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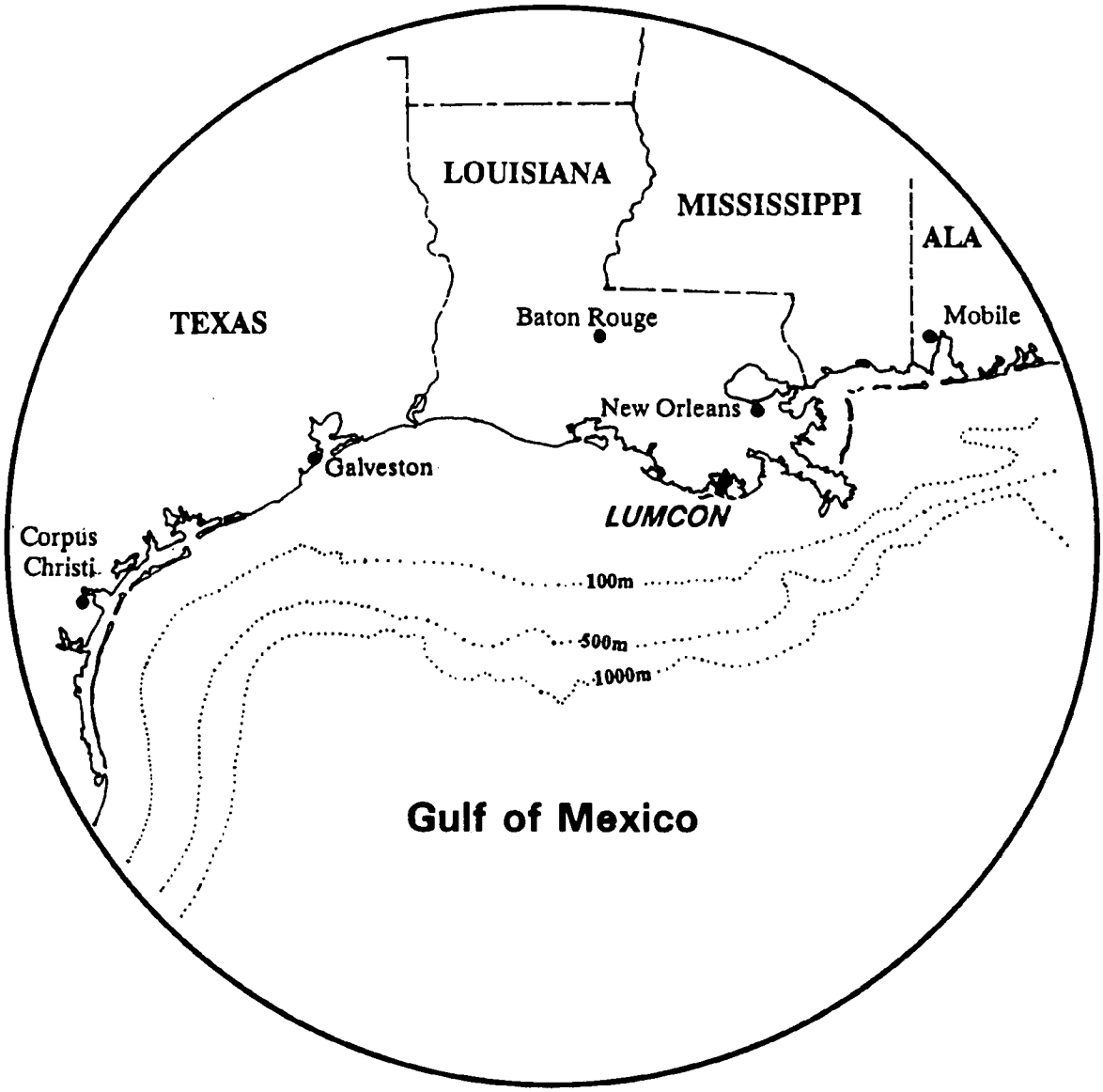
Bioavailability and Genotoxicity of Produced Water Discharges Associated with Offshore Production Operations



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Minerals Management Service
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University Research Initiative
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ABSTRACT

Discharges from offshore oil and gas production are drilling fluids, drill cuttings, and produced water. In coastal regions, effluents are discharged into fresh or brackish marshes or wetlands which are spawning sites for many economically important fish and shellfish. The assessment of ecological health impact of marine and estuarine pollution require sound technology for monitoring and assessing risk of pollutant impacts. Field-exposed organisms yielded much lower ethoxyresorufin 0-deethylase activities (EROD) than experimentally induced levels reported in microsomes of other fish species. Clear patterns of EROD induction were not evident in fish sampled from contaminated areas. EROD was highly variable in liver microsomes of all fish, regardless of the area from which they were sampled, and in some cases showed overlap between responses in control and impacted areas. Obvious correlations were not evident between antioxidant enzyme activity and distance from point source offshore dischage, contaminated coastal sites vs. reference sites, species, collection time, depth of habitat, sediment-dwelling fish vs. demersal fish and between offshore sites. Microsomal P450 was found to be mostly denatured or highly contaminated with other interfering CO-binding chromaphores. EROD, P450 reductase and CO-binding data should be reconciled before tenable conclusions of the effects of pollutant outfall can be made. Mutagen mobilization into co-solvent extracts was detectable by the *Salmonella typhimurium* assay, with tester strains varying in sensitivity. Promutagens were detectable in all extracts, via activation with Arochlor-induced rat liver S9. Mutagenicity varied among co-solvent concentrations, perhaps reflecting mobilization of different arrays of compounds. Mutagens were not detected with the *umu* gene induction assay, which uses *S. typhimurium* 1535/psk1002, whether assays were conducted in the presence or absence of fish liver S9 or microsomes. Isomer specific analyses of bioaccumulated petroleum aromatic hydrocarbons in tissues of aquatic organisms indicate the relative bioavailability of these contaminants, which may enter biological foodwebs in aquatic systems. This suggests that contaminated sediments remain as bioavailable toxic substances even after discharges are suspended. Our data confirm recognized problems in biomarker research; fish from large areas display temporal and spatial variability; biochemical responses of fishes are variable as a function of species, sex, gonadal status, season, temperature, and nutrition.

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EXECUTIVE SUMMARY

The major waste streams associated with offshore oil and gas production are drilling fluids, drill cuttings, and produced water. The ecological and human health hazards of marine and estuarine pollution make it imperative to seek new and improved technology for monitoring and risk assessment of pollutant impacts. This report presents results of laboratory and field studies directed by the co-principle investigators.

Laboratory studies of the effects of petroleum-contaminated sediments on ethoxyresorufin O-deethylase (EROD) activities the marine worm, *Nereis virens*, have been conducted. Results of initial experiments indicated induction of EROD activity in *N. virens*, exposed to petroleum-contaminated sediment from Pass Fourchon, the site of a major separation facility for both the coastal and offshore oil and gas operations in Louisiana. Worms were embedded in 20% and 50% (w/w) sediment mixtures for 7 and 14 days. EROD activity was detected in microsomal fractions extracted from excised gut tissue. EROD activity in worms exposed in 20% sediment was ~14-fold higher than controls after 7 days; after 14 days of exposure the increase in EROD was ~20-fold over controls. When exposed to 50% sediment a 27-fold induction of EROD was observed after 7 days, and after 14 days induction was ~32-fold. For unexplainable reasons these data could not be reproduced in two subsequent experiments. However, EROD activity in invertebrates is traditionally difficult to quantify. *N. virens* exhibited EROD induction in gut tissues, when exposed to benzo(a)pyrene via food (Fries and Lee, 1984), thus the data need to be further reconciled. Western blot analysis performed on the worm gut microsomes probed with rabbit anti-trout CYP1A1 polyclonal antibody did not indicate the presence of proteins with common epitopes of CYP 1A proteins.

Laboratory challenge experiments were conducted with three mutagenic agents, to evaluate the responsiveness of EROD induction in adults, larvae, and embryos of the killifish. Cyclophosphamide (CP) and ethylmethanesulfonate (EMS) are two benchmark mutagens with known genotoxic effects on *Fundulus*.; 3-methylcholanthrene (3-MC) is a classical pro-mutagen, MFO inducer and model carcinogen in mammalian studies. EROD activity was detected in microsomes from whole embryos, larvae, or adult livers. The mean EROD activity in control adult livers was 0.08 nmol resorufin produced/min/mg protein. EROD was not detected in control embryos or larvae. 3-MC exposure induced EROD to 2.8, 1.9, and 2.2 nmol/min/mg in embryos, larvae, and adults, respectively. EMS induced EROD to 1.50, 1.60, and 0.57 nmol/min/mg. CP induced to 1.25, 1.20, and 0.18 nmol/min/mg.

Field validation of the EROD assay as a biomarker in teleost fish may provide a sensitive index of environmental pollution, to detect exposure history and to qualitatively establish the bioavailability of dissolved and sediment-bound aromatic hydrocarbons. Bottom-dwelling fishes were periodically collected from offshore petroleum production platforms. S9 and microsomal fractions were prepared from pooled or individual livers, and assayed for EROD activity. EROD induction was not observed in S9 of bay whiff (*Citharichthys spilopterus*); levels in fringed flounder (*Etropus crossotus*) and shoal flounder (*Syacium gunteri*) ranged between 0.4-5.2 (mean = 2.9) pmol resorufin produced /min/mg S9 protein and 0.9-9.1 (mean = 3.6), respectively. Field exposed fishes yielded much lower EROD values than in microsomes from experimentally induced fish reported in the literature. S9 preparations were prone to inhibitory effects and a larger base of field data is needed to validate the use of EROD for detecting exposure history and qualitatively demonstrating bioavailability of dissolved and sediment-bound chemicals. Collecting trips to offshore and coastal marsh areas yielded fish specimens from both contaminated and control (pristine) locations. Pristine locations were defined as those which contained less than 50 ppb total aromatic hydrocarbons as measured by gas chromatography/mass spectrometry. Generally, microsomes from the livers of both control fish and fish from contaminated sites showed very low or no detectable EROD activity.

Clear patterns of EROD induction are not evident in fish sampled from contaminated areas. Liver microsomes of all fish, regardless of the area from which they were sampled exhibited highly variable EROD activities, which in some cases showed overlap. It is difficult to draw inferences from small samplings, but these data do point to some recognized problems in biomarker research. For example, fish from large areas display temporal and spatial variability; biochemical responses of fishes are variable as a function of species, sex, gonadal status, season, temperature, and nutrition. Thus, field studies can result in a disconcerting array of data, even though controlled laboratory studies are very predictable. An objective of biomarker research is to establish the efficacy of the use of the sentinel and this is the stage of this research.

Oxygen radicals (ROS) are produced as a normal part of all aerobic life. Many metals and organic chemicals increase ROS production in biological systems with accompanying oxidative damage. The toxicities of these chemicals are linked to enhanced generation of ROS. The consequences of enhanced ROS production are protein degradation, enzyme inactivation, lipid peroxidation, DNA damage and cell death. Superoxide dismutase (SOD) and catalase measurements of fish liver cytosol were conducted. SOD activities expressed as units/ mg cytosolic protein ranged from less than 1 or below detection limits in Fringed flounder taken from 200 m of ST 53a to 530 units/mg in Shoal flounder caught 500 m from ST 34. Fringed flounder sampled from 200 m of ST 34 had one of the higher SOD levels. In the few cases where antioxidant enzymes were shown to correlate at all with distance from a platform an inverse relationship was observed. The significance of this relationship might relate to depletion of antioxidant defenses under conditions of high xenobiotic exposure. Obvious correlations were not evident between catalase activity and distance from point sources of offshore contamination, contaminated coastal sites vs. reference sites, species, collection time, depth of habitat, sediment-dwelling fish vs. demersal fish and one offshore site vs another.

Aside from the heme-containing cytochromes P450 and b5 there are also peripheral flavoproteins, which serve as reductases for the cytochromes. These reductases are held to the membrane by a hydrophobic binding domain and they contain the prosthetic groups FMN and FAD. Microsomal flavoprotein reductases are rate-limiting components of NAD(P)H-dependent microsomal mixed function oxygenase activities and also extremely active loci of oxyradical production. There are relatively few studies of flavoprotein reductases on submammalian species. Thus, we have studied both NADH- and NADPH-dependent cytochrome *c* reduction to indicate the intra and inter species range of activities and the effects of various Gulf of Mexico field site environments on those activities. In conjunction with reductase activities, attempts were made to measure the specific content of cytochromes P450 in an effort to determine the efficacy of the microsomal preparations and for use in the evaluation of the MFO studies conducted in association with this project. NAD(P)H-dependent cytochrome *c* reductase activities were measured in liver microsomes of fish sampled from various offshore sites. These sites were further subdivided as a function of distance from the platform. NADH-dependent cytochrome *c* reductase ranged from below detectable limits in Bay Whiff caught at ST 52 to approximately 1000 nmol/min/mg microsomal protein in catfish caught at ST 34 (Table 5.1). NADH-dependent activities were consistently higher in catfish (870±170 nmol/min/mg, n = 7), irrespective of the distance from the platform at which they were caught. Fringed flounder had the next highest activities with NADH (83±44 nmol/min/mg, n = 6). Fringed flounder from ST 36, 37, 52, 53, and 54 had notably lower NADH-cytochrome c reductase activities than those from ST 53a or any of those caught from ST 34. NADPH-dependent activities ranged from 0.2 nmol/min/mg in Bay Whiff caught at ST 53 and 55 to 300 nmol/min/mg in catfish caught at ST 34. An unexpected observation was the NADH/NADPH ratios of < 1.0 found in some of the Fringed flounder and Bay Whiff microsomes from the most recently provided samples. NADH-dependent activites are generally greater than NADPH- dependent activites. The specific content of cytochromes P450 were determined in all fish samples for which we were supplied with adequate amounts of microsomal protein. In all cases microsomal P450 was found to be mostly denatured to cytochrome P420 or highly contaminated with other interfering CO-binding

chromaphores. EROD, P450 reductase and CO-binding data should be reconciled before tenable conclusions of the effects of pollutant outfall can be made.

The high degrees of variability among the different species, the lack of correlation between MFO and field parameters, and the denaturation of MFO catalyst in microsomal preparations are all contrary to laboratory conducted exposure studies. Additional major problems were the inconsistency of the catches, which were unpredictable with respect to species, lack of knowledge of the sex and gonadal status of the fish at the times in which they were caught and variable conditions of abiotic factors such as fluctuations in oxygen tension. It is not known to what extent Hurricane Andrew perturbed zones of delineation in temperature, salinity, partial oxygen pressures, sediment-water interfaces, etc. Hurricane Andrew's impact on these abiotic factors could conceivably have resulted in stresses to the organisms which were reflected in the biochemical and physiological homeostasis of the sampled organisms.

Petroleum wastes from produced water separation facilities, marketing terminals and production facilities contain a complex array of normal and branch-chained hydrocarbons, volatile (benzene, toluene, xylenes and ethyl benzene) and semi-volatile (naphthalenes, phenanthrenes, fluorenes, etc.) parent aromatic hydrocarbons, alkylated analogs of these aromatic compounds and various heterocyclic aromatic compounds, e.g. dibenzothiophenes and aza-arenes. Many of these compounds are lipophilic and thus, tend to bioaccumulate in aquatic food chains. To provide a more realistic assessment of genotoxic risk to aquatic organisms studies were conducted to evaluate the utility of co-solvent extracts from sediments in a way that can be related to bioavailability of contaminants. These studies have yielded the following: direct-acting mutagens were mobilized at all co-solvent concentrations but were detected only with strain TA 102. Mutagen mobilization into co-solvent extracts was detectable by the *Salmonella typhimurium* assay, with tester strains varying in sensitivity. Promutagens were detectable in all extracts, via activation with Arochlor-induced rat liver S9. Mutagenicity effects varied among co-solvent concentrations, perhaps reflecting mobilization of different arrays of compounds. Low co-solvent fractions enhanced extraction of water-soluble mutagens but suppressed extraction of lipophilic mutagens. In high co-solvent fractions, extraction of lipophilic mutagens was significantly increased. Increasing co-solvent fraction appeared to suppress the extraction of some water-soluble mutagens. TA 102 was the most responsive tester strain with these extracts and yielded very reproducible results. TA 98 showed no response to mutagens in these extracts. Attempts to detect mutagens with the *umu* gene induction assay, which uses *S. typhimurium* 1535/psk1002 did not detect mutagens whether assays were conducted in the presence or absence of fish liver S9 or microsomes. Chemical analyses of the co-solvent extracts by GC/MS may yield information on the identity of possible mutagens present.

The effects of sediment exposures on the indigenous organism's ability to modulate the mutagenicity of the benchmark promutagens 2-aminoanthracene (2AA) and 2-acetylaminofluorene (2AAF) was investigated by the *umu* gene induction assay. The ability of microsomes from Lake Champagne catfish and Pass Fourchon catfish to activate 100 mM 2AA was compared with that of liver microsomes from control rats and from rats pre-treated with 3-methylcholanthrene (3MC), an aromatic hydrocarbon known to induce enzymes that activate these promutagens in the rat. The rat samples were run with 200 mg of microsomal protein and the fish with 50, 100 and 200 mg of protein. The results showed a 1.6-fold and a 2.4-fold enhancement of *umu* gene induction with microsomes from control and 3MC-treated rats, respectively, but no increase was observed over background controls with the fish samples. Rat liver cytosol contains amine oxidases which are capable of activating certain arylamines to mutagens in the Ames mutagenicity assay. Experiments were conducted with cytosolic fractions from Atlantic Croaker (*Micropogon undulatus*) (Pass Fourchon), Threadfin Shad (*Dorosoma petense*) (Lake Champagne) and Pinfish (Lake Champagne) liver to determine if fish liver cytosolic fractions catalyzed arylamine activation in the *umu* mutagenicity assay and if so, was

such catalysis altered in fish from contaminated sites. Cytosolic protein was tested separately at 400 mg and 800 mg; no significant increase in gene induction was observed over background.

An objective of biomonitoring studies is to determine amounts of bioconcentratable organics present in petroleum-related waste streams and receiving environments. Several criteria for the methods must be met, including: a wide dynamic range (~0.01 ppb to 100 ppm)-7 orders of magnitude in concentration; compound specificity-discriminaton of individaul compounds from biological and geological matrices; compound quantitation- ability to determine concentrations of specific compounds; sufficient "fingerprinting" capability to discriminate between multiple sources of similar contaminants. Methods should allow for frequent sampling and analysis and a minimum of procedural steps to obviate against losses or contamination. Sediment bound contaminants associated with chronic produced water discharges which accumulate over time are bioavailable to the filter-feeding bivalve *Crassostrea virginica.* Accumulation was observed in three days of exposure and increased toward steady-state for 21 days or more. Petroleum-associated aromatic hydrocarbons increased in all experiments in a dose- and time-dependent fashion. Mortalities observed in higher-dose groups appeared to be contaminant-related. Some effects on accumulation of the compromised physiological state of toxicant-stressed oysters were observed in the highest dose group at 14 days. Isomer-specific analyses of bioaccumulated petroleum aromatic hydrocarbons in tissues of aquatic organisms yields information on the relative bioavailability of these contaminants, which rapidly enter biological foodwebs in aquatic systems. This suggests that contaminated sediments remain as bioavailable toxic substances even after discharges are suspended. Since some of these foodwebs potentially lead directly to man, there may be both ecological and human health risks associated with in-place, sediment-bound contamination from produced waters.

CHAPTER 1

INDUCTION OF EROD IN *NEREIS* AFTER EXPOSURE TO CONTAMINATED SEDIMENT.

FOUR INDEPENDENT EXPERIMENTS WITH TIME AND CONCENTRATION VARIABLES.

WESTERN BLOT STUDIES OF INDUCED CYP ISOFORMS.

L.A. Reily, G.W. Winston, J.C. Means, Z.-M. Yan and S.B. Dobias

INTRODUCTION

The aquatic environment is continually being challenged by pollutants, with oil spills and effluent discharges representing major sources of estuarine and marine pollution. Oil drilling operations generate very large volumes of effluent water. During the production of oil and gas, water trapped within permeable, petroleum-bearing sedimentary rocks is brought to the surface, carrying with it crude oil, drilling fluids, and various geological materials (Boesch and Rabalais, 1985; Daniels et al., 1990). This water, called produced water, is composed of a complex mixture of sub-surface water (formation water) and solid and liquid geological materials, including crude oil, and treatment chemicals, and may contain elevated levels of inorganic (trace metals) and organic (petroleum hydrocarbons) substances of a potentially toxic nature. Major waste streams of drilling fluids, drill cuttings, and produced water are associated with offshore oil and gas production. In Louisisana, produced waters were historically discharged into fresh or brackish marshes or wetlands, which also serve as spawning sites for a wide variety of species of fish and shellfish. Acute and chronic releases of petroleum hydrocarbons and other xenobiotic substances into surface waters affect biota and disrupt entire ecosystems. Moreover, bioavailability of hazardous compounds in aquatic environments increasingly presents a direct threat to man via seafood consumption. The expanding ecological and human health hazards of marine and estuarine pollution make it imperative to to seek new and improved technology for monitoring and risk assessment of pollutant impacts.

Biological monitoring methods assess toxicity of xenobiotic compounds, synergistic interactions of multiple compounds, and, moreover, involve components of uptake and accumulation phenomena. Biomonitoring regimens yield assessments of toxicant impact which are quite tangible.

Certain polychaete species are the dominant individuals in areas of oil spills, refinery effluents, oil field brine effluents, and natural oil seeps (Reish, 1971; Baker, 1976; Armstrong et al., 1979; Spies et al., 1979; Sanders et al., 1980). Though reduced in numbers after oil spills, species such as *Arenicola marina* and *Nereis diversicolor* reestablish in the sediments in spite of the continued presence of oil (Prouse and Gordon, 1976; Levell, 1976; Baker, 1976).

Exposure of vertebrates to benzo(a)pyrene or other natural or synthetic inducers causes increases in MFO activity and cytochrome P-450 content, and produces new cytochrome P-450 (Lu et al., 1973; Philpot and Bend, 1975; Bend et al., 1979; Johnson, 1979; Corbett et al., 1982; Stegeman, 1980; Payne et al., 1987). The properties of microsomal enzyme systems in

marine invertebrates have been extensively reviewed (Stegeman, 1981; Lee, 1981; Lee et al., 1983; James, 1989; Livingstone, 1989). The cytochrome P-450 mediated mixed-function oxygenase (MFO) system is responsible for the oxidation of a variety of foreign organic compounds. Lee (1981) reported that the sandworm, *Nereis virens* possesses a mixed function oxygenase system (MFO) which may provide a useful biological indicator of xenobiotic challenge. Evolutionary impetus for the MFO system may be to facilitate the elimination of foreign compounds, by converting them to more water-soluble metabolites (Lee, 1981). This apparently enhances the individual's ability to eliminate them. Animals with active hydrocarbon metabolism accumulate lower levels of parent PAHs, and the higher rates of metabolism associated with P450 induction can enhance the rates of PAH elimination (Lu et al., 1977). However, the patterns of PAH metabolism in invertebrates can differ from those found in vertebrates.

Molluscan and crustacean microsomal enzymes also transform diverse chemicals, including aromatic hydrocarbons (Jewell and Winston, 1989; James, 1989; Winston et al., 1991). Rates of hydrocarbon metabolism detected *in vitro*, in these invertebrates are 1 to 2 orders of magnitude lower than those seen in most teleost fish liver microsomes. Levels of cytochrome P-450 in mollusks and crustaceans are comparable to those seen in some untreated fish (but fish vary markedly, with exposure to inducers). Polychaete MFO has similarities to MFO systems in vertebrates and other invertebrates, involving similar cofactors and being comprised of phospholipid, cytochrome P-450 and NADPH-cytochrome P-450 reductase. MFO components and cofactors required are the same as those required by insect and vertebrate MFO systems (Lee et al., 1981). Microsomal preparations from the mussel, *Mytilus edulis*, tested *in vitro*, formed quinone derivatives from benzo(a)pyrene,whereas fish microsomes formed epoxides and ultimately yielded hydroxylated derivatives. (Stegeman, 1985; Anderson, 1978; Winston et al., 1988). Other *in vitro* metabolites formed by molluscan extracts include 7,8- and 9, 10- dihydrodiols. Fish rely predominantly on cytochrome P450 acting in epoxide formation, while PAH metabolism in mussels has been attributed to radical oxidation. (Livingstone et al., 1989; Winston et al., 1991). Benzo(a)pyrene hydroxylase, from *Nereis virens* was associated with the lower portion of the intestine, with little or no enzyme activity in the pharynx, esophagus, or upper portion of the intestine (Fries and Lee, 1984). Lee (1981) reported that full enzyme activity required NADPH, Mg^{+2}, and oxygen. Subcellular fractionation of the intestine indicated that the enzyme system was associated with the microsomal fraction (Fries and Lee, 1984) Activity was linear with time and protein, was unaffected by 0.1% Tween-80, was completely inhibited by 0.1% SDS and Triton X-100, and incubation with phospholipase C destroyed activity. These findings indicate a phospholipid component. Activity was diminished by cytochrome P450 inhibitors, e. g., CO, 7,8-benzoflavone and SKF 525-A [2(diethylamino) ethyl 2,2-diphenylpentanoate)]. Temperature optimum was 30° C; pH optimum was between 7 and 8. Fries and Lee (1984) found that benzo(a)pyrene was catalyzed by *Nereis virens* MFO to the major metabolite, 3-hydroxybenzo(a)pyrene. Also produced are small amounts of 6-hydroxy- and 9-hydroxy-derivatives, and the two diols: trans- 4,5- dihydrodiolbenzo(a)pyrene and trans-7,8-dihydrobenz(a)pyrene.

The rates and pathways of xenobiotic metabolism in fish and invertebrates can have a direct impact on the organisms themselves, and may affect other organisms via food web and commensial associations. Metabolism can activate certain carcinogens and can alter bioavailability and bioaccumulation characteristics of toxicant compounds. Thus, uptake and metabolic transformation influence the fate of xenobiotic compounds in the environment, and can determine the identity and levels of parent compound and metabolite residues which accumulate in organisms. Induction of P-450 can influence both of these aspects (Stegeman and Lech, 1991). In mammals, certain polycyclic aromatic hydrocarbons are metabolically activated by MFO, resulting in the production of unstable epoxides which damage DNA and initiate the process of carcinogenesis (Miller and Miller, 1971; Ames and Sims, 1972). Polynuclear aromatic hydrocarabons (PAHs) are ecologically significant components of crude oil. Stegeman

et al. (1988a) have noted the potential for formation of activated carcinogenic derivatives of PAHs by fish cytochrome P-450 enzymes. Metabolism of PAH is catalyzed primarily by the microsomal mixed function oxygenase (MFO) system, of which cytochrome P-450 is the key component. The MFO system generally produces PAH metabolites which are less toxic and more easily excreted than the parent compound, although, in some cases the oxidation reactions produce electrophilic species capable of DNA binding and mutagenesis (Harvey, 1983).

In the polychaete *Capitella capitata,* exposure to crude oil or the PAH, benz(a)anthracene, in the sediment resulted in the third generation having higher MFO activity than the first or second generation (Lee, 1981). These workers did not detect basal MFO levels in *C. capitata,* but noted the need for dissection of intestinal tissue, which was precluded by the small size of this species.

Molluscs are proven, useful indicators of bioavailable levels of contamination, in applications involving direct analysis of pollutant residues, which may be attributable to their low metabolic activity. But, based on present knowledge, there is little potential for using monooxygenase activity or cytochrome P-450 levels in mollusks, or crustaceans, to indicate their exposure to compounds such as the aromatic and chlorinated hydrocarbons (Stegeman and Lech, 1991). This is due to the lack of any convincing evidence for induction of specific cytochrome P-450 isozymes of monooxygenase activity by any of the hydrocarbon inducers known to be active in the vertebrates. The marine polychaete worm, *Nereis virens* exhibited ethoxyresorufin-O-deethylase (EROD) induction in the gut tissues, when exposed to benzo(a)pyrene via food (Fries and Lee, 1984). These workers also examined feral worms from contaminated and reference sites near Portland, Maine. Field-collected *Nereis virens* from heavily-oiled sediment yielded MFO activities six times greater than that of reference site specimens. Cytochrome P450 content was also higher, but not significantly so.

MFO induction has been suggested to indicate the exposure of organisms to xenobiotic challenges in the environment. Stegeman and Lech (1991) noted that P-450 induction would not detect direct-acting carcinogens and tumor promotors which are non-inducers, although such compounds can be expected to co-occur with pollutants that are inducers. Lee (1981) reported that the sandworm, *Nereis virens,* possesses a mixed function oxygenase system (MFO), which may provide a useful biological indicator of xenobiotic challenge. *Nereis virens* is promising as a bioindicator species, by virtue of its wide geographic distribution, the availability of specimens from commercial sources, and its relative ease to maintain in laboratory culture. Demonstrated responsiveness of a quantifiable MFO activity in *Nereis virens,* such as ethoxyresorufin O-deethylase (EROD), would substantiate the usefulness of this organism/assay combination as a biological indicator of pollution. This study was conducted to evaluate MFO induction in *Nereis virens,* by exposure to authentic, petroleum-contaminated sediment.

Oil spills and produced water discharges represent major sources of estuarine and marine pollution. Biomarker research is often limited to model pollutant compounds or model sediments. However, field use of biomonitoring methods involves not only toxicity of individual xenobiotic compounds, but synergistic interactions of multiple compounds and components of uptake and accumulation phenomena, as well. It is therefore essential, to include authentic environmental samples in the investigation of potential biological monitoring systems. Biomonitoring regimens yield tangible assessments of physiological effects and, additionally, often suggest food web and ecosystem consequences of pollution.

MATERIALS AND METHODS

Authentic field-collected sediments were used to challenge *Nereis virens* in microcosm exposure experiments. Natural petroleum- laden sediments were collected near the produced-water discharge of an oil separation site at Pass Fourchon, in coastal Louisiana (map, Figure 1).

The sediments for this study were collected at a site 400 m from the discharge. Clean fluvial sand was used as a bedding medium for the test organisms prior to experiments, as a control medium for zero- level exposures, and as a diluent for varying sediment concentrations. "Clean" sand was collected from a pristine, spring-fed creek in central Louisiana. Concentrated extracts of sand samples exhibited no petroleum hydrocarbons detectable by standard GC-MS methodology applied in this study.

A considerable body of information on Pass Fourchon has been assembled from laboratory studies (Boesch et al., 1989; Means et al., 1989; Daniels et al., 1990; Means et al., 1990; Rabalais et al., 1991a,b; Means and McMillin, 1993). Pass Fourchon sediment was found to contain a complicated suite of aliphatic and aromatic hydrocarbons (Figure 2). The aromatic fraction of the extract was found to contain moderate quantities of normal aromatic hydrocarbons associated with combustion sources while very high concentrations of a suite of alkylated aromatic hydrocarbons, dominated by the C-2 and C-3 alkylated naphthalenes, with lesser amounts of other alkylated naphthalenes, alkylated phenanthrenes and alkylated dibenzothiophenes. As reported previously, analyses of the produced waters revealed a complex mixture of normal alkyl- and heterocyclic- polynuclear aromatic hydrocarbons (Daniels et al., 1990). Major volatiles in the produced water were benzene and its C-1 to C-3 homologs. Mean benzene concentration was 2,000 ng/l, with various other hydrocarbons present at concentrations less than 0.5% of the benzene concentration. Benzoic acid was the most abundant organic acid, at 3100 ng/ml. Total PAHs were 520 ng/ml, and saturated hydrocarbons totalled 16,000 ng/ml. The daily discharge of produced water at the Pass Fourchon facility was approximately 18,000 bbl/d (Boesch and Rabalais, 1985). This discharge caused heavy loading of sediments in the area, with petroleum hydrocarbons. The most abundant sediment PAHs at this site were the alkyl- substituted phenanthrenes. Daniels and Means (1990) studied genotoxic effects of Pass Fourchon produced waters on fish embryos, using *Cyprinodon variegatus* as a model. They observed an increase in chromosome aberrations in exposures to decreasing dilutions, but noted that the effect was not additive with respect to produced water concentration. They further reported that a specific type of aberration was induced, i.e. chromosomal breaks and extensive alterations in ploidy.

ORGANISMS: Specimens of the omnivorous, sediment-dwelling, polychaete worm, *Nereis virens*. were obtained from commercial sources and maintained in laboratory holding tanks containing a 7 cm layer of clean sand, with 10 cm overlying water. A commercial salts mixture (Fritz Chemical Co., Dallas, TX) was used to prepare the full-strength (32 ppt) seawater used throughout the experiment.

MICROCOSMS: Exposures were conducted in microcosm arrangements, with sediment as the route of dosing. Individual and multiple- animal microcosms were both used. Individual units were preferred, as casualties observed in multiple- animal arrangements were thought to have resulted from territorial aggressiveness among worms. Adequate depth of sediment seemed to lend protection from agressiveness and from self- inflicted injuries. Worms were exposed to 0%, 20 %, and 50% levels of Pass Fourchon sediment. Exposure times were also varied in the experiments, with MFO inductions evaluated after 0, 3, 7, and 14 days. All treatments were replicated in independent duplicate or triplicate studies.

EROD ASSAYS: At the conclusion of treatments, worms were euthanized by freezing or ice-chilling, and dissected. The gut was homogenized in 2 ml TES buffer (0.05 M TRIS-HCl, 1.0 mM EDTA, 0.25M sucrose, pH 7.2) by 4 passes in a 10 ml Potter-Elvehjem tissue grinder, at 250 rpm. Microsomes were purified by multiple centrifugations of the homogenate, according to previously published techniques (Winston et al., 1989). Gross fragments and cell debris were removed at 200 X g for 5 min, and organelles and other elements were sedimented at 10,000 X g for 15 min. The microsomal fraction was pelleted at 40,000 X g for 180 min. Mixed function oxygenase activity (MFO) was evaluated in microsomal fractions, as O-dealkylation reactions from substituted alkoxyphenoxazones. Specifically, the ethoxyresorufin

O-deethylase reaction (EROD) was used to detect the presence of this enzymatic mechanism. EROD activity was assayed spectrofluorometrically, by the method of Burke and Mayer (1983) and Burke et al. (1985), as modified by Winston et al. (1988), with an excitation wavelength of 530 nm, and emission read at 585 nm. Reaction product was determined from fluorescence response of the instrument, as intensity (I). A standard curve was prepared, of resorufin diluted in the reaction matrix. Microsomal protein was determined by a modified Lowery technique,with bovine serum albumin as the standard (Winston et al., 1988). Specific activity of EROD was reported as picomoles or nanomoles of resorufin formed per minute per milligram of microsomal protein.

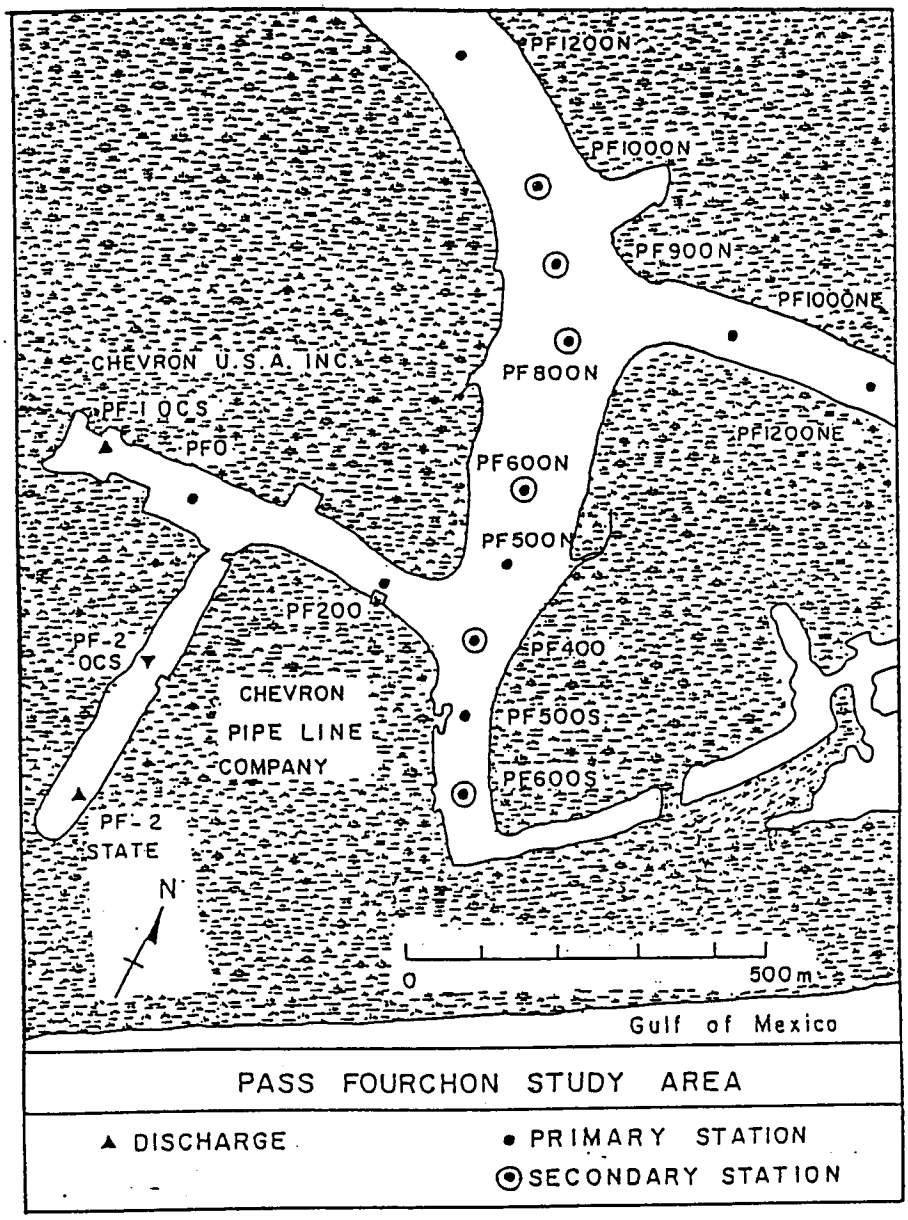

Figure 1. Map of sampling sites, Pass Fourchon, LA.

SEDIMENT DATA FOR PASS FOURCHON 400

NAPHTHALENES	ppb
2-Methylnaphthalene	3.0
1-Methylnaphthalene	1.5
2-Ethylnaphthalene	1.9
1-Ethylnaphthalene	9.1
2,6/2,7-Dimethylnaphthalene	10
1,3/1,7-Dimethylnaphthalene	48
1,6-Dimethylnaphthalene	4.7
1,4/2,3-Dimethylnaphthalene	27
1,5-Dimethylnaphthalene	25
1,2-Dimethylnaphthalene	12
2-Isopropylnaphthalene	nd
1,8-Dimethylnaphthalene	12
1,6,7-Trimethylnaphthalene	197
C3-NAPHTHALENES	9,700
C4-NAPHTHALENES	8,900

PARENT PAH	ppb
Naphthalene	1.7
Acenaphthylene	4.0
Acenaphthene	11
Fluorene	18
Dibenzothiophene	5.6
Phenanthrene	52
Anthracene	16
Fluoranthene	73
Pyrene	54
Benzanthracene	24
Chrysene	84
Benzo(b)fluoranthene	29
Benzo(k)fluoranthene	8.4
Benzo(a)pyrene	12
Indenopyrene	30
Dibenz(a,h)anthracene	17
Benzo(g,h,i)perylene	38

DIBENZOTHIOPHENES	ppb
4-Methyldibenzothiophene	68
2/3-Methyldibenzothiophene	55
1-Methyldibenzothiophene	34
1,2-Dimethyldibenzothiophene	5.9
C2-DIBENZOTHIOPHENES	8,600

PHENANTHRENES	ppb
3-Methylphenanthrene	183
2-Methylphenanthrene	87
4/9-Methylphenanthrene	177
1-Methylphenanthrene	102
3,6-Dimethylphenanthrene	102
3,5-Dimethylphenanthrene	nd
2,6-Dimethylphenanthrene	93
2,7-Dimethylphenanthrene	77
3,9-Dimethylphenanthrene	335
1,6/2,5/2,9-Dimethylphenanthrene	228
1,7-Dimethylphenanthrene	83
1,9/4,9-Dimethylphenanthrene	78
1,5-Dimethylphenanthrene	nd
1,8-Dimethylphenanthrene	32
1,2-Dimethylphenanthrene	22
9,10-Dimethylphenanthrene	nd
1,2,8-Trimethylphenanthrene	84
C3-PHENANTHRENES	23,000

TOTALS	ppb
Total Parent PAH	478
Total Alkylated PAH	52,397
Total PAH	52,875

Figure 2. Quantitative analysis of petroleum hydrocarbon contaminants in Pass Fourchon sediment collected at PF 400 site, 400 m from produced water discharge.

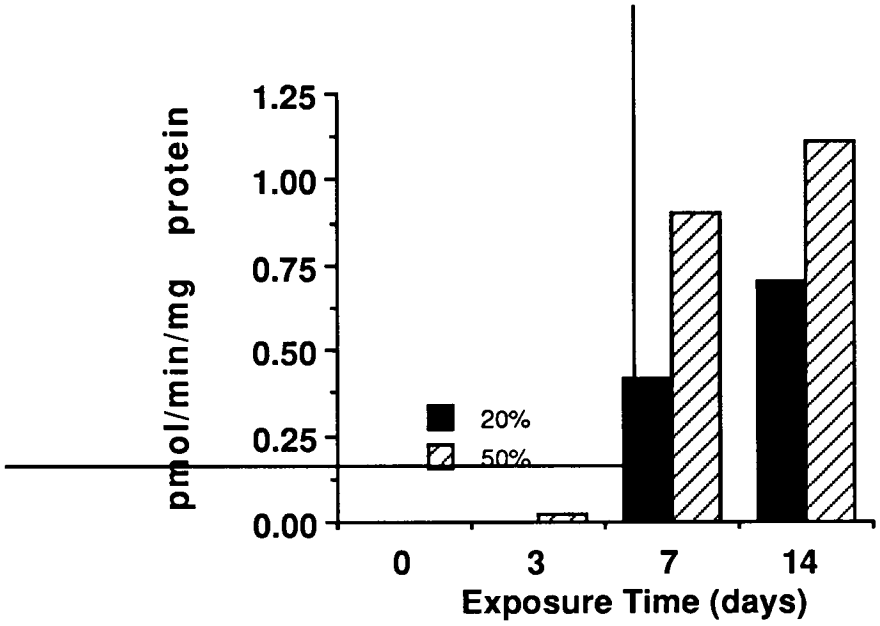

Figure 3. EROD induction in *Nereis virens* by exposure to petroleum-contaminated sediment.

Immunoblot Analyses: CYP1A is an important intermediary in mutagen activation. The P450 IA activities are inducible, and are associated with several tissues, but are most often studied in liver. In mamalian systems, the CYPIA gene family which is inducible by polycyclic aromatic hydrocarbons (PAH), consists of one subfamily of two genes: CYPIA1 and CYPIA2. Immunoblot (Western blot) comparisons were made of worm gut microsomes to known trout cytochrome P450 isozyme 1A1 (courtesy of Dr. D.R. Buhler, Department of Agricultural Chemistry, Oregon State University, Corvallis, OR) and to Arochlor-induced rat liver microsomes prepared in house (Dr. G.W. Winston, Department of Biochemistry, Louisiana State University). The significance of Western blot analysis is in its ability to indicate the presence or absence of a CYP1A ortholog or paralog in this invertebrate's system. The protein bands, separated elecrophoretically, were reacted with a purified and highly specific polyclonal anti-CYP1A1 antibody. The antibody preparation , also kindly provided to us by Dr. Buhler, was produced in rabbit, against trout CYP1A1. The bands of immune complexes were probed with an alkaline phosphatase-linked secondary anti-rabbit antibody.

RESULTS AND DISCUSSION

The EROD assay is one of the most robust MFO assays available for biomarker research, and is not elevated by normal physiological stressors, such as temperature, high or low salinity, high or low oxygen levels. The present study investigated EROD induction in the marine polychaete *Nereis virens,* by exposure to petroleum-contaminated sediments. *Nereis virens* exposed to dilutions of the Pass Fourchon sediment showed good survival rates (greater than 90%) for periods of up to ~20 days. In the first battery of experiments conducted EROD was observed to be induced in organisms exposed to authentic petroleum-contaminated sediments for

at least seven days. Unexposed control organisms had mean EROD activity of 0.03 nmol resorufin formed/min/mg microsomal protein. Sediment control organisms exhibited a similar or lower level of EROD activity. *Nereis virens* exhibited ethoxyresorufin-O-deethylase (EROD) induction in the gut tissues, when exposed in bedded, petroleum-contaminated sediments. Both dose dependent and time dependent responses were observed using this experimental approach. The worms in 3 day exposures yielded quantifiable EROD activities only in the 50% contaminated sediment dose. This activity was not significantly different from the basal level. This may indicate a lag phase in expression of enzyme activity or in the kinetics of xenobiotic uptake, or it may relate to a threshold level of tissue residues required for induction. Worms exposed in 20% Pass Fourchon sediment had EROD activities 14-fold higher than controls after 7 days and 32-fold higher after 14 days. In 50% Pass Fourchon sediment, EROD was 27- fold higher than control after 7 d, and 32-fold higher after 14 d. The levels of EROD activity were consistently higher in the 50% sediment exposure as compared to the 20% exposure. The responses of *Nereis* in these experiments had suggested sufficient bioavailability of dissolved and sediment-bound normal, alkylated and heterocyclic aromatic hydrocarbons to benthic invertebrates to elicit an induction of mixed function oxidase activity in the invertebrates. Two attempts have been made to confirm the data described in the above experiments; in both cases the results were not reproducible. Whether this represents seasonal variation in the response of the worm to the contaminants present in the sediments awaits further analysis. The irreproducibility of the results is inexplicable. Based upon this fact, no definite conclusions as to the efficacy of this marine invertebrate as a sentinel of sediment contamination can be drawn at this time.

There have been no reports of CYP1A induction in marine invertebrates. The present studies have demonstrated time- and concentration- dependent induction of EROD in the nereid model. EROD is regarded as the indicator of choice for presence of the PAH-inducible isoforms of P450, i e., CYP1A. The immunoblot analysis provides stronger evidence for the presence of a CYP1A ortholog in EROD-induced *Nereis virens*., and thus, whether it may be a component of the observed EROD activity. Western blot analysis was performed on two separate occassions with the rabbit antibody raised against trout anti CYP1A1 and compared to a BNF-induced trout liver microsomal fraction. The worm gut microsomes were loaded at 50 μg per lane and the trout liver at 10 μg per lane. The data showed strong recognition of a single protein band of about 54 kDa in the trout liver microsomal fraction, whereas no recognition of epitopes was observed with the worm microsomal fractions (Figure 4).

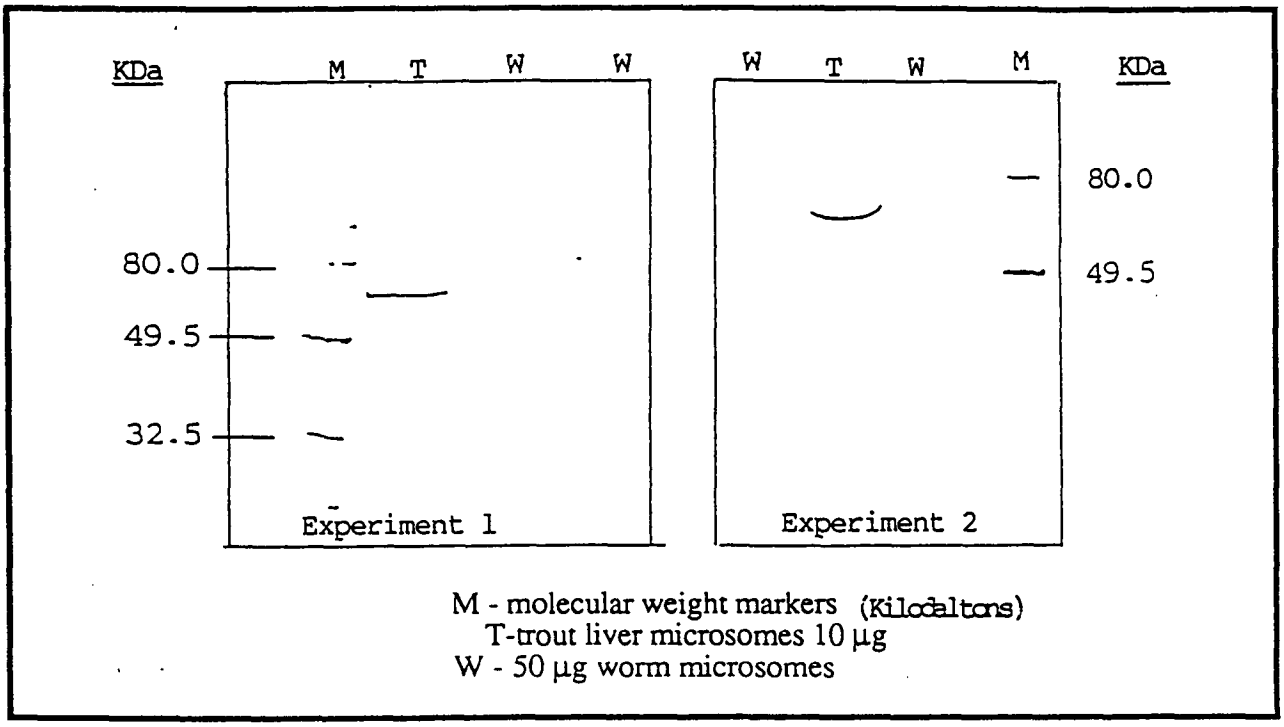

Figure 4. Western blot analysis of *Nereis* intestinal microsomes probed with trout anti-CYP 1A1.

CHAPTER 2

INDUCTION OF ETHOXYRESORUFIN O-DEETHYLASE (EROD) IN THE KILLIFISH (*FUNDULUS GRANDIS*) BY SELECTED MUTAGENS

L.A. Reily, J.C. Means, Z-M. Yan, G.W. Winston

INTRODUCTION

Major discharges of drilling fluids, drill cuttings, and produced water are associated with offshore oil and gas production. In coastal regions, these wastes are discharged into fresh or brackish marshes or wetlands which also serve as the spawning sites for a wide variety of economically important species of fish and shellfish. Pollutant impacts on early life stages of aquatic organisms and on embryonic and larval fish development are of particular significance in coastal estuaries and wetland areas. This has implications for the survival and relative fitness of exposed fish at these stages, in contaminated spawning areas which are widespread throughout coastal Louisiana. Additionally, bioavailability of hazardous compounds in aquatic environments increasingly presents a direct threat to man, via seafood consumption. The obvious ecological and human health hazards of marine and estuarine pollution make it imperative to to seek new and improved technology for monitoring and risk assessment of impacts by such pollution.

Various studies have demonstrated that exposure to benzo(a)pyrene or polychlorinated biphenyls causes increases in mixed-function oxygenase (MFO) activity and cytochrome P-450 content and produces new cytochrome P-450 (Lu et al., 1973; Philpot and Bend, 1975; Bend, et al., 1979; Johnson, 1979; Stegeman, 1980; Varanasi and Gmur, 1981; Corbett et al., 1982; Stegeman et al., 1986; Varanasi et al., 1986; Payne et al., 1987; Van Veld et al., 1988). Similarly, the cytochrome P-450-mediated MFO system exists in invertebrates. MFO is responsible for the oxidation of a variety of foreign organic compounds,which facilitates elimination of lipophilic xenobiotics by converting them to more water-soluble metabolites (Lee, 1981). Animals with active hydrocarbon metabolism accumulate lower levels of parent polynuclear aromatic hydrocarbons (PAH), and the higher rates of metabolism asssociated with P-450 induction, can enhance PAH elimination (Lu et al., 1977). MFO systems in mammals and fish are similarly comprised of phospholipid, cytochrome P-450, and NADPH cytochrome P-450 reductase (Stegeman, 1989). MFO cofactors required are also the same, although the mode of action may differ (Lee, 1981; Lee et al., 1981). Microsomal preparations from fish microsomes formed epoxides and ultimately yielded hydroxylated derivatives (Stegeman, 1985; Anderson, 1978; Winston et al., 1988). The rates and pathways of xenobiotic metabolism in fish and invertebrates can have a direct impact on the organisms themselves, and may affect other organisms via food web and commensial associations. Metabolism can activate certain carcinogens and can alter bioavailability and bioaccumulation characteristics of toxicant compounds. In mammals, certain polycyclic aromatic hydrocarbons are metabolically activated by MFO, resulting in the production of unstable epoxides which may damage DNA and initiate the process of carcinogenesis (Miller and Miller, 1971; Ames and Sims, 1972). Thus, uptake and metabolic transformation influences the fate of these compounds in the individual and in the environment, and can determine the identity and levels of parent compound and metabolite

residues which accumulate in organisms. Induction of P-450 can influence all of these aspects (Stegeman and Lech, 1991). Stegeman and others have studied the potential for formation of activated carcinogenic derivatives of PAHs by fish cytochrome P-450 enzymes (Stegeman and Lech, 1991; Stegeman et al., 1988b).

Polynuclear aromatic hydrocarbons (PAHs) are ecologically significant components of crude oil. Metabolism of PAH is catalyzed primarily by the microsomal mixed function oxygenase (MFO) system, of which cytochrome P-450 is the key component. The MFO system generally produces PAH metabolites which are less toxic and more easily excreted than the parent compound, although, in some cases the oxidation reactions produce electrophilic species capable of DNA binding and mutagenesis (Harvey, 1983).

MFO induction has been suggested to indicate the exposure of organisms to xenobiotic challenges in the environment although, Stegeman and Lech (1991) noted, P-450 induction in sentinel species would not detect direct- acting carcinogens and tumor promotors which are non-inducers, but such compounds can be expected to co- occur with pollutants that are inducers. The present studies investigate the induction of MFO in the killifish, *Fundulus grandis*, as a biological indicator of pollution. MFO evaluation was focused on detection of O-dealkylation reactions from substituted alkoxyphenoxazones, with the ethoxyresorufin O-deethylase reaction (EROD) used to detect the presence of this enzymatic mechanism. EROD has been recently identified as a sensitive sublethal index of environmental impact on several test organisms, its induction indicating: 1) the bioavailability of dissolved and sediment-bound normal, alkylated and heterocyclic aromatic hydrocarbons; 2) the ability of organisms to metabolize these compounds; and 3) the genotoxicity of the compounds and their metabolites. Laboratory challenge experiments were conducted with model compounds, to evaluate the responsiveness of EROD induction in adults, larvae, and embryos of the killifish. Three known mutagenic agents were chosen as model compounds for these studies. Cyclophosphamide (CP) and ethylmethanesulfonate (EMS) are two benchmark mutagens which have been documented with respect to their genotoxic effects on *Fundulus*.; 3-methylcholanthrene (3-MC) is classically used as a pro-mutagen, MFO inducer and model carcinogen in studies of mammalian systems.

MATERIALS AND METHODS

Specimens of the killifish, *Fundulus grandis,* were obtained from commercial sources and maintained in laboratory aquaria, at 7 ppt salinity, 21° C, and 9 hour light photoperiod. Spawning was induced by 12 h light, at 25° C. Fertilized embryos were cultivated at 25° C, 7 ppt salinity, and 12 h light. Hatchling larvae were maintained at 21° C, 7 ppt salinity and12 h light, and fed daily with live *Artemia*.

Laboratory exposure experiments were conducted with the three major developmental stages of *Fundulus grandis*, i. e. adults, embryos, and larvae. Adults were exposed for 24 h to 100 nM EMS or CP in water and sacrificed 5 d later. Because of concern over insolubility, 3-MC was administered to adults by injection (20 mg/kg, in corn oil) on days 0, 4, and 8, and they were sacrificed on day 16. Later studies have suggested that water-column exposures of adults may also be efficacious. Embryos (11 day post-fertilization) and larvae (11 day post-hatch) were exposed to 3-MC, EMS, or CP in the water, at 100 nM concentration and sacrificed 48 hours later. Stock solutions of the model mutagens were prepared in DMSO, and results were compared to DMSO- dosed controls. All experiments were duplicated in independent trials.

Adult fish were euthanized by decapitation, and dissected. Larvae, whole embryos, and adult livers were homogenized in 2 ml buffer (.05 M TRIS-HCl, 1.0 mM EDTA, 0.25M sucrose, pH 7.2) by 5 passes in a 10 ml Potter-Elvehjem tissue grinder, at 250 rpm.

Microsomes were purified by differential centrifugation of the homogenate, according to Winston et al. (1989). Gross fragments and cell debris were removed at 200 X g for 5 min, and organelles and other elements sedimented at 10,000 X g for 15 min. The microsomal fraction was pelleted at 105,000 X g for 60 min. Microsomal mixed function oxygenase activity (MFO) was evaluated by measuring the rates of ethoxyresorufin O-deethylase (EROD) spectrofluorometrically (Burke and Mayer, 1983; Burke et al., 1985), as modified by Winston et al. (1988). Excitation and emission wavelengths were 530 and 585 nm, respectively. Reaction product was determined from a standard curve, prepared of resorufin diluted in the reaction matrix. Microsomal protein was determined by the method of Lowry et al. (1951) technique, with bovine serum albumin as the standard. Specific activity of EROD was reported as nanomoles resorufin formed per minute per milligram of microsomal protein (Figure 5).

RESULTS AND DISCUSSION

EROD induction was demonstrated in *Fundulus grandis* embryos, larvae, and adults by the three model MFO inducers investigated in this study. The various developmental stages of *Fundulus grandis* , particularly the embryonic and larval stages, exhibited substantially elevated levels of MFO activity following short exposures to these mutagens. The mean EROD activity in control adult livers was 0.08 nmol resorufin produced/min/mg microsomal protein. EROD activity was not detected in control embryos or larvae. Exposure to 3-MC induced EROD activities to 2.80, 1.90, and 2.21 nmol/min/mg protein in embryos, larvae, and adults, respectively. EROD activities induced by EMS treatment were 1.50, 1.60, and 0.57 nmol/min/mg protein. CP induced activities of 1.25, 1.20, and 0.18 nmol/min/mg protein, respectively.

CP and EMS are two benchmark mutagens which have been documented with respect to their genotoxic effects on *Fundulus*. (Baksi and Means) 3-MC is classically used as a pro-mutagen, MFO inducer and model carcinogen in studies in mammalian systems. The various developmental stages of *Fundulus grandis* , and in particular the embryo and larval stages, exhibited substantially elevated levels of MFO activity, following exposure short exposures to these mutagens, as demonstrated in figure 5). EROD was detected in microsomal fractions from whole embryos, larvae, or excised adult livers. The mean EROD activity in control adult livers was 0.08 nmol resorufin produced/min/mg microsomal protein. EROD activity was not detected in control embryos or larvae. Exposure to 3-MC induced EROD activities to 2.80, 1.90, and 2.21 nmol/min/mg in embryos, larvae, and adults, respectively. EROD activities induced by EMS treatment were 1.50, 1.60, and 0.57 nmol/min/mg protein. CP induced activities of 1.25, 1.20, and 0.18 nmol/min/mg protein, respectively. Significant among these finding are the determinations of elevated EROD activities in the early development of the metabolic machinery of the embryo and larval fish. This has implications for the survival and relative fitness of exposed fish at these stages in contaminated coastal spawning areas so widespread throughout Louisiana. Also of interest is the relative activities observed in these early life stages with that found in adult livers, a primary organ involved in xenobiotic metabolism. These results suggest that there should be more emphasis on examining the metabolic competences of early life stages of fish.

Many chemical carcinogens occur as procarcinogens, and require activation by metabolic processes to yield carcinogenic derivatives. Oxidative transformations of these compounds are primarily catalyzed by microsomal monooxygenases or mixed function oxidases. The various developmental stages of *Fundulus grandis* , in particular the embryo and larval stages, exhibited substantially elevated levels of MFO activity following short exposures to the mutagens. EROD activity was not detected in control embryos or larvae.

A bioindicator system, based upon EROD induction in *Fundulus grandis,* could prove to be a valuable tool for assessment of potential impacts on both ecological and human

environmental risks associated with increased discharges of petroleum-associated hydrocarbons. As the aquatic environment is a major sink for numerous contaminants, the elucidation of biochemical responses to contaminants, by aquatic animals, becomes increasingly important. The epidemiology of highly elevated rates of idiopathic lesions and neoplasia among some populations of aquatic animals inhabiting polluted environments is, in most cases, far clearer than is generally the case for human cancers suspected of being related to environmental pollution.

The present study investigated EROD induction in the major life stages of the killifish. Experimental subjects showed good survival rates (>90% in all of the treatments used). EROD was induced in adults, larvae, and embryos exposed to the model mutagens tested. The responsiveness of EROD in the killifish indicates promise for development of this assay/ organism system, as a biomarker of contamination in marine and estuarine environments. The EROD assay is one of the most robust MFO assays available for biomarker research. It is highly selective for CYP1A, the aromatic hydrocarbon-inducible form of cytochrome P450, and is not elevated by normal physiological stressors, such as temperature, high or low salinity, high or low oxygen levels. Unexposed control organisms in the present study had mean EROD activity of 0.08 nmol resorufin formed/min/mg microsomal protein, indicating a low basal level of EROD activity.

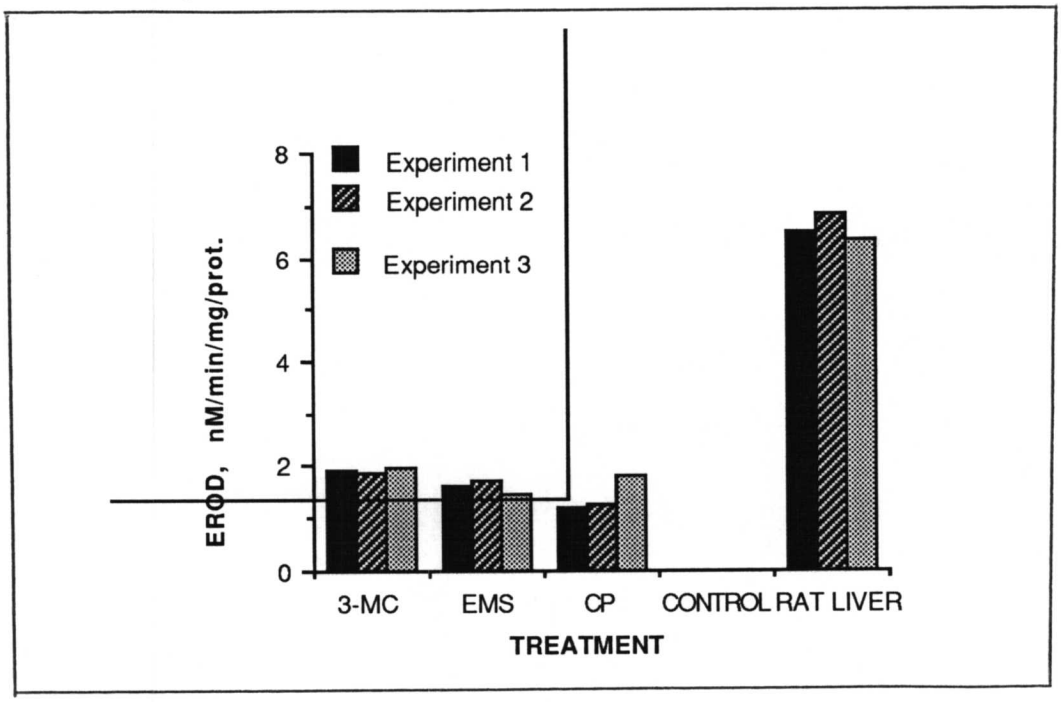

Figure 5a. EROD induction in *Fundulis grandis* larvae in independent replicate experiments.

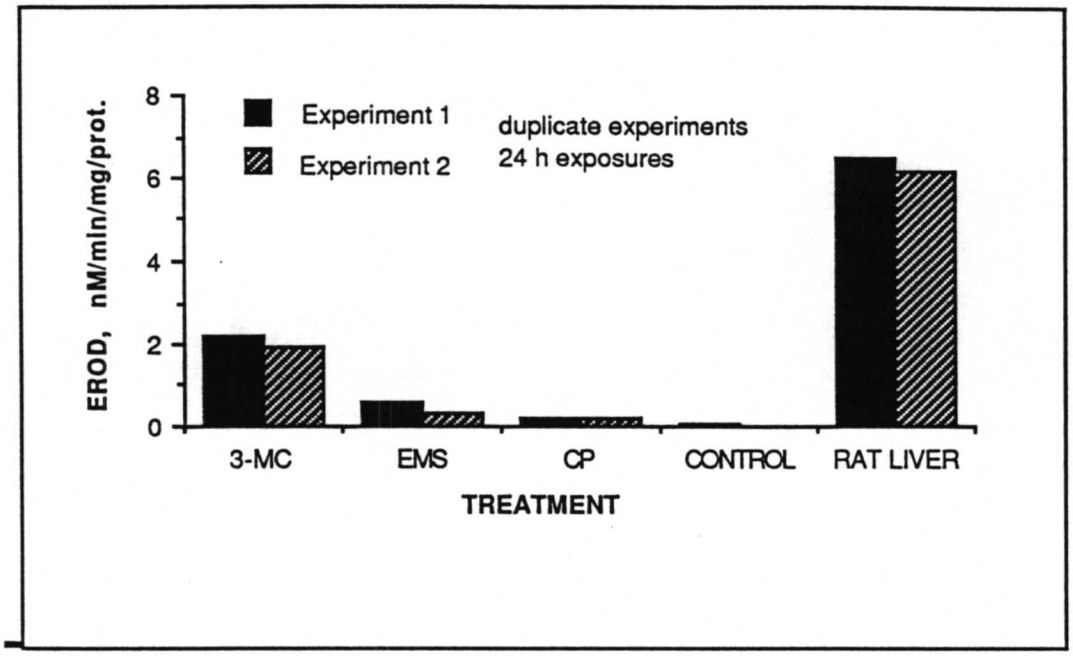

Figure 5b. EROD induction in *Fundulis grandis* adults in independent replicate experiments.

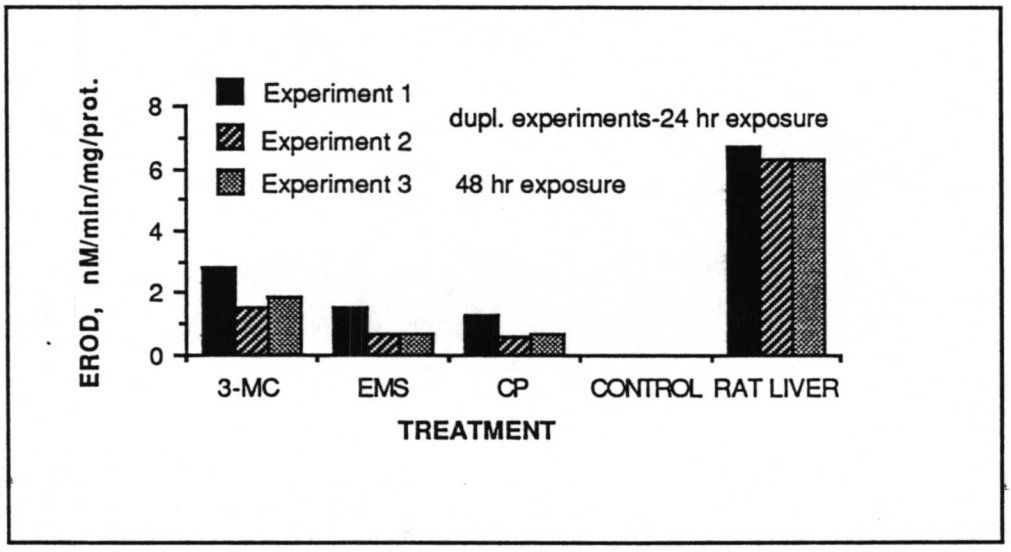

Figure 5c. EROD induction in *Fundulis grandis* embryos in independent replicate experiments.

Since the aquatic environment, particularly sediments, are major sinks for numerous contaminants, the elucidation of biochemical responses to contaminants by aquatic animals is an increasingly important area of research. The epidemiology of highly elevated rates of idiopathic lesions and neoplasia among some populations of aquatic animals inhabiting polluted environments is, in most cases, far clearer than is generally the case for human cancers suspected of being related to environmental pollution. The data generated in these studies may allow the assessment of potential impacts, as well as ecological and human environmental risks associated with increased discharges of petroleum-associated hydrocarbons.

The low background activity, the broad spatial distribution of populations of the species and responsiveness of EROD activity in *Fundulus grandis* indicates the potential usefulness of this organism/assay combination as a biomarker for environmental contamination. It is noteworthy, that induced EROD levels in *Fundulus grandis* reach a specific activity approximately one- third to one- half that observed in Arochlor-induced rat livers. Also of significance is the finding that exposures of as little as 24 h will result in a quantifiable and significant increase in the activity of EROD in the liver tissues of *Fundulus grandis*.

CHAPTER 3

FIELD INVESTIGATIONS OF THE INDUCTION OF EROD IN FISH AT ST 34 AND ST 52 PLATFORMS IN THE GULF OF MEXICO, PASS FOURCHON AND LAKE CHAMPAGNE

L.A. Reily, J.C. Means, S.B. Dobias, G.W. Winston

INTRODUCTION

Field studies for validation of MFO induction as a bioindicator in feral fish as a function of pollution gradients were conducted by contrasting EROD levels in organisms from control (pristine) areas with organisms from known polluted environments. Field validation of the EROD assay as a biomarker in teleost fish, should provide a sensitive index of environmental pollution, to detect exposure history and to qualitatively establish the bioavailability of dissolved and sediment-bound normal, alkylated and heterocyclic aromatic hydrocarbons. This would allow environmental managers to assess the potential impacts and both ecological and human environmental risks associated with discharges of petroleum-associated hydrocarbons. As the aquatic environment is a major sink for numerous contaminants, the elucidation of biochemical responses to contaminants, by aquatic animals, becomes increasingly important. The epidemiology of highly elevated rates of idiopathic lesions and neoplasia among some populations of aquatic animals inhabiting polluted environments is, in most cases, far clearer than is generally the case for human cancers suspected of being related to environmental pollution.

MATERIALS AND METHODS

The present study evaluates biochemical markers recently identified as sensitive sublethal indices of environmental impact on several test organisms through detailed studies of: 1) the bioavailability of dissolved and sediment-bound normal, alkylated and heterocyclic aromatic hydrocarbons to benthic invertebrates and demersal fish, eggs, and larvae; 2) the ability of both benthic and pelagic organisms to metabolize these compounds; and 3) the genotoxicity of the compounds and their metabolites in benthic organisms and demersal fish.

Field validation studies of MFO induction as a potential indicator in species of teleost fish were undertaken. Collecting trips to offshore and coastal marsh areas (Pass Fourchon) yielded fish specimens from both contaminated and control (pristine) locations. The bottom-dwelling fishes, Fringed flounder (*Etropus crossotus*) and Shoal flounder (*Syacium gunteri*) were trawled near offshore petroleum production platforms with known produced water discharges (contaminated) and without produced water discharges (control). Collected specimens were evaluated for ethoxyresorufin O-deethylase (EROD) induction. Excised livers were pooled according to species and collection site, and were homogenized, then centrifuged at 9000 x g. The supernates (S9 fractions) were tested for EROD) activity. A scaled-down technique, accomodating less tissue, allowed individuals to be tested. S9 fractions were prepared from pooled or individual livers, and assayed for EROD activity. Further-purified microsomal extracts from the livers of control and contaminated specimens were analyzed for EROD activity. Preparations of S9 fractions from contaminated specimens had low levels or zero EROD activities. Microsomal fractions were harvested by centrifuging the S9 fractions at 40,000 x g for 180 min. Microsomal protein was determined by a modified Lowery (1951) procedure, with

bovine serum albumin as the standard. Microsomal fractions catalyzed sufficient activity to permit quantification and comparison of EROD activities.

Additional samples of fishes from offshore sites were provided to the laboratory by the Louisiana Department of Wildlife and Fisheries. These samples were collected during LDWF offshore sampling trips, by trawling at control and impacted stations in the South Terrebonne region of coastal Louisiana. Collection dates and hepatic microsomal EROD for these samples are presented in Table 3.4. Our field investigations of EROD inductions were amply enhanced by the inclusion of these samples to our database. Sediment cores were collected from the Lake Champagne reference site and at distance increments from the Pass Fourchon produced water discharge. These samples were analyzed for PAH contents, by methods detailed in chapter 7. Data for these analyses are reported in Table 3.1

RESULTS AND DISCUSSION

Pass Fourchon sediment was found to contain a complicated suite of aliphatic and aromatic hydrocarbons (Table 3.1). As might be expected, the concentration and makeup of sediment contaminants varies as a function of distance from the discharge. The aromatic fraction of the extract was found to contain moderate quantities of normal aromatic hydrocarbons associated with combustion sources while very high concentrations of a suite of alkylated aromatic hydrocarbons, dominated by the C-2 and C-3 alkylated naphthalenes, with lesser amounts of other alkylated naphthalenes, alkylated phenanthrenes and alkylated dibenzothiophenes. As reported previously, analyses of the produced waters revealed a complex mixture of normal alkyl- and heterocyclic- polynuclear aromatic hydrocarbons (Daniels et al., 1990). Total PAHs were 520 ng/ml, and saturated hydrocarbons totalled 16,000 ng/ml. The daily discharge of produced water at the Pass Fourchon facility was approximately 18,000 bbl/d (Boesch and Rabalais, 1985). This discharge caused heavy loading of sediments in the area, with petroleum hydrocarbons. Daniels et al. (1990) reported a concentration of total sediment PAHs to be into the parts per thousand range. The most abundant sediment PAHs at this site were the alkyl- substituted phenanthrenes. Daniels et al. (1990) studied genotoxic effects of Pass Fourchon produced waters on fish embryos, using *Cyprinodon variegatus* as a model. They observed an increase in chromosome aberrations in exposures to decreasing dilutions, but noted that the effect was not additive with respect to produced water concentration. They further reported that a specific type of aberration was induced, i. e. chromosomal breaks and extensive alterations in ploidy. Analyses of MFO levels in field-collected organisms were expected to show a sharp delineation between pristine and contaminated areas. Specimens taken at various distances from pollution sources should exhibit a gradient of MFO induction responses, in accordance with decreasing pollutant concentration over distance.

Sediment concentrations of petroleum hydrocarbons were analyzed at various distances from the Pass Fourchon discharge site. These data, presented in Table 3.1, indicated that a gradient of pollutant concentrations indeed exists as a function of distance from the discharge. Lake Champagne was chosen to represent a control (pristine) area. Analyses of sediment cores from this reference site confirmed that it is relatively uncontaminated. In Table 3.1, these Lake Champagne data may be contrasted with sediment data for the pollution gradient samples from Pass Fourchon .

Specimens collected and tested from the Lake Champagne reference area included: Pompano (*Trachinotus carolinus*), Croaker (*Micropogonias undulatus*), and Southern flounder (*Paralichthys lethostigma*). EROD activity was not detected in these field-collected, control specimens (data not shown). This confirms that livers of feral fish in natural clean environments do not exhibit elevated EROD activities..

In a subsequent field collection, EROD induction was compared for contaminated (Pass Fourchon) and control (Lake Champagne) organisms. Harvested specimens were pooled by species for analyses, and hepatic EROD data are presented in Table 3.2. EROD was not detected in control Pinfish, Pompano, or Southern flounder. Levels of EROD observed in other control organisms may represent uninduced background, levels, but insufficient samples precluded substantiation of this. Suprisingly, the contaminated samples of Croaker, Anchovy, and Jack had undetectable EROD, whereas measurable levels were found in their Lake Champagne counterparts. The reason for this was not evident, although speculations might include supressive influences of specific compounds or overall xenobiotic stress in the contaminated environment. Influences in the Lake Champagne environment which might be postulated to increase EROD expression, might include dietary substances such as phenolic compounds from plants. The only well delineated sample contrast was found in the Southern flounder, in which single specimens represented the control and contaminated conditions. EROD was not detected in the control sample, but hepatic EROD in the contaminated representative was 2,500,000 pmol/min/mg. It must be emphasized that these data represent only individual organisms. Subsequent sampling trips did not produce flounder samples for replicating this comparison. Induction of EROD in the flounder, if this was not a chance phenomenon, might relate to its tendency to occupy a rather small range, whereas the scattering of EROD levels observed in the other organisms may be an artifact of their more nektonic lifestyle.

Organisms collected from contaminated environments near offshore oil platforms included specimens of Fringed flounder (*Etropus crossotus*) and Sea robin (*Prionotus tribulus*).

Table 3.1 Sediment data for Pass Fourchon and reference site

NAPHTHALENES	ppb
2-Methylnaphthalene	3.0
1-Methylnaphthalene	1.5
2-Ethylnaphthalene	1.9
1-Ethylnaphthalene	9.1
2,6/2,7-Dimethylnaphthalene	10
1,3/1,7-Dimethylnaphthalene	48
1,6-Dimethylnaphthalene	4.7
1,4/2,3-Dimethylnaphthalene	27
1,5-Dimethylnaphthalene	25
1,2-Dimethylnaphthalene	12
2-Isopropylnaphthalene	nd
1,8-Dimethylnaphthalene	12
1,6,7-Trimethylnaphthalene	197
C3-NAPHTHALENES	9,700
C4-NAPHTHALENES	8,900

PARENT PAH	ppb
Naphthalene	1.7
Acenaphthylene	4.0
Acenaphthene	11
Fluorene	18
Dibenzothiophene	5.6
Phenanthrene	52
Anthracene	16
Fluoranthene	73
Pyrene	54
Benzanthracene	24
Chrysene	84
Benzo(b)fluoranthene	29
Benzo(k)fluoranthene	8.4
Benzo(a)pyrene	12
Indenopyrene	30
Dibenz(a,h)anthracene	17
Benzo(g,h,i)perylene	38

DIBENZOTHIOPHENES	ppb
4-Methyldibenzothiophene	68
2/3-Methyldibenzothiophene	55
1-Methyldibenzothiophene	34
1,2-Dimethyldibenzothiophene	5.9
C2-DIBENZOTHIOPHENES	8,600

PHENANTHRENES	ppb
3-Methylphenanthrene	183
2-Methylphenanthrene	87
4/9-Methylphenanthrene	177
1-Methylphenanthrene	102
3,6-Dimethylphenanthrene	102
3,5-Dimethylphenanthrene	nd
2,6-Dimethylphenanthrene	93
2,7-Dimethylphenanthrene	77
3,9-Dimethylphenanthrene	335
1,6/2,5/2,9-Dimethylphenanthrene	228
1,7-Dimethylphenanthrene	83
1,9/4,9-Dimethylphenanthrene	78
1,5-Dimethylphenanthrene	nd
1,8-Dimethylphenanthrene	32
1,2-Dimethylphenanthrene	22
9,10-Dimethylphenanthrene	nd
1,2,8-Trimethylphenanthrene	84
C3-PHENANTHRENES	23,000

TOTALS	ppb
Total Parent PAH	478
Total Alkylated PAH	52,397
Total PAH	52,875

Table 3.2 Hepatic microsomal EROD levels in specimens from nearshore sites at Pass Fourchon and Lake Champagne, LA

Organism:	EROD, pmol/min/mg Champagne (Control)		Pass Fourchon (Contaminated)	
	#		#	
Hardhead catfish *(Arius felis)*	3	80-12,667*	4	204-647
Croaker *(Micropogonias undulatus)*	3	450	4	n. d.
Anchovy *(Anchoa mitchilli)*	26	741	4	n. d.
Pinfish *(Lagodon rhomboides)*	8	n. d.	16	33
Spot *(Leiostomus xanthurus)*	17	1,075	40	719-2,222
Pompano *(Trachinotus carolinus)*	1	n. d.		
Southern flounder *(Paralichthys lethostigma)*	1	n. d.	1	2,500,000
Jack *(Caranx hippos)*	1	2,000	1	n. d.

* Ranges are given where catch size permitted subsampling; n. d., not detected.

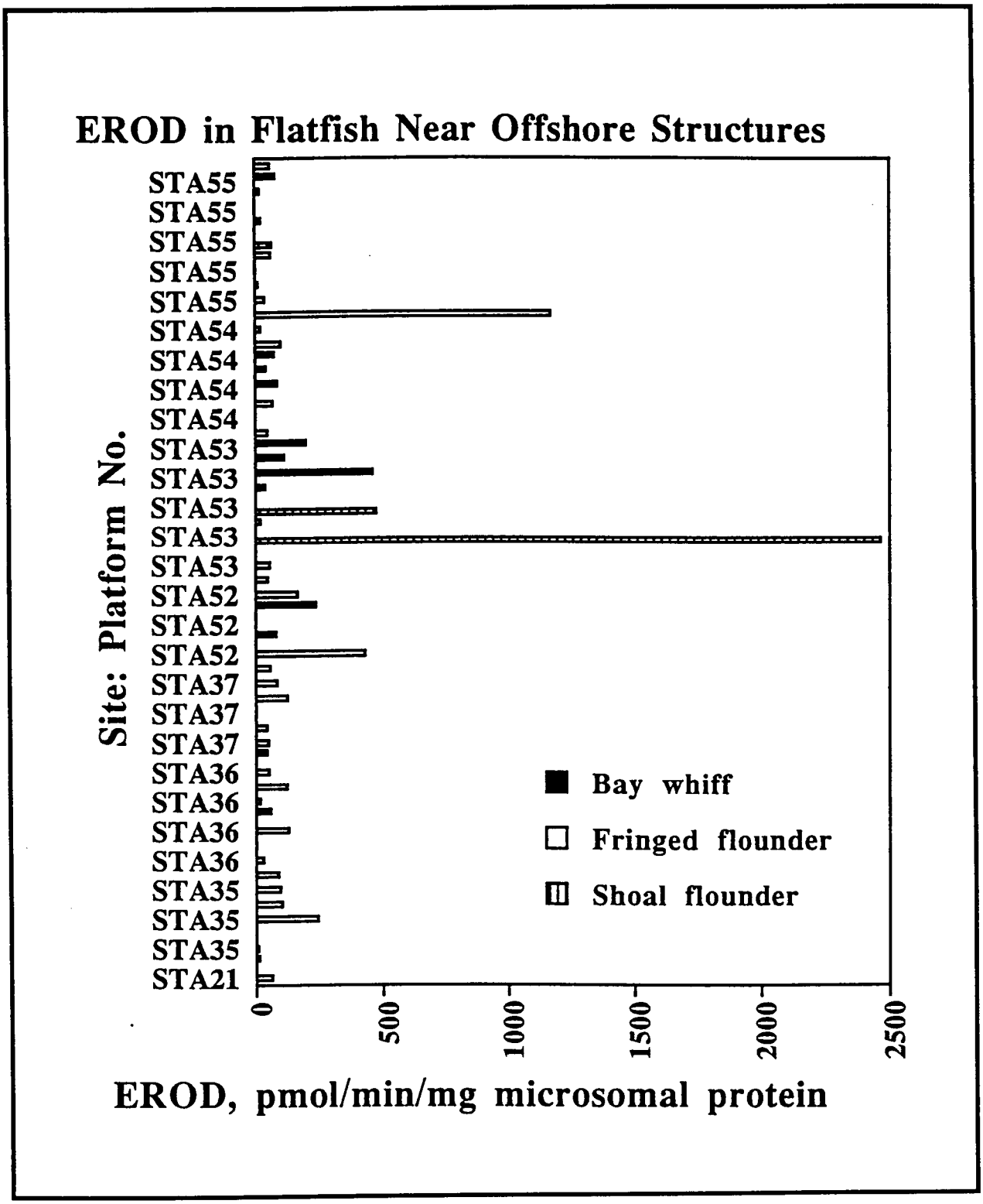

Figure 6. Induction of EROD in three species of flatfish near offshore production facilities.

Table 3.3. Hepatic microsomal EROD levels in fish collected by LDWF near offshore platforms.

Site	Species	Sample Date	EROD
ST 21	Fringed flounder	12/17/93	81.8
ST 35	Bay whiff	11/9/93	0.00
ST 35	Fringed flounder	11/9/93	0.00
ST 35	Fringed flounder	12/17/93	141
ST 35	Fringed flounder	12/17/93	331
ST 35	Fringed flounder	12/17/93	221
ST 36	Bay whiff	12/17/93	331
ST 36	Fringed flounder	11/9/93	0.00
ST 36	Fringed flounder	12/17/93	344
ST 36	Fringed flounder	11/9/93	0.00
ST 36	Fringed flounder	12/17/93	283
ST 36	Fringed flounder	11/9/93	0.00
ST 36	Fringed flounder	12/17/93	876
ST 36	Fringed flounder	11/9/93	0.00
ST 37	Bay whiff	11/9/93	0.00
ST 37	Fringed flounder	11/9/93	0.00
ST 37	Fringed flounder	12/17/93	0.00
ST 37	Fringed flounder	11/9/93	0.00
ST 37	Fringed flounder	12/17/93	620
ST 37	Fringed flounder	11/9/93	0.00
ST 37	Fringed flounder	12/17/93	766
ST 52	Bay whiff	10/14/93	0.00
ST 52	Bay whiff	11/3/93	0.00
ST 52	Bay whiff	12/10/93	0.00
ST 52	Fringed flounder	5/3/93	0.00
ST 52	Fringed flounder	12/10/93	0.00
ST 53	Bay whiff	10/14/93	0.00
ST 53	Bay whiff	12/10/93	941
ST 53	Bay whiff	12/10/93	0.00
ST 53	Bay whiff	11/3/93	0.00
ST 53	Fringed flounder	3/2/93	0.00
ST 53	Fringed flounder	5/3/93	0.00
ST 53	Fringed flounder	6/14/93	93327
ST 53	Fringed flounder	8/3/93	821
ST 53	Fringed flounder	12/10/93	0.00
ST 53	Shoal flounder	6/14/93	90125
ST 53	Shoal flounder	8/3/93	1341
ST 54	Bay whiff	11/3/93	0.00
ST 54	Bay whiff	10/14/93	0.00
ST 54	Fringed flounder	5/3/93	0.00
ST 54	Fringed flounder	3/2/93	0.00
ST 54	Fringed flounder	12/10/93	0.00
ST 54	Fringed flounder	12/10/93	81.8
ST 54	Fringed flounder	12/10/93	13.4
ST 55	Bay whiff	11/3/93	0.00
ST 55	Bay whiff	12/10/93	0.00
ST 55	Bay whiff	10/14/93	0.00
ST 55	Fringed flounder	3/2/93	0.00
ST 55	Fringed flounder	5/3/93	919
ST 55	Fringed flounder	6/14/93	372
ST 55	Fringed flounder	8/3/93	5325
ST 55	Fringed flounder	11/3/93	0.00
ST 55	Shoal flounder	8/3/93	1332

Field investigations of EROD inductions were also conducted on feral fish collected by LDWF, at control and impacted stations in the coastal region of Louisiana. Data from these studies are reported in Table 3.4 and Figure 6.

Specimens of benthic fish were collected by trawling in control areas, coastal estuaries and offshore oil platforms. Initial field studies were performed on fish species from Pass Fourchon, a coastal site, and three offshore sites designated STC1 (a control site) and two produced water sites, ST34 and ST52. EROD activity in fish liver from these offshore sites were studied as a function of distance from the point-source discharge. Although EROD readings were obtainable only on two distance points (200 and 2000 m) from the oil platform at ST34, and two (500 and 2000 M) from the platform at ST52, an apparent inverse correlation was observed in EROD activity of Fringed flounder livers from these sites (data not shown). This is not unprecedented and points to the need for immunochemical analysis with antibodies prepared against the enzymes (cytochromes P450 1A) catalyzing this activity. We have not been successful in obtaining positive western blots with monoclonal anti CYP1A antibodies prepared from cod liver or mammalian liver. The array of data does not indicate any statistically significant pattern of response to field parameters. Closer scrutiny and reconciliation of data are warranted.

A direct correlation was seen at ST34 in Sea robin (Figure 7), however the sample at the closest distance to the site (200 M) was lost during preparation of microsomes. Subsequent collections did not produce Sea robins from this distance. The activites are much lower than anticipated for the induced state but we note that baseline parameters for this species have not been reported. EROD activity (10.5) nmol/min/mg was detected in a Sea robin 1000 M from a platform at ST52. Protein analysis was not sensitive enough to obtain values for specific activity calculations at the other distance points on these and certain other samples.

The data in Tables 3.2 - 3.4 indicate that EROD activity varies by as much as four orders of magnitude among the various species from the several locations from which they were obtained. Because it is known that ethoxyresorufin degrades rapidly even in the freezer, it is standard operating procedure to recalibrate standards on a daily basis. From daily recalibrated standards new standard curves were prepared for every experiment. Figure 8 below shows a typical resorufin standard curve. Note the excellent correlation coefficient of 0.993.

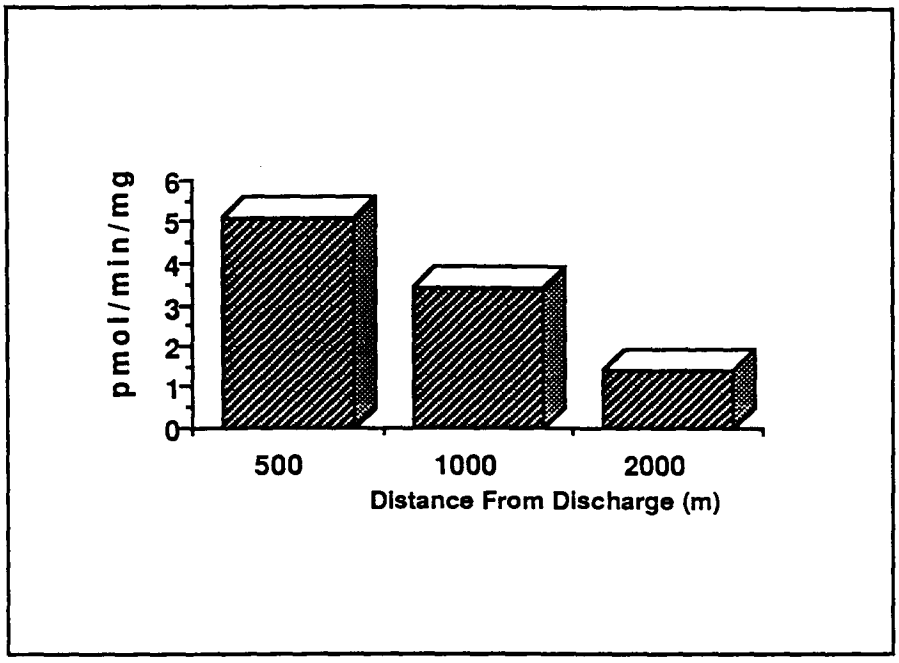

Figure 7. EROD Activity in Sea robin as a function of distance from ST 34

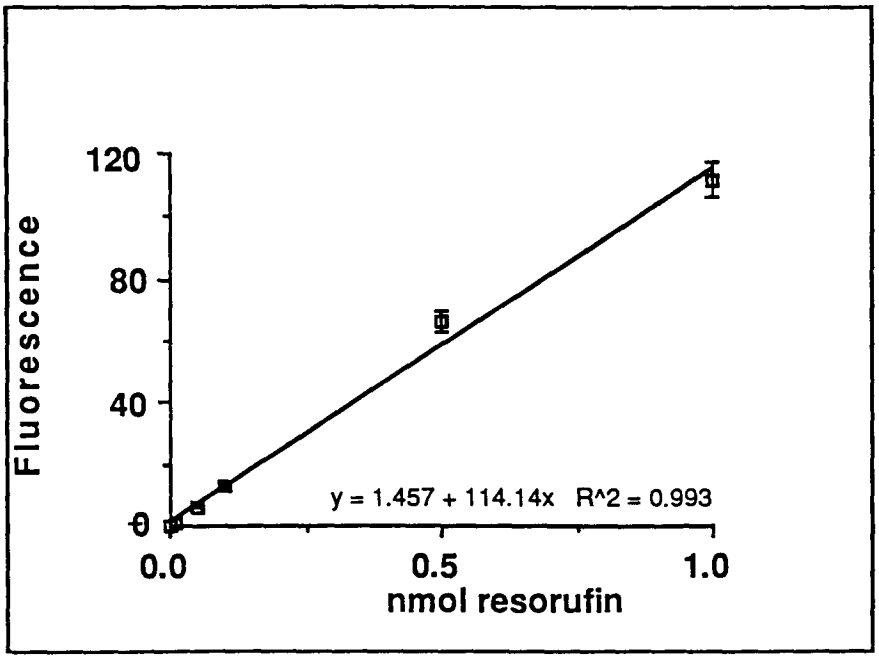

Figure 8. Typical EROD standard curve

Table 3.4 Liver microsomal EROD activities of Gulf of Mexico fish samples

Sample#	Species	Location/Distance from Platform	pmol/min/mg
301	Hardhead catfish	ST 34 100 m	14.8
302	Hardhead catfish	ST 34 50 m	BDL
303	Hardhead catfish	ST 34 1000 m	12.5
304	Hardhead catfish	ST 34 50 m	42.2
305	Hardhead catfish	ST 34 50 m	42.2
306	Hardhead catfish	ST 34 1000 m	9.8
307	Hardhead catfish	ST 34 1000 m	BDL
309	Fringed flounder	ST 53a 500 m	BDL
	Control rat		10-BDL
	3MC-treated rat		899/775
308	Fringed flounder	ST 53a 200 m	26.2
310	Shoal flounder	ST 53a 500 m	BDL
311	Fringed flounder	ST 34 200 m	7.2
312	Fringed flounder	ST 53a 1000 m	BDL
313	Fringed flounder	ST 53a 1000 m	BDL
315	Bay whiff	ST 34 1000 m	BDL
316	Bay whiff	ST 34 1000 m	BDL
317	Fringed flounder	ST 34 1000 m	2.1
318	Fringed flounder	ST 34 1000 m	BDL
319	Sea robin	ST 34 1000 m	BDL
320	Bay whiff	ST 34 1000 m	BDL
321	Fringed flounder	ST 34 500 m	1.7
322	Shoal flounder	ST 34 500 m	5.4
37	Fringed flounder	ST 34, 500 m	BDL
38	Sea robin	ST 34, 500 m	0.01
39	Fringed flounder	ST 34, 200 m	BDL
40	Fringed flounder	ST 34, 2000 m	0.04
41	Gulf menhaden	ST 34, 1000 m	0.01
42	Sea robin	ST 34, 1000 m	0.003
43	Fringed flounder	ST 34, 1000 m	BDL
44	Sea robin	ST 34, 2000 m	0.001
45	Fringed flounder	ST 52, 200 m	0.02
46	Fringed flounder	ST 34, 200 m	0.02
47	Pompano	ST 34, 200 m	BDL
70	Threadfin shad	ST CI 500	0.001
71	Sea robin	ST 52, 500 m	BDL
72	Fringed flounder	ST 52, 500 m	0.01
73	Sea robin	ST 52, 1000 m	0.01
74	Fringed flounder	ST 52, 1000 m	BDL
75	Sea robin	ST 52, 2000 m	BDL
176	Bay whiff	ST 53	32.3
177	Fringed flounder	ST 54	37.0
178	Fringed flounder	ST 52	31.7

BDL = below detection limits

Figure 9 shows a typical microsomal response curve representative of the conditions of all of our assays, which were conducted under conditions in which the assay was first order with respect to microsomal protein.

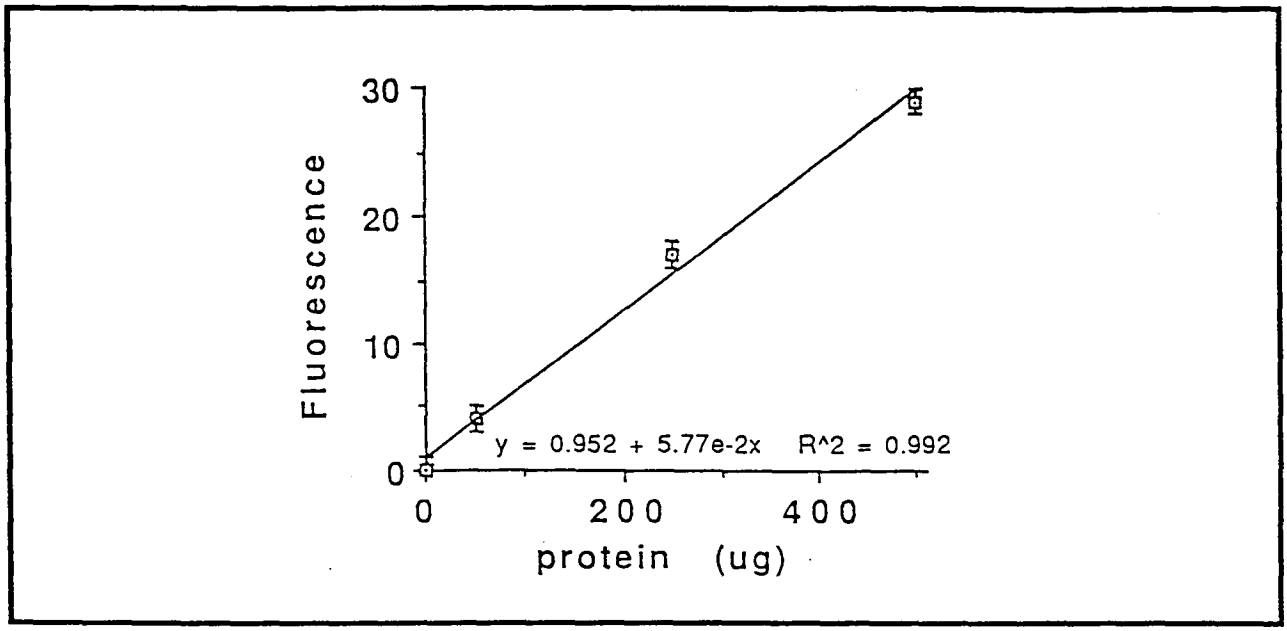

Figure 9. EROD vs. microsomal protein concentration

EROD activities ranged from below detectable limits to values of 42.2 pmol/min/mg in offshore catfish. In the coastal sites of Pass Fourchon and Lake Champagne values ranged from 0.08 to 2.5 pmol/min/mg protein. Rat liver controls from 3MC-induced rats were typically in the range of 840 ± 60 pmol/min/mg protein.

Table 3.5 Liver microsomal EROD activitites of fish species sampled from a contaminated (Pass Fourchon) and reference (Lake Champagne) coastal site in the Gulf of Mexico.

Sample#	Species	Conditions	pmol/min/mg
50	Hardhead catfish	LC	0.08
51	Hardhead catfish	LC	0.15
52	Hardhead catfish	LC	1.07
53	Hardhead catfish	LC	0.27
55	Croaker	LC	0.45
57	Anchovy	LC	0.74
62	Jack	LC	2.00
65	Spot	LC	1.08
64	Jack	PF	0.21
66	Spot	PF	2.22
67	Spot	PF	0.72
58	Anchovy	PF	BDL
59	Fringed flounder	PF	2.50
61	Pinfish	PF	0.03
49	Hardhead catfish	PF	0.65
54	Hardhead catfish	PF	0.21
48	3-MC-induced *Fundulus* adult		0.09

BDL = below detection limits of 0.001 pmol/min/mg protein

In environments where carcinogenic PAHs occur, MFO induction does not necessarily mean that the parent compound is still at high levels in the fish tissues. Studies have shown that animals with active hydrocarbon metabolism accumulate lower levels of parent PAH (Lu et al., 1977). The higher rates of metabolism associated with induction of the hydrocarbon metabolizing P-450 can enhance the rates of PAH elimination, via transformation to more soluble compounds. The detection of high levels of hydrocarbon metabolites in the bile of fish exposed to hydrocarbons is consistent with this reasoning(Statham et al., 1978; Goddard et al., 1987) Highly induced animals can be expected to have lower levels of PAHs in their tissues than either animals which are not induced or those that have inherently lower rates of hydrocarbon metabolizing activity. Numerous investigations have reported relatively high levels of PAH residues in molluscan tissues, intermediate levels in crustaceans and low levels of these compounds in fish. This was illustrated in a study by Dunn and Fee (1979), which reported levels of BaP in commercial samples of fish and shellfish. BaP content was inversely related to the associate rates of *in vitro* BaP metabolism. The potential risk associated with parent PAH carcinogens in seafood would be greater with invertebrates, which are less able to metabolize these compounds. There would still be risk associated with consumption of fish from highly contaminated sites. While fish may contain a lesser and even undetectable amounts of parent compound, carcinogenic metabolic products of these may be present.

It is difficult and often disconcerting to draw inferences from the small samplings, but the data do point to some of the recognized problems in biomarker research. These problems are: 1) samplings of fish species from large areas are quite variable from time to time and place to place and 2) biochemical responses of fishes are extremely variable as a function of species, sex, gonadal status, season, temperature, nutrition etc. Thus, field studies can result in a disconcerting array of data, even though controlled laboratory studies may be very predictable. This study sought to investigate the differences of hepatic EROD activity between pristine and contaminated areas. Significant delineation might ultimately be useful for quantitative or qualitative employment in a biomonitoring regimen. The objective of biomarker research is to establish the efficacy of the use of the sentinel.

Chapter 4

STUDIES OF PRO- AND ANTIOXIDANT PARAMETERS IN LIVER CYTOSOL AND MICROSOMES OF GULF OF MEXICO FISH SAMPLES AND OTHER MARINE ORGANISMS: EVALUATION OF SUPEROXIDE DISMUTASE, CATALASE, MICROSOMAL GENERATION OF OXYRADICALS, AND OXYGEN CONSUMPTION

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INTRODUCTION

Oxygen radicals are produced as a normal part of all aerobic life. Molecular oxygen is reduced by four electrons with the resulting production of two molar equivalents of water for each oxygen molecule that is reduced. Thus, partially reduced reactive oxygen species (ROS) are produced which can be greatly enhanced under exacerbative conditions of xenobiotic exposure. Nicotinamide adenine dinucleotide phosphate, reduced form (NAD(P)H)-dependent electron transport by microsomes from virtually all vertebrate and invertebrate species studied produces ROS including superoxide anion radical (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical (·OH) (Jewell and Winston, 1989; Winston et al., 1991). Each of these species have been implicated in toxic damage to cells including cell death (Kappus and Seis, 1981).

Hydroxyl radicals have been shown to participate in the oxidation of certain xenobiotic substrates including aliphatic alcohols, benzoate (Winston and Cederbaum, 1982), aniline (Ingelman-Sundberg and Ekstrom, 1982), benzene (Ingelman-Sundberg and Hagbjork, 1982) and salicylates (Grootveld and Halliwell, 1986). Thus, their production in biological systems is an important, often overlooked, consideration in the response of organisms to various xenobiotics. The oxidation of a variety of ·OH scavenging agents e.g., 2-keto-4-thiomethylbutyric acid (KMBA) and benzoate have been used to detect the production of potent oxidants generated by microsomes of crustaceans, molluscs, fish and mammals (Winston and DiGiulio, 1991). The sequence of reactions believed to be involved in the production of H_2O_2 in microsomal systems is outlined below.

$$2O_2 + NADPH \xrightarrow{\text{Microsomes}} 2O_2^- + NADP^+ + H^+ \quad (1)$$

$$2O_2^- + 2H^+ \longrightarrow H_2O_2 + O_2 \quad (2)$$

Eq. [2] shows the disproportionation of superoxide resulting in the production of hydrogen peroxide. Hydrogen peroxide can serve as the precursor of ·OH via the Haber-Weiss reaction (Eq. 3) (Haber and Weiss, 1934).

$$O_2^- + H_2O_2 \longrightarrow O_2 + \cdot OH + OH^- \quad (3)$$

This reaction is kinetically quite slow, therefore transition metals, especially iron generally are invoked as a catalyst for ·OH production (Eqs. 4, 5).

$$Fe(III) + O_2^- \rightarrow O_2 + Fe(II) \quad (4)$$

$$Fe(II) + H_2O_2 \rightarrow Fe(III) + \cdot OH + OH^- \quad (5)$$

The sum of Eqs. [4] and [5] is the Haber-Weiss reaction (Eq. 3). Eq. [5] is the Fenton reaction (Walling, 1975).

Metal chelates, nitroaromatics, quinones and bipyridyls (Mason, 1979; Kappus and Seis, 1981; Bus and Gibson, 1984) are known to increase ROS production in biological systems with accompanying oxidative damage. The toxic sequalae of these various classes has been linked to the proliferative generation of oxyradicals that results from these compounds uncoupling biological electron transport. The consequence of ROS production is protein degradation, enzyme inactivation, lipid peroxidation, DNA damage and cell death (Borg and Schaich, 1984). Two possible loci of O_2^- production in microsomes are the autooxidation of the oxycytochrome P450 complex (Dybing et al., 1976) and/or the autooxidation of the cytochrome P450 reductase (Winston and Cederbaum, 1986). Winston and Cederbaum (1986) showed that the oxidation of ·OH scavenging agents was dependent only upon the amount of P450 reductase in a reconstituted MFO system and not the amount of cytochrome P450. In contrast to mammalian systems, little work has been done on the mechanism involved in the production of ROS in submammalian vertebrates and invertebrates. A characteristic of invertebrate microsomal electron transport is the comparatively low specific activity of NAD(P)H-cytochrome c reductase, which is about 3-10 % of mammals (James et al., 1979; Jewell and Winston, 1989).

The presence of oxyradicals does not preclude physiological damage as they are limited by the presence of antioxidant enzymes e.g., superoxide dismutase (SOD) and catalase (Diguiseppi and Fridovich, 1984). Therefore the levels of these antioxidants were assessed as a function of produced water discharges in parallel with MFO and genotoxic assessment. The hydroxyl radical scavenging agents 2-keto-4-thiomethylbutyric acid (KMBA) and dimethyl sulfoxide were oxidized to ethylene and formaldehyde, respectively by microsomes isolated from the hepatopancreas of the Red Swamp crawfish (Jewell and Winston, 1989), the common marine mussel (Livingstone et al., 1989; Winston et al., 1990) and the Salmon (Kennish, 1989).

METHODS

Studies have been carried out to measure superoxide dismutase (SOD) and catalase levels in various species as a function of the conditions described in the previous 3 chapters with the newly developed SOD-525 assay from Bioxytech (Cedex, France). This is the newest system for measurement of SOD in biological tissues. The assay exploits the enhanced rate of autoxidation of a patented pyrogallol derivative, which offers some distinct advantages over previously published assays. One advantage is that confounding substances, which are both replete and variable in biological samples and can interfere with detection of SOD, can be diluted from the sample without loss of enzyme activity. It is also highly reproducible from one laboratory to another which suggests its utility for wide geographical distribution. Preliminary studies designed to optimize the concentrations of microsomal and cytosolic fractions of marine mussels and sea anemones on the end-point chromaphore (oxidized pyrogallol derivative; λ_{max} = 525nm) have been completed. Interferences are noted only in some concentrated biological materials but can be eliminated by dilution of samples 1:10 or greater.

RESULTS AND DISCUSSION

Table 4.1 below shows the effects of 3-methylcholanthrene (3MC) exposure on the specific activity of SOD in the sea anemone *Anthapleura elegantissima* . This anemone species is

known to contain algal symbionts, which have been shown by others to impose oxidative stress on the anemone resulting in elevated SOD levels (Dykens et al., 1992). Thus xenobiotic exposure might enhance the oxidative stress placed on this organism which may already have its antioxidant defenses compromized by the photosynthetically-generated oxygen via photosynthesis by the zooxanthellae. It can be seen in Table 4.1 that SOD levels are highest in the uv-light-exposed anemones which is consistent with adaptation by the organism to the hyperoxic conditions produced by photosynthetically generated oxygen; SOD activities are about equivalent and about 30-40% lower in the dark-reared and symbiont-depleted anemones. These data corroborate the findings of Dykens et al. (1992). Depletion of zooxanthellae resulted in lower SOD levels in the 3MC-treated anemones, however growing zooxanthellate anemones in the dark to prevent photosynthesis did not cause significant changes in SOD activity. This may be the result of oxidative stress caused by the 3MC exposure or a response by the zooxanthellae to 3MC. The general lack of responsiveness of SOD to 3MC exposure indicates the probable ineffectiveness of SOD response for use in biomonitoring, at least with anemone sentinels.

Table 4.1 SOD content of sea anemone *(Anthapleura elegantissima)* cytosols: Effect of 3-methylcholanthrene (3MC) exposure and zooxanthellate symbionts.

Conditions	SOD (units/ml)
Controls	
symbionts uv-reared	341 ± 10.7*
symbiont-depleted	231 ± 11.4
symbiont dark-reared	214 ± 14.3
3MC-exposed	
symbionts uv-reared	258 ± 29.0
symbiont-depleted	199 ± 13.6
symbiont dark-reared	226 ± 10.0

* Mean±SD of 2-3 determinations on pooled cytosol from 5-8 anemones.

NAD(P)H-dependent microsomal electron transport is a known source of oxyradical production in biological systems. The oxidation of α-keto-γ-thiomethylbutyric acid (KMBA) to ethylene is catalyzed by microsomal electron transport in the presence of iron-EDTA. The latter is used as a catalyst of the Fenton reaction to reduce hydrogen peroxide to the hydroxyl radical, which is the terminal oxidant in the reaction. Certain nitroaromatic compounds enhance oxyradical production by microsomes via a redox cycling reaction, which is linked to univalent reduction of the nitro group by microsomal flavoprotein reductases. Group IIB metals, especially Cd and Hg are inhibitors of enzymes that contain sulfhydryl groups (-SH) at the active site. Flavoprotein reductases, (e.g., cytochrome P450 reductase) are such enzymes and their inhibition by mercurials is classical. Furthermore, To better understand the effects of exposure to Group IIB metals on microsomal electron transport in marine invertebrates a pilot study was conducted in which sea anemones *(Anthapleura elegantissima)* were exposed for 5 days to 25 and 250 μg/liter of $CdCl_2$. The temperature was maintained at 25°C and salinity at 32 ppt. Following this exposure period microsomes were prepared by differential centrifugation and the ability of these microsomes to catalyze NADPH-dependent nitroaromatic reduction assessed. 4-Nitroquinoline N-oxide (4NQO) was used as a model redox cycling nitroaromatic because it is well characterized in the marine invertebrate literature and is known to stimulate oxygen consumption and KMBA oxidation by microsomes from *Mytilus edulis* digestive gland microsomes.

The data shown in Figure 10 represent preliminary findings of the effect of Cd exposure on NADPH-dependent microsomal reduction of 4NQO as measured by oxyradical generation, i.e. KMBA oxidation. It is noted that microsomes isolated from anemones exposed to either 25

or 250 µg/liter of $CdCl_2$ reduce 4NQO at rates significantly less than nonexposed controls. The lower rates of 4NQO oxidation are reflected in lower rates of KMBA oxidation and hence, ethylene production. Microsomal yields from anemones are very low as compared to those obtained from organs such as digestive gland or liver. This coupled with small amounts of starting material resulted in exhausting the supply of microsomes from the Cd study. The preliminary data warrant further study of the effects of metal exposure on microsomal electron transport. A tentative working hypothesis for future study of these effects is that Cd binding to active site -SH groups results in inefficient electron transport in the microsomal fraction of the exposed animals.

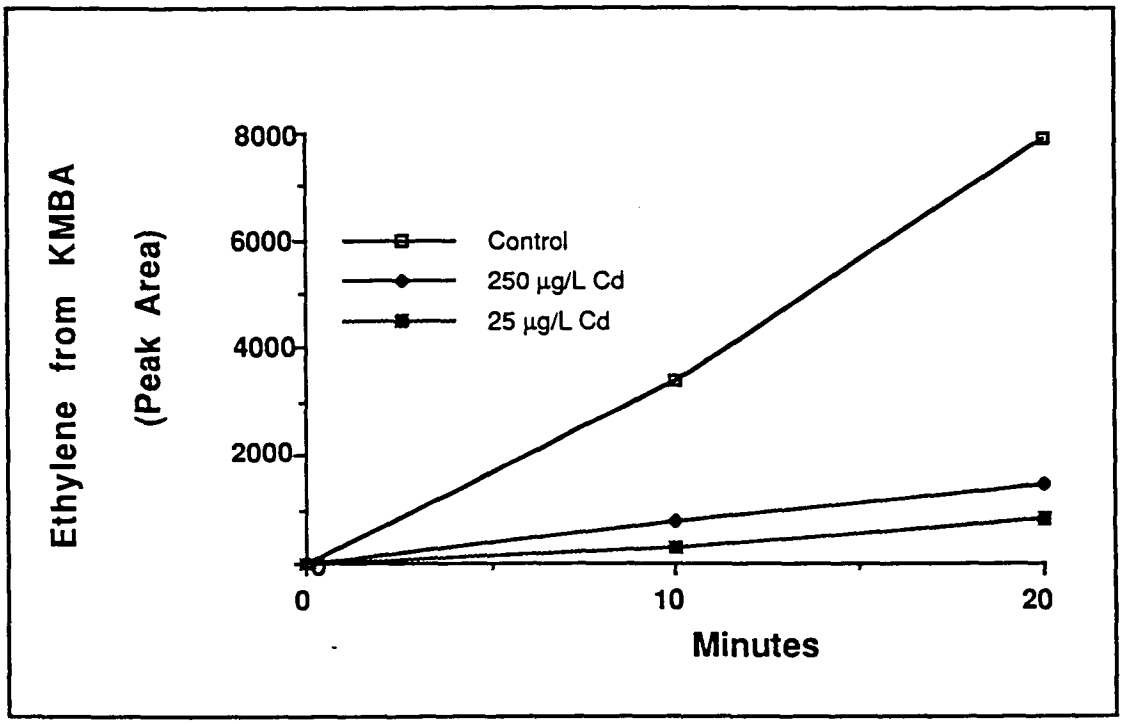

Figure 10. Nitroaromatic-stimulated oxyradical production by sea anemone microsomes: Effect of Cadmium preexposure.

A separate preliminary study to compare NADH-dependent with NADPH-dependent KMBA oxidation by sea anemone microsomes in the presence of 4NQO was conducted. With NADPH as primary electron donor KMBA oxidation was 1.33±0.14 nmol/min/mg microsomal protein and with NADH 0.96±0.11 nmol/min/mg. Based on numerous ethylene standard curves 1560 area units = 1nmol.

Exposure of sandworms to sand mixed with petroleum hydrocarbon-contaminated sediment (50:50 w/w) for 14 days did not increase SOD levels above the detection limits of the assay. This lack of response of SOD in gut tissue cytosolic fractions of the sand worm, *Nereis virens* indicates its ineffectiveness as a marker of sediment contamination.

Table 4.2 SOD Content of Gut Cytosol from *Nereis virens* exposed to contaminated sediment.

Days exposed	% contamination	SOD (units/ml)
0	0%	0.26 ± 0.16
14	0%	BDL
14	20%	BDL
14	50%	BDL

BDL = below detection limits

Results of field sample analysis of SOD in microsomes of several species of fish from various stations representing varying degrees of impact in of the Gulf of Mexico are shown in Table 4.3. There it is noted that microsomal SOD activity is below the detection limits or very near the detection limits of the assay for all samples tested. These data confirm the ineffectiveness of SOD measurements in fish as biomarkers of exposures to the kinds of contaminants in these studies in the field.

Table 4.3 Microsomal SOD content of Gulf of Mexico fish samples

Location	Species	SOD-525 units/ml
ST 34, 200m++	Fringed Flounder	BDL
ST 34, 200m	Fringed Flounder	BDL
ST 34, 500m	Sea Robin	BDL
ST 34, 1000m	Pogy	4.31±0.47
ST 34, 2000m	Sea Robin	0.03±0.01
ST 52, 500m	Sea Robin	BDL
ST 52, 1000m	Sea Robin	BDL
ST 52, 1000m	Fringed Flounder	BDL
ST 52, 2000m	Fringed Flounder	BDL
ST 52, 2000m	Sea Robin	BDL
Lake Champagne	Hardhead Catfish	BDL
Lake Champagne	Anchovy	BDL
Pass Fourchon	Anchovy	BDL
3MC*	Fundulus adult	BDL

BDL = below detection limits; * Laboratory exposure to 3-methylcholanthrene
++ numerical values distances in meters from platforms. All trawels were west of (down-current from) platforms.

Figure 11 establishes the efficacy of the SOD-525 assay as confirmed by its robustness in detecting with great precision the units of SOD activity of a wide range of concentrations of a commercially available preparation of SOD from bovine liver (Sigma Chemical Co., St. Louis, MO).

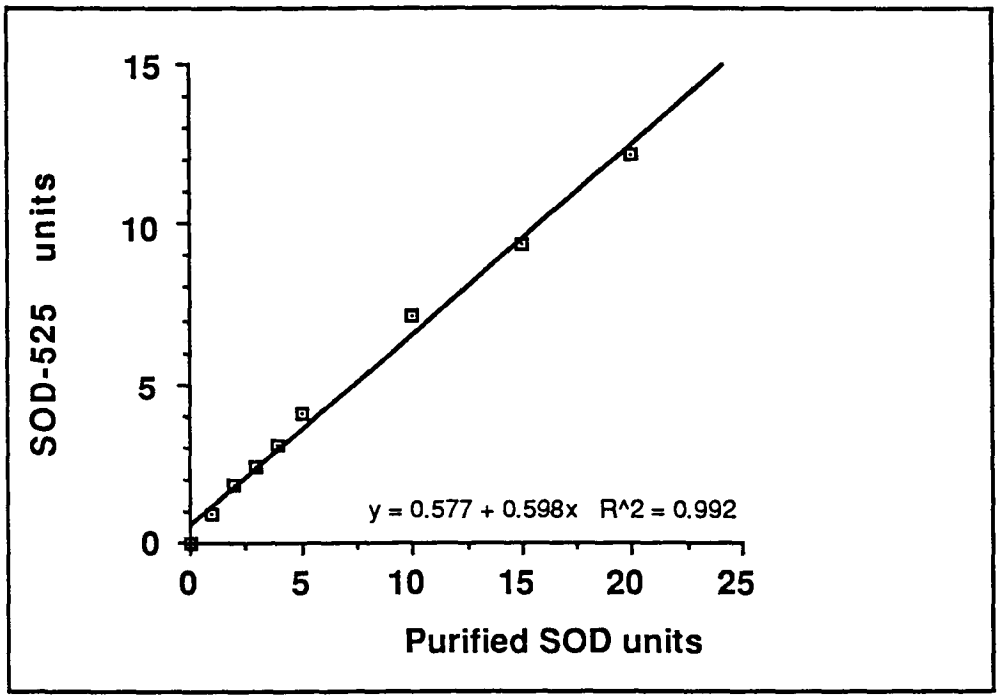

Figure 11. Detection of purified SOD by SOD-525 assay.

SOD determinations of fish liver cytosol were conducted. These data are presented in Table 4.4 where it can be seen that SOD activities were markedly higher than in the microsomal compartment. The activity expressed as units/ mg cytosolic protein ranged from less than 1 or below detection limits in Fringed flounder taken from 200 m from ST 53a platform to 530 units/mg in Shoal flounder caught 500 m from ST 34. Fringed flounder sampled from 200 m from ST 34 had one of the higher SOD levels. In the few cases where antioxidant enzymes were shown to correlate at all with distance from a platform an inverse relationship was observed. This was noted also for cytosolic catalase (see Table 4.5).

Table 4.4 Superoxide dismutase activities of liver cytosol from fish sampled from various Gulf of Mexico field sites.

Sample #	Species	Location/site	SOD (units/mg protein)
170	Fringed Flounder	ST 54 †	26.1
172	Fringed Flounder	ST 53	17.6
173	Bay Whiff	ST 55	43.4
174	Fringed Flounder	ST 54	31.6
175	Bay Whiff	ST 52	29.2
176	Bay Whiff	ST 53	21.0
177	Fringed Flounder	ST 54	26.2
178	Fringed Flounder	ST 52	10.4
301	Catfish	ST 34 100 m *	28.7
302	Catfish	ST 34 50m	5.9
303	Catfish	ST 34 1000m	25.8
304	Catfish	ST 34 50m	17.0
305	Catfish	ST 34 50m	41.5
306	Catfish	ST 34 1000m	20.5
307	Catfish	ST 34 1000m	16.6
308	Fringed Flounder	ST 53a 200m	0.23
309	Fringed Flounder	ST 53a 200m	ND
311	Fringed Flounder	ST 34 200m	437
312	Fringed Flounder	ST 53a 1000m	103
313	Shoal Flounder	ST 53a 1000m	514
314	Bay Whiff	ST 53a 1000m	ND
315	Shoal Flounder	ST 34 1000m	33.3
319	Sea Robin	ST 34 1000m	62.3
320	Bay Whiff	ST 34 1000m	334
322	Shoal Flounder	ST 34 500m	530

ND = not detected. † Samples collected by Louisiana Department of Wildlife and Fisheries; distances not specified. * Numerical values are distances in meters (m) from platforms.

Catalase is another important determinant of oxidative stress; this enzyme along with SOD is common to all forms of aerobic life. Together SOD and catalase prevent the accumulation of certain partially reduced oxygen species such as superoxide anion radical, hydrogen peroxide and thus the formation of potent oxidants such as the hydroxyl radical. Catalase activities were measured in all samples for which cytosol and microsomes were available. As was noted for the SOD results, catalase measurements are also extremely variable. In general catalase activities are higher in cytosols than in microsomes, albeit some overlap in the values between the two compartments exists. The range of variability for the microsomes was ca. 2 to 850 nmol/min/mg protein and for the cytosol ca. 6 to 22,000.

Table 4.5 Specific activities of Catalase in Gulf of Mexico fish liver microsomes.

Species	Location/Distance	Cor. Coef.*	Microsomal Protein (mg/ml)	S. A.** (nmol/min/mg)
LC† Catfish		0.980	2	11.8
LC Catfish		0.999	0.6	29.0
LC Catfish		0.994	209	6.8
LC Croaker		0.970	0.5	20.4
PF‡ Croaker		0.996	1.7	60.7
LC Pinfish		0.996	0.97	16.0
PF Pinfish		0.934	2.6	1.9
PF Jack		0.999	0.28	280.0
PF Jack		0.988	0.42	118.0
Shad ST CI 500	0.999	1.62	17.2	
Hardhead Catfish	ST 34 50m	0.998	42.3	12.7
Hardhead Catfish	ST 34 50m	0.997	45.2	8.7
Hardhead Catfish	ST 34 50m	0.994	38.2	4.0
Hardhead Catfish	ST 34 100m	0.998	36.3	191.1
Fringed Flounder	ST 34 200m	0.999	28.0	97.3
Fringed Flounder	ST 34 500m	0.997	12.4	36.6
Shoal Flounder	ST 34 500m	0.999	3.2	206.4
Hardhead Catfish	ST 34 1000m	0.982	50.5	18.8
Hardhead Catfish	ST 34 1000m	0.999	30.3	3.3
Hardhead Catfish	ST 34 1000m	0.998	19.4	4.8
Shoal Flounder	ST 34 1000m	1.000	12.8	658.0
Shoal Flounder	ST 34 1000m	1.000	12.5	422.8
Fringed Flounder	ST 34 1000m	0.999	18.6	834.6
Fringed Flounder	ST 34 1000m	0.999	17.1	715.9
Sea Robin	ST 34 1000m	0.996	13.7	98.8
Bay Whiff	ST 34 1000m	0.997	0.9	504.6
Fringed Flounder	ST 53a 200m	0.999	2.3	110.0
Fringed Flounder	ST 53a 500m	0.999	10.1	506.9
Shoal Flounder	ST 53a 500m	1.000	6.4	851.5
Fringed Flounder	ST 53a 1000m	1.000	11.5	416.4
Shoal Flounder	ST 53a 1000m	0.997	2.3	305.1
Fringed Flounder	ST 36§	0.998	2.6	95.3
Fringed Flounder	ST 37	0.981	2.62	189.1
Bay Whiff	ST 52	0.965	1.77	174.9
Fringed Flounder	ST 52	0.799	3.8	130.4
Bay Whiff	ST 53	0.989	5.71	79.3
Bay Whiff	ST 53	0.940	12.58	7.2
Fringed Flounder	ST 54	0.989	3.43	216.7

* Correlation Coefficient. ** S.A. = Specific activity. † LC = Lake Champagne. ‡ PF = Pass Fourchon. § Fish samples from stations in which distance was not specified were collected by Louisiana Department of Wildlife and Fisheries.

For the most part no obvious correlations existed between catalase activity and distance from point sources of offshore contamination, contaminated coastal sites vs. reference sites, species, collection time, depth of habitat, sediment-dwelling fish vs. demersal fish and one offshore site vs another. There was however a noteworthy exception. Fringed flounder caught 1000 and 500 m from site 53 had low cytosolic catalase activity (< 2000 units/mg), whereas the

same species from 200 m had a much higher activity (~8,800 units/mg). The significance of this trend remains to be established.

Taken as a whole, results from catalase and SOD measurements indicate that oxidative stress enzymes may not be reliable markers of exposure to contaminants. Interestingly, no pattern of catalase activity was observed between deep-dwelling fish and fish obtained from shallow areas, i.e. only a few meters. The enormous variation observed in these activities amongst the various species studied indicates highly variable capacities to cope with oxidant stress. Of particular importance would be the response of antioxidant enzymes of fish subjected to varying periods of anoxia. In such cases injury may occur when oxygen is reintroduced after periods of anoxia, i.e. reperfusion injury. Thus, the variability and extreme range of antioxidant defenses indicated by the data suggest the importance of studies relating these enzymes to various conditions of oxygen concentration as well as conditions that enhance ROS production in aquatic organisms.

Table 4.6 Specific activities of Catalase in Gulf of Mexico fish liver cytosol

Species	Location/Distance	Cor. Coef.*	Cytosolic Protein (mg/ml)	S.A.** (nmol/min/mg)
Mussel		0.992	3.18	136
Anemone		0.997	3.7	24
Fringed Flounder	ST 34 500 m	0.999	0.01	3750
Fringed Flounder	ST 34 1000 m	0.999	0.12	303
Sea Robin	ST 34 1000 m	0.995	0.19	180
Fringed Flounder	ST 34 1000 m	0.999	0.16	284
Pinfish	LC†	0.984	3.2	41
Jack	PF‡	1.000	1.0	91
Jack	PF	0.999	1.7	39
Shad	ST CI 500	0.993	6.4	27
Catfish	ST 34 50m	0.997	40.0	206
Catfish	ST 34 50m	0.996	32.4	305
Catfish	ST 34 50m	0.997	16.8	6
Catfish	ST 34 100m	0.997	26.9	286
Fringed Flounder	ST 34 200m	0.994	0.71	22200
Shoal Flounder	ST 34 500m	0.998	0.66	1276
Catfish	ST 34 1000m	0.994	25.7	199
Catfish	ST 34 1000m	0.991	11.8	362
Catfish	ST 34 1000m	0.993	11.2	656
Shoal Flounder	ST 34 1000m	0.999	3.91	1900
Sea Robin	ST 34 1000m	0.995	6.26	208
Bay Whiff	ST 34 1000m	0.959	0.18	853
Fringed Flounder	ST 53a 200m	0.999	0.48	8810
Fringed Flounder	ST 53a 500m	0.997	3.71	1410
Fringed Flounder	ST 53a 1000m	0.997	4.12	1800
Shoal Flounder	ST 53a 1000m	0.994	0.68	2670
Bay Whiff	ST 53a 1000m	0.956	---	BDL
Bay Whiff	ST 52§	0.999	3.43	936
Fringed Flounder	ST 52	0.988	15.5	750
Bay Whiff	ST 53	0.0001	18.07	122
Bay Whiff	ST 53	0.988	15.5	436
Fringed Flounder	ST 54	0.995	9.93	1499

* Correlation Coefficient. ** S.A. = Specific activity. † LC = Lake Champagne. ‡ PF = Pass Fourchon. § Fish samples from stations in which distance was not specified were collected by Louisiana Department of Wildlife and Fisheries.

Chapter 5

STUDIES OF NADH- AND NADPH-DEPENDENT MICROSOMAL ELECTRON TRANSPORT: RELATIONSHIP OF CYTOCHROME C REDUCTASE AND OXYGEN CONSUMPTION TO SPECIES AND ENVIRONMENTAL CONDITIONS OF GULF OF MEXICO FIELD SITES.

G.W. Winston and S.B. Dobias

INTRODUCTION

The microsomal mixed-function oxidase system of fish and marine invertebrates is comprised of various components of the microsomal membrane (Winston et al., 1991). Aside from the heme-containing cytochromes P450 and b_5 there are also periferal flavoproteins, which serve as reductases for the cytochromes. These reductases are held to the membrane by a hydrophobic binding domain and they contain the prosthetic groups FMN and FAD. Cytochrome P450 reductase preferentially accepts electrons from NADPH and cytochrome b_5 from NADH. Typically, microsomal NADPH-dependent reduction of cytochrome *c* (used as an artificial electron acceptor) is taken as a measure of cytochrome P450 reductase and NADH-dependent reduction is generally regarded as cytochrome b_5 reductase (Winston et al., 1991).

The importance of these enzymes is emphasized by the fact that microsomal flavoprotein reductases are rate-limiting components of NAD(P)H-dependent microsomal mixed function oxygenase activities and also extremely important loci of oxygen radical production. The oxidation of hydroxyl radical scavenging agents was shown to be dependent soley upon the concentration of cytochrome P450 reductase in a reconstituted MFO system and not the amount of cytochrome P450 (Winston and Cederbaum; 1986). Furthermore, cytochrome P450 inhibitors including CO, metyrapone and SKF-525A do not inhibit oxyradical production during microsomal electron transport as measured by the production of ethylene from KMBA (Jewell and Winston, 1989; Winston et al., 1991).

In mammalian systems, cytochrome P450 reductase is induced by only certain of the agents that classically induce cytochrome P450. Phenobarbital and Arochlor 1254 both affect increases in cytochrome P450 reductase activity of microsomes (Narayan and Winston, 1988), presumably via proliferation of smooth endoplasmic reticulum (Okey, 1990). Thus, microsomes prepared from liver of phenobarbital- and Aroclor 1254- pretreated rats have higher rates of NADPH-dependent oxygen consumption in the presence of substrates that are univalently reduced by flavoprotein reductases, e.g. nitroaromatics, quinones and metal chelates (Metosh-Dickey et al., 1994). A characteristic of invertebrate microsomal electron transport is the comparatively low specific activity of NAD(P)H-cytochrome c reductase, which is about 3-10 % of mammals (James et al., 1979; Jewell and Winston, 1989). Moreover, relatively few investigations of this important rate-limiting enzyme system has been performed with submammalian species. Thus, we have studied both NADH- and NADPH-dependent cytochrome *c* reduction to indicate the intra and inter species range of activities and the effects of various Gulf of Mexico field site environments on those activities. In conjunction with the determination of reductase activities, attempts were made to measure the specific content of cytochromes P450 in an effort to determine the efficacy of the microsomal preparations and for use in the evaluation of the MFO studies conducted in association with this project.

METHODS

Fish liver microsomes were isolated by differential centrifugation according to standard techniques in the literature (Winston et al., 1989). NAD(P)H-cytochrome *c* (P450) reductase was measured by monitoring reduction of ferricytochrome *c* (Type III from horse heart, Sigma Chem. Co., St. Louis, MO) at 550 nm (Phillips and Langdon, 1962) and quantified from the millimolar extinction coefficient, 18.5 mM-1 cm-1 for ferrocytochrome *c*. Cytochrome P450 specific content was determined according to Omura and Sato (1964).

RESULTS AND DISCUSSION

NAD(P)H-dependent cytochrome *c* reductase activities were measured in liver microsomes of fish sampled from various offshore sites. The offshore sites were further subdivided as a function of distance from the platform station. Reminiscent of data obtained on these parameters in microsomes from the Lake Champagne and Pass Fourchon fish there was extremely wide interspecies and intraspecies variability. NADH-dependent cytochrome c reductase ranged from below detectable limits in Bay Whiff caught at ST 52 to approximately 1000 nmol/min/mg microsomal protein in catfish caught at ST 34 (Table 5.1). NADH-dependent activities were consistently higher in catfish (870±170 nmol/min/mg, n = 7), irrespective of the distance from the platform at which they were caught. Fringed flounder had the next highest activities with NADH (83±44 nmol/min/mg, n = 6). When the value for fringed flounder caught at ST 53 (9 nmol/min/mg) is eliminated from the data set the mean ± SD was 98±31. Fringed flounder from ST36, 37, 52, 53, and 54 had notably lower NADH-cytochrome c reductase activities than those from ST 53a or any of those caught from ST 34. NADPH-dependent activities ranged from 0.2 nmol/min/mg in Bay Whiff caught at ST 53 and 55 to 300 nmol/min/mg in catfish caught at ST 34. An unexpected observation was the NADH/NADPH ratios of < 1.0 found in some of the Fringed flounder and Bay Whiff microsomes from the most recently provided samples. NADH-dependent activites are generally greater than NADPH-dependent activites.

Table 5.1 Microsomal NAD(P)H-dependent cytochrome *c* reductase activitites of several fish species sampled from a contaminated (Pass Fourchon) and reference (Lake Champagne) site in the Gulf of Mexico.

Location	Species	NADH	NADPH	NADH/ NADPH
		nmol/min/mg		
Lake Champagne	Hardhead Catfish	20	16	1.3
	Hardhead Catfish	55	145	0.4
	Hardhead Catfish	42	46	0.4
	Pinfish	57	7	10.0
	Spot	95	48	2.0
	Croaker 14	28	0.5	
Pass Fourchon	Hardhead Catfish	n/a	12	--
	Croaker	20	5	5.0
	Pinfish	92	4	26.3
	Jack	54	34	1.8
	Jack	8	13	0.6
	Spot	48	48	1.0
	Spot	71	38	1.9

Table 5.2 Microsomal NAD(P)H-dependent cytochrome *c* reductase activitites of several fish species sampled from varying distances from offshore oil platforms in the Gulf of Mexico.

Distance from platform	(meters)	Species	NADH nmol/min/mg	NADPH	NADH/ NADPH
ST 53a	200	Fringed flounder	108	104	1.0
		Fringed flounder	115	44	2.6
		Shoal flounder	7	14	0.5
ST 53a	1000	Fringed flounder	138	30	4.6
ST 53		Fringed flounder	9	29	0.3
ST 53		Bay Whiff	11	38	0.2
ST 34	50	Hardhead catfish	995	300	3.0
ST 34	50	Hardhead catfish	852	270	3.2
ST 34	50	Hardhead catfish	831	121	6.9
ST 34	100	Hardhead catfish	1111	203	5.5
ST 34	200	Fringed flounder	47	91	0.5
ST 34	500	Fringed flounder	80	70	1.1
ST 34	500	Shoal flounder	163	142	1.1
ST 34	1000	Shoal flounder	244	179	1.4
ST 34	1000	Bay Whiff	159	ND	-
ST 34	1000	Bay Whiff	94	54	1.7
ST 34	1000	Bay Whiff	245	92	2.7
ST 34	1000	Fringed flounder	321	85	3.8
ST 34	1000	Fringed flounder	65	12	5.4
ST 34	1000	Sea Robin	16	5	3.2
ST 34	1000	Bay Whiff	155	226	0.7
ST 34	1000	Hardhead catfish	993	177	5.6
ST 34	1000	Hardhead catfish	757	179	4.2
ST 34	1000	Hardhead catfish	555	96	5.8
ST CI	500	Threadfin shad	20	5	3.6
ST 36*		Fringed flounder	4	7	0.7
ST 37*		Fringed flounder	11	36	0.3
ST 52*		Fringed flounder	5	17	0.4
ST 52*		Bay Whiff	ND	27	--
ST 54*		Fringed flounder	2	13	1.8
ST 55*		Bay Whiff	6	38	0.2
Laboratory-treated		3-MC Fundulus Adult	260	96	2.7

*Collected by LDWF, distance not specified

The specific content of cytochromes P450 were determined in all fish samples for which we were supplied with adequate amounts of microsomal protein. In all cases microsomal P450 was found to be mostly denatured to cytochrome P420 (Figure 5.1) or highly contaminated with other interfering CO-binding chromaphores.

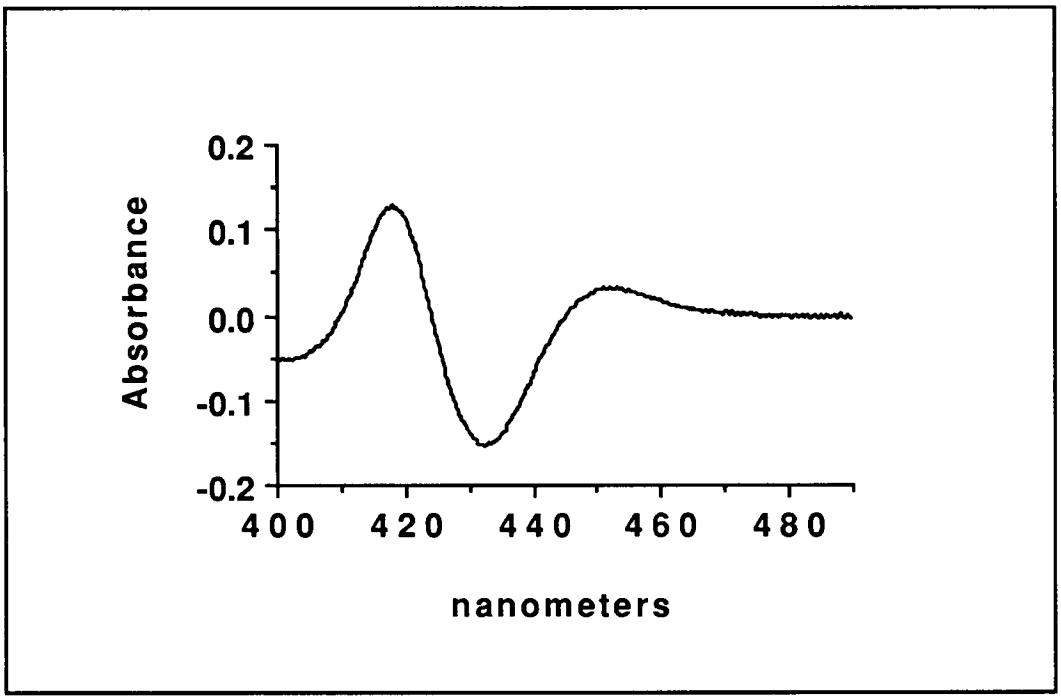

Figure 12. P450 CO-binding spectrum of fish #301 liver microsomes.

In some samples in which a positive absorbance change could be measured between 490 nm (isosbestic point for reduced, CO-liganded and reduced cytochromes P450) and 450 nm an attempt to calculate a residual P450 specific content was made. It is recognized that the accuracy of the measurements are questionable owing to the possibility of spectral interferences of other substances; the above CO-binding spectrum resembles that of cytochrome oxidase and is believed to reflect mitochondrial contamination of the microsomal preparation. Interestingly, the spectra resembled more those of S9 preparations than microsomal preparations. Table 5.2 shows cytochrome P450 specific content estimates of microsomes from fish caught at various offshore sites. These fish represent various distances from oil platforms. Table 5.2 indicates the cytochrome P450 specific content to vary from 0.027 - 0.39 nmol/mg microsomal protein. A microsomal preparation from a rat pretreated with Aroclor 1254, a polychlorinated biphenyls (PCB) mixture of coplanar and non-coplanar PCB congeners was used as a positive control; this preparation had a specific content of P450 of 1.62 nmol/mg protein, which is in good agreement with the literature (Figure 13).

A possible explanation for the high P420 (denatured P450) content of the fish microsomes is having to freeze liver samples prior to microsomal isolation. This has credence in light of a fresh microsomal preparation from a catfish, which showed the expected P450 CO-spectrum and had a specific content of about 300 pmol/mg protein.

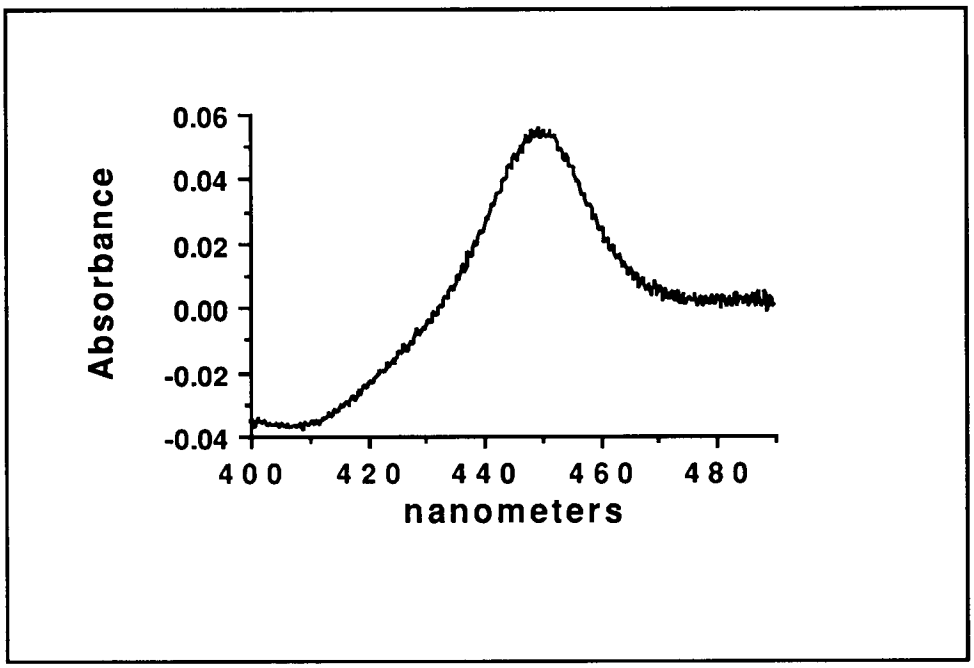

Figure 13. Cytochrome P450 CO-binding spectrum in microsomes of PCB-induced rats.

Table 5.3 Cytochrome P450 specific content of sampled Gulf of Mexico fish.

Species	Location/Distance from platform	P450 Specific Content (nmol/mg protein)
PCB-treated rat		1.630
Hardhead catfish	ST 34 100 m	.391
Hardhead catfish	ST 34 50 m	.031
Hardhead catfish	ST 34 1000 m	.050
Hardhead catfish	ST 34 50 m	.184
Hardhead catfish	ST 34 50 m	.161
Hardhead catfish	ST 34 1000 m	.205
Hardhead catfish	ST 34 1000 m	.077
Fringed flounder	ST 53a 1000 m	.183
Bay Whiff	ST 34 1000 m	.024
Fringed flounder	ST 34 1000 m	.054
Fringed flounder	ST 34 1000 m	---
Sea robin	ST 34 1000 m	---

NAD(P)H-dependent oxygen consumption by Gulf of Mexico fish liver microsomes was measured by oxygen polaragraphy with a Clark-type oxygen electrode. In conjunction with these measurements the ability of the potent nitroarene mutagen, 4-nitroquinoline N-oxide (4NQO), to stimulate oxygen consumption by microsomes was evaluated to assess one-electron reduction metabolism by fish microsomes. It was of principle interest to determine if the univalent pathway of xenobiotic reductive metabolism was altered in fish microsomes as a function of the various field site parameters studied. Various species of fish caught at Pass Fourchon and Lake Champagne as well as from offshore sites were compared. The oxygen

electrode tracings presented in Figure 14 show that all of the fish microsomes studied catalyze NAD(P)H-dependent oxygen consumption. NADH-dependent rates tended to be about 2 to 3 times greater than the corresponding NADPH-dependent rates. Oxygen consumption increases with increasing concentrations of microsomal protein. This concentration dependence is variable for the different fish species. For the most part, at constant microsomal protein concentration, rates of oxygen consumption are proportional to the concentration of 4NQO. Variation in the response to 4NQO is noted, i.e. in some cases 4NQO is saturating at lower concentrations than in others.

No obvious differences were observed as a function of the field parameters. For example, microsomes (200 μg protein) from Hardhead catfish caught at Lake Champagne, a relatively pristine reference site, and from Pass Fourchon, a produced water site and contaminated shipping channel, catalyze NADH-dependent, 4NQO-mediated oxygen consumption at similar rates and with a similar dependence on toxicant concentration (Figure 14). Further, the effect of varying the microsomal protein concentration on oxygen consumption rates while holding 4NQO constant (100 μg) yields similar results with microsomes from Lake Champagne and Pass Fourchon Catfish (Figure 15).

Nitroaromatic compounds are abundant in the marine environment and represent a source of oxidative stress owing to the ability of such compounds to undergo redox cycling with concomitant production of oxyradicals. Nitroso intermediates are common in reductive metabolism of nitroarenes. Attempts to assess C-nitrosoreductase activity in the cytosolic fractions of mussels *(M. edulis)*, sea anemones *(Aiptasia pallida)* and sand worms *(Nereis virens)* were made. *p*-Nitrosophenol was used as a model C-nitroso (R-C-N=O) compound. Nitroso group reduction was measured by monitoring the disappearance of *p*-nitrosophenol at 420nm. Based on this activity, cytosol from neither the worms nor the anemones reduced *p*-nitrosophenol to *p*-aminophenol. M. edulis cytosol catalyzed NADH-dependent p-nitrosophenol reduction at a rate of ~10 mM/min/mg cytosolic protein and NADPH-dependent reduction at a rate of ~22 mM/min/mg cytosolic protein. Noteworthy is the observation that this activity is not stable and gradually disappears over a period of 3 hours on ice. Approximately 20% of the activity was lost in the first hour on ice. By 3 hours approximately 50% of the initial activity was lost. Further experiments to determine nitro(so) reductase activities of cytosol should account for this time-dependent loss of activity. It is recommended that such activities be performed whenever possible on freshly prepared cytosolic fractions. If this is not possible then liquid nitrogen freezing of cytosol should be done and activities performed on samples that have been freshly thawed.

As it presently stands, for use in toxicological assessment the data obtained for each of the microsomal mixed function oxygenase parameters on field samples are of questionable value. The high degrees of variability among the different species, the lack of correlation between MFO and field parameters, and the denaturation of MFO catalyst in microsomal preparations are all contrary to laboratory conducted exposure studies. Additional major problems were the inconsistency of the catches, which were unpredictable with respect to species, lack of knowledge of the sex and gonadal status of the fish at the times in which they were caught and variable conditions of abiotic factors such as fluctuations in oxygen tension. Variability in oxygen tension of the water column possibly contributed to the variability and highly unpredicatable nature of field samplings. Correlation of MFO parameters with oxygen tension measurements may be of heuristic value.

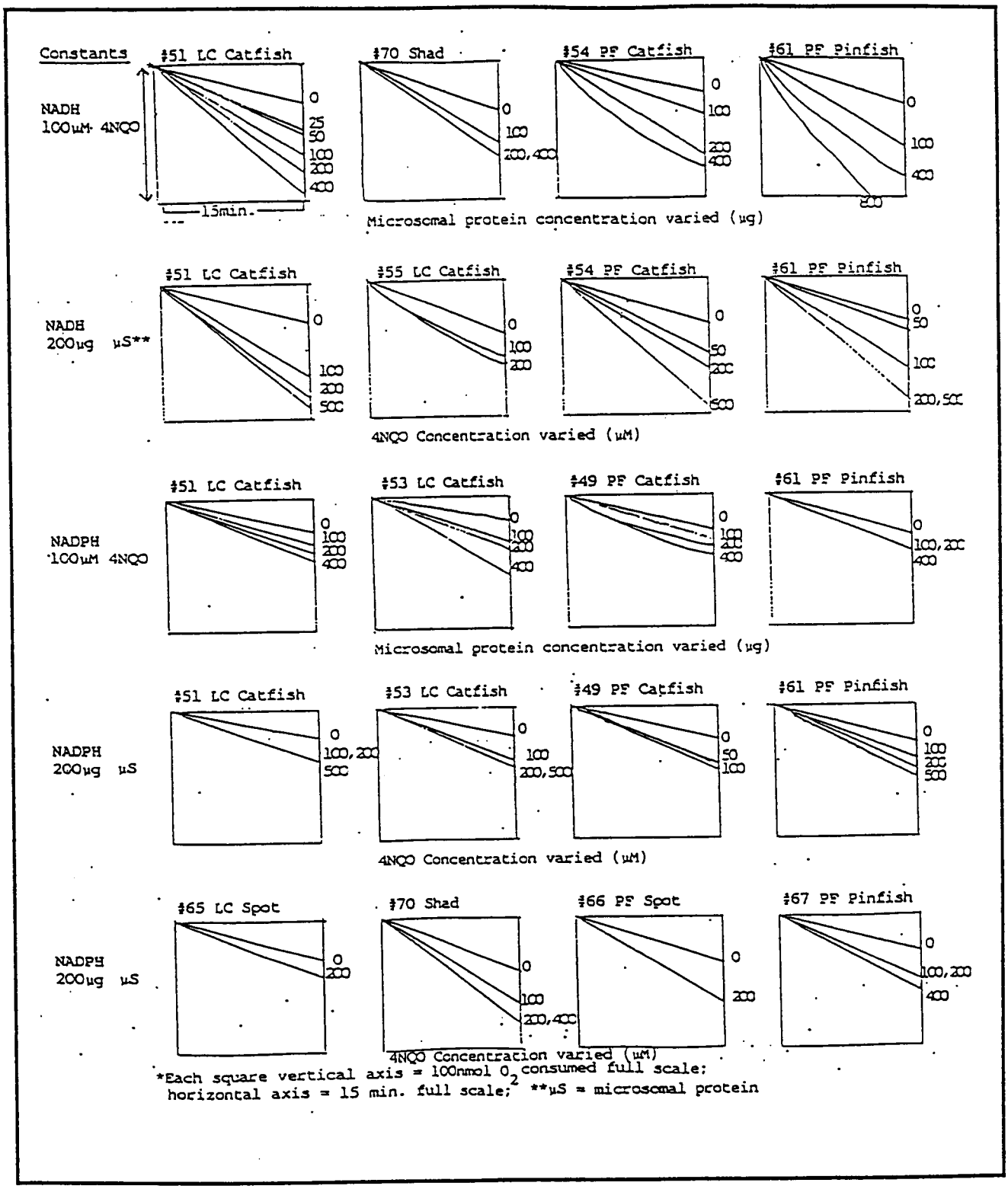

Figure 14. Polaragraphic tracings of 4NQO-mediated oxygen consumption during microsomal electron transport.

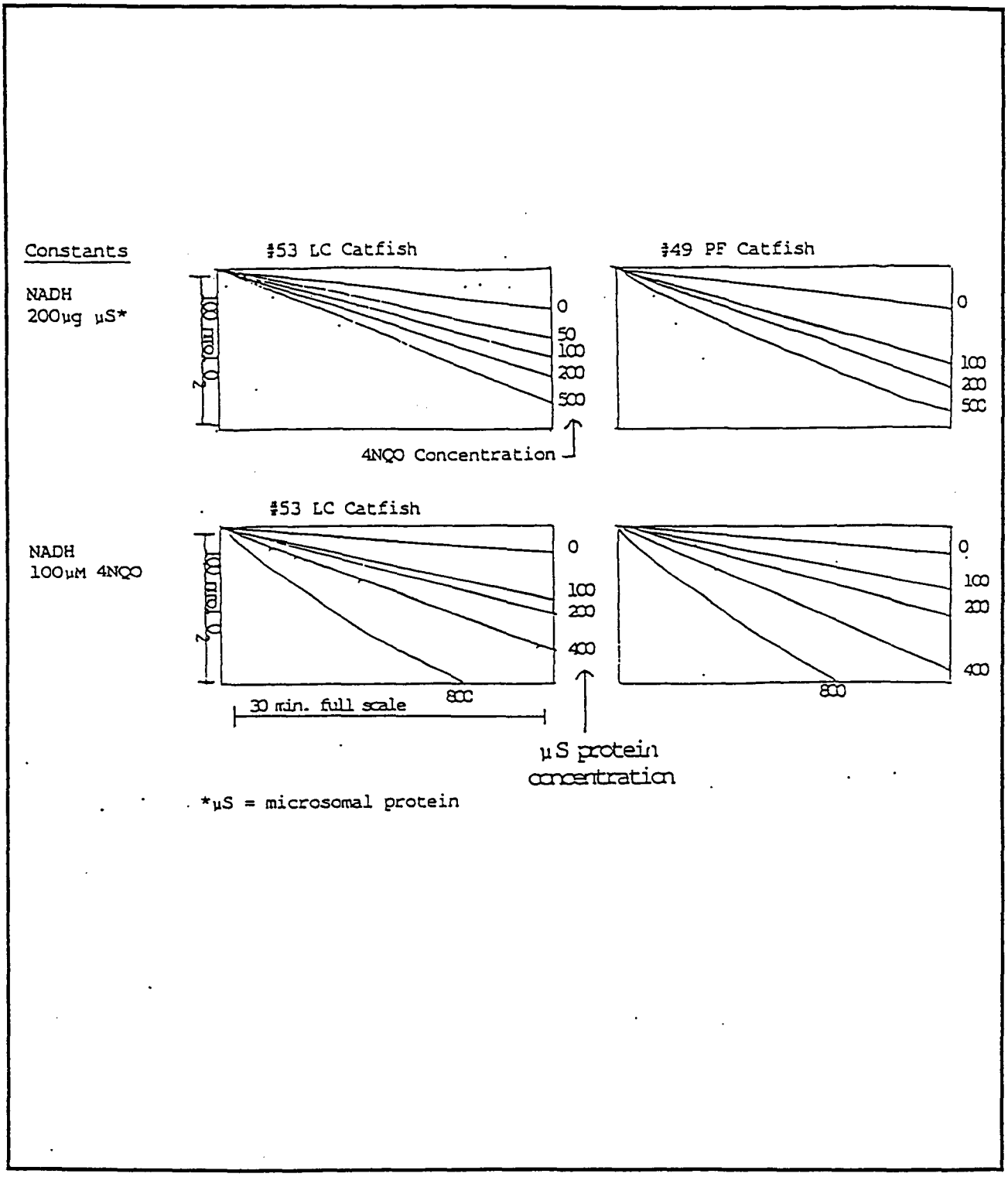

Figure 15. Comparison of NADH-dependent oxygen consumption catalyzed by LC vs PF catfish: Effect of varying microsomal protein microsomes and 4NQO concentrations.

CHAPTER 6

Part A

EVALUATION OF THE BIOCONCENTRATION POTENTIAL OF GENOTOXIC PETROLEUM INDUSTRY EFFLUENTS USING CO-SOLVENT MOBILIZATION.

J.C. Means, D.J. McMillin and L.A. Reily

INTRODUCTION

Characterization of petroleum-related wastes have been part of the scientific literature for a very long time. It is well established that environmental petroleum releases from such facilities as produced water separation facilities, marketing terminals and production facilities contain a very complex mixture of normal and branch chained hydrocarbons and a complex mixture of both volatile (benzene, toluene, xylenes and ethyl benzene) and semi-volatile (naphthalenes, phenanthrenes, fluorenes, etc.) parent aromatic hydrocarbons but also alkylated analogs of these aromatic compounds as well as various heterocyclic aromatic structures such as dibenzothiophenes and aza-arenes (Neff et al., 1987). Among these aromatic hydrocarbons are several compounds which by virtue of their low aqueous solubilities are likely to bioaccumulate in food chains in aquatic systems (Capuzzo et al., 1987; Rabalais et al., 1991b). However, detailed quantification of the genotoxic potential of many of these constituents has been limited.

Once these substances are discharged, they are subject to a variety of physical transport processes. Further, at the molecular level many of the water insoluble or particle reactive substances in petroleum industry effluents may undergo partitioning reactions with suspended particulates, colloidal solids and flocculating materials contained in the effluents themselves as well as the receiving waters. While many of the constituents of hydrocarbon-related wastes are volatile and/or partially biodegradable and therefore present a limited risk to aquatic organisms, there is ample evidence to suggest that the vast majority of the semi-volatile components of petroleum are relatively refractory and are therefore likely to accumulate in sediments and biological tissues. Means et al. (1980 and 1982) showed that PAHs and heterocyclic analogs of PAHs were strongly sorbed to sediments and that the relative strength of sorption could be predicted from compound aqueous solubilities. Further, we have shown that these same compounds may sorb to colloidal organic matter in the water column thus facilitating transport and possibly inhibiting bioaccumulation (Means and Wijayaratne, 1982; Wijayaratne and Means, 1984; Sigleo and Means, 1990 and references therein).

Chemical analyses of environmental samples have frequently been directed toward meeting some regulatory criteria (eg. NPDES permitting requirements) and therefore much of the available literature on hydrocarbon contamination has been limited to studies which report the concentrations of only a relative few of the aromatic hydrocarbons potentially present in a petroleum industry waste (16 priority pollutant PAHs + 1-methyl and 2-methyl-naphthalene). While these compounds may be present as components in the total aromatic fraction of petroleum industry waste, recent studies of petroleum-related wastes such as produced waters, suggest that these 18 compounds may represent less than 5% of the total aromatic materials present. While the genotoxicity of a few of these compounds such as benz[a]pyrene and benzanthracene have been extensively studied and reported, the potential for genotoxic impacts of the petrogenic compounds is poorly characterized.

The hydrocarbon load from petroleum industry effluents is normally stated as one relatively crude number called oil and grease. Detailed characterizations of what compounds comprise this number are not readily available. The EPA conducted two studies in developing guidelines for effluent discharge limitations (USEPA 1979 and 1982). There were seventeen petroleum industry discharge sources that were sampled and analyzed for priority pollutants. Table 1 lists the compounds that were detected in at least one discharge. The majority of these compounds were actually detected in less than five discharges. Most other detailed analyses of petroleum industry discharges have also focused in on the EPA priority pollutant list. Although one study conducted in western Australia investigated petroleum-derived hydrocarbons in a petroleum industry effluent and in organisms 250 meters away from the discharge (Alexander et al., 1982a and 1982b). They found both in the discharge and in mullet and mussel tissue: naphthalene, methylnaphthalenes, dimethylnaphthalenes, trimethylnaphthalenes, phenanthrene, methylphenanthrenes and dimethylphenanthrenes.

Table 6A.1 Priority pollutants commonly detected in treated petroleum industry effluents *

Volatiles	**Base/Neutral Extractables**
Methylene chloride	Naphthalene
trans-1,2-Dichloroethene	Dimethyl phthalate
Chloroform	Fluorene
1,2-Dichloroethane	Diethyl phthalate
Bromodichloromethane	Phenanthrene
Trichloroethene	Anthracene
Benzene	Di-n-butyl phthalate
Tetrachloroethene	Fluoranthene
Toluene	Pyrene
Ethyl benzene	Benz(a)anthracene
	Chrysene
Acid Extractables	Bis(2-ethylhexyl) phthalate
	Benzo(a)pyrene
Phenol	Benzo(g,h,i)perylene
2,4-Dimethylphenol	
2,4-Dichlorophenol	
4-Chloro-3-methylphenol	
4-Nitrophenol	

* EPA (1979, 1982)

We proposed that a more detailed analysis of the petrogenic components as presented in the present study could yield valuable information on the potential for adverse genotoxic effects upon aquatic organisms exposed to these complex mixtures. Methodologies developed as part of this study and reported in Chapter 7 were employed to analyze of petroleum-related wastes for over 55 specific parent and heterocyclic PAHs and their alkylated homologues. These methods were utilized to characterize the exposure concentrations in sediments or water and the bioaccumulated residues found in exposed biomonitoring organisms. These data are reported in Chapter 3.

While these data provide an improved understanding of the exposure potential for petroleum-related wastes such as produced waters, they do not allow for the assessment of genotoxic risks of that exposure. Over the last two decades, investigators have utilized whole extracts of soils and sediments to evaluate genotoxic potential of particle-associated toxic substances. Typically these methods have employed exhaustive extraction of the sediment or soil with an organic solvent followed by concentration of the extract. The final extract was typically transferred to another solvent compatible with various bacterial or cell culture genotoxicity assays such as dimethylsulfoxide and applied to the assay at different dose levels to establish the presence or absence of genotoxic agents in the mixture (Dexter et al., 1979; Suzuki et al., 1982, Osborne et al., 1982 and Daniels et al., 1985). These and numerous other studies have reported dose-related responses in a number of genotoxicity assays. Among the problems reported in these investigations are the cytotoxicity of many of the extracts, the complexity of the mixtures, the high background (positive genotoxicity responses) of extracts from uncontaminated soils and sediments.

A basic conceptual limitation of these experiments has been the inability of these types of studies to relate results to actual risk. The limitation is that there has been no way to relate data on genotoxicity of sediment extracts to bioavailability and bioaccumulation potential of organisms living in contact with contaminated sediments, thus establishing risk. This study sought to combine elements of models of bioaccumulation with models of sediment/water partitioning theory to evaluate the utility of co-solvent extracts from sediments to evaluate genotoxicity in a way that can be related back to the bioavailability of the contaminants and therefore provide a more realistic assessment of genotoxic risk to aquatic organisms.

THEORY

Sediment Partitioning

The equilibrium partitioning of organic contaminants in sediment/water systems has been the focus of investigation for a long time. Polycyclic aromatic hydrocarbons and their alkylated analogs, and various classes of heterocyclic aromatics such as dibenzothiophenes and dibenzocarbazoles all have been investigated Means et al., 1980, Hassett et al., 1980, and Banwart et al., 1982). In all cases, these compounds partition between the organic carbon phase of the sediments and water in a predictable way based upon the aqueous solubility of the individual compound. Another predictive compound property was found to be the octanol-water partition coefficients of the compounds. Wijayaratne and Means (1984) established that similar partitioning occurs between organic-rich colloidal phases found in aquatic systems and water. In more recent studies, Means and McMillin (1993) showed that this partitioning was completely reversible in estuarine sediments and that the kinetics of desorption were sufficiently rapid to allow exposure of benthic organisms living in contact with the sediments.

Figure 16a shows the relationship between octanol-water partition coefficients (log P) and the values of sediment-water partition coefficients normalized for the organic carbon content of the sediment (log K_{OC}). This relationship has the following equation:

$\log K_{OC} = \log P - 0.317$ (Means et al., 1980)

Bioavailability of Selected Trace Organic Contaminants: Many of the trace metals and organic substances which have been found in previous examinations of petroleum discharges are known to be toxic both to aquatic organisms and to man NAS, (1985). In particular, many of the aromatic organic compounds are known or suspected mutagens or carcinogens EPA (1982). Therefore, a key aspect of a complete ecological and human risk

assessment for these discharges must address the bioavailability and bioaccumulation potential of the contaminants found in these discharges.

One approach which has been used in many such assessments has been the collection and analysis of indigenous populations of non-migratory species in the area of a discharge site and in a spatial gradient away from that site. Bivalve mollusks such as the blue mussel (*Mytilus edulis)* , the oyster (*Crassostrea virginica)* and the freshwater Asiatic clam (*Corbicula fluminea*) have been used successfully in several large-scale biomonitoring studies in estuaries and coastal marine environments (Farrington et al., 1983, Neff et al., 1976, NOAA, 1988). These and other studies have established the scientific validity of such biomonitoring efforts. However, adequate data on the bioavailability and bioaccumulation of petroleum industry-associated discharges of aromatic organics remain largely incomplete.

There remains a certain degree of uncertainty concerning the bioavailability of sediment-associated contaminants, particularly with respect to suspended particulates. Since many of the toxic organics found in petroleum wastes are highly particle-reactive and sorbed to suspended or bedded sediments, a more detailed examination of the bioavailability of these contaminants is required.

The vast majority of detailed bioaccumulation studies have been performed using aqueous phase exposures of a wide variety of aquatic organisms including fish, crustaceans, mollusks, etc.. From these investigations a variety of predictive equations have been derived which relate the octanol-water partition coefficient of the compound to the bioconcentration potential (BCF) of the compound in tissues. Many of these relationships have further been refined and predictive capability improved by normalization of the quantities bioaccumulated in the organisms to the lipid content of the tissues examined or the whole organism. Figure 16b shows one such relationship. In this case, the slope of the regression equation between log P and log BCF is near unity just like the sediment-water partitioning relationship discussed earlier. If we assume that exposures of aquatic organisms in sediment/water systems involves a sequential process in which the contaminant must first desorb from the sediment and then is bioavailable to be accumulated in the organism, then the two predictive relationships shown in Figure 16 can be combined to predict sediment exposures. In this figure, log K_{oc} refers to the organic carbon-normalized sediment/water partition coefficient, log BCF (lipid) refers to the lipid-normalized bioconcentration factor, and log P refers to the octanol/water partition coefficient.

Investigations have been performed by several research groups to establish a predictive relationship between sediment concentrations and bioavailable contaminant levels in organisms living in association with sediments. These experimental studies have suggested that, for a limited number of neutral hydrophobic organic compounds, including aromatic hydrocarbons, an exposure level can be estimated (McElroy and Means, 1988 and references therein, Foster, et al., 1987) from the sediment concentration normalized for organic carbon and the lipid content of the test organism using the following equation:

$$AF = \frac{\text{[toxicant sed. conc./TOC]}}{\text{[toxicant tiss. conc./lipid]}}$$

where AF is an accumulation factor which is an empirically determined constant equal to ~2.

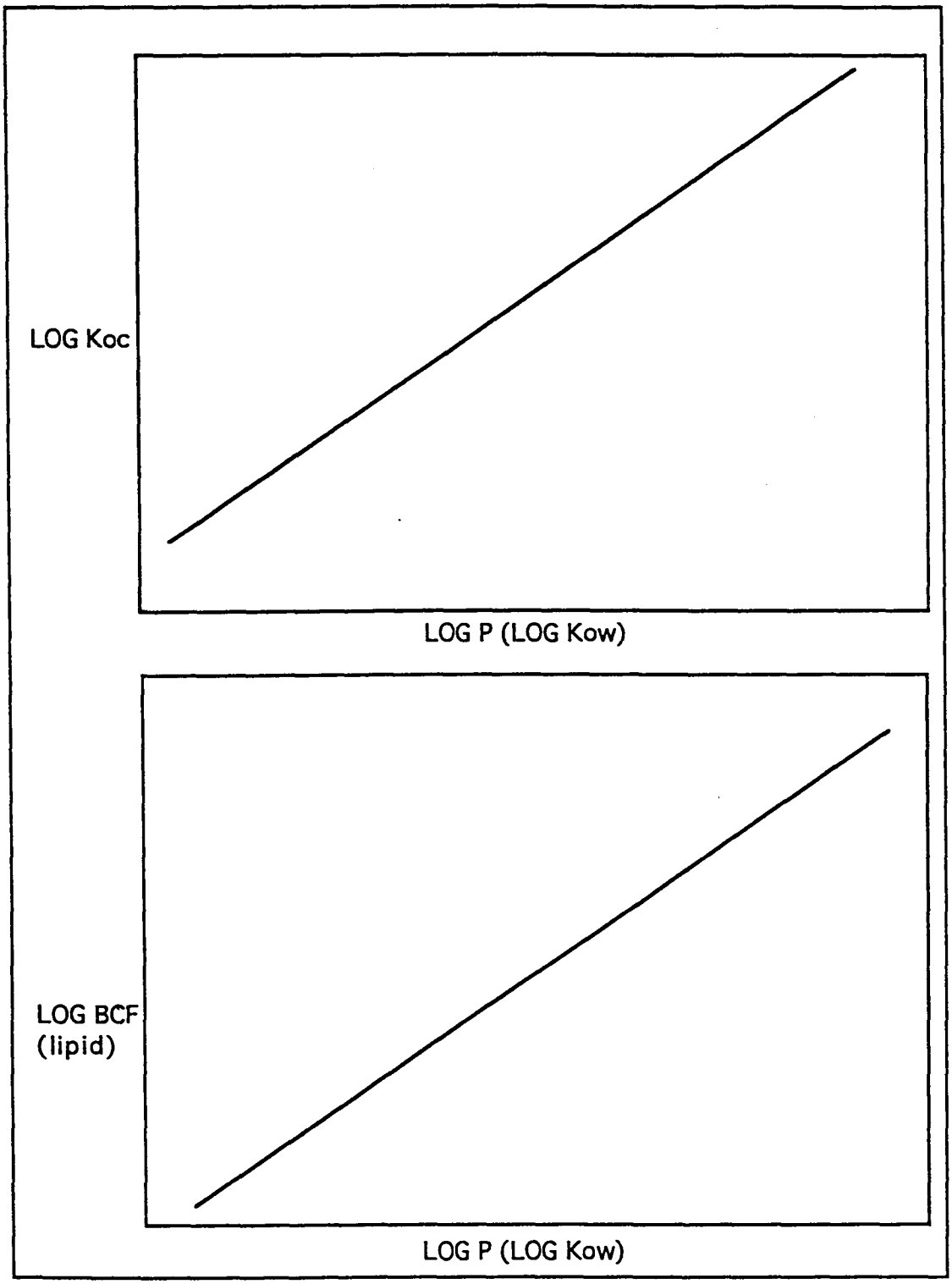

Figure 16. Relationship of Log P to Log Koc and Log BCF.

Co-solvent Theory Applied to Sediment Partitioning: Co-solvents are defined as water miscible organic solvents which when mixed in varying proportions with water result in alteration of the solubility of a wide variety of organic chemicals. Typical co-solvents include short-chain organic acids, alcohol, ketones, aldehydes, etc. As the fraction of co-solvent added to water increases, the solubility in the liquid phase of hydrophobic compounds such as PAH, chlorinated hydrocarbons, and even dioxins increases in a log-linear fashion. The slope of the relationship is dependent upon the co-solvent and the solubility of the compound.

Rao et al. (1985) combined co-solvent theory with sediment-water partitioning theory to attempt to develop predictive equations for the enhanced transport of organic contaminants in sub-surface soil environments. Means et al. (1988) investigated the effects of several co-solvents on the sediment-water partitioning of PCBs and DDT. In that study, they concluded that co-solvents predictably reduced the organic carbon normalized sediment-water partition coefficients (Koc) for several neutral hydrophobic compounds in a log-linear fashion. They further observed that of the organic co-solvents evaluated, the degree to which the hydrophobic compounds were solubilized followed the following sequence: propionic acid>acetic acid>acetone>ethanol>methanol.

Figure 17 shows the effect of three co-solvents upon the Koc of the model compound 2,4,2',4'-tetrachlorobiphenyl. As the mole fraction of each of the co-solvents was increased, the log Koc value decreased in a linear fashion. In each case, the regression lines intersect the Y-axis at a point corresponding to the log Koc measured or predicted for pure water. Figure 18 shows the sorption and desorption equilibria for the compound on two different sediments with acetic acid as the co-solvent. Importantly, both the Y-axis intercept and the slopes of the regressions remain relatively constant from sediment to sediment and regardless of the mode of approach to equilibrium (sorption vs. desorption).

The experimental results may be fit to the equation:

$$\log (K_{ocm}/K_{ocm,w}) = -\alpha\sigma f$$

where K_{ocm}, w and K_{ocm} are the mole-based, organic carbon-normalized partition coefficient for the water and co-solvent systems, f is the mole fraction of the co-solvent, and the product as represents the slope of the line. These constants relate solute-soil (α) and solvent-soil (σ) interactions.

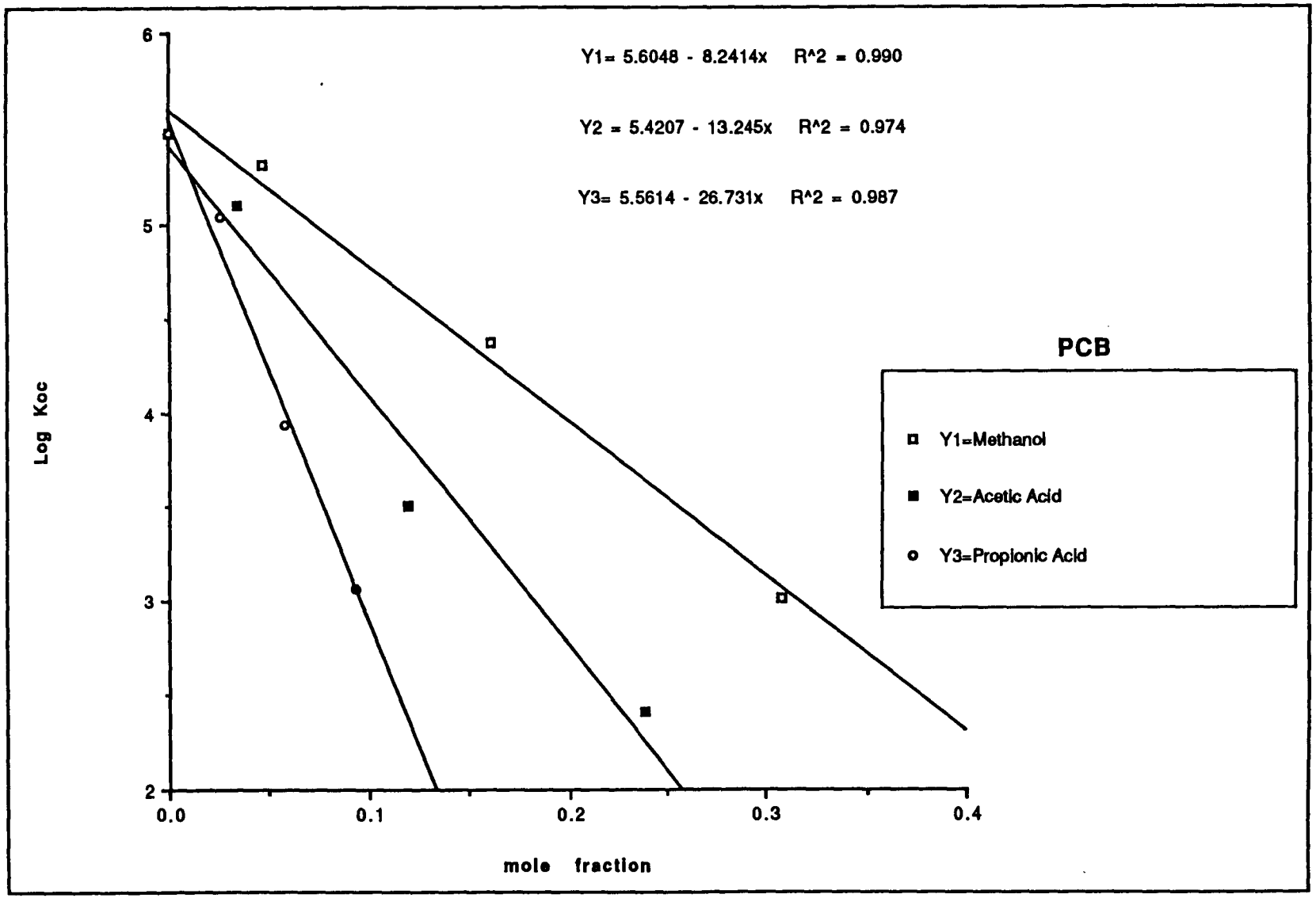

Figure 17. Effect of mole fraction of three co-solvents on desorption Koc values for hexachlorobiphenyl

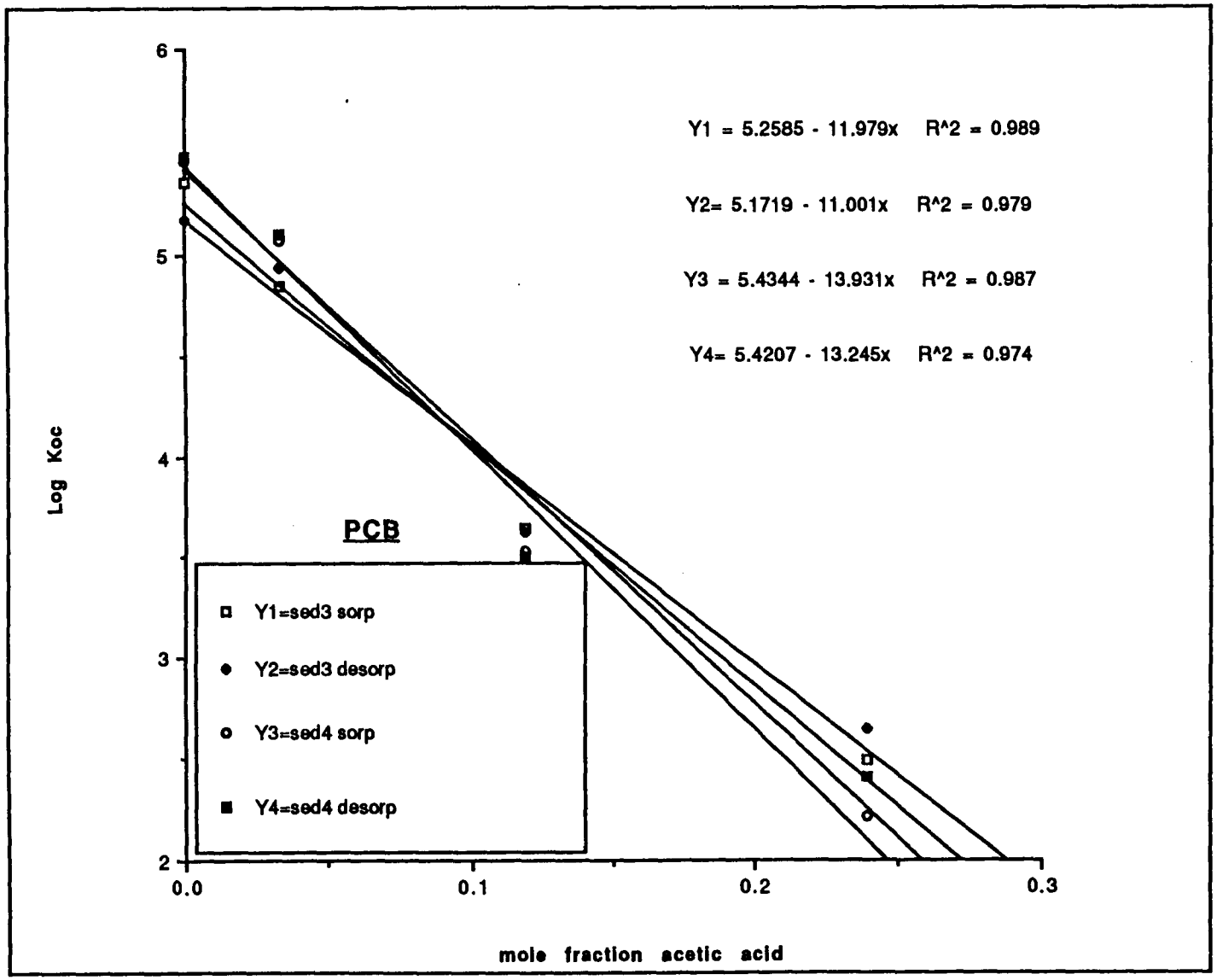

Figure 18. Effect of mole fraction acetic acid on Koc for hexachlorobiphenyl

Figure 19 shows the behavior of three compounds in a single co-solvent systems. With a given co-solvent such as acetic acid, the slopes of the regression lines remain constant while the Y-axis intercepts change in response to magnitude of the equilibrium partitioning constant Koc for the individual compound.

Co-Solvent Theory And Predictive Assessment Of Bioaccumulatable Mutagenic Compounds In Sediments: Since bioaccumulation potential has been observed to be strongly correlated with the octanol-water partition coefficient of a compound (log P), especially when tissue concentrations at steady-state (log BCF) are expressed on a lipid-normalized basis. Similarly, since sediment water partition coefficients normalized to the organic carbon content of the sediment (log Koc), which have been inversely related to bioaccumulation potential, are also strongly correlated with log P. Bioavailability of contaminants sorbed to sediments have been modeled as two-step process involving first, desorption from sediment into interstitial or overlying water, followed by bioaccumulation from the aqueous phase.

Total extracts of sediments/soils using non-polar solvents have been used to determine the total concentrations of contaminants in sediments but have been shown to be poor predictors of bioaccumulation potential from the sediment, due to differences in the relative bioavailability of different pools of the contaminants within the organic and inorganic matrix of the sediment particles. Total extracts do not reflect differences in bioavailability due to differences in log P of the several contaminants contained in a sediment. Further, the contribution of relatively water-soluble mutagenic compounds may be underestimated when total extracts are used to assess sediment mutagenicity or toxicity.

Co-solvent partitioning behavior of compounds, in which the log Koc for a compound decreases in a predictable log-linear fashion with increasing mole fraction of co-solvent, may be used to assess the relative desorption potential of different classes of sediment-bound chemicals. The resulting extracts contain amounts of different chemicals, not only in proportion to the total amounts present in the sediment but also in relation to their relative desorption potential from sediment and therefore in relation to their bioavailability and bioaccumulation potential. In addition, relatively water-soluble mutagenic compounds are included in the extracts. A further advantage of this approach is that a relatively non-toxic organic solvent such as acetic acid can be used which is volatile for concentration purposes and which is not mutagenic in bioassay systems like many chlorinated solvents.

If the co-solvent theory is working as expected in the system, then we would expect to see water soluble compounds extracted from the sediment present in the low co-solvent fraction extracts and declining in concentration at higher co-solvent fractions. Conversely, we would expect neutral hydrophobic compounds extracted from the sediments, such as aromatic hydrocarbons, to be very low in low fraction co-solvent extracts and more abundant in higher co-solvent extracts.

Figure 20 shows the concentrations of two model compounds determined in a series of acetic acid extracts with increasing mole fraction co-solvent. As expected, bisphenol, a relatively polar aromatic compound, was detected in highest amounts in the 0% acetic acid extract and at progressively lower concentrations in extracts with higher co-solvent content. Methyl naphthalenes, an abundant class of petroleum-associated alkylated aromatic compounds, were not detectable in either the 0% or 10% acetic acid co-solvent extracts but increased dramatically in concentration in 20% to 50% acetic acid co-solvent extracts.

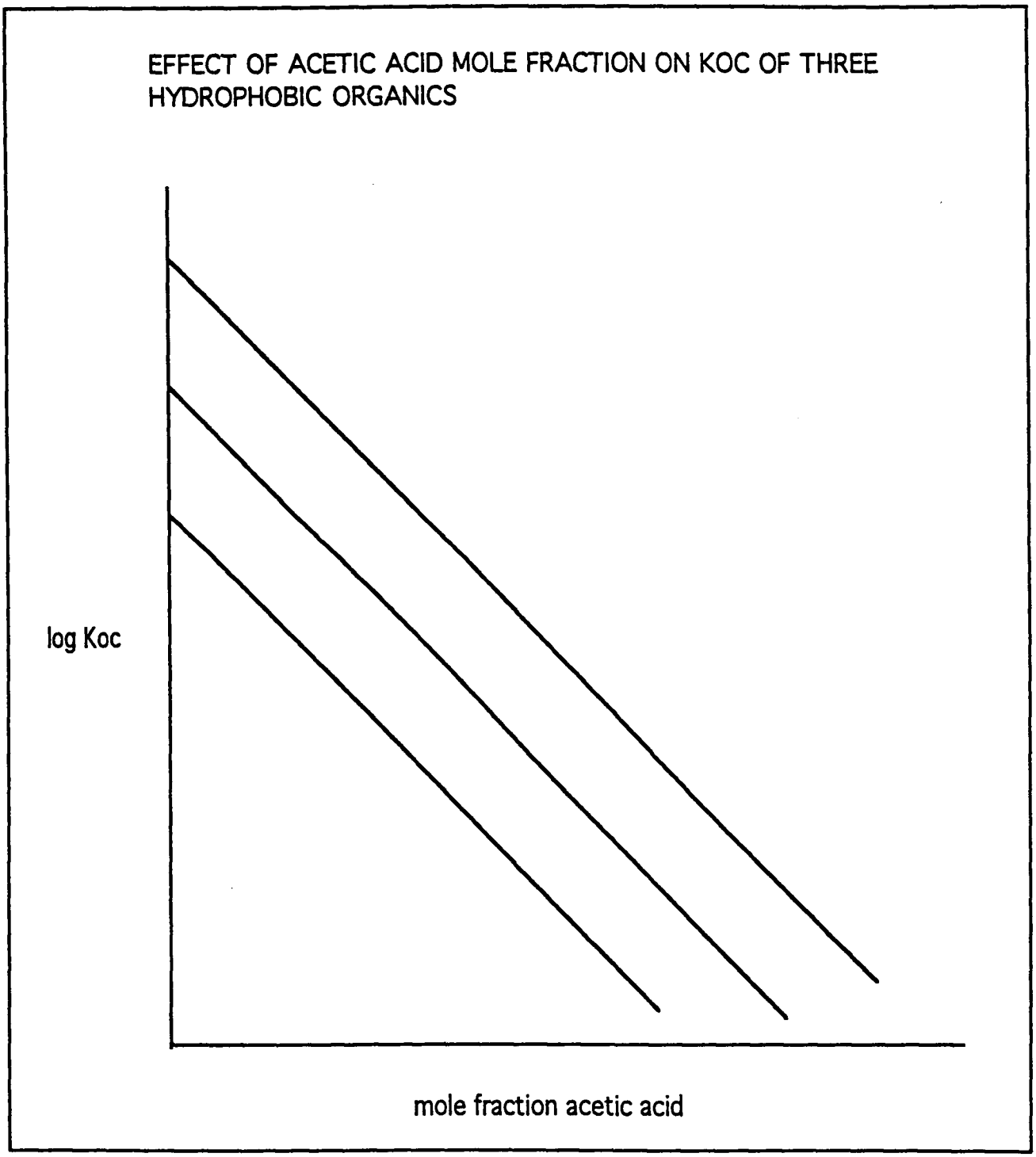

Figure 19. Effect of acetic acid co-solvent concentration upon the Koc of three model hydrophobic compounds.

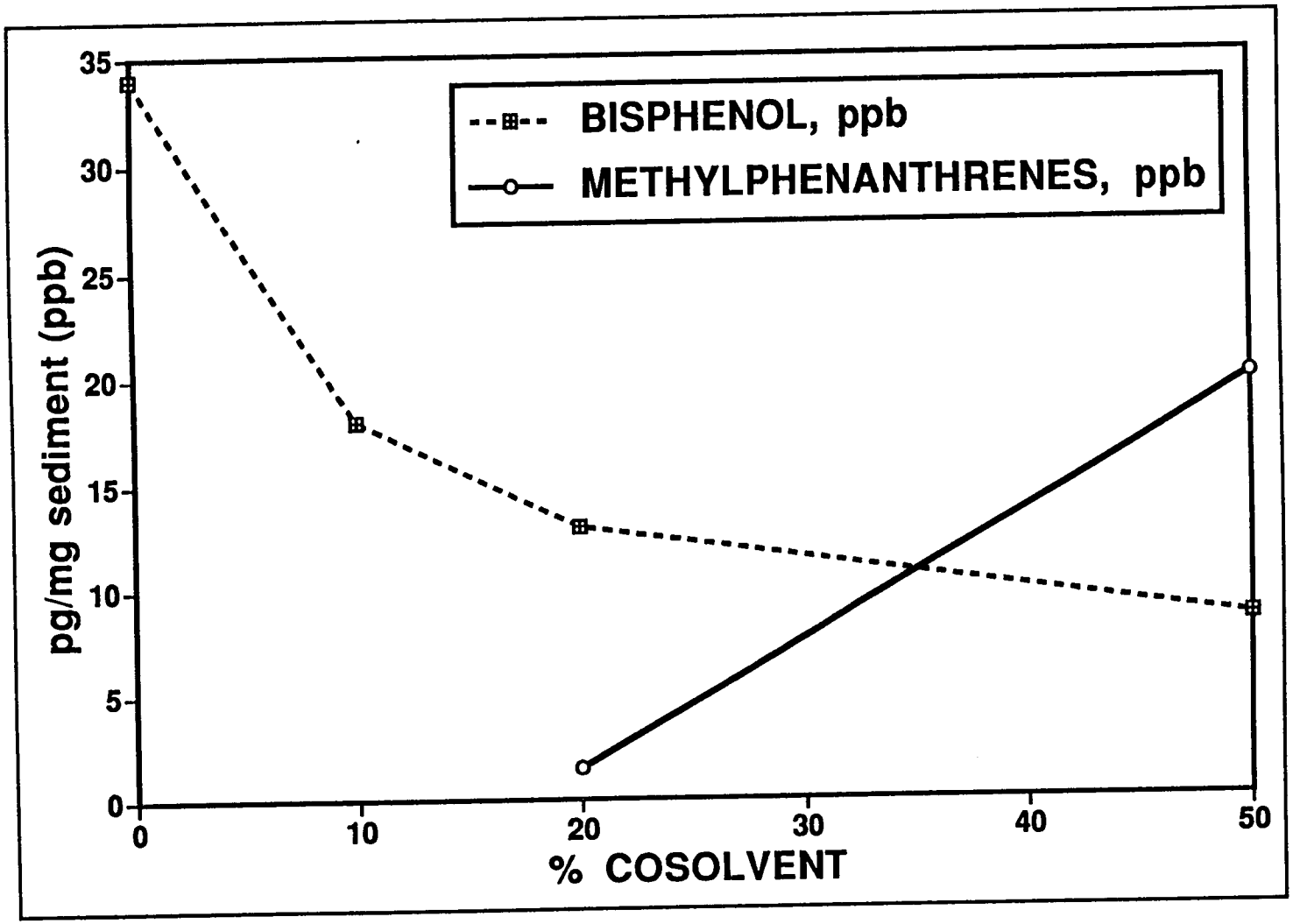

Figure 20. Co-solvent mobilization of selected pollutants in a contaminated sediment.

Based upon the theory described and the basic confirming results from chemical analyses of co-solvent extracts of petroleum-contaminated sediments, an experiment was designed to evaluate the mutagenicity of co-solvent extracts of Pass Fourchon sediments using a series of acetic acid co-solvent extracts assayed in the Ames microbial mutagenicity test.

EXPERIMENTAL METHODS

Sediment Extractions

Approximately 50 g of wet Pass Fourchon sediment were weighed and placed in a 120 mL glass bottle along with 100 mL of extraction co-solvent. The co-solvents used were 0%, 10%. 20% and 50% acetic acid in distilled water. Extractions were performed by rolling the bottle containing sediment and solvent together on a roller mill for 24 hrs, separating the phases by centrifugation and decanting the solvent. This process was repeated two times and the three extracts were combined. The solvents were concentrated to 50 ml by rotary evaporation such that 1 ml of extract would contain compounds extracted from approximately 1 g of wet sediment. These 1 ml aliquots of extracts were evaporated to near dryness under a stream of nitrogen and taken up in 1 ml of dimethylsulfoxide (DMSO). These DMSO solutions were used to apply the extracts to petri dishes for the mutagenicity assays.

Mutagenicity Testing

Mutagenicity of the co-solvent extracts of Pass Fourchon sediment was assessed using the Ames microbial mutagenicity assay, revised as described in Maron and Ames (1983). Several tester strains were used, including strains TA97a, TA98, TA100, and TA102. Each extract was assayed both in the presence and absence of a rat S-9 microsomal activating system to evaluate the presence of direct-acting and promutagens in the extracts. Each extract was evaluated over a range of doses corresponding to 0 to 50 mg of sediment (50 ul of appropriate extract dilutions in DMSO, applied to the series of plates). Plates were scored for revertant colonies per plate after incubation for 72 hr at 37 C. Results were reported as revertants per plate per mg of sediment.

RESULTS AND DISCUSSION

The Ames mutagenicity assay employs auxotrophs of *Salmonella typhimurium* which contain errors in the gene which codes for conversion of biotin to the essential amino acid histidine (Ames, 1975; Maron and Ames, 1983). The occurance of additional mutations within the gene DNA following exposure to a test mutagen, can cause shifts in the genetic reading frame which restore cells to a histidine-autotroph genotype. This process is known as reversion. Revertant colonies which grow in the absence of histidine may be enumerated as an index of the mutagenic potential of mixtures or pure compounds to which they were exposed. The mutagenicity of the cosolvent extracts was observed, to assess the various types of mutational injury detectable with the array of five tester strains. The experiments were also conducted with and without metabolic activation of promutagens by induced rat liver S9.

Consistent dose-response relationships are prerequisite to the use of Ames test data for a particular tester strain/mutagen assay system. Further, in order for the proposed co-solvent system to work, the extracts must yield consistent results in the assay system so that results can be replicated within reasonable limits of variability. Only then, can the results be used to extrapolate to real exposure levels (risks) associated with a particular sediment using the proposed co-solvent/bioaccumulation model. In order to test the level of replication achieved in these co-solvent experiments, assay series which yielded linear dose-response curves were assembled and calculated on a revertant/plate/mg basis for a range of doses tested on different

plates corresponding to 0 to 50 mg of sediment assayed using two co-solvent system extracts (0% and 50% acetic acid) in strain TA 102 and a rat S-9 activating system. Adequate replication was present in the system, yielding similar values of revertants/plate/mg sediment for all sediment doses applied to plates.

Figure 21 shows the responses obtained in this experiment. It is clear that, except for the dose equivalent to 2.5 mg of sediment from the 50% co-solvent extract, the results are reproducible for both water soluble and 50% co-solvent soluble pro-mutagens. This suggests that extrapolations using the model can be made with reasonable confidence.

Both direct acting and promutagenic substances were observed in the extracts from Pass Fourchon sediment, however, the direct acting substances were only detected with strain TA 102. Figure 22 shows the direct acting mutagenic activity of the four co-solvent extracts from Pass Fourchon. It should be noted that in this form the data for spontaneous reversions in these strains when calculated on an equivalent sediment dose level fall into a range of <1 revertant/plate/mg.

Both water soluble and more hydrophobic (eg. 20% co-solvent) direct acting mutagenic compound groups were detected with TA 102. A decrease in mutagenicity was observed in the 10% co-solvent extract relative to all other extracts tested, suggesting that the extraction of water soluble mutagens was suppressed in this extract. This is consistent with the results of chemical analyses reported before. The highest response was observed at 20% co-solvent. The relative decrease seen in the 50% co-solvent extract may be due in part to enhanced cytotoxicity of this extract to the bacteria.

Mutagenic compound groups which required metabolic activation with S-9 were detected in all three bacterial assay strains utilized. Of the three strains employed, TA 102 was the most responsive to the promutagens extracted from this sediment. Figure 23 shows the responses of strain TA97a to the four co-solvent extracts. Comparable but low levels of mutagenic activity were detected in the 0% and 10% co-solvent extracts. A three-fold increase in mutagenic activity was detected in the 20% co-solvent extract, suggesting that there were more hydrophobic than water soluble pro-mutagens which responded in this strain. A great deal of cytotoxicity was observed with the 50% co-solvent extract in this strain which suppressed responses in the assay.

Figure 24 shows the mutagenicity observed in strain TA 100. In contrast to the systems reported thus far, the greatest response in this assay was observed with the water soluble extract which contained very high levels of promutagens. The extraction of these promutagens was completely suppressed in the 10% co-solvent extract. Low levels of more hydrophobic promutagens were progressively extracted in the 20% and 50% co-solvent systems. No cytotoxicity was observed in this case with the 50% co-solvent extract. This observation suggests that the toxicity observed in other strains may be due to very high mutation frequencies rather than general cytotoxicity of the extract. This is a fairly common observation in assays of complex mixtures.

Figure 25 shows the responses observed in strain TA 102 with the four extracts. As noted earlier, the promutagens in this sediment elicited the greatest response in this assay strain. Very high frequencies of mutation were observed in the water soluble extract. Unfortunately, the 10% co-solvent extract was lost just prior to this assay and therefore no results for this extract were obtained. High levels of mutation were also seen in both the 20% and 50% co-solvent extracts applied to this strain. The slight decrease in mutagenicity was interpreted as being to the very high mutation rates leading to reduced survivorship on these plates.

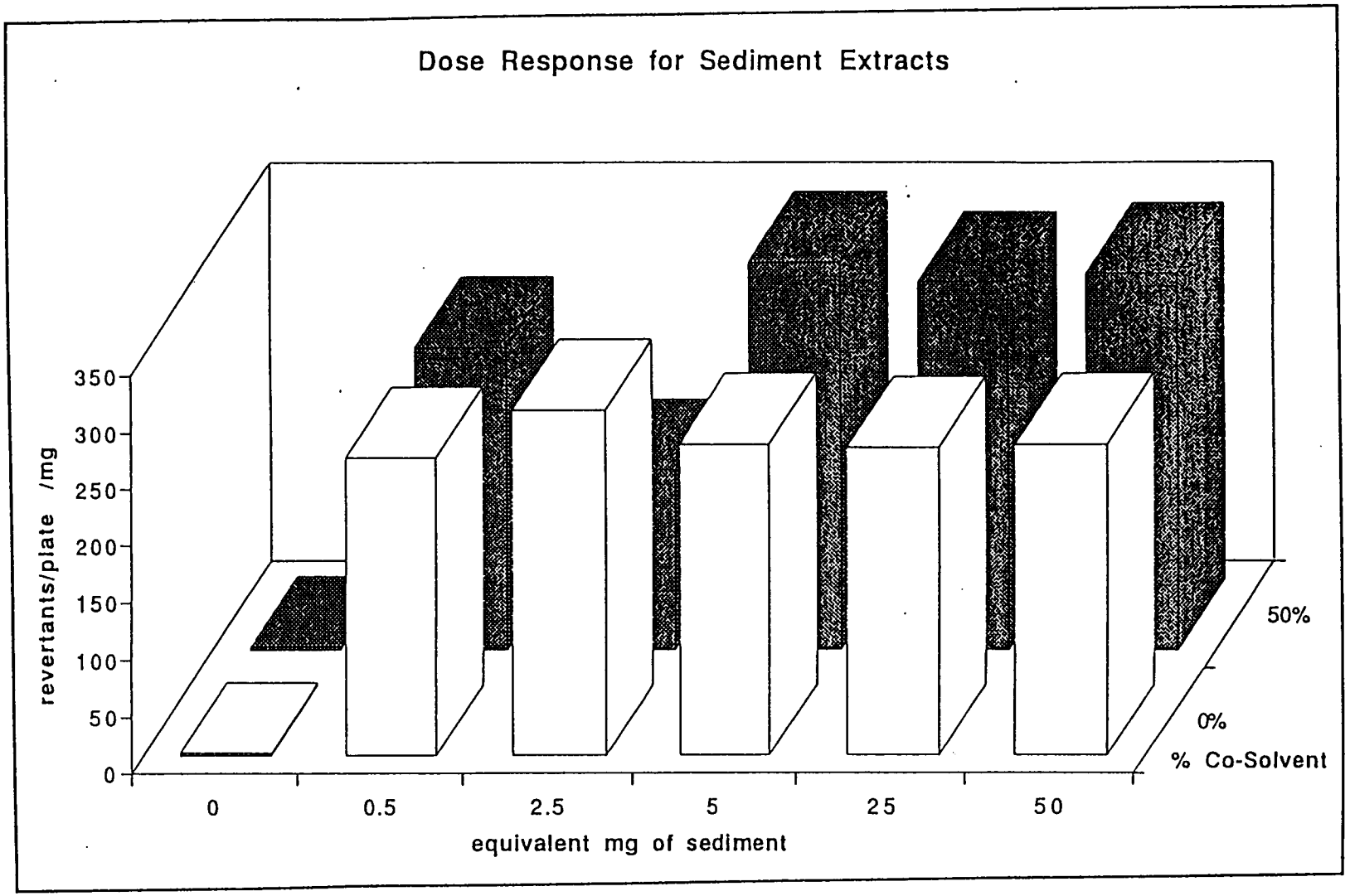

Figure 21. Dose response of mutagens extracted from sediments

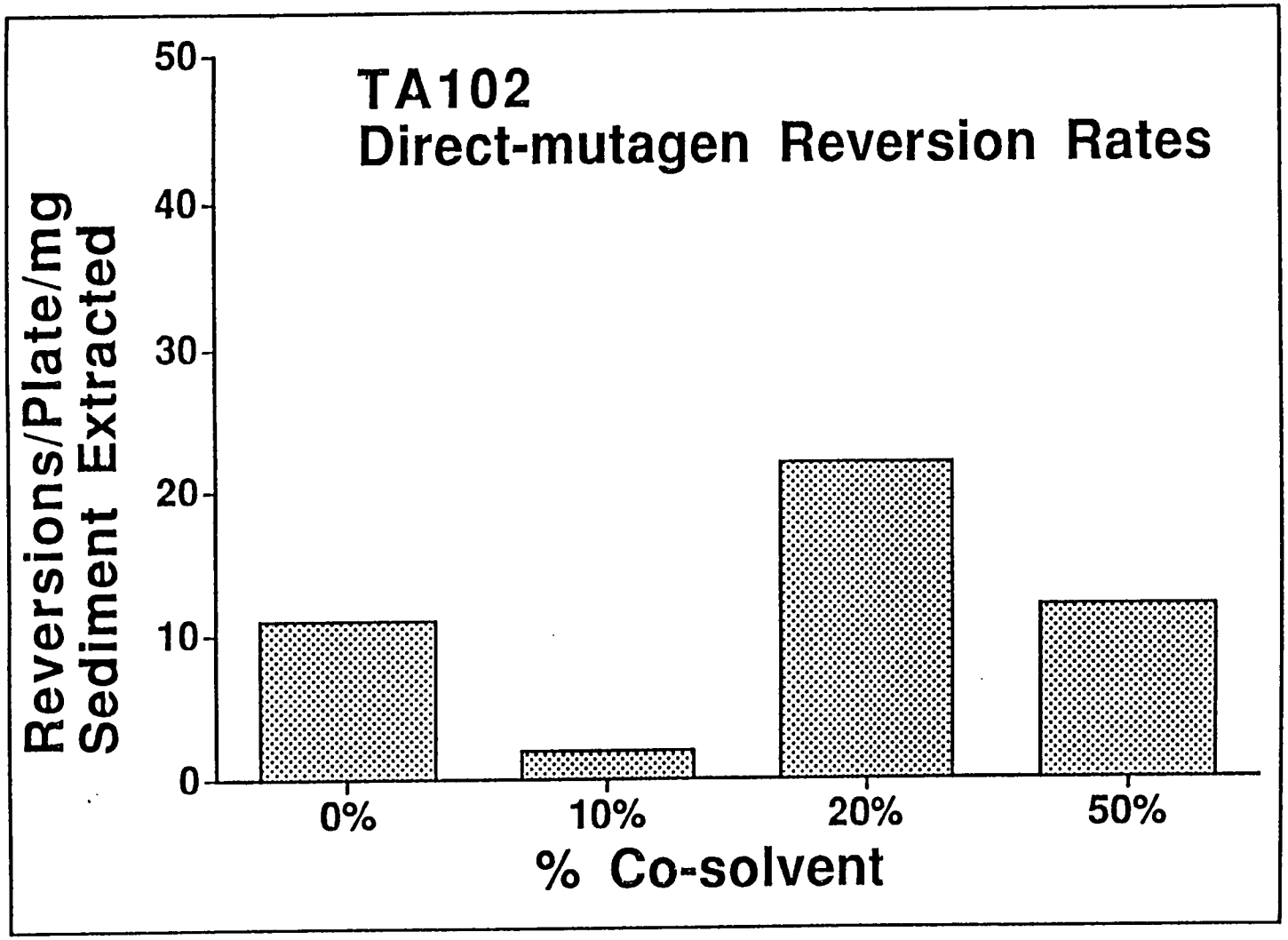

Figure 22. Reversion frequencies for direct-acting mutagens extracted at various co-solvent fractions from contaminated sediment, (TA102).

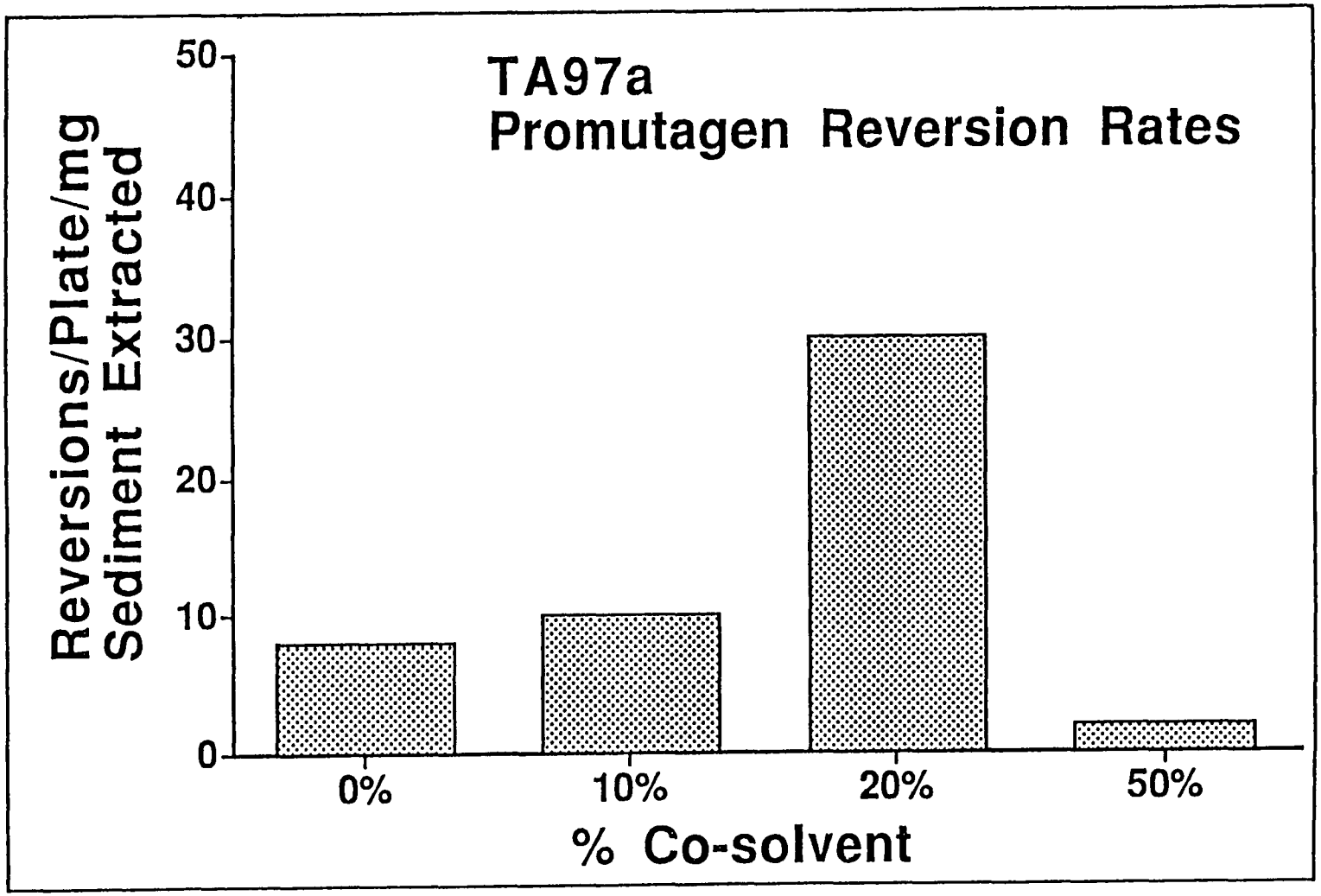

Figure 23. Reversion frequencies for promutagens extracted at various co-solvent fractions from contaminated sediment, (TA97a).

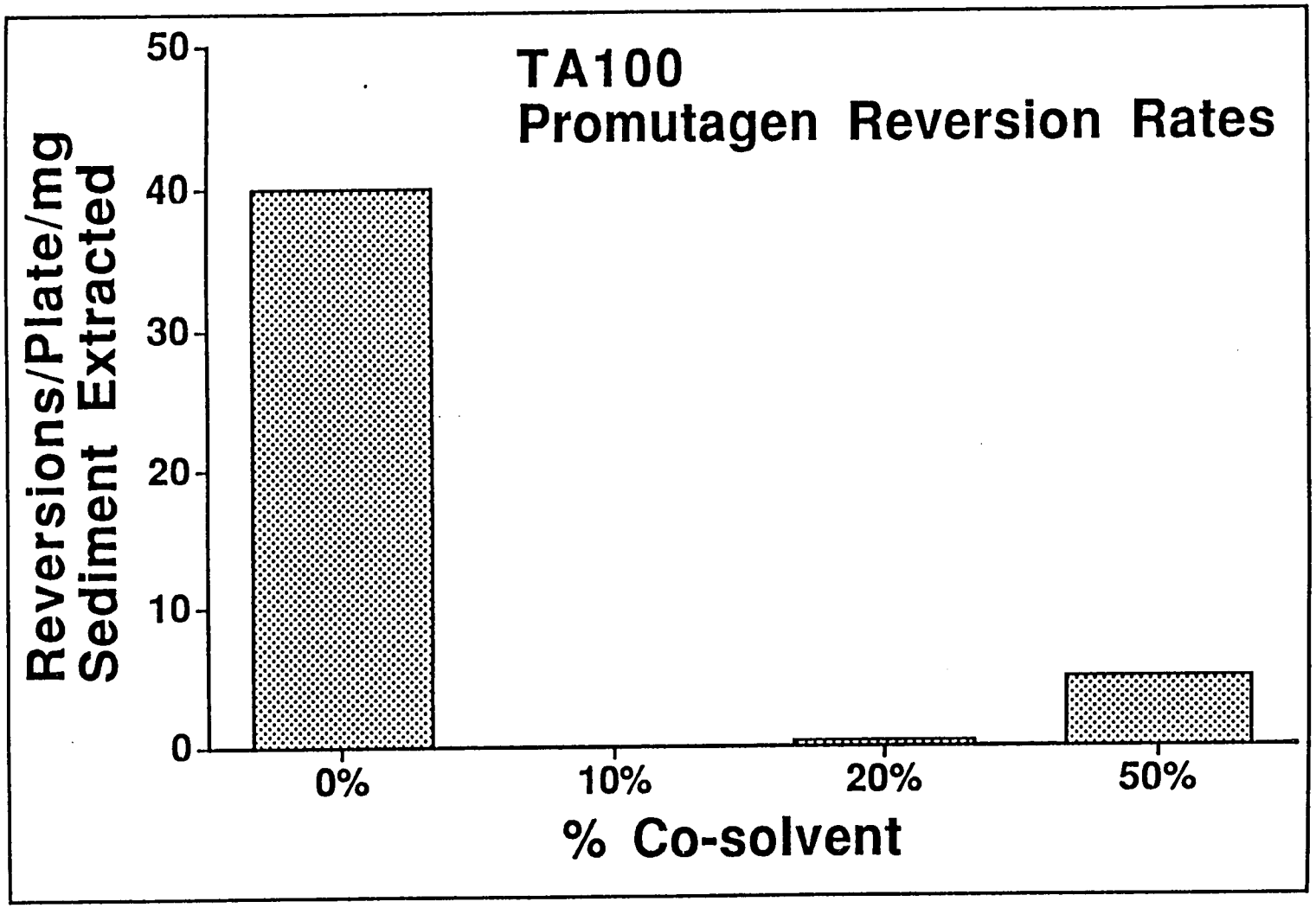

Figure 24. Reversion frequencies for promutagens extracted at various co-solvent fractions from contaminated sediment, (TA100).

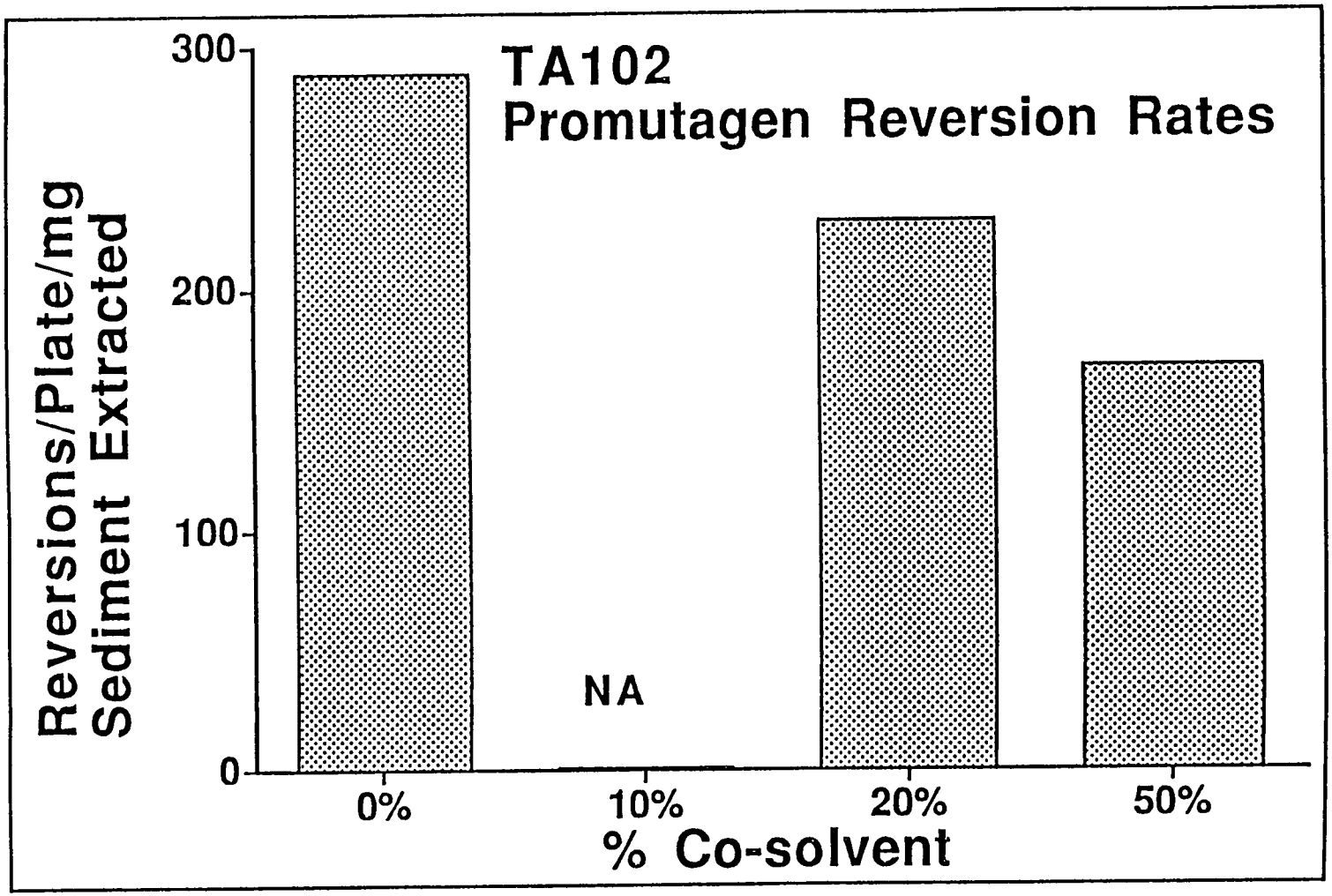

Figure 25. Reversion frequencies for promutagens extracted at various co-solvent fractions from contaminated sediment, (TA102).

Identification of Problems Associated with Measurement Methods: As with the measurement of any complex mixture of chemicals in environmental media, there are potential interferences which may influence the results. In the general case, these include but are not restricted to matrix effects in the sediments themselves such as the presence of extractable salts, nutrients and other dissolved solids (including colloids) or particulates. The presence of substances which interfere with the accurate detection and quantification of mutation frequencies or which result in matrix effects in the receptor assay organisms (effects on lipids, proteins and other biomolecules) may result in detection problems. For example, the presence of the free amino acid histidine or metabolizable peptide fragment containing histidine in sediment extracts may result in false positives in the Ames assay. In the case of petroleum industry effluents, all of these potential interferences are possible. It should be noted that all extracts, regardless of the solvent system used have the potential to contain such interferences. Further investigation of these potential interferences will need to be undertaken before the co-solvent extract model can be applied widely to soil/sediment bioassays.

Application of the Combined Co-Solvent/Bioaccumulation Model to Results: The results of the the mutagenicity assays for a given co-solvent extract reported in units of revertants/plate/mg sediment may be converted to an equivalent water exposure frequency by application of co-solvent theory and using the equation above and a value for the product of the constants as for acetic acid of 12 (Means et al., 1988). Using an accumulation factor of 2 as described above for the bioaccumulation potential (McElroy and Means, 1988), an estimate of the bioaccumulatable mutation frequency for the sediment-bound contaminants may be expressed but now the units would be revertants/plate/mg lipid in the organisms tissues.

CONCLUSIONS

Based upon the results of these experiments several conclusions were developed. They were as follows:

- Direct-acting mutagens were mobilized at all co-solvent concentrations but were detected only with strain TA 102.
- Mutagen mobilization into co-solvent extracts was detectable by the *Salmonella typhimurium* assay, with tester strains varying in sensitivity.
- Promutagens were detectable in all extracts, via activation with Arochlor-induced rat liver S9.
- Mutagenicity effects varied among co-solvent concentrations, perhaps reflecting the mobilization of different arrays of compounds.
- Low co-solvent fractions appeared to enhance extraction of water-soluble mutagens but suppress the extraction of lipophilic mutagens. In high co-solvent fractions, extraction of lipophilic mutagens was significantly increased.
- Increasing co-solvent fraction appeared to suppress the extraction of some water-soluble mutagens.
- TA 102 was the most responsive tester strain with these extracts and yielded very reproducible results. TA 98 showed no response to mutagens in these extracts.
- Chemical analyses of the co-solvent extracts by GC/MS may yield information on the identity of possible mutagens present.

CHAPTER 6

Part B

STUDIES OF GENOTOXICITY AND POTENTIAL FOR MUTAGENIC ACTIVATION IN FISH SAMPLED FROM THE GULF OF MEXICO AS EVALUATED BY THE UMU MUTAGENICITY ASSAY

G.W. Winston, S.B. Dobias and L. A. Reily

INTRODUCTION

A major objective of this project is to identify genotoxic effects in correlation with the various biochemical responses from contaminant exposure. Preliminary studies in the Ames assay suggest mutagen mobilization into the water column. Previous studies in this laboratory (Winston et al., 1989) indicated that liver microsomes from channel catfish (*Ictalurus punctatus*) caught from a contaminated river basin swamp near Baton Rouge (Devil's Swamp) were more effective catalysts of promutagen activation with certain xenobiotics than microsomes prepared from the same species obtained from the relatively pristine Ben Hur Reseach Facility at LSU, which we used as a reference site. Therefore, the effects of exposures to sediments on the indigenous organism's ability to modulate the mutagenicity of the benchmark promutagens 2-aminoanthracene (2AA) and 2-acetylaminofluorene (2AAF) in the *umu* gene induction assay were tested.

METHODS

The ability of microsomes from Lake Champagne catfish and Pass Fourchon catfish to activate 100 μM 2AA was compared with that of liver microsomes from control rats and from rats pre-treated with 3-methylcholanthrene (3MC), an aromatic hydrocarbon known to induce enzymes that activate these promutagens in the rat. The rat samples were run with 200 μg of microsomal protein and the fish with 50, 100 and 200 μg of protein.

The activation of 2AA and 2AAF to mutagenic products was determined by measuring the expression of the *umu* gene in *Salmonella typhimurium* TA1535/pSK1002 as described in Shimada and Nakamura (1987). Total β-galactosidase activity toward O-nitrophenyl-β-D-galactoside was taken as a measure of the expression of the chimeric *umu* DC-*lac* Z gene product and was thus a measure of mutagenicity (Kirchin and Winston, 1992).

Co-solvent extracts were provided by Dr. Reily, Department of Veterinary Pharmacology and Toxicology, Louisiana State University School of Veterinary Medicine. These consisted of aqueous extracts in 10%, 20% and 50% acetic acid (w/v). Aqueous extracted material was concentrated in dichloromethane (DCM), evaporated to dryness under nitrogen and reconstituted in DMSO for sue in the mutagenicity assay.

RESULTS

Figure 26 shows the results of mutagenicity assays designed to test the ability of microsomes from Lake Champagne and Pass Fourchon catfish to activate 100 μM 2AA. These data are compared with liver microsomes from untreated control rats and rats pre-treated with 3-methylcholanthrene (3MC), an aromatic hydrocarbon known to induce enzymes that activate various promutagens in mammals and fish. A 1.6-fold and 2.4-fold enhancement of *umu* gene

induction with microsomes from control and 3MC-treated rats was observed, respectively, but no increase was observed over background controls with the fish samples.

We have also shown that the cytosolic fraction of rat liver contains an amine oxidase, which is capable of activating certain arylamines to mutagens in the Ames mutagenicity assay (Traynor et al, 1992). Therefore experiments were conducted with cytosolic fractions from Croaker (Pass Fourchon), Shad (Lake Champagne) and Pinfish (Lake Champagne) liver to determine if fish liver cytosolic fractions catalyzed arylamine activation in the *umu* mutagenicity assay and if so, was such catalysis altered in fish from contaminated sites. For these studies 400 μg and 800 μg of cytosolic protein were tested separately. No significant increase in gene induction was observed over background (Figure 27).

Experiments were repeated in parallel with 200 μg of microsomal protein from control and 3MC-treated rats as positive controls. Control and 3MC-treated rat liver microsomes catalyzed 3- and 3.5-fold enhancement of gene expression with 2AA however, as in the previous experiments, no enhancement by 2AA of gene induction was seen with fish samples (Figure 28).

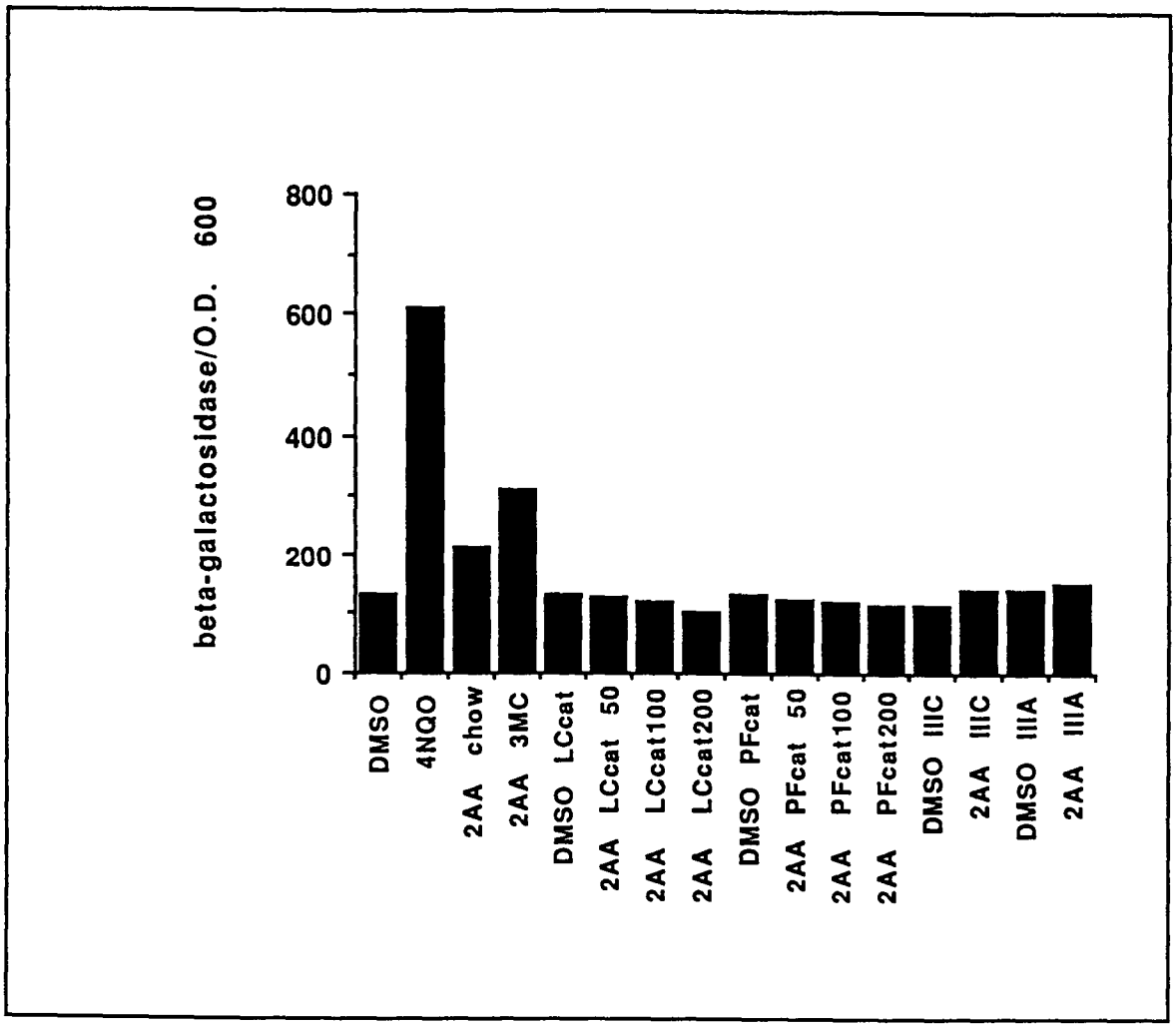

Figure 26. 2-aminoanthracene activation by liver microsomes of Pass Fourchon and Lake Champagne catfish.

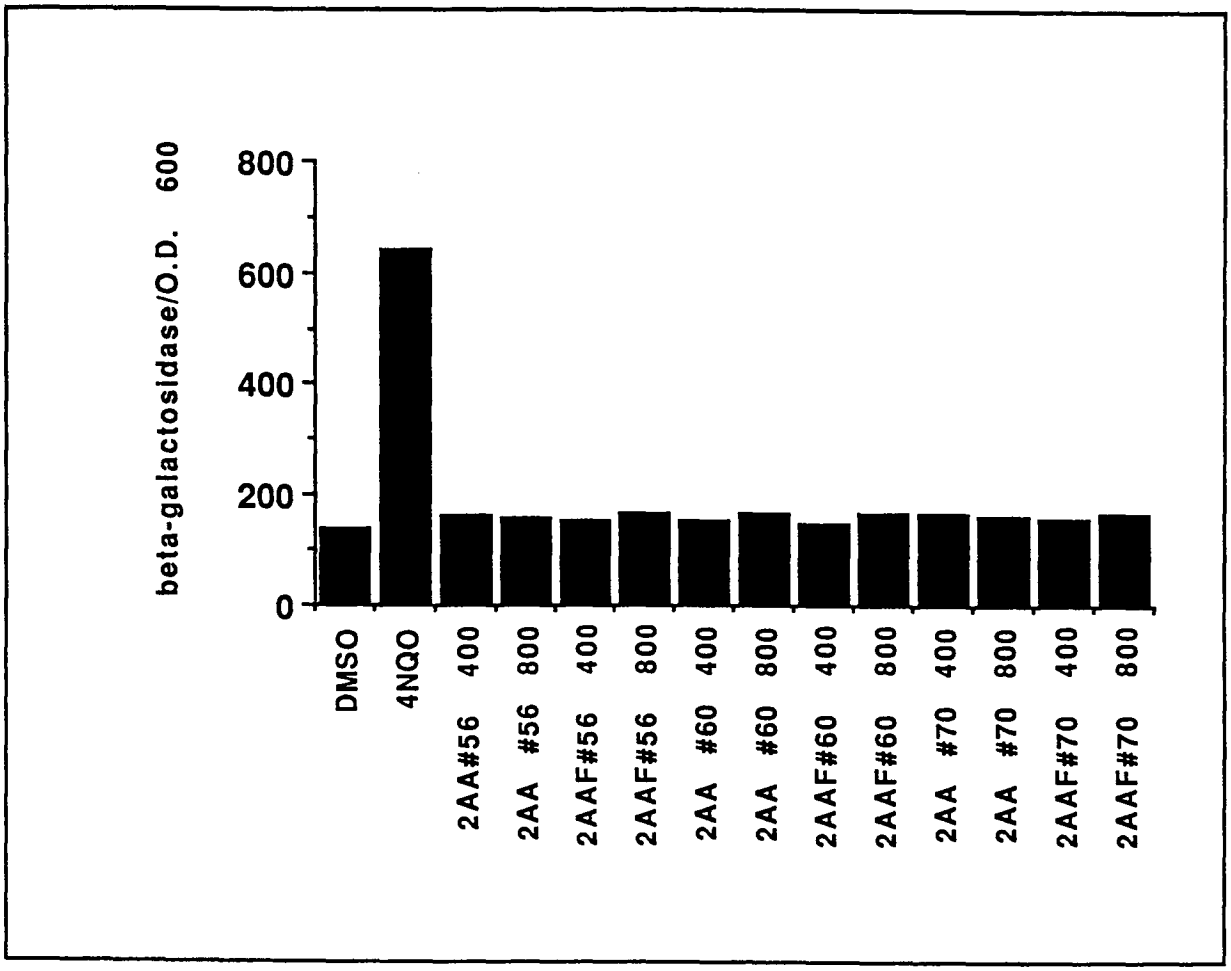

Figure 27. Effect of fish liver cytosol on umu gene induction by 2-aminofluorene and 2-acetyl aminofluorene.

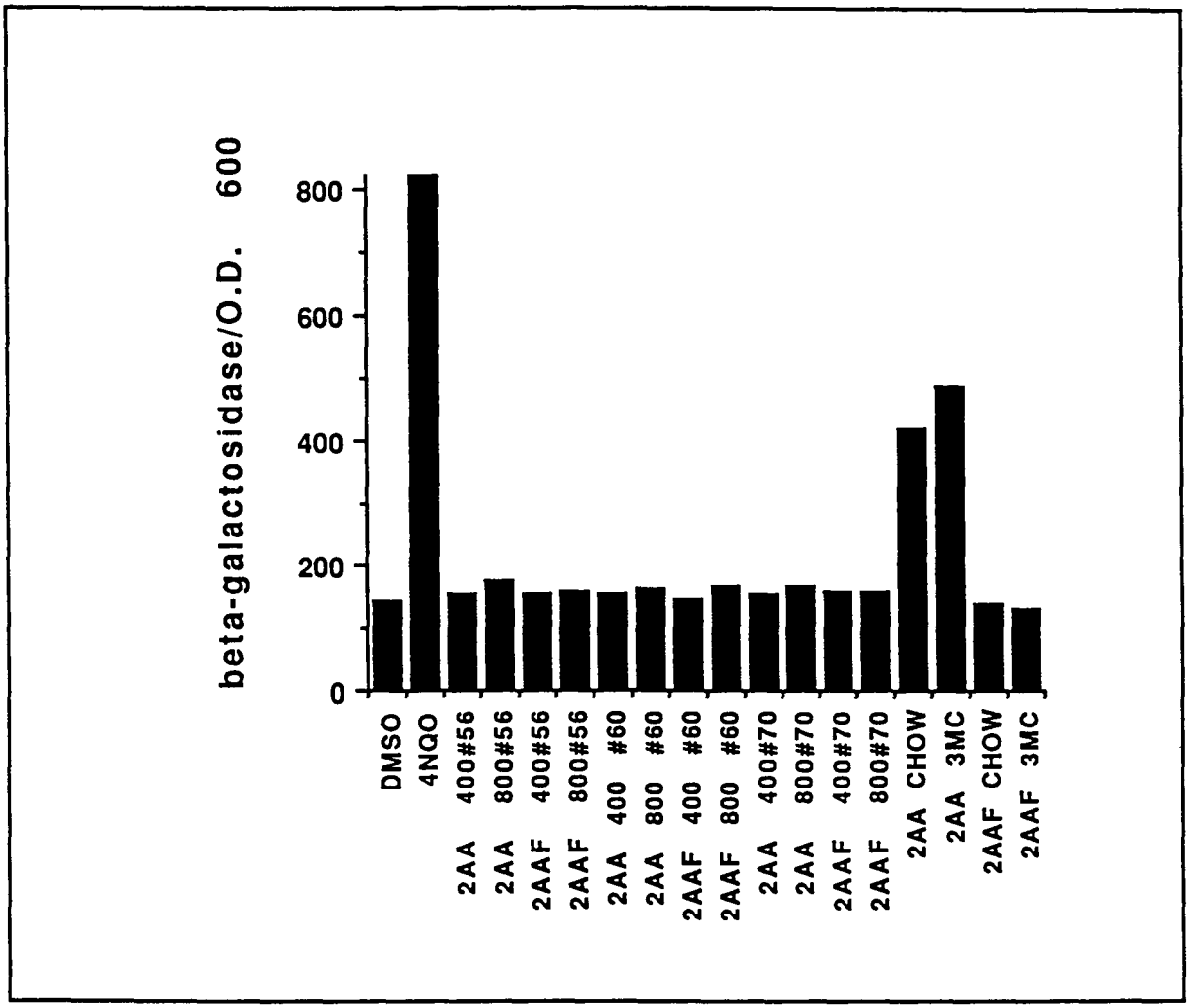

Figure 28. Arylamine activation by fish liver cytosol.

Additional experiments were performed to determine if chemicals extracted by the co-solvent method (Methods) from contaminated sediment obtained at Pass Fourchon were capable of *umu* gene induction in the *umu* mutagenicity assay. In each of these cases there was no *umu* gene induction observed with any of the samples.

CONCLUSIONS

The *umu* gene induction assay has been widely used to assess the genotoxicity of numerous toxicants including complex mixtures (Shimada and Nakamura, 1987). Thus, the *umu* gene induction assay may not be sensitive enough to evaluate mutagens in sediment extracts. Typically, promutagens that are activiated by microsomal fractions or S9 fractions in the Ames assay are also activated in the umu gene induction assay (Shimada and Nakamura, 1987). It is also known that polycyclic aromatic hydrocarbon metabolism catalyzed by fish liver microsomes is often one to three orders of magnitude lower than that of rat liver microsomes

(Stegeman, 1981). Presently, it cannot rigorously be established if the relative lack of sensitivity of the *Salmonella typhimurium* TA1535/pSK1002 strain to extracted mutagens reflects susceptibility of the strain to the promutagens in the sediments or a problem of activation threshold. Future experiments will address these possibilities.

Chapter 7

DEVELOPMENT AND APPLICATION OF (PARTS-PER-TRILLION) GC/MS ANALYSIS METHODS FOR APPROXIMATELY 60 AROMATIC HYDROCARBONS

D.J. McMillin and J.C. Means

ANALYTICAL METHOD DEVELOPMENT

Rationale For Target Analyte Selection

The compounds of interest in the study of impacts of petroleum production were selected based upon knowledge of 1) the composition of petroleum related waste effluents, 2) the potential of certain types of compounds to bioaccumulate in aquatic indicator organisms and 3) the potential of such compounds to cause adverse effects upon components of ecosystems receiving discharges of these compounds on a chronic basis.

Much of the chemical analysis of environmental samples has been directed toward meeting some regulatory criteria and therefore much of the available literature on hydrocarbon contamination has been limited to studies which report the concentrations of only a relative few of the aromatic hydrocarbons potentially present in petroleum industry wastes (16 priority pollutant PAHs + 1-methyl and 2-methyl-naphthalene) (USEPA, 1984, 1986). While these compounds may be present as components in the total aromatic fraction of petroleum refinery waste, recent studies of petroleum-related wastes, such as produced waters, suggest that these 18 compounds may represent less than 5% of the total aromatic materials present.

The hydrocarbon load from petroleum wastes is normally stated as one relatively crude number called oil and grease. Detailed characterizations of what compounds comprise this number are not readily available. The EPA conducted two studies in developing guidelines for discharge limitations for petroleum refinery effluents (USEPA 1979 and 1982). Seventeen refinery discharge sources were sampled and analyzed for priority pollutants. The majority of these pollutants were actually detected in less than five discharges. Most other detailed analyses of refinery discharges have also focused in on the EPA priority pollutant list, although one study conducted in western Australia investigated petroleum-derived hydrocarbons in a refinery effluent and in organisms 250 meters away from the discharge (Alexander et al., 1982a and 1982b). Naphthalene, methylnaphthalenes, dimethylnaphthalenes, trimethylnaphthalenes, phenanthrene, methylphenanthrenes and dimethylphenanthrenes were found both in the discharge and in mullet and mussel tissue.

In detailed investigations of the composition and environmental fate of hydrocarbons assocated with produced water discharges into fresh, estuarine and marine aquatic ecosystems, we have observed that these effluents are very complex mixtures of aliphatic, aromatic, alkylated and heterocyclic aromatic hydrocarbons (Rabalais et al., 1991a, Means et al., 1990, Means et al., 1989). In order to conduct a successful biomonitoring program, one necessary precursor must be the development and verification of sensitive and compound/element specific analytical techniques for application in tissue analysis. We have developed and applied such techniques for the analysis of petroleum-related wastes in water, sediments and tissues. To date these methods have been utilized in well over 3,000 analyses and the requisite QA/QC practices and measures have also been developed and are reported here.

The overall data objective of our biomonitoring studies is to determine the presence and amounts of bioconcentratable organics present in petroleum-related waste streams and receiving environments. In order for this data objective to be accomplished, several criteria for the methods must be met, including:

*a wide dynamic range (~0.01 ppb to 100 ppm)-7 orders of magnitude in concentration

*compound specificity-the ability to discriminate individaul compounds from both biological and geological matrices.

*compound quantitation- the ability to determine the concentrations of specific compounds, as opposed to estimates made by class

*sufficient "fingerprinting" capability to be able discriminate between possible multiple sources of similar contaminants

*the methods should be designed to allow for frequent sampling and analysis to be performed

*methods should require a minimum of procedural steps to minimize potential for losses or contamination.

Table 7.1 lists the selected target analytes and the mass fragment used for quantitative evaluation ("primary ion") and for confirmation of identity ("confirming ion"). Each of these analytes was selected from a more extensive list (over 300 potential analytes) based upon the availability of standards, their real or potential presence in effluents and/or previous bioaccumulation studies conducted in estuaries in south Louisiana and California. Each of the pollutants selected has documented or suspected implications for the ecological health of coastal marine ecosystems and also potential human health implications through foodwebs leading to man which exist in these ecosystems. In previous and ongoing studies of the fate and transport of produced water hydrocarbons under MMS funding, our research group has been able to use these techniques to follow both the chemical signature of a discharge for up to 1500 m and long-range pollutant transport in the Gulf of Mexico (Rabalais et al., 1991a, Means and McMillin, 1993, Murray, 1994).

As with the measurement of any complex mixture of chemicals in environmental media, there are potential interferences which may influence the results. In the general case these include but are not restricted to matrix effects in the discharges themselves such as the presence of salts and other dissolved solids (including colloids) or particulates; the presence of substances which interfere with the accurate detection, identification or qualification of other substances (oil and grease); matrix effects in the receptor organisms (lipids, proteins and other biomolecules); and contaminants having sources other than the facility in question which enter the samples either at the point of discharge, during sampling or during analysis. In the case of petroleum industry effluents, all of these potential interferences are possible and probable. Techniques used to overcome or minimize these problems include:

*the use of Mass Spectrometry as a means of positive identification combined with

*high-resolution capillary column gas chromotography optimized for resolution of individual sample/standard components

*extensive quality control procedures, including blanks, spikes, duplicates and the analysis of standard reference materials

*target analytes isolation through extraction and sample cleanup steps including solvent exchange and Silica gel fractionation.

Table 7.1 Selected aromatic hydrocarbon analytes

Analyte	Primary Ion	Confirming Ion
Polynuclear Aromatic Hydrocarbons		
Naphthalene	128	129
2-Methylnaphthalene	142	141
1-Methylnaphthalene	142	141
2-Ethylnaphthalene	156	141
1-Ethylnaphthalene	156	141
2,6/2,7-Dimethylnaphthalene	156	141
1,3/1,7-Dimethylnaphthalene	156	141
1,6-Dimethylnaphthalene	156	141
1,4/2,3-Dimethylnaphthalene	156	141
1,5-Dimethylnaphthalene	156	141
Acenaphthylene	152	153
1,2-Dimethylnaphthalene	156	141
2-Isopropylnaphthalene	170	155
1,8-Dimethylnaphthalene	156	141
1,6,7-Trimethylnaphthalene	170	155
Acenaphthene	153	154
Fluorene	166	165
Dibenzothiophene	184	185
Phenanthrene	178	179
Anthracene	178	179
4-Methyldibenzothiophene	198	197
2/3-Methyldibenzothiophene	198	197
1-Methyldibenzothiophene	198	197
3-Methylphenanthrene	192	191
2-Methylphenanthrene	192	191
4/9-Methylphenanthrene	192	191
1-Methylphenanthrene	192	191
3,6-Dimethylphenanthrene	206	191
3,5-Dimethylphenanthrene	206	191
2,6-Dimethylphenanthrene	206	191
2,7-Dimethylphenanthrene	206	191
3,9-Dimethylphenanthrene	206	191
1,6/2,5/2,9-Dimethylphenanthrene	206	191
1,7-Dimethylphenanthrene	206	191
1,9/4,9-Dimethylphenanthrene	206	191
1,2-Dimethyldibenzothiophene	212	211
Fluoranthene	202	101
1,5-Dimethylphenanthrene	206	191
1,8-Dimethylphenanthrene	206	191
1,2-Dimethylphenanthrene	206	191
9,10-Dimethylphenanthrene	206	191
Pyrene	202	101
1,2,8-Trimethylphenanthrene	220	205
Benzo(a)anthracene	228	226
Chrysene	228	226
Benzo(b)fluoranthene	252	253
Benzo(k)fluoranthene	252	253
Benzo(a)pyrene	252	253
Indeno(1,2,3-cd)pyrene	276	278
Dibenz(a,h)anthracene	278	276
Benzo(g,h,i)perylene	276	278

Laboratory methodology

The following is a description of sample preparation methods for various sample matrices that have been developed or modified for use in the compound-specific analysis for aromatic hydrocarbons of petrogenic origin. The methods were applied in the present study for sediment and tissue samples (Chapters 3 and 8, respectively).

Tissue Sample Preparation

Tissue samples for bioaccumulation studies (see Chapter 8) were defrosted overnight in a refrigerator. Individual samples were removed from the bags and rinsed with tap water, then shelled on a glass cutting board with a Teflon® coated knife. The shell-free flesh was rinsed with organic free water and then chopped into small pieces. The pieces were placed in a clean jar and homogenized using a Tekmar Tissumizer®. The homogenized samples were kept frozen at -20°C until sample extraction.

The extraction method is a modification of that described by MacLeod et al. (1985). Approximately 20 g of defrosted homogenized tissue was placed in an 240-ml amber glass bottle with 100 ml nanograde® dichloromethane (DCM) and four times the tissue weight of cleaned sodium sulfate (DCM rinsed). A surrogate spike of 200ng each of a mixture of deuterated standards (US-108, Ultra Sientific) was added to the bottle. The sample was extracted with a Tekmar Tissumizer® for 2 min at a speed of 24,000 rpm. The probe was rinsed with DCM and the rinsings added to the sample bottle. The extract was allowed to settle for 1 min and then filtered through a funnel filled with sodium sulfate into a labelled florence flask. The sample was extracted as above with two additional 100 ml volumes of DCM. After the third extraction, the extract was rinsed with an additional 30 ml of DCM, which was added to the flask. The sample extract was reduced in volume to approximately 2 ml with a rotary evaporator. The concentrated extract was quantitatively transferred to a 4-ml vial and concentrated to a volume of 2 ml under a stream of purified nitrogen. Reagent blanks, duplicates and matrix spiked samples were extracted to monitor laboratory technique and extraction efficiency. Matrix spikes were duplicate tissue samples to which 100 ng of the quantitative calibration standard were added instead of the deuterated surrogates mixture.

Most tissue samples contain a layer of fine white precipitate (extracted proteins) which was removed before silica column cleanup. The extracts were "cleaned" by adding 500 µl of hexane, mixing the extract well and then centrifuging for 5 min at 3,000 rpm. The solvent layer was transferred to a clean 4-ml vial and the extract was "cleaned" twice more with 1 ml of hexane. All solvent layers were combined and reduced in volume to 1 ml under a stream of purified nitrogen.

One half of the extract was separated from the polar lipids on 200 mm x 10 mm columns packed with 6.0 g of 100-200 mesh Grade 923 silica, eluted with 100 ml of 60% DCM and 40% hexane. The extract was concentrated under nitrogen to 200 µl, after which 2 µl of 2-fluorobiphenyl (100 ng/µl in hexane) was added as an internal standard to monitor the GC/MS injection performance.

Sediment Sample Preparation

Sediment cores (see Chapter 3) and water/filter samples were sealed with electric tape in the field and packed on ice for transport to the laboratory. Once in the laboratory, the cores were stored at 5°C. until processed, within one week The top 8 cm of sediment from each core was placed in a clean glass jar with a teflon-lined cap, mixed, and refrigerated until sampled for

analyses, then stored at -20°C. The extraction method was a modification of that described by MacLeod et al. (1985). Excess water was decanted from sample jars prior to thorough mixing of the sediment sample, and 15 g was weighed into an amber bottle with a teflon-lined cap. The sediment was covered with 75 ml of dichloromethane (DCM) and 50 g sodium sulfate incorporated until the sediment mixture was free-flowing. A suite of deuterated PAH was added (800 ng in hexane of six deuterated PAH) with the first solvent aliquot as a surrogate standard to monitor extraction efficiency. The jars were sealed and placed on a roller for 16, 6, and 16 hours, for a total of three extractions using the modified rock tumbler and rolling times specified by MacLeod et al. (1985). The DCM from each jar was decanted into an amber jar and refrigerated until 3 extracts were collected. The decanted DCM was filtered through sodium sulfate, and concentrated by rotoevaporation and a nitrogen stream to a final volume of 200 µl, with solvent exchange to hexane. Activated fine granular copper was added to reduce sulphur interferences.

Water Sample Preparation

Water samples (20 L) were collected in 20L polypropylene cubitainers™ (Cole-Parmer, Inc.), and transferred to a stainless steel compressed nitrogen pressurized vessel to facilitate filtration through a 149 mm 0.4 µm membrane filter with glass fiber prefilter. This concentrated the particulate phase on the membrane filters, which were stored in small glass jars held at 5°C. The filtrate was ultrafiltered using an Amicon Model DC10 equipped with hollow-fiber cartridges (MW cutoff 3000 daltons) to separate dissolved and enriched colloidal phases. The colloidal fraction was concentrated to 240 ml and stored in amber glass jars at 5°C. Prior to SPE extraction 20µl of the deuterated surrogate standard at 40 ppm in methanol was added to 4L of the ultrafiltrate (dissolved phase). A two liters aliquot was pumped through an SPE disk (Empore™, C18) housed in a teflon filter holder attached in line with a MasterFlex pump set for a flow rate of 2-3 ml/min. Extraction volume was determined by pumping time. The SPE disk was removed from the filter holder and stored in a small glass vial covered with 10 ml of methanol.

Particulate Phase: The membrane and glass fiber prefilters were placed together in tared teflon digestion bombs. The first extraction solvent was 45 ml of 2:1 hexane:acetone. A surrogate standard was added (120 ng in methanol of six deuterated PAH and dibromooctafluorobiphenyl) at the same time as the first extraction solvent, and the bombs placed in an ice-cooled sonicating bath for 6 min. After decanting, two more extractions were performed using 45 ml of hexane only. The extracts were combined and acetone removed by adding >75 ml of purified water. The top layer of hexane was removed by pipet and filtered through clean sodium sulphate. Subsequent concentration steps were the same as for sediment extracts, excluding the addition of copper. To obtain the particulate fraction weight, the teflon bombs containing the filter sets were dried at 60°C overnight in an oven modified to exchange the atmosphere with charcoal-filtered air. The weight of the filters and bombs was recorded and the samples sent to the inorganics sample preparation laboratory for acid digestion.

Colloidal Phase: Samples were transferred to separatory funnels, surrogate standard added (400 ng in methanol of six deuterated PAH and dibromooctafluorobiphenyl-DBOFBP) and extracted with DCM in triplicate, first at ambient (neutral) pH, then at acidic pH (2). The extracts from both pH levels were combined and the extract concentrated as for sediment samples, without the addition of copper.

Dissolved Phase: An additional laboratory surrogate standard (120 ng each of DBOFBP, 1,3-dimethyl-2-nitrobenzene, and triphenylphosphate in methanol) was added to the vials containing SPE disks to monitor laboratory performance. The disks were extracted three times with 20ml, 10ml, and 10ml of added DCM and using a sonicating bath for 1 minute per extraction. The extracts were combined in a separatory funnel and the DCM separated from methanol (storage

solvent) and residual water by adding excess water (>25ml) and decanting the DCM layer over clean sodium sulphate. Concentration steps were similar to colloidal samples.

Instrumental Analysis

Analytical Approach

The primary analytical tool employed in these studies was a gas chromatograph/mass spectrometer (GC/MS) system for organic analyses. This instrument system has the advantage that multiple compounds may be quantified simultaneously on a single sample. Repetitive analyses are not required. The use of multiple selected ion mass spectrometry on sample extracts allow the quantification, at sub-ppb levels, of several classes of pollutants in a single analysis without extensive sample fractionation or cleanup techniques. Thus, both pyrogenic and petrogenic PAHs may be analyzed simultaneously.

A Hewlett-Packard 5890 Gas Chromatograph equipped with a 30m by 0.25mm ID, 0.25µm DB-5 capillary column (J&W Scientific) is directly interfaced to a Hewlett-Packard 5970B Mass Selective Detector (MSD). The GC is temperature programmed from 50° to 300°C using a series of linear temperature ramps to optimally separate the analytes. The mass spectrometer is tuned prior to calibration every 16 hours using Perfluorotributylamine (PFTBA).

Retention order of the alkylated PAH isomers has been determined in our laboratory by analysis of individual "true" components obtained from Chiron Laboratories A>S> (Norway). Following initial determination of retention order, the resolution between isomers was optimized by adjusting the temperature ramp rates, while considering the effects of band-spreading. Table 7.1 presents the analytes in order of elution from the GC column, and also indicates resolved versus co-eluting isomers (positional indications separated by a "/" mark).

Quantitative reference standards were used for daily calibration of the instrument. 2-fluoro-biphenyl was added as an internal standard to each sample aliquot to monitor performance of the GC/MS instrument and injection precision. Standard response curves were prepared using 5 to 9 calibration points over several orders of magnitude of concentration for each analyte.

Linearity, Dynamic Range, and Detection Limits

Linearity and dynamic range of the GC/MS using the SIM method described above was demonstrated by two independently acquired standard curves. The first was a 5-point curve with concentrations ranging from 0.02 ng to 2.0 ng, or three orders of magnitude, and with each point replicated three times. A 9 point curve of concentrations ranging from 0.005 ng to 10 ng, with two replications of each point, represented over four orders of magnitude. Table 7.2 shows the regression statistics and calculated X-intercept (ng) values and response factors for both curves. Table 7.3 shows LOD and LOQ values (Keith et al., 1983) calculated from the average standard deviations between replicates for both curves. The 5-point curve is reproduced for routine verification of linearity and detection limits in our laboratory, since most sample values fall in this range. Also, column overload and detector shut-downs occur when values exceed 5-10 ng on column. Thus, the 9-point curve showed lower correlations than did the 5-point curve. The correlations for the 9-point curve were improved when a second-order function was calculated, and indicates losses in response were occuring at the highest concentration points. Detection limits calculated from LOD values for the target analytes are presented in Table 7.4 showing results for all matrices routinely analyzed.

Validation Study

Method detection limits in an environmental matrix were verified using seawater samples spiked in triplicate at 5, 10, 50, and 100 parts-per-trillion. Samples (2000 ml) were processed using the C18 SPE disks as described above after spiking with the standard solution prepared in methanol. Spike concentration corresponded to 0.1, 0.2, 1.0, and 2.0 ng on-column, respectively, based on concentration of extracts to a final volume of 200 µl and injection of 2µl sample onto the GC/MS. Summary statistics are shown in Table 7.5. All aromatics were detected at the lowest spike concentration in all three replicate samples, with the exception of Indenopyrene, Dibenzanthracene, and Benzoperylene. Recovery of spiked components averaged 47%, or approximately one-half of what was spiked, and indicates that the amount detected on-column was in the range of 0.05 ng, which would be nearly equivalent to the mean LOD value calculated from the 5-point standard curve. The mean standard deviations between replicates averaged 0.093 ng, or 4.7 ppt.

APPLICATION OF METHODS

The following tables and discussion show examples of data obtained using the methods that have been developed for compound specific analysis of aromatic hydrocarbons of petrogenic origin.

Historical Sediment Data For Pass Fourchon

Table 7.6 presents a brief summary of the data accumulated since 1988 for sediments in Pass Fourchon. This table shows that, while there is some variablilty over time and depth in sediment cores, the overall levels of PAH and saturated hydrocarbons remain high, and FFPI values indicate that the source is petroleum contamination. The detailed isomer-specific database begins in 1989. The values dated 1994 were obtained for this study.

Three-Phase Water Data For Coastal Zone Region Near Pass Fourchon

This data (Table 7.7) is presented as an example of the ability of the analytical methods to discriminate specific petroleum contaminant markers at sub-ppb levels, providing additional information on transport and availability of these compounds to aquatic organisms.

Table 7.2 Summary of regression statistics for two standard curves

	5-point curve REGRESSION STATISTICS (0.02-2ng, 3 replicates):						9-point curve REGRESSION STATISTICS (0.005-10ng, 2 replicates):					
ANALYTE	SLOPE	INT	CORR.	X-INT (ng)	Mean Std. Dev. (ng)	RF	SLOPE	INT	CORR.	X-INT (ng)	Mean Std. Dev. (ng)	RF
d8 NAPH	2.13E+06	2.34E+04	0.999	-0.011	0.0037	4.65E-07	3.11E+06	-2.36E+05	0.999	0.076	0.0027	3.47E-07
Naphthalene	2.74E+06	1.85E+04	1.000	-0.007	0.0062	3.62E-07	4.01E+06	-3.03E+05	0.999	0.076	0.012	2.70E-07
2-MN	2.19E+06	1.58E+04	0.999	-0.007	0.0024	4.54E-07	3.27E+06	-2.98E+05	0.998	0.091	0.0031	3.37E-07
1-MN	2.03E+06	8.10E+03	1.000	-0.004	0.0035	4.90E-07	3.04E+06	-2.61E+05	0.998	0.086	0.0037	3.60E-07
2-EN	1.20E+06	4.96E+03	0.999	-0.004	0.0048	8.28E-07	1.83E+06	-2.42E+05	0.998	0.13	0.0064	6.28E-07
1-EN	1.14E+06	1.04E+04	0.999	-0.009	0.0091	8.70E-07	1.72E+06	-2.00E+05	0.998	0.12	0.0075	6.60E-07
2,6/2,7-DMN	4.17E+06	1.11E+02	1.000	0.000	0.014	4.80E-07	6.27E+06	-5.98E+05	0.998	0.19	0.0059	3.53E-07
1,3/1,7-DMN	5.09E+06	-8.21E+03	1.000	0.003	0.016	3.94E-07	7.51E+06	-6.46E+05	0.998	0.17	0.0059	2.92E-07
1,6-DMN	2.40E+06	1.36E+04	0.999	-0.006	0.0077	4.15E-07	3.59E+06	-3.15E+05	0.998	0.088	0.0049	3.05E-07
1,4/2,3-DMN	3.94E+06	-8.34E+02	1.000	0.000	0.013	5.08E-07	5.70E+06	-4.24E+05	0.998	0.15	0.0090	3.79E-07
1,5-DMN	2.05E+06	1.21E+04	0.999	-0.006	0.011	4.86E-07	3.06E+06	-2.86E+05	0.998	0.093	0.0054	3.60E-07
Acenaphthylene	3.14E+06	-2.35E+04	1.000	0.007	0.0065	3.21E-07	4.97E+06	-7.72E+05	0.997	0.16	0.0034	2.38E-07
1,2-DMN	2.93E+06	1.64E+03	0.999	-0.001	0.0089	3.41E-07	4.36E+06	-3.89E+05	0.998	0.089	0.0050	2.52E-07
1,8-DMN	1.80E+06	5.63E+03	0.999	-0.003	0.0041	5.54E-07	2.74E+06	-3.33E+05	0.998	0.12	0.0089	4.16E-07
d10 ACE	1.73E+06	4.65E+03	1.000	-0.003	0.0074	5.75E-07	2.63E+06	-3.46E+05	0.998	0.13	0.0044	4.37E-07
Acenaphthene	2.41E+06	9.32E+03	1.000	-0.004	0.0069	4.14E-07	3.33E+06	-1.28E+05	0.986	0.038	0.0086	3.13E-07
1,6,7-TMN	2.62E+06	1.63E+04	1.000	-0.006	0.0062	3.80E-07	4.00E+06	-5.18E+05	0.997	0.129	0.0067	2.87E-07
Fluorene	2.79E+06	1.77E+04	1.000	-0.006	0.0067	3.56E-07	4.33E+06	-6.27E+05	0.997	0.14	0.0070	2.70E-07
Dibenzothiophene	2.47E+06	6.45E+03	0.999	-0.003	0.0081	4.04E-07	3.95E+06	-8.37E+05	0.996	0.16	0.012	3.02E-07
d10 PHEN	3.56E+06	6.43E+02	1.000	0.000	0.0035	2.81E-07	5.98E+06	-9.04E+05	0.996	0.15	0.0052	1.97E-07
Phenanthrene	4.20E+06	4.04E+03	1.000	-0.001	0.0073	2.38E-07	6.87E+06	-9.78E+05	0.996	0.14	0.0039	1.70E-07
Anthracene	4.17E+06	-1.91E+04	1.000	0.005	0.010	2.41E-07	6.95E+06	-1.12E+06	0.995	0.16	0.0070	1.71E-07
4-MDBT	1.43E+06	1.04E+03	0.999	0.000	0.0044	3.49E-07	2.43E+06	-4.11E+05	0.995	0.085	0.0043	2.48E-07
2/3-MDBT	2.36E+06	5.41E+03	0.999	-0.002	0.0088	3.17E-07	4.00E+06	-6.37E+05	0.995	0.12	0.0044	2.23E-07
3-MP	1.32E+06	1.54E+05	0.931	-0.117	0.13	6.79E-07	5.45E+06	-8.76E+05	0.995	0.16	0.0049	2.18E-07
1-MDBT	1.82E+06	1.33E+04	1.000	-0.004	0.0029	2.73E-07	3.10E+06	-4.63E+05	0.995	0.075	0.0045	1.89E-07
2-MP	6.05E+06	-7.29E+04	0.999	0.018	0.012	2.51E-07	9.78E+06	-1.54E+06	0.995	0.24	0.0082	1.82E-07
4/9-MP	4.87E+06	8.95E+03	0.999	-0.003	0.012	3.08E-07	7.97E+06	-1.26E+06	0.995	0.24	0.0096	2.24E-07
1-MP	3.92E+06	9.72E+03	0.999	-0.002	0.0078	2.54E-07	6.45E+06	-1.04E+06	0.995	0.16	0.0086	1.85E-07
3,6-DMP	1.45E+06	-1.12E+04	0.999	0.004	0.0056	3.47E-07	2.48E+06	-5.45E+05	0.994	0.11	0.0051	2.58E-07
3,5-DMP	1.21E+06	-9.21E+03	0.998	0.004	0.011	4.16E-07	2.02E+06	-4.39E+05	0.994	0.11	0.0043	3.16E-07
2,6-DMP	2.43E+06	-1.36E+04	1.000	0.003	0.0036	2.07E-07	4.18E+06	-9.20E+05	0.994	0.11	0.0040	1.53E-07
2,7-DMP	1.10E+06	4.20E+03	0.999	-0.004	0.013	9.08E-07	1.87E+06	-3.81E+05	0.994	0.20	0.013	6.71E-07
3,9-DMP	2.00E+06	9.17E+01	1.000	0.000	0.0046	2.49E-07	3.41E+06	-6.74E+05	0.994	0.099	0.0044	1.83E-07
1,6/2,5/2,9-DMP	5.32E+06	-2.44E+04	1.000	0.006	0.011	2.36E-07	8.86E+06	-1.49E+06	0.995	0.21	0.0088	1.70E-07
1,7-DMP	2.32E+06	-2.83E+03	1.000	0.001	0.011	4.32E-07	4.07E+06	-8.89E+05	0.994	0.22	0.0076	3.14E-07
1,9/4,9-DMP	2.55E+06	-1.55E+04	1.000	0.005	0.0082	2.96E-07	5.02E+06	-8.72E+05	0.997	0.13	0.0067	1.81E-07
Fluoranthene	4.94E+06	2.72E+04	0.999	-0.006	0.013	2.01E-07	1.06E+07	-1.24E+06	0.997	0.12	0.0073	1.07E-07
1,2-DMDBT	1.65E+06	1.65E+04	0.999	-0.008	0.0084	4.80E-07	3.50E+06	-4.44E+05	0.998	0.10	0.0081	2.62E-07
1,5-DMP	1.15E+06	2.10E+03	1.000	-0.002	0.0096	8.71E-07	1.99E+06	-3.57E+05	0.995	0.18	0.014	6.13E-07
1,8-DMP	1.78E+06	5.61E+03	1.000	-0.002	0.0038	2.80E-07	3.21E+06	-6.52E+05	0.993	0.10	0.0056	1.95E-07
1,2-DMP	1.56E+06	3.47E+03	0.999	-0.001	0.0063	3.20E-07	2.78E+06	-6.11E+05	0.993	0.11	0.0084	2.30E-07
9,10-DMP	1.54E+06	8.68E+03	0.999	-0.003	0.0078	3.22E-07	2.65E+06	-5.40E+05	0.994	0.10	0.0041	2.37E-07
Pyrene	5.89E+06	-2.49E+04	0.999	0.004	0.012	1.70E-07	1.03E+07	-1.61E+06	0.993	0.16	0.0045	1.15E-07
1,2,8-TMP	3.77E+06	-1.28E+04	0.999	0.003	0.013	2.66E-07	7.16E+06	-1.42E+06	0.992	0.20	0.011	1.74E-07
Benzanthracene	5.48E+06	-1.49E+05	0.999	0.027	0.017	1.88E-07	9.66E+06	-1.05E+06	0.992	0.11	0.0087	1.16E-07
d12 CHRYS	4.60E+06	-8.54E+04	0.999	0.019	0.019	2.21E-07	8.30E+06	-8.31E+05	0.994	0.10	0.012	1.34E-07
Chrysene	5.33E+06	-7.87E+04	0.999	0.015	0.015	1.91E-07	9.40E+06	-8.75E+05	0.993	0.093	0.014	1.17E-07
Benzo (b) fluor	3.35E+06	-2.39E+05	0.991	0.071	0.059	3.21E-07	1.06E+07	-5.71E+06	0.965	0.54	0.015	2.06E-07
Benzo(k),fluor	4.49E+06	-2.15E+05	0.998	0.048	0.015	2.34E-07	1.15E+07	-4.21E+06	0.975	0.37	0.018	1.38E-07
Benzo(a)pyrene	2.59E+06	-1.38E+05	0.994	0.053	0.046	4.07E-07	8.36E+06	-5.37E+06	0.966	0.64	0.021	3.35E-07
d12 PERYL	2.03E+06	-7.42E+04	0.995	0.036	0.042	5.10E-07	6.32E+06	-2.96E+06	0.972	0.47	0.027	2.98E-07
Indenopyrene	8.86E+05	-1.37E+05	0.981	0.154	0.058	1.33E-06	3.42E+06	-3.08E+06	0.969	0.90	0.23	2.95E-06
Dibenzanthracene	6.06E+05	-8.46E+04	0.988	0.140	0.063	1.92E-06	2.61E+06	-2.55E+06	0.967	0.97	0.65	1.48E-05
Benzoperylene	7.18E+05	-6.22E+04	0.992	0.087	0.026	1.53E-06	2.51E+06	-1.89E+06	0.978	0.76	0.029	1.63E-06
MEANS	2.79E+06	-1.94E+04	0.997	0.009	0.015	4.57E-07	5.07E+06	-1.06E+06	0.992	0.200	0.024	6.14E-07
MIN	6.06E+05	-2.39E+05	0.931	-0.117	0.002	1.70E-07	1.72E+06	-5.71E+06	0.965	0.038	0.003	1.07E-07
MAX	6.05E+06	1.54E+05	1.000	0.154	0.132	1.92E-06	1.15E+07	-1.28E+05	0.999	0.974	0.652	1.48E-05

Table 7.3 Limit of detection, limit of quantitation, and sensitivity values calculated from standard deviations of standard curves

	5-PT CURVE			9-PT CURVE		
ANALYTE	LOD (3*S.D.ng)	LOQ (10*S.D.ng)	95% CI (ng) STDS	LOD (3*S.D.ng)	LOQ (10*S.D.ng)	95% CI (ng) STDS
d8 NAPH	0.011	0.037	0.010	0.008	0.027	0.008
Naphthalene	0.019	0.062	0.017	0.035	0.116	0.033
2-MN	0.0073	0.024	0.007	0.009	0.031	0.009
1-MN	0.010	0.035	0.010	0.011	0.037	0.011
2-EN	0.014	0.048	0.013	0.019	0.064	0.018
1-EN	0.027	0.091	0.026	0.022	0.075	0.021
2,6/2,7-DMN	0.041	0.14	0.039	0.018	0.059	0.017
1,3/1,7-DMN	0.049	0.16	0.047	0.018	0.059	0.017
1,6-DMN	0.023	0.077	0.022	0.015	0.049	0.014
1,4/2,3-DMN	0.038	0.13	0.036	0.027	0.090	0.026
1,5-DMN	0.034	0.11	0.032	0.016	0.054	0.015
Acenaphthylene	0.020	0.065	0.018	0.010	0.034	0.010
1,2-DMN	0.027	0.089	0.025	0.015	0.050	0.014
1,8-DMN	0.012	0.041	0.012	0.027	0.089	0.025
d10 ACE	0.022	0.074	0.021	0.013	0.044	0.013
Acenaphthene	0.021	0.069	0.020	0.026	0.086	0.024
1,6,7-TMN	0.019	0.062	0.017	0.020	0.067	0.019
Fluorene	0.020	0.067	0.019	0.021	0.070	0.020
Dibenzothiophene	0.024	0.081	0.023	0.036	0.121	0.034
d10 PHEN	0.011	0.035	0.010	0.016	0.052	0.015
Phenanthrene	0.022	0.073	0.021	0.012	0.039	0.011
Anthracene	0.030	0.10	0.028	0.021	0.070	0.020
4-MDBT	0.013	0.044	0.013	0.013	0.043	0.012
2/3-MDBT	0.026	0.088	0.025	0.013	0.044	0.013
3-MP	0.40	1.3	0.373	0.015	0.049	0.014
1-MDBT	0.0086	0.03	0.008	0.013	0.045	0.013
2-MP	0.035	0.12	0.033	0.025	0.082	0.023
4/9-MP	0.037	0.12	0.035	0.029	0.096	0.027
1-MP	0.023	0.078	0.022	0.026	0.086	0.024
3,6-DMP	0.017	0.056	0.016	0.015	0.051	0.014
3,5-DMP	0.033	0.11	0.032	0.013	0.043	0.012
2,6-DMP	0.011	0.036	0.010	0.012	0.040	0.011
2,7-DMP	0.039	0.13	0.037	0.038	0.127	0.036
3,9-DMP	0.014	0.046	0.013	0.013	0.044	0.013
1,6/2,5/2,9-DMP	0.033	0.11	0.031	0.026	0.088	0.025
1,7-DMP	0.032	0.11	0.031	0.023	0.078	0.021
1,9/4,9-DMP	0.025	0.082	0.023	0.020	0.067	0.019
Fluoranthene	0.038	0.13	0.036	0.022	0.073	0.021
1,2-DMDBT	0.025	0.084	0.024	0.024	0.081	0.023
1,5-DMP	0.029	0.096	0.027	0.042	0.141	0.040
1,8-DMP	0.011	0.038	0.011	0.017	0.056	0.016
1,2-DMP	0.019	0.063	0.018	0.025	0.084	0.024
9,10-DMP	0.023	0.078	0.022	0.012	0.041	0.012
Pyrene	0.037	0.12	0.035	0.013	0.045	0.013
1,2,8-TMP	0.039	0.13	0.037	0.034	0.112	0.032
Benzanthracene	0.05	0.17	0.049	0.026	0.087	0.025
d12 CHRYS	0.06	0.19	0.054	0.036	0.120	0.034
Chrysene	0.05	0.15	0.044	0.041	0.136	0.038
Benzo (b) fluor	0.18	0.59	0.168	0.045	0.150	0.042
Benzo(k),fluor	0.045	0.15	0.042	0.053	0.178	0.050
Benzo(a)pyrene	0.14	0.46	0.131	0.064	0.212	0.060
d12 PERYL	0.13	0.42	0.118	0.080	0.267	0.076
Indenopyrene	0.17	0.58	0.164	0.692	2.307	0.652
Dibenzanthracene	0.19	0.63	0.179	1.956	6.519	1.843
Benzoperylene	0.078	0.26	0.074	0.087	0.290	0.082
MEANS	0.046	0.155	0.044	0.072	0.241	0.068
MIN	0.007	0.024	0.007	0.008	0.027	0.008
MAX	0.396	1.320	0.373	1.956	6.519	1.843

Table 7.4 Method detection limits for three sample matrices

	LOD	MATRIX		
	(3*S.D.ng)	TISSUES	SEDIMENTS	WATERS
ANALYTE		ppb	ppb	ppb*
AVE. SAMPLE AMT.		20.45g	7.42g**	2000ml
Naphthalene	0.019	0.091	0.25	0.00093
2-MN	0.0073	0.035	0.098	0.00036
1-MN	0.010	0.051	0.14	0.00052
2-EN	0.014	0.070	0.19	0.00071
1-EN	0.027	0.13	0.37	0.0014
2,6/2,7-DMN	0.041	0.20	0.56	0.0021
1,3/1,7-DMN	0.049	0.24	0.67	0.0025
1,6-DMN	0.023	0.11	0.31	0.0012
1,4/2,3-DMN	0.038	0.18	0.51	0.0019
1,5-DMN	0.034	0.17	0.46	0.0017
Acenaphthylene	0.020	0.095	0.26	0.00098
1,2-DMN	0.027	0.13	0.36	0.0013
1,8-DMN	0.012	0.060	0.17	0.00061
Acenaphthene	0.021	0.10	0.28	0.0010
1,6,7-TMN	0.019	0.091	0.25	0.00093
Fluorene	0.020	0.098	0.27	0.0010
Dibenzothiophene	0.024	0.12	0.33	0.0012
Phenanthrene	0.022	0.11	0.29	0.0011
Anthracene	0.030	0.15	0.41	0.0015
4-MDBT	0.013	0.065	0.18	0.00066
2/3-MDBT	0.026	0.13	0.36	0.0013
3-MP	0.40	1.9	5.3	0.0198
1-MDBT	0.0086	0.042	0.12	0.00043
2-MP	0.035	0.17	0.48	0.0018
4/9-MP	0.037	0.18	0.50	0.0018
1-MP	0.023	0.11	0.31	0.0012
3,6-DMP	0.017	0.082	0.23	0.00084
3,5-DMP	0.033	0.16	0.45	0.0017
2,6-DMP	0.011	0.053	0.15	0.00054
2,7-DMP	0.039	0.19	0.52	0.0019
3,9-DMP	0.014	0.067	0.18	0.00068
1,6/2,5/2,9-DMP	0.033	0.16	0.45	0.0017
1,7-DMP	0.032	0.16	0.44	0.0016
1,9/4,9-DMP	0.025	0.12	0.33	0.0012
Fluoranthene	0.038	0.19	0.51	0.0019
1,2-DMDBT	0.025	0.12	0.34	0.0013
1,5-DMP	0.029	0.14	0.39	0.0014
1,8-DMP	0.011	0.055	0.15	0.00057
1,2-DMP	0.019	0.092	0.25	0.00095
9,10-DMP	0.023	0.11	0.32	0.0012
Pyrene	0.037	0.18	0.50	0.0019
1,2,8-TMP	0.039	0.19	0.53	0.0020
Benzanthracene	0.052	0.25	0.70	0.0026
Chrysene	0.046	0.23	0.62	0.0023
Benzo (b) fluor	0.18	0.87	2.4	0.0089
Benzo(k),fluor	0.045	0.22	0.60	0.0022
Benzo(a)pyrene	0.14	0.68	1.9	0.0070
Indenopyrene	0.17	0.85	2.3	0.0087
Dibenzanthracene	0.19	0.93	2.6	0.0095
Benzoperylene	0.078	0.38	1.1	0.0039
MEANS	0.050	0.24	0.67	0.0025
MIN	0.0072	0.035	0.097	0.00036
MAX	0.40	1.9	5.3	0.020

*MDL (ppb)=LOD*200ul final vol. / (sample amt * 2ul inj'd)
**typical dry wt. of 15g wet sediment

Table 7.5 Percent recovery of analytes spiked at four concentrations

ANALYTE	5 PPT			10 PPT			50 ppt			100 ppt			SUMMARY STATISTICS		
	#1	#2	#3	#1	#2	#3	#1	#2	#3	#1	#2	#3	AVE	MIN	MAX
d8 NAPH	54%	62%	42%	42%	33%	34%	25%	23%	38%	27%	25%	43%	37%	23%	62%
Naphthalene	369%	351%	287%	181%	130%	113%	52%	47%	74%	42%	36%	64%	146%	36%	369%
2-MN	108%	103%	73%	66%	54%	43%	32%	32%	45%	31%	36%	50%	56%	31%	108%
1-MN	96%	91%	71%	61%	47%	41%	33%	32%	45%	31%	37%	51%	53%	31%	96%
2-FBP-IS	122%	96%	148%	103%	98%	98%	111%	155%	140%	115%	102%	127%	118%	96%	155%
2-EN	40%	44%	35%	34%	27%	27%	28%	26%	38%	27%	35%	42%	34%	26%	44%
1-EN	40%	43%	38%	35%	26%	31%	29%	26%	38%	28%	36%	44%	35%	26%	44%
2,6/2,7-DMN	54%	49%	37%	37%	33%	29%	29%	28%	37%	28%	36%	43%	36%	28%	54%
1,3/1,7-DMN	60%	50%	38%	35%	35%	29%	31%	29%	40%	29%	37%	45%	38%	29%	60%
1,6-DMN	55%	53%	40%	37%	35%	29%	31%	29%	40%	29%	37%	45%	38%	29%	55%
1,4/2,3-DMN	41%	41%	34%	36%	30%	29%	32%	29%	42%	30%	39%	47%	36%	29%	47%
1,5-DMN	49%	45%	44%	36%	32%	35%	33%	31%	42%	30%	39%	47%	38%	30%	49%
Acenaphthylene	46%	48%	38%	38%	32%	35%	38%	34%	50%	35%	45%	57%	41%	32%	57%
1,2-DMN	48%	43%	41%	36%	28%	28%	33%	30%	41%	30%	39%	46%	37%	28%	48%
1,8-DMN	45%	58%	43%	41%	37%	41%	36%	32%	48%	33%	42%	50%	42%	32%	58%
d10 ACE	47%	46%	37%	39%	34%	33%	37%	33%	48%	34%	42%	52%	40%	33%	52%
Acenaphthene	69%	58%	48%	42%	44%	39%	37%	36%	49%	35%	43%	54%	46%	35%	69%
1,6,7-TMN	63%	55%	42%	41%	38%	38%	38%	33%	45%	34%	41%	48%	43%	33%	63%
Fluorene	74%	60%	55%	47%	45%	43%	44%	40%	54%	38%	45%	57%	50%	38%	74%
Dibenzothiophene	62%	64%	50%	52%	42%	46%	47%	42%	57%	44%	45%	58%	51%	42%	64%
d10 PHEN	61%	53%	47%	48%	40%	45%	48%	42%	64%	45%	45%	60%	50%	40%	64%
Phenanthrene	111%	98%	77%	71%	63%	93%	53%	48%	65%	47%	46%	61%	70%	46%	111%
Anthracene	51%	52%	39%	42%	38%	41%	47%	41%	61%	45%	44%	59%	47%	38%	61%
4-MDBT	70%	82%	58%	55%	48%	53%	49%	43%	63%	46%	44%	59%	56%	43%	82%
2/3-MDBT	55%	64%	43%	49%	42%	43%	49%	43%	65%	45%	44%	59%	50%	42%	65%
3-MP	57%	57%	46%	50%	42%	47%	50%	44%	65%	47%	44%	60%	51%	42%	65%
1-MDBT	67%	65%	45%	49%	46%	46%	49%	43%	62%	46%	44%	60%	52%	43%	67%
2-MP	54%	54%	41%	47%	38%	44%	48%	42%	63%	46%	43%	58%	48%	38%	63%
4/9-MP	57%	58%	41%	48%	41%	46%	49%	43%	64%	47%	44%	60%	50%	41%	64%
1-MP	55%	55%	47%	52%	44%	50%	49%	44%	67%	46%	44%	59%	51%	44%	67%
3,6-DMP	54%	59%	53%	49%	43%	47%	50%	44%	66%	48%	46%	59%	52%	43%	66%
3,5-DMP	43%	57%	46%	50%	39%	47%	50%	44%	64%	46%	45%	58%	49%	39%	64%
2,6-DMP	86%	ndl	76%	66%	63%	93%	51%	44%	70%	48%	47%	61%	64%	44%	93%
2,7-DMP	47%	nd	26%	40%	35%	nd	49%	43%	65%	46%	44%	57%	45%	26%	65%
3,9-DMP	72%	77%	48%	55%	48%	56%	50%	44%	67%	47%	46%	59%	56%	44%	77%
1,6/2,5/2,9-DMP	56%	55%	41%	45%	42%	43%	48%	41%	64%	47%	45%	57%	49%	41%	64%
1,7-DMP	62%	62%	42%	48%	44%	43%	47%	41%	63%	46%	43%	55%	50%	41%	63%
1,9/4,9-DMP	49%	69%	46%	54%	46%	50%	54%	46%	71%	46%	41%	65%	53%	41%	71%
Fluoranthene	65%	63%	68%	58%	44%	54%	57%	49%	77%	51%	47%	67%	58%	44%	77%
1,2-DMDBT	65%	64%	52%	53%	45%	51%	50%	46%	73%	49%	44%	63%	55%	44%	73%
1,5-DMP	67%	58%	42%	49%	44%	45%	49%	42%	65%	49%	46%	60%	51%	42%	67%
1,8-DMP	63%	58%	41%	47%	49%	45%	48%	42%	64%	47%	44%	56%	50%	41%	64%
1,2-DMP	104%	69%	62%	49%	63%	51%	49%	42%	65%	47%	45%	58%	59%	42%	104%
9,10-DMP	56%	59%	56%	51%	49%	45%	51%	41%	66%	48%	46%	58%	52%	41%	66%
Pyrene	53%	59%	52%	49%	45%	46%	52%	43%	67%	49%	46%	61%	52%	43%	67%
1,2,8-TMP	53%	53%	44%	45%	46%	40%	46%	35%	59%	42%	41%	50%	46%	35%	59%
Benzanthracene	41%	57%	40%	42%	43%	39%	51%	27%	57%	42%	38%	55%	44%	27%	57%
d12 CHRYS	58%	63%	44%	50%	46%	41%	48%	26%	50%	38%	33%	46%	45%	26%	63%
Chrysene	43%	62%	46%	44%	47%	43%	50%	25%	52%	37%	32%	48%	44%	25%	62%
Benzo (b) fluor	33%	51%	32%	37%	40%	30%	50%	12%	46%	38%	29%	53%	38%	12%	53%
Benzo(k),fluor	26%	50%	33%	33%	42%	27%	42%	9%	34%	25%	20%	36%	31%	9%	50%
Benzo(a)pyrene	42%	51%	45%	35%	49%	26%	50%	11%	40%	30%	23%	47%	37%	11%	51%
d12 PERYL	35%	64%	44%	43%	46%	35%	48%	11%	40%	26%	23%	44%	38%	11%	64%
Indenopyrene	nd	nd	nd	3%	19%	10%	22%	7%	21%	14%	10%	22%	14%	3%	22%
Dibenzanthracene	nd	nd	nd	11%	10%	2%	13%	3%	16%	7%	6%	19%	10%	2%	19%
Benzoperylene	nd	nd	nd	13%	22%	3%	23%	9%	20%	13%	11%	20%	15%	3%	23%
MEAN RECOVERY	59%	61%	48%	47%	42%	42%	44%	36%	55%	39%	38%	53%	47%	36%	61%
Parent PAH	78%	80%	65%	47%	44%	40%	43%	28%	49%	35%	33%	49%	49%	28%	80%
Alk. Naphthalenes	58%	56%	45%	41%	35%	33%	32%	30%	42%	30%	38%	47%	41%	30%	58%
Alk. DBT's	64%	69%	49%	52%	45%	48%	49%	44%	66%	47%	44%	60%	53%	44%	69%
Alk. Phens.	61%	60%	47%	50%	46%	50%	49%	43%	65%	47%	44%	58%	52%	43%	65%
TotalAlk. PAH	61%	62%	47%	48%	42%	44%	43%	39%	58%	41%	42%	55%	48%	39%	62%
Surrogates	47%	52%	39%	42%	36%	35%	37%	25%	43%	31%	31%	46%	39%	25%	52%

Table 7.6 Historical data for sediments from the Pass Fourchon 400m (from discharge) sampling site

DATE	DEPTH (CM)	TOTAL PAH ppb	SATURATED HC ppb	FFPI	Reference
Oct-87	0-5	7,700	260,000	0.91	1
	5-10	4,200	184,000	0.83	
	10-15	6,800	210,000	0.89	
	15-20	1,000	250,000	0.76	
Jan-88	0-5	43,000	650,000	0.93	1
Feb-89	0-2	21,000	490,000	0.92	2
	2-5	22,000	570,000	0.91	
	5-10	20,000	490,000	0.92	
	10-15	21,000	630,000	0.91	
	15-20	7,900	140,000	0.92	
	20-27	31,000	580,000	0.93	
May-89	0-2	35,000	680,000	0.90	2
Oct-89	0-2	22,000	760,000	0.91	2
Feb-90	0-2	91,000	1,700,000	0.94	2
	2-5	36,000	930,000	0.93	
	5-10	61,000	1,000,000	0.95	
	10-15	20,000	750,000	0.88	
	15-20	14,000	660,000	0.87	
	20-25	12,000	510,000	0.87	
	25-30	17,000	471,000	0.93	
	30-35	12,000	310,000	0.80	
	35-40	22,000	490,000	0.96	
1994		53,000	NA	0.98	3

1 Boesch & Rabalais, 1989
2 Rabalais et al, 1991. Vol. III
3 this report

Table 7.7 Application of analytical methods to aqueous and particulate phases of water and sediments in coastal waters near Pass Fourchon

ANALYTE	DISSOLVED PHASE		PARTICULATE PHASE		SEDIMENT
	SURFACE	BOTTOM	SURFACE	BOTTOM	(BEDDED)
	pg/ml	pg/ml	ng/g	ng/g	ng/g
Naphthalene	134	96	2673	2420	2.2
2-MN	223	141	132	96	2.5
1-MN	125	80	53	40	2.2
2-EN	26	12	27	14	1.0
1-EN	5.1	TR	TR	TR	3.2
2,6-/2,7-DMN	62	32	65	36	nd
1,3/1,7-DMN	43	26	38	27	2.2
1,6-DMN	28	17	33	22	1.8
1,4/2,3-DMN	19	9.3	24	12	2.3
1,5-DMN	32	18	9.3	7.6	nd
Acenaphthylene	TR	TR	nd	TR	0.63
1,2-DMN	7.4	4.4	9.0	TR	0.39
2-IPN	13	8.9	nd	nd	NA
1,8-DMN	nd	nd	nd	nd	NA
Acenaphthene	19	15	13	6.2	0.51
1,6,7-TMN	9.2	nd	24	19	1.0
Fluorene	13	11	12	7.2	nd
Dibenzothiophene	6.3	7.6	nd	7.6	0.71
Phenanthrene	17	13	11	38	5.6
Anthracene	nd	TR	nd	0.28	0.92
4-MDBT	TR	TR	nd	15	1.3
2/3-MDBT	nd	nd	nd	6.0	nd
3-MP	1.5	nd	nd	13	3.0
1-MDBT	nd	nd	nd	0.12	NA
2-MP	1.0	nd	nd	17	1.8
4/9-MP	2.7	3.0	nd	nd	1.9
1-MP	nd	nd	nd	3.4	0.98
3,6-DMP	nd	nd	nd	6.3	1.1
3,5-DMP	nd	nd	nd	nd	
2,6-DMP	nd	nd	nd	7.3	0.77
2,7-DMP	nd	nd	nd	20	0.83
3,9-DMP	nd	nd	nd	2.9	2.8
1,6/2,5/2,9-DMP	nd	nd	nd	2.1	1.7
1,7-DMP	nd	nd	nd	1.9	2.1
1,9/4,9-DMP	nd	nd	nd	nd	1.6
1,2-DMDBT	nd	nd	nd	nd	nd
Fluoranthene	1.4	TR	55	128	4.9
1,5-DMP	nd	nd	nd	nd	nd
1,8-DMP	nd	nd	nd	2.4	0.60
1,2-DMP	nd	nd	nd	nd	0.78
9,10-DMP	nd	nd	nd	nd	nd
Pyrene	1.0	1.1	57	115	6.6
1,2,8-TMP	nd	nd	nd	nd	nd
Benzanthracene	4.3	nd	47	77	3.4
Chrysene	2.3	nd	62	118	4.3
Benzo(b)fluor	nd	nd	333	1297	9.6
Benzo(k)fluor	nd	nd	nd	nd	nd
Benzo(a)pyrene	nd	nd	nd	381	3.7
Indenopyrene	nd	nd	TR	418	4.7
Dibenzanthracene	nd	nd	nd	TR	TR
Benzoperylene	nd	nd	77	113	4.9
Estimated Totals					
C1-Naphthalenes	347	221	185	136	4.7
C2-Naphthalenes	222	119	206	120	11
C3-Naphthalenes	96	6.5	124	123	NA
C4-Naphthalenes	nd	5.9	20	37	NA
C1-Dibenzothiophenes	TR	TR	nd	21	1.30
C2-Dibenzothiophenes	8.5	8.6	nd	64	NA
C1-Phenanthrenes	5.1	3.0	nd	34	7.7
C2-Phenanthrenes	nd	nd	nd	43	17
C3-Phenanthrenes	6.7	nd	nd	17	NA

CHAPTER 8

INVESTIGATION OF THE BIOACCUMULATION POTENTIAL OF 60 INDIVIDUAL AROMATIC PETROLEUM-ASSOCIATED HYDROCARBONS IN THE OYSTER *CRASSOSTREA VIRGINICA*

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INTRODUCTION

Owing to their chemical structures and inherent physiochemical properties, a number of anthropogenically generated organic chemicals are bioaccumulated by marine organisms via various routes of uptake. *Crassostrea virginica* are bottom dwelling-filter feeders and tend to burrow slightly into the sediment along the benthic zone. Petroleum hydrocarbons, which are considered toxicologically relevant, are being released into the water of coastal Louisiana and the Gulf of Mexico due to the ongoing extraction of petroleum. Once released, the chemicals are sorbed onto the sediment and as our study shows, become bioavailable to benthic bottom feeders. Concentrations of contaminants will vary from site to site but where uptake does not cause a physiologically compromised state or death, introduction to aquatic food webs is probable.

MATERIALS AND METHODS

Microcosm Exposures

Live oysters (*Crassostrea virginica*), with an average shell length of 5 cm, from Black Bay, LA were purchased from a local seafood store (Baton Rouge, LA.) The oysters were then brought to our aquatic laboratory where they were placed in a large holding tank for acclimation. In the holding tank, oysters were submersed in sea water (pre-dissolved FRITZ Super Salt - a synthetic sea water salt mixture plus carbon filtered/ dechlorinated water 11 ppt salinity; 21-23°C) for a period of 4.5 days. Oysters were acclimated to a 12-hr. day and night. Spawning began on day 1 and ceased around day 4. During the daylight hours of this period, 1 day old prepared sea water was constantly flushed through the holding tank to discourage spawning and to keep ammonia levels to a minimum. Holding tank was aerated with 2 aquaria air pumps of appropriate pumping capacity with air stones attached during the duration of the acclimatization. A liquid diet for invertebrate marine animals by Hawaiian Marine Imports, Inc. was chosen as the diet and their first feeding was on day 3 in the holding tank.

Twenty, 10-gallon aquarium tanks (glass sides and bottoms) were set up as follows. First, sediment obtained from the contaminated area as well as sediment from the control area was hand pressed through a 2 mm sieve to remove stones, shell hash and debris. Four separate dilutions were then prepared using a mixture of the contaminated and control sediments in varying amounts to yield a 0% contaminated batch, comprised solely of sediment from Lake Champagne; a 12.5% contaminated batch; 25%; and 50% with the latter percentages indicating % of contaminated sediment from Pass Fourchon accordingly.

Oysters were exposed to dilutions of sediment contaminated with 52 ppm total petroleum aromatic hydrocarbons to study the rates and magnitude of bioaccumulation. A period of 28 days was chosen for the exposure study based upon previous experiments which indicate that the organism can achieve steady-state within that period. (McElroy and Means, 1988). Four concentrations of contaminated sediment from Pass Fourchon (52 ppm total petroleum aromatic hydrocarbons) were prepared by mixing with "clean" sediment from Lake Champagne (< 0.015 ppm total petroleum aromatic hydrocarbons) and rolling in jars for 24 hr. to ensure homogeneity. The resulting mixtures, ~3000 kg total (wet wt.), were comprised of 0%, 12.5%, 25%, and 50% of contaminated sediment, respectively. Oysters (4-5 per chamber) were held in separate aquaria and samples were taken on days 0, 3, 7, 14, 21, and 28 to assess uptake of the aromatic hydrocarbons from the sediments by the oysters. Overlying water in the tanks was exchanged every two days to prevent the accumulation of metabolic wastes. Feeding occurred immediately after each water exchange. Mortality was noted every two days and recorded.

Oysters were recovered from the tank, rinsed and frozen at -40°C until analyses were performed. Tissues were prepared for hydrocarbon analysis by thawing, followed by removal of the soft tissues from the shell and rinsing with distilled water to remove any residual sediment.

Chemical Analysis

Tissue Sample Preparation

Oysters samples were defrosted overnight in a refrigerator. Individual samples were removed from the bags and rinsed with tap water, then shelled on a glass cutting board with a Teflon® coated knife. The shell-free flesh was rinsed with organic free water and then chopped into small pieces. The pieces were placed in a clean jar and homogenized using a Tekmar Tissuemizer®. The homogenized samples were kept frozen at -20°C until sample extraction.

The extraction method is a modification of that described by MacLeod et al. (1985). Approximately 20 g of defrosted homogenized tissue was placed in an 240-ml amber glass bottle with 100 ml nanograde® dichloromethane (DCM) and four times the tissue weight of cleaned sodium sulfate (DCM rinsed). A surrogate spike of 200 ng each of a mixture of deuterated standards (US-108, Ultra Scientific) was added to the bottle. The sample was extracted with a Tekmar Tissuemizer® for 2 min. at a speed of 24,000 rpm. The probe was rinsed with DCM and the rinsings added to the sample bottle. The extract was allowed to settle for 1 min. and then filtered through a funnel filled with sodium sulfate into a labeled florence flask. The sample was extracted as above with two additional 100 ml volumes of DCM. After the third extraction, the extract was rinsed with an additional 30 ml of DCM, which was added to the flask. The sample extract was reduced in volume to approximately 2 ml with a rotary evaporator. The concentrated extract was quantitatively transferred to a 4-ml vial and concentrated to a volume of 2 ml under a stream of purified nitrogen. Reagent blanks, duplicates and matrix-spiked samples were extracted to monitor laboratory technique and extraction efficiency. Matrix spikes were duplicate tissue samples to which 100 ng of the quantitative calibration standard was added instead of the deuterated surrogates mixture.

Most tissue samples contained a layer of fine white precipitate (extracted proteins) which was removed before silica column cleanup. The extracts were "cleaned" by adding 500 µl of hexane, mixing the extract well and then centrifuging for 5 min. at 3,000 rpm. The solvent layer was transferred to a clean 4-ml vial and the extract was "cleaned" twice more with 1 ml of hexane. All solvent layers were combined and reduced in volume to 1 ml under a stream of purified nitrogen.

One half of the extract was separated from the polar lipids on 200 mm x 10 mm columns packed with 6.0 g of 100-200 mesh Grade 923 silica, eluted with 100 ml of 60% DCM and 40% hexane. The extract was concentrated under nitrogen to 200 µl, after which 2 µl of 2-fluorobiphenyl (100 ng/µl in hexane) was added as an internal standard to monitor the GC/MS injection performance. Instrumental analysis (GC/MS) was performed as described in Chapter 7.

Sediment Sample Preparation

Sediment samples from the microcosms were sealed in glass jars with a Teflon-lined cap, mixed, and refrigerated at -20°C until processed for analyses. The extraction method was a modification of that described by MacLeod et al. (1985). Excess water was decanted from sample jars prior to thorough mixing of the sediment sample, and 15 g was weighed into an amber bottle with a Teflon-lined cap. The sediment was covered with 75 ml of dichloromethane (DCM) and 50 g sodium sulfate incorporated until the sediment mixture was free-flowing. A suite of deuterated PAH was added (800 ng in hexane of six deuterated PAH) with the first solvent aliquot as a surrogate standard to monitor extraction efficiency. The jars were sealed and placed on a roller for 16, 6, and 16 hours, for a total of three extractions using the modified rock tumbler and rolling times specified by MacLeod et al. (1985). The DCM from each jar was decanted into an amber jar and refrigerated until 3 extracts were collected. The decanted DCM was filtered through sodium sulfate, and concentrated by rotoevaporation and a nitrogen stream to a final volume of 200 µl, with solvent exchange to hexane. Activated fine granular copper was added to reduce sulfur interferences. Instrumental analysis (GC/MS) was performed as described in Chapter 7.

RESULTS AND DISCUSSION

Mortality

Significant mortality was observed in the two highest dose rates. Table 8.1 shows the mortality data for all exposures. After two separate experimental attempts at sustaining viability in the 25% and 50% exposure chambers for longer than 14 days, we were unable to maintain enough live organisms in order to assess the rate of uptake and bioaccumulation of the petroleum hydrocarbons for those periods due to the mortality. Survivorship in the control sediments and 12.5% contaminated dose tanks was satisfactory to obtain bioaccumulation data. Our conclusion in this case is that the concentrations of petroleum HC's were too great to maintain viability at the high dose levels for more than 14 days. Maintenance of the laboratory experimental conditions proved adequate to sustain life in all other chambers.

Table 8.1 Mortality data

% of oysters surviving at collection date

	Dose 0%	12.50%	25%	50%
DAY 0	100%	100%	100%	100%
DAY 3	100%	75%	75%	100%
DAY 7	100%	75%	25%	25%
DAY 14	75%	50%	25%	25%
DAY 21	75%	25%	0%	0%
DAY 28	25%	50%	0%	0%

Sediment Contaminant Chemistry

Both of the sediments used in these experiments are typical of fine-grained sediments found in coastal regions of Louisiana. The contaminated sediment from Pass Fourchon is a sandy silt, ~70% sand, 25% silt and < 5% clay. The Lake Champagne sediment is also a sandy silt, 38.5% sand, 56.6% silt and <5% clay. The total organic carbon content of the two sediments were 2.0% for the Pass Fourchon and 4.0% for the Lake Champagne sediments, respectively.

Total aromatic hydrocarbon content was measured in both sediments. The undiluted Pass Fourchon sediment contained 52.8 ppm of total aromatics of which only 0.5 ppm were parent aromatic compounds. The vast majority of the aromatic fraction was composed of alkylated analogs of parent, and heterocyclic aromatics typically associate with petroleum sources, including produced water discharges (Means and McMillin, 1993). In contrast, the Lake Champagne reference sediment contained only 0.015 total aromatic hydrocarbons of which <0.001 ppm were petroleum-associated materials.

Table 8.2 shows the concentrations of each individual compound determined in the sediments and reported on a dry-weight basis. The values are consistent with data obtained at this site in previous investigations (Rabalais et al., 1991a; Means and McMillin, 1993).

Bioaccumulation

The filter-feeding benthic organism [oyster (*Crassostrea virginica*)] was exposed to four dilutions of a contaminated estuarine sediment [52 ppm total petroleum aromatic hydrocarbons]. An assessment of the amount of bioaccumulation of 60 individual aromatic hydrocarbons, measured by gas chromatography/mass spectrometry (GC/MS) revealed that accumulation was both time and dose dependent. A summary of the concentrations of each individual compound detected in each set of oysters at each time and dose level are presented in Appendix A. Selected data, showing major trends, are presented here.

Figure 29a shows the accumulation of high molecular weight parent PAH from the 0% contaminated dose sediment (pure reference sediment). Chrysene and benzo(b and k) fluoranthene were not detected in any samples. Fluoranthene and benzanthracene were found at low levels in the tissues of acclimated but unexposed organisms. The levels of these two compounds appeared to decrease through day 14 of the experiment but were elevated after 21 days of exposure. Pyrene, absent at the early time points, was observed at day 21 in the tissues at low levels.

Table 8.2 Sediment data for Pass Fourchon and reference site

SAMPLE	L.CHAMP	PF400
Units, dry wt. basis	ppb*	ppb
Naphthalene	nd	1.7
2-Methylnaphthalene	nd	3.0
1-Methylnaphthalene	nd	1.5
2-Ethylnaphthalene	nd	1.9
1-Ethylnaphthalene	nd	9.1
2,6/2,7-Dimethylnaphthalene	nd	10
1,3/1,7-Dimethylnaphthalene	nd	48
1,6-Dimethylnaphthalene	nd	4.7
1,4/2,3-Dimethylnaphthalene	nd	27
1,5-Dimethylnaphthalene	nd	25
1,2-Dimethylnaphthalene	nd	12
2-Isopropylnaphthalene	nd	nd
1,8-Dimethylnaphthalene	nd	12
1,6,7-Trimethylnaphthalene	nd	197
Acenaphthylene	nd	4.0
Acenaphthene	nd	11
Fluorene	nd	18
Dibenzothiophene	nd	5.6
Phenanthrene	1.0	52
Anthracene	nd	16
4-Methyldibenzothiophene	nd	68
2/3-Methyldibenzothiophene	nd	55
1-Methyldibenzothiophene	nd	34
3-Methylphenanthrene	nd	183
2-Methylphenanthrene	1.0	87
4/9-Methylphenanthrene	nd	177
1-Methylphenanthrene	nd	102
3,6-Dimethylphenanthrene	nd	102
3,5-Dimethylphenanthrene	nd	nd
2,6-Dimethylphenanthrene	nd	93
2,7-Dimethylphenanthrene	nd	77
3,9-Dimethylphenanthrene	nd	335
1,6/2,5/2,9-Dimethylphenanthrene	nd	228
1,7-Dimethylphenanthrene	nd	83
1,9/4,9-Dimethylphenanthrene	nd	78
1,5-Dimethylphenanthrene	nd	nd
1,8-Dimethylphenanthrene	nd	32
1,2-Dimethylphenanthrene	nd	22
9,10-Dimethylphenanthrene	nd	nd
Fluoranthene	4.5	73
1,2-Dimethyldibenzothiophene	nd	5.9
Pyrene	2.7	54
1,2,8-Trimethylphenanthrene	nd	84
Benzanthracene	3.0	24
Chrysene	1.9	84
Benzo(b)fluoranthene	nd	29
Benzo(k)fluoranthene	nd	8.4
Benzo(a)pyrene	nd	12
Indenopyrene	nd	30
Dibenz(a,h)anthracene	nd	17
Benzo(g,h,i)perylene	nd	38
C3-NAPHTHALENES	nd	9,700
C4-NAPHTHALENES	NA	8,900
C2-DIBENZOTHIOPHENES	nd	8,600
C3-PHENANTHRENES	nd	23,000
Total Parent PAH	12	478
Total Alkylated PAH	0.50	52,397
Total PAH	13	52,875
FFPI (missing C3-DBTs)	0.06	0.98

*mean of lab duplicates

Figure 29b shows the same group of parent PAH accumulating from the 25% contaminated sediment. In this exposure, each of the compounds detected was observed to increase with time through day 14, except for benzanthracene which decreased and then increased at day 14. Chrysene was never observed in the 0% dose, but was found at significant levels in tissues exposed to 25% contaminated sediment.

Figure 29c shows the accumulation of high molecular weight PAH in oysters exposed at the 50% dose. Each of the compounds, including the benzofluoranthene isomers, were observed in these tissues after 7 to 14 days. The levels of PAH accumulated were much higher than any of the lower exposure doses and each of the compounds was observed to increase with time except benzanthracene, which decreased and then increased as reported at the other dose levels. At the 50% dose level, tissue concentrations at 7 days were approximately 5 times higher than seen at the lower doses at that time point. It is interesting to note that between day 7 and day 14 of this experiment, the concentrations of PAH declined in the oyster tissues. We interpret this as being a reflection of the physiologically compromised nature of the organisms, since mortality was so high at this exposure level.

Figure 30(a-d) shows the bioaccumulation pattern of low molecular weight PAH in oysters at four sediment doses. In the 0% dose system, a pattern similar to that observed with the high molecular weight PAH of decreasing followed by increasing PAH concentrations. At the 12.5% dose, a general pattern of declining PAH concentrations with time was observed. At the 25 and 50% dose levels, these compounds appear to increase in tissues with time but there is no clear dose dependent pattern of increasing tissue levels.

The bioaccumulation behavior of the more abundant alkylated aromatic hydrocarbons present in the sediments give a more consistent pattern tissue distributions with both time and sediment dose. Figure 31(a-d) show the bioaccumulation of C3-phenanthrenes in oyster tissues as a function of time and dose. In the 0% dose, where sediment C3-phenanthrenes were not detected, these compounds were not detected in the day 0 oysters, but were detected at very low but consistent levels (~12 ppb) in tissues samples after day three. At the 12.5% and 25% dose levels, there was a rapid, dose-dependent increase in C3-phenanthrenes in the oyster tissues to 14 days. Only oysters at the 12.5% dosage level survived to 28 days, and these showed declining PAH levels, again probably due to compromised physiology. At the 50% dose level, tissue concentrations increased even more rapidly to high levels. After only 7 days, the tissue concentrations were comparable to the levels observed in the 25% dose level after 14 days. The highest concentration reached for this compound group was 670 ppb, wet weight, which is equivalent to 33,000 ppb on an average lipid-weight basis.

Figure 32(a-d), shows the bioaccumulation pattern of C2-phenanthrenes, another group of alkylated PAH which were absent from the reference sediment but very concentrated in the contaminated sediment. A dose-dependent increase and time-dependent increase in tisssue residues of each of the individual dimethyl phenanthrenes was observed in the oyster tissues. As with several of the contaminant groups at the high level dose, tissues residues declined between day 7 and day 14, and after 14 days in the 12.5% dosage level. This was attributed to the toxicity of the sediments to the oysters with time.

Another compound group, which is unique to petroleum-related contaminated sediments are the alkyl dibenzothiophenes. This group of heterocyclic aromatic hydrocarbons were absent from the reference sediment and relatively abundent in the contaminated sediment. Figure 33(a-d) show the bioaccumulation of the C2-dibenzothiophenes in oyster tissues. Total dimethyl dibenzothiophenes showed a dose and time dependent increase in tissue residues, similar to the trend seen for C2- and C3-phenanthrenes.

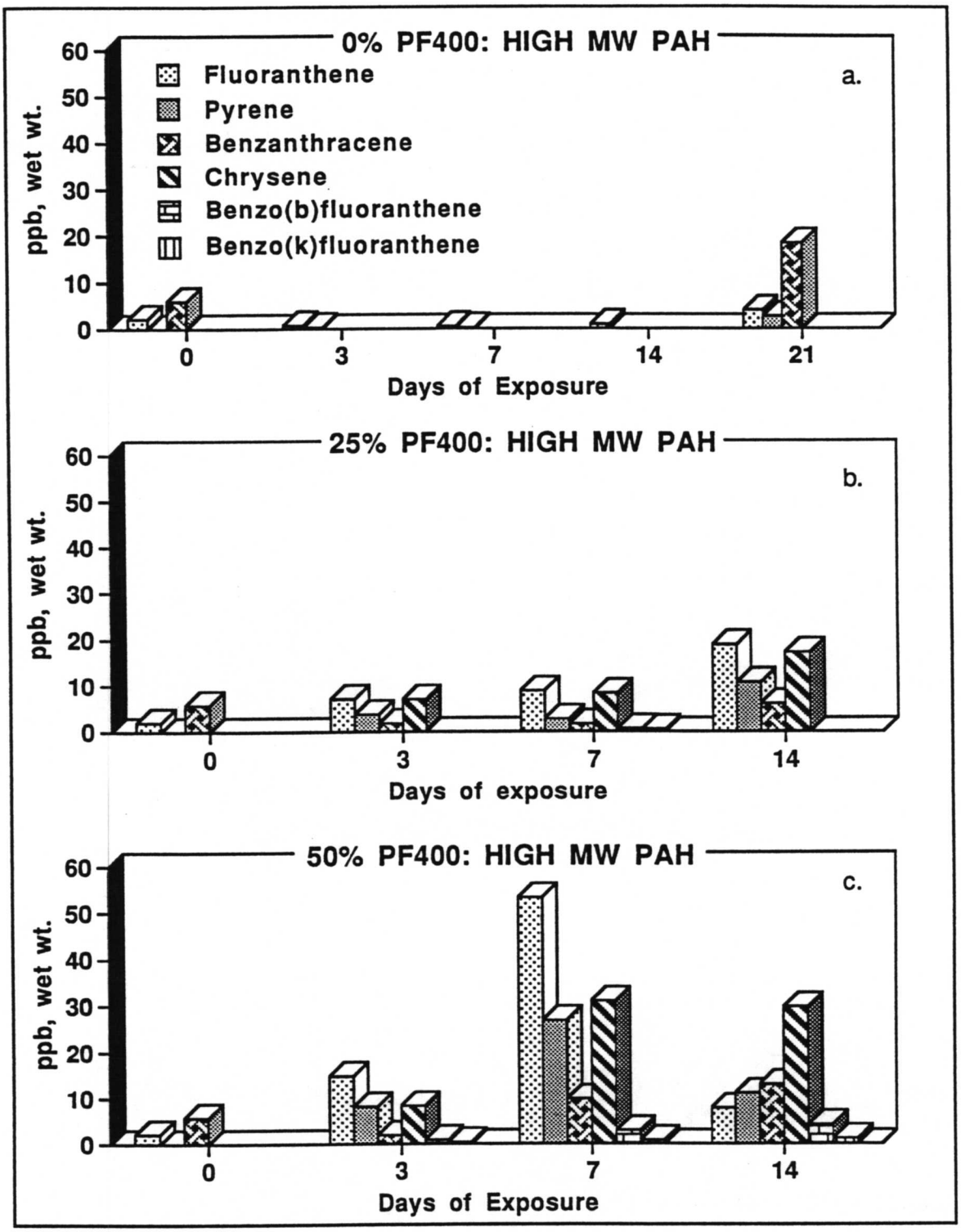


Figure 29. Concentrations (ppb) of high molecular weight PAH bioaccumulated at 3 dose levels.

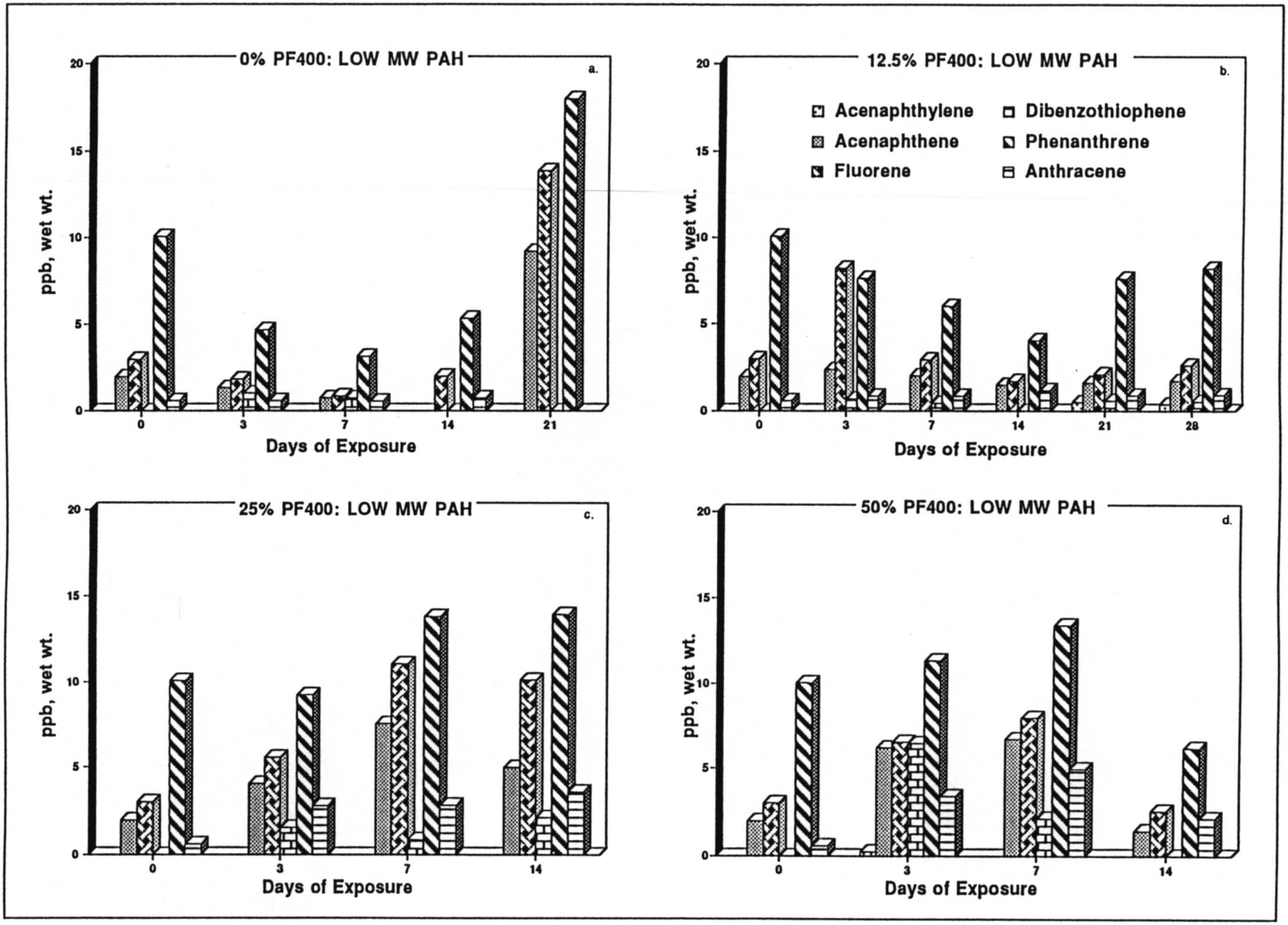
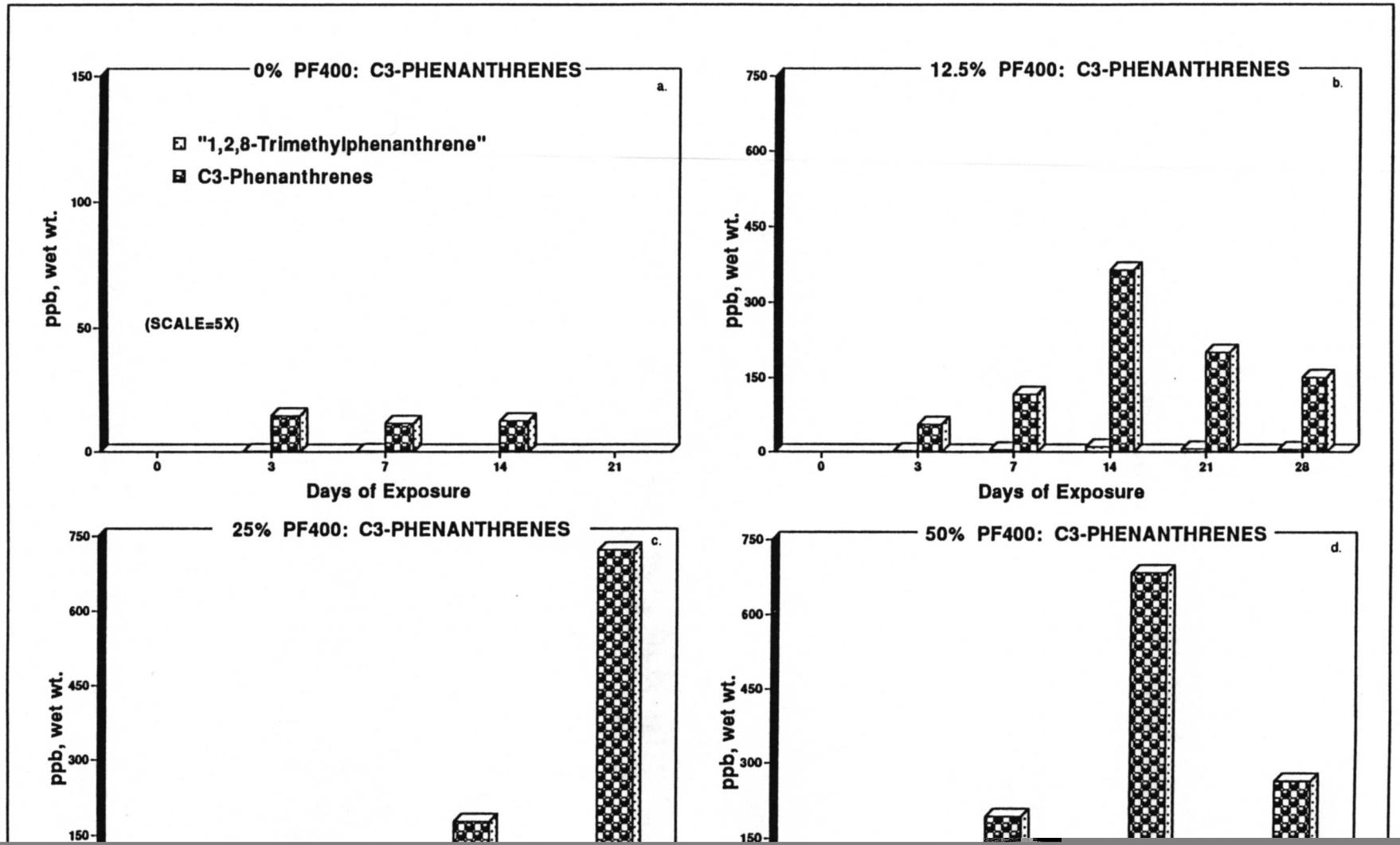


Figure 30. Concentrations (ppb) of low molecular PAH bioaccumulated at 4 dose levels.


0% PF400: C3-PHENANTHRENES
a.
"1,2,8-Trimethylphenanthrene"
C3-Phenanthrenes
(SCALE=5X)
ppb, wet wt.
150
100
50
0
0
3
7
14
21
Days of Exposure
12.5% PF400: C3-PHENANTHRENES
b.
ppb, wet wt.
750
600
450
300
150
0
0
3
7
14
21
28
Days of Exposure
25% PF400: C3-PHENANTHRENES
c.
ppb, wet wt.
750
600
450
300
150
50% PF400: C3-PHENANTHRENES
d.
ppb, wet wt.
750
600
450
300
150

Other compound groups were also examined and followed trends observed with the preceeding groups discussed. They include: methyl dibenzothiophenes, C-2 naphthalenes, C3-naphthalenes and C4-naphthalenes. These data are contained in Appendix A.

CONCLUSIONS

The sediment-bound contaminants associated with chronic produced water discharges which accumulate over time were found to be bioavailable to the filter-feeding bivalve *Crassostrea virginica.* Accumulation could be observed in as little as three days of exposure. Petroleum- associated aromatic hydrocarbons increased in a dose- and time-dependent fashion. Mortalities observed in the higher dose groups appeared to be contaminant related and effects on accumulation due to the compromised physiological state of intoxicated oysters was observed after 14 days in the highest dose group, and after 21 days at the 12.5% dosage level.

Isomer-specific analyses of bioaccumulated petroleum aromatic hydrocarbons in tissues of aquatic organisms yield information on the relative bioavailability of this important group of contaminants. These chemicals have been shown to rapidly enter biological foodwebs in aquatic systems as the result of exposure to contaminated sediments even in the absence of a continuous source of fresh contaminants. This suggests that these contaminated sediments will remain a source of bioavailable toxic substances even after discharges are suspended at a site. Since some of these foodwebs potentially lead directly to man, there are both ecological and human health risks associated with in-place, sediment-bound contamination from produced waters.

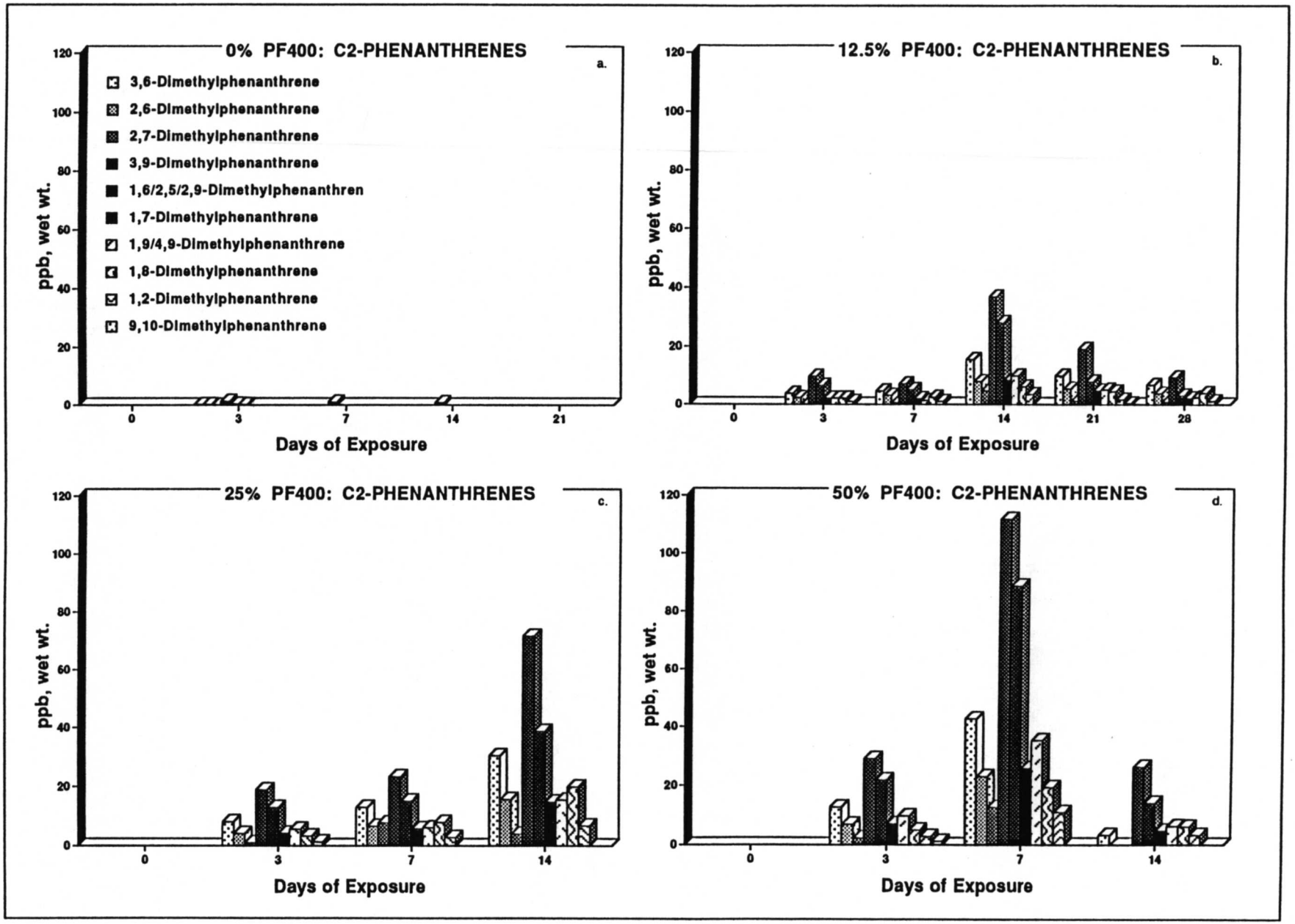


Figure 32. Concentrations (ppb) of C2-phenanthrenes bioaccumulated at 4 dose levels.

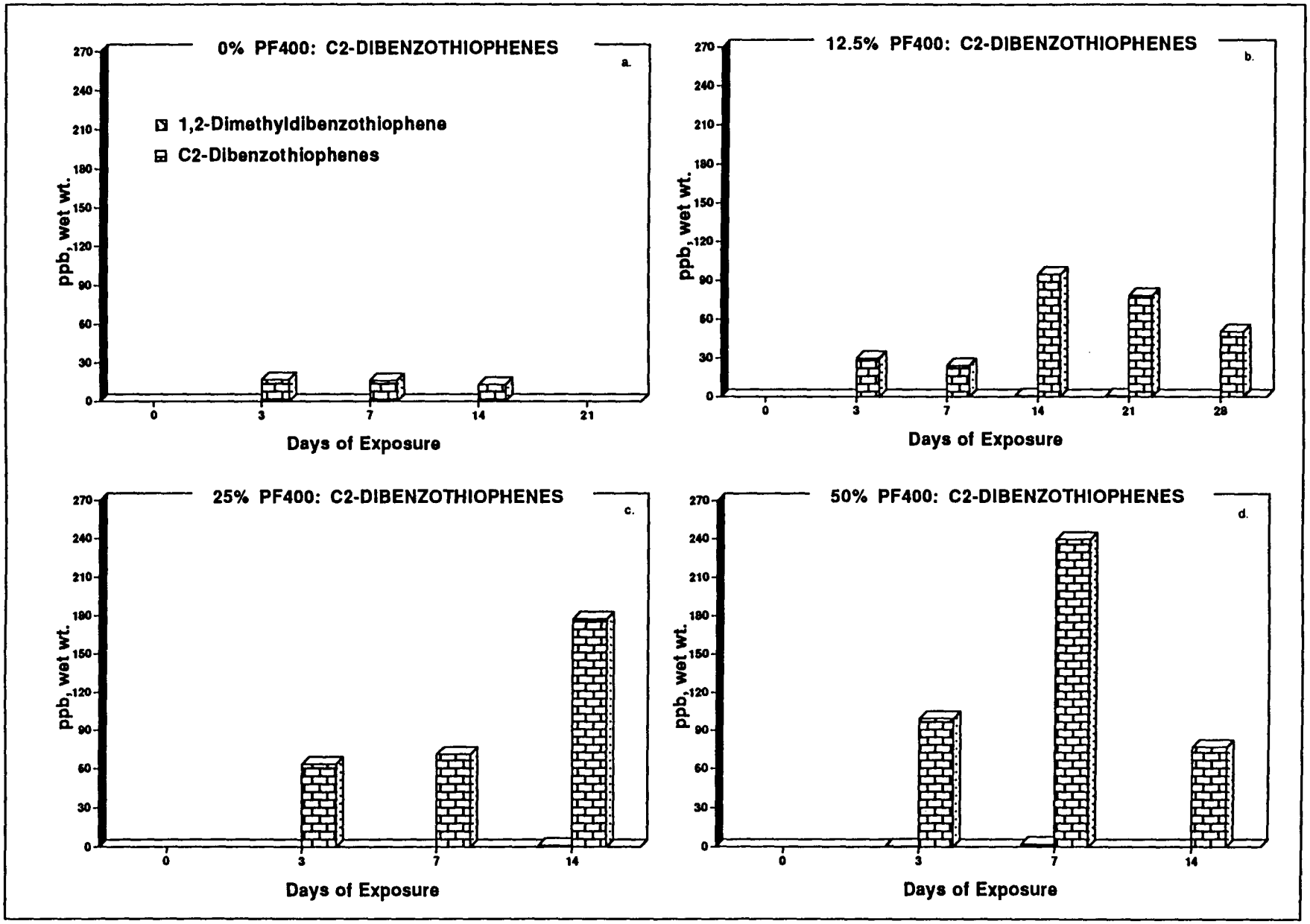


Figure 33. Concentrations (ppb) of C2-dibenzothiophenes bioaccumulated at 4 dose levels.

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Appendix A: Parent and alkylated PAH concentrations (ng/g) in oyster tissue from organisms exposed to varying levels of contaminated Pass Fourchon sediment for 28 days.

EXPOSURE LEVEL	0% LEVEL (CONTROL)							12.5% LEVEL					25% LEVEL			50% LEVEL		
SAMPLING DAY	0	0-dup.	3	3-dup.	7	14	21	3	7	14	21	28	3	7	14	3	7	14
ANALYTE	ng/g	ng/g	ng/g	ng/g	ng/g	ng/g	ng/g	ng/g	ng/g	ng/g	ng/g	ng/g	ng/g	ng/g	ng/g	ng/g	ng/g	ng/g
Naphthalene	nd	nd	16	20	23	13	261	69	16	9.6	14	21	30	82	90	37	42	21
2-Methylnaphthalene	nd	nd	1.5	3.0	1.5	5.1	139	24	2.8	1.4	4.1	4.9	7.5	29	8.3	2.4	12	5.7
1-Methylnaphthalene	nd	nd	0.81	1.7	0.78	1.9	65	15	1.6	0.96	3.3	4.1	4.7	19	5.1	1.4	6.0	2.6
2-Ethylnaphthalene	nd	nd	0.24	nd	0.13	nd	nd	nd	0.47	nd	0.91	1.0	1.2	3.9	nd	0.29	1.9	nd
1-Ethylnaphthalene	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	0.59	0.72	0.48	nd	nd	0.16	nd	nd
2,6/2,7-Dimethylnaphthalene	nd	nd	0.91	0.93	0.70	nd	nd	nd	1.1	nd	1.6	1.7	1.9	14	4.6	1.8	4.3	nd
1,3/1,7-Dimethylnaphthalene	nd	nd	0.53	0.93	0.34	1.4	26	7.1	0.74	0.55	1.5	1.6	2.7	8.0	2.6	2.4	4.5	1.7
1,6-Dimethylnaphthalene	nd	nd	0.39	0.68	0.24	1.1	20	5.7	0.51	0.20	0.99	1.1	1.3	5.4	2.0	0.65	2.3	1.5
1,4/2,3-Dimethylnaphthalene	nd	nd	0.32	nd	0.18	nd	nd	nd	0.57	0.60	1.0	1.0	1.6	5.3	2.6	2.5	3.7	nd
1,5-Dimethylnaphthalene	nd	nd	0.16	nd	nd	nd	nd	nd	0.28	0.35	0.36	0.44	1.5	3.0	0.55	1.8	2.2	nd
1,2-Dimethylnaphthalene	nd	nd	0.16	nd	nd	nd	nd	nd	0.26	nd	0.62	0.70	0.73	2.8	nd	0.79	0.97	nd
2-Isopropylnaphthalene	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	0.09	nd	nd	nd	nd	nd	nd	nd
1,8-Dimethylnaphthalene	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	0.14	nd	nd	nd	nd	nd	nd	nd
1,6,7-Trimethylnaphthalene	nd	nd	0.59	0.59	0.25	nd	nd	28	1.8	9.5	2.8	1.8	11	28	19	21	49	6.2
C3-NAPHTHALENES, est.	nd	nd	3.5	9.9	1.8	nd	nd	112	9.2	36	13	8.5	49	138	80	77	203	39
C4-NAPHTHALENES, est.	nd	nd	3.1	nd	1.5	nd	nd	193	20	95	33	17	50	262	184	81	320	57
Acenaphthylene	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	0.48	0.38	nd	nd	nd	0.24	nd	nd
Acenaphthene	nd	3.9	0.76	1.9	0.74	nd	9.2	2.4	2.0	1.5	1.6	1.7	4.0	7.5	5.0	6.2	6.7	1.4
Fluorene	nd	6.0	0.94	2.7	0.86	2.0	14	8.2	2.9	1.7	2.1	2.6	5.6	11	10	6.5	8.0	2.5
Dibenzothiophene	nd	nd	1.1	0.96	0.75	nd	nd	0.68	0.45	nd	0.58	0.53	1.5	0.86	2.1	6.5	2.1	nd
Phenanthrene	13.2	6.9	3.2	6.1	3.1	5.3	18	7.6	6.0	4.0	7.6	8.2	9.2	14	14	11	13	6.2
Anthracene	nd	1.2	0.36	0.79	0.55	0.76	nd	0.85	0.85	1.2	0.87	0.90	2.8	2.8	3.6	3.4	5.0	2.1
4-Methyldibenzothiophene	2.7	1.9	3.9	2.2	1.8	nd	nd	2.5	1.2	5.3	2.7	1.8	5.7	4.0	14	18	20	2.0
2/3-Methyldibenzothiophene	nd	nd	0.87	nd	nd	nd	nd	nd	0.36	2.2	0.75	0.55	2.1	1.3	3.6	8.3	6.9	nd
1-Methyldibenzothiophene	nd	nd	1.0	nd	nd	nd	nd	nd	0.44	2.6	0.93	0.40	2.8	0.98	5.0	9.1	11	nd
3-Methylphenanthrene	nd	nd	0.78	0.67	nd	nd	nd	3.3	1.2	7.6	2.6	1.7	8.0	5.3	10	15	30	nd
2-Methylphenanthrene	nd	nd	0.36	0.38	nd	nd	nd	0.79	0.58	1.6	1.5	1.4	1.5	1.1	3.0	3.7	4.7	nd
4/9-Methylphenanthrene	nd	nd	0.50	nd	nd	nd	239	1.9	nd	4.9	3.0	1.9	7.1	3.8	12	15	36	nd
1-Methylphenanthrene	nd	nd	0.60	0.50	nd	nd	nd	1.8	nd	4.1	1.6	1.1	4.1	2.3	6.4	9.4	17	327
3,6-Dimethylphenanthrene	nd	nd	0.35	nd	nd	nd	nd	3.8	4.6	15	9.9	6.8	8.1	13	30	13	43	3.3
3,5-Dimethylphenanthrene	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
2,6-Dimethylphenanthrene	nd	nd	0.43	nd	nd	nd	nd	2.3	3.1	7.9	5.4	3.7	4.0	6.6	16	6.8	23	nd

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EXPOSURE LEVEL	0% LEVEL (CONTROL)							12.5% LEVEL					25% LEVEL			50% LEVEL		
SAMPLING DAY	0	0-dup.	3	3-dup.	7	14	21	3	7	14	21	28	3	7	14	3	7	14
ANALYTE	ng/g	ng/g	ng/g	ng/g	ng/g	ng/g	ng/g	ng/g	ng/g	ng/g	ng/g	ng/g	ng/g	ng/g	ng/g	ng/g	ng/g	ng/g
2,7-Dimethylphenanthrene	nd	nd	nd	nd	nd	nd	nd	nd	0.51	4.8	1.1	0.78	0.99	7.7	3.9	2.2	13	nd
3,9-Dimethylphenanthrene	nd	nd	1.2	1.3	0.96	0.83	nd	9.6	7.0	37	19	9.4	19	23	72	29	112	26
1,6/2,5/2,9-Dimethylphenanthrene	nd	nd	0.62	nd	nd	nd	nd	6.5	5.0	28	7.8	3.2	13	15	39	22	89	14
1,7-Dimethylphenanthrene	nd	nd	0.37	nd	nd	nd	nd	2.1	1.9	8.3	4.7	2.2	4.2	5.7	15	7.0	26	4.7
1,9/4,9-Dimethylphenanthrene	nd	nd	nd	nd	nd	nd	nd	2.1	1.4	9.9	5.1	2.4	5.6	6.1	15	9.6	35	6.3
1,5-Dimethylphenanthrene	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	5.6	1.7	nd	nd	nd	nd	nd	nd
1,8-Dimethylphenanthrene	nd	nd	nd	nd	nd	nd	nd	2.1	2.5	6.0	4.5	4.0	3.3	7.8	20	4.9	19	6.0
1,2-Dimethylphenanthrene	nd	nd	nd	nd	nd	nd	nd	0.95	1.0	3.5	1.7	1.1	1.3	2.7	6.7	2.7	11	3.3
9,10-Dimethylphenanthrene	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	0.36	nd	nd	nd	nd	1.14	nd	nd
Fluoranthene	2.6	1.6	0.48	0.86	0.44	0.87	4.0	4.2	2.8	12	6.0	0.27	7.2	8.9	19	15	53	7.9
1,2-Dimethyldibenzothiophene	nd	nd	nd	nd	nd	nd	nd	nd	nd	0.64	0.67	nd	nd	nd	1.2	0.61	1.8	nd
C2-DIBENZOTHIOPHENES, est.	nd	nd	19	15	16	13	nd	30	24	94	78	50	63	71	177	99	240	77
Pyrene	nd	nd	0.22	0.33	0.14	nd	2.7	1.7	1.5	7.2	3.8	0.21	3.8	2.8	11	8.1	27	11
1,2,8-Trimethylphenanthrene	nd	nd	0.21	nd	0.12	nd	nd	1.4	4.4	10	6.0	5.3	2.4	4.0	26	5.8	20	6.7
C3-PHENANTHRENES, est.	nd	nd	11	19	12	13	nd	56	117	363	200	150	90	176	723	194	684	264
Benzanthracene	7.1	4.6	nd	nd	nd	nd	18	nd	2.0	3.6	2.0	1.5	1.9	1.6	6.2	1.9	9.8	13
Chrysene	nd	nd	nd	nd	nd	nd	nd	2.3	3.0	12	9.0	4.9	7.1	8.4	17	8.4	31	30
Benzo(b)fluoranthene	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	1.6	1.1	nd	0.59	nd	0.91	3.0	4.3
Benzo(k)fluoranthene	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	0.58	0.19	nd	0.52	nd	0.40	0.78	1.2
Benzo(a)pyrene	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	0.21	nd	nd	nd	nd	nd	nd	nd
Indenopyrene	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Dibenz(a,h)anthracene	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Benzo(g,h,i)perylene	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd

The Department of the Interior Mission

As the Nation's principal conservation agency, the Department of the Interior has responsibility for most of our nationally owned public lands and natural resources. This includes fostering sound use of our land and water resources; protecting our fish, wildlife, and biological diversity; preserving the environmental and cultural values of our national parks and historical places; and providing for the enjoyment of life through outdoor recreation. The Department assesses our energy and mineral resources and works to ensure that their development is in the best interests of all our people by encouraging stewardship and citizen participation in their care. The Department also has a major responsibility for American Indian reservation communities and for people who live in island territories under U.S. administration.

The Minerals Management Service Mission

As a bureau of the Department of the Interior, the Minerals Management Service's (MMS) primary responsibilities are to manage the mineral resources located on the Nation's Outer Continental Shelf (OCS), collect revenue from the Federal OCS and onshore Federal and Indian lands, and distribute those revenues.

Moreover, in working to meet its responsibilities, the **Offshore Minerals Management Program** administers the OCS competitive leasing program and oversees the safe and environmentally sound exploration and production of our Nation's offshore natural gas, oil and other mineral resources. The MMS **Royalty Management Program** meets its responsibilities by ensuring the efficient, timely and accurate collection and disbursement of revenue from mineral leasing and production due to Indian tribes and allottees, States and the U.S. Treasury.

The MMS strives to fulfill its responsibilities through the general guiding principles of: (1) being responsive to the public's concerns and interests by maintaining a dialogue with all potentially affected parties and (2) carrying out its programs with an emphasis on working to enhance the quality of life for all Americans by lending MMS assistance and expertise to economic development and environmental protection.