HAZARD EVALUATION OF SOIL CONTAMINANTS FROM AN ABANDONED OIL REFINERY SITE WITH AQUATIC ANIMALS AND PLANT TOXICITY TESTS

By

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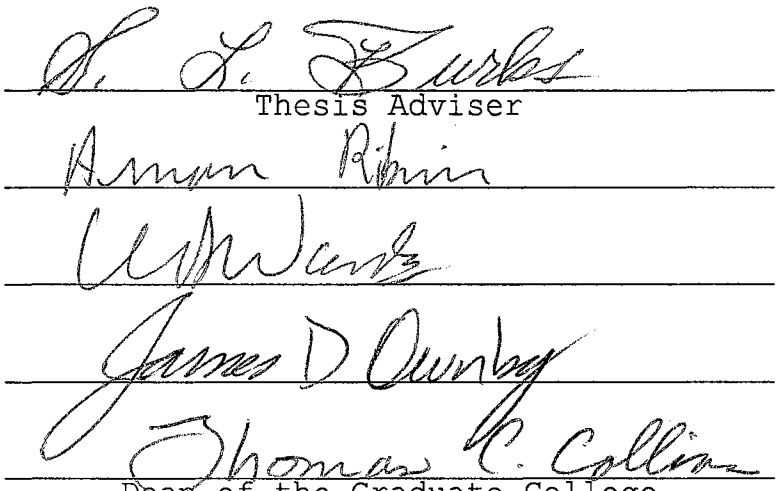

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CHAPTER I

INTRODUCTION

The overall goal of this project was to use short-term acute lethality, partial chronic lethality, and sublethal deleterious responses of aquatic organisms for characterizing the potential toxic effects of contaminants at an abandoned oil refinery. A battery of toxicity tests were used to evaluate potential effects of contaminated soil and leachates of soil on rice seed germination, rice shoot elongation, *Ceriodaphnia dubia* acute survival, fathead minnow acute survival, Microtox® acute response, 7 day *Ceriodaphnia dubia* survival and reproduction, and 7 day fathead minnow survival and growth. This study was conducted in collaboration with a team of investigators simultaneously performing evaluations of the effects of the contaminants upon population structure, physiology, cytotoxicity, and biochemistry of resident small mammals at the abandoned oil refinery. The toxicity tests presented in this portion of the study estimate the short-term integrated effects of complex petrochemical waste mixtures on aquatic organisms and terrestrial plants.

Toxicity is one of the principal characteristics used to identify and characterize hazardous waste sites. Toxicity tests measure the aggregate toxicity of all

constituents in a complex mixture including additive, synergistic, and antagonistic effects. The Environmental Protection Agency (EPA) uses both toxicity based approaches and/or chemical specific approaches to estimate the toxicity of hazardous wastes. In the toxicity-based approach, toxicity tests are used to measure toxicity directly. The toxicity-based approach was developed for measuring and assisting in the regulation of toxic complex effluents discharged to surface waters (U.S. EPA 1985). It was also used to identify and characterize toxic wastes under regulations enforced by the Resource Conservation and Recovery Act (RCRA) of 1976 (Millemann and Parkhurst 1980) and the Superfund Act (Greene et al. 1988).

Toxicity tests are necessary to determine if any adverse effects may be caused by the contaminants within the wastes. Toxicity tests are generally classified as either acute (short-term) or chronic (long-term) depending on the length of exposure of the organisms to the contaminated media. Acute toxicity tests are probably the best means for conducting a preliminary assessment of the distribution and extent of toxic conditions at a site. They are relatively quick, easy, and inexpensive to conduct. On the other hand, acute toxicity tests may not be adequate indicators of potential effects at critical life stages or responses to longer term exposure to contaminants. Thus the absence of an acute toxic response cannot be interpreted as the absence of a toxicity problem. Chronic toxicity tests are generally more sensitive than are acute tests, and can be used to

define "no effect" levels. In addition, chronic tests provide a better index of field population responses and more closely mimic actual exposure in the field. Several short-term partial-chronic tests have been designed to estimate long-term effects. Tests such as the 7-day *Ceriodaphnia* sp. survival and reproduction test and the 7-day fathead minnow survival and growth test are widely used to predict chronic toxicities of chemical and mixtures (Mount and Norberg 1984, 1986; Rand and Petrocelli 1985). Fish early life stage tests generally provide good estimates of the potential effects that might occur during full life cycle chronic exposures (McKim 1977, 1985; Macek and Sleight 1977).

A battery of toxicity tests is recommended for assessing relative responses of different species to the hazardous waste sites and rank the sites with respect to their degree of toxic potential (Warren-Hicks et al. 1989). The toxicity tests selected for this study represent a battery of single-species bioassays that are standardized tests used in assessments for hazardous waste site-soil and sediment samples. This study describes toxicity test methods for water flea survival and reproduction (*Ceriodaphnia dubia*), fish survival and growth (fathead minnow, *Pimphales promelas* Rafinesque), rice seed germination (*Oryza sativa* L.), rice root elongation, and Microtox® (luminescence bacterial test to measure light inhibition). The battery of test methods was applied to soils, and elutriates from soils for the purpose of

measuring both short term acutely lethal and partial chronic sublethal effects.

Protein biomarkers induced by stress can serve as diagnostic screening tool in environmental monitoring. Rice (*Oryza sativa*) plants which are somewhat tolerant to environmental changes showed alteration of several polypeptides when exposed to NaCl, drought, low and high temperature (Lim et al. 1992; Koga et al. 1991; Mathias and Walbot 1989), anoxia (Mujer et al. 1993), and heavy metals (Kaneta et al. 1986; Illangovan and Vivekanandan 1990). Protein biomarkers will serve as additional tools to determine if adverse effects still exist in the ecosystem in cases where toxicity tests or field tests may fail to show any adverse effects. Alteration of proteins observed in rice plant tissue on exposure to contaminated soils could be used for monitoring and for site characterization of hazardous waste sites.

The overall objective was to evaluate the potential hazard of the contaminants at an abandoned oil refinery upon the aquatic ecosystems within the vicinity. The specific tests used to accomplish the overall objective included; 1) To estimate phytotoxicity of the soil at the selected contaminated areas within the refinery, 2) to determine potential for leaching at the selected contaminated areas within the refinery, 3) to assess the relative toxicity of each of the three contaminated areas in the refinery, 4) to determine alteration of proteins in rice plant tissues which were exposed to contaminated soil, and 5) to evaluate if

shoot and root inhibition observed in the rice plants correlate with change in protein pattern in the plant tissues.

Three contaminated and three uncontaminated sites from an abandoned oil refinery near Cyril, Oklahoma were selected for this study. In coordination with other co-investigators a set of trap grids and enclosures were established upon the contaminated sites, designated as 2, 3, and 4, and the uncontaminated sites, designated as 1, 5, and 6. The trap grids (3-5 m^2) were used for collecting small feral rodents from the site and the enclosures (12'square) were used for confining populations of lab-raised white-footed deer mice on site for controlled mesocosm exposures (McMurtry, 1993). Soil sampling was performed in the trap grids and also within the enclosures (mesocosm study areas) placed within the refinery.

The following null hypotheses were used as guidelines for the design of specific sets of toxicity tests and statistical significance analyses;

1. There are no significant difference between each of the three uncontaminated grids and the three contaminated Grids for *Ceriodaphnia* survival and reproduction, rice shoot growth, rice root growth, and Microtox®.
2. There are no significant differences between each of the three uncontaminated enclosures and the three contaminated enclosures for *Ceriodaphnia* survival and reproduction, fathead minnow larval survival and growth, rice shoot growth, rice root growth, and

Microtox®.

3. There are no significant differences between proteins in root and leaves of rice plants exposed to contaminated sites (grids and enclosures) and uncontaminated grids.

CHAPTER II

LITERATURE REVIEW

This chapter describes how toxicity tests were used as biomonitoring tools along with chemical analysis in hazardous site evluations. Protein biomarkers studies are added in this chapter because they served as additional tools for hazard evaluation in this study. Toxicity of Petrochemical wastes, and bioassessment of hazardous wastes are also included in this chapter.

Toxicity Tests

The primary goal of U. S. Congressional mandates to Environmental Protection Agency's (EPA) via RCRA, CERCLA, and TOSCA is to identify, rank, and remediate hazardous waste sites to protect human health and environment. Toxicity is one of the principal characteristics used to identify and evaluate hazardous waste site. EPA uses both toxicity based approach and/or chemical specific-approach to assess the potential hazard of wastes. In the toxicity-based approach, toxicity tests are used to measure toxicity directly. The toxicity-based approach was developed for measuring and assisting in the regulation of toxic complex efflents discharged to surface waters (U.S. EPA 1985). It was also used to identify and characterize toxic wastes

under regulations enforced by the Resource Conservation and Recovery Act (RCRA) of 1976 (Millemann and Parkhurst 1980) and Superfund Act (Greene et al. 1988). In the chemical-specific approach, chemical analysis and water quality criteria are used to estimate toxicity.

Although chemical analysis is a critical element of a superfund site assessment, the fact remains that chemical analysis alone cannot be used to define the toxicity of chemical mixture due to chemicals that are present at levels below the analytical detection limits, biodegradation metabolites that may be difficult to identify and the interactions of components of a complex mixtures. Both Tokiwa et al. (1980) and Garner et al. (1986) suggest that nitropolynuclear aromatic hydrocarbons present at levels that were below the detection limits of the instrument used, were responsible for a large portion of the mutagenic activity of air samples.

Chemical analysis often fails to provide sufficient information to characterize potential for leaching of hazardous constituents into groundwater. The components of a complex mixtures may interact to provide synergistic, antagonistic, or additive effects (Williams and Weisburger 1977). Chemical analyses for complex mixtures, especially for organics can be more expensive than toxicity testing. The specific chemicals analyzed in complex mixtures may not include many toxic chemicals actually present. The quantity of benzo(a)pyrene (BAP) in emissions from a firefighter training facility accounted for only 20 percent of total

mutagenic activity of the sample (Atlas et al. 1985). In addition Flessel et al. (1988) indicated that less than 2 percent of the mutagenic activity of an air sample could be accounted for by the BAP content. A toxicity assessment was done for the Western Processing site, Kent, WA. and by using a battery of single species and multimedia toxicity tests, the results indicated that toxicity was indeed present at various locations, despite the chemical analysis of water samples which suggesting that toxicity was not evident (Miller et al., 1985).

Industrial and municipal effluents often contain large number of potentially toxic pollutants which can move through treatment systems virtually unaltered. Often these are pollutants for which little or no toxicity data exist. Therefore National Pollution Discharge Elimination System (NPDES) permit policy implemented an integrated approach that included use of whole effluent toxicity test combined with chemical-specific analyses.

It is not always clear from chemical data which compounds are causing toxicity in a complex hazardous waste mixtures. Two bioassays *Salmonella typhimurium* (Ames) and *Panagrellus redivivus* (a nematode) had been used in concert with chemical fractionation to identify cores of lake sediments by Samoiloff et al. (1983). They found that the "most toxic fractions contained none of the priority toxic chemicals." The bioavailability of toxic chemicals is directly evidenced by toxicity tests but not with chemical analyses. Therefore, chemical data may over or

underestimate the toxicities of single chemicals.

Toxicity tests integrate the effects of all constituents into a single measurement. In addition toxicity test endpoints integrate the bioavailability and interactions of multiple toxicants into a single measurement. Thus toxicity tests play an important role in and of themselves in site assessments, and potentially link the occurence of contamination, as evidenced by an elevated chemical concentration, to biological effects (Warren-Hicks et al. 1989). Thomas et al. (1986) identified the most likely toxicant present in the complex mixture on the basis of the toxicity expressed by different components of the test battery.

Results from toxicity tests are specific to the site of sample collection, and thus can be mapped to define gradients and zones of toxic conditions. Such maps, in addition to toxic responses can serve as a guide to the design of field surveys and other sampling programs. A close correspondence between spatial patterns of toxicity and spatial patterns of effects measured in field surveys provides strong evidence for the importance of toxic contaminants in controlling the status of ecological communities at the site. Thomas et al. (1986) used toxicity test results to develop an assessment of the spatial distribution of toxicity at Rocky Mountain Arsenal site in Colorado. Several investigators have concluded that the toxicity-based approach is better for estimating potential toxicity of complex chemical mixtures of unknown

composition, such as hazardous waste site samples (Bergman et al. 1986; U.S. EPA 1985; Greene et al. 1988).

There is an extensive multiplicity of compounds and sources contaminating both terrestrial and aquatic systems. The major problem in such systems is to determine which specific components pose the greatest long- and short-term risks to biological systems, including man. The question of what chemicals are present is secondary; The primary objective is to establish which compounds in a particular system pose the greatest risk. In complex mixtures, however, it may be impossible to determine which chemical or chemicals are causing toxicity. Toxicity-based identification and chemical fractionation methods have been used to identify the cause of toxicity in complex mixtures (Parkhurst 1986; U.S. EPA 1985; U.S. EPA 1988).

Federal and state regulatory and consulting laboratories routinely use toxicity tests for biomonitoring water and wastewaters. These tests use organisms such as fathead minnow, *Daphnia magna* or *Daphnia pulex*, and *Selenastrum capricornutum*. In studies where effluent and ambient toxicity tests were used for predicting biological impact there were significant correlation of the field impact and toxicity data (Dickson et al. 1992; Mount et al. 1986). Significant correlation ($P = .05$) was obtained with the *Ceriodaphnia* data and the periphyton, macroinvertebrates and fish species richness (Mount et al. 1986). In a study concerning agricultural drains a toxicity identification evaluation was conducted using *Ceriodaphnia dubia* to

identify the suspected toxicants both methyl parathion and carbofuran (Norberg-King et al. 1991).

Data on the toxicity of liquid or solid samples from hazardous waste sites provide a cost effective and rapid means for ranking the potential environmental hazards of the sites (Greene et al. 1988). One of the important uses of toxicity test is for site characterization of environmental conditions in and near hazardous waste sites. Toxicity test results can provide valuable information relevant to the decision process associated with cleanup action. Toxicity tests are valuable monitoring tools because they provide cost-effective, rapid assessment of potential toxicity.

Three types of endpoints were derived from the acute and chronic toxicity tests: 1) Percent response of the test organisms exposed to soil; 2) a concentration-percent response relationship for test organisms exposed to extracts of soil; and 3) measures of the lowest observed effect concentration (NOEC) for *Ceriodaphnia dubia* reproduction and fathead minnow growth. Acute toxicity test results were expressed as LC50's (the concentration of a chemical which is estimated to be lethal to 50% of the test organisms) or EC50's (the concentration of a chemical that is estimated to have a sublethal effect to 50% of the test organisms). Chronic test results are often expressed as estimates of acceptable concentrations or toxicity threshold concentrations. The NOEC (no-observed-effects-concentration), is the highest test concentration that caused no statistically significant difference in effects

upon the treatments when compared with the controls. The LOEC (lowest-observed-effects-concentration), is the lowest concentration that caused the effects upon the treatments to be statistically significantly different from the control.

Protein Biomarkers

In addition to toxicity tests biomarkers could be used to evaluate exposure or effects of environmental contaminants. Biomarkers are measurements of physiological and biochemical responses in individual organisms. Biomarkers serve as sensitive indicators of exposure to contaminants and/or sublethal stress. Based on the magnitude and pattern of biomarker responses, the test species serve as predictors of long-term effects on health of population or the integrity of the ecosystem. A major advantage of the biomarker approach is that biochemical and cellular events tend to be more sensitive, less variable, and highly conserved. Biomarkers provide evidence of exposure to compounds that do not bioaccumulate or are rapidly metabolized. The biomarkers express the cumulative effect of toxicant interactions in molecular or cellular targets. The rapidly responding biochemical markers can serve as valuable short-term indicators of the direction of toxic exposure and potential effects at monitoring sites. In a stream near Oak Ridge, Tennessee levels of genotoxic damage had been decreasing significantly in response to improvements in waste treatment at an industrial facility

discharging into the stream (Shugart 1990). Information on the short-term responses to toxic exposure could aid in management decisions regarding the effectiveness of any remedial activities and the rates of recovery of affected sites.

Biomarker measurements in organisms living on or near hazardous waste sites or industrial facilities can help provide data needed to assess the potential exposure and effects from contaminants at the sites. Biomarker measurements showed significant differences along a gradient going downstream from the industrial discharge, and correlated well with community level effects (Loar et al. 1988). Biomarker measurements have proved to be a useful and informative component of the National Pollution Discharge Elimination System permit for government facilities in Oak Ridge, Tennessee (Adams et al. 1989). Biomarkers measurment had demonstrated a clear improvement in water quality (Adams et al. 1989; Shugart 1990). Biomarkers can be sensitive and cost-effective measures demonstrating the effectiveness of remedial action.

Changes in gene expression associated with stress response are extremely rapid and result in synthesis and accumulation of proteins. Stress proteins are part of the primary cellular protective response from environmental stress, are induced by a wide variety of environmental stressors, and are highly conserved in all organisms from bacteria to man (Schlesinger et al. 1982). Protein

biomarkers induced by stress can serve as diagnostic screening tool in environmental monitoring.

Stress proteins have been induced by a wide variety of stressors including heavy metals (Hammond et al. 1982; Caltabiano et al. 1986), Xenobiotics (Cochrane et al. 1989), oxidative stress (Kapoor and Lewis 1987), anoxia (Spector et al. 1986), salinity stress (Ramagopal 1987), teratogens (Bournias-Vardiabasis et al. 1983; Bournias-Vardiabasis and Buzin 1986), and hepatocarcinogens (Carr et al. 1986). In *Neurospora*, cells indicated different translational patterns when exposed to heat shock, arsenite, and oxidative stress (Kapoor and Lewis 1987). A pattern of 11 proteins was translated in response to heat shock, whereas in response to arsenite only the 70 KDa and a unique arsenite specific protein of approximatly 40 KDa were translated. Hydrogen peroxide exposure led to the synthesis of 70 KDa and 80 KDa proteins. The heavy metals Cu and Cd and thiolreactive agents induced the synthesis of 100, 90, and 70 and a 32 KDa stress protein, heme oxygenase (Caltabiano et al. 1986). Lead induced stress response in rat fibroplasts and epithelial cells (Shelton et al. 1986). Salinity stress induced the synthesis of low molecular stress proteins from 20-30 KDa in barley plants (Ramagopal 1987). Biomarkers are applicable to broad range of organisms when exposed to a wide variety of stress conditions in their environment. Biomarkers have been correlated with decreased physiological functions and survival of the organism.

Biomarkers have been used to identify exposure to specific contaminants and can be measured in a cost efficient manner. The photosynthetic microorganism *Euglena gracilis* responded to cadmium and synthesized small glutathione-like peptides which bind to metals (Shaw III et al. 1989). In response to metals Cd, Cu, Hg, Zn, and Pb plant cell cultures of *Agrostis gigantea* and *Brassica capitata* had produced metal-binding proteins, phytochelatins (Grill 1987) and Cd induced phytochelatins 776 Da complex in *Datura innoxia* (Rauser 1987) and 4000-8000 Da complex in *Zea mays* (Bernhard and Kagi 1987). Stress proteins satisfy many of the conditions as a potential biomarker and are ideal candidates for developing a biomarker approach to environmental monitoring.

Petrochemical Wastes

Petroleum products have been identified as soil contaminants at many hazardous waste sites. Such chemicals and compounds as benzene, xylene, polyaromatic hydrocarbons (PAHs), toluene, and metals are potential contaminants of public health concern when petroleum products have been released into the environment. Petroleum products are complex mixtures of many petroleum hydrocarbons. Upon release of a petroleum product to the environment, certain fractions of similar compounds may quickly be lost via volatilization, dissolution/leaching, or biodegradation. Petroleum hydrocarbons are degraded by microbial metabolism

in which the hydrocarbon oxidized to carbon dioxide and water. The heavy metals found in petroleum products will tend to adsorb to soil particles, making the metals relatively immobile. On the other hand volatile organic compounds, such as benzene and toluene will tend to evoporate and volatilize from the surface soil into the air or migrate from subsurface soils into the groundwater.

Toxicity of an individual compound in a petroleum mixture may be quite different from the toxicity reported for the same compound acting alone. The carcinogen 7-12 dimethylbenzanthracene was applied to mouse skin at less than carcinogenic dose. Subsequent dermal administration of Dodecane and Tetradecane resulted in the promotion of skin tumors (Sice 1966). Octadecane and Eicosane induce tumor more rapidly than dodecane and tetradecane and are active both as cocarcinogens and promoters (Horton et al. 1976). Dodecane and decane have been found to be cocarcinogens with benzo(a)pyrene (Horton et al. 1957). Thus the constituents of petroleum products can have altered toxicity when they are combined into a mixture such as No.2 fuel oil.

The health effects of refined petroleum products are due to four major groups of hydrocarbon components. The alkane (paraffin) compounds of refined petroleum products are primarily aliphatic hydrocarbons from C3 to C8. Alkanes have potent narcotic action when inhaled at high doses. Straight chain alkanes are, in general more toxic than branched chain isomers (NRC 1982; USDH 1977). Acute alkane intoxication involves a transient depression of the central

nervous system (CNS). Polyneuropathy developed in animals and humans following chronic intoxication by alkanes (USDH 1977). Alicyclic hydrocarbons, which include saturated and unsaturated naphthenes or cycloparaffins, have toxic effects similar to aliphatic hydrocarbons. Alicyclic compounds and alkenes (olefin, unsaturated aliphatics) constituents from petroleum products are anesthetic and are CNS depressants.

The aromatics, which include benzene, alkyl derivatives of benzene (toluene and xylene), and polynuclear aromatic hydrocarbons (such as anthracene, phenanthrene) have been considered most toxic components of refined petroleum (NRC 1982; IARC 1983). Humans are more likely to be exposed to these compounds, since the aromatics are more water soluble than other hydrocarbon compounds in petroleum products. Benzene has the highest health impact, since it is volatile and water soluble, and is a human carcinogen. Toluene, the xylenes, and the alkylated benzene derivatives are less toxic than benzene but are quite water soluble and still pose significant toxicity to humans (Beck et al. 1984).

Following its release into the environment, the fuel constituents may become transformed, and this may enhance or reduce their toxicity. The burning of fuel will result in the formation of mutagenic and carcinogenic PAHs. PAHs form during the incomplete combustion of organic compounds. Photooxidation products of oils are of significant concern since these products have been shown to have increased toxicity over the parent compound. Photooxidation of No.2 fuel oil produces water soluble compounds that are more

toxic to yeast, algae, fish, and shrimp than water soluble compounds from No.2 fuel oil that have not been exposed to sunlight (Scheier and Gominger 1976; Larson et al 1979; Callen and Larson 1978). MacFarland (1988) indicated that n-hexane is metabolized by oxidation at the 2 and 5 carbon atoms to the hydroxyl group and then to the keto group, resulting in the intermediate metabolite methyl n-butyl ketone and active neurotoxin 2,5-hexanedione.

Gasoline is produced from crude petroleum by a variety of refining and manufacturing processes. Gasoline contains a large number of chemicals, some of which are carcinogenic. Benzene, a significant component of gasoline and other petroleum products is well known and has been shown to be a carcinogen in both animals and humans. Benzene causes leukemia and cancers of the lung, liver, lymph, stomach, oesophagus, nasopharynx, and intestine (Mehlman 1992). It is generally considered that the the only absolutely safe concentration for benzene is zero (Drinker 1948). Benzene is currently classified by EPA and the International Agency for Research on Cancer (IARC) as a human carcinogen. Butadiene (BD) is a component of gasoline and motor oil. In humans BD causes increase in lymphomas, leukemias, and other cancers of hematopoietic systems and organs. BD is also a potent alkylating agent, directly toxic to developing embryos and damages progeny after parental exposure (Mehlman and Legator 1991).

Oil Pollution: Field Impact

Greater incidence of morphological deformities were observed in the benthic communities of the St.Clair River impacted by a petrochemical complex near Sarnia, Ontario (Canada) (Dermott 1991). Larvae of *Chironomids* showed high incidence of morphological deformities in their mouth parts.

Land application of waste (also called land treatment of waste or land farming) has potential effect such as reduction of waste mass and toxicity. Potential negative effects include the contamination of the environment and entry of toxic chemicals into the food chain. Environment Canada is sponsoring several research studies which assess the treatability of oily wastes by land application and the environmental acceptability of this practice (Visser et al. 1987).

Studies concerning the land application of diesel-based drilling mud residues were initiated to assess the effects of drilling mud residue application rates on degradation of oil and selected hydrocarbons and to evaluate phytotoxicity. Incubation studies were performed in which two sources of diesel-based drilling mud residue were applied to two soil types (Visser et al. 1987). The composition of the diesel-based drilling mud residue was typical of lightly weathered diesel fuel, with 85% to 90% aliphatic hydrocarbons, 8% to 13% aromatic hydrocorbons and about 2% polar materials. Rates of degradation varied between the two sources of mud and was lower in finer textured clay soil than in the clay loam. The concentration of normal alkanes decreased to 50%

rapidly and 95% of low molecular weight aromatics such as naphthalene disappeared rapidly within 30 days. Concentrations of aromatic hydrocarbons in the 516 to 212 molecular weight range were reduced by 98% after 240 day incubation period. The soil/waste mud mixtures were initially inhibitory to seed germination and plant growth. After 5 weeks of incubation, the mixtures no longer inhibited germination, but plant growth was reduced probably due to changes in soil physical characteristics and nutrient availability.

A field study was established in four locations in Alberta in 1982 to determine the efficiency of land treatment for the degradation of oil in oilfield sludges (Danielson et al. 1987). These sites had received oily waste application for a three year period, and oil and metal concentrations had been monitored in soil and groundwater. The decreased plant productivity was related to residual oil content in the soil. The oil content in soil ranged from 2.6% to 8.6%. Plant growth was good on plots with 3.5% oil or less, but poor on plots with oil content of over 5%. Residual oil contained 15% to 18% asphaltenes, 40% to 50% resins, 16% to 23% aliphatics, and 11% to 16% aromatics. Shallow groundwater beneath and downgradient from the land treatment sites contained elevated levels of chloride and nitrate.

Hazardous Waste Sites

In the past, hazardous wastes have been disposed of with little or no attention paid to site location, safety measures, or maintenance of records. This has led to numerous instances of contamination that have severely damaged the environment and threatened human health. For two and one half decades, hundreds of tons of toxic waste were dumped into an unfinished canal built by William T.Love in Niagara Falls, New York. Approximately 22,000 tons of chemical waste were buried between 1938 and 1951 at the hazardous waste site (Kim et al. 1980) including 6,900 tons of lindane, 2000 tons of chlorbenzenes, 2,400 tons of benzyl chloride, and 200 tons of trichlorophenol contaminated with unknown amounts of dioxin (TCDD). The canal was covered when full; houses and a school were built near and above the canal. In the later 1970s, alarmed by unusual health symptoms, residents of the Love Canal area called the attention of government officials to hazardous substances floating to the surface, seeping into the basement of houses, and migrating from the site (U.S. EPA 1980).

Over 24,000 uncontrolled hazardous waste sites, have been identified by the EPA (U.S.EPA 1986). The Resource Conservation and Recovery Act (RCRA) of 1976 was the first comprehensive Federal legislation to deal with the hazardous wastes issue. Superfund was established by the Comprenhensive Environmental Response, Compensation, and Liability Act (CERCLA), Public Law 96-510, enacted in

December 1980. The United States Environmental Protection Agency (US EPA) is responsible for managing the Superfund Program. It can be used to provide both short-term actions and long-term cleanup, with the latter generally undertaken only at inactive waste sites listed on the National Priorities List (NPL) (U.S. EPA 1990). Environmental monitoring during and following remedial actions is an important part of CERCLA and RCRA assessments. Biomonitoring is a valuable tool for site evaluation as indicated in the following studies.

The polycyclic aromatic hydrocarbons (PAH) and other organic components from an abandoned American Creosote Works (ACW) in Penscola, Florida had been transported from the original waste containment ponds into the groundwater and toward Pensacola Bay (Goerlitz et al. 1985). Bioaccumulation of PAHs in caged bivalve molluscs, placed near the discharge of a perennial stream flowing into Pensacola Bay, was confirmed (Elder and Dresler 1988). A concentration of 100% streamwater caused significant teratogenic responses in fish embryos and larvae and groundwater caused significant embryo toxic or teratogenic responses at concentrations of 100, 10, and 1% (Middaugh et al. 1991). Exposure of juvenile fish to creosote-contaminated sediments from the perennial stream for 43 days resulted in a 50-fold increase in liver cytochrome P-450 levels, compared to controls (Schoor et al. 1991).

Drake superfund site, Lock Haven Clinton Co. Pennysylvania is an inactive chemical manufacturing facility

located 1 km south of a creek. The herbicide 2,3,6-trichlorobenzene acetic acid (trade name Fenac) had been manufactured at the site and remains a major on-site and off-site contaminant (Greene 1991). In selected locations the sludge had been buried to a depth of 4-7 m. Both mutagenicity and acute toxicity were evaluated with the *Salmonella*/microsome assay and the Microtox® assay with the core samples obtained from major areas affected by the waste management activities at the facility (Donnelly et al. 1991). An increased mutagenic response was corresponded to increased level of herbicide in one location. The aqueous extract of the contaminated soil induced a positive response in *Salmonella* and Microtox® assays, *Salmonella* mutagenic response as 737 net revertants and Microtox® EC-50 as 0.74-2.5 toxic units. In addition the aqueous soil extracts provided an indication of the presence of mutagens that could leach into ground water. Groundwater samples collected from the site also indicated that contaminants have migrated into the groundwater.

Standard bioassays were used to locate the contaminants from a wood treatment site in Mississippi (Athey et al. 1989). The site was contaminated with creosote and pentachlorophenol used for wood treatment on the site. The toxicities of sediment, sediment elutriate, and whole water samples collected from a creek adjacent to the wood treatment were estimated with bioassays using alga (*Selenastrum capricornutum*), *Daphnia magna*, Microtox® (*Photobacterium phosphoreum*), earthworm test (*Eisenia*

foetida) seed germination test and root elongation test (lettuce, *Lactuca sativa*) and compared with estimated concentration of creosote and related materials obtained from the same samples by infrared spectroscopy. The results of the study indicated that the infrared measurements of creosote were not accurate predictors of biotoxicity. Different bioassay organisms had different sensitivities to the mixture of contaminants resulting from the wood treatment operations. Of the bioassays, *Daphnia* and Microtox® were more sensitive to the contaminants from the wood treatment operations.

The Rocky Mountain Arsenal site at Commerce City, Colorado has been used for the manufacture of antipersonnel gases, insecticides and herbicides, and as an ordinance testing area. Over the years myriad organic and inorganic compounds were disposed of through ditches to a series of interconnecting holding basins. Thomas et al. (1986) used standard laboratory bioassays for a toxicity assessment of the Rocky Mountain Arsenal site. Soil toxicity estimated by earthworm mortality and seed germination contributed to the site assessment of the Rocky Mountain Arsenal. Toxicity test with unknown chemical composition from the Rocky Mountain Arsenal site showed that lettuce seed germination phytoassay was the most sensitive.

A toxicity assessment was completed for the Western Processing site in Kent, WA (Miller et al. 1985). Toxicity tests selected for this site evaluation included the earthworm test on soil as well as the algal, root

elongation, and daphnid short-term test which were completed on surface waters and soil eluates. The battery of single species, multimedia toxicity test contributed significantly to the evaluation of the Western Processing site.

CHAPTER III

TEST SPECIES

This chapter deals with the test species significance in the toxicity tests particularly for multiple contaminants present in sediments, aqueous wastes, and soil. The test species used in this study were *Ceriodaphnia*, fathead minnow, rice and Microtox®. The various instances these test species used for multimedia contaminants are included and also some of the disadvantages in using these test species are also specified.

Ceriodaphnia

Daphnia species are considered the most sensitive among several aquatic species tested (APHA 1976). *Daphnia* toxicity tests have been used for a variety of purposes including generating lethality data for pure compounds and complex mixtures, as well as estimating maximum allowable toxicant concentrations. In cases where *Daphnia* species were the most sensitive genus tested, these data have been utilized in setting water quality criteria (APHA 1976). *Daphnia* toxicity data have been used to evaluate the potential hazards of new chemicals, and production decisions have been based on these and other data (Kimerle et al. 1978). Because of their sensitivity to industrial

effluents, daphnids have been suggested for screening toxicity tests for industries to monitor discharges and measure the efficiency of waste treatment facilities (Buikema et al. 1980).

The cladoceran *Ceriodaphnia* inhabits larger streams, reservoirs and lakes, and it is widespread in many types of habitats. *Ceriodaphnia* has a short generation time and is easy to culture and maintain in the laboratory. Four cladoceran species; *Daphnia magna, D.pulex, Simocephalus vetulus* and *Ceriodaphnia reticulata* were tested in Lake Superior water and their 48 h LC50 were compared (Mount and Norberg 1984). In that test, *Ceriodaphnia* was found to be more sensitive than *Daphnia magna*. According to Mount and Norberg (1984), a three-brood life cycle test completed in 7 days for *Ceriodaphnia* is convenient when samples to be tested are limited in volume. Time is especially important, such as on-site effluent testing. According to Geiger et al. (1980) a 7-day growth impairment test is a viable predictor of subchronic or chronic effects of reproduction. Short-term methods are used to estimate the chronic toxicity of effluents and receiving waters to the fathead minnow (*Pimphales promelas*) and *Ceriodaphnia dubia* (U.S. EPA. 1982; Peltier and Weber 1984).

Warren-Hicks et al. (1989) suggest that acute test methods used for whole effluent testing and whole sediment testing are directly applicable to hazardous waste site assessment. In addition short term tests developed to estimate chronic toxicity of effluents and receiving waters

are standardardized test methods for hazardous waste site evaluation (Greene et al. 1988).

Whole effluent toxicity tests are used to predict instream response of the aquatic community. Eagleson et al. (1990) conducted in 43 freshwater flowing systems *Ceriodaphnia dubia* chronic toxicity test procedures and standardized qualitative sampling of benthic macroinvertebrates. In 88% of the comparisions there was agreement between both measures.

The toxicity of sediment in Waukegan (Illinois) and Indiana (USA) harbors sites were evaluated using a multitrophic level test battery (Burton et al. 1989). The test battery consisted of the following assays: *Daphnia magna* 48-h survival, *Ceriodaphnia dubia* 48-h survival, *Hyatella azteca* 48-h survival, *Selenastrum capricornutum* 48-h growth inhibition, and enzymatic activity of indigenous microbial community. The test battery effectively discriminated between sites in most cases. *Ceriodaphnia dubia* was the most sensitive species among the test battery. The acute toxicity response patterns verified sites containing the highest levels of contaminants at Waukegan harbor and Indiana harbor (Burton et al. 1989).

In situ exposures of *Ceriodaphnia dubia* were conducted in a stream impacted by several effluents, a combined sewer overflow and a creosote treatment operation (Sasson and Burton, 1991). The sediment contained high levels of metals and polynuclear aromatic hydrocarbons. Fish and macroinvertebrate community indices were depressed.

Ceriodaphnia dubia were placed in sediment exposure chambers in the stream for 48 h and also exposed to sediments that were simultaneously collected in the laboratory. *In situ* sediment exposures proved to be sensitive indicators of both degraded and nondegraded stream conditions.

The *Daphnia* toxicity test method has some limitations. Many industrial effluents are turbid and colored and in such cases filtration may be required. Filtration removes indegenous microfauna which can interfere with daphnia survival. Filtration also removes toxic suspended particles and thus reduces sample toxicity. Some effluents are high in biochemical oxygen demand and low in oxygen. Aeration can increase volatilization of the sample organics. The hard reconstituted water used as dilution water may alter the toxicity of the sample.

Fathead Minnow

Fathead minnow, *Pimphales promelas* is an important forage fish in the aquatic food chain. It belongs to the *Cyprinidae* family, which is the largest group of fresh water fish in North America. It is easily cultured in the laboratory and is highly prolific. It has been extensively used in life cycle chronic test and is considered to be a consistently sensitive species for use in toxicity studies (McKim 1977; Benoit et al. 1982). It is one of the recommended species to test freshwater effluents (Weber et al. 1989).

Several studies have reported that early developmental stages of fish were more sensitive to linear alkylbenzene sulfonate (LAS) surfactants than to other life stages during acute toxicity tests (Marchetti 1965; Pickering 1967; McKim et al. 1975). Pickering and Thatcher (1970) also observed during a complete life cycle exposure of fathead minnows to LAS that newly hatched fry were more sensitive than all other life stages. The embryos and newly hatched fry stages were also extremely sensitive to many chemicals (Pickering and Thatcher 1970 ; Macek et al. 1976; Benoit et al. 1982). Studies conducted on copper and cadmium indicated that fish larvae and early juvenile stages of all fresh water species tested were more sensitive to copper and cadmium than the embryos (Mckim et al. 1978; Eaton et al. 1978).

Sauter et al. (1976) reported that egg and fry studies are an effective, reliable means of assessing chronic toxicity of certain compounds to selected freshwater fish

species. In their study eggs and fry of rainbow trout, lake trout, channel catfish, bluegill, white sucker, northen pike and walleye were continuously exposed for a maximum of 30 and 60 days to a series of concentrations of metals such as lead, chromium, copper, and cadmium. Observations of the hatchability of eggs and survival and growth of fry were made after expoure of the fry of these species to metals. Maximum Acceptable Toxicant Concentration (MATC) estimated from these relatively short duration of egg and fry tests were found generally similar and in some cases, nearly identical to those estimated from chronic studies of much greater duration.

Fathead minnow is currently the most commonly used species for early life stage tests and a large database has been established for acute, partial life cycle chronic tests using the embryo and larval stages (McKim 1977; Macek and Sleight 1977; USEPA 1985). Tests such as 7-day short term chronic tests are widely used to predict the chronic toxicities of chemicals and mixtures (McKim 1985). Short term tests have been developed to estimate toxicity of effluents and receiving waters (Horning and Weber 1985; Weber et al. 1989; ASTM 1988) and these tests are recommended for hazardous waste site assessment (Warren-Hicks et al. 1989). Fathead minnow short term test methods were also employed in other tests such as sediment and ambient water quality as cited below.

Estuarine and freshwater species were used to identify toxic ambient areas in the Chesapeake Bay watershed (Hall et

al. 1992). Fish larval survival growth tests were conducted in the ambient water transported to the laboratory with sheepshead minnow, grass shrimp, and fathead minnow. *Ceriodaphnia dubia* survival and reproduction test and copepod, *Eurytemora affinis* life cycle tests were also performed. In the multispecies test different species displayed varying sensitivity to different types of contaminants in the water. Overall the results indicated that selected ambient areas of the Chesapeake Bay watershed are toxic, based on various biological indicators.

Toxicity identification evaluation studies were made with various aqueous fractions to determine the toxicity of Illinois River bulk sediment (Schubauer-Berigan and Ankley 1991). Toxicity tests with *Ceriodaphnia dubia* , fathead minnow, *Hyatella azteca*, and *Lumbricus variegatus* were conducted. Both sediment and pore water were toxic to all species. Fathead minnow sensitivity to the pore water was similar to *Ceriodaphnia dubia*. The elutriate was only toxic to fathead minnow. Toxicity test procedures indicated that pore water contained ammonia, metals, and nonpolar organics. Pore water was identified as a major component of the sediment contaminants responsible for toxicity.

The fathead minnow test method has some limitations. Some test samples are turbid and/or colored and filtration may be required for conducting the tests. Sample filtration may remove suspended particles and can reduce toxicity. Some samples are high in biochemical demand and low in oxygen. Aeration can cause volatilization of organics and

modify the toxicity of the sample.

Rice

Rice (*Oryza sativa* L.) is economically important as food and forage and ecologically important in terms of family size, distribution, and abundance. It is one of the recommended cereal crop by the Organization for Economic Cooperation and Development (OECD 1984) for ecotoxicity testing. It is also ecologically relevant to aquatic environments because its habitat is typically in wetlands or shallow water. Rice is a promising species for toxicity testing based on long shelf-life, high germination rate and sensitivity to toxicity. Rice had shown sensitivity to wide variety of chemicals such as heavy metals, organic pollutants, effluents, air pollutants, UV radiation, and air pollutants as mentioned in the following studies.

Mercury in toxic concentrations had been reported to interfere with growth and cause marked inhibition of alpha-amylase and ribonuclease in germinating rice seeds (Mukherji and Ganguly 1974). In another study when rice seeds were germinated in 6.10^{-4} M concentration of mercuric chloride rice growth was inhibited and three fold increase in Indoleacetic acid (IAA) oxidase was observed when compared to the control (Mukherji and Nag 1976). Lidon and Henriques (1992) determined the threshold concentration of copper and its effects on Mn and Fe concentrations in rice plant tissues by growing rice plants hydroponically. The root length of rice was maximum for the 0.002 and 0.01 mg/L Cu treatment. At higher copper treatment, root length was inhibited. Mn and Fe concentrations in the shoot decreased

at Cu concentration above 0.05 mg/L and Fe concentration in the root decreased at Cu concentration above 0.05 mg/L. The estimated threshold toxic concentration was 35.1 ug/g. Wang (1991) demonstrated ammonia toxicity to rice by exposing rice seedlings to un-ionized ammonia. Un-ionized ammonia-N at the rate of 8.85 mg/L inhibited rice seedling growth by 27% in a static renewal method. Toxic effects of metals such as arsenite and arsenate on rice root elongation were compared under various levels of phosphate application (Tsutsumi 1982). The toxicity of arsenite was almost independent of added phosphate whereas that of arsenate was antagonistically reduced by phosphate application. MSMA (Monosodium methanearsonate) is a cost-effective herbicide widely used as a postemergent broadcast to cotton. A study was conducted to simulate the drift of MSMA onto rice fields by foliar application of MSMA (Wauchope et al. 1982). Arsenic residues in the rice foliage increased upto 21 ppm which was above the background concentration (1.5 ppm) and also an exponential increase in the arsenic concentration in the grain was observed.

When lettuce and rice were exposed to pretreated industrial waters the results of lettuce and rice root elongation tests indicated that pretreated industrial samples were more toxic to rice than duckweed (Wang 1990). The seed germination method was employed to test phytotoxicity of an pretreated effluent sample collected from an acid bath of a metal engraving plant to ten plant species (Wang and Keturi 1990). Among nine species tested,

rice was the most sensitive, with an IC50 (inhibition concentration) value of 12% effluent concentration. When rice seedlings were exposed to effluents released from pulp and paper industries at different exposure times, the longer exposure (24 hrs) produced severe adverse effects on germination, growth, chlorophyll concentation, carbohydrate and protein contents (Misra and Behera 1990). The protein content was the most sensitive macromolecule affected. Root elongation of rice plants was strongly inhibited when rice was grown in canal water drained from tropical deep peat area surrounded by woods in Thailand which contained a high concentration of phenolic compounds, low pH, and a low Cu concentration (Tadano et al. 1992)

The physiological and biochemical effects of salinity on rice plant were studied in salt tolerant and salt sensitive rice varieties (Wang et al. 1988). It was found that the drastic reduction of net photosynthetic rate caused by salinity in susceptible variety resulted in growth reduction. The quantity of nucleic acids were reduced sharply with prolonged salinity treatment.

Rice plants were exposed to 0.25 ppm SO_2 and 0.25 ppm O_3 and a mixture of both gases i.e. 0.25 ppm SO_2 + 0.25 ppm O_3 (Kats et al. 1985). An increase in electrolyte loss was observed in leaf strips of rice when exposed to ozone alone and was least affected by sulfur dioxide exposure. Rice plants also showed synergistic response to pollutant mixture. In another study exposure to 0.1 ppm O_3 reduced the dry weight of the whole rice plants by 50% after 5 to 6

weeks exposure (Nouchi et al. 1991). In a field study when rice plants were grown near to a fertilizer plant grain yield was reduced and total sulfur and fluoride contents in the rice leaves were higher for plants grown in the polluted environment than in the rice plants grown distant from the fertilizer plant (Anbhazhagan and Bhagwat 1991).

Enhanced ultraviolet-B (UV-B, 280-320 nm) radiation, which could be caused by stratospheric O_3 depletion, has been demonstrated to profoundly affect plants. When rice (*Oryza sativa* L.) cultivars were exposed to UV-B radiation for four weeks plant height, leaf area, dry weight, net assimilation rate were significantly reduced (Dai et al. 1992).

Rice plants can withstand to some extent adverse soil conditions such as flooded soil and anoxic environment. Most plants are sensitive to anoxic condition and acidic soil. In those circumstances rice adaptability may underestimate environmental toxicity.

Microtox®

Many enzyme, bacterial and algal tests have been developed for monitoring or screening of toxicant/genotoxicant effects in effluents, waters and sediments (Bitton and Dutka 1986). The majority of these tests are rapid, simple, and reproducible. The tests are inexpensive and require little space and time as compared to fish and *Cladoceran* tests (Dutka and Bitton 1986). The bioassay is based on the measurement of the activity of a luminescent bacteria (*Photobacterium phosphoreum*) which emits light under normal metabolic conditions. Any simulation or inhibition of metabolism affects the intensity of the light output. By accurately measuring the light output in control and toxicant-treated samples, EC-50 values (the concentration of toxicants causing 50% reduction in light emission) can be determined. Microtox® is a standardized test method used in environmental assessment of hazardous waste sites (Warren-Hicks et al. 1989)

The first commercial toxicity test using luminescent bacteria was described by Bulich (1979). This bacterial system was unique in that the test organisms were hydrated, freeze-dried preparations. An important characteristic of any bioassay method is the precision or reproducibility of the test method. The quantitation of precision for the Microtox® bioassay was earlier described by Greene and Bulich in 1981 (personal communication). A study in which sodium pentachlorophenate was used as a toxicant by Greene and Bulich showed 5 and 15-min coefficient of variations

were +/- 11 and +/- 12% respectively, for 30 determination. The Duluth Environmental research Lab tested 68 chemicals using the Microtox® system (Curtis et al. 1982). Seven of these chemicals were tested in duplicate and one in triplicate. The replicates deviated from the mean EC50 values by about 10%. Canadian water quality scientists published a comparative study which included the Microtox® system (Qureshi et al. 1982). Pure chemicals and complex effluents were tested with *Daphnia*, rainbow trout, *Spirillum volutans* (bacterial inhibition test), and Microtox®. The Microtox® reproducibility was reported to be better than the other test methods, which ranged from +/- 13 to +/- 26%. Microtox® was compared with fish and *Daphnia* to assess wastewater treatment effectiveness (Casseri et al. 1983). The authors reported the Microtox® data which ranged from +/- 5 to 10% to be very reproducible not only for duplicated tests, but also for testing conducted at different times on split samples.

A general advantage of bacterial toxicity test methods is getting the response of a large population of organisms being measured, compared to the relatively small populations of higher life forms. The advantage not only contributes to improved precision, but to resolution as well. Strosher et al. (1980) evaluated the toxicity of numerous components of oil well drilling fluids. He reported that the Microtox® test reproducibility quantitated small differences in toxicity, which were beyond the resolving ability of rainbow trout test.

The most commonly used acute bioassay to assess toxicity of aquatic samples is the 24-h to 96-h LC50 test (Peltier and Weber 1985). Several studies which have compared the Microtox® bioluminescent assay with fish toxicity test have been reported. Lebsack et al. (1981) tested fossil fuel process waters with 5-min Microtox® test with 96-hr fish lethality test. Using Microtox® and a 96-hr flow-through rainbow trout test, 15 different samples were tested. Microtox® EC50 data were plotted against the rainbow trout LC50 data. A linear regression analysis of the data indicated a correlation coefficient (r) of 0.82. Toxicity of 68 organic chemicals to fathead minnows and the Microtox® test was evaluated by Duluth Environmental Research Laboratory (Curtis et al. 1982). LC50 values from 96-hr flow-through fish test were compared with Microtox® 5-min EC50 values. When the data for all 68 chemicals were compared, the R^2 value was 0.72. De Zwart and Slooff (1983) quantitated the relative sensitivity of Microtox®, *D.magna*, and *D.pulex* for 18 pure compounds. On the average, *D.magna* was 2.5 times and *D.pulex* 3.5 times more sensitive than the Microtox®. These authors compared Microtox® 5-min EC10 data with least concentration causing toxic effects on *Daphnia*. When Qureshi et al. (1982) compared 48-hr *D.magna* LC50 data with Microtox® 5-min EC50 data from 11 chemicals, the *Daphnia* were more sensitive for 7 of the compounds. The correlation coefficient for the data was calculated at 0.67. For nine complex effluents tested with both methods, the results were quite similar with an r value of 0.8. Casseri

et al. (1983) tested leachates and treated and untreated phenolic wastewater samples using *Daphnia* and Microtox®, and he found good agreement between both test methods. All the samples tested, displayed EC50 or LC50 values of less than 5%. The correlation coefficient for the data was 0.9.

In addition to effluent monitoring different Microtox® applications have been developed. Microtox® was used to identify the most toxic fractions from petrochemical process water (Peake and Maclean 1982; Delistraty 1984). The toxic fraction was further analyzed in order to identify the toxic compounds. Neiheisel et al. (1983) described the use of Microtox® to quantitate toxicity reduction through a conventional wastewater treatment system. Mathews and Bulich (1984) devised and field tested a toxicity reduction test system to predict land treatability of hazardous organic wastes. Microtox® was used to quantitate the optimum land-loading rates of solid organic waste materials. Too high a loading rate inhibited normal biodegradation of waste material and risked the leaching of toxicants into groundwater.

Microtox® assay is utilized for simple rapid and relatively inexpensive aquatic toxicity tests to provide data and results that can be used as an indicator toxicity measure for conventional tests such as acute lethality rainbow trout and daphnid assays (Blaise et al. 1985). The Microtox® assay procedure required the addition of 2 percent sodium chloride to the test sample because of the marine origin of the luminescent bacterium (*Photobacterium*

phosphoreum). Such a modification of the test sample, particularly from freshwater environments with sodium chloride, might be considered as reducing the sample integrity. Likewise, the pH of the Microtox® diluent can affect the ionization, solubility, or volatility of a toxicant. The color of certain industrial effluents could produce a problem in the determination of a representative Microtox® response and EC50 values. In such instances, colored substances in an effluent would affect or mask the light output from the luminescent bacteria. Turbidity or suspended solids in a sample or water hardness may also affect the light output measurements in the Microtox® test.

CHAPTER IV

MATERIALS AND METHODS

This chapter presents description of the study site and soil sampling method and methods of soil sample preparation for a battery of toxicity tests. The toxicity test methods include water flea (*Ceriodaphnia dubia*) survival and reproduction, fathead minnow (*Pimphales promelas*) survival and growth, rice (*Oryza sativa* L.) seed germination, rice root elongation, and Microtox®. This battery of test methods were applied to soils and/or extracts of soil for the purpose of measuring short-term acute and chronic effects. In addition to the toxicity test methods, protein alteration in rice plants was determined by methods such as rice seedling growth and proteins analysis which includes the extraction and quantification of proteins, and separation of plant proteins by gel electrophoresis. Finally the method used to analyze the toxicity data is also included.

Study Site

The study site was an old oil refinery abandoned by Oklahoma Refining Company (ORC) located in Cyril, at Caddo county, Oklahoma. The refinery site covered approximately 63 ha which included the main processing plant facility in addition to an array of unlined earthen ponds and storage

pits, and 3.4 ha soil farm waste treatment facility. Refining crude oil into petroleum products for distribution had been performed at Cyril, Oklahoma since early 1920's. This practice had produced considerable quantities of waste materials requiring treatment, storage, and disposal. From 1920 to 1984 process waste were placed in over 50 impoundments, many unlined. Also, some process waste was applied to the soil and treated in a land-farming operation. Preliminary sampling inspection reports in 1986 and 1987 indicated that the soil and ground water at the site were contaminated with petroleum-related organic compounds (including benzene, toluene, xylene, and phenols), heavy metals (including lead, chromium, arsenic, nickel, beryllium, and cobalt), and also acid and caustic waste. Discharges from the site have been observed in Gladys Creek which borders the site (USEPA, March 29 1990, Fed. Reg. 55 161).

Soil Sampling

Soil samples were collected from predefined grids covering known areas of contamination (Table 1 & Figure 1) All 6 grids (3-5 m^2)were ecologically similar to each other with respect to vegetative structure and composition.

Preliminary toxicity tests were conducted in December 1991. Soil samples were collected in the three contaminated Grids 2, 3, 4 and in one of the reference site Grid 1. Soil sampling was conducted in six enclosures (mesocosm areas,12 sq.feet) for toxicity tests in May 1992.

Table 1: Cyril Refinery, Oklahoma, Sampling Site Locations, and Site Description

Site	Site Location	Site description
Grid 1	North side and upstream and upslope of the Refinery.	Reference site. Uncultivated land and Refinery property.
Grid 2	Within the Refinery. Asphalt drum storage area.	Site of storage pits filled with asphalt wastes.
Grid 3	Within the Refinery. Area surrounding the oil/sludge traps.	Area with wastes disposed in unlined impoundments.
Grid 4	Within the Refinery. Soil Farm area.	Land treatment area used for processing sediments from oil/ sludge traps.
Grid 5	Private property, 1 mile southwest of the refinery.	Reference site, Farm land used for cultivation.
Grid 6	Private property, 4 miles east of the Refinery.	Reference site. uncultivated land.
Enclosure 1	Placed in Grid 1.	Reference site. Refinery property, uncultivated land.
Enclosure 2	Within the Refinery, placed in leaded gasoline tank area.	Site of leaded gasoline storage tank that was demolished due to continuous leakage and the soil is soaked with gasoline.
Enclosure 3	Within the Refinery, placed near API separator.	Phytotoxic site.
Enclosure 4	Within the Refinery, placed in the land farm area.	Area used for processing sediments from the oil settlement ponds.
Enclosure 5	Adjacent to the southern border of the Refinery.	Reference site. Property owned by town of Cyril, uncultivated land.
Enclosure 6	Adjacent to Enclosure 5.	Reference site. Property owned by town of Cyril, uncultivated land.

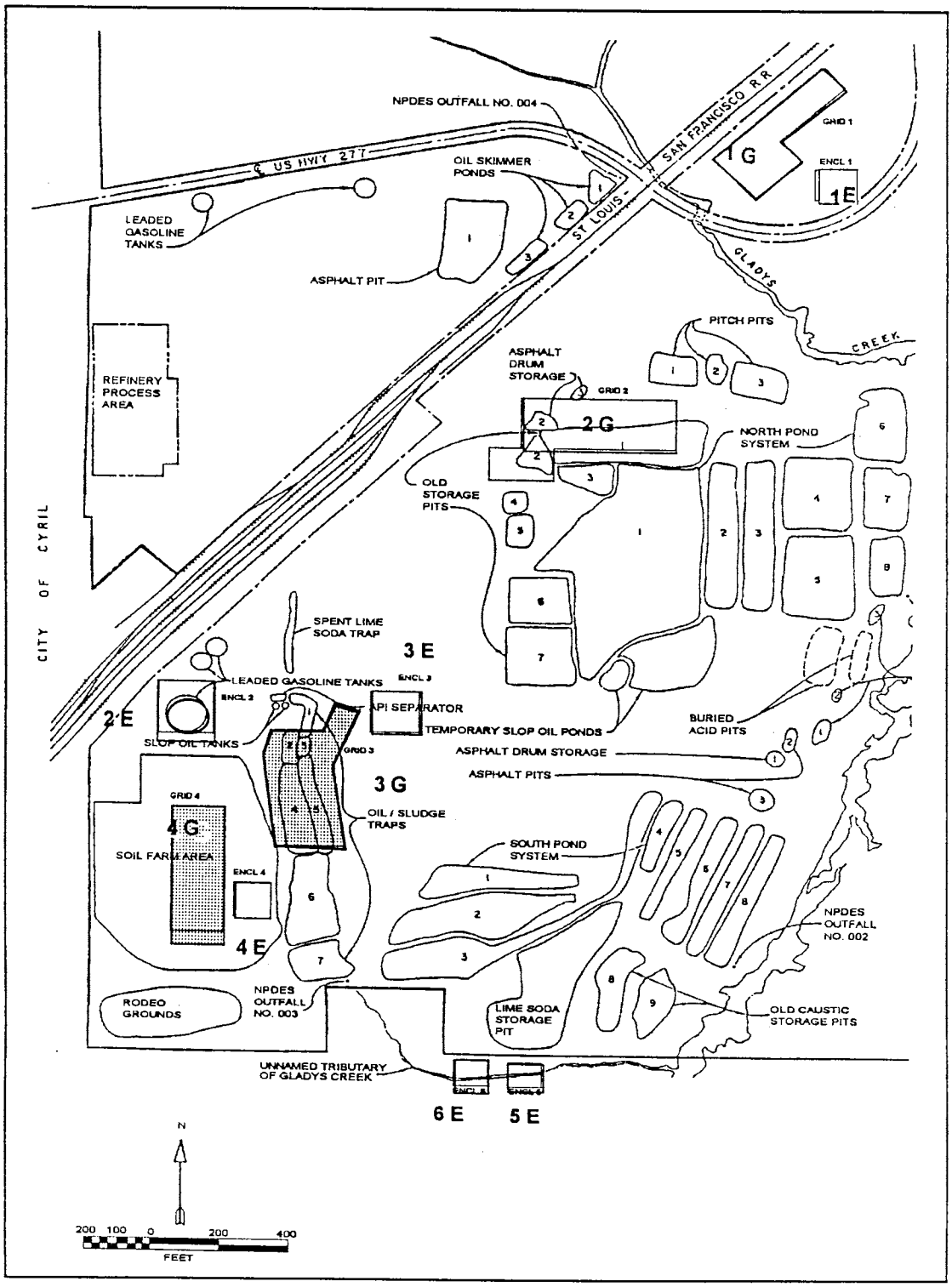

Figure 1: Map of Cyril Refinery, Oklahoma and Sampling Areas.

Soil samples were collected during April 1992 in the Grids, during May 1992 in the Enclosures, and during January 1993 in both the Grids and Enclosures. About 10-15 surface soil samples were taken from each area with a shovel. Soil samples within each area were composited and aliquots were transported in sealed buckets to the lab and stored at 4 degree celsius.

Experimental Subjects and Handling

Both *Ceriodaphnia dubia* and fathead minnow cultures were maintained in the Water Quality Research laboratory, Oklahoma State University. Rice seeds were obtained from Rice Research of USDA in Beaumont, Texas. Seeds were graded for seed germination and root elongation tests.

Methods of Sample Preparation

Soil from each area were homogenized, air dried and grounded for seed germination test. Aqueous soil extracts were made according to Dredged Material and Testing Manual US-ACE method (US-ACE 1991). Aqueous soil extracts were used for toxicity assays with *Ceriodaphnia dubia*, fathead minnow and rice. For *Ceriodaphnia* and fathead minnow tests soil extracts were made with hard reconstituted water and for rice soil extracts were made with deionized water and for Microtox® soil extracts were made with reagent grade water.

Soil Extraction Procedure

Soil sample and water were combined in a ratio of 1:4 on a volume basis at room temperature. This was accomplished by volumetric displacement. The mixture was stirred in the rotary extractor for 18 hrs. After 18 hrs the mixture was allowed to settle for 1 hr. The supernatant was clarified by further centrifuging and filtering.

The battery of toxicity tests used include:

1. *Ceriodaphnia* survival and reproduction
2. Fathead minnow larval survival and growth
3. Rice seed germination
4. Rice root elongation
5. Microtox®

7-day Ceriodaphnia Survival and Reproduction Test

The test was performed according to the effluent toxicity test methods described by Weber et al. (1989). The tests were initiated with <24 h old neonates, a neonate per container, and 10 replicate containers per treatment level (Table 2). The organisms were exposed to 100% aqueous soil extracts and compared with hard reconstituted water control. Dilutions (0.5 factor) were used for toxic soil. The test was conducted at 25 degrees celsius (16:8, light:dark). Dissoved oxygen and pH were measured at the beginning and end of each 24-h exposure period. Conductivity, alkalinity, and hardness were measured in the test samples. After seven days, *Ceriodaphnia* LC50, NOEC were calculated.

Table 2: Summary of Test Conditions for *Ceriodaphnia* (C) Survival and Reproduction and Fathead Minnow (F) Survival and Growth Short-Term Chronic Tests.

Test type:	Static renewal
Temperature (C):	25.0 +/- 1.0 C
Light Quality:	GE daylight fluorescent
Light intensity:	50-100 ft-c
Photoperiod:	16 h light, 8 h dark
Test Chamber size:	(C) 30 ml (F) 600ml
Test solution volume:	(C) 15 ml (F) 250 ml
Renewal of test conc.	Daily
Age of test organisms:	(C) 2-24 h old (F) 24 h old
Number of test organisms per chamber:	(C) 1 (F) 10
Number of replicate chambers per treatment:	(C) 10 (F) 4
Feeding regime:	(C) 0.1 ml food suspension daily (F) 0.1 ml brine shrimp twice/day
Dilution water:	Reconstituted hard water
Test duration:	7 days
Effects measured:	(C) survival and reproduction (F) survival and growth
Test acceptability:	(C) > 80% survival and average of 15 young/female in the control (F)> 80% survival and > 0.25 mg average dry wt in surviving controls

7-day Fathead minnow Survival and Growth Test

The test was performed according to the effluent toxicity test methods described by Weber et al.(1989). The test was initiated with <24 old larvae and dilutions (0.5 factor) were used for toxic soil (Table 2). Dissoved oxygen and pH were measured at the beginning and end of 24-h exposure period. Conductivity, alkalinity, and hardness were measured in the test samples. The tests were conducted at 25 degree celsius and photoperiod (16:8, light:dark). After seven days fathead minnow LC50, NOEC for survival and growth were calculated.

Rice Seed Germination Test

The test was performed according to Modified Neubauer Seed Germination Phytoassay (Thomas and Cline 1985). Seed germination was performed on air dried soil. Rice seeds were soaked with 50 percent Chlorax solution for 20 minutes and rinsed with sterile water four times. Soils (100 g) were placed in a bottom half of a plastic petri dish (100mm wide x 20 mm depth). Twenty seeds were placed on the soil and pushed into the soil with a glass rod (Table 3). The soil was hydrated with deionized water to 80% water holding capacity (Greene et al. 1988). Fifty grams of sterile silica sand (16 mesh) was used to cover the hydrated soil. The petri dish was enclosed in a Ziploc plastic bag (1 gallon size) and placed in a controlled environment at 25 degree celsius and photoperiod (16:8, light:dark) and light

Table 3: Summary of Test Conditions for Rice (*Oryza sativa*) Seed Germination Toxicity Test.

Test type:	Static
Temperature (C):	28.0 +/- 1.0 C
Light quality:	Fluorescent
Light intensity:	4300 +/- 430 lux
Photoperiod:	16 h light, 8 h dark
Test vessel type and size:	The bottom halves of plastic petri dish, 100 mm wide and 20 mm height, placed in Ziploc storage bag (1 gallon)
Test soil mass:	100 g
Test soil moisture content:	80% of water holding capacity
Renewal of test materials:	None
Age of the test organisms:	Seeds
Number of test organisms per chamber:	20
Number of replicate chambers per dilution:	6
Dilution factor:	0.5
Test duration:	10 days
Effects measured:	Germination and shoot growth
Test acceptability:	90% germination in controls

intensity (4300 lux) for 10 days. Soil pH and temperature were recorded at the beginning and end of the test. Germination response, recorded on days 5, 7, and 10 by visual observation through the plastic bag, was considered successful when the seeds emerged 1 cm above the soil. After 10 days shoots were severed above the soil and shoot dry weights were determined. Germination percentage at different exposure period was estimated. LC50 and EC50 were calculated for seed germination and shoot growth.

Rice Root Elongation Test

The test was performed by method described by Greene et al. (1988) (Table 4). Rice seeds were exposed to aqueous soil extracts (100%). Dilutions (0.5) were used for toxic soil. Ten rice seeds were placed between blotters 2"x4" in dimension. One blotter was laid on a flat surface, and using forceps ten seeds were placed embryo upwards in a row with the tip of the seeds laid level with the blotters. The row of seeds was held in place by a narrow strip (1 x 4 cm) of a clean tissue (Kimwipe) placed over them. A second blotter of similar size was placed over the seeds and the first blotter to form a sandwich (Myhill and Konzak 1967). The blotters were pressed together and supported vertically between cross-cut 1/8" wide slots in racks (10 slots/rack). The seed rack was constructed from 4" OD PVC pipe sawed in longitudinal halves of 4" in length. The seed rack was placed in a round bottom glass chamber and 500 ml of soil extract was added to the chamber. The bottom of the

Table 4: Summary of Test Conditions for Rice (*Oryza sativa*) Root Elongation Toxicity Test.

Test type:	Static
Temperature (C):	28.0 +/- 1.0 C
Light quality:	Fluorescent
Photoperiod:	16 h light, 8 h dark
Test vessel type and size:	Seed racks with slots and placed in glass chambers and enclosed in Ziploc storage bags (2 gallon)
Test solution volume:	500 ml
Dilution water:	Deionized water
Renewal of test materials:	None
Age of the test organisms:	Seeds
Number of test organisms per chamber:	50
Number of replicate chambers per dilution:	3
Dilution factor:	0.5
Test duration:	5 days
Effect measured:	Root length and root dry weight

blotters were immersed in the test solution. The glass chambers containing the seed rack were enclosed in a Ziploc plastic bag (2 gallon size) and placed at 25 degree celsius and photoperiod (16:8, light:dark) and light intensity (4300 lux). Three replicates and 50 seeds per replicate were used for each soil extract. Temperature and pH of the test solution were measured at the beginning of the test. After five days the single primary or seminal root length was measured from the point of attachment to the seed and root dry weight was determined. The EC50 was calculated for both root length and dry weight.

Microtox®

The method was used as an additional assessment of the soil toxicity. The assays were performed with a Microtox® model 500 (Microbics Corporation, Carlsbad, CA), according to the instructions described in the Microtox® manual. The Microtox® reagent (freeze-dried lyophilized luminescent bacteria) was added to reconstituted solution. The reconstituted solution was used to hydrate the freeze dried bacteria. It was specifically prepared distilled water, free of organic compounds. The soil samples were extracted with reagent grade distilled water. Soil extracts (100%) were used and for toxic soil dilutions were used for the test. The soil extracts were mixed with Microtox® osmotic adjustment solution containing 22% sodium chloride to adjust osmotic pressure of the sample. The diluent blank containing 2% sodium chloride was used to compare the test

samples. The blank and samples were equilibrated to 15 degree celsius in the incubator wells. Luminescent bacterial suspension was added to blank and samples. Light measurements were recorded after 5 minutes exposure period. EC50 and NOEC values were calculated.

Rice Seedling Root Growth Test

Rice seeds were germinated in blotters and three day old seedlings were transferred to glass jars containing about 450 ml of aqueous soil extracts. One hundred percent soil extracts were used and for toxic soil namely Enclosure 3 site soil (batch 1 collected in 1992) different dilutions were made by mixing with reference Grid 1 soil extract. Seedlings were grown on screens placed on neck of glass jars that were coated with silica beads. Approximately 200 seedlings per treatment and two replicates per treatment were used in this study. The glass jars were enclosed in plastic bags (1 gallon size) and jars were placed at 25 degree celsius, light 4300 lux and photoperiod (16:8, light:dark) for five days. After five days 10 roots were selected randomly from replicate/treatment and the primary or seminal root lengths were measured. The remaining rice roots from each treatment were rinsed with distilled water and stored in plastic bags at -80 degree celsius for protein analysis. For rice leaf protein analysis rice plants were grown in contaminated soils (batch II 1993) for 10 days and the leaves were severed and stored under -80 degree celsius.

Rice Protein Analysis

The rice roots and leaves were homogenized with pre-chilled extraction buffer (0.05M Tris (pH 6.8), PMSF (phenyl methyl sulfonyl fluoride)). The homogenate was filtered through two layers of miracloth. The crude extracts were placed in Eppendorf tubes and centrifuged at 14000 rpm for 10 minutes. The supernatant was drawn into another Eppendorf vial and stored at -20 degree celsius. About 20 ul of sample was transferred to a tube containing 5 ml of Biorad coloring reagent (Biorad Laboratories, Richmond, California) for protein quantification. A standard curve was constructed with four concentration of bovine serum albumin. Absorbance was recorded for the protein samples and compared with the standards. The protein amount was quantified for both the root and leaf samples with known standards.

The proteins from the root and leaf samples were analyzed by SDS-PAGE (sodium dodecylsulfate polyacrylamide gel electrophoresis) as described by Laemmli (1970). Mini gels were prepared with the Bio-rad mini electrophoresis system. The acrylamide matrix for separating the protein consisted of 12.0% acrylamide separating gel. The separating gels were allowed to polymerize prior to the addition of the stacking gel. A 1.5 cm 4.0% acrylamide stacking gel with a 10-well comb was layered over the separating gels. Two gels were casted simultaneously.

The protein samples (75 ul) were dissolved in 4x sample buffer (25 ul) (Hurkman and Tanaka, 1986) and boiled for 2

minutes and centrifuged. Each sample was gently loaded into a well, or lane, of the polymerized stacking gel. Molecular weight standards were loaded into one well. The power supply was connected to the gel was run at about 100 volts for two hours. The gels were run until the dye fronts were about 1 cm from the end of the gel. At completion of the run the gels were removed from the glass plates by gently twisting one of the 1.5 mm teflon spacers, lifting the separated plate from the gel and then gently removed the gel.

The silver staining method was adopted from procedure described by Morrissey (1981). The two gels were immersed in mixture of 50 percent methanol and 30 percent acetic acid for 30 minutes. Thorough agitation was done throughout the procedure. After 30 minutes, gels were transferred to a mixture of 5 percent methanol and 7 percent acetic acid for 30 minutes. Then the gels were fixed with 10 percent glutaraldehyde for 30 minutes. After 30 minutes the gels were rinsed and soaked with distilled water overnight. The next day the gels were soaked in 5 ug/ml dithiothreitol for 30 minutes. Then the gels were treated with 0.1 percent silver nitrate for 30 minutes. After 30 minutes the gels were rinsed with distilled water and soaked in the developer (50 ul of 37 percent formaldehyde in 100 ml 3 percent sodium carbonate) until the desired level of staining was obtained. Staining was stopped by adding 5 ml of 2.3M citric acid directly to the developer and agitating for 10 minutes. The stained gel was washed several times in distilled water over

a 30 minute period. The stained gels were stored in plastic bags.

Data Analysis

LC50 (the concentration lethal to 50% of the organisms within the test period) was calculated for *Ceriodaphnia* survival, fathead minnow larval survival and seed germination toxicity tests that measuring mortality. The EC50 (the concentration reduces the average response of the test organisms by 50% within the test period) was calculated for rice shoot and root growth test. Data from partial chronic toxicity test with fish and *Ceriodaphnia* followed the decision process described by Weber et al. (1989). First the data were tested for normality using the Shapiro-Wilk test and then the data was tested for homogeneity of varience using Barlett's test. A t-test was used to compare the three reference sites (uncontaminated) with the blanks such as hard reconstituted water for the *Ceriodaphnia* toxicity tests, deionized water for the rice root elongation test and sterile sand used as blank for the rice seed germination test. If the reference (uncontaminated) site was found to be significantly different from the blank then the reference site was not used to compare contaminated sites. Either the blank or the reference sites that were not significantly different from blank were used to compare the contaminated areas.

Dunnett's procedure consisting of an analysis of variance (ANOVA) and comparision of the treatment means with

the control means was used. In cases where the number of replicates for each concentration were not equal, a t-test was performed with Bonferroni adjustment for multiple comparision. When the data were not normally distributed Steele's Many-one Rank Test a multiple comparision method was used for comparing several treatments with a control. When the data were not normally distributed and the number of replicate was not same for all concentration the Wilcoxon Rank Sum Test, was used for comparing the treatment with a control. Probit analysis was used to analyze mortality data and to estimate LC50 or EC50. Rice shoot growth and root growth inhibition proportion data were used to calculate EC50 by probit methods.

CHAPTER V

RESULTS

This chapter describes the various responses observed among the test species which were exposed to multiple contaminants in soil collected from the oil refinery. This chapter includes interpretation of the toxicity data observed in this study. The results of the protein analysis were also described and how they were related to toxicity effects were discussed. Finally the conclusions drawn from the toxicity tests and protein biomarker test were included in the chapter.

Toxicity Tests

The battery of toxicity tests indicated that different bioassay organisms exhibited differential sensitivity to the soil contaminants at the Cyril Refinery site. Grid 2 had no significant toxic effect to all organisms tested in this study (Table 5). The three contaminated enclosures show differential responses among the organisms tested (Table 6 and 7). Aqueous extracts of soil collected from the contaminated grids 2,3, and 4 and uncontaminated grids 1,5, & 6 were not toxic to *Ceriodaphnia* survival and reproduction test. Whereas Aqueous elutriates of soil from Enclosure 3

Table 5: Toxicity Test Results from Soil Samples collected from Grids at the Cyril Refinery Site. Percent Inhibition in Elutriate of Soil and Soil (100%).

Toxicity test	Year	G1[r]	G2[c]	G3[c]	G4[c]	G5[r]	G6[r]
Ceriodaphnia survival[e1]	1992	NE	NE	NE	NE	NE	NE
	1993	NE	NE	NE	NE	NE	NE
Ceriodaphnia reproduction[e1]	1992	NE	NE	NE	NE	22*	NE
	1993	NE	NE	NE	NE	42*	47*
Rice seed germination[s2]	1992	NE	NE	NE	NE	20*	14*
	1993	NE	NE	10*	8*	24*	NE
Rice shoot dry weight[s2]	1992	NE	NE	22*	25*	NE	NE
	1993	NE	NE	21*	30*	24*	NE
Rice root length[e3]	1992	NE	NE	NE	NE	NE	NE
	1993	NE	NE	NE	NE	14*	NE
Rice root dry weight[e3]	1992	NE	NE	27*	NE	26*	NE
	1993	NE	NE	NE	NE	NE	NE
Microtox[e4]	1992	19*	NE	24*	20*	58*	29*
	1993	24*	NE	NE	NE	23*	23*

G = Grids
[r]reference grid (uncontaminated)
[c]contaminated grid
[e]soil elutriate 100%
[s]soil 100%
[1]7-day test
[2]10-day test
[3]5-day test
[4]5-minute test
NE no biologically significant effect
*percent inhibition from control

Table 6: Toxicity Test Results from Soil Samples Collected from Enclosures at Cyril Refinery Site, Oklahoma. Percent Inhibition in Elutriates of Soil and Soil (100%).

Toxicity test	Year	E1[r]	E2[c]	E3[c]	E4[c]	E5[r]	E6[r]
Ceriodaphnia survival[e1]	1992	NE	NE	NA	NE	NE	NE
	1993	NE	100*	NA	NE	NE	NE
Ceriodaphnia reproduction[e1]	1992	NE	78*	NA	NE	NE	NE
	1993	NE	100*	NA	NE	29*	17*
Rice seed germination[s2]	1992	NE	NE	44*	NE	20*	14*
	1993	NE	NE	46*	NE	NE	NE
Rice shoot dry weight[s2]	1992	NE	31*	81*	13*	NE	NE
	1993	NE	21*	71*	NE	NE	NE
Rice root length[e3]	1992	NE	24*	91*	NE	NE	NE
	1993	NE	NE	19*	NE	NE	18*
Rice root dry weight[e3]	1992	NE	44*	54*	NE	NE	NE
	1993	NE	27*	25*	NE	NE	NE
Microtox[e4]	1992	25*	NE	NA	NE	37*	29*
	1993	23*	NE	NA	NE	18*	29*

E = Enclosure
[r]reference enclosure (uncontaminated)
[c]contaminated enclosure
[e]soil elutriate 100%
[s]soil 100%
[1]7-day test
[2]10-day test
[3]5-day test
[4]5-minute test
NE no biologically significant effect
NA not applicable
Ceriodaphnia 0% survival in.01% soil elutriate (1992)
0% survival at 20% in soil elutriate (1993)
Microtox 9% light inhibition in 1% soil elutriate (1992)
26% light inhibition in 1% soil elutriate (1993)
* percent inhibition from control

Table 7: LC50 (Confidence Interval), EC50, and NOEC Responses Caused by Soil Contaminants in Enclosure 2 and 3, Cyril Refinery, Oklahoma

Toxicity test	Year	LC50*		EC50*		NOEC*	
		E2	E3	E2	E3	E2	E3
Ceriodaphnia survival[e 1]	1992	NE	NA	-	-	NE	0.005*
	1993	NA	12.85* (7.5-17.7)	-	-	12.5*	5*
Ceriodaphnia reproduction[e1]	1992	-	-	-	-	X	0.001*
	1993	-	-	-	-	12.5*	0.001*
Fathead minnow survival[e1]	1992	NE	0.06* (.05-.08)	-	-	NE	0.01*
	1993	NE	>20*	-	-	NE	5*
Rice seed germination[s2]	1992	NE	X	-	-	-	-
	1993	NE	NA	-	-	-	-
Rice shoot dry weight[s2]	1992	-	-	-	X	-	-
	1993	-	-	-	NA	-	-
Rice root length[e3]	1992	-	-	-	14* (6.7-25.4)	-	-
	1993	-	-	NE	-	-	-
Rice root dry weight[e3]	1992	-	-	X	NA	-	-
	1993	-	-	-	-	-	-
Microtox[e4]	1992	-	-	NE	>5*	NE	0.5*
	1993	-	-	NE	>5*	NE	0.5*

*% soil elutriate or soil
[e]soil elutriate 100%
[s]soil 100%
[1]7-day test
[2]10-day test
[3]5-day test
[4]15-minute test
NE no biological effect
X test not done
NA not applicable, unable to calculate LC50.
E3 1992 showed all or none effect for *Ceriodaphnia* survival.
E2 1993 showed all or none effect for *Ceriodaphnia* survival.
E3 1993 showed all or none response for seed germination.
E3 1993 no increase in shoot or root dry weight in different dilutions.
E3 1992 & 1993 Microtox samples were colored and turbid > 5%.

caused high toxicity to *Ceriodaphnia* and fathead minnow. Enclosure 4 soil had no toxic effect to aquatic organisms tested in this study. Enclosure 3 soil samples collected in 1992 caused 100% mortality of Ceriodaphnia exposed to 0.01% aqueous extract of soil. Though at 0.01% of the soil extract there was 0% survival at the next concentration tested namely 0.005% there was 100% survival and LC50 could not be calculated by probit method (Table 7). The soil extract 0.001% had no significant effect on *Ceriodaphnia* reproduction.

The 1993 Enclosure 3 soil samples were comparatively less toxic than 1992 soil samples and *Ceriodaphnia* survival LC50 had increased to 12.85% in 1993 soil samples. But sublethal effects still persisted at low concentration as seen in partial chronic test and significant effect on *Ceriodaphnia* neonate production was observed in 0.01% soil extract. The no observed effect concentration in *Ceriodaphnia* reproductive test was 0.005%. Enclosure 2 soil though not extremely toxic like Enclosure 3 still had significant effect on organisms tested. *Ceriodaphnia* reproduction was significantly affected by the Enclosure 2 soil extract (Table 6). The Enclosure 2 soil collected in 1992 had suppressed 78% *Ceriodaphnia* reproduction. Enclosure 2 soil collected in 1993 caused 100% mortality in 100% soil extract and 0% *Ceriodaphnia* reproduction in 50% soil extract. The abnormality such as shedding the eggs by the adult was observed in 50% soil elutriate. There was no significant effect on *Ceriodaphnia* reproduction in 12.5%

soil extract. Enclosure 4 soil has no toxic effect on aquatic organisms tested in this study.

Enclosure 3 soil samples collected in 1992 also caused significant toxic effect to fathead minnow larval survival and 100% mortality was observed in 0.1% soil extract. The NOEC for fish survival was 0.01% which was two fold higher than observed in *Ceriodaphnia* survival. But no significant effect upon fish larval growth was observed. The Enclosure 3 soil samples collected in 1993 was similar to *Ceriodaphnia*, i.e., exhibited low toxicity to fish. No significant effect was observed in 5% soil extract. Fish survival was reduced to 25% in 20% soil extract. The soil extract at 10% was a very dark brown color which could not be removed by filtration or centrifugation. The color could not be removed by activated carbon absorption or passage through a nonpolar absorbant such as C18. Toxicity tests with daphnids and fish could not be conducted above 20% soil extract, since the color prevented visual observation of daphnid neonates as well as fish larvae and thus determination of toxic effects. The Enclosure 2 soil extract (100%) had no significant effect on fathead minnow larval survival.

The soil samples collected in 1992 and 1993 from the contaminated Grids 3 and 4 never showed any toxic effect to rice seed germination, but significant effects were observed on rice shoot growth (Table 5). The effect upon shoot growth of Grid 3 and 4 may be due to either a lack of nutrients or possibly contaminants inhibitory on plant

growth. Grid 3 soil collected in 1992 also caused 27% suppression of root growth in 100% soil elutriate.

Initially the seed germination test was conducted for five days and then the test was extended up to 10 days to determine the maximum germination rate and also to determine toxic effects on plant growth. For example the germination was 0% in Enclosure 3 Soil (100%) on day 5, but it increased above 50% on day 10 (Figure 2). When Enclosure 3 soil was diluted with sand, the toxic effect was reduced at low dilutions on day 5 (Figure 3). Though 100% soil showed less than 50% germination on day 10, the next dilution namely 50% soil showed no significant toxic effect and LC50 could not be calculated by probit method. The shoot growth was suppressed to 81% in soil collected in 1992 and shoot growth was suppressed to 71% in soil collected in 1993. Though shoot dry weight decreased 71% in 100% soil collected in 1993 it did not increase at low dilutions (0.5) due to nutrient deficiency in sand and an EC50 could not be calculated.

Enclosure 3 soil collected in 1992 also caused significant effect on rice root growth, i.e., rice root length EC50 value was 14%. In addition some morphological abnormalities in root growth were also observed such as roots appearing stubby and stout. Though root dry weight was suppressed to 54% in the soil extract (100%) it did not increase in different dilutions (0.5 factor) and EC50 could not be calculated. Enclosure 3 soil sample collected in 1993 was less toxic than 1992 soil sample and caused 19%

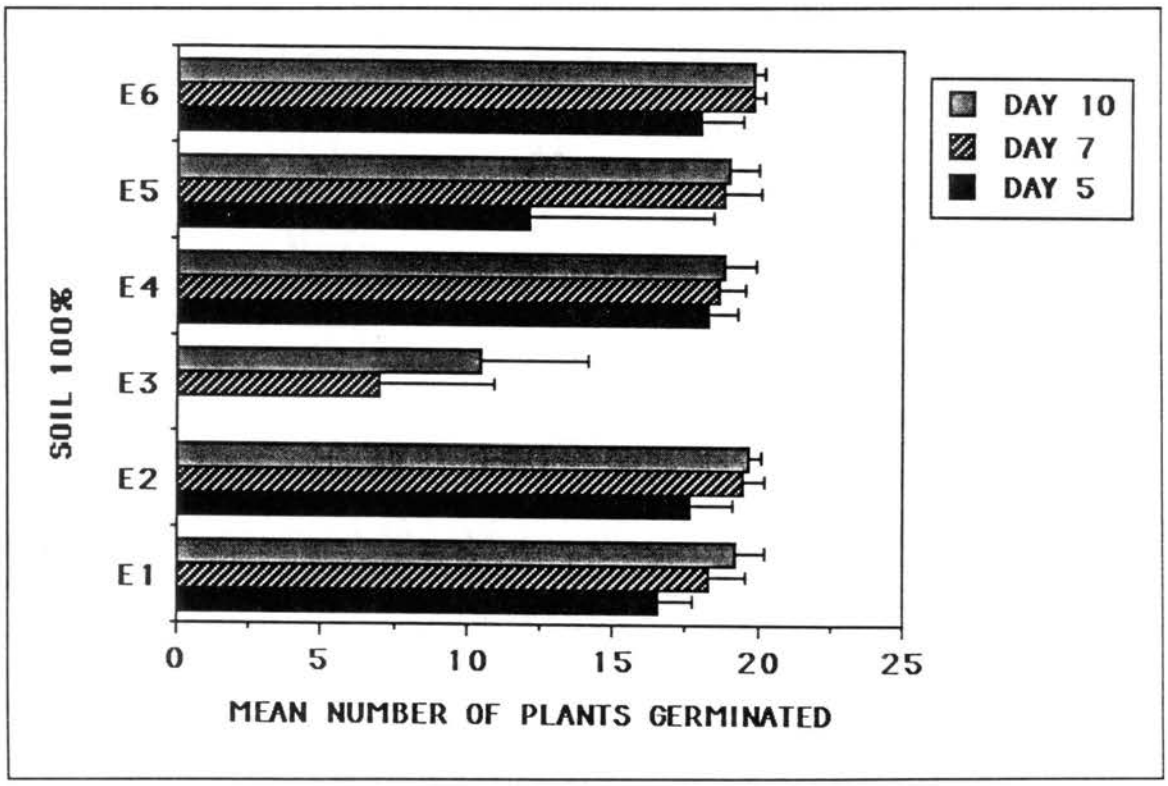

Figure 2: Rice Seed Germination in Soil (100%) Collected from Enclosures at Cyril Refinery, Oklahoma.

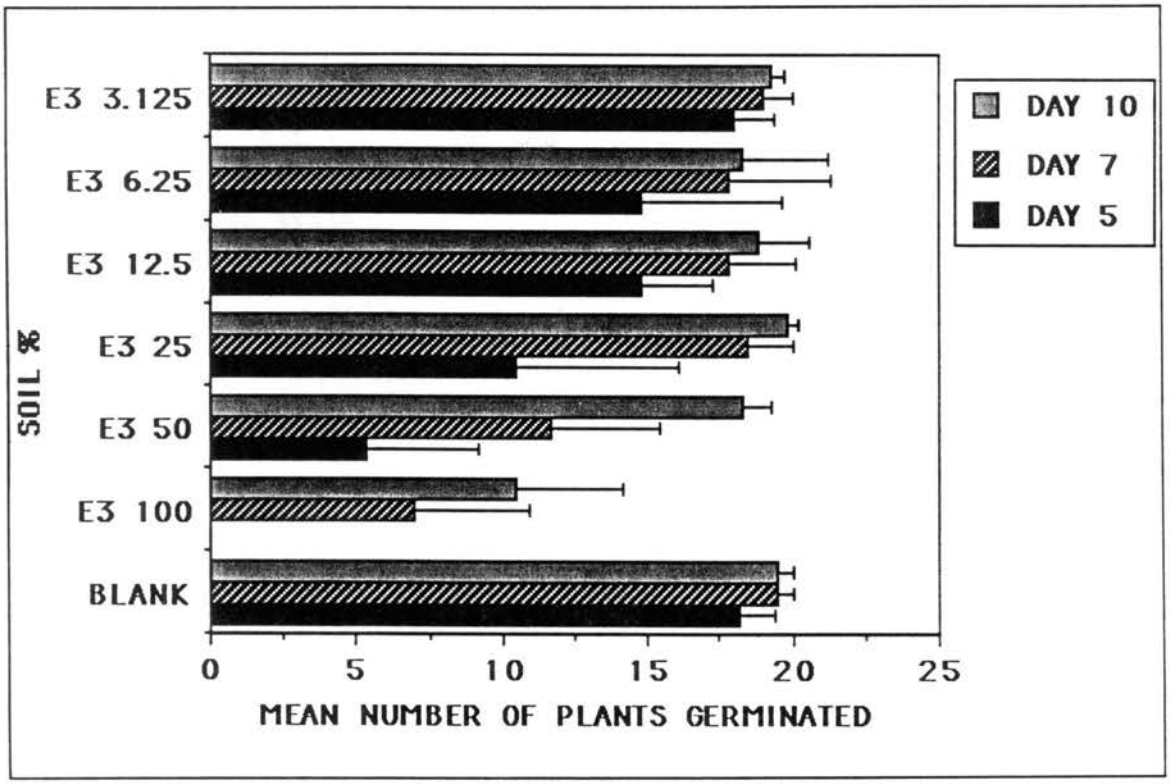

Figure 3: Rice Seed Germination in Soil Collected from Enclosure 3 at Cyril Refinery.

suppression in root length in 100% soil extract and 25% suppression of root dry weight in 100% soil extract.

The soil toxicity in 1993 was reduced relative to the previous year as indicated by acute and partial chronic tests with aquatic animals. But phytotoxic effects persisted in the two year soil samples. The physical chemical properties of leachates of Enclosure 3 soil collected in 1992 indicate presence of multiple nonpolar to semi-polar toxicants that pose a threat to aquatic and terrestrial communities. Though Enclosure 2 soil (100%) did not cause any significant effect on seed germination, rice shoot growth was inhibited to 31% and 21% in soil collected in 1992 and 1993. Enclosure 2 soil collected in 1992 had more effect on root dry weight (44%) than on root length (24%). Enclosure 2 soil collected in 1993 had no significant effect on root length but suppressed 27% root dry weight. Enclosure 4 soil had no toxic effect to organisms tested in this study and the only toxic effect was suppression of rice shoot dry weight to 13% in soil collected in 1993.

When compared to *Ceriodaphnia* and fish bioassays Microtox® was found to be least sensitive to the oil refinery soil (Table 5 and 6). Though soil collected from contaminated Grids 3 and 4 in 1992 diminished the phosphoresence to 20 and 24% in 100% soil extract the soil samples collected in 1993 did not cause any significant effect. Enclosure 3 soil collected in 1992 and 1993 caused 9 and 26% light inhibition in 1% soil extract and no

significant effect was observed in 0.5% soil extract. Above 5% the Enclosure 3 soil extracts were colored and turbid and prohibiting calculation of an EC50. The reference soil extracts were also colored by dissolved substances which could not be removed by sequential centrifugation or filtration with 37 microns pore size pads and therefore the reference soils also caused light diminution when compared to Microtox diluent control (Table 5 and 6). In contrast contaminated soil extracts were colorless. Microtox® diluent control was used to compare the contaminated soil elutriates.

The bioassay results indicate that selected sites at the refinery have contaminants that can pose threat to both aquatic and terrestrial communities. Enclosure 3 seems to be the most toxic site among the selected sites. Contaminants seems to be concentrated to produce both lethal and sublethal effects. The aqueous soil extracts would preferentially dissolve polar and semipolar contaminants but might not adequetly solubulize non polar organics and the process of centrifugation and filtering might have removed suspended particles that could be more toxic. The contaminants could be both toxic polar organics and toxic inorganics in the aqueous soil elutriates that were toxic to aquatic animals and plants tested in this study. Seed germination and shoot growth suppression could be due to presence of phytotoxic chemicals as well as due to nutrients deficiencies in the soil. As the plants had direct contact with the soil they were exposed to more toxic pollutants

than aquatic animals tested in this study. Yet in this study aquatic organisms tested had shown more sensitivity than rice plants. Thomas et al. (1986) and Miller et al. (1985) had reported in earlier studies that different sensitivity exhibited among the test species was useful for ranking the hazardous waste sites for cleanup operations. Ceriodaphnia was the most sensitive species of all the bioassay organisms tested in this study.

The bioassays conducted in the two year study had shown that some of the selected sites had exhibited more toxicity than the previous year. The 1993 soil samples caused 100% *Ceriodaphnia* mortality whereas the 1992 soil samples had no significant effect on *Ceriodaphnia* survival. Soil samples collected in 1993 had a noxious odor and more gasoline was concentrated in the soil elutriates than observed in the previous year. For example Enclosure 2 soil samples collected in 1993 contained visibly greater quantities of gasoline and were more toxic to *Ceriodaphnia* than the soil samples collected in 1992. In contrast the 1993 Enclosure 3 soil was less toxic to aquatic organisms than the previuos year. These temporal variations may have been due to climatic variability. In 1993 soil sampling in January was followed by heavy rains in the area which could have shifted the contaminants. The contaminants in the soil samples also possibily differed due to random sampling method.

In this study the Enclosure 3 soil posed a problem. Enclosure 3 soil was sticky and hard to mix and the aqueous elutriate was turbid and highly colored. The elutriate was

so highly colored it could not be tested at > 5% solution volume diluted with control water. Rice germination test had an advantage having a direct contact with soil and seeds had more exposure to almost all contaminants in soil than the toxicity tests with aquatic animals using elutriates of soil. But soil may lose volatile organics when air dried. Though the Enclosure 3 soil elutriate was turbid and unclear root elongation test could still be performed to determine the toxicity of soil elutriate (100%). As the enclosure 3 soil collected in 1992 was more toxic than 1993 root growth suppression was more pronounced in soil samples collected in 1992 than in 1993.

In this study three reference sites were used to compare three contaminated sites. Sometimes the reference (uncontaminated) sites exhibited physical and chemical parameters that caused adverse effects to the test battery. When more than one reference site was used reference site suitable for the entire test battery should be employed. A laboratory control can be used for preliminary screening of the reference sites and those reference sites that produce ill effects to the test battery can be eliminated from the study. In this study rice had shown sensitivity to the multiple contaminants present in the refinery site soil and therefore rice seeds could be used in the test battery employed for a hazardous waste site evaluation. As this study provides information on short-term acute effects as well as partial chronic effects, both lethal and "no effect" concentration of the wastes could be used for site

characterization.

Protein Biomarkers

The contaminated Grids 3 and 4 caused significant effect on rice shoot growth when compared to the uncontaminated sites, namely the reference Grids 1 and 6 (Fig.4). Contaminated Grid 2 did not cause any significant toxic effect on shoot growth. Rice shoot growth was inhibited to 21% in Enclosure 2 soil. The most significant effect was caused by Enclosure 3 soil and shoot growth was suppressed to 71% in Enclosure 3 soil.

Root length of the seedlings was greatly inhibited in Enclosure 3 soil collected in 1992 than in Enclosure 3 soil collected in 1993 (Fig.5). Root growth did not increase in dilutions though the 100% soil elutriate was diluted with the uncontaminated Grid 1 soil elutriate up to 25 percent. The elutriate was very toxic so addition of nutrients by mixing reference soil elutriate still did not overcome growth inhibition. The roots also appeared thick and stubby. Whereas root growth was stimulated to 12.5% in the II batch (1993) Enclosure 3 soil elutriate (100%) when compared to the reference Grid 1 (Fig.5). Though the soil elutriate was promoting root growth shoot growth was inhibited in soil to 71 percent. This could be due to the seeds having direct contact to all contaminants present in the soil. The contaminated sites Enclosure 2 and Grid 2 did not cause any significant toxic effects on root growth.

Figure 6 shows the results of one dimensional SDS-

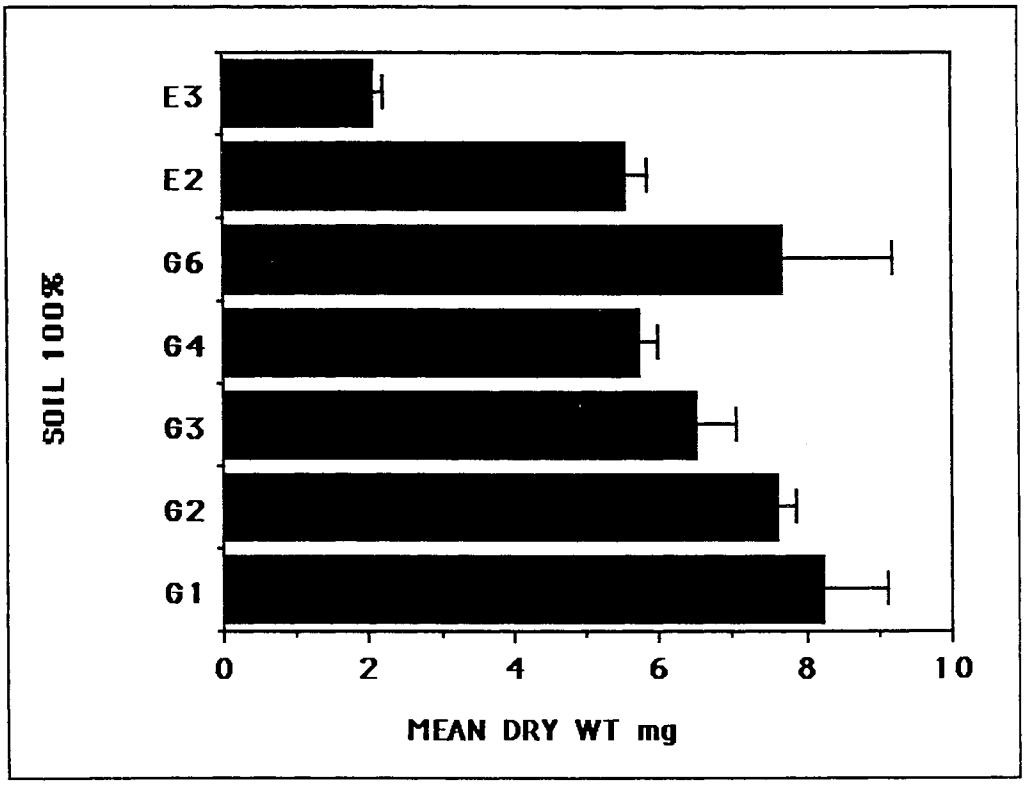

Figure 4: Rice Shoot Growth in Contaminated Soil (Grids and Enclosures, Cyril, Refinery, Oklahoma.

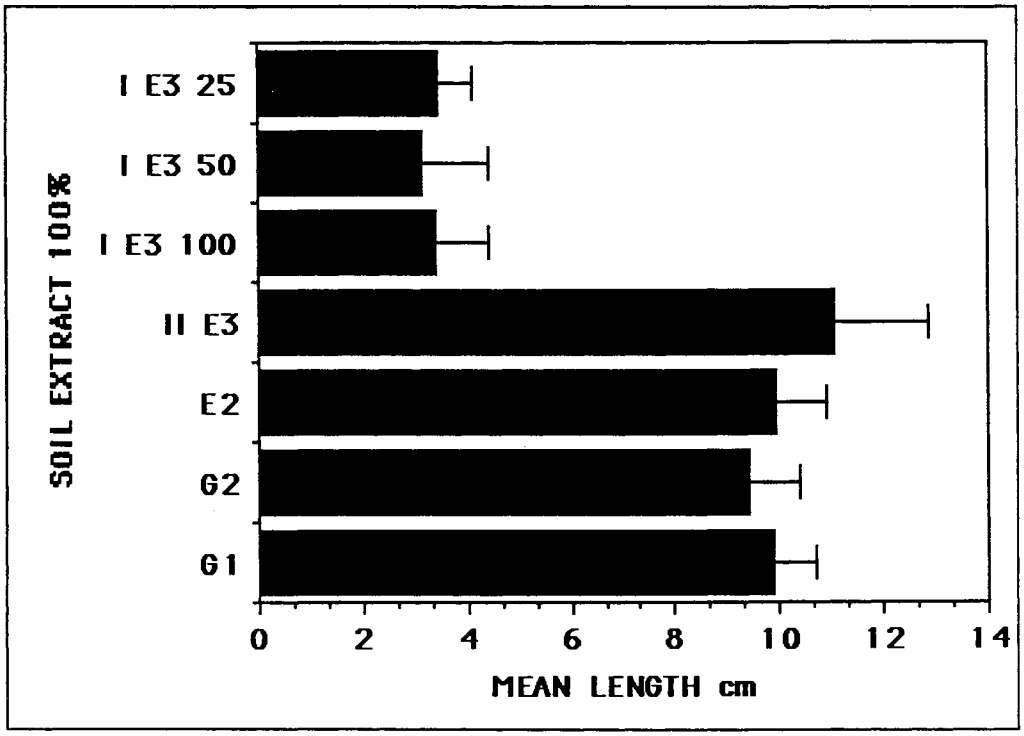

Figure 5: Root Growth of 3-Day Rice Seedling Grown for Five Days in the Aqueous Extracts of Soil (Grids and Enclosures, Cyril Refinery, Oklahoma)

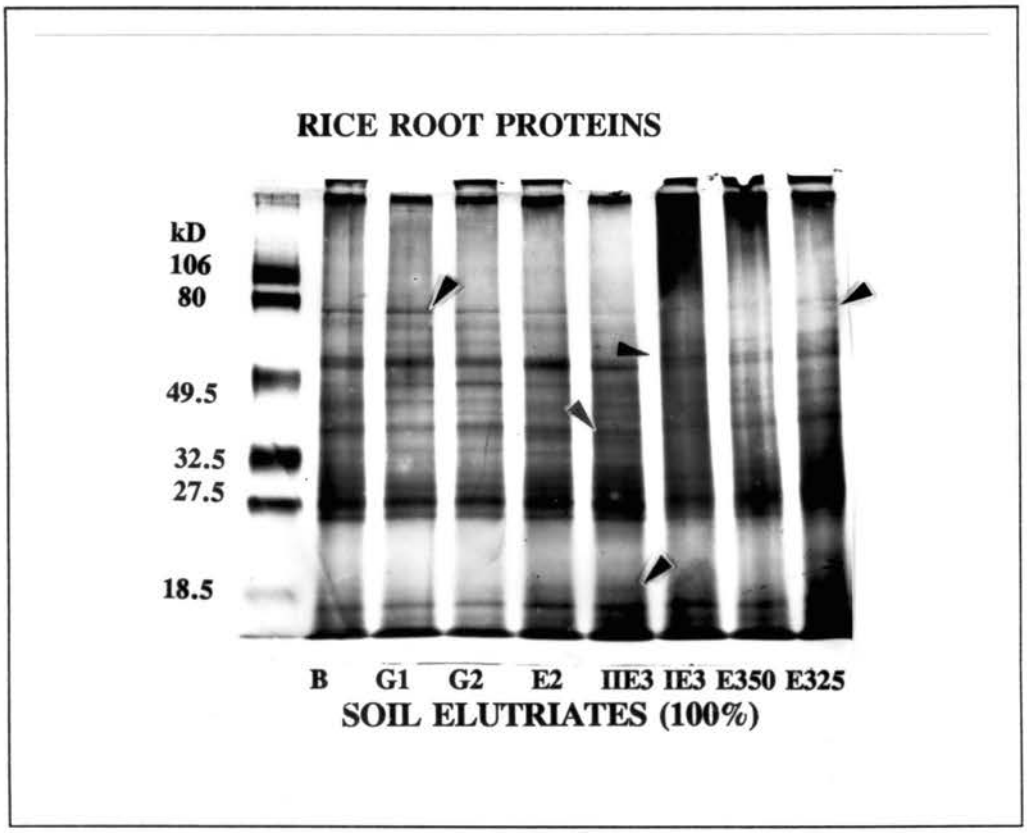

Figure 6: Root Proteins of Rice Plants After Exposure to Aqueous Extracts of Contaminated Soil (Grids and Enclosures, Cyril Refinery, Oklahoma)

polyacrylamide gel electrophoresis of rice root proteins. Marked changes in patterns of protein were observed in seedlings treated with Enclosure 3 batch 1 (1992) soil elutriate. Proteins were almost suppressed in roots treated with 100 percent Enclosure 3 soil elutriate. Polypeptides 74, 70, and 56 KDa were suppressed in roots exposed to I batch Enclosure 3 soil elutriate while they were present in roots exposed to reference Grid 1 soil elutriate and to the blank, deionized water control.

Polypeptides were also induced in roots treated with contaminated soils. Polypeptide 34 KDa was induced in roots exposed to contaminated site soils Grid 2, Enclosure 2, and Enclosure 3 batch 2 (1993). Enclosure 3 batch 2 which had stimulatory effect on root growth had induced polypeptides 34, and 19 KDa in the roots. In this study proteins were induced when both shoot and root growth were inhibited. Induction of proteins also occurred independent of toxic effect. Both roots as well as leaves have changes in protein pattern in plants exposed to contaminated soil elutriates. Polypeptide 98 KDa was suppressed in leaves of the plants exposed to batch 1 Enclosure 3 soil elutriates (Fig.7).

Changes in protein pattern were also observed in leaves of rice plants grown in contaminated soils batch 2 (1993). Polypeptide 94 KDa was highly induced in leaves of plants grown in contaminated soils Grid 3 and Enclosure 3 (Fig.8). Polypeptide 51 KDa was suppressed in leaves exposed to Enclosure 2 and Enclosure 3 site soils when compared to the

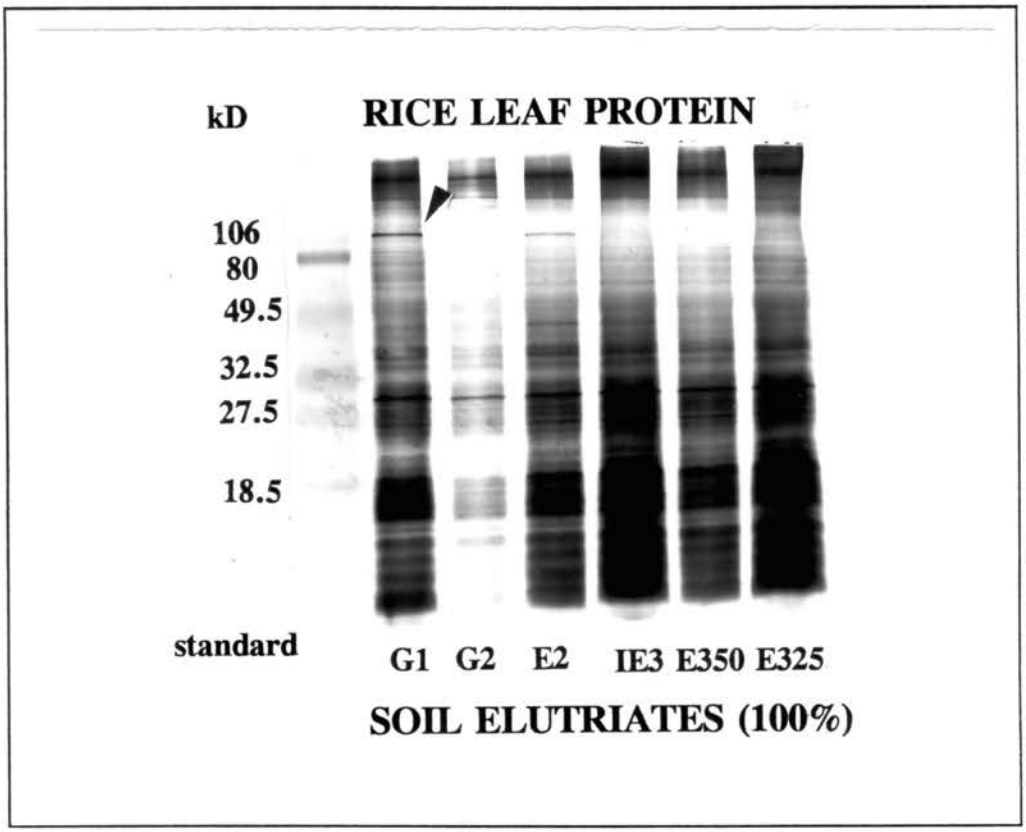

Figure 7: Leaf Proteins of Rice Plants After Exposure to Aqueous Extracts of Contaminated Soil (Grids and Enclosures, Cyril Refinery, Oklahoma.

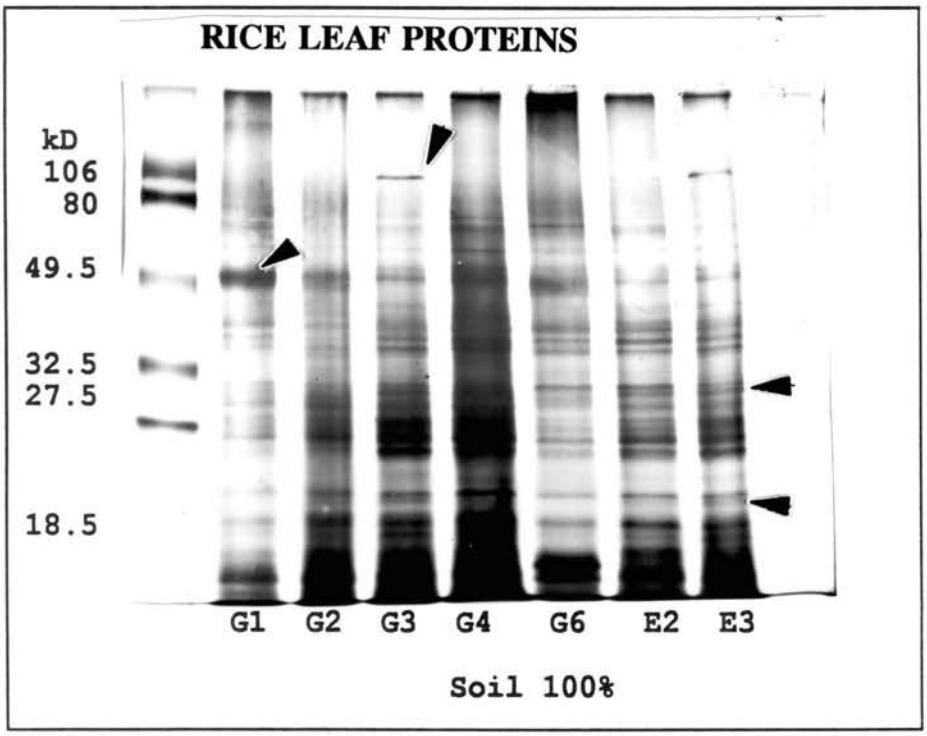

Figure 8: Leaf Proteins of Rice Plants After Exposure to Contaminated Soil (Grids and Enclosures, Cyril Refinery, Oklahoma)

leaves exposed to reference Grids 1 and 6. The leaves of plants exposed to contaminated soils namely Grid 2, Grid 3, Enclosure 2, and Enclosure 3 all had highly induced polypeptides 29, and 21 KDa when compared to reference Grids 1 and 6. Interestingly the proteins in leaves exposed to reference Grid 1 soil and soil elutriate were too different) which may be due to the different media of exposure.

The changes in the patterns of protein synthesis observed in rice plants in this study could be due to the effect of multiple contaminants present in the refinery soil. Polypeptide 94 KDa was highly induced in the rice plants grown in Grid 3 and Enclosure 3 soils which could be due to similar contaminants in both soils. The Grid 2 soil had no significant effect on shoot and root growth but still had induced polypeptides in roots and leaves. Polypeptides were found to be induced even after 10 days of plant growth in the contaminated soil which shows plants tolerance to environmental toxicity. Protein biomarkers will serve as additional tools to determine if adverse effects still exist in the ecosystem in cases where toxicity tests or field tests may fail to show any adverse effects. Protein biomarkers could be employed as biomonitoring tools and for site characterization of hazardous waste sites.

Conclusion

Enclosure 3 soil collected in 1992 was found to be more toxic than other contaminated sites to all organisms tested in this study. Enclosure 3 soil collected in 1993 was less

toxic to aquatic organisms than the previous year. The Enclosure 2 soil collected in 1993 was more toxic to *Ceriodaphnia* survival than the soil collected in the previous year. *Ceriodaphnia* was the most sensitive species of all the bioassay organisms tested in this study. Rice plant shows sensitivity to the multiple contaminants present in this study.

Polypeptides were induced in rice leaves and roots treated with toxic soils. Enclosure 3 batch 1 soil elutriate was toxic and inhibited both shoot growth and root growth and also suppressed polypeptides in roots. Enclosure 3 batch 2 soil inhibited shoot growth but it was found to have stimulatory effect on root growth and induced many polypeptides in root. Polypeptides were induced in rice leaves and roots treated with all contaminated soils. Grid 2 had no toxic effect on shoot and root growth but still induced polypeptides.

The Null Hypothesis number 1 was accepted since there were no differences between uncontaminated sites (Grids and Enclosures) and the contaminated sites (Grids and Enclosures) for toxicity tests such as *Ceriodaphnia* survival and reproduction , fathead minnow larval survival and growth, rice shoot growth, and rice root growth, and Microtox. The Null Hypothesis number 2 was judged to be incorrect for both toxicity tests and protein biomarker studies. The Null hypohesis number 2 was rejected at alpha 0.05 and the alternate hypothesis was accepted. There were significant difference between two of the three

uncontaminated grids namely Grid 1 and 6 and two of the three contaminated grids namely Grid 3 and 4 tested for rice shoot growth, rice root growth, and Microtox. Only *Ceriodaphnia* survival and reproduction tests were not significally different in the contaminated Grids when compared to the uncontaminated Grids. The uncontaminated Enclosures were also significantly different from contaminated Enclosures namely Enclosure 2 and Enclosure 3 for *Ceriodaphnia* survival and reproduction, fathead minnow larval survival, rice seed germination, rice shoot growth, and rice root growth. The rice proteins were also different in the contaminated sites (Grids and Enclosures) when compared to uncontaminated Grids namely Grid 1 and 6.

This two year study indicated that toxic chemicals still existed in the selected areas of the refinery and elicited some acute and chronic adverse effects from the aquatic animals and plants tested in this study. The Co-investigators in this superfund project used the similar contaminated sites for mammalian field studies and observed adverse effects on cotton rats. The above laboratory studies performed on the refinery site soil and other field studies conducted in the similar sites soil at the refinery indicates that the refinery site soil still poses a threat to the ecosystem and further cleaning and monitoring the site is necessary to remove the contaminants and for future use of the refinery.

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APPENDIXES

Appendix A

Table A1: *Ceriodaphnia* Survival and Mean Number of Neonates Produced in Extracts of Soil Collected from Grids, Cyril Refinery (1992).

Soil*	Survival %	Mean number of neonates	Significance at P=0.05
Grid 1 (Reference)	100	15.7	
Grid 2 (contaminated)	100	17.67	
Grid 3 (contaminated)	100	17.1	
Grid 4 (contaminated)	100	17.3	
Grid 5 (Reference)	100	13.2	**
Grid 6 (Reference)	100	18.6	

*soil extracts (100%) compared with reconstituted hard water control.
7-day test
Number of replicates = 10.

Table A2: *Ceriodaphnia* Survival and Mean Number of Neonates Produced in Extracts of Soil Collected from Grids, Cyril Refinery (1993).

Soil*	Survival %	Mean number of neonates	Significance at P=0.05
Grid 1 (Reference)	100	18.3	
Grid 2 (contaminated)	100	20.1	
Grid 3 (contaminated)	100	25.5	
Grid 4 (contaminated)	100	26.3	
Grid 5 (Reference)	100	16.4	**
Grid 6 (Reference)	100	17.9	**

*soil extracts (100%) compared with reconstituted hard water control.
7-day test,
Number of replicates = 10

Table A3: *Ceriodaphnia* Survival and Mean Number of Neonates Produced in Extracts of Soil Collected from Enclosures, Cyril Refinery (1992).

Soil*	Survival %	Mean number of neonates	Significance at P=0.05
Enclosure 1 (Reference)	100	15.7	
Enclosure 2 (contaminated)	100	3.5	**
Enclosure 3 (contaminated)	0		**
Enclosure 4 (contaminated)	100	13.0	
Enclosure 5 (Reference)	100	26.8	
Enclosure 6 (Reference)	100	25.7	

*soil extracts (100%) compared with reconstituted hard water control.
7-day test
Number of replicates = 10

Table A4: *Ceriodaphnia* Survival and Mean Number of Neonates Produced in Extracts of Soil Collected From Enclosures, Cyril Refinery (1993).

Soil*	Survival %	Mean number of neonates	Significance at P=0.05
Enclosure 1 (Reference)	100	18.7	
Enclosure 2 (contaminated)	0		**
Enclosure 3 (contaminated)	0		**
Enclosure 4 (contaminated)	100	23.4	
Enclosure 5 (Reference)	100	21.9	
Enclosure 6 (Reference)	100	25.78	

*soil extracts (100%) compared with reconstituted hard water control.
7-day test
Number of replicates = 10

Table A5: *Ceriodaphnia* Survival and Mean Number of Neonates Produced in Extracts of Soil Collected from Enclosure 2, Cyril Refinery (1992 & 1993).

Soil*	Survival %	Mean number of neonates	Significance at P=0.05
Enclosure 2 (1992)			
	100	3.5	**
Enclosure 2 (1993)			
6.25	100	25.6	
12.5	100	27.2	
25%	100	14.5	**
50%	100	0	**
100%	0		**

*soil extract % compared with reconstituted hard water control.
7-day test
Number of replicates = 10

Table A6: *Ceriodaphnia* Survival and Mean Number of Neonates Produced in Extracts of Soil Collected from Enclosure 3, Cyril Refinery (1992 & 1993).

Soil*	Survival %	Mean number of neonates	Significance at P=0.05
Enclosure 3 (1992)			
0.001%	100	22.6	
0.005%	100	10.6	**
0.01%	0		**
0.1	0		
1.0	0		
Enclosure 3 (1993)			
0.005	100	22.4	**
0.01	100	23.4	
0.05	100	15.4	**
0.1%	100	16.2	**
0.5%	100	14.4	**
1.0%	100	7.1	**
5 %	100	13.78	**
10	100	9.3	**
20	0		

*soil extracts % compared with reconstituted hard water control.
7-day test
Number of replicates = 10

Appendix B

Table B1: Fathead Minnow Larval Survival and Growth in Extracts of Soil Collected from Enclosure 3, Cyril Refinery (1992 & 1993).

Soil*	Survival %	Significance at P=0.05	Dry weight mg	Significance at P=0.05
Enclosure 3 (1992)				
0.01 %	0.97		0.44	
0.05 %	0.66	**	0.57	
0.1 %	0.25	**	0.62	
Enclosure 3 (1993)				
0.01 %	0.95		0.42	
0.05 %	0.92		0.51	
0.1 %	0.82		0.59	
0.5 %	0.91		0.50	
5%	0.97		0.37	
10%	0.94		0.33	
20%	0.76		0.32	

*soil extract % compared with reconstituted hard water control.
7-day test
Number of replicates = 4

Appendix C

Table C1: Rice Seed Germination in Soil Collected From Grids and Enclosures, Cyril Refinery (1992).

Soil*	Germination %		
	Day 5	Day 7	Day 10
Grid 1 (Reference)	73.35	86.5	92.5
Grid 2 (contaminated)	95	97.5	97.5
Grid 3 (contaminated)	80.85	88.35	90.85
Grid 4 (contaminated)	82.5	90.85	93.35
Grid 5 (Reference)	55	75.8	83.3
Grid 6 (Reference)	53.3	75	77.5
Enclosure 1 (Reference)	66.5	89.15	90.0
Enclosure 2 (contaminated)	30.85	70.0	88.35
Enclosure 3 (contaminated)	2.5	27.5	55.0
Enclosure 4 (contaminated)	77.5	88.35	92.5
Enclosure 5 (Reference)	82.5	90.8	91.6
Enclosure 6 (Reference)	88.3	92.5	95

*site soil 100%
Number of replicates = 6

Table C2: Rice Seed Germination in Soil Collected from Grids and Enclosures, Cyril Refinery (1993).

Soil*	Germination %		
	Day 5	Day 7	Day 10
Grid 1 (Reference)	73.35	91.5	93.35
Grid 2 (contaminated)	72.5	91.5	95.0
Grid 3 (contaminated)	73.35	85.85	87.5
Grid 4 (contaminated)	76.5	85.85	89.15
Enclosure 1 (Reference)	90.85	95.85	98.35
Enclosure 2 (contaminated)	88.35	97.5	98.35
Enclosure 3 (contaminated)	0	35	52.5
Enclosure 4 (contaminated)	91.5	93.35	94.15

*site soil 100%
Number of replicates = 6

Table C3: Rice Shoot Growth in Soil Collected from Grids and Enclosures, Cyril Refinery (1992)

Soil*	Dry Weight mg	Significance at P=0.05
Grid 1 (Reference)	7.69	
Grid 2 (contaminated)	7.26	
Grid 3 (contaminated)	5.98	**
Grid 4 (contaminated)	5.75	**
Grid 5 (Reference)	6.27	
Grid 6 (Reference)	7.73	
Batch 2 1992		
Enclosure 1 (Reference)	8.70	
Enclosure 2 (contaminated)	6.03	**
Enclosure 3 (contaminated)	1.64	**
Enclosure 4 (contaminated)	7.60	**
Enclosure 5 (Reference)	9.26	
Enclosure 6 (Reference)	8.63	

*site soil 100%
Number of replicates = 6

Table C4: Rice Shoot Growth in Soil Collected from Grids and Enclosures, Cyril Refinery (1993).

Soil*	Dry weight mg	Significance at P=0.05
Grid 1 (Reference)	8.23	
Grid 2 (contaminated)	7.61	
Grid 3 (contaminated)	6.49	**
Grid 4 (contaminated)	5.73	**
Grid 5 (Reference)	6.27	
Grid 6 (Reference)	7.73	
Enclosure 1 (Reference)	7.03	
Enclosure 2 (contaminated)	5.55	**
Enclosure 3 (contaminated)	2.06	**
Enclosure 4 (contaminated)	7.63	

*site soil 100%
Number of replicates = 6

Appendix D

Table D1: Rice Root Growth (Length) in Extracts of Soil Collected from Grids and Enclosures, Cyril Refinery (1992).

Soil*	Length cm	Significance at P=0.05
Grid 1 (Reference)	7.46	
Grid 2 (contaminated)	8.22	
Grid 3 (contaminated)	6.91	
Grid 4 (contaminated)	7.03	
Grid 5 (Reference)	6.04	**
Grid 6 (Reference)	6.4	
Enclosure 1 (Reference)	7.62	
Enclosure 2 (contaminated)	5.77	**
Enclosure 3 (contaminated)	0.7	**
Enclosure 4 (contaminated)	7.67	
Enclosure 5 (Reference)	7.23	
Enclosure 6 (Reference)	5.97	

*soil extract 100%
Number of replicates = 3
10-day test

Table D2: Rice Root Growth (Dry Weight) in Extracts of Soil Collected from Grids and Enclosures, Cyril Refinery (1992).

Soil*	Dry weight mg	Significance at P=0.05
Grid 1 (Reference)	1.44	
Grid 2 (contaminated)	1.34	
Grid 3 (contaminated)	1.05	**
Grid 4 (contaminated)	1.41	
Grid 5 (Reference)	1.06	**
Grid 6 (Reference)	1.18	
Enclosure 1 (Reference)	1.46	
Enclosure 2 (contaminated)	0.82	**
Enclosure 3 (contaminated)	0.67	**
Enclosure 4 (contaminated)	1.42	
Enclosure 5 (Reference)	1.33	
Enclosure 6 (Reference)	1.08	

*soil extract 100%
Number of replicates = 3
10-day test

Table D3: Rice Root Growth (Length) in Extracts of Soil Collected from Grids and Enclosures, Cyril Refinery (1993).

Soil*	Length cm	Significance at P=0.05
Grid 1 (Reference)	5.85	
Grid 2 (contaminated)	5.61	
Grid 3 (contaminated)	5.94	
Grid 4 (contaminated)	5.23	
Grid 5 (Reference)	4.47	
Grid 6 (Reference)	6.33	
Enclosure 1 (Reference)	5.32	
Enclosure 2 (contaminated)	4.93	
Enclosure 3 (contaminated)	4.71	**
Enclosure 4 (contaminated)	5.17	
Enclosure 5 (Reference)	5.51	
Enclosure 6 (Reference)	4.75	**

*soil extract 100%
Number of replicates = 3

Table D4: Rice Root Growth (Dry Weight) in Extracts of Soil Collected from Grids and Enclosures, Cyril Refinery (1993).

Soil*	Dry weight mg	Significance at P=0.05
Grid 1 (Reference)	1.04	
Grid 2 (contaminated)	1.08	
Grid 3 (contaminated)	1.1	
Grid 4 (contaminated)	1.05	
Grid 5 (Reference)	0.82	
Grid 6 (Reference)	1.11	
Enclosure 1 (Reference)	0.98	
Enclosure 2 (contaminated)	0.82	**
Enclosure 3 (contaminated)	0.84	**
Enclosure 4 (contaminated)	0.99	
Enclosure 5 (Reference)	1.04	
Enclosure 6 (Reference)	0.8	**

*soil extract 100%
Number of replicates = 3

Appendix E

Table E1: Rice Shoot Growth in Soil Collected from Enclosure 3, Cyril Refinery (1993)

Soil %	Dry weight mg	Significance at P=0.05
Encl.1 100 (control)	7.028	
Encl. 3 100	2.063	**
Encl. 3 50	3.947	**
Encl. 3 25	4.943	**
Encl. 3 12.5	4.328	**
Encl. 3 6.25	3.732	**
Encl. 3 3.125	3.947	**

10-day test
Number of replicates = 6

Table E2 - Rice Root Growth in Extracts of Soil Collected from Enclosure 3, Cyril Refinery (1992).

Soil*	Mean length cm	Significance at P=0.05
Enclosure 1 (Reference)	7.62	
Enclosure 3 100%	0.7	**
Enclosure 3 50%	1.62	**
Enclosure 3 25%	2.72	**
Enclosure 3 12.5%	4.1	**
Enclosure 3 6.25%	5.8	**
Enclosure 3 3.125%	5.81	**

*soil extract %
5-day test
Number of replicates = 3

Appendix F

Table F1: Root Growth of 3-day Rice Seedling Grown for Five Days in Extracts of Contaminated Soil, Cyril Refinery.

Soil*	Root length(cm)	Significance at P=.05
Grid 1[a] (Reference)	9.86	
Grid 2[a] (contaminated)	9.43	
Enclosure 2[a] (contaminated)	9.91	
Enclosure 3[a] (contaminated)	11.1	
Enclosure 3[b] 100% (contaminated)	3.40	**
Enclosure 3[b] 50% (contaminated)	3.16	**
Enclosure 3[b] 25% (contaminated)	3.47	**

*soil extract %
[a]1993 soil
[b]1992 soil
5-day test
Number of replicates = 2

Appendix G

Table G1: Microtox Response to Extracts of Contaminated Soil, Cyril Refinery (1992 & 1993).

Soil extract 100%	Light readings %	Significance at p=0.05
Blank (Microtox diluent)	93.5	
Grid 2[a]	111.75	
Grid 3[a]	71.0	**
Grid 4[a]	75.75	**
Enclosure 2[a]	106.25	
Enclosure 3[a] 5%	71.25	**
Enclosure 3[a] 1%	85.0	**
Grid 2[b]	90.3	
Grid 3[b]	95.0	
Grid 4[b]	101.0	
Enclosure 4[b]	91.25	
Blank	94.0	
Grid 5[a]	39.0	**
Grid 6[a]	66.5	**
Enclosure 1[a]	70.5	**
Enclosure 5[a]	59.0	**
Enclosure 6[a]	66.5	**
Grid 1[b]	71.0	**
Enclosure 1[b]	72.5	**
Enclosure 2[b]	98.75	
Enclosure 3[b] 5%	68.75	**

[a]1992 soil extract
[b]1993 soil extract
5-minute test
Number of replicates = 4

Appendix H

Table H1: Rice Growth - Chemical Data of Extracts of Soil and Soil Collected from Cyril Refinery.

Soil	Soil pH (23 C)		Soil elutriate pH (23 C)		Conductivity ms*/cm	
	1992	1993	1992	1993	1992	1993
Grid 1	6.7	6.4	7.4	6.9	0.13	0.13
Grid 2	7.65	7.4	7.8	8.35	0.45	0.10
Grid 3	8.1	6.6	7.6	8.05	0.6	-
Grid 4	7.4	7.7	7.9	8.1	0.43	0.3
Grid 5	6.45	6.25	7.3	7.8	0.67	-
Grid 6	6.5	6.8	7.1	7.4	0.14	-
Encl.1	6.5	6.2	6.5	6.9	0.1	-
Encl.2	8.7	7.5	7.65	8.7	0.66	0.75
Encl.3	9.5	9.1	8.45	-	3.74	2.15
Encl.4	8.15	7.8	7.8	8.45	-	0.6
Encl.5	7.9	8.0	-	8.15	-	-
Encl.6	7.7	7.7	-	8.35	-	-

*millisemens/cm

Appendix I

Table I1: Ceriodaphnia Toxicity Test- Chemical Data of Extracts of Soil Collected from Cyril Refinery (1992 & 1993).

Soil extract 100%	pH	Conductiv. us*/cm	DO (T C)	Alkali.	Hardn.
1992					
Grid 1	7.4	509	-	78	140
Grid 2	8.1	698	-	184	256
Grid 3	8.1	879	7.5 (23)	246	412
Grid 4	8.2	755	8.0 (24)	212	302
Grid 5	7.7	1657	7.6 (23)	90	900
Grid 6	7.3	504	7.4 (23)	62	
174					
Enc. 1	7.9	444	8.0 (24)	61	114
Enc. 2	8.2	961	7.4 (23)	382	190
Enc. 3					
.005%	7.8	572	7.2 (23)	110	172
0.01%	7.9	568	7.3 (24)	132	180
0.05%	7.9	585	7.6 (23)	136	132
0.10%	7.85	581	6.7 (24)	114	184
1.0%	8.0	589	8.0 (23)	112	174
Enc. 4	8.4	818	8.0 (24)	210	220
Enc. 6	8.2	761	8.2 (23)	146	230
1993					
Grid 1	8.1	423	8.0 (22)	60	108
Grid 2	8.3	499	8.0 (22)	82	172
Grid 3	8.1	752	8.8 (22)	224	286
Grid 5	8.1	1345	8.5 (23)	78	574
Grid 6	8.0	494	8.2 (24)	76	90
Enc. 1	7.9	490	8.3 (23)	70	126
Enc. 2					
50%	8.0	647	6.4 (24)	198	254
25%	8.1	578	6.6 (25)	170	178
Enc. 4	8.4	649	7.3 (24)	184	268
Enc. 5	8.4	583	8.4 (24)	158	290
Enc. 6	8.4	548	9.0 (24)	168	296

*microsemens/cm

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