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Monooxygenase induction and lethality as endpoints in aquatic toxicology

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MONOOXYGENASE INDUCTION AND LETHALITY AS ENDPOINTS IN AQUATIC TOXICOLOGY

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DEDICATION

This dissertation is dedicated to my parents, Edwin and Dorothy, to my wife, Monica, and to my daughter, Liana. Without their help, guidance, love, and support this work would never have been completed. Thanks for everything.

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ABSTRACT

Spot, *Leiostomus xanthurus*, were exposed to suspended sediments (≈20 mg/L) contaminated with polycyclic aromatic hydrocarbons (PAH) in a laboratory flow-through system to evaluate the applicability of hepatic ethoxyresorufin O-deethylase (EROD) induction as an indicator of PAH exposure. PAH sources tested were coal-tar creosote (CTC), a low molecular weight fraction of creosote (LMWF), and a high molecular weight fraction of creosote (HMWF).

A standard 96-h acute toxicity test was conducted to ensure that PAH concentrations tested in induction studies were sub-acutely toxic. The 96-h LC50 for spot was 1740 μg PAH/L (95% confidence interval=1480-2060 μg PAH/L). The lowest concentration producing an observable effect in 96 h was 560 μg PAH/L; no effects were observed for spot exposed to 250 μg PAH/L for 96 h.

Induction of hepatic EROD activity occurred rapidly in fish exposed to high environmentally realistic concentrations of CTC or the HMWF, but not the LMWF. Maximal induction (30-fold) occurred in fish exposed for 48 h to 150 μg PAH/L. Induction was concentration-dependent up to 150 μg PAH/L; at 320 μg PAH/L induction was 14-fold. EROD activity decreased upon further exposure; by day 7, EROD activity was not significantly different than that on day 0. EROD activity in fish exposed to 16 μg PAH/L was not consistently higher than that in control fish.

Spot exposed to at least 70 μg PAH/L from CTC or the HMWF experienced severe fin erosion, epidermal lesions, and mortality beginning a few days after maximal EROD induction occurred. No relationship between EROD induction and whole animal responses is implied, only that EROD induction did precede any high order effects.

These results indicate complications to the use of EROD activity as a sensitive, reliable indicator of PAH exposure. The toxicity of CTC may inhibit or interfere with continued induction of EROD activity, but neither the toxicity nor inducing capability is associated with the LMWF. The lack of exposure-dependent EROD induction indicate there could be difficulties in interpreting field studies, where fish have unknown exposure histories.

Key Words: Acute toxicity; Biomonitoring; Creosote; Cytochrome P-450; EROD activity; *Leiostomus xanthurus*; Mixed-function oxygenase; PAH; Spot

MONOOXYGENASE INDUCTION AND LETHALITY AS ENDPOINTS IN AQUATIC TOXICOLOGY

GENERAL INTRODUCTION

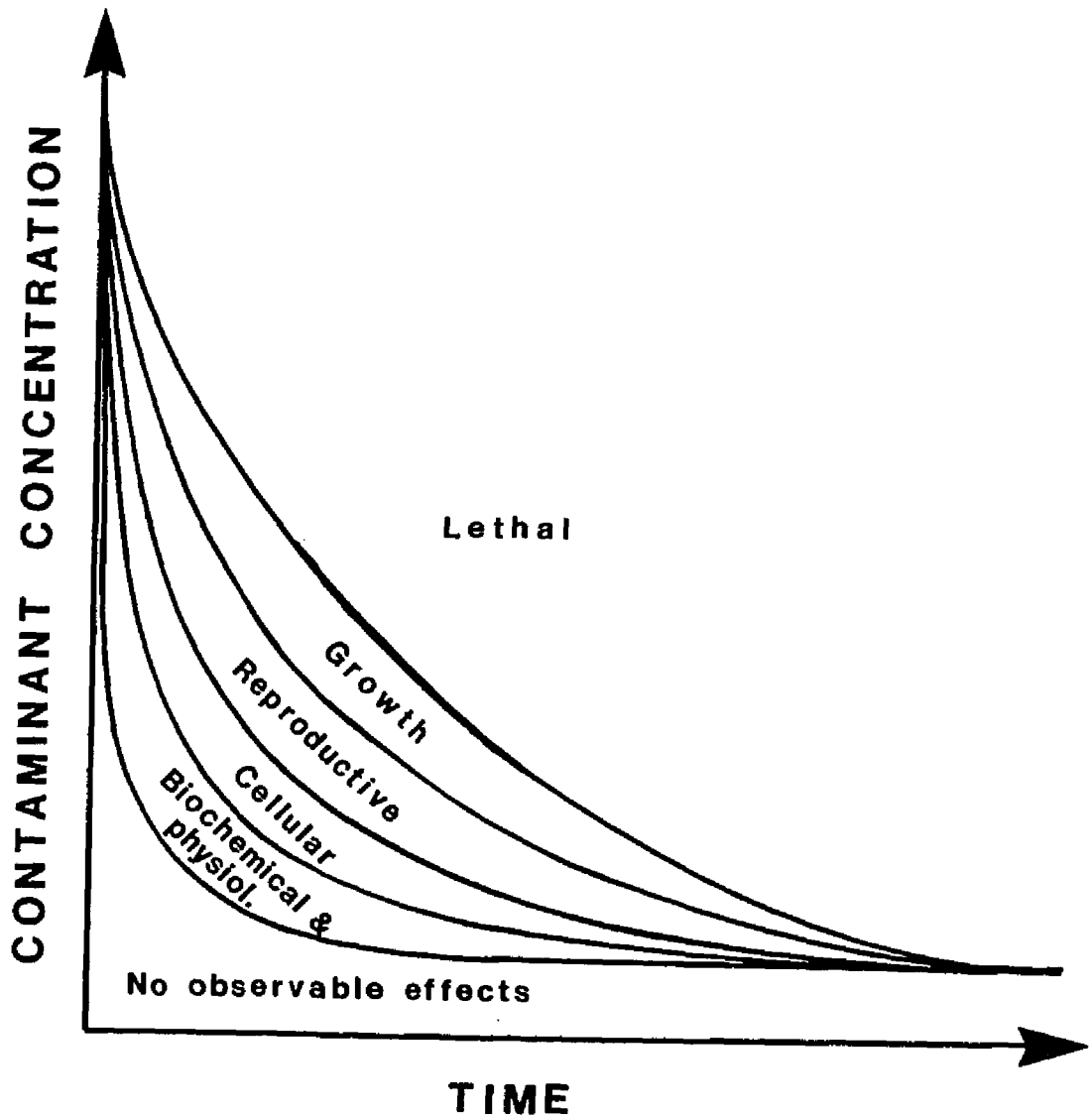
One of the most basic principles in toxicology is that all substances are toxic if given in sufficient quantity (Doull and Bruce, 1986). This concept is adequate in mammalian toxicology because isolated doses of a chemical are often administered in order to elucidate mechanisms of toxicity and establish means for the prevention and treatment of toxic doses. In environmental and aquatic toxicology the effects of continuous administration of chemicals must be considered. Time becomes an essential factor in establishing the relationship between the concentration of a chemical and the resulting effects. Exposure can be defined as an integration of the chemical concentration available to an organism over time. The response of an organism is therefore a function of exposure, instead of dose.

Whole animal responses, or high order effects, have typically been used as endpoints in aquatic toxicology. Whole animal responses include mortality, gross abnormalities, behavioral changes, and changes in growth rate (Mehrle and Mayer, 1985). These responses may not be particularly relevant to environmental situations, because severe impairment decreases an animal's ability to escape predators. Impaired animals may therefore be misrepresented in field collections.

The processes that occur upon exposure to chemicals can be considered as a continuum of effects (Figure 1). For most toxic responses, a threshold exposure exists, below which no effects are observable and the animal continues to exist without any change in homeostasis. At extremely high contaminant concentrations, disruption

FIGURE 1

Hypothetical continuum of chemical effects including time as a factor.


CONTAMINANT CONCENTRATION
Lethal
Growth
Reproductive
Cellular
Biochemical & physiol.
No observable effects
TIME

of physiological processes can occur so rapidly that mortality occurs essentially immediately. Exposures exceeding the threshold result in metabolic changes that can eventually affect cellular function, reproductive success, growth, and survival. Impaired reproduction, growth, or survival could, in turn, affect the structure of a population and ultimately the structure of a community or ecosystem.

An additional concern in environmental toxicology is the assessment of biological responses to complex chemical mixtures, and not necessarily a mixture of only one class of compounds. Polluted areas tend to be contaminated with more than one chemical, as well as more than one class of chemical. A chemical class is a group of compounds of the same basic nature. Polycyclic aromatic hydrocarbons (PAH), polychlorinated biphenyls (PCB), and heavy metals are examples of chemical classes. The presence of more than one chemical, whether they are of the same class or not, can result in interactions, both within and external to an organism, that affect an organism's response to the chemicals. Interactions can be synergistic, additive, or antagonistic (Marking, 1985).

Biochemical Indicators of Chemical Exposure

The first detectable effects that occur in response to chemical stresses are biochemical changes. Some important criteria involved in selecting a biochemical indicator of chemical exposure are: 1) the response should be sensitive to environmentally realistic levels of chemical stress in an exposure-dependent manner, 2) the response should not be sensitive to other environmental stresses, 3) the response should be detectable before any high order effects occur, 4) the response should indicate that high order effects will occur upon continued exposure, 5) the response should be applicable to a large number of chemicals, and 6) the response should be easy and inexpensive to measure (Neff, 1985).

Several metabolic processes have been considered for their utility in assessing exposure to chemical stress. These include cytochrome P-450 dependent monooxygenases (Payne, 1976), metallothioneins (Roch *et al.*, 1982), δ-aminolevulinic acid dehydratase (Hodson *et al.*, 1977), and stress (heat shock) proteins (Bradley, 1990). Each of these biochemical systems does not necessarily provide an appropriate endpoint for assessing exposure to all chemicals, and their applicability and relevance to assessing effects on the organismal, population, and community levels has yet to be established.

If the purpose is to detect a biochemical response to one particular chemical or chemical class, then the applicability of the response to the chemical or chemical class must first be established in the laboratory. If the response is applicable, then other parameters that may affect the response should be investigated. Other parameters that affect biochemical responses to contaminants may be of a physical, chemical, or biological nature. These parameters are not always easy to distinguish and may interact to affect the response.

Temperature, for instance, may affect the response, but the mechanism may be multifaceted. Both the aqueous solubility of a chemical (May *et al.*, 1978) and membrane fluidity (Hazel and Prosser, 1974) are temperature dependent. Membrane fluidity affects both the uptake of chemicals into a cell (Spacie and Hamelink, 1985) and the activity of membrane-bound enzymes (Ingelman-Sundberg, 1986). Therefore, the mechanism by which temperature affects the response is not necessarily clear.

Particulate matter provides another example of how basic parameters can interact to affect a biochemical response. The chemicals of interest may adsorb to sediment to different degrees and not all be biologically available to the same extent (Varanasi *et al.*, 1985). Benthic organisms and demersal fish may have greater susceptibility to sediment-bound pollutants than pelagic organisms (Adams, 1987). Fish

that forage through contaminated sediment stand to have increased risks compared to pelagic fish that remain in the water column.

The biochemical response to a chemical may be affected by other chemicals in the environment or the organism. These other chemicals may be additional contaminants or natural components. An additional contaminant that disrupts membrane function can affect the uptake rate of the chemical of interest (Viarengo, 1985). Once inside the cell, additional contaminants may interfere directly or indirectly with the biochemical response being measured. Natural chemicals in the environment, such as the salts present in seawater or dissolved organic acids, may affect the biochemical response through interactions with the contaminant (May *et al.*, 1978). Normally present endogenous compounds may interfere with the response of the biochemical system to xenobiotic chemicals (Stegeman *et al.*, 1982). Endogenous compounds that are produced in response to other stressors, such as corticosteroids (Davis and Parker, 1986; Robertson *et al.*, 1987; Robertson *et al.*, 1988), may also interfere with the biochemical system under study.

Cytochrome P-450 Monooxygenase

A biochemical response that has received substantial attention as a possible indicator of chemical exposure is the induction of cytochrome P-450 dependent monooxygenase (MO) activity. Cytochrome P-450 (P-450) is a family of enzymes that catalyze a wide range of biological reactions involving a diverse group of organic substrates (Coon and Persson, 1980). Many compounds that induce P-450 are also MO substrates (Guengerich, 1988). Induction of MO activity by organic xenobiotic compounds has led several scientists to suggest that MO activity may be used to monitor polluted environments (Payne and Penrose, 1975; Kurelec *et al.*, 1977; Stegeman, 1978).

P-450 is part of an electron transport chain found in both prokaryotes and eukaryotes (Hodgson, 1979). In vertebrates, there are

two basic systems. The mitochondrial P-450 system catalyzes reactions with endogenous substrates, most notably steroid hormones (Hodgson, 1979). The microsomal P-450 system, located in the endoplasmic reticulum, catalyzes reactions with organic xenobiotics as well as endogenous compounds, including bile acids, bilirubin, and fatty acids (Ahmad, 1979). P-450 is most abundant in the liver, but has been found in most tissues studied to date (Guengerich, 1988).

The P-450 electron transport chain uses molecular oxygen as the final electron acceptor. Unlike the respiratory electron transport chain, the atoms of the oxygen molecule end up in two different molecules: water and an organic substrate (Ortiz de Montellano, 1986). Thus, P-450 dependent monooxygenases are often referred to as a mixed-function oxygenases, or less precisely, mixed-function oxidases.

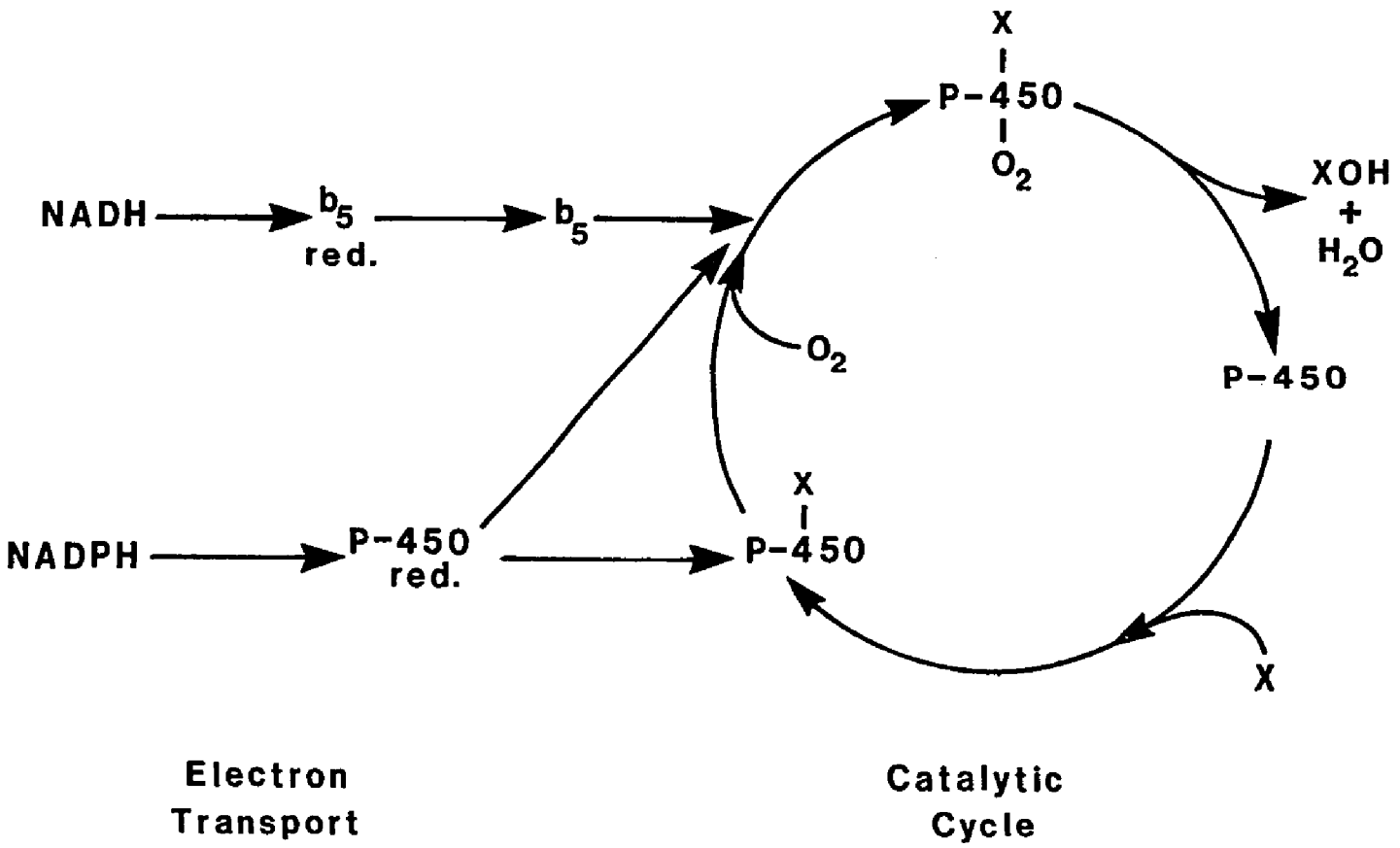
Mechanism of P-450 Catalysis

P-450 is capable of transferring only one electron at a time through the oxidation and reduction of its single iron atom containing protoporphyrin IX prosthetic group (McMurry and Groves, 1986). This is the site where a molecule of oxygen is bound, reduced, and activated. Another area of the P-450 molecule serves to bind a substrate thus forming a ternary complex (Miwa and Lu, 1986). Differences in the substrate binding sites of various forms of P-450 account for its substrate diversity.

The catalytic sequence for P-450 dependent monooxygenation begins with the binding of a substrate by P-450 (Figure 2). This is followed by the transfer of an electron from cytochrome P-450 reductase to the P-450-substrate complex. Molecular oxygen is then bound, forming a ternary complex and a second electron is transferred to the complex. The second electron can come from either cytochrome P-450 reductase or cytochrome b_5. Next, the oxygen-oxygen bond is broken and one oxygen

FIGURE 2

Catalytic sequence and microsomal electron transport chain (adapted from Peterson and Prough, 1986 and Estabrook *et al.*, 1978). b_5 = cytochrome b_5, b_5 red. = cytochrome b_5 reductase, P-450 = cytochrome P-450, P-450 red. = cytochrome P-450 reductase, X = organic xenobiotic.


NADH
b5
red.
b5
NADPH
P-450
red.
P-450
X
P-450
O2
XOH
+
H2O
O2
P-450
X
X
Electron
Transport
Catalytic
Cycle

atom is incorporated into the substrate while the other atom forms water. Finally, P-450 and the oxygenated substrate dissociate (Ortiz de Montellano, 1986). The exact nature of the reactive oxygen species is unknown (McMurry and Groves, 1986).

Reactions Catalyzed by P-450

The mechanism discussed above describes the process by which monooxygenation of organic xenobiotics occurs and most of the reactions involving P-450 are oxidative. P-450 is, however, also capable of catalyzing reduction reactions. When oxygen tension is low, reducible substrates, such as alkyl halocarbons and azo compounds, can compete with molecular oxygen as the terminal electron acceptor (Ortiz de Montellano, 1986).

The following types of oxidative reactions can be catalyzed by P-450: aliphatic hydroxylation, aromatic oxidation, alkene epoxidation, nitrogen dealkylation, oxidative deamination, oxygen dealkylation, nitrogen oxidation, oxidative desulfuration, oxidative dehalogenation, and oxidative denitrification (Wislocki *et al.*, 1980). The following types of reductive reactions can be catalyzed by P-450: nitro reduction, azo reduction, tertiary amine N-oxide reduction, arene oxide reduction, and reductive dehalogenation (Wislocki *et al.*, 1980).

Multiplicity and Induction of P-450

In vertebrates, and at least some invertebrates (Quattrochi and Lee, 1984), P-450 is not a single entity, but exists as multiple forms that are individual gene products (Nebert *et al.*, 1989). Based on homology, the forms are grouped into subfamilies and families. Not all forms are induced by the same substrates or catalyze the same reactions, but there is considerable overlap in both inducibility and catalytic function (Black and Coon, 1986).

The P-450 I gene family is induced by certain PAH, including benzo[a]pyrene (BaP) and 3-methylcholanthrene (3-MC), as well as other planar aromatic organic compounds such as certain PCB, polychlorinated dibenzodioxins (PCDD), and polychlorinated dibenzofurans (PCDF). The P-450 I gene family has only one subfamily (P-450 IA). Hepatic IA cytochromes have been referred to by many different names including P-448 (general), P-450d (rat), P-450 LM_4 (rabbit), P_3-450 (mouse), and P-450E (fish), depending on the animal studied, the laboratory where the research was done, and the purification and characterization method used (Negishi and Nebert, 1979; Imai *et al.*, 1980; Guengerich *et al.*, 1982; Klotz *et al.* 1983). The use of "P-450" throughout the remainder of this treatise will refer to PAH-inducible forms of cytochrome P-450, particularly cytochrome P-450 IA1.

Induction of P-450 is believed to be regulated by a cytosolic protein, termed the Ah receptor (Ah stands for arylhydrocarbon). After an inducer binds to the receptor, the complex can activate P-450 genes. The exact process involved in activation is unknown (Bresnick, 1980; Eisen, 1986; Ioannides and Parke, 1990). Activation leads to increased transcription and synthesis of P-450 apoproteins, the Ah receptor, and some other enzymes (Ioannides and Parke, 1990). The P-450 apoprotein interacts with heme to form the holoenzyme.

Measurement of MO Activity

P-450 is frequently measured in terms of its catalytic activity. The monooxygenation of aromatic hydrocarbons by P-450 usually results in hydroxylation, although epoxidation can result because epoxides are intermediates in the hydroxylation process (Guroff *et al.*, 1967). As the catalyst for aromatic oxidations, P-450 is referred to as arylhydrocarbon hydroxylase (AHH). This reaction has classically been measured with BaP, so the enzyme has often been referred to as benzo[a]pyrene hydroxylase (BaPH).

Ethoxyresorufin (7-ethoxy-3H-phenoxazine-3-one) is the most specific substrate known for cytochrome P-450 IA1 (Ioannides and Parke, 1990). Dealkylation of ethoxyresorufin to form resorufin (Figure 3) is easily measured by spectrophotometric (Klotz *et al.*, 1984) or spectrofluorometric (Burke and Mayer, 1974) methods. As the catalyst for this dealkylation reaction, P-450 is referred to as ethoxyresorufin O-deethylase (EROD).

Metabolism of PAH

P-450 is an essential enzyme in the metabolism of PAH. Metabolism of PAH occurs in two phases (Figure 4) with each phase leading to products that are more polar and therefore, more water soluble and more readily excreted than the original substrates (Jakoby, 1980). Phase I reactions are catalyzed by P-450 and yield phenols and epoxides (Buhler and Williams, 1989). Phenols can be further metabolized by P-450 to yield diols and quinones. Many Phase I products undergo further metabolism, or Phase II reactions, before excretion (Foureman, 1989). Phase II involves the formation of readily excretable metabolites such as sulphates, quinones, glucuronides, and glutathione conjugates. For PAH, Phase II also includes the formation of diol epoxides, some of which are the ultimate carcinogenic and mutagenic derivatives of PAH (for reviews see Oesch, 1984; Dipple, 1985; Eisenstadt, 1985; Thakker *et al.*, 1985).

MO Activity as an Indicator of Chemical Exposure

Increases in MO activity following injection of PAH-type inducers have been measured in many species of fish (Buhler and Rasmusson, 1968; Pohl *et al.*, 1974; Chambers and Yarbrough, 1976; Bend *et al.*, 1977; Statham *et al.*, 1978; Elcombe and Lech, 1979; James *et al.*, 1979; Stegeman *et al.*, 1979; Bodine *et al.*, 1985). Induction of the teleost P-450 system has also been measured following exposure to water soluble

FIGURE 3

O-deethylation of ethoxyresorufin.

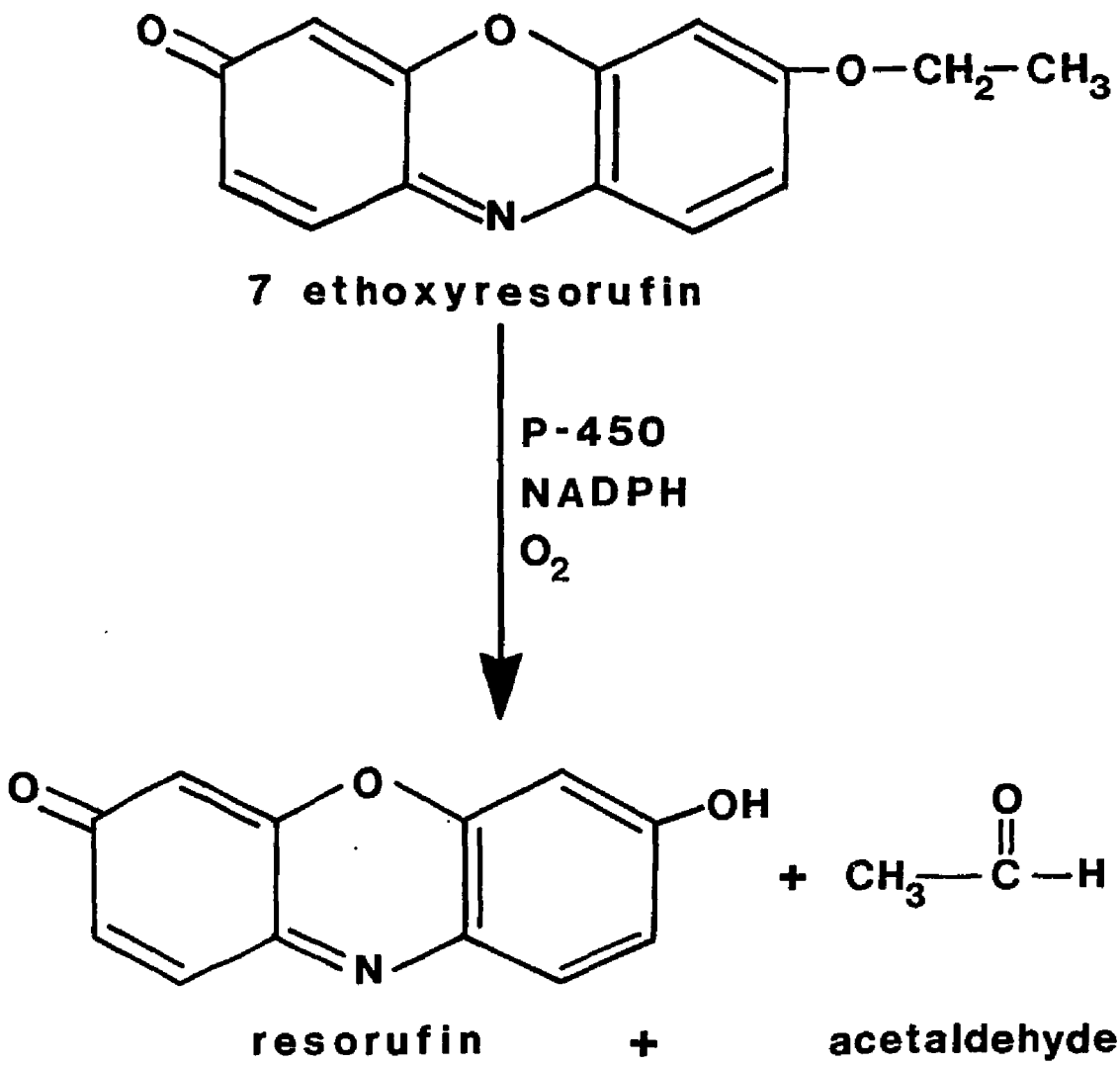
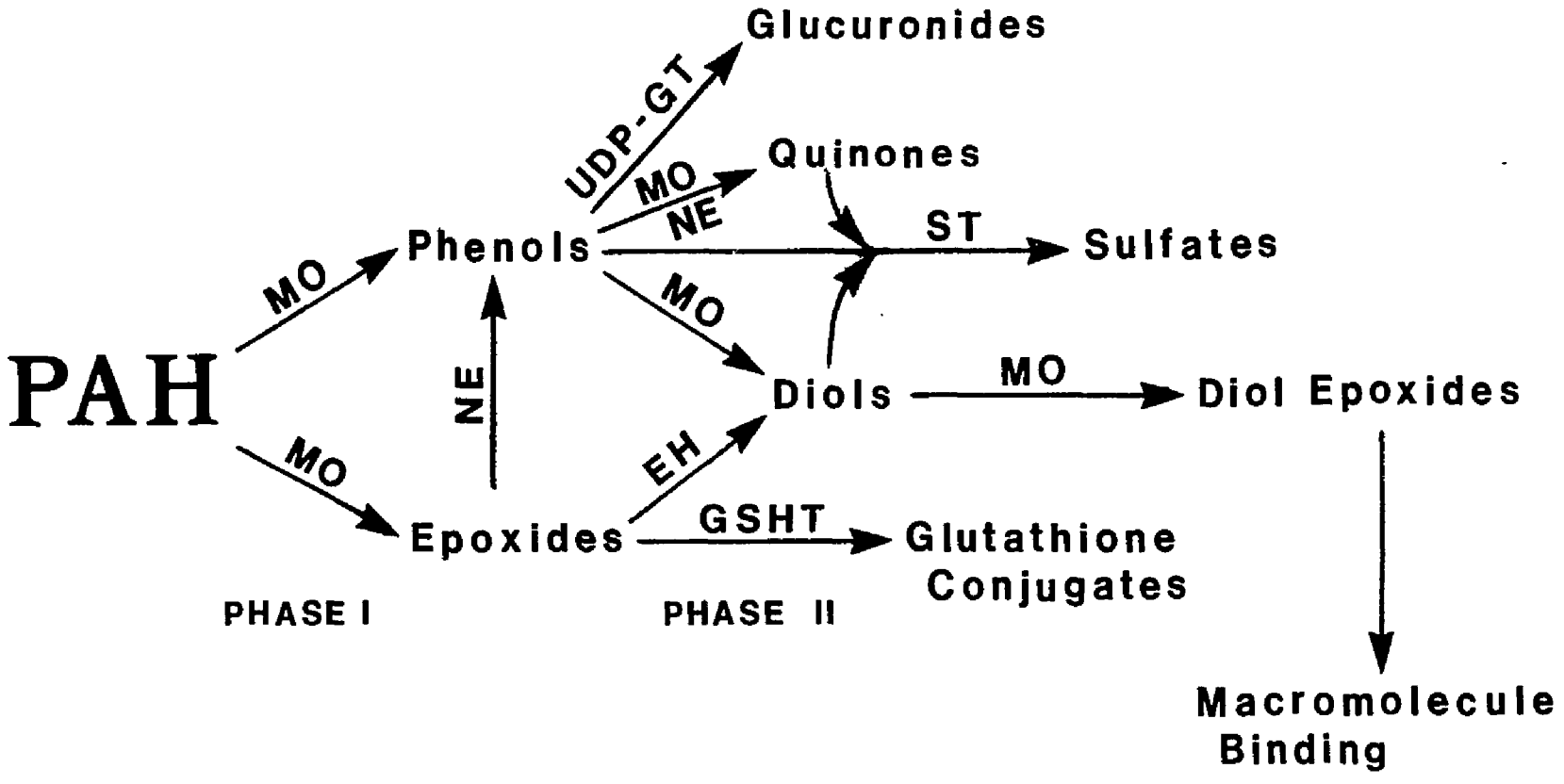

O
O
O—CH2—CH3
N
7 ethoxyresorufin
P-450
NADPH
O2
O
O
OH
N
+
O
CH3—C—H
resorufin
+
acetaldehyde

FIGURE 4

Metabolism of PAH (adapted from Gelboin, 1980). EH = epoxide hydrolase, NE = non-enzymatic, MO = monooxygenase, GSHT = glutathione transferase, ST = sulfotransferase, UDP-GT = UDP-glucuronosyltransferase.


PAH
MO
MO
Phenols
NE
NE
UDP-GT
Glucuronides
MO
Quinones
ST
Sulfates
MO
Diols
MO
Diol Epoxides
EH
Epoxides
GSHT
Glutathione
Conjugates
PHASE I
PHASE II
Macromolecule
Binding

fractions of petroleum products, dispersed oils, or individual PAH in solution (Payne and Penrose, 1975; Kurelec *et al.*, 1977; Walton *et al.*, 1978; Payne and Fancey, 1982; Jensen and Knudsen, 1983; Goddard *et al.*, 1987; Steadman *et al.*, 1991a).

Because PAH and other hydrophobic contaminants readily adsorb to particulate matter (Gearing *et al.*, 1980), the response of MO activity to sediment-bound contaminants has also been investigated (Payne and Fancey, 1982; Collier *et al.*, 1986; van der Weiden *et al.*, 1989; Schoor *et al.*, 1991). One method for exposing fish to contaminated sediments is to place fish in an aquarium with sediment layered on the bottom. This is a difficult exposure to quantify because the concentration of the contaminant on the sediment and the amount of sediment in suspension may change over time. An alternative exposure method uses a continuous flow of contaminated sediment in suspension (Sinnett and Davis, 1983). A flow-through system has been used to expose bivalves to contaminated sediments (Pruell *et al.*, 1987).

The results of many field studies (Payne and Penrose, 1975; Payne, 1976; Burns, 1976; Kurelec *et al.*, 1977; Spies *et al.*, 1982; Kezic *et al.*, 1983; Davies *et al.*, 1984; Payne *et al.*, 1984; Förlin *et al.*, 1985; Luxon *et al.*, 1987; Addison and Edwards, 1988; Van Veld *et al.*, 1990) suggest that induction of MO activity may be a sensitive, reliable biochemical indicator of exposure to many organic pollutants (for a review see Payne *et al.*, 1987), despite several major pitfalls revealed in laboratory studies. Several researchers (Nava and Engelhardt, 1982; Goddard *et al.*, 1987; Steadman *et al.*, 1991a) have found that induction of MO activity in fish exposed to PAH was not dose-dependent, which casts doubt on the suitability of P-450 induction as a quantitative indicator of contamination in the environment. Differences between the sexes and seasonal variations in MO activity contribute a large degree of variance to basal activity values (Walton *et al.*, 1983; George *et al.*, 1990). Temperature has generally been shown to affect basal MO

activity and the induction process (Stegeman, 1979; Koivusaari, 1983). Competing endogenous substrates (Stegeman *et al.*, 1982; Walton *et al.*, 1983) and heavy metals (Fair, 1986; George and Young, 1986) can interfere with the induction response. At present, there is no information concerning the effect of salinity on induction.

Creosote

Creosote is a distillate of coal tar made by high-temperature carbonization of bituminous coal (Nestler, 1974) resulting in a complex mixture of PAH. Creosote is not defined in terms of its chemical composition, but rather its physical and chemical characteristics, which are dependent upon the distillation temperature, carbonization temperature, and the type of equipment used (Nestler, 1974). Creosote is usually mixed with coal tar or heavy petroleum prior to its use as a wood preservative (Webb, 1980). The toxic properties of PAH account for creosote's value as a wood preservative. Unlike most pesticides, which are widely dispersed during application, creosote is pressure impregnated into wood inside closed systems (Webb, 1980).

Straight run creosote (distillation range 175-450°C) contains over 160 compounds (Nestler, 1974). Over 90% of these compounds are PAH and heterocyclic analogs (hereafter use of "PAH" will include heterocyclic compounds), with minimal aliphatic substitution (Nestler, 1974). In contrast, crude and refined oils typically contain 10-30% PAH with a fair degree of aliphatic substitution (Neff and Anderson, 1981). Typical analyses of creosote reveal that only 17 compounds are present in quantities greater than 1% of the total PAH (Table 1); BaP represents approximately 0.1% of the total PAH.

There are over 700 creosote-contaminated sites throughout the United States (Mueller *et al.*, 1989). Several of the country's major waterways, such as Puget Sound, Chesapeake Bay, and Pensacola Bay, have tributaries that are heavily contaminated with creosote (Malins *et al.*,

TABLE 1

THE MOST ABUNDANT COMPOUNDS IN CREOSOTE[a]

Compound	Molecular Weight	% of Total
Phenol	94	1.49
m/p-cresol	108	1.26
Indene	116	2.01
Naphthalene	128	26.1
2-methylnaphthalene	142	4.17
1-methylnaphthalene	142	1.71
Biphenyl	154	1.03
Acenaphthene	154	5.50
Fluorene	166	4.24
Carbazole	167	1.27
Dibenzofuran	168	3.27
Phenanthrene	178	10.0
Anthracene	178	3.07
Cyclopenta[d,e,f]phenanthrene	190	1.51
Fluoranthene	202	5.58
Pyrene	202	4.85
Chrysene	228	1.21

[a] Compounds greater than 1% of the total PAH. Courtesy of Koppers Co., Pittsburgh, PA.

1985; Merrill and Wade, 1985; Elder and Dresler, 1988). Minimal amounts of creosote leach from creosoted timber (Baileys and Webb, 1987). Most input of creosote to the environment is from process wastewater, leakage from dump sites and storage tanks, and spills (Merrill and Wade, 1985).

The physical properties of creosote reflect the properties of its PAH constituents. The aqueous solubilities of PAH are low and decrease with increasing molecular weight (Eganhouse and Calder, 1976; May *et al.*, 1978). Solubility of naphthalene in distilled water at 25°C is usually estimated to be about 30 mg/L (Eganhouse and Calder, 1976; May *et al.*, 1978). Solubility of the two monomethylated naphthalenes is approximately 25 mg/L (Eganhouse and Calder, 1976). The solubility of phenanthrene is only about 1 mg/L (Eganhouse and Calder, 1976; May *et al.*, 1978) and that of chrysene about 0.002 mg/L (May *et al.*, 1978). Upon input to the environment, PAH preferentially forms surface slicks or sorbs to sediment and suspended particulate matter rather than dissolving in the aqueous phase (Gearing *et al.*, 1980).

Spot

Spot, *Leiostomus xanthurus* Lacépède, is an abundant, commercially important, catadromous sciaenid fish found along the Atlantic and Gulf coasts of the United States (McCambridge and Alden, 1984). Spot spawn in near-shore waters during winter (Dawson, 1958). Young-of-the-year and yearling spot migrate to estuarine nursery areas in spring and leave by winter (Chao and Musick, 1977). Spot mature sexually near the end of their second year (Dawson, 1958). Spot reside in estuarine areas during important, formative life stages. The land surrounding estuaries tends to be highly urbanized and industrialized, which leads to higher concentrations of anthropogenic pollutants in estuaries than in offshore habitats (O'Connor and Huggett, 1988).

Spot predominantly feed on infaunal and epibenthic polychaetes as evidenced by stomach content analysis of feral fish (Chao and Musick,

1977). The morphology of the mouth enables spot to be particularly adept at feeding on benthic infauna (Chao and Musick, 1977). During feeding, spot scoop up sediment with their lower jaw and expel the sediment through the mouth and over the gills (personal observation of aquarium held fish). Sediment and organic matter is typically found in the stomach of feral spot (Chao and Musick, 1977). Therefore, ingestion of or contact with contaminated sediment may represent significant routes of exposure for spot.

Objectives

The major objectives of this research project were:

1. To develop and evaluate a flow-through system for exposing fish to suspended, contaminated sediments.

2. To determine the short-term toxicity of suspended, creosote-contaminated sediments to spot.

3. To evaluate hepatic EROD activity of spot as a sublethal indicator of exposure to suspended, creosote-contaminated sediments.

SHORT TERM TOXICITY OF CREOSOTE-CONTAMINATED SEDIMENTS

INTRODUCTION

To characterize the effects of a chemical or mixture on an organism, a first step is to determine the acute or short-term toxicity. Short term toxicity in fish is often defined in terms of lethality. The concentration of a chemical that causes mortality in 50% of a test population is termed the median lethal concentration (LC50). This term must additionally be identified by the length of the exposure over which the toxic action is exerted, such as the 96-h LC50.

Acute toxicity studies with several fish species indicate that creosote is only moderately toxic to fish (Bionomics, 1974a, b; Borthwick and Patrick, 1982). Many pesticides are acutely toxic to fish at concentrations below 100 μg/L (Nimmo, 1985), whereas the lowest 96-h LC50 reported for fish exposed to creosote was 200 μg/L (Bionomics, 1974a). There are no known studies that have examined the influence of particulate matter on creosote toxicity.

Bioavailability of Sediment-Bound Contaminants

In order for a chemical to cause toxicity, the chemical must first enter the organism, or in other words, the chemical must be bioavailable. If a chemical is bioavailable, then an organism should respond to the chemical over some range of concentrations (Rodgers *et al.*, 1987). In fish, uptake can occur via several routes: 1) directly from water through the integument or gills, 2) from contaminated food via absorption in the digestive tract, and 3) from contaminated sediments (Spacie and Hamelink, 1985). The route of uptake from

contaminated sediments may either be from desorption of the chemicals into water, which are then bioavailable, or through ingestion of contaminated particles (Opperhuizen and Stokkel, 1988). The relative importance of these various routes of exposure remains a topic of considerable debate and no single route may be most significant for all chemicals.

The bioavailability of sediment-bound contaminants is controlled by several factors. Partitioning of a hydrophobic chemical onto sediment is a function of the organic carbon content of the sediment, the aqueous solubility of the chemical, and the concentration of suspended sediment (Rodgers *et al.*, 1987). Anderson *et al.* (1987) consider the organic carbon content of the sediment to be the most significant factor. If the range of organic carbon content in natural suspended or bed sediments is narrow (Rodgers *et al.*, 1987) and the aqueous solubility of a given compound is a constant, the most variable factor controlling bioavailability would be the concentration of suspended sediment.

Suspended sediment concentration is extremely variable. There is a systematic increase in suspended sediment concentration from surface to bottom (Patten *et al.*, 1966). There is a systematic decrease in suspended sediment concentration from rivers to offshore (Schubel, 1977). Superimposed on these systematic variations is extreme temporal variation (Schubel, 1977).

If the concentration of suspended particulate matter is sufficiently high, then contaminated sediments can serve as a substantial source of hydrophobic chemicals (Opperhuizen and Stokkel, 1988). Spot prevented from having direct contact with PCB-contaminated sediment do not significantly accumulate PCB (Rubinstein *et al.*, 1984). If spot are allowed to disturb the sediment and swim among the suspended particles, then significant accumulation of PCB occurs (Rubinstein *et al.*, 1984).

Bioavailability of contaminants from sediments is also related to the aqueous solubility of the contaminants (Zitko, 1974). The aqueous solubilities of PAH decrease with increasing molecular weight and can differ by several orders of magnitude for PAH with two aromatic rings and PAH with five aromatic rings (May *et al.*, 1978). When sorbed to sediment, PAH with different aqueous solubilities are bioavailable to different degrees (McCain *et al.*, 1978; Varanasi and Gmur, 1981). Low molecular weight PAH are more soluble and therefore more bioavailable than high molecular weight PAH (Varanasi and Gmur, 1981). This relationship between solubility and bioavailability may not be true when uptake is via ingestion.

Substituent groups can affect the solubility of a compound and therefore the bioavailability of the compound. Acridine is more soluble than its strictly hydrocarbon analog, anthracene, on account of the nonbonding pair of electrons on the nitrogen atom (Wingrove and Caret, 1981). The bioavailability of acridine is only slightly affected by the presence of sediments (Southworth *et al.*, 1979). On the other hand, alkyl and halide substituents decrease the solubility of a compound (Wingrove and Caret, 1981). The bioavailability of chlorinated hydrocarbons can be greatly affected by the presence of sediment (Eaton *et al.*, 1983).

The influence of particles on bioavailability depends on several factors. The aqueous solubility of the contaminant controls both the partitioning onto particles and desorption rates (Opperhuizen and Stokkel, 1988). Partitioning of contaminants to sediments can serve to reduce the effective concentration of contaminants, but at the same time the sediments serve as a sink for the contaminants (McElroy *et al.*, 1989). Particle type (Anderson *et al.*, 1987) and amount in suspension (Opperhuizen and Stokkel, 1988) are also extremely important. An increase in the suspended sediment concentration leads to increased

desorption of the contaminant, which then becomes readily bioavailable (Opperhuizen and Stokkel, 1988; McElroy *et al.*, 1989).

Objectives

The objectives of this short-term experiment were:

1. To develop and evaluate a flow-through exposure system in which fish could be exposed to contaminated suspended sediments.
2. To determine if creosote-contaminated sediments in suspension provide a bioavailable source of PAH as evidenced by an acutely toxic response.
3. To determine the 96-h LC50 for spot exposed to suspended creosote-contaminated sediment so that subsequent experiments could test concentrations known to be sub-acutely toxic.

MATERIALS AND METHODS

Fish

Young-of-the-year spot were collected from shoals in the lower York River, VA using a 16-foot semi-balloon otter trawl (1 inch stretched mesh, 1/4 inch mesh liner). Tow duration was usually 5 min at 1-2 knots. Fish showing obvious signs of trawl damage or distress were eliminated from the collection. Fish were transported to the laboratory in 60-gallon plastic trash cans. Water in the trash cans was recirculated using a 12 V bilge pump (Model 800, Rule Industries, Gloucester, MA) and aerated by suction through a "Y" fitting.

Fish were held outside the laboratory in 1000-L tanks receiving York River water at a flow rate of 5 L/min. Stocking density never exceeded 0.4 fish/L. Fish were fed a daily ration of 2% of their body weight/day (Salmon Crumbles #3, Zeigler Bros., Gardners, PA) and the

amount adjusted every two weeks. Growth was observed under these conditions, but was not quantified with this population of fish.

Four weeks before beginning the experiment, 60 spot were transferred into each of four 160-L aquaria in the laboratory (0.4 fish/L). York River water was supplied to each aquarium at a flow rate of 1 L/min. The water temperature was increased 1°C/day from an ambient temperature of approximately 15°C to the test temperature of 20°C. Acclimation at the test temperature lasted two weeks. The heating and holding tanks were vigorously aerated. The photoperiod was 14-h light, 10-h dark. Fish were denied food for 48 h prior to the start of the test.

Sediment Preparation

Reference sediment was collected from the York River, near the Virginia Institute of Marine Science, in approximately 10 m water depth (plankton buoy station; Boesch et al., 1976), using a Smith-McIntyre grab sampler. The sediment was sieved through a 400-μm mesh stainless steel sieve to remove large particles. The sediment was allowed to settle in plastic buckets or glass jars and stored covered at 12°C. Enough sediment was processed at one time for all experiments.

Prior to use, approximately 2 L of sediment were placed in a plastic bucket and resuspended in 1-μm filtered York River water at a dry weight concentration between 0.19 and 0.33 g/mL. The sediment was stirred with a plastic propeller connected to an overhead stir motor (Model 102, Talboys Engineering Corp., Emerson, NJ) to ensure homogeneity. Actual sediment concentration was determined for each resuspended sediment batch from five 5-mL samples that were dried overnight at 60°C. Each sediment batch lasted a few days depending on the amount of sediment added. When not being used the resuspended sediment was stored tightly covered at 12°C.

Sediment Characterization

Sediment grain size was determined by a modification of the method described by Folk (1980). Approximately 30 g wet weight sediment was removed from each of three sediment batches and stored at -20°C until analysis. The samples were thawed, 10 mL of 10% Calgon® added as a dispersant, and sonicated for 5-10 min. Each sample was wet-sieved through a 62.5-μm sieve to separate sand and gravel from silt and clay. The sand and gravel retained on the sieve were dried overnight at 65-75°C and passed through a 2-mm sieve to separate sand from gravel. Gravel, which was retained on the 2-mm sieve, and sand, which passed through the 2-mm sieve were weighed individually.

The silt and clay, which had passed through the 62.5-μm sieve, were thoroughly mixed in a 1-L glass cylinder and allowed to settle overnight. The temperature in the cylinder was measured (this is required to determine the time interval for removing the clay containing sample). The sediment was resuspended with a plunger for at least 20 s. Then, 20 s after stirring, a 20-mL sample was removed from a depth of 20 cm. This sample contained silt and clay. The sediment was then allowed to settle for 122 min and a 20-mL sample was removed from a depth of 10 cm. This sample contained only clay. The two samples were dried overnight at 65-75°C and the dry weights of the samples corrected for the amount of dispersant used. The amount of clay was determined directly from the sample containing only clay. The amount of silt was determined from the sample containing both silt and clay after correcting for the amount of clay.

The percent particulate carbon and nitrogen in the sediment was determined electrolytically using a nitrogen-carbon analyzer (NCA; Model NA-1500, Carlo Erba Strumentazione, Milano, Italy) according to the manufacturer's instructions. A composite sample of three sediment batches (30 mL containing approximately 5 g dry weight sediment) was mixed with an equal volume of 10% HCl and stored at 4°C until analysis.

The sample was dried overnight at 105°C. Between 10 and 30 mg of sediment was weighed into a tin cup and placed in the combustion chamber of the NCA. Analysis of the sample and data acquisition were controlled automatically by a computer program (E.A.G.E.R. 100) that is an integrated part of the nitrogen-carbon analyzer. Only a single determination of percent particulate carbon and nitrogen was made.

Creosote Stock Preparation

Two 18-L glass bottles covered with black plastic were used for the stock suspensions; one for creosote-contaminated sediment and one for reference sediment. A hole was drilled in the side of each bottle near the top. A 4-mm glass tube extended through the hole to the bottom of each bottle so that the suspension could be withdrawn.

The bottle used for the creosote-contaminated sediment received 130.9 g dry weight sediment; the bottle for the reference sediment received 327.3 g. These amounts were determined to yield a final sediment concentration of 20 mg/L based on the set flow rates (see Exposure System, below).

The bottles were then filled with 9 L of 1-μm filtered York River water and stirred with stainless steel propellers driven by overhead motors (Model D226, Fasco Distributing Co., Ozark, MO). Motor speed was controlled by a rheostat (Model L10B, The Superior Electric Co., Bristol, CT) set at 30% power, enough to keep the sediment in suspension. After the sediment had stirred for about 10 min, 26.2 g of creosote (marine grade, coal-tar distillate, Koppers Industries, Pittsburgh, PA) was added to the bottle for the creosote-contaminated sediment (200 mg creosote/g dry weight sediment). The bottles were stirred another 10 min, and then the volume was increased to 18 L. Stock suspensions were stirred continuously and renewed daily.

Exposure System

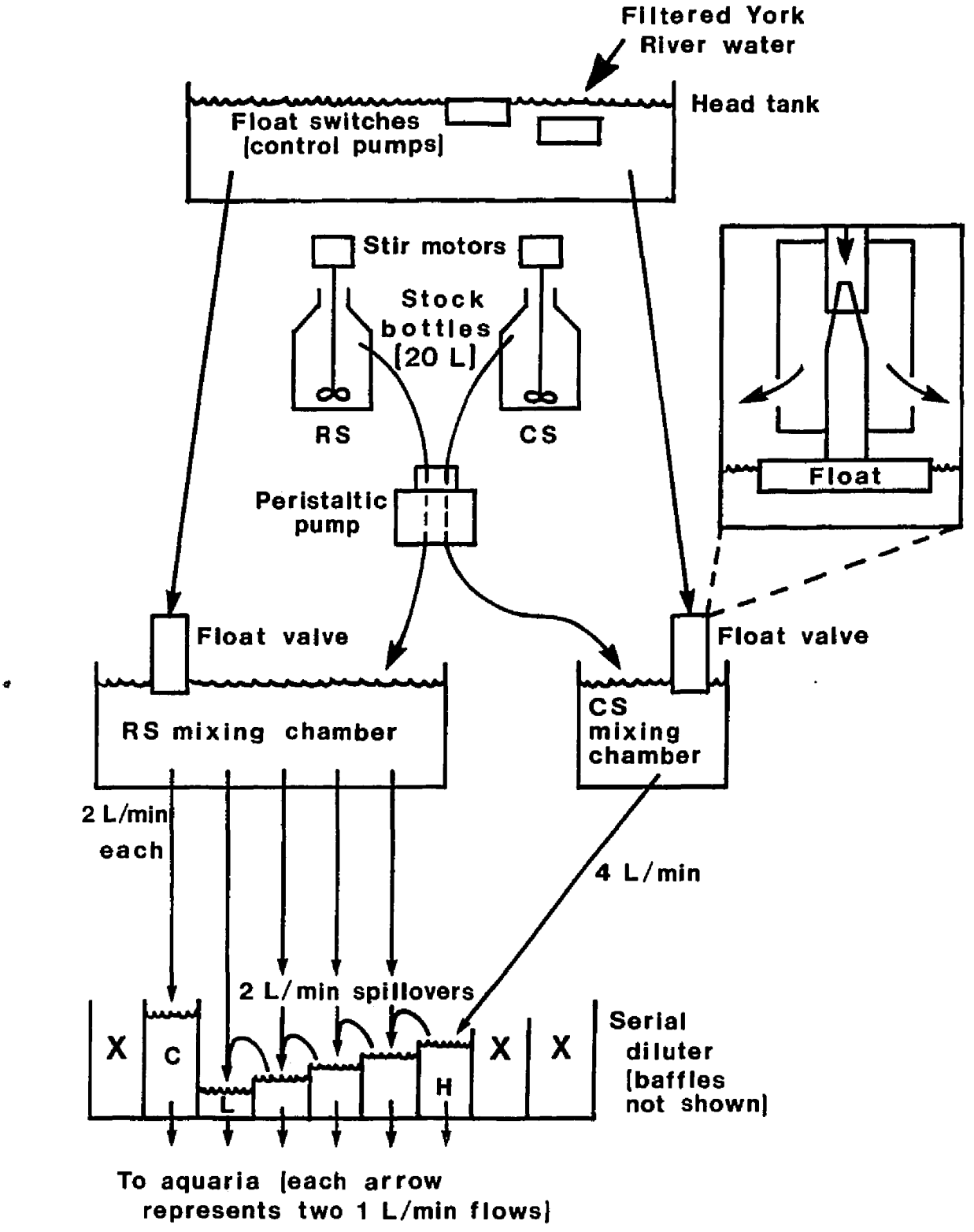
Spot were exposed to creosote-contaminated sediments in a flow-through system (Figure 5). York River water filtered to 50 μm was collected in a reservoir. Two magnetic drive pumps (Model MDXT-3, March Manufacturing, Glenview, IL) pumped water from the reservoir through fiber-wound filters (two sets of one 10-μm filter followed by two 1-μm filters) to a vigorously aerated head tank. Two float switches (Phipps Water Alarm Co., Portland, OR) were mounted one above the other in the head tank to control the operation of the pumps. When both switches were down, the pumps turned on; when both switches were up, the pumps turned off. Use of the two switches allowed the pumps to be off for a few minutes before turning back on. A flapper valve constructed of polyvinyl chloride (PVC) pipe and silicone rubber prevented the pumps from losing their prime. A thermostatically controlled 9000 W heater (Glo-Quartz Titanium, Electric Heater Co., Mentor, OH) set at 20°C was situated in the head tank.

Water from the head tank was siphoned into two glass mixing chambers. A 135 mm X 135 mm X 150 mm chamber, which received the creosote-contaminated sediment, drained through a glass tube fitted into the bottom. The tube was calibrated for a flow rate of 4 L/min. A 580 mm X 160 mm X 140 mm chamber, which received the reference sediment, drained through five glass tubes fitted into the bottom. Each tube was calibrated for a flow rate of 2 L/min. Water flow to the mixing chambers was metered by float-controlled needle valves (see inset, Figure 5) to maintain a constant hydrostatic pressure (80-mm water column in both chambers). The stock sediment slurries were added to the mixing chambers at a rate of 11 mL/min using a peristaltic pump (Model 1203, Harvard Apparatus, South Natick, MA).

The sediment-laden water from the mixing chambers flowed through glass tubing to a glass serial diluter. The diluter was 580 mm X 175 mm X 150 mm and divided into nine 60-mm wide compartments. Two

FIGURE 5

Flow diagram of exposure system for short-term toxicity test.


Filtered York River water
Head tank
Float switches (control pumps)
Stir motors
Stock bottles (20 L)
RS
CS
Peristaltic pump
Float
Float valve
Float valve
RS mixing chamber
CS mixing chamber
2 L/min each
4 L/min
2 L/min spillovers
X
C
L
H
X
X
Serial diluter (baffles not shown)
To aquaria (each arrow represents two 1 L/min flows)

compartments extended all the way to the top of the diluter. One of these was used for the control; the other was not used. The remaining seven compartments were a series separated by walls decreasing in height by 10-15 mm between successive compartments. This allowed excess water in the compartment with the highest wall to spill into the next compartment, and so on. Glass baffles were placed in the compartments to aid in mixing. The last compartment in the series was fitted with a glass standpipe that allowed excess water to drain out of the system. Only five of the serial compartments were used.

Each compartment of the diluter drained through two glass tubes fitted into the bottom. The tubes were calibrated for a flow rate of 1 L/min. The creosote-contaminated sediment went to the first compartment of the diluter. Since 4 L/min of water entered the first compartment and only 2 L/min could flow out through the calibrated glass tubes, 2 L/min had to spill over into the next compartment where it was mixed with 2 L/min of water carrying reference sediment, thus creating a 50% dilution of creosote, but not of sediment. This process continued through three more compartments producing the five test concentrations. Based on the amount of creosote added (assuming 100% PAH) and the flow rates used, the nominal PAH concentrations for this experiment were 250, 500, 1000, 2000, and 4000 μg/L. The compartment used for the control received only water containing reference sediment.

Water from the diluter flowed through glass tubing to 38-L glass aquaria. The 12 aquaria were arranged on a partially covered wet table in two blocks: shaded and non-shaded. Each treatment was randomly assigned to one aquarium in each block. The aquaria were connected with glass siphons to external PVC standpipes to maintain a constant water level in the aquaria; the volume of water in the aquaria was approximately 32 L. The aquaria had glass covers and were vigorously aerated. The exposure system was allowed to equilibrate for 5 days prior to the beginning of the test.

Experimental Procedure

A standard 96-h acute toxicity test was conducted according to established guidelines (APHA, 1985; ASTM, 1989; Weber *et al.*, 1988). On the first day of the test (day 0), 10 spot were randomly assigned to each test aquarium. Temperature, salinity, dissolved oxygen, pH, and flow rate were measured and recorded daily for each aquarium. Temperature was measured to the nearest degree Celsius with a stem thermometer and salinity to the nearest part per thousand with a refractometer. Dissolved oxygen concentration was measured to the nearest tenth of a part per million with an oxygen meter (Model 57, Yellow Springs Instrument Co., Yellow Springs, OH) and pH to the nearest hundredth of a pH unit with a pH meter (Model EA920, Orion Research, Cambridge, MA). The meters were calibrated daily according to the manufacturer's instructions. Flow rate was measured by collecting the flow to an aquarium for 1 min and measuring the volume. Flow rate was used to determine if the diluter was functioning properly.

A 1-L water sample to determine creosote concentration (total resolvable PAH) was collected daily from the highest, middle, and lowest nominal concentrations and from the control. Intermediate concentrations were not sampled for PAH analysis because of limitations in the number of samples to be processed. Sampling alternated between the blocks of treatments. Samples were collected in glass graduated cylinders. Processing of the samples for PAH analysis began immediately and was completed within 48 h (See Chemical Analysis, below). No attempt was made to separate the aqueous and suspended sediment fractions.

A 0.5-L water sample was collected daily from each treatment to determine sediment concentration. Samples were collected in a glass flask from the same block sampled for PAH analysis. The water was filtered through a pre-weighed 0.45-μm filter (47 mm, Type HA, Millipore Filter Corp., Bedford, MA). The flask and the filter holder were each

rinsed onto the filter three times with deionized water. The filter and sediment were dried overnight at 60°C, allowed to cool 5 min, and weighed.

Survival was recorded every 12 h, even though dead fish were removed as soon as observed. A fish was considered dead when it no longer responded to prodding with a blunt instrument. Dead fish were visually inspected and measured at the time of removal. Standard and total lengths were measured to the nearest millimeter and weight to the nearest hundredth of a gram. At the end of the experiment, all surviving fish were anaesthetized with ethyl *m*-aminobenzoate methanesulfonic acid (MS-222, 30 mg/L, Aldrich Chemical Co., Milwaukee, WI) and then inspected and measured as above. Fulton's Condition Factor (K) was calculated for all fish according to Bagenal and Tesch (1978) as:

$$K = \frac{100W}{L^3}$$

where W is wet weight in grams and L is total length in centimeters. Tail length was calculated as the difference between the standard and total lengths.

Chemical Analysis

Water samples were analyzed for total resolvable PAH by a modification of the U.S. Environmental Protection Agency Method 625 (Longbottom and Lichtenberg, 1982). The major modification was the extraction of only acid-neutral compounds for analysis. The alkaline fraction, whether taken before or after the acidic fraction had low recoveries because of formation of an emulsion. Analysis of both acidic and alkaline fractions indicated that most compounds were neutral and

were extracted in whichever fraction was collected first. Acid-neutral extracts have been used previously for the analysis of creosote (Borthwick and Patrick, 1982).

All solvents used were of high purity (Burdick & Jackson Brand, Baxter Healthcare, Muskegon, MI). All glassware was scrupulously cleaned with laboratory detergent and then allowed to soak overnight in 5% Contrad 70® (Curtin Matheson Scientific, Houston, TX). Glassware was rinsed in hot tap water followed by rinses with 3 N HCl and deionized water (Milli-Q Reagent Grade Water using a distilled water feed, Millipore Filter Corp.). Immediately prior to use, glassware was rinsed consecutively with methanol, acetone, UV-hexane, and dichloromethane (DCM).

Water samples were transferred to 2-L separatory funnels. DCM was used to rinse the sample containers into the separatory funnels. A 1-L deionized water blank was also prepared. The samples were acidified by the addition of 2 mL H_2SO_4 (diluted 1:1 with deionized water); 20 μg 1,1'-binaphthyl in acetone was added as an internal standard. The samples were extracted three times with 60 mL DCM. The organic layer from each extraction was combined in a 250-mL round-bottom flask and concentrated to approximately 2 mL under a vacuum at 40°C using a rotary flash evaporator (Buchler Instruments, Fort Lee, NJ). The concentrated samples were transferred to 15-mL graduated conical tubes and further concentrated under nitrogen to 1 mL. These samples were stored in the dark at 4°C for 24 h before analysis by gas chromatography.

The gas chromatograph (Model 3700, Varian Instrument Group, Palo Alto, CA) was fitted with a 1701 cross-linked glass capillary column prepared according to the method of Grob and Grob (1961) as revised by Grob *et al.* (1982). The column was 30 m long, had an inner diameter of 0.32 mm, and a film thickness of 0.25 μm. Injection of a 1-μL sample was in the splitless mode (Lee *et al.*, 1984) with an initial column temperature of 75°C. High purity helium carrier gas was input at a flow

rate of 30 cm/s. When the solvent peak began to elute, the split valve was turned on and the oven temperature programmed to increase at a rate of 6°C/min to an upper temperature of 300°C. The upper temperature was then maintained for 5 min. The injector temperature was 310°C. Flame ionization detection was used with 30 mL H_2/min and 300 mL air/min at a temperature of 330°C. The attenuation was set at 8 X 10^{-11}.

Data from the detector was collected on a computerized data acquisition system (Model 3350A, Hewlett Packard Co., Avondale, PA). Quantification was based on the internal standard with the assumption that all compounds extracted with the same efficiency and were detected with the same sensitivity. Identifications were based on an aromatic retention index established from chromatography of the source creosote and checked daily with a set a standards. Identification of individual compounds in the source creosote was by mass spectrometry. Selected samples were also analyzed by gas chromatography-mass spectrometry (GC-MS) to verify the identification of individual compounds.

For the GC-MS analysis, the gas chromatograph (Model 3300, Varian Instrument Group) was fitted with a DB-5 fused silica column (J & W Scientific, Folsom, CA). The column was 30 m long, had an inner diameter of 0.32 mm, and a film thickness of 0.25 μm. The carrier gas and temperature program were set as above. The gas chromatograph was connected via an open interface at 340°C to a magnetic sector mass spectrometer (Model 21-492B, E. I. du Pont de Nemours & Co., Wilmington, DE) in electron ionization mode. The source temperature was 310°C and the source ionization energy was 70 eV. The magnet scan rate was 1 s/decade. Data were collected on a computerized data acquisition system using a combination of custom written software and a GC-MS data system (Model ELQ400-2, Extrel Corp., Pittsburgh, PA). The mass spectra were identified by comparison with published spectra of known purified compounds (McLafferty and Stauffer, 1989).

Statistical Analysis

Temperature, salinity, pH, dissolved oxygen, sediment concentration, and fish standard length, total length, weight, condition factor, and tail length were analyzed using randomized block analysis of variance (Zar, 1984) with a significance level of 0.05. Daily water quality measurements were considered to be time weighted repeated measures. If there were no significant differences between blocks, then blocks were pooled for *a posteriori* testing. A two-tailed Dunnett's test (Zar, 1984), with a significance level of 0.05, was used to identify treatments that differed from the control.

PAH concentration data were analyzed by non-parametric randomized block analysis of variance (Zar, 1984). These data were treated as above, except that a one-tailed Steel's many-one rank test (Steel, 1959) was used to identify treatments with PAH concentrations that were significantly greater than the control.

Survival data were analyzed non-parametrically by the conventional Spearman-Karber method (Hubert, 1984). An LC50 with 95% confidence intervals (CI) was calculated independently for both blocks as recommended by Stephan (1977). Fisher's exact test, with a significance level of 0.05, was used to determine the lowest concentration producing significant mortality (Siegel, 1956).

RESULTS

Sediment Characterization and Concentration

No gravel was found in the sediment used for these tests. The size distribution of the sediment particles was 6.6% (SD = 0.6) sand, 63.5% (SD = 13.1) silt, and 29.9% (SD = 13.3) clay. The sediment contained 0.24% particulate organic nitrogen and 1.77% particulate organic carbon.

Suspended sediment concentration was not significantly different between blocks or among treatments (Table 2). Mean sediment concentration for all treatments was 23.6 mg/L (N = 36, SD = 4.7).

PAH Concentration

Total resolvable PAH concentration was significantly higher for all concentrations measured than for the control (Table 2). Over 100 compounds were quantified in water samples collected from the highest concentration; most of these were identified by GC-MS. Almost 60% of the total PAH at the highest concentration was attributable to six compounds: naphthalene, acenaphthene, fluorene, phenanthrene, fluoranthene, and pyrene. These same six compounds accounted for almost 70% of the total PAH in the lowest concentration (Table 3).

Total measured PAH concentration was over 80% of the nominal concentration for the highest concentration, but was only about 50% of the nominal concentration for the two lower concentrations measured. The percent of the nominal concentration represented by the six most abundant PAH in creosote also decreased with decreasing concentration (Table 2).

Hydrographic Data

Temperature, salinity, dissolved oxygen, and pH were relatively constant with few, if any, differences among treatments (Table 4). Temperature was 19°C and did not vary within the scope of the measurement. Salinity fluctuated between 21 and 22 g/kg. There were no significant differences between blocks or among treatments for either temperature or salinity.

Dissolved oxygen was always above 90% of saturation and pH was approximately 7.9. There were no significant differences between blocks for dissolved oxygen and pH, but there were differences among treatments ($P = 0.012$) for both parameters (Table 4). Dissolved oxygen was

TABLE 2

PAH and Sediment Concentration for Short-Term Toxicity Test

Nominal PAH (μg/L)	Total Resolvable PAH (μg/L)	Sum of Selected PAH[a] (μg/L)	Sediment Concentration (mg/L)
Control	BLD[b,c]	BLD	25.6 (4.4)[d]
250	110 (40)[e]	73 (27)	20.9 (4.5)
500	250[f]	160[f]	22.3 (5.3)
1000	560 (200)[e]	350 (120)	21.2 (2.6)
2000	1400[f]	810[f]	24.5 (3.9)
4000	3300 (950)[e]	1900 (540)	29.6 (5.6)

[a] Naphthalene, acenaphthene, fluorene, phenanthrene, fluoranthene, and pyrene.

[b] Below the detection limit (<2.0 μg/L).

[c] Values are means of 5 daily measurements taken alternately between replicate treatments. Standard deviations are in parentheses.

[d] No significant differences between blocks or among treatments. Randomized block ANOVA (α = 0.05).

[e] Significantly greater than the control. One-tailed Steel's many-one rank test (α = 0.05).

[f] Estimated as the geometric mean of adjacent values.

TABLE 3

The Six Most Abundant PAH in Short-Term Toxicity Test

Compound	Molecular Weight	% of Total at 3.3 mg/L	% of Total at 110 µg/L
Naphthalene	128	21 (2.6)[a]	21 (8.4)
Acenaphthene	154	7.7 (0.62)	8.1 (3.1)
Fluorene	166	6.4 (0.59)	6.3 (2.5)
Phenanthrene	178	12 (0.63)	15 (5.7)
Fluoranthene	202	6.6 (0.91)	11 (3.5)
Pyrene	202	5.3 (0.75)	6.9 (2.5)
Total		59.0	68.3

[a] Values are means of 5 daily measurements taken alternately between replicate treatments. Standard deviations are in parentheses.

TABLE 4

Hydrographic Data for Short-Term Toxicity Test

PAH (μg/L)	Temperature (°C)	Salinity (g/kg)	Dissolved Oxygen (mg/L)	pH
CONTROL	19.0 (0.0)[a]	21.6 (0.5)	8.2 (0.1)	7.89 (0.04)
110	19.0 (0.0)	21.6 (0.5)	8.2 (0.1)	7.90 (0.03)
250	19.0 (0.0)	21.6 (0.5)	8.2 (0.1)	7.93 (0.02)[b]
560	19.0 (0.0)	21.6 (0.5)	8.1 (0.1)	7.93 (0.02)[b]
1400	19.0 (0.0)	21.6 (0.5)	8.0 (0.1)[b]	7.91 (0.03)
3300	19.0 (0.0)	21.6 (0.5)	8.0 (0.2)[b]	7.89 (0.05)

[a] Values are means of 5 daily measurements from each of two replicate treatments. Standard deviations are in parentheses. No significant differences between replicate treatments for any water quality parameter. Randomized block ANOVA ($\alpha = 0.05$).

[b] Significantly different from the control value. Two-tailed Dunnett's test ($\alpha = 0.05$).

statistically different from that in the controls for both the 1400 and 3300 μg PAH/L treatments. The 250 and 560 μg PAH/L treatments had pH that was statistically different from that in the controls.

Fish Size and Condition Factor

There were no significant differences between blocks or among treatments for standard length, total length, wet weight, condition factor, or tail length (Table 5). Mean standard length was 10.1 cm (SD = 0.5), mean total length was 12.1 cm (SD = 0.6), and mean wet weight was 24.35 g (SD = 4.06). Mean condition factor was 1.37 (SD = 0.08). Mean tail length was 19.8 mm (SD = 1.5).

Behavioral Observations and Mortality

Upon placing the fish in the test aquaria, spot exposed to 1400 and 3300 μg PAH/L showed immediate signs of distress as evidenced by increased ventilation rates. Death, however, was a slow process. At 3300 μg PAH/L, the first mortalities did not occur until after 48 h. Prior to death, fish swam irregularly, eventually only able to maintain themselves in the water column for brief periods. Once no longer able to swim, fish typically continued ventilation on the bottom of the tank for many hours, sometimes as long as a day before dying.

Survival for control fish was 100% during the 96-h exposure. The highest concentration with no observable effects was 250 μg PAH/L. Significant mortality (100%) occurred only at 3300 μg PAH/L. At 1400 μg PAH/L, mortality averaged 25%. Except for one apparently healthy fish in each replicate treatment, all survivors at 1400 μg PAH/L were near death or swimming abnormally at 96 h. There were no mortalities at any of the other PAH concentrations tested. One fish at 560 μg PAH/L was near death after 96 h.

The 96-h LC50 for the shaded block was 1670 μg PAH/L (95% CI = 1290-2160 μg PAH/L). The 96-h LC50 for the non-shaded block was

TABLE 5

Fish Measurements for Short-Term Toxicity Test

PAH (μg/L)	Standard Length (cm)	Total Length (cm)	Wet Weight (g)	Condition Factor[a]	Tail Length[b] (mm)
CONTROL	10.2 (0.6)[c]	12.1 (0.7)	24.6 (4.4)	1.36 (0.06)	19.8 (1.2)
110	10.3 (0.5)	12.3 (0.6)	25.7 (4.1)	1.37 (0.07)	20.0 (1.7)
250	10.1 (0.6)	12.1 (0.7)	24.1 (4.8)	1.35 (0.08)	19.5 (1.6)
560	10.0 (0.4)	12.0 (0.5)	23.3 (3.7)	1.35 (0.07)	19.4 (1.6)
1400	10.1 (0.5)	12.0 (0.5)	24.7 (3.7)	1.41 (0.09)	19.6 (1.4)
3300	10.0 (0.5)	12.0 (0.6)	23.8 (3.6)	1.36 (0.09)	20.4 (1.2)

[a] Condition Factor = (100 * Wet Weight) / Total Length^3

[b] Tail Length = Total Length - Standard Length

[c] Values are means of 10 fish per each of two replicate treatments. Standard deviations are in parentheses. No significant differences between replicates or among treatments. Randomized block ANOVA (α = 0.05).

1820 μg PAH/L (95% CI = 1460-2280 μg PAH/L). Because these values agreed within 10%, the survival data was pooled and an overall LC50 was calculated (Figure 6). The overall 96-h LC50, based on measured concentrations, was 1740 μg PAH/L (95% CI = 1480-2060 μg PAH/L).

DISCUSSION

Sediment Characterization

The sediment used in this experiment was predominantly silt and clay. In their original description of the sediment collection site, Boesch *et al.* (1976) reported finding 20-30% very fine sand. The paucity of sand in this study probably reflects the fact that the sediment collected for this study was sieved prior to use and analysis.

Organic carbon content of natural sediments is usually between 0 and 3% (Wetzel, 1975; Diaz *et al.*, 1985; deFur *et al.*, 1987; Unger *et al.*, 1988). The sediment used to expose spot to creosote was 1.8% organic carbon, well within the normal range.

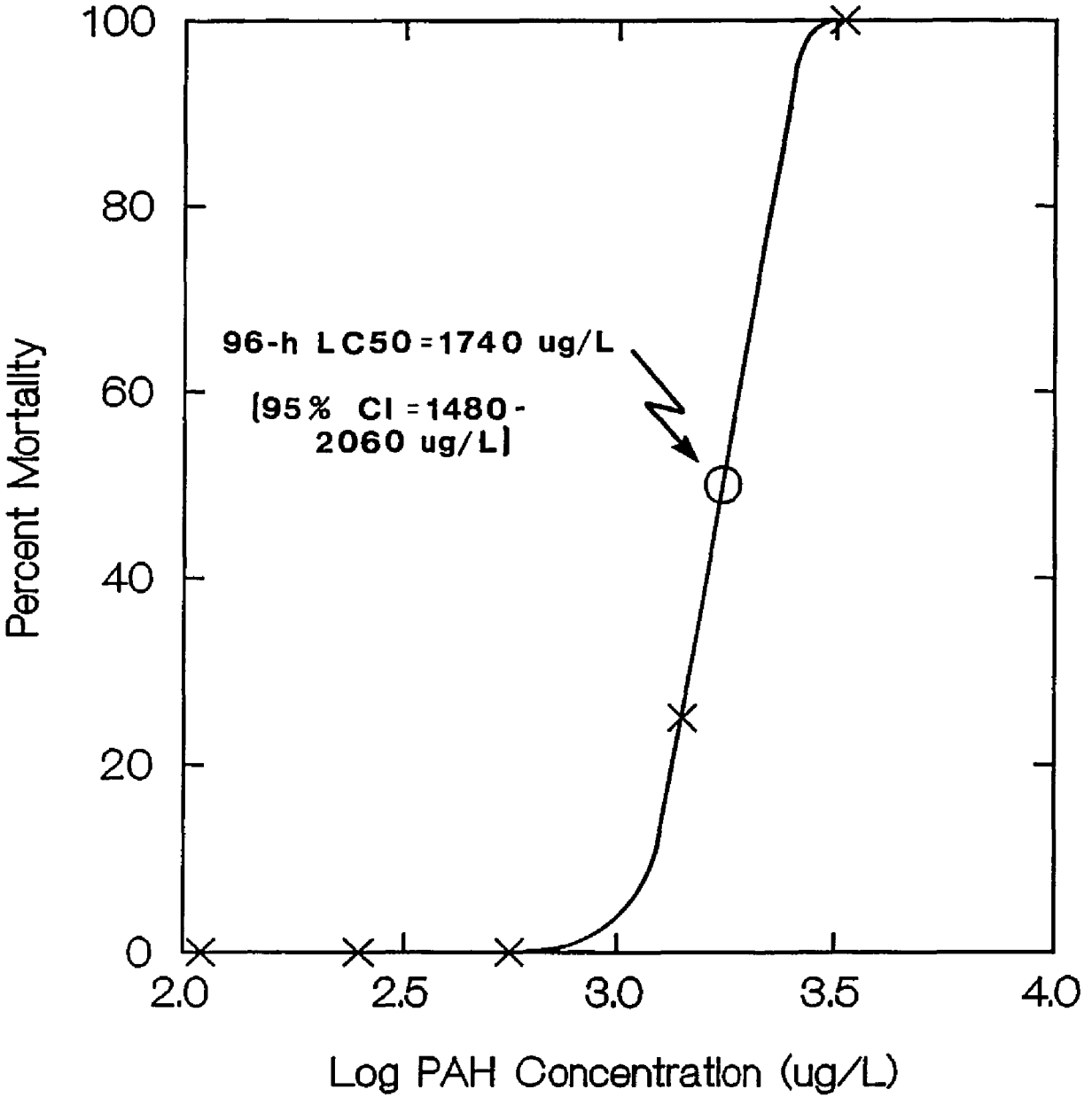
Small sediment particles provide more surface area for the adsorption of hydrophobic chemicals than large particles. High organic content allows for maximal partitioning of hydrophobic chemicals onto sediment (Rodgers *et al.*, 1987). Together, the small particle size and high organic carbon content provide optimal conditions for the adsorption of PAH to sediment. In natural situations, sorption of PAH to particles may be different than in this experiment.

Sediment Concentration

Rivers discharging into Chesapeake Bay have a tremendous range in suspended sediment concentrations, both with respect to depth and time. Patten *et al.* (1966) found suspended sediment concentrations in the York River, VA ranged from approximately 10 mg/L at the surface to over 200 mg/L near the bottom. Schubel (1977) measured suspended sediment concentrations in surface waters of several areas of Chesapeake Bay.

FIGURE 6

Percent mortality for short-term toxicity test. X = mean of two replicate treatments, O = 96-h LC50.


100
80
60
40
20
0
Percent Mortality
96-h LC50 = 1740 ug/L
(95% CI = 1480 - 2060 ug/L)
2.0
2.5
3.0
3.5
4.0
Log PAH Concentration (ug/L)

Surface concentrations near the bay mouth were approximately 7 mg/L. Values at the mouth of the Susquehanna River, MD were typically about 10 mg/L, but exceeded 10,000 mg/L following tropical storm Agnes in 1972. These results indicate that the suspended sediment concentration used as a creosote-carrier during this experiment (23.6 mg/L) was well within the normal range for Chesapeake Bay and its tributaries. Suspended sediment concentration varied as much as 25%, which may explain some of the variation in the measured PAH concentrations.

Sinnett and Davis (1983) developed an automated system for maintaining constant sediment concentration. Their system used a nephelometer, or transmissometer, suspended in a test chamber to measure sediment concentration. The nephelometer was connected to a microprocessor that controlled an air pump. The air flow served two purposes: to mix the stock sediment slurry and to "bubble" some of the slurry into the test chamber. When turbidity in the test chamber decreased, the air pump was turned on and sediment was added to the test chamber.

Pruell *et al.* (1987) used the system designed by Sinnett and Davis (1983) to expose mussels (*Mytilus edulis*) to sediments contaminated with PCB and PAH. The intended sediment concentration was 15 mg/L. Even with the automated system, sediment concentration varied by over 45%. Total PAH concentration varied by approximately 40%. The concentration of biphenyl varied by over 100%. In the present experiment sediment concentration varied by 25% and total PAH concentration varied by 30-35%. The compound with the largest variation in concentration was naphthalene, which varied by approximately 50% in the 560 μg PAH/L treatment. Thus, the relatively simple exposure system used in the present experiment outperformed previously used systems for exposing aquatic organisms to suspended, contaminated sediments.

Differences in Hydrographic Parameters

In the aquaria receiving the two highest PAH concentrations, dissolved oxygen concentrations were statistically lower than in the control aquaria. This may be the result of chemical oxygen demand or increased respiration of the fish. The fish exposed to the two highest PAH concentrations were noticeably ventilating at higher rates than any of the other fish. The difference in dissolved oxygen concentrations may have been statistically significant, but the biological significance was probably negligible because dissolved oxygen concentrations were always above 90% of saturation in all treatments.

The aquaria receiving 250 and 560 μg PAH/L had statistically higher pH than the control aquaria. Again, this difference was probably not biologically significant. The difference was only 0.04 pH units. Differences of this magnitude are not known to affect biological systems (Lehninger, 1982).

Nominal Versus Measured PAH Concentration

Nominal PAH concentrations were calculated based on the amount of creosote added to the sediment stock solution and the various flow rates, with the assumption that 100% of creosote is PAH (including heterocycles). Analyses of the creosote stock, in this laboratory and by the creosote manufacturer, indicate that this is not a bad assumption.

Mueller *et al.* (1989) reported that creosote contains 85% PAH and 5% heterocycles, with the remaining 10% being phenolic compounds. Mueller *et al.* did not experimentally determine the composition of creosote and failed to reference their information. Koppers Industries and Allied Chemical, two of the largest creosote manufacturers in the United States, remove most of the phenol, cresols, and xylenols following the distillation process. The mixture of these compounds was

at one time sold as an insecticidal oil, but the compounds are now purified for sale individually.

One of the specifications for creosote is low (<1.0%) water solubility. This would be hard to achieve if creosote contained 10% phenolic compounds. Aqueous solubility of phenol is 82 g/L at 15°C in deionized water (Verschueren, 1983). Solubility of cresols ranges from 20-30 g/L depending on the isomer and temperature (Verschueren, 1983). These high solubilities suggest that more than 1% of creosote would be water soluble if the phenolic fraction were not removed.

Measured PAH concentrations were lower than the nominal concentrations and, as PAH concentration decreased, the difference between measured and nominal concentrations increased. The change in the difference between nominal and measured concentrations can be attributed to several phenomena: 1) the flow rates to the diluter may have created more than a 50% dilution with each step, 2) volatile compounds may have been preferentially lost during the extra steps involved in producing the dilutions, 3) the discrepancy may have been an artifact of the method used to quantify total PAH, 4) extraction efficiency may be greater at high PAH concentrations than at low concentrations, 5) the surface area of the exposure system relative to volume increased with each successive dilution and creosote may simply have adsorbed to the walls of the diluter, and 6) the amount of sediment that settled in the diluter may have increased with increasing dilution.

Flow rates were measured daily and never differed from the desired value by more than 10%. Any deviations from the desired value were immediately corrected. Thus, it is unlikely that the change in the difference between measured and nominal concentrations was a function of diluter flow rates.

The composition of the creosote, as evidenced by the six most abundant compounds, did not change much between the highest and lowest concentrations. If compounds had been lost through volatilization, then

the contribution of the low molecular weight components should have decreased and the contribution of the high molecular weight components should have increased. Since this was not the case, volatilization of low molecular weight compounds was also probably not the reason for the change in the relationship between measured and nominal PAH concentrations.

For the highest concentration, approximately 100 compounds were quantified to arrive at a total PAH value. Many of these compounds were sufficiently dilute in the lowest concentration that they were below the limit of detection and consequently no longer contributed to the total PAH concentration. This would cause an apparent decrease in the percent of the nominal concentration represented by the total measured PAH, but the sum of the six most abundant compounds, which were above the detection limit at all test concentrations, also decreased at a greater rate than the nominal concentration. Therefore, the change in the difference between measured and nominal concentrations was not an artifact of the quantification method.

Siron and Giusti (1990) have shown that extraction efficiency of organic compounds is affected by the amount of particulate matter in the sample. This does not explain the discrepancy between nominal and measured concentrations because suspended sediment concentration was constant. Extraction efficiency was not necessarily equal at all PAH concentrations. At high PAH concentrations there may be a thicker coating of creosote on the sediment particles than at low PAH concentrations. A larger percentage of the thick coating might have been extracted by the simple liquid-liquid extraction technique used in this study than was extracted from the thin coating. One of the assumptions of the quantification method used was that the internal standard extracted with the same efficiency as the quantified compounds. Use of an internal standard should account for any discrepancies in the extraction efficiency among samples. Determination of the relationship

between the extraction efficiency of the internal standard and that of all the compounds quantified in this experiment was beyond the scope of this study.

Adsorption of PAH to the walls of the exposure system or losses resulting from the settlement of sediment may also be reasons for the increased difference between nominal and measured concentrations as dilution occurred. Visual inspection of the exposure system revealed that material continually adsorbed to the surfaces of the system and sediment accumulated in corners where the water flow was insufficient to keep the sediment in suspension. Regardless of the cause, total PAH concentration was quantified from readily extractable compounds in water samples collected from the test aquaria and therefore represents the best estimate of PAH exposure.

Use of Sediment as a Carrier

The high octanol-water partition coefficients of most of the components in creosote indicate that creosote entering an aquatic environment would preferentially sorb to sediment (Leo *et al.*, 1971). Pockets of creosote were found in a sediment core from the Elizabeth River, VA (Lu, 1982). Therefore, exposing fish to creosote-contaminated sediment, even at extremely high concentrations, can be considered environmentally realistic.

The liquid-liquid extractions used to recover PAH do not necessarily extract all of the PAH sorbed to the sediment, nor does it provide an estimate of aqueous PAH concentration. Dose, therefore, was defined in terms of extractable PAH. Different results may be obtained if dose is based only on the amount of PAH actually in solution.

Several researchers have used contaminated sediments as a means of exposing fish (Southworth *et al.*, 1979; Eaton *et al.*, 1983; Rubinstein *et al.*, 1984) and invertebrates (Pruell *et al.*, 1987; Swartz *et al.*, 1989) to various hydrophobic chemicals. A few laboratory studies have

exposed fish to sediment collected from areas contaminated with PAH presumed to be of a creosote origin (Hargis *et al.*, 1984; Roberts *et al.*, 1989; Schoor *et al.*, 1991).

One problem associated with studies using natural sediments is obtaining reference sediment that is free of contaminants. Sediment collected from the same locality as the sediment used in the present study has been shown to have a moderate degree of PAH contamination (R. Hale, Virginia Institute of Marine Science, personal communication). The analytical method used in the present study failed to detect significant concentrations of PAH, possibly because a simple liquid-liquid extraction was used instead of a vigorous extraction procedure. Nevertheless, the sediment used in the present study was used for all treatments at equal concentrations and thus provided a true control rather than a reference treatment.

To my knowledge, the only studies that have investigated the toxicity of creosote to fish used either organic solvents as carriers or dispersed the creosote in water instead of using contaminated sediments to deliver the creosote (Bionomics, 1974a, b; Borthwick and Patrick, 1982). McLeese and Metcalfe (1979) exposed lobsters (*Homarus americanus*) and shrimp (*Crangon septemspinosa*) to creosote, but fail to mention the method by which the creosote was administered. Laboratory studies using petroleum products as a source of PAH have typically used water accommodated fractions (Anderson *et al.*, 1974; Walton *et al.*, 1978) or dispersed oil (Anderson *et al.*, 1974; Steadman *et al.*, 1991a, b), although oiled sediments have been used to expose fish (McCain *et al.*, 1978; Payne and Fancey, 1982).

A chemical can be considered bioavailable if an organism responds to the chemical over some range of concentrations (Rodgers *et al.*, 1987). By this definition, creosote-contaminated sediments provide a bioavailable source of PAH. Observed responses included increased ventilation rates, swimming abnormalities, listlessness, and death.

Toxicity of Creosote

The conventional Spearman-Karber method was used to calculate the 96-h LC50 and 95% confidence interval for spot exposed to creosote-contaminated sediment. This method is appropriate because only one concentration yielded partial mortality (Stephan, 1977). A trimmed Spearman-Karber estimator would produce similar results because there were no anomalous mortality responses (Hamilton *et al.*, 1977). The moving average method would also be appropriate, but does not allow for determination of a 95% confidence interval.

In 1974, the American Coke and Coal Institute contracted Bionomics (EG&G, 790 Main Street, Wareham, MA), a private toxicology company, to perform standard acute toxicity tests with marine grade creosote. The first of these studies (Bionomics, 1974a) was a flow-through test using an intermittent-flow proportional diluter (Mount and Brungs, 1967). Creosote of unspecified origin was added in acetone. Two test organisms, bluegills (*Lepomis macrochirus*) and rainbow trout (*Salmo gairdneri* = *Oncorhynchus mykiss*), were used. The highest concentration tested with bluegills was 1000 μg creosote/L, which resulted in 30% mortality. The 96-h LC50 calculated for rainbow trout was 200 μg creosote/L (95% CI = 130-320).

The second study (Bionomics, 1974b) involved a static test with rainbow trout. Creosote was dispersed in water and added to each test chamber. No mortality was observed at 560 μg creosote/L; 100% mortality was observed at 750 μg creosote/L. These results are quite different from those of the first study, but static and flow-through tests often yield different results (Parrish, 1985).

Results from both of the Bionomics' studies were expressed in terms of nominal concentrations, which were calculated based on the amount of creosote added. In the one case (flow-through, rainbow trout) where an LC50 was calculated, probit analysis was used. This was an

appropriate method because the data set included three concentrations at which partial mortalities occurred (Stephan, 1977).

Acute toxicity of marine grade creosote has also been determined for the sheepshead minnow (*Cyprinodon variegatus*) in both static and flow-through tests (Borthwick and Patrick, 1982). The creosote used was obtained from the U.S. EPA Pesticides Laboratory (Beltsville, MD). For the static tests, creosote was added in triethylene glycol. For the flow-through tests, creosote was added in acetone using an undescribed metering pump. Calibrated siphons were used to control diluent water flow rate.

Based on the amount of creosote added, Borthwick and Patrick (1982) calculated a 96-h LC50 for both tests using probit analysis, but did not provide sufficient information to determine if this method was appropriate. The 96-h LC50 for the static test was 720 μg creosote/L (95% CI = 660-790 μg creosote/L); the 96-h LC50 for the flow-through test was 3500 μg creosote/L (95% CI = 2900-4200 μg creosote/L).

For the flow-through test, Borthwick and Patrick (1982) also measured actual concentrations. Two acid-neutral extractions using 1:1 petroleum ether:diethyl ether (v/v) were combined, concentrated, and analyzed by gas chromatography with flame ionization detection. Quantification was based on eight chromatogram peak heights compared to a 250 ng injection of creosote as a standard.

There are several problems with Borthwick and Patrick's (1982) measurement of creosote. First, three extractions are usually done to ensure optimal recovery. Second, no internal standard was used to account for extraction efficiency. Finally, quantification was based on peak height, rather than peak area. Use of peak heights can lead to erroneous values if peak width is not constant.

Measured concentrations for Borthwick and Patrick's (1982) experiment were 28-52% of nominal concentrations with no systematic discrepancy. Using the average difference between measured and nominal

concentrations for the two concentrations surrounding the 96-h LC50 value, a 96-h LC50 based on measured creosote concentration can be estimated as 1300 μg creosote/L.

These previous acute toxicity tests with creosote provide little data with which to compare the values obtained for spot. The Bionomics' (1974a, b) studies only report creosote concentration based on the amount added. The actual creosote concentration could be quite different. The 96-h LC50 estimated from Borthwick and Patrick's (1982) actual concentration data is only slightly lower than the 96-h LC50 determined in the present experiment (1740 μg PAH/L) for spot. The value determined for spot may be higher than that determined by Borthwick and Patrick (1982) because of differences in the susceptibility of the species used or partial unavailability of the sediment-bound creosote.

One other study provides some information for comparison. Roberts *et al.* (1989) exposed spot to several dilutions of bed-load sediment collected from the Elizabeth River, VA. The sediment in this river is highly contaminated with PAH, most of which probably came from several wood treatment facilities along the banks (Merrill and Wade, 1985; Bieri *et al.*, 1986). A 5-cm layer of sediment was placed in each test aquarium. Spot were placed in cages that were pushed slightly into the sediment. Fish had direct contact with the sediment, but were prevented from burying themselves.

The 96-h LC50, in terms of percent Elizabeth River bed-load sediment was approximately 55% (Roberts *et al.*, 1989). Total resolvable PAH measured in 100% Elizabeth River sediment was 21,200 μg PAH/g dry weight sediment. Assuming a near bottom suspended sediment concentration of 200 mg/L, the 96-h LC50 based on suspended sediment translates to 2340 μg PAH/L in suspension. This value is only slightly higher than the 96-h LC50 determined for spot using creosote on suspended sediments, possibly reflecting differences in the

bioavailability of the freshly contaminated sediment used in the present experiment and the natural sediment, which has been in contact with the creosote for a long time.

Extrapolation of a 96-h LC50 to long-term sub-acutely toxic concentrations is plagued with uncertainty (Rand and Petrocelli, 1985). One method of extrapolation is to determine an incipient LC50 (the concentration at which 50% of a test population can live indefinitely), based on the LC50 at various time points during a short-term test (Rand and Petrocelli, 1985). With creosote, acute mortality was continuing at 96-h, so that an incipient LC50 calculated from the present results would be of little value. An alternative to determining an incipient LC50 is to base long-term exposures on the lowest concentration producing a response in a short-term test. At 560 μg PAH/L, only one fish in the present experiment showed any signs of being affected at 96 h. Thus, 560 μg PAH/L is a reasonable highest test concentration for a long-term experiment.

RESPONSE OF EROD ACTIVITY TO CREOSOTE-CONTAMINATED SEDIMENTS

INTRODUCTION

There have been several studies concerned with sublethal effects of PAH believed to be of creosote origin. These studies have either examined fish collected in areas where creosote is a major contaminant or have used sediments from these areas to expose fish in the laboratory. These studies, however, fail to establish a direct causal relationship between observed effects and creosote because creosote may not be the only contaminant present.

The severity of observed effects appears to be related to the degree of PAH contamination on the sediments. Integumental lesions, fin and gill erosion, reduced hematocrit values, pancreatic and liver alterations, and mortality have been reported for fish exposed to high PAH concentrations (2,500-3,900 μg PAH/g dry weight sediment) in the laboratory (Hargis *et al.*, 1984). Hepatic lesions including hepatocellular neoplasms, foci of cellular alteration, cholangiolar proliferation, and hepatocellular adenomas have been reported in fish collected from sites contaminated with 120-2,200 μg PAH/g dry weight sediment (Malins *et al.*, 1985; Vogelbein *et al.*, 1990). Induction of hepatic EROD activity and increased P-450 (P-450 IA1 as determined by immunological techniques) concentration has been reported for fish collected at sites contaminated with 3-96 μg PAH/g dry weight sediment (Van Veld *et al.*, 1990). Earlier research at some of the same locations sampled by Van Veld *et al.* (1990) failed to show consistent induction of BaPH activity over time (Roberts *et al.*, 1987). An increase in P-450 (P-450 IA1 as determined by immunological techniques) concentration has

also been reported for fish exposed to creosote contaminated sediments in the laboratory, but comparisons with this data are difficult because total PAH concentration was not reported (Schoor *et al.*, 1991).

Objective

The objective of this experiment was to determine the exposure-dependence of hepatic EROD activity in spot exposed to sub-acutely toxic concentrations of creosote-contaminated sediments in suspension.

MATERIALS AND METHODS

Fish

Fish were collected and transported to the laboratory as described in the previous chapter. Holding conditions were also as previously described with the following exceptions. The holding tanks were located inside the laboratory in a room not used for toxicity testing. Water passed through a head tank equipped with a heater before entering the holding tank. Minimum water temperature was 20°C. Three weeks prior to the beginning of the test, holding temperature was increased at a rate of 1°C/day to the test temperature of 25°C. A sample of 40 fish was measured (total and standard lengths) and weighed every two weeks for the first ten weeks to determine growth rates under holding conditions. The holding period was approximately 5 months. A 14-h light, 10-h dark photoperiod was maintained during the entire holding period. Fish were fed daily throughout the holding period.

Creosote Stock Preparation

Creosote stock preparation was similar to that described in the short-term toxicity chapter with the following exceptions. Polycarbonate bottles covered with black plastic were used instead of glass bottles for the following reason. During the short-term test, the stir motors required frequent adjustment because the propellers would

bang against the glass; a longer exposure period meant increased risk of breakage. To reduce the risk of breakage, stainless steel receptacles, which would hold the end of the propeller shaft in place, were fastened inside the glass bottles with epoxy cement. The receptacles came loose after a few days. As an alternative, polycarbonate bottles were fitted through the bottom with a stainless steel socket head cap screw (1/2 inch X 1 inch). The socket head served as the receptacle for the propeller shaft. Silicone rubber gasket material was used to prevent leakage around the screw.

The bottle used for the creosote-contaminated sediment received 65.5 g dry weight sediment; the bottle for the reference sediment received 163.6 g. These amounts were half of that used in the short-term study because six, instead of twelve, aquaria were being used. Only 3.3 g of creosote was added to the bottle for the creosote-contaminated sediment (50.4 mg creosote/g dry weight sediment) because the concentrations to be tested were lower than for the short-term test.

Exposure System

The flow-through system described in the previous chapter was modified as follows. York River water was pre-filtered through two high-rate sand filters (Model HR-31, Purex Corp., Industry, CA); one filter contained 1-mm filter aggregate, the other contained activated carbon. The water was further filtered through fiber-wound filters (two sets of a 10-μm filter, followed by a 1-μm filter, followed by an activated carbon filter). Water flow to the head tank was regulated by an electric ball valve (3/4 inch Electomni, Asahi/America, Medford, MA), which was controlled by two float switches in the head tank.

A 12,000 W heater (Glo-Quartz Titanium, Electric Heater Co.) situated in-line was controlled by a miniature microprocessor temperature controller (Model CN9111, Omega Engineering, Stamford, CT). Input to the controller was from a copper-constantan thermocouple (Type

T, Omega Engineering) located down-line of the heater. Upon detecting a temperature that was more than 5°C above or below the test temperature, the controller was programmed to initiate a shut-down sequence, which consisted of closing the electric ball valve, terminating power to the heater and peristaltic pump, and activating a telephone alarm system (Sensaphone Model 1000, Phonetics, Media, PA). A third float switch, located near the bottom of the head tank, was used to detect a loss of water supply. This third float switch also initiated the shut-down sequence.

Water exited the head tank through holes drilled in the side of the head tank near the bottom. Sediment was diluted to the desired concentration in the same mixing chambers used in the short-term study. The glass tubes exiting the mixing chambers were replaced with tubes calibrated to lower flow rates than used in the short-term test; the mixing chamber for the creosote-contaminated sediment had a tube calibrated to 2 L/min and the mixing chamber for the reference sediment had five tubes each calibrated to 1 L/min.

Nominal PAH concentrations of 62.5, 125, 250, 500, and 1000 μg/L were produced using the same serial diluter used in the short-term test. Each compartment of the diluter was fitted with a single glass tube calibrated for a flow rate of 1 L/min. Water from the diluter flowed through glass tubing to six 208-L glass test aquaria. The aquaria were placed on a totally covered wet table and each treatment was randomly assigned to an aquarium. External standpipes were used to maintain a constant water level in the aquaria; water volume in each aquarium was approximately 160 L. The aquaria had plexiglass covers. Black plastic was draped around the aquaria to isolate the fish from activity in the room. All waste water was passed through a 20-μm fiber-wound filter followed by a filter containing approximately 3 cubic feet of activated carbon.

Experimental Procedure

The experiment was divided into two phases. In the first phase (exposure phase), spot were exposed to creosote-contaminated sediments for up to 14 days. Samples of four fish were periodically removed and analyzed for hepatic EROD activity. After 14 days, the exposure system was cleaned and no more creosote-contaminated sediment was used. The experiment continued for an additional 14 days (recovery phase) during which sampling continued.

On the first day of the test (day 0), 58 spot were randomly assigned to each test aquarium. Each fish was measured (total and standard lengths) and weighed prior to being placed in an aquarium. Four randomly selected fish from each treatment were set aside for the day 0 samples. On days 1, 2, 4, 7, 10, and 14, four fish were removed from each aquarium with a dip net. Fish were inspected for gross abnormalities, measured (total and standard lengths), and weighed. Fish were sacrificed by rapidly severing the spinal chord. The liver was excised, rinsed in ice-cold 150 mM KCl, wrapped in labelled aluminum foil, and frozen in liquid nitrogen. Livers were stored at -80°C for up to 5 months (see Microsomal Preparation, below, for stability of P-450 during storage).

After sampling on day 14, the exposure system was cleaned as follows. The mixing boxes and the diluter were washed with laboratory detergent and rinsed sequentially with copious amounts of tap water, 3 N HCl, and acetone. The stock bottle containing the creosote-contaminated sediment was replaced with a clean bottle containing reference sediment. New tubing, to deliver stock sediment to the mixing chamber was installed. All delivery lines were rinsed several times with acetone and then water. With the fish still in the aquaria, the inside surfaces of the aquaria were cleaned with a scrubbing pad. Aquaria were drained to within 10 cm of the bottom and then allowed to refill with water

containing reference sediment. Sampling of fish continued on days 15, 17, 19, 23, and 28 as described above.

Temperature, salinity, dissolved oxygen, pH, and flow rate were measured and recorded daily for each aquarium as in the short-term test. Aquaria were inspected at least daily and any dead fish were removed, measured, and weighed. A 1-L water sample to determine creosote concentration (total resolvable PAH) was collected from the highest, middle, and lowest concentrations and from the control 2 days prior to starting the test and on days 3, 6, 9, 13, 16, 20, 24, and 29. A 0.5-L water sample to determine sediment concentration was collected from each treatment on the same days.

Microsomal Preparation

Livers were indiscriminately removed from the freezer, unwrapped, and allowed to thaw briefly at room temperature. Each liver was placed in approximately 10 ml of ice-cold buffer containing 100 mM potassium phosphate (pH 7.4), 1 mM dithiothreitol, 1 mM EDTA, 20% glycerol, and 0.1 mM phenylmethyl sulfonyl fluoride (PMSF; added from a 100 mM stock solution in ethylene glycol monomethyl ether). Livers were homogenized with a few short bursts of a Polytron tissue homogenizer (Model PT 10 20 350D, Brinkmann Instruments, Westbury, NY) equipped with a foam reducing generator (Model PTA 10TS, Brinkmann Instruments).

All centrifugations were performed at 4°C with a refrigerated centrifuge (Sorvall RC 28S, E.I. du Pont de Nemours & Co.) using a titanium rotor (Sorvall F-28/13, E.I. du Pont de Nemours & Co.). In between centrifugations all samples were kept on ice. The homogenates were centrifuged at 13,000 X *g* for 10 min. The supernatant fraction was decanted into a clean centrifuge tube being careful not to include the lipid pellicle and centrifuged again at 13,000 X *g* for 10 min. The supernatant fraction from the second centrifugation was transferred to another clean centrifuge tube and centrifuged at 100,000 X *g* for 60 min.

The supernatant fraction from the third centrifugation was removed with an aspirator. The pellet was gently resuspended in homogenization buffer and centrifuged at 100,000 X *g* for 60 min. The supernatant fraction from the fourth centrifugation was removed with an aspirator. The pellet (microsomal fraction) was gently resuspended in a few hundred microliters of homogenization buffer without PMSF, passed once through a 22 gauge syringe needle, transferred to a labelled cryogenic vial, and frozen in liquid nitrogen. Microsomal fractions were stored at -80°C for up to 2 months. Dithionite-reduced CO difference spectra (Omura and Sato, 1964) were obtained from randomly selected microsomal samples to ensure that no degradation of P-450 occurred during sample storage or microsomal preparation. Little or no degradation of P-450 was observed.

EROD Analysis

EROD activity was measured spectrophotometrically using a modification of the method of Klotz *et al.* (1984). A 1-mL reaction mixture containing 100 mM Tris-HCl (pH 8.0), 100 mM NaCl, 2 μM 7-ethoxyresorufin (Molecular Probes, Eugene, OR), and approximately 150 μg of microsomal protein was prepared being careful not to introduce any bubbles. The mixture was divided equally between two 0.5 mL optical glass cuvettes (1-cm path length). The cuvettes were placed in a dual-beam spectrophotometer (Model UV-160, Shimadzu Corp., Kyoto, Japan) and the instrument adjusted to zero absorbance at 572 nm. The reaction was initiated by the addition of 0.5 mM NADPH to the sample cuvette, again being careful not to introduce any bubbles. The reaction temperature was 25°C. The change in absorbance at 572 nm was monitored for up to 5 min. On each day the assay was performed, a previously measured sample was also assayed to ensure consistent results.

Triplicate analyses were performed for each sample. EROD activity (in pmoles/min/mg protein) was calculated by the formula:

$$\text{EROD Activity} = \frac{\Delta A_{572}\ \text{min}^{-1}\ \text{cm}^{-1} * V * 10^9\ \text{pmoles mmoles}^{-1}}{73.2\ \text{mM}^{-1}\ \text{cm}^{-1} * P}$$

where ΔA_{572} is the average change in absorbance per minute at 572 nm for the three measurements, V is the volume in the reaction cuvette in liters, and P is one-half of the amount of microsomal protein (in milligrams) added to the reaction cuvette. One-half of the amount of protein added is used in the calculation because only one-half of the protein is in the reaction cuvette. The extinction coefficient used for resorufin (73.2 mM^{-1} cm^{-1}) was that determined by Klotz *et al.* (1984). A detection limit of 30 pmoles/min/mg was determined based on the fluctuation of absorbance at 572 nm with no protein added.

Protein Analysis

Microsomal protein concentration was measured by the method of Lowry *et al.* (1951). Briefly, 1 mL of reaction mixture containing 2% Na_2CO_3, 0.1 N NaOH, 0.01% $CuSO_4*5H_2O$, and 0.02% potassium tartrate was added to a 200 μL sample containing 5-100 μg of microsomal protein. After 10 min, 100 μL of 1 N Folin and Ciocalteu's phenol reagent was rapidly added and immediately mixed. Color development was allowed to proceed for at least 30 min. Absorbance at 750 nm was measured against a blank prepared with 200 μL deionized water instead of the protein.

A series of crystalline bovine serum albumin standards were analyzed along with the samples. Protein concentration for the microsomal samples was calculated using the equation generated from linear least-squares regression of the absorbance of the standards against the concentration of the standards.

Chemical Analysis

Water sample analysis for total resolvable PAH was the same as described in the short-term toxicity chapter with the following exceptions. The extracts were concentrated to 0.5 mL because of the lower concentrations being measured. This required that only 10 μg 1,1'-binaphthyl be added as the internal standard. A DB-5 fused silica column, as described for the GC-MS analysis in the short-term toxicity chapter, was used for all gas chromatography.

Statistical Analysis

Temperature, salinity, pH, and dissolved oxygen data were analyzed using Kruskal-Wallis analysis of variance by ranks (Zar, 1984) with a significance level of 0.01. Non-parametric statistics were used because the hydrographic data failed to meet the assumptions of normality and homoscedasticity required for ANOVA (Underwood, 1981). Daily water quality measurements were considered to be time weighted replicate values. Sediment concentration, and initial fish standard length, total length, wet weight, condition factor, and tail length data were analyzed using one-way analysis of variance (Zar, 1984) with a significance level of 0.05.

PAH concentration data from the two phases of the experiment were analyzed separately using Kruskal-Wallis analysis of variance by ranks with a significance level of 0.01. The measurements at a given concentration were considered to be replicate values. A one-tailed Steel's many-one rank test (Steel, 1959), at a significance level of 0.05, was used to identify treatments with PAH concentrations that were significantly greater than those of the control.

EROD activity and final fish size (total, standard, and tail lengths and wet weight) and condition factor data were analyzed in three groups: days 0-7, days 7-14, and days 14-28. This was done because mortality at the highest concentration precluded fish from being sampled

after day 7 and mortality at the second highest concentration precluded fish from being sampled after day 14. Dividing the analyses and overlapping the data sets results in the experimentwise significance level being higher than stated. To compensate for the increased probability of erroneously rejecting a true null hypothesis (Type I error), a significance level of 0.01 was used for these analyses.

EROD activity data were analyzed using an extended Kruskal-Wallis analysis of variance by ranks (Zar, 1984) and final fish size and condition factor data were analyzed using two-way analysis of variance (Zar, 1984). PAH concentration and length of exposure were the factors evaluated. A one-tailed Steel's many-one rank test was used to identify treatments for which EROD activity was significantly greater than for the control and Dunn's multiple range test (Zar, 1984) was used to identify significant differences in EROD activity among sampling days. A one-tailed Dunnett's test (Zar, 1984) was used to identify treatments for which final fish size and condition factor were significantly lower than for the controls and Tukey's test (Zar, 1984) was used to identify significant differences in final fish size and condition factor among sampling days. All *a posteriori* tests were conducted with a significance level of 0.05.

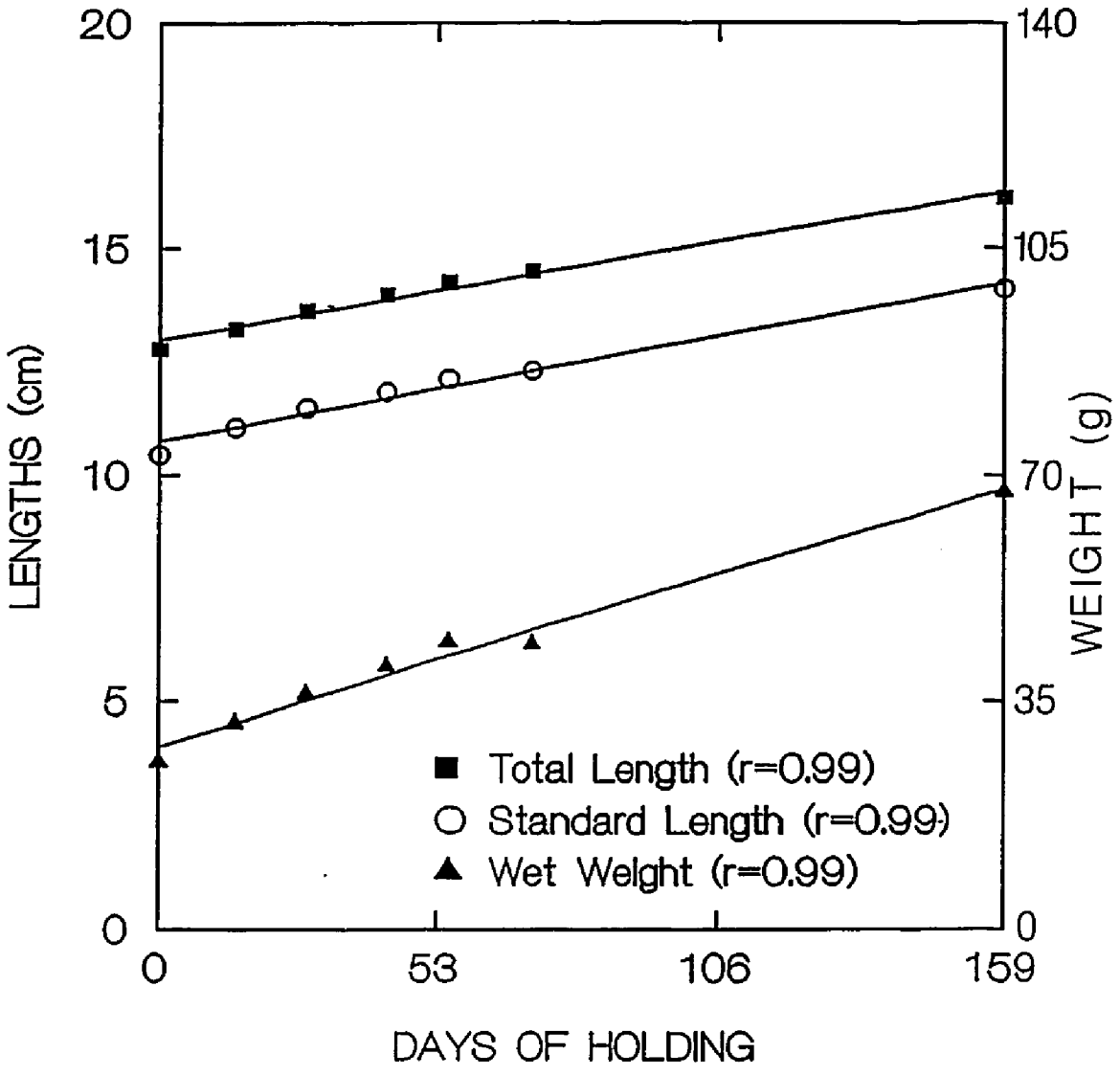
RESULTS

Growth During Holding Period

Total and standard lengths and wet weight increased linearly ($r = 0.99$) during the holding period (Figure 7). Total length increased an average of 0.21 mm/day. Standard length increased an average of 0.22 mm/day. Wet weight increased an average of 0.25 g/day. Condition factor increased from 1.23 ($N = 40$, $SD = 0.07$) at the beginning of the holding period to 1.59 ($N = 348$, $SD = 0.08$) at the beginning of the experiment.

FIGURE 7

Growth of spot during 5 month holding period prior to EROD response test.


20
15
10
5
0
140
105
70
35
0
LENGTHS (cm)
WEIGHT (g)
0
53
106
159
DAYS OF HOLDING
Total Length (r=0.99)
Standard Length (r=0.99)
Wet Weight (r=0.99)

Sediment Concentration

Sediment concentration was not significantly different among treatments (Table 6). Sediment concentration for all treatments was 16.5 mg/L (N = 54, SD = 3.6).

PAH Concentration

During the exposure phase, total resolvable PAH concentration was significantly higher in all treatments than in the control (Table 6). Almost 50% of the total PAH concentration was attributable to six compounds: naphthalene, acenaphthene, fluorene, phenanthrene, fluoranthene, and pyrene (Table 7).

In terms of percent of the nominal concentration, total measured PAH concentrations were relatively constant with dose. Total measured PAH concentration was about 30% of the nominal concentration for the highest concentration (Table 6). For the lowest PAH concentration, measured values were about 25% of the nominal concentration. The six most abundant PAH in creosote represented approximately 15% of the nominal concentration at all concentrations (Table 6).

During the recovery phase, PAH concentration rapidly decreased. On day 16, PAH concentration was 17 μg/L in the aquarium used for the 76 μg PAH/L treatment and 4.9 μg/L in the aquarium used for the 16 μg PAH/L treatment. Almost 67% of the measured PAH was due to phenanthrene. By the end of the experiment, PAH was not detectable.

Hydrographic Data

Temperature, salinity, dissolved oxygen, and pH were relatively constant with only minor differences among treatments (Table 8). Temperature ranged from 24-26°C. Salinity ranged from 17-19 g/kg. Dissolved oxygen always exceeded 70% of saturation and pH was approximately 7.9.

TABLE 6

PAH and Sediment Concentration for EROD Response Test

Nominal PAH (μg/L)	Total Resolvable PAH (μg/L)	Sum of Selected PAH[a] (μg/L)	Sediment Concentration (mg/L)
Control	BLD[b,c]	BLD	17.6 (2.0)[d]
63	16 (3.8)[e]	7.3 (0.6)	17.0 (3.2)
125	35[f]	16[f]	16.5 (4.0)
250	76 (20)[e]	36 (7.7)	14.7 (4.5)
500	150[f]	73[f]	16.3 (4.2)
1000	320 (75)[e]	149 (36)	16.6 (3.7)

[a] Naphthalene, acenaphthene, fluorene, phenanthrene, fluoranthene, and pyrene.

[b] Below the detection limit (<0.2 μg/L).

[c] Values are means of 5 measurements. Standard deviations are in parentheses.

[d] Values are means of 9 measurements. Standard deviations are in parentheses. No significant differences among treatments. One-way ANOVA ($\alpha = 0.05$).

[e] Significantly greater than the control. One-tailed Steel's many-one rank test ($\alpha = 0.05$).

[f] Estimated as the geometric mean of adjacent values.

TABLE 7

The Six Most Abundant PAH in EROD Response Test

Compound	Molecular Weight	% of Total at 320 µg/L	% of Total at 16 µg/L
Naphthalene	128	4.5 (1.0)[a]	11 (3.9)
Acenaphthene	154	4.6 (0.81)	4.5 (1.2)
Fluorene	166	4.1 (0.86)	3.4 (1.3)
Phenanthrene	178	15 (1.2)	12 (3.4)
Fluoranthene	202	12 (1.6)	9.9 (0.91)
Pyrene	202	7.2 (0.55)	6.0 (0.92)
Total		47.7	46.8

[a] Values are means of 5 measurements. Standard deviations are in parentheses.

TABLE 8

Hydrographic Data for EROD Response Test

PAH (μg/L)	Temperature (°C)	Salinity (g/kg)	Dissolved Oxygen (mg/L)	pH
CONTROL	24.7 (0.6)*	18.0 (0.4)	6.9 (0.3)	7.81 (0.10)
16	24.7 (0.6)	18.0 (0.4)	6.9 (0.3)	7.83 (0.11)
35	24.7 (0.6)	18.0 (0.4)	7.0 (0.3)	7.86 (0.09)
76	24.7 (0.6)	18.0 (0.4)	6.9 (0.5)	7.84 (0.13)
150	24.7 (0.6)	18.0 (0.4)	6.9 (0.4)	7.83 (0.09)
320	24.7 (0.6)	18.0 (0.4)	7.0 (0.3)	7.87 (0.10)

* Values are means of daily measurements. Standard deviations are in parentheses. No significant differences among treatments for any water quality parameter. Kruskal-Wallis analysis of variance by ranks using daily measurements as 24-h time weighted replicates (α = 0.01).

Distribution of Fish at Beginning of Test

There were no significant differences among fish assigned to any of the treatments for any of the parameters measured (Table 9). Mean standard length was 14.1 cm (SD = 0.9), mean total length was 16.1 cm (SD = 0.9), and mean wet weight was 67.5 g (SD = 12.8). Mean condition factor was 1.59 (SD=0.08). Mean tail length was 20.3 mm (SD = 2.0).

At the end of the experiment, there were three more fish in the 16 μg PAH/L aquarium than there should have been based on the number placed in the aquarium and the number sampled. There was one less fish in the 35 μg PAH/L aquarium and two less fish in the 76 μg PAH/L aquarium than there should have been. Only one aquarium was ever uncovered at a time suggesting that the fish were misplaced at the beginning of the experiment rather than having jumped from one aquarium to the other.

To evaluate the effect misplacement of fish may have had on the initial distribution of fish, the initial data set was modified in two ways: 1) the appropriate number of the most deviant measurements for each parameter from the treatments missing fish were reassigned to the treatment with extra fish and 2) the appropriate number of the least deviant measurements for each parameter from the treatments missing fish were reassigned to the treatment with extra fish (Table 10). The first approach maximizes the variance for the treatment with extra fish, while the second approach maximizes the variance for the treatments missing fish. The new data sets were reanalyzed. Using the modified data sets, there were still no significant differences among fish assigned to any of the treatments for any of the parameters measured.

Observations, Mortality, and Gross Pathology

During the exposure phase, fish at all PAH concentrations tested did not appear to eat while control fish fed normally. A few days into

TABLE 9

Initial Fish Measurements for EROD Response Test

PAH (µg/L)	Standard Length (cm)	Total Length (cm)	Wet Weight (g)	Condition Factor[a]	Tail Length[b] (mm)
CONTROL	14.1 (0.8)[c]	16.1 (1.0)	66.7 (12.3)	1.58 (0.10)	20.0 (2.5)
16	14.2 (0.7)	16.2 (0.8)	68.9 (10.9)	1.60 (0.09)	20.4 (2.0)
35	14.1 (0.9)	16.1 (1.0)	67.2 (13.2)	1.58 (0.07)	20.2 (2.0)
76	14.2 (0.8)	16.2 (0.9)	69.4 (12.5)	1.60 (0.08)	20.6 (1.7)
150	13.9 (1.0)	16.0 (1.1)	65.1 (13.9)	1.57 (0.09)	20.5 (2.1)
320	14.1 (0.9)	16.2 (1.0)	67.7 (13.6)	1.58 (0.08)	20.4 (1.8)

[a] Condition Factor = (100 * Wet Weight) / Total Length^3

[b] Tail Length = Total Length - Standard Length

[c] Values are means of 58 fish per treatments. Standard deviations are in parentheses. No significant differences among treatments for any measurement. One-way ANOVA ($\alpha = 0.05$).

TABLE 10

Initial Fish Measurements for EROD Response Test (Modified)

Modification	PAH (µg/L)	N	Standard Length (cm)	Total Length (cm)	Wet Weight (g)	Condition Factor[a]	Tail Length[b] (mm)
Most Deviant[c]	16	61	14.2 (0.8)[d]	16.1 (0.9)	68.7 (12.7)	1.60 (0.10)	20.2 (2.3)
	35	57	14.1 (0.8)	16.2 (0.9)	66.4 (12.2)	1.59 (0.06)	20.3 (1.7)
	76	56	14.2 (0.8)	16.3 (0.8)	70.3 (11.7)	1.60 (0.07)	20.6 (1.6)
Least Deviant[e]	16	61	14.2 (0.7)	16.2 (0.8)	68.9 (10.7)	1.60 (0.08)	20.4 (2.0)
	35	57	14.1 (0.9)	16.1 (1.0)	67.2 (13.4)	1.58 (0.07)	20.2 (2.0)
	76	56	14.2 (0.8)	16.2 (0.9)	69.4 (12.7)	1.60 (0.08)	20.5 (1.7)

[a] Condition Factor = (100 * Wet Weight) / Total Length^3

[b] Tail Length = Total Length - Standard Length

[c] Original data set modified by reassigning the two most deviant measurements from the 76 µg/L PAH treatment and the one most deviant measurement from the 35 µg/L PAH treatment to the 16 µg/L PAH treatment.

[d] Values are means. Standard Deviations are in parentheses.

[e] Original data set modified by reassinging the two least deviant measurements from the 76 µg/L PAH treatment and the one least deviant measurement from the 35 µg/L PAH treatment to the 16 µg/L PAH treatment.

the recovery period all remaining fish appeared to resume normal feeding habits.

Mortality was first noted on day 5 in the 320 μg PAH/L treatment. The peak of mortality at this concentration was on days 7 and 8. By day 10 there were no fish left to sample from the 320 μg PAH/L treatment. At 150 μg PAH/L, mortality began on day 6. Peak mortality at 150 μg PAH/L was on days 10 and 11. After sampling on day 14 there were no more fish in the 150 μg PAH/L treatment. Mortality in the 76 μg PAH/L treatment also began on day 6, but only 7 fish died at this concentration, the last one on day 18, 4 days after exposure had ceased. Mortality at 76 μg PAH/L was not severe enough to prevent fish from being sampled on any of the scheduled dates. There were no mortalities at 16 or 35 μg PAH/L or in the control.

Epidermal lesions and fin erosion began to appear shortly after mortality started, and only at the concentrations that were sufficient to cause mortality. Not all fish that died had lesions or eroded fins. Some fish with lesions survived until the end of the experiment. At 76 μg PAH/L, severe fin erosion was noted as late as day 19, but by the end of the experiment the remaining fish appeared to have normal fins. Most lesions were on mid-lateral surfaces although some lesions occurred around the opercula. The most severe lesions occurred on fish exposed to 76 μg PAH/L. Hemorrhaging in the absence of lesions was observed at the base of fins and around the mouth and nares. No lesions or fin erosion were noted on fish exposed to 16 or 35 μg PAH/L or on control fish. No cataracts were observed in any of the fish exposed in this experiment.

One fish, sampled on day 17 from the aquarium receiving 16 μg PAH/L, had a parasitic isopod (*Lironeca ovalis* Say) on its gills. The parasite was not firmly attached and was removed prior to processing. All measurements for this fish, including EROD activity, were within the range of values for the other fish sampled from the 16 μg PAH/L

treatment on day 17. No parasites or evidence of parasites were found on any other fish used in this study.

Fish Size and Condition

No significant differences were detected for total length, standard length, wet weight, or condition factor with respect to sampling day or treatment. Significant differences among sampling days and treatments were detected in the tail length data.

During the exposure phase of the experiment (Figure 8), tail length was dependent on length of exposure ($P = 0.006$) and PAH concentration ($P < 0.001$). No effect was observed until day 7 when fish from the two highest PAH concentrations had tails that were significantly shorter than control fish. Mean tail length for fish from these two treatments was 15.1 mm (SD = 1.6) on day 7. On days 10 and 14, no fish remained to be sampled at the highest PAH concentration tested. Only fish from the second highest PAH concentration (150 μg PAH/L) had tails that were significantly shorter than control fish.

During the recovery phase of the experiment (Figure 9), tail length was again dependent on length of exposure ($P = 0.003$) and PAH concentration ($P < 0.001$). Using Tukey's multiple comparison test, however, no significant differences among sampling days could be determined. Therefore, for each day, all treatments were compared to the control using one-tailed Dunnett's tests. Only fish that had been exposed to 76 μg PAH/L had significantly shorter tails than those of control fish. The differences were detected only on days 17 and 19. The maximum difference occurred on day 19, when tail length of fish exposed to 76 μg PAH/L averaged 15.3 mm (SD = 2.1).

EROD Activity

During the exposure phase of the experiment (Figure 10) EROD activity was dependent on length of exposure ($P < 0.001$) and PAH

FIGURE 8

Tail length of spot during 14 day exposure to creosote-contaminated sediment. Horizontal lines are ± 1 SD of mean tail length for all control fish sampled during the 14 days. * = significantly different from control on day indicated (one-tailed Dunnett's test, $\alpha = 0.05$). ND = no data.

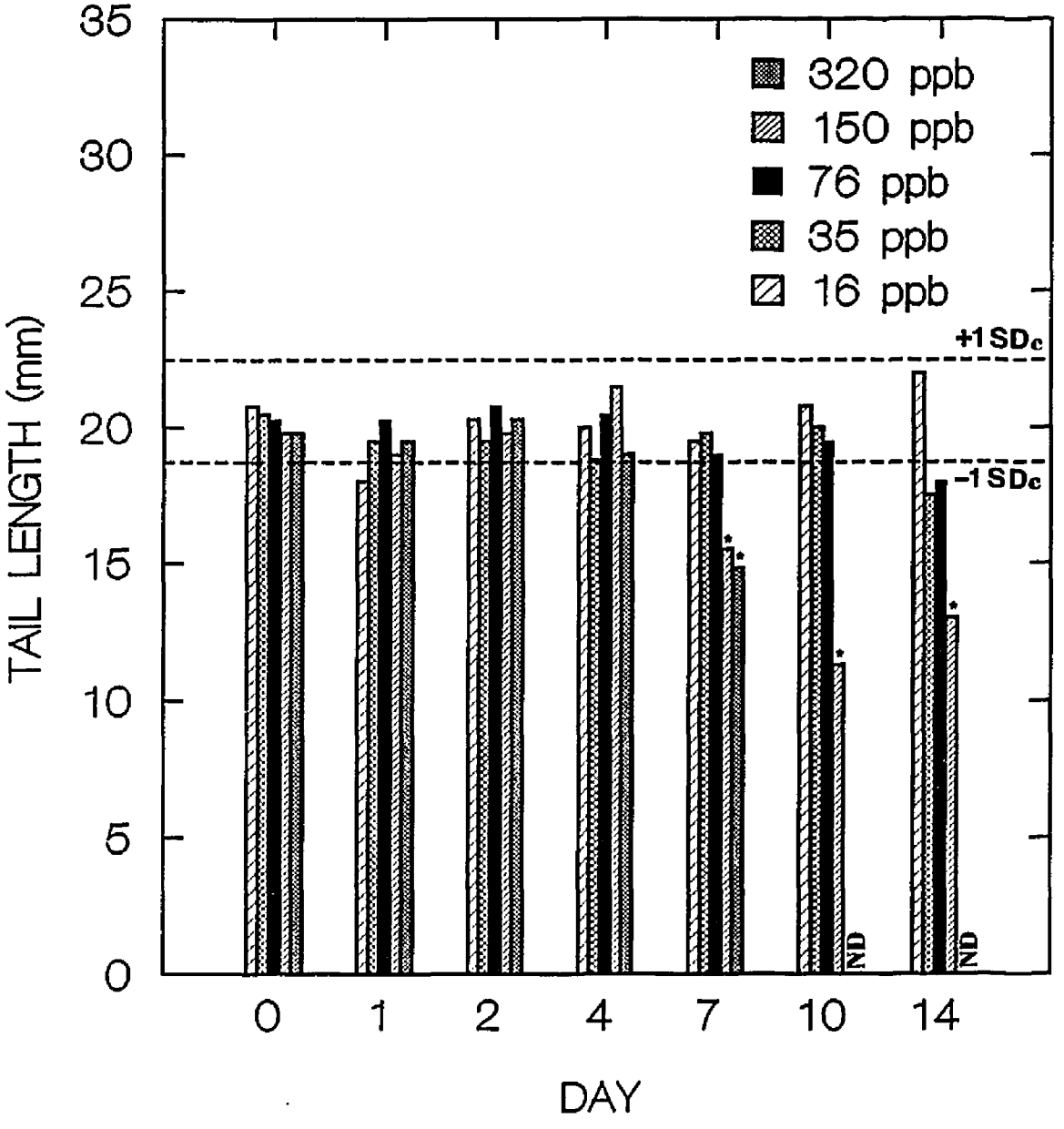

35
30
25
20
15
10
5
0
TAIL LENGTH (mm)
320 ppb
150 ppb
76 ppb
35 ppb
16 ppb
+1SDc
-1SDc
*
*
*
*
ND
ND
0
1
2
4
7
10
14
DAY

FIGURE 9

Tail length of spot during 14 day recovery period after exposure to creosote-contaminated sediment. Horizontal lines are ± 1 SD of mean tail length for all control fish sampled during the 14 days.
* = significantly different from control on day indicated (one-tailed Dunnett's test, $\alpha = 0.05$).

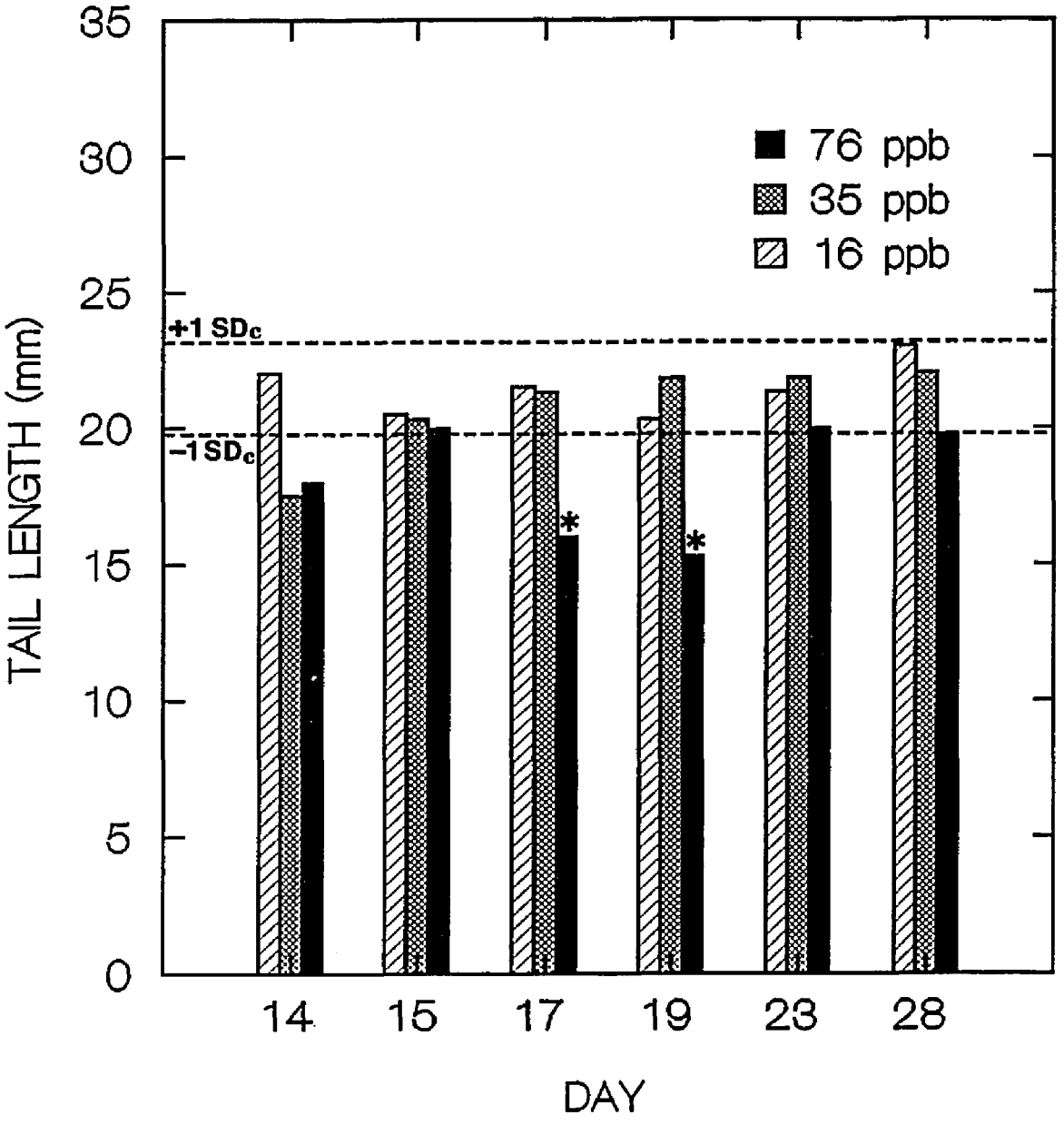
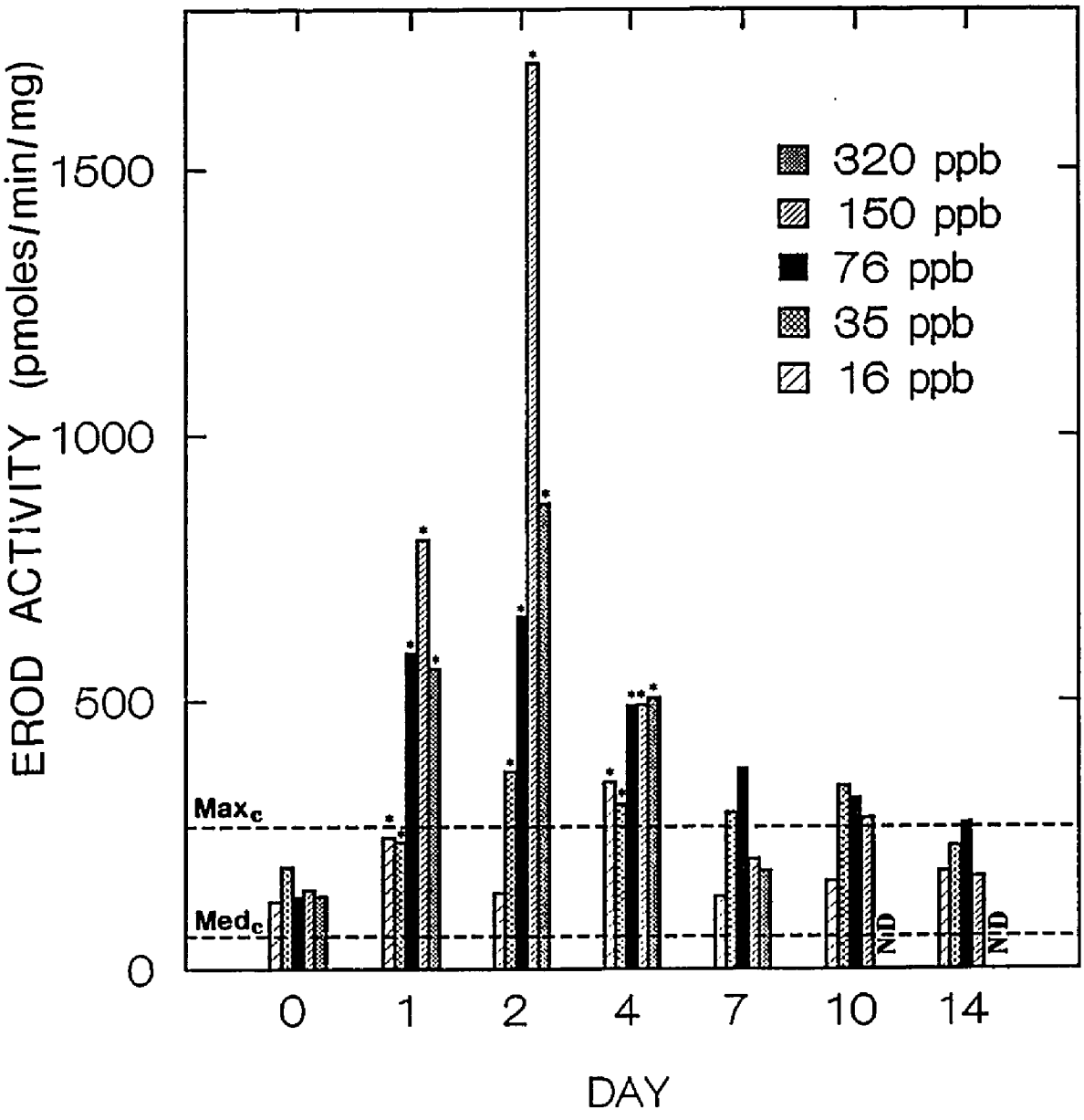

35
30
25
20
15
10
5
0
TAIL LENGTH (mm)
+1 SDc
−1 SDc
76 ppb
35 ppb
16 ppb
*
*
14
15
17
19
23
28
DAY

FIGURE 10

Hepatic EROD activity of spot during 14 day exposure to creosote-contaminated sediment. Lower horizontal line is median EROD activity for all control fish sampled during the 14 days. Upper horizontal line is maximum EROD activity for all control fish during the 14 days. * = significantly different from control on day indicated (one-tailed Steel's many-one rank test, $\alpha = 0.05$). ND = no data.


EROD ACTIVITY (pmoles/min/mg)
1500
1000
500
0
320 ppb
150 ppb
76 ppb
35 ppb
16 ppb
Max$_c$
Med$_c$
ND
ND
0
1
2
4
7
10
14
DAY

concentration ($P < 0.001$). Median EROD activity in control fish during the exposure period was 62 pmoles/min/mg (Interquartile Range [IQR] = below detection limit-166). At 35, 76, 150, and 320 μg PAH/L, EROD activity increased during the first two days of exposure. Maximal induction was almost 30-fold at 150 μg PAH/L. There was 14-fold induction at 320 μg PAH/L, 10-fold induction at 76 μg PAH/L, and 6-fold induction at 35 μg PAH/L. At 16 μg PAH/L, EROD activity was significantly higher than activity in control fish only on days 1 and 4. EROD activity sharply declined by day 4. By day 7, EROD activity was not significantly different than that on day 0.

During the recovery phase of the experiment (Figure 11), EROD activity was not dependent on length of exposure ($0.01 < P < 0.025$) or PAH concentration ($0.01 < P < 0.025$). Median EROD activity for all fish during the recovery period was 75 pmoles/min/mg (IQR = below detection limit-150).

DISCUSSION

Growth of Spot

Several studies have determined growth rates for feral young-of-the-year spot (Table 11). Increases in standard length for feral fish ranged from 0.23 to 0.54 mm/day. During the holding period prior to the present experiment, the increase in standard length was 0.22 mm/day. This value is only slightly lower than values obtained from feral fish, suggesting that the holding conditions prior to the present experiment were more than adequate.

Sediment Concentration

The suspended sediment concentration in this experiment (16.5 mg/L) was lower than that used in the short-term test (23.6 mg/L), but was still within the range of suspended sediment concentrations reported for Chesapeake Bay and its tributaries (Patten *et al.*, 1966;

FIGURE 11

Hepatic EROD activity of spot during 14 day recovery period after exposure to creosote-contaminated sediment. Lower horizontal line is median EROD activity for all control fish sampled during the 14 days. Upper horizontal line is maximum EROD activity for all control fish during the 14 days. No significant differences between recovering and control fish (one-tailed Steel's many-one rank test, $\alpha = 0.05$).
BLD = below the limit of detection.

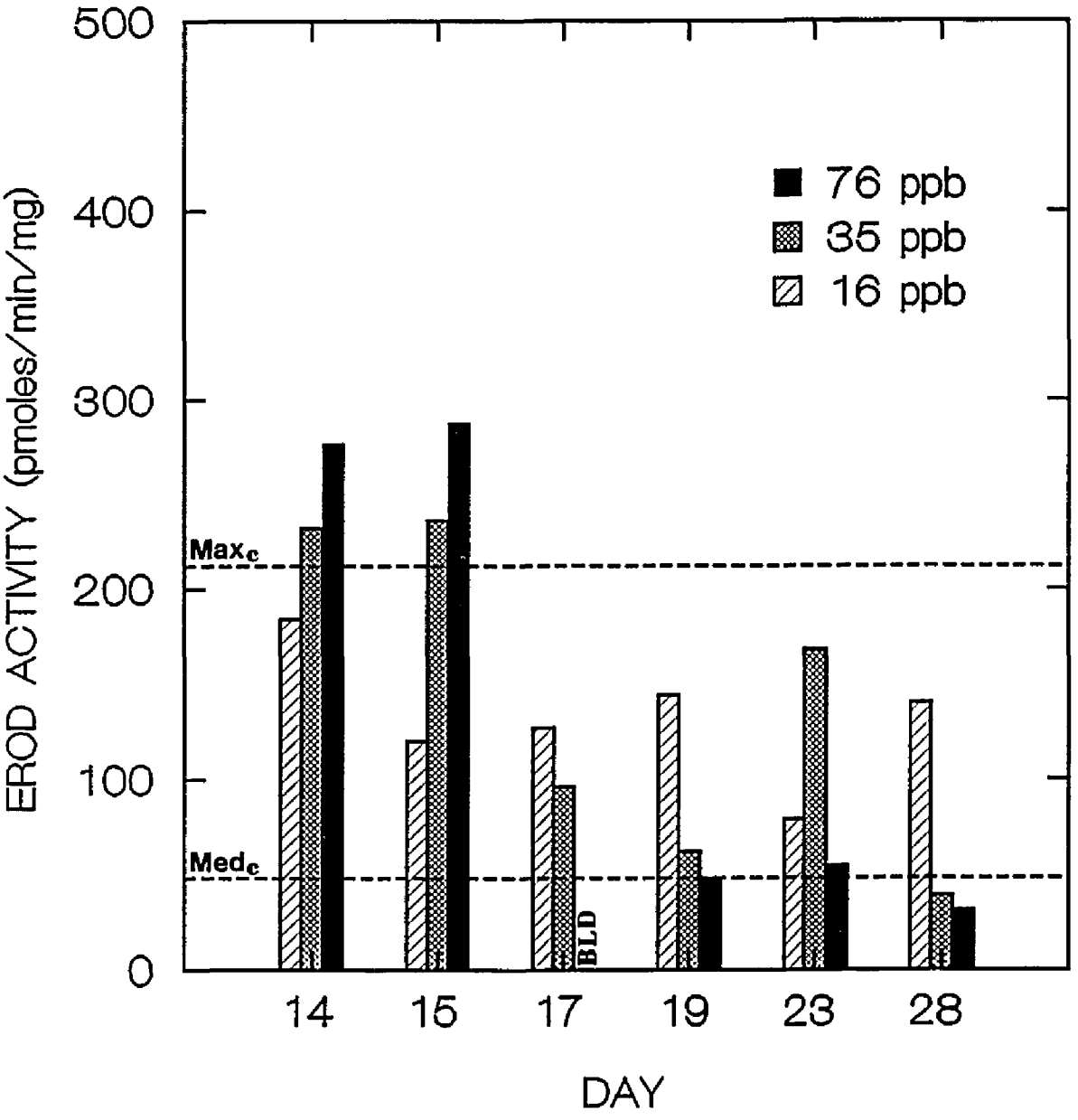

500
400
300
200
100
0
EROD ACTIVITY (pmoles/min/mg)
76 ppb
35 ppb
16 ppb
Max$_c$
Med$_c$
BLD
14
15
17
19
23
28
DAY

TABLE 11

Growth Rates of Feral Young-of-the-Year Spot

Location	Growth Rate (mm Standard Length/day)	Reference
Chesapeake Bay	0.35	Hildebrand and Schroeder (1928)
Beaufort, NC	0.39	Hildebrand and Cable (1930)
York River, VA	0.54	Pacheco (1957)
Cape Fear, NC	0.23	Weinstein and Walters (1981)
James River, VA	0.38	McCambridge and Alden (1984)
North Inlet, SC	0.30	Beckman and Dean (1984)

Schubel, 1977). Because sediment concentration can affect bioavailability (Opperhuizen and Stokkel, 1988) the actual percentage of bioavailable PAH may differ between the two experiments. Nevertheless, the gross pathological effects and mortality clearly indicate that PAH was bioavailable.

PAH Concentration and Composition

The measured PAH concentration as a percent of nominal concentration was lower in this experiment (25-30%) than in the short-term test (50-80%). The suspended sediment concentration was lower than expected indicating that less stock suspension may have been delivered than expected. Low PAH concentration may also have been due to the use of polycarbonate bottles for the creosote stock suspension. Dark stains were noted on the inside of the stock bottles, indicating that creosote adsorbed to the polycarbonate instead of being carried with the sediment to the exposure aquaria.

The composition of PAH in the exposure aquaria was also different for the two experiments. This may be due to several factors. The components of creosote may differentially adsorb to the polycarbonate stock bottles, resulting in a compositional change of the PAH delivered to the aquaria. The major difference, however, was in the contribution of naphthalene, which represented less of the total PAH in this experiment (4.5-11%) than in the short-term experiment (21%). The aqueous solubility of naphthalene is higher than that of other PAH constituents, suggesting that adsorption of naphthalene would be less than that for other PAH. On the other hand, naphthalene is more volatile than other PAH constituents. The decrease in percent naphthalene may have been due to the loss of naphthalene from the creosote source, although the source container was always stored tightly covered when not in use. Finally, because naphthalene is very volatile, losses during analysis may not have been consistent.

Some PAH persisted into the recovery period despite vigorous cleaning. When the aquaria were cleaned, the sediment that was stuck to the walls of the aquaria was not totally removed from the aquaria. The sediment was scrubbed off the walls of the aquaria, but remained in the aquaria. Draining the aquaria removed some, but not all, of this sediment. The remaining sediment was left to flush out of the system. Turn-over time for the aquaria was approximately 3 h, indicating that the sediment should have been flushed out rather quickly. Some contaminated sediment may have stuck to the gills of the fish and provided another source of PAH during the recovery period, but no appreciable accumulation of sediment was noted on the gills of fish sampled the day after the cleaning (day 15). PAH that was adsorbed directly to the walls of the aquaria may not have been totally removed by the cleaning procedure. Thus, several sources of PAH remained during the recovery period. These sources were depleted by the end of the experiment since no PAH was detectable on the last sampling day.

Epidermal Lesions and Mortality

Epidermal lesions were not noted in the short-term test, but were observed in this experiment. Similar lesions occurred in a similar time frame after exposing spot to PAH-contaminated sediment from the Elizabeth River, VA (Hargis *et al.*, 1984). In the present experiment, no cataracts were observed in fish exposed to creosote-contaminated sediments. Cataracts were observed in spot exposed to PAH-contaminated sediments in one particular experiment conducted outdoors (Hargis *et al.*, 1984)

Mortality was higher than expected based on the results of the short-term toxicity test. This is another example of the difficulties in extrapolating short-term results to long-term effects (Macek *et al.*, 1978). It is possible that starvation was a factor in the mortalities,

although Roberts *et al.* (1989) reported over 90% survival after starving spot for 11 days.

Fin Erosion

Caudal fin erosion was expected to be reflected in three of the measured parameters: total length, condition factor, and tail length. Only tail length provided a basis for quantifying caudal fin erosion. Failure to detect changes in total length may have resulted from trying to detect a small change in a relatively large measurement. Condition factor may have remained essentially constant if both total length and wet weight decreased.

EROD Activity

Statistical Considerations

Conservative statistical analysis of the EROD activity data was warranted (Helsel, 1990). Approximately 15% of the values were below the detection limit (censored data), preventing typical summary statistics, such as means and standard deviations, from being calculated. There are several approaches for estimating censored values, but all require poorly substantiated assumptions (Helsel, 1990).

As an alternative to using the mean as a measure of central tendency, median values were used. Median values can be calculated for data sets containing censored data provided less than half of the values are below the detection limit. IQR was used as an alternative to standard deviation as a measure of dispersion. The IQR can be determined for data sets containing censored data provided less than a quarter of the values are below the detection limit. For control fish in this experiment, over a quarter of the EROD activity values were below the detection limit so the IQR encompassed the detection limit.

Dose-Response

The relationship between dose and MO activity remains a controversial topic. Dose-dependent increases in MO activity have been reported (Gerhart and Carlson, 1978; Van Veld *et al.*, 1988, 1990). Other researchers have reported that MO activity is only dose-dependent to an extent, with activity reaching a plateau at high doses (Elcombe and Lech, 1978; Addison *et al.*, 1981). Other researchers have reported dose-dependent increases in MO activity to an extent, with activity then decreasing as dose is increased further (Nava and Engelhardt, 1982; Goddard *et al.*, 1987; Steadman *et al.*, 1991a).

Lack of a dose dependency as reported by Nava and Engelhardt (1982), Goddard *et al.* (1987), and Steadman *et al.* (1991a) was also found in the present experiment using spot exposed to creosote-contaminated sediments. Maximal induction of hepatic EROD activity was at 150 μg PAH/L. Induction at 320 μg PAH/L was approximately half that found at 150 μg PAH/L, although there was considerable variation and overlap in EROD activity values at these two concentrations.

Two of the experiments for which a dose-dependency was reported (Gerhart and Carlson, 1978; Van Veld *et al.*, 1988) spanned a much larger range of doses than the other studies, in terms of both absolute range and the orders of magnitude spanned. Spot collected from various areas of the Elizabeth River, VA had mean hepatic EROD activity that was highly correlated ($r = 0.95$) with sediment PAH concentration (Van Veld *et al.*, 1990). Fish from sites with 16-96 μg PAH/g sediment had EROD activities that were statistically indistinguishable because coefficients of variation were between 40 and 70% (Van Veld *et al.*, 1990). When doses are less than an order of magnitude apart, variation in MO activity may obscure differences among treatments. Various inducing compounds and routes of administration may produce different responses further complicating the dose-dependency controversy. Lack of

a dose-dependent response for MO activity would prevent the quantitative use of MO activity as an indicator of environmental contamination.

Time-Response

Induction of MO activity is typically a rapid response. A single administration of inducing agents by i.p. injection leads to maximal induction of hepatic BaPH activity within 3-4 days (James and Bend, 1980; Fingerman *et al.*, 1983). BaPH activity decreases shortly after maximum activity is reached (James and Bend, 1980; Fingerman *et al.*, 1983). Rapid induction of MO activity also occurs in response to continuous exposure. There is, however, some controversy concerning the persistence of the response over prolonged exposures.

Continued increases in MO activity have been reported for fish exposed to crude oil for as much as 30 days (Payne and Penrose, 1975; Collodi *et al.*, 1984). Other researchers have found that MO activity increases rapidly during the first few days of exposure and then levels off (Kurelec *et al.*, 1977; Van Veld *et al.*, 1988). Other researchers have reported rapid increases in MO activity followed by decreases in activity upon continued exposure (Nava and Engelhardt, 1982; Steadman *et al.*, 1991a). BaPH activity in fish collected from PAH contaminated sites varied over time and was not consistently higher than that in fish from a reference site (Roberts *et al.*, 1987).

The present experiment supports the results of Nava and Engelhardt (1982) and Steadman *et al.* (1991a). Hepatic EROD activity in spot exposed to creosote-contaminated sediments increased during the first 2 days (there were no samples taken on day 3). Activity then rapidly decreased, so that by day 7 activity was similar to that measured prior to exposure.

As with the dose-dependency, various inducing compounds and routes of administration may produce different time-responses. The dependence of MO activity on length of exposure is a crucial element in the

application of MO activity to environmental monitoring. In field collected samples, rarely is the length of exposure a known factor. Lack of a relationship between MO activity and length of exposure would result in difficulties in interpreting data obtained from feral fish.

Effect of Starvation

The effect of starvation on MO activity has been reported by several authors. Most authors have reported decreases in MO activity for starved fish. Cunner (*Tautogolabrus adspersus*) starved for 8 weeks had 50% less hepatic BaPH activity than fed control fish (Walton *et al.*, 1978). In rainbow trout, starvation for 6 weeks had no effect on hepatic BaPH or EROD activities, but after 12 weeks starvation, both measurements of activity were approximately 60% lower than activity in fed controls (Andersson *et al.*, 1985). The difference between starved and fed fish was not apparent after i.p. injection of an inducing agent (ß-Naphthoflavone). Spot starved for 5 days had decreases in hepatic EROD and BaPH activities of 60% and 75%, respectively, as compared to fed controls (Van Veld *et al.*, 1988).

In contrast, after starving eelpouts (*Zoarces viviparus*) for six months, Jensen and Knudsen (1983) found hepatic naphthalene hydroxylase activity that was 80% higher than activity in newly caught fish. These results are questionable because the newly caught fish were not a proper control.

In the present experiment, spot exposed to PAH did not appear to eat, whereas control fish fed normally. The decrease in hepatic EROD activity by the fourth day of exposure to PAH may have been due to starvation since no decrease in EROD activity was noted in control fish.

Inhibition of EROD Activity

Degradation of P-450 results in decreased enzymatic activity, whereas low EROD activity may or may not indicate low P-450

concentration. Degradation of P-450 by heme oxygenase results in decreased P-450 concentration and enzymatic activity (Ariyoshi *et al.*, 1990). Phosphorylation of P-450 inhibits MO activity and may also be involved in P-450 degradation (Jansson *et al.*, 1990). Several mechanisms can inhibit MO activity without loss of hemoprotein, including cofactor depletion, ligand binding to the heme moiety, and competition for the substrate binding site (Testa, 1990). Substrate binding site inhibitors can be reversible or irreversible and can be exerted directly by a compound or only after metabolic transformation.

Some substrates, such as 2,3,4,7,8-pentachlorodibenzofuran (Kuroki *et al.*, 1986) and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (Voorman and Aust, 1989), have been shown to bind tightly to the substrate binding site of certain forms of P-450. Blocking the substrate binding site prevents metabolism of other substrates. While chlorinated dibenzofurans and dibenzodioxins are not known constituents of creosote or coal tar (Kruber *et al.*, 1955), dibenzofuran is a major constituent (2.9%) and alkylated dibenzofurans are minor components. Creosote also contains dibenzothiophene (0.7%) and carbazole (1.3%), which are the sulfur and nitrogen analogs of dibenzofuran, respectively.

In the present experiment, no attempt was made to measure the concentration of the form(s) of P-450 responsible for EROD activity. Actual concentrations of an enzyme can be measured using immunological techniques, but these methods have limitations that are frequently overlooked. Milstein (1984) has urged researchers to be cautious in interpretation of measurements made using monoclonal antibodies because of cross-reactivity resulting from various factors: the same determinant on unrelated compounds, similar determinants on related compounds, divergent and convergent determinants on evolutionarily related compounds, and coincidental expression of determinant shape by unrelated compounds. Heteroclytic antibodies, which are made against one compound but preferentially recognize a different compound, can

further complicate matters (Milstein, 1984). Given the structural similarity of many of the forms of P-450 (Black and Coon, 1986, 1987), interpretation of P-450 immunological data should be done with the utmost care.

Effect of Non-Inducing Compounds

Creosote is a complex mixture containing over 160 organic compounds. All of these compounds are not necessarily capable of inducing MO activity. Phenanthrene, pyrene, fluoranthene, and chrysene, all of which are major creosote constituents, were not capable of inducing hepatic BaPH activity in rainbow trout (Gerhart and Carlson, 1978). Of the PAH tested by Gerhart and Carlson (1978), only BaP caused significant induction.

Rossi and Neff (1978) determined the acute toxicity of several PAH to a marine polychaete (*Neanthes arenaceodentata*). For the compounds tested, PAH with three or less aromatic rings were more toxic than PAH with four or more aromatic rings. Low aqueous solubility may limit the bioavailability of high molecular weight PAH (Neff and Anderson, 1981).

In the present experiment, induction of EROD activity followed by a sharp decline in activity may reflect an interaction between inducing agents and highly toxic compounds. Induction is a rapid response, whereas the PAH concentrations tested were lower than the 96-h LC50. Therefore, the possibility exists for induction to occur before the highly toxic components exert an effect. The decrease in EROD activity may then be due to the toxicity of major non-inductive creosote constituents.

TOXICITY OF SEDIMENTS CONTAMINATED WITH FRACTIONS OF CREOSOTE

INTRODUCTION

The multitude of compounds present in creosote raises the possibility that each response to creosote exposure that was observed in the previous experiment may be caused by different compounds. To date, little data are available to evaluate this possibility. Low molecular weight PAH were substantially more toxic than high molecular weight PAH in one study (Rossi and Neff, 1978). On the other hand, of the compounds in creosote, only BaP has been shown to significantly induce BaPH activity (Gerhart and Carlson, 1978).

PAH are found in the environment as complex mixtures from many sources. The composition of PAH in the environment is not necessarily identical to the composition of the original source. After input to the environment, PAH are affected by many factors including evaporation, dissolution, photooxidation, and adsorption to particulate matter (McElroy *et al.*, 1989). The various PAH are differentially affected by these processes, thus fractionating the source material. High molecular weight PAH typically associate with sediments because of their hydrophobic nature.

Objectives

The objective of this experiment was to determine if the toxic properties of creosote are attributable to low molecular weight PAH, high molecular weight PAH, or both. The following responses, which occurred following exposure to whole creosote, were evaluated:

1. Mortality.
2. Epidermal lesions and fin erosion.
3. Induction of EROD activity.
4. Lack of sustained EROD induction.

MATERIALS AND METHODS

Fish

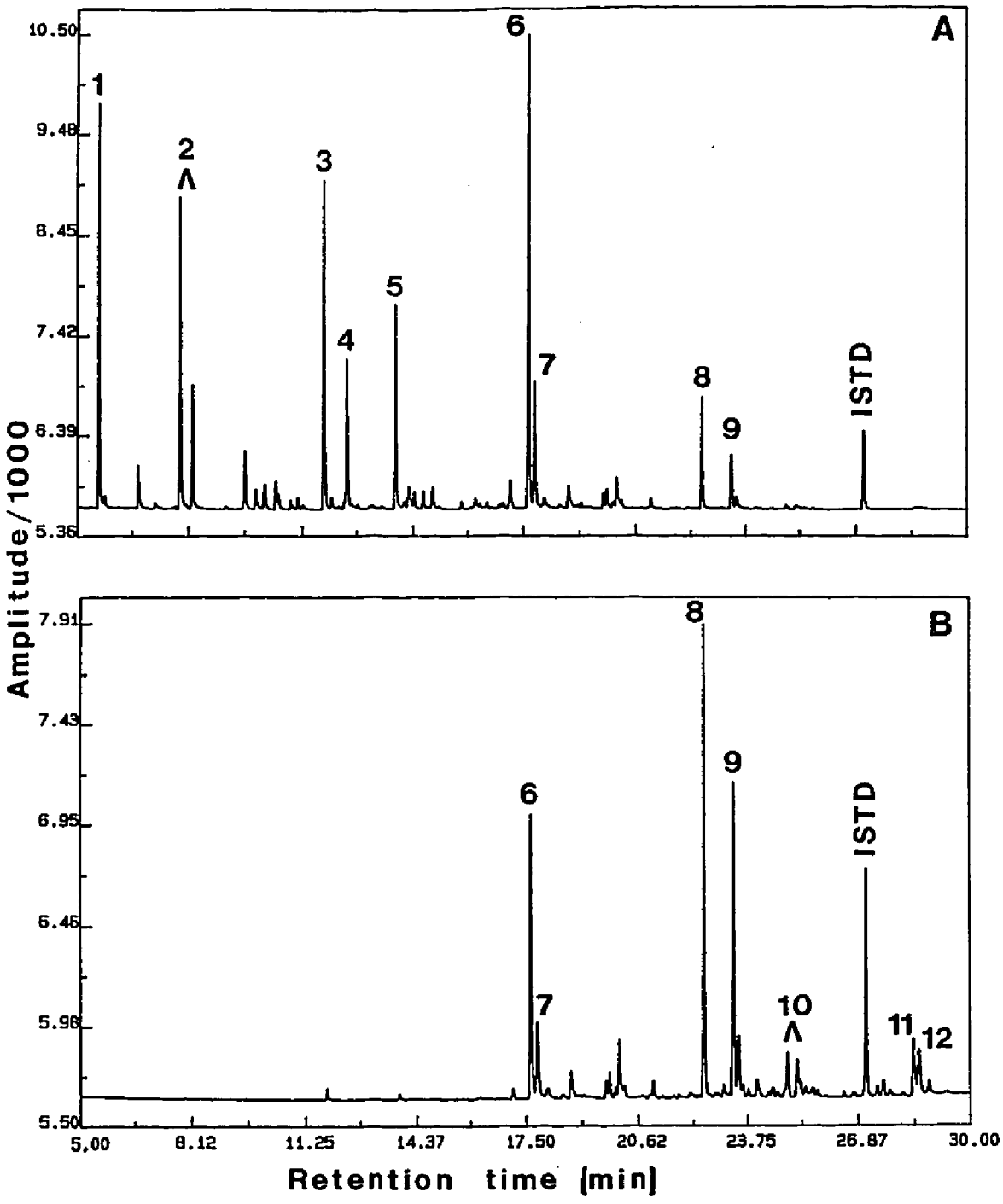
Fish were collected and transported to the laboratory as described in the previous chapter. Holding conditions were also as previously described. A sample of 28-30 fish was measured (total and standard lengths) and weighed periodically during the holding period to determine growth rates under holding conditions. The pre-test holding period was approximately 5 weeks.

Creosote Fractionation

Three 100-g samples of marine grade creosote were redistilled by the manufacturer as part of the routine procedure for verifying specifications. The distillates at 210, 235, 270, 315, and 355°C were combined and considered to be the low molecular weight fraction (LMWF). The residue at 355°C, which was paste-like after cooling, was dissolved in a minimal amount of acetone and considered to be the high molecular weight fraction (HMWF). The LMWF from the original three samples were pooled following distillation; the HMWF from the original three samples were also pooled. Gas chromatography of the two fractions revealed that certain PAH, particularly phenanthrene, anthracene, fluoranthene, and pyrene, occurred in both fractions (Figure 12). In the LMWF, compounds heavier than pyrene were not detectable (<0.9 μg/L). In the HMWF, compounds lighter than phenanthrene were not detectable (<0.7 μg/L).

FIGURE 12

Chromatograms of (A) low molecular weight fraction of creosote and (B) high molecular weight fraction of creosote. 1. Naphthalene, 2. Methylnaphthalenes, 3. Acenapthene, 4. Dibenzofuran, 5. Fluorene, 6. Phenanthrene, 7. Anthracene, 8. Fluoranthene, 9. Pyrene, 10. Benzofluorenes, 11. Benzo[a]anthracene, 12. Chrysene, ISTD = 1,1'-Binaphthyl.


A
B
1
2
3
4
5
6
7
8
9
10
11
12
ISTD
10.50
9.48
8.45
7.42
6.39
5.36
7.91
7.43
6.95
6.46
5.98
5.50
Amplitude/1000
5.00
8.12
11.25
14.37
17.50
20.62
23.75
26.87
30.00
Retention time (min)

Fraction Stock Preparation

Three polycarbonate bottles were used for the stocks: one for each of the creosote fractions and one for reference sediment. Each bottle received sediment slurry containing 32.8 g dry weight sediment. The bottles were filled as previously described with the following exception. Instead of adding creosote (or nothing for the reference sediment), one bottle received 1 mL of HMWF, one bottle received 345 μL of LMWF plus 1 mL of acetone, and the third bottle received 1 mL of acetone. Stocks were renewed daily.

Exposure System

The exposure system used was similar to that described in the previous chapter except that the mixing chambers and serial diluter were not used and only three exposure aquaria were required. Filtered York River water entered a distribution chamber that was identical to the mixing chamber used for the reference sediment in the previous experiments. Water exited the distribution chamber through three glass tubes, each calibrated for a flow rate of 1 L/min. Stock suspensions, at a flow rate of 11 mL/min, were added to the water just down-line of the distribution chamber.

Experimental Procedure

On the first day of the test (day 0), 53 spot were randomly assigned to each test aquarium. Each fish was measured (total and standard lengths) and weighed prior to being placed in an aquarium. Eight randomly selected fish from each treatment were set aside for the day 0 samples. On days 2, 4, 7, and 10, eight fish were removed from each aquarium and processed as described in the previous chapter. Livers were stored at -80°C for up to 2 months.

Temperature, salinity, dissolved oxygen, pH, and flow rate were measured and recorded daily for each aquarium as in the previous

experiments. Aquaria were inspected at least daily and any dead fish were removed, measured, and weighed. A 1-L water sample to determine total PAH concentration was collected from each aquarium twice prior to starting the test (days -5 and -1) and on days 3, 6, and 9. A 0.5-L water sample to determine sediment concentration was collected from each treatment on the same days with the exception that sediment concentration was not measured 5 days prior to starting the experiment.

Microsomal Preparation and Analyses

Microsomal fractions were prepared as described in the previous chapter and stored for up to 1 month prior to analysis. EROD and protein assays were performed as described in the previous chapter. Determination of PAH concentration was also performed as described in the previous chapter.

Statistical Analysis

Data were analyzed as in the previous experiment with the following exceptions. PAH concentration data were not analyzed in two groups because the present experiment only had an exposure phase. EROD activity and final fish measurement data were not analyzed in groups because mortality did not preclude fish from being sampled at any time. Multiple comparison tests (Dunn's test for PAH concentration and EROD activity data and Tukey's test for final fish measurement data) were used to identify differences in the various parameters among treatments. The previous experiment used *a posteriori* tests that only compared treatments to controls, whereas multiple comparison tests allowed for identification of differences in the various parameters between the LMWF and HMWF.

RESULTS

Growth During Holding Period

Total and standard lengths and wet weight increased linearly during the holding period (Figure 13). Total length increased an average of 0.19 mm/day ($r = 0.91$). Standard length increased an average of 0.23 mm/day ($r = 0.98$). Wet weight increased an average of 0.19 g/day ($r = 0.96$). Condition factor increased from 1.29 ($N = 30$, $SD = 0.04$) at the beginning of the holding period to 1.39 ($N = 153$, $SD = 0.07$) at the beginning of the experiment.

Sediment Concentration

Sediment concentration was not significantly different among treatments (Table 12). Sediment concentration for all treatments was 19.5 mg/L ($N = 12$, $SD = 1.4$).

PAH Concentration

Total resolvable PAH concentrations were significantly higher in both the LMWF and HMWF exposures than in the control (Table 12). Total resolvable PAH concentration was not significantly different between the LMWF and HMWF exposures (Table 12). The six most abundant compounds in whole creosote (naphthalene, acenaphthene, fluorene, phenanthrene, fluoranthene, and pyrene) accounted for approximately 70% of the total PAH in both the LMWF and HMWF exposures. The amount of the creosote fractions added to the sediment suspensions was normalized to the amount of phenanthrene in each fraction, with the goal being an equal amount of phenanthrene in both exposures. Phenanthrene concentration was 14 μg/L ($N = 5$, $SD = 3.1$) in the LMWF exposure and 16 μg/L ($N = 5$, $SD = 9.2$) in the HMWF exposure.

FIGURE 13

Growth of spot during 5 week holding period prior to fraction test.

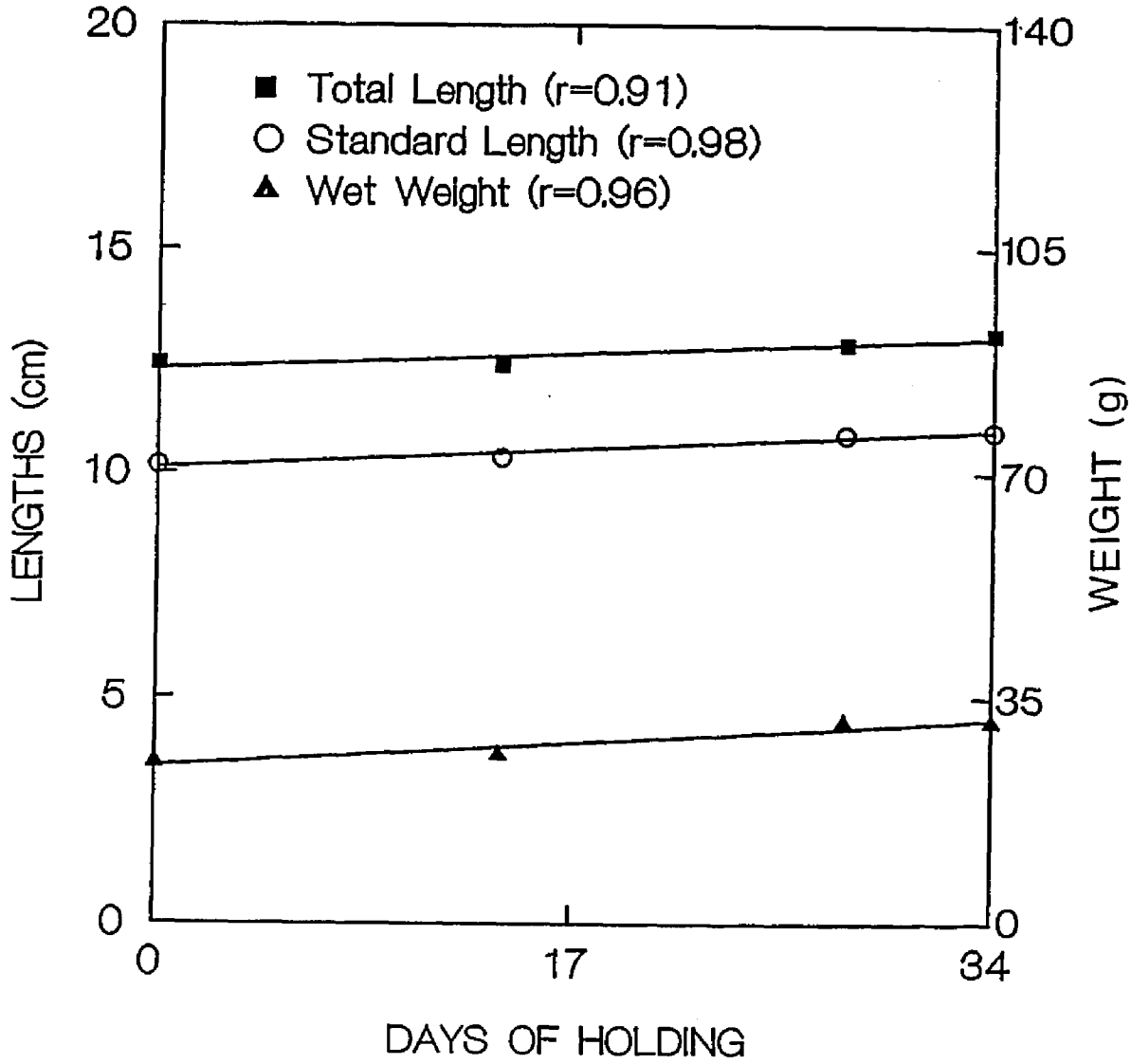

20
15
10
5
0
140
105
70
35
0
0
17
34
■ Total Length (r=0.91)
○ Standard Length (r=0.98)
▲ Wet Weight (r=0.96)
LENGTHS (cm)
WEIGHT (g)
DAYS OF HOLDING

TABLE 12

PAH and Sediment Concentration for Fraction Test

Treatment	Total Resolvable PAH (μg/L)	Sum of Selected[a] PAH (μg/L)	Sediment Concentration (mg/L)
Control	BLD[b,c]	BLD[b,c]	20.2 (2.2)[d]
LMWF[e]	49 (9.5)[f]	38 (6.9)	19.5 (0.8)
HMWF[g]	72 (42)[f]	53 (30)	18.8 (0.6)

[a] Naphthalene, acenaphthene, fluorene, phenanthrene, fluoranthene, and pyrene.

[b] Below the limit of detection (<0.4 μg/L).

[c] Values are means of 5 measurements. Standard deviations are in parentheses.

[d] Values are means of 4 measurements. Standard deviations are in parentheses. No significant differences among treatments. One-way analysis of variance ($\alpha = 0.05$).

[e] Low molecular weight fraction.

[f] Significantly different from the control, but not from other treatment. Dunn's test ($\alpha = 0.05$).

[g] High molecular weight fraction.

The concentration of individual PAH constituents other than phenanthrene was quite different for the LMWF and HMWF treatments (Table 13). The majority of PAH in the LMWF exposure was lighter than phenanthrene, while the majority of PAH in the HMWF exposure was heavier than phenanthrene. Naphthalene and the methylated naphthalenes were conspicuously absent from the routine water samples, which were collected from the aquaria prior to renewing the stock suspensions.

On day 3, shortly after preparing fresh stock suspensions, a water sample was collected from the delivery line leading to the aquarium receiving the LMWF. The concentrations of naphthalene, 2-methylnaphthalene, and 1-methylnaphthalene in this sample were 6.4, 5.9, and 1.8 μg/L, respectively. On day 11, even though the experiment had ended, fresh stock suspensions were prepared and delivered to the aquaria as done during the experiment. Five water samples were collected from the aquarium receiving the LMWF, one every 3 h. The major compounds present (acenaphthene, dibenzofuran, fluorene, and phenanthrene) showed no systematic change over time. Naphthalene and the methylated naphthalenes were originally below the detection limit (<0.4 μg/L). In the sample collected after 3 h, the concentrations of naphthalene, 2-methylnaphthalene, and 1-methylnaphthalene were 4.2, 3.3, and 1.4 μg/L, respectively. The concentrations of these compounds decreased with time so that by 12 h naphthalene was again undetectable and the methylated naphthalenes were each present at a concentration of approximately 1 μg/L.

Hydrographic Data

Temperature, salinity, dissolved oxygen, and pH were relatively constant with only minor, if any, differences among treatments (Table 14). Temperature ranged from 24-26°C. Salinity fluctuated between 20 and 21 g/kg. There were no significant differences among treatments for either temperature or salinity. Dissolved oxygen was always above 80%

TABLE 13

The Six Most Abundant PAH in Fraction Test

Compound	Molecular Weight	% of Total in LMWF[a]	% of Total in HMWF[b]
Naphthalene	128	BLD[c]	BLD
Acenaphthene	154	14 (5.3)[d]	2.9 (1.9)
Fluorene	166	12 (.75)	3.4 (2.3)
Phenanthrene	178	28 (1.9)	22 (2.0)
Fluoranthene	202	9.0 (1.9)	26 (3.1)
Pyrene	202	3.4 (.64)	15 (.90)
Total		66.4	69.3

[a] Low molecular weight fraction.

[b] High molecular weight fraction.

[c] Below the limit of detection (0.4 μg/L).

[d] Values are means of 5 measurements. Standard deviations are in parentheses.

TABLE 14

Hydrographic Data for Fraction Test

Treatment	Temperature °C	Salinity (g/kg)	Dissolved Oxygen (mg/L)	pH
CONTROL	24.8 (0.7)[a]	20.3 (0.4)	6.9 (0.1)	7.9 (0.1)
LMWF[b]	24.8 (0.7)	20.3 (0.4)	6.9 (0.1)	7.9 (0.1)
HMWF[c]	24.8 (0.7)	20.3 (0.4)	6.6 (0.2)[d]	7.7 (0.1)[d]

[a] Values are means of daily measurements. Standard deviations are in parentheses.

[b] Low molecular weight fraction.

[c] High molecular weight fraction.

[d] Significantly lower than other treatments. One-tailed Steel's many-one rank test ($\alpha = 0.05$).

of saturation and pH ranged from 7.6 to 7.9. The aquarium receiving the HMWF had significantly lower dissolved oxygen and pH than those for the other two treatments.

Distribution of Fish at Beginning of Test

There were no significant differences among fish assigned to any of the treatments for any of the parameters measured (Table 15). Mean standard length was 11.0 cm (SD = 0.5), mean total length was 13.1 cm (SD = 0.5), and mean wet weight was 31.4 g (SD = 4.2). Mean condition factor was 1.39 (SD = 0.07). Mean tail length was 21.3 mm (SD = 1.1).

Observations, Mortality, and Gross Pathology

Control fish ate normally throughout the experiment. Fish exposed to either creosote fraction also appeared to eat normally.

Mortality only occurred in the aquarium receiving the HMWF. Mortality was first noticed on day 8 and increased through the end of the experiment. A total of 10 fish had died by day 10. All dead fish had moderate to severe fin erosion and hemorrhaging. There were no mortalities in the aquarium receiving the LMWF or in the control aquarium.

Fin erosion and hemorrhaging were noticed on every fish sampled from the HMWF exposure on days 7 and 10. Prevalence of epidermal lesions for fish exposed to the HMWF increased from 13% on day 7 to 63% on day 10. No fin erosion was noticed on fish exposed to the LMWF. On day 7, two fish that had been exposed to the LMWF had slight hemorrhaging indicative of the onset of lesions. On day 10, 38% of the fish sampled from the LMWF exposure had epidermal lesions. All of the lesions on fish exposed to the LMWF were restricted to the area around the opercula and mouth. Lesions and fin erosion were not found on control fish.

TABLE 15

Initial Fish Measurements for Fraction Test

Treatment	Standard Length (cm)	Total Length (cm)	Wet Weight (g)	Condition Factor[a]	Tail Length[b] (mm)
Control	11.1 (0.4)[c]	13.2 (0.5)	32.4 (3.9)	1.41 (0.07)	21.3 (1.1)
LMWF[d]	10.9 (0.5)	13.0 (0.6)	30.9 (4.7)	1.39 (0.06)	21.1 (1.4)
HMWF[e]	10.9 (0.4)	13.1 (0.5)	30.9 (3.8)	1.38 (0.06)	21.5 (0.9)

[a] Condition Factor = (100 * Wet Weight) / Total Length^3

[b] Tail Length = Total Length - Standard Length

[c] Values are means of 53 fish for each treatment. Standard deviations are in parentheses. No significant differences among treatments for any measurement. One-way analysis of variance ($\alpha = 0.05$).

[d] Low molecular weight fraction.

[e] High molecular weight fraction.

Fish Size and Condition

No significant differences were detected for standard length with respect to sampling day or treatment. Significant differences among sampling days and treatments were detected in the total length, condition factor, and tail length data. Significant differences in wet weight were detected only with respect to treatment.

Total length was dependent on length of exposure ($P = 0.009$) and treatment ($P < 0.001$). Differences in total length were not detected until day 10, at which point the total lengths of fish exposed to the HMWF were significantly shorter than those of fish exposed to the LMWF or control fish (Figure 14).

Condition factor was also dependent on length of exposure ($P < 0.001$) and treatment ($P = 0.006$). Differences in condition factor were not detected until day 10 when condition factors of fish exposed to the HMWF were significantly higher than those of fish exposed to the LMWF or control fish (Figure 15).

Tail length was also dependent on length of exposure ($P < 0.001$) and treatment ($P < 0.001$). Differences in tail length were detected starting on day 7. On days 7 and 10, fish exposed to the HMWF had significantly shorter tails than fish exposed to the LMWF or control fish (Figure 16).

Wet weight was only dependent on treatment ($P < 0.001$). Control fish (33.35 g, SD = 4.67, N = 40) were significantly heavier than LMWF-exposed fish (30.10 g, SD = 4.43, N = 40) and HMWF-exposed fish (29.06 g, SD = 3.86, N = 40). There was no statistical difference between fish exposed to either of the creosote fractions.

EROD Activity

EROD activity was dependent on treatment ($P < 0.001$) and length of exposure ($0.001 < P < 0.005$). Median EROD activity in control fish was 89 pmoles/min/mg (IQR = 61-129). EROD activity in fish exposed to the

FIGURE 14

Total length of spot during 10 day exposure to creosote fractions. Horizontal lines are ± 1 SD of mean total length for all control fish sampled during the 10 days. * = significantly different from control and other treatment on day indicated (Tukey's test, $\alpha = 0.05$).

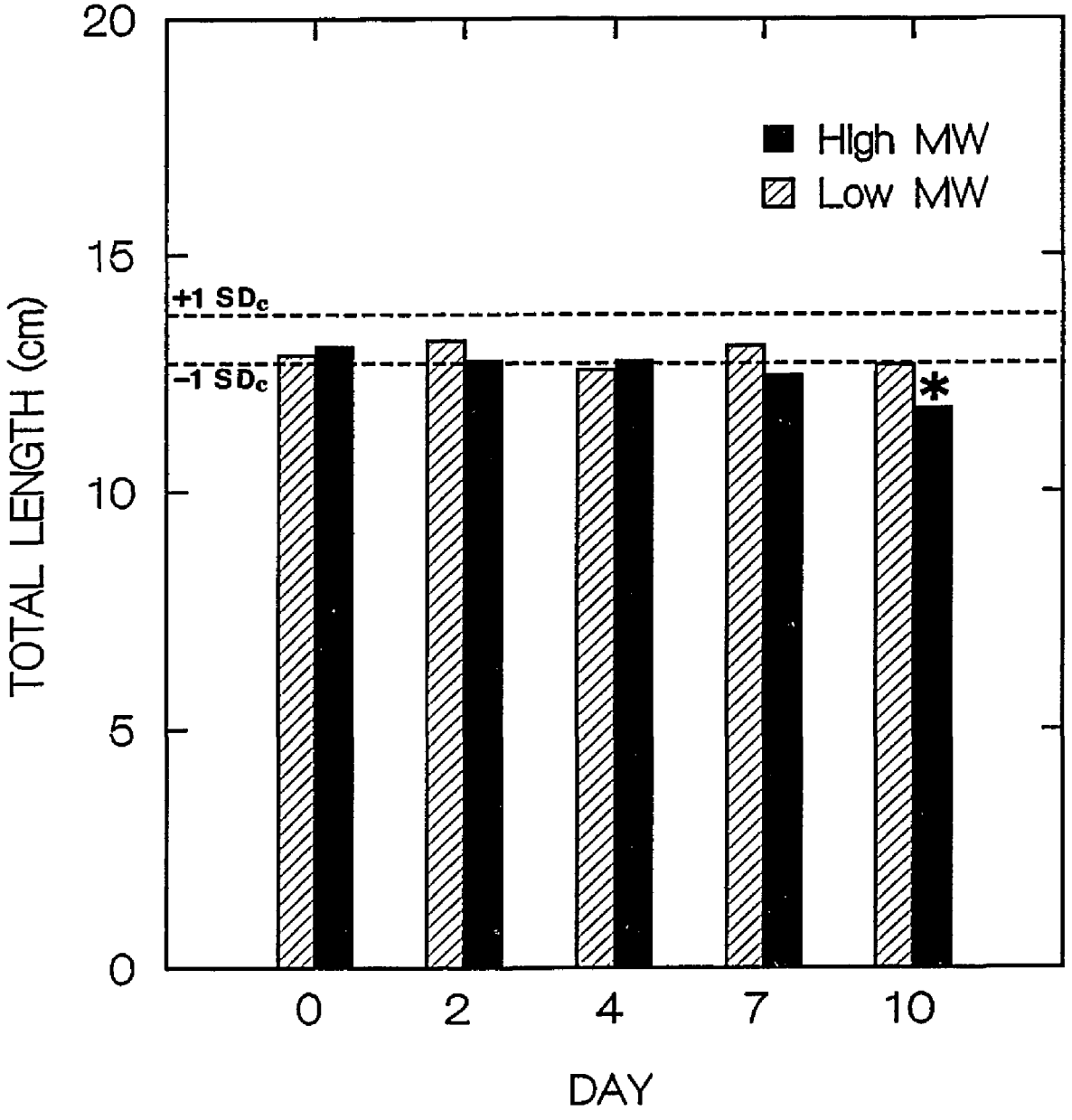

20
15
10
5
0
TOTAL LENGTH (cm)
+1 SDc
-1 SDc
High MW
Low MW
*
0
2
4
7
10
DAY

FIGURE 15

Condition factor of spot during 10 day exposure to creosote fractions. Horizontal lines are ± 1 SD of mean condition factor for all control fish sampled during the 10 days. * = significantly different from control and other treatment on day indicated (Tukey's test, $\alpha = 0.05$).

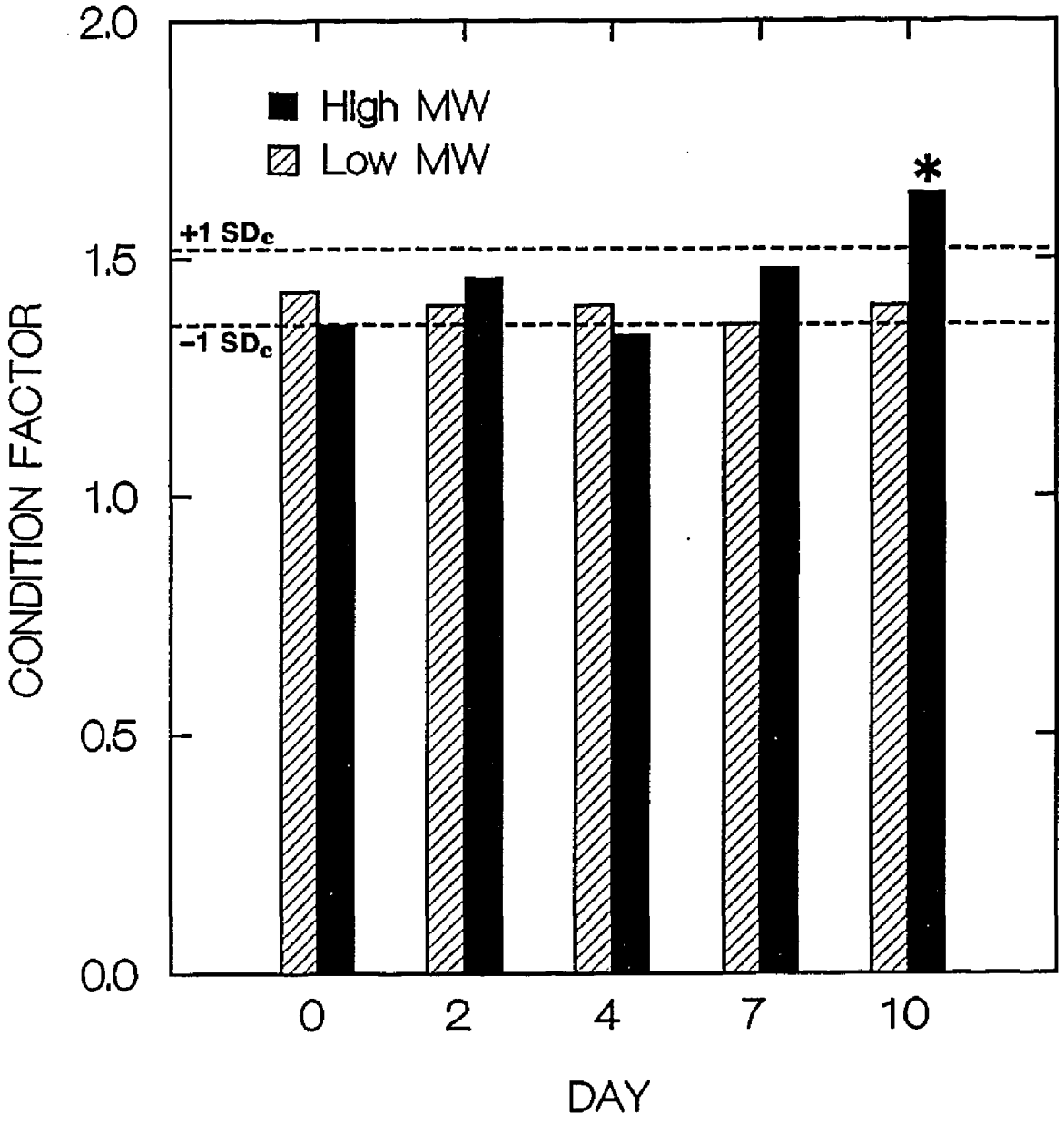
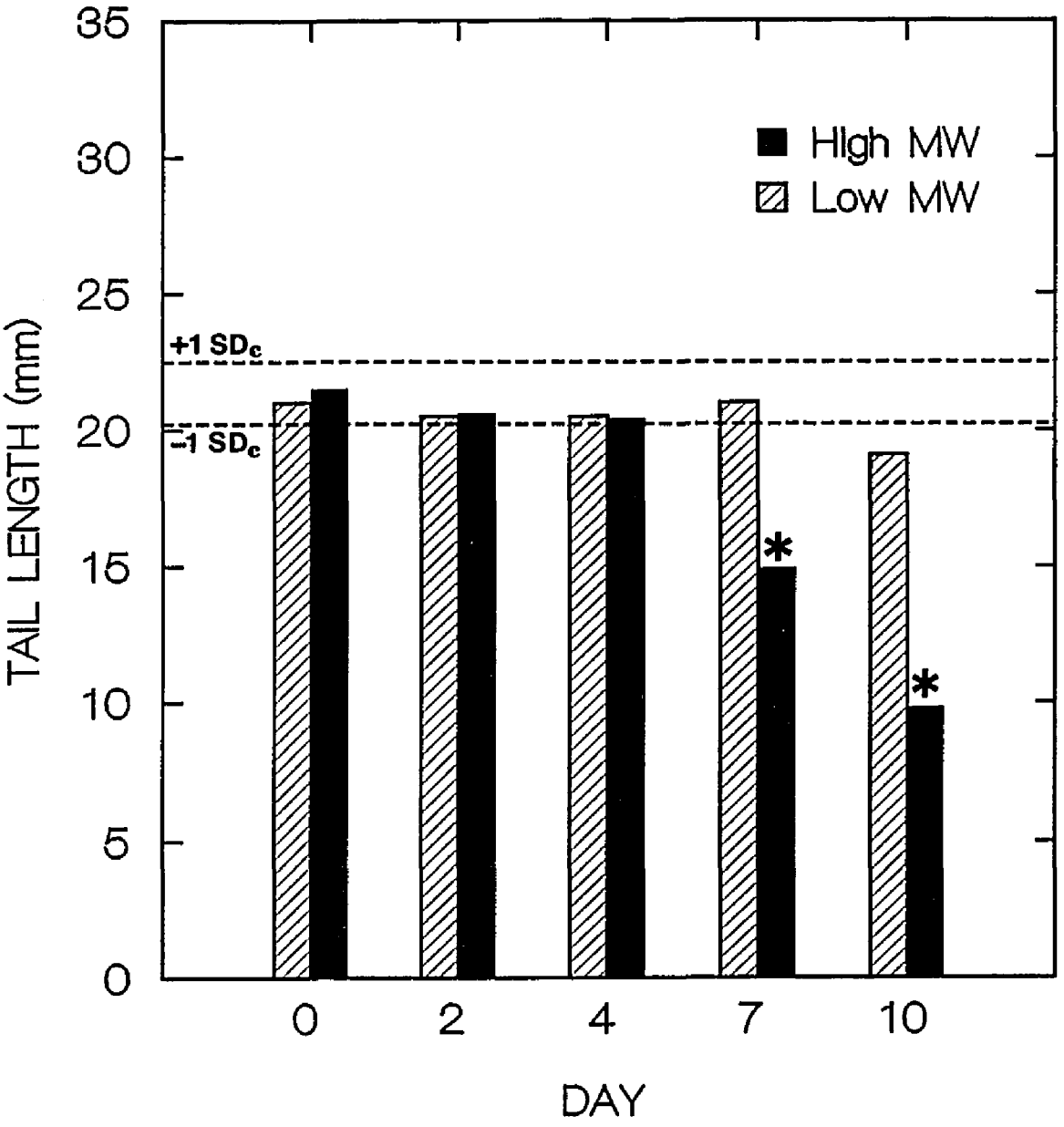

2.0
1.5
1.0
0.5
0.0
CONDITION FACTOR
High MW
Low MW
+1 SDc
−1 SDc
*
0
2
4
7
10
DAY

FIGURE 16

Tail length of spot during 10 day exposure to creosote fractions. Horizontal lines are ± 1 SD of mean tail length for all control fish sampled during the 10 days. * = significantly different from control and other treatment on day indicated (Tukey's test, $\alpha = 0.05$).


35
30
25
20
15
10
5
0
TAIL LENGTH (mm)
+1 SDc
-1 SDc
High MW
Low MW
*
*
0
2
4
7
10
DAY

HMWF increased to a maximum on day 2 and then decreased (Figure 17). Maximal induction was 9-fold. By day 7, EROD activity was not significantly different from that on day 0. EROD activity in fish exposed to the LMWF was always higher than that in control fish, but the difference was not statistically significant.

DISCUSSION

Growth of Spot

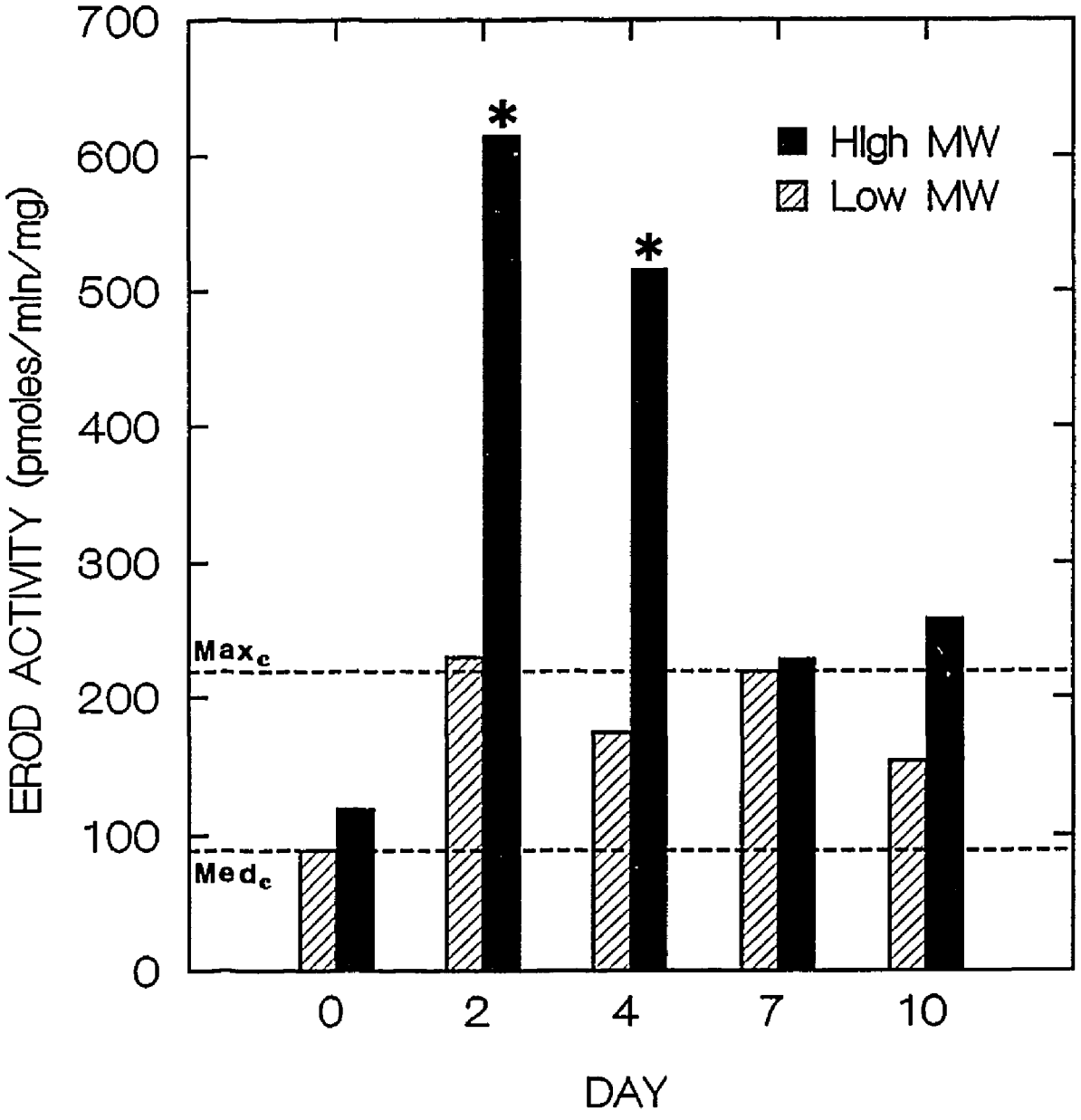
The growth rate of spot prior to the present experiment was similar to the growth rate prior to the previous experiment. Slightly lower growth rates were measured prior to the present experiment in terms of total length and wet weight (0.19 mm/day and 0.19 g/day, respectively) than prior to the previous experiment (0.21 mm/day and 0.25 g/day, respectively). Growth rate in terms of standard length was essentially identical for both holding periods. The increase in standard length of 0.23 mm/day compares favorably with growth rates obtained from feral spot populations (Hildebrand and Schroeder, 1928; Hildebrand and Cable, 1930; Pacheco, 1957; Weinstein and Walters, 1981; Beckman and Dean, 1984; McCambridge and Alden, 1984).

Sediment Concentration

The average suspended sediment concentration used in the present experiment (19.5 mg/L) was intermediate to the concentrations achieved in the previous experiments. Suspended sediment concentration in the short-term toxicity test was 23.6 mg/L and that in the induction experiment using whole creosote was 16.5 mg/L. The suspended sediment concentration in the present experiment was approximately equal to the average suspended sediment concentration for all the experiments combined (19.2 mg/L, SD = 5.3, N = 102). Coefficients of variation for individual treatments have typically been 20-30%. Given this inherent variability in the exposure system, the average values obtained for each

FIGURE 17

Hepatic EROD activity of spot during 10 day exposure to creosote fractions. Lower horizontal line is median EROD activity for all control fish sampled during the 10 days. Upper horizontal line is maximum EROD activity for all control fish during the 10 days. * = significantly different from control and other treatment on day indicated (Dunn's test, $\alpha = 0.05$).


700
600
500
400
300
200
100
0
EROD ACTIVITY (pmoles/mln/mg)
Max$_c$
Med$_c$
*
*
High MW
Low MW
0
2
4
7
10
DAY

of the experiments are all within the range expected. These values also reflect realistic suspended sediment concentrations (Patten *et al.*, 1966; Schubel, 1977).

PAH Concentration and Composition

The LMWF used in this experiment was essentially devoid of any PAH heavier than phenanthrene. The HMWF was essentially devoid of any PAH lighter than phenanthrene. Phenanthrene was chosen as the cutoff because the aqueous solubilities of PAH with molecular weights higher than that of phenanthrene can be several orders of magnitude less than the aqueous solubilities of the compounds lighter than phenanthrene (Eganhouse and Calder, 1976).

Neff and Anderson (1981) have suggested that the aqueous solubility of PAH is the dominant factor influencing the toxicity of PAH. PAH with molecular weights up to and including that of phenanthrene are much more toxic than PAH with molecular weights higher than that of phenanthrene (Rossi and Neff, 1978). The partitioning of PAH in the environment reflects the aqueous solubilities of PAH. On sediments collected from creosote-contaminated sites, the percent of total PAH represented by PAH with low aqueous solubilities is typically enriched compared to whole creosote (Bieri *et al.*, 1986; Huggett *et al.*, 1987; Roberts *et al.*, 1989).

Total PAH concentrations for the fraction exposures were similar to the minimum concentration of whole creosote (76 μg PAH/L) that yielded pronounced induction of EROD activity. The concentrations of the major PAH constituents in the LMWF exposure (acenaphthene, fluorene, and phenanthrene) were intermediate to the concentrations of these components at whole creosote concentrations of 150 and 76 μg PAH/L. The concentrations of the major PAH constituents in the HMWF exposure (phenanthrene, fluoranthene, and pyrene) were also intermediate to the

concentrations of these components at whole creosote concentrations of 150 and 76 μg PAH/L.

Differences in Hydrographic Parameters

Slight differences in dissolved oxygen concentration and pH were found between the aquarium used for the HMWF exposure and the other two aquaria. As discussed in the chapter on short-term toxicity, these differences, while statistically significant, are probably not biologically significant.

Epidermal Lesions and Mortality

The prevalence of epidermal lesions was higher for fish exposed to the HMWF than for those exposed to the LMWF. There was a distinct difference in the location of the lesions produced by each fraction. Fish exposed to the LMWF had lesions surrounding the mouth and opercula, whereas fish exposed to the HMWF typically had lesions on the sides of the body. Some fish exposed to the HMWF also had lesions around the opercula.

In the previous experiment, lesions were primarily observed on mid-lateral surfaces. Hargis *et al.* (1984) reported similar lesions on spot exposed to PAH-contaminated sediment. The high prevalence of mid-lateral lesions on fish exposed to the HMWF suggests that high molecular weight PAH, typically associated with sediment, are capable of causing severe penetrating lesions. The absence of lesions typical of the LMWF in fish exposed to whole creosote remains puzzling.

One might argue that compounds that were common to both fractions were responsible for the formation of lesions. The difference in the location of lesions resulting from exposure to the two fractions does not support this hypothesis. Instead, there appear to be different compounds in each fraction capable of producing distinct types of

lesions. Exposure of fish to subsets of these fractions or purified compounds may be of help in identifying the responsible agents.

Mortality only occurred in fish exposed to the HMWF. This was unexpected because earlier work (Rossi and Neff, 1978) indicated that low molecular weight PAH were more toxic than high molecular weight PAH. Rossi and Neff (1978) did find that the toxicity of PAH increased with increasing molecular weight up to phenanthrene. Methylphenanthrenes and fluoranthene were also quite toxic. Phenanthrene was present in both fractions at equal concentrations, which suggests that if phenanthrene were the major toxic agent then both fractions should have had equal toxicity. On the other hand, the compounds slightly heavier than phenanthrene (methylphenanthrene and fluoranthene) occurred at much higher concentrations in the HMWF than in the LMWF. The presence of these moderate molecular weight compounds may significantly increase the toxicity of the fraction.

Condition of Fish

Control fish were heavier than fish exposed to either of the creosote fractions. Length of exposure was not a significant factor indicating that the difference was probably not due to exposed fish failing to eat. Although the difference was not statistically significant, the average weight of fish assigned to the control treatment was slightly higher than the average weight of fish assigned to either of the fraction exposures. The difference seen in the sampled fish may reflect the difference in the initial distribution. After sampling on day 10, there was no large discrepancy in the weight of the remaining fish.

Unlike the previous experiment, caudal fin erosion in the present experiment was evidenced by changes in total length, condition factor, and tail length. Obviously, shorter tails would be reflected in decreases of total length and tail length. Condition factor, which is

inversely proportional to total length cubed, would be expected to increase as total length decreased, provided weight remained constant. The three measures were not equally sensitive. Changes in total length and condition factor were only detected on day 10. Tail length was the most sensitive indicator of caudal fin erosion with changes being detected as early as day 7.

EROD Activity

EROD activity in fish exposed to the LMWF was never significantly different from that in control fish indicating a lack of inducing agents in the LMWF. Both trimethylnaphthalene and phenanthrene have been shown to be incapable of inducing BaPH activity in rainbow trout (Gerhart and Carlson, 1978).

The response of EROD activity to the HMWF was very similar to the response to whole creosote. EROD activity in fish exposed to either the HMWF or whole creosote increased to a maximum on day 2. Median activity on day 2 of fish exposed to 72 μg PAH/L from the HMWF was 615 pmoles/min/mg (IQR = 567-663). Median activity on day 2 of fish exposed to 76 μg PAH/L from whole creosote was 659 pmoles/min/mg (IQR = 648-669). EROD activity in fish exposed to either the HMWF or whole creosote decreased after day 2 and by day 7 was not significantly different from EROD activity on day 0.

GENERAL DISCUSSION

MO Activity as an Indicator of PAH Exposure

Several criteria have been proposed for evaluating the utility of biochemical responses as indicators of chemical exposure (Neff, 1985). These will be addressed individually in the following subsections.

Sensitivity to Environmental Concentrations

Biochemical indicators need to be sensitive to environmentally realistic concentrations of chemicals. In the induction experiment using whole creosote, the lowest PAH concentration tested was 16 μg PAH/L. In terms of PAH on sediment, the lowest concentration tested was 800,000 ng PAH/g dry weight sediment, which is over twice the highest concentration reported for the Elizabeth River, VA, one of the most heavily PAH contaminated rivers in the world (Bieri *et al.*, 1986). At this lowest concentration tested, EROD activity was only sometimes marginally higher than activity in control fish. Therefore EROD activity does not appear to be very sensitive.

The above comparison is not necessarily a good one. Exposures in the experiments presented here used suspended sediment concentrations of approximately 20 mg/L, well within the range reported for Chesapeake Bay and its tributaries (Patten *et al.*, 1966; Schubel, 1977). Patten *et al.* (1966) noted that suspended sediment concentrations increase with depth and that near bottom suspended sediment concentrations are probably closer to 200 mg/L than 20 mg/L. Suspending the most contaminated Elizabeth River sediment (approximately 400,000 ng PAH/g dry weight sediment) at a sediment concentration of 200 mg/L would produce a

suspended PAH concentration of approximately 80 μg PAH/L. The PAH concentrations tested in the present work encompassed this value. Based on this comparison, induction of EROD activity is sensitive to environmentally realistic PAH concentrations.

Exposure-Dependency

Both the time and dose elements of exposure are crucial. If the response is not dose-dependent, the indicator cannot be used quantitatively. If the response varies temporally, the significance of the response is questionable.

The dose-dependency of MO induction is questionable. The present research along with others (Elcombe and Lech, 1978; Addison *et al.*, 1981; Nava and Engelhardt, 1982; Goddard *et al.*, 1987; Steadman *et al.*, 1991a) did not find a true dose-dependent response for P-450 activity. The lack of dose-dependency has always been seen at high concentrations, which may not be environmentally realistic.

Other research (Gerhart and Carlson, 1978; Van Veld *et al.*, 1988) has suggested that induction of MO activity is dose-dependent, even at high doses, when BaP is administered in the diet or by i.p. injection. A dose-dependent response was also suggested in a field study (Van Veld *et al.*, 1990), where the PAH concentration gradient spanned four orders of magnitude. High variance (coefficients of variation up to 70%) was associated with high EROD activity in the field caught fish, preventing statistical distinction of a dose-dependent response.

Decreases in EROD activity after only a few days of exposure found in the present study and by Steadman *et al.* (1991a) present an additional problem. In the field, the length of time that fish are at a particular location is usually impossible to determine. If induced EROD activity is not maintained under controlled laboratory exposures, then difficulties could be encountered in interpreting field collected data.

Sensitivity to Other Environmental Stresses

Biochemical indicators need to be insensitive to other environmental stress. Environmental stresses other than PAH contamination were not addressed in the work presented here, but some stresses have been addressed by other researchers. Other stresses have generally been shown to effect MO activity.

Temperature has been shown to alter both basal MO activity and the induction response. Fish acclimated to low temperatures consistently have higher basal BaPH activity than fish acclimated to high temperatures (Dewaide, 1971; Stegeman, 1979; Koivusaari, 1983; Ankley *et al.*, 1985). In contrast, the effect of temperature on induction of MO activity is inconclusive. BaPH activity in mummichogs acclimated to low temperatures did not increase following an i.p. injection of BaP (Stegeman, 1979). BaPH activity in sheepshead minnows acclimated to low temperatures did increase following an i.p. injection of 3-MC, but induction was not as pronounced and took longer than induction in sheepshead minnows acclimated to high temperatures (James and Bend, 1980). A longer time course for induction at low temperatures was also noted with channel catfish, but the level of induction was independent of temperature (Fingerman *et al.*, 1983).

The effect of heavy metals on MO activity has also been investigated. An inhibitory effect of heavy metals on mammalian MO activity is well documented (Nebert and Gelboin, 1968; Yoshida *et al.*, 1976). Many heavy metals induce the activity of the heme degrading enzyme, heme oxygenase (Maines and Kappas, 1975, 1976), which may lead to decreased MO activity (Ariyoshi *et al.*, 1990). Inhibition of EROD and BaPH activities by heavy metals has been reported for fish (Fair, 1986; Förlin *et al.*, 1986; George and Young, 1986). Metals can also affect membrane permeability by disrupting the ionic balance of cells (Viarengo, 1985).

Relationship to High Order Effects

In order to be a valuable indicator, biochemical responses should be detectable before any high order effects occur and should indicate that high order effects will occur upon continued exposure. This has yet to be adequately demonstrated for MO activity, but theoretically P-450 is required for the metabolic activation of procarcinogenic PAH to ultimate carcinogens. The role of P-450 in acute toxicity of PAH is not known.

In the present research, changes in MO activity occurred rapidly upon exposure to creosote and the high molecular weight fraction of creosote. The induction of MO activity, while ephemeral, did precede any high order effects. Length of exposure appeared to be more important than PAH concentration. Except at the two lowest PAH concentrations tested, gross abnormalities and death began to occur a few days after the peak of MO induction. No cause and effect is implied here. In other words, induction of MO activity does not necessarily cause gross abnormalities and death. On the other hand, P-450 does provide a mechanism for the activation of PAH to products that are more reactive than the original compounds (Jakoby, 1980).

Applicability to Many Chemicals

The applicability of monitoring MO activity as an indicator of toxicity for large numbers of compounds is questionable. The Ah receptor, which mediates induction of P-450 (Ioannides and Parke, 1990), has high affinity for planar polycyclic aromatic compounds (Eisen, 1986). Certain PCB congeners are potent P-450 inducers in fish (Lidman *et al.*, 1976; Melancon *et al.*, 1981; Spies *et al.*, 1982; Kobayashi *et al.*, 1987) as well as certain PCDD and PCDF (Hahn *et al.*, 1989; van der Weiden *et al.*, 1989). The results presented here may not be applicable to organic xenobiotics other than creosote.

Most compounds in creosote are planar PAH. The model inducer 3-MC is a PAH, but is not present in creosote. Not all of the compounds found in creosote have been tested for their ability to induce MO activity. Of the PAH tested in fish and found in creosote, only BaP was a potent inducer of MO activity (Gerhart and Carlson, 1978). BaP represents approximately 0.1% of the total PAH in creosote.

From the study presented here that tested two fractions of creosote, no compounds in the low molecular weight fraction were inducers of MO activity. If there are compounds in creosote other than BaP that are capable of inducing MO activity, then the compounds are of high molecular weight. Testing all of the compounds present in the high molecular weight fraction would be an arduous task, but may be necessary if monitoring MO activity is to be applied to creosote-contaminated sites.

Ease and Expense of Detection

Measuring MO activity, especially EROD activity, is relatively easy. The cost of the EROD and protein analyses are inexpensive compared to chemical analyses for a multitude of organic compounds. The real comparison lies in the application of biochemical monitoring. If fish are to be collected in the field and analyzed in the laboratory, then results can never be totally conclusive because of the effects of other environmental factors, the unknown exposure history of the fish, and the lack of a true control. If water or sediment is brought to the laboratory and fish are exposed under controlled conditions, then the cost of the application rises. In the case of creosote, a long test with mortality or gross abnormalities as endpoints would be less expensive than a short test with MO induction as the endpoint.

Conclusions

1) A relatively simple flow-through system can be used to expose aquatic organisms to contaminants that are adsorbed to suspended sediments. Using this system with a demersal fish, suspended sediment concentrations varied up to approximately 25%. Using creosote as a PAH source, concentrations of individual PAH varied by no more than 50%. At the highest concentration tested, variation in the concentration of individual PAH was less than 15%.

2) Creosote-contaminated sediments can serve as a source of bioavailable PAH. This was evidenced by biological effects including mortality, fin erosion, formation of epidermal lesions, and induction of EROD activity.

3) The 96-h LC50 for juvenile spot exposed to creosote-contaminated sediments is 1740 μg PAH/L (95% CI = 1480-2060 μg PAH/L).

4) After a 2 day exposure, hepatic EROD activity in spot increases with increasing PAH concentration up to a point, then activity decreases with increasing concentration. Maximal induction of EROD activity was found at 150 μg PAH/L. With increased exposure time, EROD activity decreases, so that after 7 days of exposure EROD activity is not significantly different from that prior to exposure.

5) At 76, 150, and 320 μg PAH/L, induction of EROD activity was followed by mortality, fin erosion, and appearance of epidermal lesions within a few days.

6) A high molecular weight fraction of creosote produces results similar to that obtained with whole creosote with respect to induction of EROD activity, mortality, fin erosion, and epidermal lesions.

7) A low molecular weight fraction of creosote is much less toxic than whole creosote and does not induce EROD activity at 50 μg PAH/L.

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