Development of a method to reduce the spread of the ascidian *Didemnum vexillum* with aquaculture transfers

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The colonial ascidian *Didemnum vexillum* was discovered in Shakespeare Bay (New Zealand) in 2001 and now poses a serious threat to the aquaculture industry. I assess several techniques to eliminate *Didemnum* from Greenshell™ seed-mussels (*Perna canaliculus*) in order to reduce the spread of the pest species with aquaculture transfers. Simple approaches based on fresh-water immersion proved ineffective or impractical in controlling *Didemnum*, so different chemical treatments were evaluated. Initial trials were conducted using acetic acid at concentrations ranging from 0.1 to 10% for a range of exposure times. However, at concentrations or exposure times tolerated by seed-mussels, *Didemnum* colonies survived with, on average, ~80% mortality. These results led to the testing of other chemicals, and sodium hypochlorite (bleach) was identified as a potential candidate. It was determined that dipping *Didemnum* in a 0.5% solution of bleach for 2 min was a 100% effective method of treatment that also left seed-mussels relatively unaffected.

Keywords: acetic acid, aquaculture, bleach, *Didemnum vexillum*, fresh water, *Perna canaliculus*, vector management.

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Introduction

Invasive ascidians can have serious impacts on aquaculture operations by smothering equipment and stock, making aquaculture production labour-intensive and, in some cases, cost-prohibitive (US Geological Survey, 2003). Ascidians may also reduce the exchange of food, oxygen, and waste on aquaculture farms, which can lead to poor health and high rates of mortality. The colonial ascidian *Didemnum vexillum* (hereafter *Didemnum*) was discovered in Shakespeare Bay (New Zealand) in 2001 (Kott, 2002), and it has subsequently become a significant fouling pest on mussel farms to which it has spread in the region, a vital one for aquaculture (Coutts, 2005). As a result of the threat to aquaculture, a control programme is under way that is attempting to eradicate localized populations of *Didemnum* and to prevent their spread within and between mussel-farming regions. Managing human-mediated vectors of spread is critical to the success of the programme, because vessel (recreational and commercial) movements and transfers of mussel-farming equipment (e.g. ropes, floats) and seed-mussels from *Didemnum*-infested areas appear to be key vectors for the spread of the species (Coutts, 2005).

Previous work has investigated the development of methods to treat *Didemnum* and other biofouling pests on vessel hulls, aquaculture equipment, and mussel-farming seed stock. These include the use of encapsulation techniques, water-blasting, air-drying, fresh- and hot-water immersion, and dilute acetic acid (the active ingredient in vinegar; Carver *et al.*, 2003; Forrest and Blakemore, 2006; Forrest *et al.*, 2007). Whereas equipment management is relatively straightforward, treatment of seed-mussels requires methods that are effective against pests but that do not affect the mussel stock adversely. Forrest and Blakemore (2006) found that fresh-water immersion for 1–2 d achieved 100% mortality of the kelp *Undaria pinnatifida*, without adversely affecting mussel health. Katayama and Ikeda (1987) demonstrated that *Didemnum moseleyi* soaked in fresh water survived for up to 2 h in winter but were dead after as little as 15 min in summer; cultured oysters (*Crassostrea gigas*) were unaffected. Forrest and Blakemore (2006) suggested that seed-mussels be treated in fresh water while being transported. However, although fresh water is environmentally friendly, the logistic difficulties for implementation at a field scale mean that faster-acting chemical methods may be necessary to treat *Didemnum*.

Several studies have found that acetic acid can eliminate other colonial ascidians (*Botryllus schlosseri* and *Botrylloides leachi*), as well as solitary species such as *Ciona intestinalis* and *Styela clava* (Carver *et al.*, 2003; Coutts and Forrest, 2005). Although Carver *et al.* (2003) demonstrated that *Mytilus edulis* >20 mm were typically unaffected by acetic acid, Forrest *et al.* (2007) proved that acetic acid could affect Greenshell™ seed-mussel survival adversely under certain conditions. As an alternative, the use of sodium hypochlorite (bleach) has been used to control fouling organisms in industrial water cooling systems of power stations (Rajagopal *et al.*, 1996) and has been tested as a possible treatment method for other species (Anderson, 2003; Williams and Schroeder, 2004; Coutts and Forrest, 2005). Here, the efficacy of fresh water, acetic acid, chlorine, and other compounds are

investigated as potential means of eliminating *Didemnum* from Greenshell™ seed-mussels in order to prevent the transfer of the fouling pest between regions.

Methods

Laboratory trials with acetic acid

Laboratory trials with acetic acid were initially conducted to determine the effect of a range of concentrations of acetic acid and of dipping times on seed-mussel survival. The aim was to assess methods that could be used on *Didemnum* in the field, but that would not adversely affect seed-mussels. Seed-mussels (20–50 mm) were declumped (to mimic industry practice) with ~80 placed in each mesh bag and dipped in solutions of acetic acid for several periods ranging from 5 s to 10 min (Table 1). The dipping time ranged from a short immersion (5 s, 10 s, and 1 min) in 10% acid to an exposure of up to 10 min at 0.1–1% acid. A trial was also conducted to compare mussel mortality in 4% acetic acid in seawater and fresh water (dipped for 2 min). A transport period of 24 h was simulated to reflect industry practice that involves leaving declumped seed-mussels out of the water for 1–2 d before being reseeded. There were three replicates for each treatment in these laboratory trials. Mussel mortality was assessed two weeks after treatment.

Field trials with fresh water and acetic acid

Field experiments examined the effects of fresh water and acetic acid (Table 1). For all experiments, each treatment unit consisted of ~130 declumped mussels (20–60 mm) placed in a labelled mesh bag. The fresh-water treatments, and acetic acid Experiments A and B (Table 1), had 2–3 larger mussels covered in *Didemnum* (total area $\sim 13 \times 13\ cm^2$) placed within mesh bags containing seed-mussels. Samples were exposed to variable treatment regimes (Table 1), then each bag was tied at random onto a rope ~40 cm apart at a water depth of 1–2 m. Controls were subjected to the same dipping times and transport periods as the *Didemnum*-infected mussels, but were dipped in seawater. There were three replicates for each treatment in this and other field trials, described below. Samples were collected two weeks later, and seed-mussel and *Didemnum* mortality was recorded.

The effect of fresh water on *Didemnum* was evaluated when mesh bags (as above) were dipped in fresh water for three periods (2, 5, and 10 min) with four durations of transport (Table 1). To assess the effects of acetic acid, three experiments were conducted (Experiments A–C; Table 1). Experiment A examined the effect of acetic acid ($\leq$2%) on seed-mussel and *Didemnum* mortality, in combination with various treatment and transport times, whereas Experiment B tested the effect of higher concentrations of acetic acid but with no transport phase.

Table 1. Summary of treatments on mussels and/or *Didemnum* with concentrations, treatment times, rinse/no rinse, transport times, and number of replicates per treatment.

Treatment	Species	Concentration (%)	Treatment times	Rinse	Transport (h)	replicates
Field trials with fresh water						
Fresh water	Mussels and *Didemnum*	n/a	2, 5, and 10 min	No	1, 5, 12, and 24	3
Laboratory trials with acetic acid						
Acetic acid	Mussels	0.1, 0.5, and 1%	2, 5, and 10 min	No	24	3
Acetic acid	Mussels	4% (salt and fresh water)	2 min	No	24	3
Acetic acid	Mussels	10%	5 and 20 s, and 1 min	No	24	3
Field trials with acetic acid						
Acetic acid (Experiment A)	Mussels and *Didemnum*	0.1, 0.5, 1, and 2%	20 s, and 2 and 10 min	No	1, 6, 18, and 41	3
Acetic acid (Experiment B)	Mussels and *Didemnum*	1, 2, and 4%	1, 3, and 5 min	Yes	0	3
Acetic acid (Experiment C)—Spraying	Mussels and *Didemnum*	2, 4, and 10%	3 s	No	1, 4, 20, and 26	3
Laboratory and field trials with bleach and other compounds						
Lime (Calcium oxide)	*Didemnum*	5 and 10%	20 s and 2 min	Yes	0	3
Sodium metasilicate	*Didemnum*	3 and 6%	20 s and 2 min	Yes	0	3
Caustic soda (Sodium hydroxide)	*Didemnum*	3 and 6%	20 s and 2 min	Yes	0	3
Bleach (Sodium hypochlorite)	*Didemnum*	0.5 and 2%	20 s and 2 min	Yes	0	3
Bleach[a]	Mussels and *Didemnum*	0.1, 0.25, 0.5, and 1%	30 s and 2 min	Yes	0	3
Bleach	Mussels and *Didemnum*	0.1, 0.25, and 0.5	30 s and 2 min	No	5 and 24	3
Bleach, sprayed	*Didemnum*	0.5%	3 s	No	n/a	3

[a]Two brands of bleach were tested, Janola™ and 30 Seconds Outdoor Cleaner™. n/a, not applicable.

As an alternative to immersion-based approaches, Experiment C tested the efficacy of spraying acetic acid over mussels and *Didemnum*. This method was tested because it is likely to be relatively easy for mussel farmers to retrofit a spray-based system for seed-mussel treatment. For Experiment C, *Didemnum* was cut into small (2×2 cm) pieces to simulate the declumping process, each treatment consisting of sufficient *Didemnum* to cover an 8×8 cm^2 area, which was placed in a mesh bag with ~130 seed-mussels. Each bag was spread flat in a bin (80×40 cm) and sprayed with acetic acid, using a 7-l pressure garden sprayer with a spray nozzle, with four simulated transport times (Table 1). Controls followed the same procedure, but were sprayed with seawater.

Laboratory and field trials with other compounds

A pilot-scale investigation was undertaken to examine how four additional chemicals affected *Didemnum* survival. These were calcium oxide (lime, CaO), sodium metasilicate (silicic acid, Na_2SiO_3), sodium hydroxide (caustic soda, NaOH), and sodium hypochlorite (bleach; NaClO). Experimental solutions were diluted to target concentrations in 2 l of seawater. *Didemnum* was spread on a pre-measured board, and a $\sim 10 \times 10$ cm^2 area was placed into a labelled mesh bag. Each bag was then dipped in the appropriate chemical concentration for either 20 s or 2 min (Table 1), with three replicates of each treatment. Controls consisted of bags dipped in seawater for each dipping time. Once the dipping was completed, each bag was immediately secured to a rope and immersed in seawater 1.5 m deep. Samples were removed for analysis 10 d later. Mussel mortality was not examined at this stage, because it was deemed important to identify which chemicals, in what concentrations, would kill *Didemnum*.

The pilot trials suggested that bleach may be an effective treatment, so an experiment examined how various concentrations affected both seed-mussel and *Didemnum* mortality. A layer of *Didemnum* ($\sim 10 \times 10$ cm^2 area) was placed within a mesh bag with ~60 seed-mussels. Each bag was then dipped in 0.1, 0.25, or 0.5% bleach concentrations for 30 s or 2 min, either rinsed or not rinsed in seawater, with a 5 or 24 h simulated transport period (Table 1). Controls were dipped in seawater and followed the same rinsing regimes and transport times. The pH value (Hanna Instruments HI−98128) and chlorine concentration (Merckoquant Chlorine test kit) were recorded prior to the samples being dipped. Following the transport period, samples were re-immersed in the sea, and mussel and *Didemnum* mortality assessed two weeks later.

To examine any difference in the effectiveness of brands of bleach, samples were dipped in two brands (Janola™ and 30 Seconds Outdoor Cleaner™) at concentrations of 0.1, 0.25, 0.5, and 1% for either 30 s or 2 min, and immediately tied to rope in a 250 l tank with aerated seawater (Table 1). *Didemnum* mortality was assessed 5 d later.

Differences in mussel and *Didemnum* mortality were analysed using analysis of variance. "Concentration", "dipping time", and "transport time" were the factors used (where appropriate) in the model. Observed differences between means were tested for statistical significance using Tukey's studentized range test.

Results

Didemnum mortality increased with longer dipping times in fresh water: 74% mortality for 2 min, 84% for 5 min, and 87% with a 10 min dip, but it was not 100% effective. However, mussel mortality was extremely low for fresh-water treatments, averaging from 0.9 to 1.6%, comparable with seawater controls.

Mussels dipped in 0.1 and 0.5% acetic acid, followed by simulated 24 h transport without rinsing, had very low mortality (<1.5%) in laboratory trials, regardless of dipping time (Figure 1). Mussels dipped in 1% acetic acid (for three time intervals) and left out of water for 24 h showed 5–10% mortality. However, very high mussel mortality (69–87%) occurred when the material was dipped in 10% acetic acid for as little as 5 s. Additional work found that mussels dipped in 4% acetic acid for 2 min (in either seawater or fresh water), then left out of water for 24 h without rinsing, suffered mortality ranging from 57% (± 3.4 s.e.) to 75% (± 5 s.e.), respectively (Figure 1). There was a significant difference in mussel mortality between the 0.1% treatment and all other treatments ($p < 0.05$). Further, there was a significant difference between the 1 and the 4% and 10% treatments ($p < 0.05$), but no significant difference between the 4 and 10% treatments.

In field trials with acetic acid, the treatments with ≤2% acid (Experiment A) had heavy *Didemnum* mortality (average 77%). However, no treatment consistently ensured 100% mortality of *Didemnum*, and variability among replicates within each treatment was high. In Experiment B, samples dipped in acetic acid, then immediately resubmerged in seawater (i.e. no transport phase), showed sequentially greater *Didemnum* mortality with increasing acid concentration (Figure 2). Greater *Didemnum* mortality was observed with increased dipping time in the 2% acid treatment, but this was not evident for the 1 and 4% acid concentrations (Figure 2). There was a significant difference between the controls and the 1, 2, and 4% concentrations, and between the 1 and 4% concentrations ($p < 0.05$). There was no significant difference for *Didemnum* mortality between transport times, with similar mortality between 1 and 41 h, regardless of acid concentration. *Didemnum* mortality for the controls and the 0.1 and 0.5% concentrations of acetic acid were similar and exhibited little effect. When *Didemnum* was sprayed with acetic acid (Experiment C), mortality levels were 75% (2% acid concentration), 81% (4% acid), and 80% (10% acid). Unsprayed control mortality was 65%, indicating that handling and cutting *Didemnum* had a negative impact on survival. For the acetic acid spray trials, seed-mussel mortality was typically <5%.

The pilot study with 0.5% bleach resulted in 100% *Didemnum* mortality in both the 20 s and 2 min dip with no transport phase. The 6% sodium hydroxide (NaOH) was the only other treatment to result in 100% mortality, other chemicals being relatively ineffective (Figure 3). Additional bleach experiments demonstrated that a 0.5% concentration resulted in 100% *Didemnum* mortality when dipping lasted either 30 s or 2 min, with no transport phase (Figure 4). There was no significant difference between *Didemnum* mortality for the 30 s and 2 min treatments. Another trial showed that *Didemnum* samples dipped in a 0.25% solution for 2 min suffered 100% mortality, but that this high rate of mortality was not achieved for a 30 s dipping time. The 0.1% bleach concentration resulted in relatively high *Didemnum* mortality, indicating that, even at these low concentrations, bleach does have some effect (Figure 4). Controls consistently resulted in low *Didemnum* mortality, and there was no difference in mortality between the two brands of bleach tested. The controls differed significantly from the bleach treatments ($p < 0.5$), but there was no significant difference between different bleach concentrations.

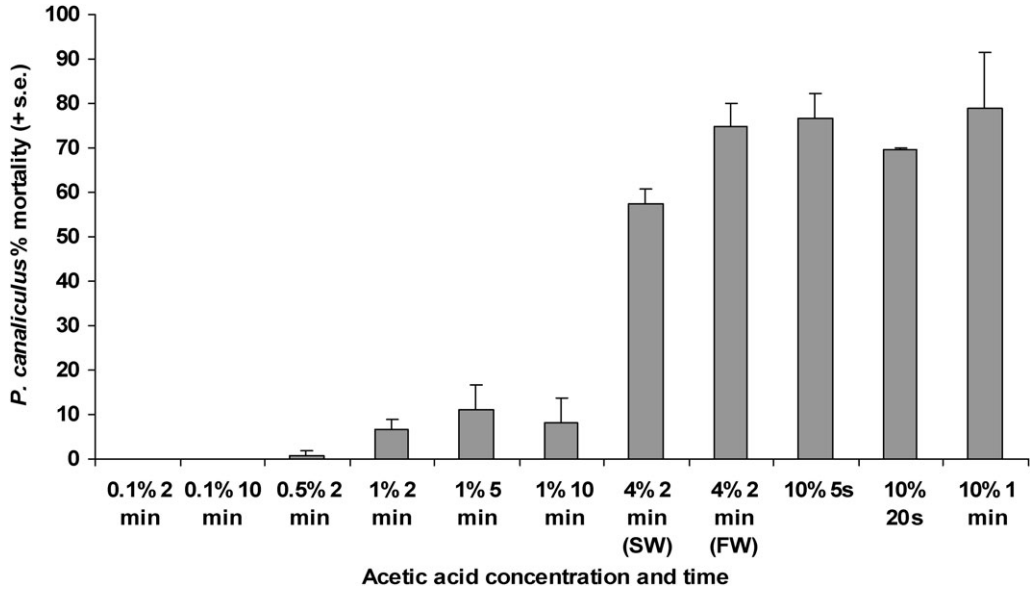

Figure 1. Average *P. canaliculus* seed-mussel mortality (+ s.e.) with various acetic acid concentrations and dipping times, with a 24 h transport period (SW, seawater; FW, fresh water).

Seed-mussel mortality was similar between treatments that were rinsed or not rinsed (following dipping), and between the 6 and 24 h transport period tests. Mortality increased slightly with higher concentrations of bleach, up to 6% in a 0.5% solution (Figure 5).

Discussion

Here, I have described investigations into a treatment method that could be used on seed-mussels to eliminate *Didemnum* (and perhaps other unwanted pests). Fresh-water treatments were tested because mussels can tolerate fresh-water immersion for several days (Lützen, 1999). However, this treatment was unsuccessful in the present study. Solitary ascidians, such as *S. clava*, are able to withstand immersions in fresh water for at least 1 h, presumably by closing their siphons for extensive periods (Coutts and Forrest, 2005). Therefore, a 10 min fresh-water dip may not have been long enough to have any effect on *Didemnum*. Although a dipping time longer than 10 min may be impractical for farmers at growing sites, Forrest and Blakemore (2006) suggested that seed-mussels could be immersed in bins of fresh water while being transported between aquaculture regions. However, the water needed to be exchanged during transport to maintain salinity at $\leq$1 psu, creating logistic difficulties for implementation at a field scale.

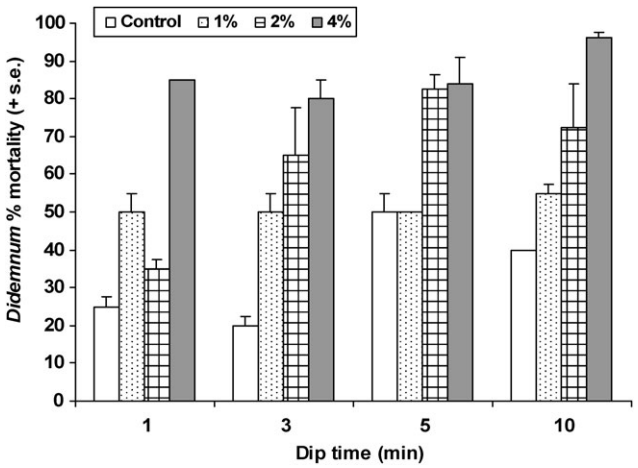

Figure 2. Average *Didemnum* mortality (+ s.e.) with three acetic acid concentrations and a control, and four dipping times (min).

In terms of chemical treatment alternatives to fresh water, there was sufficient evidence to suggest that acetic acid would be an effective solution. For example, Forrest *et al*. (2007) demonstrated that 4% acetic acid eliminated colonial ascidians (*B. schlosseri* and *Botrylloides leachi*), which are morphologically and functionally similar to *Didemnum*. However, the present study found acetic acid to be ineffective at eliminating 100% of the *Didemnum*, at least at concentrations where mussel mortality would be acceptable to mussel farmers. In order to control populations, 100% *Didemnum* mortality is required, because even small fragments can bud and start a new colony (Bullard *et al*., 2007a; Valentine *et al*., 2007).

Mussels dipped in acetic acid at concentrations of $\geq$4% and transported for 24 h suffered high mortality, even in the case of a 5 s treatment at 10%. This suggests that the unrinsed acetic acid residue has an effect on mussels during transport. In this regard, it is surprising that any residual acetic acid effect did not also lead to complete mortality of *Didemnum*. A possible explanation may be the acidic nature of *Didemnum*'s test. Other *Didemnum* colonies have a surface pH of $<$3 (Pisut and Pawlik, 2002; Bullard *et al*., 2007b), so because the pH of 4% acetic acid is only slightly higher than that (2.4), *Didemnum* may be naturally tolerant.

In contrast to the acetic acid results, a 0.5% bleach solution may be useful in treating *Didemnum* when transferring seed-mussels. Other workers have also found bleach to be effective at eliminating fouling pests (Ferguson, 2000; Rajagopal *et al*., 2002; Anderson, 2003; Coutts and Forrest, 2005). A subsequent trial has also

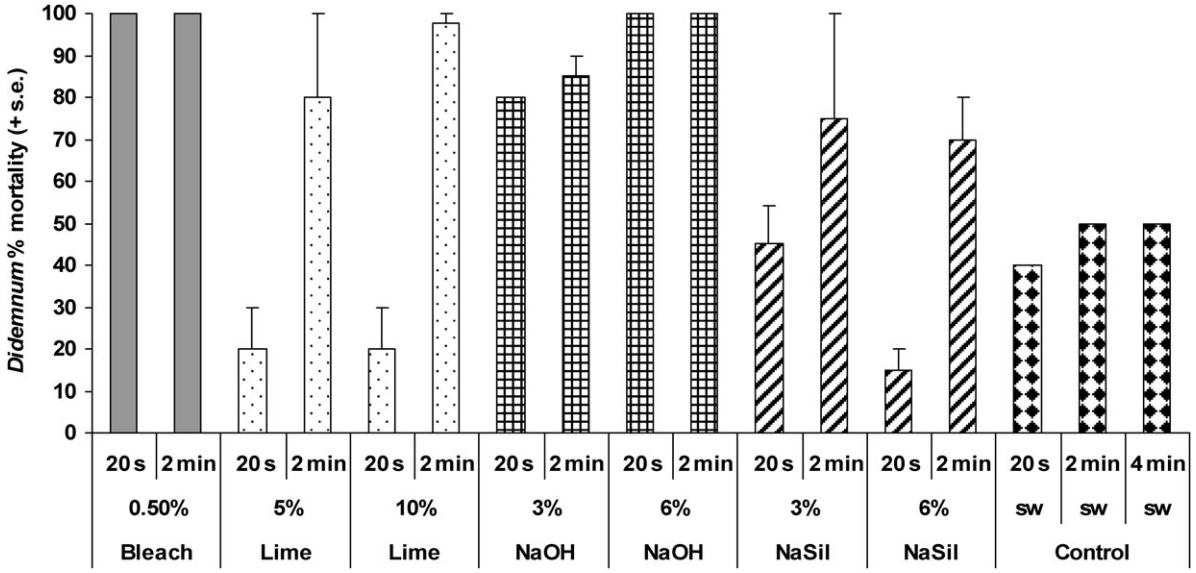

Figure 3. *Didemnum* mortality with four chemicals, bleach, lime, sodium hydroxide (NaOH), and sodium metasilicate (NaSil), and controls, at different concentrations for several dipping times (SW, seawater).

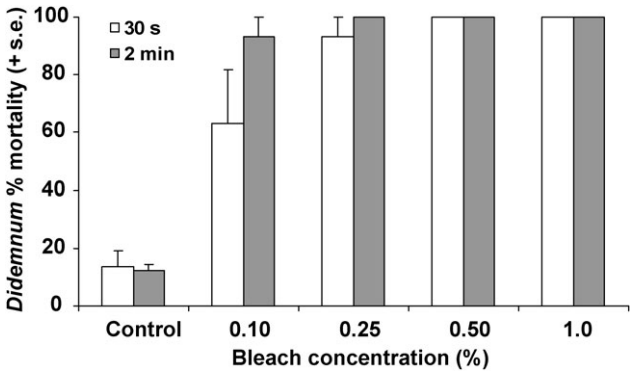

Figure 4. *Didemnum* mortality for four bleach concentrations at two dipping times (no transport period).

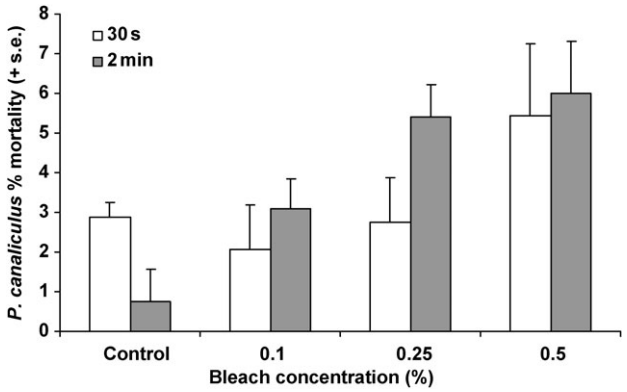

Figure 5. Seed-mussel mortality (+ s.e.) for three bleach concentrations and a control at two dipping times.

demonstrated that 0.5% bleach effectively eliminated *Didemnum* when sprayed onto colonies exposed on wharf piles at low tide (CMD, unpublished data). It is possible that the byssus threads (or byssus gland) of mussels may be affected by exposure to bleach, possibly resulting in them falling off ropes more easily. Although the bleach treatments used here did not appear to affect the byssal attachment of mussels, the longer term effects of bleach treatments on the health and functioning of the byssus gland and the strength of the byssus threads should be a part of any future research.

In relation to seed-mussel transfer, to ensure complete *Didemnum* mortality, it is proposed that a 0.5% bleach solution be used and that samples be dipped for at least 2 min. This concentration is suggested because it is safe to use, environmentally friendly, and repeated dipping of seed-mussels in bleach can result in the consumption of chlorine by organic material. Therefore, even if the original concentration is lowered to 0.25%, *Didemnum* will be eliminated. An alternative may be the use of a higher concentration of chlorine concentration (i.e. 1%) with a shorter dipping time (30 s), because the results presented here suggest that mussels may survive such a treatment. The next phase in this work is to test the efficacy of bleach at an industry scale, and to devise a protocol for maintaining the concentration of chlorine with repeated dipping.

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