

1-1-2013

Trophic Response to Polycyclic Aromatic Hydrocarbons and Copper In Tidal Flats of North Inlet, South Carolina

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TROPHIC RESPONSE TO POLYCYCLIC AROMATIC HYDROCARBONS AND COPPER
EXPOSURE IN TIDAL FLATS OF NORTH INLET, SOUTH CAROLINA

by

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Bachelor of Science
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Submitted in Partial Fulfillment of the Requirements

For the Degree of Master of Science in

Biological Sciences

College of Arts and Sciences

University of South Carolina

2013

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DEDICATION

This work is dedicated to my parents, Brad and Lorna Muggelberg, without the support of whom I would not be where I am today. They have been extraordinary role models and inspire me every day. Their encouraging words have helped me through many trying times and their praise of my accomplishments have made all sacrifices worthwhile.

I also dedicate this thesis to the memory of my beloved aunt and godmother, Lynn Muggelberg, who was taken from us too soon but taught me that my life is my own and mine to cherish. I always admired her independence and selflessness, which persisted even during her four month battle with cancer as she put on a brave face to ease the distress of our family. My graduate school experience has brought out some of her in me and I hope to someday be as strong of a woman as she proved to be.

ACKNOWLEDGEMENTS

I would like to thank my advisor, Dr. Jay Pinckney, for all of his guidance throughout the past two years. I have learned so much and grown as a scientist under his supervision, for that I cannot even begin to express all of my gratitude. With him helping me stay focused on my goals I was able to complete my “ambitious” project on the proposed time-scale, a feat I take pride in. I would also like to thank my committee members, Dr. Blaine Griffen and Dr. Michele Harmon for all of their advice and support devising and executing my experiments.

I would also like to acknowledge all of the people that helped me while conducting my research at the Belle W. Baruch Institute for Marine and Coastal Science. Dennis Allen and Paul Kenny worked with me to design an experimental set-up to ensure containment of all contaminants used and advocated approval of my project by the Belle W. Baruch Foundation. Without their support I would not have had the wonderful experience of spending my summer at the field lab, enjoying the beauty of the research reserve. Stephen Forehand was a tremendous help to me throughout the entire execution of my project, aiding with electrical issues, construction of mesocosm components and other set-up details.

Many thanks are owed to my lab partners, Bridget Bachman, Erik Lachenmyer, Doug Bell, Lauren Hehmen, Michelle Zimmerlin and Isaac Hagenbuch for providing me with their thoughts, insights and encouraging words during stressful times.

Finally, I would like to acknowledge my funding sources, the F. John Vernberg Bicentennial Fellowship in Marine Sciences and the Slocum-Lunz foundation, that provided much of the needed financial support for this project.

ABSTRACT

The trophic link between benthic microalgae (BMA) and fiddler crabs is critical for the ecosystem functioning of estuaries and alterations in this linkage by anthropogenic activities could have cascading impacts on food webs and biogeochemical cycling in these sensitive habitats. Singular and interactive effects of two common pollutants in aquatic ecosystems, polycyclic aromatic hydrocarbons (PAHs) and copper (Cu), were investigated by exposing field collected sediment communities to the contaminants and measuring changes in BMA biomass and community composition in a bioassay design. The consequential impacts on the food web were then explored by examining the effects of PAHs and copper on food preference and feeding rates of sand fiddler crabs (*Uca pugilator*). No significant overall change in BMA biomass (as chlorophyll *a*) after 10 days was observed between treatments. However, the trends in the algal biomass responses throughout the experiment, as well as the significantly greater change in BMA biomass between 4 and 10 days after exposure in the Cu treatment compared to the controls, suggest a complex sediment community response. The abundance of diatoms relative to cyanobacteria (the fucoxanthin to zeaxanthin ratio) increased significantly in Cu and Mix (PAHs + Cu) treatments compared to controls, possibly due to cyanobacterial sensitivity. Fiddler crabs grazed on sediments of the Mix (PAHs + Cu) treatment significantly less than they did upon controls during a food choice experiment. In addition, the feeding rates of crabs exposed to Cu only, PAHs only and to PAHs + Cu (the Mix treatment) were significantly lower than those in the Water control when no

alternative food choice was provided. Because fiddler crabs are important bioturbators, a reduction in feeding, (and therefore sediment processing) in contaminated areas could have significant impacts on the chemistry of surficial sediments which subsequently influences sediment communities and marsh grass growth. Reduced feeding would likely also reduce resources for crab growth and reproduction, effect crab health, and eventually could result in a reduction in crab abundance. Sublethal exposure of intertidal communities to PAHs and Cu may result in subtle alterations in the trophodynamics of BMA and fiddler crabs that have the potential to affect multiple levels of biological organization.

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INTRODUCTION

In the past several decades, the human population density of coastal South Carolina, and many other coastal areas, has increased dramatically and this trend is expected to continue (Crossett et al., 2004; Wilson and Fischetti, 2010). Between 1980 and 2003, total U.S. coastal population increased by 28% (or 33 million people) (Crossett et al., 2004) and with this increase in population inevitably comes a great deal of urban expansion, residential development and impervious land cover. Such development poses great threats to estuarine and coastal ecosystems which provide habitats and nurseries for commercially valuable fish and shellfish, as well as many ecologically important species (Courrat et al., 2009). The close proximity of estuarine tidal creeks to urban development and human activities results in both chronic and acute exposure to runoff containing harmful pollutants such as pesticides, heavy metals and polycyclic aromatic hydrocarbons (PAHs) (Kucklick et al., 1997; Sanger et al., 1999 a, b; Holland et al., 2004; Van Dolah et al., 2008; Garner et al., 2009). These pollutants may individually impact marine organisms or can act in combination with one another. As generally the primary waterways to receive urban runoff in coastal areas, tidal creeks are among the first aquatic habitats to show symptoms of degradation. This relationship makes tidal creeks ideal sites to investigate the potential impacts of anthropogenic stressors on estuarine communities.

PAHs and copper are both routinely introduced into estuarine environments by a variety of sources and higher concentrations of these pollutants have been recorded in tidal creeks associated with urban areas compared to those with less developed watersheds (Sanger et al., 1999a,b; Van Dolah et al., 2008; Garner et al., 2009). PAHs are a group of organic, hydrophobic compounds characterized by fused aromatic rings. They are components of fossil fuels and are also released upon incomplete combustion of such fuels. PAHs are emitted as a mixture of individual compounds (Zhu et al., 2004; Tobiszewski and Namieśnik. 2012) and estuarine pollution sources for these contaminants can include urban runoff, direct fuel spills or leaks and air deposition (Kucklick et al. 1997; Ngabe et al., 2000; Kose et al., 2008; Nikolaou et al., 2009). Due to their hydrophobic nature, PAHs readily adsorb to inorganic and organic particles and so can accumulate within the sediments of estuaries (Nikolaou et al., 2009; Tobiszewski and Namieśnik. 2012). Copper, a heavy metal, also accumulates in the sediments of estuarine habitats upon introduction by anthropogenic sources due to its adsorption to organic matter and other particles (Helland and Bakke, 2002). The primary source of copper contamination in many marine habitats is boating activity, as many anti-fouling paints used on boats and docks are copper based. Other sources such as sewage discharge, urban runoff, industrial inputs and drainage from copper-based algacide treated ponds may also contribute to the accumulation of this heavy metal in sediments (Thomson et al., 1984; Matthiessen et al., 1999). Concentrations of PAHs and copper in sediments will likely continue to rise in coastal habitats in coming years because the primary sources of these contaminants are related to urban expansion and associated

anthropogenic activities. For this reason, the potential ecological impacts of these contaminants on estuarine habitats merit experimental study.

Benthic microalgae (BMA) form the foundation of many estuarine food webs (MacIntyre et al., 1996; Miller et al., 1996; Sullivan and Currin, 2000; Consalvey et al., 2004), therefore, changes in this food source due to toxic contaminant effects could have dramatic impacts on higher trophic levels. Fiddler crabs consume organic matter in sediments, including BMA (Miller, 1961; Crane, 1975; Meziane et al., 2002), and so may be directly affected by changes in quantity or quality BMA. These crabs play very important roles in salt marsh ecosystems as a food source for fish, birds and other crustaceans (Crane, 1975; Montague, 1980; Grimes et al., 1989) and also serve as important bioturbators with their burrowing and feeding activities (Katz, 1980; Montague, 1980; Hoffman et al., 1984; Bertness, 1985; Grimes et al., 1989; Meziane et al., 2002; Smith et al., 2009; Sayão-Aguiar et al., 2012). Because BMA and fiddler crabs are a trophic link critical for the ecosystem functioning of salt marshes, alterations in this linkage by anthropogenic activities could have significant trophic and energetics impacts on estuarine food webs.

The first objective of this study was to examine the potential effects of PAHs and copper (singly and in combination) on the biomass and community composition of BMA. A second objective of this study was to investigate effects of PAHs and copper (singly and in combination) on the food preference and feeding rates of fiddler crabs through exposure to contaminated sediments and food source (BMA).

While numerous studies have examined the effects of either PAHs or copper on only BMA or crustaceans (McLeese, 1975; Steele et al., 1992; Carman et al., 1995; Cid et

al., 1995; Piehler et al., 2003; Vijayavel and Balasubramanian, 2006; Alsterberg et al., 2007), the current study adopted a more realistic approach of investigating potential impacts of non-point pollution. The effects of two contaminants (PAHs and copper) not only singly, but also in combination were examined, as it is very unlikely that only one type of contaminant will be present in these systems. In the relatively few studies that have been done where the effects of multiple pollutants were explored, generally only one trophic level was focused upon. This study, however, evaluates potential effects of these pollutants on two trophic levels to provide further insight into the responses at a larger scale. By employing this experimental design involving multiple pollutants and trophic levels, a more accurate representation of community responses to anthropogenic stressors can be obtained.

CHAPTER 1: RESPONSE OF BENTHIC MICROALGAE TO POLYCYCLIC AROMATIC HYDROCARBON AND/OR COPPER EXPOSURE

1.1 INTRODUCTION

As coastal populations grow (Crossett et al., 2004; Wilson and Fischetti, 2010) and urbanization of coastal areas increases, the introduction of harmful pollutants into the environment will likely also increase. Runoff due to increased impervious ground cover associated with urbanization carries a variety of pollutants, including pesticides, polycyclic aromatic hydrocarbons (PAHs) and heavy metals, to nearby water systems, particularly tidal creeks due to their close proximity to developed areas (Kucklick et al., 1997; Sanger et al., 1999 a, b; Holland et al., 2004; Van Dolah et al., 2008; Garner et al., 2009). The contamination of estuarine environments is cause for great concern as these habitats are essential for the survival of a diversity of organisms, serving as nurseries and feeding grounds for many ecologically and economically important fish, crustacean and bird species (Courrat et al., 2009).

Benthic microalgae (BMA) are the foundation for many estuarine food webs and their productivity is vital to ecosystem functioning (MacIntyre et al., 1996; Miller et al., 1996; Sullivan and Currin, 2000; Consalvey et al., 2004). BMA can account for as much as a third of the total carbon fixed in some estuarine environments, at times exceeding the phytoplankton production and macroalgal production (Pinckney and Zingmark, 1993; Consalvey et al., 2004). They can support a great deal of secondary production as BMA

are directly fed upon by amphipods, gastropods, polychaetes, fish, crustaceans and many meiofauna species (Miller et al., 1996; Sullivan and Currin, 2000). For that reason, effects of pollutants on BMA, such as reduced productivity or contamination of this food source, may have cascading effects on the food web. BMA also function in sediment stabilization, the degree of which is dependent on BMA density and mucilage secretion, thus a significant reduction in BMA biomass resulting from pollutant exposure could potentially affect sediment dynamics as well (Holland et al. 1974; de Brouwer et al., 2005). The various roles that BMA play in estuarine habitats make them key links between biological compartments as well as biogeochemical cycles, therefore, the effects of anthropogenic influences on these microorganisms merit investigation.

PAHs and copper are two common pollutants of estuarine habitats, the concentrations of which are associated with the degree of development within the watershed, as urbanized areas generally have elevated concentrations relative to rural or suburban areas (Sanger et al., 1999 a, b; Holland et al., 2004; Van Dolah et al., 2008; Garner et al., 2009). As compounds found in petroleum products and released upon combustion of fossil fuels, PAHs can be introduced into the estuarine habitats both directly (through oil spills or leaks from boats) and indirectly (through urban runoff or air deposition following car exhaust emissions) (Kucklick et al. 1997; Ngabe et al., 2000; Kose et al., 2008; Nikolaou et al., 2009). Urban runoff can be a source of copper contamination as well, but the use of copper-based biocide paints on boats and docks serves as the major contributor to high concentrations of that heavy metal in coastal areas (Thomson et al., 1984; Matthiessen et al., 1999). PAHs and copper can accumulate in the sediments of estuarine environments due to their adsorption to organic and inorganic

particles (Helland and Bakke, 2002; Nikolaou et al., 2009; Tobiszewski and Namieśnik, 2012) and so pose a threat to benthic organisms.

Pollutant effects on BMA can vary greatly. Conflicting results with regards to the effect of PAHs on BMA biomass have been observed in multiple studies and seem to depend on contaminant concentration, medium contaminated (water vs. sediments) and duration of exposure (Carmen et al., 1997; Piehler et al., 2003; Wang and Zheng, 2008; Wang et al., 2008; Petersen et al., 2009). While some experiments have shown indirect stimulation of BMA growth after the introduction of PAHs (Carmen et al., 1997; Petersen et al., 2009), others have demonstrated direct toxic effects of PAHs on phytoplankton and BMA, primarily involving adverse effects on cell membranes and induction of oxidative stress (Piehler et al., 2003; Wang and Zheng, 2008; Wang et al., 2008). Likewise, heavy metals (especially copper) are also known to induce oxidative stress in algae (Pinto et al., 2003; Yu et al., 2007; Sabatini et al., 2009), and copper may reduce photosynthesis as well as inhibit growth at varying concentrations (Cid et al., 1995; Hadjoudja et al., 2009; Levy et al. 2009). Aside from effects individual contaminants can have on BMA, interaction between multiple contaminants may induce complex biological responses. Synergistic toxic effects on algae and higher plants have been observed upon their exposure to mixtures of copper and organic pollutants (such as PAHs), but such responses can be contaminant ratio dependent (Babu et al., 2001; Babu et al., 2005; Wang et al. 2008). The varying results between studies highlight the importance of simulating natural conditions and utilizing environmentally relevant concentrations in bioassays to accurately assess potential biological responses to contaminants.

In addition to the potential effects on overall BMA biomass, variation in group (e.g., diatoms vs. cyanobacteria) and species-specific responses to these stressors would promote alterations in community composition (structure) and trophic transfer (function). Cyanobacteria seem to be more sensitive to various pollutants than other algal groups. Exposure to PAHs has greater negative effects, evidenced by greater percent reduction in cell counts, on cyanobacteria compared to diatoms (Piehler et al., 2003). Copper sulfate (a commonly used algaecide) is often added to lakes and ponds to control algal blooms and at levels that result in rapid, dramatic declines in cyanobacteria biomass, chlorophytes, diatoms and chrysophytes are able to survive, replacing cyanobacteria as dominant algal groups after copper sulfate treatment (Witaker et al., 1978). At a finer scale, the inhibition of some enzymes occurs at lower copper concentration in cyanobacteria than in chlorophytes, indicating differences between groups with respect to responses to copper exposure (Hadjoudja et al., 2009). The greater sensitivity of cyanobacteria to chemical pollutants would suggest possible BMA community shifts after contaminant introduction, potentially altering benthic dynamics.

The current study investigated the effects of PAHs and copper, singly and in combination, on BMA biomass and BMA community composition. Based on the numerous reported negative impacts of these two pollutants on BMA, it was hypothesized that at the environmentally relevant concentrations used in this study 1) BMA biomass (as chlorophyll *a* concentration) will decrease when exposed to either contaminant (PAHs or copper) and there will be a synergistic effect on the BMA biomass when exposed to both contaminants; 2) BMA community composition (as determined by photopigment concentrations) will be significantly altered in all contaminant treatments,

evidenced by significant increases in the fucoxanthin to zeaxanthin (biomarker pigments of diatoms and cyanobacteria, respectively) ratio. Due to the roles BMA play in salt marsh ecosystems, results from this study could have implications for higher trophic levels as well as biogeochemical processes.

1.2 METHODS

Study Site

North Inlet (33.3° N, 79.1° W) is a bar-built estuary within the boundaries of the North Inlet – Winyah Bay National Estuarine Research Reserve (NI – WB NERR) in Georgetown, SC. This estuary contains extensive tidal creeks and experiences regular semi-diurnal tides, with a mean tidal amplitude of 1.4 meters. Major contributors to the primary production of North Inlet include *Spartina alterniflora* (the dominant marsh grass in North Inlet), as well as BMA. North Inlet is considered a relatively pristine with more than 90% of its watershed in a naturally forested state.

Sediment Collection & Set-up

Polypropylene plastic containers with interior dimensions of 43 x 35 x 19 cm containing field collected sediments served as mesocosm tanks for this experiment. Sediment bricks that were the length and width of the tanks and 5 cm deep were collected from a tidal flat located near the Clambank research site within the NI – WB NERR (Figure 1.1; Figure 1.2) at low tide with a flat-edged shovel. This site was selected due to its relatively pristine condition, resulting in limited contaminant exposure of the natural benthic community prior to the experiment. Sediments for all tanks of a single replicate were collected within a three meter span of the tidal flat at the same distance away from

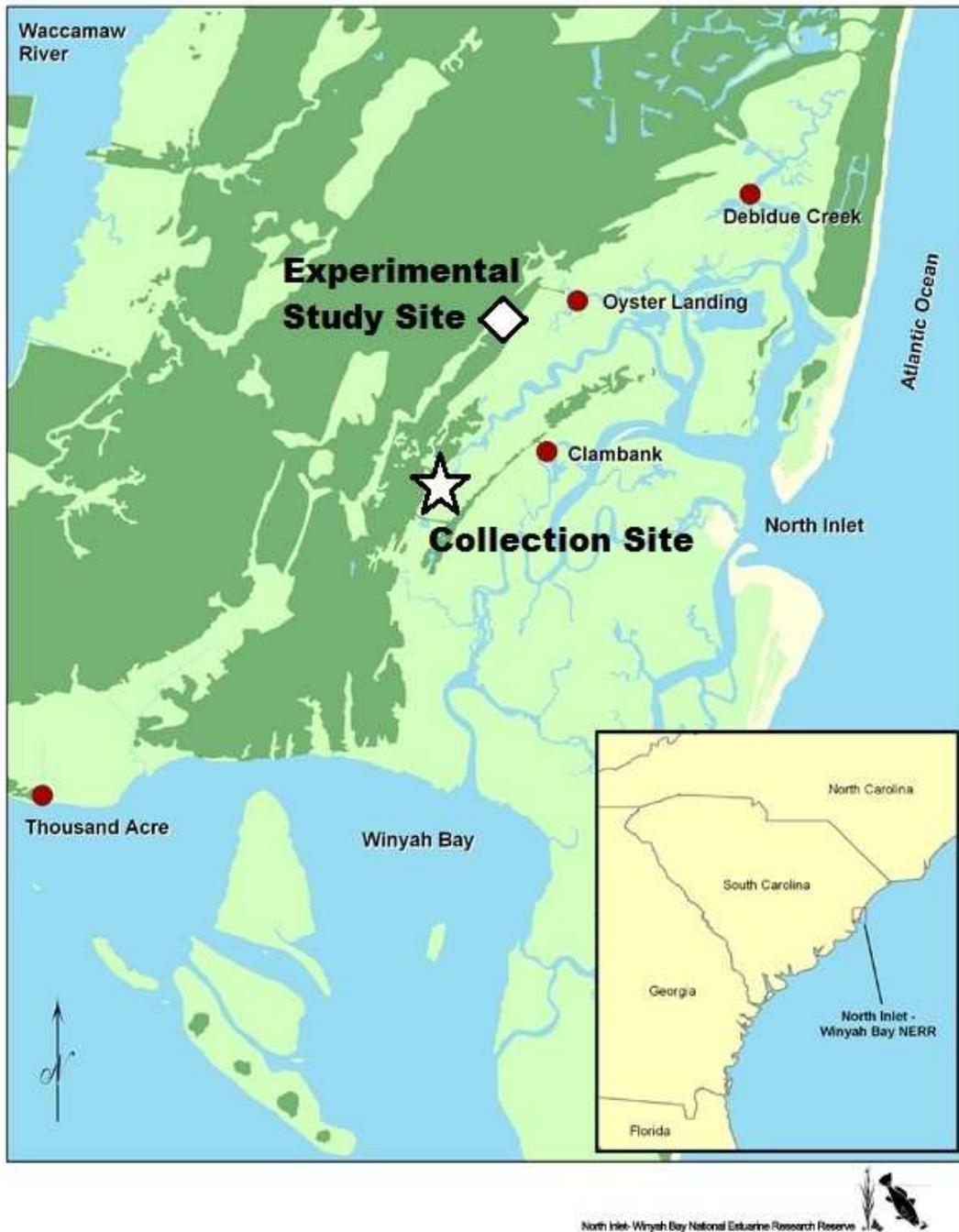


Figure 1.1 Location of research area in Georgetown, SC. Sediments were collected from a tidal flat near Clambank throughout the study and transported back to the main research facility at the Belle W. Baruch Institute for Marine and Coastal Science where mesocosms were set up and experiments took place.



Figure 1.2. Collection site photo. Tidal flat within North Inlet, SC.

the bank to reduce spatial variability in sediment grain size and organic content/composition. Four experimental replicates were carried out; Replicate 1 and Replicate 2 were performed June 23 - July 3, 2012, Replicate 3 and Replicate 4 were performed July 13 – 23, 2012.

Sediment bricks were transferred to the mesocosm tanks and immediately transported to the experimental study site behind the main research facility at the Belle W. Baruch Institute for Marine and Coastal Science (Figure 1.1). The site was exposed to full sun from 10:00 am - 3:30 pm and experienced ambient temperatures. A closed-loop water system design composed of a seawater reservoir, an Aqua Lifter AW-20 aquarium vacuum pump and a mesocosm tank was utilized for the set-up of this study. Components of each mesocosm were connected by plastic tubing, and pumps were controlled by timers to simulate semi-diurnal tides (Refer to Figure 1.3 for diagram of mesocosm set-up).

Dosing of Mesocosms

Two days after sediment brick collection and mesocosm set-up, surface sediments (0 – 2 cm) were collected from the original collection site during low tide using a flat-edged shovel. The sediments were immediately transported back to the research facility where they were sieved through 2 mm mesh and then homogenized for 2 minutes using a steel mixing paddle attached to a drill.

The volume of sediment required to provide three 2 mm thick layers over the exposed surface (corrected for sediments removed around the drain area) of the sediment brick in a mesocosm tank (780 ml sediment/tank) was dosed with the appropriate

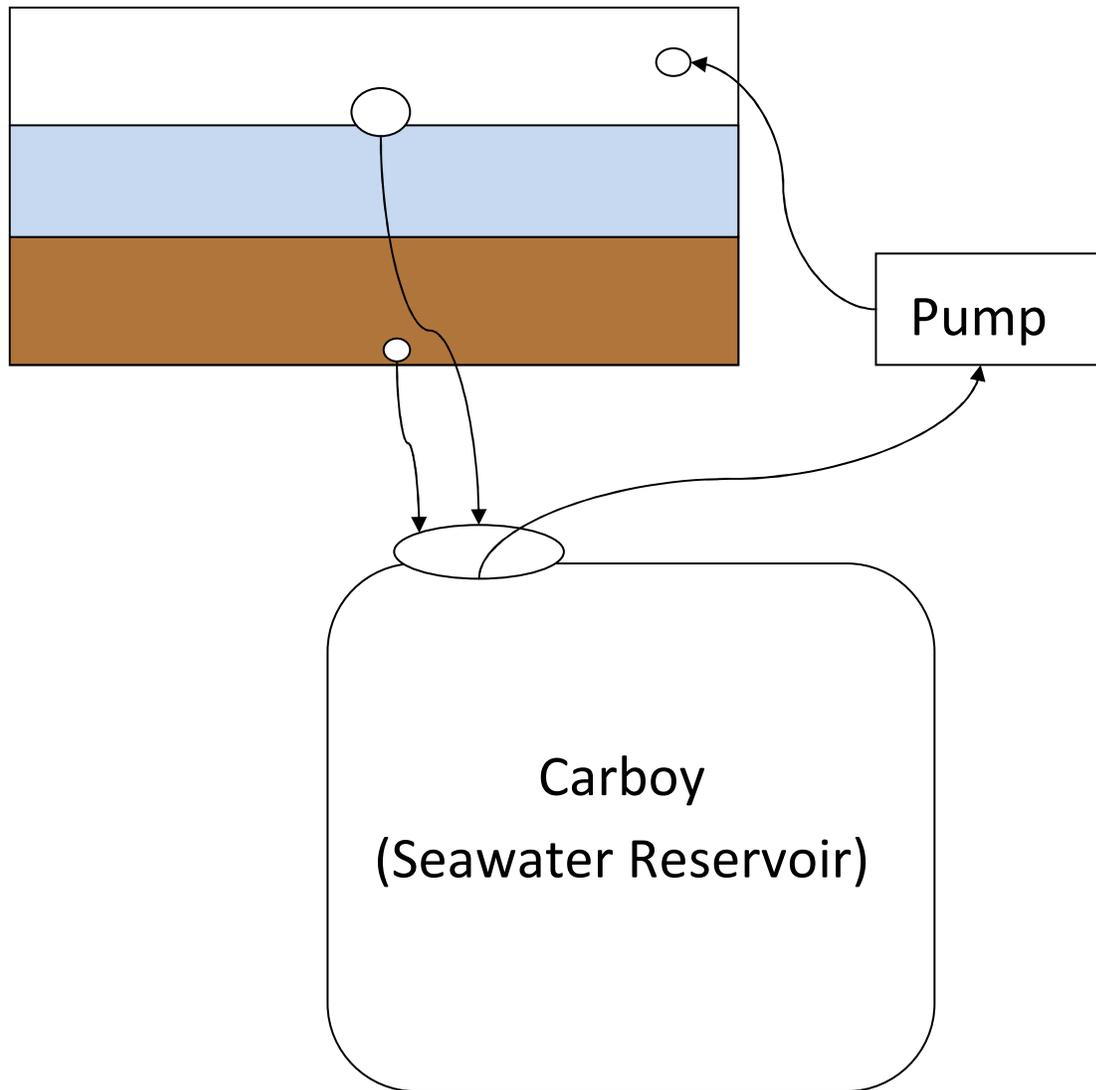


Figure 1.3 Diagram of mesocosm set-up. The large rectangle represents the mesocosm tank, containing collected sediments (brown coloration) and several centimeters of water (during a simulated high tide) (blue coloration). Circles represent holes in the tank functioning either as drains or the point of water introduction. Curved lines represent tubing, with arrow heads indicating the direction of water flow through the tubing. Note the relative size of the holes in the mesocosm tank. The hole corresponding to the point of water introduction is larger than that of the bottom drain, which allowed for water accumulation while pumps were on (to simulate high tides). The much larger top drain allowed for rapid drainage once the waterline reached that level, preventing overflow and limiting the depth of water overlaying the sediments to 7 cm. While pumps were off, water gradually exited the bottom drain until tanks were empty, simulating a low tide.

components based on the designated treatment for that mesocosm (See Table 1.1 for dosing details). For those treated with contaminants, dosing consisted of dissolving the contaminants and adding the solution(s) to the sediments, yielding concentrations within the range observed in urbanized coastal areas of South Carolina (4.77 – 37.03 µg total PAHs/g dry sediment and 58.4 – 93.7 µg Cu/g dry sediment) (Sanger et al. 1999 a, b). The target concentrations of dosed sediments were 10 µg total PAHs/g dry sediment (ratio of individual PAH compounds used in the dosing mixture based on Sanger et al., 1999b) and 70 µg Cu/g dry sediment (for the appropriate treatments) (Table 1.1). It was assumed that contaminant concentrations of field collected sediments from the relatively pristine site of North Inlet were almost negligible (0.07 – 0.12 µg total PAHs/g dry sediment and 2.2 – 11.4 µg Cu/g dry sediment (Sanger et al. 1999 a, b)), compared to the target concentrations. All contaminants were purchased from Fisher Scientific (pyrene, 98+%, Cat No.: AC180830250; phenanthrene, 98+%, Cat No.: AC130090050; anthracene, 99%, Cat No.: AC104861000; copper (II) sulfate, Cat No.: AA1417836). The Acetone treatment was used as a control for the PAHs and Mix treatments (as acetone was used for the solvent of PAHs) and the Water treatment was used as an overall control (i.e. no added contaminants). Fluid volume added was kept constant through all treatments (Table 1.1).

Dosed sediments were homogenized with the mixing paddle for 1 minute to ensure even distribution of any contaminants and fluid added. The mixing paddle was thoroughly rinsed with acetone and water in between homogenizing sediments for each treatment to avoid cross-contamination. After homogenization, 260 ml aliquots of dosed sediments (the volume required for a single 2 mm layer of sediment in a mesocosm tank)

Table 1.1 Sediment dosing details.

Treatment	Contaminant Added	Solvent Used	Fluid Volumes Added	Concentrations of Pollutants Added
Water	None	N/A	40 ml deionized water	N/A
Acetone	None	N/A	20 ml 100% acetone 20 ml deionized water	N/A
PAHs	Mixture of PAHs (included pyrene, phenanthrene and anthracene)	100% acetone	20 ml 100% acetone 20 ml deionized water	10 µg total PAHs/g dry sed (composed of 6 µg pyrene/g dry sed; 3 µg phenanthrene/g dry sed; 1 µg anthracene/g dry sed)
Cu	Copper (as 5 H ₂ O · CuSO ₄)	Deionized water	40 ml deionized water	70 µg Cu/g dry sed
Mix	Mixture of PAHs (included pyrene, phenanthrene and anthracene) Copper (as 5 H ₂ O · CuSO ₄)	100% acetone (for PAHs) Deionized water (for 5 H ₂ O · CuSO ₄)	20 ml 100% acetone 20 ml deionized water	10 µg total PAHs/g dry sed (composed of 6 µg pyrene/g dry sed; 3 µg phenanthrene/g dry sed; 1 µg anthracene/g dry sed) 70 µg Cu/g dry sed

were divided among glass jars which were then covered in foil and stored in a refrigerator until used. This was done to prevent photodegradation and reduce biodegradation of contaminants during storage.

After dosing was complete, one aliquot of each treatment was removed from storage and homogenized by thorough stirring. A stainless steel spatula was used to spread a 2 mm thick layer of the dosed sediments over the sediment brick in the designated mesocosm tank for each treatment during the day-time simulated low tide. Separate spatulas were used for each treatment to avoid cross-contamination.

Three additions of dosed sediments were added in this way; one addition a day on Day 1 (the day of sediment dosing), Day 2 and Day 3. Two millimeter layers were used to dose the mesocosms in order to prevent smothering the existing BMA in the sediment bricks. As BMA are known to migrate in sediments (MacIntyre et al., 1996; Consalvey et al., 2004), it was thought that by applying only thin layers of dosed sediments at a time, the BMA would be able to migrate to the surface of those sediments before the next layer was added. This is a more than reasonable assumption as BMA vertical migration rates have been reported to be $612 - 1008 \mu\text{m h}^{-1}$ (Consalvey et al., 2004).

All beakers and utensils used during this process were acid-washed in 10% HCl and thoroughly rinsed with deionized water before the next use.

Sampling and Maintenance

Core tubes with a 1.0 cm^2 interior area were used to take sediment samples for chlorophyll *a* (chl *a*) concentration analysis using fluorometry and for biomarker pigment analysis by high performance liquid chromatography (HPLC) throughout the 10 day

exposure period of this experiment (See Table 1.2 for sampling schedule details). On sampling days for fluorometry, five samples were taken from each treatment and sectioned at 2 mm below surface level (as this is where the majority of photosynthetically active BMA reside (MacIntyre et al., 1996)) using a sectioning tool that pushes the sediment from the bottom of the core at 1 mm intervals with each turn of its handle. Sectioned samples were stored in 20 ml scintillation vials until processing within several hours of sectioning. Five samples from each treatment were also collected for HPLC at the beginning and end of the experiment. These samples were sectioned at a 5 mm depth and stored in 1 ml microcentrifuge tubes at -80°C until analyzed. A depth of 5 mm was selected for the HPLC samples to determine whether or not BMA community composition changed over time within the majority of the layer of contaminated sediment (since 6 mm total layer of contaminated sediment was applied to the collected sediment brick).

Throughout the 10 day experiment, mesocosms were routinely checked for clogged drains, malfunctioning pumps and leaks. Freshwater was regularly added to the carboy reservoirs to offset evaporative water loss from the mesocosms and to maintain salinity (initial salinity was ~ 30). Mesocosms were protected from animal disturbances (such as raccoons and birds) by a domed cage made of hardware cloth and were protected from heavy rain events with plastic tarps to prevent the washing away of dosed surface sediments into the reservoirs or the clogging of drains with sediments.

Table 1.2 Sampling schedule.

	Day 0	Day 4	Day 7	Day 10
Details of Day	The day before first addition of dosed sediments; initial samples taken	The day following final application of dosed sediments		Final sampling day
Samples taken; Sectioning depth	5 samples taken for fluorometry; sectioned at 2 mm depth 5 samples taken for HPLC; sectioned at 5 mm depth	5 samples taken for fluorometry; sectioned at 2 mm depth	5 samples taken for fluorometry; sectioned at 2 mm depth	5 samples taken for fluorometry; sectioned at 2 mm depth 5 samples taken for HPLC; sectioned at 5 mm depth

Sample Analysis

Ten milliliters of HPLC-grade 100% acetone was added to samples taken for chl *a* concentration analysis by fluorometry. The samples were agitated through vigorous shaking for 30 seconds and stored at -20°C for roughly 16 hours during extraction. Fluorescence of the extract was measured with a Turner TM Trilogy Laboratory Fluorometer (model #: 7200-000) using the non-acidification method (Welschmeyer, 1994).

Once removed from -80°C storage, samples for biomarker pigment analysis by HPLC were freeze dried overnight. To each sample, 1 ml 90% acetone and 50 µl of synthetic carotenoid β-apo-8'-carotenal (Sigma, cat. no. 10810) (an internal standard) were added. Samples were then sonicated for 30 seconds and stored at -20°C for 24 hours during extraction. A centrifuge was used to spin down the samples and the supernatant (sample extract) was filtered through a 0.45-µm PTFE filter (Gelman Acrodisc). The filtered extract (450 µl) and an ion-pairing solution (1.00 M ammonium acetate) (113 µl) were dispensed into amber glass autosampler vials which were immediately loaded into the refrigerated (4°C) autosampler for analysis.

Samples (250 µL) were injected into a Shimadzu HPLC equipped with a monomeric and polymeric reverse-phase C₁₈ column in series. A nonlinear binary gradient consisting of the solvents 80% methanol:20% 0.50 M ammonium acetate and 80% methanol:20% acetone was used for pigment separations. Absorption spectra and chromatograms (440 ± 4 nm) were acquired using the Shimadzu software and pigment peaks were identified based on retention times and absorption spectra (Pinckney et al. 1996).

Statistical Analysis

A linear regression was performed on the chl *a* concentrations within samples from Day 0 and Day 10, measured by HPLC (independent variable) and fluorometric (dependent variable) methods in order to determine if there was linear relationship between values obtained by both methods.

Change in chl *a* concentration, determined by fluorometry, was compared between treatments for Day 0 – Day 4, Day 4 – Day 10 and Day 0 – Day 10. Separate single-factor ANOVAs were performed for each of the three time intervals to test for differences between treatments. Due to the inherent spatial variability of BMA abundance in sediments (MacIntyre et al., 1996), an average from the five samples taken within each tank on each sampling day was used as the representative value for that tank and day combination (See Equation 1.1). The Ryan-Einot-Gabriel-Welsch F (R-E-G-W-F) post-hoc test was used for multiple comparisons of means when significant treatment effects were observed.

In order to identify any change in benthic algal community composition, changes (as the difference between final and initial values) in fucoxanthin (a biomarker for diatoms) and zeaxanthin (a biomarker for cyanobacteria) concentrations, as well as changes in the fucoxanthin to zeaxanthin ratio (Fuco:Zea Ratio) were compared between treatments. The average concentration (for each pigment), as well as the average Fuco:Zea Ratio, from the five samples taken within a mesocosm tank on a single sampling day was used as the representative value for that tank in the appropriate statistical analysis. A multivariate analysis of variance (MANOVA) followed by individual univariate analyses, with the changes in fucoxanthin concentration and

zeaxanthin concentration as response variables, were used for statistical analysis of the biomarker pigment concentrations. A single-factor ANOVA using the change in Fuco:Zea Ratio as a response variable was employed to compare changes in community composition between treatments. The R-E-G-W-F post-hoc test was used for multiple comparisons of means when significant treatment effects were observed.

IBM SPSS Statistics software (Version 21) was used for all analyses in this experiment. Tests were performed to verify that the data met assumptions of normality (K-S test), homogeneity (Levene's test) and independence of variance (Durbin-Watson statistic) when appropriate.

1.3 RESULTS

Relationship between HPLC and fluorometric measurements

The linear regression analysis determined that there was a significant linear relationship between chl *a* concentrations measured with HPLC and fluorometric methods (linear regression, $p < 0.001$, adj. $r^2 = 0.624$) (Figure 1.4). The linear relationship is expressed by Equation 1.1.

$$y = 0.375x + 1.396 \quad \text{(Equation 1.1)}$$

Where y = fluorometric values and x = HPLC values.

BMA biomass

Results from the single factor ANOVA indicated that there was no significant difference between treatments with respect to overall change (difference between final and initial values) in chl *a* concentrations (i.e. BMA biomass) as determined by

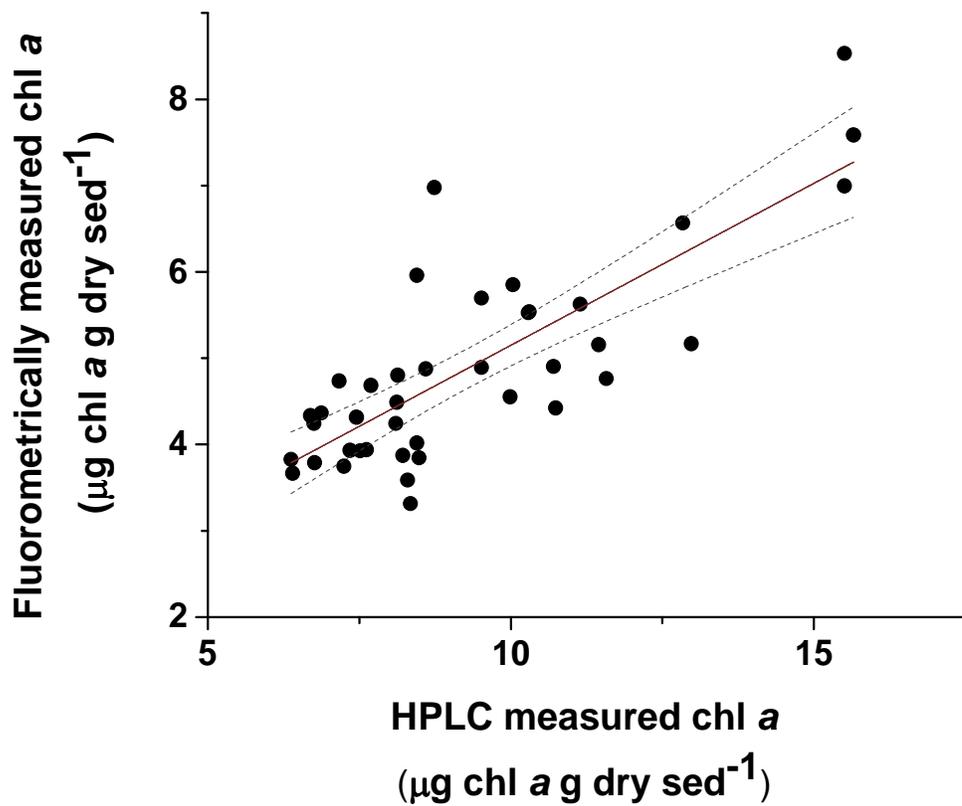


Figure 1.4 Relationship between chl *a* concentrations measured by HPLC and fluorometric methods. Sediment samples analyzed for chl *a* concentration by HPLC and fluorometry were sectioned at 5 mm and 2 mm depths, respectively. Linear fit line is represented by a solid line, dashed lines represent 95% confidence interval (n = 4).

fluorometric methods (ANOVA, $p = 0.325$) during this 10 day experiment (Figure 1.5). There were, however, notable trends in the chl *a* concentrations throughout the experiment. An initial decrease between Day 0 and Day 4 was observed in all of the treatments. This was then followed by an increase in chl *a* concentrations until the end of the experiment (Day 4 – Day 10) (Figure 1.6). No significant difference was measured between treatments with respect to the change in chl *a* concentration during the period of decline (ANOVA, $p = 0.673$), (Figure 1.7). However, there was a significant treatment effect on the change in chl *a* concentration between Day 4 and Day 10 (ANOVA, $p = 0.032$), when concentrations appeared to increase in all treatments (Figure 1.8). The increase in the Cu treatment was significantly greater than the increase in the Water control. Two homogeneous groups were revealed by the R-E-G-W-F post-hoc test: Water, Acetone, PAHs and Mix ($p = 0.066$); Acetone, PAHs, Mix and Copper ($p = 0.133$).

BMA community composition

The BMA community composition was affected by treatments that included copper as well (Cu and Mix treatments). The MANOVA using fucoxanthin concentration and zeaxanthin concentration as response variable indicated that there was a significant treatment effect on combination of variables analyzed (Roy's Largest Root, $p < 0.001$). Subsequent univariate analyses of the data revealed no significant difference between treatments with respect to the change in fucoxanthin concentration (ANOVA, $p = 0.114$) (Figure 1.9), but there was a significant treatment effect on the change in zeaxanthin concentration (ANOVA, $p < 0.001$). The R-E-G-W-F post-hoc test identified four

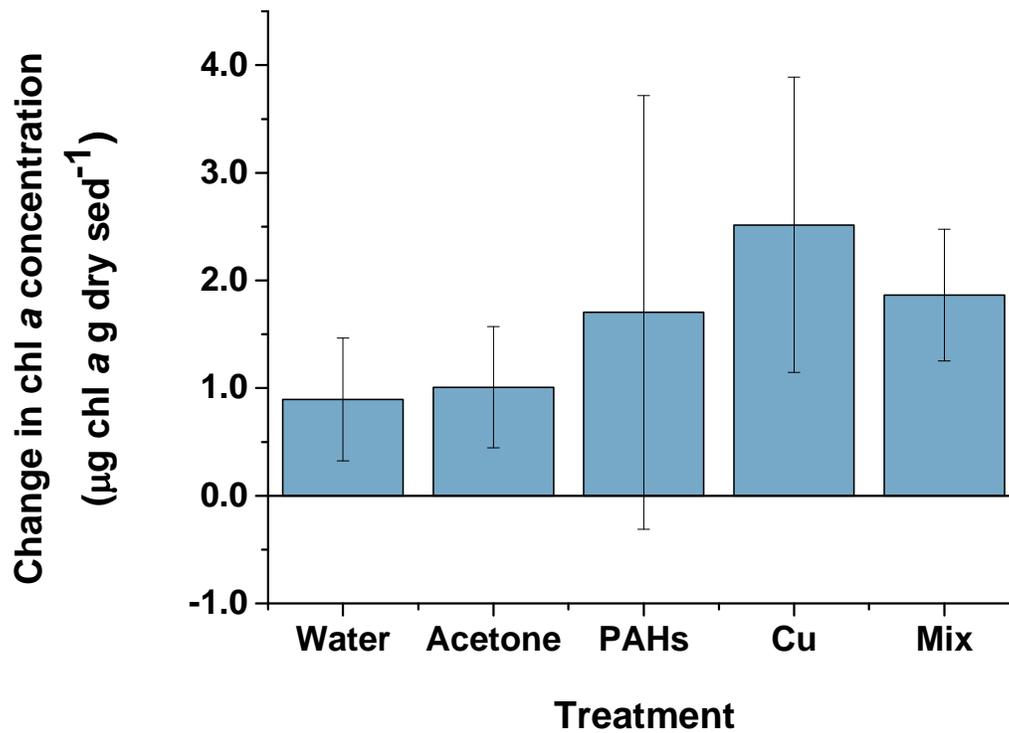


Figure 1.5 Change in chl *a* concentration within dosed sediments from Day 0 to Day 10. The reported change is the difference between Day 10 and Day 0 chl *a* concentrations within the top 2 mm of dosed sediments in mesocosm tanks, measured by fluorometric methods. Water and Acetone treatments were used as controls for contaminant (PAHs, Cu and Mix) treatments. The Mix treatment was dosed with both PAHs and Cu. Values represent means \pm 1 SD (n = 4).

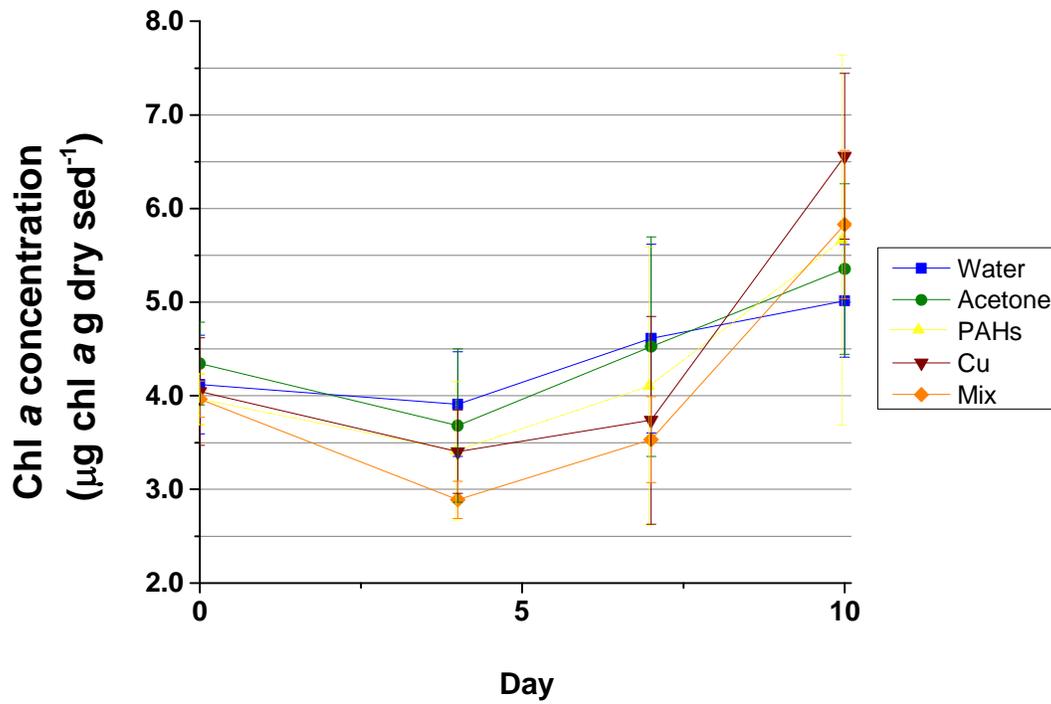


Figure 1.6 Change in chl *a* concentration within dosed sediments over time. Chl *a* concentration values represent those within the top 2 mm of dosed sediments in mesocosm tanks, measured by fluorometric methods. Water and Acetone treatments were used as controls for contaminant (PAHs, Cu and Mix) treatments. The Mix treatment was dosed with both PAHs and Cu. Values represent means \pm 1 SD ($n = 4$).

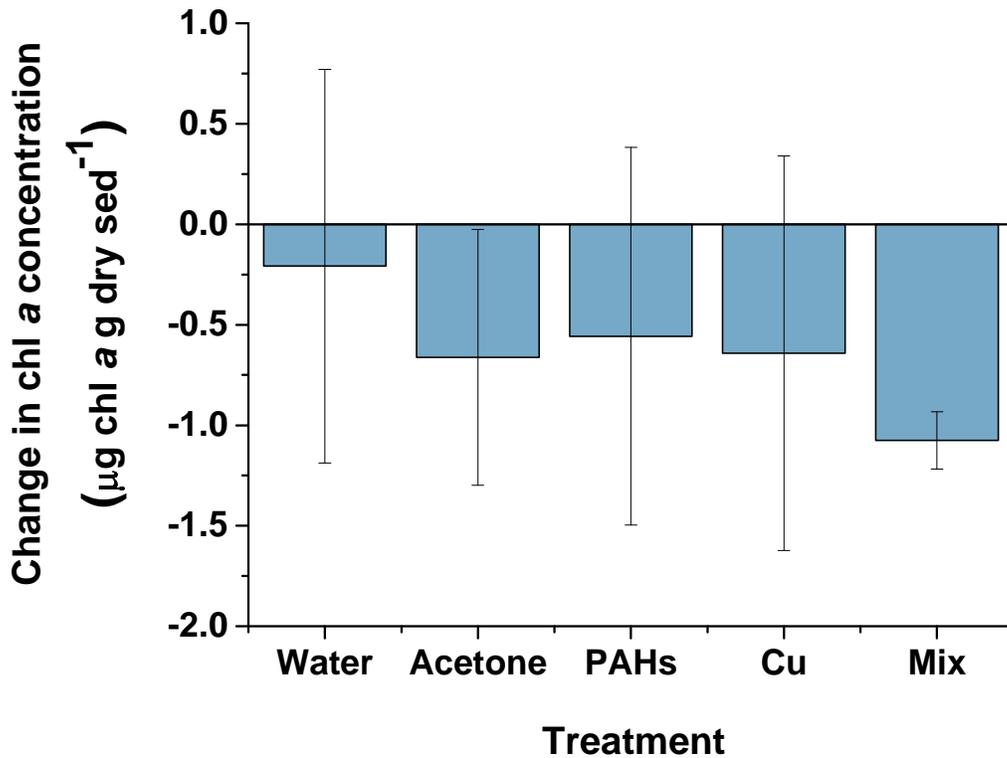


Figure 1.7 Change in chl *a* concentration within dosed sediments from Day 0 to Day 4. The reported change is the difference between Day 4 and Day 0 chl *a* concentrations within the top 2 mm of dosed sediments in mesocosm tanks, measured by fluorometric methods. Water and Acetone treatments were used as controls for contaminant (PAHs, Cu and Mix) treatments. The Mix treatment was dosed with both PAHs and Cu. Values represent means \pm 1 SD ($n = 4$).

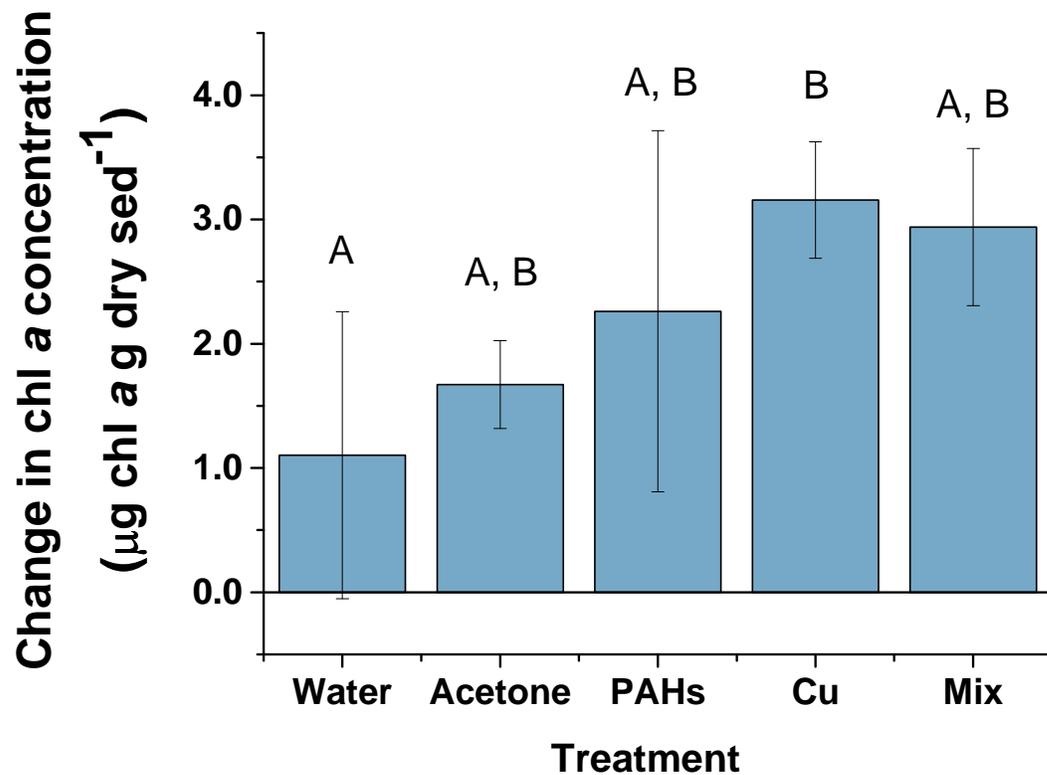


Figure 1.8 Change in chl *a* concentration within dosed sediments from Day 4 to Day 10. The reported change is the difference between Day 10 and Day 4 chl *a* concentrations within the top 2 mm of dosed sediments in mesocosm tanks, measured by fluorometric methods. Water and Acetone treatments were used as controls for contaminant (PAHs, Cu and Mix) treatments. The Mix treatment was dosed with both PAHs and Cu. Letters above bars indicate homogeneous groups. Values represent means \pm 1 SD ($n = 4$).

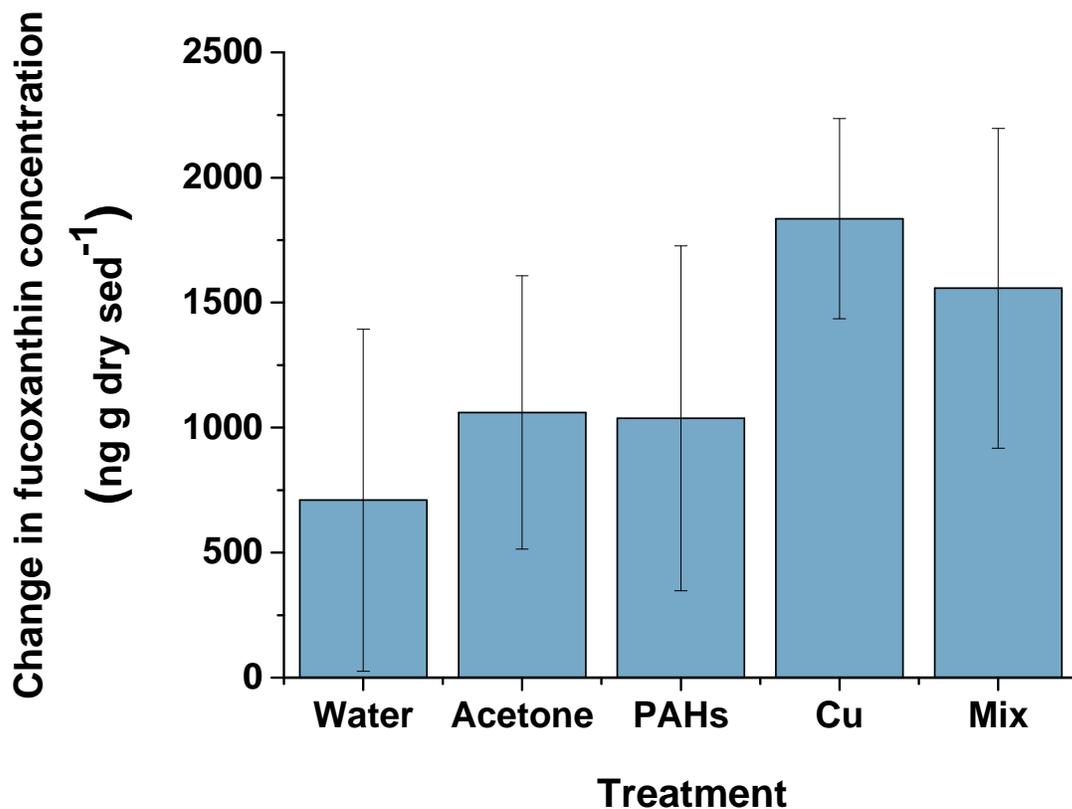


Figure 1.9 Change in fucoxanthin concentration within dosed sediments. The reported change is the difference between Day 10 and Day 0 fucoxanthin concentrations within the top 5 mm of dosed sediments in mesocosm tanks, measured by HPLC. Water and Acetone treatments were used as controls for contaminant (PAHs, Cu and Mix) treatments. The Mix treatment was dosed with both PAHs and Cu. Values represent means \pm 1 SD (n = 4).

homogeneous groups within the zeaxanthin data: Cu and Mix ($p = 0.645$); Mix and Acetone ($p = 0.068$); Acetone and PAHs ($p = 0.460$); PAHs and Water ($p = 0.133$). All treatments (except Cu) showed an increase in zeaxanthin concentration over the experiment (Figure 1.10). As a result, the change in the Cu treatments was significantly lower than that in the Water control, Acetone control and PAHs treatment. Though there was a slight increase in zeaxanthin concentration in the Mix treatment, this change was significantly lower than those observed in the Water control and PAHs treatment. The Water control also showed a significantly greater increase in zeaxanthin concentration than the Acetone control.

Linked to these differences between treatments with respect to pigment concentration, there was a significant treatment effect on the change in Fuco:Zea Ratio (ANOVA, $p = 0.001$) and three homogeneous groups were recognized by the R-E-G-W-F post-hoc test: Water, PAHs and Acetone ($p = 0.397$); PAHs, Acetone and Mix ($p = 0.058$); Mix and Copper ($p = 0.482$). Copper showed a significantly greater increase in the ratio than the Water control, Acetone control and PAHs treatment and the Mix treatment showed a significantly greater increase in the ratio than the Water control (Figure 1.11).

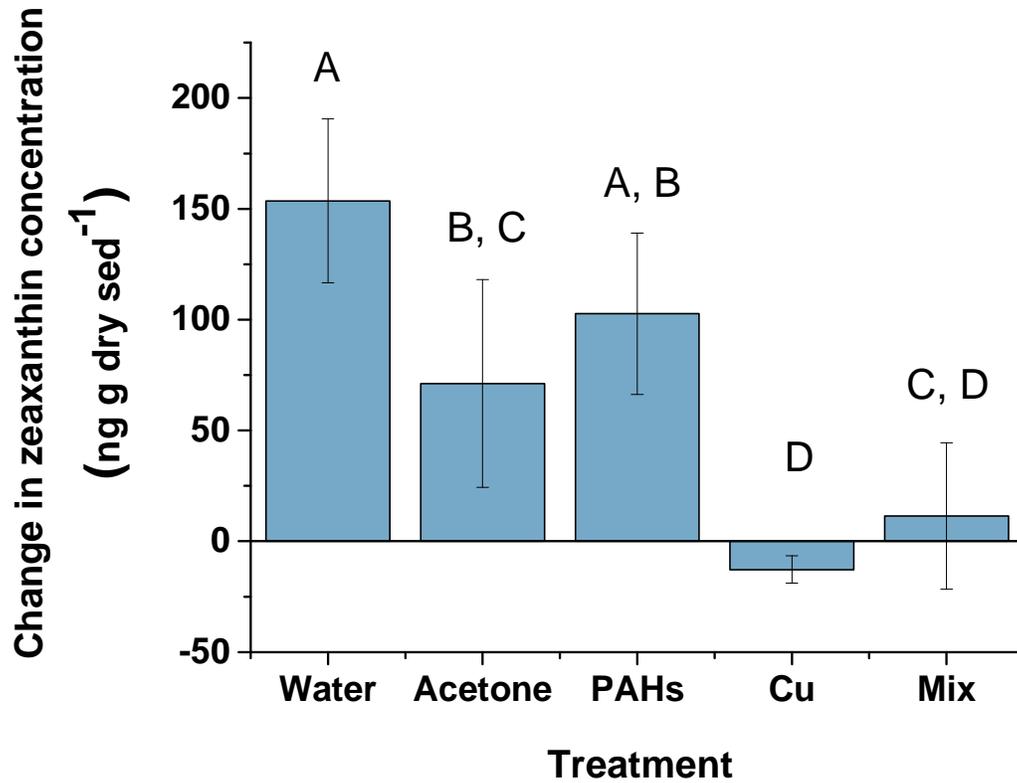


Figure 1.10 Change in zeaxanthin concentration within dosed sediments. The reported change is the difference between Day 10 and Day 0 zeaxanthin concentrations within the top 5 mm of dosed sediments in mesocosm tanks, measured by HPLC. Water and Acetone treatments were used as controls for contaminant (PAHs, Cu and Mix) treatments. The Mix treatment was dosed with both PAHs and Cu. Letters above bars indicate homogeneous groups. Values represent means \pm 1 SD (n = 4).

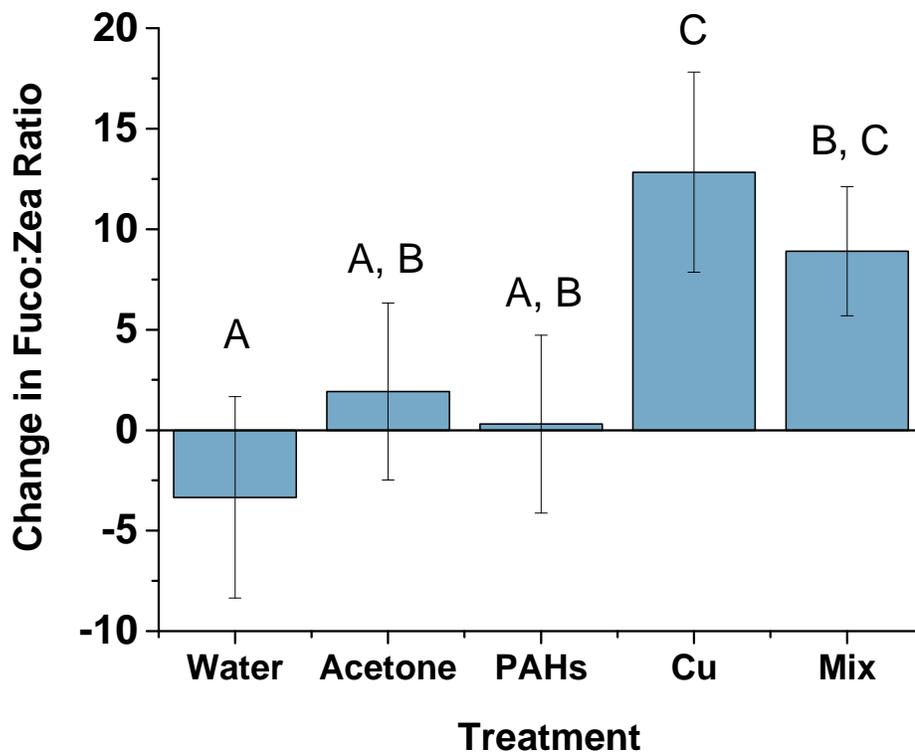


Figure 1.11 Change in Fuco:Zea Ratio within dosed sediments. The reported change is the difference between Day 10 and Day 0 fucoxanthin to zeaxanthin ratios within the top 5 mm of dosed sediments in mesocosm tanks, measured by HPLC. Water and Acetone treatments were used as controls for contaminant (PAHs, Cu and Mix) treatments. The Mix treatment was dosed with both PAHs and Cu. Letters above bars indicate homogeneous groups. Values represent means \pm 1 SD (n = 4).

1.4 DISCUSSION

The results from this study investigating the effects of PAHs and/or Cu on BMA biomass and BMA community composition suggest a complex benthic community response. Although HPLC provides a more accurate measurement of chl *a* concentration than fluorometry (Pinckney et al., 1994), it requires a great deal more processing time. For this reason, fluorometry was used as the primary method of measuring chl *a* in this study. The linear regression revealed a significant linear relationship between HPLC and fluorometric measurements of chl *a* concentrations, indicating that the samples analyzed by fluorometric methods can be used to make valid comparisons between treatments, in relative terms. Furthermore, the samples used for pigment analysis by HPLC were sectioned at a 5 mm depth, while those analyzed by fluorometry were sectioned at a 2 mm depth and the difference in sediment sample volume is likely a contributing factor to the higher chl *a* concentrations obtained by HPLC.

The chl *a* concentration measurements indicate that there was no significant treatment effect on the overall change in algal biomass during the 10 day experiment (Figure 1.5). This was contrary to my hypothesis that algal biomass in all contaminant treated tanks (PAHs, Cu and Mix) would decline due to the toxic effects of each of the contaminants tested and that there would be a synergistic effect upon exposure to both pollutants. Closer examination of the data throughout the experiment provides some insights into the possible dynamics of the sediment community.

Figure 1.6 shows an initial decreasing trend in chl *a* concentration in all treatments, but there was no significant difference between treatments with respect to the change in chl *a* concentration during that time frame. This indicates that there were no

significant toxic effects of the contaminants on BMA, using BMA biomass as the response variable. Conversely, other work has shown copper to have direct toxic effects on algae such as inducing oxidative stress and ultrastructural impairment of cells likely due to the interference of copper with the cell membrane (Pinto et al., 2003; Wong and Zheng 2008; Sabatini et al., 2009). As a result, primary productivity and overall biomass can greatly be reduced (Cid et al., 1995; Alsterberg et al., 2007; Wong and Zheng 2008; Araújo et al., 2010). The copper concentration used in this study was higher than the EC₅₀ for microalgal growth inhibition demonstrated by Moreno-Garrido et al. (2003) for the benthic pennate diatom, *Cylindrotheca closterium*, but no such effects were observed here. Microalgal sensitivity to copper is affected by interactions between species, with multispecies assemblages exhibiting higher EC₅₀ values than single-species populations (Yu et al., 2007; Levy et al., 2009). It is possible, therefore, that the concentration of copper used in the present study was too low to cause dramatic reductions in biomass (as observed in other studies) in the natural, multispecies sediment community utilized here. Furthermore, much of the copper in this experiment was likely adsorbed onto sediment and organic particles (Helland and Bakke, 2002) whereas other studies have exposed algal cultures in aqueous solutions (Cid et al., 1995; Wong and Zheng 2008; Araújo et al., 2010) in which the copper would be more bioavailable and therefore have stronger effects. Numerous studies have also shown direct toxic effects of PAHs on BMA (Piehler et al., 2003; Wang and Zheng, 2008; Wang et al., 2008; Peterson et al. 2009), however, these differed from the current study in the PAH compounds used (Piehler et al., 2003; Wang and Zheng, 2008; Wang et al., 2008; Peterson et al., 2009) and/or the medium that was contaminated (water vs. sediment, respectively) (Wang and Zheng, 2008; Wang et

al., 2008). Individual PAH compounds vary in toxicity and can act synergistically or antagonistically with other PAH compounds, resulting in complex effects dependent on the PAHs involved (Wang et al., 2008). Therefore, the lack of significant PAH toxic effects may be due to the relatively low concentration, the combination of compounds and the mode of exposure used in this experiment. PAHs and copper have the potential to negatively affect BMA biomass, but no such effects were measured in this experiment, possibly due the methods employed.

Alternatively, toxic effects may have been induced by the contaminants but remained undetected due to the sampling schedule used in this experiment. PAHs and copper can induce oxidative stress, inhibit growth or reduce biomass in BMA communities within 24 hours of exposure (Cid et al., 1995; Wang and Zheng, 2008; González et al., 2009). However, in the present study, sediment samples were not collected until four days after initial contaminant introduction. By that time in the experiment, BMA communities may have begun to recover (in terms of biomass) from initial contaminant effects as they have been shown to be resilient to disturbances (Piehler et al., 2003; Alsterberg et al., 2007; González et al., 2009). Alsterberg et al. (2007) revealed that light utilization efficiency and primary production of benthic microalgae were significantly reduced within 24 hours of exposure to a copper-containing antifouling biocide, but by Day 3 of the experiment, there was no significant difference between treatments (control vs. exposed) with respect to primary production, indicating a rapid physiological recovery of the exposed BMA. Similarly, Piehler et al. (2003) and González et al. (2009) demonstrated recovery of diatom density and chl *a* concentration, respectively, within several days to levels close to or greater than those

observed for controls. These results provide support for the hypothesis that in the present study, BMA recovery may have occurred before sampling on Day 4. The trend in Figure 1.6 suggests average chl *a* concentrations for all contaminant treatments were lower than those of the controls at Day 4, hence, it is possible that there were immediate toxic effects due to contaminant exposure, but rapid BMA recovery diminished the evidence of those effects (relative change in chl *a* concentration) before the first samples after exposure were taken. Further experimentation implementing more frequent sampling would be needed determine if PAHs and/or copper at the environmentally relevant concentrations used in this study produce any negative impacts on BMA biomass within very short time periods after exposure.

After the period of decline (Day 0 – Day 4), chl *a* concentrations increased in every treatment until the end of the experiment (Day 4 – Day 10), with final values exceeding initial values (Figure 1.6). The general increase was likely due to the exclusion of large grazers, such as fiddler crabs and gastropods, from the mesocosms. The biomass increase in the Cu treatment from Day 4 to Day 10 was significantly greater than that in the Water control suggesting a contaminant effect (Figure 1.8). A possible explanation is that the greater increase was the result of toxic copper effects on meiofauna, which thereby decreased grazing pressure on BMA. Several studies have found that copper significantly alters the meiofauna community composition and reduces overall meiofauna abundance (Dalto et al., 2006; Alsterberg et al., 2007). More specifically, nematodes (the most predominant meiofauna group found in North Inlet tidal flats (Findlay 1981; Montagna et al., 1983; Coull, 1985)) show a reduction in abundance, intrinsic rate of natural increase and development rate (Vranken and Heip, 1986; Korthals et al., 1996;

Gyedu-Ababio and Baird, 2006) upon copper exposure, but these responses are species specific. In the study by Alsterberg et al. (2007), a significant reduction in meiofauna abundance from exposure to a copper containing antifouling agent was not seen until 9 days after contamination, indicating a somewhat latent effect. The significant contaminant effect on chl *a* concentration in the present study was not detected until after Day 4 (more specifically, the greatest increase occurred between Day 7 and Day 10) (Figure 1.6). Therefore, the possibility that a reduction in grazing pressure due to toxic effects on meiofauna allowed for a significant increase in BMA biomass at that point in the experiment within copper treated tanks is supported by the Alsterberg et al. (2007) study. The trends in the data (dramatic alterations in slope and steep slope from Day 7 – 10) suggest the BMA population did not reach steady state by the end of the 10 day experiment. Differences between the Cu treatment and control, therefore, may have reached an even greater magnitude over longer time periods, especially if sufficient time was allowed for the developmental effects of copper on meiofauna to exert an influence. The implications of these results could be of ecological significance in that meiofauna are involved in the breakdown of detritus, the cycling of nutrients and serve as important bioturbators as well as food sources to higher trophic levels (Coull, 1999). Thus a reduction in meiofauna abundance could have impacts on biogeochemical cycles and food webs (through both bottom-up and top-down effects) of estuarine habitats.

If there was a significant toxic effect on meiofauna due to copper exposure, resulting in reduced grazing pressure and subsequent increases in BMA biomass, the Mix treatment (which had a copper component) should have also exhibited a significantly greater increase in BMA biomass (as chl *a*) relative to the Water control. Though that

was not the case (which appears to be due to the high degree of variability in the data from the Water and Mix treatments), the change in chl *a* from Day 4 – Day 10 in the Mix treatment was very close to and not significantly different than that of the Cu treatment (according to the post-hoc test) (Figure 1.8). This suggests there may have been some degree of a contaminant effect. The significance of that effect in the Mix treatment may have been reduced due to an antagonistic interaction between the two contaminants with respect to meiofauna response. While copper can have toxic effects (Vranken and Heip, 1986; Korthals et al., 1996; Dalto et al., 2006; Gyedu-Ababio and Baird, 2006; Alsterberg et al., 2007), some nematode species show an increase in abundance (Mahmoudi et al., 2005) and grazing rate (Carman et al., 1997) after PAH exposure. Alternatively, as organic compounds can form complexes with copper, interaction between the contaminants affecting bioavailability (and therefore toxicity to meiofauna) may be another explanation for the slightly less stimulatory effect on BMA observed in the Mix treatment (Newell and Sanders, 1986; Meador, 1991; Breault et al., 1996; Wright and Mason, 2000; Renella et al., 2004). This result exemplifies the potential complexity behind multiple stressor interactions and the difficulty in interpretation, and subsequent prediction, of organismal responses.

Though other studies have shown toxic effects of PAHs on overall meiofauna abundance and grazing rate (Carmen et al. 1997; Sundbäck et al., 2010), there was no evidence (as a significant increase in chl *a* concentration in sediments) of such effects in this experiment. This is possibly due to the lower PAH concentration and less toxic PAH compound combination used in this study compared to others. Sundbäck et al. (2010) utilized a PAH concentration 5x that used in the current study and Carmen et al. (1997)

exposed communities to diesel, which is comprised of a suite of many PAHs (Dobbins et al., 2006). The lack of evidence for toxic effects of PAHs on meiofauna in this study contrasts with the findings of other work, but may be attributed to the dosing specifics.

While there was no significant treatment effect on overall change (Day 0 – Day 10) in algal biomass based on chl *a* concentrations, there was a significant shift in the BMA community composition due to copper exposure. The initial Fuco:Zea Ratios for all treatments were ~ 10:1, but by Day 10 that ratio roughly doubled in the Cu and Mix treatments. These dramatic increases in the Fuco:Zea Ratio suggest an increase in relative diatom to cyanobacteria abundance in those tanks that had been treated with copper (Cu and Mix treatments). The shift in this ratio for those treatments is the product of much smaller changes in the zeaxanthin concentrations (a decrease in the Cu treatment and only a slight increase in the Mix treatment) relative to the increases in fucoxanthin concentrations in both treatments. The changes in zeaxanthin concentration were significantly less in the Cu and Mix treatments than several other treatments (including the Water control) (Figure 1.10), while the change in fucoxanthin concentration showed no significant difference between treatments (Figure 1.9). At the concentration used in this study, PAHs had no effect on the Fuco:Zea Ratio or the respective pigment concentrations relative to the control. It appears that exposure to copper solely drove the changes in BMA community composition in this experiment.

The significantly lower changes in zeaxanthin concentration in the Cu and Mix treatments compared to the Water control indicates a cyanobacterial sensitivity to copper contamination. Similar implications have been drawn from the results of other studies (Witaker et al., 1978; Hadjoudja et al., 2009) and the susceptibility of cyanobacteria to

negative copper effects may be related to their morphology and physiology.

Cyanobacteria are prokaryotes, possessing no membrane-bound organelles and are hence, less structurally complex than eukaryotic algae. Hadjoudja et al. (2009) proposed that this lack of compartmentalization in cyanobacterial cells allows for easier access of copper to the photosystem, contributing to their greater sensitivity to this heavy metal than other algal groups. Nitrogen-fixation, a process essential to the functioning of some cyanobacteria but not required by other microalgae, is inhibited by copper (Horne and Goldman, 1974) making that physiological process another potential factor adding to the susceptibility of cyanobacteria to copper. The sensitivity of algal groups to copper varies but seems to be greater in the prokaryotic cyanobacteria, possibly due to a combination of structural (morphological) and physiological (biochemical) traits they possess.

The implication that cyanobacteria are significantly affected by copper exposure could be of great importance in coastal areas where they dominate, including regions along the coast of New England, Europe and California (Sullivan and Currin 2000, Currin et al. 2011). Cyanobacteria can not only play important roles in salt marsh ecosystems as primary producers, but some also have the ability to fix N_2 , making them a valuable part of the nitrogen cycle in estuarine environments (Stal and Krumbein, 1981; Stal et al., 1985; Rejmánková and Komárková, 2005; Currin et al., 2011). Cyanobacteria can also form thick mats in areas where they occur in high abundance, which aid in stabilizing the sediments and preventing erosion (Noffke et al., 2001; Stal, 2003). The reduction in abundance of these microorganisms, therefore, could be detrimental to ecosystem functioning in some coastal areas if copper contamination reached the levels used in this study.

The results from this study emphasize the complexity of biological responses in sediment communities upon the exposure to multiple contaminants. Changes in BMA biomass showed no indication of toxic effects on the primary producers, but possible alleviation of grazing pressure by meiofauna as a result of copper-only contamination is speculated as the cause for greater increases in BMA biomass in the Cu treatment relative to controls. In addition, shifts in BMA community composition observed in copper treated sediments (Cu and Mix treatments) suggested a sensitivity of cyanobacteria to copper contamination. Overall, Cu only treatments had stronger effects on BMA biomass and the shifts in community composition than the Mix treatments that had been dosed with the same levels of copper, suggesting some mediating effect of PAHs in the Mix treatment. As the interaction between contaminants can vary depending on conditions and algal responses to stressors may be altered by the presence of other organisms in the community (Yu et al., 2007; Levy et al., 2009), this study highlights the importance of utilizing natural communities and environmentally relevant contaminant concentrations and ratios to accurately represent expected responses in the field.

CHAPTER 2: FIDDLER CRAB FEEDING RESPONSES TO SEDIMENTS CONTAMINATED WITH POLYCYCLIC AROMATIC HYDROCARBONS AND/OR COPPER

2.1 INTRODUCTION

Fiddler crabs (*Uca* spp.) are ubiquitous salt marsh residents and their biomass is usually the highest of all macro-invertebrates in those habitats (Montague, 1980). These decapods play very important roles in salt marsh ecosystems as a food source for fish, birds, and other crustaceans. Many of these predators are only temporary marsh residents and their consumption of fiddler crabs coupled with subsequent relocation could function as an energy export from marsh habitats (Montague, 1980; Grimes et al., 1989).

Fiddler crabs also serve as bioturbators since some of the most important activities for the survival of a fiddler crab, and for the functioning of the salt marsh ecosystem, involve the manipulation of sediments. Burrowing by these crabs provides refuge and also aerates the sediments, altering the sediment hydrology and chemistry which in turn can stimulate the growth of marsh grasses (Katz, 1980; Montague, 1980; Bertness, 1985; Grimes et al., 1989; Smith et al., 2009). While foraging during low tide, fiddler crabs bring sediments to the buccal cavity with the minor chelae where specialized mouth parts are utilized to scrape microorganisms from sediment particles (Miller, 1961; Meziane et al., 2002). After ingestion of the organic matter (along with fine sediment particles), the crabs deposit the non-ingested particles back onto the

substrate in small irregular shaped balls (feeding pellets) (Miller, 1961). The deposit feeding of fiddler crabs, therefore, results in additional bioturbation that oxygenates the sediments, altered grain size distribution and decreased organic content of sediments (Hoffman et al., 1984; Meziane et al., 2002; Sayão-Aguiar et al., 2012). These alterations in sediment characteristics can also affect sediment chemistry as well as microbial activity. The bioturbation associated with fiddler crab activity (burrowing and feeding) has both biological and physical impacts important for salt marsh ecosystem health.

Because fiddler crabs are deposit feeders that consume organic matter in sediments, including benthic microalgae (BMA), these decapods could be directly affected by changes in quantity or quality of BMA that result from anthropogenic disturbances. Increased urbanization in coastal areas has been associated with relatively high concentrations of various pollutants in tidal creeks (Sanger et al., 1999 a, b; Holland et al., 2004; Van Dolah et al., 2008; Garner et al., 2009). Polycyclic aromatic hydrocarbons (PAHs), found in petroleum products and released upon combustion of fossil fuels, and copper are two common contaminants in estuarine habitats that can be introduced by a number of human activities. Urban runoff, oil spills or leaks and air deposition of PAHs released during combustion processes serve as frequent PAH sources to the environment (Kucklick et al. 1997; Ngabe et al., 2000; Kose et al., 2008; Nikolaou et al., 2009). Copper-based biocide paint, commonly used on boats and docks, is one of the main sources of copper in coastal habitats. In addition, urban runoff, sewage discharge and drainage from copper-based algaecide treated ponds can also contribute to marine copper levels (Thomson et al., 1984; Matthiessen et al., 1999). The adsorption of PAHs and copper to particles (both organic and inorganic) leads to accumulation of these

contaminants in coastal sediments (Helland and Bakke, 2002; Nikolaou et al., 2009; Tobiszewski and Namieśnik, 2012). As such, there is potential for PAHs and copper to affect benthic organisms, including fiddler crabs and their food sources. BMA biomass, as well as BMA community composition, can be negatively impacted by PAHs and copper (Cid et al., 1995; Piehler et al., 2003; Wang and Zheng, 2008; Wang et al., 2008). Because these primary producers form the foundation of many estuarine food webs, subsequent effects on trophic energy transfer and on organisms at higher trophic levels due to contaminant exposure of this food source are possible.

The chemosensory system plays a significant role in the behavior of fiddler crabs, and has evolved to be somewhat specialized. The feeding of fiddler crabs is mediated by this system which involves sensory receptors located on the minor chelae and legs of the crab (Robertson et al., 1980). At low tide, fiddler crabs probe the sediments with their appendages, evaluating the organic content based on chemical cues received by the sensory receptors immersed in the sediments (Robertson et al. 1980). Feeding is initiated when the sensory stimulation exceeds a threshold, thus the feeding response is dependent upon food density (Robertson et al. 1980). Aside from the ability to detect differences in food quantity, Robertson et al. (1981) showed sand fiddler crabs (*Uca pugilator*) can also distinguish between different chemical stimulants. Feeding behavior is elicited by compounds associated with natural crab food sources (particularly BMA and detritus), while compounds associated with food sources of other crustaceans (flesh) do not induce strong responses (Robertson et al., 1981). Additionally, according to Rittschof and Buswell (1989) various fiddler crab species exhibit feeding preferences towards different hexose sugars. Taken together, these studies suggest fiddler crab feeding responses are

very sensitive to chemical stimuli and these crabs have the ability to detect chemical differences (as quantity and quality of stimuli) in sediments.

Proper functioning of the chemosensory system, therefore, is essential to fiddler crab survival. Chemical contaminants can often interfere with crustacean chemosensory, resulting in altered behavior or a lack of responses to natural chemical stimuli. There has been some evidence that other crustacean groups have the ability to detect various pollutants (including PAHs and copper) and show avoidance of them (McLeese, 1975; Hellou et al., 2005; De Lange et al., 2006). In addition, a reduction in feeding behavior has been observed for lobsters, crayfish and blue crabs due to PAH exposure (Atema and Stein, 1974; Pearson and Olla, 1980; Gauthier 2012) as well as in prawn and lobsters after copper exposure (McLeese, 1975; Santos et al., 2000). Persistent alterations in feeding responses as a result of chronic exposure to these contaminants in natural habitats may have detrimental impacts on the health of crustaceans. In the case of fiddler crabs, impaired health and a decline in bioturbation due to reduced feeding activity could impact the functioning of the entire ecosystem. The strong influence of the chemosensory system over fiddler crab behavior may lead to avoidance of chemically contaminated habitats if the contaminants are detected or an alteration (perhaps even an inhibition) in feeding behavior if the contaminants disrupt functioning of the chemosensory system.

The first objective of this experiment was to investigate the effects of chemical contaminants (as PAHs and/or copper) within sediments on feeding preference/avoidance of fiddler crabs using a food choice experiment. Based on results from other studies showing crustacean avoidance of PAHs and copper (McLeese, 1975; Hellou et al., 2005; De Lange et al., 2006) and the known sensitivity of the fiddler crab chemosensory

system, it was hypothesized that fiddler crabs would avoid feeding in contaminated sediments, with higher avoidance of those contaminated with multiple pollutants (PAHs and Cu). Two possible mechanisms leading to this result were postulated and evaluated in this experiment. First, any avoidance of crab feeding in contaminated sediments may have been related to food quantity (BMA biomass) and quality (BMA community composition). Differences in these parameters were expected between treatments (controls and various contaminant treatments) and it was predicted that BMA biomass would be significantly reduced (and BMA community composition would be altered) due to contaminant exposure (Witaker et al., 1978; Cid et al., 1995; Wang and Zheng, 2008; Wang et al., 2008). Since fiddler crab feeding is dependent on food density, it was proposed that this could lead to a reduction or avoidance of crab feeding in those treatments. The second possible mechanism that could lead to avoidance of contaminant treatments was chemical contaminant interference with proper chemosensory functioning. Because fiddler crab feeding is mediated by the chemosensory system, a disruption in receiving and/or the processing of natural stimuli (from food sources) could inhibit crab feeding, hence resulting in avoidance of those sediments.

The second objective of this study was to determine if the presence of chemical contaminants (PAHs and/or copper) in sediments would affect the feeding rate of fiddler crabs when no alternative food choice was provided. Based on the chemosensory disruptions PAHs and copper induced in other crustacean studies, it was hypothesized that the feeding rates of fiddler crabs exposed to either contaminant would be lower than those of the controls, and multiple pollutants will have a synergistic effect.

The crucial roles fiddler crabs and their feeding activities play in salt marsh ecosystems make research centered around the impacts of contaminants on fiddler crab feeding behavior essential for predicting responses at larger scales.

2.2 METHODS

Study Site

North Inlet (33.3° N, 79.1° W) is a bar-built estuary within the boundaries of the North Inlet – Winyah Bay National Estuarine Research Reserve (NI – WB NERR) in Georgetown, SC. This estuary contains extensive tidal creeks and experiences regular semi-diurnal tides, with a mean tidal amplitude of 1.4 meters. Major contributors to the primary production of North Inlet include *Spartina alterniflora*, as well as BMA. North Inlet is considered a relatively pristine with more than 90% of its watershed in a naturally forested state.

Sediments and sand fiddler crabs (*Uca pugilator*) used for the crab food choice and crab feeding rate experiments were collected from this site due to its relatively pristine condition, ensuring limited contaminant exposure of the natural benthic community prior to the experiment.

Crab food choice: Sediment collection

Polypropylene plastic containers with interior dimensions of 43 x 35 x 19 cm containing field collected sediments served as mesocosm tanks for this experiment. Sediment bricks that were the length and width of the tanks and 5 cm deep were collected from a tidal flat located near the Clambank research site within the NI – WB NERR (Figure 2.1; Figure 2.2) at low tide with a flat-edged shovel. Sediments for all tanks of a

single replicate were collected within a three meter span of the tidal flat at the same distance away from the bank to reduce spatial variability in sediment grain size and organic content/composition. Sediment bricks were transferred to the mesocosm tanks and immediately transported to the experimental study site behind the main research facility at the Belle W. Baruch Institute for Marine and Coastal Science (Figure 2.1). The site was exposed to full sun from 10:00 am - 3:30 pm and experienced ambient temperatures.

Crab food choice: Dosing and sediment incubation

To determine if crabs avoid feeding in contaminated sediments, crab food preference was examined by presenting fiddler crabs with sediments that had been dosed with the treatments listed in Table 2.1 yielding contaminant concentrations within the range observed in urbanized coastal areas of South Carolina (4.77 – 37.03 μg total PAHs/g dry sediment and 58.4 – 93.7 μg Cu/g dry sediment) (Sanger et al. 1999 a, b). The Acetone treatment was used as a control for the PAHs and Mix treatments (as acetone was used for the solvent of PAHs) and the Water treatment was used as an overall control (i.e. no added contaminants). All contaminants were purchased from Fisher Scientific (pyrene, 98+%, Cat No.: AC180830250; phenanthrene, 98+%, Cat No.: AC130090050; anthracene, 99%, Cat No.: AC104861000; copper (II) sulfate, Cat No.: AA1417836).

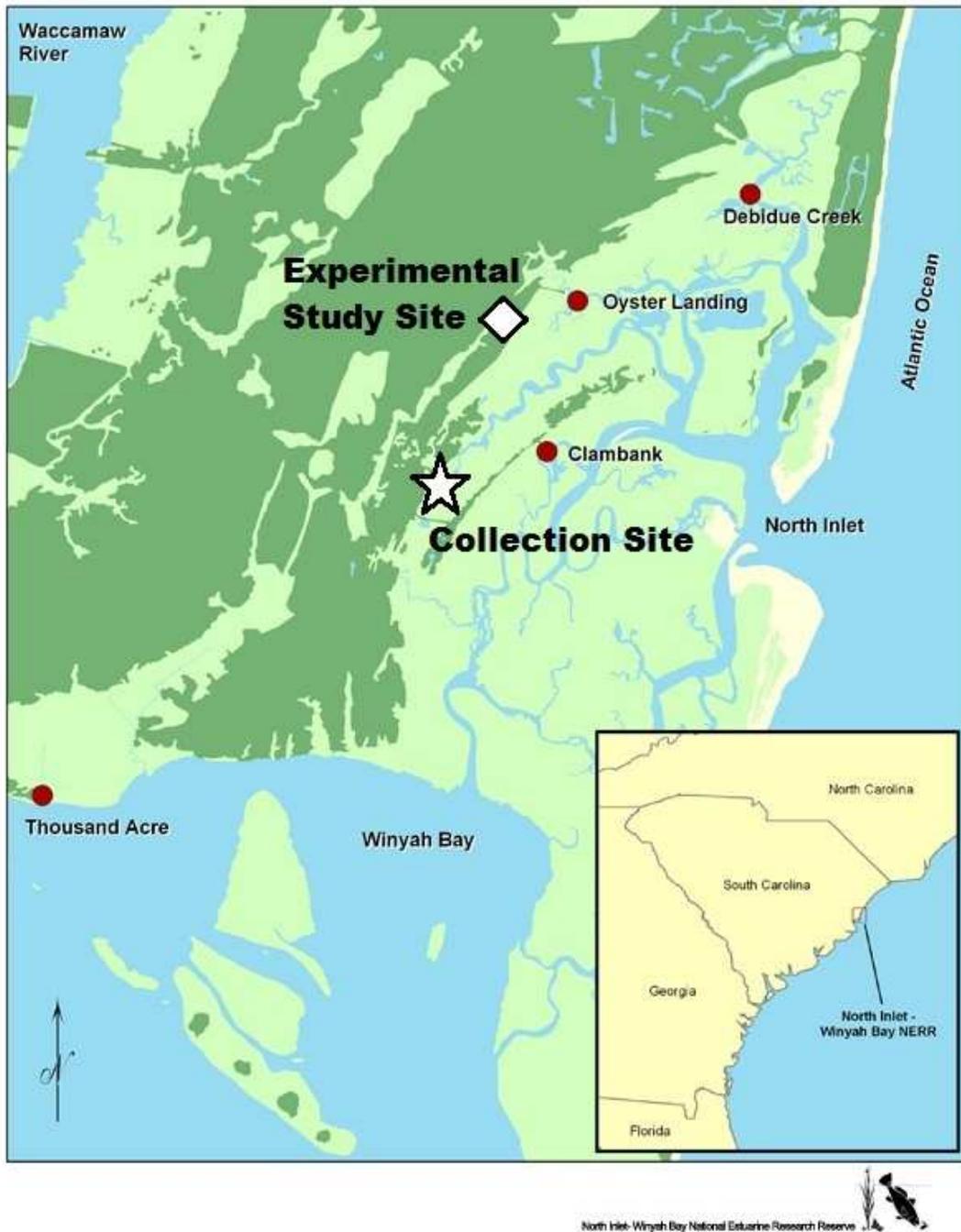


Figure 2.1 Location of research area in Georgetown, South Carolina, USA. Sediments were collected from a tidal flat near Clambank throughout the study and transported back to the main research facility at the Belle W. Baruch Institute for Marine and Coastal Science where mesocosms were set up and experiments took place.



Figure 2.2 Collection site photo. Tidal flat within North Inlet, SC

Table 2.1 Sediment dosing details.

Treatment	Contaminant Added	Solvent Used	Fluid Volumes Added	Concentrations of Pollutants Added
Water	None	N/A	40 ml deionized water	N/A
Acetone	None	N/A	20 ml 100% acetone 20 ml deionized water	N/A
PAHs	Mixture of PAHs (included pyrene, phenanthrene and anthracene)	100% acetone	20 ml 100% acetone 20 ml deionized water	10 µg total PAHs/g dry sed (composed of 6 µg pyrene/g dry sed; 3 µg phenanthrene/g dry sed; 1 µg anthracene/g dry sed)
Cu	Copper (as 5 H ₂ O · CuSO ₄)	Deionized water	40 ml deionized water	70 µg Cu/g dry sed
Mix	Mixture of PAHs (included pyrene, phenanthrene and anthracene) Copper (as 5 H ₂ O · CuSO ₄)	100% acetone (for PAHs) Deionized water (for 5 H ₂ O · CuSO ₄)	20 ml 100% acetone 20 ml deionized water	10 µg total PAHs/g dry sed (composed of 6 µg pyrene/g dry sed; 3 µg phenanthrene/g dry sed; 1 µg anthracene/g dry sed) 70 µg Cu/g dry sed

The sediments were dosed by adding the appropriate components (depending on the designated treatment) directly to an aliquot of field collected surface sediments. After thorough mixing, the dosed sediments were spread over top of the previously collected sediment brick in the designated mesocosm for each treatment. Application of dosed sediments took place over three days, with only a 2mm thick layer being applied at a time.

The dosed sediments were incubated in the mesocosm tanks, which simulated semi-diurnal tides and were exposed to natural sunlight (refer to Figure 2.3 for mesocosm set-up details). On Day 10 of the incubation, core tubes with a 1.0 cm² interior area were used to take sediment samples for chlorophyll *a* (chl *a*) concentration analysis using fluorometry and for biomarker pigment analysis by high performance liquid chromatography (HPLC). Five samples were taken for fluorometry and sectioned at 2 mm below surface level (as this is where the majority of photosynthetically active BMA reside (MacIntyre et al., 1996)) using a sectioning tool that pushes the sediment from the bottom of the core at 1 mm intervals with each turn of its handle. Sectioned samples were stored in 20 ml scintillation vials until processing, which took place within several hours of sectioning. Five samples were also collected for HPLC. These samples were sectioned at a 5 mm depth and stored in 1 ml microcentrifuge tubes at -80°C until analyzed.

After the 10 days of incubation, the top 2-3 mm of sediments were scraped off the brick of sediments in each mesocosm and sealed in a glass jar until use later that day to prevent drying out. Four replicates of this experiment were conducted; Replicate 1 and Replicate 2 were performed June 23 - July 5, 2012, Replicate 3 and Replicate 4 were performed July 13 – 25, 2012.

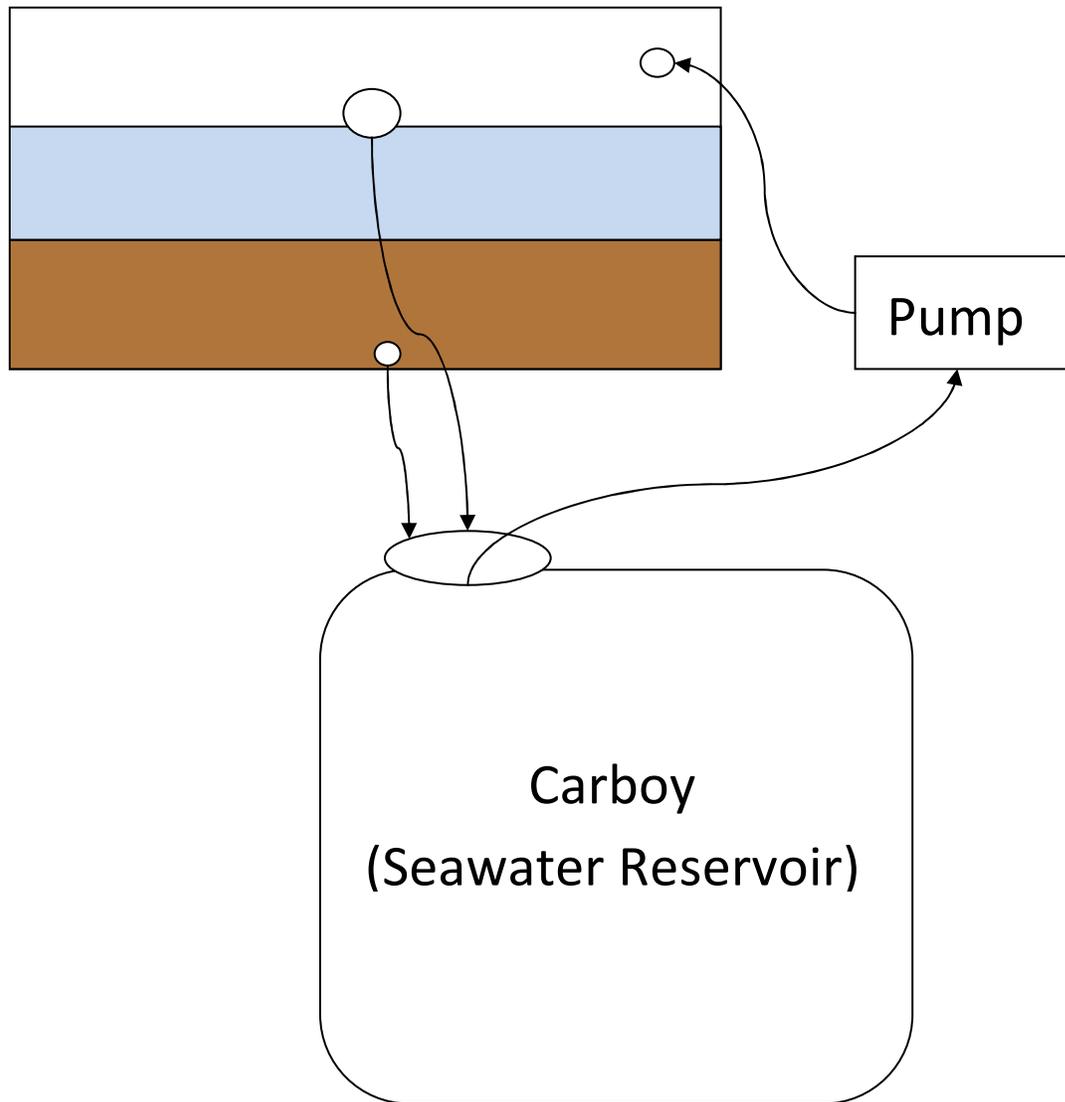


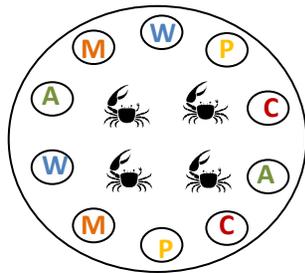
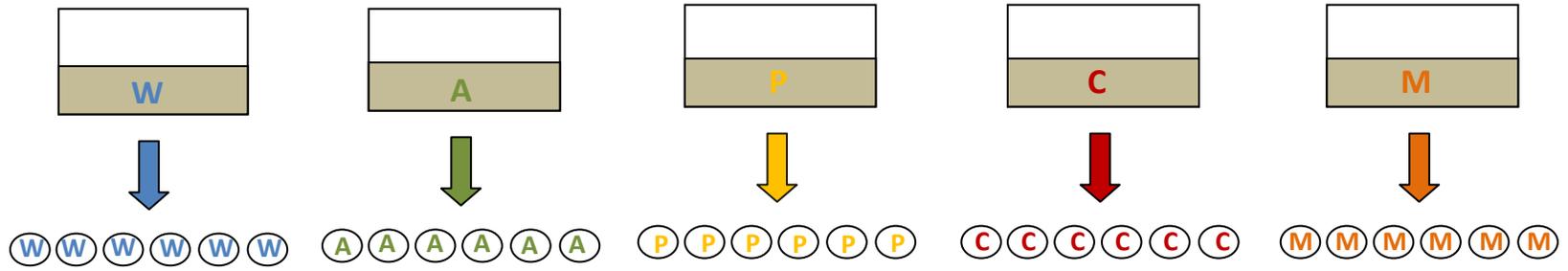
Figure 2.3 Diagram of mesocosm set-up. Pumps were controlled by timers, allowing for simulation of semidiurnal tides. The large rectangle represents the mesocosm tank, containing collected sediments (brown coloration) and several centimeters of water (during a simulated high tide) (blue coloration). Circles represent holes in the tank functioning either as drains or the point of water introduction. Curved lines represent tubing, with arrow heads indicating the direction of water flow. Note the relative size of the holes in the mesocosm tank. The hole corresponding to the point of water introduction is larger than that of the bottom drain, which allowed for water accumulation while pumps were on (to simulate high tides). The much larger top drain allowed for rapid drainage once the waterline reached that level, preventing overflow and limiting the depth of water overlaying the sediments to 7 cm. While pumps were off, water gradually exited the bottom drain until tanks were empty, simulating a low tide.

Crab food choice: Experiment set-up

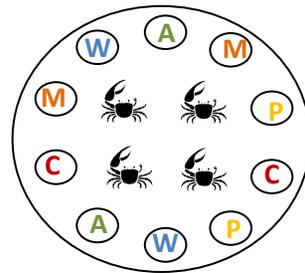
A circular arena was constructed of poster board (attached to a poster board base) for the food choice experiments. Three arenas were used for each of the four replicates. Sediments collected from each mesocosm were homogenized by thorough stirring and used to fill two petri dishes for each of the three arenas corresponding to that specific replicate (for a total of 10 dishes per arena; resulting from 5 treatments x 2 dishes per treatment) (see Figure 2.4; Figure 2.5). Prior to being filled, each petri dish was labeled on the bottom with the treatment name in order to prevent any observer bias throughout the experiment. All 10 petri dishes were arranged along the border of the arena and spaced 5.5 cm apart. As the label of each dish was not visible during placement, the order of the dishes was random and unknown during the experiment. Dishes were numbered 1-10 on the poster board base for data recording purposes. Four large male fiddler crabs (~22 mm carapace width) were then introduced into the center of the arena and allowed to feed for 24 hours. Four petri dishes filled with water were also added to the center of the arena to prevent desiccation of the crabs. These crabs had been captured from the tidal flat collection site previously described (Figure 2.1; Figure 2.2) and starved for 48 hours prior to their introduction into the arena.

Crab food choice: Data collection

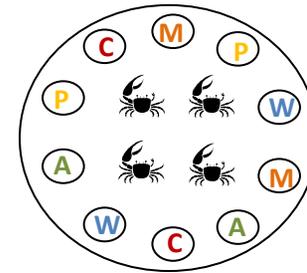
Observations about which (if any) dish a crab was occupying were made ten times in every arena throughout the experiment (the number of the dish was noted) as a measure of the amount of time spent associated with sediments of each treatment. After the 24 hour feeding period, the crabs were removed from the arena. The number of



Arena 1



Arena 2



Arena 3

Figure 2.4 Diagram of food choice experiment set-up. Letters represent the different treatments: W = Water; A = Acetone; P = PAHs; Cu = Copper; M = Mix. Rectangles represent the incubation mesocosms dosed sediments were collected from, then distributed into petri dishes (the small circles). Ten petri dishes (two from each treatment) were randomly arranged around the perimeter of the food choice arena (the large circles). Four fiddler crabs () were introduced into the middle of each arena and allowed to feed for 24 hours. Four replicates were run using this set-up.



Figure 2.5 Crab food choice experiment photo. Dosed sediments were distributed into petri dishes which were arranged around the perimeter of a circular arena. Four male fiddler crabs were then introduced into the arena and allowed to feed from the dishes for 24 hours.

feeding pellets (non-ingested sediment clumps) inside or within a 2 cm radius of each dish was counted. Because fiddler crabs produce these small pellets as they extract and ingest organic matter (Miller, 1961), they were used as a relative measure of the amount of feeding that took place in each dish. Pellets were destroyed after being counted to prevent re-counting. This method of evaluating foraging intensity by counting feeding pellets is similar to that employed by Robertson et al. (1980, 1981).

Crab food choice: Sediment sample analysis

Ten milliliters of HPLC-grade 100% acetone was added to samples taken for chl *a* concentration analysis by fluorometry. The samples were agitated through vigorous shaking for 30 seconds and stored at -20°C for roughly 16 hours during extraction. Fluorescence of the extract was measured with a Turner™ Trilogy Laboratory Fluorometer (model #: 7200-000) using the non-acidification method (Welschmeyer, 1994).

Once removed from -80°C storage, samples for biomarker pigment analysis by HPLC were freeze dried overnight. To each sample, 1 ml 90% acetone and 50 µl of synthetic carotenoid β-apo-8'-carotenal (Sigma, cat. no. 10810) (an internal standard) were added. Samples were then agitated for 30 seconds with a sonifier and stored at -20°C for 24 hours during extraction. A centrifuge was used to spin down the samples and the supernatant (sample extract) was filtered through a 0.45-µm PTFE filter (Gelman Acrodisc). The filtered extract (450 µl) and an ion-pairing solution (1.00 M ammonium acetate) (113 µl) were dispensed into amber glass autosampler vials which were immediately loaded into the refrigerated (4°C) autosampler for analysis.

Samples (250 μ L) were injected into a Shimadzu HPLC equipped with a monomeric and polymeric reverse-phase C₁₈ column in series. A nonlinear binary gradient consisting of the solvents 80% methanol:20% 0.50 M ammonium acetate and 80% methanol:20% acetone was used for pigment separations. Absorption spectra and chromatograms (440 \pm 4 nm) were acquired using the Shimadzu software and pigment peaks were identified based on retention times and absorption spectra (Pinckney et al. 1996).

Crab food choice: Statistical analysis

Because Replicates 1 and 2 were conducted at the same time and exposed to slightly different environmental conditions than Replicates 3 and 4, there may have been inherent differences with respect to food quantity or quality (BMA biomass or community composition, respectively) linked to the abiotic factors incubations were subjected to. Additionally, since Replicates 1 and 2 took place several weeks before Replicates 3 and 4, crabs were collected at slightly different points in the summer as well. Due to the potential for effects related to the time at which replicates were conducted, the replicates were identified as belonging to two groups (Replicates 1 and 2 were considered Group 1 and Replicates 3 and 4 were Group 2).

The total number of crab observations and total number of feeding pellets associated with the sediments of each treatment were calculated for each of the three arenas per replicate. An average of those values for each replicate was determined for each treatment and used for the statistical analysis (See Equation 2.1 and 2.2).

$$W_{x,y,1} + W_{x,y,2} = W_{x,y,\text{Total}} \quad (\text{Equation 2.1})$$

Where W = value (# of observations or # of feeding pellets) for a dish, x = replicate number, y = arena number, the third subscript represents the Dish # for that specific treatment and $W_{x,y,\text{Total}}$ = total value (# of observations or # of feeding pellets) for a treatment of Replicate x in Arena y .

$$(W_{x,1,\text{Total}} + W_{x,2,\text{Total}} + W_{x,3,\text{Total}}) / 3 = W_{x,\text{Avg}} \quad (\text{Equation 2.2})$$

Where W = value (# of observations or # of feeding pellets) for an arena that was derived from Equation 2.1, x = replicate number, the second subscript denotes arena number, $W_{x,\text{Avg}}$ = value used in statistical analysis for Replicate x . These equations were used to derive the values to be statistically analyzed for each treatment in every replicate.

Each data set (number of observations and number of feeding pellets) was analyzed with a two-factor ANOVA using treatment and “replicate group” as the main factors. “Replicate group” was used as a block factor in the ANOVAs to control for any inherent differences due to replicate timing.

Analyses were performed to delineate possible influences of food quantity (BMA biomass) and food quality (BMA community composition) on fiddler crab feeding (measured as number of feeding pellets produced). A multifactor analysis of variance (MANOVA) followed by individual univariate analyses were performed using representatives of BMA biomass (chl a) and community composition (fucoxanthin to zeaxanthin ratio or Fuco:Zea Ratio) as the response variables and treatment as the main

factor. These measurements were obtained from the sediment samples collected on Day 10 of the incubation, which were subsequently used for the food choice experiment. An analysis of covariance (ANCOVA) was also performed using treatment as the main factor, chl *a* and Fuco:Zea Ratio as covariates and number of feeding pellets as the response variable.

Due to the inherent spatial variability of BMA in sediments (MacIntyre et al., 1996), an average from the five samples taken within each treatment on each sampling day was used as the representative value (chl *a* concentration or Fuco:Zea Ratio) for that treatment and day combination in the appropriate statistical analysis.

IBM SPSS Statistics software (Version 21) was used for all analyses in this experiment. Tests were performed to verify that the data met assumptions of normality (K-S test) and homogeneity (Levene's test). The Ryan-Einot-Gabriel-Welsch F (R-E-G-W-F) post-hoc test was used for multiple comparisons of means when significant treatment effects were observed.

Crab feeding rate: Sediment collection & set-up

Prior to sediment collection, a divider made of a plastic mesh and enforced with strips of clear Lexan along the perimeter was inserted into each of the plastic mesocosm tanks (interior dimensions of 43 x 35 x 19 cm) in a position that would result in equal exposed surface sediment area on each side. Sediment bricks were collected from the tidal flat in North Inlet (Figure 2.1; Figure 2.2) similar to the manner previously described, with sediments being deposited into the tanks on each side of the divider to firmly hold the mesh in place. All bricks were collected the same distance from the bank

and within a 7 m span of the sandflat to reduce spatial variability in sediment grain size and organic content/composition.

Tanks were transported back to the experimental study site and connected to the pump/carboy closed-loop system used to simulate semi-diurnal tides (see Figure 2.3). The pumps were set to turn off at 9 am and on at 3 pm. This ensured that the water would be drained out of the tanks during the time of the day when they would be exposed to direct sunlight, preventing heating of the water beyond tolerable limits of the crabs. A partial “ceiling” made of clear Lexan was attached on top of the tanks adjacent to the divider on the side the crabs would be contained in to prevent their escape. This collection and set-up day was designated Day 0. Four replicates of this experiment were set up and ran simultaneously in mid-July, 2012.

Sediments of mesocosms were dosed with the treatments listed in Table 2.1 the day following collection using the procedure previously described for the food choice experiment (dosed sediments were applied to bricks in mesocosms Day 1 – Day 3).

Crab feeding rate: Sampling

Sediment samples were collected in order to compare the concentration of chl *a* (proxy for BMA biomass) in an area where crabs had been allowed to feed to an area they had been excluded from to determine relative feeding rates in each treatment.

Core tubes with a 1.0 cm² interior area were used to take sediment samples for chl *a* concentration analysis using fluorometry. Five samples were taken on each side of the divider on Day 4 during the simulated low tide and sectioned at 2 mm below surface level (as this is where the majority of photosynthetically active BMA reside (MacIntyre et al.,

1996)). Sectioned samples were stored in 20 ml scintillation vials until processing, which took place within several hours of sectioning. After sampling, four large male fiddler crabs (~ 22 mm carapace width) that had been captured from the sediment collection site and starved for 48 hours, were introduced into one side of each mesocosm tank (see Figure 2.6 for set-up). The following day, additional samples for fluorometry were collected on each side of the divider (5 per side) within each tank. Sample analysis by fluorometry took place using the procedure previously described.

Crab feeding rate : Statistical analysis

In order to determine the relative feeding rate of the crabs in each treatment, an average of the chl *a* concentration from the five samples collected was calculated for each side of a tank for the initial and final (after 24 hours of feeding) samples. To correct for any starting differences in chl *a* concentration between the two sides of any tank, the initial difference between the crab exclusion and crab exposed sides was subtracted from the final difference between the two sides. This was then divided by the number of feeding crabs (4) to find a relative feeding rate per crab per day (See Equation 2.2).

$$((x_2 - y_2) - (x_1 - y_1))/4 = z \quad \text{(Equation 2.2)}$$

Where x_1 = initial chl *a* concentration on crab exclusion side, y_1 = initial chl *a* concentration on crab exposed side, x_2 = final chl *a* concentration on crab exclusion side, y_2 = final chl *a* concentration on crab exposed side, z = relative crab feeding rate.



Figure 2.6 Crab feeding rate experiment photo. Mesocosm tanks containing field collected sediments were set-up and dosed with treatments. Four male fiddler crabs were introduced into one half of each mesocosm and allowed to feed for 24 hours. Measurements of sediment chlorophyll *a* concentration on each half of the tanks (crab exposed and crab excluded) were compared to determine the degree of feeding by the crabs.

A single-factor ANOVA using treatment as the main factor and feeding rate as the response variable was utilized to compare the relative feeding rates between the five treatments. IBM SPSS Statistics software (Version 21) was used for all analyses in this experiment. Tests were performed to verify that the data met assumptions of normality (K-S test) and homogeneity (Levene's test). The R-E-G-W-F post-hoc test was used for multiple comparisons of means.

2.3 RESULTS

Crab Food Choice

The trends shown by the average number of crab observations for each treatment suggest crabs spent less time in contaminant treated sediments, but statistical analysis revealed there was no significant difference between treatments (ANOVA, $p = 0.062$). Additionally, no significant difference between the Replicate Groups was observed for this data set (ANOVA, $p = 0.697$) (Figure 2.7).

Analysis of the feeding pellet data revealed a significant treatment effect ($p = 0.008$), as well as a significant difference between Replicate Groups (Replicates 1 and 2 produced significantly less feeding pellets than Replicates 3 and 4) ($p < 0.001$).

According to the post-hoc test, there were three homogeneous groups: Mix, Cu and PAHs ($p = 0.069$); Cu, PAHs and Water ($p = 0.152$); PAHs, Water and Acetone ($p = 0.616$).

Significantly less feeding pellets were produced by crabs in the Mix dishes than in the Water and Acetone control dishes (Figure 2.8). There was also significantly less pellets produced in the Cu treatment than in the Acetone control, but no significant

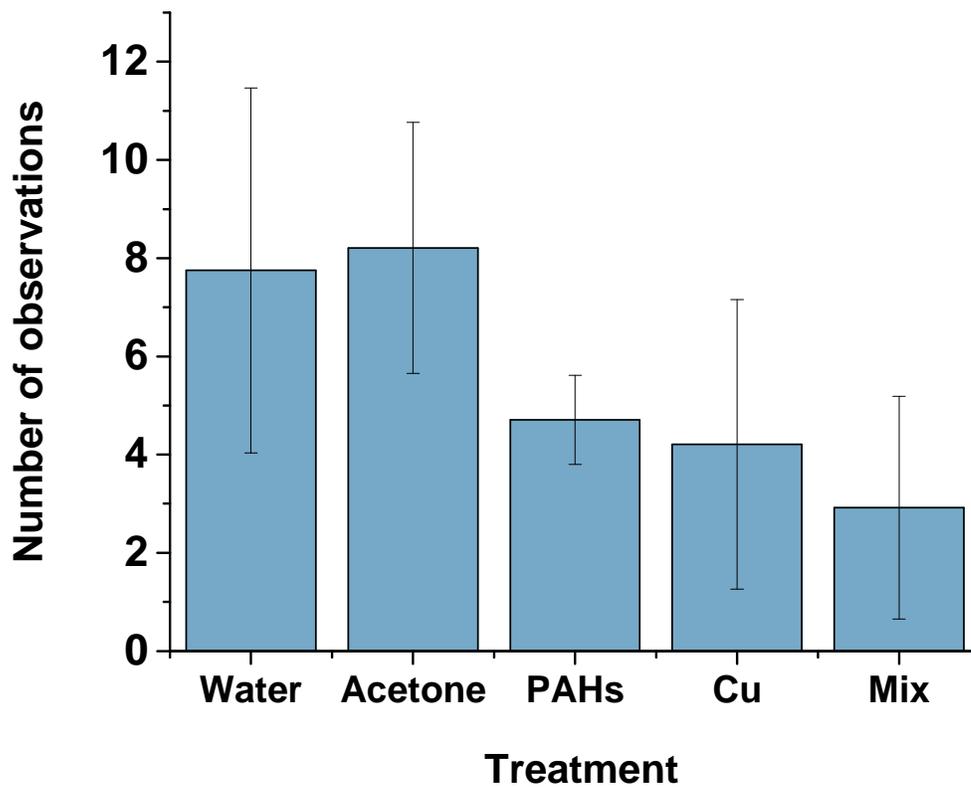


Figure 2.7 Number of times a crab was observed occupying dosed sediments. Observations were made ten times during the 24 hour food choice experiment, during which any dishes one the four crabs occupied were noted. Water and Acetone treatments were used as controls for contaminant (PAHs, Cu and Mix) treatments. The Mix treatment was dosed with both PAHs and Cu. Values represent means \pm 1 SD (n = 4).

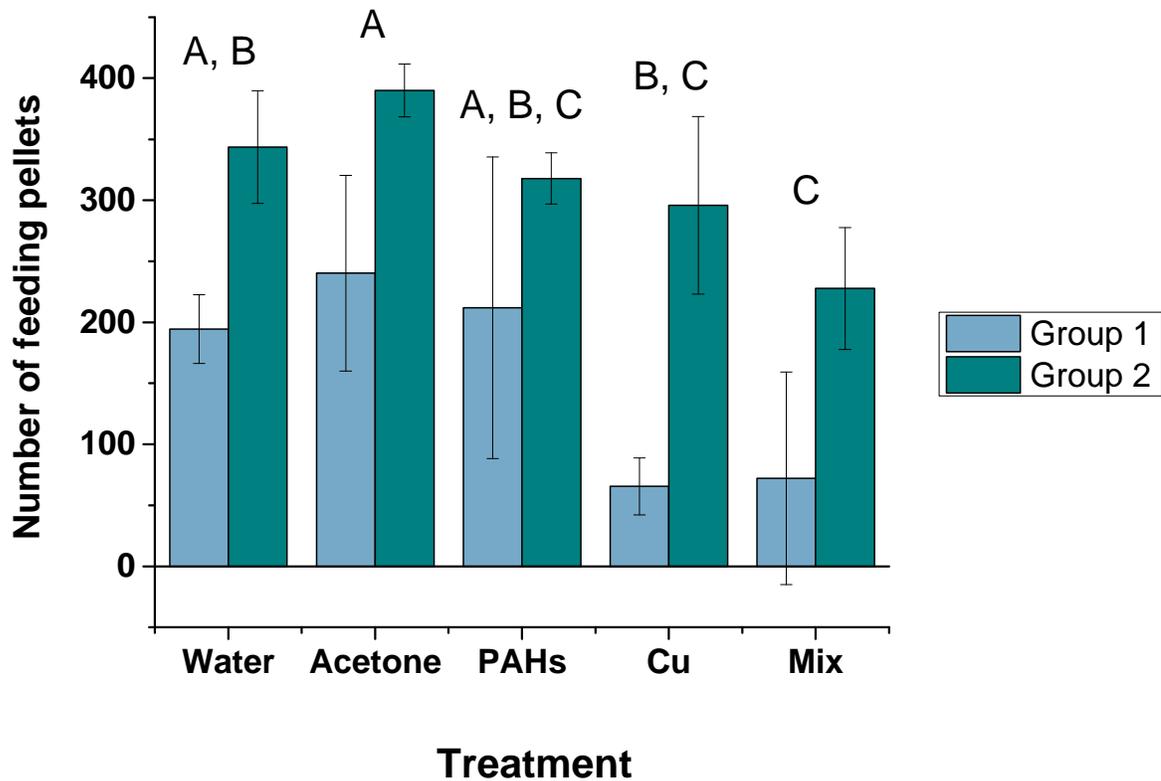


Figure 2.8 Number of feeding pellets produced by crabs within dosed sediments. Replicates 1 and 2 were run concurrently and Replicates 3 and 4 were run concurrently. Due to variability in the environmental conditions replicates were exposed to and the differences in collection dates of the crabs, the replicates were divided into two groups (Group 1 = Replicate 1 and Replicate 2; Group 2 = Replicate 3 and Replicate 4) for the analysis and replicate group was used as a block factor. Water and Acetone treatments were used as controls for contaminant (PAHs, Cu and Mix) treatments. The Mix treatment was dosed with both PAHs and Cu. Letters above bars for a treatment indicate homogeneous groups. Values represent means \pm 1 SD (n = 2).

difference was observed between Cu and its control, Water (Figure 2.8). Due to the high degree of burrowing observed in various Water and Acetone control dishes, some of the feeding pellets produced may have been destroyed or buried throughout the 24 hour feeding period. Therefore, the number of feeding pellets in those dishes should be considered conservative and the actual significance of the treatment effect may be greater than the analysis indicates.

The MANOVA using chl *a* and Fuco:Zea Ratio as response variables indicated that there was a significant treatment effect for that combination of variables (Roy's Largest Root, $p = 0.001$). Subsequent univariate analyses revealed no significant difference between treatments with respect to chl *a* concentrations at the beginning of the food choice experiment (ANOVA, $p = 0.423$), but that there was a significant difference in Fuco:Zea Ratios between treatments (ANOVA, $p = 0.005$). Post-hoc analysis (R-E-G-W-F test) identified three homogeneous groups with respect to Fuco:Zea Ratio: Water, PAHs and Acetone ($p = 0.729$); PAHs, Acetone and Mix ($p = 0.099$); Mix and Cu ($p = 0.745$). The Fuco:Zea Ratio was significantly higher in the Cu treatment than in the Water control, PAHs treatment and Acetone control, while the ratio in the Mix treatment was significantly higher than that of the Water control. However, neither chl *a* concentration, nor Fuco:Zea Ratio had any significant effect on the number of feeding pellets produced by crabs (ANCOVA, $p = 0.803$ and $p = 0.671$, respectively).

Crab Feeding Rate

Fiddler crabs expressed lower feeding rates in contaminated mesocosms than in the controls. A significant treatment effect was observed ($p = 0.001$) and three

homogeneous groups were identified through the analysis: Mix, Cu and PAHs ($p = 0.590$); Cu, PAHs and Acetone ($p = 0.115$); Acetone and Water ($p = 0.164$). The feeding rate of crabs in the Mix tanks was significantly lower than the feeding rate of crabs in the Acetone and Water control tanks. Crabs also exhibited significantly lower feeding rates in both the Cu and the PAHs treatments when compared to the Water control. There was no significant difference between the PAHs treatment and its Acetone control (Figure 2.9). Average feeding rates of crabs in the contaminated treatments were only 12 – 32% of those in the Water control.

2.4 DISCUSSION

Crab Food Choice

The results from the crab food choice experiment showed significant crab avoidance of PAH + copper (Mix treatment) contaminated sediments and what seemed to be a varied degree of avoidance of copper contaminated sediments, which could have important ecological implications and partially supported the hypothesis. Though crabs did not avoid spending time in contaminated sediments (Figure 2.7), based on the feeding pellet data they did avoid feeding in the Mix treatment dishes (Figure 2.8). While there appeared to be a significantly less feeding in the Cu treatment dishes for Replicate Group 1 (Replicates 1 and 2) compared to controls as well, this was not true for Replicate Group 2 (Replicates 3 and 4) resulting in no significant difference overall between that treatment and its control (Water). It could be interpreted that crabs do avoid feeding in copper contaminated sediments, but that the degree of this response can vary. In the sediment incubation period, the tanks of Replicates 3 and 4 were exposed during a sudden rain

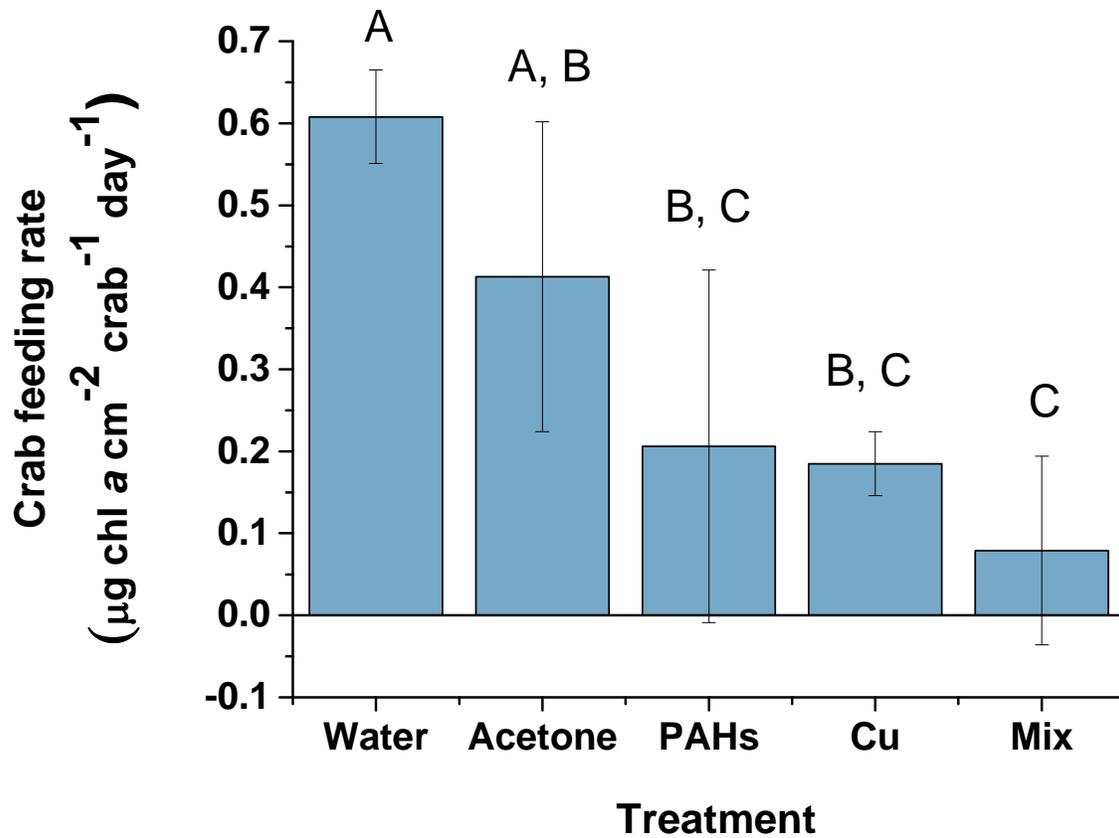


Figure 2.9 Crab feeding rate within dosed sediments. Feeding rate was determined by comparing chl *a* concentrations in sediment samples from an area where crabs had been allowed to feed for 24 hours to samples from a crab exclusion area. Water and Acetone treatments were used as controls for contaminant (PAHs, Cu and Mix) treatments. The Mix treatment was dosed with both PAHs and Cu. Values represent means \pm 1 SD (n = 4).

event, after which it was observed that some of the surface sediments (those to which the contaminants had been applied) were washed to the drain of the tank. This loss of contaminated sediment could explain the reduced response of the crabs in Replicate Group 2 relative to that of Replicate Group 1 when sediments from those tanks were later used in the food choice experiment. Taken together, the results from this experiment seem to suggest avoidance of feeding by fiddler crabs in sediments contaminated with a copper component.

The avoidance of copper contaminated sediments was likely due to the presence of the heavy metal in the sediments interacting with the chemosensory of the crabs, rather than a response to changes in the food source (BMA) induced by copper exposure. Fiddler crab feeding is induced when chemical cues associated with organic matter are detected by the crab's sensory receptors and the feeding behavior has been shown to be highly dependent on food density (Robertson et al., 1980). As there was no significant difference between treatments with respect to the chl *a* concentration in the sediments used for the food choice experiment (ANOVA, $p = 0.423$), chl *a* concentration had no significant effect on the number of feeding pellets produced (ANCOVA, $p = 0.803$). This, therefore, implies that the reduced feeding (shown by the number of feeding pellets) in the Mix dishes (and to some degree the Cu dishes) was not in response to the quantity of food available in those dishes. There was, however, a significantly higher Fuco:Zea Ratio, and therefore a different BMA community composition (with a lower relative abundance of cyanobacteria), in the Cu and Mix treatments compared to the controls. It could be argued that the avoidance towards those treatments was related to food quality. Though cyanobacteria have been shown to induce a greater feeding response in fiddler

crabs than diatoms (Robertson et al., 1981), it is unlikely that the shift towards greater diatom relative abundance in the Cu and Mix treatments would reduce feeding as dramatically as observed in this experiment as diatoms are usually already the dominant BMA group in Atlantic salt marsh sediments (Sullivan and Currin, 2002). This conclusion is further supported by the ANCOVA results that revealed BMA community composition (Fuco:Zea Ratio) had no significant effect on the number of feeding pellets produced by crabs (ANCOVA, $p = 0.671$). By ruling out the effects of food quantity and quality as the drivers for crab avoidance of the Mix (and to some extent the Cu) dishes, the results suggest the avoidance was induced by the presence of the chemical contaminant(s).

A great deal of crustacean behavior is dependent on chemical cues received from the surrounding environment, thus, the presence of chemical contaminants in salt marsh habitats has the potential to alter normal fiddler crab behavior. Various crustacean groups are able to detect and show avoidance of heavy metals, including copper (McLeese, 1975; Maciorowski et al., 1980; Lopes et al., 2004; Seuront, 2010), suggesting the response (avoidance) of the fiddler crabs to copper treated sediments (Mix and Cu treatments) in the present study was possibly due to a chemosensory detection of the contaminant. Additionally, since initial feeding is induced when the sensory receptors of the crab are adequately stimulated, but continued feeding is regulated by stimulation of the mouth parts (Robertson et al., 1981) if the chemical contaminant caused some unpleasant sensory stimulation or “taste”, the crab avoidance may have been related to that as well. Alternatively, feeding avoidance of copper contaminated sediments may have been due to chemosensory disruption rather than detection of the heavy metal. As

fiddler crabs are dependent on the proper functioning of their chemosensory system to detect food sources, and thereby elicit feeding behavior, interference with crab chemosensory by contaminants could dramatically impact crab feeding. Copper disrupts chemosensory in a variety of taxa spanning numerous trophic levels. Pyle and Mirza (2007) demonstrated that leeches have difficulty finding food and that daphnia and fish seem unable to detect predators, both of which are chemosensory mediated survival mechanisms, after exposure to sublethal levels of copper. Similarly, feeding behavior is reduced by copper exposure in lobsters, crayfish and prawn (McLeese, 1975; Steele et al., 1992; Santos et al., 2000), indicating a diminished ability to sense naturally stimulating chemical cues in these crustaceans. The mechanism for copper interference with proper chemosensory functioning is unknown, but some theories have been postulated. Heavy metals may bind to receptors, preventing binding of natural stimuli or the metals may inhibit transmission of the signal to central nervous system (Pyle and Mirza, 2007). In their study examining the effect of copper on salmon chemosensory, McIntyre and colleagues (2008) provide evidence that the former mechanism is responsible for chemosensory interference of copper, but much is still unknown. Whatever the mechanism, copper seems to have strong effects on chemosensory mediated behavior in a variety of organisms, including fiddler crabs based on the data collected during the present study.

The results from this food choice experiment have implications for fiddler crab responses to contamination of their natural habitat. The avoidance of feeding in copper and PAH + copper contaminated sediments (Cu and Mix treatments) suggests that given the opportunity, crabs will migrate out of areas that are contaminated with these

chemicals at or above the levels used in this study in search of more suitable habitats. These concentrations have already been measured in urban areas (Sanger et al. 1999a,b), and as coastal populations continue to increase, it is very likely these levels (and some higher) will become more common.

As fiddler crabs are an extremely important part of the salt marsh ecosystem, the emigration of these decapods could have detrimental effects. Fiddler crabs aerate sediments with their burrowing, which stimulates the growth of marsh grasses (Bertness, 1985) and they also serve as prey to many different fish, bird and even crustacean species (Grimes et al., 1989). These crabs help maintain sediment community dynamics as well, reducing the biomass of BMA and meiofauna by as much as 20% and 60%, respectively, in one tide cycle (Reinsel, 2004) and thereby imposing some top-down control over the populations of those two benthic groups. The relocation of fiddler crabs from tidal creeks in response to increased levels of contamination would, therefore, likely upset many biological balances and impact ecosystem functioning.

Alternatively, the continued living of crabs in contaminated areas would increase their exposure to the contaminants. Because there was no avoidance shown towards the PAH treatment sediments, it can be inferred that the crabs did not detect the contaminants or that chemosensory was not disrupted at the concentration used in this study. Therefore, the results suggest that fiddler crabs would not emigrate out of areas with that level of PAHs in the sediments. Crabs did not seem to avoid burrowing in PAH-only contaminated sediments (based on bioturbation observations) and therefore would probably also continue normal burrowing activity in the field, increasing the likelihood of accumulating the contaminants in their tissues through direct contact with contaminated

sediments. Furthermore, no reduced feeding (measured by the number of feeding pellets) was observed in the PAH treatment compared to the controls. Since during fiddler crab feeding sediment particles (to which PAHs quickly adsorb (Nikolaou et al., 2009; Tobiszewski and Namieśnik, 2012)) are placed directly into the buccal cavity and the smallest are also ingested along with the food particles (Sayão-Aguiar et al., 2012), the crabs are directly exposed to the contaminants in that way as well. These modes of direct exposure resulting from the intimate association fiddler crabs have with the sediments increases the likelihood of contaminants impacting these crustaceans.

PAHs are known to bioaccumulate in the tissues of a variety of organisms, including fiddler crabs (Baumard et al., 1998; Granberg and Selck, 2007; Chase et al., 2013), which can affect the health of the individual as well as its predators. The hepatopancreas, or digestive gland, of crabs is an invaluable organ that stores lipids and is involved in detoxification (Sousa and Petriella, 2007) and so is extremely susceptible to harm by contaminants. PAHs can damage the hepatopancreas at a cellular level as well as reduce the activity of respiratory enzymes and induce oxidative stress (Vijayavel et al., 2004; Vijayavel and Balasubramanian, 2006; Sousa and Petriella, 2007), compromising the energy storage ability and overall health of the individual. The accumulation of PAHs in fiddler crabs has the potential to affect those organisms that prey upon them as well. Trophic transfer of PAHs between aquatic invertebrates has been demonstrated (Filipowicz et al., 2007; Carrasco Navarro et al., 2013) and as PAH metabolites are sometimes more toxic than the parent compound (Hawkins et al., 2002; Šepič et al., 2003; Lee and Landrum, 2006) effects on higher trophic levels may be significant as well.

While the results from this study seem to show no significant PAH effect on fiddler food choice or burrowing, there are still potentially important ecological implications.

The results from this experiment indicate that fiddler crabs do show avoidance of sediments contaminated with environmentally relevant levels of a combination of PAHs and copper. Fiddler crabs may also avoid copper-only contaminated sediments to some degree but this response seems to vary. The results of this experiment suggest, fiddler crabs may emigrate from areas with PAH and copper levels at or above those used here, which could have detrimental effects on ecosystem functioning. The lack of avoidance of PAH-only contaminated sediments implies fiddler crabs would continue to occupy PAH contaminated sediments in the field, potentially diminishing the health of individuals as well as impacting the health of predators through fiddler crab accumulation and subsequent trophic transfer of PAHs.

Crab Feeding Rate

During the feeding rate experiment, fiddler crabs showed a reduced rate in all three contaminant treatments (PAHs, Cu and Mix) compared to the Water control, supporting part of the hypothesis. Though the rate in the Mix treatment was not significantly lower than the rates in the PAHs or Cu treatments, the average feeding rate in the Mix treatment was roughly half of that seen in the singular contaminant treatments, possibly the result of a non-additive combined effect. This was contrary to the hypothesized interaction between multiple pollutants, as it was expected that there would be a synergistic effect. A possible explanation for this non-additive combined effect is that the bioavailability of one (or both) contaminant(s) may have been slightly decreased

due to the presence of the other contaminant (Newell and Sanders, 1986; Meador, 1991; Breault et al., 1996; Wright and Mason, 2000; Renella et al., 2004), thereby resulting in a less than additive response. In any case, the impacts of these contaminants on fiddler crab feeding rate were dramatic (with 68 – 88% reductions observed in contaminant treatments compared to controls) and have physiological as well as ecological implications.

The results suggest the fiddler crabs were either able to detect the contaminants or that the contaminants interfered with the crabs' chemosensory system, preventing the detection of food particles (as previously discussed for the Crab Food Choice experiment). It seems that the reduction in feeding rate upon exposure to the contaminants was most likely due to a disruption of normal chemosensory functioning. The crabs of this experiment had been starved for 48 hours to ensure they would be hungry and in search of food upon introduction into the dosed mesocosm tanks. Because no alternative food choice was provided in this experiment (i.e. crabs had no opportunity to forage on uncontaminated sediments if they were assigned to "contaminated" mesocosms) a mere detection of a foreign chemical probably would not be enough to deter feeding to the degree observed in this experiment after a 48 hour starvation period. Many other studies have demonstrated an impairment of chemosensory functioning in both vertebrates and invertebrates when exposed to the PAHs or copper (Hara et al., 1976; Baldwin et al., 2003; Krång, 2007; Pyle and Mirza, 2007; McIntyre et al., 2008; Seuront, 2010) providing support for chemosensory disruption being the mechanism responsible for the reduction in fiddler crab feeding rate in the present study.

A reduction in crab feeding rate in contaminated sediments compared to non-contaminated sediments could have severe effects on the individual, and also potentially the population as a whole. Because fiddler crab feeding is already time limited in the field (foraging time is limited by the amount of time the sediments are exposed during low tide) (Reinsel and Rittschof, 1995) a lowered feeding rate would result in reduced food intake. This, in turn, limits resources available for growth and reproduction. Crab fecundity is strongly linked to food availability (McKillup and McKillup, 1994; Sampedro et al., 1997; Delevati Colpo and Negreiros-Fransozo, 2003) and a lack of adequate food intake can prevent female crabs from becoming ovigerous (Micheli, 1993; McKillup and McKillup, 1994). Declines in fecundity, may in time result in a dramatic reduction in the population size. The feeding rates in all of the contaminant treated sediments were greatly reduced in the present study, with average rates in the PAHs, Cu and Mix treatments being only 12 – 32% of the Water control, which is likely enough of a reduction to affect the health and fecundity of an individual. As female fiddler crabs exhibit greater chemosensory sensitivity than males (Weissburg and Derby, 1995; Weissburg et al., 1996), their feeding response to contaminated sediments may be even more dramatic than that demonstrated by the males used in this study.

Fiddler crab feeding has a significant effect on both biotic and abiotic aspect of salt marsh environments. These crabs graze down bacterial, BMA and meiofauna populations, potentially stimulating new growth by reducing densities (Montague, 1980; Hoffman et al., 1984; Reinsel, 2004) and in the process oxygenate the substratum and alter grain size composition of the sediments (Hoffman et al., 1984; Sayão-Aguiar et al., 2012). A reduction in individual crab feeding rate would affect this trophic interaction

and relieve some of the top-down control exerted by fiddler crab feeding, potentially disturbing balances within the sediment community. Additionally, if the population size were to decrease as a result of reduced reproduction as proposed above, the bioturbation by crab burrowing and the sediment processing by crab feeding, two very important processes in salt marshes (Montague, 1980; Bertness, 1985), would be reduced as well, possibly to a great degree depending on the intensity of the effect on the population. If the contaminant effects on crab feeding rate endured, the severity of the effects observed in this study suggest the potential for disturbances in trophic balances and important ecosystem processes.

This experiment has demonstrated that environmentally relevant concentrations of PAHs and copper in sediments negatively impact the feeding rate of fiddler crabs, an outcome that would likely have detrimental effects in the field. It is postulated that this reduction in feeding rate of the crabs was the product of a chemosensory detection of, or disruption by, the chemical contaminants as has been observed in other studies. The implications of this study span several levels of biological organization, as individual crab health, population size and ecosystem functioning could all be affected by consequences associated with a reduction of fiddler crab feeding rate.

CONCLUSION

The impacts of multiple contaminants on more than one trophic or biological level are difficult to predict due to the numerous unknown variables and the many ways contaminants can interact with one another. By utilizing mesocosms with field collected sediments containing natural microorganism communities and simulating natural environmental conditions, this study revealed insights into the possible effects of PAHs and copper on salt marsh communities at concentrations that have already been observed in urban areas along the coast of South Carolina. The response of benthic microalgae to the contaminants suggest the toxic effects of the singular contaminants and a combination of the two are not severe enough at the levels tested to cause any significant reduction in BMA biomass, but meiofauna grazers may be significantly affected by copper contamination. BMA community composition showed significant responses to copper contaminated sediments as well (as the Cu and Mix treatments), possibly indicating cyanobacteria sensitivity to the heavy metal.

The fiddler crabs that graze upon BMA also showed significant responses to copper and PAHs + copper (Mix treatment) contaminated sediments by reducing their feeding (both when there was an alternative food source and when there was not). Fiddler crabs also showed less feeding in PAH contaminated sediments, but this response may be dependent on the potency of the contaminant and whether the sediments are exposed only once (so the PAHs eventually degrade) or are continually exposed (therefore maintaining,

or increasing the concentration). This conclusion is based on the lack of response in the food choice experiment (when crab introduction took place several days after sediment dosing) and the significant response in the feeding rate experiment (when crab introduction occurred one day after dosing). There seemed to be a non-additive combined effect on crab feeding rate when exposed to a combination of the two contaminants compared to when they were exposed to only one, suggesting an interaction between contaminants or their mode of action on crab response.

The implications of this study range from the individual to the ecosystem level. If meiofauna are impacted by copper contamination as suggested by the results in the BMA response experiment, biogeochemical processes and sediment community dynamics may be affected. Individual fiddler crab health will likely be impacted by PAHs accumulation within their tissues if PAH contaminated sediments are not avoided and later trophic transfer of metabolites could affect the health of crab predators. Migration of crabs out of areas contaminated with copper would dramatically reduce the degree of bioturbation and sediment processing, potentially negatively influencing ecosystem functioning. Fiddler crab individual health would also probably suffer by a reduction in food intake in response to sediment contamination, which could translate into a decline in population size if reproduction is impacted. This, in turn, could possibly affecting the salt marsh ecosystem as a whole if burrowing and fiddler crab feeding are sufficiently reduced.

It should be acknowledged that the contaminant concentrations presently in the environment may differ from those levels reported by Sanger et al. and used in this study, as that source is over a decade old. Current levels probably now exceed the Sanger et al. (1999 a,b) values due to increased anthropogenic activities, thus those concentrations are

likely conservative estimates for present levels in estuarine habitats of South Carolina. Therefore, the results of this study may represent effects at what are now considered relatively low contaminant concentrations in urban areas.

There are many potential impacts of PAHs and copper on salt marsh systems suggested here, however, more experimentation and field studies will be needed to clarify some of what is still unknown.

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