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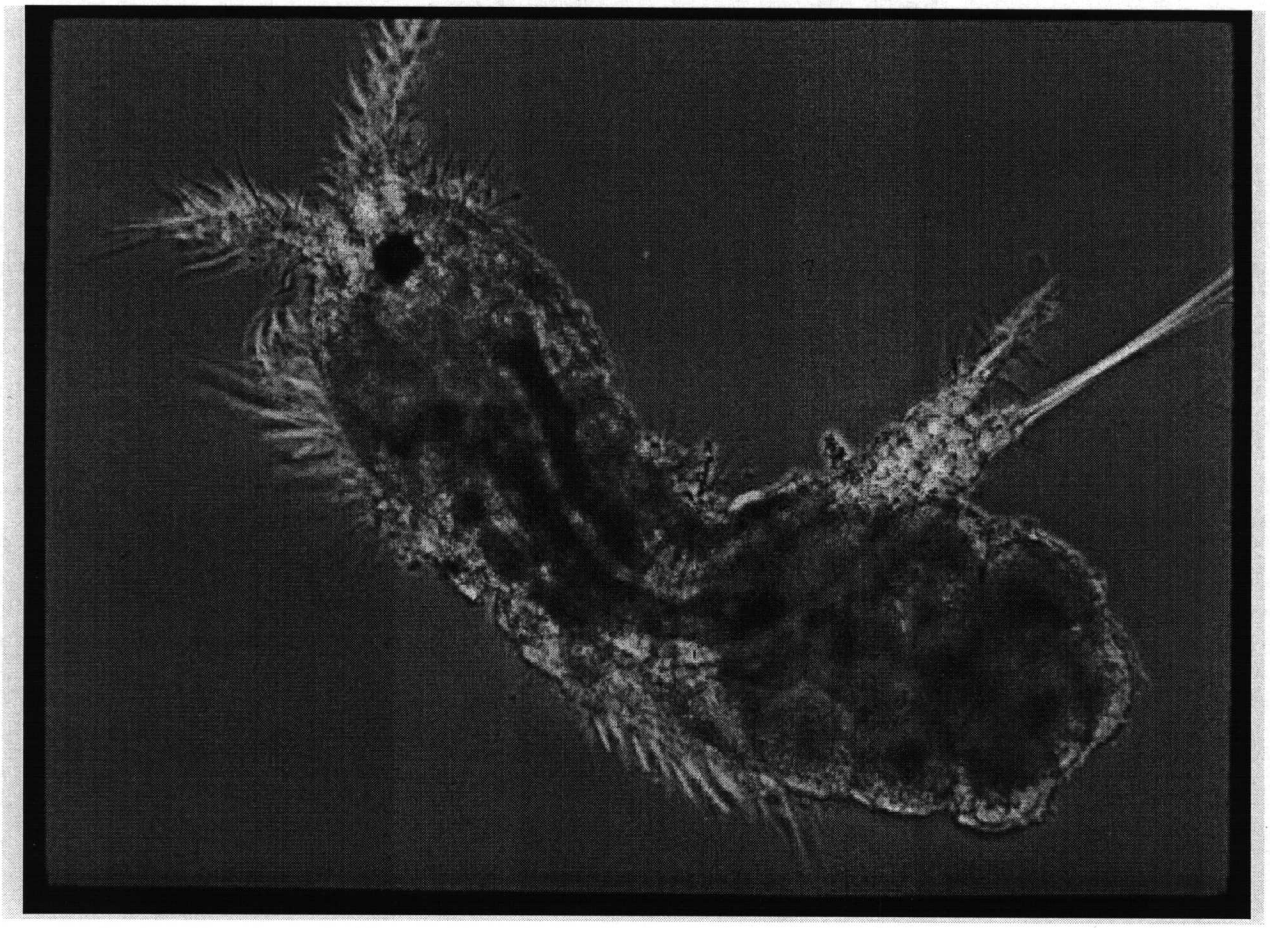

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Development and Application of a Sublethal Toxicity Test to PAH Using Marine Harpacticoid Copepods

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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 analysis. This research project was designed to improve our understanding of the acute and sublethal effects of PAHs to benthic invertebrates. Sublethal bioassay protocols for benthic harpacticoid copepods were developed, and two species of harpacticoids were exposed to a range of concentrations of sediment-amended PAHs; the single compounds fluoranthene and phenanthrene as well as a complex mixture (diesel fuel).

The harpacticoid copepods *Schizopera knabeni* and *Nitocra lacustris* were tested using several bioassay approaches. Reproductive assays, feeding assays and avoidance tests were conducted in addition to lethal tests for *S. knabeni.* Test protocols yielded repeatable results and statistically sound data. Adult harpacticoids were found to be relatively tolerant to PAHs in10-day exposures (e.g., the LC_{50} for *S. knabeni* was 345 μg/g PAH). Species-specific differences in sensitivity were detected. Early life history stages were much more sensitive than adults in one species but not in the other. Concentrations of PAH as low as 26 μg/g PAH decreased copepod offspring production, egg hatching success, and embryonic and early-stage development, demonstrating the high sensitivity of life history-related endpoints. In addition, grazing on microalgae was significantly impaired at concentrations as low as 20 μg/g PAH after short exposures (< 30 h). Finally it was demonstrated that harpacticoids can actively avoid contamination. These protocols are proving useful to address other questions regarding contaminant effects on benthic organisms.

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CHAPTER 1. INTRODUCTION

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. Species belonging to the meiobenthic group Harpacticoida meet the above requirements and potentially serve as important indicators.

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 but retained on a 0.062 mm sieve), with important ecological roles in marine ecosystems. Their densities often reach 10^5 - 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 (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 (Moore and Bett, 1989; Peterson et al., 1996). 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 (*e.g.*, Chandler, 1990; DiPinto *et al.*, 1993; Chandler *et al.,* 1994; Green *et al.*, 1996).

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). 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. 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.

Besides causing short-term mortality, the toxicity of PAHs is manifested as alterations in a variety of biological functions. Sublethal effects 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). This wide range of deleterious effects from PAH detected at concentrations much lower than 96-h LC_{50}s demonstrates that mortality is not a sensitive indicator of toxicity. Unfortunately, accounts of the sublethal toxicity of sediment-associated PAHs are few (Landrum *et al.*, 1994; 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. Extrapolation of sublethal effects observed with relatively few planktonic organisms to all benthic species has been considered inappropriate. 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.

This research project is divided into two portions. First, a bioassay technique for harpacticoids was developed and used to determine the toxicity of sediment-associated phenanthrene, fluoranthene and diesel fuel to the estuarine harpacticoid copepod *Schizopera knabeni* Lang. Phenanthrene (molecular weight, 178) and fluoranthene (molecular weight, 202) were selected as PAH congeners for their concentration in contaminated estuarine sediments, and for being reportedly toxic to sediment-dwelling invertebrates (Kennish, 1992). 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. 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.

When copulating pairs of *Schizopera knabeni* were exposed to phenanthrene in sediment exposures for 14 d in the above experiments, decreased offspring production was observed at sublethal concentrations. 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 the second component of this research was to explore ways in which the harpacticoid toxicity assay might be applied to specific applications to better understand the mechanisms by which contaminants influence populations. Thus, the sublethal effects of sediment-associated PAHs on harpacticoid copepods were subsequently investigated 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 followed by 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-day 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.

CHAPTER 2. MATERIALS AND METHODS

2.1 Experiments with *Schizopera knabeni*

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 *Chaetocerous muelleri* (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 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 Elemental Analyzer, was 1.5 % after acidification with HCl to remove inorganic carbonate. The stock sediment was stored at 4°C.

Phenanthrene and fluoranthene 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 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 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 Iatroscan (as described in Carman *et al.*, 1996) and determined to be 19, 45, 93, 130, 185 and 370 μg/g.

In 96-h toxicity tests, test units (28 x 45 mm carrier glass vials) 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 pipette 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 water-soaked paper towels which created a humid environment to retard evaporation from experimental dishes). They were kept overnight in the dark at 25 ^{0}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.

In a 30-h grazing bioassay designed to determine the effects of PAHs on *S. knabeni* grazing rates, sediment exposed copepods were fed radiolabeled microalgae. An inoculum of *Thallasiossira weissflogii* in log-phase growth was added to 350 ml of F/2 media at 25 ‰ in Erlenmeyer flasks containing 200 μCi (7.4 x 10^6 Bq) of $NaH^{14}CO_3$ (specific activity 60 Ci/mM). Cultures were sealed to prevent loss of label as $^{14}CO_2$ 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 liquid scintillation counter.

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, 451μg/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 10^5 cells; 2.5 DPM per cell). In the diesel-fuel experiment, each vial received 0.5 ml of radiolabeled cells (~ 2 x 10^5 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 tissue solubilizer. After 24 h, 100 µl of 1.2 N HCl (to neutralize the tissue solubilizer) and 10 ml of liquid scintillation cocktail were added and ^{14}C 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.

In a 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.

A 6-h avoidance/preference experiment was designed 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 H_2O) 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. 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 pipette 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.

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. Point estimates for grazing rate and reproductive output was calculated using linear interpolation combined with bootstrapping, or ICp method. 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.

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.

2.2 Experiments with *Schizopera knabeni* and *Nitocra lacustris*

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‰ ASW fully renewed fortnightly.

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 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 pipette 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).

Phenanthrene was amended to the stock sediment by spiking as above. 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.

In all bioassays, copepods were exposed to sediment treatments in 50 x 35 mm crystallizing dishes filled with 25 ml of ASW. Eight ml of sediment treatment was dispensed to the bottom of each dish 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 and oxygen concentration 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 how 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 *Chaetocerous 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.

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 ($\alpha = 0.05$). 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. Point estimates for reproductive output were calculated using the ICp method. With p=25, IC_{25} estimates the contaminant concentration causing a 25% reduction in the measured endpoint in relation to the control.

CHAPTER 3. RESULTS

3.1 Experiments with *Schizopera knabeni*

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

3.1.1 96-h Survivorship Bioassay

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 (Figure 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 (Figure 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). In the diesel experiment (Figure 3.1), mortality was significantly different from the control in all treatments except at 45 μg/g. The LC_{50} was 194 μg/g (Table 3.1).

3.1.2 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 (Figure 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_{25} 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 (Figure 3.2). The IC_{25} 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 (Figure 3.2). All diesel-fuel treatments were significantly different from the control. The IC_{25} value obtained was 19.3 μg/g (Table 3.1).

3.1.3 14-d Reproduction Output Bioassay

Mean offspring production was calculated on a per-surviving-female basis; accordingly, sample sizes differed among treatments (Figure 3.3). Total offspring 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

Table 3.1. Toxicity values from bioassays performed with *Schizopera knabeni.* All values expressed as μg/g. Numbers in parenthesis indicate 95% confidence intervals.

Value	Phenanthrene	Fluoranthene	Diesel Fuel
4-d LC50	473 (401 - 588)	> 2,100	194 (182-199)
Grazing IC25	25.5 (19.3 - 35.8)	65.1 (16.9 - 94.5)	19.3 (9.9 - 39.1)
Total reproduction IC25	26.9 (19/2 - 75.7)	21.8 (17.4 - 32.9)	47.1 (11.7 - 71.3)
Realized reproduction IC25	25.9 (17.8 - 67.8)	18.4 (16.1 -22.1)	18.2 (10.1 - 58.5)

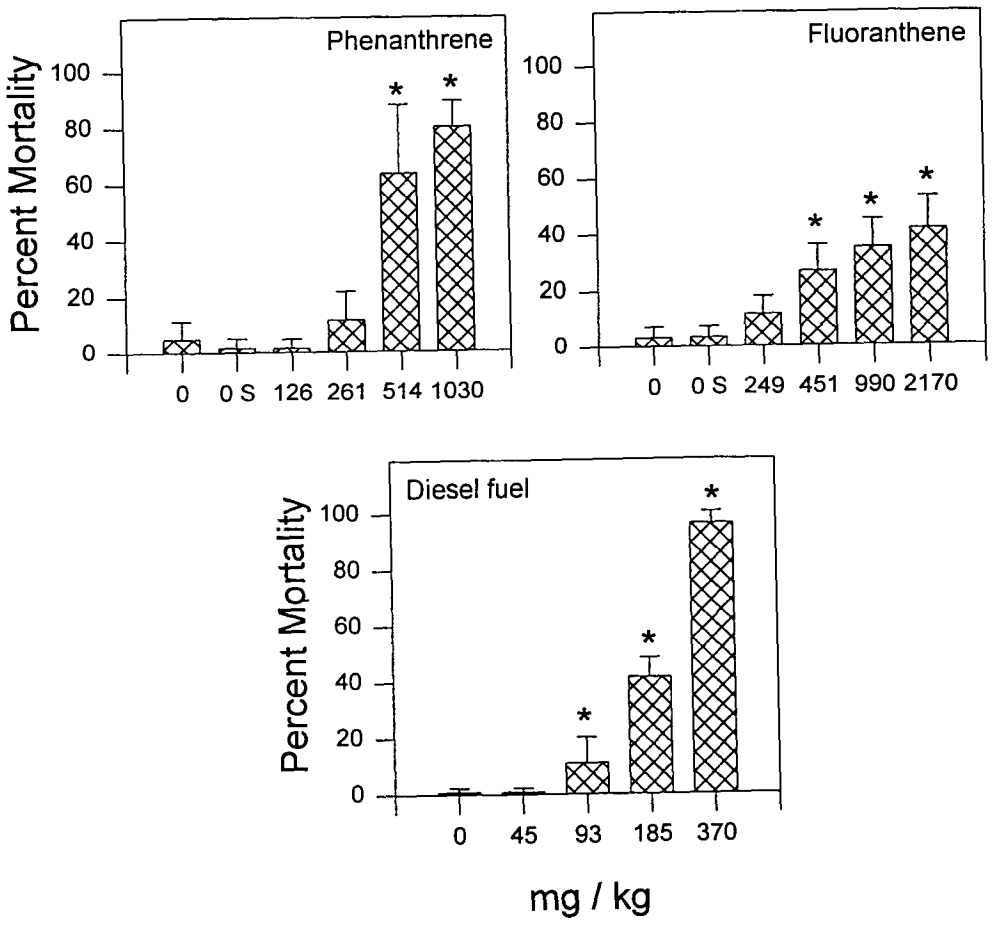

Figure 3.1. Mortality of *Schizopera knabeni* in the phenanthrene, fluoranthene and diesel fuel 96-h bioassay. Error bars show 1 SD of the mean ($n = 4$); 0 S = solvent control; * represents significance difference ($\alpha = 0.05$) from the control (or combined control) mean.

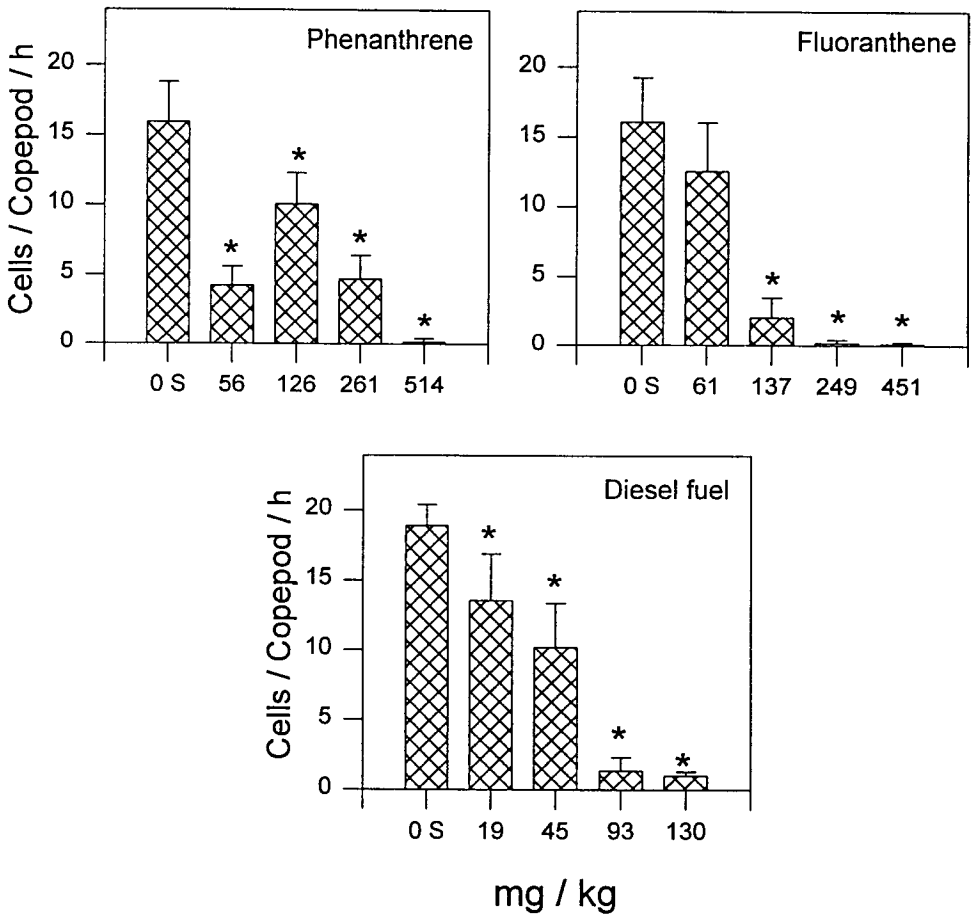

Figure 3.2. Grazing rate of *Schizopera knabeni* in the phenanthrene, fluoranthene and diesel fuel 30-h bioassay. Error bars show 1 SD of the mean ($n = 4$); 0 S = solvent control; * represents significance difference ($\alpha = 0.05$) from the control mean.

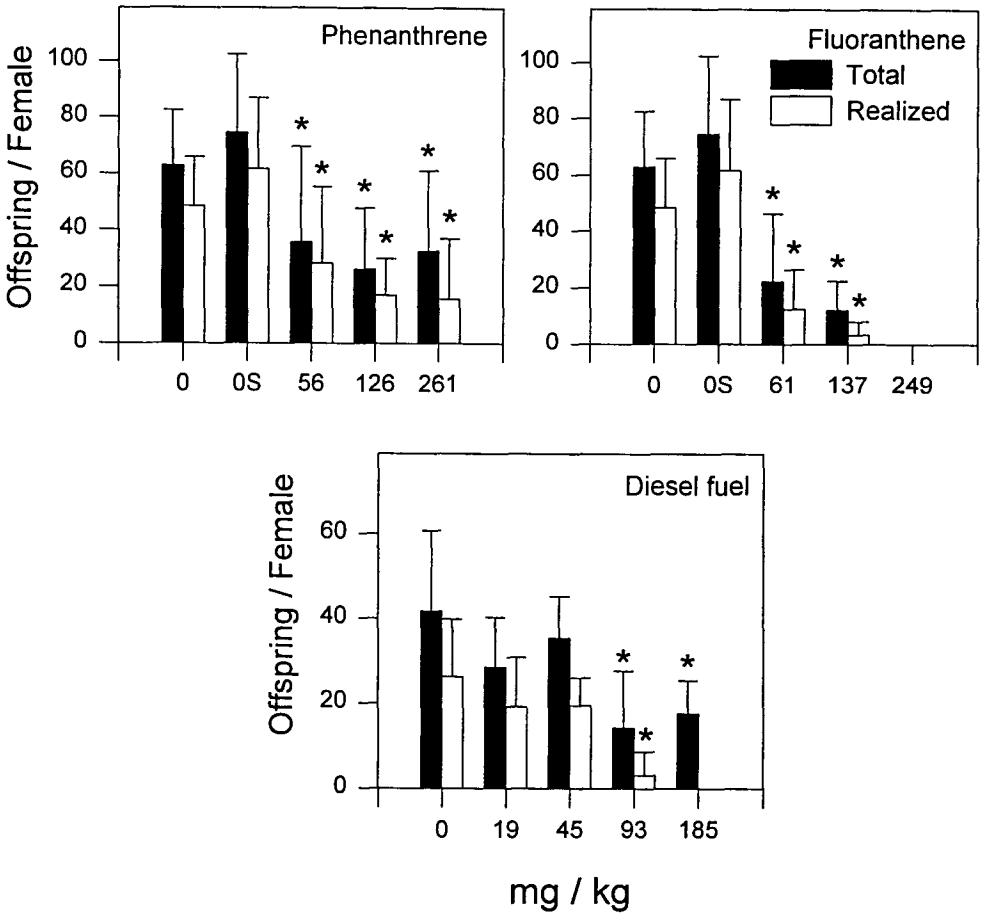

Figure 3.3. Number of total and realized offspring produced by individual copulating pairs of *Schizopera knabeni* in the phenanthrene, fluoranthene and diesel fuel 14-d bioassay. Error bars show 1 SD of the mean ($n = 4$); 0 S + solvent control; * represents significance difference ($\alpha = 0.05$) from the control (or combined control) mean.

mortality was observed in the controls. Mean number of total offspring was higher in the solvent than in the full control, but not significantly (Figure3.3). 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 (Figure 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 (Figure 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 (Figure 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. 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 (Figure 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 (Figure 3.4), but clutch size was not significantly different among treatments for any contaminant ($p > 0.25$).

3.1.4 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

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.

Phenanthrene: 40:8 (11.1) % found burrowed					
Treat.	0	56 μg/g	126 μg/g	261 μg/g	514 μg/g
%	46.8 (10.8)	19.1 (12.6)	15.6 (9.8)	12.6 (7.9)	5.9 (4.4)
Fluoranthene: 67.5 (12.2) % found burrowed					
Treat.	0	137 μg/g	249 μg/g	451 μg/g	990 μg/g
%	47.0 (17.8)	17.0 (11.5)	9.7 (4.7)	12.9 (5.1)	13.4 (8.2)
Diesel fuel: 36.7 (18.1) % found burrowed					
Treat.	0	45 μg/g	93 μg/g	130 μg/g	185 μg/g
%	68.7 (12.4)	13.3 (8.0)	9.9 (9.6)	6.2 (4.0)	2.0 (2.1)
Control experiment: 76.8 (2.9) % found burrowed					
Treat.	I	II	III	IV	V
%	18.2 (6.8)	20.1 (2.9)	25.1 (9.4)	20.7 (5.5)	16.0 (5.7)

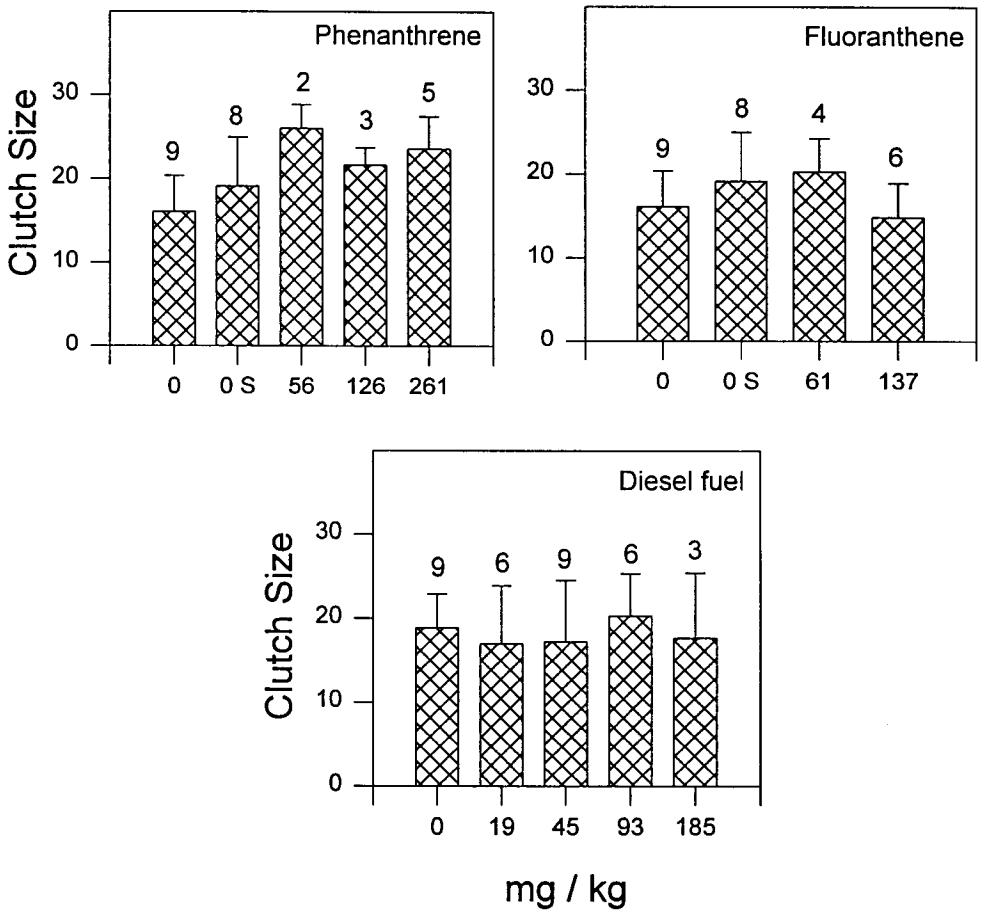

Figure 3.4. Clutch size of ovigerous *Schizopera knabeni* at termination of the phenanthrene, fluoranthene and diesel fuel 14-d bioassay. Error bars show 1 SD of the mean ($n = 4$); numbers above error bars indicate number of ovigerous females in each treatment; 0 S = solvent control.

contamination is indicated by the decrease in the fraction of burrowed copepods with increasing contamination observed with the three contaminants. Moreover, in the 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" ($p = 0.50$).

3.2 Experiments with *Schizopera knabeni* and *Nitocra lacustris*

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 ± 0.9 (mean ± standard deviation) clutches (1 every 2.4 days) with 16.9 ± 1.3 nauplii hatching from each brood. Nauplius production over 10 days was 73.5 ± 15.3, and clutch size measured at day 10 was 22.6 ± 0.6. *Nitocra lacustris* produced an average of 3.7 ± 0.5 broods, 15 ± 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 ‰) experiments. Mean DO levels were 5.5 ± 1.5 mg/L at day 0 and 5.2 ± 1.2 mg/L at day 10.

3.2.1 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 (Figure 3.5). 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.

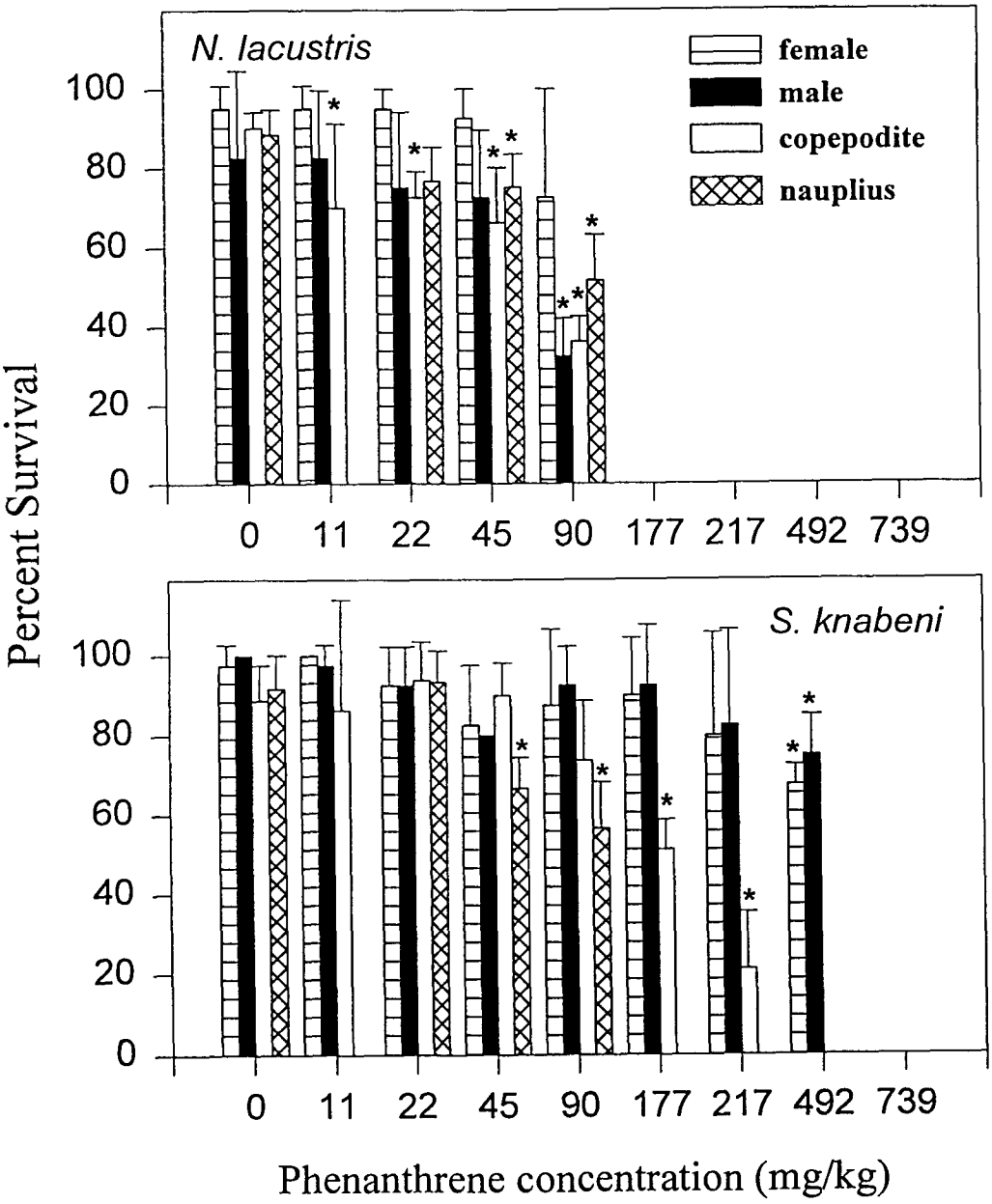

Figure 3.5. Survival of *Nitocra lacustris* and *Schizopera knabeni* life stages (adult female, adult male, copepodite, and nauplius) exposed to sediment-associated phenanthrene for 10 d. Error bars show 1 SD of the mean ($n = 4$); * represents significance difference ($\alpha = 0.05$) from control mean.

Table 3.3. Toxicity values from bioassays performed with *Schizopera knabeni* and *Nitocra lacustris*. Numbers in parenthesis indicate 95% confidence intervals. All values expressed as μg/g.

Value	*Schizopera knabeni*	*Nitocra lacustris*
Male LC50	349 (291–417)	72 (62-83)
Female LC50	345 (291–407)	105 (95-116)
Copepodite LC50	172 (155–190)	43 (36-52)
Nauplii LC50	84 (74–96)	71 (65-77)
Total offspring IC25	48 (7–74)	65 (24-105)
Real. Offspring IC25	26 (0–57)	40 (23-69)

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 μg/g. The LC_{50} values were 105 μg/g for females, 72 μg/g for males, 43 μg/g for copepodites, and 71 μg/g for nauplii (Table 3.3). 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 (Figure 3.5). 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 LC_{50} values were 345 μg/g for females, 349 μg/g for males, 172 μg/g for copepodites and 84 μg/g for nauplii (Table 3.3). 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 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 LC_{50}'s suggests significant overall differences of sensitivity among nauplii, copepodites and adults. The LC_{50}'s of adult females and males were practically identical.

3.2.2 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 copepodites. 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 (Figure 3.6). Realized offspring was 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 (Figure 3.6) 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 3.3). 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 (Figure 3.6). 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 (Figure 3.6). 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 (Figure 3.6) 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 3.3). 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.

3.2.3 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 (Figure 3.7), the fraction of surviving copepods that attained the adult stage in 10 d was highest in

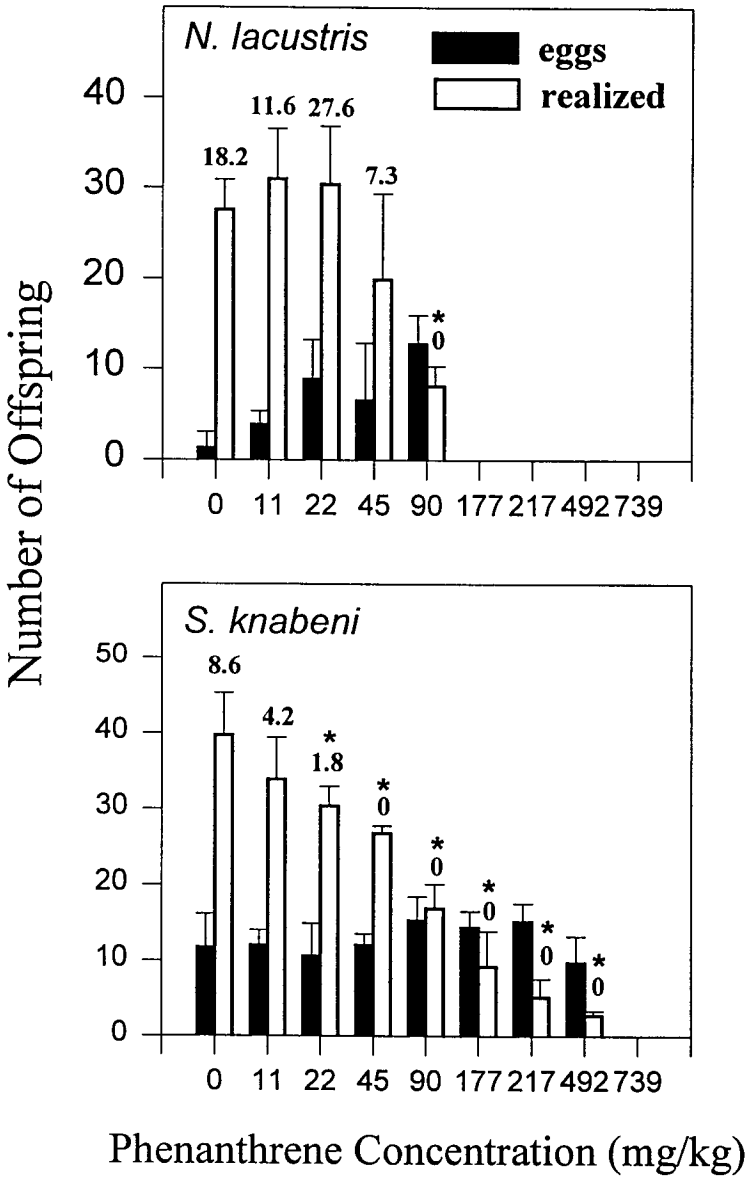

Figure 3.6. Number of offspring produced by *Nitocra lacustris* and *Schizopera knabeni* exposed to sediment-associated phenanthrene for 10 d. Offspring were fractioned into eggs carried by surviving females and realized offspring (nauplii plus copepodites) present at test termination. Error bars show 1 SD of the mean (n = 4); * represents significance difference ($\alpha = 0.05$) from control mean for realized offspring; numbers over bars indicate the average percent of realized offspring comprised of copepodites.

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 (. 7), 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 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 (Figure 3.8), 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 (Figure 3.8), 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 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 (Figure 3.9). 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 (Figure 3.9).

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.

3.2.4 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 (Figure 3.10). 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 (Figure 3.10). Mean number of nauplii per clutch was significantly lower than in the control at the 45, 90 and 177 μg/g treatments. Although 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.

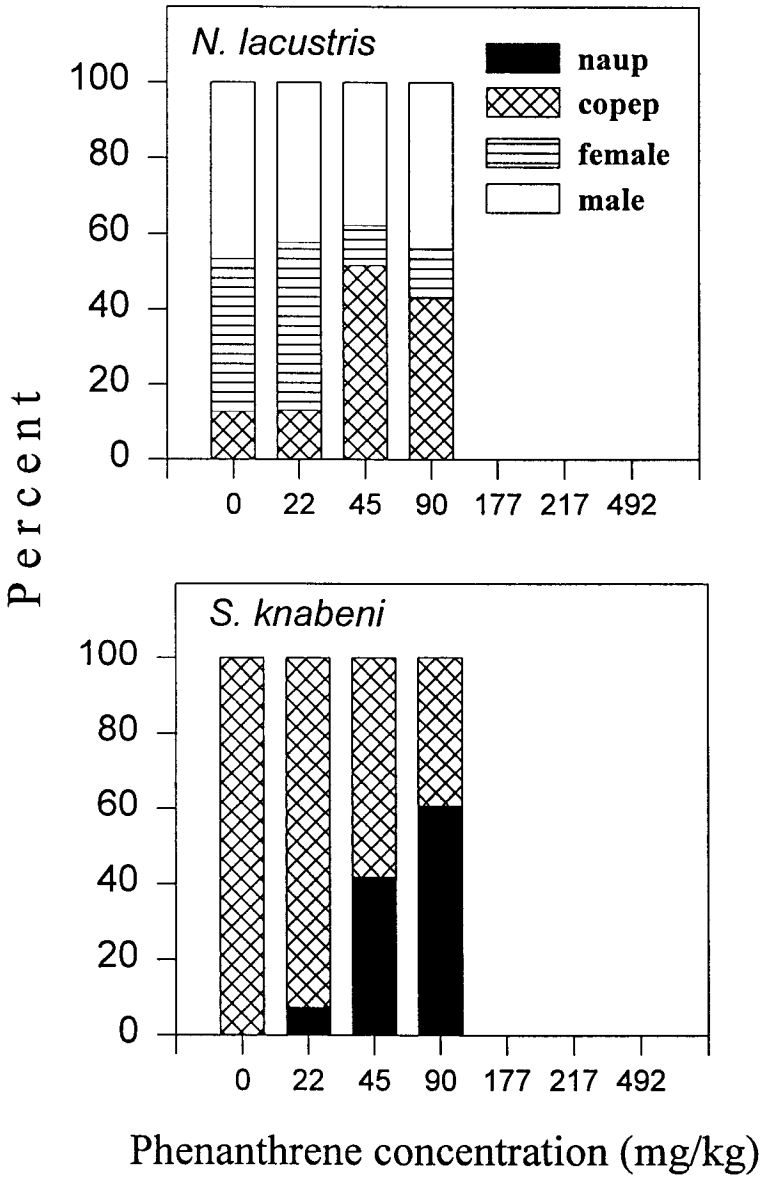

Figure 3.7. Distribution of surviving *Nitocra lacustris* and *Schizopera knabeni* among life stages in the 10-d nauplius bioassay. Naup = nauplius; copep = copepodite; female = adult female; male = adult male. No nauplii survived exposure to the 177, 217 and 492 mg/kg treatments.

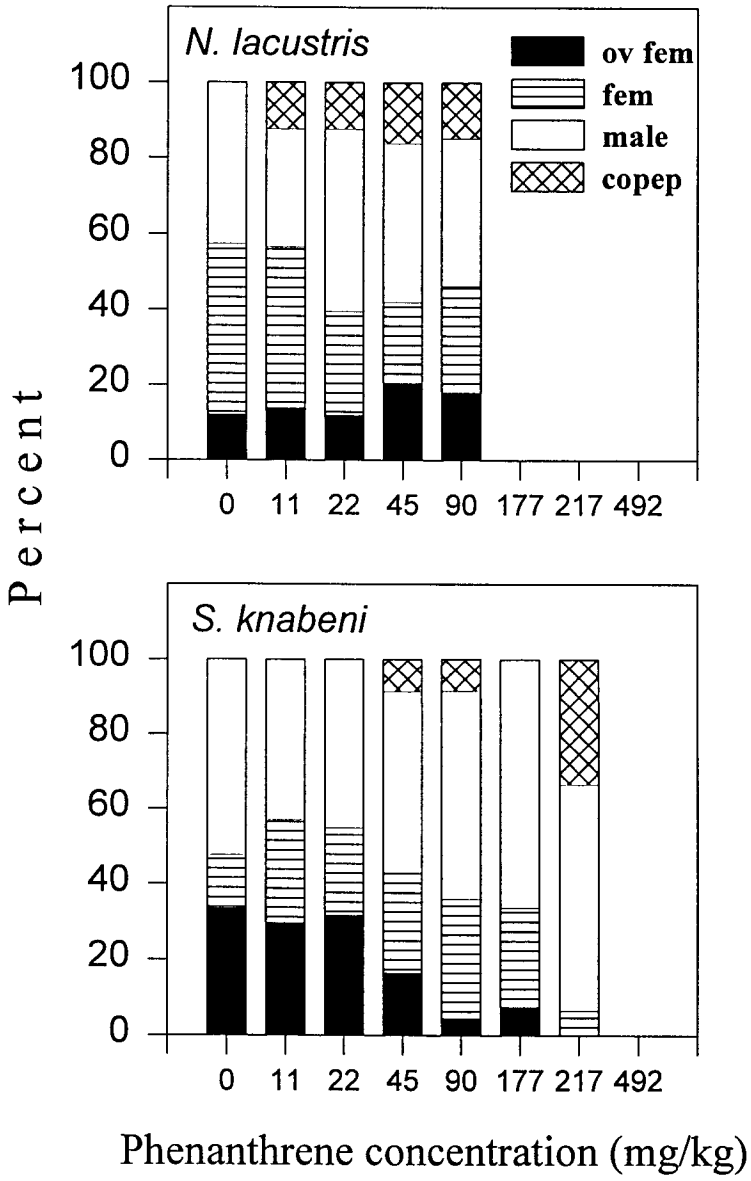

Figure 3.8. Distribution of surviving *Nitocra lacustris* and *Schizopera knabeni* among life stages in the 10-d copepodite bioassay. Ov fem = ovigerous female; fem = non-ovigerous adult female; male = adult male; copep = copepodite. No copepodite *N. lacustris* survived exposure to the 177, 217 and 492 mg/kg treatments and no copepodite *S. knabeni* survived exposure to the 492 mg/kg treatment.

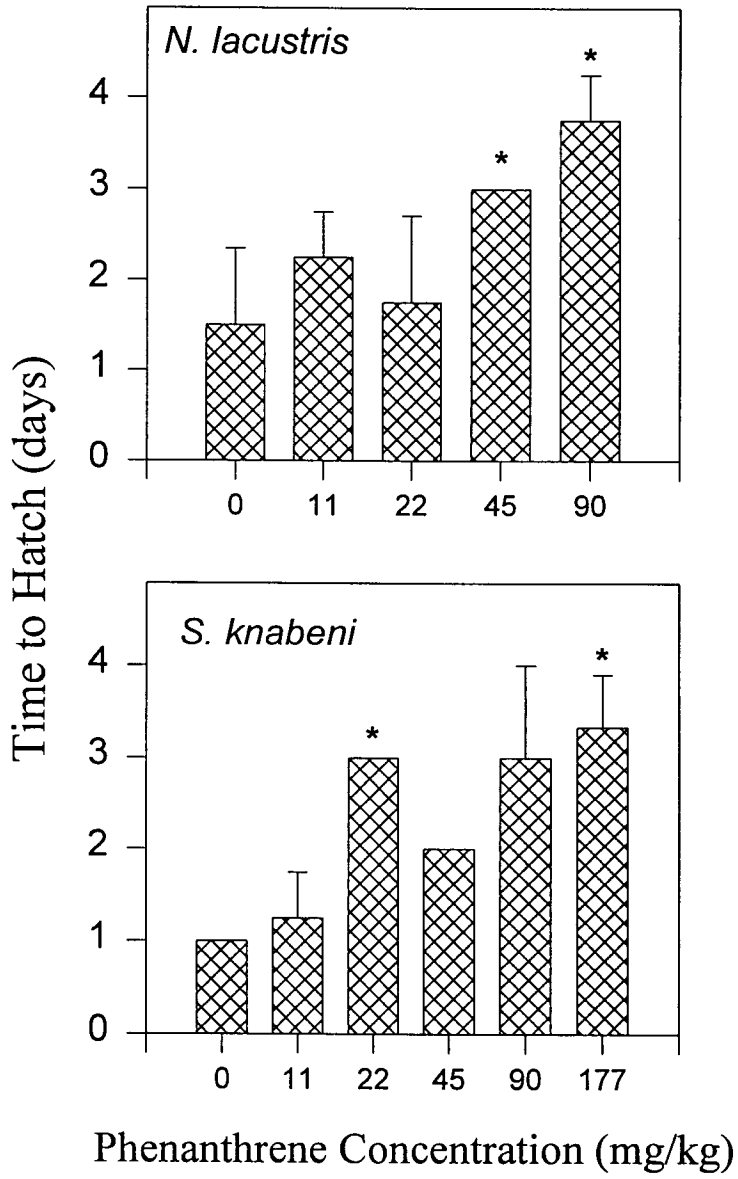

Figure 3.9. Time to hatch for eggs carried by *Nitocra lacustris* and *Schizopera knabeni* ovigerous females obtained in the 10-d copepodite bioassay. Time is expressed in days from the beginning of the observation period. Error bars show 1 SD of the mean ($n = 4$); * represents significance difference ($\alpha = 0.05$) from control mean

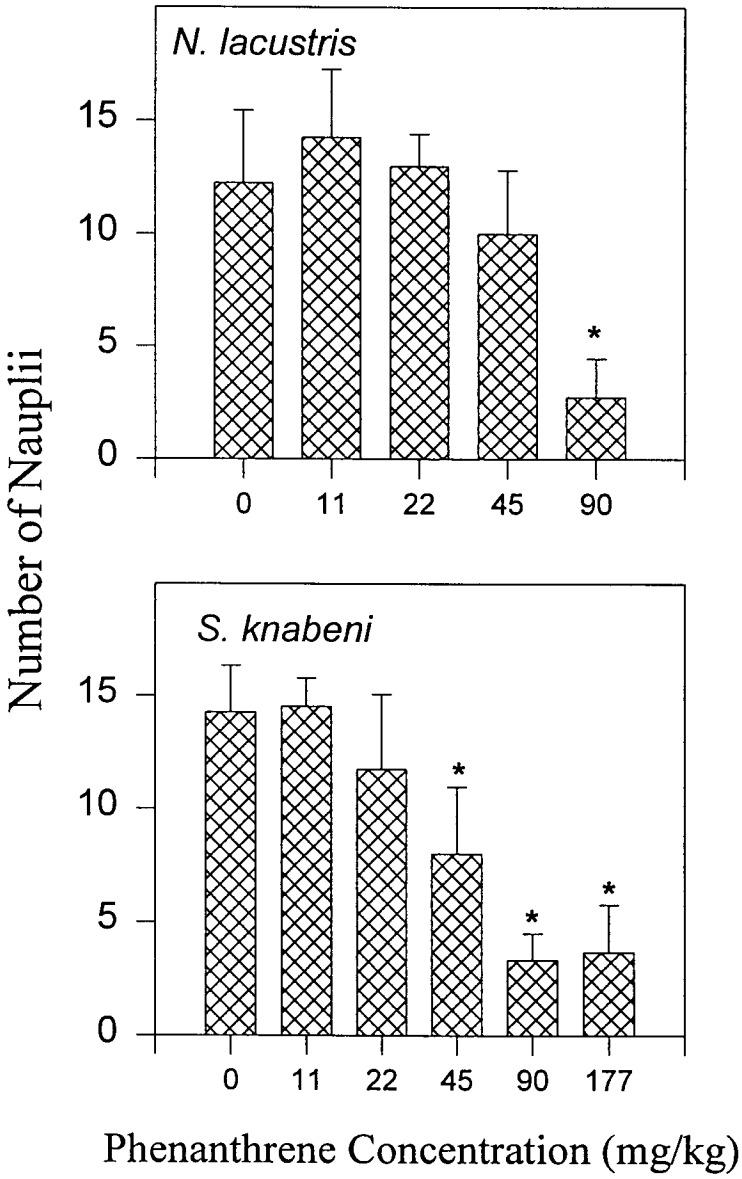

Figure 3.10. Hatching success of *Nitocra lacustris* and *Schizopera knabeni* ovigerous females obtained in the 10-d copepodite bioassay. Hatching success is expressed as the number of nauplii hatching per female. Error bar show 1 SD of the mean (n = 4); * represents significance difference (α + 0.05) from control mean.

CHAPTER 4. DISCUSSION

Diesel fuel was more toxic to *S. knabeni* in sediment exposures than the PAH congeners phenanthrene and fluoranthene. The higher toxicity of diesel fuel was probably due to the elevated concentration of naphthalene and its alkyl derivatives (Carman *et al.*,1996). 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, 1985). However, the effects of PAH are generally expected to be additive (Swartz *et al.* (1995), and predictions of PAH effects are often based on total PAH levels (Long *et al.*, 1995).

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. 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 (Bitton *et al.*, 1996).

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.

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 (Capuzzo *et al.*, 1984) and phenanthrene (Laughlin and Neff, 1979), and appear to be related to the molting process in crustaceans. Copepod naupliar stages were consistently the most sensitive in exposures to other contaminants (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 (DiPinto *et al.*, 1993; Carman and 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 (U.S.EPA, 1993), 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* (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.

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 LC_{50} 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. 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 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.

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 LC_{50}s

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 (r_m) under optimal conditions is therefore expected at relatively low PAH concentrations. Likewise, population decreases are therefore expected in field populations of benthic harpacticoids inhabiting sediments contaminated with PAH at levels typical for polluted estuaries; the concentrations of total PAH 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 although much higher values (100's μg/g) have been reported (Kennish, 1992). Total PAH concentrations in sediments near a produced water outfall in Pass Fourchon, Louisiana, have been reported to reach levels as high as 90 μg/g (Rabalais et al., 1991).

The results of this study suggest that for *S. knabeni* offspring production is the most sensitive life-cycle variable (IC_{25} = 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 (LC_{50} = 43 μg/g), followed by offspring production (IC_{25} = 40 μg/g), and egg hatching (18% reduction at 45 μg/g). These results suggest that species- and age class-specificity to contaminant effects will likely prove common among harpacticoid copepods, making predictions of contaminant effects tenuous.

The development of a useful toxicity test for harpacticoids is an important step to improved understanding of contaminant effects (Lotufo, 1996). Harpacticoids have been shown to be a group sensitive to contaminant effects as abundance, species diversity and genetic diversity may all be influenced (Peterson et al., 1996; Street and Montagna, 1996). A direct result of these tests and results provided here is better information for the development of sediment quality criteria. Also important, however, is the use of techniques (including culture techniques and bioassays) to determine the mechanisms behind contaminant effects. For example, a recent laboratory experiment using *Nitocra lacustris* suggested reductions in genetic diversity found at contaminated sites may be due to selective pressures associated with exposure to PAHs (Street at al., 1998). Finally, the potential also exists to determine ecotoxicological dynamics in copepods to better understand organismal/physiological responses to contaminants (Lotufo 1998a; b).

CHAPTER 5. LITERATURE CITED

Anderson, J.W. 1977. Responses to sublethal levels of petroleum hydrocarbons: are they sensitive indicators and do they correlate with tissue contamination? In *Fate and effects of petroleum hydrocarbons in marine organisms and ecosystems.* Wolfe D.A., ed. Pergamon Press, New York, NY, p. 95-114.

Bitton, G., Rhodes, K. and Koopman, B. 1996. Ceriofast (TM): An acute toxicity test based on *Ceriodaphnia dubia* feeding behavior-short communication. *Environ. Toxicol. Chem.,* 15: 123-125.

Burton, G.A., Nelson, M.K. and Ingersoll, C.G. 1992. Fresh water benthic toxicity tests. In *Sediment Toxicity Assessment.* Burton, G.A., ed. Lewis, Ann Arbor, MI, p. 213-240.

Capuzzo, J.M., Lancaster, B.A. and Sasaki, G.C. 1984. The effects of petroleum hydrocarbons on lipid metabolism and energetics of larval development and metamorphosis in the American Lobster (*Homarus americanus* Milne Edwards). *Mar. Environ. Res.,* 14: 201-228.

Carman, K.R., J.W. Fleeger, J.C. Means, S. Pomarico and D.J. McMillin. 1995. Experimental investigation of the effects of polynuclear aromatic hydrocarbons on an estuarine sediment food web. *Mar. Environ. Res.* 40: 289-318.

Carman, K. R., Means, J. C., Pomarico, S. M. 1996. Response of sedimentary bacteria in a Louisiana salt marsh to contamination by diesel fuel. *Aquat. Microb. Ecol.*, 10: 231-241.

Carman, K. R. and Todaro, M.A. (1996). Influence of polycyclic aromatic hydrocarbons on the meiobenthic-copepod community of a Louisiana salt marsh. *J. Exp. Mar. Biol. Ecol.*, 198: 37-54.

Chandler, G.T. 1990. Effects of sediment-bound residues of the pyrethroid insecticide fenvalerate on survival and reproduction of meiobenthic copepods. *Mar. Environ. Res.* 29: 65-76.

Chandler, G.T., Coull, B.C. and Davis, J.C. 1994. Sediment-phase and aqueous-phase fenvalerate effects on meiobenthos - implications for Sediment Quality Criteria development. *Mar. Environ. Res.,* 37: 313-327.

Coull, B.C. 1988. Ecology of the marine meiofauna. IN *Introduction to the Study of Meiofauna.* Higgins, R..P., Thiel, H., eds. Smithsonian Institution Press, Washington D.C., p. 18-38.

Coull, B.C. 1990. Are members of the meiofauna food for higher trophic levels? *Trans. Am. Microsc. Soc.,* 109: 233-246.

Coull, B.C. and Chandler, G.T. 1992. Pollution and meiofauna: Field, laboratory and mesocosm studies. *Oceanogr. Mar. Biol. Ann. Rev.,* 30: 191-271.

Decho, A.W. and J.W. Fleeger. 1988. Microscale dispersion of meiobenthic copepods in response to food-resource patchiness. *J. Exp. Mar. Biol. Ecol.* 118: 229-244.

DiPinto, L.M., Coull, B.C. and Chandler, G.T. 1993. Lethal and sublethal effects of the sediment-associated PCB aroclor 1254 on a meiobenthic copepod. *Environ. Toxicol. Chem.,* 12: 1909-1918.

Green, A.S., Chandler, G.T. and Piegorsch, W.W. 1996. Life stage specific toxicity of sediment associated chlorpyrifos to a marine, infaunal copepod. *Environ. Toxicol. Chem.,* 15: 1182-1188.

Kennish, M.J. 1992. Ecology of estuaries: Anthropogenic effects. *Ecology of Estuaries: Anthropogenic Effects*. CRC Press.

Kukkonen, J. and Landrum, P.F. 1994. Toxicokinetics and toxicity of sediment-associated pyrene to *Lumbriculus variegatus* (Oligochaeta). *Environ. Toxicol. Chem.,* 13(9): 1457-1468.

Landrum, P.F., Dupuis, W.S. and Kukkonen, J. 1994. Toxicokinetics and toxicity of sediment-associated pyrene and phenanthrene in *Diporeia* spp.: Examination of Equilibrium Partitioning Theory and residue-based effects for assessing hazard. *Environ. Toxicol. Chem.,* 13: 1769-1780.

Laughlin, R.B.J. and Neff, J.M. 1979. Interactive effects of salinity, temperature and polycyclic aromatic hydrocarbons on the survival and development rate of larvae of the mud crab *Rhithropanopeus harrisii. Mar. Biol.,* 53: 281-291.

Long, E.R., D. Macdonald, S.L. Smith and F.D. Calder. 1995. Incidence of adverse biological effects within ranges of chemical concentrations in marine and estuarine sediments. Environmental Management. 19: 81-97.

Lotufo, G.R. 1996. Toxicity of Polycyclic Aromatic Hydrocarbons to Benthic Organisms. Ph. D. Dissertation, Louisiana State University, Baton Rouge, LA. 180 pp.

U.S. Environmental Protection Agency. 1993. Sediment quality criteria for the protection of benthic organisms: Phenanthrene. EPA 822/R/93/014. Washington, DC.

Van Wezel, A.P. and Opperhuizen, A. 1995. Narcosis due to environmental pollutants in aquatic organisms: residue-based toxicity, mechanisms, and membrane burdens. *Critical Reviews in Toxicology.* 25: 255-279.

Wade, T.L., Atlas, E.L., Brooks, J.M., Kennicutt, M.C.I., Fox, R.G., Sericano, J., Garcia-Romero, B. and DeFreitas, D. 1988. NOAA Gulf of Mexico status and trends program: trace organic contaminant distribution in sediments and oysters. Estuaries. 11: 171-179.

The Department of the Interior Mission

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

The Minerals Management Service Mission

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

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

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