# Investigation of the Edwards protocol's effectiveness on dreissenid mussel veligers

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**Abstract and Study Justification**

A treatment of 750 mg/L potassium chloride (KCl) for 1 h prior to a 25 mg/L 2 h formalin treatment for a total treatment time of 3 h, the Edwards protocol, has been recommended to be used in hatchery hauling tanks to kill zebra or quagga mussels that might be present in the water. However, studies conducted to confirm this protocol have found it ineffective in killing all veligers in some circumstances. Several hypotheses are proposed to explain the possible reasons for the discrepancies. The variation may be due to water quality, physiological differences between quagga and zebra mussels, lack of recovery time provided in the initial study to assure veligers were dead, chemical effectiveness of analytical grade KCl versus industrial grade (muriate of potash), and temperature effects on toxicity response. Further criticism of the Edwards protocol included testing with low fish loading densities that were not representative of more typical fish hauling protocols.

Our study evaluated these hypotheses in a hatchery hauling truck and in laboratory trials. Zebra mussel veligers from the Mississippi River were tested in a hauling truck with and without fish at two more realistic fish loading densities at temperatures similar to the original study. Additional laboratory trials were conducted with zebra mussel veligers at four temperatures in three water conductivities: ambient, and two elevated levels, created by supplementing source water with sodium chloride. Additional laboratory studies were conducted with quagga mussel veligers at the same four temperatures, and at an ambient conductivity similar to the highest conductivity tested with zebra mussel veligers. Quagga mussel trials also compared the toxicity response of analytical grade with industrial grade KCl. All trials evaluated the proportion of live and dead veligers after a short recovery time with the use of a differential stain to aide in the determination of the status of the veliger.

We found the Edwards protocol was effective with and without fish at temperatures of approximately 27°C, and in water with a conductivity of 0.37 mS/cm, similar to conditions in the original study. In trials without fish, no live veligers were detected after the treatment was applied. Trials with fish also showed the protocol was successful. The presence of fish accelerated veliger mortality, but we failed to observe 100% mortality in tests conducted for 3 h at 24°C in well water with a higher (0.62 mS/cm) water conductivity using a high density (95 g/L) of fish. Laboratory studies with zebra mussel veligers supported the trend of reduced mortality with lower temperature and with higher conductivity. Few differences were observed between the response of quagga mussel veligers exposed to analytic grade or industrial grade KCl and mortality was accelerated with increasing water temperature. The life stage of the veligers was also a factor affecting mortality. The population of zebra mussels tested was composed primarily of D-shaped and small umbonal veligers, whereas the populations of quagga mussels tested were a mix of D-shaped, umbonal, and pediveliger sized veligers. In the treatments, large umbonals and pediveligers were more resistant to the chemical toxicant, and had high survival, whereas the D-shaped and small umbonal veligers were dead after treatment for both zebra and quagga mussels.

Results from our study help explain the differences between the original and the other studies of this protocol. Sodium concentration and conductivity, water temperature, and veliger size class

all are factors affecting mortality, and should be identified in advance to assure a successful treatment.

## Introduction

To reduce the risk of invasive zebra mussel veligers (*Dreissena polymorpha*) being transported out of infested aquaculture facilities with fish hauling, Edwards et al. (2000, 2002) tested the effectiveness of chemical treatment protocols applied to transport water. They suggested that a 1 h pretreatment with 750 mg/L potassium chloride (KCl) followed by addition of 25 mg/L formalin for 2 h (Edwards protocol) in fish hatchery trucks transporting fish. In the Edwards et al. (2000) study, the method effectively killed D-shaped and post-D-shaped sized veligers at 20°C and Edwards et al. (2002) found that the protocol killed zebra mussel veligers at testing temperatures of 27.6°C without fish and with fathead minnows *(Pimephales promelas*) (6 g/L loading density) in a fish hauling truck without causing fish mortality. They reported this protocol was an effective method with and without fish present to assure 100% mortality of zebra mussel veligers.

Studies conducted to replicate the Edwards protocol in different settings to ensure that the protocol was effective on other species, in other locations, and under different water conditions showed mixed results. Sykes (2009) tested the Edwards protocol on quagga mussel (*Dreissena rostriformus bugensis*) veligers at Willow Beach National Fish Hatchery, Willow Beach, Arizona on the Colorado River (WBNFH). Sykes exposed veligers in well water to KCl at 22°C for 1 h, removed the veligers from the solution and placed them into beakers with 25 mg/L formalin for 2 h. Veligers were immediately observed for response to touch or motion of velum cilia or internal organs. Sykes (2009) found that all exposed veligers survived and increasing the concentration of KCl did not change the results. Tests were repeated with well water at 18°C, without removing the veligers from the KCl solution and formalin was added to the beaker after the 1 h pretreatment and only 12% mortality was achieved. Increasing concentrations of KCl were tested, including 4,250 mg/L with 100 mg/L formalin, and veliger survival was 100% after the veligers were placed into a recovery bath for at least 2 h at a testing temperature of 18°C. Sykes speculated that water quality, species difference, or recovery period were the likely factors responsible for the discrepancy from the Edwards studies.

Pucherelli et al. (2014) conducted further tests of the Edwards protocol on quagga mussels at Lake Mead Fish Hatchery on the Colorado River. Their procedures investigated the quagga mussel response to the Edwards protocol in static beakers with three source waters, Pueblo Reservoir, Pueblo Hatchery well water, and Lake Mead water at 13°C. They reported a mixed response and failed to find 100% treatment mortality in any of the water sources. Most recently, Howell et al. (2015) repeated the Edwards protocol on zebra mussels in a fish transport truck without fish at 23°C with water from Lyon State Fishing Lake, Kansas with veligers from Melvern Lake, Kansas. They found the treatment was not effective on zebra mussels, and settled mussels were found in treated tanks up to 10 months after treatment.

A number of potential hypotheses are suggested to explain why the Edwards protocol worked for Edwards et al. (2000; 2002) in the Great Lakes region, but not for Sykes (2009), Pucherelli et al. (2014) and Howell et al. (2015). Edwards et al. (2000; 2002) did not use a recovery method to assess mortality post treatment. Sykes (2009) and Pucherelli et al. (2014) conducted their studies in the Colorado River basin with quagga mussels and Howell et al. (2015) tested zebra mussel veligers in the Missouri River basin. The water from these locations had different conductivity due to variation in dissolved minerals when compared with water from the Great Lakes, a factor reported by Moffitt et al. (2016). Quagga mussel veligers could also respond differently from zebra mussels. Furthermore, these studies were conducted at temperatures from 13 to 27.6 °C, likely affecting the mortality response.

Edwards et al. (2002) tested the protocol in the presence of fish, but used loading densities (6 g/L) much lower than those used to transport fish in the Mississippi River basin (Jay Rudacille, Warm and Cool water Fish Culture Supervisor Iowa Department of Natural Resources, personal communications, June 2016). The higher loading densities could increase the organic content in the water and limit the effectiveness of formalin on the mussels (Meinelt et al. 2005). Another variable in studies was use of analytical grade KCl for laboratory studies compared to use of muriate of potash for truck treatments.

The purpose of this study was to pursue some of the factors likely affecting veliger mortality during use of the Edwards protocol. This study investigated the mortality of zebra mussels from the Mississippi River in fish hauling tanks with and without fish at a two fish loading densities as recommended by hatchery personnel to simulate a low and high density. The other variables hypothesized to effect the Edwards protocol, such as temperature and conductivity, were tested using static exposures in laboratory tests. Studies were conducted at four different temperatures and three different conductivities of water with zebra mussel veligers. The Edwards protocol was also tested on quagga mussels at the four temperatures, at one conductivity, and effect of industrial versus analytical grade KCl was investigated.

## Methods

### *Study Location and Collection*

*Zebra Mussels*— Tests were conducted at Fairport State Fish Hatchery, Fairport, IA (FFH) in the Lucille A. Carver Mississippi Riverside Environmental Research Station (LACMRERS) laboratory of University of Iowa in August 2016. Zebra mussel veligers were collected from the dock structure at Fairport Landing Marina, IA (41.4357°N; 90.9016°W). Plankton tow nets (35 µm-mesh) were used to filter river water in 5 min tows on the day of testing. Contents of the cod end were poured through a 300 µm sieve into 3.8 L containers and transported to the LACMRERS for laboratory trials or to the hatch house for fish truck tank trials.

Source water used in tests was obtained from the Mississippi River or well water. River water was pumped into a reservoir and then serially passed through a sand filter system and a 25 µm and 10 µm sock filter system (filtered river water). The fish truck tanks or the containers used for

laboratory studies were filled with filtered river water the day before testing and either maintained at original temperature, 26°C, or brought to the laboratory testing temperature. Tests with well water were brought up to testing temperature from 17°C.

*Quagga Mussels*— Tests were conducted at KASF Consulting Laboratory, Henderson, NV (KASF lab) in September 2016. Colorado River water and veligers were collected over three days at Las Vegas Boat Harbor, Lake Mead NRA, Boulder City, NV (36.0301°N; 114.7711°W). The testing water was filtered through a 35 µm filter into 3.8 L containers (filtered lake water). Numerous 15 m vertical tows were conducted to collect veligers using a 53 µm-mesh plankton net. Contents of the cod end were poured through a 300 µm sieve into 2-3.8 L containers and transported to KASF lab.

*Experimental Design*

*Edwards Protocol Preparation*— To minimize density biases between collection containers for the fish hauling truck treatments at FFH, the contents of plankton tows were transferred into a 22.7 L bucket and constantly mixed with a bucket aerator. When 20 L of plankton tows were collected, five 3.8 L containers were filled with 0.8 L successively at a time with the veliger concentrate. These containers were brought back to the hatch house and placed into an empty raceway with the lid off until used to start the experiment. A random sample of 0.8 L of veliger concentrate was retained from the bucket and enumerated to characterize the number of live veligers used for testing.

To prepare for the static laboratory studies, the veligers were poured onto a 63 µm sieve and then back flushed into a container with filtered water. The veliger concentrate was well mixed before three 1 mL aliquots were removed and examined with light microscopy to determine the average number of live veligers per mL. Veliger concentrate was assessed for density of live (physically moving or ciliary movement or organ activity of veliger), dead (cracked shells or degraded tissue), or empty shell (with no tissue and shell remaining). If veliger concentrate contained >1% dead to live veliger density, the plankton collection was not used. The veliger concentrate was divided into testing containers to achieve at least 500 live veligers per beaker, ranging in size from D-shaped (>63 µm) to pediveligers (<300 µm) per replicate in 10 mL of filtered water. This division was done within 1 h after density evaluation.

*Chemical Sources*— All treatments were conducted similarly throughout this study. KCl was added to zebra and quagga mussel test containers and fish hauling tanks to achieve 750 mg/L KCl active ingredient (muriate of potash; 98.8% KCl, Mosaic Global Sales, LLC, Lithia. FL; lot #102944). Analytical grade KCl (99.9% KCl, CAS 7447-40-7; FW 74.55, Macron Chemical Company, Center Valley, PA, lot #66919) was used in the comparison study with quagga mussel tests. After an hour, formalin (Parasite-S, Western Chemical, Ferndale, WA; lot # G180629-1) was added to achieve 25 mg/L. Formalin was added at full strength concentration into the fish hauling tanks, and 1.2 mL of a freshly prepared 1 to 10 diluted stock concentration of formalin was added to the zebra and quagga test containers. Total treatment time was 3 h. To elevate the sodium content in the zebra mussel veliger studies, the test water was supplemented with additions

of 0.1 M sodium chloride (NaCl; CAS 7647-14-5; FW 58.44, EMD Millipore, Billerica, MA, lot #XH04N) to achieve specific conductivity measurements of 0.5 and 1.0 mS/cm.

*Fish Hauling Truck Trials*— An Iowa Department of Natural Resources fish hauling truck was used for this study, which had eight individual 750 L (200 gallon) tanks with Fresh-flo aerators. Five tanks were used for each trial, three with fish and two without fish (Table 1). The five tank treatments were conducted on the same day and three trials of each tank treatment were conducted. Testing tanks were filled 2/3 full with filtered river water (500 L) the night before testing; and the three treatment tanks were dosed with 750 mg/L potash and mixed (Table 1). For trial three, the three fish containing tanks were filled with hatchery well water instead of filtered river water. Aerators were turned on in the morning to remix the water solution while water chemistry readings occurred.

Fish were added the morning of testing; for trial 1 and 2 channel catfish (*Ictalurus punctatus*) were used in the high density treatments (95 g/L; 0.8 lbs/gal) and koi (*Cyprinus carpio*) were used for trial 3. A mixture of goldfish (*Carassius auratus*) and bluegill (*Lepomis macrochirus*) were used in all three trials of the low density treatment (35 g/L; 0.3 lbs/gal).

**Table 1**. Tank treatment description with type of fish species used for each testing tank in the fish hauling truck trials at Fairport State Fish Hatchery in August 2016.

| Tank Treatment | Description | Loading Density (g/L) | Fish Species Used |
|---|---|---|---|
| Control | No chemical | No fish | None |
| Edwards | KCl and formalin | No fish | None |
| Edwards low density fish | KCl and formalin | 35 g/L | Goldfish/Bluegill |
| Edwards high density fish | KCl and formalin | 95 g/L | Catfish; Koi |
| Control high density fish | No chemical | 95 g/L | Catfish; Koi |

Testing was initiated when a 3.8 L container of concentrated veligers was transferred into the testing tank. Each container contained at least 8,000 veligers. In the control tanks, no KCl was added and equal amount of filtered river water was added instead of formalin to the testing tank. Treatment start times were staggered to allow for end of treatment processing.

After the 3 h treatment period, water was removed from the tank and passed through a plankton tow net (35 µm-mesh) to collect the veligers. For trial 1, the water was drained from the gate valve at the bottom of the tank with a net in front of the drain opening; the tanks with fish were drained 1/2 to 2/3, then fish were removed and then the rest of the tank was drained through the plankton net. Tanks were rinsed with filtered river water and the rinse was collected in the plankton net. Trials 2 and 3 used a different method of collection to reduce the large amount of organic material present in the tanks containing fish and to assure that tanks were not rinsed with the filtered water that could be contaminated. A subsample of the water was removed with three, 5 cm diameter PVC pipes with check valves at the bottom held together with hose clamps. Approximately 40% of the water in the tank was removed with this device to reduce the amount of organic material collected. The pipes were emptied into a 19 L bucket, which was then poured into the plankton tow net for veliger collection. Separate and disinfected nets were used for each

tank. Contents of the plankton net cod end were poured into a 0.9 L glass container and taken to the lab for veliger processing.

Within 5 min of final collection, the veligers were taken to the LACMERS lab where the veliger collection was passed through 35 µm filters and rinsed with aged laboratory well water three times. The contents of the filters were then stained with fast green and veligers were assessed for survival using microscopy. All equipment was disinfected with a 1% solution of Virkon® Aquatic between tanks.

*Laboratory Trials of Temperature and Conductivity*—Tests with zebra mussel veligers were conducted with re-filtered river water (filtered river water filtered again through a 35 µm filter) at three levels of conductivity labeled low (0.37 mS/cm), medium (0.5 mS/cm) and high (1.0 mS/cm). Re-filtered river water was supplemented with NaCl to achieve the targeted medium and high specific conductivity test waters (Table 2). Test containers, 0.9 L glass jars, were filled with aerated re-filtered river water that was pre-adjusted to testing conductivity and temperature for each testing combination. Four temperatures were tested: 27, 23, 18, and 15 °C (Table 2). Water temperatures were controlled with a chiller using a water bath surrounding the test jars. Temperature was monitored with water quality probes and logged with temperature loggers. Test jars were seeded with zebra mussel veligers from the stock concentrate to obtain a final volume of 500 mL prior to initiating the trial. Veligers were maintained at room temperature in re-filtered river water prior to seeding the test jars. Treatment start times were staggered to allow for end of treatment processing.

**Table 2.** Treatment matrix of variable for zebra mussel veliger studies conducted at Fairport State Fish Hatchery, IA in August 2016 for the control and Edwards protocol with muriate of potash. Three replicates of each conductivity and temperature combination were evaluated for the treatment and control to test a total of 24 treatment combinations.

| Conductivity (mS/cm) | Temperature °C |
|---|---|
| 0.37 | 15, 18, 23, 27 |
| 0.5 | 15, 18, 23, 27 |
| 1.0 | 15, 18, 23, 27 |

To initiate the trial, 750 mg/L potash was added to each Edwards treatment jar and stirred into solution, and the protocol for addition of 25 mg/L formalin after 1 h was followed. Treatment jars with veligers but with no addition of KCl or formalin served as controls. For each treatment combination of temperature and conductivity, three replicate jars were evaluated.

At the end of the test interval, the contents of each test jar were poured over a 10 µm filter and rinsed three times with aged laboratory well water. The contents of the filters were then stained with fast green and veligers were assessed for survival.

*Laboratory Trials of Temperature and KCl Grades*— Tests with quagga mussel veligers were conducted similarly, but jars were filled with 35 µm filtered lake water that was adjusted to testing temperature and seeded with quagga mussel veligers for a final volume of 500 mL. Four temperatures were tested: 27, 23, 18, and 15 °C (Table 3). Temperatures were maintained by

using a chiller and water bath to cool to target temperatures as above. Test solutions were maintained at target temperatures before veligers were added. In these trials only the ambient conductivity was tested (1.0 mS/cm), but analytical grade KCl and muriate of potash (industrial grade) were both tested. Veligers were maintained at room temperature in re-filtered river water prior to seeding the test jars. Treatment start times were staggered to allow for end of treatment processing. Controls were tested concurrently and contained live veligers but no KCl or formalin. Trials were conducted using three replicates per combination of treatment and temperature.

**Table 3.** Matrix of treatments for the quagga mussel study conducted at KASF laboratory in September 2016. Three replicates of each control and treatment at each temperature were evaluated for a total of 12 treatment combinations.

| Treatment | Temperatures °C |
|---|---|
| 750 mg/L Analytical KCl + 25 mg/L Formalin | 15, 18, 23, 27 |
| 750 mg/L Muriate of Potash + 25 mg/L Formalin | 15, 18, 23, 27 |

At the end of the test interval, the contents of each test container were poured over a 35 µm filter and rinsed three times with filtered lake water. The contents of the filters were then stained and veligers were assessed for survival.

*Veliger Processing and Analysis*

Veligers in the filters were placed into ~5 mL of 0.4% aqueous solution of fast green FCF (Harleton, Gibbstown, NJ, lot 4287G) for 20 min (Stockton-Fiti and Claudi *in review*). The contents were then washed with aged LACMRERS lab water (zebra mussel tests) or filtered lake water (quagga mussel tests) until dye was not visibly present and then back flushed from the filter into a recovery beaker with <5 mL filtered water until the sample was evaluated with a microscope. A 2 mL sample of veliger concentrate was removed from each recovery beaker with a disposable pipet and visually evaluated using a gridded Sedgewick-Rafter counting cell and compound microscope and were scored. Scoring levels included live (non-stained with mantle intact), dead (stained mantle), and open shells (non-stained but with no mantle intact).

Zebra mussel veliger concentrate from each treatment were held for an additional 24 h post treatment. From the fish truck trials all treatments from trials 1 and 2 and from the zebra mussel veliger studies controls from the 28°C were placed into 0.8 L jars after analysis with 0.7 L of re-filtered river water at room temperature (20°C). The samples were aerated and analyzed 24 and 48 h post treatment with the fast green method. Further time points were not conducted due to 0% survival in the control treatments. Counts and conditions were taken from the initial observations after being stained and a short recovery time.

*Characterization of Test Organisms*

Throughout testing at FFH, veligers were identified and counted as either zebra or quagga mussel and characterized by size class to determine population structure. Life stages were identified and counted as straight-hinged (D-shaped), small and large umbonal, and pediveliger. Morphological determination of the veligers were made using the characteristics described in Nichols and Black (1993) with a compound microscope (OMAX M8333Z 40X-2500X). Photographs were taken periodically of samples using a compound microscope and camera (OMAX A35140U3); these were evaluated for veliger health status and measurements of the prodissoconch. Measures of the prodissoconch included shell length (umbo to ventral margin axis) and height (anterior to posterior axis) as described by Martel et al. (1995).

To validate morphological determinations, veligers (20 vials with 2-3 veligers per vial) were collected with a micropipette and placed into a 1.5 mL tube with 100% ethanol for DNA analysis. Both morphologically typical and atypical veligers were collected. Preserved samples were sent for DNA analysis to the Reclamation Detection Laboratory, Reclamation Technical Service Center Laboratory, Denver, Colorado. The laboratory processed the samples using the polymerase chain reaction method (PCR) for dreissenid mussel DNA (Denise Hosler and Jacque Keele, personal communications, 1 September 2016). In brief, the process involved removing approximately 1 mL of ethanol from each sample after which samples were centrifuged to create a pellet. The DNA was then extracted from the pellet with a Qiagens DNeasy Blood and Tissue kit. Following the DNA extraction, all samples were analyzed using both zebra mussel or quagga mussel cytochrome oxidase I PCR primers. After the PCR reaction was completed, the PCR products were analyzed by gel electrophoresis, and the gels were scored for absence and presence of bands of each species. In addition, each gel contained both a positive and negative control for quality assurance.

Adult zebra mussels were collected from Fairport marina docks where veligers were collected and retained in 70% isopropanol for later measurement of shell length, height, and thickness (Beggel et al. 2015). They were characterized by morphological characteristics as described in Nichols and Black (1993).

*Water Quality, Metal and Ion Analyses*

Water quality parameters, such as temperature, pH, specific conductivity, salinity, and total dissolved solids (TDS) were measured with multiprobe instruments, a YSI 556 (YSI, Yellow Springs, OH) and dissolved oxygen was measured with a Hach HQ40d LDO probe (Hach, Loveland, CO) for all of the source waters at FFH (Table 4). The water quality parameters monitored for the filtered Lake Mead water at KASF lab were temperature, dissolved oxygen, pH, conductivity, salinity and TDS (Table 4) using a Hach HQ40d with a LDO 101, CDC401, and PHC705 probes (Hach, Loveland, CO). To obtain specific conductivity from the Hach conductivity measurements, the conductivity readings were adjusted as follows:

$$\frac{C}{1+\left(0.0191(T-25)\right)};$$

where *C* is conductivity (mS/cm) and *T* is temperature (°C), as described in Carlson (2015). Water quality measurements were collected prior to KCl addition, after KCl addition, after formalin addition, and prior to completion of the trial. Conductivity and salinity readings were used to verify the dosage of KCl.

**Table 4.** Temperature, pH, specific conductivity, salinity and total dissolved solids of source waters (filtered Mississippi river water, Fairport State Fish Hatchery well, Lucille A. Carver Mississippi Riverside Environmental Research Station (LACMRERS) laboratory, and filtered Lake Mead water used in trials testing zebra and quagga mussel veligers.

| Water Source | Temp (°C) | pH | Sp. Cond (mS/cm) | Salinity (ppt) | TDS (mg/L) |
|---|---|---|---|---|---|
| Mississippi river | 25.5 | 8.15 | 0.371 | 0.18 | 0.241 |
| FFH well | 24.2 | 8.03 | 0.612 | 0.30 | 0.398 |
| LACMRERS lab | 21.2 | 7.30 | 0.687 | 0.33 | 0.446 |
| Lake Mead | 23.5 | 8.41 | 1.033 | 0.51 | 0.510 |

In the fish hauling truck tanks, temperature loggers (HOBO, Onset Corporation, Bourne, MA) were placed in some of the tanks over the entire testing period and were placed in the water bath and in the laboratory to monitor temperatures during laboratory studies. Dissolved oxygen was monitored and adjusted during the fish truck trials to remain above 5 mg/L and below 150% saturation, when readings were out of range the amount of oxygen supplemented to the tank was adjusted.

Water samples were removed from each water source at FFH and quantified for a suite of metals (Appendix Table A1). The total quantities of barium, cadmium, calcium, chromium, cobalt, copper, iron, magnesium, manganese, molybdenum, nickel, potassium, sodium, vanadium, and zinc of representative samples were analyzed using inductively coupled plasma (ICP) protocols at the University of Idaho Analytical Sciences Laboratory. One water sample was also taken for each treatment in the fish hatchery truck tanks for each replicate for metal and ion analysis.

*Statistical Analysis*

We compared the proportion of live, dead, and empty veligers in each of the studies to determine differences in treatments between the trials, replicates or testing variables with chi-square tests of independence. Chi-square tests were also conducted on live and dead plus empty (DE) veligers for the studies to determine if the empty shells were influencing the outcomes. When no differences were observed the dead and empty counts were combined into one term (DE) and further chi-squares tests were conducted to determine relationship for multiple variables. Using log-linear categorical models we compared the proportions of live and DE to determine the relationship of the treatment variables, temperature, conductivity, and treatment for the zebra veliger laboratory studies (Stokes et al. 2000). We also used this type of analysis to compare the quagga mussel veligers study to the zebra mussel laboratory study with the same conductivity

and chemical treatment to determine relationship between species and temperature. Frequency tests and log linear models were conducted using SAS 9.4 (SAS Institute, Cary, NC).

Water quality was summarized for mean temperature, dissolved oxygen, pH, conductivity, salinity, and TDS. Metals and ions of the source waters were summarized by parameter and means were calculated. A general linear model was used to evaluate the relationships between trials, replicates, or different measurement times for each of the measured water quality parameters and metal ion concentrations to determine differences. The GLM procedure was conducted in R 3.1.3 (R Development Core Team, 2015) with package *lme4* (Bates et al. 2014). Tukey's HSD was used to determine how the variables related to each other if significant differences were found using package *multcompView* (Graves et al. 2012). Correlations were assessed with the package *Hmisc* (Harrell 2016).

## Results

### *Fish Hauling Truck Trials*

The Edwards protocol was effective in causing mortality in all of the trials. However, variations in temperature influenced mortality across trials. Test temperatures for trial 1 and trial 2 was 27.7°C and trial 3 was 25.0°C. In trial 1 and trial 2, the veligers in the control showed >10% mortality, but veliger mortality dropped to 3.5% in trial 3 (Tables 5 and 6). The proportion of veligers that were live, dead, and empty in the control tests were different across trials ($\chi^2$=12.7, *P*= 0.013) and was also different when the proportion of veligers live and DE (dead and empty combined) were compared ($\chi^2$=59.3, *P*<0.001), showing that temperature influenced mortality in the controls. The Edwards protocol treatment with no fish was successful in killing the veligers (Table 5) at both temperatures tested in the trials. In trial 1, there was potential for contamination of veligers from the rinse water at the end of treatment and methods were modified for trial 2 and 3 to eliminate this source of veliger contamination. Trial 2 and 3 achieved 100% mortality and there was no further contamination and there were no significant differences in the proportions of live, dead and empty or live and DE veligers between the trials.

Adding fish to the treatments increased veliger mortality, but decreased the number of recoverable veligers (Table 5). The proportion of veligers live, dead, and empty or the live and DE in the high density fish control was not significantly different between the trials ($\chi^2$=4.1, *P*= 0.393 and $\chi^2$=1.5, *P*= 0.484, respectively) and resulted in higher mortality than the control with no fish, indicating that fish did have an effect on veliger survival in a fish hauling tank treatment. When chemical was added with either low or high fish density in the tank, the mortality of the veligers increased compared to the control with high fish density. In the low fish density treated tanks, for all three trials, 100% mortality was achieved with the Edwards protocol and there were no significant differences between the trials ($\chi^2$=4.2, *P*= 0.123). However, in the high fish density treated tanks, trial 3 did not have complete mortality in contrast to trial 1 and 2 (Table 5). In trial 3, the proportion of live and DE was not significantly different between the treatments ($\chi^2$=3.7, *P*= 0.159), but was significantly different when calculated with live, dead, and empty ($\chi^2$=14.2, *P*= 0.007). This indicated that the empty shells were influencing this treatment, but number of

empty shells did not affect the other treatments; the low sample counts for the fish trials was reducing the power of analysis. Trial 3 was conducted at a lower temperature, 24.2°C with well water instead of filtered river water, which had higher concentrations of sodium (Table 6).

**Table 5.** Number of zebra mussel veligers observed in the fish truck trials for each treatment and control conducted at Fairport State Fish Hatchery in August 2016. Proportions of veligers DE (dead and empty) compared to the total counted are presented as percent mortality.

| Trial | Treatment | Live | Dead | Empty | DE | Total | Mortality (%) |
|---|---|---|---|---|---|---|---|
| 1 | Control | 96 | 14 | 6 | 20 | 116 | 17.2 |
| | Edwards (no fish) | 1* | 116 | 16 | 132 | 133 | 99.3 |
| | Edwards low density fish | 0 | 20 | 7 | 27 | 27 | 100 |
| | Edwards high density fish | 1* | 21 | 10 | 31 | 32 | 96.9 |
| | Control high density fish | 1 | 9 | 8 | 17 | 18 | 94.4 |
| 2 | Control | 165 | 13 | 8 | 21 | 186 | 11.3 |
| | Edwards (no fish) | 0 | 99 | 12 | 111 | 111 | 100 |
| | Edwards low density fish | 0 | 18 | 19 | 37 | 37 | 100 |
| | Edwards high density fish | 0 | 8 | 23 | 31 | 31 | 100 |
| | Control high density fish | 6 | 7 | 12 | 19 | 25 | 76 |
| 3 | Control | 112 | 4 | 0 | 4 | 116 | 3.5 |
| | Edwards (no fish) | 0 | 88 | 15 | 103 | 103 | 100 |
| | Edwards low density fish | 0 | 6 | 4 | 10 | 10 | 100 |
| | Edwards high density fish | 1 | 4 | 9 | 13 | 14 | 92.9 |
| | Control high density fish | 3 | 4 | 4 | 8 | 11 | 72.7 |

*indicates potential contamination of test system from rinse water used only in trial 1.

Temperature, pH, salinity, and TDS were not significantly different between the filtered river water for trial 1 and 2; only specific conductivity was significantly different (($F_{(1,5)}$=70; $P$<0.001); Table 6). All of trial 3 water quality measurements for the filtered river water were significantly different from trial 1 and 2 and well water quality measurements were significantly different from the filtered water (all *P-values*<0.01; Table 6). Sodium and potassium concentrations were high in the well water, which explained the high specific conductivity, salinity and TDS.

**Table 6.** Temperature, pH, specific conductivity, salinity and total dissolved solids (TDS) of filtered Mississippi river water and Fairport State Fish Hatchery well water added to testing tanks measured with the YSI meter; and sodium and potassium concentrations measured through inductively coupled plasma analysis from fish hauling truck trials conducted August 2016.

| Water Source | Trial | Temp (°C) | pH | Sp. Cond (mS/cm) | Salinity (ppt) | TDS (mg/L) | Sodium (mg/L) | Potassium (mg/L) |
|---|---|---|---|---|---|---|---|---|
| Filtered River | 1 | 27.69 | 8.34 | 0.347 | 0.16 | 0.235 | <10 | 3.4 |
| Filtered River | 2 | 27.79 | 8.21 | 0.355 | 0.17 | 0.231 | <10 | 2.5 |
| Filtered River | 3 | 25.03 | 8.79 | 0.312 | 0.15 | 0.203 | <10 | 2.4 |
| Well | 3 | 24.20 | 8.03 | 0.612 | 0.30 | 0.398 | 33 | 7.0 |

Observing the veligers through the organic filtrate that was observed in tanks with fish (e.g. sludge) was extremely hard and few veligers were counted (Figure 1). The sludge was composed of fish mucus, scales, and sediment or organic content of the water. Veliger collection methods post-treatment were modified for trial 2 and trial 3 to minimize the amount of sludge collected in the treatments with fish. Because of the difficulty observing veligers in trial 2, we changed the treatment water to well water for trial 3. The fish continued to produce high amounts of mucus and were covered in the turbidity of the Mississippi river water upon loading into the treatment tank, and we still had high amounts of sludge in our collections.

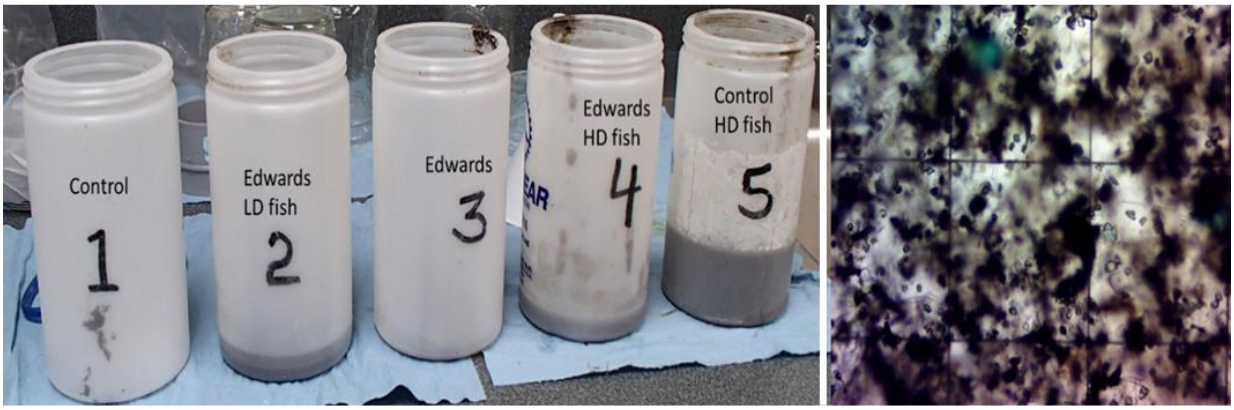


**Figure 1.** Sludge collected in plankton tow net of the five treatments from trial 1of the fish hauling truck trials conducted August 2016 and the view of the control high fish density sample stained with fast green at 40X magnification.

Once treatments were initiated, temperature, dissolved oxygen, pH, conductivity, salinity, and TDS did not significantly change over the treatment period. Temperature was significantly different between trials ($F_{(2, 57)}$=392.8, *P*=<0.001), which the temperature of trial 3, 24°C, was lower than trial 1 and 2, 27°C (Table 7). Dissolved oxygen concentrations for the different treatments or trials were not significantly different from each other and all readings were within tolerance limits for zebra mussel veligers for fish survival greater than 24% and less than 200% air saturation (Boyd et al. 1978; Sprung 1987; Choi et al. 2013; Stockton-Fiti 2015). The pH readings were significantly different between treatments ($F_{(4,54)}$=16.5, *P*=<0.001), where Tukey's HSD showed that the fish treatments had lower pH readings, 7.97, than the tanks without fish, 8.46. Significant differences between the trials pH readings were observed (($F_{(2, 56)}$=4.95, *P*=0.01); Table 7); but these pH readings were still within the tolerance limits of zebra mussel veligers (Sprung 1993). Since specific conductivity, salinity, and TDS were highly correlated, the resulting significant variables were similar for the treatments. The measurements of specific conductivity, salinity, and TDS were only significantly different by treatment (all *P values*<0.001), where control was less than the treated tanks (Table 7). Analysis with Tukey's HSD showed that the specific conductivity, salinity, and TDS of the control was significantly different from the control high density fish tanks. There were no significant differences between trials for the specific conductivity, salinity or TDS measurements.

**Table 7.** Mean temperature, dissolved oxygen (DO), pH, specific conductivity, salinity, and total dissolved solids (TDS) of each treatment separated out for each trial with standard deviation in parenthesis for the fish truck trials conducted August 2016. Water quality measurements taken post addition of KCl, post veliger addition, post formalin addition, and end of treatment were combined as there were no significant differences between the measurements using GLM procedures.

| Trial | Temp °C | DO mg/L | DO% | pH | Sp. Cond mS/cm | Salinity ppt | TDS mg/L | Na+ mg/L | K + mg/L |
|---|---|---|---|---|---|---|---|---|---|
| **Control** | | | | | | | | | |
| 1 | 27.40 (0.12) | 9.59 (2.38) | 124 (30) | 8.32 (0.05) | 0.345 (0.001) | 0.16 (0.0) | 0.224 (0.001) | <10 | 3.4 |
| 2 | 27.63 (0.34) | 7.96 (0.07) | 102 (1) | 8.39 (0.13) | 0.350 (0.008) | 0.17 (0.0) | 0.227 (0.006) | <10 | 2.5 |
| 3 | 24.64 (0.33) | 9.93 (2.44) | 121 (30) | 8.57 (0.15) | 0.314 (0.001) | 0.15 (0.0) | 0.204 (0.0) | <10 | 2.4 |
| **Edwards** | | | | | | | | | |
| 1 | 27.12 (0.14) | 8.03 (0.08) | 103 (1) | 8.48 (0.07) | 1.841 (0.057) | 0.93 (0.0) | 1.197 (0.005) | 11 | 390 |
| 2 | 27.33 (0.59) | 8.00 (0.11) | 103 (1) | 8.47 (0.10) | 1.785 (0.038) | 0.90 (0.02) | 1.160 (0.025) | <10 | 380 |
| 3 | 24.51 (0.34) | 8.87 (0.64) | 108 (8) | 8.56 (0.08) | 1.731 (0.042) | 0.88 (0.02) | 1.125 (0.028) | 10 | 370 |
| **Control High Density Fish (95 g/L)** | | | | | | | | | |
| 1 | 27.42 (0.32) | 9.69 (2.31) | 125 (30) | 7.62 (0.41) | 0.433 (0.057) | 0.21 (0.03) | 0.281 (0.037) | 14 | 20 |
| 2 | 27.00 (0.59) | 8.73 (0.89) | 111 (11) | 7.90 (0.21) | 0.392 (0.026) | 0.19 (0.01) | 0.255 (0.017) | 11 | 7.0 |
| 3 | 24.30 (0.11) | 9.18 (0.37) | 112 (5) | 8.09 (0.05) | 0.619 (0.007) | 0.30 (0.0) | 0.403 (0.005) | <10 | 2.3 |
| **Edwards Low Density Fish (35 g/L)** | | | | | | | | | |
| 1 | 27.17 (0.18) | 9.01 (0.12) | 116 (1) | 7.96 (0.28) | 1.828 (0.011) | 0.92 (0.01) | 1.188 (0.007) | 14 | 390 |
| 2 | 27.01 (0.58) | 9.72 (1.67) | 124 (22) | 8.15 (0.18) | 1.750 (0.036) | 0.88 (0.02) | 1.135 (0.021) | 12 | 360 |
| 3 | 24.26 (0.19) | 10.51 (0.21) | 128 (3) | 8.30 (0.17) | 1.959 (0.035) | 1.00 (0.02) | 1.274 (0.022) | 17 | 370 |
| **Edwards High Density Fish (95 g/L)** | | | | | | | | | |
| 1 | 27.40 (0.28) | 10.07 (5.56) | 131 (73) | 7.69 (0.43) | 1.831 (0.030) | 0.92 (0.02) | 1.190 (0.020) | 17 | 380 |
| 2 | 27.08 (0.54) | 9.60 (0.84) | 119 (12) | 7.98 (0.28) | 1.729 (0.028) | 0.87 (0.01) | 1.124 (0.018) | 11 | 350 |
| 3 | 24.19 (0.14) | 8.65 (0.38) | 105 (5) | 8.07 (0.08) | 1.956 (0.042) | 0.99 (0.02) | 1.271 (0.027) | 16 | 370 |

The sodium in the treatment tanks with filtered river water without fish did not change and the addition of potash did not increase the sodium concentrations in the treated tanks. In treatments

containing fish, the sodium concentrations were higher (Table 7). However, in trial 3 in treatment tanks with fish the sodium content decreased from the original amount of 33 mg/L (Table 6). Potassium levels were low in the control water and increased with presence of fish in the control high density fish treatment. In the treated tanks, where potash was added, the potassium concentrations were around 370 mg/L. Presence of fish did not change the amount of potassium as much as the change in sodium concentration.

Concentrations of cadmium, chromium, cobalt, copper, molybdenum, nickel, vanadium and zinc were all below detection levels. Barium, calcium, iron, magnesium, and manganese had detectable concentrations. These metal concentrations did not change significantly with the presence of fish or addition of potash (Table 8). The largest difference in the metal levels was river water compared to well water; barium ($F_{(1, 13)}$=163.2, $P$<0.001), calcium ($F_{(1, 13)}$=32.07, $P$<0.001), and magnesium ($F_{(1, 13)}$=27.07, $P$=0.002) were all significantly higher than river water (Table 8). Iron and manganese concentrations did not vary over any factor: water source, replicate, treatment, or presence of fish in the treatment. Presence of these metals did not influence the results of the study.

**Table 8.** Mean and standard deviation in parenthesis of metals in the testing water at the end of treatment for the fish truck trials conducted August 2016, divided out by water source (filtered Mississippi river water and Fairport State Fish Hatchery well water), treatment and presence of fish.

| | **Barium (mg/L)** | **Calcium (mg/L)** | **Iron (mg/L)** | **Magnesium (mg/L)** | **Manganese (mg/L)** |
|---|---|---|---|---|---|
| | | | **Filtered River** | | |
| **Edwards** | | | | | |
| No Fish (n=3) | 0.05 (0.01) | 32.0 (4.58) | 0.19 (0.04) | 13.3 (2.08) | 0.11 (0.08) |
| Fish (n=4) | 0.06 (0.0) | 34.8 (1.71) | 0.25 (0.21) | 14.5 (0.58) | 0.15 (0.12) |
| **Control** | | | | | |
| No Fish (n=3) | 0.04 (0.0) | 33. 7 (0.58) | 0.14 (0.05) | 14.3 (0.58) | 0.07 (0.05) |
| Fish (n=2) | 0.05 (0.0) | 33.5 (0.71) | 0.09 (0.0) | 15. 0 (0.0) | 0.08 (0.08) |
| | | | **Well** | | |
| **Edwards** | | | | | |
| Fish (n=2) | 0.87 (0.0) | 71. 0 (2.83) | 0.33 (0.2) | 26.5 (0.71) | 0.15 (0.01) |
| **Control** | | | | | |
| Fish (n=1) | 0.50 | 40 | 0.14 | 16 | 0.06 |

Channel catfish used in this study were infected with columnaris disease (*Flavobacterium columnare*) and had been treated using 35% hydrogen peroxide following Aquatic Animal Drug Approval Partnership Program (AADAP) protocols in the days before the study (Melanie Harkness, Fairport State Fish Hatchery, personal communication April 2017). Additional fish were obtained from a private fish hatchery to supplement densities. Densities of catfish were adequate to stock the high density tanks during trials 1 and 2 (Figure 2), but about 34 kg of catfish were lost over the weekend and the densities were not high enough fish to conduct trial 3

with catfish. Koi were used as a replacement. Hatchery personnel reported the catfish mortality to be caused by the infection of columnaris and not due to treatment, as these treatments had not caused fish mortality in the past. In the low density treatments, goldfish mixed with some bluegill were used during all three replicates and no mortality events occurred (Figure 2).

**Figure 2.** Example fish from the goldfish and bluegill low density treatments and channel catfish used for the high density treatments.

*Temperature and Conductivity Laboratory Trials*

We observed greater than 85% mortality of zebra mussel veligers with the Edwards protocol treatment while control mortality was less than 8% (Table 9). There were no significant difference in the proportion of live, dead, and empty veligers of the controls between the three conductivities ($\chi^2$=4.6, *P*= 0.334). Treated veligers showed a significant difference in the proportion of live, dead, and empty veligers between the three conductivities tested ($\chi^2$=175.6, *P*<0.001) indicating that conductivity was a significant variable in treatment mortality. Control and treatment mortalities were significantly different from each other at all four temperatures; 15 ($\chi^2$=1600.9, *P*<0.001), 18 ($\chi^2$=1941. 4, *P*<0.001), 23 ($\chi^2$=2081. 2, *P*<0.001), and 27 ($\chi^2$=1941, *P*<0.001) °C. This showed that temperature was an important variable in veliger mortality to treatment.

Mortality of zebra mussel veligers was observed with the Edwards protocol at all temperatures and adjusted background conductivities, which the proportion of live to DE veligers was significant (Table 9; all $\chi^2$ *P* values <0.001). The mortality of the zebra mussel veligers did increase with temperature and decreased with increased background conductivity (Table 9; Figure 4). Likelihood ratios showed that the conductivity, temperature and treatment were not as significant individually, but the interaction terms between conductivity and temperature, conductivity and treatment, and temperature and treatment were highly significant (Table 10). Thus there was a combination of variables showing effect on the treatment success.

**Table 9.** Number of zebra mussel veligers observed from testing of the Edwards protocol at four different temperatures in three different background water conductivities with standard deviation in parenthesis at Fairport State Fish Hatchery in August 2016. Proportion of veligers dead and empty (DE) compared to the total counted presented as percent mortality with chi-square analysis for difference between treatments for each temperature and conductivity variable tested.

| Treatment | | Live | DE | Mortality % | $\chi^2$ | *P* |
|---|---|---|---|---|---|---|
| | **0.37 mS/cm** | | | | 576. 5 | <0. 001 |
| | Control | 107 (6.1) | 5 (3.6) | 4.4 (3.0) | | |
| | Treated | 3 (1.2) | 107 (5.5) | 97.6 (0.9) | | |
| | **0.5 mS/cm** | | | | 515.1 | <0.001 |
| 15°C | Control | 113 (17.6) | 8 (2.1) | 6.9 (1.6) | | |
| | Treated | 9 (4.4) | 106 (14.6) | 92.4 (2.4) | | |
| | **1.0 mS/cm** | | | | 441. 8 | <0.001 |
| | Control | 106 (19.2) | 9 (3.1) | 7.7 (3.2) | | |
| | Treated | 17 (5.5) | 106 (7.6) | 86.5 (3.9) | | |
| | **0.37 mS/cm** | | | | 717.0 | <0.001 |
| | Control | 118 (26.4) | 7 (0.6) | 6.1 (1.5) | | |
| | Treated | 0 (0.0) | 142 (39.2) | 100 (0.0) | | |
| | **0.5 mS/cm** | | | | 619.1 | <0.001 |
| 18°C | Control | 107 (7.5) | 6 (4.0) | 5.43 (2.9) | | |
| | Treated | 3 (3.0) | 126 (41.3) | 97.6 (2.8) | | |
| | **1.0 mS/cm** | | | | 567.4 | <0.001 |
| | Control | 117 (9.5) | 5 (0.6) | 3.9 (3.2) | | |
| | Treated | 7 (2.1) | 106 (16.6) | 86.5 (3.9) | | |
| | **0.37 mS/cm** | | | | 712. 8 | <0.001 |
| | Control | 138 (26.5) | 5 (1.0) | 3.6 (1.1) | | |
| | Treated | 0 (0.0) | 114 (9.2) | 100 (0.0) | | |
| | **0.5 mS/cm** | | | | 624.1 | <0.001 |
| 23°C | Control | 113 (22.0) | 8 (4.0) | 6.4 (2.3) | | |
| | Treated | 0 (0.0) | 117 (22.4) | 100 (0.0) | | |
| | **1.0 mS/cm** | | | | 661.1 | <0.001 |
| | Control | 138 (56.9) | 10 (6.1) | 6.7 (2.0) | | |
| | Treated | 0 (0.0) | 110 (10.4) | 100 (0.0) | | |
| | **0.37 mS/cm** | | | | 616.5 | <0.001 |
| | Control | 108 (6.8) | 8 (1.2) | 7.1 (0.7) | | |
| | Treated | 0 (0.0) | 120 (12.8) | 100 (0.0) | | |
| | **0.5 mS/cm** | | | | 688.7 | <0.001 |
| 27°C | Control | 119 (9.3) | 9 (2.6) | 6.9 (1.5) | | |
| | Treated | 0 (0.0) | 134 (25.4) | 100 (0.0) | | |
| | **1.0 mS/cm** | | | | 599.0 | <0.001 |
| | Control | 113 (10.5) | 6 (3.2) | 5.4 (3.1) | | |
| | Treated | 0 (0.0) | 105 (4.0) | 100 (0.0) | | |

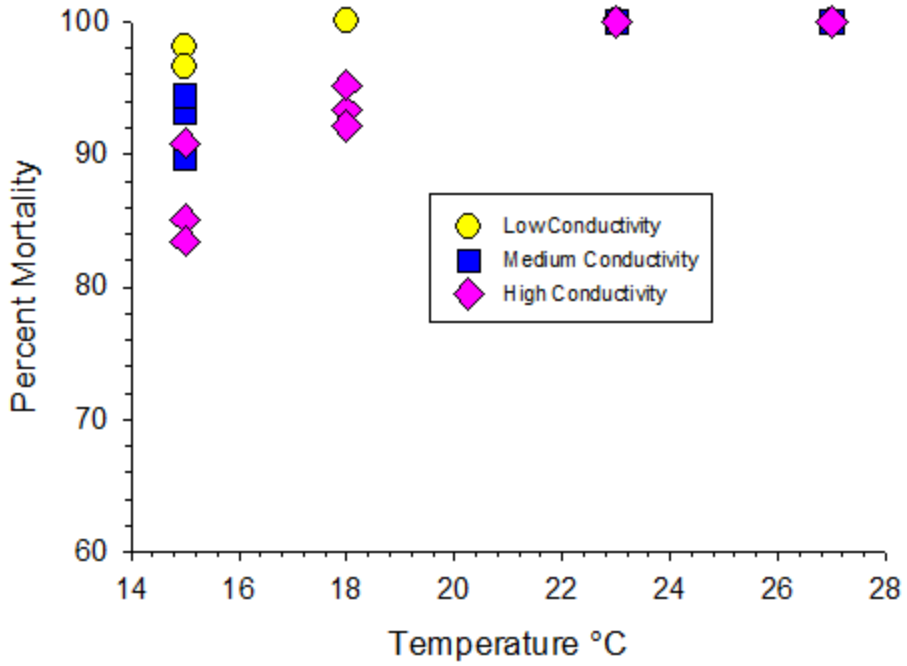


**Figure 4.** Percent mortality of zebra mussel in the three different background conductivity waters at the four testing temperatures exposed to the Edwards protocol at Fairport State Fish Hatchery in August 2016.

**Table 10.** Summary of the maximum likelihood analysis of variance log-linear comparisons of independence for frequency of live and DE (dead and empty) veligers for the zebra mussel veligers exposed to the Edwards protocol at four different temperatures in three different background water conductivities at Fairport State Fish Hatchery in August 2016.

| **Source** | **Degrees of Freedom** | $\chi^2$ | ***P*** |
|---|---|---|---|
| Conductivity | 2 | 1.6 | 0.441 |
| Temperature | 3 | 7.5 | 0.059 |
| Cond.*Temp. | 6 | 19.8 | 0.003 |
| Treatment | 1 | 2.6 | 0.107 |
| Cond.*Treat. | 2 | 6.5 | 0.038 |
| Temp.*Treat. | 3 | 18.7 | 0.000 |
| Cond.*Temp.*Treat. | 6 | 10.0 | 0.123 |

Water quality measures throughout the testing period stayed consistent. There were no significant differences between replicates. Temperature was within 0.5 to 1 °C of desired temperature throughout testing. Dissolved oxygen was kept within levels that were suitable for veliger survival, i.e. greater than 24% and less than 200% air saturation (Sprung 1987; Choi et al. 2013; Stockton-Fiti 2015). The measured dissolved oxygen concentration had a trend of decreasing with increasing temperature, which was expected (Table 11). There were significant

differences in pH for the different testing temperatures ($F_{(3, 278)}$ =11.7; $P$<0.001), but there was not a trend of higher temperature leading to higher pH (Table 11). The pH throughout testing was within tolerance ranges for veliger survival (Sprung 1993). Specific conductivity, salinity and TDS were highly correlated with each other and had the same trends. Specific conductivity did show a significant difference between treated and control ($F_{(1, 280)}$=294.5; $P$<0.001; Table 11), which was expected. There was also a significant difference between the different background conductivities ($F_{(2, 279)}$=21.63; $P$<0.001), which was also expected. There were no significant differences of readings in the stage of testing once KCl was added to the treatment and the specific conductivity measurements were not significantly different over the different temperatures tested.

**Table 11.** Summary of mean temperature, dissolved oxygen (DO), pH, specific conductivity, salinity, and total dissolved solids taken throughout testing in the zebra mussel veliger tests at four testing temperatures conducted August 2016. Standard deviations for temperature was <0.5°C and all other measurement were <0.1 unit of measurement.

| Treatment | | Temp °C | DO mg/L | pH | Sp. Cond mS/cm | Salinity ppt | TDS mg/L |
|---|---|---|---|---|---|---|---|
| | **0.37 mS/cm** | | | | | | |
| | Control | 14.94 | 9.87 | 8.20 | 0.381 | 0.18 | 0.246 |
| | Treated | 14.86 | 9.83 | 8.16 | 1.796 | 0.91 | 1.168 |
| | **0.5 mS/cm** | | | | | | |
| 15°C | Control | 14.68 | 9.87 | 8.20 | 0.542 | 0.26 | 0.352 |
| | Treated | 14.69 | 9.87 | 8.15 | 1.957 | 1.00 | 1.272 |
| | **1.0 mS/cm** | | | | | | |
| | Control | 14.75 | 8.19 | 8.16 | 1.010 | 0.50 | 0.656 |
| | Treated | 14.70 | 8.43 | 7.96 | 2.369 | 1.23 | 1.541 |
| | **0.37 mS/cm** | | | | | | |
| | Control | 18.34 | 9.26 | 8.22 | 0.376 | 0.18 | 0.244 |
| | Treated | 18.29 | 9.24 | 8.16 | 1.753 | 0.89 | 1.139 |
| | **0.5 mS/cm** | | | | | | |
| 18°C | Control | 18.41 | 9.24 | 8.23 | 0.518 | 0.25 | 0.337 |
| | Treated | 18.55 | 9.24 | 8.15 | 1.910 | 0.98 | 1.242 |
| | **1.0 mS/cm** | | | | | | |
| | Control | 18.45 | 9.27 | 8.23 | 1.004 | 0.50 | 0.653 |
| | Treated | 18.49 | 9.23 | 8.13 | 2.396 | 1.24 | 1.557 |
| | **0.37 mS/cm** | | | | | | |
| | Control | 22.91 | 8.72 | 8.41 | 0.354 | 0.17 | 0.230 |
| | Treated | 22.91 | 8.69 | 8.25 | 1.735 | 0.88 | 1.128 |
| | **0.5 mS/cm** | | | | | | |
| 23°C | Control | 22.95 | 8.74 | 8.44 | 0.522 | 0.25 | 0.339 |
| | Treated | 22.83 | 8.67 | 8.38 | 1.877 | 0.95 | 1.220 |
| | **1.0 mS/cm** | | | | | | |
| | Control | 22.97 | 8.68 | 8.40 | 1.030 | 0.51 | 0.670 |
| | Treated | 22.91 | 8.65 | 8.38 | 2.432 | 1.25 | 1.581 |
| | **0.37 mS/cm** | | | | | | |
| | Control | 26.87 | 7.58 | 8.02 | 0.400 | 0.19 | 0.259 |
| | Treated | 26.29 | 8.54 | 8.08 | 1.780 | 0.90 | 1.157 |
| | **0.5 mS/cm** | | | | | | |
| 27°C | Control | 26.49 | 7.48 | 7.91 | 0.540 | 0.26 | 0.350 |
| | Treated | 26.21 | 7.59 | 7.98 | 1.898 | 0.96 | 1.233 |
| | **1.0 mS/cm** | | | | | | |
| | Control | 26.73 | 7.47 | 7.96 | 1.006 | 0.50 | 0.654 |
| | Treated | 26.45 | 7.28 | 7.93 | 2.399 | 1.23 | 1.560 |

*Temperature and KCl Grade Laboratory Trials*

Mortality of greater than 50% was achieved with quagga mussels when exposed to the Edwards protocol (Table 12; Figure 5). Control mortality was high in some of the treatments and not in others, which corresponded to collection day. The trials at 27°C were completed from day 1 collection day and average veligers per replicate was 1,000. The 15 and 23 °C trials were completed from collection day 2 where 3,000 veligers were added per replicate and had similar control mortality; and the 18°C trials were completed from collection day 3 and were dosed with about 2,000 veligers per replicate, but had the highest control mortality (Table 12). Most of the control mortality from the 18°C treatment was attributed to the high number (44% of sample) of empty shells present in the sample. These were most likely present in the veliger concentrate prior to testing, but the control counts did not show this. Chi-square analysis of the proportion of live, dead and empty showed that there were no significant differences between the replicates in the controls (Table 12). However both analytical and potash treatments at 15°C and the analytical treatments at 18 and 23 °C had significant differences in the replicates. These treatments had a large number of veligers counted in one of the replicates and also were from a collection day that had very high number of veligers in the concentrate.

**Table 12.** Mean number of quagga mussel veligers observed from the testing of the Edwards protocol at four different temperatures with use of analytical or potash KCl at KASF laboratory September 2016. Proportion of veligers DE (dead and empty) compared to the total counted presented as percent mortality with standard deviation in parenthesis and chi-square analysis for difference between replicates for each treatment and temperature variable tested.

| Treatment | | Live | Dead | Empty | Total | Mortality % | $\chi^2$ | *P* |
|---|---|---|---|---|---|---|---|---|
| 15°C | Control | 250.0 | 6.3 | 3.0 | 259.3 | 3.6 (1.0) | 1.6 | 0.445 |
| | Treated Analytical | 105.3 | 143.3 | 1.0 | 249.7 | 57.3 (7.9) | 11.6 | 0.003 |
| | Treated Potash | 115.0 | 152.0 | 2.3 | 269.3 | 56.1 (9.6) | 22.4 | <0.001 |
| 18°C | Control | 124.0 | 15.7 | 18.3 | 158.0 | 20.9 (4.9) | 3.0 | 0.223 |
| | Treated Analytical | 87.3 | 60.7 | 127.7 | 275.7 | 68.1 (6.5) | 10.8 | 0.005 |
| | Treated Potash | 73.3 | 74.0 | 107.3 | 254.7 | 71.3 (2.5) | 1.6 | 0.442 |
| 23°C | Control | 245.7 | 4.0 | 2.7 | 252.3 | 2.7 (1.6) | 5.2 | 0.076 |
| | Treated Analytical | 120.0 | 160.3 | 2.0 | 282.3 | 58.2 (6.5) | 10.2 | 0.006 |
| | Treated Potash | 74.3 | 147.0 | 1.7 | 223.0 | 66.7 (3.6) | 2.6 | 0.275 |
| 27°C | Control | 106.0 | 15.7 | 2.0 | 123.7 | 14.3 (2.0) | 0. 8 | 0.678 |
| | Treated Analytical | 34.7 | 97.7 | 5.3 | 137.7 | 74.9 (3.4) | 1.5 | 0.466 |
| | Treated Potash | 33.3 | 85.0 | 5.7 | 124.0 | 72.8 (3.6) | 1.5 | 0.463 |

At 23°C, the chi-square analysis showed that there was a significant difference in the proportion of live to DE quagga mussel veligers between the analytical and potash treatments ($\chi^2$=13.3, *P*<0.001). This was the only significant difference and there was no trend seen in the data to show that analytical KCl was different from potash KCl in these treatments (Figure 5). Proportion of live to DE quagga mussel veligers increased as temperature increased for the control, treated analytical and treated potash treatments. Differences in these proportions were

significant (control: $\chi^2$=176.1, $P$<0.001; treated analytical: $\chi^2$=133.1, $P$<0.001; and treated potash: $\chi^2$=63.8, $P$<0.001). Temperature was an important variable in predicting mortality of the quagga mussel regardless of treatment.

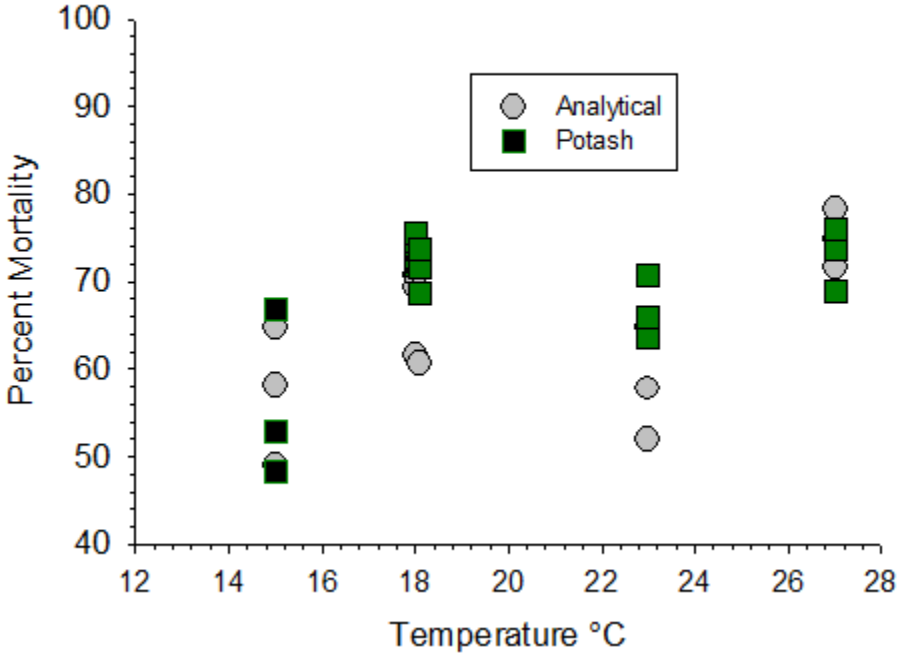


**Figure 5.** Percent mortality of quagga mussel veligers at each temperature tested at KASF laboratory in September 2016 comparing the use of analytical grade to potash (industrial grade) KCl in the Edwards protocol.

Measurements of water quality were consistent through testing period, and no significant differences were detected between replicates. Temperature was within 1°C of desired temperature throughout testing. Dissolved oxygen remained within ranges suitable for veliger survival, i.e. above 24% and below 200% air saturation (Table 13; Sprung 1987; Choi et al. 2013; Stockton-Fiti 2015). As expected, the dissolved oxygen readings decreased with increased temperature. There were significant differences in pH for the different testing treatments ($F_{(2, 141)}$ =5.335; $P$=0.006), where average control pH was slightly lower than treatment pH (Table 13). The pH throughout testing was within tolerance ranges for veliger survival (Sprung 1993) and within standard error of the Hach meter readings. Specific conductivity, salinity and TDS were highly correlated with each other and had the same trends. Specific conductivity did show a significant difference between treated and control ($F_{(2, 141)}$ =69.83; $P$<0.001), which was expected, but there was no difference between the potash treatment and analytical KCl treatment ($F_{(1, 94)}$ =0.006; $P$=0.937). There were no significant differences of readings in the stage of testing once KCl was added to the treatment, and the specific conductivity measurements were not significantly different across tested temperatures.

**Table 13**. Summary of mean temperature, dissolved oxygen, pH, specific conductivity, salinity, and total dissolved solids taken throughout testing in the quagga mussel veliger tests at four testing temperatures conducted September 2016. Standard deviations for temperature was <0.5°C and all other measurement were <0.1 unit of measurement.

| Treatment | | Temp °C | DO mg/L | pH | Sp. Cond mS/cm | Salinity ppt | TDS mg/L |
|---|---|---|---|---|---|---|---|
| 15°C | Control | 15.4 | 8.30 | 8.41 | 1.011 | 0.50 | 0.501 |
| | Analytical KCl | 15.3 | 8.43 | 8.44 | 2.384 | 1.24 | 1.220 |
| | Potash KCl | 15.3 | 8.48 | 8.43 | 2.227 | 1.22 | 1.200 |
| 18°C | Control | 18.2 | 8.17 | 8.41 | 1.018 | 0.51 | 0.504 |
| | Analytical KCl | 18.3 | 8.08 | 8.41 | 2.368 | 1.22 | 1.207 |
| | Potash KCl | 18.3 | 8.14 | 8.40 | 2.395 | 1.24 | 1.226 |
| 23°C | Control | 22.7 | 7.96 | 8.43 | 1.030 | 0.51 | 0.509 |
| | Analytical KCl | 22.7 | 8.00 | 8.42 | 2.366 | 1.22 | 1.203 |
| | Potash KCl | 22.7 | 7.98 | 8.42 | 2.389 | 1.23 | 1.216 |
| 27°C | Control | 26.8 | 7.49 | 8.38 | 1.039 | 0.51 | 0.511 |
| | Analytical KCl | 26.7 | 7.60 | 8.40 | 2.392 | 1.22 | 1.211 |
| | Potash KCl | 26.8 | 7.64 | 8.41 | 2.430 | 1.24 | 1.231 |

*Confirmation of Veliger Identity and Characteristics*

Veliger collections consisted of a variety of life stages. D-shaped veligers ranged in size from 57 to 120 µm in shell length and 83 to 131 µm in shell height for both species (Table 14). Small umbonals had a size range that overlapped slightly with the surrounding size classes, but both species had similar ranges for height and length (Figure 6). There were only a small number of large umbonals present in the photographs used for measurement in the zebra mussel treatments and were not prevalent throughout testing, but size ranges were similar with the quagga mussel size ranges for this life stage (Figure 6). Pediveligers had a height greater than 200 µm and length greater than 190 µm; they were present in only the quagga mussel collections. Filtrate from the 300 µm filter from the zebra mussel collection tows was analyzed, and only D-shaped and small umbonals were present.

**Table 14**. Measurements with standard deviation in parenthesis of zebra and quagga mussel veligers by life stage from photo analysis from fish truck trials, zebra and quagga mussel laboratory studies conducted August through September 2016.

| | Zebra mussels | | Quagga mussels | |
|---|---|---|---|---|
| | **Length** | **Height** | **Length** | **Height** |
| **D-shaped** | N=176 | | N=87 | |
| Mean (µm) | 86.9 (11.7) | 104.5 (10.1) | 91.7 (13.1) | 106.5 (13.9) |
| Range (µm) | 57-107 | 83-131 | 60-119 | 83-131 |
| **Small Umbonal** | N=47 | | N=96 | |
| Mean (µm) | 123.9 (15.4) | 140.4 (15.4) | 138.3 (20.4) | 148.6 (20.3) |
| Range (µm) | 100-156 | 119-190 | 95-179 | 107-197 |
| **Large Umbonal** | N=4 | | N=133 | |
| Mean (µm) | 187.8 (36.0) | 194 (34.7) | 197.7 (19.5) | 203.6 (17.6) |
| Range (µm) | 142-226 | 147-226 | 155-267 | 155-250 |
| **Pediveliger** | | | N=103 | |
| Mean (µm) | | | 247.4 (23.9) | 253.0 (22.2) |
| Range (µm) | | | 190-321 | 202-321 |

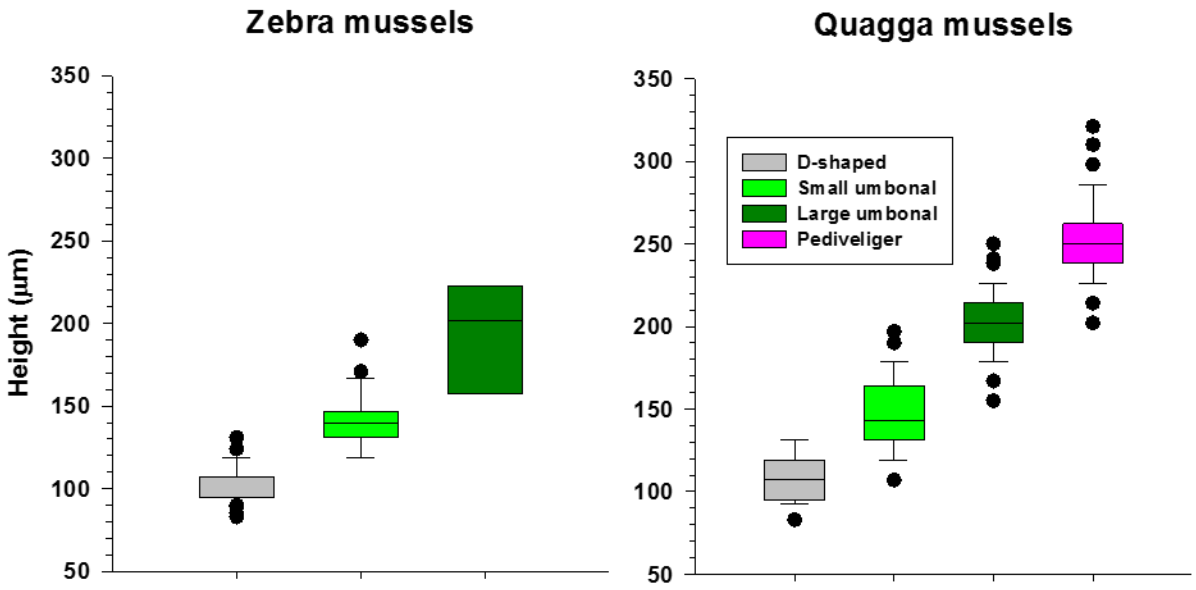


**Figure 6.** Boxplots for zebra and quagga mussel veliger height by life stage of zebra and quagga mussel veligers from photo analysis from fish truck trials, zebra and quagga mussel laboratory studies conducted August through September 2016.

The population structure of zebra mussel veligers tested were 75.1% D-shaped, 19.5% small umbonal and 5.4% large umbonal veligers (Table 15). Life stage distribution was evenly distributed across the different life stages in the quagga mussel population, where 20.7% were D-shaped veligers, 23.1% small umbonal, 31.7% large umbonals and 24.5% were pediveligers (Table 15). Observationally, we noticed that many of the large sized veligers were alive where

the small sized quagga mussel veligers were dead. Photos of the samples confirmed that some of the large umbonals and many of the pediveligers were living, but the smaller sized veligers were dead (Table 15).

**Table 15.** Proportion of zebra and quagga mussel veligers tested characterized by size class to determine population structure and mortality of quagga mussels by size class from photo analysis from fish truck trials, zebra and quagga mussel laboratory studies conducted August through September 2016.

| | D-shaped (%) | Small umbonal (%) | Large umbonal (%) | Pediveligers (%) |
|---|---|---|---|---|
| Zebra mussels (N=1031) | 75.1 | 19.5 | 5.4 | 0.0 |
| Quagga mussels (N=1881) | 20.7 | 23.1 | 31.7 | 24.5 |
| Mortality | 100 | 100 | 79.2 | 26.2 |

When the response to treatment of zebra mussel veligers was compared to the response of quagga mussels at the same conductivity treatment with potash KCl, zebra mussels had significantly higher proportional mortality than quagga mussels at all tested temperatures (15°C: $\chi^2$=304.3, $P$<0.001; 18°C: $\chi^2$=68.5, $P$<0.001); 23°C: $\chi^2$=142.0, $P$<0.001; 27°C: $\chi^2$=98.8, $P$<0.001). Likelihood ratios showed that the temperature ($\chi^2$=71.1, $P$<0.001) and species ($\chi^2$=187.9, $P$<0.001) were significant, and the interaction term between these variables was also significant ($\chi^2$=65.0, $P$<0.001). Temperature was a significant variable in both species response. The life stage differences tested between the two species explained differences in mortality, where larger life stages were the most resistant to treatment.

Veligers used during testing at FFH were identified morphologically as zebra mussels. This was confirmed with the morphological determination of adult mussels collected and measured at the plankton tow site (Table 16). Results from DNA analysis confirmed that all veligers from the Mississippi River tested were zebra mussels; no quagga mussel DNA was present in the samples. The veligers from the Lake Mead water were quagga mussels determined by morphological characteristics, and only quagga adult species have been found in the Colorado River system (Fuller 2017).

**Table 16.** Zebra mussel adult measurements of length (longest antero-posterior distance), height (longest dorso-ventral distance) and thickness (widest section of whole mussel shell) collected from Fairport Landing Marina, IA August 2016 (Beggel et al. 2015). Standard deviations are listed in parentheses.

| Variable | N | Mean (mm) | Minimum (mm) | Maximum (mm) |
|---|---|---|---|---|
| Length | 106 | 9.59 (1.41) | 5.09 | 12.65 |
| Height | 106 | 5.23 (0.74) | 3.10 | 7.08 |
| Thickness | 106 | 4.90 (0.71) | 2.85 | 6.87 |

## Discussion

The Edwards protocol was successful in killing zebra mussels at two temperatures tested: 27 and 24 °C in fish hauling truck trials. There was an issue of contamination of live veligers in the treated tanks in trial 1 due to using rinse water that was not veliger free, but methods were revised for trials 2 and 3. The revised methods resulted in no further contamination problems and 100% mortality of treated zebra mussel veligers. Control mortality was high at the 27°C testing temperature, which could have potentially inflated treatment success; upper lethal limits for zebra mussel veligers is 31°C (McMahon 1996). At the high treatment temperature, treatment was successful, but the veligers were stressed as control mortality was high. In trial 3, when temperatures were lower, the Edwards protocol treatment was successful in killing all veligers and control mortality dropped to 4%.

Presence of fish did influence the zebra mussel survival in the tank, and veliger mortality in the control tanks containing fish was greater than 70%. During treatment with the Edwards protocol, the fish added to treatment success, especially at high temperatures. High and low fish densities did not significantly change the outcome of the treatment. In trial 3, one live zebra mussel was found in the high fish density treated tank. Survival of the veliger in this treatment could have been attributed to the lower testing temperature and higher conductivity (i.e. 33 mg/L sodium concentration) of the testing water, or presence of a high fish density that inhibited the chemical action of formalin. Fish mortality occurred throughout the trials, though the fish were being treated for an outbreak of *Flavobacterium columnare* prior to study initiation. Mortality of fish by the treatment could not be differentiated from the mortality from the infection, though hatchery personnel that have used the Edwards protocol prior to this study have not reported fish mortality from treatment (Andy Fowler and Melanie Harkness, Fairport State Fish Hatchery, personal communications, August 2016).

In the temperature and conductivity laboratory studies, mortality of zebra mussel veligers was observed with the Edwards protocol at all temperatures and adjusted background conductivities. Mortality of the zebra mussel veligers increased with temperature and decreased with increased background conductivity. Interaction between conductivity and temperature were highly significant and both were important variables in the success of the Edwards protocol treatment on zebra mussels. These laboratory studies would have predicted that there would have been 100% mortality in the trial 3 high fish density treated tank; at a testing temperature of 24°C and the well water at 0.61 mS/cm conductivity as the 23°C 0.5 and 1.0 mS/cm conductivity tests showed 100% mortality. Since there was not 100% mortality, there might have been an interaction with decreased potency of formalin due to the high fish density. Further investigation into fish density effect on treatment protocol is warranted given these results.

Overall mortality was lower in the quagga mussel veliger studies than in the zebra mussel veliger laboratory studies for the same temperature and similar conductivity. At the same conductivity, 1.0 mS/cm, quagga and zebra mussel mortality showed a temperature dependence. The other variable was the size of the veligers used for testing. In the zebra mussel studies, the veligers were D-shaped or small umbonal with a few large umbonals present; and in the quagga mussel studies the veligers were almost evenly distributed between all of the life stages (Table 15).

Mortality differences between the zebra and quagga mussel studies were attributed to differences in life stage rather than difference in species response. The same mortality was achieved within the same life stage tested across the two species. When larger life stages were present in the tests, the treatment mortality decreased. Further testing of larger life stages of zebra mussel veligers is warranted to confirm these associations.

Showing that life stage was an important variable in the effectiveness of the Edwards protocol, along with temperature and conductivity helped explain much of the discrepancy in the previous studies conducted. Many hypothesis surfaced to explain the possible reasons for the discrepancies, including lack of recovery time used by Edwards et al. (2000; 2002); water quality differences including temperature and conductivity; physiological difference in response between quagga and zebra mussels; or differences in chemical effectiveness (i.e. analytical vs industrial grade KCl). It was also recommended that a successful treatment work in the presence of fish and at densities that are standard for fish transport protocols by hatcheries.

Both Sykes (2009) and Pucherelli et al. (2014) stated a need for a recovery period for the treated mussels before confirmation of mortality of the veligers could be assessed with confidence. Edwards et al. (2002) did not use a recovery period and assessed veliger mortality based on observation of no movement of cilia or organs. Sykes (2009) and Pucherelli et al. (2014) examined veligers after a recovery period for movement of cilia or organs and degradation of tissues. Mollusk toxicity studies that have used potassium as a toxicant have been shown to relax the tissues and cause the cessation of movement, and when potassium was removed from the system the movement restarted (Fisher et al. 1991; O'Donnell et al. 1996; Medler et al. 1999; Sykes 2009; Pucherelli et al. 2014; Moffitt et al. 2016). This study used fast green dye to aide in mortality assessment with a short recovery period. Fast green FCF, a synthetic food dye (Food Green 3), at 0.4% concentration has been used as a stain to determine mortality of quagga mussel veligers exposed to KCl with precise results (Whitledge et al. 2015; Moffitt et al. 2016; Stockton-Fiti and Claudi *in review*). In aquaculture, the fast green stain has been used to differentiate tissue damage in Chinook salmon (Elliott et al. 2009) and health of mussel spat prior to distribution from the nursery (Webb and Heasman 2006; Elliott et al. 2009). In these trials, the dye stained dead tissues and allowed for rapid analysis and recording of mortality with a compound microscope. We achieved similar results as Edwards et al. (2000 and 2002) with zebra mussels in laboratory and fish truck hauling trials without fish. With the quagga mussel studies, our results were most similar to Pucherelli et al. (2014) where at least 50% mortality was achieved. The use of the fast green stain to determine the health of the veligers was comparable to using a recovery period. When zebra mussel veligers were held in recovery for 24 h or more, all the control veligers were dead, making the 24 h recovery protocol not feasible at the high temperatures and in the high productivity water. Additional studies with quagga mussels by Stockton-Fiti and Claudi (*in review*) have shown that a 24 h recovery period introduces confounding factors of determining true survival as control survival significantly decreased and treatment mortality increased after a 24 h recovery period. Procedures in this study included removing the veligers from the toxicant, rinsing the toxicant away and leaving the veligers in water without toxicant for at least 45 min before health status was observed. In the studies conducted by Sykes (2009) and Pucherelli et al. (2014) veligers were pipetted out of toxicant and

transferred into clean water for the recovery period of 2 to 96 h. In those studies, toxicant was not rinsed from the veligers, only diluted and no differential stain was used.

We found that water temperature and conductivity were important factors in determining the effectiveness of the Edwards protocol. The Edwards protocol was conducted at 27°C, but Sykes (2009) and Pucherelli et al. (2014) conducted their experiments in much lower temperatures, 22 and 18 or 13 °C, respectively. In this study, mortality of zebra mussels was dependent on the temperature and conductivity of the treatment. The effect of temperature on treatment efficacy is supported by increased toxicity of formalin with increased water temperatures (Francis-Floyd 1996) coupled with increased mussel metabolism at higher temperatures, which makes toxicants more effective (Prasada Rao and Kahn 2000). Moffitt et al. (2016) conducted studies using different water sources from around North America testing KCl at 960 mg/L to determine mortality of quagga mussel veligers. They found that mean time to 50% mortality (LT50) was 2.7 hours at 22°C in low conductivity water (0.37 mS/cm) with low sodium concentrations (25 mg/L). The time to LT50 increased with increased conductivity of the water to the point where no mortality was observed in high conductivity waters on the Colorado River (1.08 mS/cm) within a 24 hour exposure period. This study showed that as conductivity (specifically sodium concentration) increased, zebra mussel veliger mortality decreased. The inverse relationship between sodium concentrations and veliger mortality explains why the Edwards protocol worked in some river basins, but not in the highly saline Colorado River where studies were conducted by Sykes (2009) and Pucherelli et al. (2014).

Although different species, quagga mussels have been found in similar niches in the environment and respond similarly to toxicants as zebra mussels (Karatayev et al. 1998; Jones and Ricciardi 2005). Sykes (2009) and Pucherelli et al. (2014) conducted their experiments on quagga mussels, but the Edwards protocol and Howell et al. (2015) studies were tested on zebra mussels. Quagga mussels have been found in deeper depths, tolerate larger oxygen extremes, and feed on different sized particles (Karatayev et al. 1998; Jones and Ricciardi 2005). Zebra mussels have been found to have a higher tolerance to lower pH treatments than quagga mussels (Claudi et al. 2013). It is possible that there could be other physiological differences that make the quagga mussel more resistant to a potassium chloride treatment. However, we maintain that the life stage tested is the most likely contributing factor to the differences in mortality. Edwards et al. (2000, 2002) tested on D-shaped and post D-shaped zebra mussel veligers, whereas Sykes (2009), Pucherelli et al. (2014), and Howell et al. (2015) had a mixed sample of life stages and Sykes (2009) and Howell et al. (2015) protocols selected for larger life stages.

We detected one treatment temperature, 23°C, where the industrial grade had significantly different results than the analytical grade KCl used during treatment. However, there were no trends observed showing either industrial or analytical grade KCl leading to higher mortality. There were significant differences between replicates at this treatment due to large numbers of veligers counted in one of the replicates. The variation of response to treatment and variation of included life stages was the most likely reason for this significant difference. Further testing with more replicates and control of life stage tested is recommended to determine if there is an actual

significant difference between analytical and industrial grades of KCl, but this study did not show that there was any difference at the temperatures tested using the Edwards protocol.

Our treatment results from the truck trials with fish were comparable to the results obtained by Edwards et al. (2002) at the 27°C testing temperature. The Edwards et al. (2002) study used a low density of fish, 6 g/L fathead minnow, and was able to report over 200 veligers observed. Our lowest density of fish used for treatment was 35 g/L goldfish and koi and reported observing 74 total veligers. The high turbidity of the Mississippi River water and the use of higher fish densities, which produced more mucus in the treatment tanks, led to a lower number of veligers recovered and observed. Edwards et al. (2002) also observed low control mortality of veligers with fish present. We observed very high control mortality with fish present >70%.

In this study, the low number of recovered individual veligers and a high control mortality in the fish containing treatments showed that the presence of fish increased the mortality of the zebra mussels. Ammonia produced by the fish, their mucus and scales, organic matter and natural turbidity of the Mississippi River water led to an increased amount of sludge that accumulated in the bottom of the fish tanks. Veligers have a low tolerance to ammonia, but studies have not been completed to determine tolerance rates. Fish produce ammonia when stressed and in the hauling tanks it accumulates (Wedemeyer 1996). The mucus of fish has been proposed to help in ionic and osmotic regulation, disease resistance, protection and many other uses (Shepard 1994). We hypothesize that a combination of interactions occurred between the veligers and the sludge as fish mucus and turbidity in the water could have decreased the veligers ability to swim and was toxic to the veligers. Few veligers were found in the water column after treatment duration and the sludge was so thick and difficult to look through that even empty shells were not found. Survival of veligers in the sludge would have been minimal as the veligers do poorly in high turbidity water (Fenske et al. 2014; Ernandes-Silva et al. 2016). Further investigation is recommended in determining the role of fish mucus and ammonia concentrations in veliger survival.

We observed that in fish truck trials, the concentrations of sodium and potassium changed relative to trials without fish present. Stress in fish causes increased permeability of the surface epithelia, such as gills, to water and ions (Wendelaar Bonga 1997). Thus, during stress, fish loose ions from their blood and try to replace it with ions in the water, explaining the differences observed in ion concentrations of sodium and potassium in the trials. Typical treatment for stressed fish is to add sodium chloride to the water to minimize the loss of ions and maintain osmotic balance (Wedemeyer 1996). However, our results indicated that the addition of sodium chloride to the treatment would make the Edwards protocol ineffective in killing veligers. Edward et al. (2000) also noticed that addition of NaCl to this protocol resulted in lower veliger mortality and recommended against adding NaCl if treating for veligers.

We found that the success of the Edwards protocol was dependent on conductivity with emphasis on sodium concentrations of water used, temperature, and life stage of veliger being treated. Sykes (2009) and Pucherelli et al. (2014) studies were conducted at colder temperatures than the Edwards protocol (2000; 2002), in higher conductivity water, and with a variety of life stage of mussels. Edwards tested on D-shaped and post D-shaped zebra mussel veligers at 20 and 27 °C

in the Great Lakes region, which had low conductivity readings and low sodium levels (Moffitt et al. 2016). The study conducted by Sykes (2009) was conducted in high conductivity water 1.0 mS/cm, at 22 and 18 °C and they selected veligers out of the veliger concentrate and testing system, which most likely resulted in a higher proportion of large umbonals and pediveligers in the treatments and in the final observation of health status. Pucherelli et al. (2014) study was conducted at 13°C, with Lake Mead water (~1.0 mS/cm) and Pueblo Reservoir and Pueblo Hatchery well water (unknown conductivity), with a mixed sample of life stages. The colder temperatures, higher conductivities and larger life stage of veligers used explains why the treatment was not successful in the Sykes (2009) and Pucherelli et al. (2014) studies. Howell et al. (2015) study resulted in zebra mussel veliger settlement after treatment, which is explained by the having larger life stages present in the treatment that were less susceptible to treatment.

## Conclusions and Recommendations

This study helped clarify some of the confusion around the Edwards protocol. We found that veliger life stage, temperature, and water conductivity specifically sodium concentration were all important variables to consider when using the Edwards protocol to treat veliger infested waters. Temperatures should be above 23°C and conductivity less than 0.5 mS/cm to ensure an efficacious treatment. For efficacious treatments below 23°C, low specific conductivity measurements, around 0.37 mS/cm would be essential. Veliger life stage should be D-shaped to small umbonal size for an effective treatment. For the Edwards protocol to be used in a fish hauling truck treatment these variables need to be analyzed prior to treatment.

At FFH, an optimal period of using the Edwards protocol would be from June through August (Figure 7). Information regarding veliger life stage would need to be collected to use the Edwards treatment protocol after August. At low temperatures, high conductivities, or large veliger life stages, higher concentrations of KCl and formalin with longer duration times are recommended to achieve a successful treatment (Sykes 2009; Pucherelli et al. 2014).

Pucherelli et al. (2014) recommended using a 12 h KCl pretreatment at 1,500 mg/L followed by a 2 h dose of 50 mg/L formalin in Pueblo Hatchery well water (sodium concentration unknown) at 13°C, but these concentrations and duration times may be toxic to the fish species being transported. Sykes (2009) also conducted elevated dosage treatments, up to 4,250 mg/L KCl and 100 mg/L formalin, and did not achieve 100% mortality in Colorado River water (sodium concentration approximately 100 mg/L) at 18°C. Further research into use of higher concentrations of chemical for treatment at different temperatures, sodium concentrations, and veliger life stage are recommended if the Edwards protocol is to be recommended for use in scenarios where the temperature is below 23°C, specific conductivity is higher than 0.37 mS/cm and veliger life stage is large umbonal or pediveliger. These dosages and duration times would also need to incorporate fish toxicity data at recommended fish densities and include feasibility information for hatchery personnel to conduct the treatment.

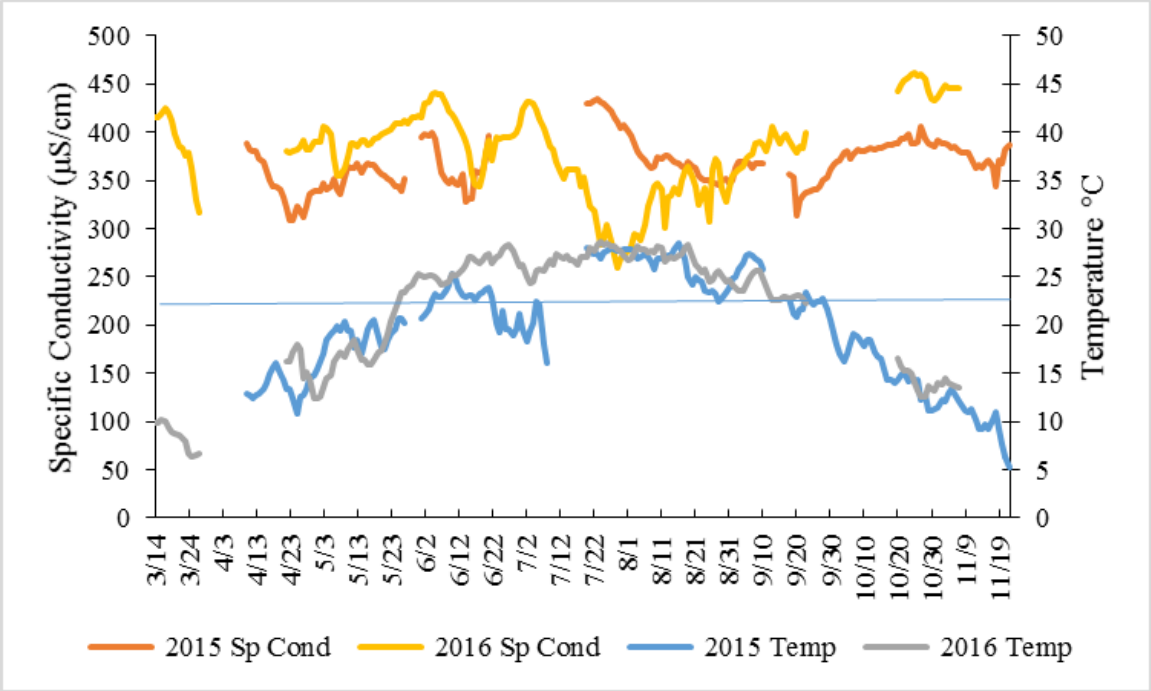


**Figure 7.** Seasonal data of temperature and specific conductivity for the Mississippi River at Fairport State Fish Hatchery collected by the University of Iowa water quality sonde.

We also recommend that additional research focus on tolerance of veligers to high ammonia concentrations and the interaction of fish mucus on veliger mortality. We found that there was high mortality of veligers associated with being in the transport tanks with fish at high densities (95 g/L), but could not determine if the veliger mortality was caused by ammonia concentrations produced by the fish or from contact with fish mucus and high turbidity of the testing water. Combining results from a fish effect study with rinsing fish in mussel-free water for 12 h as recommended by Pucherelli et al. (2014) could be an effective way of eliminating veligers from transported fish. Fish could be loaded into a mixed cell raceway that was supplied with mussel-free water prior to transport, which would remove veligers from the water column and fish, creating mussel free fish (Stockton et al. 2016). There are many ways to obtain mussel free water, including using a source that is not infested, filtering out veligers sized particles, such as with a hydrocyclone (Nielson et al. 2012), or chemical water treatment with a neutralizer added prior to use (Van Benschoten et al. 1993).

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## Appendix

Table A1. Metal ion analysis from inductively coupled plasma (ICP) protocols at the University of Idaho Analytical Sciences Laboratory of source waters (filtered Mississippi river water, Fairport State Fish Hatchery well, and Lucille A. Carver Mississippi Riverside Environmental Research Station (LACMRERS) laboratory) used in this study. Analysis results for the filtered Lake Mead water are from Moffitt et al. (2016) and NR=not reported. The reported measures for chromium, cobalt, iron, vanadium and zinc were below detection limit for all source waters and not reported in the table.

| Water Source | Barium (mg/L) | Cadmium (mg/L) | Calcium (mg/L) | Copper (mg/L) | Magnesium (mg/L) | Manganese (mg/L) | Molybdenum (mg/L) | Nickel (mg/L) | Potassium (mg/L) | Sodium (mg/L) |
|---|---|---|---|---|---|---|---|---|---|---|
| Mississippi River | 0.043 | < 0.020 | 33 | < 0.020 | 14 | 0.020 | < 0.25 | < 0.050 | 2.4 | < 10 |
| FFH Well | 0.50 | < 0.020 | 40 | < 0.020 | 16 | 0.059 | < 0.25 | < 0.050 | 2.3 | < 10 |
| LACMRERS Lab | < 0.020 | < 0.020 | 0.36 | 0.17 | 0.054 | < 0.005 | < 0.25 | < 0.050 | 1.1 | 170 |
| Lake Mead | 0.126 | 0.004 | 79 | NR | 27.6 | 0.005 | 0.054 | 0.009 | 4.6 | 100 |