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Research Article

A preliminary investigation into biosecurity treatments to manage the invasive killer shrimp (*Dikerogammarus villosus*)

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Abstract

Following the detection of the invasive killer shrimp, *Dikerogammarus villosus* (Dv) at two sites in the UK in September 2010, an effective biosecurity system is required to prevent further spread. This study investigated the application of several treatments as potential biosecurity measures with a view to their application on Dv-infected fomites. For each treatment, adult Dv were submerged for 15 minutes at different concentrations to determine the maximum lethal concentration, and for each effective treatment for different times to assess a minimal lethal time (LT_{50}). Sodium hypochlorite (50,000 mg/Lmg/L), FAM30® (6 ml/l), Virkon S® (1% solution) and water at high temperature (45 °C) were found to cause 100% mortality within 15-min exposure, while carbonated water caused narcosis in 100% of animals within a few seconds of exposure. Due to various drawbacks in the use of sodium hypochlorite, FAM30® and Virkon S® (e.g. health and safety, legal use) they were not recommended as biosecurity treatments, whereas both water at high temperature and carbonated water showed promise. However, further investigation and field based research are required before these techniques can be fully realised as methods of control.

Key words: aquatic invasive species, control management, high temperature, chemicals, submersion exposure

Introduction

The killer shrimp, *Dikerogammarus villosus* (Dv; Sowinsky, 1894) is a large gammarid of Ponto-Caspian origin and is regarded as one of the most damaging invasive species in Europe, being one of the "Top 100" invasive alien species in Europe (DAISIE 2009; Rewicz et al. 2014). Dv exhibits several biological characteristics that contribute to its environmental impact: long reproductive period, early sexual maturity, short generation time, high growth rates, short duration of embryonic development, large number of eggs, large reproductive capacity, highly predatory and tolerant of a wide range of environmental conditions (Dick and Platvoet 2000; Devin et al. 2003, 2004; Kley and Maier 2006; Pöckl 2009). These biological characteristics have made Dv an effective invasive species with only a few individuals required to establish new populations in recipient ecosystems (Devin et al. 2004). Dv has invaded and spread over much of mainland Europe (currently found in 17 countries) where it has out-competed a number of native species (Šporka 1999; Devin et al. 2001; Müller et al. 2002; Casellato et al. 2006; Gruszka and Woźniczka 2008). In addition, the size of the prey (small fish, fish larvae etc.) does not seem to be an obstacle for Dv, with the species occupying a trophic niche above that of native gammarids (Dick et al. 2002; Devin et al. 2003; Schmit and Josens 2004; Casellato et al. 2007).

In the UK, at the time the present study was conducted, Dv had been discovered at Grafham Water, Cambridgeshire, England, in September 2010 and subsequently in Cardiff Bay and Eglwys Nunydd Reservoir near Port Talbot, both in Wales (MacNeil et al. 2010; NNSS (GB Non-native species secretariat) 2016). After the study, in March 2012, a further population was found during dedicated monitoring at Barton Broad, in the Norfolk Broads, England, and another in 2015, at Pitsford Reservoir, England. All

of the invaded sites in the UK are used for a number of recreational activities including sailing and angling, with members of the public using equipment at these sites that may subsequently be used at other freshwater venues in Great Britain. Similarly to other "piggy-backing" aquatic invasive species (Johnson and Carlton 1996; Johnson et al. 2001; Kerfoot et al. 2011), Dv has been found to readily attach to equipment used in water, such as sailing vessels, wetsuits, and fishing nets (MacNeil et al. 2012; Bacela-Spychalska et al. 2013; Bacela-Spychalska 2015). These fomites (inanimate objects capable of carrying organisms and hence transferring them between water bodies) pose the potential risk of spreading Dv to un-invaded ecosystems, as it has been shown to survive for at least 16 days in damp conditions (Anderson et al. 2015).

It has already been recognised that few individuals may be required to establish a new population. It is therefore important that an effective biosecurity system is implemented at infected waters to prevent further spread. The alert caused by Dv lead to the "Stop the spread – Check, Clean, Dry" campaign to be implemented in the UK; raising both species-specific and more generic awareness activity (NNSS). Currently physical removal of Dv from fomites is being used in conjunction with visual inspections, but there is concern that the effectiveness of these measures could potentially decrease with time. Therefore, to increase levels of biosecurity and to reduce the potential for human error, methods to treat fomites with the aim of removing Dv are required. Ideally methods should meet the following criteria: (i) cause mortality (ideally 100%) in Dv within a short exposure time, (ii) can be applied either as a dip and/or spray, (iii) is usable near drinking water, (iv) is easily disposed of, (v) does not require a specific licence for use, (vi) can be used for this purpose and at a sufficient rate without infringement of appropriate legislation, (vii) is readily available, (viii) can be used by members of the public without the use of protective equipment, (ix) will not cause damage to the fomites on which it is used, (x) has a long "shelf life" and (xi) can be easily prepared by a person with little or no training. While this list is a "gold standard", finding a treatment that meets all these criteria is unlikely.

The study presented here examined several treatments with a view to determining the most effective biosecurity measure for the control of Dv, while meeting the "gold standards" presented above. In addition, the limitations of each of the treatments are discussed, in addition to how the most viable treatments could be applied and incorporated into a biosecurity programme.

Methods

Collection and maintenance of animals

In March–April 2011, *Dikerogammarus villosus* (Dv) were collected by hand from both Grafham Water (52º17′54.73″N; 0º18′51.43″W) and Cardiff Bay (51º27′46.8″N; 3º9′50.4″W) and transported on damp tissue paper in water tight containers to Cefas, Weymouth Laboratory (Dorset, UK). Dv from the different populations were held in separate stock tanks under biosecure conditions. Prior to experimentation, animals were left to acclimatise for at least five days in a controlled environment (14–15 °C; 12L:12D – hours light:hours dark) in 30 L tanks with a constant fresh flow-through supply of dechlorinated mains water (water hardness 140 mg/L $CaCO_3$, 7.2–8.2 pH, < 0.04 mg/L NH_3, < 1 mg/L NO_2, <80 mg/L NO_3). Biosecurity measures were put into place to prevent the escape of any live animals from the holding facility (a chemical treatment, two physical barriers, a failsafe system and an ozone plant). Dv were fed alternate days with coarse fish pellets and frozen blood worms. Stock tanks were cleared regularly of detritus, with mortalities and moults (shed exo-skeletons) counted and recorded to ensure all animals were accounted for. During the study period, precopula pairs and recently hatched juveniles were observed in the stock tanks; these were separated from the main population with a view of collecting juveniles to determine the least susceptible life stage to the treatments tested.

Chemicals/Treatments

Hydrochloric acid (1M), Hydrogen peroxide (30%), and Sucrose were obtained from Sigma (Poole, UK). Acetic acid (> 95%), HPLC grade Methanol (> 95), Urea obtained from Fisher Scientific (Loughborough, UK), with Citric acid from Acros organics BVBA (Belgium) and Artificial marine salt from Tropic Marin® (Germany). Commercial solutions were also purchased of: Sodium hypochlorite (10–15%) from Kilco Ltd (Lockerbie, UK), FAM30® (Alcohol ethoxylate 20–25%, Sulphuric acid 5–10%, Phosphoric acid 5–10% and Iodine 1–5%) from Evans Vanodine International (Preston, UK), Virkon S® (Potassium peroxomonosulphate 50%, Sulphamic acid 5% and Sodium alkyl benzene sulphonate 15%) as powder from Antec International Ltd (Sudbury, UK) and Carbonated water (as sold, pH = 5.1) from a local supermarket (Weymouth, UK).

Table 1 provides details concerning the tested concentrations for each treatment. Using dechlorinated mains water (from the same source used for the Dv stocks), the different tested solutions were serial

Table 1. Summary of the treatments applied to *Dikerogammarus villosus* for toxicity tests.

Treatment	Tested solutions		Maximum lethal concentration test	LT_{50} test[a]
	Number	Concentration		
Sodium hypochlorite	11	200, 300, 450, 675, 1012.5, 1000, 2000, 4000, 5000, 10000 and 50000 mg/L	✓	✓[b]
Salinity	8	5, 10, 20, 30, 35, 40, 80 and 160 g/L	✓	×
Virkon S®	5	1, 2, 4, 8 and 10 g/L	✓	✓[b]
pH (Hydrochloric acid)	5	7, 6, 5, 4 and 3	✓	×
FAM30®	4	1, 2, 4 and 6 ml/L	✓	✓[b]
Temperature	5	30, 35, 40, 45 and 50 °C	✓	✓[b]
Acetic acid	2	1 and 10%	✓	×
Methanol	2	1 and 10%	✓	×
Citric acid	2	15 and 150 mg/L	✓	×
Urea	2	1 and 10 g/L	✓	×
Hydrogen peroxide	1	100 mg/L	✓[b]	×
Carbonated water	1	as sold	✓[b]	×
Sucrose	2	10 and 100 g/L	✓	×

[a] LT_{50} = lethal time where 50% of the animals were killed.
[b] only one concentration tested.

diluted from the highest concentration to obtain a broad range of concentrations. Carbonated water was tested as sold. For the different water temperatures tested, hot tap water (> 60 °C) was diluted with cold (14–15 °C) dechlorinated mains water (from the same source used for the Dv stock) until the target temperature was reached.

Experimental protocols

Control chart

Sodium hypochlorite was chosen as a reference toxicant as it is a routinely-used disinfectant in the Weymouth laboratory and has been found to be toxic to other arthropod species (e.g., *Gammarus fasciatus* (Ewell et al. 1986) and *Daphnia magna* (Ewell et al. 1986; Santos et al. 2007)). A control chart was created to monitor the sensitivity of the test organisms and to assess precision and accuracy of the performed toxicity tests.

Pre-study trials

Adult and juvenile (less than one week old) Dv were exposed separately to sodium hypochlorite at 200, 300, 450, 675 and 1,012.5 mg/L (following protocol described in next section) to determine which life stage was least sensitive. Similarly, adults from populations in Grafham Water and Cardiff Bay were separately exposed to 5,000, 10,000 and 50,000 mg/L of sodium hypochlorite to determine if there was any difference in the response of animals from the two populations. Adult mean total length was 14.34 ± 0.128 mm, juveniles were too small to measure accurately, without excessive handling, resulting in potential stress, but all were less than 7 mm in length.

Maximum lethal concentration test

Survival experiments were developed to determine a maximal lethal concentration (100% mortality) of different concentrations of the treatments on Dv when applied as a dip application. A 15-min exposure period was used as this was considered to be the maximum time that any method could be applied by potential end users.

On the day of the assay, solutions were prepared (see Table 2 for details) and stored in ambient conditions (14–15 °C). For each concentration and water control, five Dv were placed into a container (90 × 45 × 48 mm with a perforated base) placed into a 300 ml Pyrex glass basin (Fisher Scientist, Loughborough, UK) filled with 250 ml of treatment. The animals (within the container) were left in the treatment for 15 minutes, with observations being made and recorded every five minutes throughout the exposure period. Observations made consisted of recording the behaviour of Dv in response to stimuli (gentle poke), when they were not swimming. Dv were considered dead when they were stationary with no twitching of antennules, antenna or pereopods. After 15 minutes of exposure to the treatment, the plastic container containing the animals was removed from the Pyrex glass basin, rinsed briefly in dechlorinated mains water and moved to a recovery basin (Pyrex glass basin as described above) containing 250 ml dechlorinated mains water. The behaviour of Dv was recorded every 15 minutes for 60 minutes after the end of the exposure period (recovery period). Water chemistry was recorded before and after the experiment, with no changes from those described above under "Collection and maintenance of animals".

Table 2. Percentage mortality of *Dikerogammarus villosus* after exposure (15 minutes) to a range of concentrations of different treatments and after recovery in dechlorinated mains water (60 minutes) (see also Supplementary material Table S1).

Treatment	Concentration		Percentage of mortality	
	Nominal	Measured[a]	Exposure	Recovery
Sodium hypochlorite (mg/L)	0	–	0	0
	200	–	0	60
	300	–	0	60
	450	–	0	100
	675	–	0	100
	1012.5	–	0	80
	1000	–	60	100
	2000	–	20	100
	4000	–	20	100
	5000	–	0	100
	10000	–	100	100
	50000	–	100	100
Virkon S® (g/L)	0	–	0	0
	1	–	0	0
	2	–	0	0
	4	–	60	100
	8	–	40	100
	10	–	80	100
FAM30® (ml/L)	0	–	0	0
	0.25	–	0	80
	1	–	60	100
	2	–	100	100
	4	–	100	100
	6	–	100	100
Temperature (°C)	20	21.6 (22–21.5)	0	0
	30	28.4 (30–27.1)	0	0
	35	31.1 (33.2–29.4)	60	80
	40	36.3 (40.5–32.9)	100	100
	45	38.9 (44.7–35.9)	100	100
	50	43.2 (49.2–38.3)	100	100
Carbonated water	0	–	0	0
	as sold	–	100	0

[a] measured concentrations when available, thus only for temperature: average of the temperatures recorded every five minutes during the exposure period; in brackets: temperature at the start and end of the exposure.

Lethal Time (LT_{50}) test

Once a maximal lethal concentration had been established, survival experiments were carried out to determine a minimal lethal time (LT_{50}), that is the minimal time of required exposure to the treatment at the lethal concentration to cause 50% mortality.

The experiments followed the same method as previously described. Similarly, solutions of the treatments at the lethal concentration were prepared on the day and five Dv per container were used for each treatment. Treatments consisted of different fixed periods of exposure (from 5 s to 12 min), with the water control being dechlorinated mains water for the longest time period. Sodium hypochlorite at 50,000 mg/L (see results) was used as a reference toxicant (positive control) and run in parallel; the time where > 50% mortality was achieved was recorded. At the end of each exposure period, the container with the Dv in was rinsed and placed in dechlorinated mains water for 60 minutes. Observations were made at the start and the end of the exposure, and every 15 minutes during the recovery period.

The experiment was repeated three times (i.e. three replicates) for each treatment where a maximal lethal concentration was determined, except for sodium hypochlorite where five replicates were run (as a reference control treatment).

Termination of the tests

At the end of the test, all the animals were measured end to end with a Vernier calliper. Any live animals were dispatched with a lethal dose of sodium hypochlorite before being securely disposed of.

Data analysis

All statistical analyses were carried out using Sigmaplot (version 12.0, Systat Software Inc.). Size differences between and within experiments were tested using by Kruskal-Wallis as the data did not followed a normal distribution (Shapiro-Wilk, $p > 0.05$). Chi-square tests (N-1 two proportion test) were run to compare differences in the percentage of mortality between life stages and populations. Survival data were used to generate LT_{50}, which was calculated by non-linear regression. LT_{50} is given as the mean of the replicates with the minimal and maximal values in brackets.

Results

Control parameters

Water parameters (temperature, pH, lux, dissolved O_2, total alkalinity and hardness) were as those recorded for stock tank water presented under "Collection and maintenance of animals" above, unless being deliberately manipulated such as with temperature and pH treatments.

All animals tested were identified as *Dikerogammarus villosus* (Dv) and measured on average 14.3 mm (± 0.128 SE), ranging from 9.9 to 19.7 mm. Although there were significant differences between the experiments conducted ($H = 50.0$, $df = 8$, $p < 0.001$) no significant variation in sizes was found within each experiment (for all $p > 0.05$).

Pre-study results

Juvenile Dv were found more susceptible than adults to sodium hypochlorite ($X^2 = 31.65$, $p = 0.001$); no adult Dv died, while most of the juveniles were dead from 200 mg/L onwards (data not shown). Therefore, only adult Dv (least susceptible life stage) were used in all further trials.

No significant difference was observed in the response of Dv from either population confirming that both populations (Grafham Water and Cardiff Bay) could be used in the trials ($X^2 = 0.1$, $p = 0.752$; data not shown).

Maximum lethal concentration test

Only two tested concentrations (10,000 and 50,000 mg/L) of sodium hypochlorite resulted in 100% mortality within the 15-minute exposure period (Table 2). Given the rapid effect, 50,000 mg/L was chosen as the maximal lethal concentration and also as the reference concentration for the control chart. Interestingly, 100% mortality was still observed in lower concentrations (> 675 mg/L) within 60 minutes' recovery period after the animals had been returned to dechlorinated mains water.

For citric acid, urea, hydrogen peroxide and sucrose, no mortalities were observed after exposure or during the recovery period (Table S1). Therefore, no LT_{50} tests were carried out for these chemicals.

For salinity, only 40% mortality was induced at the highest concentration of 160 g/l (measured at 133.6 g/L), approximately 3.5 times higher concentration than that of normal sea water (Table S1). There were also no significant mortalities observed during the recovery period. Given the need to remove salt from fomites subsequent to treatment to avoid possible corrosion, making the practicality of treatment more complicated and as a result of the lack of mortalities, even at high concentrations, the testing of salinity was stopped. Similarly, methanol exposure resulted in 20% mortality in the higher concentration (10%) and thus no LT_{50} was determined (Table S1).

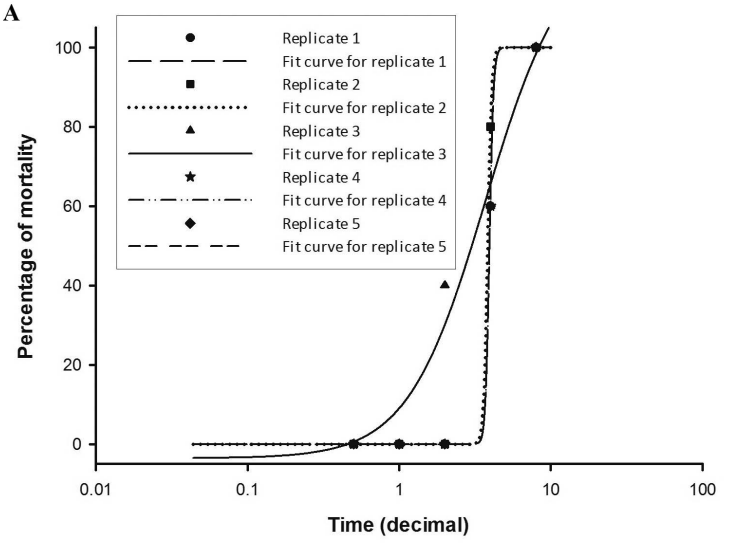
Within the tested pH range, mortality was not induced within the 15-minute exposure period and only 20% mortality was observed at the end of the recovery period for two concentrations (Table S1). Given that lowering the pH further (< 3) would have caused significant problems with the potential application of this treatment, it was decided not to continue testing the possible effects of pH in this manner. Acetic acid exposure resulted in 100% mortality, however the pH measured at 10% solution was 2.28 (Table S1). For the same reasons as given for pH treatment, no further LT_{50} tests were carried out for acetic acid.

For Virkon S®, maximal mortality was not observed during the 15-minute exposure but was after 60 minutes of the recovery period in dechlorinated mains water for three concentrations – 4, 8 and 10 g/L (Table 2). The concentration of 10 g/L was chosen for the LT_{50} tests.

FAM30® induced 100% mortality at 2, 4 and 6 ml/l within the 15-minute exposure period (Table 2). At 1 ml/L 100% mortality was observed 30 minutes into the recovery period. As a quicker response was observed in tests using the 6 ml/l solution, this concentration was chosen for estimating a LT_{50}.

Temperatures higher than 30 °C appeared to have a significant effect on the behaviour of Dv, with exposures to temperatures higher than 36 °C resulting in 100% mortality within the 15-minute exposure period (Table 2). Dv exposed to water temperatures higher than 40 °C died almost immediately. Giving the variation in the measured temperatures, the temperature of the tested water for calculating LT_{50} was aimed at 50 °C to ensure that 40–45 °C will be achieved.

A



B

Exposure time (min)	Percentage of mortality at the end of recovery period				
	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Replicate 5
8 (in aquaria water)	0	0	0	0	0
0.5	**100**	**100**	80	**100**	**100**
1	**100**	**100**	**100**	**100**	**100**
2	**100**	**100**	**100**	**100**	**100**
4	**100**	**100**	**100**	**100**	**100**
8	**100**	**100**	**100**	**100**	**100**

Figure 1. A: Determination of the LT_{50} (lethal time) of *Dikerogammarus villosus* after exposure to sodium hypochlorite at 50,000 mg/L (n = 5 replicates). The x axis is in a log scale and presents the time in decimal.
B: Percentage mortality of Dv recorded at the end of the recovery period in dechlorinated mains water (60 min).

The initial response to carbonated water, showed 100% "mortality" (as defined in the method) within only a few seconds of exposure (Table 2). Interestingly, all the animals recovered within 60 minutes of the recovery period in dechlorinated mains water.

Lethal Time (LT50) test

Sodium hypochlorite

Dv were exposed to 50,000 mg/L of sodium hypochlorite for 30 seconds, 1, 2, 4 and 8 minutes (Figure 1A). Initially only the 8-minute exposure period resulted in 100% mortality, while maximal mortality was observed for all the exposure durations within 60 minutes of the recovery period where the animals were returned to dechlorinated mains water (Figure 1B).

A LT_{50} was determined at 3.837 (3.708–3.951), i.e., in terms of minutes and seconds 03:52 (03:42–03:57). All the LT_{50} recorded during the LT_{50} tests for each chemical were pooled together with the previous determined LT_{50} for sodium hypochlorite to form a control chart (Figure 2). The overall LT_{50} calculated was 3.381 (2.500–4.170) i.e., 03:22 (02:30–04:10). All the data were within the acceptance range (± 2 standard deviations) and the coefficient variation was 15.7%, showing that the results were comparable.

Virkon S®

Dv were exposed to 10 g/L of Virkon S® for 30 seconds, 1, 2, 4 and 8 minutes with 2 replicates, and for 30 seconds, 2, 4, 8 and 12 minutes for 1 replicate (Figure 3A). Only for the 12-minute exposure was 100% mortality observed; however, maximal mortality was observed during the recovery period for all replicates, except for the 30 second duration exposure (Figure 3B). There was a high variability in the behavioural response of Dv, particularly during the recovery period. From this set of data, a LT_{50} of 7.728 (7.640–7.903) i.e., 07:43 (07:38–07:54) was calculated.

FAM30®

Dv were exposed to 6 ml/L of FAM30® for 15 and 30 seconds, as well as 2, 4 and 6 minutes (Figure 4A). Maximal mortality was only observed when

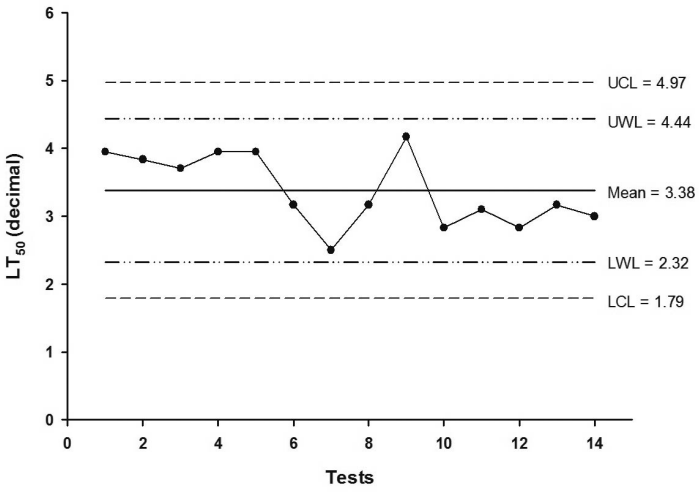
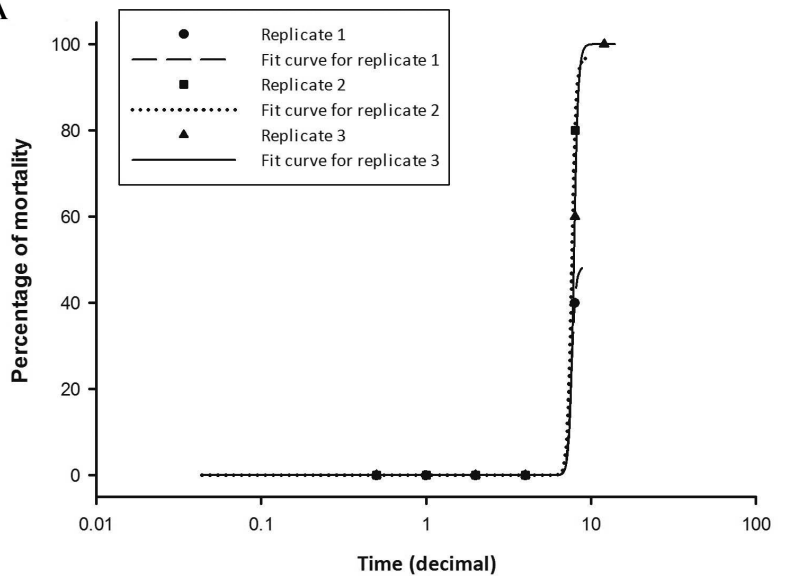


Figure 2. Control chart with sodium hypochlorite as a reference toxicant, to check for the toxicity test performance. LT_{50} = lethal time where 50% of the animals were killed. UWL (Upper Warning Limit = + 2 SD (standard deviation)), LWL (Lower Warning Limit = −2 SD), UCL (Upper Control Limit = + 3 SD), LCL (Lower Control Limit = −3 SD).

A

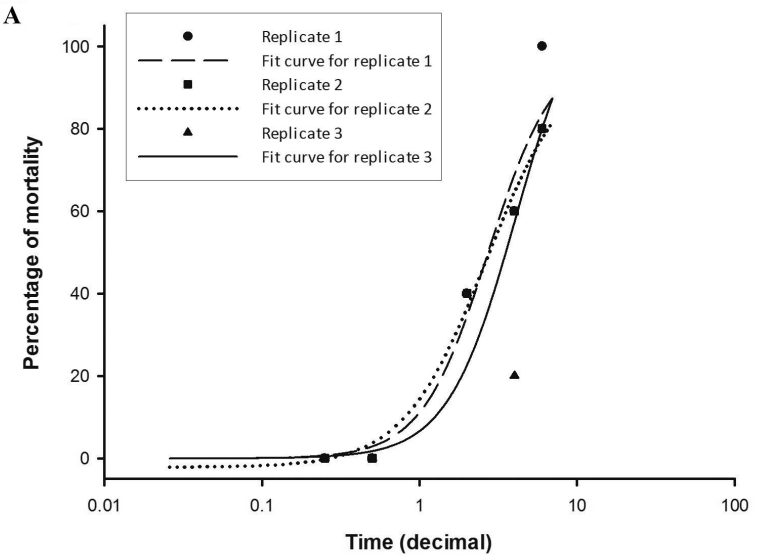


B

Exposure time (min)	Percentage of mortality at the end of recovery period		
	Replicate 1	Replicate 2	Replicate 3
8 (in aquaria water)	0	0	0
0.5	60	60	40
1	80	**100**	
2	**100**	**100**	80
4	80	**100**	**100**
8	**100**	**100**	80
12			**100**

Figure 3. A: Determination of the LT_{50} (lethal time) of *Dikerogammarus villosus* after exposure to Virkon S® at 10 g/l (n = 3 replicates). The x axis is in a log scale and presents the time in decimal. B: Percentage mortality of Dv recorded at the end of the recovery period in dechlorinated mains water (60 min).

A



B

Exposure time (min)	Percentage of mortality at the end of recovery period		
	Replicate 1	Replicate 2	Replicate 3
6 (in aquaria water)	0	0	0
0.25	**100**	80	**100**
0.5	**100**	80	80
2	80	80	**100**
4	**100**	80	**100**
6	**100**	**100**	**100**

Figure 4. A: Determination of the LT_{50} (lethal time) of *Dikerogammarus villosus* after exposure to FAM30® at 6 ml/L (n = 3 replicates). The x axis is in a log scale and presents the time in decimal.
B: Percentage mortality of Dv recorded at the end of the recovery period in dechlorinated mains water (60 min).

exposed for 6 minutes. When the animals were returned to dechlorinated mains water, however, maximal mortality was reached for every duration in at least one replicate, and all replicates for 6 minutes' exposure (Figure 4B). An average LT_{50} of 3.538 (2.643–5.241) i.e., 03:32 (02:38–05:14) was calculated.

High temperature

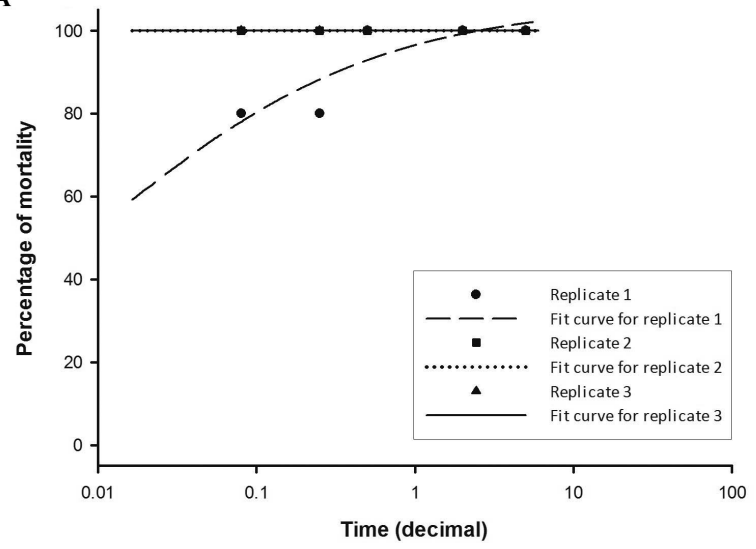
Finally, Dv were exposed to 50 °C freshwater for 5, 15 and 30 seconds, as well as 2 and 5 minutes (Figure 5A). Temperatures were measured across each time period; from the start to the end of exposure; they were on average 48.2, 49.9 and 49.6 °C for replicates 1, 2 and 3, respectively, which were higher than previously recorded for the determination of the maximum lethal concentration test. 100% mortality was achieved rapidly (< 30 s) and all the animals were confirmed dead after one-hour recovery in dechlorinated mains water (Figure 5B). It was therefore difficult to estimate a mean LT_{50}, as for two replicates, mortality was instant, giving a LT_{50} of less than 1 ms (0.0000000066 in decimal time). The LT_{50} for replicate 1 was calculated as 0.010 i.e., 38 ms.

Discussion

Biosecurity is an integral part of invasive species management and mitigation (Simberloff et al. 2013). Seeking effective biosecurity measures that meet some or all of the ideal golden standards, as presented in the introduction, will provide valuable information for the development of tools to use in preventing the further spread of the target species. Similar studies to the one presented here have been conducted, examining the effectiveness of a range of candidate treatments for use as potential biosecurity measures (e.g. Barbour et al. 2013). Screening a wide range of potential candidate treatments is an essential starting point providing valuable information, enabling further, more targeted research effort, and refinement of delivery mechanisms.

Thirteen products were tested for their potential lethal effect against the killer shrimp *Dikerogammarus villosus* (Dv). Seven treatments (salinity, pH, methanol, citric acid, urea, hydrogen peroxide and sucrose) caused no mortality or relatively low mortality of Dv at the safe concentrations used. Although acetic

A



B

Exposure time (min)	Percentage of mortality at the end of recovery period		
	Replicate 1	Replicate 2	Replicate 3
5 (in aquaria water)	0	0	0
0.08	80	**100**	**100**
0.25	**100**	**100**	**100**
0.5	**100**	**100**	**100**
2	**100**	**100**	**100**
5	**100**	**100**	**100**

Figure 5. A: Determination of the LT_{50} (lethal time) of *Dikerogammarus villosus* after exposure to water at 50 °C (n = 3 replicates). The x axis is in a log scale and presents the time in decimal. B: Percentage of mortality of Dv after recovery in dechlorinated mains water (60 min).

acid was effective, it was thought impracticable as a fomite disinfectant due to its high acidity, even at a low concentration. The remaining five promising treatments (sodium hypochlorite, FAM30®, Virkon S®, temperature and carbonated water) were effective and their potential suitability discussed. Table 3 presents a summary of the five most promising treatments in relation to the gold standards as set out in the introduction.

Sodium hypochlorite, FAM30® and Virkon S® were effective at high concentrations, while showing some delay in their lethal effect, as seen during the one-hour recovery period. The toxicities of sodium hypochlorite have been evaluated against other species, including adult zooplankton, benthic invertebrates and species present in ballast water, being toxic to resting eggs of *Artemia* sp. (LC_{90}: 86.5 ± 3.0 mg/L) and *Daphnia mendotae* (LC_{90}: 78.3 ± 1.6 mg/L) (Raikow et al. 2007). Santos and colleagues (2007) estimated a LC_{50} (48 h) of 5,550 mg/L in *Daphnia magna*. FAM30® is an iodine-based product; *D. magna* was found sensitive to iodine solution with $LC_{50} \geqslant$ 0.16 mg/L (Laverock et al. 1995). Finally, according to the safety data sheet for Virkon S®, its main ingredient, potassium peroximonosulphate, is toxic to *Daphnia* sp. at 3.5 mg/L (LC_{50}). However, at the concentrations required for their effectiveness in our study, it would be impossible to use near drinking water safely and the disposal of used treatment may be problematic. Barbour et al. (2013) suggested the use of Virkon® Aquatic as an alternative as it contains inert ingredients to make it more suitable for aquatic application. All three products are not listed as insecticides under the Biocidal Products Directive (98/8/EC), thus either they have to be licensed for this application, ministerial permission sought under emergency authorisation or extension of approval obtained from the manufacturer if to be used as a biosecurity treatment against Dv within the EU. Other studies conducted within the EU examining biosecurity measures for use in combating aquatic invasive species (e.g. Barbour et al. 2013) have recommended the use of Virkon®, without mention of the possible legal issues in relation to its use. Further

Table 3. Summary of the five most promising treatments applied to *Dikerogammarus villosus* in relation to the gold standards.

	Gold standards for the treatments	Sodium hypochlorite 50 g/L	Virkon S® 10 g/L	FAM30® 6 ml/L	High temperature 45°C	Carbonated water as sold
i	cause mortality (ideally 100%) in Dv within a short exposure time	++	++	++	+++	+++[a]
ii	can be applied either:					
	a) as a dip	+++	+++	+++	+++	+++
	b) as a spray	+++	+++	+++	+	-
iii	is usable near drinking water	---	---	---	+++	+++
iv	is easily disposed of	---	---	---	+++	+++
v	does not require a specific licence for use	---	---	---	+++	+++
vi	can be used for this purpose and at a sufficient rate without infringement of appropriate legislation	---	---	---	+++	+++
vii	is readily available	+	+	+	+++	+++
viii	can be used by members of the public without the use of protective equipment	---	-	-	++	+++
ix	will not cause damage to the fomites on which it is used	-	-	-	++	+++
x	has a long 'shelf life' as prepared for treatment	-	-	-	---	--
xi	can be easily prepared by a person with little or no training	-	-	-	++	+++

+: meet the criteria; -: does not meet the criteria; degrees by which the criteria have been met expressed by the number of relevant symbols presented: 1 low, 2 medium and 3 high, i.e. '+++' indicates that the criterion is highly met for this treatment.

[a] Although not causing mortality, narcosis was still induced in 100% of treated animals and is viewed by the authors as possibly as valuable in application of biosecurity as inducing mortality.

examination of the legality of use will have to be examined before such products can viably be recommended for use as an invasive species control measure. In addition to these significant drawbacks, however, FAM30® stains while Virkon S® and sodium hypochlorite have a bleaching effect, potentially resulting in damage to treated equipment. On the other hand, fomites that could be left to soak in 450 mg/L sodium hypochlorite for prolonged periods could effectively be treated for the removal of Dv in this manner (Table 2).

Data on acute upper lethal temperatures of invasive species are rare and usually studies have investigated gradual-increased temperatures to mimic conditions such as flushing intake pipes (Beyer et al. 2011). In the present study, we looked at a thermal shock treatment, as it was thought to be more efficient and adaptable for use in the treatment of fomites. Water at high temperature (> 35°C, measured) caused 100% mortality within 15 minutes, and in fact at > 45°C (measured), all the Dv were killed almost instantly (< one second). Other aquatic species have been shown to be sensitive to rapid exposure of hot water: e.g., zebra mussels (*Dreissena polymorpha*), quagga mussels (*D. rostriformis bugensis*) and spiny waterflea (*Bythotrephes longimanus*), dying after 5 min at 43 °C and within 1 min at 49 °C (Beyer et al. 2011); eggs from spiny waterfleas did not hatch after 5 min at 50 °C and after 1 min at 60 °C (Branstrator et al. 2013). Similarly Anderson and colleagues (Anderson et al. 2015) showed that hot water at 45 °C caused 99% mortality in seven invasive non-native species, of which Dv was one, but after 15-minutes submersion. No data were given for a shorter exposure period, and therefore this study is a refinement of the results presented in relation to Dv by Anderson et al. (2015).

Water at high temperature presents the potential of an effective biosecurity treatment, that can be used near drinking water, disposed of easily, does not require protective clothing to use, or a specific licence, contrary to other products tested here. However, water temperature exceeding 52 °C poses serious risk of severe burns to adults and children. At 49 °C it takes 10 minutes and at 52 °C it takes two minutes to cause full thickness burns of adult skin (Moritz and Henriques 1947). At 55 °C and higher a child's skin burns in one quarter of the time it takes to burn an adult's skin, as it is thinner and more vulnerable (Feldman 1983). Therefore, guidance on the use of hot water as a biosecurity measure would need to be specific in how the treatment is applied to avoid health risks. Given the potential health risks of higher water temperatures and the effectiveness of water heated to 45 °C, guidance should be based around this temperature. It is unlikely that

equipment would be damaged if treated with heated water at this temperature; however, it could potentially be difficult and expensive to maintain water at a high enough temperature for prolonged periods of time, especially in the field. If large enough volumes of water, suitable for the treatment of fomites could be heated and maintained at 45 °C then this could effectively be used to treat smaller fomites as a dip (nets, wetsuits, boots, waders etc). This may prove to be a difficult method of application as maintaining water above 45 °C could prove difficult and costly in the field. A more practical approach to the application of heated water for the treatment of personal fomites, would be to recommend application in a home environment where equipment can be submerged in a bath or large container containing heated water. While not ideal, as this would involve the removal of fomites from locations where Dv or other invasive species may be present, it would be a viable means of preventing the transfer of organisms between sites, if for example they are visited on separate days. For the treatment of larger items, such as boats, where the submersion of the fomite is not a viable option, a more suitable means to deliver heated water may be as a spray. The delivery of much higher temperature water (> 70 °C) at a high rate could potentially allow the delivery of water > 45 °C to the surface of the fomite in sufficient quantity, although this would vary considerably with environmental conditions. The use of high pressure steam cleaners to treat fomites removed from infected sites would not only provide a mechanism by which high temperature water could be applied, but also a mechanical means of removing any Dv as well. In Wisconsin (USA), 16 locks on the Lower Fox River were to be repaired and maintained (plans from 2005 to 2017). Back in 2007, a proposal was made by the Fox River Navigational System Authority (FRNSA) for one of the locks, Rapide Croche Lock, to become a Boat Transfer and Aquatic Invasive Species Cleansing Station (Fox River Navigation System Authority (FRNSA) 2014). After a visual inspection, boats will be rinsed with high pressure water (~ 2000 psi) to dislodge any organisms adhering to the hull, and then be placed in a 110 °F (~ 43 °C) water bath for a minimum of 10 minutes to kill any remaining organisms. The project has been estimated at $3.8 million and has started in beginning of 2017.

Carbonated water, although not causing mortality was effective at immobilising Dv in a short period of time as a dip. The subsequent recovery of Dv during the recovery period demonstrates the narcotising effect of carbonated water (Gannon and Gannon 1975). The narcotising effect observed could be effective as a potential control mechanism, reducing the chance of Dv finding and attaching to fomites. CO_2 has been used as an alternative anaesthesia in fish (Mitsuda et al. 1982; Bernier and Randall 1998; Kugino et al. 2016), but has also some application in aquatic invertebrates (Ross and Ross 2008), particularly crustacea (Mestre et al. 2011; Aguilar-Alberola et al. 2012; Premarathna et al. 2016). Carbonated water was better than chlorobutanol to obtain living entocytherids, that were "knocked down" from infected crayfish, in 5, 10 and 15 minute submersion baths (Mestre et al. 2011). CO_2 is listed as an insecticide (for use against storage pests) so it would be legal to use it against Dv and it is readily available, safe to use around drinking water and is easily disposed of. As a biosecurity measure, small equipment such as nets, wet suits, paddles etc could be dipped effectively for few seconds in water with CO_2-bubbled through it. A potential drawback to this method would be that Dv may remain attached to fomites even if narcotised, as they seem to readily attach to most surfaces via their setae (Bacela-Spychalska et al. 2013), and then recover once removed from the carbonated water, potentially being transferred to other waters. However, gravity alone was efficient enough for Dv to detach from wet rope (Bacela-Spychalska et al. 2013). In addition vigorous shaking, while submersed in carbonated water, may also aid in dislodging Dv from items (Bacela-Spychalska et al. 2013; Bacela-Spychalska 2015). Carbonated water could potentially be used as a preventive measure with CO_2 bubbling into water in specific areas during the launching and landing of boats; this may prevent the attachment of Dv to fomites as they are locally narcotised. This would require that air-lines are placed in areas, possibly under the substrate, that would require maintenance and could possible impinge on the movement of boats in and out of the area. However, if combined with habitat modification, then Dv could be excluded/narcotised from certain areas reducing the potential for their attachment to fomites. A potential drawback of these methods would be the use of pressurised CO_2 where pressurised cylinders would be required to deliver the CO_2. However, it may be possible to use dry ice, which may overcome many of these obstacles; it is cheap, easy to transport, but does pose risks when being handled.

Conclusions

While a number of potential treatments have been tested at a preliminary level, and demonstrated to be effective at killing Dv, only hot water and carbonated water present viable solutions when compared with the gold standards presented here (see Table 3). The present study provided evidence that water at high

temperature and carbonated water may be effective biosecurity measures against Dv in a short time (less than one minute). Both treatments have limitations, which could be overcome with further investigations into effective application in the field. At the very least, hot water presents a viable means of easily and safely treating personal equipment where facilities allow.

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Supplementary material

The following supplementary material is available for this article:

Table S1. Percentage mortality of *Dikerogammarus villosus* after exposure to a range of concentrations of different treatments and after recovery in dechlorinated mains water.

This material is available as part of online article from:
http://www.reabic.net/journals/mbi/2018/Supplements/MBI_2018_Sebire_etal_Table_S1.pdf