

## Research Article

## Effects of the biopesticide Zequanox<sup>®</sup> on reproduction and early development of the fathead minnow (*Pimephales promelas*)

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### Abstract

The biopesticide, Zequanox<sup>®</sup>, is registered for dreissenid mussel control in open water systems in the United States. Previous toxicity trials with nontarget organisms, including several young-of-the-year fish species and invertebrates, demonstrated selectivity of Zequanox for dreissenid mussels, but data are lacking on the treatment-related effects on reproduction and early life stage development of fish. The present study evaluated the effects of Zequanox on spawning and early life stages of the fathead minnow, *Pimephales promelas*, after exposure to the maximum approved concentration [100 mg active ingredient (AI)/L] and exposure duration (8h) for open water application. The results showed no significant treatment-related effect of Zequanox on survival, condition, or cumulative egg production (21 d) in adult fathead minnow. Eggs ( $\leq 24$ -h old) exposed to Zequanox developed to the eyed-stage at a similar rate to that of untreated eggs. Additionally, Zequanox did not have a significant effect on survival and growth (90 d) of newly hatched larvae ( $\leq 24$ -h old). Zequanox may be an option for control of dreissenid mussels in localized open water habitats where concerns exist regarding reproduction and recruitment of cyprinids and related species.

**Key words:** *Pseudomonas fluorescens*, molluscicide, fathead minnow, spawning, egg development

### Introduction

Zebra mussels (*Dreissena polymorpha* Pallas, 1771) and quagga mussels (*D. rostriformis bugensis* Andrusov, 1898) are nuisance invasive species that have expanded their range throughout the United States and into Canada since their arrival in 1985 (Mackie and Claudi 2010). These mussels have adversely affected aquatic communities on a number of levels. The high filtration capacity of dreissenid mussels has caused a shift from a pelagic to a benthic food web in areas of the Great Lakes, resulting in alterations in diet and concomitant reduction in condition of some fish species (Vanderploeg et al. 2002; Pothoven and Madenjian 2008; Nalepa et al. 2009). Settlement of dreissenid mussels in high densities has degraded fish spawning shoals (Marsden and Chotkowski 2001) and caused the decline and disappearance of native mussels from habitats throughout

the Great Lakes' region (e.g., Nalepa et al. 1996; Ricciardi et al. 1996; Schloesser et al. 1998; Strayer and Malcom 2007). Dreissenid infestations of water bodies used by aquaculture facilities have altered fish management activities by compromising or eliminating sources of brood stock and eggs for aquaculture (OMNR 2005; Sykes 2009). Consequently, there is an urgent need to develop control measures for these invasive dreissenids that are targeted and have minimum nontarget toxicity.

One potential molluscicide to control dreissenid mussels in open water is the biopesticide, Zequanox<sup>®</sup>. The product is a commercial formulation of a specific strain (CL145A) of the ubiquitous soil bacterium, *Pseudomonas fluorescens*, that has shown selective toxicity to dreissenid mussels and low toxicity to a range of nontarget species (Marrone Bio Innovations [MBI] 2012a; Molloy et al. 2013a, b; Waller et al. 2016). Zequanox is registered by the

US Environmental Protection Agency (USEPA) for control of dreissenid mussels in point source discharge and open water systems (Marrone Bio Innovations 2012b; registration number 84059-15). The maximum approved open water application is 100 mg active ingredient (AI)/L for 8 h. Previous studies have demonstrated that the potency of Zequanox significantly decreased after 8 h in water, and was near control levels within 24 h after hydration (Molloy et al. 2013a). Toxicity tests with bluegill (*Lepomis macrochirus* Rafinesque, 1810), largemouth bass (*Micropterus salmoides* Lacépède, 1802), and brown trout (*Salmo trutta* Linnaeus, 1758) showed no lethality at 100 mg AI Zequanox/L in a 72-h static exposure (Molloy et al. 2013b). Luoma et al. (2015a) tested fingerlings of a variety of warm-, cool- and coldwater fish species in a continuous 24-h exposure to Zequanox concentrations ranging from 50 to 300 mg AI/L. Significant differences in toxicity occurred among species; for example, rainbow trout (*Oncorhynchus mykiss* Walbaum, 1792) and lake sturgeon (*Acipenser fluvescens* Rafinesque, 1817) were highly sensitive to the biopesticide (LC50 = 19.2 mg AI/L and 8.9 mg AI/L, respectively), while the LC50 value exceeded 100 mg AI/L for six other species. However, Luoma et al. (2015a) applied Zequanox continuously for three times longer than the expected exposure duration (i.e., 24-h continuous dosing). Anderson et al. (2012) tested eggs and embryos of fathead minnows in a continuous 28-d exposure to concentrations of Zequanox that ranged from 2.3 to 23.9 mg AI/L and found no effect on hatchability of eggs but significant effects on survival and condition of fry. There are no published studies on the treatment related effects of Zequanox to spawning adults, eggs, and early life stages of fish after a single application at the expected exposure duration (8 h) and concentration (100 mg AI/L).

Fathead minnows are well-established test organisms for measuring toxicant effects on survival and reproductive fitness of fish (Ankley et al. 2001; Jensen et al. 2001; Kahl et al. 2001). Reproductive maturity of males and females can be established by the appearance of secondary sex characteristics; males develop a dorsal pad and various sizes and numbers of nuptial tubercles. Fathead minnows are fractional spawners and females may produce clutches of 50–250 eggs every 3–5 days at 25 °C (Gale and Buynak 1982; Jensen et al. 2001; USEPA 2002; Thorpe et al. 2007). Unfertilized eggs can be distinguished by an opaque or clear appearance and a white spot where the yolk has precipitated (USEPA 2002). Fertilized eggs undergo cleavage to the blastula stage within 3–4 h of spawning. The eyed-stage

develops within 48–72 h and can be used to distinguish unfertilized or undeveloped eggs from developing larvae. Eggs hatch within about 96 h of fertilization (USEPA 1996; Thorpe et al. 2007).

The goal of the present study was to determine the treatment-related effects of Zequanox on the survival and reproductive success of fathead minnows at expected open-water application rates. Trials were conducted in an outdoor mesocosm to evaluate effects of 8-h exposure to 50 and 100 mg AI Zequanox/L on: 1) survival and condition of adult fathead minnows, 2) cumulative egg production, 3) egg development to the eyed-stage, and 4) survival and growth of newly hatched larvae.

## Methods

### *Test system*

Trials were conducted in an outdoor mesocosm that consisted of four 0.004-ha concrete ponds, each containing nine independently plumbed 1000-L circular livestock tanks (high density polyethylene, 175 cm diameter × 64 cm height) (Figure 1A). Pond water was pumped from a 0.1 ha earthen pond, passed through a 400 µm filter, and delivered to a head-box system for distribution to the test and rearing tanks. Tanks were filled to a volume of 980 L. Daily and diurnal fluctuations in water temperature of the tanks were minimized by filling the concrete ponds with well water and by covering the ponds with black shade cloth. Flow rate was adjusted to approximately 3.8 L/min per tank (~ 6 tank exchanges/day). Aeration was supplied during the post-exposure rearing period through individual airstones in each tank that were connected to a regenerative blower. Aeration was not supplied during Zequanox exposure to simulate conditions in an open water application and assess treatment-related effects on dissolved oxygen concentrations.

### *Zequanox treatment and concentration verification*

Two concentrations of Zequanox (50 and 100 mg AI/L) and an untreated control were tested at the expected environmental exposure duration (8 h). The test material, Zequanox, was a spray-dried powder formulation, made of 50% active ingredient (MBI-401 SDP, Marrone Bio Innovations, Davis, CA). A dosing stock was prepared from test tank water and the appropriate weight of dry Zequanox and added to the test tank within 5 minutes of preparation. Water flow to each test tank was stopped during the 8 h treatment; at the end of the exposure period, tanks were drained to half-volume and refilled to full



**Figure 1.** Test system. **A)** Mesocosm test system consisting of nine independently plumbed 1000-L tanks within each of four 0.004-ha concrete ponds. Water was supplied to test tanks from a 0.25-ha earthen pond. Concrete ponds were filled with water and covered with shade cloth to moderate diurnal temperature fluctuation. **B)** Spawning substrates with newly deposited eggs on the concave surface. A digital photograph was taken of substrates for enumeration of newly deposited and eyed-eggs. **C)** Incubation of spawning substrates in the test tank atop a bubble wand. Photographs by D. Waller.

volume to speed removal of Zequanox. Water flow was resumed for the post-exposure period.

Mid-column water samples were collected from each test tank for Zequanox concentration analysis at 0, 1, 2, 4, 6, and 8 h of exposure and at 1, 4, 8 and 16 h post-exposure. The concentration of Zequanox (AI) in each test tank was determined by comparing the spectrophotometric absorbance (660 nm wavelength)

of the sample to a linear regression created from Zequanox standard solutions (Beckman UV/Vis Spectrophotometer, Model DU 800, Beckman Coulter, Indianapolis, IN). Confirmatory post-test efficacy verification of Zequanox was completed at MBI, Davis, CA. Results of all efficacy trials met the quality control standards set forth by MBI for the product (i.e.,  $\geq 70\%$  dreissenid mussel mortality).

### Water chemistry

Dissolved oxygen, pH and temperature were measured daily in each tank during the pre- and postexposure period and at 1 h and 8 h during the exposure period. Dissolved oxygen was measured with a YSI<sup>®</sup> 550A dissolved oxygen meter (YSI, Inc., Yellow Springs, OH). The pH was determined with a Beckman Coulter<sup>®</sup> φ410 pH meter and probe (Beckman Coulter, Inc., Fullerton, CA). Temperature was measured with a ThermoMapen<sup>®</sup> digital thermometer (ThermoWorks, American Fork, UT). Total hardness (mg/L as CaCO<sub>3</sub>) was determined by titrimetric method with Manver Red indicator (USEPA 1983). Total alkalinity (mg/L as CaCO<sub>3</sub>) was determined by titrimetric method to an endpoint of pH 4.5 (APHA 2012). Conductivity was measured with a Fisher Accumet<sup>®</sup> conductivity meter (Fisher Scientific, Pittsburg PA). Hardness and alkalinity were measured from one replicate tank of each treatment before exposure and once during the exposure period. During the post-exposure period, hardness and alkalinity were measured weekly on the source water. Conductivity was measured in each tank before and once during the exposure period.

### Adult spawning trial

Adult fathead minnows, 9 to 16 mo-old, were obtained in June 2014 from fish culture facilities at the Upper Midwest Environmental Sciences Center (UMESC). Fish were sedated with Aquic-S<sup>®</sup>20E (16 mg AI eugenol/L), hand sorted by sex, and transferred into a partitioned raceway. Males were identified by the presence of tubercles on the head, a black spot on the dorsal fin and/or a dark band behind the head. Females were identified by a lack of the aforementioned features and the appearance of the ovipositor (Flickinger 1969). Female and male fish were randomly distributed to each of nine adult tanks (three replicate tanks per treatment) with a target sex ratio in each tank of 2.5 females:1.0 males (n=40 female and 15 male fish). However, external sexual characteristics became well developed during the spawning trial and indicated errors in the initial sexing of individuals and the final number of females per tank ranged from 30–40. In the analysis of spawning, the number of females per tank was based on sex determination at the conclusion of the trial. Mean total length of females was 62.8 mm (range 50.0–76.1 mm; SD = 4.1) and of males was 70.1 mm (range 56.9–85.0 mm; SD = 5.3). Mean wet weight of females was 2.67 g (range 1.59–3.83 g; SD = 0.48) and of males was 4.11 g (range 2.14–7.70 g; SD = 0.89).

The following day, 10 spawning substrates were placed into each adult tank to monitor pre-exposure egg production. Spawning substrates were constructed of a 15-cm length of 10-cm inner diameter polyvinyl chloride (PVC) pipe that was cut in half lengthwise (Figure 1B). Substrates were observed daily for 5 days to verify that fish in each tank were in spawning condition and to provide an estimate of pre-exposure egg production. After the 5-d pre-exposure spawning period, Zequanox was applied to the adult tanks as described in the section *Zequanox treatment and concentration verification*. Following Zequanox exposure, adult fish mortality and egg production were monitored for 21 days. Spawning substrates were removed daily from the adult tanks and examined for egg deposition. A typical spawn for a female fathead minnow is 50–250 eggs (USEPA 2002). Therefore, to ensure an adequate sample size for statistical analysis of egg development, only substrates with ≥50 eggs were retained. Substrates with <50 eggs were recorded as partial spawns, the eggs were removed, and the substrate was returned to the adult tank. All substrates with ≥ 50 eggs were photographed for enumeration of eggs. To standardize the sample size and reduce variability in egg viability spawning date (i.e., first versus last spawn of a female) only the first ten spawned substrates (1–10) with ≥50 eggs in each adult tank were retained for measuring development of eggs to the eyed-stage. When a substrate was removed from the adult tank, it was immediately replaced to maintain the total number of substrates at 10 per tank.

Substrates 1–10 were transferred to a 1000-L rearing tank, corresponding to the adult tank, in an adjacent concrete pond. Spawning substrates in the rearing tanks were incubated in an upright position atop a mounted grill grate (57 cm diameter × 30 cm height). A 120-cm bubble wand was used to maintain flow over the eggs and reduce development of fungus (Figure 1C). Additionally, substrates were immersed in a fungicidal treatment (1667 mg/L formalin bath for 15 min) on 3 consecutive days (Schnick 1973). Substrates in the rearing tanks were photographed again at 48–72 h to enumerate the number of eyed-eggs and assess fertilization and development.

At the conclusion of the 21-day post-exposure spawning period, adult fish were euthanized in tricaine methanesulfonate (MS-222), sexed, measured, and weighed. The final count of live females per tank (range = 25 to 40, mean = 33, SD = 4) was used in analyses of egg production. Condition factor of adult fish was calculated as  $K = 100 \times (W/L^3)$  where K = condition, W = wet weight (g) and L = total length (cm) (modified from Williams 2000).

Egg counts were made from digital images using image analysis software (NIS Elements<sup>®</sup>). Cumulative egg production per live female in a tank was enumerated over the 21-d post-exposure period. Percent eyed-eggs was determined for the ten substrates in the rearing tanks and was defined as: (number of eyed-eggs on substrate/number of eggs deposited on substrate) × 100.

#### *Egg trial*

Mature fathead minnows were transferred from indoor culture facilities into three outdoor 0.04 hectare (ha) concrete ponds in early June 2014. About 150–180 spawning substrates were placed into each pond and checked at about the same time each day for egg deposition. Substrates with >50 eggs (≤24-h old) were placed into a cooler of pond water and immediately transferred to the mesocosm for distribution to test tanks. Ten substrates were randomly distributed to each of the nine test tanks (n = 3 tanks/treatment). Within 4 h of initial substrate collection from the pond, Zequanox was applied to the test tanks using the same procedure as described in the section *Zequanox Treatment and Concentration Verification*. At 1-h post-exposure, all substrates in a test tank were removed and simultaneously immersed in a formalin bath, as described in the section *Adult Spawning Trial*, returned to the test tank, and incubated on a grill grate with aeration (Figure 1C). Substrates were individually photographed on Day 0 and Day 3 for enumeration of initial egg deposition and number of eyed-eggs, respectively. Egg counts were made from digital images as previously described. Percent eyed-eggs was defined as: (number of eyed-eggs on substrate/number of eggs deposited on substrate) × 100.

#### *Larvae trial*

Adult fathead minnows were transferred from indoor culture facilities at UMESC to two outdoor ponds in early June 2015. Spawning substrates were placed throughout the ponds and checked at the same time each day for newly deposited eggs. When sufficient eggs were found deposited on one day, substrates were placed into a cooler of pond water and transferred to a wet lab. Eggs were stripped from substrates by immersion in a de-adhesion solution of sodium sulfite (15 g/L) for approximately 3 minutes followed by immersion in a bucket of well water, gentle rinsing with a spray bottle, and collection of sloughed eggs (Ringle et al. 1992). Eggs were transferred to an upwelling incubator jar and maintained rolling at 23 °C with water flow of ~7.5 L/min. Newly hatched larvae

(≤24-h old) were collected on Day 4 and randomly distributed (n = 300 per replicate) to each of 18 test tanks (6 replicates per treatment) in the outdoor mesocosm.

For ease of observation, a 100-L stainless steel vessel (35 cm × 60 cm × 60 cm, H × L × W) served as the test tank during the exposure period. Larvae were allowed to acclimate overnight and the following day Zequanox was applied to the test tank using the same procedure as described in the section *Zequanox Treatment and Concentration Verification*. At the end of the 8-h exposure, the contents of the test tank were poured into a 1000-L rearing tank. Daily observations of mortality in each rearing tank were recorded for 90 days.

On days 44 and 90 post-exposure, condition factor was determined from a subsample of 20 fish per tank. Fish were euthanized in MS-222 (250 mg/L) then weighed (wet weight, 0.01 g) and measured (total length, 0.1 mm). Total fish survival was determined on day 90. Each rearing tank was drained and fish were transferred to a 9.5 L bucket and euthanized by overdose in MS-222. The total number of fish was counted and a total wet weight was obtained for each rearing tank.

#### *Data analysis*

In every analysis, the tank was treated as the experimental unit. All statistical analyses were performed using SAS Version 9.3 (SAS Institute, Inc, Cary, NC) and statistical significance was defined at  $\alpha < 0.05$ . Analysis of water chemistry (dissolved oxygen, pH, temperature, alkalinity, water hardness, and conductivity) and Zequanox exposure concentration were summarized with simple descriptive statistics. Egg production per female and the total and partial number of spawned substrates per female (adult trial) were compared across treatments with Kruskal-Wallis nonparametric test (Proc nparway1). Percent eyed-eggs (adult and egg trial) and percent mortality of adults at 21 d and larvae at 90 d were analyzed with a general linear mixed model (Proc glimmix) with treatment as a fixed effect, tank as a random effect, and a binomial logistic regression (logit link function) with random intercepts. A scale parameter was added to the model using the random\_residual\_statement. Responses of each treatment group were individually compared to the control group using a two-sided least squares means (LSD) comparison test. Condition factor of adult fish (21 d post-exposure) and condition factor and wet weight of fry (44 and 90 d post-exposure) were modeled using a mixed effects model (Proc mixed) with treatment as a fixed effect and tank as a random effect. Adult condition factor (by

**Table 1.** Mean (standard deviation) water quality and chemistry measurements during Zequanox trials of fathead minnow adults, eggs, and newly hatched larvae. The pH range is shown in parentheses (n = 3 replicate tanks for adult and egg trials; n=6 replicate tanks for larvae trial).

Water quality parameter	Zequanox concentration (mg AI/L)	Exposure (8 h)		
		Adult trial	Egg trial	Larvae trial
DO (mg/L)	Control	9.3 (2.2)	9.2 (1.3)	9.0 (0.6)
	50	8.1 (0.5)	8.8 (1.7)	8.7 (0.6)
	100	7.9 (0.6)	8.6 (1.8)	8.7 (0.5)
Temp (°C)	Control	26.2 (1.6)	26.0 (1.0)	24.9 (1.8)
	50 mg	26.3 (1.7)	26.0 (1.1)	24.7 (1.7)
	100 mg	26.3 (1.7)	26.1 (1.0)	24.9 (1.8)
pH	Control	8.94 (8.85–9.10)	8.86 (8.54–9.22)	8.75 (8.39–9.26)
	50	8.77 (8.64–8.88)	8.75 (8.57–8.93)	8.63 (8.31–9.25)
	100	8.63 (8.33–8.87)	8.59 (8.55–8.64)	8.60 (8.02–9.25)
Post-exposure observation				
DO (mg/L)	Control	8.9 (2.2)	9.3 (1.0)	8.1 (0.9)
	50	8.9 (2.2)	9.3 (1.0)	8.1 (0.9)
	100	8.9 (2.1)	9.2 (1.0)	8.1 (0.9)
Temp (°C)	Control	22.3 (1.5)	21.5 (1.6)	21.7 (2.4)
	50	22.3 (1.5)	21.5 (1.6)	21.7 (2.4)
	100	22.3 (1.5)	21.5 (1.6)	21.6 (2.4)
pH	Control	8.78 (8.13–9.62)	8.89 (8.31–9.94)	8.53 (7.77–9.57)
	50	8.79 (8.14–9.61)	8.90 (8.31–9.94)	8.55 (8.04–9.57)
	100 L	8.78 (8.13–9.60)	8.89 (8.33–9.92)	8.53 (8.02–9.63)
Alkalinity (mg/L) <sup>1</sup>	NA <sup>2</sup>	131.6 (8)	101.6 (14)	99.7 (12)
Hardness (mg/L) <sup>1</sup>	NA <sup>2</sup>	172.4 (12)	126.7 (18)	131.0 (9)
Conductivity (µS/cm)	NA <sup>2</sup>	392.7 (18)	276.7 (42)	373.0 (187)

<sup>1</sup>Reported as mg/L CaCO<sub>3</sub><sup>2</sup>Means were determined from test tank and headbox samples

sex) of the control groups was compared to that of the treatment groups using a two-sided LSD comparison test.

## Results

### *Water quality and Zequanox concentration*

Mean measurements of water quality were similar for the three trials (Table 1). Mean temperature ranged from 24.7–26.3 °C during the exposure and from 21.5 °C to 22.3 °C during the post-exposure period of the three trials. A diurnal fluctuation in pH occurred in all adult and rearing tanks in the three trials that ranged from a morning low of about 8.10 to a peak of about 9.60 at late afternoon (Table 1). Dissolved oxygen concentrations decreased slightly during the 8-h exposure in the treatment tanks compared to the control tanks, but remained >7.9 mg/L (>90% saturation) in all tanks (Table 1).

The mean concentration of Zequanox was similar among replicates in the three trials (Figure 2). Based on absorbance measurements, the concentration of Zequanox in the low treatment (50 mg AI/L) tanks in each trial averaged 41.7, SD 4.3 (adult), 46.8, SD 6.1 (egg), and 44.7, SD 2.6 mg AI/L (larvae) (Figure

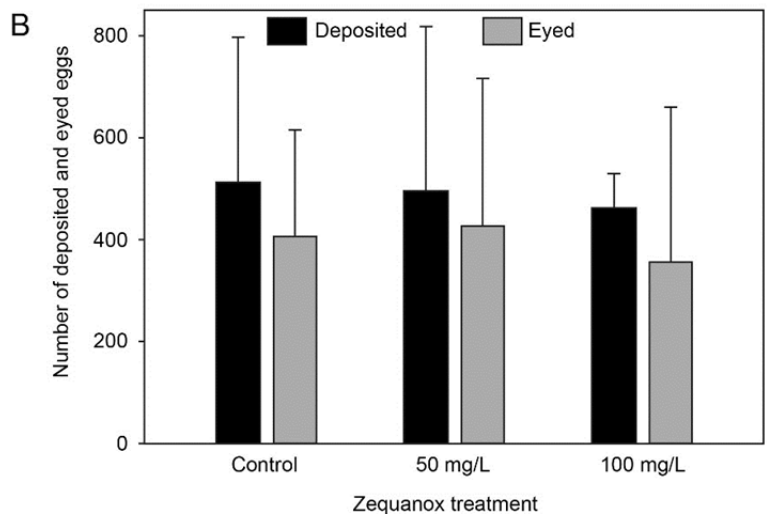
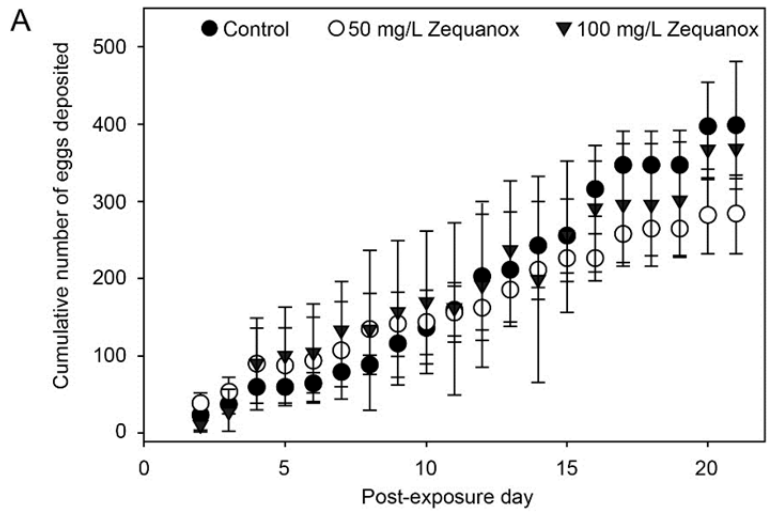
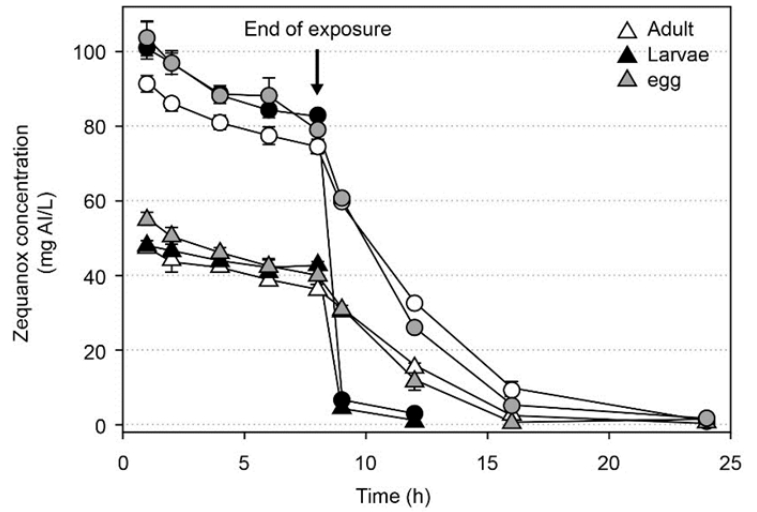
2). The concentration of Zequanox in the high treatment (100 mg AI/L) tanks in each trial averaged 82.1, SD 6.7 (adult), 91.2, SD 9.3 (egg) and 90.8, SD 7.9 mg AI/L (larvae) (Figure 2). Differences in measured concentrations of Zequanox among life stage trials are partly attributed to volume differences of the test tanks between the adult/egg trials and fry trials and settling of the product during the 8-h exposure period. Assuming a degradation rate of 0.0576 in a static test (USEPA 2014), the geometric mean concentration of active ingredient in our trials over 8 h was 38–44 mg AI/L in the low treatment and 73–82 mg AI/L in the high treatment.

### *Adult spawning trial*

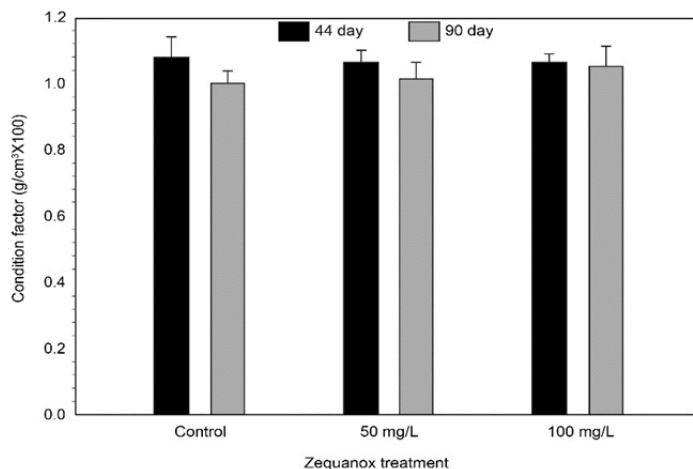
Pre-exposure spawning occurred in every adult tank. The number of spawning substrates with eggs deposited ranged from 4 to 13 per tank and the total number of eggs ranged from 2156 to 8974 per tank. Mean number of eggs per substrate was 700, SD 163.

A spawning event occurred on every day of the 21-d post-exposure spawning period in at least one tank and spawning occurred consistently throughout the 21-d period (Figure 3A). The total number of spawned

**Figure 2.** Mean Zequanox concentration (mg active ingredient/L) over time as determined by spectrophotometry. The exposure was terminated at 8 h (arrow) and water flow resumed to the tanks. n = 3 replicate tanks in adult and egg trials; n = 6 replicate tanks in larvae trial. Error bars represent the standard deviation.



**Figure 3.** Adult trial: A) Cumulative 21-d mean egg production per female fathead minnow after 8-h Zequanox treatment. B) Mean egg deposition and development to eyed-stage of fathead minnow eggs after 8-h Zequanox treatment. n = 10 substrates per tank, 3 tanks per treatment. Error bars represent the standard deviation.



**Figure 4.** Larvae trial: Mean condition factor of fathead minnow fry 44 and 90 days after 8-h Zequanox treatment.  $n = 20$  fish per tank, 6 tanks per treatment. Error bars represent the standard deviation.

**Table 2.** Mean length and wet weight (standard deviation) of fathead minnow larvae at 44 d and 90 d after 8-h exposure to Zequanox ( $n = 20$  larvae per tank; 6 replicate tanks per treatment).

Zequanox concentration (mg AI/L)	Day 44		Day 90	
	Total length (mm)	Wet weight (g)	Total length (mm)	Wet weight (g)
Control	31.4 (4.1)	0.35 (0.14)	43.5 (5.1)	0.85 (0.31)
50	32.6 (3.0)	0.38 (0.11)	44.0 (4.6)	0.89 (0.28)
100	30.8 (3.1)	0.32 (0.11)	41.9 (5.3)	0.81 (0.32)

substrates ranged from 27–43 per tank. Mean total spawns per tank ( $n = 3$ ) was 35, SD 7 (control), 33, SD 2 (50 mg AI/L) and 36, SD 7 (100 mg AI/L) and did not differ among treatments (chi-square = 0.63,  $p = 0.73$ ,  $df = 2$ ). Mean total spawns per female was 1.0, SD 0.2 (control), 0.9, SD <0.1 (50 mg AI/L) and 1.0, SD 0.2 (100 mg AI/L) and did not vary significantly among treatment (chi-square = 4.2,  $p = 0.12$ ,  $df = 2$ ). Mean cumulative egg production per female ranged from 283 eggs, SD 62 (50 mg AI/L) to 409 eggs, SD 84 (control) and did not differ significantly among treatments (chi-square = 3.82,  $p = 0.15$ ,  $df = 2$ ). Additionally, there was no treatment-related effect on fertilization and development to the eyed-stage ( $F = 2.26$ ,  $p = 0.11$ ,  $df = 2$ ) (Figure 3B). Although there was a trend downward in percent eyed-eggs in the high treatment, differences among treatments were not significant ( $F = 2.26$ ,  $p = 0.11$ ,  $df = 2$ ).

Total mortality of adult fish at 21-d post exposure ranged from 0–12% ( $n=0$  to 7 fish per tank). Mean mortality in the three treatments was 4.9%, SD 2.7 (controls), 3.6 SD 1.8% (50 mg AI/L) and 2.5 % SD 2.9 (100 mg AI/L) and was not significantly different among treatments ( $F = 0.59$ ,  $p = 0.59$ ,  $df = 2$ ); Mean mortality of females ranged from 1.1–2.9% and for males ranged from 3.7–8.0% and did not differ between control and treated groups (females,  $F = 0.45$ ,

$p = 0.66$ ,  $df = 2$ ; males,  $F = 0.49$ ,  $p = 0.63$ ,  $df = 2$ ). Mean condition factor of fish at 21-d post-exposure did not differ (within sex) between control and treated groups (females,  $F = 0.93$ ,  $p = 0.93$   $df = 2$ ; males  $F = 1.33$ ,  $p = 0.26$ ,  $df = 2$ ). Mean condition factor of females was 1.07, SD 0.03 (control), 1.07, SD 0.01 (50 mg AI/L) and 1.08, SD 0.03 (100 mg AI/L). Mean condition factor of males was 1.15, SD 0.01 (control), 1.18, SD 0.04 (50 mg AI/L) and 1.22, SD 0.06 (100 mg AI/L).

#### Egg trial

Zequanox treatment did not have a significant effect on development of eggs to the eyed-stage. The percent of eye-eggs ranged from 92.6%, SD 11.0 (50 mg AI/L) to 94.8%, SD 5.5 (control) and was not significantly different between the control and treated groups ( $F = 0.19$ ,  $p = 0.82$ ,  $df = 2$ ).

#### Larvae trial

Zequanox treatment of newly hatched fathead minnow larvae did not have a significant effect on 90-d survival. Cumulative mean survival ranged from 81.4%, SD 3.2 (50 mg AI/L) to 83.7%, SD 5.1 (100 mg AI/L) and did not differ significantly between control and treated groups ( $F = 0.65$ ,  $p = 0.54$ ,  $df = 2$ ). Mean length and weight of fry at 44-d and



90-d post-exposure was similar among treatments (Table 2). Additionally, no treatment-related effects on 44-d ( $F = 0.29, p = 0.75, df = 2$ ) and 90-d ( $F = 1.21, p = 0.30, df = 2$ ) condition factor of fry were detected between control and treatments (Figure 4).

## Discussion

By all measures used in the present study, Zequanox had no negative effect on spawning of fathead minnows during or after exposure (Figure 3A, B). Zequanox is composed primarily of organic particulates and produces a highly turbid suspension in the water column. Eggs were found on spawning substrates on Day 1 post-exposure indicating that turbidity did not prevent spawning or fertilization during the 8-h exposure (Figure 3A). There was no direct or lingering effect of Zequanox on egg deposition and larval development at the concentrations and exposure duration that were tested (Figures 3A, B and 4). Females continued to deposit eggs during the post-exposure period and development of those eggs to the eyed-stage was similar in all treatments (Figure 3B).

In the only other study to test the effects of Zequanox on egg development, Anderson et al. (2012) exposed fathead minnow eggs from 24-h after deposit to 28-d post-hatch in a continuous flow-through system to Zequanox concentrations that ranged from 2.25 to 23.9 mg AI/L. Zequanox exposure had no effect on hatching success, but survival and growth were significantly reduced at 28 d post-hatch. The no effect concentrations (NOEC) for larval post-hatch survival and growth were 3.63 and 2.25 mg AI/L, respectively (Anderson et al. 2012).

We found no treatment-related effect on survival or condition of larvae that were exposed 24-h post-hatch to Zequanox. Differences in toxicity effects between the present study and that of Anderson et al. (2012) are likely related to exposure pattern (8-h static vs 24-h continuous), combined with developmental stage of the fish. In our study, larvae were transitioning from the yolk-sac to exogenous feeding stage and may not have ingested Zequanox particulates during the 8-h treatment. Exposure of older, feeding larvae (e.g. >56 h post-hatch) with a similar treatment regime is needed to determine whether toxicity varies with exposure duration and/or larval stage.

The mode of action of Zequanox is through ingestion of the bacterium and subsequent degradation of the digestive epithelium (Molloy et al. 2013c). Organisms that are susceptible to the toxic component in Zequanox may have reduced growth and condition rather than overt mortality. We found no treatment-related effects on condition or survival

of adult fathead minnow from static exposure to a mean concentration of 82.1 mg AI/L for 8 h. The effects of Zequanox on survival and condition factor vary among fish species. Luoma et al. (2015a) reported reduced condition factor at 22 d post-exposure in several fish species for which the LC50 value was >100 mg AI /L in a 24 h exposure. For example, the 24-h LC50 for largemouth (*Micropterus salmoides*) was 173.9 mg AI/L and of smallmouth bass (*M. dolomieu*) was 139.4 mg AI/L, but condition factor was reduced at 75.3 and 33.4 mg AI/L, respectively (Luoma et al. 2015a). In the same study, salmonids (rainbow trout, *Oncorhynchus mykiss* and brook trout, *Salvelinus fontinalis*) and lake sturgeon (*Acipenser fulvescens*) were the most sensitive species. Luoma et al. (2015a) reported that condition factor was reduced at the lowest concentrations tested ( $\leq 40.3$  mg AI/L) and the 24-h LC50 value of all three species was <100 mg AI/L; however, Molloy et al. (2013b) found no mortality of brown trout (*Salmo trutta*) or bluegill (*Lepomis macrochirus*) from static exposure to 100 mg AI/L for 72 h. Likewise, 24-h LC50 values for yellow perch (*Perca flavescens*), bluegill and walleye (*Sander vitreus*) were >150 mg AI/L and no difference in condition factor was detected at <138 mg AI/L by Luoma et al. (2015a). The exposure pattern of Molloy et al. (2013c) and Luoma et al. (2015a) varied from the present study and are not directly comparable to our results. A continuous application of Zequanox for 24 h was used by Luoma et al. (2015a) to generate standard LC50 values for species' comparison but this dosing regime would be impractical and costly in open water. Molloy et al. (2013c) tested fish in both static and continuous exposures for up to 72 h, which exceeds the current approved application of 8 h by nine-fold.

The mesocosm system and application method used in the present study simulate a small-scale control scenario for dreissenid mussels in static, open water environments, such as a pond, embayment, or other quiescent bodies of water. For example, Meehan et al. (2014) and Whitley et al. (2015) used a similar application regime to evaluate the use of Zequanox for dreissenid control in a canal and lake, respectively. Target sites were enclosed by a barrier or membrane to prevent dispersion of Zequanox during the exposure period. Zequanox was applied to achieve 150 mg AI/L near the benthic zone, with additional product added within 1–2 h to replace lost or diluted product. The barriers remained in place for 16–24 h after application. Turbidity (a measure of Zequanox concentration) at 24 h, before removal of the barrier, was reduced 50–80% (Meehan et al. 2014) and 75–90% (Whitley et al. 2015) below the peak treatment concentration. In the present study,

the concentration of Zequanox in treatment tanks decreased to control levels by 24 h. Additionally, the maximum concentrations of Zequanox that were maintained during the 8 h exposure were below the maximum allowable concentration (100 mg AI/L) for open water application (Figure 2). The duration and dose of Zequanox exposure tested in the present study may underestimate that to which fish would be exposed in an open water application. On the other hand, exposure to a single dose of Zequanox for more than 8 h may have negligible effect on toxicity to fish. Molloy et al. (2013a) reported that the potency of Zequanox decreases significantly within 8 h after wetted and degrades to control levels by 24 h at 20 °C. Molloy et al. (2013b) reported no treatment-related mortality of 3-month-old fathead minnows to live (100 mg AI/L, 72 h) or killed (50 mg AI/L, 24 h) cells of *P. fluorescens* in extended exposures at temperatures similar to those tested in the present study (21–23 °C).

Field efficacy trials with Zequanox achieved 75% (Meehan et al. 2014) to 90% mortality (Whitledge et al. 2015) of dreissenid mussels in the application areas. Luoma et al. (2015b) applied a single treatment of 50 and 100 mg AI/L Zequanox within lake enclosures to successfully reduce zebra mussel burden on native mussels by 53–68% without significant mortality to unionid mussels. The results of the aforementioned studies indicate that a single application of Zequanox is unlikely to eradicate dreissenids from a body of water. Depending on the management objectives for a water body, repeated Zequanox treatments may be required to maintain dreissenids below target densities. In such situations, the effects of multiple Zequanox treatments to fish and other nontarget organisms may need to be monitored when establishing an appropriate treatment schedule.

Field studies, combined with non-target safety data, can be used to identify uses for Zequanox in an integrated pest management program. For example, a potential application for Zequanox in fishery management is removal of dreissenid mussels from critical fish spawning shoals in open water. Spawning areas can be delineated and potentially enclosed to maintain effective concentrations for the exposure period. Although our results indicate that spawning activity and egg hatchability are not reduced by Zequanox exposure to fathead minnows, similar data are needed for those species that are more sensitive to Zequanox, such as salmonids, as well as for larval stages of more tolerant species. Consideration should be given to the time of year of Zequanox treatment to reduce exposure of spawning adults, sensitive stages, and species of concern.

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