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DEVELOPMENT OF A DREISSENA BIOASSAY TO ASSESS THE TOXICITY OF CONTAMINANTS ACROSS TWO LIFE-HISTORY STAGES

by

CAROLINE ADDIS

THESIS

Submitted to the Graduate School

of Wayne State University,

Detroit, Michigan

in partial fulfillment of the requirements

for the degree of

MASTER OF SCIENCE

2016

MAJOR: BIOLOGY

Approved By:

_____

Advisor Date

DEDICATION

I dedicate this work to my two children, Xavier and Sebastian Bell. Their unquenchable curiosity about the world is a daily reminder of why I chose to delve into the sciences in the first place. Also I dedicate to Jeffrey Bell for being supportive throughout my education; my mother-in-law Jolan and late father-in-law Jon for helping out with the kids, and my own beloved parents John and Linda for their love, guidance, and general parental awesomeness.

“For small creatures such as we, the vastness is bearable only through love.”—Carl Sagan.

ACKNOWLEDGEMENTS

This completion of this work was made possible with contributions of time and energy of many individuals whom I'd like to thank. First, my advisor Dr. Donna Kashian who first sparked my fascination with aquatic ecology in the course of my undergraduate studies and inspired me to continue my education in this field. Without her professionalism, expertise, and patience, it would have not been possible. In addition to teaching me essential research and laboratory skills, she taught me to never give up on myself while learning how to balance work and family time management. She also showed me the importance of not being afraid to ask questions and seek out assistance when needed, a vital lesson for both career and daily life.

I would also like to acknowledge the Department of Biological Sciences for providing assistance through granting me Graduate Teaching Assistant positions, during which I gained confidence for public speaking as well as an ability to break down scientific concepts into easy-to-understand elements. Thank you to the rest of my thesis committee, Dr. Dan Kashian and Dr. Jeff Ram for their valuable feedback. From the United States Geological Service Columbia Environmental Research Center, I thank Dr. Edward Little for requesting our assistance with this project, and Holly Puglis and Erinn Beahan for working with me at the onset to begin development of the assay techniques. Finally, a huge thanks to all of my labmates and good friend Jordan Sinclair for all their help.

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CHAPTER 1: DEVELOPMENT OF DREISSENID VELIGER BIOASSAY

Introduction

The biofouling species *Dreissena polymorpha* was unintentionally introduced to the Great Lakes in the 1980s along with its close congener, *Dreissena bugensis*. Since their introduction, these species have changed the freshwater r ecosystems of the region and have spread throughout the continental United States (Benson, 2011). They have caused immense ecological and economic damage to the watersheds they colonize. Ecologically, they have negatively affected populations of native unionids (Schloesser et al., 1998; Gillis and Mackie, 1994), and fish populations including lake whitefish (Dermott et al, 2005), alewife, and salmonids (Pothoven and Madenjian, 2008). Economically, estimates of costs related to dreissenid mussel control in North America have been proposed to be as high as one billion dollars (Pimental et al., 2005).

Most dreissenid research to date has predominantly focused on adult mussels, their environmental impacts and control methods. However, despite all the negative impacts associated with dreissenid mussels, they may provide a useful and inexpensive test organism for toxicological analyses. Locations of established populations are well documented and easy to locate, collect, and maintain in the laboratory at little cost. These are important traits for choosing model organisms for toxicity testing (Coler and Rockwood, 1989). Furthermore, because they are longer lived than some organisms commonly used in freshwater toxicity testing (i.e. *Ceriodaphnia* and *Daphnia*), dreissenid mussels can provide a longer reflection of environmental conditions.

Adult mussels have a high filtration rate which results in relatively rapid bioconcentration of chemicals (Reeders et al, 1989; Gossiaux et al, 1998). For these reasons, adult dreissenids can be used as bioindicators for lipid-soluble metals and contaminants in freshwater systems (Kraak et al, 1991; Johns and Timmerman, 1998). In addition, Nowicki et al. (2014) identified a gradient of toxicity with increasing proximity to sediment associated with a polychlorinated biphenyl (PCB) spill using adult dreissenids,

demonstrating their usefulness as a bioindicator of toxicity.

Veligers, dreissenid larvae, may also prove to be a useful bioindicator of contaminants. Chemicals have varying degrees of aqueous solubility; those with low K_{ow} (a ratio that expresses a chemical's tendency to distribute itself between equal volumes of octanol and water) will partition into the water, while those with high K_{ow} are hydrophobic and will partition into the sediment. Veligers are planktonic and are exposed to dissolved compounds with low K_{ow} values in the water column that may not be associated with the sediments. In contrast, the adults are sessile and primarily benthic, thus exposed to sediment-bound chemicals with high K_{ow}. In addition, the two life history stages are likely to have different sensitivities. Aquatic larvae are often more sensitive to contaminants than their adult counterparts due to morphological, physiological, and behavioral differences (Mohammed, 2013). However, at this time there is little information on larval dreissenid sensitivity to contaminants. A few previous studies have examined the lethality of molluscicide chemicals in an attempt to identify a control measure for invasive mussels by examining the effects of the compounds on non-isolated veligers. For example, Mackie and Kilgour (1994) investigated the effectiveness of alum on eradicating dreissenid veligers through an assay on many individuals in a single volume of water in a shared petri dish. Likewise, veligers of the mussel species *Mytilus edulis* were exposed to tributyltin, with percentage mortality determined by sampling 100 veligers at a time from each vessel (Beaumont and Budd, 1984). These experiments were designed to evaluate an extermination method and not as a means of evaluating toxicity.

The objective of this work was to develop a multiple life-history stage dreissenid bioassay. To validate this technique, the toxicity of a surfactant mixture (Chemguard First Class firefighting foam) was tested on the veliger and adult stage of dreissenid mussels. This type of chemical is commonly mixed with water and applied during aerial wildfire fighting operations. With lethality as the chosen assessment endpoint, I hypothesized that the veligers would have greater sensitivity and thus exhibit significantly

lower survival than the adults. The integrated examination of multiple life stages proved a more comprehensive measure of contamination than adult assays alone.

Methods

Veliger collection and isolation

Dreissenid veligers were collected from the Detroit River from a pier on Belle Isle (42.358 N, 82.978 W). While nearly impossible to visually differentiate between the two at this stage, PCR analysis has shown this location to be populated by both *Dreissena polymorpha* and *Dreissena bugensis* veligers (Ram et al, 2011). Collection was performed using a 63 μm Wisconsin plankton net tossed perpendicular to a sea wall and allowed to drift for five minutes before steadily retrieving in a hand-over-hand motion. Water samples containing the veligers were concentrated by swirling the codend before carefully rinsing into 1000 mL Corning® PYREX® bottles. This process was repeated at least five times to ensure a suitable quantity of veligers. In addition, one liter of river water was also collected to be filtered through 20 micron mesh for use in rinsing, dilutions, and controls. Physiochemical parameters of temperature and dissolved oxygen, conductivity, and pH readings were taken on site using YSI ProODO, Oakton CON 11, and Mettler Toledo EL2 meters, respectively. The samples were immediately transported back to the laboratory on ice.

Back at the laboratory, samples were rinsed twice with filtered lake water through a 210 μm nylon mesh to remove larger organisms and detritus. The filtrate containing the veligers was then filtered through an 80 μm mesh to remove smaller particles. The remaining material was carefully rinsed from the mesh into a clean 50-mL glass beaker. This filtering was done to isolate particles between 80 and 210 μm, which encompasses most D-stage and the larger (umbonal) veligers (Ackerman, 1994). The veligers were then allowed to acclimate in an environmental chamber at 23 ºC for at least 24 hours before use in assays.

Acute veliger assay

The veligers were emptied from the 50-ml beaker into 60x15 mm glass Petri dishes and examined at 20x magnification under cross-polarized light to differentiate veligers from other particles. Under cross-polarized light, veligers display a characteristic black "X". Veligers were gently isolated by placing the tip of a 10 µL Eppendorf micropipette directly adjacent to a random individual and slowly drawing it inside, taking care to never come into direct physical contact as this was shown to easily damage the developing shells. For the acute veliger assay trial, five concentrations (10, 20, 30, 40, and 50 g/L diluted with filtered river water) of Chemguard First Class were separately tested against controls with 12 replicates each (n=12), for a total of 24 wells filled per acute assay. Each concentrations was tested individually against the control to allow for enough time to evaluate mortality. Each treatment well was filled with 300 µL of the appropriate treatment, and 300 µL of filtered river water added to the controls. A viable veliger was then carefully placed into each well, and each sequentially checked for approximately 10 seconds for 2.5 hours or until 100% mortality occurred, whichever came first. Mortality was determined when no swimming or cilia movement was observed. After microscopic observation, the plates were immediately removed from the microscope lights as to not overheat the veligers.

Chronic veliger assay

The same general procedure as the acute veliger assay was followed for the chronic veliger assay with the following modifications. All concentrations were tested simultaneously in the same microtiter plate. For the chronic assay seven concentrations (0.001, 0.1, 0.25, 0.5, 0.75, 1, and 10 mg/L with a food source *Chlamydomonas reinhardtii*) were tested plus control (n=12) for a total of 96 wells filled per assay. Individual veligers were checked daily for 6 days by observing for 30 seconds each, the entire tray taking about an hour to examine. Wells were refreshed on the third day with new solution to minimize water and chemical loss due to evaporation, remove waste, and refresh food resources. To do so, the fluid

in each well was carefully drawn off with a micropipette and replaced with fresh solution.

Acute Adult Assay

For the acute adult assays, ten adults (n=10) for each treatment (0, 10, 20, 30, 40, and 50 g/L) were placed in individual vials with 30 mL of treatment and randomized, for a total of 60 vials. Aeration was performed through gentle stirring at every check. The entire experiment was incubated at 20 °C and examined in 15 minute intervals. A mussel was determined to be dead if open and not responding to the stimuli of stirring or a gentle prodding with a pipette tip.

Chronic Adult Assay

Chronic adult assays were performed in a similar manner, using lower concentrations than the acute assays (0, 0.001, 0.1, 0.25, 0.5, 0.75, 1, and 10 mg/L with food source *Chlamydomonas reinhardtii*) for a total of 80 units. Vials were stirred and checked daily for mortality, with solutions refreshed on the third day by carefully pouring off and replenishing the fluids.

Results

Acute Assays

All acute treatment concentrations reached 100% veliger mortality within 20 to 40 minutes of exposure, with zero mortality in the control group. Average times to death for 10, 20, 30, 40, and 50 g/L were 23.3, 12.9, 10.0, 9.6, and 9.6 minutes, respectively. Adult mussels at these concentrations took between 30 and 150 minutes to reach 100% mortality, with zero control mortality. Average time to death for 10, 20, 30, 40, and 50 g/L were 63, 36, 19.5, 16.5, and 18 minutes (figure 1).

ANOVA (α=0.05) was performed to compare the times to death between the two life stages [$F(1, 108) = 17.3$, $p = 0.0000038$)]. The assumption of homogeneity of variances tested with Levene's F test was violated, and Welch's t-tests for unequal variances were conducted for each assay to determine specific differences between veliger and adult mean times to death. These showed that mortality for veligers occurred significantly sooner compared to adults for each concentration (p=0.037 for 10 g/L,

p=0.035 for 20 g/L, p=0.022 for 30g/L, and p=0.0048 for both 40 and 50 g/L). The 15-minute LC_{50} was 16 g/L for veligers and 67 g/L for adults based on probit analysis (Finney 1971).

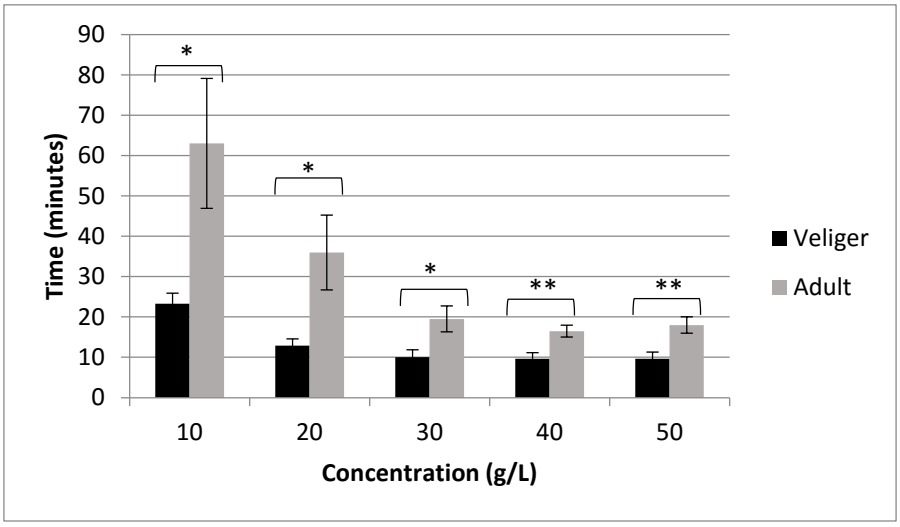


Figure 1. Average time to death of veligers and adults. Mortality was quantified at five-minute intervals for veligers (n=12) and 15 minute intervals for adults (n=10). Bars indicate standard error. *p<0.05, **p<0.01, ***p<0.001

Chronic Assays

For the 6-day chronic assay, 100% of veligers exposed to 0.25, 0.5, 0.75, 1 and 10 mg/L died, as well as 92% of those exposed to 0.1 mg/L. No adult mussels exposed to these concentrations expired. Chronic exposure assays were analyzed with regard to proportion dead after six days with Fisher's exact tests. These showed veligers to be significantly more sensitive at all concentrations over 0.001 (p = 0.000034 at 0.1 mg/L, p = 0.0000016 at all higher concentrations). Probit determined the 5-day LC_{50} to be 0.2 mg/L for veligers, and could not calculate an LC_{50} for adults because no mortality was observed.

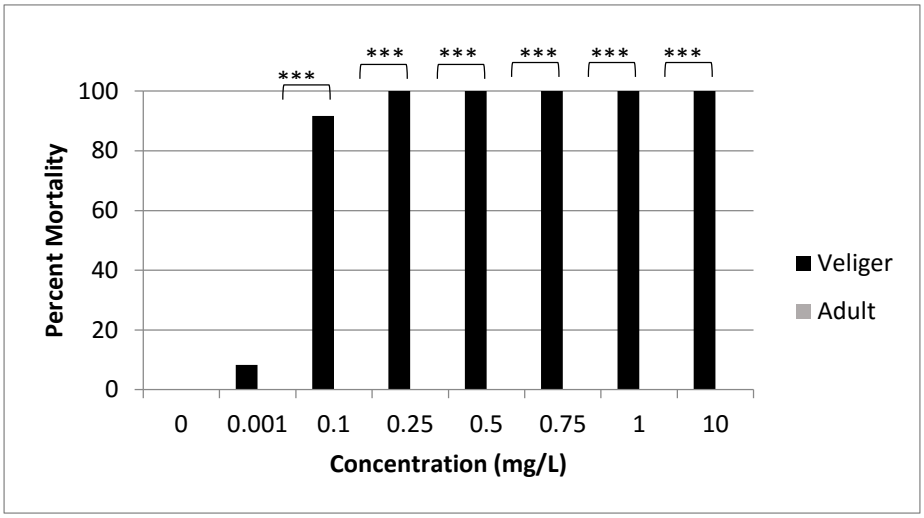


Figure 2. Percent mortality at the end of 6-day assay. Adults (n=10) and veligers (n=12) were examined daily. No adult mortality was observed.

Discussion

There are several challenges encountered while working with dreissenid veligers. Their small size makes the traditional bioassay methods used for larger organisms such as adult mussels, *Daphnia* and ceriodaphnia impractical. It proved difficult and time consuming to locate an individual veliger, smaller than 200 microns, in one of the relatively large vessels normally used for such assays. Using large vessels also necessitated excessive handling of the veliger as it had to be removed from the vial to be microscopically examined during each mortality check. The use of microtiter well plates mitigated these obstacles and allowed for expeditious microscopic examination with minimal disturbances. Veligers are delicate and care must be taken to handle themgently as to not damage their developing shells. Therefore it is necessary to avoid direct pipette contact during transfers. They are also sensitive to heat, so the well plates must not linger under the microscope light to avoid overheating. Following these precautions, zero or near-zero control mortality can easily be attained.

This novel assay was successful in examining toxicity across two dreissenid life history stages. Veligers were significantly more sensitive to Chemguard First Class than adults at every concentration

over 0.001 mg/L (chronic assays: $p<0.0001$ for 0.1, 0.25, 0.5, 0.75, 1 and 10 mg/L; acute assays: $p=0.037$ for 10 g/L, $p=0.035$ for 20 g/L, $p=0.022$ for 30g/L, and $p=0.0048$ for both 40 and 50 g/L). The low mortality in the control demonstrates that the observed effect was elicited by the toxicant and not due to handling of the test organisms.

As an early life history stage, veligers may be more sensitive to certain chemicals due to morphological and physiological differences. For example, the thick shell of the adult mussel can close to protect soft tissue from exposure for days at a time (Jenner and Janssen-Mommen 1993) but at the veliger stage the shell is developing and thin. Most aquatic larvae also have underdeveloped organ systems and homeostatic mechanisms to deal with pollutants, and have relatively large surface-area-to-volume ratios that increase exposure (Mohammed, 2013). Increased vulnerability at the larval stage is an important factor to consider when developing invasive mussel control methods, since significantly lower concentrations of molluscicide may be required to eradicate veligers compared to adults. Most chemical agents used for dreissenid mitigation have detrimental effects on non-target species and human health (Mussalli et al., 1992), and targeting veligers may help to minimize these exposures. However, relatively few toxicological studies have examined early life stage dreissenid mussels. The new assay method described here is a straightforward and inexpensive means of examining toxicity of dreissenid veligers, and could easily be adapted for the toxicological study of other species too small and delicate for conventional assay techniques.

While dreissenids have become a detrimental invasive species in North America, they may also be useful for performing toxicological assessments. Dreissenids are an exceptional test organism because they exhibit both benthic and planktonic life history stages with varying degrees of sensitivity to contaminants. Populations are not difficult to locate because their locations are well monitored and documented, and they are easy to collect in large quantities and maintain in the laboratory. Aquatic toxicology tests often use model organisms such as fish or *Daphnia* sp., and benthic organisms are

equally important to incorporate into research. The marine blue mussel (*Mytilus edulis)* has been toxicologically studied extensively; however, there is no comparable freshwater bivalve model in common use, and dreissenids can help fill that role.

CHAPTER 2: EXAMINING THE TOXICITY OF SEVERAL CLASS "A" FOAMS ON DREISSENID VELIGERS AND ADULTS

Introduction

Fire control is often a necessity to protect human health and property; however, the influx of potentially harmful substances to combat fires creates a unique ecological and environmental concern. A variety of fire suppression chemicals has been applied in the United States since the 1950s (USFS, 2001), and many of the places they are applied are often relatively pristine ecosystems inhabited by endangered and threatened species (Little and Calfee, 2000). There are two main classifications of these chemicals: fire retardant chemicals and fire suppressant foam concentrates (Backer et al., 2004) that are often mixed with water and dumped onto wildfires. Both can have negative impacts on the environments into which they are introduced, from intentional wildfire application as well as accidental spills. For example, the first fire retardants widely applied were borate salts, which are no longer used because of high phytotoxicity and soil sterilization effects (Fenton, 1959). Hardy et al. (1962) found that ammonium-containing retardants were the most effective chemicals to use for forest firefighting, specifically ammonium sulfate and diammonium phosphate. However, high fish mortality was subsequently observed in streams adjacent to treated areas, prompting investigations into toxicity to aquatic organisms (Blahm 1973). George (1970) found that such mortality from these retardants was caused by increased levels of available ammonium (NH_4) and ammonia (NH_3) in the streams, produced from the dissociation of ammonium salts from the retardant. The levels of the more toxic un-ionized form of ammonia (NH_3) can be further exacerbated with increasing stream pH (Norris and Webb, 1978).

Ammonium-based products are referred to as long-term fire retardants (LTRs), and their mechanism of action is through the conversion of coated cellulose ($C_6H_{10}O_5$) into 6C and $5H_2O$ while preventing the emission of any combustible hydrocarbons. In contrast, fire-suppressing Class A foams are considered less toxic to terrestrial ecosystems but are more toxic to aquatic species, and are primarily composed of surfactants and solvents instead of nitrogenous compounds. Foams work by cooling the fires and coating the fuels to prevent contact with oxygen.

One toxic component of foam suppressants is anionic surfactant, which can prevent organisms from obtaining oxygen through the lowering of water surface tension (McDonald et al. 1996) and slow development (Abel, 1974). McDonald (1996) showed that both the newer foams and LTRs could adversely affect aquatic ecosystems through toxic effects on invertebrates, a common food for many fish. However, foam toxicities in invertebrates can be 10-20 times higher in *Daphnia magna* and 2-5 times more in amphipod *Hyalella azteca* (McDonald et al, 1996; McDonald et al, 1997), compared to that of LTRs. In addition, to indirect effect on fish through toxicity to their food Minshall and Brock (1991) suggested that these foams can cause fish kills following spills into rivers. Researchers have also found the foams are between 10 and 258 times more toxic to fathead minnows than LTRs (Gaikowski et al 1996b), and at least 10 times more toxic to early stages of rainbow trout (Gaikowski et al, 1996a). Buhl and Hamilton (1997) compared toxicities of several retardants and foams and found the common foam Phos-Chek WD881 to be substantially more toxic to Chinook salmon juveniles than any of the three tested retardants (96 h LC_{50} of 7-13 mg/L, compared to Phos-Chek D75-F at 218–305 mg/L Fire-Trol GTS-R at 218–412 mg/L and Fire-Trol LCG-R at 685–1,195 mg/L). Most of the studies examining the toxicity of foams on aquatic organisms have focused on lethal effects, yet little is known about potential sub-lethal effects such as reproduction rates resulting from low-concentration exposure to these foams.

Routine fire suppression activities conducted during forest fires often involve the use of fire retardants and fire suppressant foams. These are often prepared at a location near the fire by mixing these

chemicals with water from natural sources such as lakes, ponds, and streams, or reservoirs for helicopter dip-tank applications. The mixture is then dropped from aircraft onto the fire. The operations can result in thousands of gallons of water obtained from one watershed to be applied in another. Such inter-watershed transfers result in the movement of organisms. These organisms may include invasive species, including plants, parasites, pathogens, and invertebrates. Although such releases often do not impact aquatic resources because they are applied on combustible substrates in terrestrial systems, occasionally misapplications and runoff result in their introduction to aquatic systems and may potentially spread aquatic invasive species. In addition to directly dumping organisms between nearby water bodies, some species such as dreissenid mussels may remain in the equipment for up to 28 days (Choi et al. 2013) and become introduced during the next firefighting event many miles away. To prevent this, it is recommended that the equipment be disinfected with either hot (>140 ^{o}F) water or chemical disinfectants (quaternary ammonium compounds and bleach).

There is a possibility that the chemicals used in retardants, foams and water enhancers are toxic to these invasive species, yet little is known about the sensitivity of the broad range of invasive organisms to these products. If they are toxic at the concentrations and durations used in aerial firefighting, this information can be valuable for aquatic invasive species (AIS) risk assessment. No research to date has examined effects of these chemicals on *Dreissena* species which continue their westward invasion of North American watersheds.

Importance of **Dreissena Spp.**

Dreissena polymorpha (zebra mussel, or ZM) and *Dreissena bugensis* (quagga mussel, or QM) are believed to have been introduced in the 1980s via the ballast water of transoceanic ships into Lake St Clair, located between Windsor and Detroit. Since their introduction, these species have significantly changed the freshwater ecosystems of the Great Lakes region and have continued to spread to the Pacific coast. They have been implicated in the crash of native unionid populations because of physical

infestation on the hosts' shells (Schloesser et al., 1998; Gillis and Mackie, 1994). In addition to this direct effect, they have significant ramifications that extend throughout the food web. In the Great Lakes, the phenomena of sparser and smaller lake whitefish (*Coregonus clupeaformis*) is blamed on the dreissenids, and these mussels are believed to have caused the important prey amphipod *Diporeia* to decrease from 5365 to 329 m^{-2} between 1995 and 2005 (Nalepa et al., 2009). This decline resulted from both competition with amphipods for food, and the excretion of pseudofeces with rejected food particles that are toxic to amphipods (Pothoven et al, 2001; Dermott et al., 2005). In addition, the voracious plankton-filtering ability of these dreissenids contributed to the loss of the planktivorous alewife (*Alosa pseudoharengus*), ultimately resulting in a decline of commercially valuable salmonids (Pothoven and Madenjian, 2008). Dreissenids can completely change the chemistry of a water body (Makarewicz et al., 2000), and have been blamed for an increase in frequency and extent of toxic algae through the effect of selective filtration of more palatable species (Vanderploeg et al. 2001). In addition to the severe environmental and ecological damage they inflict, they have caused considerable economic impacts. They infest water intake pipes, water filtration facilities and electrical plants; the resulting damage and control efforts cost an estimated $1 billion annually (Pimental et al 2005).

The spread of *Dreissena* mussels is largely attributed to human activities including ballast water introduction and overland transport on recreational boats, as well as natural movement via waterways. Once *Dreissena* mussels establish in a lake, spread is facilitated by outflowing streams carry free-swimming *Dreissena* larvae (veligers) downstream to uninvaded lakes. For example, mussels in Lake Michigan produced veligers that drifted into the Illinois and Mississippi rivers and those from Lakes St. Clair, Erie, and Ontario invaded the Detroit, Niagara, and St. Lawrence Rivers, respectively (Griffiths et al., 1991). An additional mechanism of spread, particularly in the western United States, may include aerial transport of water from airlifts during wildfire firefighting activities (USDA 2014).

In order to obtain a comprehensive understanding as to how pollutants such as firefighting foams

affect dreissenid populations, it is important to determine acute toxicity and sublethal reproductive effects on adults as well as the toxicity to veligers. Few studies to date have examined any toxicant effects on early life stage dreissenids. In these experiments, I investigated adult and veliger stage mortality at acute and chronic exposures, as well as adult spawning effects for three firefighting foam concentrates.

Methods

I tested three commonly used firefighting foam concentrates (Chemguard First Class, or CG; Phoschek First Response, or PFR; and Phoschek WD881, or PWD) on adult and larval stage dreissenids. Lethality was investigated for both acute high concentrations, such as those mixed in aircraft tanks or immediately following an accidental concentrate spill, as well as chronic low concentrations that may persist in small waterbodies after contamination. In addition to examining the lethality endpoint, spawning intensity of adults was also measured.

Firefighting Foams

Three class A firefighting foams were provided by the United States Geological Survey (USGS). Chemguard First Class FC (Chemguard Inc.) consists of 11.5% diethylene glycol monobutyl ether, and a proprietary blend of alkyl sulfates, ethoxylates, amphoterics, solvents and corrosion inhibitors (Chemguard Inc, 2011), Phoschek WD881 contains 60-80% alpha-olefin sulfonate solution, 10-30% 2,4-pentanediol, 2-methyl, 1-5% lauryl alcohol, and 1-5% d-limonene (ICL Performance Products LP, 2011). Phoschek First Response contains 45-75% alpha-olefin sulfonate solution, 7- 22% 2,4-pentanediol, 2-methyl , 1-4% d-limonene, 1-4% lauryl alcohol, and 22-27% water (ICL Performance Products LP, 2011). The 96-hr LD_{50} for rainbow trout has been established for Chemguard as 130 mg/L, PS WD881 as 10.8 mg/L, and PS-FR as 16.8 mg/L.

Veliger collection and isolation

Dreissenid veligers were collected from the Detroit River off a pier on Belle Isle (42.358 N, 82.978 W). Collection was performed using a 63 μm Wisconsin plankton net tossed perpendicular from

shore and allowed to drift for five minutes before retrieval, then rinsed into 1000 mL Corning® PYREX® bottles. This was repeated at 15 times to ensure a suitable quantity of veligers. Three liters of river water were also collected and for use in controls and dilutions. Physiochemical parameters of temperature and dissolved oxygen, conductivity, and pH readings were taken on site using YSI ProODO, Oakton CON 11, and Mettler Todedo EL2 meters. The samples were brought to the laboratory on ice and rinsed through a series of 80 and 210 μm nylon meshes to isolate veliger-sized particles from larger organisms and detritus, then gently rinsed into a 100 mL beaker with clean filtered river water. The veligers were then allowed to acclimate at 23 ºC. for 24 hours in an environmental chamber.

Acute veliger assays

The plankton samples were placed in a 60x15 mm glass Petri dish and examined at 20x magnification under cross-polarized light to discern veligers from other particles. They were gently isolated by placing the tip of a 10 μL Eppendorf micropipette adjacent to a random individual and slowly drawing it inside, taking care to never come into direct physical contact.

. Solutions were prepared through gentle swirling of the beaker to avoid excessive foaming. For the acute veliger assay trial, five concentrations (10, 20, 30, 40, and 50 g/L diluted with filtered river water) of each formula were separately tested against controls with 12 replicates each (n=12), for a total of 24 wells filled per assay. Each acute concentration was tested individually against a control. Every treatment well was filled with 300 μL of the dilution, and 300 μL of filtered river water added to the controls. Each was then sequentially checked for approximately 10 seconds for 2.5 hours or until 100% mortality occurred, whichever came first. Individuals were determined to be dead when all swimming and ciliary movement ceased. The plates were removed from the microscope whenever not under observation to avoid heating the organisms.

Chronic veliger assays

Chronic assays followed the same procedure as acute, with a few modifications. All treatment concentrations were tested simultaneously in the same plate. For the chronic assay seven concentrations (0.001, 0.1, 0.25, 0.5, 0.75, 1, and 10 mg/L with food source *Chlamydomonas reinhardtii*) were tested plus control (n=12) for a total of 96 wells filled per assay. Individual veligers were checked daily for 6 days which took about an hour at 30 seconds per well. Solutions were refreshed on day three.

Acute adult assays

Ten adults (n=10) for each treatment (0, 10, 20, 30, 40, and 50 g/L) were placed in individual 50 mL vials with 30 mL of treatment and randomized, for a total of 60 vials for each of the three chemicals tested. Jars were gently stirred during checks to ensure proper aeration. Vials were incubated at 20 °C and checked in 10 to 15 minute intervals from the time of exposure. Death was determined through lack of response to stimulus and shell remaining open. Each individual took about 10 seconds to check for a total of 10 minutes to observe all 60 vials per chemical.

Adult Chronic Assay

The chronic assay was performed in a similar manner as the acute assay, using lower concentrations (0, 0.001, 0.1, 0.25, 0.5, 0.75, 1, and 10 mg/L with food source *Chlamydomonas reinhardtii*) for a total of 80 vials for each of the three chemicals. Each was stirred and checked daily for mortality, with solutions refreshed on day three. In addition to examining the lethality endpoint, adults exposed to the sublethal concentrations were chemically induced to spawn with a 10^{-3} M serotonin (5-hydroxytryptamine, 5-HT) solution on day 6 following methods described by Ram et. al, (1993). One modification was made to the Ram et al. (1993) method to quantify male spawning intensity was to use a light spectrophotometer at 600 nm to assign absorbance ranges to each cloudiness score value of 0-4 (Table 2). This was done in an attempt reduce visual subjectivity and improve repeatability. Female spawning was characterized by quantifying dreissenid egg production following exposure to serotonin,

as observed in a 25 mm diameter vial under 10x power inverted microscope. Ranges were slightly increased compared to those described in Ram et al. (1993) to account for exceptionally high fecundity of test subjects (Table 3).

Table 2. Absorbance of serotonin-induced sperm clouds measured at 600 nm with a spectrophotometer, and the corresponding scores of 0-4.

Absorbance at 600nm	Score
>0.050	0
0.050-0.150	1
0.151-0.250	2
0.251-0.350	3
>0.351	4

Table 3. Scores assigned dreissenid egg production following exposure to serotonin, as observed in a 25 mm diameter vial under 10x power inverted microscope. Slightly modified from Ram et al. (1993) to account for exceptionally high fecundity.

Quantity of eggs	Score
0	0
Eggs covering < 1/3 the vial bottom	1
Eggs covering 2/3 of the bottom the vial bottom	2
Eggs covering between 2/3 and 100% of the vial bottom	3
More than one layer of eggs	4

Results

Acute Assays

A two-way ANOVA was conducted on the influence of two factors (three treatment chemicals and five concentrations) on mean times to death for veligers (figure 2A). The assumption of normality and was tested and met with a Q-Q plot. All effects were statistically significant at the 0.05 significance level. The main effect for chemical type yielded an F ratio of $F(2,165) = 128.3$, $p < 0.001$, indicating a

significant difference between CG (M = 13.1, SD = 8.3), PFR (M = 76.1, SD = 30.2), and PWD (M = 55.9m, SD=28.6) The main effect for concentration was also significant [$F(4, 165) = 9.7$, $p < 0.001$], with no interaction effects between the two factors ($p = 0.06$). Tukey post hoc tests showed CG to cause veliger mortality significantly faster than both PFR ($p < 0.0001$ at 10, 20, 30, and 40 g/L; $p = 0.0014$ at 50 g/L) and PWD ($p < 0.0001$ at 10, 20, and 30 g/L; $p = 0.001$ at 40 g/L; $p = 0.02$ at 50 g/L). PFR and PWD did not differ in acute veliger assays at any concentration except 40 g/L ($p = 0.0003$).

Survival times for adults did not differ significantly between CG and PWD at any concentration (figure 2B), and CG was only significantly faster than PFR at 30 g/L ($p = 0.009$). PFR caused faster mortality than PWD at only one concentration tested as well ($p = 0.01$ at 20 g/L).

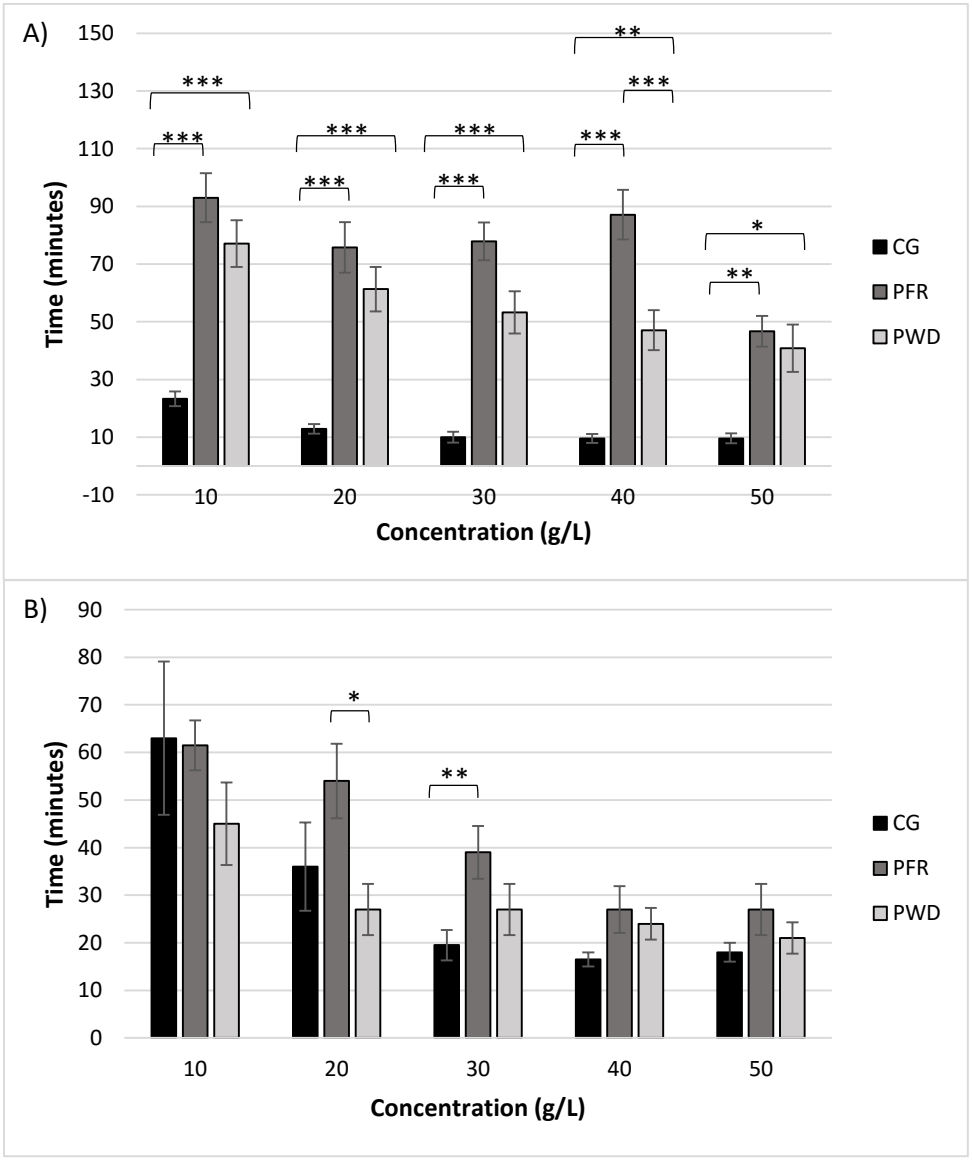


Figure 3. A) Veliger (n=12) and B) adult (n=10) average time to death for acute assays of Chemguard(CG), Phoschek First Response (PFR) and Phoschek WD881 (PWD). Error bars show standard error. Asterisk brackets indicate significance: *p<0.05, **p<0.01, ***p<0.001

Chronic Assays

Chronic assays were analyzed with regard to proportion dead at the end of the 6-day assay using Fisher's exact test. CG caused higher proportions of veliger mortality at the end of 6-day chronic assays than PFR for all concentrations over 0.001 mg/L (p = 0.0001 at 0.1 mg/L; p = 0.00001 at 0.25 mg/L, p = 0.0001 at 0.5 mg/L; p = 0.0001 at 0.75 mg/L; p = 0.0003 at 1 mg/L and p = 0.005 at 10 mg/L) and PWD (p = 0.0006 at 0.10 mg/L, p = 0.0003 at 0.25 mg/L; p = 0.0003 at 0.50 mg/L; p = 0.001 at 0.75 mg/L; p = 0.005 at 1 mg/L, and p = 0.013 at 10 mg/L). No significant difference between PFR and PWD was observed at any chronic concentration. Mortality of adults was insignificant for all treatments. Spawning intensity was analyzed with a nonparametric Kruskal-Wallis test which showed no significant effects on serotonin-induced spawning intensity.

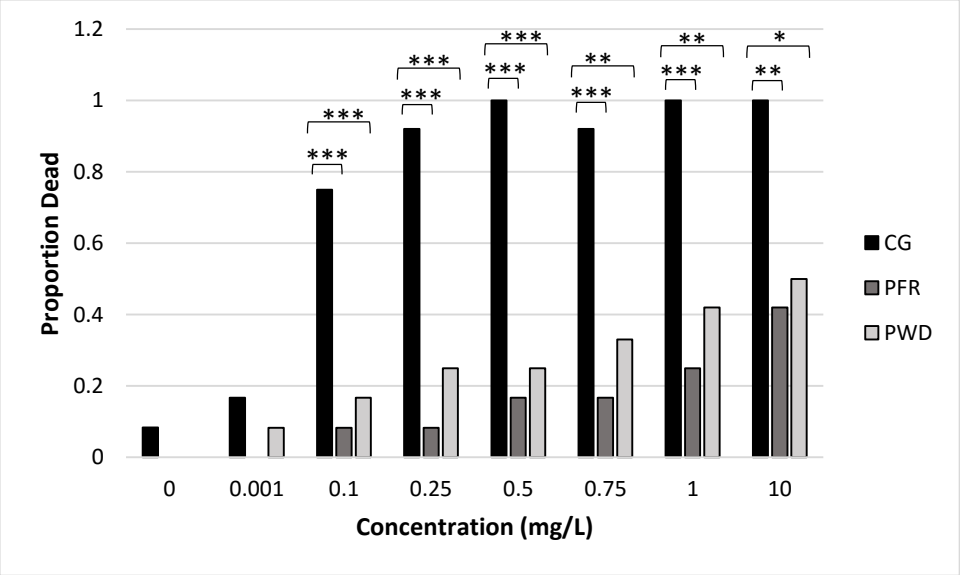


Figure 4. Veliger (n=12) percent mortality following 6-day chronic exposures of Chemguard (CG), Phoschek First Response (PFR) and Phoschek WD881 (PWD). *p<0.05, **p<0.01, ***p<0.00.1

Discussion

More than 73,000 wildfires occur in the Unites States in any given year, and chemical suppression of these fires costs an average of $917,000,000 annually (USDA, 2011). Some ecosystems depend on these naturally occurring events; however an estimated 80% of all wildfires are caused by human activity (USFS, 2001). These events can have devastating impacts on property, as well as environmental and human health. When managing forest fires, care must be taken to choose the appropriate formula for the ecosystem affected and water bodies avoided as much as possible. Foams are applied at concentrations up to 1% are diluted by streams but may persist at levels toxic to aquatic organisms in small lentic water bodies, particularly for invertebrates that spend time on the water surface (Poulton, 1994). For fires on National Forest System lands, the United States Forest Service and NOAA Fisheries coordinate to provide current data for "avoidance area" maps on which fire retardant and foam application is strongly discouraged. These maps include habitats of threatened, endangered, and sensitive species. In addition, a 300-foot buffer zone is required on both sides of waterways (USDA, 2011b). Retardants containing phosphorus are not likely to affect water bodies if applied farther away than 9 feet from the water's edge due to soil adherence (Norris et al., 1978), but little data exists regarding the ability of firefighting foams to migrate underground. Fire suppressing foams may have the benefit of being less toxic to terrestrial ecosystems than the long-term retardants, but have much worse impact on aquatic organisms. Highly soluble chemicals such as these have a low K_{ow}, causing them to partition into the water column. While not bioaccumulative, the surfactants in these foams can effectively suffocate aquatic species. The use of dreissenid mussels as model subjects enabled an examination of responses of both sensitive planktonic organisms that swim in the pelagic zone, as well as the heartier adults who dwell on the benthos.

In these experiments, three firefighting foam formulations were evaluated for toxicity on adult and veliger life stages of dreissenid mussels utilizing a novel larval assay technique. As hypothesized, the veliger stage experienced overall higher mortality than adults at both chronic and acute concentrations

of these chemicals. Chemguard was shown to cause higher mortality at acute concentrations than either Phoschek WD881 or Phoschek First Response for veligers, but almost no significant difference occurred at these concentrations for adult mussels. Adult mussels for the chronic assays exhibited very low mortality, and no effect was seen on spawning intensity following the 6-day exposures. These findings highlight the importance of developing a complete analysis of not only the toxicological effects on adult populations exposed to fire suppression applications, but larval effects as well.

Climate change is expected to increase the severity and frequency of forest fires in certain regions (Backlund et al, 2008). This may in turn contribute to a positive feedback loop for climate change, by shifting the balance of stored biomass carbon to the atmosphere and lead to more wildfires. In addition, increasing temperature has significant effects on the toxicities of contaminants. Warmer temperatures are often correlated with heightened aquatic toxicity of many toxicants due to factors such as increased uptake, altered metabolism, and the stress of lowered dissolved oxygen (Noyes et al, 2009). Thus, understanding the ecological impact of chemical fire suppression practices will likely become increasingly important as climate change progresses.

While fire suppressant foams are considered an improvement from the traditional retardant formulations, their significantly higher toxicity towards aquatic species, especially in early life stages, is an important factor to consider when engaging in firefighting operations. This information may be useful in risk assessment of spreading invasive dreissenid veligers between watersheds, especially as these species continue to invade westward into areas commonly affected by wildfires. Foams are dispersed at concentrations up to 1%, and that is sufficient for Chemguard to kill all veligers in our experiments in under 30 minutes. However, aircraft will often pick up water, mix the concentrate, and aerially apply it in half of that time. Comprehensive analysis on the toxicity of firefighting chemicals is not only useful for assessing potential effects on the spread of invasive species, but also for understanding the broader ecological impact on the aquatic ecosystems that can be affected in wildfire management.

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ABSTRACT

DEVELOPMENT OF A DREISSENA BIOASSAY TO ASSESS THE TOXICITY OF CONTAMINANTS ACROSS TWO LIFE-HISTORY STAGES

by

CAROLINE ADDIS

Advisor: Dr. Donna R. Kashian

Major: Biological Sciences

Degree: Master of Science

Dreissenid mussels (*Dreissena polymorpha* and *D. bugensis*) have rapidly become widespread and ubiquitous in North America since their introduction into the Great Lakes in the 1980s. The resulting environmental and economic impacts of their invasion have been extensive, negatively affecting biodiversity and costing millions of dollars in control efforts and damage to power generation and water treatment facilities. Although dreissenids are often associated with negative impacts, they may present a practical tool for toxicology studies. The typically sessile behavior of the benthic adults coupled with the planktonic nature of the veligers allow for a more complete evaluation of water quality than previous single species toxicity tests that focus on a single life history stage. Both Dreissenid veliger and adults are relatively easy to collect and maintain in the laboratory, making them useful test organisms for toxicological studies. Current bioassays used to evaluate contaminants typically utilize only the adult stage of a single species. I developed novel acute and chronic bioassays for the veliger stage of dreissenid mussels to evaluate several firefighting foam formulations commonly applied to structural and forest fires: Chemguard First Class, Phoschek First Response, and Phoschek WD881. Toxicity assays using veligers were conducted in 96-well microtiter plates and compared with adults exposed to the same concentrations in 50 mL vials. Adult spawning intensity was also quantified as a sublethal measure of

toxicity. Results show veliger stage dreissenids to be significantly more sensitive to these chemicals than their adult counterparts ($p<0.01$). Each firefighting foam formulation had a significantly different effect on survival time for acute veliger trials ($p<0.01$). Chemguard caused acute veliger mortality significantly faster than either of the other two chemicals on average ($p<0.05$), while overall mortality for Phoschek WD881 and Phoschek First Response did not differ significantly ($p>0.05$). Chronic veliger assays also showed Chemguard to cause the fastest mortality of the three formulas at ($p<0.05$). Chemguard mortality was higher in acute adult assays compared to Phoschek First Response ($p<0.05$). Chronic adult exposure of these formulas had no significant effect on mortality or spawning intensity.

AUTOBIOGRAPHICAL STATEMENT

I developed an appreciation for nature and earth sciences at an early age. One could usually find me exploring streams in our nearby forest or gathering interesting stones or pressed wildflowers for my collections. Although I started postsecondary education interested in the digital arts, I found myself engrossed in a geology class and changed paths to earn a B.S. in Environmental Science before deciding to pursue a graduate degree in biology. During the course of my education I became a nontraditional student and expanded my family, and became involved with volunteering and community work in Detroit. Along with some other amazing ecology graduate students, I also helped develop a non-profit group that cleaned up and replanted native trees and shrubs in many abandoned blighted lots around the city. It has been a long road, but my two boys keep me going. Some of the best parts of being a scientist are being able to answer virtually all the "whys" they throw at me, and seeing them get excited about all the wonders of the universe. I'm looking forward to beginning this new chapter of my life and career, and hope to continue to work towards the betterment of the community and environment.