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Safety of the molluscicide Zequanox® to nontarget macroinvertebrates *Gammarus lacustris* (Amphipoda: Gammaridae) and *Hexagenia* spp. (Ephemeroptera: Ephemeridae)

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Abstract

Zequanox® is a commercial formulation of the killed bacterium, *Pseudomonas fluorescens* (strain CL145A), that was developed to control dreissenid mussels. In 2014, Zequanox became the second product registered by the United States Environmental Protection Agency (USEPA) for use in open water environments as a molluscicide. Previous nontarget studies demonstrated the safety and selectivity of *P. fluorescens* CL154A, but the database on the toxicity of the formulation (Zequanox) is limited for macroinvertebrate taxa and exposure conditions. We evaluated the safety of Zequanox to the amphipod *Gammarus lacustris lacustris*, and nymphs of the burrowing mayfly, *Hexagenia* spp. at the maximum approved concentration (100 mg/L active ingredient, A.I.) and exposure duration (8 h). Survival of animals was assessed after 8 h of exposure and again at 24 and 96 h post-exposure. Histopathology of the digestive tract of control and treated animals was compared at 96 h post-exposure. The results showed no significant effect of Zequanox on survival of either species. Survival of *G. lacustris* exceeded 85% in all concentrations at all three sampling time points. Survival of *Hexagenia* spp. ranged from 71% (control) to 91% at 8 h, 89–93% at 24 h post-exposure, and 70–73% at 96 h post-exposure across all treatments. We saw no evidence of pathology in the visceral organs of treated animals. Our results indicate that application of Zequanox at the maximum approved concentration and exposure duration did not cause significant mortality or treatment-related histopathological changes to *G. lacustris* and *Hexagenia* spp.

Key words: *Pseudomonas fluorescens*, molluscicide, nontarget, macroinvertebrate, amphipod, Ephemeroptera

Introduction

Zebra mussels, (*Dreissena polymorpha* Pallas, 1771) and quagga mussels, (*Dreissena rostriformis bugensis* Andrusov, 1898) were introduced to North American freshwaters through the release of ballast water from transoceanic vessels entering the Great Lakes (Carlton 2008). Since their arrival in the 1980s, dreissenid mussels have expanded from the east to west coast of the United States and into Canada (USGS 2015), significantly altering the dynamics of the freshwater systems where they have established (Higgins and Vander Zanden 2010; Mayer et al. 2014; Colvin et al. 2015). The ecological and economic impacts of dreissenids continue to mount in North America, as well as in invaded systems in Europe (Nalepa and Schloesser 2014).

A suite of treatment options is available to control dreissenid populations in closed systems of intake lines and cooling systems (Claudi and Mackie 1994; Mackie and Claudi 2009; Glomski 2015). However, there are currently only two molluscicides registered by the United States Environmental Protection Agency (USEPA) to control dreissenids in open water. Earthtec QZ® (Earth Science Laboratories, Inc., Bentonville, AR) is a copper-based product that has demonstrated effectiveness for killing dreissenid mussels; however, exposures of up to 96 h are required to kill adult mussels (Claudi et al. 2014) and the product can be toxic to fish and other aquatic invertebrates (USEPA 2008). Zequanox® (Marrone Bio Innovations (MBI), Davis, CA), the most recently approved molluscicide for dreissenid control, requires a shorter application period (e.g., 8 h), reportedly

has fewer nontarget impacts, and toxicity of the aquaeous product degrades within 24 h (Marrone Bio Innovations 2012a; Molloy et al. 2013a). The active ingredient of Zequanox is killed cells of a specific strain (CL154A) of the common soil bacterium, *Pseudomonas fluorescens.* Researchers at the NewYork State Museum found *P. fluorescens* CL154A to be toxic and relatively selective for dreissenid mussels (Molloy et al. 2013a, b, c). The toxic component of the bacterium has not been reported, but its mode of toxicity is lysis and degradation of the digestive gland and stomach epithelium of the mussels when ingested (Molloy et al. 2013b). Initial non-target toxicity trials were conducted using the unformulated cells (live and dead) of *P. fluorescens* with a variety of invertebrates that included seven species of unionid mussels, the ciliate *Colpidium colpoda (*Ehrenberg, 1838), the cladoceran *Daphnia magna,* (Straus, 1820), and the amphipod *Hyallela azteca* (Saussure, 1858) (Molloy et al. 2013c). Exposure durations of 24–72 h were tested at concentrations that were efficacious to dreissenids (100 or 200 mg/L active ingredient, A.I.). Mortality was insignificant in all species, except *H. azteca;* however, mortality (3–27%) in the amphipod was considered unrelated to *Pf*-CL145A toxicity. Following commercial production of *Pf*-CL145A by MBI as Zequanox, additional non-target trials were conducted to expand the database on selectivity of the product for dreissenids (Marrone Bio Innovations 2012a). Meehan et al. (2014) tested the duck mussel (*Anodonta*), a non-biting midge (*Chironomus plumosus* Linnaeus, 1758), and the white-clawed crayfish (*Austropotamobius pallipes* Lereboullet, 1858) in 72-h static tests and found that Zequanox was safe to these species at concentrations that equaled (100 mg/L A.I.) or exceeded the approved open water label (up to 750 mg/L A.I.). The most comprehensive non-target testing has been conducted on native unionid mussels including, trials on adults (Luoma et al. 2015a), newly transformed juveniles (Weber et al. 2015), and the glochidia (Luoma et al. 2015b). Adult and subadult mussels survived 24-h exposure at the maximum concentration of 100 mg/L A.I. (Luoma et al. 2015a). Juveniles and glochidia of several species were more sensitive (Luoma et al. 2015b; Weber et al. 2015), suggesting a need for further testing. Safety evaluation of Zequanox to other macroinvertebrate taxa requires an effort similar to that given to unionid mussels. An expanded database on non-target animal safety will assist resource managers in assessing risks of Zequanox exposure to the broader macroinvertebrate community in a dreissenid control program.

The goal of our study was to determine the safety of the commercial formulation of *Pf*-CL145A, Zequanox, at open-water application rates (100 mg/L A.I.) to high-value, non-target invertebrate species, the amphipod *Gammarus lacustris lacustris* (Sars, 1864) and burrowing mayflies, *Hexagenia bilineata* (Say, 1824) and *H. limbata* (Serville, 1829). *Gammarus lacustris* is distributed from the Great Lakes region into the western United States and north into most of Canada (Holsinger 1972). This is one the most abundant amphipods in the pothole region of the Upper Midwest (Kantrud et al. 1989) and is a significant dietary component for fish and waterfowl (Anteau and Afton 2006; Anteau and Afton 2008; Anteau et al. 2011). *Gammarus* feed on suspended coarse organic particulates, along with epibenthic algae, zooplankton and bacteria (Mathias and Papst 1981) and are at risk for ingestion of adsorbed Zequanox on these food items. Currently, Zequanox toxicity data for macroinvertebrate crustaceans is limited to the aforementioned laboratory tests with *A. pallipes* (Meehan et al. 2014), *D. magna* and *H. azteca* (Molloy et al. 2013c), and *Asellus aquaticus* Linnaeus, 1758 (Marrone Bio Innovations 2012a).

Hexagenia spp. are distributed throughout the United States (McCafferty 1975) and the nymphal stage represents a benthic-dwelling detritus feeder in the macroinvertebrate community of rivers and lakes. Nymphs dislodge deposited detrital material with their forelegs or beating movements of the gills and transfer particulates to the mouth parts for ingestion (Zimmerman and Wissing 1980). The pre-emergent nymph can reside in sediments for 1–2 years in northern lakes and rivers (Fremling 1960, Hilsenhoff 1981; Heise et al. 1987). Since applications of Zequanox would target the benthic zone where dreissenids densities are highest, *Hexagenia* nymphs have a high probability of exposure to and ingestion of the particles. Toxicity information on Zequanox for mayflies is limited to trials with *Pf*-CL145A on a single non-burrowing species, *Baetis* spp. (Marrone Bio Innovations 2012a).

We simulated an open-water application of Zequanox by conducting trials in outdoor mesocosms and testing maximum approved exposure concentrations (50 and 100 mg/L A.I.) and duration (8 h). In addition to comparing survival rates, we compared histological sections of the digestive tract among control and treated animals of both species in order to assess sublethal effects of Zequanox. Degradation of the digestive gland of dreissenids is evident within 24–48 h of ingesting *P. fluorescens*, though mortality may not occur for a week or more (Molloy et al. 2013b). Histological changes would be expected to occur within 96 h of exposure if the toxin in the product has a similar mode of action in macroinvertebrates.

Figure 1. Test system in outdoor concrete ponds. Two ponds held nine 1000-L tanks in a thermal water jacket. Water was supplied to test tanks from an adjacent earthen pond. Amphipod test chambers (arrow) were suspended in the test tank at mid-depth. Mayfly chambers (not visible) were placed on the tank bottom. Each pond contained three replicates of each treatment (0, 50, and 100 mg/L A.I. Zequanox).

Methods

Test animals and test system

Mayfly nymphs (mean length = 26.9 mm, standard deviation = 3.6 mm, range = 19–35 mm) were obtained from an independent bait supplier (Hilger and Sons, Inc., Antigo, WI) and identified as a mixture of *H. limbata* and *H. bilineata* (McCafferty 1975). Adult amphipods (mean length = 20.3 mm, standard deviation = 1.6 mm, range = 16.8–26.4) were obtained from a private aquaculture facility (Lincoln Bait Supply, Staples, MN) and identified as *G. lacustris lacustris* (Holsinger 1972). Before testing, mayflies and amphipods were held in separate raceways at the Upper Midwest Environmental Sciences Center (UMESC), La Crosse, WI, and supplied with a semi-recirculating chilled well water (12°C); water temperature in the raceways was increased gradually to 14°C over 24 h and maintained at this temperature during the holding period. Mayflies were contained in aluminum pans (36.8 × 27.0 × 7.6 cm; L × W × H) that were filled with 4–5 cm of sand/silt substrate. Dried alfalfa tablets (Hikari® algal wafers, Kyorin Co., Hayward, CA) and aged leaf litter were provided as a food source. Amphipods were contained in mesh cages (90 × 90 × 30 cm; L × W × H) and provided with leaf packs of aged birch leaves for cover and food. Feeding was supplemented once a day with Tetramin® flaked fish food (Tetra US, Blacksburg, VA). Animals were quarantined in the laboratory at UMESC for about 1 week and then transferred in their respective holding containers to outdoor raceways and acclimated to the test water and temperature for 1 week. The outdoor raceways were supplied with water from the same 0.10 ha earthen pond that supplied the test system.

The test system consisted of 18 1000-L circular high density polyethylene (HDPE) tanks (175 cm diameter × 64 cm height). Nine tanks were placed into each of two 0.004-ha concrete ponds (Figure 1). Test water was pumped from a nearby 0.1 ha earthen pond, filtered through a 200-µm filter to remove particulates and other invertebrates, and delivered to a head box above each concrete pond. Water was delivered from the head box to each tank at a rate of approximately 3.8 L/min. Daily and diurnal fluctuations in water temperature were minimized by filling the concrete ponds with well water to provide a thermal jacket for the test tanks and by covering the ponds with black shade cloth.

Amphipod test chambers were constructed of poly vinyl chloride (PVC) pipe (25.4 cm length, 10.2 cm inner diameter) with 1500-µm Nitex® screen covering each end. A peristaltic pump and tubing was connected to a threaded hose barb inserted at the midpoint of each chamber. To ensure that Zequanox

was mixed uniformly inside the chambers, water from the treatment tank was drawn through the peristaltic pump, into the top of the test chamber, and out each end of the chamber. A cylindrical roll (4 cm × 10 cm; diameter × length) of semi-rigid plastic mesh (3.0 mm diameter opening) was packed with aged birch leaves and placed inside each chamber to provide cover and substrate for the amphipods. Chambers were suspended in the test tank at approximately mid-depth (Figure 1). Mayfly test chambers consisted of plastic dishpans (28.5 cm × 12.1 cm; diameter × depth) filled with 2.5–3.0 cm of conditioned sand/silt substrate and dry leaf litter. The top of the chamber was covered with mesh screen (3.0 mm diameter opening) to prevent loss of mayflies during the test; the chamber was then placed on the bottom of the test tank.

Each pond contained three blocks of each treatment (0, 50, and 100 mg/L A.I. Zequanox) for a total of six replicates per treatment for each species. Treatments were randomly allocated to a tank according to a randomized block design. Amphipods and mayflies were tested simultaneously. Twenty-four hours before exposure, amphipods and mayflies were transferred from the outdoor raceways to the test tanks. Groups of 10 amphipods were removed from the raceway and distributed into 18 10-L buckets, in three separate rounds, according to a predetermined randomization schedule. Amphipods that were paired (male and female) were not selected. The 30 amphipods were then transferred into a test chamber in each test tank. Mayfly nymphs were distributed in the same manner, following a unique randomization schedule. In both species, only animals that were actively swimming were selected for testing. Ten additional animals of each species were randomly assigned to each test tank to assess mortality at 8 h and 24 h post-exposure without disturbance of the larger test group. These were placed into separate mesh bags, with aged leaf litter, and suspended mid-depth within each tank.

Exposure and assessments

The test material, Zequanox was a spray-dried powder formulation, made of 50% active ingredient (MBI-401 SDP, MBI, Davis, CA). A dosing stock was prepared for each individual tank by removing 10 L of water from the tank and adding the specified weight of dry Zequanox to the water. The stock was stirred for approximately 5 minutes using a paint mixer attachment on an electric drill. The solution was poured through a strainer and funnel into a second bucket. Undissolved test product was mechanically broken apart within the strainer using a pestle. The dosing stock was added to the test tank within 5 minutes of preparation. The stock was thoroughly mixed within each tank using a boat paddle. Control tanks (i.e., no test material added) were mixed using a boat paddle in a manner identical to the treatment tanks. Water flow to each tank was halted during the 8 h treatment and re-established for the remainder of the test period.

Water quality parameters (dissolved oxygen, pH, and temperature) were measured immediately before exposure, at 1, 4, and 8 h during the exposure, and once daily thereafter. Dissolved oxygen was measured with a YSI® 550A dissolved oxygen meter (YSI, Inc., Yellow Springs, OH). The pH was determined with a Beckman Coulter® φ410 pH meter and probe (Beckman Coulter, Inc., Fullerton, CA). Temperature was measured with a Thermapen® digital thermometer (ThermoWorks, American Fork, UT). Water flow rates (mL/min) were measured daily in each tank. Hardness and alkalinity were measured from each tank at 1 h exposure; conductivity was measured from each tank at 1 h exposure and at 96 h post-exposure. Total hardness (mg/L as $CaCO_3$) was determined by titrimetric method with Manver Red indicator (USEPA 1983). Total alkalinity (mg/L $CaCO_3$) was determined by titrimetric method to a pH endpoint of 4.5 (APHA 1995). Conductivity was measured with a Fisher Accumet® conductivity meter (Fisher Scientific, Pittsburg PA).

At the conclusion of the 8 h exposure, the mesh bags containing 10 animals of each species were removed from each tank and animals were rinsed into a shallow pan to assess immediate mortality. Mortality was defined as lack of response to probing and light stimulation with a battery-wired forceps. Up to five live animals were indiscriminately selected from each bag and preserved in Davidson's fixative (Humason 1962) for histological examination. The remaining live animals were returned to the mesh bags and assessed at 24 h post-exposure following the same procedure. At 96 h post-exposure, test chambers (i.e., PVC chambers and dishpans) were individually removed from each tank and animals were rinsed into a shallow pan to assess survival. Each tank was also siphoned through a 600-µm bag filter to collect animals that had escaped from the test chambers. Three animals were indiscriminately selected from each tank and preserved in Davidson's fixative for histological examination. The remaining live animals were retained in 70% alcohol for length measurements. Total length of amphipods was measured from the base of the antennae to the tip of the third uropod along the curve of the dorsal surface. Total length of mayflies was measured from the base of the antennae to the last abdominal segment.

Histological processing

Animals that were processed for histology were preserved in Davidson's fixative for 24 h, rinsed with water, dehydrated in a graded series of ethanol or ethyl alcohol (50–100%) and embedded in Paraplast® using a tissue processor (Thermo Scientific Shandon Excelsior, ThermoFisher Scientific, Waltham, MA). Serial sections were cut (7–10 µm) with a rotary microtome (Leica RM2035, Leica Biosystems Inc., Buffalo Grove, IL), stained with hematoxylin and eosin, and examined with a compound microscope (≤1000×). Ten samples per species were indiscriminately selected from control and 100 mg/L treatments for histological examination. Because the mode of toxicity of *Pf*-CL145A in dreissenids is necrosis of the digestive epithelium, our histological examination focused on the digestive tract of both amphipods and mayflies. In addition, we examined the hepatopancreatic ceca, anterior dorsal and rectal ceca of *Gammarus* and fat bodies of *Hexagenia* since these organs are integral to nutrient digestion, absorption and metabolism. Photomicrographs of stained sections were made using light microscopy (Nikon Eclipse E600, DSFi1 digital camera, Nikon Instruments, Inc., Melville, NY). Histological descriptions and terminology for *Gammarus* followed that of Schmitz (1967) and Schmitz and Scherrey (1983); descriptions and terminology for *Hexagenia* were derived from Csoknya and Halász (1973), Saouter et al. (1991), Gaino et al. (1997), Harker (1999), Oliveira and Cruz-Landim (2003), and Liarte et al. (2014).

Zequanox analysis

Concentrations of Zequanox in the test tanks were determined by spectrophotometric comparison to a linear regression created from Zequanox standard solutions (Beckman UV/Vis Spectrophotometer, Model DU 800). Zequanox standards of 25, 50, 100, and 150 mg/L (A.I.) were prepared from dilutions of a 2,000 mg/L (A.I.) standard stock solution. A linear zero-intercept standard curve was prepared from the 25, 50, 100, and 150 mg/L dilution stocks using triplicate standard samples. The spectrophotometer was blanked using filtered (200 µm) pond water. 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.

Confirmatory post-test efficacy verification of Zequanox was completed at MBI. Efficacy trials were conducted on *D. rostriformis bugensis*, collected from Lake Havasu, AZ, at 20°C with three replicates of 20 animals exposed to 100 mg/L A.I. Zequanox. Mean mortality of mussels was 73.3% (standard deviation 12.6%) at 15 d post-exposure, which meets the quality control standards set forth by MBI for the product.

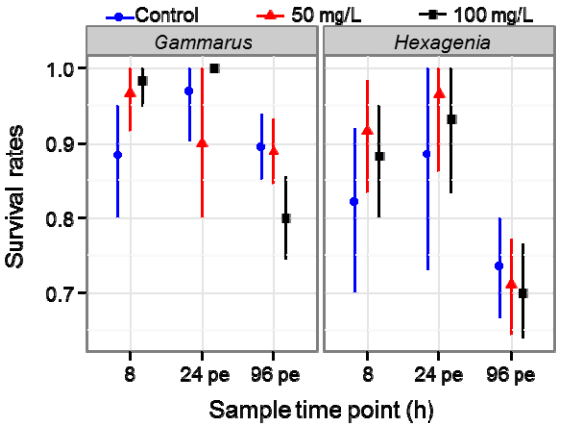

Figure 2. Survival rates of *Gammarus lacustris* and *Hexagenia* spp. in controls and Zequanox (mg/L active ingredient) treatments at each sampling time point. Exposure duration was 8 h. Survival rate (number survived/total) was assessed on a subsample of animals at 8 h (n = 10 per tank) and 24 h post-exposure (n = 5 per tank). Survival at 96 h post-exposure (pe) was assessed on 30 animals/tank. Bars represent 95% confidence limits.

Statistical analysis

Unrecovered animals were counted as mortalities in the analysis of survival. An outlier tank (control) of *Hexagenia* was removed at the 8 h time point because this tank had an extremely low recovery rate (<20%) when all other tanks had higher recovery rates (>75%). A generalized linear mixed effects model with a binomial error term (also known as a random-effect logistic regression) was used to analyze survival of each species by exposure concentration and time point (8 h exposure, 24 and 96 h post-exposure), while controlling for tank as a random effect (Bolker 2008). An interaction term was included between the two predictor variables. If the interaction was not significant, the model was re-parameterized without the interaction term. R (R Core Team 2015) was used to analyze the data. The glmmPQL function from the MASS package was used for the generalized linear mixed effects model (Venables and Ripley 2002). The ggplot2 package was used to plot the results (Wickham 2009).

Results

The results were similar for each species. Mean survival of *G. lacustris* exceeded 80% in all control and test concentrations at all three sampling time points (Figure 2). Mean survival of *Hexagenia* spp. ranged from 71% (control) to 91% (50 mg/L) at 8 h, 89–93% at 24 h post-exposure and 70–73% at 96 h post-exposure across all treatments. None of the interaction terms were significant (50 mg/L *Gammarus* $p = 0.8417$, *Hexagenia* $p = 0.7807$, $df = 15$; 100 mg/L *Gammarus* $p = 0.8684$, *Hexagenia* $p = 0.2070$, $df = 15$). In fact, the only significant terms from the analysis were the intercepts ($p<0.001$) and effect of the 96 h post-exposure time point ($p = 0.0045$, $df = 775{,}792$ *Gammarus* and *Hexagenia*, respectively). The number of organisms that died or were unrecovered was greatest at 96 h post-exposure but did not vary significantly among treatments. The contribution of unrecovered animals to total mortality in *Gammarus* was 9% in control and 50 mg/L treatments and 16% in the 100 mg/L treatment. The contribution of unrecovered animals to total mortality in *Hexagenia* was similar, ranging from 6% in control tanks to 12% in the 100 mg/L treatment tanks. In some cases, unrecovered animals had escaped from the test chamber and were later recovered when the contents of the test tank were drained. Mean recovery was not different among control and test treatments.

Concentrations of Zequanox were slightly higher than targeted in 50 mg/L tanks, averaging 54.3 mg/L at the onset of exposure and decreasing to a mean of 52.6 mg/L at 8 h (Figure 3). Concentrations in the tanks targeted for 100 mg/L treatments averaged 94.6 mg/L at 1 h and 82.3 mg/L at 8 h. The lowest mean concentration, 77.8 mg/L, occurred at 6 h. The inflection in mean Zequanox concentration from 6 to 8 h was attributed to variability in the depth of water sample collection between sampling times. After water flow was reestablished to the test tanks at the termination of the 8 h exposure, Zequanox concentrations decreased by 50% at 1 h post-exposure and were not detectable in the treatment tanks at 8-h post-exposure (Figure 3).

Water quality and chemistry were similar among tanks and treatments (Tables 1, 2). Dissolved oxygen remained >10 mg/L in all tanks at all sampling points. The pH was relatively high (>8.8) at the start of the exposure and decreased by 0.1–0.3 pH units in Zequanox treatments during the 8-h exposure; pH remained above 9 in the control tanks. During the post-exposure period, the pH remained above 8.3 and was similar among all tanks. Water temperature increased during the 8-h exposure from about 16 °C to 23.8 °C, but did not vary among tanks.

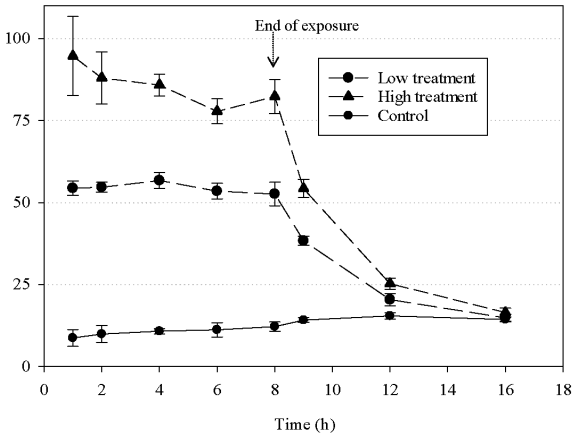

Figure 3. Mean Zequanox concentration (standard deviation) over time as determined by spectophotometry. The exposure was terminated at 8 h (arrow) and water flow resumed to the tanks.

Histology

There was no evidence of treatment-related histopathologic changes to the digestive tract or visceral organs of *G. lacutris* or *Hexagenia* spp. treated with 100 mg/L A.I. Zequanox.

Histology of amphipods: In the control animals, the midgut showed normal histological structure (Schmitz 1967), with an inner epithelium consisting of short columnar cells on a basement membrane, surrounded by circular muscle (Figure 4A). The dorsal median and rectal ceca each consisted of a monolayer of tall columnar cells, with non-vacuolated, basophilic cytoplasm (Figure 4A). The glandular epithelium of the hepatopancreatic ceca was columnar with large vacuoles near the luminal end and nuclei located near the basal end (Figure 4B). In amphipods treated with 100 mg/L Zequanox, the digestive epithelium of the stomach, midgut and hindgut were intact, with no evidence of sloughing or necrosis (Figure 4C). Cells of the hepatopancreatic ceca were comparable in appearance between control and treatment animals, with no signs of necrosis or inflammation (Figure 4D). There was no sign of hemocyte infiltration into the gut or ceca (Figure 4C, D). Furthermore, the lumen of both control and treated animals contained ingested material, indicating that the treatment did not cause purging of intestinal contents or cessation of feeding (Figures 4A, B, D).

Histology of mayflies: The midgut in control animals consisted of tall columnar epithelial cells, resting on a thin basement membrane, and underlying muscle fibers (Figure 5A, B). Using light microscopy, we could distinguish a distinct band along the apical

Table 1. Mean (standard deviation) dissolved oxygen and temperature, and pH range of each treatment group during the study period.

Water chemistry parameter	Treatment group (mg/L)	Pre-exposure[1]	Exposure period			Post-Exposure period			
			≤1 h	4 h	8 h	16 h	48 h	72 h	96 h
DO (mg/L)	0	10.42 (0.17)	11.50 (0.09)	13.52 (0.39)	16.13 (0.76)	11.55 (0.08)	13.47 (0.18)	12.70 (0.15)	11.47 (0.18)
	50	10.35 (0.08)	11.30 (0.09)	11.85 (0.19)	13.38 (0.75)	11.50 (0.06)	13.45 (0.18)	12.70 (0.09)	11.43 (0.18)
	100	10.60 (0.46)	11.25 (0.19)	11.33 (0.38)	11.38 (0.50)	11.40 (0.17)	13.30 (0.28)	12.62 (0.15)	11.38 (0.17)
Tempera-ture (°C)	0	16.00 (0.09)	17.22 (0.40)	20.62 (0.32)	22.87 (0.20)	16.57 (0.08)	16.57 (0.16)	17.77 (0.08)	18.40 (0.09)
	50	16.00 (0.09)	17.05 (0.28)	21.07 (0.80)	23.72 (0.83)	16.53 (0.05)	16.53 (0.12)	17.80 (0.11)	18.35 (0.12)
	100	16.02 (0.12)	17.28 (0.32)	21.40 (0.77)	23.87 (0.92)	16.58 (0.08)	16.63 (0.12)	17.77 (0.14)	18.38 (0.08)
pH	0	8.96-8.97	8.94-8.98	9.07-9.18	8.97-9.15	8.73-8.75	8.70-8.79	8.63-8.65	8.37-8.43
	50	8.94-8.96	8.81-8.90	8.88-8.92	8.79-8.92	8.73-8.75	8.73-8.78	8.64-8.67	8.33-8.42
	100	8.95-8.98	8.67-8.79	8.7-8.78	8.53-8.80	8.73-8.74	8.76-8.78	8.64-8.65	8.37-8.43

[1]Pre-exposure time points were measured approximately 1 h before application of Zequanox

Table 2. Mean (standard deviation) hardness, alkalinity and conductivity. Hardness and alkalinity were measured on water from each tank at 1 h; conductivity was measured on each tank at 1 h and 96 h (post-exposure).

Treatment group (mg/L)	Water chemistry parameter			
	Hardness (mg/L)[1]	Alkalinity (mg/L)[1]	Conductivity (µS/cm)[2]	
			1 h Exposure	Post-exposure
0	132.7 (1.6)	103.2 (1.2)	278 (3.6)	354 (2)
50	131.7 (0.8)	106.2 (2.5)	287 (4.0)	355 (2)
100	133.7 (0.8)	106.8 (1.5)	298 (2.7)	353 (2)

[1]Reported as milligrams per liter $CaCO_3$
[2]Temperature compensated to 25 °C

surface indicative of the brush border. The peritrophic membrane, an extracellular sheath, was visible in some sections. Fat bodies were seen throughout the body cavity, especially adjacent to the digestive tract and gonads (Figure 5A). Trophocyte cells in the fat bodies were large, ovoid, contained a large nucleus, and a cytoplasm filled with vacuoles (Figure 5A). Again, we saw no evidence of treatment-related histopathologic change in the midgut or fat bodies of treated *Hexagenia* nymphs (Figure 5C, D). The epithelium was intact and cell structure appeared normal (Figure 5D). The peritrophic membrane was observed between the gut contents and brush border of the epithelium (Figure 5C). The fat bodies were distinct with large vacuolated trophocytes, comparable to those of control animals (Figure 5C, D).

Discussion

Zequanox is composed primarily of organic particulates and produces a highly turbid suspension in the water column. Aquatic organisms may be negatively affected by a Zequanox suspension in several ways: (1) hypoxia may develop as the organic material degrades in a stagnant system (Whitledge et al. 2015), limiting gas exchange, (2) the particulate matter may cover or damage respiratory surfaces and interfere with gas exchange, and (3) the toxic component of *Pf*-CL145A may cause tissue damage and death. In our study, hypoxia was not a concern as dissolved oxygen concentrations remained >10 mg/L in all test tanks throughout the exposure period. High turbidity also did not appear to affect survival or recovery. In response to stressors, mayflies will often abandon their burrows (Fremling 1960, 1975). We could not directly observe the behavior of animals to determine whether Zequanox exposure triggered their escape from the test chambers. However, we found no difference in recovery of mayflies among control and test treatments, suggesting that Zequanox exposure did not elicit an escape response. Decreased recovery of *Gammarus* at 96 h post-exposure was partially attributed to cannibalism within the chambers (MacNeil et al. 2003; Dick 2009); however, recovery did not differ significantly among control and test treatments. Survival of test

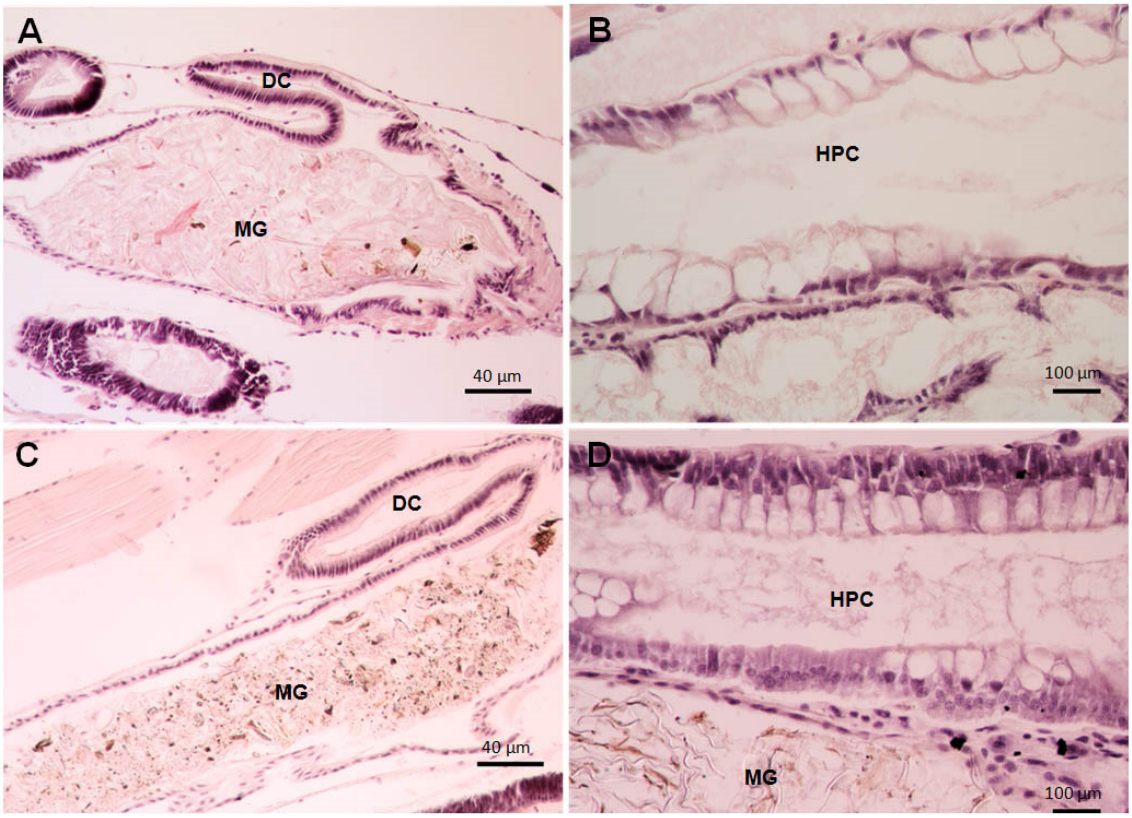

Figure 4. Photomicrographs of *Gammarus lacustris lacustris*, longitudinal sections. A) Control-midgut and dorsal cecum, B) Control-hepatopancreatic cecum, C)100 mg/L A.I. treatment-midgut and dorsal cecum, D) 100 mg/L A.I. treatment-hepatopancreatic cecum and midgut. DC=anterior dorsal cecum showing tall columnar epithelial cells without vacuoles, HPC=hepatopancreatic cecum; note basal location of nuclei and large vacuoles, MG=midgut, lined by short columnar epithelial cells. The gut lumen of animals in all treatments was filled with food.

organisms at 8 h and 24 h post-exposure was also unrelated to Zequanox treatment, suggesting that the toxic component of *Pf*-CL145A did not cause direct mortality of *Gammarus* and *Hexagenia* spp.

Ingestion of *Pf*-CL145A is necessary to produce mortality of dreissenids (Molloy et al. 2013a), as direct exposure (i.e., gills) is also not lethal. Most chemical molluscicides cause mussels to close and stop siphoning to reduce exposure to the toxin, but because of its high organic content, Zequanox is filtered out and ingested as a food source. Based on their modes of feeding, trophic uptake of Zequanox by both mayflies and amphipods was expected. *Hexagenia* nymphs are primarily detritus feeders that turn over the sediment with the forelegs (Zimmerman and Wissing 1980) and create water flow through the burrow by gill movements (Fremling 1960). They reportedly feed continuously and pass food through the short gut in 4–12 h (Zimmerman et al. 1975). As a result, mayflies could potentially ingest a significant amount of Zequanox. *Gammarus* are most likely to ingest Zequanox that has coated or been adsorbed to food items, such as the leaf litter provided in the test chambers. Histological examination of *Gammarus* and *Hexagenia* showed food in the digestive tract and confirmed that animals were eating during the trial and likely ingesting Zequanox particulates.

The toxicity of *Pf*-CL145A to dreissenids is not immediate, but is caused by gradual degradation of the digestive epithelium (Molloy et al. 2013a, b). Occurrences of mussel mortality may range from 3–21 days, depending on water temperature (Molloy et al. 2013a; Marrone Bio Innovations 2012b). Therefore, an extended post-exposure period is required to assess treatment mortality. However, Molloy et al. (2013b) reported signs of histopathology in dreissenids within 24–48 h of ingesting *Pf*-CL145A. Hemocyte infiltration was observed in the stomach lumina and digestive gland at 24 h and degradation of digestive

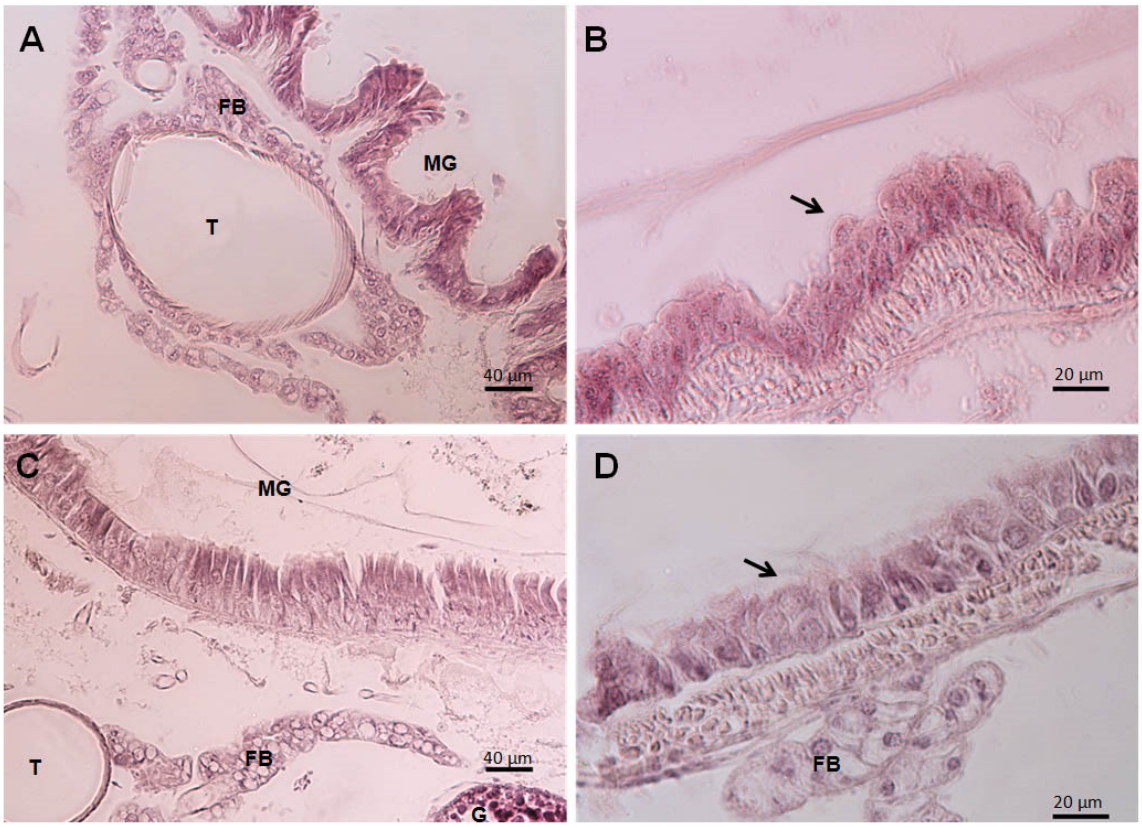

Figure 5. Photomicrographs of *Hexagenia* nymphs A) Control-midgut, tracheole and surrounding fat bodies, B) Control-gut epithelium (arrow) at 40×, C) 100 mg/L treatment-midgut, tracheole and surrounding fatbodies, D) 100 mg/L treatment-gut epithelium (arrow) at 40×. FB=fat bodies, G=gonad, MG=midgut, T=tracheole.

epithelium was evident at 48 h (Molloy et al. 2013b). An extended post-exposure period was impractical for test organisms in our study, particularly given the potential of cannibalism and escape. The histological exam was imperative for identifying tissue damage that could cause delayed mortality of the test animals. We saw no evidence in the digestive tract and associated viscera of *Gammarus* and *Hexagenia* that Zequanox exposure caused pathology similar to that reported in dreissenids. Cursory observation of other body tissues (i.e., gills, tracheoles, gonads, muscle) showed no other signs of pathology in either species. The presence of food and absence of of treatment-related histopathologic change in the digestive tract supports our conclusion that Zequanox does not cause significant mortality in *G. lacustris* and *Hexagenia* spp. nymphs.

Nontarget toxicity trials with Zequanox have been primarily conducted in indoor laboratories under controlled conditions (e.g., Molloy et al. 2013c; Luoma et al. 2015b). Mesocosm toxicity trials are a compromise between the highly controlled, but artificial environment of the laboratory and uncontrolled, natural conditions of a field application. Daily fluctuations in water temperature and pH in our mesocosm test system were greater than reported in most standard laboratory studies, but reflected commonly measured daily cycles in the earthen pond that provided source water for the test tanks. The variability in water temperature and pH did not appear to increase the sensitivity of mayflies or amphipods to Zequanox treatments. Moreover, the mesocosm environment provided a food supply and photoperiod for test organisms that more closely mimicked natural conditions. Results from mesocosm toxicity tests may better reflect the behavior of the test organism, degradation of the toxin, and fluctuations in water chemistry than laboratory-based tests (Mikó et al. 2015). However, loss of animals from cannibalism and escape from test chambers might have been reduced in a more controlled laboratory test system.

Desirable features of an agent for dreissenid mussel control include toxicity to all life stages, at a

concentration and duration of exposure that can be maintained in natural waters, safety to nontarget organisms, and rapid degradation in the environment. In addition to Zequanox, several chemical control agents that have been used for dreissenid control meet some, but not all of these features. Potassium chloride was successfully used to eradicate zebra mussels from a 12-acre quarry lake in Virginia (Fernald and Watson 2013). The quarry was isolated from other surface and groundwater connections and did not contain native species that are sensitive to potassium, such as unionid mussels. Potassium chloride (KCl) was applied for 3 weeks to achieve a concentration of 100 mg/L. Two years after application the concentration of KCl remained near 70 mg/L. Although KCl was an appropriate choice for this isolated body of water, its toxicity to other molluscs and persistence in the environment precludes its use in lakes and streams that contain native unionid mussels. The copper-based chemical, EarthTec Qz is the only molluscicide, other than Zequanox, registered by the USEPA for use in open water. Other copper-based chemicals have been used for eradication of dreissenid mussels in the United States under a Special Local Need Label issued by the USEPA. Most copper compounds can effectively kill adult dreissenids in 96 h of exposure (Watters et al. 2013; Claudi et al. 2014) and reduce veliger settling; however, copper-based products are toxic to a number of aquatic organisms including plants, fish, and other molluscs (USEPA 2008). Following application of copper sulfate to Lake Offutt in Nebraska to eradicate zebra mussels, significant mortality of fish was reported (URS 2009).

In most natural water bodies, dreissenid control efforts will require a balance between the negative effects of invasive mussels and the risk of the treatment to the native community. Amphipods and mayflies are of particular concern for resource managers because of their high value for the fishery and role as indicators of ecosystem health. Amphipods can comprise the major part of the diet for a variety of waterfowl and fish species (Anteau and Afton 2008; Pothoven and Madenjian 2008). *Hexagenia* spp. are preyed upon by a variety of fish, owing to their long-lives and multiple molts (Fremling 1960). Mayflies have routinely been considered indicators of water and sediment quality and are sensitive to point and nonpoint source pollution (Fremling 1970; Fremling and Mauck 1980; Resh and Jackson 1993; Barbour et al. 1999; Harwood et al. 2014). The existing database for nontarget tests with Zequanox indicates few negative effects of the product on the invertebrate species that have been tested.

The current study augments those data and provides evidence that ingesting *Pf*-CL145A under conditions simulating an actual application does not induce histopathologic change in tissues of the digestive tract in *Gammarus* and *Hexagenia* as it does in dreissenids. Comprehensive data on the effects of a control tool are imperative for decision-making by resource managers and for public support of control management plans. For example, anglers in Michigan were concerned that chemical treatments to control sea lamprey (*Petromyzon marinus* Linneaus, 1758) in a valued trout stream could cause mortality of mayflies and a concomitant reduction in the fishery. In order to reach agreement between anglers and managers on control application, the USFWS demonstrated that lampricide treatments did not negatively impact macroinvertebrates or fish in the stream (USFWS 2014). We recommend continued testing of Zequanox on additional taxa and life stages, especially if Zequanox becomes routinely used in a dreissenid control program. Further evaluation of indirect or latent effects (e.g., hypoxia, nutrient addition, increased turbidity) of treatments would further broaden our understanding of its effects on an ecosystem for informing decisions on dreissenid control strategies.

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