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EFFECTS OF DIESEL-FUEL AND COPPER CONTAMINANTS ON BENTHIC MICROALGAE

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 Oceanography and Coastal Sciences

by

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ABSTRACT

Salt marshes are dynamic, highly productive habitats and serve as nursery grounds for many commercially and economically important species. Benthic microalgae (BMA) are considered an important food source for benthos and provide the principal source of nutrition that fuels secondary production. Estuarine sediments around the world are a repository for many contaminants from anthropogenic sources. In particular, hydrocarbons and metals are ubiquitous contaminants in coastal systems. The primary goal of this study was to study the effects of diesel fuel and copper, alone and in combination, on the BMA assemblage from a coastal salt marsh. To achieve this objective, salt marsh sediments were exposed to various combinations of diesel fuel and copper during a 20-day microcosm experiment. Response of the BMA assemblage was assessed based on the analysis of photosynthetic pigments, species composition, biomass, and production of carbohydrates. Through use of a cryopreservation technique, these variables were measured in the top 2 mm of sediment at 200-μm vertical intervals. This experiment is the first attempt to measure the influence of contaminants on BMA with such high resolution. Hydrocarbons caused significant indirect effects on the BMA assemblage, such as blooms of large diatom species (*Bacillaria paxillifer*), and diesel combined with copper induced responses that differed from the effects of either contaminant alone (e.g., the stimulation of diatoms was reduced). Diversity of the diatom assemblage was reduced in the presence of diesel, and copper significantly reduced species richness. Contaminants significantly influenced water-extractable but not EDTA-extractable carbohydrate concentrations. The effect of sediments contaminated with phenanthrene and Cd was evaluated in relation to its influence on the feeding activity of a

harpacticoid copepod, *Schizopera knabeni*. My results suggest that there was no interactive effect on feeding when Cd and phenanthrene are combined. When other metals were added (Pb and Hg) to that mixture, an additive influence on feeding rate was observed.

Collectively, my results indicate that the BMA assemblage is significantly affected by diesel and copper. Contaminant effects influence BMA structure and distribution within the top 2 mm of sediment, which may have important implications for the biological and physical properties of marsh sediment.

CHAPTER 1

GENERAL INTRODUCTION

Benthic microalgae (BMA), also called microphytobenthos, are composed of several algal classes (Bacillariophyceae, Chlorophyceae, Cyanobacteria, Euglenophyceae). BMA assemblages are found in the top few millimeters of intertidal sediment in estuarine and coastal systems (Kelly et al., 2001). As the penetration of light is largely limited to the upper 0.2-2 mm, the distribution of BMA is restricted to this relatively thin surface layer (Wolff, 1979; MacIntyre et al., 1996). Many BMA are known for their ability to migrate vertically, moving to and away from the surface in response to factors such as light, tide cycles, desiccation, predation or resuspension (Admiraal et al., 1984; Pinckney and Zingmark, 1991; Paterson et al., 1998).

The main constituent of the BMA assemblage in salt marsh sediments are the motile pennate diatoms (Admiraal, 1984; Underwood & Krompkamp, 1999), particularly those species belonging to the genera *Navicula*, *Nitzschia* and *Amphora* (Drum and Webber; 1966, Sullivan, 1975, 1977, 1978). Diatoms are unicellular phototrophic algae that are characterized by a silica frustule enveloping the protoplast.

Microalgae residing within or associated with intertidal sediment are termed 'edaphic' (Sullivan and Currin, 2000). Edaphic BMA can be artificially divided into the episammic microalgae (attached more or less firmly to sand grains with motility absent or highly reduced) and epipelic microalgae (which migrate up and down within intertidal sediments in response to irradiance and tidal rhythms) (Sullivan and Currin, 2000).

Microphytobenthic diatom populations are usually composed of pennate, prostrate forms that are either episammic or epipelic (Daehnick et al., 1992; Moncreiff et al., 1992; Agatz et al., 1999; Mitbavkar and Anil, 2002). Episammic diatoms are araphid, monoraphid, or biraphid pennales or centric species of a small size that are attached to sediment particles by mucilaginous pads or stalks. The epipelic forms are biraphid pennates species that actively move through the

sediment by means of the mucilaginous secretion through their raphes (Round, 1971).

BMA have great ecological significance in estuarine sediments despite inhabiting sediments with a narrow photic zone (few mm) (Consalvey, 2004), limited periods of light exposure (Serodio and Catarino, 1999), and highly variable and potentially stressful temperatures and irradiances (Blanchard and Guarini, 1996; Perkins et al., 2001; Underwood, 2002). BMA can also influence the microstructure and properties of sediment, due to the secretion of extracellular polymeric substances (EPS) (Decho, 1990; Underwood et al., 1995), which may increase sediment stability (Paterson, 1988, 1997; Underwood, 1997; Sutherland, 1998).

Coastal sediments are complex habitats and besides the BMA, they also support many invertebrate species. The sediment dwellers can be classified according to their size ranges as proposed by Plante-Cuny and Plante (1984) into microfauna (ciliates), meiofauna (harpacticoid copepods, nematodes, ostracods) and macrofauna (mainly amphipods, isopods, gastropods, polychaetes). In addition to detritus and bacteria, BMA provide the principal source of nutrition that fuels secondary production in shallow coastal systems (Sullivan and Moncreiff, 1990; Moncreiff and Sullivan, 2001) and can contribute up to 50% of the total primary production in estuarine systems (Sullivan and Moncreiff, 1988; Cahoon, 1999; Underwood and Kromkamp, 1999).

Estuarine sediments around the world are a repository for many contaminants from anthropogenic sources. One of the main pollutants in marine environments is petroleum hydrocarbons (National Research Council, 1985). Among petroleum hydrocarbons, diesel-fuel is considered to be highly toxic because it is enriched in polycyclic aromatic hydrocarbons (PAH; National Toxicology program, 1986), the most toxic component of petroleum hydrocarbons (Clark, 1989; Kennish, 1992). Much of the high molecular weight PAH released

into aquatic environments accumulates in estuarine sediments (Kennish, 1992), and is resistant to bacterial metabolism (Clark, 1989). PAH becomes associated with suspended fine hydrophobic particles that are ultimately deposited in the benthos (Connell and Miller, 1984; Readman et al., 1984; Ko and Baker, 1995). In the long-term, estuarine sediment that contains large pools of organic matter can be a significant repository for PAH (Pereira et al., 1999).

Information about the direct effects of hydrocarbons on BMA is scarce. Most of the information comes from studies on phytoplankton. In aquatic environments, algal species exhibit differential sensitivity to toxic pollutants and petroleum hydrocarbons have been shown to be inhibitory to the growth of some sensitive species while promoting the growth of tolerant ones (Morales-Loo and Goutz, 1990), including nuisance algal species (Capone and Bauer, 1992). The response of some green algae to hydrocarbons has been examined in some detail. For example, the exposure of *Selenastrum capricornutum* to fuel oil water extracts and aromatic hydrocarbons significantly decreased the carbohydrate and protein content of algal cells (El-Dib et al., 1997; Herman et al., 1991).

When pollutants are released into aquatic environments, direct toxic effects on aquatic biota are possible (Fleeger et al., 2003). Direct toxic effects vary with the intensity and duration of exposure to a specific toxicant, but typically result in mortality and reduced abundance of species. Indirect effects of contaminants on a natural community occur when contaminant-tolerant species are influenced by the ecological changes that result when some species suffer direct toxic effects (Paterson, 2001). Single-species, laboratory-based toxicity tests can not detect indirect effects (Fleeger et al., 2003). Trophic cascades (indirect effects mediated through consumer-resource interaction) are a well studied type of indirect effect (Pace et al., 1999), and are generally considered in terms of "top-down" (predator influence on lower trophic levels) and

"bottom-up" (nutrient/food/prey influence on higher trophic levels) causes (Fleeger et al., 2003). Microalgal blooms in hydrocarbon-contaminated sediment have been interpreted as an indirect effect that is the result of mortality of meiofaunal grazers (Carman et al., 1997, 2000).

Heavy metals are another main group of contaminants in coastal sediments. Among them, copper represents one of the more prevalent and toxic metals (Babu et al., 2001). All algae have a copper micronutrient requirement for growth and reproduction; however, excess copper is highly toxic to most algae because of its oxidative potential (Joux-Arab et al., 2000). By binding with sulfhydryl groups, copper inhibits a large number of enzymes involved in photosynthetic carbon fixation, chlorophyll synthesis, electron transport, and photophosphorylation (Fisher et al., 1981; Rijstenbil et al., 1994).

Little is known about the ecotoxicological interaction of copper and diesel-fuel, and their joint-effects on BMA. Millward et al. (2004) studied the joint effects of heavy metals and diesel contamination on a salt-marsh benthic invertebrate community. They concluded that it is difficult to predict the combined effects of metals and diesel contamination on benthic communities based on knowledge of the effects of metals or diesel alone. Metals and hydrocarbon contamination in aquatic sediments could significantly affect BMA by changing their abundance and distribution and are likely to have significant effects on benthic food webs in coastal systems because of the important role of BMA as a food source for many benthic invertebrates. Therefore, effects of contaminants on BMA communities have potentially major implications for the food web that these primary producers support.

My dissertation research involved an evaluation of the influence of copper and diesel fuel on a salt marsh BMA community. Microcosms of a natural marsh community were exposed to contaminants and the response of BMA was determined using various

approaches. The fine-scale (μm) vertical distribution of photosynthetic pigments was measured using High Performance Liquid Chromatography (HPLC). Meiofaunal abundances were also examined and compared in conjunction with the photosynthetic pigments (Chapter 2).

In order to elucidate possible effects of diesel- and copper-contaminated sediment, alone and in combination, on the BMA assemblage, a taxonomic analysis was performed on the predominant component of the BMA community, the benthic diatoms (Chapter 3).

Benthic pennate diatoms are motile and can migrate vertically through the sediment because they excrete extracellular polysaccharides (EPS) (Cahoon, 1999; Stal, 2003). Therefore, In Chapter 4, the effects of contaminated sediment on the BMA biomass and the production of carbohydrates were evaluated. Two operational fractions were extracted from the samples in order to determine if contaminated sediment have an influence on their production and distribution.

Several experiments were performed to determine the effect of contaminated sediment on the feeding activity of a harpacticoid copepod (Chapter 5). Grazing rates of *Schizopera knabeni* were analyzed after exposing it to sub-lethal concentrations of phenanthrene- and Cd-contaminated sediment.

A final section (Chapter 6) summarizes the overall results and a final summary is presented.

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CHAPTER 2

BENTHIC MICROALGAL AND MEIOFAUNAL RESPONSE TO CONTAMINATED SEDIMENTS

INTRODUCTION

Benthic microalgae (BMA) play an important role in the dynamics of shallow coastal systems. BMA provide the principal source of nutrition that fuels secondary production (Sullivan and Moncreiff, 1990) and can contribute up to 50% of the total primary production in estuarine systems (Sullivan and Moncreiff, 1988; Cahoon, 1999; Underwood and Kromkamp, 1999).

Algal groups have characteristic pigments and certain assemblages occurring in different water bodies and/or sediments can be differentiated on the basis of their pigment biomarkers (Wiltshire et al., 1998). The determination of pigment biomarkers using High Performance Liquid Chromatography (HPLC, Gieskes and Kraay, 1983; Wright and Jeffrey, 1987) has been used to study the composition of assemblages of primary producers in aquatic systems. Recent studies have provided significant ecological information based on BMA pigment analyses by HPLC, allowing the estimation of both biomass, using Chlorophyll *a* (Chl *a*) concentration, as well as using accessory pigments to determine taxonomic composition (Wilthshire, 1992; Cariou and Blanchard, 1995; Brotas and Plante-Cuny, 1998; Lucas and Holligan, 1999; Buffan-Dubau and Carman, 2000; Cartaxana et al., 2003). For example, the carotenoid fucoxanthin is a biomarker for the presence of diatoms, while zeaxanthin is a biomarker for cyanobacteria (Wright et al., 1991; Bianchi et al., 1996).

Pheopigments, such as pheophytins and pheophorbides, are the degradation products of chlorophylls; pheophorbides have been used as markers for metazoan grazing (Schuman and Lorenzen, 1975; Welschmeyer and Lorenzen, 1985; Bianchi et al., 1988, 1995; Bennett et al., 1999). Thus, BMA pigments can be used to determine the composition and

heterotrophic consumption of benthic algal sources in shallow coastal systems.

Estuarine sediments around the world are a repository for many contaminants from anthropogenic sources. One of the main pollutants in marine environments is petroleum hydrocarbons (National Research Council, 1985). Among petroleum hydrocarbons, diesel-fuel is considered to be highly toxic because it is enriched in polycyclic aromatic hydrocarbons (PAH; National Toxicology program, 1986), the most toxic component of petroleum hydrocarbons (Clark, 1989; Kennish, 1992). Heavy metals are another main class of contaminants in coastal sediments. Among them copper represents one of the more prevalent and toxic of the heavy metals (Babu et al., 2001). All algae have a copper micronutrient requirement for growth and reproduction; however, excess copper is highly toxic to most algae because of its oxidative potential (Joux-Arab et al., 2000).

There is evidence that pigments can be used as an indicator of contaminant influence on BMA community structure (Bennett et al., 1999). Carman et al. (1997) reported that diesel contamination caused an increase in the Chl *a*: pheopigment ratio, which is indicative of reduced grazing pressure. Barranguet et al. (2003) showed that copper caused a significant reduction of microalgal biomass (Chl *a*) and induced a shift in the population from diatoms (fucoxanthin) to cyanobacteria (zeaxanthin).

Little is known about the ecotoxicological interaction of copper and diesel-fuel, and their joint-effects on BMA community structure. Metals and hydrocarbon contamination in aquatic sediments could significantly affect BMA by changing their community structure and are likely to have significant effects on benthic food webs in coastal systems because of the important role of BMA as a food source for many benthic invertebrates. Therefore, the effects of contaminants on BMA communities have potentially major implications for

the ecosystems that these primary producers support. Previous studies of the effects of hydrocarbon-contaminated sediments in microcosms and field settings indicate that PAHs can reduce diversity, abundance and feeding activity of meiofauna, but that these effects are dependent on taxa, and dosage of the hydrocarbon (Carman et al., 1995; Carman and Todaro, 1996; Carman et al., 1997; Millward et al., 2004).

The overall hypothesis of this study was that copper, diesel and their combination alter the community structure and vertical distribution of BMA, and that the interaction between copper and diesel causes different effects than the effects of each contaminant individually. Thus, the aim of this study was to examine the influence of diesel-fuel and copper contaminants on the fine-scale distribution of the BMA assemblage from a Louisiana salt marsh. The effects of those contaminants on the meiofaunal community were also studied. Here, I report the influence of contaminants on a benthic community structure through the analysis of BMA pigments and meiofaunal abundance. Combinations of copper and diesel were employed to determine if meiofaunal abundance and pigment concentrations and distributions were different in diesel + copper contaminated sediments from those in copper or diesel alone.

MATERIALS AND METHODS

Study Site

Microcosms of natural sediment communities (Carman et al., 1995, 1997, 2000) were collected from mudflats in a *Spartina alterniflora* saltmarsh, located in the Terrebone Bay estuary near the Louisiana University Marine Consortium in Cocodrie, Louisiana to examine the influence of diesel and copper contaminants on benthic microalgae (BMA), The tidal range in the estuary is approximately 0.3 m and its salinity ranges from 1 to 20

psu. Sediments have a median grain size of 38 μm, an organic carbon content of approximately 2.5%, and are composed primarily of silts (41%) and clays (17%) (Chandler and Fleeger, 1983). The study site is located in a region of hydrocarbon production and drilling activity, where both commercial and recreational boat traffic is high (Carman et al., 1997).

Experimental Design

The microcosm experiment was designed to study the effects of two concentrations of both copper and diesel, individually and in combination. Twenty-seven cylindrical sediment microcosms (15.2 cm i.d. polyvinyl chloride pipe with windows cut in the sides above sediment level and covered with Nitex 63-μm mesh to allow exchange of water; Carman et al. (1995) were collected from exposed sediment to a depth of 15 cm at low tide on 22 May 2002. The microcosms were transferred to wet tables with circulating water and flooded with 2 to 3 cm of ambient marsh water, which was replaced daily to excess with filtered (63 μm) marsh water. Each core was aerated gently using an aquarium air stone. The temperature was maintained at 25 ± 2 °C, and the microcosms were illuminated (70 μmol $m^{-2}s^{-1}$) on a 14:10 light: dark cycle with banks of 40-W fluorescent lights.

Treatments included a Control (no contaminants added), Low Diesel (LD), High Diesel (HD), Low Copper (LC), High Copper (HC), Low diesel-High copper (LD-HC), Low Diesel-Low Copper (LD-LC), High Diesel-High Copper (HD-HC) and High Diesel-Low copper (HD-LC). Treatment concentrations were based upon levels known to produce sub-lethal and lethal responses in benthic communities (Carman et al., 1997; Millward et al., 2001). Three replicates were used per treatment.

Preparation of Contaminated Sediment

Surface sediment was collected from the marsh and processed following the procedure described by Chandler (1986), which yields sterile sediment particles < 63 μm. The resulting sediment did not contain any diatoms. This sediment was then contaminated with diesel (Carman et al., 1996, 1997) and copper (Millward et al., 2001). Diesel fuel was added to a sediment aliquot (1 part diesel to 3 parts sediment v:v) in an amber glass bottle and tumbled on a roller mill for 10 days. The sediment was then allowed to settle overnight. Unadsorbed diesel was aspirated from the bottle and 12 ppt artificial sea water (ASW) was added. This rinsing procedure was repeated three times. The sediment-water slurry was transferred to 35-mL glass centrifuge tubes and centrifuged at 1700 g for 3 min. The supernatant was removed and replaced with fresh ASW. The sediment and water were mixed thoroughly, and then centrifuged. The supernatant was decanted again, and the process was repeated for a total of four rinses. The sediment was then recombined into a single batch and mixed to assure homogeneity. This sediment was used as the high diesel (HD) spike. A sub sample was diluted to one third of this concentration (1 part high diesel to 2 parts control sediment v:v) to produce the low diesel (LD) spiked sediment. Copper-contaminated sediments were prepared by adding the appropriate volume of a copper solution to control sediment and diesel-contaminated sediment. The copper solution was added to sediment over a period of 2 h using a dripping gravity-feed apparatus while mixing vigorously with a food blender. In this way, a homogeneous mixture of copper, within the spiking sediment, was obtained. The copper solution was added to the control and diesel-contaminated sediment in order to produce copper-amended sediment that were 10x (HC) and 5x (LC) of copper concentrations in San Diego Harbor (219 mg Kg dw^{-1},

Kennish, 1992).

Experimental Procedure

The experimental treatments consisted of the initial addition of 25 mL of contaminated sediment to the overlying water in each microcosm. An additional 2.5 mL of sediment was added each day until the end of the study (20 days). Uncontaminated sediment was added to Control microcosms. These sediment additions created a sediment layer approximately 1 mm thick, which was quickly (within a few hours) mixed into the top few mm of sediment by bioturbation of benthic fauna.

Samples were collected on days 0, 5, and 20 during the day. Sediment cores were collected using the cryolander coring method (Wiltshire et al., 1997). The cryolander was placed on the surface of the sediment, and approximately 10 mL liquid nitrogen was gently poured onto absorbent cotton. The cotton was at ambient temperature, which caused the liquid nitrogen to vaporize. The cold vapor sank through the gauze and froze the sediment surface without physical distortion. After approximately 3 min of freezing under nitrogen vapor, additional liquid nitrogen was added and the sediment was allowed to freeze for approximately 6 min. Frozen sediment samples were removed, wrapped in aluminum foil, and stored in a –80 °C freezer. Prior to sectioning, the cryolander samples were cut into pieces of approximately 1 cm^2 using a lapidary saw. Subsequently, a microtome (Cryo-Cut, American Optical Company) was used to section the top 1 mm of sediment into five 200-μm layers and the second 1 mm into 2 layers of 500 μm each. Samples were lyophilized prior to analysis of pigments.

In addition, separate core subsamples were collected for the analysis of concentrations of total sediment-associated copper and total petroleum hydrocarbons

(TPHs; 3.5 cm i.d. cores) in the top 1 cm sediment surface.

Pigment Analysis

Photosynthetic pigments were extracted by sonicating each sample for 30 s in 1.3 mL 100% HPLC acetone (Fisher Scientific), bubbled with nitrogen gas (to remove oxygen), and incubated overnight in the dark at –4 °C. The extracts were centrifuged for 10 min at 1500 g, filtered twice using syringe filters (Sun International: diameter 13 mm, pore size 0.2 μm) and stored in the dark at –80 °C.

The pigment extracts were analyzed using a Hewlett Packard 1100 High Performance Liquid Chromatography (HPLC), consisting of a 100-μL loop autosampler, a quaternary solvent delivery system coupled to a diode array spectrophotometer, and a Hewlett Packard 1046A fluorescent detector. The diode array detector was set at 436 nm for detection of carotenoid and chlorophyll pigments, and at 405 nm for detection of Pheopigments (Wright et al., 1991). The separation of pigments was performed by reverse-phase liquid chromatography using a C18, 5-μm column (250 mm x 4.6 mm i.d.) coupled to a guard column. Data analysis was performed using Hewlett Packard HPChemStation software.

Meiofauna Abundance

At the beginning (Day 0) and end of the study (Day 20), core samples were collected from microcosms for meiofaunal abundances (2.5 cm i.d. cores). Meiofauna were identified to the level of major taxa, and copepods were identified to species.

Copper Concentrations

One gram of dry, finely ground sediment was digested by refluxing with 5 mL trace-metal grade concentrated HNO_3 at 120 °C for 6 h. Following digestion, the HNO_3 volume was

decreased to1.5 mL and samples were diluted with deionized water to 35 mL. Samples were then shaken, allowed to settle for 24 h and the supernatant analyzed on an ICP-OES (Inductively Coupled Plasma, Optical Emission Spectrometer).

Hydrocarbon Concentrations

Hydrocarbons were extracted using EPA standard method 3550C (US EPA, 1996). Thirty grams of sample was spiked with a surrogate mixture (d_{14}-terphenyl, 5α-androstane, d_{10}-phenanthrene) to assess extraction efficiency, dried with anhydrous Na_aSO_4 and sonicated three times in excess dichloromethane for 15 min. Combined extracts were filtered and reduced to 1 mL. TPHs were analyzed with Gas Chromatography using a Flame Ionization Detector (GC/FID) and using EPA standard method 8015C (US EPA, 1996). Gas chromatography was performed using a Hewlett Packard 5890 GC.

Statistical Analysis

Statistical analysis was performed on log (n+1)-transformed data when necessary to fulfill requirements for parametric analysis. Normal probability plots were examined and data were subjected to Levene's test of homogeneity of variance to determine if data were consistent with the assumptions of ANOVA. Non-parametric ANOVA were performed when the assumptions were not fulfilled after transformation of data. For BMA, a Proc Mixed procedure was used to perform an analysis of variance, using a Split-Plot design with a 3 x 3 x 3 x 7 factorial arrangement and all remaining interactions, followed by Tukey's posteriori comparisons. Treatments (Diesel and Copper), and layer were treated as fixed factors while replicate was treated as a random factor. Comparisons between treatments were performed using depth-integrated biomass and carbohydrate data, unless stated otherwise. One-way ANOVA was used to determine if microalgal pigments differed

among layers within each treatment. Major meiofauna taxa and copepods were analyzed using a one-way ANOVA across all treatments, and Tukey's a posteriori comparison test for comparison of treatment effects. All statistical analyses were performed using SAS 9.1 software.

RESULTS

Contaminant Concentrations

The final concentration of TPHs in the HD contaminated sediment spike was 293 ± 1.0 mg kg sediment^{-1}, and for the LD contaminated sediment spike it was 179 ± 6.6 mg kg sediment $^{-1}$. Contaminated sediment was added to microcosms with the objective of achieving a final concentration in the top 1 cm of sediment of 29 and 18 mg kg sediment^{-1} at the beginning of the experiment. The background concentration of TPHs in the sediment, where the microcosms were collected, was 0.27 ± 0.12 mg kg sediment^{-1}. After 20 days, the concentrations of TPHs were lower than those at the beginning of the experiment. TPHs in the HD and LD combined with copper did not differ significantly from the HD and LD treatments alone (Fig. 2.1, $p = 0.10$).

The background concentration of copper in the Control sediment at day 0 was 44 ± 5 mg kg sediment^{-1}. At the beginning of the experiment the nominal concentrations of copper in the top 1 cm of sediment were 2190 mg kg sediment^{-1} for the HC and 1095 mg kg sediment^{-1} for the LC treatments. After 20 days, copper concentrations in the HC and LC treatments did not differ significantly from HDHC, LDHC, and HDLC and LDLC treatments, respectively (Fig 2.1, $p > 0.22$).

Day 0

Among the chlorophylls, Chl *a* was detected in the sediment. On day 0, Chl *a*

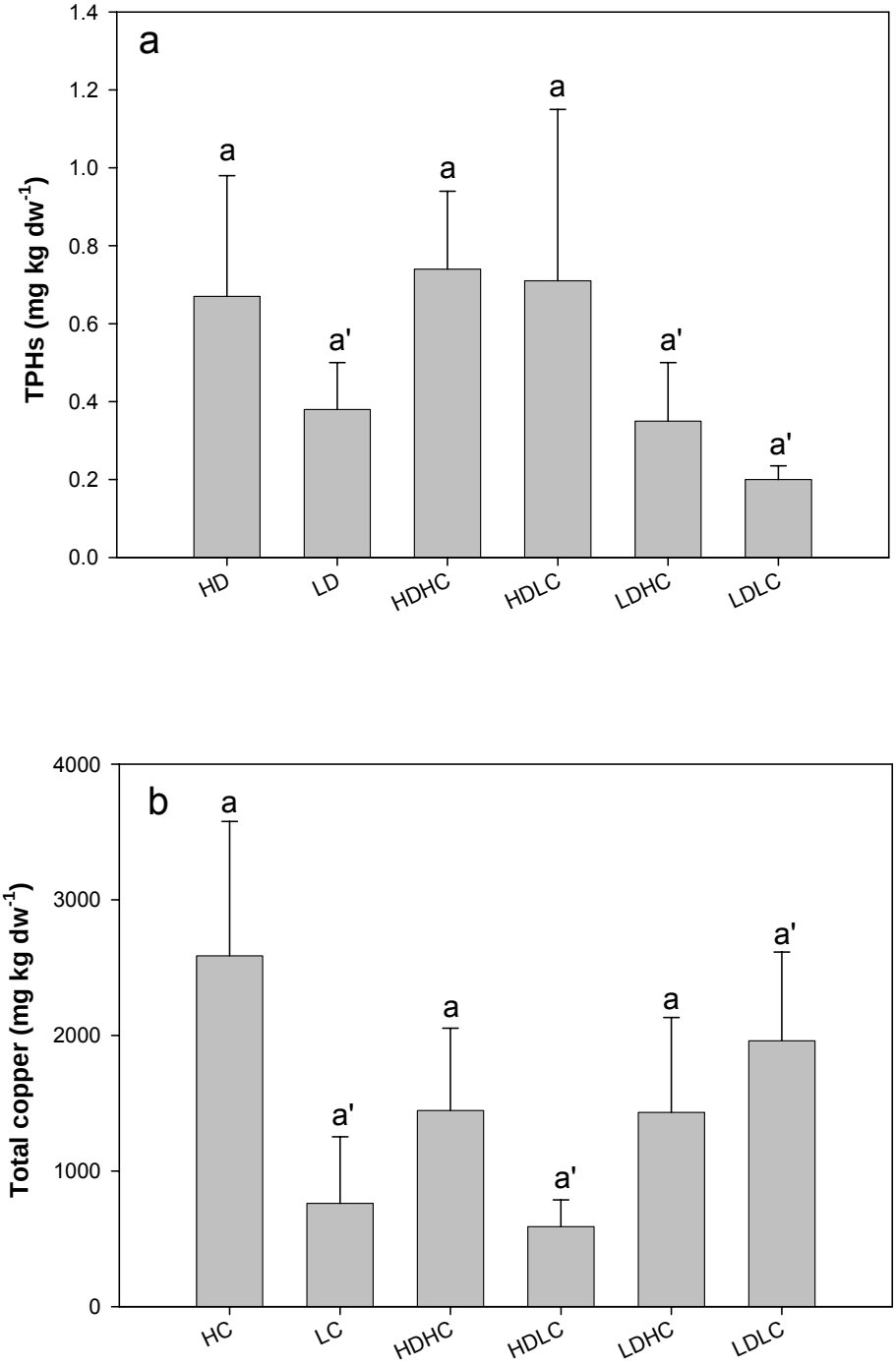


Figure 2.1. Total concentrations of petroleum hydrocarbons (TPH) (a) and copper (b) in the top 1 cm of surface sediment after 20 days. Bars represent mean + SD (n = 3).

concentration in the surface 2 mm of sediment was 231.3 ± 129.0 (SD) µg.g dry sediment^{-1} (Fig. 2.2). The accessory pigments fucoxanthin, zeaxanthin, lutein, diadinoxanthin diatoxanthin and β-carotene were also detected. Fucoxanthin was the dominant accessory pigment, and other carotenoid pigments were present in very low concentrations (Fig. 2.2). β-carotene is a carotenoid pigment that is ubiquitous in all algae. Although β-carotene was present in low concentrations and located only in the top layer (200 µm) on day 0 it is one of the most abundant carotenoids and has a very important protective role against reactive oxygen species (oxidative stress) caused by metals (Woodall et al., 1997; Pinto et al., 2003). Fucoxanthin and diadinoxanthin are marker pigments for diatoms. While fucoxanthin is a major carotenoid pigment in diatoms, diadinoxanthin is a minor pigment and it is considered to be the "low light" pigment in the xanthophyll cycle (Wiltshire, 2000). Its presence in bottom layers of sediment could indicate the presence of diatoms in reduced light conditions.

The degradation products of Chl *a* detected in the sediment were pheophytin *a* (82.0 ± 40 (SD) µg.g dry sediment^{-1}) and pheophorbide *a* (2.37 ± 2.10 (SD) µg.g dry sediment^{-1}). (Fig. 2.3). Chlorophyllide *a* was not detected.

Although there was a trend of decreasing photopigment concentration with depth in the sediment, no significant differences were detected by ANOVA among different layers for any of the pigments found (Fig. 2.4, $p = 0.23$). The distribution of fucoxanthin was very similar to that of Chl *a*. Although there were no significant differences in the vertical distribution of degradation products of Chl *a*, pheophorbide was detected only in the very top layer of the sediment (200 µm). Pheophytin was more uniformly distributed throughout the top 2 mm of sediment surface (Fig. 2.5).

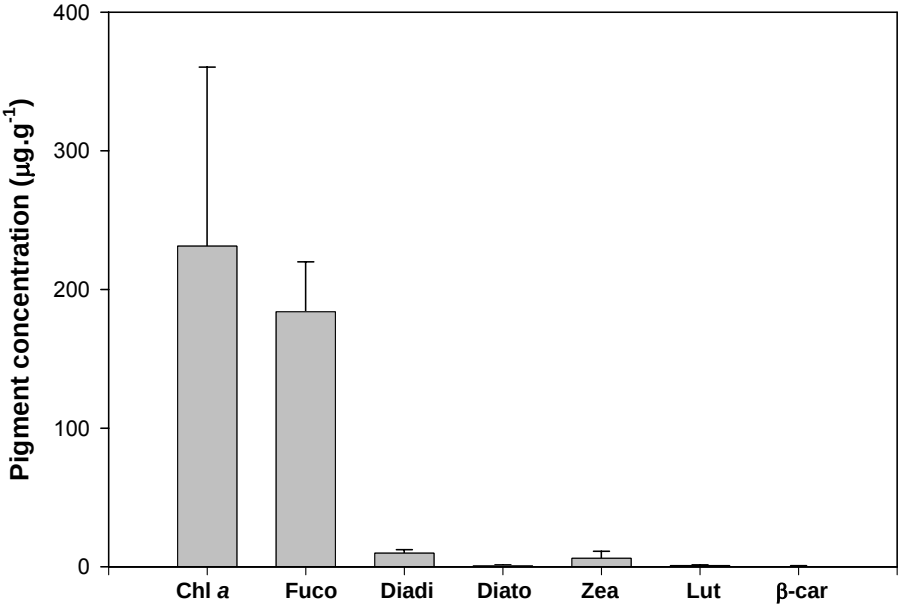


Figure 2.2. Concentrations of Chl *a* and carotenoid pigments detected in the top 2 mm of surface sediment on day 0. Bars represent means + SD (n = 3).

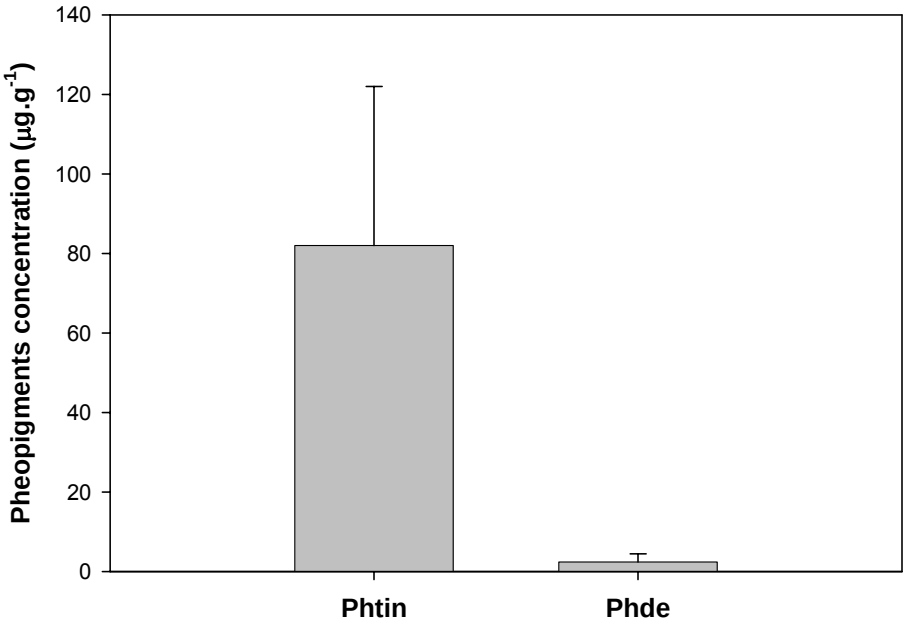


Figure 2.3. Pheopigments concentration detected in the top 2 mm of surface sediment on day 0. Phtin = pheophytin, Phde = pheophorbide. Bars represent means + SD (n = 3).

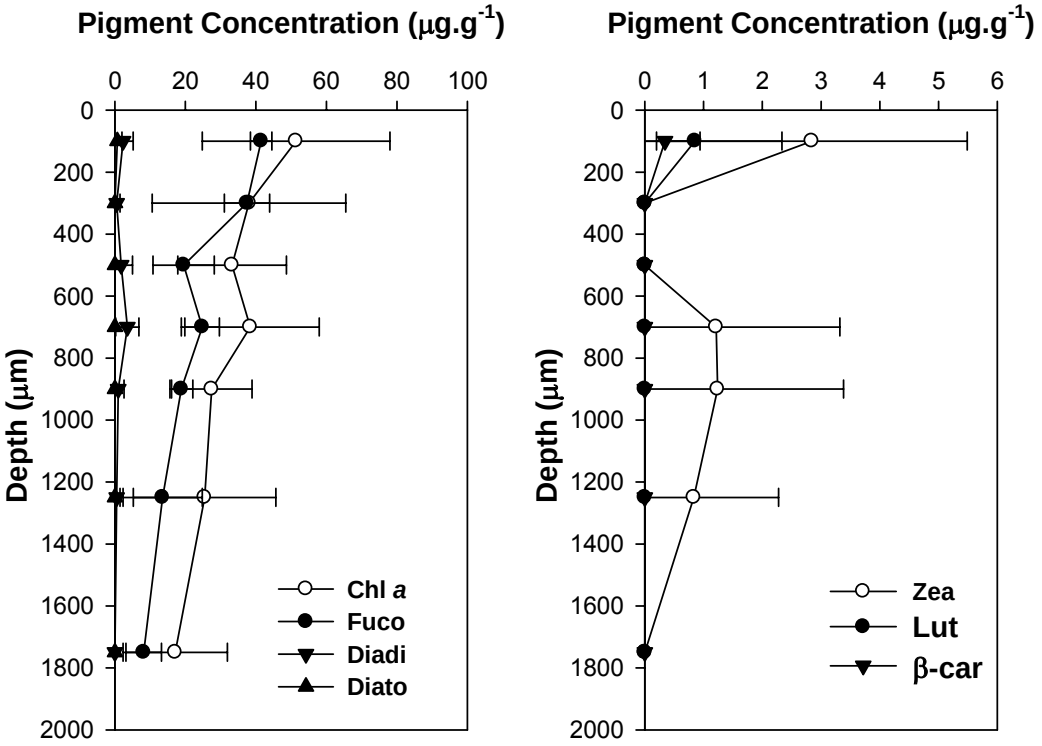


Figure 2.4. Depth profiles for Chl *a*, Fucoxanthin, Diadinoxanthin, Diatoxanthin, Zeaxanthin, Lutein and β-carotene on day 0. Fuco = Fucoxanthin, Diadi = Diadinoxanthin, Lut, = Lutein, Diato = Diatoxanthin, Zea = Zeaxanthin. Values are mean ± 1 SD (n = 3).

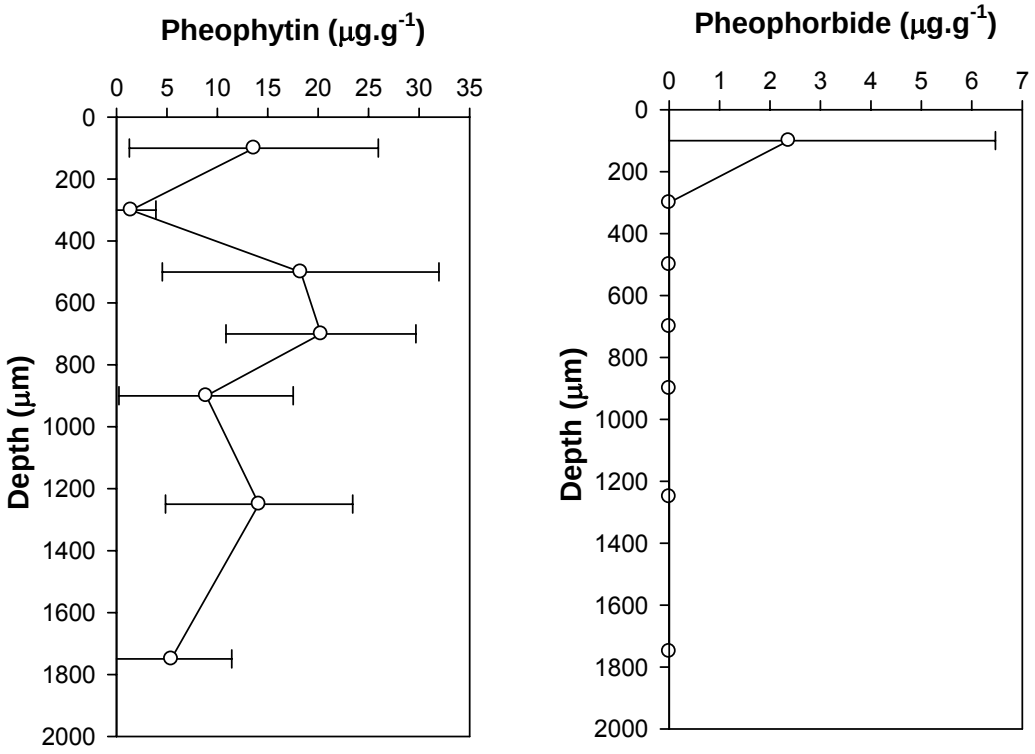


Figure 2.5. Pheopigment concentrations in the top 2 mm of sediment on day 0. Values are mean ± 1 SD (n = 3).

Meiofauna Abundance on Day 0

The meiofauna community at the beginning of the experiment included nematodes as the major group (35% of total), ostracods (31.5%), copepods (17%), nauplii (13.2%) and polychaetes, oligochaetes and chironomids (together, 3.7%). The main species of copepods found were *Enhydrosoma* sp.*, Coullana* sp., *Pseudostenhelia wellsi,* and *Halicyclops coulli.*

Day 5

When considering the entire depth profile, Chl *a* in the High Diesel (HD) treatment was significantly higher than all other treatments, including the Control ($p < 0.001$, Fig.2.6a), but Chl *a* in the LD treatment did not differ significantly from the Control ($p = 0.61$). The Chl *a* concentration in the HC and LC treatment were significantly lower than in the Control treatment (Fig. 2.6a, $p = 0.05$). Chl *a* concentration in the LD treatment did not differ significantly from LDHC and LDLC (Fig. 2.6a, $p = 0.56$). Chl *a* concentration in the HC treatment was significantly lower than in HDHC or LDHC treatments (Fig. 2.6a, $p = 0.58$). In contrast, the Chl *a* concentration in the LC treatment was not significantly different than that in HDLC or LDLC (Fig. 2.6a, $p = 0.25$).

Although fucoxanthin concentration in the HD treatment was higher than in the Control treatment, the difference was not significant (Fig. 2.6b, $p = 0.10$). However, the fucoxanthin concentration in the HD treatment was significantly higher than in HDHC and HDLC treatments (Fig. 2.6b, $p < 0.0001$). The fucoxanthin concentration in the LD treatment did not differ significantly from the Control, LDHC or LDLC treatments (Fig. 2.6b, $p > 0.10$). Fucoxanthin concentration in the HC treatment did not significantly differ from the concentrations in the HDHC and LDHC treatments (Fig 2.6b, $p > 0.26$). The fucoxanthin concentration in the LC treatment was significantly lower than in the LDLC

treatment ($p = 0.0004$), but did not differ significantly from the concentration in the HDLC treatment ($p = 0.15$). Fucoxanthin concentrations in the HC and LC treatments were significantly lower than in the Control treatment (Fig 2.6b, $p > 0.003$).

Diadinoxanthin was significantly higher in the HD treatment than in the Control ($p < 0.0001$), HDHC and HDLC treatments (Fig 2.6c, $p < 0.0017$). Diadinoxanthin in the LD treatment was also higher than in the Control ($p < 0.0001$), but did not differ significantly from the LDHC or the LDLC ($p = 0.88$). Diadinoxanthin concentration in the HC treatment did not differ significantly from the Control treatment ($p = 1.00$), but was significantly lower than in the HDHC treatment ($p < 0.0001$). Diadinoxanthin concentration was significantly lower in the LC treatment than in the HDLC and the LDLC treatments ($p < 0.0003$).

Zeaxanthin is a pigment marker of cyanobacteria. Zeaxanthin concentration was about an order of magnitude lower than fucoxanthin (Fig. 2.6d). Zeaxanthin in the HD treatment did not differ significantly from the Control ($p = 0.37$) or from the HDHC and HDLC treatments (Fig 2.6d, $p > 0.10$). In contrast, zeaxanthin in the LD treatment was significantly higher than in the Control ($p = 0.0001$), but zeaxanthin in the LD treatment did not differ significantly from the LDHC and LDLC treatments ($p = 0.27$). Thus, the presence of copper did not significantly influence the stimulatory effect of LD on zeaxanthin concentration. Zeaxanthin concentration in the HC treatment did not differ significantly from the HDHC (Fig 2.6d, $p = 0.30$) but zeaxanthin concentration was significantly lower than in LDHC treatment ($p < 0.001$). Zeaxanthin in the LC treatment did not significantly differ from HDLC ($p = 0.19$), but was significantly lower than in the LDLC treatment (Fig. 2.6d, $p < 0.0007$).

β-carotene concentration was not detected in the Control treatment and was significantly lower in the HC treatment than in the LDHC treatment (Fig. 2.6e, $p < 0.0001$). β-carotene concentration did not differ between the HC and HDHC treatments (Fig. 2.6e, $p = 0.18$). β-carotene in the LC treatment was significantly lower that in the LDLC treatment (Fig. 2.6e, $p < 0.0001$), but did not differ significantly from the HDLC treatment ($p = 0.08$). Thus, the presence of diesel in the LDHC and LDLC treatments diminished the inhibitory effect of HC and LC treatments alone. β-carotene concentration in the HD treatment was significantly higher that in the HDHC treatment ($p < 0.0001$), but did not differ significantly from the HDLC treatment (Fig. 2.6e, $p = 0.49$). β-carotene in the LD treatment did not differ significantly from the LDHC and LDLC treatments (Fig. 2.6e, $p > 0.42$).

The ratio of pheopigments to Chl *a* was used as an indirect and qualitative indication of the physiological or grazing state of a microalgal community (Struman and Lorenzen, 1975). Pheophytin:Chl *a* ratios on day 5 (Fig. 2.7) showed a significant decrease in the HD treatments compared with the Control treatment. However, that ratio in HD treatment did not differ significantly from other treatments (Fig. 2.7a, $p > 0.15$), with the exception of the LD treatment. Pheophytin:Chl *a* ratio in the HC treatment did not differ significantly from any treatment ($p = 0.65$). Although there were no significant differences in the Pheophorbide:Chl *a* ratios among all the treatments ($p = 0.21$), the highest ratio was in the HC treatment (Fig. 2.7b).

Vertical Distribution of Pigments

Significant differences in the vertical distribution of pigments within the top 2 mm of sediment were detected for both, Chl *a* and fucoxanthin in the HD treatment. In the

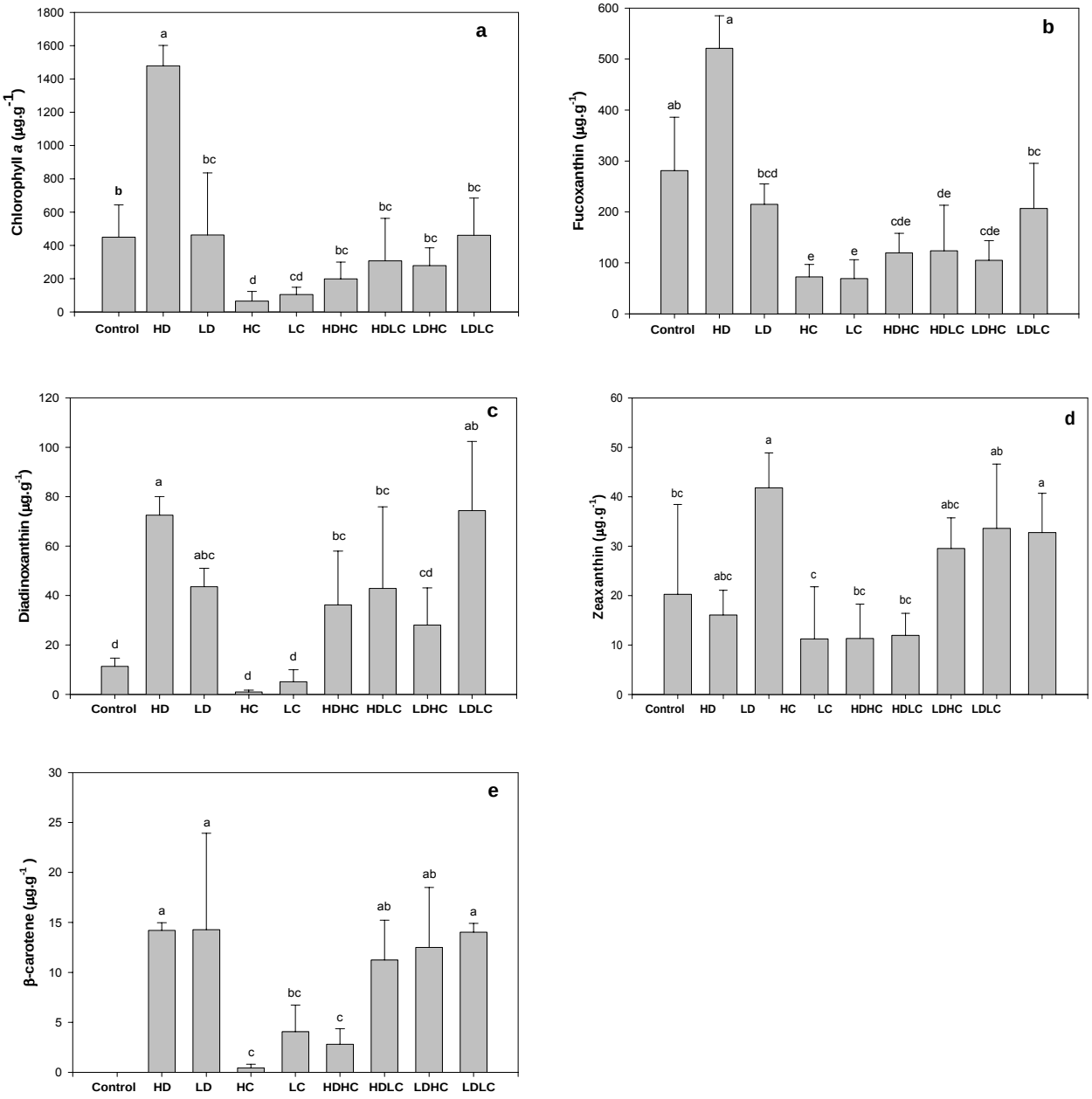


Figure 2.6. Chl *a* (a), fucoxanthin (b), diadinoxanthin (c) zeaxanthin (d) and β-carotene (e) concentration in the 2 mm of sediment surface on day 5. Values are mean + 1 SD (n = 3). Different letters indicate significant differences ($p < 0.05$). HD: High Diesel, LD = Low Diesel, HC = High Copper, LC = Low Copper, HDHC = High Diesel-High Copper, HDLC = High Diesel-Low Copper, LDHC = Low Diesel-High Copper and LDLC = Low Diesel-Low Copper.

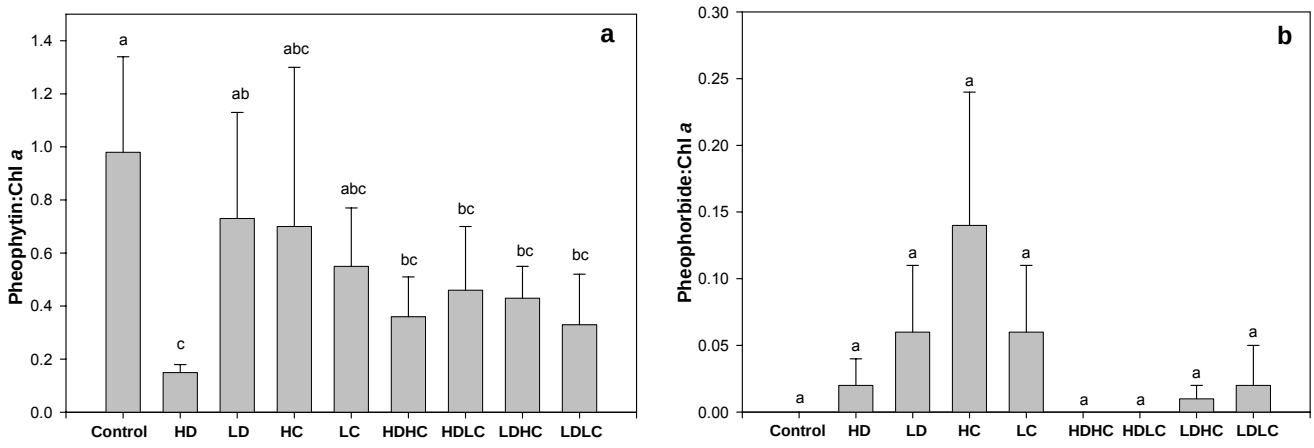


Figure 2.7. Pheophytin:Chl *a* (a), and Pheophorbide:Chl *a* (b) in the 2 mm of surface sediment on day 5. Values are mean + 1 SD (n = 3). Different letters indicate significant differences ($p < 0.05$). HD = High Diesel, LD = Low Diesel, HC = High Copper, LC = Low Copper, HDHC = High Diesel-High Copper, HDLC = High Diesel-Low Copper, LDHC = Low Diesel-High Copper and LDLC = Low Diesel-Low Copper.

three uppermost layers, Chl *a* concentration in the HD treatment was significantly higher than in the Control and HDHC and HDLC treatments (Fig 2.8a, $p < 0.0001$). Fucoxanthin concentration in the HD treatment was significantly higher in the top layer (200 μm) than in HDHC and HDLC treatments ($p < 0.0093$), and did not differ significantly from the Control (Fig 2.8b, $p > 0.85$). No significant differences were detected in the content of zeaxanthin, and diadinoxanthin:Chl *a* and pheopigment:Chl *a* ratios among different layers in any treatment.

Effects of Contaminants on Day 20

When considering the entire depth profile for day 20, Chl *a* concentration in the HD treatment was significantly higher than in other treatments, including the Control (Fig. 2.9a). Chl *a* concentration in the LD treatment did not differ significantly from Control, but was significantly higher than in the LDHC and LDLC treatments (Fig. 2.9a, $p < 0.010$). Chl *a* concentrations in the HC treatment was significantly lower than in the Control treatment ($p < 0.01$), but did not differ significantly from HDHC and LDHC treatments (Fig.2.9a, $p > 0.45$). In the same way, Chl *a* in the LC treatments did not significantly differ from the Control, HDLC and LDLC treatments (Fig. 2.9a, $p > 0.05$).

As was observed for Chl *a*, fucoxanthin concentration in the top 2 mm of sediment was significantly higher in the HD treatment than in all other treatments (Fig. 2.9b, $p < 0.0001$). Fucoxanthin in the LD treatment was significantly higher than in the Control, LDHC and LDLC treatments ($p < 0.0002$). Fucoxanthin in the HC treatment was not significantly different from that in HDHC and LDHC (Fig. 2.9b, $p > 0.54$), but was significantly lower than in the Control ($p = 0.021$). Fucoxanthin concentration in the LC treatment was not significantly different from the HDLC treatment (Fig. 2.9b, $p = 0.53$), but was significantly lower than in the Control and LDLC

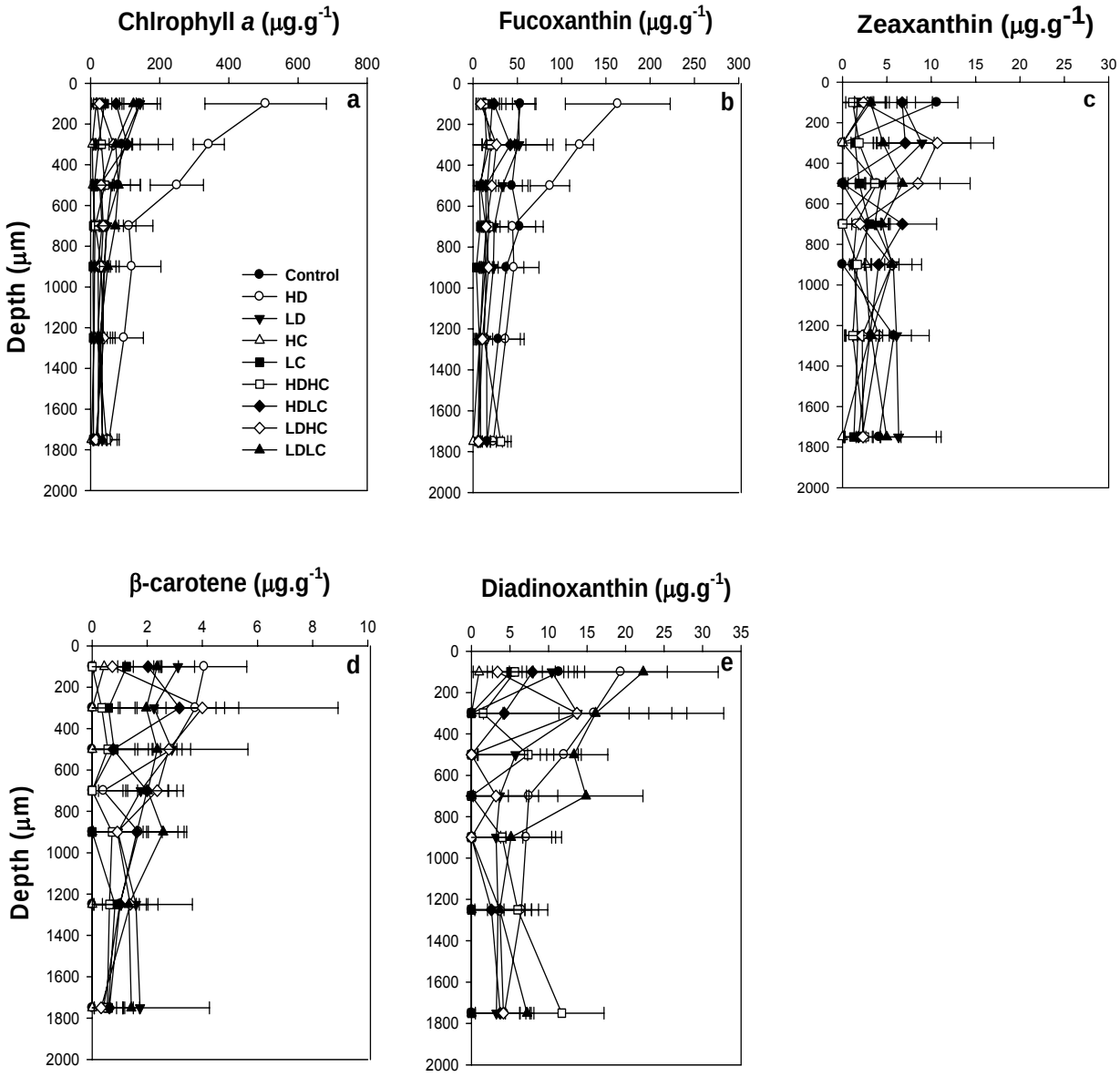
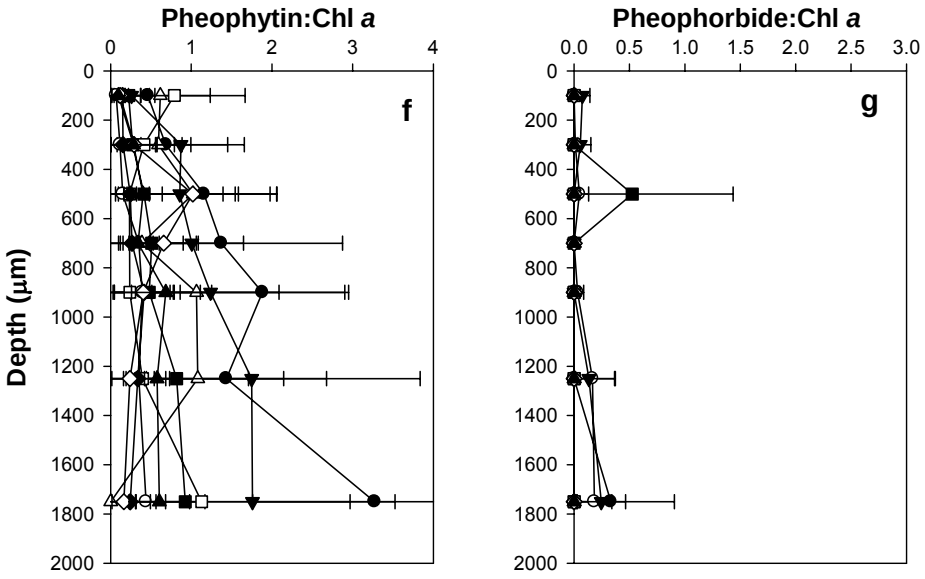


Figure 2.8. Vertical profiles of Chl *a* (a), fucoxanthin (b), zeaxanthin (c), β-carotene (d), diadinoxanthin (e), and pheopigments:Chl *a* ratios (f, g) on day 5. Values are mean ± 1 SD (n = 3). HD = High Diesel, LD = Low Diesel, HC = High Copper, LC = Low Copper, HDHC = High Diesel-High Copper, HDLC = High Diesel-Low Copper, LDHC = Low Diesel-High Copper, LDLC = Low Diesel-Low Copper.



(Figure 2.8. continued).

treatments (Fig. 2.9b, $p > 0.05$).

The diadinoxanthin concentration was significantly higher in the HD treatment than in the Control and HDHC treatments (Fig. 2.9c, $p < 0.0001$), and was also significantly higher in the LD treatment than in the LDHC treatment ($p = 0.02$). However, the diadinoxanthin concentration did not differ significantly between the HD and HDLC treatments or between LD and LDLC treatments (Fig. 2.9c, $p > 0.10$). Thus, the presence of LC influenced the diadinoxanthin concentration observed in the HD and LD treatments alone. Diadinoxanthin concentration was significantly lower in the HC treatment than in the Control (Fig. 2.9c, $p > 0.05$). There were no significant differences in the diadinoxanthin content between the HC treatment and HDHC and LDHC treatments ($p > 0.21$). Diadinoxanthin concentration in the LC treatment was significantly lower than in the HDLC treatment (Fig. 2.9c, $p < 0.0001$), but not significantly different from the LDLC treatment ($p = 0.10$).

The content of zeaxanthin in the top 2 mm of sediment was significantly higher in the HD than in the HDHC treatment (Fig. 2.9d, $p = 0.001$). Zeaxanthin in the HD treatment did not significantly differ from Control or HDLC treatments (Fig. 2.9d, $p > 0.55$). Zeaxanthin in the LD treatment was significantly higher than in the LDHC treatment (Fig. 2.9d, $p = 0.0001$), but did not differ significantly from the LDLC treatment ($p > 0.05$). The zeaxanthin concentration in the HC treatment did not differ significantly from the HDHC or LDHC treatments ($p > 0.34$), but was significantly lower than in the Control (Fig. 2.9d, $p = 0.02$). Zeaxanthin in the LC treatment did not significantly differ from the Control, HDLC, and LDLC treatments (Fig. 2.9d, $p > 0.42$).

The β-carotene concentration in the top 2 mm of sediment was significantly higher in the HD treatment than in the Control, HDHC, and HDLC treatments (Fig 2.9e, $p < 0.0001$). Similarly, the β-carotene concentration in the LD treatment was significantly higher than in

the Control, LDHC, or LDLC treatments (Fig 2.9e, $p < 0.0001$). In contrast, the β-carotene concentration in the HC treatment did not differ significantly from Control, HDHC, or LDHC treatments (Fig. 2.9e, $p > 0.40$). The β-carotene concentration in the LC treatment did not differ significantly from that in the Control and LDLC treatments (Fig. 2.9e, $p > 0.16$), but was significantly lower than in the HDLC treatment (Fig. 2.9e, $p = 0.0004$). The presence of HC in diesel treatments (HDHC or LDHC) significantly reduced β-carotene concentration in comparison to treatments with only diesel (HD and LD).

The pheophytin:Chl *a* ratio on day 20 was significantly lower in the HD treatment than in the HDHC treatment (Fig. 2.10a, $p < 0.0001$). There were no significant differences among the other treatments ($p > 0.07$).

Although there were non-significant differences in the pheophorbide:Chl *a* ratios among different treatments ($p > 0.06$), those ratios were higher in the HDHC and LDHC treatments than in all other treatments (Fig. 2.10b).

Vertical Profiles on Day 20

Pigment concentrations in individual vertical layers were also compared for each pigment. ANOVA revealed highly significant differences between Chl *a* concentration in HD treatment and all other treatments in the top five layers (Fig. 2.11a, $p < 0.0001$). Beyond 1000 μm there were no significant differences between different treatments. Fucoxanthin concentration in the HD treatment was also significantly higher than in other treatments, but only in the uppermost four layers (Fig. 2.11b, $p < 0.02$). There were no significant differences among treatments in the vertical layers for zeaxanthin, β-carotene and the pheopigments:Chl *a* ratios (Fig. 2.11c-f, $p > 0.28$).

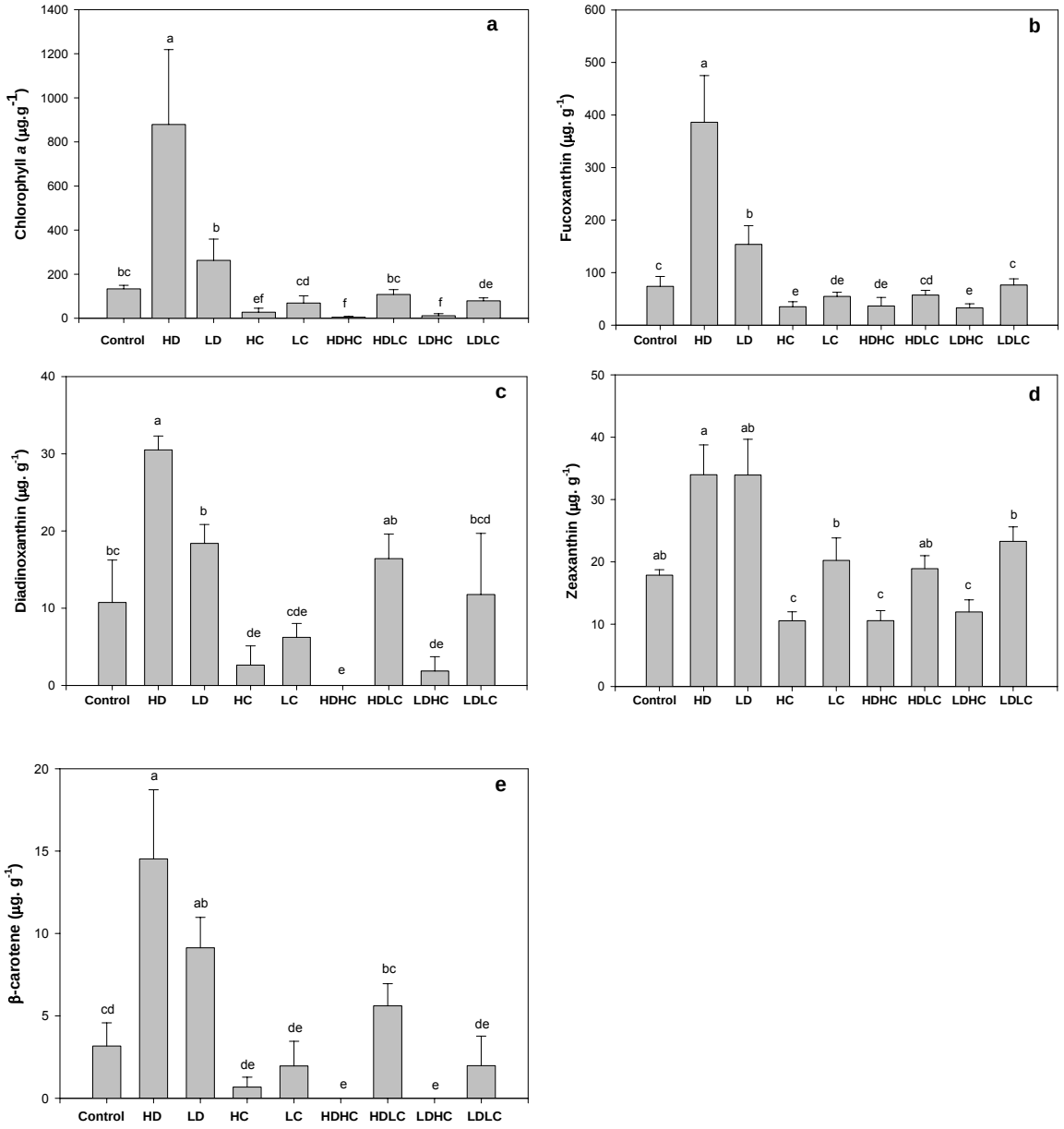


Figure 2.9. Chl *a* (a), fucoxanthin (b), diadinoxanthin (c), zeaxanthin (d), and β-carotene (e) concentration in the 2 mm of surface sediment on day 20. Values are means + 1 SD (n = 3). Different letters indicate significant differences ($p < 0.05$). HD = High Diesel, LD = Low Diesel, HC = High Copper, LC = Low Copper, HDHC = High Diesel-High Copper, HDLC = High Diesel-Low Copper, LDHC = Low Diesel-High Copper and LDLC = Low Diesel-Low Copper.

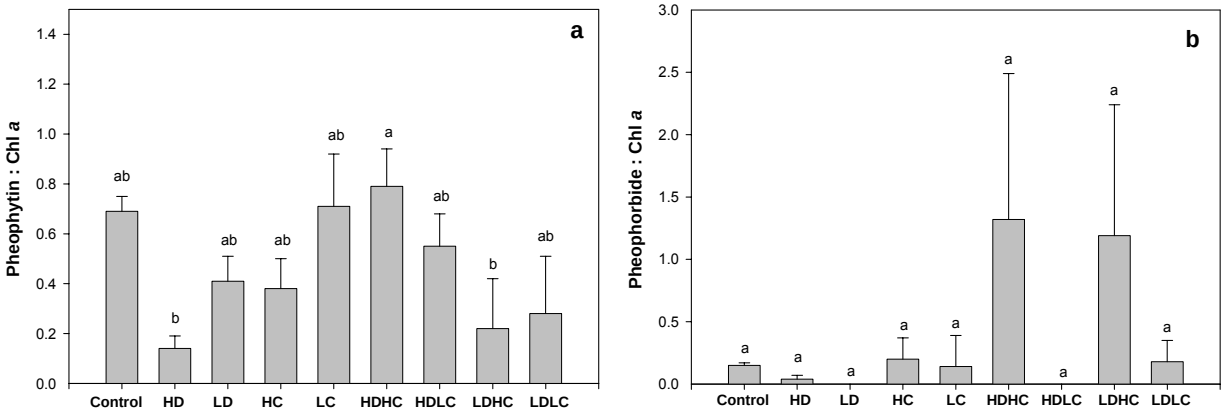


Figure 2.10. Pheophytin:Chl *a* (a), and pheophorbide:Chl *a* (b) ratios in the 2 mm of surface sediment on day 20. Values are mean + 1 SD (n = 3). Different letters indicate significant differences ($p < 0.05$). HD = High Diesel, LD = Low Diesel, HC = High Copper, LC = Low Copper, HDHC = High Diesel-High Copper, HDLC = High Diesel-Low Copper, LDHC = Low Diesel-High Copper, LDLC = Low Diesel-Low Copper.

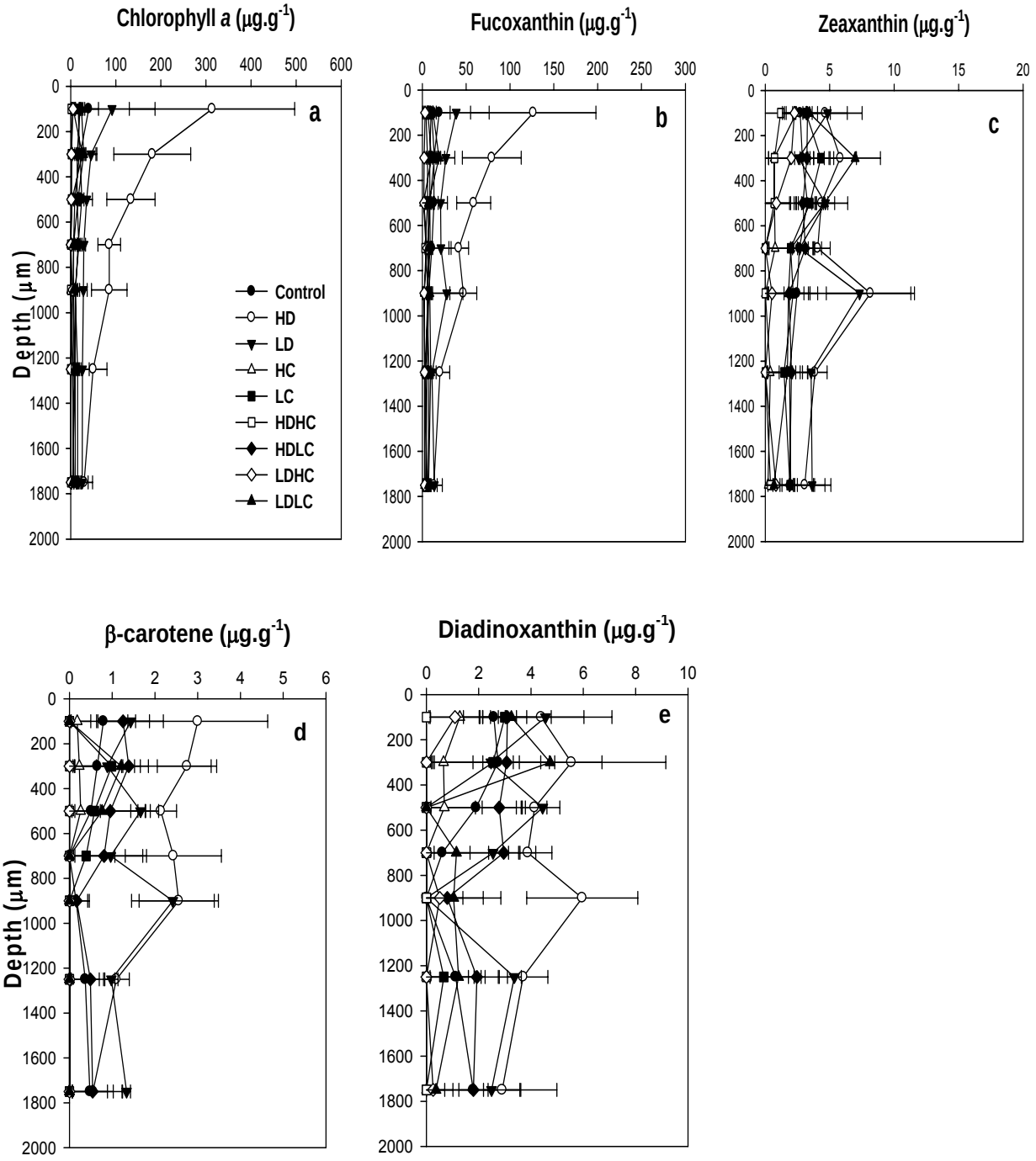
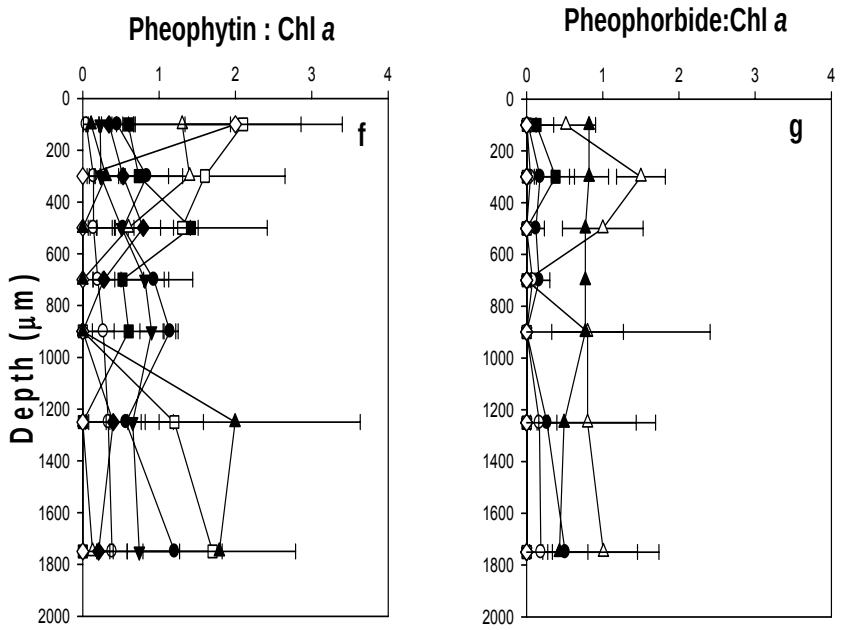


Figure 2.11. Vertical profiles of Chl *a* (a), fucoxanthin (b), zeaxanthin (c), β-carotene (d), diadinoxanthin (e) and pheophytin:Chl *a* (f) and pheophorbide:Chl *a* (g) ratios on day 20. Values are mean ± 1 SD (n = 3). HD = High Diesel, LD = Low Diesel, HC = High Copper, LC = Low Copper, HDHC = High Diesel-High Copper, HDLC = High Diesel-Low Copper, LDHC = Low Diesel-High Copper, LDLC = Low Diesel-Low Copper.



(Figure 2.11. continued).

Effects of Contaminants on Meiofaunal Abundance

Nematodes ranged from 42 to 75% of the total meiofaunal abundance among the experimental treatments (Table 2.1). ANOVA revealed significantly higher abundance of nematodes in the LD treatment than in the LDHC and LDLC treatments ($p < 0.023$). There were no significant differences among the other treatments ($p > 0.06$, Fig. 2.12). Ostracods constituted the second most abundant meiofaunal taxon found. Their abundance was significantly reduced in the LDHC treatment compared with the Control and LD treatments (Fig. 2.12, $p < 0.0019$). No significant effects were detected in the other treatments ($p > 0.11$). Nauplii were totally absent in the LDHC treatment, and differed significantly from the Control and LD treatment (Fig. 2.12, $p < 0.009$). Copepods abundance was not significantly affected by any of the treatments (Fig. 2.12, $p > 0.40$).

The two most abundant copepod species were *Coullana* sp. and *Enhydrosoma* sp. Their abundances were not significantly influenced by any treatment (Fig. 2.13). The other two species found were *Pseudostenhelia wellsi* and *Halicyclops coulli*, but these species were found in very low numbers at the beginning of the experiment (Cont d0, Fig. 2.13). *P. wellsi* was totally absent in the HC and LDHC treatments, and their abundance in the HDHC, HDLC was significantly lower than that in the HD treatment (Fig. 2.13, $p = 0.003$). Also, *P. wellsi* abundance in the LD treatment was significantly higher that that of LDHC treatment ($p = 0.0009$).

DISCUSSION

TPHs concentrations in the top 1 cm of sediment were 29 and 18 mg kg sediment^{-1} in the HD and LD treatments, respectively. These concentrations are relatively moderate concentrations (Long, 1992), but higher than the "effects range low" (ERL)

Table 2.1. Major taxa composition of the total meiofauna in different treatments. Cont 0 = Control at day 0, rest of abbreviations correspond to day 20, Cont = Control, HD = High Diesel, LD = Low Diesel, HC = High Copper, LC = Low Copper, HDHC = High Diesel + High Copper, HDLC = High Diesel + Low Copper, LDHC = Low Diesel + High Copper, LDLC = Low Diesel + Low Copper. Nema = nematodes, Ostra = ostracods, Cope = copepods, Naup = nauplii, Poly = polychaetes, Oligo = oligochaetes, Chiro = chironomids. Values represent mean of 3 replicates.

	Relative Abundance (%)							
	Nema	Ostra	Cope	Naup	Poly	Oligo	Chiro	Total 10 cm^{-2} (SD)
Cont 0	35.0	31.5	17.0	13.2	1.7	1.1	0.9	219 (93)
Cont	46.3	20.2	16.2	15.1	1.9	0.7	0.9	192 (69)
HD	56.6	11.9	14.0	15.3	0.7	0.9	1.0	196 (78)
LD	54.6	22.2	7.6	14.2	0.2	0.3	0.7	349 (97)
HC	42.1	28.6	12.7	15.4	1.5	0.0	0.8	86 (29)
LC	46.3	22.2	22	6.6	0.3	0.8	1.0	126 (12)
HDHC	45.2	22.3	20.9	10.1	1.2	0.9	0.6	115 (30)
HDLC	59.4	13.3	20.0	4.8	0.0	1.0	0.7	138 (8)
LDHC	75.1	10.5	10.5	0.0	1.7	2.8	0.0	60 (52)
LDLC	54.2	22.0	16.9	4.2	1.0	0.9	1.5	111 (5)

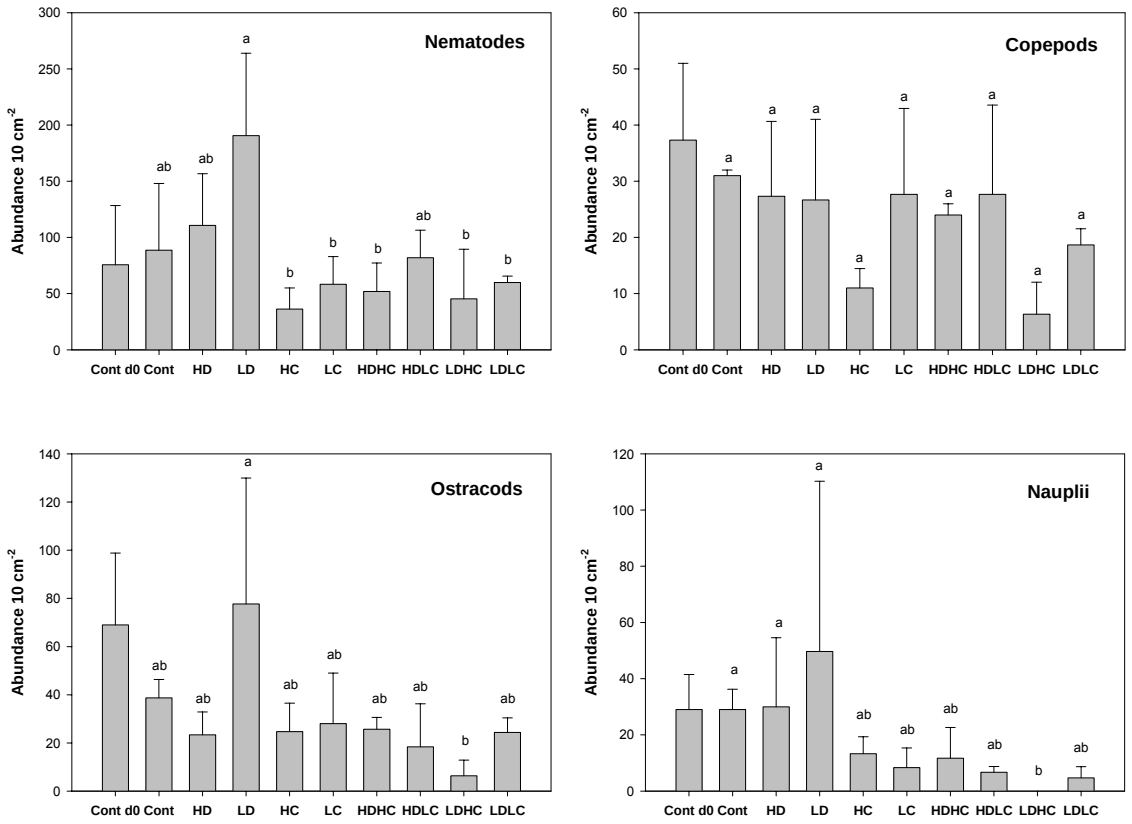


Figure 2.12. Abundance of major taxa. Cont d0 = Control on day 0, Cont = Control, HD = High Diesel, LD = Low Diesel, HC = High Copper, LC = Low Copper, HDHC = High Diesel + High Copper, HDLC = High Diesel + Low Copper, LDHC = Low Diesel + High Copper, LDLC = Low Diesel + Low Copper. Bars represent mean + 1 SD (n = 3). Different letters indicate significant differences.

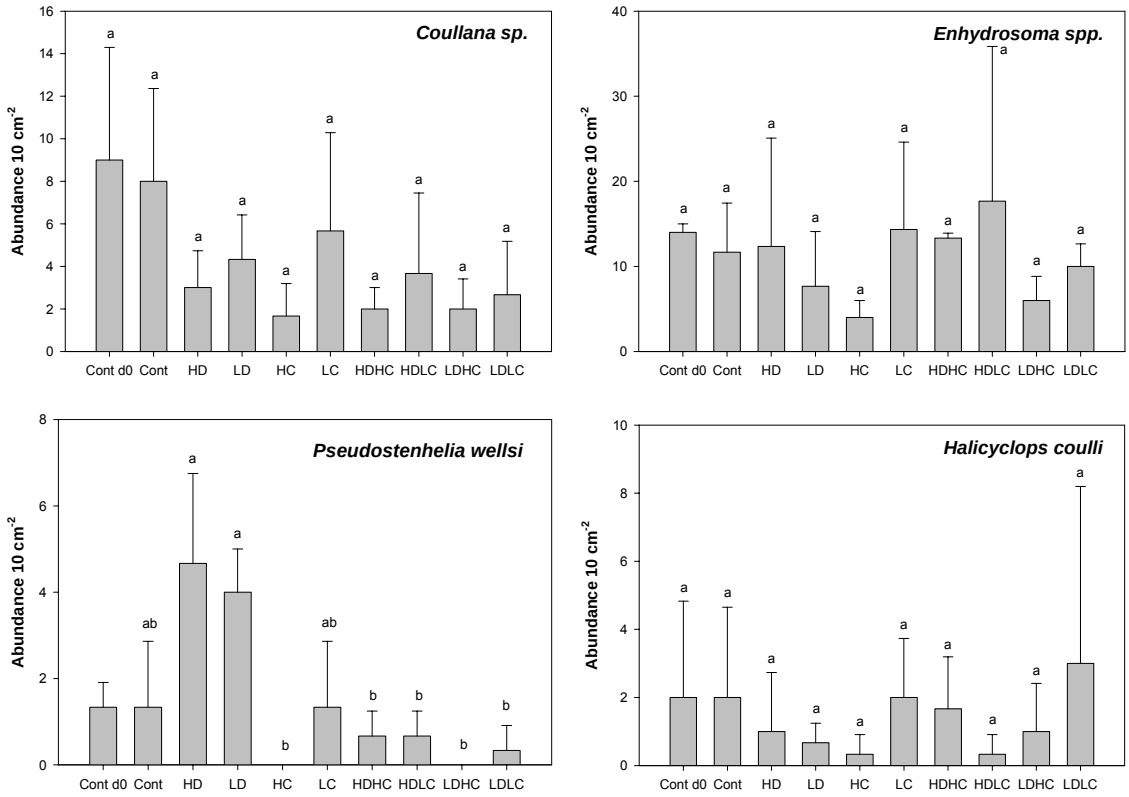


Figure 2.13. Abundances of copepod species. Cont d0 = Control at day 0, Cont = Control, HD = High Diesel, LD = Low Diesel, HC = High Copper, LC = Low Copper, HDHC = High Diesel + High Copper, HDLC = High Diesel + Low Copper, LDHC = Low Diesel + High Copper, LDLC = Low Diesel + Low Copper. Bars represent mean + 1 S.D. Different letters indicate significant differences.

concentrations for PAH reported by Long (1995). Concentrations at or above the ERL, but below the ERM (Effects Range Median), represent a range within which effects occasionally occur. At concentrations above the ERM, frequent effects are expected (Long, 1995). The background concentration of TPHs in the sediment, where the microcosms were collected, was 0.27 mg kg sediment^{-1}, and this concentration is consistent with background concentration reported previously at my site (Carman et al., 1995). After 20 days, the concentrations of TPHs in the treatments were lower than at the beginning of the experiment (Fig. 2.1), and lower than the ERL. This decrease was likely due to bacterial degradation of hydrocarbons, and has been reported previously (Bennet et al., 1999).

The background concentration of copper in the Control sediment at day 0 was 44 ± 5 mg kg sediment^{-1}. The total copper concentrations used in this study were higher than the ERM reported from Long (1995). Measured concentrations of total copper and TPHs after 20 days indicated that neither diesel nor copper had an influence on each others concentration (Fig. 2.1). The choice of copper concentrations used in this study was based on results of previous experiments (Millward et al., 2001).

At the beginning of the experiment the presence of significant concentrations of fucoxanthin indicated that the predominant algal group in the BMA community was diatoms. Fucoxanthin, diadinoxanthin and diatoxanthin are all found in diatoms (Lucas and Holligan, 1999), as well as in the prymnesiophytes. Microscopic observation of these samples indicated the presence of benthic diatoms, which were mainly species of the genera *Navicula* and *Nitzschia* (Chapter 3). The predominance of diatoms in the BMA community at this location site has been previously reported (Buffan-Dubau and

Carman, 2000) and it is typical of marine intertidal sediments (Admiraal, 1984; Underwood and Krompkamp, 1999).

Another pigment detected was zeaxanthin, which is a biomarker of cyanobacteria (Wright et al., 1991). Zeaxanthin concentration ranged from 11 to 42 $\mu g.g^{-1}$of sediment among all treatments. Concentrations of zeaxanthin reported here are one order of magnitude higher than reported previously for zeaxanthin for the same location (Carman et al., 2000), and about an order of magnitude lower than Fucoxanthin. Lutein was also detected, but Chl *b* was not. Chl *b* decays much faster than lutein (Bianchi et al., 1991), so the absence of Chl *b* in the sediment suggests that live chlorophytes were absent. Thus, the presence of lutein could indicate the presence of detrital chlorophyte material (Buffan-Dubau and Carman, 2000).

Pheophorbide was found in very low concentration. This result contrasts with other authors who have found that pheophorbides are measured in higher concentration than pheophytin and chlorophyllide *a* in sediments (Cariou-Le Gall and Blanchard, 1995; Buffan-Dubau et al., 1996; Brotas and Plante-Cuny et al., 2003). In my study, pheophytin concentrations were higher than pheophorbide, which indicates that heterotrophic activity was a major pathway for microalgal degradation (Bianchi et al., 1988, 1991; Goosen, 1999). My study differs from those cited above in the depth of my sampling. I sampledthe top 2 mm of sediment surface, while most other studies are based on cm-scale samples. The top few mm of sediment surface is an area where most of the BMA are present but also is most likely to undergo both photo-oxidation of Chl *a* and reworking by fauna. My result is consistent with that obtained by Barranguett et al. (1997), in which 1 mm deep surface slices were studied and pheophorbides were only found at low

concentration. One of the main goals of this study was to analyze the microscale distribution of the BMA community based on photosynthetic pigments. This aspect is important because the upper layers (1 – 1.5 mm) of illuminated sediment contain the photosynthetically active biomass (PAB), while deeper layers contain Chl *a* derived from photosynthetically inactive biomass (PIB) of various sources (Kelly et al., 2001). The surface layer of sediment is an area of intense biogeochemical activity, which establishes chemical and biological gradients over a microscale. To better understand these systems it is important to use a scale appropriate to the processes occurring in the sediment (Paterson, 1999).

Our results indicate that the presence of HD in sediment caused a stimulatory effect on the growth of diatoms as indicated by fuxocanthin. In contrast, HC reduced diatom biomass on both day 5 and day 20. The increase of diatoms in diesel contaminated sediment is consistent with other studies (Carman et al., 1995, 1997, 2000), which suggested that the grazing activity in diesel-contaminated sediment was reduced, contributing to enhancement of algal biomass. Meiofaunal abundance at my site at the beginning of the experiment was very low compared with previous experiments (Millward et al., 2001), but my results are consistent with previous studies (Carman et al., 1996; Millward et al., 2004), which found that nematodes and copepods were not significantly affected by diesel or copper. The copepod community was comprised of few species with low abundances and there was high variability between replicates, which could have reduced the power to detect any effect (Fleeger et al., 2006). The two most abundant species of copepods were *Coullana* sp. and *Enhydrosoma* sp. They did not respond significantly to any treatment. The other two species were *Pseudostenhelia wellsi* and

Halicyclops coulli, but these species were found in very low numbers at the beginning of the experiment. Although *Cletocamptus* sp. was missing, this species typically increases its abundance in diesel-contaminated sediment (Carman et al., 1997; Millward et al., 2004; Fleeger et al., 2006). The abundance of ostracods was significantly reduced in LDHC treatment compared with the Control treatment, which was also observed by Millward et al. (2004). The nematode abundance in the LD treatment was higher than that in the LDHC and LDLC treatments, which suggests that the presence of copper reduced the stimulatory effects of LD in contaminant mixtures.

Increased nutrient availability due to microbial activity could also play important role in diesel-contaminated sediment (Carman et al., 2000). Nyman (1999) observed that additional C respired from oiled microcosms exceeded C added as crude oil by 1.4-3.5 times and concluded that accelerated organic matter mineralization increased the rate of nutrient mineralization. Nyman (1999) suggested that such nutrient release might explain some reports of crude oil stimulating growth of emergent marsh plants (Li et al., 1985; Lin and Mendelson, 1996). PAH degraders become enriched in diesel fuel-contaminated sediment (Carman et al., 1996). The significant reduction of TPHs after 20 days of the experiment, from 27 mg kg sediment^{-1} at the beginning of the experiment down to 0.7 mg kg sediment^{-1} in HD treatment, also suggests a high rate of degradation of diesel. This implies that the contamination with diesel may be changing the microbial ecology of the sediment, and that petroleum hydrocarbons could change the biogeochemistry of the ecosystem.

Decreased Chl *a* and fucoxanthin concentrations in the HC treatment suggests that copper caused a direct toxic effect on BMA. This observation is consistent with Millward et al. (2001),

who showed that in the presence of elevated concentrations of copper (787 ppm) there was a saturation of the metal-sorbing capacity of the sediment causing an increased concentration of soluble Cu^{2+}, the more bioavailable and toxic form of copper, resulting in direct toxic effects on the entire benthic community. Toxicity of copper to diatom growth has been previously reported (Cid et al., 1995; Rijstenbil and Wijnholds, 1991). In contrast, it has been shown that *Amphora* is able to tolerate a high concentration of dissolved copper (Thomas and Robinson, 1986; Brown et al., 1988). Copper concentrations used here were higher than the ones used by Millward et al. (2001). The total copper concentration in my study in the HC treatment after 20 days was 2500 ppm.

Zeaxanthin concentrations (cyanobacteria) on day 5 indicated that the presence of LD in the HC or LC treatment reduced the effect of HC or LC alone. In contrast, the presence of HC reduced the stimulatory effect of HD or LD alone observed on day 20. Although the contribution of cyanobacteria to the total BMA biomass (Chl *a*) was low compared with that of diatoms, cyanobacteria were detected on both days 5 and 20, and showed some differential responses to copper and diesel. High concentration of copper (HC) caused a toxic effect on cyanobacteria (zeaxanthin), HD did not cause stimulation of cyanobacteria, and diesel did not have an influence in the toxic effect of copper (zeaxanthin concentration in the HC treatment did not differ from that in the HDHC treatment). It is important to point out that cyanobacteria may not be able to compete with diatoms that are known for their chemotrophic capacities and better growth at higher nutrient conditions (Stal, 2003), and this might be related to a possible release of nitrogen in diesel-contaminated sediment. The very small particle size of silt and clays that form the mud causes a very high attenuation of light and this may represent a

difficulty for cyanobacteria, and the small grain size may constitute a poor support for the gliding motility of cyanobacteria. Metals such as copper can could cause oxidative stress in microalgae (Pinto et al., 2003). When the levels of reactive oxygen species (ROS) formed exceed the ability of the antioxidant system to cope with them, damage to cellular compounds occurs (Rijsrenbil et al., 1994). β-carotene is a carotenoid that has antioxidant protection against oxidative stress (Pinto et al., 2003). My results indicate that the toxic effect of copper on day 5 may have exceeded the antioxidant capacity of β-carotene, and the presence of LD combined with HC and LC somehow diminished that toxic effect.

Pheophytin:Chl *a* ratios measured on days 5 and 20 in the HD treatment could be a result of the diatom bloom that formed in that treatment. Higher pheophorbide:Chl *a* ratios in HC on day 5 and in HDHC and LDHC on day 20 could indicate that grazing activity occurred in those treatments, as pheophorbide is widely considered to be the main degradation product of grazing activity (Hawkins et al., 1986; Bianchi et al., 1988; Klein and Riaux-Gobin, 1981).

Both diesel and copper significantly influenced the vertical distribution of Chl *a* and fucoxanthin. In particular, the Chl *a* concentration in the HD treatment in the top layers (0 - 400 μm) was significantly higher than the concentration in the bottom layers (600 - 2000 μm), resulting in a visible diatom bloom on the sediment surface. By microscopic observation of a sample from the HD treatment, I found that the predominant species of diatom in that bloom was *Bacillaria paxillifer* (Chapter 3). The BMA (diatoms) accumulated in the top layers to obtain sufficient light for photosynthesis, and by doing so they had greater exposure to contaminants, causing a significant change in their metabolism.

My results indicate that moderate concentrations of hydrocarbons can cause significant

changes in the community composition of BMA communities, and combined with significant concentrations of metals could produce different responses than either contaminant alone. The combined effect of diesel and copper is different than the effect of each contaminant alone. The potential interactions of diesel and copper have implications for the entire benthic community in the salt marsh.

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CHAPTER 3

EFFECTS OF DIESEL FUEL AND COPPER CONTAMINANTS ON THE ASSEMBLAGE COMPOSITION OF BENTHIC DIATOMS FROM A COASTAL SALT MARSH

INTRODUCTION

Benthic microalgae (BMA) contribute significantly to the total primary production of estuarine and other shallow water ecosystems (McIntyre et al., 1991; Underwood and Kromkamp, 1999). They are an important food source for benthos and provide a principal source of nutrition for secondary production (Sullivan and Moncreiff, 1990). Diatoms, cyanobacteria, and green algae are the dominant groups. Of these, diatoms are universally present and are by far the taxonomically most diverse group (Sullivan and Currin, 2000).

Descriptions of the species composition of diatom assemblages (Hustedt and Aleem, 1951; Hopkins, 1964; Aleem, 1973, Underwood, 1994) and of patterns of species distribution (Round, 1960; McIntyre and Overton, 1971; McIntyre, 1978; Sullivan, 1977a, 1977b, 1978, 1982; Admiraal, 1984; Laws, 1988; Admiraal and Peletier, 1980; Admiraal et al., 1982; Admiraal et al., 1984; Oppenheim, 1991; Laird and Edgar, 1992) have been published for several estuarine mudflat and saltmarsh habitats. The diatom assemblages in Louisiana salt marshes have not been extensively studied. However, Parsons (1998) evaluated the salt marsh sedimentary record of the landfall of hurricane Andrew on the Louisiana coast, and Cook and Whipple (1982) studied the distribution of edaphic diatom along a salinity gradient in a Louisiana coastal marsh.

Estuarine and coastal marine sediments are major sinks for anthropogenic metal and petroleum hydrocarbon contamination. It is well established that high concentrations of some metals can have toxic effects on diatoms (Fisher and Frood, 1980). Studies that examine the effects of metal toxicity on diatoms are typically laboratory-based experiments involving a single diatom species exposed to different concentrations of

metals (Mason et al., 1995). Light crude oil and diesel-based oil-cuttings can significantly influence benthic diatom species (Plante-Cuny et al., 1993), and more subtle effects on diatom assemblages have been related to hydrocarbon contamination (Morales-Loo and Goutz, 1990). Typically, marine species are more sensitive to hydrocarbon contamination than are freshwater species (Kusk, 1981). However, there is little information available regarding the impact of petroleum hydrocarbons and metals on benthic marine diatom assemblage composition.

Recent studies have addressed the influence of diesel-fuel and metals on meiofauna communities and the associated food web in Louisiana salt marshes (Carman et al., 1996, 1997, 2000; Millward et al., 2001; Millward et al., 2004), but the effects of diesel-fuel and metals on the benthic diatom community have not yet been explicitly examined in those studies. The hypothesis of this study was that diesel-contaminated sediment cause an enhancement of large diatoms, and when combined with copper this enhancement was reduced. Here, I used microcosms of natural sediment to examine the effect of diesel-fuel and copper contaminants on a benthic saltmarsh diatom assemblage.

MATERIAL AND METHODS

Study Site

Microcosms of natural sediment communities (Carman et al., 1995, 1997, 2000) were collected from mudflats in a *Spartina alterniflora* saltmarsh located in the Terrebone Bay estuary near the Louisiana University Marine Consortium in Cocodrie, Louisiana, to examine the influence of diesel and copper contaminants on benthic microalgae (BMA) assemblage composition. The tidal range in the estuary is approximately 0.3 m and salinity ranges from 1 to 20 ppt. Sediments have a median grain size of 38 μm, an organic

carbon content of approximately 2.5%, and are composed primarily of silts (41%) and clays (17%) (Chandler and Fleeger, 1983). The study site is located in a region of hydrocarbon production and drilling activity, and both commercial and recreational boat traffic is high (Carman et al., 1997).

Experimental Design

The microcosm experiment was designed to study the effects of two concentrations of both copper and diesel, individually and in combination. Twenty-seven sediment microcosms (15.2 cm i.d. polyvinyl chloride pipe with windows cut in the sides above sediment level and covered with Nitex 63-μm mesh to allow exchange of water; Carman et al., 1995) were collected from exposed sediment to a depth of 15 cm at low tide on May 22, 2002. Microcosms were transferred to wet tables with circulating water and flooded with 2 to 3 cm of ambient marsh water, which was replaced daily to excess with filtered (63 μm) marsh water. Each core was aerated gently using an aquarium air stone. Temperature was maintained at 25 ± 2 °C, and microcosms were illuminated (70 μmol $m^{-2}s^{-1}$) on a 14:10 light:dark cycle with banks of 40-W fluorescent lights.

Treatments included a Control (no contaminants added), Low Diesel (LD), High Diesel (HD), Low Copper (LC), High Copper (HC), Low diesel-High copper (LD-HC), Low Diesel-Low Copper (LD-LC), High Diesel-High Copper (HD-HC) and High Diesel-Low copper (HD-LC). Treatment concentrations were based upon levels known to produce sub-lethal and lethal responses in benthic communities (Carman et al. 1997; Millward et al. 2001). Only Control, HD, HC and HDHC treatments were analyzed to study the effects of diesel and copper contaminants on the taxonomic composition of benthic diatoms.

Preparation of Contaminated Sediment

A detailed description of the preparation of contaminated sediment is is in Chapter 2 of this dissertation. Briefly, diesel fuel was added to a sediment aliquot (1 part diesel to 3 parts sediment v:v) in an amber glass bottle and tumbled on a roller mill for 10 days. The sediment was then allowed to settle overnight. Unadsorbed diesel was aspirated from the bottle and 12 psu artificial sea water (ASW) was added. The sediment-water slurry was transferred to 35-mL glass centrifuge tubes and centrifuged at 1700 g for 3 min. The supernatant was removed and replaced with fresh ASW. The sediment and water were mixed thoroughly, and then centrifuged. The supernatant was decanted again, and the process was repeated for a total of four rinses. This sediment was used as the high diesel (HD) spike. At the beginning of the experiment the concentration of TPHs in the top 1 cm of sediment surface was 29 mg kg dw sediment^{-1}.

Copper-contaminated sediments were prepared by adding the appropriate volume of a copper solution to control sediment and diesel-contaminated sediment. Copper solution was added to sediment over a period of 2 h using a dripping gravity-feed apparatus while mixing vigorously with a food blender. In this way, a homogeneous mixture of copper within the spiking sediment was obtained. Total copper concentration at the beginning of the experiment was 2190 mg kg dw sediment^{-1}.

Experimental Procedure

Experimental treatments consisted of the initial addition of 25 mL of contaminated sediment to the overlying water in each microcosm. An additional 2.5 mL of sediment was added each day until the end of the study (20 days). Uncontaminated sediment was added to Control microcosms. These sediment additions created a sediment layer approximately

1 mm thick, which was quickly (within a few hours) mixed into the top few mm of sediment by bioturbation of benthic fauna.

Samples were collected on days 0, 5, and 20 during the day. Sediment cores were collected using the cryolander coring method (Wiltshire et al., 1997). The cryolander was placed on the surface of the sediment, and approximately 10 mL liquid nitrogen was gently poured onto absorbent cotton. The cotton was at ambient temperature, which caused the liquid nitrogen to vaporize. The cold vapor sank through the gauze and froze the sediment surface without physical distortion. After approximately 3 min of freezing under nitrogen vapor additional liquid nitrogen was added and sediment was allowed to freeze for approximately 6 min. Frozen sediment samples were removed, wrapped in aluminum foil, and stored in a –80 °C freezer. Prior to sectioning, the cryolander samples were cut into pieces of approximately 1 cm^2 using a lapidary saw. Subsequently, a microtome (Cryo-Cut, American Optical Company) was used to section the top 1 mm of sediment into five 200-μm layers and the second 1 mm into 2 layers of 500 μm each. The top layer (0 – 200 μm) was examined for diatom assemblage composition.

Sample Preparation

Diatoms were cleaned by boiling in 30% HNO_3 (Sullivan and Millie, 2001), and the sample was allowed to cool after it was concentrated to 20 mL by heat evaporation. Then 25 mL of H_2O_2 were added to further oxidize the sample plus few granules of $K_2Cr_2O_7$. Distilled water was added and after the valves settled to the bottom of the beaker (about 6 h), the sample was decanted. This rinsing with distilled water was repeated several times until all traces of HNO_3 and H_2O_2 were removed. Permanent slides were prepared in Naphrax (refractive index 1.71) and up to 300 valves were counted on each slide.

Diatoms were identified using an Olympus BX-50 light microscope at 1000x and using differential interference contrast (DIC) optics.

After a sample had been analyzed taxonomically, its species diversity was calculated employing the Shannon-Wiener informational index : $H' = -\sum_{i=1}^{S} \frac{n_i}{N} \log_2 \frac{n_i}{N}$, where n_i is the number of valves of the *i*-th taxon, N is the total number of valves, and S is the total number of taxa in the sample. In addition, the evenness index (J') was calculated as $J' = H'/H'_{max}$, where $H'_{max} = \log_2 S$ (Pielou, 1966). One way ANOVA was used to determine differences between treatments. The Bray-Curtis similarity index was used to compare assemblages between treatments. The Bray-Curtis index is also known as the Czekanowski coefficient or the percentage of similarity. The index values range from 100%, when the contribution of all taxa is identical, to 0% when both assemblages have no taxa in common (Clarke and Warwick, 1994). Taxa were identified following the descriptions by Krammer and Large Bertalot (1986, 1988), Round et al. (1990), Witkowski et al. (2000), Hustedt (1955), and Snoeijs et al. (1993-1998).

Data on the relative abundance of the 15 most abundant diatom taxa were analyzed by non parametric multidimensional scaling analysis (NMMDS) using the multivariate Plymouth Routines in Multivariate Ecological Research package (PRIMER, Plymouth Marine Laboratories, Plymouth, UK). Ranking of taxa contributing to dissimilarities between treatments were investigated using the similarity percentages procedure (SIMPER).

RESULTS

A total of 91 different diatom taxa were identified, and their relative abundance

varied between the treatments after 5 and 20 days (Table 3.1). The species richness (S) at the beginning of the experiment was 79 species. In the HC treatment, S was significantly lower than in the Control treatment ($p = 0.0021$) on day 5. There were no significant differences between the HD, HD-HC treatments and the Control (Fig. 3.1A, $p > 0.06$). Species richness in the HD-HC treatment was not significantly different than richness in the HD or HC treatments on day 5 ($p > 0.48$). Species diversity did not differ significantly among treatments on day 20 (Fig. 3.1B, $p > 0.05$).

The diversity (H') of the natural community before being exposed to contaminants was high (4.10 bits ind^{-1}), and was consistent with values reported for other North American salt marshes (Sullivan 1977, 1979). On day 5, H' in the HD treatment was significantly lower than in the Control treatment (Fig. 3.2A, $p = 0.0029$); there were no significant differences between treatments on day 5 ($p > 0.06$). There were no other significant differences in H' between treatments on day 20 (Fig. 3.2B, $p > 0.05$).

The evenness index (J') in the HD treatment was significantly lower than in other treatments on day 5 (Fig. 3.3A, $p < 0.01$); there were no significant differences in J' between other treatments on day 5 ($P > 0.10$). There were no significant differences in J' on day 20 (Fig. 3.3 (B), $p > 0.20$).

The Bray-Curtis similarity index values were between 44 and 62% (Table 3.2). Values higher than 50% suggest that the communities were more structurally similar than dissimilar.

The multidimensional scaling (MDS) ordination of the diatom assemblages on day 5 revealed distinct separation of HD from other treatments on day 5 (Fig. 3.4). The SIMPER revealed that the highest ranked dissimilarity (29%) between the HD and

Table 3.1. Relative abundance (%) of diatom taxa identified from the sediment surface (200 μm) in different treatments at day 5 and day 20 (day5/day20). "-" indicates taxon not collected, HD = High Diesel, HC = High Copper, HDHC = High Diesel-High Copper. Cont d0 = day zero.

Taxon	Cont d0	Control	HD	HC	HDHC
Achnanthes amoena Hust.	2.6	3/-	-/-	2.7/1.4	0.8/2.3
Achnanthes brevipes Kütz.	1.3	0.6/-	0.5/0.7	2.7/ -	1.5/-
Achnanthes curvirostrum Brun					
Achnanthes delicatula (Kütz) Grun.	0.7	1.2/2.7	0.5/0.7	- / -	- / -
Achnanthes submarina Hust.	1.3	1.8/4	0.5/0.7	2.7/2.9	2.3/3.5
Achnanthes temperei M.Perag.	-	- /2.7	0.5/0.7	4.1/2.9	3.1/ -
Achnanthes sp.1	0.7	1.2/ -	- /0.5	- /1.4	- / -
Achnanthes sp.2	0.5	- /0.5	- /0.8	- /2.1	- / -
Achnanthes sp.3	0.7	- / -	- / -	- /1.4	- / -
Actinoptychus senarius Ehren.	1.3	1.8/ -	- / -	- /2.9	2.3/1.2
Amphora acutiuscula Kütz.	1.3	1.8/2.7	1.0/ -	2.5/1.4	2.3/1.2
Amphora coffeaeformis Kütz.	2.0	2.4/2.7	1.0/1.4	5.5/2.9	1.5/2.3
Amphora cf. *exigua* Greg.	2.0	2.4/2.7	1.0/0.7	2.7/ -	1.5/2.3
Amphora cf. *marina* (W.Smith) Van Haurck	2.6	2.4/3.4	1.5/0.7	1.4/2.9	2.3/2.3
Amphora tenerrima Hust.	0.7	1.2/1.3	1.0/0.7	1.4/1.2	1.5/1.2
Amphora sp.1	0.7	0.6/ -	- /0.7	- / 1.0	- / -
Bacillaria .paxillifer Gmelin	2.0	0.6/11	44/33	- / -	1.5/2.3
Biremis circumteta (Meister) Lange-Bertalot & Witkowski	-	- / -	- / -	2.7/ -	2.3/ -
Caloneis westii (W.Smith) Hendey	0.7	1.2/ -	- / -	- / -	- /2.3
Campylosira alexandrica Salah	2.3	1.2/2.7	1.0/1.4	1.4/5.7	6.5/3.5
Camylosira cymbelliformis (A.Schmidt) Grun.	0.7	1.2/ -	0.5/1.4	- /2.9	6.9/3.5
Campylodiscus sp.1	0.3	- / -	- / -	- / -	- / -
Cocconeis placentula Ehren.	2.0	1.2/ -	- / -	- / -	1.5/2.3

(Table 3.1. continued).

Taxon	Cont d0	Control	HD	HC	HDHC
Cocconeis scutellum Ehren.	0.7	1.8/2.7	- / -	1.4/1.4	0.8/2.3
Cyclotella meneghiniana Kütz.	2.0	2.4/2.7	1.0/1.1	2.5/2.1	2.7/3.7
Diploneis elliptica (Kütz.) Cl.	0.7	1.2/ -	1.5/1.4	1.4/ -	- /1.2
*Diploneis smithii (*Bréb.) Cl.	2.0	1.2/ -	2.5/0.7	- / -	- /1.2
Diploneis stroemii Hust.	-	- /2.7	- / -	6.8/2.9	1.5/1.6
*Entomoneis alata(*Ehren.) Ehren.	1.3	- / -	- / -	- /1.4	- / 1.2
Epithemia adnata (Kütz) Bréb.	-	- / -	- / -	1.4/ -	1.5/ -
Fallacia pigmaea (Kütz.) Stickle & Mann	0.7	2.4/2.7	1.5/1.4	- / -	1.6/ -
Fallacia tenera (Kütz.) Stickle & Mann	2.0	0.6/ -	0.5/0.7	- / -	- / -
Fragilaria sp.1	0.7	0.6/ -	- / -	- /1.4	- / -
Gyrosigma peisonis (Grun.) Hust.	1.3	1.2/ -	- / -	- / -	0.8/ -
Gyrosigma acuminatum (Kütz.) Raben.	-	0.6/1.4	1.0/2.1	- / -	- / -
Gyrosigma balticum (Ehren.) Rabh.	0.7	1.2/ -	- / -	- / -	- / -
Gyrosigma cf. *limosum* Sterrenburg & Underwood	0.7	1.2/1.3	- / -	- / -	0.8/1.2
Gyrosigma sp.1	0.5	0.9/ -	- / -	- / -	0.8/ -
Hippodonta aff.*caotica* Witowski	-	- / -	0.5/1.4	- /1.4	- / 2.3
Navicula abunda Hust.	1.3	2.4/2.7	1.0/1.4	- /1.4	0.8/ -
Navicula accomoda Hust.	0.7	0.6/ -	2.0/2.1	- /0.7	- / -
Navicula agnita Hust.	2.0	3.0/2.7	1.5/2.1	1.4/1.4	0.8/ -
Navicula cf. *crucicula* (W.Smith) Donk.	0.7	- / -	- / -	- / -	- /1.2
Navicula cryptocephala Kütz.	0.7	1.8/1.3	1.0/2.1	1.4/ -	1.5/ -
Navicula flanatica Grun.	3.3	3.6/2.7	2.1/2.1	- / 2.1	1.6/1.2
Navicula gregaria Donk.	2.0	2.4/ -	0.5/0.7	- / -	0.8/ -
Navicula normaloides Choln.	1.3	- / -	- / 0.7	- / 2.9	- / 1.8
Navicula peregrina (Ehr.) Kütz.	0.7	1.2/2.7	1.0/0.7	- / 1.4	0.8/1.4
Navicula perminuta Grun.	0.7	1.2/1.3	- / 1.4	- / -	- / -

(Table 3.1. continued).

Taxon	Cont d0	Control	HD	HC	HDHC
Navicula phyllepta Kütz.	4.0	4.8/2.7	1.0/2.1	4.1/2.1	2.3/ -
Navicula platyventris Meist.	0.7	- / -	0.5/ -	2.7/2.9	- /1.2
Navicula salinarum (Grun.) Grun.	2.0	3.0/2.7	1.5/1.4	- / -	0.8/1.2
Navicula salinicola Hust.	2.0	3.0/2.0	1.0/ -	- / -	- / -
Navicula tripunctata (Müll.) Bory.	0.7	1.8/2.7	2.0/3.5	- /1.4	1.5/1.8
Navicula sp.1	0.7	0.3/ -	- /0.7	- /1.0	0.4/ -
Navicula sp.2	0.7	- / -	- /0.3	- /1.4	- / -
Navicula sp.3	0.5	- / -	- /0.7	- / -	- / -
Navicula sp.4	0.5	0.6/ -	1.5/2.1	- /1.4	- /-
Neodelpheneis sp.1	0.7	0.6/ -	- / -	- /2.9	0.8/1.8
Neofragilaria nicobarica Des.,Pra. &Prem.	0.5	- / -	- / -	- / -	0.8/ -
Nitzschia agnita Hust.	1.3	1.8/2.7	1.0/ -	2.1/1.4	2.3/1.2
Nitzschia aurariae Choln.	1.3	0.6/1.4	- /0.7	- /1.0	- / -
Nitzschia filiformis (W.Sm) Hust.	0.7	1.2/1.3	- / -	1.4/1.4	- /1.2
Nitzschia insignis Greg.	1.3	0.6/ -	- /1.4	- / -	- / -
Nitzschia liebetruthii Raben.	2.6	3.0/4.0	- / -	2.7/1.4	0.8/2.3
Nitzschia lorenziana Grun.	0.5	1.2/ -	0.5/0.7	- /1.4	- / -
Nitzschia parvula Lewis	0.7	1.2/1.3	1.0/1.4	4.1/2.9	- /1.2
Nitzschia perspicua Choln.	2.7	1.2/1.4	1.0/1.4	- /2.1	3.5/ -
Nitzschia cf. *rigida* (Kütz.) Peragallo	-	- / -	- / -	2.7/1.4	- / -
Nitzschia sigma (Kütz.) W.Sm.	1.3	- / -	4.1/ -	- / 1.4	- /1.2
Nitzschia cf. *subacicularis* Hustedt	-	- /2.5	- /0.7	1.4/1.4	1.5/1.2
Nitzschia sp.1	0.3	0.3/ -	- / -	- /1.2	- / -
Nitzschia sp.2	0.3	- / -	- /0.5	- /1.4	- /2.3
Opephora pacifica (Grun.) Petit	0.7	- / -	- / -	2.1/1.4	0.8/3.5

(Table 3.1. continued).

Taxon	Cont d0	Control	HD	HC	HDHC
Parlibellus cf. *hamulifer* (Grun.) E.J.Cox	-	0.6/ -	2.5/ -	- / -	2.3/1.2
Parlibellus cruciculoides Brockmann	0.7/-	- / -	- / -	- / -	0.8/2.4
Petrodictyon gemma (Ehren.) D.G.Mann	-	- / -	0.5/ -	- / 0.5	- / -
Pinnularia neomajor Kram.	-	- / -	- / -	- /1.4	- / -
Pleurosigma salinarum Grun.	-	- / -	- / -	1.4/ -	- / -
Psammodictyon panduriforme (Greg.) D.G.Mann	1.3	0.6/ -	1.5/1.4	1.4/2.1	0.8/1.2
Seminavis strigosa Hust.	-	- /1.3	1.0/1.4	2.7/ -	2.3/ -
Thalasiosira proschkinae Makarova	1.5	1.8/ -	- /0.7	- /1.4	- /1.2
Thalasiosira sp.1	0.5	0.6/1.3	0.5/0.7	2.7/2.1	0.8/2.3
Tryblionella apiculata Greg.	0.5	- / -	- /1.4	2.4/1.2	- /1.0
Tryblionella calida (Grun.) Grun.	2.0	1.2/2.7	0.5/1.4	- /1.0	- /0.6
Tryblionella gracilis W.Smith	2.0	0.8/ -	0.5/ -	- / -	2.3/1.2
Tryblionella granulata Grun.	0.8	0.6/ -	- / -	5.5/5.7	3.8/3.5
Tryblionella hungarica (Grun.) Frenguelli	2.0	1.2/ -	- / -	- /1.4	- / -
Tryblionella levidensis W.Smith	2.0	1.2/2.8	- /0.7	1.4/5.7	2.3/3.5
Tryblionella perversa (Grun.) Frenguelli	0.5	1.0/1.3	- /1.4	- /2.9	- /1.0
Tryblionella punctata W.Smith	2.6	3.0/4.0	3.6/3.5	12.3/13	10.0/10.5

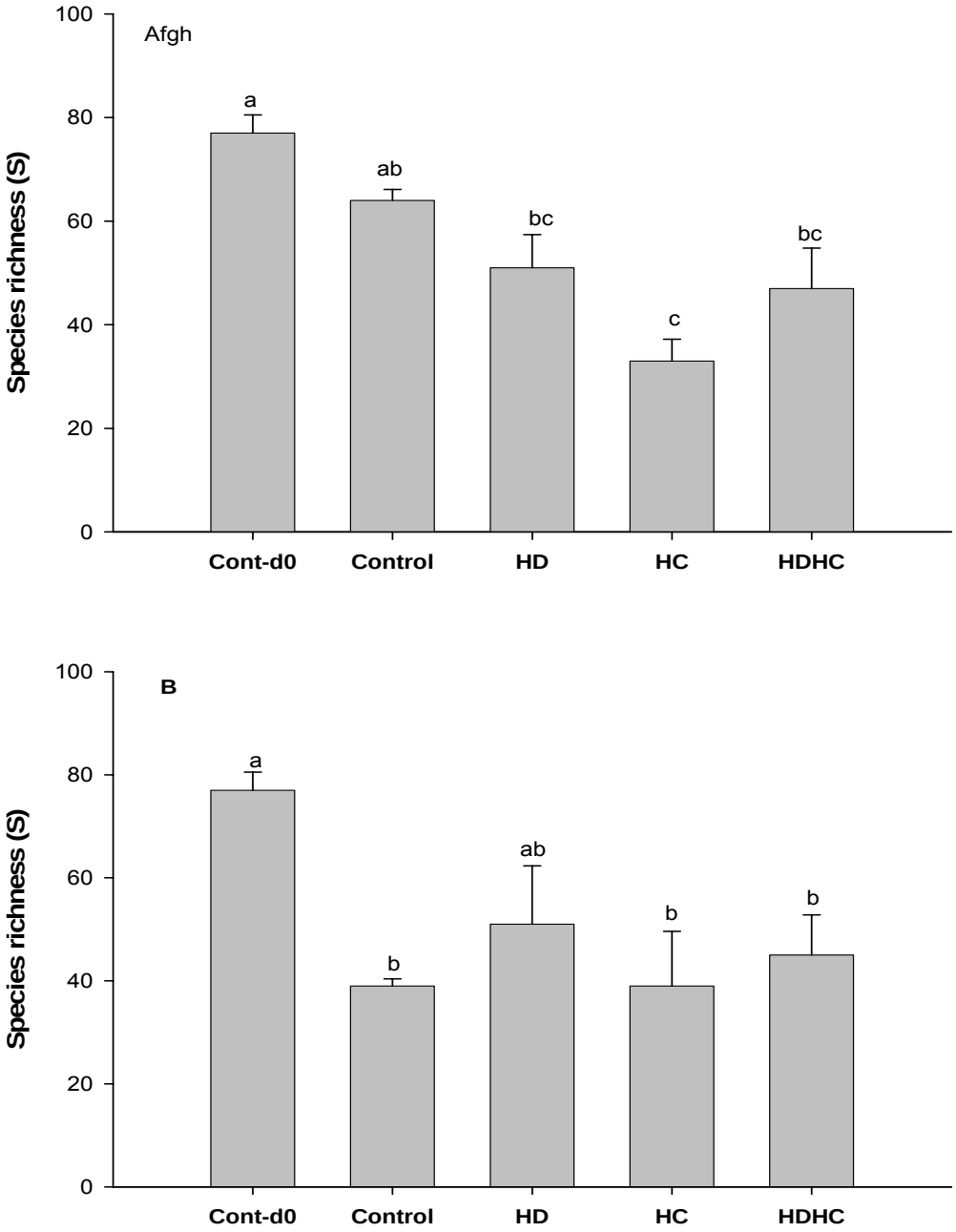

Figure 3.1 .Species richness (S) of the benthic diatom assemblage in different treatments on day 5 (A) and on day 20 (B). Cont-d0 = day 0, HD = High Diesel, HC = High Copper, HDHC = High Diesel-High Copper. Error bars represent + SD (n = 2).

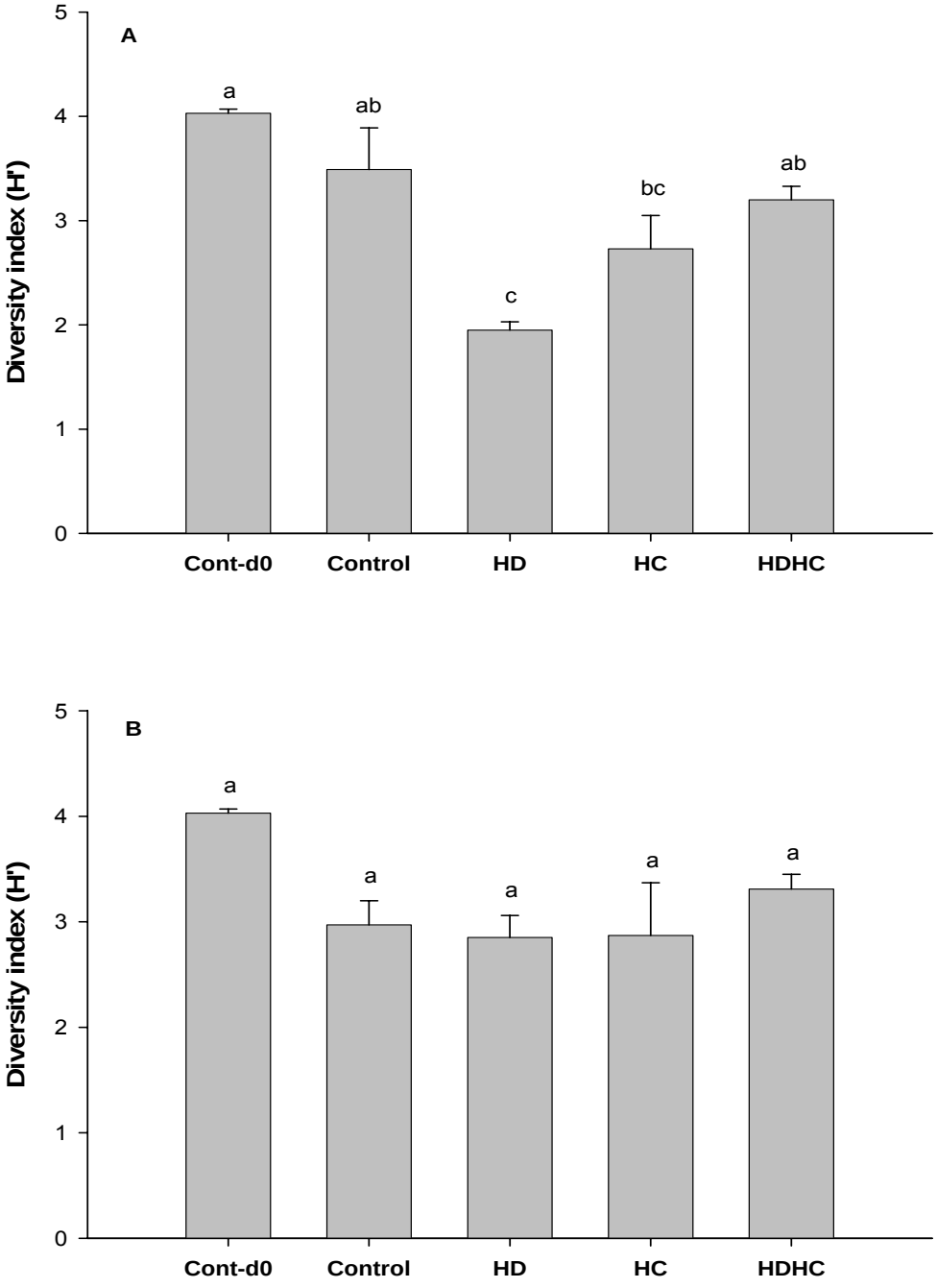

Figure 3.2 Diversity index (H') of the benthic diatom assemblages on day 5 (A) and 20 (B). Cont d-0 = day 0, HD = High Diesel, HC = High Copper, HDHC = High Diesel-High Copper. Error bars represent + SD (n = 2).

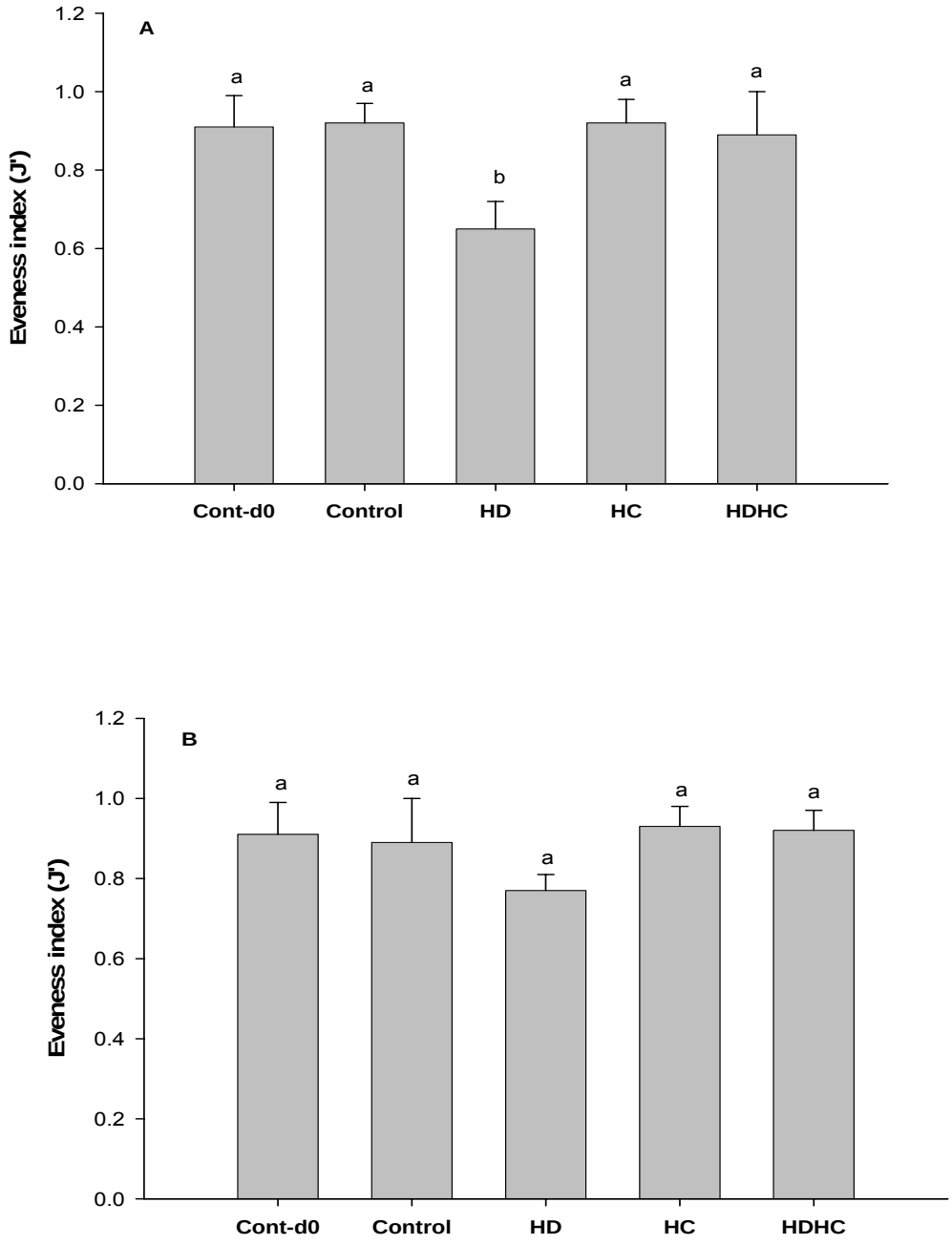

Figure 3.3 Evenness (J') of the benthic diatom assemblages in different treatments on day 5 (A) and 20 (B). Cont d-0 = day 0, HD = High Diesel, HC = High Copper, HDHC = High Diesel-High Copper. Error bars represent + SD (n = 2).

Table 3.2. Bray-Curtis similarity index between treatments. HD = High Diesel, HC = High Copper, HD-HC = High Diesel + High Copper.

	Day 5	Day 20
Control vs HD	55	60
Control vs HC	44	53
Control vs HDHC	59	51
HD vs HDHC	48	44
HC vs HDHC	59	62

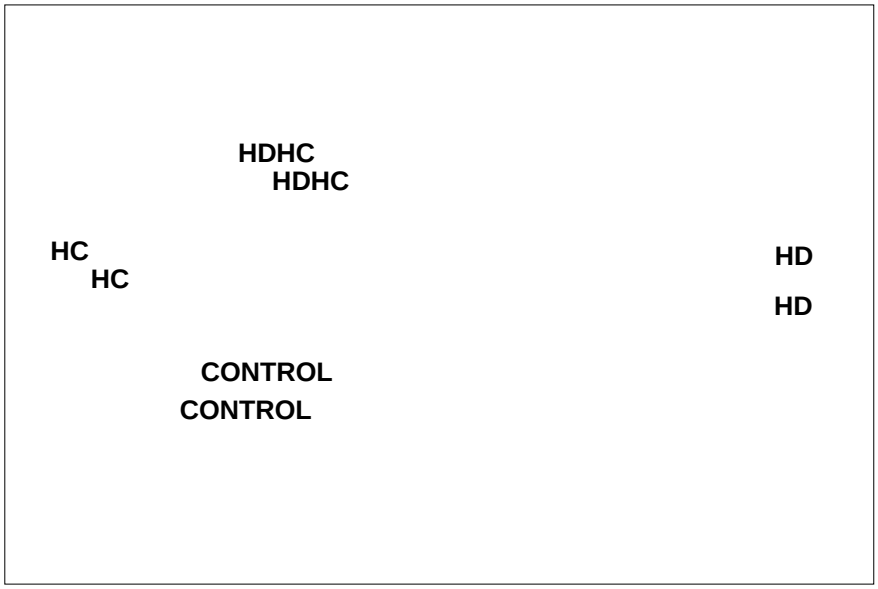

Figure 3.4. Multidimensional scaling (MDS) analysis of diatom assemblages on day 5. Stress = 0.01. HD = Diesel, HC = Copper, HDHC = Diesel + Copper.

Control treatment were due mainly to increases in abundance of *Bacillaria paxillifer* in the HD treatment. The ranked dissimilarities between the Control and HC treatments was 16% and was due to decreases in abundance of *Navicula flanatica* and *Navicula tripunctata* and increases in abundances of *Ttryblionella punctata* and *T. granulata* in the HC treatment. The highest ranked dissimilarity (27%) between the HD and HDHC treatments were due mainly to increases in abundance of *B. paxillifer* in the HD treatment. The ranked dissimilarity between the HC and HDHC treatments (15%) was due to increase of abundance of *Campylosira alexandrica* in the HDHC treatment and to the absence of *N. tripunctata* in the HC treatment.

The On day 20, the multidimensional scaling (MDS) ordination of the diatom assemblages revealed that HD still formed a separate group from the Control, and from HC and HD-HC treatments, and the Control treatment formed a separate group from the HC and HDHC treatments, which formed another group (Fig. 3.5). The SIMPER indicated that the dissimilarity (20%) between the HD and Control treatment was due to decrease in abundance of *Nitzschia liebetruthii* and increase of *B. paxillifer* in the HD treatment. The dissimilarity (17%) between the HD and HDHC treatment was due to increases of *B. paxillifer* in the HD treatment. The highest ranked dissimilarity between the HC and Control treatment (22%) was due to increases of *B. paxillifer* in the Control treatment. The absence of *B. paxillifer* and *Amphora exigua* in the HC treatment, and the decrease of *Navicula phyllepta* in the HDHC treatment contributed to the dissimilarity (16%) between HC and HDHC treatments.

DISCUSSION

The purpose of this study was to determine the influence of diesel and copper

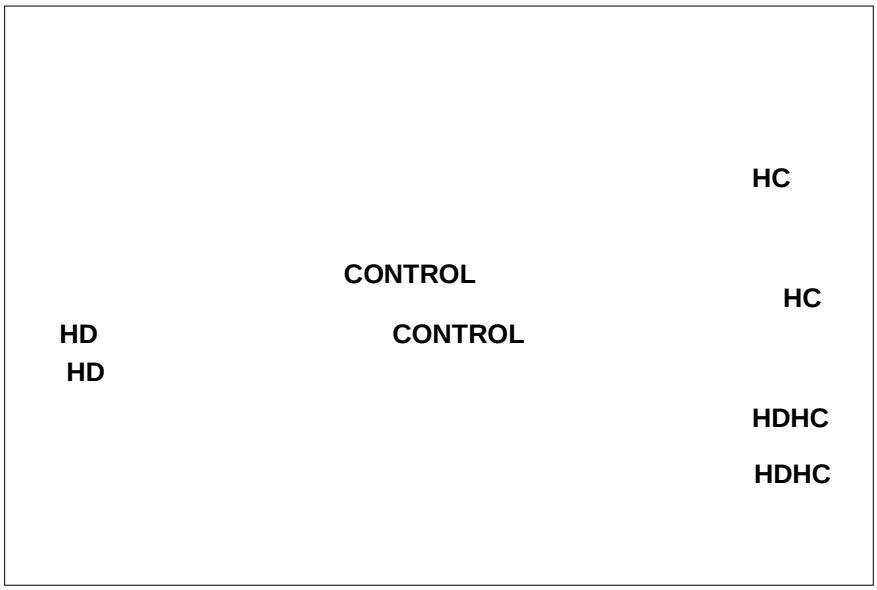

Figure 3.5. Multidimensional scaling (MDS) analysis of diatom assemblages on day 20. Stress = 0.01. HD = Diesel, HC = Copper, HDHC = Diesel + Copper.

contaminants on the assemblage composition of the benthic diatom assemblage from a Louisiana salt marsh. The benthic diatom flora at my study site (Cocodrie, Louisiana) has not been described before. All dominant taxa possessed a well-developed raphe system on at least one valve. The possession of a locomotion system has obvious advantages in an environment where deposition of sediments is a continual process and microgradients in moisture and essential growth elements may exist (Sullivan, 1978). This may explain the dominance of the flora by *Navicula* and *Nitzschia* species in this salt marsh. The diatom assemblage found in Control sediment (i.e., prior to the experimental addition of contaminants) was very similar to those of other Louisiana salt marshes (Parsons, 1998; Cook, 1982) and to salt marshes from Delaware (Sullivan 1975), New Jersey (Sullivan, 1977a) and Mississippi (Sullivan, 1978). In Control treatments there was a mixed assemblage of species comprised primarily of *Navicula* and *Nitzschia*. Sullivan (1978) has hypothesized that a uniform diatom assemblage exists along the Atlantic and Gulf Coast of North America. The diatom assemblage found in sediment surface from Cocodrie supports this hypothesis.

In the HD treatment the diatom assemblage was dominated by *B. paxillifer,* which is a large colony-forming diatom (individual cells 100 -150 μm in length); their relative abundance was 44% of the total diatom assemblage in HD treatment on day 5 and 33% on day 20. *B. paxillifer* was the only species that bloomed in the HD treatment. Previous studies have shown that in diesel-fuel contaminated sediment and in sediments where grazing was experimentally reduced there was a significant enhancement of large diatoms (Carman et al., 2000), which suggested that grazing pressure was primarily responsible for that enhanced algal biomass. Results from feeding experiments involving *Coullana*

sp., an abundant harpacticoid copepod at my study site, indicated that *Coullana* did not feed preferentially on *B. paxillifer* (S.Silva, unpublished data). However, there are many other other copepod species that might consume large diatoms. Furthernematodes have the ability to puncture diatom frustules and remove cell contents (Romeyn et al., 1983; Moens and Vincx, 1997). Nematode abundance at my study did not significantly change in the HD treatment (Chapter 2), and previous experiments involving this benthic community have indicated that grazing by nematodes is relatively minor (Carman et al., 1996). Consequently, factors other than grazing activity were probably involved in the bloom of *B. paxillifer* observed in my study.

Microalgal blooms in diesel–contaminated sediments have been related to enhanced nitrogen availability (Carman et al., 2000). Enhanced microalgal growth depletes the excess, naturally available NH_4^+ within a few days (Carman et al., 2000; Fleeger et al., 2006), at which time the microbial community becomes N-limited. Further growth of the microbial community is maintained by the enhanced NH_4^+ production in diesel–contaminated sediments (Nyman, 1999; Carman et al., 2000). I am unaware of previous studies that have examined the response of *B. paxillifer* to different nutrients, but my results suggest that this might be a fruitful area of study. The increase of only *B. paxillifer* in diesel–contaminated sediment suggests that the motility, size, and growth form (colony-forming) gives an advantage to this species in diesel-contaminated sediments and that *B. paxillifer* is a diesel-tolerant species. The average diversity value of the natural benthic assemblage studied was 4.10 bits ind^{-1} and this value decreased significantly in the HD treatment compared with the Control treatment. Diversity values in Mississippi salt marshes ranged from 3.35 to 4.26 bits ind^{-1} (Sullivan, 1978). In a

Delaware marsh H' ranged from 4.03 to 4.69 bits ind^{-1}, whereas in a New Jersey marsh H' ranged from 3.96 to 4.83 bits ind^{-1} (Sullivan, 1977). The predominance of *B. paxillifer* in the HD treatments probably caused the decrease in H' and in J' on day 5. Diversity and Evenness in treatments containing copper did not differ significantly from Controls, although the number of taxa significantly decreased on day 5 and 20 in the HC treatment. However, BMA biomass was significantly decreased in contaminated sediment with HC and HD-HC (Chapter 4).

Bray-Curtis similarity values indicates that benthic diatom assemblages were structurally similar in all treatments. The highest similarities were observed in HC and HD-HC treatments (58%) and Control and HD-HC treatments (59%) on day 5. Control and HD treatments, as well as HC and HDHC treatment had the similarity indexes of 60 and 62%, respectively on day 20. These results indicate that the benthic diatom assemblages were more similar than dissimilar in HC and HD-HC treatments on day 5 and 20. The most abundant diatom species in those treatments were *T. punctata* and *T. granulata.* Control and HD treatment were more structurally similar, probably because *B. paxillifer* relative abundance was 11% in the Control treatment and 33% in the HD treatment. The abundance of other species as *N. flanatica* and *N. tripunctata* decreased in the HC treatment, indicating the differential response of the diatom assemblage to copper-contaminated sediment.

Long-term exposures to inorganic chemical stress, such as that produced by copper, can cause a replacement of metal-sensitive species by metal-tolerant ones (Foster, 1982; Deniseger et al., 1986). Therefore, an early reduction in overall abundance can be followed by recovery from growth of pollution-tolerant species. Although the diversity

was not significantly decreased in HC and HDHC treatments, there was an increase in the abundance of *T. punctata* and *T. granulata* compared with the Control treatment. It is possible that this is a tolerant taxon. Metal tolerance would enable these species to increase in abundance as the result of reduced competition from contaminant-sensitive species.

It has previously been suggested that small algal species become dominant in communities exposed to chemical stress (Kinross et al., 1991). Increased abundances of small *Navicula* spp. have been related to organic enrichment and eutrophication (Kelly and Whitton, 1995) as well as zinc and cadmium pollution (Ivorra et al., 1999). In my study *Navicula* species did not increase in the presence of contaminants, and the major change observed in HD treatments was from increased abundance of *B. paxillifer*, a very large diatom. Thus, my results do not support the hypothesis that chemical stress favors small algal species.

Consistent with my result of decrease in a diversity index (H') in the HD treatment, this index has been previously reported to vary as a result of exposure to petroleum hydrocarbons (Vargo et al., 1982; El-Dib et al., 2001). In the same way, compositional differences with changes in the presence or absence of species have previously been observed between control communities and those exposed to either light crude oil or diesel-based oil cuttings (Plante-Cuny et al., 1993). In my study *B. paxillifer* was absent only in the HC treatment.

Metal contamination has also been shown to influence the structure of diatom communities, resulting in lower diversity and richness but increased dominance values (Crossey and La Point, 1988). Within my study copper caused a reduction of species

richness compared with the Control treatment, but did not an influence the diversity index. In contrast, the combined effect of diesel and copper (HD-HC) did not cause a significant effect on diversity or richness on day 5 compared with Control, which could indicate that the combination of diesel and copper caused an effect on the physiology of the microalgae.

Most previous studies of benthic diatom responses to chemical contaminants have examined freshwater, not marine species (Ivorra et al., 1999; Ivorra et al., 2002; Ruggiu et al., 1998). Total petroleum hydrocarbon concentrations used here caused a significant change in the composition of diatom community by causing a bloom of *B. paxillifer.* In contrast, copper did not change significantly the diversity of the diatom assemblage. My results show that the presence of diesel and copper contaminants in sediments could significantly affect the structure of the benthic diatom community, and therefore significant effects in the entire benthic community, including the microbial community. My results also show the need for autecological studies of specific benthic diatom species, such as *B. paxillifer.*

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CHAPTER 4

EFFECTS OF DIESEL-FUEL AND COPPER CONTAMINANTS ON THE BIOMASS AND CARBOHYDRATE PRODUCTION BY BENTHIC MICROALGAE

INTRODUCTION

Benthic microalgae (BMA) are composed of chlorophytes, euglenoids, cyanobacteria and diatoms that inhabit the top centimeters of intertidal sediment in estuarine and coastal systems (Kelly et al., 2001). Diatoms are typically the dominant taxon (Admiraal, 1984; Underwood & Krompkamp, 1999). BMA provide the principal source of nutrition that fuels secondary production in shallow coastal systems (Sullivan and Moncreiff, 1990) and can contribute up to 50% of the total primary production in estuarine systems (Sullivan and Moncreiff, 1988; Cahoon, 1999; Underwood and Kromkamp, 1999). BMA have great ecological significance in estuarine sediments despite inhabiting sediments with a narrow photic zone (mm) (Consalvey, 2004), limited periods of light exposure (Serodio and Catarino, 1999), and highly variable and potentially stressful temperatures and irradiances (Blanchard and Guarini, 1996; Perkins et al., 2001; Underwood, 2002). BMA can also influence the microstructure and properties of sediment, due to the secretion of extracellular polymeric substances (EPS) (Decho, 1990, Underwood et al., 1995), which may increase sediment stability (Paterson, 1988; Sutherland, 1998; Underwood and Paterson, 2003).

The motile epipelic diatoms secrete copious amounts of EPS, over the plasmalemma and into the surrounding sediment, as a function of movement (Edgar and Pickett-Heaps, 1984). Furthermore, low-temperature scanning electron microscopy (LTSEM) has revealed biofilms of epipelic diatoms dominating the surface of many intertidal sediments (Paterson, 1995). The extracellular carbohydrates exudates produced by benthic diatoms can be methodologically separated into different fractions using precipitation and ultrafiltration techniques (Smith and Underwood, 2000; de Brouwer and Stal, 2001). Water extractable fractions have been term "colloidal carbohydrates" (Underwood et al., 1995; Smith and Underwood, 1998; Taylor and

Paterson, 1998; Staats et al., 1999). In diatom-rich bofilms, around 20-25% of the colloidal carbohydrates material present is EPS (Underwood and Paterson, 2003). EDTA-extractable fractions constitute the material that is more tightly bound to the sediment, probably through bridging with divalent cations (Stal, 2003).

Estuarine sediments around the world are a repository for many contaminants from anthropogenic sources. One of the main pollutants in marine environments is petroleum hydrocarbons (National Research Council, 1985). Among petroleum hydrocarbons, diesel-fuel is considered to be highly toxic because it is enriched in polycyclic aromatic hydrocarbons (PAH; National Toxicology program, 1986), the most toxic component of petroleum hydrocarbons (Clark, 1989; Kennish, 1992). Much of the high molecular weight PAH released into aquatic environments accumulates in estuarine sediments (Kennish, 1992) and is resistant to bacterial metabolism (Clark, 1989). PAH becomes associated with suspended fine hydrophobic particles and are ultimately deposited in the benthos (Connell and Miller, 1984; Readman et al., 1984; Ko and Baker, 1995). In the long term, estuarine sediment that contains large pools of organic matter can be a significant repository for PAH (Pereira et al., 1999).

Information about the direct effects of hydrocarbons on BMA is scarce. Most of the information comes from studies on phytoplankton. In aquatic environments, algal species exhibit differential sensitivity to toxic pollutants and petroleum hydrocarbons have been shown to be inhibitory to the growth of some sensitive species while promoting the growth of tolerant ones (Morales-Loo and Goutz, 1990), including nuisance algal species (Capone and Bauer, 1992). The response of some green algae to hydrocarbons has been examined. For example, the exposure of *Selenastrum capricornutum* to fuel oil water extracts and aromatic hydrocarbons gnificantly decreased the carbohydrate and protein content of algal cells (El-Dib et al., 1997;

Herman et al., 1991).

Various studies have shown that PAHs can have direct (toxic) or indirect (ecological) effects on BMA communities (Bott et al., 1978; Farke et al., 1985; Plante-Cuny et al., 1993). For example, PAHs indirectly benefit BMA production and abundance via reduced grazing by meiofauna and macrofauna (Farke et al., 1985; Plante-Cuny et al., 1993; Carman et al., 1997, 2000). Also, PAH may affect BMA in a negative manner; Piehler et al. (2003) reported a decrease in primary productivity and alteration in photophysiology of BMA in the presence of PAHs.

Heavy metals are another main class of contaminants in coastal sediments. Among them, copper represents one of the more prevalent and toxic (Babu et al., 2001). All algae have a copper micronutrient requirement for growth and reproduction; however, excess copper is highly toxic to most algae because of its oxidative potential (Joux-Arab et al., 2000). By binding with sulfhydryl groups, copper inhibits a large number of enzymes involved in photosynthetic carbon fixation, chlorophyll synthesis, electron transport, and photophosphorylation (Fisher and Jones, 1981; Rijstenbil et al., 1994).

Little is known about the ecotoxicological interaction of copper and diesel-fuel, and their joint-effects on BMA. Millward et al. (2004) studied the joint effects of heavy metals and diesel contamination on a salt-marsh benthic invertebrate community. They concluded that it is difficult to predict the combined effects of metals and diesel contamination on benthic communities based on knowledge of the effects of metals or diesel alone. Metals and hydrocarbon contamination in aquatic sediments could significantly affect BMA by changing their abundance and distribution and are likely to have significant effects on benthic food webs in coastal systems because of the important role of BMA as a food source for many benthic invertebrates. Therefore, effects of contaminants on BMA communities have potentially major implications for the ecosystem that

these primary producers support. The overall hypothesis of this study was that the presence of diesel and copper cause an influence in the production of carbohydrates by BMA, and that influence could be visualized in a micro-scale. Also, I hypothesized that the combination of diesel and copper cause different effect than either contaminant individually. Thus, the purpose of this study was to examine the influence of diesel-fuel and copper contaminants on the ecophysiology of the BMA community from a Louisiana salt marsh. Here, I report the influence of contaminants on carbohydrates and BMA biomass. Combinations of copper and diesel were employed to determine if responses to mixed contaminants were distinct from responses to either copper or diesel alone.

MATERIALS AND METHODS

Study Site

Microcosms of natural sediment communities (Carman et al., 1995, 1997, 2000) were collected from mudflats in a *Spartina alterniflora* saltmarsh located in the Terrebone Bay estuary near the Louisiana University Marine Consortium in Cocodrie, Louisiana to examine the influence of diesel and copper contaminants on benthic microalgae (BMA), Tidal range in the estuary is approximately 0.3 m and salinity ranges from 1 to 20 psu. Sediments have a median grain size of 38 μm, an organic carbon content of approximately 2.5%, and are composed primarily of silts (41%) and clays (17%) (Chandler and Fleeger, 1983). The study site is located in a region of hydrocarbon production and drilling activity, and both commercial and recreational boat traffic is high (Carman et al., 1997).

Experimental Design

The microcosm experiment was designed to study the effects of two concentrations of both copper and diesel, individually and in combination. Twenty-seven sediment microcosms

(15.2 cm i.d. polyvinyl chloride pipe with windows cut in the sides above sediment level and covered with Nitex 63-μm mesh to allow exchange of water; Carman et al., 1995) were collected from exposed sediment to a depth of 15 cm at low tide on May 22, 2002. Microcosms were transferred to wet tables with circulating water and flooded with 2 to 3 cm of ambient marsh water, which was replaced daily to excess with filtered (63 μm) marsh water. Each core was aerated gently using an aquarium air stone. Temperature was maintained at 25 ± 2 °C, and microcosms were illuminated (70 μmol $m^{-2}s^{-1}$) on a 14:10 light: dark cycle with banks of 40-W fluorescent lights.

Treatments included a Control (no contaminants added), low diesel (LD), high diesel (HD), low copper (LC), high copper (HC), low diesel-high copper (LD-HC), low diesel-low copper (LD-LC), high diesel-high copper (HD-HC) and high diesel-low copper (HD-LC). Treatment concentrations were based upon levels known to produce sublethal and lethal responses in benthic communities (Carman et al., 1997; Millward et al., 2001). Three replicates were used per treatment.

Preparation of Contaminated Sediment

Surface sediment was contaminated with diesel (Carman et al., 1996, 1997) and copper (Millward et al., 2001). Diesel fuel was added to a sediment aliquot (1 part diesel to 3 parts sediment v:v) in an amber glass bottle and tumbled on a roller mill for 10 days. The sediment was then allowed to settle overnight. Unadsorbed diesel was aspirated from the bottle and 12 psu artificial sea water (ASW) was added. This rinsing procedure was repeated three times. The sediment-water slurry was transferred to 35-mL glass centrifuge tubes and centrifuged at 1700 g for 3 min. The supernatant was removed and replaced with fresh ASW. The sediment and water were mixed thoroughly, and then centrifuged. The supernatant was decanted again, and the

process was repeated for a total of four rinses. The sediment was then recombined into a single batch and mixed to assure homogeneity. This sediment was used as the high diesel (HD) spike. A sub sample was diluted to one third of this concentration (1 part high diesel to 2 parts control sediment v:v) to produce the low diesel (LD) spiked sediment.

Copper-contaminated sediments by adding copper solutions to sediment over a period of 2 h using a dripping gravity-feed apparatus while mixing vigorously with a food blender.

Experimental Procedure

Experimental treatments consisted of the initial addition of 25 mL of contaminated sediment to the overlying water in each microcosm. No contaminated sediment was added to the Control treatment. An additional 2.5 mL of sediment was added each day until the end of the study (20 days). Uncontaminated sediment was added to Control microcosms. These sediment additions created a sediment layer approximately 1 mm thick, which was quickly (within a few hours) mixed into the top few mm of sediment by bioturbation of benthic fauna.

Samples were collected on days 0, 5, and 20 during the day. Sediment cores were collected using the cryolander coring method (Wiltshire et al., 1997). The cryolander was placed on the surface of the sediment, and approximately 10 mL liquid nitrogen was gently poured onto absorbent cotton. The cotton was at ambient temperature, which caused the liquid nitrogen to vaporize. The cold vapor sank through the gauze and froze the sediment surface without physical distortion. After approximately 3 min of freezing under nitrogen vapor additional liquid nitrogen was added and sediment was allowed to freeze for approximately 6 min. Frozen sediment samples were removed, wrapped in aluminum foil, and stored in a –80 °C freezer. Prior to sectioning, the cryolander samples were cut into pieces of approximately 1 cm^2 using a lapidary saw. Subsequently, a microtome (Cryo-Cut, American Optical Company) was used to

section the top 1 mm of sediment into five 200-μm layers and the second 1 mm into 2 layers of 500 μm each. Samples were lyophilized prior to analysis of Chlorophyll *a* and carbohydrates (n=3). In addition, separate cores (3.5 cm i.d.) subsamples were collected for the analysis of concentrations of total sediment-associated copper and total petroleum hydrocarbons. The top 1 cm of the core was used for the analysis.

Copper Concentrations

One gram of dry, finely ground sediment was digested by refluxing with 5 mL trace-metal grade concentrated HNO_3 at 120 °C for 6 h. Following digestion, the HNO_3 volume was decreased to1.5 mL and samples were diluted with deionized water to 35 mL. Samples were then shaken, allowed to settle for 24 h and the supernatant analyzed on an ICP-OES (Inductively Coupled Plasma, Optical Emission Spectrometer)

Hydrocarbon Concentrations

Hydrocarbons were extracted using EPA standard method 3550C (US EPA, 1996). Thirty grams of sample was spiked with a surrogate mixture (d_{14}-terphenyl, 5α-androstane, d_{10}-phenanthrene) to assess extraction efficiency, dried with anhydrous Na_aSO_4 and sonicated three times in excess dichloromethane for 15 min. Combined extracts were filtered and reduced to 1 ml. TPHs were analyzed with Gas Chromatography using a Flame Ionization Detector (GC/FID) and using EPA standard method 8015C (US EPA, 1996). Gas chromatography was performed using a Hewlett Packard 5890 G**.**

Chlorophyll *a* Analysis

Photosynthetic pigments were extracted by sonicating each sample for 30 s in 1.3 mL 100% HPLC acetone (Fisher Scientific), bubbled with nitrogen gas (to remove oxygen), and incubated overnight in the dark at –20 °C. The extracts were centrifuged for 10 min at 1500 g,

filtered twice using syringe filters (Sun International: diameter 13 mm, pore size 0.2 μm) and stored in the dark at –80 °C. Pigment extracts were analyzed using a Hewlett Packard 1100 High Performance Liquid Chromatography (HPLC) consisting of a 100-μL loop auto sampler, a quaternary solvent delivery system coupled to a diode array spectrophotometer, and a fluorescent detector. More detailed information about HPLC configuration and extraction methods can be found in Buffan-Dubau and Carman (2000).

Carbohydrate Analysis

Carbohydrate analysis was performed according to de Brouwer and Stal (2001). Freeze-dried samples were extracted with 400 μL distilled water for 1 h at 30 °C. The extract was centrifuged for 5 min at 6000 x *g*, and the colloidal carbohydrates fraction of the supernatant was determined. Subsequently, the pellet was incubated with 500 μL 0.1 M Na_2EDTA for 16 h at room temperature. The extract was centrifuged for 5 min at 6000 x *g* and the EDTA-extractable carbohydrate fraction of the supernatant was assayed.

Carbohydrate was measured spectrophotometrically using the phenol-sulfuric acid method (Dubois et al., 1956). Glucose was used as the reference. Two hundred μL of 5% (w/v) phenol and 1 mL concentrated sulfuric acid were added to 200 μL sample, and the mixture was incubated for 35 min. Results were expressed as $\mu g\ g^{-1}$ dry wt of sediment.

Statistical Analysis

Statistical analysis was performed on log (n+1)-transformed data. A Proc Mixed procedure was used to do an analysis of variance, using a Split-Plot design with a 3 x 3 x 3 x7 factorial arrangement and all remaining interactions, followed by Tukey's posteriori comparisons. Treatments, day, and layer were treated as fixed factors while replicate was treated as random factor. Comparisons between treatments were performed using depth-integrated

biomass and carbohydrate data, unless stated otherwise. One-way ANOVA was used to determine if microalgal biomass (Chl *a*) differed among layers within each treatment. All statistical analyses were performed using SAS 9.1 software.

RESULTS

Contaminant Concentrations

The final concentration of TPHs in the HD contaminated sediment spike was 293 ± 1.00 (SD) mg kg sediment^{-1}, and for the LD contaminated sediment spike it was 179 ± 6.6 (SD) mg kg sediment $^{-1}$. Contaminated sediment was added to microcosms with the objective of achieving a final concentration in the top 1 cm of sediment of 29 and 18 mg kg sediment^{-1}. The background concentration of TPHs in the sediment, where the microcosms were collected, was 0.27 ± 0.12 mg kg sediment^{-1}. After 20 days, the concentrations of TPHs were lower than those at the beginning of the experiment. TPHs in the HD and LD combined with copper did not differ significantly from the HD and LD treatments alone (Chapter 2, Fig. 2.1, $p > 0.10$).

The background concentration of copper in the Control sediment at day 0 was 44 ± 5 mg kg sediment^{-1}. The nominal concentrations of treatments containing copper at the beginning of the experiment in the top 1 cm of sediment were 2190 mg kg sediment^{-1} for the HC and 1095 mg kg sediment^{-1} for the LC treatments. After 20 days, the HC and LC treatments did not significantly differ from HDHC, LDHC, LDHC and LDLC treatments (Chapter 2, Fig 2.1, $p > 0.22$).

Benthic Microalgal Biomass (Chl a)

Day 5

ANOVA revealed highly significant effects of copper, diesel, sediment layer and their

interactions on Chl *a* concentration at day 5 (Table 4.1). When considering the entire depth profile, Chl *a* in the High Diesel (HD) treatment was significantly higher than all other treatments ($p < 0.0003$, Fig. 4.1). The Chl *a* concentration in the HC treatment was significantly lower than in the Control, HDHC and LDHC treatments ($p < 0.05$).

Vertical distributions of Chl *a* on day 5 were analyzed within each treatment to determine if contaminants influenced the vertical profile of BMA. In the Control treatment, Chl *a* concentration was highest in the top layer and declined down to a depth of 1000 μm, from where it remained almost constant to 2000 μm (Fig. 4.2). There was a significant difference between the top layer (0-200 μm) and the bottom two layers (1000-1500, and 1500- 2000 μm) ($p < 0.02$, Table 4.2), but there were no significant differences between other layers ($p > 0.20$, Table 4.2).

Chl *a* concentration in the HD treatment decreased steeply from the top layer (200 μm) with increasing depth (Fig. 4.2). Chl *a* concentrations in first two layers (200 - 400 μm) were not significantly different from each other ($p > 0.1490$), but there was a significantly difference between the top layer (200 μm) and bottom layers (600 -2000 μm) ($p < 0.0033$, Table 4.2). In the Low Diesel (LD) and High Copper (HC) treatments, and in all treatments containing the mixtures of diesel with copper (HD-HC, HD-LC, LD-HC, LD-LC), Chl *a* concentration did not differ significantly among layers ($p > 0.073$, Table 4.2). Copper eliminated significant HD differences between the top and bottom layers. In the LC treatment, Chl *a* concentration was not significantly different between the 200 and 400 μm layers ($p = 0.1973$), but Chl *a* concentration in the top layer (200 μm) was significantly higher than Chl *a* in the bottom layers (600- 2000 μm, $p < 0.05$, Table 4.2).

As previously noted, there was a significant interaction between Diesel, Copper and Layer factors (Table 4.2). Therefore, comparisons between different treatments were performed

Table 4.1. Summary results from ANOVA performed on Chl *a* concentration on day 5.

Effect	F	p-value
Copper	19.91	< 0.0001
Diesel	11.72	0.0005
Copper*Diesel	7.62	0.0009
Layer	17.03	< 0.0001
Copper*Layer	7.55	< 0.0001
Diesel*Layer	3.41	0.0003
Copper*Diesel*Layer	2.58	0.0005

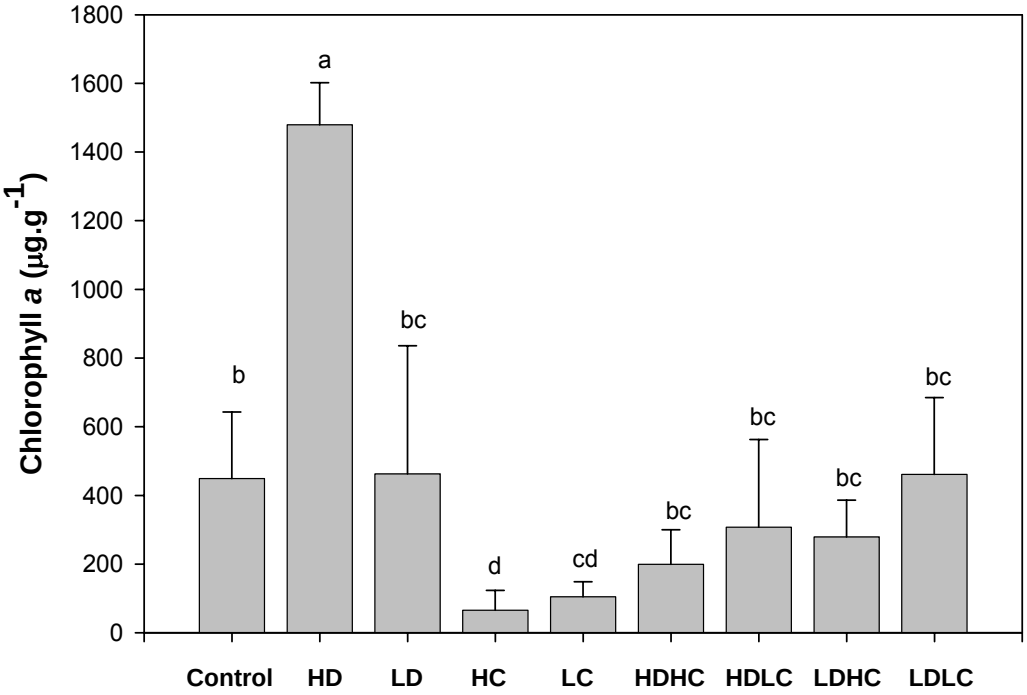


Figure 4.1. Chl *a* concentrations in the top 2 mm of sediment on day 5. Values are means + 1 SD (n = 3). Different letters indicate significant differences ($p < 0.05$). HD = High Diesel, LD = Low Diesel, HC = High Copper, LC: Low Copper, HDHC = High Diesel-High Copper, HDLC = High Diesel-Low Copper, HC = Low Diesel-High Copper, LDLC = Low Diesel-Low Copper.

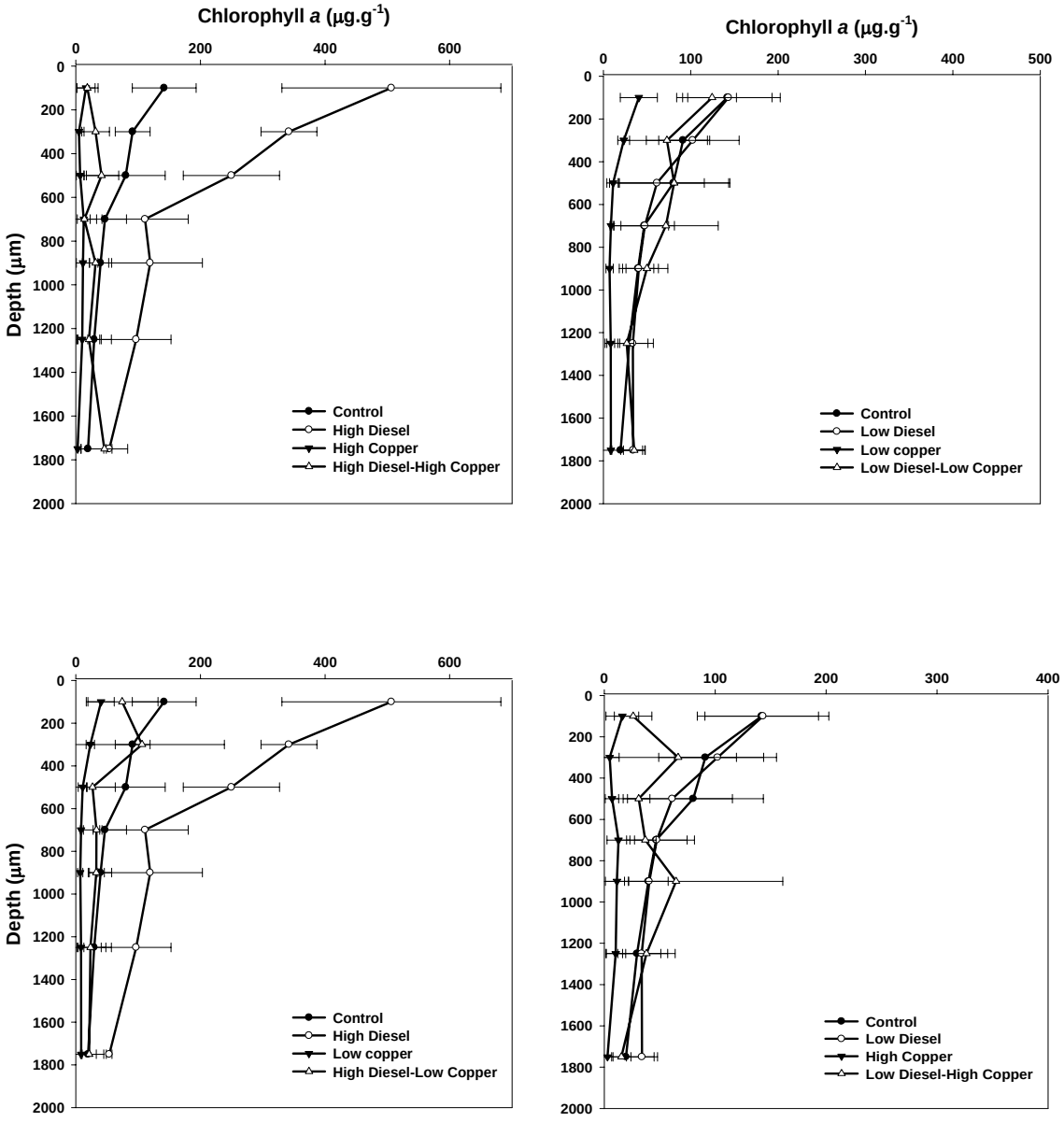


Figure 4.2. Vertical profiles of Chl *a* on day 5. Values are means ± 1 SD (n = 3).

Table 4.2. Tukey's pairwise comparisons of chlorophyll-*a* between different layers in each treatment on day 5. Different letters indicate significant differences, ns = no significant differences.

	Layers (μm)						
	0-200	200-400	400-600	600-800	800-1000	1000-1500	1500-2000
Control	a	ab	ab	ab	ab	b	b
HD	a	ab	bc	bc	bc	c	c
LD	ns						
HC	ns						
LC	a	ab	b	b	b	b	b
HC	ns						
HDLC	ns						
LDHC	ns						
LDLC	ns						

by layer (Table 4.3). Chl *a* concentration in the HD treatment was significantly higher than in all other treatments in each of three uppermost layers (Fig 4.2, $p < 0.0001$, Table 4.3). From layers spanning 800 to 2000 μm, there were no significant differences among treatments ($p > 0.05$).

Day 20

As observed in Day 5, ANOVA revealed that Chl *a* concentrations were significantly influenced by the effects of copper, diesel, layer and their interactions on day 20 (Table 4.4). When considering the entire depth profile only HD treatment differed significantly from the rest of the treatments; Chl *a* concentration in HD treatment was significantly higher than all other treatments, including Control (Fig. 4.3). Chl *a* concentration in the LD treatment did not significantly differ from Control, but did differ significantly from all other treatments (Fig. 4.3). Chl *a* concentrations in HC, HD-HC and LD-HC treatments were very low and they differed significantly from most of the other treatments (Fig 4.3). The distribution of Chl *a* was also examined within each treatment by layer. In Control sediment significant differences in Chl *a* concentration were observed only between the top layer (200 μm) and the three bottom layers (1000-2000 μm, Table 4.5). Chl *a* concentration in HD treatment was significantly higher in the top layer (200 μm) than the bottom layers (1500-2000 μm, Table 4.5). There were no significant differences between Chl *a* concentration in layers up to 800 μm (Table 4.4). Chl *a* concentration in the HD treatment decreased steeply from the surface to a depth of 800 μm, from where it remained constant with increasing depth (Fig. 4.4). As observed on day 5 there were no significant differences in Chl *a* concentration among different layers in the LD and HC treatments ($p > 0.32$, Fig. 4.4, Table 4.5). Chl *a* concentration in the LC treatment did not differ significantly among layers ($p = 0.31$, Table 4.5).

Among the combinations of diesel and copper, only the combination of LD-LC showed

Table 4.3. Tukey's pairwise comparisons of chlorophyll-*a* between different treatments by layer on day 5. Different letters indicate significant differences, ns = no significant differences.

Layer (μm)	Control	HD	LD	HC	LC	HDHC	HDLC	LDHC	LDLC
0-200	a	b	a	a	a	a	a	a	a
200-400	a	b	a	a	a	a	a	a	a
400-600	a	b	a	a	a	a	a	a	a
600-800	ns								
800-1000	ns								
1000-1500	ns								
1500-2000	ns								

Table 4.4. Summary results from multiple comparisons ANOVA performed on Chl *a* on day 20

Effect	F	p-value
Copper	29.83	< 0.0001
Diesel	11.46	0.0006
Copper*Diesel	10.97	0.0001
Layer	10.09	< 0.0001
Copper*Layer	6.15	< 0.0001
Diesel*Layer	2.35	0.0103
Copper*Diesel*Layer	3.16	< 0.0001

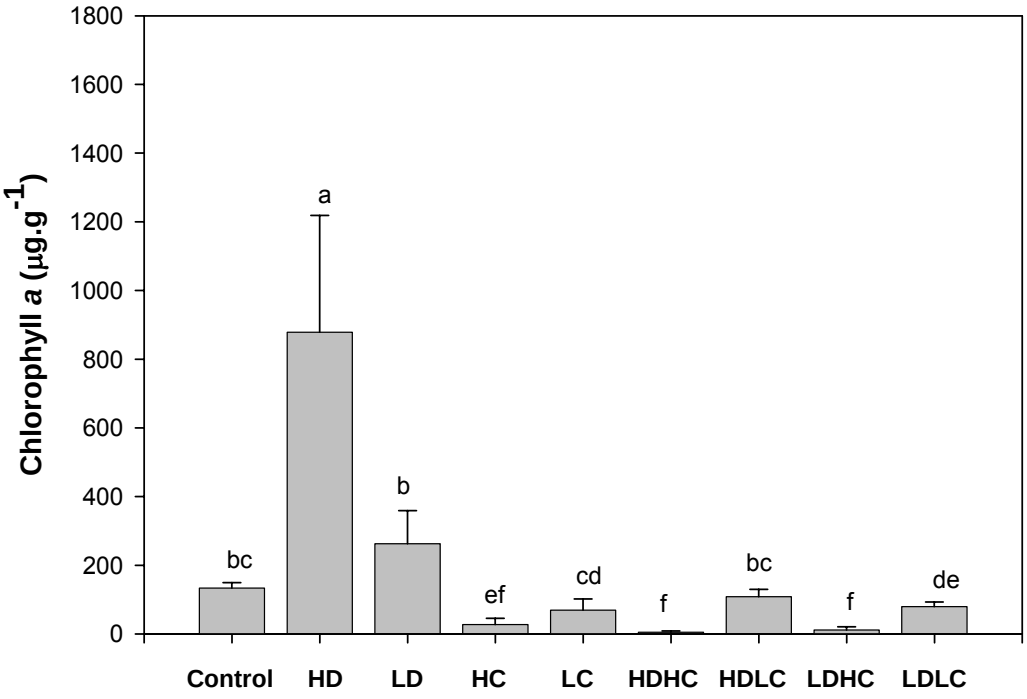


Figure 4.3. Chl *a* concentrations in the top 2 mm of sediment on day 20. Values are means + 1 SD (n = 3). Different letters indicate significant differences ($p < 0.05$). HD = High Diesel, LD = Low Diesel, HC = High Copper, LC = Low Copper, HDHC = High Diesel-High Copper, HDLC = High Diesel-Low Copper, LDHC = Low Diesel-High Copper, LDLC = Low Diesel-Low Copper.

significant differences among layers (Fig. 4.4, Table 4.5). Chl *a* concentration in the top layers (0-400 μm) were significantly higher than the bottom layers (800-2000 μm, $p < 0.04$). Copper eliminated the significant differences among layers observed in HD treatment.

As in day 5, all factors and their interactions (copper, diesel, and layer) had a significant effect on Chl *a* concentration (Table 4.4). Therefore, another analysis was performed to compare treatments by layer. The analysis revealed highly significant differences between Chl *a* concentration in HD treatment and all other treatments in the top five layers ($p < 0.0001$, Table 4.6). There were no significant differences between the other treatments in other layers ($p > 0.99$, Table 4.6).

Production of Carbohydrates on Day 5

ANOVA revealed highly significant effects of copper, diesel, copper-diesel interaction and layer on water-extractable carbohydrates concentration on day 5. There were no significant interactions between layer and copper or diesel (Table 4.7). Tukey pair-wise comparisons revealed significant differences between the LC and the Control and HDLC treatments (Fig. 4.6, $p < 0.003$) and between the HC treatment and the Control, LDHC and HDHC treatments (Fig. 4.6, $p < 0.05$). No significant differences were detected among the other treatments ($p > 0.06$). In the case of EDTA-extractable carbohydrates no significant effect was observed for copper or diesel, or their interaction with layer (Fig. 4.6, Table 4.8). There was a significant difference in the vertical distribution of both water- and EDTA-extractable carbohydrates among different layers in all sediment samples, as indicated by the significant effect detected in the Layer factor (Table 4.7, 4.8). Both fractions of carbohydrates were generally evenly distributed throughout the top 800 μm of sediment, then, gradually decreased to a depth of 2000 μm (Fig. 4.6).

When considering the entire depth profile (2 mm) there were no significant differences

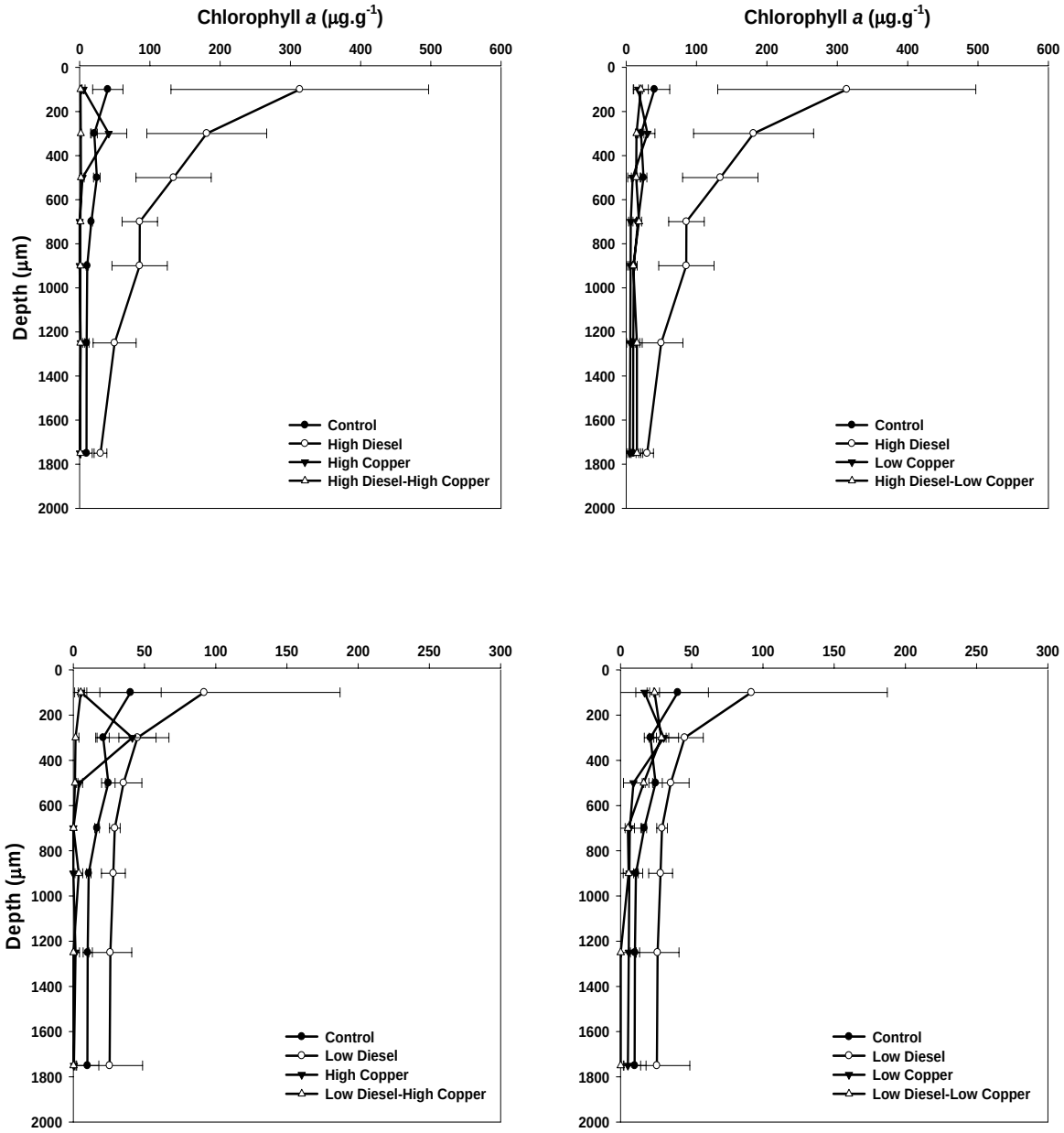


Figure 4.4. Vertical profiles of Chl *a* on day 20. Values are means ± 1 SD (n = 3).

Table 4.5. Tukey's pairwise comparisons of chlorophyll-*a* between different layers in each treatment on day 20. Different letters indicate significant differences, ns = no significant differences.

Layers (μm)	0-200	200-400	400-600	600-800	800-1000	1000-1500	1500-2000
Control	a	ab	ab	ab	b	b	b
HD	a	ab	ab	ab	ab	b	b
LD	ns						
HC	ns						
LC	ns						
HDHC	ns						
HDLC	ns						
LDHC	ns						
LDLC	a	a	ab	bc	bc	c	c

Table 4.6. Tukey's pairwise comparisons of chlorophyll-*a* between different treatments by layer on day 20. Different letters indicate significant differences, ns = no significant differences.

Layer (μm)	Control	HD	LD	HC	LC	HDHC	HDLC	LDHC	LDLC
0-200	a	b	a	a	a	a	a	a	a
200-400	a	b	a	a	a	a	a	a	a
400-600	a	b	a	a	a	a	a	a	a
600-800	ac	b	c	a	ac	a	ac	a	ac
800-1000	a	b	a	a	a	a	a	a	a
1000-1500	ns								
1500-2000	ns								

between different treatments for water-extractable carbohydrates (Fig. 4.5, $p = 0.36$) and EDTA-extractable carbohydrates (Fig. 4.5, $p = 0.35$). EDTA-extractable carbohydrate concentrations were generally higher compared to the concentration of water-extractable carbohydrate in all treatments (Fig. 4.5).

Production of Carbohydrates on Day 20

Both water- and EDTA-extractable carbohydrate concentrations were significantly influenced only by layer on day 20 (Table 4.9, 4.10). Considering the entire depth profile (2 mm) water- and EDTA-extractable carbohydrate concentrations did not differ significantly among treatments ($p > 0.05$, Fig. 4.7).

As observed on day 5, EDTA-extractable carbohydrate concentrations were generally higher compared to the concentration of water-extractable carbohydrate in all treatments (Fig. 4.8). Although no significant differences were observed in water- and EDTA-extractable carbohydrates, both fractions of carbohydrate were evenly distributed from the sediment surface down to 1000 μm, then the concentration declined in the bottom layers (1000-2000 μm); the only exception was the HD treatment, where there was an increase of carbohydrates in the 400 μm layer (Fig. 4.8).

Relationship between Water-extractable Carbohydrate and Chlorophyll *a*

Linear regression analysis of $\log_{10}$-transformed data showed a statistically significant relationship between Chl *a* and water-extractable carbohydrate in HD ($r^2 = 0.65$, $p = 0.0002$, $n = 3$), LD ($r^2 = 0.39$, $p = 0.0024$, $n = 3$) and HC ($r^2 = 0.27$, $p = 0.02$, $n = 3$) treatments on day 5 (Fig. 4.9). There was also a statistically significant linear relationship between Chl *a* and EDTA-extractable carbohydrate in HD ($r^2 = 0.63$, $p < 0.0001$, $n = 3$) and LD ($r^2 = 0.52$, $p = 0.002$, $n = 3$)

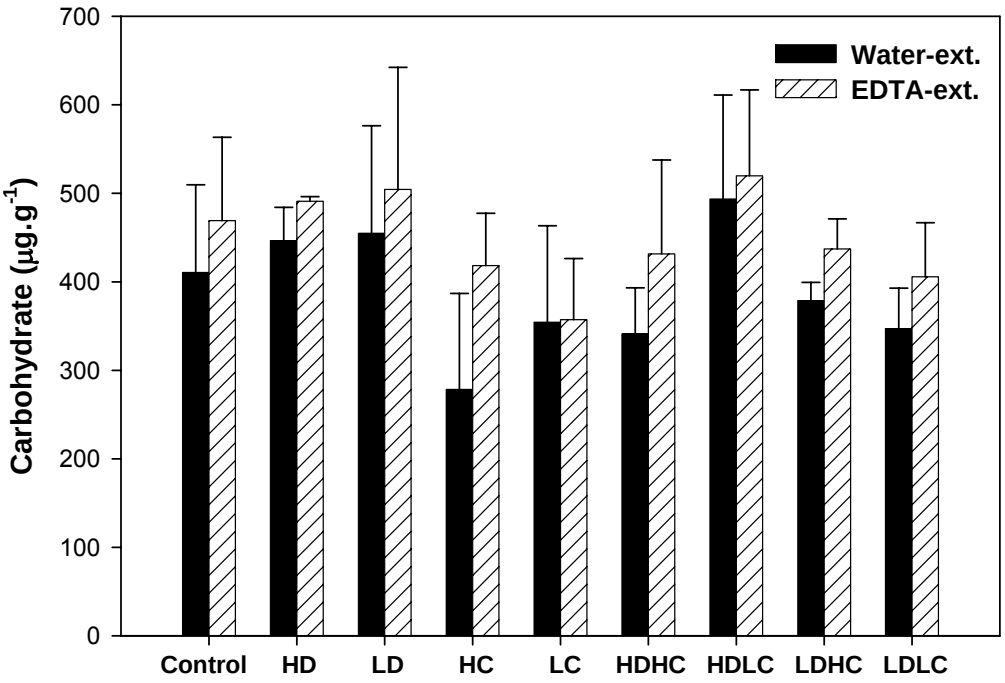


Figure 4.5. Water- and EDTA-extractable carbohydrates on day 5. Bars are means + 1 SD (n = 3). HD = High Diesel, LD = Low Diesel, HC = High Copper, LC = Low Copper, HDHC = High Diesel-High Copper, HDLC = High Diesel-Low Copper, LDHC = Low Diesel-High Copper, LDLC = Low Diesel-Low Copper.

Table 4.7. Summary results from multiple comparisons ANOVA performed on water-extractable carbohydrates on day 5

Effect	F	p-value
Copper	15.90	< 0.0001
Diesel	4.76	0.0104
Copper*Diesel	5.36	0.0006
Layer	9.86	< 0.0001
Copper*Layer	1.76	0.0636
Diesel*Layer	1.64	0.0895
Copper*Diesel*Layer	0.99	0.4900

Table 4.8. Summary results from multiple comparisons ANOVA performed on EDTA-extractable carbohydrates on day 5.

Effect	F	p-value
Copper	2.72	0.0793
Diesel	9.89	0.7125
Copper*Diesel	7.23	0.1533
Layer	3.14	0.0140
Copper*Layer	1.06	0.4219
Diesel*Layer	0.45	0.9285
Copper*Diesel*Layer	0.82	0.6896

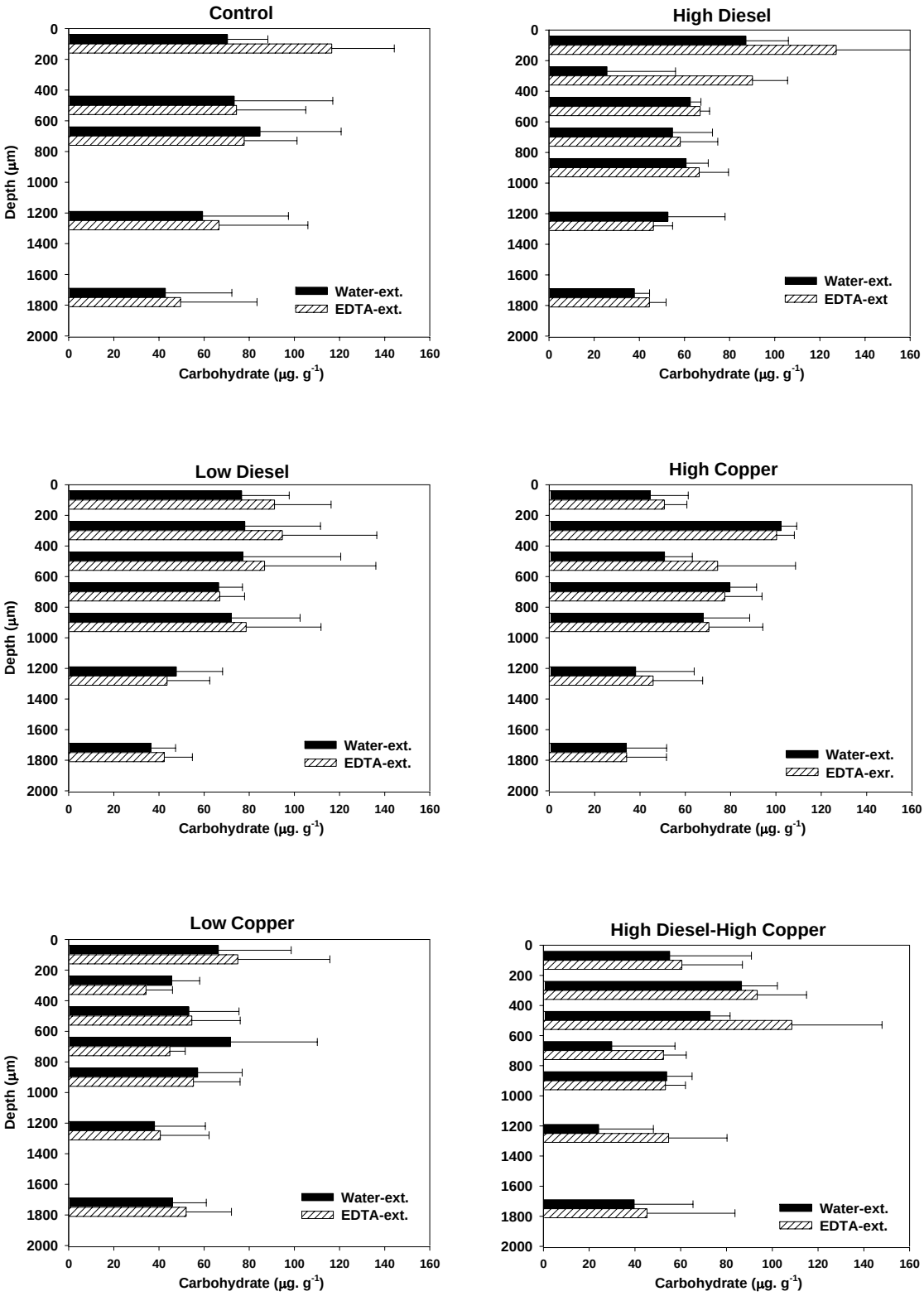
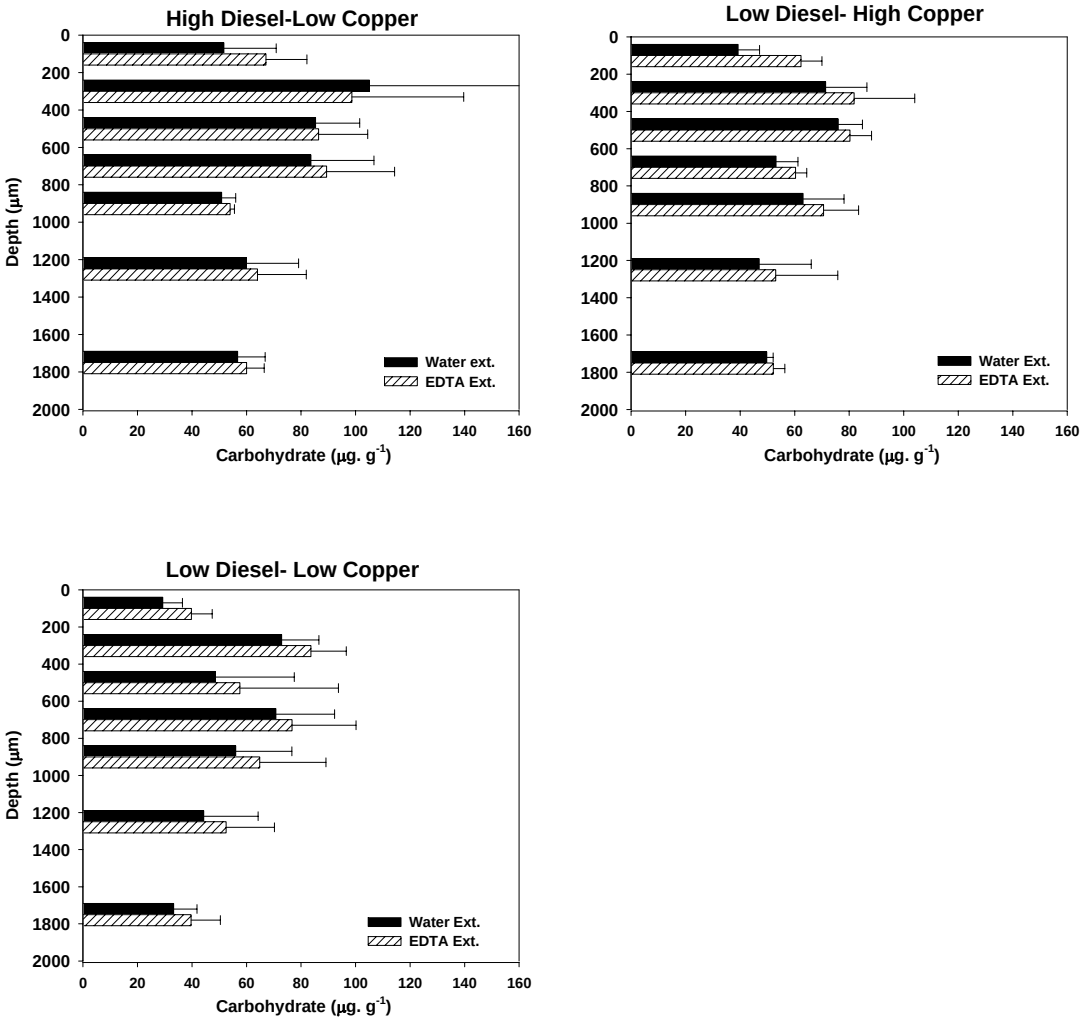


Figure 4.6. Carbohydrate concentration on day 5. Bars are means + 1 SD (n = 3).



(Figure 4.6. continued).

Table 4.9. Summary results from multiple comparisons ANOVA performed on water-extractable carbohydrates on day 20.

Effect	F	p-value
Copper	2.81	0.0656
Diesel	2.31	0.1024
Copper*Diesel	4.52	0.0723
Layer	13.82	< 0.0001
Copper*Layer	1.26	0.2508
Diesel*Layer	0.77	0.6843
Copper*Diesel*Layer	0.62	0.9148

Table 4.10. Summary results from multiple comparisons ANOVA performed on EDTA-extractable carbohydrates on day 20

Effect	F	p-value
Copper	1.38	0.5160
Diesel	3.19	0.3679
Copper*Diesel	3.76	0.3669
Layer	4.74	0.0001
Copper*Layer	0.43	0.9504
Diesel*Layer	0.38	0.9699
Copper*Diesel*Layer	0.36	0.9981

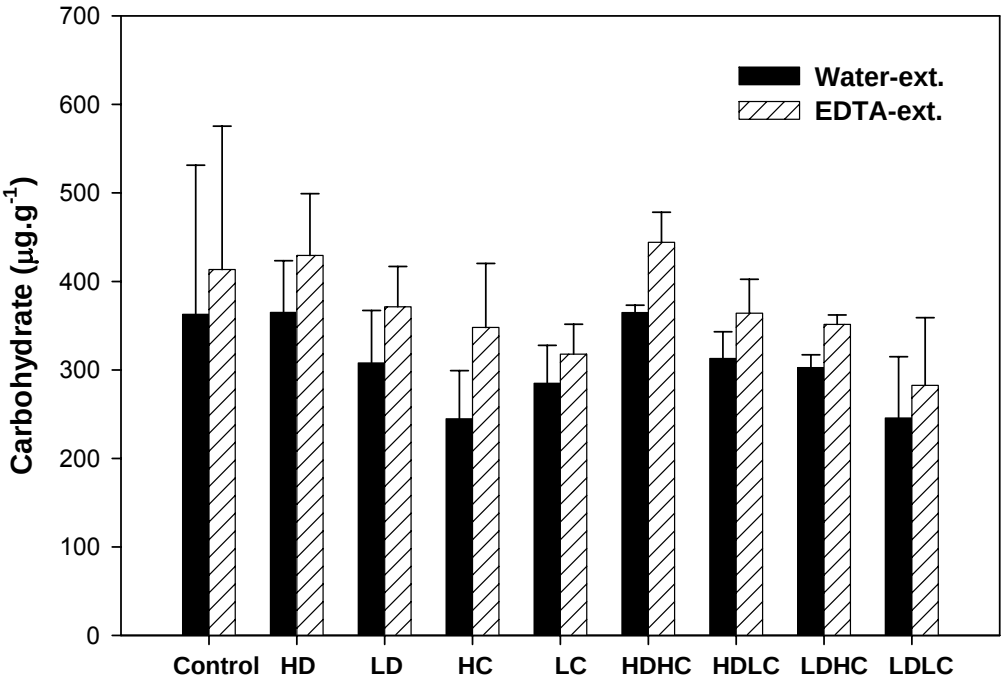


Figure 4.7. Water- and EDTA-extractable carbohydrates on day 20. Bars are means + 1 SD (n = 3). HD = High Diesel, LD = Low Diesel, HC = High Copper, LC = Low Copper, HDHC = High Diesel-High Copper, HDLC = High Diesel-Low Copper, LDHC = Low Diesel-High Copper, LDLC = Low Diesel-Low Copper.

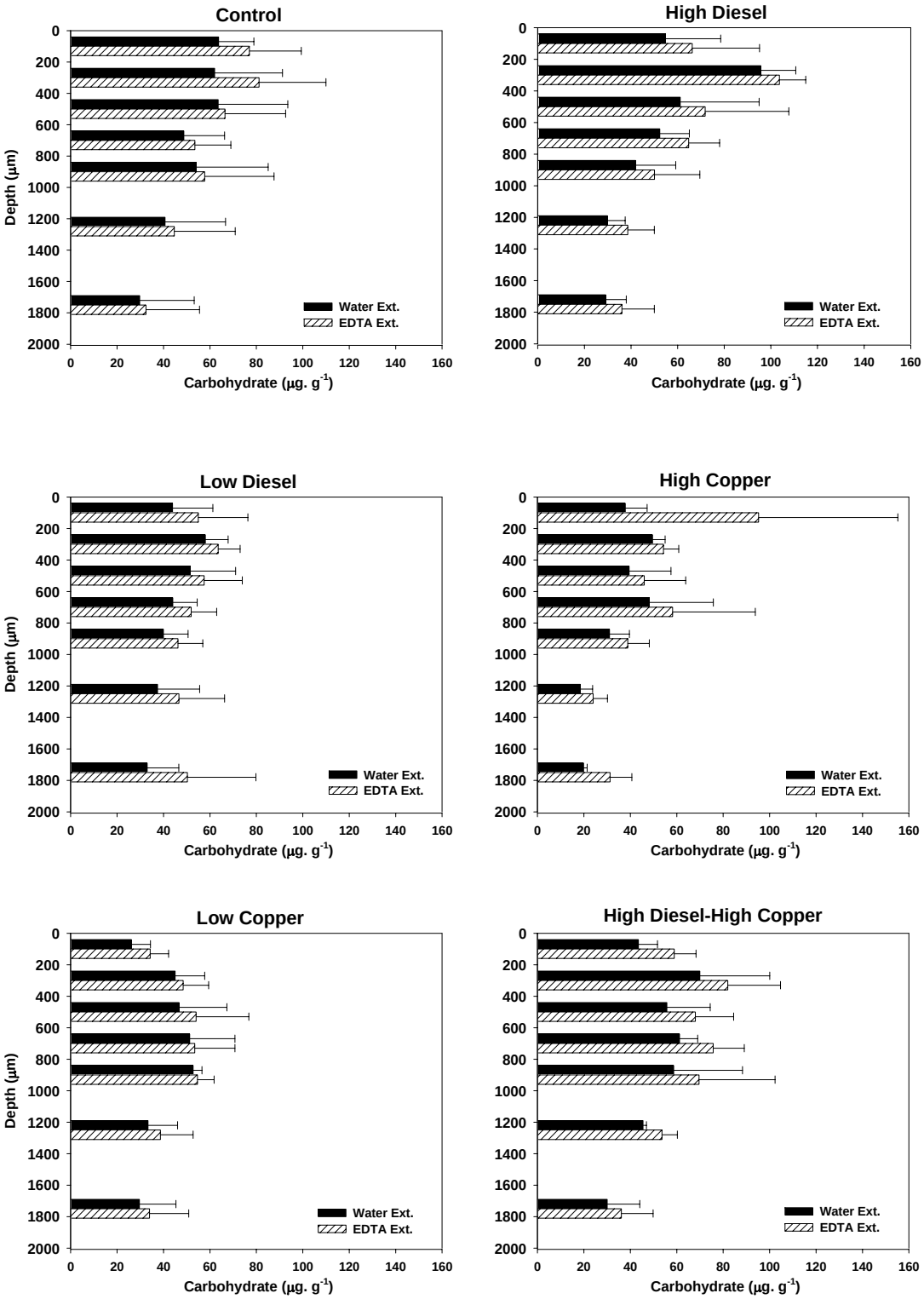
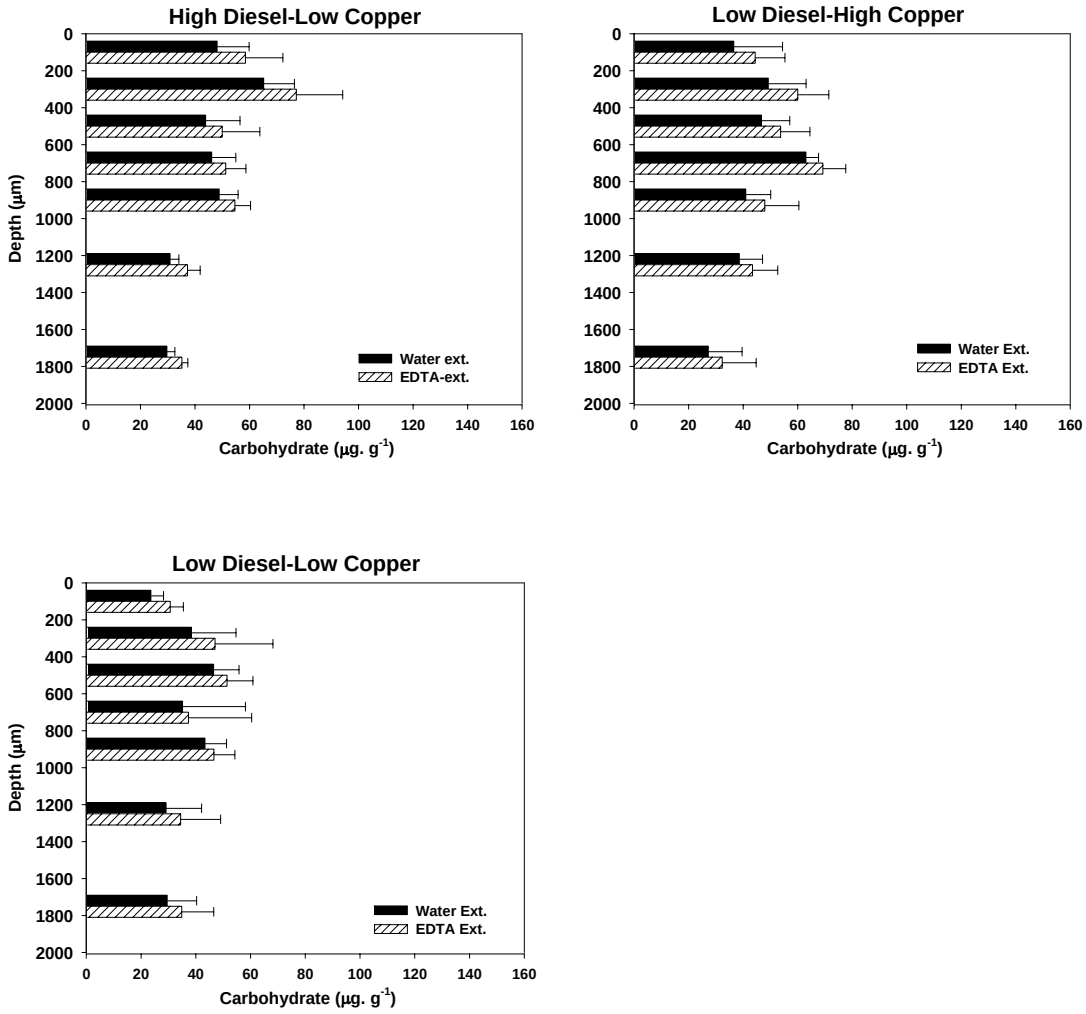


Figure 4.8. Carbohydrate concentration on day 20. Bars are means + 1 SD (n = 3).



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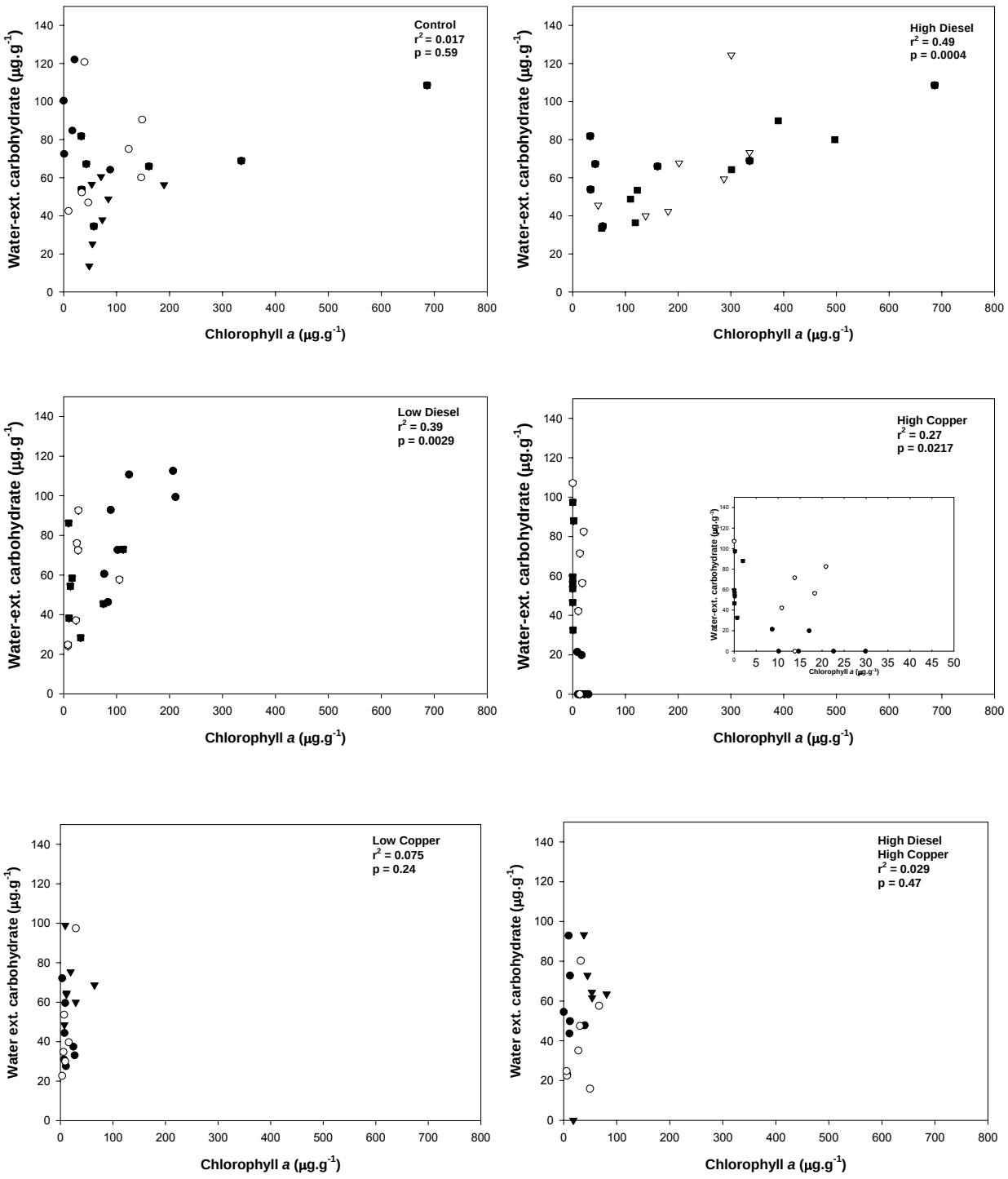
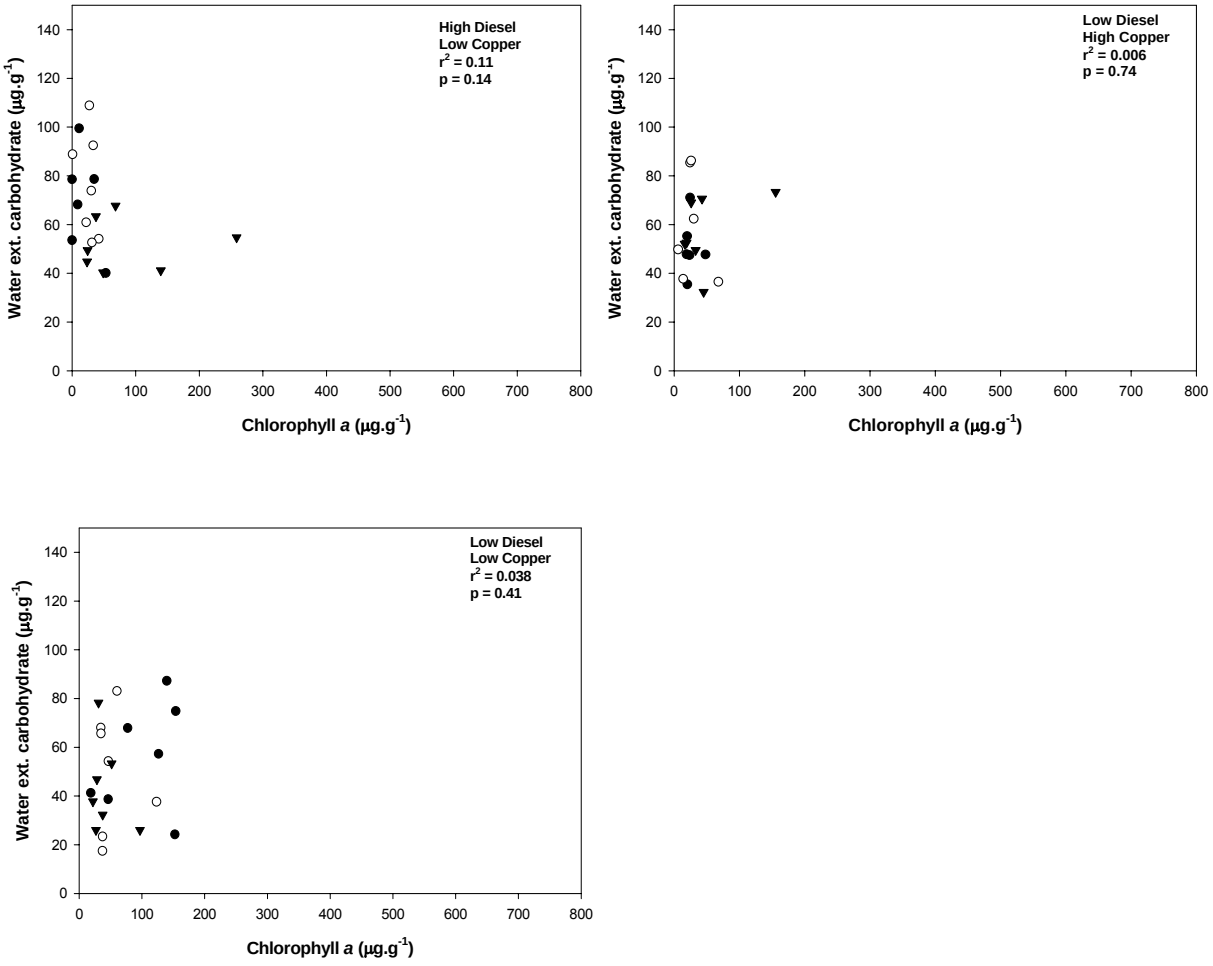


Figure 4.9 Relationship between water-extractable carbohydrate and Chlorophyll *a* on day 5. Different symbols indicate 3 different replicates.



(Figure 4.9. continued).

on day 5 (Fig. 4.10). There were no significant linear relationships between the water-extractable carbohydrate and Chl *a* in other treatments on day 5, and no significant relationships were observed in any treatment on day 20.

DISCUSSION

The main goal of this study was to determine if diesel-fuel and copper contaminants influence the carbohydrate production and the vertical distribution of BMA community in a Louisiana salt marsh. By studying the effects of those contaminants on BMA, we can further understand fundamental relationships between BMA and the grazers that feed upon them. Thereis evidence from previous experiments that the BMA assemblage was influenced by contaminated sediment (Carman et al., 1995, 1997, 2000), but these experiments were not conducted using the fine-scale approach I used here.

Contaminant Concentrations

TPHs concentrations in the top 1 cm of sediment were 29 and 17 mg kg sediment^{-1} in the HD and LD treatments, respectively. These concentrations are relatively modest concentrations (Long, 1992), but higher than the "effects range low" (ERL) concentrations for PAH reported by Long (1995). Concentrations at or above the ERL, but below the ERM (Effects Range Median), represent a range within which effects occasionally occur. At concentrations above the ERM, frequent effects are expected (Long, 1995). The background concentration of TPHs in the sediment, where the microcosms were collected, was 0.27 mg kg sediment^{-1}, and this concentration is consistent with background concentration reported previously at my site (Carman et al., 1995). After 20 days, the concentrations of TPHs in the treatments were lower than at the beginning of the experiment (Fig. 2.1), and lower than the ERL. This decrease

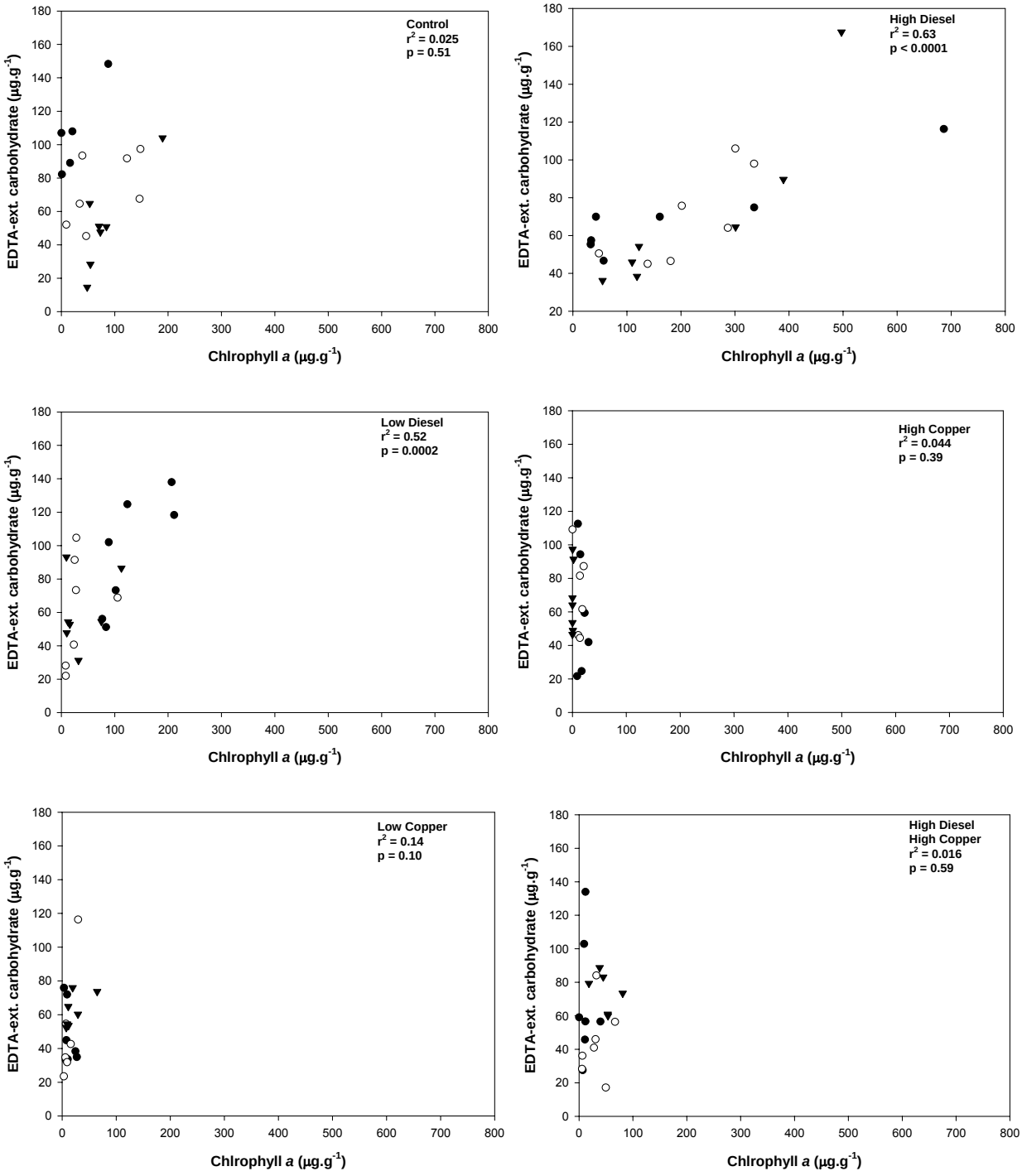
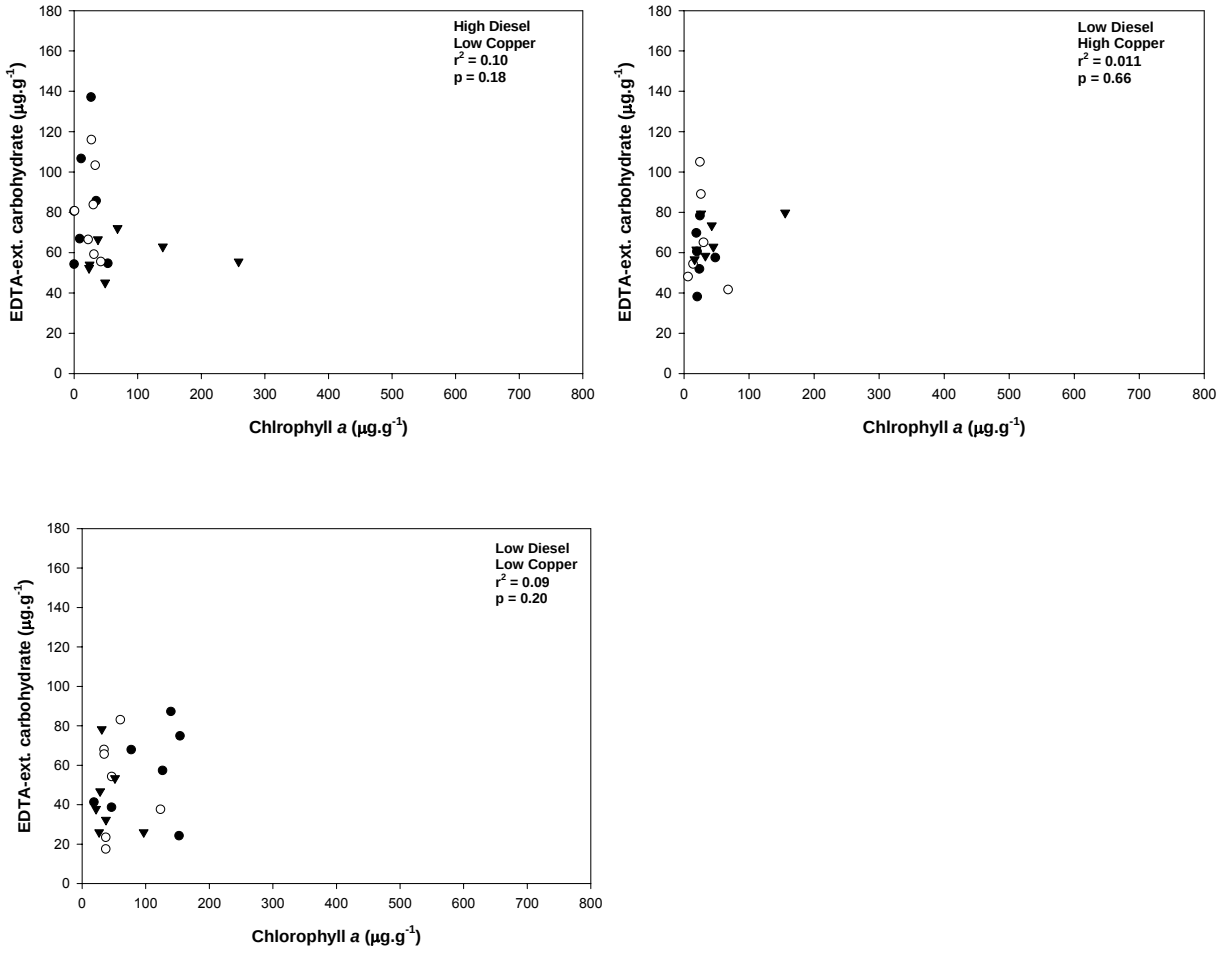


Figure 4.10. Relationship between EDTA-extractable carbohydrate and Chlorophyll *a* on day. Different labels indicate 3 different replicates.



(Figure 4.10. continued).

was likely due to bacterial degradation of hydrocarbons, and has been reported previously (Bennet et al., 1999).

The background concentration of copper in the Control sediment at day 0 was 44 ± 5 mg kg sediment^{-1}. The total copper concentrations used in this study were higher than the ERM reported from Long (1995). Measured concentrations of total copper and TPHs after 20 days indicated that neither diesel nor copper had an influence on each others concentration. The choice of copper concentrations used in this study was based on previous observations that exposures to 1200 ppm caused a decrease in abundance of copepods and ostracods, but had no significant effect on nematodes (Millward et al., 2001). The literature dealing with the effects of contaminants on BMA is scarce. Underwood and Paterson (1993) showed that in a biocide-treated site, the decline in Chl *a* concentrations corresponded to a decline in the dominant species of diatoms. Diatoms exposed to heavy metals often appear to alter their growth, forming closely adpressed cushion-like colonies (Miles, 1984). Barranguet et al. (2002) noted that heavy metals compromised the photo-physiological performance of diatoms. Also, it has been shown that the rate of movement of epipelic forms varies with the concentration of the metal (Underwood and Paterson, 2003). In one study, diatoms showed a consistent decrease in locomotive ability as the concentration of metal in the overlying medium increased (Underwood and Paterson, 2003).

Chl *a* on Day 5 and 20

The surface layer of sediment is an area of intense biogeochemical activity that establishes chemical and biological gradients over a micro-scale (Kelly et al., 2001). The 200-µm scale of resolution examined here allows distinctions to be made between

Chl *a* measured within and below the photic zone of the sediment, which is usually limited to 1-1.5 mm in muddy sediment (Paterson et al., 1998). In addition, it has been shown that the study of sediment surfaces on a micro-scale enhances the understanding of erosional processes and the cohesive properties of sediments (Yallop et al., 1994; Paterson, 1995).

The fine-scale distribution of BMA and their exudates has been studied previously (Taylor et al., 1999; Kelly et al., 2001; Paterson et al., 2000, 2001; de Brouwer and Stal, 2001; Perkins et al, 2001; Underwood et al., 2005). Taylor and Paterson (1998) observed a log-linear decrease in colloidal carbohydrate with depth in the upper 2 mm of sediment. Similarly, both Chl *a* and colloidal carbohydrates content were greater in the surface 0.2 mm of the sediment, and decreased with depth in three European estuaries (Kelly et al., 2001). De Brouwer et al. (2001) showed that the production of carbohydrate was confined to the top 1 mm of sediment, and was highest in the top 0.2 mm. While the fine-scale distribution of BMA has been studied in natural conditions, the influence of contaminants on the microscale distribution of BMA has not been previously studied. This study represents one of the first studies evaluating the potential effects of contaminants on a microscale distribution of BMA.

When considering the entire 2 mm of sediment surface, the main finding in the present study was the significant increase of Chl *a* concentration in the HD treatment and the significant reduction of Chl *a* in the HC treatment on both day 5 and 20. The dramatic increase of Chl *a* in HD treatments agrees with results from previous studies (Carman et al., 1995, 1997), which suggested that the grazing activity in diesel-contaminated sediment was reduced, contributing to enhancement of algal biomass. Nutrient increase due to microbial activity could play also important role in diesel-contaminated sediment (Carman et al.,

2000). Results from my study suggest also a high degradation of diesel, because there was a significant reduction of TPHs after 20 days of the experiment, from 20 mg kg sediment^{-1} at the beginning of the experiment to a final concentration of 0.7 mg kg sediment^{-1} in the HD treatment (Chapter 2). This implies that the contamination with diesel may be changing the microbial ecology of the sediment, and that petroleum hydrocarbon could change the biogeochemistry of the entire ecosystem.

The response of algal species to different petroleum hydrocarbons can vary (Megharaj et al., 2000). For example, heavy-duty marine diesel oil (at 10% concentration) has been shown to prevent the growth of a marine microalgae *Isochrysis* sp., whereas crude oil at a similar concentration caused little effect on the growth of the same microalga (Ansari et al., 1997). In my study a bloom of *Bacillaria paxillifer* Gmelin occurred in the HD treatment (Chapter 3), suggesting this species is diesel-tolerant, or that conditions (release of nutrients- discussed in Chapter 2) created after the addition of diesel were appropriate to stimulate its growth.

While there was a significant increase of Chl *a* in the HD treatment, its concentration in the HC treatment was significantly decreased. Elevated concentration of copper in the sediment (2190 ppm) presumably caused a direct toxic effect on BMA by reducing cell division. This observation was consistent with that of Millward et al. (2001), who showed that in the presence of elevated concentrations of copper (~1,200 ppm) there was a saturation of the metal-sorbing capacity of the sediment. This caused an increased concentration of the more bioavailable and toxic form of copper (the free ionic form Cu^{2+}), resulting in direct toxic effects on the whole benthic community. Toxicity of copper on diatom growth has been previously reported (Tadros et al., 1990). Copper can also interfere with the action of the oxidizing site on photosystem II

(PSII) and has an inhibitory effect on photosynthesis (Cid et al., 1995). In contrast, it has been shown that *Amphora* is able to tolerate a high concentration of dissolved copper (Thomas and Robinson, 1986).

In the HDHC and HDLC treatments the presence of copper reduced the magnitude of the diatom bloom observed in the HD treatment. The biomass in all combinations of diesel with HC was higher than in the HC treatment on day 5, but lower than that in the HD treatment. These results imply that the direct toxic effect of copper stops the stimulation of growth observed in the HD treatment. After 20 days Chl *a* concentrations in the HC, HDHC, and LDHC treatments were drastically reduced relative to the Control. It is possible that after 20 days the diesel added to the sediment was lost to volatilization and/or microbial degradation and the toxic effect of copper was the stronger contaminant. The HC concentration (2500 ppm) was sufficiently toxic to suppress any stimulative effect of diesel. In contrast, the lower copper concentration used in the HDLC and LDLC treatments did not negatively affect the biomass of benthic microalgae, because Chl *a* concentrations were similar to those in the Control treatment on day 5. Chl *a* concentration in the LDLC treatments on day 20 was lower than in the LD treatment and the Control treatments, suggesting that the concentration of copper in the LDLC treatment caused an effect on reproduction, preventing BMA growth.

Both diesel and copper significantly influenced the vertical distribution of Chl *a*. In particular, the Chl *a* concentration in the HD treatment in the top layers (0 - 400 μm) was significantly higher than the concentration in the bottom layers (600 - 2000 μm), resulting in a visible diatom bloom on the sediment surface. The BMA accumulated in the top layers (400 μm) to obtain sufficient light for photosynthesis, but in doing so may have been exposed to higher diesel and copper concentration.

The vertical distribution of Chl *a* in the mixtures of diesel and copper did not differ significantly among different layers of the sediment, possibly because in the diesel + copper treatments the low biomass and high spatial variation influenced the distribution of biomass in the top 2 mm of sediment. This result is consistent with De Brouwer and Stal (2001), who found that when BMA biomass was low, no specific pattern in the vertical distribution of biomass was observed. The vertical distribution of Chl *a* in the HD treatment significantly differed from that in Control and in the combination of diesel and copper, because high levels of biomass were concentrated in the surface layers.

Carbohydrates on Day 5

Benthic epipelic (mud-dwelling) diatoms are known to produce copious amounts of Extracellular Polymeric Substances (EPS) consisting mainly of carbohydrates (Hoagland et al., 1993). The excretion of EPS plays a role in the movement of epipelic benthic diatoms (Edgar and Pickett-Heaps, 1984). It is assumed that a close positive relationship exists between epipelic diatom biomass and water-extractable (colloidal) carbohydrate concentrations (Smith and Underwood, 1998; De Brouwer and Stal, 2001). Therefore,I analyzed the distribution and concentration of carbohydrates produced by BMA.

Sediment carbohydrates were operationally separated into two fractions: water-extractable carbohydrates and EDTA-extractable carbohydrates. Water-extractable carbohydrate represents any extracellular polysaccharides (EPS) that are not tightly bound to the sediment, but which may be associated with the cells (Stal, 2003). Previous studies have shown that this fraction is composed of 80% monosaccharides and 20% polysaccharides (Underwood et al., 1995). EDTA-extractable carbohydrate is the material that is more tightly bound to the sediment, probably through bridging with divalent cations.

Water-extractable carbohydrates, which have been suggested to be indicative of microphytobenthic production (Underwood and Smith, 1998), were present in lower amounts than EDTA-extractable carbohydrates in all treatments including the Control. Water-extractable carbohydrates may represent a more dynamic fraction and be a strong indicator of epipelic diatom metabolism (Taylor et al., 1999). Several studies analyzing carbohydrate dynamics in natural conditions have also reported lower amounts of water-extractable carbohydrates compared to EDTA-extractable carbohydrates (Brouwer and Stal, 2001; Underwood et al., 1995; Yallop and Paterson, 1994). The difference in the amounts of both carbohydrate fractions could reflect intracellular sugars from benthic organisms and sugars in complex structures, such as cellulose, occurring in the sediment (Taylor et al., 1999). It has also been suggested that water-extractable carbohydrates are recently produced and have a short residence time, so that the carbohydrate is either washed away during immersion (Smith and Underwood, 1998; de Winder et al., 1999; Taylor et al., 1999) or that it is utilized by heterotrophic consumers (van Duyl et al., 1999; Middelburg et al., 2000). The negatively charged groups of polymers of carbohydrates interact with the charged surface of sediment particles and result in a strong binding (Stal, 2003). EDTA-extractable carbohydrates are recalcitrant and therefore accumulate in the sediment (de Winder et al., 1999), which is consistent with my observation of high amounts of EDTA-extractable carbohydrates in all treatments including the Control.

Contaminants significantly influenced water-extractable but not EDTA-extractable carbohydrate concentrations. The vertical distribution of both carbohydrate fractions did not differ significantly with depth in the 2 mm sediment surface. My results agree with those of de Brouwer et al. (2003) who found that carbohydrate content showed little variation with depth in one study performed in three intertidal mudflats in Western Europe (de Brouwer et al., 2003).

The similar distribution and concentration of both carbohydrate fractions in the 2 mm surface of the sediment could indicate a common source of these carbohydrates.

Although there was a significant increase in algal biomass in the HD treatment, the concentration of both water- and EDTA-extractable carbohydrate did not increase significantly compared with Control treatment. Similarly, de Brouwer et al. (2003) found that in the presence of diatom biofilms, carbohydrate contents were not elevated in the upper 0.5 cm relative to the deeper sediment layers. There is evidence that in the presence of a microalgal bloom, the excretion of extracellular carbohydrates as part of the mechanism of diatom motility is probably small, compared to that produced through unbalanced photosynthetic growth (de Brouwer and Stal, 2001). Possibly, the amount of carbohydrates produced in the HD treatment was related to a species-specific effect observed in that treatment. As previously mentioned, in the HD treatment the predominant species in the diatom bloom was *B. paxillifer*, and this particular species probably does not need to excrete carbohydrates, as it would laid on the surface due to their size. Because of its unusual behaviour, *B. paxillifer* forms colonies in which the single cells move along each other (Gebeshuber et al., 2004), allowing entire colonies of five to 30 cells to expand and contract rhythmically and in coordination (Kapinga and Gordon, 1992). It is also possible that the relatively low amounts of carbohydrates in the HD treatment was a consequence of high rates of bacterial metabolism (Goto et al., 2001; van Duyl et al., 1999; Yallop et al., 2000) or consumption by grazers (de Deckere et al., 2002). Diatom species may vary in their production of carbohydrates. For example, *Gyrosigma limosum* and *Nitzschia sigma* have been associated with high carbohydrate concentrations (Underwood et al., 1998), while carbohydrate production by *B. paxillifer*,

Achnanthes longipes, and *Navicula pelliculosa* is relatively low (Holland et al., 1974). My results with *B. paxillifer* are consistent with those studies.

Water-extractable carbohydrate was significantly lower in the HC treatment than in the LDHC or HDHC treatments. This could be attributed to the low biomass present in the HC treatment compared with that in the LDHC and HDHC treatments, where the presence of diesel decreased the magnitude of the toxicity of copper, and probably caused a lower growth of BMA. Although the distribution of carbohydrates in HC and HDHC treatments did not vary significantly with depth, there was a lower concentration of carbohydrates in the top layer (0-200 μm). In the same way, biomass was lower in the top layer than in bottom layers in the HDHC treatment. It is possible that the presence of copper had an influence on the migration of benthic diatoms, because most of the carbohydrate was located at the 200-400-μm layer, suggesting that diatoms migrated downward the sediment. Paterson and Underwood (2003) found that the locomotive ability of diatoms decreased when they were exposed for 24 h to increasing concentrations of copper. This effect may be due to reduced production and secretion of locomotive EPS by copper toxicity, or because of changes in EPS conformation and viscosity caused by the availability of metal ions, or more probably, a combination of both factors (Underwood and Paterson, 2003).

My results indicated that there was a significant relationship between Chl *a* and water-extractable carbohydrates in the presence of contaminants. The strongest relationship was observed in the HD treatment, probably because there was a rapidly growing BMA assemblage here, which developed as a bloom in several days after the addition of diesel. The relationship between water-extractable carbohydrates and Chl *a* was less strong in the treatments where BMA growth was reduced (i.e. HC treatment). De Deckere et al. (2002) observed a significant

relationship between colloidal (water-extractable) and EDTA-extractable and Chl *a* in natural conditions. I speculate that BMA were responding to environmental stress caused by the contaminant, through variation in the nature and quantity of carbohydrates produced. We do not have data regarding the chemical composition of the carbohydrates produced in these treatments, but that information could be useful to explain different responses observed.

Multiple factors may play a role in determining the extracellular carbohydrate contents in diesel- and copper-contaminated sediment. The extracellular carbohydrate content in intertidal sediments is the result of complex interactions between biological, sedimentological and physical processes, rather than being determined by a straightforward correlation with BMA biomass (de Brouwer et al., 2003). Other sources of carbohydrates from bacteria (Decho, 1990), meiofauna and macrofauna (Meadows et al., 1990) could be also be important. The production of both water- and EDTA-extractable carbohydrates has been found to depend on migratory patterns (Perkins et al., 2001; Serodio et al., 1997; Smith and Underwood, 2000), photosynthetic processes (de Brouwer and Stal, 2001; 2002, Staats et al., 2000), nutrient depletion (Staats et al., 2000) and physiological status of BMA (de Brouwer and Stal, 2002; de Brouwer et al., 2002, Smith and Underwood, 2000; Staats et al., 1999; Sutherland et al., 1998; Yallop et al., 2000), all of which are under environmental control.

In summary my study indicates that moderate concentrations of diesel and high concentration of copper contaminants significantly affected the BMA assemblage of a Louisiana salt marsh. Diesel and copper altered the vertical distribution and biomass of BMA in the top 2 mm of sediment surface, and the combined effect of diesel + copper was different than the effect of either contaminant alone. Bottom-up indirect effects are common and changes in BMA due to toxicants could influence higher trophic levels (Fleeger et al., 2003). Petroleum

hydrocarbons may cause an alteration in the microbial activity in sediments contaminated with diesel, which may increase nutrient concentrations, causing an stimulation of BMA.

The relationship between BMA biomass and carbohydrates concentration is complex. Reduced biomass in copper and diesel + copper treatments were associated with reduced levels of carbohydrates. However, carbohydrates did not significantly increase in diesel-contaminated sediment where biomass was significantly increased; this result is probably related to a species-specific effect on the production of carbohydrates. Further research is needed to chemically characterize of carbohydrate production by natural BMA assemblage from specific species of diatoms to better understand the dynamics of carbohydrate production by the microflora.

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CHAPTER 5

EFFECTS OF PHENANTHRENE- AND METAL-CONTAMINATED SEDIMENT ON THE FEEDING ACTIVITY OF THE HARPACTICOID COPEPOD *SCHIZOPERA KNABENI*

INTRODUCTION

Aquatic sediments often contain mixtures of contaminants, including heavy metals and polycyclic aromatic hydrocarbons (PAH) (Daskalakis and O'Conner, 1995; Kennicutt et al., 1996). Heavy metals and PAHs have very different modes and sites of toxic action; metal toxicity is usually expressed at the cellular level as a non-specific binding with reactive macromolecules that interferes with physiological processes such as respiration and oxidative phosphorylation (Di Giulio et al., 1995). In contrast, PAH disrupt membrane function due to non-polar narcosis, and many are potent mutagens, carcinogens and teratogens (Rand et al., 1995).

Biota may respond to contaminant mixtures in different ways because the combined toxicity of contaminants may be expressed in an additive or non-additive manner. Additive toxicity is called "dose/concentration addition" and refers to the non-interactive process in which the toxicity of the chemical mixture is proportional to the sum toxicity of each individual contaminant (Price et al., 2002; Casee et al., 1998; Broderius, 1991). Non-additive toxicological effects occur when individual contaminants interact and modify the overall magnitude or nature of toxicity (Cassee et al., 1998). Non-additive interactions are manifested as effects that are either significantly greater (synergistic) or less (antagonistic) than expected compared to the effect of individual contaminants alone (Cassee et al., 1998; Broderius, 1991). Another possible manifestation of contaminant mixtures is known as "response addition" or independence, which occurs when the contaminants in a mixture have no influence on each other's toxic effect (Price et al., 2002; Cassee et al., 1998; Broderius, 1991). Independent toxicity in contaminant mixtures has been demonstrated for dissimilarly acting organic compounds

including industrial contaminants (Broderius et al., 1995) and pharmaceutical compounds (Faust et al., 2000).

In the Gulf of Mexico, metals and PAHs are found in elevated concentrations near oil-production platforms (Kennicutt et al., 1996; Peterson et al., 1996). Metals associated with barium ore (used in drilling fluids), including Cd, Hg and Pb, occur at concentrations that potentially have lethal or sublethal toxic effects on marine biota, based on single-compound exposures (Kennicutt et al., 1996). These metals appear to be persistent, especially in low-energy environments around oil platforms. PAHs are released with produced waters, and elevated concentrations have been found around oil platforms (Kennicutt et al., 1996). Both metals and hydrocarbons tend to partition from the water column into benthic sediments (Di Toro et al., 1991; Di Toro and McGrath, 2000), and may therefore be available to the benthic fauna.

Even though some work has been done on contaminant interactions and we have a theoretical framework for interpreting their effects, very little is known about the sublethal effects of these contaminants in the salt marsh habitats. Sublethal endpoints could be more sensitive indicators of the toxicity of contaminated sediments than measurement of mortality. The hypothesis of this study was that the mixture of metals and phenanthrene has an additive effect on the grazing rate of *Schizopera knabeni*. Thus, the objective of this study was to investigate the effects of sub-lethal concentrations of metal-hydrocarbon mixtures (Cd, Hg, Pb and phenanthrene) on the feeding rate of the harpacticoid copepod, *Schizopera knabeni*, in sediment exposures.

MATERIALS AND METHODS

Test Organism

The meiobenthic harpacticoid copepod *Schizopera knabeni* was used as a test organism. *S. knabeni* has been maintained in monospecific culture since 1993 (J. Fleeger, personal communication). The culture was initiated using animals obtained from a salt-marsh at Port Fourchon, Louisiana (Lotufo, 1997). Copepods were kept at room temperature in a 1000 mL Erlenmeyer flask containing 600 mL of 20 psu artificial seawater (ASW), which was completely renewed every 14 days. The copepods were fed once a week with T-*Isochrysis* paste (Brine Shrimp Direct).

Contaminated Sediment Preparation

Sediment was collected from the upper 2 cm of a mudflat in a *Spartina alterniflora* salt marsh near Cocodrie, Louisiana. This sediment was processed following Chandler (1986) to reduce the organic matter content and to generate a more uniform particle size distribution. The sediment was autoclaved after processing and liquid slurry was created by homogenizing the sediment with an appropriate volume of 20 psu ASW. The final wet: dry ratio was 1.31:1. The total organic carbon (TOC) content of the processed sediment was measured using a Perkin Elmer 2400 CHN Series II elemental analyzer (Norwalk, CT, USA) and found to be 3.70% ± 0.34%. Samples were refluxed for 6 h in concentrated HCl to eliminate inorganic carbonate and oven-dried at 70˚C prior to TOC analysis. The sediment was stored in sealed containers at 4^{o}C.

Phenanthrene (98% purity, Aldrich Chemical Co. Milwaukee, WI, USA) was amended to processed sediment by dissolving the chemical in HPLC-grade hexane and then volatilizing the solvent in an ultra-high-purity nitrogen gas stream to coat the inside

walls of round opaque glass loading jars (Reible et al., 1996). The appropriate mass of wet sediment to achieve a targeted concentration was then added to each loading jar and tumbled on a mechanical roller mill at room temperature (~22^{o}C) for 21 d to homogenize and age the phenanthrene-sediment complex. Amended sediment was stored at 4˚C for no more than 7 d prior to use. The putative concentration of phenanthrene was 110 ppm. That concentration was based on previous experiments by Lotufo (1997), who showed that phenanthrene alone caused a significant reduction in the grazing rate of *Schizopera* after 30 h of exposure at a concentration of 55 ppm, and suggested that higher concentrations of phenanthrene were needed to greatly reduce the feeding rate.

Lead, cadmium and mercury (as chloride salts; 98% purity, Sigma Chemical Co. Saint Louis, MO, USA) were amended to processed sediment for bioassays. When used in mixtures, Pb, Cd and Hg were always amended in a ratio of 5:3:2 respectively. The required amount of each compound was dissolved in deionized water, and then added to a specific mass of wet sediment to achieve a targeted sediment concentration. To ensure homogenization, the metal-rich solution was added by slow dripping via a gravity-fed apparatus to 39 g of wet sediment in a glass jar undergoing mixing with a food mixer (Millward et al., 2001). The amended sediment was stored at 4˚C for no more than 7 d until the initiation of the experiment. Phenanthrene was added to the sediment prior to the addition of metals.

Microalgae Labeling

An inoculum of *Isochrysis* in log-phase growth was added to 600 mL of f/2 culture media at 20 psu. After 3 days, 250 μCi of $NaH^{14}CO_3$ was added (specific activity 50mCi/mmol, American Radiolabeled Chemicals). Cultures were sealed to prevent the

loss of the label as $^{14}CO_2$, and maintained at 21 °C, 14/10 h light /dark cycle. Cultures were monitored every 48 h for cell density and label incorporation, and grown until the ^{14}C in the cells became constant (6 or 7 days). Unincorporated ^{14}C was removed by repeated centrifugation and decantation of supernatant and rinsing with 20 psu artificial seawater (ASW). Cell density was determined by direct count using a Neubauer haemocytometer, and ^{14}C in algal cells was determined using a liquid scintillation counter (Packard Tri-Carb 2900 TR Liquid Scintillation Counter).

Grazing Experiment

To determine the effect of sediment contaminated with phenanthrene and metals on the feeding rate of *Schizopera knabeni*, copepods were fed ^{14}C-labeled *I. galbana*. In the first experiment, adult female copepods were exposed to sediment contaminated with phenanthrene alone, Cd alone, and a mixture of phenanthrene and Cd. In a second experiment *S. knabeni* was exposed to phenanthrene alone, a metal mixture alone (Pb, Cd, and Hg in a 5:3:2 ratio), and phenanthrene combined with that mixture of metals. I selected Cd for this experiment because Cd is a highly toxic metal that is environmentally ubiquitous and associated with industrial and urban contamination. Experimental units consisted of crystallizing dishes filled with 25 mL of ASW and 3 mL of contaminated sediment. Four replicates were used per treatment. The phenanthrene concentration was kept constant at 110 ppm for both experiments and the total metal concentrations for the metal mixture were between 100 and 400 ppm. Cd concentrations were in the range of 50 to 200 ppm.

Dishes were placed in loosely covered plastic containers underlined with water-soaked paper towels, which created a humid environment to retard evaporation from the

experimental units. Ten adult females were added to each crystallizing dish. Four test units were used as control treatment (no contaminant added) and 3 formaldehyde-killed units were used to determine the copepod incorporation of label by means other than feeding (poison control). After an incubation period of 48 h, each dish was inoculated with 330 μL of radiolabeled cells (2.27×10^7cell mL^{-1}, 0.19 dpm $cell^{-1}$). After 4 h, copepods were formaldehyde killed, concentrated on a 125-μm sieve and sorted under a stereo microscope.

Copepods were placed in scintillation vials and solubilized with 200 μL TS-2 tissue solubilizer. After 24 h, 200 μL HCl was added to neutralize the tissue solubilizer, and 6 mL of Biosafe II liquid scintillation cocktail was added and the radioactivity was determined by liquid scintillation counting. Radioactivity was converted to the amount of algal cells consumed by dividing the mean radioactivity of copepods by the mean radioactivity per algal cell. Grazing rates were expressed as the number of cells ingested per copepod over the 4 h grazing period.

Statistical Analysis

All data from each experiment were analyzed using a one-way analysis of variance (ANOVA). *A posteriori* comparisons were performed using the Tukey test ($\alpha = 0.05$). All analyses were carried out using SAS 9.1 software.

RESULTS

Cadmium alone and Cd combined with phenanthrene significantly decreased grazing rates of *S. knabeni* ($p < 0.05$) at Cd concentrations above 50 ppm (Fig. 5.1). No grazing was observed in 100, 150 and 200 ppm Cd alone and phenanthrene combined ith Cd at these concentrations. Although there was a 32% reduction in the grazing rate

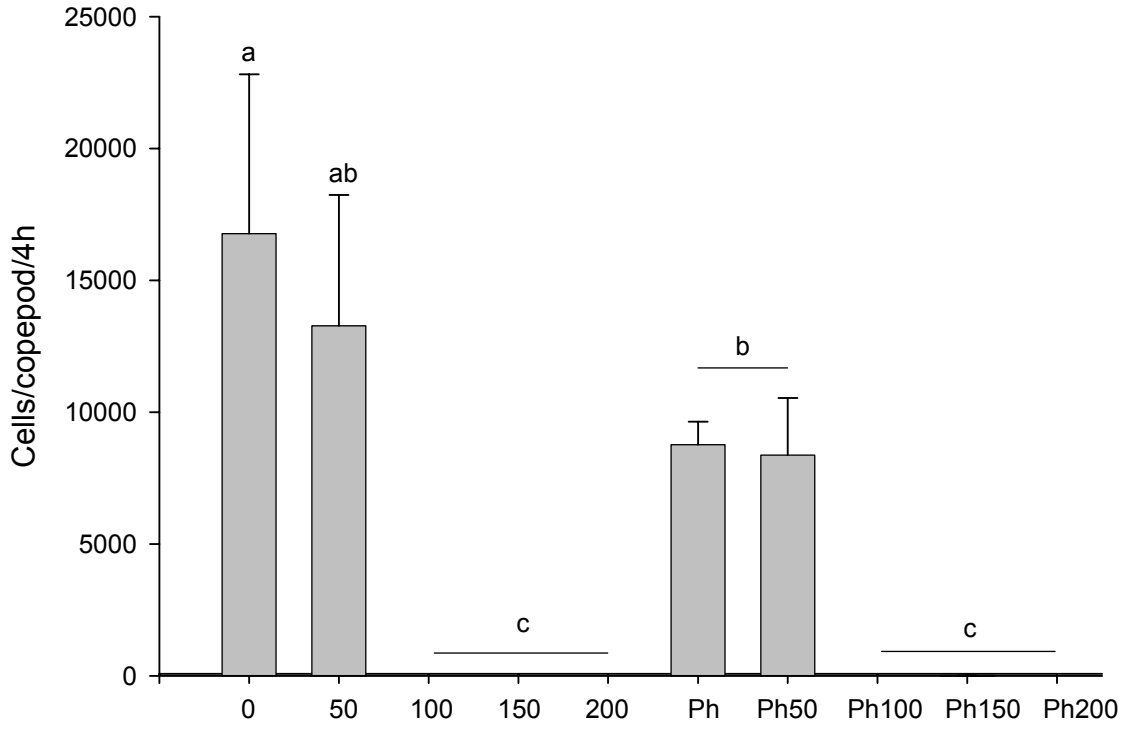

Figure 5.1. Grazing rates of *Schizopera knabeni* in phenanthrene- and Cd-contaminated sediment. Error bars are + 1 SD (n = 4). Different letters indicate significant difference between treatments ($\alpha = 0.05$). 0 = Control, 50 through 200 indicates concentration of Cd (ppm), Ph = phenanthrene alone, Ph50 through Ph200 indicates phenanthrene (concentration kept constant at 110 ppm) combined with Cd.

at 50 ppm of Cd, this rate was not significantly different from the Control ($p = 0.09$). The mixture of 50 ppm Cd plus phenanthrene caused a 62% of reduction in the grazing rate, which was significantly lower than grazing in the control ($p < 0.0003$). Phenanthrene alone also caused a significant decrease (55% reduction) in grazing rates relative to the Control ($p = 0.0002$). Grazing rates in the 50 ppm Cd treatment did not differ from those in the phenanthrene plus 50 ppm Cd treatment ($p = 0.43$) or those in phenanthrene alone ($p = 0.10$).

Grazing rates in the mixture of metals at concentrations higher than 100 ppm were significantly lower than in the Control (Fig. 5.2, $p = 0.003$). The grazing rate at the lowest metals concentration (100 ppm) was not significantly different from the Control ($p = 0.74$). The reduction in the grazing rate in 100 and 200 ppm of metal mixture were 5% and 37%, respectively. Grazing at the highest concentrations of metals (300 and 400 ppm) was totally suppressed. Phenanthrene alone (110 ppm) also caused a significant decrease (31%) in grazing rates ($p = 0.03$) compared with the Control. The mixture of phenanthrene and all three metals caused a significant decrease in grazing rates compared to the Control ($p < 0.0001$). There was a reduction of 33% in grazing rates in phenanthrene combined with 100 ppm of metals, and this reduction increased to 41% in 200 ppm of metals. The grazing rate was totally suppressed in the highest concentrations of metals combined with phenanthrene. Grazing rates in phenanthrene combined with 100 and 200 ppm of metals did not differ significantly from grazing rates in phenanthrene alone ($p > 0.08$) and from grazing rates in metals alone at 100 and 200 ppm ($p > 0.05$)

In a previous experiment (not presented here) samples were analyzed immediately after the completion of the grazing experiment (prior to formaldehyde

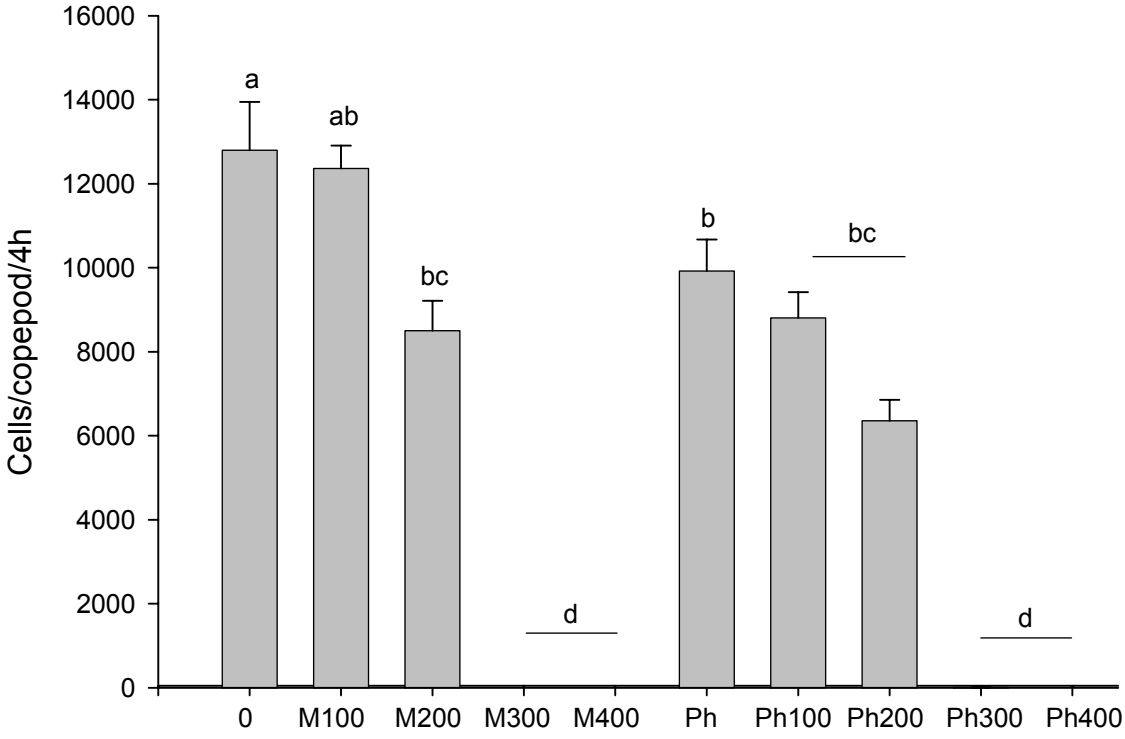

Figure 5.2. Grazing rates of *Schizopera knabeni* in phenanthrene- and metals-contaminated sediment (5:3:2 ratio of Pb:Cd:Hg). Error bars are + 1 SD (n = 4). Different letters indicate significant difference between treatments ($\alpha = 0.05$). 0 = Control, M100 through M400 indicates concentration of metals, Ph = phenanthrene alone, Ph 100 through Ph400 indicates phenanthrene (concentration kept constant at 110 ppm) combined with metals.

addition), and I observed that copepods were alive. Thus, the analysis is based on the assumption that copepods were alive, and therefore capable of grazing, during the experiments.

DISCUSSION

The purpose of this study was to determine if combinations of metals and phenanthrene had different effects on the grazing rate of *S. knabeni* than either metals or phenanthrene alone. Cd was used as a model contaminant in a first experiment of joint toxicity with phenanthrene. In a second experiment a mixture of metals (Pb, Cd and Hg) was combined with phenanthrene to determine if responses to the joint action of phenanthrene and metal mixtures were different from responses to phenanthrene and Cd alone.

Studies on joint metal-PAH toxicity have shown complex results including additive, independent and interactive toxic effects (Casee et al., 1998; Broderius, 1991). Laboratory studies frequently suggest that the joint effects of contaminants with different modes and sites of toxicity, such as metals and PAHs (i.e. phenanthrene), equals the sum of the responses to the individual contaminants alone (Swartz et al., 1995; US EPA, 1986). I therefore examined if the combined effect of metals and phenanthrene could be predicted by extrapolating the cumulative effects of individual contaminants.

By comparing the percent reduction in grazing rates obtained from my study with the values predicted according by the additive theory of toxicity, I observed that in the Cd + phenanthrene contaminated sediment (Fig. 5.3) the percent reduction in grazing rates was significantly lower (62 ± 21%, $p = 0.02$) than the predicted value (87 ± 16%, sum of percent reduction in Cd- and Phenanthrene-alone). These results are evidence of

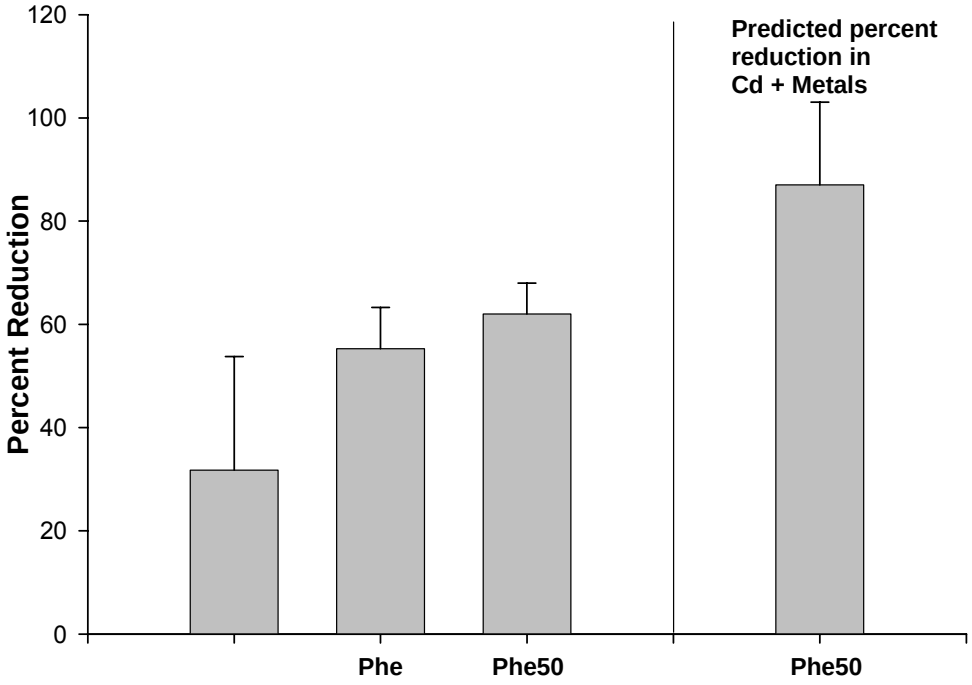

Figure 5.3. Percent reduction (observed and predicted) of grazing rates in *S. knabeni* in Cd- (50 ppm) and phenanthrene- (110 ppm) contaminated sediment. Error bars are + 1 SD (n = 4). 50 = Cd concentration in ppm, Ph = phenanthrene alone, Ph50 indicates phenanthrene combined with 50 ppm of Cd.

a non-additive toxic effect of Cd-phenanthrene contaminated sediment on grazing rates of *S. knabeni.*

The grazing rate observed in the mixture of Cd (50 ppm) and phenanthrene was not significantly different from the grazing rate in sediment contaminated with phenanthrene or Cd alone. The presence of Cd had no influence on the toxic effect of phenanthrene and vice versa.

Grazing rates were significantly reduced in phenanthrene alone and phenanthrene combined with Cd at 50 ppm. These responses are consistent with independent toxicity, which occurs when each contaminant in a mixture has no influence on the toxic effects of other contaminants (Escher et al., 2002; Altenberger et al., 2005; Faust et al., 2000; Cassee et al., 1998). In a similar study, Gust and Fleeger (2005), showed independent joint toxicity of phenanthrene and Cd on the feeding rate of the tubificid oligochaete *Ilyodrilus templetoni*, and suggested that phenanthrene-induced reductions in the feeding rate can reduce exposure to sediment-bound Cd in bulk deposit feeders. Also, Viarengo et al. (1987) demonstrated that phenanthrene had no effect on Cd-metallothionein binding or total metal body burden in the marine mussel *Mytilus galloprovincialis* when it was exposed to the contaminants in aqueous solution. Although there is a clear trend of a synergistic interaction in many contaminant mixtures (Gust, 2005), my results indicate independent toxic effects of phenanthrene and Cd in *S. knabeni*.

Grazing rates were totally suppressed at higher concentrations of Cd-alone and Cd combined with phenanthrene. Also, the same suppression in grazing rates occurred in the mixture of metals-alone (300 and 400 ppm) and when combined with phenanthrene. The concentrations of metals mixture (300 and 400 ppm) correspond to concentrations of Cd

that completely suppressed grazing (90 and 120 ppm). Grazing rates at the lowest concentration of metal mixture (100 ppm), which corresponds to 30 ppm of Cd, were not significantly reduced. These results suggest that Cd had a major contribution in the response obtained in the grazing rates of *S. knaben* in contaminated sediments.

The predicted percent reduction in grazing rates in the metals + phenanthrene contaminated sediment was not significantly different from the observed values (Fig. 5.4, $p = 0.26$). In the metals (100 ppm) + phenanthrene contaminated sediment, the percent reduction in grazing rates was close (33 ± 5%) to the predictive value (36 ± 15%, sum of percent reduction in Cd- and Phenanthrene-alone). Although the percent reduction in grazing rates in the metals at 200 ppm + phenanthrene treatment was lower (41 ± 2%) than the predicted value (68 ± 31%), there was no significant difference between them ($p = 0.83$) . Because the reduced grazing rate in the mixture of phenanthrene and metals was close to the sum of the effects of each contaminant alone, these observations were consistent with an additive influence.

Different feeding strategies in meiofauna have been related to different toxicological responses to metal contaminated sediment (Millward et al., 2001). The mode of feeding of *S. knabeni* probably consists of selective deposit feeding, as has been found in similar species (Lotufo, 1997). Selective deposit feeders select fine, organically enriched particles, which sorb a major fraction of available metals due to a high surface area (Selk et al., 1999); therefore, selective deposit feeding likely results in high concentrations and rates of metal exposure.

It has been shown that petroleum hydrocarbons cause a decrease in the feeding rate in animals with different modes of feeding, including suspension feeding copepods

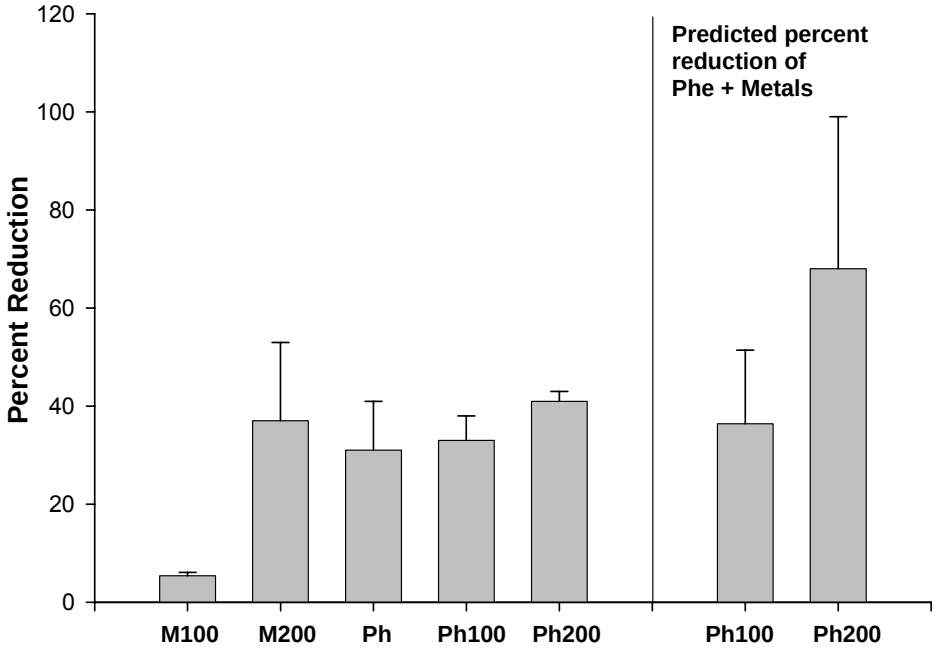

Figure 5.4. Percent reduction (observed and predicted) of grazing rates in *S. knabeni* in metals- (200 and 200 ppm) and phenanthrene- (110 ppm) contaminated sediment. Error bars are + 1 SD (n = 4). M100 and M200 = metals concentration in ppm, Ph = phenanthrene alone, Ph100 and Ph200 indicates phenanthrene combined with metals.

(Cowles and Remillard, 1983), cladocerans (Geiger and Buikema, 1981), bivalves (Donkin et al., 1989), and deposit-feeding oligochaetes (Lotufo and Fleeger, 1996). Phenanthrene significantly decreased feeding in tubificid oligochaetes (Lotufo and Fleeger, 1995). Sediment-associated PAH (phenanthrene, fluoranthene and diesel fuel) inhibited feeding by *S. knabeni* after an exposure of 30 h and at a concentration well below the 96 h LC50 values (Lotufo, 1997). In the current work, copepods were exposed to contaminants for 48 h and decreased grazing was observed in the phenanthrene concentration used (110 ppm), which is below the LC50 of 426 ppm (Marlbourough et al., in preparation).

It is possible that decreased feeding in phenanthrene-contaminated sediment was the result of lethargy caused by narcotization. Exposure to hydrophobic contaminants, including PAH, can cause narcosis. Narcosis is the result of a disruption of membrane function resulting in decreased activity and a diminished ability to react to stimuli that can ultimately lead to death (Van Wezel and Opperhuizer, 1995). Little is known about the effects of metals and hydrocarbon mixtures on feeding rate of copepods. Apparent synergisms and antagonisms in the amphipod *Hyalella azteca* exposed to Cd and phenanthrene may result from exposure-mediated effects in sediment that are unrelated to toxicological interactions (Gust and Fleeger , 2005).

My results suggest that there is no interactive (synergism or antagonism) effect on feeding when Cd and phenanthrene are combined. When other metals are added (Pb and Hg) to the mixture of Cd and phenanthrene, an additive influence on feeding rate is the result. Gust and Fleeger (2006) suggested that contaminant mixtures may elicit unexpected interactive effects facilitated by modifying exposure. Marlborough et al., (in

preparation) have shown reduced synergism when phenanthrene is combined with Cd in the presence of Pb and Hg for *S. knabeni*, suggesting that increasing complexity of contaminants may increase likelihood that their mixture could produce a predictable response.

Significant reductions in *S. knabeni* grazing rates were observed at concentrations much lower than those eliciting a significant mortality after 4 days exposure (LC_{50}, Malbourough et al., in preparation). When comparing those concentrations from both endpoints (grazing rates and lethality), my results showed evidence that grazing rate has a higher sensitivity as a sublethal endpoint than lethality, although a direct comparison is not possible because the duration of exposure was different in both cases.

Lotufo (1997) demonstrated that quantification of feeding rate in sediment exposures, previously measured only for deposit feeders (Stromgren et al., 1993; Lotufo and Fleeger, 1996), is also feasible with selective feeders. My study supports those findings and shows that the use of grazing rate as a sub-lethal endpoint is a sensitive and useful indicator of the toxicity of contaminated sediments.

Results obtained in this study indicate that phenanthrene and cadmium-contaminated sediments cause sub-lethal effects that could be missed when other endpoint indicators are used. Although the joint toxicity of Cd and phenanthrene did not show any type of interaction (synergism or antagonism) there are many studies that report an interaction, so it is possible that responses in grazing experiments to joint toxicity of contaminants are species-specific.

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CHAPTER 6

SUMMARY AND CONCLUSIONS

The main goal of this study was to analyze the effects of contaminated sediments on the benthic microalgae assemblage and meiofaunal abundance from a coastal salt marsh through the use of a microcosm experiment. In addition, I analysed the effects of contaminated sediment on the feeding rate of a harpacticoid copepod.

In Chapter 1 a general introduction concerning the role of benthic microalgae in coastal sediments was presented. Benthic microalgae have great ecological significance in estuarine sediments despite inhabiting sediments with a narrow photic zone (1-2 mm), and limited periods of light exposure. BMA can also influence the microstructure and properties of sediment, due to the secretion of extracellular polymeric substances (EPS), which may increase sediment stability. BMA provide the principal source of nutrition that fuels secondary production in shallow coastal systems and can contribute up to 50% of the total primary production in estuarine systems

Considering the importance of benthic microalgae in supporting benthic food webs, I performed a series of experiments to determine how they were affected by contaminated sediments. One of the main aspects of this study was the use of the cryolander device, which allowed obtain undisturbed sediment samples for micro-scale analysis. This aspect is important because the upper layers (1 – 1.5 mm) of illuminated sediment contain the photosynthetically active biomass, while deeper layers contain Chl *a* derived from photosynthetically inactive biomass of various sources. The use of fine-scale sampling in this study allowed us to obtain more accurate information about BMA activity in the sediment in response to the presence of contaminants, especially in the sediment surface characterized with high chemical and biological gradients.

Results in Chapter 2 indicated that moderate concentrations of hydrocarbons caused an enhancement of BMA biomass (Chl *a*) and was mostly diatoms (fucoxanthin). This

result support previous work which suggests that diesel enhanced BMA biomass due to a reduction in grazing activity or release of nutrients caused by degradation of hydrocarbons. The vertical stratification of BMA was greatly accentuated in presence of diesel. In contrast, cyanobacteria (measured as zeaxanthin concentration), was not significantly affected by diesel on day 5, but a stimulation of cyanobacteria was detected on day 20. This result suggests the possibility that cyanobacteria may play a direct role in the metabolism or degradation of hydrocarbons. The significant reduction of TPHs after 20 days of the experiment in HD treatment suggests a high rate of degradation of diesel. This implies that the contamination with diesel may be changing the microbial ecology of the sediment, and that petroleum hydrocarbons could change the biogeochemistry of the ecosystem.

High levels of copper caused a direct toxic effect on BMA biomass, mainly on diatoms and on day 5 the significant reduction of biomass in presence of copper was eliminated by the presence of diesel, but after 20 days diesel did not inhibit toxic effect of copper. Results from this study suggest that cyanobacteria could be more tolerant to high levels of copper, because they excrete copper complexing ligands reducing the availability of copper.

Meiofaunal abundance at my site at the beginning of the experiment was very low nematodes and copepods were not significantly affected by diesel or copper. The copepod community was comprised of few species with low abundances and there was high variability between replicates, which could have reduced the power to detect any effect.

In Chapter 3 the taxonomic composition of the main component of the benthic microalgae community was studied in the presence of diesel and copper contaminants.

Results from this study show that the presence of contaminants changed the abundance and diversity of the benthic diatom assemblage originally mainly in the HD treatment. A bloom of *Bacillaria paxillifier* in the HD treatment could indicate that this diesel-tolerant species or could be related to enhanced nitrogen availability. Enhanced microalgal growth depletes the excess, naturally available NH_4^+ within a few days, at which time the microbial community becomes nitrogen-limited. Further growth of the microbial community is maintained by the enhanced NH_4^+ production in diesel – contaminated sediments. I am unaware of previous studies that have examined the response of *B. paxillifer* to different nutrients, but this result suggests that this might be a fruitful area of study.

Copper significantly reduced species richness and altered the abundance of certain taxa but did not affect assemblage diversity. Results obtained in copper-contaminated sediment indicated that *Tryblionella granulata* and *T. punctata* are tolerant to copper and *Navicula flanatica* and *Navicula tripunctata* sensitive to copper, indicating the differential response of the diatom assemblage to copper-contaminated sediment.

In Chapter 4 reported the effects of diesel and copper contaminants on benthic microalgae biomass and production of carbohydrates. These results indicate that water-extractable carbohydrates were present in lower amounts than EDTA-extractable carbohydrates in all treatments including the Control. This result could indicate that water-extractable carbohydrates are recently produced and have a short residence time or that it is utilized by heterotrophic consumers. Contaminants significantly influenced water-extractable but not EDTA-extractable carbohydrate concentrations and the vertical distribution of both carbohydrate fractions did not differ significantly with depth in the 2 mm sediment surface.

The relationship between BMA biomass and carbohydrates concentration is complex. Reduced biomass in copper and diesel + copper treatments were associated with reduced levels of carbohydrates, caused by reduced growth of BMA. However, carbohydrates did not significantly increased in diesel contaminated sediment where biomass was significantly increased; this result was related to species specific effect that probably influences the production of carbohydrates. Carbohydrates produced by BMA are rich in organic matter and therefore an important source of carbon for different organisms in the benthic food web, but at the same time could be a transfer of copper to higher trophic level.

The goal of Chapter 5 was to determine if combinations of metals and phenanthrene had different effects on the grazing rate of the harpacticoid copepod *Schizopera knabeni* than either metals or phenanthrene alone. Cd was used as a model contaminant in a first experiment of joint toxicity with phenanthrene. In a second experiment a mixture of metals (Pb, Cd and Hg) was combined with phenanthrene to determine if responses to the joint action of phenanthrene and metal mixtures were different from responses to phenanthrene and Cd alone. My results suggest that there is no interactive (synergistic or antagonistic) effect on feeding when Cd and phenanthrene are combined. When other metals are added (Pb and Hg) to the mixture of Cd and phenanthrene, an additive influence on feeding rate is the result.

In summary, my results indicate that the BMA assemblage is significantly affected by diesel and copper, and effects are detected on a microscale level. The BMA community is a key component of the highly active sediment surface, where many biological and physical properties of the upper 2 mm of the sediment surface are closely

related. The presence of diesel and copper in the sediment surface caused changes in the microscale geochemistry (i.e. photosynthesis, respiration, degradation).

BMA contribute significantly to the total primary production of estuarine and other shallow water systems, therefore BMA are consequently an important carbon source for benthic heterothrophs and can significantly affect the exchange of oxygen and nutrients across the sediment-water interface. Benthic microalgae are responsible for 50% or more of the carbon assimilated by consumer organisms. These organisms are in turn preyed upon by a variety of transient fish and bird species, therefore the relationship of BMA with their potential consumers could also be affected by contaminants.

VITA

Soraya Silva was born in Punto Fijo, Falcon State, Venezuela, to Felix S. Silva and Carmen C. de Silva. Soraya went to the Universidad del Zulia (LUZ), in Maracaibo, Venezuela, to study Licenciatura in biology. During that period she worked in the "Instituto para la Conservacion del Lago de Maracaibo" (ICLAM) to perform her undergraduate thesis under the guidance of Dr. Pedro Gutierrez. After graduation in 1990 she received a scholarship from the Consejo Nacional de Investigaciones Cientificas (CONICIT) to pursue a master's degree in microbiology under the guidance of Dr. Joseph Jay Ewald. During her master's studies, she worked in the Microalgae Culture Laboratory of the Department of Biology at LUZ, and participated in several research interdisciplinary projects related to toxicological assays using microalgae. After graduating in 1996, she received a fellowship from the "Fundacion Gran Mariscal de Ayacucho" (FUNDAYACUCHO) in Venezuela, to pursue doctoral studies in Louisiana State University, where she started in the Spring 2000 under the supervision of Dr. Kevin Carman. Currently, Soraya is a candidate for the Doctor of Philosophy degree and will graduate on August 11, 2006.