SUPERCRITICAL CARBON DIOXIDE VS. AQUEOUS EXTRACTION OF CONTAMINATED SOILS AS A PREPARATIVE PROCEDURE FOR ACUTE TOXICITY TESTING

By

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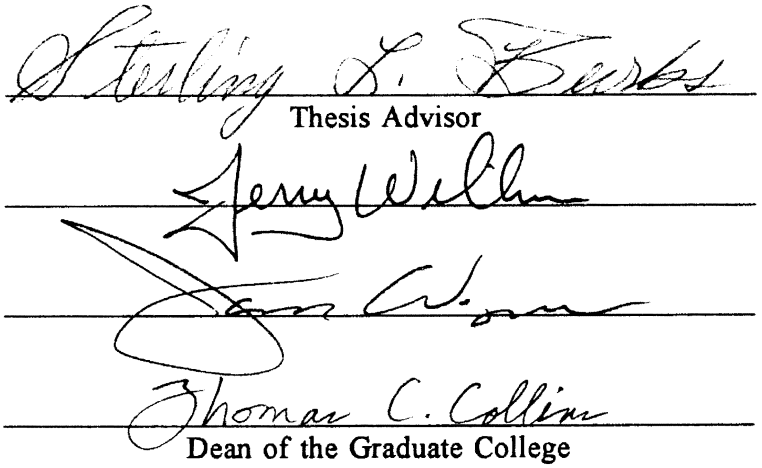

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CHAPTER I

INTRODUCTION

Extraction procedures for soil analysis are useful research tools. However, most extraction techniques have unfavorable side-effects, such as solvent toxicity, or give an incomplete assessment of the soil's toxic nature. The purpose of this experiment is to assess a new extraction procedure for measuring contaminated soil toxicity. Conventional toxicity tests, using common test organisms, were used to evaluate the success of the procedure.

Hazardous waste sites have plagued mankind since before the Industrial revolution. Mining spills occurring in Europe during the 1700s began to create serious contamination problems. As the centuries progressed, the quantity and variety of the chemical wastes increased, including wastes from the steel and coal industries as well as petroleum refinery wastes. During the 1970s, hazardous wastes gained public and political attention with the problems that surfaced with the Love Canal waste disposal site in Niagara Falls, New York [1].

In dealing with the concerns of environmental and hazardous waste problems, several important laws were passed in the 1970s. These included the Toxic Substances Control Act of 1976; the Clean Water Act of 1977; and the Resource, Recovery, and

Conservation Act (RCRA) of 1976, which was later amended by the passage of the Hazardous and Solid Waste Amendments (HSWA) of 1984. This legislation charged the U.S. Environmental Protection Agency (EPA) with protecting human health and the environment from improper management and disposal of hazardous wastes [1].

These reforms were still not enough to address the problems of actual or potential releases of hazardous materials at uncontrolled or abandoned waste sites. In order to deal with these problems, the Comprehensive Environmental Response, Compensation, and Liability Act (CERCLA) of 1980 was established. As of 1989, CERCLA, also known as Superfund, had spent almost $4.5 billion on 1,173 sites, with another 30,844 potential sites waiting [1].

Petroleum refineries are possibly one of the most complex and potentially toxic of all waste sites. Spills, leaks, wastewater discharge, vapor/gas emissions, and similar releases related to petroleum refining are contributing to environmental contamination. Soil is the major environmental media that is being contaminated by these releases [2].

According to RCRA, petroleum dispensing facilities and petroleum products are specifically exempt from most hazardous waste regulations. Refinery soils can only be considered hazardous if they fail one of the four hazardous characteristic tests: ignitability, corrosivity, reactivity, or toxicity. Of these four tests, only the toxicity test usually meets the hazardous characteristics definition required by RCRA. The soil can only be considered toxic if its leachate failed the EP Toxicity test for one of eight metals or six pesticides [3]. The Toxicity Characteristic Leachate Procedure (TCLP) will add a number of organic contaminants to the list, but like the EP Toxicity test, the only measure

of toxicity is contaminant concentration. Unfortunately, this type of toxicity evaluation does not take into account possible additive effects of hundreds of contaminates which, individually, do not exceed EPA's toxic threshold levels. With these tests, no direct measurement of soil or leachate toxicity to test organisms exists.

Several methods exist for determining soil toxicity with test organisms. Some require field studies, while others rely on laboratory analysis. Tests which involve direct exposure to the soil require several days, weeks, and even months before relevant data can be obtained. Several terrestrial bioassays include the *Eisenia foetida* (earthworm) 14-day Soil Acute Toxicity Test and the Seed Germination Toxicity Test which takes 120 hours to complete. In some cases, *in situ* tests are performed which make use of the resident species within a hazardous site. These *in situ* tests require many man-hours and are influenced by environmental variables which may not be associated with the toxic compounds found within the area. Aqueous extracts of contaminated soils allow for indirect toxicity measurement using such tests as the 48-hour *Daphnia magna* or the fathead minnow (*Pimephales promelas*) short-term toxicity test. Shortcomings exist with the aqueous extracts. Since only those compounds which exhibit an affinity for water are leached, the tests do not give a complete assessment of soil toxicity [4].

The need for quick, sensitive, and accurate testing methods for determining biological toxic responses to waste sites has become increasingly important. The heterogenous nature of soil requires that several locations be tested within a given area in order to evaluate correctly the toxic level of the site. By the nature of their existence, refinery soils are continuously exposed to complex organic contaminants, including

various polycyclic aromatic hydrocarbons. Extraction methods must be able to remove semi-polar to non-polar organics in order to test the true toxic nature of the contaminated soils. These methods must also produce non-toxic controls in order to measure accurately soil contamination effects.

Supercritical fluid extraction (SFE) may provide a favorable alternative which will fit the above requirements. Abundant research over the past few years concerned the ability of supercritical carbon dioxide to extract organics from various matrices, including soil. Due to the inherent nontoxic nature of CO_2, use of this procedure should circumvent the toxic residues found with traditional extraction solvents. Adapting this application for preparing samples for organismal toxicity testing will provide much needed information regarding complex organic toxicity.

In order to test the true value of an SFE sample, comparisons must be made with more traditional extraction procedures, which can be applied effectively to the same test organisms. The U.S. Army Corps of Engineers (USACE) has developed an aqueous extraction procedure for sediment which can be used for testing toxicity with aquatic organisms. Aqueous extraction has been chosen for this comparison due to the contrasting effect that water exhibits with semi- to non-polar organic contaminants.

Two different bioassay tests were used for establishing toxicity. One method used the Microtox® Toxicity Test System. Publications concerning the usefulness of Microtox® in testing organic contamination are numerous and have become widely accepted. For comparison, a more traditional 48-hour acute toxicity test, with *Ceriodaphnia dubii*, was performed concurrently.

In order to determine an estimate of the organic toxicity associated with a waste site, high performance liquid chromatography (HPLC) analysis was conducted on each extract. These analysis visually illustrate the organic content of the extractions. Linear regression analysis between the log of the LC50 of the *Ceriodaphnia* bioassays and the log of the inverse peak area of sections based on octanol/water partition coefficients (K_{ow}) may provide a useful tool in estimating which areas of an organic extract correlate most with the acute toxicity results. Acute toxicity associated with individual chemical compounds have been correlated with the octanol/water partition coefficient for that chemical [5]. By dividing the HPLC chromatograph of each toxic sample into retention time sections based on an estimate of the log K_{ow} and using the section area as concentration, a correlation with the sample toxicity may be provided. The following null hypothesis for each objective of this study will be tested:

1. H_o: Microtox® EC50 toxicity responses of SFE extraction of soils are not significantly different from responses of aqueous extracts of soils using the USACE sediment method.
2. H_o: 48 hour acute *Ceriodaphnia* LC50 toxicity responses of SFE extraction of soils are not significantly different from responses of aqueous extracts of soils using the USACE sediment method.
3. H_o: Supercritical carbon dioxide leaves a toxic residue in blank extractions.
4. H_o: Peak area of sections of the HPLC chromatograms does not correlate with the observed acute toxicity level of the bioassays.

CHAPTER II

LITERATURE REVIEW

The classic definition for supercritical fluid is a dense gas that has been heated beyond its critical temperature. Beyond this temperature the fluid can no longer be liquified by pressure. The density of a supercritical fluid approaches that of a liquid making it a potent solvent. Since it is less viscous than a liquid, it can diffuse more readily. Compounds such as carbon dioxide have a low critical temperature and pressure and can be easily maintained. This makes them ideal candidates for extracting organic pollutants such as polycyclic aromatic hydrocarbons (PAHs) from environmental samples [6].

The history of supercritical carbon dioxide dates back to the late nineteenth century. Dr. Thomas Andrews cited observations of the critical properties of CO_2 at a lecture to the Royal Society in 1869. The values he reported, 30.9° C and 73 atm, agree closely to the present values of 31.1° C and 72.8 atm [7]. A graphic representation of the supercritical phase is shown in Figure 1. Although the practice of supercritical fluids has been known for more than 100 years, the need for a cheap, efficient extraction tool did not really become necessary until the late 1970s. The toxic characteristics of extraction solvents such as carbon tetrachloride and methylene chloride and the subsequent disposal

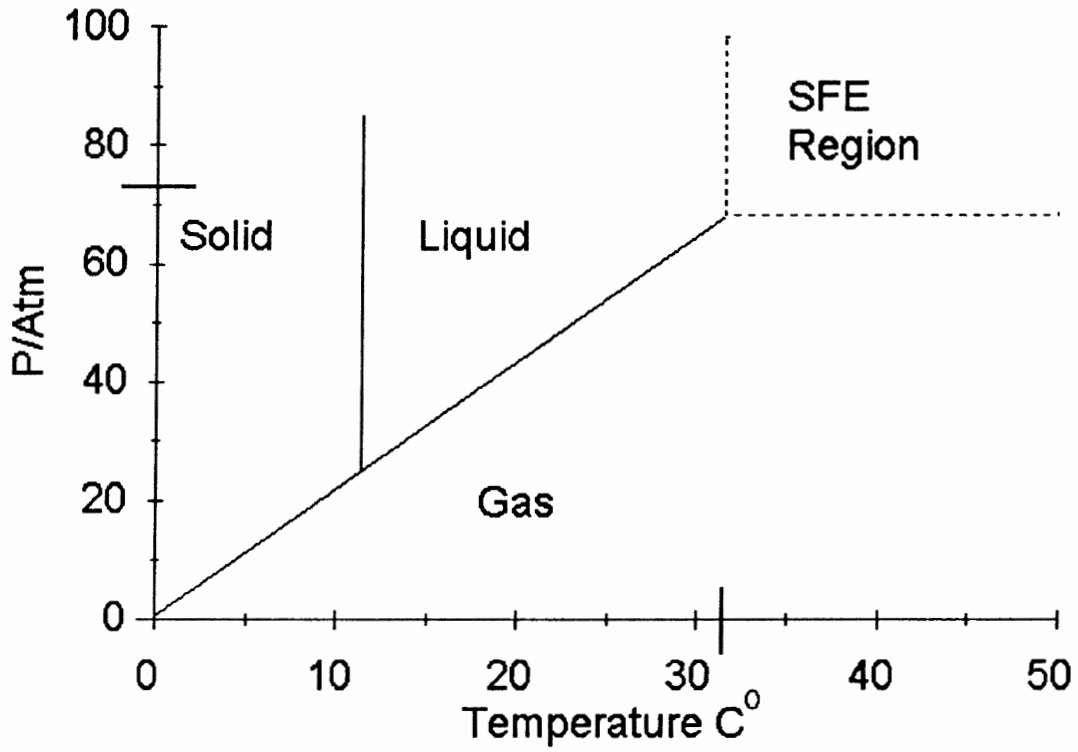

Figure 1. Pressure-temperature phase diagram of carbon dioxide listing gas, liquid, solid and supercritical sections.

problems associated with them necessitated the rapid development of supercritical extraction techniques.

Traditional methods of extraction such as Soxhlet have been valuable tools for quantifying chemical contamination of many solid samples including soils. Unfortunately, Soxhlet extraction is both time-consuming and labor-intensive. Several comparative studies have been reported [8,9] between Soxhlet and supercritical extraction techniques. Extraction times with SFE are generally less than 2 hours and yield recoveries equivalent to Soxhlet extractions that take up to 48 hours. Most SFE applications use nontoxic, nonflammable CO_2 instead of solvents that emit hazardous and/or flammable vapors used by Soxhlet. Also, thermally unstable samples which could deteriorate under Soxhlet conditions can be extracted with SFE at moderate conditions. Research on the recovery of PAHs using SFE extractions show that the more volatile PAHs from naphthalene through pyrene gave high percent recoveries (relative to Soxhlet). Higher molecular weight PAHs required additional extraction time to achieve similar recoveries [8]. Another traditional method of soil extraction involves the use of sonication, as described in the U.S. EPA Method 3550. A recent study has compared extraction efficiencies between sonication, Soxhlet and SFE methods. Recovery rates of base-neutral and acid compounds from soil were calculated at an average of 80.2% for SFE, 66.4% for Soxhlet and 58.6% for sonication. In addition to superior recovery, the SFE method used only 15 ml of methylene chloride as compared to several hundred milliliters of solvent for both Soxhlet and sonication extraction [9].

Supercritical fluid extraction offers other benefits in addition to those stated above.

By manipulating the temperature and pressures of the extraction, SFE can emulate several types of solvent characteristics. Increasing pressure can enhance solvent strength by making it less polar. Increases in temperature will increase vapor pressure and facilitate extraction of the analyte into the flowing CO_2. Typical solvent counterparts to supercritical CO_2 include cyclohexane, ethylene, carbon tetrachloride, and trimethyl pentane. The solvent properties of SFE carbon dioxide can be changed at any time during the extraction process in order to collect different classes of analytes [10]. The range of equivalent solvent characteristics of SFE at 6000 psi and various temperatures is impressive (Table 1). Supercritical fluid extraction has been shown to be a valuable technique used by analytical laboratories in an effort to improve their sample analysis cycle. Its usefulness has been proven in several areas including chemical, pharmaceutical and environmental applications [11]. Technical limitations for SFE require samples to be solid to semi-solid in order for the process to effective. Under these conditions, soil samples fit the requirements very well.

Much SFE experimentation has focused on extracting organics from soils. Laboratory studies conducted by Isco Inc. have shown applicability for extracting diesel fuel and PCBs from soil samples [12]. The summary of this experiment indicated good recovery of the diesel fuel from the soil sample. The range of recovered solutes varied from C_{14} to C_{20}. One interesting side note to come from this research indicated that diatomaceous earth was more useful as a dispersant for SFE than magnesium sulfate when extracting soils with some degree of moisture.

Extraction of petroleum hydrocarbons by SFE has become an extremely useful tool

Table 1. Solvent equivalents of supercritical carbon dioxide at a constant 6000 psi and various temperatures

Temperature (Deg C)	Density (g/ml)	Solubility (Hildebrand)	Equivalent solvent
40.00	0.967	8.248	cyclohexane
43.00	0.958	8.164	cyclopentane
46.00	0.948	8.079	xenon
49.00	0.937	7.990	ethylene
55.00	0.917	7.814	carbon tetrafluoride
59.50	0.902	7.687	nonane
68.50	0.870	7.419	propane
70.00	0.865	7.375	hexane
77.50	0.840	7.157	butane
80.50	0.829	7.070	pentane
83.50	0.819	6.985	trimethyl pentane
89.50	0.800	6.821	methane

since Freon-13 was banned because of hazardous vapors in the lab. One method has been developed by Viorica Lopez-Avila *et al.*[13]. In order to test the applicability of the procedure, soil samples were spiked with various petroleum hydrocarbons ranging from kerosene to heavy gas oil. Recoveries of these spiked samples ranged from 90 to 137%.

The effectiveness of the SFE process was indicated by the development of a new EPA approved method which addressed the supercritical extraction of total petroleum hydrocarbons (TPH) in soil and solid waste. The EPA Draft Method 3560 was approved in July 1992 by the SW-846 Working Committee of the U.S. EPA Office of Solid Waste [14]. This method provides the first federally recognized procedure for extracting organic contaminants from soils. Extraction pressures and temperatures were provided as well as equipment and material recommendations [15]. Recovery rates were not included with this draft method but the process was based in part on the work done by Viorica Lopez-Avila,*et al* [13].

Aquatic macroinvertebrates have been used for toxicity testing of priority pollutants for many years. In 1980, *Daphnia magna* were used to conduct toxicity evaluations on selected pollutants to help EPA develop national water quality criteria [16]. Chemicals tested included fluoranthene, naphthalene, phenol, nitrobenzene and over 80 other chemicals. EPA has published several manuals dealing with acute toxicity testing procedures using aquatic macroinvertebrates. The use of macroinvertebrate bioassays has been studied for use in evaluating remedial action at hazardous waste sites. Macroinvertebrates, specifically *Daphnia*, have shown toxicity in 86% of eluates of soil

samples from the waste site samples [17]. Acute toxicity testing of soil eluates using macroinvertebrates was also described in EPA's Ecological Risk Assessment of Hazardous Waste Sites manual [4]. The test is a standard 48 hour static non-renewal exposure used to calculate the lethal concentration for half of the population (LC50). Specific acute toxicity test procedures for use of *Ceriodaphnia* in 48 hour static toxicity tests of effluents have been described in another of EPA's manuals [18]. The American Society for Testing and Materials (ASTM) also established guidelines for conducting acute toxicity tests using macroinvertebrates [19]. ASTM guidelines suggest 24 to 96 hour exposure to groups of five or more organisms. The recommended invertebrates are daphnids, referring to all species in the family *Daphnidae*. Actual test species should be selected on basis of availability; past successful uses; and ease of handling in the laboratory.

Ceriodaphnia play an important role in EPA's Toxicity Reduction Evaluations (TIE/TRE) [20]. In the section dealing with toxicity identification evaluations, *Ceriodaphnia* are valuable in the testing of effluents, because of their toxic response. The 48 hour LC50's were recommended in the screening portion of Phase I (toxicity characterization) procedures. Phase II (toxicity identification) procedures also used *Ceriodaphnia* to help determine effluent fraction toxicity. Effluent organic contaminates are concentrated on a C_{18} solid phase extraction column and then eluted with methanol.

This methanol fraction was then mixed with dilution water at a 1.5% concentration. This dilution was recommended as a safe concentration for both *Ceriodaphnia* and fathead minnow. The methanol LC50 of *Ceriodaphnia* was >3.0% [20].

The Microtox® Toxicity Test System has been in use for many years and has become well established as an acute toxicity indicator. Studies by Dutka *et al* [21] compared Microtox to several other microbiological assays. The Microtox® test uses luminescent bacteria (*Photobacterium phosphoreum*) to assess toxic response by the alteration in intensity of light output. EC50 levels are calculated from the concentration of toxicant causing a 50% reduction in light from a base level. Dutka's study showed the Microtox® system to be a sensitive toxicity assay procedure which has a quick turnaround time and can also serve as a potential early warning test. There are, however, some problems with reproducibility indicated in the study.

Microtox® was used in a performance comparison study by Reteuna, *et al* [22]. This test compared Microtox® to the oxygen consumption of activated sludge assay and the Glucose U-^{14}C mineralization assay. The compounds screened for toxic activity included metals, amines, halogenated alkanes, chlorophenols, aromatic hydrocarbons, surfactants and pesticides. While no single assay performed best in every toxicity evaluation, the Microtox® system appeared to be competent in the detection of aquatic pollution and in the screening of solid waste effluents and/or leachates.

As the data on general toxicity applications became more available, Microtox® studies have begun concentrating on more specific applications. A study of the possible effects of petroleum hydrocarbon spills and leaks into the groundwater system was conducted by Eisman, *et al* [23]. Microtox® was used as a rapid bioassay for analysis of the toxicity of petroleum fuels and components. Many toxicity test have been performed with single chemicals, but environmental contamination usually involves

mixtures of chemicals, sometimes in excess of 300 components, such as petroleum fuels. Eisman, *et al* studied the toxic responses of Microtox® to diesel, unleaded gas, aviation fuels, as well as alkanes, cycloalkanes, alkylbenzenes and polynuclear aromatic hydrocarbons. Only the 5 minute exposures were used to establish an EC50 calculation as extending the time to 15 minutes did not significantly alter the EC50 values. In this case, the Microtox® system was an effective and rapid means of assessing toxicity of fuels, fuel components and the water soluble fractions of these components.

Research comparing the acute toxicity of both solvent and aqueous extraction of contaminated soils using the Microtox® assay system was conducted by Donnelly, *et al* [24]. The solvent extracts of the soil were prepared with a Tecator Soxtec® extractor using 20 grams of soil and 25 ml of methylene chloride or methanol. The solvent extracts were then evaporated and the residue dissolved in dimethyl sulfoxide (DMSO) for use in the Microtox® bioassay. The aqueous extracts of the soil samples were prepared using a modification of the sediment extraction method described in the Army Corp of Engineers manual on the Criteria for Dredged and Filled Material [25]. Toxicity for the Microtox® assay was calculated by EC50 values. Although Donnely's study focused primarily on the correlation between toxic responses and mutagenic activity, there was evidence to support the viability of using solvent and aqueous extracts of soil to assess hazard and contamination potential for groundwater.

Many times, solvent extraction can be the only way to collect certain contaminants from samples. Problems are encountered with the inherent toxicity associated with most organic solvents. The two solvents most associated with studies involving Microtox® are

DMSO and methanol. Campbell, *et al* [26] studied the toxicity comparisons between methanol and DMSO as used for exchange solvents with Microtox®. No significant differences were found between these two solvents. In one study designed to assess testing procedures and applications, Ribo, *et al* [27] found that, in general, addition of an organic solvent as a carrier will increase the solubility of a hydrophobic contaminant. This usually results in increased toxicity due to the toxic effects of most solvents. It was found that methanol can be added to samples in ranges from 5% to 10% without affecting the results.

There has been much emphasis in recent years in associating the octanol/water partition coefficient (K_{ow})with the environmental fate of organic chemicals. Relationships are found with water solubility, soil/sediment adsorption coefficients, and bioconcentration factors for aquatic life [28]. K_{ow} is defined as the ratio of a chemicals concentration in the octanol phase to the concentration in the aqueous phase of a two-phase octanol/water system. K_{ow} values for organic chemicals range from 10^{-3} to 10^{7}. Various methods have been described for calculating K_{ow} for a compound based upon the effect of polarity or solubility of specific chemical constituent groups. One of the easier methods for calculating K_{ow} is based upon high performance liquid chromatography and retention time (HPLC/RT) analysis. Calculation of K_{ow} using HPLC\RT involves only simple linear regression [28].

There are several HPLC\RT test methods which have developed procedures for calculating the K_{ow} for individual chemicals. ASTM [19] has a procedure where the log K_{ow} can be estimated for chemicals over a range of 0 to 8. Retention times are provided

by passing the chemical or chemicals through a C18 HPLC column using a mobile phase of 85% methanol and 15% water. This ratio of methanol to water is primarily a starting point and can be modified to improve separation. Calibration is provided by 5 to 10 reference compounds of known K_{ow}. Linear regression analysis was then performed by plotting the log retention time of 5 to 10 reference compounds of known K_{ow} versus the log K_{ow}. An estimate of the log K_{ow} of the test compound can then be calculated based on the retention time. Other researchers have used similar methods in obtaining estimates of K_{ow}. Klein, *et al* [29] has stated that using an appropriately buffered methanol/water eluent with a water content of at least 25% and at least 6 references with known partition coefficients, the log K_{ow} of a test substance can be obtained within $\pm$ 0.5. Klein used reverse phase (C18) high performance liquid chromatography due to the good correlation of the retention time of a test compound with the octanol/water partition coefficient. Estimates of a test compound's K_{ow} were calculated with linear regression from a log K_{ow}-log retention time plot. Klein found that the reference compounds should cover the log K_{ow} range from 0 to 8. Estimation of the partition coefficient by means of HPLC has proven to be a reliable method within the range of log K_{ow} 0 to 6. The determination of the log K_{ow} can be determined with a deviation of less than 0.5 from the traditional Shake-Flask value.

Numerous studies have been performed in an attempt to correlate K_{ow} values with toxicity responses of several test organisms. Since the toxicity and bioconcentration factors of certain contaminants seem related, it would appear that LC50s or EC50s for a given chemical could be predicted given a chemical's K_{ow}. An impressive study

conducted by Kaiser, *et al* [5] made an attempt to investigate the correlation of the acute toxicity of 267 compounds to six aquatic species and one terrestrial species. Most of the toxicity data for this study was obtained from literature and there were certain limitations induced by incompleteness of data sets. Significant correlation was found between the LC50s of *Daphnia magna*, fathead minnows and Microtox® EC50 values. The toxicities also showed collinearity with the octanol/water partition coefficient for all species accept the common Norway rat.

Other studies have tried to be more selective in their choice of test organisms and chemical compound. Koldway, *et al* [30] tested the acute toxicity of *para*-substituted phenols to 8-day old larval American flagfish and tried to correlate the LC20 and LC50 to previously report toxicity for Microtox® and with the log octanol/water partition coefficient. Neither the LC20 or LC50 values correlated significantly to Microtox® or K_{ow} values. Koldway believes that there is a danger in using "low level" biota techniques or simple quantitative structure-activity correlations when attempting to predict toxicity of specific chemicals to higher organisms such as fish.

Several other articles have been written by T.W. Schultz concerning the structure-toxicity relationships of many different compounds to test organisms. Schultz, *et al* [31] showed relationships between the log K_{ow} of aliphatic aldehydes to several test organisms, including *Tetrahymena pyriformis* (ciliate), *Poecilia reticulata* (guppy), *Lactuca sativa* (lettuce) and fathead minnows. The equations needed to arrive at a correlation between the log K_{ow} of the chemical and the EC50 and IGC50 of the tested organisms turned out to be different for each species.

CHAPTER III

MATERIALS AND METHODS

Experiment Location and Sampling Procedures

The Cyril Oil Refinery, located in Caddo County, Oklahoma, has been in the business of refining crude oil since the early 1920s. The refinery was shutdown in the 1970s because of economics. Since its closing, the Environmental Protection Agency has funded several investigations into the environmental consequences caused by the refinery. The latest study being funded by the Air Force Office of Scientific Research and is conducted by several laboratories within the Zoology Department at Oklahoma State University.

Test sites at the refinery consisted of three control locations, located off of the refinery and three suspected contaminated sites within the refinery. Control locations were designated as Enclosures 1, 5, and 6. The contaminated locations were designated as Enclosures 2, 3, and 4. A map of the facility was provided by Nova Engineering Inc. through a previous study conducted in 1981 (Figure 2).

Soil samples were collected in August, 1993. The samples were collected from the surface to approximately 60 cm below the surface. Sample containers were 2 liter Nalgene TCLP bottles. The soil samples were stored at 4° C until extraction.

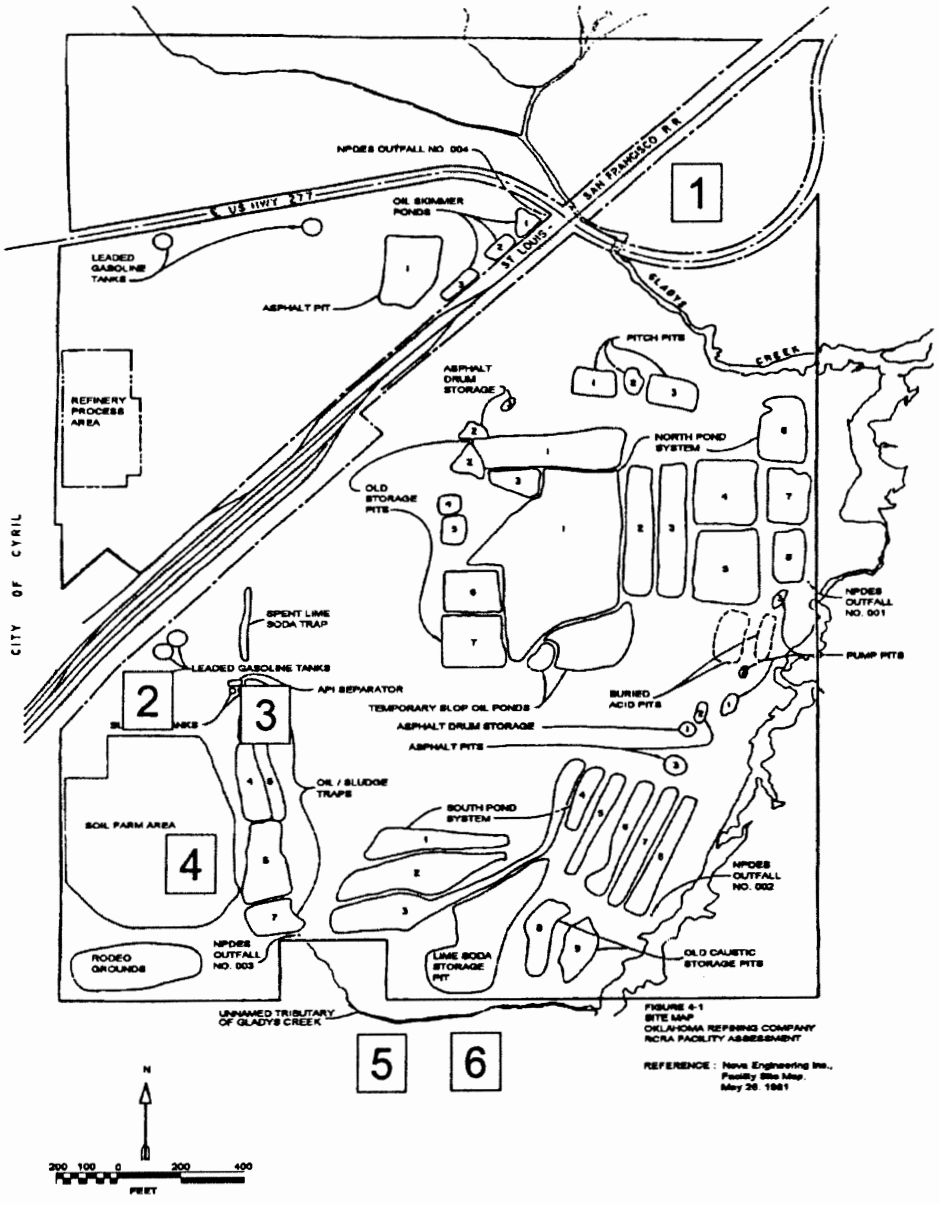

Figure 2. Map of Cyril refinery and enclosure locations.

Supercritical Fluid Extraction Equipment

The primary equipment used in this research project was the supercritical fluid extractor. Due to budget constraints, it was decided to manufacture one from individual components, rather than purchase one from a commercial vendor. Several designs were described in journals dealing with SFE. After discussions with people directly involved with building supercritical extractors, it seemed the best design would be based on a commercial extractor built by the Dionex Corporation. Dr. Bruce Richter and Michael Hey of Dionex provided information concerning manufacturers of the equipment necessary to build the extractor. The pump was a Haskell air driven liquid pump, model MS-110, slightly modified by eliminating the piston return spring. The pressure gauge was rated to 15,000 psi and is available through Autoclave Engineers. Tubing for the extractor was 1/16 inch O.D. stainless steel rated at 10,000 psi. The sample vessel was a 19 ml SFE container from Keystone Scientific, also rated to 10,000 psi. The stainless steel restrictor tube was purchased from Isco, Inc. and has a flow rate of 3 ml/min at 5000 psi. The CO_2 used in this experiment was purchased from Air Products. It was an SFE grade liquid CO_2 with an 1800 psi helium headspace. The heat source for the SFE was a Timberline HPLC column heater with a range of temperature control from ambient to 100°C. A programmable thermocouple meter with a range of -199.9° C to 400° C $\pm$ 1° C was used to monitor the temperature of the column heater (Table 2). The actual design of the extractor was kept simple to provide ease of troubleshooting (Figure 3).

Table 2. Supercritical extraction parts and vendors.

Part	Part #	Vendor	Price
Haskell liquid pump	579 05-MS-110-C12	Womack Machine Supply, Tulsa, OK	$400.00
Pressure gauge	P-0483CG	D.D. Frederick, Tulsa, OK.	$405.00
Sample vessel 11mm X 200mm	67020	Keystone Scientific, Bellefonte, PA	$300.00
Restrictor	68-3867-038	Isco Inc., Lincoln, NE	$100.00
Column heater	25964	Timberline (Alltech Associates, Inc.)	$925.00
Temperature gauge	DP465-TC	Omega Engineering Inc.	$229.00
Couplings	SMN10-316SST	D.D. Frederick Tulsa, OK.	$90.00
Tubing (10 ft) (1/16 in OD .031 in ID)	MS15-070	D.D. Frederick Tulsa, OK.	$45.00
SFE grade CO_2 (1800 psi helium headspace)	360-034327	Air Products and Chemicals. Oklahoma City, OK.	$313.00

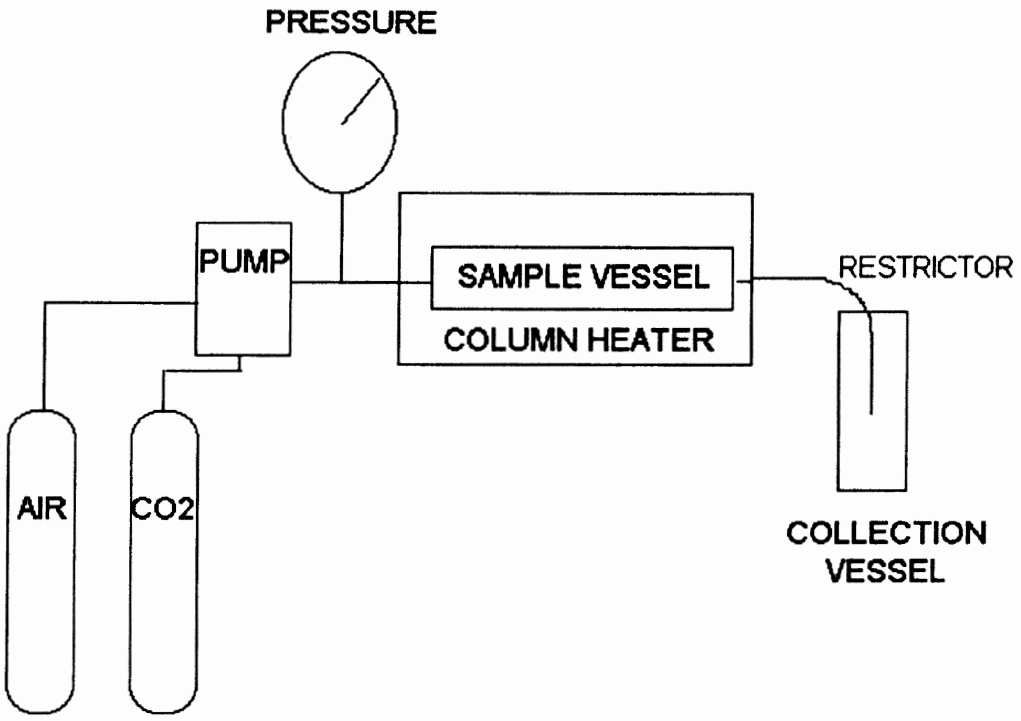

Figure 3. Schematic of a supercritical fluid extractor.

Supercritical Carbon Dioxide Extraction

The procedure used for sample preparation and supercritical extraction was based on EPA's Method 3560 [15] with necessary modifications due to the primitive nature of the homemade extractor. Soil samples were prepared by sifting and discarding obvious twigs and stones from the sample. Sample size was 15 ml of soil in volume and combined with 5 ml of diatomaceous earth, as a drying agent. The soil and drying agent were uniformly distributed with a mortar and pestle. The outlet end of the sample vessel was packed with a small quantity of glass wool and then packed with the sample mixture, making sure that no void volume was left. The sample vessel was then sealed and refrigerated at 4°C overnight. This overnight chilling helps the extraction efficiency, according to the EPA Method 3560. The actual sample extraction procedure started at 89°C and 4000 psi for 20 min. The pressure was then raised to 5000 psi for an additional 20 min. The final step was to raise the pressure to 6500 psi for another 50 min. Flow rates of the escaping CO_2 gas was approximately 600 ml/min at 4000 psi. Flow at 6000 psi was approximately 1200 ml/min. Collection of the extract was accomplished by bubbling the CO_2 extract directly into 10 ml methanol contained in a 15 ml tube. At the end of the extraction period, the final volume of methanol was about 1 ml. When necessary, additional methanol was be added to the tube during extraction to keep the collection tube from drying. At the end of the extraction period, the volume of methanol was brought up to 1.5 ml volume and was stored in an sealed amber glass vial at 4°C. The extract, at this point, was ready for HPLC and toxicity analysis.

Three separate extractions were performed upon samples from each enclosure to

test reproducability. A total of 18 supercritical extractions were performed on the six test locations (Figure 4).

In order to assess any toxicity which may be related to the SFE equipment or process itself, it was necessary to create control blank extracts. For this purpose, sand was substituted for the soil in the procedure described above. The sand was washed and rinsed with methanol and was baked at 100°C. Control blanks were run after the third extraction of each test site.

Soil samples from control enclosure 5 were spiked with reference standards to obtain extraction efficiency of the method. The reference standards run for this experiment included acenapthene, phenanthrene, pyrene, chrysene and benzo(a)pyrene.

Clean-up between extractions consisted of washing and scrubbing the sample vessel with Microclean® detergent and reagent grade water, followed by a rinse with methanol. The vessel was then placed in an oven at 100°C for a minimum of 90 min. Each end cap and frit of the vessel was back-flushed with methanol and oven dried. The restrictor tubing was connected to a high pressure pump which forced methanol through the tube. The restrictor was flushed for a minimum of 20 min. A spare set of sample vessels and restrictors were purchased so that extractions could be continued while cleaning previously used equipment.

Aqueous Extraction

The method for aqueous extraction of soils was a modified version of the method described by the U.S. Army Corps of Engineers [25]. The soil sample was mixed with

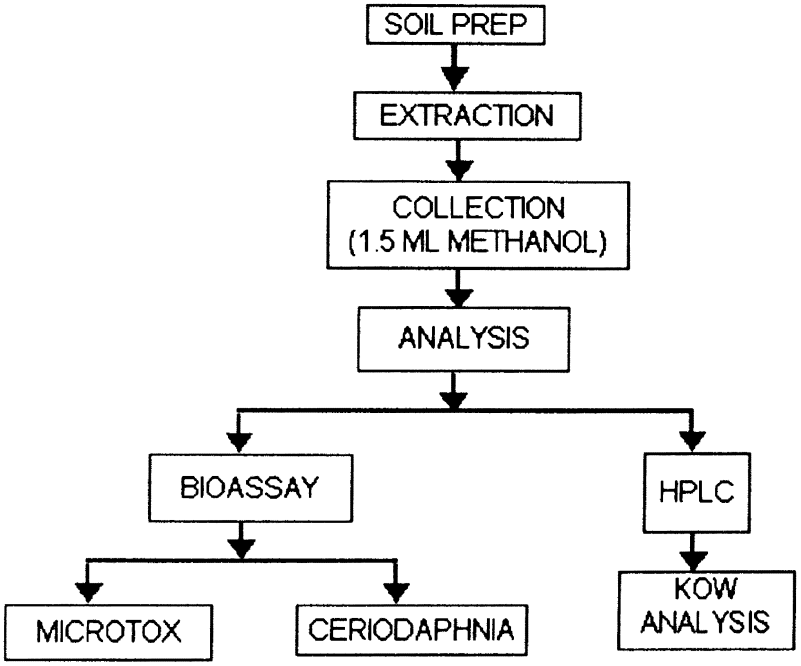

Figure 4. Flow diagram of the supercritical carbon dioxide extraction and analysis procedure.

moderately hard reconstituted water in a 1:4 vol/vol mix. This mix was then rotated at 20 rpm for a period of 8 to 12 hr. After rotation, the sample was centrifuged at 10,000 rpm for 20 min. and then filtered through a Whatman 47 mm GF/F glass microfibre filter. At this point, the extract was ready for toxicity analysis (Figure 5).

HPLC analysis of any organic content of the aqueous extract requires exposure to a solid-phase extraction (SPE) process [32]. The EPA method for SPE is slightly modified. Methanol was used in place of methylene chloride, as the extracting solvent, in order to facilitate direct HPLC injection.

The number of aqueous extractions was kept the same as for the supercritical extractions. There were three extractions per sample location for a total of 18 aqueous extractions. Method blanks were run with each extraction as a control. The control blanks are moderately hard reconstituted water that has been rotated, centrifuged and filtered along with the other samples.

Microtox® Assay

Toxicity testing for both SFE and aqueous extractions was performed with the Microtox® Model 500 Toxicity Test System. The basic protocol was described within the Microtox® manual [33]. Sample preparation for the SFE extracts was as follows: 150 ul of the methanol extract was added to 10 ml of Microtox® diluent to make up the 1.5% methanol dilution. This concentration was considered the 100% sample. Serial 1:1 dilutions were then made so that subsequent concentration were 50%, 25%, 12.5%, etc. Highly toxic samples required further dilution. Four replicates of each dilution were

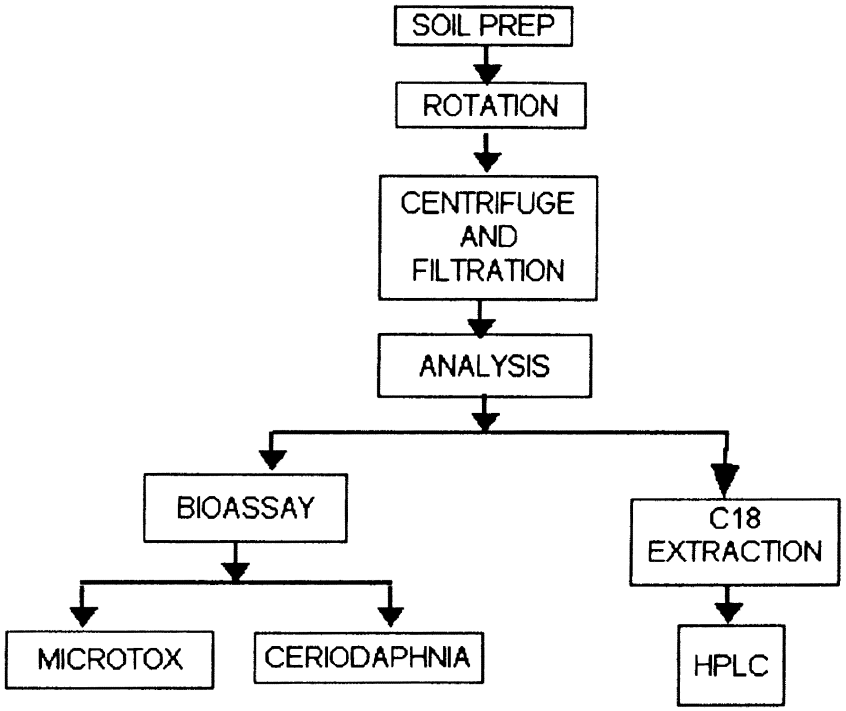

Figure 5. Flow diagram of the aqueous extraction and analysis procedure.

prepared for testing. A Microtox® control blank of 1.5% pure methanol to diluent was prepared to offset any effect from the methanol and to set the light measurement of the machine. SFE control blanks made from sand extraction were run with each test sample. This was to detect any toxic effects of CO_2 or SFE equipment on the bacteria. Light emission was recorded and EC50 values calculated. Lowest observed effect concentrations (LOEC) were also calculated for each test site. The SFE control blank values were used as the control for these statistical calculations in order to offset possible CO_2 effects.

The Microtox® manual procedure was used for basic toxicity testing of the aqueous extracts. 100 *u*l of Microtox® Osmotic Adjusting Solution (MOAS) was added to 1 ml of sample and mixed. Adjustments were made to bring the highest concentration tested to 91% of the original sample. Subsequent 1:1 dilutions were made, bringing the following concentrations to 45.5%, 22.75% etc. Light emission values were recorded and compared to a diluent control. Values for EC50 and LOEC were calculated.

Ceriodaphnia Assay

The 48 hour acute toxicity test followed the general guidelines established by the EPA TIE/TRE procedures [20]. Sample preparation for the SFE samples required 300 ul of the methanol sample added to 20 ml of moderately hard reconstituted water(MHR). This 1.5% concentration was set as the 100% sample. Serial dilutions were then made as 1:1 dilutions. Each concentration was made in triplicate for replicate testing. Control blanks were prepared using 1.5% dilution of pure methanol in 10 ml of MHR. Additional

control blanks were prepared using the SFE sand blanks.

The preparation for *Ceriodaphnia* testing of the aqueous extractions was a dilution of the pure extracts. The 100% concentrations are pure aqueous extracts. Serial 1:1 dilutions were made by the addition of pure MHR. Triplicate samples were made for each dilution, as with the SFE samples.

Ceriodaphnia neonates, less than 24 hr old, were exposed to the SFE and aqueous test concentrations. Ten neonates each were placed into test chambers and after 48 hr, mortality was documented and the lethal concentration for 50% of the population was calculated (LC50). The LOEC for each test site was also calculated.

High Performance Liquid Chromatography Analysis

HPLC analysis was based on a combination of methods. One method used the isocratic procedure of the ASTM K_{ow} method (19) which used 85% methanol and 15% water. The other method was a modified version combining parts of one procedure recommended by EPA's TIE/TRE manual [20] and another procedure listed in the EPA 40 CFR as Method 610 [34]. A gradient mobile phase beginning with a 50/50 mix of acetonitrile and water and increasing to 100% acetonitrile within 30 min and maintaining that at 100% for an additional 20 min was used for elution. The chromatography equipment was a Waters Model 510 Dual pump system with Maxima chromatography software. Detection was performed by a Waters 484 Tunable Absorbance Detector running at 254 nm. The separation column was a 25 X 4.6 cm J&W Scientific Accubond ODS(C18) with a 5u particle size. Injections of 50 ul for all of the SFE samples and the

C18 extracted aqueous samples were analyzed for organic content using both HPLC methods. Control soils spiked with reference standards were also analyzed to determine extraction efficiency.

Chromatograph Sectioning

After the samples were resolved by HPLC, the next step was to divide the chromatographs into sections that estimate log K_{ow}. In order to accomplish this, the HPLC must be used to generate the linear regression equation needed to correlate retention time with the log K_{ow} of reference standards. The ASTM method [19] was the procedure of choice for building the regression. The ASTM isocratic HPLC method was used to correlate log retention time versus log K_{ow}. In addition to the isocratic HPLC correlation, the gradient HPLC method was also used to calculate regression analysis based on log K_{ow} and retention time. The gradient method is being studied since its application is used extensively in TIE/TRE analysis and could provide a valuable tool if correlation is proven successful.

Since the toxicants contained in the refinery soils were suspected of being semi-polar to nonpolar in nature, it was decided to use standards which ranged from log K_{ow} of 2 to 6. The standards run with both the isocratic and gradient HPLC method were: toluene, acenapthene, phenanthrene, pyrene, chrysene, and benzo(a)pyrene. The log K_{ow} values for these chemicals were provided by EPA's RREL Treatability Database software [35]. Analysis was performed with both the isocratic (methanol/water) method and the gradient (acetonitrile/water) method. Three separate injections were made and the average

retention times were used with the linear regression analysis (Table 3). Linear regression analysis, along with the respective r^2 values, was calculated for the isocratic method (Figure 6). The same procedure was used for the gradient method (Figure 7). Once the linear regression equations were calculated, the sample chromatographs were sectioned into areas representing log K_{ow} values from 1 to 7. There are eight sections per chromatograph using this method. Section 1 represents log K_{ow} values from 0.1 to 1. Section 2 represents log K_{ow} values from 1.1 to 2 and so on. The retention time breaks between log K_{ow} sections were calculated for both HPLC methods (Table 4). A typical sectioning of the isocratic method is shown in Figure 8. Similar sectioning results of the gradient HPLC method can be seen in Figure 9. The peak area of each section was then treated as a concentration value (*u*volts*seconds) and each section's value was then compared, alone and in addition with following sections, to the *Ceriodaphnia* LC50 values of the SFE extracts.

The log of the average *Ceriodaphnia* LC50 values for each contaminated site was calculated. The inverse of the average area of each section of the chromatographs acquired from each contaminated site was calculated. The log of that inverse value was then derived. These values were then added in a series of progressional combination of sections. Beginning with section 1, as a starting point, each proceeding section was then added to section 1 to create multiple combinations of peak areas. Section 1 is one peak area, section 1 plus section 2 is the second peak area, section 1 plus section 2 plus section 3 is the third peak area combination. This procedure continues until section 1 through 8 are added together. After section 1 is used, section 2 is chosen as the starting point and

Table 3. Retention times of standard injections

		Gradient HPLC		Isocratic HPLC	
Standards	Log Kow	Average	SD	Average	SD
Toluene	2.69	12.81	0.47	5.23	0.08
Acenapthene	3.92	16.91	0.32	6.29	0.08
Phenanthrene	4.46	20.75	0.24	8.44	0.16
Pyrene	5.18	24.34	0.16	11.65	0.34
Chrysene	5.61	26.71	0.12	14.82	0.51
Benzo(a)pyrene	5.98	30.69	0.04	23.59	0.98

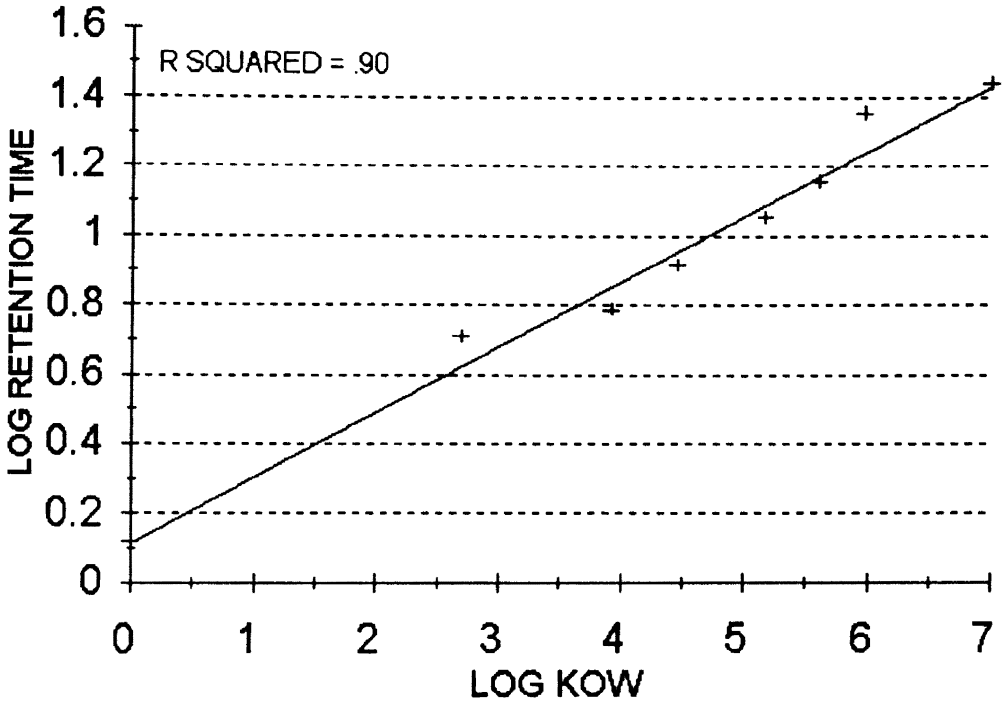

Figure 6. Linear regression of standards in isocratic HPLC method

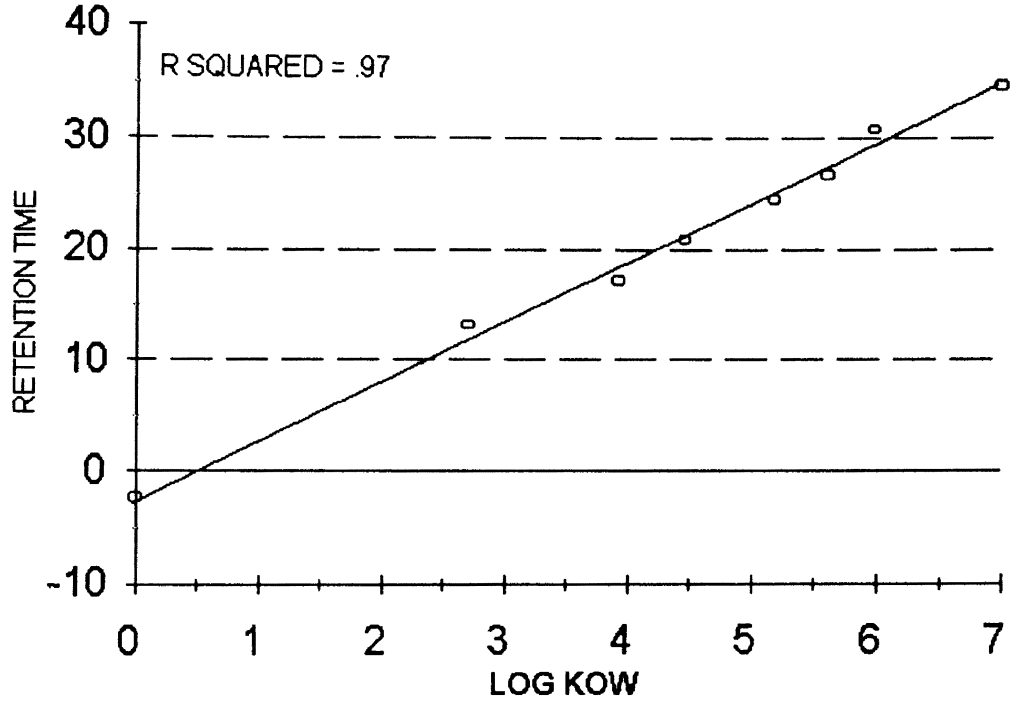

Figure 7. Linear regression of standards in gradient HPLC method

Table 4. Retention time breaks and estimated log K_{ow} for chromatograph sections

Section	Est. LogK_{ow}	Isocratic HPLC (min)	Gradient HPLC (min)
1	0.1-1	2.03	3.03
2	1.1-2	3.14	8.29
3	2.1-3	4.85	13.56
4	3.1-4	7.50	18.83
5	4.1-5	11.60	24.10
6	5.1-6	17.93	29.36
7	6.1-7	27.71	34.63

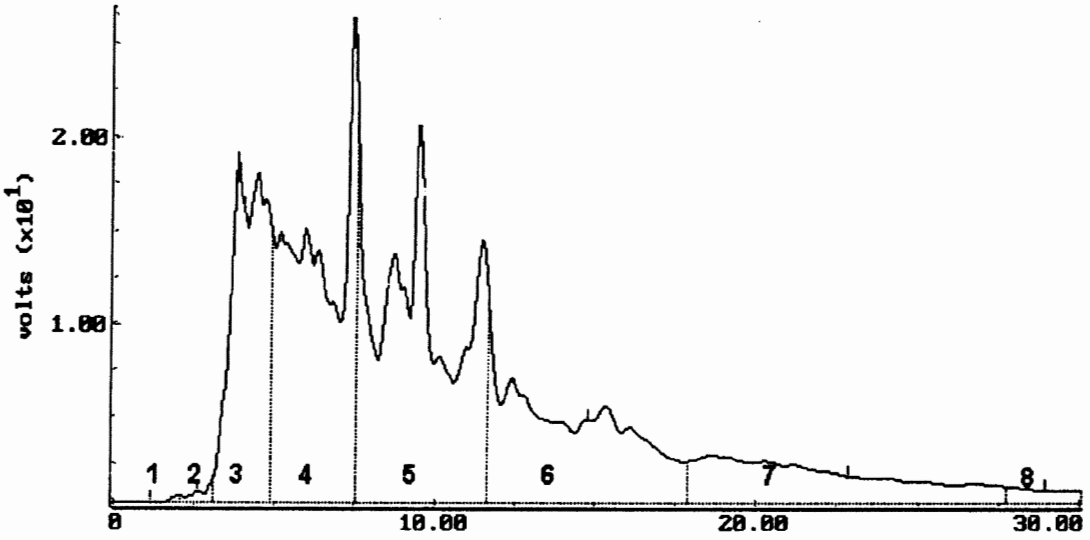

Figure 8. Sectioning of enclosure 3 with the isocratic HPLC method

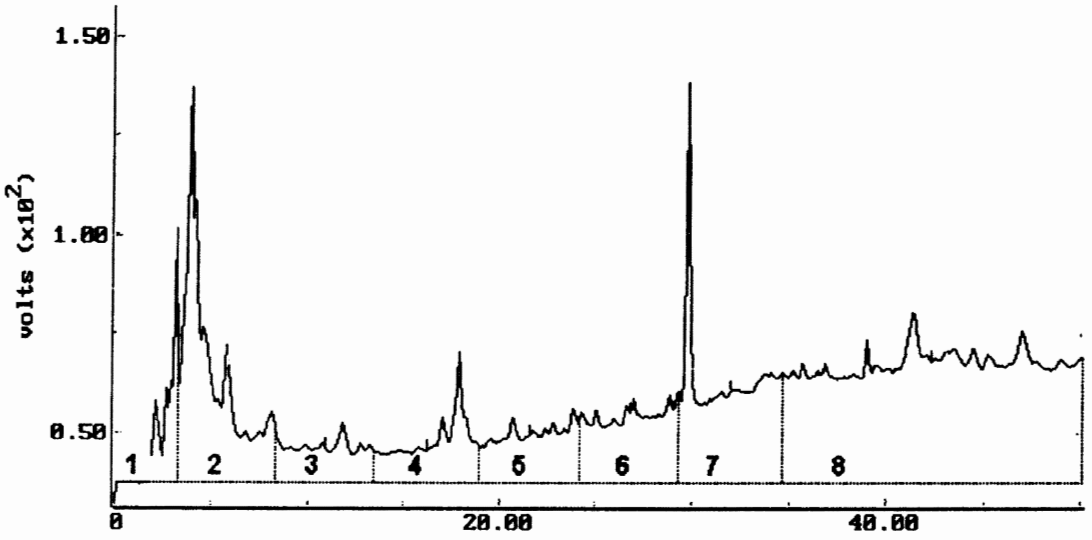

Figure 9. Sectioning of enclosure 3 with the gradient HPLC method.

the process is repeated until sections 2 through 8 are added together. This process was repeated using each consecutive section as a starting point. This process is used separately on each chromatograph from enclosures 2, 3, and 4.

A linear regression analysis was then performed, plotting log LC50 values versus the log of the inverse of the peak areas of the sectional combinations obtained from each contaminated site and r^2 values were determined. The value of the r^2 is the coefficient of determination. It is determined by dividing the sum of squares due to regression by the sum of squares of the corrected total. This coefficient of determination is a measure of the goodness of fit of the data within the linear regression. The closer the value is to 1, the better the fit of the data [36]. The r^2 values from these regressions were then graphed for each section combination (Figure 10). An r^2 value of .90 or better was chosen as a conservative indication of the best correlation between log LC50 and the sectional areas of the chromatographs. The log K_{OW} of the sections were then looked at to determine which ranges of octanol/water partition coefficients corresponded with the correlation. Both the isocratic method and the gradient method were used in this comparison (Figure 11).

Statistical Methods

The statistical analysis of the toxicity data was provided by commercially available software. The EC50 of the Microtox® assays was calculated by the software included in the test system. The LOEC values were calculated with the Toxstat software designed by D.D. Gulley *et al* [37]. For the LC50 values involving *Ceriodaphnia* toxicity, a

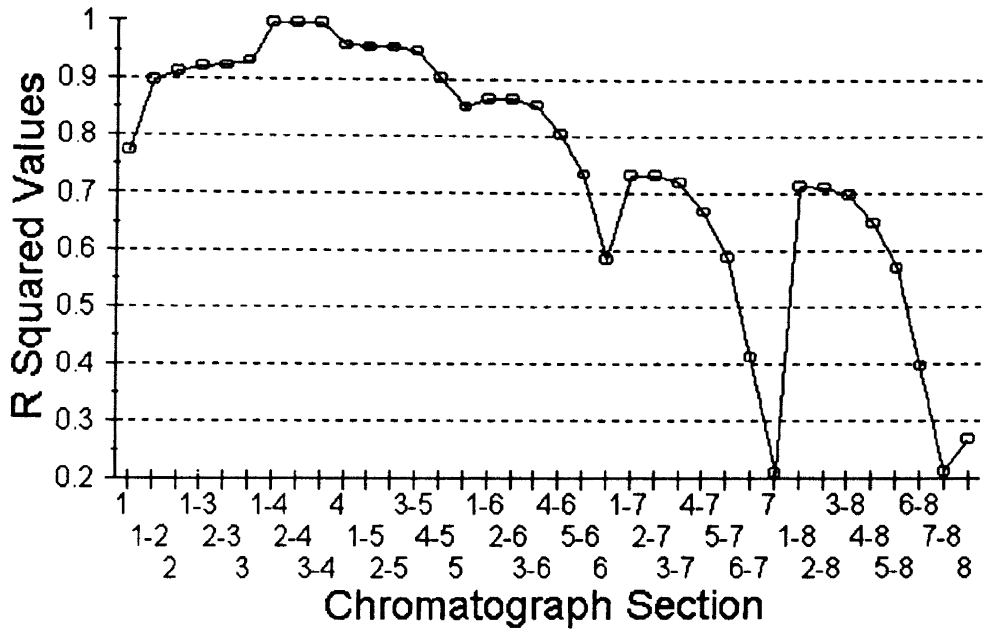

Figure 10. Isocratic chromatograph section combinations vs r^2 values.

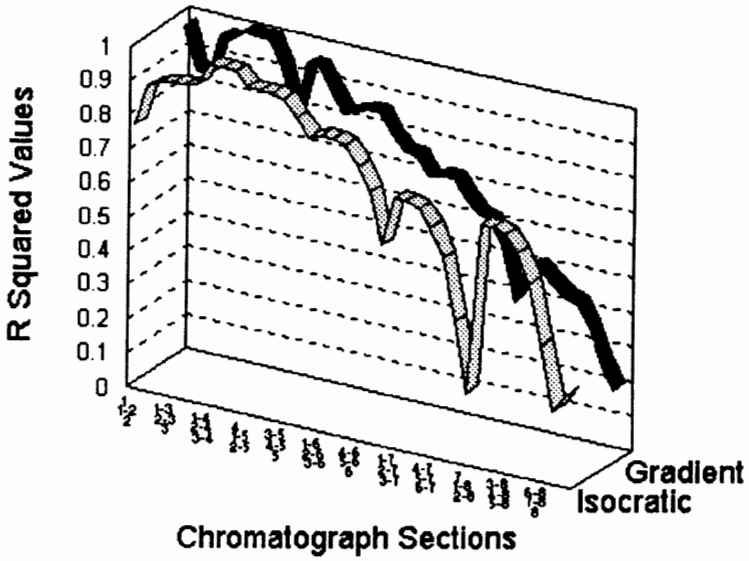

Figure 11. Comparison of r^2 correlation between isocratic and gradient HPLC method.

program provided by the EPA was used [38]. Comparisons of the EC50, LC50, and LOEC values between the aqueous extracts and the SFE extracts were determined by the Least Significant Difference (LSD) analysis of the SAS® software [39]. This procedure is useful when making multiple comparisons. All data was evaluated at $\alpha = 0.05$. Correlation between the log inverse of the peak areas of the sections of the chromatographs and the log LC50 values was calculated using simple linear regression.

CHAPTER IV

RESULTS

In general the supercritical fluid extraction (SFE) procedure was an improvement over conventional solvent extraction procedures or aqueous extraction procedures with respect to time expended on extractions. The time required for preparation of sample, extraction, and clean-up of the SFE was approximately 4 hours per sample. In contrast, aqueous extractions could require up to 30 hours per sample and conventional solvent extraction with a micro Soxhlet devise could require from 4 to 8 hours per sample. SFE was also a good method for specifically targeting moderately non-polar to strongly non-polar organics as the extraction compounds when these classes of contaminants are suspected of being the primary source of toxicity. The greatest advantage of the SFE procedure was the absence of a solvent residue in the extract, so the extract could be used directly in toxicity tests without solvent residue effects. This permits SFE extracts to be used directly for toxicity tests, without time consuming steps to remove solvent residues.

Extraction Efficiency

The efficiency of the SFE equipment was determined by the amount of reference compounds recovered from spiked soil samples. In general, the higher molecular weight

and more lipophilic compounds were recovered at a higher percentage than the lower molecular weight compounds. Acenapthene had a recovery rate of about 24%, while benzo(a)pyrene was recovered at near 100% (Table 5). The reasons for the low recovery of the smaller molecular weight compounds could be numerous, but it was suspected that these compounds, having a lower boiling point, were lost while concentrating the methanol by evaporation.

HPLC Analysis

HPLC analyses of SFE extracts indicate extraction of organic contaminants with polarity characteristics ranging from moderately non-polar to strongly non-polar, based upon comparisons with compounds of known K_{ow}. It was anticipated that SFE would extract more non-polar lipophilic compounds than the aqueous procedure, therefore comparisons of the HPLC chromatograms should yield some insight to classes of contaminants at the different study sites.

HPLC analysis of the SFE samples provided evidence of significant differences among the three contaminated enclosures. An overlay of the chromatograms for enclosures 2, 3, and 4 show the differences in the amount of organic contamination (Figure 12). HPLC analysis of control enclosures 1, 5, and 6 showed no detectable quantities of organic contamination. Chromatographic analysis of the solid phase extraction of the aqueous samples revealed some organic contamination for enclosures 2 and 3. The aqueous extract for enclosure 4 did not show any organic contaminants with the HPLC analysis (Figure 13).

Table 5. Recovery efficiency of SFE from a soil sample spiked with PAHs

Compound	Spike (ug)	Recovery (ug)	SD	% Recovered
Acenapthene	9.2	2.25	0.11	24.5
Phenanthrene	3.0	1.33	0.02	44.4
Pyrene	9.2	7.66	0.19	83.3
Chrysene	3.0	2.91	0.08	97.2
Benzo(a)pyrene	3.0	2.88	0.05	96.1

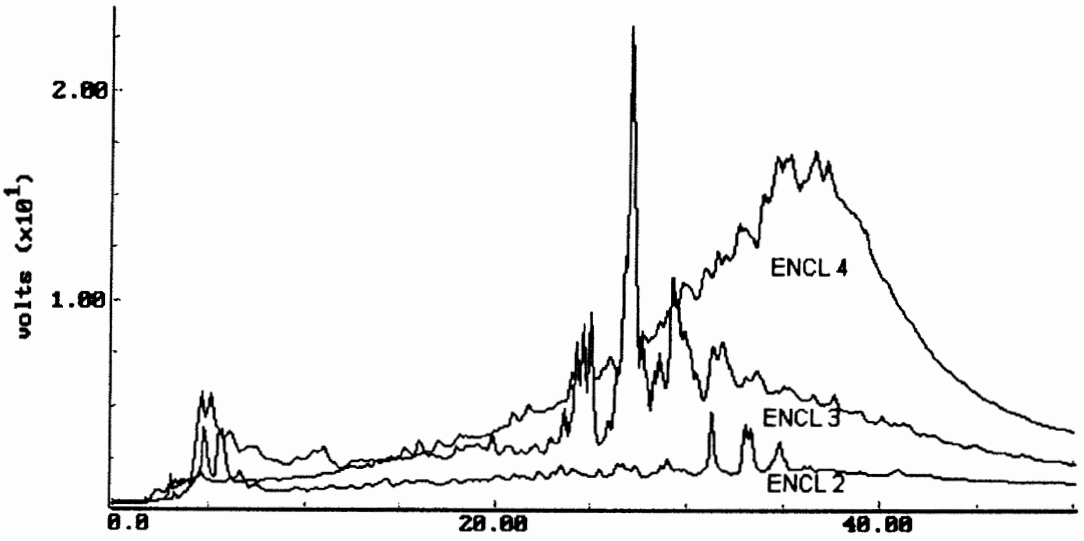

Figure 12. Overlay of SFE chromatograms of enclosures 2, 3 and 4

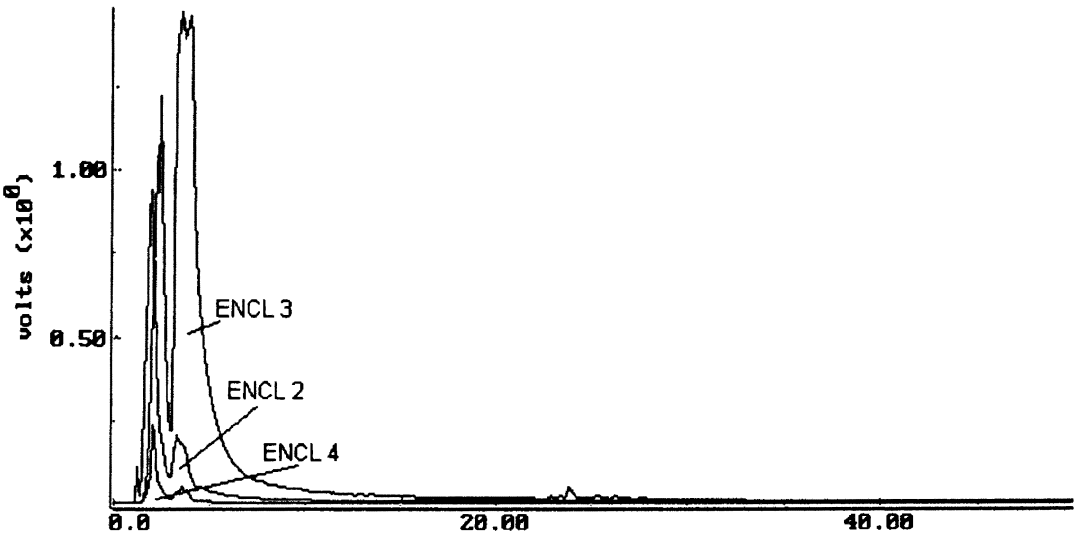

Figure 13. Overlay of aqueous chromatograms of enclosures 2, 3, and 4.

Bioassay Results

One of the anticipated advantages of the SFE procedure was the capability of producing extracts from contaminated soils, which could be tested for toxic effects without further preparation. The SFE extracts, collected in methanol, were directly diluted for bioassay testing. The aqueous extracts required additional preparations, such as centrifuging and filtering before bioassay tests could be performed. The bioassay results for both methods were tabulated (Tables 6 and 7) and comparisons using the LSD method in the SAS® software were then made for each test site and for both methods of extraction.

SFE Blank Effectiveness

No significant differences were detected between the SFE and aqueous control blanks with the *Ceriodaphnia* 48 hour acute tests. The survival percent for the SFE extracts were averaged at 96.67% compared to the control blank survival of 100%. The analysis of variance procedure (ANOVA) calculated the F value at 1.00 and the LSD 9.25 (APPENDIX D).

Statistical analysis of the SFE blanks showed significant decrease in light emission compared to the control blanks with the Microtox® assay. The average light emission for the Microtox® samples for the SFE blanks was 84.7 compared to an average of 93.05 for the control blanks. The F value from the ANOVA was 27.81 and the LSD was 3.192 (APPENDIX E). Although the differences do not appear to be large, a non-toxic blank cannot be claimed for the SFE extracts when using the Microtox® assay. The decrease

Table 6. Average LOEC and LC50 values for *Ceriodaphnia* assays as a percent of actual concentration.

	Aqueous *Ceriodaphnia*				SFE *Ceriodaphnia*			
Site	LC50	SD	LOEC	SD	LC50	SD	LOEC	SD
Encl 1	100	0	100	0	100	0	83.3	23.57
Encl 2	36.37	3.73	50	0	22.9	9.60	20.83	5.89
Encl 3	2.86	1.96	3.19	2.47	0.13	0.049	0.13	0.045
Encl 4	100	0	100	0	2.25	0.200	2.6	0.74
Encl 5	100	0	100	0	100	0	100	0
Encl 6	100	0	100	0	100	0	100	0

SD = Standard deviation.

Table 7. Average LOEC and EC50 values for the Microtox® assays as a percent of actual concentration.

	Aqueous Microtox®				SFE Microtox®			
Site	EC50	SD	LOEC	SD	EC50	SD	LOEC	SD
Encl 1	91	0	91	0	100	0	75	35.35
Encl 2	13.61	3.29	2.36	0.67	87.33	17.91	10.42	2.94
Encl 3	4.49	2.35	0.24	0.08	10.3	2.72	1.04	0.37
Encl 4	91	0	91	0	100	0	20.83	20.62
Encl 5	91	0	91	0	100	0	83.3	0
Encl 6	91	0	91	0	100	0	54	36.03

SD = Standard deviation.

in light emission by the bacteria can be compensated for by using the SFE blank as the control group when comparing results with SFE samples. Based on the results of the Microtox® comparisons, the conclusion is to accept H_O: Supercritical carbon dioxide leaves a toxic residue in blank extractions.

Supercritical Carbon Dioxide Extraction

All of the contaminated sites were significantly more toxic than the control sites as indicated with the SFE *Ceriodaphnia* 48 hour acute tests. There was no significant difference in the LOEC values between the contaminated sites (APPENDIX F). The average LC50 values showed enclosure 3 (0.13%) to be more toxic than enclosures 2 (29.2%) and 4 (2.25%). There was no significant difference in toxicity between 3 and 4 (APPENDIX G). The average values of the LC50s indicated that enclosure 3 was ranked the most toxic, followed by enclosures 4 and 2.

The LSD comparisons of the SFE Microtox® assays showed differences between the EC50 and LOEC values. With the EC50 values, only enclosure 3 showed a significant increase in toxicity from the control sites. The EC50 values of enclosures 2 and 4 were not significantly different from the control site values (APPENDIX H). All contaminated enclosures were significantly more toxic than the control enclosures, with respect to the LOEC values. However, no significant differences existed between the contaminated sites (APPENDIX I). Based on the average values of the EC50s, enclosure 3 (10.29%) appeared to be the most toxic, followed by enclosure 2 (87.3%). Enclosure 4 (>100%) appeared to be the least toxic, based on the EC50 value.

The results of the *Ceriodaphnia* assays (APPENDIX A) indicate that they were more sensitive to the contaminants extracted through SFE than was Microtox® (APPENDIX B).

Aqueous Extraction

Microtox® assay results with the aqueous extracts showed a distinct difference from the SFE samples. LSD comparisons indicated no significant difference between the control sites and enclosure 4. Only enclosures 2 and 3 were more toxic compared to the control sites. Results indicate that enclosure 3 was more toxic than enclosure 2. This was true for both the LOEC (APPENDIX J) and EC50 (APPENDIX K) values.

The *Ceriodaphnia* tests showed the same results as did the Microtox® assays. Enclosure 4 was not different from the control sites using either the LOEC (APPENDIX L) or LC50 (APPENDIX M) values. Enclosure 3 was the most toxic site, followed by enclosure 2. Again, this was true for both the LOEC and LC50 values. With the aqueous extracts, there did not appear to be any difference in the ranking of the toxicity when comparing Microtox® to *Ceriodaphnia*.

Comparison of Supercritical and Aqueous Results

The comparisons between the aqueous and SFE LC50s of the *Ceriodaphnia* acute tests were more clearly defined (APPENDIX N). With all LC50s >100%, none of the control sites were significantly different when comparing average aqueous and SFE LC50s. With the contaminated sites, enclosure 2 showed no significant difference

between aqueous (36.3%) and SFE (22.9%), but did exhibit toxicity. The average LC50 values for enclosure 3 did not show any significant differences between aqueous (2.86%) and SFE (0.31%) either, but was still the most toxic based on the LC50 values. Enclosure 4 was the most interesting result of the experiment. The aqueous extracts (100%) showed no signs of toxicity, when compared to the controls. The SFE extract (2.25%) was significantly more toxic when compared to control sites, second in toxicity only to the SFE extract of enclosure 3. Because of the significant increase in toxicity of the SFE extract compared to the aqueous extract of enclosure 4, the conclusion was to reject H_O: 48 hour acute *Ceriodaphnia* LC50 toxicity responses of SFE extraction of soils are not significantly different from responses of aqueous extracts of soils using the USACE sediment method.

Comparing the Microtox® EC50s of the aqueous extracts to the SFE extracts had certain inherent problems. The highest concentration tested with the aqueous extracts was 91% due to the necessity of adding MOAS to the sample. With the SFE samples, the 1.5% sample dilution into diluent was considered the 100% concentration. Since all calculated EC50s for the control sites and enclosure 4 were greater than the highest concentration tested, the use of an LSD comparison was not necessary. The average EC50 of enclosure 2 did show a significant increase in the toxicity of the aqueous extract (13.6%) compared with the SFE extracts (87.33%). The ANOVA showed an F value of 32.7 and an LSD of 35.75 (APPENDIX O). This difference may be the result of problems associated with the color correction procedure necessary because of the color of the aqueous extracts for enclosure 2. Enclosure 3 showed no significant difference

between the average EC50s of the aqueous (4.48%) and SFE extracts (10.29%). Although there was no difference in toxicity, it was suspected that the cause of the toxicity may be due to different contaminates. Comparisons of the HPLC chromatographs between the SFE and aqueous extracts indicated different organic contaminants, based on retention time (Figures 12 and 13). Since the aqueous extract for enclosure 2 was significantly more toxic than for the SFE extract, the conclusion was to reject H_O: Microtox® EC50 toxicity responses of SFE extraction of soils are not significantly different from responses of aqueous extracts of soils using the USACE sediment method.

Correlation of HPLC Peak Area and Toxicity

Correlation between HPLC analysis and toxicity was entirely dependent on the assumption of organics contaminants as the source of the toxicity. The aqueous extraction method can provide the addition of other contaminants, such as metals, water soluble organics, and inorganic toxicants, which can cause a significant difference in this correlation analysis. This being the case, only the SFE extracts were used in the comparison.

The Microtox® EC50 data was sporadic at best and the toxicity results were much different from the *Ceriodaphnia* data. The *Ceriodaphnia* tests seemed to have more reliable data. It was decided to use the *Ceriodaphnia* results for this correlation analysis, based on the LC50 values and the HPLC results from the SFE extracts of the contaminated sites.

Only those peak areas which contained combinations of sections 1 to 5 (log K_{ow}

estimates of 0.1 to 5), showed an r^2 correlation to log LC50 values greater than 0.90 (APPENDIX C). Other combinations which contained sections 6, 7, or 8 (log K_{ow} estimates of 5.1 to >7) had r^2 values of less than 0.90. It seems that the correlation drops dramatically as the estimated log K_{ow} increases.

Based on the presence of r^2 correlation of greater than 0.90 between specific combinations of chromatograph sections and the *Ceriodaphnia* LC50 data, it is concluded to reject H_o: Peak area of sections of the HPLC chromatograms does not correlate with the observed acute toxicity level of the bioassays.

CHAPTER V

DISCUSSION

Supercritical carbon dioxide provides a fast and effective method for extracting contaminated soils. The extracts can be used for both chemical analysis and toxicity testing. SFE can be a useful supplement to the more traditional methods. Toxicity was established by the SFE method with all three suspected contaminated sites. Additionally, all three contaminated sites had differing levels of toxicity and different chromatographic signatures. The SFE affinity for compounds which may not be collected with other extraction methods will make SFE a necessary laboratory procedure for use with environmental risk assessments.

The aqueous extracts did provide an indication of toxicity for enclosures 2 and 3. The lack of moderately non-polar to strongly non-polar organic compounds, as evidenced in the HPLC analysis, leads to a belief that the toxic components of those enclosures are of a partially or completely water soluble nature. The complete lack of toxicity of enclosure 4 is a good indication of the failure of aqueous extraction to provide a more complete indication of the true toxic nature of a hazardous waste site.

Microtox® evaluations were useful in determining the presence of toxicity in some cases, but the SFE enclosure 4 extract indicated an EC50 of greater than 100%

concentration. The *Ceriodaphnia* tests of enclosure 4 showed severe toxicity. This difference indicated that the bioluminescent bacteria test was not as sensitive to some contaminants. Also, it was judged to be inconsistent when compared to the organismal toxicity tests.

Supercritical carbon dioxide was capable of extracting toxic contaminants from soil samples where aqueous extraction methods failed. The ecological significance could not be ascertained with the current experimental design but SFE procedures would appear to offer new opportunities for determining fate and effect of nonpolar contaminants at hazardous waste sites. However, there are still problems which need to be addressed. The cause of the toxicity associated with the SFE blanks, detected by the Microtox® system, needs to be studied further. Addition of the 1.5% methanol extracts did not alter the pH of the diluent. There may be some toxicity associated with the equipment or with the matrix used for the blank extractions. Admittedly, the SFE extractor used here is somewhat primitive. The low recovery rates for acenapthene and phenanthrene may be improved with the addition of a condenser at the vent of the collection tube. The addition of a booster pump to the extractor could provide faster extraction times and increase recovery with better flow rates. Addition of a co-solvent pump could also increase contaminant recovery.

Dividing the chromatographs into sections based on estimated log K_{ow} provided a means to check correlation of portions of the whole chromatograph to toxicity. The use of standards with known K_{ow} provided a method by which other laboratories could repeat chromatograph division regardless of the specific type of HPLC equipment available. The

correlation of the combinations of the peak areas of different sections to toxicity provides a clue to the possible existence of additive effects of contaminants. After correlation is established, the sections with the best correlations were identified and their estimated log K_{ow} was reviewed. The analysis provided good information to establish a correlation between the peak areas of the estimated log K_{ow} sections of a chromatograph to the log LC50 of the *Ceriodaphnia* SFE bioassays. The best correlations with acute toxicity were sections with log K_{ow} ranging between 1 and 5. These ranges agree with a study by Veith et al [40] which indicate a good linear correlation between narcotic chemicals with log K_{ow} less than 4 and acute 96 hour fathead minnow bioassays. An addition of a fluorescence detector more sensitive to aromatic hydrocarbons than the UV/Vis detector used in this study should provide additional data to substantiate the present findings.

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APPENDIXES

APPENDIX A

Ceriodaphnia bioassay results.

Site	Units	Aq A	Aq B	Aq C	SFE A	SFE B	SFE C
Encl 1	LOEC	100	100	100	50	100	100
	LC50	100	100	100	100	100	100
Encl 2	LOEC	50	50	50	12.5	25	25
	LC50	34.55	32.99	41.56	9.69	26.79	32.23
Encl 3	LOEC	0.195	3.125	6.25	0.189	0.094	0.094
	LC50	0.31	3.2	5.08	0.2	0.1	0.09
Encl 4	LOEC	100	100	100	1.56	3.125	3.125
	LC50	100	100	100	1.99	2.27	2.48
Encl 5	LOEC	100	100	100	100	100	100
	LC50	100	100	100	100	100	100
Encl 6	LOEC	100	100	100	100	100	100
	LC50	100	100	100	100	100	100

APPENDIX B

Microtox® bioassay results.

Site	Units	Aq A	Aq B	Aq C	SFE A	SFE B	SFE C
Encl 1	LOEC	91	91	91	100	25	100
	EC50	91	91	91	100	100	100
Encl 2	LOEC	2.84	1.4	2.84	12.5	12.5	6.25
	EC50	10.27	12.48	18.09	62	100	100
Encl 3	LOEC	0.178	0.355	0.178	1.563	0.781	0.781
	EC50	3.33	7.76	2.36	12.85	11.51	6.53
Encl 4	LOEC	91	91	91	50	6.25	6.25
	EC50	91	91	91	100	100	100
Encl 5	LOEC	91	91	91	100	100	100
	EC50	91	91	91	100	100	100
Encl 6	LOEC	91	91	91	100	50	12
	EC50	91	91	91	100	100	100

APPENDIX C

Correlation data of chromatograph areas to *Ceriodaphia* LC50 values as r^2 values.

	r squared	
Chromatograph section	Isocratic HPLC	Gradient HPLC
1	0.772	0.972
2	0.912	0.846
3	0.932	0.997
4	0.960	0.785
5	0.851	0.838
6	0.584	0.698
7	0.209	0.386
8	0.267	0.192
1-2	0.895	0.855
1-3	0.922	0.969
1-4	0.999	0.993
1-5	0.957	0.947
1-6	0.865	0.852
1-7	0.734	0.710
1-8	0.713	0.498
2-3	0.923	0.969
2-4	0.999	0.991
2-5	0.956	0.945
2-6	0.864	0.850
2-7	0.733	0.708
2-8	0.712	0.497
3-4	0.997	0.918
3-5	0.949	0.878

APPENDIX C (cont)

	r squared	
Chromatograph section	Isocratic HPLC	Gradient HPLC
3-6	0.854	0.794
3-7	0.720	0.657
3-8	0.699	0.460
4-5	0.901	0.824
4-6	0.802	0.756
4-7	0.669	0.622
4-8	0.649	0.432
5-6	0.735	0.750
5-7	0.588	0.607
5-8	0.568	0.416
6-7	0.411	0.541
6-8	0.395	0.358
7-8	0.214	0.254

APPENDIX D

ANOVA and LSD analysis for Ceriodaphnia control blanks and SFE blanks.

Analysis of Variance Procedure

Dependent Variable: Percent Survival

Source	DF	Sum of Squares	Mean Square	F Value	Pr>F
Model	1	16.6667	16.6667	1.0	0.374
Error	4	66.6667	16.6667		
Corrected Total	5	83.3333			

R-Squared	C.V	Root MSE	% Survive Mean
0.200	4.151	4.082	98.333

Source	DF	Anova SS	Mean Square	F Value	Pr>F
Assay	1	16.6667	16.6667	1.0	0.374

T tests (LSD) for variable: % Survival
Alpha= 0.05 df= 4 MSE = 16.667
Critical Value of T= 2.78
Least Significant Difference= 9.25

Means with the same letter are not significantly different

T Grouping	Mean	N	Assay
A	100.00	3	Ceriodaphnia control
A	96.67	3	Ceriodaphnia SFE blanks

APPENDIX E

ANOVA and LSD analysis for Microtox® control blanks and SFE blanks.

Analysis of Variance Procedure

Dependent Variable: Percent Light

Source	DF	Sum of Squares	Mean Square	F Value	Pr>F
Model	1	616.6944	616.6944	27.81	0.0001
Error	34	754.0556	22.1781		
Corrected Total	35	1370.7500			

R-Squared	C.V	Root MSE	% light Mean
0.449	5.296	4.709	88.917

Source	DF	Anova SS	Mean Square	F Value	Pr>F
Assay	1	616.6944	616.6944	27.81	0.0001

T tests (LSD) for variable: % light mean
Alpha= 0.05 df= 34 MSE = 22.1781
Critical Value of T= 2.03
Least Significant Difference= 3.19

Means with the same letter are not significantly different

T Grouping	Mean	N	Assay
A	93.056	18	Microtox® control
B	84.778	18	Microtox® SFE blanks

APPENDIX F

ANOVA and LSD analysis for Ceriodaphnia LOEC SFE control and contaminated sites.

Analysis of Variance Procedure

Dependent Variable: LOEC

Source	DF	Sum of Squares	Mean Square	F Value	Pr>F
Model	5	35063.3209	7012.6624	47.48	0.0001
Error	12	1772.4721	147.7060		
Corrected Total	17	36835.7931			

R-Squared	C.V	Root MSE	LOEC Mean
0.952	23.7062	12.153	51.149

Source	DF	Anova SS	Mean Square	F Value	Pr>F
Assay	5	35063.3209	7012.6624	47.48	0.0001

T tests (LSD) for variable: LOEC
Alpha= 0.05 df= 12 MSE = 147.706
Critical Value of T= 2.18
Least Significant Difference= 21.62

Means with the same letter are not significantly different

T Grouping	Mean	N	Assay
A	100.00	3	Enclosure 5
A	100.00	3	Enclosure 6
A	83.33	3	Enclosure 1
B	20.83	3	Enclosure 2
B	2.60	3	Enclosure 4
B	0.13	3	Enclosure 3

APPENDIX G

ANOVA and LSD analysis for Ceriodaphnia lc50 values of control and contaminated sites.

Analysis of Variance Procedure

Dependent Variable: LC50

Source	DF	Sum of Squares	Mean Square	F Value	Pr>F
Model	5	37600.527	7520.1055	154.27	0.0001
Error	12	584.9467	16.6667		
Corrected Total	17	38185.474			

R-Squared	C.V	Root MSE	LC50 Mean
0.985	12.633	6.982	55.263

Source	DF	Anova SS	Mean Square	F Value	Pr>F
Assay	5	37600.527	7520.1055	154.27	0.0001

T tests (LSD) for variable: LC50
Alpha= 0.05 df= 12 MSE = 48.7456
Critical Value of T= 2.18
Least Significant Difference=12.42

Means with the same letter are not significantly different

T Grouping	Mean	N	Assay
A	100.00	3	Enclosure 1
A	100.00	3	Enclosure 6
A	100.00	3	Enclosure 5
B	29.21	3	Enclosure 2
C	2.25	3	Enclosure 4
C	0.13	3	Enclosure 3

APPENDIX H

ANOVA and LSD analysis for Microtox® EC50 SFE control and contaminated sites.

Analysis of Variance Procedure

Dependent Variable: EC50

Source	DF	Sum of Squares	Mean Square	F Value	Pr>F
Model	5	19381.588	3876.3178	47.23	0.0001
Error	12	984.8461	82.0705		
Corrected Total	17	20366.435			

R-Squared	C.V	Root MSE	EC50 Mean
0.9516	10.922	9.059	82.938

Source	DF	Anova SS	Mean Square	F Value	Pr>F
Assay	5	19381.588	3876.3177	47.23	0.0001

T tests (LSD) for variable: EC50
Alpha= 0.05 df= 12 MSE = 82.0705
Critical Value of T= 2.18
Least Significant Difference= 16.116

Means with the same letter are not significantly different

T Grouping	Mean	N	Assay
A	100.00	3	Enclosure 1
A	100.00	3	Enclosure 6
A	100.00	3	Enclosure 5
A	100.00	3	Enclosure 4
A	87.33	3	Enclosure 2
B	10.29	3	Enclosure 3

APPENDIX I

ANOVA and LSD analysis for Microtox® LOEC for SFE control and contaminated sites.

Analysis of Variance Procedure

Dependent Variable: LOEC

Source	DF	Sum of Squares	Mean Square	F Value	Pr>F
Model	5	27108.031	5421.6063	9.681	0.0007
Error	12	6719.1545	559.9255		
Corrected Total	17	33827.186			

R-Squared	C.V	Root MSE	LOEC Mean
0.8013	48.852	23.663	48.337

Source	DF	Anova SS	Mean Square	F Value	Pr>F
Assay	5	27108.031	5421.6063	9.681	0.0007

T tests (LSD) for variable: LOEC
Alpha= 0.05 df= 12 MSE = 559.9255
Critical Value of T= 2.18
Least Significant Difference= 42.096

Means with the same letter are not significantly different

T Grouping	Mean	N	Assay
A	100.00	3	Enclosure 6
A	83.33	3	Enclosure 5
A	75.00	3	Enclosure 1
B	20.83	3	Enclosure 4
B	10.42	3	Enclosure 2
B	1.04	3	Enclosure 3

APPENDIX J

ANOVA and LSD analysis for Microtox® LOEC for Aqueous control and contaminated sites.

Analysis of Variance Procedure

Dependent Variable: LOEC

Source	DF	Sum of Squares	Mean Square	F Value	Pr>F
Model	5	32192.197	6438.4394	55057.40	0.0001
Error	12	1.40333	0.1164		
Corrected Total	17	32193.600			

R-Squared	C.V	Root MSE	LOEC Mean
0.999	0.559	0.341	61.1099

Source	DF	Anova SS	Mean Square	F Value	Pr>F
Assay	5	32192.197	6438.4394	55057.40	0.0001

T tests (LSD) for variable: LOEC
Alpha= 0.05 df= 12 MSE = 0.1169
Critical Value of T= 2.18
Least Significant Difference= 0.6084

Means with the same letter are not significantly different

T Grouping	Mean	N	Assay
A	91.00	3	Enclosure 1
A	91.00	3	Enclosure 6
A	91.00	3	Enclosure 5
A	91.00	3	Enclosure 4
B	2.36	3	Enclosure 2
C	0.24	3	Enclosure 3

APPENDIX K

ANOVA and LSD analysis for Microtox® EC50 for Aqueous control and contaminated sites.

Analysis of Variance Procedure

Dependent Variable: EC50

Source	DF	Sum of Squares	Mean Square	F Value	Pr>F
Model	5	26988.746	5397.7492	1320.36	0.0001
Error	12	49.0569	4.0881		
Corrected Total	17	27037.802			

R-Squared	C.V	Root MSE	% Survive Mean
0.998	3.174	2.022	63.683

Source	DF	Anova SS	Mean Square	F Value	Pr>F
Assay	5	26988.746	5397.7492	1320.36	0.0001

T tests (LSD) for variable: EC50
Alpha= 0.05 df= 12 MSE = 4.0881
Critical Value of T= 2.18
Least Significant Difference= 3.596

Means with the same letter are not significantly different

T Grouping	Mean	N	Assay
A	91.00	3	Enclosure 1
A	91.00	3	Enclosure 6
A	91.00	3	Enclosure 5
A	91.00	3	Enclosure 4
B	13.61	3	Enclosure 2
C	4.48	3	Enclosure 3

APPENDIX L

ANOVA and LSD analysis for Ceriodaphnia LOEC for aqueous control and contaminated sites.

Analysis of Variance Procedure

Dependent Variable: LOEC

Source	DF	Sum of Squares	Mean Square	F Value	Pr>F
Model	5	24839.940	4967.9881	3250.97	0.0001
Error	12	18.3379	1.5282		
Corrected Total	17	24858.278			

R-Squared	C.V	Root MSE	LOEC Mean
0.999	1.636	1.236	75.531

Source	DF	Anova SS	Mean Square	F Value	Pr>F
Assay	5	24839.940	4967.9881	3250.97	0.0001

T tests (LSD) for variable: LOEC
Alpha= 0.05 df= 12 MSE = 1.5282
Critical Value of T= 2.18
Least Significant Difference= 2.199

Means with the same letter are not significantly different

T Grouping	Mean	N	Assay
A	100.00	3	Enclosure 1
A	100.00	3	Enclosure 6
A	100.00	3	Enclosure 5
A	100.00	3	Enclosure 4
B	50.00	3	Enclosure 2
C	3.19	3	Enclosure 3

APPENDIX M

ANOVA and LSD analysis for Ceriodaphnia LC50 for aqueous control and contaminated sites.

Analysis of Variance Procedure

Dependent Variable: LC50

Source	DF	Sum of Squares	Mean Square	F Value	Pr>F
Model	5	27530.702	5506.1405	1241.5	0.0001
Error	12	53.2193	4.4349		
Corrected Total	17	27583.922			

R-Squared	C.V	Root MSE	LC50 Mean
0.998	2.876	2.105	73.205

Source	DF	Anova SS	Mean Square	F Value	Pr>F
Assay	5	27530.702	5506.1405	1241.5	0.0001

T tests (LSD) for variable: LC50
Alpha= 0.05 df= 12 MSE = 4.434
Critical Value of T= 2.18
Least Significant Difference= 3.746

Means with the same letter are not significantly different

T Grouping	Mean	N	Assay
A	100.00	3	Enclosure 1
A	100.00	3	Enclosure 6
A	100.00	3	Enclosure 5
A	100.00	3	Enclosure 4
B	36.37	3	Enclosure 2
C	2.86	3	Enclosure 3

APPENDIX N

ANOVA summary and LSD analysis for Ceriodaphnia aqueous vs. sfe LC50 results of test sites.

Location	LSD	F Value	Pr>F	Mean	T Grouping
	0	9999.9	0.0		2.78
Aqueous Encl 1				100.00	A
SFE Encl 1				100.00	A
Aqueous Encl 6				100.00	A
SFE Encl 6				100.00	A
Aqueous Encl 5				100.00	A
SFE Encl 5				100.00	A
	0.3941	9999.9	0.0		2.78
Aqueous Encl 4				100.00	B
SFE Encl 4				2.24	C
	20.224	3.42	0.138		2.78
Aqueous Encl 2				36.367	D
SFE Encl 2				22.903	D
	3.852	3.88	0.120		2.78
Aqueous Encl 3				2.86	E
SFE Encl 3				0.31	E

APPENDIX O

ANOVA summary and LSD analysis for Microtox® aqueous vs. sfe EC50 results of test sites.

Location	LSD	F Value	Pr>F	Mean	T Grouping
	0	9999.9	0.0		2.78
Aqueous Encl 1				91.00	A
SFE Encl 1				100.00	B
Aqueous Encl 6				91.00	A
SFE Encl 6				100.00	B
Aqueous Encl 5				91.00	A
SFE Encl 5				100.00	B
Aqueous Encl 4				91.00	A
SFE Encl 4				100.00	B
	35.757	32.77	0.0046		2.78
Aqueous Encl 2				13.61	C
SFE Encl 2				87.33	D
	7.056	5.23	0.084		2.78
Aqueous Encl 3				4.48	E
SFE Encl 3				10.29	F

VITA

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Master of Science

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