MINNEHAHA CREEK WATERSHED DISTRICT

QUALITY OF WATER QUALITY OF LIFE

MEMORANDUM

DATE: January 26, 2017
TO: MCWD Board of Managers
FROM: Eric Fieldseth, MCWD AIS Program Manager
RE: Zebra mussel veliger control study

In 2016, the MCWD received AIS grant funding from Hennepin County to work with researchers from the University of Minnesota to evaluate a potential new method to prevent and control zebra mussels. Typically, prevention focuses on target vectors (e.g., boats, docks and lifts) and not sources of spread such as infested waters. This research aimed to evaluate the use of low doses of the copper-based molluscicide EarthTec QZ for control of veligers. Where previous treatments have targeted adult mussels with higher levels of copper (0.3 to 1 ppm Copper), this research focused on targeting veligers at levels less than 0.1 ppm.

There are two potential benefits of this strategy to prevent spread:

- Controlling veligers would likely reduce population sizes, resulting in fewer juveniles and adult zebra mussels that might be transported by docks, lifts and boats. *(Zebra mussels typically live less than 5 years and rely on veligers for population growth – if veligers are reduced, populations would reduce over time)*
- Veliger control would reduce concentrations of live larvae in residual water of boats.

Summary of Results

- The in-lake enclosures were effective at maintaining desired doses for ~16-hour exposure times. Managing in-lake enclosures can be difficult; there are no pre-made devices that can be used. These enclosures could be replicated by other researchers for similar use.
- With ~16-hour exposure time, half of the veligers could be controlled by a rate of 0.018 ppm. Almost complete control could be had with 0.478 ppm at 17 hours. Keep in mind, complete control of adult zebra mussels can take 10 days at a range of 0.3 to 0.5 ppm. Longer exposure time testing is needed to determine how many hours it takes to get complete control of veligers, and at the lowest dose possible.
- Methods and protocols for sampling dead zebra mussels in the field, and laboratory methods for determining whether dead or alive, were improved. This is an area not well developed; we found out veligers sink to the bottom of the lake pretty quickly upon dying, so traps were created to sample these. Determining if a microscopic veliger is dead is also not so simple, and protocol for doing such were improved upon in this research that will be utilized in future research.

Next Steps

Longer exposure times need to be tested so we can have a better idea of the lowest dose of pesticide, over the shortest time duration, that can yield good control on zebra mussel veligers. The U of M will be applying for another year of funding from Hennepin County to continue building upon this work. The MCWD would be listed as a partner and help with logistics and some field work if funding was granted.

Summary Report 2016

Project Title: Field evaluation of toxicity of low-dose molluscicide treatments for zebra mussel veliger larvae—potential applications in lake management

Prepared by:

Michael A. McCartney
Research Assistant Professor,
Minnesota Aquatic Invasive Species Research Center
Department of Fisheries, Wildlife and Conservation Biology
University of Minnesota,
135 Skok Hall, 2003 Upper Buford Circle
Saint Paul, MN 55108
mmccartn@umn.edu
Office: 612-301-7703

Prepared for:

Minnehaha Creek Watershed District

December 31, 2016

I. Executive summary

Rationale and Background

Prevention of spread of zebra mussels usually targets vectors (e.g. boats, docks and lifts) and not sources of spread (i.e. infested waters), but research and management with zebra mussel pesticides opens new options for prevention aimed at source water bodies. EarthTec QZ™ (hereafter, EarthTec) is a copper sulfate formulation approved for use as a mollusk pesticide (i.e. molluscicide). Applications to adult zebra mussel infestations in open waters and industrial and municipal facilities (e.g. dams, water treatment facilities) in Minnesota (MN) and elsewhere show EarthTec to be a potent molluscicide. In previous laboratory studies (none under field conditions) copper sulfate products have been found to be lethal to zebra and quagga mussel larval stages (known as "veligers") at doses much lower than those lethal to adult mussels. This implies that low-dose treatments of EarthTec might be used to kill veliger larvae in open waters. If this approach were found to be effective, low dose treatments might be applied to larger lake areas (e.g. whole bays, harbors). Potential benefits would include **lowering ecologic and economic impacts** by **reducing zebra mussel population sizes,** as well as **preventing spread** by reducing densities of veliger larvae in lakes, and therefore **lowering the risk of transport of larvae and mussels by recreational boaters**. This research provides the first-ever attempt to study EarthTec toxicity to veliger larvae under close-to-natural conditions in field experiments within a zebra-mussel infested lake.

Objectives

1. **To develop methods for estimating toxicity of EarthTec to the larval "veliger" stages of zebra mussels**, *Dreissena polymorpha*, under field conditions in Lake Minnetonka, Hennepin County, Minnesota (MN).
2. **To estimate EarthTec concentrations required to kill 50% and 99% of veligers (LC_{50} and LC_{99}, respectively) in the lake by analyzing dose/response curves** in overnight (~ 16-hour) exposures. We make these estimates in multiple impermeable "pens" or enclosures that hold moderate volumes (about 1200 gallons each) of lake water containing naturally reproduced veligers, then add increasing doses to each closure.
3. To adapt the methods developed above to study the toxicity of EarthTec to zebra mussel larvae over increasing durations of exposure—using so called **exposure-time trials to estimate the time required to kill 50% and 99% of veligers (LT_{50} and LT_{99}, respectively)** at a low to moderate dose**.**
4. **To conduct laboratory trials that expose zebra mussel veligers to EarthTec** to estimate LC and LT values under laboratory conditions.

Major Findings

1. Relatively inexpensive, PVC framed enclosures wrapped in plastic tarp and shrink-wrap were durable and effective at maintaining doses of EarthTec for ~16-hour exposure durations. Recovering larvae from enclosures, as expected, was challenging but our most successful approach used "larval traps" to collect dead larvae as they settled toward the lake bottom, as well as live larvae in the overlying water column. Fast Green vital dye staining assays, combined with microscopic observations of larval motility were adequate to score larval mortality, although there is room for improvement of

these methods. These and several other field and laboratory methods are described in greater detail in this report.

2. **We produced statistically robust estimates of LC_{50} and LC_{99} from dose/response curves** in overnight exposures over each of 2 weeks in July-August. The most reliable of these ca. 17-hour exposures yielded values for **LC_{50}** = 0.018 and **LC_{99}** = 0.478 [ppm free copper (Cu^{2+}) concentration: Figs. 9-10, Table 9]. These doses are *considerably lower than* Cu^{2+} *concentrations (roughly 1 ppm) used to treat MN lakes for adult mussels (see pp. 21-22 in this report for further discussion, and Tables 9-10 for more detail on comparisons of toxicity)* .
3. **Exposure-time trials** at a moderate dose (0.100 ppm Cu^{2+}) showed a trend of increase in mortality over a short span of exposure durations (4, 12, 16 hours) but statistical analysis was unable to estimate the LT_{50} and LT_{99} values. Uncertainty was due to logistical issues created by an unexpectedly early (mid-August) crash in veliger counts within Robinson's Bay, which compromised our ability to produce reliable estimates. In future work (see below) exposure-time trials should be repeated in larger enclosures and earlier in the season to give the best chances of producing these crucial estimates.
4. **Our laboratory trials to expose zebra mussel veligers to EarthTec** were not successful at estimating LC and LT values under laboratory conditions. Like Objective 3, these were even more compromised by very low veliger concentrations in eastern bays in Minnetonka in the 3rd week of August, and these experiments must be repeated.

Conclusions and Management Implications

Our results suggest that low-dose treatments of EarthTec in open waters will be effective at killing veliger larvae. This means that the benefits of toxicity to veligers will be realized as an incidental by-product of treatments that target adults (these have been conducted in MN at the maximum Cu^{2+} concentration of 1 ppm). We would estimate that low-dose treatments at the lowest recommended dose in open waters (0.060 ppm Cu^{2+}: US EPA 2015) would produce 20.2 % survival of veligers after 17-hour exposure (Table 9B), so low dose treatments to intentionally target veligers would be effective. In future research (below), experiments at these low doses should be attempted to estimate mortality during the typical week long treatment regimen used in open waters, and for longer durations.

Future Directions

We will describe, in an upcoming proposal to Hennepin County AIS Prevention Grants program (2017) a research plan to use larger lake area treatments, and to set these up in Lake Minnetonka in the earlier season (early June to late July). The goal of these new experiments will be to produce statistically robust exposure-time estimates of LT_{50} and LT_{99}. These values are crucial for management, because they will estimate the level of mortality in the larval population to be expected in treatment durations of 1-week or more, at doses low enough to produce minimal non-target impacts to native organisms. We will also propose to repeat the laboratory experiments to provide controlled laboratory measures for comparison to results from the field trials.

Field studies of toxicity of low-dose EarthTec QZ™ to zebra mussel veliger larvae Enclosures being placed into position in Robinson's Bay, Lake Minnetonka, Hennepin Co. MN.
July 20, 2016 Image from Eric Fieldseth

II. Background and introduction

Zebra mussels (*Dreissena polymorpha*) were first introduced to North America, into the Great Lakes in the mid-1980s, in ballast water discharge from trans-Atlantic ships (Hebert et al. 1989, Carlton 2008). By 2010, *D. polymorpha* was found in more than 600 lakes and rivers across 26 U.S. states (Benson 2010). It is one of the world's most economically and ecologically damaging aquatic invasive species. Economic costs of damage and control of zebra and quagga mussels (a close relative, *D. rostriformis,* which has replaced zebra mussels in the lower Great Lakes), have been estimated to total as much as $1 billion annually in North America (Pimentel et al. 2005). Costs to North American power generating stations and drinking water treatment plants alone were estimated to be about $18 million per year from 1989-2005 (Connelly et al. 2007, Strayer 2009). Zebra mussels clog water intake pipes of industrial facilities (Prescott et al. 2013), compete with and smother native bivalve species (Padilla and Karatayev 1997, Lucy et al. 2014), and restructure aquatic food webs (Higgins and Zanden 2010, Bootsma and Liao 2013, Mayer et al. 2014). The ability of zebra mussels to spread rapidly and to colonize new water bodies results from their high reproductive output, and from their planktonic larval stage (known as a veliger) that during its roughly 3-week larval life, spent feeding on algal plankton and developing in the water column, can drift huge distances before settling down on lake or stream bottoms. The ability of adult mussels to attach to hard surfaces using fibers known as byssal threads (Hebert et al. 1989, Mackie 1991) and the tendency for mussels to reach such high densities that their total filtering capacity can remove 50% or more of the biomass of phytoplankton at the base of aquatic food webs (Higgins and Zanden 2010, Strayer 2010) have also led to the great success and impact of this highly damaging aquatic invader.

In Minnesota, zebra mussels were likely introduced into Duluth/Superior Harbor on Lake Superior in 1989. Over the next 5 years, they began to they spread inland via the major river systems (Mississippi River, St. Croix River and other tributaries). This was followed somewhat later (post-2003) by their colonization of inland lakes. In August 2016, the MN DNR (http://www.eddmaps.org/midwest/tools/infestedwaters/) confirmed 122 water bodies as infested with zebra mussels and listed (without confirming) another 130 water bodies as infested due to short waterway connections between them and the confirmed infested waters. These water bodies, 252 in total, represent less than 2% of Minnesota's > 11,000 lakes and rivers. Infestations of inland waters follow two major routes—spread downstream between interconnected waterways, and spread overland via human pathways associated with boating or other waterway-related activities.

Efforts to prevent spread of zebra mussels between water bodies are most often targeted at vectors of transport (e.g., boats, docks and lifts). Targeting sources (i.e. infested waters) is typically not considered, in large part because few management options exist for zebra mussel control. Attempts to treat zebra mussel infestations in open waters using pesticides (reviewed in Lund et al., in revision) commonly have eradication as the goal. This is a challenging endpoint, because due to costs and non-

target toxicity risks, treatments are rarely lake wide (but see Offut Air Force Base 2009, Fernald and Watson 2014), and conversely partial-lake treatments have not often eradicated mussels (reviewed in Lund et al., in revision). An alternative approach would be to suppress populations, rather than eradicate. This proposed research, for the first time, provides an evaluation of the feasibility of a prevention and population suppression program that attempts to kill veliger larvae in lakes.

There are two potential benefits of this strategy to prevention of spread. First, controlling veligers would likely reduce adult population sizes, resulting in fewer juveniles and adults that might be transported e.g. on docks, lift and boats. And second, veliger control would reduce concentrations of live larvae in "residual waters" transported by recreational boats (Dalton and Cottrell 2013, Kelly et al. 2013, Montz and Hirsch in press). Laboratory studies (Fisher et al. 1994, Kennedy et al. 2006, Watters et al. 2013, Marrone Bio Innovations 2015) have shown that molluscicides are more toxic to veliger larval stages than to adults—particularly in the case of copper sulfate products. This suggests that low-dose applications in expanded lake areas could be used to decrease larval survival and therefore lower the risks that larvae would be transported to uninfested waters. Reducing larval survival might also be used to reduce recruitment (i.e., larval settlement). Controlling recruitment (via bay-wide treatment in larger lakes or lake-wide treatments in smaller lakes) could reduce ecological and economic harm with minimal non-target impacts. And given the short life span of adults (<< 5 years in North America) and give that population growth and persistence over time depends on annual recruitment (Nalepa et al. 1995, Stoeckel et al. 1997, Stoeckel et al. 2004, Strayer and Malcom 2006, Wimbush et al. 2009), this strategy might be used to control population growth. Evaluation of this notion first requires field trials of veliger larval toxicity of candidate molluscicides, but these trials have not been done. We performed field assays of veliger larval toxicity of a potent copper sulfate product that has been recently used to treat zebra mussel infestations. Field-testing was done in Lake Minnetonka during the reproductive season of 2016 in experimental enclosures, with some limited lab testing conducted the goal to fill in knowledge gaps.

III. Materials and Methods

Overview of study design

We used multiple, cube-shaped PVC-framed treatment enclosures, each holding approximately 1200 US gallons (4542 L) of lake water containing veliger larvae—produced from natural reproduction of the zebra mussel population in Robinson's Bay, Lake Minnetonka (Hennepin Co., MN: Fig. 1). These enclosures were designed to retain EarthTec QZ™ molluscicide (hereafter, EarthTec: Earth Science Laboratories, Bentonville, Arkansas), a formulation of copper sulfate pentahydrate, at or near target concentrations for treatment periods of ≤ 1 day (typically overnight or 16 hours). The veligers contained in these enclosures consisted of a range of sizes, ages and developmental stages; of these we could quickly discriminate and score *D-stage* veligers

and *umbonal* veligers. D-stage (straight-hinged stage) are the youngest veliger stage, 1-3 days post fertilization (Ackerman et al. 1994) and about 80-120 µm longest shell length in our samples, whereas umbonal veligers have a visible larval shell hinge or umbo (includes umbonal veliconcha through pediveliger stages: Ackerman et al. 1994): about 150-300 µm longest shell length in our samples.

Our field experimental approach screens for toxicity of EarthTec, under near-natural conditions, to the veliger larval population that was present in Robinson's Bay in late summer 2016. We conducted two weeks of trials in which we varied the dose applied to each enclosure (to **construct a dose/response curve**) in **weeks 1 and 2**. We followed in **week 3** with trials in which we **varied exposure time** (to examine the levels of survival over time at a fixed, intermediate dose). In each week, we included **controls to examine veliger survival within untreated enclosures** (as a reference that includes enclosure effects on survival), and **controls to examine veliger survival in ambient conditions outside of enclosures** (as a way to examine effects of laboratory processing and processing time on veliger survival.)

Construction and deployment of treatment enclosures

Enclosures were deployed off Robinson's Bay Beach (N 44.9433, W093.5221) in water at approximately 1.8 m (6 ft.) depth. Minnehaha Creek Watershed District (MCWD) was responsible for the construction and deployment of these structures. Enclosures were built from tubular (5 cm diameter) PVC, and measured 1.52 m (5 ft.) L X 1.52 m W X 2.43 m (8 ft.) H. To seal them from water leakage and loss of EarthTec over the duration of these trials as much as possible, enclosure frames were wrapped in plastic "shrink wrap," followed by plastic tarp material. The frames were anchored to the lakebed using sections of rebar, and sand bags were laid on the crossbeams on the lake bottom to provide further support and to keep the enclosures in position.

MCWD staff kept the enclosures on shore during times when treatments were not being conducted. Then, by wading and snorkeling, they moved the 6 enclosures into position. Our goal was to allow at least 4 hours prior to initiating a trial to permit debris and sediment to settle. This was mostly successful, although the scoring of some trials was compromised by debris, particularly during a heavy rain event in the last week of the exposure-time trials. Also, during the trials run 07/18/16 through 7/22/16 (Week 1), sand, mud and organic material slowed and made scoring of dead and live larvae more ambiguous, but this occurred due to re-suspension of lake bottom sediments by the pump used in Week 1 trials (see below).

Week 1 trials, overview

These trials were designed to develop field methods and to provide an initial test of the toxicity of EarthTec to veligers at concentrations far more dilute than target concentrations (1 ppm free Cu^{2+}) that have been typically used in treatment of adult

populations in Minnesota lakes. This initial test was accomplished by constructing a dose/response curve of mortality vs. decreasing concentrations of EarthTec. Exposure time was approximately 16 hours (overnight). Trials in week 1 attempted two approaches to recover veligers from field enclosures, which was of course a major logistical challenge. On the first day, we used the gentlest approach, and that was to simply mix the contents of the enclosures with a paddle, and then harvest veligers using multiple plankton tows. The problem with this procedure (see results) was that total recovery of veligers declined as EarthTec dose increase, suggesting inefficient recovery of dead veligers that had settled to the bottom and were not resuspended by manual mixing. Therefore, on the second and third days, we used a gas powered water pump (Honda WX10) to mix the contents of the enclosure and to re-suspend dead veligers that had settled onto the lakebed. This procedure also showed the same negative correlation between dose and recovered veliger concentration (see results). While we did not have the data in July to address this (i.e. the veliger concentration counts had not yet been done), we found that pumping re-suspended lakebed sediment and organic material slowed the scoring of larval mortality to such an extent that we turned to alternative methods in weeks 2 and 3.

Dosing: week 1

Each step listed below in the dosing and harvest procedures was conducted on board an MCWD AIS program boat. Further details of week 1 trials at dosing: Table 1.

1. Test copper concentrations were chosen to represent a concentration series over half-log increments [0, 0.01, 0.03, 0.10, 0.33, and 1.0 ppm copper (Cu^{2+})]. A random number generator was used to assign enclosures 1-6 to each of these 6 concentrations.
2. We moved the boat to enclosure 1, and added to it the volume of EarthTec (Table 1) using a Pipetman P-1000 automated pipet (Gilson Inc., Middleton, WI).
3. We recorded the dosing time, then mixed the contents of the enclosure vigorously using a canoe paddle for approximately 3 minutes.
4. **Copper analysis.** A 125 ml water sample was then taken for free Cu^{2+} analysis by Inductively Coupled Plasma (ICP) Atomic Emission Spectrometry in the Research Analytical Laboratory (RAL: http://ral.cfans.umn.edu) at the University of Minnesota (UMN). The RAL uses Method 200.7 [US Environmental Protection Agency (EPA) 1983] in a 15-element ICP analysis on a fee per sample basis. Samples from the field were not pre-filtered and were not digested, so we measured free Cu^{2+} dissolved in the surrounding water column (i.e. not taken up, for example, by phytoplankton). One sample was taken at dosing after the contents of the enclosures were mixed, and another was taken after the EarthTec treatment duration had elapsed (i.e. at harvest). Water samples for Cu^{2+} analysis were stored at 4°C in sealed Nalgene polypropylene bottles, in a

cooler containing ice packs. MCWD also checked Cu^{2+} using a LaMotte (Chestertown MD) 1200 Copper Calorimeter on site—immediately after dosing and at harvest.

5. **Other physical data.** MCWD also routinely took surface water temperature within the enclosures before and after treatment, and they measured water depth at the deep end and shallow end of the enclosures (often enclosures were slightly tilted due to slope of the lake bed at the treatment site: see photo on pg. 4 of this report). True water depth was reported as the midpoint of these two measures, and used to calculate adjusted treatment concentrations, which slightly deviated from intended (target concentrations) because the latter were calculated assuming exactly 6 ft (1.8 m) depth. MCWD also recorded dissolved oxygen (DO) during the first week of the trials to examine the magnitude of change in DO over 16 hours, which was found to be minor (see results) even in 1-ppm treatments.
6. Steps 1-5 were repeated for the next 5 enclosures.

Harvest: week 1

Further details of week 1 trials at harvest: Table 2.

1. The boat was moved in the vicinity of the 6 enclosures. Using the Honda water pump, 147 L of lake water was pumped (at approximately 40 L/min), through a plankton net which was lowered into a 170 L (45 gallon) plastic drum on board the boat. The net (Aquatic Research Instruments, Hope ID, USA) was 30 cm in diameter, 120 long, 50-micron Nitex mesh size, fitted to a ballast-weighted cod end with 50-micron Nitex windows.
2. The filtrate (50 micron filtered lake water: FLW) was collected, and used to fill 7 x 20 L polypropylene carboys to a volume of ~ 19 L each. These carboys were floated within the enclosures to maintain them at lake surface water temperature for the duration of field sampling.
3. We next moved the boat to an site near the enclosures in approximately 2.43 m (8 ft.) depth. First we obtained veligers for the **ambient control trial (carboy # 0).** This control was used to check for mortality (and any increase in mortality over time) of non-chemically treated veligers in the lab, due to handling and maintaining them throughout the duration of the day's lab work. We also compared these estimates to the mortality estimate from the control enclosure (O ppm Cu^{2+}), in which enclosure effects were present but the water was not treated with EarthTec.

4. We took 5 x 0.914 m (3 ft.) deep plankton tows (from 2.43 m lake-depth), and combined the contents of the cod-end from the 5 tows, or 7.3 m (24 ft.) pooled depth (517 L pooled volume) into carboy 0, using squirt bottles containing FLW to rinse the net and cod end. Carboy 0 was filled to 20 L total using FLW then placed in ambient surface water.
5. The boat was moved to **enclosure #1**. Following temperature measurement and copper sampling (above), enclosure contents were thoroughly mixed in order to resuspend dead veligers, by pumping with the Honda water pump. To do so, we dropped the inlet hose to 0.5 m off the lake bottom in the enclosure, and expelled water through the outlet hose, which was placed within the enclosure and allowed to "snake" freely as water flowed from it. This created a strong circulatory flow for about 3 minutes (at a pump flow rate of approximately 50 L per minute, or 1/3rd maximum).
6. We then took 6 X 1.22 m plankton net tows within enclosure #1, following the procedure used in step #4 for the ambient control.
7. Steps 5 through 6 were repeated for each of the 5 remaining enclosures. Carboys were stored in the lake until all enclosures were sampled. Then we returned to the laboratory at MCWD (about a 10 minute drive) for laboratory sample processing.

Week 2 trials, overview

Week 2 trials were also run, like week 1 trials, to test a concentration series of EarthTec for toxicity to veligers in field enclosures, using 16-hour exposures. Week 1 samples were difficult to score due to re-suspended material in the carboys that interfered with counting and scoring zebra mussel veligers. Mineral sediment interferes with the cross-polarized light microscopy (CPLM) used to view veligers, because like veligers, sand grains are bi-refringent under CPLM. Organic debris makes viewing larvae by CPLM more difficult, and it obstructs attempts to view them non cross-polarized light (to visualize the Fast Green stained samples: see methods below). It seemed apparent that pumping to mix the enclosures was responsible, particularly for the fine particle size sediment in the samples that was re-suspended into the water column. Another possible drawback of water pumping was that it could potentially lift veligers off the lake bottom that have died previously, for reasons unrelated to treatment conditions within the enclosures. Instead, we found an clear decline in recovery of veligers in treated compared to untreated enclosures (see Results), suggesting that larvae killed by EarthTec were settling to the lake bottom and were not being completely re-suspended by water circulation created by the pump.

A solution was devised that involved the use of larval traps to capture dying larvae as they sank toward the lake bottom. A trap lowered to a position above the lakebed at the start of a treatment could also be used to filter the water column above the trap (in a manner similar to a plankton net). When the trap is retrieved at harvest, it is drawn slowly to the surface, during which the overlying cylinder of water is filtered and the retained material is combined with any material captured by settling into the trap.

Larval trap construction

I built traps from 20 L (Home Depot) paint buckets with lids that seal with flexible gaskets. With a jigsaw, I cut out (flush to their edges) the lid and the bottom surface of the bucket to open the bucket to water flow. Next, I cut a 40 cm X 40 cm square of 54-micron Nitex mesh. This mesh was then carefully placed over the top of the bucket. Then the lid was attached carefully, and the Nitex smoothed to remove any slack in the material, such that the filter was pulled evenly taught (like a drum skin) over the lid opening of the bucket. The bucket has a handle to which I attached a 2 lb. SCUBA dive weight, so that the handle swung below the bucket to weight the filter end towards the lake bottom. The finished trap was 37 cm high, with a 30 cm diameter opening towards the lakebed (the exposed filter diameter) and 25 cm diameter towards the water surface. This top opening of the trap also defines the diameter of the column of water sampled above the trap.

Dosing: week 2

Further details of week 2 trials at dosing: Table 3.

Steps 1-5 in this protocol were identical to the dosing protocol for week 1, Step 6 now reads as follows:

6. A larval trap was suspended 0.5 m off the lake bottom in the center of the enclosure, from 2 polypropylene lines, each run through a pair of holes drilled on opposite sides near the top of the trap. The trap lines were then tied to the top crossbeams on the enclosure frame, and their lengths adjusted such that the filter plane held parallel to the water surface. Steps 1-5 in the procedure were repeated for the next 5 enclosures.

Harvest: week 2

Further details of week 2 trials at harvest: Table 4.

Each step in the harvest protocol is rewritten below for clarity

1. The boat was moved in the vicinity of the 6 enclosures. Using the Honda water pump, 147 L of lake water was pumped (at approximately 40 L/min), through a plankton net, which was lowered into a 170 L (45 gallon) plastic drum on board the boat. The net was a 30 cm diameter, 120 long, 50 micron Nitex mesh size, fitted to a ballast-weighted cod end with 50 micron Nitex windows (Aquatic Research Instruments, Hope ID, USA).
2. The filtrate (50 micron filtered lake water: FLW) was collected, and used to fill 7 X 20 L polypropylene carboys to a volume of ~ 19 L each. These carboys were floated within the enclosures to maintain them at lake surface water temperature for the duration of field sampling.
3. The boat was moved to **enclosure #1**. Following temperature measurement and copper sampling (above), we retrieved the larval trap by untying the lines. One person each on 2 opposite sides of the enclosure frame retrieved the trap slowly towards the surface at a rate of 0.3 m per second, much like a vertical plankton tow.
4. The trap was placed on the boat deck, filter surface down, and the inner contents washed onto the mesh using squirt bottles filled with FLW. The filter was folded and carefully placed within the corresponding carboy (1-6) such that no material was lost. The volume of the carboy was "topped off" to 20 L with FLW and the carboy was placed into the enclosure to maintain ambient surface water temperature.
5. Steps 1 through 4 were repeated for each of the 5 remaining enclosures. Carboys were stored in the lake until all enclosures were harvested.
6. At the end of the harvesting, we moved the boat to an area towards the center of the lake and off Robinson's Bay beach, in approximately 5 m (16 ft.) water depth. Here we obtained veligers for the **ambient control trial (carboy # 0)**. See week 1 for the purpose of this trial. We took 1 X 2.75 m (9 ft.) deep plankton tow and rinsed the contents of the cod-end into carboy 0, using squirt bottles containing FLW to rinse the net and cod end. Carboy 0 was filled to 20 L total using FLW then brought into the boat. Then we returned to the laboratory at MCWD for laboratory sample processing and scoring of veliger mortality.

Week 3 trials, overview and protocols

Further details of week 3 trials at dosing: Table 5, at harvest: Table 6.

Week 3 trials were designed to test the effect of increasing exposure times on toxicity of EarthTec to veligers. We chose a mid-concentration (0.1 ppm Cu^{2+}) and tested 3 enclosures at this dose, plus 3 enclosures at 0 ppm (controls). Protocols were identical to week 2 for dosing and harvest, except that exposure times of 4 hours, 12 hours, and 24 hours were used. A new set of treated and control enclosures were established for each exposure time. This strategy was chosen (instead of withdrawing samples over time from the same enclosures) due to perceived logistical issues with loss of larvae from the enclosures due to harvest.

Scoring of veliger mortality

We used a Fast Green staining procedure (Link et al. 2013, Stockton-Fiti and Claudi 2016) to distinguish live from dead zebra mussel veligers. This method relies upon the tendency for Fast Green to stain dead tissue by binding to ends of partially degraded collagen fibers. The dye was reported to stain dead larvae in a diffuse pattern throughout the soft tissues within the larval shell; live larvae, in contrast, often show no staining, or a discrete "gut spot" where the dye has stained digested food material held within the larval gut. It was found to reliably stain dead veligers and to greatly shorten observation times required to distinguish live from dead larvae that were killed by exposure to potassium chloride (potash) molluscicides (Link et al. 2013, Stockton-Fiti and Claudi 2016). Potash is initially narcotizing to zebra mussels, and observers have found that immobile larval stages (as well as juveniles and adults) treated with potash would recover after immersion in untreated water. While copper sulfate has not been reported to be narcotizing, we sought a method to unambiguously differentiate live and dead larvae and to lessen scoring times, due to the fact that we found larval survival in controls to decline steadily throughout the day. Motility of larvae, of cilia on the velum, or movement of internal organs has often been used in zebra mussel larval mortality assays (Fisher et al. 1994, Kennedy et al. 2006, Watters et al. 2013), but we found some larvae to require 1-2 minutes of observation or more to make these determinations under 50 X magnification on a stereomicroscope.

Unfortunately, we found the Fast Green assay to be unreliable as an endpoint when considered alone. We continued to use the stain because we found that it did in fact lessen scoring time, but we opted for an approach that used multiple criteria to distinguish dead from live larvae. Below are the multiple criteria and the decision key that we adopted. These rules were selected after protracted observation of immotile and motile larvae that were stained using the protocol on the following page.

Key for scoring mortality

A. Umbonal larvae

1. Motile larva, ciliary motion on velum, movement of heart, gut or other internal organs: alive
2. Immotile (upon first encounter): go to step 3
3. Staining results
    a. Discrete "gut spot": alive
    b. Diffuse partial staining of internal organs: dead
    c. Diffuse, complete staining of internal organs: dead
    d. No staining: observe 2 minutes for organ or ciliary motion
        i. Motion: alive
        ii. No motion: dead

B. D-stage larvae

1. Motile larva, ciliary motion on velum or other internal organs: alive
2. Immotile: dead.
3. Staining results. The "gut spot" was seen sporadically, even in highly active D-stage larvae, perhaps because the gut was empty of food in many D-stages. We found D-stage larvae to rarely be stained green with a diffuse pattern, but the few that were stained were always immotile.

Laboratory sample processing, staining and scoring of larval mortality

1. We prepared a 4% Fast Green staining solution by adding 1g Fast Green (Sigma Chemical, St. Louis, MO) to 25mL FLW, and vortexing vigorously, then decanted 25 mL stain into a staining dish (13.5 cm weigh boat).
2. After mixing by inversion of the carboy, we filtered ~8L water containing veligers from carboy 0 (about 1/2 of the carboy) through a 54 micron Nitex filter attached by silicon to a 12 cm diameter PVC collar.
3. Then we immersed the filter collar in the stain, where it was soaked for 30 min with gentle agitation, at the start and periodically.
4. We removed the sample from the stain and washed it in each of three 1L beakers filled with 700 ml FLW, until water color was evenly mixed in each beaker, flushing the filter collar carefully up and down in the wash water.
5. The time that the sample was removed from the wash was recorded, and then the sample was transferred by aid of a squirt bottle into a spiral-maze labeled glass petri dish for counting. (For samples that were full of debris or had a high density of organisms, it was often necessary to count the sample in portions).

6. The petri dish was observed under cross polarized light microscopy (CPLM: (Johnson 1995) using a Meiji RZ-APO stereomicroscope fitted with a polarizing plate and a rotating polarizer. We located veligers using cross-polarized light, and examined each veliger under polarized and cross-polarized light for signs of life, using the scoring key above. We did not record empty or shell damaged veligers, or fragments, which were never abundant.
7. The first 100 veligers encountered were placed into 4 categories using the above scoring key:
    a. Umbonal live
    b. Umbo dead
    c. D-stage live
    d. D-stage dead
8. We filtered the remaining water out of the sample and used Modified Buffered Ethanol (MBE: 70% ethanol, 30% aqueous buffer = 50 mM Tris-HCl, 2.5 mM EDTA pH 8.0) to rinse all material into a 20mL scintillation vial. We labeled these vials using the same format used for water samples taken for copper analysis (e.g. label 1A01E: Enclosure (and carboy) **1**, **A**ugust **01**, **E**vening sample). Evening and morning labels were used to distinguish when the sample was taken during the day. This was useful, for example when due to scheduling, a set of enclosures was dosed and a set was harvested on the same day.
9. Steps 2-8 were repeated for the second half of the contents of carboy 0 at (when this used for a second measure of mortality at the end of the day. These steps were also repeated to score carboys 1-6. In each case, we transferred the entire contents of each carboy, and the material rinsed off the larval trap filter when this was used (see below) into the filter collar so that all material of each carboy was preserved in MBE for later estimation of larval density.

Estimation of larval density

Veliger density is an important estimate to make because it provides an indication of the seasonal reproductive status of the population. Veliger sampling must always consider seasonality of reproduction within sampled water bodies. In our case in 2016, we were sampling into mid-August—a month during which veliger concentrations are likely to decline as spawning activity wanes. Data from MCWD surveys of Lake Minnetonka veliger concentrations over the months May-October in 3 years (MCWD 2011, 2012, 2013) confirm, depending on the sampled year, that August is a time of seasonal decline in veliger counts in the bays that they sampled (Lower Lake North, Wayzata and Gray's Bays) that are nearest to Robinson's Bay.

Therefore, we followed trends in veliger concentration in our "ambient control" plankton samples in Robinson's Bay, and checked concentrations in Wayzata Bay in mid August as part of our work to collect veligers for laboratory toxicity studies. [The ambient controls are most representative of densities in the surrounding bay, since ambient controls will not include any of the enclosure effects on veliger concentrations that we encountered (see results)]. Measures of veliger density are very relevant to our procedures in the laboratory. When samples dropped to their lowest densities, we found that counting and scoring larvae for toxicity testing became too difficult and time consuming to complete in a reasonable time frame, leading to higher mortality in controls and our inability to discern effects of the EarthTec, particularly at the lower concentrations that are the focus of this study. This drop in quality with density also occurred because low-density samples must be concentrated more in the lab to allow counting, which shortens the time during which they can be maintained in the laboratory without incurring high levels of mortality in the controls. In addition, our original goal was use the veliger counts taken from density estimates to estimate the proportion of the larval population that consisted of D-stage and umbonal veligers.

To estimate larval density, the material in the 20 ml scintillation vial (from Laboratory sample processing: step 9 above) was poured through a 50-micron Nitex filter, then the filter was rinsed with deionized water (or FLW in some cases) to remove the ethanol. The sample was transferred into a graduated cylinder and resuspended in a total volume of water to allow the veligers to reach a density convenient for counting (determined by trial and error with a given set of samples). Typically, 6 ml volumes were used to make the counts, in triplicate. To do so, the sample in the graduated cylinder was mixed by pouring it between 2 cylinders, then 6 ml was withdrawn with a P5000 (Rainin) Pipetman automated pipetor, and expelled into a 100 or 150 mm glass petri dish with a spiral pattern drawn on the plate bottom. Veligers were counted under cross-polarized and plane-polarized light and placed into 2 categories (D-stage and umbonal: see pg. 5). Total veliger counts and numbers in each category were tallied and concentrations of all counts per liter were calculated, using the dilution factor used to prepare the samples for counting, and the volumes in the towed or pumped sample, or the volume sampled by the larval trap. For the latter, we assumed that volume of water in the cylinder of water above the trap at its position in the enclosure was the volume sampled.

Data analysis

All statistical analyses used *JMP Pro* v 12.0.1 (SAS Institute Inc., Cary NC). For exploring the recovery of larvae from experiments in week 1, I used nonlinear regression of veliger density recovered (Y-variable: veligers counts per liter) on the ppm target dose of Cu^{2+} (X-variable). Excellent fits to negative exponential decay functions were obtained. Fit was assessed according to the Akaike Information Criterion (corrected for small sample sizes), which showed the lowest values (i.e. best fit) to a simple 2-parameter function. More complex non-linear functions, such as one that includes a parameter for an asymptote (i.e. a 3-parameter negative exponential decay function) did not significantly

improve fit. To compare slope and intercept parameters across the fitted functions, I used a comparison of means test, which tests whether the parameters for each of the functions are equal to an overall mean, with upper and lower decision limits set using $\alpha = 0.05$.

Recovery from larval traps in week 2 did not show the steep non-linear decline ("exponential decay") in recovery seen in week 2. Instead the relationships showed very shallow slopes, so I tested whether recovery changed in a linear fashion with increasing dose using linear regression. Tests evaluated whether the linear regression coefficients (regression of recovery on target dose) were different from 0.

For copper concentrations, I addressed the correspondence between readings from the colorimeter and target dose, and between ICP readings and target dose, using linear regression. Values of r^2 and adjusted r^2 were examined to compare this correspondence for concentrations measured at dosing and for concentrations measured at harvest. Correspondence between colorimeter readings and ICP readings was evaluated by calculating the Pearson's *r* between the two data sets, with values (in ppm Cu^{2+}) measured at harvest.

Analysis of dose-response data proceeded as follows. In all analyses of dose-response, survival data was probit-transformed, a typical approach in toxicology. Probit transformation was accomplished by taking the inverse of the standard normal cumulative distribution function of (p), where p = proportion of surviving larvae (with 0.0001 added to all values of p to deal with trials where p =0). First I tested for effects of gear types used to harvest larvae on estimates of larval survival, using analysis of covariance (ANCOVA) on the week 1 data. The dependent variable was probit transformed survival, and the linear covariate was (log-transformed) target dose, with "gear" (plankton net or pump) used as a fixed treatment effect. First I tested the equality of slopes of the two lines (Fig. 8) and found slopes to be homogeneous (this amounts to testing the significance of the interaction between the covariate and the fixed treatment (gear effect). The full ANCOVA (Table 8), with this non-significant interaction removed, was used to test the significance of gear type when adjusted for differences in values of the dose of copper (the linear covariate). This amounts to testing whether the two lines differ in elevation (i.e. have different Y-intercepts), which they did. This implies that pump sampling revealed greater larval mortality than net sampling. A simple explanation is that the pump re-suspended settled, dead larvae from the lake bottom in the enclosure. The other explanation, that the pump caused mortality is less parsimonious, and we did not see fragment of larvae in any of the pumped samples suggesting that the pump was particularly destructive to larvae. We moved on to using the larval traps in any case, so further exploration of this issue was not warranted.

Analysis of dose-response in week 2 data also used probit transformed survival data as the independent (Y) variate as well. Linear regression of these values on log

transformed dose was used to generate the prediction formula (Table 9B), and t tests were used to examine whether slope and intercept values were significantly different from 0. The prediction formula was used to estimate the survival of veliger larvae at high and low doses of EarthTec (Table 9B: the Y-values generated were back transformed from the probit transforms). To estimate LC_{50} and LC_{99} values, I used inverse prediction, which generates the upper and lower 95% confidence intervals as well (Table 9C).

Analysis of exposure-time trials in week 3 used probit transformed survival data as the independent (Y) variate as well, and tested for the effects of treatment group—0.1 ppm EarthTec (treated), 0 ppm EarthTec (untreated), exposure time — 4, 12, 17 hours, and the interaction term—treatment group x exposure time in a Model I Two-Way ANOVA.

IV. Results and discussion

Evaluation of field experimental protocols—harvest methods

The protocols used (Materials and Methods, sections D-E) influenced our ability recover veliger from the water column in field enclosures (Figs 2 and 3). In week 1, we attempted to harvest larvae from enclosures by plankton tow (7/19/16) and by pumping water from the enclosure through a plankton net (7/20 and 7/22/16). On each date, we mixed the contents of the enclosure prior to harvest. On 7/19/16, mixing was manual (by paddle) and on 7/20 and 7/22/16, we used the pump to mix the enclosure contents. Regardless of the details, each date showed a sharp, non-linear decline in the density of veligers obtained in plankton tows or pumped samples (termed "recovery" below), following 16-hour treatment with EarthTec (termed "at harvest" below). Nonlinear regression of veliger density recovered (Y-variable) on the ppm dose of Cu^{2+} (X-variable) yielded highly significant fits to 2-parameter negative exponential decay functions. The formulae for these best-fit lines were:

$$y = a * e^{-bx}$$

where y = veligers/L, x = ppm Cu^{2+}.

The r^2 values from non-linear regression were 0.927, 0.752 and 0.980 (for 7/19, 7/20, and 7/22/16, respectively). The slopes of these regressions (i.e. the *b* parameters in the formula above) were not significantly different from one another (Table 7B). To interpret these analyses, note that the decision limits for regression coefficients were not exceeded (last column, Table 7B), meaning that each of the curves had an equal slope. This result indicates that the drop in recovery was the same regardless of the details of the gear used with this approach. While it is possible that this pattern could be taken advantage of to invent an assay for dose/response in the field, we were concerned about possible bias in the loss of larvae. For example, it is likely that larvae not recovered in the higher doses were more often dead, whereas recovered larvae were more often alive (and still suspended in the water column).

We concluded that moving to an approach in which we captured larvae in traps was warranted. The notion underlying this approach is that trap sampling would be an effective and unbiased way to sample dead and live larvae. A trial that has low mortality should have few larvae passively settling into the trap, and relatively more live larvae captured from the overlying water column as the trap is retrieved. Conversely, a trial with high mortality should show a relatively high number of larvae from that same water column, captured in the trap as they die and sink to the bottom. One possible issue with the traps is lack of knowledge about the volume of water sampled. For example, it is likely that there is horizontal water motion within the enclosures such that there is some sampling of water outside the cylinder of water above the trap. We assumed 0 net horizontal transport of water—i.e. that any gain from horizontal water motion into the cylinder of water above the trap is balanced by loss due to horizontal motion out (see methods for calculation of volumes sampled by traps).

Analysis of larval densities from the trials in which we used larval traps showed that they did in fact solve the issue of bias encountered with pump-sampling in week 1; i.e. density of larvae recovered in week 2 trials did *not* depend on dose of copper applied. Fig. 3 (plot A) shows no trend in recovery of larvae with increasing doses of EarthTec. Linear regression analysis confirmed that none of the regression lines (Fig 3) had slopes that were significantly different from 0. Tests for the significance of regression coefficients verify this: $F_{[1, 4]} = 0.103$ (8/2/16 data), $F_{.05\,[1, 4]} = 0.159$ (8/3/16 data), and $F_{.05\,[1, 4]} = 2.204$ (8/4/16 data); $P >> 0.05$ in each test. Therefore, with trap sampling we have no concern that recovery is dependent upon, and biased by differences in the dose applied. We did find that larval traps within enclosures recovered fewer larvae per unit volume than was achievable from plankton tows in ambient control trials (Fig. 3, see blue shaded box and figure legend for explanation). It cannot be determined whether this is due to enclosure effects on density of larvae (as we saw in week 1: Fig. 2) or to some unknown inefficiencies in capture by the trap of larvae as they settled.

Analysis of copper concentrations

We had 48 measures of copper concentration from the hand-held LaMotte colorimeter that can be compared to the target (intended) dose of copper that we added to the enclosures. These 48 measures were collected at dosing and at harvest. Plotting these data on a linear scale (Fig. 4) reveals that there is more spread in the data (particularly at higher doses) at the time of dosing, suggesting that incomplete mixing leads to spatial variation in concentrations of copper within enclosures; this variation declines by the time of harvest. This is borne out by regression analysis; the r^2 values for regressions of the colorimeter data on target dose were 0.431 at dosing and 0.894 at harvest. The ANOVA in the linear regression analysis was highly significant in both cases ($P < 0.001$); $F_{[1, 47]} = 34.95$ and $F_{[1, 47]} = 387.9$ for dosing and harvest, respectively. The slopes of these lines also show a decline from dosing to harvest, in large part due to declines in the measured values at the highest dose (Fig. 4). Also discernible on this graph is the large number of readings at the lowest doses with the colorimeter that are negative values—showing that the hand held meter fails to accurately read at the lowest doses. This can

also be seen in the plot of colorimeter values vs. ICP values at harvest (Fig. 5). Values from the lab ICP and from the meter are highly correlated with each other (Pearson's r = 0.949), but the metered values are unreliable at the lowest copper concentrations. When the "true value" (by ICP analysis) lies between 0 and 0.1 ppm, the LaMotte meter often delivers a negative reading (Fig. 5), meaning that it is not well suited to monitoring low doses of copper such as would be important in these low dose treatment regimens. It may be possible to gain better sensitivity with other reagent sets.

Otherwise the ICP data, for which there are only 60 values to examine from the field enclosures (30 at dosing, 30 at harvest) showed some similar patterns to the data from the meter. Again, values declined at harvest (Fig. 6), and showed far less scatter (r^2 = 0.934, $P < 0.05$) compared to values measured at dosing (r^2 = 0.204, $P < 0.05$). This again reflects the mixing issue and the patchiness at dosing within the enclosures. Examination of a plot of the ICP values on a logarithmic scale (Fig. 7) shows that, at the highest doses, the actual values measured by ICP were always lower than the target intended dose. This is a real phenomenon that we readily observed in the field. Maintenance of 1-ppm copper dose was never obtained after overnight treatment (see Tables 2 and 4); only about half this dose was achievable. For further analysis of dose/response relationships in week 2 trials, we used the midpoint between the value of ICP-measured copper concentration at dosing and the value at harvest. Analysis of week 1 trials—because we decided not to budget for ICP analysis for this week, due to mixed results and to the exploratory nature of the work in week 1—required a decision to use colorimeter data, or to use the target doses (adjusted for the true volumes of the enclosures). Because the colorimeter delivered negative values at doses ≤ 0.03 ppm, we opted to use the target concentrations.

Larval staining assay results

We found the Fast Green staining assay to not reliably stain larvae that were clearly dead. After 30 minutes staining in Fast Green dye (followed by transfer to fresh FLW), we selected several larvae that lacked any sign of motility, and viewed these for several minutes to confirm that they were not motile and that they showed no signs of movement in internal organs, nor any ciliary motion. Larvae with no motility after protracted observation were often found to not stain with Fast Green. We found the diffuse staining pattern in dead larvae reported by (Stockton-Fiti and Claudi 2016) to be present in a minority of non-motile larvae, and staining was often partial—i.e. only a portion of the internal tissue was stained. In fact, the clearest pattern we found was the presence of the "gut spot" in vigorously swimming larvae that had not been treated with EarthTec. These live larvae never stained with the diffuse pattern. Furthermore, highly active live umbonal-stage larvae not exposed to EarthTec *nearly always* showed the gut spot, whereas highly active D-stage larvae not exposed to EarthTec did not always show the gut spot. On higher magnification, we concluded that these unstained live D-stage larvae had empty guts. With all of this information in hand, we assembled the criteria used in the key for scoring live and dead larvae. It is possible that the mixed results for Fast Green in EarthTec-exposed larvae can be explained by aversive

behavior—closing of the shell valves in response to copper could render the larvae not readily stained by Fast Green. This seems reasonable in that the dye appears to not stain the veliger larvae quickly in any case. Interestingly, the dye is capable of intensely staining internal tissues in several of species of crustacean zooplankton that, also interestingly, appeared to show a whole range of (apparently species-differential) toxicity responses to EarthTec. It is clear that more work is needed to develop a better assay for discriminating dead from live larvae after treatment with EarthTec. For now, we would advocate an examination of larvae under higher magnification using a compound scope in the laboratory. Under a stereomicroscope, Fast Green staining + our scoring key was acceptable alternative.

Analysis of concentration (dose/response) trials: field enclosures

We present results from week 1 first, because of uncertainty with regard to the consequences of the pattern of loss of larvae with increasing doses of EarthTec. The trials run on the first day (7/18/16: Table 1) showed a very shallow decrease in larval survivorship with increasing dose of EarthTec (Fig. 8A: "net"). The methods used on this day (manual mixing of the enclosure, followed by plankton tow to collect larvae) may not have efficiently recovered larvae that died and settled to the bottom of the enclosure. In support of this statement is the earlier finding of declining recovery of larvae from the enclosures with increasing dose. Also we compared the findings from day 1 (7/18/16) to the findings from days 2 and 3 (7/19 and 7/22/16), on which we saw a much greater mortality response to dose (Fig. 8A: "pump"). These data are also plotted with dose log-transformed (X-axis) and response probit-transformed (Y-axis: Fig. 8B). Further analyses of these data by ANCOVA (see Data Analysis for rationale, and Table 8 for results of the ANCOVA) confirm that there were highly significant differences between the gear types—i.e. intercepts of the two regression lines show strong significant differences, with net sampling revealing a higher level of larval survivorship than pump sampling, at the same EarthTec doses. Log (dose) was a highly significant linear covariate, and slopes of the regressions were not different (Table 8)—both of these are assumptions required for ANCOVA.

Next we examined the analysis from week 2 data, in which larvae were collected using traps. We found a stronger dose response in these data. The proportion of veligers surviving dropped to levels near zero at the highest doses, following overnight (mean of 17 hours and 19 minutes) exposure to EarthTec in the field enclosures in week 2 (Fig. 9). Survival data from week 2 also provided an excellent fit in linear regression of probit-transformed survival data on log dose (Fig. 10, Table 9). The linear regression on dose was highly significant (F_s = 44.589, P < 0.001) and explained over 70 % of the variation in veliger survival (r^2 = 0.735) in these enclosures under field conditions. Toxicity estimates for overnight exposure in enclosures were LC_{50} = 0.0189 ppm (18.9 µg/L) and LC_{99} = 0.479 ppm (479 µg/L) Cu^{2+}.

These values can be compared to toxicity estimates for copper sulfate products from the literature cited earlier in this report (Table 10: Kennedy et al. 2006, Claudi et al. 2014).

Useful comparisons can be made; albeit with some caution due to differences between the present study and assays that have previously been conducted. We focus on the comparisons, however, that allow us to address the main goal of this study, and that is to evaluate whether larvae are more sensitive to copper products than later life stages, and whether this pattern holds up under field conditions. First, lethal concentration estimates (LC_{50} and LC_{99} values, expressed in µg/L Cu^{2+}) for zebra mussels can be compared across life stages (Table 10A and B). In larvae compared to trochophores, similar-duration estimates (17.3 hour compared to 24 hour exposures for trochophores) yielded values that are 1.57 – 2.7 fold higher (i.e. *less toxic)* for the larval LC_{50}, and 10.2 - 23.9 fold higher for the larval LC_{99}. This indicates that in fact the larval stage is less sensitive than the earlier, trochophore embryonic stage. In adults compared to larvae, exposure durations which should bias downward—i.e. make more conservative, any differences observed (17.3 hour for larvae compared to 48 hour exposures for adults), yielded values that are still 64.2 fold higher (i.e. *less toxic)* for adult compared to larval values for the LC_{50}, and 18.2 fold higher for adult compared to larval values for the LC_{99}. Admittedly, comparisons are imperfect—i.e. values for earlier (trochophore) and later (adult) stages were estimated using other products (copper sulfate and Cutrine® -Ultra algicide), which can influence bioavailability and thus toxicity. Nevertheless, our study indicates that embryo and larval stages of zebra mussels are far more sensitive than adults to copper products, and these life stage differences persisted under field conditions within a lake.

One other useful comparison can be made, and that is the comparison across life stages for estimates of time to 100% mortality in all studies of EarthTec, that have been performed at comparable doses, to date. Again there are differences between studies (see Table 10C for details) that confound these comparisons, but we have another opportunity here to examine the hypothesis of greater sensitivity of earlier life stages. The comparisons show (Table 10C) that at comparable doses of EarthTec (equivalent to 500 – 610 µg/L of Cu^{2+}), veliger larvae in our study experienced 100% mortality in $1/10^{th}$ the time required to kill 100% of the adults in laboratory studies at Christmas Lake—a lake within 5 kilometers of Robinson's Bay on Lake Minnetonka. Time to 100% mortality in Robinson's Bay was $1/5^{th}$ the time to 100% mortality of zebra mussels in a study at Davis Dam on a California reservoir (Claudi et al. 2014), again indicating the greater sensitivity of larvae to EarthTec. We would expect that these differences would be further magnified if adults were to be studied in field conditions, in which we expect toxicity to be less, but that direct comparison must await further study. A single study of EarthTec toxicity to quagga mussel veligers (Watters et al. 2013), however, demonstrates the marked differences in EarthTec mortality in comparisons between laboratory and field studies that we can expect to see. In that study, Watters et al. (2013) reported 100% quagga mussel veliger mortality in the laboratory assays after just 6 minutes exposure (1.4% of the exposure duration required for zebra mussel veligers in our field enclosures). The quagga mussel study used an EarthTec dose equivalent to 600 µg/L Cu^{2+}; very similar to our dose (Table 9C). We turn now to examine results from exposure time trials in our own field research.

Analysis of exposure-time trials: field enclosures

Exposure time trials were set up in week 3, during which we were successful at testing two additional exposure times (4 hour and 12 hour: Tables 5 - 6). We also set up a 24-hour exposure time trial, but this trial (harvested on 8/11/16) was abandoned due to weather at harvest. A lightning storm created hazardous work conditions. The field crew returned later that day to harvest the larval traps, yielding a 28-hour exposure time, but in the lab we found that the samples were so full of sediment and debris, they were very difficult to score and initial counts of mortality in the controls were difficult to interpret. Therefore, we have available data from 4, 12, and 17-hour exposures (the latter from week 2) to analyze for exposure time effects on veliger survival.

We observed three patterns when plotting these data (Fig. 11). First, mean survival in the control (0 ppm) trials in week 3 was quite low; around 45-50% while it was higher in the previous week (> 60%). Second, there was considerable variation around this mean value (as seen by the wide error bars, Fig. 10). And third, there appeared to be a modest trend of decrease in survival over time from 4 to 17 hours, but again this was partially obscured by the high mortality in the controls in week 3 trials (4 and 12 hours).

Two-way ANOVA on these data confirmed our suspicions—there were no statistically significant effects of exposure time, of treatment group, and the interaction effect was also not significant (Table 11). Future research needs are clearly the greatest for a re-examination of the effects of exposure time. The trend observed suggests that they will be discernible in these field experiments. However, our suspicion is that we need to extend the total duration over which exposure time is examined—to a scale of days and not hours—both for logistical reasons and for management implications. EarthTec open water treatments typically last a week when adults are targeted, and it is possible that low-dose treatments could extend through several weeks of the reproductive season (e.g. during peak settlement). The experimental protocol will require expanding the size of the enclosures used. Enclosures of roughly 150 ft^3 dimensions are not large enough to readily allow exposures over 24 hours. We conclude this because (1) there is a discernible drop in DO after 1 day, and more importantly (2) control mortalities are considerable in these enclosures. We also would conclude that field experiments earlier in the season, when veliger concentration are higher, would increase the signal: noise ratios. There was a clear increase in control mortality, even in the ambient controls, as we progressed through August and veliger concentrations in the bay declined.

Results and analyses not shown in this summary version:

1. *Larval density patterns in ambient control sample*
2. *Analyses of the proportion of D-stage and umbonal veliger*
3. *Results from the laboratory assays in week 4*

V. Acknowledgements

Jill Sweet and Eric Fieldseth with the Minnehaha Creek Watershed District (MCWD) AIS Program arranged to purchase materials, designed and built the enclosures, and scrapped an earlier prototype to end up with structures that worked for the duration of this study. Without their lead on the field portions of the project, and without their assistance during dosing and harvest of the field experiments, again we could not have completed the work. We are also grateful to the "in-lake crew" (Jill, Marcie, et al.) at MCWD who worked long hours and tolerated the grinding work schedule required to move these enclosures back and forth, lakeshore to offshore, so that we could run this large number of replicate trials. MCWD provided boat support and access to laboratory facilities and the district office near Lake Minnetonka, which simplified logistics considerably. Maxwell Kleinhans at the University of Minnesota (UMN) contributed to virtually all aspects of this study. Max assisted with fieldwork, laboratory toxicity assays, laboratory processing and scoring of veligers, and helped with getting water samples analyzed. The Research Analytical Laboratory provided ICP analysis. Funding was provided by an AIS Prevention Program grant from Hennepin County to MCWD; UMN subcontract # CON000000061500. Private donations to the Minnesota Aquatic Invasive Species Research Center provided a portion of the salary funds.

VI. Tables

Table 1. EarthTec QZ enclosures in Robinson's Bay, Lake Minnetonka, MN. Parameters measured at dosing: Dose/response trials, week 1.

Target $[Cu^{2+}]$ (ppm)	[EarthTec QZ] (ppm)	Enclosure Number	Date	Dosing Time	Measured Depth[1]	Enclosure dimension s^2 (ft^3)	Enclosure volume (L)[2]	Enclosure volume (gal)[2]	EarthTec QZ Applied (mL)	Nominal $[Cu^{2+}]$[2]	[Cu2+] at dosing - colorimeter (ppm)	[Cu2+] from ICP analysis[3] (ppm)	DO at dosing, (mg/L)	Water Temp at dosing, measured (°C)	Mean temp at dosing (°F) (°C)
1	16.7	3	7/18/16	18:36	N/A	150.0	4247.5	1122.1	70.8	1.000	2.26	N/A			79.5
0.3	5.6	6	7/18/16	18:45	N/A	150.0	4247.5	1122.1	23.6	0.333	0.16	N/A			26.4
0.1	1.8	1	7/18/16	18:30	N/A	150.0	4247.5	1122.1	7.9	0.111	0.05	N/A		26.41	
0.03	0.62	2	7/18/16	18:33	N/A	150.0	4247.5	1122.1	2.6	0.037	0.03	N/A			
0.01	0.21	4	7/18/16	18:39	N/A	150.0	4247.5	1122.1	0.9	0.013	-0.02	N/A			
Control	0	5	7/18/16	18:42	N/A	150.0	4247.5	1122.1	0.0	0.000	0	N/A			
1	16.7	3	7/19/16	16:50	N/A	150.0	4247.5	1122.1	70.8	1.000	0.46	N/A	8.5	25.6	78.0
0.3	5.6	1	7/19/16	16:31	N/A	150.0	4247.5	1122.1	23.6	0.333	0.32	N/A	8.2	25.5	25.5
0.1	1.8	6	7/19/16	17:07	N/A	150.0	4247.5	1122.1	7.9	0.111	0.17	N/A	8.7	25.4	
0.03	0.62	2	7/19/16	16:43	N/A	150.0	4247.5	1122.1	2.6	0.037	0.05	N/A	8.9	25.7	
0.01	0.21	5	7/19/16	17:00	N/A	150.0	4247.5	1122.1	0.9	0.013	0.04	N/A	9.0	25.5	
Control	0	4	7/19/16	16:55	N/A	150.0	4247.5	1122.1	0.0	0.000	0.02	N/A	8.1	25.5	
1	16.7	1	7/21/16	16:09	6'7"	164.6	4660.5	1231.2	70.8	0.911	1.43	N/A			
0.3	5.6	5	7/21/16	16:19	6'10"	170.8	4837.5	1277.9	23.6	0.292	0.2	N/A			
0.1	1.8	6	7/21/16	16:22	6'8"	166.7	4719.5	1246.8	7.9	0.100	0.05	N/A			
0.03	0.62	2	7/21/16	16:12	6'7"	164.6	4660.5	1231.2	2.6	0.034	-0.06	N/A			
0.01	0.21	3	7/21/16	16:15	6'10"	170.8	4837.5	1277.9	0.9	0.011	0.02	N/A			
Control	0	4	7/21/16	16:17	6'10"	170.8	4837.5	1277.9	0.0	0.000	0.01	N/A			

Notes

[1]Actual depth measures were not taken on 7/18 and 7/19/16

[2]Enclosure dimensions, volumes and nominal $[Cu^{2+}]$ for 7/18 and 7/19/16 are estimated assuming 6' 0" depth

[3]ICP (Inductively Coupled Plasma Atomic Emission Spectrometry) analyses of Cu^{2+} concentration were not obtained for July trials

Column headings: $[Cu^{2+}]$ = concentration of copper (cupric) ion; [EarthTec QZ] = concentration of EarthTec QZ; "Nominal" $[Cu^{2+}]$ was calculated to express the intended dose, adjusted to the true enclosure volume; DO = dissolved oxygen. Colorimeter used on site to estimate $[Cu^{2+}]$ is a LaMotte copper colorimeter.

Table 2. EarthTec QZ enclosures in Robinson's Bay, Lake Minnetonka, MN. Parameters measured at harvest: Dose/response trials, week 1.

Target $[Cu^{2+}]$ (ppm)	[EarthTec QZ] (ppm)	Enclosure Number	Harvest Date	Harvest Start Time	Harvest End Time	Target Exposure Time (hr:min)	Actual Total Exposure Time (hr:min)	$[Cu^{2}+]$ at harvest, meter (ppm)	$[Cu^{2+}]$ at harvest, ICP analysis (ppm)	Water Temp at harvest, measured	Mean Temp at harvest (°F) (°C)	DO at harvest (mg/L)
1	16.7	3	7/19/16	11:38	11:46	16:00	17:02	0.39/0.42	N/A	25.52	78.1	8.47
0.3	5.6	6	7/19/16	12:15	12:26	16:00	17:30	0.14	N/A	25.59	25.6	8.01
0.1	1.8	1	7/19/16	10:30	11:09	16:00	16:00	0.14	N/A	25.56		8.7/8.42
0.03	0.62	2	7/19/16	11:20	11:36	16:00	16:47	0.02	N/A	25.66		7.65
0.01	0.21	4	7/19/16	11:50	12:00	16:00	17:11	-0.01	N/A	25.61		7.73
0	0	5	7/19/16	12:03	12:12	16:00	17:21	0.02/0.01	N/A			
1	16.7	3	7/20/16	10:05	10:18	16:00	17:15	0.58	N/A	24.8	76.7	7.5
0.3	5.6	1	7/20/16	8:52	9:05	16:00	16:21	0.16	N/A	24.8	24.8	7.7
0.1	1.8	6	7/20/16	11:00	11:12	16:00	17:53	0.12	N/A	24.8		7.2
0.03	0.62	2	7/20/16	9:15	9:25	16:00	16:32	0.04	N/A	24.8		7.3
0.01	0.21	5	7/20/16	10:48	10:58	16:00	17:48	0.01	N/A	25		7.7
0	0	4	7/20/16	10:23	10:45	16:00	17:28	0.01	N/A	24.8		6.9
1	16.7	1	7/22/16	9:25	9:30	16:00	17:16	0.62/0.47	N/A	79.6 F	79.6	
0.3	5.6	5	7/22/16	10:10	10:17	16:00	17:51	0.13	N/A	26.4 C	26.4	
0.1	1.8	6	7/22/16	10:19	10:30	16:00	17:57	0.05	N/A			
0.03	0.62	2	7/22/16	9:35	9:40	16:00	17:23	0.01	N/A			
0.01	0.21	3	7/22/16	9:48	9:54	16:00	17:33	0.01	N/A			
0	0	4	7/22/16	9:58	10:09	16:00	17:41	0.06/0.05	N/A			

Notes

ICP (Inductively Coupled Plasma Atomic Emission Spectrometry) analyses of Cu^{2+} concentration were not obtained for July trials

Column headings: Harvest start time and end times are given to document that plankton tow and pump sampling from enclosures was much more time consuming that larval trap sampling (in August)

Table 3. EarthTec QZ enclosures in Robinson's Bay, Lake Minnetonka, MN. Parameters measured at dosing: Dose/response trials, week 2.

Target [Cu^{2+}] (ppm)	[EarthTec QZ] (ppm)	Enclosure Number	Date	Dosing Time	Measured Depth	Enclosure dimensions (ft^3)	Enclosure volume (L)	Enclosure volume (gal)	EarthTec QZ Applied (mL)	Nominal [Cu^{2+}]	[Cu2+] at dosing, meter (ppm)	[Cu2+] at dosing, ICP analysis (ppm)
1	16.7	5	8/1/16	15:46	6'4"	158.3	4483.5	1184.4	70.8	0.947	0.850	0.820
0.3	5.6	4	8/1/16	13:39	6'6"	162.5	4601.5	1215.6	23.6	0.307	0.600	0.258
0.1	1.8	1	8/1/16	15:15	6'6"	162.5	4601.5	1215.6	7.9	0.102	0.050	0.094
0.03	0.62	2	8/1/16	15:23	6'6"	162.5	4601.5	1215.6	2.6	0.034	0.050	0.037
0.01	0.21	3	8/1/16	15:31	6'7"	164.6	4660.5	1231.2	0.9	0.012	0.010	0.014
0	0	6	8/1/16	15:53	6'4"	158.3	4483.5	1184.4	0.0	0.000	0.050	0.004
1	16.7	6	8/2/16	16:38	6'5"	160.4	4542.5	1200.0	70.8	0.935	4.910	0.507
0.3	5.6	5	8/2/16	16:32	6'6"	162.5	4601.5	1215.6	23.6	0.307	0.230	0.219
0.1	1.8	2	8/2/16	16:14	6'7"	164.6	4660.5	1231.2	7.9	0.101	0.180	0.069
0.03	0.62	3	8/2/16	16:22	6'7"	164.6	4660.5	1231.2	2.6	0.034	0.110	0.036
0.01	0.21	1	8/2/16	16:09	6'7"	164.6	4660.5	1231.2	0.9	0.012	-0.020	0.008
0	0	4	8/2/16	16:27	6'4"	158.3	4483.5	1184.4	0.0	0.000	0.000	0.017
1	16.7	2	8/3/16	15:21	6'1"	152.1	4306.5	1137.7	70.8	0.986	0.110	0.400
0.3	5.6	6	8/3/16	15:37	5'11"	147.9	4188.5	1106.5	23.6	0.338	0.060	0.032
0.1	1.8	1	8/3/16	15:20	6'1"	152.1	4306.5	1137.7	7.9	0.109	0.390	1.710
0.03	0.62	3	8/3/16	15:26	6'	150.0	4247.5	1122.1	2.6	0.037	-0.010	0.017
0.01	0.21	4	8/3/16	15:31	6'5"	160.4	4542.5	1200.0	0.9	0.012	-0.040	0.011
0	0	5	8/3/16	15:35	5'11"	147.9	4188.5	1106.5	0.0	0.000	-0.040	0.016

Table 4. **EarthTec QZ enclosures in Robinson's Bay, Lake Minnetonka, MN. Parameters measured at harvest: Dose/response trials, week 2.**

Target $[Cu^{2+}]$ (ppm)	[EarthTec QZ] (ppm)	Enclosure Number	Harvest Date	Harvest Start Time	Target Exposure Time (hr:min)	Actual Total Exposure Time (hr:min)	$[Cu^{2}+]$ at harvest, meter (ppm)	$[Cu^{2+}]$ at harvest, ICP analysis (ppm)	Water Temp at harvest (°F) (°C)	DO at harvest (mg/L)
1	16.7	5	8/2/16	8:42	16:00	16:56	0.460	0.402	78.5	
0.3	5.6	4	8/2/16	8:36	16:00	18:57	0.190	0.167	25.8	
0.1	1.8	1	8/2/16	8:17	16:00	17:02	0.080	0.065		
0.03	0.62	2	8/2/16	8:23	16:00	17:00	0.080	0.025		
0.01	0.21	3	8/2/16	8:30	16:00	16:59	0.040	0.012		
0	0	6	8/2/16	8:49	16:00	16:56	-0.010	0.004		
1	16.7	6	8/3/16	8:57	16:00	16:19	0.290	0.257	81.9	
0.3	5.6	5	8/3/16	8:51	16:00	16:19	0.220	0.191	27.2	
0.1	1.8	2	8/3/16	8:39	16:00	16:25	0.070	0.068		
0.03	0.62	3	8/3/16	8:42	16:00	16:20	0.020	0.026		
0.01	0.21	1	8/3/16	8:34	16:00	16:25	-0.030	0.013		
0	0	4	8/3/16	8:47	16:00	16:20	0.070	0.018		
1	16.7	2	8/4/16	10:03	16:00	18:42	0.490	0.378	80.5	
0.3	5.6	6	8/4/16	10:18	16:00	18:41	0.130	0.105	26.9	
0.1	1.8	1	8/4/16	9:59	16:00	18:39	0.040	0.058		
0.03	0.62	3	8/4/16	10:06	16:00	18:40	0.020	0.023		
0.01	0.21	4	8/4/16	10:11	16:00	18:40	0.060	0.010		
0	0	5	8/4/16	10:14	16:00	18:39	-0.010	0.012		

Table 5. EarthTec QZ enclosures in Robinson's Bay, Lake Minnetonka, MN. Parameters measured at dosing: Exposure time trials, week 3.

Target $[Cu^{2+}]$ (ppm)	[EarthTec QZ] (ppm)	Enclosure Number	Date	Dosing Time	Measured Depth	Enclosure dimensions (ft^3)	Enclosure volume (L)	Enclosure volume (Gal)	EarthTec QZ Applied (mL)	Nominal $[Cu^{2+}]$	[Cu2+] at dosing, meter (ppm)	[Cu2+] at dosing, ICP analysis (ppm)	Water temp at dosing (°F) (°C)
0.1	1.8	1	8/8/16	9:20	6'2"	154.2	4365.5	1153.2	7.9	0.109	N/A[4]	0.034	78.0
0	0	2	8/8/16	9:26	6'3"	156.3	4424.5	1168.8	0	0.000	N/A[4]	0.006	25.6
0.1	1.8	3	8/8/16	9:30	6'6"	162.5	4601.5	1215.6	7.9	0.103	N/A[4]	0.145	
0.1	1.8	4	8/8/16	9:33	6'4"	158.3	4483.5	1184.4	7.9	0.106	N/A[4]	0.079	
0	0	5	8/8/16	9:37	6'7"	164.6	4660.5	1231.2	0	0.000	N/A[4]	0.001	
0	0	6	8/8/16	9:40	6'5"	160.4	4542.5	1200.0	0	0.000	N/A[4]	0.001	
0.1	1.8	1	8/9/16	19:02	6'4"	158.3	4483.5	1184.4	7.9	0.106	0.140	0.103	79.0
0	0	2	8/9/16	19:06	6'2"	154.2	4365.5	1153.2	0	0.000	-0.010	0.003	26.1
0.1	1.8	3	8/9/16	19:10	6'8"	166.7	4719.5	1246.8	7.9	0.100	0.110	0.059	
0	0	4	8/9/16	19:15	6'5"	160.4	4542.5	1200.0	0	0.000	0.020	0.003	
0	0	5	8/9/16	19:18	6'	150.0	4247.5	1122.1	0	0.000	0.020	0.002	
0.1	1.8	6	8/9/16	19:20	6'6"	162.5	4601.5	1215.6	7.9	0.103	0.160	0.029	
0	0	1	8/10/16	9:24	6'2"	154.2	4365.5	1153.2	0	0.000	-0.020		79.2
0	0	2	8/10/16	9:29	5'10"	145.8	4129.5	1090.9	0	0.000	-0.020		26.2
0.1	1.8	3	8/10/16	9:34	5'7"	139.6	3952.6	1044.2	7.9	0.120	0.100		
0.1	1.8	4	8/10/16	9:38	6'1"	152.1	4306.5	1137.7	7.9	0.110	0.040		
0	0	5	8/10/16	9:43	6'5"	160.4	4542.5	1200.0	0	0.000	0.040		
0.1	1.8	6	8/10/16	9:46	6'5"	160.4	4542.5	1200.0	7.9	0.104	0.250		

Notes:

[4]Meter readings were not available for 8/8/16 because we ran out of reagent on site.

We also did not analyze Cu^{2+} concentrations for the trial begun on 8/10/16 because this trial was abandoned due to weather at harvest.

Table 6. EarthTec QZ enclosures in Robinson's Bay, Lake Minnetonka, MN. Parameters measured at harvest: Exposure time trials, week 3.

Target $[Cu^{2+}]$ (ppm)	[EarthTec QZ] (ppm)	Enclosure Number	Harvest Date	Harvest Start Time	Target Exposure Time (hr:min)	Actual Total Exposure Time (hr:min)	$[Cu^{2}+]$ at harvest, meter (ppm)	$[Cu^{2+}]$ at harvest, ICP analysis (ppm)	Water Temp at harvest (°F) (°C)	DO at harvest (mg/L)
0.1	1.8	1	8/8/16	13:28	4:00	4:08	-0.040	0.058	81.0	
0	0	2	8/8/16	13:36	4:00	4:10	-0.060	0.006	27.2	
0.1	1.8	3	8/8/16	13:41	4:00	4:11	0.110	0.060		
0.1	1.8	4	8/8/16	13:45	4:00	4:12	0.020	0.060		
0	0	5	8/8/16	13:50	4:00	4:13	0.050	< 0.001		
0	0	6	8/8/16	13:53	4:00	4:13	0.020	0.002		
0.1	1.8	1	8/10/16	7:30	12:00	12:28	0.100	0.063	81.0	
0	0	2	8/10/16	7:33	12:00	12:27	0.000	0.004	27.2	
0.1	1.8	3	8/10/16	7:37	12:00	12:27	0.010	0.056		
0	0	4	8/10/16	7:41	12:00	12:26	0.020	0.062		
0	0	5	8/10/16	7:45	12:00	12:27	0.050	0.002		
0.1	1.8	6	8/10/16	7:48	12:00	12:28	0.000	0.041		
0	0	1	8/11/16		24:00					
0	0	2	8/11/16		24:00					
0.1	1.8	3	8/11/16		24:00					
0.1	1.8	4	8/11/16		24:00					
0	0	5	8/11/16		24:00					
0.1	1.8	6	8/11/16		24:00					

The trial harvested on 8/11/16 was abandoned due to weather at harvest. A lightning storm created hazardous work conditions. The field crew harvested the larval traps, but in the lab the samples were full of sediment and debris, which made them impossible to score.

Table 7. Parameter estimates and multiple comparisons between non-linear regression coefficients, analysis of veliger recovery, week 1

A. Parameter Estimates

Parameter	Group	Estimate	Std Error	Lower 95%	Upper 95%
a_1	07/19/2016	24.697053	2.3357441	20.119079	29.275028
b_1	07/19/2016	-17.97191	5.3209986	-28.40087	-7.542942
a_2	07/20/2016	12.783439	2.1638015	8.5424663	17.024413
b_2	07/20/2016	-11.82108	6.656013	-24.86663	1.224466
a_3	07/22/2016	8.509584	2.4541867	3.6994664	13.319702
b_3	07/22/2016	-23.34678	20.345438	-63.22311	16.529544

B. Comparison of means for values of *b* (regression coefficients)

Group	Lower Decision Limit	b	Upper Decision Limit	Limit Exceeded
07/19/2016	-28.1304	-17.9719	-3.63976	No
07/20/2016	-31.2027	-11.8211	-0.56747	No
07/22/2016	-62.7064	-23.3468	30.93623	No

Table 8. Probit analysis of dose/response in field enclosures, week 1. Analysis of covariance (ANCOVA)

Source of variation	***df***	***SS***	***MS***	F_s
Sampling gear type (net, pump)	1	9.4829	9.4829	30.726***
Log dose (covariate)	1	5.4159	5.4159	17.548***
Error	15	4.6294	0.30863	
Total	17	19.414		

***$P < 0.001$

Homogeneity of slopes test was not significant [i.e. test of significance of interaction term (Log dose x Gear type)]:

$F_{.05[1,15]} = 2.5107$, $P > 0.05$

Table 9. Probit analysis of dose/response in field enclosures, week 2. Regression statistics and toxicity estimates.

A. ANOVA table: regression

Source of varation	*df*	*SS*	*MS*	F_s
Explained: linear regression	1	18.516	18.516	44.589***
Error	16	6.644	0.415	
Total	17	25.160		

****P* << 0.001

B. Regression statistics

Line of fit (prediction formula):
Probit (p + .0001) = $a - b_{Y \cdot X}$ [Log midpoint (Cu^{2+})], where
p = proportion of surviving veligers

Parameter estimates

Term	Estimate	Std Error	t_s	*P*
a	-2.855	0.3588	-7.958	< 0.001
$b_{Y \cdot X}$	-1.655	0.2479	-6.677	< 0.001

Estimated levels of veliger mortality at recommended EarthTec doses

ppm Cu^{2+}	Estimated (17-hour) veliger survival	Recommended dose rate in open waters	EarthTec QZ™ label reference
1.0	0.002	Maximum	Environmental Protection Agency (US EPA), 2015
0.060	0.202	Lowest	EPA 2015

C. Toxicity estimates

Probit (prop. Surviving veligers)	Log estimate (log (ppm))]	Std Error	Lower 95%	Upper 95%	Estimate (ppm)
0	Log (LC_{50}) = -1.72491	0.1106875	-1.94186	-1.50797	LC_{50} = 0.0188
-2.32635	Log (LC_{99}) - 0.319762	0.1745578	-0.661889	0.0223649	LC_{99} = 0.4788

Table 10. Comparison of toxicity estimates for EarthTec (this study) to estimates from earlier studies of toxicity of copper sulfate products to dreissenid mussels.

A. Lethal concentration (LC_{50}) estimates

Chemical agent	Species[1]	Assay description	Life stage	Duration	Estimate[2]	Reference
EarthTec	ZM	Field (in-lake enclosures)	Veliger (D-stage to umbonal)	17 hour	18.9	Present study
$CuSO_4$	ZM	Laboratory culture	Trochophore (72 hr post-fertilization	24 hour	7	
Cutrine-Ultra	ZM	Laboratory culture	Trochophore (72 hr post-fertilization)	24 hour	12	
Cutrine-Ultra	ZM	Laboratory (glass chambers)	Adult	48 hour	1214	

B. Lethal concentration (LC_{99}) estimates

Chemical agent	Species[1]	Assay description	Life stage	Duration[2]	Estimate[2]	Reference
EarthTec	ZM	Field (in-lake enclosures)	Veliger (D-stage to umbonal)	17 hour	479	Present study
$CuSO_4$	ZM	Laboratory culture	Trochophore (72 hour post-fertilization	24 hour	20	
Cutrine-Ultra	ZM	Laboratory culture	Trochophore (72 hour post-fertilization)	24 hour	47	
Cutrine-Ultra	ZM	Laboratory (glass chambers)	Adult	48 hour	8780	

[1]ZM = zebra mussel
[2]Durations for toxicity exposures in previous studies were those closest to exposure durations in the present study
[3]Toxicity estimates are in µg/L free Cu^{2+}

C. Time to mortality estimates for EarthTec

Time to 100% mortality

Chemical agent	Species[1]	Assay description	Life stage	Dose[2]	Time[3]	Reference
EarthTec	ZM	Field (in-lake enclosures)	Veligers (Lake Minnetonka, Hennipen Co. MN)	500-610	17 hour: 19 minutes	Present study
EarthTec	ZM	Laboratory (plastic chambers in aquaria)	Adults (Christmas Lake, Hennepin Co. MN)	500	168 hour	Lund et al. in review
EarthTec	ZM	Laboratory (mesh bags in glass beakers)	Adult (Davis Dam, CA reservoir)	500	84	Claudi et al. 2014
EarthTec	QM	Laboratory (glass petri dishes)	Veligers (Lake Mead, NV)	600	6 minutes	Waters et al. 2013

[1]ZM = zebra mussel, QM = quagga mussel

[2]Doses are in are in μg/L free Cu^{2+}

[3]Time to 100% mortality in the present study is not based on statistical fitting of mortality (e.g. time-to-event functions), but on single trials at this high dose, and the time might be overestimated (since earlier times were not examined). Mortality in Lund et al. (in review) was recorded on days 0,2, 3, 4, 7 and 8 and 100% mortality was first reached at day 7 in aquaria.

Table 11. Two-way ANOVA: effects of exposure time on veliger survival from field enclosure experiments.

Source of variation	*df*	*SS*	*MS*	F_s
Treatment (Control vs. 0.1ppm Cu^{2+})	1	1.0801	1.0801	2.2861 *ns*
Exposure Time	2	0.0641	0.0320	0.0678 *ns*
Treatment x Exposure Time	2	0.3962	0.3962	0.4193 *ns*
Error (within subgroups)	12	5.6695	0.4724	
Total	17	7.2099		

ns = not significant ($P > 0.05$)

VII. Figures

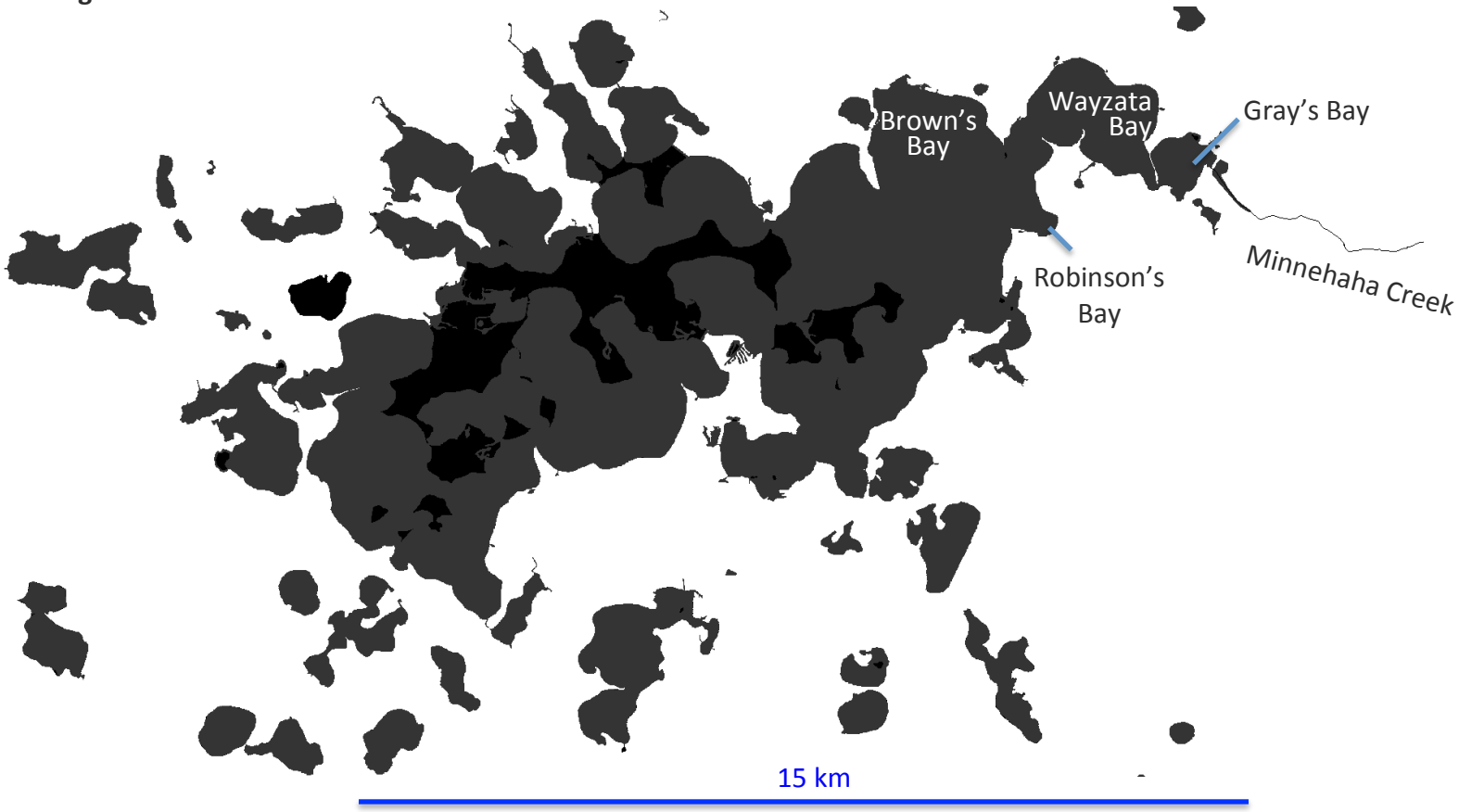

Figure 1A. Map of Lake Minnetonka, Hennepin Co. MN, showing the location of Robinson's Bay and other nearby bays in the eastern portion of the lake. The map was created from MN lake outline maps, an ArcGIS layer available from MN Geospatial Commons (https://gisdata.mn.gov).

Figure 1B. Detailed map of Robinson's Bay. Red squares mark approximate location of enclosures used in the veliger toxicity study. The black squares mark the approximate positions where **ambient control** plankton tows were taken during weeks 1 (lower square) and weeks 2 and 3 (upper square). The white cross marks the location of Robinson's Bay Beach. This topographic map was downloaded from the USGS National Hydrography Viewer

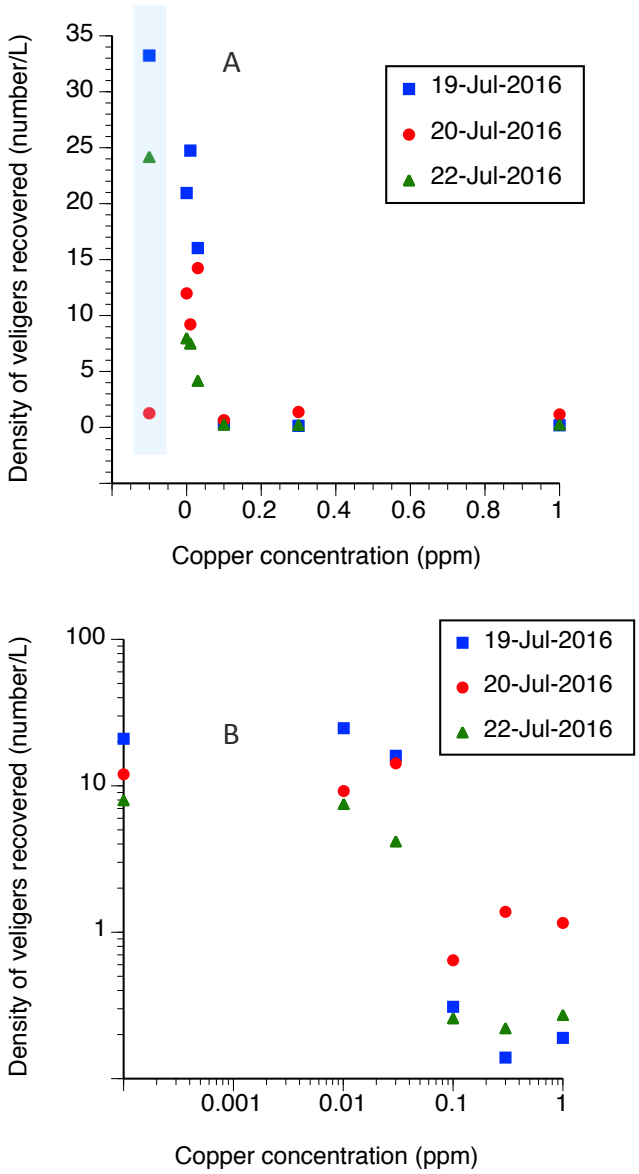

Figure 2. Density of veliger larvae harvested from week-1 enclosures (using plankton tows and pump-harvesting). A. Plot with linear axes, **B.** Plot with logarithmic axes to better visualize the steep decline in veliger density as a function of dose from 0 (points on the Y-axis) to 1 ppm Cu^{2+}. Copper concentration (in ppm) on the X-axes represents the intended target concentration. Data points to the left of zero in plot A (in blue shaded box) represent veliger density in a vertical plankton tow (7/19/16) or in samples pumped (7/20 and 7/22/16) from outside of, but in the immediate vicinity of the enclosures (see **ambient control trial 0:** Materials and Methods E.3).

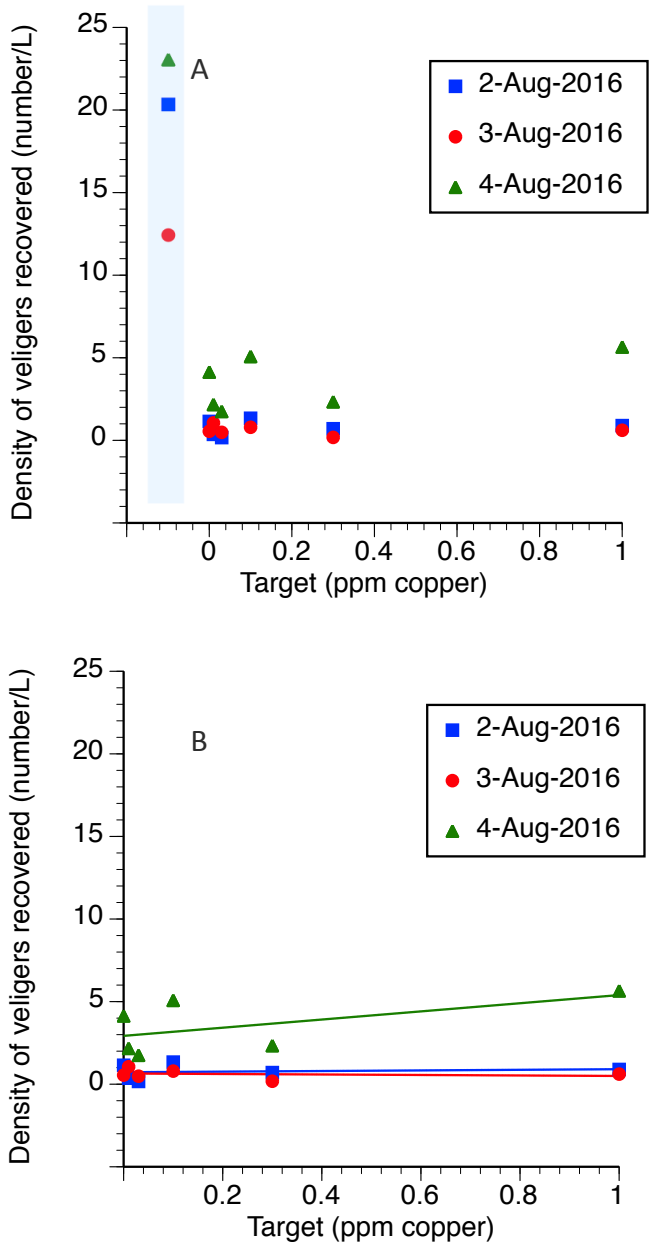

Figure 3. Density of veliger larvae harvested from week-2 enclosures (using larval traps). A. Plot with linear axes, **B.** Plot with linear regression lines added. Copper concentration (in ppm) on the X-axes represents the intended target concentration. Data points to the left of zero (in blue shaded box, plot A) represent veliger density estimated from vertical plankton tows from outside of the enclosures in Robinson's Bay (see **ambient control trial 0:** Materials and Methods I.6). None of the regression lines in B have slopes that are significantly different from zero, confirming the lack of dependence of veliger recovery on copper dose.

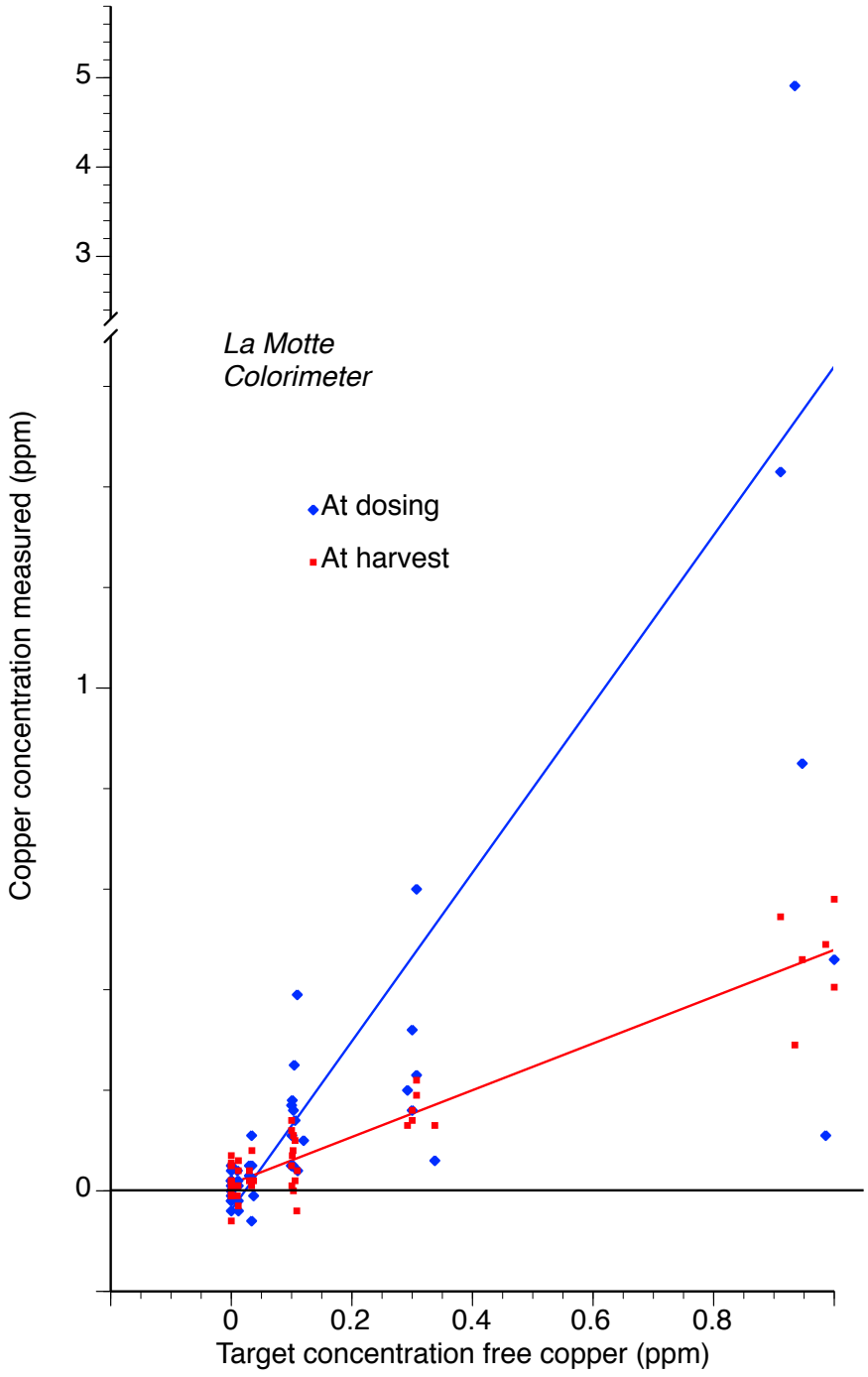

Figure 4. Plot of copper concentrations measured with the LaMotte colorimeter as a function of intended (target) concentration of free copper added to the enclosures. The black horizontal line marks the zero line for measured values; note the numerous negative values measured with the meter at doses of 0, 0.01, and .03 ppm. The colored lines are from linear regression. See text for further explanation.

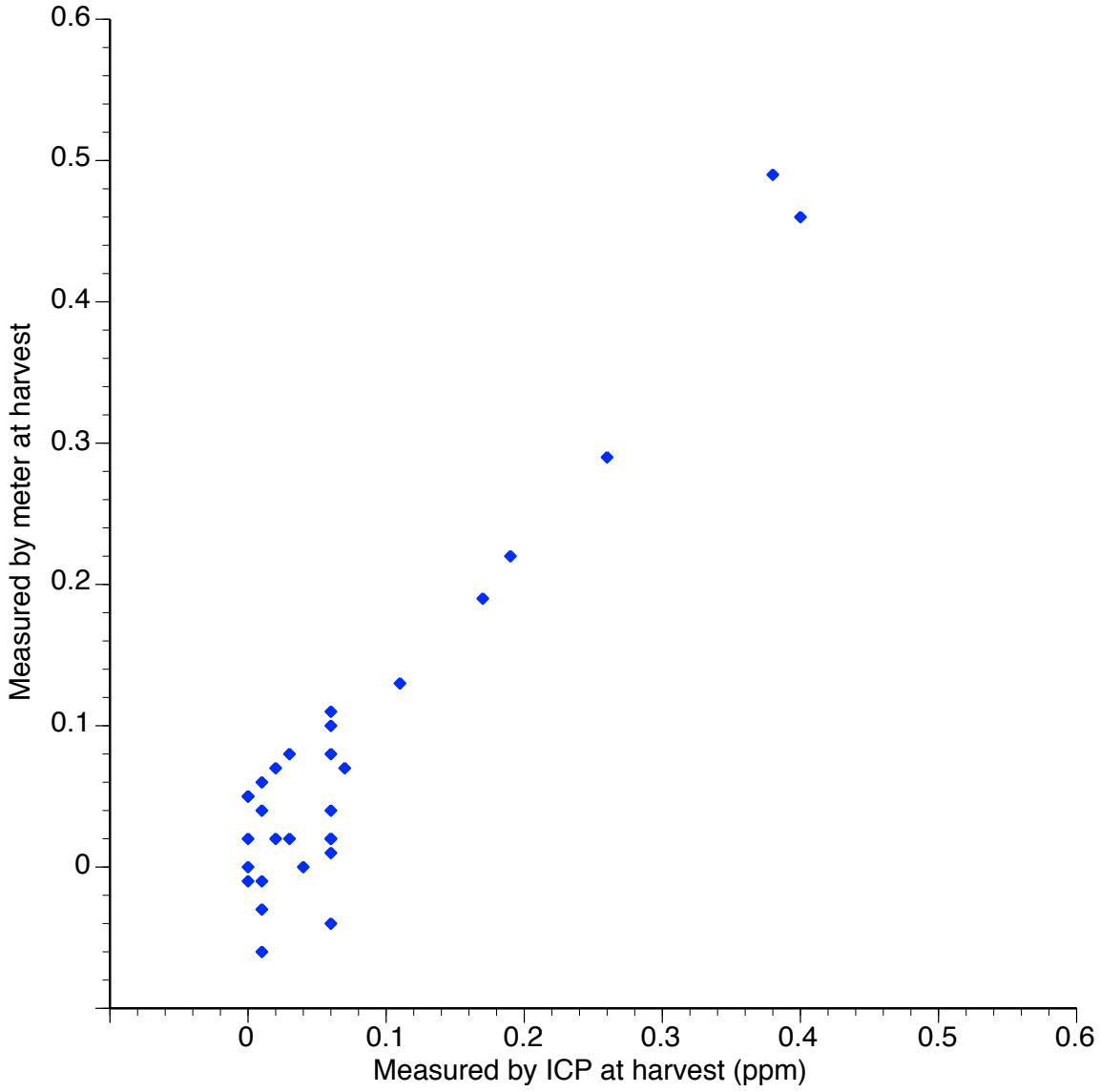

Figure 5. Plot of correspondence between copper concentrations measured with the LaMotte colorimeter by laboratory ICP spectroscopy analysis at harvest. These two sets of values are highly correlated (see text for further explanation).

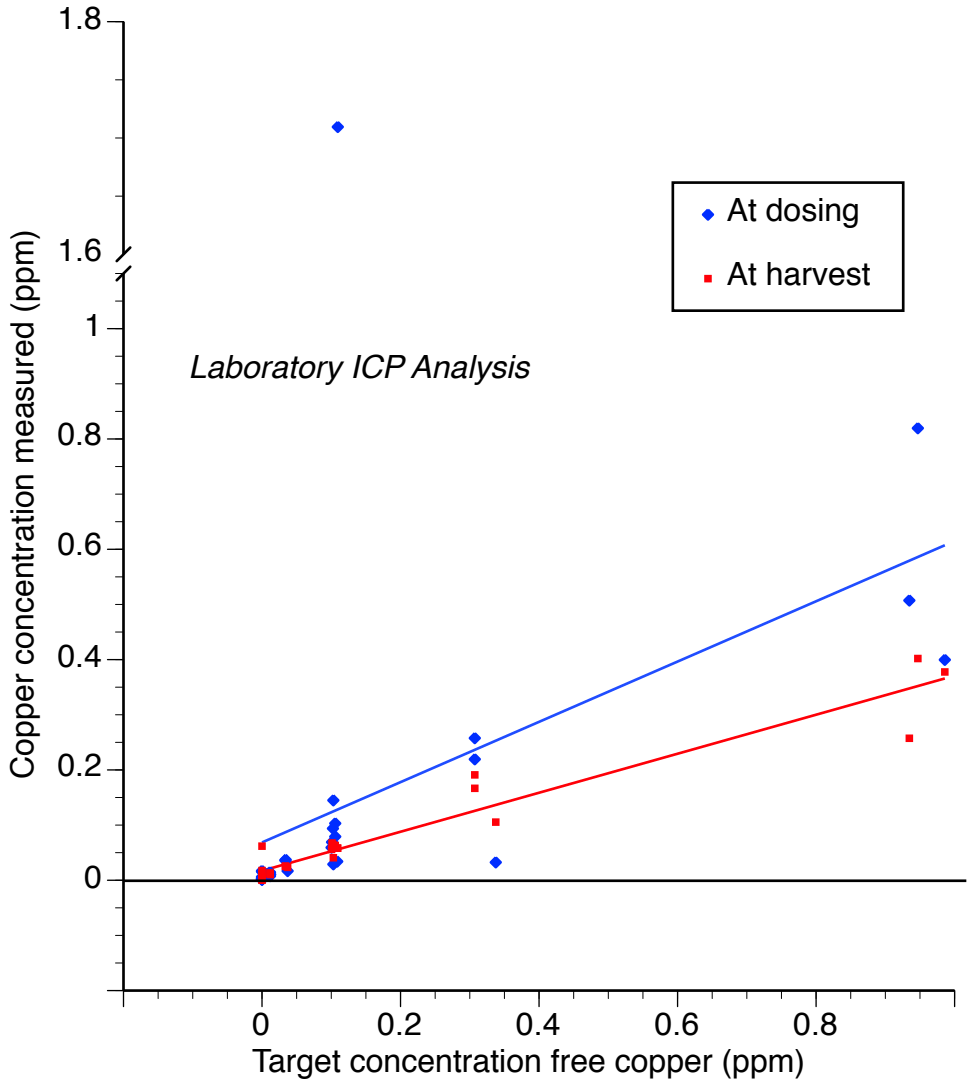

Figure 6. Plot of copper concentrations measured in the laboratory ICP analysis as a function of target concentration of free copper added to the enclosures. The black horizontal line marks the zero line for measured values. The colored lines are from linear regression. See text for further explanation.

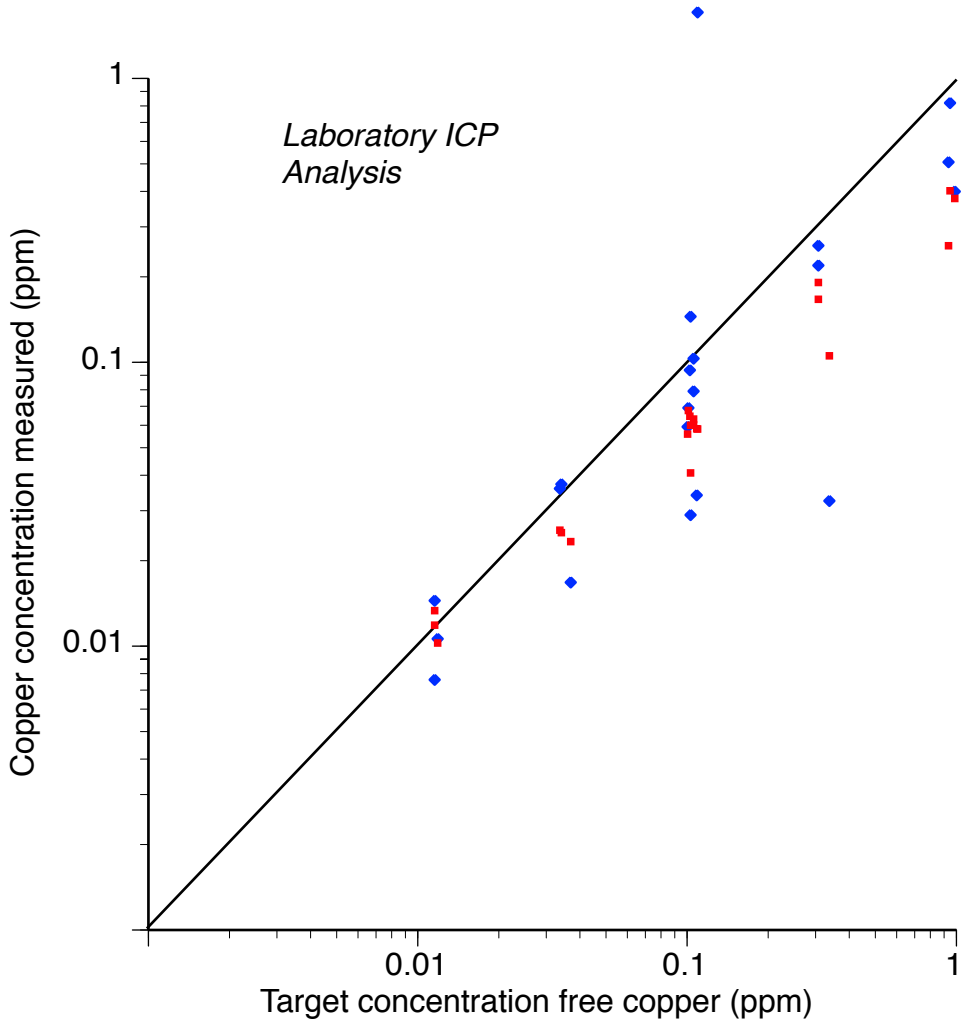

Figure 7. Plot of copper concentrations measured in the laboratory ICP analysis as a function of target concentration of free copper added to the enclosures. The black diagonal marks the line where target doses and measured values would be equal; note measured concentration at the two highest doses are all below this line. The colored lines are from linear regression. See text for further explanation.

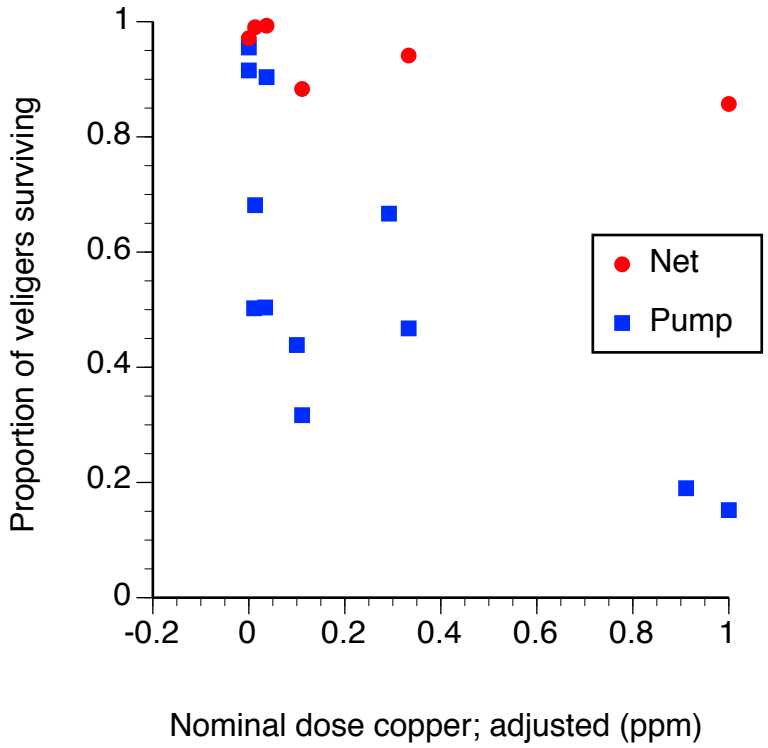

Figure 8A. Dose/response plot of data from field enclosures, week 1. The nominal concentration of copper (adjusted for actual enclosure volumes) is plotted as the dose (X-axis). The proportion of surviving veligers is calculated across all stages (D-stage + umbonal veligers combined: Y-axis). Net: data from 7/19/16, in which enclosures were manually mixed prior to sampling by plankton tow. Pump: data from the two dates (7/19 and 7/22) on which enclosures were mixed by water pump then sampled by pumping measured volumes of the mixed water through a plankton net.

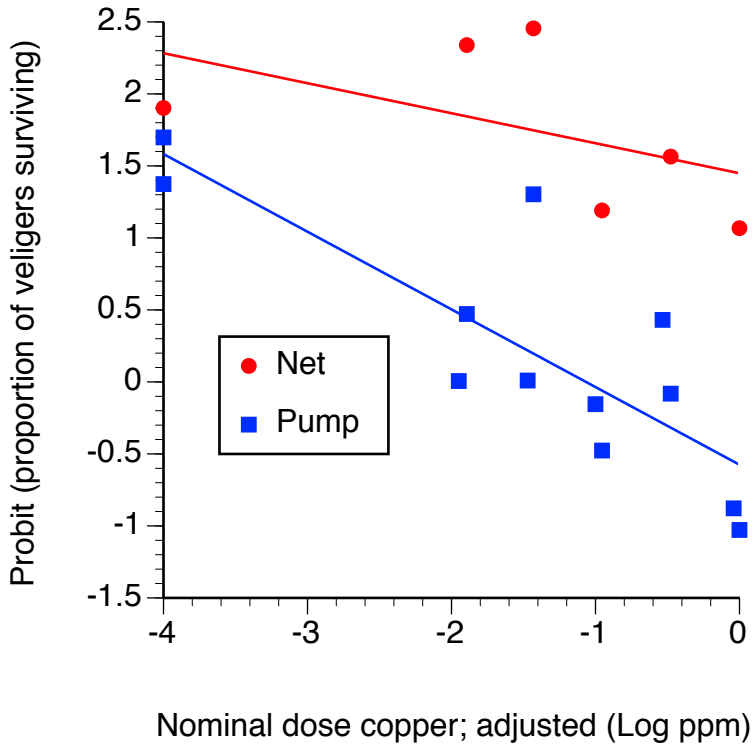

Figure 8B. Dose/response plot of data from field enclosures, week 1. The nominal concentration of copper (adjusted for actual enclosure volumes) is plotted as the dose (log transformed, X-axis). The proportion of surviving veligers is estimated from all stages (D-stage + umbonal veligers combined), and is probit transformed (Y-axis). Net: data from 7/19/16, in which enclosures were manually mixed prior to sampling by plankton tow. Pump: data from the two dates (7/19 and 7/22) on which enclosures were mixed by water pump then sampled by pumping measured volumes of the mixed water through a plankton net. See text for analysis of these data by ANCOVA.

.

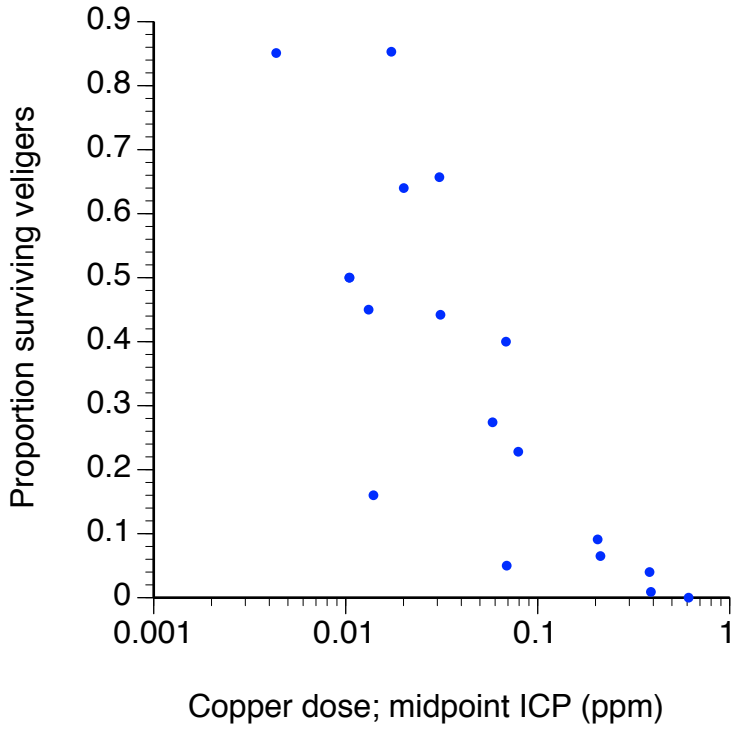

Figure 9. Dose/response plot of data from field enclosures, week 2. The midpoint between concentrations of copper at dosing and at harvest is used (X-axis). The proportion of surviving veligers is estimated from all stages (D-stage + umbonal veligers combined). These same data were used to generate the plot below (Fig. 10).

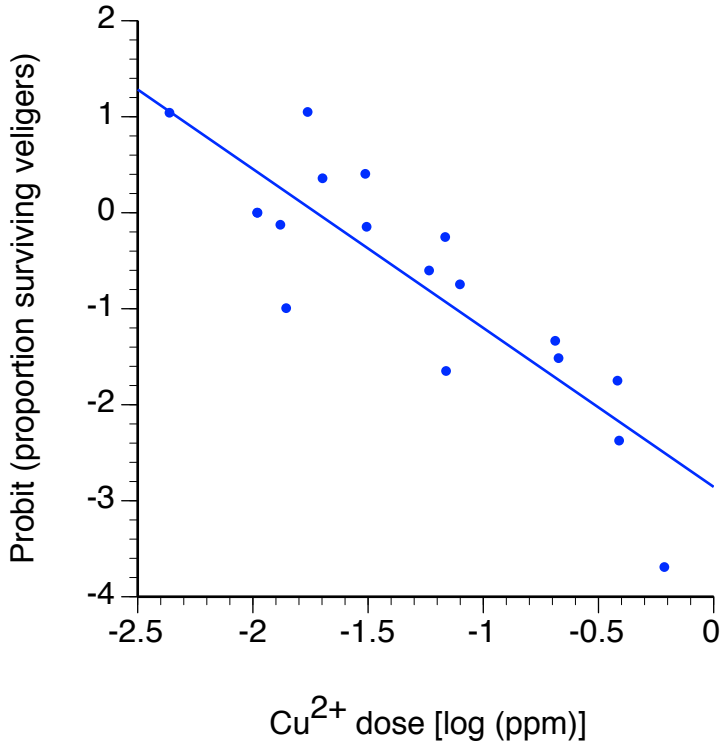

Figure 10. Dose/response analysis of data from field enclosures, week 2. Dose is the midpoint between concentrations of copper at dosing and at harvest (X-axis). The proportion of surviving veligers (*p*) is estimated from all stages (D-stage + umbonal veligers combined), and is probit-transformed [probit (*p* + 0.0001): Y-axis].

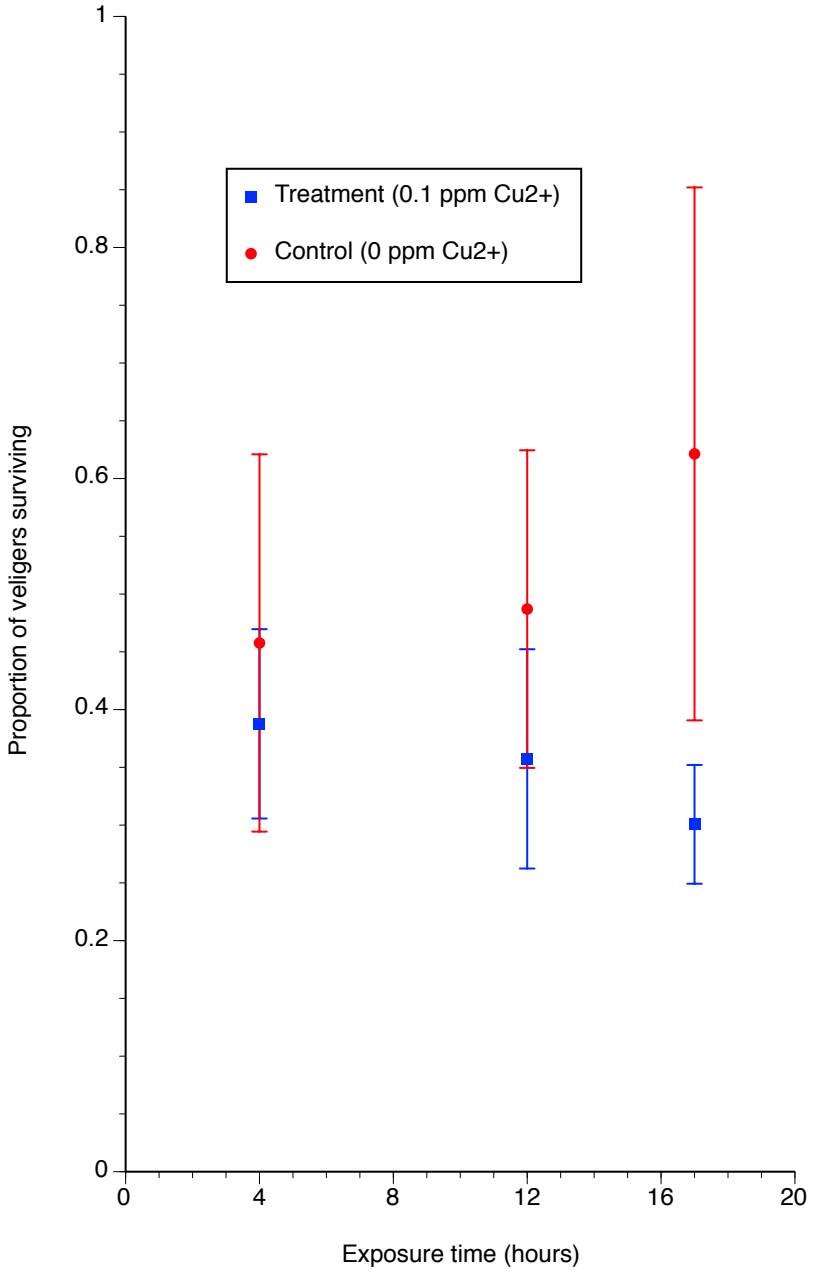

Figure 11. Exposure-time analysis of data from field enclosures, weeks 2 and 3. The mean proportion of veligers surviving (Y-axis) is plotted as a function of exposure time (rounded to the nearest hour). Error bars represent one standard error. The 17-hour exposure time trial showed higher survival in the 0-ppm controls, because it was harvested in week 2, during which we observed higher control survival in all cases compared to week 3. Nevertheless, there appears to be a trend of decreasing survival with exposure time over the time span studied in the EarthTec treatment trials. See text for statistical analysis of this trend.

VIII. References

Ackerman, J. D., B. Sim, S. J. Nichols, and R. Claudi. 1994. A review of the early life history of zebra mussels (Dreissena polymorpha): comparisons with marine bivalves. Canadian Journal of Zoology **72**:1169-1179.

Benson, A. J. 2010. Chronological history of zebra and quagga mussels (Dreissenidae) in North America, 1988 - 2010. Pages 1 - 24 *in* T. F. Nalepa and D. W. Schloesser, editors. *Quagga and Zebra Mussels: Biology, Impacts and Control.* CRC Press.

Bootsma, H., and Q. Liao. 2013. Nutrient cycling by dreissenid mussels: controlling factors and ecosystem response. Quagga and zebra mussels: biology, impacts, and control, 2nd edn. Taylor & Francis, London:555-574.

Carlton, J. T. 2008. The zebra mussel *Dreissena polymorpha* found in North America in 1986 and 1987. Journal of Great Lakes Research **34**:770-773.

Claudi, R., P. T.H., S. Mastisky, and H. Coffey. 2014. Efficacy of copper based algaecides for control of quagga and zebra mussels. RNT Consulting, Picton, Ontario Canda.

Connelly, N. A., C. R. O'Neil, B. A. Knuth, and T. L. Brown. 2007. Economic impacts of zebra mussels on drinking water treatment and electric power generation facilities. Environmental Management **40**:105 - 112.

Dalton, L. B., and S. Cottrell. 2013. Quagga and zebra mussel risk via veliger transfer by overland hauled boats. Management of Biological Invasions **4**:129-133.

Fernald, R. T., and B. T. Watson. 2014. Eradication of zebra mussels (*Dreissena polymorpha*) from Millbrook Quarry Virginia: Rapid response in the real world. Pages 195-213 *in* T. F. Nalepa and D. W. Schloesser, editors. *Quagga and Zebra Mussels: Biology, Impacts and Control*. CRC Press, Boca Raton, FL.

Fisher, S., H. Dabrowska, D. Waller, L. Babcock-Jackson, and X. Zhang. 1994. Sensitivity of zebra mussel (*Dreissena polymorpha*) life stages to candidate molluscicides. Journal Of Shellfish Research **13**:373-377.

Hebert, P. D., B. Muncaster, and G. Mackie. 1989. Ecological and genetic studies on *Dreissena polymorpha* (Pallas): a new mollusc in the Great Lakes. Canadian Journal of Fisheries and Aquatic Sciences **46**:1587-1591.

Higgins, S. N., and M. J. V. Zanden. 2010. What a difference a species makes: a meta–analysis of dreissenid mussel impacts on freshwater ecosystems. Ecological Monographs **80**:179-196.

Marrone Bio Innovations. 2015. Zequanox®: for biological quagga and zebra mussel control in open waters for recreational and environmental rehabilitation. Pages 1-13. Marrone Bio Innovations, Davis CA.

Johnson, L. E. 1995. Enhanced early detection and enumeration of zebra mussel (*Dreissena* spp.) veligers using cross-polarized light microscopy. Hydrobiologia **312**:139-146.

Kelly, N. E., K. Wantola, E. Weisz, and N. D. Yan. 2013. Recreational boats as a vector of secondary spread for aquatic invasive species and native crustacean zooplankton. Biological Invasions **15**:509-519.

Kennedy, A. J., R. N. Millward, J. A. Steevens, J. W. Lynn, and K. D. Perry. 2006. Relative Sensitivity of Zebra Mussel (*Dreissena polymorpha*) Life-stages to Two Copper Sources. Journal of Great Lakes Research **32**:596-606.

Link, C., R. Claudi, and C. Tarborelli. 2013. Objective method for determining mortality in larval and juvenile lifestages of dreissenid mussels. Page 152 18th International Conference on Aquatic Invasive Species (ICAIS). ICAIS, Niagara Falls, Ontario, CA.

Lucy, F., L. Burlakova, A. Karatayev, S. Mastitsky, and D. Zanatta. 2014. Zebra mussel impacts on unionids: a synthesis of trends in North America and Europe. *Quagga and Zebra Mussels: Biology, Impacts and Control*, 2nd edn. Ann Arbor, MI.

Lund, K., Cattoor, K.B., Fieldseth, E., Sweet, J. and M.A. McCartney. In revision. Zebra mussel (*Dreissena polymorpha*) eradication efforts in Christmas Lake, Minnesota. Lake and Reservoir Management.

Mackie, G. 1991. Biology of the exotic zebra mussel, *Dreissena polymorpha*, in relation to native bivalves and its potential impact in Lake St. Clair. Pages 251-268 Environmental assessment and habitat evaluation of the upper Great Lakes connecting channels. Springer.

Mayer, C., L. Burlakova, P. Eklöv, D. Fitzgerald, A. Karatayev, S. Ludsin, S. Millard, E. Mills, A. Ostapenya, and L. Rudstam. 2014. Benthification of freshwater lakes: exotic mussels turning ecosystems upside down. *Quagga and Zebra Mussels: Biology, Impacts, and Control*, 2nd ed. CRC Press, Boca Raton:575-586.

Montz, G., and J. Hirsch. in press. Veliger presence in residual water – assessing this pathway risk for Minnesota watercraft. Management of Biological Invasions **7**.

Nalepa, T. F., J. A. Wojcik, D. L. Fanslow, and G. A. Lang. 1995. Initial Colonization of the Zebra Mussel (Dreissena polymorpha) in Saginaw Bay, Lake Huron: Population Recruitment, Density, and Size Structure. Journal of Great Lakes Research **21**:417-434.

Offutt Air Force Base. 2009. Final Summary Report: Zebra Mussel Eradication Project, Lake Offutt Nebraska. Offutt Air Force Base, Lake Offutt, Nebraska.

Padilla, D. K., and A. Y. Karatayev. 1997. The effects of *Dreissena polymorpha* (Pallas) invasion on aquatic communities in eastern Europe. Journal Of Shellfish Research **16**:187-203.

Pimentel, D., R. Zuniga, and D. Morrison. 2005. Update on the environmental and economic costs associated with alien-invasive species in the United States. Ecological Economics **52**:273-288.

Prescott, T. H., R. Claudi, and K. L. Prescott. 2013. Impact of dreissenid mussels on the infrastructure of dams and hydro electric power plants. Quagga and Zebra Mussels: Biology, Impacts, and Control:244-257.

Stockton-Fiti, K., and R. Claudi. 2016. Use of a differential simple stain to confirm mortality of dreissenid mussels in field research experiments. 19th International Conference on Aquatic Invasive Species. ICAIS, Winnepeg, MB Canada.

Stoeckel, J. A., C. R. Rehmann, D. W. Schneider, and D. K. Padilla. 2004. Retention and supply of zebra mussel larvae in a large river system: importance of an upstream lake. Freshwater Biology **49**:919-930.

Stoeckel, J. A., D. W. Schneider, L. A. Soeken, K. D. Blodgett, and R. E. Sparks. 1997. Larval dynamics of a riverine metapopulation: implications for zebra mussel recruitment, dispersal, and control in a large-river system. Journal of the North American Benthological Society **16**:586-601.

Strayer, D. L. 2009. Twenty years of zebra mussels: lessons from the mollusk that made headlines. Frontiers in Ecology and the Environment **7**:135-141.

Strayer, D. L. 2010. Alien species in fresh waters: ecological effects, interactions with other stressors, and prospects for the future. Freshwater Biology **55**:152-174.

Strayer, D. L., and H. M. Malcom. 2006. Long-term demography of a zebra mussel (Dreissena polymorpha) population. Freshwater Biology **51**:117-130.

U. S. Environmental Protection Agency. 1983. Method 200.7: Methods for Chemical Analysis of Water and Wastes. (EPA-600 4-79-020) Environmental Monitoring and Support Laboratory, Office of Research and Development, Cincinnati, OH 45268.

U. S. Environmental Protection Agency. 2015. Amended master label for EarthTec. EPA Reg. Number 64962-1, Washington, D.C., pp 1-21.

Watters, A., S. L. Gerstenberger, and W. H. Wong. 2013. Effectiveness of EarthTec® for killing invasive quagga mussels (*Dreissena rostriformis bugensis*) and preventing their colonization in the Western United States. Biofouling **29**:21-28.

Wimbush, J., M. E. Frischer, J. W. Zarzynski, and S. A. Nierzwicki-Bauer. 2009. Eradication of colonizing populations of zebra mussels (*Dreissena polymorpha*) by early detection and SCUBA removal: Lake George, NY. Aquatic Conservation: Marine and Freshwater Ecosystems **19**:703-713.