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An independent report undertaken for the  
Department of Agriculture, Fisheries and  
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Pests Group



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## Summary

The introduction and establishment of introduced marine species has been known to cause deleterious impacts on natural resources, industrial infrastructure and human welfare (Carlton 2002); the mitigation of these impacts is an ongoing process. The primary mechanisms for the majority of introductions to Australian waters are from ballast water deposition and biofouling. Strategies for the mitigation of introductions from biofouling, such as antifouling paints, can be effective and are currently used widely. However, an effective means for the mitigation of fouling in vessels internal seawater systems is needed.

This study investigated the efficacy of two chemical treatments, Quatsan and vinegar, at killing the native bivalve *Saccostrea glomerata* (Gould, 1850) (Family Ostreidae, common name: Sydney Rock oyster), under simulated internal seawater system conditions. Of interest during the experiment was determining whether the chemicals were effective biocidal agents at different concentrations under different exposure periods and against different densities of oysters.

The following points summarise the major experimental findings of this investigation:

- The chemical treatment Quatsan was ineffective under experimental conditions.
- The combination of vinegar at a concentration of 10% over an exposure period of 12 hours was the most effective treatment.
- Increasing the chemical concentration of vinegar produced an inverse response in efficacy of killing oysters.
- A chemical concentration of 10% vinegar may be used with 75% efficacy consistently as a treatment against bivalves fouling internal seawater systems of small vessels.

## 1. Introduction

The introduction and establishment of invasive marine species (IMS), also known as introduced marine pests or species, causes fundamental, and typically deleterious, impacts on fisheries and ecosystem resources, industrial infrastructure and development and human welfare (Carlton 2002). The ecological and economic impacts that result from pest species invasions are often irreversible and typically cumulative (Bax et al. 2003) as is evidenced by the increase of introduced taxa reported from Australian waters in the last decade from 55 in 1990 (Pollard & Hutchings 1990a,b) to over 250 introduced and cryptogenic marine species in 2004 (Neil et al. 2004). Most of these introductions are believed to have been unintentional and linked to commercial shipping, fishing vessels and aquaculture (e.g. Hillman 1999, Thresher 1999 CRIMP <http://www.csiro.au/~spinks/crimp/index.html>). Other activities facilitating the spread of unwanted IMS include those related to the aquarium trade, movements of refugee vessels, cruising yachts and the relocation of drilling rigs, floating docks and military vessels (e.g. Coles & Eldredge 2002, Hutchings et al. 2002, Minchin & Gollasch 2002). Although species may be introduced by many different vectors, the primary mechanisms for the majority of introductions to Australian waters are through ballast water deposition and biofouling.

At present the Australian Ballast Water Decision Support System (DSS) is utilised to manage the risk of introducing invasive marine species to Australian waters via this vector. The DSS is a risk based assessment and decision tool which, prior to international ballast entering Australian waters, is used to assess the risk of the ballast to be carrying pest taxa of concern (currently 12 species) and identifies appropriate management options for the ballast to mitigate the risk of species introduction. The current management option for high risk ballast involves exchanging ballast water at sea. The suitability and efficacy of this management tool at preventing incursions from ballast water deposition are being assessed through the development of the National System for the Prevention and Management of Introduced Marine Pest Incursions (the “National System”).

The Australian Government Department of Agriculture, Fisheries and Forestry (DAFF) is the lead agency responsible for developing practical policy approaches to address the issue of introduced marine pests in Australian waters. The Introduced Marine Species Program within DAFF coordinates the development and implementation of the National System. Responsibility of implementation is shared between the Federal, State and Northern Territory Governments. The National System identifies key activities and responsibilities of Australian and State or Territory Government agencies in monitoring and managing IMS, their vectors and the risks associated with IMS introduction. The National System will be developed by October 2006 and comprises three main components:

- A prevention regime providing a single, nationally consistent approach to minimising the risk of new incursions of marine pests into Australia;
- Emergency responses to new incursions; and
- Ongoing management and control of established populations of marine pests in Australia.

Supporting arrangements for monitoring, communications, research and development, evaluation and review will form part of the National System.

In conjunction with examining the efficacy and suitability of utilising the DSS for management of IMS risks associated with ballast water deposition, the National System is also identifying and examining processes for mitigating the introduction of biofouling. To date risks associated with the introduction of marine pests through biofouling vectors or other vectors have received comparatively little attention. Currently Australia, as a whole, does not have effective controls governing the translocation of invasive species via biofouling. Implementation of the National System will provide a national approach to preventing introductions, or decreasing risk of spread of introduced taxa, through biofouling vectors.

Biofouling introductions may occur through fouling of gear such as fishing nets and anchors or through fouling of vessel hulls and/or vessel internal seawater systems. Fouling of vessel hulls and fishing nets/gear may be mitigated in many cases through appropriate vessel and net/gear maintenance regimes and/or use of appropriate anti-fouling paint (for hulls). However, appropriate means for reducing/mitigating the introduction of biofouling taxa via vessel internal seawater systems (e.g. cooling systems, seacocks, etc.) are needed.

Experience in the Northern Territory and Queensland has demonstrated the real risk represented by biofouling as a vector for marine pests. In 2001 Asian green mussels (*Perna viridis*) and Caribbean tube worm (*Hydroides sanctaecrucis*) were introduced to Trinity Inlet, Cairns, Queensland as a result of biofouling with the likely source identified as an apprehended Foreign Fishing Vessel. Similarly, the invasive black-striped mussel (*Mytilopsis sallei*) incursion in Darwin in 1999 is attributed to its introduction in the biofouling of an internationally travelled non-trading vessel. This incursion prompted the Northern Territory Government to develop border control inspection/treatment protocols for vessels entering the lock-accessed marinas in Darwin as a prevention strategy, minimising the risk of further incursions via biofouling. In addition to visual inspections of external hull surfaces, this protocol requires that internal seawater systems are inspected/treated for potential biofouling. The types of vessels using the marinas encompass non-trading vessels less than 25m in length and commercial fishing and tour vessels less than 25m in length. This is currently the only strategy in Australia to prevent incursions via biofouling.

In recent history a number of apprehended vessels escorted into Northern Territory waters and inspected under Northern Territory border control protocols have been found to have either black-striped mussel or Asian green mussel present on their hulls or biofouling vessel internal seawater systems (pers.comm. A. Marshall, 2005). Such inspections have demonstrated that the inspection of externally visible surfaces for marine pests only is not a true indication of what may be resident within the pipe work of vessels and therefore a risk for introduction. The internal seawater systems of a 25m motor cruiser, which had current and effective antifouling and was free from external biofouling, were inspected and found to harbour over 200 recognised invasive juvenile bivalves, including the pests Asian green mussel (*Perna viridis*) and Asian bag mussel (*Musculista senhousia*). The internal seawater systems of this vessel were treated to kill these taxa prior to allowing the vessel entry into one of the lock-accessed marinas in Darwin.

The Northern Territory protocol for the treatment of internal seawater systems for potential marine pests involves introducing a 5% (in seawater) detergent solution (Conquest) into the pipe work of the vessel, and closing the seacocks to retain the solution in the system for a minimum period of 14 hours. Vessel owners are not charged for this service. Conquest is used as it was tested by Bax et al. (2002) and shown to an effective agent against black striped mussel (1% v/v in 19 and 33 ppt salinity seawater gave LT 100 of 7 hours).

Non-oxidising disinfectant solutions such as Conquest contain quaternary ammonium compounds (QAC) as the active biocidal agent. Benzalkonium chloride is the biocidal agent in Conquest. Non-oxidising chemicals, such as QAC's, have specific activities against biofouling which are not well understood. In contrast, oxidising chemicals, such as chlorine, have non-specific activity against biofouling and have been widely tested as a treatment option. Chlorine has been adopted by power station industries globally to control fouling of cooling water intakes (Rajagopal et al. 2002, Chou et al. 1999, Waller et al. 1993), but its use has inherent risks associated with its release to the environment. For example, chlorine can form harmful compounds, such as trichloromethanes, which are considered carcinogenic. Although non-oxidising QAC's have not had the same volume of research on release to the environment as oxidising chemicals, some studies demonstrate that when non-oxidising chemicals, such as benzalkonium chloride, are released to the environment they are not readily broken down and can persist for long periods in their active form (Claudi & Mackie 1994). Alternative treatment options such as organic acids (e.g. vinegar) have had little attention as biofouling treatments or Molluscicides. However, when released to the open environment the oxidising chemical acetic acid is readily converted to carbon dioxide and water by microbial activity making it a potentially attractive treatment option against biofouling.

Straight chlorination has been used to kill biofouling communities in marine piping systems and has been utilised widely with apparent consistency as a control mechanism for biofouling (Jenner et al. 1998). However, recent research by Rajagopal et al. (2002) on the efficacy and consistency on the application of oxidising chemicals, such as chlorine, for killing common tropical bivalve fouling species demonstrated variation in mortality among species. In this study the Asian green mussel was shown to be the most tolerant of species tested to chlorination at both low and high concentrations. As such, the use of chlorination to kill taxa of concern for Australian waters (such as the Asian green mussel) may not be suitable.

Of further concern with using chemicals to treat biofouling in internal seawater systems is the impact the effluent water/treatment solution may have on the surrounding marine environment if released. In recognition of the risks to the environment of releasing biocidal chemicals, environmental protection agencies in Australia have placed restrictions on the use of chemicals as biocidal agents and their release into the environment after treatment must adhere to strict guidelines.

The strategy to prevent incursions to Australian waters via biofouling under the National System will be through the development of best practice guidelines and codes of conduct for various sectors for certification procedures, treatment and inspection protocols. These measures will in some cases be mandatory (e.g. for international vessels <25m under the *Quarantine Act 1908*) but for most sectors be voluntary, at least initially. Adoption of

best practice guidelines in each state and Territory may be aligned or developed and/or implemented with relevant legislation to assist adoption of such guidelines.

The proposed prevention component of the National System addressing the risk of introducing marine pests species via biofouling on and in small international vessels (<25m) has considered incorporating the use of Northern Territory protocols. However, concerns have been expressed about:

- the paucity of evidence regarding the efficacy of the treatment utilised by the Northern Territory (being Conquest, active ingredient benzalkonium chloride)
- the impact of the chemicals on the environment and their acceptance under MARPOL (International convention for the prevention of marine pollution from ships)
- the level of inconvenience afforded masters of the vessels as a result of the 14 hour delay, and
- the potential impact of the cost of the new regulations on the master of the vessel.

If a strategy was adopted under the National System to treat vessel internal seawater systems to kill biofouling and minimise the risk of introduction via this vector, the strategy must be demonstrably effective against all biofouling species of concern, particularly key bivalve species which have previously been demonstrated to be resistant to particular treatments.

The implementation of mandatory risk based assessment of internal seawater system treatment will depend on the findings of investigations targeting the efficacy of chemical treatment at killing biofouling taxa of concern and the suitability of the treatment for release to the environment/another facility. To go towards addressing this need for implementation of a treatment protocol under the National System, this project was developed to examine the efficacy of the use of QAC's (active ingredient benzalkonium chloride) based on the Northern Territory's current treatment protocol against other, suitable, treatment solutions.

## 2. Scope of project

The scope of this project was to determine the efficacy of different treatment solutions, concentrations and treatment periods on inflicting 100% mortality on bivalves (at different densities) as a first step towards the assessment of the efficacy of using chemical treatments against any marine pests potentially inhabiting internal piping of vessels. The model taxa used for this experiment was the native bivalve species *Saccostrea glomerata* (Gould, 1850) (Family Ostreidae, common name: Sydney Rock oyster).

Native rock oysters are similar in their ecology to many introduced bivalve taxa. Although not within the same family (Mytilidae) as many of the recently introduced pest bivalves (*Perna viridis*, *Mytilopsis sallei*), rock oysters are similar to mytilids in that they have similar salinity and temperature tolerances (Prasard 1999, Rajagopol et al. 2002). While they vary from mytilids in their method of attachment to substrata (they cement to hard substrates whereas mytilids attach via byssus) rock oysters have been found to occur in similar habitats to many mytilids and have the potential to settle on

engineered structures including concrete piles and vessel hulls including internal seawater systems.

Members of both the Mytilidae and Ostreidae families have similar habitats and co-exist in many hard substrate areas from the littoral zones to subtidal zones. In particular, these groups both possess the ability to survive periods of air exposure, fluctuations in salinity and temperature and high turbidity commonly experienced in estuarine areas of high anthropogenic activity (Neil 2000, Ruesink et al. 2005). Given their similar ecologies and morphologies these rock oysters provide a suitable model bivalve to test treatment solutions that would also effect Mytilids and other potentially introduced taxa.

### 3. Methods

#### 3.1 Main Study

##### 3.1.1 Experimental Design

In the absence of a suitable non-native test subject commercially grown *Saccostrea glomerata*, harvested from the same cohort and size class, were sourced from Toulkerrie (Blue Hole) oyster farm located within Morton Bay north east of Brisbane, Queensland, Australia. Oysters were air freighted to the laboratory facilities at the Queensland Department of Primary Industries and Fisheries, Northern Fisheries Centre located in Cairns, Queensland, Australia.

The experiment consisted of three trials with one third of the experimental replicates chosen at random and matched with appropriate controls run within each trial. Delivery of oysters was staggered to overcome logistical complications including, but not limited to:

- oyster availability from the farm
- freighting of large numbers of oysters
- holding and maintaining oyster nutrition and water quality.

This approach minimised the likelihood that any decline in oyster condition would have occurred as a result of handling and movement of the oysters from a farm (growth) environment to a laboratory environment. As such, it was considered that oysters used in each trial were of equal health condition and that the experimental set up did not render any particular group of oysters more susceptible to mortality as a result of any treatment.

Following receipt of each delivery, oysters were transferred to holding baskets (20 oysters per basket) (Plate 1) acclimated in the laboratory in seawater for 24 hours in a flow-through system prior to treatment (Plate 2).

For each trial, following acclimation oysters



**Plate 1.** Holding basket for oysters during acclimation. Five baskets holding 20 oysters each were stacked within the holding tank.

were allocated to treatment vessels. At this time all oysters were examined and only responsive oysters (closed rapidly when handled) were utilised in the treatment. After allocation to individual treatment vessels equipment used to handle the oysters and the hands of technical staff and were cleaned to mitigate the possibility of contamination between samples. Disruption of oysters was minimised to remove any potential effects of handling.

For this experiment the efficacy of various concentrations of two treatment solutions, Quatsan (active compound: Benzalkonium chloride 7.3%) and Vinegar (active compound: acetic acid 6% non-verified, commercially available, food grade white vinegar) (refer to Appendix 1 for manufacturers details), were tested on oysters over two exposure periods: 6 and 12 hours. A pilot study was conducted to examine several potential treatment solutions and chemical concentrations, and their suitability for testing under this experimental design. Pilot study results went towards development of this experimental design and are provided in Appendix 1. Each treatment solution was tested across six replicates at a density of 20 and 10 oysters and across nine replicates at a density of 2 oysters, with appropriate controls for each solution. The experimental design is presented in Table 1.



**Plate 2.** Fibre-glass holding and acclimation tanks (1500 l capacity).

**Table 1.** Experimental design for main experiment consisting of two chemicals at various concentrations treated on oysters at a density of 2, 10 or 20 over a 6 or 12 hour exposure period.

Chemical	Solution concentration in seawater	Treatment Period					
		6 hours			12 hours		
		Density (# oysters / vessel)		Density (# oysters / vessel)		Density (# oysters / vessel)	
		20	10	2	20	10	2
Quatsan (Benzalkonium chloride)	5%	6 reps	6 reps	9 reps	6 reps	6 reps	9 reps
	<i>Controls</i>	6 reps	6 reps	9 reps	6 reps	6 reps	9 reps
	10%	6 reps	6 reps	9 reps	6 reps	6 reps	9 reps
	<i>Controls</i>	6 reps	6 reps	9 reps	6 reps	6 reps	9 reps
Vinegar (acetic acid)	10%	6 reps	6 reps	9 reps	6 reps	6 reps	9 reps
	<i>Controls</i>	6 reps	6 reps	9 reps	6 reps	6 reps	9 reps
	25%	6 reps	6 reps	9 reps	6 reps	6 reps	9 reps
	<i>Controls</i>	6 reps	6 reps	9 reps	6 reps	6 reps	9 reps
	50%	6 reps	6 reps	9 reps	6 reps	6 reps	9 reps
	<i>Controls</i>	6 reps	6 reps	9 reps	6 reps	6 reps	9 reps
	100%	6 reps	6 reps	9 reps	6 reps	6 reps	9 reps

Following completion of the above experiment it was decided to further examine the effects of vinegar at a concentration of 100%. Oysters for the post hoc experiment of 100% vinegar was sourced from the same farm, cohort and size class as the oysters used in the former experiment. The experiment was conducted using the same methods (i.e. handling, acclimation and analysis) and experimental design (Table 1).



**Plate 3.** Treatment vessel containing treatment solution and oysters.

Individual treatment vessels had a total capacity of 9 litres. Each vessel was filled to a volume of 3 litres with the appropriate treatment solution prior to the addition of oysters. Following acclimation the treatment density of oysters (2, 10 or 20) were submerged beneath the solution for either a 6 or 12 hour exposure period (Plate 3). The addition of oysters to the treatment solutions was staggered through time to ensure that when oysters were due to be removed from each solution and examined for mortality, that the period

of treatment was strictly adhered to and not impacted by the time taken to examine the treatments.

### 3.1.4 Mortality

After each experimental replicate had been exposed to the treatment solution for the appropriate exposure period, the oysters were examined for mortality (Plate 4). The criterion used to assess oyster mortality was shell valve gape with no response of exposed mantle tissues to external stimuli. If an oyster was observed to respond to external stimuli it was deemed to be alive, if no response to external stimuli was observed oysters were deemed to be dead.



**Plate 4.** Laboratory staff conducting examination of oysters for mortality immediately after the completion of exposure period.

### 3.1.5 Water quality and effluent water analysis

Water quality parameters of the treatment solutions were measured prior to the allocation of oysters to the treatment vessels and immediately after the removal of oysters at the end of the exposure period. Parameters measured were salinity, pH, temperature and dissolved oxygen (Plate 5).

Effluent water samples (volume 100mL) were collected from Quatsan treatments and sent to Leeder



**Plate 5.** Laboratory staff recording water quality data immediately after a treatment exposure period has ended.

Consulting, a National Association of Testing Authorities (NATA) accredited commercial laboratory, to determine the level of active benzalkonium chloride remaining in the effluent water post treatment period and to determine toxicity using Microtox (EC<sub>50</sub>). A combination of Quatsan concentrations of 5% and 10%, exposure periods of 6 and 12 hours and density of 2, 10 and 20 were randomly selected for analysis. At least two samples of all concentrations, exposure periods and densities were tested for concentration of active compound in effluent water and toxicity. This was compared, for reporting, against acceptable limits for disposal of this chemical in Australian water systems. When released to the environment acetic acid (vinegar) through microbial action readily breaks down to form non-toxic non-persistent compounds and therefore, effluent water from vinegar samples was not considered for microtox analysis.

### **3.1.6 Data interpretation**

The efficacy of two treatments, vinegar and Quatsan, on the mortality of the oyster *Saccostrea glomerata* under different experimental treatment conditions (concentration of chemical, density of oysters, period of exposure) was examined using univariate statistical techniques including Analysis of Variance (ANOVA) and post hoc application of Tukey's Least Significant Difference (LSD) test. For analysis, mortality means were adjusted for natural mortalities using Abbott's formula, then arcsin-transformed. Analyses were performed using the statistical analysis package GENSTAT v8.0. Data is presented and interpreted using graphical representation. Efficacy of the post hoc experiment of 100% vinegar was analysed and interpreted independently from, but using the same methods, as the above. Results of the analysis were compared between the two experiments to examine the effects of 100% vinegar against the former experiment.

The concentration of benzalkonium chloride remaining in effluent water samples and the toxicity of this solution is examined graphically. Concentration and toxicity of benzalkonium chloride needed no further analysis due to analysis providing a clear indication of concentration and toxicity.

## 4. Results

### 4.1 Mortality

Very low mortality of *S. glomerata* (<0.2%) was experienced as a result of air freighting of oysters from the farm source to the laboratory facility and acclimation in seawater (mean  $\pm$  SD;  $6.2 \pm 1.0$  mg l<sup>-1</sup> dissolved oxygen,  $7.9 \pm 1.2$  pH,  $33.9 \pm 0.8$  salinity,  $25.0 \pm 1.6$  temperature). Mortality within experimental control treatments was negligible with less than 1% of oysters acting as controls (actually 0.17%) dying during the experiment.

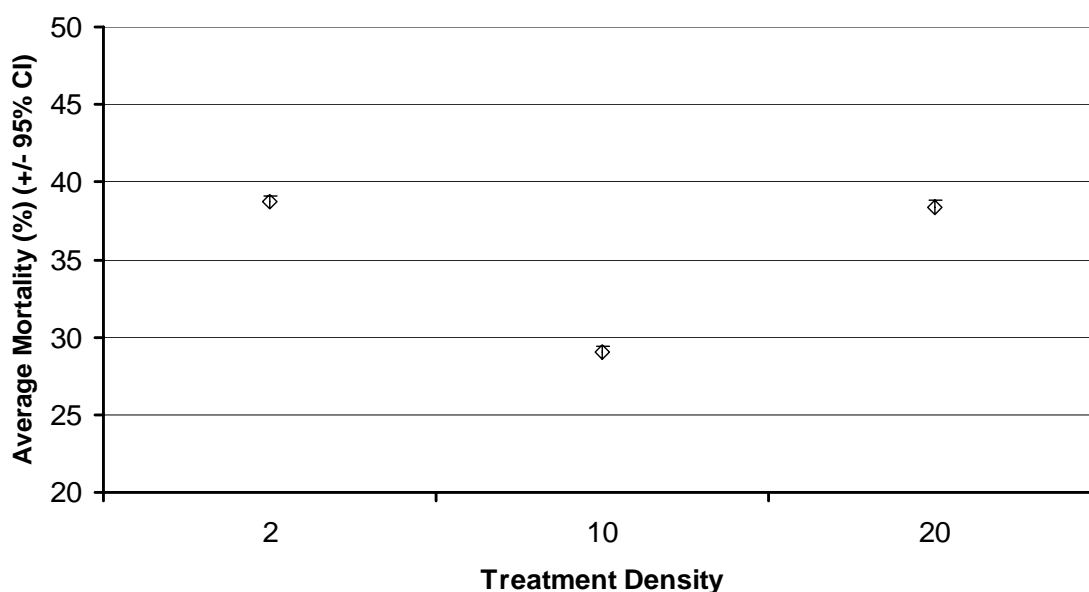
Mortality of oysters was analysed using ANOVA to determine whether any treatment factors effected survivorship of oysters significantly. A probability threshold of  $P < 0.05$  was set for this (i.e.  $\alpha = 0.05$ ). No significant differences were detected across experiments and therefore this factor was blocked for analysis. From the ANOVA significantly different responses in mortality between different “exposure periods” and “chemicals” (type and concentration) were detected. However, a significant interaction was detected between chemical (type and concentration) and exposure period for Quatsan and vinegar (Table 2) and this interaction was investigated further to determine how these variables influenced oyster mortality. Response in mortality was also significantly different between the different densities. No other factors in the analysis provided significant differences in the level of mortality of oysters in each treatment and as such only factors of significance are further examined here using Tukeys LSD test and presented graphically to assist in interpretation of results.

**Table 2.** ANOVA results demonstrating significance of experimental factors on levels of mortality of *S. glomerata* at  $P < 0.05$ . Analysis was performed on mean mortality of various densities of *S. glomerata* treated with different chemicals at different concentrations (Quatsan at 5% and 10% and vinegar at 10%, 25% and 50%). No significant difference across experiment was found and therefore factors presented in the ANOVA table are blocked for experiment. Significant interactions are denoted with the letter s and non-significant interactions with the letters ns.

Analysis of variance						
Source of variation	d.f	s.s.	m.s.	v.r.	F pr.	Sig*
Experimental stratum	2	1286.7	643.4	3.19		
Experimental Units						
Chemical x Concentration	4	38421.5	9605.4	47.64	<.001	s
Exposure	1	10350	10350	51.33	<.001	s
Density	2	1365.7	682.9	3.39	0.036	s
Chemical and Concentration x Exposure	4	2770.1	692.5	3.43	0.01	s
Chemical and Concentration x Density	8	1111.3	138.9	0.69	0.701	ns
Exposure x Density	2	26.8	13.4	0.07	0.936	ns
Chemical and Concentration x Exposure and Density	8	2923.7	365.5	1.81	0.079	ns
Residual	148	29841.4	201.6			
Total	179	88097.2				

Analysis of variance demonstrated significantly lower mortality of *S. glomerata* at a density of 10 individuals per treatment than at a density of 2 and 20 individuals per treatment (Figure 1). Although significant, actual difference in percentage mortality between 2, 10 and 20 density was very small, with 2 and 20, 38.71% and 38.41% mortality respectively and a density of 10 oysters, 29.02% mortality at a 95% confidence interval of  $\pm 0.396$ . These differences were, however, coupled with very low variation in

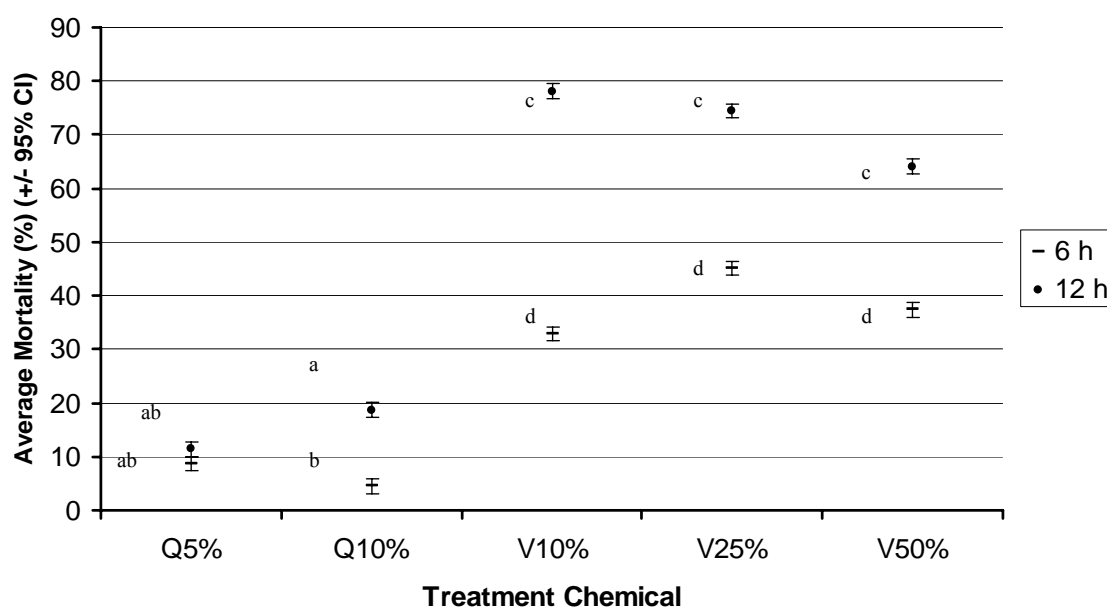
response of each treatment, which is likely driving the significant response for this variable (Figure 1). Biologically, however, a difference between ~30% mortality of oysters and ~40% mortality of oysters has minimal importance when considering the differing levels of density examined in the context of this experiment. As such, although there was a significantly different effect on mortality of varying density of oysters subject to treatment, the actual range of responses across low, medium and high densities there was between ~30-40% mortality; no one chemical was more effective at very low or very high densities of oysters.



**Figure 1.** Average mortalities of *S. glomerata* expressed as a percentage for each treatment of density (2, 10, 20). The original percentages were arcsined for analysis. The percentages in this figure are back-transformed values with a 95 % confidence interval of  $\pm 0.396$  at  $P < 0.05$ .

The significant interaction of chemical (concentration and type) and exposure period in average mortality of oysters was examined post hoc with a Tukey's LSD test to better understand which factors were driving the significant interaction. The treatment period of 12 hours showed significantly higher mortality of *S. glomerata* than the treatment period of 6 hours for all chemicals at all concentrations except 5% Quatsan. Of note, however, is that significantly higher mortality of *S. glomerata* occurred from vinegar treatments at all concentrations than Quatsan treatments at all concentrations over both treatment periods (6 and 12 hours). The responses within the vinegar treatment were not, however, consistent with mortality of oysters at 12 hours being significantly greater than at 6 hours. These significant responses are indicated on Figure 2 below; results of the Tukey's test are indicated by letters – different letters indicate data that differed significantly.

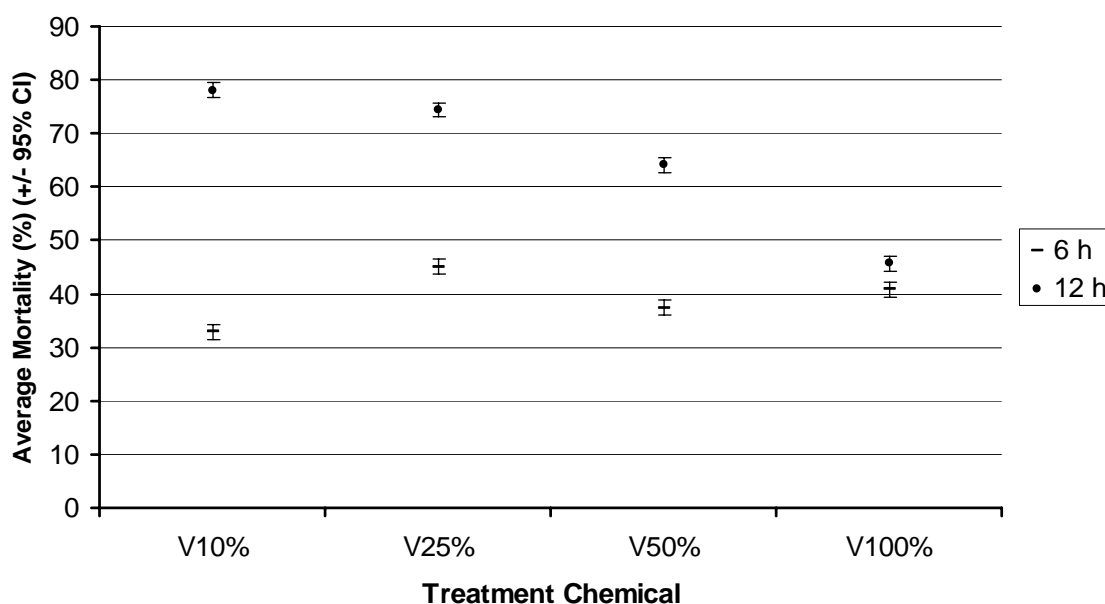
Quatsan was, in comparison to vinegar, highly ineffective at killing oysters during the experiment (Figure 2). The very low level of mortality experienced for 5% Quatsan treatments did not differ between exposure periods, while there was a difference for 10% Quatsan. 10% Quatsan at 12 hours exposure was the most effective Quatsan treatment, however, on average, less than 20% of oysters subject to this treatment were killed (Figure 2).



**Figure 2.** Average Percent mortalities for Chemical (type and concentration) x Exposure Time interaction, averaged over 3 densities (2,10,20) and over 3 trials. The original percentages were arcsined for analysis, and significance testing using Tukeys LSD test. The percentages in this table are back-transformed values with a 95 % confidence interval of  $\pm 1.35$  at  $P < 0.05$ . Means followed by a different letter differ significantly.

Experimental results demonstrated vinegar to be the most effective chemical at killing *S. glomerata* under the experimental conditions (determination of such being the purpose of the experiment) and as such this information is presented in more detail.

The most effective concentration/exposure period combination of vinegar at killing *S. glomerata* during the experiment was 10% for 12 hours exposure. Although Tukey's LSD test did not detect significant interaction between vinegar concentrations over 12 hours there was an apparent decrease in mortality with an increase in concentration over the treatment period (Figure 3). The strong response demonstrated by vinegar in the main experiment led (post hoc) to a secondary experiment involving vinegar at a concentration of 100% shortly after the main experiment had ceased and before data analysis had started. When data for vinegar at a concentration of 100% is presented graphically in comparison with data for vinegar from the main study the downward trend in mortality continues such that 100% vinegar for 12 hours exposure returned a similar level of mortality as 100% vinegar for only 6 hours exposure (Figure 3). There appears to be no clear or similar trend with vinegar over a 6 hour exposure period. The decreasing trend in mortality over increasing concentrations of vinegar is likely to reflect an increasing occurrence of a physiological response by the oysters. At detection of high, dangerous, concentrations of vinegar the oysters likely close their valves sealing the shell from further treatment entering the shell and enter into an anoxic phase.



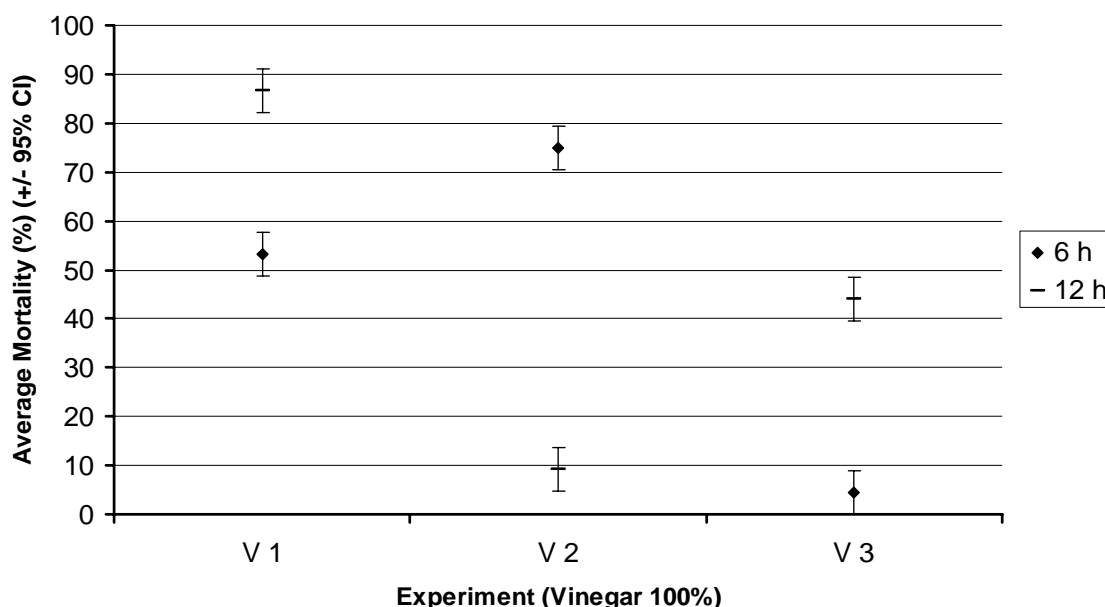
**Figure 3.** Average Percent mortalities for vinegar at concentrations of 10%, 25%, 50% and 100% for exposure periods of 6 and 12 hours, averaged over 3 densities (2,10,20) and over 3 trials. The original percentages were arcsined for analysis. The percentages in this figure are back-transformed values with a 95 % confidence interval of  $\pm 1.35$  at  $P < 0.05$ . Average mortality for 100% vinegar calculated independently of all other vinegar concentrations and added to this figure post hoc.

As for the previous experiment, mortality of oysters subject to a concentration of 100% vinegar was analysed using ANOVA to determine whether differing levels of mortality were experienced under different exposure periods and densities. A probability threshold of  $P < 0.05$  was again used. A significant interaction was detected between experiment and exposure period (Table 3).

Significant differences in mortality of *S. glomerata* exposed to 100% vinegar were found (ANOVA) between trials 1, 2 and 3 and exposure period (Table 3). The source of significant difference between the trials and exposure periods is likely to be variation in response by the oyster population when exposed to the vinegar treatment at a very high concentration (Figure 4). In some cases, as shown in trial 1, 100% vinegar can affect up to 88% mortality over 12 hours. However, this may be as little as 5% over 6 hours, as shown in trial 3 (Figure 4). This experiment, and the highly variable responses, demonstrates the significant level of variation the treatment oysters may express when subject to very high concentrations of treatment chemicals. Some oysters may effectively close their shells against such chemical treatments, while others may be ineffective at doing such and suffer mortality as a result. While varied, these results do show that treatment of taxa such as oysters with catastrophic chemical levels can lead to highly varied success, and 100% effective mortality would likely only be reached by ensuring treatment was maintained beyond the taxa's anoxic period capabilities (i.e. through suffocation).

**Table 3.** ANOVA results demonstrating significance of experimental factors on levels of mortality of *S. glomerata* at  $P < 0.05$ . Analysis was performed on mean mortality of various densities of *S. glomerata* treated with 100% vinegar. \*Significant factors are denoted with the letter s and non-significant interactions with the letters ns.

Analysis of variance						
Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.	Sig*
Experimental Units						
Experimental	2	5807.2	2903.6	12.89	<.001	s
Exposure	1	72.7	72.7	0.32	0.577	ns
Density	2	523.2	261.6	1.16	0.337	ns
Experiment x Exposure	2	9315.3	4657.6	20.68	<.001	s
Experiment x Density	4	654.6	163.6	0.73	0.586	ns
Exposure x Density	2	11.4	5.7	0.03	0.975	ns
Experiment x Exposure x Density	4	1087	271.8	1.21	0.344	ns
Residual	17(1)	3829.5	225.3			
Total	34(1)	20762.2				

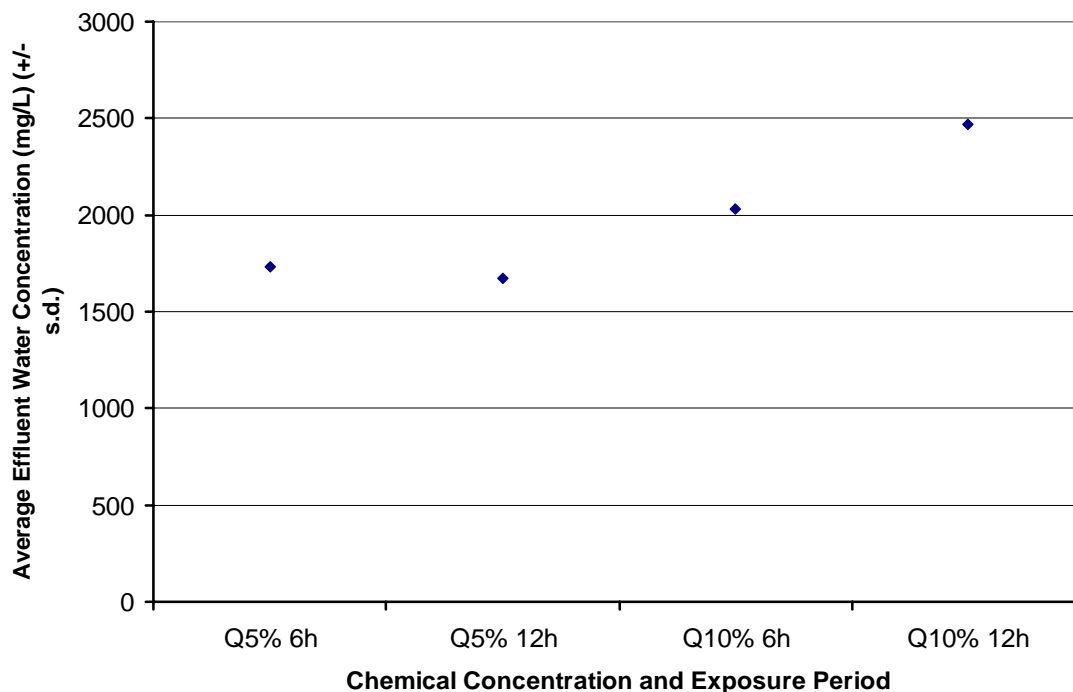


**Figure 4.** Average Percent mortalities for vinegar at a concentration of 100% for exposure periods of 6 and 12 hours over three trails and averaged over 3 densities (2,10,20). The original percentages were arcsined for analysis. The percentages in this figure are back-transformed values with a 95 % confidence interval of  $\pm 4.50$  at  $P < 0.05$ .

## 4.2 Water quality

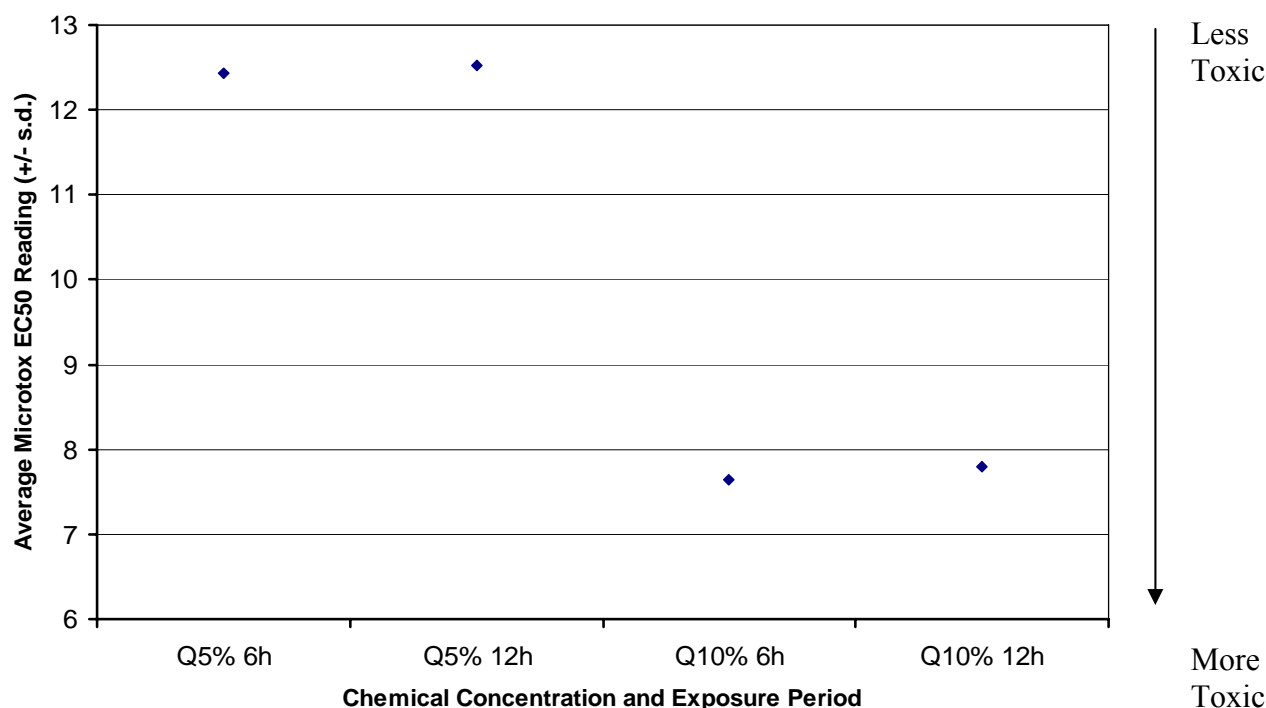
To determine whether it would be possible to release (to the natural environment) effluent waste water from Quatsan treatments post treatment of the oysters, effluent waste water was analysed for the active compound benzalkonium chloride (Q = quatsan) and this is presented in (Figure 5). Quatsan at a concentration of 10% returned a higher mean concentration of benzalkonium chloride regardless of exposure period in comparison to Quatsan 5%. A combination of 10% quatsan and exposure period of 12 hours returned

the highest mean concentration followed by 10% quatsan and exposure period 6 hours, 5% quatsan and exposure period 12 hours, 5% quatsan and exposure period 6 hours (Figure 5).



**Figure 5.** Average effluent water concentration ( $\pm$  s.d.) of benzalkonium chloride ( $\text{mg l}^{-1}$ ) obtained from Quatsan treatment water at 5% and 10% over exposure periods of 6 and 12 hours.

To determine the toxicity of the effluent water concentration of benzalkonium chloride, Microtox  $\text{EC}_{50}$  analysis (concentration of effluent producing a 50% reduction in light emitted from bioluminescent bacteria) was undertaken and is presented in (Figure 6). A higher Microtox reading indicates the sample concentration is less toxic. Effluent waste water from both Quatsan treatments of 10% concentration were more toxic than effluent waste water from Quatsan concentrations of 5%. A combination of 10% Quatsan and exposure period of 6 hours returned the lowest mean  $\text{EC}_{50}$  in comparison to 10% Quatsan and exposure period of 12 hours, 5% Quatsan and exposure period 12 hours and 5% Quatsan and exposure period 6 hours.



**Figure 6.** Average EC<sub>50</sub> (Microtox) (± s.d.) for Quatsan effluent water at 5% and 10% over exposure periods of 6 and 12 hours. An EC<sub>50</sub> of 14 is less toxic than an EC<sub>50</sub> of 1.

To return each treatment combination of concentration and exposure to a non-toxic level (EC<sub>50</sub> = 100) to enable waste water to be released to a natural environment, each treatment waste water solution would need to be multiplied by its corresponding dilution factor (Table 4). Formulae for calculating the appropriate dilution factor is provided in Appendix 2.

**Table 4.** Mean Microtox (EC<sub>50</sub>) analysis of Quatsan (Q = Quatsan) effluent water (± s.d.) and corresponding dilution factor.

Chemical concentration and exposure period	Microtox EC <sub>50</sub>		
	mean	± s.d.	dilution factor
Q5% 6h	12.43	2.42	8050
Q5% 12h	12.53	1.89	7985
Q10% 6h	7.65	1.6	13080
Q10% 12h	7.79	0.54	12845

For example, based on the above Microtox mean value (sample volume = 100ml) and using the calculation for dilution factor (Appendix 2), 805 litres of clean sea water would be needed to dilute a 5% solution of benzalkonium chloride used to treat oysters for a 6 hour period back to acceptable levels for release into the environment. In the case of a more toxic treatment such as the sample of Quatsan 10% over 12 hours a 100ml treatment would need to be diluted by 1284.5 litres of clean sea water before the solution could be released to the open environment.

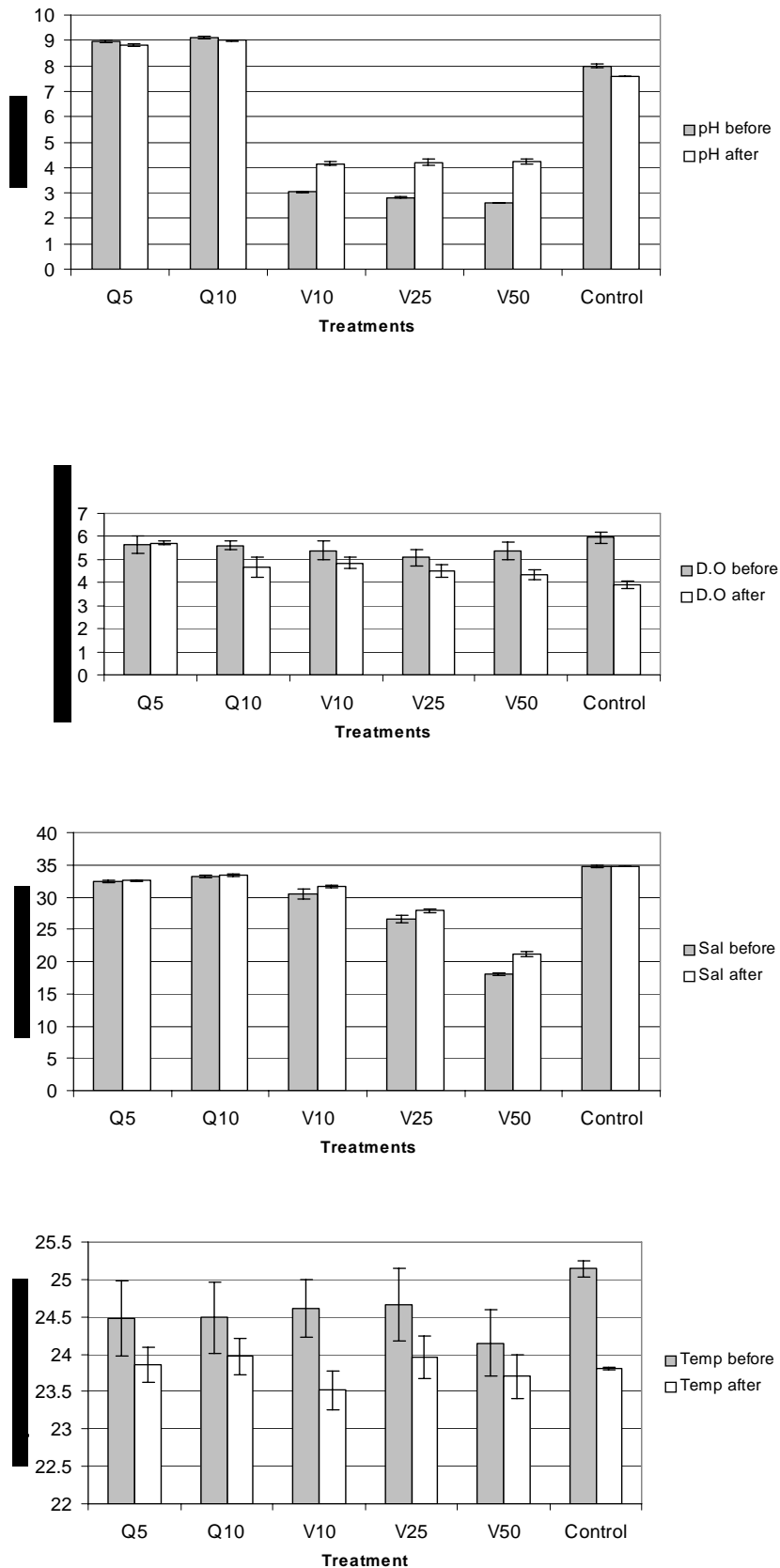
Water quality data was collected from treatment solutions immediately before the addition of oysters and immediately after the removal of oysters at the end of the exposure period and are presented in Figure 7. This was undertaken to determine whether any of the above responses may have been also influenced by a change in a water quality parameter. Notably, all treatments including controls behaved similarly across all parameters and as such it is not expected that any one water quality parameter effected the above noted responses to treatment solutions.

As would be expected, pH of vinegar treatments increased post addition of vinegar. There was no detectable difference in pH post addition of Quatsan to treatments, although pH in control solutions did decline slightly (Figure 7a).

Dissolved oxygen declined slightly for all treatments, including controls, except for a 5% Quatsan treatment (Figure 7b). Again, a decline in dissolved oxygen during this experiment was expected as oysters being subject to treatment would have continued to consume oxygen from the treatment solutions. That more oxygen was consumed, on average, under higher concentrations of Quatsan and vinegar may indicate a greater level of stress on the oysters in these treatments. The greatest level of oxygen consumed was, however, in the controls where very little (<1%) mortality was experienced during the experiment.

Salinity did not fluctuate as a result of experimental treatment (Figure 7c) except for the addition of 50% vinegar, where salinity increased. As the test oysters have a wide salinity tolerance range the mild differences in the average level of salinity noted between the different treatments is not considered to have had any influence on the different responses of the oysters to the different treatments.

Temperature declines in all treatments during the experiment (Figure 7d). This is likely a reflection of the ambient environments influence on each of the treatment vessels. As controls also experienced a decline in temperature with very little mortality (<1%), and as these taxa are known to have wide temperature tolerance range, it is not expected that this decline influenced the different responses of the oysters to the different treatments.



**Figure 7.** Average water quality data ( $\pm$  s.e.) for pH (a.), dissolved oxygen (b.), salinity (c.) and temperature (d.) for each treatment immediately before the addition of oysters at the start of the exposure period and immediately after the removal of oysters at the end of the exposure period.

## 5. Discussion

The introduction and establishment of introduced marine species has been known to cause deleterious impacts on natural resources, industrial infrastructure and human welfare (Carlton 2002) and the mitigation of these impacts is an ongoing process. The primary mechanisms for the majority of introductions to Australian waters are from ballast water deposition and biofouling. Strategies for the mitigation of biofouling, such as antifouling paints, can be effective and are currently used widely. However, an effective means for the mitigation of fouling in vessels internal seawater systems is needed.

This study investigated the efficacy of two chemical treatments at killing the native bivalve *Saccostrea glomerata* under simulated internal seawater system conditions. Of interest during the experiment was determining whether the chemicals were effective biocidal agents at different concentrations under different exposure periods and against different densities of oysters. Density of oysters was shown to be a significant factor in mortality with a density of 10 oysters having, on average, a 9% lower mortality to all treatments at both 6 and 12 hour exposure periods compared to a density of 2 or 20 oysters. Average mortality of oysters at low (2) and high (20) densities were comparable. This response does not appear to fit a dose/response relationship; a density of 10 oysters in a treatment volume of three litres may be the optimal density for resistance to treatments in this experiment. However, in the context of this experiment, this variation between different densities has minimal biological importance. Although there was a significantly different effect on mortality of varying density of oysters subject to treatment, the actual range of responses across low, medium and high densities was between ~30-40% mortality; no one chemical was more effective at very low or very high densities of oysters.

Other factors of interest in the experiment were exposure period and chemical (type and concentration). An exposure period of 12 hours produced higher mortality regardless of chemical; vinegar was the most effective agent in this experiment at killing the test taxa. Vinegar at a concentration of 10% was shown to have the highest mortality over an exposure period of 12 hours and there was an inverse trend of declining efficacy with increasing concentration of vinegar. Although this trend was not significant, the low variation noted in the data suggests that consistently high mortality (approximately 78%) of fouling taxa such as the test organisms could be expected if a 10% vinegar solution was used as a fouling treatment within internal seawater systems. While not 100% effective at killing all taxa within a treatment vessel, 10% vinegar was highly effective over a 12 hour period.

There was a trend of decreasing mortality of oysters with an increase in concentration of vinegar during the 12 hour treatment period. This is likely to be a physiological response by the oyster to increasing concentration of vinegar. During this experiment oysters responded to increasing concentrations of vinegar by closing their valves to prevent the treatment solution from entering the shell and affecting their tissue and functioning. Oysters, mytilid bivalves, and many other fouling taxa that have intertidal habits, typically possess an ability to close their shells/valves and persist in an anaerobic state for a period of time. This is typically a response to unfavourable, increasing, salinity and/or temperature conditions as a result of being exposed during low tide periods (Neil 2000).

However, many taxa have also been shown to demonstrate similar behaviour patterns when subject to treatment with chemicals that may negatively effect the taxa's tissues and functioning (Jenner et al. 1998, Rajagopal 2002). Oysters may differ in their individual ability to remain closed to the surrounding environment for various reasons including individual fitness or individual variation in shell damage, thereby preventing valves from closing completely.

During this experiment large variations in mortality of oysters subject to high, catastrophic, doses of vinegar were experienced. In some 100% vinegar treatments over half the exposure population of *S. glomerata* persisted, while in other 100% treatments less than 10% of the exposure population persisted. And during the experiment where *S. glomerata* was exposed to 100% vinegar, there was no particular trend or a consistent rate of mortality across different treatments and different replicates (trials) of each treatment, indicating an extremely varied response to this treatment. This finding supports the suggestion that oysters used in this experiment had individually differing anaerobic fitness capabilities. As these oysters were all sourced from a single farm environment and cohort, and as mortality within controls was less than 1%, it is suggested that natural variations in shell morphology are most likely responsible for differing abilities to close shell valves and prevent the chemical treatments effecting the oysters. Handling of the shells after receipt from the farm was undertaken with care and while some damage may have been inflicted that affected this result, possibly before shells were received, it is unlikely given the highly varied responses to the different treatments and the consistent survival of the controls.

A 5% Conquest solution (active ingredient benzalkonium chloride) in seawater at an exposure period of 14 hours is currently used in the Northern Territory border control as a biofouling treatment for internal seawater systems. In this experiment, treatment exposure period was restricted to a 12 hour period for logistical reasons and to test its suitability for application. Mortality of *S. glomerata* from Quatsan treatments was low within 12 hour treatments with a concentration of 10% being the most effective, but still only producing less than 20% efficacy of killing the test taxa following 12 hours of exposure. This low level of efficacy of this treatment was not noted during a pilot study (Appendix 1), however, the scale of the pilot study was highly restricted with very few replicates. The results noted here were consistent with little variation and, as such, are taken as stronger indications of how this chemical would perform if utilised in the field. Examination of efficacy of this treatment chemical was not undertaken beyond 12 hours exposure. It may be that a treatment period of greater than 12 hours produces a very different result than that presented here, with much greater mortality. This requires further investigation. However, determination of the suitability of this chemical for release to the natural environment, under relevant Australian standards, post treatment of vessels was also a goal of this experiment. This examination revealed that a 5% solution of Quatsan could be safely released to the environment only by ensuring proper dilution of the solution occurred.

The active compound in Quatsan, benzalkonium chloride, recorded high concentrations in post treatment effluent water analysis and returned very high toxicity in effluent water from treatments containing 5% and 10% Quatsan. Microtox analysis indicated effluent water from 10% Quatsan over 6 and 12 hour treatment to be the most toxic of all treatments, and would need to be diluted approximately 13000 times to be released safely to the open environment. This dilution effect could be experienced if effluent water from

a vessel was released to a vast body of water that may be held in a bay or inlet area, however, strong consideration would need to be given to this practice to ensure that appropriate dilution was occurring without negatively effecting other taxa that may be present within the release area. It would be better practice to release waste water to an onshore dilution facility, ensuring it was appropriately diluted prior to releasing the water to the environment.

Increasing the concentration of Quatsan treatments would also increase the dilution factor required before releasing effluent water to the open environment. On that basis higher concentration of Quatsan were not tested post hoc.

QAC's, such as benzalkonium chloride, are more generally known as cationic surfactants. A cationic surfactant has a hydrophobic and hydrophilic segment of the molecule and when in solution tends to congregate at interfaces rather than spread throughout the body of the solution. Therefore, QAC's are popular amongst antibacterial coating products (Chou et al. 1999) and have found limited success as biofouling agents (Jenner et al. 1998, Chou et al. 1999). This character of surfactants could be an important consideration when applied to the treatment of biofouling, such as this, where the treatment solution is required to be in contact with oyster tissue. Although not tested in this investigation the effectiveness of surfactants may be less in biofouling situation as the bulk of the treatment chemical will be at interfaces and less available to make contact with the test oysters.

As noted previously, QAC's are considered to be non-oxidising chemicals, meaning the general mode of action does not involve oxidation of the soft tissue. The mode of action for these chemicals is not fully understood and requires more research. However, non-oxidising chemicals have been developed for bacterial disinfection, algal control and as Molluscicides. The primary concern with their use in these instances is persistence in the environment after discharge and cost of the chemical (Claudi & Mackie 1994). Jenner et al. (1998) reports that quaternary ammonium compounds are not metabolised by marine organisms but can persist for long periods. Neutralisation of non-oxidising chemicals is required due to toxicity to non-target organisms. Clay or bentonite is usually used to detoxify quaternary ammonium compounds (Jenner et al. 1998).

If Quatsan was to be considered for use as a treatment for biofouling in vessel internal seawater systems, it should be noted that the effectiveness of such non-oxidising chemicals is dependant on water temperature. Typically effectiveness increases with increasing temperature (Jenner et al. 1998). The mean treatment temperature for Quatsan throughout the experiment was  $24.49^{\circ}\text{C} \pm 0.48$  s.e. (before treatment) and  $23.97^{\circ}\text{C} \pm 0.24$  s.e. (after treatment). It would be expected that some variation in the mortality of bivalves treated with Quatsan would be experienced across geographic locations where water temperature was warmer or colder than that experienced in this experiment. As such, Quatsan may be more effective in tropical areas than temperate ones. Indeed, Quatsan may be more effective in different tropical areas, such as Darwin compared to Cairns. However, based on the results of this experiment, usage of Quatsan in preference to a treatment such as vinegar would need further investigation and justification.

Acetic acid is the active ingredient organic acid in vinegar and can be manufactured in its pure form using various methods e.g. oxidation of acetylene and water or produced in diluted form as a product of bacterial fermentation i.e. vinegar. Little information is

available on the effects of vinegar or acetic acid on the environment. Toxicological information available from the acetic acid materials safety data sheet reports that this material is expected to be slightly toxic to aquatic life with an LC50/96-hour values for fish between 10 and 100 mg/l. Vinegar has not been shown to persist in the environment for longer than 10 days and breaks down readily into non-toxic fundamental compounds of water and carbon dioxide (<http://www.bu.edu/es/labsafety/ESMSDSs/MSAcetic.html>).

A primary goal of this experiment was to determine whether various chemical treatments and concentrations would be suitable for use in treating vessel internal seawater systems for biofouling. A maximum 12 hour treatment period was chosen both for laboratory logistical considerations and also as it presented a sensible option for implementation in practice. Vessels being subject to an internal seawater system treatment would need to remain immobile during the treatment period and, as such, the longer the treatment period, the greater the inconvenience to the vessel. This could lead to a decrease in compliance / agreement to any particular method that may be implemented. A QAC was tested during this experiment as it is currently used in the Northern Territory. Vinegar was also tested as this is widely regarded as a natural disinfectant and performed well under pilot study conditions. Vinegar is also relatively inexpensive, easily accessible in large volumes, even in remote locations, and is widely recognised by the public as not being harmful to handle. These features could go towards facilitating the accepted use of such a 'household' article as a biofouling border control agent.

The experimental results clearly demonstrated that Quatsan was highly ineffective at killing the test taxa. In contrast, while not 100% effective, a 10% vinegar solution exposure for a 12 hour period did produce consistently good results at killing over 75% of the test taxa. As such, this treatment option could be utilised with some knowledge of the level of risk associated with using this option. However, some consideration would have to be given to the high variation of effectiveness within vinegar treatments at a concentration of 100% in trial two and the overall variation within each vinegar treatment of trial one. The varied results in the 100% trial suggest that it would be necessary to ensure vinegar concentrations were accurately measured to ensure efficacy for any treatment application.

Further investigation may need to consider if successive treatments of vessel pipes with low concentrations of vinegar would be more effective than a 12 hour treatment of 10%. Results here indicated that increasing concentrations of treatment chemicals do not produce better mortality results. Future research may consider repeated doses of low concentrations or longer exposure time to low doses, although increased costs would be incurred through such a strategy. Using a treatment period of greater than 12 hours may also be problematic in terms of implementation as vessels being treated may not be able to be held inactive for extensive periods of time. It could be that a low dosage of a chemical such as 25% vinegar for 6 hours (45% effective in this experiment), with flushing and immediate re-dosing for a further 6 hour period may produce a much greater efficacy. This is yet to be investigated, as are the potential increased costs for application compared to a single dose application.

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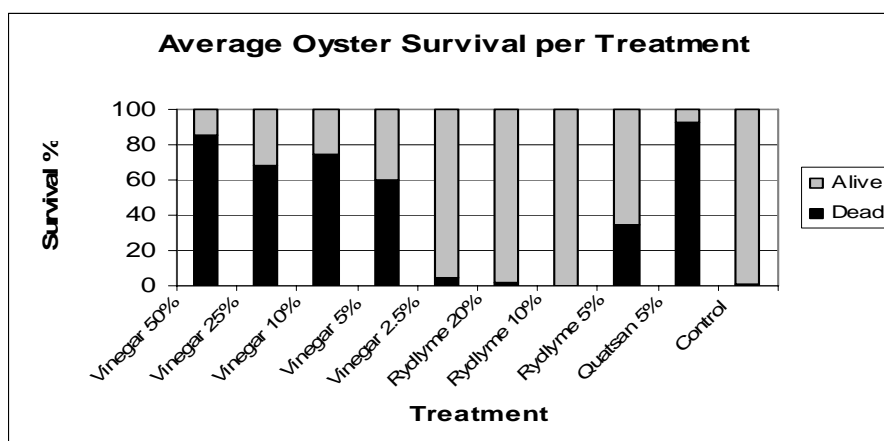
## 7. Appendix 1

### 1.0 Justification for the proposed experimental design

#### Pilot Study

The preliminary efficacy of various concentrations of three treatment solutions, Quatsan (benzalkonium chloride), Rydlyme (aqueous hydrochloric acid) and Vinegar (acetic acid), were tested on *Saccostrea glomerata* at a density of 10 oysters over a 12 hour treatment period to enable refinement of the most appropriate treatment solution/s and their concentrations for the main study.

Results indicated quatsan to be the most effective killing agent of oysters at a concentration of 5% over a treatment period of 12 hours with overall mortality of 93% followed by vinegar at a concentration of 50% with an overall mortality of 85% (Figure 1a). All other treatments were less effective killing agents with Rydlyme the worst performing chemical with an overall mortality of 2%, 0% and 35% at a concentration of 5%, 10% and 20% respectively over the 12 hour treatment period (**Figure X**). Overall mortality of <0.2% was recorded for controls with no significant change in water quality before or after the treatment period.



**Figure 1a.** Average oyster survival after treatment with Quatsan, Rydlyme and vinegar at various concentrations over a 12 hour treatment period and a stocking density of 10 oysters.

Based upon results from the pilot study it was proposed and accepted that the following changes be made to the originally proposed design for the main project:

- Rydlyme, a third treatment solution, not be used as a treatment for the main project.
- Treatment concentrations of Quatsan be 5% and 10% not 2.5% and 5%. It is expected that a concentration of less than 5% would result in less mortality than that recorded for 5% over twelve hours. 100% mortality is the goal and as such a higher, not lower concentration should be trialled.
- Vinegar be trialled at concentrations of at least 10%, 25% and 50%. A concentration of 5% or less would result in mortality less than 60% based on the finding of the pilot study.

- That the number of replicates of the low density treatment (2 oysters) be increased. Instead of 6 replicates of the low density treatment for each experimental solution combination, this be increased to 9 replicates. Six replicates would provide a response from 12 oysters per treatment combination for the experiment. Nine replicates per treatment providing a response from 18 oysters for each the experimental combination, providing capacity to have greater confidence in the result.

## 8. Appendix 2

### 2.0 Manufacturer details

#### **Vinegar**

Palms white vinegar  
Palm Foods Company  
18 Production Street  
Wacol, QLD 4076

#### **Quatsan**

Northern Chemicals Pty, Ltd.  
157 Hartley Street  
Cairns, QLD 4870

### 2.1 Calculation for Microtox dilution factor

The equation for calculating the dilution factor of EC<sub>50</sub> effluent water is as follows (figures obtained from **Table 4**):

$$\text{Non-toxic EC}_{50} = (\text{EC}_{50} / 1000) \times \text{dilution factor}$$

Example 1

$$\begin{aligned} \text{Non-toxic EC}_{50} &= (\text{Q10\% 6h} / 1000) \times \text{dilution factor} \\ &= (7.65 / 1000) \times 13080 \end{aligned}$$

$$\text{Non-toxic EC}_{50} = 100$$