EFFECTS OF COPPER AND TEMPERATURE ON THE LIFE STAGES OF THE INVASIVE ZEBRA MUSSEL

By

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INTRODUCTION

The zebra mussel (*Dreissena polymorpha*) and the closely related quagga mussel (*Dreissena rostriformis bugensis*) are two of the most invasive organisms to enter freshwater systems in North America. These freshwater bivalves have successfully made their way into numerous lakes and rivers across the United States after entering via the Great Lakes region in the 1980s (Lund et al. 2018). Their distribution expands as far south as New Orleans and in the case of quagga mussels as far west as Lake Mead by 2007 (Lake-Thompson 2019).

Zebra mussels spread throughout the United States by transport along natural waterways such as rivers, streams, and human-made reservoirs, and by commercial or recreational boat traffic in ballast water attached to improperly cleaned boats. Zebra mussels exhibit an r-selected reproductive strategy consisting of external fertilization and planktonic larval stages (veligers) that allow them to be spread rapidly (McMahon 2002). Their high rates of reproduction, high survival rate, and dispersal have allowed zebra mussels to spread over a large portion of North American waterways (Ackerman 1995, Ram & McMahon 1996).

The life cycle of zebra mussels begins with the external fertilization of gametes that are released into the water column by dioecious individuals (Figure 1). After fertilization, the embryos progress into the larval stage. The embryo develops into a free-swimming, planktonic veliger and develops a velum and small D-shaped shell. A velum is a ciliated organ for feeding and locomotion and is the characteristic feature of the veliger stage (Ackerman 1995). The veliger grows to approximately 280 μm in diameter before it goes through metamorphosis into the juvenile stage in which the velum is lost and functionally replaced by gills for respiration and feeding. The D-shaped shell is also lost and replaced with 2 adult shells attached by a hinge (Ackerman 1995). Development of these morphological traits signals sexual maturity. In order for zebra mussel control to be effective, all three life stages should be targeted, especially the gametes and larvae that are the most sensitive to chemical treatments.

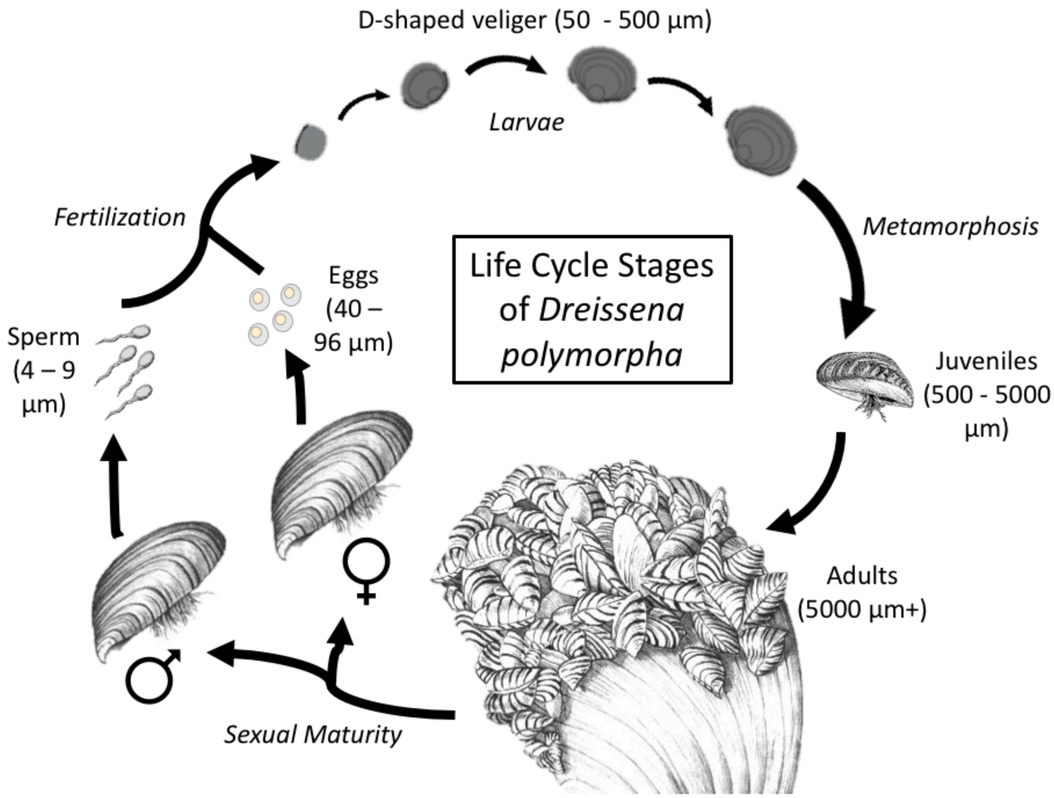

Figure 1. The life history stages of *Dreissena polymorpha* with key developmental transitions in italics.

Adult zebra mussels are one of the most environmentally harmful aquatic invasive species and have significant economic impacts. The mussels clog water intake pipes and disrupt equipment that is used for daily operations of electricity-generating plants and water treatment facilities (Watters et al. 2013). After a zebra mussel population becomes established in a lake, there are significant capital costs and yearly operational maintenance costs in order to prevent further impairment of the functionality of the lake including interference with use of water for power plants and domestic water supply. Mussel infestations also impact industries such as commercial fisheries (Lake-Thompson 2019) The United States Army Corps of Engineers estimates that the economic impact of zebra and quagga mussels in North America exceeds $1 billion a year (US Army Corps of Engineers 2002). This cost will only increase with further spread of zebra and quagga mussels to uninfested bodies of water (Turner et al 2011).

Zebra mussels also alter the ecosystem of a waterbody by their efficient filter feeding capabilities. Their large populations can filter large volumes of water rapidly. For example, in Lake Michigan, it is estimated that the current mussel population can filter the entirety of the lake volume in four to six days (Qualls et al. 2007). Zebra mussels remove suspended particles such as phytoplankton, bacterioplankton, and detritus, increasing water clarity, which has cascading effects on all components of the lakes food web and water chemistry (Claudi & Mackie 1994). Zebra mussel infestation dramatically reduces the availability of food and resources for native filter feeding organisms, many of which are endangered. There have been massive reductions in numbers of native mussels in range and populations, including endangered mussels like the unionid, following invasion by zebra mussels (Ricciardi et al 1998, Schloesser & Nalepa 1994).

Given the detrimental impacts of dreissenid mussels, effective treatment methods are essential. Chemicals are commonly used for controlling zebra and quagga mussel biofouling. The most popular and least expensive option used in closed systems is chlorine, which kills adult mussels and prevents the larvae from settling onto substrates (Van Benschoten 1995). Adult zebra mussels can sense noxious chemicals, and they respond by closing their shells and ceasing filter feeding in order to avoid the potential threat (Van Benschoten 1995). As a result, the established adult forms of zebra mussels is the most difficult life stage to eradicate.

EarthTec QZ is a copper compound that is an effective chemical control method for zebra and quagga mussels (Watters et al. 2013, Claudi et al. 2014, Lund et al. 2018, Barbour et al. 2018, Hammond & Ferris 2019, Lake-Thompson & Hoffman 2019). The biologically active ingredient in EarthTec is the cupric ion form of copper, Cu^{2+}, and is made by combining copper sulfate pentahydrate with Earth Science Laboratory's base acid. Earthtec is commonly used in lakes, ponds, reservoirs, and in municipal wastewater and drinking water systems as an algicide and bacteriacide. Earthtec is registered with the US EPA as a drinking water additive. Zebra mussels do not detect EarthTec as a threat and readily ingest it. Earthtec is an effective molluscicide at low doses that are safe for the surrounding areas (Watters et al. 2013). Earthtec has a unique formula that allows the copper to remain in solution longer which increases its bioavailability (Watters et al. 2013). Low doses of Earthtec can have powerful effects on invasive mussel populations and when used strategically as a management tool can reduce mussel transfer between waterways.

Because copper toxicity on mussels is known to increase with increasing water temperature (Rao and Khan 2000, Mersch et al 1993), the amount of EarthTec needed to effectively treat a population can vary based on the environment or the weather at the time of treatment. Increased copper concentrations in the water can increase the respiration rate, decrease the oxygen consumption rate, and increase the rate of metabolism by zebra mussels in water ranging from 20 to 25°C compared to water at 15°C (Rao & Khan 2000). The increased toxicity of copper with temperature may be the result of changes to physiological processes such as respiratory and circulatory rates in order to accommodate the increased rate of cellular metabolism (Cairns et al 1971, Rao & Khan 2000). This increase in metabolism and respiration at high water temperatures leads to increased inflow of water to obtain more oxygen, which ultimately results in increased uptake of dissolved copper (Rao & Khan 2000).

Zebra mussel larvae are more susceptible to chemical treatments than adults (Sprung 1993, Stoeckel & Garton 1993, Mackie & Kilgour 1994, Van Benschoten et al 1993). Relative tolerances are chemical-specific and have not been determined for EarthTec QZ. Because of this, zebra mussel larvae are a viable target for chemical control. Chemical treatment of adult zebra mussels can become expensive, which could become an economic barrier for treatment. One remedy is to manage the more sensitive life-stages, like the larval veligers and the gametes, in order to prevent adults from establishing. This would minimize release of excess chemicals entering the environment, the risk to other species, and the cost of the chemical needed.

The objective of this study was to determine the effectiveness of EarthTec QZ to treat the various life stages of zebra mussels (gametes, veligers, and juveniles/adults). Additionally, the relationship between temperature and EarthTec QZ effectiveness was examined. These findings will aid the design of the most efficient and cost-effective treatment strategies for zebra mussel control and prevention using EarthTec QZ for different stages of the zebra mussel lifecycle.

METHODS

We examined the effects of EarthTec QZ on various life stages of zebra mussels at different temperatures and concentrations of EarthTec QZ. The life stages that were examined included the gametal, larval, juvenile and adult stages.

Collection

The adult mussels used for this study were collected from Lake Bridgeport, Texas. Clusters of adult mussels were removed from various hard surfaces including rocks, equipment, and docks. This was done by cutting their byssal threads in order to minimize disturbance and damage to the mussels. The mussels were stored in coolers filled with lake water, transported to the laboratory, and placed in aerated aquaria maintained at 12°C. Mussels were fed Shellfish Diet 1800 (Reed Mariculture). Mussels were transferred from the aquaria and acclimated to reach the appropriate temperature prior to each experiment. To prevent unintentional release, all water and equipment that came into contact with mussels were chlorinated following approved protocols prior to disposal.

The veligers used for this study were also collected from Lake Bridgeport, Texas using a 60-µm mesh plankton net. The veligers were transported back to the laboratory following the same procedure as the adults. The veligers were identified using cross-polarized light under a dissecting scope and transferred by pipette to acrylic dishes for each experiment. The veligers were used for experiments within 24 hours of collection.

EarthTec QZ

EarthTec QZ has a primary stock concentration of 59,400 mg/L and was diluted using artificial pond water (Dietz et al 1995) to achieve the desired copper concentrations. Solutions were prepared as needed throughout each experiment.

<u>Gametal Stage Experiments</u>

In vitro Spawning Induction

Thirty adult zebra mussels measuring at least 8 mm in length were induced to spawn to retrieve eggs and sperm for experiments following standard lab protocols (Misamore et al. 1994). The adult mussels were isolated in individual vol cups from the holding tanks and acclimated for 24 hours at room temperature. Individual mussels were placed in glass test tubes and induced to spawn via exposure to a 1 mM serotonin in artificial pond water for 20 to 30 minutes (Ram et al. 1993). The animals were then removed from the serotonin, rinsed with distilled water, and placed into clean artificial pond water. Male mussels were typically spawned within 20 minutes and females spawned after 45 minutes. Sperm and eggs from were removed for use in each experiment. Only gametes obtained from the clean pond water with no serotonin present were used for the experiments. Experiments were conducted at 20 $\pm$ 2°C (room temperature).

Fertilization Trials

Following spawning, sperm (0.5 mL) was collected from an adult male and was added to 0.5 mL of 2x EarthTec in pond water. The final concentrations of EarthTec within the sperm mixture were 0 ppm, 0.5 ppm, 1.0 ppm, and 2.0 ppm. The sperm remained in the Earthtec solution for 10 minutes prior to being used for fertilization. The sperm was then mixed with 0.5 mL of eggs from 1 female in a 5-mL beakers.

At various timepoints (5, 20, 90 minutes) following insemination, 0.25 mL subsamples of the egg + sperm mixtures were collected and fixed using a 1:1 ratio of 4% paraformaldehyde in mussel buffer (Figure 2, Misamore et al 1994). Five replicate fertilizations involving different males and females were performed.

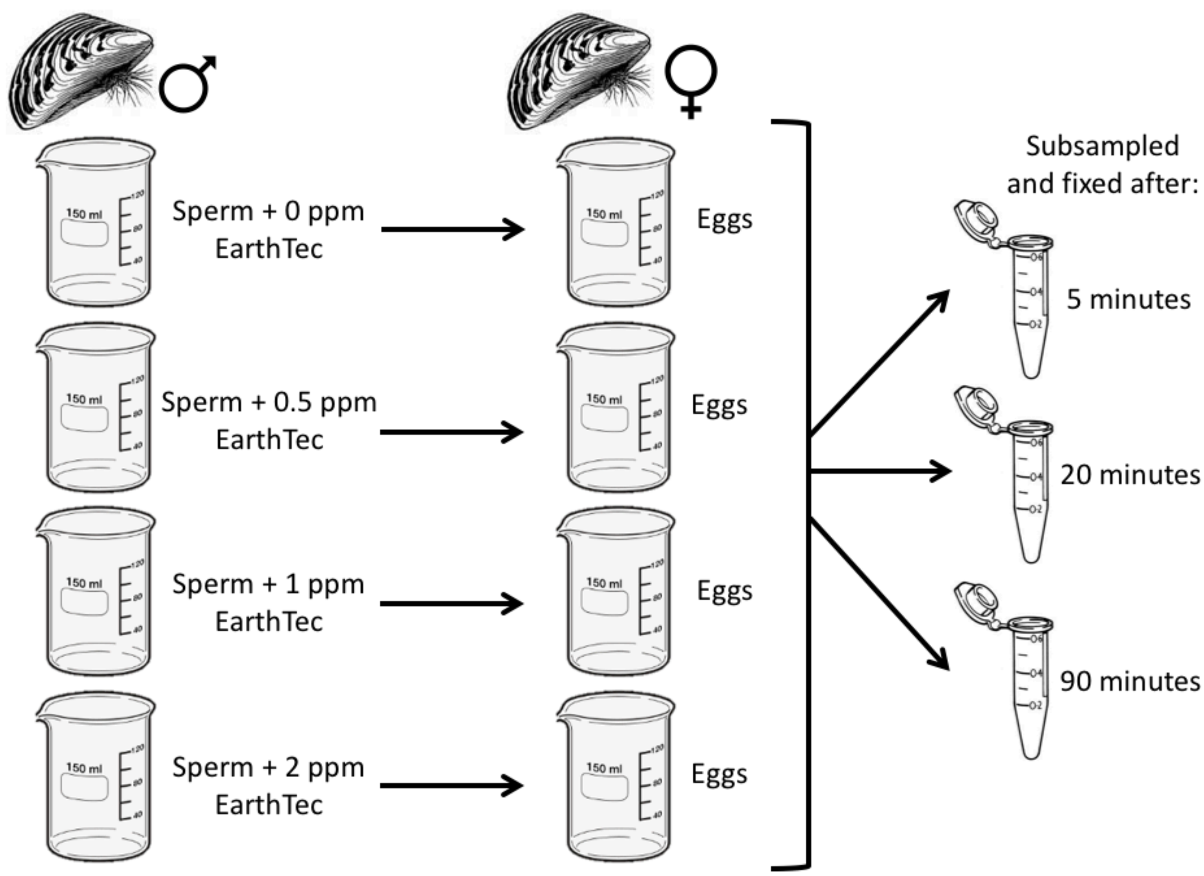

Figure 2. Subsampling process of eggs and sperm with various doses of EarthTec for fertilization experiments.

To determine the effect of EarthTec on sperm binding, 5-minute postinsemination, samples were examined under 10X and 40X using a Zeiss Axioscope with DIC optics. Using an equatorial focus, the number of bound sperm was determined for each egg (Figure 3). A total number of 50 eggs per trial were counted.

Egg Activation

Prior to fertilization and egg activation, zebra mussel eggs are arrested at metaphase I. Following egg activation by sperm entry, meiosis resumes leading to the formation of the first polar body, forming at 15 minutes post-insemination (Misamore et al 1994). To determine if treated sperm were able to activate eggs, samples from the 20-minute post-insemination time were examined to determine if polar body formation had occurred. A total of 50 eggs per trial were counted for analysis.

Embryonic Cleavage

Zebra mussel zygotes typically cleave at 60 minutes post-insemination (Misamore et al 1994). To determine if treated gametes were able to undergo 1st cleavage, eggs from the 90-minute post-insemination timepoint were scored for cell cleavage (Figure 3). A total of 50 eggs per trial were counted for analysis.

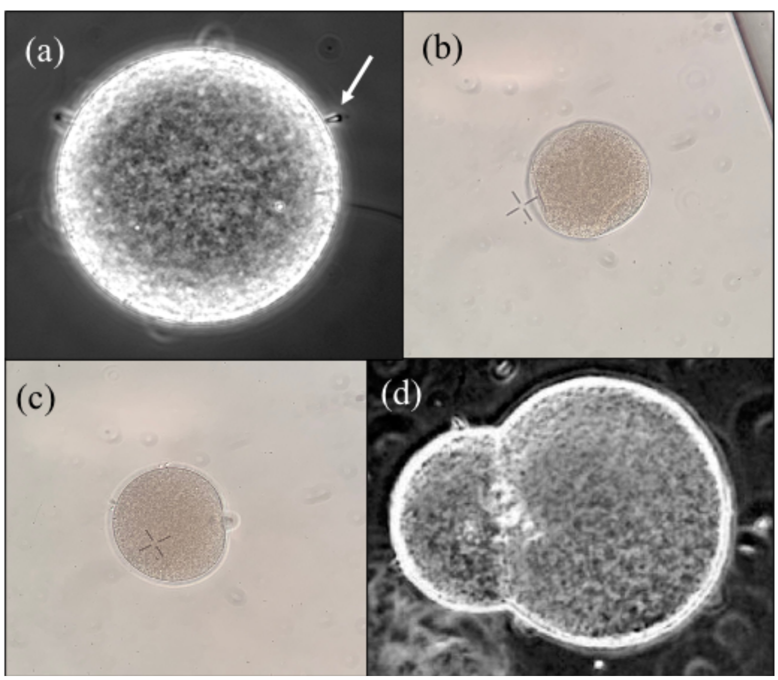

Figure 3. Example of zebra mussel egg (a) with sperm attached, (b) without polar body formation, (c) with first polar body formation, and (d) undergoing first cleavage.

Developmental Progression

In order to determine egg cleavage progression, fifty eggs from each treatment concentration were classified as either a 1 (a single egg), a 2 (single egg with visible polar body formation), or a 3 (egg exhibiting cleavage).

<u>*Larval Stage Experiments*</u>

Veliger Mortality Experiments

Ten zebra mussel veligers were pipetted into each well of a 6-well acrylic multi-well plate, each containing 1 mL of artificial pond water. A proportionate amount of EarthTec QZ was added to each well to achieve the appropriate experimental concentrations. The concentrations of EarthTec used were: 0 ppm, 0.5 ppm (0.03 ppm Cu^{2+}), 1.0 ppm (0.06 ppm Cu^{2+}), 1.5 ppm (0.09 ppm Cu^{2+}), 2 ppm (0.12 ppm Cu^{2+}), 3 ppm (0.18 ppm Cu^{2+}), 10 ppm (0.60 ppm Cu^{2+}), 25 ppm (1.5 ppm Cu^{2+}), and 50 ppm (3.00 ppm Cu^{2+}). The veligers were held in the EarthTec QZ solutions for 24 hours at 20 $\pm$ 2°C (room temperature).

Mortality was determined for each veliger after 24 hours of exposure to the EarthTec QZ. Veliger mortality was based on the absence of movement of the velum cilia or other internal organs.

<u>*Adult and Juvenile Stage Experiments*</u>

Adult and Juvenile Mortality by Temperature

For each treatment, five beakers containing 10 adult or juvenile mussels each were monitored for mussel survival in the presence of EarthTec (Figure 4). Mussels were exposed to three concentrations of EarthTec (0.5, 1, and 2 ppm) or only artificial pond water, used as a control, at five temperatures (10, 15, 20, 25, 30 °C). These temperatures and EarthTec concentrations are expected ranges for treating waters in north Texas.

Temperatures were maintained by placing glass beakers in water baths maintained at the appropriate temperature. The water baths consisted of 30-gallon coolers filled approximately half full. For cooler temperatures, the cooler was placed in a 8°C cold room and heated to the desired temperatures (10, 15°C) using Hygger Titanium Digital Aquarium Heaters. Other water temperature trials were placed in an environmental chamber at 20°C and heated to a desired temperature as needed with Hygger heaters. All water baths had Sun Microsystem submersible powerhead pumps to circulate the water to ensure uniform temperature. Temperatures were monitored using Inkbird Temperature data loggers.

Each beaker was aerated with an airstone. All adult mussels were acclimated overnight to reach the appropriate temperature before being added to the beakers containing EarthTec. Mussels were fed and water was changed weekly. Existing water in beakers was removed and replaced with new water with appropriate concentration of EarthTec.

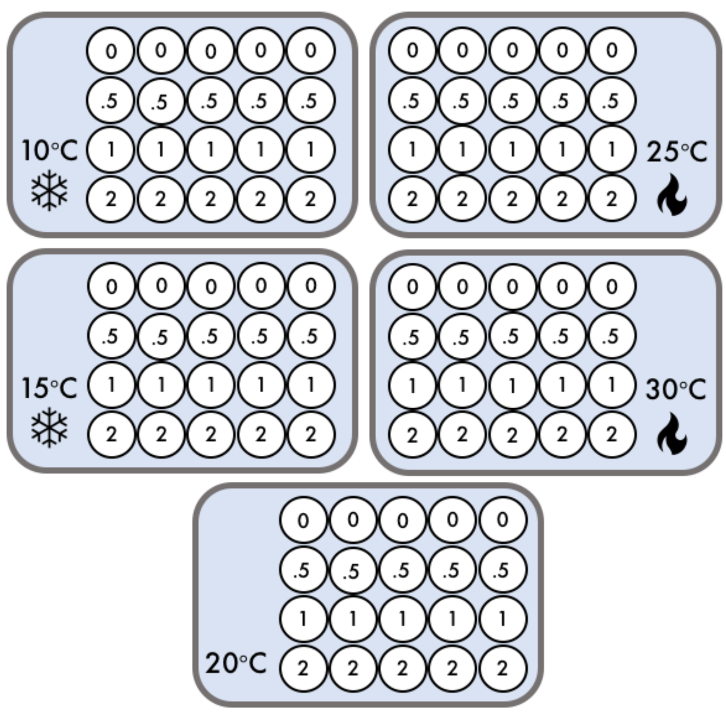

Figure 4. Experimental design for the interaction between EarthTec concentration and temperature. Circles represent beakers of mussels with the EarthTec concentration indicated by number (0 = control, .5 = 0.5 ppm, 1 = 1 ppm, and 2 = 2 ppm). Each jar contained 10 mussels.

Mussel mortality was determined using the method of Abbott (1925). Based on Abbott's method, the mussels were pronounced dead when they no longer respond to a stimulus by closing their shells (Abbott 1925). In this 4x5 factorial experiment, mortality was assessed for each adult daily for 62 days and dead mussels were removed and counted.

Higher Dosage Experiments

To determine higher threshold levels, this experiment was repeated following the same procedures but using higher concentrations of EarthTec including 0 ppm (0 ppm Cu^{2+}), 4 ppm (0.24 ppm Cu^{2+}), 6 ppm (0.36 ppm Cu^{2+}), 8 ppm (0.48 ppm Cu^{2+}), and 10 ppm (0.60 ppm Cu^{2+}) at 15°C and 20°C. In this 5x2 factorial experiment, mortality was monitored for 72 hours.

Adult Reproduction

Forty adult zebra mussels were isolated into individual specimen cups and acclimated overnight to room temperature. The animals were randomly assigned into 4 treatment groups with 10 mussels each: the control group, serotonin only, EarthTec only, and a combination of serotonin and Earthtec. The final concentration of EarthTec was 1 ppm (0.06 ppm Cu^{2+}) and all mussels were kept at 20°C (room temperature).

All of the animals were examined after three hours and again at 48 hours. At the three-hour mark, the mussels were checked to determine the effect of Earthtec on the animals' ability to spawn based on the presence of either eggs or sperm released into the water. The mussels were checked again after 48 hours to determine the effect of Earthtec in conjunction with serotonin on mussel mortality.

Statistical Analysis

To determine if mean sperm attachment differed among EarthTec dosage, I used a one-way analysis of variance (ANOVA), followed by a Tukey-Kramer post hoc test. I used a

Kruskal-Wallis non-parametric test followed by a post hoc test, to determine if mean egg cleavage progression differed among EarthTec dosage. To determine if veliger mortality was related to variation in EarthTec concentration, I log-transformed the proportion of dead veligers and regressed the proportion of veliger mortality against EarthTec concentration. For each of the EarthTec concentration groups, I used a 4x5 factorial ANOVA to compare the main effects of EarthTec concentration and water temperature and the interaction effect between concentration and temperature on mortality. For all tests, statistical significance was determined at $p < 0.05$. Statistical tests were performed using Minitab v18.

RESULTS

<u>*Gametal Stage Experiments*</u>

EarthTec QZ effects on sperm binding

Sperm attachment to the perimeter of the eggs varied significantly (Table 1, Figure 5; ANOVA, $F_{(3,16)} = 4.295$, $p < 0.05$) with EarthTec concentration. In the control group (0 ppm), on average (mean ± SE), approximately 5.248 ± 1.69 sperm were attached to each egg. In the 0.5, 1.0, and 2.0 ppm groups, only an average of 1.876 ± 0.53, 1.832 ± 0.79, and 0.536 ± 0.16 sperm respectively attached to each egg. The 2.0 ppm concentration of EarthTec had the most significant impact on the sperm's ability to bind to the eggs when the EarthTec was present (Tukey-Kramer post hoc, $p < 0.05$). Many of the sperm displayed reduced motility but sperm immotility did not reach 100%.

Table 1. Mean number of zebra mussel sperm attached to eggs at different doses of EarthTec. 50 eggs from 5 females were examined per concentration.

EarthTec	Copper level (µg/L)	Sperm (Mean ± SE)
0 ppm	0 µg/L	5.248 ± 1.69
0.5 ppm	30 µg/L	1.876 ± 0.53
1.0 ppm	60 µg/L	1.832 ± 0.79
2.0 ppm	120 µg/L	0.536 ± 0.16

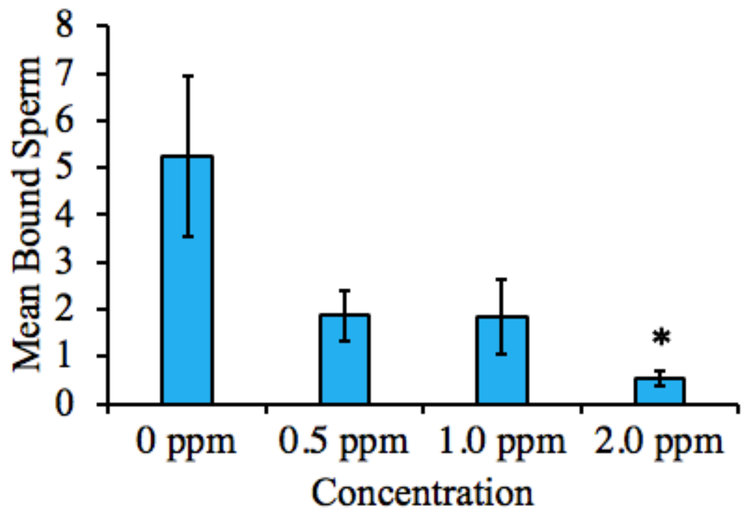

Figure 5. Mean number of zebra mussel sperm bound to eggs (mean ± SE) at 4 concentrations of EarthTec. Concentrations that are significantly different from the others (ANOVA, $p < 0.05$) indicated by "*". Data also presented in Table 1.

EarthTec QZ effects on developmental progression

The eggs from each concentration were sorted into 3 categories: (1) egg exhibiting no cleavage; (2) activated egg with a polar body only; or (3) egg visibly exhibiting cleavage. There was not a significant difference seen in the average progression of egg development among concentrations of EarthTec with the exception of the 2.0 ppm group (Figure 6; Kruskal-Wallis non-parametric, $H_{(3)} = 118.92$, $p < 0.001$). On average, a majority of the eggs progressed to form a polar body with many also exhibiting cleavage (Table 2). In the 2.0 ppm group, many eggs did not progress far enough in the development process to even develop a polar body and appeared as just single, unfertilized eggs.

The proportion of eggs that progressed to each developmental category differed with each concentration of EarthTec. In the groups where EarthTec was present, there was a higher proportion of eggs that did not progress. Many eggs, especially from the 2.0 ppm group, were not able to cleave or even form a polar body. The was a lower proportion of eggs categorized as a 2 or 3 in the experimental groups compared to the control group (Figure 7). In comparison to the control group, the groups with EarthTec present appeared to be inhibited to progress through development as they normally would.

Table 2. Average cleavage progression category of zebra mussel eggs achieved at different doses of EarthTec.

EarthTec	Copper level (µg/L)	Category (Mean ± SE)
0 ppm	0 µg/L	2.26 ± 0.224
0.5 ppm	30 µg/L	2.12 ± 0.268
1.0 ppm	60 µg/L	2.096 ± 0.321
2.0 ppm	120 µg/L	1.412 ± 0.284

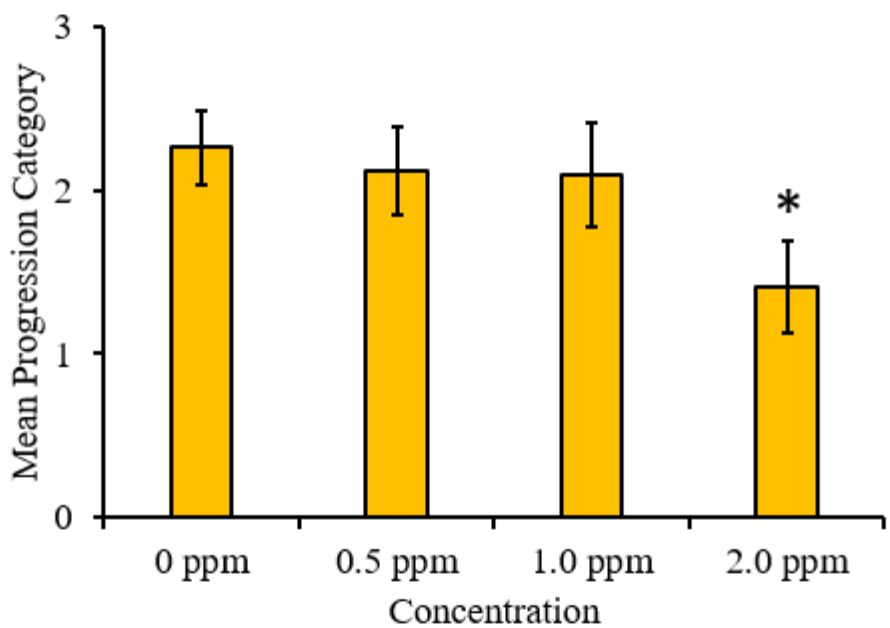

Figure 6. Mean categorization of zebra mussel eggs (mean ± SE) post-fertilization for 4 concentrations of EarthTec. Eggs were sorted into 3 categories: (1) egg exhibiting no cleavage; (2) activated egg with a polar body only; or (3) egg visibly exhibiting cleavage. Concentrations that are significantly different from the others indicated by “*”. Data also presented in Table 2.

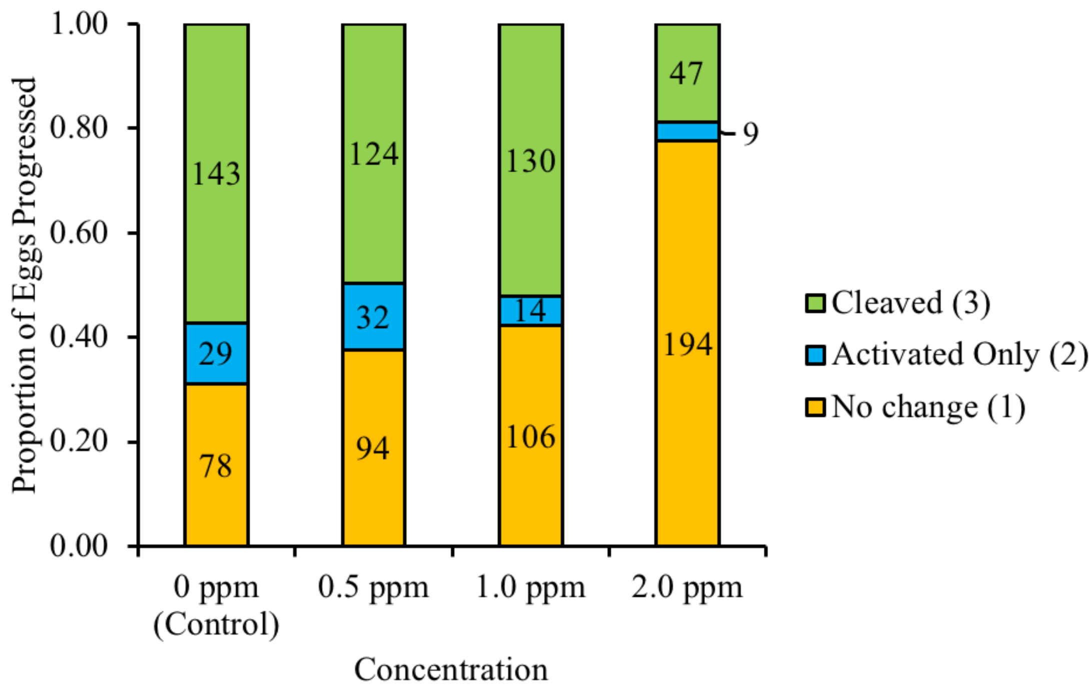

Figure 7. Proportion of eggs categorized as a 1, 2, or 3 for four concentrations of EarthTec. Eggs were sorted into 3 categories: (1) egg exhibiting no cleavage; (2) activated egg with a polar body only; or (3) egg visibly exhibiting cleavage.

Larval Stage Experiments

EarthTec QZ effects on veliger mortality

Doses of 1.5 ppm and greater were effective for killing approximately 100% of veligers within 24 hours (Table 3, Figure 7). After 24 hours, a large majority of the veligers in the control groups and the 0.5 and 1.0 ppm groups were alive.

EarthTec doses as little as 1.5 ppm were able to achieve increased mortality rates compared to lower concentrations, indicating that veligers are more sensitive than adult zebra mussels. Proportion of veliger mortality varied significantly with treatment ($p < 0.01$). Treatment concentration and mortality are positively correlated.

Table 3. Proportion of zebra mussel veligers found dead after 24 hours for 10 concentrations of EarthTec. n = 37 groups of 10 veligers each.

EarthTec	Proportion dead
0 ppm	0.2, 0.4, 0.5, 0.7, 0.8
0.25 ppm	0
0.5 ppm	0, 0, 0, 0, 0.2, 0.2
1.0 ppm	0, 0.1
1.5 ppm	0.3, 0.8, 1, 1, 1, 1
2.0 ppm	0.8, 0.9
3.0 ppm	0.4, 1
10.0 ppm	1, 1, 1, 1
25.0 ppm	0.9, 1, 1, 1
50 ppm	1, 1, 1, 1

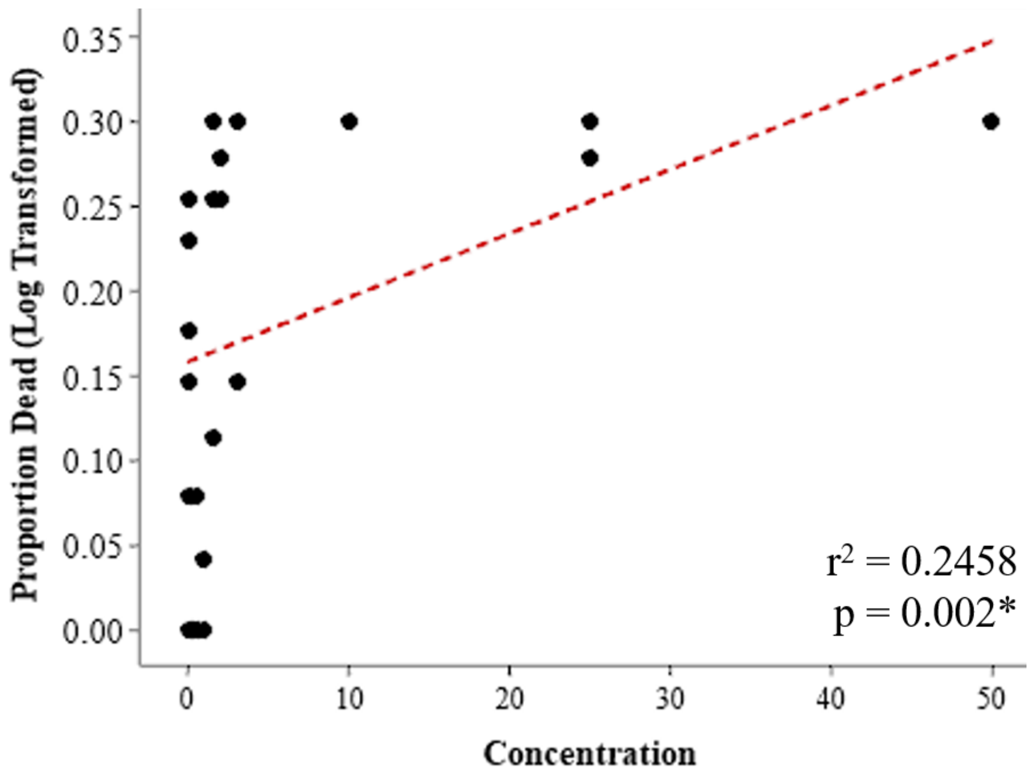

Figure 8. Relationship between the proportion of dead zebra mussel veligers and EarthTec concentration. Dotted line indicates the best fit regression line. Slope of regression significantly different than zero (*p < 0.01*).

<u>*Adult and Juvenile Stage Experiments*</u>

Adult and Juvenile Mortality by Temperature

GROUP 1 (2.0 ppm maximum)

A 4x5 factorial ANOVA was conducted to compare the main effects of EarthTec concentration and water temperature and the interaction effect between concentration and temperature on mortality. The effect of temperature on mortality was significant at the 0.05 significance level (Figure 9; ANOVA, $F_{(3,16)} = 127.41$, $p < 0.01$). There was no significant effect of concentration on mortality (ANOVA, $F_{(4,16)} = 1.86$, $p > 0.05$). The interaction between concentration and temperature on morality was not significant (ANOVA, $F_{(1,16)} = 0.31$, $p > 0.05$). After the 62-day-long experimental period, the application of the various concentrations of EarthTec to the test groups resulted in varying levels of response both between concentrations and between tank temperatures (Figure 9). Overall, increased concentration of EarthTec along with increased temperature resulted in a greater percentage of mean mortality.

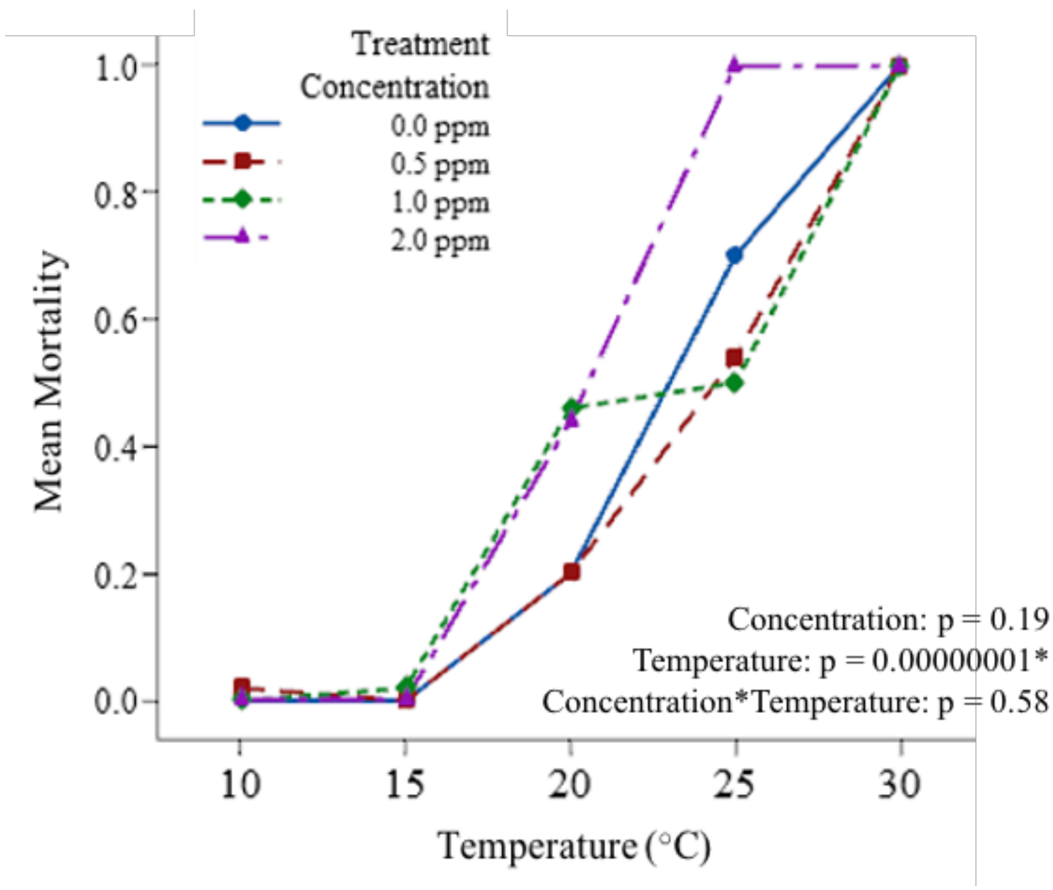

Figure 9. Interaction plot of the main effects of temperature and concentration on mean mortality. Main effect of temperature present ($p < 0.01$) indicated by "*".

Mortality in the control group was higher in the higher temperature groups compared to the colder temperature groups. The mean survival decreased for the same temperatures but in the groups with increasing EarthTec concentrations for the 20°C, 25°C, and 30°C. Mean mortality remained the same for the 10°C and 15°C groups, regardless of EarthTec concentration (Table 4).

The time to achieve 100% mortality of adult mussels decreased with increasing EarthTec concentration for mussels kept in at least 20°C water. In the adult control group, 0.0 ppm, 0% of the mussels had died in the 10°C and 15°C water throughout the course of the 62 days while 20%, 70%, and 100% of the mussels had died in the 20°C group, 25°C group, and the 30°C, respectively.

This same trend was seen in the 10°C and 15°C tanks for the other 3 EarthTec concentration groups as well. By the end of the experimental time frame, 100% of the mussels kept in the 30°C water had died, including in the control group.

- In the 0.5 ppm group, 20% of the mussels had died over the course of the 62 days in the 20°C group, followed by 54% mortality in the 25°C group. The 30°C group reached 100% mortality at the 40-day mark.
- In the 1.0 ppm group, 46% of the mussels had died over the course of the 62 days in the 20°C group, followed by 50% mortality in the 25°C group. The 30°C group reached 100% mortality at the 34-day mark.
- In the 2.0 ppm group, 44% of the mussels had died over the course of the 62 days in the 20°C group, followed by 100% mortality in the 25°C group. In this group, both groups of mussels in water greater than 20°C resulted in 100% mortality.

The 25°C group reached 100% mortality at the 54-day mark while the 30°C group reached 100% mortality at the 6-day mark.

Table 4. Mortality counts (± SE) for zebra mussel juveniles/adults after 62 days at different doses of EarthTec at different water temperatures. Number of days in which 100% mortality was reached also included for groups in which completely mortality was achieved.

EarthTec	Copper level (μg/L)	Water temperature (°C)	Mortality (Mean ± SE)	Days for 100% mortality
0 ppm	0 μg/L	10 °C	0	
		15°C	0	
		20°C	20 ± 9.49%	
		25°C	70 ± 10%	
		30°C	100 ± 0%	42
0.5 ppm	30 μg/L	10°C	0	
		15°C	2 ± 2%	
		20°C	20 ± 13.04%	
		25°C	54 ± 15.68%	
		30°C	100 ± 0%	40
1.0 ppm	60 μg/L	10 °C	0	
		15°C	2 ± 2%	
		20°C	46 ± 22.27%	
		25°C	50 ± 13.42%	
		30°C	100 ± 0%	34
2.0 ppm	120 μg/L	10°C	0	
		15°C	0	
		20°C	44 ± 22.93%	
		25°C	100 ± 0%	54
		30°C	100 ± 0%	6

GROUP 2 (10.0 ppm maximum)

Doses of 6, 8, and 10 ppm of EarthTec were effective for killing a majority of the adult mussels within 48 hours at both 15°C and 20°C. After 72 hours, at 15°C, 100% of the adult mussels were dead in the 6 ppm group. At 20°C, 100% of the adult mussels were dead in the 8 ppm group after 72 hours (Table 5). A 5x2 factorial ANOVA was conducted to compare the main effects of EarthTec concentration and water temperature and the interaction effect between concentration and temperature on mortality. The effect of concentration on mortality was significant at the 0.05 significance level (Figure 10; ANOVA, $F_{(4,6)} = 8.50$, $p < 0.05$). There was no significant effect of temperature on mortality (ANOVA, $F_{(1,6)} = 0.19$, $p > 0.05$). The interaction between concentration and temperature on morality was not significant (ANOVA, $F_{(1,6)} = 0.04$, $p > 0.05$).

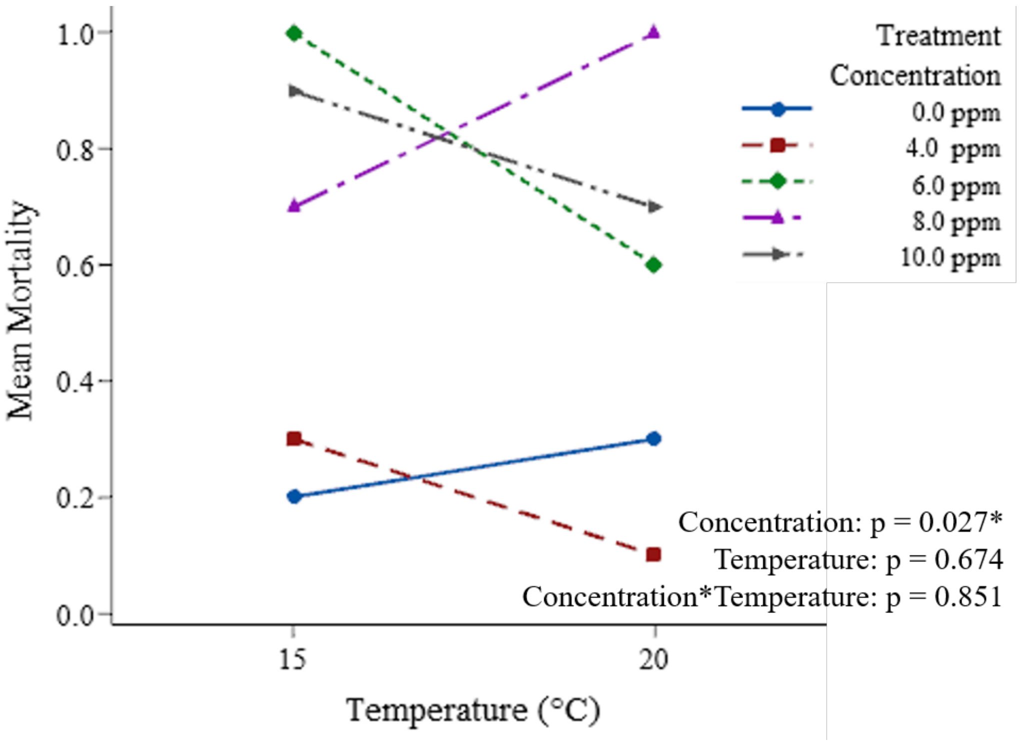

Figure 10. Interaction plot of the main effects of temperature and concentration on mean mortality. Main effect of concentration present ($p < 0.05$) indicated by "*".

Table 5. Mortality counts for zebra mussel juveniles/adults after 72 hours at different (high) doses for EarthTec at different water temperatures.

EarthTec	Copper level (µg/L)	Water temperature (°C)	Mortality
0 ppm	0 µg/L	15°C	20%
		20°C	30%
4.0 ppm	240 µg/L	15°C	30%
		20°C	10%
6.0 ppm	360 µg/L	15°C	100%
		20°C	60%
8.0 ppm	480 µg/L	15°C	70%
		20°C	100%
10.0 ppm	600 µg/L	15°C	90%
		20°C	70%

Adult Reproduction

After 3 hours, none of the adult mussels in the control group or those that were exposed to EarthTec alone exhibited spawning. Of the adult mussels that were induced to spawn in the presence of serotonin alone, 40% successfully released gametes into the water. Of the adults that were induced to spawn by serotonin in addition to EarthTec, 40% of the adults released gametes (Table 6, Figure 11).

Table 6. Proportion of adult zebra mussels that released gametes within 3 hours after spawning induction and proportion of those mussels that were dead after 24 hours. The four groups included the control mussels in pond water only, mussels that were exposed to serotonin only, EarthTec only, or a combination of EarthTec and serotonin.

Group	Proportion Spawned	Proportion Dead
Control	0	0.1
Serotonin Only	0.4	0.1
EarthTec Only	0	0
EarthTec + Serotonin	0.4	0.3

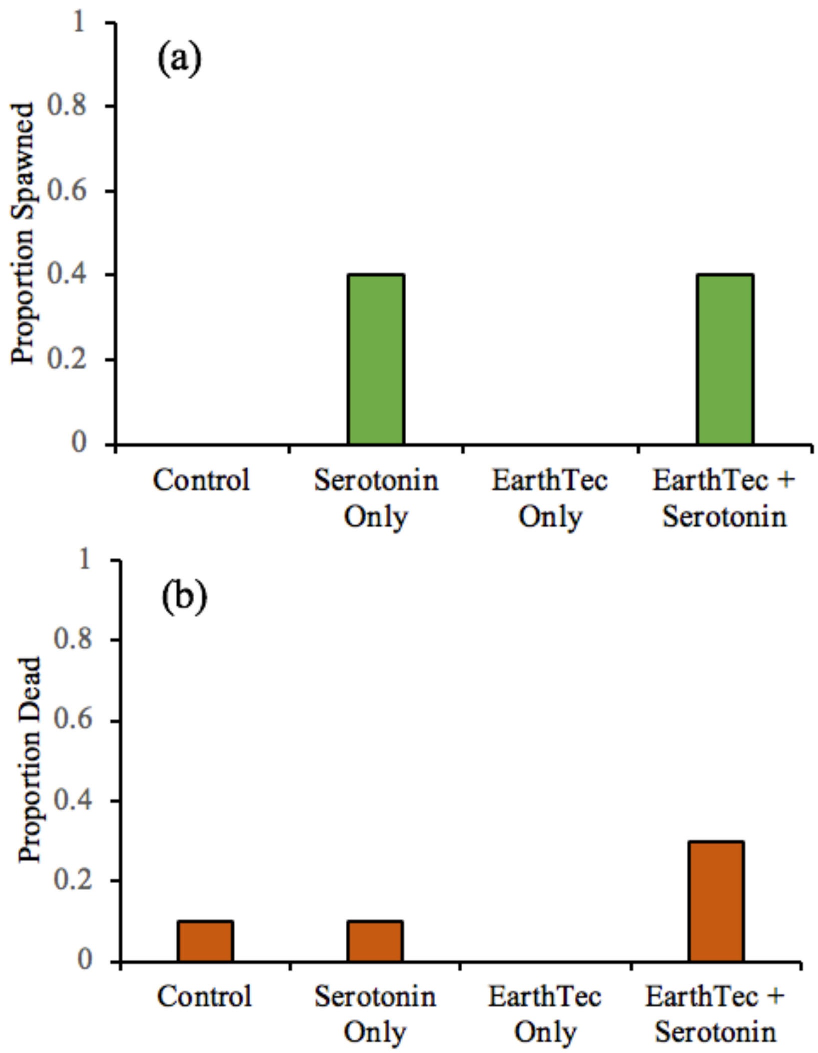

Figure 11. Proportion of adult zebra mussels from the four experimental groups that (a) were able to spawn and (b) died after 24 hours. The four groups include the controls, those that were exposed to serotonin only, to EarthTec only, and a combination of EarthTec and serotonin.

After 48 hours, the control group and the serotonin only groups did not exhibit a significant amount of mortality (<10% of the mussels died). In the group that was exposed to EarthTec only, none of the mussels died within 48 hours. Of the mussels that were exposed to serotonin along with EarthTec, 30% of the mussels were dead within 48 hours.

DISCUSSION

Gametal Stage Experiments

EarthTec QZ effects on sperm binding

While several studies have examined the effects of copper on veligers and adult mussels, no published studies have examined the effects on gametes. Sperm displayed reduced motility presumably due to the presence of copper, a common spermicide. Copper reduces sperm motility and is the major component in the most common form of intrauterine contraceptive (Jecht & Bernstein 1973, Roblero et al 1996). Sperm were able to bind to eggs at lower copper concentrations. This may be due in part to insufficient levels of copper in the water to inhibit sperm motility and binding. This may also be due to insufficient time to affect sperm motility before coming in contact with the egg. Increasing the length of preincubation in copper prior to exposing to the eggs may result in decreased sperm motility and binding, even in lower doses. However, this increased copper exposure prior to eggs may not be biologically tractable. Although the time between spawning and fertilization in *in situ* zebra mussels is unknown, it is generally assumed that fertilization occurs quickly after gamete release. A 30-minute separation between copper exposure and egg encounter is not possible in natural spawning populations. Furthermore, it is possible that the sperm did not reach 100% mortality due to the sheer number of sperm that is released by a single zebra mussel male in a spawning event.

This information is valuable, and it can be presumed that at an increased concentration of EarthTec, most of the sperm will be killed or immobilized and unable to fertilize the eggs. This shows that when EarthTec is present, especially at concentrations above 2.0 ppm, the ability for sperm to bind to an egg is reduced. This could essentially render a population of zebra mussels sterile and, provided no new input of mussels or veligers, allow the population of mussels to die off in an enclosed waterbody.

EarthTec QZ effects on developmental progression

Although sperm binding did decrease with higher (2.0 ppm) levels of EarthTec, sperm entry into the egg, egg activation, and subsequent zygote cleavage will be able to occur. EarthTec did not seem to have a significant effect on fertilization progression up to the point of egg cleavage for eggs in the presence of 0.5 or 1.0 ppm. However, EarthTec did have an impact on egg progression post-fertilization on eggs that were exposed to 2.0 ppm. This suggests that the effects of copper on subsequent development were not as significant after sperm binding compared to prior to sperm binding. This correlates with the primary role that copper is believed to play as a contraceptive in inhibiting sperm function prior to fertilization (Jecht & Bernstein 1973, Roblero et al 1996).

Ultimately, this indicates that during zebra mussel spawning season, exposing the gametes in the water column to at least 2.0 ppm of EarthTec can reduce the breeding population by acting as a contraceptive. This might suggest that treating with slightly higher doses of EarthTec during reproductive times would help reduce mussel populations by preventing new reproductive input. Treatment of the gametes and larvae that are virtually impossible to see with the naked eye are of a high priority in order to preserve water bodies that have not already been infected. The gametes and larvae should be targeted at an equal amount to the adults.

Larval Stage Experiments

EarthTec QZ effects on veliger mortality

Zebra mussel veligers were susceptible to EarthTec, particularly at doses greater than 1.5 ppm. As anticipated, increasing concentrations of EarthTec resulted in increased veliger mortality. The unexpectly high levels of mortality in the controls (0ppm) indicated the difficulties of working with veligers particularly in a laboratory setting. Additional replicates should decrease the overall mortality rate, further emphasizing the significant effect of EarthTec on veligers.

Similar studies have examined the effects of EarthTec and other forms of copper on dreissenid mussels. Similar to our results, Watters et al (2013) found that 3 ppm EarthTec resulted in 100% mortality within 30 min in quagga veligers. Kennedy et al. (2006) found 50% mortality in zebra mussel veligers treated with 13 μg/L Cu (0.217 ppm) after 24 hours which increased to 100% at 300 μg/L Cu (5.0 ppm) in less than one hour. This study parallels similar studies, that veligers are more sensitive to copper treatment than adults. This is even true for the lower concentrations used in the present study – which more closely mirror practical large-scale dosage applications.

Adult and Juvenile Stage Experiments

Adult and Juvenile Mortality by Temperature

Lower dosages of EarthTec were not as effective in treating adult mussels in comparison to the higher dosages at all water temperatures (ANOVA, $F_{(1,16)} = 0.31$, $p > 0.05$). Regardless of EarthTec concentration, temperature had a significant effect on mortality (ANOVA, $F_{(3,16)} = 127.41$, $p < 0.01$). These results also indicate that 30°C exceeds the threshold of water temperature that is survivable by zebra mussels. This potentially means that once the mussels are already stressed by temperature, an EarthTec concentration of 2.0 ppm can kill almost 100% of the mussels very quickly. In very warm weather, like the summer in Texas, zebra mussels can be treated very quickly with 2.0 ppm EarthTec and eliminated in about a week (Luoma et al. 2018). These results confirm those of other studies that demonstrate that copper toxicity increases with temperature (Rao & Khan 2000, Mersch et al 1993, Luoma et al 2018, Lund et al 2018, Watters et al 2013).

Adults are likely more resistant than the larvae regardless of EarthTec presence due to the high mortality rate of larvae that is common of a broadcast spawning species. Adults also have the advantage of having more protective shells that they can close in the presence of noxious chemicals for days and sometimes up to weeks to avoid ingesting any potentially damaging material in the water (Van Benschoten 1995).

High Dosage Experiment

Concentrations of 6, 8, and 10 ppm of EarthTec were effective for producing high mortality rates after 72 hours compared to lower concentrations, regardless of water temperature. In order to treat an established adult population of zebra mussels with EarthTec in less than 48 hours, one would need an incredibly high concentration that would no longer be economically feasible and would be potentially damaging to the ecosystem.

Earthtec QZ effect on adult reproduction

Serotonin is known to increase siphoning rates and induce spawning in zebra mussels (Ram et al 1993). Like most bivalves, zebra mussels can sense noxious chemicals in the water and will stop siphoning and close their shells. Dietz and Lynn (1995) proposed that the addition of serotonin might increase effectiveness of chemical control mechanism by forcing mussels to resume siphoning in the presence of noxious chemicals. We performed a primarily proof of concept study using a combination of EarthTec and serotonin. We found that when the mussels are exposed to EarthTec alongside serotonin, they are more likely to take in the EarthTec.

Interestingly, some were still able to spawn but at a reduced rate relative to controls. While commercial application would be difficult given costs associated with serotonin application combined with environmental regulations, this study does demonstrate that combining chemical treatment with in vitro spawning techniques, increased mortality is observed.

Potential implications for practical application

The EarthTec concentrations used in this study ranged from 0.25 ppm (15 µg/L Cu) to 50 ppm (3000 µg/L Cu), with most of the experiments using between 0-2 ppm. Most of these treatments are substantially lower than the allowed copper concentration accepted by the EPA for drinking water standards of 21.6 ppm or 1300 µg/L Cu. The bulk of the concentrations used in this experiment are low enough to not pose a risk to the water quality in drinking water treatment facilities. The higher concentrations used, such as 25 and 50 ppm, despite being effective, would not be safe or economically feasible. The lower concentrations, however, may be a valuable means of treatment for multiple life stages of zebra mussels.

Chemical control by EarthTec may be the most effective in proactively managing further invasive spread of the mussels by negatively affecting the gametes and the veligers. This would hinder their ability to survive long enough to form a new destructive adult population in uninfested waters.

EarthTec could be used to treat ballast water tanks in commercial or recreational boats in order to avoid transfer between waterways and diminish the time period currently required to completely dry out an individual boat before entering another waterway. Treatment with EarthTec may be effective for facilities that are vulnerable to damage such as power station inlets and water treatment plants. This may be particularly true where increased temperatures will increase the effectiveness of the treatment. Variability in application dosage strategies at large scale facilities to correspond to reproductive cycles or elevated temperatures may help maximize the effectiveness of the treatments. Specific applications during he more sensitive early life stages would prevent the recruitment of new mussels into industrial facilities or downstream waterways. Similarly, management strategies can also be affected by time of year. In the summer, less of the chemical is likely needed to treat zebra mussels effectively. This would be particularly true for warmer climates. In the winter, more of the chemical is needed to have a similar effect. Optimizing treatment based on water temperature would help minimize application concentrations. Optimizing control by EarthTec by using doses that are effective on the different life stages would decrease the risk to non-target species in the surrounding ecosystem and decrease the amount of the chemical needed, thus reducing cost while lowering the overall release of copper into these facilities.

CONCLUSION

The copper-based algalcide solution, EarthTec QZ, was evaluated for its efficacy as a means of control of adult, juvenile, and larval *Dreissena* mussels. EarthTec QZ was also examined for its effects on zebra mussel gametes and its consequences on fertilization and development. This study was conducted to determine the feasibility for facilities such as water treatment plants, specifically in North Texas, to use EarthTec QZ to treat current populations of mussels and prevent further spread. This study included a range of copper dosages along with a range of water temperatures in order to determine the efficacy of the chemical under different temperatures.

Of the conditions tested, EarthTec appears to be an effective treatment to kill adult zebra mussels at temperatures above 20°C over the experimental period of 62 days. Adult zebra mussels showed a high rate of mortality in the higher temperatures when EarthTec was present.

High concentrations of Earthtec also had a negative effect on zebra mussel veligers at 20°C where mortality is seen within 24 hours.

Adult mussels that were induced to spawn in the presence of EarthTec have no issues releasing gametes, but many died within 24 hours. EarthTec also had an inhibiting effect on the ability for zebra mussel sperm to attach to the surface of eggs when fertilization is induced. Fertilized eggs that were exposed to EarthTec did not progress normally through development.

In this study, the copper product is seen to have negative repercussions on the various life stages that were examined. This information provides valuable insights to the versatility of the chemical to treat zebra mussels at any stage of colonization. EarthTec can be used to treat zebra mussels that have already entered a system and also potentially prevent further spread.

The results of this study align with those found by Rao and Khan (2000), which state that copper increases in toxicity to zebra mussels with increasing temperature. In the warmer waters, the negative effects of EarthTec were exacerbated in all of the zebra mussel life stages examined.

Chemical options for zebra mussel treatment in open water bodies are limited. Therefore management of zebra mussels on the scale of water treatment facilities should be optimized (Claudi and Mackie 1994). Treatments with EarthTec should be optimized with doses that are most effective for the current water temperature and state of settlement (Kennedy et al. 2006). This would minimize the risk to non-target species and minimize the amount of the chemical that is required, which would decrease overall the cost of treatment. For comparison, for warmer water temperatures of above 20°C, a concentration of EarthTec as low as 0.5 ppm resulted in increased adult mussel mortality. However, the larval stages of zebra mussels were also killed with at least 0.5 ppm of EarthTec present but in a shorter amount of time. If larval zebra mussels that haven't developed into adults are present in the system, less of the chemical is needed over time in order to eradicate the population.

A higher mortality of reproductive aged adult mussels would ultimately lead to a decrease in reproductive success of the population (Kennedy et al. 2006). Given this, less adults would be releasing gametes thus further decreasing the risk of spread by larvae in the water column. This shows that a good time for treatment would be dosing during the reproductive season, specifically during spawning events.

In the presence of EarthTec, sperm did not reach 100% mortality so fertilization could still take place. However, there is a potential for sperm viability to decrease due to premature acrosomal reactions, decreased motility, or DNA damage which should be investigated further.

If water was treated using some of the concentrations of EarthTec used in this study, the copper concentration may pose a risk to non-target fauna such as the endangered unionid mussel. The fate of copper through the drinking water/wastewater cycle and subsequent release back into the environment should be considered. This study provides important implications in the design of the management plans for zebra mussels and other invasive species using EarthTec.

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ABSTRACT

EFFECTS OF COPPER AND TEMPERATURE ON THE LIFE STAGES OF THE INVASIVE ZEBRA MUSSEL

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Dreissena polymorpha, zebra mussels, are an invasive species of freshwater bivalves that have recently spread into bodies of water across North America via the Great Lakes. Zebra mussels are mainly spread throughout the United States by their free-swimming larvae called veligers that are moved from waterbody to waterbody by human boat traffic, attributing to the success of their invasive spread. Once an adult zebra mussel population is established, they proliferate quickly and cause many problems to the ecosystem and cause damage to boating and water treatment equipment by tightly attaching to many hard surfaces. Zebra mussels have recently entered many Texas waterways, indicating that they have possibly adapted to conditions outside of originally expected for a cold-water species that are not representative of the Great Lakes region. The focus of this study was to look at various environmental factors which may affect zebra mussel survival and reproduction including temperature and the effects of a copper-based molluscicide, EarthTec QZ, as a potential mechanism of control. Zebra mussel survival and reproductive success were examined in various experiments to gain an overall understanding of the effects at all zebra mussel life stages.