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ARTICLE

Disinfection of Three Wading Boot Surfaces Infested with New Zealand Mudsnails

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Abstract

New Zealand mudsnails *Potamopyrgus antipodarum* (NZMS) have been introduced into many continents and are easily transported live while attached to wading and other field gear. We quantified the relative attachment by different life stages of NZMS to felt, neoprene, and rubber-soled boots exposed to two densities of NZMS in experimental exposure totes. Attachment by NZMS occurred on boots of all surfaces, but the highest numbers of all life stages occurred on boots with felt surfaces. We found a 15–20-min bath application of 20 g/L Virkon Aquatic was a reliable tool to disinfect boot surfaces infested with NZMS and other aquatic invertebrates. Our studies support that spray application of this disinfectant was not reliable to provide complete mortality of attached adult NZMS or neonates. Wading gear surfaces exposed to repeated bath disinfections showed little deterioration. Our results provide strong evidence that bath disinfections with Virkon Aquatic are helpful to assure biosecurity in field and hatchery settings, but applications should be coupled with cleaning procedures to remove organic materials that can deactivate the reagent.

Many nonindigenous invertebrates and microorganisms have been introduced throughout the world via ships and boats, wading gear, and human activities (Mills et al. 1993; Ricciardi and Rasmussen 1998; Johnson et al. 2001; Briski et al. 2012; Root and O'Reilly 2012). New Zealand mudsnails *Potamopyrgus antipodarum* (NZMS) have been introduced and become established in many continents, and these transplants are speculated to be associated with human activities such as recreational fishing, boating, and aquaculture operations (Bowler 1991; Loo et al. 2007; Bruce and Moffitt 2010; Alonso and Castro-Díez 2012). Studies of the effects of NZMS on the aquatic environment after their introductions show a range of outcomes, probably due to the specifics of the aquatic community and the wide habitat tolerance of the snails (reviewed in Alonso and Castro-Díez 2008, 2012). Some introductions appear to have few consequences on the community (Brenneis et al. 2011; Moffitt and James 2012), others show population density effects on native snails (Lysne and Koetsier 2008), and others result in major trophic shifts (Hall et al. 2006; Riley et al. 2008).

Chemical reagents are useful tools to reduce the spread of NZMS from infested areas on wading gear, but chemical treatments must be effective, safe, and practical for the proposed applications (Dwyer et al. 2003; Hosea and Finlayson 2005). In the USA several chemical disinfectants and biocides have been evaluated for their effectiveness in killing NZMS on contaminated surfaces and gear in field and hatchery operations. Hosea and Finlayson (2005) examined the effectiveness of a large suite of compounds on snail mortality: grapefruit seed extract (GSE), benzethonium chloride (BZCl, Alpha Aesar), Clorox, copper sulfate pentahydrate, Formula 409 degreaser and

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disinfectant, potassium permanganate, isopropyl alcohol, Pine-Sol, and household ammonia. All substances tested by Hosea and Finlayson (2005), except for copper sulfate and Formula 409, caused damage to wading gear. Schisler et al. (2008) explored the efficacy of formulations of quaternary ammonium compounds (QACs), including common Formula 409 and Sparquat 256, but did not test their effects on gear. Oplinger and Wagner (2009a, 2010) tested several compounds and explored their use in the context of fish safety and regulatory concerns. Any approved use must consider both human and environmental safety. Copper sulfate has potentially harmful effects on aquatic species (McGeer et al. 2000; Shaw et al. 2012), and residues of QACs, which have wide use in disinfectants and other industrial applications, have come under increasing scrutiny by scientists and regulators (Boethling 1984; García et al. 2001; EPA 2010; Sarkar et al. 2010). Researchers stress the need for additional testing of disinfecting protocols and chemicals to consider a variety of environmental conditions and target species to improve tools that can be used safely to reduce the risks of transport of viable invasive invertebrates, microorganisms, and pathogens in field and hatchery settings (Schisler et al. 2008; Britton and Dingman 2011).

Highly secure facilities such as hatcheries routinely use disinfectants on equipment and wading gear to limit the spread of diseases caused by bacteria and viral agents. The disinfectant, Virkon Aquatic (reformulated from Virkon S in 2007; Mainous et al. 2010) is registered by the U.S. Environmental Protection Agency (USEPA) and labeled specifically for use in aquaculture facilities as a surface disinfectant against bacterial, fungal, and viral pathogens (DAHS 2011a). Virkon Aquatic is composed of a triple salt of potassium monopersulphate acting as an oxidizing agent, sulfamic acid and malic acid, sodium hexamethaphosphate buffer, and sodium alkyl benzene sulphonate as a surfactant (Western Chemical 2012), and its breakdown products are nontoxic salts. The chemical oxidizes proteins and other components of cell protoplasm, resulting in inhibition of enzyme systems and loss of cell-wall integrity (Curry et al. 2005). In unpublished studies in our laboratory, we found that concentrations of Virkon Aquatic at 20 g/L were effective in killing all life stages of NZMS when target snails were placed in glass beakers and immersed for 20 min (see Stockton 2011).

The efficacy of Virkon S and Virkon Aquatic as disinfectants has been evaluated in several studies reported by Mainous et al. (2010). As a dry powder it is easily stored and transported, and Virkon S was used in the Antarctic for disinfecting boots of visitors to reduce the risk of translocation of microbial pathogens to the sensitive ecosystem (Curry et al. 2005). Other studies demonstrated a reduction in numbers and species of zooplankton (Schmidt et al. 2009) and killing of mollusks, including Sydney rock oyster *Saccostrea glomerata* veligers (Dove and O'Connor 2007), red-rim melania *Melanoides tuberculata* (Mitchell et al. 2007), and faucet snails *Bithynia tentaculata* (Mitchell and Cole 2008). Root and O'Reilly (2012) evaluated the efficacy of 10-g/L Virkon solutions to kill the freshwater diatom *Didymosphenia geminata* in laboratory studies, and Johnson et al. (2003) reported its efficacy against the amphibian chytrid fungus *Batrachochytrium dendrobatidis*. Vertebrates may be more tolerant to exposure, as Schmidt et al. (2009) found tadpoles of the European common frog *Rana temporaria* and the common toad *Bufo bufo* survive limited exposures to 4 g/L Virkon even after a 1-week exposure.

International, national, and state and provincial agencies have increased their attention to developing guidance documents for international trade of livestock and commodities and their potential effects on human health (Firestone and Corbett 2005; Perrings et al. 2010), but there are few, if any, harmonized protocols for disinfecting contaminated gear in field and hatchery settings (Root and O'Reilly 2012). When measures are in place, instructions generally include cleaning and drying of equipment and wading gear, but this step may not be completely reliable. As a precaution, several states, provinces, and countries have banned or are considering prohibitions on the use of felt-covered boots for field and hatchery use because of the risks of transport of invasive species and pathogens (CANS 2012).

The objectives of our study were to test felt, neoprene, or rubber-surfaced wading boots to determine (1) the likelihood of infestation by NZMS, (2) the effectiveness of disinfecting with spray or bath applications of Virkon Aquatic, (3) the effect of the proposed disinfection procedures on the durability of wading gear, and (4) the effectiveness of solutions of Virkon Aquatic when contaminated with organics.

METHODS

Experimental Organisms

New Zealand mudsnails were collected from springs at Hagerman National Fish Hatchery (HNFH), Idaho, and shipped in coolers to the University of Idaho fisheries wet laboratory. Upon arrival, the snails were washed through a 2.0-mm and 0.85-mm sieve to separate snails from sediments. The NZMS samples, which included other aquatic invertebrates from the springs (additional snails and insects), were transferred into 2-L containers with dechlorinated, aged, well water equilibrated to 15°C. A portion of the water in each container was changed every other day. The NZMS were retained in the laboratory for no more than 3 weeks. The culture containers also included some algae and vascular plants such as pondweed *Potamogeton* spp. as food sources for the invertebrate cultures in the laboratory. Temperature in the room was maintained at a target level of 15°C throughout trials and recorded at 15-min intervals with HOBO data loggers (Onset Computer Corporation, Bourne, Massachusetts). A natural photoperiod for the latitude of HNFH was maintained in the test room.

Test Substance

Virkon Aquatic (lot 2258523 or 2258515; Western Chemical, Ferndale, Washington) was used for testing. We weighed Virkon Aquatic (0.01 g) to make 10- or 20-g/L solutions (1% and 2%) in deionized water. Test reagents were then placed into acid-washed totes or flasks until tested. At least 1 h was allowed for

all solutions to acclimate to test temperature and to activate fully. The concentrations were verified with Virkon Aquatic test strips (Western Chemical, Ferndale, Washington) to ensure chemical was mixed and active.

Experimental Design

Colonization of three wading-boot surfaces.—A controlled 3 × 2 factorial designed study was conducted to determine the colonization likelihood of NZMS on three different wading-boot surfaces: neoprene, felt, and rubber. We evaluated the rate of infestation onto the wading surfaces in exposures to two densities, 50 or 100 g wet weight per tote (55 × 37 × 43 cm deep; volume, 114 L), of mixed culture of NZMS with small numbers of other aquatic invertebrates.

To test the rate of colonization, we placed one foot from each of three different wading-boot surfaces (three boots per tote) into a tote with live NZMS. The arrangement of test surfaces was rotated (from left to right) for each replicate test to account for placement variation in our analysis. The boots were left in the colonization tote for 30 min and were then removed carefully and placed into a cleaning bath tote where all organisms attached to the boots were carefully removed, recovered, and enumerated. The numbers of adult, juvenile, and neonate NZMS and other aquatic invertebrates were recorded, and the organisms were identified and then destroyed. The colonization testing was replicated three times at both densities of NZMS.

Efficacy of spray versus bath application.—To compare the mortality of NZMS after a bath or spray exposure we conducted controlled experiments at 15°C. Two test solutions of Virkon Aquatic (10 and 20 g/L) were used. A bath exposure was conducted with 100 mL of test substance introduced into a 150-mL acid-washed glass beaker. A spray test was conducted on 10 adult-sized NZMS in a 30-mm-diameter glass petri dish. A spray application consisted of two sprays (∼2 mL) from a 1-L spray bottle of test solution onto the snails in the petri dish. To serve as controls, we treated snails in beakers and petri dishes with water and no chemical. The test durations were 15, 20, or 30 min for bath applications and 20, 30, or 40 min for spray applications. We increased the exposure time for the spray applications to determine whether the added time would result in additional mortality. Each trial was repeated three times for each concentration and exposure time.

At the end of each exposure, the snails were removed from the test system by pouring the test chemical and snails through a sieve. Snails were rinsed three times with clean, aged, laboratory water and placed into small 250-mL plastic cups containing aged water for recovery. Mortality of test organisms was observed immediately and at 24 h, and final assessment was conducted after 48 h by counting the number of live and dead NZMS in each cup with the aid of a dissecting microscope. We observed snails for movement or probed individuals to elicit movement or tactile response. Neonates released from test snails were counted and assessed for mortality. We recorded temperature, pH, and conductivity in replicate test and control beakers containing NZMS before and after the test duration with a YSI 556 MPS multiprobe (YSI, Yellow Springs, Ohio).

Disinfection of three wader surfaces infested with NZMS.—To determine the efficacy of disinfections and to evaluate whether the multiple bath disinfections of infested surfaces affected wader durability, we tested felt, neoprene, and rubber wader boot or wading bootie surfaces in laboratory simulations. We obtained 18 waders of each type from regional field or hatchery operators (54 total). All waders tested were in good condition at the start of testing, but the equipment had been used (to simulate a normal field or hatchery setting). Waders were cut at the knee to leave only the boot or bootie. They were numbered with unique codes prior to the experiments and washed to ensure that there was no contamination of the NZMS container. Before and at the end of each experiment, all boots were inspected and any damage, such as cracks, holes, stains, tears, and discoloration, was recorded. At the end of the testing the boots were inspected for change in condition and the damage was ranked as (1) none if there were no cracks, discoloration, loss of flexibility, tearing, or stitching failure, and the gear could still repel water, (2) mild if the wading gear had slight discoloration, small cracks, or tearing, and the gear still repelled water, or (3) severe if the wading gear showed signs of high discoloration, flexibility loss, large cracks and tears, or stitching failure, and the boots did not repel water or leaked significantly.

To allow for a volitional laboratory infestation of the wader boots, we placed adult NZMS (>3 mm) into a 114-L tote (55 × 37 × 43 cm) 2 d before testing. Before each trial, we removed any snails from the sides of the tote and swirled the contents to spread out the snails on the bottom surface. Three replicate boots or booties of the same surface were then placed into each tote for a trial. The inside of boots was weighted down with plastic bags containing bricks or gravel to ensure that the boots were touching the bottom of the experimental tote. The wader boots were allowed to be colonized by NZMS for 20–60 min until at least 10 or more snails could be observed on the surface of each one.

After colonization, the boots or booties were transferred directly into a disinfection bath with 20 g/L Virkon Aquatic or with tap water (as control). The boots were then retained for 15, 20, or 30 min in the disinfection (or control) bath and removed. Boots were then placed into a rinse sequence of three baths each containing clean tap water. Rinses lasted approximately 5–10 s. Boots were inspected to ensure all snails were removed. Each rinse bath was then sieved, and any NZMS that fell off in the rinse system were placed into a recovery cup. The disinfection baths were also emptied, and all organisms were sieved to collect NZMS that had dropped off from the boots. All snails recovered from test or rinse systems were again rinsed in three separate baths and placed into recovery cups with aged laboratory water. We repeated our trials until we tested at least 100 NZMS infested on each boot type and test interval. As in other experiments, we assessed mortality and enumerated all NZMS in recovery cups immediately, 24 h, and 48 h postexposure. Any

neonates released from test snails were counted at each time interval and assessed for mortality.

Deterioration of waders following repeated disinfecting.—We conducted a brief experiment to examine the effect of repeated disinfection exposures without rinsing on waterproof characteristics of new neoprene and nylon breathable waders. Two pairs of nylon breathable and one pair of neoprene waders (CADDIS Systems, La Pine, Oregon) were exposed to a 20-g/L Virkon Aquatic solution repeatedly for a 20-min soak at 15°C to follow the deterioration. All material below the front chest pocket was immersed in the experimental tote. After each exposure, waders were examined for holes, discoloration, tears, failure of water proofing, or any other signs of wear. One pair of nylon breathable waders was rinsed for comparison. The waders were hung up in the laboratory to dry fully between trials. Waders were then worn by subjects in a large tank with water for at least 5 min to assess leakage, and disinfections were repeated until waders exhibited damage.

Effect of organic contaminants on oxidizing activity.—To determine the effect of organic contaminants on the effectiveness of the reagent, we followed the decay of the Virkon Aquatic activity with additions of *Sphagnum* peat moss, stream mud, and NZMS. The peat moss was obtained from a garden supply company, the stream mud was collected from a nearby creek, and the NZMS were from our laboratory We determined the proportion of organic content of the peat moss and stream mud by drying representative samples at 105°C to a constant weight. We used the loss-on-ignition method to determine the amount of organic material (ASTM 2007). The proportion of organic material was calculated as the difference between the initial dry weight and final sample ash weight divided by the initial sample dry weight to determine the percentage of organics (Schumacher 2002).

To study the deterioration of the test solution (active Virkon Aquatic) with and without contamination, we prepared solutions of 10 and 20 g/L of Virkon Aquatic. For tests of peat moss and stream mud, we placed 1 L of the 10- or 20-g/L concentration into a glass beaker with 10 g of dried peat moss or stream mud (1% addition). Solutions were stirred with a magnetic stir plate to assure continuous mixing. At selected intervals (0.5, 2, 4, 24, and 48 h), we removed triplicate 15- or 30-g samples from each test system and placed them into separate beakers for analysis of activity. As a control, we placed a solution of 10 and 20 g/L Virkon Aquatic without organic materials and measured similarly at longer intervals (0, 4, 24, 48, 72, 120, and 168 h). Tests were repeated three times over 3 weeks.

To study the effect of NZMS as an organic contaminant on active chemical, 100-mL samples of a 20-g/L Virkon Aquatic solution were poured into individual 150-mL glass beakers for two test treatments (with or without 10 NZMS). We removed samples and tested two replicate samples from these beakers, as above, at daily intervals for 7 d.

We determined the available oxidizing agent (active Virkon Aquatic) in all experiments with the chlorine (residual) iodometric method I (Method 4500-ClB; Clesceri et al. 1996). The iodometric method is a titration that uses the principle that chlorine will liberate free iodine from potassium iodide solutions at pH 8 or less, where the liberated iodine is titrated with a standard solution of sodium thiosulfate with starch as the indicator. To accomplish these tests, a 15-g sample of the 20-g/L solution or a 30-g sample of the 10-g/L Virkon Aquatic solution was added to a test beaker. Approximately 1 g potassium iodide (lot 143188, Sigma Aldrich) and 10 mL of a 200-mL/L sulfuric acid solution was added and mixed. The sample was immediately titrated with 0.1 N sodium thiosulfate (lot 100705, Fisher Scientific) until the yellow color was almost gone. Then 2–4 mL of a 1% starch indicator (lot 0155-03, Fisher Scientific) was added to make a blue color and titration continued until the solution remained clear for at least 30 s. The percent available active oxidizer (%AO) was calculated with the following equation provided by Thomas P. Tufano, DuPont Chemical Solutions Enterprise (personal communications):

$$\%\ \mathrm{AO} = \frac{\mathrm{V_1} \times \mathrm{N_{thio}} \times 100 \times 152.17\ \mathrm{g/mole\ KHSO_5} \times 16\ \mathrm{g/mole\ O_2}}{1{,}000\ \mathrm{mL} \times W_s(\mathrm{g}) \times 2e^- \times 152.17\ \mathrm{g/mole\ KHSO_5}}, \quad (1)$$

where $\mathrm{V_1}$ = volume of sodium thiosulfate (mL) $\mathrm{N_{thio}}$ = normality of sodium thiosulfate, and W_s = weight of Virkon Aquatic sample tested.

Statistical Analysis

All statistical analyses were conducted using SAS version 9.2 (SAS Institute, Cary North Carolina) and significant differences were reported as *P*-values. We used a general linear model to test for significant differences in the number of adult, juvenile, and neonate NZMS, other aquatic invertebrates, and total invertebrates colonizing each of the three boot surfaces that could be attributed to the boot surface, the two test densities, and their interaction. When we detected a significant effect of boot surface, we used Tukey's honestly significant difference test to separate the treatment means. We used a general linear model to test the proportion of total to live NZMS remaining on the three boot surfaces after the three periods of disinfection to determine differences over time. We used repeated measures ANOVA to determine the associations between the dependent variable of percent active oxidizer over time and organic constituents with concentration of Virkon Aquatic combined as treatments as follows: stream mud in 10 g/L, stream mud in 20 g/L, peat moss in 10 g/L, and peat moss in 20 g/L. The time intervals were 0.5, 2, 4, 24, and 48 h. A polynomial transformation was used to account for the uneven time intervals.

RESULTS

Colonization of Three Wader Surfaces

We observed NZMS of all sizes, including neonates, and other aquatic invertebrates on the wader surfaces after 30 min

TABLE 1. Summary of ANOVA of counts of invertebrates colonizing three types of boot surfaces after 30-min exposure in totes with cultures of New Zealand mudsnails (NZMS) and other invertebrates at two densities. Each test of boots and density was repeated nine times.

Source	df	Sum of squares	F-value	P
All invertebrates including NZMS				
Density	1	930.454	17.82	<0.001
Surface	2	777.463	7.45	0.001
Density × Surface	2	71.352	0.68	0.507
All NZMS				
Density	1	56.333	1.85	0.177
Surface	2	357.389	5.86	0.004
Density × Surface	2	18.722	0.31	0.736
Adult NZMS				
Density	1	14.815	0.95	0.333
Surface	2	242.130	7.75	<0.001
Density × Surface	2	2.796	0.09	0.915
Juvenile and neonate NZMS				
Density	1	13.370	1.47	0.227
Surface	2	46.741	2.58	0.081
Density × Surface	2	7.407	0.41	0.666
Other invertebrates excluding NZMS				
Density	1	528.898	30.68	<0.001
Surface	2	84.019	2.44	0.093
Density × Surface	2	19.241	0.56	0.574

TABLE 2. Summary of single-degree comparisons of least-squares means (Table 1) of New Zealand mudsnails (NZMS) and other invertebrates by boot surface and invertebrate grouping. Means with significant differences at $P <$ 0.05 are accompanied by different letters.

Surface	Least-squares mean	Separation
All Invertebrates including NZMS		
Felt	12.722	z
Neoprene	8.111	y
Rubber	6.361	y
All NZMS		
Felt	6.639	z
Neoprene	3.778	y
Rubber	2.250	y
Adult NZMS		
Felt	4.417	z
Neoprene	3.167	z
Rubber	0.806	y
Juvenile and neonate NZMS		
Felt	2.222	
Neoprene	0.611	
Rubber	1.444	
Other invertebrates excluding NZMS		
Felt	6.083	
Neoprene	4.333	
Rubber	4.111	

of exposure. We found significant differences in the numbers attributable to type of wader surface (Table 1), but no significant interactions between the density of NZMS at exposure and surface. The effect of wader surface was $0.10 > P > 0.05$ for models of numbers of juvenile and neonate NZMS and numbers of invertebrates excluding NZMS (Table 1). We found counts were significantly higher on felt surfaces for totals of all invertebrates and all NZMS ($P < 0.05$). We found that the least-squares mean number of organisms on rubber surfaces was lowest, except for juvenile and neonate NZMS (Table 2).

Spray versus Bath

A bath of 20 g/L Virkon Aquatic for 30 min was effective in killing all stages of NZMS (Figure 1). In shorter exposure times, we observed some snails surviving or live neonates released during recovery. Live neonates were observed released from adult NZMS in control and 10-g/L bath applications, and live neonates were found in recovery containers of all concentration and exposure times tested with sprays (Table 3). The pH, temperature, and conductivity of the test solutions were consistent and varied little over the trials. The temperature of all solutions averaged 15.4°C before and after testing, and pH averaged 7.16 in controls and 2.4 in solutions of 10 and 20 g/L Virkon Aquatic, respectively. Total conductivity increased with increasing concentrations of Virkon and averaged 8,123 mS/cm in 10 g/L and 14,500 mS/cm in 20 g/L. Conductivity of control water was 268 μS/cm.

Disinfection of Three Wader Surfaces Infested with NZMS

All NZMS on wader surfaces exposed to baths with 20 g/L Virkon Aquatic were killed regardless of exposure time. No neonates were found in recovery baths (Table 4). The pH, temperature, and conductivity of the test solutions remained consistent throughout all trials. Some mortality of NZMS was observed

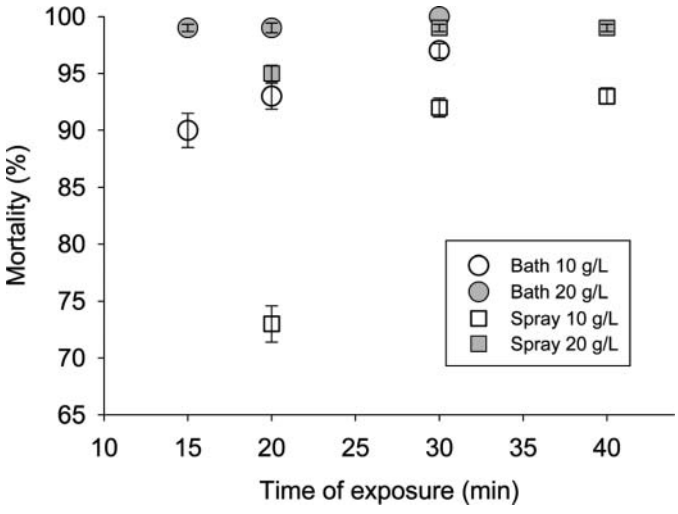

FIGURE 1. The percent mortality (±SE) of New Zealand mudsnails after simulated spray or bath exposures with 10 and 20 g/L of Virkon Aquatic. Control mortality ranged from 0% to 4%.

TABLE 3. Summary of mortality of adult New Zealand mudsnails and number of live neonates released after spray versus bath applications of Virkon Aquatic using three exposure times and two concentrations (10 and 20 g/L).

Test	Concentration (g/L)	Time (min)	Number dead	Total number tested	Percent mortality (SE)	Number of live neonates
Bath	Control (0)	15	2	90	2 (0.04)	19
		20	0	90	0 (0)	19
		30	1	90	1 (0.03)	22
	10	15	81	90	90 (0.15)	5
		20	85	92	92 (0.09)	7
		30	89	92	97 (0.03)	5
	20	15	89	90	99 (0.03)	0
		20	89	90	99 (0.03)	0
		30	90	90	100 (0)	0
Spray	Control (0)	20	0	90	0 (0)	22
		30	0	89	0 (0)	26
		40	0	90	0 (0)	23
	10	20	67	91	74 (0.17)	27
		30	84	91	92 (0.09)	9
		40	84	90	93 (0.07)	3
	20	20	86	91	95 (0.07)	6
		30	89	90	99 (0.03)	2
		40	89	90	99 (0.03)	1

TABLE 4. Summary of mortality in disinfections of boots naturally infested with New Zealand mudsnails (NZMS). The total mortality of NZMS and number of live neonates recovered from soles of boots by concentration and time in bath exposure to Virkon Aquatic is provided. TNTC = too numerous to count (>300).

Boot surface	Concentration (g/L)	Exposure (min)	Number of replicates	Total number NZMS tested	Number dead	Percent mortality (SE)	Number of live neonates
Felt	Control	15	3	150	9	6 (3.4)	6
		20	2	116	3	3 (1.9)	5
		30	3	108	5	5 (2.7)	8
	20	15	3	174	174	100 (0)	0
		20	2	233	233	100 (0)	0
		30	3	247	247	100 (0)	0
Neoprene	Control	15	1	967	5	0.5	TNTC
		20	2	267	4	1.5 (0.2)	TNTC
		30	1	235	4	2	TNTC
	20	15	3	152	152	100 (0)	0
		20	1	216	216	100 (0)	0
		30	3	730	730	100 (0)	0
Rubber	Control	15	4	56	0	0 (0)	13
		20	4	48	2	4 (5.9)	30
		30	4	113	7	6 (3.8)	31
	20	15	4	233	233	100 (0)	0
		20	4	207	207	100 (0)	0
		30	4	532	532	100 (0)	0

TABLE 5. Percent active oxidizer (AO) (and SE) for each concentration of Virkon Aquatic with no organic material, 10 g/L peat moss, or 10 g/L Paradise Creek stream mud for each time tested. NT = not tested.

	Mean% AO (SE)					
	10 g/L Virkon Aquatic			20 g/L Virkon Aquatic		
Time (h)	No organic material	Peat moss	Stream mud	No organic material	Peat moss	Stream mud
0	12.81 (1.85)	NT	NT	12.37 (3.52)	NT	NT
0.5	NT	12.20 (3.49)	11.73 (0.87)	NT	9.31 (0.78)	10.44 (0.11)
2	NT	10.42 (1.15)	9.66 (0.16)	NT	8.87 (0.46)	9.67 (0.28)
4	10.98 (0.39)	10.10 (0.81)	10.01 (0.42)	10.28 (0.06)	8.34 (0.08)	9.39 (0.24)
24	10.65 (0.52)	7.27 (0.38)	8.35 (0.41)	9.96 (0.22)	6.76 (0.31)	8.51 (0.16)
48	10.79 (0.26)	6.00 (0.27)	4.10 (0.16)	9.30 (0.07)	4.85 (0.12)	7.56 (0.19)
72	11.21 (0.20)	NT	NT	9.29 (0.07)	NT	NT
120	11.23 (0.19)	NT	NT	9.00 (0.01)	NT	NT
168	11.10 (0.53)	NT	NT	9.01 (0.19)	NT	NT

in control solutions. We detected no damage to felt, rubber, or neoprene wading gear over repeated disinfections with 20 g/L Virkon Aquatic. Preobservation and postobservation tests on damages revealed that the holes, cracks, stains, discoloration, and tears did not increase in severity throughout the study. We found no waterproofing failure in any of the tested boots or booties.

Deterioration of Waders with Repeated Disinfecting

The crotch of the neoprene waders started to leak after 65 exposures to Virkon Aquatic with rinsing. Testing continued with the neoprene waders until the legs leaked water, and after 77 exposures, both booties leaked around the seams. The nylon breathable waders that were not rinsed leaked behind both knees after 29 exposures to Virkon Aquatic, and waterproofing sealant appeared to disintegrate. After 30 exposures, the breathable waders that were rinsed leaked at the crotch. We repeated testing with these waders until the legs leaked, at 43 exposures, with disintegration of the glue occurring around the seams. We detected no discoloration, tears, or holes in the waders, but damage occurred in the seam or waterproofing glue.

Effect of Organic Contaminants on Oxidizing Activity

Organic material caused a decrease in oxidizing activity of Virkon Aquatic solutions over time of exposure (Table 5; Figure 2). Tests above 9.0% were considered active compound (Jeffery Odle, DuPont Animal Health Solutions, Wilmington, Delaware, personal communications). The organic fractions of peat moss and creek mud were 92.9% and 3.9%, respectively. The peat moss deactivated the 10- and 20-g/L solutions rapidly (Figure 2). The stream mud deactivated the 10-g/L Virkon Aquatic solution more than the 20-g/L solution, and the differences were apparent at 48 h.

We found a significant time and treatment effect on the oxidizing activity (Time $F = 56.22$, $P < 0.001$; Treatment $F = 3.39$, $P = 0.018$). The data fit the model, satisfying the criteria for the sphericity. We found that the linear part of the polynomial model was significant ($P < 0.05$), but the quadratic, cubic, or quartic trends were not significant ($P > 0.10$). The oxidizing activity of the 20-g/L solution of snails and no snails in Virkon Aquatic over 0–144 h of exposure remained above 9.0%; the means (SE) were 9.72% (SE = 0.322) and 9.70% (SE = 0.599) in solutions with and without NZMS, respectively.

DISCUSSION

Our studies validated the already published account that felt surfaces on wading gear are likely to transport aquatic invertebrates and other microorganisms including fish disease pathogens (Gates et al. 2008, 2009; Bothwell et al. 2009;

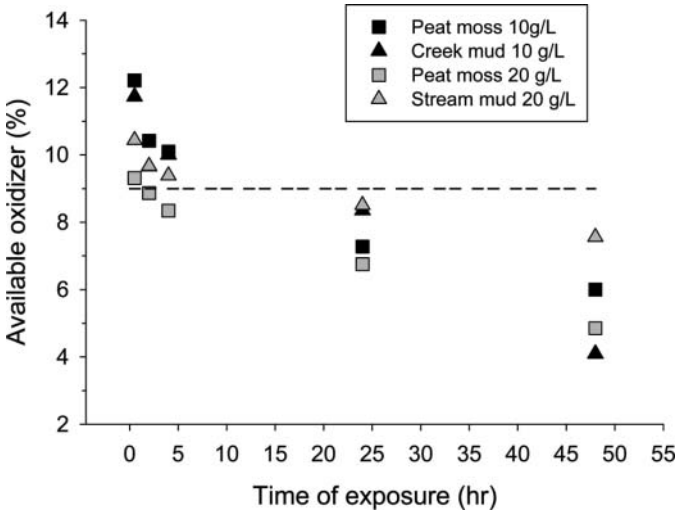

FIGURE 2. The percent available oxidizer over time in solutions of 10 and 20 g/L Virkon Aquatic with 10 g peat moss or stream mud. Virkon Aquatic is considered active at percent available oxidizer above 9.0 (horizontal dashed line).

Waterkeyn et al. 2010). We found NZMS infested all boot surfaces including rubber soles, although numbers were lower on the rubber surfaces. The need for regulations and informed behavior regarding wading gear transport cannot be overestimated. Gates et al. (2009) found that anglers wearing boots and waders potentially transported an average of 16.78 g of sediments during angling trips in Montana. Gates et al. (2008) reported that the interstitial spaces of felt boots were the largest when compared with lightweight nylon, neoprene, and rubber. Waterkeyn et al. (2010) found a suite of organisms were contained in mud on footwear and wading suits of 25 field workers and biologists but did not relate the gear type. They found 18 taxa on the footwear and wading suits, including protozoans, brine shrimp *Artemia* sp., ostracods, rotifers, cladocerans, large freshwater branchiopods, bryozoans, turbellarians, and nematodes.

In our small-scale tests comparing spray applications of NZMS in petri dishes with submersion in laboratory beakers, we found a disinfection bath of Virkon Aquatic for 30 min was needed to kill all NZMS and neonates. Spray applications provided only 99% mortality after 30–40 min before rinsing, and live neonates were present in the recovery containers. Oplinger and Wagner (2009b) found that 15-min spray applications of copper sulfate, hydrogen peroxide, Clorox Commercial Solutions Formula 409 cleaner–degreaser–disinfectant, and Hyamine 1622 were 100% effective on NZMS, but they did not report examination of recovery systems for neonates. In other studies of NZMS, live neonates were often released rapidly in the recovery water systems from dying adults (Bruce and Moffitt 2010). Schisler et al. (2008) reported that compromised NZMS that survived exposure often released live neonates in the recovery bath. Bath disinfection is recommended for most other reagents tested on NZMS (Hosea and Finlayson 2005; Schisler et al. 2008). Spray applications do not cover all surfaces with the same amount or concentration of disinfectant for the required time, and there are more likely to be inconsistencies in the individual delivery methods and spray apparatus.

We validated that the concentration and durations of exposure to Virkon Aquatic in our small laboratory test systems were exceptionally effective on boots and booties infested with NZMS. Mortality was rapid, and no snails were observed crawling in the disinfectant bath. Instead, we observed NZMS falling off when the boots or booties were placed into test solutions. Very few NZMS were found in the rinse tubs and those found were dead, even after a 15-min exposure. The effectiveness of the shorter exposure time in tests with infested surfaces could be attributed to the fact that most snails are attached with their foot to gear and the chemical reached them rapidly. In the laboratory bath tests, the NSMS had closed opercula.

Although our tests of deterioration of boots and waders were limited, our studies support that Virkon Aquatic was not as harsh on waders and boots as observed with some other chemicals. Hosea and Finlayson (2005) found deterioration of boots and waders after seven exposures to chemicals such as bleach and Pine-Sol. We observed disintegration in the glue of wader seams after approximately 30 exposures of 20 min each to a 20-g/L Virkon Aquatic solution. Neoprene seam glue lasted longest, failing after 67 exposures. We recommend additional testing of equipment to evaluate seam glues and the effects on different equipment manufacturers' products.

The 20-g/L concentration that was effective in our tests is in line with concentrations recommended for disinfecting surfaces and gear for target bacteria, fungi, and parasites of 10 or 20 g/L for at least 5 min (DAHS 2011a; Western Chemical 2012). For aquaculture facilities already using Virkon Aquatic disinfectant protocols to protect against pathogens, the expansion of this tool to reduce risks of infestation with NZMS can be easy. However, additional tests will probably be needed to expand the label claim to meet the USEPA criteria for invasive mollusks.

We documented that Virkon Aquatic solutions were deactivated by organic material after 4–24 h of exposure. We found stream mud deactivated 10 g/L Virkon Aquatic at a faster rate than the peat moss. Although this was not expected, stream mud is a heterogeneous mixture containing metals and organic material, and thorough mixing did not make the mixture homogenous. Since Virkon Aquatic is broken down by metal salts (DAHS 2011b), there could have been a different composition of organic material and metal salts in the mud. Another explanation for the different decomposition rates between 10- and 20-g/L solutions was that a concentration of 20 g/L had a higher amount of active ingredients to prolong the reaction than did the concentration of 10 g/L. The peat moss had a high percent organic material and deactivated the 20-g/L solution faster than the stream mud. Disinfecting protocols recommend removal of organics and debris during or before decontamination procedures (Hosea and Finlayson 2005; Schisler et al. 2008; CDFG 2012). We did not test the effectiveness of the solutions below the suggested limit of activity of 9%. Further tests could be conducted to explore the safety limits of compromised solutions.

Banning different gear types has been proposed as one solution for controlling the spread of aquatic invasive species, specifically *Didymosphenia geminata* and *Myxobolus cerebralis* (Spaulding and Elwell 2007; Gates et al. 2008; Bothwell et al. 2009; Kilroy and Unwin 2011). Wading gear provides a pathway for NZMS and other nonindigenous organisms to spread (Dwyer et al. 2003; Richards et al. 2004; Hosea and Finlayson 2005). However, our research demonstrates that banning felt-soled wading boots will not eliminate the risk of introduction. Moveover, precaution must be exercised when using chemical disinfectants to ensure efficacy and safety of the environment and worker (Oplinger and Wagner 2009a, 2010). Many chemicals tested are not safe near the water and have high aquatic toxicity. Although other studies have documented the efficacy of a range of QACs as disinfectants (Hosea and Finlayson 2005; Schisler et al. 2008; Oplinger and Wagner 2009b), there are concerns about the widespread use of QACs and their potential effect on aquatic and soil systems (García et al. 2001; Li and Brownawell 2010). The QACs are used in numerous industrial and household applications, including in fabric softeners,

detergents, disinfectants, preservatives, and personal care products. Their persistence has increased concerns regarding their genotoxic effects (Ferk et al. 2007). We found that Virkon Aquatic was effective on boots after a short duration of bath exposure. The chemical is deactivated quickly by organic material and is minimally corrosive on gear. However, prerinsing gear to remove soil, excess organic material, and large organisms is an essential step before using any disinfectant.

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