Development of Targeted Delivery Techniques for Zequanox®

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Abstract

The effects of water temperature and concentration on the physical characteristics of Zequanox®, a dead-cell spray-dried powder formulation of *Pseudomonas fluorescens* (strain CL145A) used for controlling invasive dreissenid mussels (zebra mussel, *Dreissena polymorpha*, and quagga mussel, *Dreissena bugensis*), were investigated to determine optimal temperature-specific concentrations and delivery techniques for use during open-water subsurface Zequanox applications. Temperature-controlled laboratory tests evaluated viscosity, settling, stratification, and buoyancy of various concentrations of Zequanox suspension in water to select an optimal target viscosity for Zequanox applications. A two-step linear regression procedure was used to create a temperature-specific Zequanox prediction model from the viscosity data. The prediction model and subsurface application techniques were validated by conducting three independent outdoor pond trials at temperatures of ~9, 14, and 20°C. During these outdoor trials, subsurface applications of Zequanox at concentrations predicted by the model were performed and water samples were collected at varying depths and analyzed via spectroscopy to determine Zequanox concentration and dispersion. Although the predicted Zequanox concentrations and delivery techniques used resulted in successfully maintaining lethal Zequanox concentrations in the bottom 7.5 cm of the water column for the duration of the exposure, a revised prediction model is also provided for more accurately selecting temperature-specific Zequanox concentrations.

Introduction

Dreissenid mussels, including the zebra mussel, *Dreissena polymorpha*, and the quagga mussel, *Dreissena bugensis*, are bivalves native to the Black, Caspian, and Azov Seas of the Ponto-Caspian region of Eurasia (Spidle et al., 1994; Gollasch and Leppäkoski, 1999). Invasive zebra mussels and quagga mussels were introduced to North America in the mid to late 1980s (Roberts, 1990; Spidle et al., 1994), likely as free swimming veligers discharged in ballast waters of oceanic freight ships (Griffiths et al., 1991). Adult zebra mussels were first discovered in 1988 on Lake St. Clair (Hebert et al., 1989) and they have continued a rapid invasion throughout North American waterways. Dreissenid mussels are highly efficient invaders because of their high reproductive fecundity, planktonic larval dispersal, ability to attach to most surfaces by the use of byssal threads (Birnbaum, 2011), lack of major ecological constraints, and new infestations being aided by anthropogenic means (Gollasch and Leppäkoski, 1999; Ludyanskiy et al., 1993). Severe biofouling is an economically harmful characteristic of dreissenid

[1] U.S. Geological Survey.

mussels that has been responsible for significant financial costs to many industries located on infested waterways (Ludyanskiy et al., 1993). Epizoic colonization of already imperiled native unionid species is one of the greatest ecological effects of dreissenid mussels (Hebert et al., 1989; Schloesser et al., 1996). Currently, eradication of established populations of dreissenid mussels in large systems is not feasible, but implementation of an Integrated Pest Management (IPM) program that combines physical, mechanical, biological, chemical, and cultural control mechanisms could be used to decrease the ecological and economic impacts of this invader (Culver et al., 2013). One product that could be incorporated into an IPM program for controlling dreissenid mussels is Zequanox®, a dead-cell spray-dried powder formulation of *Pseudomonas fluorescens* (strain CL145A). The New York State Museum Field Research Laboratory first isolated *Pseudomonas fluorescens*, strain CL145A, and discovered that when ingested by dreissenid mussels, it induces mortality by degrading the epithelial cells within the mussel's digestive system (Molloy et al., 2013). Marrone Bio Innovations (Davis, CA) acquired the rights to *Pseudomonas fluorescens*, strain CL145A, and developed the product, Zequanox, which was registered by the U.S. Environmental Protection Agency for controlling dreissenid mussels in defined discharge water systems in 2012 and for open-water use in 2014 (EPA Reg. No. 84059-15). Previous research investigated the use of Zequanox in laboratory and field settings for dreissenid mussel control (Luoma et al., 2015a; Luoma et al., 2015c) and non-target animal exposure-related impacts (Luoma et al., 2015b; Luoma et al., 2015d). The work conducted by Luoma et al. (2015a) demonstrated the potential for subsurface Zequanox applications by conducting successful applications in 350-L tanks; however, the effects of temperature and concentration on the dispersion of Zequanox suspensions were not investigated in that study.

Subsurface Zequanox applications are desirable because they would (1) significantly reduce the amount of Zequanox required for treatment, subsequently reducing cost; (2) decrease the potential for exposure, reducing risk to non-target species; and (3) decrease nutrient input inherently related to the Zequanox product. Fundamental challenges related to subsurface Zequanox applications include the potential for premature Zequanox migration/dilution and the significant impact of water temperature on the viscosity of Zequanox suspensions. Therefore, the present study was conducted in an attempt to create a standardized procedure for selecting the appropriate concentration of Zequanox in suspensions created for subsurface applications over a range of water temperatures. This included conducting a series of temperature-controlled laboratory tests that evaluated viscosity, settling, stratification, and buoyancy of various concentrations of Zequanox at different water temperatures. Results of these laboratory tests were used to estimate the optimal Zequanox suspension viscosity for subsurface application at a range of environmental temperatures. The laboratory-derived, temperature-dependent Zequanox concentration selection protocol was then evaluated by conducting a series of outdoor tests at three different environmental temperatures.

Study Overview

Laboratory

Laboratory tests were conducted in an environment-controlled chamber at four temperatures (7, 12, 17, and 22°C). At each temperature, four Zequanox suspensions (ranging from 5–25% w/v) were evaluated to determine (1) the effects of temperature and (2) the effects of Zequanox concentration on the viscosity, settling, stratification, and buoyancy of Zequanox suspensions. The Zequanox suspensions were prepared in well water by mixing with a household immersion blender and the viscosity of the suspensions were measured using cup viscometers.

Zequanox suspensions were injected with 1-mL syringes into a series of twelve 100-mL graduated cylinders (four Zequanox suspensions X three replicates) and initial observations of air entrainment were followed by observations of settling and stratification throughout the 8-hour exposure period. The laboratory observations were used to determine a target Zequanox suspension viscosity for subsurface applications. A two-step linear regression procedure was used to predict temperature-concentration combinations that would result in the target Zequanox suspension viscosity at water temperatures ranging from 7 to 22°C.

Pond

The laboratory-derived, temperature-concentration prediction model was verified by conducting a series of replicated outdoor pond tests at three water temperatures (~9, 14, and 20°C). Zequanox suspension concentrations were selected from the prediction model and the prepared suspensions were applied to three replicated 9-m^2 test enclosures that were placed in 0.004-ha concrete ponds. Water samples were collected from three depths (7.5, 30, and 60 cm from pond bottom) in each enclosure 1, 2, 4, and 8 hours after Zequanox application and analyzed via spectroscopy to determine Zequanox concentrations. Zequanox concentrations were compared by sampling depth using a General Linear Model procedure in SAS version 9.3.

Materials and Methods

Test Article

The test article was Zequanox®, a commercially available spray-dried powder formulation of *Pseudomonas fluorescens*, strain CL145A (*Pf*-CL145A) produced by Marrone Bio Innovations (Davis, CA). Zequanox is formulated to contain 50% weight to weight *Pf*-CL145A as the active ingredient (A.I.). The test article, lot number 401P130918C, was expired for use in dreissenid mussel control applications; however, the physical characteristics of the test article are not affected by biological activity and therefore the test article was deemed acceptable for use in this study (Megan Weber, Zequanox Product Development Manager at MBI, pers. comm., 2014). Concentrations of test article in suspensions are reported as % weight to volume (w/v) and spectroscopy measurements were conducted and are reported based on mg A.I./L.

Test Systems

Laboratory

Laboratory tests were conducted within a 22-m^2 environmental chamber at the Upper Midwest Environmental Sciences Center (UMESC), which maintained the temperature ±1°C. Twelve 100-mL polymethylpentene graduated cylinders (Fisher Scientific, Hampton, NH, cat. no. 03-007-33) filled with temperature-appropriate well water were used as the observation system. The test article application system consisted of disposable 1-mL syringes (Becton, Dickinson and Co., Franklin Lakes, NJ, mfr. no. BD309628) held at a fixed height in the water column by positioning the syringe in an acrylic mounting block, which was placed on top of the graduated cylinders (Fig. 1).

Viscosities of Zequanox suspensions were measured with cup viscometers (Cole-Parmer Viscosity Cups #1, 3, 4, and 5, Cole-Parmer Instrument Co., Vernon Hills, IL; EZ Zahn Viscosity Cup #2, Gardco, Pompano Beach, FL).

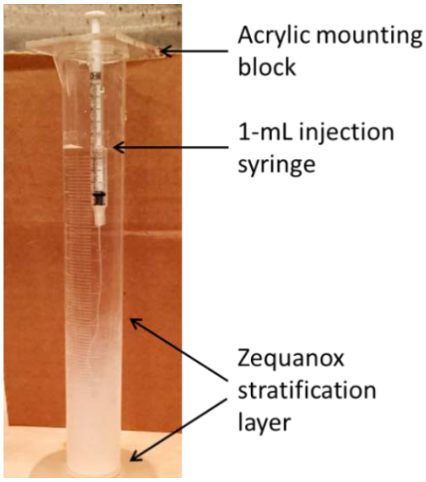

Figure 1. An example of laboratory test system replicate showing Zequanox stratification.

Pond

Pond tests were conducted in replicate 9-m^2 test enclosures positioned in independent 0.004-ha concrete ponds (Fig. 2a). The enclosures were assembled by interconnecting four welded aluminum frame panels (3.0 x 1.8 m, L x H) that were covered with an impermeable 30-mil ethylene propylene diene monomer (EPDM) pond liner membrane. Panels were constructed with 0.3-m EPDM bottom-sealing skirts that contained ballast chain and each assembled enclosure had multiple sand bags (n = 16, ~12 kg each) placed on the skirts to aid in creating the bottom seal.

Water sampling systems were constructed for each enclosure and consisted of peristaltic pumps fitted with dual channel pump heads (Masterflex Digi-Staltic pump drive, model 77310-01 and Easy-Load II, model 77202-60, respectively, Cole-Parmer Instrument Co., Vernon Hills, IL), peristaltic tubing (Masterflex Silicone Tubing L/S 16, item no. EW-96400-16, Cole-Parmer Instrument Co., Vernon Hills, IL), vinyl airline tubing (4.8-mm inside diameter [ID]), and positioning rods (3 vertical aluminum rods [0.95-cm diameter] welded to a horizontal 1.5-m piece of aluminum angle [5.1 x 5.1 cm]) that were used to secure the tubing at the desired sampling locations and depths (7.5, 30, and 60 cm from bottom) for a total of nine sampling locations within each pond (Fig. 2b).

Zequanox delivery systems, with similar construction to water sampling systems, were used to apply Zequanox to each enclosure replicate. Peristaltic pumps were calibrated to deliver 40 mL/minute/tube and used to deliver Zequanox to 16 locations in each enclosure (Fig. 2b). The Zequanox was pumped through tubing that terminated 90 cm from pond bottom with T-shaped hose barb fittings mounted to aluminum positioning rods (four stands, each constructed from four vertical aluminum rods welded to a horizontal 1.8-m length of aluminum angle).

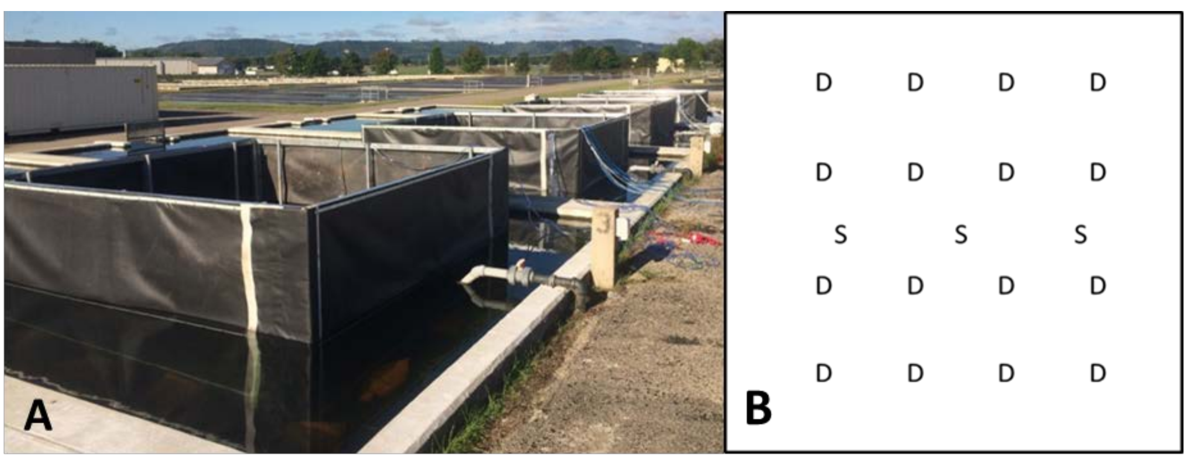

Figure 2. Test enclosures in outdoor concrete pond complex (A, left); plan view of treatment enclosure with delivery (D) and sampling (S) apparatus placement (B, right). Each delivery point terminates 90 cm from pond bottom, whereas each sampling point terminates 7.5, 30, and 60 cm from pond bottom.

Test Article Applications, Observations, and Data Analysis

Laboratory

At each of four test temperatures, a series of twelve 100-mL graduated cylinders were filled with 100 mL of temperature-acclimated well water and labeled by Zequanox concentration and replicate number (n = 4 Zequanox suspensions X 3 replicates). Four suspensions, with Zequanox concentrations ranging from 5 to 25% (w/v), were prepared in 500-mL batches by mixing Zequanox and well water with an immersion blender for one minute. Equivalent masses of Zequanox from each suspension (ranging in volume from 0.25 to 1.00 mL) were drawn into 1-mL syringes and placed on top of the graduated cylinders using an acrylic mounting block. The mounting blocks secured the syringes in a fixed position, with the tip of the syringe terminating at the 80-mL graduation mark. Syringe plungers were depressed at a consistent rate to administer Zequanox into the graduated cylinders.

Observations

Prior to application, viscosities of prepared Zequanox suspensions were measured in triplicate using an appropriate-sized cup viscometer to obtain efflux time in Zahn seconds, which was then converted to viscosity in centistokes (cSt) using conversion equations provided by the viscometer manufacturer. Buoyancy upon initial application, followed by settling and depth of stratification layer observations were also made 1, 2, 4, and 8 hours after application. Stratification layers were observed using mL graduations on the cylinders and were converted into distance with a digital caliper, in mm, and also into percentage of water column.

Water temperature, pH, hardness, and alkalinity were measured in triplicate on water samples collected prior to Zequanox application following internal UMESC protocols (UMESC SOPs AEH 186, 712, and 706). Data analyses for water chemistry were limited to simple summary statistics; comparative statistics were not generated. Stratification and settling observations were numerically ranked based on defined data ranges and were used to determine a target Zequanox suspension viscosity for subsurface applications.

Qualitative stratification and settling observations were numerically ranked (Table 1), assessed, and used, in combination with buoyancy observations, to estimate an acceptable target Zequanox suspension viscosity for subsurface applications of 180 cSt.

Two-Step Linear Regression Prediction Model

A two-step linear regression procedure was used to predict temperature-concentration combinations that would result in Zequanox suspensions with a viscosity of 180 cSt at water temperatures ranging from 7 to 22°C. The first step involved plotting viscosity data versus Zequanox suspension concentrations from each test temperature and then fitting linear regressions. With the exception of the 12°C trial, viscosity measurements ≥ 575 cSt were excluded when creating these temperature-specific linear regression models. Inclusion of a 575-cSt observation, from the 12°C test, was required to maintain at least three data points for the creation of each model. The second step involved selecting Zequanox concentrations corresponding to a viscosity of 180 cSt at each test temperature. These data were then plotted and a second linear regression model was created and used to predict the concentration of Zequanox, which would result in a suspension with a viscosity of 180 cSt for any water temperature ranging from 7 to 22°C.

Table 1. Numerical ranking of qualitative Zequanox settling observations and stratification layer data.

[Numerical rankings for settling: heavy (0), medium/heavy (1), medium (2), light (3), and very light (4); numerical rankings for stratification layer: 0 to 5% (0), 6 to 25% (1), 26 to 50% (2), 51 to 75% (3), 76 to 100% (4)]

Zequanox concentration (% w/v)	Mean numerical ranking		Zequanox concentration (% w/v)	Mean numerical ranking	
	Settling	Stratification layer		Settling	Stratification layer
	7ºC			17ºC	
5	1.0	2.0	5	3.0	2.8
7.5	0.0	0.4	10	0.0	1.8
10	0.0	0.0	15	0.0	1.2
12.5[1]	0.0	0.8	20[1]	0.0	0.0
	12ºC			22ºC	
5	2.0	2.2	10	4.0	3.0
10	0.0	0.0	15	1.0	2.6
15[1]	0.0	0.0	20[1]	0.0	2.6
20[1]	0.0	0.4	25[1]	0.0	1.0

[1]In all replicates, suspensions were observed to float upon application due to air entrainment; these concentrations were considered unsuitable when selecting optimal Zequanox concentration and viscosity.

Pond

Prior to initiation of pond trials, a non-replicated scale-up test of the pond delivery system was conducted in a 350-L laboratory test tank. During this testing, Zequanox suspensions previously observed to be non-buoyant during laboratory testing were found to be buoyant, and as a result, premature mixing within the water column was observed. Air entrainment during suspension preparation was speculated to be the cause for buoyancy. Therefore, a silicone-based aquaculture defoaming agent (Proline Foam Eliminator, Pentair Aquatic Eco-Systems Inc., Apopka, FL) was added to all prepared suspensions at 0.1% (v/v).

For each of the three outdoor pond trials, the ponds were filled with well water and allowed to acclimate to temperatures of ~9, 14, or 22°C for a minimum of 48 hours prior to Zequanox application. Three treatment ponds and one control pond were randomly assigned to the four enclosures for each temperature trial. On each trial day, water temperature was measured and the laboratory-derived temperature-concentration model was used to predict the concentration of Zequanox required for a suspension to achieve the target viscosity of 180 cSt. The predicted concentrations of Zequanox ranged from 7.8 (9°C) to 15.2% w/v (22°C). For each treated enclosure, the appropriate volume Zequanox suspension required to treat the water in the bottom 60 cm of the test enclosure at 100 mg A.I./L was prepared by mixing Zequanox into pond water with an immersion blender for ~90 seconds and approximately 15 minutes after preparation, the viscosity of each suspension was measured in triplicate as previously described. Zequanox suspensions were then applied to the test enclosures at a rate of 40 mL/minute through each of the 16 delivery tubes. Care was taken to bleed the application lines of air prior to beginning the Zequanox applications and to prevent air from entering the application lines during the application process. The time required for application ranged from ~15 to 28 minutes per enclosure because the volume of Zequanox suspension required to achieve the target concentration of 100 mg A.I./L varied for each temperature.

Concentration Verification and Water Chemistry

Water sampling tubing was flushed for several minutes immediately prior to sample collection. Water samples were drawn through all nine sampling tubes in an enclosure simultaneously, resulting in triplicate samples being collected from each depth (7.5, 30, and 60 cm from bottom). Samples were pooled by depth and then analyzed for concentration via spectroscopy by comparing the absorbances of samples to a linear regression created from known concentrations of Zequanox A.I. (50, 100, 200, and 300 mg A.I./L) using a Beckman DU Series 800 spectrophotometer at 660 nm. A General Linear Model (SAS Version 9.3, SAS Institute, Inc., Cary, NC) was used to compare Zequanox concentrations by temperature at various depth combinations (7.5 cm only; 7.5 and 30 cm combined; and 7.5, 30, and 60 cm combined).

Water temperature, pH, dissolved oxygen, hardness, and alkalinity were measured in each test enclosure prior to Zequanox application following internal UMESC protocols (UMESC SOP AEH 186, 304, 712, and 706). Surface water temperature was measured in each test enclosure at 1, 4, and 8 hours. To prevent mixing of the stratified Zequanox into the water column, pH and dissolved oxygen were measured near the bottom of the water column upon termination (8 hours). Data analyses for water chemistry were limited to simple summary statistics; comparative statistics were not generated.

Results and Discussion

Laboratory

Mean water quality parameters (pH, temperature, hardness, and alkalinity) for the laboratory trials are summarized in Table 2. For all four laboratory trials, water hardness and alkalinity ranged from 187 to 190 mg/L and 141 to 146 mg/L, respectively, and pH ranged from 7.80 to 8.01. Viscosities of the Zequanox suspensions are summarized by test temperature in Table 3. The first-step temperature-specific viscosity regression models and the second-step temperature-concentration prediction models are displayed in Figs. 3 and 4. The correlation coefficients of the first-step regression models ranged from 0.929 to 0.987 (Fig. 3) and the correlation coefficient of the second-step regression model was 0.83 (Fig. 4).

Table 2. Water quality parameters observed in the laboratory trials.

	Mean water chemistry parameter (SD)			
Target temperature (ºC)	Observed temperature (ºC)	pH[1] (standard units)	Hardness (mg/L as $CaCO_3$)	Alkalinity (mg/L as $CaCO_3$)
7	7.2 (0.0)	7.90 (0.01)	189 (1)	144 (1)
12	12.2 (0.0)	7.94 (0.02)	187 (0)	144 (1)
17	17.0 (0.1)	7.80 (0.01)	188 (1)	141 (1)
22	21.6 (0.1)	8.00 (0.01)	189 (1)	146 (1)

[1]pH values were log transformed prior to calculating mean values; standard deviations were calculated from observed pH values.

Table 3. Mean viscosity of Zequanox suspensions prepared during the laboratory trials.

Zequanox concentration (% w/v)	Mean viscosity in centistokes (SD)	Zequanox concentration (% w/v)	Mean viscosity in centistokes (SD)
7ºC		17ºC	
5	10 (1)	5	4 (0)
7.5	110 (4)	10	83 (2)
10	255 (7)	15	316 (9)
12.5	1058 (23)	20	583 (27)
12ºC		22ºC	
5	8 (1)	10	13 (1)
10	170 (5)	15	58 (2)
15	583 (35)	20	236 (7)
20	>1725[1]	25	454 (9)

[1]Over range of #5 viscometer cup.

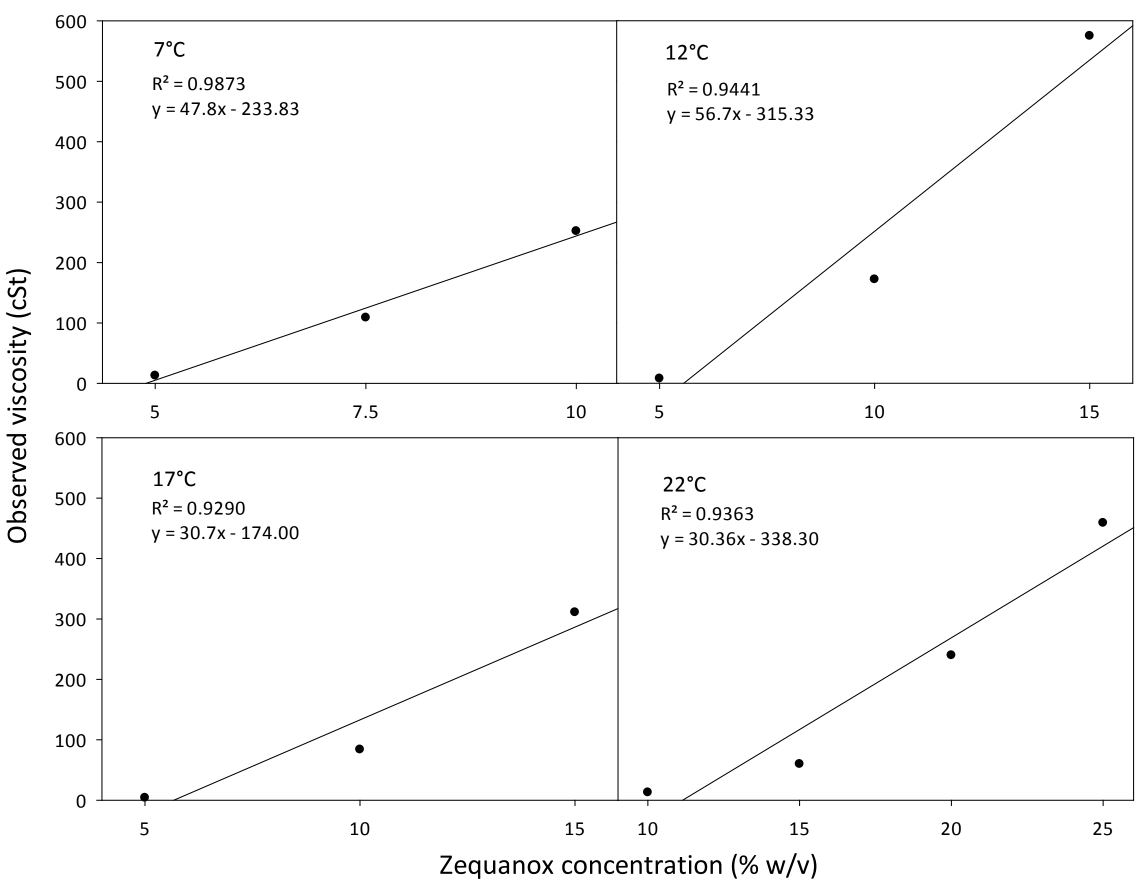

Figure 3. First-step linear regressions plotting Zequanox concentrations and viscosities from laboratory tests.

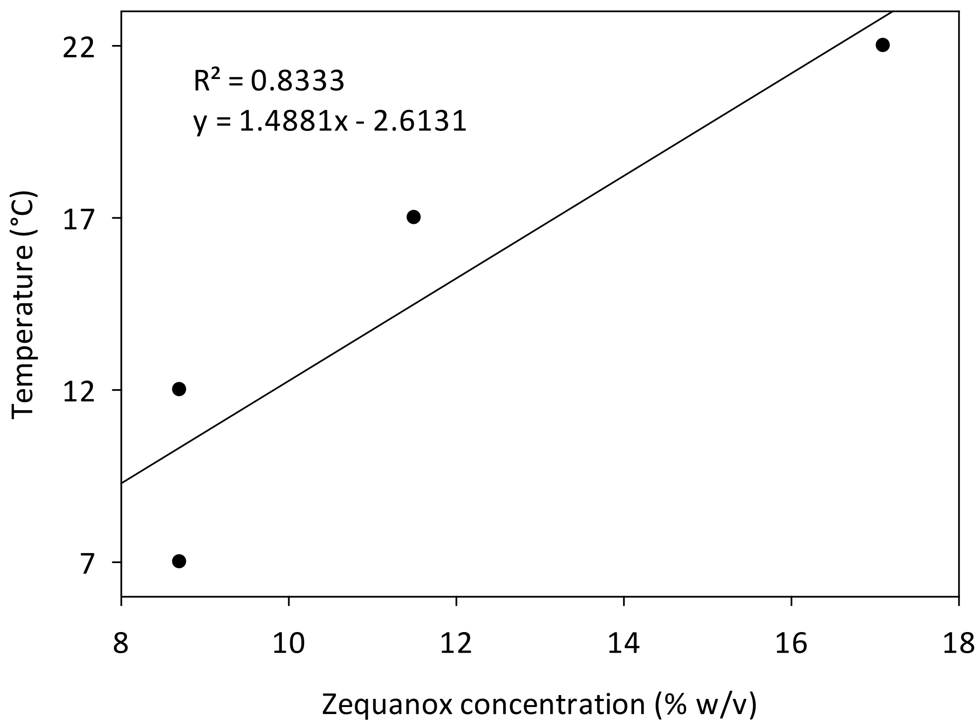

Figure 4. Second-step linear regression used to predict Zequanox concentrations at the target viscosity (180 cSt) for temperatures ranging from 7 to 22°C. This prediction regression was used to determine Zequanox concentrations in suspensions used for applications in outdoor pond tests.

Pond

Mean pre-exposure water quality parameters (pH, temperature, dissolved oxygen, hardness, and alkalinity) for the pond trials are summarized in Table 4. Individual pre-exposure hardness and alkalinity measurements in all three pond trials ranged from 178 to 194 and 137 to 145 mg/L, respectively; pH ranged from 7.87 to 8.08; and dissolved oxygen ranged from 7.79 to 10.58 mg/L. Water quality parameters during the exposure period (temperature [1-, 4-, and 8-hour]; pH and dissolved oxygen [8-hour only]) for the pond trials are summarized in Table 5. Individual exposure period dissolved oxygen and pH measurements in all three pond trials ranged from 7.84 to 8.09 and 7.49 to 10.27 mg/L, respectively. Application of Zequanox to the enclosures had no appreciable impact to dissolved oxygen and pH for the duration of the exposure period. Throughout the exposure period, the dissolved oxygen, pH, alkalinity, and hardness remained at acceptable levels for aquaculture according to Timmons and Ebeling (2013).

Table 4. Pre-exposure water quality parameters observed during the pond trials.

Target temperature (ºC)	Mean water chemistry parameter (SD)				
	Observed temperature (ºC)	pH[1] (standard units)	Dissolved oxygen (mg/L)	Hardness (mg/L as $CaCO_3$)	Alkalinity (mg/L as $CaCO_3$)
9	8.6 (0.2)	7.97 (0.08)	10.32 (0.16)	179 (1)	138 (1)
14	13.8 (0.1)	8.05 (0.02)	8.07 (0.19)	193 (1)	145 (1)
20	19.7 (0.1)	7.96 (0.02)	8.43 (0.23)	181 (1)	139 (1)

[1]pH values were log transformed prior to calculating mean values; standard deviations were calculated from observed pH values.

Table 5. Exposure period water quality parameters observed during the pond trials.

Target temperature (ºC)	Mean water chemistry parameter (SD)				
	1-hour temperature (ºC)	4-hour temperature (ºC)	8-hour temperature (ºC)	8-hour pH[1] (standard units)	8-hour dissolved oxygen (mg/L)
9	8.4 (0.2)	8.5 (0.2)	8.6 (0.2)	8.01 (0.01)	10.09 (0.17)
14	14.1 (0.2)	15.3 (0.2)	15.4 (0.1)	7.95 (0.08)	8.92 (0.42)
20	20.1 (0.1)	20.7 (0.2)	20.2 (0.1)	7.98 (0.07)	8.15 (0.83)

[1]pH values were log transformed prior to calculating mean values; standard deviations were calculated from observed pH values.

Mean viscosities of Zequanox suspensions during the pond trials were 49, 133, and 275 cSt for 9, 14, and 20°C tests, respectively. The observed variance from the target of 180 cSt was likely influenced by the addition of defoaming agent, which was not used in the laboratory tests; error in the prediction regression model; and difficulty in obtaining precise viscosity measurements with cup viscometers. Although there was considerable variance in the observed viscosities of the applied Zequanox suspensions, each pond trial maintained concentrations near or above the target concentration of 100 mg A.I./L for the entire exposure duration near the water/substrate interface (7.5 cm). Observed mean Zequanox concentrations for all three pond tests were as follows: 7.5-cm samples ranged from 98.7 to 138.5 mg A.I./L, 30-cm samples ranged from 23.8 to 94.2 mg A.I./L, and 60-cm samples ranged from 1.9 to 30.5 mg A.I./L (Fig. 5). For the 7.5-cm samples, significantly higher Zequanox concentrations were detected in the 20°C trial compared to the 9 and 14°C trials ($P < 0.001$) and no difference was detected between the 9 and 14°C trials ($P = 0.58$). All trials maintained lethal levels of Zequanox at a depth of 7.5 cm for the duration of the exposure, and the increased concentrations observed in the 20°C trial correlates with the significantly higher viscosity observed in this trial. When comparing the results of this trial to the mortality of zebra mussels observed in a Zequanox study conducted by Luoma et al. (2015a), the concentration of Zequanox measured in the 30-cm samples remained above lethal levels in the 9°C trial for the duration of the exposure and for approximately 4 and 3 hours during the 14 and 20°C trials, respectively. All samples measured at the 60-cm depth were likely below lethal levels for the duration of the exposure period. Although the 9°C trial maintained lethal levels in a larger portion of the water column than did the 14 and 20°C trials, wave action and other disturbances to the Zequanox stratification layer in field applications would likely result in more rapid Zequanox dispersion than was observed in our pond trials. Therefore, the observed mean viscosity of 49 cSt indicates that the Zequanox suspension applied during the 9°C pond trial was likely too diluted and should have been closer to the selected viscosity of 180 cSt.

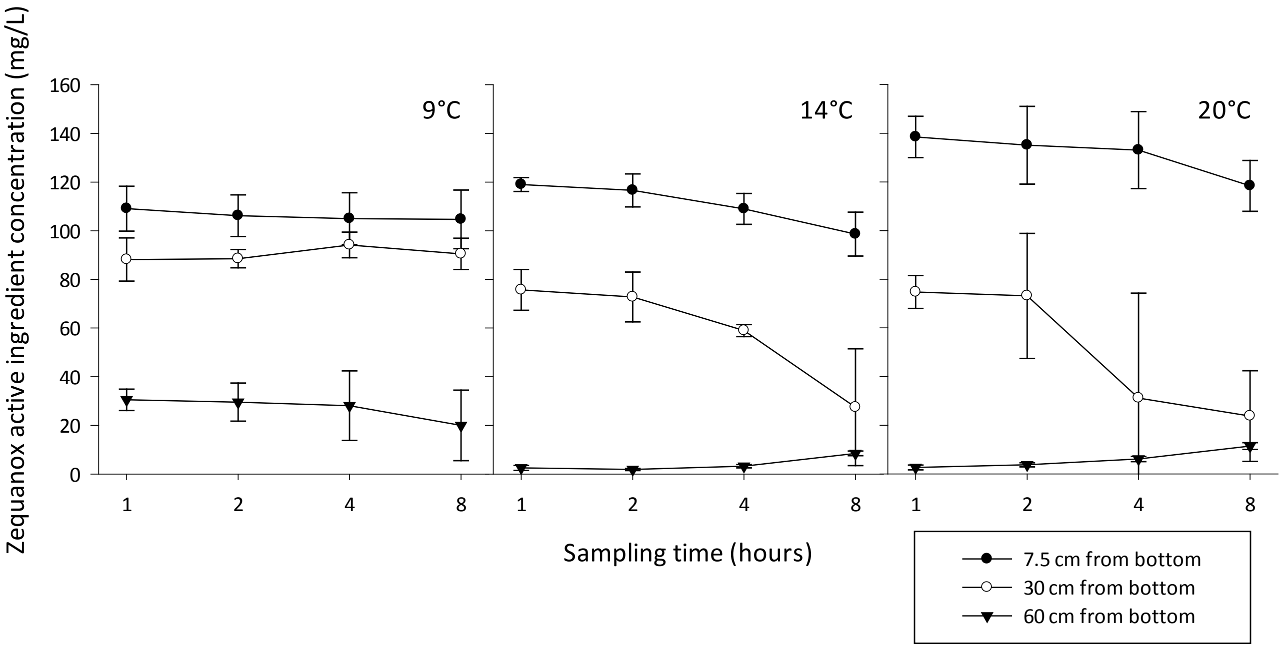

Figure 5. Mean Zequanox active ingredient concentrations observed by temperatures in outdoor pond trials.

Revised Prediction Model

At the coldest pond temperature evaluated (9ºC), the two-step linear regression model used in the study predicted a concentration of Zequanox that yielded a suspension that was too thin ($\bar{x}$ = 49 cSt), and at the warmest pond temperature evaluated (20ºC), it predicted a concentration of Zequanox that yielded a suspension that was too viscous ($\bar{x}$ = 269 cSt). A reexamination of the methods used to create the prediction model determined that creating first-step temperature-specific models using SigmaPlot (Version 13, Systat Software, Inc., San Jose, California) with 2-parameter power regression equations ($y = ax^b$) was superior than the linear method because it provided a better fit, as demonstrated by correlation coefficients > 0.99, while including all data points for each temperature (Fig. 6). The optimal viscosity of 180 cSt was entered into each new power model to determine optimal Zequanox concentration at each temperature and a new second-step logarithmic temperature-concentration prediction model ($y = a\ ln(x) + b$) was generated (Fig. 7). The Zequanox concentrations predicted to achieve the target viscosity of 180 cSt, using both the original linear and the revised logarithmic models, are presented in Table 6. The revised logarithmic model predicts Zequanox concentrations closer to the target viscosity of 180 cSt than the original linear model by increasing and decreasing the Zequanox concentration for the lower and higher temperatures, respectively. Additional multiple linear regressions were developed to model the relationships between viscosity, temperature, and Zequanox concentration using both logarithmic and square root transformations; however, these models failed to predict Zequanox concentrations as well as the revised two-step logarithmic temperature-concentration model at either the lower, higher, or both ends of the temperature spectrum evaluated and therefore these models were rejected.

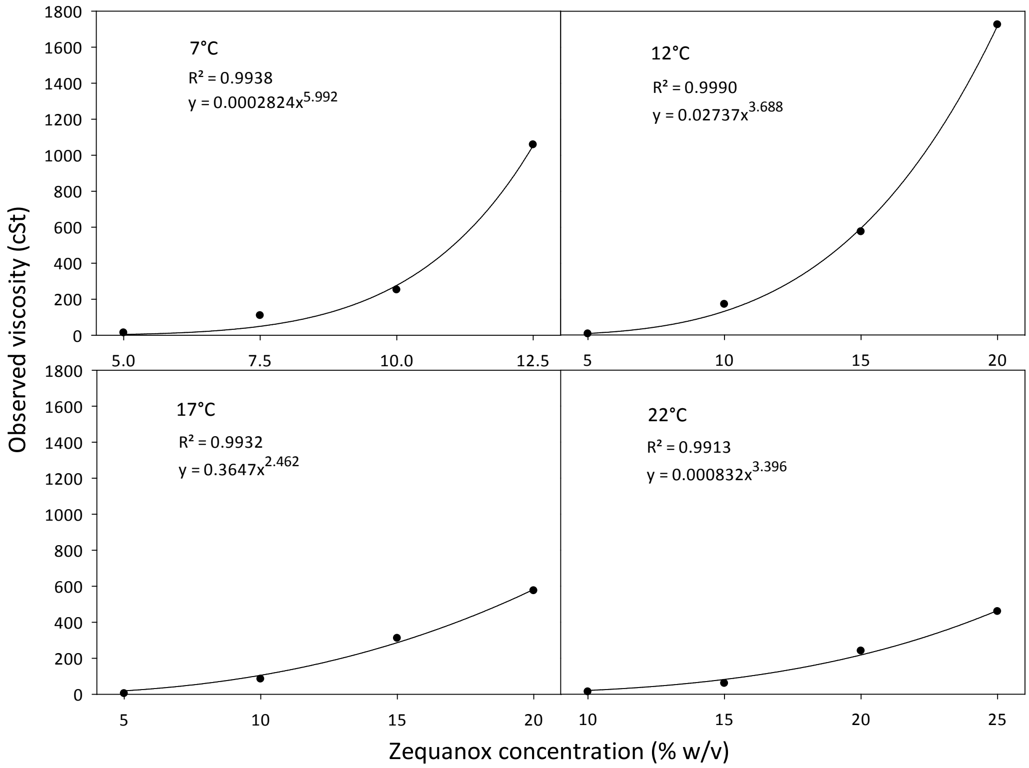

Figure 6. Revised first-step power regressions plotting Zequanox concentrations and viscosities from laboratory tests.

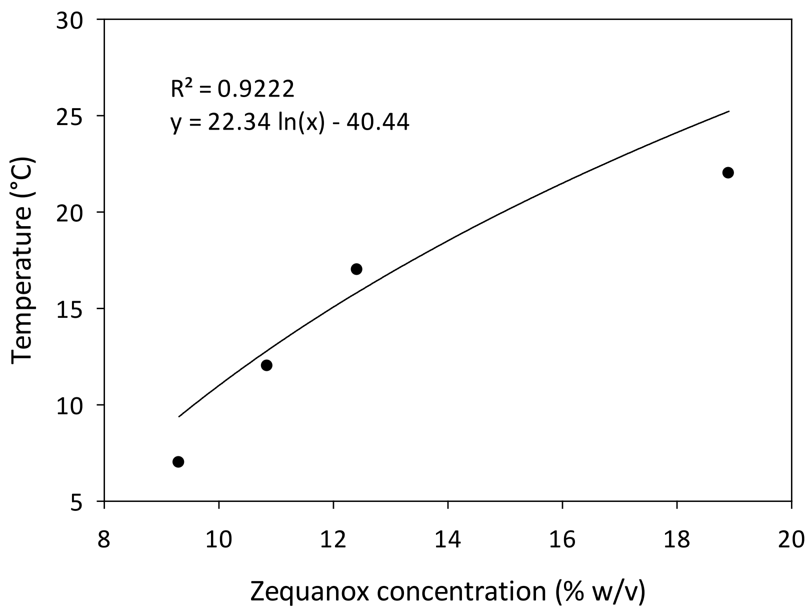

Figure 7. Revised second-step logarithmic regression derived from concentrations produced by revised first-step power regressions. This regression should predict Zequanox concentrations closer to the target viscosity (180 cSt) for temperatures ranging from 7 to 22°C.

Table 6. Predicted Zequanox concentrations required to achieve a suspension viscosity of 180 cSt for temperatures ranging from 7 to 22°C.

Water temperature (ºC)	Predicted Zequanox concentration (% w/v)		Water temperature (ºC)	Predicted Zequanox concentration (% w/v)	
	Linear[1]	Logarithmic[2]		Linear[1]	Logarithmic[2]
7	6.5	8.4	15	11.8	12.0
8	7.1	8.7	16	12.5	12.5
9	7.8	9.1	17	13.2	13.1
10	8.5	9.6	18	13.9	13.7
11	9.1	10.0	19	14.5	14.3
12	9.8	10.5	20	15.2	15.0
13	10.5	10.9	21	15.9	15.6
14	11.2	11.4	22	16.5	16.4

[1]Predicted Zequanox concentrations from the two-step linear model used in the study.

[2] Revised two-step power regression and logarithmic model predicted Zequanox concentrations.

Conclusion

The laboratory trials clearly demonstrated that temperature greatly impacts the viscosity of Zequanox suspensions, and achieving precise viscosity measurements of Zequanox suspensions is difficult with cup viscometers. The laboratory trials and preliminary scale-up tests showed the importance of eliminating air entrainment within Zequanox suspensions, which can result in Zequanox mixing throughout the water column. The use of a silicone-based aquaculture defoaming agent in the Zequanox suspensions appeared to reduce air entrainment, thereby increasing the potential for successful subsurface applications by allowing the use of more viscous suspensions. The results of this study, including the revised prediction model and the methods for subsurface delivery of Zequanox, will aid resource managers engaged in Zequanox applications. The use of subsurface Zequanox applications should be limited to quiescent waters in order to maintain a stratified Zequanox layer to achieve satisfactory dreissenid mussel control. This research provides foundational information for additional research related to subsurface Zequanox applications including refinement of the Zequanox concentration prediction procedure.

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