

# **Verification of the effectiveness of KCl on zebra mussel veligers**

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## Introduction

Quagga mussels *Dreissena rostriformis bugensis* and zebra mussels *Dreissena polymorpha* are reported to be particularly sensitive to potassium toxicity (Fisher et al. 1991; Fernald and Watson 2014; Moffitt et al. 2016). Concentrations of 100 ppm KCl, as potash, have been proposed as a preferred rapid response tool to control and eliminate infestations of adult quagga and zebra mussels in contained areas within the Pacific Northwest because of its low toxicity to non-target organisms (Fisher et al. 1991; Heimowitz and Phillips 2014). An infestation of zebra mussels in Millbrook Quarry, Virginia was eliminated with a potash treatment and provides an excellent model treatment study (Fernald and Watson 2014). Other deployments of KCl in infested areas have been conducted in Lake Winnipeg, Manitoba and Christmas Lake, Minnesota with varying success due to incomplete treatments in the entire infested area.

Recent studies by Moffitt et al. (2016) with quagga mussels showed that concentrations of 100 mg/L KCl were effective in killing byssal stage mussels in low sodium surface waters from Idaho and Lake Ontario (mean survival 4.9 and 6.9 d, respectively). Their studies further illustrated that the toxicity response of quagga mussels was related to the sodium content of the source water, and the relative toxicity in different source waters could be modeled with veligers exposed to more elevated test concentrations. They conducted short term exposures in a nearly 10 fold elevated concentration (960 mg/L), and found 960 mg/L KCl caused 100% mortality to quagga mussel veligers in low sodium (less than 30 mg/L) waters within 5 hours whereas nearly all veligers survived for 24 h when tested in water with sodium >100 mg/L.

Although quagga and zebra mussels are closely related species, differences in their ecology and tolerance to stressor are reported. 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). Other physiological differences could exist between zebra and quagga mussels that may affect the mortality response to potassium chloride. The objectives of this study were to evaluate the mortality of zebra mussel veligers to concentrations of 960 mg/L and relate the response to sodium concentrations in the test systems.

## Methods

### *Study Location, Source Water and Test Organisms Collections*

Tests were conducted at Fairport State Fish Hatchery, Fairport, IA in the Lucille A. Carver Mississippi Riverside Environmental Research Station (LACMRERS) laboratory of Iowa State University in August 2016. Source water used in tests was obtained from the hatchery reservoir. The reservoir water was pumped from the Mississippi River, and passed serially through a sand filter system and a 25 µm and 10 µm sock filter system. Testing water was collected the day before testing, and filtered through a 300 µm and a 35 µm filter into 3.8 L containers. Water was brought to room temperature (~ 20°C) in the laboratory.

Dreissenid veligers were collected from the Mississippi River from a dock structure at Fairport Landing Marina, IA, (41.4357°N; 90.9016°W). Plankton tow nets (35 µm mesh) were used to

filter water in 5 min tows on the day of testing. The contents of the cod end were poured through a 300  $\mu\text{m}$  sieve into 3.8 L containers and transferred to the lab. In the lab, the veligers were poured onto a 63  $\mu\text{m}$  sieve and then back flushed into a container with filtered river source water. The veliger concentrate was well mixed and a three 1 mL aliquots were removed and examined with light microscopy to determine the average number of veligers per mL. Using the density of veligers from sampling, adjustments were made to provide a total number of concentrated veligers of 400 to 500 individuals per replicate.

### *Identification of Test Organisms*

Throughout testing, zebra mussel veligers were identified, counted and characterized by size class to determine population structure. Samples were examined and life stages were identified and counted as straight-hinged (D-shaped), umbonal, and pediveliger. The population of zebra mussel veligers tested were 83% D-shaped and 17% small umbonal veligers (n=655). Morphological determination of the veligers were made using the characteristics described in Nichols and Black (1993) with a compound microscope. Photographs of samples were later analyzed in detail to provide measurements of size and distribution (Table 1). Straight-hinged larvae all had a D-shape and ranged in size from 57 to 107  $\mu\text{m}$  in shell length and small umbonals ranged in size from 100 to 156  $\mu\text{m}$  (Table 1). Large umbonals or pediveligers (>200  $\mu\text{m}$ ) were not present in the collection used for testing. Filtrate off the 300  $\mu\text{m}$  filter was analyzed and only D-shaped and small umbonals were present.

Adult zebra mussels were collected from Fairport marina docks where veligers were collected and retained in 70% isopropanol for later measurement of shell length and height (Beggel et al. 2015). They were characterized by morphological characteristics as described in Nichols and Black (1993) (Table 1).

**Table 1.** Measurements of zebra mussels tested by life stage observed with standard deviation listed in parenthesis.

	<b>Length</b>	<b>Height</b>
<b>D-shaped (N=176)</b>		
Mean ( $\mu\text{m}$ )	86.9 (11.7)	104.5 (10.1)
Range ( $\mu\text{m}$ )	57-107	83-131
<b>Small Umbonal (N=47)</b>		
Mean ( $\mu\text{m}$ )	123.9 (15.4)	140.4 (15.4)
Range ( $\mu\text{m}$ )	100-156	119-190
<b>Adult (N=106)</b>		
Mean (mm)	9.59 (1.41)	5.23 (0.74)
Range (mm)	5.09-12.65	3.1-7.08

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% EtOH for DNA analysis. Both 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 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 and samples were centrifuged to create a pellet. The DNA was then extracted from the pellet with a Qiagen 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 bands. In addition, each gel contained both a positive and negative control to ensure for quality assurance.

*Water Quality, Test Substance, and Experimental Design*

The filtered river water quality was measured with a YSI 556 multi-probe (YSI Yellow Springs, OH). The baseline river water had specific conductivity of 0.37 mS/cm. To elevate the sodium content, 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, 1.0 and 1.5 mS/cm. After the waters were adjusted to provide each specific conductivity treatment (Table 2), 1.2 g/L of potassium chloride (KCl) (CAS 7447-40-7; FW 74.55, Macron Chemical Company, Center Valley, PA, lot 66919) was added to create a stock solution after methods of Moffitt et al. (2016). Experiments were conducted in replicated 150 mL glass beakers, with 3 beakers for each chemical and control treatment and exposure time interval. Each test beaker was seeded with at least 400 veligers. For each specific conductivity treatment, the volume of the test container was brought to 20 mL with test water without KCl. The test began when 80 mL of the KCl stock solution specific for each treatment was added. For controls 80 mL of treatment specific conductivity adjusted filtered river water was added to each beaker. Duration of exposure for trials ranged from 3 to 24 hr (Table 1). The 3 and 24 hr treatments for the 0.37, 0.5, and 1.0 mS/cm background conductivity waters were conducted with a veligers collected on 8/13 in the morning; the 12 hr treatments were conducted with veligers collected on 8/13 in the evening. 6 hr treatments and the 1.5 mS/cm tests were conducted with veligers collected on 8/17 in the morning.

**Table 2.** Matrix of test treatment conductivity and intervals of exposure. Three replicate beakers were tested at each interval and test treatment (960 mg/L KCl and control with no added KCl).

<sup>a</sup>baseline conductivity with no addition of NaCl.

Specific Conductivity (mS/cm)	Test exposure interval for KCl and control
0.37 <sup>a</sup>	3, 6, 12 hr
0.5	3, 6, 12 hr
1	3, 6, 12, 24 hr
1.5	24 hr

Temperature, pH, specific conductivity, salinity, and total dissolved solids (TDS) were measured at the end of each test exposure. Water samples from each of the stock solutions, were analyzed

to quantify the total quantities of metals (barium, cadmium, calcium, chromium, cobalt, copper, iron, magnesium, manganese, molybdenum, nickel, potassium, sodium, vanadium, and zinc) using inductively coupled plasma (ICP) protocols. The stock solutions for the 0.37 and 0.5 mS/cm were also submitted to determine the negative ion concentrations of the treatment water, including bromide, chloride, fluoride, nitrate-N, nitrite-N, o-Phosphate-P, and sulfate. All water analytes were measured at the University of Idaho Analytical Sciences Laboratory, Moscow, ID.

To assess mortality of test organisms, the contents of each beaker at each test interval were poured through a 10  $\mu$ m sieve and then placed into a 0.4% aqueous solution of fast green FCF (Harleton, Gibbstown, NJ, lot 4287G) for 20 min. The contents were then rinsed with lab water to remove excess dye and then placed into a recovery beaker and held for (10 to 60 min) until analysis by microscopy. To evaluate survival, samples of ~ 200 veligers were removed from each recovery beaker with disposable pipets, and evaluated with a gridded Sedgewick-Rafter counting cell and compound microscope (total magnification of 40 and 100 $\times$ ) and were scored. Scoring levels included live (non-stained with mantle intact), dead (stained mantle), and open empty shells (non-stained but with no mantle intact). At least 100 veligers were assessed per replicate.

### *Statistical Analysis*

The proportion of live, dead, plus open empty shells was summarized for each replicate, treatment, and interval. Empty shells were included in the assessment of dead veligers due to the high decomposition rate observed during testing. This decision was based on the low numbers of empty shells in the controls, indicating that the numbers of empty shells in the treatment beakers was likely due to the effects of the treatment rather than present in the sample concentrate. In addition, an estimate of relative mortality was also calculated by subtracting the mean control mortality from the mean treatment mortality for each test exposure and duration combination. The water quality measures were summarized to provide a mean temperature, specific conductivity, salinity and pH.

The significance of the test replicates was analyzed as a covariate in linear regression models and also in categorical models of the frequency of live and dead using Fisher's exact chi-square tests. The test replicate was then removed from the models when test replicate was not significant. The relative mortality was transformed with square root of the proportion of mortality. Ordinary least squares methods were used to conduct linear regression modeling, which was used to determine significant variables to predict the mortality of the veligers using a linear mixed-effects model fitting in R 3.1.3 (R Development Core Team, 2015) with package *lme4* (Bates et al. 2014). Model significance and R-squared results were relied upon to choose the best model to determine variable significance. Correlations were assessed with the package *Hmisc* (Harrell 2016) and package *car* (Fox and Weisberg 2011) was used to determine multicollinearity of the model, which was used to find the best model. Chi-squared analysis was used to determine significant differences in the replicates and between treatment and control results and linear regression analysis was repeated in SAS 9.3 (SAS Institute, Cary, NC).

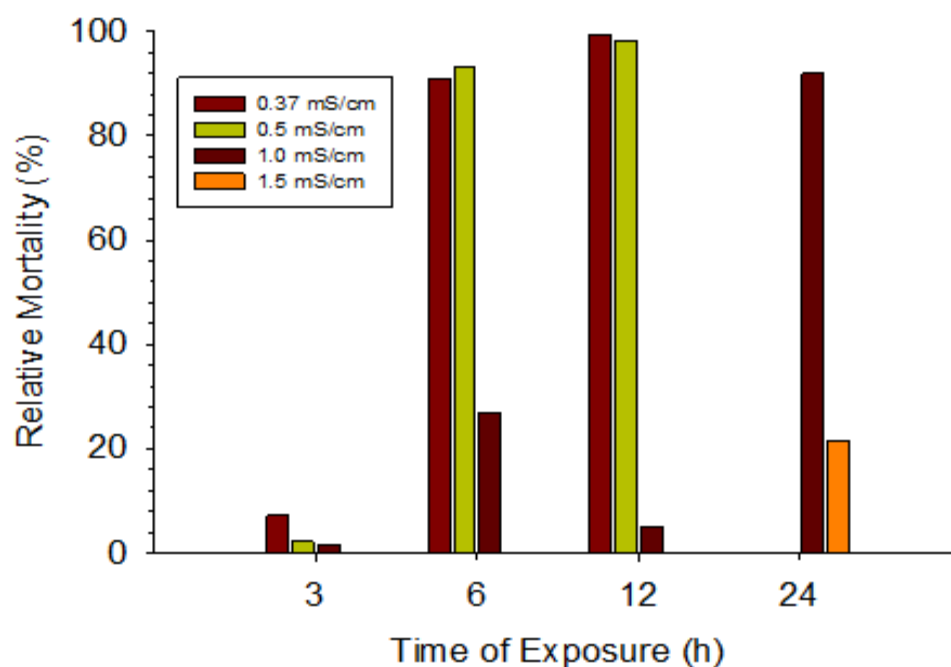
## Results

A 960 mg/L KCl dose was effective, causing mortality in zebra mussel veligers within 6 hr (Table 3) in water that was not supplemented with NaCl to achieve a higher conductivity. Time to mortality increased as conductivity and sodium increased. At the highest adjusted specific conductivity water, 1.5 mS/cm, only 32% of veligers were dead after 24 hr. Control mortality was not affected by exposure time or conductivity. Standard deviations of the mean mortality in the controls and treatment were observed. Relative mortality was used to adjust for the elevated mortalities in the controls (Table 3; Figure 1).

The frequency of live and dead in control versus test system in each treatment time interval were significantly different in all treatments, except the 3 hr 0.5 mS/cm ( $\chi^2=0.911$ ,  $p= 0.340$ ) and the 3 hr 1.0 mS/cm treatments ( $\chi^2=1.3043$ ,  $p= 0.2534$ ) (Table 3).

**Table 3.** Mean and (SD) mortality and mean relative mortality of zebra mussel veligers exposed to 960 mg/L KCl or controls without KCl for duration of exposure and conductivity treatment. Values represent the mean of three replicate beakers for each treatment and exposure time.

<b>Treatments</b>	<b>3 hr</b>	<b>6 hr</b>	<b>12 hr</b>	<b>24 hr</b>
<b>0.37 mS/cm</b>				
Control	6.64 (1.43)%	9.06 (2.53)%	0.74 (0.22)%	
960 mg/L KCl	13.81 (1.55)%	100 (0.0)%	100 (0.0)%	
<i>Relative mortality</i>	<i>7.17%</i>	<i>90.94%</i>	<i>99.26%</i>	
<b>0.5 mS/cm</b>				
Control	8.80 (1.44)%	6.56 (2.26)%	1.82 (1.68)%	
960 mg/L KCl	10.90 (1.44)%	99.69 (0.53)%	100 (0.0)%	
<i>Relative mortality</i>	<i>2.1%</i>	<i>93.13%</i>	<i>98.18%</i>	
<b>1.0 mS/cm</b>				
Control	5.29 (0.23)%	6.14 (1.54)%	1.95 (1.92)%	8.00 (0.55)%
960 mg/L KCl	6.91 (3.91)%	33.09 (8.62)%	6.98 (1.88)%	100 (0.0)%
<i>Relative mortality</i>	<i>1.62%</i>	<i>26.95%</i>	<i>5.03%</i>	<i>92.00%</i>
<b>1.5 mS/cm</b>				
Control				10.41 (1.22)%
960 mg/L KCl				32.02 (4.42)%
<i>Relative mortality</i>				<i>21.61%</i>



**Figure 1.** Plot of the relative mortality of zebra mussel exposed to 960 mg/L KCl at each exposure time tested for each adjusted conductivity tested.

Only one treatment 6 hr 1.0 mS/cm 960 mg/L KCl had a significant difference between the replicates ( $\chi^2=8.39$ ,  $p=0.016$ ), and also the highest standard deviation of all the treatments (8.62%; Table 3). One replicate had much higher mortality than the other two, but if removed the resulting mortality was 28%, and still higher than mortality in the 12 hour sample. Removal of these data did not change model fit and thus all data were retained for analysis. There were no significant differences between replicates in the controls.

Specific conductivity, salinity and TDS increased from the control levels with additions of KCl (Table 4). Average temperature of this study was at 20.4 ( $\pm 0.4$ ) °C, and the mean pH was 8.24. No significant differences were determined in measures of water chemistry for each exposure duration by each treatment.

The sodium concentrations in NaCl supplemented water were reflected in the increased measured amount found in the ICP analysis (Table 4). In the KCl treatment, the stock solution had higher potassium concentrations over the control water for each treatment. The increased measurements of specific conductivity, salinity and TDS, plus the metals analysis indicated the test solution was prepared correctly.

**Table 4.** Summary of mean water quality measurements with standard deviation in parenthesis in treatments of the different adjusted waters for the test of toxicity to 960 mg/L KCl, with the measured sodium and potassium concentrations from the ICP analysis.

	Temp (°C)	pH	Sp. Cond. (mS/cm)	Salinity (ppt)	TDS (mg/L)	Sodium (mg/L)	Potassium (mg/L)
<b>0.37 mS/cm</b>							
<b>Control</b>	20.42 (0.45)	8.27 (0.16)	0.367 (0.004)	0.18 (0.01)	0.241 (0.007)	<10	2.6
<b>960 KCL</b>	20.38 (0.37)	8.22 (0.14)	2.121 (0.078)	1.08 (0.05)	1.379 (0.051)	<10	590 <sup>@</sup>
<b>0.5 mS/cm</b>							
<b>Control</b>	20.44 (0.41)	8.32 (0.17)	0.502 (0.011)	0.24 (0.01)	0.326 (0.007)	35	2.7
<b>960 KCL</b>	20.40 (0.43)	8.27 (0.25)	2.299 (0.092)	1.18 (0.05)	1.494 (0.059)	35	600 <sup>@</sup>
<b>1.0 mS/cm</b>							
<b>Control</b>	20.48 (0.45)	8.11 (0.45)	0.979 (0.026)	0.48 (0.01)	0.637 (0.017)	130	2.7
<b>960 KCL</b>	20.49 (0.48)	8.23 (0.29)	2.764 (0.64)	1.44 (0.04)	1.798 (0.042)	130	590 <sup>@</sup>
<b>1.5 mS/cm</b>							
<b>Control</b>	20.20 (0.04)	8.31 (0.06)	1.522 (0.015)	0.77 (0.01)	0.989 (0.010)	220	2.7
<b>960 KCL</b>	20.23 (0.05)	8.22 (0.11)	3.358 (0.055)	1.77 (0.03)	2.191 (0.051)	220	570 <sup>@</sup>

<sup>@</sup>Stock concentration measurement.

The best model to predict mortality included exposure time and background specific conductivity ( $F(2, 8) = 5.2, p < 0.036$ ), with an  $R^2$  of 0.57. Slope of the background specific conductivity was negative and a significant parameter and exposure time had a significant positive slope (Table 5). Specific conductivity was used in modeling; sodium and TDS were not used in the model due to all three being significantly correlated to each other (Table 6). Temperature and pH were highly correlated to each other and both variables were removed from the model as there were no significant differences in the measurements across the treatments. This model showed that as background conductivity increased the mortality decreased and as exposure time increased mortality increased.

**Table 5.** Summary of linear regression analysis of the best model to predict square root transformed mortality.

Variable	B	SE B	t value	p-value	F Value	p-value
<b>Exposure Time</b>	0.041	0.014	2.92	0.019	2.29	0.168
<b>Sp. Cond</b>	-0.82	0.289	-2.85	0.022	8.11	0.021
<b>Intercept</b>	0.788	0.189	4.16	0.003		



**Table 6.** Correlation matrix for the variables considered for model building, where asterisk values indicated  $p$ -value was  $<0.05$ .

<b>Variable</b>	<b>Sp. Cond</b>	<b>Sodium</b>	<b>Temp</b>	<b>pH</b>
<b>Exposure Time</b>	0.6	0.59	-0.13	0.04
<b>Sp. Cond</b>		1*	-0.02	0
<b>Sodium</b>			-0.04	-0.03
<b>Temp</b>				0.88*

Many metals in the test water were low, and cadmium, chromium, cobalt, copper, iron, molybdenum, nickel, vanadium and zinc levels were below detection limit. For the negative ion analysis, bromide, fluoride, nitrite-N, o-phosphate-P concentrations were either not detected or below detection limits (Table 7).

**Table 7.** Concentration of metals and negative ions in mg/L for the testing water from the Mississippi River, where ND=non detection.

<b>Metal</b>	<b>Mississippi River</b>	<b>Metal</b>	<b>Mississippi River</b>	<b>Negative Ion</b>	<b>Mississippi River</b>
Barium	0.047	Magnesium	17	Bromine	ND
Cadmium	< 0.020	Manganese	0.10	Chloride	17
Calcium	39	Molybdenum	< 0.25	Fluoride	< 0.20
Chromium	< 0.050	Nickel	< 0.050	Nitrate-N	0.11
Cobalt	< 0.010	Potassium	2.6	Nitrite-N	ND
Copper	< 0.020	Sodium	< 10	o-Phosphate-P	ND
Iron	< 0.10	Vanadium	< 0.020	Sulfate	22
		Zinc	< 0.020		

## Discussion

Zebra mussels were highly sensitive to 960 mg/L KCl. Increasing the amount of sodium in the water before adding KCl provided protection for the mussels as it decreased the mortality of zebra mussels. Treatment water quality was within normal ranges for conducting the tests and the associated mortality observed was due to the toxicity of KCl. Water quality also did not change over treatment time. The model of the data indicated that as background specific conductivity increased mortality decreased and increased exposure time increased mortality.

There was high variation in the health of the veligers at collection time. We used the relative mortality when modeling the data to account for some of this variation. While the model parameters may not be specific to the effect, the trend and directionality that was shown in the model held true for all testing.

Water quality measurements for dissolved oxygen and pH were considered within tolerance limits for zebra mussel survival (Sprung 1993) and should not have affected the health of the

veligers. The metals and ion analysis showed calcium levels were within tolerance ranges for zebra mussel survival and growth (Sprung 1993).

Presence of heavy metals, such as manganese, or toxic algae in the water may have been responsible for the increased mortality in the morning collections. The metal ion analysis from the treatment water did come back with elevated levels of manganese, when compared to the waters that support quagga mussels from the Moffitt et al. (2016) study, but no research was available to determine the toxicity of that heavy metal on the veligers (Claudi and Prescott 2011). However, the 1.5 mS/cm water had very low measured manganese levels (0.03 mg/L) and still had high control mortality. The presence of a thin green layer of algae at the top of the concentrated veliger collections may have been related to toxic algal bloom, but further research would need to be conducted to validate this hypothesis and not other variables that may have caused elevated mortality.

We consider these results to be valid despite the control mortality and variation in the mortality with the different exposure times. Testing of additional exposure times would help fit a probit model to the mortality response and improve the precision of the relationship between KCl toxicity, salinity and exposure time to zebra mussels.

The levels of potassium in the stock solutions measured with ICP were slightly lower than expected; the actual potassium concentration of a 1.2 g/L stock solution should have been 624 mg/L plus background. The concentration of potassium in the tested samples ranged from 570-600 mg/L (Table 4). Potassium is an ion that does not readily bind to other ions in aqueous solutions, therefore it should have been available for ICP analysis. Using this assumption the stock solution was an average concentration of 1.13 g/L KCl, which would equate to a 904 mg/L testing concentration. However, the testing water was from the Mississippi River and had bacteria and algae in it. The bacteria and algae could have easily removed the potassium from the water, making the ion unavailable for ICP analysis (Iyer et al. 2015; Golby et al 1990; Poole et al. 1990). Water samples were removed at the end of testing, stored refrigerated for up to three week then frozen before analysis. The samples were analyzed up to a month after sampling date allowing time for algae and bacteria to remove the potassium from the water. Another way to check if the solutions were done correctly was to look at the chloride concentrations. Chloride concentration should have been 576 mg/L for the KCl portion added, and with the additional measured background chloride concentration, 17 mg/L, the FFH 0.37 mg/L specific conductivity plus the 1.2 g/L KCl should have been 593 mg/L chloride; the actual reading was 630 mg/L. This indicated that the dosing was higher than 1.2 g/L KCl and was instead at 1.28 g/L KCl, which was very close to expected. Both the bacteria and algae could have affected measurements as they achieved homeostasis.

This study was conducted with the same methods as the Moffitt et al. (2016) study, to make sure that zebra mussel veligers were similarly susceptible to potassium chloride and that the toxic effect of potassium chloride was diminished by the addition of sodium in the treatment system. We found that zebra mussels were similar in response to the toxicity of potassium chloride and that the addition of sodium decreased the toxicity of potassium chloride.

These data should be used in preparing for a potassium chloride treatment on zebra mussel infested water. It is important to ensure that the background specific conductivity and sodium levels are known and potassium chloride treatments be adjusted to account for increased sodium and specific conductivity levels. Small scale studies should be conducted to determine the best minimum dose before larger treatments are conducted to dose the area with the minimum amount of toxicant and still be effective.

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