Evaluating the effectiveness of hot water as a decontamination method to prevent the accidental movement of aquatic invasive species.

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1. Summary

- Boats and equipment used for recreational water sports activities are thought to be potential vectors for the spread of aquatic Invasive Non-Native Species (INNS) in the UK. In 2010, the Department of Environment, Food and Rural Affairs (Defra) launched the *Check Clean Dry* biosecurity campaign to raise awareness of aquatic INNS and to encourage water sports participants to prevent accidentally translocation of species between sites on their kit by adopting good biosecurity practices.
- Here, we test the effectiveness of the *Check Clean Dry* advice at killing a range of high impact aquatic INNS on anglers keep nets. Specifically, we test whether hot water at 45˚C for 15 minutes is effective as a consistent method by which people can 'Clean' their kit.
- We put seven aquatic INNS including four plants (*Lagarosiphon major, Myriophyllum aquaticum, Hydrocotyle ranunculoides* and *Crassula helmsii*) and three animals (*Dreissena polymorpha, Dikerogammarus villosus* and *Hemimysis anomala*) into individual angling keep nets and exposed them to one of four treatments: hot water only, hot water and drying, drying only or control (no treatment). The hot water treatment involved submerging the nets in water at 45˚C for 15 minutes while the drying treatment consisted of laying the damp nets out on trays in a 14 ± 1˚C room to simulate a shed or outhouse.
- The hot water treatment and hot water and drying treatment resulted in 99% and 97% mortality respectively across all species within one hour while it took 7.52 days to reach LT_{90} with the drying treatment and a projected 17.16 days to reach LT_{90} for the control treatment.
- The hot water treatment caused significantly higher mortality than drying at all time points (1 hour: $X^2 = 117.24$ $p<0.001$; 1 day $X^2 = 95.68$, $p<0.001$; 8 days $X^2 = 12.16$, $p<0.001$ and 16 days $X^2 = 7.58$, $p<0.001$). Although less effective than cleaning, drying caused significantly higher mortality than the control treatment from day 4 ($X^2 = 8.49$, $p<0.01$) onwards and killed 80% of invaders after 8 days and 90% after 16 days.
- Compared to the other species, *Crassula helmsii* was particularly resistant to the biosecurity treatments. Mortality occurred 24 hours after hot water treatment for *C.helmsii* while mortality took <1 hour to occur after hot water treatment in the other species, and the drying treatment only resulted in 60% mortality in *C.helmsii* after 16 days.
- Adult American signal crayfish (*Pacifastacus leniusculus*) were also exposed to hot water treatments to determine minimum lethal temperature and time. An exposure time of 5 minutes at 40°C was the lowest temperature that caused 100% mortality. Although 100% mortalities were observed when exposed for 1 minute to 60°C, this water temperature could degrade watersports equipment and has the potential to cause burns in children.

- Based on our evidence, we suggest that Defra advocates the use of hot water as part of the national *Check Clean Dry* biosecurity campaign to help to prevent the accidental spread of invasive species by recreational water users. The advice that 'the submersion of personal kit into water that is as hot as you can comfortably put your hand into for a minimum of 15 minutes' is sufficient to kill a range of invasive species. We also believe that the method is suitable biosecurity tool for other water equipment and the field kit used by ecologists and environmental researchers.

2. Introduction

The threat that Invasive Non Native Species (INNS) pose to global biodiversity loss is considered to be second only to habitat destruction since INNS have devastated terrestrial, freshwater and marine ecosystems across all continents (Mack *et al.* 2000). Freshwater systems are particularly vulnerable to the introduction of INNS due to their exposure to a multiple transport pathways along which new species can be either accidentally or intentionally introduced, and because the resilience of freshwater ecosystems is already reduced by pollution, agricultural run-off and altered hydrology (Strayer 2010).

Recent research indicates that fishing, boating and leisure activities are collectively responsible for almost 40% of aquatic species introductions into Europe (Gallardo & Aldridge 2013). These pathways commonly include the release of boat ballast water and the stocking and subsequent escape of non-native fish or crustaceans introduced for aquaculture or sport. However, they also include the accidental transfer of invasive plants and invertebrate species "hitchhiking" on personal equipment such as angling nets, bait buckets, wet suits and waders used during recreational activities (Ludwig & Leitch 1996; Buchan & Padilla 1999; Johnson, Ricciardi & Carlton 2001; Gates *et al.* 2008; Stebbing, Sebire & Lyons 2011; Stasko *et al.* 2012; Bacela-Spychalska *et al.* 2013). Such accidental transfer is thought to have been responsible for new introductions , as well as facilitating the secondary spread of species, once introduced (Johnson *et al.* 2001; Bothwell *et al.* 2009; Kilian *et al.* 2012). The fact that many invasive species can survive for many days if not weeks in damp environments – for example, zebra mussels can survive outside water for at least 5 days (Ricciardi, Serrouya & Whoriskey 1995) and killer shrimp (*Dikerogammarus villosus*) for 15 days (Fielding 2011) -- further increase the likelihood that aquatic INNS could survive the accidental transfer of contaminated kit from a source population to a new water body.

In the UK, freshwater ecosystems contain seven of the UK Environment Agency's 10 'most wanted' INNS (Environment Agency 2011) and are thought to be threatened by a further 11 (Gallardo &

Aldridge 2013). The connectivity of these waterways through both river networks and human activities facilitate the rapid spread of aquatic INNS once they are introduced (Rahel 2007) and make eradication virtually impossible (Mack *et al.* 2000; Kolar & Lodge 2001). Preventing these species from being introduced in the first place is considered to be a far more effective management strategy (Vander Zanden *et al.* 2010; Caplat & Coutts 2011). The ecological impacts of these INNS range from habitat degradation, to competition with native species, to the introduction of pathogens and disease (Prenter *et al.* 2004; Hatcher & Dunn 2011; Okamura & Feist 2011). Moreover, these species can have enormous economic burdens on the tourism, water and power industries (Williams *et al.* 2010).

There are an estimated 4 million recreational anglers in the UK (Environment Agency 2004) and recent research suggests that 50% of anglers and 53% of canoeists travel to two or more water bodies within a fortnight without cleaning their equipment between uses (Anderson *et al.* 2014). These group may pose a considerable pathway for the spread of aquatic INNS should their biosecurity practices be lax.

In order to improve biosecurity practices among recreational water users, the *Check, Clean, Dry* campaign was launched in the UK by Defra in 2010, soon after the first discovery of the killer shrimp (*D. villosus*) in 2009. The objective of the campaign is to promote good biosecurity practices amongst water users to prevent the introduction and spread of *D.villosus*, as well as other aquatic INNS. The campaign provides broad guidance for water users to adopt:

"Check your equipment and clothing for live organisms – particularly in areas that are damp or hard to inspect.

Clean and wash all equipment thoroughly. If you do come across any organisms, leave them at the water body where you found them.

Dry all equipment and clothing – some species can survive for many days in damp conditions. Make sure you don't transfer water elsewhere." (DEFRA 2013)

However, specific advice, especially in reference to cleaning of equipment, is required.

It is important that any cleaning treatment recommended to UK water users is easy and economical for people to source, requires no specific training or protective equipment to use and has no impact on the environment when disposed (potentially in large volumes) (Kilroy *et al.* 2006). Moreover, the recommended cleaning treatment needs to be effective at killing a wide range of aquatic INNS as it is unrealistic to expect water users to know (or identify) which invasive species are present in different water ways or to use multiple treatments for different species.

Preliminary research conducted by the Centre for Environment, Fisheries and Aquaculture Science (Cefas) indicated that sodium hydrochloride (household bleach), iodine (FAM30) and Virkon S (Potassium peroxomonosulphate 50%, Sulphamic acid 5% and Sodium alkyl benzene sulphonate 15%) were all effective at causing mortality (LT_{90}) of *D.villosus* in dip form within 10 minutes (Stebbing *et al.* 2011). However, none of them could be recommended for use by the general public due to risks associated with use near drinking water reservoirs, potential to cause irritation in the absence of protective clothing or the potential to damage the personal equipment that they were used on (Stebbing *et al.* 2011). In addition, the use of chemical disinfectants is controlled under the Biocidal Products Directive 98/8 EC. For a chemical to be used in the control of *D. villosus* it would require listing under Product Type 18 of the Directive. Currently there are no listed disinfectants for use in the control of *D. villosus.* To list a product for a specific usage, research would have to be conducted; however, this would be both time consuming and expensive.

The Cefas study did however indicate that submersion in hot water at 45˚C was sufficient to cause 100% mortality in *D.villosus* within 15 minutes and this advice has since been adopted as the biosecurity advice of given to water users by the Norfolk Broads Authority (Broads Authority 2013).

Thermal control is considered to be one of the most efficient, environmentally sound and cost effective methods by which to prevent the accidental spread of aquatic INNS (O'Neill & MacNeill 1991; Beyer, Moy & Stasio 2010; Stebbing *et al.* 2011; Perepelizin & Boltovskoy 2011). In addition to *D.villosus*, previous studies indicate that hot water can also cause 100% mortality in zebra mussels, quagga mussels and spiny water fleas (Beyer *et al.* 2010) as well as the invasive algae *Didymo germinata* (Kilroy *et al.* 2006) suggesting that this treatment has the potential to be effective across a range of taxonomic groups.

The aim of this research is to test whether the *Check Clean Dry* advice being recommended by Defra is effective at killing a range of aquatic INNS on angling nets. We also aim to evaluate whether hot water at 45˚C is effective at prevent the survival of a range of aquatic INNS including plant and invertebrate species which pose an existing or future threat to UK waterways, with the goal of providing an effective and consistent message to water users about what to clean their kit with. We will primarily be assessing the effectiveness of hot water as a dip suitable for personal equipment used by anglers such as keep nets and waders.

3. Methods

Experiments to evaluate the effectiveness of drying and the use of hot water as a treatment for decontaminating angling nets from aquatic INNS were performed during October 2013 and

February/March 2014 at 14˚C at the Faculty of Biological Sciences, University of Leeds. The crayfish pilot experiment was performed during March 2014 at the Centre for Fisheries, Environment and Aquaculture Science (Cefas) laboratories in Weymouth.

3.1 SPECIES SELECTION

The experiment was repeated with seven aquatic INNS currently present in the UK representing a range of taxa: zebra mussels (*Dreissena polymorpha*), killer shrimp (*Dikerogammarus villosus)*, bloody red shrimp (*Hemimysis anomala*), floating pennywort *(Hydrocotyle ranunculoides)*, curly water-thyme (*Lagarosiphon major)*, Australian stone crop (*Crassula helmsii*), and parrot's feather (*Myriophyllum aquaticum*). Adult American signal crayfish (*Pacifastacus leniusculus)* were also used in a pilot experiment, as a proxy for juvenile crayfish. This is because signal crayfish were not accessible at the time of year when the experiment was undertaken, and because if a treatment kills an adult signal crayfish with a calcified carapace, it is likely that it will kill the juvenile too. The species were selected due to their classification as high impact invaders by the UK Technical Advisory Group for the EU Water Framework Directive. The animals and plants used in the experiment were collected from sites across the UK using hand searching (*D.villosus*, *D. polymorpha* and *H. anomala*) or from UK retailers of aquatic pond plants where it was unfeasible to collect wild specimens (*L. major*, *H. ranunculoides, M. aquaticum, C. helmsii*).

3.2 EXPERIMENTAL PROTOCOL: CLEAN (HOT WATER) & DRY EXPERIMENT

Once collected, the animals/plants were stored in separate aerated tanks of dechlorinated, aerated tap water in a climate controlled room (14 ± 1˚C, light: dark cycle 12h: 12h) for at least 48 hours before being subjected to a treatment. This allowed them to acclimatise to laboratory conditions and to recover from any collection or transport-induced stress. The experiments were performed under the same temperature condition which were chosen to reflect the conditions in a garage or shed, the conditions in which most anglers store their equipment.

At the start of the experiment, plants were removed from the tank and cut into fragments of approximately 60mm to simulate a fragment of plant that might become broken off and tangled up in an angling net. All of our plant species are vegetative reproducers and care was taken to include the reproductive part of the plant in each fragment, for example root nodes were included in each fragment of floating pennywort. A FluorPen was used to determine the equivalent variable fluorescence: maximal fluorescence (F_V:F_M) ratio in the aquatic plants. This ratio is commonly used as an index of plant stress (Willits & Peet 2001). Only those with scores of at least 0.7 (healthy) were included in the experiment (Dan, Sankaran & Saxena 2000).

D.polymorpha, *H.anomala* and *D.villosus* were randomly selected from the tank to prevent bias towards particular sizes. *D.polymorpha* ranged in size from 8.0mm – 22.0mm (median 16.0mm), *D.villosus* ranged between 8.7 and 20.9mm (median 11.2mm) and *H.anomala* ranged between 10.5 and 13.8mm (median 12.5mm). As the data were non-normally distributed, we used Kruskal Wallis tests to confirm that there were no significant differences in the size ranges of *D.polymorpha* ($H = 2.1$, $df = 3$, $p = 0.55$), *D.villosus* ($H = 3.17$, $df = 3$, $p=0.36$) or *H.anomala* ($H = 7.39$, $df = 3$, $p = 0.06$) assigned to different treatments.

The selected individuals then were observed and only those swimming normally (*D.villosus* and *H.anomala*) or siphoning water and responding to stimuli (*D.polymorpha*) (Beyer *et al.* 2010) were used in the experiment. The maximum body lengths of *D.villosus* and *D.polymorpha* were measured using callipers and recorded.

The experiment was designed to mimic the conditions of an anglers keep net. As such, each animal or plant fragment was placed in a mesh bag measuring 50mm x 50mm which had been created out of mesh from a popular coarse angling keep net (http://www.keepnetsdirect.com/#/3m-black-carp-kn/4547129087). The bags were sealed with duct tape and submerged in dechlorinated tap water for 1 hour in order to absorb the amount of water retained in a net during a typical angling trip (L Anderson, unpublished data). Once damp, the nets were subjected to one of four treatments (Table 1). For the hot water treatment, a 15 minute exposure period was selected as this period of time has shown to be effective at killing *D.villosus* in previous studies (and subsequently been implemented as biosecurity advice) and because this is the maximum period of time that a treatment could realistically be applied in the field (Stebbing *et al.* 2011).

Table 1. Summary of experimental set up. Repeated for each of six species.

Treatment	Description	Number of individuals checked at each time point				
		24 h	48 h	4 days	8 days	16 days
Clean (hot water) only	50 mesh nets submerged in waterbath at 45˚C for 15 minutes. Nets put inside individual (unsealed) plastic bags and stored in climate controlled room at 14 ˚C.	10	10	10	10	10
Clean (hot water)and dry	50 mesh nets submerged in water bath at 45˚C for 15 minutes. Mesh nets laid out on tray at 14 ˚C.	10	10	10	10	10
Dry only	50 mesh nets laid out on trays in climate controlled room at 14 ˚C.	10	10	10	10	10
Control	50 mesh nets put inside individual (unsealed) plastic bags and stored in climate controlled room at 14 ˚C.	10	10	10	10	10

Animals/plants were observed and recorded as alive/dead at six time points after the initial treatment: 1 hour, 24 hours, 48 hours, 4 days, 8 days, and 16 days. The time units were chosen to represent time intervals during which angling kit might be stored for between uses (i.e. if an animal survived for 16 days, it is probable that they could survive on the net of an angler who went fishing once a fortnight). Because the plants and animals had to be handled and/or exposed to water to test for survival, separate batches of 10 animals tested at each time point. Having been tested, individuals were not returned to the experiment.

3.3 TESTING SURVIVAL

Zebra mussels were assumed dead if their shells gaped and they did not respond to stimuli either immediately after the experiment or after 1 hour recovery in a container of dechlorinated water (Ricciardi *et al.* 1995; Beyer *et al.* 2010; Comeau *et al.* 2011). *D.villosus* and *H. anomala* were considered dead if they were discoloured (or had begun to decompose) and neither responded to stimuli nor swam after being put in a container of dechlorinated water for 1 hour. For the plants, a FluorPen was used at the end of the experiments to measure the variable to maximal fluorescence of leaves (F_v:F_m). This measurement is widely used as an indication of plant stress (Willits & Peet 2001), and plants with F_v:F_m values of 0.3 of below were considered to be dead (Dan *et al.* 2000).

3.4 EXPERIMENTAL PROTOCOL: CRAYFISH PILOT EXPERIMENT

Adult signal crayfish (*Pacifastacus leniusculus*) were held in mixed sex 250 litre flow through holding tanks maintained at ambient photoperiod and temperature. A water bath was prepared and maintain at either 30, 40, 50 or 60°C (±1°C). At the beginning of the pilot experiment a single animal was removed from the holding tank, sexed and carapace length measured. It was then placed into the water bath for either 5 minutes, 1 minute or 5 seconds for one of the temperatures. The animal was then removed and placed into fresh ambient water for a recovery period of up to 30 minutes. Behavioural observations were made during this recovery period at 1 and 30 minutes of the recovery period (see Table 3). This was repeated 5 times for time temperature combinations (see Table 2). No animal was used more than once in any trial. The carapace length of animals used varied between 3cm and 7cm with a median length of 4.5cm. ,

Table 2. Experimental design of crayfish experiment. Numbers show the number of crayfish exposed to each treatment combination.

Temperature/Duration	5 minutes	1 minute	5 seconds
30˚C	5	-	-
40˚C	5	5	-
50˚C	5	5	5
60˚C	5	5	5

Behaviour was assessed systematically by i) observation (approaching animal without touching), ii) touch (trying to move animal), iii) righting ability (manually turning animal upside down, and observing the ability of the animal to right itself). The scoring system used is shown in Table 3. The 5 behavioural score for each trial was totalled for the observations made at 1 and 30 minutes (maximum of 20) and expressed as a percentage for the purpose of interpretation.

Table 3. Behavioural scale used to assess crayfish. Reaction = meral spread, tail flip or backing away. Walking = no real reaction to stimulus but upright and locomotion observed in response to stimulus.. Movement = no locomotion but movement of pleopods, gnathopods or perepods. Twitching = movement of any body part, usually small and infrequent. Still = no movement observed, confirmation of death is no response to an eye flick.

Score	Observation	Touch	Turn over
4	reaction	reaction	cannot be turned or immediately rights
3	walking	walking	rights within a minute
2	movement	movement	movement, can't right
1	twitching	twitching	twitching (test with eye flick)
0	still	still	still (test with eye flick)

4. Results

4.1 CLEAN (HOT WATER) & DRY EXPERIMENT

Mortality differed between treatments and increased over time for all treatments. The hot water treatment and hot water and drying treatment resulted in 99% and 97% mortality respectively within one hour while it took 7.52 days to reach LT_{90} with the drying treatment and a projected 17.16 days to reach LT_{90} for the control treatment.

More specifically, the hot water treatment resulted in 100% mortality in six of our seven species and 90% mortality in the seventh species (*C.helmsii*) within 1 hour, regardless of whether the nets were allowed to dry or remained damp afterwards. The hot water and dry treatment showed similar results, with 100% mortality across 6 of the 7 species and 80% mortality in *C.helmsii* after 1 hour. A much longer time period was required for the drying treatment to cause mortality, with 19% of individuals subjected to the drying treatment still alive after 8 days and 10% still alive after 16 days. In the control treatment, mortality was low, with 70% of individuals alive after 7 days and 30% still alive after the full 16 days, including individuals of all species except *H.anomala*.

Generalised linear models with binomial errors revealed that there was a significant difference in mortality between treatments after 1 hour (Estimate = 1.28, SE = 0.15, Z = 8.4, $p<0.001$), 1 day (Estimate=2.36, SE = 0.26, Z = 9.02, $p<0.001$), 8 days (Estimate=0.698, SE = 0.14, Z = 4.75, $p<0.001$), and 16 days (Estimate=0.624, SE = 0.17, Z = 3.59, $p<0.001$), as shown in Figure 1. Species was not a significant predictor of mortality at any of the four time points (binomial GLM >0.05). To compare differences between treatments, we compared survivorship between pairs of treatments at key time points to explore their relative effectiveness at causing mortality (Table 4). There were no significant differences in survivorship between the hot water and dry treatment and hot water only treatment at any of the time points, indicating that drying kit after submersion in hot water has no additional benefit (Table 4). The hot water only treatment killed a significantly higher proportion of individuals than the drying or control treatments at every time point (Table 4 and Figure 1).

Table 4. Results of paired X^2 tests to compare the proportion of individuals (all species combined) which had died between treatments after 1 hour, 1 day, 8 days and 16 days. Figures show X^2 value. NA = result was the same for both treatments so X^2 tests could not be performed. *p<0.05, **p<0.01, ***p<0.001

Treatment comparison	1 hour	1 day	8 days	16 days
CLEAN (HOT WATER) ONLY vs. CLEAN (HOT WATER) AND DRY	NA	2.31	NA	NA
CLEAN (HOT WATER) ONLY vs. DRY ONLY	117.24***	95.68***	12.16***	7.58**
CLEAN (HOT WATER) ONLY vs. CONTROL	113.77***	101.37***	70.77***	43.44***
DRY ONLY vs. CONTROL	NA	0.05	34.34***	25.20***
CLEAN (HOT WATER) AND DRY vs. CONTROL	110.03***	86.96***	70.77***	43.44***

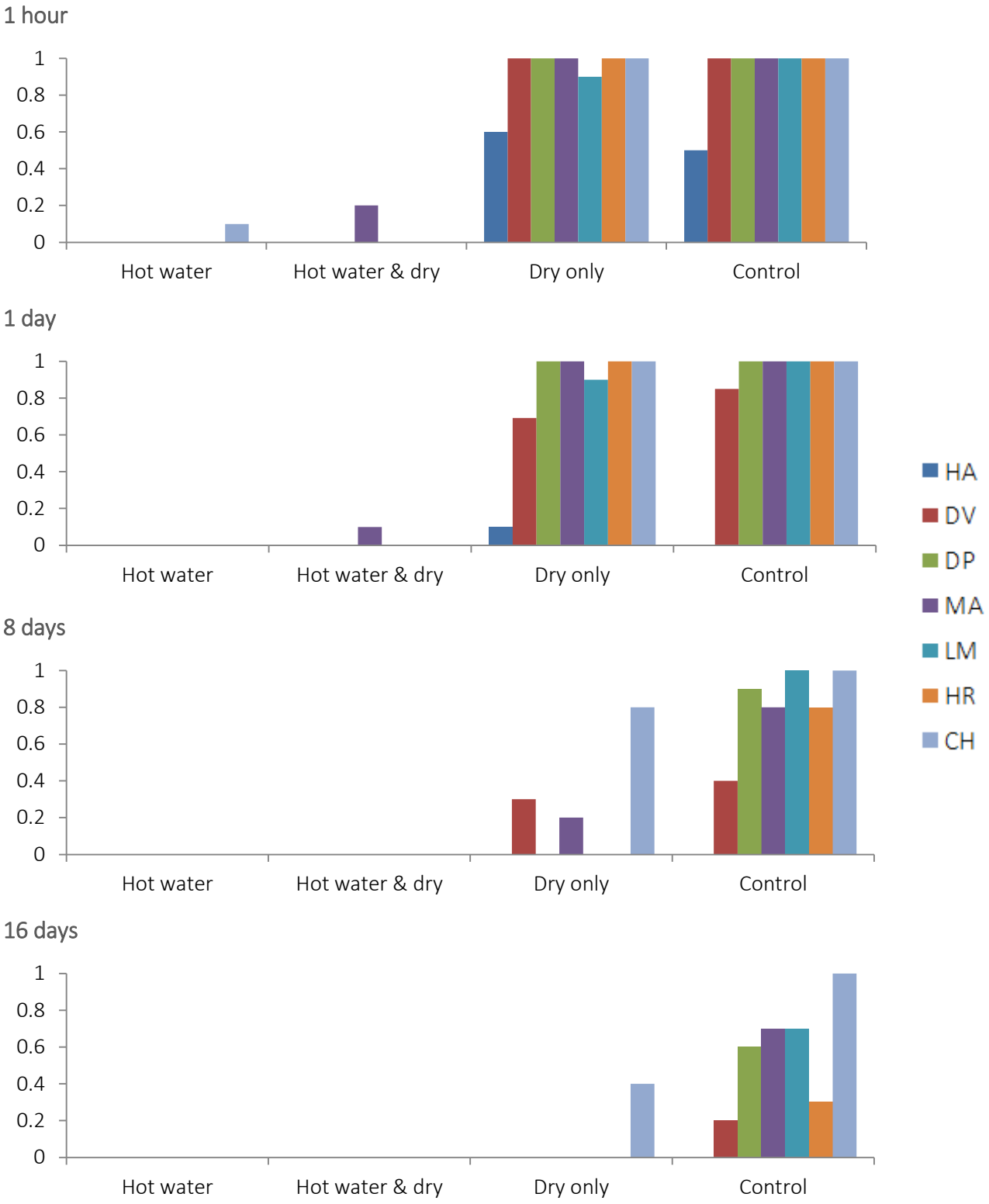

Figure 1. Proportion of individuals surviving at four key time points after treatment: 1 hour, 1 day, 8 days and 16 days. Bars show different species: HA = *Hemimysis anomala*, DV = *Dikerogammarus villosus*, DP = *Dreissena polymorpha*, MA = *Myriophyllum aquaticum,* LM = *Lagarosiphon major,* HR = *Hydrocotyle ranunculoides*, CR = *Crassula helmsii*.

Although hot water is clearly the most effective treatment, it may not always be available to recreational water users. Therefore, to explore whether drying alone was sufficient to decontaminate

kit in the absence of hot water, we plotted dose response curves to compare survivorship and calculate LT50 and LT90 for the drying and control treatments (Figure 2 and Table 5).

Table 5. Figures show how projected time taken for each species to reach 50% and 90% mortality in the control and drying treatments. *As none of the *C.helmsii* died during in the control experiment, we were unable to accurately calculate its projected survival under the control treatment. We therefore excluded this species from the mean calculation and T-tests.

Species	LT 50 (days)		LT90 (days)	
	Drying treatment	Control	Drying treatment	Control
C.helmsii	15.42	>100*	22.53	>100*
H.ranunculoides	4.13	13.35	4.34	19.04
L.major	2.25	16.31	3.21	17.14
M.aquaticum	6.19	18.52	8.73	27.65
H.anomala	0.15	0.10	0.95	0.10
D.polymorpha	4.81	16.93	6.62	23.46
D.villosus	3.43	6.45	8.54	15.59
MEAN	6.93	11.94*	7.52	17.16*

Despite not being as effective at causing mortality as hot water (Figure 1), drying caused significantly higher mortality than the control treatment from day 4 ($X^2 = 8.49$, $p<0.01$) onwards (Table 5), at which point the nets had dried out completely with no significant difference between their day 4 mass and their starting (dry) mass ($t = 1.17$, $p>0.05$). Most species exposed to the drying treatment reached LT90 in one week (7.52 days), while aquatic plants such as *L.major* and *H.ranunculoides* only survived for 3-4 days when they were allowed to dry out (Table 5). In contrast, our projections indicate that *C.helmsii* could survive over 23 days of drying (Table 5). Independent samples T-tests confirmed that the drying treatment took significantly less time than the control to cause 50% mortality ($t = -2.76$, $df = 10$, $p < 0.05$) and 90% mortality ($t = -2.89$, $df = 10$, $p < 0.05$) than the control treatment.

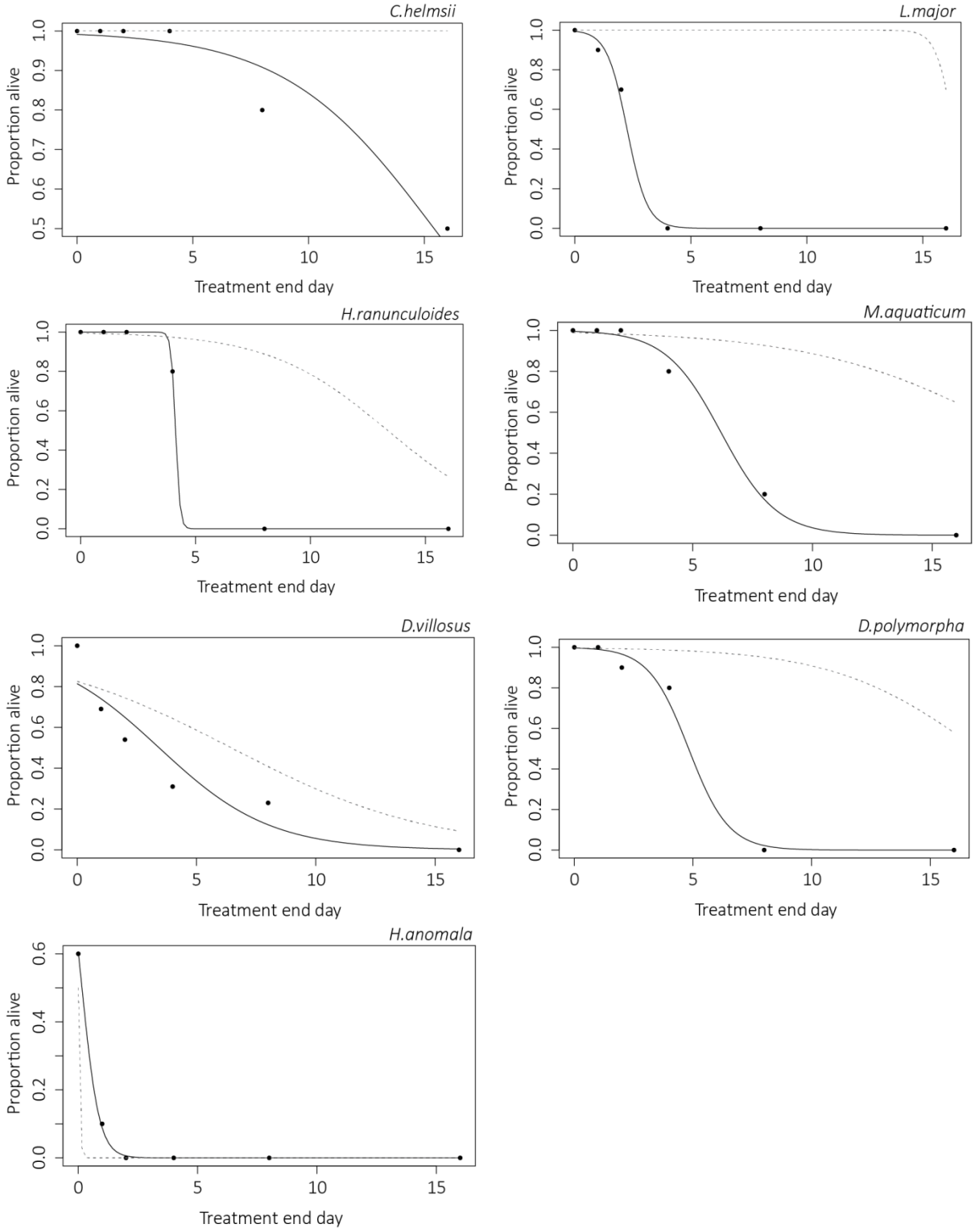

Figure 2. Dose response curves showing projected survival over time for drying and control treatments. The solid line shows projected for the drying treatment and the dashed line for the control.

4.2 RESULTS OF CRAYFISH PILOT EXPERIMENT

Table 6 presents the result of the crayfish pilot experiments. The data is expressed as a percentage of the maximum behavioural score (20) that could be recorded for each temperature/time combination for the 5 animal exposed. Estimated lethal temperature is also presented.

No mortalities were observed when exposed to any temperature tested (50 and 60°C) for 5 seconds. At 50°C chronic behavioural effects were observed but the animals recovered fully after 30 minutes. At 60°C chronic effects were also observed with degradation in animal behaviour during the recovery period.

With 1 minute of exposure mortalities were observed at 60°C (30 minutes after exposure). With a degradation of behaviour observed at 50°C, but recovery being observed during the recovery period when exposed to 40°C.

With 5 minutes of exposure, mortalities where observed in all animals exposed to 60, 50 and 40°C and recovery observed at 30°C post exposure.

Table 6. Results of behavioural index for 5 second, 1 minute and 5 minutes heat exposure experiment in crayfish. Figures expressed as percentage of crayfish in each treatment group. Recovery was measured 1 minute and 30 minutes after treatment ended.

Exposure	5 minutes		1 minute		5 seconds	
Recovery	1m	30m	1m	30m	1m	30m
60°C	0	0	35	0	80	50
50°C	0	0	40	25	80	100
40°C	0	0	75	100		
30°C	80	95				
Estimated. $LT(temperature)_{50}$	35°C		52°C		60°C	

5. Discussion

The aim of this experiment was to test whether the *Check Clean Dry* advice recommended by Defra is effective at killing a variety of different aquatic INNS and animals which threaten the UK. Specifically, we were interested in determining whether hot water (45˚C for 15 minutes) which had previously shown to be effective at killing *D.villosus* (Stebbing *et al.* 2011) was a suitable suggestion for the 'Clean' stage of the *Check Clean Dry* biosecurity protocol. While we were not able to experimentally test the 'check' element of the protocol, we investigated 'cleaning'and 'drying' by comparing cleaning (using hot water), cleaning (using hot water) and drying, and drying only to a control treatment (i.e. doing nothing).

Our results clearly demonstrate that submerging water sports equipment in 45˚C water for 15 minutes is an extremely effective method for killing a range of invasive animals and plants in a short time frame. Hot water caused 99% mortality across the seven invasive species used in our experiment within 1 hour. Moreover, hot water was effective regardless of whether or not the net which the invader was in was subsequently dried, or remained damp. The use of hot water is consistent with Defra's existing *Check Clean Dry* messaging.

Adult crayfish are unlikely to remain attached to equipment without being noticed, but were used in this study as a proxy for juvenile crayfish. With 100% mortality observed with 5 minutes exposure at 40°C, the suggestion of exposing water sport equipment in 45°C water for 15 minutes is considered more than sufficient to cause mortality in juvenile crayfish.

In the absence of hot water, drying was still found to be a significantly more effective treatment than doing nothing and caused 90% mortality in a mean of 7.52 days, suggesting that it would be suitable as a biosecurity treatment for anglers who go fishing once a fortnight or less frequently. However, drying is a more subjective biosecurity treatment and our results support previous studies which show that complete desiccation is required for it to be effective (Jerde *et al.* 2012; Poznanska *et al.* 2013), making it an unsuitable decontamination method for use by anglers who go fishing frequently.

Our previous research suggests that 64% of anglers use their equipment in more than one catchment within a fortnight and that 12.5% do so without drying it between uses (Anderson *et al.* 2014). Despite some mortality, six of the seven species (all except *H.anomala*) in our control group were able to survive for at least 16 days in damp conditions, demonstrating the clear biosecurity threat posed by damp equipment which is used at multiple sites within a fortnight. Several of the species in our experiment were not previously thought to be able to survive for this long out of water: *D.villosus* has only been reported to survive for 15 days out of water (Fielding 2011) and zebra mussels for 3-5 days

(Ricciardi *et al.* 1995). We also provide evidence that aquatic plants including *H.ranunculoides* and *M.aquaticum* can survive out of water for at least 16 days which, to the best of our knowledge, has not been previously reported.

Crassula helmsii and *M.aquaticum*, were the only two species to survive submersion in hot water after 1 hour, although all individuals were dead one day after treatment. If these species were present at a site, hot water could only be suggested as a suitable treatment to cause 100% mortality if an angler was going fishing on consecutive days of the weekend or less frequently. Particular caution should be taken when using recreational equipment in areas where these plants are known to be present.

Whilst not as effective as hot water at any time point, drying caused significantly higher invasive species mortality than doing nothing (control) if a net was allowed to dry for 4 days or longer. Our results indicated that drying caused 90% mortality in an average of 7.5 days in all species except *Crassula helmsii*, suggesting that for anglers who go fishing once a fortnight or less frequently in areas where *C.helmsii* is not present, drying would be sufficient to decontaminate their kit.

In contrast, all treatments except drying resulted in 100% mortality of *H.anomala* within 1 day. We suspect that this is because this species is particularly fragile and that handling in the lab/physical damage by the nets resulted in mortality. Our results therefore indicate that it is unlikely that this species would survive transport in an angling net and that water-based transfer methods (such as ballast water) may be a more important vector for this species. Further research is needed to confirm this.

We also suggest that further research is conducted to test the effectiveness of hot water as a treatment to kill aquatic pathogens, such as *Aphanomyces.astaci,* the causal agent of crayfish plague and *Batrachochytrium dendrobatidis,* the causal agent of chytrid disease in amphibians. This would be of significant use in demonstrating hot water as a single 'catch all' biosecurity message for both invasive species and aquatic pathogens.

5.1 CONCLUSION & SUGGESTIONS

Hot water fulfils the criteria for an effective biosecurity treatment. Not only does it cause 99% mortality within an hour, it is environmentally sound and cost effective (O'Neill & MacNeill 1991; Beyer *et al.* 2010; Stebbing *et al.* 2011; Perepelizin & Boltovskoy 2011) and the temperature we are recommending, 45˚C, is below the temperature at which hot water is thought to be able to cause burns in children (52 ˚C) (Feldman *et al.* 1998) making it safe to use by children as well as adults.

We have provided evidence that hot water is effective at killing a range of high impact invasive species in a short time frame. As such, we suggest that Defra advocates the use of hot water (45˚C for 15minutes) as part of their national *Check Clean Dry* biosecurity awareness campaign. In addition to anglers, we suggest that this method is adopted by water sports participants with wetsuits or equipment that can easily be submerged, as well as ecologists, environmental scientists and field centre staff and volunteers who use nets, waders and other equipment to undertake freshwater fieldwork in the UK. As a final precaution, we suggest that the hot water is disposed of onto the ground, rather than into a water body or drain, after cleaning is complete.

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