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Research Article

Effects of temperature and exposure duration on four potential rapid-response tools for zebra mussel (*Dreissena polymorpha*) eradication

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This study was contributed in relation to the 20th International Conference on Aquatic Invasive Species held in Fort Lauderdale, Florida, USA, October 22–26, 2017 (http://www.icais.org/html/previous20.html). This conference has provided a venue for the exchange of information on various aspects of aquatic invasive species since its inception in 1990. The conference continues to provide an opportunity for dialog between academia, industry and environmental regulators.

Abstract

Zebra mussels (*Dreissena polymorpha*) have continued their spread within inland lakes and rivers in North America despite diligent containment and decontamination efforts by natural resource agencies and other stakeholders. Identification of newly infested waterways by early detection surveillance programs allows for rapid response zebra mussel eradication treatments in some situations. Previous eradication treatments have occurred over a broad range of water temperatures which have influenced the efficacy of molluscicides. Natural resource managers will benefit from knowledge regarding the impacts of water temperature and exposure duration on the toxicity of molluscicides to zebra mussels. In particular, temperature specific data are needed to inform the selection of an effective molluscicide and the proper dose that will induce 100% zebra mussel mortality. We evaluated the influences of temperature and exposure duration on the toxicity of two U.S. EPA-registered (EarthTec QZ and Zequanox) and two nonregistered (niclosamide and potassium chloride) molluscicides to zebra mussels at water temperatures of 7, 12, 17, and 22 °C. Our results indicate that treatment options for the eradication of zebra mussels in waters ≤ 12 °C include 336 h or longer treatments with EarthTec QZ and KCl as well as treatments with niclosamide ≥ 24 h in duration. In waters ≥ 17 °C, multiple toxicant and exposure duration combinations are potentially effective for zebra mussel eradication. On-site or *in situ* zebra mussel bioassays are a useful tool for the evaluation of treatment efficacy.

Key words: dreissenid, control, toxicant, molluscicide

Introduction

Zebra mussels (*Dreissena polymorpha* Pallas, 1771) are native to the Black, Caspian, and Aral Seas of Eastern Europe and they have become widely established within the United States since their introduction in the mid-1980s (Gollasch and Leppäkoski 1999; Benson 2013). Their high fecundity and microscopic free-swimming larval life stage allow for rapid natural and anthropogenic dispersion (Mackie and Claudi 2010; Birnbaum 2011). Many pathways have been implicated for overland dispersal; however, human-mediated mechanisms, including recreational watercraft, have been identified as the main source (Gollasch and Leppäkoski 1999; Johnson et al. 2008). The likelihood of zebra mussels spreading from an established population to a nearby uninfested waterbody is greatly enhanced by the "dispersion kernel phenomenon", which is a function of infestation probability and distance from a source population (Havel et al. 2015). The significant harm to aquatic ecosystems and severe economic losses associated with the zebra mussel invasion in North America are well documented in the scientific literature (Higgins

and Vander Zanden 2010; Mackie and Claudi 2010; Nalepa and Schloesser 2013; Mayer et al. 2014; Colvin et al. 2015). Zebra mussels demonstrate a classic invasion curve, in which there is a lag phase prior to a rapid growth phase (Crooks and Soulé 1999). Eradication is nearly impossible once an invasive species population surpasses the lag phase (Crooks and Soulé 1999; Bax et al. 2001). Therefore, early detection and rapid response (EDRR) to invasive species introductions are vital for eradication (Westbrooks 2004; Anderson 2005; Locke and Hanson 2009).

The majority of zebra mussel control strategies in North America have been developed for industrial water conveyance systems and control methods have included toxicants (oxidizing and non-oxidizing chemicals) and physical means such as filtration, ultraviolet radiation, and antifouling coatings (Strayer 2009; Mackie and Claudi 2010; Nalepa and Schloesser 2013; Glomski 2015; Wong and Gerstenberger 2015). A limited number of zebra mussel control treatments have been attempted in open-water environments. Perhaps the most well-known example is the successful treatment of a highly-infested, 4.9-ha quarry lake (Millbrook Quarry, Prince William County, VA) in 2006 (Fernald and Watson 2013). Several partial-lake EDRR zebra mussel eradication treatments have been attempted in Manitoba, Minnesota, and Nebraska but long-term success has not been achieved. The lack of success of these treatments may have been influenced by the timing of the applications because water temperature has been shown to have a substantial and overriding effect on the sensitivity of zebra mussels to toxicants (Rao and Khan 2000; Costa et al. 2008). A better understanding of the influences that water temperature and exposure duration have on the efficacy of zebra mussel toxicants may improve the success of future zebra mussel eradication treatments.

The United States Environmental Protection Agency (U.S. EPA) has registered two pesticides for use as open-water zebra mussel toxicants: EarthTec QZ, a copper-based product; and Zequanox, a killed-cell bacteria-based product. EarthTec QZ has demonstrated toxicity to both zebra and quagga mussels (*D. bugensis* Andrusov, 1897); however, limited information is available regarding the influence of water temperature on toxicity (Watters et al. 2013; Claudi et al. 2014). Research by Rao and Khan (2000) indicates temperature influences the toxicity of copper to zebra mussels. EarthTec QZ toxicity is likely to be similarly influenced by temperature since the active ingredient is copper. Zequanox also has demonstrated toxicity to zebra and quagga mussels, yet, the influence of temperature has not been specifically investigated or documented (Luoma et al. 2015a, b; Whitledge et al. 2015). Niclosamide (5-Chloro-N-(2-chloro-4-nitrophenyl)-2-hydroxybenzamide) and potassium chloride (KCl) are not registered for zebra mussel control; however, past and present use in aquatic systems suggest that either has potential for registration with the U.S. EPA for use as a zebra mussel toxicant. Several formulations of bayluscide, the ethanolamine salt formulation of niclosamide, are registered by the U.S. EPA to kill sea lamprey (*Petromyzon marinus* Linnaeus, 1758) in tributaries to the Great Lakes. Furthermore, products containing niclosamide are routinely used for snail control in tropical and subtropical areas (McDonald and Kolar 2007; Dai et al. 2008). The effects of temperature on toxicity was not thoroughly reported in studies examining the seasonal differences of the susceptibility of zebra mussels to bayluscide (Kilgour and Baker 1994; Costa et al. 2008). Potassium was discovered to be toxic to zebra mussels in the early 1990s and KCl was the most selective toxicant out of 18 chemicals evaluated for use as a zebra mussel toxicant (Fisher et al. 1991; Waller et al. 1993). Potassium, typically applied as muriate of potash, has a history of use in zebra mussel eradication treatments, including the Millbrook Quarry eradication where approximately 131,000 kg of potash was applied over 3 weeks to maintain a potassium concentration of 100 mg/L (Fernald and Watson 2013). The influence of water temperature on the toxicity of potassium to zebra mussels has been documented (Fisher et al. 1991; Wildridge et al. 1998; Costa et al. 2008); however, these studies did not report concentrations that would induce 100% zebra mussel mortality.

Literature is lacking on the concentrations and exposure durations of toxicants required to induce 100% mortality of zebra mussels at various water temperatures. This information is necessary to determine treatment options for the eradication of localized zebra mussel infestations. Understanding toxicant-specific limitations, required treatment parameters, and potential impacts to nontarget animals will allow natural resource managers to determine the feasibility of zebra mussel eradication treatments. The goal of this project was to determine the treatment parameters required for successful zebra mussel eradication treatments at various water temperatures. Our objectives included determining the effects of water temperature and exposure duration on the toxicity of four zebra mussel toxicants: EarthTec QZ, niclosamide, potassium chloride, and Zequanox. Specifically, we sought to determine the toxicant-specific dose required to induce 100% zebra mussel mortality at water temperatures of 7, 12, 17 and 22 °C with various exposure durations.

Table 1. Test animal source, collection date, and test initiation date by temperature and test article.

Temperature (°C)	Test article(s)	Test animals		Test initiation
		Source	Collection	
7	EarthTec QZ, Niclosamide, KCl	Lake Minnetonka	11/2/2016	11/7/2016
	Zequanox	Lake Minnetonka	11/2/2016	11/9/2016
12	EarthTec QZ, Niclosamide	Lake Minnetonka	10/12/2016	10/17/2016
	KCl	Lake Minnetonka	11/2/2016	1/9/2017
	Zequanox	Lake Minnetonka	10/12/2016	10/19/2016
17	EarthTec QZ, Niclosamide, KCl	Lake Minnetonka	9/22/2016	9/26/2016
	Zequanox	Lake Minnetonka	9/22/2016	9/28/2016
22	EarthTec QZ, Niclosamide, KCl	White Bear Lake	9/8/2017	9/11/2017
	Zequanox	Lake Minnetonka	11/2/2016	12/7/2016

Methods

Test animals

Zebra mussel test animals were acquired from two different lakes for this study (Table 1). The primary source was Robinson's Bay in Lake Minnetonka (Hennepin County, MN). Zebra mussels (10–25 mm) were collected at four discrete times between September and November, 2016, when water temperatures were 7, 12, 17, and 22 ± 2 °C. In September 2017, Lake Minnetonka zebra mussels were deemed unsuitable due to high mortality observed within the lake. Therefore, test animals collected in 2017 were obtained from White Bear Lake (Ramsey County, MN) and used to repeat EarthTec QZ, KCl, and niclosamide exposures at 22 °C because previous tests failed to achieve 100% mortality. Zebra mussels were hand collected and removed from substrate by severing their byssal threads with a scalpel. Zebra mussels were transported to the U.S. Geological Survey's Upper Midwest Environmental Sciences Center (La Crosse, WI) in sealed plastic fish shipping bags containing temperature-acclimated well water and an oxygen overlay. The zebra mussels were maintained at the desired test temperatures in a 350-L holding tank located in a climate-controlled environmental chamber. When the temperature of collection and testing varied (i.e. 12 °C KCl and the 22 °C Zequanox exposures; Table 1) the zebra mussels were acclimated at a rate of ≤ 3 °C per day and held at the test temperature for ≥ 96 h prior to testing (ASTM 2007). The holding tank was aerated and supplied with 2 L/min of tempered fresh well water inflow. Feeding was consistent with established recommendations and feed was continuously supplied to the inflow at 2.0 mg/L as dry weight (ASTM 2013). The diet consisted of a 5:3 ratio of Shellfish Diet and Nanno 3600 Instant Algae® (Reed Mariculture, Inc., Campbell, CA). Test animals were fasted 12 h prior to and through the exposure period.

Zebra mussels were tested within 1 week of collection (except for the 12 °C KCl and the 22 °C Zequanox exposures; Table 1). Zebra mussels were deemed acceptable for testing by observing resistance to gentle pressure applied to the adductor muscle. Groups of 20 zebra mussels were then placed into individually labelled soft plastic mesh bags (Hubert Co. Harrison, OH) that were suspended in the water column from a length of wooden dowel and held open by a piece of polyvinyl chloride pipe (~2.5 cm long × 7.6 cm i.d., Figure 1A). The mesh bags containing zebra mussels were randomly assigned a treatment, labelled, and held overnight in a 60-L fiberglass tank that was supplied with ~ 2 L/min of tempered well water. Lastly, zebra mussels were transferred to the appropriate test chambers immediately after the addition of the test articles (ASTM 2007).

To assess the adequacy of the feeding and holding regimen, the body condition of mussels from each unique test animal cohort was determined by comparing the individual relationship of the soft tissue dry weight to the shell length at three times: prior to exposure (≤ 144 h), at the termination of the 336-h exposures, and at the termination of the post-exposure holding period. Pre-exposure condition assessments were conducted on mussels indiscriminately selected from the holding tank and the exposure and post-exposure termination condition assessments were conducted on groups of mussels from negative control test chambers. At each sample time, the groups of 40 untreated zebra mussels were individually measured and their soft body tissue dry weight was obtained after drying for 24 h at 60 °C.

Body condition (K) of each animal was calculated with a modified equation from Luoma et al. (2018):

$$K = W/L \times 100$$

Where,

W = soft tissue dry weight (mg), and
L = shell length (mm).

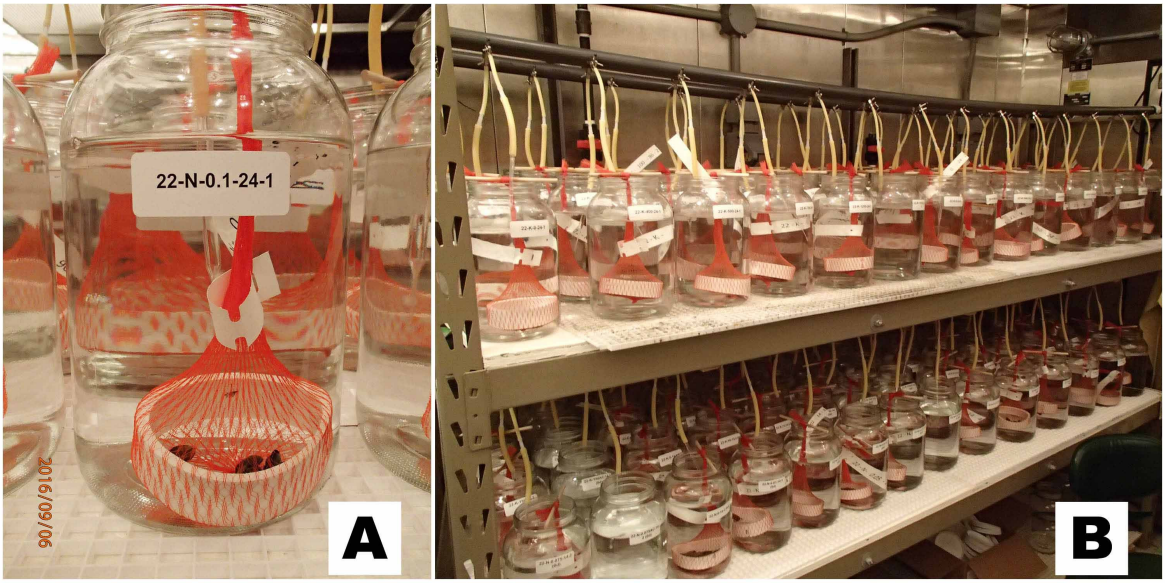

Figure 1. Test chamber with a mesh bag containing zebra mussel test animals (A), and a series of replicated test chambers positioned in a climate-controlled environmental chamber (B). USGS photo.

Test system

The test system consisted of a replicated series of 3.8-L glass jars (test chambers) containing 3 L of well water (Figure 1B). Test animal loading in the test chambers was estimated to be ≤ 0.104 g/L of body tissue dry weight based on calculations from mussels analyzed for body condition. Aeration was uniformly provided to each test chamber through a glass Pasteur pipet (ASTM 2013). Test chambers were the experimental units and four replicate chambers were used for each unique combination of test article, concentration, temperature, and exposure duration.

Test articles

Four test articles were evaluated: EarthTec QZ (5% copper as active ingredient; Earth Science Laboratories, Inc., Bentonville, AR), potassium chloride (KCl; ≥ 99% ACS reagent grade, 52.4% potassium as active ingredient; EMD Millipore Corp. Billercia, MA), bayluscide (3.2% granules, 2.7% niclosamide as the active ingredient; The Coating Place Inc., Verona, WI), and Zequanox (50% *Pseudomonas fluorescens*, strain CL145A, as active ingredient; Marrone Bio Innovations, Davis, CA).

Treatment administration and exposures

Treatment exposures were conducted in 2016 and 2017 as detailed in Table 1. Dosing solutions were prepared by diluting known masses of test article in known volumes of well water. Dosing solutions were applied to obtain a series of four (Zequanox) or five (EarthTec QZ, niclosamide, and KCl) test concentrations including an untreated control. Target concentrations of Zequanox were consistent across all temperatures and target concentrations of other toxicants varied based on the test temperature and exposure duration. Toxicant concentrations were determined from preliminary range-finding trials and were targeted to achieve replicates with low, medium to high, and complete test animal mortality. Target concentrations of Zequanox (0.5, 1, and 2 times the maximum label concentration) were consistent across all temperatures and exposures durations because the mode of action requires ingestion (Molloy et al. 2013a). After test article application, test chambers were thoroughly mixed with a glass rod prior to addition of the test animals. Exposure durations of 24, 96, 336 h in the EarthTec QZ, niclosamide, and KCl tests were chosen to provide concentration-mortality relationship data for both short and long term exposures. Exposure durations of 8, 12, and 24 h in the Zequanox tests were chosen because the toxicity of the active ingredient degrades approximately 30 h after hydration (Mayer 2011). All exposures 96 h or less were static for the duration of the exposure. The 336-h exposures were static renewals with complete test water replacement (i.e. new test water with fresh toxicant added) at 96-h intervals. The mesh bags with zebra mussels were triple rinsed with clean well water upon exposure termination and then transferred

into 60-L fiberglass tanks for post-exposure holding. The tanks were supplied with 0.5 ± 0.1 L/min of freshwater inflow and the feeding regimen was restored.

Treatment verification

Test article concentrations were verified after addition of the test articles using microwave plasma atomic emission spectroscopy (MP-AES; EarthTec QZ [as copper], KCl [as potassium]), liquid chromatography-mass spectroscopy (LC-MS; niclosamide), or UV-VIS spectroscopy (Zequanox) with methods modified from Truong and Carduro (2014), Doran and Stevens (2014), and Waller et al. (2016), respectively. Copper and potassium concentrations were verified using an Agilent 4100 MP-AES (Agilent technologies Inc., Santa Clara, CA) and comparing the emitted intensity and resulting peak area of samples to a five-point quadratic regression (copper) or a six-point linear regression (potassium) created with analytical standards that bracketed the exposure concentrations. All EarthTec QZ and KCl samples were filtered with a 0.45-µm polypropylene syringe filter (catalog number 28145-485; VWR International, Radnor, PA) acidified with 1% nitric acid, and stored in the dark until analyzed. Concentrations of copper and potassium were converted and reported as product concentrations using percent molar mass (EarthTec QZ: 5.0% copper; KCl: 52.4% potassium).

Niclosamide concentrations were verified using an Agilent 1290/6460 Liquid Chromatograph Triple Quadrupole Mass Spectrometer (Agilent technologies Inc., Santa Clara, CA) (LC-MS; Agilent technologies Inc., Santa Clara, CA) and comparing the abundance of ions present in the samples to a 5-point quadratic regression created with niclosamide analytical standards that bracketed the exposure concentrations. Samples were diluted 1:10 with methanol and then centrifuged on a Beckman Avanti 30 centrifuge (Beckman Coulter Inc., Brea CA) at 12,000 relative centrifugal force for 10 minutes prior to injection on the LC-MS. Zequanox concentrations were verified using a Beckman DU 800 spectrophotometer (Beckman Coulter Inc., Brea CA) and comparing sample absorbance at 660 nm to a zero-intercept, 5-point linear regression created with standards that bracketed the exposure concentrations.

Water chemistry

Dissolved oxygen (DO; mg/L), pH, and temperature (°C) were monitored daily in each test chamber and post-exposure holding tank. DO and pH were measured using a Hach portable water quality meter (model HQ40d) fitted with an IntelliCAL™ luminescent optical DO probe (model LDO 10101) and an IntelliCAL™ pH probe (model PHC 10101 or PHC 70501; Hach Company, Loveland, CO). Temperature was measured with a Thermapen® digital thermometer (model Mk4; ThermoWorks Company, American Fork, UT). Total hardness, alkalinity, and specific conductance were measured on replicate samples of the common test water source at each test temperature. Total hardness (mg/L as $CaCO_3$) was measured by the EDTA titrimetric method (method 2340C; APHA 2012). Alkalinity (mg/L as $CaCO_3$) was measured by titrating to an endpoint of pH 4.5 (method 2320B; APHA 2012), and specific conductance (µS/cm at 25 °C) was measured with a Fisher Accumet® conductivity meter (model AP 75; Fisher Scientific Company, Pittsburg, PA).

Total ammonia nitrogen (TAN) concentrations were measured at the termination of each exposure period to assess the impacts of test animal loading and test article addition on water quality. TAN was measured in negative control test chambers (test animals but no test article) and in positive control test chambers (highest test article concentration but no test animals). TAN concentrations were measured using a Hach model DR 3900 spectrophotometer and TNTplus™ test vials (Hach Company, Loveland, CO).

Survival assessments and post-exposure holding

Interim survival assessments were completed for test chambers observed to have significant numbers of dead test animals to mitigate adverse impacts to water quality. Only test animals with advanced tissue decomposition were removed during the interim assessments. Interim assessments were not necessary for any 24-h or 7 °C exposures. Final survival assessments were completed for each test replicate upon termination of the post-exposure holding period. During the final assessments, test animals were considered dead by absence of a response to gentle stimulus or by lack of resistance to gentle pressure applied to the adductor muscle.

The durations of the post-exposure holding periods were metabolically standardized using accumulated daily water temperature units (°C × number of days), and ranged from 216 to 221 temperature units. Holding periods, therefore, varied temporally and ranged from 10 to 31 days for the 22 and 7 °C exposures, respectively.

Data analysis

Water chemistry analyses were limited to simple descriptive statistics calculated using Microsoft Office®

Professional Plus 2013 Excel (Version 15.0.4833.1000 [64-bit]). Zebra mussel body condition analyses were performed using SAS software version 9.3 (SAS 2010). Significance was declared at $\alpha \leq 0.05$ and the individual zebra mussels were the experimental unit. The relationship of zebra mussel soft tissue dry weight per unit shell length measured prior to exposure, after 336 h of fasting during the exposure period, and at the termination of the post-exposure hold period were analyzed with linear regression models (Proc glimmix). Separate body condition analyses were completed for each unique cohort of mussels from each test temperature and exposure combination that were initiated > 2 d apart (n = 6; Tables 1 and 2). A normal distribution was assumed and sampling time served as the categorical predictor variable. Post-hoc comparisons were made among sampling times using a two-sided least square means comparison test. Exposure concentration descriptive statistics were calculated using SAS software (SAS 2010).

The lethal concentration of test article predicted to cause 50 and 99% mortality of the test animals (LC50 and LC99, respectively) and the corresponding 95% fiducial limits were calculated with SAS software (SAS 2010). Calculations were completed using a probit regression analysis (Proc probit) for each unique treatment combination. The mean, or the weighted mean (336-h exposures), test article concentrations were used as numeric predictor variables. A Gompertz distribution was used to allow for asymmetry in the mortality curves.

Results

Water chemistry

Mean water quality parameters during the tests met acceptability criteria established for aquaculture (Timmons and Ebling 2013). Water quality parameters were similar among treatment replicates and individual DO measurements in all trials remained above the 4.0 mg/L criteria for conducting tests with freshwater mussels (ASTM 2013). The only individual DO measurements that were below 5.0 mg/L were in the highest Zequanox concentration replicates during the 22 °C 24-h exposure. Temperature and pH were similar across treatment replicates. Individual pH measurements for all tests ranged from 7.21 to 8.92. Individual temperature measurements varied ≤ 1.8 °C between replicates, except during the 7 °C trial when the temperature rose to about 10 °C for less than 24 h during the post-exposure holding period due to a chiller malfunction. Specific conductance of the source water ranged from 392 to 414 µS/cm and the alkalinity and hardness of the source water ranged from 141 to 145 mg/L and 186 to 200 mg/L (as $CaCO_3$), respectively. TAN concentrations in all negative (i.e. untreated) control test chambers remained well below the acceptable criteria for cool and warmwater aquaculture species and the U.S. EPA's chronic criterion magnitude (Timmons and Ebling 2013, U.S. EPA 2013). TAN concentrations in the KCl and niclosamide positive control (i.e. treated) test chambers were well below chronic criteria. TAN concentrations in the EarthTec QZ positive control test chambers were likely inflated due to interference caused by the color of the test article; however, none exceeded the chronic criterion magnitude (U.S. EPA 2013). The only TAN measurements that exceeded the chronic criterion magnitude were in Zequanox exposures and the only measurements that exceeded the acute criterion magnitude were the Zequanox 22 °C 24-h exposure replicates (U.S. EPA 2013).

Test animal condition

With one exception, the condition of the test animals decreased during the exposure period and increased during the post-exposure holding period (Table 2). In some instances, the condition of test animals at the end of the exposure period was significantly less than the condition prior to exposure ($n = 3$, $p < 0.01$, $df = 114–117$). In all trials, the condition of test animals at the end of post-exposure holding period was significantly greater than the condition at the end of the exposure period ($p < 0.01$, $df = 114–117$). The condition of the test animals at the end of the post-exposure holding period was greater than the condition prior to exposure for all trials, except for the 22 °C exposures conducted in 2017 (EarthTec QZ, niclosamide, and KCl). The increase in test animal condition observed between the pre-exposure and post-exposure sampling times was significant in four of the six cohorts ($p < 0.01$, $df = 116–117$).

Treatment verification, lethal concentrations and zebra mussel mortality

The standard curve regression coefficient of determination (r^2) was ≥ 0.99 for all EarthTec QZ, KCL, niclosamide, and Zequanox analyses. Nominal treatment concentrations were used for the 17 °C niclosamide test calculations because of an instrument malfunction during treatment concentration verification. Calculated LC50s, LC99s, and the corresponding 95% fiducial limits are presented in Figures 2–5 for each combination of test article, temperature, and exposure duration. LC50s were not reported for treatment combinations with < 45% mortality in

Table 2. Mean (SD) condition of zebra mussels by sampling time and test temperature.

Temperature (°C)	Test articles	Sampling time		
		Pre-exposure	Exposure termination	Post-exposure termination
7	EarthTec QZ, niclosamide, KCl, Zequanox	0.0624 [a,b] (0.0195)	0.0577 [a] (0.0131)	0.0688 [b] (0.0188)
12	EarthTec QZ, niclosamide, Zequanox	0.0623 [a] (0.0191)	0.0516 [b] (0.0142)	0.0729 [c] (0.0179)
12	KCl	0.0537 [a] (0.0134)	0.0574 [a] (0.0154)	0.1000 [b] (0.0233)
17	EarthTec QZ, niclosamide, KCl, Zequanox	0.0479 [a] (0.0124)	0.0370 [b] (0.0095)	0.0512 [a] (0.0138)
22	Zequanox	0.0537 [a] (0.0211)	0.0489 [a] (0.0121)	0.0694 [b] (0.0171)
22	EarthTec QZ, niclosamide, KCl	0.0846 [a] (0.0166)	0.0603 [b] (0.0137)	0.0710 [c] (0.0163)

[a, b, c] Values in each row with the same letter are not significantly different.

Table 3. Minimum lethal dose of toxicants required to induce 100% zebra mussel mortality at specific water temperatures (a ">" symbol indicates observed mortality was > 90% and < 100% at the specified concentration; NE = treatment deemed not effective, observed mortality was ≤ 90%).

Temperature (°C)	EarthTec QZ, niclosamide, and KCl				Zequanox	
	Exposure duration (h)	EarthTec QZ (mg/L)	Niclosamide (mg/L)	KCl (mg/L)	Exposure duration (h)	Zequanox (mg/L)
7	24	NE	> 0.552	NE	8	NE
	96	> 58.8	> 0.189	NE	12	NE
	336	11.3	0.054	> 586	24	NE
12	24	> 150.4	0.182	NE	8	NE
	96	25.5	0.066	NE	12	NE
	336	4.5	0.053	165	24	NE
17	24	> 47.6	> 0.200[a]	> 2,071	8	NE
	96	9.5	0.100[a]	422	12	NE
	336	2.0	0.075[a]	147	24	> 323
22	24	> 49.6	0.181	> 3,066	8	NE
	96	21.5	0.137	220	12	> 315
	336	5.8	> 0.092	125	24	> 310

[a] Nominal concentration

the highest concentration. Likewise, LC99s were not reported for treatment combinations with < 90% mortality in the highest concentration. The amount of data collected for these treatment combinations were insufficient to calculate these LC values because of low test animal mortality and they were reported accordingly. Fiducial limits were incalculable for some treatment combinations because of the limited number of concentrations evaluated and variance in the observed mortality among the treatment replicates. The concentration of each test article that induced 100% mortality at each test temperature and exposure duration are reported in Table 3. The highest concentrations of test article tested (preceded by a > symbol) were reported for treatments that achieved mortality greater than 90% but less than 100%. Treatments were reported as not effective (NE) for all treatment combinations for which observed mortality was ≤ 90%.

EarthTec QZ toxicity was both temperature and exposure duration dependent (Figure 2, Table 3). Temperature moderately influenced EarthTec QZ toxicity, particularly at the coldest temperature evaluated. Nearly twice the amount of EarthTec QZ was required to induce 100% mortality in the 336-h exposures conducted at 7 °C compared to those conducted at temperatures ≥ 12 °C (11.3 vs ≤ 5.8 mg/L, respectively). Similarly, 96 h of exposure at 7 °C did not produce 100% mortality at over twice the concentration which induced 100% mortality at 12 °C (58.8 vs 25.5 mg/L, respectively). Exposure duration

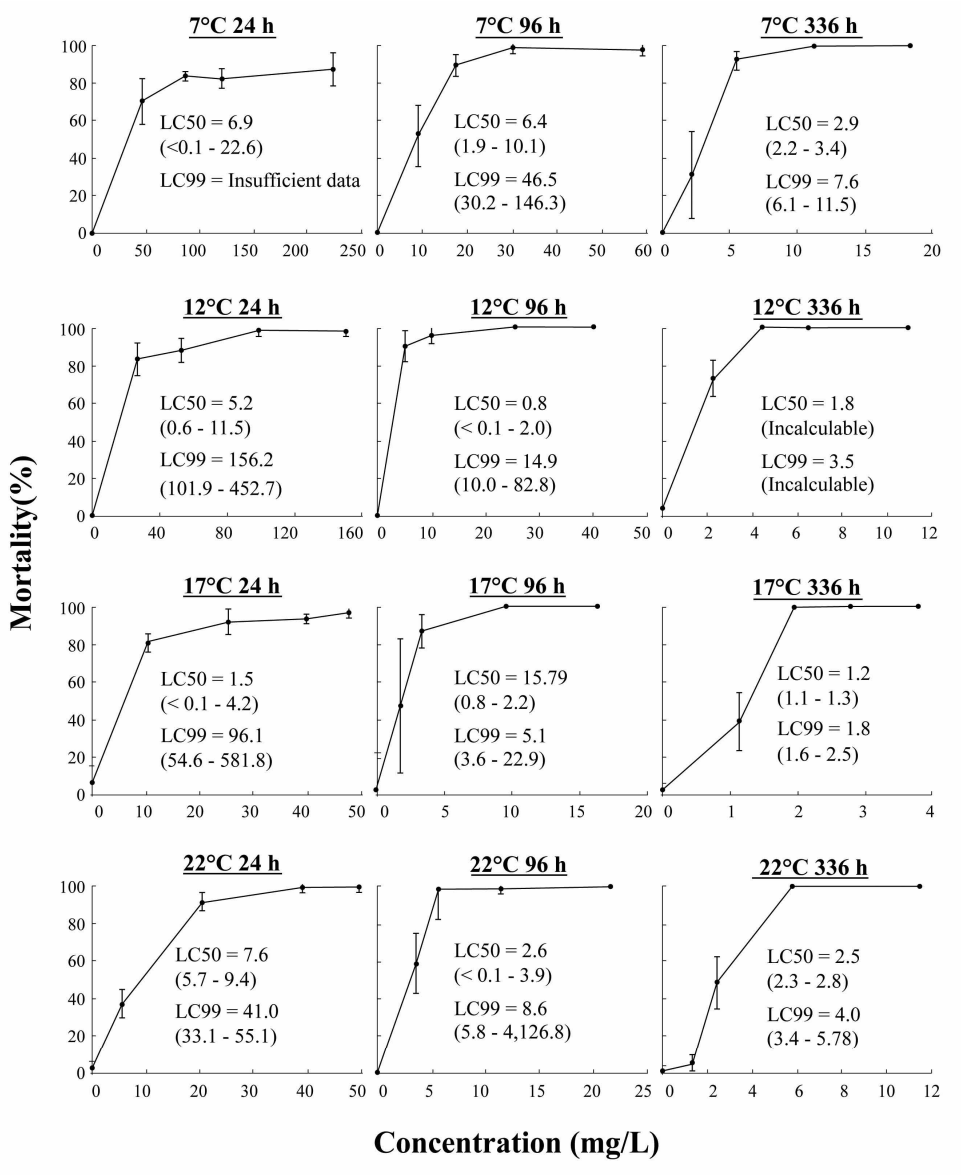

Figure 2. EarthTec QZ temperature and exposure duration mortality curves and the predicted LC50s and LC99s (95% fiducial limits) observed during laboratory trials.

dramatically influenced EarthTec QZ toxicity across all temperatures. For example, at 7 °C, exposure to 11.3 mg/L for 336 h resulted in 100% mortality; whereas, increasing the exposure concentration 5 and 20 times during the 96 and 24 h exposures, respectively, failed to induce 100% mortality.

Temperature and exposure duration moderately influenced niclosamide toxicity (Figure 3, Table 3). The greatest impact of temperature on niclosamide toxicity was in the 24-h exposure at 7 °C, where over twice the amount of niclosamide was required to induce similar mortality compared to the 24-h exposures conducted at warmer temperatures (0.552 vs ~ 0.200 mg/L, respectively). Exposure duration impacted niclosamide toxicity; however, the increase in toxicity caused by increased exposure duration were not similar at each temperature. Ten times the amount of niclosamide was required at 7 °C to induce similar mortality in the 24-h exposures compared to the 336-h exposures (0.552 vs 0.054 mg/L, respectively). Whereas, approximately three times the niclosamide concentration in the 24-h exposures yielded similar

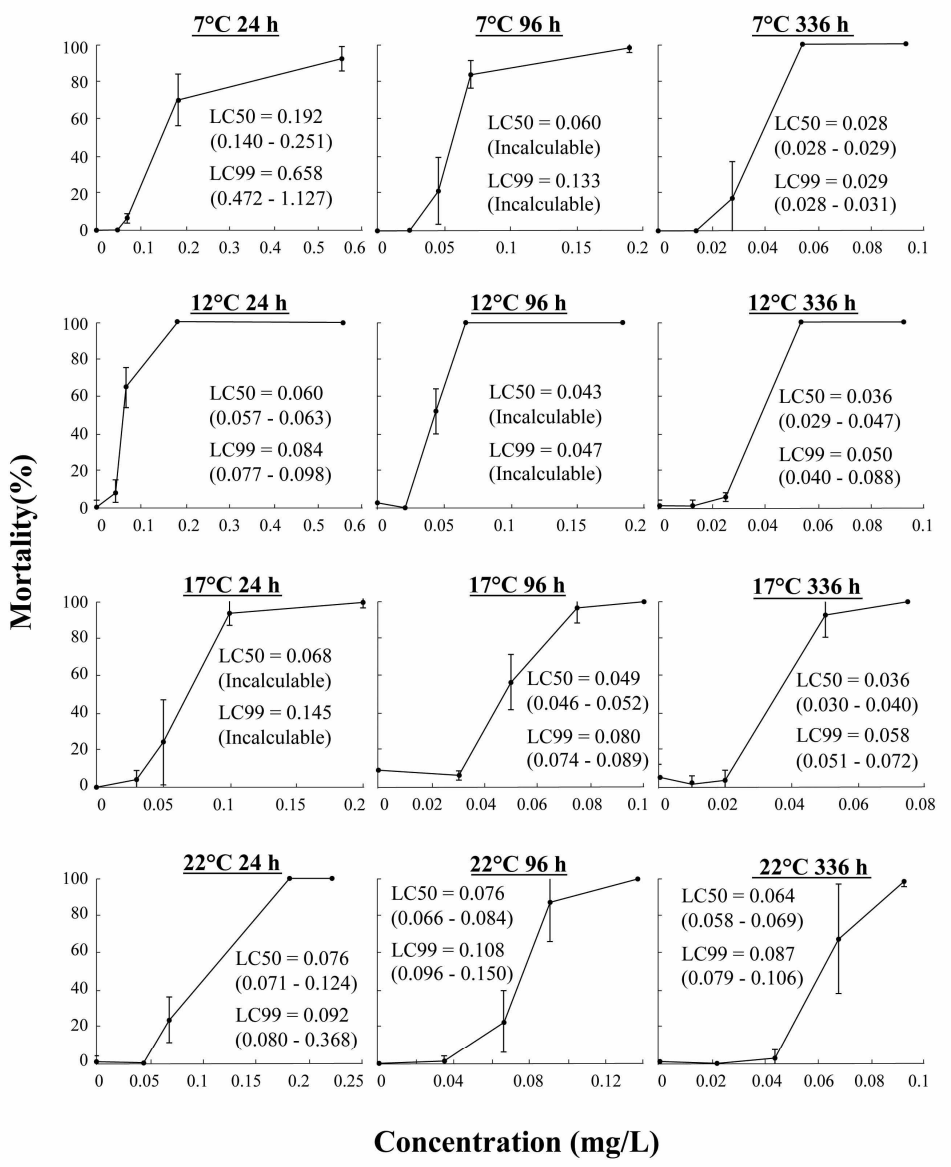

Figure 3. Niclosamide temperature and exposure duration mortality curves and the predicted LC50s and LC99s (95% fiducial limits) observed during laboratory trials.

mortality as the 336 h exposures when temperatures were between 12 and 22 °C. Likewise, when temperatures were ≥ 12 °C, a 1.25 to 1.5 increase in niclosamide concentration achieved similar mortality with 96 h of exposure compared to 336 h of exposure at the same temperature (0.066–0.137 vs 0.053–0.092 mg/L).

Temperature and exposure duration dramatically impacted the toxicity of KCl (Figure 4, Table 3). Substantially less KCl was required at warmer temperatures to induce similar mortality. For instance, 336 h of exposure to 586 mg/L of KCl at 7 °C produced 98.8% mortality; whereas, 336 h of exposure to 125 mg/L at 22 °C induced 100% mortality. More strikingly, lengthening the exposure duration from 24 to 336 h greatly reduced the amount of KCl needed to cause similar mortality. For example, 24 h of exposure to 2,070 mg/L of KCl at 17 °C yielded 93.8% mortality; whereas, 336 h of exposure to 147 mg/L at 17 °C caused 100% mortality.

Zequanox toxicity was impacted by both temperature and exposure duration (Figure 5, Table 3).

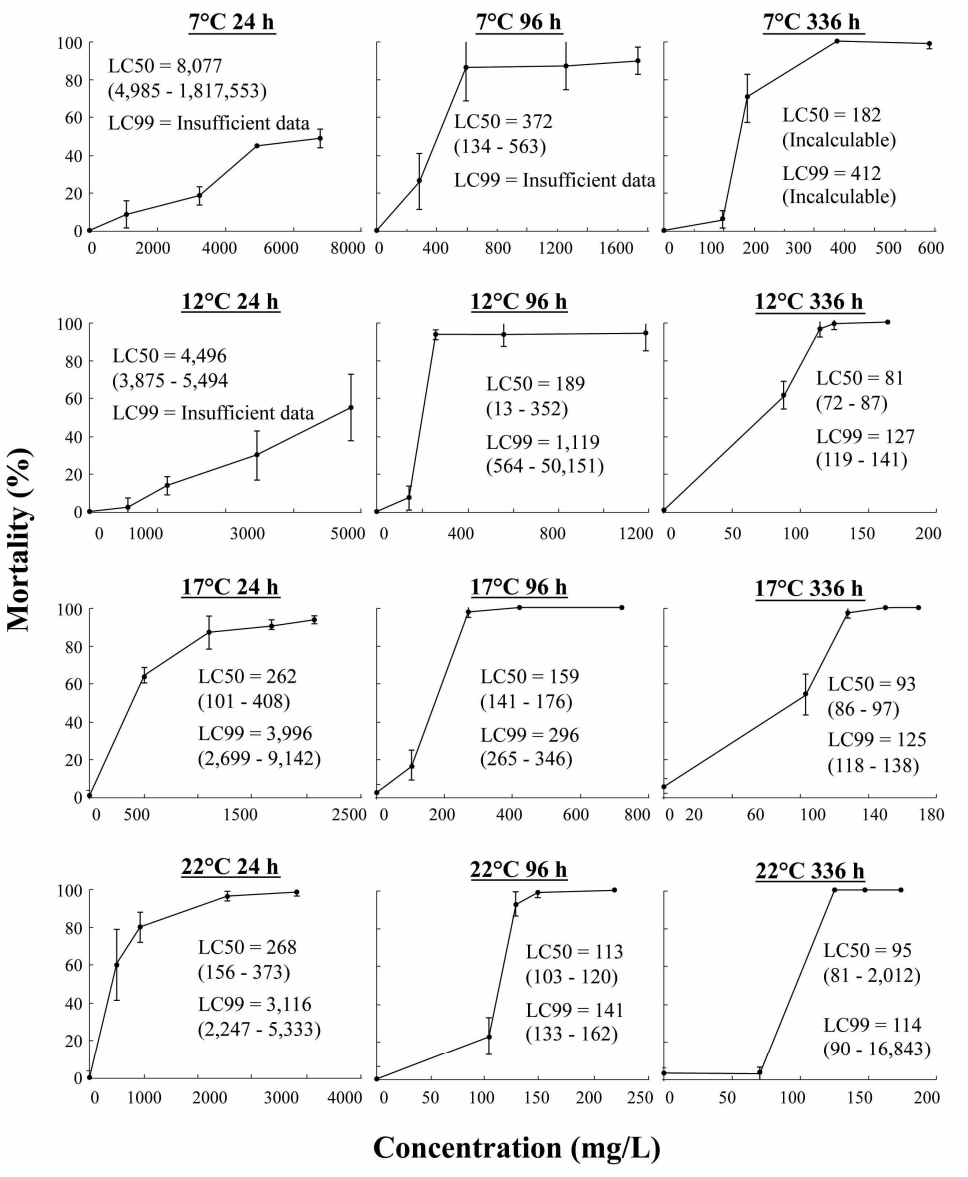

Figure 4. Potassium chloride temperature and exposure duration mortality curves and the predicted LC50s and LC99s (95% fiducial limits) observed during laboratory trials.

Mortality of zebra mussels was < 78% in all Zequanox exposures conducted ≤ 12 °C. Mortality of zebra mussels in Zequanox exposures conducted at 17–22 °C reached 100% in only one treatment combination (24 h, 166 mg/L treated group at 17 °C). However, 24 h of exposure to 323 mg/L at 17 °C yielded 98.8% mortality. At 22 °C, exposure to Zequanox for 24 h at ≥ 158 mg/L induced mortality ≥ 92.5%. Increasing the exposure duration from 8 to 24 h increased observed mortality approximately 11 to 30% across all temperatures.

Discussion

Our findings show the likelihood for successful zebra mussel eradication increases at higher water temperatures and with longer exposure durations. Our data suggest three of the four toxicants that were evaluated are viable candidates for zebra mussel eradication: EarthTec QZ, KCl, and niclosamide. These toxicants demonstrated the consistent ability to induce 100% zebra mussel mortality at multiple temperatures and exposure durations. Zequanox, as

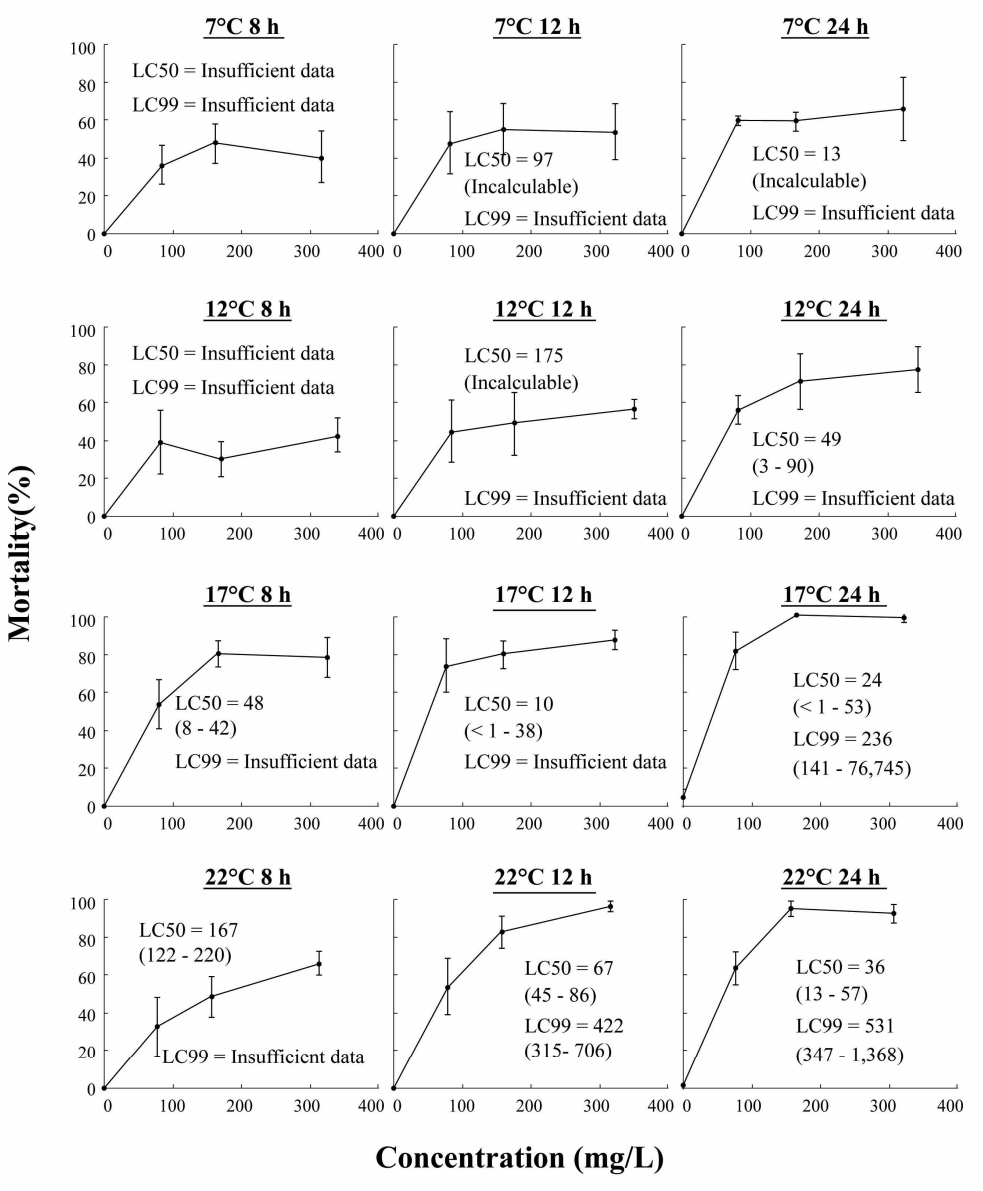

Figure 5. Zequanox temperature and exposure duration mortality curves and the predicted LC50s and LC99s (95% fiducial limits) observed during laboratory trials.

presently formulated and as tested under our laboratory conditions, did not consistently achieve 100% mortality.

In general, we found that lower toxicant concentrations were required at warmer temperatures and with longer exposures. Exceptions include the 22 °C EarthTec QZ and niclosamide exposures. In those tests, more EarthTec QZ and niclosamide were required at 22 °C to achieve 100% mortality compared to the similar tests conducted at colder temperatures (Table 3). The 22 °C EarthTec QZ, niclosamide, and KCl tests were conducted in 2017 with test animals collected from White Bear Lake in Minnesota; whereas, all other tests were conducted in 2016 with mussels collected from Lake Minnetonka in Minnesota (Table 1). Variations observed in temperature-related toxicity trends were likely due to population level variances with regard to toxicant susceptibility (ASTM 2007).

Our data suggest that effective eradication treatments in cold to cool waters (i.e. 7 to 12 °C) include 336 h or longer treatments with EarthTec QZ or KCl and niclosamide treatments ≥ 24 h in duration

(Table 3). In warmer waters (i.e. ≥ 17 °C), effective treatment options increase to also include 96-h treatments with EarthTec QZ or KCl (Table 3). When feasible, longer treatments conducted in warm waters would likely be more cost effective and may reduce nontarget animal impacts.

Selection of a toxicant for use in a zebra mussel eradication treatment requires careful consideration of the impacts to nontarget organisms. Various aquatic species that have demonstrated sensitivity to copper (e.g. EarthTec QZ) could be adversely impacted during eradication treatments (Waller et al. 1993; Yanong 2010). Niclosamide would likely cause substantial nontarget animal mortality at the minimum lethal concentrations we found effective for inducing 100% zebra mussel mortality (≥ 0.053 mg/L). Dawson (2003) reports turbellarians, oligochaetes, and leeches are some of the most sensitive aquatic invertebrates to niclosamide, all having 24-h LC50s < 0.05 mg/L. The niclosamide Reregistration Eligibility Decision document lists a range of acute invertebrate impacts between 0.034 and 50 mg/L (U.S. EPA 1999). Furthermore, niclosamide has demonstrated high toxicity to fish with LC50s ranging from 0.03 to 0.23 mg/L and toxicity did not increase proportionately with exposure duration (Marking and Hogan 1967; U.S. EPA 1999). Conversely, the mammalian toxicity of niclosamide has been reported as practically nontoxic (U.S. EPA 1999).

Effective and practical KCl concentrations for zebra mussel eradication treatments were observed during the 336-h exposures at 12, 17, and 22 °C and during the 96-h exposures conducted at 22 °C. Complete mortality was observed in these exposures at ≤ 220 mg/L of KCl, which is approximately 115 mg/L as potassium. The target potassium concentration of 100 mg/L during the Millbrook Quarry eradication was maintained for 3 weeks and nonmollusk species were reported as continuing to thrive throughout the exposure (Fernald and Watson 2013). Waller et al. (1993) reported KCl 48-h LC50s ≥ 720 mg/L for channel catfish and rainbow trout, a further indication of a safety margin for fish.

Zequanox has demonstrated selectivity for zebra mussels with few reported impacts to nontarget species when applied at a dose consistent with the product label. (Molloy et al. 2013b; Meehan et al. 2014; Luoma et al. 2015b; Waller et al. 2016; Waller and Luoma 2017). Due to the reported selectivity, Zequanox may be a reasonable tool for zebra mussel management; however, latent impacts have been reported in lake trout exposed to a label consistent dose of Zequanox in a laboratory trial (Luoma et al. 2018). Additionally, Zequanox applications that are contained for an extended period may reduce dissolved oxygen to levels that would impact nontarget animals (Lund et al. 2018).

Accurate information on the extent of the infestation, the ability to maintain treatment concentrations, and knowledge of the water chemistry profile are essential elements for an acceptable zebra mussel eradication treatment. An eradication attempt would likely not be warranted if zebra mussels are already widespread or if maintaining an effective treatment dose is not feasible. Water chemistry can impact the toxicity of various toxicants. For example, potassium toxicity has been shown to be reduced by elevated sodium concentrations and the solubility and toxicity of copper-based products are affected by alkalinity and hardness (Masuda and Boyd 1993; Perschbacher and Wurts 1999; Moffit et al. 2016). The bioavailability of copper is known to be influenced by several other competing water constituents; therefore, the U.S. EPA developed a biotic ligand model (BLM) to standardize the comparison of copper toxicity (U.S. EPA 2007). The BLM model requires several input parameters including temperature, pH, dissolved organic and inorganic carbon as well as major geochemical cations (calcium, magnesium, sodium, and potassium) and anions (chloride, sulfate). The collection of robust water chemistry data and other site specific variables during eradication treatments will aid in the transferability of results.

Registration status and the urgency of treatment (e.g. due to spawning, etc) should also be considered when selecting a toxicant for a zebra mussel eradication treatment. EarthTec QZ should be considered for urgent treatments because the permitting requirements are likely less than alternate toxicants. Niclosamide is a U.S. EPA-registered pesticide that is used for sea lamprey control in the United States but it is not registered for zebra mussel control. Niclosamide has been used to control swimmers itch in Michigan, Minnesota, and Wisconsin and it is regularly used in tropical areas to control schistosomiasis (Marking 1992; McDonald and Kolar 2007; Dai et al. 2008). The current registration status and use of niclosamide in aquatic systems may expedite the permitting processes for its use as a zebra mussel toxicant. Similarly, KCl was successfully used in the Millbrook Quarry eradication and it has been used in other unsuccessful zebra mussel rapid response treatments in Minnesota and Manitoba despite not being registered by the U.S. EPA as a molluscicide. The efficacy of KCl as a molluscicide in addition to a safety margin for some nontarget species may lead to eventual U.S. EPA-registration or treatment-specific permits for use as zebra mussel toxicant.

Our laboratory trials provide evidence for the selection of an effective toxicant and dose for zebra mussel eradication. However, consideration should be given to site specific variables (aquatic vegetation, nontarget species, substrate, water chemistry, etc.) and population level differences in zebra mussel sensitivity that may influence toxicant selection and treatment efficacy. Since efficacy is somewhat dependent on site specific conditions, the use of a robust on-site or *in situ* zebra mussel bioassay would be useful for the confirmation of treatment efficacy and the use of bioassays has been deemed necessary in previous eradication attempts (Lund et al. 2018).

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