Perforation with and without vinegar injection as a mitigation strategy against two invasive tunicates, *Ciona intestinalis* and *Styela clava*

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Abstract

Ciona intestinalis and *Styela clava,* two nuisance species for Prince Edward Island's blue mussel industry, were treated with individual perforations using nails or hypodermic needles. Other treatments using the same species included simultaneous perforations using perforation devices with low, medium and high needle density, either with or without vinegar injections. Mortality levels estimated for all ranges of individual perforations were significantly higher than mortality levels estimated in control groups during treatments conducted at laboratory facilities. Mortality of *C. intestinalis* reached 100% for 60 individual perforations or injection of 0.05 mL of vinegar. In *S. clava,* 100 individual perforations resulted in 100% mortality. Two applications of the high-density perforation device resulted in 80% mortality of *C. intestinalis*. During field testing, two applications of the same high-density needle device did not significantly decrease *C. intestinalis* wet weight, regardless of the addition of vinegar. The field applicability of perforation upon tunicates fouling mussel socks was at least in part limited by the uneven surface created by the mussels and the possible inhibition of bacterial growth caused by low water temperatures. Perforation and vinegar injection showed to be successful in laboratory trials and should be further studied with different perforation devices under field conditions.

Keywords

Mortality, needle, acetic acid, biofouling, blue mussel

Introduction, Hypotheses and Problems for Management

Fouling of aquaculture gear by two solitary tunicates, *Ciona intestinalis* (L., 1758) and *Styela clava* (Herdman, 1881), has negative effects on the blue mussel (*Mytilus edulis* L., 1758) farming industry on Prince Edward Island (PEI), Canada. These effects include loss of crop from fall-off due to tunicate biomass accumulation on the mussels (Bourque et al. 2007) and increased costs associated with labour, equipment and fouling mitigation. A number of tunicate fouling mitigation strategies, including both physical and chemical methods, have been tested with variable levels of success (reviewed in Locke et al. 2009; Carver et al. 2003). Many of these methods have negative impacts on the environment, mussels, or other organisms colonizing the socks (e.g. Locke et al. 2009; Paetzold et al. 2008), or are not cost-effective. These issues have prompted the exploration of alternative mitigation strategies (Locke et al. 2009), particularly against *C. intestinalis* and *S. clava.*

C. intestinalis has immune system elements that are comparable to those of other invertebrates and even some vertebrates (Di Bella & De Leo 2000) in that they trigger an inflammatory-like response to the injection of alien soluble proteins (Parrinello & Patricolo 1984). The formation of a white circular capsule can be observed in the tunic of these species (De Leo et al. 1996; Parrinello & Patricolo 1984). Healing processes can result in damage to the surrounding tissues (Parrinello & Patricolo 1984), the extent of which is dependent on the dosage and nature of the substance injected (Parrinello et al. 1990). By targeting these inflammatory and healing processes, mortality can be caused, thus identifying a new mitigation strategy. The objective of this study was to assess the applicability of two mitigation measures against invasive tunicates: tunic perforation and vinegar (5 % acetic acid) injection. The first part of this study included laboratory trials assessing the effects of perforation and vinegar injection on the survival of *C. intestinalis* and *S. clava*. The second part evaluated the efficacy of perforation with or without

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vinegar at reducing *C. intestinalis* biomass on mussel socks in the field. The null hypothesis for all experiments was that tunicate perforation with and without vinegar injection does not result in *C. intestinalis* and *S. clava* mortality.

Methods

Tunicate collection and holding

Adult C. *intestinalis* (20-70 mm long from incurrent siphon to base) and S. *clava* (30-70 mm long excluding the stalk) were collected off mussel leases in various PEI estuaries and transported either to the Atlantic Veterinary College (AVC) in Charlottetown, or the Portable Aquatic Laboratory (PAL), located on the Marine Terminal in Georgetown (Fig. 1). Tunicate length was measured on relaxed animals on a flat surface using a ruler or calipers (+/- 0.1 mm).

At the AVC, tunicates were held in aerated tanks with recirculating artificial seawater (Instant Ocean®, salinity 25 ppt, temperature 13.1-14.7 ºC, ammonia 0-0.138 ppm, nitrite 0.025-0.100 ppm, nitrate 19.0-70.0 ppm and pH 7.11-7.50). Tunicates were fed with condensed algal paste (Innovative Aquaculture Ltd., Canada) once a day. In contrast, holding tanks at the PAL were fed by flow-through seawater from the adjacent harbour (13.7-22.4 ºC and 27-29 ppt) without additional aeration or feeding. Lighting at both facilities was controlled according to the daylight patterns of the season.

Only healthy tunicates were selected for the trials. Tunicates

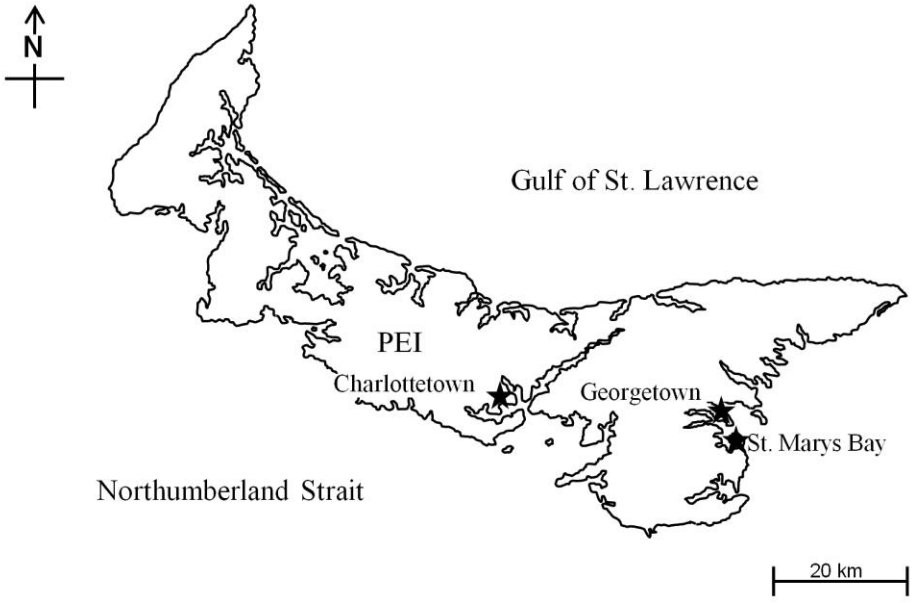

Figure 1. Approximate location of the field site (St. Marys Bay) and the laboratories (AVC in Charlottetown and PAL in Georgetown) were the perforation trials were conducted.

Resumen

Ciona intestinalis y *Styela clava*, dos especies nocivas para la industria de mitílidos en Prince Edward Island, fueron tratadas con perforaciones de clavos o agujas hipodérmicas. Otros tratamientos aplicados a las mismas especies incluyeron perforación simultánea utilizando dispositivos con densidades de agujas baja, media y alta, ya sea con o sin la inyección de vinagre. En el laboratorio, los niveles de mortalidad estimados en tunicados tratados con perforaciones individuales fueron significativamente más altos que los niveles medidos en grupos control. La mortalidad de *C. intestinales* alcanzó un 100% con 60 perforaciones o la inyección de 0.05 mL de vinagre. En comparación, en el caso de *S. clava*, sólo 100 perforaciones resultaron en un 100% de mortalidad. Cuando el dispositivo de perforación fue aplicado dos veces, se alcanzo un 80% de mortalidad en *C. intestinalis*. Sin embargo, en los ensayos de terreno la aplicación del dispositivo dos veces no disminuyó significativamente el peso húmedo de *C. intestinalis*, sin importar si esto estuvo o no asociado a la inyección de vinagre. En terreno, la aplicabilidad del dispositivo de perforación sobre tunicados adheridos a los mitílidos estuvo limitada por la heterogeneidad de la superficie de los mitílidos y la posible inhibición de crecimiento bacteriano debido a las bajas temperaturas. En general, nuestros resultados muestran que la perforación y la inyección de vinagre fueron exitosas en el laboratorio pero ambos deberían estudiarse más a fondo con la ayuda de dispositivos de perforación en condiciones de terreno.

Palabras clave

Mortalidad, aguja, ácido acético, bio-ensuciar, mejillón azul

were considered healthy if they actively and consistently filtered water and contracted their bodies if one or both siphons were touched. A total of 4 to 274 tunicates were equally separated into compartments of a divided tray (6 or 12 compartments) with water circulating throughout. When possible, treatments were randomly assigned to different compartments to control for the effect of location within the holding tank.

Perforation of C. intestinalis and S. clava in the laboratory

Various treatments consisting of individual or simultaneous perforations with our without vinegar were applied to adult C.*intestinalis* (Tables 1-3) and adult S. *clava* (Tables 4-5). All treatments were performed out of water unless otherwise indicated. For individual perforations, a 20 Gauge (G) hypodermic needle was inserted through the tunic into the viscera region of a tunicate lying on a flat surface either above or under water. To mimic a possible future commercial application technique, perforation devices (Fig. 2) were constructed to apply multiple perforations simultaneously. The device consisted of needles that were pushed through a 1.8 cm thick sheet of styrofoam and the lid of a plastic container. A second sheet of styrofoam and another lid were placed over the needle bases to

Holding facility	Treatment grouping	# of perforations	# of replicate trials
AVC[a]	Control	0	6
	0<x≤30	15	1
		20	2
		25	1
		30	4
	30<x≤50	35	1
		45	3
		50	2
	50<x≤70	55	5
		70	2
	70<x≤100	85	2
		100	3
PAL[a]	Control	0	12
	0<x≤30	5	2
		10	2
		20	3
		30	2
	30<x≤40	35	3
		40	4
	40<x≤50	42	2
		45	5
		50	1
PAL[b]		45	2
		50	3
		60	2

Table 1. Number of individual perforations applied to adult *C. intestinalis* either above or underwater in re-circulating tanks at the Atlantic Veterinary College (AVC) or in flow-through tanks at the Portable Aquatic Laboratory (PAL). Treatment groupings indicate which perforation frequencies were pooled for statistical purposes, so that the sum of the number of replicate trials is equal to the sample size per treatment grouping (e.g. for AVC 0<x≤30, N=8). [a] Perforations applied above water; [b] Perforations applied below water

Perforation device type	# of needles cm^{-2}	Approx. # of simultaneous perforations	# of replicate trials
Low-density	0.5	4	1
Medium-density	1.1	9	1
High-density	2.4	21	2
High-density twice	2.4	42	2

Table 2. Needle density and the approximate number of needles perforating each *Ciona intestinalis* during the application of multiple simultaneous perforations of C. *intestinalis* using various devices. Treatments were performed at the Portable Aquatic Laboratory.

Vinegar application	Injection volume	# of replicate trials	Treatment description
Injection (single needle)	0.05 mL	1	
	0.5 mL	2	
	0.2 mL	1	
	1 mL	2	
	2 mL	2	
	3 mL	2	
	4 mL	2	
Dip (multiple needles, high-density device)	n/d	2	Dipped and perforated once
	n/d	2	Dipped and perforated twice
Injection (multiple needles, high-density device)	~ 4 mL	2	0.2 mL per needle, injected during perforation once

Table 3. Individual and multiple perforations of *C. intestinalis* with vinegar. Treatments were performed at the PAL.

hold the needles in position. Lids were connected with screws at opposing corners. Individual vinegar injections were applied by attaching a 5 mL syringe with the desired volume of vinegar to the 20 G needle. Using the perforation devices, vinegar was either applied by dipping the device needle-deep into a vinegar bath before each application or by attaching tuberculin 1 mL syringes filled with 0.2 mL vinegar each to all needles on a perforation device and pressing on all the needles simultaneously during perforation using the lid of the same type of container used to construct the device.

Following treatment, each tunicate was immediately returned to its compartment in the holding tanks for observation to assess mortality after 7 days. Mortality was defined as a change in tunic colour and decomposition of the tunicate after one or more days of no filtering activity and no reaction to touching the siphons. The number of tunicates per treatment group varied depending on tunicate availability from 4 to 60 for *C. intestinalis* and 4 to 10 for *S. clava*. Treatments were repeated in replicate trials to increase sample size. Because of limited success in obtaining and sustaining healthy adult *S. clava* throughout the study, not all replicate trials could be

Figure 2. Perforation devices used to apply multiple simultaneous perforations to tunicates. (A) Low density: 0.5 needles/cm^2, (B) medium density: 1.1 needles/cm^2, and (C) high density: 2.4 needles/cm^2.

completed for this species. *S. clava* mortality occurred in conjunction with an unidentified white growth on the tunic, and mortalities were observed in controls. Thus, the majority of the experiments and results reported here focus on *C. intestinalis*.

Application of the high-density perforation device in the field

The short-term effect of perforation with and without vinegar on tunicates attached to mussel socks was assessed in a 1-week field trial starting in mid-October. Water temperature ranged from 7.8 to 8.4 °C, salinity from 25.4 to 25.8 ppt and dissolved oxygen from 6.5 to 6.7 mL/L throughout the week-long field trial. Mussel socks fouled with *C. intestinalis* were collected from a mussel lease in St. Marys Bay (Fig. 1) and divided into 40 cm sections. Five of these sections were perforated twice on all sides using the high-density perforation device. A second group of 5 sock sections was perforated twice on all sides with the device being dipped in vinegar before every application. A third set of sock sections were left untreated (controls). Water quality parameters were recorded throughout the trial using a water quality meter (WET Labs, OR, USA). One week post-treatment, all mussel sock sections were retrieved. Only the central 15 cm of each section were processed to avoid any edge effect. All *M.*

Holding facility	# of perforations	# of replicate trials
AVC	0 (control)	4
	30	1
	55	1
	70	3
	77	2
	85	3
	100	2
PAL	0 (control)	2
	70	2
	77	2
	85	2

Table 4. Individual perforations of adult *S clava*. All perforations were performed out of water.

Perforation device type	# of needles/ cm^2	Approx. # of simultaneous perforations	# of replicate trials
Low-density	0.5	4	2
High-density	2.4	21	2

Table 5. Multiple perforations of *S. clava* with vinegar. Treatments were performed at the PAL.

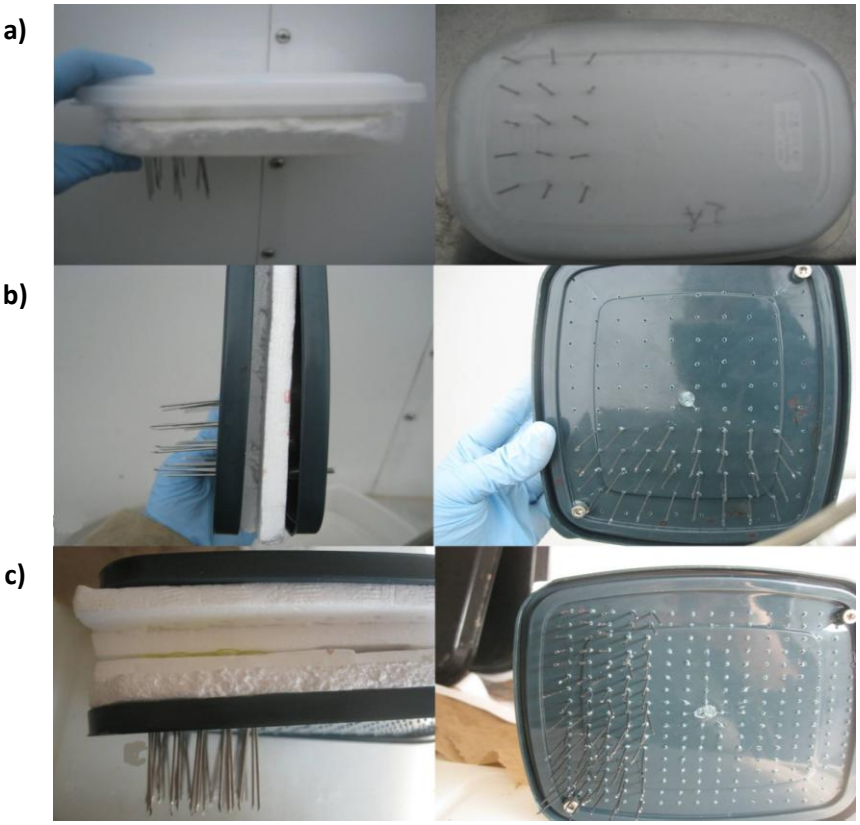

Figure 2. Perforation devices used to apply multiple simultaneous perforations to tunicates. (a) Low density: 0.5 needles/cm^2, (b) medium density: 1.1 needles/cm^2, and (c) high density: 2.4 needles/cm^2.

edulis and *C. intestinalis* from each sock section were counted and weighed (wet weight). *C. intestinalis* were subsequently frozen and thawed to measure uncontracted length.

Statistical analyses

For the laboratory data, *C. intestinalis* % mortality for each compartment (4-60 individual *C. intestinalis*) was considered a single replicate. Mortality values from replicate trials were combined into the following arbitrarily chosen ranges of individual numbers of perforations: at AVC, control (0 perforations), 0<x≤30, 30<x≤50, 50<x≤70, and 70<x≤100 (were x represents the number of individual perforations); at the PAL control, 0<x≤30, 30<x≤40, and 40<x≤50 perforations (Table 1). Differences among treatment groups were determined using one-way ANOVA following assumption testing (normality, homogeneity of variances). Data were transformed to meet the normality assumption if necessary. Subsequent pair-wise comparisons between controls and each treatment group were made using Tamhane's post-hoc test which does not assume homogeneity of variance of the data. *S. clava* data were omitted from statistical comparisons due to an insufficient number of replicates. The effects of some special treatments such as vinegar injections and underwater perforations were not analyzed statistically because of limited availability of tunicates and the resulting low sample sizes.

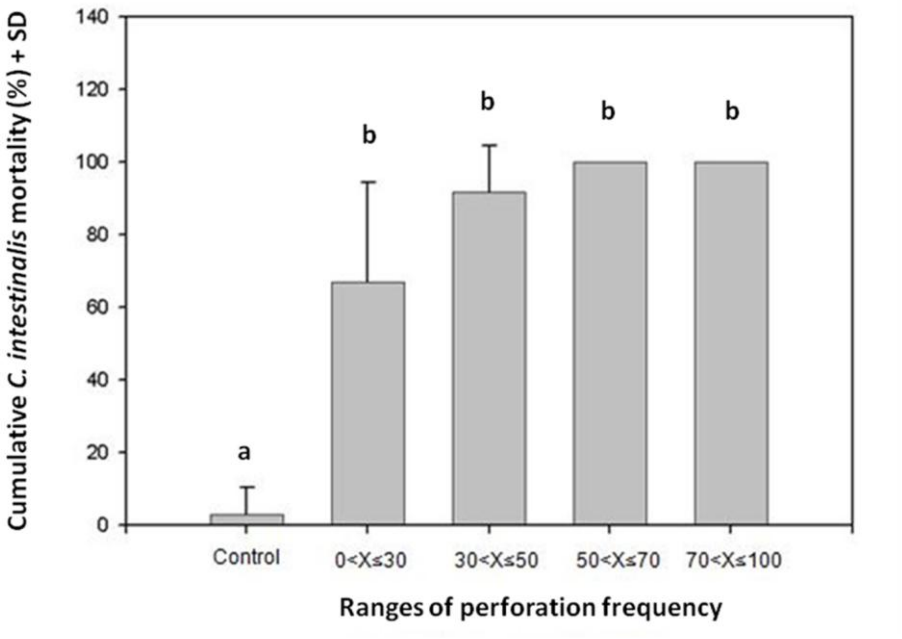

Figure 3. Average (+1 SD) cumulative mortality of C*. intestinalis* 1 wk after perforation with 20 G needles ranging from 0 (control) to 100 individual perforations (see Table 1 for details on treatment groupings). Tunicates were held in recirculating artificial seawater at the Atlantic Veterinary College. Significant differences between groups are indicated by different letters.

For the field trial, abundance, length and wet weight of *C. intestinalis* and mussels were compared among the treatment groups using one-way ANOVA if assumptions for parametric tests (normality of data and homogeneity of variances) were met. Data were not normally distributed and they were inverse transformed to ensure normality. Power analysis was performed when type II error was suspected (*C. intestinalis* biomass). A permutation test (which is independent of the distribution of the data) with 1000 randomizations was used to confirm ANOVA results in cases when normality assumptions could not be met and p -values were close to 0.05. Since permutation tests confirmed all ANOVA p-values, only the latter are reported in this paper. Statistical analyses were performed using SPSS Statistics® 17.0 and Minitab® 15 for Windows.

Results

Effect of individual perforations in the laboratory

C. intestinalis reacted to perforation from carpet, small, medium and large nails and 20 G, 25 G and 28 G needles by contraction of the siphon

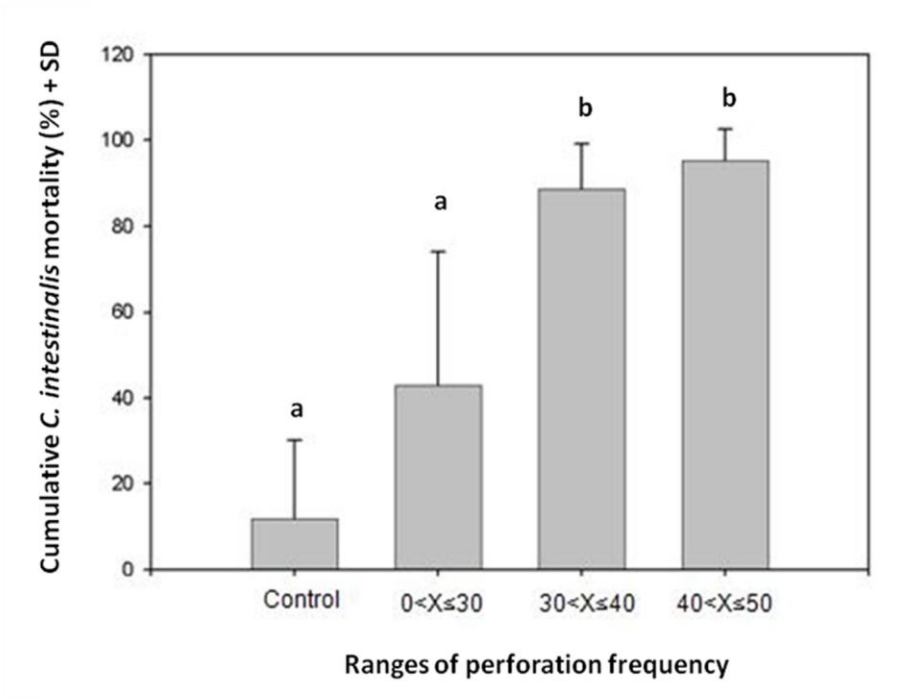

Figure 4. Average (+1 SD) cumulative mortality of *C. intestinalis* held in a flow -through system (Portable Aquatic Laboratory) 1 wk following perforation with a 20 G hypodermic needle. Perforations were applied with a single needle on individual tunicates, ranging from 0-50 repeated individual perforations (see Table 1 for details on treatment groupings). Significant differences between groups are indicated by different letters.

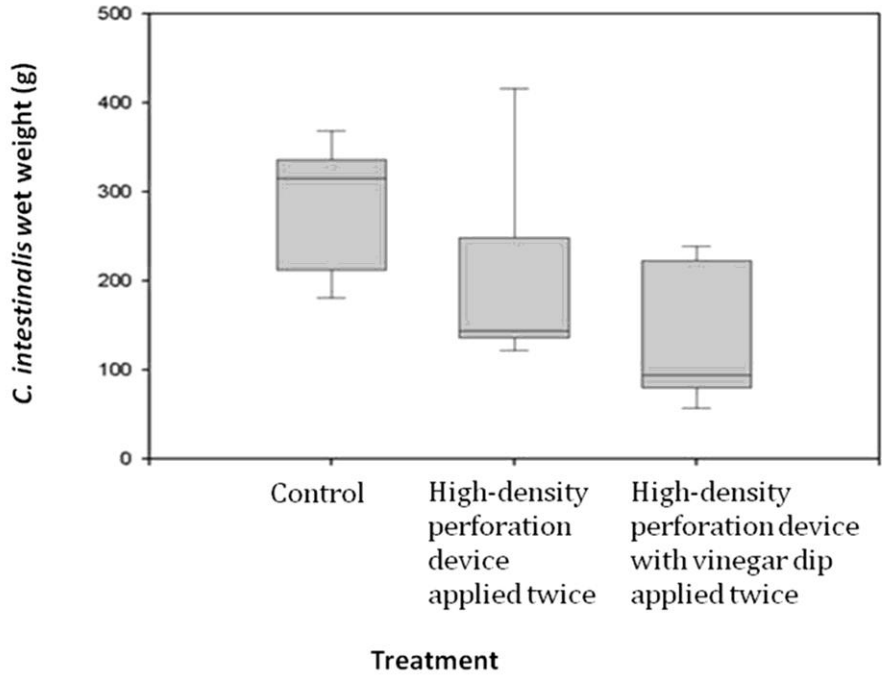

Figure 5. Wet weight of *C. intestinalis* on 15 cm mussel sock sections one week after treatment. Five mussel sock sections each were either left untouched (control), or treated twice with a high-density perforation device with or without vinegar application. Box blot depicts sample minimum, 1st quartile, median, 3rd quartile and maximum.

and body muscles. Perforation wounds in its tunic could not be visually observed. However, round white capsules were observed in some *C. intestinalis* individuals at the base of the tunic at probable perforation sites irrespective of the type of perforator used. *Styela clava* reacted to perforation by contraction of the body. Cross-sections of a perforated *S. clava* that appeared harder than normal after 6 d post-treatment revealed a bright yellow visceral mass that was not observed in untreated *S. clava.*

In the recirculation system at AVC, percent mortality was significantly lower for control *C. intestinalis* than on perforated ones (ANOVA, $p<0.05$; Tamhane's comparisons; control versus $0<x\leq30$, $p<0.05$; control versus $30<x\leq50$, $p<0.05$; control versus $50<x\leq70$, $p<0.05$; control versus $70<x\leq100$, $p<0.05$; (Fig. 3). Any number of perforations above 50 resulted in 100% tunicate mortality. For *S. clava*, 85 perforations resulted in 50% mortality, and 100 perforations caused 100% mortality (data not shown). Below 85 perforations, mortality levels were inconsistent. For instance, 77 perforations resulted in 20% mortality, whereas 70 perforations resulted in either 30 or 70% mortality (data not shown).

In the flow-through system at the PAL, mortality was significantly higher among *C.*

Treatment device and application frequency		n	% Mortality
Perforation devices	Low density, applied once	45	7
	Medium density, applied once	44	11
	High-density, applied once	19	21
	High-density applied twice	12	33
Perforation devices and vinegar	High-density with vinegar dip, applied once	10	45
	High-density with vinegar dip applied twice	20	80
	High-density with vinegar injection, applied once	12	100

Table 6. *C. intestinalis* mortality caused by the application of perforation devices (low, medium and high) once, twice and in conjunction with vinegar. For application of the high-density perforation device with vinegar injection, 0.2 mL was taken in a syringe attached to each needle of the device. The number of tunicates in each trial is indicated by n.

intestinalis perforated >30 times than among the controls (ANOVA, $p<0.05$; Tamhane's, $p<0.05$ for the ranges $0<x\leq30$ and $30<x\leq40$; Fig. 4). Fewer than 30 perforations did not cause significant mortality among *C. intestinalis* compared to controls (Tamhane's, $p>0.05$). *C. intestinalis* mean mortality caused by perforations with a single needle was slightly higher in the PAL compared to the AVC, 72% and 53%, respectively. When *C. intestinalis* were perforated underwater, 50 perforations were necessary to cause 100% mortality. Mortality decreased to 90% at 45 underwater perforations.

Effect of multiple-needle perforation devices with and without the injection of vinegar in the laboratory

A single application of any of the three perforation devices (low, medium and high needle-densities) on individual *C. intestinalis* resulted in up to 21% mortality (Table 6). Two applications of the high-density perforation device increased mortality to 33%. A single application of the high-density perforation device dipped in vinegar increased mortality to 35 %. However, when the high-density device was dipped in vinegar and applied twice, *C. intestinalis* mortality increased up to 80%. Vinegar injection of 0.2 mL per needle via the high-density perforation device into the tunic of *C. intestinalis* caused 100% mortality (Table 6).

Preliminary trials of single vinegar injections into the tunic of *C. intestinalis* resulted in 100% mortality for injection volumes of 0.05 to 4 mL. As early as 2 d post-treatment, all *C. intestinalis* injected with vinegar had a liquefied visceral mass that exited the tunic through the siphon openings when the animals were handled. The low and high-density perforation devices applied once without vinegar resulted in *S. clava* mortality of 15% and 20%, respectively (data not shown).

Effect of perforation with or without vinegar application in the field

On average, the wet weight of *C. intestinalis* perforated with or without vinegar application was 28 % and 51 % lower than on control socks, respectively (Fig. 5), but this difference was not significant (ANOVA, $p>0.05$). Neither abundance (ANOVA, $p>0.05$) nor length (ANOVA, $p>0.05$) of *C. intestinalis* differed between treated and control sock sections (data not shown). Likewise, *M. edulis* wet

weight (ANOVA, p>0.05) and abundance (ANOVA, p>0.05) were not significantly different among treatment groups (data not shown).

Discussion

C. intestinalis mortality caused by individual perforations

Perforations with a 20 G needle resulted in substantial tunicate mortalities. Specifically, 50 individual perforations or more could effectively cause 100% tunicate mortality. These results contradict previous evidence that perforation is ineffective at causing tunicate mortality (K. Gill, pers. comm.) and suggest that perforation could be a useful mitigation strategy against tunicate fouling."

There were no white capsules observed in the tunics of *C. intestinalis* treated with the perforation device with and without vinegar application. Possible reasons for this are that the large number of perforations caused a shock (similar to bacterial septic shock) to the immune system that prevented it from encapsulating so many wounds. This in turn raises two questions: (1) why was mortality not consistently 100% in groups perforated a large number of times, and (2) why were capsules not observed in those individuals that did not die? Further research into the pathology of mortality caused by perforation is necessary to answer those questions.

The occurrence of round white capsules in *C. intestinalis* post-perforation appears to be similar to the inflammatory reaction caused by the injection of alien cells into a tissue (De Leo et al. 1996; Parrinello & Patricolo 1984). Possibly, the encapsulation of the substance injected was initiated by pathogens that gained an entry-pathway into the tunic via the perforation. The encapsulation likely began with the movement of morula cells, phagocytes and granulocytes toward the wound site (De Leo et al. 1981). Normally, the encapsulation is followed by a melanization healing process (De Leo et al. 1996; 1997). If an exaggerated reaction from the lysozymes and granulocytes occurs as a result of the alien substance injected in the tunic, severe damage to the tissue is expected (Parrinello et al. 1984).

Differences in *C. intestinalis* percent mortality between those perforated at the PAL and at the AVC may be caused by different microbial communities. The AVC tanks have recirculated water that may concentrate pathogens and result in higher mortalities compared to the flow-through seawater system at the PAL.

S. clava mortality caused by individual perforations

S. clava laboratory trials showed that twice as many perforations were necessary to cause 100% mortality compared to *C. intestinalis* (100 vs. 50 perforations). Perforations were made through the tunic of both *C. intestinalis* and *S. clava* so it is unlikely that the differences in the composition of their tunics (gelatinous in *C. intestinalis* and leathery in *S. clava*) contributed to the difference in number of perforations necessary to cause 100 % mortality. Possibly, *S. clava* has better healing capabilities and can therefore survive more perforations than *C. intestinali.* In fact, the literature suggests that leukocytes make up a slightly higher proportion of total hemocyte counts in *S. clava* than in *C. intestinalis* (reviewed in Wright & Cooper 1983). Leukocytes include phagocytes (Wright & Cooper 1983) which are involved in the healing process of injected substances in *C. intestinalis* (Parrinello & Patricolo 1984).

Applicability of perforation in the field

The field application of the high-density perforation device with or without vinegar on tunicate-fouled mussel socks reduced *C. intestinalis* biomass but did not result in any significant differences compared to the control group. The low sample size (n = 5) along with the inherent

variability of natural substrates (mussel socks) may have contributed to this result, as statistical power was low (55 %). Other alternative causes were proposed for such unexpected results. First, the decreased efficacy of perforation in field conditions may have been due to the uneven mussel sock surface, time of year and damage to the perforation device from continuous use. Compared to the cutting boards used as surfaces in laboratory tests, the surface of a mussel sock is uneven and with multiple crevices between mussels. This factor alone likely limited the number of actual perforations into the *C. intestinalis* tunic. While the perforations could have still weakened the tunicates and potentially lead to mortality, a trial duration of over a week might have been necessary to detect a slower mortality rate. Second, time of the year with its associated low temperature may also have been a factor in the reduced mortality observed during the field trial. Water temperature throughout the 1 wk trial ranged from 7.8 to 8.4ºC. While these temperatures are below the optimal growth temperature range for *C. intestinalis* (10-30°C; Dybern 1965; Carver et al. 2003) and might therefore reduce their healing ability, the growth and pathogenicity of aquatic microbes would also be negatively affected. Observations from previous experiments indicate that decomposition onset is delayed during the cold winter months (C. Arens, pers. comm.). Thus, while the uneven surface still represents a logistic issue, it is expected that the effects of perforation on *C. intestinalis* will be much higher if applied in warmer months.

Third, the perforation device was damaged during successive applications to mussel socks, with needles bending and breaking off. In future experiments, this problem would need to be addressed, possibly by using stronger needles or a more flexible mechanism that allows needles to retract when they encounter an elevated point on the surface. Since the high-density perforation device only resulted in approximately 20 % mortality for both tunicate species when applied once, even in laboratory applications on an even surface, the device either needs to be re-designed or applied repeatedly to achieve a greater number of perforations per tunicate (21 perforations estimate for a single use of the high density perforation device). In the current design, needle density was limited by the diameter of the needle base. This connector must either be removed or a different type of needle must be used to increase the needle-density of the device. Alternatively, using three or more applications of the high-density device would increase mortality levels as shown in the higher *C. intestinalis* mortality achieved by two applications of the device compared to a single application (70 % and 20 % mortality, respectively).

Successful application of the perforation treatment is expected to have the added benefit of not instantly removing tunicates from the mussel sock as is the case with the currently used high-pressure washing treatment. Instead, a layer of dead tunicates would remain on the mussel sock, preventing settlement of new tunicate recruits by occupying the available space. Even settlement on dead tunicates would not present a problem since decomposition would eventually lead to the natural removal of the dead tunicates and their attached fouling. By contrast, high-pressure washing seems to have a priming effect, creating bare space that can instantly be recolonized by tunicates and other fouling organisms (C. Arens, pers. comm.).

Effect of addition of vinegar

Vinegar injection into the visceral mass of *C. intestinalis* was more successful in terms of % mortality compared to individual perforations. 100% mortality was achieved with a volume as low as 0.05 mL injected with a single needle. Vinegar injection through the high-density perforation device, volume estimated at 4mL, also produced

100% mortality. One application of the high-density perforation device used in conjunction with a vinegar dip caused 45% mortality while two applications with vinegar dip resulted in 80% mortality. The percent mortalities obtained were similar to experiments using vinegar spray and immersion against *C. intestinalis* (Carver et al. 2003) and *S. clava* (A. Coutts & B. Forrest, pers. comm.). Under laboratory conditions, a 5% acetic acid spray or dip for 1 minute followed by rinsing in seawater resulted in 100 % mortality of *C. intestinalis* (Carver et al. 2003). Immersion of *S. clava* in 5% acetic acid for 1 or more minutes resulted in 100% mortality as well (A. Coutts & B. Forrest, pers. comm.).

Potential effects on mussel sock epifauna

Methodologies assessed during this study have associated potential negative impacts. Vinegar treatments such as immersion or spray have resulted in mortality of other epifauna living on mussel socks (Locke et al. 2009; Paetzold et al. 2008). The injection of vinegar directly into solitary tunicates, *C. intestinalis* and *S. clava,* reduces the dispersion of acetic acid on the mussel sock. Limiting the mortality of other epifauna is beneficial in that it may control the settlement of invasive species by favouring the settlement of competitive species (Osman & Whitlach 1992) and allowing predation on recently settled tunicates to occur (Osman & Whitlach 1995). During injection, the minimal amounts of vinegar that is not introduced into the visceral mass of the tunicate would not have a negative impact on the epifauna.

For example, Davies (1991) suggested that fish, *Atherina* (*Hepsetia*) *boyeri* Risso, 1810, can perceive the presence of acids in the water and swim away from the source, thus preventing injury. The same applies to mobile epifauna but not necessarily to epifaunal species that attach to the mussel socks and cannot swim away. These species may cease filtering and protect themselves from their surroundings by retracting into their bodies or shells until vinegar dispersal.

Conclusions

Considering the positive results obtained in laboratory trials, it is strongly suggested the development of further studies that include the optimization of the perforation device and treat tunicates in the field during warmer months. Individual perforations, although effective, are time consuming and unpractical for the industry. Since the device used for the field trial was damaged due to repetitive use on the mussel sock, stronger needles should be used in conjunction with a shock-system for the needles, for example springs. Such an adaptation would buffer the needles if they hit a hard substrate such as mussel shells but would allow the piercing of soft tissues such as the tunic of *C. intestinalis*. If perforation could be successfully used at the commercial level, it would provide a mitigation strategy against tunicates with none of the side effects associated with large scale chemical treatments. In addition, the underwater applicability of this treatment would eliminate the need of lifting mussel lines from the water, thus reducing substantial labour costs.

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