Toxicity of Polycyclic Aromatic Hydrocarbons to Benthic Invertebrates.

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TOXICITY OF POLYCYCLIC AROMATIC HYDROCARBONS TO BENTHIC INVERTEBRATES

A Dissertation

Submitted to the Graduate Faculty of the Louisiana State University and Agricultural and Mechanical College in partial fulfillment of the requirements for the degree of Doctor of Philosophy in

The Department of Zoology and Physiology

by

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# TABLE OF CONTENTS

| ACKNOWLEDGMENTS | .......... | ii |
| ABSTRACT | .......... | iv |
| CHAPTER | .......... |
| 1 GENERAL INTRODUCTION | LETHAL AND SUBLETHAL TOXICITY OF POLYCYCLIC AROMATIC HYDROCARBONS TO AQUATIC INVERTEBRATES | 1 |
| 2 TOXICITY OF SEDIMENT-ASSOCIATED PYRENE AND PHENANTHRENE TO *LIMNODRILUS HOFFMEISTERI* (OLIGOCHAETA: TUBIFICIDAE) | 18 |
| 3 TOXICITY OF SEDIMENT-ASSOCIATED PAHs TO AN ESTUARINE COPEPOD: EFFECTS ON SURVIVORSHIP, FEEDING, REPRODUCTION, AND BEHAVIOR | 51 |
| 4 EFFECTS OF SEDIMENT-ASSOCIATED PHENANTHRENE ON SURVIVAL, DEVELOPMENT AND REPRODUCTION OF TWO SPECIES OF MEIOBENTHIC COPEPOD | 79 |
| 5 BIOACCUMULATION AND TOXICITY OF SEDIMENT-ASSOCIATED FLUORANTHRENE IN MEIOBENTHIC COPEPODS USING THE CRITICAL-BODY-RESIDUE APPROACH | 112 |
| 6 SUMMARY AND CONCLUSIONS | 154 |
| REFERENCES | 161 |
| APPENDIX: LETTER OF PERMISSION | 179 |
| VITA | 180 |
ABSTRACT

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous contaminants in aquatic environments. Because of their hydrophobicity, PAHs accumulate in bed sediments and pose a risk to the benthos. Information on the toxicity of sediment-associated PAHs is, however, limited and more information is needed to improve sediment quality-criteria and ecological-risk assessments. My research focused on improving our current knowledge of the acute and, especially, the sublethal effects of sediment-associated PAHs to aquatic invertebrates. Representatives of two phylogenetically distinct benthic invertebrate groups, freshwater tubificid oligochaetes and estuarine meio-benthic harpacticoid copepods, were used. Organisms were exposed to a range of concentrations of sediment-amended contaminants in laboratory bioassays. Individual PAH congeners (pyrene, fluoranthene, and phenanthrene) and a complex mixture (diesel fuel) were employed.

The tubificid oligochaete Limnodrilus hoffmeisteri was tolerant to PAH effects on survival in 10-d exposures. Because of its feeding mode (head-down, bulk-deposit feeder), egestion rates were used as surrogates for ingestion rates. PAHs significantly reduced sediment ingestion at concentrations much lower than lethal. This impact had direct effects on the flux of contaminants from sediments to the water column. Offspring production was also decreased at relatively low PAH concentrations, whereas burrowing avoidance did not occur.

Harpacticoid copepods display many traits desirable for sediment-toxicity testing. The estuarine species Schizopera knabeni, Nitocra lacustris and Coullana sp.
were used. Adult harpacticoids were relatively tolerant to PAHs in 10-d exposures. Species-specific differences in sensitivity were detected. Early life stages were much more sensitive than adults in some species, but not others. Low concentrations of PAHs decreased copepod-offspring production, egg hatching-success, and embryonic and early-stage-development rate, demonstrating the high sensitivity of life-history related endpoints. In addition, grazing on microalgae was significantly impaired at low concentrations after short exposures (<30 h). Finally, it was demonstrated that harpacticoids can actively avoid contaminated sediments.

Harpacticoids were shown to uptake and eliminate PAHs efficiently. Lethal and sublethal effects were related to the tissue concentration of PAH. Lethal doses were within the range predicted to cause death by narcosis in animals. Low tissue concentrations were associated with significant reductions in reproduction and feeding.
CHAPTER 1

GENERAL INTRODUCTION
LETHAL AND SUBLETHAL TOXICITY OF POLYCYCLIC AROMATIC HYDROCARBONS TO AQUATIC INVERTEBRATES
CHARACTERIZATION AND SOURCES

Polycyclic (or polynuclear) aromatic hydrocarbons (PAHs) occur in most urbanized coastal areas of the world, as well as in freshwater and terrestrial systems. They accumulate in sediments and present a potentially serious hazard to the benthos. Many benthic species bioaccumulate PAHs at high levels and exposure to higher trophic levels may occur through the aquatic food chain. PAHs encompass a broad range of compounds with two or more benzene rings; side groups (substituted PAHs) also occur. In general, PAHs have low solubility in water, high melting points, and low vapor pressure. As solubility increases, melting and boiling points increase, and vapor pressure decreases with increasing molecular weight (MW) (Albers, 1995). Molecular weight varies from 128.2 (naphthalene) to 276.3 (indeno[1,2,3-c,d]pyrene). PAHs are derived from petroleum sources, combustion products, and natural synthesis by organisms (LaFlamme and Hites, 1978). Crude oils contain 0.2 to 7% PAHs and this fraction increases with specific gravity. Kerosene, gasoline, and diesel oil are rich in bicyclic PAHs such as naphthalene and its substituted forms, but have relatively low concentrations of tricyclic and larger PAHs. Heavy oil products such as bunker c oil and asphalt contain higher fractions. Shale oil and coal-derived crudes tend to have the highest concentrations, as much as 15% (Neff, 1985).

The presence of PAHs in the environment is usually associated with human activities, although forest fires, volcanoes, and petroleum seeps may cause localized accumulations. When substituted PAHs predominate, the source is petroleum based. A predomination of unsubstituted PAHs, especially of high molecular weight, indicate
burning as the source (Meador et al., 1995a). A major source of petroleum hydrocarbons originates from oil spills, and much research has been concerned with the fate and effects of the aromatic hydrocarbons in oil. Petroleum-based PAHs enter the coastal environment through accidental releases of petroleum products and infusion from natural seepage. Combustion-derived PAHs enter aquatic environments through aerosol infiltration from combustion of various materials, both natural (e.g., forest fires), and anthropogenic (e.g., fossil fuel combustion). Naturally derived PAHs are produced by bacteria, fungi, and plants including phytoplankton (LaFlamme and Hites, 1978).

**TOXICITY IN AQUEOUS EXPOSURES**

Due to their high hydrophobicity, PAHs are classified as non-polar narcotics. Like other narcotics, PAHs do not affect specific organs, organ systems or biochemical pathways. Rather, they cause a reversible disfunction called general narcosis, or Narcosis I. The exact mechanisms of narcosis are not known, but theories propose that narcotic molecules disturb membrane phospholipids and proteins and the interaction of the two so that membranes become inoperative (Van Wezel and Opperhuizen, 1995). Inoperative membranes reduces overall activity in exposed organisms, and eventually leads to paralysis and death. In the water-soluble form, the toxicity of individual PAHs to animals increases as molecular weight increases up to 202 (e.g., fluoranthene, pyrene). Beyond MW of 202, a rapid decline in solubility reduces potential PAH concentrations to less-than-lethal levels. However, sublethal effects can result from exposure to these very low concentrations of high-MW compounds (Neff, 1985).
Besides the toxicity of untransformed PAH, a major concern is the ability of reactive metabolites of high-MW PAHs to bind to cellular proteins and DNA. The resulting biochemical disruptions and cell damage lead to mutations, developmental malformations, tumors, and cancer (Menzie et al., 1992).

In invertebrates, the ability to metabolize PAHs varies widely within and among phyla. Overall, invertebrates biotransform and eliminate PAHs much less efficiently than vertebrates (Meador et al., 1995a). Bivalves metabolize PAHs to a very limited extent, and therefore bioaccumulate PAHs at high levels. Polychaetes and crustaceans exhibit a range in the ability to biotransform PAH, and pronounced differences are found among phylogenetically related species (Meador et al., 1995a). With crustaceans, for example, the blue crab Callinectes sapidus and the amphipod Hyalella azteca are able to extensively metabolize PAHs, while a different crab (Uca pugnax) and a different amphipod (Diporeia sp.) biotransform PAHs to a very limited extent. Livingstone (1994) provides a comprehensive review of xenobiotic metabolism in invertebrates.

Most of the toxicity of the water-soluble fraction (WSF) of hydrocarbon complex mixtures, such as crude-oil or fuel oils, have been attributed to PAHs, especially naphthalenes. The WSF of diesel fuel contains approximately 90% PAHs, 30% of which are naphthalenes. PAHs comprise a lower fraction of total hydrocarbons in WSF of crude oils (Anderson et al., 1974; Anderson, 1977). Lethal toxicity of WSF of crude or refined oils have been reported for all major invertebrate groups and is best expressed as lethal median concentration (LC₅₀) (Rice et al., 1977; Kennish, 1992).
Because of the co-occurrence of a multitude of compounds in complex mixtures, interpretation and comparison of toxicity data are difficult. When using single PAH congeners, however, cause and effect relationships can more easily be established. Toxicity of PAH single compounds has been investigated using a variety of invertebrate species. Differences in sensitivity to the lethal effects of PAH congeners in short-term exposures can be assessed by comparing LC_{50} values available in the literature (see Neff, 1985 and Kennish, 1992 for summaries). Only crustaceans and annelids have been tested to date, and the former are most susceptible to PAHs.

Besides causing short-term mortality, the toxicity of PAHs is manifested as alterations in a variety of biological functions. Sublethal effects of the WSF of crude or No. 2 fuel oil in macro-invertebrates include changes in respiration and metabolic rates, disruption of ionic and osmotic regulation, changes in energy metabolism and behavior, and decreased growth, molting rate, egg-hatching success and offspring production (Anderson, 1977 and references therein; Donahue et al., 1977; Linden, 1977; Tatem et al., 1978; Cucci and Epifanio, 1979; Laughlin and Neff, 1978; Stickle et al., 1984, 1985; 1987; Wang and Stickle 1987, 1988; Nagarajah et al., 1985; Chapman et al., 1988; Fisher and Foss, 1993; Barre et al., 1994). In addition, the WSF of crude oils decreases life span, feeding and egg production rates and egg viability in copepods (Berdugo et al., 1977; Ustash, 1979; Cawies and Remillard, 1983).

The sublethal toxicity of individual PAHs in aqueous solution has also been the subject of numerous investigations. Effects have been detected at concentrations well below the reported 96-h LC_{50} for several compounds. The most studied PAH congener
is naphthalene, for which reported effects are as follows: decrease in life span, brood size, and overall offspring production of a planktonic copepod (Ott et al., 1978); disruption of ion exchange across the epithelia of the gills of blue crabs (Sabourin, 1982); decreased crawling activity of intertidal snails (Mackey and Hodgkinson, 1996); increased duration of larval development in Dungeness crabs (Caldwell et al., 1977); and decreased feeding rate of blue mussels (Donkin et al., 1989). Reports on the sublethal toxicity of phenanthrene include decreased growth and larval developmental rate and increased respiration rate of mudcrabs (Laughlin and Neff, 1979, 1980), decreased growth, molting, offspring production and feeding rate in daphnids (Geiger and Buikema, 1981, 1982), and decreased feeding rate in blue mussels (Donkin et al., 1989). The toxicity of naphthalene was also assessed in three of the above studies (Laughlin and Neff, 1979; Geiger and Buikema, 1981, 1982) and was determined to be negligible when compared to phenanthrene. Although benzo(a)pyrene was not toxic in short-term exposures, it impaired larval development in sea urchins (Ceas, 1974).

**PAH IN SEDIMENTS**

In general, PAHs are more hydrophobic as molecular weight increases. As hydrophobicity increases, thermodynamic equilibrium tends to favor partitioning of the PAHs molecules to more non-polar environments, such as the lipid of an organism or the organic carbon (OC) fraction of sediment particles. Hydrophobicity can be expressed as the octanol-water partition coefficient \( K_{ow} \). The log \( K_{ow} \) of PAH congeners ranges from 3.3 to 7 (Meador et al., 1995a). The \( K_{ow} \) is the dominant physical parameter that explains a substantial amount of partitioning behavior exhibited.
by PAHs in the aqueous environment. Non-polar contaminants such as PAHs accumulate in fine-grained sediments because of their hydrophobicity and partitioning to organic-coated particles (Means et al., 1980). Fine-grained or muddy sediments are characteristic of estuaries, and many commercially-important species reside in these important nursery-ground habitats. When anthropogenic activities such as sewage disposal, manufacturing, shipping, and recreational boating occur near estuaries, contaminants such as PAHs can accumulate in the sediments to levels much higher than those found in more pristine areas (Marcus et al., 1988). The concentrations of PAHs in sediment can range from a few ng/g (ppb) to very many µg/g (ppm). Wade et al. (1988) reported PAH concentrations in the Gulf of Mexico ranging from below detection (5 ng/g) to 37 µg/g. Higher concentrations (up to 2.8 mg/g) have been reported to the Elizabeth River system in the Chesapeake Bay, Eagle Harbor, Washington, and a site near a coking plant in Nova Scotia, Canada. Total PAH concentrations in sediments near a produced water outfall in Pass Fourchon, Louisiana, has been reported to reach levels as high as 90 µg/g (Rabalais et al., 1991).

**TOXICITY OF SEDIMENT-ASSOCIATED PAHS**

There are three potential sources or paths by which sediment-associated PAHs reach benthic organisms: the sediments themselves, ingestion of sediment particles or prey, and the overlying and interstitial water (via uptake across respiratory surfaces and body walls). Detrimental impacts of chemicals in sediment may result from any combination of these. The relative importance of each route may change with both the compound and a number of modifying factors affecting bioavailability. The prevailing
theory states that uptake from porewater is the main route of PAH exposure for animals that live below the sediment surface or closely associated with the sediment-water interface (Meador et al., 1995a). However, some studies suggest that ingestion of contaminated sediment can be a major contributor to body burden of hydrophobic compounds (Boese et al., 1990; Weston 1990; Meador et al., 1995b).

The bioavailability of hydrophobic compounds has been intensely investigated, and theories have been developed to explain why different sediments with similar amounts of contaminants vary widely in their toxicity. The Equilibrium Partitioning (EqP) Theory states that the chemical concentration in sediment and in the surrounding water are related as a function of the chemical's hydrophobicity (DiToro et al., 1991). The theory relies on observations that non-polar chemicals binds preferentially to OC. Non-polar contaminants partition between the OC of the solid phase and the surrounding interstitial water in a predictable way. The theory assumes that the contaminant in the solid (sediment OC), aqueous (porewater) and lipid (organism) phases are in equilibrium and that toxicity is independent of exposure route (DiToro et al., 1991). The EqP theory has been used for generating sediment-quality criteria (SQC) to predict impacts of PAHs on benthic organisms and derive "safe" levels of given contaminants. Using partitioning coefficients ($K_{ow}$) and toxicity data from aqueous exposures with planktonic organisms, safe sediment concentrations standards (normalized for OC) were created for phenanthrene, fluoranthene and acenaphthene (U.S.EPA, 1993a,b,c). The use of this theory was further expanded into a predictive model for assessing the toxicity of sediments contaminated with mixtures of PAH

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congeners (Swartz et al., 1995). The model predicts the toxicity of PAH-contaminated sediments solely by measuring the concentrations of their PAH single-compounds on an OC-normalized basis, rather than reliance on bioassays.

Accounts of the lethal toxicity of PAH single compounds in sediment exposures are few compared to those using aqueous exposures. Test species investigated are limited to estuarine and freshwater amphipods and freshwater chironomids. Experiments have been performed using sediments with different levels of OC in order to examine predictions of the EqP approach for phenanthrene, acenaphthene, and fluoranthene. Based on 10-d sediment toxicity tests, OC-normalized sediment concentrations or interstitial-water concentrations were much better predictors of toxicity than bulk sediment concentrations (Swartz et al., 1990; DeWitt et al., 1992; Suedel et al., 1993; U.S.EPA 1993 a,b,c).

Acceptance of the EqP theory is not universal however. Several investigators have criticized it as an oversimplification of the numerous factors involved in determining bioavailability. In several instances the EqP approach failed to predict lethal toxicity in sediment exposures (e.g., Nebeker et al., 1989; Chandler et al., 1994; Landrum et al., 1994; Kane Driscoll et al., in press). For example, in a major departure from the EqP theory predictions, low mortality was observed when a meiobenthic copepod was exposed to aqueous fenvalerate (an organic pesticide) 7300 x higher than predicted porewater LC₅₀s. In addition, organic carbon alone did not solely account for the differences in bioavailability of chemicals observed with various sediments and compounds. Some recent investigations indicated that besides sediment OC, a third
phase, the dissolved OC in the pore water, must also be accounted for, adding complexity to the predictive model. The fraction of chemicals bound to dissolved OC was usually not bioavailable to benthic organisms (Hoke et al., 1994; Ankley et al., 1994a; Meador et al., 1995b). Furthermore, it has been demonstrated that factors other than OC content (such as C:N ratio) are involved in contaminant partitioning (Landrum and Faust, 1994).

**SUBLETHAL TOXICITY ASSESSMENT OF SEDIMENT-ASSOCIATED CONTAMINANTS**

As stated above, a wide range of deleterious effects from PAH have been detected at concentrations much lower than 96-h LC₅₀s, demonstrating that mortality is not a sensitive indicator of toxicity. Unfortunately, accounts of the sublethal toxicity of sediment-associated PAHs are few. Reported effects are limited to impaired burrowing activity in the amphipods *Diporeia* spp. (Landrum et al., 1994) and the oligochaete *Lumbriculus variegatus* (Kukkonen and Landrum, 1994). In addition, cause-and-effect relationships for sublethal toxicity have never been addressed in a quantitative way for sediment-associated PAHs. The bioavailability of sediment-bound, non-polar contaminants to benthic organisms is a complex issue involving numerous factors other than just partitioning to the porewater (Landrum et al., 1992, 1994). Toxicity information derived from water-only exposures therefore has been considered a poor surrogate for predicting the toxicity of contaminated sediments (e.g., Chandler et al., 1994; Landrum et al., 1994). Furthermore, extrapolation of sublethal effects observed with relatively few planktonic organisms to all benthic species has been considered...
inappropriate (Iannuzzi et al., 1995). Single-compound-PAH-sediment-quality criteria for the protection of benthic organisms rely on such extrapolations. Improved understanding of the sublethal toxicity of PAHs to benthic organisms is highly desired.

CRITICAL BODY RESIDUE APPROACH FOR TOXICITY ASSESSMENT

The paradigm established in aquatic toxicology for water exposures has been to relate the dose in the external environment (e.g., water) to that in the organisms, which is proportional to the dose at the receptor (McCarty, 1986). Therefore, toxicity has been widely expressed as the external concentration related to the observed endpoints (e.g., LC₅₀). However, it is the dose at the receptor, and not in the surrounding media that determines the response. When the source of exposure is water, the internal dose can be assumed to be equivalent to the external dose because there is only one route of accumulation, and behavioral and physicochemical modifications that reduce exposure are minimal. In sediment exposures, however, several factors are involved. Contaminant uptake takes place via multiple routes, mainly bulk ingestion of particles, and integumental uptake via the overlying water and porewater. The relative importance of each route depends on the chemistry of the contaminant and organism behavior. Burrowing avoidance, for example, reduces exposure to the porewater and can be associated with modified feeding behavior (Landrum et al., 1994). Given such complexities, relating effects to only a single-source concentration (the basic premise of the EqP theory), appears unreasonable.

An alternative approach to account for bioavailability differences among sediments is based on the dose; not the external concentration, but rather the internal
tissue concentration that is associated with an effect. The critical-body-residue (CBR) approach improves the interpretation of toxicity data by explicitly considering bioavailability, accumulation kinetics, uptake from food, and effects of metabolism (Landrum et al., 1992; McCarty and Mackay, 1993). It states that the molar tissue concentration causing a toxic effect (e.g. mortality) is similar for chemicals with the same mode of action and relatively constant across species. For non-polar narcotics, for example, tissue concentrations varying from 2 to 8 μmol/g wet weight were associated with lethality in several fish species and daphnids (McCarty et al., 1992; Pawlisz and Peters, 1993a). PAH body residues also have been measured in sediment-exposed organisms and used to generate LD_{90} values or lethal CBR (Landrum et al., 1991; Landrum et al., 1994; Kane Driscoll et al., in press). PAH tissue concentrations, much lower than those associated with mortality, have been associated with decreased filtration rate in mussels (Donkin et al., 1989) and reduced reproductive output in polychaetes (Emery and Dillon, 1996). Using a dose-response relationship, the CBR approach offers compelling advantages for risk assessment. Tissue concentrations measured from contaminated sites would be related to CBRs determined in laboratory experiments, allowing an accurate prediction of the potential deleterious effects associated with exposures. The sole use of lethal endpoints would limit the predictive ability of this approach by setting critical levels unlikely to be observed, even at the most contaminated sites. Further research is necessary for the generation of sublethal critical residues for exposures to PAH and other non-polar contaminants.
TEST ORGANISMS

Burton et al. (1992) listed eight criteria to be considered when selecting test species for sediment toxicity assays, including behavior in sediment (e.g., feeding), sensitivity to test materials, ecological relevance, geographical distribution, taxonomic relation to indigenous species, acceptability for use in toxicity assessment, availability, and tolerance to natural geochemical sediment characteristics such as grain size. Test-organisms belonging to two major taxonomic groups were used in experiments described in this dissertation: tubificid worms (Annelida: Oligochaeta) and meiobenthic harpacticoid copepods (Crustacea: Copepoda). Species belonging to these two groups meet the above requirements and have been successfully used in toxicity-testing with a variety of contaminants, both organic and inorganic, as discussed below.

Harpacticoid copepods are benthic and epibenthic crustaceans, typically the second most abundant group in the omnipresent meiobenthos (metazoans passing through a 0.5 mm sieve), with important ecological roles in marine ecosystems. Their densities often reach $10^3 - 10^6$ m$^{-2}$, and as such, harpacticoids have a significant importance to marine food webs (Coull, 1988). Meiobenthic copepods are critical to the diet of selected juvenile fish and macro-crustaceans (Gee, 1989; Coull, 1990). Studies of the effect of pollution on benthos often conclude that harpacticoids are more sensitive than other groups (e.g., nematodes) to a variety of contaminants (Coull and Chandler, 1992). Demand for more laboratory-oriented research comes from the increasing recognition of meiofauna community structure as a sensitive bioindicator of pollution in the benthic environment (Heip, 1980; Sanduli, 1986; Vinks and Heip, 1987;
Moore and Bett, 1989; Warwick et al., 1990; Somerfield et al., 1994; Somerfield et al., 1995). Harpacticoids display many traits that make them well suited for sediment toxicity studies: high abundance, intimate association with the sediment, a predominantly sessile life-style, and ease of laboratory culturing. Life cycle is short (2 to 4 weeks) and all larval stages are infaunal for most species, making them excellent test organisms for assessing the effects of contaminants on life-history traits in sediment exposures. Their suitability for the examination of lethal and sublethal toxicity of sediment-associated compounds has been confirmed by the studies of Chandler and co-workers (e.g., Chandler, 1990; DiPinto et al., 1993; Green et al., 1996).

Deleterious effects of petroleum hydrocarbons on meiobenthic copepods have been reported from field and microcosm studies (Coull and Chandler, 1992; Carman and Todaro, 1996). Lethal effects have been demonstrated from the aqueous phase of petroleum hydrocarbons in laboratory exposures of individual harpacticoid species (Coull and Chandler, 1992). In addition, petroleum hydrocarbons have been shown to decrease algal-consumption rate in a meiobenthic copepod at sublethal concentrations (LaCaze and Ducreux, 1987).

Tubificid oligochaetes are a major component of the benthos in some freshwater environments. High abundance of worms have been reported from areas of heavy organic pollution and low oxygen concentration, suggesting that opportunistic species such as Tubifex tubifex and Limnodrilus hoffmeisteri may serve as bioindicators of anthropogenic disturbance (Chapman and Brinkhurst, 1984; Lauritsen et al., 1985). Tubificids are bulk deposit feeders and ingest sediment continuously in a conveyor-belt
fashion. Experimental procedures have been developed for accurately collecting egested material for analysis (Appleby and Brinkhurst, 1970; Kaster et al., 1984). This feeding pattern promotes a thorough mixing of the top 5-15 cm of sediment in freshwater systems and largely enhances solute transport across the sediment-water interface (Bosworth and Thibodeaux, 1990). This phenomenon, collectively termed bioturbation, has been reported to significantly increase the flux of metals and organic contaminants from sediment into the water column (Karickoff and Morris, 1985; Reynolds, 1987; Reible et al., 1996).

Tubificids have been extensively used in toxicity testing of freshwater sediments. Besides survivorship (Wiederholm et al., 1987; Wiederholm and Dave, 1989; Reynolds, 1994), sublethal endpoints have been successfully employed, and include burrowing avoidance (McMurth, 1984; Keilty et al., 1988a; White and Keilty, 1988) and reproduction and population growth (Milbrink, 1987; Wiederholm et al., 1987; Wiederholm and Dave, 1989; Reynolds et al., 1991; Reynolds, 1984; Day et al., 1995a; Day et al., 1995b; Casellato et al., 1992). Tubificid worms are sexually reproducing hermaphrodites. Following fertilization, cocoons are formed and attached to the substrate. Direct development of the larvae follows. Effective reproduction can occur in artificial settings, making tubificids conducive to laboratory culture. Detailed protocols for conducting toxicity tests using *T. tubifex* offspring production as a sensitive endpoint have been developed (Reynolds et al., 1991) and successfully employed in toxicity assessments of field-collected sediment samples (Reynolds et al., 1991; Reynolds 1984; Day et al., 1995a,b). The toxicity of sediment-associated
PAHs has been investigated with *Lumbriculus variegatus*, a lumbriculid oligochaete (Kukkonen and Landrum, 1994; Ankley et al., 1994b; Monson et al., 1995), but never with tubificid species.

This dissertation is divided into four major research chapters detailing the toxicity of PAHs to two phylogenetically and ecologically distinct benthic invertebrate groups, oligochaetes and harpacticoid copepods. Chapter 2 is scheduled to be published in the journal *ENVIRONMENTAL TOXICOLOGY AND CHEMISTRY* volume 15, number 9 (1996). It describes the toxicity of sediment-associated PAH congeners to a deposit-feeding freshwater oligochaete and explores feeding and reproduction as test endpoints. Chapter 3 is in submission to the journal *MARINE ENVIRONMENTAL RESEARCH*. It describes the toxicity of PAH congeners and a complex hydrocarbon mixture to the harpacticoid copepod *Schizopera knabeni*. It also promotes the use of meiobenthos in sediment-toxicity assessment by employing new sublethal endpoints, such as a grazing and a behavior bioassay. Chapter 4 explores in detail toxic effects of PAHs on life-history-related endpoints such as reproductive output, egg hatching, and early-stage development and survival in harpacticoids. Comparison between two species were made. Chapter 5 employed a different approach, the critical body residue, to evaluate PAH hazardous effects in sediment exposures. Two functionally different copepod species were used (*S. knabeni* and *Coullana* sp.), and aspects of the bioaccumulation of PAH in meiobenthic copepods are described.

The overall objective of this dissertation was to improve our knowledge of the toxicity of sediment-associated PAHs to benthic invertebrates. Toxicity experiments
were conducted under controlled laboratory conditions, providing accurate cause-and-effect relationships. Toxicity at the sublethal level was explored in greater detail in an attempt to enhance the understanding of deleterious effects on ecologically relevant functions. Research conducted for this dissertation also aimed to improve the use of tubificids and meiobenthic copepods as test-organisms in sediment exposures. Contamination levels and endpoints examined aspired to provide a better understanding of effects at the community level observed in contaminated field sites and experimental mesocosm manipulations.
CHAPTER 2

TOXICITY OF SEDIMENT-ASSOCIATED PYRENE AND PHENANTHRENE TO
LIMNODRILUS HOFFMEISTERI (OLIGOCHAETA: TUBIFICIDAE)*

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INTRODUCTION

Tubificids are a major component of the benthos in most freshwater environments. They are exceedingly abundant in polluted areas, and the eutrophic species *Tubifex tubifex* and *Limnodrilus hoffmeisteri* serve as indicators of organic pollution and low dissolved oxygen (Chapman and Brinkhurst, 1984; Lauritsen et al., 1985). Oligochaetes are increasingly being used to test the toxicity of freshwater sediments; *Lumbriculus variegatus* in studies of chemical bioaccumulation (U.S.EPA. 1994) and *T. tubifex* in toxicity assessment (McMurthy, 1984; Milbrink, 1987; Wiederholm et al., 1987; Wiederholm and Dave, 1989; Reynoldson et al., 1991; Reynoldson, 1984; Day et al., 1995a; Day et al., 1995b) are especially important. Investigations of the toxicity of sediment-associated contaminants using tubificid oligochaetes have employed several endpoints including survivorship (Wiederholm et al., 1987; Wiederholm and Dave, 1989; Reynoldson, 1994) burrowing avoidance (McMurthy, 1984; Keilty et al., 1988a; White and Keilty, 1988a), reproduction and population growth (Milbrink, 1987; Wiederholm et al., 1987; Wiederholm and Dave, 1989; Reynoldson et al., 1991; Reynoldson, 1994; Day et al., 1995a,b; Casellato et al., 1992).

Tubificids feed continuously in a conveyor-belt fashion by ingesting particles in bulk at depth and defecating on the sediment surface. Such bioturbation has been reported to significantly increase the flux of metals and organic contaminants from sediment into the water column (Karickoff and Morris, 1985; Reynoldson, 1987; Reible et al., 1996). Several studies have used egestion rate as a surrogate of tubificid feeding.
activity (Wachs, 1967; Appleby and Brinkhurst, 1970; Kaster et al., 1984; Klump et al., 1987). A decrease in egestion was observed when *L. hoffmeisteri* was exposed to sediment contaminated with a mixture of pyrene, phenanthrene and dibenzofuran (Reible et al., 1996) suggesting that feeding rate may serve as a sublethal endpoint. In addition, tubificid worms exhibit the characteristics of an annelid hermaphrodite. Sperm must be exchanged for viable fertilization, cocoons are formed and attached to substrate, and development is direct. Reproduction has been demonstrated to be a sensitive endpoint in sediment toxicity tests using tubificids (Reynoldson et al., 1991; Reynoldson, 1994; Day et al., 1995a,b), but has not been applied in toxicity studies using spiked sediment.

Few studies have investigated the lethal toxicity of sediment-associated individual polynuclear aromatic hydrocarbons (PAHs) to benthic organisms. Phenanthrene and acenaphthene were lethally toxic to the amphipods *Eohastorius estuarius* and *Leptochirius plumosus* (U.S.EPA, 1993a; 1993b). Phenanthrene was slightly toxic to the amphipod *Diporeia* spp. (Landrum et al., 1994). Fluoranthene was lethally toxic to the amphipod *Hyalella azteca*, the midge *Chironomus tentans*, the channel catfish *Ictalurus punctatus*, and to the amphipod *Rhepoxynius abronius* (U.S.EPA, 1993c). Pyrene was lethally toxic to *Diporeia* spp. (Landrum et al., 1994), and the low lethal toxicity to *L. variegatus* was associated with strong burrowing avoidance behavior (Kukkonen and Landrum, 1994). No other PAH congener has been used in toxicity tests with benthic organisms in single compound sediment exposures.
The purpose of this study was to assess the toxicity of two PAH congeners (pyrene and phenanthrene) to *L. hoffmeisteri*. Phenanthrene and pyrene are among the PAH congeners of most concern to the environment and they differ in their chemical properties, phenanthrene being more water soluble and less hydrophobic. Unexpectedly low lethal toxicities, as predicted by the equilibrium partitioning theory (DiToro *et al.*, 1991), were observed when *Diporeia* spp. and *Lumbriculus variegatus* were exposed to high sediment concentrations of phenanthrene or pyrene (Landrum *et al.*, 1994; Kukkonen and Landrum, 1994). PAHs, especially in the higher range of molecular weight (e.g. pyrene), may not be lethally toxic to sediment-dwelling organisms either because the interstitial water concentration, even at saturation, falls below that required to elicit mortality or by failure to bioaccumulate at levels expected to cause mortality by non-polar narcosis. Sublethal toxicity have been demonstrated for PAHs, but solely in water-only exposures (Geiger and Buikema, 1981; 1982; Laughlin and Neff, 1979; 1980). Lethal effects on *L. hoffmeisteri* were assessed in 10- and 28-d exposures, and sublethal effects were investigated using feeding rate and reproductive output as endpoints.

**MATERIAL AND METHODS**

**Laboratory culture and stock sediment**

Sediment was collected from the margins of a local drainage system. In the laboratory, it was placed in beakers and fine sediment (<125 µm) was added to the top. Overnight, tubificids migrated to this layer and were aspirated, washed, and placed in
culture. They were subsequently identified by R.O. Brinkhurst (Aquatic Resources Center, Franklin, TN) as a mono-specific collection of *L. hoffmeisteri*. Glass aquaria, 50-cm x 25-cm x 30-cm, with a medium consisting of sediment from the collecting site mixed with the <125 µm fraction of stock sediment collected from Bayou Manchac, Baton Rouge, LA was used to culture *L. hoffmeisteri*. The sediment was covered with artificial pond water (APW) (0.5 mM NaCl, 0.2 mM NaHCO₃, 0.05 mM KCl, 0.4 mM CaCl₂) and aerated. A food supplement (mixed-grain baby cereal) was added once a month.

Stock sediment was sieved to <125 µm and allowed to settle for one week at 4°C. Overlying water was removed by aspiration, replaced with APW and fully mixed with the sediment. The sediment organic carbon (SOC), measured on a Perkin Elmer (Norwalk, CT) 2400 CHN elemental analyzer, was 0.7 % after acidification with HCl to remove inorganic carbonate. The fraction of combustible solids, measured by loss on ignition at 550°C for 5 hours, was 4.3 %. Stock sediment was stored at 4°C, in the dark, for future use in the experiments.

**10-d egestion and survivorship bioassay**

Pyrene or phenanthrene (98% purity, Aldrich Chemical Co, Milwaukee, WI) were amended to the stock sediment by spiking. Settled sediment was fully homogenized with the overlying water and 715 g of wet sediment (dry-to-wet-ratio = 0.35) was transferred to 1 L Teflon-coated jars. After equilibration to room temperature and stirring, the appropriate amount of pyrene or phenanthrene, carried in a constant volume of acetone, was spiked dropwise on the slurry while stirred for 15 min with a
hand blender. Phenanthrene was spiked in 1 ml of acetone and pyrene in 5 ml of acetone. Mixing continued for 15 min. The required amount of spiked PAH was calculated on a dry weight basis to attain target concentrations. A solvent control was prepared by adding acetone only and a full control was prepared without spiking. To reduce the amount of solvent, the overlying water was removed by aspiration and replaced with APW and the sediment fully homogenized every 24 h, three times. Sediments were stored in the dark at 4°C for 3 weeks. The overlying water was removed, the sediment homogenized and samples were taken for contaminant concentration and dry-weight measurements 24 h before use in experiments (Table 2.1). Pyrene concentrations ranged from 98 to 841 µg/g and phenanthrene concentrations from 47 to 612 µg/g.

*Limnodrilus hoffmeisteri* was removed from the culture by sieving. Visibly mature individuals (noted by their gonads) of approximately the same size were selected. To measure the effects of pyrene and phenanthrene on daily fecal production (egestion rate), upright defecation chambers were assembled after Kaster *et al.* (1984). Four 50-ml polypropylene centrifuge vials (11.5 x 2.7 cm) were filled with 40 ml of each sediment treatment. Fifteen randomly selected worms were placed on the sediment surface of each vial. All vials were kept for 24 h in the dark. After this period, all the worms burrowed and some of the sediment settled. The overlying water was removed with a pipet. The sediment surface was then covered with a thin layer of polyester aquarium floss and a circle of wetted cheese cloth with a diameter a little larger than that of the vial. The floss and cheese cloth were held in place by a PVC
Table 2.1. Average sediment contaminant concentrations (μg/g) measured at initiation (day 0) and termination (day 10) of the 10-day pyrene and phenanthrene egestion and survivorship bioassay, percent loss of contaminants during exposure period, and percent solids content as measured on day 0.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Pyrene</th>
<th>Phenanthrene</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 0</td>
<td>Day 10</td>
<td>% Loss</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0 S*</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>98</td>
<td>95</td>
<td>3</td>
</tr>
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<td>124</td>
<td>119</td>
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<td>210</td>
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<td>435</td>
<td>354</td>
<td>19</td>
</tr>
<tr>
<td>841</td>
<td>816</td>
<td>3</td>
</tr>
</tbody>
</table>

* 0 S = Solvent control
b Sample lost.
split-ring with outer diameter adjusted to tightly fit the vial inner diameter. Each vial was filled with APW, placed on a rack and kept in the dark inside an environmental chamber at 25°C. After a few hours, *L. hoffmeisteri* began to extrude their posterior ends through the floss and cloth and to eliminate fecal pellets. Feces produced during the initial 24 h were discarded. Thereafter, feces were washed into a jar daily for nine consecutive days. Chambers were washed in random order and re-filled with APW. Feces were filtered through pre-weighed 8 μm Millipore membrane filter. Filters were dried overnight at 80°C and weighed to determine fecal dry weight. Immediately preceding feces collection, each chamber was observed with a magnifying lens and the number of worms extruding their posterior ends recorded. After the last fecal collection, worms were forced to retreat to deeper layers by disturbance with a probe. The ring, cheese cloth and floss were carefully removed, washed, and examined for worms. Approximately 3 ml of sediment was removed from one chamber per treatment for contaminant content analysis. The sediment from each chamber was washed through a 125 μm sieve and the retained worms removed intact and enumerated for survivorship determination. They were placed individually in well plates overnight to allow for gut clearance. After washing, the recovered worms from each chamber were assembled together and placed on pre-weighed aluminum trays for dry weight determination.

The number of worms recovered from the chambers at experiment termination ranged from 1 to 15 (see results). The daily egestion measured from each chamber was standardized by our best daily estimate of the number of worms alive in that chamber.
and egestion rates were calculated on a per worm basis (see discussion). This number was estimated by the daily observations of the number of active worms (those extruding their posterior ends) in each chamber and/or by the number of worms recovered at the end of the experiment. As an example, the number of active *L. hoffmeisteri* recorded in the chamber # 2 of the 203 μg/g phenanthrene treatment was 1 - 5 - 3 - 5 - 8 - 11 - 8 - 9 from day 1 to 9. The number of worms recovered at the end the experiment was 9 in this chamber, so during the 10-d period, the number of live worms decreased from the initial 15 to 9 worms. But at day 7, 11 worms were alive and active in the chamber, since they were observed in the chambers. Therefore, the best estimate of the number of live worms at each day was 11 - 11 - 11 - 11 - 11 - 11 - 9 - 9. The daily fecal dry weight determined for that chamber was divided by the best estimate of the number of living worms.

**Recovery experiment**

Fifteen randomly selected worms retrieved from the 841 μg/g pyrene treatment and from the 202 μg/g phenanthrene treatment were used in a recovery experiment. Following retrieval, each group of 15 worms was placed in a new defecation chamber containing the solvent-control sediment treatment. Feces produced during the initial 24 h were discarded and those produced thereafter were collected daily for three consecutive days and dry weight determined as above.

**5-d egestion bioassay**

Results from the 10-d experiment indicated that the effects of pyrene and phenanthrene on egestion were detectable after the first 48 h of exposure and that feces
collection over a restricted number of days virtually eliminated time effects (see below).

Thus we repeated the egestion rate experiment using a lower range of sediment concentrations (pyrene measured concentrations were 10, 15, 24, 46 and 91 and phenanthrene 6, 9, 20, 47, 71 and 171 µg/g) but for fewer days. Both compounds were spiked in a constant volume of 1 ml of acetone. A solvent control treatment was also prepared. The procedures were the same as in the 10-d bioassay, except for the test duration; feces were discarded on days 1 and 2 and retained on days 3 and 4 only.

**Sediment egestion vs. Body dry weight relationship**

Daily fecal egestion rate by *L. hoffmeisteri* has been expressed on a dry weight basis normalized by averaged post-experimental measurements of body weight (Appleby and Brinkhurst, 1970; Kaster *et al.*, 1984). In order to determine if this was the best way to standardize data from our egestion bioassays, we examined the relationship between sediment egestion and body dry weight. One hundred and fifty *L. hoffmeisteri* encompassing a size range similar to that used in the egestion bioassays were obtained from culture and placed individually in well plates. After 24 h for gut clearance, individuals were assigned a number and their wet weight determined. All worms were ranked according to wet weight, from lowest to highest. The first 15 worms in the rank were assembled together, the following 15 worms formed another group, and so forth until 10 groups were obtained. Each group was assigned to a defecation chamber containing the <125 µm fraction of untreated stock sediment. After 24 h, feces were collected for five consecutive days. Feces were poured directly into pre-weighed aluminum trays for dry weight determination without filtration. Intact
worms were recovered following the last feces collection and placed in distilled water for gut clearance. They were then thoroughly washed, counted and placed on pre-weighed aluminum trays for dry weight determination.

**Burrowing avoidance and reproduction bioassay**

Four 50-ml centrifuge vials were filled with 40 ml of each sediment treatment used in the 10-d egestion rate bioassay (Table 2.1). Eight randomly selected mature worms were placed on the sediment surface of each vial. Each vial was carefully filled with 5 ml of APW, and kept in the dark, inside an incubator at 25°C. The sediment surface within each chamber was examined periodically to record the number of burrowed worms following Keilty *et al.* (1988a). An individual worm was considered unburrowed if greater than an estimated 75% of its body was exposed to the sediment surface. Observations were made at 0.17, 0.5, 1, 2, 4, 8, 12, 24, 48, 72, 96 h and 7, 14, 21 and 28 days. The overlying water in each chamber was replaced weekly with fresh APW. After 28 days, the sediment from each chamber was washed on a 63 μm sieve, poured into a petri dish and adults counted and removed. The remaining material was carefully observed on counting dishes and the cocoons (hatched and unhatched) and juveniles were enumerated to estimate the reproductive output (Reynoldson *et al.*, 1991).

**Chemical analysis**

Concentration of each sediment contaminant in sediment was determined in duplicate. Sediment samples (approximately 2 g wet weight) were weighed and placed in Teflon-coated 100 ml jars. At the same time, samples of 2 g of wet sediment were
placed in pre-weighed aluminum trays for solids content determination (dry-to-wet-weight ratio). Samples were then desiccated with anhydrous sodium sulfate and extracted by adding 40 g of acetone:hexane (1:1, v/v). Samples were sonicated for 20 min. An aliquot of solvents was pipetted into a 2 ml volumetric tube and fully evaporated using an ultra-high purity nitrogen stream. It was exchanged to 2 ml acetonitrile:hexane (9:1, v/v). This solution was transferred to vials for analysis by UV detection high performance liquid chromatography (Hewlett-Packard Model 1090 HPLC; Meriden, CT) with acetonitrile:water (6:4, v/v) carrier and 150x4.6 mm column packed with Envirosep-PP (a reversed phase C8 column). Average recovery of pyrene and phenanthrene by this process was 93.3 and 92.7%, respectively.

Statistical analysis

The daily egestion data from the 10-d and 5-d bioassays was analyzed using a split-plot design. A two-way ANOVA tested for the significance of three different effects on the amount of egested feces: the main-unit effect (or the effect of sediment contamination), the subunit effect (or the effect of time), and the effect of the interaction of these two factors. Of principal interest was the effect of sediment contamination on egestion rate. But this effect can only be analyzed separately from the time effect if the interaction of the two factors is non-significant. In the case of significant interactions, multiple comparisons within a split-plot design were performed by calculating least significant differences according to Steel and Torrie (1960). When the interaction effect was not significant but sediment contamination level effect was, contaminant treatments were compared to control treatments using Dunnett's one-tailed
t-test (\(\alpha = 0.05\)). Survivorship and reproduction were analyzed with one-way ANOVA. Contaminant treatments were compared with control treatments using Dunnett's one-tailed t-test (\(\alpha = 0.05\)). Mean solvent-control values were compared to mean full control values using a two-tailed t-test (\(\alpha = 0.05\)). When a significant difference was detected between the two means, only the solvent-control mean was used. When no difference was detected, the data from the two controls were combined. Body dry weight data from the daily egestion experiments were analyzed using one-way ANOVA, and treatments were compared using Bonferroni's two-tailed t-test (\(\alpha = 0.05\)). Linear regression was performed to obtain the relationship between sediment egestion and worm body dry weight. LC\(_{50}\) values were computed using probit analysis. Point estimates for daily egestion and reproductive output was calculated using linear interpolation combined with bootstrapping, or IC\(_p\) method (U.S.EPA, 1994). Two-way ANOVA using the split-plot design and simple linear regression analysis were performed using SAS\(^\text{\textregistered}\) (Release 6.03 Edition; Cary, NC). One way ANOVA, multiple comparison t-tests, LC\(_{50}\) values and IC\(_{25}\) values were computed using TOXCALC\textsuperscript{TM} version 5.0 (Tidepool Software; McKinleyville, CA). Confidence intervals for IC\(_{25}\) values were calculated with 1000 resamples.

RESULTS

10-d egestion and survivorship bioassay

Sediment contaminant concentrations were measured for all treatments at the initiation and termination of the experiment. The average loss of pyrene and
phenanthrene from the sediment was 5.8% and 54.2% respectively. The average solids content in the sediment treatments was 47.3% in the pyrene experiment and 51.6% in the phenanthrene experiment (Table 2.1).

Mortality occurred over the 10-d sediment exposure (Fig. 2.1). Mortality was low (< 20%) in the control and solvent-control chambers. The ANOVA showed no difference in the mean mortality among all pyrene treatments, as mortality was < 15%. A significant difference was found among treatments in the phenanthrene experiment. A one-tailed Dunnett's multiple comparison test showed that the mean mortality in the 203 μg/g and higher treatments were significantly higher than in the combined controls. The 10-d LC₅₀ for phenanthrene was 297.5 μg/g (Table 2.2).

In the pyrene experiment, average control and acetone control sediment egestion ranged from 6.2 to 14.7 mg feces dry weight/worm between days 1 and 9 (Fig. 2.2). For the first two days, sediment egestion for all pyrene treatments was greatly reduced, averaging between 0.4 to 2.3 mg. However, for all pyrene treatments there was a pronounced general increase of sediment egestion with time, with averages always lower than controls, except for the 98 μg/g treatment on day 6. In the phenanthrene experiment, sediment egestion was very similar in the control and acetone control treatments, ranging from 10.5 to 24.2 mg between day 1 and 9, increasing steadily from days 1 to 7. Sediment egestion was greatly reduced for all phenanthrene treatments, with averages remaining well below controls (Fig. 2.2). A general increase of egested sediment with time was also observed, especially for the two lowest concentrations. The split-plot ANOVA demonstrated significant effects of sediment-contamination.
Table 2.2. Toxicity values from bioassays performed with *Limnodrilus hoffmeisteri* (numbers in parenthesis indicate 95% confidence intervals)

<table>
<thead>
<tr>
<th>Value</th>
<th>Pyrene</th>
<th>Phenanthrene</th>
</tr>
</thead>
<tbody>
<tr>
<td>10-d LC50</td>
<td>nd*</td>
<td>297.5 (252.2 - 348.3)b</td>
</tr>
<tr>
<td></td>
<td></td>
<td>42,500 (36,029 - 49,757)c</td>
</tr>
<tr>
<td>10-d Egestion IC25</td>
<td>51.6 (37.2 - 86.1)b</td>
<td>24.5 (18.3 - 33.1)b</td>
</tr>
<tr>
<td></td>
<td>7,371 (5,314 - 12,306)c</td>
<td>3,500 (2,614 - 4,729)c</td>
</tr>
<tr>
<td>5-d Egestion IC25</td>
<td>58.9 (32.1 - 89.4)b</td>
<td>28.4 (10.0 - 41.3)b</td>
</tr>
<tr>
<td></td>
<td>8,414 (4,586 - 12,771)c</td>
<td>4,057 (1,429 - 5,900)c</td>
</tr>
<tr>
<td>Reproduction IC25</td>
<td>59.1 (38.3 - 112.5)b</td>
<td>40.5 (12.1 - 165.5)b</td>
</tr>
<tr>
<td></td>
<td>8,443 (5,571 - 16,071)c</td>
<td>5,790 (1,729 - 23,623)c</td>
</tr>
</tbody>
</table>

* nd = not determined.

b Values reported in μg/g.

c Values reported in μg/gCor.

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Fig. 2.1. Mortality of *Limnodrilus hoffmeisteri* versus sediment concentration in the pyrene (A) and phenanthrene (B) 10-d bioassay. Initial number of worms = 15. Error bars show 1 SD of mean (n = 4); 0 S = solvent control; * represents significant difference (α = 0.05) from combined control mortality.
Fig. 2.2. Daily sediment egestion by *Limnodrilus hoffmeisteri* in each sediment concentration in the pyrene (A) and phenanthrene (B) 10-d bioassay. Initial number = 15. Sediment concentrations are expressed in µg/g. Error bars omitted for clarity; 0 S = solvent control.
level, time, and interaction for both pyrene and phenanthrene (p < 0.005). Least significant differences calculated to compare means of each treatment to the solvent-control treatment at each day level indicated that, in the pyrene experiment, the solvent-control was significantly different from the control treatment only at days 2, 6, 7, and 9. The 98 μg/g pyrene treatment was significantly different from the solvent-control treatment only from days 1 - 4 and 9. The remaining pyrene treatments were significantly different from the solvent-control on all days. In the phenanthrene experiment, the solvent-control was not significantly different from the control treatment and all phenanthrene levels were significantly different from the solvent-control on all days.

Daily egestion averaged for days 1-9 decreased with increasing contaminant concentration for both pyrene and phenanthrene (Fig. 2.3). The average egestion for days 3-4 closely followed the trend for the average of days 1-9, indicating that this bioassay could be simplified by collecting on certain days that may be representative of long-term trends. The ANOVA obtained using the average egestion for days 3 and 4 showed significant sediment treatment effect (p < 0.001). A Dunnett's multiple comparison test revealed that all contaminant treatments were different from either of the controls. The IC_{25} values obtained from mean egestion over days 3 and 4 were 51.6 μg/g for pyrene and 24.5 μg/g for phenanthrene (Table 2.2).

The mean body dry weight per worm was determined from the assemblage of worms recovered from each defecation chamber at the end of the experiment (Table 2.3). ANOVA indicated a significant difference among the body dry weight in both the
Table 2.3. Average body dry weight (mg) of *Limnodrilus hoffmeisteri* recovered from each sediment treatment (µg/g) at the end of the 10-d egestion bioassay

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Pyrene</th>
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<tbody>
<tr>
<td></td>
<td>0</td>
<td>0 S*</td>
<td>98</td>
<td>128</td>
<td>210</td>
<td>435</td>
<td>841</td>
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<tr>
<td>Average</td>
<td>0.683</td>
<td>0.693</td>
<td>0.52</td>
<td>0.51</td>
<td>0.523</td>
<td>0.51</td>
<td>0.56</td>
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<td>Standard</td>
<td>0.11</td>
<td>0.04</td>
<td>0.05</td>
<td>0.05</td>
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<td>0.1</td>
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<td></td>
<td>0</td>
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<td>47</td>
<td>102</td>
<td>143</td>
<td>203</td>
<td>396</td>
</tr>
<tr>
<td>Average</td>
<td>0.528</td>
<td>0.583</td>
<td>0.613</td>
<td>0.640</td>
<td>0.615</td>
<td>0.380</td>
<td>0.513</td>
</tr>
<tr>
<td>Standard</td>
<td>0.026</td>
<td>0.122</td>
<td>0.109</td>
<td>0.104</td>
<td>0.097</td>
<td>0.105</td>
<td>0.104</td>
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<tr>
<td>deviation</td>
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<td></td>
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</tr>
</tbody>
</table>

*0 S = solvent control*
Fig. 2.3. Daily sediment egestion by *Limnodrilus hoffmeisteri* versus sediment concentration in the pyrene (A) and phenanthrene (B) 10-d bioassay. Initial number of worms = 15. Means were taken either from daily egestion from days 1-9 or days 3-4. Error bars show 1 SD of mean (n = 4); 0 S = solvent control.
pyrene ($p = 0.021$) and phenanthrene ($p < 0.001$) experiments. For pyrene, a Bonferroni's multiple comparison test indicated no significant differences among sediment treatments. For phenanthrene, significant differences in dry weight were detected only at $612 \mu g/g$, which was significantly lower than at all other sediment treatments.

**Recovery experiment**

The daily fecal production of worms transferred from the $841 \mu g/g$ pyrene treatment and from the $203 \mu g/g$ phenanthrene treatment into solvent-control sediment increased quickly. Following transfer from pyrene, sediment egestion increased 2.8 times on day 1 and reached a peak of 17.3 mg on day 3. Worms from the phenanthrene treatment egested 7 times more sediment on day 1. Egestion increased slowly over time, reaching a maximum of 9.9 mg on day 3.

**5-d egestion bioassay**

No mortality occurred during the 5-d exposure in either pyrene or phenanthrene. Egested sediment accumulated from days 3 and 4 (Fig. 2.4) was analyzed using a split plot design. The ANOVA showed a non-significant interaction between sediment treatments and time ($p = 0.66$ for pyrene and $P = 0.43$ for phenanthrene). There was a significant treatment effect ($p < 0.001$) in both experiments. A t-test showed a significant difference between control and solvent control in both the pyrene and phenanthrene experiments, with the solvent-control yielding the lowest average. A Dunnett's multiple comparison test showed that only the $91 \mu g/g$ pyrene and the 47, 71, and $171 \mu g/g$ phenanthrene treatments were significantly different from the
Fig. 2.4. Daily sediment egestion by *Limnodrilus hoffmeisteri* versus sediment concentration in the pyrene (A) and phenanthrene (B) 5-d bioassay. Initial number of worms = 15. Error bars show 1 SD of mean \((n = 4)\); 0 S = solvent control; * represents significant difference \((α = 0.05)\) from solvent control mean.
solvent-control treatment. The IC_{50} values were 58.9 µg/g for pyrene and 28.4 µg/g for phenanthrene (Table 2.2). ANOVA indicated no significant difference among body dry weight of worms recovered from any treatment in both experiments.

**Sediment egestion vs. body dry weight relationship**

A significant relationship between average body dry weight and sediment egestion in *L. hoffmeisteri* was found (Table 2.4). Linear regression of average daily egestion by body dry weight over 4 consecutive days yielded the following equation:

\[
\text{average daily sediment egestion (mg)} = 111.8 + 22.2 \times \text{body dry weight (mg)} \quad (R^2 = 0.577).
\]

**Burrowing avoidance and reproduction bioassay**

Observations of the sediment surface were made throughout the 28-d experiment. In the pyrene experiment, all 15 *L. hoffmeisteri* burrowed within 10 min and remained burrowed throughout the observation period. In the phenanthrene experiment, all worms also burrowed within 10 min. However, in the period between 2 and 96 h, a maximum of 4 worms emerged out of the sediment in some chambers of 143 µg/g and higher. By 96 h and thereafter, all worms were found burrowed in the sediment.

After 28 days, cocoons (hatched and unhatched) and juveniles were recovered and enumerated from each chamber. The relative number of unhatched cocoons was low in all treatments, reaching a maximum of 10% of the total offspring and hatching success was not examined as a test endpoint. All statistical analyses were made on the total number of offspring produced (unhatched cocoons plus juveniles). Since mortality
Table 2.4. Wet- and dry weight (mg) of groups of 15 *Limnodrilus hoffmeisteri* assigned to defecation chambers and their average daily sediment egestion (mg dry feces).

<table>
<thead>
<tr>
<th>Chamber</th>
<th>Wet weight</th>
<th>Dry weight</th>
<th>Average egestion</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.93</td>
<td>0.37</td>
<td>206.3</td>
</tr>
<tr>
<td>2</td>
<td>1.52</td>
<td>0.42</td>
<td>247.0</td>
</tr>
<tr>
<td>3</td>
<td>2.17</td>
<td>0.47</td>
<td>200.3</td>
</tr>
<tr>
<td>4</td>
<td>2.42</td>
<td>0.55</td>
<td>206.0</td>
</tr>
<tr>
<td>5</td>
<td>2.67</td>
<td>0.56</td>
<td>234.0</td>
</tr>
<tr>
<td>6</td>
<td>3.54</td>
<td>0.63</td>
<td>211.3</td>
</tr>
<tr>
<td>7</td>
<td>2.92</td>
<td>0.63</td>
<td>206.7</td>
</tr>
<tr>
<td>8</td>
<td>3.38</td>
<td>0.65</td>
<td>294.3</td>
</tr>
<tr>
<td>9</td>
<td>4.21</td>
<td>0.82</td>
<td>352.7</td>
</tr>
<tr>
<td>10</td>
<td>5.95</td>
<td>1.02</td>
<td>325.0</td>
</tr>
</tbody>
</table>

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occurred during the 28-d sediment exposure, the number of offspring recovered from each chamber was standardized by the number of adults enumerated at the experiment termination.

In the pyrene experiment (Fig. 2.5), offspring were produced in all treatments and adult survival was high (> 88%). The average number of offspring per worm was highest in controls, ranging from 19.6 to 23.6. Offspring production was slightly lower in the acetone control and greatly reduced in all pyrene treatments, with averages tending to decrease with increasing pyrene concentration. In the phenanthrene experiment (Fig. 2.5), adult survival was high in the controls and treatments in which offspring were produced (> 81%). The few adults that survived exposures to the 396 \( \mu g/g \) and 612 \( \mu g/g \) treatments produced no offspring. Average offspring production was highest in the solvent control treatment (reaching a maximum of 34.5 offspring per worm), was slightly lower in the full control and decreased with increasing phenanthrene concentration. The ANOVA revealed significant difference among sediment treatments (p < 0.001) in both pyrene and phenanthrene experiments. There was no significant difference between the controls in both experiments. Dunnett's multiple comparison test of all pyrene treatments against the pooled control treatments showed that offspring production was significantly decreased for all concentrations. Dunnett's multiple comparison test of all phenanthrene treatments against the combined control treatments showed that offspring production was significantly decreased at the 102, 143 and 203 \( \mu g/g \) treatments. The IC\textsubscript{50} values were 59.1 \( \mu g/g \) for pyrene and 40.53 \( \mu g/g \) for phenanthrene (Table 2.2).
Fig. 2.5. Offspring production by *Limnodrilus hoffmeisteri* versus sediment concentration in the pyrene (A) and phenanthrene (B) reproduction bioassay. Initial number of worms = 8. Error bars show 1 SD of mean (n = 4); 0 S = solvent control; * represents significant difference (α = 0.05) from combined control mean. Numbers indicate percent adult survival.
Pyrene caused low mortality of *L. hoffmeisteri* (<20%) even at high sediment concentrations (up to 841 µg/g) in both 10 and 28-d exposures. Phenanthrene caused significant mortality only at high concentrations, yielding a 10-d LC₅₀ of 297.5 µg/g. Despite low lethal effects, significant sublethal toxic effects of pyrene and phenanthrene were detected as a decrease in sediment ingestion rate and offspring production by *L. hoffmeisteri* as indicated by low IC₅₀ values. The measured effects followed the expected concentration-response relationship, with increasing deleterious effects occurring with increasing contaminant concentrations. Sublethal effects of phenanthrene on aquatic invertebrates, reported for water-only exposures, include decreased growth, development and filtering rate of daphnids (Geiger and Buikema, 1981, 1982) decreased growth and development of juvenile mud crabs (Laughlin and Neff, 1979; 1980). Reports of pyrene sublethal effects are restricted to burrowing avoidance in *L. variegatus* (Kukkonen and Landrum, 1995).

Sediment contaminant concentration was measured at the initiation and end of the 10-d egestion rate experiment. Phenanthrene concentrations were greatly reduced during the 10-d exposure while pyrene concentrations declined very little. The sharper decline in phenanthrene sediment concentration was consistent with previous studies using freshwater sediment of similar organic carbon content (Landrum *et al.*, 1991; 1994). *Limnodrilus hoffmeisteri* created a network of tubes and burrows in the sediment matrix, increasing the surface area of sediment in contact with water. Daily exchange of overlaying water and adsorption to polypropylene in the experimental
chambers probably contributed to the depletion of contaminants from the sediment phase. Flux rate of PAHs from sediment into the water column has been shown to be correlated with the compound's water solubility and inversely related with its $K_{ow}$ (Reible et al., 1996; Miller et al., 1985). Phenanthrene is approximately 10 times more water soluble than pyrene. Microbial biodegradation is another possible source of phenanthrene depletion from the sediment.

Pyrene and phenanthrene sediment exposures at high concentrations resulted in low mortality of $L$. hoffmeisteri over 10 or 28 days. Pyrene did not cause significant mortality even at sediment concentrations much higher than that necessary to yield pore water saturated with contaminant, as predicted by the equilibrium partitioning theory (DiToro et al., 1991). Low pyrene lethal toxicity has been previously reported in sediment exposures using $Lumbriculus variegatus$ (Kukkonen and Landrum, 1994) and $Diporeia$ spp. (Landrum et al., 1994). Phenanthrene was lethally toxic only at high concentrations in the sediment. Results from the 10-d exposure indicate that $L$. hoffmeisteri is much less sensitive to this PAH than marine amphipods. The 10-d LC$_{50}$ value for $L$. hoffmeisteri (expressed as $\mu$g/g SOC), is 5.16 times and higher than for any amphipod tested (U.S.EPA, 1993b). The freshwater amphipod $Diporeia$ spp. also exhibited non-significant mortality at high phenanthrene concentrations (Landrum et al., 1994).

Sediment concentration has proven to be a poor representation of dose for mortality by PAHs, which was better expressed as body residue (Landrum et al., 1991; Landrum et al., 1994; Kukkonen and Landrum, 1994). Low lethal toxicity at high PAH
concentration in the sediment has been explained by a failure of PAHs to bioaccumulate at levels expected to cause mortality by non-polar narcosis (Landrum et al., 1994; Kukkonen and Landrum, 1994). Failure of body burden to reach lethal doses may be explained by behavioral modification of exposure, such as burrowing avoidance, which decreases organism contact with interstitial water and particle ingestion rate (Kukkonen and Landrum, 1994). This was not the case in this study, since most *L. hoffmeisteri* were observed fully burrowed in contaminated sediments throughout the exposure periods. However, high contaminant concentrations decreases sediment ingestion rate (Keilty et al., 1988b,c; this study) even of fully burrowed organisms, and may cause a decrease in bioavailability and consequently bioaccumulation.

Phenanthrene and pyrene significantly decreased feeding by *L. hoffmeisteri* at sublethal concentrations. Two experiments were performed using different ranges of contaminant concentrations. The 10-d egestion experiment was compared with the 5-d egestion experiment using the average daily egestion from days 3 and 4 combined. The IC$_{25}$ values from the 10-d experiment was only slightly lower than that from the 5-d experiment, indicating a similar dose-response pattern for both experiments, demonstrating good replicability of the bioassay. Effects of contaminants on the feeding rate of aquatic organism have been investigated using egestion rate as a surrogate with a variety of organisms. For example, the deposit feeder bivalve *Abra alba* greatly reduced feeding when exposed to sediment containing encapsulated diesel fuel high in PAHs (Stromgren et al., 1993), and significant inhibition of feeding rate of the lugworm *Nereis versicolor* occurred when the overlying water was contaminated.
with kepone (Rubinstein, 1979). Thus, we found the use of egestion rate to assess the effects of sediment-spiked contaminants on tubificid oligochaetes sensitive and replicable. It employs a simple test-protocol and reliable results can be obtained in few days.

Time interacted significantly with contamination to influence feeding by *L. hoffmeisteri* during long exposures. All contamination levels yielded sediment egestion significantly lower than the controls for the first 4 days of the egestion experiment. Only the lowest pyrene treatment was not significantly lower than solvent-control beyond day 4, since averages approached and even exceeded control levels. Mean sediment egestion from days 3 and 4 combined were very close to averages from days 1-9 and were significantly lower than controls. Averages from day 3 and 4 were representative of the general trend of decreased feeding over 10 days. By virtually eliminating the time effect of longer exposures and therefore simplifying the statistical analysis and interpretation of results, collecting egested sediment over 2 days following a 3 day acclimation period was the most efficient procedure to access the effects of PAH congeners on the feeding activity of *L. hoffmeisteri*.

Feeding activity was significantly increased when *L. hoffmeisteri* from contaminated sediment were transferred to solvent-control sediment. Worms recovered faster from the inhibitory effects of pyrene, and feeding rates attained control levels in few days. Individuals transferred from phenanthrene-contaminated sediment increased feeding dramatically, but it never reached control levels. This rapid increase was probably associated with PAH rapid elimination from body tissues via ingestion of
uncontaminated sediment. Kukkonen and Landrum (1994) determined rapid elimination of pyrene body burdens in *L. variegatus* placed in clean sediment, with a half life of 27 h. Recovery to normal feeding rate was also observed when the bivalve *Abra alba* exposed to aromatic hydrocarbons was transferred to clean sediment (Stromgren *et al.*, 1993). Rapid recovery suggests that pyrene and phenanthrene decreased the feeding activity *L. hoffmeisteri* due mostly due to a reversible narcotizing effect and less to permanent effects on physiological processes.

Mortality occurred throughout the duration of the 10-d bioassay, and the number of surviving worms used for dry weight determination ranged from 15. Therefore, the biomass of worms which were actively feeding during the early period of the experiment and died thereafter was not measured and should not be estimated as part of the weight of a group of worms composed of very few individuals. Consequently, egestion rates were normalized by the number of living worms in each chamber rather than by post-experimental measures of average worm biomass. Our procedure of randomly assigning worms to defecation chambers created a non-biased distribution of worm biomass per sediment treatment, and the number of potentially active (or surviving) worms in each defecation chambers was estimated daily. Furthermore, the linear relationship established between daily sediment egestion and body dry weight indicated that only 57.7% of the variation on levels of daily egestion was explained by body dry weight.

*Tubifex tubifex* offspring production has been reported as a sensitive toxicity test endpoint (*e.g.*, Reynolds *et al.*, 1991; Reynolds, 1994; Day *et al.*, 1995a). Using
procedures modified from Reynoldson et al., (1991), we detected a significant sublethal
effects of pyrene and phenanthrene on the reproductive output of L. hoffmeisteri.
expressed as a decrease in the numbers of offspring (unhatched cocoons and young)
produced relative to control levels. The concentrations used were the same as in the 10-
d egestion bioassay. When compared with the egestion experiments, the reproduction
bioassay yielded higher IC$_{50}$ values (only slightly with pyrene and over 1.5 time with
phenanthrene) with considerably wider confidence intervals. The width of the
confidence interval as calculated by the bootstrap method is related with the variability
of the data and the reliability of the ICp value is in question when the range is too broad
(U.S.EPA, 1994).

Bioturbation by deposit feeders has profound ecological implication in
contaminated sites by affecting the stratigraphic redistribution of sediment particles and
by increasing the rate at which contaminants flux into the overlying water.
Concentration of benzo(a)pyrene in the overlying water increased in sediment reworked
by chironomids in a microcosm experiment (Clements et al., 1994). The presence of a
tubiculous polychaete Nereis virens in the sediment also increased the flux of
benzo(a)pyrene from the sediment and enhanced microbial degradation of this
compound (McElroy et al., 1990). Bioturbation by the polychaete Nereis diversicolor
significantly influenced the fate of hydrocarbons (crude oil) in the sediment by
increasing downward movement into deeper layers and flux into the water column (Gilbert
et al., 1994). Tubificid worms bioturbate sediments by building tubes and altering its
porosity and by reworking sediment in a conveyor-belt fashion, moving sediment
ingested at sub-surface to the sediment-water interface as fecal pellets. The effects of tubificid activity on the flux of organic contaminants into the overlying water has been documented and modeled (Karickoff and Morris, 1985; Reible et al., 1996). The flux of pyrene, phenanthrene and dibenzofuran increased by a factor of 4 to 6 when L. hoffmeisteri was added to sediment microcosms (Reible et al., 1996). Sediment reworking activity correlates directly to feeding activity in conveyor-belt-fashion deposit feeders and have been investigated using tubificid worms (Karickoff and Morris, 1985; Reynoldson, 1987; Reible et al., 1996; Keilty et al., 1988b,c). This study and previous investigations demonstrated that the presence of organic contaminants in the sediment depresses deposit feeders reworking rates. Keilty et al. (1988c), using a \(^{127}\)cesium marker layer burial technique, determined that a long-term exposure to endrin significantly decreased the reworking rates of L. hoffmeisteri at concentrations 2 orders of magnitude lower than the 96-h LC\(_{50}\). The reworking activity of Nereis diversicolor was significantly reduced in the presence of Arabia light crude oil (Gilbert et al., 1994).

It is strongly recommended that changes in egestion (reworking) rate be accounted for in investigations of flux of contaminants into the overlying water in the presence of bioturbating organisms. Ideally, these effects should be included in models that predict flux in natural settings.
CHAPTER 3

TOXICITY OF SEDIMENT-ASSOCIATED PAHs TO AN ESTUARINE COPEPOD: EFFECTS ON SURVIVORSHIP, FEEDING, REPRODUCTION, AND BEHAVIOR
INTRODUCTION

PAHs are among the most carcinogenic, mutagenic and toxic contaminants found in aquatic systems (Kennish, 1992). The lethal toxicity of single-PAH compounds to aquatic invertebrates has been the subject of numerous investigations (Kennish, 1992; Neff, 1985). In aqueous exposures, PAH sublethal effects include decreased growth, development time, reproductive output, feeding rate, life-span and oxygen uptake (Tatem, 1977; Ott et al., 1978; Laughlin and Neff, 1979; Laughlin and Neff, 1980; Geiger and Buikema, 1981; 1982; Crider et al., 1982; Donkin et al., 1989). Refined petroleum derivatives rich in PAHs, such as diesel fuel, are also toxic. Besides causing mortality, aqueous exposures to these mixtures impaired feeding, reproduction, development, and behavior of planktonic and benthic invertebrates (Barnett and Kontogiannis, 1975; Laughlin et al., 1978; Tarkpea and Svanberg, 1982; Nagarajah et al., 1985; Barre et al., 1994; Mackey and Hodgkinson, 1996).

Due to their high hydrophobicity, PAHs in the water column are rapidly sequestered into the organic matrix of suspended and bed sediments resulting in chronic sediment contamination. Despite the risk imposed by PAH-contaminated sediments to infaunal organisms, relatively few investigations have addressed the toxicity of sediment-associated PAHs (Swartz et al., 1990; Suedel et al., 1993; 1996; U.S.EPA, 1993a,b,c; Kukkonen and Landrum, 1994; Landrum et al., 1994). PAH sediment-concentration in polluted sites (Kennish, 1992) are usually below those reported as lethally toxic, and, unfortunately, accounts on sublethal toxicity of PAHs in sediment exposures are even fewer in number. Effects include decreased sediment ingestion rate
and offspring production in oligochaetes (Lotufo and Fleeger, 1996) and impaired burrowing activity of gastropods (Chapman et al., 1988), amphipods (Landrum et al., 1994), and oligochaetes (Kukkonen and Landrum, 1994). Ecologically relevant sublethal effects of organic compounds such as decreased feeding rate and reproductive output have been detected at sediment concentrations much lower than LC₃₀s, indicating greater sensitivity for toxicity assessment (e.g. DiPinto et al., 1993; Lotufo and Fleeger, 1996). More information on the sublethal or chronic toxicity of sediment associated PAHs is necessary to improve existing sediment-quality criteria for single PAH compounds (U.S.EPA 1993a, b, c), and predictive models for the toxicity of PAH mixtures in field-collected sediments (Swartz et al., 1995).

Harpacticoid copepods are typically the second most abundant taxon in the omnipresent meiofauna (metazoans passing through a 0.5 mm sieve). They have a significant role in estuarine food webs, especially as a critical food source for a variety of juvenile fish and shellfish (Coull, 1990). Harpacticoids display many traits that make them well-suited for sediment-toxicity assessment: high abundance, small size, intimate association with the sediment, infaunal larvae, a predominantly sessile life-style, and ease of laboratory culturing. Numerous field and mesocosm studies have demonstrated adverse effects of petroleum hydrocarbons on harpacticoids (Coull and Chandler, 1992; Carman and Todaro, 1996). Few laboratory studies have been conducted, all using aqueous exposures and involving complex mixtures (Coull and Chandler, 1992). Sediment-toxicity testing, however, is important for the interpretation of contamination impacts on field populations, especially as meiofauna community structure is being
recognized as a sensitive bioindicator of pollution in the benthic environment (Heip, 1980; Sandulli, 1986; Vincks and Heip, 1987; Moore and Bett, 1989; Warwick et al., 1990; Bett and Moore, 1992; Somerfield et al., 1994; Somerfield et al., 1995).

Here, the toxicity of sediment-associated phenanthrene, fluoranthene and diesel fuel to the estuarine harpacticoid *Schizopera knabeni* Lang was investigated. Phenanthrene and fluoranthene were selected as PAH congeners for their concentration in contaminated estuarine sediments, and for being reportedly toxic to sediment-dwelling invertebrates (Kennish, 1992; U.S.EPA, 1993b,c). Diesel fuel is a refined petroleum product comprised of hundreds of saturated and aromatic hydrocarbons. It is more toxic to estuarine crustaceans than most crude oils because of its high relative abundance in di- and tri-cyclic aromatic hydrocarbons (Anderson et al., 1974). Because *S. knabeni* has a short life-cycle (ca. 21 d), with all stages contained within the sediment, and is easily cultured in laboratory, it is suitable to examine effects on reproduction. Effects on survival were assessed in short-term exposures (4 d), and sublethal effects were investigated using grazing rate and offspring production as endpoints. The ability of *S. knabeni* to detect and avoid exposure to PAH-contaminated sediment was assessed using preference arenas.

**MATERIAL AND METHODS**

**Test organism**

*Schizopera knabeni* used in all experiments was obtained from a mono-specific laboratory culture started with a stock collected in October 1993 from the surface
sediment from an intertidal mud flat of a *Spartina alterniflora* salt-marsh at Port Fourchon, Louisiana. It was cultured sediment-free in 500 ml Erlenmeyer flasks at room temperature with 25% artificial seawater (ASW) fully renewed fortnightly. Copepods were fed twice a week with a mixture of *Chaetoceros muelleri*, strain Chaet 10 (a planktonic diatom) and Microfeast Plus Larval Diet®. Copepods were harvested by sieving the culture medium through a 125 μm mesh, and sorted under a stereo microscope.

**Sediment contamination: phenanthrene and fluoranthene**

Sediment was collected from the top 2 cm of a mud flat in a *Spartina alterniflora* salt marsh at Cocodrie, Louisiana. The typical total PAH concentration in this sediment was 0.26 mg/kg (Carman et al., 1995). Stock test-sediment was prepared by sieving the mud through a 45 μm mesh. The sediment that went through the sieve (< 45 fraction) was allowed to settle overnight at 4 °C, the supernatant removed by aspiration, and the sediment autoclaved. The sediment dry-to-wet-weight ratio was determined by oven drying (80 °C) and adjusted to 0.15 by homogenizing the sediment with the appropriate volume of 25 % ASW. The sediment organic carbon (SOC) of the resultant slurry, measured in duplicate on a Perkin Elmer (Norwalk, CT) 2400 CHN Elemental Analyzer, was 1.5 % after acidification with HCl to remove inorganic carbonate. The stock sediment was stored at 4°C.

Phenanthrene and fluoranthene (98% purity, Aldrich Chemical Co, Milwaukee, WI) were amended to the stock sediment by spiking. Stored sediment was fully homogenized with the overlying water and 133 g of wet sediment (20 g dry weight) was

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transferred to 250 ml beakers and vigorously stirred under vortex. The appropriate amount of phenanthrene (PHN) or fluoranthene (FLN), carried in 0.2 ml of acetone was spiked on the slurry and stirred for 4 h. The required amount of spiked phenanthrene or fluoranthene was calculated on a dry weight basis. A solvent control (0 S) was prepared by adding acetone only and a full control was prepared with no spiking. Sediments were stored in the dark at 4°C for 3 to 5 weeks, fully homogenized with the overlying water. Fluoranthene and phenanthrene sediment concentrations were measured by reverse phase HPLC as described in Lotufo and Fleeger (1996) before use in experiments and determined to be 56, 126, 261, 514, 1030, µg/g (PHN) and 61, 137, 249, 451, 990, 2170 µg/g (FLN).

**Sediment contamination: diesel fuel**

Sediment was collected and processed as described above, sieved to < 125 µm, and allowed to settle overnight. Two liters of sieved sediment were tumbled on a roller mill for 10 days with 600 ml of diesel fuel obtained from a commercial vendor. The bottle was then removed from the tumbler and sediment allowed to settle overnight. Diesel was aspirated from the bottle and 1 L of 15 % ASW was added. The mixture was tumbled again (overnight), allowed to settle, and the supernatant aspirated. This procedure was repeated three times (total of four rinses). The sediment/water slurry was transferred to 35-ml glass centrifuge tubes and centrifuged at 1700 x g for 3 min. The supernatant was removed and replaced with fresh ASW. Sediment and water were mixed thoroughly then recentrifuged. The supernatant was decanted and again, and the process was repeated for a total of four rinses via centrifugation. Sediment was then
recombined into a single batch and mixed to assure homogeneity. Final sediment was
analyzed by GC/MS for total PAH which was determined to be 687 µg/g. The absolute
concentrations of major PAH classes is provided in Carman et al. (1996).
Contaminated sediment was then sieved to < 45 µm and diluted with < 45 µm non-
contaminated sediment creating a dilution series. Total PAH concentrations were
measured using an latroscan (as described in Carman et al., 1996) and determined to be
19, 45, 93, 130, 185 and 370 µg/g.

96-h survivorship bioassay

Test units (28 x 45 mm carrier glass vials; Kimble, Toledo, OH) were filled with
10 ml of ASW using a volumetric flask. One ml of sediment treatment was dispensed
with minimal disturbance to the bottom of each vial using a 1-ml Finnpipette® creating a
2-3 mm sediment layer. Four replicates were used per sediment treatment (PHN: 0, 0 S,
126, 261, 514, 1030 µg/g; FLN: 0, 0 S, 137, 249, 451, 990, 2170 µg/g; DIE: 45, 93,
185, 370 µg/g). Five additional replicates containing control sediment were prepared
for water-quality measurements. Vials were placed in random order inside moisture
chambers (loosely covered plastic containers underlined with soaked paper towels
which created a humid environment to retard evaporation from experimental dishes).
They were kept overnight in the dark at 25 °C in an incubator with no illumination.
Fifteen adult female S. knabeni were introduced to each experimental unit and vials
were returned to the incubator. Five additional replicates containing control sediment
were prepared for water-quality measurements. After 96 h, the contents of all vials
were sieved through a 45-µm mesh and retained copepods were enumerated as live or
dead. Salinity and dissolved oxygen (DO) were measured at initiation and termination of the experiment using a Reichert refractometer (Cambridge Instruments, Buffalo, NY) and an Orion (Boston, MA) model 820 oxygen meter.

30-h grazing bioassay

To determine the effects of PAHs on *S. knabeni* grazing rates, sediment exposed copepods were fed radiolabeled microalgae. An inoculum of *Thalassiossira weisflogii* (UTEX-Collection, University of Texas at Austin) in log-phase growth was added to 350 ml of F/2 media at 25 °C in Erlemeyer flasks containing 200 μCi (7.4 x 10⁶ Bq) of NaH¹⁴CO₃ (specific activity 60 Ci/mM, American Radiolabeled Chemicals, St. Louis, MO). Cultures were sealed to prevent loss of label as ¹⁴CO₂ and maintained at 25°C, 16/8 h light/dark cycle, and pH of 7.5. Cultures were monitored every 48 h for cell density and label incorporation and grown until the radioactivity in the cells became constant (7-d). Algal cells were then harvested by centrifugation followed by aspiration and replacement of supernatant with 25 % ASW (performed twice) to ensure removal of unincorporated label. Cell density was determined by direct count and radioactivity in algal cells using a Beckman LS 6000IC liquid scintillation counter (Paloalto, CA).

Test units were prepared, with 4 replicates per sediment treatment (PHN: 0 S, 56, 126, 261, 514 μg/g; FLN: 0 S, 61, 137, 249, 451 μg/g; DIE: 0, 19, 45, 93, 130 μg/g), and 5 adult females added to each vial as described for the survivorship experiment. Four test units containing control sediment and five formalin killed adult females were used to determine copepod incorporation of label other than by feeding (dead control). After a period of 24 h (starvation and acclimation period), each vial was inoculated with
radiolabeled cells and returned to the incubator (25 °C). The phenanthrene and fluoranthene experiments were performed simultaneously with each vial receiving 0.7 ml of radiolabeled cells (~2 x 10⁶ cells; 2.5 DPM per cell). In the diesel-fuel experiment, each vial received 0.5 ml of radiolabeled cells (~2 x 10⁶ cells; 1 DPM per cell). After 6 h (grazing period), copepods were formalin killed and retrieved from test-vials. The five copepods from each replicate were placed together in a scintillation vial and solubilized in 200 µl of TS-2 tissue solubilizer (Research Products International Corps., Mount Prospect, IL). After 24 h, 100 µl of 1.2 N HCl (to neutralize the tissue solubilizer) and 10 ml of Biosafe II liquid scintillation cocktail (Research Products International Corps., Mount Prospect, IL) were added and ¹⁴C activity was determined by liquid scintillation counting. Radioactivity was converted to number of ingested algal cells by subtracting total counts by the mean radioactivity in dead control copepods and dividing by the mean radioactivity per algal cell.

14-d reproductive output bioassay

The effects of PAHs on the reproductive output of S. knabeni were assessed by exposing individual copulating pairs to sediment treatments. Test units were prepared, with 10 replicates per sediment treatment (PHN: 0, 0 S, 56, 126, 261 µg/g; FLN: 0, 0 S, 61, 137, 249 µg/g; DIE: 0, 19, 45, 93, 185 µg/g), as described for the survivorship experiment. The phenanthrene and fluoranthene experiments were conducted simultaneously and shared the same set of controls. Five additional replicates containing control sediment were prepared for water-quality measurements (salinity and DO). One copulating pair was added to each vial. Copulating pairs consisted of an
adult male clasping a pre-adult female (copepodite V) or, less frequently, an adult female. Each vial received food at day 0 as a single dose of 0.1 mg of Microfeast Plus Larval Diet® and were placed in moisture chambers inside an incubator at 25°C with no illumination for 14 d. At test termination, the contents of all vials were sieved through a 45-μm mesh and the retained material was washed into a plastic dish, examined for adult survival, preserved with 4% buffered formalin, and stained with Rose Bengal. Subsequently, females were separated and realized offspring (nauplii and copepodites) were enumerated. Egg sacs were detached intact from gravid females and examined for clutch size determination.

6-h avoidance/preference experiment

In order to determine whether S. knabeni actively discriminates contaminated sediment and avoids exposure to it, burrowing preference experiments were conducted. The experimental apparatus was a modification of that used by Decho and Fleeger (1988). Crystallizing dishes (150x75 mm) were filled with liquified agar (15 g agar to 1 L H₂O) to a depth of 1 cm. Five cylindrical jars (30 mm in diameter) were placed equidistantly from the center and among themselves near the periphery of the dish. After solidification, the jars were removed leaving five circular wells in the agar. The dish was filled with 500 ml of 25% ASW and each well was randomly assigned to one sediment treatment (PHN: 0 S, 56, 126, 261, 514 μg/g; FLU: 0 S, 137, 249, 451, 990 μg/g; DIE: 0, 45, 93, 130, 185 μg/g) and filled with minimal disturbance, until flush with the agar substrate and. The experimental dish was left to settle for 6 hours. A plastic cylindrical tube open on both sides was then pressed against the agar in the
center of the dish. A group of 150 adult *S. knabeni* were poured into the tube, which was removed after 5 min, setting the copepods free on the agar surface. For each contaminant, this experiment was conducted in the dark simultaneously in seven arenas. After a period of 12 h, sediment from each well was collected using a 50-ml transfer pipet and sieved. Retained copepods were formalin killed and enumerated. Copepods remaining in the bath water were also removed and enumerated. A control experiment was performed as above using non-contaminated sediment (0 S) only and each well randomly assigned a number from 1 to 5.

**Statistical analysis**

*Schizopera knabeni* mortality, grazing rate, number of offspring and clutch size were analyzed using one-way ANOVA. Contaminant treatments were compared with control treatments using Dunnett's one tailed t-test ($\alpha = 0.05$). Mean solvent control values were compared to mean full-control values using a two-tailed t-test ($\alpha = 0.05$) and data were combined if no difference was detected. $LC_{50}$ values were computed using the Trimmed Spearman-Karber method (Hamilton *et al.*, 1977). Point estimates for grazing rate and reproductive output was calculated using linear interpolation combined with bootstrapping, or ICp method (U.S.EPA, 1994). With $p=25$, IC$_{25}$ estimates the contaminant concentration causing a 25% reduction in the measured endpoint in relation to the control(s). All the analyses were performed using TOXCALC™ version 5.0 (Tidepool Software; McKinleyville, CA).

A multivariate analysis of variance was performed to analyze data from the preference/avoidance experiments due to a lack of independence among treatments.
Pairwise contrasts of each contaminant treatment against the control were performed. Multivariate analysis were conducted using SAS® software (Release 6.03 Edition, Cary, NC).

RESULTS

A concentration-dependent response was obtained in all experiments. ANOVA indicated significant PAH-treatment effects ($p < 0.05$) in all 96-h survivorship, grazing rate and reproductive output experiments.

96-h survivorship bioassay

The salinity remained unchanged throughout the duration of the experiment (25%). Mean DO levels were 4.9 mg/L (SD = 0.24) at 0 h and 4.8 mg/L (SD = 0.16) after 96 h.

Mortality ranged from 0 to 13% in the control replicates for all experiments, but mortality increased with increasing PAH sediment concentration. In the phenanthrene and fluoranthene experiments, mortality was not significantly different between full and solvent control. In the phenanthrene experiment (Fig. 3.1), mortality at the two highest concentrations (514 and 1030 µg/g) was significantly different from the controls. The $LC_{50}$ was 473 µg/g (Table 3.1). In the fluoranthene experiment (Fig. 3.1), all treatments except 249 µg/g were significantly different from the controls, but mortality never exceeded 50%. Probit and Trimmed Spearman-Karber analysis to estimate an $LC_{50}$ were unsuccessful due to low mortality in the concentrations used ($LC_{50} > 2,100$ µg/g).
Table 3.1. Toxicity values from bioassays performed with *Schizopera knabeni*. All values expressed as \( \mu g/g \). Numbers in parenthesis indicate 95% confidence intervals.

<table>
<thead>
<tr>
<th>Value</th>
<th>Phenanthrene</th>
<th>Fluoranthene</th>
<th>Diesel Fuel</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-d LC50</td>
<td>473 (401-588)</td>
<td>&gt; 2,100</td>
<td>194 (182-199)</td>
</tr>
<tr>
<td>Grazing IC25</td>
<td>25.5 (19.3-35.8)</td>
<td>65.1 (16.9-94.5)</td>
<td>19.3 (9.9-39.1)</td>
</tr>
<tr>
<td>Total reproduction</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IC25</td>
<td>26.9 (19.2-75.7)</td>
<td>21.8 (17.4-32.9)</td>
<td>47.1 (11.7-71.3)</td>
</tr>
<tr>
<td>Realized reproduction</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IC25</td>
<td>25.9 (17.8-67.8)</td>
<td>18.4 (16.1-22.1)</td>
<td>18.2 (10.1-58.5)</td>
</tr>
</tbody>
</table>

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Fig. 3.1. Mortality of *Schizopera knabeni* in the phenanthrene, fluoranthene and diesel fuel 96-h bioassay. Error bars show 1 SD of the mean (n= 4); 0 S = solvent control; * represents significance difference (α = 0.05) from the control (or combined control) mean.
In the diesel experiment (Fig. 3.1), mortality was significantly different from the control in all treatments except at 45 μg/g. The LC<sub>50</sub> was 194 μg/g (Table 3.1).

**Thirty-h grazing bioassay**

Grazing rates, expressed as the number of algal cells ingested per hour, were significantly decreased by sediment-associated PAHs at sublethal concentrations. Phenanthrene (Fig. 3.2) significantly reduced grazing rates at all levels compared to the control. The mean grazing rate at the lowest concentration (56 μg/g) was lower than grazing at the next two higher concentrations. The IC<sub>25</sub> value obtained was 25.5 μg/g. The grazing rate at the lowest fluoranthene concentration (61 μg/g) was lower than but not significantly different from the control. However, rates were sharply reduced at 137 μg/g treatment and feeding virtually ceased at the two highest concentrations, 249 and 514 μg/g (Fig. 3.2). The IC<sub>25</sub> value obtained was 65.1 μg/g (Table 3.1). Grazing rates gradually decreased with increasing diesel-fuel concentrations in the sediment, attaining averages below 10% of control levels at the two highest concentrations, 93 and 130 μg/g (Fig. 3.2). All diesel-fuel treatments were significantly different from the control. The IC<sub>25</sub> value obtained was 19.3 μg/g (Table 3.1).

**14-d reproduction output bioassay**

The salinity remained unchanged throughout the experiment duration (25 %). Mean DO levels were 4.36 mg/L (SD = 0.34) at 0 d and 4.18 mg/L (SD = 0.44) after 10 d.

Mean offspring production was calculated on a per-surviving-female basis; accordingly, sample sizes differed among treatments (Fig. 3.3). Total offspring
Fig. 3.2. Grazing rate of *Schizopera knabeni* in the phenanthrene, fluoranthene and diesel fuel 30-h bioassay. Error bars show 1 SD of the mean (n = 4); 0 S = solvent control; * represents significance difference (α = 0.05) from the control mean.
consisted of eggs contained in a pair of attached sacs, plus realized offspring (nauplii and copepodites) recovered after 14 d. The same set of controls was used for both phenanthrene and fluoranthene treatments; no adult mortality was observed in the controls. Mean number of total offspring was higher in the solvent than in the full control, but not significantly (Fig 3.3). In the phenanthrene experiment adult survival was 70% in all treatments. Mean number of total offspring at 56, 126 and 261 µg/g was significantly reduced to 52, 38 and 48% of the pooled control level, and mean realized offspring production was reduced to 51, 31 and 29%, respectively (Fig. 3.3). The IC_{25} value obtained was 26.9 µg/g for total and 25.9 µg/g for realized offspring (Table 3.1). All phenanthrene treatments were significantly different from the pooled controls.

In the fluoranthene experiment, adult survival was 80% at 61 µg/g, 100% at 137 µg/g and 0% at 249 µg/g treatment. Mean total offspring production at 61 and 137 µg/g was significantly reduced to 32 and 18% of the pooled control level, and mean realized offspring production was reduced to 21 and 6%, respectively (Fig. 3.3). The IC_{25} value obtained was 21.8 µg/g for total and 18.4 µg/g for realized offspring (Table 3.1).

In the diesel-fuel experiment, adult survival was 100% in all treatments, except at 185 µg/g (30%). The mean total and realized offspring produced at 19 and 45 µg/g were lower than in the control, but not significantly so (Fig. 3.3). The mean number of total offspring at 93 and 185 µg/g was significantly reduced to 34 and 42% of the control, respectively. Mean number of realized offspring was reduced to a greater extent: 12% of the control at 93 µg/g and no realized offspring production at 185 µg/g.
Fig. 3.3. Number of total and realized offspring produced by individual copulating pairs of *Schizopera knabeni* in the phenanthrene, fluoranthene and diesel fuel 14-d bioassay. Error bars show 1 SD of the mean (n = 4); 0 S = solvent control; * represents significance difference (α = 0.05) from the control (or combined control) mean.

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The IC$_{25}$ value obtained was 47.1 µg/g for total and 18.2 µg/g for realized offspring (Table 3.1).

Average brood size was determined for ovigerous females surviving the exposure period. Replication ranged from 2 to 9 (Fig. 3.4). Control and solvent-control females for the phenanthrene and fluoranthene experiments carried an average of 16.1 and 19.1 eggs, respectively. Control females in the diesel experiment carried an average of 18.9 eggs. The average number of eggs per clutch ranged from 14.8 to 26 in females exposed to PAHs (Fig. 3.4), but clutch size was not significantly different among treatments for any contaminant (p > 0.25).

**6-h avoidance/preference experiment**

The fraction of copepods introduced to preference arenas that burrowed into sediment was estimated from the mean number of copepods recovered from sediment wells (Table 3.2). The fraction was lowest in the diesel-fuel experiment, with only 36.7% of the copepods recovered from sediment wells, and highest in the control experiment (76.8%). From the total number of copepods found burrowed, the fraction found in each sediment treatment was calculated. When exposed to uncontaminated sediment and four levels of contaminated sediment, most copepods actively burrowed into non-contaminated sediment. The fraction of burrowed copepods recovered from control sediment was approximately 47% in the phenanthrene and fluoranthene experiment and 68.7% in the diesel-fuel experiment. Detection and avoidance of contamination is indicated by the decrease in the fraction of burrowed copepods with increasing contamination observed with the three contaminants. Moreover, in the
Fig. 3.4. Clutch size of ovigerous *Schizopera knabeni* at termination of the phenanthrene, fluoranthene and diesel fuel 14-d bioassay. Error bars show 1 SD of the mean (n = 4); numbers above error bars indicate number of ovigerous females in each treatment; 0 S = solvent control.
Table 3.2. Results from the avoidance/preference experiments. For each experiment, numbers indicate the mean percentage of burrowed copepods recovered from each sediment treatment. Numbers in parenthesis indicate 1 standard deviation of the mean.

<table>
<thead>
<tr>
<th>Phenanthrene: 40.8 (11.1) % found burrowed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treat.</td>
</tr>
<tr>
<td>%</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Fluoranthene: 67.5 (12.2) % found burrowed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treat.</td>
</tr>
<tr>
<td>%</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Diesel fuel: 36.7 (18.1) found burrowed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treat.</td>
</tr>
<tr>
<td>%</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Control experiment: 76.8% (2.9) found burrowed</th>
</tr>
</thead>
<tbody>
<tr>
<td>“Treat”: I</td>
</tr>
<tr>
<td>%</td>
</tr>
</tbody>
</table>
control experiment, when only uncontaminated sediment was used, copepods were found uniformly distributed among the five wells (range from 16 to 25.1%), indicating no preference. In the phenanthrene and diesel-fuel experiments, Wilks' lambda statistics indicated significant differences among treatments (p < 0.05) and the contrasts indicated that the all phenanthrene treatments were significantly lower than the control (p <0.01). In the fluoranthene experiment, Wilks' lambda statistics indicated a marginal significant difference among treatments (p = 0.054) and the contrasts indicated that all fluoranthene treatments were significantly lower than the control (p <0.05). In the control experiment, Wilks' lambda statistics indicated no significant differences among "treatments" (p = 0.50).

DISCUSSION

Although all compounds caused mortality, diesel fuel was more toxic to \textit{S. knabeni} in sediment exposures than the PAH congeners phenanthrene and fluoranthene. In a short-term exposure (4 d), fluoranthene and phenanthrene caused significant mortality only at very high concentrations (≥ 451 μg/g). Mortality remained below 50% even at fluoranthene concentrations as high as 2170 μg/g. The higher toxicity of diesel fuel was probably due to the elevated concentration of naphthalene and its alkyl derivatives (Anderson, 1974; Carman \textit{et al.}, 1996), which are expected to become more rapidly bioavailable in the pore water. In the 14-d exposure, complete adult mortality occurred at 249 μg/g fluoranthene, but phenanthrene caused low mortality at concentrations as high as 261 μg/g; thus fluoranthene was more lethally toxic than
phenanthrene. Higher toxicity of fluoranthene was expected, as PAH toxicity increases with increasing molecular weight (Neff, 1995). *Schizopera knabeni* was more tolerant to phenanthrene and fluoranthene effects on survival than benthic amphipods and chironomids (Swartz *et al.*, 1990; DeWitt *et al.*, 1992; Suedel *et al.*, 1993; 1996; U.S.EPA 1993b,c). No other crustacean or meiofaunal organism has been tested in sediment exposures to PAH congeners.

Although significant mortality was observed only at high PAH concentrations in 96-h exposures, signs of narcotization were observed in individuals retrieved from all contamination levels. Copepods retrieved from the 96-h exposure displayed different degrees of lethargy, from slow and erratic swimming to complete immobilization. A paralyzed copepod would still be enumerated as live if it displayed body contraction or appendage movement upon contact with a probe. All live copepods from the highest phenanthrene and fluoranthene treatments were incapable of normal swimming.

The effects of sediment-associated PAH on *S. knabeni* grazing on microalgae was assessed in a short-term exposure (30 h). Overall, a negative impact of PAH on grazing increased with increasing contaminant concentration in the sediment, following an expected concentration-response relationship. Strong impairment of feeding was detected at concentrations well below those that caused mortality in the 96-h exposures. Feeding almost completely ceased at concentrations much lower than the phenanthrene and diesel fuel 96-h LC$_{50}$s. For example, feeding was decreased by 85% at 137 µg/g of fluoranthene, whereas at 2,100 µg/g only 42% mortality was observed in 96 h. Petroleum hydrocarbons (crude oil, water soluble fraction, naphthalene) have been
observed to decrease the feeding rate in a variety of animals with a diversity of feeding
modes, including suspension feeding copepods (Berdugo et al., 1977; Spooner and
Corkett, 1979; Berman and Heinle, 1980; Cowles, 1983; Cowles and Remillard, 1983;
Lacaze and Ducreux, 1987) cladocerans (Geiger and Buikema, 1981), bivalves (Donkin
et al., 1989), deposit-feeding oligochaetes (Lotufo and Fleeger, 1996), and bivalves
(Stromgren et al., 1993). Feeding activity has been increasingly used as a toxicity-test
endpoint. Recently developed tests using planktonic invertebrates, such as cladocerans,
rotifers and ciliates allowed sensitive and repeatable detection of decreased or halted
feeding in exposures as short as one hour (Fernandez-Casalderrey et al., 1994; Bitton et
al., 1995; Juchelka and Snell, 1995; Bitton et al., 1996). Narcosis due to exposure to
hydrophobic contaminants, including PAH, is described as a disturbance of membrane
function resulting in decreased activity and a diminished ability to react to stimuli that
can ultimately lead to death (van Wezel and Opperhuizen, 1995). If one assumes that
decreased feeding rate is a direct consequence of increased lethargy, it seems quite
possible that feeding rate could be used as a measurement of narcotization. However,
decreased feeding may be related to the direct interference of hydrocarbons with
mechanisms of food perception via blockage of receptors, as speculated with planktonic
copepods (Cowles, 1983).

PAHs adversely affected S. knabeni reproduction at sublethal concentrations.
Offspring production over 14 days was significantly reduced at the lowest phenanthrene
and fluoranthene concentrations used (approximately 60 µg/g) and 93 µg/g of diesel
fuel in sediment. Ovigerous females from all treatments were examined and no
significant difference in clutch size was detected in any of the experiments. However, a consistent decline in number of realized offspring (nauplii plus copepodites) was observed at increasing concentrations of PAH in the sediment. Therefore, reduced offspring production was due not to decreased clutch size, but most likely to a combination of a reduction in the number of broods produced, reduced egg hatching success, and reduced larval survival. Petroleum hydrocarbons have been reported to affect the reproduction of other copepod species. Reduced life span, total number of eggs produced during the entire reproductive life, mean brood size, and rate of egg production followed a short-term exposure of *Eurytemora affinis* to the water soluble fraction (WSF) of high aromatic heating oil and naphthalene (Berdugo *et al.*, 1977). The WSF of Louisiana crude oil significantly reduced brood size but not the number of broods produced by *Nitocra affinis* Ustach, 1979), and severely decreased hatching success and naupliar survival but not rate of egg production in *Centropages hamatus* (Cowles and Remillard, 1983). A decrease in naupliar production was also observed when *Tigriopus californicus* was exposed to urban sediment contaminated with aromatic and chlorinated hydrocarbons and heavy metals (Misitano and Schiewe, 1990). Copepod reproductive output has proved to be a sensitive endpoint to assess the sublethal toxicity of sediment-associated contaminants. Egg production and brood size of *Microarthridion littorale* and *Paronychochamptus wilsoni* were significantly reduced at sublethal concentrations of the pyrethroid insecticide fenvalerate (Chandler, 1990) and the reproductive output of *M. littorale* was significantly decreased at PCB (Aroclor 1254) sediment concentrations as low as 5% of the concentration required to
significantly affect mortality (DiPinto et al., 1993). Differential features such as high fecundity and short developmental and generation time make harpacticoids exceptional test-organisms for assessing contaminant effects on specific population and life-history traits.

Results from the avoidance/preference experiments clearly indicate that *S. knabeni* actively avoids contaminated sediment and selects non-contaminated sediment in standing water. The fraction of total copepods found burrowed in sediment wells was likely related to contaminant hydrophobicity. In the experiment performed with diesel fuel, which contains a large fraction of low-molecular-weight aromatic hydrocarbons, an average of 36.7% of the individuals introduced in the arena were recovered from sediment, whereas with fluoranthene, the most hydrophobic contaminant used, 67.5% of the copepods were found burrowed. This finding suggests that the concentration of PAHs in the overlying water may contribute to the overall burrowing avoidance of copepods. PAH concentration in the overlying water was probably highest with diesel-contaminated sediment and burrowing may have been inhibited even into non-contaminated sediment. Avoidance of exposure to fluoranthene in the water phase has been demonstrated with fathead minnows (Farr et al., 1995). The presence of contaminants, including crude oil, in the sediment has been reported to inhibit burrowing in numerous invertebrate species (*e.g.* Olla, 1983, 1984; White and Keilty, 1988; Ort et al., 1995). Few studies, however, have employed preference arenas, where both uncontaminated and contaminated sediments are present (Pynnönen, 1996; Gossiaux et al., 1993).
Experimental addition of hydrocarbons to sediment in field and mesocosm studies have shown strong and consistent negative impacts on harpacticoids at the population level (Coull and Chandler, 1992; Carman and Todaro, 1996; Carman et al., submitted). Changes in community structure occurs as a result of population-level effects. Significant changes in species relative abundances indicates differential sensitivity to hydrocarbon contamination (Carman et al., submitted). Results from this study suggest that not only mortality, but also a decrease in fitness resulting from impaired reproduction and feeding can be directly related to decreased harpacticoid density in mesocosm and field settings. Avoidance behavior in harpacticoid copepods also has the potential to alter abundance patterns. Many harpacticoids move through the near bottom water to disperse to new sites (Palmer, 1988). In addition, harpacticoids are generally most abundant in favorable locations (Sun et al., 1993), probably due to active movement during no or low flow conditions (Fleeger et al., 1995). Therefore, it is speculated that harpacticoids avoiding contaminated sediment will either actively search for or be more likely to be carried by currents to more favorable (i.e., less contaminated) locations. Fleeger et al. (1996) concluded that the low abundance of two harpacticoid species in colonization trays contaminated with crude oil was the result of differential migration away from contaminated sites. Increased predation may also contribute to population decline in contaminated sites, since non-burrowed and narcotized infauna constitute easy prey (Pihl et al., 1992).

In conclusion, S. knabeni was sensitive to both lethal and sublethal toxicity of sediment amended PAHs. Although broad differences in short-term lethal toxicity were
detected, with diesel fuel being more toxic than phenanthrene or fluoranthene, sublethal
toxicity took place at a more similar range of concentrations across contaminants and
endpoints (i.e., realized offspring production and grazing rate). It should be pointed
out, however, that if point estimates were expressed in a molar basis instead of dry-
weight, it would become evident that the sublethal toxicity of fluoranthene is greater
than that of phenanthrene. Sublethal effects of phenanthrene, diesel, and above all
fluoranthene took place at concentrations much lower than LC$_{50}$s and in the range
reported for sediment collected from numerous estuaries (Kennish, 1992). As
evidenced from this study and others (e.g. Lotufo and Fleeger, 1996; DiPinto et al.,
1993), sublethal endpoints can provide ecologically relevant and sensitive assessment
of the toxicity of sediment-associated organic contaminants. Their use in risk
assessment of contaminated sediments as well as for deriving sediment quality criteria
(SQC) is encouraged.
CHAPTER 4

EFFECTS OF SEDIMENT-ASSOCIATED PHENANTHRENE ON SURVIVAL, DEVELOPMENT AND REPRODUCTION OF TWO SPECIES OF MEIOBENTHIC COPEPODS
INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are among the most carcinogenic, mutagenic and toxic contaminants found in aquatic systems. An estimated $2.3 \times 10^6$ metric tons of PAHs enter aquatic systems every year (Kennish, 1992). Phenanthrene is a medium-molecular-weight PAH composed of three benzene rings and is among the PAHs of highest concern to the environment (USEPA, 1993b). Reports on the toxicity of phenanthrene to aquatic invertebrates in aqueous exposures include effects on survival (Rossi and Neff, 1978; Laughlin and Neff, 1979; USEPA, 1993b) and sublethal effects such as decreased developmental rate or molting impairment (Laughlin and Neff, 1979; Geiger and Buikema, 1982), increased respiratory rate (Laughlin and Neff, 1980), and decreased reproduction and growth (USEPA, 1993b; Emery and Dillon, 1996). Due to their high hydrophobicity, phenanthrene and other PAHs in the water column are rapidly sequestered into the organic matrix of suspended sediment and bed sediments resulting in chronic sediment contamination. Reports of the toxicity of sediment-associated phenanthrene to infaunal invertebrates are scarce and include lethal toxicity to amphipods (USEPA, 1993b), and decreased survival, feeding rate and reproductive output of tubificid worms (Lotufo and Fleeger, 1996) and harpacticoid copepods (see Chapter 3).

Impairment of reproduction is recognized as a key variable in hazard assessments of the effects of xenobiotics at the concentrations encountered in the natural environment (Chapman, 1995). Offspring production is widely employed as a sensitive endpoint for toxicity testing with aqueous media. In addition, the early life
stages of aquatic species are frequently the most sensitive to toxicants (McKim, 1985; Green et al., 1996). Exposure of larval stages of many species, especially fish and cladocerans, throughout their developmental period is common practice in aquatic toxicology. Copepods have been employed in life-table studies (Daniels and Allan, 1981; Allan and Daniels, 1981; Bechmann, 1994) and in early-life-stage exposures (Verriopoulus and Moraitou-Apostopoulou, 1982; O'Brien et al., 1988; Hutchinson et al., 1994) in sediment-free media. Harpacticoid copepods are well suited for sediment-toxicity assessment due to their intimate association with the sediment and ease of culture. Most species undergo a short life cycle and all larval stages are infaunal, making them excellent test organisms for assessing the effects of contaminants on life-history traits in sediment exposures. Investigations employing copepod life-history-related endpoints in sediment exposures are scarce and include reproductive output (Chandler, 1990; Strawbridge et al., 1992; DiPinto et al., 1993) and life-stage-specific survival (Green et al., 1996).

When copulating pairs of Schizopera knabeni were exposed to phenanthrene in sediment exposures for 14 d, decreased offspring production was observed at sublethal concentrations (see Chapter 3). Phenanthrene did not reduce clutch size, but the reduction in reproductive output could have been due to changes in egg-production rate, hatching success or early-stage survival, alone or in an additive manner. The objective of this research was to investigate sublethal effects of sediment-associated PAHs on harpacticoid copepods using life-history related endpoints. Toxicity to two meiobenthic copepod species, S. knabeni and Nitocra lacustris, was compared. Both harpacticoid
copepod species inhabit the upper zone of intertidal mud-flats of salt marshes. Their reproductive cycle starts with a male clasping a female and spermatophore placement. Fertilized eggs are extruded in egg-sacs and hatch while still attached to the female. They undergo six larval stages (nauplii) and six juvenile stages (copepodites). Both species complete a full life cycle (egg to egg) in less than 21 days at 25 °C and are easily cultured under laboratory conditions. Ten-d exposures, starting with different life stages (nauplius, copepodite, or adult), were conducted to investigate phenanthrene effects on life-stage-specific survival, development, offspring production, and hatching success.

**METHODS**

**Test-organisms**

*Schizopera knabeni* Lang and *N. lacustris* (Schmankevitsch) were obtained from mono-specific laboratory cultures started with stock collected from the surface sediment from intertidal mudflats of a *Spartina alterniflora* salt-marsh at Port Fourchon, Louisiana. *Schizopera knabeni* has been cultured since October 1993 and *Nitocra lacustris* since February 1995. They were cultured sediment-free in 500 ml Erlenmeyer flasks at room temperature with 25% artificial seawater (ASW) fully renewed fortnightly. Copepods were fed twice a week with a mixture of *Chaetoceros muellert*, strain Chaet 10 (a planktonic diatom), and Microfeast Plus Larval Diet®.

To better understand the reproductive cycle of *S. knabeni* and *N. lacustris*, copulating pairs of *S. knabeni* and non-copulating pairs of *N. lacustris* were...
individually placed in small dishes containing uncontaminated 25% ASW in 6 replicates. Copulating pairs consisting of an adult male clasping a pre-adult female (copepodite V) were very abundant for *S. knabeni* but very rare for *N. lacustris*. Copepods were fed Microfeast Plus Larval Diet® and kept at 25°C. Dishes were examined daily for 10 d, and adults were removed and transferred to new dishes and produced nauplii were enumerated. Individuals at distinct life stages were used in 10-d bioassays to determine life-stage-specific responses to contamination. Copepods were removed from the culture flasks with a pipet and copulating pairs or single adults (males and non-ovigerous females) were sorted under a stereo microscope and used in the male/non-ovigerous-female bioassays (see below). One- or two-day old nauplii were obtained by placing approximately 100 ovigerous females retrieved from the culture flasks into loosely covered crystallizing dishes. They were fed *Chaetocerous muelleri*, and eggs started hatching within a few hours. After 48 hours, all females were removed and nauplii were sorted and immediately used in the nauplius bioassays (see below). Eight- or nine-day old copepodites were obtained using the same procedure, except that offspring were raised for seven extra days following the removal of the adults and fed *Chaetocerous muelleri* every other day. At the end of this period, all offspring had metamorphosed to early copepodite stages and were immediately used in the copepodite bioassays (see below).

**Sediment dosing**

Sediment was collected from the top 2 cm of a mudflat in a *Spartina alterniflora* salt marsh near Cocodrie, Louisiana. The typical total PAH concentration in this
sediment was 0.26 mg/kg (Carman et al., 1996). Stock test-sediment was prepared by sieving the mud through a 45-μm mesh. The sediment that went through the sieve was retained. After settling overnight at 4 °C and removal of the supernatant by aspiration, the sediment was autoclaved. The dry-to-wet-weight ratio was determined by oven drying (80 °C) and adjusted to 0.15 by homogenizing the sediment with the appropriate volume of 25 % ASW. The sediment organic carbon (SOC) of the resultant slurry, measured in duplicate on a Perkin Elmer (Norwalk, CT) 2400 CHN Elemental Analyzer, was 1.5 % after acidification with HCl to remove inorganic carbonate. The stock sediment was stored at 4°C.

Phenanthrene (98% purity, Aldrich Chemical Co, Milwaukee, WI) was amended to the stock sediment by spiking. Sediment was fully homogenized with overlying water and 150 g of stock sediment (wet weight) was transferred to 500 ml beakers and vigorously stirred. The appropriate amount of phenanthrene, carried in 0.2 ml of acetone, was added to the slurry and stirred for 4 h. The required amount of spiked phenanthrene was calculated on a dry-weight basis. A control was prepared by adding 0.2 ml of acetone only. To reduce the amount of solvent, the overlying water was removed by aspiration and replaced with fresh ASW and the sediment homogenized every 24 h, twice. Sediments were stored in the dark at 4°C for 3 to 6 weeks. Phenanthrene sediment concentrations were measured by reverse phase HPLC before being used in experiments, as described in Lotufo and Fleeger (1996), and determined to be 11, 22, 45, 90, 177, 217, 492, 739 μg/g.
**Toxicity bioassays**

In all bioassays, copepods were exposed to sediment treatments in 50 x 35 mm crystallizing dishes (Kimble, Toledo, OH) filled with 25 ml of ASW. Eight ml of sediment treatment was dispensed to the bottom of each dish using a 5-ml Finnpipette creating a 3-4 mm sediment layer. Food was added to each dish as 0.3 mg of Microfeast Plus Larval Diet mixed in 0.1 ml of ASW. Dishes were placed in random order inside moisture chambers (loosely covered plastic containers underlined with soaked paper towels that created a humid environment to retard evaporation from experimental dishes). They were kept overnight in the dark at 25 °C in an incubator with no illumination before test organisms were added.

Ten-d bioassays were conducted to determine stage-specific sensitivity to fluoranthene lethal effects as well as fluoranthene sublethal effects on reproduction and development. Separate experiments were conducted using *S. knabeni* and *N. lacustris*. Four replicates were used per concentration. Three bioassays were conducted for each species using male/non-ovigerous-female pairs, early-age nauplii, or early-age copepodites. For all bioassays, copepods were introduced to experimental units, which were then returned to moisture chambers and kept inside an incubator at 25 °C with no illumination for 10 d. At test termination, the contents of all dishes were sieved through a 45-μm mesh and the retained material was washed into a plastic cell-culture dish. Five additional experimental units containing control sediment were prepared for determinations of salinity (Reichert refractometer, Cambridge Instruments, Buffalo,
NY) and oxygen concentration (Orion 820 oxygen meter, Boston, MA) at initiation and termination of the experiment.

Male/female bioassays were initiated with 10 copulating pairs of S. knabeni or 10 non-copulating adult pairs (single males and non-ovigerous females) of N. lacustris per replicate. At test termination, surviving adults and produced offspring were fixed with 4% buffered formalin, and stained with Rose Bengal. Ovigerous females were subsequently sorted and nauplii and copepodites were enumerated. Egg sacs were detached intact from ovigerous females and examined for clutch-size determination. Nauplius bioassays were initiated with 15 individuals (one- or two-d old) per replicate; all phenanthrene concentrations were used, except for 11 and 739 µg/g. Copepodite bioassays were initiated with 20 individuals (eight- or nine-d old) per replicate; all treatments were used except for 739 µg/g. At test termination, all surviving individuals were enumerated and examined for developmental stage (nauplius, copepodite, adult male, or adult female) in both nauplius and copepodite bioassays.

In order to determine phenanthrene effects timing and success of egg hatching, ovigerous females obtained from the copepodite bioassays were individually placed in tissue culture dishes (35 x 10 mm) half filled with ASW (no sediment), fed Chaetoceros muelleri, and observed every 8 h until eggs hatched. Nauplii were enumerated when their presence was detected in the dishes. One ovigerous female from each replicate of the copepodite bioassay was used. The 90 and 177 µg/g treatments from the S. knabeni bioassay yielded single ovigerous females from only 3 replicates and the 217 µg/g did not yield any ovigerous female. Because S. knabeni ovigerous
females of the same age were carrying their first brood of eggs, their age at first reproduction could be determined. This determination was not possible for *N. lacustris* because not all females were carrying their first brood when the observation period was initiated.

**Statistical analysis**

Copepod survival in each life-stage experiment, reproductive output, clutch size, proportion of surviving copepods at different life stages in the nauplius and copepodite bioassays, time for egg hatching and egg hatching success were analyzed using one-way analysis of variance (ANOVA). Phenanthrene treatments were compared with control treatments using Dunnett’s one tail t-test (*α* = 0.05). LC$_{50}$ values were computed using the Trimmed Spearman-Karber method (Hamilton *et al.*, 1977). In order to compare and test for significant differences in the sensitivities of the four life stages (nauplius, copepodite, adult male, adult female) to phenanthrene, survivorship data for each copepod species was analyzed using a split-plot design. A two-way ANOVA using the Mixed Procedure tested for the significance of three different effects on copepod survival in the presence of phenanthrene: the main unit effect (or the effect of life stage), the subunit effect (or the effect of contamination level), and the effect of the interaction of these two factors. Data for the 11 μg/g treatment were not available for naupliar stages and therefore was removed from the analysis. Of principal interest was the comparison of overall survival under each life stage in a pairwise fashion (female versus male; female versus copepodite; female versus nauplius; male versus copepodite; male versus nauplius; copepodite versus nauplius). However, if the interaction between
life stage and contamination level was significant, the above pairwise comparisons were tested at each concentration level using $t$-tests ($\alpha = 0.05$), and a Bonferroni adjustment for multiplicity. When the interaction effect was not significant but the effect of life stage was, pairwise comparisons among life stages were performed, again using the Bonferroni correction for multiplicity. For the two-way ANOVA, the observed survival proportions were transformed via the angular transformation (Green et al., 1996):

$$y = \arcsin \left( \frac{r + 0.375}{n + 0.75} \right)$$

where $r$ is the observed number of survivors and $n$ is the total number of organisms exposed in each replicate. Point estimates for reproductive output were calculated using linear interpolation with bootstrapping, or ICp method (U.S.EPA, 1994). With $p=25$, IC$_{25}$ estimates the contaminant concentration causing a 25% reduction in the measured endpoint in relation to the control. Statistical analysis were performed using TOXCALC™ version 5.0 (Tidepool Software; McKinleyville, CA) and SAS® software (Release 6.03 Edition, Cary, NC).

RESULTS

Daily observations of S. knabeni mating pairs in uncontaminated water indicated that egg clutches were first extruded at day 2 and 3 and hatched at days 3 and 4; each female produced $4.4 \pm 0.9$ (mean ± standard deviation) clutches (1 every 2.4 days) with $16.9 \pm 1.3$ nauplii hatching from each brood. Nauplius production over 10 days was $73.5 \pm 15.3$, and clutch size measured at day 10 was $22.6 \pm 0.6$. Nitocra lacustris produced an average of $3.7 \pm 0.5$ broods, $15 \pm 1.6$ nauplii per brood, and a total of
56.3 ± 11 nauplii over ten days. Clutch size at day 10 was 17.5 ± 2.3. Four groups of 15 one-day-old nauplii of each species were also observed daily. For *S. knabeni*, the copepodite stage was attained by 7-8 days. Sexual maturity was attained quickly; eggs were extruded at 17-18 days and hatched at 18-20 days. For *N. lacustris*, the copepodite stage was attained at the age of 5-7 days; eggs were extruded at 14-16 days and hatched at 16-18 days.

The analysis of variance indicated significant phenanthrene-treatment effects (*p* < 0.05) on survivorship, offspring production, and egg hatching in *N. lacustris* and *S. knabeni*. The salinity remained unchanged throughout 10-d (25 ± 6 °) experiments. Mean DO levels were 5.5 ± 1.5 mg/L at day 0 and 5.2 ± 1.2 mg/L at day 10.

**Lethal toxicity: life-stage sensitivity**

For *N. lacustris*, female mortality was lowest in the control (5%) and ranged from 5 to 27.5% at concentrations from 11 to 90 µg/g (Fig. 4.1). Male mortality was lowest in the control and 11 µg/g (17.5%), averaged 25% at 22 and 45 µg/g, and reached 67.5% at 90 µg/g; mortality was significantly different from the control at 90 µg/g. All individuals during exposure to ≥177 µg/g and higher concentrations. Average copepodite mortality was lowest in the control (10%) and tended to gradually increase with increasing concentrations of phenanthrene, ranging from 27.5% at 22 µg/g to 63.75% at 90 µg/g; it was statistically different from the controls in all phenanthrene treatments. Average naupliar mortality was lowest in the control treatment (12%) and increased with increasing phenanthrene concentrations; it was significantly different from the control at 45 and 90 µg/g and all individuals died during exposure to ≥177
Fig. 4.1. Survival of *Nitocra lacustris* and *Schizopera knabeni* life stages (adult female, adult male, copepodite, and nauplius) exposed to sediment-associated phenanthrene for 10 d. Error bars show 1 SD of the mean (n = 4); * represents significance different (α = 0.05) from control mean.
The LC₅₀ values were 105 µg/g for females, 72 µg/g for males, 43 µg/g for copepodites, and 71 µg/g for nauplii (Table 4.1). Results from the two-way ANOVA on copepod survival indicated a non-significant interaction between life stages and contamination level (p = 0.21) and a significant life-stage effect (p < 0.01); female survival was significantly higher than survival at all other life stages. Adult male, copepodite and naupliar survival were not significantly different among each other.

For *S. knabeni*, average adult mortality was low in controls (2.5% for females and 0% for males) and ranged from 0 to 32.5% at concentrations from 11 to 492 µg/g. Mortality of males and females was significantly higher than in the control only at 492 µg/g, and all adult copepods died during the exposure to 739 µg/g (Fig. 4.1). Average copepodite mortality was low (<13%) in the control and phenanthrene concentrations ranging from 11 to 45 µg/g and gradually increased at higher concentrations, ranging from 27 to 79%; it was significantly different from the control at 177 and 217 µg/g, and all copepods died during exposure to 492 µg/g. Average naupliar mortality was low (<10%) in the control and at the 22 µg/g treatment and increased at higher phenanthrene concentrations; it was significantly different from the control at 45 and 90 µg/g, and all copepods died during exposure to ≥177 µg/g. The LC₅₀ values were 345 µg/g for females, 349 µg/g for males, 172 µg/g for copepodites and 84 µg/g for nauplii (Table 4.1). Results from the two-way ANOVA on copepod survival indicated a significant interaction between life stage and contamination-level effects (p < 0.01). Adult male and female survival were not significantly different at any contamination level; naupliar survival was significantly lower than copepodite survival at 45 µg/g and higher.
Table 4.1. Toxicity values from bioassays performed with *Schizopera knabeni* and *Nitocra lacustris*. Numbers in parenthesis indicate 95% confidence intervals. All values expressed as μg/g.

<table>
<thead>
<tr>
<th>Value</th>
<th><em>Schizopera knabeni</em></th>
<th><em>Nitocra lacustris</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Male LC50</td>
<td>349 (291-417)</td>
<td>72 (62-83)</td>
</tr>
<tr>
<td>Female LC50</td>
<td>345 (291-407)</td>
<td>105 (95-116)</td>
</tr>
<tr>
<td>Copepodite LC50</td>
<td>172 (155-190)</td>
<td>43 (36-52)</td>
</tr>
<tr>
<td>Nauplii LC50</td>
<td>84 (74 - 96)</td>
<td>71 (65-77)</td>
</tr>
<tr>
<td>Total offspring IC25</td>
<td>48 (7-74)</td>
<td>65 (24-105)</td>
</tr>
<tr>
<td>Real. offspring IC25</td>
<td>26 (0-57)</td>
<td>40 (23-69)</td>
</tr>
</tbody>
</table>
concentrations, and lower than adult male and female survival at 90 µg/g and higher concentrations. Copepodite survival was significantly lower than adult male survival at ≥ 90 µg/g and lower than female survival at ≥ 177 µg/g. The lack of overlap in the 95% CI for the calculated LC₅₀s suggests significant overall differences of sensitivity among nauplii, copepoides and adults. The LC₅₀s of adult females and males were practically identical.

Sublethal toxicity: offspring production

Offspring were produced in the course of 10-d in the male/female bioassay. Adult mortality took place in most replicates and increased with phenanthrene concentration. In order to more accurately assess sublethal effects on reproductive output, average offspring production was calculated on a per-surviving-female basis. Realized offspring consisted of nauplii and early-stage copepoides. Total offspring consisted of realized offspring plus the eggs forming a sac (N. lacustris) or a pair of sacs (S. knabeni) attached to the female. The number of eggs found at test termination is not a measure of total egg production, but represents the standing stock of eggs after 10 d.

For N. lacustris, the fraction of surviving females that were ovigerous at test termination varied significantly and increased with increasing phenanthrene concentration and ranged from 8% in the control to 80% at 90 µg/g (data not shown). Only the fraction of ovigerous females in the 90 µg/g treatment was significantly higher than in the control. Mean number of realized offspring was highest at 11 and 22 µg/g, followed by the control, 45 and 90 µg/g treatments (Fig. 4.2). Realized offspring was
Fig. 4.2. Number of offspring produced by *Nitocra lacustris* and *Schizopera knabeni* exposed to sediment-associated phenanthrene for 10 d. Offspring were fractioned into eggs carried by surviving females and realized offspring (nauplii plus copepodites) present at test termination. Error bars show 1 SD of the mean (n = 4); * represents significance different (α = 0.05) from control mean for realized offspring; numbers over bars indicate the average percent of realized offspring comprised of copepodites.
significantly lower than in the control only at 90 μg/g. The mean fraction of total offspring comprised of realized offspring was highest in the control (95%) and gradually decreased with increasing phenanthrene concentration down to 39% at 90 μg/g. The mean number of eggs present at test termination increased with phenanthrene concentration (Fig. 4.2) and was significantly different from the control at 45 and 90 μg/g. The IC_{25} value was 65.5 μg/g for total offspring and 39.7 μg/g for realized offspring production (Table 4.1). The mean fraction of the realized offspring comprised of copepodites was highest at 22 μg/g (27.6%) and ranged between 7.3 and 18.2% in the remaining treatments up to 45 μg/g (Fig. 4.2). No copepodites were found in the 90 μg/g treatment. The mean fraction of copepodites at 45 and 90 μg/g was significantly lower than in the control. Clutch size ranged from 15.4 to 17.9 and was not significantly among sediment treatments (p = 0.59).

For *S. knabeni*, the fraction of surviving females that were ovigerous at test termination ranged from 60 to 81% and did not differ significantly among treatments (data not shown). Mean number of realized offspring was significantly lower than in the control for all phenanthrene treatments except 11 μg/g; it gradually decreased with increasing phenanthrene concentration, ranging from 85.2 to 6.7% of control levels (Fig. 4.2). The mean fraction of total offspring comprised of realized offspring was highest in the control (77%) and gradually decreased with increasing phenanthrene concentration down to 22% at 492 μg/g. The mean number of eggs present at test termination (Fig. 4.2) was not significantly different among sediment treatments (p = 0.147). The IC_{25} values were 48 μg/g for total offspring and 26 μg/g for realized
offspring production (Table 4.1). The mean fraction of realized offspring comprised of copepodites was 8.6% in the control and decreased significantly with increasing phenanthrene concentrations. No copepodites were found at concentrations of 45 μg/g and higher. Mean clutch size was highest in the control (19.9) and ranged from 16.6 to 18.9 in phenanthrene treatments up to 217 μg/g, none of which were significantly different from the control. Mean clutch size at the 492 μg/g treatment (12.1) was significantly lower than in the control.

**Sublethal toxicity: development**

Effects of phenanthrene on the development rate of larval and juvenile copepods over 10 d were examined in the nauplius and copepodite bioassays by identification of life stage at the end of the exposure period. In the *N. lacustris* nauplius bioassay (Fig. 4.3), the fraction of surviving copepods that attained the adult stage in 10 d was highest in the control and 22 μg/g treatments (88%) and decreased at higher phenanthrene concentrations (48% at 45 μg/g and 57% at 90 μg/g). The fraction of copepodites that attained the adult stage was significantly higher than the control at 45 and 90 μg/g. The sex ratio among the adult copepods was near 1:1 in the control and 22 μg/g treatments, with the mean proportion of males being 54 and 50%, respectively. The mean proportion of males was higher at the 45 and 90 μg/g treatments (77 and 78%, respectively), but not significantly different from the control. In the *S. knabeni* nauplii bioassay (Fig. 4.3), surviving copepods in the control replicates metamorphosed to copepodites in 10 d. However, copepods still in naupliar stages were observed at test termination in all phenanthrene treatments. The mean fraction of surviving copepods...
Fig. 4.3. Distribution of surviving *Nitocra lacustris* and *Schizopera knabeni* among life stages in the 10-d nauplius bioassay. Naup = nauplius; copep = copepodite; female = adult female; male = adult male. No nauplii survived exposure to the 177, 217 and 492 mg/kg treatments.

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attaining copepodite stages decreased with phenanthrene concentrations (93, 57, and 35% at 22, 45 and 90 μg/g, respectively). The fraction of surviving individuals comprised of copepodites at 45 and 90 μg/g was significantly lower than in the control.

In the *N. lacustris* copepodite bioassay (Fig. 4.4), all surviving copepods developed to adult stages in the control treatment; however, a fraction ranging from 12 to 17% remained in copepodite stages in all phenanthrene treatments. The fraction of individuals in the copepodite stage was not significantly different among treatments (*p* = 0.165). The proportion of females that were ovigerous was not significantly different among replicates (*p* = 0.54). Early-age nauplii were present in some replicates of all treatments, indicating offspring production by exposed copepods. The sex ratio was near 1:1 across treatments, with males comprising from 39 to 55% of the adults. In the *S. knabeni* copepodite bioassay (Fig. 4.4), all surviving copepods in the control and at 11, 22 and 177 μg/g had developed to adult stages at experiment termination. Copepodites comprised 8% of the surviving copepods at 45 and 90 μg/g and 35% at 217 μg/g. The fraction of copepodites at 217 μg/g was significantly higher than in the control. Ovigerous females were observed in all treatments except at 217 μg/g, and comprised 70% of the total number of females found in the control. This fraction tended to decrease with increasing phenanthrene concentration, ranging from 58 down to 14%, indicating delayed production of the first egg clutch. The fraction of ovigerous females at 90 and 177 μg/g was significantly different than the control. The sex ratio was near 1:1 in the control and phenanthrene concentrations up to 45 μg/g, with males comprising from 45 to 53% of the adults. The proportion of males increased gradually.
Fig. 4.4. Distribution of surviving *Nitocra lacustris* and *Schizopera knabeni* among life stages in the 10-d copepodite bioassay. Ov fem = ovigerous female; fem = non-ovigerous adult female; male = adult male; copep = copepodite. No copepodite *N. lacustris* survived exposure to the 177, 217 and 492 mg/kg treatments and no copepodite *S. knabeni* survived exposure to the 492 mg/kg treatment.
at higher concentrations, ranging from 61% at 90 µg/g up to 89% at 217 µg/g. The proportion of males was not significantly different among treatments (p = 0.216).

Observation of ovigerous females obtained in the copepodite bioassay suggested an increase in the embryo maturation period in the presence of phenanthrene. For *N. lacustris*, hatching occurred after an average period of 1.5 days from the beginning of the observation period in the control and after increasingly longer periods at increasing phenanthrene concentrations, up to a maximum of 3.8 days at the 90 µg/g treatment (Fig. 4.5). For *S. knabeni*, hatching occurred after an average period of 1 day in the control and occurred after increasingly longer periods at increasing phenanthrene concentrations, up to a maximum of 3.3 days at the 177 µg/g treatment (Fig. 4.5).

Delayed egg hatching represented an increase in the average age at first reproduction from 19 days in the control to 21.3 days at 177 µg/g.

**Sublethal toxicity: hatching success**

For *N. lacustris*, the mean number of nauplii hatching from exposed ovigerous females obtained from the copepodite bioassay ranged from 10 to 14.25 in the control and phenanthrene concentrations from 11 to 45 µg/g (Fig. 4.6). An average of only 2.75 eggs hatched from females in the 90 µg/g treatment which was significantly lower than in the control. For *S. knabeni*, the mean number of nauplii hatching from exposed females was highest in the control and at 11 µg/g (14.5). This number gradually decreased with increasing phenanthrene concentration, down to a minimum of only 3.7 nauplii per clutch at 177 µg/g (Fig. 4.6). Mean number of nauplii per clutch was significantly lower than in the control at the 45, 90 and 177 µg/g treatments. Although

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Fig. 4.5. Time to hatch for eggs carried by *Nitocra lacustris* and *Schizopera knabeni* ovigerous females obtained in the 10-d copepodite bioassay. Time is expressed in days from the beginning of the observation period. Error bars show 1 SD of the mean ($n = 4$); * represents significance different ($\alpha = 0.05$) from control mean.
Fig. 4.6. Hatching success of *Nitocra lacustris* and *Schizopera knabeni* ovigerous females obtained in the 10-d copepodite bioassay. Hatching success is expressed as the number of nauplii hatching per female. Error bars show 1 SD of the mean ($n = 4$); * represents significance different ($\alpha = 0.05$) from control mean.
clutch size was not directly measured because of the experimental design, it appeared to be similar among all *N. lacustris* and *S. knabeni* ovigerous females used in these observations.

**DISCUSSION**

Ten-d LC$_{50}$ values were obtained from tests starting with different life stages of *S. knabeni* and *N. lacustris* and indicated markedly different patterns of life-stage-specific sensitivity for the two species. For *S. knabeni*, the lowest LC$_{50}$ value was obtained when exposure was initiated with early naupliar stages, followed by early copepodite stages, adult males and pre-adult females. This apparent gradual decrease in sensitivity to phenanthrene acute effects as larval development progresses is supported by the statistical analysis. No sex-specific differences in sensitivity were apparent, as indicated by very similar LC$_{50}$ values for males and females. The LC$_{50}$ for adult copepods was approximately four times higher than that for nauplii and two times higher than that for copepodites. For *N. lacustris*, the LC$_{50}$ was lowest for copepodites and was increasingly higher for nauplii, adult male, and adult females. The statistical analysis indicates a higher tolerance of adult females but a lack of differences among all other life stages. Sex-specific differences were observed, with females significantly more tolerant than males. Higher sensitivity of the early larval stages of decapod crustaceans are reported from exposures to crude oils (Katz, 1973; Karinen and Rice, 1974; Mecklenburg *et al.*, 1977; Cucci and Epifanio, 1979; Capuzzo *et al.*, 1984) and phenanthrene (Laughlin and Neff, 1979), and appear to be related to the molting process.
in crustaceans. Higher PAH tissue burden was found in newly molted compared to intermolt crabs and is speculated to be a consequence of increased uptake of water and integument permeability at ecdysis, or decreased metabolism of PAHs during the molt process (Mothershed and Hale, 1992). Copepod naupliar stages were consistently the most sensitive in exposures to other contaminants. Exposure of different life stages of the harpacticoid copepods *Tisbe holothuriae* (Verriopoulus and Moraitou-Apostopoulou, 1982) and *Tigriopus californicus* (O’Brien et al., 1988) to copper showed that naupliar stages are the most sensitive and that susceptibility decreases with age, with an abrupt increase in tolerance occurring as individuals attain the adult stage, as observed in this study with *S. knabeni*. Nauplii of *Tisbe battagliai* were more sensitive than adults in exposures to copper, cadmium and hexavalent chromium (Hutchingson et al., 1994). Finally, naupliar *Amphiascus tenuiremis* was the most sensitive stage in sediment exposures to chlorpyrifos (Green et al., 1996). Higher tolerance of female copepods, as observed with *N. lacustris*, has been reported for other species of copepods in aqueous and sediment exposures to PCBs (Dalla Venezia et al., 1981; DiPinto et al., 1993; Carman & Todaro, 1996) and has been speculated to be related to the elimination of hydrophobic contaminants via egg production (DiPinto et al., 1993).

Overall, *N. lacustris* was more sensitive to phenanthrene acute toxicity than was *S. knabeni*. The 10-d LC$_{50}$ values for *S. knabeni* ranged from 84 to 349 µg/g, whereas with *N. lacustris* values ranged from 43 to 105 µg/g. The LC$_{50}$ values for eight species of copepods at different stages exposed to copper ranged from 19 to 762 µg/L,
indicating large species-specific differences in copepod sensitivity to toxicants other than PAHs (O'Brien et al., 1988). Phenanthrene acute toxicity in sediment exposures is only known for a very limited number of species. When the phenanthrene 10-d \( LC_{50} \) values calculated on an organic-carbon basis for \( S. \) knabeni (5,600-26,800 \( \mu \)g/g\(_{OC} \)) and \( N. \) lacustris (2,867-7000 \( \mu \)g/g\(_{OC} \)) are compared with values obtained using adults of the marine amphipods \( Eohastorious estuarinus \) (3,820-4,050 \( \mu \)g/g\(_{OC} \)) and \( Leptocheirus plumosus \) (6,490-8,200 \( \mu \)g/g\(_{OC} \)) (U.S.EPA, 1993b), it is evident that all stages of \( N. \) lacustris and the larval stages of \( S. \) knabeni are equally or more sensitive than the adult amphipods tested; adult \( S. \) knabeni are significantly more tolerant. Both \( S. \) knabeni and \( N. \) lacustris were more sensitive to phenanthrene than the oligochaete \( Limnodrilus hoffmeisteri \) (10-d \( LC_{50} = 42,500 \) \( \mu \)g/g\(_{OC} \), Lotufo and Fleeger, 1996).

Phenanthrene delayed normal larval development and metamorphosis in \( N. \) lacustris and \( S. \) knabeni. An increasingly larger fraction of copepods failed to attain more advanced stages in phenanthrene treatments in relation to controls in 10-d bioassays initiated with early naupliar or copepodite stages. In addition, \( S. \) knabeni produced their first offspring at a later age when exposed to phenanthrene at early copepodite stages, and there were indications of delayed embryonic development for both species. Decreases in developmental rate are also reported for the mud crab \( Rhithropanopeus harrisii \) in aqueous exposures to phenanthrene; exposure to naphthalene, however, slightly increased developmental rate (Laughlin and Neff, 1979).

Phenanthrene also impaired both molting and the onset of sexual maturation in \( Daphnia pulex \) (Geiger and Buikema, 1982). Delayed development and slow growth rates were
observed in larval stages of several species of decapods exposed to crude oil or No. 2 fuel oil (Capuzzo et al., 1984, and references therein). Reduced feeding and decreased scope for growth have been demonstrated in crustaceans exposed to petroleum hydrocarbons (Wang and Stickle, 1987; Chapter 3) and are likely related to delayed development and low growth rate. In addition, Capuzzo et al. (1984) showed that delayed development and lower growth rate in larval lobster exposed to petroleum hydrocarbons are related to alterations in normal patterns of lipid storage, utilization and synthesis. Oil-exposed larvae had lower levels of triacylglycerols, the major energy store, in relation to control larvae. Increased levels of sterols were also detected and were speculated to be related to decreased transformation of cholesterol into metabolic pathways including the biosynthesis of ecdysterone (Capuzzo et al., 1984). Copepod developmental rate and onset of reproduction were also delayed under aqueous exposure to a variety of contaminants, such as 2,4-dichlorophenol and 4-chlorophenol (Kuiper and Hanstveit, 1984), kepone (Alan and Daniels, 1982) and the insect growth regulator diflubenzuron (Savitz and Wright, 1994; Wright et al., 1996).

The sex ratio of copepods in the phenanthrene treatments at the termination of the 10-d nauplius and copepodites was skewed towards males. There are several possible ways that PAHs might have influenced sex ratio in these 10-d experiments. Environmental sex determination has been reported for copepods (Fleeger and Shirley, 1990), and may be influenced by PAHs, but a more likely explanation involves the effects of PAHs in developmental rate. Male copepodites attain the adult stage earlier than females, as observed previously with other species of harpacticoid copepods.

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(Bergmans, 1981). Because development was faster in controls, copepodites of both sexes attained adult stage during the short duration of the experiment. If the exposure period were longer, female copepodites in phenanthrene treatments would probably have developed to adults as well. No previous studies have reported PAH effects on sex ratio in crustaceans.

*Nitocra lacustris* and *S. knabeni* early-stage copepodites exposed to sediment-associated phenanthrene for 10 d that developed into ovigerous females were isolated and observed in clean ASW for egg hatching. Although there was no apparent difference in clutch size, broods produced in the presence of phenanthrene hatched into significantly fewer nauplii as compared to broods from control females. Hatching success was reduced to only approximately 25% of control levels at 90 μg/g for both *N. lacustris* and *S. knabeni*, while the LC₅₀ for nauplii was 71 and 84 μg/g for the two species, indicating that the egg stage is equally or perhaps more sensitive than larval stages to phenanthrene toxicity. Cowles and Remillard (1983) showed that low egg-hatching success followed exposure of adult females of the planktonic copepod *Centropages hamatus* to crude oil. Strong inhibition of egg hatching also occurred when ovigerous females of the grass shrimp *Palaemonetes pugio* exposed to the water-soluble fraction of No. 2 fuel oil were held in clean water (Tatem, 1977). Similar results were shown by Fisher and Foss (1993) in a more elaborate experiment, where embryos of the grass shrimp were individually exposed to a similar hydrocarbon mixture; since higher temperatures caused earlier mortality after exposure, it was
speculated that two mechanisms, toxicant penetration of the egg casing and metabolism related toxicity, were operative.

Phenanthrene did not have a negative impact on *S. knabeni* clutch size, except at 492 µg/g. The number of nauplii and copepodites produced per surviving female of *S. knabeni*, however, decreased in a concentration-dependent fashion. A significant decrease occurred at 22 µg/g and the IC$_{25}$ value indicated a 25% reduction at 26 µg/g. Because neither clutch size, hatching success, or naupliar survival (LC$_{50}$ = 84 µg/g) were adversely affected at 22 µg/g, the reduction in the number of offspring was probably a consequence solely of decreased brood production rate. However, decreased hatching success and larval survivorship must have also contributed to reduce the final number of nauplii and copepodites at higher concentrations. The fraction of the realized offspring comprised of copepodites tended to decrease with increasing phenanthrene concentrations, likely due to delayed formation and hatching of the earliest broods and slow metamorphosis. Assessment of realized offspring production was the most sensitive endpoint for detecting sublethal effects of phenanthrene using *S. knabeni*. Lower point estimates were obtained when eggs were not included in the analysis (total vs. realized offspring), since unhatched eggs made up most of the offspring produced and were probably not viable at higher concentrations.

*Nitocra lacustris* offspring production was affected by phenanthrene sublethal toxicity to a lesser extent than *S. knabeni*. No effect on clutch size was observed. The IC$_{25}$ value for realized offspring production using non-ovigerous females estimates a 25% reduction in offspring production at 40 µg/g, whereas the 10-d LC$_{50}$ value

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estimates a 50% mortality of copepodites at 43 μg/g, indicating that lethal toxicity on juvenile stages is a more sensitive endpoint than offspring production. The decrease in offspring production detected in the 45 μg/g treatment was not statistically different from the control, although hatching success, naupliar mortality and copepodite mortality were expected to have contributed towards a further decrease in the number of offspring at this concentration. Phenanthrene also likely inhibited hatching, as the fraction of surviving females that were ovigerous at test termination were significantly higher at high phenanthrene concentration (90 μg/g) than in the control. The fraction of offspring attaining copepodite stages was significantly lower in phenanthrene treatments than in the control, suggesting a negative effect on developmental rate.

Reproduction impairment by petroleum hydrocarbons on planktonic copepods in aqueous exposures is well documented (Berdugo et al., 1977; Ott et al., 1978; Ustach, 1979; Cowles and Remillard, 1983; Buttino, 1994). Phenanthrene in aqueous solution decreased the rate of brood production in daphnids (Geiger and Builkema, 1982) and polychaetes (Emery and Dillon, 1996). Significant decreases in reproductive output were also observed in sediment exposures to PAH congeners. The reproductive output of individual copulating pairs of *S. knabeni* exposed to sublethal concentrations of sediment-associated phenanthrene and fluoranthene dramatically declined (Chapter 3). Reproductive output of the oligochaete *Limnodrilus hoffmeisteri* was a much more sensitive endpoint than mortality for assessing the toxicity of pyrene and phenanthrene (Lotufo and Fleeger, 1996). Decreased feeding rate and reproductive output have been detected at the same range of PAH concentrations, suggesting that offspring production
might at least be partially related to narcosis-induced reduction in feeding rate (Cowles and Remillard, 1983; Lotufo and Fleeger, 1996).

Survival and offspring production were not monitored for the entire life cycle of female copepods, preventing the application of a full life table. Results from this study nevertheless reveal that phenanthrene concentrations much lower than the adult LC₅₀s will reduce hatching success, early-stages survivorship, rate of development, sexual maturation and fecundity of benthic harpacticoids. Offspring production of *S. knabeni*, for example, was significantly decreased at concentrations as low as 22 µg/g for, whereas adult survival was significantly decreased only at 492 µg/g. A decrease in the intrinsic rate of natural increase (*rₜ*) under optimal conditions is therefore expected at relatively low PAH concentrations. Likewise, population decreases are expected in field populations of benthic harpacticoids inhabiting sediments contaminated with PAHs at levels typical for polluted estuaries (Kennish, 1992).

The results of this study suggest that, for *S. knabeni*, offspring production is the most sensitive life-cycle variable (IC₅₀ = 26 µg/g), followed probably by age at first reproduction (11% increase at 22 µg/g) and egg hatching success (56% reduction at 45 µg/g). For *N. lacustris*, results suggest that survivorship at the copepodite stages is the most sensitive variable (LC₅₀ = 43 µg/g), followed by offspring production (IC₅₀ = 40 µg/g), and egg hatching (18% reduction at 45 µg/g).

A prediction of which life-history-related endpoint is the most relevant from an ecotoxicological perspective cannot be attempted with the available data from this study. Based on complete life-cycle experiments with soil nematodes, Kammenga *et al.*
(1996) showed that although a reduction of the reproductive period by cadmium was the most pronounced (45%) among several life-cycle variables, it did not have an effect on fitness (defined as $r_m$), whereas a prolongation of the juvenile period by 7.5% or a reduction in offspring production by 22% had the greatest impact, decreasing fitness by 5%. Only a full-life table study accompanied by a deterministic model designed to relate changes in individual variables to fitness would indicate the ecological significance of each variable individually (Kammenga et al., 1996). Life-table experiments exposing copepods to individual contaminants in aqueous media have been successful (Daniels and Allan, 1981; Allan and Daniels, 1982; Bechmann, 1994). Full life-cycle sediment exposures of benthic copepods to PAHs and other contaminants accompanied by a deterministic model analysis as in Kammenga et al. (1996) is encouraged for a better understanding of the ecological implication of sublethal contaminant impact on life-history parameters.
CHAPTER 5

BIOACCUMULATION AND TOXICITY OF SEDIMENT-ASSOCIATED FLUORANTHENE IN MEIOBENTHIC COPEPODS USING THE CRITICAL-BODY-RESIDUE APPROACH
INTRODUCTION

Intense research has been devoted to improving our understanding of the toxicity of sediment-associated contaminants. Benthic organisms accumulate chemicals from sediments, thereby becoming susceptible to their deleterious effects. The manifestation of toxicity depends on species-specific sensitivity and dose. The dose, or concentration at the site of action in the organism, is directly related to the extent of exposure. When the source of exposure is water, the concentration in the exposure media represents a reasonable surrogate for the dose because there is only one route of accumulation, and behavioral and physicochemical modifications of exposure are minimal. In sediment exposures, however, complexities involving contaminant bioavailability to benthic organisms, such as chemical partitioning, and the behavior of the test organism defy simplification. The equilibrium-partitioning (EqP) theory was examined such complexities and has been used by regulatory agencies for predicting the toxicity of sediment-associated contaminants, such as PAHs (DiToro et al., 1991; U.S.EPA, 1993a,b,c). It relies on the external dose (porewater) as a surrogate for the dose at the receptor site (tissues). In several instances, however, EqP predictions have failed to predict the toxicity of nonpolar contaminants in sediment exposures (Nebeker et al., 1989; Chandler et al., 1994; Landrum et al., 1994; Kane Driscoll et al., in press). In these studies, the bioavailability of sediment-associated nonpolar compounds was probably much lower than that predicted by EqP, and as a result, mortality did not occur at sediment concentrations predicted as lethal (Landrum et al., 1994; Kane Driscoll et al., in press).
The critical-body-residue (CBR) approach has been used as an alternative to EqP, and improves the interpretation of toxicity by explicitly considering bioavailability, accumulation kinetics, uptake from food and the effects of biotransformation (Landrum et al., 1992; McCarty and Mackay, 1993). It states that the molar-tissue concentration causing a toxic effect (e.g. mortality) is similar for chemicals with the same mode of action and relatively constant among species. This approach predicts that the potency of chemicals that act by nonpolar narcosis should be essentially constant among organisms, and that lethal narcosis will occur at tissue concentrations varying from 2 to 8 μmol/g wet weight (McCarty, 1986; McCarty et al., 1992). Body residues have been measured for organisms and used to generate LD₅₀ values or lethal CBRs that were within or close to the predicted range (e.g., Landrum et al., 1991; De Bruijm et al., 1991; Pawlisz and Peters, 1993a; van Wezel et al., 1995). Research on CBR has mostly focused on manifestations of mortality or complete paralysis. The absence of mortality, however, does not necessarily mean the absence of deleterious effects. Therefore, the sole use of mortality or complete narcotization as endpoints for calculating CBR limits its power to adequately predict toxicity. Disruption of life functions (e.g., feeding, energy metabolism and reproduction) have been associated with low body residues of narcotics such as PAHs (Donkin et al., 1989; Widdows and Donkin, 1989; Emery and Dillon, 1996). These sublethal manifestations can, in turn, affect population dynamics and presumably ecosystem function. Further research is needed to better establish cause-effect relationships between tissue

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concentration of nonpolar compounds and the many sublethal manifestations of
toxicity.

Meiobenthic copepods have been successfully used as test-organisms in aquatic
hazard assessments, including sediment toxicity testing (e.g. Chandler et al., 1994;
DiPinto et al., 1993; Green et al., 1996). Several species are easily cultured under
laboratory conditions and have a short life cycle, allowing the examination of sublethal
toxic effects using life-history-related endpoints (DiPinto et al., 1993; Green et al.,
1996, Chapter 4). However, the bioaccumulation of organic contaminants by copepods
is poorly known, except for a few studies using planktonic calanoids (Corner et
al., 1976; Harris et al., 1977a, b; Wyman and Connors, 1980; McManus et al., 1983) and
one study that examined PCB bioaccumulation from sediment by a meiobenthic
copepod (Wirth et al., 1994). Despite their small size, sediment-dwelling copepods
appear to be excellent test-organisms for examining the CBR approach. The use of
radiotracers (isotopic dilution technique) greatly facilitates the determination of
contaminant-tissue concentrations and has been previously used successfully with
invertebrates as small as daphnids and amphipods (e.g., Landrum, 1982; Pawlisz and
Peters, 1993b; Landrum et al., 1994).

The present study investigates bioaccumulation and toxicity of fluoranthene, a
PAH congener, by two species of meiobenthic harpacticoid copepods in sediment
exposures. Coullana sp. is the largest and among the most abundant meiobenthic
copepods from the Gulf of Mexico coastal waters (Fleeger, personal communication).
It has been reported to feed primarily from the water column (Decho 1986; Pace and
Caiman, in press). *Schizopera knabeni* Lang, also commonly found in salt-marshes, is about one tenth the size of *Coullana* sp. and is thought to be a selective deposit feeder as has been observed in a related species (Chandler *et al.*, 1994). Short (24 h) and long-term (10-d) bioaccumulation of fluoranthene was investigated under increasing sediment concentrations. Elimination dynamics and the potential for biotransformation were also studied. The relationship between biological effects and fluoranthene concentration in the exposure media (sediment), and in the organism (body residue) was examined. In addition to mortality, sublethal effects were examined based on changes in offspring production and feeding rate.

**MATERIAL AND METHODS**

**Test organisms**

*Schizopera knabeni* was obtained from a mono-specific laboratory culture started with a stock collected in October 1993 from the surface sediment from an intertidal mud flat of a *Spartina alterniflora* salt-marsh at Port Fourchon, Louisiana. It was cultured sediment-free in 500 ml Erlenmeyer flasks at room temperature with 25% artificial seawater (ASW) that was fully renewed fortnightly. Copepods were fed twice a week with a mixture of *Chaetoceros muelleri* (a planktonic diatom) and Microfeast Plus Larval Diet®. *Coullana* sp. was collected from the surface sediment from an intertidal mud flat of a *Spartina alterniflora* salt marsh at Port Fourchon, Louisiana. Approximately 200 ovigerous females were sorted into groups of 5 individuals and placed in 50 x 35 mm crystallizing dishes containing ASW at 25%. After 48 h, adults
were removed and nauplii were pooled together in a 150 x 75 mm crystallizing dish. They were fed daily with a mixture of Isochrysis galbana and Thalassiosira wessfflogii and the water was partially renewed weekly. At the end of three weeks most copepods had developed to adulthood. Copepods of both species were harvested by sieving the culture medium through a 125-μm mesh. Retained copepods were sorted under a stereo microscope and adult females were used in the experiments.

For dry-weight determination, copepods were frozen in liquid nitrogen and rinsed in deionized water. Non-ovigerous adult females, 50 S. knabeni or 10 Coullana sp., and 300 eggs separated from ovigerous Coullana sp. females were transferred to a pre-weighed, 12-mm diameter aluminum pans (Cahn, Madison, WI), in four replicates. Samples were dried at 55 °C for 4 d. After drying, pans were transferred to a desiccator, cooled to room temperature and weighed using a Metler PM1200 (Hights Town, NJ) balance. Average dry weight was 0.76 ± 0.05 μg per individual S. knabeni non-ovigerous females, 5.75 ± 0.8 μg per individual Coullana sp. and 0.043 ± 0.007 μg per individual Coullana sp. egg. Dry weights were used to normalize fluoranthene tissue concentration.

Sediment dosing

Sediment was collected from the top 2 cm of a Spartina alterniflora mudflat near Cocodrie, Louisiana. The typical total PAH concentration in this sediment was 0.26 mg/kg (Carman et al., 1995). Stock test-sediment was prepared by sieving through a 45-μm mesh. The sediment that passed through the sieve was allowed to settle overnight at 4 °C, the supernatant removed by aspiration, and the sediment autoclaved.

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The dry-to-wet-weight ratio was determined by oven drying (80 °C) and adjusted to 0.145 by homogenizing the sediment with the appropriate volume of 25 % ASW. The sediment organic carbon (SOC) of the resultant slurry, measured in duplicate on a Perkin Elmer (Norwalk, CT) 2400 CHN Elemental Analyzer, was 1.5 % (SD = 0.15) after acidification with HCl to remove inorganic carbonate. Sediment was stored at 4 °C.

Radiolabeled [3-¹⁴C]fluoranthene (50.4 mCi/mmol) was purchased from Chemsyn Laboratories (Lexana, KS) and non-radiolabeled fluoranthene (98% purity) was purchased from Aldrich Chemical Co (Milwaukee, WI). The [¹⁴C] purity was determined to be 99% pure by thin layer chromatography (TLC), and liquid scintillation counting (LSC). TLC was performed on K6 silica gel plates (Whatman, Clifton, NJ) with hexane:benzene (4:1, v/v) as the solvent (Landrum, 1982). Fluoranthene was amended to the stock sediment by spiking to create 5 target concentrations: 0, 25, 100, 250, 690, and 2000 nmol/gram dry weight (gdw). Separate dosing solutions were prepared for each sediment concentration by adding 40 μCi of [3-¹⁴C]fluoranthene in 190 μl of acetone and the appropriate amount of a 6.7-mg/ml stock solution of non-radiolabeled fluoranthene to small vials. The final volume of each stock solution was adjusted to 360 μl by adding acetone. The 0 nmol/gdw (control) dosing solution consisted of 360 μl of acetone only and the 25 nmol/gdw dosing solution did not receive any non-radiolabeled fluoranthene. Stored sediment was fully homogenized with the overlying water and 300 g of wet sediment (20 g dry weight) was transferred to 500 ml beakers and vigorously stirred under vortex. Stock solutions were added
dropwise to each sediment slurry and stirred for 4 h. Aliquots were taken from each sediment treatment. Dry-to-wet-weight ratio was determined by oven-drying two 1-g sediment aliquots at 60 °C overnight. Total fluoranthene sediment concentrations (Table 5.1) were measured in duplicate by reverse phase HPLC as described in Lotufo and Fleeger (1996). Measured concentrations ranged from 0.95 to 1.07 of nominal concentrations. Sediments were stored in the dark at 4°C. To minimize the amount of solvent, the overlying water was removed by aspiration and replaced with ASW, and the sediment fully homogenized every 48 h, four times. Sediments were stored for 3 weeks at 4°C in the dark, and the overlying water was replaced. Total fluoranthene concentration was determined in duplicate by HPLC before use in the experiments (day 0, Table 5.1). Two 100-ml aliquots were transferred to scintillation vials, Biosafe II liquid scintillation cocktail (Research Products International Corps., Mount Prospect, IL) was added and [3-14C]fluoranthene activity was measured by liquid scintillation counting (LSC) on a Beckman LS 6000IC Liquid Scintillation Analyzer (Paloalto, CA). Samples were corrected for quench using the external standards ratio method and for background radiation by subtraction. Mean values were used to calculate a specific activity for each sediment treatment (μCi of radiolabeled fluoranthene per nmol of total fluoranthene). Specific activities were used for estimating total fluoranthene concentrations in sediment and tissue samples in all experiments.

10-d experiment

In order to examine the bioaccumulation of fluoranthene and associate tissue concentrations with mortality and offspring production, separate 10-d experiments were
performed using *S. knabeni* and *Coullana* sp. Copepods were exposed to sediment treatments in 50 x 35 mm crystallizing dishes (Kimble, Toledo, OH) filled with 25 ml of ASW using a volumetric flask. Eight ml of sediment treatment was dispensed to the bottom of each dish using a 5-ml Finnpipette\textsuperscript{8} creating a 3-4 mm sediment layer. Dishes were placed in random order inside moisture chambers (loosely covered plastic containers underlined with soaked paper towel which created a humid environment to retard evaporation from experimental dishes) and kept at 4°C in the dark for 7 d. Dishes were then kept overnight in the dark at 25 °C in an incubator with no illumination before copepods were added. In the *S. knabeni* experiment, 50 non-ovigerous adult females were added to each dish, and returned to the incubator. Four replicates per treatment were sampled at days 1, 4, and 10. Thus, the number of dishes was nine per sediment treatment. In the *Coullana* sp. experiment, 15 non-ovigerous adult females were added to each dish. Three replicates per treatment were sampled at days 1, 4 and 10. Thus, the number of dishes was 12 per sediment treatment. Lower replication was used for Coullana sp. because of limitation in the number of test-organisms. Copepods were fed after introduction into the experimental dishes and at days 3, 6 and 9. Each dish with *S. knabeni* received 1 mg of Microfeast Plus Larval Diet\textsuperscript{8} mixed in 0.5 ml of ASW, and dishes with *Coullana* sp. received 1x10\textsuperscript{6} *T. weisflogii* in 0.65 ml of ASW. At each sampling period, the contents of each dish was sieved through a 45-μm mesh. The sediment passing through the sieve was collected in a 50 ml glass beaker and kept in the dark at 4 °C. After 24 h, the overlying water was removed by aspiration. Aliquots of sediment were taken in duplicate for dry weight (1 ml) and \textsuperscript{14}C activity
determination (0.1 ml). The material retained in the sieve was examined under a stereo-
dissecting microscope. Living copepods were transferred to a plastic cell-culture dish
containing ASW, formalin killed, and transferred to a new dish with ASW to maximize
removal of sediment particles. Surviving *S. knabeni* were enumerated and groups of 15
non-ovigerous females per replicate were transferred to scintillation vials. Surviving
*Coullana* sp. were enumerated and sorted into ovigerous and non-ovigerous females.
All non-ovigerous females from each replicate were transferred to a scintillation vial.
Ovigerous females were separated from their egg sacs and transferred to scintillation
vials. All eggs obtained from each replicate were pooled, enumerated and transferred to
scintillation vials. Eggs of *S. knabeni* eggs did not contain enough tissue to be assayed
for radioactivity. Therefore, only non-ovigerous females of this species were used.
Female copepods or eggs were solubilized in 200 ml of TS-2 tissue solubilizer
(Research Products International Corps., Mount Prospect, IL). After sitting overnight
on a hot plate (60°C), 100 \( \mu l \) of 1.2 HCl (to neutralize the tissue solubilizer), 10 ml of
Biosafe II scintillation cocktail were added and \( ^{14}C \) activity was assayed by LSC for
tissue concentration determination. At the 10-d sampling period of the *S. knabeni* and
*Coullana* sp. experiment, offspring were present in all replicates, except at the 652
nmol/gdw for *Coullana* sp. and the 1652 nmol/gdw treatment for both species. Nauplii
and copepodites were preserved in 4% formalin and stained with Rose Bengal, before
enumeration.
24-h experiment

In order to examine the short-term bioaccumulation of fluoranthene, separate experiments were performed using *S. knabeni* and *Coullana* sp. Dishes with sediment were prepared as in the 10-d experiment, except that 28 x 45 mm carrier glass vials (Kimble, Toledo, OH) filled with 10 ml of ASW and 1.5 ml of sediment were used. Non-ovigerous adult females (15 for *S. knabeni* or 3 for *Coullana* sp.) were added to each dish, which were returned to the incubator. Three replicates per treatment were sampled after 3, 6, 12 and 24 h. Copepods were not fed. At each sampling period, the contents of each dish were sieved through a 45-μm mesh and the retained material examined under a stereo-dissecting microscope. Living copepods were formalin killed, enumerated, transferred to scintillation vials, solubilized, and assayed for ¹⁴C activity by LSC for determination of tissue concentration. Dead copepods were in advanced stage of decomposition, and therefore were not analyzed.

Elimination experiment

In order to examine fluoranthene elimination, non-ovigerous female copepods (300 *S. knabeni* or 60 *Coullana* sp.) were exposed to [¹⁴C]fluoranthen sediment treatment (20 nmol/gdw) in 150 ml beakers filled with 50 ml of ASW and 15 ml of sediment for 24 h. After exposure, copepods were retrieved from the sediment and divided into groups; 18 groups with 15 individuals each for *S. knabeni* and 9 groups with 3 individuals each for *Coullana* sp. Two groups of *S. knabeni* and one group of *Coullana* sp. were immediately used for radioisotopic analysis. For each species, half of the remaining groups were individually placed in 28 x 45 mm vials containing 10 ml
ASW and half were individually placed in vials containing 10 ml ASW and 1.5 ml of uncontaminated sediment for fluoranthene elimination assessment. Vials were sampled at 2, 4, 8, and 12 h. Duplicate vials per sampling period were used for *S. knabeni*. Single units were used for *Coullana* sp. due to a shortage of test-organisms. At each sampling period, copepods were retrieved from the sediment or water and assayed of $^{14}$C activity by LSC for determination of tissue concentration. The elimination rate constants ($k_e$) for both species in sediment and water was estimated from linear regressions of ln(tissue concentration) versus time. The corresponding half-lives ($t_{1/2}$) were determined in terms of $k_e$ by the formula ($t_{1/2} = 0.693/k_e$) (Meador *et al.*, 1995a).

**Grazing experiment**

In order to relate body burden with a physiological effect following a short exposure period, grazing rates, assessed over 3 h, were measured concomitantly with fluoranthene bioaccumulation over a total period of 27 h. Copepods exposed to sediment-associated $[^{14}$C$]$fluoranthene were fed $^3$H-labeled microalgae. An inoculum of *T. weisflogii* (UTEX-Collection, University of Texas at Austin) in log-phase was added to 350 ml of F/2 media at 25% in an Erlenmeyer flask containing 100 $\mu$Ci of $[^3$H$]$adenine (0.952 mCi/ml, Sigma Chemical Co). The culture was maintained at 25 °C, 16/18 h light/dark cycle, and pH of 7.5. Algal cells were harvested after 7 d by centrifugation followed by aspiration and replacement of supernatant with ASW (twice) to ensure removal or unincorporated label. Cell density was determined by direct count and $^3$H activity per algal cell was determined using LSC.
Test units were set-up as described for the 24-h bioaccumulation experiment. Non-ovigerous females, 20 for *S. knabeni* and 4 for *Coullana* sp., were added to each experimental unit. All sediment treatments were used, except 25 nmol/gdw, with 4 replicates each. Four test units containing uncontaminated sediment and formalin-killed copepods were used to determine copepod incorporation of $^3$H other than by feeding (dead control). After a 24-h sediment-exposure period, each vial was inoculated with 0.6 ml of $[^3]$Hlabeled algal cells ($0.6 \times 10^6$ cells, 0.19 DPM per cell). After a grazing period of 3 h, copepods were formalin killed and retrieved from test-vials. Copepods were placed in scintillation vials, solubilized and simultaneously assayed for $^3$H and $^1$C activity by LSC. Tritium activity was converted to number of ingested cells by subtracting the mean radioactivity in dead-control copepods and dividing by the mean radioactivity per algal cell.

**Biotransformation experiment**

In order to examine fluoranthene biotransformation, copepods (approximately 1500 adult *S. knabeni* or 60 adult female *Coullana* sp.) were exposed to $[^1]$Cfluoranthene sediment treatment (20 nmol/gdw) in 150 ml beakers filled with 15 ml of ASW and 20 ml of sediment for 96 h. After exposure, copepods were retrieved from the sediment, frozen in liquid nitrogen, and analyzed for biotransformation products using procedures modified from Landrum (1982). Three replicates with approximately 500 *S. knabeni* or 30 *Coullana* sp. were lyophilized and sonicated for 20 s in 5 ml of ethylacetate:acetone (1:4, v/v). The extract was filtered through a sodium-sulfate column and reextracted with 2 ml of hexane. All extracts were combined and
the volume reduced under a stream of nitrogen to approximately 100 µl. The extracts were analyzed by TLC using hexane:benzene (4:1) as solvents. Developed plates were divided into four sections corresponding to the Rf of fluoranthene and three other regions, including the origin. The silica gel from each section was scraped from the TLC plate and ¹⁴C activity was determined using LSC. The amount of total metabolite was determined to be the sum of all non-fluoranthene ¹⁴C on the TLC plate.

**Statistical analysis**

For both the *S. knabeni* and *Coullana* sp. experiments, one-way analysis of variance (ANOVA) was used to analyze: (1) fluoranthene sediment concentrations after spiking and at days 0, 1, 4, and 10; (2) tissue concentrations at 3, 6, 12 and 24 h and days 1, 4 and 10; and (3) offspring production and grazing rates at each sediment treatment. Treatments were compared using Bonferroni's two-tailed t-test (α = 0.05) for (1) and (2), and contaminant treatments were compared to control treatments using Dunnett's one tailed t-test (α = 0.05) for (3). Linear regression was performed to obtain the relationship (1) between sediment and tissue fluoranthene concentrations for days 1, 4 and 10; (2) between offspring production and average tissue concentration at each sediment treatment; and (3) grazing rates and tissue concentration at each replicate for all sediment treatments used. Mean lethal concentration (LC₉₀) or mean lethal dose (LD₉₀) values were computed using probit analysis. Point estimates for offspring production and grazing rates were calculated using linear interpolation combined with bootstrapping, or the ICₚ method (U.S.EPA, 1994). With p = 25, IC₂₅ estimates the contaminant concentration (in either sediment or tissues) causing a 25% reduction in the
measured endpoint in relation to the control. One-way ANOVA, multiple comparison
tests and simple linear regression analysis were performed using Sigma Stat® (Release
3.0, Jandel Corp, San Rafael, CA). Calculations of LC50 and LD50 values were
computed using TOXCALC™ version 5.0 (Tidepool Software, McKinleyville, CA).

RESULTS

Sediment

The sediment fluoranthene concentrations measured after spiking were usually
slightly higher when measured by HPLC than nominal concentrations (Table 5.1),
indicating high recovery of the spiked compound. Concentrations measured at day 0
were lower than concentrations measured after spiking. This decrease in sediment-
associated fluoranthene ranged from 0.5 to 23% across treatments and was a
consequence of the procedure used to remove the water soluble carrier (acetone). The
dry-to-wet ratio of all sediment treatments at day 0 ranged from 0.115 to 0.128. In the
S. knabeni experiment, ANOVA indicated that differences among fluoranthene
sediment concentrations at days 0, 1, 4, and 10 were not significantly different for any
sediment treatment, except for the 1652 nmol/gdw treatment, where concentration at
day 4 was significantly lower than concentration at day 1 (Table 5.1). In the Coullana
sp. experiment, statistically significant differences were detected at 90 and 652
nmol/gdw, where concentrations at days 1, 2 and 4 were significantly lower than
concentrations at day 0, and at 1652 nmol/gdw, where concentration at day 4 was
significantly lower than concentration at day 0 (Table 5.1).
Table 5.1. Average sediment fluoranthene concentrations as target (nominal), measured after spiking (spiked), before use in the 10-d experiments (day 0) and at days 1, 4 and 10 of the *S. knabeni* and *Coullana* sp. experiments. All concentrations in nmol/gdw, except for number in brackets (µg/gdw). Number in parenthesis indicate 1 standard deviation of the mean.

<table>
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<th>spiked (n = 2)</th>
<th>day 0 (n = 2)</th>
<th>day 1 (n = 4)</th>
<th>day 4 (n = 4)</th>
<th>day 10 (n = 4)</th>
<th>day 1 (n = 3)</th>
<th>day 4 (n = 3)</th>
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<td>(0.7)</td>
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<td>(7.5)</td>
<td>(7.1)</td>
<td>(6.4)</td>
<td>(2.7)</td>
<td>(6.2)</td>
<td>(2.4)</td>
</tr>
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<td>(4.0)</td>
<td>(14.4)</td>
<td>(43.2)</td>
<td>(24.4)</td>
<td>(25.8)</td>
<td>(75.6)</td>
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</tr>
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<td>(9.4)</td>
<td>(20.8)</td>
<td>(99.7)</td>
<td>(87.4)</td>
<td>(30.6)</td>
<td>(44.2)</td>
<td>(15.7)</td>
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<td>(15)</td>
<td>[334]</td>
<td>(91.1)</td>
<td>(159.5)</td>
<td>(91.8)</td>
<td>(73.1)</td>
<td>(24.8)</td>
<td>(174.1)</td>
</tr>
</tbody>
</table>
Bioaccumulation

Tissue concentration increased with increasing sediment concentration in a linear fashion for all days in both *S. knabeni* (Fig. 5.1) and Coullana sp. (Fig. 5.2) 10-d experiment. The $R^2$ ranged from 0.906 to 0.947 in the *S. knabeni* experiment and from 0.935 to 0.994 in the *Coullana* sp. experiment, indicating a tight relationship between sediment and tissue concentrations. Body burdens were not measured at the highest sediment treatments at day 10 in the *S. knabeni* experiment due to reduced number of surviving individuals.

For *S. knabeni*, body burden tended to increase from 3 to 6 h and to remain unchanged thereafter (Fig. 5.3). The mean body burden at 3 h comprised from 33 to 49% of the maximum levels attained during the 24-h exposure, and was significantly lower than at any other time period at all sediment treatments, except at 652 nmol/gdw. Mean body residues at 6, 12 and 24 h were not significantly different at any treatment. Body burdens in the 10-d experiment tended to decline slightly from day 1 through 10, except at 652 nmol/gdw (Fig. 5.3). Mean body burden at days 1, 4 and 10 were not significantly different, except at the lowest and highest concentrations. At 25 nmol/gdw, body burden at day 1 was similar to body burden at day 4 and decreased by 53% to a significantly lower mean at day 10. At 1652 nmol/gdw, mean body burden decreased by 54% at day 4 and was not measured at day 10 due to low number of surviving organisms. Overall, the analysis of the data from the 24-h and 10-d experiment together, indicated that *S. knabeni* body burden attained apparent steady-state in the course a 24 h exposure, and remained relatively constant through day 10,
Fig. 5.1. Fluoranthene tissue concentrations as a function of sediment concentration in the *Schizopera knabeni* bioaccumulation experiment after 1, 4 and 10 d of exposure. Solid lines are the best fit from linear regression; dashed lines represent 95% confidence bands. Day 1: tissue concentration (tis) = 75.6 (± 726) + 16.6 (± 0.9) x sediment concentration (sed), $R^2 = 0.95$, $p < 0.001$; day 4: tis = 67 (± 525) + 10.3 (± 0.8) x sed, $R^2 = 0.91$, $p < 0.001$; day 10: tis = -130 (± 538) + 23.9 (± 1.9) x sed, $R^2 = 0.92$, $p < 0.001$. 

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Fig. 5.2. Fluoranthene tissue concentrations as a function of sediment concentration in the Coullana sp. bioaccumulation experiment after 1, 4 and 10 d of exposure. Solid lines are the best fit from linear regression; dashed lines represent 95% confidence bands. Day 1: tissue concentration (tis) = 57.6 (± 197) + 13.3 (± 0.3) x sediment concentration (sed), $R^2 = 0.99$, $p < 0.001$; day 4: tis = 128 (± 367) + 11.6 (± 0.6) x sed, $R^2 = 0.97$, $p < 0.001$; day 10: tis = 67 (± 628) + 12.7 (1.0) x sed, $R^2 = 0.94$, $p < 0.001$. 

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Fig. 5.3. Accumulation of fluoranthene in tissues (solid lines) of *Schizopera knabeni* over time in the 24-h experiment (left) and 10-d experiment (right) in exposures to increasing sediment concentrations at measured day 0 (indicated in the upper left corner). Dashed lines correspond to percent copepod mortality at each time period in the 10-d experiment. Error bars show 1 SD of the mean.

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except for the lowest and highest concentrations, where it tended to decline from day 1 to 4. For *Coullana* sp., body burden remained relatively constant from 3 to 24 h (Fig. 5.4). Means were not significantly different at any sediment treatment. Body burden also remained relatively constant from day 1 to 10, with means not statistically different at any treatment. Steady state was apparently attained in less than 24 h. Higher variability in body burden was observed in the 24-h experiment than in the 10-d experiment, probably due to the lower number of organisms per replicate used in for $^{14}$C activity analysis, three in the 24-h and from 5 - 15 in the 10-d experiment. The time required to reach 95% steady state tissue concentration can be estimated by the elimination rate constant in sediment ($k_e$, see below) using the formula $(TSS_{95}) = 2.99/k_e$ (Meador *et al.*, 1995a). Using $k_e$ values obtained by direct measurements of copepods exposed to the 25 nmol/gdw treatment, the time for 95% steady state was 20.2 h for *S. knabeni* and 18.1 h for *Coullana* sp., confirming the visual estimation. Overall, *Coullana* sp. accumulated higher concentrations of fluoranthene on a dry-weight basis than *S. knabeni* in the 24-h experiment. However, mean fluoranthene tissue concentration was higher in *S. knabeni* at all sediment treatments in the 10-d experiment.

**Mortality**

Mortality did not take place at any sediment treatment during the 24-h experiment for either *S. knabeni* or *Coullana* sp. In the *S. knabeni* 10-d experiment, mean survival was high for all sediment treatments, except at 1652 nmol/gdw, and ranged from 93.5 to 99.5%. At the sediment treatment 1652 nmol/gdw, mean survival
Fig. 5.4. Accumulation of fluoranthene in tissues (solid lines) of *Coullana* sp. over time in the 24-h experiment (left) and 10-d experiment (right) in exposures to increasing sediment concentrations at day 0 (indicated in the upper-left corner). Dashed lines correspond to percent copepod mortality at each time period in the 10-d experiment. Error bars show 1 SD of the mean.

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was high at day 1 (95.5%) and declined to 62% at day 4 and to 3% at day 10 (Fig. 5.3). In the *Coullana* sp. experiment, survival was high at day 1 for all sediment treatments, ranging from 91.1 to 95%. At day 4, survival tended to decline at high fluoranthene sediment concentrations, ranging from 85 to 96% at sediment concentrations up to 231 nmol/gdw and decreased to 71% at 652 nmol/gdw and 64% at 1652 nmol/gdw. At day 10, survival was highest in the control (91%) and declined steadily with increasing sediment concentrations, down to 13.3% at the highest sediment treatment (Fig. 5.4); mortality was significantly different from the control at sediment concentrations of 652 and 1652 nmol/gdw. LC$_{50}$ values were calculated using mortality data only from day 10 due to high survival across concentrations at day 1 and 4. Ten-d LC$_{50}$s were determined to be 1052 nmol/gdw sediment (954 and 1142, 95% C.I.) or 213 μg/g for *S. knabeni* and 652 nmol/gdw sediment (435 and 890, 95% C.I.) or 132 μg/g for *Coullana* sp.

In the 24-h experiment, tissue concentrations up to 29.2 pmol/gdw for *S. knabeni* and 39.3 μmol/gdw for *Coullana* sp. were not associated with mortality. In the 10-d experiment with *S. knabeni*, body burden as high as 30 μmol/gdw was not associated with mortality after a 1-d exposure. After a 4-d exposure, 38% mortality at the highest fluoranthene sediment concentration was associated with a mean tissue concentration of 14.8 μmol/gdw in surviving organisms. Finally, after a 10-d exposure, 97% mortality of *S. knabeni* in the highest sediment concentration occurred but could not be associated with tissue concentration due to the low number of surviving organisms (Fig. 5.3). For *Coullana* sp., tissue concentrations as high as 18.7 μmol/gdw after a 1-d exposure were not associated with significant mortality. After 4 d, mean
body burdens of 5.2 and 14.9 μmol/gdw were associated with 29% and 36% mortality, respectively. After a 10-d exposure, mean tissue concentration of 5.5 μmol/gdw and 13.3 μmol/gdw were associated with 53% and 84% mortality, respectively (Fig. 5.4). Ten-d LD₅₀s were calculated using averaged tissue concentrations for days 1, 4 and 10 (average for days 1 and 4 for the highest sediment treatment in the S. knabeni experiment). An LD₅₀ of 14.2 μmol/gdw tissue (13 and 15.2, 95% C.I.) was calculated for S. knabeni and 6.6 μmol/gdw (4.6 and 8.8, 95% C.I.) for Coullana sp.

**Reproduction**

Offspring produced by S. knabeni after a 10-d exposure were enumerated. Most offspring consisted of nauplii in all replicates (78 - 98%). The highest average number of offspring produced per surviving female was observed in the control (12 ± 1.6), followed by the 90 nmol/gdw (11 ± 1.2) and the 25 nmol/gdw (10.6 ± 1.3) sediment treatments. Mean number of offspring per female was significantly lower than the control at the 231 nmol/gdw (6.5 ± 0.3) and decreased to close to 0 at the 652 nmol/gdw sediment treatment (0.2 ± 0.1). No surviving offspring were found at the highest fluoranthene sediment treatment. The IC₂₅ value was 148 nmol/gdw sediment (69 and 198, 95% CI) or 30 μg/g. Using the average tissue concentrations at days 1, 2 and 4, it was observed that the number of offspring produced per surviving female decreased with increasing female body burden in a linear fashion (Fig. 5.5). The high R² value (0.91) indicates a strong relationship. Decreased offspring production of approximately 50% was associated to an average body burden of 3.1 μmol/gdw and close to total reproduction impairment was associated with a body burden of 9.4 μmol/gdw. The IC₂₅...
Fig. 5.5. Offspring production per surviving female *Schizopera knabeni* and *Coullana* sp. after a 10-d exposure as a function of body residue (average over 10 d). Solid lines are the best fit from linear regression; dashed lines represent 95% confidence bands. For *S. knabeni*: number of offspring = 11.41 (± 0.38) - 1.22 (± 0.09) x tissue concentration (μmol/gdw), $R^2 = 0.92$, $p < 0.001$. For *Coullana* sp: number of offspring = 10.24 (± 1.79) - 1.75 (± 0.65) x tissue concentration (μmol/gdw), $R^2 = 0.36$, $p = 0.018$. 

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value obtained was 1.8 μmol/gdw tissue (0.3 and 2.6, 95% C.I.). Offspring produced after a 10-d exposure in the Coullana sp. experiment tended to decrease with increasing average body burden (Fig. 5.5). Due to high variation among replicates (coefficient of variation ranging from 38 to 100%), a poor linear relationship (R² = 0.2) was obtained between body burden and offspring production and ICₚ values could not be calculated. Total impairment of offspring production occurred at tissue concentrations as low as 5.5 μmol/gdw.

About 30% of the non-ovigerous Coullana sp. exposed to sediment-associated fluoranthene extruded their eggs after one day. This represented an excellent opportunity to compare the relative contaminant concentration in non-ovigerous females (with mature ovaries) and ovigerous-females that had just extruded their eggs (and therefore had empty ovaries) with eggs. At the day-1 sampling period of the Coullana sp. 10-d experiment, ¹⁴C activity was measured in non-ovigerous females, ovigerous females separated from their eggs, and eggs pooled together from each replicate. On a dry-weight basis, fluoranthene tissue concentrations in non-ovigerous females were approximately 50% higher than in ovigerous females without eggs at all sediment treatments, and tissue concentrations in the eggs alone were 3 to 4.4 times higher than in non-ovigerous females and 6 to 10 times higher than in ovigerous females (Table 5.2). The relative distribution of fluoranthene in non-ovigerous females, ovigerous females without eggs, and eggs alone at day 1 is shown in Fig. 5.6. For each ovigerous female, eggs usually contained approximately as much fluoranthene as the female’s body, although average biomass of a full clutch is estimated to be only...
Table 5.2. Tissue concentrations of *Coulana* sp. measured after a 1-d exposure to sediment-associated fluoranthene. Non-ovig. females = non-ovigerous females; ovig. fem. w/o eggs = ovigerous females minus egg sacs; nd = not determined.

<table>
<thead>
<tr>
<th>Treatment (nmol/gdw)</th>
<th>non-ovig. females (μmol/gdw)</th>
<th>ovig. fem w/o eggs (μmol/gdw)</th>
<th>eggs (μmol/gdw)</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>0.165 (0.028)</td>
<td>0.087 (0.021)</td>
<td>0.576 (0.071)</td>
</tr>
<tr>
<td>90</td>
<td>0.808 (0.135)</td>
<td>0.359 (0.088)</td>
<td>3.558 (0.336)</td>
</tr>
<tr>
<td>231</td>
<td>2.668 (0.348)</td>
<td>1.340 (0.199)</td>
<td>11.513 (1.713)</td>
</tr>
<tr>
<td>652</td>
<td>6.177 (0.561)</td>
<td>3.186 (0.215)</td>
<td>18.151</td>
</tr>
<tr>
<td>1652</td>
<td>18.915 (0.395)</td>
<td>nd</td>
<td>nd</td>
</tr>
</tbody>
</table>
Fig. 5.6. Relative bioaccumulation of fluoranthene in individual non ovigerous females (non-ovig), ovigerous females minus eggs (ovig), and total eggs per ovigerous females (eggs) in *Coullana* sp. Bioaccumulation is expressed in a relative basis as $^{14}$C activity (desintegration per min). Error bars show ± SD of the mean.
approximately 25% of the body biomass. The contaminant distribution in eggs and body of ovigerous females indicates that approximately 50% of the female body burden of a female with mature ovaries resides in the eggs and is released when eggs are extruded. Similar trends were found for days 4 and 10 (data not shown).

Grazing

In the *S. knabeni* experiment, average grazing rate, measured as the number of cells ingested per hour per copepod, was maximal in the control (59 ± 12.5 cells/h) and gradually decreased with increasing sediment concentration. Grazing at all fluoranthene treatments was significantly lower than in the control. The IC$_{50}$ value obtained was 47.6 nmol/gdw sediment (29 and 104, 95% C.I.) or 9.6 µg/gdw. Grazing rates were regressed against tissue concentration (Fig. 5.7) and indicated that grazing decreased with increasing body burden in a linear fashion. The R$^2$ value indicated that 59% of the variation in grazing rate was explained by tissue concentration. Grazing remained below 50% of the control average at tissue concentrations as low as 2.4 µmol/gdw and was almost totally impaired at tissue concentrations as low as 10.3 µmol/gdw. The IC$_{50}$ value obtained was 1.1 µmol/gdw tissue (0 and 1.4, 95% C.I.).

For *Coullana* sp., average grazing rate was highest in the control (282 ± 57 cells/h), followed by the 90 nmol/gdw sediment treatment (210 ± 42 cells/h). It decreased significantly at 231 nmol/gdw sediment (71 ± 14 cells/h) and 652 nmol/gdw sediment (107 ± 49 cells/h) and was minimal (6.6 ± 1.5 cells/h) at 1952 nmol/gdw sediment. The IC$_{50}$ value obtained was 94 nmol/gdw sediment (28 and 173, 95% C.I.). Grazing rates were regressed against tissue concentration (Fig. 5.7). Grazing tended to

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Fig. 5.7. Grazing rate of *Schizopera knabeni* and *Coullana* sp. in the after a 24-h exposure as a function of body burden (average over 10 d). Solid lines are the best fit from linear regression; dashed lines represent 95% confidence bands. For *S. knabeni*: number of cells ingested per h = 40.7 (± 4.2) - 2.8 (± 0.6) x tissue concentration (μmol/gdw), R^2 = 0.59, p < 0.001. For *Coullana* sp: number of ingested cells per h = 204.1 (± 24.2) - 17.0 (± 4.2) x tissue concentration (μmol/gdw), R^2 = 0.46, p < 0.001.
decrease with increasing body burden in a linear fashion, except for body burdens ranging from 1 to 8.5 \( \mu \text{mol/gdw} \). The \( R^2 \) indicated that 46% of the variation in grazing rate was explained by tissue concentration. Grazing remained below 50% of the control average at tissue concentrations as low as 1.1 \( \mu \text{mol/gdw} \) and was almost totally impaired at tissue concentrations as low as 9.9 nmol/gdw. The IC\(_{50}\) value obtained was 0.4 \( \mu \text{mol/gdw tissue} \) (0.3 and 1.2, 95% C.I.).

**Elimination and biotransformation**

The elimination of fluoranthene was evaluated in both sediment and water in 24-h experiments. For *S. knabeni*, elimination in water and sediment occurred at approximately the same rate constant \( (k_0) \), 0.148 (± 0.23) \( \text{h}^{-1} \) \( (R^2 = 0.83) \) in sediment and 0.142 (± 0.025) \( \text{h}^{-1} \) \( (R^2 = 0.80) \) in water (Fig. 5.8). The corresponding half-lives \( (t_{1/2}) \) were 4.68 h in sediment and 4.88 h in water. For *Coullana* sp., elimination in sediment was faster (0.165 ± 0.048 \( \text{h}^{-1} \), \( R^2 = 0.80 \)) than in water (0.094 ± 0.048 \( \text{h}^{-1} \), \( R^2 = 0.95 \)) (Fig. 5.8). The corresponding half lives were 4.2 h in sediment and 7.4 h in water. Virtually no \( ^{14}\text{C} \) activity was left in copepod tissues after an elimination period of 12 h for *S. knabeni* in both water and sediment and only 10% of the initial activity was present in *Coullana* sp. after the same period in sediment and 32% in water.

After a 96-h exposure to sediment-associated fluoranthene, the fraction of \( ^{14}\text{C} \) activity in the copepod tissues from non-fluoranthene compounds in TLC plates was determined to be 10.8 (± 8.8)% for *S. knabeni* and 12.8 (± 4)% for *Coullana* sp. These fractions are assumed to correspond to products of fluoranthene biotransformation.
Fig. 5.8. Fluoranthene elimination by *Schizopera knabeni* and *Coullana* sp. in clean (control) sediment and in clean artificial sea water. Solid lines are the best fit from linear regression; dashed lines represent 95% confidence bands. For *S. knabeni* in sediment: ln(tissue concentration, tis) = 4.84 (±0.16) - 0.15 (± 0.02) x time, $R^2 = 0.83$, p < 0.001; in water: ln(tis) = 4.98 (± 0.17) - 0.14 (0.02) x time, $R^2 = 0.80$, p < 0.001. For *Coullana* sp. in sediment: ln(tis) = 5.08 (± 0.33) - 0.16 (± 0.05) x time, $R^2 = 0.80$, p = 0.042; in water: ln(sed) = 5.54 (± 0.08) - 0.09 (± 0.01) x time, $R^2 = 0.95$, p = 0.006.

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DISCUSSION

In all experiments, $^{14}$C activity was used as a surrogate for fluoranthene concentration in copepod tissues and sediment. Degradation of the parent compound in sediments, either by light or microorganisms specialized in PAH breakdown, was unlikely because the sediment was autoclaved prior to use and all experiments were conducted in the dark. Therefore, $^{14}$C-activity was assumed to represent parent-compound concentration. Changes in sediment concentration that occurred during the 10-d exposure with *S. knabeni* were small; concentrations measured at day 10 were 3 - 13.5% lower than those measured at day 0. Greater decreases (12 - 25%) in concentrations were observed between day 0 and day 1 in the *Coullana* sp. experiment. However, only insignificant further decreases (0 - 8%) occurred between day 1 and 10. The absence of significant decreasing concentration over time suggests that degradation of fluoranthene was minimal. Conversely, the presence of metabolites was detected and quantified in copepods exposed to contaminated sediment for 96-h. Nevertheless, tissue concentrations represented molar equivalents of fluoranthene, since radioactivity was generated by parent compound molecules and by biodegradation products. Based on results from a 96-h sediment exposure, metabolites are expected to account for approximately 11% of the molar fluoranthene-equivalent concentration in *S. knabeni* and 13% in *Coullana* sp.

*Schizopera knabeni* and *Coullana* sp. both bioaccumulated fluoranthene in a concentration-dependent manner. Tissue concentrations closely correlated to sediment concentrations throughout the 10-d exposure (Figs. 5.1 and 5.2). Similarly, high
correlation coefficients have been reported for naphthalene bioaccumulation by planktonic copepods from water (Comer, 1976; Harris et al., 1977b) and fluoranthene bioaccumulation by the polychaete Capitella sp. I from sediment (Forbes et al., 1996). Overall, maximal fluoranthene tissue concentrations in S. knabeni and Coullana sp. were attained in less than 6 h and tended to remain constant throughout a 24-h period. Body burden measured after 1 d also tended to remain relatively constant throughout the 10-d exposure. Overall, bioaccumulation data indicated that apparent steady-state was reached very rapidly in both species. To allow comparisons with other species, the time to reach 50% steady state (TSS₅₀) was estimated using the elimination-rate constant. The TSS₅₀ is equivalent to the elimination half life (tₑ) (Meador et al., 1995a). Values obtained for S. knabeni and Coullana sp. in sediment, 4.7 and 4.8 h, respectively, are much lower than values reported for a variety of macro-invertebrate species, which typically range from 7 to 14 d (Meador et al., 1995a). Among freshwater invertebrates, PAH bioaccumulation was very slow in the amphipods Diporeia spp. (Landrum et al., 1994) but rapid (steady-state reached in a few h) in the amphipod Hyalella azteca (Landrum and Scavia 1983) and the midge Chironomus riparius (Leversee et al., 1982). Lower values in harpacticoid copepods are likely related to their small size and high surface-area-to-volume ratio. However, slow uptake has been reported for naphthalene in water with planktonic copepods (Harris et al. 1977b) and for PCBs in sediment with a meiobenthic copepod (Wirth et al., 1994).

The elimination of PAHs can occur by diffusion when external concentration favors outward flux, by excretion of parent or metabolite compounds, and by
biotransformation of parent compounds (Meador et al., 1995a). Crustaceans exhibit a range in ability to biotransform PAHs, and phylogenetically related species may differ in their ability of biotransform PAHs (Meador et al., 1996). While the harpacticoids used in this study do not biotransform PAHs efficiently (an average of 10.8 and 12.8% of fluoranthene $^{14}$C activity was recovered as potential metabolites in S. knabeni and Coullana sp., respectively), extensive biotransformation of naphthalene was demonstrated with planktonic copepods by Comer et al. (1976) and Harris et al. (1977a,b). The amphipods Diporeia spp. biotransform PAHs inefficiently (Landrum, 1988), while the related species Hyalella azteca biotransforms PAHs at a high rate (Landrum and Scavia, 1983). High elimination rates are typical for invertebrates that biotransform PAH efficiently, as observed with Chironomus riparius, Hyalella azteca and Capitella sp. I (Leversee et al., 1982; Landrum and Scavia, 1983; Forbes et al., 1996). Although the biotransformation of PAHs in the copepod species used in this study seem to be limited when compared to other invertebrates, formation and elimination of metabolites likely contributed significantly to the rapid decrease of $^{14}$C activity in the tissues in uncontaminated media. Elimination rates ($k_e$) were very similar in both media for S. knabeni, indicating independence from the presence of substrate. Fluoranthenel elimination was faster in sediment than in water for Coullana sp. Similar observations have been reported previously for deposit-feeding invertebrates and were related to increased elimination under active sediment ingestion compared to starvation (Landrum, 1982; Landrum and Scavia, 1983; Landrum et al., 1992; Kukkonen and Landrum 1995). The higher elimination rate in sediment for Coullana sp. was
unexpected, since it feeds primarily by filter-feeding from the near-bottom water
(Decho 1986; Pace and Carman, in press), and food was not added with either media.
In addition, there is no compelling evidence of bulk deposit feeding among
harpacticoids.

Empirical and theoretical studies suggest that lethal CBR is approximately
constant across species for compounds with a similar mechanism of toxicity, and that
lethal CBR is independent of exposure concentration and exposure time. For
compounds that exert toxicity by non-polar narcosis, lethal CBR is predicted to vary
from 2 to 8 μmol/g wet weight (McCarty, 1986). Experimental results from several
studies with fish and daphnids in water exposure support this prediction (Van Hoogen
and Opperhuizen, 1988; De Bruijn et al., 1991; van Wezel et al., 1995; Pawlisz and
Peters, 1993a). Evidence of lethal CBR for non-polar narcotics are also provided in
sediment exposures with Diporeia spp. Although 50% mortality was not reached at any
sediment concentration, rough estimates of LD₅₀ values were 6.1 μmol/g wet weight
(gww) for a PAH mixture (Landrum et al., 1991), 6.3 and 9.4 μmol/gww for pyrene
(Landrum et al., 1994) and 6.5 μmol/gww for fluoranthene (Kane Driscoll et al., in
press). For comparative purposes, LD₅₀ values obtained in the present study for
copepods can be expressed in a wet-weight basis using an average wet-to-dry-weight
ratio of 5:1 for invertebrates (Meador et al., 1995a). The fluoranthene LD₅₀ for S.
knabeni (2.84 μmol/gww) is within the range predicted by McCarty (1986) and
obtained with Diporeia spp. For Coullana sp., the LD₅₀ (1.32 μmol/gww) obtained was
lower than the predicted range. As a result of biotransformation, some of the
radioactivity in copepod tissues was shown to be associated with accumulated polar metabolites rather than the original compounds. If metabolites do not participate in narcotization, point estimates for narcotizing body burdens would be overestimated. Conversely, the presence of metabolites may contribute to higher toxicity compared to the parent compound alone. If this is the case, modes of actions other than narcosis could have been operative and point estimates for fluoranthene would be underestimated for both species. Characterization of the identity and toxicity of metabolites should be assessed to clarify toxicity relationships.

Lethal CBRs were estimated in this study using the average tissue concentration measured at days 1, 4 and 10 because of the overall lack of statistically significance of body residues among sampling periods. Tissue concentrations were measured in surviving organisms only. Therefore, the accuracy of the point estimates depends on the assumption that body residues at a given sediment concentration in live organisms closely resembles the concentration in organisms dying by non-polar narcosis. Results from this study indicate that death does not take place concomitant with tissue concentrations that reach levels associated with mortality. Tissue concentrations remained relatively constant from day 1 to 10 under exposure to fluoranthene, but mortality only took place between days 4 and 10 for S. knabeni. Short-term exposures of fish (e.g., 96-h) have been determined to be insufficient to test the toxicity of chemicals with Log $K_{ow}$ values exceeding 5 due to long uptake periods of these compounds (Mackay et al., 1992). Short-term exposures would also be inappropriate in testing the toxicity of these compounds with copepods, but not due to kinetic...
limitations, as rapid uptake was apparent. Instead, the onset of mortality in copepods does not seem to be a short-term manifestation of CBR, as appears to be the case with small fish (McCarty et al., 1992; Mackay et al., 1992) and amphipods (Landrum et al., 1991, 1994), but rather a relatively longer-term manifestation of critical doses. A possible explanation is that narcotization impairs ventilatory activity in fish and macroinvertebrates leading to acutely low oxygen levels in the tissues, while narcotization in copepods would not lead to similar effects, since gas exchange takes place by diffusion through the integument.

Although it appears that Coullana sp. is more susceptible to PAHs than S. knabeni and that both copepods are more sensitive than Diporeia spp., lipid-normalized CBRs should be used to more accurately compare sensitivity among species. Preliminary determinations of lipid content of S. knabeni and Coullana sp. from stockcultures were obtained using flame ionization detection (TLD-FID). Total lipid content was determined to be the sum of the contents of triacylglycerols, fatty acids, steroids, and wax esters. Average total lipid contents were as follows: for Coullana sp., 31.7% for non-ovigerous females, 13.2% for ovigerous females without the eggs, and 87% for the eggs; and 23.2% for S. knabeni non-ovigerous females. It has been hypothesized that sequestration of contaminants in storage-lipid tissues would remove it from the site of action (membranes for narcotics). Therefore, organisms with higher lipid content are expected to be less susceptible to toxicity manifestation (Lassiter et al., 1990; Geyer et al., 1994). According to this theory and assuming that lipid content in sediment-exposed individuals was similar to that measured from individuals from the stock
culture, *Coullana* sp. would be expected to be less sensitive than *S. knabeni*, because its average lipid content was higher. Lipid-normalized LD$_{50}$s were 61.2 μmol/g lipid for *S. knabeni* and 20.8 μmol/g lipid for *Coullana* sp., indicating that differences in sensitivity cannot be explained by lipid content.

Lipid content in *Coullana* sp. was highly associated with the reproductive status. Non-ovigerous females with mature ovaries contained approximately twice as much lipid as ovigerous females without egg sacs. Consequently, higher concentrations of fluoranthene were measured in non-ovigerous than in ovigerous females. Lipid content was much higher (on a dry-weight basis) in the eggs than in female copepods, explaining the higher contaminant concentration measured in the eggs of *Coullana* sp. (Table 5.2). Overall, 50% or more of the non-ovigerous female body burden was released with eggs at their extrusion. Similar disposition of lipophilic contaminants was observed with copepods exposed to PCBs (MacManus et al., 1983) and in crabs exposed to kepone (Roberts and Leggett, 1980).

The number of offspring produced by *S. knabeni* decreased as a function of adult average tissue concentration over 10-d, in a strong relationship between the two variables. It has been determined that PAH reduces offspring production in *S. knabeni* primarily by decreasing brood production rate and egg hatching success (see Chapter 4). Tissue concentration in adult females *S. knabeni* appears to be a reasonable surrogate for the overall effects of PAHs on reproduction. A 25% reduction in reproduction was associated with a body residue of 1.8 μmol/gdw (0.36 μmol/gww), as indicated by IC$_p$ estimation. This value corresponds to 0.13 of the 10-d LD$_{50}$ for *S.*

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The CBRs associated with chronic effects, assuming general narcosis as the mode of action, are predicted to be one order of magnitude lower than lethal CBR, and therefore ranged from 0.2 to 0.8 μmol/gww (McCarty and Mackay, 1993). A very low phenanthrene body residue (4.38 x 10⁻³ μmol/gww) in adult polychaetes was associated with reduced offspring production (Emery and Dillon, 1996). It was speculated that modes of action other than chronic narcosis, such as decreased lysosomal membrane stability and disrupted steroidogenesis, were operative. A low correlation coefficient between tissue concentration and number of offspring was obtained with *Coullana* sp. In this species, eggs hatch into planktonic nauplii, which may not have been supplied with adequate conditions (e.g., food supply) for their survival under the experimental conditions in this study. This was likely reflected in the high coefficients of variation for *Coullana* sp. offspring (38-100%) compared to those for *S. knabeni* (5-13%).

Despite these restrictions, the bioassay for *Coullana* sp. showed that total reproductive impairment occurred at tissue concentrations lower than the 10-d LD₉₀.

To better understand the relationship between exposure and narcosis, body-residue analysis should be made concomitantly with response measurements. This was not possible using mortality as an endpoint, because recently killed individuals were not available for analysis, nor with reproductive output as an end-point, because effects result from PAH exposure over the reproductive cycle during 10 d. However, concomitant measures of dose and effects were accomplished in the present study by measuring tissue residues after a 27-h exposure and simultaneously measuring, using a dual-label approach, grazing on microalgae during the final 3 h of exposure. For *S.*
knabeni, a > 50% decrease in grazing activity occurred at tissue concentrations as low as 2.4 μmol/gdw following a 24-h exposure, while mortality did not take place during the same time period at body residues as high as 32 μmol/gdw (the 10-d LD_{50} was 14.2 μmol/gdw). Similar trends were observed with Coullana sp., indicating that decreased grazing should be an expected manifestation of narcosis. Comparison of point estimates indicate that grazing is a much more sensitive endpoint than mortality for assessing biological responses to narcotization. In addition, the overall decrease in activity commonly observed in exposures to narcotics before any mortality takes place has only been qualitatively characterized. The grazing experiment performed in this study allowed not only the detection of narcotization at low doses, but also its quantification for establishing a cause-effect relationship. Similar findings were obtained by Donkin et al., 1989, who correlated the effects of non-polar compounds on the ciliary feeding activity in mussels to physico-chemical properties of the contaminants, which included PAHs. The tissue concentrations associated with a 50% reduction in feeding ranged from 0.08 to 0.24 μmol/gww for compounds with Log \( K_{ow} < 4.6 \). However, contrasting to the present study, more hydrophobic compounds (Log \( K_{ow} > 5 \)) were accumulated at much higher concentrations before feeding rate was affected (e.g., \( EC_{50} = 3.1 \) μmol/gww for fluoranthene).

The use of feeding rate as a sublethal endpoint is growing in importance for toxicity assessment (e.g., Bitton et al., 1995; Allen et al., 1995). Effects can be detected after a short of time (minutes) by exposing test organisms to a labeled food source. Feeding rates can be also measured with different functional groups, such as filter
feeders (e.g., Juchelka and Snell, 1995) and deposit feeders (Stromgren 1993; Lotufo and Fleeger, 1996), among others. Therefore, the protocol described in this study can easily be modified and used for determining sublethal CBRs for virtually any test-species that feeds continuously.

The CBR approach holds a great promise to improve ecological risk assessment of contaminated field sites. Most approaches for assessing sediment toxicity, such as the EqP, rely on the external (porewater) concentration as a surrogate for the dose at the receptor (tissues). Tissue concentrations derived by the CBR approach are proving to more accurately predict toxicity in benthic organisms compared to organic carbon-normalized sediment concentration (e.g., Landrum et al., 1991, 1994; Kane Driscoll et al., in press). The CBR approach has the potential to provide an improved link between laboratory test data and ecological effects observed in the field. Body residues can be easily measured from specimens collected from areas of concern, in both aquatic or terrestrial environment, and compared to CBRs to predict and interpret contamination impact. As demonstrated in this study, sublethal CBRs can be generated using ecologically relevant endpoints, such as reproductive output and feeding rate, that are much more sensitive than mortality. More information on sublethal CBRs is necessary, especially using organisms from different functional groups, to enhance the discrimination power of the CBR approach as a tool in ecological risk assessment.
CHAPTER 6
SUMMARY AND CONCLUSIONS
Because of their high hydrophobicity, polycyclic aromatic hydrocarbons (PAHs) accumulate in the organic carbon fraction of sediment particles becoming available to and posing a serious risk to the benthic fauna. The toxicity of PAHs in aqueous solutions has been extensively investigated using a variety of aquatic invertebrates. However, information on the toxicity of sediment-associated PAHs is very limited. My research focused on improving the current knowledge on the lethal and the sublethal effects of PAHs on aquatic invertebrates. Ecologically relevant representatives of the estuarine and freshwater fauna were used in laboratory-conducted toxicity bioassays.

Tubificid oligochaetes are a major component of the benthos in freshwater streams and lakes. *Limnodrilus hoffmeisteri* is an abundant tubificid and is considered a bioindicator of anthropogenic activity. Adults of this species were exposed to sediment-associated PAH congeners (Chapter 2). Sediment treatments with increasingly higher concentrations of phenanthrene or pyrene were prepared by spiking. Phenanthrene was acutely toxic at high sediment concentrations while pyrene caused low mortality even at excessively high sediment concentrations. Because of their feeding mode (head-down, bulk deposit feeding), PAH effects on sediment ingestion rate were accurately assessed with defecation chambers using egestion rate as a surrogate for feeding rate. As observed from daily fecal collections, PAHs decreased sediment ingestion rates at concentrations much lower than those associated with 50% mortality over a 10-d period. In contrast to closely related species exposed to similar PAH concentrations in sediment, *L. hoffmeisteri* displayed limited burrowing avoidance. These findings have direct application in modeling the role of benthic
organisms in enhancing the flux of contaminants across compartments (e.g., from sediment into the water column by bioturbation). In addition, offspring production of *L. hoffmeisteri* was decreased at sublethal concentrations of PAHs, following a concentration-response relationship similar to that observed for decreased ingestion rate.

In the subsequent chapters, harpacticoid copepods were used as test organisms in bioassays with sediment-associated PAHs. Harpacticoid copepods, the second most abundant taxon in the marine meiofauna, have undisputed ecological relevance in estuarine food webs. Harpacticoid copepods have been successfully used in sediment toxicity testing as they display desired features such as intimate association with the sediment, short generation time, and ease of laboratory culture. Three species collected from Louisiana salt marshes (*Schizopera knabeni, Nitocra lacustris* and *Coullana* sp.) were cultured sediment-free and used in all bioassays performed in this study.

In the third chapter, *S. knabeni* was exposed to increasing concentrations of two single compounds (phenanthrene or fluoranthene) or of a complex hydrocarbon mixture (diesel fuel) in sediment. Broad differences in short-term (4 d) acute toxicity were detected, with diesel fuel the most acutely toxic contaminant, followed by phenanthrene and fluoranthene. Differences were probably related to variation in bioavailability associated with hydrophobicity. Fluoranthene, the most hydrophobic of the three contaminants, caused very low mortality even at exceedingly high concentrations, similarly to pyrene acute effects on *L. hoffmeisteri*. Sublethal toxicity on offspring production was assessed in 10-d exposures using individual copulating pairs. Decreases
in the number of offspring occurred at concentrations much lower than the 4-d LC50s, especially for fluoranthene, corroborating the finding that reproductive output is much more sensitive endpoint than mortality. Assessment of contaminant effects on feeding rates over short term exposures are becoming common practice in aquatic toxicology. Significant decreases in grazing rate were detected in 30-h sediment exposures using radiolabeled microalgae. This experiment allowed a quantitative assessment of PAH narcotizing effects frequently observed in exposures to hydrocarbons and other non-polar compounds. Finally, avoidance/preference experiments were conducted in small-scale preference arenas. Schizopera knabeni actively avoids contaminated sediment and selects for non-contaminated sediment.

In order to improve the understanding of PAH effects on life-history related features, such as reproductive output, egg viability, and early-stage survival and development, further experiments were conducted using S. knabeni and N. lacustris. In separate experiments, individuals of different life stages (nauplius, copepodite, adult male, adult female) were exposed to sediment-associated phenanthrene for 10-d (Chapter 4). Overall species-specific differences in sensitivity to PAH lethal effects were observed, with N. lacustris significantly more sensitive. Strong differences in life-stage-specific sensitivity were observed for S. knabeni, with the nauplius stage the most sensitive, followed by copepodite and adults. Adult males and females of this species were equally susceptible. Differently, for N. lacustris, females were significantly more tolerant than other life stages, which were not statistically different among themselves. Decrease in offspring production were much drastic than decrease in survival in larval
stages for *S. knabeni*, but only marginally with *N. lacustris*. It was evidenced that this reduction was due to decreased clutch production rate and egg hatching success, as well as prolonged embryonic development. In addition, larval development of larval and juvenile copepods surviving the 10-d exposure was strongly retarded in both species. Overall, deleterious effects were manifested in the same range of concentrations for both species, but strong species-specific differences in the pattern of responses were evident. Therefore, caution is necessary when generalizing toxicity data obtained with limited number of species.

In all experiments described above, the PAH sediment concentration was related to observed effects in order to obtain concentration-response relationships and point estimates such as lethal median concentrations (LC$_{50}$). However, given the complexities involving the bioavailability of sediment-associated contaminants, it has been demonstrated that the dose at the receptor (e.g., tissue concentration), rather than in the surrounding media (sediment) better represents exposure and correlates to the observed manifestations of toxicity. Therefore, the accumulation of sediment associated PAH in copepods and the relationship between internal concentration and lethal and sublethal effects were investigated (Chapter 5). Adult females of *S. knabeni* and *Coullana* sp. were exposed to radiolabeled fluoranthene. Apparent steady-state was reached within hours for both species and tended to remain constant throughout a 10-d exposure. Very fast elimination of PAH in uncontaminated media was observed (half lives < 7.5 h) and probably relates to the limited but significant ability of both species to biotransform PAHs into polar, therefore easily excreted, metabolites. Mortality was detected at tissue
concentrations predicted as lethal. However, critical lethal doses were lower for
*Coullana* sp., even after normalization to lipid content, indicating higher susceptibility
to PAHs. Sublethal critical body residues were also determined. Tissue concentrations
approximately one tenth that of the critical lethal dose (LD₅₀) were associated with
decreased offspring production in both species. PAH toxicity is by a mode of action
known as general narcosis: exposed organisms become lethargic and eventually
paralyzed before death. In an attempt to correlate tissues concentrations well below
lethal to narcotization, grazing rates were measured following short-term exposures to
sediment-associated PAH. Grazing rates decreased as PAH tissue concentrations
increased, in a linear relationship. The use of dual-label (*¹⁴C-fluoranthene and *³H-
* microalgae) allowed simultaneous measurements of tissue concentrations and feeding
quantification. This approach can potentially be used with virtually any test-organism
that feeds continuously, in a way to improve our understanding of the risks associated
with exposure to non-polar compounds such as PAHs.

Environmental contamination by polycyclic aromatic hydrocarbons is
widespread and poses potentially serious consequences to aquatic biota. Dramatic
changes at the population and community levels have been reported from acute (*e.g.*, oil
spills) and chronic exposures to hydrocarbons. Organisms are affected in many different
ways, as attested by numerous field and laboratory investigations. However, relatively
little information is available on the lethal and sublethal effects of sediment-associated
PAHs, as opposed to PAHs dissolved in water. My research suggests that exposure well
below lethal levels causes reductions in reproduction of two phylogenetically and
ecologically distinct benthic animals. PAHs also cause a reduction in feeding rates that is probably directly related to narcotizing effects. Decreased grazing may have subtle ecological impacts such as an alteration in sediment microbial communities and changes in the fate of contaminants. Furthermore, my results suggest that the critical body residue approach to assess toxicity is a relatively accurate predictor of sublethal impacts. Data generated using the critical body residue approach are necessary to improve our understanding of the response to and mode of action by PAH in sediment-dwelling animals. Future studies should enhance the use of sublethal determinations of impact for establishing sediment quality criteria. Furthermore, the sublethal CBR should be generated using organisms from different functional groups in order to improve the interpretation of impacts observed in the field and to enhance its predictive power for use in ecological risk assessment of contaminated sites.
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161

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APPENDIX: LETTER OF PERMISSION

Society of Environmental Toxicology and Chemistry

7 August 1986

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VITA

Guilherme R. Lotufo was born in São Paulo, Brazil, on July 7, 1967. He attended the University of São Paulo, in Brazil, from 1986 until 1989, when he graduated with a bachelor's degree in biology. He attended the same university from 1990 until 1992, and received a master of science degree in Zoology. In 1992, Mr. Lotufo was admitted to the doctoral program in Zoology at Louisiana State University in Baton Rouge, Louisiana. In December, 1996, Mr. Lotufo will be awarded his doctor of philosophy degree.

Mr. Lotufo has six publications in the field of systematic biology, most of them concerning the meiofauna of sandy beaches in Brazil. In the summer of 1993, he received a fellowship from the Smithsonian Institution to work as a short term visitor at the National Museum of Natural History.
DOCTORAL EXAMINATION AND DISSERTATION REPORT

Candidate: Guilherme R. Lotufo

Major Field: Zoology

Title of Dissertation: Toxicity of Polycyclic Aromatic Hydrocarbons to Benthic Invertebrates

Approved:

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Major Professor and Chairman

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Dean of the Graduate School

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