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Research Article

Exposure-related effects of Zequanox on juvenile lake sturgeon (*Acipenser fulvescens*) and lake trout (*Salvelinus namaycush*)

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Abstract

The environmental fate, persistence, and non-target animal impacts of traditional molluscicides for zebra, *Dreissena polymorpha*, and quagga, *D. bugensis*, mussel control led to the development of the biomolluscicide Zequanox. Although previous research has demonstrated the specificity of Zequanox, one study indicated sensitivity of salmonids and lake sturgeon, *Acipenser fulvescens*, following non-label compliant exposures to Zequanox. This study was conducted to evaluate sublethal and lethal impacts of Zequanox exposure on juvenile lake sturgeon and lake trout, *Salvelinus namaycush*, following applications that were conducted in a manner consistent with the Zequanox product label. Fish were exposed to 50 or 100 mg/L of Zequanox as active ingredient for 8 h and then held for 33 d to evaluate latent impacts. No acute mortality was observed in either species; however, significant latent mortality was observed in lake trout that were exposed to the highest dose of Zequanox. Statistically significant but biologically minimal differences were observed in the weight (range 20.17 to 21.49 g) of surviving lake sturgeon at the termination of the 33 d post-exposure observation period. Statistically significant and biologically considerable differences were observed in the weight (range 6.19 to 9.55 g) of surviving lake trout at the termination of the 33 d post-exposure observation period. Histologic evaluation of lake trout gastrointestinal tracts suggests that the mode of action in lake trout is different from the mode of action that induces zebra and quagga mussel mortality. Further research could determine the sensitivity of other salmonid species to Zequanox and determine if native fish will avoid Zequanox treated water.

Key words: quagga mussels, zebra mussels, dreissenid, control, Pf-CL145A

Introduction

Approximately three decades after discovery in North America, zebra mussels (*Dreissena polymorpha* Pallas, 1771) have been documented in the waterways of 34 US states, 3 Canadian provinces, and 1 Mexican state (A. Benson, pers. comm.). Zebra and quagga mussels (*D. bugensis* Andrusov, 1897; collectively referred to as dreissenids) have profoundly restructured aquatic ecosystems, leading to changes in phytoplankton community structure (Higgins and Vander Zanden 2010; Sousa et al. 2014), declines in zooplankton (Higgins and Vander Zanden 2010; Adlerstein et al. 2013), amplification of pollutants (Zimmermann et al. 2005; Sousa et al. 2014), contributions to harmful algal blooms (Vanderploeg et al. 2001; Knoll et al. 2008), alteration of fisheries (Strayer et al. 2004; Hoyle et al. 2008), and extirpation of unionid mussels (Ricciardi et al. 1998; Strayer and Malcom 2007). The financial burden to remediate the impacts of dreissenids has been significant and the majority of control efforts have been treatments of industrial infrastructure with chlorine and other oxidizing chemicals (Mackie and Claudi 2010). Discharges of traditional molluscicides are frequently restricted and often require mitigation due to their persistence and impacts to non-target organisms (Nalepa and Schloesser 1993; Smythe and Miller 2003; Mackie and Claudi 2010; Glomski 2015).

A North American river sediment bacterial isolate, *Pseudomonas fluorescens*, strain CL 145A (*Pf*-CL145A), was found to be selectively toxic to

dreissenids irrespective of whether the bacterial cells were alive or dead (Molloy et al. 2013a). After ingestion by dreissenids, the *Pf*-CL145A cells degrade the digestive gland and stomach epithelium, resulting in death (Molloy et al. 2013a). Zequanox® (Marrone Bio Innovations, Davis, CA), is a commercially-prepared, spray-dried powder formulation containing 50% dead *Pf*-CL145A cells as the active ingredient (A.I.; Marrone Bio Innovations 2017). Zequanox was registered by the US Environmental Protection Agency (USEPA) in 2012 for dreissenid mussel control in industrial water conveyance systems and in 2014 for use in open-water environments.

Previous research that evaluated Zequanox exposure to freshwater unionid mussels, aquatic invertebrates, and several life stages of fathead minnows suggested that Zequanox exposure is safe to non-target animals when it is applied up to the maximum dose specified on the product label (100 mg/L A.I. for 8 h; Luoma et al. 2015a; Waller et al. 2016; Waller and Luoma 2016). However, laboratory trials conducted using a flow-through system to expose several species of native fish to a continuous application of Zequanox treated water for 24 h demonstrated impacts on the survival of lake sturgeon (*Acipenser fulvescens* Rafinesque, 1817) and on the survival and body condition of rainbow trout (*Oncorhynchus mykiss* Walbaum, 1792) and brook trout (*Salvelinus fontinalis* Mitchill, 1814; Luoma et al. 2015b).

Lake sturgeon are a long-lived, late-maturing, and intermittent-spawning species, which were reduced to remnant populations in the Great Lakes by over-exploitation and other anthropogenic influences (Hay-Chmielewski and Whelan 1997; Peterson et al. 2007). Lake sturgeon are currently a species of concern throughout their entire range and the rehabilitation of the species is a priority for tribal, state, and federal agencies (Hay-Chmielewski and Whelan 1997; DeHaan et al. 2006; Holtgren et al. 2007). The vulnerable status of lake sturgeon coupled with the Zequanox sensitivity reported by Luoma et al. (2015b) demonstrate the need to further investigate the impacts of Zequanox exposure on lake sturgeon.

Lake trout (*Salvelinus namaycush* Walbaum in Artedi, 1792) are native to the Great Lakes and are an important ecological, recreational, and commercial species. Overfishing and sea lamprey (*Petromyzon marinus* Linnaeus, 1758) predation nearly extirpated lake trout from the Great Lakes in the 1960s and the lake trout fishery has been supported by annual stocking of hatchery-reared fish since 1965 (Marsden and Chotkowski 2001). Interstitial space occlusion of spawning habitat by dreissenids combined with the deposition of dreissenid feces and pseudofeces has complicated the recovery of lake trout in the Great Lakes (Marsden and Chotkowski 2001). Hatchery-reared lake trout have a preference for shallow water spawning habitats and successful recruitment on these habitats has been correlated to a lack of dreissenids (Marsden and Chotkowski 2001; Claramunt et al. 2005). Therefore, management of dreissenids on high-value shallow water spawning habitats would likely aid in the recruitment of juvenile lake trout by reducing interstitial space occlusion and deposition of feces and pseudofeces. The sensitivity of salmonids to Zequanox reported by Luoma et al. (2015b) along with the potential exposure of lake trout during a spawning habitat rehabilitation treatment demonstrate the need to evaluate the impacts of Zequanox exposure on lake trout.

The goals of this study were to determine the acute and latent impacts of Zequanox exposure on lake sturgeon and lake trout when applied in accordance with the product label. To accomplish these goals, single, static Zequanox treatments were applied at the maximum concentration permitted by the Zequanox product label for veliger/juvenile and adult dreissenid mussel treatments (50 or 100 mg/L A.I., respectively) for 8 h. Fish survival was monitored for 33 d after exposure and fish body condition was assessed at the termination of post-exposure observation period. Results of this study have implications for natural resource managers considering the use of Zequanox for controlling various life stages of dreissenid mussels in systems where lake sturgeon or lake trout may be exposed.

Methods

Test animals and test system

Juvenile lake sturgeon (~ 3 months post-hatch) and juvenile lake trout (~ 5 months post-hatch) were used to evaluate the impacts of Zequanox exposure. All fish use, handling, acclimation and disposal adhered to standard operating procedures and a study-specific protocol approved by the Upper Midwest Environmental Sciences Center's Animal Care and Use Committee, and/or ASTM guidelines (ASTM 2014). Approximately 2 weeks prior to exposure, certified disease-free lake sturgeon were obtained from the U.S. Fish and Wildlife Service's Genoa National Fish Hatchery (Genoa, WI). Lake sturgeon were held at 22 ± 1 °C in a 3.80 m^2, 1,550 L square fiberglass holding tank which had a temperature-adjusted well water inflow of approximately 26 L/min. One week prior to exposure, lake sturgeon were acclimated to the test temperature of 20 ± 1 °C over 20 h and maintained at that temperature for the duration of the study. Certified disease-free lake trout eggs were

Figure 1. Test system used to expose juvenile lake sturgeon (*Acipenser fulvescens*) and lake trout (*Salvelinus namaycush*) to Zequanox. USGS photo.

received from the Sullivan Creek National Fish Hatchery (Brimley, MI). The eggs were hatched in a standard hatchery tray incubator and the resulting fish were maintained in a 2.90 m^2, 1,460 L rectangular fiberglass holding tank which had a well water inflow of approximately 24 L/min. Lake trout were maintained and tested at 12 ± 1 °C.

Lake sturgeon were hand fed to satiation four times per day with frozen chironomid larvae (Oregon Desert Brine shrimp Company, Portland, OR) prior to exposure and three times per day during the 33 d post-exposure observation period. Lake trout were fed a commercially prepared trout diet (Skretting starter crumble, Skretting USA, Tooele, UT) 12 h per day with an automatic fish feeder prior to exposure and hand fed to satiation three times per day on weekdays and two times per day on weekends during the 33 d post-exposure observation period. All fish were fasted 16 h prior to exposure through the exposure period. For both species, the amount of feed and timing of feeding were consistent across all test tanks.

The test system consisted of two sets of six circular fiberglass test tanks (0.97 m diameter) that contained approximately 320 L of well water (Figure 1). Each set of tanks was gravity supplied temperature-appropriate well water from a head box at a rate of 5.5 ± 0.2 L/min throughout the study, except during the exposure, when water flow was interrupted. Light aeration was provided to each test tank through a standard 2.5 cm aquarium air stone.

A total of 1,200 fish of each species were independently tested in the study. Fish (n = 100/tank) were randomly distributed to each of the 12 test tanks in groups of 25 fish approximately 18 h prior to exposure. Each group of 25 fish was indiscriminately selected from the holding tank and placed into a bucket containing approximately 4 L of water. The wet weight of each group was obtained by gently dewatering the fish into a colander and then placing the fish into another tared bucket containing approximately 4 L of water. Immediately after weighing, the fish were placed into their assigned test tanks. Mean initial fish loading in the test tanks was 1.16 ± 0.04 g/L for the lake sturgeon and 1.11 ± 0.03 g/L for the lake trout.

Exposures and assessments

Three replicated treatments, including an untreated control treatment, a 50 mg/L A.I. Zequanox treatment, and a 100 mg/L A.I. Zequanox treatment, were randomly assigned to independent test tanks (n = 4/treatment). The 8 h exposures were initiated in each test tank replicate with a single, static Zequanox application. Applications were completed by removing approximately 3 L of water from each test tank and mixing a pre-weighed aliquot of Zequanox into suspension with about 2 L of the water using a household blender. The blender was rinsed with the remaining water; then the Zequanox suspension and rinsate were applied to the appropriate test tank and gently mixed with tank water using a tank brush (Figure 2). Untreated controls were also gently mixed with a tank brush.

Water quality parameters (dissolved oxygen, pH, and temperature) were measured in each individual test tank prior to exposure, < 1, 4, and 7.5 h after Zequanox application, and once daily during the 33 d post-exposure observation period. Dissolved oxygen (DO) was measured with a YSI 550A DO meter (YSI,

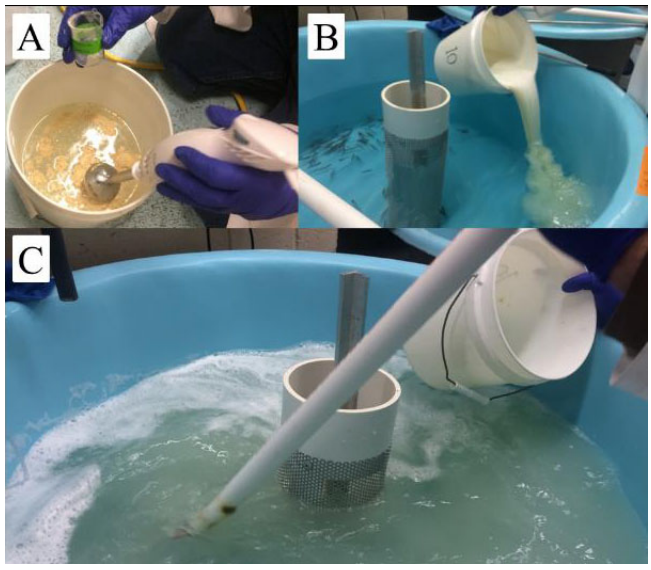

Figure 2. Examples of preparing Zequanox suspension (A), application to the tank (B), and mixing in the tank (C). USGS photo.

Figure 3. Example test specimens being measured and weighed at the conclusion of post-exposure observation period. USGS photo.

Inc., Yellow Springs, OH), pH was measured with a Beckman Coulter φ 410 pH meter (Beckman Coulter, Inc., Fullerton, CA), and temperature was measured with a Thermapen digital thermometer (ThermoWorks, American Fork, UT). Specific conductance was measured with a Fisher Accumet AP75 conductivity meter (Fisher Scientific, Pittsburg, PA) in each individual test tank during the exposure, and weekly thereafter, on pooled treatment group water samples. Total ammonia nitrogen (TAN) was measured in each individual test tank immediately prior to exposure termination using a Hach HQ40d portable water quality meter fitted with an IntelliCAL™ model ISENH318101 ion selective electrode (Hach Company, Loveland, CO). Hardness and alkalinity were measured on triplicate source water samples collected from each head box prior to exposure. Hardness and alkalinity were also measured in each test tank during the exposure, and weekly throughout the post-exposure observation period, from water samples pooled by treatment group. Total hardness (mg/L as $CaCO_3$) was determined by the EDTA titrimetric method and alkalinity (mg/L as $CaCO_3$) was determined by titrating to an endpoint of pH 4.5 (APHA 2012).

The exposures were terminated after 8 h by restoring the water flow and draining each tank approximately 75%. Mortality was assessed upon exposure termination and daily thereafter during the 33 d post-exposure observation period. All surviving test fish were euthanized at the conclusion of the post-exposure observation period by tricaine methanesulfonate (MS-222) overdose and each fish was then individually weighed and measured (lake sturgeon = fork length, lake trout = total length; Figure 3). Length-weight relationships were determined for all surviving fish by calculating a condition factor (K)

according to method described in Piper et al. (1982) using the equation:

$$K = W/L^3$$

Where,

W = weight of the fish (g), and
L = length of the fish (mm).

A subset of the surviving fish of both species from each test tank (n = 15) was indiscriminately selected and fish were examined according to the American Fisheries Society Fish Health Section's Blue Book procedures (AFS-FHS 2014) by the US Fish and Wildlife Service's La Crosse Fish Health Center. A separate subset of surviving lake trout from each test tank (n = 4) was indiscriminately selected and fish were prepared for histologic evaluation of the gastrointestinal (GI) tract due to observed mortality of lake trout during the post-exposure observation period. The GI tract of each fish was excised, placed into a standard tissue cassette, and fixed in Modified Davidson's Solution for 48 h. The cassettes were then thoroughly rinsed with and stored in 70% histological grade ethanol. The samples were transferred to a contract laboratory (Histology and Histologistics Inc., Dudley, MA) that prepared and assessed the GI tracts for treatment related impacts. The GI tracts of the fish were paraffin embedded, sectioned in 5 µm increments, mounted on glass slides, and then stained with hematoxylin and eosin. A board-certified pathologist conducted a blinded evaluation of tissue sections of the stomach and the proximal, mid, and distal intestines from each fish. Observed abnormalities were scored on a scale from NR-5, where, NR = not remarkable, 1 = minimal change, 2 = mild change, 3 = moderate change, 4 = marked change, and 5 = severe change.

Zequanox concentration analysis

Zequanox concentrations in the test tanks were determined by spectrophotometric comparison to a linear regression created from known concentrations of Zequanox on a Beckman Model DU 800 UV/Vis spectrophotometer (Beckman Coulter, Inc., Fullerton, CA). Triplicate Zequanox standards of 25, 50, 100, and 150 mg/L A.I. were prepared in well water and used to create a linear, zero-intercept standard curve. Water samples were collected from each test tank and analyzed for Zequanox concentration at 1, 2, 4, 6, and 8 h of exposure and 1 h post-exposure.

Data analyses

Water chemistry (DO, pH, temperature, alkalinity, hardness, and specific conductance) data analyses were limited to simple descriptive statistics calculated using Microsoft Office Professional Plus 2013 Excel (Version 15.0.4833.1000 [64-bit]).

The relationships between the weights of fish distributed to the test tanks were analyzed using SAS software Version 9.3 (SAS 2010) mixed linear logistic regression models (Proc mixed). Significance was declared at $\alpha = 0.05$ and the treatment group replicates (test tanks) were the experimental units. The intercept was specified as a random effect using the random statement and treatment served as the categorical predictor variable. Comparisons were made among treatment groups using a two-sided least squares means comparison test.

Statistical analyses of fish survival were performed using SAS software Version 9.3 (SAS 2010), significance was declared at $\alpha = 0.05$, and the treatment group replicates (test tanks) were the experimental units. The relationships between fish survival at the termination of the post-exposure observation period were analyzed with binomial logistic regression models (Proc glimmix). The proportion of surviving fish in each tank replicate were modeled with a binomial distribution and a logit link function. Standard error inflation resulting from sampling zero was eliminated, in accordance with Agresti (2007), by subtracting 0.1 from the observed survival in each tank before comparisons were conducted. A scale parameter was added to the model using the random residual statement and treatment served as the categorical predictor variable. Comparisons were made among treatment groups using a two-sided least squares means comparison test.

Statistical analyses of the relationships between the weight and the condition of surviving fish at the termination of the post-exposure observation period were performed using SAS software Version 9.3 (SAS 2010) and significance was declared at $\alpha = 0.05$. Analyses were completed using mixed linear regression models (Proc mixed). Treatment served as the categorical predictor variable. Test tank replicates were included as random effects nested within treatment conditions and the test tanks were the experimental units. Individual fish were treated as random effects nested within tanks. Fish weights were log transformed to correct for non-normality and non-constant variance of model residuals. Condition factors were rescaled using a multiplication factor of 100 to allow for proper model convergence. The weight and condition factor of fish in each treatment group were individually compared to the weight and condition factor of fish in the untreated control group using a two-sided least squares means comparison test.

Table 1. Mean (standard deviation) dissolved oxygen, pH, and temperature by treatment group throughout the study.

Species	Time	Control			50 mg/L			100 mg/L		
		DO (mg/L)	pH	Temp. (°C)	DO (mg/L)	pH	Temp. (°C)	DO (mg/L)	pH	Temp. (°C)
Lake sturgeon	Pre-exposure	8.12 (0.31)	8.01 (0.08)	19.9 (0.1)	8.17 (0.30)	8.01 (0.09)	19.9 (0.0)	8.17 (0.33)	8.06 (0.07)	19.8 (0.1)
	Exposure	7.92 (0.22)	8.08 (0.04)	19.4 (0.4)	7.70 (0.34)	7.97 (0.05)	19.4 (0.3)	7.93 (0.16)	7.95 (0.04)	19.3 (0.3)
	Post-exposure	7.63 (0.33)	7.90 (0.08)	19.9 (0.1)	7.66 (0.34)	7.90 (0.09)	19.9 (0.1)	7.72 (0.34)	7.93 (0.08)	19.9 (0.1)
Lake trout	Pre-exposure	9.92 (0.24)	7.96 (0.03)	12.0 (0.1)	10.04 (0.19)	7.97 (0.02)	12.0 (0.0)	9.83 (0.21)	7.95 (0.02)	12.0 (0.1)
	Exposure	9.50 (0.47)	7.92 (0.06)	12.5 (0.4)	9.65 (0.35)	7.88 (0.05)	12.5 (0.4)	9.05 (0.57)	7.75 (0.04)	12.6 (0.4)
	Post-exposure	9.62 (0.33)	7.88 (0.07)	12.4 (0.3)	9.81 (0.25)	7.90 (0.07)	12.4 (0.3)	9.66 (0.27)	7.91 (0.07)	12.4 (0.3)

Table 2. Mean (standard deviation) hardness, alkalinity, and specific conductance by treatment group throughout the study.

	Lake sturgeon			Lake trout		
	Pre-exposure[a]	Exposure	Post-exposure[b]	Pre-exposure[a]	Exposure	Post-exposure[b]
Control						
Hardness (mg/L as $CaCO_3$)	189 (2)	189 (2)	186 (3)	184 (1)	189 (2)	193 (3)
Alkalinity (mg/L as $CaCO_3$)	142 (0)	143 (0)	142 (5)	143 (2)	145 (1)	142 (2)
Specific cond. (µS/cm)	364 (0)	389 (5)	383 (10)	364 (0)	370 (2)	387 (6)
50 mg/L						
Hardness (mg/L as $CaCO_3$)	189 (2)	188 (3)	186 (3)	184 (1)	190 (2)	193 (1)
Alkalinity (mg/L as $CaCO_3$)	142 (0)	145 (1)	141 (4)	143 (2)	146 (1)	142 (3)
Specific cond. (µS/cm)	390 (2)	398 (7)	384 (10)	364 (0)	380 (0)	389 (8)
100 mg/L						
Hardness (mg/L as $CaCO_3$)	189 (2)	191 (3)	187 (3)	184 (1)	190 (1)	192 (3)
Alkalinity (mg/L as $CaCO_3$)	142 (0)	144 (1)	142 (4)	143 (2)	148 (1)	142 (3)
Specific cond. (µS/cm)	390 (1)	401 (1)	382 (7)	365 (1)	389 (1)	389 (7)

[a] Pre-exposure hardness and alkalinity were measured in triplicate from head boxes supplying water to the test system.
[b] Post-exposure hardness, alkalinity, and specific conductance were measured weekly from pooled samples collected by treatment group.

Histologic scores were analyzed by the contract laboratory using GraphPad Prism version 5 and significance was declared at $\alpha = 0.05$. Scores recorded as NR were changed to 0 and mean test tank scores were compared using a Kruskal-Wallis test. Comparisons were made among treatment groups using a Dunn's Multiple Comparison test.

Results

Water chemistry

The DO, pH, and temperature throughout the course of both trials were consistent among experimental replicates and they were well within acceptable criteria for aquaculture (Timmons and Ebling 2013; Table 1). Mean (standard deviation) DO levels during the lake sturgeon pre-exposure, exposure, and post-exposure periods remained above 8.12 (0.31), 7.70 (0.34), and 7.63 (0.33) mg/L, respectively. Mean DO levels during the lake trout pre-exposure, exposure and post-exposure periods remained above 9.83 (0.21), 9.05 (0.57), and 9.62 (0.33) mg/L, respectively. The lowest individual DO measurement (6.79 mg/L) occurred during the lake sturgeon post-exposure observation period and it was 76.7% of saturation. Individual pH measurements ranged from 7.75 to 8.15 and from 7.71 to 8.06, during the lake sturgeon and lake trout trials, respectively. Mean temperature measurements in the lake sturgeon and lake trout trials varied ≤ 0.6 °C throughout the trials.

Water hardness, alkalinity, and specific conductance were similar among the pre-exposure, exposure, and post-exposure periods as well as between species (Table 2). Mean water hardness and alkalinity for both species ranged from 184 (1) to 193 (3) and from 142 (0) to 146 (2) mg/L as $CaCO_3$, respectively, throughout the study. Specific conductance was slightly more variable with means ranging from 363 (1) to 396 (7) µS/cm across all sampling events for both species.

The difference in water temperature between species contributed to a disparity in TAN concentrations measured at exposure termination (Table 3).

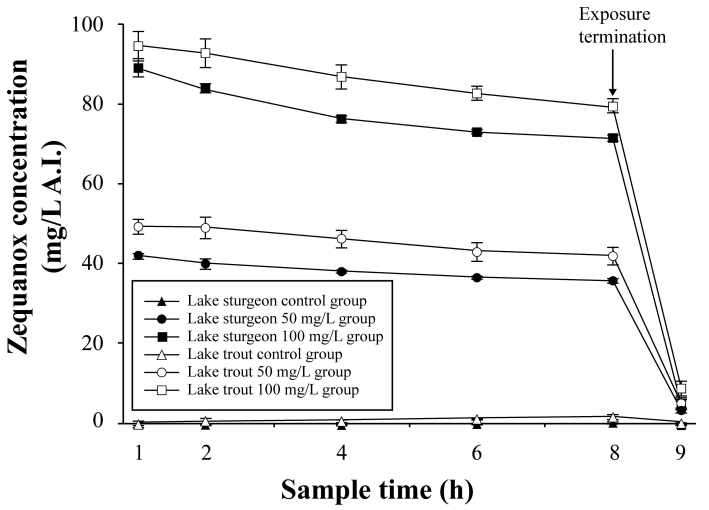

Figure 4. Zequanox active ingredient (A.I.) concentrations measured in test tank replicates throughout the exposure and 1 h post exposure (9 h).

The lake sturgeon trial had mean TAN concentrations ranging from 0.0 (0) to 2.45 (0.31) mg/L, while the lake trout trial had mean TAN concentrations ranging from 0.04 (0.02) to 1.09 (0.31) mg/L. The lake sturgeon TAN concentrations in both treated groups were below the USEPA acute criterion magnitude of 4.2 mg/L and above the chronic criterion magnitude of 0.83 mg/L at pH 8.0 and 19 °C (USEPA 2013). The TAN concentrations in the lake trout trial did not exceed the USEPA acute or chronic criterion magnitudes of 8.4 and 1.4 mg/L, respectively, at pH 7.9 and 13 °C (USEPA 2013).

Zequanox concentration analysis

Measured Zequanox concentrations were consistently lower than target and the concentrations dropped during the exposures (Figure 4). The mean Zequanox concentrations measured in the treated groups 1 h after application were 41.9 (0.7) and 89.1 (2.3) mg/L A.I. in the lake sturgeon trial, and 49.3 (1.8) and 94.6 (3.7) mg/L A.I. in the lake trout trial. After 8 h of exposure, the mean concentrations measured in the treated groups were 38.4 (2.4) and 78.8 (7.0) mg/L in the lake sturgeon trial, and 45.9 (3.7) and 87.3 (6.4) mg/L in the lake trout trial. One hour after exposure termination the concentrations in both trials were ≤ 5.0 and 8.5 mg/L in the 50 and 100 mg/L treatment groups, respectively.

Survival

No mortality was observed in any of the untreated control tanks throughout the study for either species.

Table 3. Mean (standard deviation) total ammonia nitrogen (TAN) observed at exposure termination.

Species	Treatment group	TAN (mg NH_3-N/L)
Lake sturgeon	Control	0.00 (0.00)
	50 mg/L	1.11 (0.16)
	100 mg/L	2.45 (0.31)
Lake trout	Control	0.04 (0.02)
	50 mg/L	0.72 (0.09)
	100 mg/L	1.09 (0.31)

No acute mortality or adverse impacts were observed in any treated groups of either species. No differences in lake sturgeon survival were detected among treatment groups for the duration of the study ($P > 0.12$, $df = 9$; Figure 5). Survival of lake trout did not differ between the control and 50 mg /L treated groups (100 and 99.8%, respectively; $P = 0.32$, $df = 9$); however, survival in the 100 mg/L treated group was significantly lower at study termination (53.2%; $P < 0.01$, $df = 9$; Figure 5). Substantial latent mortality was observed in the lake trout 100 mg/L treated group starting around 3 weeks after exposure (Figure 6). Mortality of lake trout in the 100 mg/L treatment group had not yet stabilized at the termination of the post-exposure observation period, as evident by graphing the daily mortality as a percentage of surviving fish (Figure 7).

Sub-lethal impacts

Analyses of mean fish weight at the time of distribution to the test tanks showed no difference ($P > 0.15$, $df = 9$)

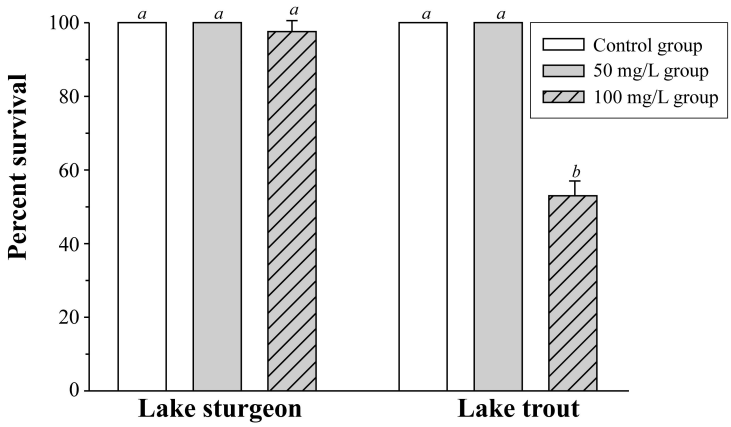

Figure 5. Survival of lake sturgeon (*Acipenser fulvescens*) and lake trout (*Salvelinus namaycush*) by treatment group 33 d after Zequanox exposure [Within each species, columns with the same letter are not statistically different].

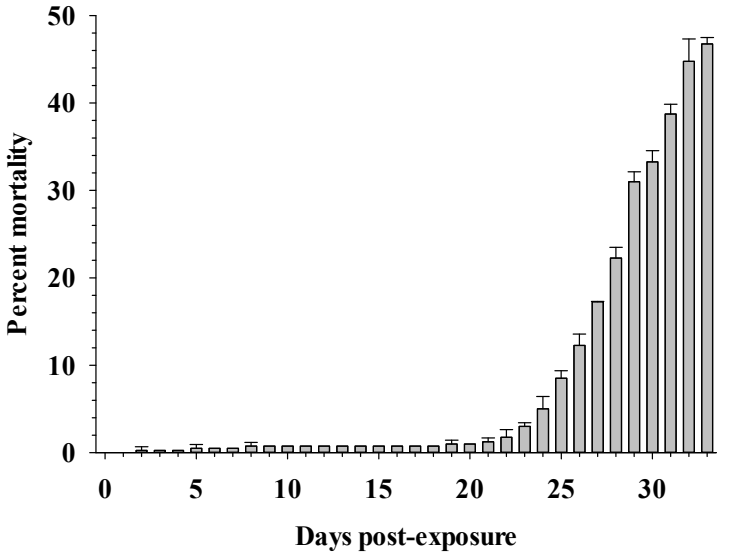

Figure 6. Cumulative mortality of lake trout (*Salvelinus namaycush*) exposed to 100 mg/L A.I. of Zequanox for 8 h.

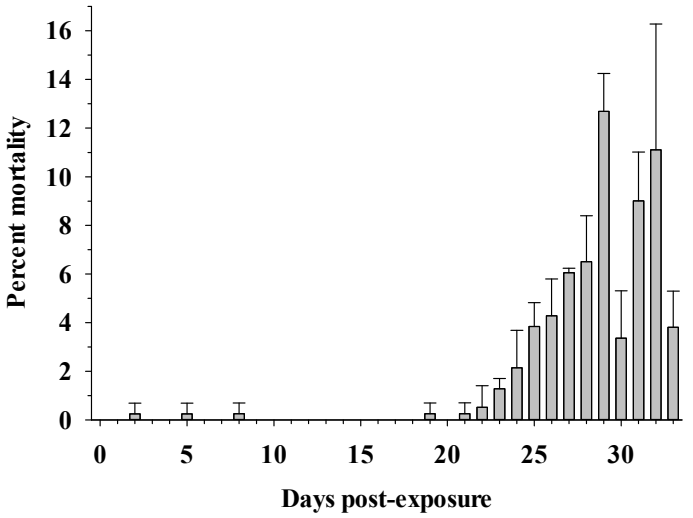

Figure 7. Daily percent mortality of lake trout (*Salvelinus namaycush*) exposed to 100 mg/L A.I. of Zequanox for 8 h.

Table 4. Mean (standard deviation) fish weight at distribution to the test tanks and fish weight, length, and percent weight gain at study termination.

Species	Treatment group	Distribution weight (g)	Termination weight (g)	Weight gain (%)	Termination length (mm)
Lake sturgeon	Control	3.81[a] (0.10)	21.49[a] (3.84)	464.0	184 (11)
	50 mg/L	3.61[b] (0.06)	20.57[b] (3.60)	469.8	181 (11)
	100 mg/L	3.70[a,b] (0.14)	20.17[b] (4.23)	445.1	179 (13)
Lake trout	Control	3.59[a] (0.10)	9.55[a] (2.26)	166.0	104 (7)
	50 mg/L	3.58[a] (0.18)	8.88[b] (1.91)	148.0	100 (7)
	100 mg/L	3.48[a] (0.03)	6.19[c] (1.55)	77.9	91 (7)

[a,b,c] For each species, values in columns with the same letter are not statistically different.

for both species, with the exception of a difference of 0.20 g between the weight of lake sturgeon in the control and the 50 mg/L treatment groups ($\bar{x}$ = 3.81 and 3.61 g, respectively) was found to be significant (P = 0.02, df = 9; Table 4). The average weight of lake sturgeon in all treatment groups increased between 445.1 and 469.8% during the study (Table 4). The weight of lake sturgeon at study termination showed differences among fish in the control and both treated groups (P < 0.03, df = 9). However, no differences were detected among fish in the treated groups (P = 0.11, df = 9). No differences were detected among the mean condition of lake sturgeon in the control and treated groups (P > 0.20, df = 9). The average lake trout fish weight increased from 77.9%, in the 100 mg/L treated group, to 166.0%, in the control group (Table 4). The weight of lake trout at study termination showed differences among fish in the control and both treated groups (P < 0.05, df = 9). Additionally, differences were detected among fish in the treated groups (P < 0.01, df = 9). Differences were detected among the condition of lake trout in the control and 50 mg /L treated group (P = 0.01, df = 9), but not between those in the control and the 100 mg /L treated groups (P = 0.06, df = 9).

No cytopathogentic effects or replicating viral agents were detected in samples of fish that were collected at study termination in either trial. Histologic examination of the lake trout GI tracts generally revealed no differences among treatments. Minimal to mild inflammation in the stomach and intestines appeared randomly distributed within all treatment groups. The only observed difference between treatment groups was in the distal intestine where the 100 mg/L treated fish had less inflammation than the control fish (Kruskal-Wallis test, p = 0.0102; Dunn's Multiple Comparison Test, p < 0.01; Figures 8 and 9). Necrosis or other signs of toxicity were not observed in any tissues examined.

Discussion

Water quality

The water quality was acceptable for rearing fish throughout the study and the parameters were similar among test replicates, with the exception of the elevated TAN concentrations in the lake sturgeon exposures. No negative impacts to lake sturgeon are attributed to elevated TAN concentrations. Previous laboratory and contained field application studies demonstrate that small Zequanox applications are not likely to have long-term water quality impacts such as ammonia toxicity (Meehan et al. 2014; Whitledge et al. 2015). However, the impacts of large-scale, open-water applications of Zequanox on water quality remain largely unknown.

Zequanox concentrations

Initial mean Zequanox concentrations measured in the test tanks were 83.8 and 89.1% of target in the 50 and 100 mg/L lake sturgeon treatment groups, respectively, and 98.6 and 94.6% in the 50 and 100 mg/L lake trout treatment groups, respectively. A standard cup style household blender was used to mix the Zequanox in the lake sturgeon trial and a household immersion style blender was used to mix the Zequanox in the lake trout trial. When wetted, Zequanox has a tendency to clump and cling to surfaces; therefore, the disparities in the Zequanox concentrations measured between the trials are attributed to the efficiency of Zequanox mixing using the different types of blenders. For both species, the decrease in measured Zequanox concentrations over the 8 h exposure ranged from 6.8 to 10.3%. The decrease in measured Zequanox concentrations was likely due to degradation, settling, and the formation of a Zequanox coagulation which was observed on the water surface.

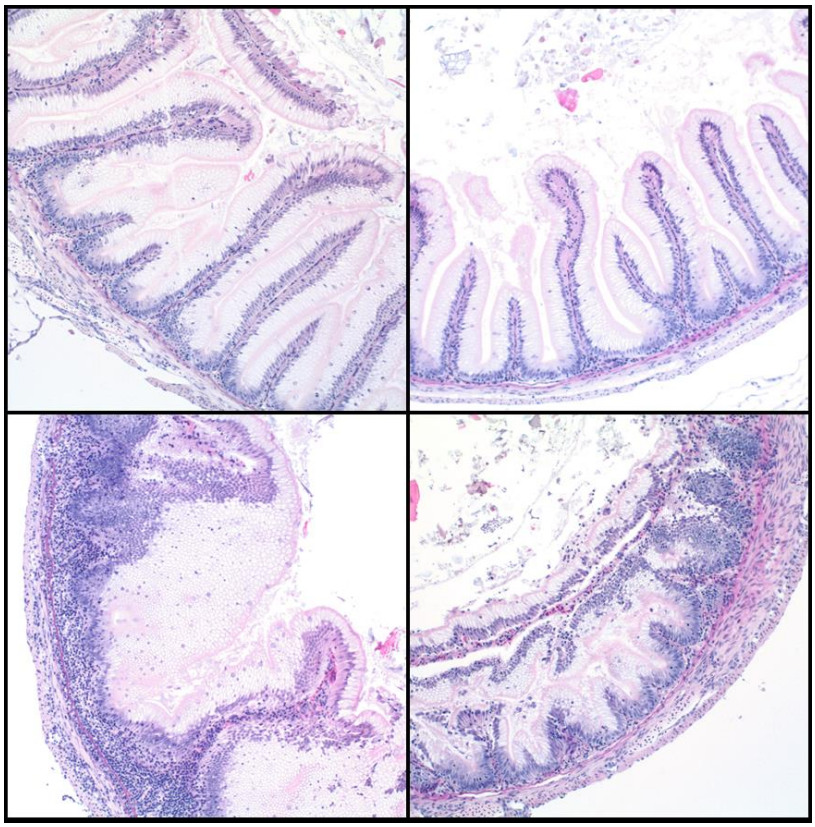

Figure 8. Representative photomicrographs with (bottom) and without (top) inflammation in the distal intestine of fish from the control (left) and the 100 mg/L A.I. Zequanox (right) treatment groups. HE, 100x magnification. Photomicrographs by Joshua Kramer, Histology and Histologics Inc.

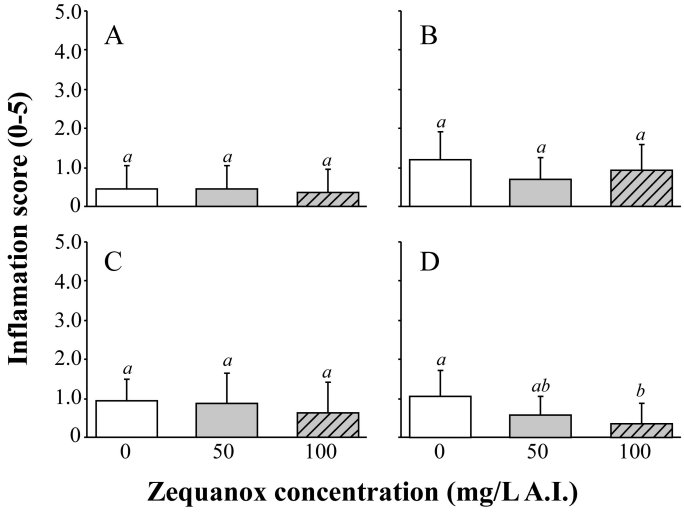

Figure 9. Mean (standard deviation) histology inflammation scores observed in stomach (A), proximal intestine (B), mid intestine (C), and distal intestine (D) tissue samples from lake trout (*Salvelinus namaycush*) 33 d after exposure to Zequanox [Within each tissue type, columns with the same letter are not statistically different].

Survival

Our results demonstrate that the length of the post-exposure observation period is critical to adequately ascertain the impacts of Zequanox exposure. Survival was not impacted in the lake trout 100 mg/L treated group for the first 3 weeks; however, impacts were readily detectable 4 weeks (28 d) after Zequanox exposure (Figures 6 and 7). Metabolic rates in fish are

highly influenced by water temperature; therefore, utilization of a temperature-based method to calculate the duration of post-exposure observation is more appropriate than a temporal-based method. Piper et al. (1982) describes a temperature unit system for predicting fish egg incubation and development which can be adapted for the standardization and comparison of toxicity trials conducted at different water temperatures. In this concept, one daily temperature unit (DTU) is assigned for every degree Celsius for 24 h. Utilizing the survival observations of the current lake trout trial to set the minimum required DTU benchmark for accurate assessment of the impacts of Zequanox exposure results in a DTU benchmark of 347 (28 d × 12.4 °C). With this method, impacts on the survival of lake sturgeon in the current study should have been observed by day 18. A study by Molloy et al. (2013b) evaluated the toxicity of killed *Pf*-CL145A cells on brown trout (*Salmo trutta* Linnaeus, 1758) at 6 ± 1 °C using a 39 d post-exposure observation period. Molloy et al. (2013b) did not detect any treatment related mortality in brown trout; however, application of the DTU benchmark to their data suggests that a 57 d post-exposure observation period would have been required to accurately assess exposure-related impacts. Molloy et al. (2013b) also conducted toxicity trials with fathead minnows (*Pimephales promelas* Rafinesque, 1820) and live *Pf*-CL145A cells at 23 °C and with bluegills (*Lepomis macrochirus* Rafinesque, 1819) and dead *Pf*-CL145A cells at 20 °C. Molloy et al. (2013b) observed no impacts to either fathead minnows or bluegills; however, the bluegill post-exposure observation period was 11 d, while the suggested DTU method would require 17 d of post-exposure observation. The fathead minnow 17 d post-exposure observation period exceeded the DTU method required 15 d post-exposure observation period. Waller and Luoma (2016) conducted outdoor mesocosm Zequanox exposures to newly hatched fathead minnow fry and adults and observed no impacts on the survival or body condition of fathead minnows 90 and 21 d after exposure, respectively. The mean temperatures during their fry and adult trials were approximately 25 and 26 °C, respectively. Therefore, the post-exposure observation periods were sufficient to determine the impacts of Zequanox exposure on the survival of fathead minnows using the DTU method. Evidence to date suggests that Zequanox exposure at a dose consistent with the open-water product label has unique impacts related to the survival of lake trout. Additional investigation into the impacts of Zequanox exposure on the survival of other salmonids may be warranted to determine if the impacts are species specific.

Sublethal impacts

The substantial weight gain of the lake sturgeon (445 to 470%) over the course of the study coupled with the minimal statistical differences in mean terminal fish weight (20.2 to 21.5 g) and length (179 to 184 mm) demonstrates that Zequanox exposure had no appreciable biological impact on the condition or growth of lake sturgeon. Our data suggests that the impacts to native fish reported by Luoma et al. (2015b) using a flow-through system should be interpreted carefully and impacts verified using Zequanox exposure patterns that would be encountered during field applications.

The weight gains of lake trout exposed to Zequanox were considerably less than those in the control group. The mean lake trout weight at study termination in the 50 and 100 mg/L treated groups were approximately 93 and 65% of those in the control group, respectively. Similarly, the mean lengths of the lake trout at study termination in the 50 and 100 mg/L treated groups were approximately 96 and 87%, respectively, of those in the control group. Our data suggests that condition is not as sensitive a measure as weight for determining sublethal impacts, and that the condition of surviving fish can be similar when there are concurrent significant impacts on weight gain and survival. The majority of growth observed in the 100 mg/L treated lake trout groups likely occurred in the first 2 weeks after exposure, during which time little mortality was observed and the fish behavior appeared normal. Poor food consumption, emaciation, and abdominal hemorrhaging were observed approximately 3 to 4 weeks after exposure in some of the lake trout exposed to 100 mg/L A.I. of Zequanox, although these observations were not specifically evaluated or analyzed.

The lack of cytopathogentic effects or replicating viral agents in fish examined at study termination provides evidence that the observed impacts in this study are due solely to Zequanox exposure. Histologic examination of lake trout detected significantly less inflammation in the distal intestines of fish in the 100 mg/L treated group compared to the control fish, but, this should not be construed as a positive finding as ample evidence indicates that exposure to 100 mg/L A.I. of Zequanox has profound effects on lake trout. The histologic examination of the lake trout provides evidence that the mode of action that causes Zequanox exposure-related impacts in lake trout is different from that which causes mortality in dreissenids. Research conducted by Molloy et al. (2013a) demonstrated that the active ingredient in Zequanox induces dreissenid mussel mortality by

causing lysis and necrosis of the digestive gland tissue and sloughing of stomach epithelium. The findings of Molloy et al. (2013a) and our observations led us to suspect and investigate impacts to the lake trout GI tract. The mode of action for Zequanox toxicity in lake trout remains indeterminate after histologic examination. Thus, further investigations are necessary to elucidate the cause of toxicity in lake trout and to determine vulnerability of other salmonids. Additional study on the potential of fish to avoid Zequanox exposure many provide valuable insight into exposure patterns that could be expected during field applications.

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