Research Article

Effect of colonial tunicate presence on *Ciona intestinalis* recruitment within a mussel farming environment

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Abstract

Aquatic invasive species decrease yields and increase costs in aquaculture operations worldwide. Anecdotal evidence from Prince Edward Island (PEI, Canada) estuaries suggested that recruitment of the non-indigenous solitary tunicate *Ciona intestinalis* may be lower on aquaculture gear where colonial tunicates (*Botryllus schlosseri* and *Botrylloides violaceus*) are already present. We tested this interspecific competition hypothesis by comparing *C. intestinalis* recruitment on un-fouled settlement plates to those pre-settled with *Botryllus schlosseri* or *Botrylloides violaceus*. *C. intestinalis* occurred on all plates after 2 month, but it was much more abundant (~80% coverage) on unfouled plates than on pre-settled plates (<10% coverage). However, *C. intestinalis* showed higher individual growth on pre-settled plates than on unfouled plates. High reproductive potential for *C. intestinalis* appears to result in rapid recruitment to control plates, but this may be impeded on pre-settled plates due to competition for space, negative settlement cues produced by the colonial tunicates, allelopathy or overgrowth.

Key words: tunicates; ascidian; aquaculture; *Botryllus schlosseri*; *Botrylloides violaceus*

Introduction

Introductions of aquatic invasive species are becoming more and more common, especially those mediated by human actions (Carlton and Geller 1993; Ruiz et al. 1997; Cohen and Carlton 1998; Ruiz et al. 2000). Often, invasive species preferentially settle on artificial substrates (Tyrrell and Byers 2007) such as ropes, nets, cages and other gear used in aquaculture operations worldwide (Lambert 2007). The impacts, both on the native populations also living on these artificial substrates (K. Ellis, pers. comm.) and the aquaculture economy, can be detrimental (e.g. Carver et al. 2003; Lutz-Collins et al. 2009). In recent years, Prince Edward Island (PEI; Canada) estuaries have experienced the introduction of 4 tunicate species: *Ciona intestinalis* (L., 1758), *Styela clava* (Herdman, 1881), *Botryllus schlosseri* (Pallas, 1766) and *Botrylloides violaceus* Oka, 1927 (Locke et al. 2007). Tunicates (class Ascidiacea) are sessile filter feeders that settle on hard substrates (e.g. wharf pilings, buoys, aquaculture gear, hard-bodied biota) after a brief period of a few hours to several days as free-swimming larvae (Berrill 1950; Millar 1952; Dybern 1965; Yamaguchi 1975; Saito et al. 1981; Jackson 2005). The blue mussel (Mytilus edulis L., 1758) aquaculture industry in Atlantic Canada has been adversely affected by these non-indigenous tunicates, especially by the solitary species *C. intestinalis* (Carver et al. 2003, 2006; Daigle and Herbinger 2009). For example, since *C. intestinalis* consists of 95% water (calculated from data in Carver et al. 2006), it adds considerable weight to mussel

socks (>3 kg m^{-1}; Ramsay et al. 2008b), leading to increased labour and crop losses. Especially in recent years, research efforts have focused on preventing or mitigating *C. intestinalis* fouling on mussel socks (e.g. Bakker et al. 2011; Paetzold and Davidson 2011; Parent et al. 2011). While mussel growers on PEI consider colonial tunicates a nuisance species, research has not shown any adverse effects of *Botryllus schlosseri* and *Botrylloides violaceus* on cultured mussels (Arens et al. 2011; N. McNair, PEI Department of Fisheries, Aquaculture and Rural Development, pers. comm.), possibly because the colonial tunicate fouling levels on assessed mussel socks were low (maximum of ~0.36 kg m^{-1}; Arens et al. 2011). The values reported here for both Ramsay et al. (2008b) and Arens et al. (2011) are based on assessments of spring-socked mussel socks in October of the socking year; thus on similar socks, colonial tunicates add 10-times less weight to the sock than *C. intestinalis*.

In Cardigan River in eastern PEI, *Mytilus edulis* is grown throughout the estuary, and *C. intestinalis* has become a serious fouler on mussel leases. Since being discovered in this estuary in 2005, this tunicate has spread rapidly throughout the ecosystem and quickly established itself as the dominant tunicate species. Ramsay et al. (2008a) also reported this phenomenon in the adjacent Brudenell River. However, anecdotal reports from mussel growers and SCUBA divers working on the leases suggest that densities of *C. intestinalis* were lower on socks heavily fouled by the colonial ascidians *Botryllus schlosseri* and *Botrylloides violaceus*.

Colonial tunicates have 2 main advantages that could lead to their dominance over solitary species on hard substrates. First, colonial larvae are larger than those of solitary species, giving them survival and growth advantages (Marshall and Keough 2005; Marshall et al. 2006). Among the study species, the larvae of *Botrylloides violaceus* are approximately 3 times larger than those of *C. intestinalis* and *Botryllus schlosseri* (Bullard and Whitlatch 2004), and colonial juveniles grow up to 4 to 6 times larger than *C. intestinalis* within the first week post-settlement (Bullard and Whitlatch 2004). Second, colonial tunicates can reproduce asexually by budding and fragmenting/ regenerating and are therefore not dependent on the time limitations of gamete production. With asexual reproduction comes the possibility of continued mat-like growth over a

surface compared to the finite shape of a solitary tunicate, though solitary species can also often be found in large groups of individuals. Studies on competitive superiority of one species over another have had differing outcomes. *Botryllus schlosseri* was a dominant competitor whose presence deterred some species from settling on the same substrate in a fouling community in one study (Grosberg 1981). However, established *Botryllus schlosseri* colonies had no effect on larval settlement by other species in a different study (Bullard et al. 2004), leading those researchers to hypothesize that invertebrate larvae do not avoid settling near superior competitors (Bullard et al. 2004; see also Osman and Whitlatch 2004). Similarly, a previous study led some of the same researchers to negate species dominance (i.e. superior ability to settle) as a factor in settlement and instead explain the success of a settler based on species richness on a substrate (Stachowicz et al. 1999). In particular, Stachowicz et al. (1999) found that more diverse communities prevented settlement by invaders, likely by occupying all available substrates and quickly filling new space, e.g. through expansion of existing colonies, such that new invaders had little chance of recruitment.

Determining the competitive interactions among the invasive tunicate species on PEI is important to better understand recruitment hierarchies of these fouling organisms on aquaculture gear. Mitigation strategies could be more effective if designed to target a dominant tunicate species or allowing a lesser nuisance species such as a colonial tunicate to outcompete a more detrimental species like *C. intestinalis*. Therefore, the objective of this study was to assess the effect of the presence of *Botryllus schlosseri* or *Botrylloides violaceus* on the recruitment and growth of the solitary tunicate *C. intestinalis*. This assessment involved an in situ study comparing *C. intestinalis* % cover, length and abundance on bare plates (controls) and plates that were pre-settled with *Botryllus schlosseri* or *Botrylloides violaceus*.

Methods

Development of colonial tunicate colonies on experimental surfaces

The first phase of the project (pre-settlement; 29 June to 20 September 2007) was conducted in 2 bays located on the north shore of PEI (Canada): St. Peters Bay (46.432°N, 62.676°W) and Savage

Figure 1. The Savage Harbour and St. Peters Bay collection sites and the study site in Cardigan River in Prince Edward Island (PEI), Canada. QC: Quebec, NB: New Brunswick, NS: Nova Scotia, NL: Newfoundland.
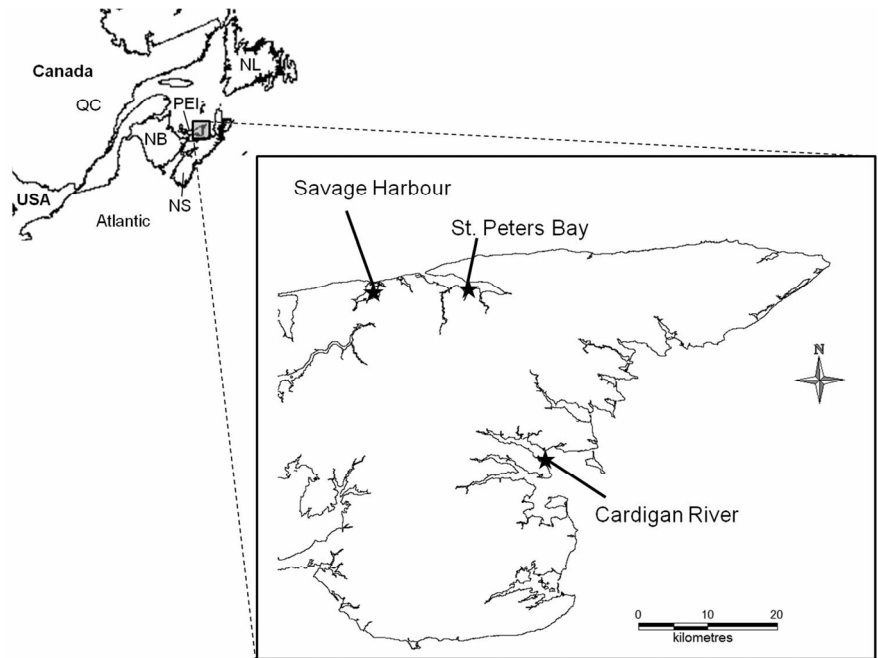
Harbour (46.416°N, 62.833°W) (Figure 1). These bays were selected as sites for pre-settling experimental substrates because both bays contained no solitary tunicates but high levels of the colonial tunicates, *Botryllus schlosseri* and *Botrylloides violaceus*, respectively (J. Hill and J.D.P. Davidson pers. obs.). The presence of high levels of the colonial tunicates allowed for rapid recruitment onto the collector plates, minimizing the possible recruitment of other unwanted epifauna and thus eliminating the need of any manipulation or "gardening" of the plates.

For this pre-settlement phase, 40 PVC collector plates measuring 10 × 10 cm were deployed in both St. Peters Bay (*Botryllus schlosseri*-dominated; Bs-plates hereafter) and Savage Harbour (*Botrylloides violaceus*-dominated; Bv-plates hereafter). PVC plates were chosen rather than more representative substrates such as mussel shells because (1) the plates present a uniformly sized, flat area ideal for image analysis using computer software and (2) this type of plate is an accepted and successful method for recruiting tunicates in PEI estuaries (e.g. Ramsay et al. 2009). The plates were deployed in sets of 5 collector plates, with plates spaced 30 cm apart. Plates were joined by rope leading through a central hole (1.3 cm diameter) and secured by knots on both sides of the plate. The collector plates were retrieved by

SCUBA divers 2 months after deployment and transported to the Cardigan River study site in ambient seawater, taking care to prevent interspecific mixing and damage to the tunicate colonies. Water temperatures at both sites ranged from 14 to 22°C (18 ± 2.5°C, mean ± SD) during the pre-settlement phase (Smith 2008).

For the second phase (*C. intestinalis* colonization; 20 September to 16 November 2007), pre-settled collector plates were transferred from St. Peters Bay and Savage Harbour to the study site in Cardigan River, an estuary located at the eastern end of PEI (Figure 1). Cardigan River (46.202°N, 62.516°W) was chosen because in addition to the presence of *C. intestinalis*, both *Botryllus schlosseri* and *Botrylloides violaceus* were present at the site (J. Hill and J.D.P. Davidson pers. obs.). The presence of the two colonial tunicates was a requirement for the acquisition of permits necessary for the transfer of the colonial-covered collector plates from St. Peters Bay and Savage Harbour.

The 27 collector plates with the highest % coverage (visual estimate) on the plate undersides from each bay were selected for use in the study. Prior to deployment in Cardigan River, plates were photographed (Sony Digicam, 7.2 megapixels) and reassembled in groups of 3 plates per collector plate array prior to

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deployment in Cardigan River. Plate orientation was maintained with the same plate side facing down during both deployments. The collector plate arrays (9 arrays per colonial species and 9 unfouled control arrays; i.e. a total of 81 plates in 27 arrays) were evenly divided among 3 adjacent mussel long lines in Cardigan River. Water temperature in Cardigan River steadily decreased from 16.6 to 5.9°C during the *C. intestinalis* recruitment phase (Smith 2008).

Laboratory analysis

At the end of the 2 mo deployment in Cardigan River, several collector sets had been lost, so only 18 Bs-plates (6 collector arrays) and 21 Bv-plates (7 collector arrays) were available for analysis. Digital photos of the plate bottoms were taken with plates lying down on a horizontal surface, and the pre- and post-recruitment phase images (September and November) were digitally scored using the software ImageJ (Version 1.38x, http://rsb.info. nih.gov/ij/) to obtain % cover estimates of *C. intestinalis* (measuring the base of the animal only, since only that part of the animals was occupying space), *Botryllus schlosseri*, *Botrylloides* violaceus and other (unidentified sessile) cover, which included mostly algae, some bryozoans and very few small mussels. In the case of stalked species (e.g. algae and bryozoans), only the base of the organism was measured as occupied space. The area around and including the central hole on each plate was omitted from analysis because the adjacent knots on the rope prevented settlement in this area. After photographing, the collector plates were subsampled to quantify *C. intestinalis* abundance and growth by scraping the tunicates off 1/16th of each plate. All subsampled *C. intestinalis* were laid out on a tray, counted, allowed to relax and extend, and measured using digital callipers (±0.01 mm). Care was taken to avoid touching the tunicates immediately prior to measuring, as the contraction would lead to erroneous length measurements.

Statistical analyses

Parametric assumptions were evaluated using normality plots and Levene's test (for homogeneity of variances among groups). When necessary, % cover data were arcsine square-root transformed to meet these assumptions. In case of minor violations of assumptions for parametric statistics, non-parametric tests were conducted to confirm the statistical results; since in all cases, non-parametric tests confirmed those results obtained with parametric tests, only the latter results are presented.

To determine whether pre-settlement conditions (i.e. colonial tunicate cover) were different at the start of the *C. intestinalis* recruitment phase in September, we used Student's t-test to compare total colonial tunicate (i.e. both colonial species combined) cover on Bs-plates and Bv-plates. We also used Student's t-test and Mann-Whitney U-test (to confirm parametric results) to compare *Botryllus schlosseri* cover on Bs-plates with *Botrylloides violaceus* cover on Bv-plates. Likewise, we compared coverage by each of the 2 colonial species on each of the pre-settled plates in November (i.e. *Botryllus schlosseri* cover on both Bs- and Bv-plates and *Botrylloides violaceus* cover on both Bs- and Bv-plates) using Student's t-test and Mann-Whitney U-test. Lastly, we determined significant increases or decreases of each tunicate species between September and November by comparing each species' cover in those months on Bs-plates or Bv-plates using Student's t-test. For *C. intestinalis*, we also compared September and November cover on bare plates.

The effect of plate depth on the collector sets (top, middle, bottom) was evaluated using 2-way ANOVA with Depth and Pre-settlement as fixed factors, but since depth did not affect any of the outcomes, the 3 plates per collector set were averaged, and a 1-way ANOVA with Pre-settlement as independent variable was used instead to increase statistical power. We determined the effect of pre-settlement (plate coverage in September) on *C. intestinalis* cover in November by comparing *C. intestinalis* cover in November among the 3 pre-settlement treatments (bare plates, Bs-plates and Bv-plates) using a 1-way ANOVA with Tukey's post-hoc test and Kruskal-Wallis test with multiple Mann-Whitney U-tests (with Bonferroni-adjusted alpha = 0.017 to correct for multiple tests) to confirm ANOVA results. Differences in length and abundance of *C. intestinalis* among the 3 pre-settlement groups (control, Bs-plates, Bv-plates) were assessed using 1-way ANOVA and Tukey's post-hoc test.

A significance level of $p < 0.05$ was used for all tests unless otherwise indicated. All statistical analyses were performed using SPSS® 15.0 for Windows.

Results

Changes in colonial tunicate assemblage over the study period

Prior to the start of the experiment, in September, the total (combined) cover of colonial tunicates was the same for both types of pre-settled plates (Bs-plates [mean ± SD]: 83 ± 20%, Bv-plates: 92 ± 5%; t_{11}=0.218, p>0.05) (Figure 2). Bs-plates from St. Peters Bay were predominantly covered by *Botryllus schlosseri* (75±18%), while Bv-plates from Savage Harbour were dominated by *Botrylloides violaceus* (86 ± 7%). All plates had similar amounts of unfouled (bare) space (Bs-plates: 7 ± 7%; Bv-plates: 8 ± 3%). On Bs-plates, about 9 ± 11% of space was covered by non-tunicate species (mussels, algae, barnacles), while on Bv plates, other species covered <0.01% of space. Statistically, there was no difference in the proportion of *Botryllus schlosseri* cover on Bs-plates and *Botrylloides violaceus* cover on Bv-plates (t_{11}= −1.519, p>0.05).

In November, *Botryllus schlosseri* cover did not differ significantly between Bs- or Bv-plates (t_{11}= −2.189, p>0.05). *Botrylloides violaceus* was significantly higher on Bv-plates than on Bs-plates (t_{11}=4.211, p<0.01). Comparing colonial tunicate cover on Bs-plates between September and November, *Botryllus schlosseri* declined significantly in cover (t_5=10.060, p<0.001), and *Botrylloides violaceus* increased significantly in the same period (t_5= −21.787, p<0.0001). On Bv-plates, however, *Botryllus schlosseri* cover did not change (t_6= −1.393, p>0.05), and *Botrylloides violaceus* decreased significantly (though not by much quantitatively) over the same period (t_6=2.531, p<0.05). Overall, colonial tunicates dominated the 2 pre-settled groups both in September and November (Figure 2).

Cover, growth and abundance of *C. intestinalis* on experimental plates

After two mo, *C. intestinalis* had recruited to all experimental plates (i.e. those pre-fouled by tunicates and bare controls) deployed in Cardigan River. Pre-settlement had a significant effect on *C. intestinalis* coverage in November, but depth did not (Pre-settlement, $F_{2,57}$=184.591, p<0.0001; Depth, $F_{2,57}$=0.017, p>0.05; Depth × Pre-settlement, $F_{4,57}$=0.303, p>0.05). *C. intestinalis* colonization by November was significantly
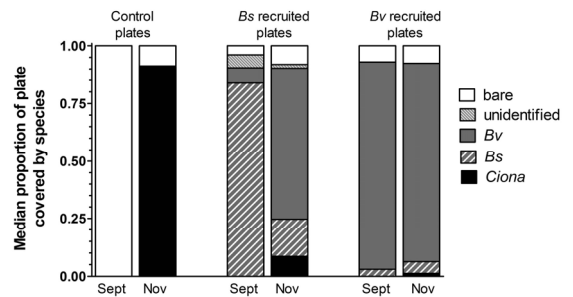

Figure 2. Cover of the solitary tunicate *Ciona intestinalis*, the two colonial tunicates *Botryllus schlosseri* (Bs) and *Botrylloides violaceus* (Bv), other fouling species (unidentified) and unfouled (bare) space on experimental PVC plates before (September) and after (November) a 2 mo long deployment at the study site, Cardigan River. Plates were pre-settled either in St. Peters Bay with *B. schlosseri* (Bs recruited plates) or in Savage Harbour with *B. violaceus* (Bv recruited plates). Medians instead of averages are shown since data could not be normalized because of the many zero-values in the control group.
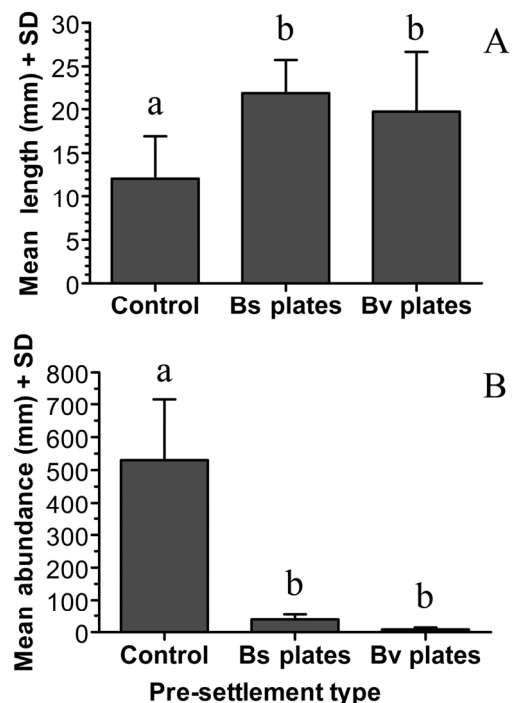

Figure 3. *Ciona intestinalis*. (A) Recruit length and (B) abundance on experimental PVC plates in November (after a 2 mo deployment) at the study site, Cardigan River. Plates were pre-settled in St. Peters Bay with *Botryllus schlosseri* (Bs-plates) or in Savage Harbour with *Botrylloides violaceus* (Bv-plates). Significant differences (1-way ANOVA, Tukey's post-hoc test, p < 0.05) between pre-settlement treatments are indicated by different letters.

higher on bare plates (79%) than on plates that had been pre-settled with colonial tunicates (1 to 9%) ($F_{2,19}$=126.668, p<0.0001; Tukey's: p<0.001) (Figure 2). *C. intestinalis* colonization on pre-fouled plates was much less (89 to 98%) than that on bare control plates (Figure 2). The species used to pre-settle the plates did not affect *C. intestinalis* colonization ($F_{2,19}$=126.6683, p<0.0001; Tukey's: p>0.05).

The length of *C. intestinalis* on control plates was significantly less (37 to 42%) than that measured on pre-settled plates ($F_{2,19}$=13.186, p<0.001; Tukey's, p<0.001 for Bs-plates and p<0.01 for Bv-plates) (Figure 3A). Length of *C. intestinalis* did not differ between Bs-plates and Bv-plates (Tukey's: p>0.05) (Figure 3A).

The presence of colonial tunicates on the plates significantly affected the abundance of *C. intestinalis* recruits ($F_{2,19}$=72.716, p<0.0001), with *C. intestinalis* abundance 14 and 72-fold higher on unfouled plates than on Bs- and Bv-plates, respectively (Tukey's: p<0.0001 for both comparisons) (Figure 3B). The abundance of *C. intestinalis* growing on Bs-plates did not significantly differ from that on Bv-plates (Tukey's p>0.05).

Discussion and conclusions

The results of our study confirm anecdotal observations by aquaculture workers and suggest that *C. intestinalis* recruitment is in some way inhibited by the presence of colonial tunicates occurring in the PEI region. After 2 mo of deployment, *C. intestinalis* was the dominant fouler on the bare plates in Cardigan River. In contrast, plates pre-settled with colonial ascidians had much lower levels of *C. intestinalis* recruitment. Possible reasons why *C. intestinalis* did not recruit well on plates pre-settled by colonial tunicates are: (1) negative settlement cues produced by the existing fouling community (Young and Chia 1981; Kingsford et al. 2002), (2) allelopathic chemical compounds from the colonial tunicates causing mortality in *C. intestinalis* recruits (e.g. tunic acidity) (Pisut and Pawlik 2002), or (3) overgrowth of *C. intestinalis* juveniles by expanding tunicate colonies (space competition) (Grosberg 1981; Kay and Keough 1981). Overgrowth of settlers seems to be the least likely reason for the observed decrease in *C. intestinalis* fouling on pre-fouled plates; while Grosberg (1981) found that *Botryllus schlosseri* and *Botrylloides violaceus*

were overgrowing many other fouling species, *C. intestinalis* was only overgrown in 1 out of 16 encounters with *Botryllus schlosseri* in his study. In addition, *C. intestinalis* on pre-fouled plates in our study were greater in length than on unfouled control plates, which may have been a response to the presence of colonial tunicates since increased length can provide a feeding advantage and prevent overgrowth (Stebbing 1973; Russ 1980). Alternatively, intraspecific competition for food and space may have prevented optimal growth of *C. intestinalis* on unfouled plates in the present study.

The identity of the pre-settling colonial tunicate did not affect the coverage by *C. intestinalis*, though due to the large decline of *Botryllus schlosseri* on Bs-plates, we cannot definitively conclude that the 2 colonial species were equally effective at reducing *C. intestinalis* colonization. *Botryllus schlosseri* cover on Bs-plates was drastically reduced by November, possibly due to regular cycles of regression during both asexual and sexual reproduction that have been reported for this species (Berrill 1935; Lauzon et al. 1992; Chadwick-Furman and Weissman 1995). Most of the lost *Botryllus schlosseri* cover was replaced by the colonial *Botrylloides violaceus* rather than *C. intestinalis* (Figure 2).

Since this paper represents results from a preliminary study with a simple design, one of the limitations was that plates were only analysed for biofouling coverage before and after the 2 months deployment in Cardigan River, therefore any shift in the fouling community on the plates within the 2 months was not recorded. It is therefore difficult to draw conclusions about the preference of *C. intestinalis* to settle surfaces covered by colonial tunicates. For example, the shift in tunicate coverage on pre-settled plates from mostly colonial to a mix of colonial tunicates and up to 9% coverage by *C. intestinalis* could have several explanations: *C. intestinalis* might have settled on bare spots on the plate and potentially outcompeted colonials in the surrounding area, creating more available space for itself. Likewise, space might become available through damage to the colonies by predators and disturbers such as fish, snails and crabs (Davis 1988; Osman and Whitlatch 1995, 2004) or natural regression of colonial tunicates (Berrill 1935; Chadwick-Furman a Weissman 1995). Merely based on the photographs, the original orientation of the plates (and thus inference on whether *C. intestinalis*

preferred to settle on bare spots only) was not possible since the fouling community on the plates had shifted too much between September and November. Further experiments are necessary to evaluate these theories.

Ciona intestinalis with its relatively small larvae (Bullard ad Whitlatch 2004) was the dominant settler on unfouled plates in this study despite reports that larger tunicate larvae such as *Botrylloides violaceus* have settlement advantages that include producing larger settlers, a faster budding rate and a larger, more dominant colony (Marshall and Keough 2003a,b; Marshall et al. 2006). Higher propagule abundance may have been the reason for the success of the smaller larvae. Estimates of *C. intestinalis* fecundity range from 12,000 over a 3 months spawning period in Nova Scotia waters (Carver et al. 2003) to 100,000 eggs over 3 to 6 months in year-round spawning populations in Japan (Yamaguchi 1975), while *Botryllus schlosseri* produces up to 50 eggs per zooid in a 2.5 months period (Chadwick-Furman and Weissman 1995). *C. intestinalis* not only produces more gametes and likely more larvae than *Botrylloides violaceus* and *Botryllus schlosseri*, but it also starts reproduction at lower water temperatures (Brunetti 1974; Carver et al. 2003; Epelbaum et al. 2009; Ramsay et al. 2009) and thus can cover bare substrates before the 2 botryllid species can spread to these surfaces or before their larvae can settle. Another reason for *C. intestinalis'* predominance on control plates may have been avoidance of these plates by colonial larvae. Both *Botryllus schlosseri* (Grosberg and Quinn 1986) and *Botrylloides violaceus* (Westerman et al. 2009) prefer to settle near con-specifics and would thus have preferred the pre-settled plates to the bare plates in our study.

While colonial tunicates are considered nuisance species in bays without solitary tunicates according to reports by PEI mussel growers, and are mitigated by using e.g. high- and low-pressure seawater spray (Arens et al. 2011), research from our laboratory (Arens et al. 2011) and provincial researchers (N. McNair, pers. comm.) has not shown any negative impact of colonial tunicates on mussel productivity, nor have we observed instances of mussels being smothered because of heavy colonial tunicate fouling on mussel socks (authors' pers. obs.). The present study presents a preliminary consideration of tolerating rather than mitigating the presence of colonial tunicate species in areas that are already invaded by colonials and

C. intestinalis, in order to reduce the amount of *C. intestinalis* biomass on mussel aquaculture gear. However, before such a strategy is implemented, future studies need to determine (1) whether and how *C. intestinalis* recruitment is inhibited by the presence of colonial tunicates, (2) whether such inhibition is effective in the long term (>6 months) and on different substrates such as mussel socks, and (3) if there are any environmental or mussel production side-effects.

Acknowledgements

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