A true test of colour effects on marine invertebrate larval settlement.

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Abstract

Past tests of settlement by marine invertebrate larvae on different colours were not designed to distinguish between responses to substrate colour and substrate brightness. Using colour vision testing methods, the design of this study provided a true test for responses to colour by including both coloured and grayscale settlement plates. The dominant fouling taxa at the test sites (the solitary ascidian, *Ciona intestinalis*; the colonial ascidians *Botryllus schlosseri* or *Botrylloides violaceous*; and a bryozoan *Bugula* sp.) showed no significant differences in settlement between blue, red or green plates. In contrast, the ascidians responded to substrate brightness with significantly lower settlement on lighter plates, while the bryozoans showed no significant preference relative to substrate brightness. The methods used here are a model for future tests of responses to colour during settlement. In addition, the contrasting responses to brightness are at odds with previous laboratory experiments that showed larvae of both ascidians and bryozoans are negatively phototactic at settlement. These results suggest that other cues must supersede any phototactic responses in the bryozoans, or alternatively that the range of light intensities near darker and lighter substrata were not part of the tests in the earlier reports.

1. Introduction

Settlement plates are used widely in marine biology, including studies of larval biology, invasive species, and biofouling (e.g., Collin et al., 2013; Ellrich et al., 2015; Humanes and Bastidas, 2015; Krishnan et al., 2015). Experiments place the plates in marine habitats and subsequently monitor the abundance or diversity of organisms or species that settle on the plate. Many of these experiments have not explicitly controlled for the effect of colour. Often, this is simply because all plates deployed are constructed from the same material, and thus any

differences in colour once the plates are deployed (due to differences in incident light) are reasonably assumed to be negligible. Other experiments, however, particularly those dealing with applied aspects of biofouling and antifouling coatings, involve plates with different materials which may be different colours (as perceived by humans). Unfortunately, it is hard to assess the prevalence of this situation, since colour is rarely reported for coatings (e.g., Vucko et al., 2012; Watson et al., 2015; Zargiel and Swain, 2014). Regardless, if coating colours do vary, controls for the effect of colour (as perceived by the settling animals) are needed to properly test the effect of the other differences among the settlement plate surfaces. A few studies have been conducted to test responses to colour, however, these have not been designed appropriately given the complicated nature of colour perception.

Tests of behavioural responses to colour must take into account the complexities of light stimuli and the proximate mechanisms underlying the responses of animals to those stimuli (Kelber et al., 2003; Skorupski and Chittka, 2011). Responses to light stimuli depend on the spectra of the stimuli, the number and spectral sensitivities of photoreceptor types, and the sensory processing circuits of the nervous system that ultimately control the behavioural responses to light. To the authors' knowledge, no previous studies of settlement responses to colour have fully articulated what aspect of colour responses was being tested (Dahlem et al., 1984; Dobretsov et al., 2013; Finlay et al., 2008; Guenther et al., 2009; Hodson et al., 2000; James and Underwood, 1994; Robson et al., 2009; Satheesh and Wesley, 2010; Su et al., 2007; Swain et al., 2006; Yule and Walker, 1984). Among studies of the sensory biology of vision, the usual starting point is to establish whether an animal has colour vision. In particular, the test must attempt to differentiate between responses driven by spectral composition differences (i.e. colour) and intensity (i.e. brightness) (Crisp, 1976). Animals can differentially respond to

different colours without having colour vision because of how light spectra may or may not match the spectral sensitivity of photoreceptor pigments. Crucially, however, an animal without colour vision should show the same differential response to achromatic light of differing overall intensities (i.e., brighter or darker light without any variation in intensities with respect to wavelength). Thus, the basic experimental design for testing for responses to colour requires treatments that can be compared to determine if differential responses arise due to differences in intensity or spectral composition. A simple approach is to test neutral gray stimuli with varying intensities that establish whether or not responses to the coloured stimuli can also be produced by stimuli that vary in intensity (as will the coloured stimuli) but not in spectral composition (unlike the coloured stimuli). This method has not been a part of previous tests of settlement responses to colour. Some experiments have included black and white stimuli, but no previous tests have included a set of gray stimuli to properly assess differential responses due to intensity alone.

Fortunately, the results of some previous studies of some taxa have not found strong responses to colour (Guenther et al., 2009; Satheesh and Wesley, 2010; e.g., Visscher and Luce, 1928), and thus the conclusions are not compromised by omitting the gray stimuli which can help distinguish colour versus intensity responses. Nonetheless, a systemic bias exists in these previous studies that lack the gray stimuli. The experiments would never have been able to conclude that any differential settlement was due to colour, and not intensity. Indeed, some studies have concluded an effect of colour on some taxa, with barnacles and tubeworms reported as having differential settlement as a consequence of colour, without including grayscale controls for intensity (Robson et al., 2009; Satheesh and Wesley, 2010; Yule and Walker, 1984). Moreover, at least one other study has concluded that responses were due to differences in intensity while only testing coloured surfaces (Su et al., 2007). Finally, yet other studies have

tested for an effect of "colour" when in fact only black and white or grayscale treatments were tested, presenting a problem of semantics rather than evidence (Dahlem et al., 1984; Darbyson et al., 2009; Dobretsov et al., 2013; Hodson et al., 2000; James and Underwood, 1994; Swain et al., 2006). Thus, given this right-royal-mess, the first motivation for this study was to introduce the practice of an unbiased experimental design, including grayscale and colour treatments as an initial step to understanding whether or not the animals in question use more than light intensity to guide settlement behaviour. The methods also attempt to remove all other potential biases, including the possibility that different coloured backgrounds could affect a human observer's ability to measure settlement.

The second motivation was more practical. In a recent study, differences were observed in settlement rates by ascidians on potential antifouling treatments with different colours (Filip et al., 2016). Thus, the second goal was empirical verification that colour does not affect settlement of the ascidians (and other dominant taxa) at the testing sites in Nova Scotia, Canada. In the current experiment, ascidians and bryozoans were the two taxa that dominated settlement on the plates. Larvae of most benthic marine invertebrates typically become photonegative as they age, presumably to promote settlement on the benthos (Thorson, 1964). In ascidians, Svane and Young (1989) have suggested the behavioural ontogenies may actually be more complicated and vary substantially between species. Nonetheless, among the ascidians present at the sites in this study, the solitary *Ciona intestinalis* Linnaeus has been shown to follow the typical pattern (Nakagawa et al., 1999). No data is available for the two colonial species present (*Botryllus schlosseri* Pallas or *Botrylloides violaceous* Oka). To the authors' knowledge, only one previous study has considered differential settlement rates by an ascidian (*Didemnum* sp.) in response to colour, and no significant responses were found (Satheesh and Wesley, 2010).

Results have been largely similar for bryozoans. Either of the two bryozoan species that may have settled on the plates in the current study (*Bugula fulva* Ryland and *B. turrita* Desor) have been recorded as initially positively phototactic, switching to negative phototaxis later in larval life (Ryland, 1977). Similar patterns have been found for a number of other bryozoans (Ramirez and Cancino, 1991; Ryland, 1977; Wendt and Woollacott, 1999). Bryozoans have not been studied with respect to colour. Thus, given these past results, fouling by both ascidians and bryozoans was expected to be higher on darker plates. In addition, a preference for colour was not expected, based primarily on the general trend that colour vision is often of lesser importance in marine habitats (Marshall et al., 2015).

2. Materials and Methods

2.1. Colour Treatments

Colour and grayscale settlement plates were created by laminating commercial paint sample cards (Table 1; Fig. 1A). The three colours were chosen to span the visible spectrum (red, green, and blue). Without information on the absorption spectrum of the photopigments of the animals tested, it is not possible to choose grays that will match the intensity of the colour treatments, and thus grays were similarly chosen to span intensities from white to black. For each plate, two cards were trimmed to include only the coloured area, and laminated end-to-end inside clear Scotch Self Laminating Pouches (LS854WS) using a Scotch Thermal Laminator (TL902-C). The colour treatment area was 7 x 20 cm, with the laminate cut to provide a 1 cm clear border to match a 9 x 25 cm polyvinyl chloride back plate. An additional clear plastic laminate (same material as above, but with nothing inside the laminate pouch) was placed over top of the colour treatment, and the three layers (clear settlement laminate, colour laminate and

back plate) were cable tied tightly together (Fig. 1B). Thus, the different colour treatments all had identical surface chemistry, and the outer clear layer could be removed for quantification of settlement without bias from differences in the background colour.

2.2. Deployment

Deployment frames were constructed from 2 inch diameter acrylonitrile butadiene styrene pipe (inside dimensions of 82 x 39 cm). Plates from each treatment (in random order) were secured with cable ties inside each frame, leaving a 1 cm gap between each of the plates and a 7 cm gap between plates and the top and bottom of the frame. Eight frames (each with the eight treatments) were deployed from docks at each of two sites (Fig. 1C). All frames were suspended at 1 m depth, with the treatment plates facing the dock (to minimize inter-frame variation in illumination due to shadows from boats and other structures). At Port Hawkesbury (Strait of Canso Yacht Club, Nova Scotia, Canada; 45.6134, -61.3653), frames were deployed on July 3rd, 2014 and sampled twice, 28 and 37 days later. At Cribbons, (Cribbons Point Marina, Nova Scotia, Canada; 45.7558, -61.8971), frames were deployed on August 12th, 2014 and sample twice, 11 and 18 days later. Sampling days were chosen based on regular monitoring of settlement and growth on the frames and an attempt to record settlement at approximately 50% coverage and just before the plates reached coverage saturation. All frames, back plates and colour treatments were reused at the second site, with new clear laminate settling surfaces attached.

2.3. Settlement Measurements

A custom constructed light box was used to provide consistent illumination for photos of the clear settlement layer from each plate. The illumination source was an inverted transparent Rubbermaid box (53 x 37 x 33 cm; length x width x height) with flexible LED tape (Canarm

LED3528TW5M, powered by a Panasonic LC-R127R2P battery) attached to the inside surface of the lid, and two plates of frosted glass attached to the upper surface of the box. The diffused light from the LEDs thus provided a uniform background illumination for the settlement surfaces placed on top of the frosted glass, while external light sources were excluded by placing the light box inside a larger, opaque Rubbermaid container. Photos were taken with a digital camera (Nikon S5100) through a hole in the centre of top of the outer opaque box.

2.4. Analysis

For each photo of a plate, individuals were counted for four different primary taxa that settled during the experiment: the solitary ascidian *C. intestinalis*, two colonial ascidians (either of *B. schlosseri* or *B. violaceous* at both sites), bryozoans (*B. fulva* or *B. turrita*., at Port Hawkesbury; differences between the two species could not be distinguished in the photos) and hydroids (species unidentified, and excluded from further analysis due to low settlement rates on most plates). Counts were performed in FIJI software (Schindelin et al., 2012), using the Cell Counter plugin. Repeated measures (for the two sampling dates at each site) multivariate analysis of variance (MANOVA) with Tukey's post-hoc comparisons ($\alpha = 0.05$) were performed in SPSS v22 (IBM) to determine if plate colour had an effect on the settlement patterns of each of the taxa found at each site. To improve normality, data were log transformed prior to analysis. Following transformation, departures from normality were restricted to just some of the *C. intestinalis* data, and for these, the analyses rely on the robust nature of the MANOVA (O'Brien and Kaiser, 1985). Deployment frame was included in the analyses as a between-subject (blocking) factor.

3. Results

3.1. Colonial ascidians: Botryllus schlosseri *and* Botrylloides violaceous

The two species of colonial ascidians settled with relatively high abundance at both sites. In both locations, no significant differences were observed among the colour treatments (Fig. 2A; Table 2). In contrast, substrate brightness had similar significant effects on settlement at both locations. Colonial ascidian density was significantly lower on the white treatment compared to all other treatments at Cribbons, and compared to all other treatments except light gray at Port Hawkesbury. Otherwise, despite a trend of increasing density as substrate brightness decreased on the second sampling day at both sites, the differences between successively darker treatments were not always significant (e.g., colonial ascidian density on lighter gray was significantly lower than on either medium gray or black, but not dark gray).

3.2. Solitary ascidian: Ciona intestinalis

The ascidian *C. intestinalis* was only observed at Port Hawkesbury. A substantial die-off of this species occurred between the two sampling dates, resulting in substantially lower abundances at the site than in previous years (pers. obs.). Nonetheless, significantly higher densities of *C. intestinalis* settled on the black and dark gray treatments compared to the white (Fig. 2B; Table 2). No significant differences between black, any of the grayscale, or colour treatments were observed.

3.3. Bryozoans: Bugula *sp.*

The bryozoan *Bugula* sp. was also only found in Port Hawkesbury. No significant differences in bryozoan densities were observed between any of the white, black, gray level or colour plates (Fig. 2C; Table 2).

4. Discussion

Substrate colour had little effect on the settlement patterns of any of the taxa observed. For *C. intestinalis*, colonial ascidians and *Bugula* sp., no significant difference in settlement density was found between any of the colour treatments (red, green, blue) at either site. Many marine animals lack colour vision, possibly since their visual systems are optimized for overall sensitivity to light (Marshall et al., 2015). Thus, it may be that the ascidian and bryozoan larvae do not need colour discrimination of substrata, but do benefit from discriminating different levels of brightness while settling.

Substrate brightness had differing effects on settlement. Both *C. intestinalis* and the two species of colonial ascidians (*B. schlosseri* and *B. violaceous*) settled at significantly lower densities on lighter plates. Given this pattern, the results from the coloured plates were as expected, with no significant difference between them and the various grayscale plates with comparable overall brightness. These results thus are all consistent with those previous studies that indicated that ascidian larvae prefer to settle on darker rather than lighter surfaces, presumably due to a photonegative response in the larvae (Darbyson et al., 2009; Dobretsov et al., 2013; Nakagawa et al., 1999; Swain et al., 2006). In contrast, no significant difference was observed between the settlement densities of bryozoans on any of the grayscale treatments, despite previous studies showing their larvae also become photonegative prior to settlement (Ryland, 1977). Thus, the two taxa provide contrasting examples of how laboratory examination of responses to specific cues may or may not predict behaviours in the field. In the field, the bryozoans may prioritize cues other than brightness, or alternatively, the range of light levels used in the laboratory experiments may not correspond to those experienced by bryozoans near to lighter versus darker substrata.

Taking into account the results here for various species of ascidians and bryozoans, future short-term studies on fouling and antifouling by these species do not necessarily require colour controls as part of their experimental design. On the other hand, substrate brightness significantly affected ascidian settlement densities. Thus, the ascidians in this study join a number of other taxa (Dobretsov et al., 2013; Swain et al., 2006) for which coating brightness could be a confounding factor in any differences among antifouling treatments. Consequently, short-term antifouling studies in the field should probably measure the reflectance spectra of coatings and consider appropriate controls: either controls with total reflectance that match that of the test coatings, or multiple controls that bracket the total reflectance of the test coatings. This practice could be included in all studies as a precautionary measure, but is particularly needed for experiments with ascidians, which have recently been recommended as a target for further antifouling studies (Aldred and Clare, 2014). Further work is required to assess whether long-term studies spanning a year or more must also incorporate controls for spectral differences among tested coatings.

The methods employed here stand as a model for any further experiments designed to truly test the effect of substrate colour on settlement (in other taxa, different colours, locations, etc.) Inclusion of both colour and appropriately selected grayscale treatments are essential for distinguishing between colour and brightness responses in the animals. Moreover, a number of earlier studies have used sample sizes of 5 or less per treatment combination (Dahlem et al., 1984; James and Underwood, 1994; Robson et al., 2009; Su et al., 2007; Swain et al., 2006). For the rapidly growing species studied here, a sample size of 10 was likely the minimal requirement to establish the validity of any trends in the face of the variation observed. Thus, in the future, robust experimental designs testing the effects of colour on settlement may require including

similar levels of replication as well as the full set of both grayscale and colour treatments. Since both considerations result in high total numbers of test plates, this will likely force some heterogeneity in the immediate environment around the plates. In particular, differences in incident light (which will affect total reflectance) may be hard to avoid. Therefore, minimizing differences among treatments by having all face the same direction relative to the sun, and using a blocked design for statistical analysis are both likely to be useful in the experimental design.

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Figure Legends

Fig. 1. Settlement plates. A. Mean (n = 8) reflectance spectra (normalized to the maximum reflectance across all plates) for the laminated white, black, gray (3 shades) and colour (red, green, and blue) treatments (see Table 2 for details). In order to approximate the reflectance spectra generated during deployment in the field, spectra were collected in shaded natural daylight, from plates submerged in seawater (1 cm depth), with an Ocean Optics S2000 spectrophotometer equipped with a QR 400-7-UV probe. B. Surface view of a white settlement plate, including the clear superficial laminate layer, laminated white card, and back plate secured to the deployment frame. C. Deployment frame with 8 settlement plates (in randomized order: blue, red, green, black, white, medium gray, light gray, dark gray). Plate dimensions: 9 x 25 cm; colour treatment area: 7 x 20 cm.

Fig. 2. Settlement densities on grayscale and coloured plates (see legend and Fig. 1) at two different sites on two different days following original deployment. A. Colonial ascidian settlement was affected by substrate brightness but not colour (at Cribbons, density was significantly less on white plates than all other plates; at Port Hawkesbury, density was significantly less on white than all other treatments except for light gray). B. Settlement by *Ciona intestinalis* was affected by substrate brightness but not colour. A significantly higher density of *C. intestinalis* was found on black plates than on white plates. C. Settlement by *Bugula* sp. was not affected by substrate brightness or colour. No significant differences occurred between densities of bryozoans on any treatments. Statistical data: homogenous subsets of treatments based on Tukey's post-hoc analyses following a repeated measures MANOVA are indicated by letters adjacent to the plate letter codes for each panel (ns, not significant). Box plot parameters: The box represents the 25th and 75th quartiles, the line

represents the median, the whiskers represent the minimum and maximum, circles represent outliers that are >1.5 times the interquartile range, and plus signs indicate extreme values that are >3 times the interquartile range.

Tables

Table 1. Normalized total reflectance intensity for the colour and grayscale treatments created from Pittsburgh Plate Glass Company paint cards. The five shades of gray cards (from white through black) provide a range of brightness values that span the brightness values of the three colour treatments. See Figure 1 for spectroscopic methods and reflectance spectra.

Colour	Paint Card	Brightness
White	Delicate White NT33	1.00
Light Gray	Flagstone NT36	0.43
Medium Gray	Dover Gray NT37	0.20
Dark Gray	Knight's Armor NT38	0.08
Black	Black Magic NT39	0.03
Red	Candy Corn CC16	0.26
Green	Wistful Walk EB15	0.14
Blue	Kimono CC4	0.25

Table 2. Repeated measures MANOVA statistics testing the effects of substrate brightness and colour on settlement patterns of colonial ascidians (either of *Botryllus schlosseri* or *Botrylloides violaceous* at both sites), *Ciona intestinalis* and *Bugula sp*. Data from each species present at each site were log transformed and tested separately, with model factors including time (repeated measures made on two different days), deployment frame (as a blocking factor), and plate colour (comprising black, white, three shades of gray and three colours; see Fig. 1 and Table 1).

Organism	Site	Effect	Statistic	P-value
Colonial	Cribbons	Time	$F_{1,47} = 977$	<0.001
		Frame	$F_{7,47} = 31.6$	<0.001
		Colour	$F_{7,47} = 15.0$	<0.001
Colonial	Port Hawkesbury	Time	$F_{1,49} = 732$	<0.001
		Frame	$F_{7,49} = 11.1$	<0.001
		Colour	$F_{7,49} = 6.23$	<0.001
C. intestinalis	Port Hawkesbury	Time	$F_{1,49} = 52.2$	<0.001
		Frame	$F_{7,49} = 3.16$	0.008
		Colour	$F_{7,49} = 3.19$	0.007
Bugula sp.	Port Hawkesbury	Time	$F_{1,49} = 420$	<0.001
		Frame	$F_{7,49} = 14.0$	<0.001
		Colour	$F_{7,49} = 0.778$	0.674

Figure 1

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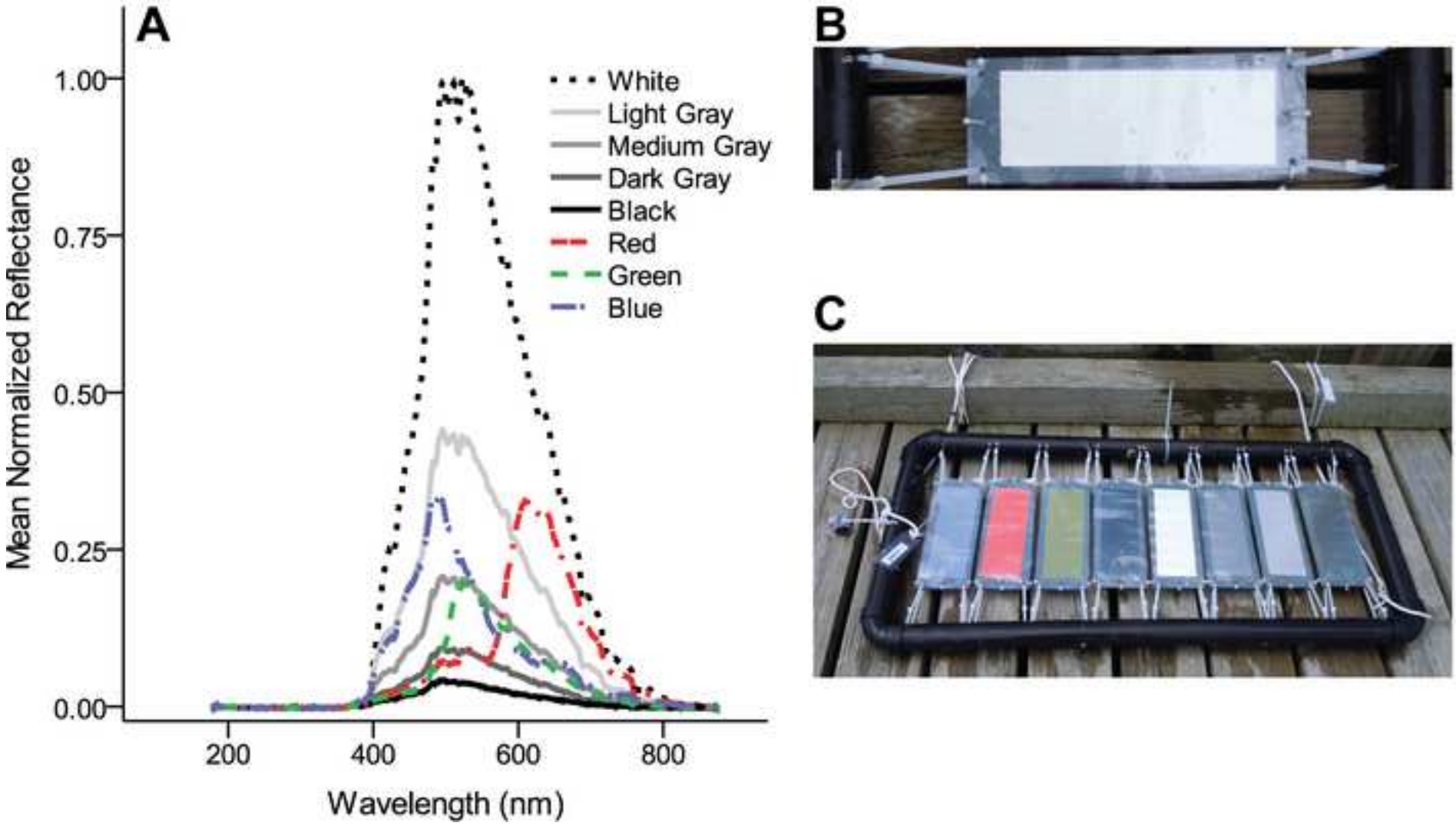
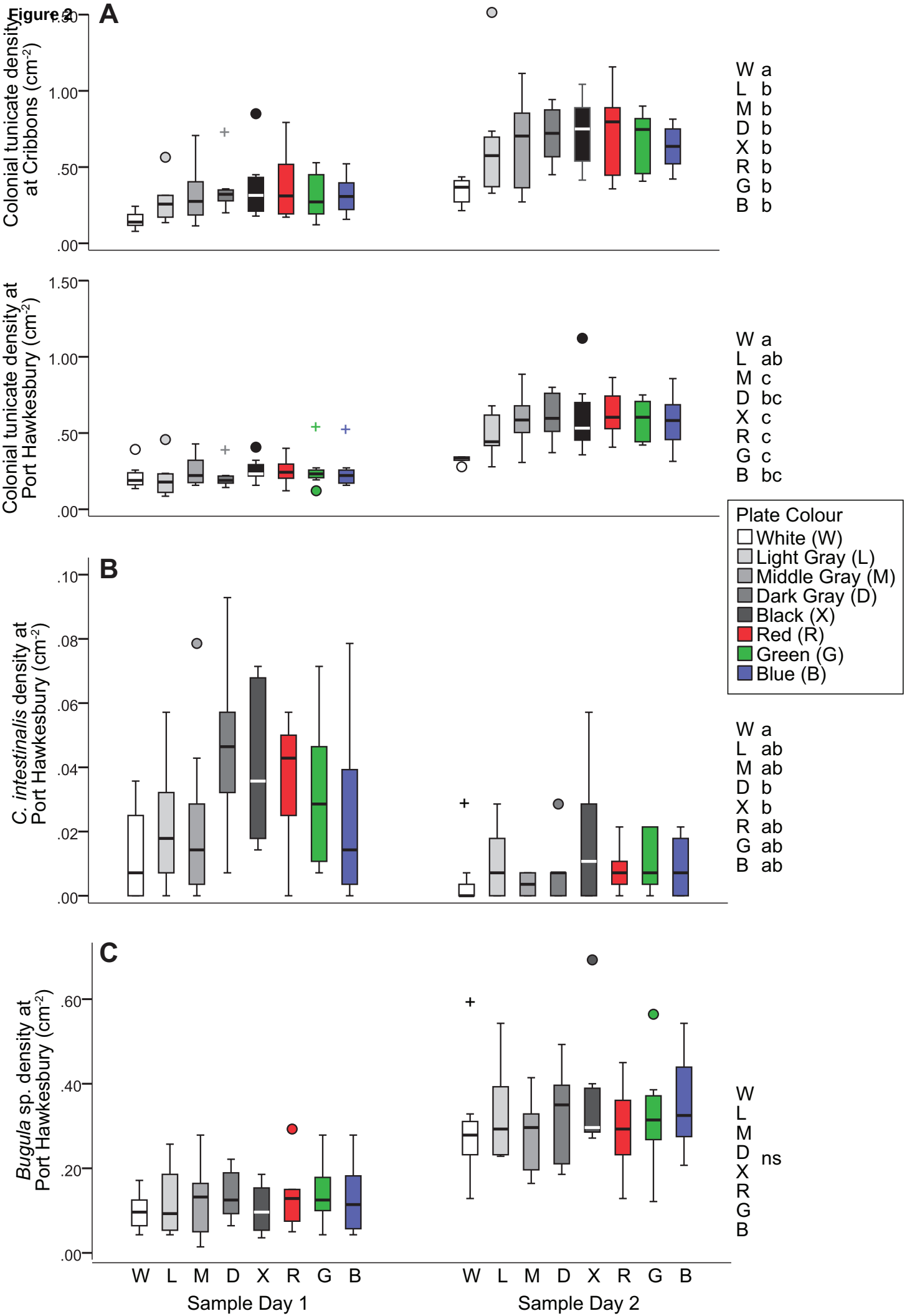


Figure 2


A
Colonial tunicate density at Cribbons (cm-2)
1.50
1.00
.50
.00
W a
L b
M b
D b
X b
R b
G b
B b
Colonial tunicate density at Port Hawkesbury (cm-2)
1.50
1.00
.50
.00
W a
L ab
M c
D bc
X c
R c
G c
B bc
Plate Colour
White (W)
Light Gray (L)
Middle Gray (M)
Dark Gray (D)
Black (X)
Red (R)
Green (G)
Blue (B)
B
C. intestinalis density at Port Hawkesbury (cm-2)
.10
.08
.06
.04
.02
.00
W a
L ab
M ab
D b
X b
R ab
G ab
B ab
C
Bugula sp. density at Port Hawkesbury (cm-2)
.60
.40
.20
.00
W
L
M
D
X
R
G
B
ns
W L M D X R G B
Sample Day 1
W L M D X R G B
Sample Day 2

Supplementary Material

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