APPLICATION OF MICROTOX ASSAY TO ESTABLISH AND EVALUATE THE EFFICACY OF *IN SITU* BURNING OF OILED MARSHES

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Application of Microtox Assay to Establish And Evaluate the Efficacy of *In Situ* Burning of Oiled Marshes

Abstract

An oil spill at Louisiana's Rockefeller Refuge presented a rare opportunity to investigate *in situ* burning as a mitigation technique in a coastal marsh environment. A subsequent monitoring study investigated the efficacy of *in situ* burning by monitoring vegetative recovery and changes in oil concentration and biodegradation. Monitoring stations were established and preburn sediment and vegetative samples collected prior to ignition of the oiled marsh. Periodic observations and sampling to monitor chemistry and vegetative recovery continued in the second year after the incident. To augment standard monitoring techniques, scientists from Louisiana State University integrated the Microtox system as a screening tool for residual oil toxicity. This paper focuses on the appropriateness of the Microtox assay to establish and evaluate the efficacy of oil spill cleanup and response activities, and specifically, *in situ* burning in a marsh environment. A strong positive correlation was observed between the light aromatic hydrocarbons and observed toxicity for a series of weathered crude oils and refined oil products. The correlations between the Total Petroleum Hydrocarbon (TPH), Total Target Aromatic Hydrocarbon (TTAH), heavy aromatic hydrocarbons, and the Microtox values were poor. Clearly, aromatic hydrocarbon composition, and not just concentration, is a key factor in assessing residual oil toxicity. Field chemistry data showed a significant change in oil contamination after the prescribed *in situ* burn and subsequent natural weathering. Comparison of the chemistry monitoring data and the Microtox data showed a poor correlation between oil loss at the Rockefeller Refuge site and sediment toxicity as measured by Microtox. Residual oil concentration did not correlate with the apparent toxicity differences observed. Microtox data was greatly influenced by poor sensitivity and background matrix effects relative to compound specific analytical chemistry techniques.

1.0 Introduction

An oil spill at the Rockefeller Refuge in South Louisiana (see Figure 1) and the subsequent application of *in situ* burning provided a "spill of opportunity" for evaluating the efficiency of the Microtox assay. Most monitoring studies rely on chemistry results such as gas chromatography/mass spectrometry (GC/MS) to assess efficacy and infer residual oil toxicity using techniques such as the Apparent Effects Threshold (Long, 1992). Our study considered the applicability of a direct toxicity measuring system to

assess changes in apparent toxicity and used the quantitative values to assess efficacy. The Microtox system was evaluated as a possible monitoring device to partly replace expensive GC/MS analyses in a comprehensive environmental monitoring system.

Monitoring is generally required as a part of any new response or spill mitigation technique to insure a positive environmental benefit. Monitoring must provide a quantitative assessment to evaluate efficacy or proof of action. The Microtox assay provides a rapid, economical toxicity assay that has been used to measure the response of the luminescent bacteria, *Photobacterium phosphorium*, to chemical agents such as aromatic hydrocarbons (AH) in bulk water and sediments (Santiago *et al,* 1993; Ramaiah and Chandramohan, 1993). The system provides a direct measure of toxic response rather than quantitative values which only infer toxicity. The end point measured by Microtox is a decrease in light intensity generated by the luminescent bacteria. The concentration of toxicant required to reduce the light intensity to 50% is called the EC_{50} value.

The project goal was to evaluate the Microtox system's applicability as an oil spill response toxicological and analytical tool. Study results validate using the assay, but several limitations were identified. This report focuses only on the Microtox study objectives. Detailed chemistry and vegetative recovery results are published elsewhere (Henry *et al.* 1996).

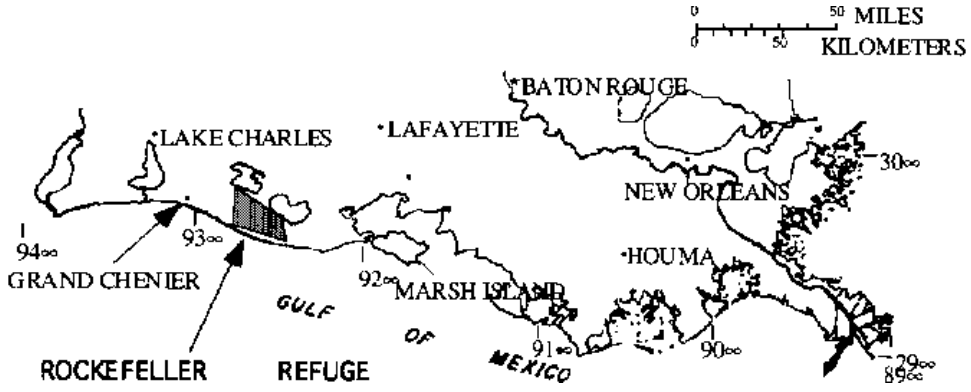

Figure 1 *Map of coastal Louisiana showing the location of the Rockefeller Refuge*

1.1 Research Objectives

The project had the following objectives:

1. Integrate the Microtox assay into the post-spill and *in situ* burn monitoring study.
2. Synthesize the experimental Microtox data in conjunction with residual oil chemistry data collected during the complementary chemical and vegetative monitoring study.
3. Establish toxicity values (EC_{50}) for laboratory weathered crude oils and refined oil products using the Microtox assay.

1.2 What is Microtox?

The Microtox assay is a rapid, economical toxicity assay that measures the response of luminescent bacteria, *Photobacterium phosphorium*, to samples being tested. This strain of bioluminescent bacteria is extremely sensitive to organic toxicants and mixtures of toxicants. Due to the complex matrix naturally found in crude oil treatment byproducts, this assay can estimate the toxicity of the remaining oil and not simply individual components in the oily residue. Research has shown the effectiveness and specificity of luminescent bacteria in determining toxic concentrations of organics and heavy metal pollutants in the marine environment (Ramaiah and Chandramohan, 1993). Data generated from Microtox screening has also been used to monitor marine pollution for organics bound to sediment (Santiago *et al*. 1993), and petroleum hydrocarbon toxicity in the water soluble fraction (Bianchini *et al*. 1988; Dasappa *et al*. 1991; Eisman *et al*. 1991). EPA documented the technique as a toxicity assay procedure for use with soil and soil-waste mixtures to establish concentration criteria for oil and sludge bioremediation (EPA Draft EP1.8:P41/3). The Microtox assay has been compared to biological species toxicity assays using rainbow trout and flathead minnow, with acceptable correlations and at considerable savings in time and cost (Kamlet *et al.* 1986). Because it is easy to use and offers lower costs for preliminary toxicity assays, this methodology can be used to determine the efficacy of response mitigative actions.

1.3 Significance of Research

Microtox is a cost effective method for assessing biological responses to toxic chemicals. When used as a monitoring tool, quantitative changes may be used to evaluate the efficacy of spill response and mitigation techniques. The approach would not be limited to *in situ* burning. The Microtox system could also be used to assess the efficacy of bioremediation, chemical cleaning, mechanical removal, and "no treatment" treatments. Further, each weathered oil assayed by Microtox had previously been characterized by GC/MS and exposed to a series of biodegradation experiments to assess

the relative degradation rates of different spilled oils (Henry *et al.* 1995; Hoff *et al.* 1995). The results not only establish Microtox values and method detection limits for a wide range of different oils, but also provide additional information to incident commanders and resource trustees who must decide whether additional cleanup, such as bioremediation, is required.

During any *in situ* marsh burn, only a portion of the oil is actually consumed by the fire. Unburned oil and a highly distilled and altered oil residue (often characterized as a "burn residue") remain in the environment. Burn residue is generally considered less acutely toxic than fresh oil due to the loss of water soluble and volatile mono- and di-aromatic hydrocarbons, such as the benzenes and naphthalenes. The fate of unburned oil and burn residue after *in situ* burning has not been extensively studied. *In situ* burning of oil spilled in marshes has been used several times over the last few years as a response and mitigation method, yet few studies have actually been conducted to evaluate the efficacy of the treatment in relationship to ecological tradeoffs and marsh recovery. Recently, Mendelssohn (1995) completed an investigation of four *in situ* burn sites; unfortunately, all of the study sites investigated lacked preburn samples. The spill at Rockefeller Refuge presented a rare opportunity to investigate *in situ* burning for mitigating oil spilled in a coastal marsh environment in conjunction with preburn sediment and vegetation samples. The monitoring study which followed was designed to investigate the efficacy of *in situ* burning by monitoring vegetative recovery and changes in oil concentration and biodegradation. The application of the Microtox assay to establish and evaluate the efficacy of *in situ* burning was integrated into the monitoring study as an alternative investigative technique.

1.4 Incident Background

On 13 March, 1995, a pipeline failure released approximately 40 barrels of condensate oil into an impounded marsh within Rockefeller Refuge in Cameron Parish, Louisiana (see Figure 1 for location). The pipeline rupture at Rockefeller Refuge involved oil and condensate crude that had not been separated from production water. The condensate oil was characterized as API 40-42 (Henry Dornak, Mobil Oil, personal communication). An estimated 50 acres of marsh were contaminated. The oiled area can be characterized as a managed brackish marsh dominated by *Distichlis spicata* (spike grass) and *Spartina patens* (salt meadow cord grass). The sensitivity and inaccessibility of the site ruled out any significant mechanical cleanup. The potential for severe weather and heavy rain also threatened to spread the oil into adjacent unoiled habitat, thus increasing the potential for wildlife exposure to the spilled oil.

Mobil Oil and the refuge manager, Tom Hess, requested permission to conduct an *in situ* burn of a section of the affected marsh measuring between 20 to 30 acres. The area to be burned was bordered on the south by a hurricane protection levee (Appendix, Photo 2). Winds were primarily from the north, creating near perfect conditions for the burn. The burn was applied to the spilled area on March 17, 1995 (Appendix, Photo 3). A

monitoring study sponsored by the Louisiana Oil Spill Coordinator's Office (LOSCO), the Louisiana Department of Wildlife and Fisheries (LDWF), and Mobil Exploration and Producing U.S. Inc. and implemented by Mendelssohn and Henry, LSU followed.

The chemistry results indicate that the overall concentration of oil as measured by TPH is approaching background levels after the *in situ* burn event and subsequent natural weathering. After seven months, oil is still present at the burn site, but at significantly lower concentrations. A greater than 90% loss in TPH was observed within the oiled and burned transect between March and April, 1995. Only a small fraction of oil loss was directly related to on-site microbial degradation; we found very slight detectable changes in selective indicators of microbial degradation, such as a relative change in the nC-18/phytane ratio. Further evaluation of the GC/MS data (or detailed chemistry results) suggests that physical transport and evaporation were the dominate processes in the reduction of the bulk oil contamination. Clearly, ecological factors which apparently inhibited the progress of natural biodegradation at the Rockefeller Refuge *in situ* burn site are poorly understood and require additional investigation. The intense heat created during the *in situ* oil burn and removal of the marsh plants may have significantly altered the normal microbiological flora and caused a lag in oil biodegradation.

1.5 Field Monitoring Stations

Three areas were originally identified for post-burn monitoring. Each area contained three separate transects: (1) two oiled and burned (OB) transects, OBa and OBb; (2) two oiled and unburned (OU) transects, OUa and OUb; and (3) two unoiled and unburned control sites (CC), transects CCa and CCb. Each OB and CC transect contained five separate monitoring stations. The OU transects were limited to only three stations because of the limited affected area. Samples were collected during five sampling periods: (1) preburn and (2) post-burn samples in March 1995; (3) middle growing season samples in July 1995; (4) end of the growing season samples in October, 1995; and (5) 13 months after the incident, April 1996. Each sampling period is identified by a number incorporated into the sample name (i.e., transect sample OBa0, OBa1, OBa2, and OBa3 identify oiled and burned Transect A samples collected preburn March, post-burn March, July, and October, respectively). After a few months of monitoring, a visually impacted area near the blowout site (BS) was identified and two additional monitoring stations were created (station BSa and BSb). It was also determined by chemical analysis that the OU transects did not represent the same degree of oiling originally present in the oiled and burned site and were essentially indistinguishable from the controls. This information coupled with a later accidental burn at the CC and OU transects lead us to drop the OU transects from the Microtox study. The BS sampling sites were added. The BS stations differed from the OB stations in that while both were burned, the BS area was subject to observable mechanical reworking in an effort to repair the damaged pipeline. As a result, subsurface oiling persisted and vegetative recovery was observably slower.

2.0 Methods and Analytical Approach

All of the OB, CC, and BS samples were analyzed for Microtox and TPH. In addition, composite samples were analyzed by GC/MS to identify Total Target Aromatic Hydrocarbon (TTAH) concentrations and specific changes in the hydrocarbon chemistry.

2.1 Microtox Analysis Method

The field samples were solvent extracted by dichloromethane following standard extraction methods for trace analysis. The sample size was 50 grams of wet sediment. Each extract was filtered through an alumina/silica gel column to remove highly polar biogenic compounds and treated with activated copper to remove inorganic sulfur. The filtered extract was subdivided into three portions for the following analyses: Microtox, gravimetric-TPH, and GC/MS. For Microtox, the solvent was exchanged into dimethyl sulfoxide (DMSO) and prepared following procedures outlined by Santiago *et al*. (1993). The DMSO soluble fraction was further diluted to 2% in a salt water solution. Each sample was assayed on a Microbic Model 500 Analyzer. Reference oils were prepared by diluting small aliquots of pure oil into DMSO following the procedure described by Santiago *et al.* (1993). Reagents and freeze-dried bacteria specified in the manual were acquired from the Microbic Corporation, Carlsbad, California. For each sample, a series of dilutions were assayed to calculate an EC_{50} value at five and 15 minutes. Phenol standards were assessed as a positive QA/QC control. Laboratory method blanks were prepared following identical procedures.

One set of field samples (April, 1996) was extracted by a water extraction technique in addition to the procedure described above. Ten grams of sediment (wet weight) were extracted into 10 ml of pure water (purified DI). The sample was centrifuged for 10 minutes at 4000 rpm to separate the extract or supernate, which was assayed by the Microtox system following the 100% sample method described in the Microtox manual.

Microtox results were synthesized on an Excel spreadsheet. Since the five minute and 15 minute EC_{50} values were nearly identical, all results reported are the mean of the two observations. All EC_{50} values were converted into Units of Sediment Toxicity (UST) values as described by Santiago *et al.* (1993). The UST value is essentially a reciprocal of the EC_{50} value normalized by sample size. A high UST value represents high toxicity (examples, the mean observed EC_{50} value for the positive control phenol was 27 ppm or a UST of 37,000; a random field sample resulted in a EC_{50} of 120,000 ppm or a UST of 8.3).

2.2 Chemistry Approach and Target Analyses

Oil is a highly complex assemblage of organic compounds that no single analytical method can fully characterize. Tracking the fate of spilled oil requires monitoring changes in bulk oil concentration and specific composition changes in the oil itself. Changes in concentration must be qualified by compositional changes in oil chemistry to assess oil fate and weathering; disappearance alone may mean that the oil simply washed away. To track oil spilled at the Rockefeller Refuge site, a tiered analytical approach was used. All samples were quantified by a gravimetric Total Petroleum Hydrocarbon (g-TPH) method to establish bulk oil concentration. Composite samples from each transect were analyzed by GC/MS to characterize composition changes in the normal hydrocarbons (alkanes) and AH.

Each sample was homogenated and 50 g subsampled for extraction. The wet sediment was dried using precleaned and conditioned anhydrous sodium sulfate. Sodium sulfate was added and mixed until a coarse, dry sandy mixture was formed. In addition to removing water as a matrix interference, the grinding of the sediment sample with sodium sulfate aids in disruption of the organic cellular material and enhances extraction efficiency. Each sample was extracted three times using pesticide grade dichloromethane solvent, and the extraction was enhanced using a heated bath-type sonicator. The extracts were combined and rotary evaporated to approximately 2 ml. The extract was then passed through a normal-phase alumina and silica-gel column to exclude any polar biogenic compounds. The extract was eluted from the column with DCM and the combination of eluent and makeup solvent resulted in a final volume of 20 ml which was further split into four vials as follows: 10 ml for g-TPH analysis, 5 ml for archive (saved for Microtox analysis), 2 ml for GC/MS analysis, and another 3 ml for repeat GC/MS analyses should any be required. Each sample was analyzed for g-TPH, and the five subsamples from each transect were composed and reduced to a final volume of either 0.25 or 2.0 ml for GC/MS analysis. Samples with moderate contamination were reduced to a final volume of only 2.0 ml.

TPH analysis provides a single gross value that approximates the concentration of bulk oil in a sediment sample. TPH values do not allow for source characterization or differentiation of natural, or biogenic-derived hydrocarbons from the spilled oil. TPH analysis is a good tool for assessing spilled oil when the oil concentration is higher than that of background hydrocarbons. To enhance the TPH information, composite samples were analyzed by a highly selective and quantitative GC/MS method. GC/MS does allow differentiation of hydrocarbons by source and can provide both specific information about a spilled oil and changes in oil chemistry due to weathering. The most useful group of target analytes in oil are the 2- to 5-ring aromatic and sulfur heterocyclic hydrocarbons and their respective alkyl-substituted homologues. Although the target aromatic hydrocarbons represent less than 5% of the bulk composition of most oils, they are essential for characterizing the petroleum source, identifying potential biological effects, determining exposure pathways, and monitoring weathering trends and degradation of the oil (Sauer and Boehm, 1991). Since hydrocarbons are naturally present in the environment, detailed chemical analyses are required to confirm the presence of oil and

differentiate the types of hydrocarbons detected in a monitoring study. To this end, aromatic hydrocarbons are extremely useful in differentiating petroleum from byproducts of combustion. Oil is characterized by PAHs composed primarily of 1-, 2-, and 3- ring aromatic compounds with a preference for alkyl-substituted alkanes. PAH resulting from incomplete combustion is characterized by 3-, 4-, and 5- ring aromatic compounds with few substituted alkyl homologues. Differences between background aromatic hydrocarbons derived from the original oil or byproducts of combustion and secondary sources of oil pollution are key elements of any long-term monitoring study.

Standard EPA methodologies are inadequate for assessing petroleum pollution since they lack key target compounds characteristic of oil. While no standardized methodology currently exists, the research community and regulatory agencies do accept gas chromatography-mass spectrometry (GC/MS) petroleum analysis for oil spill response and monitoring studies. GC/MS provides a very powerful means of separating oil constituents, and is a sensitive and highly selective tool for characterizing spilled oil samples. GC/MS procedures are widely accepted for oil spill response activities, oil fate and effects studies, and baseline pollution monitoring (Overton *et al*. 1981; Boehm and Farrington, 1984; Sauer and Boehm 1991; Sauer *et al.* 1993). Analytical methods are described in detail separately (Henry and Overton, 1993; and Roques *et al*. 1994).

Target analytes are either single compounds or isomers quantified as a single group. The target aromatic hydrocarbons listed in Table 1 exceed the EPA priority pollutant list. Many of the target analytes exist not as single compounds but as isomer groups, such as the C-2 naphthalene homologues. Quantification of the nonalkyated PAH and the saturate alkanes is based on authentic standards. The alkylated homologues are generally quantified by response factors generated by the unalkylated parent, e.g., the response factor generated for naphthalene (C-0) is used to calculate the C-1 through C-4 naphthalene homologues.

3.0 Results

Three Microtox assays were compared on samples from the Rockefeller Refuge *in situ* burn site: reference oil assays, field sediment samples assays, and aqueous extraction assays.

3.1 Reference Oil Assays

A series of commonly transported oils were analyzed by the Microtox system to assess relative toxicity differences and establish a relationship between Microtox results and oil composition. The oils tested represent a wide range of crude and refined oils. Most of the reference oils tested were weathered to simulate a spill on water that later stranded on an intertidal shoreline such as a coastal marsh. Three of the products were unweathered: gasoline (GAS), kerosene (KER), and a reference #2 fuel oil sample

collected during the North Cape incident (NNO2). GAS and KER were assayed unweathered since they are often spilled directly into sensitive areas (such as during the recent Blind River incident) and generally evaporate upon weathering. Fuel oil #2 sampled from the recent North Cape incident (NNO2) in Rhode Island was assayed unweathered since storm conditions dispersed an estimated 70 % of the spilled oil into the water column within the first few hours; the spilled oil weathered very little. Each oil, with the exception of GAS and KER, was previously analyzed by GC/MS to assess specific aromatic hydrocarbon concentrations (Henry *et al.* 1994; Hoff *et al.* 1995; Henry, 1996). The detailed chemistry data was correlated with the Microtox data to investigate what fraction of weathered oil is most responsible for the apparent toxicity measured by the Microtox system.

Figure 2 is a histogram plot of the UST values for the 14 reference oils tested. The plot presents a wide range in apparent toxicity ranging from a low of 580 UST for weathered SE-30 crankcase oil (WVAV) to a high of 48,000 UST for regular unleaded gasoline (GAS). The high values observed for GAS, KER, and NNO2 were expected. Each of the oils was unweathered and are often reported to be highly toxic (Eisman *et al*. 1991; Markarian *et al.* 1993). Gasoline is composed of only a very light petroleum distillate highly enriched with benzene, a direct acting toxicant with a relatively high water solubility (1,800 ppm). Diesel fuels are also generally recognized as being highly toxic.

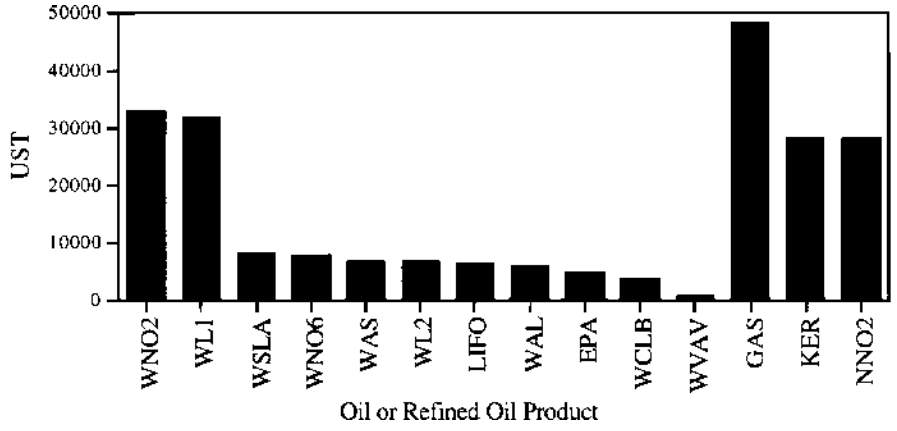

Figure 2 *UST values (relative toxicity) for the oil products tested by the Microtox System.*

Key: WNO2-Weathered #2 Fuel Oil, WL1-Weathered LAPIO #1, WSLA-Weathered South Louisiana Crude Oil, WNO6-Weathered #6 Fuel Oil (Bunker C), WAS- Weathered Alberta Sweet Medium Blend, WL2- Weathered LAPIO #2, WIFO- Weathered Intermediate Fuel Oil 380, WAL- Weathered Arabian Light Crude Oil, EPA-EPA reduced crude (EPA/NETAC Reference Oil), WCLB-Weathered Cold Lake Bitum, WVAV- Weathered Crankcase Oil, GAS-Gasoline, KER-Kerosene, and NNO2-North Cape Spill Reference #2 Fuel Oil.

The aromatic hydrocarbon constituents within oil are usually the primary contributors to oil toxicity (Sauer and Boehm, 1991). To confirm this belief, we correlated the compound specific GC/MS data with the Microtox results. Figure 3 compares the AH profile for three of the test oil cores. Differences are readily apparent. Figure 4 is a simple plot of the sum of the target analytes (TTAH) measured in ng/mg whole oil (ppm) to the UST value calculated for each test oil. GAS and KER were omitted from this comparison since they represent petroleum products whose composition is significantly different from the middle to heavy oils investigated and were not suitable for the same GC/MS target analyses. The toxicants in very light (solvent-like) hydrocarbons would likewise be different. Figure 4 exhibits some degree of correlation (r=0.630) between TTAH and UST. At least one obvious outlier, identified by the letter A, is shown. The outlier is a weathered Low API gravity Oil or LAPIO (WL2). LAPIO oils have a specific gravity greater than 1.000 relative to pure water. All LAPIOs sink when spilled into fresh water and many sink in marine sea water. A second weathered LAPIO (WL1) exhibited greater toxicity as measured by the Microtox system, and the correlation between TTAH and UST was consistent with the values observed for most of the test oils. The difference in the two LAPIO oils can be explained by Figure 5, which presents a histogram plot of the aromatic hydrocarbons quantified in both LAPIO samples. Clearly a difference in aromatic hydrocarbon composition is observable between the two oils. The outlier sample, WL2, is dominated by the heavier and less water soluble 4-ring aromatic compounds, alkylated pyrenes and chrysenes. WL1 is dominated by the 2-ring naphthalene compounds. Clearly, AH composition, and not just total AH concentration, is a key factor in assessing residual oil toxicity.

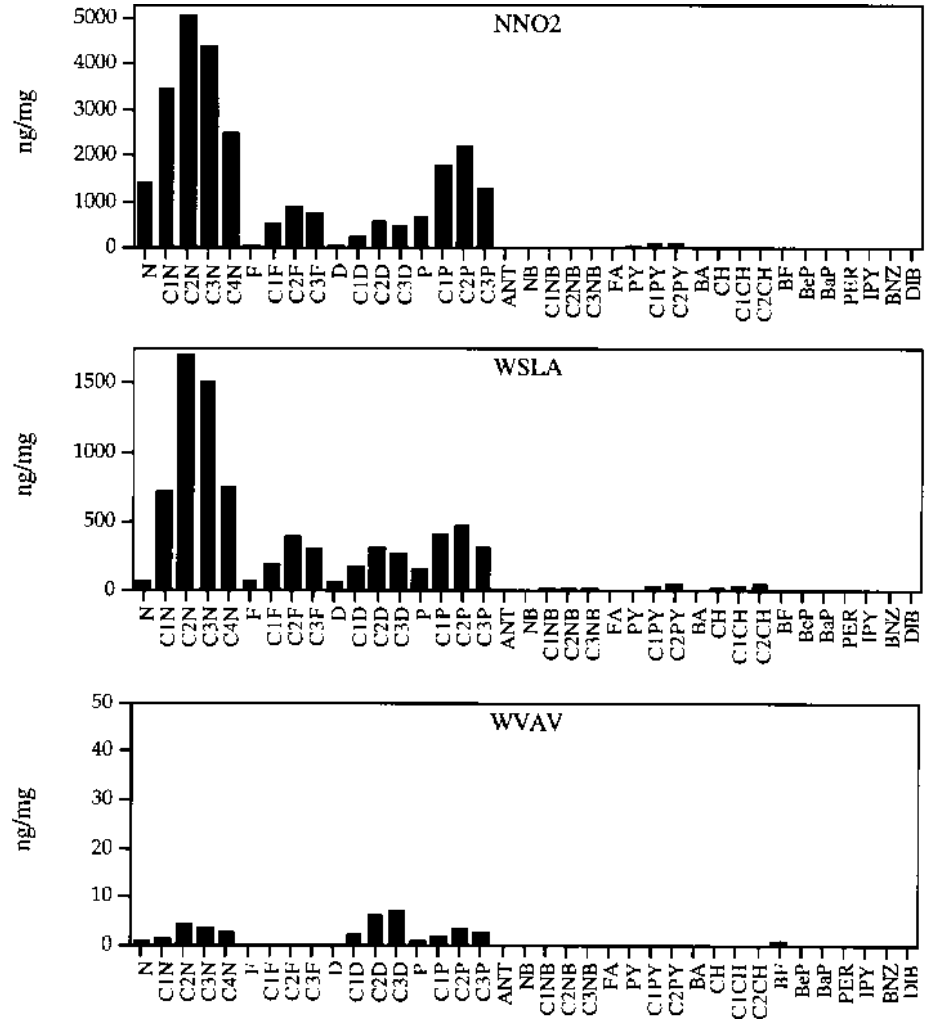

Figure 3 *Comparison of the AH profiles for North Cape #2 Fuel Oil (top), a weathered South Louisiana Crude oil (middle), and weathered lubricating oil (bottom).*

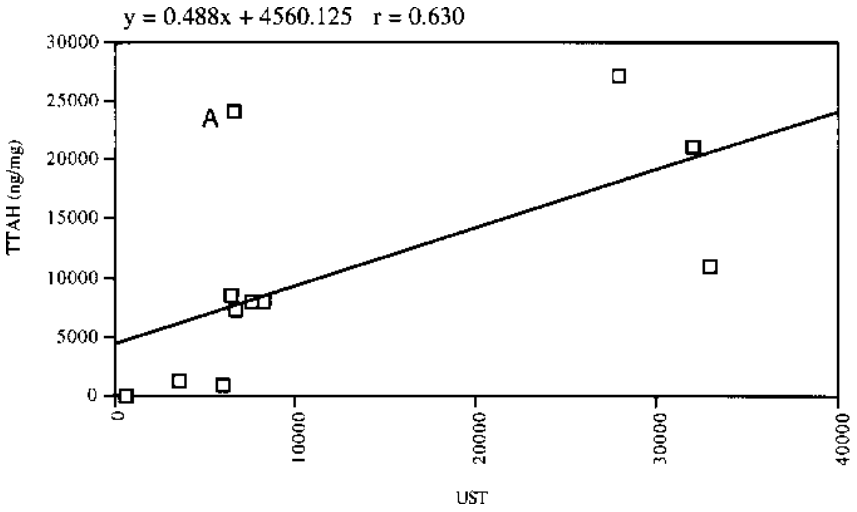

Figure 4 *Graph of TTAH versus UST for 11 reference oils analyzed.*

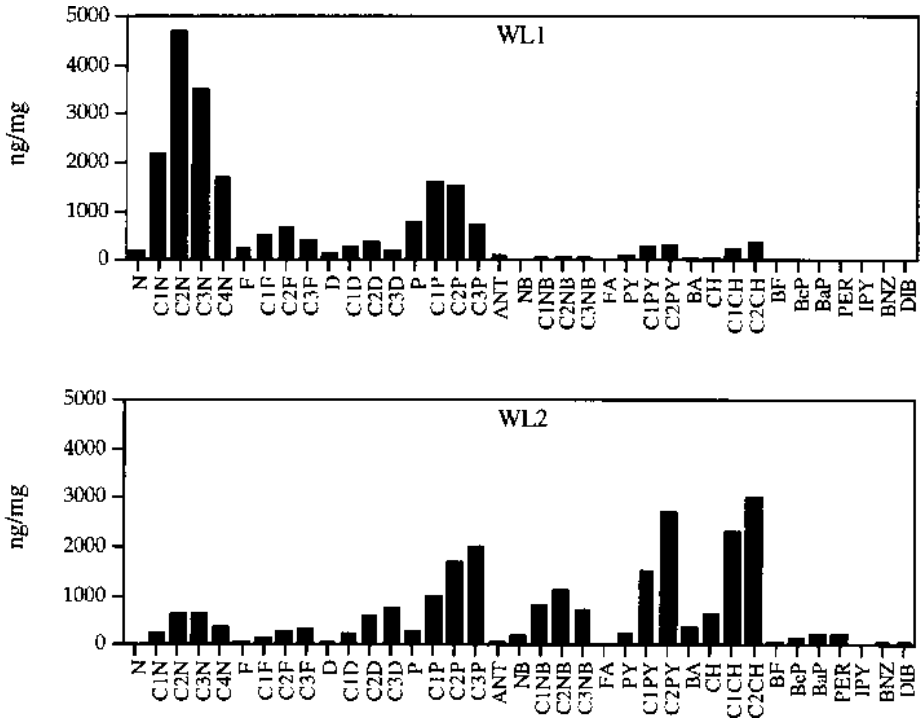

Figure 5 *AH profile comparison of two different LAPIO oils.*

Key: N-naphthalene, CxN-alkylated homologues containing x number of alkylated carbon groups, F-fluorene, D-dibenzothiophene, P-phenanthrene, ANT-anthracene, NB-naphtobenzothiophene, FA-fluoranthene, PY-pyrene, BA-benzo[a]anthracene, CH-chrysene, BF-benzo[b]fluoranthene and benzo[k]fluoranthene combined, BeP-benzo[e]pyrene, BaP-benzo[a]pyrene, PER-perylene, IPY-indeno[1,2,3-cd]pyrene, DIB-dibenz[a,h]anthracene, and BNZ-benzo[ghi]perylene.

To continue this investigation and identify which compounds or group of compounds are the primary source of the apparent toxicity, the target compound list was subdivided into the following subgroups: naphthalene and the alkylated naphthalene homologues through C-4 (N-C4N); fluorene and the alkylated fluorene homologues through C-3 (F-C3F); dibenzothiophene, phenanthrene, and related alkylated homologues (D-C3D and P-C3P); sum of the less than 3-ring compounds or Light AH (N-C4N plus F-C3F); and sum of the 3-ring and larger AH or Heavy AH (TTAH-Light AH). Figures 6 through 10 are a series of plots similar to Figure 4 and differ only in the y-coordinate or subgroups previously identified. Sample WL2, the outlier identified above, was kept in the data set for the following computations. The strongest correlation observed was for the fluorenes (r=0.939). A good correlation was also observed for the naphthalenes (r=0.785). The 3-ring dibenzothiophenes and phenanthrenes exhibited a much poorer correlation (r=0.560). The Light AHs correlated significantly better than the Heavy AHs (r=0.833 and 0.158,

respectively). For the weathered oils assayed, the Light AHs appear to be the primary toxicants to which the luminescent bacteria responded.

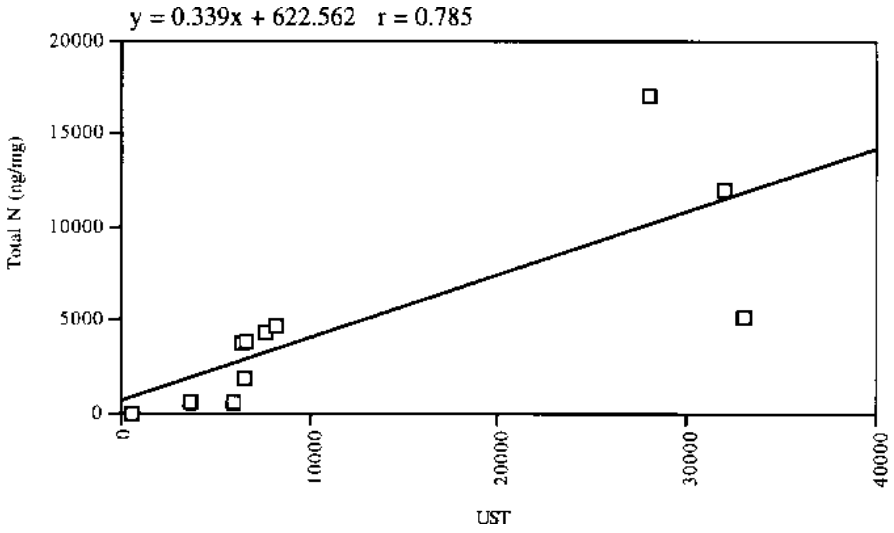

Figure 6 *Graph of naphthalene and the alkylated naphthalene homologue concentration versus UST for 11 reference oils analyzed.*

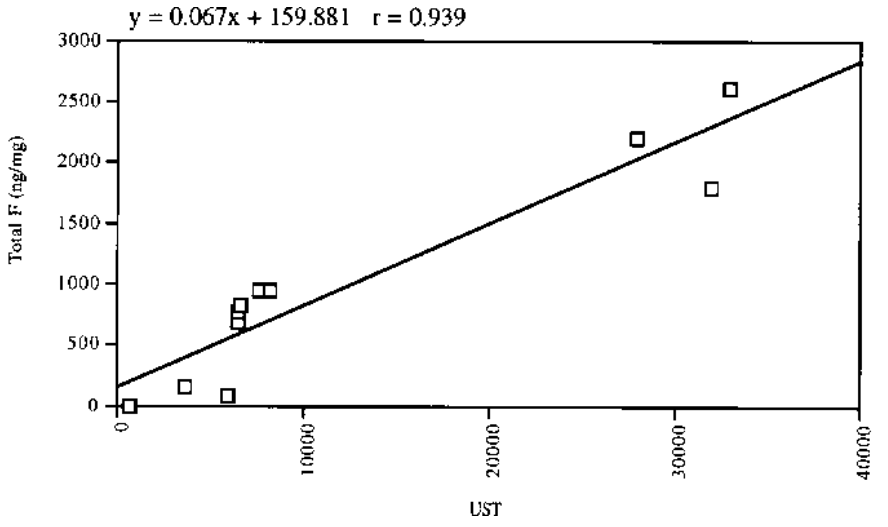

Figure 7 *Graph of fluorene and the alkylated fluorene homologue concentration versus UST for 11 reference oils analyzed.*

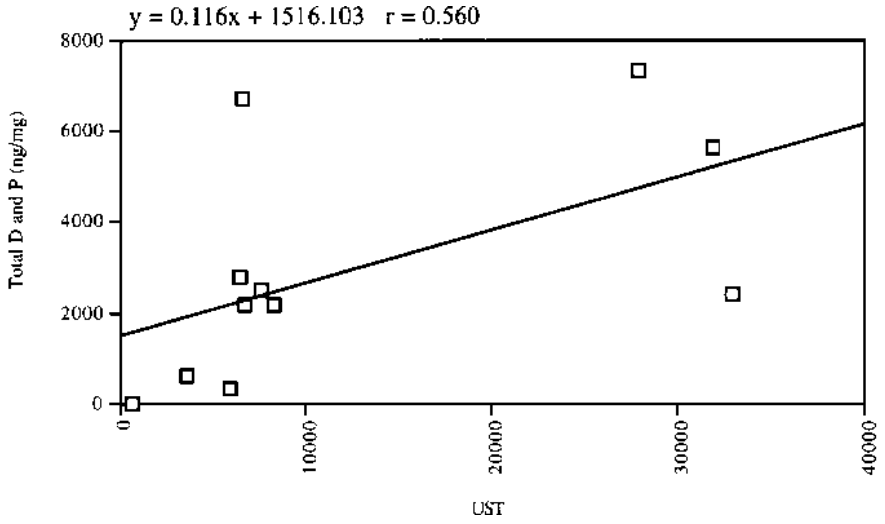

Figure 8 *Graph of dibenzothiophene and phenanthrene and associated alkylated homologue concentration versus UST for 11 reference oils analyzed.*

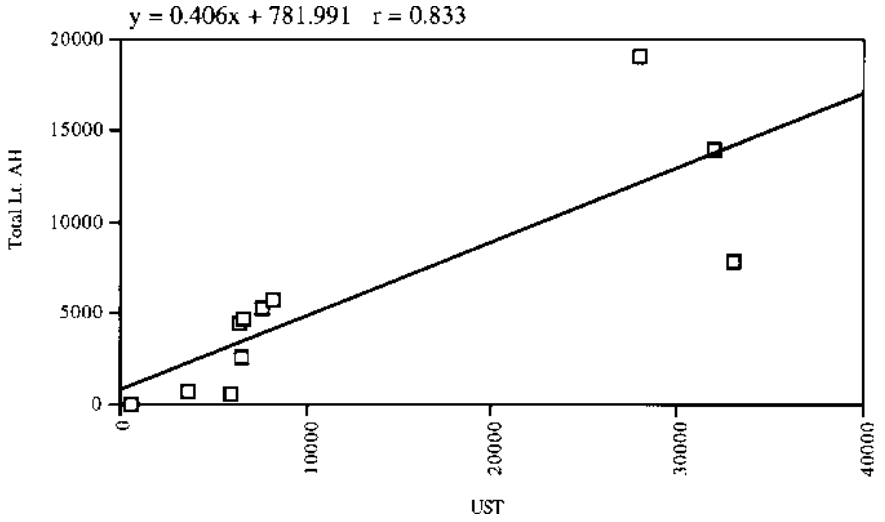

Figure 9 *Graph of the low molecular weight (light) AH concentration versus UST for reference oils analyzed.*

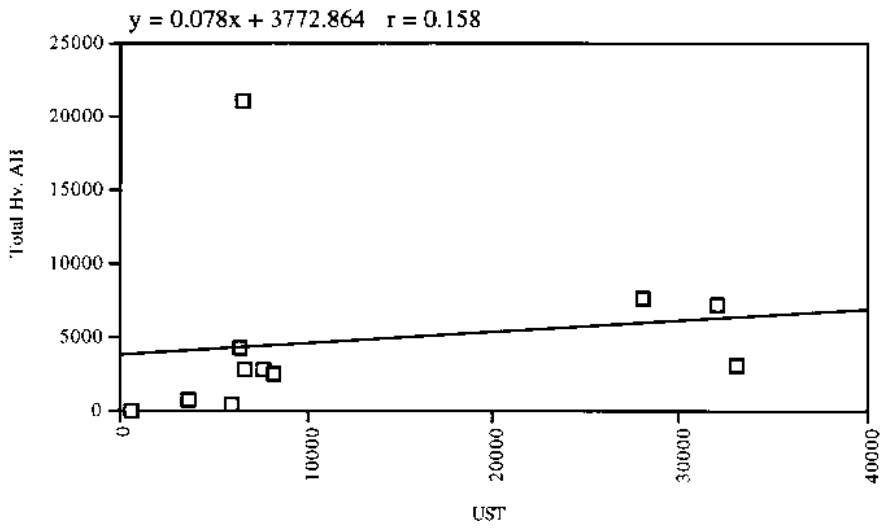

Figure 10 *Graph of the higher molecular weight (heavy) AH concentration versus UST for 11 reference oils analyzed.*

3.2 Field Sediment Sample Assays

Figures 11 through 15 are a series of histogram plots which present the UST, TPH, and TTAH values for each transect or sample station at each sampling period, preburn March 1995, post-burn March 1995, July 1995, October 1995, and April 1996, respectively. Multiple transects such as OBa and OBb were combined into a single composite value. The burn site stations (BS), oiled and burned stations (OB), control stations (CC), and laboratory method blank (BK) values are shown as mean values. The y-axis scale was kept constant in the series of figures to highlight temporal compositional changes. Error bars represent standard error values. Overall, a very high degree of site variability is observed in the data set. Figure 11 establishes the preburn values at the OB transect on 17, March 1995. Figure 12 represents the results from samples collected the day after the *in situ* burn at Rockefeller Refuge. A 60% decrease in oil concentration was observed between the preburn and post-burn sampling at the OB transect, yet no significant change in toxicity was observed between the preburn and post-burn OB samples. The mean preburn and post-burn OB UST values were essentially the same: 1.6 and 1.4, respectively. Surprisingly, the Microtox results suggest that the control transect is "more toxic" than the OB site. Observed toxicity cannot be supported by the oil chemistry data. The mean concentrations of TPH and TTAH at the OB transect were 5.0 mg/g and 4.6 ng/mg, respectively. Concentrations significantly greater than the CC

transect values were 0.11 mg/g and 0.060 ng/mg, respectively. The BK value presented is for the entire study and represents eight extraction blanks which were also assayed by Microtox following the same protocols as the field sediment samples. Relatively low toxicity values were generated by the method blanks and little variance was observed. The mean UST for the method blanks was 0.30 (standard error was 0.10, the error bars are not detectable in histogram plot shown).

Figure 13 is a comparison of the July, 1995 field data. Again, the control site exhibited a higher relative toxicity value than either the OB or BS sites, which are clearly oiled as evidenced by the TPH and TTAH values. For October, 1995 a different pattern is observed (Figure 14). The relative toxicity values as measured in UST, were the greatest for BS followed by OB and CC (4.8, 2.8, and 1.6, respectively). The differences between OB and CC fall just short of being statistically valid. Unfortunately, a marsh management experimental marsh burn extended into the control transects at our study site. New control sites were established. Data synthesized for Figures 14 and 15 include the new control sites (additional field variability). The April, 1996 data, which were compiled 13 months after the incident, suggest elevated toxicity at the OB transects relative to CC and even the BS site (Figure 15). The chemistry results do not correlate with the apparent toxicity differences observed. The BS samples exhibited significantly higher TPH and TTAH concentrations than either the OB or CC samples, yet only marginal differences are observed in the Microtox values. Figure 16 is a chromatographic comparison of the normal alkanes detected in unweathered South Louisiana Crude oil, a composite sample from the OB transect, and a composite sample from the CC transect. The depletion of the lighter alkanes, less than nC-18, is due to evaporative weathering. Clearly, normal hydrocarbons in the nC-22 to nC-36 range are present in the CC samples. The hydrocarbons detected at the CC transects have a clear odd-preference as compared to the normal distribution pattern observed in the reference oil and the OB transect. The elevated TPH values are dominated by hydrocarbons extracted from the high organic marsh sediments. The observed hydrocarbons in the CC samples are typical of biogenic-derived waxes.

Figure 17 is the TTAH histogram plot from Figure 15 with an expanded y-axis. After 13 months, the BS area exhibits elevated TTAH relative to the OB transects, and both exhibit elevated concentrations relative to the CC transects (3.5, 0.24, and 0.016 ng/mg TTAH, respectively). The TTAH values are less affected by background hydrocarbon sources such as biogenic waxes and are more selective for residual oil pollution. Figure 18 is a histogram comparison of the mean AH profiles for the April 1996, samples. The aromatic hydrocarbons at the BS site are highly similar to the original oil contamination and exhibit very little change due to weathering. The OB transect is dominated by a moderately weathered aromatic hydrocarbon profile. The CC transects are dominated by progenic sourced aromatic hydrocarbons, i.e., aromatic hydrocarbons derived from the incomplete combustion of organic material and fossil fuels. Pyrogenic or combustion-sourced aromatic hydrocarbons are ubiquitous in environmental samples. Figure 18 clearly presents GC/MS data indicating that the BS transect should exhibit the greatest Microtox response, yet the OB transect showed a greater response. The BS mean UST value was 1.4 compared to the OB mean of 2.3. Even the response of the CC transects

was higher than would be predicted by the TTAH data or aromatic hydrocarbon profile. It appears that the Microtox values are highly influenced by toxicants other than residual petroleum.

Little correlation was detected between the TPH, TTAH, or aromatic hydrocarbon subgroups values. Table 1 presents the linear fit values for field samples and oiled samples (BS and OB only). The observable values ranged between 0.176 to 0.227 for all samples and between 0.277 to 0.370 for the oiled samples only. Although a stronger correlation is apparent for the oiled transects relative to all field transects, the correlation is very poor. Figures 18 and 19 are correlation plots of: (1) the UST values, (2) the total light aromatic hydrocarbons, and (3) the mean TPH for the mean value of each transect at each sampling period for all field samples. These data also show that the Microtox values are highly influenced by toxicants other than residual petroleum.

Table 1 Linear fit values (r) for all field samples (BS, OB, and CC) and oiled samples only (BS and OB).	**All Transects**	**Oiled Transects**
TPH	0.176	0.370
TTAH	0.198	0.292
N-C4N	0.189	0.273
F-C3F	0.204	0.311
D-C3D+P-C3P	0.223	0.341
Lt AH (N-C4N+F-C3F)	0.191	0.277
Hv AH (TTAH-Lt AH)	0.227	0.351

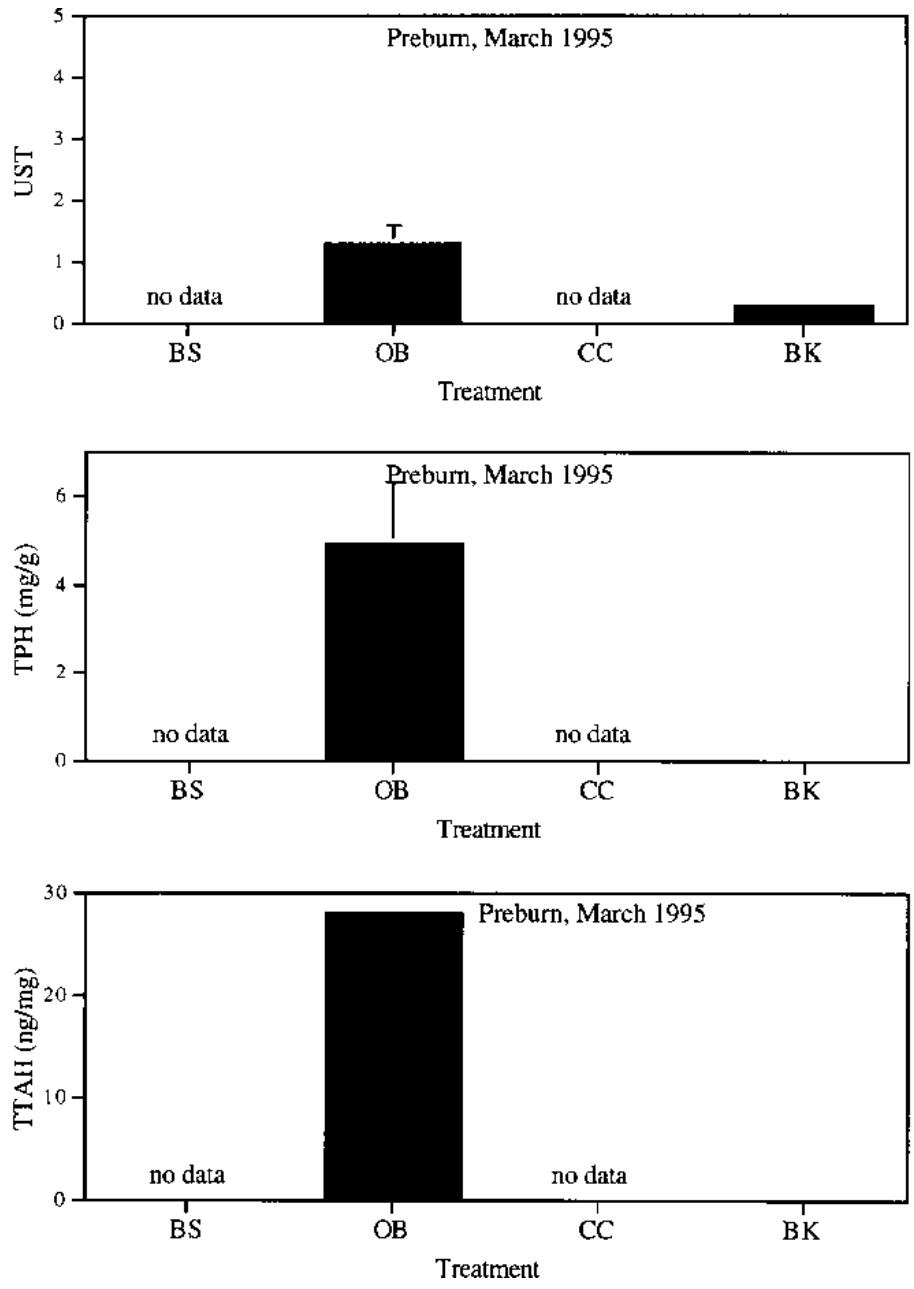

Figure 11 *Comparison of the Microtox assay results or UST (top), TPH (middle), and TTAH (bottom) for the preburn samples collected at the OB transect.*

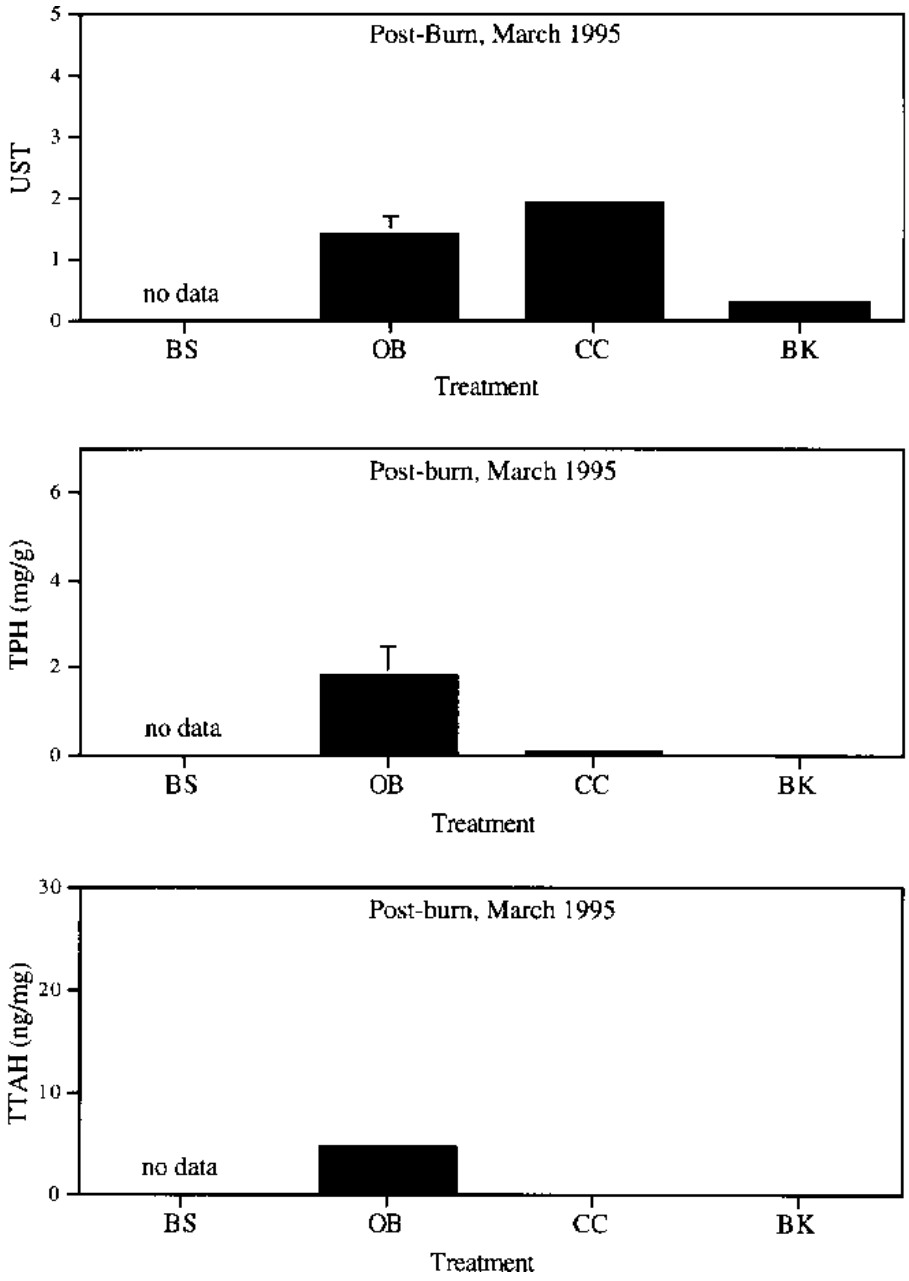

Figure 12 *Comparison of the Microtox assay results or UST (top), TPH (middle), and TTAH (bottom) for the post-burn samples collected in March, 1995.*

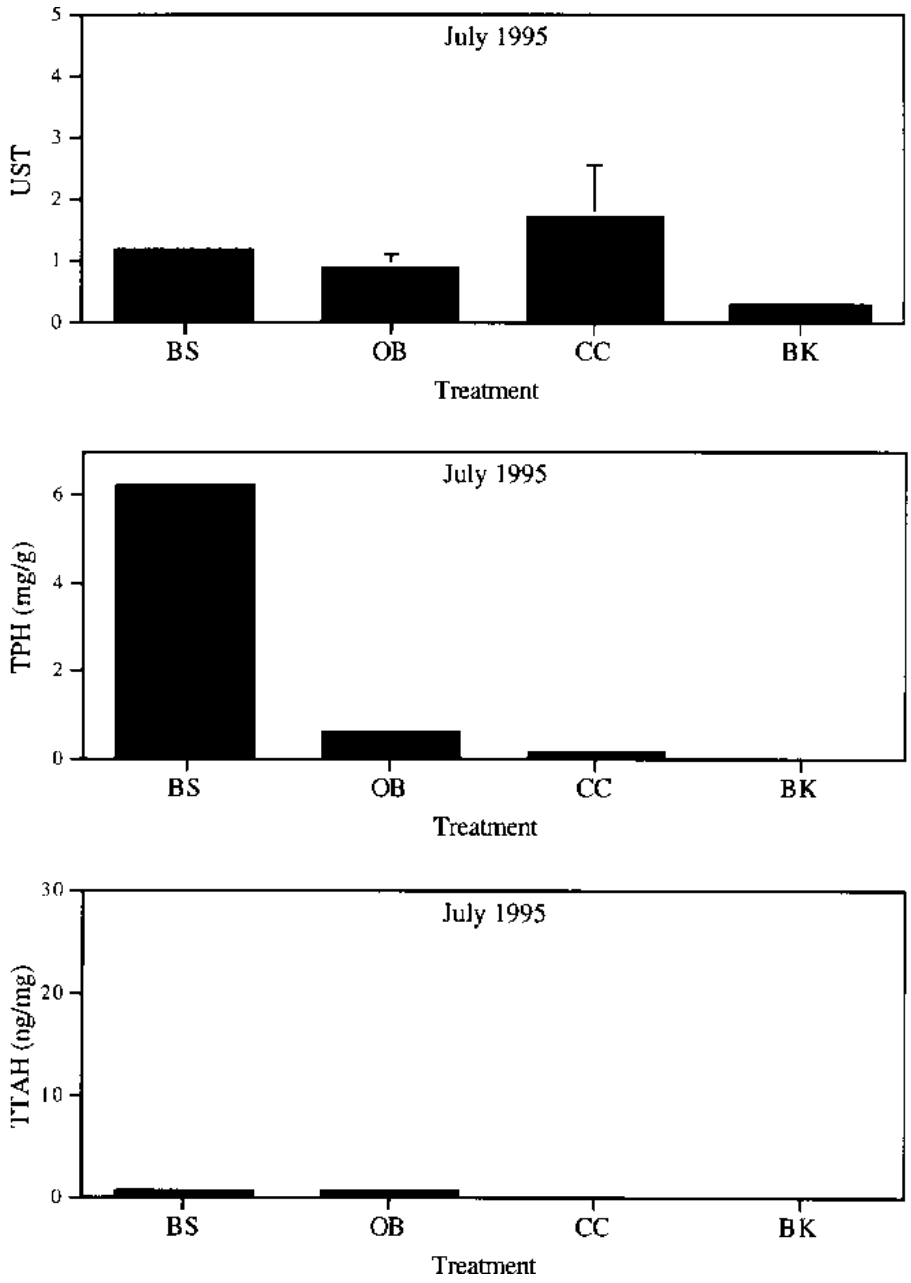

Figure 13 *Comparison of the Microtox assay results or UST (top), TPH (middle), and TTAH (bottom) for the post-burn samples collected in July, 1995.*

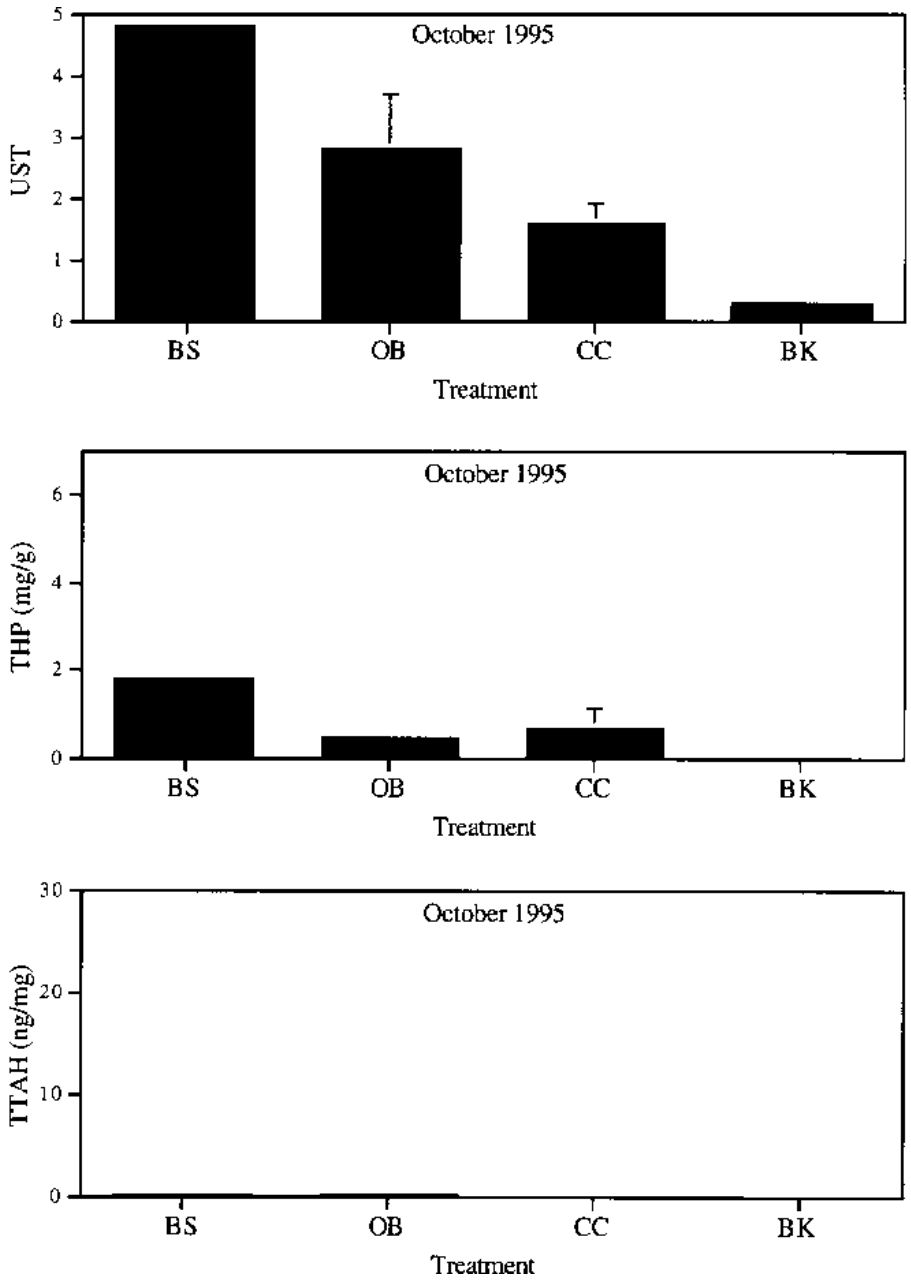

Figure 14 *Comparison of the Microtox assay results or UST (top), TPH (middle), and TTAH (bottom) for the post-burn samples collected in October, 1995.*

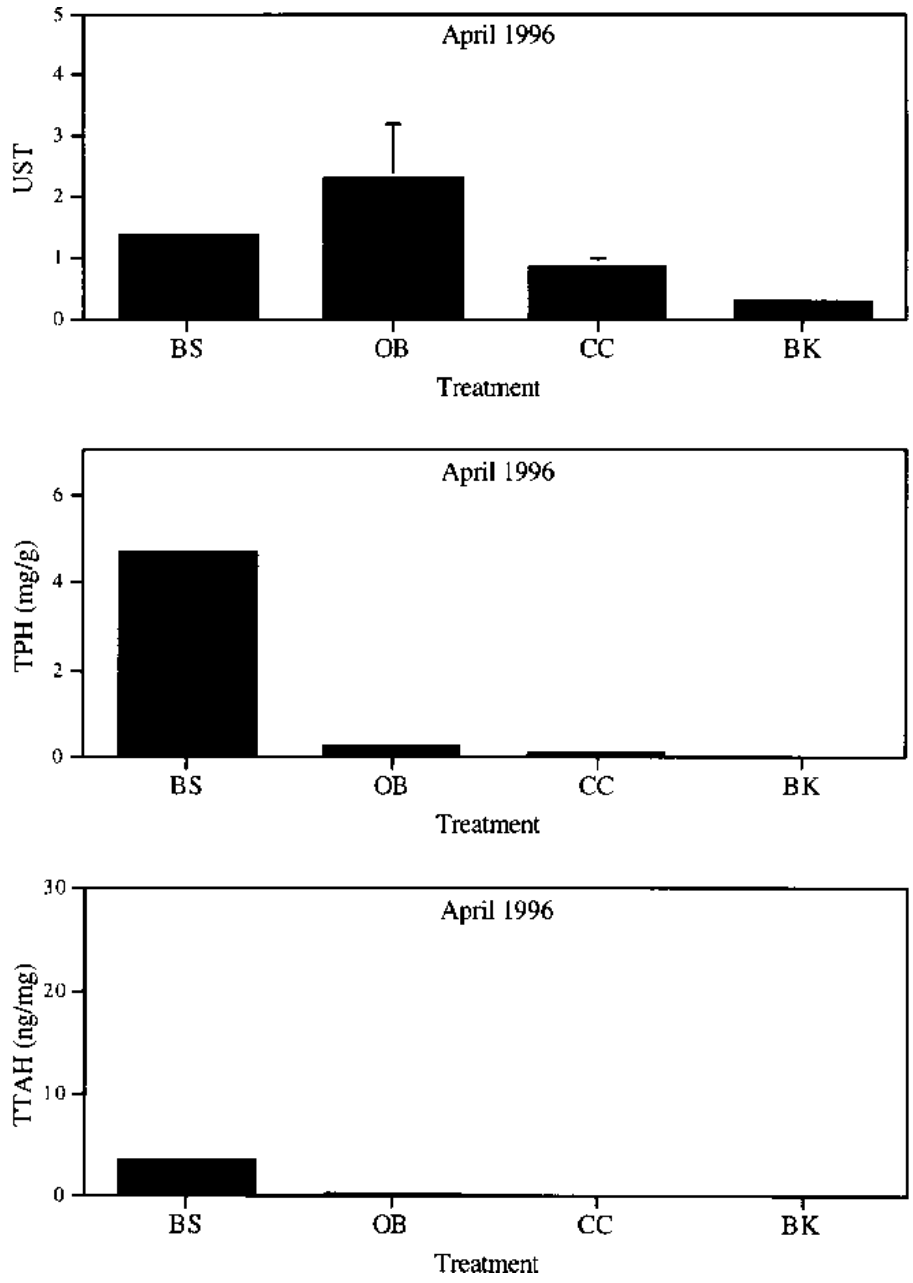

Figure 15 *Comparison of the Microtox assay results or UST (top), TPH (middle), and TTAH (bottom) for the post-burn samples collected in April, 1996.*

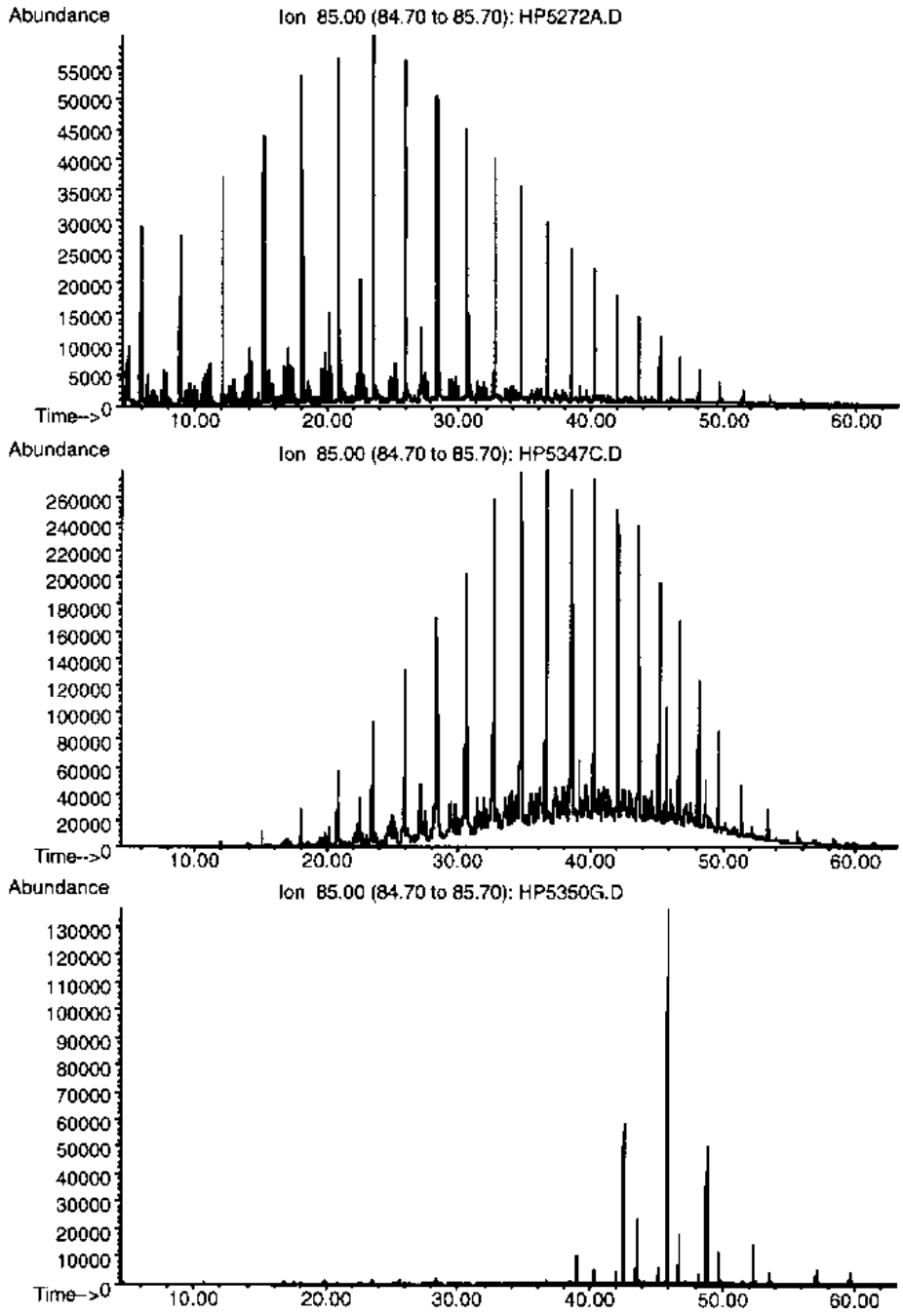

Figure 16 *Chromatographic comparison of the normal alkane distribution detected in an OB composite sample and a CC composite sample. Both samples were collected in October, 1995.*

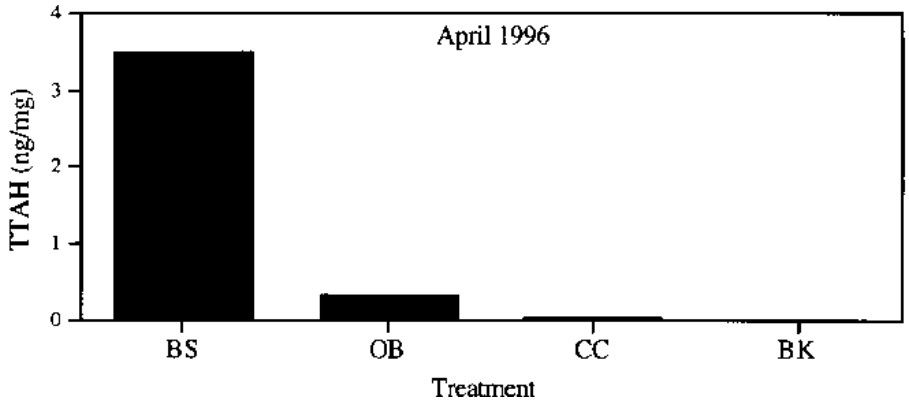

Figure 17 *Comparison of the Microtox assay results or UST for the post-burn samples collected in April, 1996.*

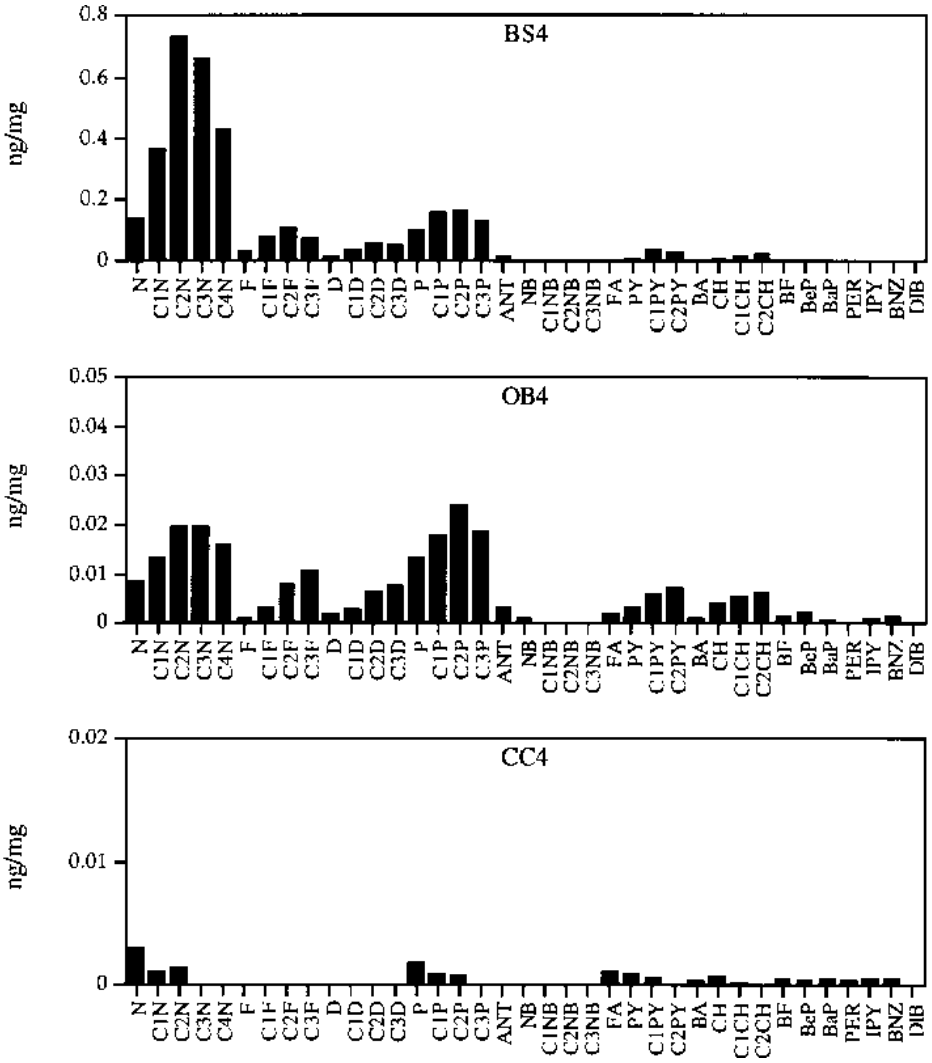

Figure 18 *Comparison of the mean AH profiles for the post-burn samples collected at the BS (top), OB (middle), and CC (bottom) transects in April, 1996 .*

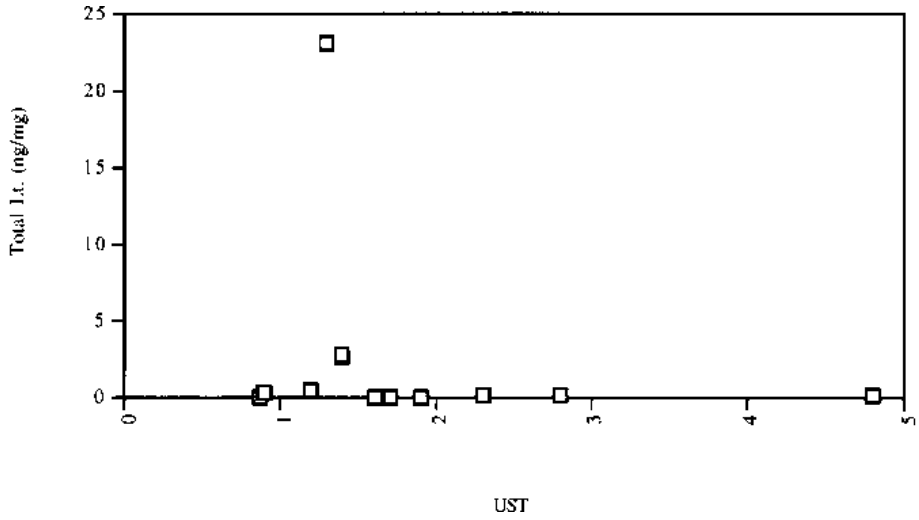

Figure 19 *Graph of lower molecular weight (light) AH concentration versus UST for the field data (mean values for BS, OB, and CC at each sampling period).*

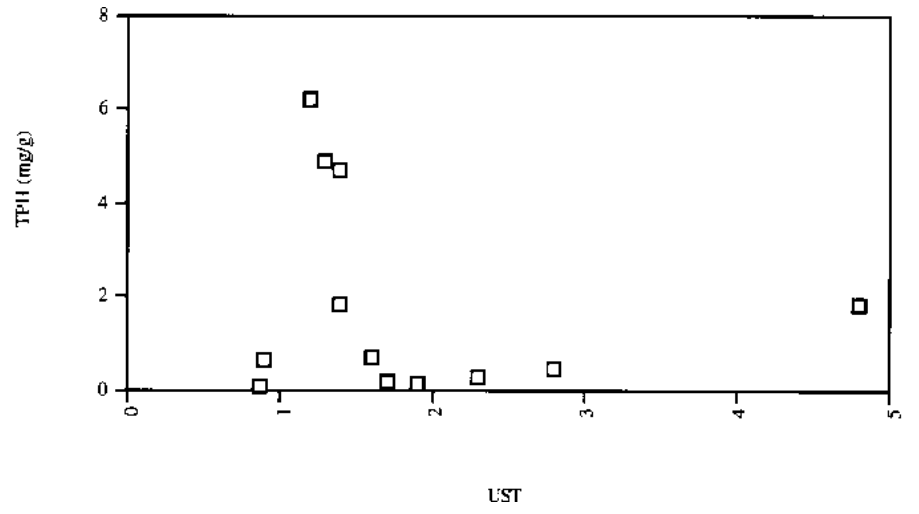

Figure 20 *Graph of TPH concentration versus UST for the field data (mean values for BS, OB, and CC at each sampling period).*

3.3 Aqueous Extraction Assays

Samples collected during April, 1996 were subjected to a DI water extraction and Microtox analysis in addition to the standard solvent extraction reported above. With the exception of one of the two burn site samples, the water extracts resulted in EC_{50} values below the method detection limit (an EC_{50} could not be established). Sample BSa4 (BS station a, April, 1996) was estimated to have a UST value of 9.6. Interestingly, this sample did qualitatively correlate well with the chemistry data. In Figure 18, the AH profile of the BS sample was relatively unweathered and remained highly enriched with naphthalene and the alykylated naphthalene homologues, compounds which are known to correlate with oil toxicity. The TTAH values for the two BS samples were 6.5 and 0.46 ng/mg TTAH, respectively. The BS sample with the lower TTAH value did not exhibit enough response to Microtox to establish a valid EC_{50} value. With the exception of BSa4, none of the samples collected in April, 1996 were above 1 ng/mg or 1 ppm TTAH. Again, a good correlation was found between the TTAH and the observed toxicity for the only sample with a TTAH concentration above 1 ppm. The minimum detection limit for weathered condensate crude oil in sediments by aqueous extraction must be above 1 ppm.

Unfortunately, not all of the water extracts were assayed. A laboratory accident resulted in several of the samples being accidentally left out at ambient temperature over a weekend. Since any data collected from the samples would be highly suspect, they were discarded. The discarded samples were all from a station with very low or no detectable residual oil contamination. Since the highly contaminated samples (except for a single sample at BS) resulted in no detectable EC_{50}, the discarded samples were also likely to be below the assay detection limit.

4.0 Discussion

The everyday use of common petroleum products spurs a wide range of safety and health concerns. Many petroleum products are very toxic while others have little or no apparent toxicity. Gasoline and diesel products require special handling and direct dermal contact should be avoided; taken internally, they are poisons. Yet, selected refined petroleum products are widely used for internal and topical applications such as intestinal lubricants and products that ease the pain of dry chapped lips, e.g., mineral oil and Vaseline. Petroleum wax or paraffin is commonly used in food preservation, e.g., a Wisconsin cheddar cheese wheel dipped in red wax. In addition, selected hydrocarbons such as waxes are biologically synthesized and are common to marsh plants. The wide distribution of USTs observed in the reference oils tested confirms that TPH or the presence of hydrocarbons alone is not a good indicator for assessing residual oil concerns. Comparison of the Microtox results and the detailed chemistry data demonstrated a poor correlation between TPH and TTAH. A subset of TTAH, the light aromatic hydrocarbons only, exhibited good correlation with observed Microtox EC_{50} values.

The Microtox system cuvettes are essentially small aquatic test chambers containing artificial sea water, or cosmetically adjusted DI water, and a small population of bacteria that emits light when the microbial animals are exposed to "natural" conditions (no habitat stress). Toxic agents added to the test water stress the luminescent bacteria and reduce light production. The greater the toxic response, the greater the reduction in light production by the bacteria. Toxic responses are generally measured as the amount of toxicant required to cause a 50% reduction in light production, also known as an EC_{50}. Measurements are generally made at five and 15 minutes after exposure to assess direct and "delayed" effects. All of the assays performed on test oils during this study exhibited little or no difference between the five and 15 minute monitoring points. As a result, the mean of the two times were used for all calculations. Eisman *et al.* (1991) reported similar results.

For a toxic response to occur in the test bacterium, there must be an exposure route. For the short 15 minute Microtox assay, the only real exposure route is direct contact through the water medium. Chemicals with relatively high water solubilities will have direct exposure to the bacteria. Chemicals with very low water solubilities will have limited actual exposure. The Microtox test is biased toward compounds with higher water solubilities that can produce a direct toxic response (a direct toxic response doesn't require activation by chemical or enzymatic reaction). Our results identified a good correlation between the concentration of low molecular weight (light), moderately water soluble aromatic hydrocarbons and a reduction in luminescence. Correlation between the heavy, essentially nonsoluble aromatic hydrocarbons and luminescence reduction was poor. The differences highlight availability and exposure pathway differences to a greater degree than an assessment of true or absolute toxicity differences. Since the system is being used to assess changes in available oil pollution as a result of mitigation techniques and natural weathering, the assay was valid for the study objectives.

The Microtox assay was surprisingly reproducible. Phenol was analyzed as a reference material periodically during the study. The phenol EC_{50} results showed a relative standard deviation of only 15%, within the values of good analytical reproducibility. Unfortunately, the natural environment is not a controlled laboratory environment. A high variability was observed in the field data for all parameters monitored (TPH, TTAH, and Microtox). The accidental burning of the experimental control sites added additional variance to the study. As a result, it was difficult to confirm any statistical differences between the field transects. Background or non-petroleum toxicity was extremely variable and significantly increased the ability of the system to detect toxic responses that were the result of residual oil pollution. Method blanks were within acceptable values. The matrix effects observed significantly reduced the potential of the Microtox system to identify changes in toxicity due to residual oil pollution. The system was being operated at its detection limit for the field samples. The petroleum chemistry data did not correlate with the observed toxicity results. Similar results have been reported by Champoux *et al.* (1990) and Jacobs *et al.* (1993).

Obviously, the Microtox response was influenced by toxicants other than residual oil pollution. Possible sources of background toxicants include biochemicals extracted from

the marsh biomass, sulfur, and metals. Sulfur has been shown to affect Microtox results (Jacobs *et al*. 1992). The water extracted sediments did not show the same background toxicity; this would suggest that the solvent extraction liberated or modified compounds naturally present such that they were more bioavailable in the Microtox assay. The solvent extraction process may have chemically altered or enhanced the exposure potential. Changes in sediment toxicity using different solvents have been reported (Ho and Quinn, 1993).

The observed toxicity at the OB transects after the burn may be dominated by residual oil and not biogenic hydrocarbons since the vegetative plants were burned. The toxicity measured at the control stations was primarily due to biogenic sources. Within six months, the OB transects had recovered some measure of vegetative cover. The toxicity results may be due to both residual oil and biogenic matrix affects. Regardless, only poor correlations were established between the field petroleum chemistry data and the Microtox results. The observed background toxicity was derived primarily from agents extracted in the marsh samples (solvents chemically modified the sample matrix). Laboratory method blanks were highly consistent and at levels significantly below the field samples.

Sample storage was not an issue when the study began, but was potentially identified as a problem in a recent paper by Becker and Ginn (1995). It appears that storage time has a significant effect on Microtox results. Overall the Microtox test varied unpredictably, and often the toxicity values increased with storage. The results reported by Becker and Ginn suggest that effects of storage time are greatest for samples with low to intermediate levels of toxicity (such as the field samples in this study). The degree to which the Rockefeller Refuge results were affected by storage time is impossible to predict. Becker and Ginn's research investigated only the water extractable fraction. The observed effect of the solvent extraction may have a much greater effect than storage time on the Rockefeller Refuge sample set. A Microtox "solid-phase" test has been developed specifically for testing solid phase or sediment samples (this assay is not currently available in our laboratory). The alternate assay may use chemical extractions to enhance the detection of toxicity of insoluble organic compounds such as the heavy AH. As this study showed, these compounds modify the sample matrix and distort results due to chemical manipulations and solvent synergism (Brouwer *et al*. 1990; Kwan and Dutka, 1992). Future investigations to assess residual oil toxicity in marsh sediments should consider using the solid-phase assay.

5.0 Summary

A strong positive correlation was observed between the light aromatic hydrocarbons and observed toxicity as measured by the Microtox system and a wide range of weathered reference oils. The correlation between the TPH, TTAH, and heavy aromatic

hydrocarbons and the Microtox values was poor. Clearly, aromatic hydrocarbon composition, and not just concentration, is a key factor in assessing residual oil toxicity.

Chemistry data showed a significant change in oil contamination after the prescribed *in situ* burn and subsequent natural weathering. Comparison of the chemistry monitoring data to the Microtox data showed a poor correlation between oil loss at the Rockefeller Refuge site and sediment toxicity as measured by Microtox. Residual oil concentration did not correlate with the apparent toxicity differences observed. Microtox data was greatly influenced by matrix effects (background toxicity). The ability of the Microtox system to quantify change directly related to the presence of residual oil pollution was limited by a lack of assay sensitivity relative to analytical chemistry, the presence of background toxicity derived from other anthropogenic and natural biogenic sources, and storage effects on sample integrity. Within the first six months, no differences were readily observed in the Microtox results between the OB and CC sites, yet the chemistry data clearly identified elevated oil contamination. As a result, the Microtox assay of solvent-extracted marsh sediment samples provided very little data to assess efficacy or recovery after the prescribed *in situ* burn at the Rockefeller Refuge.

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