

Spatio-temporal dynamics of ascidian larval recruitment and colony abundance in a non-indigenous Newfoundland population

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ABSTRACT: Spread of a non-indigenous ascidian (NIA), *Botryllus schlosseri* (Tunicata: Ascidiacea), has been detected since 2006 in coastal waters of Newfoundland. This species has been of economic concern because NIA can be a costly nuisance for bivalve aquaculture. The presence of this temperate-adapted species in Newfoundland represents an extension of its global range into subarctic waters. In 2010 and 2011, we investigated the population dynamics of *B. schlosseri* in Arnold's Cove, Placentia Bay, Newfoundland and Labrador, Canada. Experiments testing the influence of season, depth, and substrate type on larval recruitment rates were conducted within the harbour over a period of 20 mo. Seasonal cycle of colony (adult) abundance was determined from video surveys of a belt transect of the wharf. The seasonal window of recruitment was from August to mid-October (ca. 2.5 mo), constrained to temperatures $\geq 13^{\circ}\text{C}$. Peak recruitment was observed coincident with maximum seasonal temperature in September. Recruitment was greatest at 1 m depth on PVC plates. Colonies were present year-round on wharf structures and exhibited seasonal fluctuations in percent cover, from $<1\%$ in May to ca. 3% in October. Seasonal increase in colony abundance was constrained to temperatures $\geq 5^{\circ}\text{C}$. These findings provide a population-level understanding of how a temperate-adapted, non-indigenous species is established and maintained in a subarctic environment. Our results suggest that 1 sampling site may adequately represent the spatial scale of $\leq 10\text{s}$ of metres within a marina, and NIA monitoring could target specific seasons, depths, and substrate types for optimal species detection.

KEY WORDS: *Botryllus schlosseri* · Tunicate · Phenology · Depth-dependent recruitment · Substrate type · Aquatic invasive species · Newfoundland

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INTRODUCTION

Aquatic invasive species (AIS) are a global problem and a priority issue due to their negative ecological and economic impacts (Mack et al. 2000, Pimentel et al. 2005). The potential harm caused by AIS has stimulated industry, management, and policymakers to monitor for new invaders and to mitigate their impact. The rate of AIS introductions could be increasing over time due to global envi-

ronmental changes (Stachowicz et al. 2002). Arctic and subarctic waters are of particular concern because they are increasingly vulnerable to biological invasions due to (1) climatic changes that create favourable conditions (e.g. rising seawater temperatures open a new niche susceptible to invasion; Stachowicz et al. 2002), and (2) a higher volume of maritime traffic as the arctic ice sheet thins and the ice-free season becomes longer (Lambert et al. 2010).

In eastern Canada, 6 non-indigenous ascidian (NIA) species (*Ascidia aspersa* Müller, 1776, *Botrylloides violaceus* Oka, 1927, *Botryllus schlosseri* [Pallas, 1766], *Didemnum vexillum* Kott, 2002, *Diplosoma listerianum* [Milne-Edwards, 1841], and *Styela clava* Herdman, 1881) and 1 cryptogenic ascidian (*Ciona intestinalis* [Linnaeus, 1767], formerly *C. intestinalis* type B) threaten the sustainability of bivalve aquaculture because extra handling is required for bivalve products to be marketable (Carver et al. 2006a,b, Clarke & Therriault 2007, Locke et al. 2007, Sephton et al. 2011, Moore et al. 2014). *Botryllus schlosseri*, *Botrylloides violaceus*, and *C. intestinalis* have been reported in the coastal waters of insular Newfoundland (Sargent et al. 2013, Deibel et al. 2014). To date, no NIAs have been reported from Labrador and the Canadian Arctic (Ma et al. 2017). However, 1 cryptogenic ascidian, *Heterostigma* sp., was detected in arctic waters (Goldsmith et al. 2014). In Newfoundland, NIAs have been found in harbours but not yet in any aquaculture sites.

Fouling ascidians can have long-term ecological impacts on benthic communities. For example, they can be competitors for space (Claar et al. 2011) and can rapidly colonise economically important areas, such as bivalve aquaculture sites (Carman et al. 2010). In particular, the temperate species *Botryllus schlosseri* can be a dominant competitor for space by exhibiting high rates of recruitment (Anger 1978, Grosberg 1981). *Botryllus schlosseri*, which is native to the Mediterranean Sea (Carver et al. 2006a, Lejeune et al. 2011), is vertically distributed from the upper subtidal zone to a depth of 75 m or more (Rinkevich et al. 1998). Colonies of this species encrust a wide variety of natural and artificial substrates, such as bivalves, eelgrass, kelp, other ascidian species, stones, ship hulls, and wharf structures (Visscher 1928, Berrill 1950, Milkman 1967, Tyrrell & Byers 2007, Carman et al. 2009, 2016, Ben-Shlomo et al. 2010). In its native range, colonies of *B. schlosseri* are present year-round, but their abundance fluctuates seasonally (Rinkevich et al. 1998).

In Newfoundland, *B. schlosseri* was first reported in 1945 (over 100 yr after the earliest mention of this species in eastern North America, in Massachusetts [USA] in the 1830s), reported for the second time in 1975, and, via the federal monitoring programme, was reported for the third time in 2006 (Couthouy 1838, United States Navy 1951, Hooper 1975, Callahan et al. 2010). At present, *B. schlosseri* is widely distributed in Placentia Bay, Newfoundland, with lesser concentrations in 2 Conception Bay harbours and localised colonies in harbours on the south and

southeast coasts (Deibel et al. 2014, McKenzie et al. 2016). The distribution of NIA species (including *B. schlosseri*) in Newfoundland is currently known from coastal harbours and, fortunately, these species are not yet a nuisance for bivalve aquaculture operations (Deibel et al. 2014, McKenzie et al. 2016). The presence of this temperate-adapted species in Newfoundland represents its northernmost range in eastern North America and an extension of its global range into subarctic waters. This suggests that this temperate species may have evolved genetic, physiological, or phenological changes to become established in subarctic waters. Ecological information needed to guide monitoring (e.g. optimal depth and substrate type of recruitment plates) and management (seasonal timing of mitigation) is critically missing for this species as well as other NIAs invading subarctic waters. Therefore, we investigated the spatiotemporal patterns of larval recruitment and colony (adult) abundance within a population of *B. schlosseri* that has invaded a harbour in Newfoundland. We tested the hypotheses that (1) seawater temperatures of $\geq 13^{\circ}\text{C}$, which is the temperature threshold for sexual reproduction (see Westerman 2007), constrain the seasonal window of *B. schlosseri* recruitment; (2) this species exhibits differential recruitment rates among depths (1.0, 2.5, and 4.0 m below the water surface) and among substrate types (aluminium, polyvinyl chloride [PVC], wood); and (3) temperatures $\geq 6^{\circ}\text{C}$, which is the threshold for colony growth (see Epelbaum et al. 2009), constrain the seasonal window of population growth in terms of percent cover of colonies.

MATERIALS AND METHODS

Study site

The study was conducted in the harbour of Arnold's Cove, Placentia Bay, Newfoundland and Labrador, Canada ($47^{\circ} 45' \text{N}$, $54^{\circ} 00' \text{W}$). The harbour, with an area of ca. 1.5 km^2 , is near the head of Placentia Bay and is open to seas only from a southerly direction. Local ice formation during winter is rare (Catto et al. 1999). Tides in Placentia Bay are semi-diurnal, with a maximum spring tidal range of ca. 2.1 m and a neap tidal range of ca. 1.5 m. The harbour provides moorage primarily for local fishing and recreational vessels 5–15 m long. However, at times, some larger inter-provincial and international vessels are present. The focal population of *Botryllus schlosseri* was located on the wooden pilings of the perma-

ment, fixed, government wharf and on the sides, bottoms, and plastic drums of 3 attached floating docks.

Determination of recruitment rate

Larval recruitment rates were determined over a total time span of 20 mo, from 17 March 2010 to 15 November 2011. Recruitment plates (area: 10 × 10 cm) were attached to mooring lines hung from heavy chains used to anchor the 3 floating docks (Sites 1, 2, and 3) to the fixed wharf. Site 1 was associated with the floating dock that was furthest from shore, Site 2 with the middle dock, and Site 3 with the dock that was closest to shore. All plates were oriented horizontally to maximise species detection on the bottom surface of the plate because ascidian larvae exhibit negatively geotactic swimming behaviour in response to dark conditions and prior to settlement (Schmidt 1982, Collins 2013). The depths of the plates were registered from the water surface and did not vary with tidal stage. Bricks were attached to the bottom of the lines to maintain them in a vertical position and to minimise the chance of entanglement with adjacent lines (entanglement was not observed). The 3 sites (chosen to provide enough space for replicate plates) were shaded from direct sunlight during most of the day and were 15–20 m apart. Bottom depth was ca. 6 m.

First recruitment experiment

The first experiment lasted from 17 March to 8 December 2010, and was designed to determine the seasonal onset of recruitment and the depth-dependent rate of recruitment on plates made of 3 different substrates: aluminium, PVC, and wood. Aluminium plates were made by wrapping wooden plates in heavy gauge aluminium foil. PVC plates were 0.6 cm thick, smooth, and dark grey in colour. Wooden plates were made of untreated pine. A hole was drilled through the centre of each plate for attachment to mooring lines.

Before deployment, all plates were immersed for 2 wk in a large laboratory tank that contained pre-filtered (30 µm mesh) ambient seawater. This process resulted in the development of a microbial biofilm on the plates, which may facilitate the settlement of ascidian larvae (Keough & Raimondi 1996, Howes et al. 2007, Rius et al. 2010). Plates were transported to the study site in 40 l insulated containers containing 30 µm pre-filtered, ambient seawater.

The mooring 'array' at each of the 3 sites consisted of 6 polypropylene lines, each 20 cm away from its nearest neighbour. Plates were attached at 3 different depths on each line (i.e. 1.0, 2.5, and 4.0 m from the water surface). Within each depth, a plate was randomly assigned to each of the 6 mooring lines in a process that resulted in an equal number of duplicated substrate types per depth. The lines were deployed by SCUBA divers.

Exposed plates were retrieved and new plates deployed 11 times during the first experiment in 2010 (Table S1 in the Supplement at www.int-res.com/articles/suppl/m585p099_supp.pdf). The frequency of sampling was increased in September and October to improve temporal resolution during the recruitment period. The mean exposure time was 28 d deployment⁻¹. A sampling array of 18 plates was moored at each of the 3 sites for a total of 54 plates deployment⁻¹. A set of supplemental (i.e. not used in the recruitment experiments) PVC plates (n = 15) was deployed adjacent to Site 1 on 20 July 2010 to improve the temporal resolution in determining the seasonal onset of recruitment.

At the end of each deployment period, the mooring lines were retrieved by SCUBA divers. The plates were removed from the lines, and the bottom surfaces photographed in air with a 10 megapixel, Canon PowerShot G11 digital camera. The plates were then placed individually, with bottom surface upwards, in 0.7 l plastic containers filled with ambient seawater. All 54 containers were transported to the laboratory at Logy Bay in 40 l insulated containers. To avoid introducing *B. schlosseri* to Logy Bay, all water originating from Arnold's Cove was chlorinated at a minimum of 5 ppm available chlorine for at least 12 h before disposal. All field equipment was air-dried for at least 1 d before washing in fresh water.

In the laboratory, the bottom surfaces of all plates were visually inspected and photographed with the same digital camera as above, mounted on a tripod. New recruits (i.e. oozoids and juvenile colonies) of *B. schlosseri* were identified and counted using ImageJ (version 1.44). Throughout the experiment until September, a subset of plates was examined with a stereomicroscope to ensure that the photographic method did not underestimate recruitment. The relatively low fouling of plates and the resolution and quality of the images was high enough to appropriately determine recruitment. The smallest oozoids detected by the photographic method were 0.6 mm long, and the juvenile colonies ranged in diameter from 1.2–1.7 mm. Due to our lower detection limit of 0.6 mm, we did not determine larval settlement or

metamorphosis of larvae to oozoids. The length and width of each plate was determined from the images using ImageJ, and recruitment rate was converted to number of recruits $\text{m}^{-2} \text{d}^{-1}$.

Second recruitment experiment

The second experiment lasted from 8 December 2010 to 15 December 2011 and was designed to determine inter-annual variability in the time of onset of recruitment (by combining with data from the first recruitment experiment) and the depth-dependent rate of recruitment on PVC plates. Pre-deployment preparation of the PVC plates for the second experiment was identical to that for the first experiment (see above).

The mooring array at each site consisted of 2 polypropylene lines, 20 cm apart. PVC plates were attached to each line at the same depths as above for the first experiment. No randomisation was necessary as for the first experiment, as only 1 substrate type was being used.

Exposed plates were retrieved and new plates deployed 7 times during the second experiment in 2011 (Table S1). The mean exposure time was 49 d deployment⁻¹; however, exposure time was shortened to 29 d deployment⁻¹ in August and September for the determination of the seasonal onset of recruitment. A sampling array of 6 plates was moored at each of the 3 sites for a total of 18 plates deployment⁻¹. Post-retrieval plate processing, photographs, and image analysis were the same as for the first experiment (see above).

Determination of colony abundance

Twenty-eight video surveys were made from 18 March 2010 to 11 May 2011, ca. every 2 wk along a subtidal belt transect (Lam et al. 2006) of the wharf. The transect included 141 wharf pilings (all with flat surfaces of the same width) along a total length of ca. 50 m. SCUBA divers used a high-definition video camera recorder (Sony HVR-V1U; 30 frames s^{-1}) to film the pilings at a fixed distance of ca. 30 cm from the lens. An underwater light system (Amphibico Discovery G3 arc light) provided uniform illumination. Two green laser pointers (wavelength = 532 nm; BALP-LG05-B150) were affixed 30.0 cm apart on the underwater housing to provide a measurement scale in the video frames. The divers made up to 3 passes along the transect on each sampling date. The first

pass covered the intertidal zone. The second pass covered the subtidal zone. If time permitted, a third pass was made just below the second pass. The overall swimming velocity was ca. 5.6 cm s^{-1} .

Colonies of *B. schlosseri* were conspicuously present at subtidal depths. Therefore, only the video sequences of the second pass made at a subtidal depth were analysed. Ten of the total of 28 video surveys were analysed after screening for video quality. For example, sampling dates with highly turbid water or high macro-algal cover on pilings were excluded from analysis.

The number of random pilings (of the 141 pilings) required for image analysis was determined by calculating the coefficient of variation (CV) of mean percent cover of colonies until the change in CV was $\leq 5\%$ for at least 4 consecutive pilings. Consequently, ≥ 45 random pilings per sampling date were analysed for all 10 surveys.

A still image of each of the randomly selected pilings was extracted from the videos using Pinnacle Studio 12 software and converted into the JPEG file format. Image size and quality were not modified. The mean width of the pilings determined from the video frames was (mean \pm SD) $15.5 \pm 1.0 \text{ cm}$ ($n = 15$). For convenience, this piling width of 15.5 cm was used as the primary scale in the images, with the laser pointers as backup. After the scale was determined for each image, a virtual quadrat equivalent to $15.5 \times 23.3 \text{ cm}$ in the real world was superimposed on each piling using ImageJ. The left and right edges of the virtual quadrat were determined by the vertical edges of the piling, and the top and bottom edges were haphazardly determined by generating 2 horizontal lines spaced 23.3 cm apart within the image frame and positioned by default by the imaging software. Colonies were identified, the number of colonies counted, and the surface area per colony measured within the virtual quadrat. The surface area per colony was determined by hand-tracing the perimeter of the colony on a high-resolution graphics tablet (Wacom). The smallest colony that was positively identified using our technique was 0.4 cm^2 .

Environmental data

Seawater temperature, salinity, *in situ* fluorescence (later converted to total chlorophyll *a*), and turbidity were monitored with a Yellow Springs Instruments (YSI) Multiparameter Sonde (model 6600 V2) with antifouling kit. The sonde was moored 1 m below the water surface at Site 2 from 18 March 2010 to

15 November 2011. Readings were taken by the sonde every 6 h and downloaded monthly using EcoWatch (version 3.18) software. In addition, a YSI Handheld Multiparameter Probe was used ca. every 2 wk to determine the vertical profiles of temperature and salinity at Sites 1, 2, and 3 (adjacent to the recruitment plates) from the surface to 5 m depth at 1 m intervals.

Statistical analyses

All statistical analyses were done with SPSS version 19. Mean values are reported ± 1 SD. ANOVAs were conducted using generalised linear models (GLM) and type III sums of squares.

Seawater temperature, salinity, chlorophyll *a* (chl *a*) concentration, and turbidity data were smoothed by calculating daily mean values after setting negative chl *a* concentration and turbidity values to 0. Unreliable chl *a* concentration data (8 July to 11 August 2010), caused by algal growth on the *in situ* fluorescence probe, were removed prior to analysis. Environmental data were missing from 9–15 December 2010, due to maintenance of the sonde. An ANOVA (GLM with the canonical identity link function) of the transformed environmental data was used to test for variability in mean values between years and among months nested within year.

Recruitment data were transformed to natural logarithms and analysed with a 2-component conditional model. Analysis 1 consisted of data from the 2010 season, which were tested for variability in recruitment rate among sampling dates (time), sites, depths, and substrate types. Analysis 2 consisted of only PVC data from the 2010 and 2011 seasons, which were tested for variability in recruitment rate between years, among months (nested within year), among sites, and among depths. Analyses 1 and 2 were balanced factorial designs with no missing data. The first component of the conditional model considered presence and absence data and the second component considered zero-truncated data. The ANOVAs for the first component and the second component were conducted with a binomial logit GLM and by a log-normal GLM (with the log link function), respectively. ANOVAs with significant interaction terms were partitioned to interpret main effects.

The belt transect data were partitioned post hoc into 3 zones to test for spatial variability in colony abundance within the 50 m transect length. Zone 1 was relatively exposed, Zone 2 was intermediate be-

tween the exposed and sheltered zones, and Zone 3 was relatively sheltered. Each zone was composed of 47 pilings. Percent cover was transformed to natural logarithms and analysed with a 2-component conditional model (as above for the recruitment data), which tested for variability among sampling dates (time) and zones.

RESULTS

Physical environment

Late August to early September was the warmest period, with daily mean seawater temperatures $\geq 16^\circ\text{C}$, whereas late December to mid-May was the coldest period, with daily mean seawater temperatures $\leq 5^\circ\text{C}$ (Fig. 1A). The mean salinity was 31.2 ± 0.8 at the study site and ranged from a minimum of 25.1 (see description of Hurricane Igor below) to a maximum of 32.4 (Fig. 1B). Chl *a* concentration was lowest from December to June (Fig. 1C), when the mean was $0.8 \pm 1.3 \mu\text{g l}^{-1}$. Mean concentration was $1.5 \pm 3.8 \mu\text{g l}^{-1}$ from July to November. The spring bloom of phytoplankton centered on April, with a monthly mean of $1.1 \pm 1.0 \mu\text{g l}^{-1}$ in 2010 and $2.8 \pm 2.0 \mu\text{g l}^{-1}$ in 2011 (Fig. 1C). The autumn bloom of phytoplankton (from July to October) peaked in September, with a monthly mean of $2.5 \pm 4.4 \mu\text{g l}^{-1}$ in 2010 and $2.8 \pm 5.3 \mu\text{g l}^{-1}$ in 2011 (Fig. 1C).

Increased boat traffic and dockside baitfish discards during the lobster season may have contributed to higher levels of turbidity in the months of May and June (Fig. 1D). In July 2011, turbidity levels peaked around the same time that a nearby pier was under reconstruction. The source(s) of elevated turbidity (mean 3.1 ± 4.9 NTU) in October and November 2011 is unknown; however, there was increased precipitation in October and perhaps increased terrestrial runoff into the harbour.

The eye of Hurricane Igor passed almost directly over the study area on 21 September 2010 (Pasch & Kimberlain 2011). Seawater temperature rapidly declined to 7.7°C 1 wk prior to the hurricane (Fig. 1A), and salinity gradually decreased to a low of 25.1 for several days post-hurricane (Fig. 1B). Turbidity was 0 NTU before and after the hurricane (Fig. 1D); however, the daily mean turbidity attained a maximum of 8.0 ± 11.7 NTU on the day the hurricane passed over the harbour.

For the period of this study, the harbour exhibited significant inter-annual and seasonal variability in seawater temperature, salinity, chl *a* concentration,

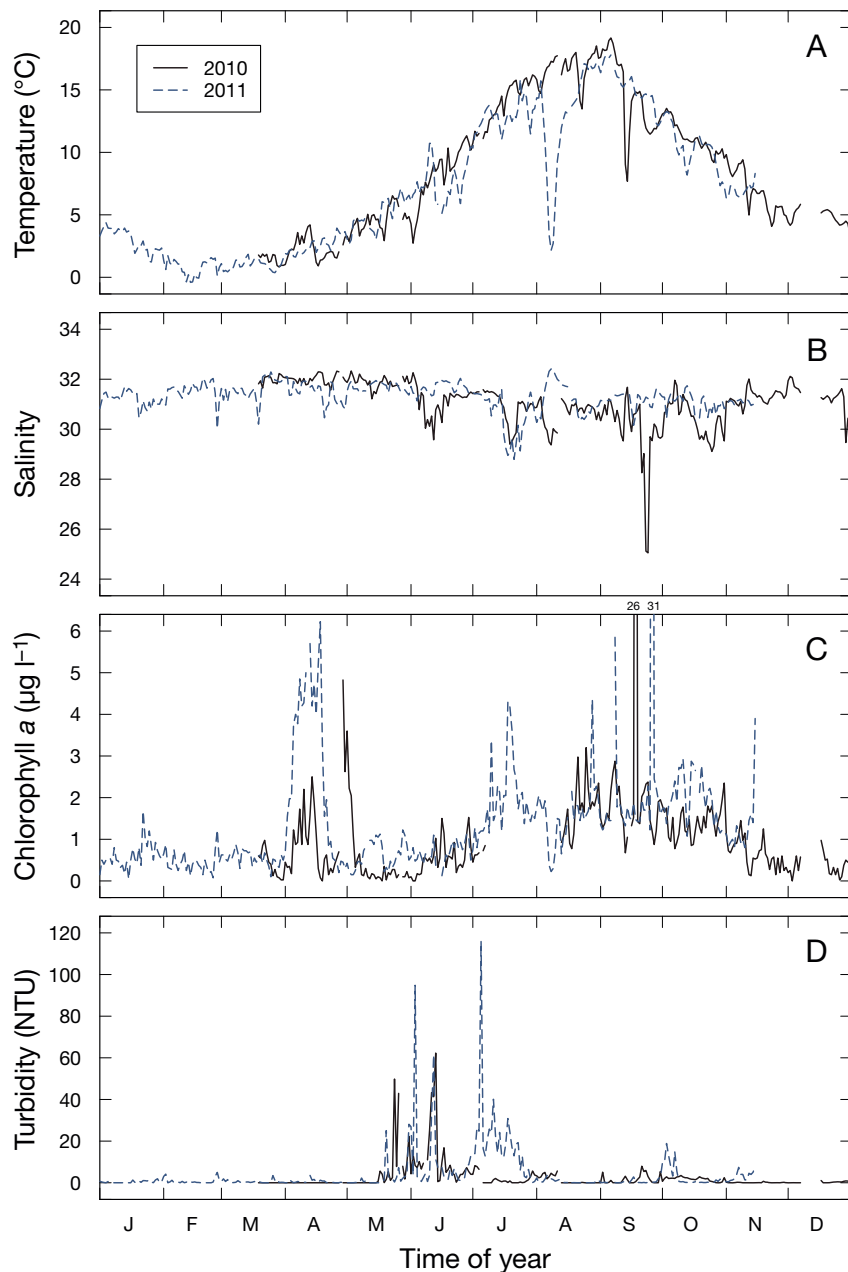


Fig. 1. Environmental data recorded by the sonde at 1 m below the water surface in Arnold's Cove, Placentia Bay, Newfoundland and Labrador, Canada. Daily mean (A) seawater temperature, (B) salinity, (C) chlorophyll *a* concentration, and (D) turbidity

and turbidity, with the seasonal scale predominant (Table S2 in the Supplement). Temperature and salinity values from the vertical profiles followed the seasonal cycle of data collected with the sonde (data not shown). According to the data from the vertical profiles, variability in temperature and salinity was significant among months and depths (range: 0–5 m), but not among sites (Sites 1, 2, and 3; Table S3).

Timing of recruitment

Recruitment of *Botryllus schlosseri* was a single conspicuous pulse, which occurred from early August to mid-October (ca. 2.5 mo; recruitment plotted on the median date of each deployment period in Fig. 2), constrained to seawater temperatures $\geq 13^{\circ}\text{C}$. The seasonal onset of recruitment was observed on 4 August 2010 on a single supplemental PVC plate (i.e. a plate not used in the first recruitment experiment) and on 1 August 2011 on several experimental PVC plates (i.e. plates that were used in the second recruitment experiment). The daily mean seawater temperature on the day of initial observation of recruitment was $15.6 \pm 0.4^{\circ}\text{C}$ in 2010 and $14.4 \pm 0.8^{\circ}\text{C}$ in 2011, which is above the 13°C threshold temperature for reproduction (Westerman 2007).

The seasonal peak recruitment periods were observed in September in 2010 and 2011, coincident with peak seasonal temperatures and chl *a* concentration (Figs. 1 & 2). Averaged over all depths, the maximum mean daily recruitment rate on PVC plates during peak recruitment was $20.0 \pm 19.0 \text{ m}^{-2} \text{ d}^{-1}$ deployment $^{-1}$ from 2 September to 1 October 2010 and $31.5 \pm 17.5 \text{ m}^{-2} \text{ d}^{-1}$ deployment $^{-1}$ from 30 August to 28 September 2011 (Fig. 2). The mean temperatures during the peak recruitment period were 14.6°C in 2010 and 15.5°C in 2011. The daily mean chl *a* concentration during the peak recruitment period was $2.4 \mu\text{g l}^{-1}$ in 2010 and $2.8 \mu\text{g l}^{-1}$ in 2011. Recruitment ended in late October 2010. The daily mean temperature during the period of final recruitment was $12.5 \pm 0.8^{\circ}\text{C}$ (Fig. 2). We were unable to determine the end of the recruitment period in 2011, as recruitment was still observed during the last deployment period of the study (plates retrieved in mid-November).

Recruitment patterns

Data from all 3 sites (1, 2, and 3) were combined to examine differences between years, among depths,

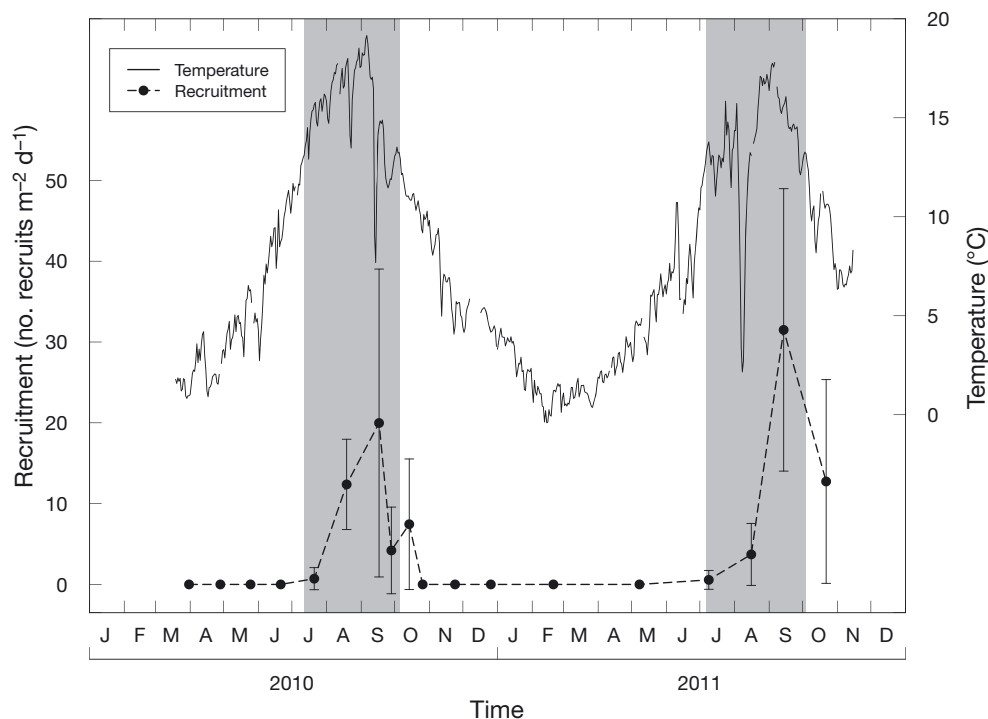


Fig. 2. Seasonal patterns of mean *Botryllus schlosseri* recruitment on PVC recruitment plates (depth- and site-averaged values) and daily mean seawater temperature. Recruitment is plotted on the median date of each deployment period. Zero values are included in the calculation of mean values. Shaded periods represent the seasonal window between the initial and final days when temperature was $\geq 13^{\circ}\text{C}$. Bars represent ± 1 SD, $n = 18$ observations per data point

and among substrate types since differences in rates among sites were not significantly different in either 2010 or 2011 (Tables S4 & S5).

Mean daily recruitment rates were greater at 1.0 and 2.5 m than 4.0 m depth and greater on PVC than on aluminium and wood substrates (Fig. 3). Maximum recruitment rates were often observed at 1.0 m depth (e.g. during peak recruitment in September) and sometimes at 2.5 m depth (Fig. 3). At any given sampling time and substrate type, mean recruitment rates at 4.0 m were the lowest (or tied for lowest) compared to shallower depths (Fig. 3). Partitioned ANOVA indicated that the seasonal cycle in mean daily recruitment rate was dependent upon depth and substrate type, with a significant effect of time (i.e. season) at 1.0 m depth, but not at 2.5 and 4.0 m depth, and on aluminium and PVC, but not on wood, substrates (Table S6). Additionally, inter-annual variability in recruitment was dependent upon depth, with a significant effect of year at 2.5 and 4.0 m, but not at 1.0 m depth (Table S7).

Colony abundance

Botryllus schlosseri on wharf pilings exhibited variability in the presence of colonies (percent of pilings colonised) seasonally (i.e. among months) and among zones (i.e. segments of the transect of wharf pilings) but not in the density and percent cover of colonies (Table S8). Colonies were present year-round and

common in the subtidal zone. No colonies were observed on the seafloor (primarily mud and silt).

Colonies of *B. schlosseri* on wharf pilings ranged from 0.4–32 cm² with a mean of 6 cm². The largest colonies (>16 cm²) were typically found in September and October. The seasonal onset of increase in mean colony size occurred between April and May in 2010 (from 4.8 to 8.2 cm²), and between February and May in 2011 (from 4.7 to 6.3 cm²). Decreases in colony size occurred between December 2010 and February 2011 (from 6.7 to 4.7 cm²). Mean colony size <6 cm² was observed from March to April 2010 and in February 2011 when temperatures were $<5^{\circ}\text{C}$. Mean colony size was ≥ 6 cm² from May to December 2010 and in May 2011 when temperatures were $\geq 5^{\circ}\text{C}$.

Increases in *B. schlosseri* cover on wharf pilings occurred in September and October 2010, and decreases occurred from December 2010 through May 2011 (Figs. 4 & 5). Due to missing data, exact timing of increase is not known in 2010, only that cover was slightly higher in September than in May. We have no data on percent cover after May 2011. Increase and decrease in colony abundance were constrained to seawater temperatures $>15^{\circ}\text{C}$ (temperature at the halfway point between May and September 2010) and $<10^{\circ}\text{C}$ (temperature in late October 2010), respectively (Fig. 5). Maximum mean percent cover was observed in October, when the maximum percent of pilings colonised was 69%, mean peak density was 47.5 ± 48.7 colonies m⁻², and mean peak

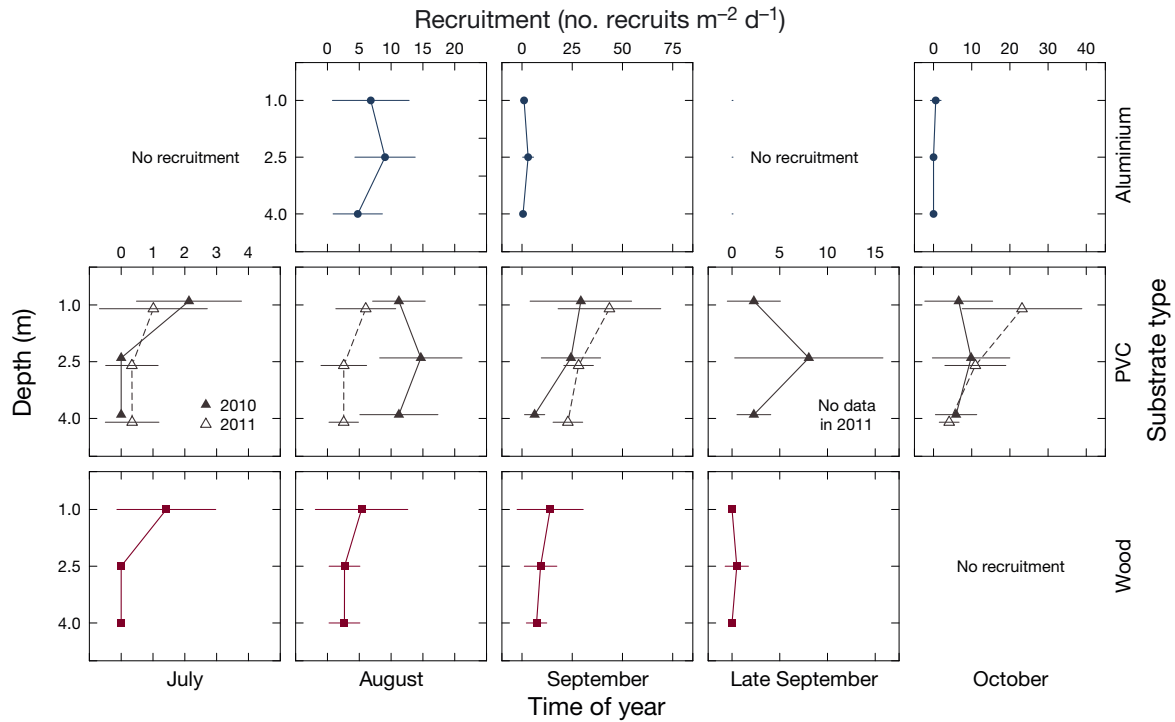


Fig. 3. Vertical recruitment patterns of *Botryllus schlosseri* from July to October in 2010 (all 3 substrate types), and 2011 (PVC only). Mean recruitment on aluminium, PVC, and wood substrates. Bars represent ± 1 SD, and $n = 6$ observations per data point

cover was $2.8 \pm 2.9\%$ (Figs. 4 & 5). The maximum recorded density and cover on a single piling were $250 \text{ colonies m}^{-2}$ and 20% , respectively, which occurred in mid-September 2010. A sharp decrease in abundance was observed in November 2010. The lowest percent of pilings colonised was 16.3% , the

lowest mean density was $6.8 \pm 19.2 \text{ colonies m}^{-2}$, and the lowest mean cover was $0.6 \pm 1.9\%$, in May 2010. Over the entire study period, the mean percent of pilings colonised was $44.8 \pm 17.3\%$ per survey date ($n = 10$), mean density was $27.1 \pm 13.5 \text{ colonies m}^{-2}$, and mean colony cover was $1.6 \pm 0.8\%$.

The abundance of colonies was greater in May 2011 than in May 2010, with inter-annual increases of 3-fold in mean density, and 2-fold in mean cover and mean percent of colonised pilings (Figs. 4 & 5).

DISCUSSION

Recruitment

Larval recruitment and survival of new colonies may have important consequences for the structure of the subarctic population of *Botryllus schlosseri* in this study. Mean and peak recruitment rates of *B. schlosseri* reported from temperate waters from Maine to Connecticut are greater than those observed from Newfoundland by 1 or 2 orders of

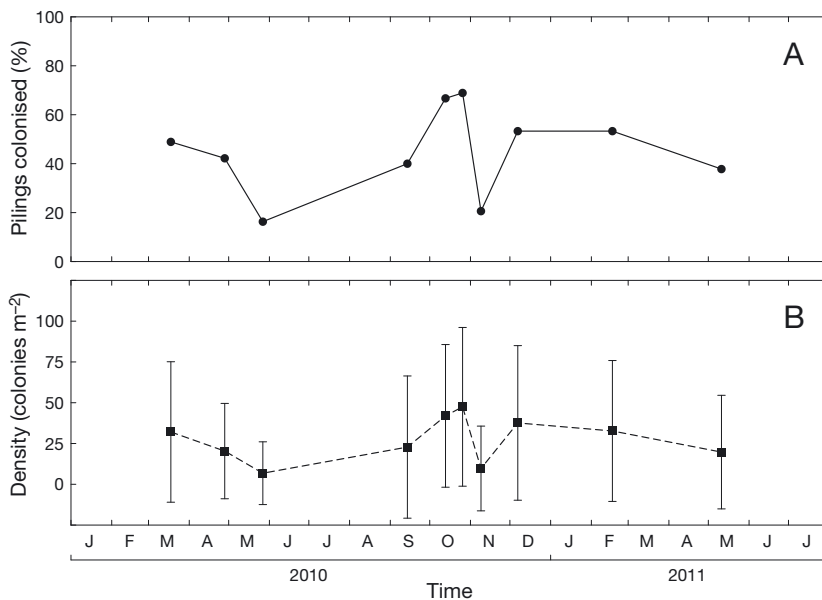


Fig. 4. Seasonal patterns of (A) percent of pilings colonised by *Botryllus schlosseri* and (B) density on wharf pilings. Zero values are included in the calculation of mean values. Bars represent ± 1 SD, $n \geq 45$ observations per data point

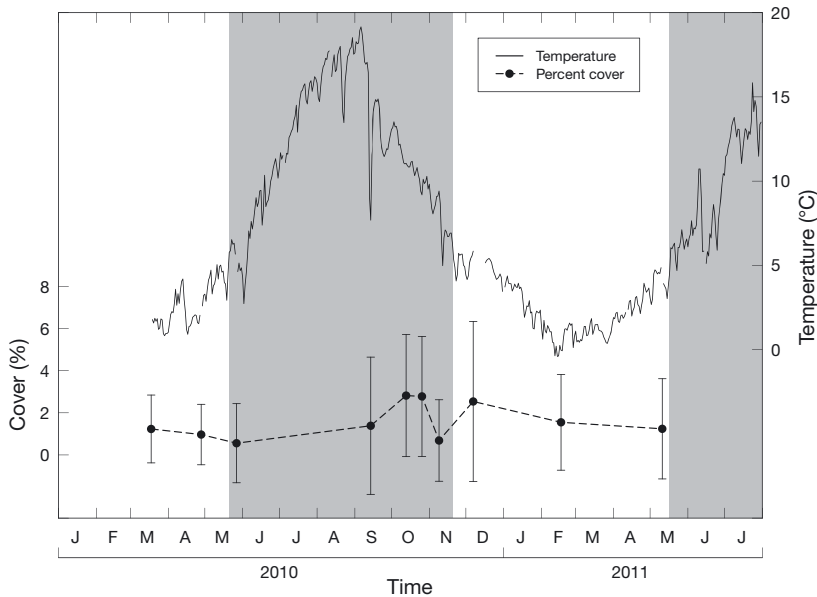


Fig. 5. Seasonal patterns of mean *Botryllus schlosseri* percent cover on wharf pilings and daily mean seawater temperature. Zero values are included in the calculation of mean values. Shaded periods represent the seasonal window between the initial and final days when temperature $\geq 6^{\circ}\text{C}$. Bars represent ± 1 SD, $n \geq 45$ observations per data point

magnitude (Table 1). Calculated from multi-year datasets, the mean recruitment rate of ca. $800\text{ m}^{-2}\text{ d}^{-1}$ in Connecticut (Whitlatch & Osman 2009), ca. $500\text{ m}^{-2}\text{ d}^{-1}$ in Massachusetts (Grosberg 1982, 1988), and ca. $500\text{--}1000\text{ m}^{-2}\text{ d}^{-1}$ in Maine (Yund & Stires 2002) are greater than in Newfoundland (ca. $10\text{ m}^{-2}\text{ d}^{-1}$).

Factors affecting recruitment rates

Season

Temperature is an important factor controlling the seasonal timing and duration of sexual reproduction in ascidians (Millar 1971, Turon 1988). The recruitment period of *B. schlosseri* in Arnold's Cove is from early August to mid-October (ca. 2.5 mo). This recruitment window is much shorter than has been reported for this species in the literature (mostly ca. 5 mo; Table 1). The shortened recruitment period is likely due to the low seawater temperature in Newfoundland, since temperature is a predominant seasonal trigger of the onset of recruitment in colonial ascidians (Westerman et al. 2009). Field observations of *B. schlosseri* indicate that the threshold for sexual reproduction is $11\text{--}12^{\circ}\text{C}$ (Brunetti et al. 1980, Westerman et al. 2009). Additional field evidence from Maine suggests that the onset of recruitment occurs in mid-June when temperature is $>12\text{--}15^{\circ}\text{C}$ (Yund & Stires 2002). In the present

study, maximum recruitment rates of *B. schlosseri* in subarctic waters were observed coincident with maximum seasonal seawater temperature in September. In its native range in the Mediterranean Sea (Carver et al. 2006a, Lejeune et al. 2011), mean seawater temperature was significantly correlated to the number of stones that were colonised, and the density of colonies of *B. schlosseri* (Rinkevich et al. 1998). Previous studies reported that recruitment of *B. schlosseri* peaked in August and early September in New England (Yund & Stires 2002, Osman et al. 2010) and in August in Germany when temperatures are warmer (Harms & Anger 1983; Table 1).

Year

In the eastern USA, *B. schlosseri* exhibited inter-annual variability of recruitment rate (study period: 1991–1999), with peak recruitment in 1996 (Terwin et al. 2002). In the same study, inter-annual fluctuations in the recruitment of the colonial ascidian *Diplosoma macdonaldi* was characterised by a high annual mean recruitment in 1995, but essentially no recruitment during the preceding and following years (Terwin et al. 2002). Variations in winter temperatures likely influenced these patterns of inter-annual variability in ascidian recruitment (Terwin et al. 2002).

Our results suggest that inter-annual variability in recruitment rate within the harbour environment was depth-dependent in the population of *B. schlosseri*, i.e. variability occurred at deeper depths but not at shallower depths. An investigation longer than 2 yr may be needed to understand inter-annual variability in recruitment patterns as it relates to depth and winter temperatures in Arnold's Cove, Newfoundland.

Site

Sites 1, 2, and 3 (associated with the 3 floating docks) in Arnold's Cove were originally chosen to provide enough space for replicate plates. There was no variability in recruitment rates of *B. schlosseri* among the 3 sites, that were $15\text{--}20\text{ m}$ apart in the harbour, even though this species is known to exhibit

Table 1. Recruitment periods and rates of *Botryllus schlosseri* that have been reported in or calculated from the literature. NL: Newfoundland and Labrador, Canada. US states are ME: Maine, NH: New Hampshire, MA: Massachusetts, CT: Connecticut, CA: California

Window	Recruitment period		Recruitment rate (recruits m ⁻² d ⁻¹)		Location	Latitude	Reference
	Peak(s)	Mean rate	Peak rate	Peak rate			
Jul–Nov	–	–	–	–	Ardrossan Harbour, Scotland	55° 39' N	Millar (1952)
Jul–Sep	Aug	40–330	–	–	Island of Helgoland, Germany	54° 11' N	Harms & Anger (1983)
	–	308 (horizontal plates)	–	–	Langstone Harbour, England	50° 49' N	Schmidt (1982)
Early Aug–mid-Oct	Sep	9–12 (PVC)	29; 44	–	Arnold's Cove, NL	47° 45' N	Present study
May–Oct	Early Jul & early Sep	500–1000	2100	–	Damariscotta River, ME	43° 56' N	Yund & Stires (2002)
	–	90	390	–	Darling Marine Center, ME	43° 56' N	Westerman (2007)
	–	50	220	–	New Castle, NH	43° 04' N	Westerman (2007)
	–	60	160	–	Salem, MA	42° 31' N	Westerman (2007)
Early Jun–late Sep	–	460–530	2100–2700	–	Woods Hole, MA	41° 31' N	Grosberg (1982, 1988)
May–Oct	–	800	17800	–	Avery Point, CT	41° 19' N	Terwin et al. (2002), Whitlatch & Osman (2009)
May–Nov	Aug	–	–	–	CT and MA	41° 04' to 41° 55' N	Osman et al. (2010)
	–	18	–	–	Bodega Bay, CA	38° 19' N	Claar et al. (2011)
Sep–May	–	9–18; 54–89	–	–	Williamstown, Australia	37° 52' S	Keough & Raimondi (1996); Keough (1998)

variability in recruitment patterns at larger spatial scales. For example, Yund & Stires (2002) reported that larval recruitment rates varied among their 5 sites along the ca. 17 km Damariscotta River estuary in Maine. Similarly, some variation in recruitment rates of the solitary ascidian *Ciona intestinalis* was reported among 3 sites ca. 1.5–2 km apart within the Brudenell estuary, Prince Edward Island, Canada (Ramsay et al. 2008). Our findings suggest that the monitoring of *B. schlosseri* using artificial plates at 1 site in Arnold's Cove would adequately represent spatial scales of ≤ 10 s of metres. Spatial variability in recruitment at scales > 10 s of metres within a coastal harbour requires further investigation and would be influenced by distance from larval source (e.g. reproductive colonies of *B. schlosseri*), larval dispersal ability, and local hydrodynamics.

Depth

Botryllus schlosseri recruitment in Arnold's Cove was greater near the water surface than at other depths. Vertical zonation of recruitment can be explained in 2 ways. Firstly, larval behaviour could explain the tendency of recruitment to peak at shallow depths. In the larva of *B. schlosseri*, the photolith is a sensory organ situated in the head region that responds to light and gravity (Sorrentino et al. 2000). The larva is initially positively phototactic and negatively geotactic (Grave & Woodbridge 1924, Grave 1937, Crisp & Ghobashy 1971, Boyd et al. 1986). Within minutes to hours of release, the positively phototactic larva transitions to a state of being non-responsive to light (Grave & Woodbridge 1924). Prior to settlement, the larva is briefly negatively phototactic and, hence, shows a preference for dark spaces (Grave & Woodbridge 1924, Crisp & Ghobashy 1971, Schmidt 1982). Secondly, vertical zonation of recruits may be related to environmental conditions at different depths. Larvae may be sufficiently sensitive to respond to environmental cues and, thus, actively select habitats where temperature and salinity are suitable along a vertical gradient. For instance, in Arnold's Cove, variability in seawater temperature and salinity was significant among depths (Table S3).

Substrate type

The materials used to construct recruitment plates for recruitment studies and monitoring programmes should be carefully considered with respect to ecologi-

cal relevance and sensitivity of species detection. Man-made structures in ports and marinas consist of a variety of materials such as wood, metals, and plastics. The diversity of materials that could be found in a coastal harbour necessitates an understanding of differential recruitment of fouling organisms (including *B. schlosseri*) on different substrate types. Additionally, the choice of substrate used to monitor for non-indigenous fouling species (e.g. ascidians, bryozoans, etc.) should be sensitive to their detection. Hence, the accumulation of the most recruitment on a specific substrate type suggests high affinity of larvae to settle.

In the present study, maximum recruitment rates of *B. schlosseri* were observed on PVC plates. Tyrrell & Byers (2007) investigated substrate preference for the same species, which was quantified in percent cover of colonies. Calculations from their data indicate that mean cover was greater on aluminium (ca. 20%) and wood (15%) than on scallop shells (4%) and PVC (3%). Based on their study, the low affinity for PVC suggests that monitoring programmes that use PVC have been systematically under-estimating colony cover, perhaps due to lower recruitment rates and/or higher mortality rates of new recruits and juvenile colonies. However, our data suggest that PVC plates would maximise the detection of *B. schlosseri*. In particular, data collected using PVC can be used to determine the timing of onset of recruitment (e.g. the present study), and the occurrence of a new invasion. Therefore, the efficacy of PVC as the substrate of choice in research studies and monitoring programmes of larval recruitment is supported. The low affinity of *B. schlosseri* on PVC in the study by Tyrrell & Byers (2007) was likely due to competition for space with another colonial NIA, *Botrylloides violaceus*. In addition to substrate type, other qualities such as surface roughness and colour can be factors affecting ascidian larval settlement on artificial substrates (Chase et al. 2016).

Colony abundance

Colonies were present year-round on wharf structures in Arnold's Cove, which indicates an established self-sustaining population in subarctic conditions. Colonies of *B. schlosseri* exhibited seasonal fluctuations in percent cover ranging from a minimum of <1% in May to a maximum of ca. 3% in October at our Newfoundland study site. This range of values is much lower than in other regions of the world where this species is established as an NIA, but comparable to values in its native range in the Mediterranean Sea. For example, colony abundance on natural stone sub-

strates in Israel was ca. 0–1.5% (Rinkevich et al. 1998). Outside its native range, the mean cover of *B. schlosseri* varies among geographic localities. In particular, along the coast of Maine, mean colony cover on artificial substrates appears to be spatially variable: near 0 in Boothbay, Freeport, and Opeeche Island, 2–2.5% in Medomak and Portland, 7–10% in Eastport and Orrs Island, and 25% in Blue Hill (McNaught & Norden 2011). After 5 mo of deployment, the cover of *B. schlosseri* on artificial substrates ranged from <25% to >75% in Dipper Harbour, New Brunswick (LeGresley et al. 2008). The variability of these values among localities suggests that colony cover, and perhaps density, may be highly variable at other localities in Newfoundland where *B. schlosseri* is present.

Interpretation of the patterns of *B. schlosseri* colony abundance (e.g. colony densities) can be affected by its mode of growth and reproductive cycle. A colony of *B. schlosseri* can grow around mussel shells or algae and appear to be multiple colonies when it is actually only 1 colony. Also, a colony undergoing blastogenic cycles—synchronised colony-wide cycles of asexual reproduction of zooids via palleal budding (Rinkevich et al. 1992, Ballarin et al. 2008)—can appear to be multiple colonies (especially in video sequences) when systems of zooids are only delicately connected by a common tunic. Colonies in contact that share 1 or both histocompatibility alleles are recognised as kin (Sabbadin & Astorri 1988, Ben-Shlomo et al. 2001, Rinkevich 2005). This results in the complete or partial integration of tissues (by resorption) of partner colonies forming a natural chimera. Thus, a chimeric colony can appear to be a single (genetically homogenous) colony when it consists of tissues from 2 or more partners (i.e. a product of 2 or more recruits). In our study population, semelparous and iteroparous colonies were not distinguished. Semelparous morphs produce only 1 clutch of larvae before death and iteroparous morphs produce 3 or more clutches (maximum 7–10; Grosberg 1988). Hence, the patterns of decreases in *B. schlosseri* colony abundance can be influenced by the reproductive cycle of semelparous morphs (death of colonies after larval release) depending on the proportion of the semelparous colonies in the population.

Factors affecting colony abundance

Season

In addition to *B. schlosseri* in Arnold's Cove, other populations in temperate waters around the world

also exhibit seasonal variation in colony abundance (Otsuka & Dauer 1982, Rinkevich et al. 1998, Yund & Stires 2002, Dijkstra et al. 2007). This variation may be related to seasonal cycles of recruitment and colony mortality, interspecific interactions (competition for space, predation, etc.), or disturbance (anthropogenic and natural disturbances). In the present study, population growth (i.e. increases in density and percent cover of colonies on wharf pilings) was observed between May and October when seawater temperatures were $\geq 5^{\circ}\text{C}$. Abundance of *B. schlosseri* decreased in November (and mean colony size in December), but later remaining colonies sustained a population that decreased gradually in abundance over the winter months when waters were $< 6^{\circ}\text{C}$.

Zone

Variability in density and percent cover of colonies of *B. schlosseri* was detected among the 3 zones along a distance of 50 m of wharf pilings in Arnold's Cove. The greatest cover on any single piling was 20%, which occurred in mid-September 2010 in the relatively exposed section of the wharf, which had the lowest cover of macro-algae. These findings suggest that there is differential long-term development of the fouling community on wharves within the spatial scale of the harbour environment.

Summary

To date, the presence of NIA species is not known from any aquaculture sites in Newfoundland (Deibel et al. 2014, McKenzie et al. 2016). However, boat connectivity between invaded Newfoundland harbours and aquaculture sites poses a threat of contaminating aquaculture sites. In particular, this threatens the sustainability of bivalve aquaculture industry due to the cost associated with removing ascidians (including *B. schlosseri*) from fouled equipment and products (Lambert 2002, Davis & Davis 2009, 2010, Locke & Carman 2009). For instance, the estimated cost of a single NIA, *Styela clava*, to aquaculture was approximately 15% of the value of the Canadian aquaculture industry (after correcting for inflated values, A. Locke pers. comm.; Colautti et al. 2006). The potential detrimental impact to the aquaculture industry in Newfoundland underscores the importance of monitoring and management of NIA species.

Management of *B. schlosseri* in Newfoundland should target mitigation efforts before the annual

onset of sexual reproduction and recruitment (i.e. no later than July) to minimise the release of larvae from viable colonies and fragmentation of colonies. Recruitment in the subarctic population in Arnold's Cove is constrained to temperatures $\geq 13^{\circ}\text{C}$, indicating that *B. schlosseri* has not adapted to settle and recruit at temperatures lower than those of its temperate range. Our results suggest that the use of 1 site to monitor for *B. schlosseri* may adequately represent spatial scales of ≤ 10 s of metres in a marina. We demonstrated that recruitment is greater at depths < 2.5 m in comparison to deeper locations, including the bottom. Therefore, mitigation efforts should target depths < 2.5 m to maximise the removal of colonies of *B. schlosseri*. In particular, floating docks, wharf pilings, and ship hulls near the water surface may be preferentially colonised. Lastly, we showed that PVC plates would maximise the detection of *B. schlosseri*, for instance, the determination of (1) the timing of onset of recruitment, and (2) the occurrence of a new invasion.

Colonies of *B. schlosseri* are present year-round in Arnold's Cove, and the population is sustained because of a relatively high cover of overwintering colonies (mean colony size was 6.7 cm^2 on wharf pilings in December 2010). These colonies (ca. 90% of autumn-recruited colonies) were observed to survive the winter and reproduce sexually the following spring (Deibel et al. 2014). The presence of *B. schlosseri* in the winter may represent a lack of predators or an adaptive response to survive in subarctic waters. Colonies are also present year-round in the native range of *B. schlosseri* in the Mediterranean Sea (Rinkevich et al. 1998), which may suggest that the subarctic population has been able to adapt to sub-zero winter temperatures in Newfoundland.

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