Efficacy of a potassium peroxymonosulfate-based disinfectant (Virkon™) against zebra mussel (Dreissena polymorpha) adults and veligers

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INTRODUCTION

Zebra mussels pose a major problem for the waterways into which they are introduced. In the United States alone, the economic impact of the species is estimated to be up to $1 billion dollars per year (Pimentel et al. 2005) and includes costs stemming from zebra mussel biofouling of intake pipes and materials. Economic impact is wide-ranging, affecting many economic sectors, including power, water, agriculture, fishing, and recreation (Connelly et al. 2007; Elliott et al. 2005; MacIssac 1996). In addition to economic impacts, zebra mussels are considered to be ecological engineers, producing changes in ecosystem structure upon introduction. Increased water clarity has been consistently reported due to their feeding on phytoplankton and has been implicated in contributing to cycles of mass macrophyte growth and die-offs. Zebra mussels have also been shown to compete with other planktivores, including smaller zooplankton (MacIssac et al. 1995), indigenous freshwater mussels (Ricardi et al. 1998), and others. These factors may be contributing to the benthification of freshwater food webs by redirecting energy and resources from pelagic to benthic zones.

The life history and lifecycle characteristics of the zebra mussel augment the species’ ability to spread to new water bodies. Aquatic recreation, considered a serious vector for zebra mussel proliferation (Johnson et al., 1996), peaks in summer months when zebra mussel reproduction is most active. Zebra mussels have incredible fecundity rates and are known to participate in mass spawning events, producing as many as 500,000 veliger larvae per square meter (Ludyanskiy et al. 1993). Adults may be transported on hulls and engines of watercraft, while immature veliger larvae are microscopic and hardy with the potential to live in live wells, bilge tanks, sinks, showers, and even engine cooling systems during overland transport of small-craft boats (Dalton and Cottrell 2013). Veligers also have the potential to survive in damp equipment and gear, including fishing nets, wet suits, rope, and clothing (Timar and Phaneuf 2009).

Many disinfection treatments exist for craft and equipment moving between water bodies, each with its own unique costs and benefits. One broad-spectrum disinfectant, Virkon Aquatic ™ (referred to hereafter as Virkon), has shown to be effective in destroying individuals of many aquatic invasive and nuisance species, including the vase tunicate (Ciona intestinalis) (Paetzold and Davidson 2011), red-rimmed melania (Melanoides tuberculate) (Mitchell et al. 2011), freshwater Asian clam (Corbicula fluminea) (Barbour, et al., 2013), quagga mussel (Dreissena bugensis) (Moffitt et al. 2015), and others.

Virkon has a long history and broad application as a disinfectant in the medical and veterinary fields and was originally developed as an effective and relatively safe treatment for microorganisms. It is composed of a strong oxidizing agent (potassium peroxymonosulfate triple salt), organic acids (malic and sulphamic acid), an inorganic buffer (sodium hexameta
phosphate), a surfactant (sodium toluenesulphonate), and sodium chloride (Antec 1994; Antec 2015). While effective on their own, the components of Virkon also interact in a regenerative cyclic reaction involving the oxidation of sodium chloride to form hypochlorous acid (Figure 1).

![Figure 1. Schematic describing the cyclical reaction of potassium peroxymonosulfate, sodium chloride, and sulphamic acid to produce hypochlorous acid. Potassium peroxymonosulfate liberates chlorine which joins with sulphamic acid to create an intermediate. The intermediate is hydrolyzed to form hypochlorous acid, which decomposes into oxygen gas, hydroxide, and chloride, which is free to once more be acted upon by the oxidizer (Antec 1994).](image)

Virkon has been shown to be relatively safe for mammals and fish, yielding LD50 values of 2,200 mg/kg in rabbits on dermal exposure and 4,123 mg/kg in rats on oral administration. A similar formulation of the product also yielded a 96h LC50 value of 53mg/L for rainbow trout (Antec 2015). In addition, the environmental impact of Virkon is considered by the distributor to be minimal (DuPont undated).

Aside from recreational boating as a vector for zebra mussel dispersal, the likelihood of introductions by lake researchers and managers themselves seems plausible. Particularly, porous or absorbent materials which remain moist for some time after use (such as anchor and mooring lines and fishing nets) can harbor living mussels and veligers for several weeks (Timar and Phaneuf 2009). Establishing an effective protocol to disinfect these types of material is an important component of a broader disinfection procedure. Since Virkon has proven to be an effective and multifaceted disinfectant for a broad spectrum of invasive species, we hypothesize that it will be effective for treating materials that potentially move between zebra mussel infested water bodies. To evaluate this, zebra mussel veligers and adults were collected from Otsego Lake and were exposed to Virkon solutions of varying concentrations and periods of time. Resulting mortality was measured.
MATERIALS AND METHODS

Phase 1: Efficacy of Virkon against zebra mussel veliger larvae

*Veliger collection and water quality assessment:*

Veligers were collected before each of five replicate trials using a series of horizontal plankton tows on the surface of Otsego Lake at one of three sites chosen for their relative shallowness and presence of a rocky lakebed (Figure 2):

Site A: South-central Otsego Lake  
N 42° 42' 35.4''  
W 74° 55' 14.1''

Site B: Blackbird Bay  
N 42° 42' 26.2''  
W 74° 55' 32.1''

Site C: North-central Otsego Lake:  
N 42° 47' 33.7''  
W 74° 53' 43.5''

Figure 2. Veligers used in this study were collected from Otsego Lake at three different sites: South-central (A), Blackbird Bay (B), and North-central (C).

Horizontal tows were performed from a boat moving at a speed of approximately 3 to 5 knots for about 15 minutes per collection. Speed and path were variable in order to sample multiple levels of the water column. A 63μm mesh plankton net and a 61μm mesh collection cup (Wildco®) were used for collection before being emptied into an opaque 1-L plastic bottle. Veliger density was ascertained immediately after returning to the lab (see below).
After veliger collection, water quality of each collection site was assessed using a multi-parameter probe (6820 V2-2 Multi-Parameter Water Quality Sonde, YSI Inc.) at a depth of 1 meter.

*Treatment preparation:*

Treatment solutions were prepared at least one week in advance of each replicate trial by dissolving the appropriate mass of solid Virkon™ Aquatic (DuPont, Lot# 1410174000) in 63μm-filtered lake water to yield 3.00, 2.00, 1.00, 0.500, and 0.250, and 0% treatments. Virkon solutions were stored in the dark at ambient laboratory temperature in 500 mL covered plastic Nalgene bottles. Before each treatment, 100mL aliquots of each solution were poured into 250mL borosilicate glass beakers.

Veligers from each sample were identified morphologically and quantified using a 1mL Sedgwick-Rafter cell at 100x magnification under cross-polarized light (Johnson 1995). To maintain a level of consistency between experimental runs, sample volumes were adjusted to attain veliger concentrations between 150-300 individuals per milliliter. Dilutions were made with filtered lake water while concentration was achieved by gravity filtration through a 63μm mesh cup.

Once brought to the appropriate concentration, 4mL of veliger-concentrated lake water sample were dispensed into each of thirty 63μm mesh-bottomed veliger holding devices (VHDs) constructed by Davis et al. (2015) which were partially submerged in a 20 x 30 x 5cm porcelain holding pan containing 1400mL of filtered lake water. Individual holding devices consisted of inverted 15mL plastic Corning® centrifuge tubes with a quarter inch (6.35 mm) hole drilled into each screw cap, with the holes being covered with 63μm Nitex mesh so that water and small particles could flow through while retaining veligers. Conical ends of each tube were cut off, leaving an open top.

VHDs were stabilized with a double layer of 3 x 3.5cm mesh steel chicken wire fastened to the top of the holding pan. When moving veligers to and from the VHDs, pipette tips were cut to increase their internal diameter to approximately 5mm to decrease the likelihood of damage to the veliger’s fragile valves. After dispensing, veligers were allowed to settle in the lake water for a minimum of 30 minutes before treatment (Figure 3).

Figure 3. Mesh-bottomed VHDs allowed veligers to be moved in and out of treatment solutions with minimal handling. Before and after treatment, VHDs were placed in a porcelain pan containing 1400mL of filtered lake water.
Treatment:

VHDs were taken out of the holding pan, rinsed with lake water, and were submerged and briefly swirled in the appropriate treatment solution. VHDs were held in their respective treatments for 5, 15, 30, 45, or 60 minutes.

At the end of the treatment, VHDs were rinsed three times and the outside patted dry before being partially submerged in a tank containing 20 L of filtered lake water, drawing water into the VHD multiple times to thoroughly rinse and dilute any residual treatment that may have remained. VHDs were patted dry and rinsed once more before being placed back into the holding pan for 1-2 hours before mortality assessment.

Treatment start times were staggered in five minute intervals among, and ten minutes between, each time class to compensate for handling time and mortality assessments.

Mortality assessment:

After 1-2 hours of holding in ambient lake water, veligers were assessed for mortality using a 1mL Sedgwick-Rafter cell at 100x magnification under cross-polarized light. A clean pipette widened to approximately 5mm internal diameter was used to transfer veligers to the cell from each VHD. During each assessment, a range of field depths was used under the microscope to observe individual veligers in full. Veligers exhibiting obvious mechanical damage (ie. cilia and/or organs showing movement with cleanly fractured valves) or those which could not be enumerated (i.e. erratic valve fragments) were ignored.

Mortality was considered in cases where internal organs and cilia were still, where internal organs were spilling out of the valves, or if the veliger was observed with empty valves altogether. Veligers with valves so darkened by oxidation that no internal structures could be observed were also considered dead.

Barring one exception, mortality assessments were conducted for each treatment until at least 30 veligers were observed. To maximize significance of the data analysis, some assessments were continued well past 30 individuals provided that the assessment had not run past 2 hours post-holding and that recovery from the holding device had not appreciably diminished.

Phase 2: Efficacy of Virkon against adult zebra mussels.

Mussel collection and setup

Adult zebra mussels were harvested from rocks collected from the source of the Susquehanna River (N 42° 41’ 58.9” W 74° 55’ 13.2”) in water 1 – 2 meters deep. Mussels were removed from rocks with a paint scraper (Davis et al. 2015) and transported to the lab for placement into mesh bags (Doc Foster CE-22541) in sets of eleven live mussels each. A target range of 15-30 mm valve length was sought during selection and bagging in order to exclude mussels in the juvenile life stage. Bags were suspended by dowels in one of two 50-L aquaria
with slow, constant flows of aerated lake water for at least 48 hours to acclimate mussels to ambient laboratory conditions.

After the acclimation period, fifteen 20-L aquarium tanks were filled with filtered lake water and dosed with Virkon to yield three replicates of the following concentrations: 1.00, 0.50, 0.25, 0.10 % with three negative control tanks filled only with filtered lake water. Each tank was lightly aerated throughout the experiment with compressed air.

Mussels in each bag were assessed for mortality before the experiment: Adult mussels with tightly closed valves were considered to be alive while those with gaping valves and no response to stimulus with a blunt probe were considered dead. Dead mussels were removed and replaced to a total of ten mussels per bag. If no dead mussels were present, one mussel was removed at random.

Treatment

Ten bags of mussels were suspended by dowels in each 20-L aquarium. One bag was removed from each tank at ten predetermined time points (5 and 30 minutes; 1, 2, 4, 6, 8, 12, 24, and 72 hours) before rinsing in a 500-L aquaculture tank filled with lake water to remove any residual Virkon. Bags were then moved to one of two 50-L aquaria with slow, constant flows of aerated lake water for 48-72 hours post-treatment. This recovery period was intended to allow those mussels that might have appeared dead to show signs of recovery, as mussels treated with disinfectants sometimes falsely appear dead immediately after chemical exposure (Pucherelli et al. 2014).

Mortality and measurement

Mussels were individually evaluated for mortality after holding in post-treatment aquaria according to the criteria listed above. Mussel lengths were obtained using a digital caliper and recorded along with mortality.

Water quality assessment

A calibrated multiparameter water quality sonde (YSI Incorporated, Model Number: 6820V2-M) was used to monitor physical and chemical parameters of water quality in each experimental, holding, and recovery tank. Data on conductivity, pH, dissolved oxygen, and temperature were obtained at 8:00 each morning.

Statistical analysis

Binomial logistic regression models were used to analyze the effects of Virkon concentration and exposure time on the mortality of zebra mussels. Mortality was analyzed using separate models for veligers and adult mussels. A Bayesian hierarchical approach was used to model variation in mortality ($p$) due to the interactive effect of Virkon concentration and exposure time ($TIME$) for each trial ($i$). To incorporate potentially different responses to exposure time between the different doses, we modeled dose as a random effect on the slope ($\beta_{\text{time}, j}$) of the linearized relationship between mortality and time:

$$\text{logit}(p_{i,j}) = \beta_0 + \beta_{\text{time}, j} \cdot TIME_i$$
The approach assumed that the number of dead mussels in each \( i \)th trial \((D_i)\) was drawn from a binomial distribution defined by the probability of mortality in each \( j \)th Virkon concentration \((p_{i,j})\) and the number of zebra mussels in each trial \((N_i)\):

\[
D_i \sim \text{Binomial}(p_{i,j}, N_i)
\]

We used uninformative prior distributions for model parameters to allow data to guide conclusions about the process of interest. We assumed a shared intercept \((\beta_0)\) among all doses because all trials started at time zero, with zero mortalities. We used a diffuse normal prior distribution with a mean of zero and a variance of 10 on the intercept.

\[
\beta_0 \sim \text{Normal}(0, 10)
\]

We hypothesized that increased exposure time would have a positive effect on the number of dead zebra mussels in each trial across all doses, but that the effect of exposure time \((\beta_{\text{time}, j})\) would vary between doses. In order to incorporate variability in the intensity of this effect (i.e. shape of the dose-response curve), we assumed that the effect of exposure time was drawn from a global population of possible effects represented by a normal distribution with hyperparameters \(\mu\) and \(\sigma^2\).

\[
\beta_{\text{time}, j} \sim \text{Normal}(\mu, \sigma^2)
\]

The mean of the global distribution for \(\beta_{\text{time}, \mu}\), was assigned a diffuse normal prior with a mean of zero and a variance of ten, and we used a uniform prior distribution on \(\sigma^2\) that ranged from zero to ten. This approach allowed us to share information across all trials to estimate hyperparameters for the global distribution of \(\beta_{\text{time}}\) to improve parameter estimation while allowing the effect of time to vary between Virkon concentrations.

We used Markov chain Monte Carlo (MCMC) methods to estimate model parameters in JAGS using the ‘R2jags’ package (Su & Yajima, 2015) in R (R Development Core Team, 2017). We used a burn-in of 3,000 runs, and simulated an additional 30,000 samples from each posterior distribution, saving every 30th sample to reduce autocorrelation between samples and increase the number of independent samples from the posterior distribution (Kruschke 2010). We ran a total of three Markov chains for each parameter, resulting in a total of 3,000 samples from which to construct posterior distributions. We assessed convergence among the three chains for each parameter using the Gelman-Rubin convergence diagnostic (Gelman & Rubin, 1992), and visually inspected plots of Markov chains to ensure adequate mixing.

RESULTS AND DISCUSSION

Phase 1: Efficacy of Virkon against zebra mussel veligers

Although water quality data collected at veliger sampling locations in the lake varied slightly over the course of the experiment, parameters measured were within the range suitable for veliger growth and survival as described by Baker et al. (1993). Mean temperature at collection was found to be 20.93 °C, mean pH was 8.15, mean conductivity was 0.18 mS/cm, and mean dissolved oxygen was 9.44 mg/mL.
Veligers exhibited 100% mortality when exposed to 3% and 2% Virkon solutions for 15 minutes or longer, or 1% Virkon solution for 30 minutes or longer. While solutions containing 0.5% Virkon or less did hinder veliger survivorship significantly, no solution in this range resulted in 100% mortality. After 30 minutes, each treatment yielded 70% mortality or higher (Figure 4).

A total of 9,343 veligers were assessed over five replicate trials, six concentrations, and six time points (Figure 4). The average number of veligers assessed in each time point, concentration, and replicate was 260 individuals.

When veligers were observed after exposure, evidence of oxidation was scalable with regard to treatment time and concentration (Figure 5). Veligers exposed to low concentrations of Virkon for shorter periods of time had little if any darkening of their valves, but a larger number were observed to be stationary or had odd, jumpy patterns of movement when compared to control individuals in the same time cohort.

Figure 4. Individual posterior predictive dose-response curves for veligers exposed to Virkon. Outer lines indicate the 95% credible interval while percentages indicate dose.
Figure 5. The effect of 3% (m/m) Virkon solution on umbonal-stage veligers over time. Veligers were exposed to a 3% (m/m) Virkon treatment over a course of 1 minute (A), 5 minutes (B), 15 minutes (C), and 30 minutes (D). A-similarly sized veliger in a solution without Virkon is also shown (A). Images B-D are of the same individual and were captured under 100x magnification with a cross-polarized light source. To minimize the effect of heat on the reaction, the light source was switched off between captures.
Phase 2. Efficacy of Virkon against adult zebra mussels

Average mussel length was 22.53 mm with a standard deviation of 2.61. Average mussel length for dead individuals was 22.55 and average length for living individuals was 22.51, suggesting no effect of size on susceptibility of adult zebra mussels to Virkon.

Adults exhibited 100% mortality following exposure to all concentrations after 48 hours and no treatment was 100% effective in any of the three replicates before that time. During the study, 11 individuals in control treatments died, with one dead individual each at the 5 and 30 minute and 24-hour time points, two at 12 hours, and three each at 38 and 72 hours.

Wider 95% credible intervals in the posterior predictions for adult mussels reflect more variation in the adult response to Virkon treatment than in veliger response.

![Figure 6. Individual posterior predictive dose-response curves for adult zebra mussels exposed to Virkon. Outer lines indicate the 95% credible interval while percentages indicate dose.](image)

Exposure to Virkon at higher dose-time sets yielded brittle, pitted, and bleached valves when compared to individuals from the control group (Figure 7). This is presumably due to Virkon’s mechanism as a strong oxidizing agent.
CONCLUSION

Due to its complex action mechanism, low toxicity, high biodegradability, and success in producing 100% mortality in two life-stages of zebra mussels, Virkon seems to be a potential candidate for disinfecting materials moving between zebra mussel infested water bodies so long as ample exposure time is provided. Mortality rates of 100% were achieved in 15 minutes with 2% or higher solutions. Since adult mussels are large and can be seen with the naked eye, a combination of manual removal of adults and treatment with a Virkon solution to rid materials of veligers may be the most practical approach, as the 48 hours of treatment for adult eradication may be too long of a time to wait or too damaging to the material being disinfected.

Like any treatment, Virkon has its drawbacks. It should not be used to disinfect any equipment or container that might contact water samples intended to be analyzed for phosphorus, as it appears to contain approximately 10% phosphate by mass (personal observation); a 1.0% solution was measured to contain approximately 1.09 g total phosphorus per liter (approximately five orders of magnitude higher than that of local surface waters). Exposure of sampling gear to a 1.0% solution required soaking in hydrochloric acid to remove phosphorus residues. According to the distributor, Virkon is only stable for 1-2 weeks in solution, is very hydroscopic, and as such must be kept completely dry when stored. It also may be deactivated by sunlight or by high concentrations of organic material (Western Chemical 2016). Its cost is moderate, at approximately $70 US (2016) for 10 lb ($15 US/kg). The SDS lists it as an irritant in its powdered form, and personal experience is that multiple water rinses are needed to remove its residue after use. Lastly, since it contains a strong oxidizer under acidic conditions, it may not be compatible for use with some metals or other materials.
Additional work that may be performed includes increasing sample size and replicates for both phases of the project to improve statistical power, performing a thorough validation of stability over time and with exposure to organic material and sunlight, conducting a materials compatibility study, and evaluating the disinfectant for use against other aquatic invasive species. According to Diggins (2001) and Costa et al. (2008), adult zebra mussels may alter their filtration rates by season and may vary by a factor of 22 between the summer and winter. The downscaling of filtration by the adult mussels in the fall and winter seasons (when phase 2 of this study was performed) may make the mussels less susceptible to chemical treatments. Because of this, the study should also be performed in the warmer months to determine whether less Virkon may be used for shorter periods of time.

REFERENCES


Su, YS. and Yajima M. 2015. R2jags (R Package version 0.5-7).


