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Efficacy of Two Approaches for Disinfecting Surfaces and Water Infested with Quagga Mussel Veligers

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ABSTRACT Disinfection tools and protocols are needed to reduce the probability of transferring invasive mollusk species as hitchhikers. Applications are needed to provide rapid disinfection of recreational equipment, boats, and tankers and other materials used in fire suppression. Moreover, tools are needed that are safe for hatchery and aquaculture operations. In two separate laboratory trials, we tested the lethality of elevated pH or a commonly used aquaculture disinfectant on quagga mussel (*Dreissena rostriformis bugensis*) veligers. We found that all reagents were effective in killing veligers. Aqueous solutions of pH 12 were created with NaOH or Ca(OH)2 and tested at 16°C and 20°C, and three aqueous concentrations of Virkon® Aquatic were tested at 20°C. We observed 100% mortality within a 10 min exposure in solutions of pH 12 prepared with Ca(OH)2 and within a 30 min exposure in solutions prepared with NaOH. We found that solutions of 5 g/L of Virkon Aquatic killed all veligers within a 10 min exposure. We concluded that all chemicals show promise as disinfectants, and use of $Ca(OH)_2$ or NaOH to elevate the pH of disinfecting solutions may provide a more economical and environmentally acceptable way to disinfect large surfaces or tanks.

Introduction

Global transportation of commodities and human movements pose challenges to natural resource managers, as the risks of transporting nonnative, invasive organisms are compounded with each vehicle and vector. In addition, many altered ecosystems are more vulnerable to invasion as natural processes can be in flux (Norbury et al. 2013; Thorp 2014). Mollusks are among the most highly successful invasive aquatic organisms, as they are armored with shells that protect them from desiccation, and have a great

capacity as engineers of systems in which they establish because of their capacity for filtration (Cuhel and Aguilar 2013). The vectors and vehicles that transport and distribute invasive mollusks to new locations include fish, birds and mammals, aquarium traders, ship ballast water, recreationalists, agency personnel, and other water users (Alanso and Castro-Diez 2008; Bowler 1991; Bruce and Moffitt 2010).

Quagga mussels, native to eastern Europe, were accidentally introduced into the Laurentian Great Lakes in North America in the 1980s in ballast water (Strayer 2009; Vanderploeg et al. 2002). The major pathway of quagga mussel introduction from the Great Lakes to other nonhydrologically connected locations in North America has been assumed to be recreational boaters (Johnson et al. 2001, 2006; Leung et al. 2006; Mueting and Gerstenberger 2011). It is hypothesized that quagga mussels were introduced into Lake Mead sometime before 2007 in bilge water or with bait or live wells in boats from the Great Lakes region (Wong and Gerstenberger 2011).

In addition to transport on recreational vessels, activities such as fire suppression and fish stocking are of concern in western North America as they move large quantities of raw water resulting in high risks of transfer of invasive mollusks (Britton and Dingman 2011; Bruce et al. 2009; Culver et al. 2000; Edwards et al. 2000). Fish hatcheries in Colorado, Arizona, and Nevada are now infested with quagga mussels, and such facilities are constrained to reduce the risk of transporting veligers with movement of fish, or equipment between locations. Western states transfer water from nearby open water sources via helibucket, pumps, and other operations for fire suppression. The U.S. Forest Service, Bureau of Land Management, and interagency fire management teams are concerned that raw water from infested areas could introduce invasive mollusks into new areas (Britton and Dingman 2011).

Once established, quagga mussels can affect ecosystems and damage infrastructure of cooling pipes, water intake pipes, head gates, and hatchery facilities (Peyer et al. 2009; Pimentel et al. 2000, 2005; Wong and Gerstenberger 2011). There is a growing demand for tools that can be used to address both small- and large-scale disinfection needs using methods that are effective and safe to humans and the environment. Despite the need for innovative disinfection tools, economic constraints for purchase of chemicals and infrastructure used in the disinfection process can be limiting factors (Karatayev et al. 2007). Often highly effective disinfectants are expensive or they have substantial environmental consequences if used in large quantities. Chemicals that can be used in large-scale applications must be less expensive and should have attributes that would allow for safe neutralization and disposal.

Developmental stages of quagga mussel veligers have different survival times, but veligers are reported to remain alive for weeks in moist environments depending on temperature (Choi et al. 2013). Britton and Dingman (2011) found that a 10 min exposure to a 3% concentration of Sparquat 256® was sufficient to kill 100% of tested veligers held 60 min post treatment. These and similar quaternary ammonia reagents have been reported effective in killing quagga mussel veligers and other invasive species in and around fish hatchery environments (Mitchell and Cole 2008; Oplinger and Wagner 2009; Waller et al. 1996). However, widespread use of quaternary ammonia compounds has caused environmental concerns as the treatments can affect aquatic and soil systems (Garcia et al. 2001; Li and Brownawell 2010; Sarkara et al. 2010), and their persistence has increased concerns regarding genotoxic effects (Ferk et al. 2007). Other compounds have been considered to reduce the risk of transporting dreissenid mussels and mitigate the effects of their biofouling abilities. Edwards et al. (2000) used KCl and formalin to reduce risks of invasive zebra mussels (*Dreissena polymorpha*). Antifouling compounds are also of great interest to protect infrastructure in aquaculture and industry but environmental consequences must be considered (Guardiola et al. 2012). Chlorine and other oxidizing reagents are effective tools for disinfection (Brady et al. 1996), but the residual compounds have environmental consequences for water supplies and aquatic organisms (Watson et al. 2012). Recent studies reported the efficacy of a proprietary copper chemical EarthTec® to kill and prevent settlement of veligers (Watters et al. 2013), but dosages tested were above those considered safe for most aquatic organisms.

Ship ballast is well recognized as a source of introduction of invasive organisms throughout the globe (Briski et al. 2012). Watten et al. (2007) have explored the use of elevated pH as a means of killing invasive species in ship ballast water and residual ballast solids. Their early studies demonstrated that pH in the range of 11–12.4 was effective in killing zooplankton with relatively short exposure requirements and that pH could be returned readily to neutral levels through the use of dilution or carbonation. The end

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product of the reaction with CO_2 (bicarbonate alkalinity) can be environmentally benign. The amount of reagent required, either lye (NaOH) or hydrated lime [$Ca(OH)_2$], for specific pH targets increased with increasing ballast salinity. This application has been particularly attractive given that elevated pH avoids ship corrosion concerns associated with certain alternative acid/oxidant ballast treatments. Reagent cost estimates for freshwater applications were attractive, which led to further testing in cooperation with the Great Ships Initiative group, Duluth Minnesota, in a series of bench-scale, pilot-scale, and shipboard-scale studies (Cangelosi 2009, 2011a,b; Cangelosi et al. 2013) conducted with support from the U.S. National Park Service, the U.S. Environmental Protection Agency, the U.S. Department of Transportation, and the American Steamship Company. Other studies support the use of hydroxide alkalinity as a tool to reduce microbial populations including fish pathogens (Starliper and Watten 2013). NaOH has long been successfully used as a dairy disinfection tool for milking equipment as an alternative to chlorine (Gleeson et al. 2013). Hydrated lime is a reagent successfully used in treatment of wastewater (Grabow et al. 1978; Scanar et al. 2001).

Virkon Aquatic (reformulated from Virkon S in 2007) is one of very few U.S. Environmental Protection Agency–registered disinfectants labeled for use in aquaculture facilities for use on bacterial, fungal, and viral pathogens (DuPont 2011; Mainous et al. 2010). Active ingredients of Virkon Aquatic are potassium monopersulfate and sodium chloride, 21.9% (Mitchell and Cole 2008). Its mode of action is oxidizing proteins and other components of cell protoplasm, resulting in the inhibition of enzyme systems and loss of cell-wall integrity (Curry et al. 2005). The recommended concentration for disinfecting most surfaces for a majority of the organisms is a 10 g/L concentration with an exposure time of at least 5 min (Western Chemical 2008).

The objectives of our study were to compare the mortality response of quagga mussel veligers in disinfectant strength solutions of aqueous pH 12 made with $Ca(OH)_2$ or NaOH and in solutions of the approved hatchery disinfectant, Virkon Aquatic. In addition, we explore the relative costs, environmental risks and benefits, and suitability for each disinfectant for use on gear, boats, tanks, and other applications.

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Methods

Study Location and Source of Organisms

All tests were conducted at Willow Beach National Fish Hatchery, Willow Beach, Arizona. Tests were conducted from October 13 to 22, 2009 (Virkon Aquatic trials) and from November 17 to 25, 2012 (elevated pH). Veligers for tests were collected with a 30 μm mesh plankton tow net hung from the grating system of a raceway head box for 30–60 min. The contents of the cod end were then filtered through a 500 μm nylon mesh screen into individual 500 mL Nalgene sample bottles. The contents of each sample bottle were allowed to settle at test temperatures for 1–3 h acclimation. Condition of veligers was assessed, and plankton tow samples with greater than 90% living active veligers were retained for testing. The veligers were concentrated by collecting settled filtrate on ~100 μm mesh nylon fabric and gently placing this into a 1.8 L Nalgene container and rinsing with squirt bottles of aerated well water at test temperatures.

Test Chemicals

We weighed ~0.6 g/L NaOH (Fisher Chemical, Pittsburgh, PA, Lot 060432) or $Ca(OH)_2$ (J.T. Baker Chemical, Phillipsburg, NJ, Lot G43364), dissolved into aerated well water to reach a final solution pH of 12. The test solutions were filtered to remove particulates, and placed into sealed Nalgene carboys to acclimate to test temperature overnight. The pH of each solution was verified with a Hach sensION platinum portable pH meter, or YSI 556 multiprobe. Virkon Aquatic (lot #2258523) (Western Chemical, Ferndale, WA) was measured (0.01 g) and mixed in volumetric flasks with well water at 18°C. Solutions were placed at test temperatures for at least 1 h to acclimate and activate. The test concentrations of 2.5, 5, and 20 g/L were verified with Virkon Aquatic test strips (Western Chemical, Ferndale, WA).

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Experimental Design

Elevated pH: A 2 mL sample from the bottom of the Nalgene container with concentrated veligers acclimated to test temperature was removed with a disposable plastic pipette. Each veliger sample was placed into a separate 120 mL beaker (4 test replicates per time interval). We then added 100 mL of test solution rapidly and recorded the starting time of exposure. Static exposures were conducted at a cool temperature (~16°C) and at room temperature (~20°C). To provide the cool temperatures, test and control beakers were maintained in a water bath (raceway trough), and temperatures were recorded continuously with data loggers (mean ± SD = 15.9°C ± 0.14°C). Room temperature tests were conducted on a laboratory bench (20.1°C ± 0.84°C). Groups of beakers were terminated at intervals of 2, 5, 10, 20, or 30 min. At the end of each time interval, the contents of each beaker were poured through a stainless steel mesh spoon lined with ~100 µm nylon mesh to collect veligers. We used well water acclimated to the test temperature to rinse residual test solution from the veligers. After rinsing, the nylon mesh with veligers was placed into a labeled glass Petri dish and rinsed to remove all veligers. Veligers were maintained in the glass Petri dish and allowed to recover for 24 h at test temperature before final assessment of mortality. As a control, we held and handled replicated beakers of veligers not exposed to test compounds at each interval. To determine surviving veligers, the glass Petri dishes were placed under a dissecting microscope and the number of live and dead veligers was counted. Veligers were considered alive if cilia was moving and moving or spinning organs and food contents could be seen inside the shell. Veligers were considered dead if shells were open or empty, body parts had crystallized, or veligers showed no reaction when disturbed.

Virkon Aquatic trials: We used a serological pipette to transfer 1 mL of concentrated veligers acclimated to room temperature from the Nalgene bottle into a 150 mL glass beaker. We then added 9 mL of stock Virkon Aquatic concentration to achieve a final concentration of 2.5, 5, or 20 g/L. Tests were conducted with two replicate beakers for each concentration and time interval. Replicate groups of veligers were assessed after 5, 10, 15, or 20 min of exposure. To evaluate survival in each test replicate, we removed 3 mL of test solution and veligers from the bottom of a test beaker and placed the contents into a 30 mm diameter Petri dish. A dissecting microscope was used to locate and enumerate veligers. After a rapid assessment of mortality, all veligers were placed in aerated well water for recovery, and final assessment of survival was recorded after 24 h with the aid of a Sedgewick rafter microscope slide and compound microscope. No movement of cilia, visible organs, and darkening or crystallization of the veliger, and lack of reaction when disturbed constituted mortality. All tests with Virkon Aquatic were conducted at room temperatures that ranged between 19°C and 22°C.

Data Analysis

We analyzed the proportion of live veligers in tests of elevated pH with a global logistic regression model that included test chemical, temperature, and exposure interval. Estimates of model fit and maximum likelihood estimates were evaluated with type III Wald chi-square effects. To discern more details of the response within each test intervals, we compared the proportions of live and dead veligers by the duration of exposure to observe chemical and temperature effects using log-linear categorical models (Stokes et al. 2000). For test intervals where only one chemical was tested, we report simple exact frequency probabilities to compare the outcome of live and dead by test temperature. In tests of Virkon Aquatic, no temperature comparisons were made, and we compared the frequency of live and dead veligers in concentrations of 2.5 and 5 g/L at exposure times of 5 and 10 min. We reported the results of exposure to 20 g/L in tabular format only. We used a probit model to estimate the LT_{50} in exposures to 2.5 g/L as there were adequate data for model convergence. All statistical analyses were conducted in SAS version 9.2 (SAS Institute, Cary, NC).

Results

We observed rapid mortality of all veligers exposed to elevated pH (Figure 30.1). We found significant effects attributed to test compound, temperature, and exposure time (all Wald χ^2 $P < 0.001$; Table 30.1). The mortality response to $Ca(OH)_2$ was more rapid and evident best in comparisons of

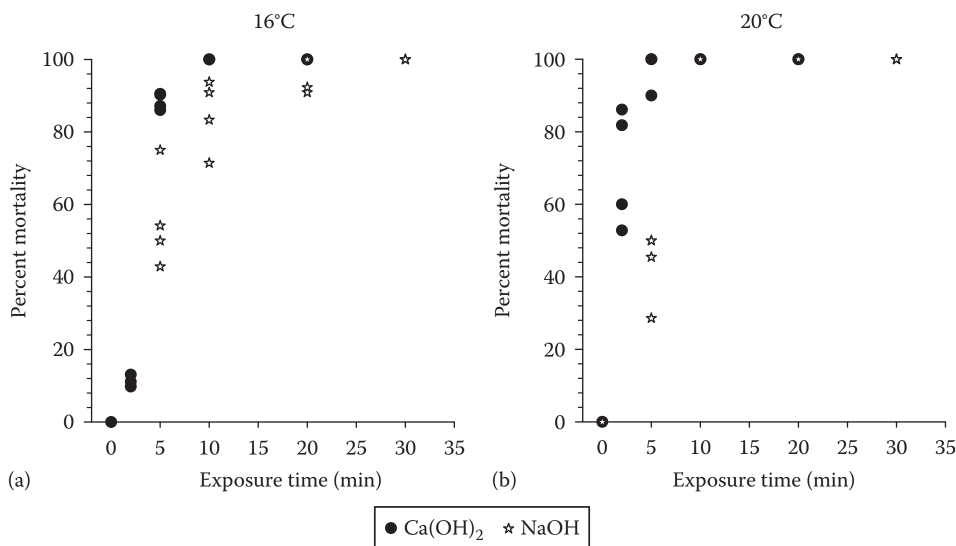

FIGURE 30.1 Summary of percent mortality of veligers versus time of exposure in replicated trials of $Ca(OH)_2$ and NaOH at pH 12 at two temperatures: 16°C Panel (a) and 22°C Panel (b).

TABLE 30.1

Summary of Logistic Regression Analysis of Proportion Live in Replicate Tests of Elevated pH 12 with Two Chemicals [$Ca(OH)_2$ or NaOH]

Parameter	DF	Estimate	Wald Chi-Square	*P*
Intercept	1	7.35	172.88	0.001
Chemical	1	−1.148	91.91	0.001
Temperature	1	−0.231	88.90	0.001
Interval	1	−0.814	225.71	0.001

Note: Maximum likelihood estimates of Wald chi-square and *P* values are provided.

responses in 10 and 12 min exposures. We found no effect of test temperature after 2 min exposure in solutions of $Ca(OH)_2$ ($\chi^2 = 1.7$, DF = 1; $P > 0.180$; Table 30.2), but the response was observed at the 5 min exposure ($P < 0.001$; Tables 30.2 and 30.3). After 10 min, the differences between test temperature were not as pronounced ($P = 0.069$), but we detected a significant test chemical by temperature interaction. The likelihood ratios of survival after a 20 min exposure to either chemical were highly significant between temperature and test chemical. In veligers exposed to pH 12 created with $Ca(OH)_2$, we recorded complete mortality at both water temperatures, but 3% of veligers held at 15°C in NaOH survived (Tables 30.2 and 30.3). Throughout our trials, we observed little to no mortality in control tests of veligers that were handled similarly, but not exposed to elevated pH (Table 30.2).

Veligers exposed to 2.5 g/L Virkon Aquatic achieved 100% mortality between 10 and 15 min (Table 30.4). Higher concentrations (5 g/L) achieved 100% mortality within 10 min. We found significant differences between the frequency of live and dead veligers between concentrations of 2.5 and 5 g/L after 5 min of exposure ($\chi^2 = 30.49$; $P < 0.001$), but no significant difference in frequency of live and dead veligers after a 10 min exposure ($\chi^2 = 2.52$; $P > 0.285$). Using the results of exposure to 2.5 g/L Virkon Aquatic, we fit a significant probit model for mortality versus exposure time (Wald $\chi^2 = 15.68$; $P = 0.001$) and estimated an LC_{50} of 6.1 min (95% CI = 4.1 − 7.3). Our test system had no effect on veliger survival, as all controls were alive during the testing and recovery time (Table 30.4).

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TABLE 30.2

Summary of the Number of Veligers Dead or Alive and Percent Survival (in Parentheses) in Tests of $Ca(OH)_2$ and NaOH and Controls by Time Interval, Temperature, and Test Compound, Replicates Combined

	16°C		20°C	
Chemical	**Dead**	**Live**	**Dead**	**Live**
2 min				
$Ca(OH)_2$	21	185	116	56
	(11)	(89)	(67)	(32)
Control	0	117	4	82
	(0)	(100)	(5)	(95)
5 min				
$Ca(OH)_2$		182	25	150
	(88)	(12)	(97)	(3)
Control	3	60	3	56
	(5)	(95)	(5)	(95)
NaOH	45	39	24	31
	(54)	(46)	(43)	(56)
Control	3	64	5	35
	4	96	88	12
10 min				
$Ca(OH)_2$	137	0	84	0
		(100)	(0)	(100)
Control	0	45	2	29
		(100)	(6)	(94)
NaOH	70	12	97	0
		(85)	(15)	(100)
Control	0	41	6	97
		(100)	(6)	(94)
20 min				
$Ca(OH)_2$	60	0	110	0
		(100)		(100)
Control	0	34	1	72
		(100)	(1)	(99)
NaOH	68	2	72	0
		(97)	(3)	(100)
Control	2	51	2	122
	(4)	(96)	(2)	(98)
30 min				
NaOH	53	0	78	0
		(100)		(100)
Control	1	40	4	92
	(2)	(98)	(4)	(96)

Note: Veligers exposed to NaOH were not scored at 2 min, and veligers exposed to $Ca(OH)_2$ were not scored at 30 min. All final assessments were made after 24 h recovery period.

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TABLE 30.3

Summary of Maximum Likelihood Analysis of Variance Log-Linear Comparisons of Independence for Frequency of Live and Dead Veligers, Tested Individually for Three Intervals of Exposure Modeled by Differences between Chemical Tested and Test Temperatures at pH 12

Source	DF	Chi-square	*P*
5 min			
Temperature	1	12.28	0.0005
Chemical	1	90.79	<0.0001
Chemical*Temp	1	0.44	0.5094
Likelihood ratio	4	306.44	<0.0001
10 min			
Temperature	1	3.32	0.0685
Chemical	1	90.79	<0.0001
Chemical*Temp	1	0.44	<0.0001
Likelihood ratio	1	45.4	<0.0001
20 min			
Temperature	1	32.67	<0.0001
Chemical	1	17.19	<0.0001
Chemical*Temp	1	0.25	0.6199
Likelihood ratio	1	78.88	<0.001

TABLE 30.4

Summary of Live and Dead Quagga Mussel Veligers in Trials of Three Concentrations of Virkon Aquatic for Durations of 5–20 min Exposure

Concentration (g/L)	Exposure (min)	Number Dead	Number Alive	Percent Mortality
2.5	5	3	7	30
	10	31	1	96.9
	15	20	0	100
	20	20	0	100
5.0	5	110	7	94
	10	80	0	100
20	5	30	0	100
0 (control)	5	0	60	0
	10	0	20	0
	15	0	20	0
	20	0	30	0

Note: Trials were conducted at room temperature, ~20°C.

Discussion

Solutions of pH 12 made with NaOH or $Ca(OH)_2$ were effective in killing veligers, although more time was needed at the cooler water temperature to achieve 100% mortality. Similar success with laboratory trials of disinfection testing a suite of microorganisms was achieved by Starliper and Watten (2013). They compared colony-forming units as an endpoint of efficacy in solutions of NaOH at pH 10–12. They achieved 100% mortality of all Gram-negative and Gram-positive cultures. In recently reported shipboard trials conducted by the Great Ships Initiative, tests with NaOH pH 12 showed large reductions in the density of live organisms ≥50 µm in the treatment discharge samples over samples analyzed in control discharge (Cangelosi et al. 2013).

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Claudi et al. (2012) evaluated the effect of a range of pH on mussel settlement and mortality and determined that dreissenid mussels have a relatively narrow range of pH tolerance, with the optimum range for settlement of pH 7.5–9.3. Other factors such as calcium, flow velocity, water temperature, dissolved oxygen, conductivity, total organic carbon, and the surface roughness scan affect the settlement of mussels (Chen et al. 2011). The rapid mortality in our tests with veligers is likely also related to the size of the organisms tested when compared with time to mortality in adult-sized mussels. The veligers in our trials ranged from approximately 180 to 220 μm in length and were characteristic of the sizes reported for Lake Mead in October and November (Gerstenber et al. 2011). Mitchell and Cole (2008) found that solutions of pH 12–13 made with hydrated lime or sodium hydroxide were not effective in killing adult-sized faucet snails after a 1 h exposure.

Our tests with Virkon Aquatic were also very effective in killing veligers. A short exposure of 5–10 min at 5 g/L killed all veligers. Our quagga mussel veliger test results support the report by O'Connor et al. (2008) that found 5 mg/L of Virkon Aquatic caused 100% abnormal embryo development in Sydney rock oysters (*Saccostrea glomerata*). Stockton and Moffitt (2013) found that a 15–20 min bath application of 20 g/L Virkon Aquatic was a reliable tool to disinfect boot surfaces infested with New Zealand mudsnails (*Potamopyrgus antipodarum*) and other aquatic invertebrates. Mitchell et al. (2007) reported 100% mortality in red-rim melania (*Melanoides tuberculata*) at concentrations greater than 1 g/L for 24 h (Mitchell et al. 2007). However, faucet snails (*Bithynia tentaculata*) exposed to concentrations of 2 g/L Virkon for 1–24 h showed little mortality (Mitchell and Cole 2008).

Our tests with Virkon Aquatic were conducted at one temperature, and additional tests are needed to establish the relationship between Virkon Aquatic efficacy and water temperature. The active ingredient in Virkon Aquatic is potassium permonosulfate and sodium chloride (Mitchell and Cole 2008; Western Chemical 2008). Potassium and sodium gradients control the neuron response in humans (Starr and Taggert 1998), and have been associated with neural responses in mollusks. A neuron response was first identified in the California sea hare (*Aplysia californica*) (Russell and Brown 1972). Fisher et al. (1991) reported that zebra mussels had a low tolerance for elevated potassium concentrations, and potassium can destroy the integrity of the mussel gill epithelium, leading to asphyxiation.

Trials conducted in our laboratory indicated that Virkon Aquatic was safe for use in and around vertebrates in aquaculture environments (Stockton 2011). Further testing of the efficacy of Virkon Aquatic on other aquatic invasive species is recommended to enable broad-spectrum use. The use of Virkon Aquatic as a disinfectant was comparable to the results obtained using Sparquat 256, but Virkon Aquatic is easily deactivated by organics into simple salts that pose little environmental risk (Stockton and Moffitt 2013).

Determining the appropriate tool for disinfection requires understanding the costs, risks, and environmental consequences of each of these treatments. At present, high-pressure hot water spray has been recommended as an effective means for boat decontamination of attached juvenile and adult mussels (Comeau et al. 2011; Zook and Phillips 2012). The addition of an elevated pH treatment could improve the effectiveness of treatment, as elevated pH residues could be effective in areas that were not adequately heated with the hot water sprays. The use of elevated pH could also be effective in treating water for fire suppression activities. The solutions were easily prepared and used approximately 0.6 g/L dissolved in well water to achieve the pH target. Natural resource interest groups and regulatory agencies have made it clear that safe and nonchemical alternatives for controlling mussel fouling are preferred (Britton and Dingman 2011).

The costs of treatment must be considered as part of any evaluation of chemical disinfection choices. The cost estimates for lye assume a market price of $250/ton for a 75% by weight solution. The cost for hydrated lime assumes a market price of $81/ton. A simple cubic meter solution of aqueous pH 12 would require approximately $0.17 of lye and $0.04 of lime. A kg of Virkon Aquatic sells from aquaculture suppliers for approximately $30, and it would take 5 kg or $150 to make a 5 g/L solution.

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