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The influence of substrate material on ascidian larval settlement

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ABSTRACT

Submerged man-made structures present novel habitat for marine organisms and often host communities that differ from those on natural substrates. Although many factors are known to contribute to these differences, few studies have directly examined the influence of substrate material on organism settlement. We quantified larval substrate preferences of two species of ascidians, *Ciona intestinalis* (cryptogenic, formerly *C. intestinalis* type B) and *Botrylloides violaceus* (non-native), on commonly occurring natural (granite) and man-made (concrete, high-density polyethylene, PVC) marine materials in laboratory trials. Larvae exhibited species-specific settlement preferences, but generally settled more often than expected by chance on concrete and HDPE. Variation in settlement between materials may reflect preferences for rougher substrates, or may result from the influence of leached chemicals on ascidian settlement. These findings indicate that an experimental plate material can influence larval behavior and may help us understand how substrate features may contribute to differences in settlement in the field.

1. Introduction

Most benthic marine organisms have a biphasic life cycle, including a pelagic larval phase and a demersal, sessile, or sedentary adult phase. Life history strategies vary widely between taxa, but pelagic larval stages often facilitate the dispersal of juvenile organisms. Larvae can spend minutes to months in the water column before metamorphosing and settling onto the seafloor and other surfaces. The site of this eventual settlement is of particular importance for sessile organisms, which are unable to change their location following substrate attachment and metamorphosis. Accordingly, the initial settlement patterns play a large role in the structuring of benthic communities, though these assemblages are also influenced by post-settlement mortality, competition, and growth (Connell, 1985; Hunt and Scheibling, 1997; Fraschetti et al., 2002). Given the influence of settlement site selection on organism survival and fitness, it is unsurprising that many sessile animals exhibit active settlement preferences (e.g., tube-building polychaetes *Hydroides dianthus* and hydroid *Ectopleura crocea*, Mullineaux and Garland, 1993; colonial ascidian *Diplosoma similis*, Stoner, 1994; hydroid *E. crocea* and barnacle *Balanus* sp., Lemire and Bourget, 1996; barnacle *Balanus crenatus*, Miron et al., 1996). Numerous factors, including light intensity (Thorson, 1964; Durante, 1991; Rius et al., 2010), proximity of prey or host organisms (Pawlik, 1992) or conspecifics (reviewed in Burke, 1986 and Pawlik, 1992), surface microtopography (Wethey, 1986; Kerr et al., 1999; Lemire and Bourget, 1996) and substrate chemical composition (Kerr et al., 1999; Bavestrello et al., 2000) are known to influence sessile organism settlement.

Many benthic taxa exhibit dramatic differences in abundance on natural and man-made structures (e.g., ascidians, Lambert, 2002; Simkanin et al., 2012), often causing resultant variation in fouling community composition on these surfaces (Holloway and Connell, 2002; Bulleri, 2005; Wilhelmsson and Malm, 2008). These community differences are frequently driven by non-native species, which are often more prevalent on man-made marine structures than on natural surfaces (Lambert and Lambert, 2003; Simkanin et al., 2012). Anthropogenic structures are at high risk of colonization by non-indigenous species, as these constructions are often located in high-traffic areas and exposed to direct influxes of non-native propagules via aquaculture, shipping, and recreational boating activities (Carlton, 1989; Carlton and Geller, 1993; Floerl and Inglis, 2005). Disparities in community structure and species composition may result from several factors that tend to vary systematically between natural and man-made substrates, including surface orientation and light exposure (Vandermeulen and Dewreede, 1982; Glasby and Connell, 2001; Thomason et al., 2002; Miller and Etter, 2008) proximity to the sea floor or water surface (Glasby and Connell, 2001; Holloway and Connell, 2002), and predation exposure (Otsuka and Dauer, 1982; Dumont et al., 2011), all of which are known to influence community development. Substrate type and composition are other factors that may contribute to observed differences in fouling on natural and man-made surfaces (Bavestrello et al., 2000). Though multiple studies have compared fouling community assemblages on different materials in the field (McGuinness, 1989; Anderson and Underwood, 1994; Tyrrell and Byers, 2007; Andersson et al., 2009; Vaz-Pinto et al., 2014), relatively few studies have directly

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examined larval settlement preferences for different materials (scyphozoa, Holst and Jarms, 2006; Hoover and Purcell, 2008).

Laboratory experiments that examine larval behavior are a valuable complement to field-based community studies and can help identify whether variation in initial settlement density and/or post-settlement mortality and growth rates are responsible for observed differences in community composition in the field. Laboratory studies have discovered that many invertebrates, including most ascidians, exhibit preferential settlement on non-illuminated surfaces (Durante, 1991; Rius et al., 2010). This adaptation may serve to encourage settlement in areas where competition with, and overgrowth by, macroalgae is less likely. Substrate microtopography, which can influence flow conditions, shear stress, and the availability of dissolved gasses and food particles (Vogel, 1996), can enhance or reduce larval settlement (Howell and Behrends, 2006; Orlov, 1996). Rougher surfaces, which have more turbulent boundary layer flows, are known to facilitate the settlement of certain organisms, including hydroids, barnacles (Mullineaux and Butman, 1991; Wright and Boxshall, 1999), bivalves (Bologna and Heck, 2000), and polychaetes (Hurlbut, 1991; Walters et al., 1999). However, many other species preferentially settle on smooth substrates (ascidians, bryozoans, polychaetes, Osman and Whitlatch, 1995a,b; barnacles, Lemire and Bourget, 1996). Material characteristics, including substrate color and chemical composition, are also known to impact settlement, though few studies have directly examined these topics (Satheesh and Wesley, 2010; Bavestrello et al., 2000).

To determine if larval settlement preferences may contribute to observed differences in ascidian abundance on natural- and man-made surfaces in the Gulf of Maine, we examined larval settlement in two commonly occurring species. These organisms, the solitary *Ciona intestinalis* (Linnaeus, 1767) and the colonial *Botrylloides violaceus* (Oka, 1927), produce larvae that are easily obtained in quantities suitable for manipulative experimentation. Larvae were exposed to settlement plates composed of concrete, granite tile, high-density polyethylene (HDPE), and polyvinyl chloride (PVC) during controlled laboratory trials. As changes in biofilm composition and thickness over time can influence larval settlement (Wieczorek and Todd, 1997), trials were conducted using plates subjected to two different durations of pre-trial soaking. In the Gulf of Maine, *B. violaceus* and *C. intestinalis* are non-native and cryptogenic, respectively. These and other ascidian species frequently dominate sessile communities on man-made marine structures in the Gulf of Maine (e.g., Dijkstra and Harris, 2009). Therefore, we hypothesized that larvae of both species would settle more frequently on artificial substrates than on a pseudo-natural substrate (granite tile).

2. Methods

2.1. Study species

Both *C. intestinalis* and *B. violaceus* commonly occur on man-made structures, also colonizing natural substrates, in the southwestern Gulf of Maine. In 2015 it was recognized that *C. intestinalis* included two distinct species, *C. intestinalis* (formerly *C. intestinalis* type B) and *Ciona robusta* (formerly *C. intestinalis* type A; Brunetti et al., 2015). Species-defining molecular or morphological characters were not assessed, but all animals used in this study were collected in New Hampshire, Maine, and Massachusetts in the fall of 2014. Only *C. intestinalis* is known to occur within this range (Zhan et al., 2010), therefore we assume that the animals studied were *C. intestinalis*. *C. intestinalis* was present in the Gulf of Maine, where it is considered cryptogenic, before the 1940s (Miner, 1950) and has become a common and often dominant fouling community constituent on floating docks in sheltered coastal areas. Fertilization of *C. intestinalis* eggs occurs in the water column following gamete release during summer and fall months. The duration of embryonic development is highly dependent upon temperature and can range from 18 h at 18–20 °C to 48 h at 12 °C (Dybern, 1965; Bullard and Whitlatch, 2004). *C. intestinalis* larvae are very small (0.88–1.28 mm in length) and nearly transparent with a larval phase ranging from 24 h at high temperatures (18–20 °C) to 5 days at low temperatures (10–12 °C; Dybern, 1965; Bullard and Whitlatch, 2004).

Our second study species, *B. violaceus*, is a colonial ascidian that is likely native to Japan and has been established in the Gulf of Maine since 1981 (Berman et al., 1992). *B. violaceus* is a common fouling organism and is found on both man-made and natural substrates including floating docks, pilings, subtidal rocks and algae, and *Mytilus edulis* shells (Carver et al., 2006). This species produces very large (length <3 mm) and often brightly colored orange, red, pink, or purple tadpole larvae that are brooded within the colony and released throughout the summer and fall (Bullard and Whitlatch, 2004). The *B. violaceus* larval phase is very short, lasting from several minutes to hours after release from the parent colony (Lambert, 1990).

2.2. Specimen collection

C. intestinalis gametes were obtained from individuals collected from HDPE floating docks at Hampton River Marina, Hampton Beach, NH and Salem Marina, Salem, MA and from HDPE floating docks and concrete and steel underwater structures at the University of New Hampshire Coastal Marine Lab Pier in Newcastle, NH. *B. violaceus* larvae were obtained from mature colonies collected from floating docks composed of HDPE at Wentworth Marina in New Castle, NH, from shallow subtidal rock surfaces and algae at Odiorne Point State Park, Rye, NH, and from subtidal rocks and algae via scuba diving at Cape Neddick, York, ME.

2.3. Experimental substrates

Four materials were selected to represent natural and man-made substrates typical of Gulf of Maine coastal systems. Concrete (commercial grade Quickrete® quick-setting cement) and black high-density polyethylene (King StarBoard® marine building material, HDPE) were chosen as experimental substrates because these materials are extremely common in developed marine areas. Gray chemical-resistant type 1 PVC was included in this study because it is a commonly used settlement plate material in scientific studies examining marine fouling communities (Osman and Whitlatch, 1995a; Stachowicz et al., 2002; Blum et al., 2007; Osman and Whitlatch, 2007; Janiak et al., 2013; Simkanin et al., 2013). Granite substrates were included because this mineral is the primary component of ledge and bedrock in the southwestern Gulf of Maine. Standardized natural rock samples could not be obtained, so the unpolished sides of dark gray granite tiles were used as pseudo-natural substrates.

2.4. Larval acquisition

2.4.1. C. intestinalis

Eggs and sperm were obtained via dissection of mature individuals and fertilized in vitro (procedure adapted from Cirino et al., 2002). A longitudinal incision was made through the tunic of each individual *C. intestinalis* and eggs were removed from the oviduct using a pipette. Eggs from 6 to 10 individuals were hydrated in dishes containing approximately 100 ml of seawater for 15–30 min before sperm addition. One drop of sperm was removed from 4 to 7 individuals and mixed with 100 ml of fresh seawater approximately 5 min before gamete mixing to promote maximum motility. A sample of the sperm mixture (~5 ml) was added to each dish of eggs and embryogenesis was monitored until larvae were fully developed. Excess sperm was removed 1 h after fertilization to minimize embryo mortality by removing several ml of water from each dish and replenishing containers with fresh seawater. The required number of larvae was removed prior to the start of trials.

2.4.2. B. violaceus

Colonies were collected in the afternoon and housed overnight in aerated 15 °C seawater aquaria under fluorescent lighting on a 12 h:12 h light:dark cycle. Larval release began rapidly upon illumination the following morning and continued for approximately 6 h. Active larvae were removed from containers using a pipette and placed into a glass holding dish as they were released from parent colonies. Larvae were counted immediately before the start of trials.

2.5. Settlement preference

Four 10 × 10 cm plates (one each of concrete, granite tile, HDPE, and PVC) were suspended with their lower surfaces approximately 2 mm below the water surface in aquaria filled with 2000 ml of seawater (Fig. 1). The position of each plate material was randomized for each larval settlement trial. At the beginning of each trial, 100 *B. violaceus* or 250 *C. intestinalis* were added to each aquarium. More *C. intestinalis* larvae were used for each trial because many more individuals were produced by in-vitro fertilization than were released by *B. violaceus* parent colonies in response to light-shock. Aquaria were left undisturbed at 15 °C until larval settlement was complete (>90% settlement as determined during pilot trials, *B. violaceus*: ~24 h, *C. intestinalis*: ~72 h). Plates were then examined and all settled individuals on the lower surface of each plate were counted. Counting techniques varied between species due to differences in settled ascidian size and color. *B. violaceus* settlers were easily identifiable and were counted without magnification. *C. intestinalis* individuals were identified by examining plates under a dissecting microscope.

2.6. Settlement preference based on substrate age

To determine if larval settlement preferences changed with substrate age, experiments were conducted using two groups of settlement plates. One set of plates were aged in seawater for 1 to 3 weeks (young) before the start of trials, while another group of plates were aged for 5 to 10 weeks (old) before the start of trials. Between 4 and 8 trials (1 trial = 1 aquarium) were performed simultaneously until at least 16 trials were completed for each species using both young and old plates. Trials were conducted when larvae and mature gametes of each species were most abundant and readily obtainable. *B. violaceus* trials were conducted between July 29 and September 18, 2014; *C. intestinalis* trials were conducted from October 10 to 24, 2014. Old and young plate trials were haphazardly conducted throughout the trial period for *B. violaceus* and *C. intestinalis*.

2.7. Settlement preference statistical analysis

Counts of settled larvae on each substrate were analyzed to examine settlement preferences of *C. intestinalis* and *B. violaceus*. Because Chi square and G-tests of goodness-of-fit can yield erroneously low *p*-values when expected values are below 5 and total sample size is less than 1000, as was the case with these data, the exact multinomial tests of goodness-of-fit were used. These analyses tested the null hypothesis that ascidian larvae were not exhibiting material-specific settlement preferences, assuming a 1:1:1:1 distribution of settled individuals among the four plate materials. Post-hoc binomial tests were used to compare the number of settled individuals on each substrate to that expected by chance. As individual binomial tests were used to

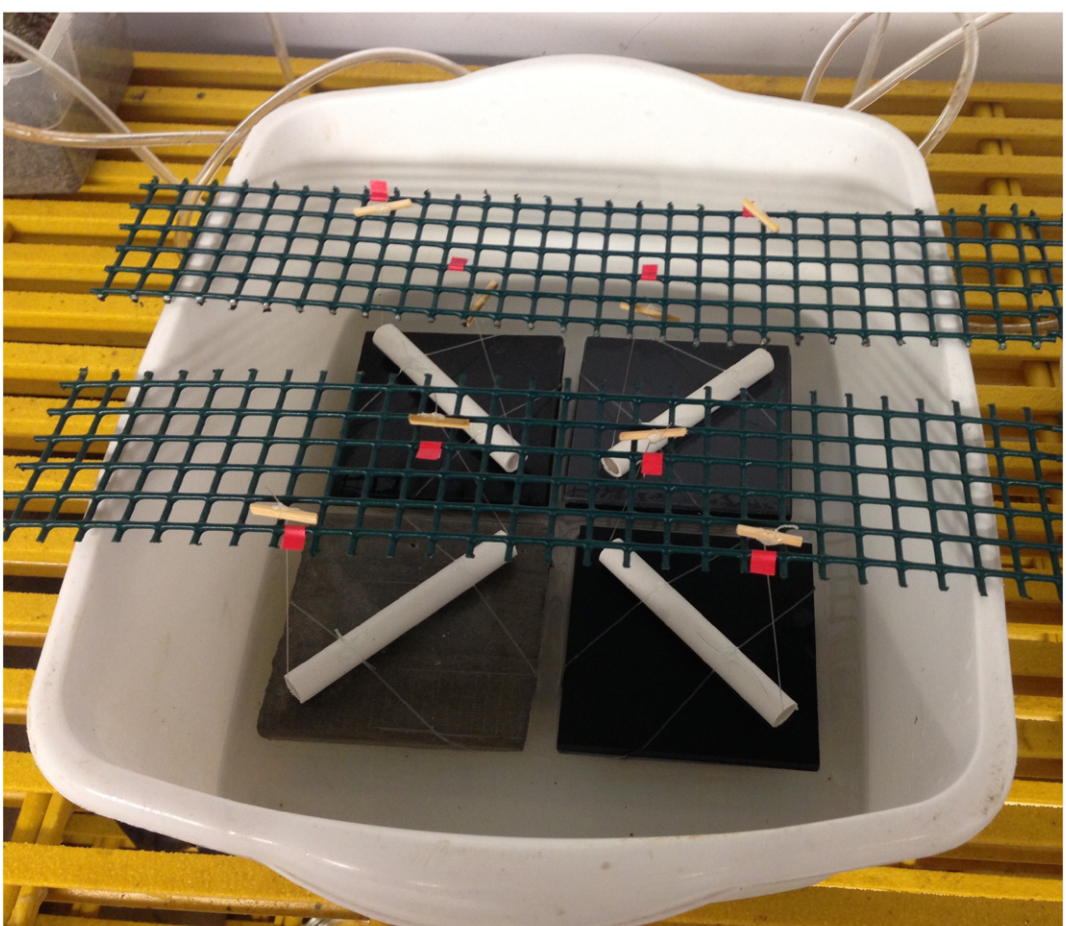

Fig. 1. Larval settlement preference experimental setup composed of four plates of different materials (concrete, granite, HDPE, PVC) with lower surfaces submerged.

Table 1
Number of ascidian larvae introduced into aquaria that settled upon experimental plates (concrete, granite, HDPE, PVC) during all trials.

Species	Plate age[a]	n	# of larvae on plates
B. violaceus	Young	16	468
	Old	19	370
C. intestinalis	Young	16	489
	Old	20	1256

[a] Duration of plate submersion in seawater before beginning of trials; young: 1–3 weeks, old: 5–10 weeks.

examine settlement on each of the four substrate materials, the significance level was adjusted from $\alpha = 0.05$ to $\alpha = 0.0125$ for these analyses to account for multiple comparisons. To determine if substrate preferences were influenced by parent organism location, counts were pooled by collection location and analyzed as above. All tests were performed in R, using the Stats (R Core Team, 2015) and Xnomial packages (Engels, 2014).

2.8. Surface roughness

A Mitutoyo Surftest SJ-400 portable surface roughness tester was used to obtain quantitative measurements of experimental settlement plate characteristics. This device measures roughness by drawing a stylus with a 2 μm-wide diamond tip across a surface for 4 mm and recording micro-scale changes in elevation. The SJ-400 generates both surface roughness profiles and standard roughness metrics, including R_a, which is the arithmetic mean of deviations in surface height from the average. A perfectly smooth surface with no imperfections (deviations from the mean surface height) would have an R_a of 0; increasing R_a values indicate greater roughness. To compare roughness among experimental substrates, six randomly placed measurements were taken on three plates of each material (concrete, granite, HDPE, and PVC). Three values were recorded from the central 4 × 4 cm area of each plate, and three values were recorded from outside this area, to evaluate if roughness varied with proximity to plate edges. R_a values were analyzed using one-way ANOVAs using SPSS statistics version 22 software.

3. Results

Tadpole larvae of both species were successfully obtained and did show settlement on the experimental substrates (Table 1). Both *C. intestinalis* and *B. violaceus* exhibited non-random settlement onto experimental substrates (exact multinomial tests, all $P < 0.05$). These preferences differed between species and plate aging treatments. When exposed to 1–3 week aged plates, *B. violaceus* settled more often on concrete panels, and less often on all other materials, than expected by chance (Fig. 2, Table 2). These patterns shifted slightly during trials using 5–10 week aged substrates; settlement onto both concrete and HDPE plates was greater than expected (Fig. 2, Table 2). Unlike *B. violaceus*, *C. intestinalis* settlement patterns changed only slightly between plate aging treatments. Significantly more individuals settled onto both 1–3 week and 5–10 week aged HDPE plates than expected if larvae were not exhibiting preferential settlement. Counts of individuals on all other materials were lower than projected, with the exception of 1–3 week aged concrete plates, which did not differ significantly from the expected value.

Parent organism collection location seemed to have limited effects on offspring settlement preferences for both species (see Supplementary materials; Figs. S1, S2, Tables S1, S2).

3.1. Surface roughness

High-magnification images (Fig. 3) and quantitative roughness profiles (Fig. 4) suggest that experimental plates differed dramatically in surface microtopography. Analysis of roughness values (R_a) obtained from each substrate material support this conclusion. Surface roughness values did not differ between outer and inner areas of settlement plates; therefore all roughness measurements (six per plate) were averaged to yield mean roughness values. HDPE was significantly rougher, and PVC was significantly smoother, than all other plate materials, with average roughness values of 15.89 ± 0.93 (mean ± SD), and 0.243 ± 0.073, respectively (Fig. 5, ANOVA, df = 3, F = 51.217, $p < 0.001$; Tukey $p < 0.05$). Granite (4.6 ± 1.02) and concrete (7.86 ± 2.88) were of intermediate roughness, between PVC and HDPE, and did not differ from one another (Fig. 5, Tukey $p = 0.136$).

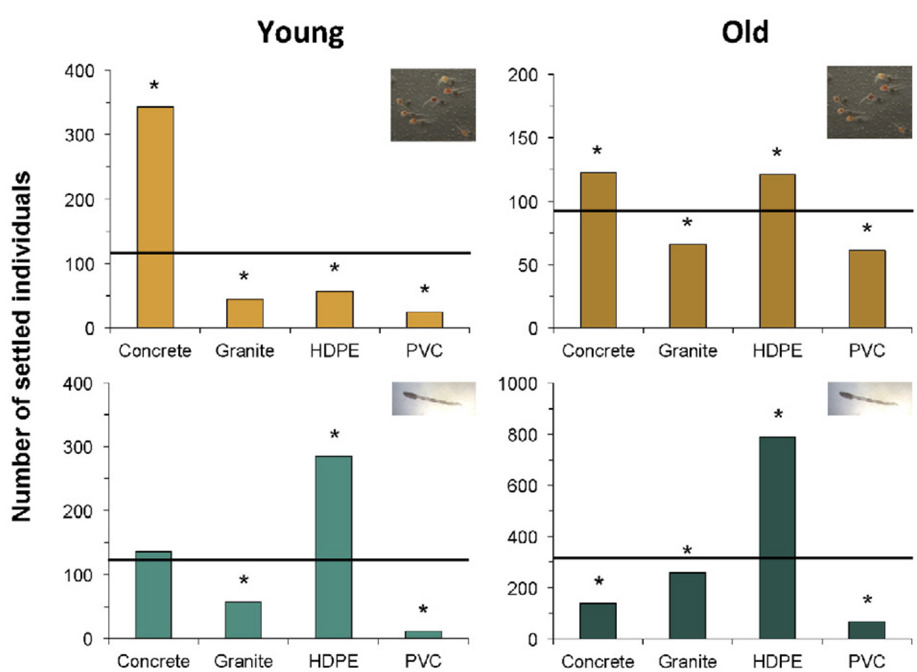

Fig. 2. Total counts of *Botrylloides violaceus* (top) and *Ciona intestinalis* (bottom) individuals settled on each of four substrate materials during single-species settlement trials. Plates were aged for 1–3 weeks (young, $N_{C.} = 16$, $N_{B.} = 16$) or 5–10 weeks (old, $N_{C.} = 20$, $N_{B.} = 19$) prior to the beginning of trials. Horizontal lines denote counts of settled individuals expected if larvae exhibited no settlement preferences. Asterisks indicate values that differ significantly from expected counts (exact binomial tests, see Table 2).

Table 2
Results of exact binomial tests of goodness-of-fit comparing observed counts of ascidians settled on experimental substrates with expected counts assuming a null model of no settlement preferences. ns: $p > 0.0125$, **$p < 0.01$; ***$p < 0.001$.

Species	Plate age[a]	N trials	Substrate[b]	Observed #	Expected #	*p*
B. violaceus	Young	16	C	343	117	***
			G	44	117	***
			H	57	117	***
			P	24	117	***
	Old	19	C	122	92.5	***
			G	66	92.5	**
			H	121	92.5	***
			P	61	92.5	***
C. intestinalis	Young	16	C	136	122.25	ns
			G	57	122.25	***
			H	285	122.25	***
			P	11	122.25	***
	Old	20	C	140	314	***
			G	260	314	***
			H	789	314	***
			P	67	314	***

[a] Duration of plate submersion in seawater before beginning of trials; young: 1–3 weeks, old: 5–10 weeks.
[b] C = concrete, G = granite, H = HDPE, P = PVC.

4. Discussion

Both *B. violaceus* and *C. intestinalis* exhibited substrate-specific settlement preferences. Though preferences differed between species, larvae only settled more frequently than expected by chance on HDPE and concrete plates (Fig. 2, Table 2). The duration of pre-trial plate submersion influenced *B. violaceus* preferences, but not *C. intestinalis* settlement patterns (Fig. 2, Table 2).

Data obtained during these trials represent active settlement preferences. All experimental substrates were suspended just below the water surface, requiring larvae to actively swim to reach these settlement sites. It can be assumed that all individuals that settled upon experimental plates were motile and physically able to contact the four substrate materials. Though settlement frequency varied by species, larval counts were greater than predicted by a random model only on concrete and HDPE plates (Table 2, Fig. 2). Surface characteristics can influence the settlement of many benthic species, and numerous organisms, including barnacles (Mullineaux and Butman, 1991; Wright and Boxshall, 1999), bivalves (Bologna and Heck, 2000), and polychaetes (Hurlbut, 1991; Walters et al., 1999) preferentially settle upon topographically complex surfaces. *B. violaceus*, and especially *C. intestinalis*, may prefer HDPE due to its greater roughness than the other experimental substrates. Boundary layer flows are more turbulent and less laminar on rougher surfaces (like HDPE) than on smooth surfaces (like PVC) (Vogel, 1996). Greater turbulence leads to increased gas exchange rates and more mixing of fluid above the substrate. These flow conditions might allow ascidians to more easily acquire food and dissolved gases when settled on rougher substrates than on very smooth materials. Larvae were not exposed to either turbulent or laminar boundary layer flows during this study, but it is possible that behavioral preferences for settlement on rough materials may be heritable and, therefore, observed even in environments with minimal or no water movement.

In addition to exposing organisms to potentially favorable boundary layer flows, settlement on rougher surfaces might be adaptive by reducing the risk of organism detachment from the substrate. Depending on the mechanism of adhesion, certain organisms may be able to form stronger attachments to rougher surfaces than to very smooth surfaces (Howell and Behrends, 2006). Although relatively little is known about the specifics of ascidian adhesion (Edlund and Koehl, 1998; Pennati and Rothbächer, 2015), both *C. intestinalis* and *B. violaceus* attach to substrates via protein-based glues. Because measured attachment strengths of colonial and solitary ascidians are considerably lower than many other benthic organisms, including mussels and barnacles (Murray

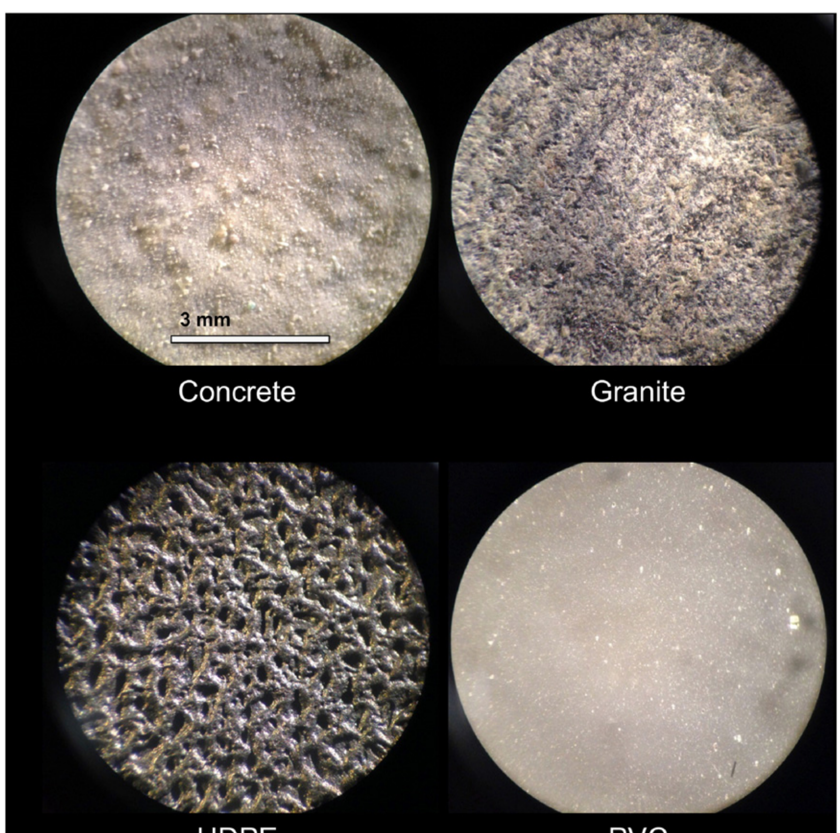

Fig. 3. Representative photographs of concrete, granite, HDPE, and PVC settlement plate surfaces under magnification. Scale bar indicates 3 mm.

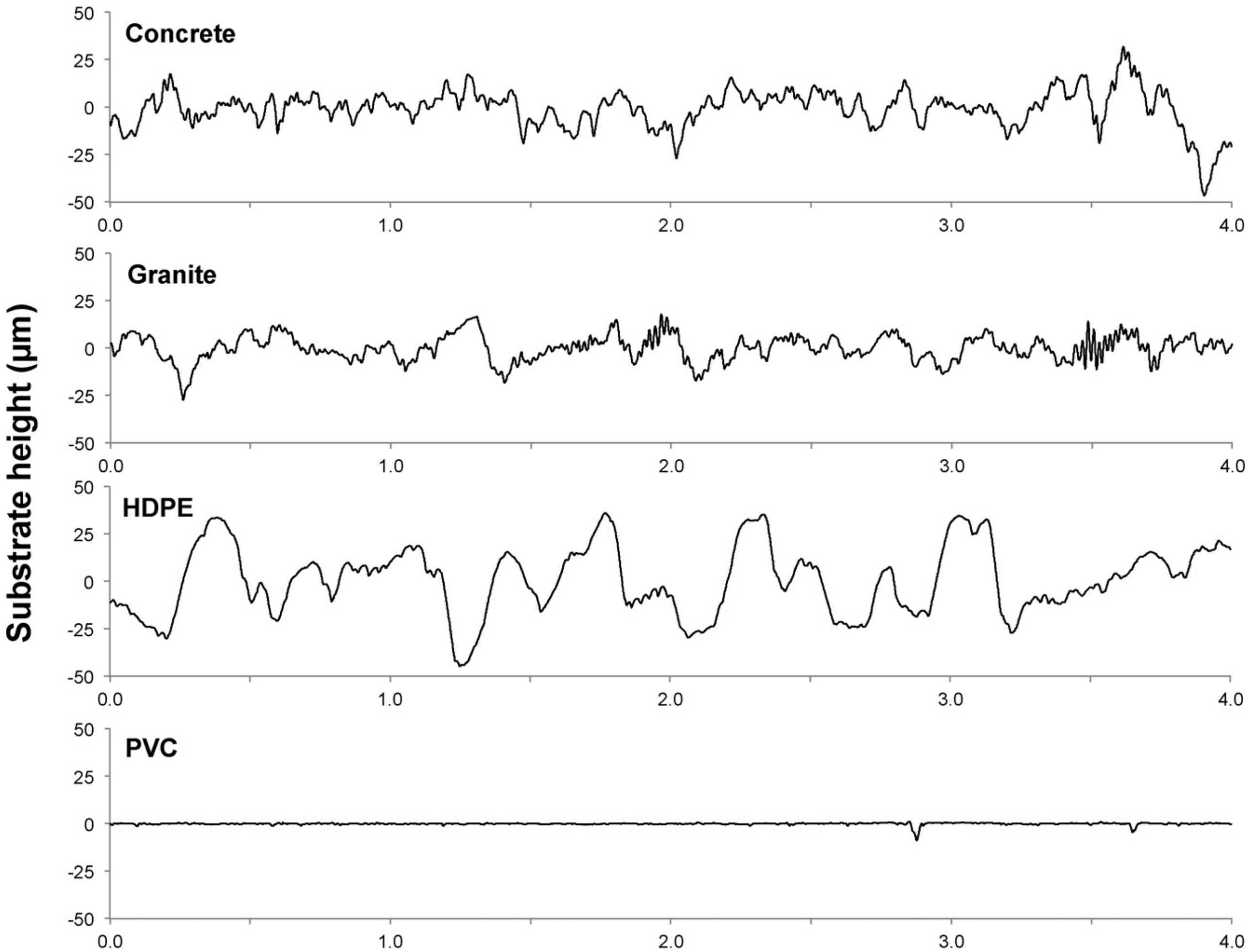

Fig. 4. Representative surface roughness profiles of concrete, granite, HDPE, and PVC settlement plates. R_a for each sample: concrete = 7.25, granite = 4.25, HDPE = 16.06, PVC = 0.25.

et al., 2012), it is possible that substrate characteristics that influence attachment strength play an important role in settlement for these animals. Though ascidians are able to reattach to surfaces through the growth of new tissue (Edlund and Koehl, 1998), this process is relatively slow, metabolically costly, and may not be common in nature, especially for solitary species like *C. intestinalis*. The relatively deep pits and valleys on the surface of HDPE plates (Figs. 3, 4)

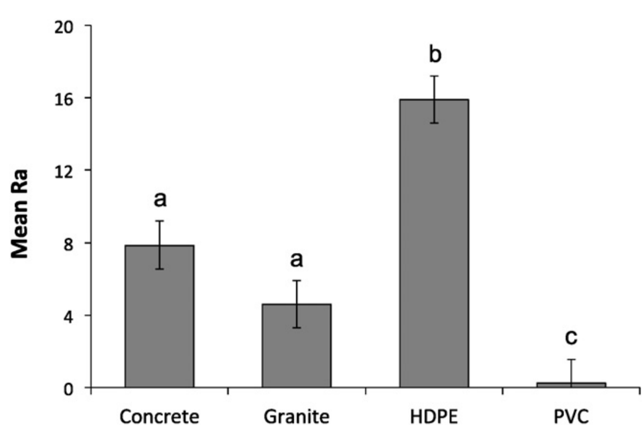

Fig. 5. Mean surface roughness of experimental plates measured using a portable surface roughness gauge. Lower R_a values indicate smoother surfaces and higher values indicate rougher surfaces. Error bars denote ± 1 SE, $n = 3$ for each substrate. Shared letters indicate roughness averages that did not differ significantly.

might allow for more secure adhesion than the other experimental materials, especially the very smooth PVC. In addition to affecting abiotic conditions, microtopography is also known to influence biofilm formation (Kerr et al., 1999; Kerr and Cowling, 2003). As biofilms can enhance or limit settlement, it is possible that indirect effects of roughness could have contributed to observed settlement patterns (Wieczorek and Todd, 1997; Maki et al., 2000).

Surface complexity alone does not seem to explain settlement patterns in *B. violaceus*. This species demonstrated a preference for concrete during trials using both 1–3 week and 5–10 week aged plates. Though concrete plates, on average, were rougher than all other materials save HDPE, there was no significant difference in R_a between concrete and granite tiles (Fig. 5). Therefore, it is possible that the chemical composition of substrate materials contributed to observed settlement patterns. Though the precise chemical components of the materials used in this study are uncertain, the composition of the four experimental substrates varied widely. Substrate material is known to influence the composition of microfouling communities, which can in turn effect macrofouling organism settlement (Marszalek et al., 1979). Additionally, mineralogical components including crystalline silica (quartz) have been found to directly impact larval settlement preferences in some species (hydroids, Bavestrello et al., 2000). Though both granite and concrete contain crystalline silica, settlement on these two substrates differed, perhaps as a result of the rate of leaching of this and other chemicals into the water column. It is also possible that other, unidentified, chemical components may have influenced settlement preferences in *B. violaceus* and *C. intestinalis*.

Plate submersion time also influenced larval settlement patterns. Both *C. intestinalis* and *B. violaceus* exhibited greater settlement on concrete in trials using 1–3 week aged plates than in trials using 5–10 week aged plates (Table 2, Fig. 2). This apparent change in the attractiveness of concrete may have resulted from decreases in chemical leaching with increased pre-trial submersion time. All settlement plates were soaked in fresh seawater for a minimum of one week before use in trials. Concrete plates, which were made specifically for this study, were observed to release a residue that formed a film on the water surface during the first several days of plate immersion. This discharge decreased with time. It is possible that both study species, and especially *B. violaceus*, were attracted to some of the chemical components that were released from concrete plates. Changes in settlement preferences between plate aging treatments might reflect decreased leaching rates of these chemicals with increased pre-trial submersion time.

It is also possible that differences in biofilm composition between 1–3 week and 5–10 week aged plates might have influenced settlement preferences. Biofilm composition and thickness, both of which can influence larval settlement, change with submersion time (Wieczorek and Todd, 1997). For example, Wieczorek and Todd (1997) found that *C. intestinalis* settlement increases with greater biofilm age. Though *B. violaceus* has not been studied in this context, it is possible that differences in biofilms, brought on by surface roughness, material chemical components, or substrate immersion time, might contribute to observed substrate preferences. However, trials examining the role of biofilms on settlement did not support this conclusion.

Larval settlement patterns varied slightly between parent organism collection locations, though it doesn't appear that parent substrate systematically influenced either *B. violaceus* or *C. intestinalis* settlement (Tables S1, S2, Figs. S1, S2). However, this study did not initially seek to examine inherited substrate preferences, necessitating further examination of this topic.

Understanding the influence of substrate material on fouling organism settlement has important implications for experimental marine ecology. Relatively few settlement and benthic community studies have incorporated multiple substrate materials, and studies that utilize only one substrate type rarely offer a justification for this choice. As substrate material can influence settlement, future studies should seek to adequately justify material choice based upon experimental goals, or utilize multiple materials. These findings also highlight the importance of future research into substrate effects in the field. If the settlement patterns observed in this study are representative of field trends, it is possible that the use of concrete and HDPE for marine construction could encourage ascidian settlement. These two materials are widely used in marine systems, and could potentially foster the spread of non-native ascidians by providing preferred settlement sites.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.marpolbul.2016.03.049.

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