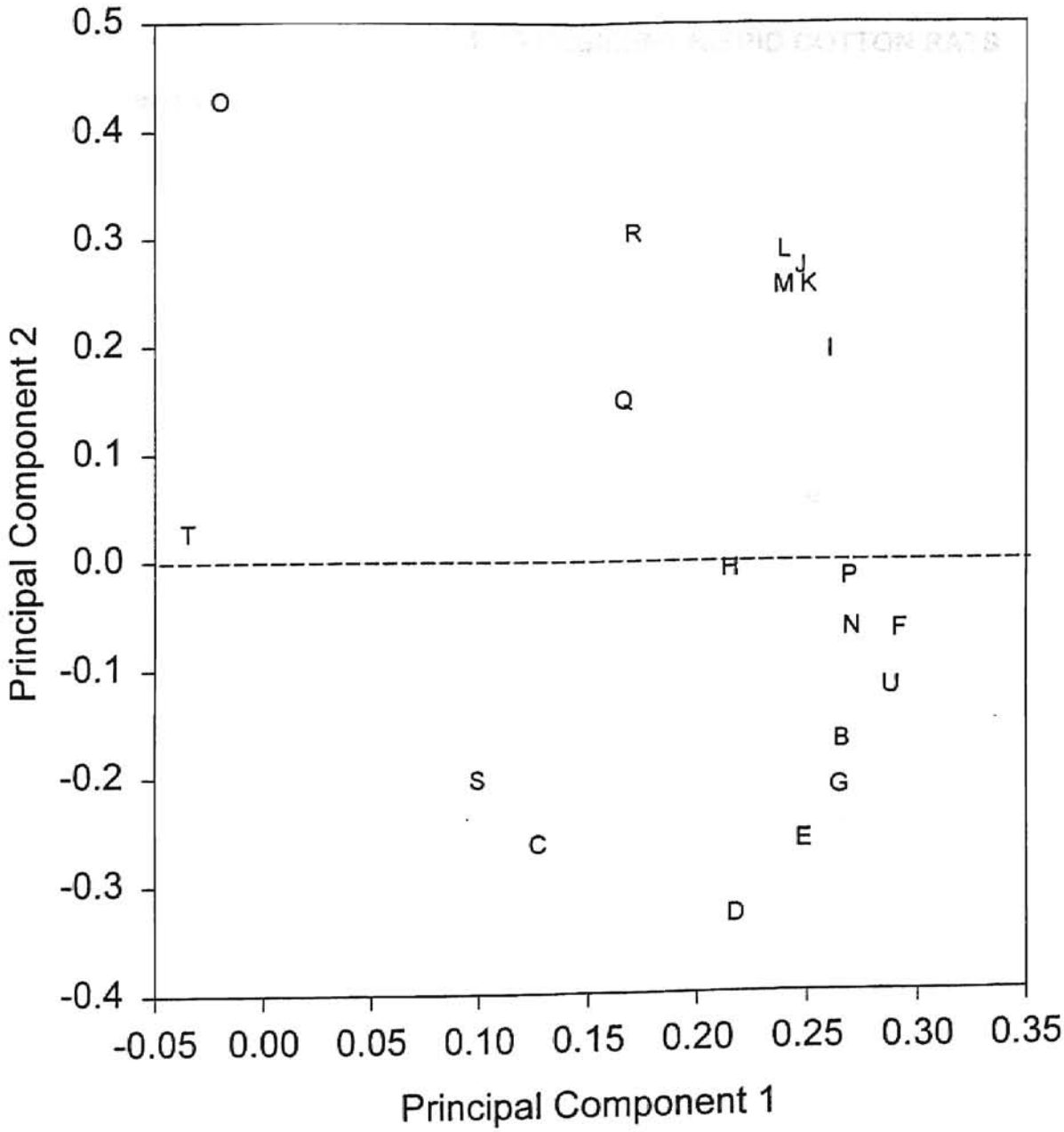
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CHAPTER III

WIDESPREAD FLUOROSIS RISKS TO RESIDENT HISPID COTTON RATS INHABITING FORMER LAND-TREATMENT FACILITIES FOR PETROCHEMICAL WASTES

ABSTRACT: Land-treatment of petroleum wastes is a widely used industrial practice, yet there has been no comprehensive evaluation of the long-term risks to human or terrestrial ecosystems from such practices. We evaluated cotton rat (Sigmodon hispidus) populations on three sites in Oklahoma that historically used land-treatment for disposal of various petroleum wastes (July 1995-March 1997). Average concentrations of fluoride in soil from these sites ranged from 878 to 4317 mg/kg. A census of resident cotton rats on land-treatment sites revealed a high incidence (40% overall) of dental lesions compared to reference populations (< 1% dental lesions). During winter there was a 34% to 65% increase compared to summer in frequency of dental lesions in cotton rats on two of the three land-treatment sites. Incidence of dental lesions on two land-treatment sites was greater (9-16%) in female cotton rats compared to males. Cotton rats from land-treatment sites had higher concentrations of fluoride in bone and greater severity of dental lesions compared to reference animals. Dental lesions were considered to be most consistent with dental fluorosis because of elevated fluoride in bone. Neither concentration of fluoride in soil nor

level of fluoride in bone was a good predictor of severity of dental lesions in cotton rats on land-treatment sites.

Keywords: Fluorosis, cotton rat, Sigmodon hispidus, dental disease, fluoride, petrochemical contamination, wildlife toxicology.

INTRODUCTION

Excessively high concentrations of fluoride from both natural and industrial sources are a continuing environmental health problem for human and wildlife populations throughout the world (WHO, 1984). Compared to human populations, wildlife and domestic livestock tend to display more severe signs of fluoride toxicity due to their higher consumption and reliance on natural vegetation and water in areas with high concentrations of fluoride (Dwivedi et al., 1997). Because of their greater exposure risks, small mammals have been useful biomonitors for fluoride contamination. For example, Boulton et al. (1994a) reported dental lesions in field voles (Microtus agrestis L.) and bank voles (Clethrionomys glareolus L.) in association with environmental exposure to contaminated soils around aluminum smelters, fluorochemical industrial works, and mine tailings. A high incidence of dental lesions consistent with dental fluorosis has been reported in cotton rats (Sigmodon hispidus) on petroleum waste sites (Paranjpe et al., 1994).

Many refineries of crude petroleum use hydrofluoric acid in the alkylation process for production of higher-octane fuels. Waste products generated from

this process are thought to be the most likely source of fluoride in soils where such wastes are land-treated for disposal. This managed technology involves controlled application of waste onto or into soil for the purpose of biodegradation of organic wastes, immobilization of inorganics, and avoiding bioaccumulation of hazardous compounds (Loehr and Malina, 1986). Over 50% of the hazardous wastes generated by the petroleum industry have been disposed of through land-treatment operations (American Petroleum Institute, 1984), yet no comprehensive assessment of their impact on terrestrial ecosystems has been completed. Initial surveys by Paranjpe et al. (1994) and McMurry (1993) suggested that fluoride toxicity risks might be high for land-treatment sites.

We explored the hypothesis that habitats where treatment of petrochemical wastes was facilitated by land-treatment techniques pose a fluorosis risk to resident populations of cotton rats. We also explored the hypothesis that the risk of fluorosis differs seasonally as was previously suggested (McMurry, 1993). To test these hypotheses, we surveyed resident cotton rat populations for incidence and severity of dental lesions, and accumulation of fluoride in bones, on three land-treatment facilities and reference sites during summer and winter in Oklahoma.

MATERIALS AND METHODS

Site Selection

We selected 3 individual units (herein referred to as land-treatment units 1, 2, and 5) in Oklahoma for study. Each unit was inhabited by a population of cotton rats that resided directly on the land-treatment waste site and another matched-reference population that resided in similar habitat < 16 km from the land-treatment site. All land-treatment sites were characteristic of a disturbed prairie ecosystem, with early successional species dominating the vegetation community. Reference sites were selected based on a visual assessment of similarity of vegetation structure and composition with their paired land-treatment site; sites were also similar with respect to topography.

These sites were chosen based on the site having been used historically for land-treating oil refinery wastes, no longer being actively cultivated, having vegetation adequate to provide suitable cover to support a resident small mammal population, and because we were able access to the site. All sites were previously or currently owned by corporations and only one of five of these land-treatment sites was a designated Superfund Waste Site (land-treatment unit 1, Oklahoma Refining Company, Cyril, OK) on the U.S. Environmental Protection Agency's National Priorities List. Treatment of petroleum wastes was discontinued on all five sites during the early to mid 1980's. Little or no prior

information existed as to duration, loading rates, and types of refinery waste products incorporated into soils on these sites.

Population Assessment

Sampling occurred (July 1995-March 1997) on all test units across two seasons, winter [January-March] and summer [July-October], to evaluate whether season influenced susceptibility or sensitivity to fluoride contamination. A permanent 70 by 70 m trapping grid with 64 trap stations evenly spaced at 10 m intervals was established on each land-treatment site and its matched-reference site. One Sherman live trap was placed at each trap station and baited with rolled oats. Cotton rats were censused for 8 periods (4 during each season) with 3 weeks separating each period within a season. Traps were set in the evening and checked between 0600 and 1000 hr for the next 4 days. Cotton rats were toe clipped with a unique identification number and information recorded on capture location, species, sex, body weight, presence or absence of dental lesions, female reproductive status (pregnant, lactating, vaginal orifice perforate or closed), and male reproductive status (scrotal or non-scrotal). We used a slight modification of the suggested method of Stafford and Stout (1983) for aging cotton rats: < 50 g = juvenile, 50 - 79.9 g = sub-adult, and > 80 g = adult.

Cotton rat incisors are normally deep, glossy, and yellow-orange in color. Presence of dental lesions was indicated by a lack of pigmentation on the upper and or lower incisors, which varied in degree from either complete (totally white)

or incomplete (striations of white) loss of pigmentation. During each population census, we inspected the incisors of each cotton rat that was captured and recorded the appearance of abnormal tooth color.

Laboratory Evaluation

Twelve adult cotton rats (6 males, 6 females) were removed from each land-treatment and reference site at the end of the last trapping period in summer and again in winter. Animals were returned to Oklahoma State University campus for further processing within 48 hours after capture. Severity of dental lesions in each cotton rat returned to the laboratory was determined by a more detailed scoring system: 0 = normal incisor color, 1 = lower incisor mildly striated; 2 = lower and upper incisor mildly to moderately striated; 3 = lower incisor severely striated to white and chalky, upper incisor mildly striated, 4 = lower incisor white and chalky, upper incisor moderate to severely striated, 5 = lower and upper incisor white and chalky.

For soil sampling land-treatment sites were sub-divided into six sample stations and matched-reference sites were sub-divided into two sample stations. At each sample station, a composite sample of soil was made from three random surface (0 to 3 cm) samples of soil obtained from within a 1-m radius of the sample station. Composite soil samples were mixed and sealed in acid-washed glass jars and transported to the laboratory for analysis. Total soil fluoride was analyzed for each composite soil sample by using fusion methods as described

by McQuaker and Gurney (1977) and Schroder (1998). Briefly, 0.5 g of soil was placed in a 100 ml nickel crucible and slightly moistened with deionized distilled water. Concentrated NaOH (19M) was added to the sample and fused in a muffle furnace at 600°C. The fusion cake was dissolved in deionized distilled water and neutralized with concentrated HCl to pH 8-9. The cooled sample was then transferred to a 100 ml volumetric flask, diluted to volume, and filtered through a 0.45 membrane filter. Sample digest (5.0 ml) was combined with 5.0 ml of TISAB II and fluoride determined with a combination ion selective electrode (Orion 960900, Orion Research Incorporated, Beverly, Massachusetts, USA); values were reported as mg/kg on a dry weight basis. Both humeri were removed from each cotton rat and adhering muscle tissue teased away. Humeri were dried to constant weight by lyophilization. Fat was removed from bones by soaking over night in petroleum ether. Bones were dried, wet digested with 5 ml trace metal grade HNO_3 in a 25 ml Teflon beaker, and refluxed on a hotplate at 95°C for 1 hour. A 1 ml aliquot was diluted to 5.0 ml with deionized distilled water and combined with 5.0 ml of TISAB II for fluoride determination with an combination ion selective electrode (Orion 960900, Orion Research Incorporated, Beverly, Massachusetts, USA) and reported as mg/kg on a dry weight basis. Aliquots of HNO_3 bone digest were analyzed for heavy metals and quantified by inductively coupled plasma emission spectroscopy (ICP; American Resources Laboratory, Fisons Maxim, Boston, Massachusetts, USA); samples were analyzed for Ba, Cr, Pb, Sr, Ti, and Zn concentrations and reported as mg/kg on a dry weight basis (Schroder, 1998).

Analysis

All data were tested for normality (PROC NORMAL; SAS, 1994) and homogeneity of variances (Levines test; Steel and Torrie, 1980). Data not meeting these assumptions were transformed (arcsine square-root or log normal) prior to further statistical analyses.

Bone concentrations of fluoride and heavy metals were analyzed using a randomized complete block design in a 2 X 2 X 3 factorial format with two treatments (land-treatment and reference sites), two seasons (summer and winter), and three land-treatment units to evaluate whether land-treatment of petrochemical wastes was associated with increased risk of fluorosis for resident cotton rat populations. Comparisons were made using PROC MIXED (SAS, 1994) with sources of variation distributed among the main factor effects (treatment and season) and the interaction terms (land-treatment unit by treatment interaction, season by treatment interaction, and land-treatment unit by season interaction); we used the treatment by land-treatment unit by season interaction as the error term in our analysis. If there were no significant interaction terms, main effects were compared with the PDIFF option for the LSMEANS statement. Significant interaction term effects were compared using the SLICE option for the LSMEANS statement. Satterthwait's approximation was used in calculating degrees of freedom for the error term. Chi-squared analysis (PROC FREQ; SAS, 1994) was used to determine if population parameters were

significantly different for presence or absence of dental fluorosis. A logistic regression model (PROC LOGISTIC; SAS, 1994) examined the relationship between body weight and presence or absence of dental fluorosis for census data. A normal regression model (PROC PROBIT; SAS, 1994) was used to describe the relationship between severity of fluorosis and bone concentrations of fluoride. An analysis of variance (PROC CATMOD; SAS, 1994) was used to evaluate whether land-treatment, season, or sex were associated a with increased risk of severity of dental fluorosis. Statistical significance for all hypotheses tests were set a priori at $\underline{P} \leq 0.05$ and all means are reported as mean (±SE).

RESULTS

Soils from both reference and land-treatment sites had detectable levels of fluoride. All land-treatment sites had mean concentrations of fluoride in soil that far exceeded the mean of three matched-reference sites, which was 121 mg/kg. Soils of land-treatment unit 5 had the highest concentrations of fluoride in soils with a mean of 4,317 mg/kg; range (6 composite samples) of 2,544 to 6,610 mg/kg. Concentration of fluoride in soil of land-treatment unit 1 averaged 2,672 mg/kg with a range between 1,001 to 5,077 mg/kg. Compared to the two other land-treatment sites in this study, land-treatment unit 2 had considerably lower concentrations of fluoride in soil with a mean of 878 mg/kg; range between 669 to1,100 mg/kg.

Analysis of cotton rat populations revealed an extremely high ($\underline{P} < 0.0001$) prevalence of dental lesions in animals from land-treatment sites compared to those from reference sites. Overall, 40% of 948 cotton rats caught in populations from land-treatment sites had dental lesions, whereas only 0.8% of 884 cotton rats from populations at reference sites had lesions characteristic of dental fluorosis (Table 1). Land-treatment sites differed significantly ($\underline{P} < 0.0001$) among each other in prevalence of dental lesions in cotton rat populations. Prevalence was greatest in land-treatment unit 1 (83%) and lowest in land-treatment unit 2 (11%). Prevalence of dental lesions in populations of cotton rats was significantly ($\underline{P} < 0.001$) influenced by season in two of the three land-treatment sites surveyed. On land-treatment unit 1 we observed a 34% increase in prevalence of lesions in cotton rat populations from summer to winter. On land-treatment unit 5 there was a 65% increase in prevalence from summer to winter.

Dental lesions in cotton rats were significantly more prevalent in females than males on land-treatment sites, especially on land-treatment unit 1 ($\underline{P} < 0.009$) and marginally ($\underline{P} < 0.06$) on land-treatment unit 2 (Table 1). Differences between females and males for prevalence of dental lesions was greatest during summer on land-treatment sites with 84% females compared to 64% males from land-treatment unit 1 and 60% females compared to 39% males on land-treatment unit 5. There was a significant correlation ($\underline{P} < 0.013$) between age of cotton rats and presence of dental lesions. However, this correlation was largely

attributed to land-treatment unit 5 ($P < 0.001$), where 70% adults, 58% sub-adults, and 45% of juveniles were positive for dental lesions.

Cotton rats returned to the laboratory for more detailed examination of dental lesions indicated severity of lesions differed significantly ($P < 0.0001$) among land-treatment units (Fig. 1). Based on dental scores (0=none to 5=severe lesions) cotton rats from the three reference sites had an average severity score < 0.13; cotton rats from land-treatment sites had average severity scores ranging from 0.88 on unit 2 to 3.1 for animals on land-treatment unit 1. Average severity scores in cotton rats on land-treatment sites differed significantly ($P < 0.0002$) between seasons with a 72% increase in severity from summer to winter.

Concentrations of fluoride in bone of cotton rats demonstrated a significant treatment by unit interaction ($P < 0.0001$). Mean concentrations of fluoride in bone of cotton rats from land-treatment sites was greater ($P < 0.0001$) than those from matched-reference sites for all land-treatment units (Fig. 1). Fluoride concentrations were greatest in animals from land-treatment unit 5, which was 1.8-to 2-fold greater than levels observed in animals from land-treatment units 1 and unit 2. There was a significant seasonal effect ($P < 0.0001$) in bone fluoride as well, with cotton rats on land-treatment sites accumulating 89% more fluoride in winter compared to summer.

Regression analysis revealed a positive relationship ($P < 0.0001$) between concentration of fluoride in bone and severity of dental lesions in cotton rats, indicating that in general, high fluoride leads to greater severity of dental lesions

(Fig. 2). However, this relationship was not strong, especially for animals with severity scores of 4 or 5. The ability to predict severity of dental lesions from concentrations of fluoride in bone was poor as indicated by the wide variation in Fig. 2.

Concentrations of Ba and Cr in bone of cotton rats did not differ by treatment with an overall mean (n = 144) concentration of 38 ± 1.6 mg/kg for Ba and 1.3 ± 0.34 mg/kg for Cr. Concentrations of Zn in bone demonstrated a significant treatment by season interaction ($P < 0.008$; Fig. 1) with 23% greater concentrations of Zn in cotton rats from land-treatment sites compared to reference animals in winter. Bone concentrations of Sr ($P < 0.0001$), Pb ($P < 0.02$), and Ti ($P < 0.02$) had a significant treatment by land-treatment unit interaction. Least squared means revealed that this significant interaction was attributed to land-treatment unit 1 which differed from its matched-reference site for concentrations of Sr, PB, and Ti in bone (Fig. 1). Concentrations of these metals in bones of cotton rats from land-treatment unit 1 were higher in those from the land-treatment site compared to reference site 1 with 4-fold higher levels of Sr ($P < 0.0001$), 2.4-fold higher levels of Pb ($P < 0.0001$), and 2.3- fold higher levels of Ti ($P < 0.002$) in Ti.

DISCUSSION

Population surveys for dental lesions in rodents residing on waste sites contaminated with fluoride are limited and most published reports of fluoride

toxicity have dealt with documenting concentrations in soil, plant, and animal tissue. Few studies have attempted to document consequences of exposure to excessive levels of fluoride in soil for wild rodent communities. Exceptions include reports of Paranjpe et al. (1994) for cotton rats residing on petroleum waste sites and Boulton et al. (1994a) for field voles (Microtus agrestis L.) and bank voles (Clethrionomys glareolus L.) residing on fluoride-contaminated soils in Europe.

Results of this study clearly establish that cotton rats have a substantial risk of developing dental lesions consistent with dental fluorosis in habitats where petroleum wastes were land-treated. Less than 1% of cotton rats from reference sites showed any sign of fluorosis and those that did had mild dental lesions. Taken alone, this indicates that cotton rats are a good in situ biomonitor for fluoride contamination at the levels experienced on the three land-treatment sites in this study and concurs with previous studies (Paranjpe et al., 1994).

Cotton rats on the three land-treatment sites demonstrated a strong seasonal influence on uptake of fluoride and prevalence and severity of dental fluorosis. This seasonal relationship was evident in cotton rats from the field census as well as those returned to the laboratory with greater risk associated with winter. Wang et al. (1995) noted a similar seasonal pattern of fluoride toxicity in Baotou goats, which they attributed to higher rates of dust-borne fluoride on and uptake by plants during the winter. A survey of a waste site containing fluorspar tailings in the United Kingdom revealed higher fluoride concentrations in herbivorous rodents and plants sampled in late winter (April)

compared to July and October (Andrews et al., 1989). We hypothesize that seasonally-induced changes in the feeding habits of cotton rats contribute to greater rates of soil ingestion and risks of fluorosis during winter. Modeling efforts by Schroder (1998) indicate that most uptake of fluoride compounds by cotton rats is from soil ingestion and dry matter deposition on plant material, and cool season grasses such as Bromus contained higher concentrations of fluoride compared to warm season grasses. Schetter et al. (1998) revealed a tendency for cotton rats to forage on germinating cool-season grasses in winter, which may inherently involve greater rates of soil ingestion. In addition to potentially higher exposures to fluoride, cotton rats are in poorer overall condition during the winter (Fleharty and Choate, 1973; Cameron et al., 1979). Stresses from nutritional and caloric restrictions may contribute to the severity of dental lesions.

An additional factor that may have contributed to seasonal differences was that cotton rats trapped in winter are relatively older than animals trapped during the summer breeding season. This may have permitted a longer exposure period for greater accumulations of fluoride in hard tissues. Previous studies have indicated that older animals can accumulate greater burdens than younger animals, however younger animals tend to accumulate fluoride at a faster rate than adults (Boulton et al., 1994b; Kierdorf et al., 1995). Nonetheless, we only observed an age-related relationship with dental fluorosis on one land-treatment site, where as expected, younger animals had lower prevalence of dental fluorosis than older animals. Prevalence of dental fluorosis was so high on one land-treatment site that animals of all ages had some fluorosis. Cotton rats are

usually not catchable in traps until well after weaning, so it remains possible that dental fluorosis is less common among the very young. Boulton et al. (1994b) indicated that small mammals generally demonstrate rapid rates of fluoride uptake immediately after weaning.

The prevalence of dental lesions in field populations, degree of uptake of fluoride, and severity of dental lesions varied substantially among the three land-treatment sites surveyed in this study. Observations during population monitoring and evaluation of animals returned to the laboratory collectively indicated that levels of fluoride in soil did not directly reflect bone burdens of fluoride or severity of dental lesions. This was clearly demonstrated in cotton rats from land-treatment unit 5 which had the highest (4,317 mg/kg) levels of fluoride in soil yet ranked far below unit 1 (2,672 mg/kg in soil) in prevalence of dental lesions in the population. Examining concentrations of fluoride in soil and bone alone, without examining animals for dental lesions, would have incorrectly lead us to predict that risks to cotton rats on land-treatment unit 5 were greater than on land-treatment unit 1. Animals from land-treatment unit 2 had the lowest frequency and severity of dental lesions, the concentration of fluoride in bones was comparable to levels on land-treatment unit 1 where prevalence and severity was greatest.

Contrary to our results, Boulton et al. (1994a; 1994b; 1995) found a much stronger relationship among soil fluoride levels, concentrations of bone fluoride, and severity of dental fluorosis for several species. Lack of other contaminants present in the soils in the above mentioned studies may have accounted for

these apparent contradictions. Soils on land-treatment sites contained numerous other contaminants besides fluoride (Loehr et al., 1992; Rafferty 1998). Sulfur and aluminum compounds have been implicated in potentiating toxic effects of fluoride (Chen et al., 1996; Caverzasio et al. 1996). Metals implicated in the enhancement of the toxic action of fluoride such as Al were not quantified in this study, but are known to occur at high levels (7,000 – 12,000 mg/kg) in refining wastes that are land-treated (Loehr et al., 1992). In addition, factors such as nutritional deficiencies (Harris and Navia, 1980) and exposure to Cd (Katsuta et al., 1996) may also induce dental lesions. Cotton rats from land-treatment unit 1 where the most severe dental lesions were observed had significantly higher bone concentrations of Sr, Pb, and Ti compared to animals from other land-treatment units.

It is apparent from this study that cotton rats have a substantial risk of developing dental fluorosis from exposure to soils and vegetation of former land-treatment facilities where petroleum-refining wastes were disposed for biodegradation. Dental abnormalities and uptake of fluoride might interfere with the acquisition and absorption of essential food items, interfering with the overall health and survival of small mammal populations. As indicated by our field survey of cotton rats residing directly on contaminated sites, a population monitoring program would be adequate to determine exposure to fluorides. Season of the year was observed to be a powerful determinant of risk and should be incorporated into any monitoring program. It also appears important to

consider possible interactions with other contaminants that may contribute to the severity of dental fluorosis.

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FIGURE LEGEND

Figure 1. Mean (± SE) severity scores of dental fluorosis for incisors (0-5) and concentrations of contaminants in bone (mg/kg) of adult cotton rats collected from land-treatment sites (n=3) and matched-reference sites (n=3) in Oklahoma. For graphs A (mean severity of fluorosis), B (bone fluoride concentration), C (bone Sr concentration), D (bone lead concentration), and F (bone Ti concentrations), values were pooled across seasons (summer and winter) and differences between land-treatment and reference sites within a land-treatment unit are indicated by an asterisk ($P < 0.05$). For graph E (bone Zn concentrations), values were pooled across treatments (land-treatment and reference) and differences between land-treatment and reference sites for season are indicated by an asterisk ($P < 0.05$).

Figure 2. A Tukey's box plot depicting the relationship between severity scores of dental fluorosis and concentration of fluoride in bone of adult cotton rats (n=144) collected from land-treatment sites (n=3) and matched-reference sites (n=3) in Oklahoma. The box represents the 25th and 75th percentiles of the column while the capped bars represent the 5th and 95th percentiles. The solid line within the box is the 50th percentile; the dashed line is the arithmetic mean. Data outside of the 5th and 95th percentiles are depicted as a black circle.

Table 1: Prevalence of dental fluorosis in populations of wild cotton rats surveyed by mark-recapture census. Three land-treatment units consisted of a land treatment site and matched reference site surveyed in Oklahoma. Number of cotton rats surveyed (n) and percentage (%) of cotton rats with dental fluorosis are indicated for season and sex categories.

	Land treatment unit											
	Unit 1				Unit 2				Unit 5			
	Reference		Land-treatment		Reference		Land-treatment		Reference		Land-treatment	
Category	n	%	n	%	n	%	n	%	n	%	n	%
Season												
Summer	179	0	91	73[b]	148	3	263	10	198	2	243	51[b]
Winter	166	0	60	98	61	0	191	13	139	0	100	84
Sex												
Female	184	0	76	90[c]	116	2	259	12	147	1	194	64[d]
Male	161	0	75	74	93	2	195	10	190	1	149	55
Total[a]	345	0	151	83*	209	2	454	11*	337	1	343	61*

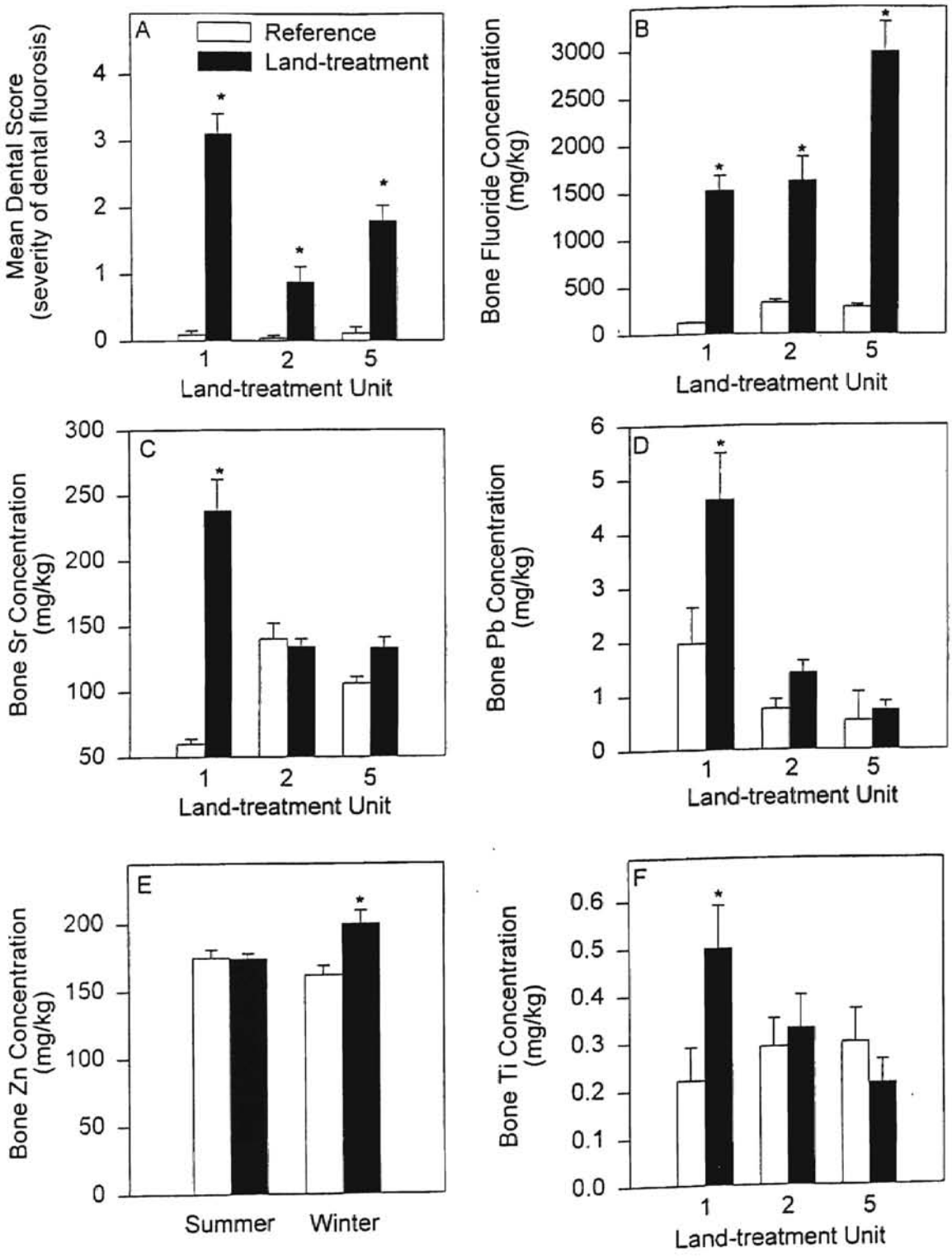
* Land-treatment significantly different ($P < 0.0001$) from reference site for that unit.

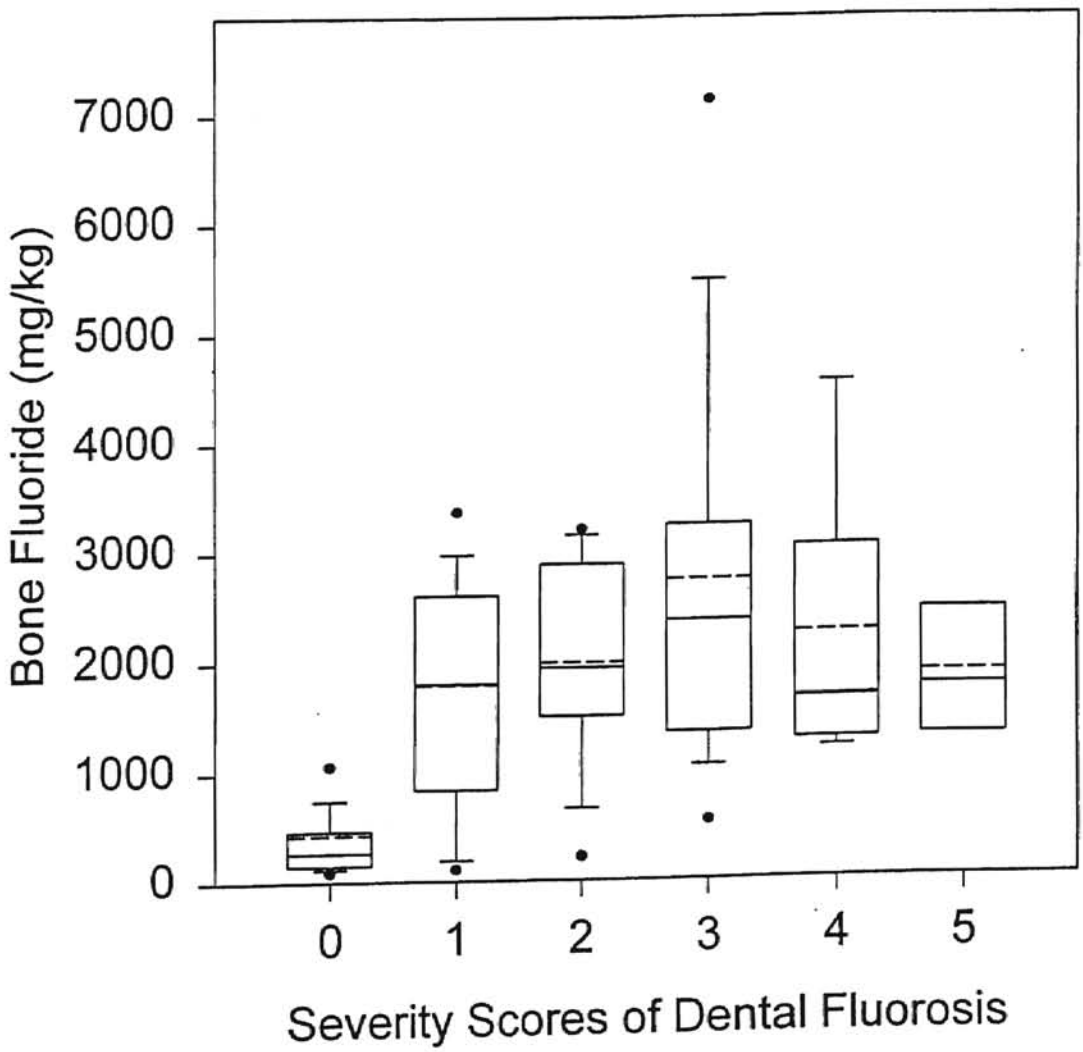
[a] Land-treatment sites differ across land-treatment units ($P < 0.0001$).

[b] Summer significantly different ($P < 0.001$) from winter for that unit.

[c] Females significantly different ($P < 0.009$) from males for that unit.

[d] Females marginally different ($P < 0.06$) from males for that unit.


A
Reference
Land-treatment
Mean Dental Score
(severity of dental fluorosis)
0
1
2
3
4
1
2
5
Land-treatment Unit
B
Bone Fluoride Concentration
(mg/kg)
0
500
1000
1500
2000
2500
3000
1
2
5
Land-treatment Unit
C
Bone Sr Concentration
(mg/kg)
50
100
150
200
250
300
1
2
5
Land-treatment Unit
D
Bone Pb Concentration
(mg/kg)
0
1
2
3
4
5
6
1
2
5
Land-treatment Unit
E
Bone Zn Concentration
(mg/kg)
0
50
100
150
200
Summer
Winter
F
Bone Ti Concentration
(mg/kg)
0.0
0.1
0.2
0.3
0.4
0.5
0.6
1
2
5
Land-treatment Unit


Bone Fluoride (mg/kg)
7000
6000
5000
4000
3000
2000
1000
0
0
1
2
3
4
5
Severity Scores of Dental Fluorosis

VITA

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