# TISSUE SPECIFIC RESPONSES OF THE ESTUARINE BIVALVE SPECIES *CRASSOSTREA VIRGINICA* AND *GEUKENSIA DEMISSA* TO HYPOXIA

by

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# ABSTRACT

BUSHRA KHAN. Tissue specific responses of the estuarine bivalve species *Crassostrea virginica* and *Geukensia demissa* to hypoxia. (Under the direction of DR. AMY H. RINGWOOD)

Aquatic ecosystems are exposed to multiple stressors simultaneously. Many estuarine sites which show diurnal dissolved oxygen (DO) and pH fluctuations are also exposed to metals which can be toxic to marine organisms. Bivalve mollusks serve as good indicators of metal pollution and water quality parameters in estuarine ecosystems. In shallow-water coastal environments, DO levels can fluctuate diurnally or during low tide exposures between hypoxia and normoxia. Dissolved oxygen and pH shifts are important environmental variables in estuarine ecosystems that may contribute to oxidative stress and tissue injury, as well as affect microbial flora and bioavailability of metals. Therefore metal exposures can cause oxidative damage that may be exacerbated by hypoxia and pH. The overall purpose of our study is to investigate the effects of hypoxia on oxidative damage and antioxidant status, and consider the potential impacts on microbial flora and metal bioavailability in Eastern oysters, *Crassostrea virginica* and Atlantic ribbed marsh mussels, *Geukensia demissa*. It is known that oxidative stress caused by these environmental factors can affect the antioxidant status of the tissues in marine organisms. In this study, bivalves were exposed to different oxygen regimes using $CO_2$ and $N_2$ gas, and minisondes were used to record water quality parameters semi-continuously over the course of the exposures. Hepatopancreas tissues were dissected and used to measure total Glutathione as a marker of overall antioxidant status and Malondialdehyde concentration as a marker of tissue damage under various

dissolved oxygen regimes. Bacterial concentrations were determined, and bacterial genomic techniques (ARISA – Automated Ribosomal Intergenic Spacer Analysis) were used to characterize microbial diversity in oyster hepatopancreas tissues. The study also included metal-contaminated sediment exposure under different dissolved oxygen regimes and metal concentrations were determined in gill and hepatopancreas tissues. Field samples were also collected from polluted and relatively clean sites and analyzed for tissue damage, antioxidant status, bacterial loads and tissue metal concentration. Differences in antioxidant and tissue damage levels in cyclic and continuous hypoxia treatments were observed, and the results indicate that reduced pH along with low DO was more damaging than low DO alone. These studies also suggest that low DO conditions can affect microbial concentrations and diversity as well as the metal bioavailability in bivalves. Increases in tissue metal concentrations were found in hypoxic regimes indicating that hypoxia plays a role in determining metal bioavailability and together these stressors can increase the susceptibility of these bivalves to oxidative stress. Species-and tissue-specific responses were observed in hypoxia as well as hypoxia combined with metal exposures. These differences emphasize the need for the use of multiple bioindicator species for overall habitat health assessment and to determine vulnerabilities of co-exisitng species. These studies suggest that the interactions between environmental stressors are important determinants of the health of bivalve populations in estuarine ecosystems.

# INTRODUCTION

The chapters in this dissertation examine the effects of a pervasive environmental stressor on two estuarine bivalve species. Chapter 1 gives an overview of hypoxia and its effects in estuarine environment. Chapter 2 compares the effects of environmental hypoxia in Eastern oysters and Atlantic ribbed marsh mussels and is formatted to be submitted to Marine Ecology Progress Series. Chapter 3 evaluates the interactive effects of multiple stressors on Eastern oysters and reports how hypoxia can affect metal bioavailability from contaminated sediments. This chapter has been formatted for submission to the Journal of Environmental Toxicology and Chemistry. My study of the interactions between hypoxia and metal contaminants led to some interesting and unexpected species-specific differences. The differences in responses of oysters and mussels to contaminated sediment exposures under hypoxia has been reported in Chapter 4, which has been formatted for the Journal of Shellfish Research. These studies also assessed the effects of hypoxia on oyster microbiome due to the commercial importance of oysters as seafood as well as the potential role of microbial communities in bivalve health. Chapter 5 outlines the effects of hypoxia on microbial communities in oysters and is currently under revision for PLOS One and is co-authored by Dr. Sandra M. Clinton, Mr. Timothy J. Hamp, Dr. James D. Oliver along with myself as the first author and Dr. Amy H. Ringwood as the principal investigator. Chapter 6 summarizes findings of my projects and their environmental implications.

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# LIST OF ABBREVIATIONS

| | |
|---|---|
| AAS | atomic absorption spectrophotometry |
| AVS | acid volatile sulfides |
| ARISA | Automated Ribosomal Intergenic Spacer Analysis |
| CAT | catalase |
| CFU | colony forming units |
| CPC+ media | colistin-polymyxin B-cellobiose |
| Cu | copper |
| DO | dissolved oxygen |
| DTNB | 5,5’-Dithiobis (2-Nitrobenzoic acid) |
| E. agar | estuarine agar |
| ERL | effects range low |
| ERM | effects range median |
| ETC | electron transport chain |
| Fe | iron |
| GPx | glutathione peroxidase |
| GSH | glutathione |
| GST | glutathione-s-tranferase |
| HP | hepatopancreas |
| ITS | intergenic spacer |
| LPx | lipid peroxidation |

| | |
|---|---|
| MDA | malondialdehyde |
| Mn | manganese |
| MnSOD | Mn superoxide dismutase |
| NADPH | nicotinamaide adenine dinucleotide phosphate |
| NC | North Carolina |
| NCDENR | NC Department of Environment and Natural Resources |
| PCA | principal component analyses |
| POC | particulate organic carbon |
| PUFA | polyunsaturated fatty acids |
| RFUs | relative fluorescence units |
| ROS | reactive oxygen species |
| SEMs | simultaneously extractable metals |
| SODs | superoxide dismutases |
| SQGs | sediment quality guidelines |
| TNB | 5'-thio-2-nitrobenzoic acid |
| TBA | thiobarbituric acid |
| UHP | ultra high purity |
| Zn | zinc |

# CHAPTER 1: ENVIRONMENTAL HYPOXIA AND POTENTIAL IMPACTS ON ESTUARINE BIVALVES

## 1. Introduction

### 1.1. Hypoxia – A Pervasive and Increasing Problem in Coastal Ecosystems

Estuaries are nutrient-rich and highly productive coastal habitats which support a wide variety of organisms. The occurrence of organisms in these habitats can be limited by the availability of oxygen (Diaz and Rosenberg 1995, Burnett 1997, Diaz and Rosenberg 2008). Environmental hypoxia is often defined as a condition where dissolved oxygen (DO) is less than 2 mg/L of water or approximately 30% saturation (Rabalais et al. 2010), but this does not account for cyclical diel patterns. Habitats characterized by hypoxia are expanding in marine coastal environments worldwide (Diaz 2001, Diaz and Rosenberg 2008). Global climate change and elevated nutrient inputs will increase the extent and severity of hypoxia in coastal ecosystems (Rabalais et al. 2010). Habitats which experience dramatic mortalities due to hypoxia are referred to as dead zones and their numbers have been growing since 1960s. Currently, dead zones cover over 245,000 $km^2$ area worldwide (Rabalais et al. 2002, Diaz and Rosenberg 2008). Total biomass lost to hypoxia in coastal zones is reported to be 9,000,000 metric tons of wet weight of organisms (Diaz et al. 2009), including seafood resources. The implications are that acceleration of hypoxia around the world poses severe threats to estuarine and marine ecosystems.

Along with hypoxia, estuaries are also exposed to other stressors including changes in pH, temperature, salinity, pollutants and pathogens which can induce oxidative stress in marine organisms (Valavanidis et al. 2006, Lushchak 2011), contributing to chronic sublethal effects as well as periodic mortality events. It is important to recognize that environmental hypoxia is nearly always accompanied by an elevation of carbon dioxide, termed hypercapnia which results in a decrease in water pH (Burnett 1997). In nutrient rich estuaries, photosynthesis during the day results in high concentrations of DO. However, at night, the absence of photosynthesis, along with respiratory consumption of oxygen and production of carbon dioxide result in hypoxic, hypercapnic waters (Cochran and Burnett 1996, Ringwood and Keppler 2002). Carbon dioxide combines with water to yield carbonic acid which further dissociates to give bicarbonate and hydrogen ions and decreased pH. Studies with bivalves have confirmed that hemolymph pH (which typically is around 7.4) decreases when external pH conditions decline (Bamber 1987, Bamber 1990) which suggests that elevated carbon dioxide in water can produce an acidosis in the body fluids of aquatic organisms.

In addition to the increase in coastal dead zones, areas with more extreme diurnal fluctuations driven by imbalances between photosynthesis and respiration are also increasing in estuaries. Diurnal fluctuations in DO that range from 7-9 mg/L ($\geq$ 100% saturation) during the day to less than 2 mg/L (<30% saturation) during the night with concomitant decreases in pH levels by more than 0.5 pH units have been reported in shallow water estuaries (Ringwood and Keppler 2002, Tyler et al. 2009), but the effects of DO cycles on cellular responses in estuarine organisms, particularly invertebrates, have not been widely studied. Published studies indicate that cyclical hypoxia affects

gene expression and reproduction in grass shrimp *Palaemonetes pugio* (Brown-Peterson et al. 2008, Li and Brouwer 2013) and oxidative balance in an estuarine crab, *Chasmagnathus granulata* (de Oliveira et al. 2005). Some field studies suggest that greater DO cycle amplitudes generate physiological stress in various fishes including staghorn sculfin *Leptocottus armatus*, pinfish *Lagodon rhomboids*, and spot *Leiostomus xanthurus* (Ross et al. 2001) as well as affect fitness in estuarine fish, *Fundulus grandis* (Cheek 2011). Additionally, studies on effects of hypoxia with concurrent increases in carbon dioxide levels (hypercapnia) on invertebrates are also limited (Burnett 1997, Willson and Burnett 2000), and it has been suggested that the effects of ocean acidification will be amplified by hypoxia in coastal ecosystems (Melzner et al. 2013). It is critical to assess the effects of environmental hypoxia on estuarine organisms and to investigate its relationships with other stressors in coastal habitats. Sensitive, diagnostic organismal and cellular biomarkers provide essential insights that are needed to fully understand and predict the interactive effects of hypoxia and other anthropogenic stressors on estuarine bivalves and other wildlife.

### 1.2. Hypoxia and Oxidative Stress

Oxidative stress occurs when there is an imbalance in generation and removal of reactive oxygen species (ROS) in cells. This balance can be perturbed under hyperoxic conditions, inflammation, ischemia-reperfusion or in the presence of limited or impaired antioxidant defenses (Auten and Davis 2009). ROS are oxygen containing ions or radicals that are produced as a result of reduction of oxygenated compounds. Oxygen readily accepts electrons produced as a result of normal oxidative metabolism in aerobically respiring organisms which leads to the production of ROS. The mitochondrial

electron transport chain (ETC) is often considered one of the biggest sources of ROS, along with other cellular contributors such as endoplasmic reticulum bound enzymes, cytoplasmic enzyme systems, NADPH oxidase of phagocytes, P450 systems, soluble oxidases and autoxidation (Halliwell and Gutteridge 1999, Auten and Davis 2009, Tahara et al. 2009). It is suggested that xanthine reductase/xanthine oxidase system can be an effective ROS producer via limited oxidation steps (Lushchak 2011). One electron reduction of oxygen leads to the production of superoxide radical ($O_2^{\cdot -}$) which in turns can accept one electron and be converted into peroxide ion, which can be reduced further to a hydroxyl radical ($OH^{\cdot}$). Mitochondrial ROS can escape from ETC and damage other cellular components. Much of the toxicity of superoxide radicals can be explained by its ability to react further with other ROS such as hydrogen peroxide ($H_2O_2$) to produce more reactive ROS (Halliwell and Gutteridge 1999).

$$O_2 + e^- \rightarrow O_2^{\cdot -} \quad (a)$$

$$O_2^{\cdot -} + e^- + 2H^+ \rightarrow H_2O_2 \quad (b)$$

$$H_2O_2 + e^- + H^+ \rightarrow OH^{\cdot} + H_2O \quad (c)$$

Cellular production of $O_2^{\cdot -}$ and $H_2O_2$ can facilitate production of more reactive and toxic $OH^{\cdot}$ in the presence of certain reduced metals such as iron and copper (Auten and Davis 2009).

$$O_2^{\cdot -} + Fe^{3+} \rightarrow O_2 + Fe^{2+} \quad (d)$$

$$Fe^{2+} + H_2O_2 \rightarrow OH^{\bullet} + OH^- + Fe^{3+} \quad (e)$$

Equation e, known as Fenton reaction serves an important role in the production of hydroxyl radicals in the presence of metals. While Fe is regarded as the most Fenton-

reactive metal, Cu is also a primary producer of ROS via this pathway, as well as other divalent metals to a lesser extent.

ROS can damage cellular components including lipids, DNA and proteins and affect antioxidant status (Kelly et al. 1998, Halliwell and Gutteridge 1999, Alves de Almeida et al. 2007). Lipid membranes are rich in polyunsaturated fatty acids (PUFA) and are often a target of ROS damage due to the presence of a bis-allylic methylene group. Autoxidation of lipids includes a chain reaction consisting of three steps: initiation, propagation and termination. Initiation leads to the production of a highly reactive carbon centered lipid radical which propagates the reaction by reacting with oxygen to yield a lipid peroxyl radical. The peroxyl radical is capable of abstracting a hydrogen atom from a number of *in vivo* sources including DNA and proteins, to form the primary oxidation product, a lipid hydroperoxide (Kelly et al. 1998, Halliwell and Gutteridge 1999). Termination of lipid peroxidation can occur by reaction of two radical species to generate a non- radical product, or by antioxidants which scavenge ROS. DNA is another important target component of oxidative damage, particularly by hydroxyl radicals as neither hydrogen peroxide nor peroxyl radicals react directly with DNA. DNA damage can include ring opening, hydroxylation, formation of DNA-protein crosslinks and strand breakage (Kelly et al. 1998, Halliwell and Gutteridge 1999). ROS can also damage proteins via oxidation and formation of protein carbonyls (Lushchak 2011).

Although, the mechanisms of hypoxia-induced oxidative stress are not clearly understood, various studies on aquatic organisms suggest that limited supply of oxygen can lead to oxidative stress (Hermes-Lima et al. 1998, Hermes-Lima and Zenteno-Savın 2002, Vidal et al. 2002, Lushchak et al. 2005, Lushchak and Bagnyukova 2006,

Lushchak and Bagnyukova 2007). It has been suggested that under hypoxic conditions, reducing equivalents can build up, increasing the chances of single electron reduction of oxygen to superoxide (Clanton 2005, Lushchak 2011). Additionally, reoxygenation following hypoxia is a major contributor in production of ROS via the ETC.

The antioxidant potential of an organism is dependent on low molecular mass non-enzymatic antioxidants like glutathione, ascorbic acid, tocopherol, and high molecular mass antioxidant enzymes such as superoxide dismutase, catalase and some specific proteins like ferritin and metallothioneins. Glutathione (GSH), a non-enzymatic antioxidant provides reducing equivalents required by glutathione peroxidase (GPx) for reduction of organic peroxides to alcohols and water, and glutathione-s-tranferase (GST) for Phase II detoxification of organic molecules. GSH also provides antioxidant protection along with acting as a reducing agent. The cysteine thiol group of GSH imparts antioxidant potential and it can directly reduce various ROS and be oxidized to GSSG in the process (Kelly et al. 1998, Halliwell and Gutteridge 1999, Lushchak 2011). Glutathione also protects cells from metal-induced oxidative damage by binding directly to metal ions as well as by scavenging oxyradicals (Mason 1996). Superoxide dismutases (SODs) are metallo-enzymes (Cu/Zn-SOD, Mn-SOD) which catalyze the dismutation of superoxide radical to hydrogen peroxide and oxygen. Hydrogen peroxide is a non-radical ROS which is quickly decomposed by the heme containing enzyme, catalase (CAT) into water and oxygen. Ultimately, under excess production of ROS, antioxidant potential may become overwhelmed which can lead to cellular damage, disruption of cellular homeostasis, and eventually cell death.

### 1.3. Hypoxia and Metal Bioavailability in Estuaries

Bottom sediments act as a sink as well as a source for metal contaminants entering aquatic ecosystems, and affect metal cycling and transformation into various chemical compounds, that affect metal bioavailability and assimilation by animals (Chapman et al. 1998, Burton and Johnston 2010). Factors such as dissolved oxygen, temperature, pH and salinity can alter the solubility of metal salts and affect metal speciation and interactions, thereby affecting bioavailability and toxicity (Chapman et al. 1998, Beck and Sañudo-Wilhelmy 2007, Namieśnik and Rabajczyk 2010, Hong et al. 2011). Surface waters can oxygenate dissolved salts of Mn and Fe which leads to precipitation of these metals as hydrated oxides (Li et al. 2007, Pakhomova et al. 2007). Other heavy metals can also co-precipitate with these hydrated oxides but free metal ions can be released in the water when there are redox changes in benthic layers especially during anoxic and reducing conditions (Namieśnik and Rabajczyk 2010). Studies show that as oxygen concentrations decrease in water, metals such as Cu, Mn, and Fe tend to flux out of the sediments (Mason 1996, Kristiansen et al. 2002, Pakhomova et al. 2007). Additionally, pH and salinity are also known to affect the release of metals from the sediments (Lopez et al. 2010, Hong et al. 2011). Metals such as Cu, Cd, Pb that can bind to carbonates get released to the porewaters and overlying waters under acidic conditions, and metals bound to hydrated oxides of Mn and/or Fe are released under reducing conditions (Tessier and Turner 1995). Microbial activity, decomposition and bioturbation also affect metal resuspension and bioavailability.

Development and associated anthropogenic activities in coastal zones continue to increase, and metal contaminants are continually introduced to estuarine ecosystems.

Sediments are a dynamic pool of chemical, physical and biological activities and assessment of their toxicity depends on the net effect of these activities (Chapman and Wang 2001). Essential metals (Cu, Zn, Fe) as well as unessential metals (Hg, Pb, Cd) can induce oxidative stress via the generation of ROS and through Fenton reactions, and overwhelm antioxidant mechanisms (Halliwell and Gutteridge 1999, Ercal et al. 2001). The effects of heavy metals on lipid peroxidation, antioxidant defenses and toxicity have been studied as valuable biomarkers of environmental pollution (Ringwood et al. 1998, Conners and Ringwood 2000, Valavanidis et al. 2006, Vlahogianni and Valavanidis 2007, Martin et al. 2008). Exposure of aquatic organisms to metal-contaminated sediments can induce oxidative stress and damage (Ringwood et al. 1999, Arnold et al. 2005, Stoiber et al. 2010) as well as perturbation of fundamental cellular processes due to metal substitutions, and affect larval development and reproductive success (Ringwood and Conners 2000, Cao et al. 2010), thereby posing significant threats to coastal wildlife. Understanding the differential sensitivity of bioindicator species as well as the bioavailability of dissolved and particulate phases of metals are essential for predicting and mitigating the impacts of metal exposures in coastal ecosystems (Strom et al. 2011). Changes in metal bioavailability under hypoxic conditions can increase the risks associated with exposure to even moderately contaminated sediments in coastal environments (Cooper and Morse 1998, Middelburg and Levin 2009).

### 1.4. Hypoxia and Bivalve Microbial Communities

Changes in pathogenic bacteria in estuarine ecosystems have been reported to be associated with water quality parameters such as DO, temperature and salinity (Blackwell and Oliver 2008, Froelich and Oliver 2013). Members of family *Vibrionaceae* are known

to be the dominant genera found in estuarine waters and in bivalve tissues, and many *Vibrio* species pose significant concerns for human health (Oliver et al. 1983). One of these, *Vibrio vulnificus* is a widely studied and naturally occurring halophilic gram-negative rod-shaped bacteria that is ubiquitous in coastal waters (Daniels 2011) and is known to cause wound infections and can potentially be fatal to infected humans (Oliver 2005).

Hypoxia can promote the growth of facultative anaerobic bacteria in coastal habitats which can be accumulated by filter-feeding organisms such as bivalves, which can then infect humans when consumed as food (Grimes 1991, Pruzzo et al. 2005). It has also been proposed that bivalves have a core microbiome that is essential to their overall health, but few studies have been done on its function and responses to stress (Zurel et al. 2011, King et al. 2012, Lokmer and Wegner 2014). Hypoxia induced increases in bacterial loads and changes in bacterial communities in bivalves could therefore be important factors that affect the incidence of seafood related illness as well as overall bivalve health that makes populations more susceptible to other stressors. Although bivalves are regarded as somewhat tolerant to hypoxia, chronic hypoxia and hypercapnia can increase their susceptibility to bacterial infections (Burnett 1997, Macey et al. 2008, Macey et al. 2008).

### 1.5. Bivalves as Bioindicators of Coastal Ecosystem Health

Bivalves such as oysters and mussels are excellent bioindicators of habitat quality and serve as model organisms worldwide to study the effects of environmental stressors. They are sessile so they cannot migrate to escape stresses unlike more mobile animals such as fish and crustaceans which can relocate to more favorable areas. The studies of

oxidative stress responses including lipid peroxidation, DNA damage and antioxidant defense systems in bivalves have been recognized as important tools for assessing environmental quality (Regoli and Principato 1995, Ringwood et al. 1999, Almeida et al. 2003, Almeida et al. 2005, Alves de Almeida et al. 2007). Two important co-existing bivalve species found in coastal NC and all along the eastern seaboard as well as the Gulf coast are eastern oysters, *Crassostrea virginica*, and marsh mussels, *Geukensia demissa*. These bivalves play important roles in maintaining ecosystem integrity, and eastern oysters are also commercially important for seafood consumption. Marsh mussels are not as widely studied as oysters or mussels of the genus *Mytilus*, so data on the effects of environmental stressors on this mussel species are scarce even though it is also considered an important ecological engineer. It has been shown that the sensitivities of different species of bivalves to one or multiple stressors can be different (Funes et al. 2006). Unlike oysters, marsh mussels have a reduced foot, which enables some limited movement, while oysters are cemented to hard surfaces and are completely sedentary as adults, often attached together in clusters. Bivalves are filter feeders i.e., they filter water through their gills and capture phytoplankton food, but marsh mussels and oysters also differ in gill ultrastructure and hence, their particle size-selection and retention efficiencies are different (Riisgard 1988). During filter feeding, they are exposed to and accumulate contaminants and microbes which make them good indicators of bioavailability and habitat quality. They sometimes also serve as passive carriers of human pathogens including indigenous bacteria as well as non-indigenous bacteria which are shed by infected animals and humans (Canesi et al. 2007), but may not adversely affect the bivalves. It is also valuable to understand the differences in sensitivities of

bivalve species which co-exist in nature, as interspecies comparisons facilitate our understanding of how marine invertebrates are affected differently by the same stressors, and strengthen our ability to identify and address broader community impacts.

Hepatopancreas (HP) tissues in bivalves serve as the digestive gland as well as a primary target tissue that concentrates toxins, contaminants and bacteria. This large organ plays important roles in detoxification as well as nutrient processing and intracellular digestion in bivalves. The HP tissues are composed of a high percentage of PUFAs which are a target of ROS during lipid peroxidation, as discussed above. Studies have shown that HP tissues tend to have higher baseline antioxidant levels than other tissues in bivalves and crustaceans (Regoli 1998, Zenteno-Savín et al. 2006). Gills, on the other hand, are important feeding and respiratory organs, which are in direct contact with the external environment and are very sensitive to oxidative damage by dissolved metals (McCarthy et al. 2013). Evaluating and understanding the effects of environmental stressors on different tissues are essential for characterizing tissue-specific effects that affect susceptibility to different causes of toxicity, and for understanding the primary factors affecting bioavailability.

2. Summary

With global climate change, water temperatures are rising and with increased nutrient loads, estuarine waters are becoming more hypoxic. Coastal hypoxia is tightly linked to eutrophication, with a variety of adverse manifestations, sometimes including predictable phases which increase in severity (Diaz and Rosenberg 2008). Deposition of organic matter promotes microbial growth and respiration and depletes DO, and increases mortalities in sensitive organisms that contribute to further accumulation of organic

matter. These processes result in hypoxia, seasonal or diel cycles that can cause chronic stress or be accompanied by mass mortalities of fish and benthic animals, sometimes characterized by boom-and-bust population cycles. These patterns have been reported in various regions all along the eastern USA and Gulf of Mexico. Hypoxic zones are expanding and are a huge threat to aquatic organisms and can lead to habitat loss as well as critical losses of seafood and marine resources. Metal contamination in coastal habitats also continues to increase with increasing development of coastal regions. The interactions between hypoxia and metal contaminants can affect metal bioavailability and increase the potential for sublethal chronic toxicity as well as mortalities. Hypoxia may also affect organismal microbiomes and microbial dynamics, potentially increasing disease problems in the organisms as well as seafood consumers, including humans. Bivalves are recognized worldwide as valuable indicator species. Understanding species-specific differences in the interactions of contaminant and hypoxia stressors and development of diagnostic indicators based on physiological and cellular biomarker responses are essential for protecting and restoring coastal resources, and promoting sustainability.

# CHAPTER 2: CELLULAR RESPONSES TO HYPOXIA IN EASTERN OYSTERS AND ATLANTIC RIBBED MARSH MUSSELS

## 1. Abstract

Hypoxic zones in coastal ecosystems are rapidly expanding as global temperatures and anthropogenic inputs continue to increase, and pose serious threats to wildlife. Environmental hypoxia (low $O_2$) is almost always accompanied by hypercapnia (high $PCO_2$), which results in decreased pH in estuaries. Hypoxia can affect cellular homeostatis and sustainability of productive coastal ecosystems by impacting fitness of a wide variety of organisms. Bivalves are ecologically important bioindicators which maintain ecosystem integrity and are susceptible to the impacts of environmental stressors such as hypoxia. The overall purpose of this study was to evaluate the effects of hypoxia on antioxidant status and tissue damage in two bivalve species - Eastern oysters, *Crassostrea virginica* and Atlantic ribbed marsh mussels, *Geukensia demissa*. Oysters and mussels were exposed to different hypoxia regimes in the laboratory, and collected from multiple field sites with varying degrees of hypoxia. Glutathione and lipid peroxidation levels were measured in hepatopancreas tissues as markers of overall antioxidant status and tissue damage respectively. Hypoxia affected tissue antioxidant and damage levels, and continuous exposure to hypercapnic hypoxia was found to be the most damaging treatment. Species specific differences in responses to hypoxia were also observed and mussels were found to be much more sensitive than oysters to hypoxia. It is critical to assess the effects of hypoxic conditions on the health of ecologically and

commercially important bivalves and to identify biomarkers of hypoxic stress in bioindicator species.

## 2. Introduction

Dissolved oxygen (DO) has been regarded as one of the most drastically changing ecological variables in marine ecosystems over the past few decades (Diaz and Rosenberg 1995, Diaz 2001, Vaquer-Sunyer and Duarte 2008). Hypoxia (often defined as DO $\leq$ 2mg/L) is a global environmental stressor in aquatic ecosystems and continues to be a threat to wildlife. According to Diaz and Rosenberg, in 2008 the number of hypoxic dead zones in the world was reported to be over 400, covering an area of 245,000 $km^2$ (Diaz and Rosenberg 2008). Increasing temperatures in coastal ecosystems and eutrophication due to anthropogenic inputs contribute to decreasing oxygen saturation potential. More recent reports on dead zone expansions suggest that climate change is further going to exacerbate hypoxic conditions worldwide (Altieri and Gedan 2014).

Coastal ecosystems support a wide variety of organisms and also serve as nurseries to larval stages of numerous species. They are exposed to diel and seasonal hypoxia (Brown-Peterson et al. 2008, Tyler et al. 2009), which is almost always accompanied by hypercapnia or high $pCO_2$ causing a decrease in pH (Burnett 1997, Ringwood and Keppler 2002). Hypercapnic hypoxia is often a result of the imbalance between photosynthetic and respiratory processes in nutrient-rich estuaries. One of the ways organisms can respond to hypoxia is to migrate away from the area, but even mobile animals like fish may face massive die offs if the expanse and severity of the hypoxic area are large (Paerl et al. 1999, Breitburg 2002). Sessile organisms with limited or no movement such as bivalves cannot escape such environmental changes, so they reflect localized conditions and long term effects of water quality degradation. Various

mechanisms of coping with periodic hypoxia including metabolic depression and energy production via anaerobic pathways have been studied in vertebrates as well as invertebrates (Burnett 1997, Le Moullac et al. 2007, Ramirez et al. 2007, Aragones et al. 2009). There is evidence for changes in gene expression, developmental abnormalities, and reduced fitness in estuarine animals exposed to hypoxia (Baker and Mann 1994, Ross et al. 2001, David et al. 2005, Brown-Peterson et al. 2008, Cheek 2011). Induction of oxidative stress as a response to limited oxygen availability has also been reported in many organisms (Hermes-Lima and Zenteno-Savın 2002, Zenteno-Savín et al. 2006, Lushchak 2011). Oxidative stress occurs when there is an imbalance in generation and removal of reactive oxygen species (ROS) in cells, as excess ROS can damage biological macromolecules and disrupt cellular homeostasis (Kelly et al. 1998, Halliwell and Gutteridge 1999, Auten and Davis 2009). This balance can be perturbed under hyperoxic conditions, inflammation, ischemia-reperfusion, or when antioxidant defenses are limited or impaired. The mitochondrial electron transport chain is typically one of the biggest sources of ROS production but other sources such as endoplasmic reticulum bound enzymes, cytoplasmic enzyme systems, NADPH oxidases of phagocytes, P450 systems, soluble oxidases and autoxidation also contribute to generation of ROS (Halliwell and Gutteridge 1999, Auten and Davis 2009, Tahara et al. 2009). It has been suggested that under hypoxic conditions reducing equivalents can build up, increasing the chances of single electron reduction of oxygen to superoxide (Clanton 2005, Lushchak 2011). Increased ROS production was observed during hypoxia possibly due to the effects on cytochrome oxidase (Chandel et al. 1998). Another process contributing to oxidative

stress under hypoxia can be due to the xanthine reductase/xanthine oxidase system, which can be an effective ROS producer via limited oxidation steps (Lushchak 2011).

Here we present the results of our field and laboratory studies on the effects of hypoxia on two ecological engineer and bioindicator species, Eastern oysters, *Crassostrea virginica* and Atlantic ribbed marsh mussels, *Geukensia demissa*. Increases in the concentrations of glutathione, the most abundant cellular antioxidant and an ROS scavenger, were reported in the hepatopancreas (HP) tissues. The study also suggests elevated lipid peroxidation, a marker of tissue damage, as an effect of hypoxic exposure. Species-specific differences in sensitivity to hypoxia were also found. Increases in total cellular glutathione (GSH) are an essential compensatory mechanism to prevent or minimize cellular damage. Short term hypoxic exposures as represented by our laboratory studies indicated increased GSH, that may be somewhat protective and facilitate their adaptation to hypoxic conditions. However, severe conditions or long term exposures could overwhelm compensatory mechanisms, disrupt cellular homeostasis and contribute to reduced long-term survivorship. Our studies suggest that hypoxia and hypercapnia increase cellular damage, alter antioxidant status and pose significant risks to estuarine bivalves that can have severe impacts on oyster health and sustainability.

## 3. Methods and Materials

### 3.1. Laboratory Exposures

Animals were collected from a clean reference site in Bogue Sound and were acclimated in the lab for 7-10 days. After the acclimation period, 10-12 bivalves were transferred to pre-conditioned 20 L polypropylene exposure buckets with 15 L of natural and artificial seawater mixture in 1:1 ratio and salinity was adjusted to 25 ppt with

distilled water. Natural, clean, low-organic, beach seawater was collected and filtered through 0.45µ filter and then mixed with artificial seawater (MBL formula, (Cavanaugh 1975). The animals were fed phytoplankton *Isochrysis galbana* and *Skeletonema costatum* every day and partial water changes were done on day four for the eight day exposures. Continuous nitrogen and cyclical nitrogen hypoxia treatments were maintained using ultra high purity (UHP)-$N_2$. Carbon dioxide (5% $CO_2$ - air mix) was used to maintain continuous hypercapnic and cyclical hypercapnic hypoxia. Carbon dioxide-induced hypoxia led to a concomitant decrease in pH, but Nitrogen-induced hypoxia did not cause a decrease in pH. For both types of cyclical hypoxic exposures, treatment buckets were aerated with filtered house air for 8 hours during the daytime, and then UHP-$N_2$ or $CO_2$ gases were used to aerate during the night. Control normoxic exposures were conducted along with every experiment and filtered house-air was used to keep the %DO levels above 60-70% and pH above 7.5 throughout the exposures. Hach Hydrolab minisonde 4a dataloggers were used to record water quality parameters (dissolved oxygen, pH, salinity and temperature) every 30 minutes throughout the duration of exposures. Representative datasonde profiles and summaries of water quality parameters are shown in Figure 2.1 and Table 2.1, respectively. At the end of 4 and 8 days, 6 animals were sacrificed from each treatment and HP tissues were frozen at -80 °C for further analyses. Data were collected from 6 different experiments over a period of 3 years.

3.2. Field Sample Collections

Animals were collected from three field sites in coastal North Carolina, USA (Figure 2.2). Bogue Sound is a reference normoxic site where DO stays between

approximately 60-100% and pH between 7.8-8.1. Two sites were sampled in Hoop Pole Creek, which is located in the Hoop Pole Reserve, Atlantic Beach NC (managed by the NC Coastal Federation). One Hoop Pole site receives limited nutrient inputs and only gets moderately hypoxic. The Hoop Outfall site is located in the headwater region of the creek near a storm drain that serves a small coastal town, and receives periodic elevated nutrient inputs. Hach Hydrolab minisonde 4a dataloggers were deployed at these sites, recording water quality every 30 minutes for a period of 7-10 days and animals were collected at the end of deployment period. Animals were brought to the laboratory in site water and were kept cool, immediately sacrificed upon arrival and HP tissues were frozen at -80 °C for further analyses. The field studies were conducted in years 2010, 2011 and 2013.

3.3. Cellular Biomarker Analyses

Hepatopancreas (HP) tissues were chosen for the study because they serve major roles in digestion and nutrient processing, and are a reservoir for toxins, contaminants and bacteria in bivalves. As a deeper tissue, HP can become more hypoxic than more superficial tissues under limited oxygen availability in water. HP tissues are also composed of a high percentage of poly unsaturated fatty acids (PUFAs) which are a primary target of ROS during lipid peroxidation.

Malondialdehyde (MDA) is one of the products of oxidation of PUFAs found in membranes and other cellular lipids. Elevated production of ROS increases total cellular MDA, and is a valuable biomarker of oxidative stress (Kelly et al. 1998, Halliwell and Gutteridge 1999). The role of glutathione (GSH) as an antioxidant in regulation of oxidative stress has been widely studied (Meister and Anderson 1983, Kelly et al. 1998)

and changes in GSH concentrations indicate disruption of cellular and physiological antioxidant status. A spectrophotometric assay was used to quantify total cellular MDA as a marker of lipid peroxidation in HP tissues. The reaction of thiobarbituric acid (TBA) with MDA forms an adduct which was quantified by a spectrophotometer (Ringwood et al. 1999, Ringwood et al. 2003). Total cellular glutathione pool, which represents the sum of reduced and oxidized forms, was quantified by a DTNB-GSSG reductase recycling assay (Ringwood et al. 1999, Ringwood et al. 2003). The HP samples were homogenized in 5% Sulfosalicylic acid (SSA) and glutathione was converted to its oxidized form (GSSG) in the homogenate. GSSG was then converted back to the reduced form (GSH) by Glutathione reductase which was coupled with recycling of 5,5'-Dithiobis (2-Nitrobenzoic acid) (DTNB). The rate of formation of the chromophore 5'-thio-2-nitrobenzoic acid (TNB) from DTNB is proportional to the concentration of GSH in the sample which was quantified spectrophotometrically. Nicotinamide adenine dinucleotide phosphate (NADPH) serves as the electron donor for the reduction reaction. Both spectrophotometric assays were run on a platereader (μQuant, Bio-Tek Instruments) using KCjunior software.

### 3.4. Data Processing and Statistics

Lipid peroxidation and GSH data were organized and summarized using Excel. One-way ANOVAs were used to compare different treatments, and student-Newman Keuls method was used to perform pairwise comparisons; normality and equal variance assumptions were confirmed (confirmed with more than 90% of the data); or if these assumptions were violated, a Kruskal – Wallis non-parametric, one way ANOVA was

used. A multiple comparisons versus control normoxic group analysis was also conducted using the Holm-Sidak method. All statistical analyses were conducted using Sigma Stat.

The data records from the minisondes used for field and laboratory studies were downloaded to Excel, and summarized. Each minisonde was verified using standards for pH, DO, salinity, and temperature prior to and at the end of the deployments. Various metrics from the data were calculated – overall means, average daily minima, average daily maximum, average daily range, and the associated standard deviations as shown in Table 2.1.

## 4. Results

### 4.1. Field and Laboratory Water Quality

The datasonde profiles from field sites show the characteristic coupling of pH and DO cycles (Figure 2.1). Most shallow water estuaries experience moderate hypoxia with diel cycles of low amplitude like Hoop Pole, especially during summer months (Figure 2.2B). More impacted sites such as Hoop Outfall (Figure 2.2C) have dramatic DO cycles that range from very low hypoxia /anoxia to supersaturation each day.

Our normoxia controls for laboratory exposures had mean DO >70% and mean pH of 7.9 (Figure 2.1). The cyclical DO and pH regimes found in the field were successfully simulated in the laboratory (Figure 2.1), going from a low of 1% to a high of 100% DO (Table 2.1). The cyclical hypoxia nitrogen exposure never experienced pHs below 7.8, unlike the cyclical hypoxia- hypercapnia exposures where pH cycles ranged from below 7 to 8. Both continuous hypoxia exposures had low DO (<15%, Table 2.1) throughout the exposures; again, high pHs occurred with the nitrogen treatments, but the hypercapnia treatments had low pHs.

4.2. Cellular Damage and Mortality

In oysters, lipid peroxidation levels (based on MDA concentrations) were not significantly affected by any of the laboratory hypoxia regimes after 4 days, but lipid peroxidation levels were significantly higher in HP tissues after 8 days for the continuous hypercapnic hypoxia and cyclical hypoxia–$N_2$ treatments ($p<0.01$); there were no significant changes in the continuous $N_2$ hypoxia treatment or the cyclical hypoxia-hypercapnia treatment (Figure 2.3A). No oyster mortalities were observed in any experiments for any of the hypoxia or control exposures. Mussels were found to be much more sensitive, as significant increases in oxidative damage were observed in three of the four hypoxia regimes after only four days (Figure 2.3B). Moreover, unlike oysters, the mussels experienced significant mortalities, 100% in some treatments by 8 days (continuous hypoxia-Nitrogen, 67% and 100% mortalities after 4 and 8 days; continuous hypercapnic hypoxia, 50% and 100% mortalities after 4 and 8 days; cyclical hypercapnic hypoxia and cyclical hypoxia nitrogen, 67% mortalities after 4 days with no further mortalities at day 8).

Oysters from all three field sites had different levels of lipid peroxidation. Bogue Sound oysters had the lowest damage levels and Hoop Outfall oysters had the highest. Mussels from Hoop Outfall also showed significantly higher damage levels than Bogue Sound mussels (Figure 2.4). While lipid peroxidation levels of mussels and oysters were very similar under normoxic conditions, the damage levels of mussels tended to be higher than the levels measured in oysters at both Hoop Pole Creek sites; and the mussels also showed much higher variation under hypoxia stress. It must be noted that Hoop Outfall

had very sparse populations of oysters and mussels compared to any other site included in this study.

4.3. Antioxidant Status

Laboratory studies indicated that total HP GSH levels were higher in hypoxia exposed oysters than in control oysters (Figure 2.5A, $p < 0.01$). Glutathione levels were elevated in all hypoxia treatments after four days, and the levels remained high, but did not increase further by 8 days, so no differences in HP GSH were observed between four and eight days for any hypoxic treatment. The control data include GSH levels from time zero (the start of the experiment), 4 days, and 8 days as there were no changes in GSH levels in the normoxic treatments. Mussels showed slightly different patterns of changes in GSH levels. No increases were observed by day 4, but GSH levels for all hypoxia treatments were significantly higher (except for continuous hypoxia hypercapnia where there was 100% mortality by day 8) (Figure 2.5B, $p<0.01$).

Oysters used for the laboratory experiments were collected from a well-oxygenated field site in the open areas of Bogue Sound, characterized by DO patterns of normoxic conditions with very minimal cycles. The GSH levels of the oysters from this site have consistently been around 1000 – 1200 nmol/ g. The GSH levels at the other two field sites targeted for these studies had elevated GSH, probably as a consequence of cyclical patterns of DO stress. Site-specific differences in GSH levels in oysters and mussels were found (Figure 2.6). Oysters from Hoop Outfall, which represents our most DO stressed site, had the highest levels of GSH among all field studies.

## 5. Discussion

With an increase in the number of dead zones, global water temperatures, and nutrient loads in marine ecosystems, hypoxia has become a key stressor for coastal organisms (Diaz and Rosenberg 2008, Rabalais et al. 2009, Rabalais et al. 2014). Environmental hypoxia can induce cellular oxidative stress and alter antioxidant status (Lushchak et al. 2005, Lushchak and Bagnyukova 2007), leading to cellular dysfunction. The concept of elevated cellular ROS production during hyperoxia and reperfusion following ischemia is widely accepted (Halliwell and Gutteridge 1999) and supports results from a number of clinical studies on aging and the roles of oxidative stress in cardiovascular diseases (Finkel and Holbrook 2000, Buttemer et al. 2010, Jacob et al. 2013, Hristova and Penev 2014). Additionally, existing literature also supports the damaging effects of environmental cycles of DO which include reoxygenation following hypoxia (Almeida et al. 2005, de Oliveira et al. 2005, Bickler and Buck 2007). A relatively new emerging complexity in free-radical biology is the production of ROS during hypoxia and several studies provide evidence for hypoxia induced ROS formation (Chandel et al. 1998, Clanton 2005, Magalhães et al. 2005, Clanton 2007, Lushchak and Bagnyukova 2007, Pialoux et al. 2009). Our studies fit this model and show that continuous hypercapnic hypoxia is the most damaging to bivalves among all other hypoxic regimes tested. Increases in cellular damage markers such as lipid peroxidation in the presence of elevated antioxidant levels such as GSH, as seen in our laboratory studies, can be indicative of the induction of compensatory mechanisms. Glutathione plays a dynamic role in ROS scavenging, metal detoxification mechanisms, drug metabolism, cell signaling pathways and cellular homeostasis (Meister and Anderson

1983, Halliwell and Gutteridge 1999). Changes in GSH concentrations represent an altered antioxidant status which can exacerbate oxidative stress and jeopardize normal cellular physiology. Studies with oysters and other bivalves indicate that exposure to contaminants such as metals or PAHs are often associated with GSH depletion, and that GSH depletion can result in increased susceptibility to toxicity (Regoli and Principato 1995, Ringwood et al. 1999, Ringwood et al. 1999, Conners and Ringwood 2000, Peña-Llopis et al. 2002). There is also some evidence that GSH levels can be affected by hypoxia. Elevated GSH levels have been reported in fish exposed to cycles of DO (Lushchak et al. 2005, Lushchak and Bagnyukova 2006). Our results indicated that GSH concentrations in oysters and mussels are affected by exposure to hypoxia and these elevated GSH levels represent short-term effects on the induction of low-molecular weight thiols as an antioxidant defense. Glutathione synthesis is catalyzed by cytosolic enzymes, γ-glutamylcysteine synthetase and GSH synthetase, and these enzymes are transcriptionally and post-transcriptionally regulated. ROS and the ratio of reduced to oxidized glutathione can play important roles in the regulation of glutathione synthetases, among a variety of other factors (Meister and Anderson 1983, Rahman and MacNee 2000, Dickinson and Forman 2002). Under sustained high oxy-radical production, cellular GSH reserves may be overwhelmed by ROS production, leading to cellular damage and decreased antioxidant potential. Our lab studies show elevated GSH levels in oysters as early as four days of exposure and this elevation prevented increases in lipid peroxidation levels. However, after eight days of exposure, the damage started mounting and elevated GSH levels were not sufficient to prevent oxidative damage. Field data, which represent more chronic responses, suggested a similar outcome where we found

tissue damage to be highest at Hoop Outfall, the site with most dramatic and severe hypoxia. Hoop Outfall undergoes dramatic DO cycles diurnally and although other factors such as nutrient inputs may also contribute to cellular damage and changes in adaptive strategies, the elevated tissue damage levels still reflect some effects of hypercapnic hypoxia.

Interestingly, under laboratory cyclical hypoxia, lipid peroxidiation damage was not higher under hypercapnic conditions compared to higher pH conditions. The cyclical hypercapnic hypoxia exposure is an environmentally relevant simulation and the organisms that live in these types of habitats are fairly well adapted to moderate diel shifts in DO and pH. Moderate decreases in pH may favor depression in metabolic rate (Larade and Storey 2002) and limit ROS production. In the absence of the moderate shifts in pH but presence of DO cycles such as our cyclical hypoxia nitrogen exposure, there may not be a suppression of metabolic rate and hence, more ROS induced damage. Although, the reduced production of ROS may minimize oxidative stress potential, hypercapnic hypoxia exposures may negatively affect immune responses of hemocytes in oysters (Boyd and Burnett 1999, Macey et al. 2008). Hemocytes play a critical role in bivalve immune system and ROS kill invading pathogens in bivalves. Under compromised immune health conditions, oysters may not be able to clear bacterial infections, which may have severe impacts on fitness. Another factor contributing to the absence of increased lipid peroxidation levels in oysters in our short term cyclical hypercapnic hypoxia laboratory exposures is that elevated GSH levels could have effectively prevented oxidative damage. While the cyclical ranges of the laboratory simulations were similar to field conditions, field sites, especially nutrient-enriched sites,

often have broader ranges, fluctuating between supersaturation and near-anoxia conditions diurnally. Therefore, despite the adaptive strategies, resident bivalve populations can be threatened in the presence of additional environmental and physiological stresses. As noted above, our most hypoxia stressed site, Hoop Outfall, has very small populations of oysters and mussels, while other regions within Hoop Pole Creek with less stressful DO regimes have extensive oyster and mussel populations.

Another important finding of these studies was the differences in the sensitivities between two co-existing bivalve species. Overall, there were some similarities in the responses of oysters and mussels to hypoxia; continuous hypercapnic hypoxia and cyclical hypoxia nitrogen were the most damaging exposures to both species. However, we found that mussels were more sensitive to cellular homeostasis disruption by hypoxia. The overall MDA levels were higher in mussels and mortalities were only reported in mussels after hypoxic exposures. Our short term studies also suggest that oysters demonstrated a greater capacity for increasing antioxidant status than mussels so their reduced ability to compensate for the oxidative damage makes them more vulnerable to hypoxia. Mussels also have a higher background variation between individuals exposed to similar water quality conditions, indicating greater differences in individual sensitivities. We conclude that although the baseline levels of glutathione and MDA in both these estuarine bivalves are comparable under non-stressed conditions; their responses to hypoxia are different. Characterizing and understanding the differences in sensitivities of two ecologically and evolutionarily related species is of critical importance for estimating the risks associated with an emerging and worsening environmental stressor like hypoxia.

In conclusion, we show that hypoxia stress, based on field as well as laboratory studies, can adversely affect antioxidant status and increase cellular damage. Our studies also show that even short term exposures to hypoxia can affect GSH levels and induce cellular damage. The differences in sensitivities of different species suggest that a suite of biomarkers in multiple indicator species should be assessed to evaluate the effects of environmental stressors on coastal and marine ecosystems. These studies further suggest that biomarkers or short-term bioassays based on biomarkers may be used in a diagnostic way to distinguish between hypoxia and contaminant stress. These studies provide valuable new insights regarding the cellular responses of two ecological engineers to the growing threat of hypoxia worldwide.

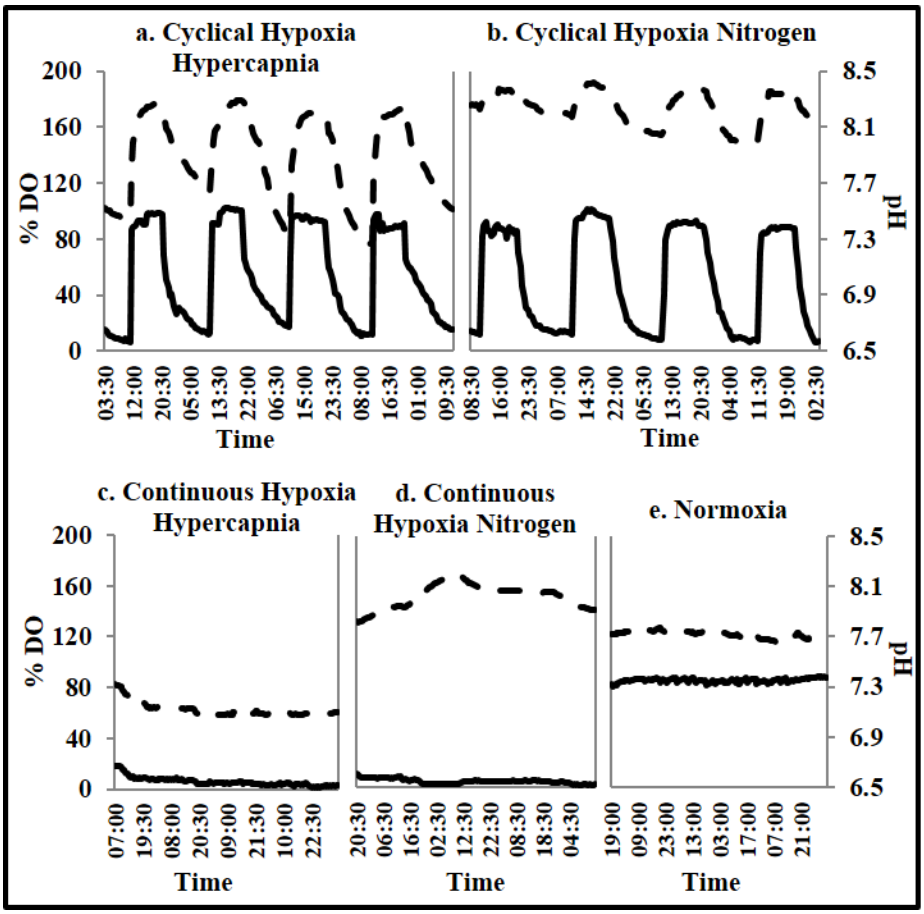


Figure 2.1.: Representative datasonde profiles measured from laboratory hypoxia exposures. Cyclical hypoxia hypercapnia (a) and continuous hypoxia hypercapnia (c) were induced by 5% carbon dioxide. Cyclical hypoxia nitrogen (b) and continuous hypoxia nitrogen (d) were induced by UHP-Nitrogen. Normoxia (e) was induced by air. Solid lines represent % dissolved oxygen (DO) and dashed lines represent pH. Laboratory exposures were conducted in the years 2010, 2011 and 2013.

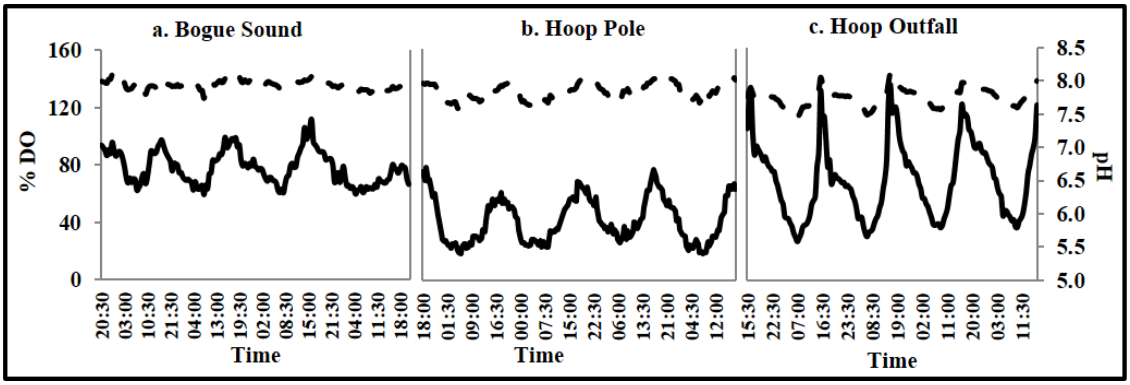


Figure 2.2.: Representative datasonde profiles for field sites - Bogue Sound (a), Hoop Pole (b), and Hoop Outfall (c). Solid lines represent % dissolved oxygen (DO) and dashed lines represent pH. Field studies were performed in the month of August in years 2010, 2011 and 2013.

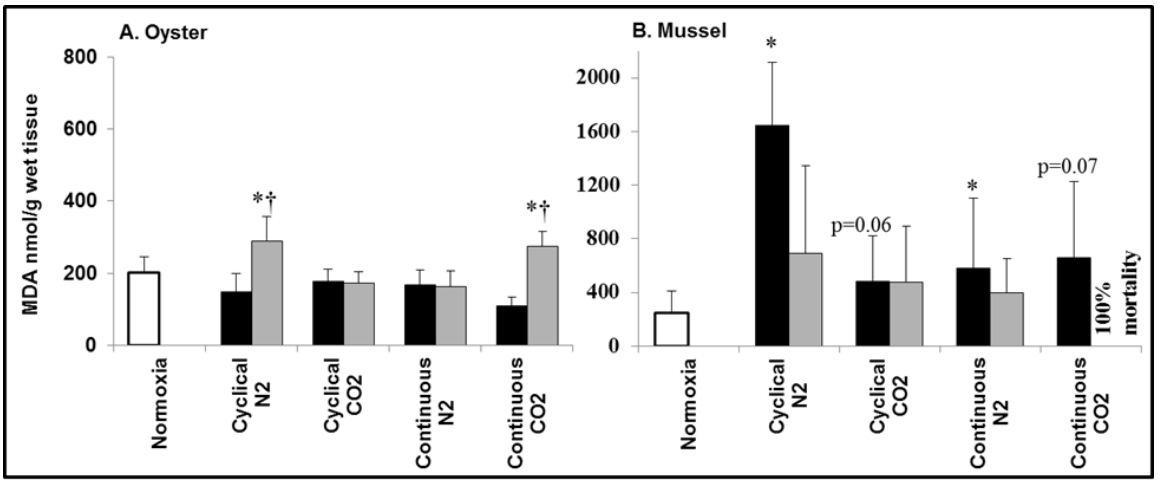


Figure 2.3.: Malondialdehyde (MDA) concentrations in hepatopancreas tissues of oysters (A) and of mussels (B). Black bars represent MDA concentrations after four days and gray bars represent MDA concentrations after eight days of hypoxic exposure. Normoxic control organisms were exposed to normoxia throughout the exposures and are represented by white bars. Asterisks (*) indicate significant increases from Normoxia, and daggers (†) indicate significant differences between four and eight days for a given hypoxic exposure. Data were combined from six experiments conducted between 2010 - 2013.Values are means plus standard deviations, N=6-28.

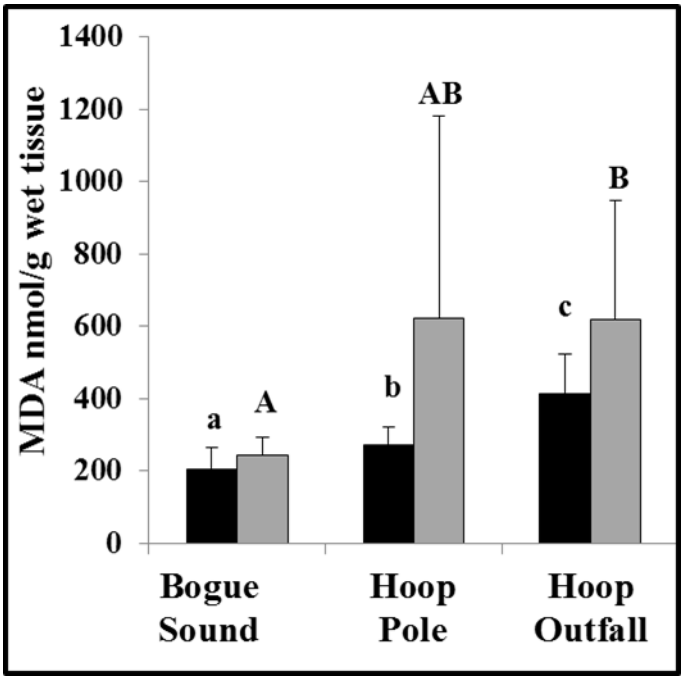


Figure 2.4.: Malondialdehyde (MDA) concentrations in hepatopancreas tissues of oysters (black) and of mussels (gray). Different letters represent statistical differences between sites; lowercase letters represent statistical differences between oysters, and uppercase letters represent statistical differences between mussels. Data were collected and averaged over multiple years between 2010 - 2013.Values are means plus standard deviations, N=8-24.

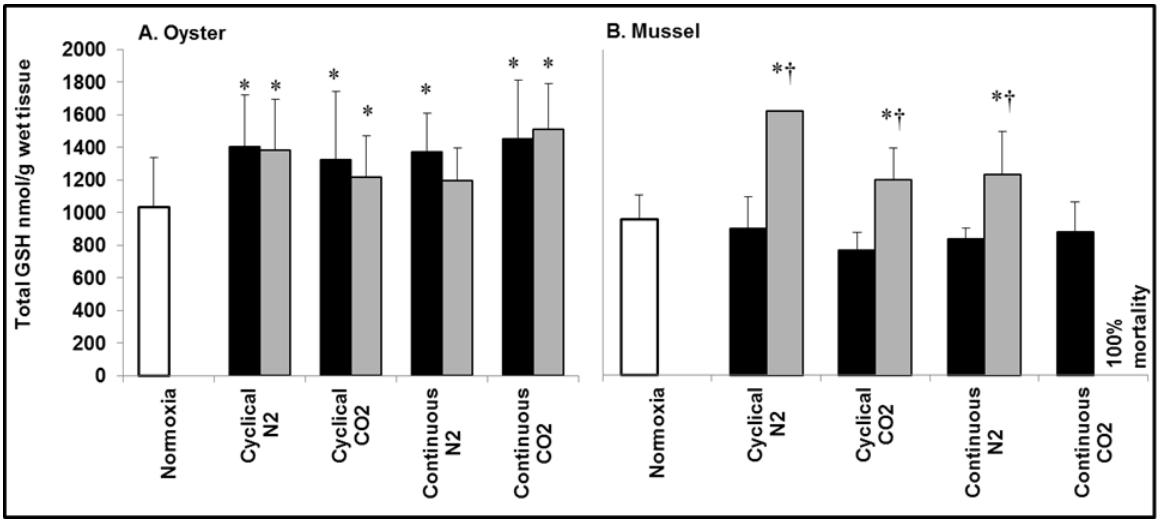


Figure 2.5.: Total glutathione (GSH) concentrations in hepatopancreas tissues of oysters (A) and of mussels (B). Black bars represent GSH concentrations after four days and gray bars represent GSH concentrations after eight days of hypoxic exposure. Normoxic control organisms were exposed to normoxia throughout the exposures and are represented by white bars. Asterisks (*) indicate significant differences from Normoxia, and daggers (†) indicate significant differences between four and eight days for a given hypoxic exposure. Data were combined from six experiments conducted from 2010 to 2013. Values are means plus standard deviations, N=6 – 28.

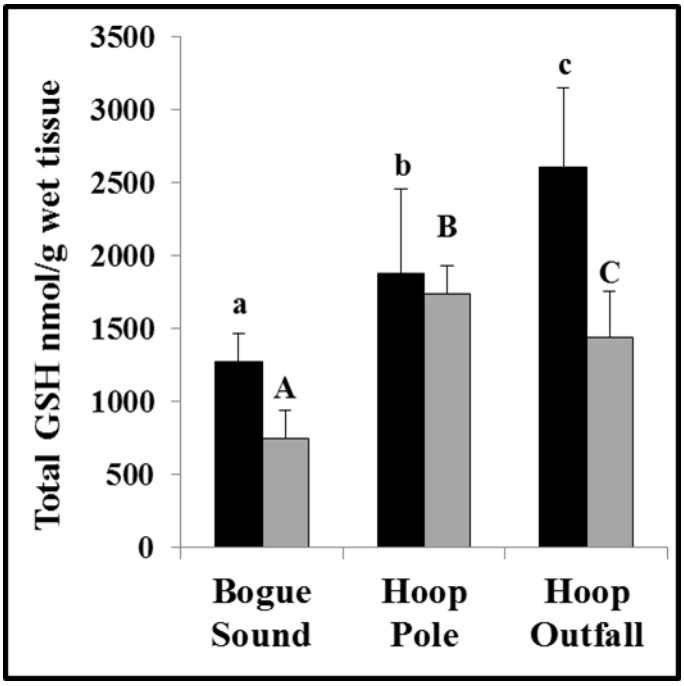


Figure 2.6.: Total glutathione concentrations in hepatopancreas tissues of oysters (black) and of mussels (gray). Different letters represent significant differences between sites; lowercase letters represent significant differences between oysters and uppercase letters represent significant differences between mussels. Data were collected and averaged over multiple years between 2010 - 2013.Values are means plus standard deviations, N=6-26

Table 2.1.: Water quality parameters from field sites and laboratory exposures. Data represent averaged values from samplings and experiments done between 2010-1013.

| | % DO Overall Mean | %DO Average Range | pH Overall Mean | pH Average Range | Overall Salinity Mean | Overall Temperature Mean |
|---|---|---|---|---|---|---|
| **FIELD SITES** | | | | | ‰ | °C |
| **Hoop Pole** | 53.7 | 24-93 | 7.79 | 7.6-8.0 | 35.5 | 29.7 |
| **Calico Creek** | 48.2 | 8-116 | 7.51 | 7.1-8.0 | 30.6 | 29.5 |
| **Hoop Outfall** | 64.1 | 26-132 | 7.74 | 7.5-8.1 | 36.2 | 29.6 |
| **LABORATORY EXPOSURES** | | | **Day4** | | | |
| **Normoxia** | 76.9 | 63-85 | 7.9 | 7.7-8.0 | 26.7 | 21.3 |
| **Hypoxia Cyclical Hypercapnic ($CO_2$)** | 49.7 | 6-92 | 7.4 | 6.6-8.0 | 25.9 | 21.9 |
| **Hypoxia Cyclical Nitrogen ($N_2$)** | 43.4 | 14-95 | 7.9 | 7.8-8.0 | 28.8 | 22.2 |
| **Hypoxia Continuous Hypercapnic($CO_2$)** | 13.7 | 9-25 | 7.0 | 6.9-7.2 | 26.3 | 22.8 |
| **Hypoxia Continuous Nitrogen ($N_2$)** | 11.5 | 5-33 | 7.6 | 7.4-7.8 | 26.8 | 22.3 |
| **Day 8** | | | | | | |
| **Normoxia** | 71.1 | 60-81 | 7.9 | 7.7-8.0 | 28.1 | 20.3 |
| **Hypoxia Cyclical Hypercapnic ($CO_2$)** | 50.1 | 6-94 | 7.5 | 6.7-8.0 | 27.1 | 21.1 |
| **Hypoxia Cyclical Nitrogen ($N_2$)** | 44.2 | 1-101 | 8.2 | 8.0-8.4 | 27.3 | 20.4 |
| **Hypoxia Continuous Hypercapnic($CO_2$)** | 4.8 | 1-18 | 7.3 | 7.2-7.5 | 28.6 | 21.6 |
| **Hypoxia Continuous Nitrogen ($N_2$)** | 9.0 | 3-24 | 7.4 | 7.3-7.6 | 26.4 | 21.5 |

# CHAPTER 3: HYPOXIA AFFECTS METAL BIOAVAILABILITY AND ANTIOXIDANT STATUS IN EASTERN OYSTERS, *Crassostrea virginica*

## 1. Abstract

Estuaries support a wide variety of organisms and are exposed to multiple stressors diurnally as well as seasonally. Fluctuations in dissolved oxygen, pH, salinity and temperature as well as heavy metal pollution from anthropogenic inputs can be challenges of living in estuaries. Habitats characterized by low dissolved oxygen (hypoxia), accompanied by increased $PCO_2$ (hypercapnia) and reduced pH are expanding in marine coastal habitats worldwide. Hypoxia and metals can induce oxidative stress in aquatic organisms via cellular production of reactive oxygen species, and interactions between them can exacerbate oxidative stress and subsequent tissue damage. Metals such as copper, zinc, manganese and iron are essential trace metals which can be toxic if bioaccumulated in the tissues. These metals are introduced in many estuarine ecosystems via industrial effluents, sewage sludge and other anthropogenic sources. Metal ions can exist in the environment complexed with organic or inorganic matter, and their bioavailability may be affected by water quality parameters such as dissolved oxygen, pH and salinity.

The overall goal of these studies was to evaluate the effects of cyclical and continuous hypoxia on metal uptake and subsequent toxicity in tissues of Eastern oysters, *Crassostrea virginica*. Oysters are bioindicators of habitat health and play critical roles in maintaining ecosystem integrity. We hypothesized that hypoxia and hypercapnia

increases bioavailability of metals from estuarine sediments, resulting in increases in metal concentrations and tissue damage in hepatopancreas and gill tissues of *C. virginica.* Tissue metal concentrations were measured after exposure to contaminated sediments under different hypoxic conditions to assess metal uptake. A lipid peroxidation assay was used to evaluate tissue damage, and total glutathione concentrations were quantified to examine antioxidant status. Tissue metal concentrations were found to be affected by hypoxia which also resulted in elevated tissue damage and altered antioxidant status. Tissue specific differences were observed in response to exposures to contaminated sediments under hypoxic conditions. Determination of the sensitivity and vulnerability of an ecologically and commercially important bivalve species to hypoxia and metals is critical for predicting impacts on estuarine ecosystems.

## 2. Introduction

Estuaries are productive, nutrient-rich ecosystems which support a wide variety of organisms. They are also exposed to multiple environmental stressors including fluctuations in temperature, dissolved oxygen (DO), pH, and salinity as well as nutrient and contaminant inputs (Wołowicz et al. 2007). Due to increasing global temperatures and anthropogenic impacts on marine coastal environments, low DO (hypoxia) regions continue to grow in size and severity (Diaz 2001, Vaquer-Sunyer and Duarte 2008, Rabalais et al. 2010). Hypoxia has been shown to play a role in metabolic depression, suppression of global protein synthesis, altered antioxidant status, growth abnormalities and mortality (Hochachka et al. 1996, Ross et al. 2001, Wu 2002, Cheek 2011). As hypoxia expands throughout coastal habitats, the interactions between hypoxia other environmental stressors such as metal contaminants will pose greater risks to overall

ecosystem health. Transition metals such as copper (Cu), manganese (Mn), Iron (Fe) and Zinc (Zn) make their way to coastal habitats via run-off, industrial waste, sewage, and boat traffic. Estuarine sediments function as depositional sites or sinks for contaminants, and sediments concentrate metals as inorganic salts or organic complexes at much higher concentrations than overlying water. In aquatic environments, metals cycle between reactive fractions and directly available fractions (Rocha et al. 2011). The reactive fractions represent metal salts such as hydroxides, carbonates, oxides, and organometallic complexes as well as metals associated with suspended organic matter. These readily exchangeable fractions bind metals until there are shifts in chemical equilibrium and/or microbial or bioturbation activities that lead to mobilization of free metal ions and metals associated with organic moieties to the surrounding water (overlying and pore waters) (Tessier and Campbell 1987, Chapman et al. 1998, Rocha et al. 2011). In anaerobic sediments, metal bioavailability can be affected by sediment sulfide concentrations, represented by Acid Volatile Sulfides (AVS). If AVS are in excess of Simultaneously Extractable Metals (SEMs), metals would be expected to precipitate as sulfides and hence, be less bioavailable to aqueous compartments (Wang and Chapman 1999). Under low AVS concentrations, particulate organic carbon (POC) binds metals readily and POC bound metals are ionized under low pH conditions. Metals bound to carbonates get released to water under acidic conditions and metals bound to hydrated oxides of Mn and/or Fe are released under reducing conditions (Tessier and Turner 1995). Dissolved oxygen and pH play important roles in cycling of metals in coastal habitats because metal mobilization is affected by redox conditions (Beck and Sañudo-Wilhelmy 2007). Decreases in DO (hypoxia) are almost always accompanied by increased carbon dioxide

(hypercapnia) which also causes a decrease in pH (Ringwood 2002). Therefore biological and chemical factors that affect sulfides, redox processes, and pH will work in combination to affect metal bioavailability to filter-feeders and other organisms, especially those that live in benthic and interstitial habitats.

Essential metals such as Cu, Mn, Fe and Zn are required in trace amounts for cellular function and growth. However, at high concentrations these metals can induce toxicity and interfere with signal transduction (Mason 1996, Leonard et al. 2004, Luoma et al. 2008). Redox active metals like Fe and Cu can participate in Fenton reactions to generate reactive oxygen species (ROS), especially hydroxyl radicals, by reacting with hydrogen peroxide (Halliwell and Gutteridge 1999) which can further damage lipids, DNA and proteins (Djuric et al. 2001, Repetto et al. 2010). Mn is also redox active and can generate hydrogen peroxide by reacting with superoxide (Halliwell and Gutteridge 1999) and it's known for its role in neurotoxicity and immune suppression as well as reduced fitness (Martin et al. 2008, Oweson and Hernroth 2009, Pinsino et al. 2010, Pinsino et al. 2012). Although Zn is not typically regarded as a redox active metal it also can induce damage to neurons and mitochondria and indirectly contribute to increased ROS production (Halliwell and Gutteridge 1999). High Zn levels can induce toxicity, especially in aquatic larvae (Fosmire 1990, Devos et al. 2012).

Filter-feeding bivalves readily accumulate free metal ions dissolved in the water and associated with resuspended organic materials which can induce toxicity (Rainbow 2007, Luoma et al. 2008). Furthermore metal toxicity increases under elevated temperatures (Cherkasov et al. 2006, Sokolova et al. 2008), and probably also with increasing hypoxic conditions as these factors affect physiological and cellular resilience

as well as the environmental flux of metals. Glutathione (GSH), the most abundant antioxidant in cells, plays key roles in cellular regulation of metals and maintaining cellular homeostasis. As an antioxidant GSH scavenges reactive oxygen species (ROS) to minimize oxidative stress. As a thiol compound, GSH readily binds metals such as Cd, Cu, Hg, etc. and can help protect cells from free metal ion induced cellular damage (e.g. Fenton chemistry mediated ROS production, and non-specific binding to essential proteins, DNA, etc.). (Halliwell and Gutteridge 1999, Dickinson and Forman 2002). Metal toxicity is often accompanied by increased production of oxyradicals, so oxidative damage and changes in antioxidants in tissues of bivalves and other organisms have been used as valuable biomarkers of heavy metal exposure and toxicity (Regoli and Principato 1995, Regoli et al. 1998, Ringwood et al. 1999, Vlahogianni et al. 2007, Vlahogianni and Valavanidis 2007).

When organisms are exposed to hypoxia and metal contaminants simultaneously, the risk of oxidative injury increases. The overall aim of this study was to assess the effects of contaminated sediment exposures under hypoxic conditions on Eastern oysters, *Crassostrea virginica*. Here we present results from short term exposures to moderately contaminated sediments under various hypoxic conditions. Changes in gill and hepatopancreas (HP) Cu and Mn levels as well as effects on antioxidant status and tissue damage levels were observed. These results indicate increased vulnerability of oyster populations to the combination of metal and hypoxia stressors.

# 3. Methods and Materials

## 3.1. Laboratory Exposures

Sediments were collected from Calico Creek, NC which is a moderately contaminated tidal creek, and press-sieved through a 2mm sieve. The sediments were added to seawater (27-30 psu, a 1:1 mixture of artificial seawater (MBL formula, (Cavanaugh 1975)) and natural, low-organic, beach seawater, 0.45µ filtered filter); 1.5 L of well mixed sediment was used in a 15 L volume of the seawater. The exposure vessels (20L polypropylene buckets) were allowed to equilibrate for 24 hours under the different treatment conditions. Oysters that were collected from a clean reference site in Bogue Sound, NC and acclimated in the lab for 7-10 days were then transferred to the exposure buckets. Water quality parameters (DO, pH, temperature, and salinity) were recorded every 30 minutes using Mini-Sonde 4a dataloggers (Hach-Hydrolab). Detailed water quality parameters for each experiment are listed in Table 3.1. Two normoxia treatments (mean DO >70%, mean pH >8.0) were maintained with and without sediments as positive and negative controls, respectively. Two hypercapnic hypoxia treatments, continuous hypoxia and cyclical hypoxia, were used to represent environmentally relevant conditions of low DO accompanied by concomitant low pH. The DO levels and pHs were maintained low throughout in continuous hypoxia hypercapnia treatments by using 5%$CO_2$ -95%air mix; for the cyclical hypoxia hypercapnic exposures, DO and pH were cycled between daytime oxygenation (filtered house air) and nighttime hypoxia using a 5%$CO_2$ -95%air mix. Nitrogen gas (UHP-$N_2$) was used to produce hypoxia treatments that were not accompanied by reduced pH. Continuous hypoxia-$N_2$ conditions were maintained at low DO and high pH throughout the exposures, and cyclical hypoxia-

$N_2$ exposures were characterized by diurnal DO cycles and high pHs throughout the exposures. Exposures were conducted for four and eight days at an average temperature of 20.3±0.7ºC and bivalves were fed *Isochrysis galbana* daily. Eight-day long exposures received a partial water and sediment change at day four.

3.2. Analytical Procedures

Gill and hepatopancreas (HP) tissues of bivalves were removed after 4 and 8 day exposures and frozen at -80ºC. Tissue metal concentrations (Cu, Mn, Fe, Zn) were determined by furnace and flame atomic absorption spectrophotometry (AAS, Perkin Elmer AAnalyst 800 equipped with Zeeman background correction). Samples were lyophilized, acid digested using trace metal grade Nitric acid and microwave digestion and then analyzed by AAS. Certified oyster reference tissue samples (NIST) were also processed as part of the data quality assurance protocols.

Lipid peroxidation (LPx) levels were measured as a biomarker of tissue damage, by assessing malondialdehyde (MDA) concentrations (Ringwood et al. 1999, Ringwood et al. 2003). Total GSH levels were measured to evaluate the effects on antioxidant status using a DTNB-GSSG reductase recycling assay (Ringwood et al. 1999, Ringwood et al. 2003). Both are spectrophotometric assays, conducted using a microplate reader (μQuant, Bio-Tek Instruments; KCjunior software) as described in Chapter 2.

3.3. Sediment Analyses

Grain size analysis was performed to assess sand and silt-clay content of the sediments using standard protocols (Ringwood et al. 1997) and were found to be 70.74 % ± 9.5 percent sand and 26.26 ± 9.5 percent silt-clay. Sediment metal concentrations were assessed using furnace and flame atomic absorption spectrophotometry (AAS, Perkin

Elmer AAnalyst 800 equipped with Zeeman background correction). Cu, Mn, Fe and Zn were measured to be 116.3 ± 50.5, 80.6 ± 25.9, 8877.7 ±935.4 and 60.5 ± 2.05 ug/g dry sediment weight. Sediment quality guidelines (SQGs) which use effects range low (ERL) and effects range median (ERM) are widely used as informal, interpretive guidelines to assess habitat health in estuarine and marine environments which further need attention. Below ERL concentrations, adverse biological effects are rarely seen; above ERM adverse biological effects are frequently seen; and between ERL and ERM, biological effects and sub-lethal toxicity can be observed occasionally (Birch and Hogg 2011). For our studies, only Cu concentrations were found to be between ERL and ERM, rest of the metals measured in this study were either below ERL or don't have suggested SQGs (Hyland et al. 2000).

### 3.4. Data Processing

All data were summarized using MS Excel. Water quality data were downloaded from the minisondes and also processed in MS excel; standards for pH, DO, salinity and temperature were confirmed before and after the exposures to ensure data quality. One-way ANOVAs were used to compare different treatments, and student-Newman Keuls method was used to perform pairwise comparisons. All statistical analyses were conducted using Sigma Stat.

## 4. Results

### 4.1. Metal Accumulation

Overall, the baseline concentrations of Mn, Fe and Zn were higher in gills than HP tissues, but Cu concentrations in gills and HP tissues were more comparable. Oysters accumulated more Cu from contaminated sediments under hypoxic conditions. In gill

tissues, significant increases in Cu concentrations were observed after four days of exposure to contaminated sediments, but in most cases, the eight day levels were not significantly different from the 4 day levels (Figure 3.1A). Only the continuous hypercapnic hypoxia treatment showed a significant increase in gill Cu accumulation from four to eight days of exposure. In the HP tissues, no increases in Cu concentrations were observed until after eight days of hypoxia exposures when compared to control oysters. In both gill and HP tissues, there was approximately a two fold increase in Cu levels of oysters exposed to hypoxia. Interestingly, the HP Cu levels in oysters exposed to hypoxia and contaminated sediments from Calico Creek used for these studies were approximately 80 ug/g, similar to the levels found in resident oysters from Calico Creek (62.5 ± 21.7 ug/g dry weight), which experiences cyclical hypercapnic hypoxia.

Zn accumulation patterns were somewhat similar to Cu. While no increases in Zn concentrations were observed in gill tissues under normoxia, the Zn concentrations of gills were elevated in all hypoxia treatments compared to controls after four days, but not significantly different from controls after 8 days (Figure 3.1B).. The HP Zn levels were significantly different from the controls after 4 days in only one treatment – the cyclical $N_2$ treatment, but by 8 days there was an overall trend of higher Zn levels in most of the treatments (normoxic as well as three hypoxic treatments; all hypoxic treatments had p values of $< 0.1$), and the highest Zn levels were observed in the continuous $N_2$ hypoxia treatment (Figure 3.1B). Cu and Zn levels were found to be positively related in HP tissues (Figure 3.2A).

Hypoxia did not affect Mn levels in oyster HP or gills (Figure 3.1C). There were slight increases in Mn HP levels after 4 days in normoxic as well as two of the hypoxic

treatments, but no significant differences were observed in any treatments at the eight day interval. Gills accumulated Fe under normoxia and hypoxia treatments. In the HP tissues, no increases in Fe concentrations were observed under hypoxia. Elevated Fe was detected for two hypoxic treatments in HP tissues (Figure 3.1D). It was interesting to note that after 4 days, gill Mn levels were high and Fe levels tended to be low; conversely after 8 days when Fe levels were high, Mn levels tended to be low. Indeed, gill Mn and Fe levels were negatively correlated (Figure 3.2B).

We also found highly significant relationships between gill Cu concentrations and overlying water DO levels (Figure 3.3A) as well as between gill Cu concentrations and water pH (Figure 3.3B). Both water quality parameters were inversely related to gill Cu levels, suggesting that with decreases in DO and pH levels, more Cu was accumulated from the sediments into oyster gills. None of the regressions for HP Cu levels and water quality parameters were significant, and there were no significant relationships between any other tissue metal concentrations; HP metal levels were more dependent on sediment concentrations. .

4.2. Antioxidant Status

Tissue specific differences were also observed in the changes in total GSH levels in oysters exposed to contaminated sediments under varying DO conditions. Gill GSH levels were significantly lower when exposed to contaminated sediments and hypoxia simultaneously for four days (Figure 3.4A). Conversely, the GSH levels of oyster HP tissues decreased slightly in the continuous hypoxia treatments after 4 days, but by 8 days, the GSH levels were elevated in all treatments, normoxic as well as hypoxic (Figure 3.4B), indicating an overall increased antioxidant compensatory response to moderately

contaminated sediments. Unlike gills, there were no differences in HP GSH levels between normoxia and hypoxia exposed oysters as the HP GSH levels were elevated in all sediment treatments after 8 days, suggesting that HP GSH levels were more dependent on sediment metal concentrations. There was a highly significant negative correlation between gill Cu concentrations and gill GSH (Figure 3.5A) and a highly significant positive correlation between HP Cu concentrations and HP GSH (Figure 3.5B). Similar relationships between GSH and gill Zn (Fig. 5C) as well as HP Zn (Figure 3.5D) were also seen, indicating that Cu and Zn accumulation in gill tissues contributed significantly to the depletion of GSH.

### 4.3. Tissue Damage and Mortality

There were no increases in lipid peroxidation in oysters exposed to contaminated sediments under normoxic conditions compared to the control conditions in either gill or HP tissues after 4 days (Figure 3.6A and 3.6B). However, lipid peroxidation levels in gill tissues were significantly elevated in all hypoxia treatments after eight days (Figure 3.6A); but in HP tissues, significant damage was only observed in the continuous hypercapnic hypoxia treatment (Figure 3.6B). Lipid peroxidation levels in HP tissues of oysters exposed to continuous hypercapnic hypoxia were also significantly different from all other hypoxic treatments.

## 5. Discussion

Sediments are a dynamic pool of metal contaminants, serving as sinks as well as sources. Bioavailable metals to benthic and filter-feeding species are partitioned among dissolved ionic forms and particle complexes based on the chemical and physical characteristics of water and sediments. Anthropogenic and industrial inputs are major

sources of heavy metals in coastal ecosystems, as well as nutrients and other factors that exacerbate hypoxia. Hypoxia, temperature, pH, and salinity can alter the solubility of metal salts and affect metal speciation in aqueous compartments, and also affect redox processes in the sediments. We present here important new findings demonstrating that hypoxic conditions, cyclical as well as continuous, can increase metal bioavailability from even moderately contaminated sediments.

Gills were found to be a sensitive target of metal accumulation and toxicity when exposed to contaminated sediments and hypoxia in combination, with significant changes observed as early as four days of exposure, especially in Cu and Zn levels. Interestingly, the Zn accumulation patterns of oyster gills behaved very similarly to Cu. Moreover, there was no significant accumulation of Cu or Zn in gills of oysters exposed to contaminated sediments under normoxic conditions over the 8 day course of the experiments, but under hypoxic conditions, gill Cu and Zn levels were over almost 2 fold higher. These increases in Cu and Zn accumulation were accompanied by perturbation of antioxidant status (GSH depletion by day 4) and oxidative damage (increased lipid peroxidation). Glutathione plays important roles as an abundant thiol in reducing Cu and Zn toxicity by binding directly to metals to minimize the availability of metal ions in cells, and also by scavenging ROS. Therefore, GSH depletion predisposed the gill tissues to oxidative stress, which then led to significant increases in lipid peroxidation by day 8 of these studies; GSH rebounded somewhat in gill tissues in response to the increase oxidative stress and depletion of GSH stores. With continued exposures, the antioxidant responses and other detoxification pathways (such as metallothioneins) may be sufficient to minimize oxidative damage. Alternatively, longer-term chronic effects may occur due

to persistent cycles of antioxidant compensation and oxidative damage or when cells are unable to compensate for oxidative damages. Therefore, hypoxia and metal contamination together can alter antioxidant status via GSH depletion, rendering organisms more vulnerable to oxidative stress. GSH depletion has also been associated with other measures of metal cytotoxicity and reproductive success in oysters and other organisms (Conners and Ringwood 2000, Ringwood and Conners 2000).

Decreased bioavailability of Cu under hypoxic conditions is often predicted as a result of its precipitation as CuS in AVS-rich sediments (Namieśnik and Rabajczyk 2010), but remobilization of Cu from sediments to aqueous compartments can occur as pH decreases (Riba et al. 2003, Lopez et al. 2010), as well as under hypoxic conditions (Riedel et al. 1999). While the sediments used in these studies were fairly fine grained (approximately 30% silt-clays), the AVS levels were probably relatively low in our experimental setups, so the oxygenation and pH changes associated with hypoxic treatments dominated the release of Cu from sediments. The responses of the gill tissues to hypoxia and contaminated sediments and the significant negative correlations between Cu concentrations and DO as well as pH suggest that the net effects of these physiochemical processes were the release of highly bioavailable $Cu^{2+}$ ions. Beck *et al*, have also shown a negative relationship between bottom water DO and labile Cu in an urban estuary which support our findings (Beck and Sañudo-Wilhelmy 2007).

In both hypercapnic treatments used in this study, elevated tissue Cu levels were observed, supporting the hypothesis that Cu complexes undergo dissolution under reduced pH conditions, releasing free ions, which are readily taken up by oysters. Since, environmental hypoxia is almost always accompanied by hypercapnia, these findings

become more relevant in the assessment of toxicity of essential metals in the coastal ecosystems. Dissolution of Cu carbonates can also facilitate free ion release from sediments. Our nitrogen studies also show elevated tissue Cu levels under continuous hypoxic conditions in the absence of low pH, which may indicate the role of hypoxia in release of Cu which has co-precipitated with iron oxides.

The tissue-specific responses that were observed may reflect important differences in exposure routes. While gills were readily affected by 4 days, significant Cu accumulation and toxicity in HP tissues were not observed until 8 days. In contrast to gills, GSH levels in oyster HP tissues were generally elevated after 8 days, and significant increases in HP lipid peroxidation damage were only observed after eight days of exposure to contaminated sediments under continuous hypercapnic hypoxia conditions. This suggests that HP tissues were able to mount a compensatory GSH response that served to minimize ROS damage under most of the exposure regimes, which is also confirmed by the positive relationships seen between HP GSH levels and Cu and Zn accumulation.

Gill responses under hypoxic conditions probably reflect significant release of highly bioavailable Cu and Zn ions. The delayed responses of HP tissues may reflect a lag between accumulation in gill tissues and transfer to HP tissues. Hepatopancreas responses may also reflect accumulation dominated by particulate components that may be less affected by redox effects but more by resuspension processes that do not require metal dissociation from organic materials. Recent studies from our laboratory regarding dissolved Ag ions and nanoparticulate Ag indicated similar short-term responses, e.g. dissolved Ag readily caused gill toxicity by 2 days of exposure, but not Ag nanoparticles;

in contrast Ag nanoparticles and small agglomerates affected HP tissues much more readily (McCarthy et al. 2013). These studies and our current field studies together also suggest that gills may have a reduced antioxidant capacity, which makes them more susceptible to oxidative damage. Overall these tissue-specific differences suggests that short term exposures to metals under hypoxic conditions, especially conditions that favor the release of highly bioavailable metal ions, may adversely affect gills more readily than HP tissues. However, long-term exposures and exposures associated with particles and resuspension processes are more likely to adversely affect HP tissues as spillover from the gill tissues occurs, and accumulation of particulate metal increases. Adverse effects on gills will impair food capture and adverse effects on HP tissues will affect nutrient processing, potentially contributing to long term effects on oyster health and reproduction (Ringwood et al. 1999, Yang et al. 2007), and declines in oyster populations.

We investigated Mn in these studies because of previously published studies with crustaceans indicating that under hypoxic conditions, Mn was readily mobilized from sediments and accumulated in gills (Baden et al. 1990). We therefore hypothesized that if this were also true for oysters, tissue Mn levels could serve as valuable indicators of hypoxia exposure. However, our results did not support this hypothesis, as gill Mn levels were not affected by hypoxia and no consistent changes were observed in HP tissues. We also found that increases in Fe, Cu and Zn concentrations negatively affected Mn accumulation. This could be due to the relatively low concentrations of Mn in the sediments used for these studies. High concentrations of Fe, Cu and Zn could have outcompeted Mn at the level of uptake. It has been shown that redox status of water affects Fe flux significantly more than Mn; and Mn fluxes are more dependent on Mn

concentrations (Pakhomova et al. 2007). Cu, Zn and Fe readily released from contaminated sediments under hypoxia and could also outcompete Mn for cellular accumulation pathways, especially shared pathways. Cu is transported inside cells via Cu transporters such as hCTR1 and DMT, and Mn can use DMT as well as transferrin (an Fe storage and transport protein). However, some evidence of Fe and Mn accumulation in HP tissues under similar hypoxic conditions was seen in our studies, suggesting that Fe and Mn fluxes are favored under hypoxic conditions as suggested by other studies as well (Pakhomova et al. 2007, Namieśnik and Rabajczyk 2010). Some of the released Fe and Mn may associate with food particles and accumulate in HP tissues via dietary routes.

In conclusion, we propose that as estuarine environments get more hypoxic and hypercapnic due to elevated global temperatures, reduced pH, and anthropogenic inputs, heavy metal bioavailability from contaminated sediments may significantly increase. We present results demonstrating important relationships between contaminated sediments, hypoxia, and metal accumulation as well as toxicity in oysters. As bioavailability increases, metals can exacerbate oxidative stress in hypoxic habitats (Ringwood et al. 1998, Ringwood et al. 1999, Rainbow 2002), and our studies reveal that exposure to contaminated sediments under hypoxic conditions increases Cu and Zn bioavailability and toxicity. Therefore sediment quality guidelines may not be sufficiently protective for habitats that experience chronic or cyclical hypoxia. Moreover, short-term laboratory sediment toxicity assays are typically conducted under fully aerated normoxic conditions, underestimating the potential for toxicity in the real world where contaminated habitats and hypoxia often co-occur. The contaminated sediments used for these studies were not highly contaminated sediments, but are typical of more moderate or low-level

contaminant conditions that commonly occur over broad expanses of coastal zones. These kinds of chronic low-level exposures and combinations of hypoxia and metals have important implications for the sustainability of oyster and other seafood resources and ecosystem health. Understanding tissue specific responses to multiple stressors is critical for assessing physiological impairment, and identifying mechanisms and pathways as well as predicting susceptibility to contaminants under environmentally relevant conditions. These studies are also essential for identifying characteristic diagnostic patterns of biomarker and contaminant responses that can be used to target potential causative factors and develop strategies for improving oyster and ecosystem health.

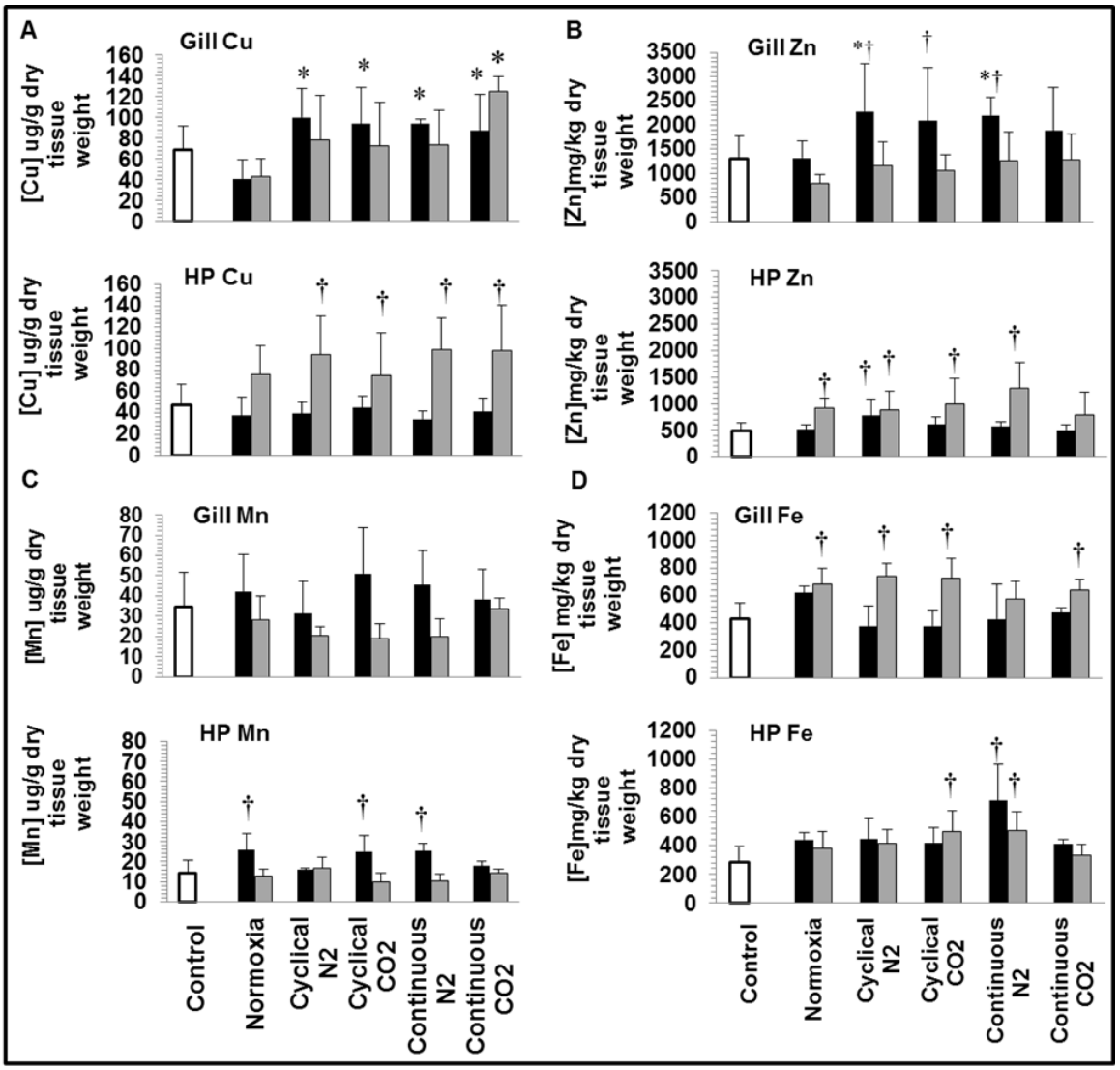


Figure 3.1.: Metal concentrations in oysters exposed to moderately contaminated sediments under normoxic and hypoxic conditions. Copper (A), Zinc (B), Manganese (C) and Iron (D) concentrations are presented for gill and hepatopancreas tissues. Black bars represent metal concentrations after four days of exposure; grey bars represent eight days of exposure. White bars represent control concentrations. Asterisks indicate significant differences from normoxia treatment and daggers show significant differences from Control (represents data from time zero and negative control oysters), n=6-12/treatment for each time point.

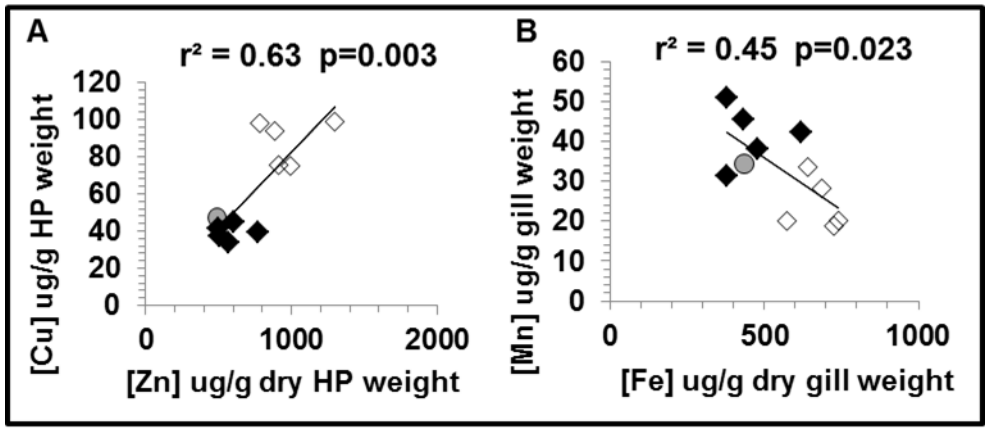


Figure 3.2.: Relationships between hepatopancreas (HP) Cu and Zn concentrations (A), and gill Mn and Fe (B) concentrations in oysters. Closed diamonds represent metal concentrations after four days of exposure and open diamonds represent eight days of exposure. Closed circle represents control levels in unexposed oysters.

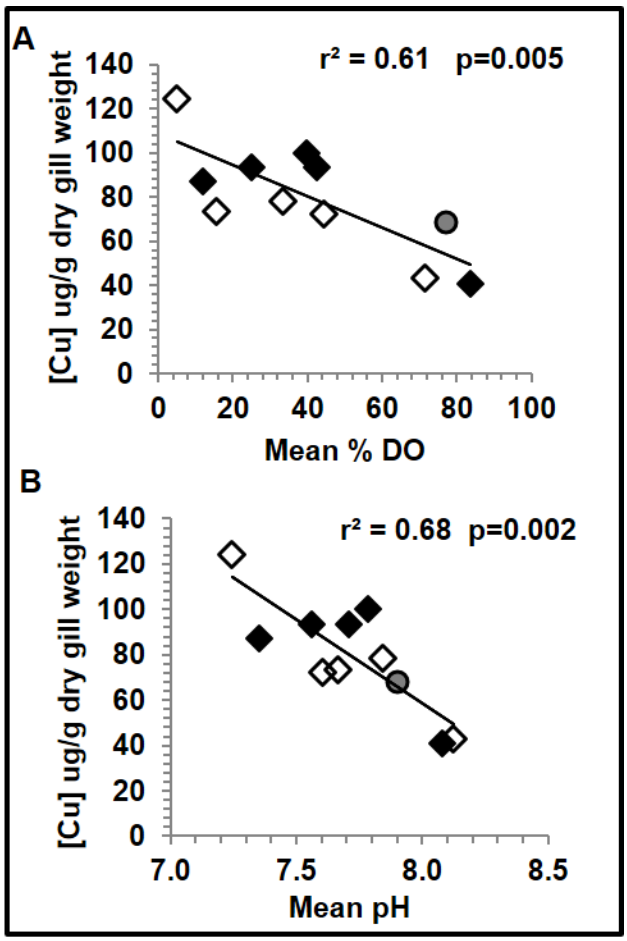


Figure 3.3.: Relationship between oyster gill Cu concentrations and (A) mean % DO and (B) mean pH. Closed diamonds represent copper concentrations after four days of exposure to contaminated sediments under different DO and pH levels and open diamonds represent eight days of exposure. Closed circle represents control levels in unexposed oysters (time zero and water-only treatments).

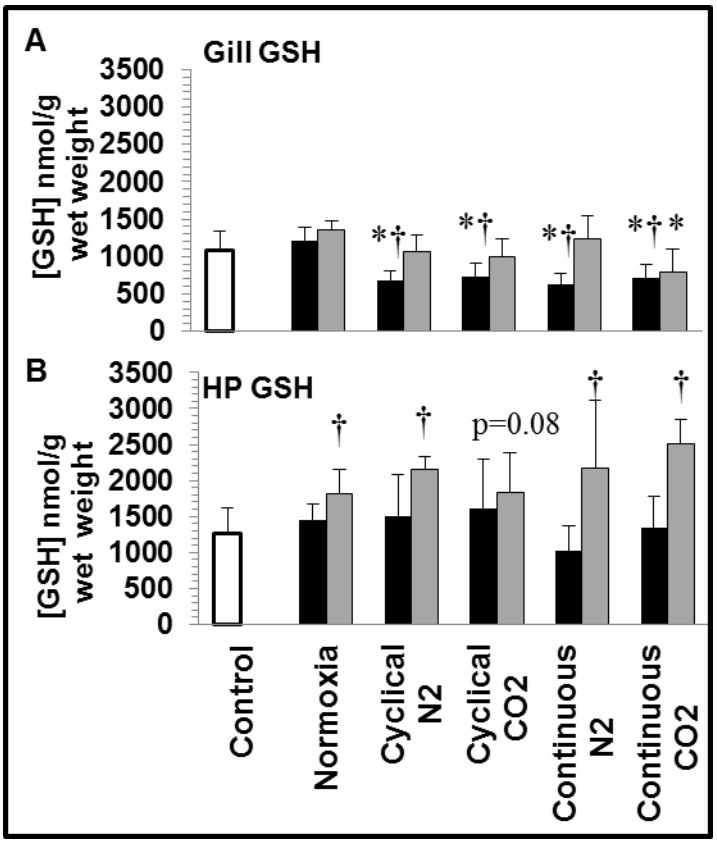


Figure 3.4.: Total glutathione (GSH) concentrations in oyster tissues, (A) hepatopancreas and (B) gills. Black bars represent GSH concentrations after four days of exposure; grey bars represent eight days of exposure and patterned bar represents control concentrations. Asterisks indicate significant differences from normoxia treatment and daggers indicate significant differences from Control (represents data from time zero and negative control oysters), n=6-12.

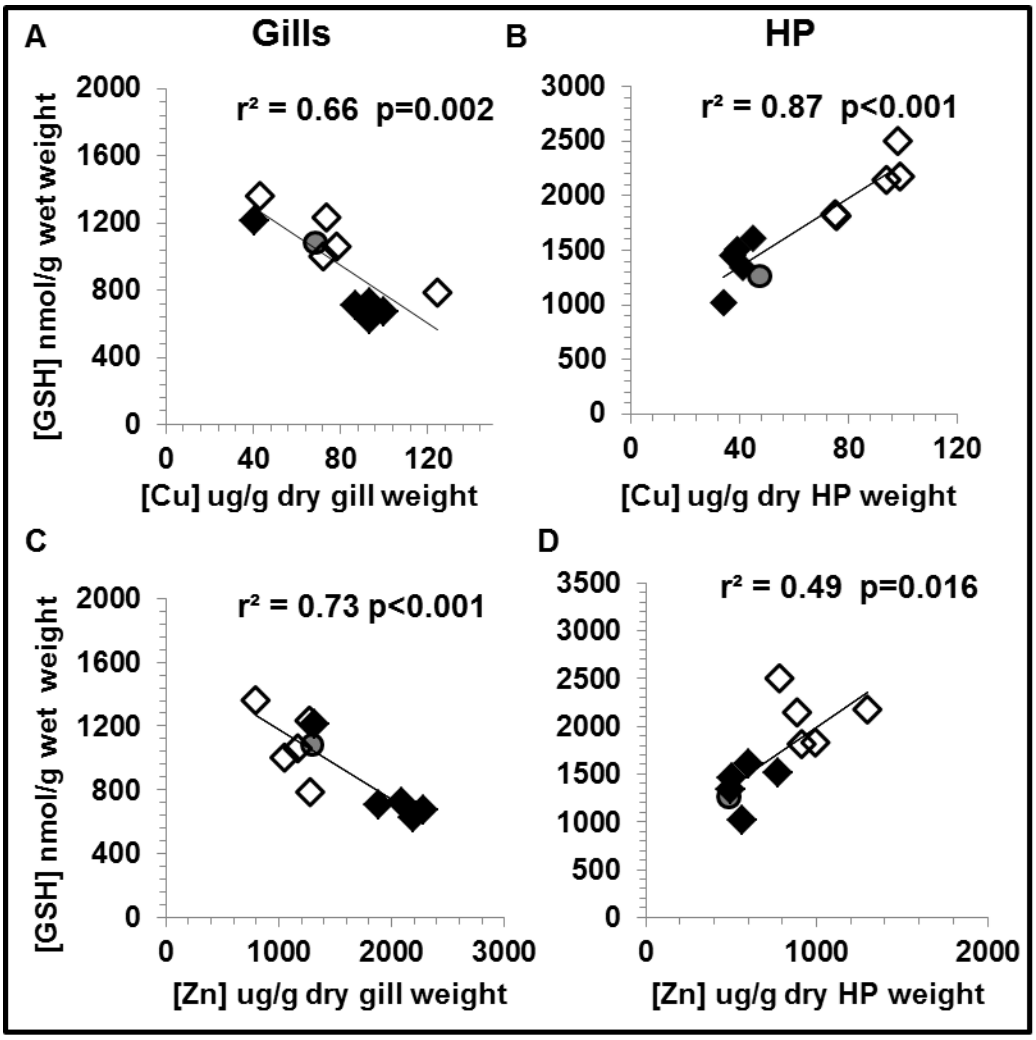


Figure 3.5.: Relationships between metal and GSH concentrations in oyster gills and HP tissues. Top panels show relationships between gill GSH and Cu concentrations (A), and HP GSH and Cu concentrations (B). Bottom panels show relationships between gill GSH and Zn concentrations (C), and HP GSH and Zn concentrations (D). Closed diamonds represent metal concentrations after four days of exposure and open diamonds represent eight days of exposure. Closed circle represents control levels in unexposed oysters.

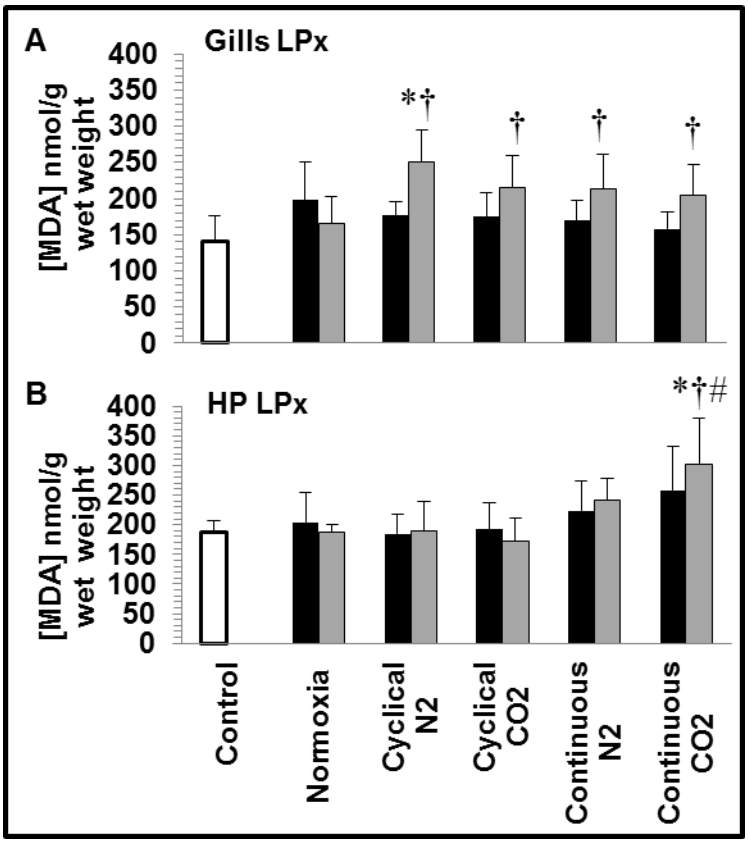


Figure 3.6.: Total MDA equivalents in oyster tissues, (A) hepatopancreas and (B) gills. Black bars represent MDA concentrations after four days of exposure; grey bars represent eight days of exposure and patterned bar represents Controls (represents data from time zero and negative control oysters), . Asterisks (*) indicate significant differences from normoxia treatment, daggers (†) indicate significant differences from control and number sign (#) indicate significant differences from all other hypoxic treatments; n=6-12.

Table 3.1.: Water quality parameters from laboratory four and eight day sediment exposures.

| | % DO Overall Mean | %DO Daily Range | pH Overall Mean | pH Daily Range | Overall Salinity Mean (ppt) | Overall Temperature Mean (°C) |
|---|---|---|---|---|---|---|
| **LABORATORY EXPOSURES** | | **Day4** | | | | |
| **Normoxia** | **83.6** | **70.6-87.2** | **8.1** | **8.0-8.2** | **28.8** | **19.2** |
| **Hypoxia Cyclical Hypercapnic (CO2)** | **39.9** | **2.8-80.6** | **7.6** | **6.8-8.0** | **29.3** | **19.7** |
| **Hypoxia Cyclical Nitrogen (N2)** | **39.9** | **12.3-83.0** | **7.8** | **7.6-7.9** | **28.9** | **19.9** |
| **Hypoxia Continuous Hypercapnic(CO2)** | **10.6** | **4.5-23.2** | **7.3** | **7.3-7.5** | **25.6** | **21.2** |
| **Hypoxia Continuous Nitrogen (N2)** | **24.7** | **10.3-44.9** | **7.7** | **7.4-7.9** | **27.5** | **20.4** |
| | | **Day 8** | | | | |
| **Normoxia** | **71.3** | **56.8-79** | **8.1** | **8.0-8.2** | **30.5** | **19.6** |
| **Hypoxia Cyclical Hypercapnic (CO2)** | **44.9** | **5.2-111.8** | **7.6** | **6.7-8.1** | **28.6** | **20.4** |
| **Hypoxia Cyclical Nitrogen (N2)** | **33.5** | **7.1-75.7** | **7.8** | **7.5-8.0** | **27.4** | **20.5** |
| **Hypoxia Continuous Hypercapnic(CO2)** | **4.4** | **0.7-19.7** | **7.3** | **7.0-7.5** | **27.7** | **21.2** |
| **Hypoxia Continuous Nitrogen (N2)** | **15.1** | **1.1-33** | **7.7** | **7.5-7.8** | **30.0** | **20.6** |

# CHAPTER 4: HYPOXIA AND ACCUMULATION PATTERNS OF MANGANESE AND COPPER FROM CONTAMINATED SEDIMENTS IN ATLANTIC RIBBED MARSH MUSSELS, *Geukensia demissa*

## 1. Abstract

Filter feeding bivalves such as oysters, *Crassostrea virgnica* and marsh mussels, *Geukensia demissa* are important bioindicator species and respond to changes in bioavailability of metals from contaminated sediments. Changes in the accumulation of metals in their tissues can be indicative of metal fluxes under varying dissolved oxygen (DO) and pH levels as well as contaminant levels. The overall goal of these studies was to evaluate the effects of hypoxia on accumulation of copper and manganese in marsh mussels. Gill and hepatopancreas tissues were harvested after eight days of exposure to moderately contaminated sediments under different hypoxic (low DO) conditions. Metal concentrations were measured to assess tissue-specific uptake of Cu and Mn. Mussels accumulated Mn under hypoxic conditions from contaminated sediments in gills; but no changes in gill or HP Cu levels were found. These unexpected results were in contrast with our previous studies with oysters that readily accumulated Cu and other metals under hypoxic conditions, which suggests selectivity at the level of uptake related to physiological differences between these two co-occurring bivalves. While the most dramatic changes were observed in gill tissues, high Mn levels in HP tissues were measured in continuous hypercapnic hypoxic conditions. Gills as well as HP Mn levels were negatively related to DO and pH, suggesting that hypoxia and hypercapnia increase

Mn bioavailability. We suggest that Mn accumulation in *G. demissa* can be used as an indicator of hypoxia, and may also be used to characterize the potential for overall increased metal bioavailability to other resident organisms, including oysters. These studies with an important but poorly studied estuarine species highlight the value of multiple bioindicators. Understanding significant species-specific differences is important for characterizing relative sensitivities to environmental stressors, and may also provide diagnostic capacity for habitat assessments.

## 2. Introduction

Estuarine sediments are a dynamic pool of nutrients and contaminants, and serve as a source as well as a sink for metals entering coastal ecosystems. Elevated metal concentrations in sediments can have adverse effects on coastal wildlife, especially to benthic communities. Metal contamination can also be biomagnified through trophic transfer. Metal partitioning and speciation in estuaries is dependent on chemical and physical characteristics of overlying water and bioturbation (Tessier and Campbell 1987, Rainbow 1997, Namieśnik and Rabajczyk 2010). Metal bioavailability is critical in determining the toxicity of contaminated sediments. Although, metals tend to associate with sediments, transient changes in water quality parameters such as diurnal changes in dissolved oxygen (DO) and pH, as well as rain events can affect metal speciation and fluxes in bottom water. Benthic filter feeding bivalves, such as marsh mussels, readily accumulate bioavailable metal ions from the water and metal-associated particulates from resuspension and bioturbation activities. Risks associated with moderately contaminated sediments may be underestimated if water chemistry is not evaluated. DO and pH often cycle diurnally in estuaries; during the day DO and pH are high, followed by a decline at

night due to imbalances between photosynthetic and respiratory processes. These diel changes can transiently affect bioavailability of metals and pose a risk to mussel populations and other estuarine organisms, which can have far reaching impacts on ecosystem integrity.

Manganese is an essential trace metal and an emerging contaminant which is introduced into coastal ecosystems via wastewater discharges, sewage sludge, and mining processes (Gibson et al. 2006, Pinsino et al. 2012). It occurs as Mn (II) and Mn(IV) and the transition between these oxidation states is largely characterized by redox potential and pH. The most bioavailable form of Mn in aquatic habitats is ionic $Mn^{2+}$. Adverse effects of high manganese levels include neurotoxicity, mortality, discoloration of gills and larval abnormalities in aquatic organisms (Baden et al. 1990, Martin et al. 2008, Pinsino et al. 2010). The overall goal of our studies was to assess tissue metal uptake from contaminated sediments under different hypoxic conditions in Atlantic ribbed mussels (also called marsh mussels), *Geukensia demissa*. We found that under hypoxic conditions, mussels readily accumulate Mn in their tissues and this accumulation is significantly related to DO and pH in the overlying water.

## 3. Methods and Materials

### 3.1. Laboratory Exposures

Mussels collected from a clean, reference site in Bogue Sound, NC were acclimated in the laboratory for 7-10 days and were transferred to buckets with pre-conditioned sediment – water mix. Sediments were collected from Calico Creek, NC which is a moderately contaminated tidal creek, and press-sieved through a 2mm sieve. Sediment Mn and Cu concentrations were 80.6± 25.9 and 116.3 ± 50.5 ug/g dry weight,

respectively (measured using furnace atomic absorption spectrophotometry, Perkin Elmer AAnalyst800). Natural, low organic beach seawater (0.45µ filtered) was added to artificial seawater (MBL formula) (Cavanaugh 1975) in 1:1 ratio, and salinity was adjusted between 27 and 30 psu. Exposure buckets were allowed to equilibrate with 1.5 L sediments per 15 L seawater under different hypoxic conditions for 24 hours before the addition of organisms. Mini-Sonde 4a dataloggers (Hach-Hydrolab) were used to record DO, pH, temperature, and salinity in the exposure buckets every 30 minutes throughout the exposures; standards for pH, DO, salinity and temperature were used to calibrate the instruments and before and after the exposures to ensure data quality. Normoxic treatments (mean DO >70%, mean pH >8.0) treatments were maintained with and without sediments as positive and negative controls, respectively. Continuous and cyclical hypoxia treatments were maintained with and without a concomitant decrease in pH. Continuous and cyclical hypoxia hypercapnia exposures were maintained using 5%$CO_2$ -95% air mix, which represents our environmentally relevant low pH exposures. Continuous and cyclical hypoxia exposures were also induced using Nitrogen gas (UHP-$N_2$), which didn't cause a decline in pH, hence representing our high pH exposures during hypoxia. Exposures were conducted for eight days and there was a partial water and sediment change at day four. All exposures were performed at an average temperature of 20.3$^{o}$C and mussels were fed *Isochrysis galbana* daily.

3.2. Analytical Procedures

Mussel gill and hepatopancreas (HP) tissues were removed and frozen at -80$^{o}$C at the end of exposures. Tissue Mn concentrations were determined by furnace atomic absorption spectrophotometry (AAS, Perkin Elmer AAnalyst 800 equipped with Zeeman

background correction). Tissues were lyophilized and acid digested (using trace metal grade Nitric acid and microwave digestion) for AAS analyses. Certified standards.were used for standard curves, and certified oyster tissue (NIST) as well as certified standards and blanks were used for QA/QC checks. Grain size analyses were also performed on sediments to obtain percent sand and silt-clay content (Ringwood et al. 1997).

3.3. Data Processing

One-way ANOVAs were used to compare different treatments, and student-Newman Keuls method was used to perform pairwise comparisons. All statistical analyses were conducted using Sigma Stat; normality and equal variance were confirmed. Water quality data were downloaded from the dataloggers and processed in Microsoft Excel.

4. Results

Manganese concentrations were significantly elevated after eight days of exposure to contaminated sediments in mussel gills under all hypoxic treatments (Figure 4.1A, $p < 0.01$). No increases in Mn gill concentrations were found in mussels exposed to contaminated sediments under normoxic conditions. No increases in gill Cu levels were seen in mussels exposed to contaminated sediments under hypoxia or normoxia (Figure 4.1B). Significant relationships with high correlation coefficients were also found between gill Mn concentrations and DO levels in overlying water (Figure 4.2A) and also between gill Mn and pH in overlying water ( Figure 4.2B). In hepatopancreas tissues, Mn concentrations were significantly higher after eight days of exposure under continuous hypoxic hypercapnic conditions (Figure 4.3A, $p<0.01$); no other hypoxia treatment showed elevated Mn levels in HP tissues. No increases were seen in HP Cu levels (Figure

4.3B). Mussel HP Mn concentrations were also significantly correlated to %DO (Figure 4.4A) and pH (Figure 4.4B) in overlying water.

## 5. Discussion

Atlantic ribbed marsh mussels play critical roles in maintaining ecosystem integrity in coastal habitats. They inhabit overlapping ecotones with oysters and the more commonly studied marine mussel, genus *Mytilus*, that is restricted to cool coastal climates. Marsh mussels, (*Geukensia demissa)*, like oysters, live in shallow estuarine habitats that extend over broad geographic ranges, from cold northeastern climates down to hot southeastern and Gulf coast areas of the US. They are typically found in intertidal habitats partially buried in marsh grass roots and sediments, and also in subtidal oyster reef habitats attached to oyster clusters by byssal threads. Marsh mussels, like other filter feeders, help keep the water clean and turbidity low, and play critical roles in benthic pelagic coupling processes. Oysters and mussels of the genus *Mytilus* are well-established bioindicators of habitat quality and their tissue metal concentrations have been used to assess the risks of metal contamination to ecosystem health (Phillips 1978, Regoli and Orlando 1993, Boening 1999, Chandurvelan et al. 2015). However, very few studies have been conducted with marsh mussels even though they have many attributes that could make them very valuable as bioindicators as well as biomodulators of ecosystem health.

Limited information on Mn concentrations, bioavailability, and toxicity exists for estuarine sediments. Our studies indicate significant increases in bioavailability of Mn to marsh mussels under hypoxic conditions. Similar patterns of increased bioavailability of Mn during hypoxic events, especially in gill tissues, have been documented in other marine organisms such as Norway lobsters, *Nephros norvegicus* (Baden et al. 1990,

Baden et al. 1995, Riedel et al. 1999). Our studies also indicated that mussel gills are a primary target tissue of Mn deposition over short term hypoxia exposures, which further suggest gills as a primary biotic ligand for bioavailable Mn ions. The Mn concentrations of gills were elevated under all hypoxia treatments, and the regression analyses indicated significant inverse relationships between DO and pH and tissue Mn levels; these analyses also suggested that DO was a slightly stronger driver than pH. A significant increase in Mn accumulation was observed in hepatopancreas tissues, but only in the most severe hypoxia treatment (continuous hypercapnic hypoxia) after 8 days suggesting that hypoxia and low pH together increase Mn bioavailability more than hypoxia alone in hepatopancreas tissues. Mn accumulation in HP tissues may also reflect more chronic responses to longer-term exposures that may eventually be observed for cyclical conditions. The regression analyses did indicate significant inverse relationships between DO and pH and hepatopancreas Mn levels, but pH was a stronger driver than DO in these tissues.

Geochemical studies also indicate increased release of Mn to overlying water during hypoxia (Riedel et al. 1999, Namieśnik and Rabajczyk 2010) . The two most common oxidative states of Mn in coastal waters are $Mn^{2+}$ and $Mn^{4+}$, representing reduced and oxidized Mn, respectively. Under normoxic conditions, insoluble Mn oxides precipitate out of the water column. However, under reduced conditions, free ionic $Mn^{2+}$ is remobilized into the water column as hydrated oxides are reduced, and becomes bioavailable to filter feeding bivalves such as marsh mussels. Our continuous and cyclical hypoxia exposures led to anaerobic conditions which facilitated Mn flux and uptake by marsh mussels. It should also be noted that the sediments used for these studies are

regarded as moderately contaminated and therefore these results highlight the risks associated with low metal burdens when combined with another environmental stress. Since no accumulation was reported in mussels exposed to contaminated sediments under normoxia, we conclude that hypoxia increases the bioavailability of Mn by potentially reducing Mn oxides. We also showed a significant increase in total Mn concentrations in HP tissues in mussels exposed to contaminated sediments under continuous hypoxia hypercapnia, which may be due in part by ingestion of Mn either adsorbed or complexed with organic particles and phytoplankton. Metal speciation and bioavailability in water has been widely studied and sediment quality guidelines have been established based on equilibrium partitioning and toxicity assays (Di Toro 1989, Tessier and Turner 1995, Ankley et al. 1996, Long et al. 1998, Birch and Hogg 2011). However laboratory toxicity assays are conducted under aerated, normoxic conditions, and equilibrium models don't always account for the potential impacts of hypoxia. There are frequently gaps between sediment chemistry based predictions and observed toxicity which limit the reliability of predictions regarding potential biological effects using models that do not integrate other major environmental factors such as hypoxia (O'Connor and Paul 2000).

Unexpectedly, Cu levels didn't show any increases in gills or HP tissues of mussels exposed to contaminated sediments under hypoxia or normoxia conditions. Overall, Cu levels were relatively low in mussels tissues, especially in the gills. Copper is a dominant contaminant in Calico Creek sediments, so the absence of Cu accumulation in mussels was very surprising. These results with both Mn and Cu in marsh mussels were in stark contrast to results with oysters, in which there were no increases in Mn levels but significant increases in Cu in oysters exposed to comparable experimental conditions

(Khan and Ringwood, In prep for ET&C), which suggest selectivity by marsh mussels for Mn uptake during hypoxia. Species-specific differences in metal accumulation may be explained based on physiological and cellular differences such as filtration rates, gill ultrastructure, cellular metal transporters and efflux pathways, etc. Marsh mussels (*G. demissa*) have some unique anatomical and biochemical adaptations to marsh and estuarine habitats. They retain very small bacterioplankton much more readily than oysters or *Mytilus* species because of structural differences in their gill laterofrontal cirri (Riisgard 1988). They can live in sulfidic marsh environments and often have high concentrations of sulfide-rich granules that probably serve to minimize toxic sulfide interactions, and also serve as sulfide storage compartments. They and/or the bacteria are also capable of using sulfides as an energy source by coupling sulfide oxidation to ATP production (Grieshaber and Völkel 1998, Parrino et al. 2000). Mn can occur as a sulfide (MnS) so the Mn sulfide complexes that form during hypoxic conditions may be readily accumulated by marsh mussels, or the high levels of sulfides in the mussel tissues may facilitate the accumulation of Mn ions as they flux out during hypoxia. Moreover, gill mitochondria serve as sulfide sinks during sulfide oxidation (Grieshaber and Völkel 1998), so Mn could serve to reduce toxic levels of circulating sulfides under hypoxic conditions, or could minimize toxicity through the increased activity of mitochondrial SOD (superoxide dismutase) which requires Mn as a co-factor to minimize oxidative stress. Overall, the increased accumulation of Mn in marsh mussels could represent a relationship between sulfide tolerance and regulation that is linked to Mn bioavailability and complexation, especially during hypoxic conditions as well as in sulfide rich environments.

These types of species-specific differences could be developed as important diagnostic indices that can be used for environmental assessments, biomonitoring, and habitat quality guidelines. Previous works from our laboratory and others have documented the importance of eastern oysters as bioindicators of contaminant exposures and effects, and their strong tendencies to accumulate Cu from contaminated sediments in gill and HP tissues. Our recent short term exposures with these same moderately contaminated sediments also indicate that eastern oysters are valuable bioindicators of Cu bioavailability. Marsh mussels on the other hand did not readily accumulate Cu, but the elevated Mn levels that we observed were linked to hypoxia, so Mn accumulation in this species may serve as a valuable indicator of environmentally relevant hypoxia stress. An important question that is often posed is – is organismal health compromised due to contaminant stress, hypoxia stress, or both? Our studies suggest that investigations with both oysters and marsh mussels, which do co-occur or could readily be caged together, could be used to consider the relative contributions of different types of environmental stressors.

In summary, we present here important new information regarding the responses of marsh mussels, *Geukensia demissa*, to moderately contaminated sediments under hypoxic (both continuous and cyclical) and normoxic conditions. Marsh mussels demonstrated a strong tendency for enhanced Mn accumulation, especially in gill tissues, under hypoxic conditions, but were resistant to Cu accumulation. These studies with marsh mussels in combination with our recent studies with oysters emphasize the effects of hypoxia on metal bioavailability from contaminated sediments and important species-specific and tissue-specific differences that can be used as valuable diagnostic indicators

of hypoxia and contaminant stress. Elevated global temperatures and anthropogenic inputs of metals contribute to depletion of oxygen from aquatic habitats and increased metal contaminants in estuaries and coastal habitats. Expanding hypoxic zones in coastal areas worldwide can therefore exacerbate metal bioavailability and toxicity of even moderately metal-contaminated sediments. Our studies highlight the potential diagnostic value of multiple bioindicator species for habitat assessments.

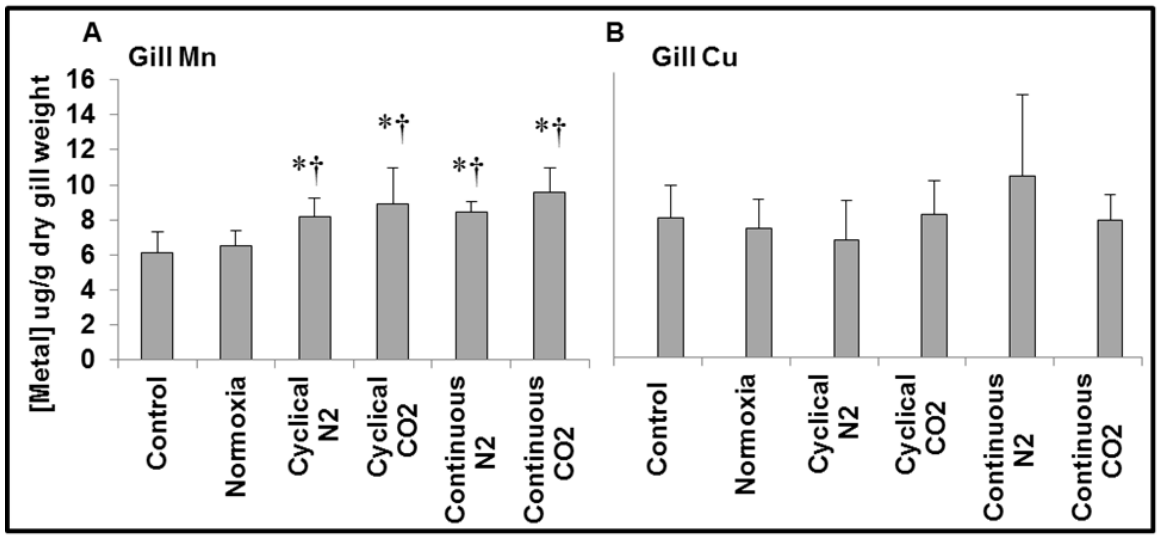


Figure 4.1.: Gill Mn (A) and Cu (B) concentrations in mussels exposed to contaminated sediments under normoxic and hypoxic conditions for eight days. Bars represent means plus standard deviations. Asterisks (*) indicate significant differences from normoxia treatment and daggers (†) indicate significant differences from control. Control represents data from time zero and negative control mussels, n=5-12.

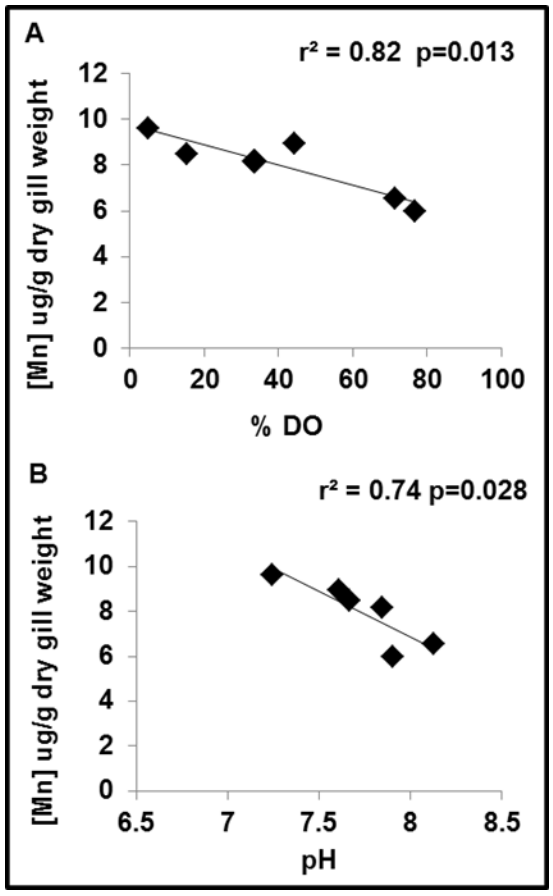


Figure 4.2.: Relationships between mussel gill Mn concentrations and mean DO % (A) and mean pH (B). Each point represents a mean of 5-12 mussels.

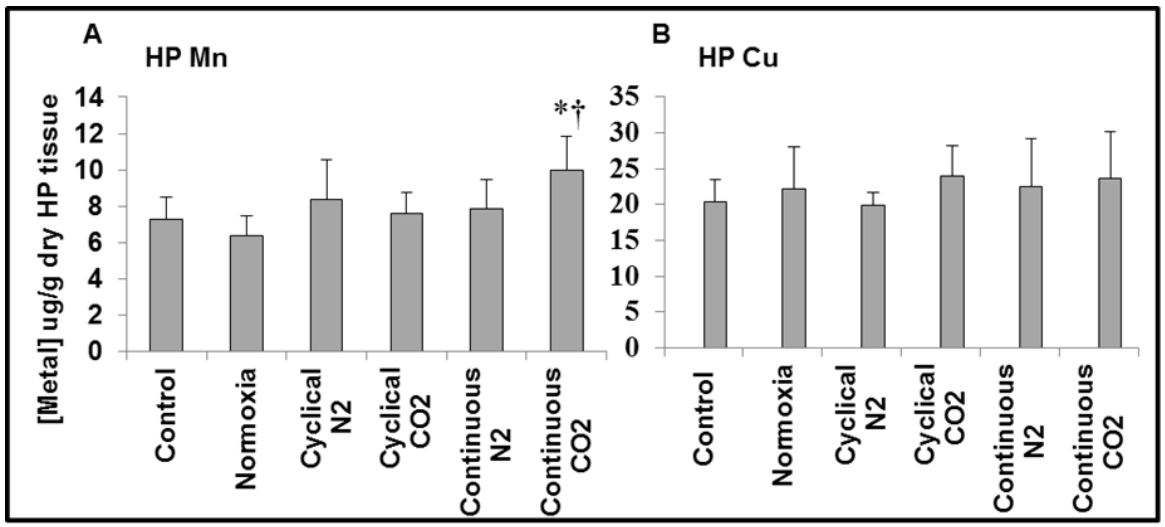


Figure 4.3.: The concentrations of Mn (A) and Cu (B) in hepatopancreas (HP) tissues of mussels exposed to contaminated sediments under normoxic and hypoxic conditions for 8 days. Bars represent means plus standard deviations. Asterisks (*) indicate significant differences from normoxia treatment, and daggers indicate (†) significant differences from control. Control represents data from time zero and negative control mussels, n=5-12.

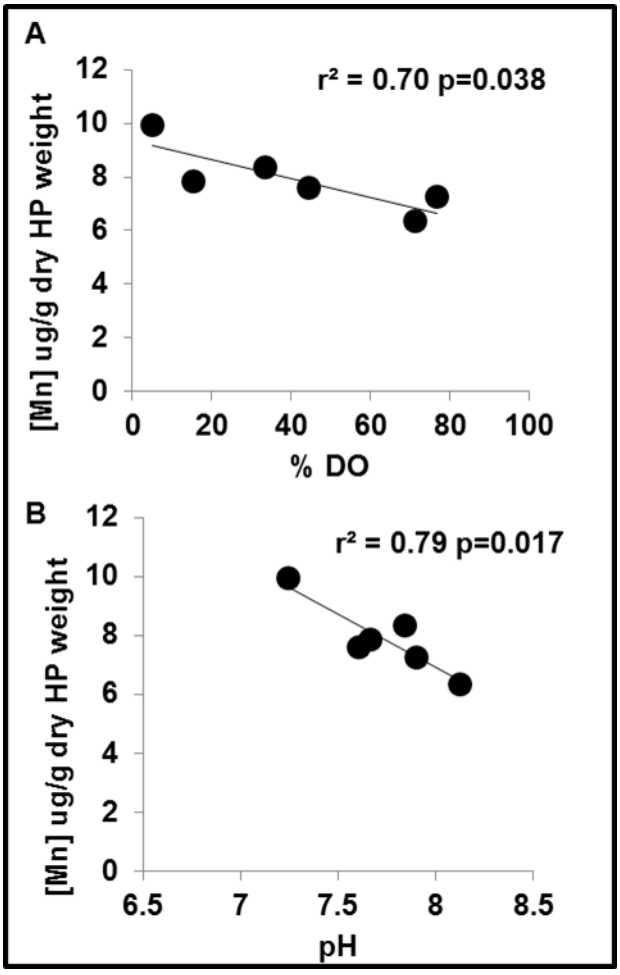


Figure 4.4.: Relationships between mussel HP Mn concentrations and mean % DO (A) and mean pH (B). Each point represents 5-12 mussels.

# CHAPTER 5: MICROBIOME DIVERSITY IN OYSTERS – POTENTIAL IMPACTS OF HYPOXIA AND A WARMING OCEAN

## 1. Abstract

Rising global temperatures and increases in hypoxic zones due to elevated water temperatures and eutrophication in estuarine ecosystems may have far reaching implications for sustainability of coastal wildlife populations. One potential impact may be changes in diversity of their constituent microbes (microbiome). We report here on changes in oyster microbiomes related to dissolved oxygen stress. The microbial composition of hepatopancreas tissues of oysters (*Crassostrea virginica*) exposed to hypoxic conditions from both laboratory and field studies was evaluated using genomic analyses as well as standard culture methods. As expected, there was evidence of increases in microbial abundance, especially in facultative anaerobes, and multivariate, principle component analyses indicated distinct bacterial subgroups related to hypoxia exposures. But in contrast to our expectations, both field and laboratory data indicated that hypoxia stress was associated with a significant increase in oyster microbiome diversity. Our studies support the hypothesis that unstressed oysters sustain a relatively small resident microbiome that can be perturbed by hypoxia. Therefore changes in oyster microbiome could interfere with nutritional processes and impact fitness as well as increase the incidence of pathogenic microbes, posing increased risks to both wildlife and human consumers of oysters.

## 2. Introduction

Hypoxia is a pervasive and growing problem in coastal ecosystems that is fueled by eutrophication and pollutant inputs (Diaz 2001, Diaz and Rosenberg 2008, Rabalais et al. 2009, Rabalais et al. 2010). Microbial communities are both modulators and targets of hypoxia dynamics, and represent multiple response levels due to their important roles as part of the organismal microbiome as well as of the environmental compartments (water and sediment). Hypoxia in coastal and estuarine ecosystems can present as sustained low dissolved oxygen (DO), but in many habitats, the regimes are characterized by diurnal DO cycles of low night-time DO and high day-time DO that are driven by photosynthetic and respiratory processes. Especially during warm periods, daytime photosynthesis by algae and phytoplankton produces high levels of oxygen that are depleted by animal and microbial respiration during the night. These DO cycles are closely tracked by pH, so as the DO decreases, the increased $PCO_2$ results in reduced pH (Burnett 1997). Therefore, these parameters function as major covariates, so as temperatures increase, both DO and pH decrease (Keeling et al. 2010, Cai et al. 2011, Gobler et al. 2014). Significant outcomes can be economically as well as ecologically catastrophic, especially when manifested as fish kills and dramatic community impacts.

Changes in water quality parameters such as temperature, salinity, and DO are associated with changes in microbial communities and increases in pathogenic bacteria in estuarine systems (Blackwell and Oliver 2008, Froelich and Oliver 2013). Therefore global increases in temperature are likely to exacerbate hypoxia issues and microbial dynamics. These interactions could contribute to compromised organismal health if there are significant shifts in the resident microbiome, as well as increases in pathogenic

species and diseases. Therefore understanding interactions between hypoxia and organismal microbiomes is essential for characterizing potential impacts of hypoxia on fitness, and predicting increases in disease-causing bacteria in seafood consumed by humans and wildlife.

The goals of this research were to evaluate the effects of hypoxia on bacterial abundance and diversity in the hepatopancreas tissues (also known as the digestive gland) of eastern oysters, *Crassostrea virginica*. These tissues play significant roles in nutrient digestion and absorption, and typically have the highest bacterial levels compared to other tissues (Kueh and Chan 1985, Froelich et al. 2010). We hypothesize that exposure to hypoxic conditions could cause significant perturbation of the oyster hepatopancreas microbiome, potentially contributing to significant chronic stress and reduced fitness of oysters as well as increasing the incidence of pathogenic bacteria. Filter-feeding estuarine bivalves, like oysters, are ecologically and commercially important, so impacts on oyster populations and increases in oyster pathogens can translate into cascade effects for consumers and other trophic levels (Daniels 2011, Oliver 2013). We present here the results of laboratory and field studies conducted over multiple years based on a combination of culture and molecular genomic techniques that were designed to test these hypotheses.

## 3. Materials and Methods

### 3.1. Animal Collection and Hypoxia Exposures

Field studies were conducted with oysters collected from multiple sites in Bogue Sound and Pamlico Sound of North Carolina (NC) during the summer months of 2010 and 2011 when hypoxic conditions are most common. Field sites included NC

Department of Environment and Natural Resources (NCDENR) sanctuary sites (West Bluff, Ocracoke, Clam Shoal, and Hatteras), and sites in the NC Coastal Federation, Hoop Pole Creek Reserve, as well as at Calico Creek, a suburban tidal creek (Figure 5.1). Oyster collections and field studies were conducted under NC State Division of Marine Fisheries scientific collection permit and the permission of the NC Coastal Federation.

For the field studies, *in situ* dataloggers (Hach Hydrolab Minisondes) were deployed for 5 – 7 days near oyster beds, and water quality parameters (temperature, salinity, pH, and DO) were recorded every 30 minutes (calibrations, pre-deployment and post-deployment checks and other quality assurance measures were used to ensure the reliability of the water quality data). Oysters were collected at the end of the datalogger deployment period to ensure that the oyster responses were coupled to the environmental conditions, kept cool (not on ice) in site water during transport to the laboratory, and processed as described under the "tissue analyses" section.

Laboratory exposure studies were designed to mimic field conditions. Oysters were collected from a reference, normoxic area in Bogue Sound, NC, held under well-aerated conditions in the laboratory for 5-7 days, and then exposed to the following treatments for 4-8 days: normoxia, diurnal cycles of hypoxia, or prolonged continuous hypoxia. The results reported here are based on three separate experiments conducted from 2010 – 2011. To consider the role of pH on responses to hypoxia, two methods were used for inducing hypoxic conditions: carbon dioxide (5% CO2 -95% air mix) was used to induce concurrent hypoxia and reduced pH, a condition known as hypercapnia that is commonly observed under real-world conditions and nitrogen gas (Ultra High Purity-$N_2$) was used to induce hypoxia with no pH reduction. Exposures were conducted in 20-L

polypropylene buckets (0.45µm filtered seawater, 27‰ salinity, 22ºC) with the in situ dataloggers recording temperature, salinity, DO, pH every 30 minutes over the full course of the experiments. Oysters were fed cultured phytoplankton (*Isochrysis galbana* and *Skeletonema costatum*) daily. Examples of the datalogger data for one of the field sites and two laboratory experiments characterized by cyclical DO patterns are provided in Figure 5.2 and detailed summaries are recorded in Table 5.1.

3.2. Tissue Analyses

Oysters from field and laboratory studies were dissected using sterile techniques (rinsed externally with water and alcohol, dissected with sterile instruments, and tissues rinsed with autoclaved artificial seawater). Subsamples of hepatopancreas tissues from individual oysters were weighed and homogenized in sterile artificial seawater, and 1/10th and 1/100th dilutions of the homogenate were plated on estuarine agar (E. agar) and on colistin-polymyxin B-cellobiose (CPC+) media. E. agar plate counts represent total bacteria in the sample and CPC+ serves as a selective media for *Vibrio vulnificus* (Oliver 2012). Plates were incubated at 37ºC and colony forming units (CFUs) were counted after incubation for 24 hours. The data were expressed as CFUs /g tissue wet weight.

Subsamples of the homogenates were also processed using ARISA (automated ribosomal intergenic spacer analysis) to characterize oyster hepatopancreas microbiomes. ARISA is a genomic approach based on the length of the intergenic spacer (ITS) region between the highly conserved 16S and 23S rRNA genes of the bacterial genome and has been successfully used to examine microbial diversity in soil and aquatic communities (Fisher and Triplett 1999, Brown et al. 2005, Danovaro et al. 2006). The ITS regions

have unique lengths for different bacterial species, and can be used for identification of taxa and estimation of diversity based on operational taxonomic units (OTUs) (Ranjard et al. 2001, Green et al. 2004). DNA was extracted from oyster hepatopancreas using the MoBio PowerSoil®DNA isolation kit according to manufacturer's instructions, and 1406F-FAM and 125R primers were used to amplify the ITS. ITSs were PCR amplified and, separated based on size by capillary electrophoresis with an Applied Biosystems 3130 Genetic Analyzer. Electropherograms were generated with a series of peaks, each of which represented an OTU. Two technical replicates were processed from each tissue sample and a peak was only identified if fluorescence was recorded in both replicates at readings of >100 relative fluorescence units (RFUs). ARISA data were binned by 2 base pairs and processed in Microsoft Excel using sequences between 250 and 900 bp for downstream analyses. The recurrent peaks for each sample were identified using PeakStudio software (McCafferty et al. 2012), and binned data were converted to a binary format and used for principal component analyses (PCA) and to estimate taxa richness. It is recognized that bacteria can have multiple copies of 16s and 23s genes which may be of different lengths for a particular species of bacterium, so distinct peaks reflect taxa, but may not be distinct species. Therefore, OTUs provide relative estimates of the number of taxa in a sample but not necessarily an exact number of distinct species. The changes in number of OTUs are used as a measure of comparing microbial diversity in this study.

### 3.3. Statistical Analyses

Sigma Stat 3.1 was used for ANOVA analyses and post-hoc pairwise analyses to identify significant differences between treatments, and for regression analyses regarding water

quality parameters and taxa richness. PCA analyses were conducted using JMP Pro 10 (SAS).

4. Results

Overall, microbial abundance and number of facultative anaerobes increased in oysters exposed to hypoxia. Unexpectedly, both field and laboratory data indicated that hypoxic stress was associated with a significant increase in the microbiome diversity in oysters.

The field water quality data reflect nutrient inputs and eutrophication in Calico Creek and Hoop Pole Creek, as Calico Creek is downstream from a sewage treatment plant and Hoop Pole Creek receives inputs from a storm-drain, but both are in relatively low-density human population areas and receive no industrial inputs. These sites experience day-time high and night-time low DO levels and just like DO, pH also follows a cyclical pattern (Table 5.1). Figure 5.1 shows the location of the field sites on the NC coast. The laboratory datalogger data show that under normoxic conditions (where DO concentrations and pH remain elevated), and under continuous hypoxic conditions (where DO saturation remains low), there is little diurnal variation (Table 5.1) unlike the cyclical hypoxic conditions. The examples in Figures 5.2A and 5.2B illustrate the tight coupling between DO and pH conditions under hypercapnic (high $CO_2$) conditions. In contrast, Figure 5.2C shows that the use of $N_2$ gas to induce hypoxia in laboratory experiments is not accompanied by concurrent decreases in pH. More comprehensive summaries for all laboratory treatments and field sites are provided in Table 5.1 illustrating the broad range of hypoxic conditions used for these studies and our success in simulating the diurnal variation of field conditions.

Regardless of the method used for inducing hypoxia in the laboratory ($CO_2$ or $N_2$), the continuous hypoxia treatments resulted in increased abundance in both total bacteria and in *Vibrio vulnificus*-like species (Figure 5.3). Moreover, bacterial abundance and richness patterns were established by four days of exposure and remained the same at eight days. Therefore these figures represent data that were pooled for both time periods and DO treatments. Note that the plots are based on log10 values, so there were significant increases, especially in the *Vibrio vulnificus*–like bacteria which exhibited an approximately eight-fold increase compared to a five-fold increase in total bacteria.

Based on the number of OTUs identified using ARISA, taxa richness also increased in oysters exposed to continuous hypoxia (Figure 5.4A). While there were slight increases in *Vibrio* abundances and OTUs with the cyclical hypoxia treatments, these were not significantly different from the controls.

The PCA analyses also indicated community differences between the treatments (Figure 5.5A). Remarkably, even though these studies were conducted over multiple years, the microbial communities in the control, normoxic oysters were very similar. It is difficult to visualize the control oyster responses in Figure 5.5A, as many of the data stack directly on top of each other (n=20). Consistent with the data in Figure 5.4A, the oyster microbiomes from the continuous hypoxia treatments also clustered as a distinct sub-group. The cyclical hypoxia treatments did form a distinct cluster, but there was considerable overlap with control oysters, and some overlap with the continuous hypoxia treatment. Therefore, data based on both traditional culture techniques and molecular analyses indicated significant changes in the oyster hepatopancreas microbiome when

exposed to continuous hypoxia, and some evidence of perturbation in oysters microbiomes exposed to cyclical hypoxia for these short durations.

Most of the field sites from Pamlico Sound are well-oxygenated, and the oysters consistently had low microbial diversity. Overall, sites with the lowest DOs tended to have higher number of OTUs, and Calico Creek and Hoop Pole Creek-1 were significantly greater than all other sites (Figure 5.4B), with two to three times greater richness. Based on the PCA analysis, the oyster microbial community in Hoop Pole Creek-1 (Hoop Outfall) and Calico Creek were very distinct from Hoop Pole Creek-2 (Hoop Pole) and all other sites (Figure 5.5B), consistent with our current and longer term data that these are the two most DO stressed sites. While both Hoop Pole Creek sites sometimes have similar overall mean DO regimes, the cycles at Hoop Pole Creek-1 are always more dramatic, frequently cycling between anoxia and supersaturation (Table 5.1). In contrast, all of the Pamlico Sound sites clustered very tightly together, suggesting similar microbial communities in the oysters collected from these sites (Figure 5.5B). Overall, the multivariate analyses indicated similar microbiome patterns within a site, with clusters related to DO conditions. Both field and laboratory data indicated greater variation in bacterial communities for oysters exposed to hypoxia compared to control and normoxic conditions. We also regard this increased variation as an important indicator of perturbation related to DO stress, whereas in control and normoxic oysters the microbiome patterns were characterized by reduced variation and convergence.

Finally, both field and laboratory data were used to conduct regression analyses between the DO conditions and microbial diversity, as indicated by the OTUs, and the results indicated a highly significant relationship ($p < 0.001$) (Figure 5.6). Regressions

based on average daily minima were also significant (p=0.002), but there were no significant relationships with pH. The laboratory controls and the well-oxygenated field sites were closely aligned on the regression. While none of the field sites used in these studies was characterized by continuous hypoxia, some were characterized by diurnal cycles similar to those used for the laboratory cyclical treatments. Together these field and laboratory data reveal a consistent trend of increased microbial diversity in oysters exposed to hypoxic conditions.

## 5. Discussion

While the general paradigm for macrofauna may typically be that reduced biodiversity is expected under stressful conditions (Rapport et al. 1985, Ritter and Montagna 1999, Jackson et al. 2001, Vaquer-Sunyer and Duarte 2008), our comprehensive studies based on both laboratory and field analyses indicate that diversity as well as abundance increased for bacterial populations of the oyster hepatopancreas tissues when exposed to dissolved oxygen stress. While the field sites used in our studies would be classified as either normoxic or cyclical, there are significant regions - especially warm, eutrophic estuaries that exhibit the Gulf "Dead Zone" conditions. At such sites oyster reef habitats can be exposed to continuous hypoxia, simulated by our laboratory studies, such that DO levels are rarely above 20-30% for sustained periods of time (Bishop et al. 2006, Diaz and Rosenberg 2008, Rabalais et al. 2009). Continuous as well as cyclical hypoxia will only be exacerbated by global warming trends as higher temperatures further reduce oxygen saturation potential.

We can only speculate at this point regarding the implications of these findings on oyster populations. Using both culture and molecular genomic approaches, numerous

studies have characterized increases in overall bacteria as well as pathogenic bacteria, and have reported bacterial diseases in oysters including cardiac edema, bacillary necrosis, nocardiosis, hinge ligament disease, and juvenile oyster disease as a result of exposure to *Vibrio* and other bacteria (Tubiash et al. 1970, Tubiash et al. 1973, Dungan et al. 1989, Friedman et al. 1998, Boettcher et al. 1999, Paillard et al. 2004). Our studies provide valuable new qualitative and quantitative data that indicate increases in microbial diversity in oysters exposed to hypoxic stress. Studies regarding oyster microbiomes are emerging but their composition and functional roles are not well understood. Recent studies aimed at characterizing oyster microbiomes have indicated dominance by some taxonomic groups that may have functional importance for oyster physiology (King et al. 2012, Lokmer and Wegner 2014). Deep-sequencing techniques are revealing exciting new information about specific taxa and lay the groundwork for considering the effects of environmental factors (King et al. 2012), but the very nature of detailed sequencing typically limits these analyses to small sample sizes. So while the techniques applied in the ARISA studies reported here are less comprehensive with regard to OTUs, they do provide valuable insights regarding relative changes in overall patterns of taxa to hypoxic conditions, and represent analyses based on a total of approximately 150 oysters from both field and laboratory studies. Our sample sizes enabled robust PCA analyses, indicating significant site and treatment patterns related to DO conditions. Our studies support the hypothesis that unstressed oysters may sustain a relatively small resident microbiome that can be perturbed by an environmental stressor, such as hypoxia. A resident oyster microbiome could provide significant nutritional value, so that under DO stress, increases in other species of bacteria would interfere with nutritional processes and

would impact fitness. It is also possible that the increased bacterial loads reflect impaired immune function in the oysters. Other investigators have shown that hemocytes from oysters exposed to hypoxia have reduced phagocytic activity, so the increases in bacterial abundances in the hepatopancreas tissues could be due to reduced clearance capacity (Boyd and Burnett 1999, Holman et al. 2004, Macey et al. 2008). Our studies further suggest that reduced removal of non-microbiome species could contribute to increases in less favorable species. Numerous published studies as well as our ongoing studies have indicated cellular and physiological effects of hypoxic exposures on oysters and other bivalves (Hochachka et al. 1996, Burnett and Stickle 2001, Wu 2002, Patterson et al. 2014), but impacts on microbiomes may reflect early responses that set the stage for reduced adaptive capacity to environmental changes.

Our studies further indicate that for oyster microbes, oxygenation conditions are stronger drivers than pH. The laboratory studies conducted using $CO_2$ and $N_2$ gases enabled consideration of the role of reduced pH and hypercapnia as well as low DO. While the bacterial responses were linked to DO levels, it was somewhat surprising that pH did not play a more significant role. Generally, the extracellular pH of bivalves under normoxic conditions ranges from 7.4 to 7.5, but as pH declines, bivalves tend to be pH conformers, so changes in hemolymph pH track changes in seawater pH and can decrease to around 7 and below. Intracellular pH tends to be lower overall, closer to 7.0, and decreases only 0.1 – 0.2 pH units when exposed to hypoxia, so most tissues exhibit high buffering capacity (Eberlee and Storey 1984, JJ and Burnett 1996, Michaelidis et al. 2005, Michaelidis et al. 2005). There are numerous studies that document the pH sensitivity of bacteria (Kashket 1987, Krulwich et al. 2011, Chiang et al. 2012), but the

relevant ranges of pHs associated with differential sensitivity of bacteria span much lower values than those observed in these studies, indicating that the decreases in water and oyster tissue pHs were not sufficient to impact microbial responses.

Characterization of changes in oyster microbiomes, as in other species, is an exciting emerging field. As we evaluate data from different studies, it is important to be aware of oyster physiology and nutritional processing. Some studies may be based on whole oyster homogenates, some on distinct tissues. Oyster intestines function differently than the typical vertebrate intestines. Nutritional processing in oysters (and most invertebrates) relies more on intracellular digestion, rather than the extracellular digestive processes and intestinal nutrient absorption that dominate vertebrate systems. In oysters, the phytoplankton, bacteria, and organic matter within the mucous mass are primarily dissociated (not fully digested) in the stomach with the aid of enzymes from a crystalline style. The bulk of the nutritional material then enters the hepatopancreas, the largest organ of the digestive system. The hepatopancreas tissues are highly branched diverticula from the anterior and posterior stomach where final digestion and nutrient absorption occurs, primarily intracellularly, via endocytosis and lysosomal processing. Oyster intestines are involved in water resorption and consolidating and transporting undigested material out via feces, rather than the absorptive processes that typify vertebrate intestines (Kennedy et al. 1996). Thus, we propose that microbiomes derived from stomach and intestinal tissues may include a relatively high incidence of more transient microbes. Hepatopancreas tissues tend to have high abundances of bacteria compared to other tissues such as gill or mantle, typically $10^5$ and greater CFUs per gram of tissue, so

it is likely that some bacteria associated with these dominant tissues represent selective microbiome species or families that contribute significantly to oyster nutrition.

We can state with greater certainty about the implications for consumers of oysters and other shellfish, including humans. *Vibrio* species are one of the most common microbes found in oysters and estuarine ecosystems (Kueh and Chan 1985, Thompson et al. 2006, Beleneva et al. 2007, Pujalte et al. 2010). The CPC+ media used in this study is selective for *Vibrio vulnificus*, which is responsible for more than 95% of all deaths resulting from seafood consumption in the United States, often within a few days of infection (Oliver 2013). Like many of the other related *Vibrio* species that can cause human illnesses (e.g. *V. cholerae*, *V. parahaemolyticus*, *V. alginolyticus)*, *V. vulnificus* is a facultative anaerobe. Our studies suggest that oysters exposed to hypoxic conditions exhibit shifts in microbiome diversity as well as microbial abundances, likely favoring facultative anaerobes. Generally, concerns about bacterial levels in shellfish are heightened after a rain event, as oysters can temporarily acquire high numbers of bacteria from significantly contaminated water. For our laboratory studies, filtered ocean water was used and no bacteria were added during the experiments, so microbial changes were dependent on sources from the oysters themselves or those naturally present in the water. These findings indicate that hypoxia, even in the absence of increased bacterial inputs from freshets and point sources, could affect the availability of safe seafood for consumption by humans and increase the potential for oysters to serve as vectors of bacteria to wildlife.

In conclusion, qualitative as well as quantitative changes in tissue microbial communities were observed in eastern oysters exposed to hypoxic conditions. Increases

in microbial diversity as well as abundances were observed in both laboratory studies and field collected oysters that were related to DO stress. While the changes were most dramatic under continuous hypoxia, there was evidence of cyclical effects. Estuarine habitats are essential shellfish and fish habitats that naturally experience periodic hypoxia stress, and while animals that live there are generally hypoxia tolerant, these ecosystems "on the edge" can also be especially susceptible to additional DO stress. Seemingly subtle but significant impacts of hypoxia on oyster microbiomes may play important roles in setting the stage for susceptibility to environmental stressors and impacting the sustainability of oyster populations.

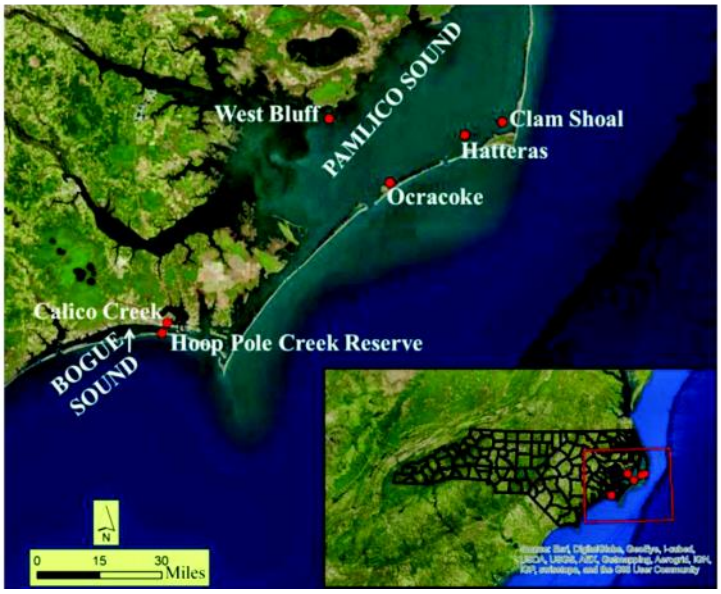


Figure 5.1.: Map of field sites in Bogue Sound and Pamlico Sound in coastal North Carolina, USA.

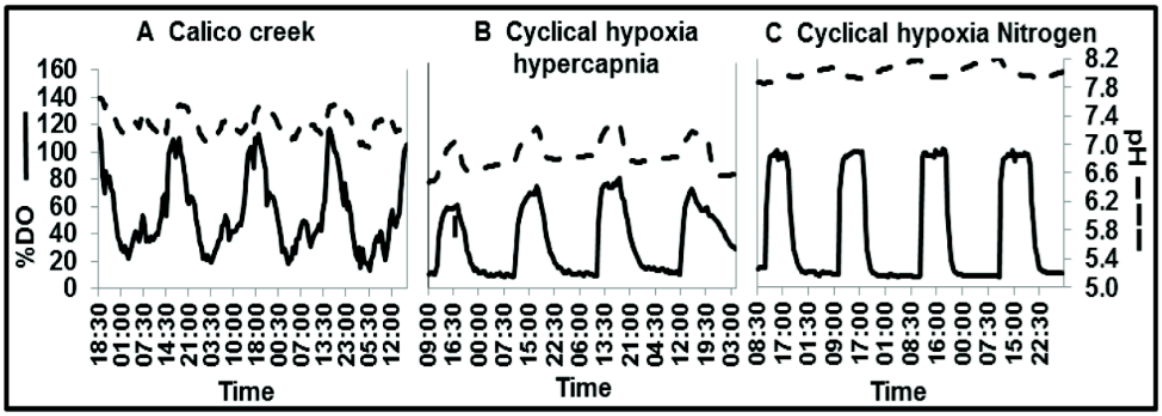


Figure 5.2.: Datasonde profiles showing %DO saturation (solid lines) and pH (dashed lines) for a field site, Calico Creek (A), laboratory $CO_2$ induced cyclic hypoxia hypercapnia (B), and laboratory $N_2$ induced hypoxia (C).

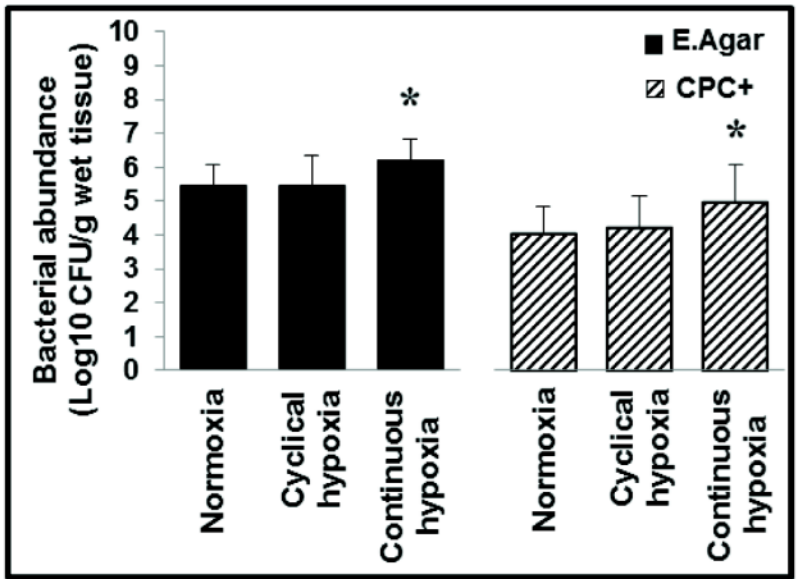


Figure 5.3.: Colony forming units (CFU) in oyster hepatopancreas tissues after laboratory hypoxic exposures. Results for estuarine agar (E. agar) used for total bacterial abundances and CPC+ selective media used to identify *Vibrio vulnificus*–like species are presented. Data are (means + standard deviations; n = 17-24 oysters per treatment). Asterisks (*) represent significant differences from normoxic and cyclic hypoxic exposures in total bacteria (p=0.001) and in presumptive *V. vulnificus* (p=0.008).

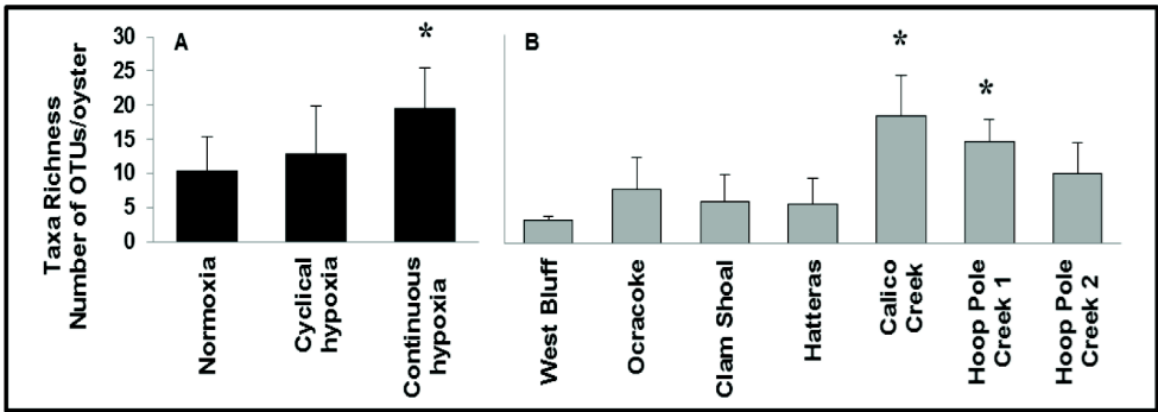


Figure 5.4.: Number of operational taxonomic units (OTUs) for microbial diversity in oyster hepatopancreas (means and standard deviations). (A) OTUs of oysters exposed to hypoxia in the laboratory. The asterisk (*) indicates significant differences from normoxic and cyclic hypoxic exposures ($p<0.001$; n = 20-27 oysters/ laboratory treatment). (B) OTUs of oysters collected from field sites in Pamlico Sound (West Bluff, Ocracoke; Clam Shoal, Hatteras) and Bogue Sound (Calico Creek, Hoop Pole Creek 1 and 2). Asterisks (*) indicate significant differences from other sites ($p<0.05$; n= 6-10 oysters/field site).

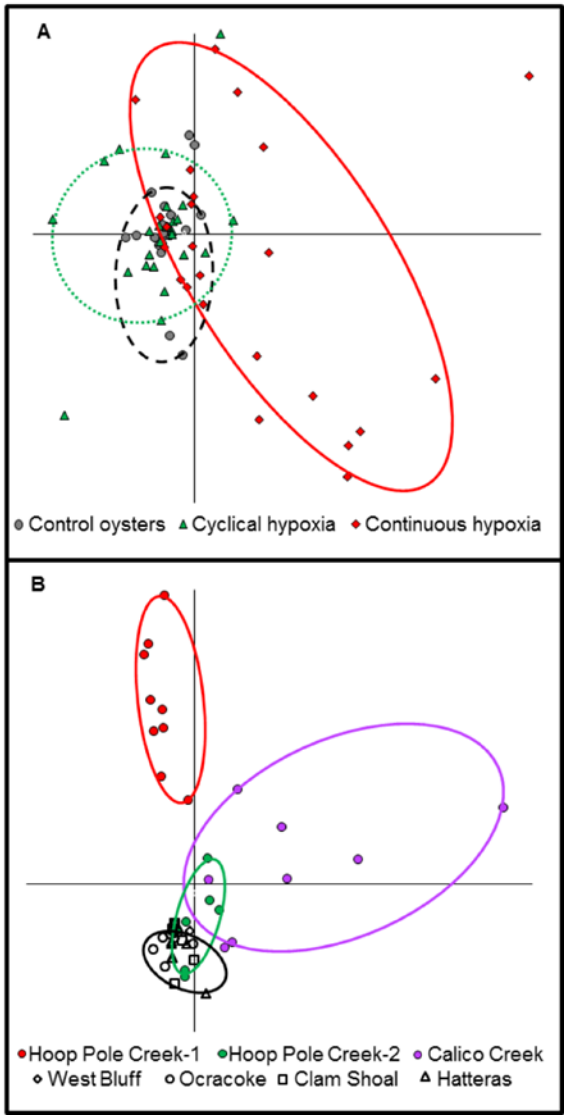


Figure 5.5.: Principal component analysis of ARISA data for microbial diversity. (**A**) Control and hypoxia-exposed oysters from the laboratory studies (each data point represents one oyster; n= 6-20 oysters/treatment from multiple experiments). Black circles represent control oysters, green triangles represent oysters exposed to cyclical hypoxia, and red diamonds represent oysters exposed to continuous hypoxia. Distinct clusters of similarity are indicated, and drawn so that no more than 2 points were excluded for each treatment. The control normoxia oysters clustered very tightly, and many of the data points are stacked on top of each other. (**B)** Microbial diversity patterns of oysters from different field sites (each data point represents one oyster; n= 6-10 oysters/site). Red circles represent oysters from Hoop Pole Creek -1, green circles represent oysters from Hoop Pole Creek -2, blue circles represent oysters from Calico Creek, and black circles represent oysters from Pamlico Sound (West Bluff, Ocracoke; Clam Shoal, Hatteras). Distinct clusters of similarity are indicated. The Pamlico Sound oysters clustered very tightly, so many of the data points stack on top of each other.

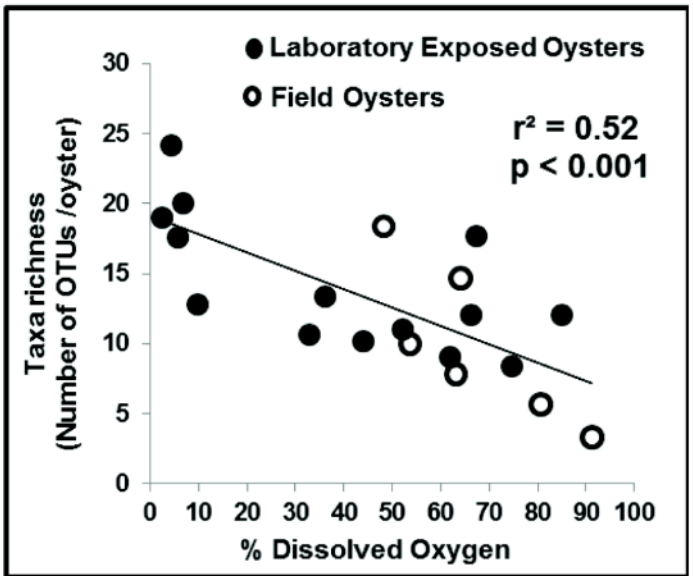


Figure 5.6.: Relationship between %DO and number of OTUs in oyster hepatopancreas tissues. Each point represents the mean number of OTUs in 6-10 oysters. The regression coefficient and p value are indicated, and are based on both field and laboratory data collected over a period of 2 years from multiple laboratory exposures and field samplings.

Table 5.1.: Water quality parameters for laboratory experiments and field sites. The overall mean % dissolved oxygen (%DO) and pH, as well as the average daily ranges, along with temperature (°C) and salinity (‰) are presented.

| | %DO Overall Mean | %DO Range | pH Overall Mean | pH Range | Temp. Overall Mean | Salinity Overall Mean |
|---|---|---|---|---|---|---|
| **LABORATORY TREATMENTS** | | | | | | |
| **Normoxia** | **72.0** | **62-80** | **7.65** | **7.6-7.7** | **20.9** | **26.4** |
| **Hypoxia Cyclical Hypercapnic ($CO_2$)** | **49.2** | **7-91** | **7.28** | **6.7-8.0** | **21.7** | **26.8** |
| **Hypoxia Cyclical ($N_2$)** | **37.0** | **7-96** | **8.00** | **7.8-8.2** | **22.3** | **27.2** |
| **Hypoxia Continuous Hypercapnic ($CO_2$)** | **5.9** | **1-18** | **7.12** | **7.1-7.3** | **23.0** | **24.9** |
| **Hypoxia Continuous ($N_2$)** | **5.9** | **2-23** | **7.47** | **7.3-7.7** | **22.0** | **27.6** |
| **FIELD SITES** | | | | | | |
| **Calico Creek** | **48.2** | **8 - 115** | **7.51** | **7.1-8.0** | **29.5** | **30.6** |
| **Hoop Pole Creek-1** | **64.1** | **26-132** | **7.74** | **7.5-8.0** | **29.6** | **36.2** |
| **Hoop Pole Creek-2** | **53.7** | **24-92** | **7.79** | **7.6-8.0** | **29.7** | **35.5** |
| **Ocracoke** | **63.1** | **22-97** | **7.82** | **7.5-7.9** | **25.2** | **24.5** |
| **Hatteras** | **80.8** | **47-107** | **8.00** | **7.7-8.4** | **24.7** | **20.1** |
| **West Bluff** | **91.2** | **84-96** | **7.83** | **7.8-7.9** | **25.9** | **20.5** |

# CHAPTER 6: SUMMARY AND PERSPECTIVES

Estuaries are predisposed to impacts of hypoxia due to high nutrient levels and diel as well as seasonal environmental fluctuations. These productive ecosystems support a wide variety of organisms and are essential to commercial seafood industries. It is imperative to evaluate the increasing risks associated with environmental stressors and to identify biomarkers of sublethal stress for biomonitoring and conservation efforts in these valuable ecosystems. Bivalves are commonly found in estuaries and they provide essential ecosystem services for maintaining habitat integrity and sustainability. Eastern oysters, *Crassostrea virginica* and Atlantic ribbed marsh mussels, *Geukensia demissa*, co-exist in the estuaries all along the eastern coast of the US and the Gulf coast. Filter-feeding and sedentary lifestyles make them excellent bioindicators.

The goals of this study were to evaluate the effects of hypoxia on oxidative stress, metal bioavailability, and microbial communities in oysters and marsh mussels. Hypoxia occurs in estuaries along with other stressors such as contaminants and pathogenic microbes so the interactions and consequences of multiple stressors must be studied to characterize potential risks to estuarine ecosystems. As hypoxia continues to be a threat to wildlife in coastal ecosystems, our understanding of its interactions with other stressors as well as cellular response biomarkers in invertebrate species is essential. Assessment of environmental risks requires an understanding of the dynamic interactions of stressors and the susceptibility of different species of organisms to stressor interaction effects.

Field sites along NC coast, like most shallow marsh estuaries, are characterized by a range of different hypoxia patterns that provided the framework for out laboratory simulations. We modeled our laboratory studies based on these patterns as well as continuous hypoxia, to evaluate responses to different kinds of hypoxic exposures. Field water quality data collected with *in situ* dataloggers confirmed cyclical fluctuations in DO and pH in some systems, sometimes going from severe hypoxia to oxygen supersaturation, with concomitant changes in pH. The laboratory studies also included treatments that simulated the typical changes in pH (e.g. CO2 gas for hypoxia treatments, hypercapnia) and treatments in which high pH conditions were maintained (e.g. N2 gas for hypoxia treatments). Overall, these hypoxia studies indicated adverse effects based on oxidative stress biomarkers, changes in microbiome communities, and increased metal bioavailability and toxicity from contaminated sediments. The continuous hypoxia hypercapnia treatment was the most damaging exposure, indicating the combined effects of low DO and low pH on estuarine bivalves, and highlighting the increasing risks of ocean acidification in marine habitats. An unexpected finding of these studies was the difference in sensitivities between oysters and mussels. Marsh mussels were more sensitive to oxidative damage under hypoxic conditions and 100% mortality was observed after eight days of exposures under hypoxic hypercapnic conditions.

Metal contamination is a common stressor in estuarine ecosystems which also simultaneously experience hypoxia, and the interactive effects of both stressors can exacerbate stress and impacts on bivalve populations. Accumulation and effects of metal contaminants have been studied in oysters but the impacts of environmentally relevant hypoxia and its effects on metal accumulation are poorly investigated. Sediments serve as

a sink as well as a source of these contaminants as the speciation and bioavailability of metals change under different water quality conditions. We investigated the effects of cyclical and continuous hypoxic conditions on metal accumulation in the tissues of oysters and mussels from contaminated sediments. Increased accumulation of Cu and Zn was observed in oysters exposed to contaminated sediments under hypoxic conditions. Our studies indicated that with decreasing DO and pH, Cu and Zn readily accumulated in gills within 4 days. Significant glutathione (GSH) depletion, which indicates reduced antioxidant capacity, was followed by increased lipid peroxidation, confirming oxidative damage due to the effects of metal exposures under hypoxic conditions. The rapid accumulation of metals in gill tissues probably indicates the sensitivity of these tissues to the free metal ions released from sediments under hypoxic conditions.

Tissue specific differences between gill and hepatopancreas tissues were also observed in oysters exposed to contaminated sediments under hypoxic conditions. Hepatopancreas (HP) tissues showed more of a lag in accumulation of Cu, Fe and Zn. Based on the roles of HP tissues in digestion and detoxification, this lag can represent either a systemic transfer of metals from gill tissues, or it may also reflect entry of metals associated with organic particles through dietary routes. The GSH levels increased in HP tissues, indicating a compensatory response to oxidative stress; but increases in lipid peroxidation damage levels were observed in HP tissues exposed to continuous hypercapnic hypoxia, indicating that the compensatory antioxidant responses were overwhelmed under these conditions. Hypercapnic hypoxia exposures were found to be the most damaging in our studies with and without sediments, which emphasizes the detrimental effects of environmentally relevant conditions of low DO and low pH

together, especially when there are even moderate elevations in sediment metal concentrations. Increased bioavailability of redox metals like Cu during hypoxia from moderately contaminated sediments can have serious implications for sites that have elevated metals but may not be classified as heavily polluted. Consistent with our previous studies, these studies also indicate that depletion of GSH can serve as a biomarker of metal contamination and increased metal bioavailability under low DO and low pH conditions.

We were again surprised at the unexpected differences between oysters and mussels. While oysters readily accumulated Cu and Zn when exposed to hypoxia and contaminated sediments, they did not accumulate Mn; conversely, marsh mussels did not accumulate Cu or Zn during these conditions, but they did accumulate Mn, especially in gill tissues. Oysters had higher Fe concentrations so it is possible that the Mn levels were not high enough to compete with Fe, as Mn can use Fe transporters and can compete for Fe binding sites. The accumulation patterns in mussels suggest selectivity for Mn under hypoxic conditions, even though sediment Fe levels were much higher than the Mn levels. Perhaps this preferential accumulation is associated with an adaptive strategy or compensatory responses. Mn is important for the production of Mn superoxide dismutase (MnSOD), which is a mitochondrial enzyme responsible for preventing mitochondrial damage by ROS generated via the electron transport chain. Increased levels of MnSOD may be an adaptation for marsh mussels which live in hypoxic environments, that from our studies, are especially sensitive to hypoxia and oxidative stress. Marsh mussels are also unique in that they tend to have very high sulfur levels in their tissues, mostly confined to granules. Some studies suggest that sulfide oxidation is coupled with ATP

production in marsh mussels as a mechanism to prevent sulfide toxicity and as an adaptation to survive in sulfidic environments. There are also significant anatomical differences in the gills of oysters and marsh mussels, such that mussels capture bacterioplankton; it is possible that there is a symbiotic relationship between mussel bacteria so that the bacteria may utilize sulfur to generate energy for themselves and/or the mussels. Mn may play a role in binding to sulfur to modulate bioavailability as well as minimize toxicity. Based on our metal accumulation data, the striking elevations in Mn levels during hypoxia in mussels can be used as a potential biomarker of hypoxia exposures.

Finally we investigated relationships between oyster microbial communities and environmental hypoxia to evaluate DO driven changes in the oyster microbiome. Surprisingly, increases in taxa richness were observed under continuous hypoxic treatments in laboratory studies, suggesting that low DO conditions favor increases in diversity of bacterial taxa colonizing the oysters. Collectively, our field and laboratory studies based on data collected over multiple years and experiments and representing analyses of more than 150 oysters using ARISA (a molecular diversity analysis technique), showed remarkably concordant patterns of increased taxa richness with increasing DO stress. While there is some current speculation about the existence of an oyster microbiome that promotes nutrient utilization and fitness, these studies do suggest that under non-stressed conditions, oysters tend to have a limited suite of bacteria that could represent a functional microbiome. Perturbations to the resident microbiome may reflect replacement of some bacterial taxa of functional importance to oyster physiology and health, which could increase susceptibility to pathogens and other stressors. Based on

more traditional bacterial plating techniques, quantitative differences between normoxic and hypoxic exposures were also observed; there were increases in total bacterial numbers and *Vibrio vulnificus*-like burdens under hypoxic conditions. These results suggest that hypoxia may foster an increase in anaerobic or facultative-anaerobic bacteria (many of which are pathogenic to humans as well as shellfish and other marine organisms). Elevated abundances of facultative anaerobes under hypoxic conditions, such as members of family Vibrionaceae, can increase the risks to oyster populations as well as consumers of seafood, including humans, potentially increasing public health risks. Therefore, our qualitative and quantitative data on oyster microbial communities sets the stage for further investigations on the effects of multiple stressors on oyster microbiomes and taxa specific responses. Together, the increases in abundances of anaerobic bacteria, and proliferation of species could affect oyster microbiomes, susceptibility to disease, and fitness. Our studies further suggest that changes in oyster microbiomes may also serve as a valuable marker of hypoxia in estuaries.

The biology and physiology of oysters and edible mussels of the genus *Mytilus* have been studied extensively and they are both well-established bioindicator species, but very little is known about marsh mussels, *Geukensia demissa*. *Mytilus* is restricted to cool climates and does not occur in the southeastern or Gulf regions of the US. Marsh mussels are not a commercial seafood (actually the high sulfur levels confer a rotten-egg smell and make them generally unpalatable to humans), but like oysters they do play vital roles in estuarine ecosystems. However, our studies indicated some very interesting and important differences in sensitivities and cellular responses in oysters and marsh mussels

that suggest that more studies with marsh mussels are needed. Marsh mussels may be especially valuable for documenting hypoxia exposures and effects.

In summary, these laboratory and field studies indicate that hypoxia can disrupt ecosystem integrity by threatening bivalve sustainability due to increases in oxidative damage, increases in metal bioavailability, and changes in microbiomes. The effects of environmental hypoxia are far reaching and its interactions with other co-occurring stressors will only exacerbate the vulnerability of these ecosystem engineers under chronic exposures. Toxic metals and pathogens can compromise seafood safety and poses risks to human consumers. As anthropogenic inputs and hypoxic conditions pose significant risks to coastal wildlife worldwide, better diagnostic tools based on cellular biomarkers in a variety of bioindicator species are essential for characterizing ecosystem health and developing effective mitigation strategies.

These studies have generated a variety of interesting and pertinent questions which warrant further investigation. The differences in responses of oysters and mussels are particularly interesting, and evaluating the underlying mechanisms will help to reveal the significance of species specific responses and their importance in biomonitoring. More detailed microbiome studies including identification of bacterial taxa using high throughput sequencing and their responses to hypoxia are needed to more fully elucidate the functional roles of microbiome communities in bivalves, and factors that increase risks of seafood borne illnesses.

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