Management of Biological Invasions (2016) Volume 7, Issue 2: 157–165
DOI: http://dx.doi.org/10.3391/mbi.2016.7.2.03

Open Access

Research Article

Tolerance of early life-stages in *Ciona intestinalis* to bubble streams and suspended particles

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Received: 5 January 2015 / Accepted: 3 September 2015 / Published online: 24 November 2015

Handling editor: Richard Piola

Abstract

There is an urgent need to develop and test potential eradication agents to prevent biofouling by ascidian tunicates in sheltered harbor's before they can spread to, and subsequently, disrupt benthic ecosystems and aquaculture ventures. We test the effects of bubble streams and suspended particles on two key stages in the early life cycle of *Ciona intestinalis* (Linnaeus, 1767); (i) Larval settlement 3 d after introducing free swimming larvae, and (ii) juvenile survival following exposure of ~ 21 d old juvenile recruits to the tested treatments for the first time. Larval settlement was effectively prevented after exposure to bubble streams at > 10 l min^{-1} or ≥ 25 Nephelometric Turbidity Units (NTU) of suspended particles. Suspended particles settling as a layer ≥ 0.3 mm thick onto the test substrate also reduced larval settlement by over 90%. None of the treatments were effective at eradicating juvenile *C. intestinalis* which proved more resistant to the tested treatments. Body size in juvenile recruits was also not significantly different across bubble stream rates. Juvenile recruits exposed to suspended particles were, however, significantly smaller than in the control treatment. In conclusion, as *C. intestinalis* settled, and grew, it became progressively more resilient to the tested treatments. Thus, continuous treatment is required during the spawning season to target larval settlement and prevent fouling by *Ciona intestinalis*.

Key words: *Ciona intestinalis*, life-cycle, settlement, recruitment, bubble streams, suspended particles

Introduction

Species introductions can negatively impact the sustainability of coastal aquaculture ventures (Bax et al. 2001; Occhipinti-Ambrogi and Savini 2003; Colautti et al. 2006). Moreover, climate change, in combination with increased vessel traffic and commercial transfers associated with the aquaculture industry, will only increase the spread of marine aquatic invasive species (AIS) (Stachowicz et al. 2002; Myerson and Mooney 2007). Currently, there is a need to develop and test potential eradication agents to prevent biofouling by invasive ascidian tunicates that disrupt benthic ecosystems and aquaculture ventures. In the last decade biofouling by temperate-zone ascidian tunicates has severely disrupted mussel cultivation in Canada, Europe, Australia, and New Zealand (Locke et al. 2007; McKindsey et al. 2007; Sephton et al. 2011).

The vase tunicate, *Ciona intestinalis* (Linnaeus, 1767), was confirmed for the first time in Newfoundland, Canada, in October 2012 (Sargent et al. 2013). A significant infestation of vase tunicate was detected in Little Bay, a sheltered embayment of Mortier Bay, Placentia Bay (Figure 1). This solitary ascidian tunicate poses a threat to the economic viability of the shellfish aquaculture industry in temperate waters around the world (e.g. Ramsay et al. 2008). It is especially important to eradicate *C. intestinalis* on wharfs and vessels before it can spread to other sites, including areas where mussels are cultivated.

Methods to limit the economic and ecosystem impact of ascidian biofouling include: i) physical detachment from the substrate by high pressure

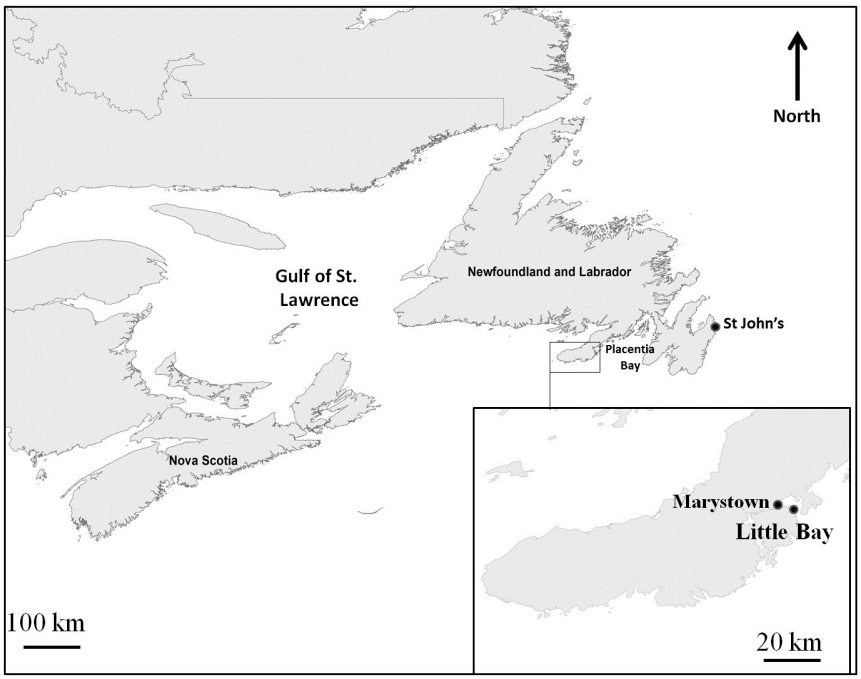

Figure 1. The Location of Little Bay (Latitude 47.163, Longitude -55.112), Newfoundland, Canada.

water jets, manual removal by divers, or suffocation after wrapping in plastic (Arens et al. 2011; Coutts and Forrest 2007); ii) chemical treatments including acidic (vinegar) or alkali (lime) washes of mussel cultivation ropes, or soaking mussels on ropes in freshwater (Forrest et al. 2007; Denny 2008; Switzer et al. 2011). iii) introduction of naturally occurring predators (Switzer et al. 2011; Atalah et al. 2013; Deibel et al. 2014). iv) application of biocide treatments, e.g. antifouling paint to ship hulls and underwater structures (Cima et al. 2008; Guardiola et al. 2012). Although some of these methods (e.g. chemical, biocide, and biological control) have been successful, it is difficult to implement them at an industry scale (see review by Deibel et al. 2014). Other promising eradication options include suffocation/starvation through sedimentation (Carver et al. 2006), and scouring by bubble streams (Bullard et al. 2010). The effectiveness of both of these possibilities was tested in our study.

Streams of air bubbles are a possible mechanism to generate and maintain high concentrations of suspended particles around a sheltered wharf and vessels. Bubbles themselves are likely an effective eradication agent as they may disrupt the settlement and recruitment of fouling organisms (Bullard et al. 2010). Bubble streams were first proposed as a means for controlling biofouling in 1946 (Smith 1946). Due to the negative effects of antifouling paints on ecosystems, there has been renewed interest in treating vessel hulls with bubble streams (Bullard et al. 2010).

Aeration by bubble streams can reduce biofouling on PVC plates by almost 100% (Bullard et al. 2010). However, as *Ciona intestinalis* was not included in the study, further research is needed to determine if bubble streams inhibit larval settlement and the establishment of juvenile recruits. It will be easier to control a population if aeration affects both processes. To aid in further commercial development, the minimum level of aeration (i.e., both intensity and time) required to prevent biofouling by *C. intestinalis* should be defined. Our study aims to address these outstanding questions.

Suspended particles may clog the feeding and respiration apparatus of ascidians (Robbins 1983; Robbins 1984; Petersen and Riisgård 1992; Carver et al. 2006) resulting in a reduction in feeding and death of adults (Robbins 1985). Early life stages could conceivably be eradicated with lower concentrations of suspended particles than are needed to kill adults (McLaughlin et al. 2013). For example, suspended particles (at 12–32 Nephelometric Turbidity Units or NTU) significantly decreased the probability of fertilization, embryo development, and metamorphosis of settling larvae, and resulted in lower survival in

5-d-old juveniles, of *Ciona intestinalis* after 18 d (McLaughlin et al. 2013). In contrast, high adult mortality of *C. intestinalis* has been observed only at concentrations of suspended particles > 650 NTU (Robbins 1985).

Developing settlers of ascidian tunicates likely remain vulnerable to suspended particles as they open their siphons and metamorphose into the first feeding stage, and thence to a juvenile. Further research is required to establish lethal sediment concentrations and exposure times to inhibit settlement and establishment of juvenile recruits. Because ascidians are thought to settle only on clean substrates (Carver et al. 2006), suspended particles settling onto the substrate could also inhibit larval settlement; but this hypothesis has never been experimentally tested.

We tested the effects of bubble streams and suspended particles on two key stages in the early life cycle of *Ciona intestinalis*; (i) settlement and, (ii) juvenile survival. Settlement, including metamorphosis into an oozooid, takes place 3 d after larvae are freely swimming at ~18ºC (Cirino et al. 2011). Juvenile recruits, here identified as juveniles beyond the 2nd ascidian stage (Cirino et al. 2011), were ~ 21 d old prior to exposure to treatments. Finally, we also examined whether consolidated particles covering previously clean substrates could reduce larval settlement.

Our findings will increase understanding of the most vulnerable early life stages to bubble streams and suspended particles. Our laboratory study will also provide guidance for scaling up eradication efforts in the field. By defining previously unknown tolerances of settling larvae and juveniles to suspended or consolidated particles, the extent to which the natural distribution of *Ciona intestinalis* is constrained by these factors can be determined.

Methods

Collecting larvae and recruits

Larvae were produced for each settlement experiment as follows. First, eggs were obtained from 5–8 adult *Ciona intestinalis*, and sperm from a further 5–10 adults. Then, a sperm solution was made in 50 ml of 0.22-µm filtered seawater. *In vitro* fertilization was performed by mixing 1 drop of sperm solution with ~ 15,000–24,000 eggs in a 2-l Pyrex dish. After approximately 1 h, the fertilized eggs were transferred to a 2-l beaker containing 0.22-µm filtered seawater, and kept in suspension with a magnetic stirring bar until the larvae hatched. The number of fertilized eggs developing into competent larvae varied from 80 to 90 %. Larvae typically hatched within 16–18 h at 22 ºC, as in Cirino et al. (2011). Free swimming larvae, not subjected to any treatment, settled within 3 d, as reported by Cirino et al. (2011). Larval concentration (ml^{-1}) was determined after averaging the number of larvae in 3, 2-ml samples from the 2-l beaker in which larvae were hatched. A known number of free swimming larvae (average 2 per ml) were subsequently transferred to each treatment beaker (see below).

Juvenile recruits were collected on strings of 15×15 cm, polyvinyl chloride (PVC) plates deployed for 3 weeks to the floating dock at the Bedford Institute of Oceanography, Dartmouth, Nova Scotia, Canada. To avoid thermal and desiccation shock after collection, PVC plates containing juvenile recruits were transferred to the laboratory in 80-l plastic coolers.

Effect of bubble stream rate

We generated bubbles during 2 experiments (the first split into 2 trials), by applying compressed air through Gardena aeration tubing (#1969 micro-pore hose, 0.5 inch ID/0.66 inch OD). Bubble stream treatment levels were 5, 10, or 20 l min^{-1} flow rate of compressed air. Free swimming larvae (2 ml^{-1}) were evenly distributed among 12, 5-l beakers (i.e., 3 beakers per bubble stream rate and a control set of 3 beakers). Each beaker contained 0.22 µm filtered seawater, and a 5-cm petri dish on which larvae settled, suspended ~ 15 cm above a length of aeration tubing. Filtered seawater in each beaker was not replaced for the duration (i.e. 3 d) of the larval settlement experiment. Settler abundance on each petri dish was determined under a stereomicroscope, 3 d after placing larvae in the 12 beakers.

Juvenile recruits growing on PVC plates were placed into 12, 20-L aquaria for 21 d. One PVC plate with ~ 150–250 juvenile recruits was assigned to each aquarium at time 0, with 3 aquaria per bubble stream rate and 3 control aquaria. Plates were suspended ~ 20 cm above a length of aeration tubing. All plates in each aquarium were photographed at the beginning of the experiment (time 0) and at weekly intervals for 3 weeks thereafter. Number and size (relaxed tunic length) of recruits at each time point was determined from digital photographs using Image J. Seawater in each aquarium was changed every 24 h at ambient temperature to provide naturally occurring food particles.

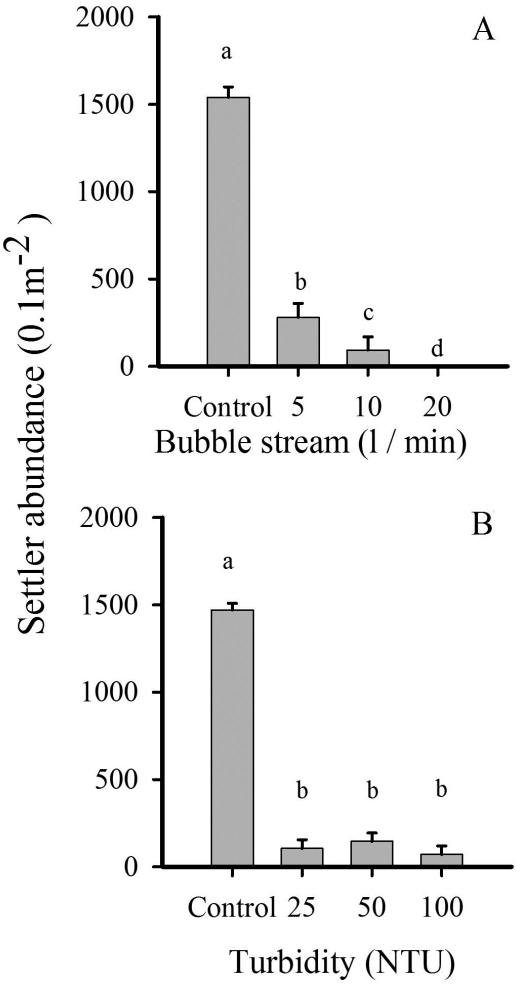

Figure 2. *Ciona intestinalis*. The effect of bubble stream rate (A) and suspended particles (B) on larval settlement 3 d after introducing free swimming larvae. Vertical lines represent 1 SEM above the mean. Treatment levels with the same letter above bars are not significantly different from each other at $p > 0.05$.

Effect of suspended and consolidated particles

Prior to each suspended particle experiment, natural benthic clay/mud (~10 g), collected from Annapolis Royal, in the Annapolis Basin, Nova Scotia, was mixed with 80 l of 0.22 µm-filtered seawater in an 95-l aquarium and left to stand for 24 h. The size frequency distribution of particles remaining in suspension after 24 h is shown in Appendix 1. This stock suspension was diluted with filtered seawater to obtain the required turbidity levels for experiments. Turbidity level (NTU) was measured with a hand-held probe (Lutron Model TU-2016). Subsamples of dilutions of the suspension, covering a range of turbidity values from 0-800 NTU, were filtered through Whatman 934-AH filter paper (1.5 µm), dried at 60ºC for 24 h, and weighed to 0.01 mg. A calibration curve was subsequently prepared to convert turbidity values (NTU) to mg l^{-1} of suspended particles (Appendix 2).

We conducted experiments with 3 concentrations of suspended particles (25, 50, and 100 NTU) and a control. Free-swimming larvae (2 ml^{-1}) were evenly distributed among 12, 500-ml beakers (3 beakers per suspended particle treatment level and 3 control beakers). A 5-cm petri dish was suspended in each beaker on which the larvae settled. Juvenile recruits growing on PVC plates were placed into 12, 20-l aquaria for 35 d. One PVC plate with ~ 150–250 juvenile recruits was assigned to each aquarium, with 3 aquaria per suspended particle treatment level and 3 control aquaria. Number of settlers, early, and juvenile recruits, and the body size of juvenile recruits, were determined as in the bubble stream trials (above). Husbandry was also conducted as in the bubble stream trials.

To determine the effect of progressively greater depths of sedimentary particles on larval settlement, suspended particles were allowed to precipitate onto a 5-cm petri dish to a depth of either 0 (control), 0.3, 1, or 3 mm. Each petri dish was placed at the bottom of a 500-ml beaker filled with filtered (at 0.22 µm) seawater. Larvae (2 ml^{-1}) were evenly distributed among 12 beakers (3 beakers per treatment level including the control). The number of settlers on each petri dish was subsequently determined under a stereomicroscope on day 3 after introduction of the larvae to the beakers. Husbandry was also conducted as in the bubble stream and suspended particle trials.

Statistical analyses

All statistical analyses were performed in the R statistical environment (R Project for Statistical Computing version 2.8.1) (R Core Team 2013). Only significant statistics are reported in the text. Settler abundance (adjusted to 0.1 m^{-2}) reflects the number of introduced larvae that successfully settled after 3d. Settler abundance was compared across treatment levels by ANOVA in a Generalized Linear Mixed Model (GLMM, package lme4 within R). Replicates and trials were nested within treatment level as appropriate. A poisson error distribution was used to accommodate count data. Where a significant main effect was detected, Tukey pairwise comparisons among treatment levels were conducted (multcomp package in R). Juvenile

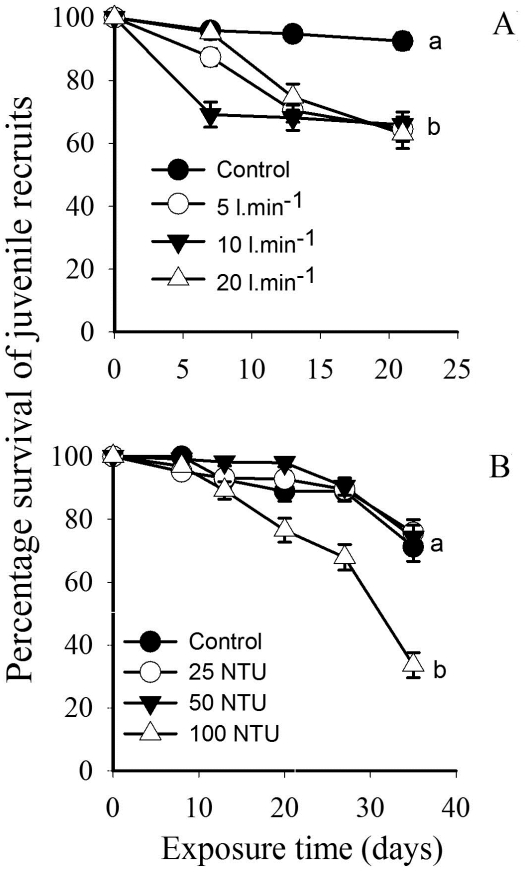

Figure 3. *Ciona intestinalis*. Juvenile survival following exposure to (A) 4 levels of bubble stream rates and, (B) suspended particle concentrations. Juvenile recruits were ~ 21 d old prior to exposure to treatments for the first time. Vertical lines represent 1 SEM. Treatment levels with the same letter are not significantly different from each other at $p > 0.05$.

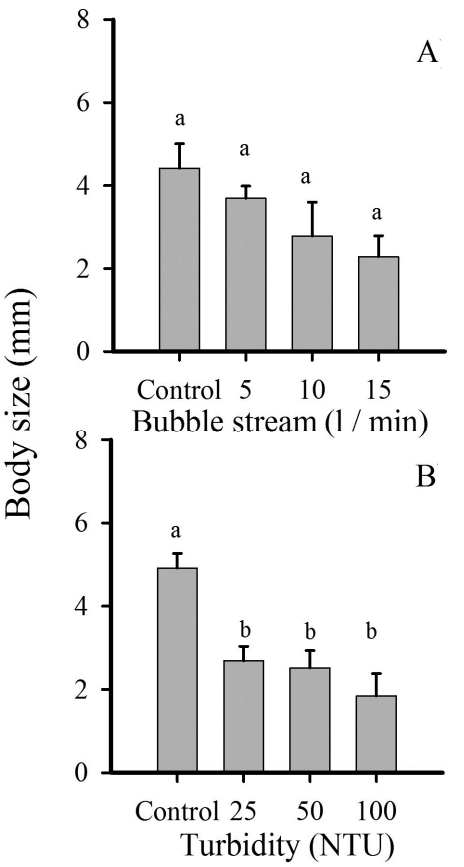

Figure 4. *Ciona intestinalis*. The effect of bubble stream rate (A) and concentration of suspended particles (B) on the body size (Mean ± 1 SEM) of juvenile recruits. Treatment levels with the same letter above bars are not significantly different from each other at $p > 0.05$.

survival (computed by the Kaplan-Meier estimator) in the bubble stream and suspended particle trials was compared across treatment levels using the log-rank test, as in Dalgaard (2002). Variation in survival among replicates did not affect interpretation of the main effect (treatment level).

The final body size of juvenile recruits was compared across treatment levels by ANOVA in a Linear Mixed Model (package lme4 within R), with replicates nested within treatment level. Where a significant main effect was detected, Tukey pairwise comparisons among treatment levels were conducted. The initial body size of juvenile recruits did not vary significantly among treatment levels (data not shown). Consequently, final body size could be used to compare growth of juvenile recruits within and among treatments.

Results

The effect of bubble stream rate on settlement and juvenile recruit survival

Increasing bubble stream rates resulted in significantly lower settler abundance (ANOVA, $F = 36$, $p < 0.01$) (Figure 2A). At 5 l min^{-1} of compressed air, settler abundance was ~ 70% lower than in the control, while at rates of 10 l min^{-1}, settler abundance was ~ 90% lower than in the control. At 20 l min^{-1} of compressed air no settlement occurred. Bubble stream rates ≥ 5 l min^{-1} resulted in significantly greater, albeit by only ~ 30%, mortality of juvenile recruits (log-rank test, $\chi^2 \geq 91$, $P<0.01$) (Figure 3A). Mortality was evident by day 14, after which survival of juvenile recruits in all treatment levels remained

relatively constant (Figure 3A). Body size in juvenile recruits was not significantly different across bubble stream rates (Figure 4A).

The effect of suspended particle concentration on settlement and juvenile recruit survival

Increasing concentration of suspended particles resulted in significantly lower settler abundance (ANOVA, $F = 101$, $p < 0.01$ (Figure 2A). Guts of settlers were clogged with particles at suspended particle concentrations > 25 NTU (Figure 5). Survival in juvenile recruits was not significantly different than in the control at the tested suspended particle concentrations ≤ 50 NTU (Figure 3B). However, significantly higher mortality of juvenile recruits was evident at 100 NTU compared with the control and suspended particle concentrations ≤50 NTU (log-rank test, $\chi^2 \geq 108$, $P < 0.01$) (Figure 3B). After 35 d, when the experiment was ended, only 30% of these juvenile recruits remained at 100 NTU. Juvenile recruits were also significantly smaller (ANOVA, $F = 13.3$, $p < 0.01$) following exposure to all suspended particle concentrations (Figure 4B).

The effect of sediment layer thickness on settlement

Larvae of *Ciona intestinalis* preferentially settled on clean substrate (ANOVA, $F = 723$, $P < 0.01$) (Figure 6). Larvae were essentially absent on substrate with even a fine layer of consolidated sediment (e.g. at ca. 0.3 mm of consolidated sediment) after 3 d of exposure. At 1 and 3mm of consolidated sediment on the substrate, no larval settlement was evident after 3d (Figure 6).

Discussion

Bubble streams

Bullard et al. (2010) reported a dramatic reduction in biofouling by benthic invertebrates after exposing PVC plates to streams of bubbles. However, *Ciona intestinalis* was not included in this study and there was no determination made on whether bubbles were inhibiting larval settlement, killing recruits, or both, and further work was recommended to address these questions (Bullard et al. 2010). Based on our findings, bubble-streams >10 l min^{-1} inhibit larval settlement, perhaps by creating turbulent currents in which tadpole larvae cannot swim to, or attach to, the substrate (Manríquez and Castilla 2007). To prevent larval settlement and subsequent fouling by *C. intestinalis,* the treated area will require continuous aeration during the spawning season. On the other hand, bubble streams did not effectively eradicate juvenile recruits; of which 70% survived. Further, juvenile growth was not significantly affected at any of the tested bubble stream rates; even though bubble streams may disrupt feeding in benthic filter feeders (Bayne et al. 1976; Menge et al. 2002; Davies et al. 2009).

Sediments

Turbidity has also been shown to affect survival and growth in ascidian tunicates (Carver et al. 2006), such that it may constrain their natural distribution (McLaughlin et al. 2013). The probability of fertilization and metamorphosis of *C. intestinalis* larvae decreases at 12–32 NTU (McLaughlin et al. 2013). In our study, the number of settlers 3 d following the introduction of larvae to the treatment was ~ 10% that of the control at ≥ 25 NTU.

Consequently, continuous exposure to suspended particles at ~ 25 NTU could effectively eradicate *C. intestinalis* as they settle on the substrate. Later life-stages that have not been exposed to suspended particles as they settle and recruit are much more resilient and thus more difficult to eradicate. In McLaughlin et al. (2013), at 5 days post settlement, ~ 40% of recruits died after they were exposed to suspended particles at ~ 22–32 NTU over 13 d. Only ~ 30% of juveniles in our study died following exposure to 100 NTU for 21 d.

As first reported by Peterson (1992), the tolerance of other invasive ascidian tunicates to suspended particles is poorly understood and requires further work. Non-indigenous ascidian tunicates (e.g. *C. intestinalis* and *Styela clava*) are conspicuously absent in a turbid environment in Prince Edward Island (Orwell Bay), despite being extremely abundant (i.e., invasive) in nearby, less turbid environments (McLaughlin et al. 2013).

In the natural environment, ascidians are often found on clean, hard substrates, and not on soft, bottom sediments. However, the effect of sedimentary particles on their settlement has never been experimentally tested. Consolidated particles may deter settlement by disrupting the orientation process that precedes settlement (Svane and Young 1989; McHenry 2005; Manríquez and Castilla 2007; Prendergast 2010), through physical abrasion (Sutherland 2006), or by creating a barrier for attachment (Kluijver and Leewis 1994; Rocha et al. 1999). In our study, depths of only 0.3 mm of clay/mud were sufficient to deter larval settlement. Thus, *C. intestinalis* settlement may be prevented if, for example, wharf pilings, and attached mussels,

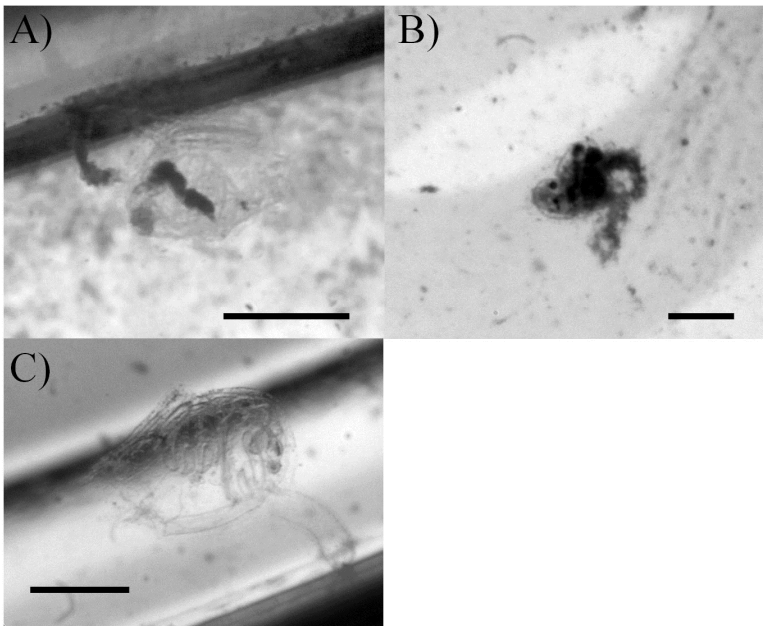

Figure 5. The effect of suspended sediment on settlers of *Ciona intestinalis*. In (A) the filtration apparatus is clogged with sediment, and in (B) the settler has died following clogging with suspended particles. Healthy settlers occurred in the control treatment (C). Black scale bar represents ~ 0.25 mm. Photomicrographs by J. Ben Lowen.

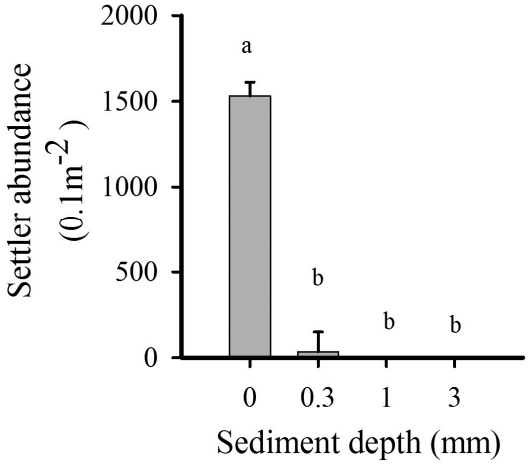

Figure 6. *Ciona intestinalis*. Effect of depth of consolidated sediment on larval settlement following ANOVA. Vertical lines represent 1 SEM above the mean. Treatment levels with the same letter above bars are not significantly different from each other at $p > 0.05$.

are coated with a fine layer of sediment following exposure to suspended particles

Timing of application

As the tested treatments were only effective on earlier life-stages, they should be timed to coincide with larval settlement during the seasonal cycle of sexual reproduction. Consequently, site-specific biological information on the seasonal cycle of reproduction is required. The seasonal reproductive window in temperate ascidian tunicates is predictably constrained by temperature limits for sexual reproduction (Millar 1952). Based on these thermal limits, the duration of sexual reproduction can be predicted from local temperature data (Lowen et al. 2013; Ma 2013). In *Ciona intestinalis*, sexual reproduction is constrained to temperatures > 6–8 ºC throughout its known range (Carver et al. 2003). Consequently, duration of treatment will be dictated by the length of the sexual reproductive window. In *C. intestinalis*, this varies from ~ 6–7 months in Nova Scotia (Vercaemer et al. 2011) to ~ 4–5 months in the colder waters of Newfoundland (Sargent 2013; Vanessa Reid pers. comm.). While it incurs a greater cost than a one-time application, a key advantage of continuously treating a site during sexual reproduction is that it will inhibit establishment of *C. intestinalis* populations and may inhibit establishment of other introduced ascidian tunicate species as well.

Future work

Future work should address the development and testing of an efficient delivery system for these treatments, which can be timed to coincide with the seasonal cycle of sexual reproduction. The application of bubble streams to vessels and structures in an enclosed harbor would be

relatively straightforward. Recent advances in aeration technology have resulted in a variety of inexpensive and efficient systems which are readily available (Bullard et al. 2010). For example, lengths of aeration piping or hose can be placed under the substrate and connected to a pump or compressor.

The application of suspended particles at > 25 NTU for an extended duration is more problematic and best suited to shallow, sheltered, enclosed harbors. A major obstacle to overcome is how to keep the particles in suspension and prevent their dilution. Installation of lengths of bubble-stream hose, as described above, may resuspend fine sediment into the water column. Here, an understanding of the bottom sediments would save time and money. For example, any suitable sediment on the bottom could simply be resuspended, rather than added artificially. If added artificially, it probably would need to be pumped into the harbor as fine slurry.

Conclusions

The tested treatments effectively prevented *Ciona intestinalis* settlement, but as settlers developed into juveniles and grew, they became progressively more resilient. Thus, further field trials with these treatments should be timed to coincide with the seasonal period of larval settlement. Because larvae settled only on clean surfaces, larval settlement may be prevented if the treated surfaces are coated with a layer of sediment (≥ 0.3 mm). These findings will help to optimize the application of bubble streams and suspended particles in further field trials. We have also generated new information that will help to determine the extent to which the natural distribution of *C. intestinalis* is constrained by suspended or benthic sediment particles.

Acknowledgements

We gratefully acknowledge the help and administrative support of Sean McNeil at the Canadian Centre for Fisheries Innovation (CCFI), and Darrell Green at the Newfoundland Aquaculture Industry Association (NAIA). This research was conducted as part of a broader project “Experimental trials to optimize the application of eradication technologies to control the vase tunicate in Little Bay, Newfoundland”. The work was funded by a CCFI grant awarded to D. Deibel with additional support from C. Couturier, Executive Director of NAIA, C. McKenzie, and the 'NSERC Discovery Grant of D. Deibel. Thank you to the three reviewers for their recommendations and guidance in the final stages of this publication.

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Supplementary material

The following supplementary material is available for this article:

Appendix 1. Normalized size frequency distribution (% of total volume) of Annapolis Basin clay/mud, as defined by a Beckman Coulter, Multisizer III.

Appendix 2. Calibration linear regression of turbidity (NTU) vs the volume concentration (mg l^{-1}) of suspended particles.

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