Cefas contract report C5256

Evaluation of a number of treatments to be used as biosecurity measures in controlling the spread of the invasive killer shrimp (*Dikerogammarus villosus*)- Final report (September 2011)

For Defra, Protected species and non-native species policy group

Paul Stebbing, Marion Sebire, Brett Lyons

Executive Summary

- The killer shrimp, *Dikerogammarus villosus* (Dv) is a large gammarid of Ponto-Caspian origin.
- Dv was discovered at Grafham Water, Cambridgeshire, England, in September 2010 and subsequently in Cardiff Bay and Eglwys Nunydd near Port Talbot, both of which are in Wales.
- It is important that an effective biosecurity system is implemented at infected waters to prevent further spread.
- This study investigates the application of a number of treatments as potential bio-security measures to prevent the further spread of Dv by fomites from infected sites.
- All the work presented here was conducted at the Centre for the Environment Fisheries and Aquaculture Science (Cefas) Weymouth Laboratory.
- This review highlighted several potential candidates for investigation, all of which were tested as part of this study: pH, salinity, iodophor (FAM30), sodium hypochlorite (NaClO), Virkon S, temperature, acetic acid, methanol, citric acid, urea, hydrogen peroxide, carbonated water and sucrose
- NaClO was selected as the reference toxicant.
- A brief review of the legislative controls on the use of biocides was conducted.
- The use of a chemical based treatment to control Dv will bring the product under the scope of the Biocidal Products Directive 98/8 EC (BPD).
- Products will need to be listed under the BPD review programme for a specific usage.
- If a product is not part of the BPD review programme for the required usage then it could not be placed on the market within the EU for that use.
- To authorise a product under the BPD for a specific usage, specific research would have to be conducted, however, this would be both time consuming and expensive, and therefore unlikely to meet the requirements for an immediate solution.
- Emergency authorisation for the use of products outside of their authorised use can be sought under regulation 15 of the Biocidal Products Regulations (2001).
- A product can receive an extension for their usage in specific situations.
- Dv were collected from both Grafham Water and Cardiff Bay.
- Dv were maintained in the Experimental Facility (EF) at Cefas, Weymouth Laboratory.
- A behavioural index was developed to determine relative sub-acute effects of the treatments tested on Dv.
- A protocol was developed to determine the effects of different concentrations of the treatments on Dv when applied as a dip application (LC_{100}).

- Once a lethal concentration (LC) had been calculated it was necessary to determine a minimal lethal time (LT_{50}).
- A protocol was developed to assess the effectiveness of certain treatments on Dv that were wrapped in netting.
- A protocol was developed to assess the potential effects of certain treatments as sprays.
- Adults were determined to be the least susceptible age group and therefore selected for all trials.
- No difference was found in the response of animals from Cardiff Bay or Grafham Water to NaClO.
- A control chart was produced for the reference toxicant (NaClO); all trials conducted were within the control chart limits.
- Of all the treatments tested NaClO (at 50,000ppm), FAM 30 (6ml/l), Virkon S (1% solution) and temperature (at 50°C) were found to cause 100% mortality within 15 minutes exposure.
- A refined exposure time was calculated (LT_{50}): NaClO of 4 minutes 20 seconds, FAM 30 of 3 minute and 10 seconds, Virkon S of 7 minute and 44 seconds, and less than 1 second for temperature.
- Carbonated water (saturated) caused narcosis in 100% of animals within a few seconds of exposure.
- In the simulated dip experiment NaClO was effective with both dry and wet recovery; temperature was effective in dry, but not wet recovery; FAM30 and Virkon S were both more effective in wet recovery.
- The only effective sprays were NaClO and FAM 30, however the ineffectiveness of temperature may have been due to the method of delivery, and could potentially to increase effectiveness.
- Due to various drawbacks in the use of NaClO, FAM 30 and Virkon S (e.g. health and safety, legal use) it was not possible to recommend as treatments.
- Recommendations are made on the potential application of temperature and carbonated water as treatments, however, further research is required before these techniques can be fully realised as methods of control.

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1.0. Introduction

The killer shrimp, *Dikerogammarus villosus* (Dv) is a large gammarid of Ponto-Caspian origin. Dv exhibits several biological characteristics which 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. 2004; Kley and Maier, 2006; Pockel 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 where it has out-competed a number of native species.

Dv was discovered at Grafham Water, Cambridgeshire, England, in September 2010 and subsequently in Cardiff Bay and Eglwys Nunydd near Port Talbot, both of which are in Wales. The prevention of the species further spread has been one of the main priorities of the Science and Technical Advisory Group (STAG), which was established to address immediate containment, associated risks, and long term risk management of Dv.

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. Dv has been found to readily attach to equipment that is used in water, such as sailing vessels, wetsuits, and fishing nets. 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.

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. 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 a chemical treatment to disinfect fomites is required. Ideally any method should meet the following criteria:

- Cause mortality (preferably 100%) in Dv within a short exposure time
- Can be applied either as a dip and/or spray
- Is usable near drinking water
- Is easily disposed of
- Does not require a specific licence for use
- Can be used for this purpose and at a sufficient rate without infringement of appropriate legislation
- Is readily available and inexpensive

- Can be used by members of the public without the use of protective equipment
- Will not cause damage to the fomites on which it is used
- Has a long ‘shelf life’
- 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 of these criteria is unlikely. This study investigates the application of a number of treatments as potential bio-security measures to prevent the further spread of Dv by fomites from infected sites. All the work presented here was conducted at the Centre for the Environment Fisheries and Aquaculture Science (Cefas) Weymouth Laboratory. A previous review of potential chemical and physio-chemical treatments was conducted by the National Centre for Environmental Toxicology (Report E3785-N750 Phase 1&2). This review highlighted several potential candidates for investigation, all of which were tested as part of this study:

1. pH
2. Salinity
3. Iodine/iodophor (FAM30)
4. Chlorine/sodium hypochlorite
5. Virkon S

In addition the following treatments were also examined:

6. Temperature
7. Acetic acid
8. Methanol
9. Citric acid
10. Urea
11. Hydrogen peroxide
12. Carbonated water
13. Sucrose

Primarily these treatments were tested as dips; in addition a number of the most effective were tested as sprays.

1.1. Selection of Reference Toxicant

During the preliminary stages of the study it was necessary to identify a chemical treatment that could be used to terminate Dv and disinfect potentially contaminated equipment with the Experimental Facility (EF). Sodium hypochlorite (NaClO) is regularly used in the EF to disinfect

equipment for disease work. Preliminary trials were conducted to assess the effectiveness of NaClO against Dv for this purpose (data not presented). NaClO was found to be effective against Dv during the initial trials. NaClO has also been found to be toxic to other arthropod species (*Gammarus fasciatus,* Ewell et al., 1986; and *Daphnia magna* Santos et al., 2007). NaClO was therefore selected as the reference toxicant.

1.2. Regulatory Reviews

The use of a chemical based treatment to control Dv will bring the product under the scope of the Biocidal Products Directive 98/8 EC (BPD). Within the BPD products are categorised into type based on their application. Any product intended for use in the control of Dv would bring it under Product Type 18 (insecticides, acaricides and control of other arthropods) of the BPD. Products will need to be listed under the BPD review programme for a specific usage (in this case Type 18) for it to be legally used for this purpose. If a product is not part of the BPD review programme for the required usage then it could not be placed on the market within the EU for that use legally. To authorise a product under the BPD for a specific usage, research would have to be conducted, however, this would be both time consuming and expensive, and therefore unlikely to meet the requirements for an immediate solution.

Emergency authorisation for the use of products outside of their authorised use can be sought under regulation 15 of the Biocidal Products Regulations (2001):

> **15.**—(1) Where a person submits an application to the Ministers for the authorisation of an unauthorised biocidal product under this regulation, the Ministers may authorise, for a period not exceeding 120 days, the placing on the market of an unauthorised biocidal product for a limited and controlled use if such authorisation appears necessary because of an unforeseen danger which cannot be contained by any other means.

However, this is for a limited time period (120 days), would require Ministerial approval and application to an unforeseen danger. Given that Dv have been in the UK for approximately a year (at time of writing) then obtaining Ministerial permission on these grounds may be difficult. Also, given the limited time period for application, an alternative solution would have to be found rapidly.

It is possible to seek an extension to the approval of a product, receiving an extension for their usage in specific situations. However, it would be the responsibility of the approval holder (usually the manufacturer) of a product to obtain an extension. It is unlikely that a manufacturer would find such an extension economically viable.

2.0. Methods and materials

2.1. Husbandry/Life Stage

2.1.1. Collection of animals

Dv were collected from both Grafham Water and Cardiff Bay. In both cases they were collected by hand with the assistance of workers at each site. The Dv were packed into water tight containers, with the Dv being placed on damp tissue paper. The containers were clearly labelled, sealed and returned to Cefas, Weymouth Laboratory on the day of collection. Dv from the different populations were held separately in the laboratory. Dv were collected from either population during the study period depending on which was most convenient. Once returned to the laboratory animals were left to acclimatise for at least 5 days prior to experimentation.

2.1.2. Bio-security and holding facilities

Dv were maintained in the Experimental Facility (EF) at Cefas, Weymouth Laboratory. The EF is a bio-secure area within Cefas, Weymouth, primarily used to work on diseases of aquatic animals. The high level of bio-security of this facility lends itself well to working on invasive species. Access to the EF is restricted to key personnel only, all of which have received training on the specific bio-security measures employed. All effluent from the EF passes through an ozone plant prior to entering the main sewage system (subsequently to a tertiary treatment plant). As the effect of ozone on Dv is unknown it was deemed necessary to implement additional bio-security measures consisting of a chemical treatment, at least 2 physical barriers and a failsafe system. The following is a brief description of the additional bio-security measures employed:

- Dv were contained in 3 flow-through tanks. These tanks contained approximately 30l of freshwater. The outflow from these tanks was situated so that a constant volume was maintained (see figure 1).
- Within each of the 3 tanks 2 containers (19.5cm x 13.0cm x 13.5cm) were submerged to approximately ¾ depth (approximately 5cm below their rim). A flow of freshwater at a rate of 30-40 ml/min entered into each container. On the side of each of the containers were fine mesh screens (Ø 1.5mm) that allowed the egress of water, but not of Dv. The water in the tanks was maintained at a temperature between 14-15°C, while oxygen levels were maintained by an airline and stone in each container (see figure 1).

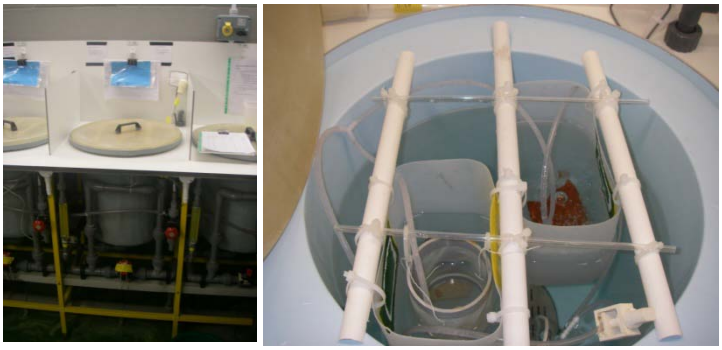

Figure 1- Pictures showing holding tanks and container set up.

- The outflow from each tank went to a water butt. Water was gathered in the butts over an 8 hour period and then treated with at least 200ppm Sodium Hypochlorite (NaClO) for at least 2.5 - 3 hours before being discharged through a fine mesh sock to the ozone plant (figure 2).

Figure 2- Water butts used for disinfection of waste water and fine mesh sock on outflow.

- All staff involved in the work were trained in the handling of Dv, taking special care to ensure that no Dv were attached to any protective clothing worn before exiting the EF.
- A fail-safe system was installed: if the flow from the tanks was blocked or increased above a rate that the outflow could not cope with, excess water would discharge into a separate water butt. This was attached to an alarm by which 24-hour on call staff would be notified of any issues with the system.
- Dv from each population were maintained in separate tanks.
- Each container in the tanks held a maximum of 150-200 individual Dv at any one time.
- Pot shards were placed in the bottom of each container as shelter and substrate.
- All experiments were conducted within the EF with no animals being removed from the EF unless dead and in fixative.

2.1.3. Maintenance of cultures

- Tanks and containers were checked daily.

- Between 3-5 coarse fish pellets were placed in each of the containers daily.
- Every other day approximately 3.22g of frozen blood worm was placed into each tank.
- Every other day each container was cleared of detritus with mortalities and moults (shed skins) counted and recorded. At no point during the study period were unexplained elevated mortalities or moult rates observed.
- During the study period precopla pairs and recently hatched juveniles were observed, these were separated from the main population to avoid predation by adults.

2.2. Behavioural index

A behavioural index was developed to determine relative sub-acute effects of the treatments tested on Dv. During preliminary trials 5 behavioural categories were described from 4 to 0. This was a linear scale describing the physical response of the animal during exposure from normal (4) through to moribund (0). Behaviour was observed after the Dv were stimulated with a pipette. The behavioural categories were:

4. Normal swimming response to stimuli.

3. Erratic swimming including tail flipping.

2. Stationary, no erratic swimming, irregular beating of pleopods or other extremities.

1. Stationary, regular twitching antennules, antenna, pleopods or walking legs otherwise not motile.

0. Stationary, very occasional twitching of antennules, antenna pleopods or walking legs. Lack of reaction was observed when stimulated.

2.3. Protocol A: Lethal Concentration (LC_{100})

A protocol was developed to determine the effects of different concentrations of the treatments on Dv when applied as a dip application. The main objective of this protocol was to determine a lethal concentration (LC) that would cause 100% mortality in Dv (LC_{100}), within a fixed time of exposure. Although technically the calculation of a LC_{100} is impossible from a dose-response sigmoid curve, the use of the term LC_{100} within this report should be read as: the lethal concentration where 100% mortalities were observed (of those tested), rather than the minimum concentration that causes 100% mortality. A 15 minute exposure period was used as this was considered to be the maximum time that any application could be applied in the field, in reality this is likely to be much shorter, but at this point in the experimentation it was deemed necessary to keep screening criteria intentionally broad. The protocol used is as follows:

Table 1 - Summary of protocol A (LC_{100}).

Study conditions	Tank approx. volume	250 ml (300ml glass crystallising dish)
	Actual temperature	14-15°C
	Day length	12:12, all studies conducted during day light hours
Experimental design	Exposure length	15 minutes in treatment
	Recovery length	Up to 80 minutes in clean freshwater
	Number of animals per tank	5
	Replicates	1
	Treatments	Multiple concentrations of the same treatment tested simultaneously
	Controls	Negative: freshwater
Measured parameters	Temperature	Measured at intervals throughout the exposure and recovery period
Endpoints	Mortality	Measured at intervals throughout the exposure and recovery period
	Behaviour	Measured at intervals throughout the exposure and recovery period (see section 3.2)
	Measurement	Length
	Identification	Confirmation of species

- Solutions of the treatment to be tested were prepared on the day of experimentation and stored in ambient conditions (14-15°C). How the solutions of each treatment were made is given as a brief description in the relevant results section. Serial dilutions were made as appropriate for the various concentrations to be tested.
- Orkney pots (90x45x48mm), figure 3, where used to contain the Dv throughout the study, allowing for them to be easily moved between exposure and recovery tanks.

Figure 3- Side and top view of Orkney pot.

- The Orkney pot containing the Dv was left in the treatment for 15 minutes, with behaviour being recorded every 5 minutes (exposure period).
- After 15 minutes of exposure to the treatment the Orkney pot containing the Dv was removed, rinsed briefly in freshwater, and moved to the recovery tank containing freshwater.
- The behaviour of the Dv was recorded every 15 minutes for up to 80 minutes after the end of the exposure period (recovery period).

2.4. Protocol B: Lethal Time Protocol (LT_{50})

Once a lethal concentration (LC) had been calculated it was necessary to determine a minimal lethal time (LT) i.e. the minimal amount of required exposure to the treatment at the lethal concentration to cause mortality. It is common practice to calculate the LT_{50} (time where 50% of mortalities are observed). The following protocol was developed to calculate a LT_{50}:

Table 2- Summary of Protocol B (LT_{50})

Study conditions	Tank approx. volume	250 ml (300ml glass crystallising dish)
	Actual temperature	14-15°C
	Day length	12:12 all studies conducted during day light hours
Experimental design	Exposure length	Integrals up to time determined in protocol A, where 100% mortality was observed during exposure
	Recovery length	Up to 80 minutes in clean freshwater
	Number of animals per tank	5
	Replicates	3 (except for reference toxicant, 6)
	Treatments	Multiple time of exposure to the same concentration tested simultaneously
	Controls	Negative: freshwater Positive: reference toxicant (NaClO at 50,000 ppm)
Measured parameters	Temperature	Measured at intervals throughout the exposure and recovery period
	pH, lux	At the end of exposure
	dO_2, total alkalinity and hardness	Only in negative control (freshwater)
Endpoints	Mortality	Measured at intervals throughout the exposure and recovery period
	Behaviour	Measured at intervals throughout the exposure and recovery period (see section 3.2)
	Measurement	Length
	Identification	Confirmation of species

- Solutions of the treatment to be tested were prepared on the day of experimentation and stored in ambient conditions (14-15°C). How the solutions of each treatment were made is given as a brief description in the relevant results section.
- The Orkney pot containing the Dv was left in the treatment for a fixed period of time, with behaviour being recorded at the beginning and end of the exposure time (exposure period).
- At the end of the exposure period the Orkney pot containing the Dv was removed, rinsed briefly in freshwater, and placed into the recovery tank containing freshwater.
- The behaviour of the Dv was recorded every 15 minutes for up to 80 minutes after the end of the exposure period (recovery period).

It should be noted that a LT_{50} instead of a LT_{100} is calculated. The choice of 50% lethality as a benchmark avoids the potential for ambiguity of making measurements in the extremes. The calculation of a LT_1 or LT_{99} would be taken from the extremes of a data set. For example, on a dose-response sigmoid curve this would be either at the beginning or end of the curve, where there are fewer data points. The calculations of lethal time from these regions of a response curve have large associated error margins making the results difficult to use as management tools. The more philosophical explanation for not calculating a LT_{100} is that it expresses an absolute certainty that is not within the power of our mortal science to deliver, because it would hold true for an infinitely large population. It is therefore a problem of inference: however many animals we observe under given conditions are a small finite number, and the assumption that the same pattern holds for yet-untested animals is a point of faith that we have identified. Mathematically, it reflects the problem of fitting a function to a bounded interval: the predicted outcome cannot sensibly be less that 0% or greater than 100%. These boundaries must therefore be singularities (like a black hole) or asymptotes where the function gets closer and closer but only arrives at 100% “at infinity”.
Despite these problems a LT_{90} has been calculated. However, it is recommended that the LT_{50} is used as the more robust management tool. It can be interpreted as the ‘average’ response of an animal within the tested population, with some extremes (the top and bottom of the response curve) responding in less time and others in more. The higher LT_{90}s calculated can therefore be attributed to mathematical artefacts, as it realistically would not take that much longer to kill another 40% of the test population (in reality the gap between an LT_{50} and LT_{100} is likely to be quite small in most cases).

2.5. Additional exposure trials protocols

2.5.1. Protocol C: net dip with wet and dry recovery

A protocol was developed to assess the effectiveness of certain treatments on Dv that were wrapped in netting. This was an attempt to replicate a realistic scenario where the Dv were trapped in netting that was then treated, then either left to dry or returned to freshwater. As fomites, such as anglers' nets could potentially be used in another water body after treatment then the potential risk of survival of Dv after treatment needs to be assessed.

- Solutions of the treatment to be tested were prepared on the day of experimentation and stored in ambient conditions (14-15°C). How the solutions of each treatment were made is given as a brief description in the relevant results section.
- 20 adult Dv were placed in netting with a mesh size of approximately 2 mm.
- The netting was folded, sealed with electrical tape, and placed in a 1inch diameter plastic pipe approximately 5cm long.
- The pipe containing the netting and Dv was then submerged into 250ml of the treatment in a 300ml glass crystallising dish for 10 seconds.
- After 10 seconds the pipe was then briefly rinsed in freshwater and then either: returned to freshwater for a 1 hour recovery period, or left to on the bench (dry recovery) for 1 hour.
- After the 1 hour recovery period the package was opened and the behaviour of the animals observed in 250ml of freshwater.
- These animals were left over night (approximately 12 hours) in freshwater and the behaviour observed again.

2.5.2. Protocol D: Spray tests

While the main aim of the work was to assess the potential effectiveness of the treatments as dips, the application in the field may also include spraying of the treatments onto fomites. A protocol was developed to assess the potential effects of certain treatments as sprays:

- Solutions of the treatment to be tested were prepared on the day of experimentation and stored in ambient conditions (14-15°C). How the solutions of each treatment were made is given as a brief description in the relevant results section.
- The prepared solution was placed into a 1L general purpose spray bottle.
- 5 adult Dv were placed into an Orkney pot which was sprayed with either 5 or 20 sprays, equating to approximately 3.75ml or 15ml of treatment respectively.
- The behaviour of the animals was observed and recorded.
- The Orkney pots containing the treated Dv were then placed on the bench for 15 minutes with the behaviour of the Dv recorded every 5 minutes in dry conditions.

- After 15 minutes the Orkney pot was then placed into another 300ml glass crystallising dish containing 250ml freshwater, with behaviour being observed every 15 minutes over a 60 minute period.

3.0. Results

It should be noted that all variables measured as part of either protocol A (temperature) and B (temperature, pH, lux, dissolved O_2, total alkalinity and hardness) were all within acceptable limits, unless being deliberately manipulated such as with temperature and pH. All animals tested were identified as Dv, with no significant variation in sizes used in or between any of the experiments conducted.

3.1. Age group susceptibility by LC_{100} (Protocol A)

To determine the most effective disinfectant to use against Dv, it was considered appropriate for the least susceptible life stage to be tested. Using protocol A, adult and juvenile (less than 1 week old) Dv were exposed to the reference toxicant (NaClO) at 200, 300, 450, 675, 1012.5ppm. The results were compared by logit regression using the statistical software Stata. It was found that there was a significant difference in response, with juvenile Dv being found to be more susceptible than adults. It was therefore decided that only adult Dv would be used in all further trials.

3.2. Population differentiation by LC_{100} (Protocol A)

Dv from populations in Grafham Waters and Cardiff Bay were used during the trial. It was important to ensure that there were no population level effects making one more resistant to the treatments being tested than the other. Adults from both populations were exposed to 5000, 10,000 and 50,000ppm of the reference toxicant (NaClO) using protocol A. The results were compared by logit regression using the statistical software Stata. No significant difference was observed in the response of Dv from either population confirming that both populations could be used in the trials.

3.3. Reference toxicant (Sodium Hypochlorite)

3.3.1. Determination of LC_{100} (Protocol A)

The lethal concentration of the reference toxicant (sodium hypochlorite, NaClO) was calculated using protocol A. A commercial stock solution (Kilco Ltd, UK) at 10-15% NaClO was diluted using dechlorinated water (from the same source used for the Dv stock). The different working solutions were serial diluted from the highest concentration. A broad range of concentrations were tested from 200 ppm to 50,000ppm (see figure 4 below).

Only 2 concentrations of NaClO tested caused mortality within the 15 minute exposure period, 10,000ppm and 50,000ppm (see figure 4). Given the significantly more rapid effect of 50,000ppm on exposed animals than the 10,000ppm, it was decided to use the 50,000ppm as the reference

concentration (LC_{100}). However, it is interesting to note that 100% mortality was still observed in lower concentrations (> 675ppm) within 60 minutes of the recovery period.

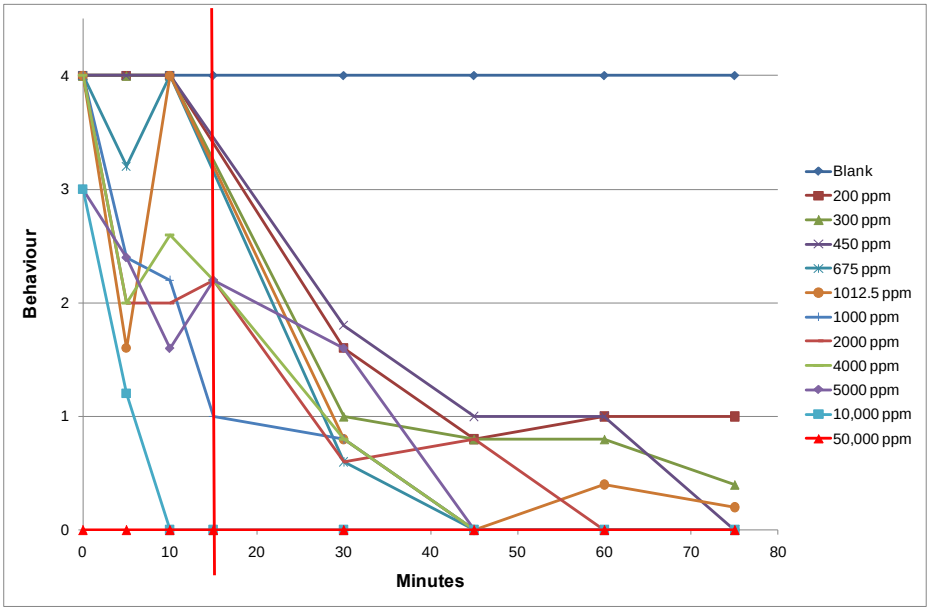

Figure 4- Graph showing range finding results for NaClO with behaviour plotted against time during exposure and recovery for concentrations between 200ppm and 50,000ppm.

The red line of the graph designates the end of the exposure period and the commencement of the recovery period.

3.3.2. Control chart for LT_{50} (Protocol B)

Using protocol B Dv were exposed to 50,000ppm of NaClO for 30 seconds, 1, 2, 4 and 8 minutes with 6 replicates of each. Figure 5 below shows the mean behavioural response of the Dv after the exposure period. It is interesting to note that initially only the 8 minute exposure period resulted in 100% mortality; however, 100% mortality was observed in all of the exposure durations within 45 minutes of being returned to freshwater.

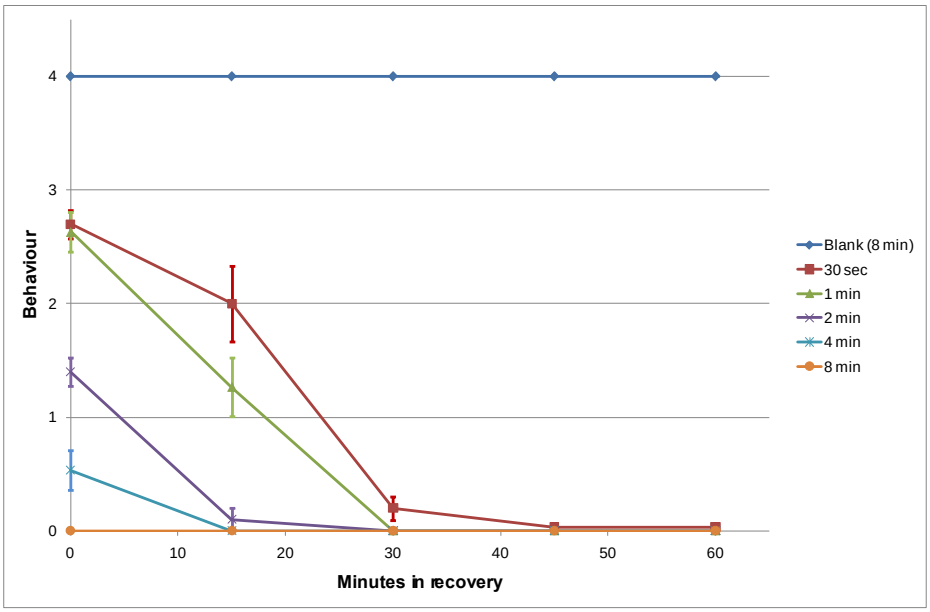

Figure 5- Behaviour results for *Dikerogammarus villosus* in recovery, after exposure to NaClO (50,000 ppm) for time periods between 30 sec and 8 min.

For each replicate (6 in total), a LT_{50} was determined from the time to death curve (Figure 6).

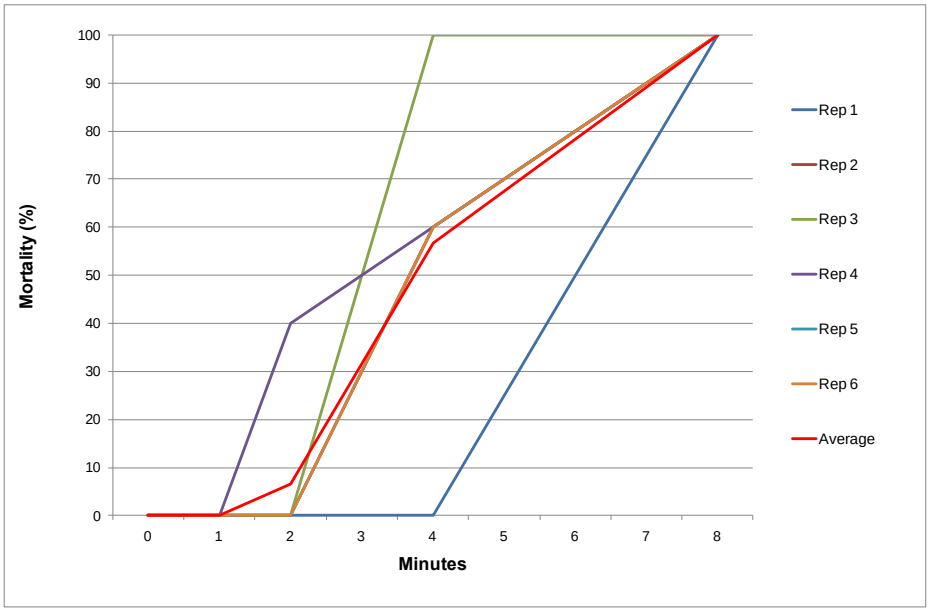

Figure 6- Time to death (minutes) percent mortality of *Dikerogammarus villosus* exposed to NaClO.

An overall average LT_{50} (4 minutes 20 seconds) and standard deviation were calculated from the LT_{50}s generated for each replicate. These data were plotted in the form of a control chart showing replicates, mean LT_{50}, ± 2 standard deviations (Figure 7). Control chart are used to monitor test precision and to assess data trends.

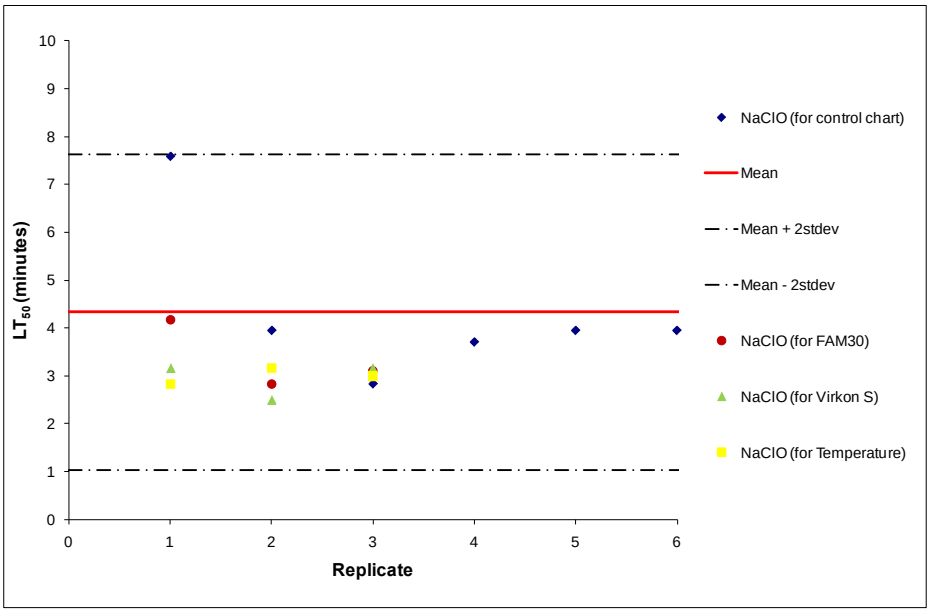

Figure 7- Control chart for sodium hypochlorite (NaClO) as a reference toxicant in toxicity assay on *Dikerogammarus villosus*.

The solid red line shows the mean value of LT_{50} from the NaClO experiment (Protocol B) and dotted lines represent ± 2 standard deviations. The diamonds represent specific LT_{50} values.

The coefficient of variation (CV) for the LC_{50}s provides a measure of test repeatability or precision; the lower the CV value the less variable the test results and the lower the frequency of false positive and false negative results. The Dv exposed to NaClO has a CV at 38.2 % (when excluding replicate 1, the CV is 13.1 %). Data from the reference toxicant control conducted alongside other treatment tests (FAM30, Virkon S and temperature) are also shown in figure 5 and are within the control chart limits.

3.4. Treatments

3.4.1. pH

Protocol A was used to test hydrochloric acid (Sigma, UK) at 1M. The pH of the dechlorinated water (from the same source used for the Dv stock) was adjusted to the different pH values (7, 6, 5, 4, and 3). During the experiment, the pH was measured, giving the following values: 7.21, 6.26, 5.3, 4.34 and 3.14. Figure 8 shows the results from the pH tests. Within the pH range tested mortality was not induced within the 15 minute exposure period. It should also be noted that no mortalities were observed during the recovery period. Given that lowering the pH further 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.

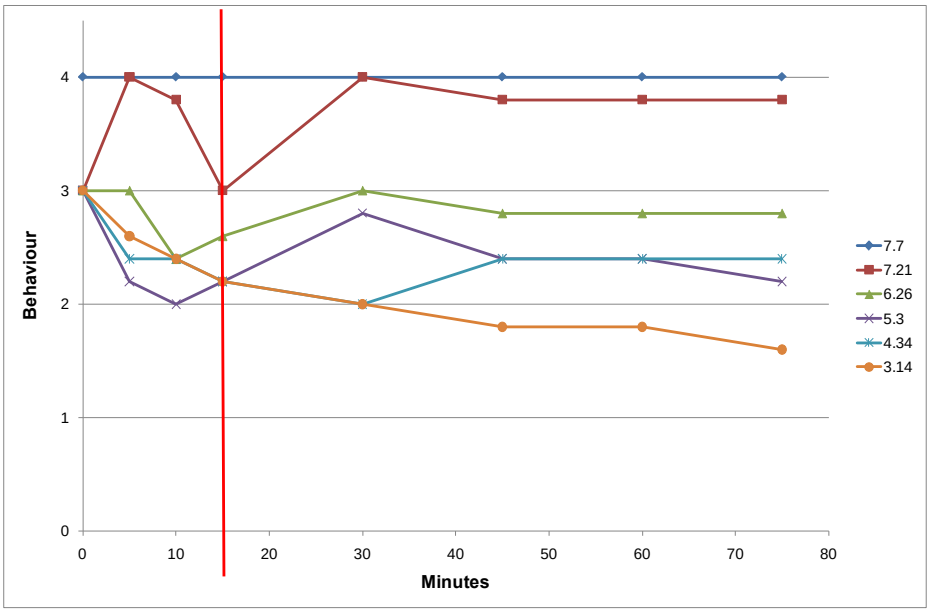

Figure 8- Graph showing range finding results for pH with behaviour plotted against time during exposure and recovery for measured values between 7.21 and 3.14 (Blank = 7.7).

The red line of the graph designates the end of the exposure period and the commencement of the recovery period.

3.4.2. Salinity

Artificial marine salt (Tropic Marin®, Germany) was tested using protocol A. Salt was weighed and then dissolved in dechlorinated water (from the same source used for the Dv stock). The different working solutions were serial diluted from the highest concentration to obtain a broad range of concentrations 5, 10, 20, 30, 35, 40, 80, and 160 g/l. The measured concentrations were: 5.29, 9.79, 19.17, 27.8, 33.9, 33.4, 66.2 and 133.6 g/l. Mortality was not induced during the exposure period even at 133g/l (approximately 3.5 times higher concentration that that of normal sea water) (see figure 9). There were also no mortalities observed during the mortality period. For these reasons the testing of salinity was stopped.

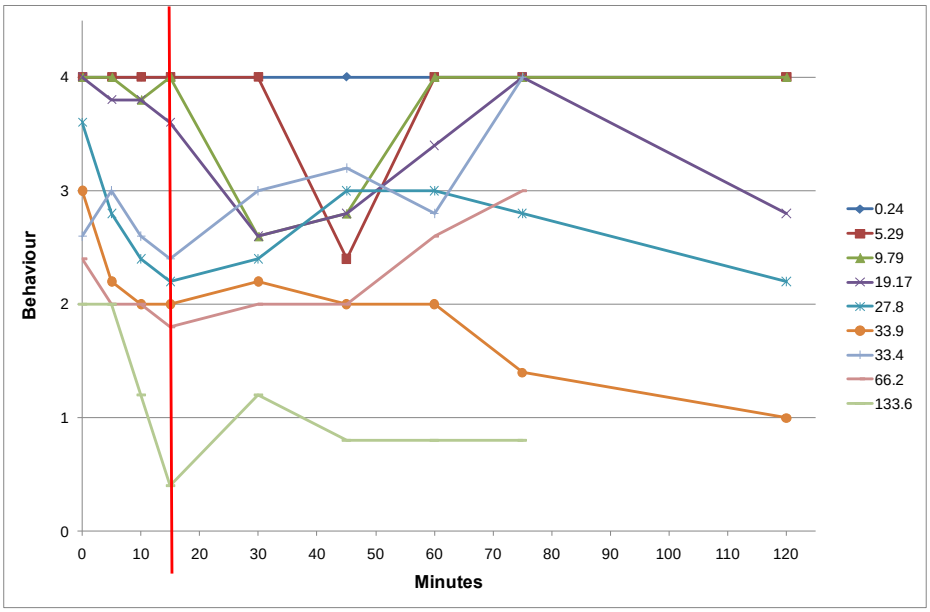

Figure 9- Graph showing range finding results for salinity with behaviour plotted against time during exposure and recovery for measured concentrations between 5.29 and 133.6 g/l (Blank = 0.24 g/l).

The red line of the graph designates the end of the exposure period and the commencement of the recovery period.

3.4.3. Iodine/iodophor (FAM30)

A commercial stock solution (Evans Vanodine International, UK) of FAM30 (Alcohol ethoxylate 20-25%, Sulphuric acid 5-10%, Phosphoric acid 5-10% and Iodine 1-5%) was diluted below the recommended dilution (1 part FAM30 to 150 parts water) using dechlorinated water (from the same source used for the Dv stock). The different working solutions were serial diluted from the highest concentration (6 ml/l i.e. 1 part FAM30 to 167 parts water). A range of concentrations were tested: 1, 2, 4 and 6 ml/l. These were tested using protocol A. FAM30 induced 100% mortality at both 4 and 6 ml/l within the 15 minute exposure period (see figure 10). At 1 ml/l 100% mortality was observed 30 minutes into the recovery period. As a quicker response was observed in tests using the 6ml/l solution it was decided that this was the concentration that would be used to calculate a LT_{50}.

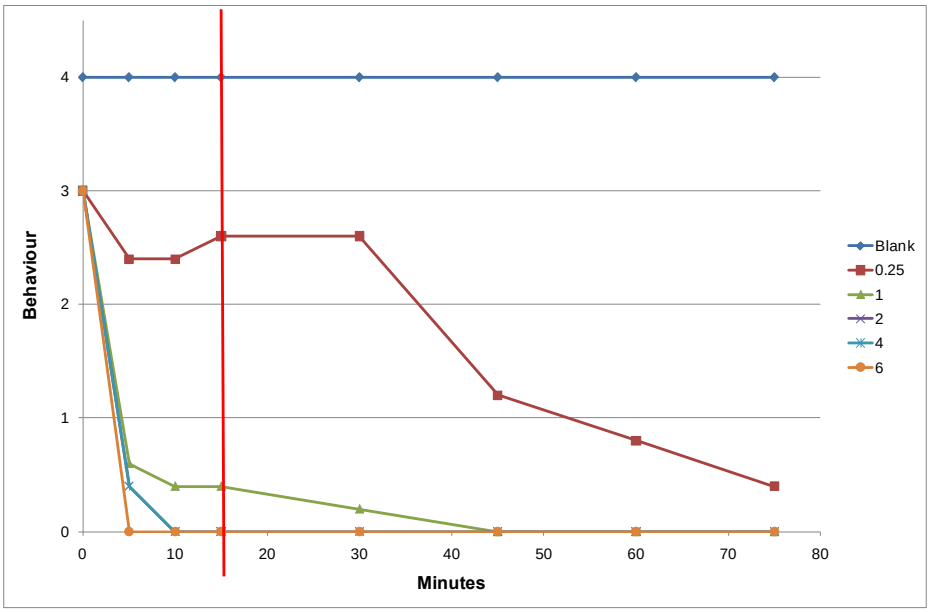

Figure 10- Graph showing range finding results for FAM30 with behaviour plotted against time during exposure and recovery for concentrations between 0.25 and 6 ml/l.

The red line of the graph designates the end of the exposure period and the commencement of the recovery period.

Using protocol B Dv were exposed to 6ml/l of FAM30 for 15 and 30 seconds, as well as 2, 4 and 6 minutes. 100% mortality was not observed at for any of the exposure times (see figure 11). Despite this, an average LT_{50} of 3 minutes and 10 seconds was calculated.

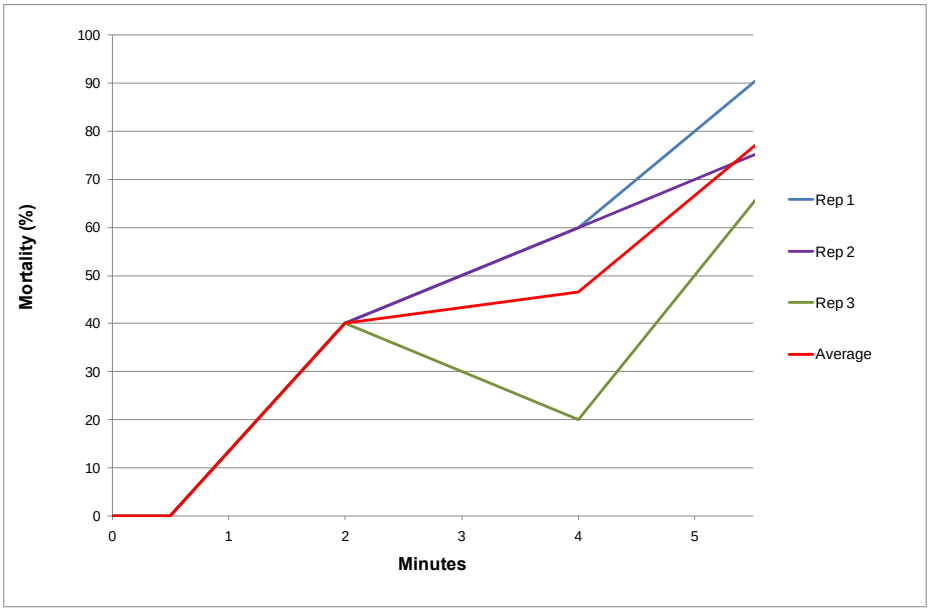

Figure 11- Time to death (minutes) percent mortality of *Dikerogammarus villosus* exposed to FAM30 at 6 ml/l.

Although 100% mortality was not observed during the exposure periods, all animals exposed were significantly affected, even when returned to freshwater during the recovery period, with 100% mortality being observed after 60 minutes with only a 15 second exposure in 2 out of the 3

replicates (see figure 12). It is not known if this decrease in behavioural response would affect their ability to locate and anchor to fomites.

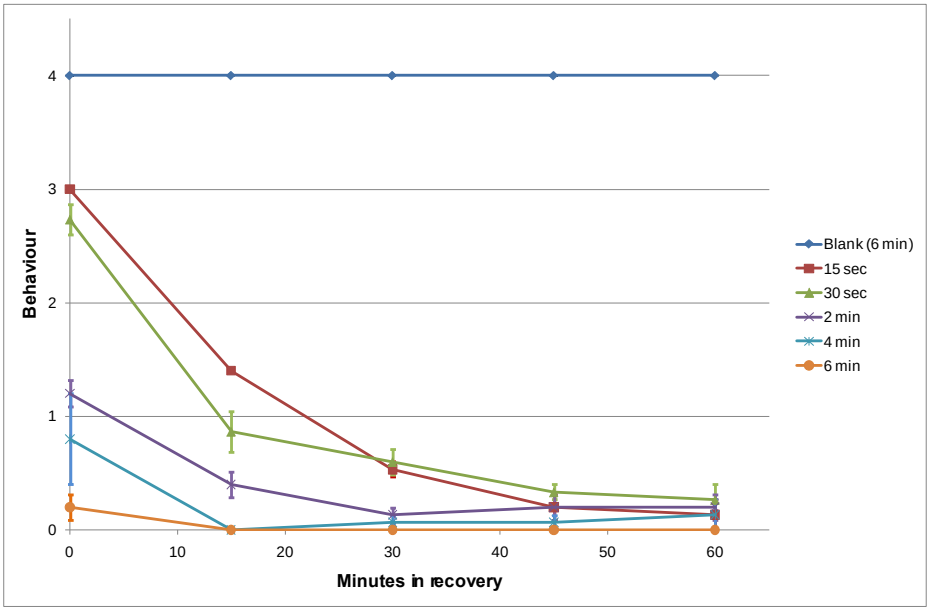

Figure 12- Behaviour results (± SE for 3 replicates) for *Dikerogammarus villosus* in recovery, after exposure to FAM30 (6ml/l) for time periods between 15 sec and 6 min.

3.4.4. Virkon S

Virkon S (Potassium peroxomonosulphate 50%, Sulphamic acid 5% and Sodium alkyl benzene sulphonate 15%) was supplied as powder (Antec International Ltd, UK). A recommended solution was made up at 1% with dechlorinated water (from the same source used for the Dv stock). The different working solutions were serial diluted from the 1% solution (10 g/l). A range of concentrations were tested: 1, 2, 4, 8 and 10 g/l. Figure 13 shows the results from these trials. Although 100% mortality was only observed during the 15 minute exposure period with the 1% solution, 100% mortalities were observed after 15 minutes in the recovery period with both the 0.4% and 0.8% solutions. The lower concentrations tested (0.1% and 0.2%) appeared to be relatively ineffectual against Dv with animals returning to normal behaviour (category 4) during the recovery period. Given the effectiveness of the 1% solution it was decided that this would be the concentration used to calculate the LT_{50} with.

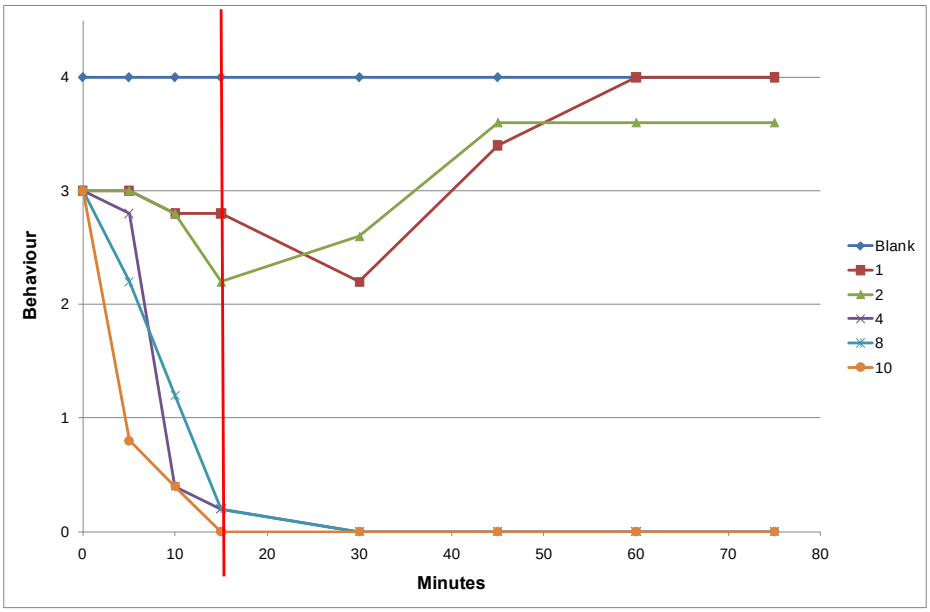

Figure 13- Graph showing range finding results for Virkon S with behaviour plotted against time during exposure and recovery for concentrations between 1 and 10 g/l (0.1 and 1% solutions).

The red line of the graph designates the end of the exposure period and the commencement of the recovery period.

Using protocol B Dv were exposed to 1% Virkon S for 30 seconds, 1, 2, 4, 8 and 12 minutes (see figure 14). From this data a LT_{50} of 7 minutes and 44 seconds was calculated.

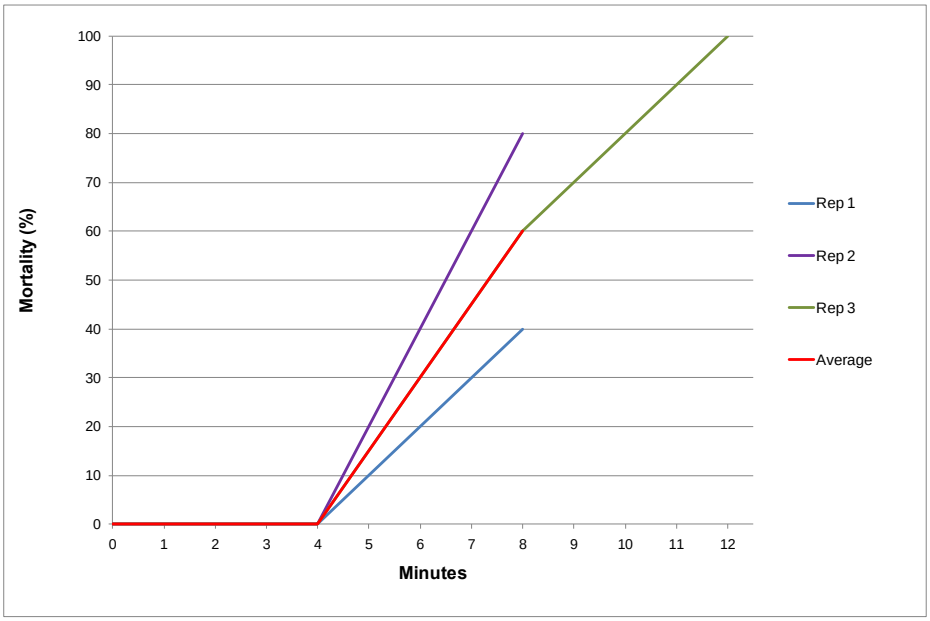

Figure 14- Time to death (minutes) percent mortality of *Dikerogammarus villosus* exposed to Virkon S at 10 g/l (1% solution).

Only after 12 minutes of exposure was 100% mortality observed; however, mortalities were observed during the recovery period for all of the exposure times apart from for 30 second exposure (see figure 15). There was a considerable amount of variation observed in the behavioural response

of Dv to Virkon S, especially during the recovery period. This was not a dose dependant response, suggesting significant variability in take up between individuals.

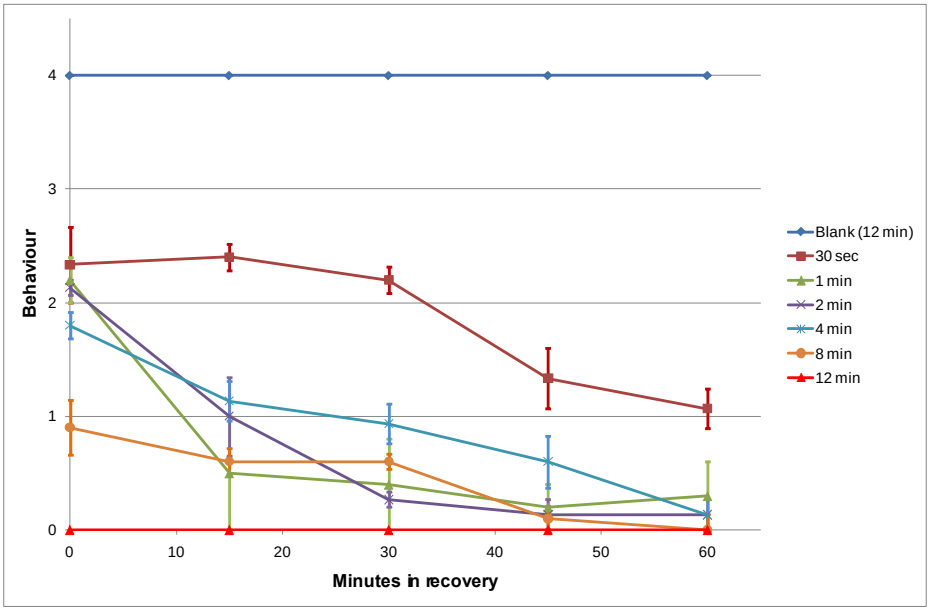

Figure 15- Behaviour results (± SE for 3 replicates) for *Dikerogammarus villosus* in recovery, after exposure to Virkon S (10 g/l) for time periods between 30 sec and 12 min.

3.4.5. Temperature

Using hot (> 60 °C) tap water, the temperature was adjusted with cold (14-15 °C) dechlorinated water (from the same source used for the Dv stock) to obtain 5 solutions: 30, 35, 40, 45 and 50 °C. These were tested using protocol A. The measured temperatures were: 28.4, 31.1, 36.3, 38.9, 43.2°C. These temperatures were the average of those recorded at the beginning and the end of the exposure period. Temperatures >30°C appeared to have a significant effect on the behaviour of Dv, with exposure to temperatures >36°C resulting in 100% mortalities within the 15 minute exposure period (figure 16). Dv exposed to water temperatures >43°C died almost immediately. This response may have been due to temperature shock. Dv maintained at higher temperatures (e.g. 30°C) may be able to withstand the sudden shock caused by submersion into water at >43°C. Due to the response of Dv to temperatures >43°C, it was decided to use water at 50°C to calculate the LT_{50}.

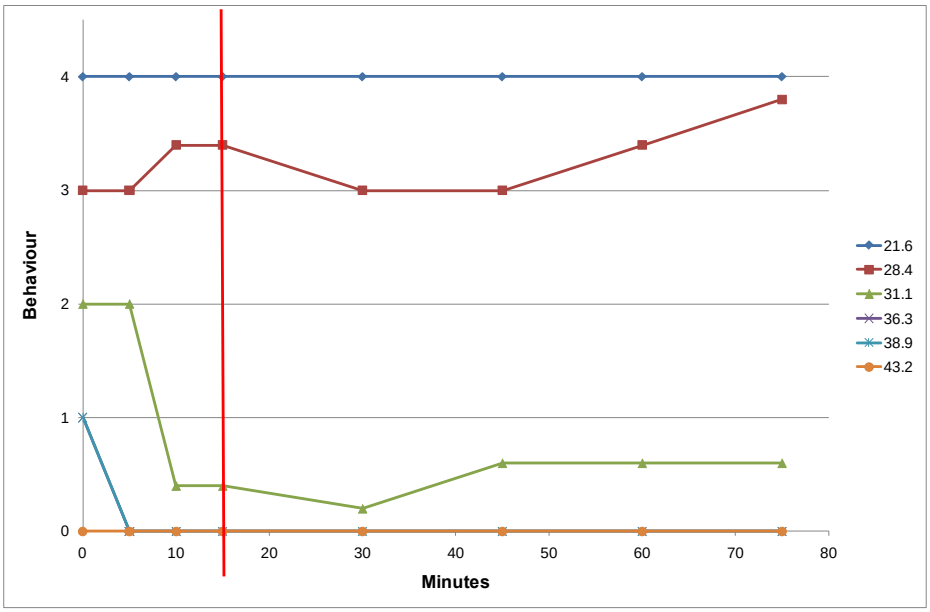

Figure 16- Graph showing range finding results for temperature with behaviour plotted against time during exposure and recovery for measured values between 28.4 and 43.2 °C (Blank = 21.6 °C).

The red line of the graph designates the end of the exposure period and the commencement of the recovery period.

Using protocol B Dv were exposed to 50°C freshwater for 5, 15 and 30 seconds, as well as 2 and 5 minutes (figure 17). From the replicates an average LT_{50} of less than 1 second was calculated.

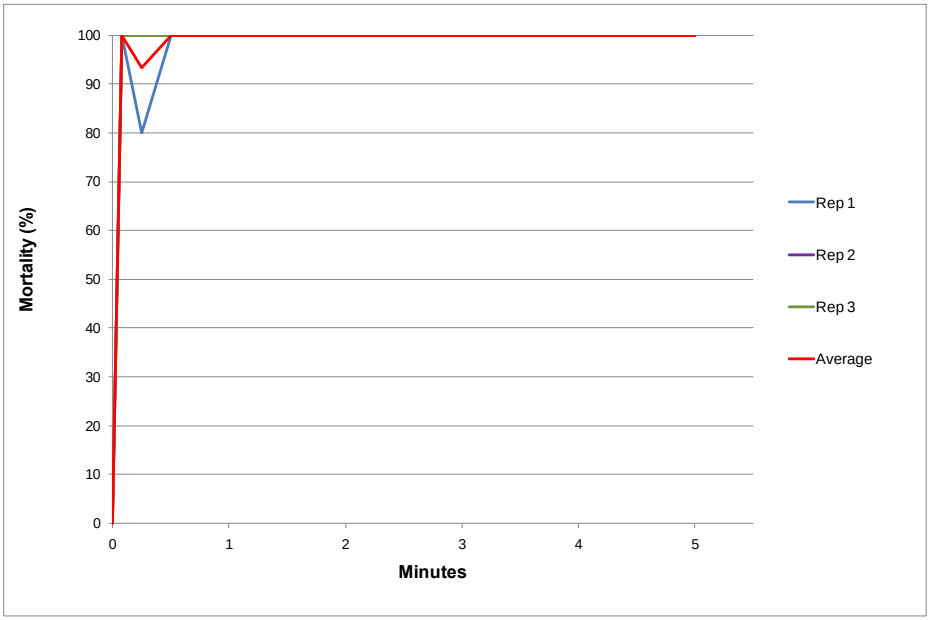

Figure 17- Time to death (minutes) percent mortality of *Dikerogammarus villosus* exposed to water at 50°C.

The behavioural response of Dv in the recovery period after exposure to water at 50°C is shown in figure 18. Although some variability in response was observed in 2 out of the 3 replicates, 100% mortality was observed for all exposure times.

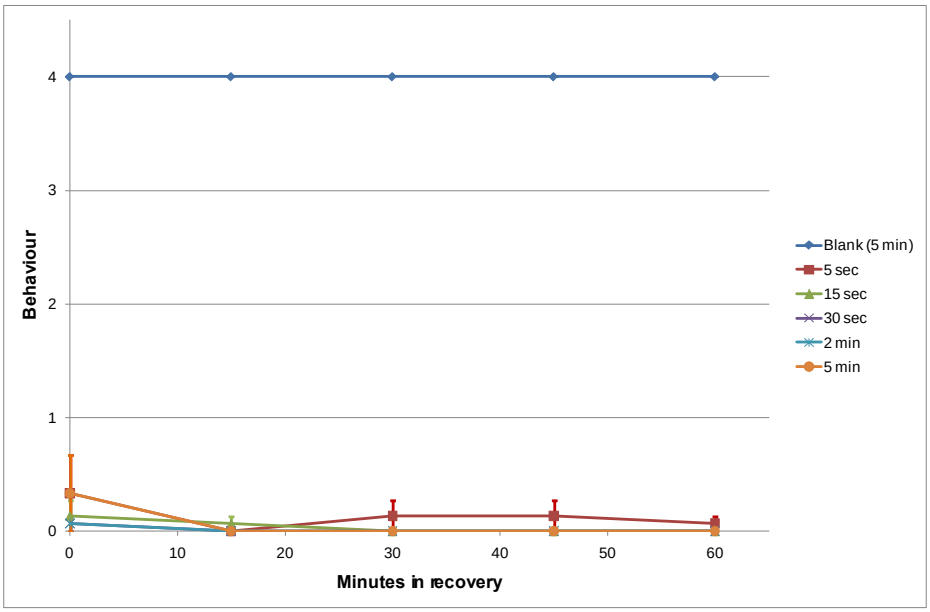

Figure 18- Behaviour results for *Dikerogammarus villosus* in recovery, after exposure to 50°C (measured at 49.3 °C) for time periods between 5 sec and 5 min.

3.4.6. Acetic acid

Acetic acid (> 95%, Fisher Scientific, UK) was diluted using dechlorinated water (from the same source used for the Dv stock) to obtain a solution at 10% and then further diluted to obtain a second solution at 1%. These concentrations were tested using protocol A (see figure 19 for results).

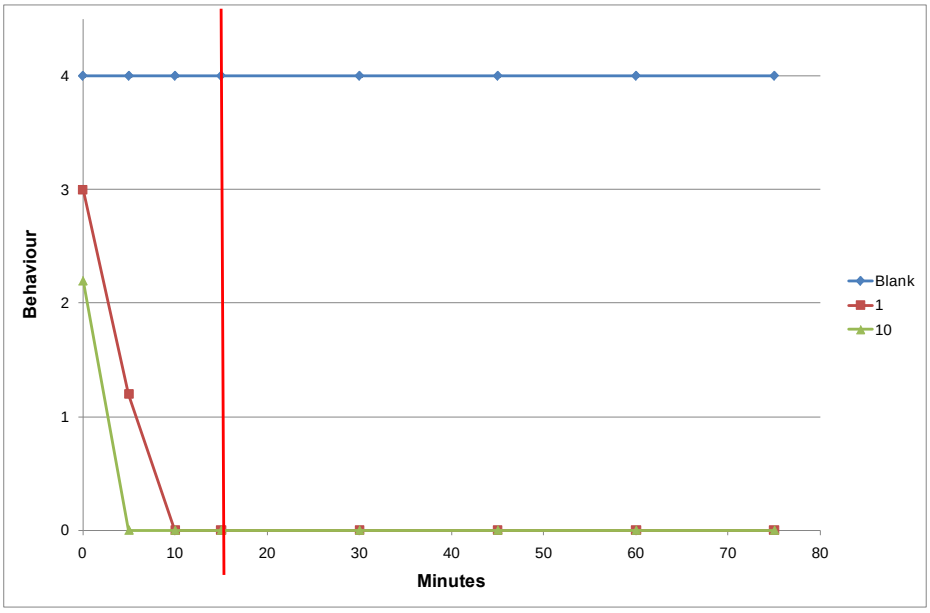

Figure 19- Graphs showing range finding results for acetic acid with behaviour plotted against time during exposure and recovery for 1 and 10% solutions.

The red line of the graph designates the end of the exposure period and the commencement of the recovery period.

Although Acetic Acid did appear to be effective against Dv with 100% mortalities observed at both concentrations, a pH of 2.28 (at 10% solution) was recorded at the end of the exposure period. Due to this, the testing of Acetic acid was not continued for the same reasons that testing of pH was stopped.

3.4.7. Methanol

HPLC grade methanol (> 95%, Fisher Scientific, UK) was diluted using dechlorinated water (from the same source used for the Dv stock) to obtain a solution at 10%. The latest was further diluted to obtain a second solution at 1%. These were tested using protocol A. As can be seen from figure 20, 100% mortalities were not observed during the exposure period at the concentrations used, it was therefore decided not to continue with testing of methanol.

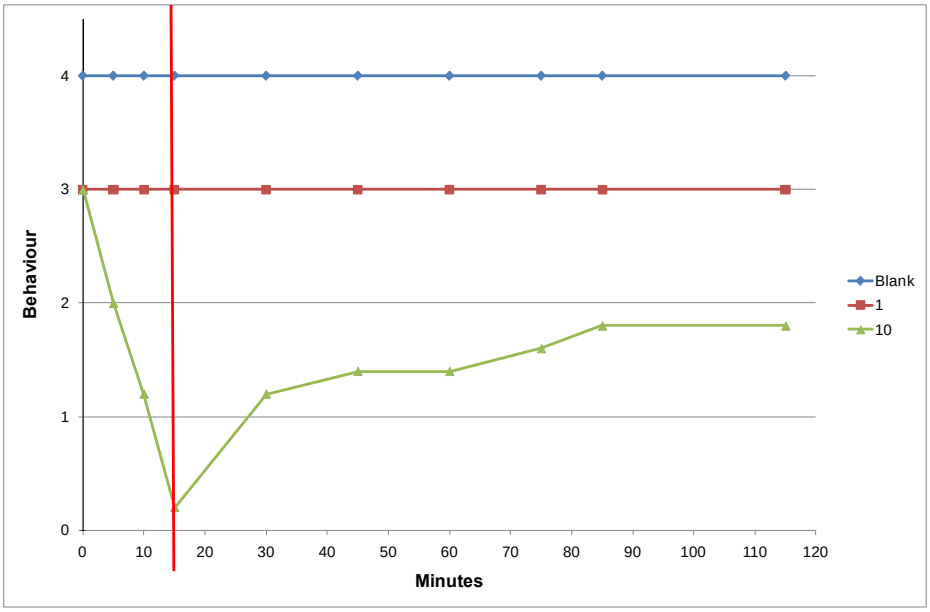

Figure 20- Graph showing range finding results for methanol with behaviour plotted against time during exposure and recovery for 1 and 10% solutions.

The red line of the graph designates the end of the exposure period and the commencement of the recovery period.

3.4.8. Citric acid

Citric acid (Acros organics BVBA, Belgium) was weighed and diluted using dechlorinated water (from the same source used for the Dv stock) to obtain a solution at 150 mg/l. The latest as further diluted to obtain a second solution at 15 mg/l. These were tested using protocol A. As can be seen in figure 21 100% mortality was not achieved during the exposure period and therefore testing was not continued.

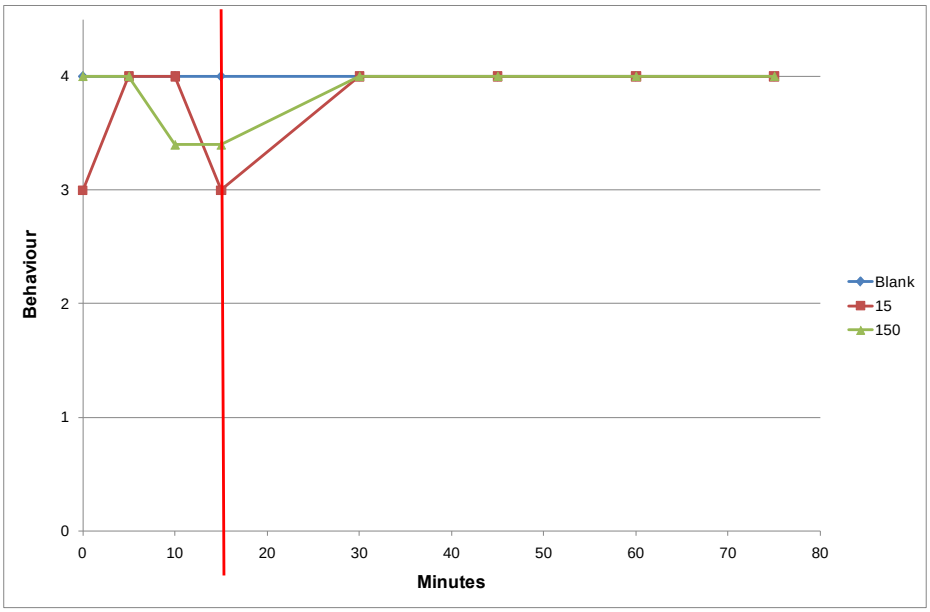

Figure 21- Graph showing r ange f inding r esults f or citric aci d with b ehaviour plotted a gainst t ime d uring exposure and recovery for 15 and 150 mg/l.

The red line of the graph designates the end of the exposure period and the commencement of the recovery period.

3.4.9. Urea

Urea (Fisher Scientific, UK) was weighed and diluted using dechlorinated water (from the same source used for the Dv stock) to obtain a solution at 10 g/l. The latest as further diluted to obtain a second solution at 1 g/l. These were tested using protocol A. 100% mortalities were not observed during the exposure period so testing was stopped.

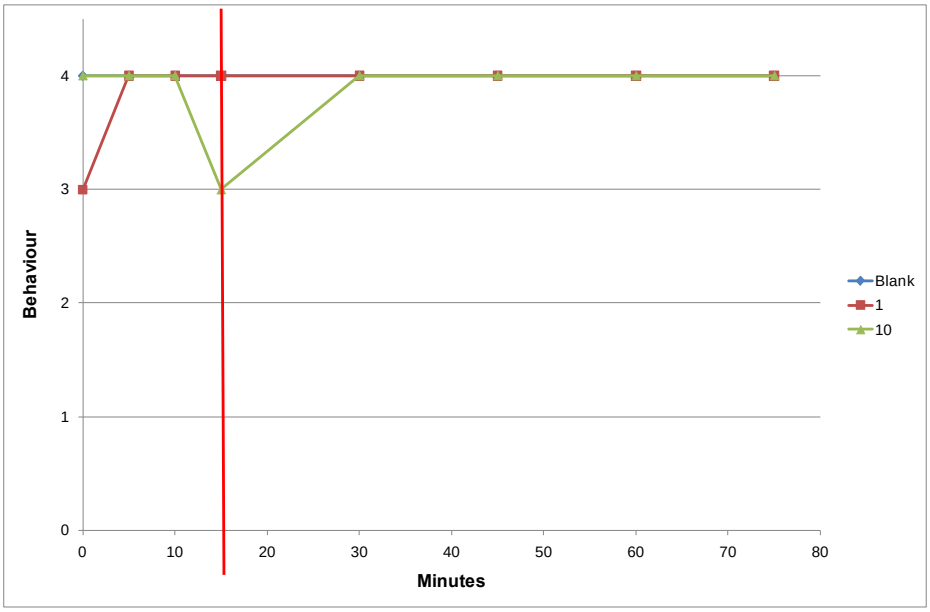

Figure 22- Graph showing range finding results for urea with behaviour plotted against time during exposure and recovery for 1 and 10 g/l.

The red line of the graph designates the end of the exposure period and the commencement of the recovery period.

3.4.10. Hydrogen peroxide

Hydrogen peroxide (30%, Sigma Aldrich,UK) was diluted using dechlorinated water (from the same source used for the Dv stock) to obtain a solution at 100 mg/l. In view of the results a further diluted solution was not tested. This was tested using protocol A. 100% mortalities were not observed during the exposure period and therefore testing was stopped.

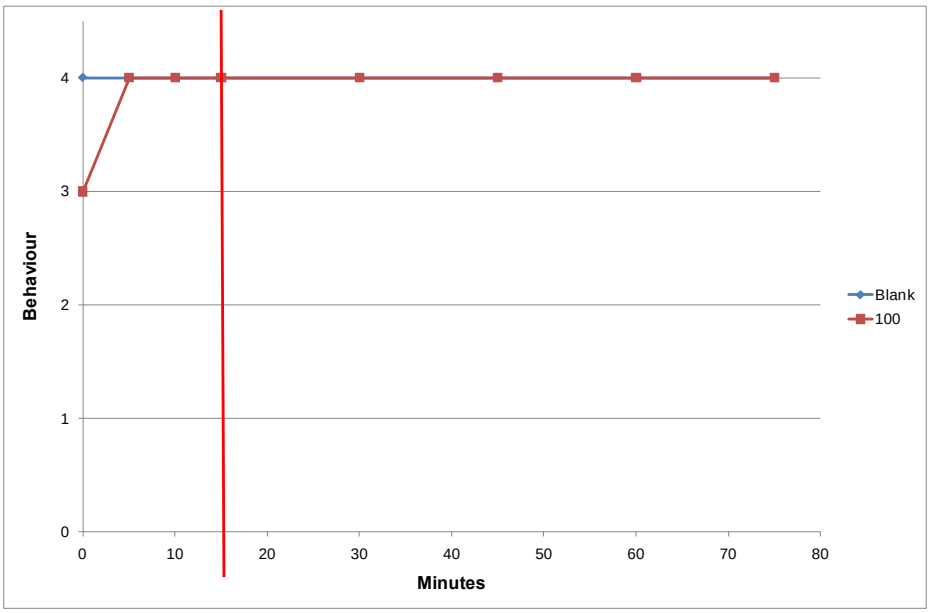

Figure 23- Graph showing results for hydrogen peroxide with behaviour plotted against time during exposure and recovery for 100 mg/l.

The red line of the graph designates the end of the exposure period and the commencement of the recovery period.

3.4.11. Carbonated water

Carbonated water (soda water: water saturated with CO_2) was purchased from a supermarket and tested as sold. This was tested using protocol A. The results from this trial are shown in figure 24. The initial response to carbonated water, showed 100% 'mortality' (behavioural category 0) was observed within only a few seconds of exposure. The subsequent recovery of Dv during the recovery period demonstrates the narcotising effect of carbonated water (Gannon and Gannon, 1975). Although this treatment did not cause mortality in Dv, the narcotising effect observed could be effective as a potential control mechanism, reducing the chance of Dv finding and attaching to fomites.

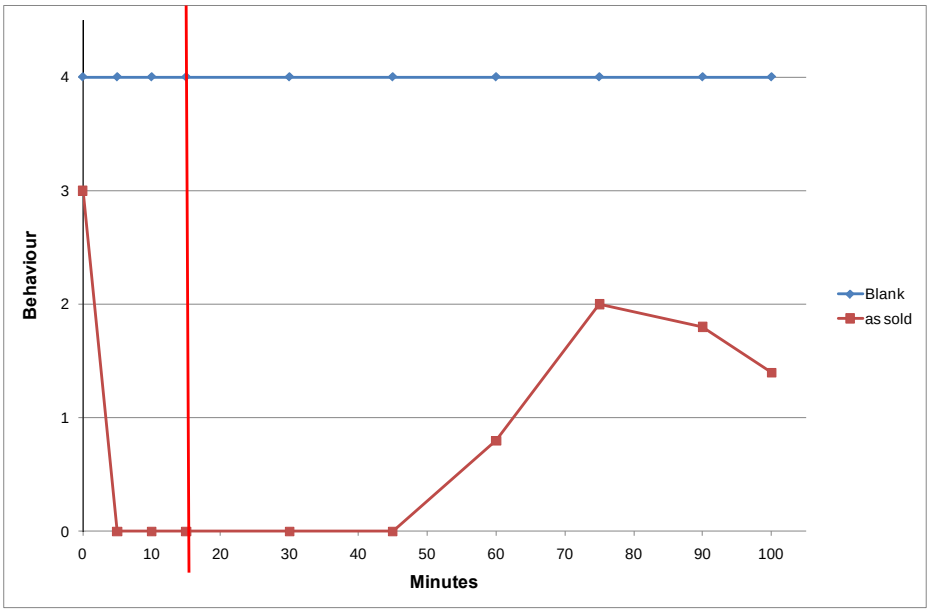

Figure 24- Graph showing results for carbonate water with behaviour plotted against time during exposure and recovery.

The red line of the graph designates the end of the exposure period and the commencement of the recovery period.

3.4.12. Sucrose

Sucrose (Sigma Aldrich, UK) was weighed and diluted using dechlorinated water (from the same source used for the Dv stock) to obtain a solution at 100 g/l. The latest was further diluted to obtain a second solution at 10 g/l. These were tested using protocol A. Although the initial effect of sucrose was significant no mortalities were observed and all Dv returned to normal behaviour in the recovery period (see figure 25).

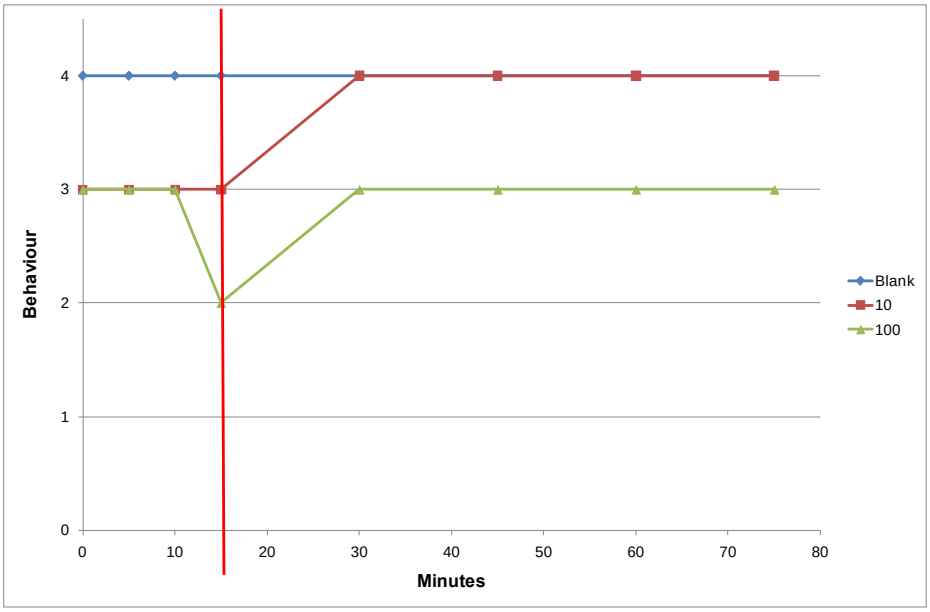

Figure 25- Graph showing range finding results for sucrose with behaviour plotted against time during exposure and recovery for 10 and 100 g/l.

The red line of the graph designates the end of the exposure period and the commencement of the recovery period.

3.5. Applications

The trials conducted using protocol A and B showed the effects on Dv when submerged in the treatment. While this provides a simulation of a dip in reality Dv attached to potential fomites could well be caught up in folds of a net or the rigging of a boat, possibly limiting the amount of exposure to the treatment. A more realistic simulation was therefore developed to test the effects of a dip. Treated fomites may also be left to dry after use or moved to a new water body, how the fomite is treated after treatment was also examined to determine effectiveness of treatments under different scenarios.

While dips are potentially effective methods of disinfecting smaller equipment, such as waders, nets, wetsuits; it is not possible to dip larger fomites, such as boats and trolleys that may come into contact with contaminated water. It is therefore important to assess the effectiveness of treatments as sprays so that they could be applied to larger objects.

3.5.1. Net dip with dry and wet recovery

Temperature (50°C), NaClO (50,000ppm) FAM 30 (6ml/l) and Virkon S (10g/l) were tested using protocol C. Figure 26 shows the results from these trials. It is interesting to note the difference in response between dry and wet recovery of the Dv to the different treatments. Temperature was shown to be an effective treatment with 15 out of 20 of the exposed Dv dead after 1 hour when the simulated fomite was left to dry (all dead after 12 hours in freshwater). In contrast, temperature was ineffective (after a 10 second exposure) after 1 hour in wet recovery.

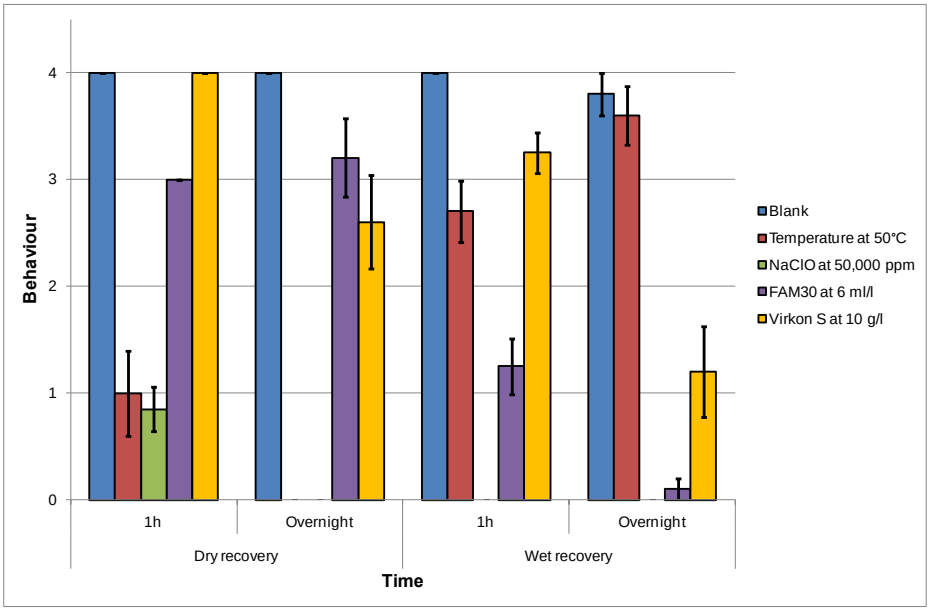

Figure 26- Behaviour results of Dikerogammarus villosus following a net dip exposure with a dry or wet recovery, for four treatments.

NaClO proved to be effective in both scenarios, especially in wet recovery where 100% mortalities were observed. FAM 30 and Virkon were ineffective in the dry recovery scenario, but were more effective in wet recovery, with FAM 30 being the more effective of the 2. It may be that the re-submersion of Dv into freshwater may increase the delivery of the already absorbed chemical to key organs speeding up the rate of mortality.

3.5.2. Spray tests

Sprays were tested using protocol D. Temperature (50°C), NaClO (50,000ppm), FAM 30 (6ml/l) and Virkon S (10g/l) were tested with both 5 and 20 spray exposure. Figure 27 and 28 shows the results from 5 and 20 sprays respectively. There was no significant difference observed in the response to either 5 or 20 sprays, suggesting that the response is not dose dependant. NaClO and FAM 30 proved to be the most effective with 100% mortalities observed within the 15 minute exposure period. Virkon S was less effective; with only a few mortalities observed within the exposure period, however, in the recovery period (when the animals were returned to freshwater) mortalities increase. This supports the theory that the effect of Virkon S is increased when the animals are re-submerged. Temperature was ineffective as a spray with no mortalities observed, and normal (category 4) behaviour observed in during the recovery period. It is thought that this was because it was difficult to maintain the temperature of the spray at 50°C by the time it had volatilised and reached the target.

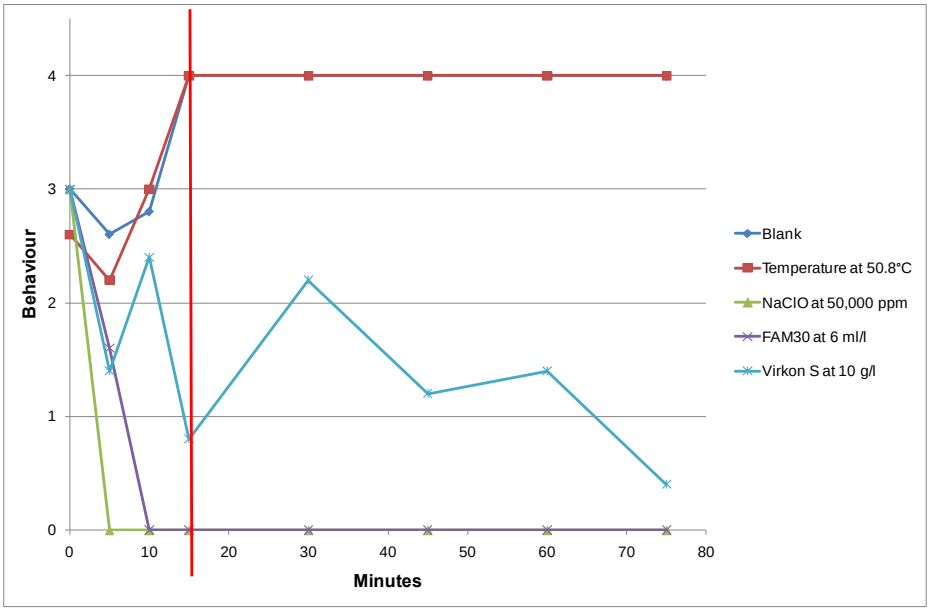

Figure 27- Spray effect (5 squirts) on *Dikerogammarus villosus* for four treatments with behaviour plotted against time during exposure and recovery.

The red line of the graph designates the end of the exposure period and the commencement of the recovery period.

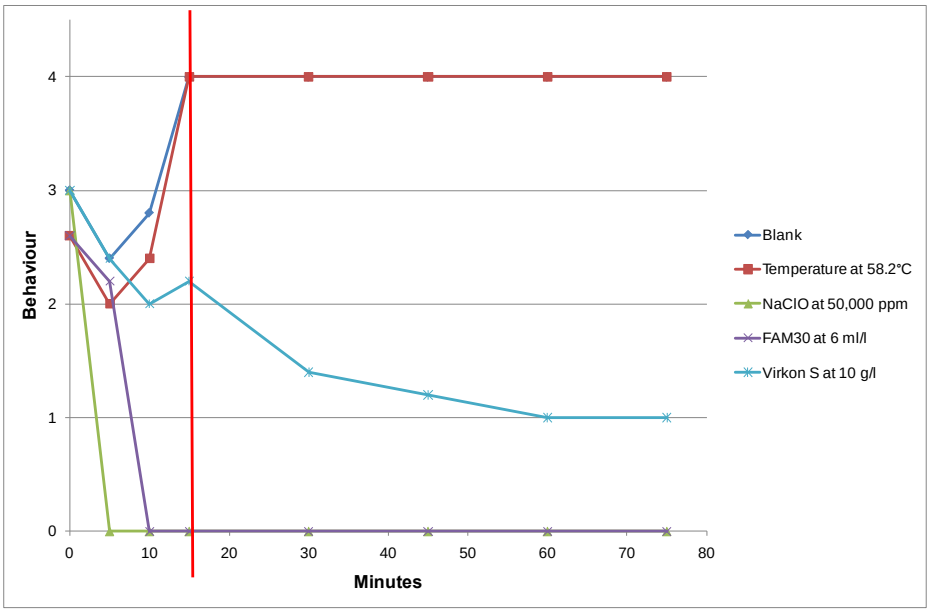

Figure 28- Spray effect (20 sq uirts) o n *Dikerogammarus villosus* for four tr eatments with b ehaviour p lotted against time during exposure and recovery.

The red line of the graph designates the end of the exposure period and the commencement of the recovery period.

4.0. Discussion and Application of techniques

Of all of the products tested NaClO, FAM 30, Virkon S, temperature and carbonated water were the most effective. The potential suitability of each of these treatments is discussed:

NaClO was shown to be effect dip and spray at 50,000ppm with a LT_{50} of 4 minutes 20 seconds (LT_{90} of 5 minutes and 29 seconds). However, it would not be possible to use this concentration near drinking water safely in the volumes that would be potentially required. Disposal of used treatment may also be problematic. NaClO is not listed as an insecticide under the BPD, and therefore it would either have to be licensed for such use or permission from the Minster sought for emergency use. Although NaClO is readily available, members of the public would not be able to use it without specific protective clothing. NaClO at 50,000ppm is lethal to human adults if 167ml or more of the solution is ingested. Prolonged skin contact at this concentration will also result in severe skin irritation. This would make this concentration of NaClO problematic to be used to disinfect equipment. Given the limitations of NaClO as either a spray or dip at this concentration, it is not recommended for use. However, fomites that could be left to soak in 200ppm NaClO for over 1 hour could effectively be disinfected in this manner.

FAM 30 was shown to be effective both as a dip and spray at 6ml/l with a LT_{50} of 3 minutes 10 seconds (LT_{90} 10 minutes and 5 seconds). It can be used near drinking water in small quantities, but with the quantities which are likely to be required would make disposal problematic. FAM30 is not an insecticides so, as with NaClO, either the product would have to be licensed for this application, Ministerial permission sought under Emergency authorisation or extension of approval obtained

from the manufacturer. FAM30 is readily available and relatively inexpensive at the concentration tested, but could not be used by members of the public without protective clothing as it is an irritant. It also stains, potentially resulting in damage to treated equipment. FAM30 does have a long shelf life, but given the other significant drawbacks, could not be recommended for use.

Virkon S at 1 % was effective as a dip with a LT_{50} of 7 minutes and 44 seconds (LT_{90} 8 minutes and 15 seconds), but was comparatively ineffective as a spray. It can be used near drinking water in small quantities, but as with FAM 30 use of large quantities may prove problematic in use near drinking water and disposal. Virkon S is not an insecticide and therefore the same case applies as with FAM 30, where either Ministerial permission, extension of approval or having the product licensed for this use. Virkon S used at the manufacturers recommended concentration of 1% is not irritating to the skin or eyes, and can therefore potentially be used without significant risks by members of the public. However, it does have a bleaching effect, which may result in damage to equipment. Virkon S does have a long shelf life if unprepared (i.e. in powder form), but once made into solution has a relatively short shelf life (3 days), also its relative effectiveness can be impaired with organic material. Because of these drawbacks, Virkon S could not be recommended for use.

Temperature (>40°C) was the most effective dip tested with a LT_{50} of less than 1 second (LT_{90} 2 seconds). However, it was not an effective spray under the scenario tested here. It can be readily used near drinking water and can be disposed of easily. No specific licence is required for it use. It can be used by members of the public without protective clothing, but it should be noted that water temperature exceeding 51.66°C poses serious risk of severe burns to adults and children. It is estimated that it takes only two seconds of exposure to water at 65.55° C and only six seconds of exposure to water at 60° C to cause a very severe burns to a child. It is unlikely that equipment would be damaged if treated with heated water. However, if could potentially be difficult and expensive to maintain water at a high enough temperature for prolonged periods of time. Due to the ease in potential use of heated water, further recommendations for this treatments use has been made below (section 5).

Carbonated water, although not causing mortality was effective at immobilising Dv in a short period of time as a dip, but was not tested as a spray. CO_2 is listed as an insecticide (for use against storage pests) so it would be legal to use it against Dv. It is readily available, safe to use around drinking water and is easily disposed of. The use of gas cylinders to carbonate water may prove to be difficult and possibly expensive. Because of the potential application of carbonated water, further recommendations are made for its use in section 5.

5.0. Recommendations and Future research

Temperature and carbonated water meet the majority of the requirements and are therefore the most suitable candidates out of those tested for application. There are a number of considerations to take into account if these treatments are to be used:

Water at a temperature of 50°C was effective as a dip. If a large enough volumes of water, suitable for the treatment of fomites could be heated and maintained at a temperature >50°C then this could effectively be used to treat smaller fomites (nets, wetsuits, boots, waders etc). This may prove to be a difficult method of application as maintaining water at above 50°C could prove difficult in the field. It may therefore be more suitable to deliver heated water as a spray. However, heated water did not prove to be effective as a spray within this project. The method of application (general purpose hand spray) may have resulted in the temperature of the water delivered to the Dv being much lower than 50°C and therefore ineffectual. The delivery of much higher temperature water (>70°C) at a higher rate than the spray used in these tests, could potentially allow the delivery of water >50°C in a higher quantity. 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.

Although carbonated water only induced narcosis in treated Dv (rather than causing mortality) this treatment could still be applied to increase biosecurity at infected sites. There are 2 potential ways in which carbonated water could be applied:

- As a disinfectant in the form of a dip or bath. This would require carbon dioxide to be bubbled through water, which is then used to dip small equipment such as nets, wet suits, paddles etc. Given the rapid response of Dv to saturated carbonated water then fomites would only have to be dipped for a few seconds for the treatment to be effective. A potential drawback to this method would be that Dv may remain attached to fomites even if narcotised. These Dv may then recover once removed from the carbonated water and potentially be transferred to other waters. One potential method to ensure that narcotised Dv would become detached from treated formites would be for the item to be vigorously plunged into the dip; however this would result in the water becoming oxygenated, resulting in a reduction in its effectiveness.
- As a preventative, where carbonated water is used to exclude/narcotise Dv from certain areas. For example, if CO_2 is bubbled into water in areas where boats are being launched and landed, then 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 both 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 over come many of these obstacles; it is cheap, easy to transport, but does pose

risks when being handled. It should be noted that fully saturated carbonated water was used in the tests reported here, the effects of unsaturated water was not investigated.

Although this work has significantly increased our understanding of the potential application of a number of treatments to the control of Dv, there are still a number of outstanding questions that would need to be answered before temperature and carbonated water could be applied effectively in the field. Given the recommendations made further research in required in the following areas:

- Further investigations into the effects of temperature on Dv.
- Assess different forms of application of temperature to fomites and feasible ways in which these can be applied in the field.
- The effects of varying concentrations of CO_2 on Dv.
- Investigation methods of carbonating water to concentrations that will effect Dv
- Methods of application of CO_2 as a control method in the field.

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Head office
Centre for Environment,
Fisheries & Aquaculture Science
Pakefield Road, Lowestoft,
Suffolk NR33 0HT UK

Tel +44 (0) 1502 56 2244
Fax +44 (0) 1502 51 3865
Web www.cefas.defra.gov.uk

Centre for Environment,
Fisheries & Aquaculture Science
Weymouth Laboratory,
Barrack Road, The Nothe, Weymouth,
Dorset DT4 8UB

Tel +44 (0) 1305 206600
Fax +44 (0) 1305 206601

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