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Environmental Stressors: Pathways Of Exposure And Aquatic Invertebrate Response

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**ENVIRONMENTAL STRESSORS: PATHWAYS OF EXPOSURE AND AQUATIC
INVERTEBRATE RESPONSE**

by

CARLY J. NOWICKI

DISSERTATION

Submitted to the Graduate School

of Wayne State University,

Detroit, Michigan

in partial fulfillment of the requirements

for the degree of

DOCTOR OF PHILOSOPHY

2014

MAJOR: BIOLOGICAL SCIENCES

Approved by:

Advisor

Date

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DEDICATION

*I dedicate this work to my husband, Mike
Who won the “doctor” race then carried me the rest of the way*

*And to you, Slugger, for always keeping me grounded and
reminding me of my strengths*

ACKNOWLEDGMENTS

This research project would not have come this far without the support and input of many people. First and foremost, I want to thank my advisor, Dr. Donna Kashian. She has been one of the most incredible and inspiring people I have ever known. She always knew the right times to give me help and when to stand back and let me learn on my own. She has made me proud of the scientist and professional that I am today. I want to thank her for not only being my advocate and mentor, but thank her for being one of my greatest friends.

To my lab family, without whom this journey would have been impossible. Each of them have shared my triumphs and failures and have always been there to lend a hand, give advice, or to bounce around ideas. Particularly I'd like to thank Cody Narlock and Karim Alame who spent many extra lab hours this past year helping me with my experiments and staying late until the work was done.

I would like to thank my dissertation committee (Dr. Dan Kashian, Dr. Dave Njus, and Dr. Jeff Ram) for their helpful comments and suggestions for my research goals and dissertation.

I would like to acknowledge the support and funding provided by the Wayne State Graduate School and Biological Sciences Department that have made my degree a success. The funding and travel opportunities, including the Thomas C. Rumble Graduate Fellowship and WSU Enhancement Awards, were instrumental to my research and professional goals. My sincere gratitude to the staff and faculty of the Biological Sciences Department who took the time to answer questions, give

encouragement, and made sure I was heading in the right direction. Also, thanks to Dr. Carl Freeman for his statistics help that has been invaluable for this research project.

Thank you to my parents and family who taught me to work hard, to never give up, and always reassuring me I can achieve my dreams. Their steady faith in me has gotten me through some of the toughest moments in my life. I thank them for encouraging me and keeping me grounded, even when my work seemed unmanageable.

A special thank you to my husband, Mike, who has shown an incredible amount of support, patience, and understanding as I worked towards my degree. He has taught me that every day is a gift and to live each day without worrying for the next. He is a true inspiration to myself and others.

TABLE OF CONTENTS

| | |
|---|------|
| Dedication | ii |
| Acknowledgments | iii |
| List of Tables | vii |
| List of Figures | viii |
| Introduction | 1 |
| <i>Environmental stressors and anthropogenic influences</i> | 1 |
| <i>Research Focus</i> | 4 |
| Chapter 1 Comparative Effects of Sediment versus Aqueous PCB Exposure on Benthic and Planktonic Invertebrates | 6 |
| <i>Background</i> | 6 |
| <i>Methodology</i> | 8 |
| <i>Results</i> | 17 |
| <i>Discussion</i> | 22 |
| Chapter 2 Comparing Oxidative Stress in Dreissenid Mussels under Different Environmental Stressors and the Role of Oxidative Stress in Environmental Tolerance | 29 |
| <i>Background</i> | 29 |
| <i>Methodology</i> | 32 |
| <i>Results</i> | 41 |
| <i>Discussion</i> | 54 |
| Chapter 3 The Effects of Multiple Stressors on Oxidative Stress in Dreissenid Mussels | 60 |
| <i>Background</i> | 60 |
| <i>Methodology</i> | 62 |

| | |
|---|-----|
| <i>Results</i> | 70 |
| <i>Discussion</i> | 81 |
| Significance | 87 |
| References | 90 |
| Abstract | 111 |
| Autobiographical Statement | 113 |

LIST OF TABLES

- Table 1.** *Measured PCB concentrations and Global Positioning System (GPS) coordinates for sediment and water collection sites in the Lange and Revere Canals, Lake St. Clair, Michigan, USA. PCB sediment and water concentrations were determined at Paragon Laboratories (Livonia, MI) using EPA Standard Method 8082A..... 17*
- Table 2.** *Physiological parameters measured at the impacted Lange Canal (I) and reference canal (R) (both located in St. Clair Shores, MI, USA) during the seven day D. bugensis PCB experiment..... 21*
- Table 3.** *Dreissena bugensis and Dreissena polymorpha were collected from two locations in the Great Lakes basin in September-October 2013 for use in environmental stressor studies. The location, physicochemical parameter measurements, and sediment and water PCB concentrations are listed for each site. Dreissena bugensis was collected on multiple days and therefore physicochemical measurements are expressed as the average \pm standard deviation..... 34*
- Table 4.** *Physicochemical parameters including dissolved oxygen (mg/L), temperature ($^{\circ}$ C), pH, and conductivity (μ S/cm) were measured in experimental chambers using a multi-parameter probe (YSI 556 Multi-Parameter Sensor) during a 4 day hypoxia stressor assay with Dreissena bugensis and D. polymorpha. Measurements are expressed as the average \pm standard deviation 51*
- Table 5.** *Dreissena bugensis and Dreissena polymorpha were collected from two locations in the Great Lakes basin in April 2014 for use in multiple stressor studies. The location, physicochemical parameter measurements, and sediment and water PCB concentrations are listed for each site 64*
- Table 6.** *Physicochemical parameters including dissolved oxygen (mg/L), temperature ($^{\circ}$ C), pH, and conductivity (μ S/cm) were measured in experimental chambers using a multi-parameter probe (YSI 556 Multi-Parameter Sensor) during a 3 day multiple stressor assay (hypoxia and PCBs) with Dreissena bugensis and D. polymorpha. Measurements were taken on days 1 and 2. Measurements are expressed as the average \pm standard deviation 77*

LIST OF FIGURES

- Figure 1.** Map of St. Clair Shores sampling sites and PCB entry-point in the Lange and Revere canals. Blue dots indicate sediment collection points. Water was collected at site L1 9
- Figure 2.** Grain size distribution for sediments collected in the Lange Canal (Lake St. Clair, Michigan) at sites L1 and L3. Each fraction was weighed and divided by the total weight of the entire sample..... 18
- Figure 3.** Total mortality of *D. pulex* exposed to A) PCB site water alone and B) PCB site sediments. For the sediment bioassay, organisms were exposed to either 100 mg of PCB-laden sediment (solid black line) or 50 mg of sediment (dotted line) 19
- Figure 4.** Percent mortality for *C. dilutus* exposed to increasing concentrations of PCB contaminated sediment collected from the Lange and Revere canals (St. Clair Shores, MI, USA) during a five day toxicity assay. Mortality was quantified on days 3, 4, and 5. Bars with the same letters were not significantly different ($\alpha = 0.050$) as determined by the Fisher's Exact Test comparing categorical mortality data among treatments. Dotted line indicates maximum mortality. Error bars represent standard deviation among treatments (n=12)..... 20
- Figure 5.** Mean mortality for *D. bugensis* transplanted into the Lange (PCB) and reference canals (St. Clair Shores, MI, USA) at both the water surface and in the sediments at the end of the 7-day field study. Error bars represent standard error (n=7)..... 22
- Figure 6.** Diagram of treatment chamber setup for examining oxidative stress in *D. polymorpha* and *D. bugensis* exposed to hypoxic and oxic conditions. Chambers were sealed on both ends and equipped with a gas inlet and outlet ports, and a water sampling port..... 37
- Figure 7.** Oxidative stress measured as (a) lipid peroxidation (LPO) and (b) antioxidant catalase activity (CAT) in *D. polymorpha* and *D. bugensis* exposed to three population density treatments: Low (1 mussel/40ml), Medium (1 mussel/20ml), and High (1 mussel/10ml) during a 12 day assay. The vertical black line between days 6 and 7 indicates the addition of 30 pre-marked mussels to all treatment jars at the end of Day 6. Initial LPO and CAT were measured in stock mussels prior to the start of the experiment on Day 0 (baseline), and mussel tissue was sampled on days 6, 7, and 12. Data points represent 95% confidence intervals of the Species*Day interaction. Points having the same letters (lowercase for *D. bugensis* and uppercase for *D. polymorpha*) indicate no differences among days at $p < 0.050$ following ANOVA and Student Newman-Keuls post-hoc tests..... 42

- Figure 8.** Oxidative stress measured as (a) lipid peroxidation (LPO) and (b) antioxidant catalase activity (CAT) in *D. polymorpha* and *D. bugensis* exposed to three population density treatments: Low (1 mussel/40ml), Medium (1 mussel/20ml), and High (1 mussel/10ml) during a 12 day assay. Data points represent 95% confidence intervals of the Species*Density interaction. Points having the same letters (lowercase for *D. bugensis* and uppercase for *D. polymorpha*) indicate no differences among days at $p < 0.050$ following ANOVA and Student Newman-Keuls post-hoc tests..... 43
- Figure 9.** Oxidative stress measured as (a) lipid peroxidation (LPO) and (b) antioxidant catalase activity (CAT) in *D. polymorpha* and *D. bugensis* exposed to PCB contaminated water (1.3µg/l) during a 7 day assay. Initial LPO and CAT were measured in stock mussels prior to the start of the experiment on Day 0 (baseline), and mussel tissue was sampled on days 1, 2, and 7. Data points represent 95% confidence intervals of the Species*Day interaction. Points having the same letters (lowercase for *D. bugensis* and uppercase for *D. polymorpha*) indicate no differences among days at $p < 0.0500$ following ANOVA and Student Newman-Keuls post-hoc tests..... 45
- Figure 10.** Oxidative stress measured as (a) lipid peroxidation (LPO) and (b) antioxidant catalase activity (CAT) in *D. polymorpha* and *D. bugensis* exposed to PCB contaminated water (1.3µg/l) during a 7 day assay. Data points represent 95% confidence intervals of the Species*PCB interaction. Points having the same letters (lowercase for *D. bugensis* and uppercase for *D. polymorpha*) indicate no differences among days at $p < 0.050$ following ANOVA and Student Newman-Keuls post-hoc tests..... 46
- Figure 11.** Oxidative stress measured as (a) lipid peroxidation (LPO) and (b) antioxidant catalase activity (CAT) in *D. polymorpha* and *D. bugensis* exposed to three temperature treatments: Low (5°C), Ambient (Amb; 20°C), and High (29 °C) during a 4 day assay. Initial LPO and CAT were measured in stock mussels prior to the start of the experiment on Day 0 (baseline), and mussel tissue was sampled on days 1, 2, 3, and 4. Data points represent 95% confidence intervals of the Species*Day interaction. Points having the same letters (lowercase for *D. bugensis* and uppercase for *D. polymorpha*) indicate no differences among days at $p < 0.050$ following ANOVA and Student Newman-Keuls post-hoc tests..... 48
- Figure 12.** Oxidative stress measured as (a) lipid peroxidation (LPO) and (b) antioxidant catalase activity (CAT) in *D. polymorpha* and *D. bugensis* exposed to three temperature treatments: Low (5°C), Ambient (Amb; 20°C), and High (29 °C) during a 4 day assay. Data points represent 95% confidence intervals of the Species*Temperature interaction. Points having the same letters (lowercase for *D. bugensis* and uppercase for *D. polymorpha*) indicate no differences among days at $p < 0.050$ following ANOVA and Student Newman-Keuls post-hoc tests..... 49

- Figure 13.** Oxidative stress measured as (a) lipid peroxidation (LPO) and (b) antioxidant catalase activity (CAT) in *D. polymorpha* and *D. bugensis* exposed to hypoxic and oxic conditions during a 4 day assay. Initial LPO and CAT were measured in stock mussels prior to the start of the experiment on Day 0 (baseline), and mussel tissue was sampled on the last day of the experiment. Data points represent 95% confidence intervals of the Species*Hypoxia interaction. Points having the same letters (lowercase for *D. bugensis* and uppercase for *D. polymorpha*) indicate no differences among days at $p < 0.050$ following ANOVA and Student Newman-Keuls post-hoc tests 52
- Figure 14.** Pearson correlations measuring the linear dependency of CAT and LPO for the four experimental stressors: (a) density, (b) PCBs, (c) temperature, and (d) hypoxia..... 53
- Figure 15.** Oxidative stress measured as (a) lipid peroxidation (LPO) and (b) antioxidant catalase activity (CAT) in *D. polymorpha* and *D. bugensis* exposed to three population density treatments, alone (control) and in combination with PCBs during a 6 day assay. The population densities examined were: Low (1 mussel/40ml), Medium (1 mussel/20ml), and High (1 mussel/10ml). Initial LPO and CAT were measured in stock mussels prior to the start of the experiment on Day 0 (baseline), and mussel tissue was sampled at the end of the experiment. Data points represent 95% confidence intervals of the Species*Density*PCB interaction. Points having the same letters (lowercase for *D. bugensis* and uppercase for *D. polymorpha*) indicate no differences among days at $p < 0.050$ following ANOVA and Student Newman-Keuls post-hoc tests 72
- Figure 16.** Oxidative stress measured as (a) lipid peroxidation (LPO) and (b) antioxidant catalase activity (CAT) in *D. polymorpha* and *D. bugensis* exposed to three temperature treatments, alone (control) and in combination with PCBs during a 3 day assay. The temperatures examined were: Low (5°C), Ambient (20°C), and High (29 °C). Initial LPO and CAT were measured in stock mussels prior to the start of the experiment on Day 0 (baseline), and mussel tissue was sampled on day 3. Data points represent 95% confidence intervals of the Species*Temperature*PCB interaction. Points having the same letters (lowercase for *D. bugensis* and uppercase for *D. polymorpha*) indicate no differences among days at $p < 0.050$ following ANOVA and Student Newman-Keuls post-hoc tests 75

Figure 17. Oxidative stress measured as (a) lipid peroxidation (LPO) and (b) antioxidant catalase activity (CAT) in *D. polymorpha* and *D. bugensis* exposed to hypoxic and oxic conditions, both alone and in combination with PCBs, during a 2 day assay. Initial LPO and CAT were measured in stock mussels prior to the start of the experiment on Day 0 (baseline), and mussel tissue was sampled on day 2. Data points represent 95% confidence intervals of the Species*Temperature*PCB interaction. Points having the same letters (lowercase for *D. bugensis* and uppercase for *D. polymorpha*) indicate no differences among days at $p < 0.050$ following ANOVA and Student Newman-Keuls post-hoc tests..... 78

Figure 18. Pearson correlations measuring the linear dependency of CAT and LPO for the three multiple stressor experiments (a) density and PCBs, (b) temperature and PCBs, and (c) hypoxia and PCBs..... 80

INTRODUCTION

Environmental stressors and anthropogenic influences

Since the Industrial Revolution, global anthropogenic influences have manipulated waterways to move goods and have drastically exacerbated the presence of environmental stressors. With the expansion of industry and the commercial movement of goods, humans have contributed to several physical, chemical, and biological stressors in aquatic environments. The detrimental physical impacts of recreational activities such as boating and wave action (Mastran et al. 1994, Burgin and Hardiman 2011, Williams et al. 2013) and the impacts of commercial overfishing (Jensen 1999, Robitaille et al. 2002) are well documented. In addition, thermal inputs from industry have reduced habitat quality and caused changes in invertebrate assemblages in aquatic environments (Verones et al. 2010, Coulter et al. 2014). One of the most detrimental human-influenced stressor in aquatic environments has been the input of numerous chemical contaminants, including industrial byproducts, fertilizers, pharmaceuticals, and personal care products. With increased agriculture and urban runoff, fertilizers, pesticides and organochlorines are having huge impacts on aquatic ecosystems (Feist et al. 2005, Mitchell et al. 2005, Tao et al. 2010).

Industrial inputs throughout the 1950s-1970s caused detrimental and sometimes irreversible effects on local bodies of water. One industrial contaminant of concern is polychlorinated biphenyls. Polychlorinated biphenyls (PCBs) are a group of chlorinated hydrocarbons that were widely used in products such as transformers, electrical equipment, and some oil-based paints due to their chemical stability, non-flammability, and insulating capabilities. During their 50+ years of use, it is estimated that

approximately 1.5 million tons of PCBs were created for industrial use and some were released into the environment as products or waste from manufacturing processes, a portion of which can be found in the environment today (Ivanov and Sandell 1992, Rantanen 1992, Cummines 1998). In 1979, the U.S. Environmental Protection Agency banned the use of PCBs for their link to adverse health effects in humans; however, PCBs continue to enter the environment through sources such as illegal dumping, hazardous waste sites, or leaking of PCB-insulated materials. Since PCBs are a legacy contaminant that is often trapped with colloidal particles of sediment and can recirculate in the environment, they are of particular concern in aquatic ecosystems. Furthermore, the strong chemical bonds of PCB congeners make them very resistant to degradation (Fiedler 1997, Li and Xie 2004). The occurrence of PCBs in aquatic organisms first appeared in reports beginning in 1966 (Fiedler 1997). The harmful effects of PCBs have been documented across many aquatic organisms including bacteria (Correa et al. 2010, Ho and Liu 2010), primary producers (Neamtu et al. 2009, Yu et al. 2011), benthic and planktonic invertebrates (Dillon and Burton 1991, Zou and Fingerman 1997, Nakari and Huhtala 2008), fish (Dillon and Burton 1991, Echols et al. 2000, Wan et al. 2010, Levensgood and Schaeffer 2011) and aquatic birds (Hegseth et al. 2011, Wang et al. 2011). Further research on the effects and mechanisms of PCBs in the environment is important for remediation of impacted sites.

Invasive species are perhaps the most detrimental biological stressors that have been severely intensified by human influence. Commercial shipping and opening of environmental corridor waterways such as the St. Lawrence Seaway and the Panama Canal have led to the movement of species and contributed to extensive habitat

degradation. The spread of harmful nuisance species has resulted in the damage of native ecosystems throughout Europe and the United States (May and Marsden 1992, Pimentel et al. 2005, Leonardos et al. 2008, Snyder et al. 2014). For example, over 180 invasive species have entered the Great Lakes watershed and have had detrimental impact on local flora and fauna (Mills et al. 1993, Ricciardi et al. 1996, Ricciardi 2006). The most notable invasion was the dreissenid mussels in the late 1980s (May and Marsden 1992). *Dreissena polymorpha* (zebra mussel) and *Dreissena bugensis* (quagga mussel) are native to the Ponto-Caspian region in southwestern Asia and have spread to Europe and the United States through ship ballast water. These species are a significant pest due to their high filtration rates, high fecundity, and competitive advantages over native species (Bruner et al. 1994, Heath et al. 1995, Wisenden and Bailey 1995, Ricciardi et al. 1996, Dermott and Kerec 1997, Bially and MacIsaac 2000, Vanderploeg et al. 2002). Dreissenid mussels have negatively impacted native unionid populations (Ricciardi et al. 1996, Bodis et al. 2014), contributed to harmful algal blooms (Malkin et al. 2008), and altered the distribution and cycling of nutrients and contaminants in the water column (Mills et al. 1993). While the dreissenid mussels have remained primarily in the Great Lakes, where abundance of substrate and open waters provides the perfect habitat for these invaders, recent studies have documented the expansion of invasive dreissenid populations into the western parts of the United States, such as the Colorado River watershed, Electric Lake in Utah, Mead and Mohave Lakes in Nevada, and even as far west as the San Justo Reservoir in California (Mueiting and Gerstenberger 2010, Benson 2014). While several studies have tried to explore the biology of these invaders and the impacts of their invasion (Mills et al. 1993, Dermott

and Kerec 1997, Karatayev et al. 1997, Bially and MacIsaac 2000), further research is needed to understand the physiology of these two species in order to facilitate management efforts.

Research Focus

Traditional methods to evaluate environmental stressors in aquatic systems involve standard toxicity bioassays, where the endpoints are typically mortality or changes in reproduction, and more recently assessment at the community level. However, traditional bioassays do not address the molecular pathways of environmental stressors that lead to lethal outcomes, and community level assessment identifies impairment after species level changes, such as losses of sensitive taxa, have already occurred in the environment. The development of a bioassessment tool that can determine if and how environmental stressors are immediately affecting an ecosystem before significant impacts occur is crucial for the management of impacted systems. One novel avenue of molecular assessment is the use of biomarkers, such as those present in the oxidative stress response. To address the molecular and physical impacts of environmental stressors in aquatic invertebrates, in Chapter 1 I compare the effects of aqueous and sediment exposure of a chemical stressor (PCBs) on benthic and planktonic invertebrates. Chapter 2 presents pathways of invertebrate tolerance to environmental stressors and I examine how physical, chemical and biological stressors affect oxidative stress in aquatic invertebrates. Finally, in Chapter 3 I examine the role of oxidative stress and invertebrate response further by determining the effects of

multiple environmental stressors on the oxidative stress response in aquatic invertebrates.

CHAPTER 1

COMPARATIVE EFFECTS OF SEDIMENT VERSUS AQUEOUS PCB EXPOSURE ON BENTHIC AND PLANKTONIC INVERTEBRATES¹

BACKGROUND

The partitioning of contaminants between the sediment and water column has large implications for toxicity to organisms. Toxicity testing for site evaluation typically quantifies contaminants separately in the water column or in the sediments; however, many aquatic organisms spend a portion of their life at the sediment-water interface where contaminants are seldom monitored, and therefore their potential exposure may be higher than previously thought. One contaminant of concern is polychlorinated biphenyls (PCBs), a group of chlorinated hydrocarbons that were widely used in industry due to their chemical stability, non-flammability, and insulating capabilities. Polychlorinated biphenyls have very high binding affinities for organic compounds and solid particulates, and low solubility in water and, therefore, are often attached to colloidal particles of sediment (Alkhatib and Weigand 2002). Adverse effects of PCBs have been documented across most major groups of aquatic organisms, from bacteria to fish (Dillon and Burton 1991, Ho and Liu 2010, Wan et al. 2010).

Due to preferential binding of PCBs to sediment, benthic organisms such as mussels, amphipods, and burrowing invertebrates have a high risk for PCB exposure (Tao et al. 2010, Van Geest et al. 2011); however, many organisms, typically classified

¹Nowicki, C. J., E. H. van Hees and D. R. Kashian (2014). "Comparative effects of sediment versus aqueous polychlorinated biphenyl (PCB) exposure on benthic and planktonic invertebrates." Environmental Toxicology and Chemistry **33**(3): 641-647.

as planktonic, experience diel vertical migration and move between the water column and the benthos. They may be exposed to higher levels of contaminants than generally reported due to elevated contaminant levels at the sediment-water interface.

It is important to understand how chemical contaminants affect aquatic invertebrates, as changes in invertebrate populations can have significant effects on higher trophic levels in aquatic food webs. Planktonic and benthic invertebrates play important ecological roles in aquatic ecosystems by contributing to the distribution of nutrients throughout the water column (Jiang et al. 2010) and as major food sources for fish (Brooks and Dodson 1965). They also contribute to the biomagnification of PCBs in aquatic systems (Rashleigh et al. 2009), being implicated in the declines of various aquatic species including seals and otters (Kannan et al. 2000) and waterfowl (Wang et al. 2011), and cause concerns of human exposure via fish consumption (Stewart et al. 1999). Other studies have modeled the transfer of PCBs in aquatic food webs and examined bioaccumulation in sediment and pore-water alone (Gobas 1993, Harrad and Smith 1998); however, fewer studies (Lohmann et al. 2004) compare the effects of sediment PCB exposure in conjunction with aqueous PCB exposure. For direct contact exposure, it remains unclear how the environmental matrix (sediment or aqueous) mediates the effect of PCB exposure. The aim of the present study is to compare the relative influence of sediment versus aqueous exposure on planktonic and benthic organisms using both field and laboratory studies, and demonstrating the potential impact of a contaminated site on local biota.

METHODOLOGY

I coupled (1) three independent laboratory bioassays using sediment and water collected from six locations at a PCB contaminated site with (2) a field experiment conducted at a reference and the PCB contaminated site to evaluate sediment- and aqueous-associated PCB exposure on benthic and planktonic invertebrates. The laboratory assays evaluated (1) aqueous PCB exposure on *Daphnia pulex*, (2) the sediment-water interface PCB exposure on *D. pulex*, and (3) sediment PCB exposure on *Chironomus dilutus*. The field experiment evaluated the toxicity of PCBs on *Dreissena bugensis* (quagga mussel) at a contaminated site at the sediment-water interface and in surface waters.

Study area

I sampled two residential boating canals known to contain PCBs: the Lange and Revere canals located in the city of St. Clair Shores, MI, USA (Figure 1). The canals discharge into Lake St. Clair, a small corridor of the Laurentian Great Lakes, and were placed on the National Priorities List and designated a Superfund Site in September 2012 by the United States Environmental Protection Agency (USEPA) due to PCB contamination. The primary source of contamination in the canals has been identified as an underground site approximately 0.6 km from the canals from which PCBs migrated into the storm sewer system and discharged into the canals (U.S. Environmental Protection Agency 2011). Recent testing in April/May 2011 at the source identified total PCB levels in the sediment ranging from 3,800 µg/g to 17,000 µg/g (DeMaria 2011). PCB concentrations at this site are similar to other Superfund sites that have

exceptionally high PCB sediment concentrations (e.g., Hudson River in New York) (Quantitative Environmental Analysis LLC 2007).

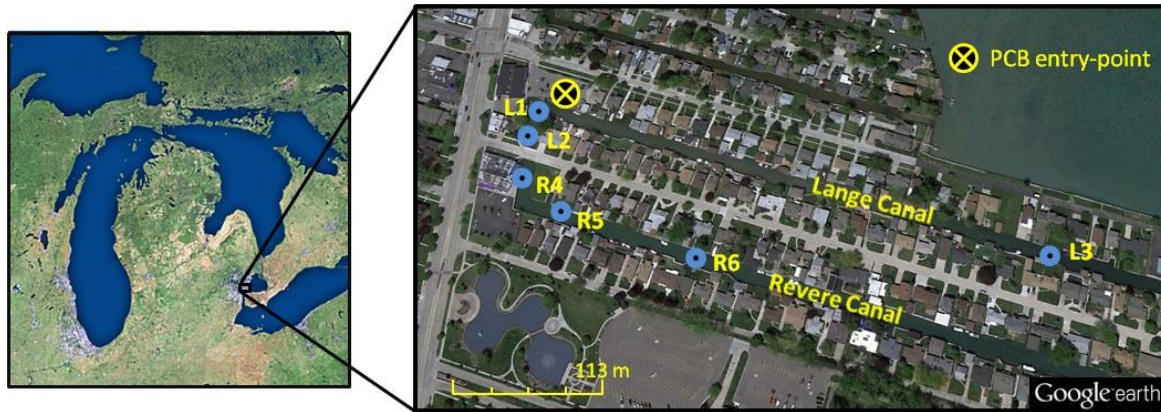


Figure 1. Map of St. Clair Shores sampling sites and PCB entry-point in the Lange and Revere canals. Blue dots indicate sediment collection points. Water was collected at site L1.

Collection of PCB sediment and water

Sediment and water were collected from six locations in the Lange and Revere canals during May 2010 for laboratory PCB analysis and toxicity bioassays. Surface sediment was collected within the first 10-15 cm of sediment using an Ekman dredge from three sites in the Lange Canal and three sites in the Revere Canal (Figure 1; Table 1). The Ekman dredge was rinsed with acetone and water and cleaned to remove residual sediments and associated PCBs between collection sites. Sediment collection progressed from the least to the most contaminated site to reduce potential contamination between samples. Collected sediment was placed in glass jars pre-rinsed with acetone and site water. Water was collected in eight 1L glass amber jars with Teflon lids from the Lange canal at site L1 (Figure 1) at approximately 0.25 meters

below the surface of the water, in an area with a water depth of approximately 1-1.5 meters.

Prior to laboratory experiments, the sediments were sieved through a 500 µm sieve to remove large debris. The sediments and water were stored in the dark at 4 °C for approximately two weeks. The excess water overlying the sediments was siphoned using a vacuum tube and the remaining sediments were funneled into a clean glass jar. Dechlorinated water was used to rinse where necessary.

PCB analysis

Water samples and sediments collected from each site were prepared for PCB analysis using USEPA standard method 3510C and analyzed for PCB concentrations using USEPA standard method 8082A (Paragon Laboratories, Livonia, MI, USA) (U.S. Environmental Protection Agency 2011). As outlined in the method (U.S. Environmental Protection Agency 2011), a measured volume of the sample was extracted with methylene chloride-acetone using a separatory funnel and then dried. A sulfuric acid/potassium permanganate cleanup reagent was then added to the sample to remove any organochlorine or organophosphorus pesticides that may have been present. Polychlorinated biphenyl concentrations were determined using gas chromatography equipped with a narrow- or wide-bore fused silica capillary column and an electron capture detector. Sediments and water samples were analyzed for a suite of PCB mixtures (i.e., Aroclors 1016, 1221, 1232, 1242, 1248, 1254, 1260, 1262, and 1268) and compared to certified standard solutions. A mixture of Aroclor 1016 and Aroclor 1260 was used as a multi-point initial calibration. The results were expressed as

“total PCBs” in $\mu\text{g/g}$ of sediment or $\mu\text{g/L}$ of water. Minimum reporting limits were 330 $\mu\text{g/kg}$ for sediment samples, and 0.10 $\mu\text{g/L}$ for water samples.

Sediment grain size distribution

In addition to determining total PCB concentration of the sediments, the grain size distribution was determined for sites L1 and L3. Wet sediment was sieved using a range of different sieve sizes (4 μm to 500 μm) and the wet fractions were dried and weighed. The finest fraction was separated using gravitation settling in a 4L graduated cylinder. The sample was allowed to settle for 24 hours. The top 20 cm (20 deciliters) were removed and the clay sized fraction allowed to dry for weighing. Distilled water was added and the process repeated until no sediment remained in the top 20 cm of the 4L cylinder. The clay size fraction was weighed, as was the silt sized fraction that settled more than 20 cm in 24 hours. Weights of each fraction were totaled to determine the total weight of the entire sediment sample. The weight of each fraction was divided by the total weight and multiplied by 100 in order to convert it to a percent grain size distribution for each fraction.

Test organisms

A *D. pulex* clone isolated from Lake Michigan and maintained in the laboratory in artificial lake water (Kilham et al. 1998) was used for the aqueous and sediment-water interface bioassays. Second instar *C. dilutus* were obtained from Aquatic Bio Systems Inc. (Fort Collins, Colorado) for use in the sediment toxicity bioassays. *Chironomus dilutus* were acclimated in four 10 L glass aquariums lined with a thin layer (~1 mm) of clean fine-grain sand and 1L of overlaying dechlorinated water. Before *C. dilutus* were

placed in the holding tanks, water chemistry was checked with specifications provided by the distributor (total alkalinity: 50-120 mg CaCO₃/L, total hardness: 96-148 mg CaCO₃/L, temperature: 22-26 °C, pH: 7.43-8.31) and adjusted accordingly. The holding tanks were aerated and placed in an environmental growth chamber at 22 ± 1 °C with a 12 h: 12 h light-dark cycle.

For the field experiment, adult *D. bugensis* were selected for the toxicity test because of their relatively sessile nature as adults, increasingly widespread distribution in temperate freshwater systems, ease of collection and maintenance in the laboratory, and ubiquity in the Lake St. Clair canals. *Dreissena bugensis* were collected from a reference site approximately 19.4 km downstream of the Lake St. Clair canals (42°21'6.05"N, 82°58'12.09"W). No detectable levels of PCBs were found in the sediment or water at the reference site where the test organisms were collected (Paragon Labs, Livonia, MI). Mussels were transported in a cooler with ice to the lab where dead mussels and shell fragments were removed; and live mussels were maintained in 10L glass aquariums filled with aerated and dechlorinated tap water. Once a week, the tanks were cleaned, dead mussels removed, and the water refreshed. The tanks were housed in an environmental growth chamber at 19±1 °C with a 12 h: 12 h light-dark cycle. All organisms were adequately fed an algal mixture of *Ankistrodesmus falcatus* and *Chlamydomonas reinhardtii*.

Laboratory bioassay 1: PCB toxicity of aqueous exposure to D. pulex

Toxicity of the site water was assessed with a modified planktonic *D. pulex* bioassay (Standard Method 8711) (American Public Health Association et al. 2005).

Two treatments consisting of forty 50 mL jars ($n = 40$) were filled with either a control treatment of artificial lake water (Kilham et al. 1998) or the exposure treatment of water collected from Site L1 (PCB concentration $1.3 \mu\text{g/L}$). Both treatments contained a 50/50 algae mixture of 2.0×10^6 cells/mL as a food source for the *D. pulex*. The jars were randomized and allowed to equilibrate overnight before adding *D. pulex*. One *D. pulex* was added to each assay jar and maintained at $20 \pm 1^\circ\text{C}$ with a 12h light: 12h dark cycle in an environmental growth chamber. Mortality was recorded daily for seven days. The assay media was replaced with fresh solutions on day four of the experiment to refresh the food source, maintain consistent contaminant levels, and remove waste.

Laboratory bioassay 2: PCB toxicity of the sediment-water interface to D. pulex

Toxicity of PCBs at the sediment-water interface was assessed using the planktonic *D. pulex* exposed to a treatment that consisted of contaminated sediment overlaid with non-contaminated artificial lake water. As such, the potential toxicity to the *D. pulex* would occur via the release of PCBs from the sediment into the overlying water. The bioassay followed a similar protocol to bioassay 1 (Standard Method 8711) (American Public Health Association et al. 2005). The experimental design consisted of a control treatment (100 mg of benzonite clay; Sigma) and two exposure treatments (50 mg and 100 mg of contaminated sediment from Site L1, $610 \mu\text{g/g}$). The sediment was allowed to settle out of solution for eight hours prior to adding one *D. pulex* to each jar. Mortality was recorded daily for five days. When *D. pulex* mortality occurred, the time to death was recorded in days and the jar was removed from the experiment.

Laboratory bioassay 3: PCB toxicity of sediments on *C. dilutus*

The toxicity of the sediment to benthic organisms was assessed using a bioassay exposing the benthic *C. dilutus* (second instar) to sediments collected from six sites in the canals along a gradient of PCB concentrations ranging from 30 to 1,100 µg/g (Figure 1; Table 1), plus a control treatment. The experimental design was similar to the standard methods 8750C and 8010F (American Public Health Association et al. 2005), however, the exposure chambers were designed to follow the *Daphnia* assay setup so as to better correlate the sediment versus aqueous PCB exposure, and the experiment would conclude upon reaching 100% mortality in the highest treatment concentration. Each treatment consisted of 12 replicates and were prepared by making a slurry with a 4:1 volume to mass ratio of water to sediment (100 g of sediment with 400 mL of oxygenated, dechlorinated water), allocating 25 mL into each 50 mL assay jar. The sediment slurry was continuously stirred to ensure that the sediment to water ratio was consistent in all assay jars. For the control sediment, I used clean, autoclaved sandbox sand (assumed to be free of PCB contamination) to allow burrowing by *C. dilutus*. Fifteen grams of sand was added to 25 mL of control water to provide a sediment depth comparable to the treatments. The jars were randomized and maintained in a cold room (4°C) for 24 hours in complete darkness to prevent photoreactivity of the PCBs and to allow the sediments to settle out of solution. Prior to the addition of *C. dilutus*, the jars were placed in an environmental growth chamber to reach the assay temperature of 22°C.

One active *C. dilutus* was inserted into each jar. All jars were visually checked to ensure that *C. dilutus* were not floating on the water surface. The experiment was

placed in an environmental growth chamber at $22\pm 1^{\circ}\text{C}$ with a 16h light: 8h dark cycle. Assay jars were checked for *C. dilutus* mortality on days 3, 4, and 5 by pouring the jar contents into a large glass plate placed over a light table, and visually searching for the organisms. A *C. dilutus* was considered dead if it did not move upon gentle prodding and/or showed signs of decay. Any missing organisms were also considered dead, as no adult *C. dilutus* were observed. If the organism was alive, the entire contents were poured back into the corresponding assay jar. Dechlorinated water was used to refill the assay jars back to the 25mL mark if water was lost because of evaporation or during the transfer. All jars were aerated every 24 hours by placing a glass pipette under the surface of the water and gently creating bubbles for one minute, without re-suspending the sediments.

Field experiment: effects of proximity to PCB-laden sediment on D. bugensis

To assess the effect of PCBs on *D. bugensis* at the sediment-water interface and throughout the water column, *D. bugensis* were placed in cages at two depths in the water column at an impacted site (Figure 1; Lange Canal at Site L2, 1,100 $\mu\text{g/g}$) and reference site (Shorewood Canal, located 2.33 km upstream of the PCB contaminated canals). Cages (20x20x10cm) were made of 0.6-cm wire mesh to allow maximum exposure to the environment while preventing the mussels from escaping. Forty pre-measured mussels (10-30 mm in length) were placed in cages attached to vertical bars. Seven cages were submerged just below the water-surface and seven cages were suspended less than 5 cm above the sediment. The caging unit was secured to a seawall. Mussel mortality was quantified for each cage on days 1- 5, and 7.

Physicochemical parameters (water depth, pH, dissolved oxygen, temperature, and conductivity) were measured daily at each site by placing probes next to the caging units at a depth approximately halfway between the water-surface and sediment cages. Alkalinity and hardness were measured at the beginning and end of the experiment. On day seven, the cage units were removed from the water and the remaining mussels were counted.

Statistical analysis

The laboratory bioassays experimental design allowed analysis of individual mortality, without confounding effects from space or nutrient competition; therefore, there was no concern for error within or between replicates. Fisher's Exact Test was used to compare the categorical data (i.e., alive or dead) among the treatments for the three laboratory experiments. For the field experiment a Pearson's chi-squared test, with a Yate's continuity correction for small data values, was used to compare dreissenid mortality between the impacted and reference sites, and sediment versus water surface exposure at each site. Differences among the treatment replicates ($n = 7$) at each site were determined using the chi-squared analysis, allowing us to pool data from replicates that were not statistically different. All statistical analyses were conducted using IBM SPSS Premium Version 20 (SPSS Inc., 2011) with $\alpha = 0.05$.

RESULTS

PCB analysis and grain size distribution

Water from the impacted site L2 had a total PCB concentration of 1.3 µg/L (Table 1). Sediment PCB concentrations ranged from 30 µg/g to 1,100 µg/g total PCBs, with the highest concentrations obtained from samples collected at sites L1 and L2, near the mouth of the storm drain implicated as the point source of the PCB contamination for the canals (Table 1; Figure 1). My analysis in 2010 indicated that Aroclor 1242 was the only PCB mixture present in all samples, and all other Aroclor mixtures were below detection limits; however, a report by the USEPA in 2011 shows that Aroclor 1016, 1248, and 1260 may also be present (Jacobson et al. 2012). Grain size distributions of sediment samples collected at sites L1 and L3 had a grain size ranging between 4 and 65 µm (Figure 2), characteristic of fine-silty sediments.

Table 1. *Measured PCB concentrations and Global Positioning System (GPS) coordinates for sediment and water collection sites in the Lange and Revere Canals, Lake St. Clair, Michigan, USA. PCB sediment and water concentrations were determined at Paragon Laboratories (Livonia, MI) using EPA Standard Method 8082A.*

| Site | PCB sediment concentration (µg/g) ^a | Location | |
|------------|--|-------------------|-------------------|
| | | Latitude | Longitude |
| L1 | 610 | 42° 28' 36.761" N | 82° 53' 18.977" W |
| L2 | 1100 | 42° 28' 35.724" N | 82° 53' 18.902" W |
| L3 | 30 | 42° 28' 33.496" N | 82° 53' 04.602" W |
| R4 | 510 | 42° 28' 34.493" N | 82° 53' 18.865" W |
| R5 | 360 | 42° 28' 33.535" N | 82° 53' 18.004" W |
| R6 | 100 | 42° 28' 32.682" N | 82° 53' 14.650" W |
| Water (L2) | 1.3 µg/L | 42° 28' 35.724" N | 82° 53' 18.902" W |

^a PCB concentrations are expressed as total PCBs

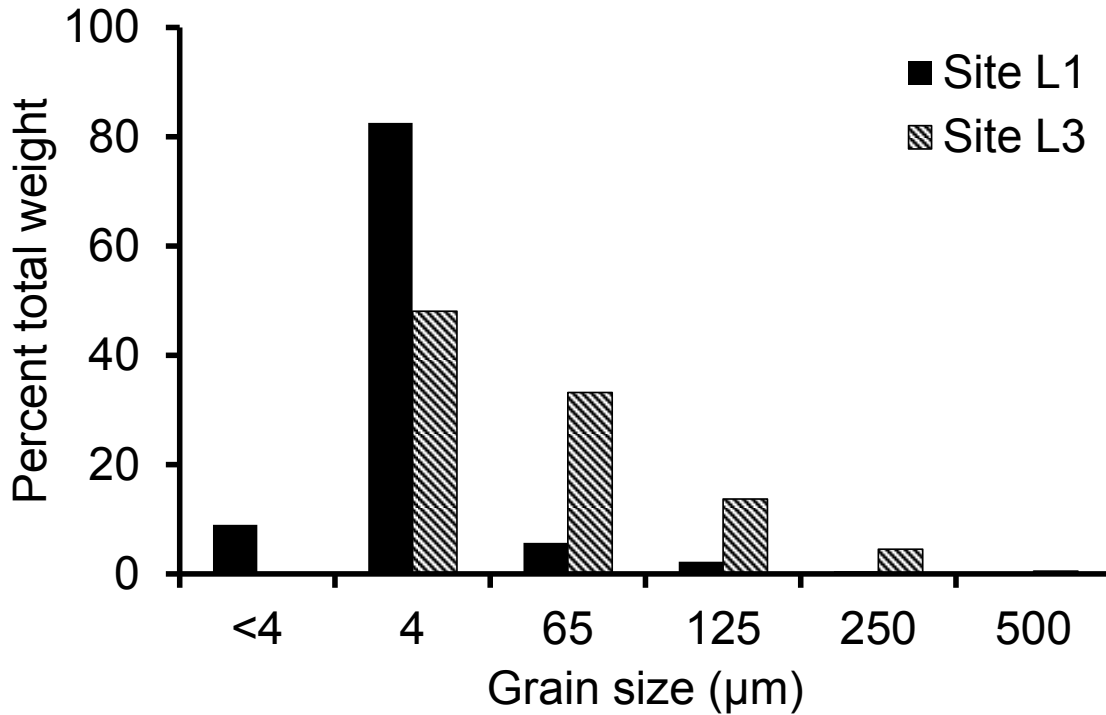


Figure 2. Grain size distribution for sediments collected in the Lange Canal (Lake St. Clair, Michigan) at sites L1 and L3. Each fraction was weighed and divided by the total weight of the entire sample.

Laboratory bioassays

Water from the contaminated canal had no significant effect on *D. pulex* mortality during the seven day assay (Figure 3A), but *D. pulex* in jars with 50 mg or 100 mg of sediment had significantly higher mortality (Figure 3B). *D. pulex* exposed to 50 mg of sediment had increased mortality after three days of exposure ($p = 0.002$; Figure 3B), and organisms exposed to 100 mg of sediment experienced increased mortality in as little as two days ($p = 0.001$; Figure 3B). On day 2, > 85% of the 100 mg treatment organisms died, while < 2% of the control had died (Figure 3B). Both treatments had a continued increase in mortality after three days until the end of the experiment ($p < 0.001$; Figure 3B). However, *D. pulex* mortality in the 100 mg treatment was significantly higher than the 50 mg treatment at day 2 ($p = 0.050$), day 3 ($p < 0.001$), and day 4 ($p =$

0.001). At the end of the five day assay, both sediment treatments had similar mortality ($p = 0.108$; Figure 3B).

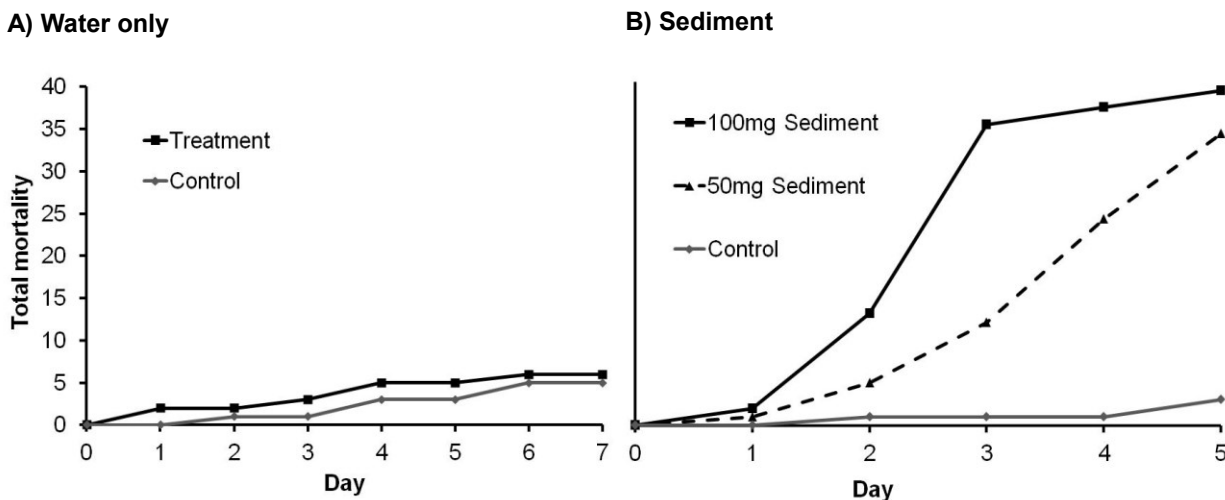


Figure 3. Total mortality of *D. pulex* exposed to A) PCB site water alone and B) PCB site sediments. For the sediment bioassay, organisms were exposed to either 100 mg of PCB-laden sediment (solid black line) or 50 mg of sediment (dotted line).

Sediment exposure also significantly increased *C. dilutus* mortality compared to the control. The assay concluded on day 5 due to complete mortality of *C. dilutus* exposed to the highest PCB sediment concentration (1100ppm). *Chironomus dilutus* in sediments with PCB levels of 610 $\mu\text{g/g}$ experienced significant mortality compared to the control after five days of exposure ($p = 0.012$; Figure 4C). Sediment PCB concentrations of 30, 100, 360, and 510 $\mu\text{g/g}$ had no significant effect on mortality compared to the control throughout the experiment (Figure 4). By day 3, 83% of *C. dilutus* died when exposed to the most contaminated sediment (1,100 $\mu\text{g/g}$; $p = 0.001$; Figure 4A), and by day 4 all organisms in the 1,100 $\mu\text{g/g}$ exposure had died (Figure 4B).

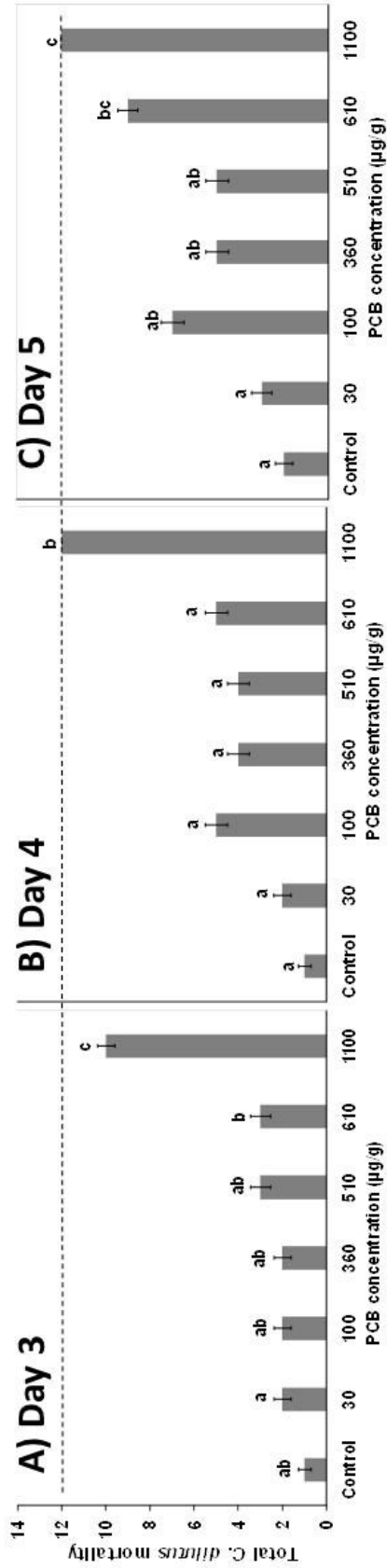


Figure 4. Percent mortality for *C. dilutus* exposed to increasing concentrations of PCB contaminated sediment collected from the Lange and Revere canals (St. Clair Shores, MI, USA) during a five day toxicity assay. Mortality was quantified on days 3, 4, and 5. Bars with the same letters were not significantly different ($\alpha = 0.05$) as determined by the Fisher's Exact Test comparing categorical mortality data among treatments. Dotted line indicates maximum mortality. Error bars represent standard deviation among treatments ($n=12$).

Field experiment

Temperature, pH, and conductivity were comparable at the impacted and reference sites, but I observed lower dissolved oxygen at the impacted site on days 1, 2, 4, 5, and 7 (Table 2). There was significantly higher mortality of *D. bugensis* at the impacted site compared to the reference site both at the surface waters ($p < 0.001$) and near the sediments ($p < 0.001$; Figure 5). After seven days, a total of 94% of *D. bugensis* survived at the reference site compared to only 57% at the impacted site. Furthermore, at the impacted site the *D. bugensis* maintained at the water surface had significantly less mortality compared to the mussels in closer proximity to the sediments ($p < 0.001$; Figure 5). At the reference site, there was no significant difference in mortality between *D. bugensis* near the surface compared to those near the sediment (Figure 5).

Table 2. Physicochemical parameters measured at the impacted Lange Canal (I) and reference canal (R) (both located in St. Clair Shores, MI, USA) during the seven day *D. bugensis* PCB experiment.

| Day | Water depth (m) | | pH | | Dissolved oxygen (mg/L) | | Water temperature (°C) | | Conductivity (µS/cm) | |
|-----------|-----------------|------|------|------|-------------------------|------|------------------------|------|----------------------|-----|
| | I | R | I | R | I | R | I | R | I | R |
| 0 (setup) | 0.99 | 0.69 | 7.64 | 8.49 | 7.49 | 8.59 | 25.3 | 25.7 | 371 | 393 |
| 1 | 0.99 | 0.70 | 7.51 | 8.39 | 6.03 | 8.15 | 26.7 | 26.3 | 398 | 390 |
| 2 | 0.99 | 0.69 | 7.32 | 7.61 | 4.04 | 5.61 | 23.6 | 23.6 | 420 | 403 |
| 3 | 0.99 | 0.71 | 7.75 | 7.78 | 7.03 | 6.40 | 24.4 | 24.0 | 378 | 405 |
| 4 | 0.98 | 0.69 | 7.29 | 7.67 | 4.10 | 6.06 | 23.2 | 22.5 | 399 | 407 |
| 5 | 0.99 | 0.69 | 7.88 | 8.12 | 4.70 | 7.80 | 23.9 | 23.4 | 410 | 403 |
| 7 | 0.98 | 0.69 | 7.62 | 8.08 | 6.15 | 6.18 | 24.8 | 25.2 | 388 | 415 |

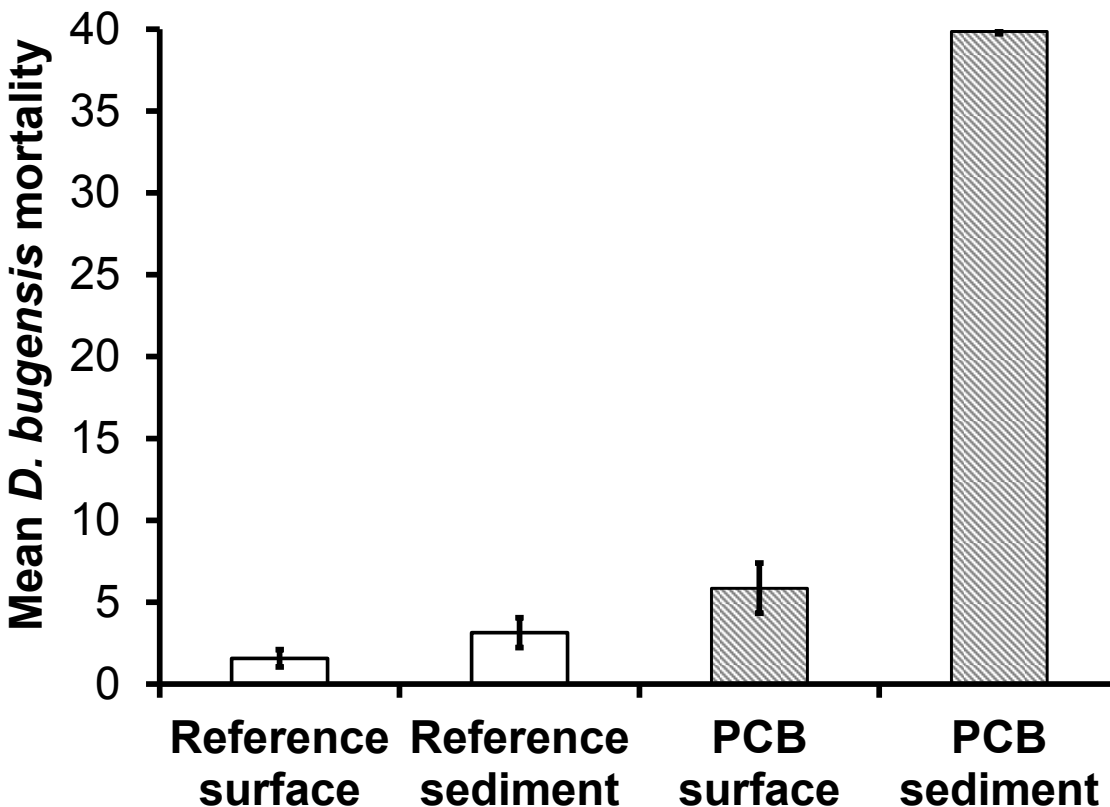


Figure 5. Mean mortality for *D. bugensis* transplanted into the Lange (PCB) and reference canals (St. Clair Shores, MI, USA) at both the water surface and in the sediments at the end of the 7-day field study. Error bars represent standard error ($n=7$).

DISCUSSION

Comparison of aqueous and sedimentary PCB exposure

Many studies of PCB effects on aquatic organisms independently examine aqueous or sedimentary exposures. The present study provides a novel comparison of PCB effects through both sediment and aqueous exposure, with a focus at the sediment-water interface. Coupled lab and field experiments support a body of literature identifying PCB contaminated sediments as the primary source of PCB toxicity in aquatic environments (Konat and Kowalewska 2001, Ho and Liu 2010). I further document that mortality increases with increased proximity to sediments.

Due to the potential for higher PCB concentration in the sediment as compared to the water column, sedimentary rather than aqueous PCBs present a much greater, environmentally relevant threat. This is supported by my results which show a greater toxicity of sediments with higher PCB concentrations in the *C. dilutus* assays, and other research highlighting the bioaccumulation and toxicity of PCBs in sediment-exposed chironomids and *Daphnia* (Santiago et al. 1993, Wood et al. 1997). Wood et al. (1997) found that bioaccumulation of sediment associated PCBs in *C. dilutus* can be three to ten times higher than other organic contaminants (i.e., polycyclic aromatic hydrocarbons). Another study found PCB concentrations in the range of 21.9-39.5 ng/g can significantly impact growth of a comparable midge species, *Chironomus riparius* (Bettinetti et al. 2003). Similar results have also been observed in other benthic-dwelling invertebrates placed on PCB contaminated sediments, such as the burrowing mayfly (*Ephoron virgo*) (de Haas et al. 2002).

Aqueous exposure can also be a significant toxicity concern as Nakari and Huhtala (2008) and Dillon and Burton (1991) found the acute toxicity of aqueous PCB exposure to *D. pulex* to be between 12.5 and 100 µg/L. These studies help explain why there was no significant mortality of *D. pulex* from the contaminated site with PCB water concentrations of 1.3 µg/L (Figure 3A). However, laboratory toxicity test may not always reflect the impacts contaminants have on individual species in their natural environment. For example, site water containing 1.3 µg/L PCBs in the laboratory experiments did not affect *D. pulex* mortality, but dreissenid mussels - considered to be more tolerant to contaminant exposure - experienced higher mortality rates in the field than when exposed to the same water in the laboratory. Polychlorinated biphenyl lethal

concentration (LC50) values in water reported for *D. pulex* range from 2.6 - 253 µg/L for 14d and 21d toxicity tests (Nebeker and Puglisi 1974), but LC50 values may not always reflect the impacts contaminants have on individual species in their natural environment. The lack of mortality of *D. pulex* exposed to site water alone is likely explained by PCB exposure below this LC50, but longer exposure times may have adverse effects on *D. pulex* survival, as other studies have found that lower aqueous PCB concentrations can impact *D. pulex* survival (Dillon and Burton 1991). In the present study, the mussels near the surface of the water and near the sediments at the impacted site may have been subjected to natural re-suspension events (i.e., storm or boating events) that would increase their exposure to contaminated sediments in the water column. The smaller grain-sized sediment particles (typically between 4 and 64µm) at the PCB contaminated sites (Figure 2) increases the likelihood of resuspension, contributes to the transport of PCBs, and increases the potential for ingestion by benthic and planktonic invertebrates, resulting in negative impacts on the aquatic ecosystem and local food web.

Planktonic invertebrates may have the ability to reduce their overall exposure with movement in the water column to areas of lower PCB concentrations, since PCB concentrations in the water column are much lower than in the sediments. This "escape-like behavior" has been documented in *D. pulex* with the insecticide carbaryl (Dodson et al. 1995). Benthic invertebrates may be at an increased risk to PCB contaminations because they dwell primarily in the sediments where physical contact is greatest and PCB concentrations are higher. Furthermore, many benthic organisms are detritivores and directly ingest sediment, making them highly vulnerable to sediment toxins, or

indiscriminately filter feed taking in suspended sediment particles (Smock 1983). In my study, dreissenid mussels experienced greater mortality even when placed just above the contaminated sediment compared to those placed higher in the water column.

Even if benthic organisms generally experience higher PCB concentrations than planktonic organisms, planktonic organisms may still be exposed to highly concentrated amounts of PCBs through their bioaccumulation via the food web (Magnusson and Tiselius 2010), or by direct contact at the sediment-water interface or through re-suspension of the sediments into the water column. For example, many planktonic invertebrates undergo diurnal vertical migration where they sometimes "bury" into the top sedimentary layers (i.e., amphipods) or hover at the sediment-water interface (Doig and Liber 2010), where they can be exposed to PCB concentrations that are higher than further up in the water column.

With a half-life range of 4.4 to 465 years, depending on the medium (e.g. water, sediment) and specific PCB composition (Sinkkonen and Paasivirta 2000), the persistence of PCBs in sediments increases their potential for severe impacts. Notably, toxicity testing for site evaluations may use water collected higher in the water column to avoid "contaminating" samples with sediment, and therefore may overlook the full suite of environmental implications as many organisms interact with both the surface and bottom waters. As such, this omission from toxicity testing likely leads to underestimates of actual impacts of exposures to PCBs in the environment.

Several factors can affect the partitioning of PCBs in the sediments and the water column, which ultimately impacts the PCB toxicity to aquatic organisms. The molecular structure of PCBs, including the molecular size and net atomic charge of attached

chlorine, hydrogen and carbon atoms, is a major factor that can affect partitioning of PCBs from the sediments to the water column (Chen et al. 2003). Similarly, environmental factors such as temperature (Chen et al. 2003) and sediment characteristics also affects PCB partitioning. For example, some studies have shown that carbon concentration and type can affect the distribution of PCBs depending on the congener (Hung et al. 2006).

Because PCB concentrations at the study site are in the parts per thousand range, similar to a few other high impact Superfund sites (Quantitative Environmental Analysis LLC 2007), the mortality to *D. bugensis* at the impacted site is likely attributed to PCBs despite lower dissolved oxygen levels at the contaminated site or the possibility of other chemical contaminants present in the sediments. This was supported by the lab studies where *C. dilutus* exhibited higher mortality from the sediments at the impacted site despite constant aeration of all treatments. The Center for Disease Control Agency for Toxic Substances and Disease Registry (ASTDR) and U.S. Geological Survey have reported other contaminants such as lead, PAHs, and other trace elements in sediments near the canals and in Lake St. Clair (Rachol and Button 2006, Agency for Toxic Substances and Disease Registry 2009); however these data do not include the same sampling sites specific to this study. Given that most urban and industrially-influenced waterways may be contaminated with multiple chemicals from many different point and non-point sources, it is possible that additional chemicals may be at levels above toxicity thresholds in these canals; however, the USEPA considers PCBs to be the sole contaminant of concern in these canals, given that 80% PCBs (800,000 mg/L)

was removed from the drain leading to the canals and USEPA sampling results support a singular focus on PCBs (U.S. Environmental Protection Agency 2009).

Ecological and remediation implications

My results demonstrate that sediment-associated PCBs can impact organism survival throughout the entire water column. Furthermore, PCB-laden sediment may affect habitat selection and distribution of benthic and planktonic invertebrates. Previous research has shown that in the environment freshwater invertebrates will preferentially select uncontaminated sediment over contaminated sediments (De Lange et al. 2006). I speculate that PCB-laden sediments might result in dreissenid mussels selecting habitats higher in the water column, which can include anthropogenic structures such as drinking water intake pipes, and seawalls, and may result in losses in revenue associated with cleaning.

Timely and successful restoration efforts can reduce the spread of PCB-laden sediments to additional locations. Sediment PCB levels for the canal study were assessed before approximately 17 weirs (small dams) were placed along the storm sewer drain to trap PCB-laden sediments and prevent them from entering the canals. In August 2012, I found that sediment PCB concentrations have nearly doubled in the canals (2100 µg/g; Paragon Laboratories, Livonia, MI, USA; unpublished data), since the remediation efforts. Consequently, it appears the weirs were ineffective in stopping sediment migration into the canals and preventing further sediment contamination.

My research indicates that in addition to evaluating contaminant levels in the water column, remediation efforts should consider the potentially toxic nature of the

sediment-water interface when evaluating a site. Toxicity tests performed from waters collected at the surface may underestimate the toxicity of waters closer to the sediments; likewise, sediment toxicity tests may overestimate toxicity to organisms that reside primarily in the sediment-water interface, such as sessile organisms attached to rocks that do not typically bury in the sediments. I recommend analyzing water at the sediment-water interface in conjunction with traditionally planned evaluations to obtain a more complete characterization of toxicity throughout the water column and to help set remediation goals.

CHAPTER 2

COMPARING OXIDATIVE STRESS IN DREISSENID MUSSELS UNDER DIFFERENT ENVIRONMENTAL STRESSORS AND THE ROLE OF OXIDATIVE STRESS IN ENVIRONMENTAL TOLERANCE

BACKGROUND

Community level assessment of benthic organisms has long been used to assess the health of surface waters (Hilsenhoff 1987, Swift et al. 1996, Wesolek et al. 2010). However, current assessment methods have several limitations. First, once impacts are observed at the community level, damage to the system has already occurred. Ideally, a monitoring or assessment tool should detect negative impacts prior to changes occurring at the community level. Second, traditional methods that evaluate toxicity use basic toxicity bioassays including endpoints such as mortality, modified behavior, or changes in reproduction. These traditional methods do not address the molecular or physical mechanisms of environmental stress that may lead to lethal outcomes. Biomarkers may be a useful tool to address these limitations, capturing impacts prior to observed changes in the community, and demonstrating the molecular effects of these impacts. The oxidative stress response is a mechanism by which aquatic organisms mitigate environmental stressors, such that monitoring this response through biomarker analysis may be a useful, early-detection tool for biomonitoring aquatic habitats and evaluating organism tolerance to environmental stressors.

Most living organisms depend on oxygen for metabolic and energy processes; however, oxygen can also cause cellular damage by inhibiting enzymes and developing

reactive oxygen species (Balentine 1982, Halliwell and Gutteridge 1984). Reactive oxygen species (ROS) are highly reactive derivatives of oxygen formed during aerobic metabolism and enzymatic reactions in the body, specifically during times of stress. If ROS are not eliminated then they can readily react with other cellular components and cause cellular damage (McCord 2000). Organisms have evolved mechanisms for inducing antioxidant production to remove damaging oxygen radicals and protect against oxidative damage (Martins et al. 1991). Organisms that can rapidly up-regulate antioxidants may be more tolerant of environmental stressors than organisms with slower and less robust antioxidant defense mechanisms.

Oxidative stress is used as a biomarker for environmental stress in terrestrial and marine ecosystems (Rico et al. 2009). In terrestrial ecosystems, heavy metals may cause oxidative stress in protozoa, birds and snails (Berglund et al. 2007, Rico et al. 2009, Radwan et al. 2010, Hegseth et al. 2011). A meta-analysis on oxidative stress studies of terrestrial animals revealed an overall increase in oxidative stress when an organism is exposed to a pollutant (Isaksson 2010). In marine and freshwater systems, waters contaminated from industrial practices are linked to oxidative stress in mussels (Livingstone et al. 1992, Verlecar et al. 2008), sea urchins (Corsi et al. 2011), aquatic insects (Barata et al. 2005, De Block and Stoks 2008), and fish (Nunes et al. 2008, Padmini and Rani 2009). Physical stressors also may contribute to oxidative stress in aquatic animals. For example, an increase in temperature may cause an increase in oxygen consumption and metabolism, resulting in increased ROS byproducts (Lushchak 2011). Similar toxicological responses to oxidative stress in mammalian and fish model systems suggests that both of these models can be useful for understanding

the mechanisms of oxidative stress (Kelly et al. 1998); however, freshwater invertebrate models have yet to be examined.

Invasive dreissenid mussels have previously been used as important bioindicators for freshwater contaminations (Kraak et al. 1991, de Lafontaine et al. 2000, Camusso et al. 2001, Binelli et al. 2004, Richman and Somers 2010) and may serve as a potential model for examining the role of oxidative stress in environmental tolerance. *Dreissena polymorpha* and *D. bugensis* have similar ecological niches and are significant pest species due to their high filtration rates, high fecundity, and competitive advantages over native species in both the Great Lakes (Heath et al. 1995, Wisenden and Bailey 1995, Ricciardi et al. 1996, Dermott and Kerec 1997, Bially and MacIsaac 2000, Vanderploeg et al. 2002) and in Europe (Karatayev et al. 1997, Imo et al. 2010, Bodis et al. 2014), and their contribution to contaminant cycling in the water column (Bruner et al. 1994). While *D. polymorpha* were the first to establish in the Great Lakes in significant numbers (Hebert et al. 1989), in the last decade *D. bugensis* has become the dominant dreissenid mussel in several regions of the Great Lakes (Mills et al. 1999, Ricciardi and Whoriskey 2004). Numerous studies have tried to elucidate the cause of this displacement, including characteristics such as body size and respiration rates (Stoeckmann 2003, Karatayev et al. 2011), tolerance to depth and temperature changes (Mitchell et al. 1996), habitat preference, or differences in filtration rates (Diggins 2001); however, very few studies have addressed differences in tolerances between the two species at the molecular level.

Several studies in *D. polymorpha* reveal the success of using oxidative stress to evaluate chemical toxicity for a wide range of chemical pollutants including polyaromatic

hydrocarbons, pesticides, heavy metals, and pharmaceuticals (Faria et al. 2009, Riva et al. 2010, Parolini et al. 2011). However, studies on *D. bugensis* could not be found and experiments comparing oxidative stress in the two dreissenid species have yet to be done. The present study examines the mechanisms of the oxidative stress response to identify a correlation between tolerance to environmental stressors and oxidative stress in *Dreissena bugensis* and *D. polymorpha*.

METHODOLOGY

To examine the role of oxidative stress in environmental tolerance, I performed a series of assays in which dreissenid mussels were analyzed for oxidative stress after exposure to 1) high population densities, 2) a chemical stressor (polychlorinated biphenyls: PCBs), 3) extreme temperatures, and 4) hypoxia. The length of all experimental assays served as a proxy for the severity of the stressor and typically ended when significant mussel mortality was observed.

Mussel collection

Dreissena bugensis were collected off fishing piers on Belle Isle in the Detroit River, Michigan (42°21'6.05"N, 82°58'12.09"W) on multiple days in September-October 2013, and *D. polymorpha* were collected from the upper Rouge River in Livonia, Michigan (42°22'0.09"N, 83°24'37.06"W) during one sampling event in October 2013. These sites are approximately 36 km apart. Physicochemical parameters (dissolved oxygen, pH, conductivity, and temperature) were measured at each collection site (Table 3). Chemical analysis of sediment and water using USEPA standard methods

3510C and 8082A indicate PCB concentrations below detection limits at both collection locations (U.S. Environmental Protection Agency 2011) (Paragon Laboratories, Livonia, MI, USA; Table 3).

Mussels were transported on ice to the laboratory where dead mussels and shell fragments were removed; live mussels were lightly cleaned and placed in aerated 10-liter glass aquariums filled with dechlorinated tap water at a density of 1 mussel per 40-45 ml of water. The tanks were maintained in an environmental growth chamber at 20 ± 1 °C with a 12 h: 12 h light-dark cycle. Once a week, the tanks were cleaned, dead mussels removed, and the water refreshed. All organisms were fed a 50/50 algal mixture of *Ankistrodesmus falcatus* and *Chlamydomonas reinhardtii*. Mussels were maintained in stock tanks for at least three weeks before use in experiments.

Prior to the start of all experiments (Day 0), 20 mussels from stock tanks of each species were extracted, rapidly dissected, and the tissue combined into four pools of five mussels each. The pooled tissue was then measured for oxidative stress (described below) to establish baseline values for lipid peroxidation and antioxidant levels (catalase) for each species. These Day 0 measurements will be referred to as baseline values.

Table 3. *Dreissena bugensis* and *Dreissena polymorpha* were collected from two locations in the Great Lakes basin in September-October 2013 for use in environmental stressor studies. The location, physicochemical parameter measurements, and sediment and water PCB concentrations are listed for each site. *Dreissena bugensis* was collected on multiple days and therefore physicochemical measurements are expressed as the average \pm standard deviation.

| | <i>Dreissena bugensis</i> | <i>Dreissena polymorpha</i> |
|--|--|---|
| Location | Detroit River, Belle Isle, MI (42°21'6.05"N, 82°58'12.09"W) | Rouge River, Livonia, MI (42°22'0.09"N, 83°24'37.06"W) |
| Dissolved oxygen (mg/L) | 10.34 \pm 0.982 | 9.25 |
| Temperature (°C) | 14.84 \pm 6.726 | 12.3 |
| Conductivity (μ S/cm) | 292.2 \pm 155.2 | 773 |
| pH | 8.249 \pm 0.489 | 8.65 |
| Sediment PCB concentration (μ g/Kg) | < 330 (detection limit) | < 330 (detection limit) |
| Water PCB concentration (μ g/L) | < 0.10 (detection limit) | < 0.10 (detection limit) |

Stressor Assay #1: Density

To examine the effects of density on oxidative stress in dreissenid mussels, mussels of each species (15-25 mm in length) were placed in 800 ml glass jars filled with 360 ml of aged tap water with three different mussel density treatments (N = 16): low (9 mussels/360 ml); medium (18 mussels/360 ml); and high (36 mussels/360 ml). The medium treatment was used as a control because mussel stock tanks were maintained at the identical density (18 mussel/ 360 ml). Experiment jars were equipped with air stones and placed in an environmental growth chamber maintained at 20 \pm 1°C with a 12h:12h light-dark cycle. The assay was run for 12 days; mussels were sampled

on days 1, 6, 7, and 12, by removing a subset of jars ($n = 4$) from each treatment for each species and analyzed for oxidative stress following methods described below. After day 6 sampling, 30 pre-marked mussels (15-25 mm in length) were added to each assay jar to add an additional level of density stress.

Stressor Assay #2: PCBs

Water was collected from a known PCB-contaminated site in St. Clair Shores, Michigan (42°28'36.83"N, 82°53'18.77"W) using fifteen 1 L glass amber jars with Teflon® lids at approximately 0.25 m below the surface of the water, in an area with a water depth of approximately 1-1.5 m. The jars were stored in a dark cold room (4°C) for up to two weeks until use in the experiments. Chemical analysis using USEPA standard methods 3510C and 8082A (U.S. Environmental Protection Agency 2011) (Paragon Laboratories, Livonia, MI, USA) revealed total PCB concentrations at 0.73 µg/L in the water.

During a 7-day assay, experimental jars were filled with either 400 ml of PCB water (1.3 µg/l), or 400 ml of control water (aged-tap water). Thirty mussels were placed in each jar so that half of the treatment jars contained *D. bugensis* and the other half contained *D. polymorpha* ($N = 12$). Mussels used in the experiment were 15-25 mm in length. All experiment jars were aerated with air stones and placed in an environmental growth chamber maintained at $20 \pm 1^\circ\text{C}$ with a 12h:12h light-dark cycle. A subset of jars ($n = 4$) was removed from each treatment for each species and analyzed for oxidative stress on days 1, 2, and 7 following methods described below.

Stressor Assay #3: Temperature

To examine the effects of temperature on the dreissenid oxidative stress response, *D. polymorpha* and *D. bugensis* were placed in experimental growth chambers set at three different treatment temperatures to create three experimental treatments: 1) low (5°C), 2) ambient (20°C), and 3) high (29 °C). Ten mussels of either *D. polymorpha* or *D. bugensis* (15-25 mm in length) were placed in the jars so that half of the treatment jars contained *D. bugensis* and the other half contained *D. polymorpha* at each treatment temperature. All assay jars contained 400 ml of aged tap water and were equipped with air stones. At the start of the experiment, all chambers started at the ambient temperature, and then were slowly increased or decreased by 4-6°C each day for three days, until reaching the final treatment temperature. Mussels were sampled every day for oxidative stress by removing a subset of jars (n = 4) from each treatment for each species and analyzed for oxidative stress following methods described below. The assay was concluded on day 4 due to observed mortality (1-2 mussels) in some of the high temperature treatment jars.

Stressor Assay #4: Hypoxia

The effect of hypoxia on mussel oxidative stress was examined in sealed oxygen chambers equipped with gas intake and outlet ports, and a water sampling port (Figure 6). Oxic treatment chambers were created by aerating chambers with filtered (0.2 µm EMD Millipore Millex filter) room-air and had a consistent dissolved oxygen reading of 9-10 mg/l. The hypoxic treatment chambers were aerated with filtered (0.2 µm EMD Millipore Millex filter) nitrogen gas, and oxygen levels never exceeded 3 mg/l.

Experimental chambers were placed in an environmental growth incubator and maintained at $20 \pm 1^\circ\text{C}$ with a 12h:12h light-dark cycle. Prior to the start of the assay, 2 L of aged tap water was added to each treatment chamber and allowed to aerate for 24 hours before the mussels were added. Thirty mussels of *D. bugensis* or *D. polymorpha* (15-25 mm in length) were placed in each treatment chamber. Physicochemical water parameters were monitored every 24 hours by removing 200 ml of water from the water sampling port and using a multi-parameter probe (YSI 556 Multi-Parameter Sensor) to measure dissolved oxygen, temperature, pH, and conductivity. Chamber water was replenished with aerated aged tap water stored at $20 \pm 1^\circ\text{C}$. The assay lasted for 4 days and mussels were sampled ($n = 4$) for oxidative stress on day 4.

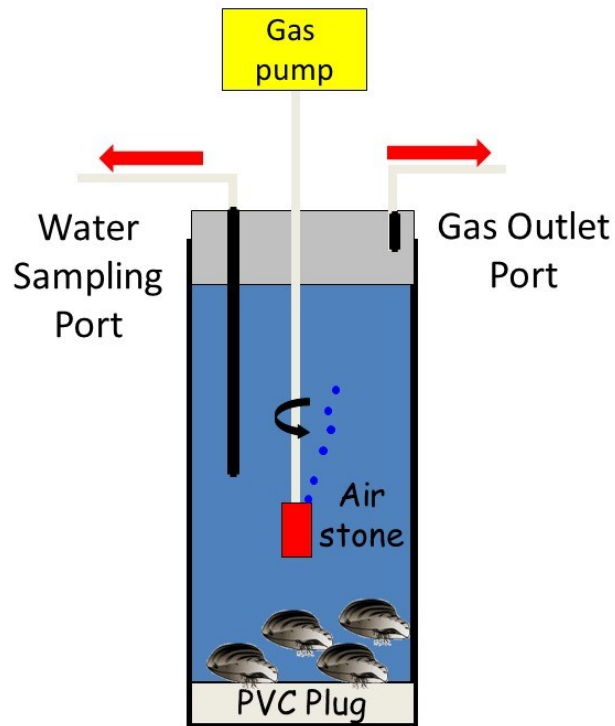


Figure 6. Diagram of treatment chamber setup for examining oxidative stress in *D. polymorpha* and *D. bugensis* exposed to hypoxic and oxic conditions. Chambers were sealed on both ends and equipped with a gas inlet and outlet ports, and a water sampling port.

Tissue preparation

On each sampling day, replicate jars of each species from each treatment were removed and five mussels were randomly selected from each jar for analysis. Immediately after collection, total mussel weight was measured and the soft tissue of each mussel was dissected and weighed. The soft tissue of all five mussels was pooled, immediately frozen in liquid nitrogen, and stored at -80°C until further analysis.

Mussel tissue was digested by grinding the frozen tissue for 2 minutes with cold mortar and pestle placed on ice. The ground tissue was then homogenized in a 1:5 weight: volume ice cold phosphate buffer (100 mM, pH 7.4, containing 150 mM KCl and 1 mM EDTA) (Faria et al. 2009) by grinding again with a mortar and pestle (1 min). Homogenized tissue was carefully transferred to 15 ml centrifuge tubes and centrifuged at 10,000 g for 30 minutes. The supernatant was then directly used for protein and biomarker analysis.

Protein analysis

To standardize biomarker measurements for mussels of different sizes and species, total protein concentrations were determined for each pooled mussel sample using a Coomassie Plus (Bradford) Assay Kit (Thermo Scientific). Tissue samples were diluted with 100 mM phosphate buffer prior to analysis. Samples were analyzed in triplicate at 595 nm on a 96-well microplate reader and plotted on a standard curve to determine the total protein concentration of each sample.

Measuring oxidative stress: Lipid peroxidation and catalase

When ROS react with the lipid membrane, lipid peroxides are formed and they quickly decompose to form a series of compounds (Halliwell and Gutteridge 1984). One common compound is malondialdehyde (MDA), which is widely used as a measurement of lipid peroxidation (LPO). To determine the level of oxidative stress damage in mussel tissue, lipid peroxidation was measured as the direct quantitative measurement of MDA using an OxiSelect TBARS (Thiobarbituric Acid Reactive Substances) Assay Kit where higher levels of MDA indicate higher oxidative damage. Samples were analyzed in duplicate by mixing 100 μ l of sample/standard with 100 μ l SDS Lysis Solution in glass centrifuge tubes, followed by the addition of 250 μ l of TBA reagent and each tube was covered. Samples were incubated at 95°C for one hour, cooled to room temperature, and then centrifuged at 3000 rpm for 15 minutes. The resulting supernatant was analyzed at 532 nm using a spectrophotometer and compared to an MDA standard curve. Results were expressed as concentration of MDA per gram of wet tissue weight.

I used the enzymatic antioxidant catalase (CAT) to measure the antioxidant response of mussel tissue, as it is a conserved antioxidant in all organisms and it has been previously used to determine oxidative stress activation in aquatic invertebrates (Barata et al. 2005, Barata et al. 2005, De Block and Stoks 2008, Faria et al. 2009, Fernandez et al. 2010). Catalase minimizes oxidative stress in the body by converting the reactive oxygen species H_2O_2 to H_2O and O_2 (Halliwell and Gutteridge 1984). Catalase was measured as the decrease in absorbance due to H_2O_2 consumption (extinction coefficient $40 M^{-1}cm^{-1}$) according to methods by Aebi (1974). The tissue homogenate is added to a reaction mixture of 80 mM phosphate buffer (pH 6.5) and 50

mM H₂O₂ with a total reaction volume of 1 ml for 1 min and measured at 240 nm on a spectrophotometer. Results were expressed as μmol of CAT per mg of protein.

Statistical analysis

Multivariate analysis of variance (MANOVA) using the Pillai's Trace test statistic was used to determine significance of overall oxidative stress measured by the two dependent variables, catalase and lipid peroxidation. The MANOVAs identified significance between species, amongst treatments, and over time, and the significance of independent variable interactions (e.g., species*stressor, or species*day). Box's M tests were performed to confirm homogeneity of covariances among groups prior to multivariate tests. One-way independent measures ANOVAs followed by Student Newman-Keuls (SNK) post-hoc comparison tests were used to compare the individual significance of lipid peroxidation levels and catalase response among treatments, between species, and to compare days. Prior to analysis, a Levene's Test was used to determine variance homoscedasticity among groups, and where appropriate, the data was log-transformed to decrease heterogeneity and meet the assumptions for parametric testing. The Pearson correlation coefficient was used to measure the degree of linear dependency of CAT and LPO for each stressor examined with the two-tailed correlation significance at $\alpha = 0.01$. All statistical analyses were conducted using IBM SPSS Premium Version 22 (SPSS Inc., 2011) and $\alpha = 0.05$.

RESULTS

Stressor #1: Density

Dreissena bugensis and *D. polymorpha* had different antioxidant responses (catalase, CAT) during the density stressor assay ($p < 0.001$, ANOVA), but oxidative cellular damage (lipid peroxidation, LPO) was similar for both species ($p = 0.246$, ANOVA) and independent of density ($p = 0.990$, ANOVA; Figures 7 and 8). *Dreissena bugensis* had a slight decrease in LPO on Day 6 compared to baseline values ($p < 0.050$, SNK), but these values returned to baseline on Day 7 and Day 12 after the addition of more mussels (Figure 7a). Catalase activity was similar to baseline values on days 6, 7, and 12 of the experiment for both species ($p > 0.050$, SNK), but *D. bugensis* had a higher CAT response than *D. polymorpha* throughout the experiment ($p = 0.002$, ANOVA), including higher baseline values ($p < 0.050$, SNK; Figure 7b; Figure 8b). The addition of 30 pre-marked mussels at the end of Day 6 reduced overall mussel stress, indicated by a decrease in CAT activity from Day 6 to Day 7 for *D. bugensis* ($p < 0.050$, SNK) and a decrease in CAT activity from Day 6 to Day 12 for *D. polymorpha* ($p < 0.050$, SNK; Figure 7b). There was no significant change in the LPO response in either species after the addition of more mussels on Day 6 ($p > 0.050$, SNK; Figure 7a). While the CAT response differed between the species, CAT activity was independent of density and both species responded to increased density in the same manner ($p = 0.062$, ANOVA) as CAT activity was the same for low, medium, and high population densities for both species ($p > 0.050$, SNK; Figure 8).

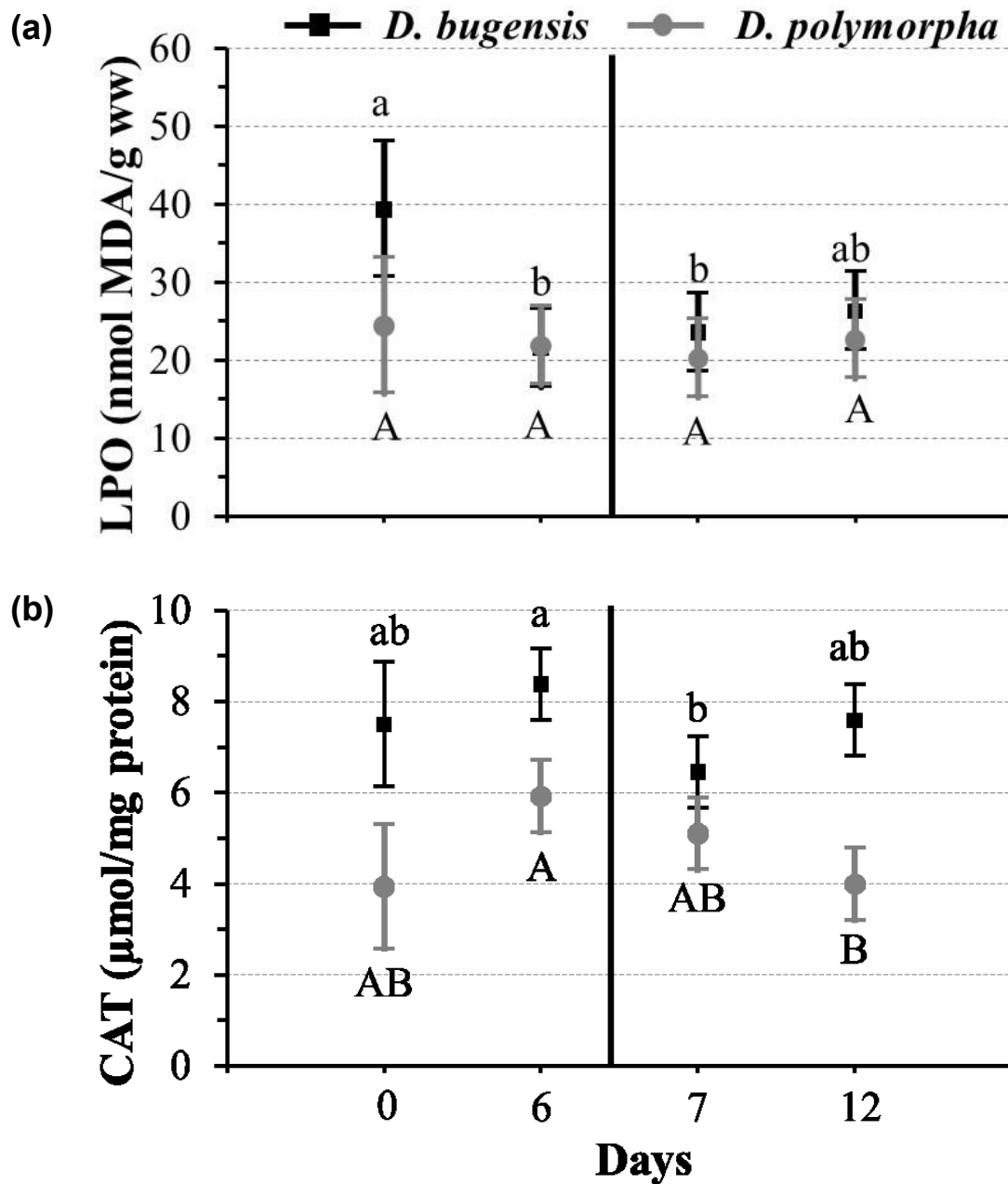


Figure 7. Oxidative stress measured as (a) lipid peroxidation (LPO) and (b) antioxidant catalase activity (CAT) in *D. polymorpha* and *D. bugensis* exposed to three population density treatments: Low (1 mussel/40ml), Medium (1 mussel/20ml), and High (1 mussel/10ml) during a 12 day assay. The vertical black line between days 6 and 7 indicates the addition of 30 pre-marked mussels to all treatment jars at the end of Day 6. Initial LPO and CAT were measured in stock mussels prior to the start of the experiment on Day 0 (baseline), and mussel tissue was sampled on days 6, 7, and 12. Data points represent 95% confidence intervals of the Species*Day interaction. Points having the same letters (lowercase for *D. bugensis* and uppercase for *D. polymorpha*) indicate no differences among days at $p < 0.050$ following ANOVA and Student Newman-Keuls post-hoc tests.

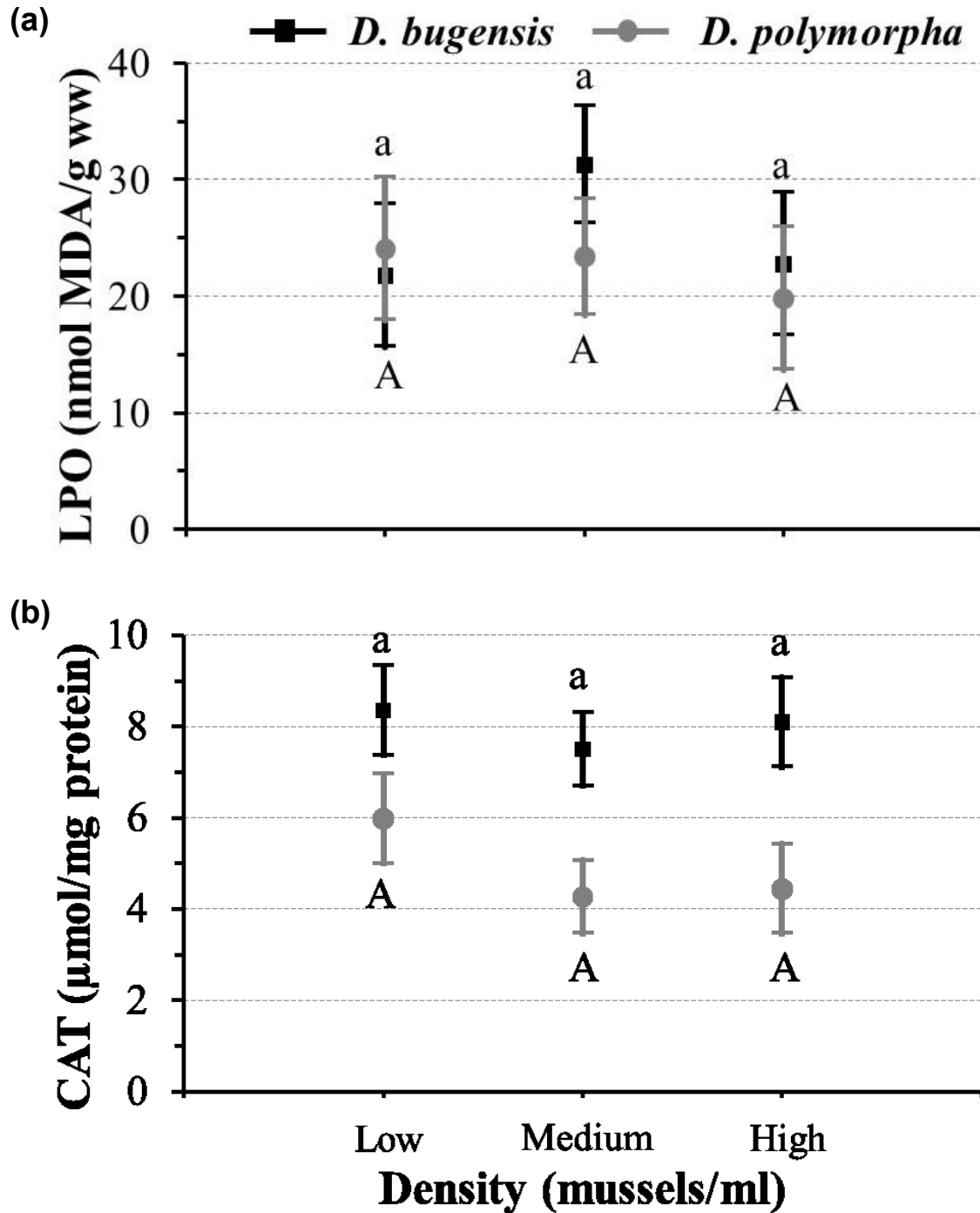


Figure 8. Oxidative stress measured as (a) lipid peroxidation (LPO) and (b) antioxidant catalase activity (CAT) in *D. polymorpha* and *D. bugensis* exposed to three population density treatments: Low (1 mussel/40ml), Medium (1 mussel/20ml), and High (1 mussel/10ml) during a 12 day assay. Data points represent 95% confidence intervals of the Species*Density interaction. Points having the same letters (lowercase for *D. bugensis* and uppercase for *D. polymorpha*) indicate no differences among days at $p < 0.050$ following ANOVA and Student Newman-Keuls post-hoc tests.

Stressor #2: Polychlorinated biphenyls

Dreissena bugensis and *D. polymorpha* exhibited a significantly different response to PCB-contaminated water ($p = 0.019$, MANOVA). *Dreissena polymorpha* had overall greater cellular damage than *D. bugensis* throughout the 7-day experiment ($p < 0.001$, ANOVA; Figures 9a and 10a). Specifically, LPO was nearly two times greater in PCB-exposed *D. polymorpha* compared to *D. bugensis* on all experiment days, except Day 0 (baseline). *Dreissena polymorpha* had significant increases in LPO on Day 1 compared to baseline values ($p < 0.050$, SNK; Figure 9a) in the PCB exposure ($p = 0.012$, ANOVA; Figure 10a). *Dreissena bugensis* exposed to PCBs had LPO values similar to the controls ($p > 0.050$; SNK; Figure 10a) and did not deviate from baseline values throughout the experiment ($p > 0.050$, SNK; Figure 9a).

Unlike the average LPO response, the CAT activity for *D. bugensis* and *D. polymorpha* exposed to PCBs were nearly the same throughout the experiment ($p = 0.917$, ANOVA; Figure 9b) and were not significantly different from the controls ($p = 0.653$, ANOVA; Figure 10b). Catalase values did not deviate from baseline values for either species ($p > 0.050$, SNK; Figure 9a).

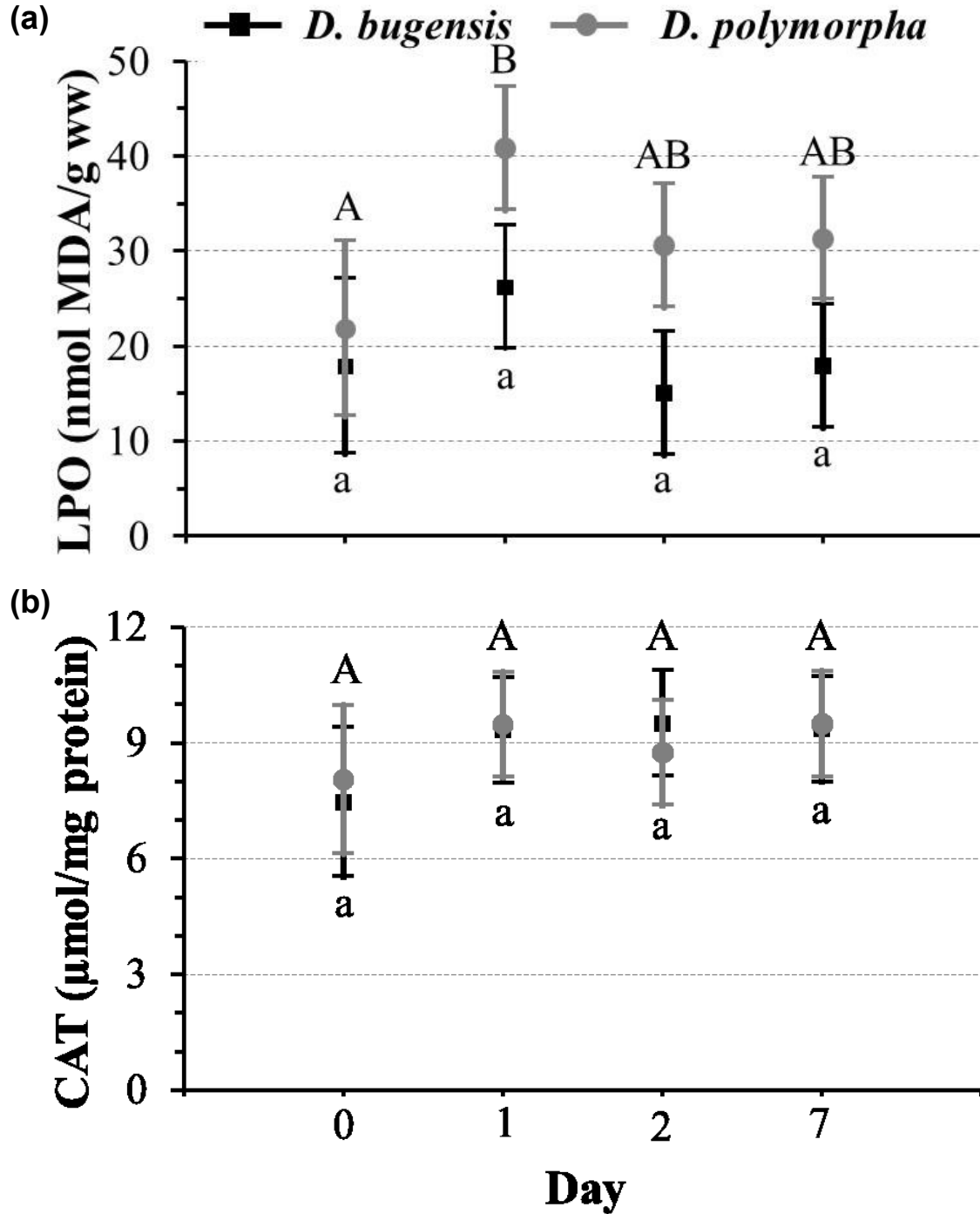


Figure 9. Oxidative stress measured as (a) lipid peroxidation (LPO) and (b) antioxidant catalase activity (CAT) in *D. polymorpha* and *D. bugensis* exposed to PCB contaminated water ($1.3\mu\text{g/l}$) during a 7 day assay. Initial LPO and CAT were measured in stock mussels prior to the start of the experiment on Day 0 (baseline), and mussel tissue was sampled on days 1, 2, and 7. Data points represent 95% confidence intervals of the Species*Day interaction. Points having the same letters (lowercase for *D. bugensis* and uppercase for *D. polymorpha*) indicate no differences among days at $p < 0.050$ following ANOVA and Student Newman-Keuls post-hoc tests.

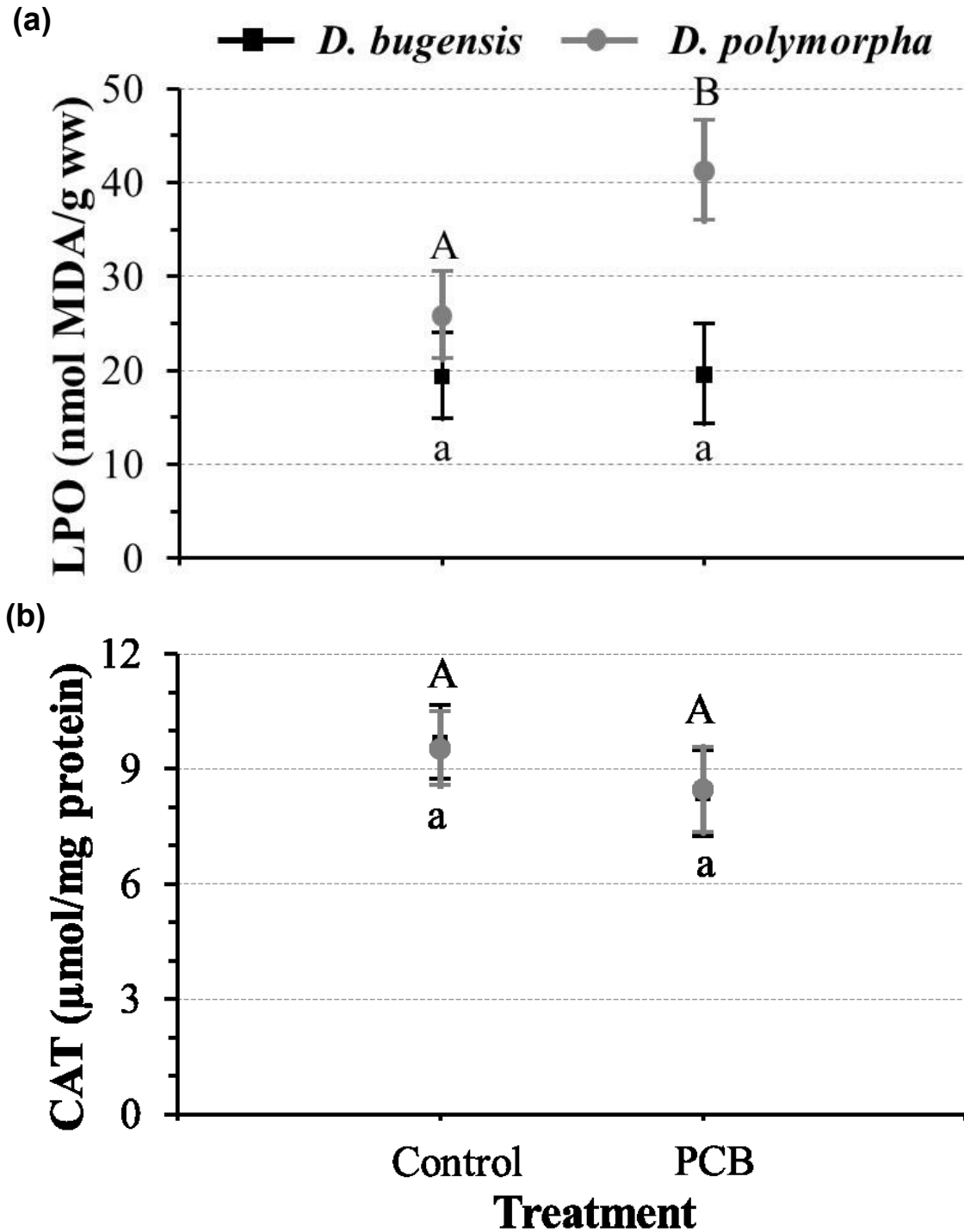


Figure 10. Oxidative stress measured as (a) lipid peroxidation (LPO) and (b) antioxidant catalase activity (CAT) in *D. polymorpha* and *D. bugensis* exposed to PCB contaminated water (1.3μg/l) during a 7 day assay. Data points represent 95% confidence intervals of the Species*PCB interaction. Points having the same letters (lowercase for *D. bugensis* and uppercase for *D. polymorpha*) indicate no differences among days at $p < 0.050$ following ANOVA and Student Newman-Keuls post-hoc tests.

Stressor #3: Temperature

During the four-day temperature stressor assay, *D. bugensis* tissue had more LPO ($p < 0.001$, ANOVA) and higher levels of CAT activity than *D. polymorpha* across all treatments throughout the experiment ($p < 0.001$; ANOVA; Figures 11 and 12). However, the higher LPO values observed in *D. bugensis* were near baseline values ($p > 0.050$, SNK), except on Day 4 when temperatures were the most extreme and oxidative damage actually decreased in *D. bugensis* ($p < 0.050$, SNK; Figure 11a). This decrease is accounted for in the lower LPO values observed in *D. bugensis* exposed to low and high treatment temperatures compared to the ambient temperature ($p < 0.050$, SNK) and baseline values ($p < 0.050$, SNK). In fact, the low and high temperature mussels had $> 25\%$ lower LPO compared to the ambient temperature mussels ($p < 0.050$ and $p < 0.050$, respectively, SNK; Figure 12a). In addition, *D. bugensis* had increased CAT activity on Days 1, 3, and 4 compared to baseline values ($p < 0.050$, SNK; Figure 11b); however, this response was independent of temperature as the CAT response in *D. bugensis* was similar across all treatment temperatures ($p = 0.370$, ANOVA; Figure 12b). *Dreissena polymorpha* did not experience any changes in LPO or CAT activity from baseline values throughout the experiment and the species' responses were independent of temperature.

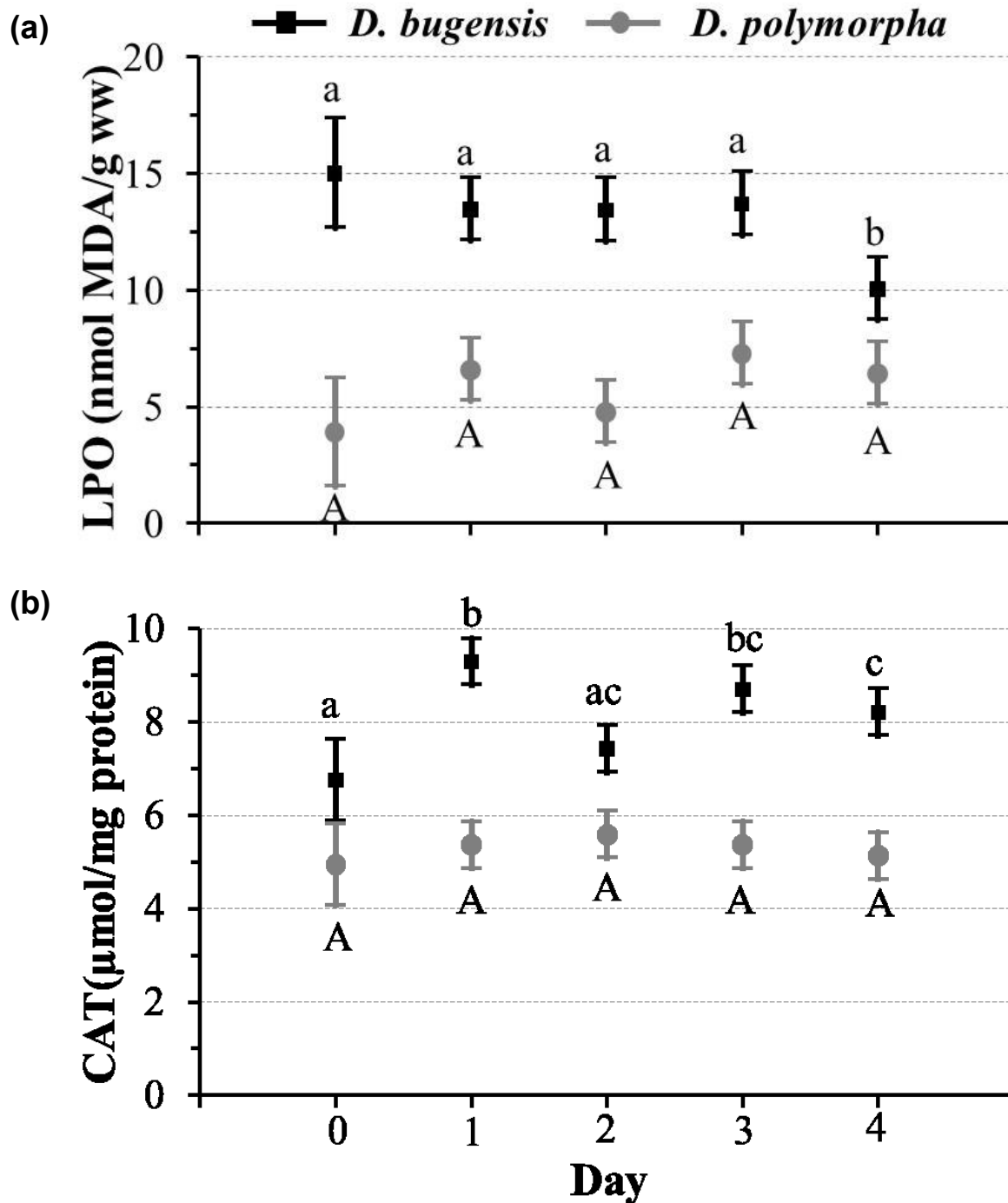


Figure 11. Oxidative stress measured as (a) lipid peroxidation (LPO) and (b) antioxidant catalase activity (CAT) in *D. polymorpha* and *D. bugensis* exposed to three temperature treatments: Low (5°C), Ambient (Amb; 20°C), and High (29 °C) during a 4 day assay. Initial LPO and CAT were measured in stock mussels prior to the start of the experiment on Day 0 (baseline), and mussel tissue was sampled on days 1, 2, 3, and 4. Data points represent 95% confidence intervals of the Species*Day interaction. Points having the same letters (lowercase for *D. bugensis* and uppercase for *D. polymorpha*) indicate no differences among days at $p < 0.050$ following ANOVA and Student Newman-Keuls post-hoc tests.

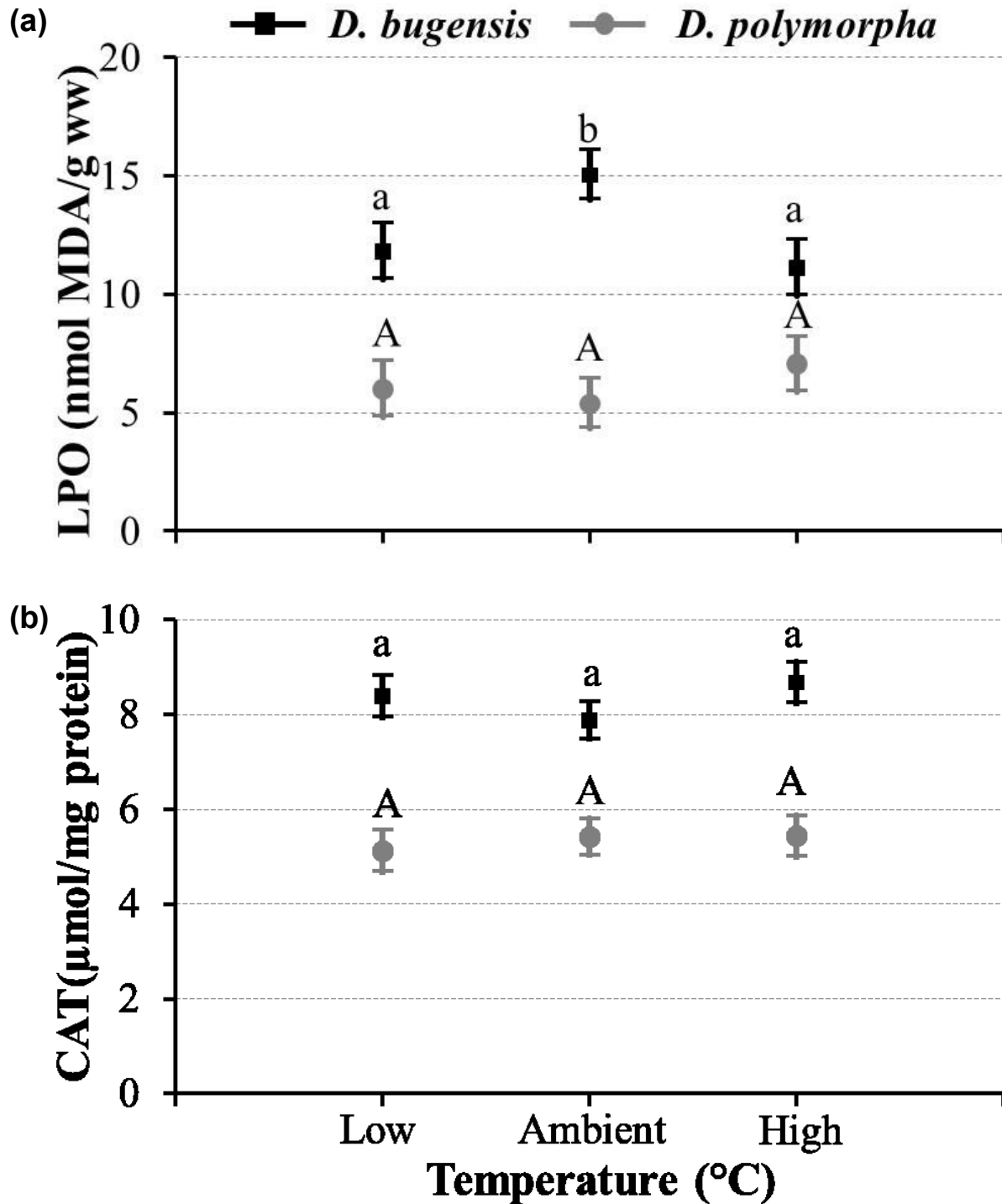


Figure 12. Oxidative stress measured as (a) lipid peroxidation (LPO) and (b) antioxidant catalase activity (CAT) in *D. polymorpha* and *D. bugensis* exposed to three temperature treatments: Low (5°C), Ambient (Amb; 20°C), and High (29°C) during a 4 day assay. Data points represent 95% confidence intervals of the Species*Temperature interaction. Points having the same letters (lowercase for *D. bugensis* and uppercase for *D. polymorpha*) indicate no differences among days at $p < 0.050$ following ANOVA and Student Newman-Keuls post-hoc tests.

Stressor #4: Hypoxia

Physicochemical parameters between the treatments were similar throughout the entire experiment (temperature 18.7 – 19.0°C; pH 7.99 - 9.05; conductivity 200 – 263 $\mu\text{S/cm}$), except for the anticipated disparity in dissolved oxygen readings between oxic and hypoxic treatment chambers (Table 4). The oxic treatment chambers maintained a dissolved oxygen range between 8.74 - 9.52 mg/L, and the hypoxic treatment chambers maintained a dissolved oxygen range between 1.77 - 2.95 mg/L (Table 4).

Oxidative stress in dreissenid mussels exposed to hypoxic conditions was significantly different between the two species ($p < 0.001$, MANOVA). *Dreissena polymorpha* exposed to hypoxic conditions had a 3-fold increase in LPO compared to *D. bugensis* ($p < 0.001$, ANOVA), and compared to oxic conditions ($p = 0.012$, ANOVA) and baseline values ($p < 0.001$, ANOVA; Figure 13a). In contrast, *D. bugensis* LPO response was similar to baseline values for both oxic ($p > 0.050$, SNK) and hypoxic conditions ($p > 0.050$, SNK), even though there was a marginal increase in LPO in oxic chambers (Figure 13a). The two species had similar LPO responses for baseline values ($p > 0.050$, SNK) and for oxic conditions ($p > 0.050$, SNK; Figure 13a).

D. polymorpha exposed to hypoxic conditions had no change in CAT activity compared to baseline values ($p > 0.050$, SNK; Figure 13b). However, *D. polymorpha* had twice as much CAT activity under oxic conditions compared to *D. bugensis* ($p < 0.001$, ANOVA; Figure 13b). Both species had similar CAT activity under hypoxic conditions ($p = 0.228$, ANOVA).

Table 4. Physicochemical parameters including dissolved oxygen (mg/L), temperature (°C), pH, and conductivity ($\mu\text{S/cm}$) were measured in experimental chambers using a multi-parameter probe (YSI 556 Multi-Parameter Sensor) during a 4 day hypoxia stressor assay with *Dreissena bugensis* and *D. polymorpha*. Measurements are expressed as the average \pm standard deviation.

| Treatment | Day | Dissolved oxygen (mg/L) | Temperature (°C) | pH | Conductivity ($\mu\text{S/cm}$) |
|------------------------------|-----|-------------------------|------------------|-----------------|-----------------------------------|
| <i>D. bugensis</i> oxic | 1 | 8.74 \pm 0.31 | 19.0 \pm 0.27 | 8.21 \pm 0.15 | 218 \pm 3.56 |
| | 2 | 9.52 \pm 0.19 | 18.9 \pm 0.45 | 8.24 \pm 0.07 | 217 \pm 3.39 |
| | 3 | 9.41 \pm 0.15 | 19.0 \pm 0.36 | 7.99 \pm 0.37 | 220 \pm 2.07 |
| | 4 | 8.88 \pm 0.19 | 18.8 \pm 0.25 | 8.21 \pm 0.10 | 220 \pm 2.66 |
| <i>D. bugensis</i> hypoxic | 1 | 2.22 \pm 0.42 | 18.9 \pm 0.24 | 8.98 \pm 0.22 | 213 \pm 4.23 |
| | 2 | 1.75 \pm 0.32 | 18.9 \pm 0.24 | 9.02 \pm 0.16 | 217 \pm 2.07 |
| | 3 | 1.93 \pm 0.11 | 18.9 \pm 0.34 | 9.05 \pm 0.06 | 221 \pm 2.74 |
| | 4 | 2.39 \pm 0.49 | 18.7 \pm 0.23 | 8.90 \pm 0.08 | 226 \pm 3.74 |
| <i>D. polymorpha</i> oxic | 1 | 8.79 \pm 0.20 | 18.8 \pm 0.26 | 8.13 \pm 0.13 | 202 \pm 3.25 |
| | 2 | 9.44 \pm 0.17 | 18.8 \pm 0.37 | 8.12 \pm 0.03 | 202 \pm 3.88 |
| | 3 | 9.28 \pm 0.23 | 18.9 \pm 0.27 | 8.18 \pm 0.09 | 202 \pm 3.62 |
| | 4 | 8.76 \pm 0.34 | 18.7 \pm 0.28 | 8.17 \pm 0.06 | 200 \pm 3.14 |
| <i>D. polymorpha</i> hypoxic | 1 | 2.21 \pm 0.14 | 18.9 \pm 0.23 | 8.83 \pm 0.13 | 221 \pm 3.10 |
| | 2 | 2.00 \pm 0.50 | 18.8 \pm 0.28 | 8.69 \pm 0.25 | 250 \pm 55.0 |
| | 3 | 2.15 \pm 0.21 | 18.9 \pm 0.31 | 8.89 \pm 0.18 | 231 \pm 4.93 |
| | 4 | 2.23 \pm 0.20 | 18.9 \pm 0.18 | 8.76 \pm 0.30 | 263 \pm 68.0 |

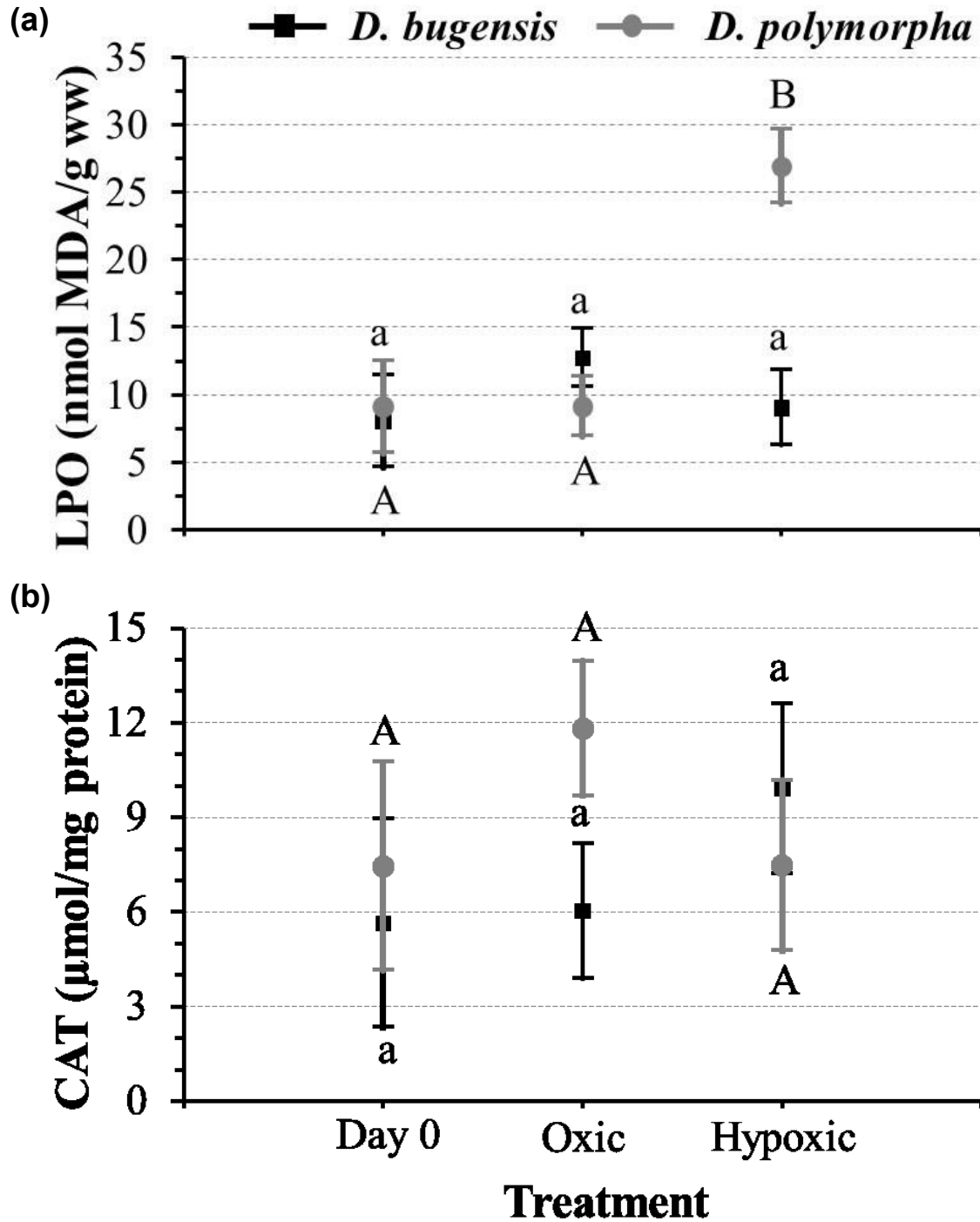


Figure 13. Oxidative stress measured as (a) lipid peroxidation (LPO) and (b) antioxidant catalase activity (CAT) in *D. polymorpha* and *D. bugensis* exposed to hypoxic and oxic conditions during a 4 day assay. Initial LPO and CAT were measured in stock mussels prior to the start of the experiment on Day 0 (baseline), and mussel tissue was sampled on the last day of the experiment. Data points represent 95% confidence intervals of the Species*Hypoxia interaction. Points having the same letters (lowercase for *D. bugensis* and uppercase for *D. polymorpha*) indicate no differences among days at $p < 0.050$ following ANOVA and Student Newman-Keuls post-hoc tests.

Correlation between CAT and LPO

Pearson correlations revealed a moderate and positive correlation between LPO and CAT with changes in temperature ($r = 0.559$, $p < 0.001$; Figure 14c). The other three stressors (density, PCBs, and hypoxia) did not result in a significant correlation between CAT and LPO, although the correlation between these indicators was only marginally insignificant ($p = 0.057$) for PCBs (Figure 14a,b,d).

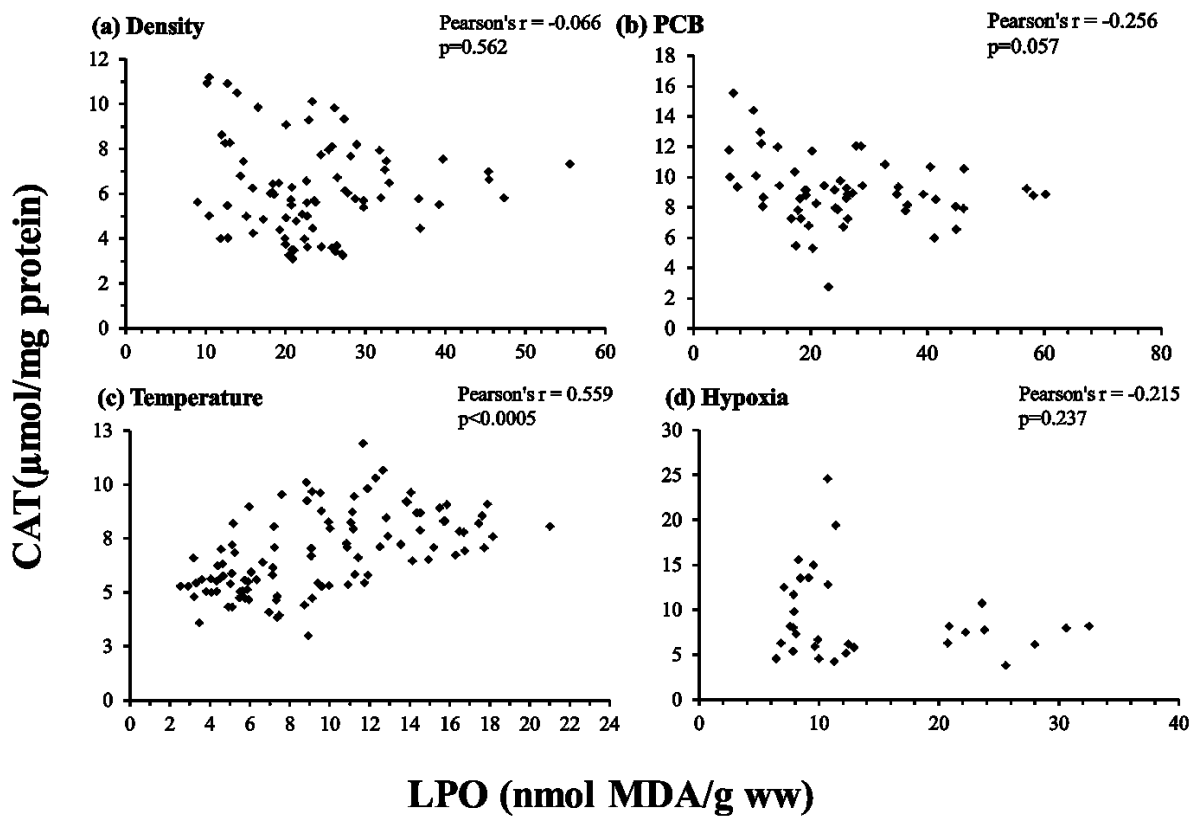


Figure 14. Pearson correlations measuring the linear dependency of CAT and LPO for the four experimental stressors: (a) density, (b) PCBs, (c) temperature, and (d) hypoxia.

DISCUSSION

Comparing oxidative stress in D. bugensis and D. polymorpha

Biological, chemical and physical stressors affect levels of oxidative stress and antioxidant response in dreissenid mussels. *Dreissena bugensis* was more tolerant of the measured environmental stressors (had less oxidative stress) compared to *D. polymorpha*. This supports findings from other studies identifying a superior tolerance of *D. bugensis* compared to *D. polymorpha* in tolerance to extreme temperatures (Karatayev et al. 2011) and higher survivorship and abundance at greater depths in hypoxic hypolimnetic waters (Mills et al. 1993, Ricciardi and Whoriskey 2004). While these studies focused on the survivorship and abundance of dreissenid mussels, the work presented here evaluated the physiological mechanisms that may contribute to the differences in survival and habitat selection between these two species. Furthermore, results from this study provide a possible mechanism for the superior tolerance of *D. bugensis* over *D. polymorpha* in aquatic habitats.

Dreissena polymorpha exhibited greater oxidative damage and stress when exposed to PCBs and hypoxia, whereas *D. bugensis* remained relatively unaffected. Catalase values in *D. polymorpha* were similar to other whole tissue stress studies involving chemical exposure (Parolini et al. 2014). My results further support the hypothesis that *D. polymorpha* is a poor oxygen regulator and cannot tolerate environments with low oxygen availability (Alexander and McMahon 2004), while the lower respiration rates of *D. bugensis* requires less oxygen consumption (Stoeckmann 2003). Also, oxidative stress in *D. polymorpha* when exposed to PCBs provides a further understanding of physiological impacts of PCBs in freshwater invertebrates.

Polychlorinated biphenyls have known adverse effects in many aquatic organisms (Dillon and Burton 1991, Ho and Liu 2010, Wan et al. 2010). They have been implicated as known neurotoxins, immunodepressants, and endocrine disruptors, and can be responsible for changes in organism metabolism and antioxidant production (Kester et al. 2000, Weisglas-Kuperus et al. 2000, Walkowiak et al. 2001, Schantz et al. 2003, Faria et al. 2009, Faria et al. 2010, Leroy et al. 2010, Hegseth et al. 2011). Notably, both species had similar CAT activity in the PCB treatments compared to controls. Similar results were reported by Faria et al. (2009) in which *D. polymorpha* exposed to PCBs had increased lipid peroxidation but no change in the CAT response. Some studies suggest that chemical contaminants may increase ROS to the point in which some of these ROS may actually *inhibit* or decrease catalase activity (Kono and Fridovich 1982). Other antioxidant enzymes such as superoxide dismutase and glutathione peroxidase have been successfully used to measure antioxidant response in benthic invertebrates (Barata et al. 2005, Faria et al. 2009) and may be useful to verify antioxidant response in future studies.

Dreissena bugensis may have an overall greater amount of catalase than *D. polymorpha* and may therefore better respond to environmental stress. *Dreissena bugensis* had higher CAT activity in the density and temperature experiments, including higher baseline values, compared to *D. polymorpha*. Given that there was no evidence of oxidative damage in *D. bugensis* in the density and temperature experiments, the high catalase values are not likely due to stress. Instead, *D. bugensis* may be more efficient in upregulating catalase, allowing it to respond more quickly to environmental stressors, as suggested by the temperature assay. The correlation between LPO and

CAT in the temperature assay suggests that the increase in CAT in *D. bugensis* may be responsible for the rapid decrease in oxidative damage by the assay's end. While it has been postulated that *D. polymorpha* prefer warm, shallow waters, and *D. bugensis* prefer deeper, cooler waters (Dermott and Munawar 1993, Mills et al. 1993), the results of this study support recent studies showing *D. bugensis* having higher abundance in areas with thermal discharges (Mitchell et al. 1996). Mitchell et al. (1996) argues that *D. bugensis* actually selects for deeper, cooler sites in Lake Erie because these sites are warmer over winter due to inverse stratification of the lake. My results suggest that *D. bugensis* are more fit under hypoxic conditions and in temperature extremes compared to *D. polymorpha* because of their ability to manage stress through antioxidant response. *Dreissena polymorpha* may not be capable of a rapid or sufficient CAT response and therefore may not be able to recover from oxidative damage when exposed to some environmental stressors (e.g. PCBs or hypoxia). A robust antioxidant response may be another potential adaptive advantage that *D. bugensis* has developed over *D. polymorpha*. In some regions, this advantage may allow *D. bugensis* to dominate. My research has provided a foundation for future research to compare CAT and other antioxidants between these two species.

Dreissenid mussels are a significant economic threat to industrial and recreational activities because they can clog water intake systems and biofoul shorelines. In fact, invasive mussels have cost approximately \$1 billion per year amount in recreation and commercial revenue (U.S. Army Corps of Engineers 2002). The results of this study are important for understanding the physiology and population

dynamics of invasive dreissenid mussels and have huge implications for management efforts.

Oxidative stress and resilience

A comparison of oxidative stress in two similar dreissenid species with potentially inherent differences in tolerance may allow some inferences about the role of oxidative stress in environmental tolerance and resilience to disturbances. Organisms that have a greater antioxidant potential may be more capable of resisting disturbances and persisting in disturbed environments. The oxidative stress response may be a mechanism of resilience (Holling 1973) that is present in all organisms and the robustness of the antioxidant response determines how an organism can respond to changes in the environment. The potentially strong CAT activity in *D. bugensis* allows this species to respond rapidly to environmental stressors and decrease oxidative damage, giving *D. bugensis* superior resilience and tolerance to environmental disturbances. However, biological systems have a threshold in which they can be overwhelmed and lose the ability to recover (Walker et al. 2004). If a stress persists, the oxidative stress system may be overloaded with ROS and the balance between ROS and antioxidants may become compromised, allowing oxidative damage to occur (Halliwell and Gutteridge 1999). This mechanism explains the high oxidative damage observed in *D. polymorpha* exposed to PCBs or hypoxic conditions. The potentially weaker antioxidant response in *D. polymorpha* may result in this species being less resilient to changes in the environment compared to *D. bugensis*.

Oxidative stress may also impact how aquatic communities respond to disturbance frequencies and influence diversity. As the Intermediate Disturbance Hypothesis dictates, species diversity is maximized when occurrence of ecological disturbances are moderate, not frequent or rare. Intermediate disturbances may allow organisms with different oxidative stress responses to coexist in the environment. Rare disturbance events may allow organisms with strong oxidative stress responses to dominate. Frequent disturbances may overwhelm the oxidative stress response and allow only the most tolerant organisms to dominate or may result in a crash of the entire ecosystem. In areas where *D. polymorpha* and *D. bugensis* cohabitate, there may be intermediate stressors that are influencing the two populations. However, in areas where there are frequent hypoxic events, or chemical inputs, *D. bugensis* may be able to dominate. Again, this may be a potential mechanism for the displacement of *D. polymorpha* by *D. bugensis* in the Great Lakes region.

My results suggest that species with higher antioxidant potentials will have greater tolerance to stress, and therefore the presence/absence of species with high antioxidants may provide an indication of site conditions and environmental stress. Aquatic invertebrates play an important role in nutrient and contaminant cycling and as important food sources for higher consumers (Jackson et al. 1998, Bott and Standley 2000, Daley et al. 2011). Insight into how pollutants directly impact aquatic invertebrates at the molecular level can contribute to a better understanding of how pollutants affect aquatic ecosystems as a whole. Just as aquatic invertebrates are used as model organisms for lethal and sub-lethal toxicity testing (West et al. 1993, Schubauerberigan et al. 1995, Nakari and Huhtala 2008, Ingersoll et al. 2009, Bernatowicz and Pijanowska

2011), aquatic invertebrates also have the potential to serve as a sub-lethal model system to understand how environmental stressors affect stress. Oxidative stress biomarkers in aquatic invertebrates may be used to develop a novel and rapid bioassessment metric for evaluating environmental stress in aquatic environments prior to negative impacts occurring in the community. By understanding the oxidative stress potential of stressors in an aquatic environment, researchers will be able to better predict how aquatic communities will respond to disturbances such as contaminants or changing temperatures. With climate change scientists predicting increased temperatures (Magnuson et al. 1997) and the continued input of industrial and commercial contaminants into the environment, the results of this study provide insight into how these environmental stressors will affect the physiology of aquatic organisms.

CHAPTER 3

THE EFFECTS OF MULTIPLE STRESSORS ON OXIDATIVE STRESS IN DREISSENID MUSSELS

BACKGROUND

Environmental stressors seldom act independently in aquatic systems. Stressors can be any combination of chemical, biological or physical disturbances that interact in different and unpredictable ways. For example, when two stressors act on an aquatic system the overall biological effect may be additive, by which the total effect is the sum of the effects of the two individual stressors. This type of effect is typically observed in aquatic systems polluted with multiple contaminants, such as heavy metals (Renella et al. 2003). Physical and biological stressors can also act in an additive manner to influence community biodiversity (Williams et al. 2013). On the other hand, two stressors may have an antagonistic relationship in which one stressor inhibits the effect of the other, leading to an overall reduced or neutral impact on the environment. For example, cyanobacterial blooms and the insecticide carbaryl independently have adverse effects on *Daphnia pulex* reproduction; however, in combination, the effect on *D. pulex* is significantly reduced (Asselman et al. 2013). In many cases, the effect is synergistic, in which the combined effect of the two stressors is much greater than the sum of the stressors acting alone. In fact, a meta-analysis of marine and coastal ecosystem research found that the overall interaction effect of anthropogenic stressors across all studies was synergistic (Crain et al. 2008). A synergistic effect can have unexpected detrimental impacts on aquatic organisms (Davies et al. 2004, Rosa and

Seibel 2008, Wolf and Nugues 2013), and may sometimes be missed in single stressor research studies. As such, it is critical to investigate the effects of multiple stressors in organisms, as opposed to the standard methods of studying individual stressors alone.

Organisms that are adapted to tolerate specific stressors may be less adapted to deal with others, and therefore are at a disadvantage when dealing with the presence of multiple stressors in the environment (Wilson 1988). For example, Kashian et al. (2007) found that metal-tolerant invertebrates had functional and behavioral impacts when exposed to metals in conjunction with ultraviolet light. When aquatic organisms experience a disturbance such as a chemical stressor or sudden changes in environmental conditions, the organisms can respond in different ways to combat the stressor, such as behavioral avoidance of the stressor (Courtney and Clements 1998, Clements 2004), entering into a dormant state (McAllen et al. 1999), or storing chemical contaminants in a biologically inactive part of the body, such as the shell or bone (Wallace et al. 2003). In addition, most organisms enact molecular pathways to protect against chemical exposure (Barata et al. 2005, Valavanidis et al. 2006). Antioxidants may play a large role in tolerance in aquatic organisms by reducing the reactive oxygen species produced during contaminant exposure (Kelly et al. 1998, Livingstone 2001, Rout and Shaw 2001, Barata et al. 2005, Wei et al. 2011), and species with higher levels of essential antioxidants may be more tolerant to stressors in the environment.

Dreissenid mussel species, *Dreissena bugensis* and *D. polymorpha*, have been invasive nuisance species in the Great Lakes region since the 1980s (Hebert et al. 1989, Ricciardi et al. 1996). *Dreissena bugensis* has been considered more robust and more tolerant of the two species, leading to *D. bugensis* displacement of *D. polymorpha*

in the Great Lakes (Mills et al. 1999, Stoeckmann 2003, Ricciardi and Whoriskey 2004, Karatayev et al. 2011). In addition, *D. bugensis* may be more tolerant to chemical and physical stressors (e.g., PCBs, hypoxia, and density) due to less oxidative damage and higher antioxidant levels (Chapter 2). In light of the preponderance of multiple stressors in the environment, there remains a need to understand oxidative stress in *D. polymorpha* and *D. bugensis* to better understand how the two species will respond to continued changes/disturbances in the environment. Further understanding of tolerance differences between the two species will be valuable for evaluating control measures of dreissenid mussels in regions where they are invasive. The objective of this study is to quantify the molecular oxidative effects of single versus multiple stressors in dreissenid mussels and determine how these changes in the oxidative stress pathways affect mussel tolerance.

METHODOLOGY

To investigate the impact of multiple stressors on oxidative stress in aquatic invertebrates, I exposed *Dreissena bugensis* and *D. polymorpha* to environmental conditions that paired physical or biological stressors with a chemical stressor (polychlorinated biphenyls: PCBs). I then quantified the oxidative biomarker response. The three stressor combinations examined were: 1) high mussel population density and PCBs, 2) extreme temperatures and PCBs, and 3) hypoxia and PCBs.

Test organism and PCB collection

Dreissena bugensis were collected off fishing piers on Belle Isle in the Detroit River, Michigan (42°21'6.05"N, 82°58'12.09"W) and *D. polymorpha* were collected from the upper Rouge River in Livonia, Michigan (42°22'0.09"N, 83°24'37.06"W) in April 2014. These sites are approximately 36 km apart. Physicochemical parameters (dissolved oxygen, pH, conductivity, and temperature) were measured at each collection site (Table 5). Chemical analysis of sediment and water using USEPA standard methods 3510C and 8082A indicate PCB concentrations below detection limits at both locations (U.S. Environmental Protection Agency 2011) (Paragon Laboratories, Livonia, MI, USA; Table 5).

Mussels were transported on ice to Wayne State University where dead mussels and shell fragments were removed; live mussels were maintained in 10 L glass aquariums filled with aerated and dechlorinated tap water at a density of 1 mussel per 40-45 ml of water. Once a week, the tanks were cleaned, dead mussels removed, and the water refreshed. The tanks were housed in an environmental growth chamber at 20 ± 1 °C with a 12 h: 12 h light-dark cycle. All organisms were fed a 50/50 algal mixture of *Ankistrodesmus falcatus* and *Chlamydomonas reinhardtii*.

Prior to the start of all experiments (Day 0), mussels from stock tanks of each species were sampled and the tissue was pooled and measured for oxidative stress to establish baseline values for lipid peroxidation and antioxidant levels (catalase) for each species. These Day 0 measurements will be referred to as baseline values.

Table 5. *Dreissena bugensis* and *Dreissena polymorpha* were collected from two locations in the Great Lakes basin in April 2014 for use in multiple stressor studies. The location, physicochemical parameter measurements, and sediment and water PCB concentrations are listed for each site.

| | <i>Dreissena bugensis</i> | <i>Dreissena polymorpha</i> |
|------------------------------------|--|---|
| Location | Detroit River, Belle Isle, MI (42°21'6.05"N, 82°58'12.09"W) | Rouge River, Livonia, MI (42°22'0.09"N, 83°24'37.06"W) |
| Dissolved oxygen (mg/L) | 11.4 | 11.1 |
| Temperature (°C) | 9.50 | 10.4 |
| Conductivity (µS/cm) | 200 | 1170 |
| pH | 7.59 | 7.50 |
| Sediment PCB concentration (µg/Kg) | < 330 (detection limit) | < 330 (detection limit) |
| Water PCB concentration (µg/L) | < 0.10 (detection limit) | < 0.10 (detection limit) |

Water was collected from a known PCB-contaminated site in St. Clair Shores, Michigan (42°28'36.83"N, 82°53'18.77"W) using fifteen 1 L glass amber jars with Teflon® lids at approximately 0.25 m below the surface of the water, in an area with a water depth of approximately 1-1.5 m. The jars were stored in a dark cold room (4°C) for a maximum of two weeks until use in the experiments. Chemical analysis using USEPA standard methods 3510C and 8082A (U.S. Environmental Protection Agency 2011) (Paragon Laboratories, Livonia, MI, USA) revealed total PCB concentrations at 1.3 µg/L in the water.

Multiple Stressor Assay #1: Density and PCBs

During a 6-day assay, 800 ml glass assay jars were filled with either 360 mL of aged tap water (control) or PCB contaminated site water (1.3 µg/l). Half of the jars were then populated with *D. bugensis* and the other half with *D. polymorpha*. Prior to the start of the experiment, all mussels were measured and only mussels ranging 15-25 mm in length were used in the study. The number of mussels added to each jar depended on the density treatment. The treatments (N=4) included: 1) low density (9 mussel/360ml), 2) medium density (18 mussel/360ml), 3) high density (36 mussel/360ml), 4) low density (9 mussel/360ml) and PCBs, 5) medium density (18 mussel/360ml) and PCBs, and 6) high density (36 mussel/360ml) and PCBs. Hereafter, these treatments will be referred to as LOW DENSITY, MED DENSITY, HIGH DENSITY, LOW DEN+PCB, MED DEN+PCB, and HIGH DEN+PCB. The MED DENSITY treatment was designated as a control because mussel stock tanks were maintained at the identical density (18 mussel/360 ml). Mussels were collected for sampling on day 6 by removing a subset of jars (n = 4) from each treatment for each species and analyzed for oxidative stress following methods described below.

Multiple Stressor Assay #2: Temperature and PCBs

To examine the effects of temperature in combination with PCBs on the dreissenid oxidative stress response, assay jars were filled with either 400 ml of aged-tap water (control) or PCB contaminated site water (1.3 µg/l). Ten mussels (15-25 mm in length) were placed in each jar so that half of the assay jars contained *D. bugensis* and the other half contained *D. polymorpha*. Assay jars were then placed in

environmental growth chambers set at three different treatment temperatures to provide the following treatments (N=12): 1) low temperature (5°C), 2) ambient temperature (20°C), 3) high temperature (29 °C), 4) low temperature (5°C) and PCBs, 5) ambient temperature (20°C) and PCBs, and 3) high temperature (29 °C) and PCBs. Hereafter, these treatments will be referred to as LOW TEMP, AMB TEMP, HIGH TEMP, LOW TEMP+PCB, AMB TEMP+PCB, and HIGH TEMP+PCB. Environmental growth chambers had a 12h:12h light-dark cycle. At the start of the experiment, all chambers started at the ambient temperature and then were slowly increased or decreased by 4-8°C each day for two days until reaching the final treatment temperature. Mussels were examined on Day 3 for oxidative stress following methods detailed below. The assay concluded on Day 3 due to observed mortality (1-2 mussels) in some of the HIGH TEMP+PCB jars.

Multiple Stressor Assay #3: Hypoxia and PCBs

To determine the combined effects of hypoxia and PCBs on mussel oxidative stress, I used sealed oxygen chambers equipped with gas intake and outlet ports, and a water sampling port (Figure 6), to create four different treatment conditions: 1) oxic, 2) hypoxic, 3) oxic and PCBs (Oxic + PCB), and 4) hypoxic and PCBs (Hypoxic + PCB). Oxic treatments were aerated with filtered (0.2 µm EMD Millipore Millex filter) room-air, and dissolved oxygen was maintained between 9-10 mg/l. The hypoxic chambers were aerated with filtered (0.2 µm EMD Millipore Millex filter) nitrogen gas and oxygen levels maintained below 3 mg/L. Experiment chambers were placed in an environmental growth incubator at 20 ± 1°C with a 12h:12h light-dark cycle. Prior to the start of the

assay, 2 L of either aged tap water or PCB site water (1.3 µg/L) was added to each treatment chamber and allowed to aerate for 24 hours before the mussels were added. Thirty mussels of either *D. bugensis* or *D. polymorpha* (15-25 mm in length) were placed in each treatment chamber. Physicochemical water parameters were monitored every 24 hours by removing 200 mL of water from the water sampling port and using a multi-parameter probe (YSI 556 Multi-Parameter Sensor) to measure dissolved oxygen, temperature, pH, and conductivity. Chamber water was replenished with aerated aged-tap water or PCB site water (1.3 µg/L) stored at $20 \pm 1^\circ\text{C}$. The assay ran for two days and mussels were sampled for oxidative stress on day 2. Preliminary studies indicated that after 2 days in hypoxic conditions mussel mortality increases significantly (unpublished data).

Tissue preparation

On each sampling day, replicate jars of each species from each treatment were removed and five mussels were randomly selected from each jar for analysis. Immediately after collection, total mussel weight was measured and the soft tissue of each mussel was dissected and weighed. The soft tissue of all five mussels was pooled, immediately frozen in liquid nitrogen, and stored at -80°C until further analysis.

Mussel tissue was digested by grinding the frozen tissue for 2 minutes with cold mortar and pestle placed on ice. The ground tissue was then homogenized in a 1:5 weight: volume ice cold phosphate buffer (100 mM, pH 7.4, containing 150 mM KCl and 1 mM EDTA) (Faria et al. 2009) by grinding again with a mortar and pestle (1 min). Homogenized tissue was carefully transferred to 15 ml centrifuge tubes and centrifuged

at 10,000 g for 30 minutes. The supernatant was then directly used for protein and biomarker analysis.

Protein analysis

To standardize biomarker measurements for mussels of different size and species, total protein concentrations were determined for each pooled mussel sample using a Coomassie Plus (Bradford) Assay Kit (Thermo Scientific). Tissue samples were diluted with 100 mM phosphate buffer prior to analysis. Samples were analyzed in triplicate at 595 nm on a 96-well microplate reader and plotted on a standard curve to determine the total protein concentration of each sample.

Measuring oxidative damage: lipid peroxidation

When reactive oxygen species (ROS) react with the lipid membrane, lipid peroxides are formed and they quickly decompose to form a series of compounds (Halliwell and Gutteridge 1984). One common compound is malondialdehyde (MDA), which is widely used as a measurement of lipid peroxidation. To determine the level of oxidative stress damage in mussel tissue, lipid peroxidation was measured as the direct quantitative measurement of MDA using an OxiSelect TBARS (Thiobarbituric Acid Reactive Substances) Assay Kit where higher levels of MDA indicate higher oxidative stress. Samples were analyzed in duplicate by mixing 100 μ l of sample/standard with 100 μ l SDS Lysis Solution in glass centrifuge tubes, followed by the addition of 250 μ l of TBA reagent and each tube was covered. Samples were incubated at 95°C for one hour, cooled to room temperature, and then centrifuged at 3000 rpm for 15 minutes.

The resulting supernatant was analyzed at 532 nm using a spectrophotometer and compared to an MDA standard curve. Results were expressed as concentration of MDA per gram of wet tissue weight.

Activation of the oxidative stress response: catalase

I used the enzymatic antioxidant catalase (CAT) to measure the oxidative stress response of mussel tissue, as it is a conserved antioxidant in all species and it has been previously used to determine oxidative stress activation in aquatic invertebrates (Barata et al. 2005, Barata et al. 2005, De Block and Stoks 2008, Faria et al. 2009, Fernandez et al. 2010). Catalase minimizes oxidative stress in the body by converting the reactive oxygen species H_2O_2 to H_2O and O_2 (Halliwell and Gutteridge 1984). Catalase was measured as the decrease in absorbance due to H_2O_2 consumption (extinction coefficient $40 M^{-1}cm^{-1}$) according to methods by Aebi (1974). The tissue homogenate was added to a reaction mixture of 80 mM phosphate buffer (pH 6.5) and 50 mM H_2O_2 with a total reaction volume of 1 ml for 1 min and measured at 240 nm on a spectrophotometer. Results were expressed as μ mol of CAT per mg of protein.

Statistical analysis

Multivariate analysis of variance (MANOVA) using the Pillai's Trace test statistic was used to determine significance of overall oxidative stress measured by the two dependent variables, catalase and lipid peroxidation. The MANOVAs identified significance between species, amongst treatments, over time, and in independent variable interactions (e.g., species*stressor, or species*day). Box's M tests were

performed to confirm homogeneity of covariances among groups prior to multivariate tests. One-way independent measure ANOVAs followed by Student Newman-Keuls (SNK) post-hoc comparison tests were used to compare differences among days, and the individual significance of lipid peroxidation levels and catalase response among treatments and between species ($\alpha = 0.05$). Prior to analysis, a Levene's Test was used to determine variance homoscedasticity among groups, and where appropriate, the data was log-transformed to decrease heterogeneity. The Pearson correlation coefficient was used to measure the degree of linear dependency of CAT and LPO for each stressor examined with the two-tailed correlation significance at $\alpha = 0.01$. All statistical analyses were conducted using IBM SPSS Premium Version 22 (SPSS Inc., 2011).

RESULTS

Density and PCBs

While the results of the single stressor studies revealed an overall positive effect of population density on the oxidative damage (LPO) of dreissenid mussels (Chapter 2, Figure 6 & 7), I observed a considerably different response when the mussels were exposed to high population densities in conjunction with the added stressor of PCBs (Figure 15). Both *D. polymorpha* and *D. bugensis* had a *decrease* in LPO under HIGH DENSITY conditions compared to baseline values ($p = 0.002$, ANOVA). The LPO response was independent of density for both species ($p = 0.630$, ANOVA) in the control treatments. However, with the combined stress of density and PCBs, *D. bugensis* experienced significantly higher LPO damage in the HIGH DEN-PCB treatments compared to mussels exposed to the single HIGH DENSITY stressor ($p =$

0.001, ANOVA; Figure 15a). *Dreissena bugensis* had more than double the LPO compared to *D. polymorpha* when both species were exposed to the HIGH DEN-PCB conditions ($p < 0.001$, ANOVA; Figure 15a). While this higher LPO response in *D. bugensis* under HIGH DEN-PCB conditions was marginally insignificant to baseline values ($p > 0.050$; SNK), it was significantly different from the decreases observed in the LOW DEN+PCB and MED DEN+PCB treatments ($p < 0.050$, SNK; Figure 15a). Conversely, *D. polymorpha* exposed to PCBs had a decrease in LPO in the MED DEN-PCB and HIGH DEN-PCB treatments compared to baseline values ($p < 0.050$, SNK), but the response was independent of density because there was no significant difference in average LPO among LOW DEN+PCB, MED DEN+PCB, or HIGH DEN+PCB treatments ($p = 0.422$, ANOVA; Figure 15a).

Population density alone did not have an impact on the antioxidant response (CAT) of either species at the end of the experiment as LOW DENSITY, MED DENSITY, and HIGH DENSITY CAT values were similar to baseline values for both species ($p > 0.050$, SNK; Figure 15b). However, there was a significant difference in response between mussels exposed to PCBs versus control mussels ($p < 0.001$, ANOVA) and a difference in the activity between *D. bugensis* and *D. polymorpha* ($p < 0.050$, SNK). *Dreissena bugensis* had a significant decrease in CAT activity with the addition of PCBs ($p < 0.050$, SNK) while *D. polymorpha* had a significant increase ($p < 0.050$, SNK) compared to baseline values, regardless of the population density (Figure 15b). *Dreissena bugensis* exposed to PCBs had nearly half the CAT activity of control mussels ($p < 0.001$, ANOVA). In contrast, CAT activity in *D. polymorpha* exposed to PCBs was double that of control mussels ($p < 0.001$, ANOVA; Figure 15b).

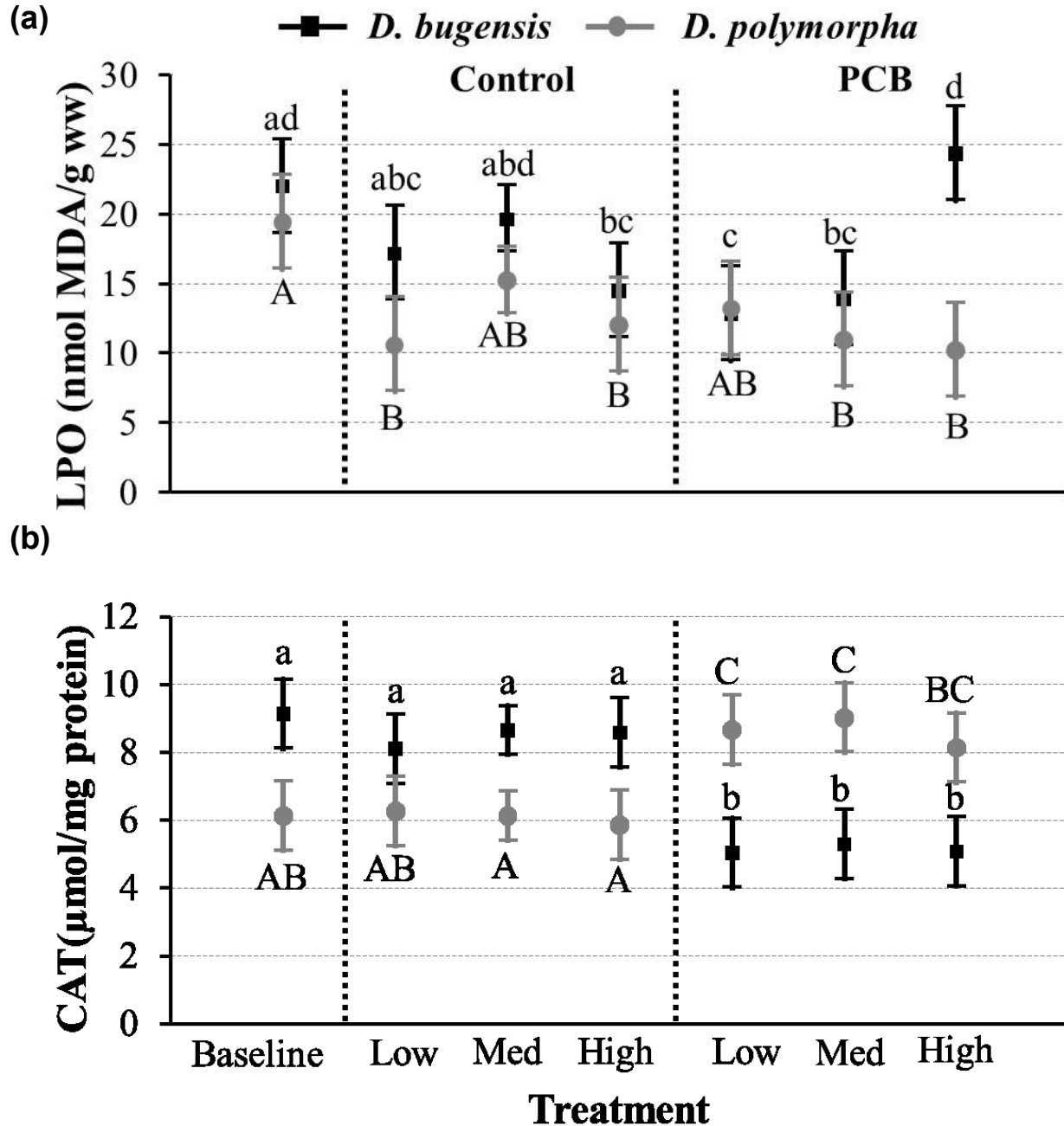


Figure 15. Oxidative stress measured as (a) lipid peroxidation (LPO) and (b) antioxidant catalase activity (CAT) in *D. polymorpha* and *D. bugensis* exposed to three population density treatments, alone (control) and in combination with PCBs during a 6 day assay. The population densities examined were: Low (1 mussel/40ml), Medium (1 mussel/20ml), and High (1 mussel/10ml). Initial LPO and CAT were measured in stock mussels prior to the start of the experiment on Day 0 (baseline), and mussel tissue was sampled at the end of the experiment. Data points represent 95% confidence intervals of the Species*Density*PCB interaction. Points having the same letters (lowercase for *D. bugensis* and uppercase for *D. polymorpha*) indicate no differences among days at $p < 0.050$ following ANOVA and Student Newman-Keuls post-hoc tests.

Temperature and PCBs

Over the duration of the multiple stressor assay in which mussels were exposed to extreme temperatures (5°C and 29°C) in combination with PCBs, combined treatments resulted in a significant increase in LPO damage and CAT activity in *D. bugensis* but had little effect on *D. polymorpha* ($p = 0.006$, MANOVA); whereas, single stressor treatments had no negative effects on either species ($p > 0.487$, ANOVA; Figure 16). LOW TEMP and HIGH TEMP treatments resulted in *decreases* in LPO in *D. bugensis* compared to baseline values ($p < 0.050$, SNK); however, LPO in *D. bugensis* in HIGH TEMP+PCB treatments was almost double the baseline values ($p = 0.028$, ANOVA) and seven times higher than the HIGH TEMP only mussels ($p < 0.001$, ANOVA; Figure 16a). Also, the HIGH TEMP+PCB treatment had higher LPO compared to the LOW TEMP+PCB and AMB TEMP+PCB treatments ($p < 0.050$ and $p < 0.050$, SNK, respectively). The LOW TEMP+PCB treatment had greater oxidative damage than the LOW TEMP treatments ($p < 0.050$, SNK), but was not significantly different than baseline values ($p > 0.050$, SNK; Figure 16a). The high LPO observed in the HIGH TEMP+PCB treatment in *D. bugensis* was also much higher than the LPO observed in *D. polymorpha* under the same conditions ($p < 0.050$, SNK). *Dreissena polymorpha* had no change in LPO under single or multiple stressor conditions compared to baseline values ($p < 0.050$, SNK). Average LPO values remained similar among temperature treatments ($p > 0.050$, SNK) and PCB versus control treatments ($p > 0.050$, SNK) in *D. polymorpha*.

When comparing the antioxidant response of *D. bugensis* and *D. polymorpha* exposed to extreme temperatures with PCBs, CAT activity was similar to baseline values for both species ($p < 0.050$; Figure 16b). However, *D. bugensis* had a slight

increase in CAT activity under LOW TEMP+PCB compared to LOW TEMP conditions ($p<0.050$) and under MED TEMP+PCB compared to MED TEMP conditions ($p<0.050$; Figure 16b). *Dreissena polymorpha* had lower CAT than *D. bugensis* in the HIGH TEMP, LOW TEMP+PCB, and MED TEMP+PCB treatments ($p<0.001$). *Dreissena polymorpha* had no change in CAT activity when comparing single stressor versus multiple stressor treatments ($p<0.050$), nor differences in CAT response among different temperatures ($p=0.299$).

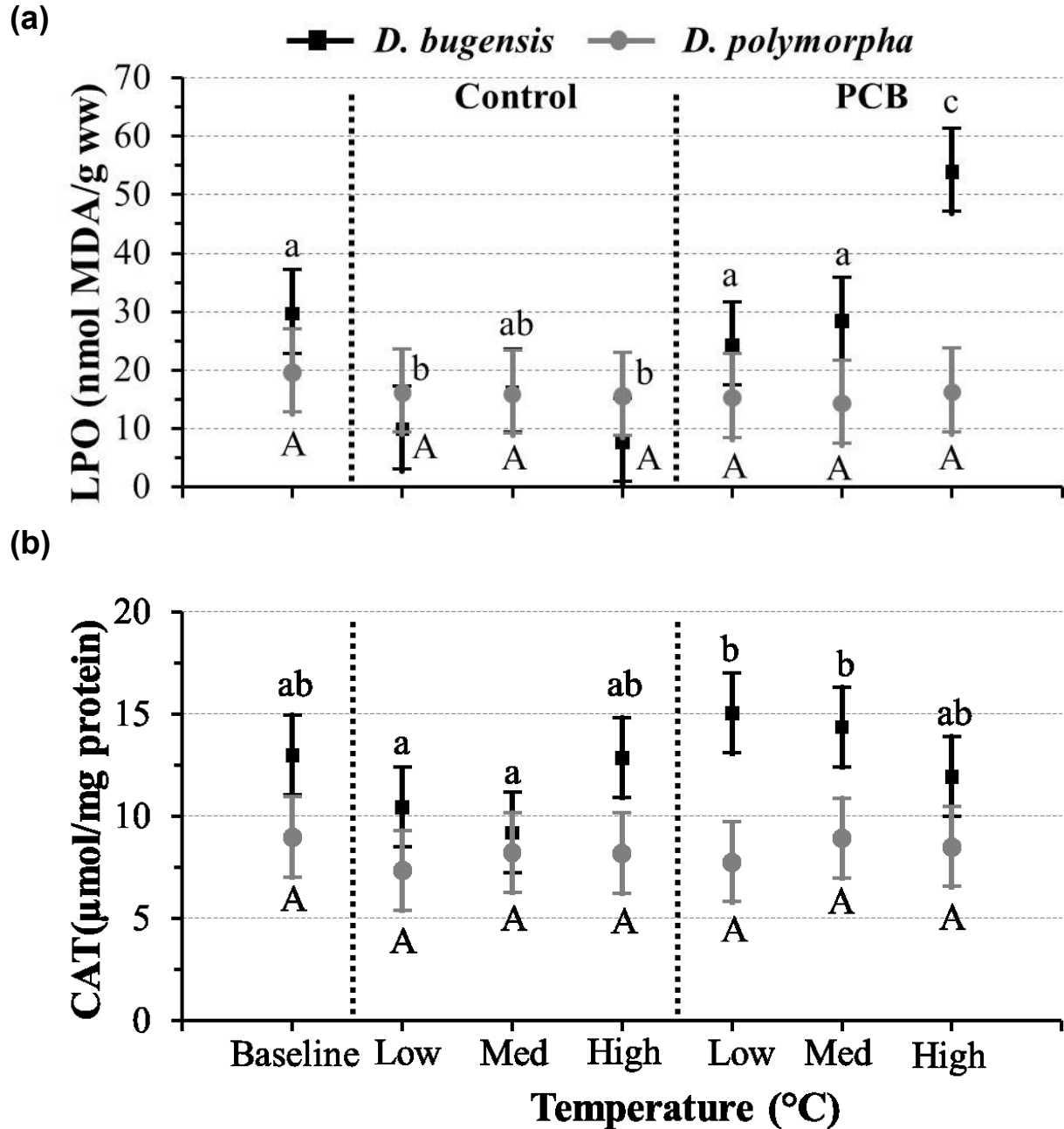


Figure 16. Oxidative stress measured as (a) lipid peroxidation (LPO) and (b) antioxidant catalase activity (CAT) in *D. polymorpha* and *D. bugensis* exposed to three temperature treatments, alone (control) and in combination with PCBs during a 3 day assay. The temperatures examined were: Low (5°C), Ambient (20°C), and High (29 °C). Initial LPO and CAT were measured in stock mussels prior to the start of the experiment on Day 0 (baseline), and mussel tissue was sampled on day 3. Data points represent 95% confidence intervals of the Species*Temperature*PCB interaction. Points having the same letters (lowercase for *D. bugensis* and uppercase for *D. polymorpha*) indicate no differences among days at $p < 0.050$ following ANOVA and Student Newman-Keuls post-hoc tests.

Hypoxia and PCBs

Physicochemical parameters between the treatments were similar throughout the entire experiment (temperature 18.3 – 19.1°C; pH 7.78 - 9.31; conductivity 235 – 256 $\mu\text{S}/\text{cm}$; Table 6). The oxic treatment chambers maintained a dissolved oxygen range between 8.25 - 9.29 mg/L, and the hypoxic treatment chambers maintained a dissolved oxygen range between 1.51 - 2.54 mg/L (Table 6).

When dreissenid mussels were exposed to hypoxic conditions both alone and in combination with PCBs, there was an overall decrease in LPO in *D. polymorpha* for all treatments ($p < 0.050$, SNK), with the lowest LPO in the Hypoxic and the Hypoxic+PCB conditions (Figure 17a). The Hypoxic and Hypoxic+PCB treatments had half the amount of LPO compared to Oxic treatments ($p < 0.001$, ANOVA). *Dreissena bugensis* had a decrease in LPO in the Hypoxic treatment compared to baseline values ($p < 0.050$, SNK); however, Oxic, Oxic+PCB and Hypoxic+PCB treatments were similar to baseline values ($p > 0.050$, SNK). *Dreissena polymorpha* had significantly lower LPO than *D. bugensis* in Oxic treatments ($p < 0.001$, ANOVA), but similar LPO measurements for the other treatments ($p < 0.001$, ANOVA; Figure 17a).

In *D. bugensis*, CAT activity was significantly higher in Hypoxic ($p < 0.050$, SNK), Hypoxic+PCB ($p < 0.050$, SNK), and Oxic+PCB ($p < 0.050$, SNK) treatments compared to the Oxic treatment and baseline values (Figure 17b). However, the Hypoxic versus Hypoxic+PCB treatments had similar CAT activities, and the presence of PCBs did not have a significant effect on CAT activity ($p = 0.065$, ANOVA) or LPO response ($p = 0.642$, ANOVA) for either species. *Dreissena polymorpha* had no change in CAT activity across all treatments, and except for higher baseline CAT values ($p < 0.050$, SNK), had similar responses compared to *D. bugensis* in all treatments ($p > 0.050$, SNK).

Table 6. Physicochemical parameters including dissolved oxygen (mg/L), temperature (°C), pH, and conductivity (µS/cm) were measured in experimental chambers using a multi-parameter probe (YSI 556 Multi-Parameter Sensor) during a 3 day multiple stressor assay (hypoxia and PCBs) with *Dreissena bugensis* and *D. polymorpha*. Measurements were taken on days 1 and 2. Measurements are expressed as the average ± standard deviation.

| Treatment | Day | Dissolved oxygen (mg/L) | Temperature (°C) | pH | Conductivity (µS/cm) |
|------------------------------------|-----|-------------------------|------------------|-------------|----------------------|
| <i>D. bugensis</i> oxic | 1 | 8.90 ± 0.28 | 19.0 ± 0.33 | 8.09 ± 0.25 | 245 ± 2.00 |
| | 2 | 9.03 ± 0.16 | 19.0 ± 0.57 | 8.28 ± 0.02 | 245 ± 2.89 |
| <i>D. bugensis</i> hypoxic | 1 | 2.16 ± 0.44 | 19.1 ± 0.22 | 8.91 ± 0.22 | 245 ± 1.15 |
| | 2 | 1.91 ± 0.51 | 19.0 ± 0.07 | 8.91 ± 0.54 | 244 ± 0.00 |
| <i>D. bugensis</i> oxic + PCB | 1 | 8.83 ± 0.08 | 18.6 ± 0.11 | 8.16 ± 0.30 | 249 ± 8.89 |
| | 2 | 8.97 ± 0.43 | 18.8 ± 0.33 | 8.43 ± 0.02 | 251 ± 9.29 |
| <i>D. bugensis</i> hypoxic + PCB | 1 | 2.33 ± 0.34 | 18.9 ± 0.18 | 9.06 ± 0.18 | 256 ± 2.08 |
| | 2 | 1.88 ± 0.38 | 18.7 ± 0.75 | 9.31 ± 0.01 | 256 ± 7.02 |
| <i>D. polymorpha</i> oxic | 1 | 8.25 ± 0.34 | 18.7 ± 0.04 | 7.78 ± 0.53 | 242 ± 4.73 |
| | 2 | 9.11 ± 0.39 | 18.5 ± 0.75 | 8.36 ± 0.11 | 245 ± 15.6 |
| <i>D. polymorpha</i> hypoxic | 1 | 2.54 ± 0.68 | 18.8 ± 0.35 | 8.76 ± 0.19 | 243 ± 4.04 |
| | 2 | 1.87 ± 0.51 | 18.8 ± 0.20 | 8.98 ± 0.16 | 247 ± 1.53 |
| <i>D. polymorpha</i> oxic + PCB | 1 | 9.06 ± 0.22 | 19.0 ± 0.71 | 8.29 ± 0.05 | 241 ± 0.58 |
| | 2 | 9.29 ± 0.60 | 18.3 ± 0.57 | 8.33 ± 0.02 | 235 ± 5.29 |
| <i>D. polymorpha</i> hypoxic + PCB | 1 | 2.27 ± 0.26 | 19.0 ± 0.11 | 8.88 ± 0.09 | 249 ± 9.85 |
| | 2 | 1.51 ± 0.11 | 18.8 ± 0.39 | 9.09 ± 0.03 | 251 ± 9.29 |

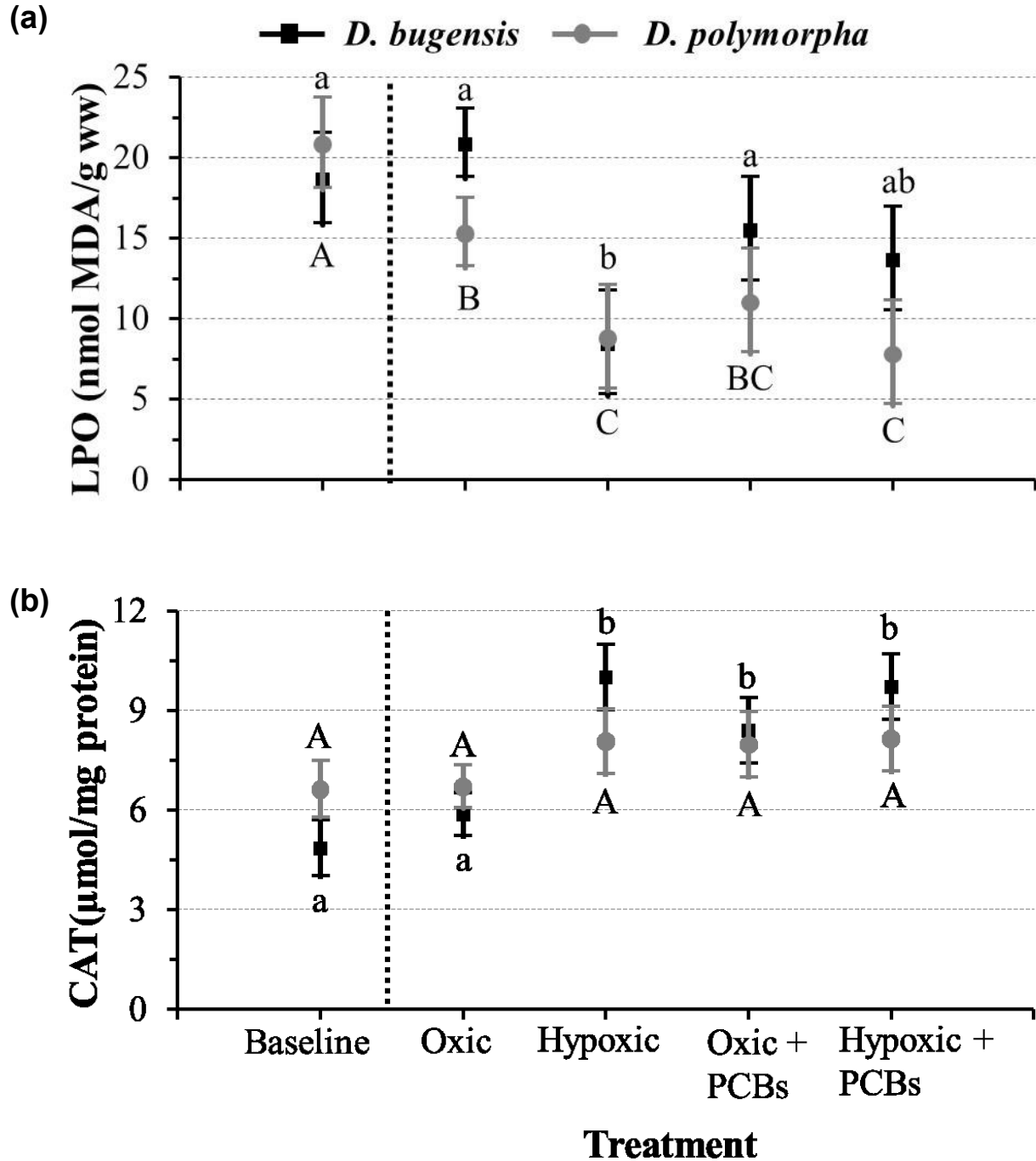


Figure 17. Oxidative stress measured as (a) lipid peroxidation (LPO) and (b) antioxidant catalase activity (CAT) in *D. polymorpha* and *D. bugensis* exposed to hypoxic and oxic conditions, both alone and in combination with PCBs, during a 2 day assay. Initial LPO and CAT were measured in stock mussels prior to the start of the experiment on Day 0 (baseline), and mussel tissue was sampled on day 2. Data points represent 95% confidence intervals of the Species*Temperature*PCB interaction. Points having the same letters (lowercase for *D. bugensis* and uppercase for *D. polymorpha*) indicate no differences among days at $p < 0.050$ following ANOVA and Student Newman-Keuls post-hoc tests.

Correlation between CAT and LPO

Catalase and LPO had a strong negative correlation ($r = -0.543$, $p = 0.002$) in the hypoxia and PCB experiment (Figure 18c). Under the temperature and PCBs and the density and PCBs experiments, there was no significant correlation between LPO and CAT (Figure 19a and Figure 19b).

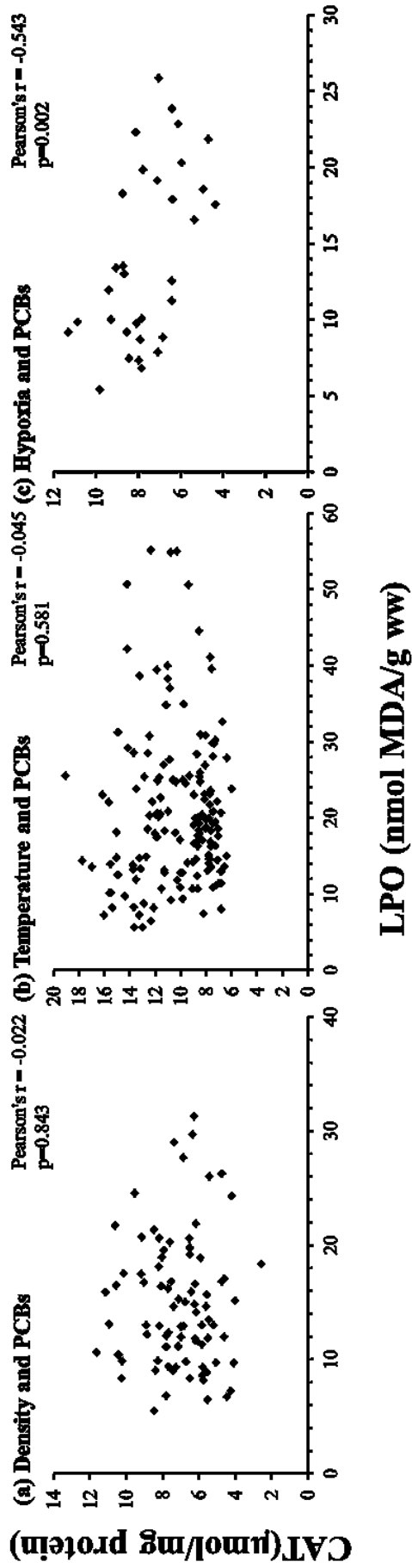


Figure 18. Pearson correlations measuring the linear dependency of CAT and LPO for the three multiple stressor experiments (a) density and PCBs, (b) temperature and PCBs, and (c) hypoxia and PCBs.

DISCUSSION

The results of my research reveal a significant physiological differentiation in oxidative stress mechanisms between two inherently similar dreissenid species, *D. bugensis* and *D. polymorpha*. This difference in antioxidant response and oxidative damage shows a potential role of oxidative stress in dreissenid tolerance to environmental stressors and has implications for understanding dreissenid mussel ecology and distribution. *Dreissena bugensis* has several competitive advantages over *D. polymorpha* in the Great Lakes including greater tolerance to depth and temperature shifts (Mitchell et al. 1996) and hypoxic conditions (Stoeckmann 2003), and physical differences in size (Karatayev et al. 2011) and filtration rates (Diggins 2001). Furthermore, *D. bugensis* has shown evidence of a possibly stronger oxidative stress response (higher CAT activity) and greater tolerance than *D. polymorpha* to single stressors such as PCBs and hypoxic conditions (Chapter 2); therefore, it was expected that *D. bugensis* would show greater tolerance compared to *D. polymorpha* when exposed to multiple environmental stressors. However, the results of this study show that when *D. bugensis* was exposed to multiple stressors there was a detrimental impact on the organism's ability to manage stress, indicated by increased oxidative damage in the high density plus PCB and the high temperature plus PCB experiments. On the other hand, *D. polymorpha* had similar stress responses between single stressors and multiple stressor treatments. The higher baseline CAT levels and robust CAT response previously observed in *D. bugensis* in high population density treatments (Chapter 2) is diminished with the added stress of PCBs. Some research suggests this decrease in catalase is the result of a chemical contaminant (i.e. PCBs) increasing ROS

to the point where catalase is actually inhibited (Kono and Fridovich 1982). However, *D. polymorpha* does not experience this same decrease in CAT with the addition of PCBs; therefore, this dramatic drop in CAT in *D. bugensis* may be associated with an overwhelmed antioxidant response under the combined stress of high population density and PCBs.

The added stress of PCBs with high temperatures appeared to have an overall synergistic effect on oxidative damage in *D. bugensis*. The effect of PCBs alone had a marginal mean of 19.5 nmol MDA/g ww (Chapter 2), while high temperatures had a mean of 7.7 nmol MDA/g ww, which would give an anticipated combined additive effect of about 27.2 nmol MDA/g ww. Since the results of the HIGH TEMP+PCB treatments reveal a LPO mean of 53.9 nmol MDA/g ww in *D. bugensis*, which is nearly double the LPO values of the additive effect, the multiple stressor effect of high temperatures and PCBs may be synergistic.

Cost of tolerance

Populations from contaminated sites often are more resistance and less sensitive to contaminants than populations from uncontaminated sites (Klerks and Weis 1987, Posthuma and Vanstralen 1993, Kashian et al. 2007). My results suggest that the oxidative stress pathway may play a role in this type of tolerance. For example, if a population of organisms is exposed to an environmental stressor for an extended period of time, the population may adapt to that stressor and develop an increased capacity to rapidly produce high levels of antioxidants to increase survival and develop resistance to additional perturbations. However, in natural systems there are many potential

confounding environmental variables, including water chemistry, temperature, species interactions and combinations of multiple stressors, which may affect the ability of the oxidative stress response to neutralize reactive oxygen species resulting from environmental stress. The dramatic difference in oxidative stress in *D. bugensis* in single stressor versus multiple stressor treatments demonstrates this effect, and reveals a potential cost of this species' tolerance to single stressors. The cost of tolerance mechanism has been attributed to an organism reallocating resources and energy to combat the environmental stressor, leaving less energy for critical functions such as growth and reproduction (Congdon et al. 2001). Studies on the effects of multiple stressors and the cost of tolerance due to resource reallocation in aquatic invertebrates have increased (Courtney and Clements 1998, Clements 1999, Kashian et al. 2007, Blake and Duffy 2010), but the molecular effects of this tolerance phenomenon remain poorly studied and understood. The physiological mechanisms explored in this study show the role of oxidative stress in this type of resource allocation. While previous research demonstrated that *D. bugensis* can tolerate single stressors such as extreme temperatures (Karatayev et al. 2011) or hypoxic condition (Stoeckmann 2003) better than *D. polymorpha*, this tolerance may have been at the expense of resources needed for the antioxidant response of *D. bugensis* to additional stressors (i.e. PCBs). The results of this study reveal the potential role of oxidative stress biomarkers as a way to measure changes in tolerance and capture the molecular effects of resource reallocation in multiple stressor environments.

Influencing tolerance through oxidative stress

Tolerance to environmental stressors may be achieved through the addition of antioxidants via the oxidative stress pathway. An increase in chemical tolerance due to elevations in antioxidants has been extensively studied in aquatic plants (Roy et al. 1994, Sivaci et al. 2007, Abraham and Dhar 2010, Wei et al. 2011). Wei et al. (2011) found that the algae *Clamydomonas reinhardtii* had increased mercury tolerance when the antioxidant heme-oxygenase-1 was over expressed in transgenic algae. In addition, a study on aquatic fungi found that copper and zinc tolerance is dependent on the fungi efficiently initiating the antioxidant defense system (Azevedo et al. 2007). In the marine worm, *Glycera dibranchiate*, the addition of antioxidants to the environment reduced organism sensitivity to sulfide (Ortega et al. 2008). Also, doses of the antioxidant Vitamin E can protect against lipid peroxidation caused by ultraviolet radiation, and extend the lifespan of the rotifer *Asplanchna brightwelli* (Sawada and Enesco 1984, Sawada et al. 1990). Antioxidants are also important in neutralizing paraquat toxicity in terrestrial insects (Driver and Georgeou 2003). The superior tolerance of some nuisance species, such as dreissenid mussels, may be attributed to robust antioxidant levels in response to environmental stressors. Pro-oxidants may be used to dampen the tolerance of nuisance species and may be a potential management tool. For example, the non-radical ROS, hydrogen peroxide, has been shown to cause increased antioxidant response and oxidative damage in digestive glands in marine mussels at relatively low concentrations (Cavaletto et al. 2002). Further research is needed on using pro-oxidants to manipulate tolerance, given the inherent role of oxidative stress in

invertebrate tolerance to environmental stressors, and the significant negative impacts of invasive species globally.

Future applications: Oxidative stress as a biotic index

Oxygen radicals are found in all organisms and antioxidant defense mechanisms are conserved in all eukaryotic cells (Halliwell and Gutteridge 1999). The unique ability of oxidative stress to be measured in all aquatic organisms makes it an ideal indicator for community stress. At the community level, tolerance to environmental stressors is the response of all individuals in the community (Clements 1999, Millward and Klerks 2002, Kashian et al. 2007). A community's tolerance to a specific stressor will increase as species develop mechanisms to cope with the stressor, or more sensitive species are eliminated and the more tolerant species remain, a phenomenon referred to as the pollution-induced community tolerance model (Blanck and Wangberg 1988, Dahl and Blanck 1996).

Currently, the potential impact of a stressor on an aquatic system is most often evaluated using several physical indicators such as water quality parameters (pH, dissolved oxygen, temperature, etc.), or biological indicators such as indicator species and community indices (e.g. Shannon-diversity Index) (Watzin and McIntosh 1999). Typically, combinations of these methods are used to evaluate ecological conditions. However, the traditional biological indicator approach mostly depends on the presence or absence of species following a disturbance event, but it does not reveal the mechanism of action by which the disturbance affects aquatic organisms. The results of this study may provide molecular techniques to assess how organisms will respond to

environmental stressors in aquatic ecosystems. For example, stress in aquatic invertebrate communities could be used to develop an index of biological integrity similar to that of the Hilsenoff Biotic Index (Hilsenhoff 1987) using oxidative stress. Species that exhibit high quantities of lipid peroxidation and catalase would be considered less tolerant, and species with the lowest quantities would be considered highly tolerant. Species can be arranged along a gradient of high oxidative stress (low tolerance) to low oxidative stress (high tolerance) and assigned stress values. To calculate the overall stress of the entire community, the stress values of each species can be multiplied by the proportion of those species in the community and summed over all species to get the community stress value. To measure community oxidative stress in the environment, researchers would collect a subsample of the aquatic invertebrates in a community, quantify the number of individuals in each species, assign oxidative stress values to each group, and predict community oxidative stress. An oxidative stress assessment may be more protective of current Indices of Biological Integrity (IBI), as a measure of stress may be obtained prior to shifts in community composition due to a stressor. This type of index can have important implications for management and restoration of impact systems affected by single and multiple stressors.

SIGNIFICANCE

This research advances the knowledge of environmental contaminant exposure and stress mechanisms in aquatic organisms. Results from the aqueous versus sediment exposure experiments revealed that the primary matrix of PCB exposure for benthic and planktonic organisms is through the sediments. Although the sediments contributing to toxicity is not a novel finding, my research showed the importance of evaluating the sediment water interface and its role in chemical exposure. These findings were verified by an *in situ* field experiment using *D. bugensis*. Chemical contaminations in aquatic ecosystems have direct application to public health, as municipal drinking water intakes are often located within freshwater lakes and rivers. The U.S. EPA enforces a maximum contaminant level for PCBs in public drinking water at 0.0005 ppm, and any concentrations in excess of 50 ppm are considered hazardous waste. The PCB concentrations of the St. Clair Shores canals are over 20 times the level of hazardous waste materials and over 2 million times the appropriate level for safe public drinking water. Results of this study imply that even if the PCB inputs are stopped, the contamination from the sediments will continue to be toxic to invertebrate communities. It is critical for management efforts at PCB-impacted sites to include a more integrated sampling regime that not only monitors surface waters but also monitors toxicity at the sediment-water interface.

The results of this study will also make significant contributions to the understanding of detrimental cellular effects associated with environmental stressors, and deepening the understanding of mechanisms contributing to invasive mussel success. The potentially weaker antioxidant response in *D. polymorpha* may result in

this species being more susceptible to disturbance, giving *D. bugensis* a competitive edge in some disturbed environments. By comparing the environmental tolerances and oxidative stress response of *D. bugensis* and *D. polymorpha*, I established a potential role for oxidative stress in environmental tolerance. While this study focuses on aquatic invertebrates, it has implications for vertebrate species that utilize these waters, such as fish and humans. Toxic effects of oxygen radicals are observed in all organisms including bacteria, plants, and animals including humans (Halliwell and Gutteridge 1999). Furthermore, antioxidant defense enzymes, such as catalase are present in virtually all eukaryotic cells and in several prokaryotes as well (Halliwell and Gutteridge 1999). The conservation of the oxidative stress mechanism allows for this endpoint to be easily extrapolated for other organisms, including humans. In fact, several studies are examining links between oxidative stress and Parkinson's disease, Alzheimer's, diabetes, rheumatoid arthritis, and cancer (Halliwell and Gutteridge 1999, Hong et al. 2009, Butterfield et al. 2010, Sriraksa et al. 2012).

Most importantly, the results of this research provide the foundation to develop a novel and rapid bioassessment metric for evaluating oxidative stress within an entire community of species. By understanding the oxidative stress potential of organisms in a community, researchers will better predict how aquatic communities respond to disturbances such as chemical contaminants. Organisms that can upregulate their antioxidant potential may be more capable to resist disturbances and survive in the environment than organisms with lower antioxidant levels. With invasive species, such as dreissenid mussels, having global impacts to native flora and fauna, exploring oxidative stress mechanisms may be important to understanding why these organism

are able to persist. This research has expanded the knowledge of these processes so scientists and managers can develop better methods for remediation efforts of impacted systems.

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ABSTRACT**ENVIRONMENTAL STRESSORS: PATHWAYS OF EXPOSURE AND AQUATIC
INVERTEBRATE RESPONSE**

by

CARLY J. NOWICKI**December 2014****Advisor:** Dr. Donna Kashian**Major:** Biological Sciences**Degree:** Doctor of Philosophy

The need to monitor freshwater and detect impairments prior to observable impacts is crucial to maintain species diversity and ecosystem function. Therefore, understanding the contribution of various matrices (i.e., sediment and water) to chemical exposure is critical for remediation of impacted sites.

To evaluate various matrices of chemical exposure, I focused on the organic pollutant polychlorinated biphenyls (PCBs) due to their relatively ubiquitous nature, high toxicity, and adverse effects on humans and wildlife. In complementary laboratory and field experiments, I compared the effects of aqueous versus sedimentary exposure of PCBs on invertebrates. In the laboratory, organisms exposed to PCB-contaminated sediments experienced greater mortality than those exposed to PCB-contaminated water and in the field experiments those closer to the sediments experienced greater mortality than those near the surface demonstrating the importance of evaluating toxicity at the sediment-water interface.

Beyond identifying the location of highest chemical exposure, it is important to monitor a protective endpoint that can indicate impacts before notable damage occurs. Oxidative stress (OS) can be used as a sub-lethal physiological impairment that if identified can indicate an environmental stressor prior to species losses. I examined OS and its potential role in stressor tolerance using two invasive mussel species, *Dreissena bugensis* and *D. polymorpha*. Mussels were evaluated for OS via lipid peroxidation and catalase activity following exposure to four stressors (e.g., high densities, temperature, hypoxia, and PCBs) both alone and in combination. *Dreissena bugensis* had a stronger OS response than *D. polymorpha* in single stressor conditions ($p < 0.050$); however, in multiple-stressor treatments *D. bugensis* had increased oxidative damage and was less tolerant to additional stressors. My results establish a correlation between tolerance to environmental stressors and oxidative stress in invertebrates, and demonstrate that *D. bugensis* competitive tolerance may come at the cost of the ability to respond to additional stressors via the antioxidant response. This study advances the ability to monitor aquatic systems. It identifies the sediment-water interface as an area of greater chemical exposure than surface waters and presents a novel and sensitive assay that can detect stressors early in the impact stages and help predict changes in aquatic communities.

AUTOBIOGRAPHICAL STATEMENT

Nature and the outdoors have always been and forever will be a huge part of my life. My father, through fishing and beach combing on the Great Lakes, taught me to always find peace “on the water.” My mother, with her passion for the wildlife in her impressive gardens, taught me to listen to nature and appreciate its intricate beauty. I even fell in love with, and eventually married, the “nature boy” from my school. However, it wasn’t until I was older when I grasped the severity of human impacts on the environment. My father told me stories of growing up down river near the steel mills and automobile plants and when he saw the heavily-polluted Rouge River catch fire. I too grew up on the Rouge and remember being warned about the mutated frogs and fish I caught in the stream. Even at a young age, I was bothered that it wasn’t “safe” for me to play in the water. When I began my graduate career, I knew I wanted to address these environmental issues and understand human impacts on nature. Now, the skills, knowledge, and professional relationships that I developed in graduate school have made me a strong research scientist that can help shape the future of the environmental field.

I believe that in the near future our society will need to transition from an anthropocentric view of the environment to a more ecocentric view where we are reorganizing our systems to produce less pollution and conserve resources, including water. It is a mistake to separate humans from nature. We are all a part of the environment and have a responsibility to protect it for our own sake, for the sake of the flora and fauna that share this planet, and for future generations. Rachel Carson said it best in her book *Silent Spring*:

“We stand now where two roads diverge. But unlike the roads in [Robert Frost’s](#) familiar poem, they are not equally fair. The road we have long been traveling is deceptively easy, a smooth superhighway on which we progress with great speed, but at its end lies disaster. The other fork of the road — the one less traveled by — offers our last, our only chance to reach a destination that assures the preservation of the earth.”

I have decided to take the road less traveled. Will you?