THE ECOLOGY AND DEMOGRAPHY OF THE INVASIVE ASCIDIAN *BOTRYLLOIDES VIOLACEUS* IN THE COOS ESTUARY

by

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Introduction

Estuarine fouling communities

Man-made structures such as docks, pilings, and floats serve as hard surfaces (or substrata) on which sessile marine invertebrates can grow (Railkin 2004; Nydam & Stachowicz 2007). Assemblages of invertebrates on artificial substrata are known as fouling communities, which are common in bays and harbors, and exhibit a wide diversity of estuarine species dominated by sponges, ascidians, bryozoans, hydroids, sessile polychaetes, barnacles, and mussels. Such communities are often characterized by high biomass due to the many strategic advantages of coastal habitats: warmer temperatures and greater illumination of shallow surface water increases primary productivity and therefore food availability for filter-feeding invertebrates. Also, harbors often have abundant, hard substrata off the bottom (e.g. floating docks) on which sessile organisms can grow nearer to the surface where higher current velocity improves feeding and efficiently removes waste from filter-feeders. Moreover, these substrata are typically close enough to the bottom to be settled by larvae from benthic communities but allow species to evade benthic predation (Railkin 2004). These characteristics of coastal fouling sites make fouling communities highly susceptible to invasion by non-native species.

Estuarine habitats are among the most heavily invaded ecosystems on Earth due to the high frequency of non-native species introduction, anthropogenic disturbance, and proximity of fouling structures to commercial shipping ports (Nydam & Stachowicz 2007). Commercial boat traffic facilitates two primary vectors (methods of transport)

for marine species invasion: ship hull fouling and the uptake in ballast water (Hewitt 1993; Carlton & Geller 1993; Carlton 1999; Moyle 1999). Ships can transport thousands of tons of ballast water from one bay to another, potentially introducing numerous marine species to a new habitat (Carlton 1999). Non-native species are also transported to estuarine habitats on aquaculture equipment moved within and between systems, and via intra-bay recreational boat traffic (Hewitt 1993). Introduced species may perish in a new habitat if conditions are inhospitable for the species, but anthropogenically-disturbed estuarine ecosystems have a high rate of survival of introduced species (Moyle 1999). While artificial substrata in the ecosystem benefit many native estuarine species, these structures confer a competitive advantage to non-native species, since they provide refuge from benthic predators (Gittenberger & Moons 2011; Simkanin *et al.* 2013).

The development of estuarine fouling communities depends on several factors related to the settlement and growth of fouling species. Recruitment events, which vary with species and season, drive community composition, as different species initially settled on bare substrate can lead to different "climax" communities (Sutherland 1974; Osman 1977; Sutherland & Karlson 1977; Sams & Keough 2012). However, competitive interactions between fouling species can have a greater impact on community development in those dominated by long-lived colonial species (Sams & Keough 2012). Of primary importance are overgrowth interactions, or the competitive displacement of one species by another (Osman 1977; Sutherland & Karlson 1977; Buss & Jackson 1979; Russ 1980; Russ 1982; Sebens 1986). Such interactions are not necessarily lethal, but can lead to the spatial dominance of a superior competitor in a

community (Russ 1982; Sebens 1986). A fouling species' distribution and abundance depends heavily on their ability to overgrow or resist overgrowth by other species (Jackson 1979). The competitive abilities of common fouling taxa typically follow the following hierarchy: ascidians ≥ sponges > bryozoans > barnacles, polychaetes, amphipods, and hydroids. However, the relative competitive ability of particular species of different taxa can form more complicated networks that do not necessarily fit into this hierarchy (Russ 1982). Furthermore, changes in the relative size of two competitors can lead to reversals in the outcome of an interaction, and often species will interact in a "standoff," in which neither species overtakes the other (Russ 1982; Sebens 1986).

Effects of invasive species on native estuarine species

Vitousek (1990) outlines three primary ways invasive species can affect ecosystems: 1) invasive species can modify the physical and chemical conditions of a habitat by their acquisition and use of resources, 2) invasive species can alter the composition of major trophic groups in either a top-down or bottom-up fashion, and 3) invasive species can change the frequency and intensity of disturbances to typical community development. While outright extinction of native species has never been attributed to invasive species in estuarine systems, invasive species can assume a "keystone" role by modifying fouling community structure and dynamics (Carlton 1993; Cox 1999). Invasive species have been responsible for both declines and growth in native species populations: the invasive mussel *Mytilus galloprovincialis* outcompetes native mussels in southern California, Europe, and South Africa (Carlton *et al.* 1999), and invasive ascidians *Diplosoma listerianum* and *Didemnum vexillum* promote population growth of native sea star *Henricia sanguinolenta*, a common

predator, in the Gulf of Neddick (Dijkstra *et al.* 2013). The dominance of non-native species over native species depends on diversity and resource use of the fouling community, and can result in either increases or decreases in species richness (Stachowicz & Byrnes 2006). In either case, non-native species can alter the ecological character of the fouling communities they invade (Ruesink *et al.* 2006). As a result, some cases of declining ecosystem health have driven local efforts to eradicate invasive species such as the zebra mussel (*Dreissena polymorpha*).

Process of successful invasion

Many factors impact the development of fouling communities and may limit the successful invasion of non-native species into a system. These factors can be categorized as abiotic (proximity to the ocean, hydrodynamics, temperature, and salinity) and biotic (competition, predation, and recruitment) (Nydam & Stachowicz 2007). The physiological tolerance or adaptations of an invasive species to abiotic conditions are among the most important factors in determining whether the species can invade a habitat (Cox 1999; Hengeveld 1999; Moyle 1999, Sandlund *et al.* 1999). In estuaries, salinity is a significant abiotic factor impacting species survival at the interface between marine and freshwater systems (Connell 1972). The invasion of estuarine systems follows a step-wise process which often includes a "lag" between initial introduction and population explosion (Sandlund *et al.* 1999). During the lag period, the population of a species is small and minimally impacts the native ecosystem (Cox 1999). Three mechanisms might explain this lag period: 1) the nature of population growth can cause an inherent lag that is simply the time necessary for a species to expand its population to a size with high colonization potential, 2) a natural

or anthropogenic change in a factor that previously limited an invasive species (such as habitat and food resources, climate, or inter- or intra-specific interactions) may increase the suitability of a habitat for an invasive species, and 3) genetic factors related to the reduced fitness of a non-native species in a novel environment may require successive generations to pass before a species can be competitive (Cox 1999; Crooks & Soule 1999). In response to any of these mechanisms, the growth rate of an invasive species may be "released," at which point the species becomes aggressively expansive and can dominate the environment (Crooks & Soule 1999; Sandlund *et al.* 1999). Often this occurs by way of overgrowth, in which a species outcompetes others for space using physical and chemical aggression, bulldozing and smothering, or successful competition for food resources (Sebens 1986). It is during this time that invasive species insert themselves into the normal process of succession, the development of community composition beginning with initial settlers on a substratum, followed by secondary and tertiary species that colonize and compete to occupy the final "climax" stage of an established fouling community (Railkin 2004).

Shifts in abiotic water quality conditions associated with climate change, including rising sea surface temperatures, often make habitats more favorable for invasive species and less favorable for natives. A negative feedback loop accelerates these patterns: invasives may simplify and homogenize habitats by outcompeting native species, which reduces the resilience of communities to changes in water quality conditions and makes them more susceptible to future invasions (Mooney & Hofgaard 1999). One major impact of climate change on marine communities is expected to be the shifting of recruitment timing; shifting minimum and maximum temperatures may

allow more tolerant invasive species to get a "head start" on native species by recruiting earlier and occupying more space than native species. This can change the order in which species colonize space and may therefore also alter successional patterns and shift dominance in fouling communities to invasive species (Stachowicz *et al.* 2002; Agius 2007).

Invasive colonial ascidians

According to Sutherland (1977), a non-native species need only have two of the following three life history characteristics in order to be a successful invader: a high recruitment rate, the ability to settle on top of other organisms, and a long lifespan. Colonial ascidians (Phylum: Chordata) are a particularly invasive group of marine organisms due to their reproductive capability, plastic life history and growth rates, and competitive dominance via epibiotic settlement and overgrowth (Stoner 1992; Railkin 2004), all of which relate to Sutherland's first two requirements for invasion.

Colonial ascidian recruitment

In contrast to solitary ascidians, colonial ascidians have high fecundity and brood internally fertilized, large lecithotrophic (feeds only on its yolk) swimming larvae with short dispersal periods and chemical defense mechanisms or deterrent coloration to compensate for their conspicuousness (Young & Bingham 1987; Svane & Young 1989; Tarjuelo & Turon 2004, Young *et al.* 2006). Larvae actively select suitable substrata for settlement using a well-developed nervous system and adhesive papillae, allowing larvae to detach and reattach at sites more suitable for their survival (Svane & Young 1989, Young *et al.* 2006). Due to the development of oozooids (the first zooid, or

colonial unit, in a colony) in the larvae, these species rapidly metamorphose upon settlement and reach maturity early (Railkin 2004). These features reduce the risk of mortality of colonial ascidian larvae during both planktonic (pre-settlement) and post-settlement phases (Tarjuelo & Turon 2004). Once settled, colonial ascidians grow rapidly via lateral asexual budding of the oozooid (the first zooid, or primary unit, in a colony) into blastozooids and exhibit high epibiotic potential, the ability to overgrow organisms in all other groups (Russ 1982; Railkin 2004; Young *et al.* 2006; Epelbaum *et al.* 2009b; Kurn *et al.* 2011). Growing adult colonies are much less susceptible to "eliminating factors," such as predation and water quality conditions, than larvae, because larvae must carefully select settlement locations with tolerable abiotic conditions (Vázquez & Young 1996; Railkin 2004).

Colonial ascidian competitive dominance

Colonial ascidians can quickly outcompete native species for space on hard artificial surfaces and thereby alter the species composition of fouling communities by dominating the assemblage (Jackson 1977; Van Dolah *et al.* 1988; Railkin 2004; Simkanin *et al.* 2013). Ascidians are superior competitors to all other common fouling taxa except for sponges, which have comparable competitive abilities (Russ 1982; Sebens 1986). Invasive colonial ascidians have altered the native species composition by dominating fouling communities in Long Island Sound (Osman & Whitlatch 1995), the Gulf of Maine (Harris & Tyrrell 2001), and several Dutch harbors (Gittenberger & van der Stelt 2011). Colonial ascidians can resist their own overgrowth using various chemical defenses in their tunic, a body wall matrix of proteins and carbohydrates that encompasses the entire organism (Mackie & Singla 1987). Though resistant to

overgrowth by many other taxa, biotic competition on colonial ascidians can limit their growth rate and competitive ability: the absence of competition increased the growth rate of *Didemnum perlucidum* nine-fold, and it increased female gonad production for further sexual reproduction of the species (Dias *et al.* 2008). Colonial ascidians respond positively to disturbances in a habitat, such as the clearing of primary substrate, which makes them especially invasive in estuarine fouling communities (Altman & Whitlatch 2007).

In addition to their ability to compete for space, colonial ascidians may also outcompete other filter-feeding species for food. Each zooid in a colonial ascidian has an inhalant siphon through which the organism pumps water to take in food, and colonial ascidians can efficiently consume nanoplankton too small for consumption by other filter-feeders. As a result, colonial ascidians can better survive habitats with low particle concentration and dominate by competing for the same food resource as cultured shellfish and native fouling species on the docks and aquaculture equipment they overgrow (Petersen 2007).

Botrylloides violaceus **invasion ecology**

Botrylloides violaceus (Oka 1927) (Chordata: Ascidiacea), is a particularly invasive colonial ascidian, well-known as a "biofouling nuisance species" (Bock *et al.* 2011). Initially introduced from the NW Pacific, this species has invaded harbors and ports around the world, including the East and West coast of North America, Australia, Italy, the Netherlands, and the U.K. (Zaniolo *et al.* 1998; Carver *et al.* 2006; Gittenberger 2007; Minchin 2007). In *B. violaceus,* oval or oblong zooids are arranged in rows or loops to form an encrusting, sponge-like mat that grows on hard substrata

such as rocks, artificial structures, or other fouling species such as *Mytlius* spp. (Dijkstra & Harris 2009; Epelbaum *et al.* 2009b). Like other colonial ascidians, *B. violaceus* can easily overgrow and outcompete both native and other non-native species for space, allowing it to become abundant and in fouling communities (Berman *et al.* 1992; Dijkstra & Harris 2009; Simkanin *et al*. 2013). While the population growth rate of *B. violaceus* is projected to remain stable with increasing effects of climate change, the competitive dominance of this species will likely increase with warming ocean temperatures due to its ability to outcompete most species it encounters and tolerate a wide range of temperatures and salinities (Cockrell & Sorte 2013). Several vectors have been proposed as mechanisms for the global spread of *B. violaceus*, including boat hull fouling, ballast water, movement of aquaculture equipment, and epibiotic growth on mobile crustaceans (Bock *et al.* 2011). Due to the short life span of this species, natural propagule dispersal and ballast water transport are likely not causes of large-scale spread of the species (Bock *et al.* 2011), but may influence intra-site distribution.

Botrylloides violaceus colonies grow laterally via asexual budding of new zooids, which is occasionally aided by colonial fragmentation in which colonies reattach to substrata after physical separation and grow as two or more individual colonies (Edlund & Koehl 1998; Epelbaum *et al.* 2009b). Attempts to remove this invasive species at aquaculture facilities using high-speed pressure washing has, in some cases, exacerbated the invasion since *B. violaceus* can reattach to the substratum and grow after fragmentation (Bock *et al.* 2011). Colonies grow asexually during the spring and summer and hibernate in the winter, during which time colonies recede in size and presumably do not sexually reproduce (Hewitt 1993; Epelbaum *et al.* 2000;

Stachowicz *et al.* 2002; Dijkstra & Harris 2009; Dijkstra *et al.* 2011). Hibernation permits coexistence with other fouling taxa by freeing up primary substrata, since *B. violaceus* has no predators to limit its population growth (Carver *et al.* 2006; Simoncini & Miller 2007; Whitlatch & Osman 2009).

In addition to asexual budding, *Botrylloides violaceus* also reproduces sexually, fertilizing internally and brooding embryos until large tadpole larvae are released into the water column from shared excurrent siphons (Young *et al.* 2006; Epelbaum *et al.* 2009b). This short larval stage lasts from minutes to hours, allowing for a potential dispersal distance between one and 100 meters depending on surrounding water currents (Epelbaum *et al.* 2009b; Simkanin *et al.* 2013). Sexual reproduction is typically seasonal and observed in some populations from June to late September (Hewitt 1993; Epelbaum *et al.* 2000; Stachowicz *et al.* 2002; Dijkstra *et al.* 2011), though year-round recruitment has been documented (Powell 1970; Ross & McCain 1976). This species boasts high recruitment success due in part to its ability to settle epibiotically in habitats with limited primary substrata (Hewitt 1993).

Coos Estuary

Due to the prominent industrial shipping industry in Coos Bay, the Coos Estuary (OR) has been heavily altered and is lined with public and private docks that host communities of fouling organisms suited to local water conditions (Hewitt 1993). There are at least 60 non-native species in the Coos Estuary, representing seven of the eight phyla of encrusting organisms in equal proportion of native to non-native in each phylum (Annelida is the sole phylum in this group without any known non-native species in the bay) (Carlton 1989; Hewitt 1993). At least 32 of these non-native species

reside in the South Slough National Estuarine Research Reserve (Ruiz *et al.* 1997). However, native species are thought to be restricted to these marine sites in the lower bay, while brackish upper bay sites are completely dominated by non-natives (Hewitt 1993). Species introductions have been attributed to both ballast water and oyster aquaculture transportation, the latter particularly contributing to South Slough invasions of ascidians such as *Botryllus schlosseri* and *Botrylloides violaceus* (Hewitt 1993; Cox 1999).

Botrylloides violaceus has been documented in the Coos Estuary since at least the 1980s, but there have been few attempts to monitor or study the resident *B. violaceus* population since then (Hewitt 1993). The current distribution of *B. violaceus* appears to differ from that observed several decades ago. In a 2010 rapid assessment of invasive ascidians in the Coos Estuary, *B. violaceus* occupied the Charleston Boat Basin and "appeared" non-threatening to the ecosystem (Lambert & Lambert 2011). At the beginning of the present study, the Charleston Inner Boat Basin (IBB, Figure 1) hosted a large population of *B. violaceus* in the summer of 2015, while relatively few colonies occupied the nearby Outer Boat Basin (OBB). No colonies occupied the upper reaches of the bay at sites such as Isthmus Slough (IS), where colonies resided several decades ago (Hewitt 1993). While management and eradication plans have attempted to remove *B. violaceus* from boats, docks, and aquaculture facilities in other harbors (Carver *et al.* 2006; Arens *et al.* 2011), no such programs currently exist in the Coos Estuary. The South Slough National Estuarine Research Reserve aims to determine which region of the estuary is the most susceptible to invasions (Rumrill 2006). Local fishing communities have focused management efforts on other invasive species such as the

European green crab (*Carcinus maenas),* the colonial ascidian *Didemnum vexillum*, the zebra mussel (*Dreissena polymorpha*), and the Asian marsh snail (*Assiminea parasitologica*) (Behrens Yamada *et al.* 2005; Laferriere *et al.* 2010).

Inspired by large variation in *Botrylloides violaceus* abundance between two neighboring dock sites (IBB and OBB), my purpose was to document the present distribution of *B. violaceus* at five sites at either end of the Coos Estuary and study its ecology so as better understand the intensity of this invasion and what, if any, threat it poses to the natural ecosystem. Three factors may explain the current distribution of *B. violaceus* in the Coos Estuary: 1) abiotic conditions that vary along a spatial gradient throughout the estuary (water temperature, salinity, and current speed) may naturally restrict the species to a particular region of the bay, 2) competitive or predatory relationships with other fouling organisms may prevent the species from surviving at sites it can tolerate physiologically and 3) limited natural dispersal of the short-lived larval stage and limited anthropogenic dispersal may restrict spread among fouling sites. Grey (2011) modelled the influence of temperature, salinity, and direct species interactions on the survival and growth rate of *B. violaceus,* and determined that temperature and salinity are the best predictors of survival for this species. As salinity has been shown to restrict the distributions of another invasive ascidian (*Didemnum vexillum*) to the marine conditions of the Charleston Boat Basin (Chapman *et al.* 2011), I hypothesized that physiological tolerance limits *B. violaceus* to lower estuarine salinities and warmer estuarine temperatures limits the spread of the species to upper bay sites. Moreover, I hypothesized that significant variation in flow velocity due to

variable currents can further explain large differences in abundance among nearby marine study sites in the Coos Estuary.

Processes associated with climate change are projected to cause wetter winters and drier summers in the Coos Estuary, which could alter estuarine circulation and salinity and increase the dominance and/or expand the distribution of species with wide abiotic tolerance ranges, such as *Botrylloides violaceus* (Sutherland & O'Neill 2016). Warmer temperatures are projected to facilitate increases in *B. violaceus* abundance, but increased precipitation and resulting decreases in salinity may create unsuitable conditions for *B. violaceus* (Grey 2011). Determining key factors that either limit or permit the spread of this invasive species will enable us to predict and combat the future spread of *B. violaceus* in the Coos Estuary and beyond.

In the intertidal zone, physical factors such as wave action and salinity tend to set the distributional limits of species, whereas biological interactions only become limiting when physical factors are less harsh (Connell 1972). My study explored the biotic relationships between *Botrylloides violaceus* and other fouling organisms to determine, if abiotic factors permit its survival, the direct impact of *B. violaceus* on native species in the Coos Estuary. Furthermore, I observed species interactions in order to elucidate how *B. violaceus* fits into successional patterns of fouling community development (native ascidians are typically thought to serve as "early successional" organisms) (Schmidt & Warner 1986; Todd & Turner 1988). By exploring this, I assessed the threat *B. violaceus* poses to native biodiversity in the Coos Estuary in order to suggest whether management programs may be necessary to minimize the spread of this species.

Methods

Study sites

The Coos Estuary (43° 20' 44" N, 124° 19' 13" W, Figure 1) is the second largest estuarine system in Oregon, at 54 km^2 in area, and is a well-mixed, drowned river mouth characterized by mixed semi-diurnal tides as well as seasonal upwelling and downwelling (Hewitt 1993; Roegner & Shanks 2001, Rumrill 2006). This estuary is comprised of two subestuaries: South Slough, which forks off of the main estuary to the south of the estuary mouth, and Coos River, which provides seasonally-fluctuating freshwater input to the estuary from the southeast at the other end of the system (Hewitt 1993; Sutherland & O'Neill 2016). During the dry summers, freshwater input is low and the Coos Estuary is dominated by well-mixed saltwater, and during the wet winters, the estuary forms a salt-wedge (Sutherland & O'Neill 2016).

I selected five dock sites in the Coos Estuary for study, based on their accessibility for periodic field work, popularity for recreational and industrial boat use, and distribution (Figure 1), in order to document the distribution of *Botrylloides violaceus* at two regions of the estuarine gradient in the bay: near the mouth and near the sloughs of the upper bay. The Charleston Inner and Outer Boat Basin (IBB and OBB) are two dock systems located near the mouth of the Coos Estuary, which may be the first fouling communities accessible to non-native species introduced by boats entering the bay. Charleston Boat Basin boasts a diverse fauna high in non-native species due to heavy boat traffic and proximity to heavily-invaded South Slough oyster grounds (Hewitt 1993). IBB is small and used only for small recreational boat docking

and small OIMB research vessels. IBB is sheltered from weather and the strong water currents of the Coos Estuary mouth by a large cement breakwater (Appendix A: Figure A) (Marshall *et al.* 2006). OBB is the larger of the two basins, utilized by larger fishing boats and more exposed to the strong estuarine current. The other marine study site is the Charleston Shipyard (CSY), located near IBB and OBB at the mouth of South Slough. These docks are used by large fishing boats. The upper bay study sites are the Coos Bay City Docks (CB), a public marina used by both large fishing vessels and small recreational boats, and Isthmus Slough (IS), a small private dock where two tugboats are docked, one of which remained docked for the entirety of the study. Both CB and IS occupy mesohaline portions of the estuary (Rumrill 2006). At these five sites, I studied fouling communities on floating docks, all of which were made of cement except for those at IS; the IS docks are composed of visibly rusting metal. Differences in fouling substrate material can influence the species assemblages that develop (Connell & Glasby 1999).

Seasonal quadrat surveys

To chart diversity and space occupation of fouling species assemblages over time I surveyed the five dock sites seasonally for a year (once each in summer, fall, winter, and spring). For the first survey (summer 2015), I used a random number generator to select 20 random locations on the dock systems at each site. At IBB and OBB, I randomly selected dock finger (pre-numbered in the harbor) (Appendix A: Figure B), side of the dock finger, and then a distance along the dock finger. At CSY and CB, I randomly selected dock (there are two at each), dock side, and then distance along the dock. At IS I randomly selected dock side and distance along the dock. I used

a measuring tape to locate each designated distance along each dock or dock finger. I surveyed the same dock locations in all four seasons (Appendix A: Figures B-F).

To survey each site, I photographed a quadrat of the submerged vertical dock wall at each selected dock location. I used a Canon PowerShot s500 camera housed in an underwater housing with attached quadrat frame to keep all quadrats a constant size of 21.5 × 16.2 cm, with the highest vertical point of the quadrat frame one to two cm below the surface of the water. In order to maximize visibility of target macrofauna in the quadrats, I removed as much kelp and algae as possible from the dock wall prior to taking quadrat photographs. Grey (2010a) determined that this survey methodology (off the sides of floating docks) shows comparable results to surveys of less-accessible dock undersides. Due to camera malfunction in August 2015, I photographed quadrats during the summer surveys at CB and IS using a Pentax Readies Optio WG-2 Waterproof Camera, with a protruding plastic stick attached to the bottom to standardize the distance of the camera to the wall and photograph quadrats 16.2 cm tall. This camera took wider pictures than the Canon, so I removed the extra width prior to analysis so all photographs in the study had the same dimensions of 21.5 × 16.2 cm. In addition, at each quadrat I measured sea surface temperature (SST) using a standard thermometer held approximately 10 cm under the surface of the water, surface salinity using a handheld refractometer, and instantaneous current flow velocity using the average of ten readings from a Marsh-McBurney Model 2000 Flowmeter.

I identified organisms in the quadrat photographs to the lowest possible taxonomic group, often to the species level. After calibration of photographs to 21.5cm × 16.2 cm, I used automated segmentation (50-pixel resolution) in photoQuad software

(Trygonis & Sini 2012) to detect and compute absolute and percent cover occupied by each segment and taxon. When automated detection was impossible due to photograph quality or similarity in color of neighboring organisms, I manually outlined taxa in the program.

I used PRIMER 6.0 software (Clarke & Gorley 2006) to compare species assemblages and abiotic conditions at quadrats in each season. I omitted quadrats for which I was unable collect both biotic (percent cover acquired from photograph analysis) and abiotic (temperature, salinity and flow velocity) data. For each season, I generated Draftsman plots to assess the spread of the raw abiotic log- and square root-transformed data. These transformations failed to remove skew from the data and create plots with random data spread, so I left abiotic data untransformed for subsequent analyses. I used the "Normalize" function in PRIMER to normalize abiotic data since temperature, salinity, and flow velocity are each measured using different scales and units. I analyzed the similarity of abiotic variables by generating a resemblance matrix based on Euclidean distance. I ran a Principal Components Analysis on this resemblance matrix to determine which abiotic variable contributed most to variation among the quadrat sites.

In addition, I used PRIMER to generate a resemblance matrix based on Bray-Curtis similarity of the biotic data (percent cover of each taxon in each quadrat). I square-root transformed the biotic data to account for less common taxa. I used this resemblance matrix to construct a multi-dimensional scaling (MDS) plot to display spatial similarity between the species assemblages in each quadrat. I compared abiotic and biotic patterns by conducting RELATE analysis: a Spearman rank correlation

between the abiotic resemblance matrix (Euclidean distance) and biotic resemblance matrix (Bray-Curtis similarity) to determine similarity at a significance level of $p = 0.01$. I then used the BIOENV BEST analysis to perform a nonparametric Mantel test comparing rank correlation coefficients between the abiotic and biotic matrices to determine which environmental variables correlated best to biotic data (significance level of $p = 0.01$). In the winter and spring surveys, there were quadrats (three and one, respectively) at IS that had 0% cover and had to be omitted from the construction of MDS plots. In these cases, I calculated two RELATE and BEST statistics: one with empty quadrats omitted and the other with all quadrats included.

I also specifically analyzed *B. violaceus* cover across sites and seasons by conducting a two-way ANOVA test ($\alpha = 0.05$) comparing the influence of site and season using R software (R Core Team 2013). I created all figures (except for MDS plots) in SigmaPlot 13 (Systat Software, San Jose, CA).

Time-integrated flow measurements

In the seasonal quadrat surveys, I incorporated variable current speeds by taking instantaneous measurements of flow velocity at each quadrat. Because I assessed all quadrats at a given site within a short period of time, these instantaneous measurements provide a reasonable comparison of flow velocity among quadrats at each given site and allowed for the inclusion of data in the Principal Components Analysis for abiotic conditions. However, estuarine currents vary with time and tide, and since I conducted quadrat surveys at different sites on different days (though consistent for each season), these instantaneous measurements do not accurately compare flow velocity across study sites.

In order to more adequately assess differences in current flow among the five study sites in the Coos Estuary, I measured the dissolution of chalk clod cards deployed at each site over the same four-day period, as a proxy for flow velocity. Using a method similar to that described by Bingham (1990), I used silicone adhesive to glue hemispherical pieces of carpenter's chalk to individual plexiglass plates, which dried for 24 hours before I weighed each plate. I attached three plates to a flat piece of old dock wood, and deployed two of these sets of clod cards at each site. Clod cards hung at a depth of 1 m with a ten-pound weight (either two bricks or a gallon water jug filled with sand) attached to the wood to weight it down. I selected the two deployment locations at each site to represented the estimated maximum variation in current flow at that site, based on orientation and visible current flow (Appendix A: Figures L-P). I retrieved the clod cards after four days of immersion, and let the clod cards air dry for two days in the lab before reweighing each plate. I deployed clod cards once in December 2015 and once in April 2016 to consider seasonal variation in current flow associated with weather patterns and freshwater input.

After running Levene's tests to confirm equal variances among mass lost due to dissolution, I used R to run two ANCOVA tests ($\alpha = 0.05$) for each trial in order to assess whether dissolution varied among sites and whether this was impacted by any slight variation in initial chalk mass (treated as the covariate); one test evaluated the effect of site on dissolution, and the other evaluated the effect of individual clod card unit, which incorporated intra-site variation. I then ran a Tukey HSD test to determine which sites, if any, had significant differences in dissolution between them.

Salinity Tolerance Experiment

Adult colonies

I collected adult *Botrylloides violaceus* colonies from the Charleston Inner Boat Basin by removing *Mytilus* spp. shells from the docks and carefully peeling off colonies in the lab using a standard scalpel. I gently fragmented colonies into two-eight cm^2 chunks and placed each colony individually into a 250-mL beaker of sea water. Prior to salinity treatment, I isolated beakers from water flow for one to two days to allow colonies to reattach to the beakers. In some trials, I expedited attachment by adhering colonies to glass beakers with Super Glue and placing beakers in flow-through aquaria for one day for colony acclimation. I then gave each beaker a different 200 mL salinity treatment (5, 10, 15, 20, 25, 30, 32/33 (control), and 35 psu, achieved by diluting sea water with tap water) and maintained these beakers at a temperature of ~15°C for seven days. I did not change water during the trial. The "control" salinity was undiluted seawater that varied between 32-33 psu depending on the trial. The total number of colonies treated at each salinity level is indicated in Table 1. A single colony at each of the treatment levels made up one "trial," and I conducted five trials over a period of several months. I omitted particular treatment levels from some trials because I was unable to collect enough suitable colonies from the field. I fed colonies every other day with Shellfish Diet (2 billion cells/mL).

Botrylloides violaceus colonies follow a predictable pattern of regression prior to their death, which is observable in the narrowing of blood vessels and slowed blood flow, the shrinking and darkening of zooids, and the disorganization of previously arranged zooids. This process is actually reversible; colonies may "hibernate" *in situ*

during suboptimal environmental conditions. However, colonies that regressed in this experiment typically proceeded to the final, irreversible stage: the disintegration of the tunic and colony tissue (Epelbaum *et al.* 2009b). I assessed colonies prior to treatment, after one day and after seven days for seven mortality indicators associated with colonial regression: size of colony, amount of attachment to substrate (for non-glued colonies), shape and color of zooids, appearance of siphons, presence of siphon contraction, ampullae condition, and amount of clear tissue.

Juvenile colonies

I collected larvae and newly settled (less than one day old) juvenile *Botrylloides violaceus* from beakers containing adult colonies. I used a standard-size dropper to collect free-swimming larvae, and I used a small needle to gently lift adhesive papillae of juvenile colonies and remove them from the glass beaker walls. Upon collection, I placed young *B. violaceus* into individual glass finger bowls filled with seawater and left juveniles to settle for one to two days, at which point most individuals successfully reattached to the bowls. Then, I placed finger bowls with attached juveniles inside beakers each filled with a different 200 mL seawater salinity treatment (5-35 psu). The "control" salinity varied between 32 or 33 psu depending on the trial, since the ambient salinity of seawater fluctuated. The total number of colonies treated at each salinity level is indicated in Table 1. A single colony at each of the treatment levels made up one "trial," and I conducted five trials over a period of several months. I omitted particular treatment levels in some trials due to variable larval release from adult colonies in the lab and limited usable juvenile colonies. I assessed juveniles prior to treatment (Day 0), and after one and seven days for seven mortality traits: number and

color of zooids, number of ampullae, size of juvenile, number of buds, number of siphons, and presence of siphon contraction. I fed colonies every other day with Shellfish Diet (2 billion cells/mL).

I plotted the percent survival of juvenile and adult colonies at each treatment level using SigmaPlot 13.

Temperature Tolerance Experiment

Adult colonies

I gently fragmented adult *Botrylloides violaceus* colonies into four cm^2 fragments and adhered each to the bottom of a scintillation vial with Super Glue. I placed vials on their sides, so that colonies rested vertically, in a flow-through aquarium for one day to allow colonies to acclimate. Then, I placed vials in an aluminum thermal gradient block (Figure 2) for one week. Vials are incubated in holes in the block, with running hot water at one end and running cold water at the other to create a temperature gradient of 18-28°C across ten vials. These temperatures reflect the upper range *B. violaceus* could encounter in the Coos Estuary and allowed for the assessment of the upper thermal tolerance limit of adult colonies. The total number of colonies treated at each temperature level is indicated in Table 1. A single colony at each of the treatment levels made up one "trial," and I conducted four trials over a period of several months. I omitted particular treatment levels in some trials because I was unable to collect enough suitable colonies from the field. To maintain food and oxygen levels in the tubes, I changed the water in each tube every other day. I assessed colonies prior to treatment (Day 0), after one day and after seven days for eight mortality indicators: size of colony,

attachment to substrate (for non-glued colonies), shape and color of zooids, appearance of siphons, presence of siphon contraction, ampullae condition, and clear tissue presence.

Juvenile colonies

I collected newly released larvae and newly settled (less than one day old) juveniles from beakers containing adult *Botrylloides violaceus* colonies from the Charleston Inner Boat Basin and placed larvae and juveniles in scintillation vials to sit for one-two days to allow for successful attachment. Then, to facilitate assessment of juvenile colonies under a microscope after treatment, I placed only vials with juveniles settled on the bottom of the vial in the thermal gradient block (Figure 2) for one week. The total number of colonies treated at each temperature level is indicated in Table 1. A single colony at each of the treatment levels made up one "trial," and I conducted five trials over a period of several months. I omitted particular treatment levels in some trials due to variable larval release by adult colonies in the lab and limited usable juvenile colonies. To maintain food and oxygen levels, I changed the water in each tube every other day. I assessed colonies prior to treatment (Day 0), after one day and after seven days for seven mortality indicators: number and color of zooids, number of ampullae, size of juvenile, number of buds, number of siphons, and presence of siphon contraction.

I plotted the percent survival of juvenile and adult colonies at each treatment level using SigmaPlot 13.

Transplant Experiment

I constructed transplantation containers using PVC filtration equipment (Figure 4a), creating a sealed mesh compartment that both permitted filter feeding of *Botrylloides violaceus* colonies and contained any larvae they released. I collected adult colonies of *B. violaceus* from the Charleston Inner Boat Basin using a scraper, and placed one colony into each transplant container. In each trial, I deployed containers by hanging them each off a selected dock wall and weighing each down with a single eight-ounce fishing weight attached to the container bottom. I deployed three containers each at IBB (control), OBB, CB, and IS, and two of the deployment locations at each site were the same locations as clod card deployment (Appendix A: Figures L-P). In trial 1, I left colonies to freely settle in the container during deployment at a depth of 1 m. In trial 2, I left colonies to freely settle in the container during deployment at a depth of 0.5 m to minimize the accumulation of sediment on the bottom of the container, which smothered colonies and prevented survival in trial 1. In trial 3, I superglued colonies to pieces of large plastic mesh, which I inserted vertically in the container to maintain colonies at a height and orientation in the container so that any sedimentation in the containers would not smother colonies (Figure 3b). For each trial, I documented survival of colonies after one week. Survival of colonies after one week in trial 3 warranted re-deployment of colonies for three more weeks, after which I documented long-term survival results.

Settlement Plates

I deployed 24 15 × 15 cm plexiglass settlement plates with one rough (sanded) and one smooth face at ~0.5 m depth at the five study sites (six each at IBB and OBB,

and four each at CSY, CB, and IS) (Appendix A: Figures G-K). Plates hung from ropes secured to docks at each site, each weighed down with an eight-ounce fishing weight. Every three weeks from August to December 2015, as well as on several later sporadic sampling opportunities through May 2016, I retrieved plates from the water and photographed both sides of each plate. I held organisms on the settlement plate out of the water for less than one minute before they were returned, to avoid adverse effects on the settled organisms.

I identified organisms in the quadrat photographs to the lowest possible taxonomic group, often to the species level. After calibration of photographs to 12.5 × 12.5 cm, I used automated segmentation (50-pixel resolution) in photoQuad software (Trygonis & Sini 2012) to detect and compute absolute and percent cover occupied by each segment and taxon. When automated detection was impossible due to poor photograph quality or similarity in color of neighboring organisms, I manually outlined taxa in the program.

I used R to analyze and SigmaPlot 13 to plot *B. violaceus* abundance, growth, and recruitment on the settlement plates.

Results

Seasonal abiotic conditions at Coos Estuary study sites

Average temperature, salinity, and flow velocity at each site during each survey varied with season (Tables 2, 3, and 4). Since I collected data for these three variables using instantaneous measurements at the time of each quadrat survey, average conditions at each site may not necessarily represent the average conditions for that season, since conditions can fluctuate on even an hourly basis (Hickey & Banas 2003). I addressed this shortfall for flow velocity measurements by deploying clod cards at study sites in the winter and spring, but I collected no other temperature and salinity measurements. However, instantaneous temperature and salinity measurements from the quadrat surveys still followed a seasonal cycle (Tables 2 and 3), suggesting that my data captured fluctuation on the seasonal scale which can be compared to seasonal fluctuation in *Botrylloides violaceus* abundance.

In addition, no randomly-selected quadrat at OBB were located at the ends of the docks (Appendix A: Figure C), locations which typically experience the fastest current flow, so the full range of current variability at this site was not captured by instantaneous flow measurements. However, this was also accounted for in the clod card flow measurements; I deployed one of the two sets of clod cards near the end of the OBB dock (Appendix A: Figure M). Instantaneous abiotic measurements permitted the comparison of individual quadrat locations within each study site using Principal Components Analysis.

Principal Components Analyses (PCA) of temperature, salinity, and current speed measured at each seasonal survey indicate that different combinations of abiotic factors contributed to differences among sites during each season. In the summer, temperature and salinity were inversely correlated and made up Primary Component 1 (PC1), which accounted for 56% of the variation in abiotic measurements across sites. Figure 4a shows clear overlap in abiotic conditions between upper bay sites CB and IS, with lower bay sites each forming distinct clusters, but CSY being the most isolated. In the fall, PC1 was comprised of all three abiotic measurements, and made up 81.4% of the variation in abiotic data. CB and IS conditions overlapped in the PCA plot but were distinct from CSY, IBB, and OBB, which each had distinct clusters representing significantly different combinations of abiotic conditions (Figure 4b). In the winter, PC1 made up 65.5% of the variation in abiotic data, and was comprised of all three abiotic factors. IS and CB were distinct from each other, but conditions at IS overlapped with those at IBB. OBB and CSY had distinct and isolated clusters (Figure 4c). In the spring, temperature and salinity were inversely correlated. PC1 is made up of all three factors and made up 69.8% of the variation in abiotic data. There is slight overlap between CSY and OBB clusters, as well as between IBB, CB, and IS (Figure 4d).

Site-specific current flow throughout Coos Estuary

Dissolution of clod cards deployed in December 2015 varied significantly among the five study sites (ANCOVA, $\alpha = 0.05$, $p < 0.01$) (Table 10). However, the covariate of initial chalk mass also significantly impacted dissolution across sites ($p < 0.01$). A Tukey HSD test ignoring initial chalk mass showed significant differences in dissolution between the following site pairs: CSY-CB ($p < 0.05$), CSY-OBB ($p < 0.05$),

and CSY-IBB ($p < 0.01$). Dissolution did not vary significantly between any other site pairs. Dissolution also varied significantly among individual clod card units (i.e. CB1, CB2, etc.) (ANCOVA, $\alpha = 0.05$, $p < 0.05$), and initial chalk mass significantly affected dissolution ($p < 0.01$) (Table 11). A Tukey HSD ignoring initial chalk mass showed no significant differences in dissolution between any pair of clod card units at the same site (all p values > 0.05).

Dissolution of clod cards deployed in April 2016 varied significantly among the five study sites (ANCOVA, $\alpha = 0.05$, $p < 0.001$) (Table 12). Initial chalk mass had no significant impact on site dissolution differences ($p > 0.05$). Dissolution also varied significantly among individual clod card units (ANCOVA, $\alpha = 0.025$, $p < 0.001$) (Table 13), even when I used a stricter α value of 0.025 due to unequal variances indicated by the results of Levene's test ($p < 0.001$). A Tukey HSD test ignoring initial chalk mass showed significant differences in dissolution among pairs of clod card units (both intra- and inter-site dissolution differences). Levene's test showed equal variance in the dissolution of individual clod card units, so I assessed this ANCOVA and Tukey test using $\alpha = 0.05$. Site pairs showed significantly different dissolution (Table 14).

In both December and April, site had a statistically significant impact on dissolution, a proxy for current velocity due to relative mass loss over time, among five sites in the Coos Bay. In December, the initial mass of chalk clod cards significantly impacted dissolution. In April, initial chalk mass had no effect on dissolution, and comparisons of clod card units show significant differences in dissolution between IBB and CB, IS, OBB, and CSY1. Dissolution varied significantly within both IBB and

OBB, and dissolution at OBB1 was significantly different from CB, IS, CSY2 and OBB2.

Seasonal biotic conditions at Coos Estuary study sites

Taxa documented at each of the sites during any seasonal quadrat survey are listed in Table 5. Average taxon richness per quadrat at each site and seasonal survey ranged from two to seven taxa (Table 6). Taxon richness varied significantly with both season and site (two-way ANOVA, $\alpha = 0.05$, $p < 0.001$) (Table 7). Average taxon richness was greatest across all sites during the fall, and across all seasons, average taxon richness was greatest at IBB (Figure 5). Species accumulation plots incorporating all sites in each season show that sampling efforts accounted for nearly all of the richness present (at my scale of detection) at the sampling sites, with the spring survey yielding the highest richness and nearing an asymptote of around 23 taxa (Figure 6).

MDS plots relating the species composition of quadrats in each seasonal survey show variability in species assemblages among sites and seasons (Figure 7). During the summer, quadrats from upper bay sites CB and IS and lower bay sites CSY and IBB all formed distinct clusters unique to each other. OBB quadrats overlapped with other lower bay sites, IBB and CSY. Correlation between these biotic patterns and abiotic conditions is weak (RELATE analysis, $\rho = 0.506$, $\alpha = 0.01$). A combination of temperature and current speed best correlate to species composition data in the summer (BEST analysis, $\rho = 0.576$, $\alpha = 0.01$). During the fall, quadrats from all sites were more tightly clustered in the MDS plot than in the summer, and only IS had a distinct cluster that failed to overlap with any other site. CSY, CB, and IBB quadrats each form clusters distinct from each other, but all overlap with quadrats from OBB. Correlation between

these biotic patterns and abiotic conditions is weak (RELATE analysis, $\rho = 0.232$, $\alpha = 0.01$). Salinity best correlates to biotic data (BEST analysis, $\rho = 0.259$, $\alpha = 0.01$).

There was considerable overlap of species composition among sites during the winter season as well, as upper bay sites IS and CB overlapped and were collectively distinct from the lower bay sites. IBB and CSY quadrats formed distinct clusters, but both overlapped with quadrats from OBB. Correlation between these biotic patterns and measurements of temperature, salinity, and current speed were weak both when I incorporated all quadrats (RELATE analysis, $\rho = 0.357$, $\alpha = 0.01$) and when I incorporated only quadrats with >0% cover (RELATE analysis, $\rho = 0.446$, $\alpha = 0.01$). Temperature best correlated with biotic data (BEST analysis, $\rho = 0.362$, $\alpha = 0.01$). The spring survey showed a similar pattern of species composition among sites as the winter survey, but showed less overlap among upper bay sites and more overlap among the lower bay sites. Biotic and abiotic patterns were weakly correlated, regardless of whether I incorporated all quadrats (RELATE analysis, $\rho = 0.365$, $\alpha = 0.01$) or only those with >0% cover (RELATE analysis, $\rho = 0.379$, $\alpha = 0.01$). A combination of temperature and current speed best correlated to the biotic patterns (BEST analysis, $\rho = 0.422$, $\alpha = 0.01$).

In summary, across all seasons, sites each had distinct combinations of temperature, salinity, and current speed variables. The abiotic factors contributing most to the variation in abiotic conditions among sites varied with season. Upper bay sites (IS and CB) had consistently clustered species assemblages relative to those of the lower bay sites. Fall was the only season when the species assemblages of site CB overlapped with those in lower bay sites, and this may correspond with observably higher

abundance of *Botrylloides violaceus* at CB during the fall survey. IBB and CSY clusters were always distinct from each other, but both consistently overlapped with OBB. Fall and spring surveys showed the most overlap and most closely-associated site clusters, which could be due to the intermediate temperature and salinity conditions during these two seasons. However, RELATE analysis showed no strongly significant correlation between abiotic variation and variation in species composition of the quadrats in any season, so increased clustering in fall and spring cannot be explained abiotically. BEST analysis showed that the particular abiotic conditions that correlated most strongly with biotic data changed with season, and this relationship was never very significant.

Seasonal distribution of *Botrylloides violaceus* in the Coos Estuary

Botrylloides violaceus occupied all study sites except Isthmus Slough, and occupied those sites during almost every season. The species was only absent from CB during the summer and from OBB during the spring. Average percent cover of *B. violaceus* at each site varied with season, and peaked in the fall (Figure 8).

Using a standard α value of 0.05, the mean percent cover of *Botrylloides violaceus* varied significantly with season and site (two-way ANOVA, $p = 0.03$) (Table 8). Unequal variance in mean percent cover due to the lack of *B. violaceus* cover in any quadrat in one or two sites during each season (which failed to improve upon transformation) necessitated evaluating ANOVA tests with a stricter α value, given that successfully transforming data to improve variances would only increase p values and evaluating the test using a stricter α value of 0.025 allows for greater confidence in significant findings. Using a stricter α value of 0.025 to account for unequal variance, the relationship of season and site with *B. violaceus* percent cover is insignificant.

However, the average percent cover of *B. violaceus* varied significantly with both season and site separately when $\alpha = 0.025$ is used (two-way ANOVA, $p < 0.01$).

Though an insignificant relationship when assessed with a necessarily stricter α value, the interaction of site and season on *Botrylloides violaceus* percent cover with site at each season is illustrated in Figure 9. If no interaction existed, changes in *B. violaceus* percent cover would be represented by parallel lines; instead, IS and OBB patterns deviate from those of IBB, CB, and CSY (Figure 9a). As such, during fall, winter, and spring, mean *B. violaceus* percent cover varied among sites. Summer is the only season in which OBB had the highest average percent cover; IBB dominated in percent cover during all other seasons, though CB (which had no *B. violaceus* cover during the rest of the year) had comparable percent cover to IBB during the fall, showing similarity in *B. violaceus* presence between the upper and lower bay. None of the seasonal patterns in *B. violaceus* percent cover across sites showed parallel trends, demonstrating again the interaction between site and season on *B. violaceus* abundance (Figure 9b). CB, CSY, and IBB all showed the highest percent cover of *B. violaceus* during the fall, followed by spring, summer, then winter. IS never had any *B. violaceus* cover, and OBB departed from the trend by hosting the highest percent cover during the summer, followed by winter, fall, and then spring.

In all four seasonal surveys, quadrats with *Botrylloides violaceus* spatially overlapped sites without *B. violaceus* in the MDS plots (Figure 10). The presence of this species at a particular quadrat location or site does not appear to biotically distinguish that site from those without the species, in terms of either species abundance or diversity.

Salinity tolerance of adult and juvenile *Botrylloides violaceus*

Adult colonies subjected to salinity treatments for 24 hours survived salinities of 15 psu and above, with a survival rate of at least 50% (Figure 11). A few colonies also survived salinities of 5 and 10 psu, suggesting that field survival in these conditions is possible, but that salinities of 15 psu or higher are more optimal for survival in the short term. At least 50% of juvenile *Botrylloides violaceus* individuals survived every salinity treatment (from 5-40 psu) when exposed for 24 hours, and all juveniles survived salinities of 20 psu and above. Juvenile colonies can therefore survive in any of the salinities tested for a 24-hour period.

After seven days of experimental salinity treatment, only three adult colonies survived: one at 10 psu, one at 30 psu and one at 35 psu. Adult colonies should have at least survived the control salinity (32-33 psu), so it is evident that experimental design flaws prevented colony survival irrespective of salinity level. Hypoxic conditions in the trial beakers may have caused early mortality in the colonies, since beakers containing juvenile colonies with lower respiration demands successfully survived. After seven days of experimental salinity treatment, no juvenile colonies survived salinities of 15 psu or below, and at least 60% of individuals survived salinities of 25 psu and above. This suggests that *Botrylloides violaceus* juvenile colonies have a long-term salinity tolerance level of 25 psu.

Temperature tolerance of adult and juvenile *Botrylloides violaceus*

Adult colonies subjected to temperature treatments for 24 hours survived temperatures up to 25°C, with a survival rate of at least 50% (Figure 12). A few

colonies survived temperatures of 27 and 28°C, suggesting that survival at this temperature is possible, but optimal temperature for survival is 25°C and below. At least 50% of juvenile *Botrylloides violaceus* individuals survived every trial temperature (from 18 to 30°C).

No adult *Botrylloides violaceus* colonies survived temperature treatments for seven days. As with salinity trials, hypoxic conditions likely developed despite regularly changing the water in the vials, thus preventing the survival of adult colonies with higher respiratory demands. However, juvenile colonies demonstrated survival even at high temperatures after seven days of treatment. Despite only 40% survival at 19 and 21 °C, colonies survived temperatures up to 27°C at rates of at least 50% (often 100%). Juvenile colonies also survived 30°C at a rate of 50% even though survival at 28°C was low, suggesting juvenile *B. violaceus* has an upper thermal tolerance limit of around 27°C, but can potentially survive up to 30°C (I did not test colonies at 29°C). Because the dissolved oxygen concentration of seawater decreases with increases in water temperature, it is possible that colonies tested in higher temperatures faced hypoxic conditions, contributing to their unreliable survival. However, I changed the water in temperature trial tubes every other day in an attempt to prevent any such effects. Clearer trends in long-term survival of the higher temperature range could be achieved with increased trials. Salinity and temperature tolerance levels for adult and juvenile colonies are summarized in Table 9.

In situ site-specific survival of transplanted *Botrylloides violaceus*

Trial 1

Only one *Botrylloides violaceus* colony survived the first nine-day transplant deployment, in the container located at the most sheltered site in IBB (Table 15). However, it is evident that transplanted colonies released larvae prior to morality, as juvenile colonies settled on the interior of mesh containers deployed in IBB, IS, and CB. Survival of these young colonies at the experimental upper bay sites suggested that given proper transplantation, adult colonies may also survive in the upper bay. Almost all transplanted colonies settled on the bottom of the transplant containers despite attempts to deploy colonies vertically on the mesh sides of the containers. Survival of these juvenile colonies suggests that settlement of the transplanted colonies in the bottom of the transplant containers subjected them to heavy sedimentation at sites with strong currents, which smothered the colonies and prevented their survival. This also indicated that *B. violaceus* recruitment can occur late into the fall and is not restricted to the spring season as previous literature has reported (Hewitt 1993; Epelbaum *et al.* 2000; Stachowicz *et al.* 2002; Dijkstra *et al.* 2011).

Trial 2

Only one colony survived the eight-day deployment of trial 2, a colony transplanted at IBB (control). Sedimentation in transplantation containers was noticeably less than in trial 1, but did vary in intensity among the deployment sites. Colonies still settled at the bottom of transplant containers, and appeared to again have

been smothered due to their position below the mesh where water flow minimized sedimentation.

One colony at IBB, one colony at OBB, and all colonies transplanted at both IS and CB released larvae during trial 2 which settled on the mesh sides of the transplant container. All settled juvenile colonies appear to have survived, though observation of the zooids and standard mortality measurements of juvenile colonies were difficult to observe clearly without a microscope. The survival of these juvenile colonies again suggests that poor survival of transplanted colonies was an artifact of the transplant containers rather than an effect of the transplant site.

Trial 3

After the initial seven days of trial 3, at least one transplanted colony at each study site survived. Orienting colonies vertically in the transplant containers by gluing them on inserted plastic mesh with cyanoacrylate adhesive ensured that colonies were exposed to water flow through the mesh, thereby avoiding sedimentation. Survival of colonies confirms expected results based on the survival of juvenile colonies at all sites in the previous trials.

After a month (35 days) of deployment, all colonies died. The uniformity of this result is likely due to the timing of this experiment; colonies were transplanted in mid-October and retrieved in mid-November, during the seasonal transition during which *Botrylloides violaceus* populations begin to regress for the winter, as documented in seasonal quadrat surveys. The mortality of colonies after one month of deployment may reflect seasonal patterns of *B. violaceus* survival rather than sedimentation or site-specific abiotic conditions, as evidenced by uniform mortality. However, transplanted

colonies in IBB released larvae during the month-long deployment, which settled on the interior of the mesh container and developed into juvenile colonies. Survival of these colonies indicates that abiotic conditions may still have been suitable for survival at this site, even though transplanted adult colonies died, and it shows that *B. violaceus* can may continue to sexually reproduce late into the fall. Sedimentation of the transplant containers over the course of this month was heaviest at the upper bay sites, but even among transplant containers at the same site, sedimentation levels varied.

Settlement of fouling organisms on plates deployed throughout the Coos Estuary

All taxa documented on settlement plates are listed in Table 5. Of all taxa documented in this study, I found 14 only in quadrat surveys, and five only on settlement plates. This suggests that the species assemblages developing on settlement plates consisted of a unique set of species compared to assemblages on docks walls documented in quadrat surveys, either due to the attraction of unique species to the particular substrate of the settlement plates, or because settlement plate assemblages were in an earlier successional stage during the nine-month sampling period, which established dock wall fouling communities had surpassed. Overall taxon richness on settlement plates steadily increased over the deployment period for all five sites, and plates deployed at OBB consistently harbored the greatest number of taxa (Figure 13). Few taxa settled during the first 10 days of plate deployment (sampling date 08/26/15), but *Botrylloides violaceus* was one of these early settlers. Timing of initial settlement and percent cover of all settlers are depicted in Figure 14.

Settlement and space occupation of *Botrylloides violaceus* on settlement plates

Botrylloides violaceus colonies only settled on plates deployed at IBB, OBB, and CSY. The space occupied by *B. violaceus* ranged from 0.01% to nearly 100% cover, depending on season and site (Figure 15). Mean percent cover of *B. violaceus* on settlement plates varied significantly with sampling date and site together, with plates showing the highest percent cover in May and at IBB (two-way ANOVA, $\alpha = 0.025$, $p < 0.001$) (Table 16). This relationship was the same regardless of whether I included only sites with *B. violaceus* cover (IBB, OBB, CSY) or included all five study sites. However, site alone did not have a significant impact on *B. violaceus* percent cover when I included only sites with *B. violaceus* (Table 17). I used a stricter α to compensate for the unequal variance in *B. violaceus* percent cover (even after attempted transformation) confirmed via Levene's test. The total number of *B. violaceus* colonies settled on plates at each site peaked in the fall and the spring for all sites, with a peak in settlement at OBB on 12/6/15 and at IBB on 5/22/16 (Figure 16a). Of all *B. violaceus* colonies present on plates at each sampling date, the relative proportion of colonies at each site varied with sampling date (Table 18). IBB had the highest proportion of colonies during early fall, late winter and spring. OBB had the highest proportion of colonies in late fall, and had an equal proportion of colonies with IBB during early fall and early winter. Periods of high *B. violaceus* proportion at OBB did not only occur during periods of high overall abundance; December and May sampling dates had comparably high *B. violaceus* abundance across all sites but the two dates had opposite proportionality with respect to IBB and OBB.

New *Botrylloides violaceus* colonies settled on plates throughout the entire sampling period, indicating that recruitment occurred year-round (Figure 16b). On most sampling dates, the highest portion of new colonies were found on plates at IBB, though the level of newly immigrated colonies was equal at IBB and OBB on sampling days in October, November, and February. The number of new colonies settled on plates peaked at OBB on 12/6/15, and on 5/22/16 for IBB and CB. Loss of colonies from settlement plates also occurred throughout the entirety of settlement plate deployment, and IBB showed the greatest number of lost colonies from August through December (Figure 16c). The highest rates of colony loss occurred during the winter months, as expected according to the seasonality of the species (Burighel *et al.* 1976; Hewitt 1993), but many colonies remained settled and grew during these periods, and the proportion of colonies at IBB and OBB during this season remained nearly 50/50. OBB showed a large decrease in number of colonies present at the January sampling date, which immediately followed the large influx of new colonies at OBB in December. Colony loss remained highest at OBB through May (though it tied with IBB in February), when an even larger increase in immigration to IBB occurred.

Lateral growth rates of *Botrylloides violaceus* on settlement plates

Growth of *Botrylloides violaceus* colonies varied significantly among sampling intervals (time between two sampling dates), with the greatest rate of average growth per colony (cm^2/week) occurring between February 20 and May 22, 2016 (two-way ANOVA, $\alpha = 0.05$, $p < 0.001$) (Table 19), particularly at IBB (Figure 17a). This sampling period was marked by a large increase in *B. violaceus* percent cover, as many colonies grew to cover the entire settlement plate. Average weekly growth rate per

colony in each sampling period is given in Table 20. Growth rate also varied significantly with season; however, "summer" data is inadequate for comparison since it consisted only of the first settlement plate sampling in September when colonies had only just begun to settle. Growth rate during the spring was significantly greater than rates in the fall and winter (two-way ANOVA, $\alpha = 0.05$, $p < 0.001$) (Table 21). Site had no significant influence on growth rate (two-way ANOVA, $\alpha = 0.05$, $p > 0.05$) (Table 19), despite all spring colony growth occurring at IBB (Figure 17b). Colonies settled on plates at CSY during winter sampling dates were the only colonies that had a negative average growth rate.

Interaction between *Botrylloides violaceus* and other fouling organisms

I observed competitive interactions between *Botrylloides violaceus* and many other fouling organisms on deployed settlement plates (Table 22). *Botrylloides violaceus* always interacted positively (it overgrew the other taxon) with brown encrusting bryozoan, orange bryozoan, *Balanus* spp., spirorbid polychaetes, and other ascidian settlers. *Botrylloides violaceus* showed a mix of positive and negative (another taxon overgrew *B. violaceus*) interactions with *Botryllus schlosseri* and hydroid species, and showed only negative or neutral interactions with *Halichondria bowerbanki.*

Discussion

Overview

This study provides the first documentation of *Botrylloides violaceus* distribution in the Coos Estuary since Hewitt (1993). *Botrylloides violaceus* occupied space on floating dock walls in both the lower and upper bay, but at one upper bay site (Isthmus Slough) the species was completely absent. The reasons for the absence of this invasive ascidian from Isthmus Slough, while it was present at the Coos Bay City Docks, remain unknown, as abiotic conditions (salinity, temperature, and flow velocity) at Isthmus Slough fell within the tolerance range for this species and transplanted colonies survived at the site. Abiotic conditions do not appear to restrict *B. violaceus* from the upper bay as hypothesized, but measured differences in flow velocity between otherwise similar sites may contribute to large variation in *B. violaceus* abundance between sites.

In this study, I also documented several demographic characteristics of *Botrylloides violaceus* in the Coos Estuary. New *B. violaceus* recruits settled throughout the entire time period spanning August 2015 through May 2016, demonstrating the capability of this species to sexually reproduce year-round. Asexual growth peaked in the spring, suggesting that during a period of peak recruitment of other fouling organisms, *B. violaceus* can confer a competitive advantage through lateral overgrowth. Through observations of interactions between *B. violaceus* and the fouling species it encountered on settlement plates, it is clear that the species dominates fouling assemblages in the bay, capable of overgrowing every species it encountered except

Halichondria bowerbanki. Through asexual and sexual reproduction, *B. violaceus* has secured a dominant position in the fouling communities of docks in the Coos Estuary.

Botrylloides violaceus spatial distribution

Isthmus Slough was the only site in this study completely devoid of *Botrylloides violaceus*. Survival of transplanted colonies to this site suggests that adult colonies can survive the abiotic conditions of IS, at least during the fall season. Lack of an established population in IS contrasts with the documented establishment of *B. violaceus* in this region of the bay. The movement of a private dock covered in *B. violaceus* from South Slough to Isthmus Slough in the summer of 1990 introduced the species and within several months, the species covered over half of the transplanted dock and 20% of the surrounding encrusting communities (Hewitt 1993). While the spatial relationship of this transplanted dock to my study dock in Isthmus Slough is unknown, some factor or combination of factors has clearly prevented *B. violaceus* from maintaining a population at the study site, if not the entire Slough, in the decades since this introduction. Initial blooms of *B. violaceus* upon introduction followed by decreases in abundance have been observed in other ecosystems (Carver *et al.* 2006), but some factors must vary between IS and other study sites in the Coos Estuary where introduction has led to spatial dominance. I tested several of these potential factors in my study.

In addition to the complete absence of *Botrylloides violaceus* from IS during this study, the species was noticeably absent from OBB during the spring and from CB during the summer, the two seasons when it was expected *B. violaceus* would be in greatest abundance. Growth of *B. violaceus* on settlement plates in the spring

demonstrates that *B. violaceus* was present at OBB during that season, but was just absent from all established survey quadrat locations. A greater abundance of *B. violaceus* was expected in quadrat surveys since the proportion of *B. violaceus* on settlement plates at OBB peaked in the spring (Figure 15), and abundance peaks at nearby Point Adams and North Jetties in April according to Hewitt (1993). Furthermore, while *B. violaceus* demonstrated year-round recruitment on settlement plates and this capacity is well-documented (Powell 1970; Ross & McCain 1976), recruitment has been limited to summer months in other studies, with recruitment shown to begin in June and peak in July in the Coos Estuary (Hewitt 1993; Epelbaum *et al.* 2000; Stachowicz *et al.* 2002; Dijkstra *et al.* 2011). Absence of *B. violaceus* from CB only during the season of peak *B. violaceus* recruitment suggests some conditions permitting settlement of the species in CB may be unsuitable in the summer, but the presence of *B. violaceus* at CB during the rest of the year demonstrates that the species can reach the upper bay and establish new populations, though in significantly lower abundance than the lower bay sites.

Tolerance of *Botrylloides violaceus* to abiotic conditions

For many marine and estuarine species, minimum salinity tolerance is the primary factor that sets the distribution (Carlton 1979). The mixing of freshwater and saltwater inputs into an estuary create a salinity gradient which leads to biological zonation of species occupying locations within the estuary that fall within a tolerable salinity range (Bulger *et al.* 1993). For ascidians in coastal estuarine environments, salinity can also act temporally: on a seasonal scale, salinity changes due to the onset of heavy winter rains can stress and kill solitary ascidians (Nydam & Stachowicz 2007).

As such, salinity conditions at a given site in an estuary can permit the establishment of an ascidian such as *Botrylloides violaceus*, but variation over time may impact the presence or abundance of the species at a given point in the year.

Temperature, in addition to salinity, is a primary factor that can limit the performance and spatial distribution of colonial ascidians (Osman & Whitlatch 2007; Epelbaum *et al.* 2009a). Due the role of enzyme kinetics in the function of lateral cilia on ascidian stigmata, feeding clearance rates drop when ascidians are subject to temperatures outside their optimal range, particularly when these temperatures are higher than optimal (Petersen 2007). Since temperature fluctuates throughout each day and throughout the year, larval substrate selection is vital: the site of larval settlement must be restricted to regions in which water quality conditions will not fluctuate above or below the physiological tolerance range since adult colonies cannot move to a new site (Vázquez & Young 1996). Variation in temperature from year to year can also affect recruitment levels in marine invertebrates, as greater recruitment has been documented in colder years (Stachowicz *et al.* 2002).

In contrast to most ascidians, *Botrylloides violaceus* and *Botryllus schlosseri* have wide temperature and salinity tolerance ranges, as well as phenotypic plasticity that allows them to alter their growth and reproduction depending on water quality conditions (Lambert 2005; Carver *et al.* 2006). These characteristics have permitted the widespread invasion of these species in bays and harbors around the world. However, while *B. violaceus* has a global temperature range of 0.6-29.3°C, abiotic tolerance varies substantially between populations just 50 km apart in the Gulf of Maine (Grosholz 2002; Zerebecki & Sorte 2011). Because of this, study of the specific abiotic tolerance

of the Coos Estuary population was necessary to determine whether spatially and temporally-variable estuarine conditions naturally limit the distribution of *B. violaceus* in this ecosystem. I hypothesized that the maximum temperature and minimum salinity tolerance of *B. violaceus* would limit the distribution of this marine species to the cooler, marine waters of the lower Coos Estuary.

Juvenile *Botrylloides violaceus* colonies in my study reliably survived temperatures up to 27°C for seven days. This is two degrees higher than the documented survival range of 5-25 °C by Epelbaum *et al.* (2009b). The highest sea-surface temperature measured at field study sites with established *B. violaceus* populations was 17.8 °C at IBB during the summer (Table 3), well below the measured temperature tolerance of the species. The highest overall field temperatures measured were 20.6 and 21.5 °C during the summer at CB and IS respectively, and while these temperatures fall well below the temperature tolerance measured in the lab, these two sites were marked by an absence of *B. violaceus* during this season. The species was absent from IS year-round, and the high survival rate of colonies experimentally subjected to substantially higher temperatures suggests temperature cannot explain the presence of *B. violaceus* at CB and absence from IS, nor the variation in *B. violaceus* abundance among study sites. A temperature tolerance level of 27°C falls within the range of global temperatures tolerated by *B. violaceus* (above), but is higher than documented temperatures that have yielded competitive advantages for the species: Stachowicz *et al*. (2002) observed higher growth rates of *B. violaceus* than native ascidian *Botryllus schlosseri* at temperatures of 19.1-23.3°C. Prolonged elevated temperatures have been attributed to the build-up of local *B. violaceus* populations due

to an increased number of annual temperature-dependent reproductive cycles (Dijkstra *et al.* 2011). Furthermore, *B. violaceus* is more tolerant of warmer summer temperatures and warmer, shallower embayments than other invasive ascidians, including *Didemnum vexillum* (McCarthy *et al.* 2007; Osman & Whitlatch 2007). My experimentally-derived tolerance level of 27°C (at which point 50% of my colonies survived) corresponds exactly to the LT_{50} documented for the U.S. East coast, which was found to be different from the West coast (25°C) due to differences in habitat temperatures of these two ocean systems (Sorte *et al.* 2011). Local differentiation of the Coos Estuary population may explain a higher tolerance level than other West coast populations.

Experimental results also demonstrate that juvenile *Botrylloides violaceus* colonies can reliably survive salinities as low as 25 psu, though a few colonies did survive at 20 psu. The salinity tolerance range of 20-32 psu reported by Epelbaum *et al.* (2009b) and the survival of colonies in the field at salinities lower than 20 psu (Table 2) suggest my laboratory experiments, likely due to low sample size, inadequately demonstrate the salinity tolerance ability of *B. violaceus* colonies in the Coos Estuary. Salinity levels measured at CB were lower than 20 psu during the fall, winter, and spring (Table 2), but I documented *B. violaceus* at CB during fall (large colonies), winter (several small colonies) and spring (one colony). As such, salinities even as low as 13.9 psu (documented at CB during the winter) do not appear to prevent the survival of this species in the field, but at a certain level may influence abundance.

Historically, salinity levels in IBB have reached a minimum of 17 psu in January, and while this is lower than winter levels observed in this study, a substantial population of *Botrylloides violaceus* has developed at this site (Hewitt 1993). Salinity

lower than 15.6 psu at IS in the spring, then, cannot solely explain the absence of *Botrylloides violaceus* there. Successful transplantation of colonies at IBB, OBB, CB, and IS during the fall season demonstrates the ability of *B. violaceus* to tolerate a combination of fall temperatures and salinities in the upper estuary, which remain well below the upper temperature limit but can approach and fall below the experimentally-derived lower salinity limit of 25 psu. While the survival of adult colonies in variable salinity in the lab failed, it is possible that with improved methodology, a difference in salinity tolerance could be demonstrated between adult and larval *B. violaceus,* which could explain discrepancies between survival of juveniles and colonies observed in the field. For sessile organisms, it is critical that larvae select optimal settlement locations since sessile adult individuals cannot move to avoid fluctuations in salinity and other conditions. As a result, the lower salinity tolerance limit of many ascidian larvae, including *Ciona intestinalis,* is higher than adults of the same species, ensuring their settlement at sites adults can tolerate (Vázquez & Young 1996). This could explain the absence of *B. violaceus* from a site with successful adult transplantation.

In addition to temperature and salinity, I hypothesized that current speed would vary throughout the bay and could contribute to variation in *Botrylloides violaceus* abundance between sites such as IBB and OBB, which had similar salinity and temperature conditions but significant variation in *B. violaceus* percent cover for most of the year. Effective current flow is necessary to reduce "smothering" by the accumulation of sediment on top of adult and juvenile ascidians (Turner *et al.* 1997), as well as enabling consistent food availability; as flow increases around an ascidian, the depleted layer created by consumption of particles by the organism decreases and the

rate of feeding can increase (Railkin 2004). However, current velocities above some speed can impede ascidian feeding and growth rate due to the increased difficulty of particle capture. For this reason, fouling is generally impossible at current velocities faster than 1.5 meters per second (Railkin 2004). I never measured water current velocities faster than 0.5 m/s during seasonal quadrat surveys, so water flow at these Coos Estuary study sites do not appear to reach this critical speed to prevent the survival of adult colonies. However, water current is also responsible for the dispersal of ascidian larvae, so it is more likely variation in current velocity among sites impacts the species by altering the settlement success of *B. violaceus* larvae.

The large tadpole larvae of *Botrylloides violaceus* have a short-lived planktonic stage prior to settlement and metamorphosis, and thus have a limited dispersal potential that varies with current speed (Olson 1985; Young 1985). Though these larvae actively swim using a long flexible tail, the swimming velocities of most invertebrate larvae are typically slower than current speeds and do not contribute significantly to horizontal transport (Chia *et al.* 1984). The ability of *B. violaceus* larvae to swim, combined with sensory and adhesive structures, allows for substrate selectivity based on light, substrate orientation, substrate type or rugosity, or chemical induction by adults of the same species (Svane & Young 1989; Bingham & Young 1991; Railkin 2004). Once an invasive fouling species such as *B. violaceus* is established in a community, secondary dispersal proceeds as currents carry larvae from the new source population within the bay or estuary in question and along coastlines to other embayments (Carlton 1999). While strong current flow enables the transport of larvae, physical settlement of larvae on substrates is impacted by the thickness of the slower boundary layer along the

substrates (Railkin 2004). For this reason, currents above some critical speed could impede the ability of larvae to settle in this boundary layer. While flow varied significantly among the five study sites in the Coos Estuary in both December and April, this variation did not occur between IS and other study sites, indicating that current speed cannot explain variation in *B. violaceus* presence and absence in the bay. Similarity in flow between IS and other sites suggests that given a source population with either sufficient dispersal potential or human transport to IS, and given all other features of IS are suitable for *B. violaceus* survival, ambient current speeds would permit the expansion of this population via settlement onto the IS dock. This is supported by the observation of *B. violaceus* population expansion via larval recruitment in Isthmus Slough from an introduced population in 1990 (Hewitt 1993).

However, current flow did vary significantly between IBB and OBB, two adjacent sites with significantly different *Botrylloides violaceus* cover in all seasons but summer. While OBB currents flow at speeds suitable for larval settlement, faster currents in OBB may prevent heavy *B. violaceus* settlement by sweeping away some larvae with the incoming and outgoing estuarine current. In contrast, slower current speeds at IBB suggest larvae released at this site may be transported in a flow pattern that remains largely contained within the basin. Species assemblages at IBB and OBB were similar enough to overlap on the corresponding MDS plot for each seasonal survey (Figure 7), as there were no taxa unique to either of the sites; variation existed only between the relative abundance of *B. violaceus* and other taxa present in the two regions of the Charleston Boat Basin. Physical study of the recruitment and settlement process of *B. violaceus* at IBB and OBB is necessary to elucidate effects of variation in

current speed on settlement and abundance of the species in the two sites, but it seems likely that the abundance of *B. violaceus* is so much higher in IBB than in OBB, a site similar in location, boat traffic, human use, species composition, and all other water quality conditions, due to slower current speeds and the containment of water in IBB as opposed to the faster current that sweeps past OBB.

The role of *Botrylloides violaceus* in Coos Estuary species assemblages

Upper bay sites hosted distinctly unique species assemblages compared to those at lower bay sites (Figure 7). While many invasive species are found at fouling sites in both regions of the Coos Estuary, upper bay sites are thought to be dominated by introduced estuarine species, whereas all native biodiversity in the estuary occupies marine sites in the lower bay (Hewitt 1993). Taxon richness at IS over the course of all four seasons was comparable to richness at CB and OBB (Figure 5), but the IS species assemblage was characterized by a high abundance of *Molgula manhattensis* and the presence of species unique to that site (*Diadumene lineata* and *Ectopleura crocea*). The comparability of taxon richness at IS to other sites, and the presence of *Botrylloides violaceus* at both the most diverse (IBB) and least diverse (CSY) sites, shows that differences in richness do not appear to make certain sites more susceptible to *B. violaceus* invasion. However, variation in taxon presence and abundance may explain or be explained by the distribution and abundance of *B. violaceus* at these five sites in the estuary.

Sebens (1986) described four major factors that determine the importance of a species in fouling communities: 1) the ability to competitively dominate other species by overgrowing them; 2) the ability to resist overgrowth by other species; 3) the

frequency of a species relative to its competitors; and 4) the potential growth rate of the species. Monitoring of *Botrylloides violaceus* colonies on settlement plates elucidated patterns in these factors.

Botrylloides violaceus *growth rate*

In the seasonal quadrat surveys, *Botrylloides violaceus* occupied the highest percent cover at CSY in the fall, which may be a result of higher recruitment rates in the spring and summer months (Hewitt 1993; Stachowicz *et al.* 2002; Dijkstra *et al.* 2011). However, by May, prior to documented peak recruitment, many settlement plates had 100% *B. violaceus* cover, often made up of a single colony, suggesting that relatively high abundance during this time was a result of asexual growth and successful competition. The spring sampling date was the last in a nine-month long monitoring program, and it was expected that greater *B. violaceus* coverage would take time to develop on the introduced substrata. Deployment timing could influence the seasonal timing of high *B. violaceus* coverage and therefore dominance on settlement plates and explain differences between this successional pattern and those observed in the seasonal quadrat surveys of established fouling communities on the sides of floating dock walls (Underwood & Anderson 1994). However, standardized growth rates of colonies throughout the entire settlement deployment were fastest in the February-to-May interval, suggesting that irrespective of the timing of colony settlement (colonies settled throughout the deployment), *B. violaceus* takes over space at a faster rate during the spring. This corresponds to another peak in *B. violaceus* abundance at IBB and CSY in the spring quadrat survey, and the fact that colonial ascidians tend to dominate spatially during periods of peak fouling species recruitment (Stachowicz *et al.* 2002).

Botrylloides violaceus grows via asexual reproduction, or the blastogenesis of new zooids, and is known to exhibit high variability in growth rate (as well as survivorship and longevity) (Brunetti *et al.* 1980; Epelbaum *et al.* 2009b). Growth rate depends heavily on temperature, though the impacts of increased temperature on growth are decidedly mixed (Saito *et al.* 1981; Grosberg 1988; Westerman *et al.* 2009). In the Coos Estuary, *B. violaceus* appear to follow the observations of Carver *et al.* (2006) and Lord & Whitlatch (2015), who documented increasing *B. violaceus* growth rates in warmer temperatures. As a result, *B. violaceus* dominates the substrate of marine fouling communities during periods of warmer water temperatures, and as this species and other invasive ascidians have increased in abundance, seasonal patterns of peak abundance have shifted from late fall to summer (Dijkstra *et al.* 2007). According to Yamaguchi (1975), however, doubling rates of *B. violaceus* decreased substantially when temperatures increased by 10°C. Temperature at marine study sites in the Coos Estuary, where *B. violaceus* grew most rapidly and was most abundant, did not fluctuate more than 7°C, and perhaps a larger increase in temperature would prove detrimental to growth processes. However, seasonal temperature increases associated with increases in growth rate, specifically at IBB, may be related to increases in primary productivity and therefore food abundance for *B. violaceus.*

Competitive domination by Botrylloides violaceus

Botrylloides violaceus exhibited competitively dominant interactions with several common fouling organisms over the course of settlement plate deployment, and exhibited complete spatial dominance (100% cover) on many plates. While *B. violaceus*

was the first species to settle on several plates, the most common early colonizers were spirorbid polychaetes, *Balanus* spp., and brown and orange encrusting bryozoans. On settlement plates deployed in Australia, *Balanus* and *Spirorbis* were also abundant early colonizers, while encrusting bryozoans dominated spatially later in the successional sequence (Chalmer 1982). These taxa were also the only groups in the Coos Estuary with which *B. violaceus* had solely positive (competitively dominant) interactions, and similar interactions have been observed previously (Hewitt 1993). Frequent overgrowth of encrusting bryozoans, as observed in the Coos Estuary, is common in colonial ascidians (Todd & Turner 1988; Hewitt 1993). The overgrowth of bryozoans and other primary colonizing species by *B. violaceus* can dramatically shift the successional patterns of a developing fouling community by changing the order in which species immigrate to an area (Hewitt 1993). Succession in fouling communities typically follows a standard pattern in which fast-growing organisms such as hydroids, bryozoans, colonial species, polychaetes, and sea anemones settle after the development of a microbial film on the substrate but before the growth of larger, slow-growing invertebrates like mollusks, sponges, and solitary ascidians (Railkin 2004). For most of the duration of settlement plate deployment in this study, plates remained in the first macrofouling stage: domination by fast-growing species. It was during this stage that I observed most interactions with *B. violaceus*, including both positive and negative interactions with invasive ascidian *Botryllus schlosseri,* and hydroid species. However, settlement of *Mytilus* spp. on several plates during the last sampling period (spring) suggests species assemblages on the plates were progressing to the second macrofouling stage during this time. As successional models suggest, inception of this stage would

bring about the decline of colonial species like *B. violaceus* in favor of larger invertebrates, and the competitive dominance of *Halichondria bowerbanki* over *B. violaceus* on settlement plates showed evidence for this. However, the frequent observation of large *B. violaceus* colonies overgrowing swaths of *Mytilus* spp. in the seasonal quadrat surveys suggests that while *B. violaceus* may suffer competition from some secondary settlers after competitively dominating the initial colonization of bare substrate, the species also confers a competitive ability later in succession by settling and growing epibiotically upon *Mytilus* spp. and other slow-growing fouling organisms. For this reason, *B. violaceus* is a competitively dominant species in the Coos Estuary, and has been for some time (Hewitt 1993).

Some studies have shown that bryozoans such as *Schizoporella* eventually replace colonial ascidians as primary occupiers of substrate after ascidians, including *Botrylloides violaceus*, seasonally senesce and uncover overgrown, living bryozoans (Todd & Turner 1988; Hewitt 1993; Nydam & Stachowicz 2007). In this study, *B. violaceus* abundance peaked in the fall after high recruitment and growth in the spring and summer, and then occupied little space during the winter. This is evidence for seasonal "hibernation" and regression which has been observed in other *Botrylloides* species (Burighel *et al.* 1976) and which is consistent with seasonal cohorts observed by Wagstaff (2017). Overgrowth interactions, then, follow seasonal patterns that depend on timing of peak growth and recruitment of dominant species (Chalmer 1982; Sebens 1986; Railkin 2004). For example, in some studies the spatial dominance of *B. violaceus* on settlement panels peaks in April and regresses by August, while other studies show the greatest amount of overgrowth of bryozoan species by *B. violaceus*

between July-August and September-October (Todd & Turner 1988; Hewitt 1993). These observed summer peaks in *B. violaceus* settlement are associated with periods of low recruitment in native species of ascidian, bryozoan, and other fast-growing taxa that would compete with *B. violaceus* for space (Stachowicz & Byrnes 2006).

Furthermore, the competitive dominance of *Botrylloides violaceus* is enabled by the limited predation pressures faced by this species (Carver *et al.* 2006). Predation is a critical process that prevents a dominant species from monopolizing space, halting successional replacement prematurely, and reducing the diversity of a species assemblage (Connell 1972; Russ 1980; Sebens 1986). In a closely related species, *Botrylloides nigrum,* fish predation on juveniles eliminated competition between ascidians and other fouling organisms (Russ 1980), but juvenile *B. violaceus* does not appear to be threatened by such predation (Osman & Whitlatch 1995). *Botrylloides violaceus* faces few, if any, predators in its invaded habitat, perhaps due to chemical defenses and a short window of vulnerability after settlement and before rapid metamorphosis (Pisut & Pawlik 2002; Tarjuelo *et al.* 2002; Carver *et al.* 2006). The species is immune to urchin grazing, and experimental exclusion of potential predators such as chitons, gastropods, and flatworms did not affect *B. violaceus* abundance or recruitment as it did for several native species (Carver *et al.* 2006; Grey 2010b). However, other predator exclusion experiments have shown the opposite result, likely due to different specific predators tested, and these results are supported by the skewed distribution of *B. violaceus* on fouling structures as opposed to the benthos (predator exclusion) (Simkanin *et al.* 2013). While *B. violaceus* grows freely in fouling communities without the impacts of predation, other fouling organisms face both

overgrowth by *B. violaceus* and predation pressures, further exacerbating the spatial dominance of this invasive species.

Resistance to overgrowth

Few species overgrow *Botrylloides violaceus,* which has allowed it to become competitive in many fouling communities. Of the taxa encountered by *B. violaceus* on settlement plates in this study, it demonstrated the capacity to overgrow all except *Halichondria bowerbanki*, also an invasive species in the Coos Bay. Sponges typically colonize space later in the successional hierarchy, and are less frequently overgrown by related species *Botrylloides nigrum* (Russ 1980). In this study, *B. violaceus* was also overgrown by *Botryllus schlosseri* and hydroid species, though it overgrew or remained neutral with these species just as often (Table 22). Colonial ascidians *B. schlosseri, Didemnum vexillum* and *Diplosoma listerianum* have traditionally dominated *B. violaceus* on the U.S. East coast, but these assemblages have become dominated by *B. violaceus* in recent decades as water temperatures have increased (Dijkstra *et al.* 2007; Lord & Whitlatch 2015). Reduction in the growth rate of *D. vexillum* in warmer water coupled with the maintenance or increase in *B. violaceus* growth rate in the same conditions could increase the competitive success of *B. violaceus* in these communities (McCarthy *et al.* 2007). Interactions between colonial ascidians competing for substrate are more frequent than between other taxa, presumably due to their propensity to dominate spatially, a trait well adapted to invading new and disturbed habitats (Schmidt & Warner 1986). All four of the competitively dominant colonial ascidians listed above are invasive in the Coos Estuary (Ruiz *et al.* 2000), but the absence of *D. vexillum* and *D. listerianum* at survey sites and on settlement plates allowed only for the limited

exploration of the relationship between *B. violaceus* and *B. schlosseri* in this study. On shared substrate and in water of higher salinity, *B. violaceus* dominates *B. schlosseri*, a species which typically tolerates lower salinities than *B. violaceus* (Gittenberger & Moons 2011). However, in the Coos Estuary, I only observed *B. schlosseri* at marine sites; sites with lower salinities in the upper bay either had only *B. violaceus* or neither colonial ascidian.

Botrylloides violaceus dominated Coos Estuary fouling communities via successful overgrowth of many native and invasive species. Lack of *B. violaceus* at IS, either on the docks or on deployed settlement plates, prevented the observation of interactions with the invasive species that dominate this site, so it is unclear whether biotic competition between *B. violaceus* and species such as *Diadumene lineata* and *Ectopleura crocea* would permit the establishment of a *B. violaceus* population upon introduction. Hewitt (1993) rejected the notion that competitive domination alone allowed for the establishment of so many invasive species in the Coos Estuary, so while this characteristic seems to allow *B. violaceus* to persist at many dock sites, other factors may work concurrently to allow or prevent the establishment of this species and determine its distribution.

Not only does *Botrylloides violaceus* alter community structure by directly overgrowing native and other non-native species, but its presence and competitive dominance can deter larvae from settling nearby (Grosberg 1981). Most marine fouling invertebrates are susceptible to overgrowth by other species; the growth of primary and secondary species on a substrate increases the surface area available for the settlement, or epibiosis, of secondary and tertiary species. However, many sponge and ascidian

species, including *B. violaceus*, are resistant to the settlement of larvae by releasing bioactive substances that larvae avoid during settlement (Hewitt 1993; Railkin 2004). I observed no epibiosis on *B. violaceus* throughout settlement plate deployment or during seasonal quadrat surveys, which supports the observations of Hewitt (1993) in the Coos Estuary. Epibiosis can inhibit the growth of the species being settled on, so by avoiding this, *B. violaceus* may be able to more rapidly take over bare and occupied substrate than other species while also preventing the settlement of species in on the surface it occupies (Railkin 2004). In this way, *B. violaceus* introduction and establishment in fouling communities can drastically impact native biodiversity and abundance.

Frequency of Botrylloides violaceus *relative to competitors*

Only one species consistently overgrew *Botrylloides violaceus* during this study: *Halichondria bowerbanki*. Because of this, it is less useful to compare the abundance of *B. violaceus* to its competitors than it might be for other species with a greater potential for overgrowth. However, the presence and abundance of *B. violaceus* relative to the abundance and diversity of the species assemblages of the Coos Estuary can potentially demonstrate the impact of this invasion on the native ecosystem. If the presence of *B. violaceus* had a significant impact on the presence and abundance of other taxa typically found in the species assemblages at a particular site, we would expect quadrats with *B. violaceus* to differ significantly in species diversity and abundance. This is not shown in the seasonal quadrat surveys (Figure 10); quadrats with *B. violaceus* presence are well dispersed and overlap with quadrats without *B. violaceus.* While the MDS plot for the fall survey appears to have greater general clustering of quadrats with *B. violaceus*,

those quadrats heavily overlap with sites without *B. violaceus*, suggesting those quadrats were biologically similar irrespective of *B. violaceus* presence.

Implications of dispersal

At the outset of this study, I hypothesized abiotic conditions such as temperature, salinity, and current speed would naturally limit the distribution of *Botrylloides violaceus* in the bay and prevent establishment of this species in Isthmus Slough. The above results suggest that temperature and salinity cannot explain this distribution, unless larval tolerance differs from settled individuals, which remains untested. Furthermore, *B. violaceus* demonstrated competitive dominance over almost every other species or taxon it encountered, and potential biotic exclusion of *B. violaceus* from the unique species assemblage in Isthmus Slough merits exploration. If neither abiotic factors or interspecific interactions contribute to the distribution of this species, then I hypothesized *B. violaceus* would be limited in estuarine distribution by its larval dispersal. *Botrylloides violaceus* has a short-duration larval stage and thus a short dispersal distance, varying with current flow velocity. If *B. violaceus* were absent from both upper-bay sites (CB and IS), this would suggest that the dispersal distance of the species prevented settlement in regions of the bay too distant from source populations in either the Charleston Boat Basin or intermediate "stepping stone" docks and bridges between the lower and upper bay (Floerl *et al.* 2009). However, the presence of *B. violaceus* at CB during three seasons demonstrates the ability of larvae to reach the upper bay; whether these larvae were released by adults in the lower bay and brought upstream via currents, released from adults in fouling communities near the upper bay that were not assessed in this study, or released by adults introduced to the

upper bay via boat transport, is unclear. Study of the specific dispersal distance of *B. violaceus* larvae in the Coos Estuary is necessary to determine whether larvae can travel all the way through the estuary from lower-bay source populations, whether other less visible populations in the bay may serve as intermediate sources, or whether boats or mariculture practices continue to introduce the species to the upper bay, which may merit stricter regulations on boat and equipment cleaning prior to intra-bay travel. Regardless, the presence of *B. violaceus* at CB suggests that the current means of larval dispersal should permit spread from CB to IS. Because of this, and the tolerance of *B. violaceus* to other abiotic conditions of the upper bay, some other factor or factors must explain the absence of *B. violaceus* in Isthmus Slough.

Invasive species control

Oduor (1999) proposes several methods to biologically control an invasive species, namely: 1) the introduction and inoculation of a natural predator; 2) the augmentation of the population of a natural predator; and 3) the conservation of natural predator populations. Biological control of invasive species is an appealing antifouling strategy, as it avoids the physical and chemical disturbance of native and potentially commercially-viable species that can occur in other methods of eradication (Arens *et al.* 2011). However, since *Botrylloides violaceus* lacks natural predators that could be used to biologically manage this species, efforts to eradicate *B. violaceus* from fouling communities have required other more intrusive means, including freshwater, brine, and acetic acid immersion of structures fouled with *B. violaceus* (Arens *et al.* 2011). Anti-fouling paints are a common technique for deterring the settlement of many fouling organisms; however, this strategy provides only a temporary solution, as this paint

eventually flakes off, permitting the settlement of fouling species again (Railkin 2004). Therefore, this method requires repeated reapplication, and the chemicals in this paint may be detrimental to the health of the native community (Bryan *et al.* 1986; Tolosa *et al.* 1996). The development of anti-fouling microstructure materials that deter settlement physically rather than chemically is promising (Flemming 2003), but the effectiveness of these materials for preventing *B. violaceus* settlement remains unexplored.

The most common antifouling treatment for management of *Botrylloides violaceus* is the use of pressurized seawater to physically remove colonies from fouling structures. While this method has proven effective in specific cases, and does not impact the growth of commercially-valuable mussels in the community, successful eradication of *B. violaceus* using this method depends on the abundance of the species, the timing of treatment, and a host of environmental factors. Also, this method may facilitate increased settlement of *B. violaceus*; for one, the process of high-pressure spraying removes other biomass from fouling structures, "priming" the substrate for successful settlement of *B. violaceus*. Furthermore, the ability of *B. violaceus* to survive and spread after fragmentation of colonies allows the species to thrive after this type of physical disturbance (Arens *et al.* 2011). Further exploration of antifouling measures is necessary for the eradication of *B. violaceus* from communities on which it has a large negative impact on native biodiversity and aquaculture practices.

The management of vectors is of primary importance in preventing the further spread of the species and limiting the population to its present distribution (Crooks & Soule 1999). Traditional protocols for managing ballast water of ships entering and

exiting ports from the open ocean, thereby potentially introducing new species to fouling communities, include the requirement of deballasting in the open ocean (where coastal species in ballast water will theoretically die) and reballasting in the open ocean for release at the destination port (where oceanic species in ballast water will theoretically die) (Carlton 1999). However, ballast water is only one of many methods of introduction of *Botrylloides violaceus* into bays and harbors; for the Coos Estuary specifically, the species is known to have invaded via oyster mariculture practices. Transport of equipment within a bay system may permit the spread of the species, and practices should be implemented to reduce potential intra-bay transport on such commercial structures. Intra-bay transport via direct fouling on recreational boats in the Coos Estuary is already managed via recreational boat-cleaning requirements.

Conclusions

In this study, I have shown that invasive ascidian *Botrylloides violaceus* has a local distribution that extends to the upper reaches of the Coos Estuary but does not reach Isthmus Slough, despite the presence of the species there almost 40 years ago. This population tolerates a wide range of salinities and temperatures throughout the estuary, and can physiologically tolerate conditions that would permit the spread of this species to uninvaded fouling sites, including Isthmus Slough. The increased difficulty of larval settlement at sites with faster current speed may explain variation in *B. violaceus* abundance between otherwise similar sites within the Charleston Boat Basin. Current speeds at IS suggest larval settlement is possible at this site, given a viable population source such as CB, and successful transplantation demonstrates the abiotic suitability of the habitat for adult colonies.

Therefore, some other factor or factors must contribute to the absence of *Botrylloides violaceus* from the IS dock site. *Botrylloides violaceus* is a highly competitively dominant fouling species that demonstrated its ability to overgrow hydroids and other ascidian species in this study, but exploration of its relationships with *Ectopleura crocea* or *Diadumene lineata* (species unique to IS) may reveal that those species impact *B. violaceus* settlement or survival in some way. However, it is also possible that differences in substrate material may determine where *B. violaceus* can settle; ascidian larvae display high selectivity for substrate type (Svane & Young 1989; Bingham & Young 1991; Railkin 2004), and the dock walls at IS are made of metal while the dock walls at all other study sites are made of cement.

The ability of *Botrylloides violaceus* to tolerate a wide array of abiotic conditions, as well as its competitive dominance in the ecosystem, has allowed the species to firmly establish populations in the Coos Estuary, as well as many other bays on the U.S. West Coast. The fouling communities of the Coos Estuary are dominated by many non-native marine invertebrates, so the impacts of *B. violaceus* on these species assemblages have not prompted local management efforts. However, projected implications of climate change on the Coos Estuary suggest that *B. violaceus* may become more dominant in fouling communities as abiotic conditions become unsuitable for native species (Sutherland & O'Neill 2016). Attempts to eradicate *B. violaceus* from the Coos Estuary could prevent the homogenization of fouling communities expected to result from increased competitive dominance of *B. violaceus*. However, suitable eradication methods must be developed for the successful elimination of this invasive species. In the meantime, improved management of maricultural equipment and

transport can minimize the spread of *B. violaceus* throughout the bay. My research has elucidated the distribution and demography of the *Botrylloides violaceus* population in the Coos Estuary and determined factors which contribute to the invasion and abundance of this species in local fouling communities.

Tables

Life stage	Salinity (psu)									
	5	10	15	20	25	30	32	33	35	40
Juvenile	4	5	5	5	5	5	3	2	3	2
Adult	5	5	5	5	5	5	4	3	3	3

Life stage	Temperature (°C)													
	16	17	18	19	20	21	22	23	24	25	26	27	28	30
Juvenile	0	0	3	5	2	5	4	5	5	3	3	2	4	2
Adult	2	4	4	4	4	0	4	4	2	4	0	4	4	0

Table 1: Number of colonies (n) treated at each salinity and temperature level in laboratory tolerance experiments.

Salinity tolerance experiments consisted of five trials for both adult and juvenile colonies; temperature tolerance trials consisted of five trials for juvenile colonies and four trials for adult colonies. Deviation of n from the number of trials is a result of the inability to collect enough colonies to test every treatment level in some trials.

Site	Summer Mean ± SD (n)	Fall Mean ± SD (n)	Winter Mean ± SD (n)	Spring Mean ± SD (n)
IBB	34.9 ± 3.7 (20)	37.8 ± 2.2 (20)	24.4 ± 1.2 (19)	26.7 ± 1.6 (20)
OBB	37.0 ± 1.3 (20)	34.9 ± 1.1 (19)	28.9 ± 0.7 (19)	30.4 ± 0.8 (20)
CSY	34.8 ± 0.4 (20)	33.3 ± 0.7 (19)	29.6 ± 1.2 (20)	32.8 ± 1.9 (20)
IS	31.3 ± 0.9 (20)	25.1 ± 0.2 (20)	15.5 ± 3.4 (20)	23.7 ± 1.3 (20)
CB	31.6 ± 0.5 (20)	18.9 ± 0.7 (20)	13.9 ± 1.2 (20)	15.6 ± 0.6 (20)

Table 2: Average surface salinity (psu) measured at study sites in seasonal quadrat surveys.

Site	Summer Mean ± SD (n)	Fall Mean ± SD (n)	Winter Mean ± SD (n)	Spring Mean ± SD (n)
IBB	17.8 ± 0.7 (20)	14.5 ± 0.2 (20)	11 ± 0.0 (19)	14.5 ± 0.4 (20)
OBB	14.4 ± 0.7 (20)	13.0 ± 0.1 (19)	11.9 ± 0.2 (19)	12.6 ± 0.7 (20)
CSY	13.0 ± 0.0 (20)	13.0 ± 0.0 (19)	12.6 ± 0.4 (20)	12.7 ± 1.1 (20)
IS	21.5 ± 0.5 (20)	12.3 ± 0.2 (20)	11.2 ± 0.3 (20)	17.6 ± 0.8 (20)
CB	20.6 ± 0.3 (20)	12.2 ± 0.4 (20)	11 ± 0.0 (20)	16.4 ± 1.1 (20)

Table 3: Average surface temperature (°C) measured at study sites in seasonal quadrat surveys.

Site	Summer Mean ± SD (n)	Fall Mean ± SD (n)	Winter Mean ± SD (n)	Spring Mean ± SD (n)
IBB	0.02 ± 0.01 (20)	0.02 ± 0.01 (20)	0.04 ± 0.02 (19)	0.02 ± 0.01 (20)
OBB	0.04 ± 0.03 (20)	0.02 ± 0.01 (19)	0.03 ± 0.02 (19)	0.02 ± 0.02 (20)
CSY	0.41 ± 0.04 (20)	0.12 ± 0.05 (19)	0.25 ± 0.10 (20)	0.16 ± 0.08 (20)
IS	0.11 ± 0.02 (20)	0.17 ± 0.04 (20)	0.04 ± 0.03 (20)	0.04 ± 0.03 (20)
CB	0.08 ± 0.04 (20)	0.20 ± 0.12 (20)	0.13 ± 0.05 (20)	0.03 ± 0.02 (20)

Table 4: Average instantaneous flow velocity (m/s) measured at study sites in seasonal quadrat surveys.

Taxa	Observation site					
	IBB	OBB	CSY	CB	IS	Settlement plates
Phylum Chordata						
Botrylloides violaceus	×	×	×	×		×
Botryllus schlosseri	×		×	×		×
Molgula manhattensis	×	×	×	×	×	×
Distaplia occidentalis	×					
Styela clava		×		×		
Ascidian spp.		×				
Ascidian settlers						×
Phylum Porifera						
Halichondria bowerbankii	×	×	×	×	×	×
Haliclona sp. A	×	×	×			×
Peach sponge			×			
Orange sponge	×	×	×			
White sponge			×			
Tethya californiana		×				
Phylum Ectoprocta						
Brown encrusting bryozoan	×	×		×		×
Red encrusting bryozoan	×			×		×
Orange bryozoan	×	×	×	×		×
White encrusting bryozoan	×		×			
Yellow encrusting bryozoan	×		×			×
Pink encrusting bryozoan	×		×			
Phylum Mollusca						
Mytilus spp.	×	×	×	×	×	×
Limpets	×	×		×		
Chitons	×	×	×	×		×
Clams	×	×	×	×	×	
Oysters	×	×	×	×	×	
Scallops	×	×			×	×
Dialula sandiegensis	×					
Doris montereyensis			×			
Janolus fuscus			×			×
Nudibranch eggs	×	×				×
Snails						×
Phylum Arthropoda						
Balanus spp.	×	×	×	×	×	×
Crabs	×	×	×	×		×
Phylum Cnidaria						
Metridium senile	×	×	×	×	×	×
Anthopleura xanthogrammica		×	×			
Diadumene lineata		×			×	×
Ectopleura crocea					×	×
Hydroids						×
Phylum Annelida						
Serpulids	×	×	×			
Spirorbid polychaetes						×
Sabellids		×	×	×		
Polychaetes						×

Table 5: Fouling taxa documented at Coos Estuary study sites in seasonal quadrat surveys and on settlement plates (deployed from August 2015 – May 2016) at any site. "×" indicates presence of taxon at a site in any seasonal survey or on a settlement plate at any site.

Site	Taxon richness (S)			
	Summer	Fall	Winter	Spring
IBB	5.4	6.6	4.6	5.6
OBB	3.3	4.6	3.9	4.3
CSY	2.0	3.1	3.9	2.5
CB	2.9	4.5	2.9	4.2
IS	3.7	5.2	3.2	2.9

Table 6: Mean taxon richness documented per quadrat in seasonal surveys at five Coos Estuary study sites.

Factor	df	SS	MS	F	p	η^2
Season	3	87.4	29.1	19.6	<0.001	0.2
Site	4	261.9	65.5	44.0	<0.001	0.3
Season × Site	12	84.8	7.11	4.88	<0.001	0.1
Residuals	330	490.9	1.5			

Table 7: Two-way ANOVA table for the effect of season and site on taxon richness in seasonal quadrat surveys.

Significance level (α) = 0.05.

Factor	df	SS	MS	F	p	Partial η^2
Season	3	187.3	62.44	5.74	<0.001	0.04
Site	4	171.0	42.88	3.93	<0.01	0.04
Season × Site	12	2554.7	21.23	1.95	0.03	0.06
Residuals	344	3743.0	10.99			

Table 8: Two-way ANOVA table for the effect of season and site on *Botrylloides violaceus* percent cover measured in seasonal quadrat surveys.

Significance level (α) = 0.025.

Experiment	Life stage	
	Juvenile	Adult
Salinity, 24 hours	5 psu	15 psu
Salinity, 7 days	25 psu	N/A
Temperature, 24 hours	30°C*	25°C
Temperature, 7 days	27°C**	N/A

Table 9: Salinity and temperature tolerance levels of juvenile and adult *Botrylloides violaceus* colonies after one and seven days of treatment.

Experimental design limited my ability to collect adequate data on salinity and temperature tolerance for adult colonies in the long-term (seven days). *Botrylloides violaceus* colonies tolerated lower salinities and higher temperatures than field conditions measured in seasonal quadrat surveys (Tables 2 and 3). *30°C was the highest temperature tested in this experiment, so it is possible juvenile colonies can survive even higher temperatures after 24 hours. **Juvenile colonies also survived 30°C at a rate of 50%, but I declare the threshold at 27°C because only one colony survived at 28°C.

Factor	df	SS	MS	F	p
Site	4	140.01	35.00	5.79	<0.01
Initial chalk mass	1	60.46	60.46	10.00	<0.01
Residuals	19	145.07	6.05		

Table 10: ANCOVA table for the effect of site and initial chalk mass on the dissolution of chalk clod cards in December 2015.

Significance level (α) = 0.05.

Factor	df	SS	MS	F	p
Clod card unit	9	156.14	17.35	2.65	0.04
Initial chalk mass	1	64.79	64.79	9.88	<0.01
Residuals	19	124.61	6.56		

Table 11: ANCOVA table for the effect of clod card unit and initial chalk mass on the dissolution of chalk clod cards in December 2015.

Significance level (α) = 0.05.

Factor	df	SS	MS	F	p
Clod card unit	8	223.22	27.90	122.37	<0.001
Initial chalk mass	1	0.93	0.93	4.09	0.06
Residuals	17	3.88	0.23		

Table 12: ANCOVA table for the effect of site and initial chalk mass on the dissolution of chalk clod cards in April 2016.

Significance level (α) = 0.05.

Factor	df	SS	MS	F	p
Site	4	170.14	42.54	15.46	<0.001
Initial chalk mass	1	0.09	0.09	0.03	0.86
Residuals	21	57.80	2.75		

Table 13: ANCOVA table for the effect of clod card unit and individual chalk mass on the dissolution of chalk clod cards in April 2016.

Significance level (α) = 0.025.

Site pairings	p
CB2-CB1	0.88
CSY1-CB1	0.71
CSY2-CB1	0.97
IBB1-CB1	<0.001
IBB2-CB1	<0.001
IS1-CB1	0.46
OBB1-CB1	<0.001
OBB2-CB1	0.99
CSY1-CB2	1.00
CSY2-CB2	0.31
IBB1-CB2	<0.001
IBB2-CB2	<0.001
IS1-CB2	0.05
OBB1-CB2	<0.001
OBB2-CB2	0.38
CSY2-CSY1	0.19
IBB1-CSY1	<0.001
IBB2-CSY1	<0.001
IS1-CSY1	0.02
OBB1-CSY1	<0.001
OBB2-CSY1	0.24
IBB1-CSY2	<0.001
IBB2-CSY2	<0.001
IS1-CSY2	0.96
OBB1-CSY2	<0.001
OBB2-CSY2	1.00
IBB2-IBB1	<0.001
IS1-IBB1	<0.001
OBB1-IBB1	0.99
OBB2-IBB1	<0.001
IS1-IBB2	<0.01
OBB1-IBB2	<0.001
OBB2-IBB2	<0.001
OBB1-IS1	<0.001
OBB2-IS1	0.93
OBB2-OBB1	<0.001

Table 14: Post-hoc Tukey HSD test for clod card dissolution between all pairs of clod card units in April 2016.

Significance level (α) = 0.05.

Site/Dock	Survival of transplanted colonies			
	Trial 1 (9 days)	Trial 2 (8 days)	Trial 3 (7 days)	Trial 3 (35 days)
IBB I59	Yes	No	No	No
IBB G80	Container lost	N/A	N/A	N/A
IBB H27	Partial	Yes	Yes	No
OBB D/E	No	No	Yes	No
OBB C27	No	No	Yes	No
OBB B	No	Partial	No	No
IS 1	No	No	No	No
IS 2	No	No	Container lost	N/A
IS 3	No	No	Yes	No
CB 1	No	No	No	No
CB 2 (end)	Partial	No	No	No
CB 2 (ramp)	Partial	No	Yes	No

Table 15: Survival of *Botrylloides violaceus* colonies transplanted to the upper and lower Coos Estuary.

Colonies in trial 1 deployed at a depth of 1.0 m, all other trials deployed at depth of 0.5 m. In trial 3, colonies maintained in a vertical position via manual adhesion to plastic mesh inside transplant containers. Trial 3 colonies assessed at seven days, then re-deployed for total of 35 days. In cases of transplant container loss, no colonies could be deployed at that site in subsequent trials.

Factor	df	SS	MS	F	p	Partial η^2
Date	7	9149.7	1307.09	17.58	<0.001	0.27
Site	4	1077.8	269.44	3.62	<0.01	0.04
Date × Site	28	7590.6	271.09	3.65	<0.001	0.23
Residuals	340	25278.6	74.35			

Table 16. Two-way ANOVA table for the effect of sampling date and site on *Botrylloides violaceus* cover on settlement plates deployed at all five study sites in the Coos Estuary.

Significance level (α) = 0.025.

Factor	df	SS	MS	F	p	Partial η^2
Date	7	11340.7	1620.10	15.72	<0.001	0.31
Site	2	458.9	229.45	2.23	0.11	0.02
Date × Site	14	5184.3	370.31	3.59	<0.001	0.17
Residuals	242	24934.9	103.04			

Table 17. Two-way ANOVA table for the effect of sampling date and site on *Botrylloides violaceus* cover on settlement plates at study sites with *B. violaceus* settlement on plates only (IBB, OBB, and CSY).

Significance level (α) = 0.025.

Site	Summer	Fall				Winter		Spring
	08/26/15	10/04/15	10/25/15	11/15/15	12/06/15	01/18/16	02/20/16	05/22/16
CB	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
CSY	0.0	25.0	0.0	12.5	7.1	5.3	0.0	4.3
IBB	100.0	50.0	50.0	37.5	31.0	47.4	53.8	74.5
IS	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
OBB	0.0	25.0	50.0	50.0	61.9	47.4	46.2	21.3

Table 18: Distribution of *Botrylloides violaceus* colonies settled on plates (in percent of total colonies present) at each sampling date.

Plates deployed on 08/16/2015.

Factor	df	SS	MS	F	p	Partial η^2
Sampling interval	6	50.20	8.37	4.98	<0.001	0.44
Site	2	5.034	2.52	1.50	0.24	0.07
Sampling interval × Site	5	3.89	0.78	0.46	0.80	0.06
Residuals	38	63.85	1.68			

Table 19. Two-way ANOVA table for the effect of sampling interval and site on *Botrylloides violaceus* lateral growth rate on settlement plates.

Significance level (α) = 0.05.

Sampling interval	Average growth (cm^2/week) ± SD	Colonies (n)
8/26 - 10/04/15	0.09	1
10/4 - 10/25/15	0.64 ± 0.66	2
10/25 - 11/15/15	0.88 ± 1.18	8
11/15 - 12/06/15	0.667 ± 1.15	18
12/6/15 - 1/18/16	0.43 ± 1.29	9
1/18 - 2/20/16	0.60 ± 0. 81	8
2/20 - 5/22/16	3.67 ± 2.11	6

Table 20: Average net growth of *Botrylloides violaceus* colonies (cm^2 per week) on settlement plates during each sampling interval (irrespective of site).

Factor	df	SS	MS	F	p	Partial η^2
Season	3	49.80	16.60	11.30	<0.001	0.44
Site	2	5.17	2.59	1.76	0.18	0.07
Season × Site	2	3.33	1.66	1.13	0.33	0.05
Residuals	44	64.67	1.47			

Table 21. Two-way ANOVA table for the effect of season and site on *Botrylloides violaceus* lateral growth rate (cm^2 per week) on settlement plates.

Significance level (α) = 0.05.

Plate	Taxon								
	BOTV	BOTS	ASC	HAL	BBRYO	OBRYO	BAL	HYD	SPIRO
CSY5 R									
CSY5 S									
CSY6 R								-	
CSY6 R								+	
CSY6 S								+	
IBB G22 R									
IBB G22 S									
IBB G36 R	B	B		-	+	+			
IBB G36 S	B	-		B					+
IBB H33 R					+				+
IBB H33 S					+		+		+
IBB H77 R									+
IBB H77 S									+
IBB I27 R		B			+				+
IBB I27 S	+	B			+				+
IBB I55 R				B					+
IBB I55 S									B
OBB B37 R	+	+	+			+	+		
OBB B37 S	+						+		+
OBB B43 S									
OBB B51 R	+								+
OBB B51 S	+			B			+	B	+
OBB B53 R					+				
OBB B53 S									+
OBB D58 S									B

Table 22: Interaction table for competitive interactions between *Botrylloides violaceus* and other fouling taxa on settlement plates.

"+" indicates a competitive interaction in which *B. violaceus* overgrew the space occupied by the other organism and gained spatial cover. "-" indicates a competitive interaction in which the other organism overgrew space occupied by *B. violaceus* and *B. violaceus* lost spatial cover. "B" indicates a border interaction: the edge of the *B. violaceus* colony bordered the edge of the other organism but the outcome of this interaction is unknown. Species key: BOTV = *Botrylloides violaceus;* BOTS = *Botryllus schlosseri;* ASC = Ascidian settlers; HAL = *Halichondria bowerbanki;* BBRYO = Brown bryozoan; OBRYO = Orange bryozoan; BAL = *Balanus* spp.; HYD = Hydroid spp.; SPIRO = Spirorbid polychaetes

Figures

Figure 1: Five dock study sites in the Coos Estuary (43° 20' 44" N, 124° 19' 13" W).

Lower bay sites (marine): Inner Boat Basin (IBB), Outer Boat Basin (OBB), and Charleston Shipyard (CSY). Upper bay sites (mesohaline): Coos Bay City Docks (CB) and Isthmus Slough (IS). IBB and OBB are two separate basins in the Charleston Boat Basin.

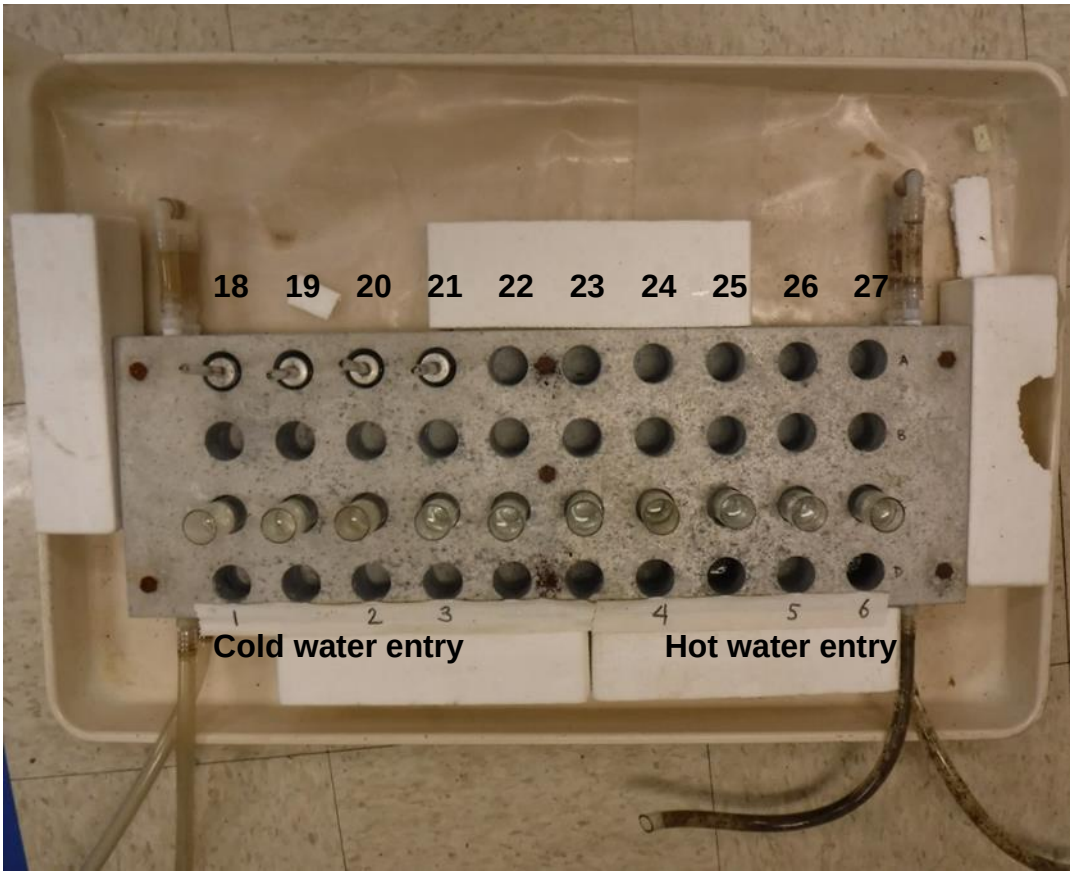

Figure 2: Aluminum thermal gradient block used for temperature tolerance experiments.

Hot water flowed in from one end and cold water flowed in from the other to create a temperature gradient (the approximate temperature of each vial is shown, in °C).

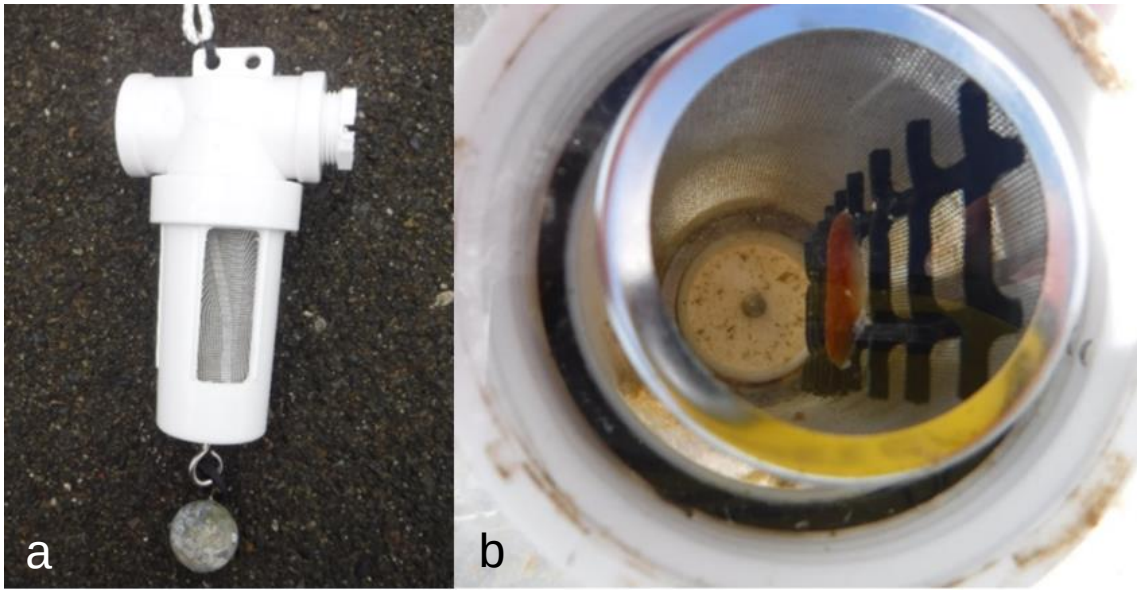

Figure 3: Container used for *Botrylloides violaceus* colony transplantation to study sites in the Coos Estuary.

Figure 3a: *Botrylloides violaceus* colony transplant container. Figure 3b: Interior of the transplant container configured for maintaining colonies in vertical orientation for trial 3.

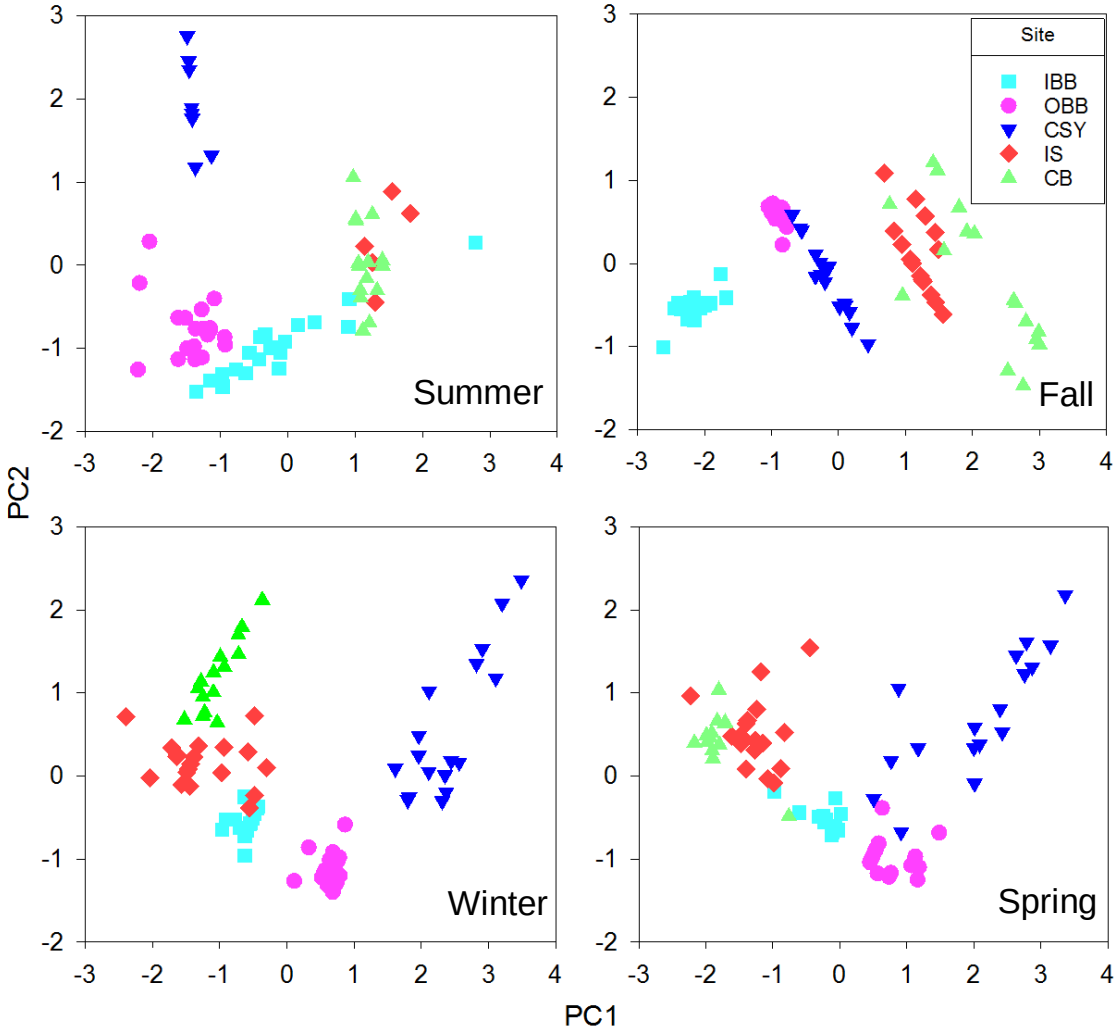

Figure 4: Principal Components Analysis relating abiotic variables (salinity, temperature, and current speed) measured at study sites in seasonal quadrat surveys.

Summer: PC1 accounts for 56% of the variation in the abiotic data. Fall: PC1 accounts for 81.4% of the variation in the abiotic data. Winter: PC1 accounts for 65.5% of the variation in the abiotic data. Spring: PC1 accounts for 69.8% of the variation in the abiotic data.

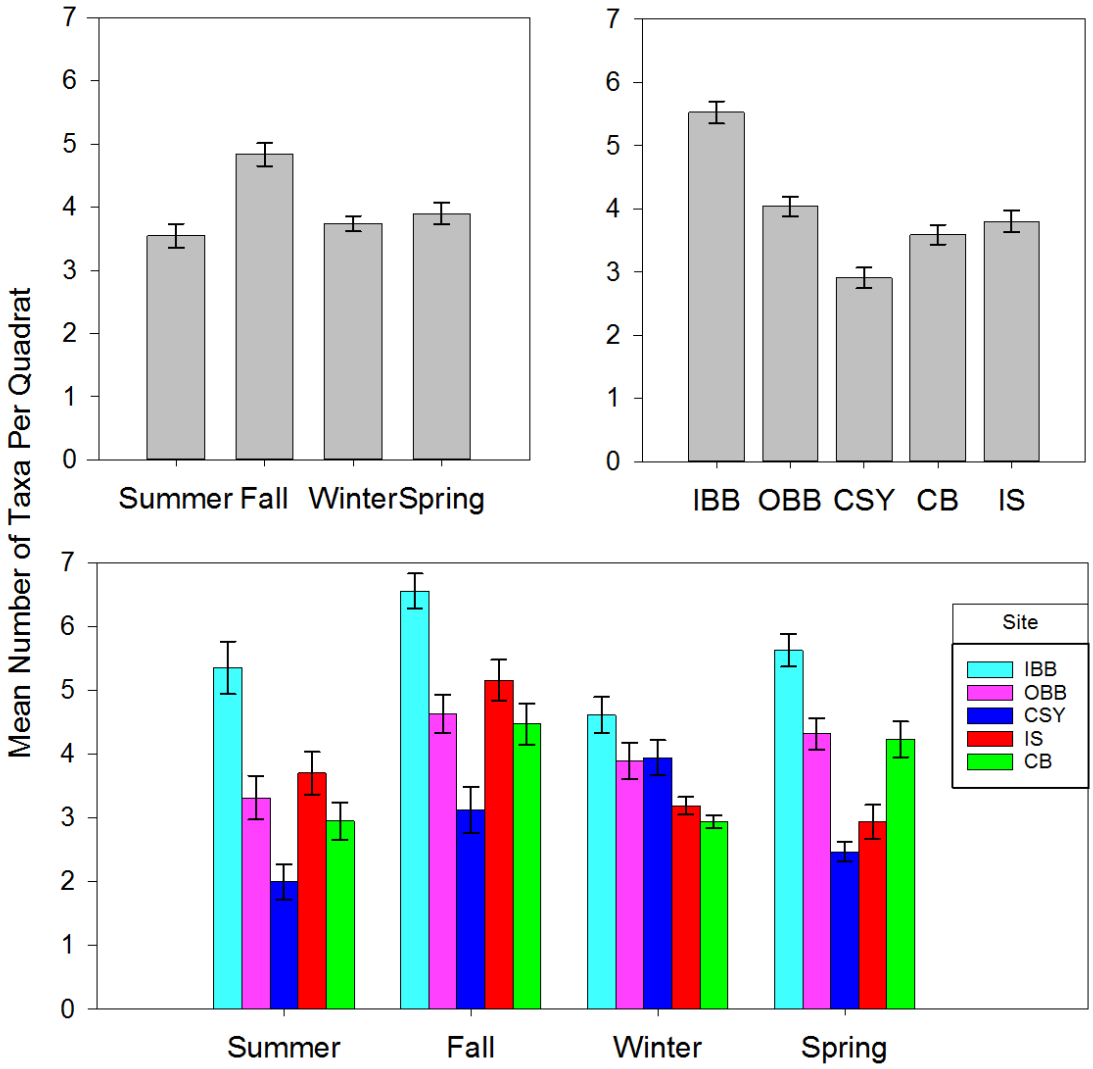

Figure 5: Mean number of taxa documented on each quadrat in seasonal surveys.

Brackets indicate standard error. Mean number of taxa varied significantly with site and season (two-way ANOVA, $\alpha = 0.05$, $p < 0.01$).

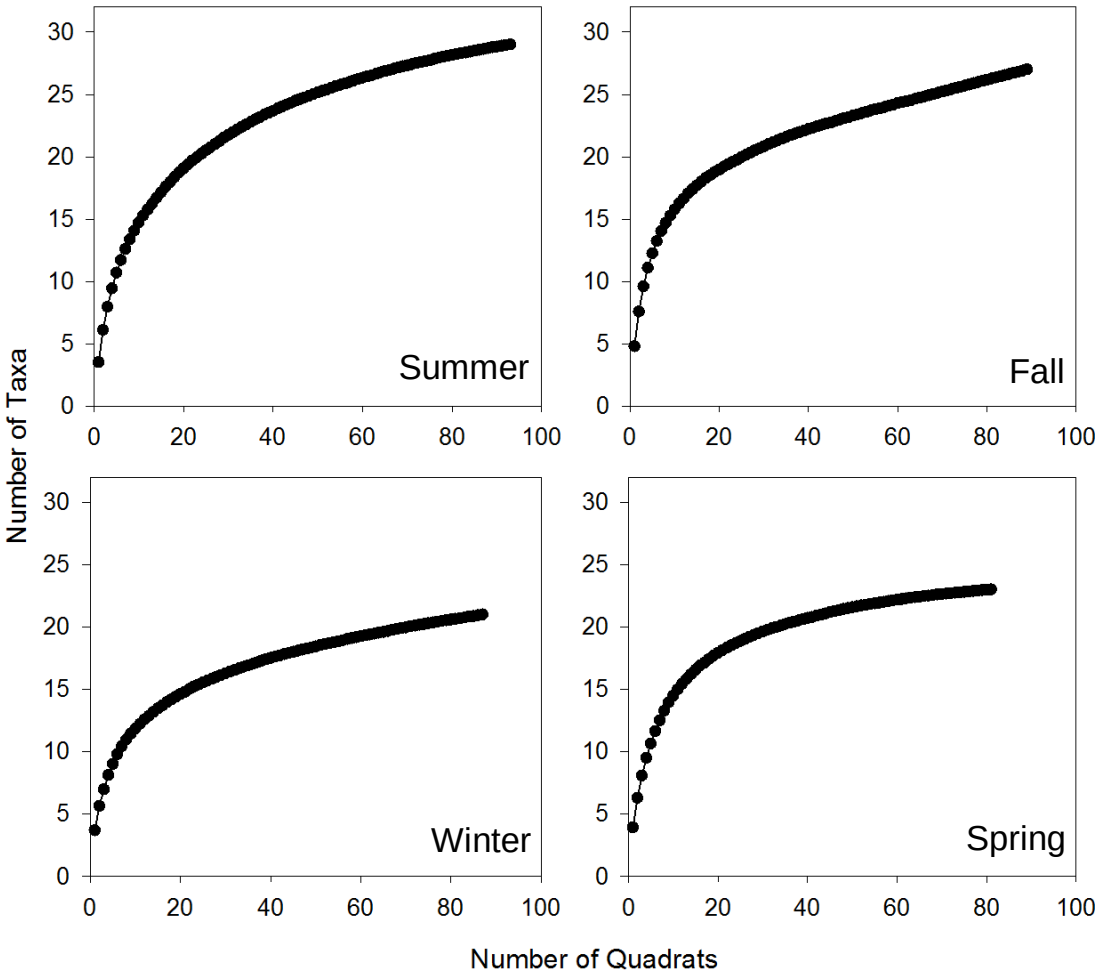

Figure 6: Taxon accumulation plots showing the total number of taxa documented at each seasonal quadrat survey.

Sampling efforts accounted for nearly all of the richness present (at my scale of detection) at the sampling sites, with the spring survey yielding the highest richness and nearing an asymptote of around 23 taxa.

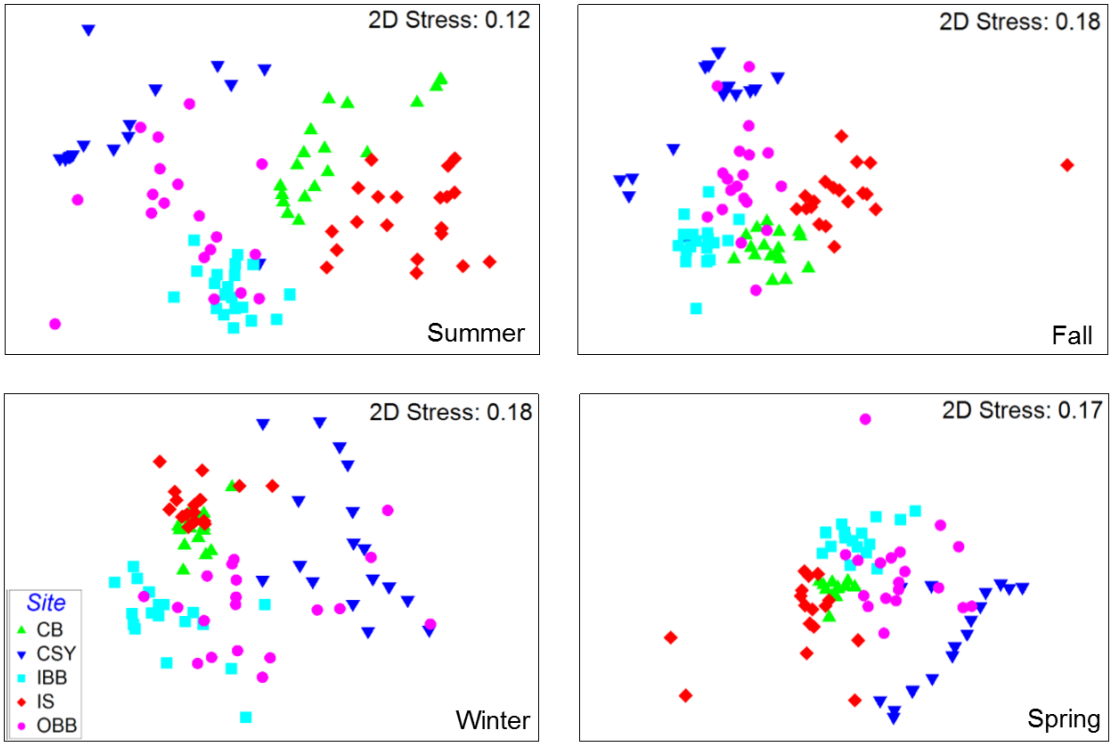

Figure 7: Multi-dimensional scaling (MDS) plot relating the species composition of each quadrat in seasonal quadrat surveys.

Plots constructed from a Bray-Curtis similarity matrix of square root-transformed percent cover data. Three quadrats at IS had 0% cover in the winter survey, and one quadrat at IS had 0% cover in the spring survey, which were omitted from the dataset in order to construct this plot. Upper bay sites (CB and IS) consistently form clusters distinct from those of lower bay sites, indicating their unique species composition.

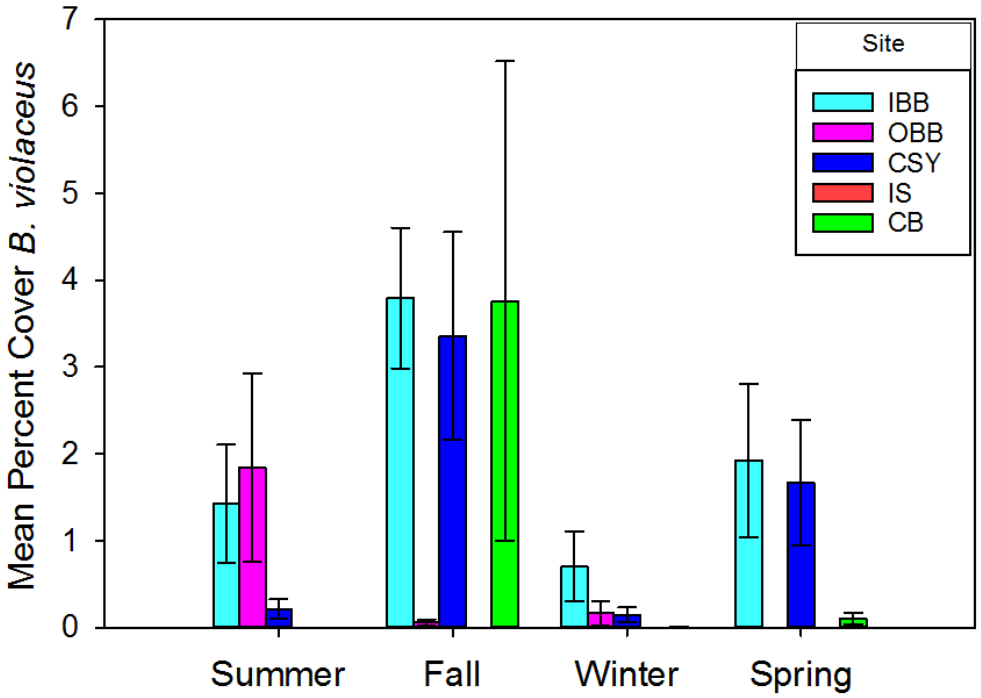

Figure 8: Mean percent cover of *Botrylloides violaceus* at each site and season in seasonal quadrat surveys.

Brackets indicate standard error. Mean percent cover varied significantly with site (two-way ANOVA, $\alpha = 0.025$, $p < 0.01$) and season (two-way ANOVA, $\alpha = 0.025$, $p < 0.01$) separately.

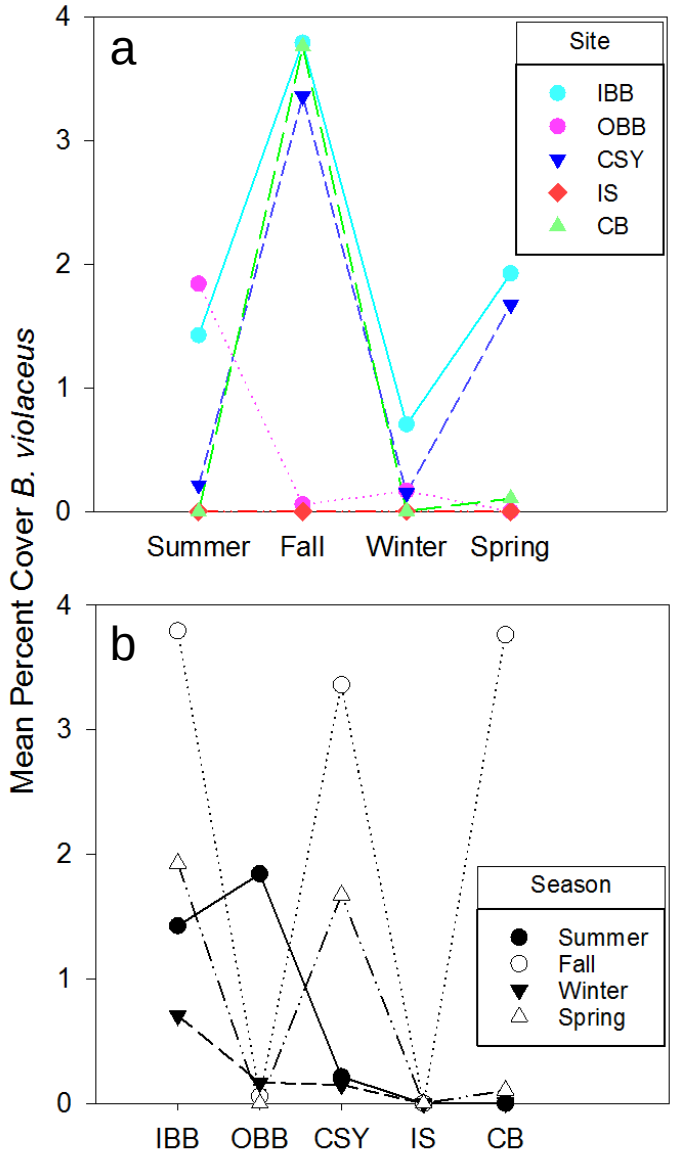

Figure 9: Two-way ANOVA interaction plot for the effect of site and season on average *Botrylloides violaceus* percent cover in quadrat surveys.

9a: Patterns in *B. violaceus* percent cover at OBB and IS deviate from those of the other three sites, demonstrating interaction between site and season on average *B. violaceus* percent cover. 9b: Non-parallel patterns in *B. violaceus* percent cover across sites during each season demonstrates interaction between site and season on average *B. violaceus* percent cover.

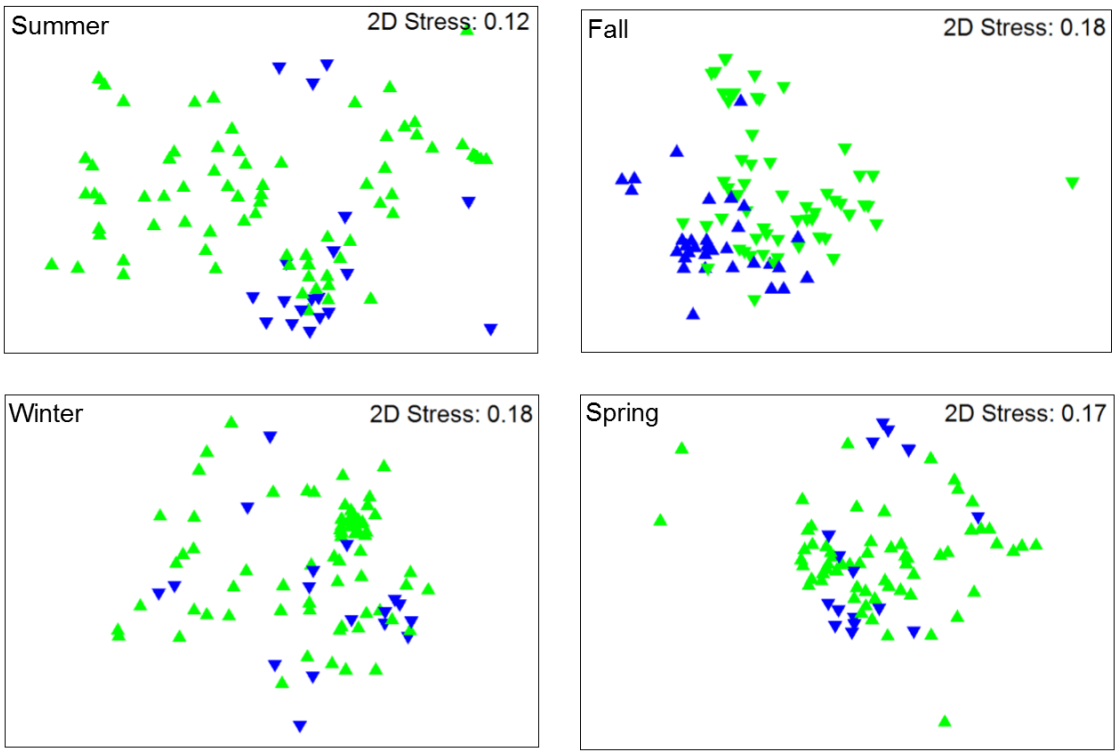

Figure 10: Multi-dimensional scaling (MDS) plot relating the species composition of quadrats with and without *Botrylloides violaceus* in seasonal quadrat surveys,

Quadrat locations with *Botrylloides violaceus* presence are indicated in blue; quadrat locations without are represented in green. Quadrats with *B. violaceus* do not form distinct clusters from those without, so the presence of this species cannot distinguish the species composition of one site from another.

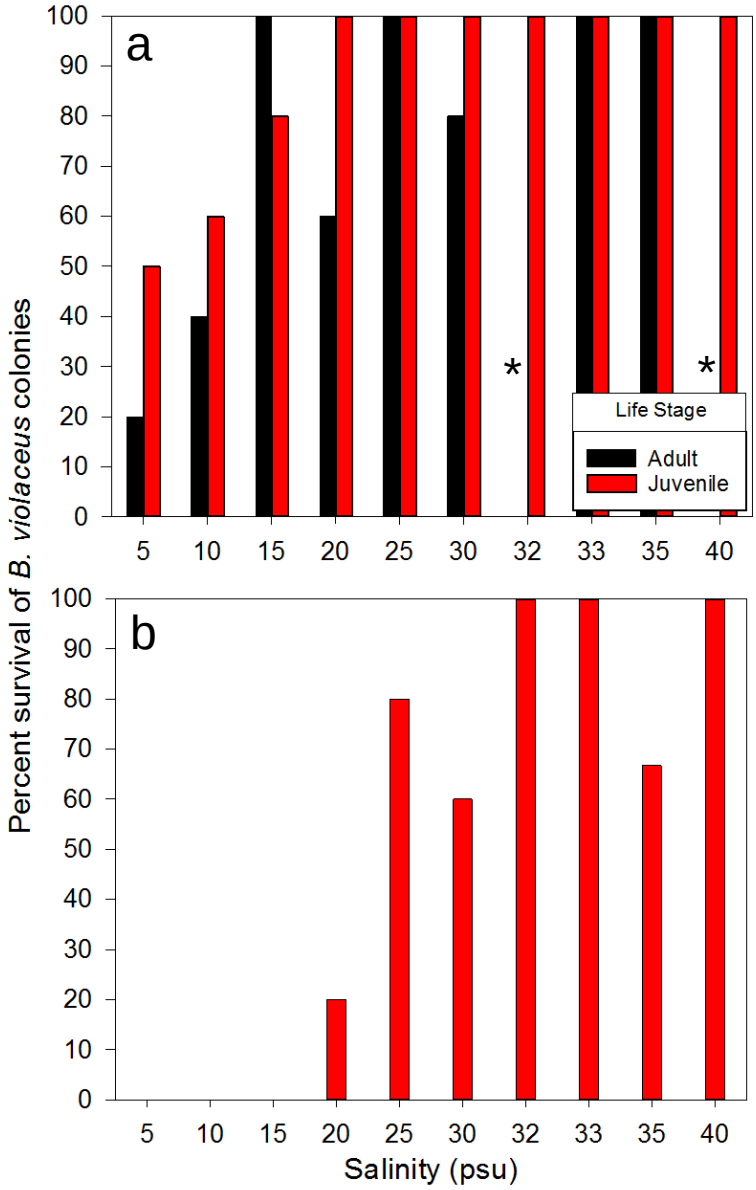

Figure 11: Percent survival of *Botrylloides violaceus* colonies subjected to a) 24-hour and b) seven-day salinity treatment.

Asterisks indicate salinity levels not tested for a given life stage. 11a: After 24 hours, juvenile colonies survived every salinity treatment, and adults survived salinities of 15 psu and above (greater than 50% survival). 11b: After seven days, juvenile colonies survived salinities of 25 psu and above. No adult colonies survived seven days of treatment, likely due to hypoxic conditions that developed in treatment water which was not changed during the treatment.

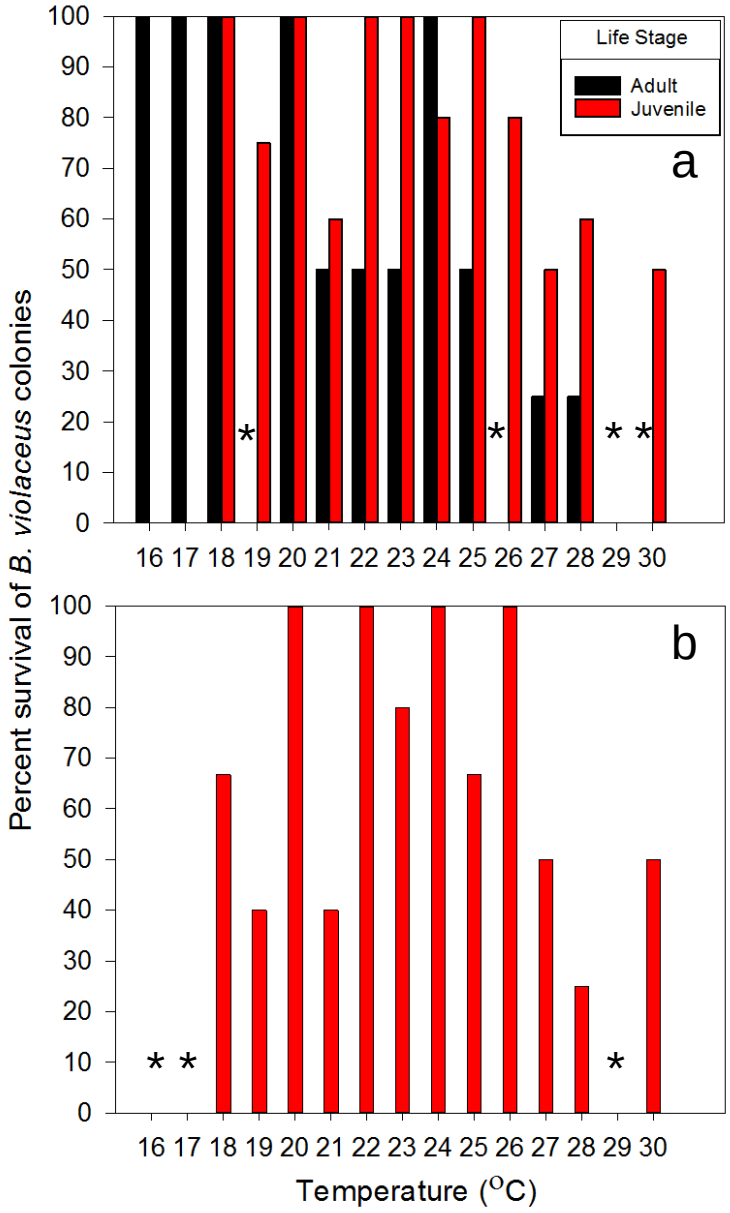

Figure 12: Percent survival of *Botrylloides violaceus* colonies subjected to a) 24-hour and b) seven-day temperature treatment.

Treatment levels ± 0.5 °C. Asterisks indicate temperatures not tested for a given life stage. 12a: After 24 hours, juvenile colonies survived every temperature treatment and adult colonies survived temperatures up to 25 °C (greater than 50% survival). 12b: After seven days, juvenile colonies survived temperatures up to 27°C, and showed 50% survival at 30°C. No adult colonies survived seven days of treatment, likely due to hypoxic conditions that developed in treatment water which was not changed during the treatment.

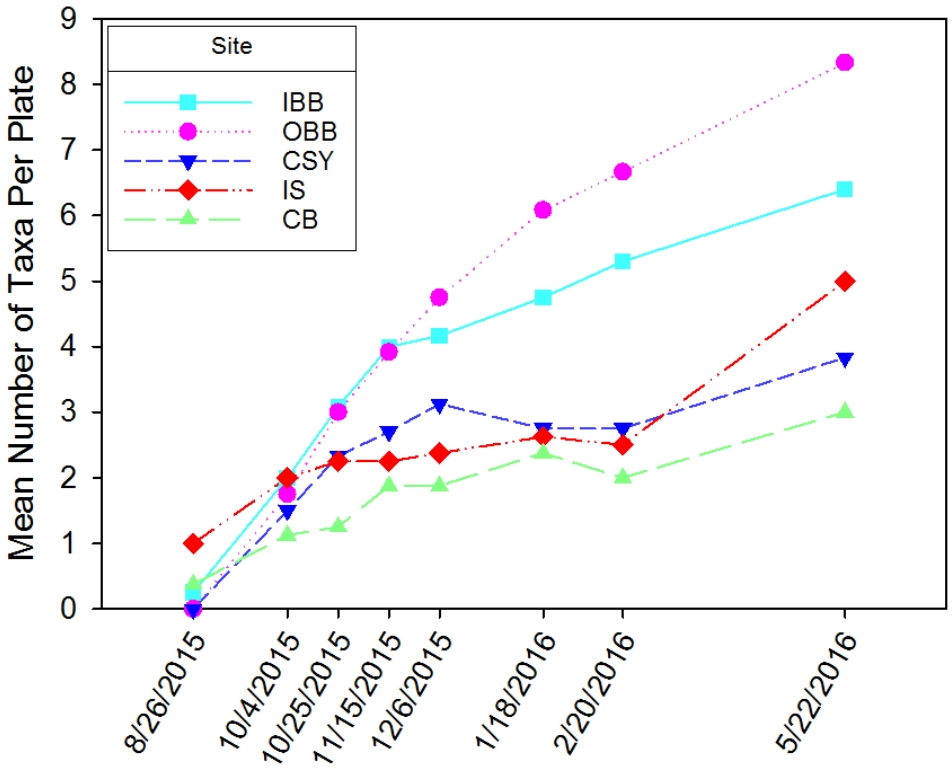

Figure 13: Mean number of taxa present on settlement plates at each sampling period.

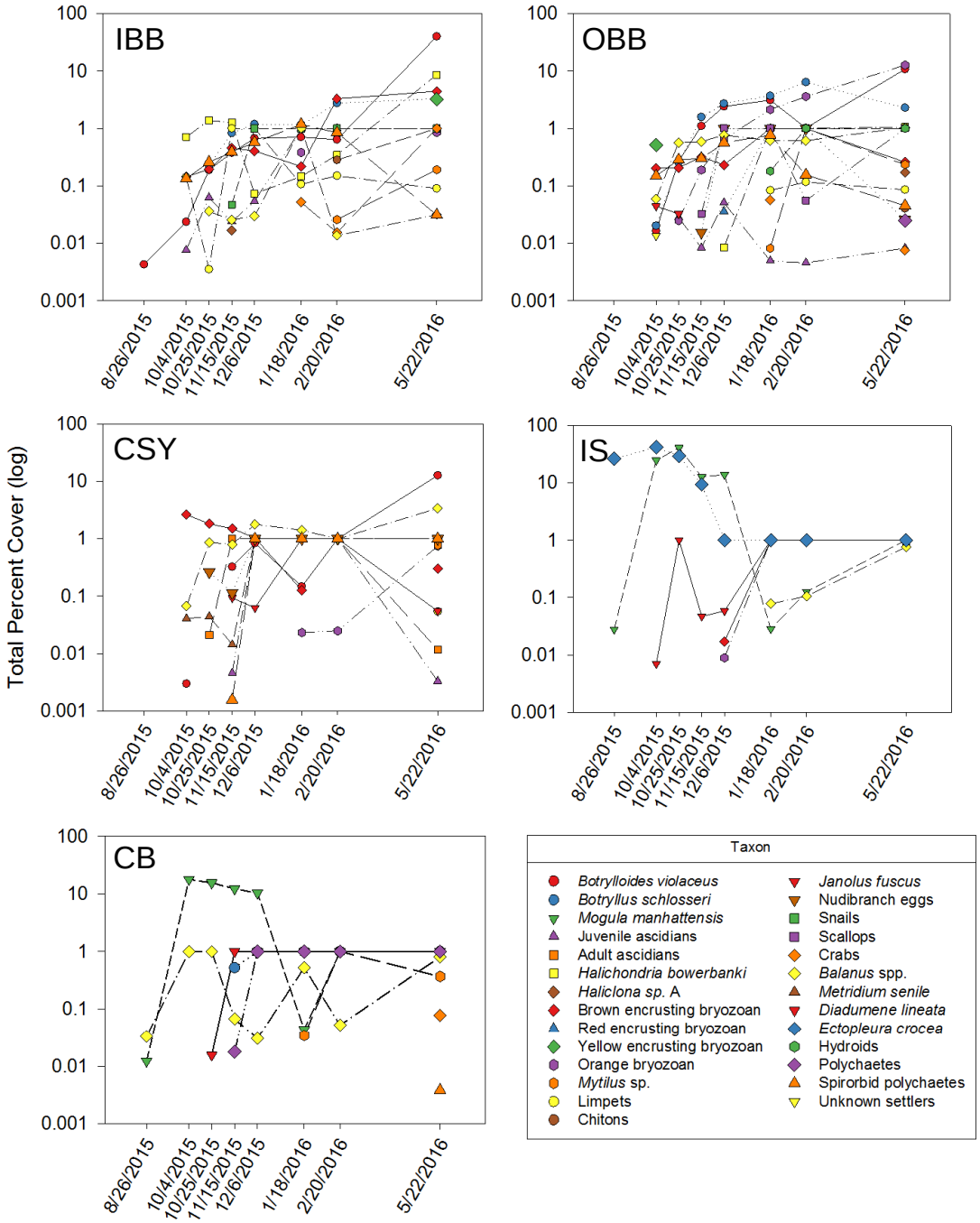

Figure 14: Total percent cover of each taxon present on settlement plates at each sampling date.

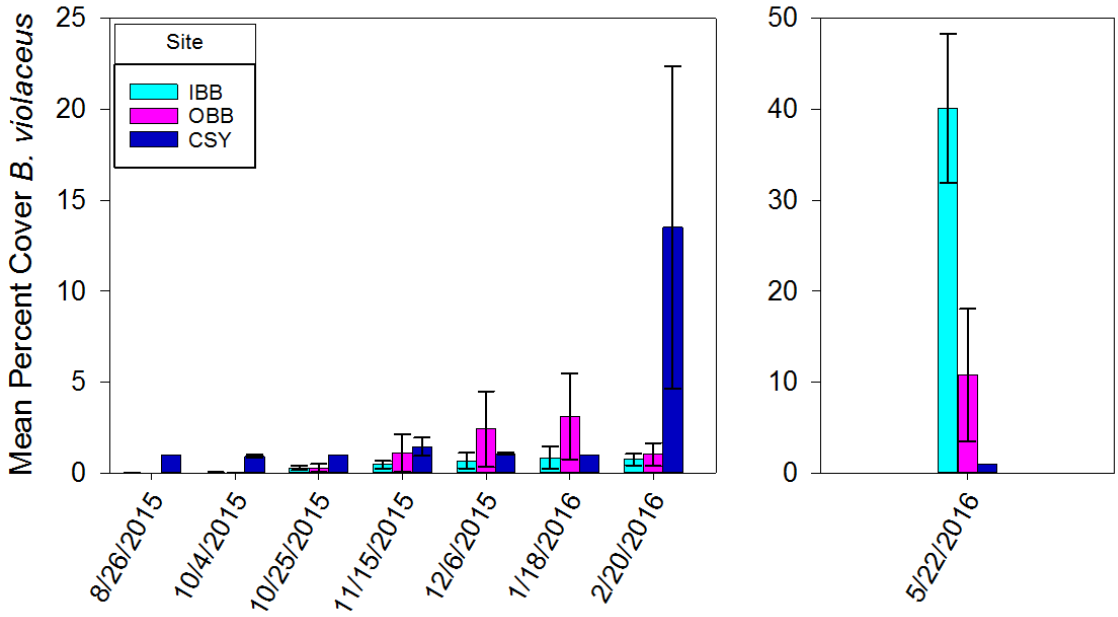

Figure 15: Percent cover of *Botrylloides violaceus* on settlement plates at each site and sampling date.

Only sites with *Botrylloides violaceus* settlement are shown. Site and date together had a significant effect on *B. violaceus* percent cover (two-way ANOVA, $\alpha = 0.025$, $p << 0.01$).

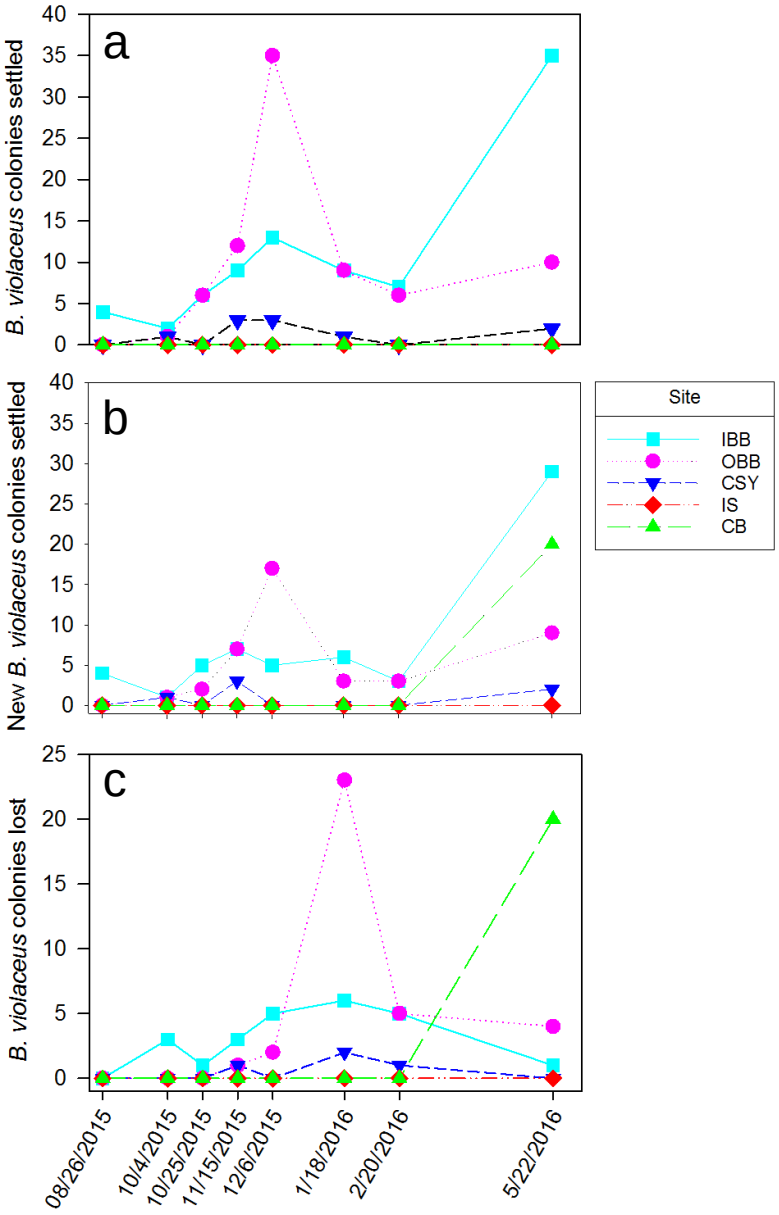

Figure 16: Frequency distribution of *Botrylloides violaceus* colonies on settlement plates at each site on each sampling date.

16a: Total number of *B. violaceus* colonies on settlement plates at each site on each sampling date. *Botrylloides violaceus* never settled on plates at CB or IS. 16b: Number of new *B. violaceus* colonies settled on plates at each site on each sampling date. Initial *B. violaceus* settlement varied in time and in number of colonies among sites and among plates at the same sites. Immigration of new colonies occurred year-round at IBB and OBB. 16c: Number of *B. violaceus* colonies lost from plates at each site on each sampling date.

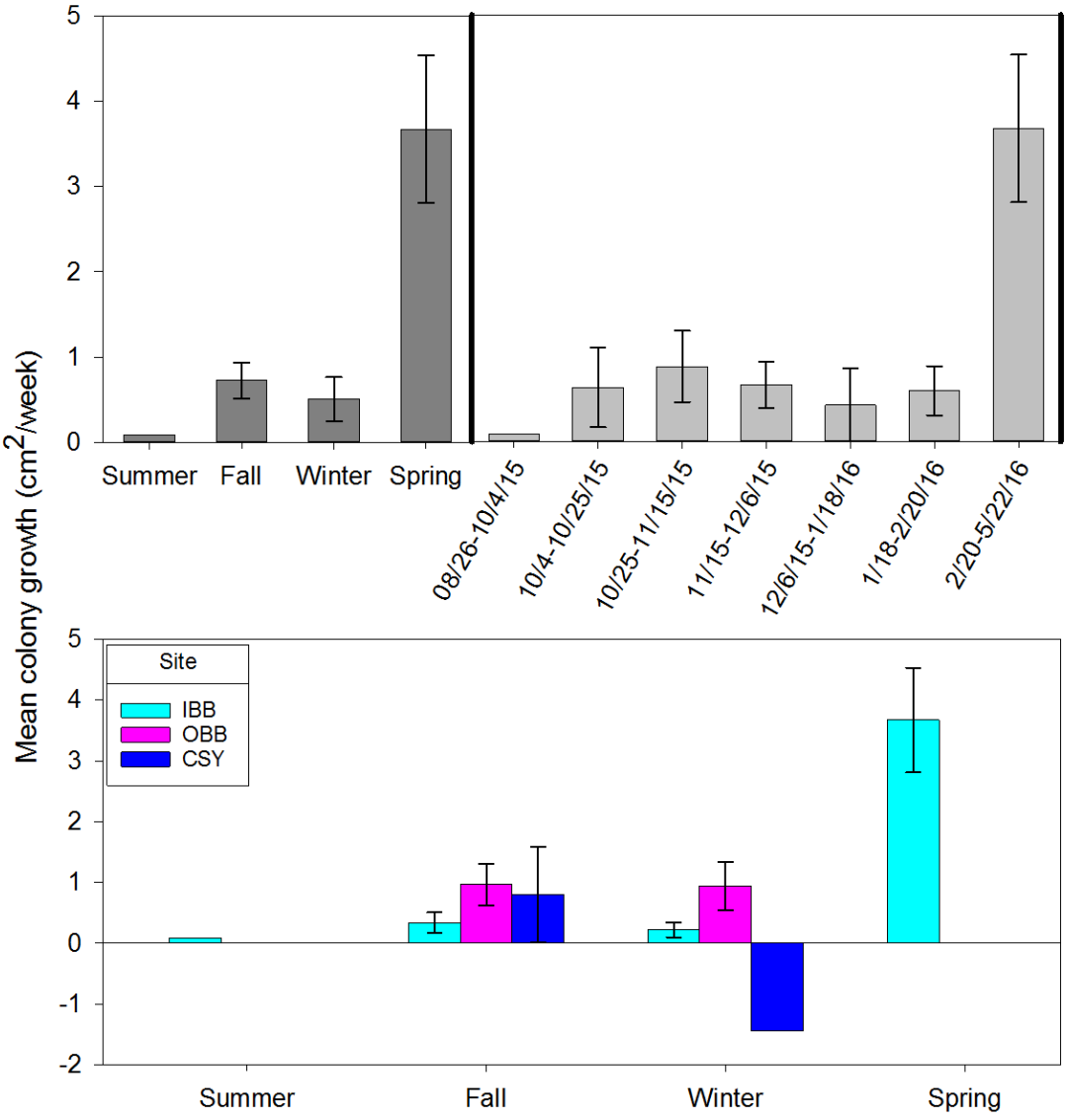

Figure 17: Average *Botrylloides violaceus* colony growth (cm^2 per week) on settlement plates during each season and sampling interval (irrespective of site).

Sites without *Botrylloides violaceus* settlement are omitted (CB and IS). Summer data includes only settlement 10 days after initial deployment of settlement plates. Growth varied significantly with sampling interval (two-way ANOVA, $\alpha = 0.05$, $p < 0.001$) and season (two-way ANOVA, $\alpha = 0.05$, $p < 0.001$). The interaction between site and season had no significant effect on average growth (two-way ANOVA, $\alpha = 0.05$, $p > 0.05$).

Appendix A: Deployment Maps

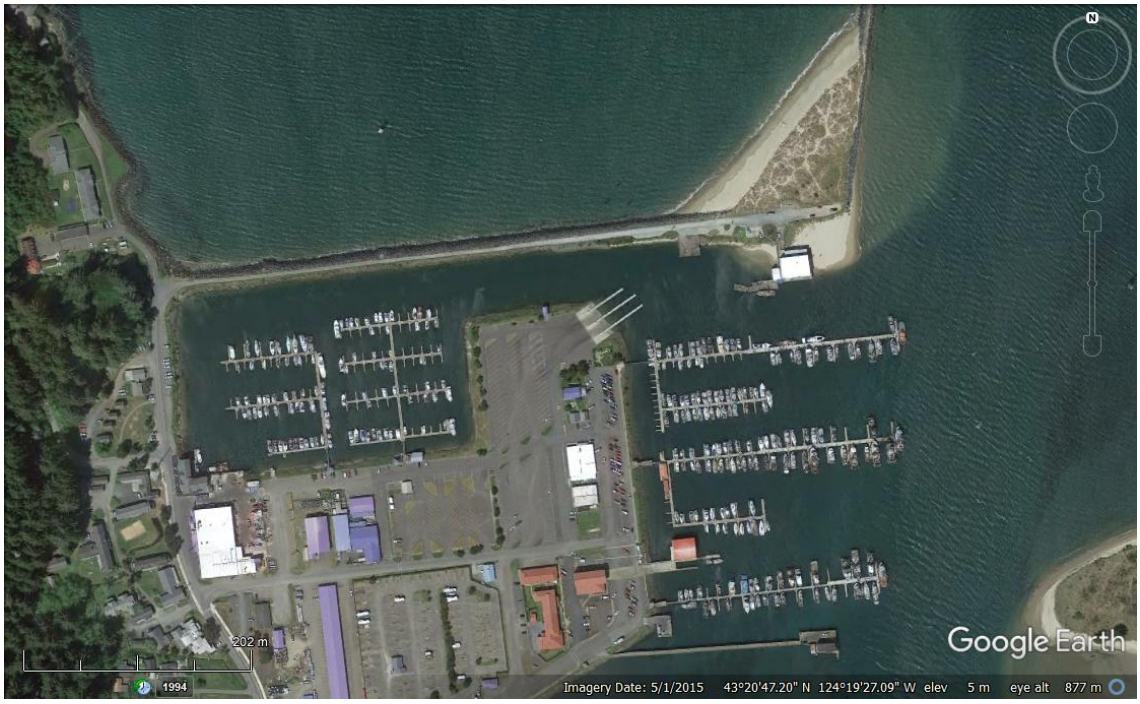

Figure A: Charleston Boat Basin.

Inner Boat Basin (IBB) on left, with large cement breakwater along its north side. Outer Boat Basin (OBB) on right, with incoming marine water flowing south along the east side of the boat basin.

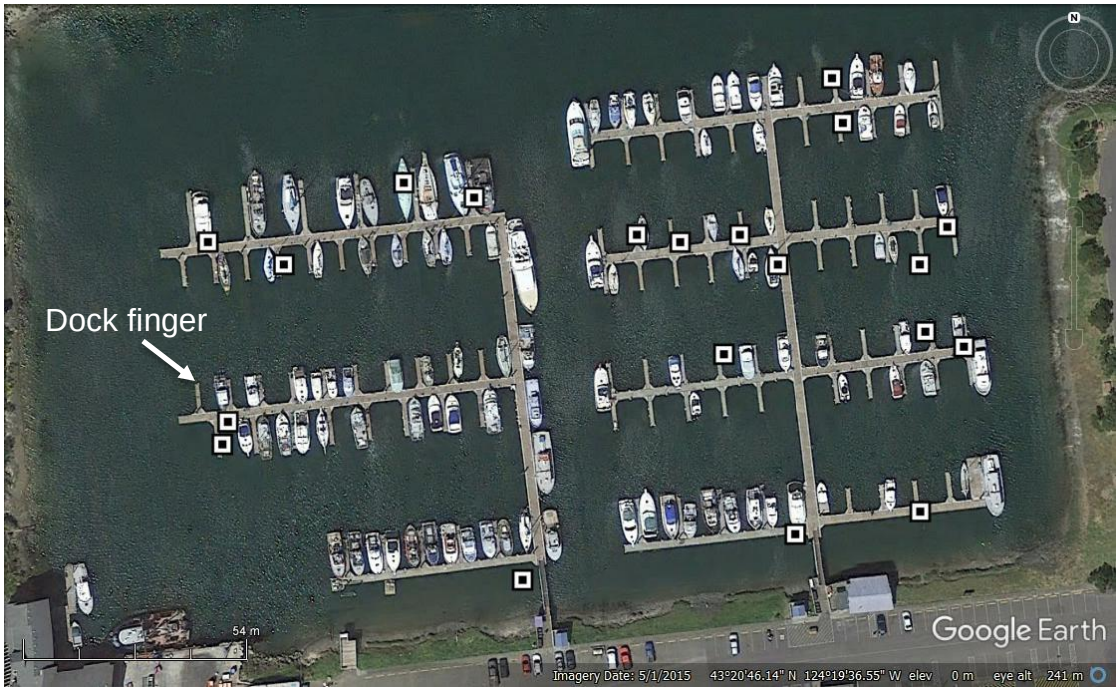

Figure B: Map of seasonal survey quadrat locations at Inner Boat Basin (IBB).

Quadrat locations indicated by white boxes.

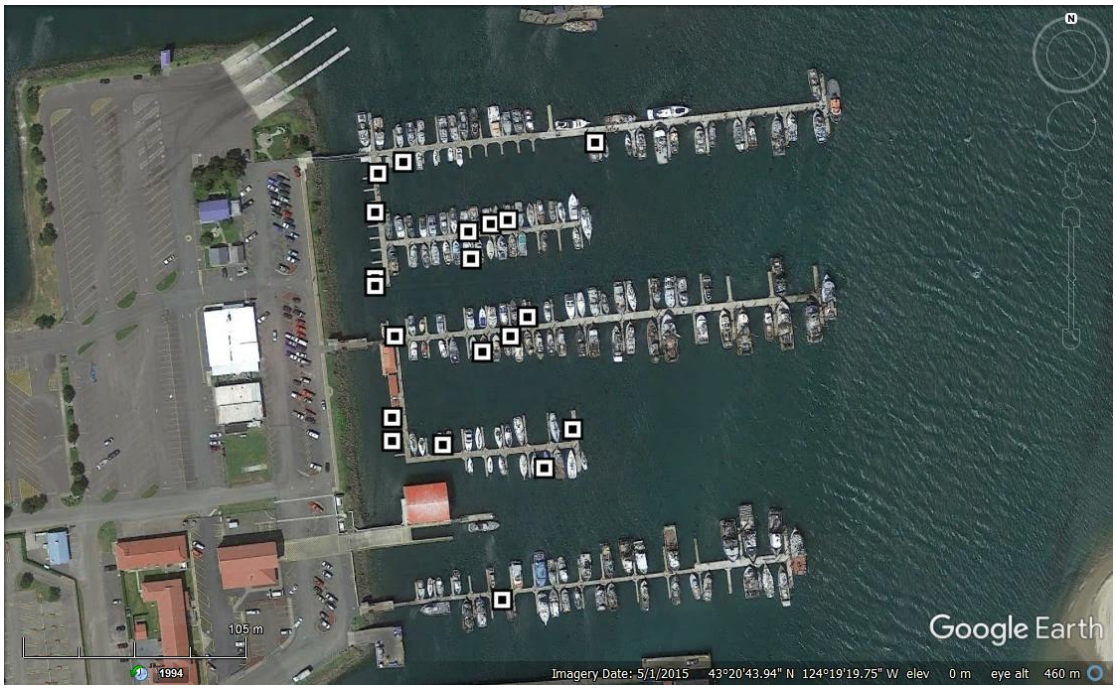

Figure C: Map of seasonal survey quadrat locations at Outer Boat Basin (OBB).

Quadrat locations indicated by white boxes.

Figure D: Map of seasonal survey quadrat locations at Charleston Shipyard (CSY).

Quadrat locations indicated by white boxes.

Figure E: Map of seasonal survey quadrat locations at Coos Bay City Docks (CB).

Quadrat locations indicated by white boxes.

Figure F: Map of seasonal survey quadrat locations at Isthmus Slough (IS).

Quadrat locations indicated by white boxes.

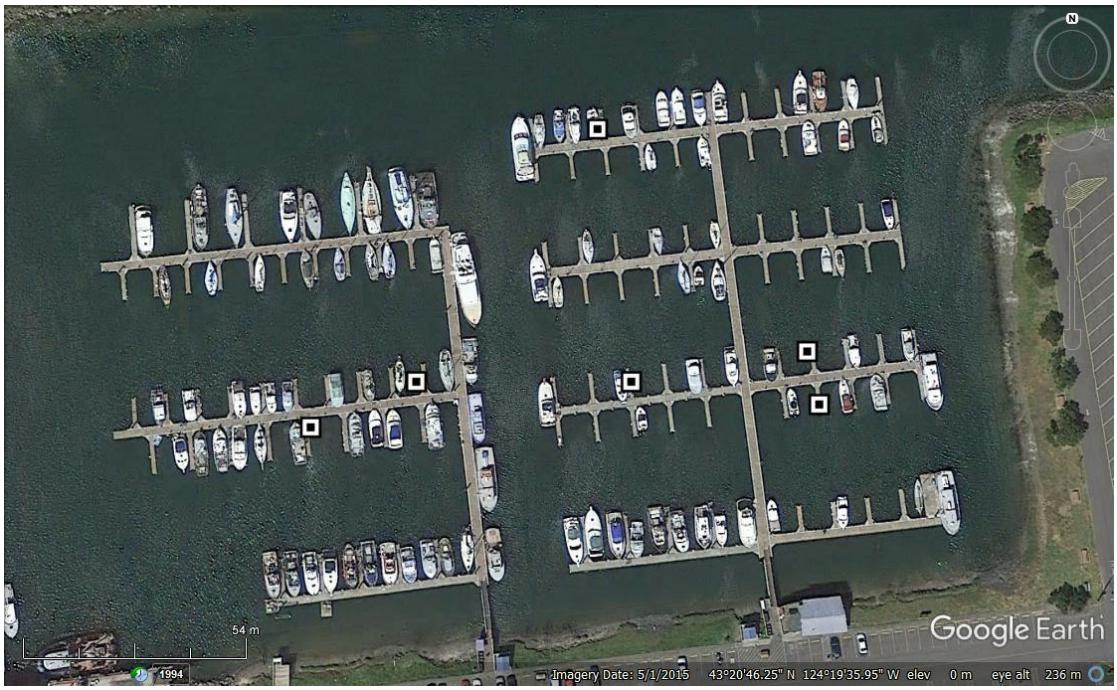

Figure G: Map of settlement plate deployment locations at Inner Boat Basin (IBB).

Settlement plate locations indicated by white boxes.

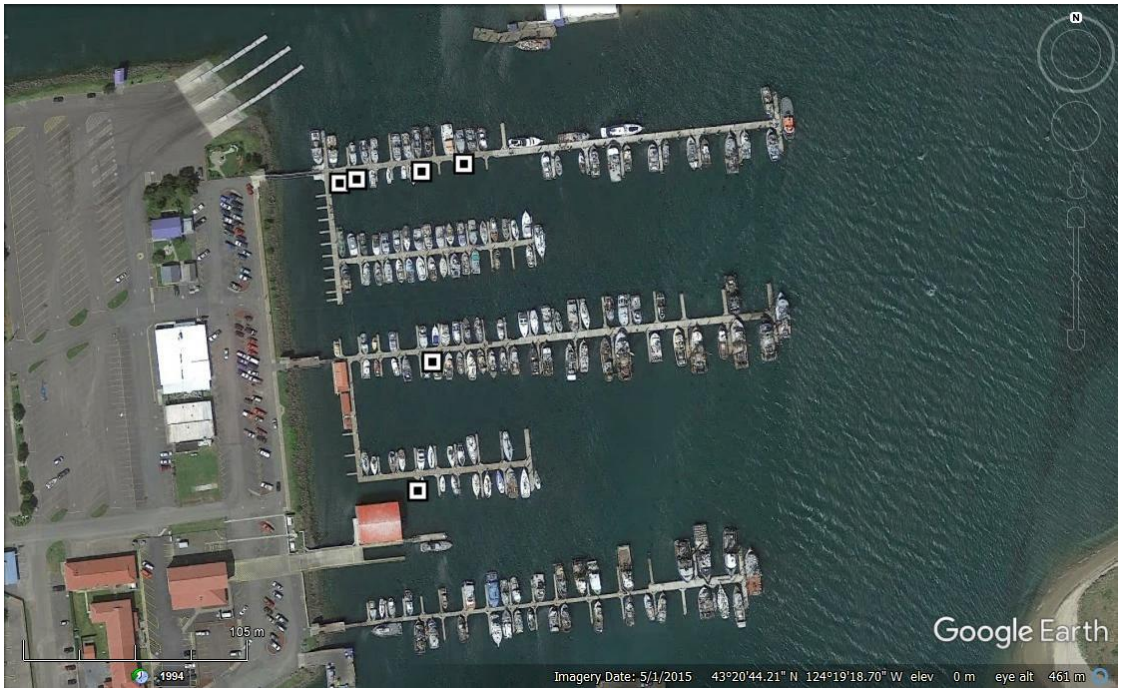

Figure H: Map of settlement plate deployment locations at Outer Boat Basin (OBB).

Settlement plate locations indicated by white boxes.

Figure I: Map of settlement plate deployment locations at Charleston Shipyard (CSY).

Settlement plate locations indicated by white boxes.

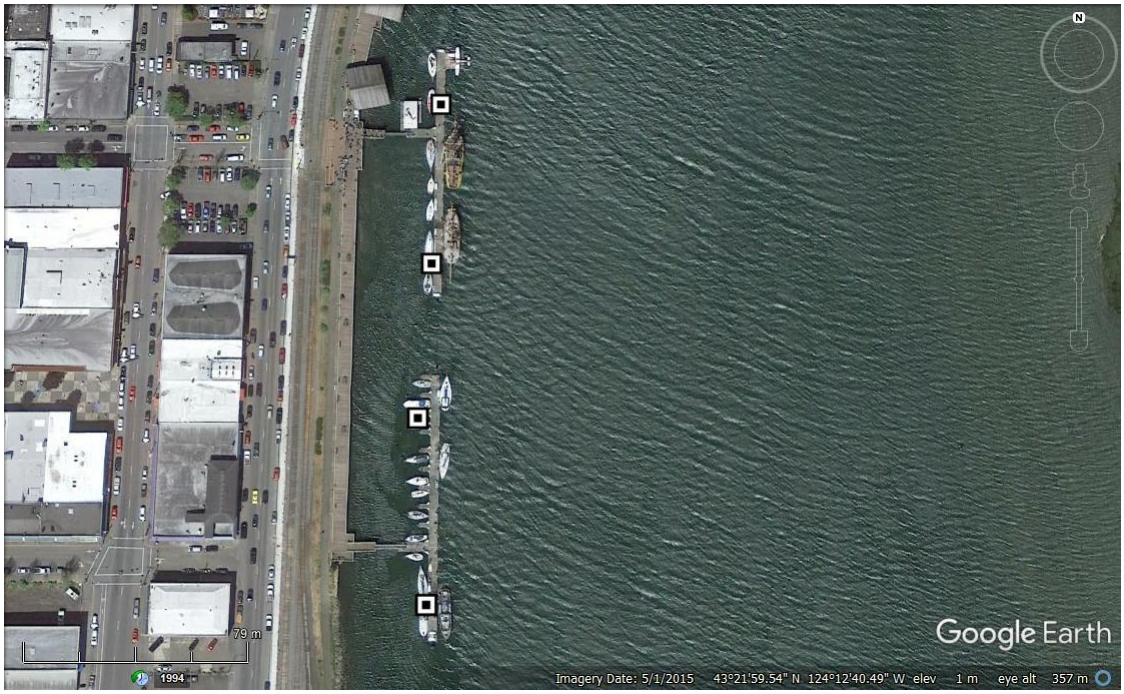

Figure J: Map of settlement plate deployment locations at Coos Bay City Docks (CB).

Settlement plate locations indicated by white boxes.

Figure K: Map of settlement plate deployment locations at Isthmus Slough (IS).

Settlement plate locations indicated by white boxes.

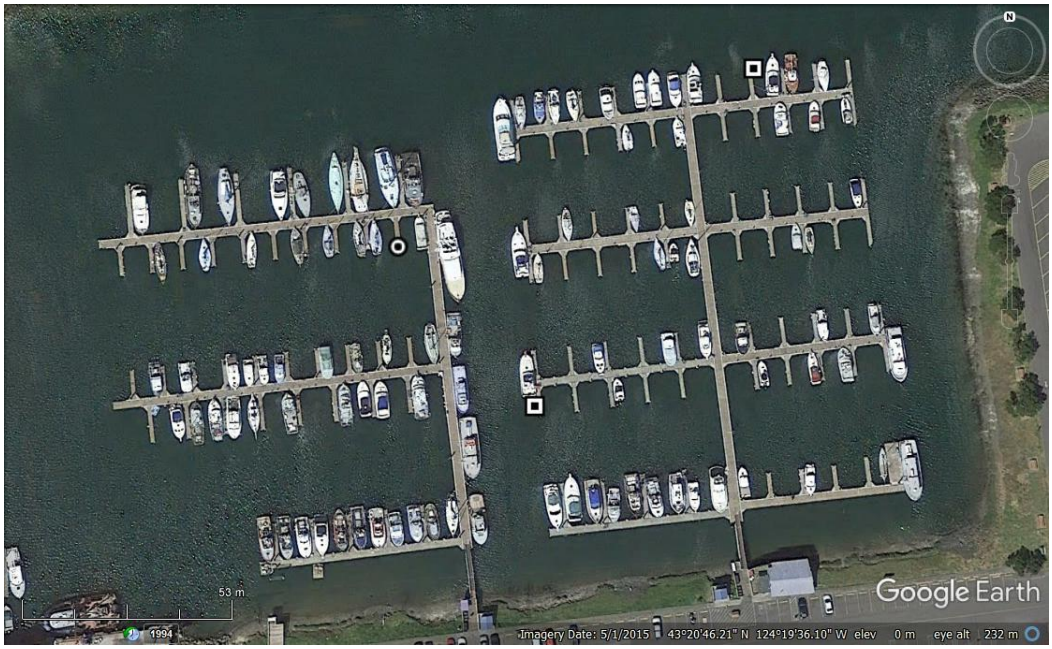

Figure L: Map of clod card and transplant deployment locations at Inner Boat Basin.

Clod card sites indicated by white squares; transplant sites indicated by white squares and circles.

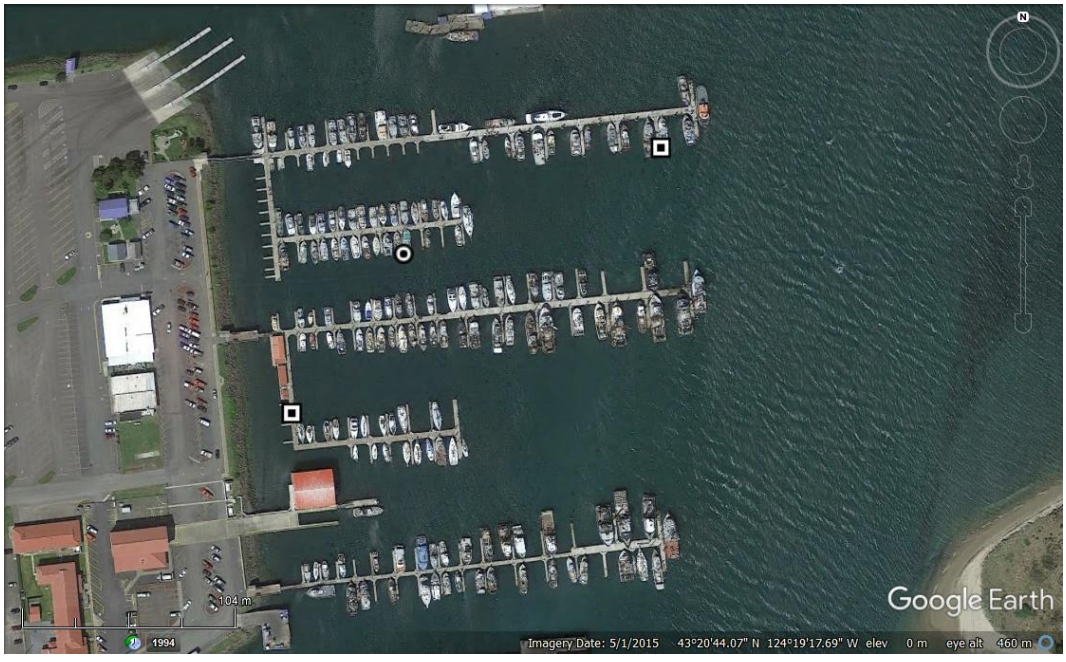

Figure M: Map of clod card and transplant deployment locations at Outer Boat Basin.

Clod card sites indicated by white squares; transplant sites indicated by white squares and circles.

Figure N: Map of clod card deployment locations at Charleston Shipyard.

Clod card deployment locations indicated by white squares.

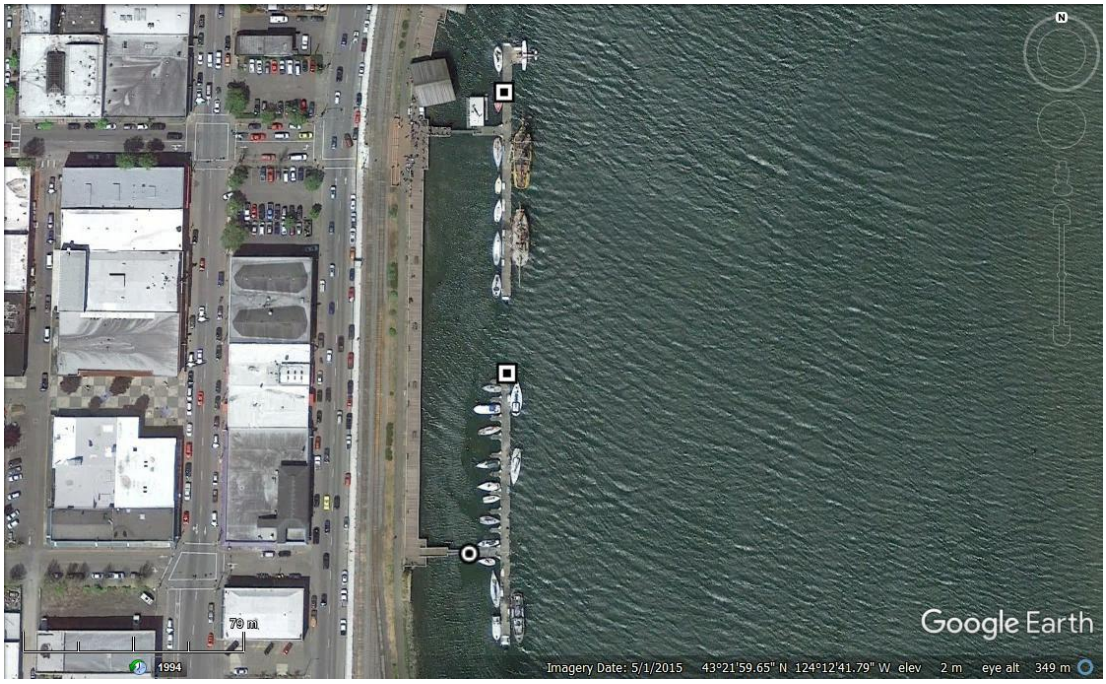

Figure O: Map of clod card and transplant deployment locations at Coos Bay City Docks.

Clod card sites indicated by white squares; transplant sites indicated by white squares and circles.

Figure P: Map of clod card and transplant deployment locations at Isthmus Slough.

Clod card sites indicated by white squares; transplant sites indicated by white squares and circles