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Research Article

Avoidance of cold-, cool-, and warm-water fishes to Zequanox® exposure

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Abstract

Zequanox® is a biopesticide registered by the U.S. Environmental Protection Agency (USEPA) and the Canadian Pest Management Regulatory Agency for controlling dreissenid mussels with demonstrated selective toxicity. However, some research has indicated that Zequanox may impact the body condition and survival of some non-target species. We assessed avoidance behaviors of two species of cold-, cool-, and warm-water fishes to Zequanox at the maximum concentration allowed by the USEPA label (100 mg/L as active ingredient). Naïve, juvenile fish (n = 30 per species) were individually observed in a two-flume choice tank through which Zequanox-treated and untreated water simultaneously flowed in an unobstructed arena. Individual fish were observed during an untreated control period (20 min) and two Zequanox-exposure periods (20 min each). Treatment was alternated between arena sides to account for potential side bias in the test subjects. Positional data were collected and tabulated in real time with EthoVision® XT software. Zequanox concentrations and water quality properties (pH, dissolved oxygen, temperature, and specific conductance) were monitored during each trial. Analysis of treatment response was performed using a contrast within linear mixed-effects models. Our results indicate that Brook Trout, Lake Trout, and Bluegill avoided Zequanox-treated water, Yellow Perch were indifferent to Zequanox-treated water, and Lake Sturgeon and Fathead Minnow were attracted to Zequanox-treated water. These results combined with existing species sensitivity literature may help inform resource managers of potential treatment-related risks.

Key words: non-target species, behavioral response, molluscicide avoidance, biopesticide

Introduction

Zequanox® is a biopesticide manufactured by Marrone Bio Innovations, Inc. (Davis, California) that is labeled for dreissenid mussel control in defined-discharge and open-water systems. Zequanox is a spray-dried powder containing 50% (w/w) killed *Pseudomonas fluorescens* (Strain CL145A) cells, a common soil bacterium, as the active ingredient. The molluscicidal action of Zequanox degrades the digestive tract of dreissenid mussels via a secondary metabolite produced by *P. fluorescens*, resulting in mussel death (Molloy et al. 2013b, c).

Considerable research has been done to understand the toxicity of Zequanox to dreissenid mussels and non-target organisms (Meehan et al. 2014b; Luoma et al. 2015, 2018a; Whitledge et al. 2015; Waller et al. 2016). Zequanox has demonstrated selectivity for dreissenid mussels over native organisms (Molloy et al. 2013a) however, much of this work focused on mortality and less on sublethal effects. Laboratory exposure of Lake Trout (*Salvelinus namaycush* Walbaum in Artedi, 1792) demonstrated sublethal effects including emaciation and hemorrhaging in addition to substantial delayed mortality (Luoma et al. 2018a). Temperature-dependent toxicity to zebra mussels (*Dreissena polymorpha* Pallas, 1771) has been noted, likely due to the influence of temperature on filtration rates and metabolism (Lei et al. 1996; Luoma et al. 2018b).

Zequanox has been used in zebra mussel eradication efforts, such as Christmas Lake (Hennepin County, Minnesota) where multiple toxicants were used between 2014 and 2015 in attempts to eradicate zebra mussels within temporary barriers (Lund et al. 2018). Successful eradication was reported within the Zequanox-treated area after 11 days; however, zebra mussels were later found outside of the treated area, resulting in the expansion of the treatment area and the use of different toxicants. Lund et al. (2018) also reported a decrease in dissolved oxygen during the treatment, from 7.8 mg/L to 0.1 mg/L, identifying a risk to non-target organisms. The first open-water application without containment barriers was performed in 2017 at Round Lake (Emmet County, Michigan; Luoma et al. 2019) and had limited success largely due to the barrier-free applications and subsequent mixing with surrounding lake water. Thus far, successful open-water Zequanox treatments have been partial-lake treatments involving containment barriers, which act to keep the molluscicide within designated treatment areas (Meehan et al. 2014a; Weber 2015; Whitledge et al. 2015; Luoma and Severson 2016; Lund et al. 2018).

Avoidance and attraction behaviors in fish are well studied. Methods and apparatuses, similar to those used in this study, have been used to investigate the effects of various environmental cues and toxicants (Bisson and Bilby 1982; Abreu et al. 2016; da Rosa et al. 2016). Jutfelt et al. (2017) used similar methods as we have when examining the avoidance of predatory cues. Some of the substances examined under similar conditions include pharmaceuticals, herbicides, pesticides, predatory cues, and suspended solids (Bisson and Bilby 1982; Morgan et al. 1991; Tierney 2016; da Rosa et al. 2016; Jutfelt et al. 2017). The overall concept is relatively simple; the test subject was provided with two choices, either swim in water to which Zequanox was applied or in the control water, while their residence time in both areas was observed and quantified.

Zequanox treatments greatly increase turbidity with the addition of suspended solids. Fish behavior and physiological stress reactions to turbidity and suspended solids are well studied (Newcombe and MacDonald

Table 1. Mean (standard deviation) and range of test organism wet weight and total length.

Species	Wet weight (g)		Total length (mm)	
	Mean (sd)	Range	Mean (sd)	Range
<i>Cold-water</i>				
Brook Trout	1.82 (0.478)	1.07–3.10	31 (4.5)	53–72
Lake Trout	2.89 (0.752)	1.91–4.67	74 (5.4)	67–86
<i>Cool-water</i>				
Lake Sturgeon	2.67 (0.733)	1.12–4.02	96 (9.3)	76–116
Yellow Perch	1.42 (0.337)	0.848–2.25	56 (4.0)	48–64
<i>Warm-water</i>				
Bluegill	2.71 (0.805)	1.14–4.13	57 (5.3)	45–65
Fathead Minnow	1.55 (0.373)	0.938–2.39	55 (4.0)	48–63

1991; Bilotta and Brazier 2008). Changes in turbidity drive different behaviors depending on species and life stage. In extreme cases, high concentrations of suspended solids have been associated with mortality in certain species (Kemp et al. 2011). The associated increase in suspended solids during a Zequanox treatment may affect aquatic organisms not well adapted to such stressors; however, the risks associated with Zequanox treatments to aquatic organisms would be diminished if sensitive species demonstrate avoidance.

Materials and methods

Test animals

Juvenile fish reared at the Upper Midwest Environmental Sciences Center (La Crosse, Wisconsin) were acclimated to test temperature at a rate of change ≤ 3 °C per day. Test fish were fed commercially available feed (frozen chironomid larvae for the Lake Sturgeon and trout and salmon starter diet for other species) to apparent satiation twice per day and the holding tanks were cleaned daily. Water quality properties (dissolved oxygen, pH, and temperature) were measured and recorded daily in the holding tanks. Test animals were indiscriminately selected daily for trials. After each trial, fish were euthanized in a solution of tricaine methanesulfonate (MS-222) and measured for total length and weight (Table 1).

Test system

A two-flume choice tank (Loligo Systems, Viborg, Denmark; Figure 1), equipped with valves to alternate treated sides within the tank and flow meters to maintain equal flow to both sides, was used to conduct behavioral trials. The choice tank was supplied with temperature-controlled well water from two aerated headboxes. A Zequanox stock for each test trial was made by mixing 315.0 g of Zequanox into 2.835 L of well water with an immersion blender. The stock was continuously stirred on a magnetic stir plate to maintain a homogeneous mixture, kept cool with a freezer pack, and was delivered to the treatment-designated headbox via a peristaltic pump. The stock was delivered at approximately 48 mL/min and adjusted

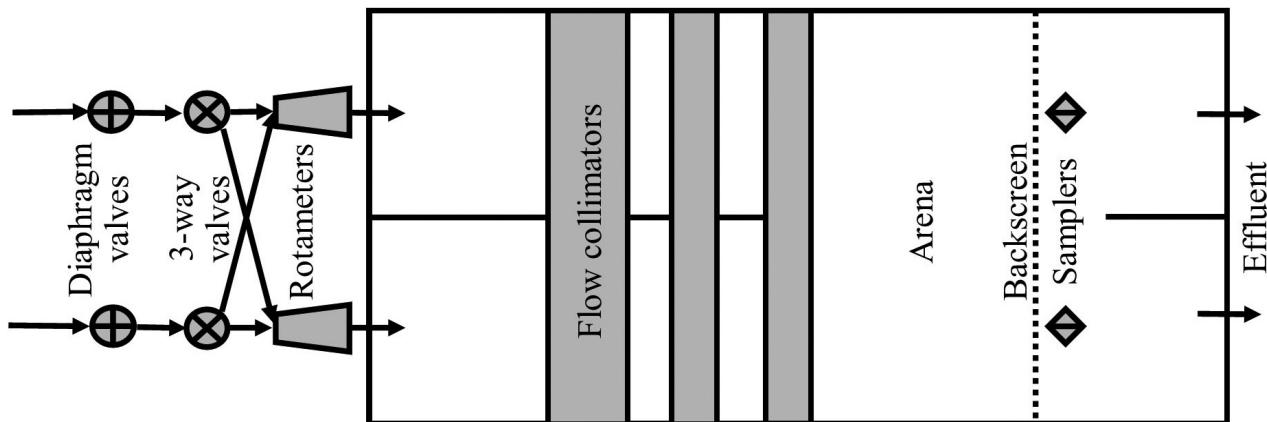


Figure 1. Overhead schematic of the avoidance system. Source water entered from the left and flow rates were controlled with diaphragm valves and monitored on side-specific rotameters. A cross-over system included a set of 3-way valves and plumbing to switch treatment sides during trials. Flow collimators of decreasing sizes on both sides of the choice tank delivered near laminar flow conditions to the arena. Fish were contained in the arena by the collimator upstream and by a backscreen on the downstream side. The samplers included a pH probe, dissolved oxygen probe, and peristaltic tubing to monitor test conditions from outside the curtain without disturbing the test individual.

as required to maintain the target concentration of 100 mg/L as active ingredient. An airstone aided mixing within the headbox. Flow to each side of the choice tank was maintained at approximately 25 L/min. A series of baffles upstream from the arena created near-laminar flow conditions which minimized mixing between the treated and untreated sides without the use of a barrier. The test arena was shortened from its original size to 16.0 × 38.5 × 10.0 cm (length × width × water depth) by moving the backscreen upstream to maintain a clear transition between the treated and untreated sides. A black-plastic curtain was installed surrounding the choice tank to minimize disturbance. Within the enclosure, a light source producing a natural spectrum was installed and controlled with a dimmer switch to maintain approximately 6 lux. A camera (Model: scA1300-60gm; Basler AG, Ahrensburg, Germany) equipped with varifocal lens (Model: H3Z4512CS-IR; Computar, Cary, North Carolina) was installed above the arena and linked to a computer running EthoVision XT software (Noldus Information Technology, Inc., Leesburg, Virginia). EthoVision XT software was used to track and tally fish location throughout the trials at a frequency of 7.5 frames per second.

Water quality meters (Model: HQ40d; Hach Company, Loveland, Colorado) were equipped with dissolved oxygen and pH probes that were suspended, along with peristaltic sampling lines, mid-water column in each side of the choice tank immediately downstream from the test arena. Dissolved oxygen and pH were measured in each side at the midpoint of each trial period (control and two treatment periods). A peristaltic pump located outside the curtain continuously sampled water through the lines suspended in the choice tank. Samples collected via peristaltic pump were used to measure specific conductance and Zequanox concentration at the midpoint of each treatment period. One trial per species ($n = 6$) was sampled at shorter intervals for Zequanox to understand the fluctuations in

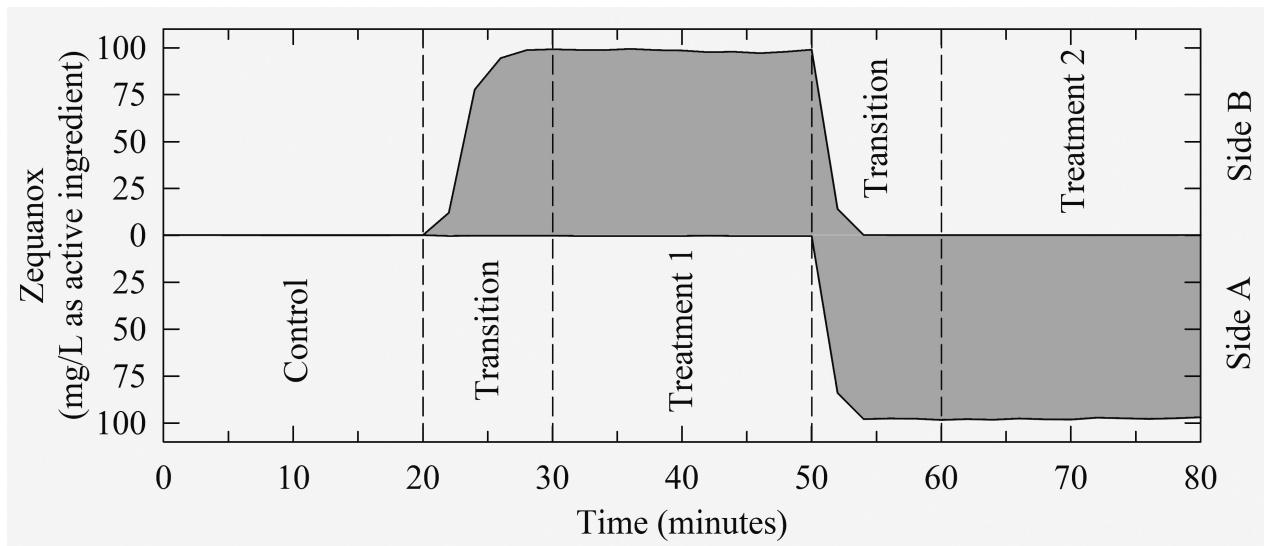


Figure 2. Representative trial format for showing side B (above centerline) treated first. Zequanox concentrations in mg/L as active ingredient (shaded area) for each side of the choice tank. One trial per species ($n = 6$) was sampled every 5 minutes during the control period and every 2 minutes thereafter. The control period was preceded by a 10-minute acclimation period.

concentration throughout the trial. All Zequanox concentrations were determined by comparison to a standard curve, generated daily from concentration standards bracketing the test concentration. Concentrations were determined with a spectrophotometer (Model: DR3900; Hach Company, Loveland, Colorado) measuring light absorption at a wavelength of 660 nm across a 2.54 cm light-path. Specific conductance was also measured in the samples with a conductivity meter (Model: AP75; Fisher Scientific, Waltham, Massachusetts). Temperature was measured from the effluent of both sides. The system was flushed with clean water for a minimum of 30 min between each trial. Water from each headbox was tested daily for alkalinity and total hardness throughout the study.

Experimental design

Six fish species were tested at three temperatures, consisting of two cold-water species, (Brook Trout (*Salvelinus fontinalis* Mitchell, 1814) and Lake Trout; 12 °C), two cool-water species (Lake Sturgeon (*Acipenser fulvescens* Rafinesque, 1817) and Yellow Perch (*Perca flavescens* Mitchell, 1814); 17 °C), and two warm-water species (Bluegill (*Lepomis macrochirus* Rafinesque, 1819) and Fathead Minnow (*Pimephales promelas* Rafinesque, 1820); 22 °C). For each species, 30 fish were individually subjected to a 90 min trial. Each trial consisted of a 20 min control period and two, side-specific 20 min treatment periods, each preceded by a 10 min transition period; this format was modeled closely after suggestions by Jutfelt et al. (2017; Figure 2). The side of the choice tank occupied by the fish at the end of the control period was selected as the side to be treated first. Zequanox delivery was initiated at the end of the control period and treated sides were switched between the treatment periods to reduce the effects of side bias.

Data analysis

Individual fish served as the experimental units for all analysis. Effect sizes were estimated with a 95% confidence interval (CI). The arena side choice by fish during each period (control and the two treatment periods) were compared. The proportion of time in avoidance of Zequanox-treated water for each individual was analyzed with a generalized linear mixed model in statistical software R version 3.6.1 with *lme4::lmer* (Bates et al. 2015; R Core Team 2019). The proportion of time spent on each side of the arena was determined by dividing the number of detections (binomial; presence or absence, 1 or 0, respectively) by the total number of observations. Additionally, the number of side changes per exposure period was tallied for each individual and summarized by species to compare activity in response to Zequanox exposures.

The two sides of the tank were identified as A and B for the sole purpose of assigning treatment and assessing behavioral response. The response variable for each model is the proportion of time in avoidance. Avoidance refers to the proportion of time in the untreated side (i.e. side B when the treatment is on side A or side A when the treatment is on side B). For the control period, avoidance is defined as the proportion of time the fish was present in the side that was treated first. Setting the control avoidance in this manner conservatively biases the proportion of time in avoidance higher for the control setting since the Zequanox was introduced to the side the individual was in at the end of the control period.

A separate model was fit for each fish species in which the experimental condition (i.e. control, side A treated, or side B treated) was the primary “within” subject predictor variable and individual fish were the random subjects. Treatment order within each trial, either side A or side B treated first, was used as a block variable. Estimated effect size for each species was computed using a contrast of mean avoidance during treatment periods to the “avoidance” during the control period (Equation 1).

$$x = \frac{(A+B)}{2} - C \quad \text{Equation 1}$$

Where, x = effect size for individual

A = proportion of time in avoidance during side A treatment

B = proportion of time in avoidance during side B treatment

C = proportion of time in the first treated side during the control period

Results

Dissolved oxygen remained above recommended concentrations for each temperature group for all trials (Table 2; Tidwell 2012). Specific conductance was slightly higher in the Zequanox-treated water (mean: 413 µS/cm at 25 °C) when compared to the untreated water (396 µS/cm at 25 °C). Alkalinity and

Table 2. Zequanox concentrations and water quality properties for avoidance trials in the treated and untreated sides by test temperature.

Trial Temperature (°C)	Treated			Untreated		
	Mean	Min.	Max.	Mean	Min.	Max.
Zequanox (mg/L as active ingredient)						
12 (Cold)	95.930	74.210	108.71	0.216	0	2.310
17 (Cool)	99.936	90.042	109.56	0.179	0	0.622
22 (Warm)	99.053	90.516	107.59	0.251	0	0.685
Dissolved oxygen (mg/L)						
12	9.36	9.05	9.64	9.44	8.96	9.72
17	8.24	6.32	9.04	8.81	8.09	9.18
22	7.12	4.21	8.22	7.91	6.70	8.40
pH						
12	7.78	7.68	7.92	7.92	7.81	8.09
17	7.75	7.66	7.90	7.88	7.79	8.08
22	7.76	7.67	8.02	7.88	7.78	8.03
Specific conductance ($\mu\text{S}/\text{cm}$ at 25 °C)						
12	412	362	459	395	342	440
17	411	387	439	392	364	425
22	417	397	434	400	376	436

total hardness were similar throughout all trials, averaging 150 mg/L as CaCO_3 and 195 mg/L as CaCO_3 , respectively. No differences in pH were detected between the treated and untreated sides, although the pH in the treated side was slightly lower. Zequanox concentrations in the treated side averaged 95.4, 99.9, and 99.1 mg/L as active ingredient in the 12, 17, and 22 °C tests, respectively. The treated and untreated flumes were well separated as indicated by negligible Zequanox concentrations in the untreated side which averaged 0.2, 0.2, and 0.3 mg/L as active ingredient in the 12, 17, and 22 °C trials, respectively. For all trials, the maximum observed Zequanox concentration in the untreated sides was 2.3 mg/L as active ingredient.

Three types of behavioral responses were detected: avoidance, attraction, and indifference (Figure 3). Compared to the control periods, there was an increase in the proportion of time in avoidance for Lake Trout (95% CI: 0.20 to 0.29), Bluegill (95% CI: 0.19 to 0.27), and Brook Trout (95% CI: 0.34 to 0.43) when the Zequanox was present. Fathead Minnow and Lake Sturgeon exhibited decreased avoidance (i.e. attraction) to the Zequanox treatment compared to the control periods (95% CIs: -0.45 to -0.75 and -0.23 to -0.52, respectively). Yellow Perch behavior was indifferent between the control and treatment periods (95% CI: -0.15 to 0.13).

The number of side changes for each trial period were also tallied and compared (Table 3). There was a noticeable decline in the number of side changes for the trout species during the exposure periods when compared to the control period. In contrast, the other species tested did not exhibit as large a change in activity between the control and exposure periods. A high degree of variation within many of the species indicates a wide range of responses regarding the number of times individuals entered or challenged the Zequanox treated water.

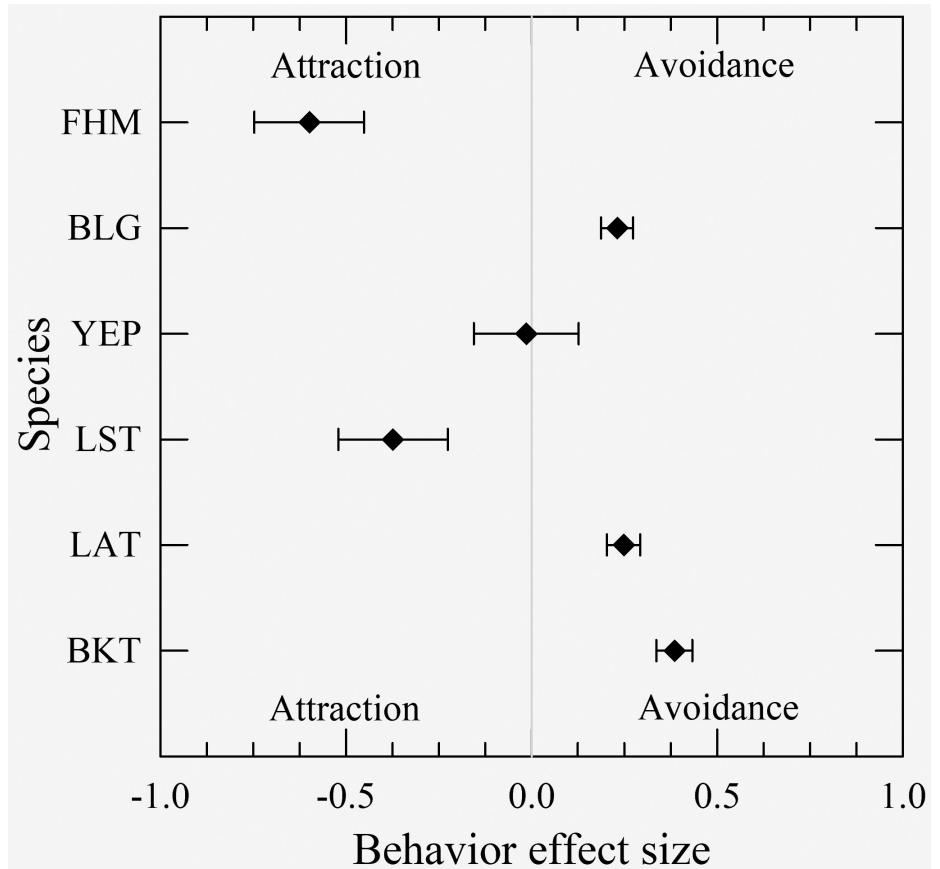


Figure 3. Comparison of behavior effect sizes and confidence intervals by species assessing the response to Zequanox exposure in a two-flume choice tank. Species are Fathead Minnow (FHM), Bluegill (BLG), Yellow Perch (YEP), Lake Sturgeon (LST), Lake Trout (LAT), and Brook Trout (BKT). Diamonds represent the species' mean behavior effect sizes and the error bars represent the 95% confidence interval. Negative values for the behavior effect sizes are considered an attraction response and positive values are considered an avoidance response.

Table 3. Mean (standard deviation) number of side changes by species during avoidance trials ($n = 30/\text{species}$).

Species	Control period	Exposure period	
		Period 1	Period 2
<i>Cold-water</i>			
Brook Trout	101.9 (36.2)	42.1 (24.6)	39.8 (18.1)
Lake Trout	120.9 (69.0)	83.7 (36.2)	83.7 (35.9)
<i>Cool-water</i>			
Lake Sturgeon	30.8 (37.9)	40.1 (50.6)	29.7 (40.5)
Yellow Perch	18.7 (25.0)	10.6 (15.9)	23.1 (20.2)
<i>Warm-water</i>			
Bluegill	21.3 (12.6)	35.6 (11.5)	30.9 (10.5)
Fathead Minnow	24.3 (32.9)	21.9 (36.2)	18.5 (32.6)

Discussion

Zequanox sensitivity may be a predictor in the avoidance behavior of fish exposed to Zequanox-treated water. Lake Trout were shown to be sensitive to Zequanox in a study by Luoma et al. (2018a) and they exhibited the strongest avoidance behavior in our study. The same pattern is true for Brook Trout; Luoma et al. (2015) calculated an 24h-LC₅₀ for Brook Trout of 104.6 mg/L, near the 100 mg/L maximum active ingredient concentration

allowed per the product label, and they also exhibited an avoidance behavior (Marrone Bio Innovations 2015). In contrast, Fathead Minnow exhibited an attraction to Zequanox-treated water and they have been shown to be resilient to Zequanox exposure at several life stages (Waller and Luoma 2017). Juvenile Lake Sturgeon were also noted as being resilient to Zequanox exposure by Luoma et al. (2018a) and they also exhibited an attraction response in this study. As an exception, Bluegill exhibited an avoidance response to Zequanox exposure and have been shown to be tolerant to Zequanox exposure (Luoma et al. 2015). However, Bluegill activity (i.e. the number of side changes) increased slightly during the exposure periods compared to the control indicating that the fish were actively venturing into the turbid Zequanox treatment. Yellow Perch were the only species tested to be indifferent to Zequanox exposure. Wellington et al. (2010) reported that increased turbidity had no effect on Yellow Perch prey consumption. Yellow Perch indifference to Zequanox-treated water may be rooted in their ability to function in both clear and turbid water in addition to their tolerance to Zequanox exposure (Wellington et al. 2010; Luoma et al. 2015). Support for this hypothesis includes the lack of differences in the number of side changes during treated and untreated conditions, indicating that Yellow Perch moved throughout the arena in similar fashions with or without Zequanox being present. These observations lead us to infer that Zequanox-sensitive species may avoid treated waters; thereby, reducing the risk to the more vulnerable species.

Elevated suspended solids can produce physiological stress for fishes including clogging and abrading gill tissues which can be exploited by opportunistic pathogens, disrupt osmotic function, and decrease dissolved gas exchange (Sutherland and Meyer 2007; Bilotta and Brazier 2008; Kemp et al. 2011). Although the effects of turbidity and suspended solids related to a Zequanox treatment on fish health are unknown, they are likely minimal in comparison to chronic or extreme exposures to suspended solids because the Zequanox label restricts open-water applications to a maximum of 8 hours in a 2-week period (Marrone Bio Innovations 2015).

The ability of sensitive species to detect and avoid Zequanox, either as a reaction to chemosensory cues, turbidity cues, or both, provides some insight for resource managers as they evaluate treatment-related risks. The attraction behavior of Fathead Minnow and Lake Sturgeon do not necessarily equate to an increased risk since these species have been found to be resilient to Zequanox exposure (Waller and Luoma 2017; Luoma et al. 2018a). The avoidance of Zequanox-treated water by sensitive species such as Lake Trout and Brook Trout could reduce the effect to these species if there is an avenue of egress from the treatment area.

Behavioral drivers such as spawning, foraging, and seasonal migrations warrant consideration when planning a Zequanox treatment. Smaller-bodied species and juveniles are known to use turbidity as refugia from

predation (Bisson and Bilby 1982; Utne-Palm 2002). Further, Fathead Minnow have been shown to alter their behavior in association with turbidity based on food availability and predatory pressures (Chiu and Abrahams 2010). Brook Trout have been shown to alter the utilization of habitat in turbid conditions by abandoning overhead cover and decreasing their association with the bottom (Gradall and Swenson 1982). A thorough understanding of the fish community and habitats within a waterbody being considered for treatment would further delineate potential treatment-related risk. Additional mitigation steps to reduce risk to fish species could include altering treatment timing and limiting treatment area to provide refugia outside the treatment.

In conclusion, our data indicate that sensitive fish species may avoid Zequanox treatments, which may reduce the overall risk to these species. Pairing avoidance data with toxicological data and a thorough understanding of fish community structure and behavior patterns may assist resource managers in the development of treatment risk assessments and treatment strategies.

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Ethics and permits

The study protocol was reviewed and approved, prior to conducting research, by the Animal Care and Use Committee at the Upper Midwest Environmental Sciences Center. Authors and researchers affiliated with this project complied with the Animal Welfare Act (7 U.S.C. 2131 et seq.) and with the protocols governing the use of test animals at the Upper Midwest Environmental Sciences Center.

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