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Sensitivity Of Quagga Mussels (dreissena Rostriformis Bugensis) To Cyanobacteria At Multiple Life History Stages

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SENSITIVITY OF QUAGGA MUSSELS (*DREISSENA ROSTRIFORMIS BUGENSIS*) TO CYANOBACTERIA AT MULTIPLE LIFE HISTORY STAGES

by

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Advisor   Date

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DEDICATION

I dedicate this to all of those who came before me and fought so that I could be here;
To my mentors who cared enough to encourage me
To my family who was there for me unconditionally
To Matt, Popcorn, and Marge who complete my home

And to all the marginalized and underrepresented students who come after me,
I will always fight for you.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Quagga mussels (*Dreissena rostriformis bugensis*) originate from the brackish waters of the Ponto-Caspian region and have since invaded fresh waters in Europe and North America (Ram et al. 2012). Their voracious filter feeding limits plankton in the water column and shifts nutrients and other resources to the benthic zone (Higgins and Vander Zanden 2010). Dreissenid mussels can filter feed on microzooplankton, such as rotifers and small copepods, which can cause shifts in the zooplankton community that favor larger animals (Pothoven et al. 2013). Likewise, phytoplankton dynamics in lakes infested with zebra (*Dreissena polymorpha*) and quagga mussels have also undergone drastic changes. Dreissenids are often responsible for decreasing primary productivity, even in nutrient rich systems, and phytoplankton production is shifted to nearshore, benthic species and cyanobacteria (Allinger and Reavie 2013). Michigan lakes invaded by dreissenid mussels have approximately three times higher concentrations of cyanobacteria species when compared to univaded lakes (Sarnelle et al. 2012). Through selective filter feeding and production of pseudofeces, dreissenid mussels can promote the size and frequency of harmful cyanobacteria blooms (Vanderploeg et al. 2001). Cyanobacteria are ubiquitous, with *Microcystis* being the most common and pervasive genus worldwide (Svrcek and Smith 2004). Many species of cyanobacteria produce toxins, which can be harmful to humans and wildlife (Carmichael 1994; Paerl and Otten 2013). Cyanotoxins can target the hepatic, neural, and dermal systems as well as cellular structure and genetic material of animal species (Svrcek and Smith 2004). While the lethal and sublethal effects of cyanobacteria and related toxins have been documented for humans and many aquatic terrestrial species (Carmichael and

Boyer 2016), it is unknown how nuisance phytoplankton may impact dreissenid populations, which have been highly successful invasive species.

Quagga mussels and their congeneric species, the zebra mussel, first arrived in the United States via ballast water transport to the Great Lakes in the late 1980s (Ram et al. 2012). Both dreissenid species have caused excessive economic and ecological damages since their invasion into North American waters. Dreissenid infestations have cost millions of dollars, with some estimates reaching up to one billion dollars (Pimentel et al. 2005; Higgins and Vander Zanden 2010). Dreissenid mussels form dense colonies and can clog pipes in public works facilities that rely on water intake from infested waters, costing an estimated $11-16 million per year in mussel removal and equipment maintenance (Connelly et al. 2007).

Ecologically, dreissenid mussels have also fundamentally altered freshwater ecosystems. They have displaced populations of many native Unionidae mussels, some of which are threatened or endangered (Higgins and Vander Zanden 2010). Dreissenid mussel invasions have been successful in part due to their high rates of filter feeding and prolific spawning that results in planktonic larvae, which disperse in the water column and establish new populations away from their origin (Ram et al. 2011). Initially, zebra mussels were quick to establish dense populations and expanded their range rapidly through connecting waterways and recreational watercraft transport; however in recent years quagga mussels have begun displacing existing zebra mussel populations and have started to invade the western United States (Ram et al. 2012). Quagga mussels have been known to eventually become the dominant species when coexisting in the same habitat as zebra mussels (Karatayev et al. 2011). Zebra and quagga

mussels share many similarities, however key differences exist between the two species that can influence their invasive ranges and population dynamics in novel freshwater systems. Quagga mussels have a wider habitat range than zebra mussels, and are able to reproduce both in the cold, deep profundal zone and the warmer, more littoral zone of large lakes (Baldwin et al. 2002). Quagga mussel populations have been recorded as deep as 130 m in the Great Lakes and have a lower thermal tolerance and lower food requirements for growth and reproduction than zebra mussels (Mills et al. 1996; Baldwin et al. 2002). As a result of lower thermal tolerance and food necessity than zebra mussels, quagga mussels have a longer spawning season that begins earlier in the year (Ram et al. 2012). These subtle differences between zebra and quagga mussels may give the latter a substantial advantage in becoming the dominant invasive bivalve in North America.

So far, eradication and control of dreissenid mussels has been largely unsuccessful. While Zequanox is a dreissenid specific, lethal product that is effective in certain control situations (Molloy et al. 2013), thus far it has not been applicable for large-scale, open water use. Chlorine is often used as a control for small scale industrial uses, but it is not effective for large-scale, open water use because of environmental restrictions on residual chlorine and its broad toxicity to non-target organisms (Aldridge et al. 2006). As quagga mussels are relatively recent invaders to the freshwaters of North America, there has been a rapid response to try to mitigate their economic and ecosystem consequences. However, a paucity of information exists on the sensitive life history stages of these animals and their interactions with naturally occurring harmful cyanobacteria. Furthermore, much of the existing literature focuses on the zebra

mussel, and the two species may have varying tolerance levels to environmental stressors (Nowicki et al. 2018), and thus more quagga mussel specific research on their physiology and sensitivities warrants further investigation.

While frequent and severe cyanobacteria blooms and healthy quagga mussel populations seem to exist in North American freshwater systems, determining sublethal and reproductive impacts of cyanobacteria and their related toxins on quagga mussels can provide novel insight on the management and population dynamics of this pervasive species. To address how cyanobacteria impact quagga mussel reproduction, Chapter 1 will evaluate quagga mussel sperm motility in the presence of several cyanobacteria cultures and Chapter 2 will address how cyanobacteria influence spawning and fertilization rates. Then, Chapter 3 will focus on the next life stage of dreissenid mussels, the planktonic larval stage, to assess mortality of this sensitive life stage to cyanobacteria and cyanotoxins. Finally, in Chapter 4 I examine the role that evolutionary traits play in stress tolerance of quagga mussels with exposure of *Microcystis aeruginosa* and its associated toxin, microcystin.

Table 1. Summary of cyanobacterial species and isolates that were exposed to bivalves in experiments. The "chapters" column refers to the chapters that describe how each isolate was used in experiments. Sperm motility was tested in Chapter 1; spawning and fertilization were tested in Chapter 2; veliger mortality was tested in Chapter 3; filtration rate and oxygen consumption were tested in Chapter 4.

Species	Isolate identifier	Origin	Chapter(s)
Anabaena flos-aquae	CPCC64	Lake Ontario	1,2,3
Aphanizomenon flos-aquae	APHH	Hamilton Harbour, Lake Ontario	1,2,3
Dolichospermum lemmermanii	LE11-02	Lake Erie, East Basin	1,2,3
Gloeotrichia echinulata	ASGV	Unknown	2
G. echinulata	BQ11-03	Bay of Quinte, Lake Ontario	1,2,3
Lyngbya wollei	LSC14	Lake St. Clair, Michigan, USA	2
Microcystis aeruginosa	UTEX LB2385	Little Rideau Lake, Ontario, Canada	1,2,3,4
M. aeruginosa	LEMS	Lake Erie	1,2,3,4
M. aeruginosa	BQ11-02	Bay of Quinte, Lake Ontario	1,2,3
M. aeruginosa	ZUR-HINDAK	Unknown	1,2,3
M. aeruginosa	LSC-13-02	Lake St. Clair	1,2,3
M. wesenbergii	LE13-01	Lake Erie western basin	1,2,3
Planktothrix suspensa	1163PL1	Sandusky Bay, Lake Erie	1,2,3

CHAPTER 1: SUBLETHAL EFFECTS OF CYANOBACTERIA ON SPERM OF A BROADCAST SPAWNING BIVALVE (QUAGGA MUSSEL: *DREISSENA ROSTRIFORMIS BUGENSIS*)

Introduction

Freshwater phytoplankton blooms, including harmful algal blooms (HABs), have been increasing in size and frequency, and have been appearing ahead of their historical norm in response to a changing climate (Thackeray et al. 2010; Paerl and Otten 2013). Freshwater HABs, almost exclusively associated with cyanobacteria, may have the potential to disrupt sensitive life history stages of aquatic animals. Cyanobacteria blooms can exert both lethal and sublethal consequences to aquatic organisms, which can interrupt physiological processes, including reproduction (Boegehold et al. 2018).

As HABs occur more frequently with an earlier seasonal onset, overlap with broadcast spawning events, which may occur from spring through fall, becomes more likely. Broadcast spawning species in aquatic systems, such as some fish and invertebrates, release gametes directly into the water column, resulting in external fertilization (Ripley and Caswell 2008). This reproductive strategy allows for higher fecundity than brood spawners, but comes at a cost of lower juvenile survival and potentially leaves gametes susceptible to environmental conditions, such as annual phytoplankton blooms (Ripley and Caswell 2008). While broadcast spawning is common in marine invertebrates, it is rare in freshwater invertebrates (Misamore and Lynn 2000). As such, environmental limitations to broadcast spawners in freshwater systems have rarely been studied. Dreissenid mussels that have invaded freshwaters of North America and Europe are broadcast spawners whose spawning season can

overlap with summer cyanobacteria blooms (Ram et al. 2011; Quinn and Ackerman 2012), resulting in the potential for reproductive impairments associated with cyanobacteria exposure.

Invasive zebra mussels (*Dreissena polymorpha*) and quagga mussels (*D. rostriformis bugensis*) originate from the Ponto-Caspian region and have been proliferating at an alarming rate in invaded areas (Ram and Palazzolo 2008). Dreissenid mussels have been successful invaders in part due to their high fecundity and reproductive success, which can lead to high population densities (Sprung 1991). Although zebra mussels and quagga mussels are congeneric species and share similar invasion traits, key differences between the species exist. Much of the research on invasive *Dreissena* species focuses on zebra mussels due to their rapid expansion post-invasion where they colonized the Laurentian Great Lakes within a few years of their introduction (Ram et al. 2012). Recently, quagga mussels have been displacing zebra mussel populations in the Laurentian Great Lakes region and are spreading westward in the United States and Europe at a greater rate (Ram et al. 2012; bij de Vaate et al. 2013). With quagga mussels currently a more pressing threat to uninvaded freshwater systems, there is a growing need for quagga mussel specific research.

Much of the research on quagga mussels has focused on determining factors that contribute to the displacement of zebra mussels by quagga mussels. Several studies found that quagga mussels have several competitive advantages over zebra mussels, including lower respiration rates and a greater tolerance to low oxygen levels (Stoeckmann 2003; Alexander and McMahon 2004). However, while quagga mussels have some competitive advantages over zebra mussels, they also exhibit higher

sensitivity to certain stressful conditions and may be exposed to a unique set of environmental conditions compared to zebra mussels. Evaluating oxidative stress biomarkers, Nowicki and Kashian (2018) found evidence suggesting that quagga mussels may be more sensitive to multiple stressors, such as high temperatures and PCBs, than zebra mussels. Karatayev et al. (2015) documented that quagga mussels can colonize a greater range of habitats, occupying colder and deeper habitats and attaching to softer substrates. In addition, Ram et al. (2011) found that quagga mussels exhibit a longer spawning season than zebra mussels, which begins in late spring when water temperatures reach 10°C and can last throughout the early fall. This expanded spawning season can potentially coincide with the expanding seasonal occurrence of HABs. Boegehold et al. (2018) provided evidence that quagga mussel spawning and fertilization can be adversely affected by cyanobacteria, but the extent of toxicity on quagga mussel gametes is yet unknown. Therefore, quagga mussels may be the better of the two dreissenid species for toxicity tests because they are both a greater nuisance than zebra mussels and also may be more sensitive to certain stressors.

Traditional measures of toxicity, such as the median lethal concentration (LC_{50}), are simplistic and may not accurately reflect the full range of impacts environmental stressors can have on an organism. A sperm cell may remain active during a cyanobacteria bloom, but may be significantly impaired and unable to fertilize an egg; therefore alternative toxicity assays can help fully address environmental contaminants. Evaluation of sublethal endpoints such as reproduction and development can reveal how contaminants have population and ecosystem level effects that are not directly associated with mortality. For example, the use of sea urchin embryo testing to evaluate

the toxicity of marine waters is common worldwide and uses endpoints of larval growth and development (Saco-Álvarez et al. 2010). Other studies have demonstrated that low concentrations of environmental contaminants may not be enough to cause mortality in *Daphnia*, but can impact growth, development, and swimming behavior (Dodson and Hanazato 1995; Kashian and Dodson 2002; Zein et al. 2014). Evaluating sublethal endpoints addresses the complexity of ecosystem interactions. Thus, sublethal endpoints related to reproduction may be useful to address environmental dynamics between cyanobacteria and broadcast spawning species.

Using a novel laboratory method to evaluate environmental interactions between broadcast spawning bivalves and cyanobacteria, I investigated the possible sublethal effects of cyanobacteria have on quagga mussel reproduction. Specifically, I evaluated sperm motility by quantifying distance, velocity, and acceleration of spermatozoa released from quagga mussels in the presence of eleven cyanobacteria cultures. I hypothesized that cyanobacteria will reduce the motility of sperm from the broadcast spawning quagga mussel, revealing cyanobacteria toxicity on previously unstudied reproductive endpoints. Furthermore, the current study presents some of the first available research on the impacts of cyanobacteria on freshwater broadcast spawning species.

Methods

Cyanobacteria culture

Cyanobacteria were cultured in 2L wide mouthed Erlenmeyer flasks containing WC media (Guillard and Lorenzen 1972). Cultures were maintained in a 21±1°C environmental chamber on a 16:8 light:dark cycle at 60 $\mu E\ m^{-2}\ s^{-1}$ light intensity.

Cyanobacteria were harvested during the exponential growth phase and stored at 5°C until use in experiments. *Microcystis aeruginosa* (UTEX) was purchased through the Culture Collection of Algae at The University of Texas at Austin, *M. aeruginosa* (GLERL) was provided by the Great Lakes Environmental Research Lab, and *Anabaena flos-aquae*, *Aphanizomenon flos-aquae*, *Dolichospermum lemmermanii*, *Gloeotrichia echinulata*, *M. aeruginsa* (BQ11-02, ZUR-HINDAK, and LSC-13-02), *M. wesenbergii* and *Planktothrix suspensa* were provided by Environment and Climate Change Canada.

Quagga mussel collection and culture

Quagga mussels were collected from the Detroit River on Belle Isle State Park, Detroit, MI (42.358° N, 82.978° W). Mussels at this site have been confirmed both morphologically and genetically as quagga mussels (Ram et al. 2011). Mussels were collected once a week throughout their spawning season from March to September 2014-2015 by scraping from a seawall at depths 1-3 m below the surface using a steel scraper. Mussels were transported in a cooler to the laboratory where dead mussels and shell fragments were removed. Mussels measuring 15-35mm were lightly cleaned of debris under running tap water and placed in 10L glass aquaria filled with dechlorinated tap water and aerated. Mussels were maintained at a density of one mussel per 20-30ml of water. Once per week the tanks were cleaned, dead mussels removed, and the water refreshed. The tanks were housed in an environmental growth chamber at 18±1°C with an 18h: 6h light-dark cycle. All mussels were fed *Ankistrodesmus falcatus*, a green alga used to maintain dreissenid cultures that does not affect reproduction (Boegehold et al. 2018).

Sperm Motility Bioassays

While previous studies used fluorescent dye and flow cytometry to analyze sperm mortality in zebra mussels (Favret and Lynn 2010), an optical bioassay was used in this study to provide measurements on sperm movement exposed to cyanobacteria. Using an optical bioassay allowed us to track multiple endpoints of motility for individual sperm, techniques that are not yet available with the use of flow cytometry (Ortega-Ferrusola et al. 2017). Such methods are undocumented in the current literature for use on sperm, but can provide more detailed data on how environmental stressors influence delicate life history stages such as reproduction. However, our methods were based on similar optical screening assays developed by Zein et al. (2014) to quantify sublethal effects of contaminants on the behavior and movement of a zooplankton species, *Daphnia pulex*.

To investigate sublethal toxicity, quagga mussel sperm motility was analyzed by optically aided imaging software that tracked individual sperm movement in response to exposure to cyanobacteria solutions. Fresh sperm were collected from mussels that had been induced to spawn following methods described in Ram et al. (1993). Mussels placed in individual vials containing 9 mL WC media were exposed to 10^{-3} M 5-HT serotonin and carefully observed until males began to release sperm. Sperm from individual males was then exposed to either cyanobacteria (treatment) or WC media (control) (n=5), so that 10-20 sperm from five randomly selected mussels was recorded for each treatment. Immediately after release, 1mL of solution containing sperm from an individual mussel was placed in 30mL of treatment. Immediately after exposure to cyanobacteria, a subsample of the sperm solution was pipetted onto a slide using hanging drop preparation for microscopy. Live video recordings of the sperm samples

were recorded so that 20-30 individual sperm were visible in the frame to reduce interference. This improved methodology allows for visible inspection of individual sperm cells, which is limited in flow cytometry techniques. Dreissenid sperm is most motile within the first thirty minutes after gamete release (Misamore et al. 1996), and thus our technique also allowed us to quickly record sperm movement without having to expose them to dyes that may be needed for flow cytometry.

A recording from each of the five individual mussel replicates per treatment was obtained using an Infinity 2-1 Luminera monochrome video camera at 400X. Sperm treatments were recorded for 5 seconds (175 frames) for optimum resolution and frame rate. The video recordings were then analyzed using Image Pro-Premier 9.1 (MediaCybernetics) video processing software. Images were calibrated with the software to provide 2D measurements in micrometers. Between 10-20 individual sperm from each mussel were tracked per recording. Only sperm that remained in the field of view for the duration of the recording were tracked. Three parameters were quantified for each sperm track: cumulative distance traveled, mean velocity, and mean acceleration. Cumulative distance for each individual sperm was calculated by summing the distance each sperm moved between frames over the duration of each 5s video. Velocity and acceleration were recorded as the means of each of those endpoints over the course of the video recording.

Statistical Analysis

Track data for sperm motility in each treatment was analyzed using a one-way MANOVA for the three sperm motility endpoints; cumulative distance, mean velocity, and mean acceleration, as the dependent variables and exposure group (i.e.

cyanobacteria solution or WC media control) as the independent variable. Univariate ANOVA tests were then performed to determine differences in each individual endpoint between the treatment and the control. Data was checked for normality using a Shapiro-Wilk test. When data did not meet requirements for normality, it was log transformed. A Levene's Test determined that all data was homoscedastic. All statistical analyses were performed using R (Version 3.3.1; R Core Team 2015).

Results

Of eleven cyanobacteria cultures tested, four had significant impacts on sperm motility endpoints (Table 2). Cyanobacteria reduced the distance, velocity, and acceleration by more than 50% of the control in some assays. Although I exposed quagga mussel sperm to five isolates of *Microcystis aeruginosa*, not all isolates significantly reduced sperm motility. The cumulative distance sperm travelled decreased when exposed to *Aphanizomenon flos-aquae* (p=0.019), *M. aeruginosa* (UTEX; p=0.005), and *M. aeruginosa* (ZUR-HINDAK; p=0.047) (Table 2, Fig. 1). Likewise, sperm velocity was reduced in the presence of *A. flos-aquae* (p=0.006), *Microcystis aeruginosa* (UTEX; p=0.005), and *M. aeruginosa* (ZUR-HINDAK; p=0.047) (Table 2, Fig. 2). Because velocity is a calculation based on distance travelled, it is expected that the same cyanobacteria that reduced distance would also reduce velocity, although differences were not proportional between distance and velocity measurements in similar treatments. However, acceleration, which is a function of velocity, was not impacted by the same species that reduced distance and acceleration. Sperm acceleration was significantly lower than the control when exposed to *M. wesenbergii* (p=0.015) (Table 2, Fig. 3).

Table 2. Statistical results of the MANOVA to analyze the effects of cyanobacteria isolates on three sperm motility endpoints (distance, velocity, and acceleration) and the univariate ANOVA post-hoc tests to determine statistical differences for each endpoint; p-values are reported; bolded values indicate p<0.05.

Cyanobacteria Culture	MANOVA	Univariate ANOVA		
		Distance	Velocity	Acceleration
Anabaena flos-aquae (CPCC64)	0.623	0.596	0.596	0.737
Aphanizomenon flos-aquae (APHH)	0.091	**0.023**	**0.006**	0.968
Dolichospermum lemmermanii (LE11-02)	0.485	0.317	0.992	0.373
G. echinulata (BQ11-03)	0.453	0.320	0.320	0.479
Microcystis aeruginosa (UTEX)	0.232	**0.022**	**0.021**	0.598
M. aeruginosa (LEMS)	0.764	0.283	0.999	0.759
M. aeruginosa (BQ11-02)	0.301	0.067	0.067	0.338
M. aeruginosa (ZUR-HINDAK)	**0.022**	**0.047**	**0.047**	0.412
M. aeruginosa (LSC-13-02)	0.862	0.056	0.056	0.517
M. wesenbergii (LE13-01)	0.083	0.400	0.400	**0.015**
Planktothrix suspensa (1163PL1)	0.503	0.126	0.126	0.815
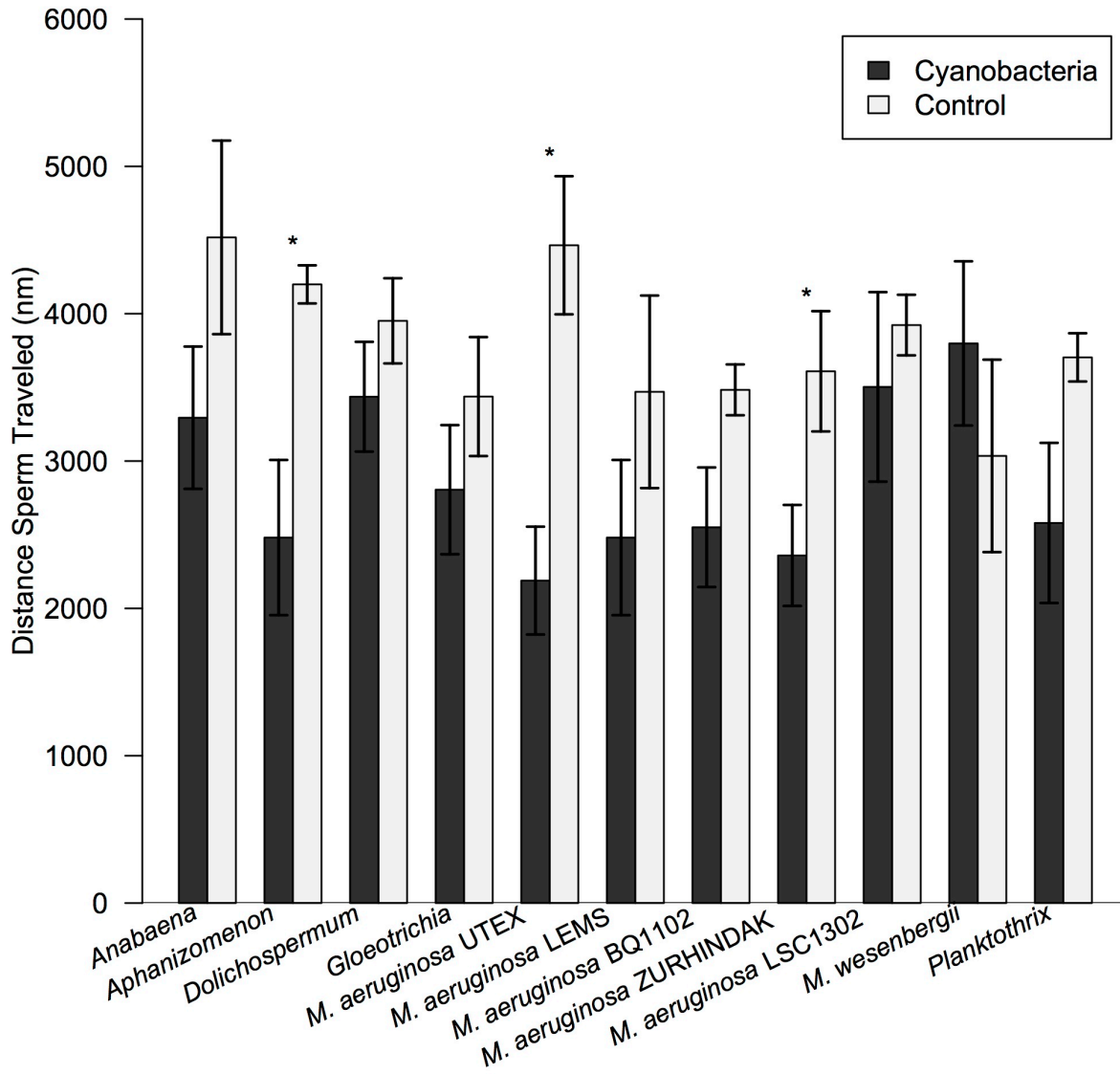
Figure 1. Mean distances that sperm travelled (nm) in each of the eleven cyanobacteria treatments compared to the WC media control. Error bars represent standard deviation of the mean. The distance sperm travelled decreased when exposed to *Aphanizomenon flos-aquae*, *Microcystis aeruginosa* (UTEX), and *M. aeruginosa* (ZUR-HINDAK); significant differences are indicated by asterisks ($P<0.05$; ANOVA).
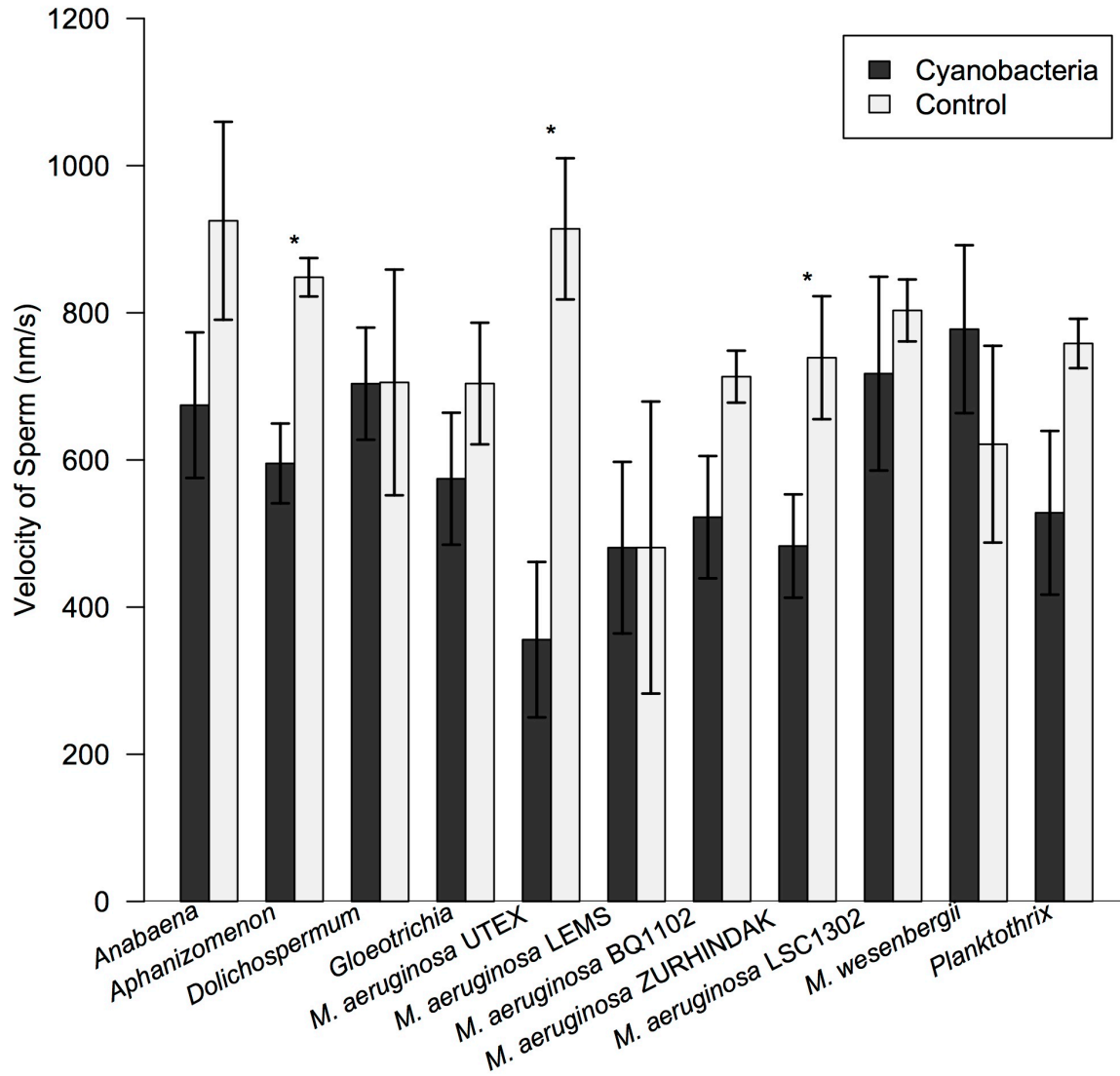
Figure 2. Mean velocity of sperm (nm/s) in each of the eleven cyanobacteria treatments compared to the WC media control. Error bars represent standard deviation of the mean. Sperm acceleration was reduced when exposed to *Aphanizomenon flos-aquae*, *Microcystis aeruginosa* (UTEX), and *M. aeruginosa* (ZUR-HINDAK); significant differences are indicated by asterisks (*P*<0.05; ANOVA).
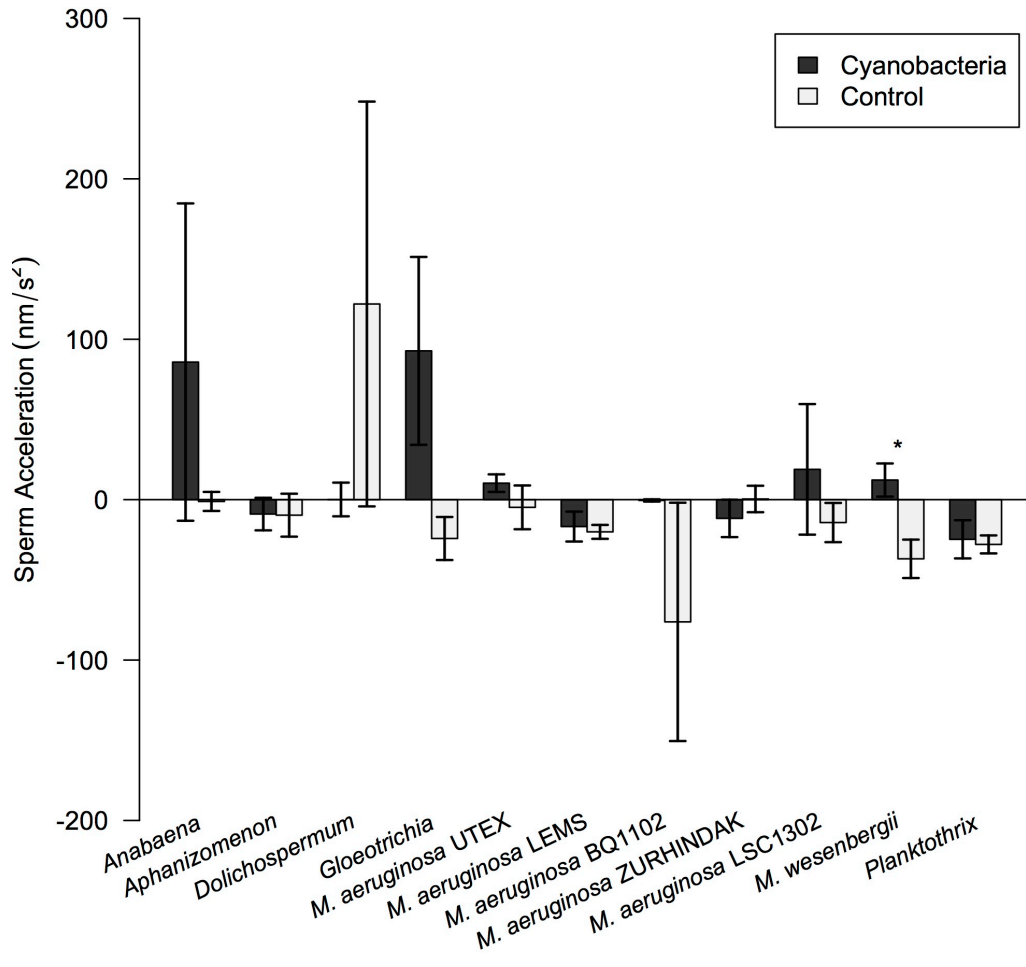
Figure 3. Mean acceleration of sperm (m/s^2) for each of the eleven cyanobacteria treatments compared to the WC media control. Error bars represent standard deviation of the mean. Sperm acceleration was impacted when exposed to *M. wesenbergii*; significant differences are indicated by asterisks (*P*<0.05; ANOVA).

Discussion

The freshwater cyanobacteria species *Aphanizomenon flos-aquae,* two strains of *Microcystis aeruginosa,* and *M. wesenbergii* negatively affected sperm motility. These three species of cyanobacteria are capable of producing toxins and can be found worldwide (Barker et al. 2000; McDonald and Lehman 2013; Michalak et al. 2013). Results from this study document the adverse effects of some freshwater cyanobacteria on bivalve sperm and align with findings in marine systems, where nuisance and toxic phytoplankton blooms have negatively affected invertebrate spermatozoa. Paralytic shellfish toxin producing dinoflagellate blooms cause cellular changes in the sperm of Pacific oysters, which can ultimately impact fertilization (Haberkorn et al. 2010; Nelly et al. 2013). Brown tides have been documented to cause juvenile recruitment failure of marine bivalves, such as the hard clam (Bricelj and MacQuarrie 2007), which could be due to fertilization failure from immotile sperm. Likewise, a red tide outbreak off the coast of North Carolina led to failed recruitment of bay scallops, caused by possible spawning interference (Summerson and Peterson 1990) while blooms of the haptophyte *Chrysochromulina polylepis* reduced fertilization and development in both ascidians and blue mussels off the coast of Sweden (Granmo et al. 1988). Gametes are particularly sensitive to environmental conditions, and externally released sperm must overcome a variety of physical, chemical, and biotic challenges to reach an egg in the water column (Ripley and Caswell 2008; Serrão and Havenhand 2009).

Reduced sperm motility has the potential to impact fertilization rates, which in turn can reduce population growth rates and juvenile recruitment in mussels frequently exposed to cyanobacteria (Summerson and Peterson 1990). Sperm contact with the

egg typically occurs seconds after sperm are released, at which time they rapidly bind to egg surface (Misamore and Lynn 2000). Thus, a loss in sperm motility during a cyanobacteria bloom may cause the sperm to fail reaching and binding to an egg. Some cyanobacteria species are benthic, and therefore more likely to be in direct proximity to spawning quagga mussels, while other species, such as *Microcystis*, are buoyant and accumulate along the surface layer of a water body. However, abrupt temperature changes and strong winds can cause vertical distribution of buoyant cyanobacteria species. For example, in the summer of 2014, the drinking water of the City of Toledo, Ohio, located on the shore of Lake Erie, became contaminated with microcystins for a two-day period. Although intake pipes at the water treatment plant are located subsurface, turbulent water conditions forced *Microcystis* and its associated toxin, microcystin, to be distributed throughout the water column (Rowe et al. 2016). The potential for quagga mussels to be exposed to cyanobacteria, especially in shallow eutrophic lakes such as Lake Erie, is likely to occur throughout the spawning season.

Boegehold et al. (2018) demonstrate additional impairments to quagga mussel reproduction, where several species of cyanobacteria inhibited spawning and reproduction. The release of quagga mussel gametes was reduced in the presence of *Aphanizomenon flos-aquae* whereas fertilization rates decreased with exposure to *Anabaena flos-aquae*, *Dolichospermum lemermanii*, *Gloeotrichia echinulata*, and two cultures of *Microcystis aeruginosa* (Boegehold et al. 2018). The current study affirms that sperm motility reduction in the presence of cyanobacteria, specifically *M. aeruginosa*, can lead to a reduction in fertilization rates in laboratory studies. However, as more species of cyanobacteria were responsible for reduced fertilization rates than

were for reduced sperm motility, factors other than sperm movement may be responsible for reduced reproduction in quagga mussels.

While dreissenid spawning may be influenced by phytoplankton and their associated kairomones, cyanobacteria-mussel interactions are relatively unknown (Ram et al. 1996). Sublethal impacts of other environmental stressors have been tested on sperm function in zebra mussels and marine bivalves. Seaver et al. (2009) irradiated zebra mussel sperm with UVB and made qualitative observations on sperm movement, finding that increased UVB radiation renders sperm immobile. Nice (2005) also used qualitative, visual methods to assess reduction in sperm motility in the Pacific oyster after nonylphenol exposure. Pesticides that enter aquatic systems can negatively impact zebra mussel sperm, as demonstrated using mortality and mitochondrial function endpoints (Favret and Lynn 2010). Bacchetta and Mantecca (2009) used histopathological analyses to determine that DDT pollution in an Italian lake disrupted the biological mechanisms involved with sperm release. While these studies have used a range of qualitative and quantitative methods to assess the toxicity of environmental stressors on sperm, the optical bioassay methods in the current study are unique in that I tracked individual sperm movement to measure precisely how cyanobacteria can inhibit motility. These methods are a technological advancement from qualitative, visual observations made in previous studies by Nice (2005) and Seaver et al. (2009). Furthermore, the interactions between freshwater broadcast spawners and cyanobacteria have not been studied until now.

Interactions between dreissenid mussels and phytoplankton may feature complexities beyond reproductive functioning. While dreissenids have caused overall

decreases in plankton, cyanobacteria such as *Microcystis aeruginosa, Aphanizomenon* spp., and *Anabaena* spp. have flourished in some invaded lakes (Vanderploeg et al. 2001; Higgins and Vander Zanden 2010). However, ecosystem changes brought upon by the dreissenid invasion are negatively impacting zebra and quagga mussels. Compared to optimal nutritional availability, zebra mussel condition tends to be lower when feeding conditions are poor, such as in the summer during *Microcystis* blooms and in areas that have experienced increased cyanobacteria abundance (Vanderploeg et al. 2009). Additionally, low quality food sources, such as the cyanobacterium *Aphanothece* sp., might negatively affect gametogenesis and reproduction in zebra mussels (Wacker and von Elert 2003).

The compounds produced by cyanobacteria impair sperm motility and other reproductive endpoints in dreissenid mussels are unknown. Reduced sperm motility occurred once the sperm entered the cyanobacteria solution, and adult quagga mussels and the spermatozoa inside the mussel gonad were not exposed to any experimental stressors. The same mechanisms of sperm motility reduction may be environmentally relevant as quagga mussel sperm may be exposed to cyanobacteria only as it enters the water column outside of the mussel gonad. Possible inhibitory effects on sperm motility reduction could either be related to the physical resistance of sperm travelling through the cyanobacteria solution or due to the degradation of the spermatozoa itself. Many cyanobacteria species, including the ones used in the current study, are capable of producing exocellular polymeric substances (EPS) under environmental stress and disturbance (Tourney and Ngwenya 2014). In colony forming cyanobacteria, such as *Microcystis*, EPS is an adhesive, gelatinous material that binds individual cells together

(Li et al. 2013; Xu et al. 2014). Production of EPS in the cyanobacteria cultures responsible for reduction in sperm motility may have physically limited sperm movement within the experimental media.

Likewise, cyanobacteria produce secondary metabolites, such as toxins (Carmichael and Boyer 2016), which may have acted to degrade the spermatozoa on contact, rendering them immotile. Cyanotoxins vary by producer species, chemical structure, and toxicity, and can cause adverse effects and even mortality in humans and wildlife (Carmichael and Boyer 2016). Chemical characteristics such as pH, cation concentration, and other compounds alter zebra mussel sperm movement (Ciereszko et al. 2001). A pH between 7.0-9.0 is optimum for maximum sperm motility in zebra mussels, and acidic conditions severely limit sperm function (Ciereszko et al. 2001). Other chemicals, such as those produced by cyanobacteria, may also influence the aquatic landscape, rendering it inhospitable to quagga mussel sperm. While I did not expose sperm to isolated cyanotoxins in this study nor did I analyze our cultures for toxin production, future research on this topic should include investigation of commonly produced cyanotoxins on sperm motility.

Reproductive events in broadcast spawners have evolved to respond to environmental conditions so that gamete release occurs during favorable times (Quinn and Ackerman 2012), and poor environmental conditions may suppress broadcast spawning in freshwater species other than dreissenid mussels. For example, several freshwater fish species are broadcast spawners. However, environmental conditions that impact broadcast spawning in freshwater fish is still largely unknown. Much of what is known about sicklefin chub reproduction is from laboratory experiments and

captive spawning attempts (Albers and Wildhaber 2017). Broadcast spawning minnows in the U.S. Great Plains may be more fecund during flood recession when nutrient enrichment and concentration are highest (Hoagstrom and Turner 2015), indicating that favorable environmental conditions are a factor in their reproduction. Broadcast spawning fish such as lake sturgeon, gar, perch, and suckers spawn during the spring while lake whitefish and cisco are fall broadcast spawning species (Lane et al. 1996). Although cyanobacteria blooms in temperate regions are typically dominant from mid to late summer until fall, rising global temperatures are extending blooms into spring (Paerl and Otten 2013), possibly rendering both spring and fall broadcast spawners susceptible to sperm toxicity. Continued and increased disruption of broadcast spawning in freshwater systems may have long-ranging environmental impacts on population and ecosystem dynamics.

CHAPTER 2 CYANOBACTERIA REDUCE QUAGGA MUSSEL (*DREISSENA ROSTRIFORMIS BUGENSIS*) SPAWNING AND FERTILIZATION SUCCESS[1]

Introduction

Quagga (*Dreissena rostriformis bugensis*) and zebra (*D. polymorpha*) mussels originate from the Ponto-Caspian region and have become highly invasive in North American and European freshwaters in part due to their reproductive vigor (Ram et al. 2012). Reproductive success influences the spread and establishment of invasive species and can be directly and indirectly controlled by food availability (Wacker and von Elert 2003). Historically, dreissenid spawning spans from April-September when water temperatures are greater than 10°C for quagga mussels and 12°C for zebra mussels (Ram et al. 1993, Ram et al. 2011). Similar to marine mussels, dreissenids are broadcast spawners; males and females simultaneously release gametes into the water column where external fertilization occurs, resulting in free-swimming, planktonic larvae (Quinn and Ackerman 2012). A single, sexually mature female zebra mussel can release more than one million eggs in a single spawning event, while males can release up to ten billion sperm (Sprung 1991). Furthermore, zebra mussels can spawn multiple times per season (Ram et al. 1996), allowing great potential for dreissenid populations to increase in number and distribution. As quagga mussels continue to invade North America, displacing existing invasive zebra mussels and native unionid mussels (Ram et al. 2012), understanding their reproductive behavior could inform population models and lead to the development of control tactics that disrupt reproduction.

In marine systems, there are documented cases of invertebrate reproduction influenced by algae. For example, Miyazaki (1938) and Himmelman (1975) found that marine algae can induce spawning in some oysters and invertebrates while Starr et al.

(1990) noted a similar phenomenon in marine mussels and urchins. This response is a possible adaptation to ensure that developing larvae have plentiful food (Kashian and Ram 2014). Similar research does not exist in the freshwater literature. Only one observational study documenting an increase in dreissenid gamete and veliger abundance following a green algae bloom (Kashian and Ram 2014) suggest phytoplankton may influence broadcast spawners in freshwater systems. Dreissenid mussels have a similar reproductive strategy to marine invertebrates whose spawning is influenced by phytoplankton (Starr et al. 1990, Sprung 1991), and thus I hypothesize that phytoplankton kairomones may also influence gamete release in dreissenids. Likewise, if palatable algae can induce spawning in some bivalves, then less palatable cyanobacteria may inhibit spawning.

Cyanobacteria are an unpalatable food source that can produce toxins and may inhibit dreissenid reproduction through the production of kairomones or other means. Although dreissenid mussel populations can survive cyanobacteria blooms, they may experience sublethal stress that affects condition and egg mass. Dreissenids can actively detect and reject cyanobacteria, such as *Microcystis aeruginosa*, suggesting that cyanobacteria are not a preferred food source, and overall mussel condition decreases with increasing cyanobacterial concentration (Vanderploeg et al. 2001, Vanderploeg et al. 2009, Tang et al. 2014). In addition, Wacker and von Elert (2003) found that female dreissenids had lower egg mass when exposed to cyanobacteria, which are low in fatty acids, compared to more nutritious algae. Furthermore, Dionisio Pires et al. (2003) found that toxic cyanobacteria affected survival and clearance rates in dreissenid veligers more than a non-toxic strain of similar fatty acid composition.

These studies suggest that cyanobacteria are a poor food source and may thus be detrimental to dreissenid reproduction, although I was unable to identify any literature connecting cyanobacteria blooms with declines in dreissenid mussel population.

Our objective was to determine if cyanobacteria and/or an associated toxin inhibit spawning intensity and fertilization success of quagga mussels. Understanding the environmental triggers of quagga mussel reproduction will provide a better understanding of their population ecology and identify possible targets for population control. I hypothesized that cyanobacteria would inhibit dreissenid reproduction. Specifically, I predicted that cyanobacteria exposure would reduce gamete release and fertilization success of quagga mussels.

Methods

Quagga mussel collection and culture

For all experiments, mature adult quagga mussels were collected during their spawning season from the Detroit River, Michigan, USA from March to September of 2014-2016. Mussels measuring between 15-35 mm were stored in multiple 20 L aquaria filled with dechlorinated tap water and kept in an environmental chamber at $18\pm1°C$ with a 16:8 light:dark cycle. Approximately 100-150 mussels were kept in each aerated aquarium, which were cleaned and refilled with fresh water weekly. To ensure that mussels were sexually mature for reproduction experiments, a subset of 10-25 mussels from each collection was tested for spawning using a known spawning inducer, 10^{-3} M 5-HT serotonin, according to methods from Ram et al. (1993). If >50% of the subset spawned, then that collection was deemed suitable for experimentation. In addition, for each collection the remainder of the mussels not immediately used for experimentation

or spawning assessment were stored in a dark cold room (5°C) to slow metabolism while maintaining reproductive viability until needed for future experiments (Stoeckel et al. 2004). Mussels were acclimated for at least 48 hours in an 18±1°C environmental chamber before use in experiments. Three times per week, mussels were fed 4.0 x 10⁶ cells/mL of a cultured green alga, *Ankistrodesmus facaltus*, which is commonly used to sustain laboratory conditioned quagga mussels (Sarnelle et al. 2012). Furthermore, I determined through experimental assays that *A. facaltus* does not affect spawning (p=0.408) or fertilization (p=0.082; where fertilization was higher in *A. facaltus* solution) in quagga mussels.

Cyanobacteria & Microcystin Preparation

In a series of bioassays, thirteen cyanobacteria isolates (Table 1) cultured in WC media (Guillard and Lorenzen 1972) and the purified toxin microcystin-LR, a common toxin produced by cyanobacteria, were evaluated for their effects on mussel reproduction. Cyanobacteria were tested at concentrations between 88 and 544 µg/L chlorophyll-*a*, which is within the concentrations reported for dense algal blooms worldwide (Yuan et al. 2014, Golnick et al. 2016, Sayers et al. 2016, Duan et al. 2017). Testing the isolated toxin allowed us to determine if any observed effects on reproduction were due to factors or toxins other than microcystin-LR.

Cyanobacteria cultures were kept in a 21±1°C environmental chamber on a 16:8 light:dark cycle at 60 µE m^{-2} s^{-1} light intensity. Cyanobacteria were harvested during the exponential phase and densities were measured using chlorophyll *a* abundance through a light spectrophotometer at 665 nm based on methods from Biggs and Kilroy (2000). Microcystin-LR was used to test the effects of cyanobacteria toxins on spawning and

fertilization because it is the most commonly produced toxin congener globally (Dawson 1998). Microcystin-LR was purchased from Beagle Bioproducts (Columbus, Ohio, USA; >95% purity by high performance liquid chromatography) and reconstituted with acetone. An equivalent amount (3-6 mL/L) of acetone was added to the control solution to maintain any effects caused by acetone exposure. All assays were run a minimum of two times to account for possible differences in seasonal variation in spawning. All cyanobacteria used had consistent effects across all assays.

Spawning Assays

The effects of the individual cyanobacteria cultures and the toxin microcystin-LR were tested in the presence of a known spawning inducer, 5-HT serotonin, using bioassay methods outlined in Ram et al. (1993). Serotonin was used to induce spawning over other methods because it is the most efficacious strategy to induce dreissenid spawning and using serotonin as a spawning inducer guaranteed that a sufficient number of mussels spawned for each experiment (Schwaebe et al. 2013). The use of serotonin also ensured that any inhibitory effects from cyanobacteria were able to overpower strong stimulatory cues. For all treatment assays, individual mussels were simultaneously exposed to serotonin and cyanobacteria by being placed in 20 mL scintillation vials (n = 25) with 9 mL of cyanobacteria solution and 1 mL of 10^{-2} M serotonin for a final concentration of 10^{-3} M serotonin (Table 2). Mussels in experimental control vials (n=25) were exposed to the same concentration of serotonin in WC media to ensure that any observed effects were not due to cyanobacteria culture medium. Concentrations of 5 and 20 µg/L microcystin-LR were also tested. These two concentrations were chosen because they represent a standard range of microcystin

concentrations in bloom conditions (Carmichael and Boyer 2016). Each microcystin-LR treatment assay consisted of individual mussels exposed to the toxin and 10^{-3} M serotonin in solution with dechlorinated tap water (n=25). Experimental controls for the microcystin-LR assay contained 10^{-3} M serotonin with an equivalent amount of acetone used to reconstitute the microcystin-LR in a solution of dechlorinated tap water (n=25).

For all assays, individual vials containing mussels were observed for four hours after the addition of serotonin, after which spawning was recorded as either positive or negative for each individual mussel (binary response; Table 2). Qualitative notes were taken on the intensity of spawning, based on Ram et al. (1993), and on the motility of sperm. Spawning was confirmed by extracting a small sample of liquid from each vial and inspecting for expelled sperm and eggs under a microscope at 200X magnification. Non-spawning animals were dissected and sexed following the experiment to determine if gonads were sexually mature. If >10% of unspawned gonads were not mature, then the experiment was discarded and later repeated until at least 90% of mussels used in experiments were sexually viable. If a cyanobacteria culture inhibited spawning, varying concentrations of that culture were tested to determine a threshold concentration for spawning response. Individual mussels in the control and the cyanobacteria treatment were categorized as one of two nominal variables: successful and unsuccessful spawning. Data were sorted in contingency tables and analyzed using a Fisher's exact test for dichotomous data. All analyses were performed using R (R Core Team, 2015).

Fertilization Assays

Fertilization was assessed using an assay method adapted from Hardege et al. (1997) and Fong et al. (1995). Twenty mussels were induced to spawn in individual vials

containing dechlorinated tap water following methods described above. Upon spawning, oocytes collected from at least three individual females were pooled and distributed in 24-well plates containing 1.5 mL of cyanobacteria solution in treatment assays or WC media for the control assays. To test effects of microcystin, dechlorinated tap water solutions containing concentrations of 5 and 20 µg/L microcystin-LR were added to treatment wells, whereas control plates contained dechlorinated tap water with an equivalent amount of solvent used to dissolve the toxin (3-6 mL/L). Eggs were added to the treatment and control wells to achieve a density of approximately 200 oocytes/well. Fresh sperm was then collected from at least three mussels, pooled, and distributed to the wells containing oocytes. The sex of quagga mussels cannot be determined without dissection of the gonad, consequently making it impossible to separate males and females before spawning induction. Gamete release by male quagga mussels tends to precede that of the females, therefore to ensure the availability of fresh, motile sperm for use in assays, a second set of mussels was induced to spawn after oocytes had been collected if females did not begin spawning within 30 minutes of males. Likewise, sperm was only added to oocytes if both gametes were produced within 30 minutes of each other. Fertilization was quantified via visual mitosis of oocytes at a magnification of 200X after exposure to spermatozoa for at least 2 hrs. Twelve random wells were chosen from each 24-well plate where approximately 100 eggs were assessed for fertilization success (n=12). After oocytes were counted as either fertilized or not, percentage of fertilized oocytes per well was calculated. Tests were rejected if average fertilization rates were less than 40% in the control. During fertilization assays, I found that successful fertilization rates in controls ranged from 40-75%, with the percent of

eggs becoming successfully fertilized never reaching 100%. The mean ratio of fertilized eggs in the treatment and control was compared using an ANOVA with Tukey post-hoc tests. The Shapiro-Wilk test was used to check for normality and to determine if the data met the requirements for parametric comparisons. The Shapiro-Wilk test indicated data met the requirements for parametric comparisons (W=0.939, p=0.440) and thus no data transformations were necessary. All analyses were performed using R (R Core Team, 2015).

Results

Spawning

Of the 13 cultures tested, only one, *Aphanizomenon flos-aquae,* significantly inhibited spawning in quagga mussels (Table 3). Spawning success was reduced when exposed to certain concentrations of *A. flos-aquae* (Fig. 4). *Aphanizomenon flos-aquae* did not inhibit spawning at a concentration of 127 µg/L chlorophyll-*a* (p=0.387). However, spawning was reduced at a concentration of 140 µg/L (p=0.045), and at higher concentrations of 284 and 544 µg/L (p<0.001). Mussel spawning inhibition in *A. flos-aquae* was not proportional to the concentration of cyanobacteria, however. While 20% of mussels spawned in the 284 µg/L treatment, 36% spawned in the higher concentration of *A. flos-aquae* of 544 µg/L. This can be attributed to natural variance in biological specimens, and spawning rates were consistently below 50% in the three highest concentrations of *A. flos-aquae*. Gamete release was not affected by 5 or 20 µg/L microcystin-LR (Table 3).
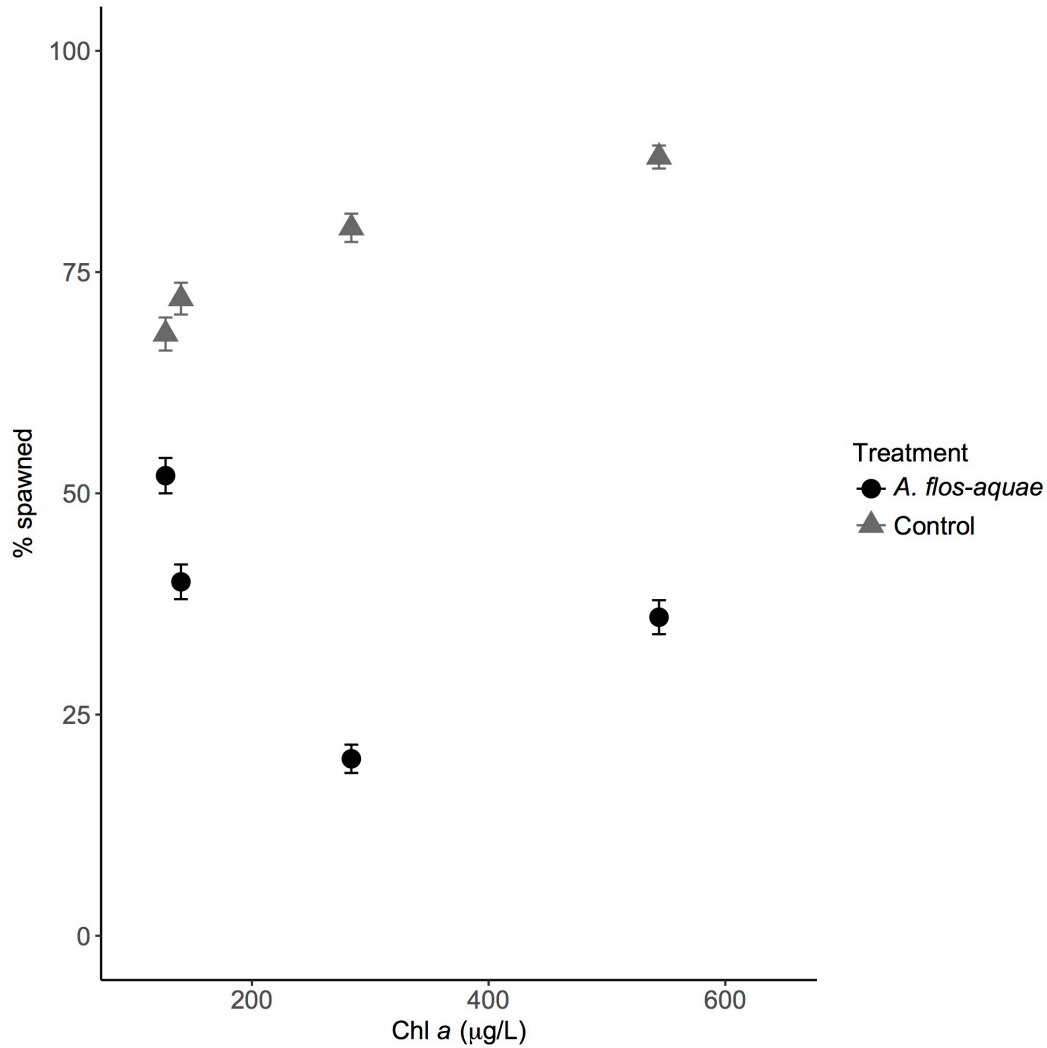
Figure 4. Percentage (±SD of binomial data) of mussels that spawned when exposed to 1 of 4 concentrations (127, 140, 284, and 544 µg chlorophyll *a*/L) of *Aphanizomenon flos-aque* compared to those that spawned in the WC media controls (*n* = 25). Each assay was run independently with its own control.

Table 3. Mean experimental concentration and results from spawning assays conducted with cyanobacteria and microcystin-LR. Multiple results are reported for *Aphanizomenon flos-aquae* to demonstrate that spawning is inhibited only at higher concentrations. *p*-values are from Fisher' exact tests. Bold indicates *p* < 0.05.

Species and isolate	Concentration (µg/L)	% spawned (*n* = 25)		*p*
		Treatment	Control	
Anabaena flos-aquae (CPCC64)	420	64	71	0.762
Aphanizomenon flos-aquae (APHH)	544	36	88	**<0.001**
A. flos-aquae (APHH)	284	20	80	**<0.001**
A. flos-aquae (APHH)	140	40	72	**0.045**
A. flos-aquae (APHH)	127	52	68	0.387
Dolichospermum lemmermanii (LE11-02)	385	92	92	1.000
Gloeotrichia echinulata (BQ11-03)	88	76	76	1.000
G. echinulata (ASGV)	217	70	50	0.187
Lyngbya wollei (LSC-14)	n/a[a]	63	87	0.072
Microcystis aeruginosa (UTEX)	371	93	87	0.671
M. aeruginosa (LEMS)	445	96	88	0.609
M. aeruginosa (BQ11-02)	151	56	71	0.377
M. aeruginosa (ZUR-HINDAK)	211	52	80	0.072
M. aeruginosa (LSC-13-02)	187	88	92	0.667
M. wesenbergii (LE13-01)	380	84	80	1.00
Planktothrix suspensa (1163PL1)	453	52	76	0.140
Microcystin-LR	5.00	70	60	0.741
Microcystin-LR	20.00	70	60	0.741

[a] Because of the morphology of *L. wollei*, which forms dense mats of benthic clusters, an absorbance reading was not produced.

Fertilization

Seven of the thirteen cyanobacteria cultures tested inhibited quagga mussel fertilization: *Anabaena flos-aquae* (F=37.51, p<0.001)*, Dolichospermum lemmermanii* (F=464.5, *p<0.001*)*, Gloeotrichia echinulata* (F=11.99, p=0.001)*, Lyngbya wollei* (F=10.46, p= 0.002)*, Microcystis aeruginosa* (LEMS; F=96.59, p<0.001), *M. aeruginosa* (BQ11-02; F=102.4, p=<0.001) and *M. wesenbergii* (p=0.028) (Table 3; Fig. 2). Quagga mussel sperm became visibly immotile in these cultures and appeared to aggregate within the cyanobacteria. The purified cyanobacteria toxin, microcystin-LR, did not affect fertilization in assays that tested 5 or 20 µg/L concentrations (Table 4; Figure 5).
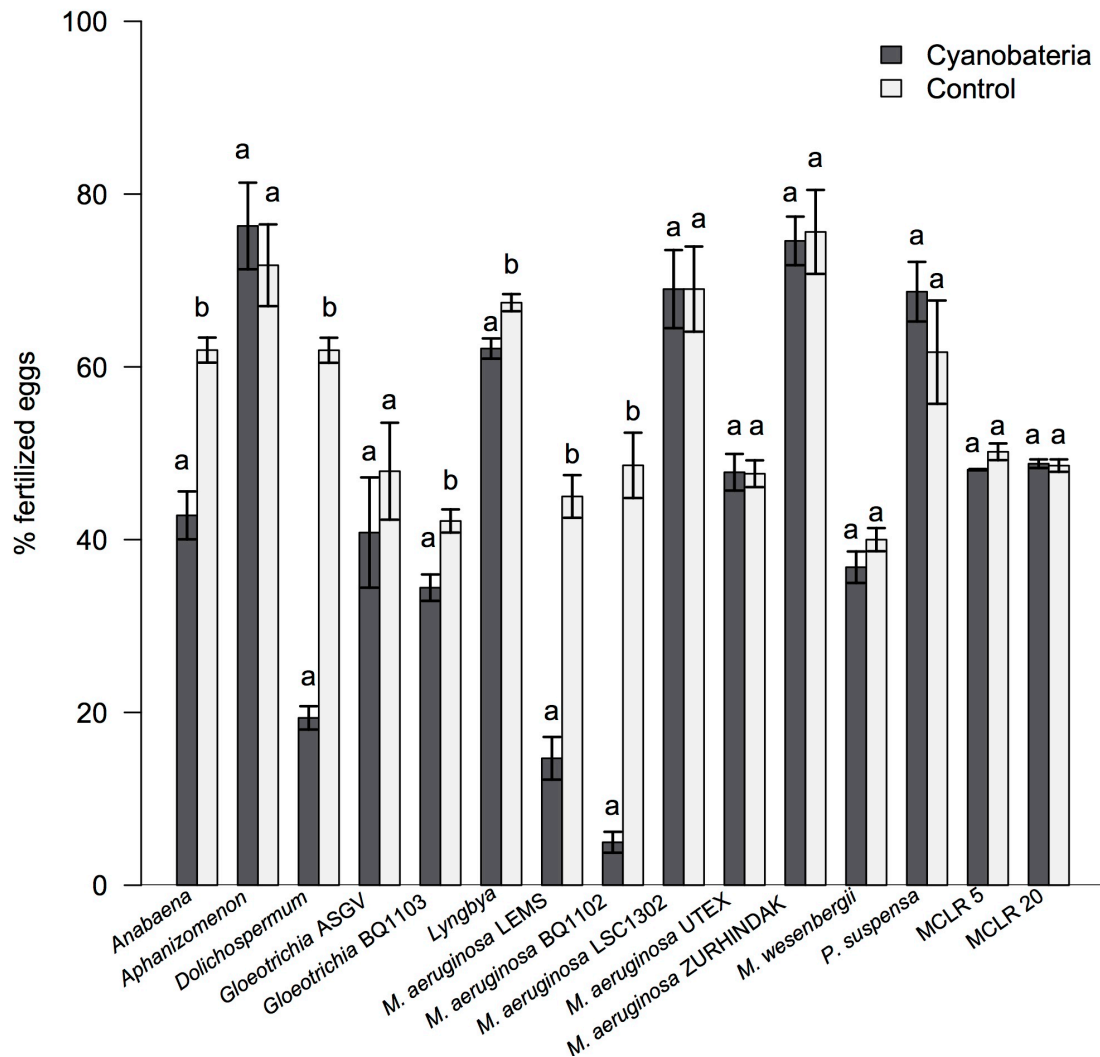
Figure 5. Mean (±SE) % fertilization of quagga mussel oocytes in cyanobacteria and toxin solutions and controls reported as % total eggs fertilized (*n* = 12). Each fertilization assay was completed independently, so each treatment is compared to its own control. Treatments and controls with the same letter are not significantly different. Cyanobacteria labelled on the *x*-axis correspond with cyanobacteria identified in Table 1. MCLR = microcystin-LR at the 2 concentrations tested (5 and 20 µg/L).

Table 4. Experimental concentration and results from fertilization assays conducted with cyanobacteria and microcystin-LR. P-values refer to paired Student's *t*-test. Bold indicates *p* < 0.05.

Species and Isolate	Concentration (µg/L)	Mean % fertilized in (*n*=12)		*p*
		Treatment	Control	
Anabaena flos-aquae (CPCC64)	417	43	62	**<0.001**
Aphanizomenon flos-aquae (APHH)	214	76	72	0.528
Dolichospermum lemmermanii (LE11-02)	192	19	62	**<0.001**
Gloeotrichia echinulata (ASGV)	341	41	48	0.612
G. echinulata (BQ11-03)	331	34	42	**0.001**
Lyngbya wollei (LSC14)	*n/a	62	67	**0.002**
Microcystis aeruginosa (UTEX)	291	48	48	0.950
M. aeruginosa (LEMS)	152	15	45	**<0.001**
M. aeruginosa (BQ11-02)	502	5	49	**<0.001**
M. aeruginosa (ZUR-HINDAK)	411	75	76	0.880
M. aeruginosa (LSC-13-02)	140	70	70	0.988
M. wesenbergii (LE13-01)	381	37	40	0.549
Planktotrhix susepnsa (1163PL1)	263	69	62	0.338
Microcystin-LR	5.00	48	50	0.076
Microcystin-LR	20.00	49	49	0.869

*Because of the morphology of *L. wollei*, which forms dense mats of benthic clusters, an absorbance reading was not produced.

Discussion

Several cyanobacteria species inhibited reproductive success in quagga mussels by disrupting spawning or fertilization. These results build off the findings of Wacker and von Elert (2003) and Sprung (1991) who concluded that dreissenid gonad development and reproduction is influenced by the nutritional quality of phytoplankton. While a direct link between phytoplankton and broadcast spawning has not yet been identified in dreissenid mussels, the results of this study indicate that the presence of cyanobacteria can impede reproduction. This effect could be caused by kairomones or other metabolites produced by cyanobacteria, as research has shown that dreissenid mussels and phytoplankton can influence one another. Although quagga mussels spend the majority of their life cycle as sessile, benthic adults, a population of dreissenid mussels can fundamentally alter a lake system because of their high rate of filter feeding. There is evidence that dreissenid mussels can shift nutrients from the pelagic zone to the benthic and littoral zones, altering phytoplankton communities in the process (Higgins and Vander Zanden 2010, Vanderploeg et al. 2017). Furthermore, dreissenid mussel feeding can be influenced by the quality and availability of phytoplankton, whereas the presence of cyanobacteria can lower feeding rates and worsen overall mussel condition (Vanderploeg et al. 2017).

All cyanobacteria species tested are commonly found in freshwater systems throughout the world and are capable of producing cyanotoxins (Barker et al. 2000, McDonald and Lehman 2013, Michalak et al. 2013). The concentrations of cyanobacteria tested in this study were within range of recorded dense bloom concentrations, including those of surface scums, indicating quagga mussel

reproduction could be impaired during a bloom event. A recent study using remote sensing set cyanobacteria bloom threshold in the Laurentian Great Lakes at 18 µg/L chlorophyll *a* while cyanobacteria blooms in Lake Taihu, China can reach concentrations of greater than 500 µg/L chlorophyll *a* (Liu et al. 2010, Sayers et al. 2016). I also tested environmentally relevant concentrations of the common cyanobacteria produced toxin, microcystin-LR, at 5 and 20 µg/L; however, the toxin did not affect spawning or fertilization. The World Health Organization (WHO) sets limits for microcystins in drinking water at 1 µg/L and recreational guidelines at 20 µg/L (World Health Organization 2004). A study on the history of cyanobacteria blooms in the Laurentian Great Lakes places typical microcystin concentrations at a range of 0-20 µg/L, with severe blooms producing over 200 µg/L (Carmichael and Boyer 2016). Although interactions between quagga mussels and the naturally occurring cyanobacteria communities are complex, there is a paucity of information regarding their dynamics.

Clonal and population variation within the same species of cyanobacteria can produce distinct genotypes which cause unpredictable interactions with other species and environmental stressors. While two isolates of *M. aeruginosa* reduced quagga mussel fertilization rates, two other isolates did not, which is consistent with previous findings of intraspecific variability of cyanobacteria species on quagga mussel physiology and behavior. For example, dreissenid mussel feeding behavior varies when exposed to different genotypes and cell sizes of *M. aeruginosa*, whereas dreissenids will selectively filter feed some *M. aeruginosa* cells and not others, regardless of microcystin production (Vanderploeg et al. 2001, Sarnelle et al. 2012, White and Sarnelle 2014). Other clonal species, such as *Daphnia*, also experience physiological

variability where genetically distinct clones of the same *Daphnia* species display varied responses when exposed to environmental stressors (Baird et al. 1990, DeMille et al. 2016). Furthermore, genotype x genotype interactions between *Microcystis* and *Daphnia* result in an array of interspecific interactions, stemming from clonal dissimilarities in either species (Lemaire et al. 2012). Genetic variability among clonal species from different locations may depend on the unique environmental conditions from which they originate. *M. aeruginosa* blooms in Lake Erie and Lake Huron, two hydrologically connected lakes, possess unique genotypes and can generate varying types and concentrations of intracellular chemicals (Dyble et al. 2008). Since all quagga mussels used in this study were taken from the same geographic location and likely the same population pool, variable responses in fertilization are likely due to genetic differences in the cyanobacteria cultures and not due to genotypic variation in quagga mussels.

The production of exocellular polymeric substances (EPS) by cyanobacteria are subject to genotypic and environmental variation which may explain the variability of distinct cyanobacteria cultures within the same species to disrupt fertilization (Tourney and Ngwenya 2014). Exocellular polymeric substances compounds are produced under environmental stress and disturbance, and all cyanobacteria species used in this study are capable of producing these compounds (Tourney and Ngwenya 2014). In *Microcystis*, EPS binds cells together to create aggregated colonial formations (Li et al. 2013, Xu et al. 2014). As cyanobacteria produce more adhesive EPS in response to environmental conditions, the diameter of cell aggregates increases (Li et al. 2013). It is possible that the visibly immotile sperm in the presence of cultures that inhibited

fertilization could have been caused by excess EPS production, physically preventing sperm from reaching eggs. However, more studies are needed to determine the cause of fertilization inhibition by cyanobacteria in quagga mussels.

While EPS is potentially the cause for fertilization disruption, this may not explain why *Aphanizomenon flos-aquae* hindered spawning in quagga mussels, but other primary or secondary metabolites could be responsible. Although *A. flos-aquae* produces cyanotoxins, I do not suspect this chemical caused spawning inhibition, as the isolated toxin microcystin-LR did not affect spawning rates in our laboratory studies. No known information is available regarding *A. flos-aquae* and its effects on dreissenids, however the invasive freshwater clam, *Corbicula fluminea,* experienced lower growth rates when fed *A. flos-aquae* compared to green algae treatments (Basen et al. 2011). Furthermore, in a chronic exposure study, two species of *Daphnia* experienced higher mortality, delayed maturation of females, and decreased fecundity and population growth rates in the presence of *A. flos-aquae* (De Figueiredo et al. 2004). Although it is currently unknown how *A. flos-aquae* inhibits quagga mussel spawning, a kairomone could be the cause. Marine invertebrates, including bivalves, show reproductive responses to chemicals produced by phytoplankton. Starr et al. (1990) recognized that certain diatom species induced sea urchins and marine mussels to spawn and were later able to isolate and characterize the chemical substance responsible (Starr et al. 1992). If similar mechanisms exist between cyanobacteria and dreissenid mussels, identification of such chemicals could be useful in quagga mussel control programs.

This study demonstrates that some cyanobacteria reduced quagga mussel reproduction, but further research is needed to determine specific causes and

ecological consequences of this phenomenon in nature. The ecological consequences could be significant if blooms occur during mussel spawning because successful reproduction may be inhibited. In North America and Europe, dreissenid mussels typically exhibit two spawning peaks, occurring in early and late summer (Borcherding 1991, Ram et al. 1993), the latter of which can potentially coincide with cyanobacteria blooms. Warmer year-round temperatures and heavy rain events driven by global climate change can not only increase the frequency and size of cyanobacteria blooms, but can cause blooms to form earlier in the year (Paerl and Otten 2013). Likewise, I have observed dreissenid mussel spawning in the Detroit River during the last 15 years where spawning has occurred as early as March and as late as October (Ram et al. 2011). A pattern of cyanobacteria blooms consistently overlapping with quagga mussel spawning could lead to prolonged impacts on mussel populations that intersect with lowered ingestion, potential intoxication, and lowered mussel condition and production of gametes. Dreissenid populations could experience lower recruitment of juveniles during periods of spawning overlap with cyanobacteria blooms, leading to reduced population growth rates. Since I have identified naturally occurring phytoplankton that inhibit spawning and reproduction in quagga mussels, there is a potential to isolate and characterize the compound responsible for this effect.

If cyanobacteria limit dreissenid reproduction in freshwater environments, a chemical tool for reducing dreissenid reproduction might be derived from cyanobacteria to be used in tandem with other control efforts. Integrated pest management techniques have been applied to other nuisance and invasive species, such as sea lamprey. For example, pheromones produced by sea lamprey (*Petromyzon marinus*) have been

characterized, synthesized, and are now used to lure adults into traps for removal (Johnson et al. 2015). In recent control efforts, synthetic sea lamprey pheromones that attract the animals to traps may be used in conjunction with chemical lampricide and sterile release programs (Johnson et al. 2015, Dawson et al. 2016). Ideally, allelochemicals isolated from cyanobacteria for use in quagga mussel control would be species specific, able to be used in low concentrations (Johnson et al. 2015), and have short half-lives (Sorensen and Vrieze 2003). These characteristics are important in terms of management to ensure that non-target species are unaffected, and that the chemical is not retained in the water body or sediments. A single control method has not proven effective for invasive quagga mussels so far, and thus a multi-step approach may be warranted. Developing a mechanism to limit quagga mussel reproduction using naturally occurring cyanobacteria may aid in slowing existing population growth and preventing new establishment in unfouled waterbodies.

CHAPTER 3 EFFECTS OF CYANOBACTERIA EXPOSURE ON DREISSENID VELIGER SURVIVAL

Introduction

Environmental factors influence successful reproduction and development in invasive dreissenid mussels; therefore, studies of such factors could provide insights for effective management and provide information on factors impacting dreissenid population recruitment. Early life stages of *Dreissena polymorpha* are more susceptible to mortality from copper exposure than adults (Kennedy et al. 2006), suggesting that dreissenid larvae may be the life stage most vulnerable to chemical control tactics. Treatment at the veliger stage may be more effective than exposing adult mussels to molluscicides as chemical treatments for adult mussels often require a high concentration of chemical application which can impact non-target organisms (Waller et al. 1993). Naturally occurring environmental stressors such as harmful cyanobacteria blooms may also interfere with the sensitive veliger life stage of dreissenid mussels.

Quagga (*Dreissena rostriformis bugensis*) and zebra (*D. polymorpha*) mussels are sessile, benthic bivalves that begin their early life stage as free-swimming larvae called veligers (Quinn and Ackerman 2012). Veligers range in size from ~60-280 μm in length and make up part of the microzooplankton in aquatic habitats (Kennedy et al. 2006; Ackerman et al. 1994). The early life history stages of both zebra and quagga mussels are unique to freshwater systems as native freshwater bivalves do not have a free-swimming larval veliger stage (Ackerman et al. 1994). This type of reproduction and life history strategy results in high mortality rates where veligers are prone to consumption by larger filter feeders and are especially sensitive to environmental conditions, including food availability (Sprung 1991; Liebig and Vanderploeg 1995). The

sensitivity of veligers to environmental conditions is such that mortality rates for dreissenid larvae can be as high as 99% (Stanczykowska 1977). While optimal environmental conditions can promote the development and survivability of dreissenid veligers, exposure to environmental stressors, such as cyanobacteria blooms, during development could lead to lower growth and survivability and reduced population recruitment.

Availability of phytoplankton is crucial to the success of populations of dreissenid mussels, as dreissenid larvae must reach a certain size correlating with their developmental stage before they can settle and metamorphose into adults (Vanderploeg et al. 1996). Prolonging this stage leaves the sensitive veligers unprotected for extended periods of time (Stoeckel et al. 2004). Veligers, which may only be 80µm long 96 hours after fertilization, depend on small phytoplankton cells for consumption (Vanderploeg et al. 1996; Wright et al. 1996a). Dreissenid veligers can compose up to 25% of non-predatory zooplankton, filtering 180 mL/mg per day, feeding on particles ranging from 1-15 µm (Lazareva et al. 2016). Dreissenid veligers selectively filter out phytoplankton from the natural seston, and are susceptible to potentially toxic cyanobacteria cells (Dionisio Pires et al. 2003, 2004).

Dreissenid veligers are typically present in the warmer seasons when water temperatures reach at least 10°C, with peak abundance occurring in mid-summer (Ram et al. 2011; Lazareva et al. 2016; Fig. 6). The abundance and quality of food during the spawning and larval development season can affect mortality rates. Dreissenid veligers require food high in polyunsaturated fatty acids (PUFAs) for growth and metamorphosis (Wacker and von Elert 2002). However, veliger abundance in eutrophic lakes can co-

occur with summer cyanobacteria blooms where the presence of nutritionally poor or toxic phytoplankton may suppress veliger growth or prevent larvae from settling to the adult stage. Over the past few decades, cyanobacteria blooms have been increasing globally due to eutrophication and global climate change. Warmer global temperatures have caused cyanobacteria blooms to occur as early as May, with their duration lasting longer than historic norms (Huisman et al. 2018). Cyanobacteria tend to contain much less PUFAs than green algae, making them a less nutritional food source for developing veligers (Dionisio Pires et al. 2004). Cyanobacteria are also capable of producing toxins, which can be harmful to humans and wildlife (Carmichael 1994) and may further exacerbate veliger mortality rates. Therefore, the aim of this study was to determine cyanobacteria toxicity on dreissenid veligers by assessing the concentrations of eleven cultures and the purified toxin microcystin-LR causing 50% (LC_{50}).
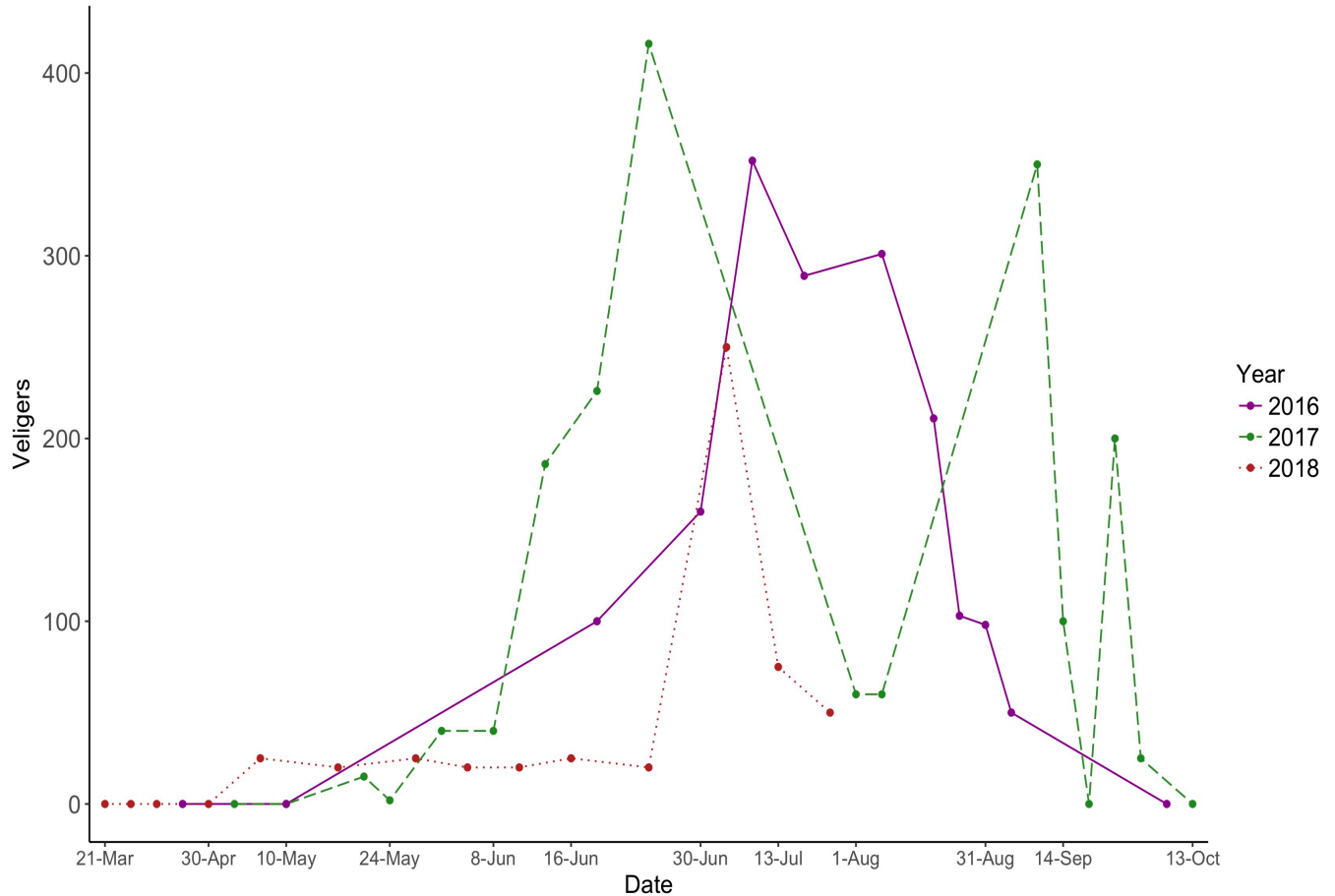
Figure 6. Veliger densities from phytoplankton samples collected from the Detroit River at Belle Isle State Park from 2016-2018.

Methods

Specimen collection and culture:

Dreissenid veligers were collected from the Detroit River on Belle Isle State Park, Detroit, MI (42.358 N, 82.978 W). As dreissenid veligers are planktonic and are not easily identified by species at this life stage, veligers collected at this site were identified down to the genus *Dreissena*, although Ram et al. (2011) genetically identified that >90% of veligers at this site were *D. rostriformis bugensis*. During each sample collection, in-situ water chemistry measurements of temperature, dissolved oxygen, conductivity, and pH were recorded using an YSI multi-meter. Veligers were collected by

tossing a 63 μm mesh Wisconsin plankton net off a pier and was allowed to be carried by the current before retrieval. All planktonic organisms were rinsed from the 63 μm net into a 1L Pyrex glass bottle using water obtained on site. Plankton samples were taken from 0-2 m depth. This process was repeated five times to ensure an adequate amount of veligers were collected for experiments. Detroit River water was also collected and taken back to the laboratory where it was filtered using a 0.22 μm membrane filter and stored at 5°C to be later used for rinsing and dilutions. At the laboratory, plankton samples were rinsed with filtered Detroit River water through a 210 μm nylon mesh to isolate veligers from large particulates and then filtered through an 80 μm mesh to isolate the veligers from smaller particulates. Veligers at growth stages appropriate for toxicological studies typically measure between 80 μm and 210 μm (Ackerman et al. 1994), and filtering plankton samples through the nylon mesh ensured the veligers used in this study were in that size range. Veligers were then rinsed off the mesh and collected in a glass beaker where they acclimated for 24 hours before use in a 23 ± 1°C environmental chamber using a 12:12 light and dark cycle.

All cyanobacteria were cultured in WC media (Guillard and Lorenzen 1972) and maintained in a 21 ± 1 °C environmental chamber on a 16:8 light:dark cycle at 60 μE m^{-2} s^{-1} light intensity (Table 1). Cyanobacteria were harvested during the exponential phase and densities were quantified using chlorophyll a abundance through a spectrophotometer at 665 nm (Biggs and Kilroy 2000). Microcystin-LR was purchased from Beagle Bioproducts (Columbus, Ohio, USA; >95% purity by high performance liquid chromatography) and reconstituted with acetone. An equivalent amount (1–3

mL/L) of acetone was added to the 0 µg/L microcystin-LR solution to maintain any effects caused by acetone exposure.

6-Day Toxicology Assays

Dose response curves for eleven cyanobacteria cultures, representing seven species, and the purified toxin microcystin-LR, a common toxin produced by cyanobacteria, were determined in a series of toxicology assays to identify lethal concentrations to dreissenid veligers. Five concentrations ranging from 40-360 µg/L chlorophyll *a* were tested to determine the lethal concentration of cyanobacteria needed to cause mortality in 50% of the veligers tested (LC_{50}). Microcystin-LR was used to determine the LC_{50} of cyanobacteria toxins on veligers because it is the most commonly produced cyanotoxin congener globally (Dawson 1998). Microplate wells (96-well plates) were filled with 300 µL of the appropriate treatment. For cyanobacteria assays, six dilutions of each isolate were tested (0, 43, 88, 178, 268, & 358 µg/L chlorophyll *a*) where the 0 µg/L concentration served as the control and consisted of filtered Detroit River water diluted with *Chlorella minutissima*, a green algae sufficient for veliger growth (Vanderploeg et al. 1996; n=12). A starvation control where no phytoplankton was added was used to determine if veliger mortality was caused by cyanobacteria or lack of food availability. Mortality from microcystin-LR exposure was tested at concentrations of 0, 1, 2.5, 5, 7.5, 10, 15, & 20 µg/L. The 0 µg/L concentration served as the control and consisted of filtered Detroit River water. Veligers in microcystin-LR assays were fed *C. minutissima*. Veligers were placed into a 60x15 mm glass Petri dish and examined using an inverted microscope under 200x magnification. Cross-polarized light was used to illuminate plankton with calcareous shells and identify veligers from other plankton as

a distinct black "X" easily identifies them (Johnson 1995). A single, live veliger was randomly chosen from the acclimated sample and carefully placed into each well of a 96-well microplate using a 10 µL Eppendorf micropipette. Each veliger was checked for mortality daily for 6 days by observing each well for 30 seconds. Veligers were marked as dead when veliger was not swimming and cilia were no longer beating.

Statistical Analysis

A probit analysis (USEPA 1991) was used to analyze mortality data for all veliger assays to determine the lethal concentration at which 50% of the organisms died (LC_{50}). A Fisher's Exact test was used to see where there is a significant difference in mortality at each concentration compared to the control at the end of each assay (Stewart-Malone et al. 2015). All analyses were performed using R (version 3.3.1; R Project for Statistical Computing, Vienna, Austria). Script for the function used to analyze LC_{50} concentrations can be found in Pacheco and Rebelo (2013).

Results

Sensitivity of veligers to cyanobacteria varied among cultures (Table 5, Fig. 7). Veligers were most sensitive to the UTEX strain of *Microcystis aeruginosa* (LC_{50}=15.06 µg/L chlorophyll-*a*), and least sensitive to the ZUR-HINDAK strain of *M. aeruginosa* (LC_{50}=135.05 µg/L chlorophyll-*a*; Table 5, Fig. 7). The LC_{50} values for the other cyanobacteria species fell between 15.06-135.06 µg/L chlorophyll-*a* (Table 5, Fig. 7). The LC_{50} of microcystin-LR was 13.03 µg/L (Table 5). In all assays, the *Chlorella minutissima* control experienced maximum mortality of 8%, and mortality in the starvation control typically reached 100% mortality by day 4. Veliger mortality rates

corresponded positively with increasing concentrations of cyanobacteria in the assays, indicating that mortality was the result of a dose response and not starvation.

Table 5. The concentration (µg/L) of cyanobacteria isolates and microcystin-LR which cause 50% mortality in dreissenid larvae (LC_{50}). LC_{50} values were generated using a probit model and are reported with lower and upper confidence concentrations.

Treatment	LC_{50} (µg/L)	Lower Confidence	Upper Confidence	χ^2	*P* value
Anabaena flos-aquae	83.02	55.27	110.76	0.25	1.27E-12
Aphanizomenon flos-aquae	100.11	56.80	143.42	5.44	2.16E-08
Dolichospermum lemermanii	79.23	24.41	134.04	13.45	5.76E-06
Gloeotrichia echinulata	66.90	20.08	113.73	10.95	1.52E-07
Microcystis aeruginosa UTEX	15.06	0.01	100.11	24.08	1.50E-04
M. aeruginosa LEMS	58.81	9.61	108.00	9.21	5.62E-07
M. aeruginosa BQ11	74.66	32.12	117.21	4.60	5.75E-05
M. aeruginosa ZUR	135.05	90.73	179.38	13.86	5.05E-08
M. aeruginosa LSC	87.48	50.21	124.76	3.01	1.94E-09
M. Wesenbergii	70.13	35.14	105.13	7.10	4.94E-07
Planktothrix suspensa	115.58	68.46	162.69	11.98	3.75E-07
Microcystin LR	13.03	5.20	20.86	15.95	3.06E-02
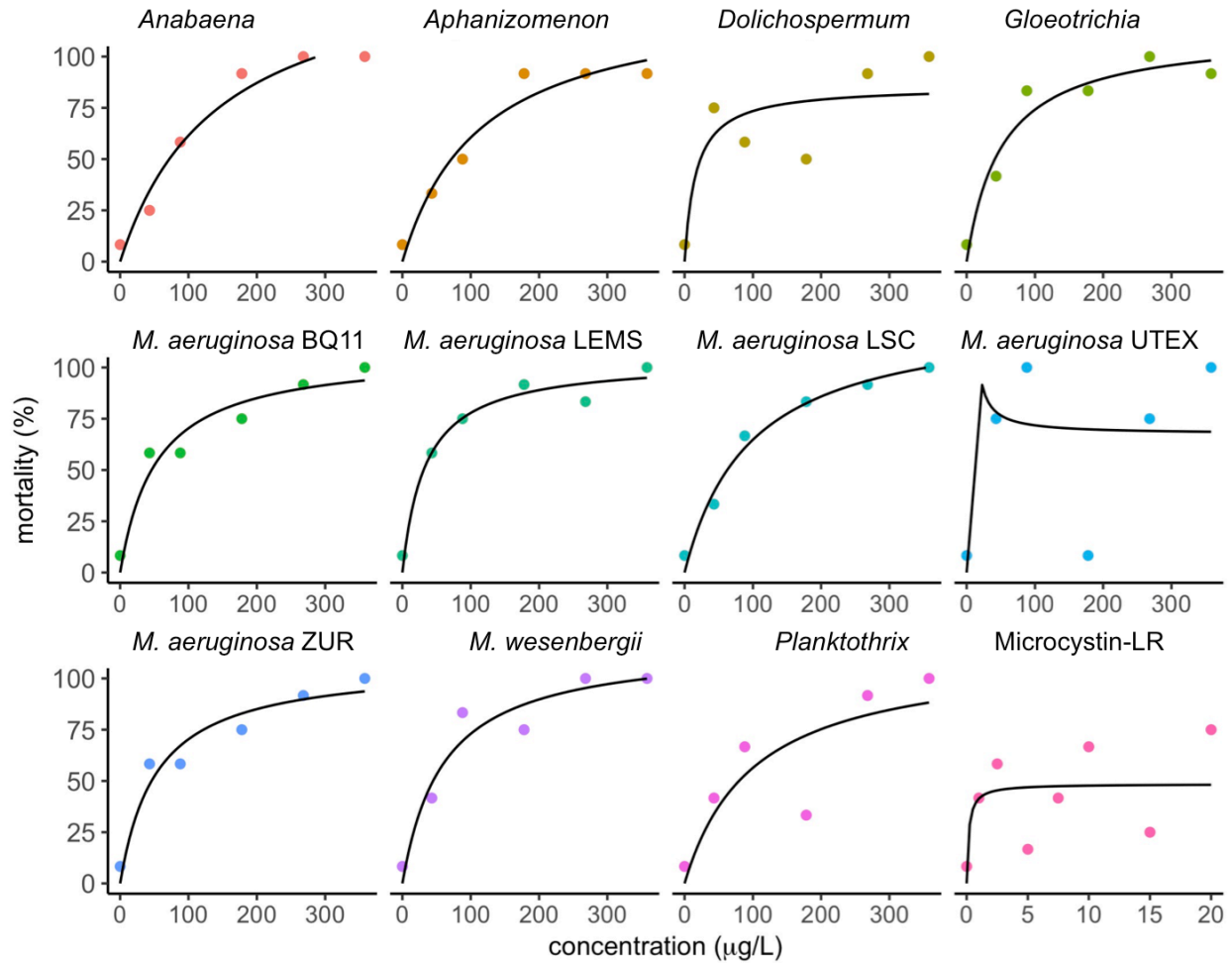
Figure 7. Dose response curves of the eleven cyanobacteria species and microcystin-LR used in determining lethal concentrations for dreissenid veligers. LC_{50} values are reported in Table 5.

Discussion

Results from the current study demonstrate varying toxicities of cyanobacteria isolates on dreissenid veligers in laboratory studies. Our results support previous findings that dreissenid veligers have lower survivability when fed cyanobacteria over other phytoplankton (Dinisio Pires et al. 2003; Vanderploeg et al. 1996). Results from this study correspond to plankton samples analyzed from a lake affected by a cyanobacteria bloom. In September 2017 during a cyanobacteria bloom on Lake Erie,

dreissenid veligers were found in abundance (>100/L) at sites where chlorophyll *a* levels were <5.0 µg/L, while less than 15 veligers/L were recorded where chlorophyll *a* concentrations exceeded 20 µg/L (Boegehold, observational data). In the Great Lakes basin, dreissenid veligers have been collected in plankton samples at depths ranging from 2-60 m (Bowen et al. 2018), with potential to coincide with both buoyant and benthic cyanobacteria. This indicates that results from this study may translate to the field, although a full investigation should be done to make this correlation. The LC_{50} concentrations for cyanobacteria reported in the current study are within range of global cyanobacteria bloom conditions. In the Laurentian Great Lakes, the threshold for sensing cyanobacteria blooms is 18 µg/L chlorophyll *a* while cyanobacteria blooms in Lake Taihu, China can reach concentrations of greater than 500 µg/L chlorophyll *a*, and have been as high as 936 µg/L (Liu et al. 2010, Yuan et al. 2014 Sayers et al. 2016). The LC_{50} concentrations of microcystin were also within range of what is typically seen in lakes that experience regular cyanobacteria blooms (Carmichael and Boyer 2016).

Results from the current study suggest that cyanobacteria have poorer nutritional quality than other phytoplankton such as green algae. This was demonstrated by low mortality in the *Chlorella minutissima* control and increasing mortality corresponding with increased cyanobacteria concentrations. Vanderploeg et al. (1996) report zebra mussel veligers fed the cyanophyte *Synechococcus* sp. did not survive to the juvenile stage, but maintained high survival rates when fed the chlorophyte *C. minutissima*. Furthermore, when veligers were fed a mixture of cyanobacteria and green algae, survival was reduced by half compared to when they were fed only green algae. The authors attribute this difference in survivability to the amount of long chain

polyunsaturated fatty acids (PUFAs) present in the phytoplankton, where *C. minutissima* had a higher percentage of this essential nutrient than *Synechococcus* (Vanderploeg et al. 1996).

Juvenile and adult bivalves fed cyanobacteria also have lower growth rates when compared to animals fed green algae. When newly settled juvenile zebra mussels were collected and fed a high PUFA strain of green algae, they had significantly higher growth rates than mussels fed a low PUFA producing cyanobacteria (Wacker and von Elert 2002). Asian clams, *Corbicula fluminea*, had higher growth rates when fed phytoplankton high in polyunsaturated fatty acids, such as *Scenedesmus* and *Cryptomonas* over cyanobacteria species *Synechococcus, Anabaena,* and *Aphanizomenon* sp. (Basen et al. 2011). Wright et al. (1996a) were successful in rearing quagga mussel veligers to metamorphosis by feeding them algae rich in fatty acids, indicating that phytoplankton availability is important for dreissenid growth and survivability. Phytoplankton cell size also affects dreissenid veliger feeding rates. Different stages of veliger development may be more or less susceptible to cyanobacteria, as some cells may be too large for them to feed on. Veligers used in the current study were between 80-210 µm, and thus were adequately sized to feed on the species they were fed. In previous studies, phytoplankton that was ingested by dreissenid veligers was generally <6 µm (Vanderploeg et al. 1996; Dionisio Pires et al. 2003). Although some of the species used in the current study are filamentous (i.e. *Anabaena flos-aquae, Aphanizomenon flos-aquae*, and *Planktothrix suspensa*), Dionisio Pires et al. (2003) report dreissenid larvae grazed upon *M. aeruginosa* cells, which have an equivalent spherical diameter of 3.8 µm. In the current study, I also observed that *M.*

aeruginosa (UTEX) was toxic to veligers at low concentrations. Individual cell size of *M. aeruginosa* is small enough for veligers to ingest so intracellular chemicals may have been responsible for the higher mortality. Perhaps some cyanobacteria are too large for veliger ingestion, and therefore do not increase mortality the same way that ingestible cyanobacteria cells do.

Production of cyanotoxins is one of the major concerns over harmful algal blooms. Toxins produced by cyanobacteria during bloom events can negatively impact human health and wildlife (Carmichael and Boyer 2016). Dionisio Pires et al. (2003) fed zebra mussel (*D. polymorpha*) larvae toxic and non-toxic strains of *M. aeruginosa* and found that veligers had higher mortality rates when exposed to the toxic strain, and preferentially fed on the nontoxic *M. aeruginosa* at higher rates than the green algae, *Chlamydomonas reinhardtii*. Adult dreissenid mussels can experience sublethal effects to microcystin exposure. Adult mussels produce large amounts of pseudofeces consisting of undigested food particles when fed a highly toxic strain of *M. aeruginosa* (Juhel et al. 2006). Other freshwater species are susceptible to the negative effects of cyanotoxins as well. In a South American reservoir, *M. aeruginosa* and microcystin-LR decreased survival and inhibited recruitment of larvae of the invasive bivalve *Limnoperna fortunei* (Boltovskoy et al. 2013). Juvenile crayfish and juvenile unionid mussels survival rates decreased when exposed to increasing concentrations of microcystins (Clearwater et al. 2012). As the purified toxin microcystin-LR was lethal to veligers at concentrations typically seen within cyanobacteria blooms, it is possible that cyanotoxins and other metabolites couple with poor nutritional quality are responsible for veliger mortality.

Intraspecific variability within cyanobacteria can result in the production of varying types and concentrations of cyanotoxins. In the current study, this is demonstrated by varying LC_{50} values for different isolates of *M. aeruginosa*. In a quagga mussel reproduction study, Boegehold et al. (2018) report that two isolates of *M. aeruginosa* reduced quagga mussel fertilization rates, two other isolates did not, indicating potential genetic differences in cultures of the same cyanobacteria species. Adult dreissenid mussel feeding behavior similarly varies when exposed to different genotypes and cell sizes of *M. aeruginosa*, whereas dreissenids will selectively filter feed some *M. aeruginosa* cells and not others, regardless of microcystin production (Vanderploeg et al. 2001, Sarnelle et al. 2012, White and Sarnelle 2014). Environmental conditions can influence genetic variability among clonal species originating from different locations. Even in Lake Erie and Lake Huron, two hydrologically connected lakes, blooms of *M. aeruginosa* have been recorded as possessing unique genotypes that generate varying types and concentrations of metabolites (Dyble et al. 2008). Therefore, sensitivity of dreissenid larvae may be dependent on genetic expression of local cyanobacteria populations.

Finally, with the abundance of dreissenid mussels in freshwater systems throughout the world, veligers could be used as model organisms for toxicity testing because they are more sensitive to environmental conditions than the adult stage of their lives. Dreissenid resistance to environmental stressors, such as salinity, increases with larval age (Wright et al. 1996b). As veligers have been proven to be susceptible to a wide range of stressors at low concentrations, they could replace sensitive native species with low populations as the benchmark organism for environmental

contaminants. Additionally, targeting early larval development of dreissenid mussels may be an effective management tactic as adult mussels have been difficult to control. Wright and Magee (1997) were successful in using extracts from the plant *Phytolacca dodecandra* as a molluscicide on quagga mussel veligers, but did not observe the same rates of mortality in adult mussels. The veliger life stage of dreissenid mussels is currently under studied, but both toxicology and management fields could benefit from further investigations of dreissenid larvae.

CHAPTER 4 SENSITIVITY OF ASIAN CLAMS (*CORBICULA FLUMINEA*) AND QUAGGA MUSSELS (*DREISSENA ROSTRIFORMIS BUGENSIS*) TO CYANOBACTERIA

Introduction

Invasion of freshwater habitats by non-native animal species is a major threat to global aquatic systems (Strayer 2010). While few introduced species become invasive, those that are successful invaders may thrive in new habitats through plastic responses or broad physiological tolerances (Lee and Gelembiuk 2008). Mollusks represent a significant proportion of invasive species in North America and can thrive in areas of poorer water quality than native species (Karatayev et al. 2009). The Asian clam (*Corbicula fluminea*) and quagga mussel (*Dreissena rostriformis bugensis*) are two bivalves that have been introduced to a wide distribution of freshwater systems outside of their native ranges. Asian clams and quagga mussels pose a serious threat to native biodiversity and ecosystem functioning, impacting food webs, nutrient cycling, and freshwater economies (Sousa et al. 2008, Higgins and Vander Zanden 2010, Strayer 2010). Both species tend to prefer habitat that is frequently disturbed and highly variable, often invading areas prone to anthropogenic use (McMahon 1982, Lee and Gelembiuk 2008). While Asian clams and quagga mussels share many similar traits regarding their ecosystem impacts and life histories that facilitate invasion, their unique evolutionary histories may result in important physiological differences related to stress tolerance in freshwater systems.

Asian clams originate from freshwater areas in Asia, Africa, and Australia and may have been intentionally introduced to North America as a food resource (Sousa et al. 2008). The first North American specimens of Asian clams were found in British

Columbia, Canada in 1924 as empty shells, and live collections were retrieved from the Columbia River in Washington, USA in 1938 (Mills et al. 1993). By the 1960s, Asian clams had spread throughout the United States via connected waterways and human transport (McMahon 1982) and by the 1980s, the distribution of the genus *Corbicula* included South America and Europe (McMahon 2002). Successful invasion and dispersal of *Corbicula* is closely linked with human activities and life history characteristics, such as rapid growth and high fecundity (Sousa et al. 2008). Although Asian clams may not demonstrate extreme stress tolerance (Sousa et al. 2008), their origin from freshwater systems may give them a physiological advantage over other freshwater invasive species that originate from brackish or saline systems.

Like Asian clams, quagga mussels have become globally invasive in many freshwater systems. However, quagga mussels originated from the brackish waters of the Ponto-Caspian region, where invasive populations most likely arose from the northern estuaries of the Black and Caspian Seas (Lee and Gelembiuk 2008). Byssate bivalves are extremely uncommon in freshwater systems, with dreissenid mussels being one of the few exceptions (Karatayev et al. 2009). Quagga mussels began their range expansion out of the Ponto-Caspian region in the 1940s, and in 2004 they were discovered in Romania, initiating their expansion into Western Europe aided by human vectors and connected water bodies (bij de Vaate et al. 2013). In 1991, quagga mussels were first reported in Lake Ontario, accidentally transported via ballast water, but may have been in the Great Lakes – St. Lawrence River drainage since 1989 (Mills et al. 1993, Ricciardi and MacIsaac 2000). Although zebra mussels (*Dreissena polymorpha*), a congeneric species, were the first documented dreissenid invaders to freshwaters of

North America, quagga mussels have recently been displacing existing North American zebra mussel populations and are expanding their range westward (Ram et al. 2012). Quagga mussels can tolerate a range of brackish salinities, which may have aided in their transport and invasion in North America (Ricciardi and MacIsaac 2000). Because of their preference for saline environments, quagga mussels that populate freshwater systems may be at a physiological disadvantage compared to other alien species, such as Asian clams, that originate from freshwater systems.

Both Asian clams and quagga mussels are globally invasive species with overlapping distribution in many colonized areas (Karatayev et al. 2007). Invasions from brackish or saline environments to freshwaters are rare due to challenges in maintaining ionic and osmotic regulation of body fluids (Lee et al. 2012). Baltic organisms that invade freshwaters develop behavioral and physiological mechanisms to cope with low salinity conditions, which can result in increased metabolic costs (Lapucki and Normant 2008). Obtaining essential ions may be more difficult and energetically expensive for freshwater invasive species from historically brackish origins than for those native to freshwaters, and thus may lead to reduced stress tolerance (Lee 2016). Quagga mussels may experience context dependent changes in physiology based on habitat salinity, which may result in quagga mussels being more susceptible to additional environmental stressors, such as toxins and poor food quality, than Asian clams.

Harmful cyanobacteria blooms are becoming more frequent and severe in freshwater systems worldwide (Paerl and Paul 2012). Cyanobacteria can potentially produce toxins, which can cause public health crises, illness, and even death in some animals (Carmichael and Boyer 2016). Furthermore, cyanobacteria tend to be poorer in

nutritional quality to filter feeding organisms than other types of phytoplankton (Vanderploeg et al. 1996). Cyanobacteria can lead to reduced growth rates in both Asian clams and quagga mussels (Wright et al. 1996a; Bassen et al. 2011). Cyanobacteria is demonstrably less desirable than other types of phytoplankton for Asian clams and quagga mussels, and is also an environmental threat that both species face in their invaded range. Thus, cyanobacteria can be useful as an experimental stressor to examine metabolic differences between quagga mussels and Asian clams.

The objective of this study was to determine if Asian clams and quagga mussels experience different physiological responses to environmental stressors. I exposed each species to the cyanobacteria, *Microcystis aeruginosa,* and its associated toxin, microcystin, and measured responses in oxygen consumption, filtration rate, and two oxidative stress biomarkers, catalase and lipid peroxidation. I hypothesized that the quagga mussels would have a higher stress response than Asian clams because the physiological stress of maintaining ionic balance would cause them to be more susceptible to additional environmental stressors. Increased knowledge of stress tolerance between the two species can help resource managers make better predictions about range expansion for these two species and can provide greater insights into the dynamics of species invasions.

Methods

Organism Collection and Culture Maintenance

Quagga mussels (*Dreissena rostriformis bugensis*) and Asian clams (*Corbicula fluminea*) were collected in Wayne County, Michigan from two distinct sampling locations 48 km apart. Both species were collected between February 2016 and

January 2017. Adult quagga mussels were retrieved from the Detroit River on Belle Isle State Park, Detroit, MI (42º21'0.62"N, 82º58'11.5"W) where they were scraped from a seawall at depths of 1-3 m below the surface. Asian clams were gathered by hand from the Rouge River in Plymouth, MI (42º22'14.2"N, 83º26'21.1"W). Live, adult specimens of both species were transported back to the laboratory in a cooler containing site water. At the laboratory, specimens were immediately cleaned of excess debris under running tap water and stored separately by species in aquaria filled with dechlorinated tap water. Animals were maintained at a density of 1 mussel per 20-30ml of water. Once per week the aquaria were cleaned, dead mussels removed, and the water refreshed. Aquaria were stored in an 18±1°C environmental chamber with a 16:8 light:dark cycle. Animals were fed three times per week with 4.0×10^6 cells/mL of laboratory cultured *Ankistrodesmus falcatus* (UTEX #748), a green alga commonly used for maintenance of aquatic filter feeding animals (Boegehold et al. 2018). *Ankistrodesmus falcatus* was also used as the control algae in experiments.

Two independent cultures of *Microcystis aeruginosa* were evaluated for adverse impacts on quagga mussels and Asian clams (Table 1). The two isolates are identified by unique markers where one strain is referred to as *M. aeruginosa* (UTEX) and the other is referred to as *M. aeruginosa* (LEMS). Genetic testing of both isolates reveal that neither strain produces cyanotoxins. Both cyanobacteria isolates were cultured in WC media (Guillard & Lorenzen 1972) and maintained in an environmental chamber at 19±1°C with 16:8 light/dark cycle at 60 $\mu E\ m^{-2}\ s^{-1}$ light intensity. *A. falcatus* was grown in COMBO media (Kilham et al. 1998) under a light intensity of 120 $\mu E\ m^{-2}\ s^{-1}$.

Oxygen Consumption Experiments

Oxygen consumption in quagga mussels and Asian clams was measured in the presence of two strains of *M. aeruginosa*, using *A. falcatus* as the control. Ten animals of each species were placed in 1L Erlenmeyer flasks. Within each treatment, a flask was prepared without mussels to correct for any oxygen production or consumption by the phytoplankton. Flasks were filled with 1L of appropriate solution to minimize airspace in the container while maintaining a low animal density (100 mL/animal; n=5). Experimental solutions consisted of phytoplankton mixed with dechlorinated tap water that was aerated for at least 24 hrs prior to experiment. Dissolved oxygen (DO, mg/L) was measured in each flask following methods adapted from Fernandez-Sanjuan et al. (2013). Dissolved oxygen in each flask was measured at the initiation of the experiment (t=0). After initial measurement, flasks were immediately covered with Parafilm to prevent gas exchange in and out of the flask. Subsequent DO concentrations were recorded at 24, 48, 72 and 96 hours after t=0. Upon termination of the experiment, the soft tissue of quagga mussels and Asian clams was removed and dried in a 60°C oven for 24 hours and weighed. Oxygen consumption was measured as differences in dissolved oxygen between time points during the exposure period. Oxygen measurements in the flasks without animals were used as a correction factor for the appropriate treatments, and oxygen levels in these flasks remained constant throughout the experiment. Measurements were taken with a YSI ProDO optical DO probe.

Oxygen consumption data was tested for normality using a Shapiro Wilk Test (W=0.911, p<0.001) and was log transformed prior to analysis to reduce heterogeneity of variances. A Levene's Test determined that all data was homoscedastic. Data was then analyzed using an ANOVA with Tukey post-hoc tests. These tests were used to

compare both *Microcystis* treatments to the *Ankistrodesmus* control within species and across time points. Data was also analyzed to compare both quagga mussels and Asian clams in similar treatments across time periods. All statistical analyses were performed using R using $\alpha = 0.05$ to determine significance in statistical analysis (Version 3.3.1; R Core Team, 2015).

Filtration Rate Experiments

Filtration rate of Asian clams and quagga mussels was measured in response to two isolates of *M. aeruginosa,* using *A. falcatus* as the control. Five animals per jar (n=5) were placed in 500 mL Erlenmeyer flasks filled with 500 mL of phytoplankton solution containing dechlorinated tap water. For each of the treatments and the control, a separate jar was prepared without animals to adjust for any settlement or photosynthesis of phytoplankton that could contribute to false filtration measurements. Aerators were placed in each treatment jar to maintain aerobic conditions and homogeneity of solutions. Immediately after addition of algae, 50 mL of solution was filtered through 47mm Whatman GF/F filter and chlorophyll-*a* was extracted and measured using hot ethanol extraction techniques (Biggs and Kilroy 2000). This was repeated at three hours after the initial algae addition. The animals remained in the jars in algae solution overnight. At t=24, 48, 72, and 96 hours after initiation of experiment, animals were placed in clean jars with 500 mL fresh solution and the previous steps were repeated. At the end of the experiment, soft tissue was removed from the animals and dried at 60°C for 24 hrs to measure dry weight (g). Filtration rate was calculated using the formula $F=(V/nt)*\ln(C_i-C_f)$, where C_i and C_f are initial and final chlorophyll-*a* concentrations (µg/L), respectively, t is time (hours), V is the volume of phytoplankton

solution (L), and n is the combined dry weight (g) of the five animals in each jar (White and Sarnelle 2014; Vanderploeg et al. 2001). Filtration rate data was tested for normality using a Shapiro Wilk test (W=0.916, p=0.022). A Levene's Test determined that all data was homoscedastic. To reduce heterogeneity of variance, data was log-transformed prior to analysis. Data was analyzed using a 2-way ANOVA to test effects of the two *Microcystis* treatments with increasing exposure time. The 2-way ANOVA was tested individually for each species and a Tukey post-hoc test was used to examine differences within treatments between days. ANOVA tests with Tukey post-hoc tests were also used to evaluate differences between species in similar treatments at each time point and to compare filtration rates in each species in the *Microcystis* treatments to the *Ankistrodesmus* control at each time point. All statistical analyses were performed using R using α = 0.05 to determine significance in statistical analysis (Version 3.3.1; R Core Team, 2015).

Oxidative Stress Response

Neither of the strains of *M. aeruginosa* used in the filtration rate and oxygen consumption studies produce cyanotoxins, and thus I wanted to analyze the cellular effects of the purified toxin, microcystin-LR, on quagga mussels and Asian clams. To examine the effects of microcystin-LR, two oxidative stress biomarkers, catalase (CAT) and lipid peroxidation (LPO), were measured in quagga mussels and Asian clams exposed to the toxin for 96 hours. These two biomarkers were chosen because they are widely conserved in the animal kingdom and well-studied (Nowicki and Kashian 2018; Faria et al. 2009). Catalase is an enzyme that reduces reactive H_2O_2 to water, and lipid peroxidation is a marker of tissue damage caused by reactive oxygen species (Faria et

al. 2009). Both quagga mussels and Asian clams were exposed to dried microcystin-LR (purchased from Beagle Bioproducts, Columbus, Ohio, USA; >95% purity by high performance liquid chromatography) at concentrations of 100 µg/L and 400 µg/L. Treatments consisted of 800 mL glass jars filled with 500 mL of solution (n=10), each containing ten animals. Dried microcystin was reconstituted using a small volume of acetone, and an equivalent volume of acetone was placed in the control jars to ensure that the solvent did not impact oxidative stress levels (maximum amounts of 1mL/L acetone). Aerators were placed in each treatment jar to maintain aerobic conditions throughout the experiment. Oxidative stress response was measured on day 0 for a baseline response and again at 24, 48, 72, and 96 hours from initiation. Animal tissue was analyzed following methods from Nowicki and Kashian (2018). Five animals from each treatment were randomly sacrificed from replicate jars within each treatment for analysis at each time point. Soft tissue from quagga mussels and Asian clams were dissected, weighed, and frozen in liquid nitrogen and stored at -80°C until analysis. Prior to analysis, tissues were homogenized and digested in phosphate buffer. LPO was quantified by measuring malondialdehyde (MDA), a stable byproduct of LPO, using an OxiSelect thiobarbituric acid reactive substances (TBARS) assay kit (ThermoFisher Scientific). CAT was measured as a decrease in absorbance of H_2O_2 consumption and standardized with total protein from each sample, quantified with Coomassie Plus (Bradford) Assay Kit (Thermo Fisher Scientific).

Three-way 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) in response to the

independent variables (species, day, toxin concentration). The MANOVAs identified significance between species, amongst treatments, over time, and in independent variable interactions (e.g., species*toxic concentration, or species*day). Box's M tests were performed to confirm homogeneity of covariances among groups prior to multivariate tests. One-way independent measure analysis of variance (ANOVA) 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. 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. All statistical analyses were performed using R using α = 0.05 to determine significance in statistical analysis (Version 3.3.1; R Core Team, 2015).

Results

Oxygen Consumption

Quagga mussels respired at rates approximately 0.5 mg/L per day per gram dry weight higher than Asian clams over the course of 96 hours in both *Microcystis aeruginosa* treatments (Table 6, Fig. 8; LEMS p=0.002, UTMS p<0.001) and in the *Ankistrodesmus facaltus* control (Table 6, Fig. 8; p=0.017). Over the course of 96 hours, quagga mussels consumed on average 0.5 mg/L per day per gram dry weight more oxygen in the UTMS treatment compared to the control (Table 6, Fig. 8; p=0.011). Quagga mussels also respired *less* oxygen in the LEMS treatment than the control between 48-72 hours (Table 6; p=0.008). There was no difference in oxygen

consumption by Asian clams in either of the *M. aeruginosa* treatments compared to the

control over the course of the experiment (Table 6, Fig. 8; p>0.05).

Table 6. Tukey post-hoc tests from analysis of variance (ANOVA) were used to determine the differences in oxygen consumption over five time periods. *P*-values are reported for the two *Microcystis aeruginosa* cultures (UTMS and LEMS) for each of the two bivalve species tested and the control (Asian clams, AC; quagga mussels, QM) and comparing the two bivalve species within similar treatments (AC-QM). Bold values indicate significance at α = 0.05.

Time Period	UTMS			LEMS		
	AC	QM	AC-QM	AC	QM	AC-QM
0-24 hrs	0.812	0.110	**0.001**	0.556	0.851	**0.002**
24-48 hrs	0.563	0.373	0.387	0.545	0.499	**0.002**
48-72 hrs	0.993	0.451	**0.028**	0.934	**0.008**	**0.003**
72-96 hrs	0.999	0.398	0.999	0.999	0.064	0.089
0-96 hrs	0.999	**0.011**	**0.002**	0.727	0.436	**<0.001**
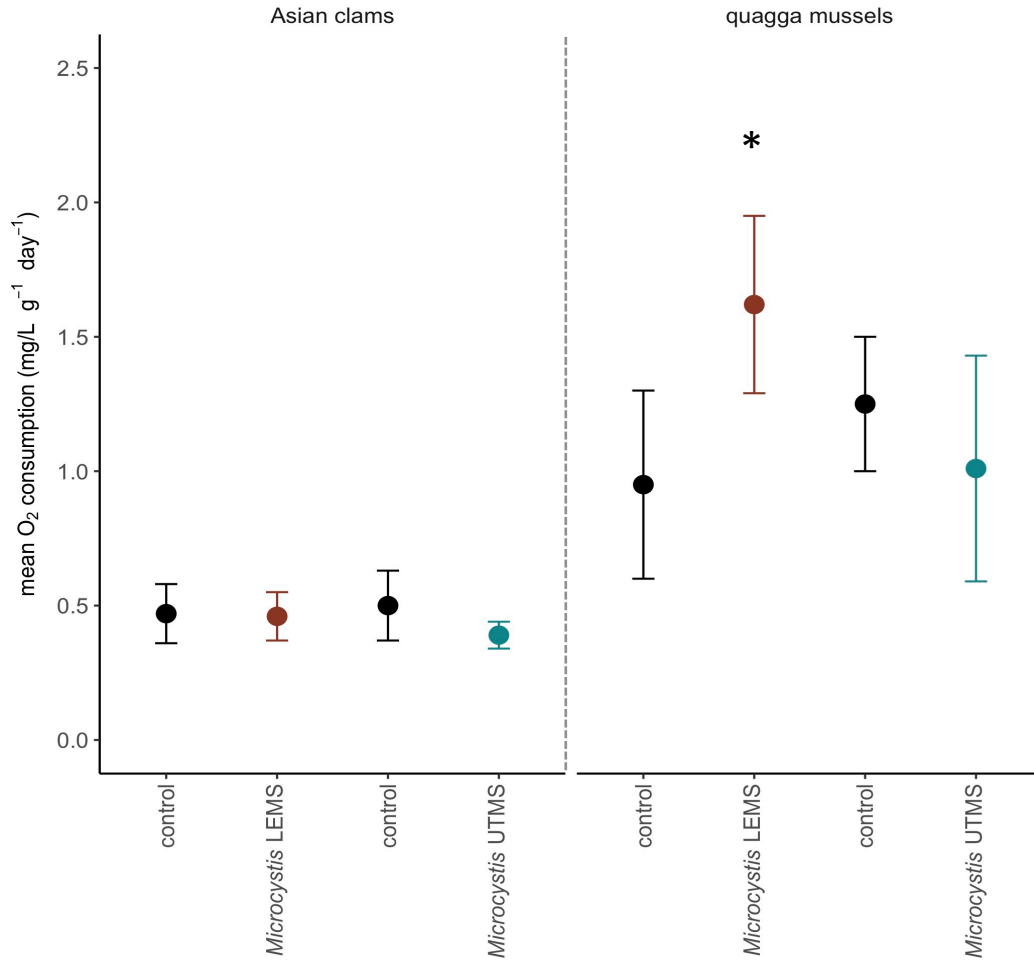
Figure 8. Mean oxygen consumption rates (± SE) over a 96-hour period for Asian clams and quagga mussels exposed to two strains of *Microcystis aeruginosa* and *Ankistordesmus facaltus* (control). Asterisk represents significant difference from the control (ANOVA with Tukey post-hoc tests; p<0.05).

Filtration Rate

Filtration rate in both species was variable in both *Microcystis aeruginosa* treatments and trends were dependent on the duration of exposure (Table 7, Fig. 9; 2-way ANOVA, p<0.05). Both species maintained consistent rates of filtration in the *Ankistrodesmus* control throughout the experiment (Table 7, Fig. 9; p>0.05). Filtration rate between the quagga mussels and Asian clams only differed on Day 4 when quagga mussels filtered double the amount of *M. aeruginosa* (UTMS) than Asian clams (Table 7,

Fig. 9; p=0.029). Asian clams filtered a higher amount of both LEMS (p=0.003) and UTMS (p=0.005) on Day 1 and a higher amount of UTMS (p=0.009) and LEMS (p=0.039) on Day 3 (Table 7, Fig. 9). Asian clams filtered a consistent amount of *Ankistrodesmus* throughout the four-day experiment (Table 7, Fig. 9; p>0.05). However, filtration rate was impacted by both *Microcystis* strains. Filtration rate in the UTMS treatment was lower on Day 2 compared to Day 1 (Table 7, Fig. 9; p=0.047). Asian clams exposed to LEMS experienced lower filtration rates on Day 2 (p<0.001) and Day 4 (p=0.011) compared to Day 1, and had a slight decrease in filtration rates from Day 1 to Day 3 (p=0.05) (Table 7, Fig. 9). Quagga mussels also filtered a consistent amount of *Ankistrodesmus* throughout the experiment (Table 7, Fig. 9; p>0.05). While quagga mussel filtration rates did not vary with exposure to UTMS (p>0.05), filtration rates in the LEMS treatment decreased from Day 1 to Day 2 (p=0.022) and from Day 1 to Day 3 (p=0.003) (Table 7, Fig. 9).

Table 7. Two-way analysis of variance (ANOVA) with Tukey post-hoc tests were used to determine significant differences in filtration rate of each species (Asian clams, AC; quagga mussels, QM) exposed to each strain of *Microcystis aeruginosa* (UTMS and LEMS) and between species within similar treatments. Bold values indicate significance at α = 0.05.

Day	UTMS			LEMS		
	AC	QM	AC-QM	AC	QM	AC-QM
1	**0.005**	0.738	0.231	**0.003**	**<0.001**	0.918
2	0.547	0.899	0.086	0.999	0.981	0.927
3	**0.009**	**0.002**	0.260	**0.039**	0.858	0.994
4	0.999	0.223	**0.029**	0.999	0.188	0.071
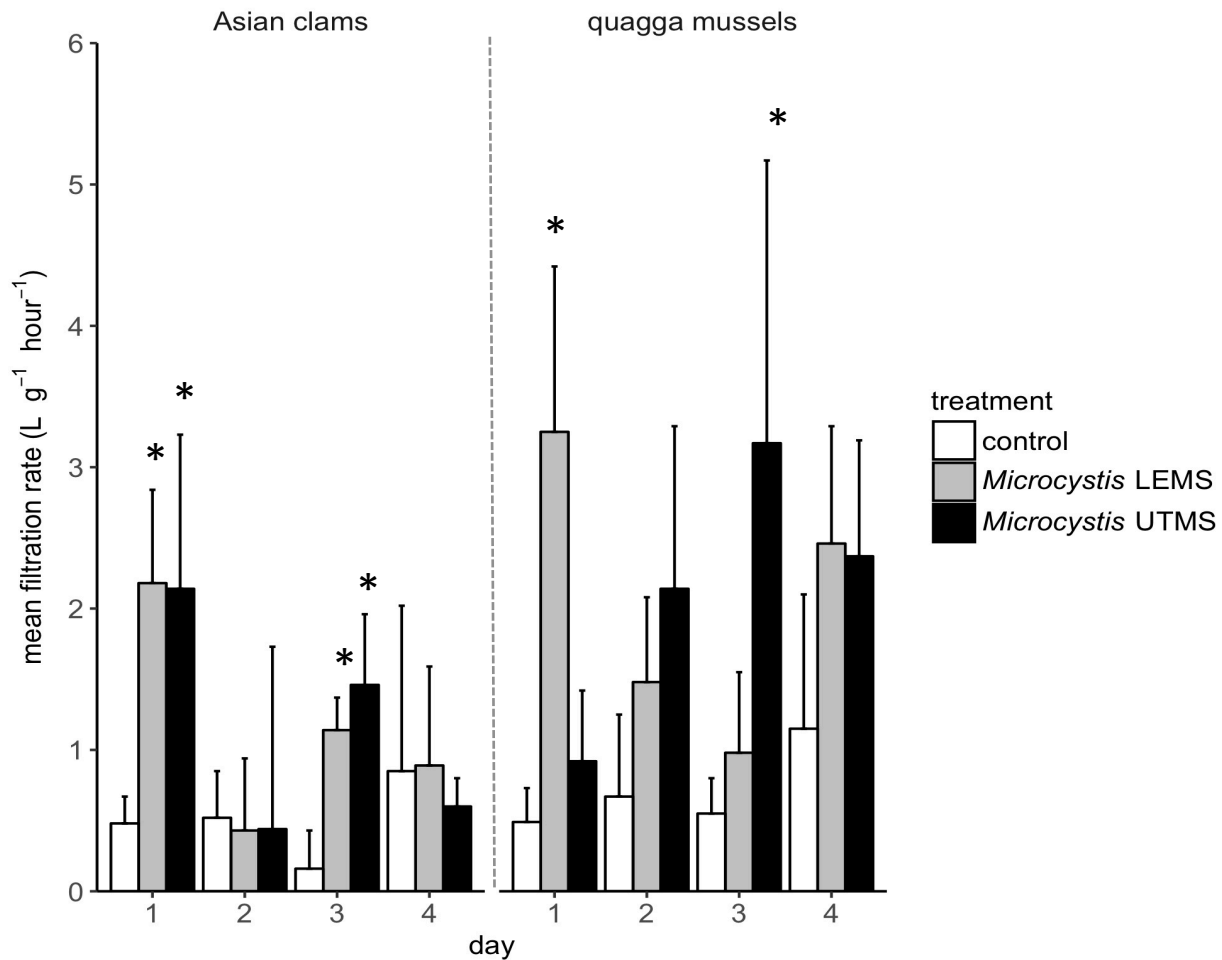
Figure 9. Mean filtration rate (+SE) of Asian clams and quagga mussels exposed to two strains of *Microcystis aeruginosa* and *Ankistrodesmus facaltus* (control) over a 4-day period. Asterisks represent significant differences from the control (2-way ANOVA with Tukey post-hoc tests; $p<0.05$).

Oxidative Stress

Quagga mussels maintained higher levels of oxidative damage (lipid peroxidation, LPO) and antioxidant response (catalase, CAT) than Asian clams throughout the experiment and across treatments (MANOVA, $p<0.001$; Table 8, Fig. 10). Quagga mussels also had a higher baseline (t= 0 hrs) level of CAT than Asian clams (ANOVA, $p=0.002$), but there was no difference in baseline levels of LPO (ANOVA, $p>0.05$). Treatment was also a

factor that resulted in a change of oxidative stress levels (MANOVA, p<0.001; Table 8, Figs. 10, 11), however the trend was contrary to my hypothesis. LPO response in quagga mussels was highest in the control, where the control was significantly higher than the 400 µg/L treatment throughout the experiment (SNK, p=0.009; Table 8, Fig. 11). Catalase response in quagga mussels did not differ between the control and either concentration of microcystin-LR (p>0.05; Table 8, Fig. 10). Cumulative catalase response in Asian clams was elevated in the control compared to the 100 µg/L treatment (SNK, p=0.003) and the 400 µg/L treatment (SNK, p=0.001) (Table 8, Fig. 10). However, LPO damage in Asian clams did not vary among treatments (ANOVA, p=0.189; Table 8, Fig. 11). While oxidative stress response in both treatments and the control was consistent throughout the study on days one through four (SNK, p>0.05), the baseline LPO on day zero was higher than days one (p=0.019), two (p=0.002), and three (p=0.003) for Asian clams (Table 8).
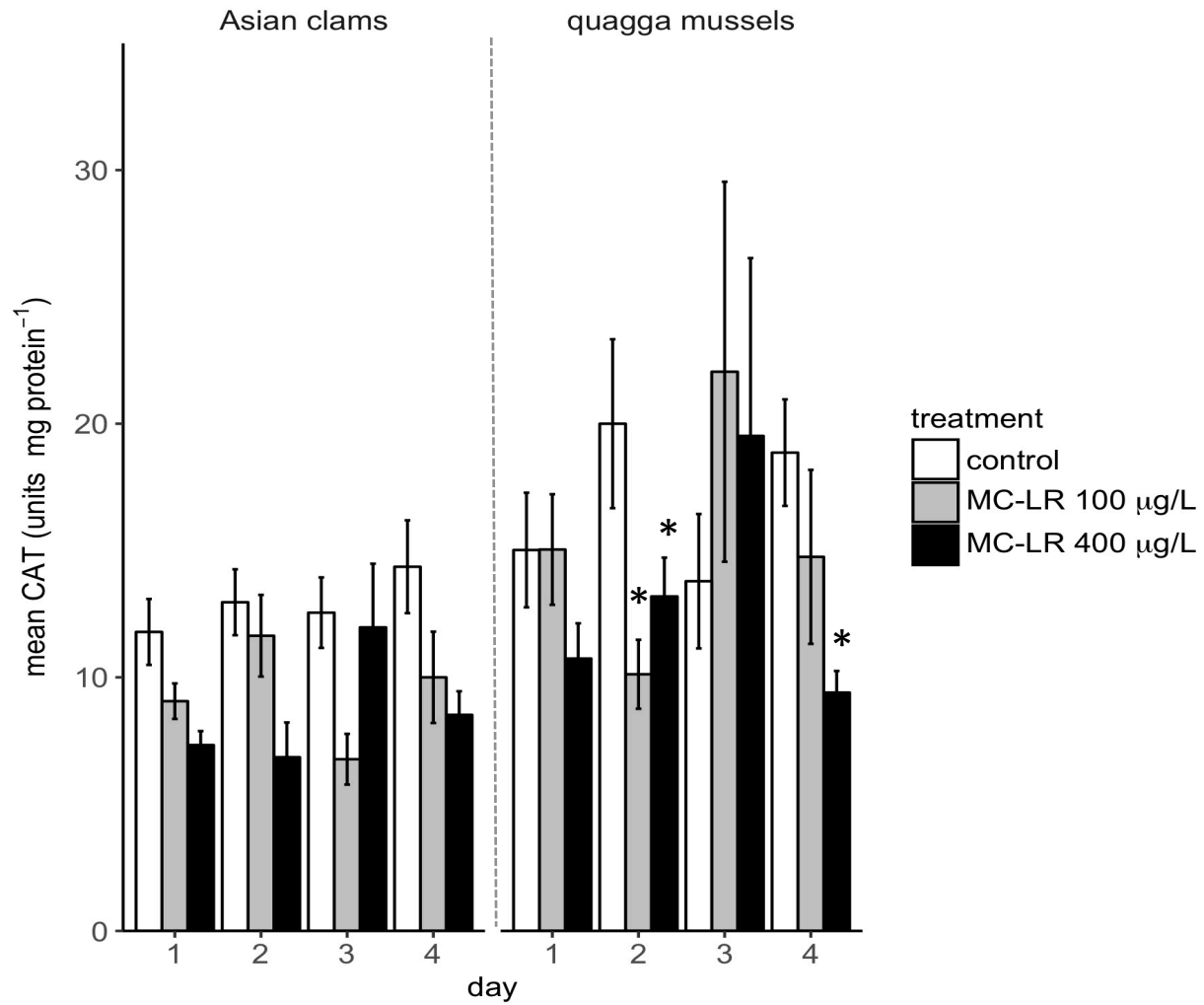
Figure 10. Mean catalase response (± SE) of Asian clams and quagga mussels exposed to microcystin-LR over a 4-day period. Asterisks represent significant differences from the control (univariate ANOVA; p<0.05).
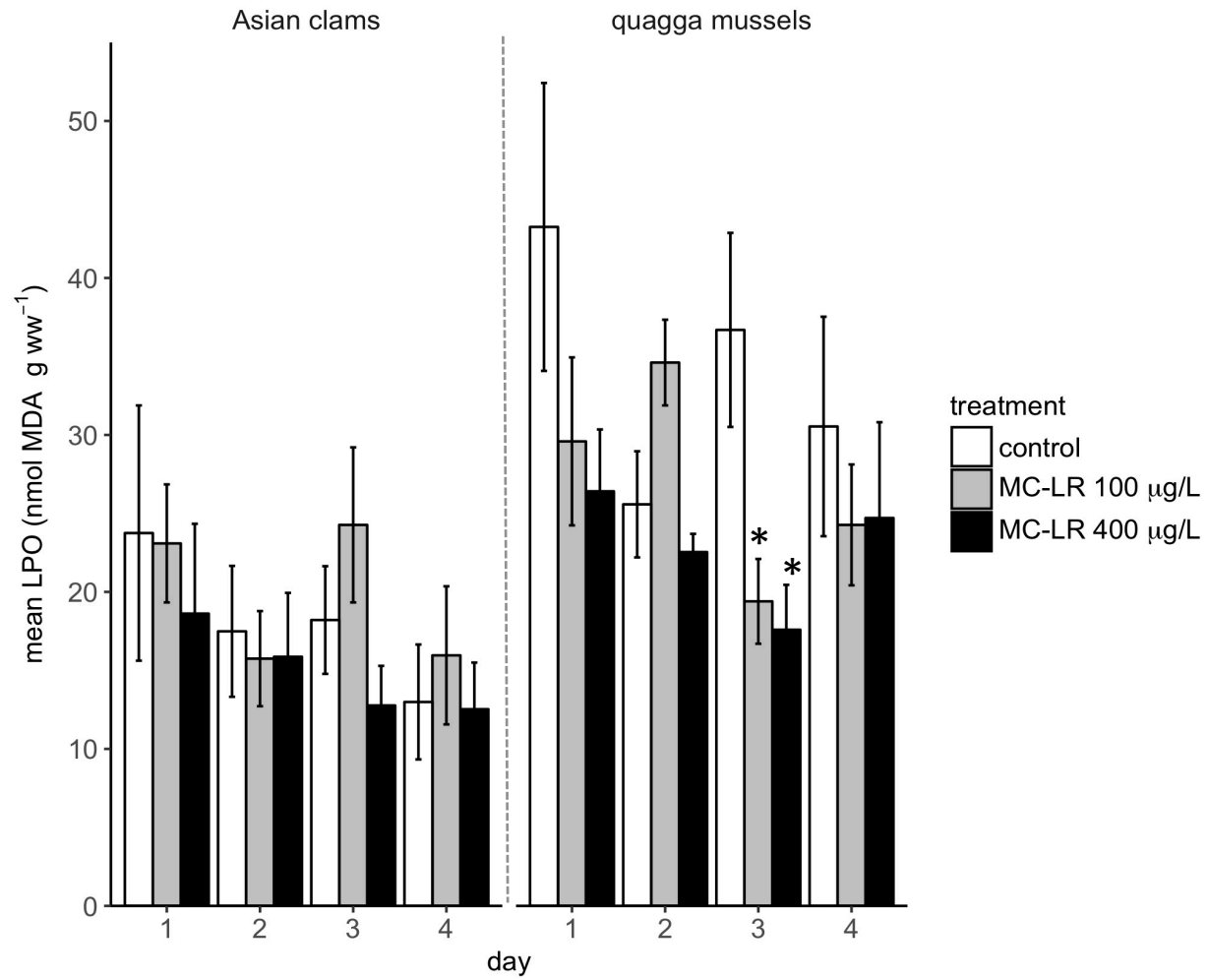
Figure 11. Mean lipid peroxidation (± SE) of Asian clams and quagga mussels exposed to microcystin-LR over a 4-day period. Asterisks represent significant differences from the control (univariate ANOVA; p<0.05).

Table 8. Three-way multivariate analysis of variance (MANOVA) followed by independent one-way analysis of variance (ANOVA) was used to determine the significance of oxidative stress measured as catalase (CAT) and lipid peroxidation (LPO) in *Dreissena rostriformis bugensis* and *Corbicula fluminea* during single stressor experiments conducted with 100 µg/L and 400 µg/L microcystin-LR. Bold values indicate significance at $\alpha = 0.05$.

Statistical Test	*F*-value	*P*-value
Levene's Test (CAT, LPO)	8.329, 0.604	**0.005**, 0.439
Box's M Test		
3-Way MANOVA		
Treatment	19.082	**<0.001**
Day	3.530	**<0.001**
Species	34.776	**<0.001**
*Treatment*Day*	1.619	0.143
*Treatment*Species*	0.085	0.918
*Day*Species*	0.931	0.492
*Treatment*Day*Species*	0.334	0.919
ANOVA (CAT, LPO)		
Treatment	15.427, 17.452	**<0.001, <0.001**
Day	0.436, 7.117	0.782, **<0.001**
Species	25.821, 34.158	**<0.001, <0.001**
*Treatment*Day*	2.914, 0.817	**0.038,** 0.487
*Treatment*Species*	0.172, 0.002	0.679, 0.963
*Day*Species*	0.820, 1.361	0.515, 0.252

Discussion

Although freshwater invasion by brackish species is becoming more common due to global anthropogenic activities (Lee and Charmantier 2012), species such as the quagga mussel (*Dreissena rostriformis bugensis*) may face greater physiological challenges adapting to certain habitats than their freshwater counterparts. Results from this study demonstrate that quagga mussels maintain higher rates of oxidative stress response, filtration rates, and oxygen consumption than Asian clams (*Corbicula fluminea*), an invasive freshwater bivalve originating from freshwater systems. Our results support previous findings indicating that freshwater invasions by species from saline or brackish habitats are more stressful than freshwater invasions by freshwater species.

Acquiring ions to maintain osmotic balance against steep concentration gradients becomes challenging as animals from more saline environments colonize waters more dilute in essential ions because aquatic invertebrates must maintain body fluid that resembles the ionic composition of the surrounding environment (Lapucki and Normant 2008, Lee and Charmantier 2012, Lee et al. 2013, Lee 2016). Hemolymph fluid regulation of brackish species in freshwaters is energetically costly, resulting in higher oxygen consumption and food consumption rates (Lapucki and Normant 2008). For example, invasive populations of the copepod, *Eurytemora affinis*, another Ponto-Caspian invader, have undergone rapid evolution of ion transport activity and associated enzymes in the Laurentian Great Lakes (Lee et al. 2012). These populations experienced an increase in ion uptake capacity and hyper-regulation of hemolymph osmolality in freshwaters, along with increased food and oxygen consumption to survive

in dilute environments (Lee et al. 2012). Furthermore, salinity changes in habitats of the isopod *Idotea chelipes* also resulted in higher metabolic costs related to increased enzyme activity involved in osmoregulation and active maintenance of homeostasis (Lapucki and Normant 2008). Quagga mussels maintained higher rates of all three studied stress markers, oxygen consumption, filtration rate, and oxidative stress biomarkers, throughout the experiment, potentially due to their need for higher metabolic regulation under slightly stressful conditions compared to the Asian clam.

Stress response is energetically expensive for organisms. Respiration rates in zebra mussels can increase during contaminant exposure (Fernández-Sanjuan et al. 2013). Zebra mussel oxygen consumption is also affected by stressful conditions such as turbidity (Alexander et al. 1994). Oxygen consumption did not vary in Asian clams exposed to different phytoplankton and quagga mussels consumed overall higher rates of oxygen than Asian clams in both treatments and the control. Although Asian clams require high oxygen habitat (Sousa et al. 2008), Matthews and McMahon (1999) report that Asian clams are more tolerant of hypoxic conditions than zebra mussels, surviving for up to seven times longer at low oxygen conditions. Our findings also correspond with our hypothesis that quagga mussels would need to consume more oxygen than Asian clams to maintain a higher metabolism in the face of fresh water habitats.

Contrary to initial predictions, filtration rate was equal to or *higher* in the *Microcystis aeruginosa* treatments compared to the *Ankistrodesmus facaltus* control for both species over the entire experiment. The present study exposed animals to non-toxic *M. aeruginosa,* and as such any changes in filtration rate were due to factors other than toxins. Higher feeding rates on cyanobacteria may not indicate a preference for

that food source, but can be related to the poor quality of nutrition in which animals must consume more of it to achieve similar nutrition rates as more preferred algae. While researchers initially thought dreissenid mussels preferentially rejected *M. aeruginosa* cells, some studies demonstrate that they will feed on the cyanobacteria under certain conditions (Sarnelle et al. 2005, Vanderploeg et al. 2013, White and Sarnelle 2014). Asian clams will also feed on non-toxic strains of *M. aeruginosa* at the same rate as the green algae, *Scenedesmus obliquus* (Liu et al. 2009), however Asian clams seem to prefer *Ankistrodesmus* to another cyanobacterium, *Planktothrix adardhii* (Marroni et al. 2014). Liu et al (2009) recorded higher filtration rate in Asian clams fed both toxic and non-toxic strains of *M. aeruginosa* compared to the green alga, *Scenedesmus obliquus.* They also observed more waste products generated by Asian clams fed *M. aeruginosa*, indicating that it is not assimilated as efficiently as other phytoplankton and thus a high rate of filtration may not indicate preference. Pham et al. (2015) also observed higher filtration rates of *Corbicula leana* in the presence of toxic and non-toxic *M. aeruginosa* than in the green alga, *Chlorella* sp. These studies, along with the results of the current study, indicate a complex relationship between filter feeding bivalves and potentially toxic cyanobacteria that is not completely explained by testing filtration rate alone. Future studies should include additional test endpoints related to filtration feeding, such as food assimilation, waste production, and production of pseudofeces to determine preference over phytoplankton species.

Quagga mussels maintained higher rates of filtration throughout the experiment than Asian clams. Filter feeding by invasive populations of both Asian clams and quagga mussels can lead to a reduction in phytoplankton, causing the two species to

outcompete native mussels (Marescaux et al. 2016). Productive freshwater ecosystems with high quality food promote survival and development of brackish invaders while species originating from freshwater conditions are less dependent on high food concentrations for survival in introduced freshwater ranges (Lee et al. 2013). Ponto-Caspian species accustomed to brackish waters have voracious filter feeding capacities in freshwaters, possibly due to the additional challenge of having less ionoregulatory capacities than their freshwater counterparts (Lee 2016). There is evidence that filtration rates in Asian clams are more sensitive than dreissenids to seasonal variation (Marescaux et al. 2016), which corresponds with the hypothesis that quagga mussels must maintain a higher base rate of filter feeding to tolerate freshwater systems.

Oxidative stress response was less dependent on exposure treatment than oxygen consumption and filtration rate. Quagga mussels demonstrated reduced catalase rates in response to microcystins. Excessive stress, which produces reactive oxygen species, can sometimes act to *inhibit* enzymatic response to cellular stress (Kono and Fridovich 1982; Faria et al. 2009). Bigot et al. (2011) exposed Asian clams to metals copper and cadmium and recorded a reduced catalase response, indicating that toxic levels of certain contaminants might hinder an organism's ability to detoxify. Burmester et al. (2012) reported that catalase activity was unaffected in zebra mussels (*Dreissena polymorpha*) exposed to microcystin-LR, but the toxin did affect the antioxidant enzymes superoxide dismutase (SOD) and glutathione S-transferase (GST). There was not as much variation in oxidative stress biomarkers as hypothesized, and future studies should also incorporate the quantification of the antioxidant glutathione (GSH) and glutathione S-transferase (GST) as they may be responsible for detoxifying

and excreting microcystins (Pham et al. 2015). In comparison to Asian clams, zebra mussels have demonstrated a catalase response to certain anthropogenic stressors. Two separate studies showed that paracetamol (acetaminophen) elicits a catalase response in zebra mussels, but not Asian clams (Parolini et al. 2010; Brandão et al. 2011), indicating that Asian clams may be more tolerant of paracetamol.

While catalase response in invasive bivalves is highly variable, lipid peroxidation damage did not increase with microcystin exposure. However, other studies have demonstrated that lipid peroxidation in invasive dreissenid mussels and Asian clams can be induced by other chemical contaminants. Lipid peroxidation in zebra mussels was found to be extremely sensitive to drugs such as ibuprofen, where concentrations as low as 1 µg/L reduced overall mussel condition (weight g/ shell length mm) and increased LPO (André and Gagné 2017). Faria et al. (2009) observed higher lipid peroxidation corresponding with gametogenesis and, higher rates of CAT and LPO corresponding with high metals concentrations (Pb, Zn, As). Furthermore, Asian clams also experienced an increase in lipid peroxidation when exposed to ibuprofen, as well as other drugs such as carbamazepine, novobiocin, and tamoxifen, but were not sensitive to caffeine (Aguierre-Martinez et al. 2015). Four day exposure to microcystin was not stressful enough to cause lipid peroxidation damage in Asian clams or quagga mussels, and other cyanobacteria factors other than toxins might be responsible for inducing stress responses in these animals. Pham et al. (2015) indicate that *Corbicula leana* experienced elevated oxidative stress levels when exposed to both a toxic and a non-toxic strain of *M. aeruginosa*, indicating that microcystin may not be the main cause of physiological stress in these two species.

Prolonged exposure to contaminants may result in acclimations and avoidances to stress. Bivalves can tolerate stressors by detoxifying contaminants, selective filter feeding, and shutting their valves for a short period of time (Dionisio Pires et al. 2004). Furthermore, Lee et al. (2017) found that populations of the copepod, *Eurytemora affinis,* were better adapted to crude oil after the 2010 Deepwater Horizon oil spill compared to populations collected prior to the spill. Other environmental factors, such as seasonal variability and ion concentrations, can cause fluctuations in oxidative stress response (Wojtal-Frankiewicz et al. 2017), which could alter stress response in mussels. Temperature effects can also confound physiological stress of these invasive bivalves. Quagga mussels have a lower temperature tolerance, surviving in waters that are subject to freezing, freezing temperatures are fatal to Asian clams (Karatayev et al. 2007). On the other hand, Asian clams can tolerate higher temperature ranges, extending to 37°C, while quagga mussels are susceptible to mortality and increased stress responses at temperatures about 30°C (Karatayev et al. 2007, Nowicki and Kashian 2018). Although these experiments were performed at ambient temperature (approximately 22°C) in the laboratory, stress levels between Asian clams and quagga mussels could differ in field conditions, especially in temperature extremes. Asian clams and quagga mussels have overlapping ranges in invaded areas such as North America and Europe (Karatayev et al. 2007). Differences in physiological tolerances to environmental stressors between quagga mussels and Asian clams could determine which species dominates in certain water bodies.

SIGNIFICANCE

This research demonstrates the physiological and reproductive consequences of quagga mussels exposed to cyanobacteria and their associated toxins. Results from these studies fill an important gap in the current literature regarding the sensitivity of several quagga mussel life history stages that can be used to better manage the spread and control of invasive quagga mussel populations. The experiments conducted in the sperm motility assays explored novel methodologies for assessing toxicity of environmental stressors on sublethal reproductive endpoints. Using an optical bioassay, I was able to visualize, record, and measure the motility of live, freshly expelled sperm. This study was also the first of its kind to analyze the effects of cyanobacteria on sperm of an aquatic invasive species. This study has implications that reach beyond the scope of invasive dreissenid management. The premise and methodologies from this study can also be used to explore how broadcast spawning freshwater fish reproduction is affected by environmental stressors.

Results from the spawning and fertilization assays help support prior hypotheses that phytoplankton influences dreissenid spawning (Kashian and Ram 2013). While other studies have demonstrated that broadcast spawning species in marine systems are affected by phytoplankton (Miyazaki 1938; Himmelman 1975; Starr et al. 1990), this is one of the first studies that demonstrate similar mechanisms in freshwater species. These experiments can lead to the development of a dreissenid-specific chemical control method that targets their reproduction, an approach that has not been explored yet in management. Alongside reproduction, early stages of development are especially sensitive life history stages. Understanding the extent of these sensitivities is another

unexplored area of research that could potentially lead to more effective management techniques. Furthermore, methods from the veliger assays can be used in toxicity testing of contaminants as they are a sensitive life stage that can be easily collected from invaded water bodies.

Finally, the physiological differences in tolerance to *Microcystis aeruginosa* and microcystin-LR between freshwater invasive species that originate from brackish or freshwaters builds upon previous work that demonstrates brackish to freshwater invasions are stressful (Lee et al. 2012, 2013). This work begins to address the evolutionary factors that are associated with species invasions and niche partitioning between populations of invasive species. Broadening our knowledge of invasive species interactions with environmental stressors can aid in predicating the spread and population dynamics of freshwater species in novel systems.

These experiments examine relationships between cyanobacteria and dreissenid mussels. Information gained from this work can contribute to new and effective invasive species control methods. Knowing when quagga mussels are stressed or sensitive to environmental conditions may be able to inform resource managers when to employ control methods. Furthermore, methods and results from these studies can inspire future research on how nuisance phytoplankton impacts reproduction and early development of native and commercially valuable freshwater species.

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ABSTRACT

**SENSITIVITY OF QUAGGA MUSSELS (*DREISSENA ROSTRIFORMIS BUGENSIS*)
TO CYANOBACTERIA AT MULTIPLE LIFE HISTORY STAGES**

by

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Major: Biological Sciences

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Quagga mussels (*Dreissena rostriformis bugensis*) originate from brackish waters of the Ponto-Caspian area and are nuisance invasive species in North American and European freshwaters. Their invasion has caused major economic and ecological damages in the Laurentian Great Lakes. Through selective filter feeding, quagga mussels have promoted the growth of cyanobacteria. Harmful cyanobacteria blooms have the potential to produce toxins, which can be toxic to humans and wildlife. Although quagga mussels can increase the prevalence of cyanobacteria blooms, it is unknown how they are physiologically affected by cyanobacteria and understanding these dynamics can be useful in the management of this invasive species. To evaluate the effects of cyanobacteria on quagga mussels, I evaluated reproduction, early development, and physiological and cellular stress in several laboratory studies. Sperm motility was assessed using a novel optical bioassay to record sperm movement and measure distance, velocity, and acceleration of individual sperm cells in the presence of eleven cyanobacteria cultures and the purified cyanotoxin, microcystin. Sperm motility was reduced in the presence of four cyanobacteria cultures, but was unaffected by

microcystin. Next, I evaluated spawning success by inducing quagga mussels to spawn using serotonin and exposing them to thirteen cyanobacteria cultures and found one culture of *Aphanizomenon flos-aquae* hinders gamete release. I then combined quagga mussel sperm and eggs in cyanobacteria solution to determine if cyanobacteria would prevent successful fertilization and found six cultures reduced fertilization rates compared to the control. To determine how cyanobacteria influence the sensitive larval veliger stage of dreissenid mussels, I collected veligers off shore from the Detroit River, MI, USA and observed mortality in the presence of five concentrations of eleven cyanobacteria cultures and microcystin. In a series of bioassays, I then determined a dose response curve for each cyanobacteria culture and microcystin and found that veligers experience high mortality rates when exposed to environmentally relevant concentrations of cyanobacteria and cyanotoxins. Results from these experiments can be used to create management techniques that target invasive quagga mussels at their most sensitive life history stages. Finally, I explored how the brackish origins of quagga mussels impact their metabolism and oxidative stress response in freshwater systems by comparing them to another freshwater invasive bivalve, the Asian clam (*Corbicula fluminea*) when exposed to cyanobacteria and microcystin stressors. My results support previous work that brackish to freshwater invasion can act as a context dependent form of stress and result in higher respiration and filtration rates and altered oxidative stress response. The findings from these studies can be used to predict invasive species distribution and range expansion and can lead to more effective control of nuisance populations.

AUTOBIOGRAPHICAL STATEMENT

I grew up in a pretty diverse family and I spent my childhood outdoors endlessly fascinated with nature. However, the outdoor spaces available to me were heavily impacted by human development. I quickly became an environmental advocate and decided to pursue higher education in ecological sciences. The diversity of my family influenced me to always look for ways to incorporate other fields into my science. It has also led me to be an advocate for social and racial justice. One of my biggest goals moving forward with my career in freshwater science is to make the field more inclusive to under-represented and marginalized people. I also want to build a career using my skills and education to investigate how environmental health is linked the human health, focusing on urban centers and environmental racism. I truly believe that the greatest science is done through collaboration with people from different academic and personal backgrounds. I also hope to never stop learning, growing, and having fun, both in my personal life and throughout my scientific career. While there have been numerous struggles and even times where I wanted to quit, there is nothing I would rather be doing than science.