A TALE OF TWO TUNICATES: *DIDEMNUM VEXILLUM* AND *BOTRYLLOIDES VIOLACEUS* AS BIOFOULING AGENTS IN AQUACULTURE

by

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THESIS ABSTRACT

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Title: A Tale of Two Tunicates: *Didemnum vexillum* and *Botrylloides violaceus* as Biofouling Agents in Aquaculture

Invasive colonial tunicates pose substantial economic threat to the shellfish aquaculture industry, but their population dynamics and ecological impacts are highly variable and region-specific. This thesis contributes to our regional understanding of two such tunicates in Oregon. The first chapter explores the population dynamics of *Didemnum vexillum*, one of Oregon's top 100 most dangerous invasive species, at an oyster farm. From May 2011 to 2016 the population fluctuated extensively, though did not exhibit any net growth over the study period. In the second chapter, I demonstrate that *Botrylloides violaceus* had no impact on the growth, condition, or organic composition of oysters and mussels grown in a simulation of longline aquaculture. Together, these studies paint a cautiously positive outlook for the shellfish aquaculture industry in Oregon.

This thesis includes previously unpublished co-authored material.

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CHAPTER I

GENERAL INTRODUCTION

The two content chapters within this thesis stand alone, though the general introduction and conclusion provide thematic cohesion. I commence each chapter with more detailed introductions. While this inherently presents some redundant background information, it facilitates the chapters' publication as journal articles. Both chapters address invasive tunicates and longline shellfish aquaculture, though each focuses on a specific tunicate: *Didemnum vexillum* (Chapter II; co-authored with Bruce Hansen, Steve Rumrill, and Aaron Galloway) and *Botrylloides violaceus* (Chapter III).

Invasive Colonial Tunicates

Invasive species—organisms whose populations expand beyond their historical range to a degree that causes damage to environmental, economic, or human health (Clinton 1999)—possess a suite of traits that confer their invasiveness. Such traits include rapid growth, short time to sexual maturity, the ability to reproduce sexually and asexually, high fecundity, long annual reproductive period, release from predation pressure, and a tolerance to broad environmental conditions, or phenotypic plasticity (Sakai et al. 2001). Numerous colonial tunicates have successfully established in South America (Rocha 2009; Ben-Shlomo et al. 2010), New Zealand (Kott 2002; Fletcher et al. 2013a), Europe (Izquierdo-Muñoz 2009; McKenzie et al. 2017), and both coasts of North America (Carver et al. 2006; Dijkstra et al. 2007a; Lambert 2009; McKenzie et al. 2017). These invasions were successful due to the coupling of the tunicates' traits and their anthropogenic transport via ballast water, ship hulls, or aquaculture materials (Carlton and Geller 1993; Ruiz et al. 1997, 2000; Hulme 2009). In the systems these tunicates

have established, they are prominent members of and dominant competitors for space in marine fouling communities (Edwards and Stachowicz 2010, 2011)—altering and threatening the native fouling community diversity (Jackson 1977; Dijkstra et al. 2007b; Simkanin et al. 2013; Kaplan et al. 2017), as well as shellfish aquaculture. This thesis addresses two of these invasive colonial tunicates, *Didemnum vexillum* Kott (2002) and *Botrylloides violaceus*, and their impacts as biofouling agents in the context of longline shellfish aquaculture.

Here, I seek to briefly introduce the natural history and ecology of ascidians in general, and the invasive colonial tunicates *D. vexillum* and *B. violaceus* (Table 1) specifically. I will use the terms "ascidian" and "tunicate". Note, however, that all ascidians are tunicates, but not all tunicates are ascidians. Subphylum Tunicata (Urochordata)—whose member organisms have bodies surrounded in a gelatinous, acellular, and cellulose-like tunic—also includes the planktonic Classes Appendicularia and Thaliacea. For more comprehensive reviews of ascidians and their larvae, see Millar (1971), Berrill (1975), Cloney (1987), Svane and Young (1989), and Lambert (2005); McKenzie et al. (2017) and Carver et al. (2006) have produced extensive reviews and descriptions of *D. vexillum* and *B. violaceus*, respectively.

The tadpole larvae of ascidians are short-lived and lecithotrophic, relying exclusively on their egg yolks for energy (Cloney 1987). Colonial ascidian larvae are large and vulnerable to visual predators, and some have therefore developed color deterrents or chemical defenses (Young and Bingham 1987; Svane and Young 1989; Lindquist et al. 1992). Some, though not all, ascidian larvae use oral papillae to sense, select, and adhere to their ultimate substratum (Svane and Young 1989). Once they attach

Table 1. Taxonomic classification of *Didemnum vexillum* and *Botrylloides violaceus*.

Rank	Taxon	
Phylum	Chordata	Chordata
Subphylum	Urochordata/Tunicata	Urochordata/Tunicata
Class	Ascidiacea	Ascidiacea
Order	Aplousobranchia	Pleurogona
Family	Didemnidae	Styelidae
Genus	*Didemnum*	*Botrylloides*
Species	*Didemnum vexillum*	*Botrylloides violaceus*

and metamorphose, adult ascidians use a ciliary mucus apparatus to filter and feed on phytoplankton, as well as other suspended particulates and bacteria (Millar 1971; Lambert 2005). In temperate regions they are reproductively most active during summer, when primary productivity is highest. The distributions of ascidians are primarily influenced by temperature, salinity, light, and hydrodynamics (Lambert 2005).

Ascidians are either solitary or colonial, wherein multiple zooids share common tissue. Colonial ascidians may be social, with stolons that connect zooids each surrounded by a separate test, or compound, with morphologically and genetically identical zooids contained within a common tunic. All ascidians reproduce sexually. In addition to sexual reproduction, all colonial tunicates also exhibit growth by asexual reproduction via budding, or blastogenesis (Cloney 1987). Therefore, many colonial ascidians are strong competitors for space, especially in disturbed habitats (Ayling 1981; Altman and Whitlatch 2007) or as epibionts that can settle on, overgrow, and smother other organisms. Together, these abilities make colonial tunicates—such as *D. vexillum* and *B. violaceus*—optimal invaders (Sutherland and Karlson 1977).

Didemum vexillum: Biology and Ecology

Didemnum vexillum is native to Japan (Lambert 2009; Stefaniak et al. 2012), though it was only recently first described outside its native range in New Zealand (Kott 2002). Due to the diversity of the genus *Didemnum*, the identification of *D. vexillum* is challenging. As a result, researchers used the nomenclature "*Didemnum* sp. A" and "*Didemnum vexillum*" interchangeably for this cryptic species prior to 2009, wherein Stefaniak et al. (2009) demonstrated with genetic analyses that they are indeed the same species. The individual zooids are small—approximately 1-2 mm (Kott 2002; Daniel and Therriault 2007; Lambert 2009)—and colonies can fuse and form chimeras (Smith et al. 2012). The zooids' gut loops, eggs, embryos, and calcareous spicules contained within the colony tunic surface result in yellow, tan, or cream-colored colonies (Kott 2002; Lambert 2009; McKenzie et al. 2017). At the tough tunic surface, each zooid possesses a six-lobed oral siphon through which it feeds (Millar 1971; Lambert 2005; Fig. 1a, b). Additional distinguishing features of *D. vexillum* include: the dark lines around their irregular zooid groupings where spicules are absent (McKenzie et al. 2017); the nine coils of vas deferens surrounding their testis (Kott 2002; Lambert 2009); and, as larvae, the six pairs of lateral ampullae and three adhesive ampullae (Lambert 2009).

Comparable to other invasive species, *D. vexillum* exhibits a wide tolerance to numerous environmental parameters, including temperature (-2 to 24ºC; Valentine 2009), salinity (10-36‰; Bullard and Whitlatch 2009; Gröner et al. 2011), depth (0-81m; Valentine et al. 2007b), habitat (Bullard and Whitlatch 2009), and settlement substrate (including over loose cobble, artificial structures, established benthic invertebrate communities, or eelgrass; Valentine et al. 2007a; Daley and Scavia 2008; Bullard and

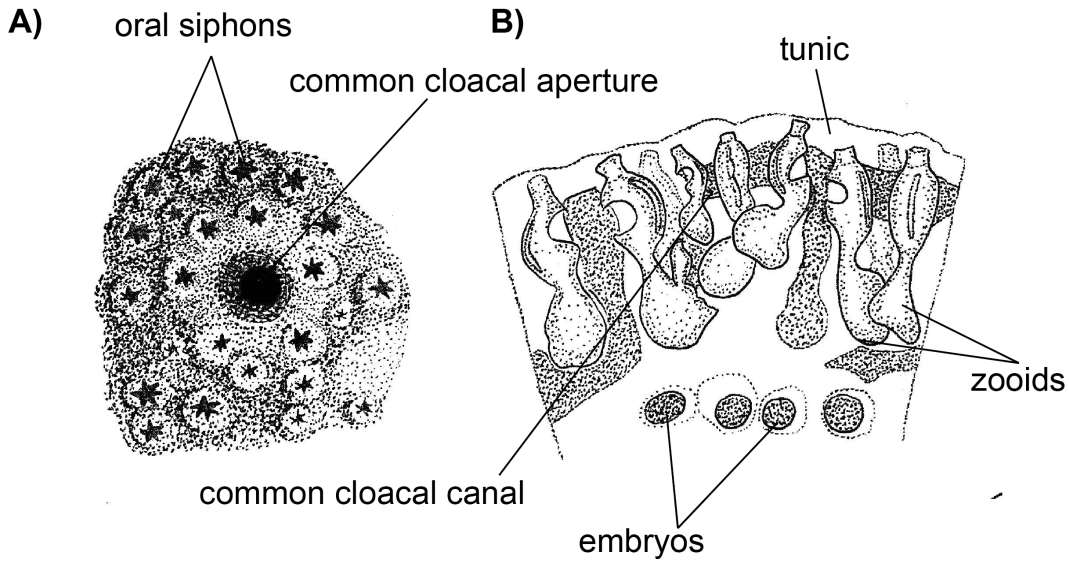

Figure 1. *D. vexillum* illustration depicting **A)** colony surface and **B)** a lateral section through the colony.

Whitlatch 2009; Carman and Grunden 2010). Growth rates may slow or stop during unfavorable conditions (e.g., cold), however, and colonies may even exhibit regression—but not necessarily complete death (McKenzie et al. 2017). Conversely, during the warm months *D. vexillum* colonies can expand at remarkable rates. For example, Valentine and colleagues (2009) observed a 6- to 11-fold increase in colony surface area in just 15 days. Further, *D. vexillum* has few predators in its introduced range, likely a mechanism of both its low surface pH (3.8 ± 0.2 [$\overline{x}$ ± 1 SD]; Morris et al. 2009) and chemical defenses (Bullard and Whitlatch 2009), which have been reported for several other didemnids (Lindquist et al. 1992) and *Didemnum* species (Pisut and Pawlik 2002). Despite these defenses, some littorine snails (Valentine et al. 2007b; Carman 2009), chitons (Kleeman 2009), and green urchins (Epelbaum et al. 2009b) have been observed feeding on *D. vexillum* colonies. And while Forrest et al. (2013) provide compelling evidence that native benthic predation is key in preventing the spread of *D. vexillum* from anthropogenic habitat to adjacent natural habitats in New Zealand, the overall potential for predators to interfere with its establishment is limited (Epelbaum et al. 2009b).

Ultimately, its *D. vexillum*'s abilities to compete for space, alter habitat complexity, and grow on numerous natural and artificial substrates—from the undersides of boats and marinas to cobble or over other organisms in the fouling community—that makes it such a pervasive invader (Daniel and Therriault 2007; Osman and Whitlatch 2007).

The plasticity of growth of *D. vexillum* also strongly aids in its success as an invader. Colonies may form lobular tendrils in low-current waters, or encrusting mats under stronger current conditions (Fig. 2a, b). It is thought that the tendril formation enhances the likelihood of asexual reproduction via fragmentation (Reinhardt et al. 2012). These fragments can remain suspended for up to 30 days, and settle and attach as new sister colonies (Lambert 2005; Bullard et al. 2007; Stefaniak et al. 2009; Morris and Carman 2012). With regards to sexual reproduction, *D. vexillum* is like all didemnids in that it is hermaphroditic and ovoviviparous. McKenzie et al. (2017) describe *D. vexillum*'s sexual reproduction in great detail. Briefly, sperm are released through the common cloacal opening of a colony, then enter the oral siphon of another zooid and fertilize the eggs therein; larvae brood within the tunic of the zooid for several weeks and are then released, again through the common cloacal apertures (Lambert 2009; Fletcher and Forrest 2011; McKenzie et al. 2017), and eventually settle in shaded areas (Millar 1971; Monniot et al. 1991; Fletcher and Forrest 2011). Larval recruitment is dependent on local environmental conditions, but occurs at temperatures of 14-20ºC (Valentine 2009). Salinities of 26-30‰ and temperatures of 14-18ºC are most conducive to growth (Gittenberger 2007; Bullard and Whitlatch 2009).

Initial reports suggested that *D. vexillum* hitchhiked on oyster shells and spat and spread from Japan as early as the 1960s (McKenzie et al. 2017). Lambert (2009) later

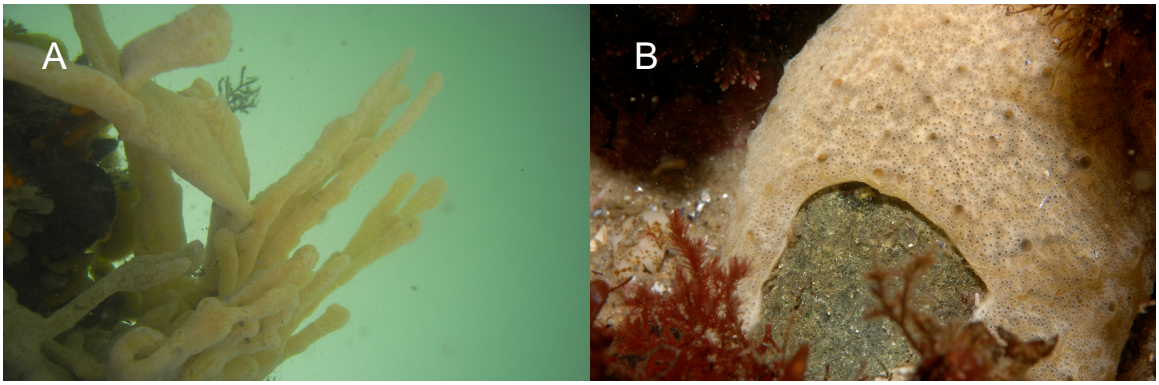

Figure 2. Didemnum vexillum in **A)** tendril (Moss Landing, CA, 2013) and **B)** encrusting formations (Sandwich, MA, 2006). Photo credits: Joshua Lord and Dann Blackwood, respectively.

rejected this hypothesis due to a lack of reports indicating its sudden appearance before the 1970s, instead suggesting that fouled ship hulls and sea chests were the primary vectors of introduction, with secondary spread via recreational boating. *D. vexillum* has since established itself globally (Table 2). Due to the short-lived nature of the planktonic tadpole larvae of ascidians, it is unlikely that ballast water transport is a significant vector—though the possibility of larvae settling inside the hull, forming reproductive adult colonies, and releasing larvae at the port of destination should not be ruled out entirely. It is more probable that *D. vexillum* spreads as a result of rafting upon colonized macroalgae or eelgrass fronds (Carman and Grunden 2010; Fletcher et al. 2013c; Carman et al. 2014), unregulated recreational boating (Clarke Murray et al. 2011; Roche et al. 2015), fouled aquaculture gear (Coutts and Forrest 2007; Denny 2008), or via a 'stepping stone' network of closely spaced artificial substrates (López-Legentil et al. 2015).

***Botrylloides violaceus*: Biology and Ecology**

Botrylloides violaceus is too believed to be endemic to Japan (Oka 1927; Berrill 1950), though its native range is considered as Siberia to Southern China. Saito et al. (1981) updated the description of *B. violaceus* following confusion in discerning several

Botrylloides spp. from one another. *B. violaceus* zooids are larger (2-4 mm) than those of *Didemnum vexillum*, and are distributed around a common cloacal aperture in elongated, irregular and ovular double rows (Fig. 3a). The monochrome colonies vary greatly in hue—from bright orange to maroon, purple, yellow, or cream (Saito et al. 1981; Lambert and Lambert 2003; Fig. 3b)—and the color may be light-dependent (Berrill 1947). Its zooids share a common vascular system and cloacal cavity, which maximizes zooid density (Taneda and Watanabe 1992). Four large and small branchial tentacles surround the zooids' oral siphons in alternation, and their testis lobes form a rosette (Fig. 4, need to draw). *B. violaceus* colonies are generally encrusting, but can also form thick lobes and projections. More comprehensive morphological descriptions are available in Saito et al. (1981), Carver et al. (2006), and Dorning (2017a). Their tunic is fragile, and easily torn.

The whole-body regeneration abilities of *B. violaceus* and related botryllid ascidians have been studied and described at length (e.g., Rinkevich et al. 1995; Voskoboynik et al. 2007; Brown et al. 2009). Extraordinarily, *Botrylloides leachi* can regenerate from as little as a small fragment of a blood vessel with several totipotent stem cells (Rinkevich et al. 1995). *B. violaceus* primarily reproduces asexually via lateral budding, during which a parent zooid is absorbed and replaced by new buds (Berrill 1947). Less frequently, asexual reproduction occurs via fragmentation and reattachment (Epelbaum et al. 2009c; Bock et al. 2011); a sophisticated genetic allorecognition system determines whether two colonies may fuse together (Rinkevich 2005). *B. violaceus* is viviparous and hermaphroditic, and five days after ovulating, the mother zooids disintegrate (Mukai et al. 1987). The larvae that are released from sexual reproduction are 2-3 mm in length, and possess 24-32 ampullae (Saito et al. 1981). Asexual reproduction

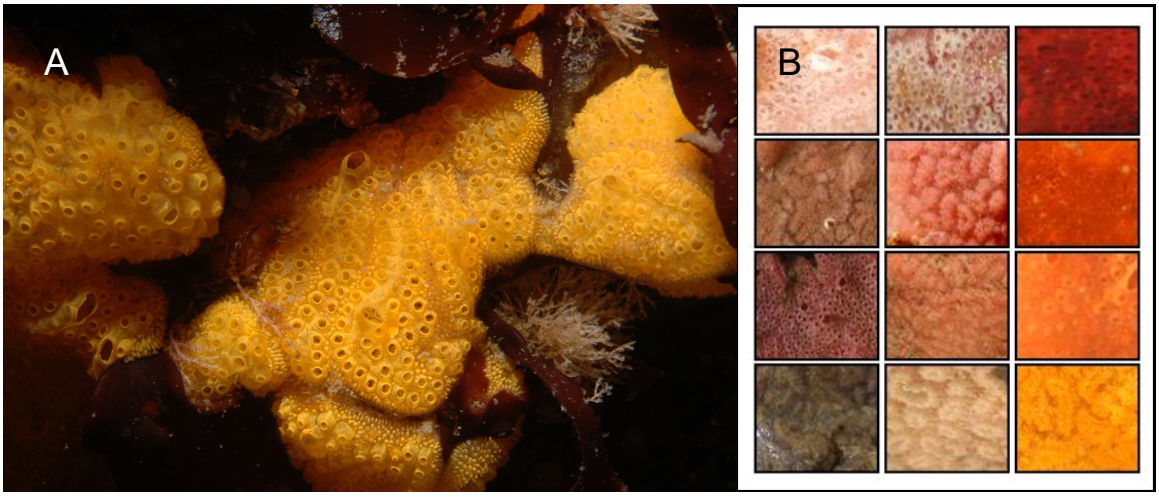

Figure 3. *Botrylloides violaceus* **A)** colonies (Sandwich, MA, 2006) and **B)** color variants. Photo credits: Dann Blackwood and Adrienne Pappal, respectively.

occurs during the spring and summer, whereas sexual reproduction generally occurs from June through September; hibernation occurs through the winter (Stachowicz et al. 2002; Epelbaum et al. 2009a; Dijkstra et al. 2011).

B. violaceus' tolerance to a variety of environmental conditions is consistent with its cosmopolitan distribution (Carver et al. 2006). Specifically, *B. violaceus* can survive conditions of 5-25ºC and 14-38‰ and grow at 15-25ºC and 20-38‰, though it performs best at 20-25ºC and 26-38‰ (Epelbaum et al. 2009a; Dorning 2017b; Lord 2017); further, Dijkstra et al. (2008) report that *B. violaceus* is not tolerant of tidal salinity fluctuations that regularly include salinities below 15‰. In their regeneration study, Brown et al. (2009) also found that colonies maintained at 11ºC developed at a slower rate than those at 16ºC. The doubling time of *B. violaceus* decreased nearly three-fold when the temperature at which it was held increased from 15 to 25ºC (Yamaguchi 1975). Perhaps most notable is *B. violaceus*' high survival in waters polluted with heavy metals and sewage (Lambert and Lambert 2003). These tolerances increase its competitive advantage, especially in an increasingly human-influenced world.

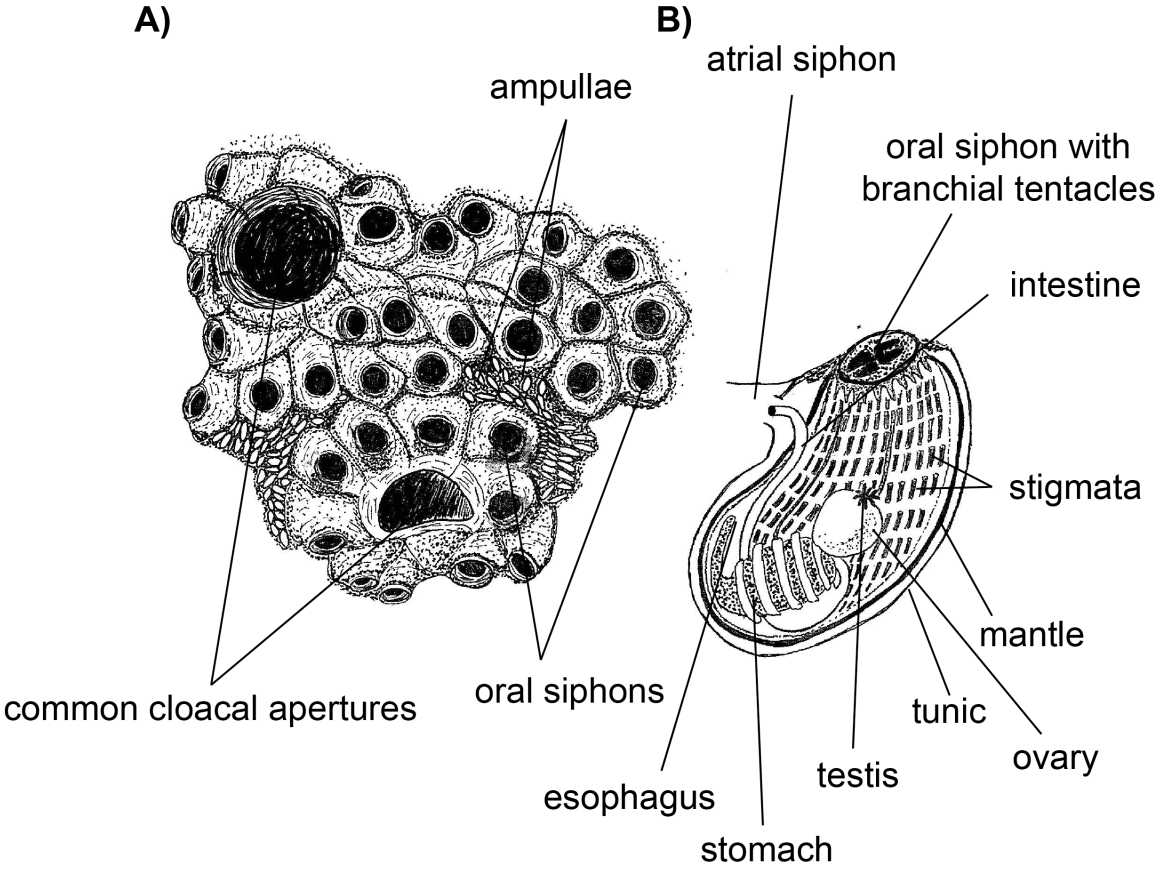

Figure 4. *Botrylloides violaceus* illustration showing **A)** colony surface and **B)** individual zooid anatomy.

As with other colonial ascidians, *B. violaceus* outcompetes native and non-native assemblages for space (Dijkstra and Harris 2009; Gittenberger and Moons 2011; Simkanin et al. 2013). Like *D. vexillum*, *B. violaceus* colonies prefer an inverted or vertical orientations, as a horizontal upward position leaves them susceptible to detrital settlement and smothering (Yamaguchi 1975). Predators have been observed consuming *B. violaceus* in controlled laboratory studies (Yamaguchi 1975; Osman and Whitlatch 2004; Epelbaum et al. 2009b), though predation alone has a limited ability to suppress fouling populations of *B. violaceus*. Simkanin et al. (2013) reported that the native predators on rocky shores prevented its infiltration from nearby floating docks into the

natural habitat. The authors also note, however, that increased propagule pressure or decreased health of the rocky reef community could eventually lead to a *B. violaceus* colonization of the site. The global distribution of *B. violaceus* is similar to that of *D. vexillum* (Table 2), likely because its vectors of introduction also include recreational boating (Berman et al. 1992; Lambert and Lambert 2003), the hulls of slow-moving commercial ships and barges (Carver et al. 2006), rafting on eelgrass (Locke et al. 2007), crustaceans (Bernier et al. 2009), or other floating debris, and bivalve aquaculture transfers (Bullard and Carman 2009). It is this last transfer method that most concerns the present work.

Shellfish Aquaculture and Invasive Colonial Tunicates

Aquaculture is an increasingly critical mechanism for meeting the growing demand for food by a global population projected to reach 9.7 billion by 2050 (United Nations 2015), particularly in developing maritime nations. For the first time in 2014, farmed fish for human consumption surpassed that of wild-caught (FAO 2016)—though this estimate may be biased by substantial underreported fisheries catch data (Pauly and Zeller 2017). Nevertheless, both the capture and aquacultural production of bivalve shellfish in the United States specifically has increased rapidly over the past half century from ~31,000 metric tons in 1950 to ~141,000 metric tons in 2010 (Campbell 2011; Campbell and Pauly 2013; Sea Around Us 2016). In 2013, sales of molluscs produced by aquaculture in the United States reached 329 million USD, to which Oregon and Washington combined contributed 160 million USD, or nearly half (USDA 2014). In addition to their economic worth, farmed bivalves provide numerous ecosystem services, including biogeochemical benthic-pelagic coupling (Newell 2004), water filtration

Table 2. First observations of *D. vexillum* and *B. violaceus* in countries outside of their native ranges to date, from where they have since continued to spread.

	Didemnum vexillum			*Botrylloides violaceus*		
Country	**Year first observed**	**Site of first observation**	**Citation**	**Year first observed**	**Site of first observation**	**Citation**
Australia	NA	NA	NA	2003	Moreton Bay, Queensland	Kott 2003
New Zealand	2001	near Tauranga and Whangamata Harbours	Kott 2002; Kleeman 2009; Lambert 2009	NA	NA	NA
Netherlands	1991	Dutch Delta	Ates 1998; Gittenberger 2007; Stefaniak et al. 2009	1999	Oosterschelde	Gittenberger 2007
Belgium	NA	NA	NA	1999	Zeebrugge	(Vanreusel et al.)
France	1968*, 1998	maybe Glénan archipelago, confirmed port of Le Havre	Lafargue 1968; McKenzie et al. 2017	NA	NA	NA
Ireland	2005	Malahide Estuary	Minchin and Sides 2006	2005-06**	Malahide and Carlingford marinas	Minchin 2007
United Kingdom	2008	Holyhead Harbour, north Wales	Griffith et al. 2009	2004	Gosport, Southampton, Hamble, Poole, Exmouth & Queen Anne's Battery in Plymouth	Arenas et al. 2006
Spain	2008	Santander, Baiona, Maoña, Corme-Porto, Gijón	El Nagar et al. 2010	2010-13**	Ria de Arosa, Galicia	Noreña et al. 2014
Italy	2012	Venitian Lagoon	Tagliapietra et al. 2012	1990s**	Venitian Lagoon	Zaniolo et al. 1998
Canada	East Coast: 2012; West Coast: 2003	Parrsboro, Nova Scotia; Okeover Inlet, British Columbia	Daniel and Therriault 2007; Therriault and Herborg 2008; Moore et al. 2014	East Coast: 2001 West Coast: 1992	Lunenburg and Mahone Bay, Nova Scotia; French Creek on Vancouver Island	Carver et al. 2006; Cohen 2011
United States	East Coast: 1988*, 2000; West Coast: 1993	Damariscotta River, ME; San Francisco, CA	Bullard et al. 2007	East Coast: 1992; West Coast: 1945†,1970s**	east coast: Great Bay Estuary, Gulf of Maine; west coast: Southern California, San Francisco Bay, Willapa Bay, Puget Sound	Van Name 1945; Fay and Vallee 1979; Berman et al. 1992
Mexico	2004-2005**	San Quintin Bay	Rodriguez and Ibarra-Obando 2008	1994-2000**	Ensenada, Baja California	Lambert and Lambert 2003

*potential, but unconfirmed first occurrence; **specific year not specified in publication; † misidentified as another *Botrylloides* spp.; NA = no recorded presence

(Coen et al. 2007), habitat creation, and refugia from predators (Grabowski and Peterson 2007). Considering the collapse of 85% of oyster reefs globally (Beck et al. 2011), the aquaculture of oysters and other filter-feeding bivalves may damper the loss of these services.

Shellfish aquaculture has been of significant cultural importance to Native Americans for millennia (Waselkov 1987; Cannon 2000), and recent research highlights the sustainability of their subsistence practices (Lepofsky and Caldwell 2013; Rick et al. 2016). In the 18th and 19th centuries, however, European colonizers overharvested the native oysters *Crassostrea virginica* and *Ostrea lurida* on the east and west coasts of the United States, respectively (Baker 1995; Kirby 2004). After the *O. lurida* populations declined, the colonizers transplanted *C. virginica* and later the Pacific oyster (*Crassostrea gigas*) to West Coast estuaries (MacKenzie et al. 1997). *C. gigas* is now the most commonly farmed bivalve in the region; the mussels *Mytilus* spp. and several clam species are also farmed. Numerous pressures now pose threat to shellfish aquaculture, including: ocean acidification (Gazeau et al. 2010; Barton et al. 2012), disease (Lafferty et al. 2015), harmful algal blooms (Shumway 1990), eutrophication (Dumbauld et al. 2009), and invasive species—including colonial ascidians.

As epibionts and fouling organisms, invasive colonial tunicates pose a growing threat to the shellfish aquaculture industry because they smother bivalves and cover the gear used to grow them (Switzer et al. 2011). The added weight can destroy the gear, including cages and nets or lines, as well as the crop contained therein or on. Revenue loss may also result from increased production and processing costs or stock mortality; broadly, conservative estimates price biofouling control at 5-10% of all production costs

(Fitridge et al. 2012). *Didemnum vexillum* specifically was estimated to cost 807,000 USD in damages to the green-lipped mussel industry in New Zealand (Sinner and Coutts 2003)—at least in part because it reduces the density of smaller, more recently seeded and therefore more vulnerable, mussels (Fletcher et al. 2013b). Further, *D. vexillum* deterred larval settlement of the bay scallop *Argopecten irradians irradians*, which Morris et al. (2009) believed to be a harbinger for the commercially grown scallop *Placopecten magellanicus* in Georges Bank. Auker (2010) suggested that *D. vexillum* inhibits *Mytilus edulis* growth by covering their lip margins and siphons, thereby interrupting their ability to filter feed, and Zajac et al. (1989) posited that *Botrylloides* sp. depleted food from its surrounding community, thereby negatively impacting its survival. Anecdotal evidence intimates, however, that *Botrylloides violaceus* does not decrease the meat yield of the mussels it fouls (Carver et al. 2006). These contradictory findings may reflect the fact that the actual filtering capacity for colonial tunicates is challenging to measure and remains largely undetermined for numerous species (Daniel and Therriault 2007), or that the impacts are dependent on both the specific members in the epibiont-basibiont relationship, as well as their geographic location.

Many invasive species exhibit rapid adaptation to local climate conditions (Sakai et al. 2001). A population of *B. violaceus* from eastern North America, for example, experienced 50% mortality at ~28ºC, compared to a 50% mortality at ~25.5ºC for a population from western North America, which the authors contributed to local adaptation to climate change (Sorte et al. 2011). In general, climate change is anticipated to favor invasive species (Stachowicz et al. 2002), including *B. violaceus*, whose thermal reproduction barriers are projected to soon disappear (Dijkstra et al. 2017). Conversely,

Lord (2017) predicts that *D. vexillum* may be a less potent invader under warming conditions, owing largely to it being a cooler-water species. Climate change is geographically variable, as are the biotic responses and adaptations it elicits. Therefore, to elucidate the current extent of the invasion and predict the future threats invasive colonial tunicates may pose to shellfish aquaculture, it is critical that the tunicate population dynamics and impacts on the specific bivalves being grown be investigated on a regional scale.

The remaining chapters of this thesis seek to fulfill these objectives in Oregon. The first study (Chapter II) describes the seasonal variation of a *D. vexillum* population at an oyster farm in Winchester Bay, Oregon from May 2011-2016. In Chapter III, I examine whether *B. violaceus*[1] has any impact on the growth, condition index, or macromolecular organic composition of *C. gigas* and *Mytilus trossulus*. While there is currently no mussel aquaculture in Oregon, a robust industry exists in Washington state's Puget Sound, as well as British Columbia. Some bivalve growers in the Pacific Northwest claim that, while invasive colonial tunicates are a nuisance because of the extra labor their fouling induces, they do not impact the quality of the shellfish itself (Gordon King of Taylor Shellfish Farms, pers. comm.). To my knowledge, this claim has not been tested empirically in the Pacific Northwest.

[1] Originally, I intended to use *D. vexillum* as the model organism for both chapters. The *D. vexillum* population in the Charleston Boat Basin, however, has dwindled (pers. obs.), and was concerned with propagating it further with my field experiment. Because *D. vexillum* is also particularly challenging to work with in a laboratory setting, I opted to use *B. violaceus*, whose Charleston Boat Basin population is thriving.

CHAPTER II

SEASONAL DYNAMICS OF A *DIDEMNUM VEXILLUM* POPULATION IN OREGON: A FIVE-YEAR SUMMARY

I gratefully acknowledge the SCUBA divers on the US Forest Service dive team that helped collect the field survey data used in this chapter. This work would not have been possible without Umpqua Aquaculture's cooperative efforts and support. While I analyzed these data and wrote this chapter alone, Drs. Edward Davis and Alan Shanks provided valuable guidance with statistical analyses and data visualization. Drs. Bruce Hanson, Steven Rumrill, and Aaron Galloway are my manuscript collaborators and co-authors; for their project planning, acquisition and sharing of data, and critical feedback of the manuscript I am thankful.

Introduction

Invasive species have dramatically altered the structure and function of several marine ecosystems, particularly coastal and estuarine habitats (Grosholz 2002). The effects of invasive species in the United States cost an estimated 120 billion USD per year (Pimentel et al. 2005); preventing and mitigating these effects are priorities of the National Oceanographic and Atmospheric Administration (NOAA; Daley and Scavia 2008). In a time where most aspects of global change are anticipated to favor invasive species (Dukes and Mooney 1999), understanding the biology of invasive species is of primary concern.

The colonial tunicate *Didemnum vexillum* Kott (2002) was first recorded on the United States West Coast in San Francisco Bay in 1993 (Bullard et al. 2007). *D. vexillum* is native to Japan (Stefaniak et al. 2012), but has become a global invader, plaguing

shellfish aquaculture in New Zealand (Fletcher et al. 2013b), the Northwest Atlantic (Carman et al. 2010; Sephton et al. 2011), the Northeast Pacific (Switzer et al. 2011), and the Mediterranean (Ordóñez et al. 2015). A fouling organism, *D. vexillum* is of particular concern for shellfish aquaculture, as it can smother crop and gear (Fig. 5a, b), depress growth rates (Fletcher et al. 2013b), and deter larval settlement (Morris 2009). Conservative estimates put the global cost of biofouling control to the aquaculture industry at 1.5-3 billion USD annually (Fitridge et al. 2012). In New Zealand, *D. vexillum* specifically caused 807,000 USD in damages to the green mussel (*Perna canaliculus*) aquaculture industry (Sinner and Coutts 2003).

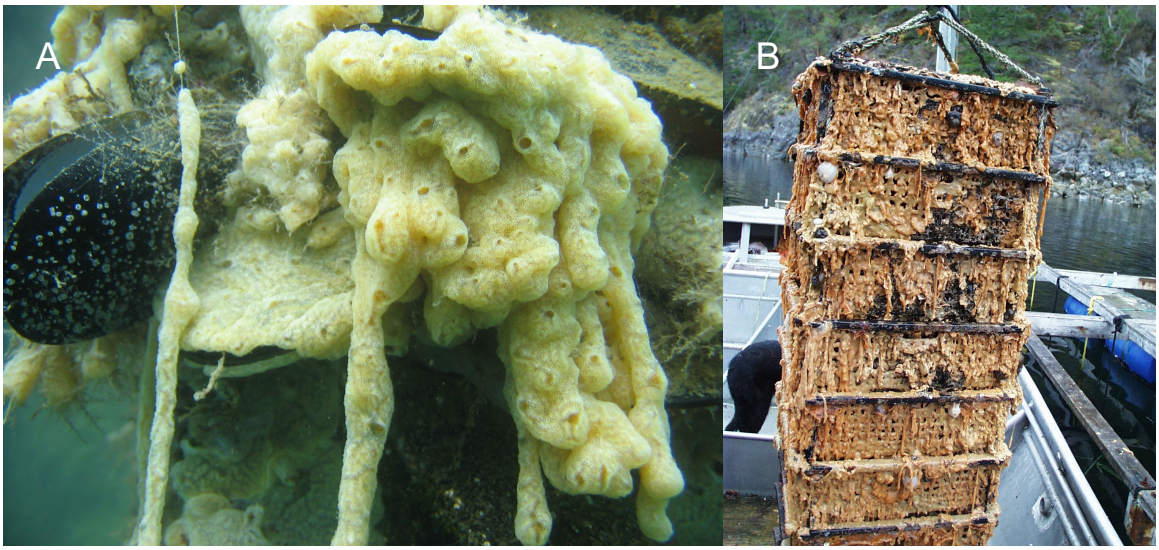

Figure 5. *Didemnum vexillum* colonies **A)** forming tendrils and encrusting mussels in New Zealand and **B)** fouling aquaculture gear in British Columbia. Photo credits: Paul Barter and Gordon King, respectively.

D. vexillum is an ecosystem engineer (Wallentinus and Nyberg 2007), and can fill diverse niches because it can survive in a wide range of environmental conditions. For example, it is found in temperatures from -2 to 24ºC (Valentine 2009), a wide range of salinities (10-36‰; Bullard 2009; Gröner et al. 2011), depths (0-81m; Valentine et al. 2007), habitats (estuarine to outer coast; Bullard 2009), and settlement substrates,

including artificial structures (Daley and Scavia 2008), loose cobble (Valentine et al. 2007b), eelgrass (Carman and Grunden 2010), and over healthy established benthic invertebrate communities (Bullard and Whitlatch 2009). During the winter, *D. vexillum* colonies exhibit regression, but not complete death (Valentine et al. 2007b). In other studies, this regression pattern has been strongly correlated to seasonal fluctuations in temperature (Gröner et al. 2011) and salinity (Fletcher et al. 2013a), but resistance to these fluctuations varies across populations (Valentine 2009; Fletcher and Forrest 2011; Gröner et al. 2011).

There are two known populations of *D. vexillum* in Oregon: one in the Charleston Marina, and one in Winchester Bay. Both populations were first observed in 2010 (Rumrill et al. 2014), and oysters are farmed at the latter site. *D. vexillum* may have arrived to Winchester Bay by way of oyster transfers, on which numerous invasive species are known to hitchhike (Mineur et al. 2007), and to the Charleston Marina via recreational boating, one of the current most common vectors of aquatic invasive species (Clarke Murray et al. 2011; Roche et al. 2015). To our knowledge, there is currently no published research about the extent or ecology of *D. vexillum* in Oregon. Addressing and assessing the risks of invasive species is a key conservation issue outlined in the Oregon Conservation Strategy (Oregon Department of Fish and Wildlife 2016), and *D. vexillum* is listed as one of the state's top 100 most dangerous invasive species (Oregon Invasive Species Council 2016). Furthermore, state officials recently rated *D. vexillum* as 'High Risk,' with a factor score of 12.5 out of 15. One of the concerns raised in this risk assessment was that *D. vexillum* would soon generate sufficient propagule pressure to

colonize new sites, as many of the diverse conditions and habitats in which *D. vexillum* can survive also exist in Oregon (Rumrill et al. 2014).

The objective of this study is to characterize the seasonal variation and general extent of the *D. vexillum* invasion in its primary Oregon foothold—Winchester Bay. We hypothesized that, in congruence with other studies, *D. vexillum* cover would be greater in fall than in spring, and that this cover is directly correlated with salinity (Gröner et al., 2011) and temperature (Valentine 2009; Fletcher and Forrest 2011; Fletcher et al. 2013a). To test these hypotheses, divers performed subtidal surveys of Winchester Bay *D. vexillum* cover biannually from 2011 to 2016. To our knowledge, this study is both the first to analyze *D. vexillum* in Oregon, and spans the longest monitoring period of an *in situ D. vexillum* population to date.

Methods

Monitoring occurred in the South Jetty 'Triangle' at the mouth of the Umpqua River in Winchester Bay, OR (43°39'54.5"N 124°12'40.3"W; Fig. 6), where the Umpqua Aquaculture company operates its longline oyster farm. The lines are attached to floats, allowing for constant submersion regardless of tidal fluctuation (Fig. 7). A United States Forest Service SCUBA team performed subtidal Triangle surveys biannually in May and October from 2011-2016. Due to periodic lapses in funding, divers did not perform surveys in May 2015 and October 2016. During each survey, divers followed vertical subtidal oyster culture lines (May 2011, n = 11; October 2011, n = 14; May 2012, n = 14; October 2012, n = 12; May 2013, n = 20; October 2013, n = 17; May 2014, n = 22; October 2014, n = 17; October 2015, n = 23; May 2016, n = 18) from the bottom to the surface, along which they counted and measured *Didemnum vexillum* colonies. The

divers randomly chose new lines to observe each survey. We used an *in situ* diver-based survey approach so as to minimize disturbance to the gear and product owned by Umpqua Aquaculture.

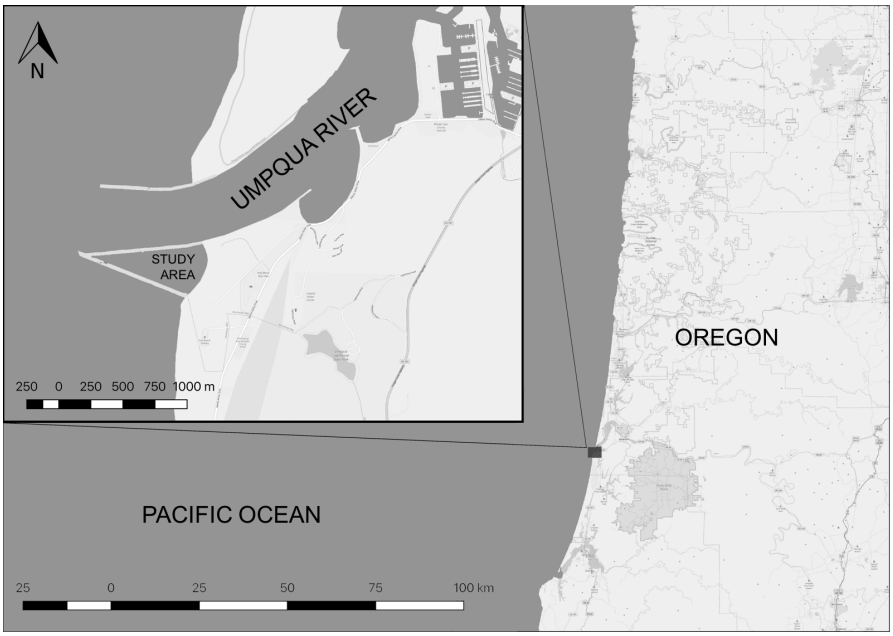

Figure 6. Map of South Jetty "Triangle" study area at the interface of the Umpqua River and Pacific Ocean in Winchester Bay, OR. Dark grey represents inlayed map area. Figure rendered in QGIS (QGIS Development Team 2018).

To account for the multi-dimensional structure of the oyster clumps and the *D. vexillum* colonies encrusting them, the divers measured along the vertical contours of the clumps, rather than following a linear path. Here, we define 'colony size' as the measured distance of continuous *D. vexillum* on an oyster line, and 'colony abundance' as the number of these continuums. Note, however, that it is probable that within such a measurement that genetically different colonies exist, as *D. vexillum* colonies have the ability to fuse and form chimeric colonies (Smith et al. 2012; Rinkevich and Fidler 2014). While surveying, the divers measured each line and colony to the nearest centimeter.

They recorded the depths at which the *D. vexillum* cover began and ended, as well as some other intermittent depths, from their dive computers.

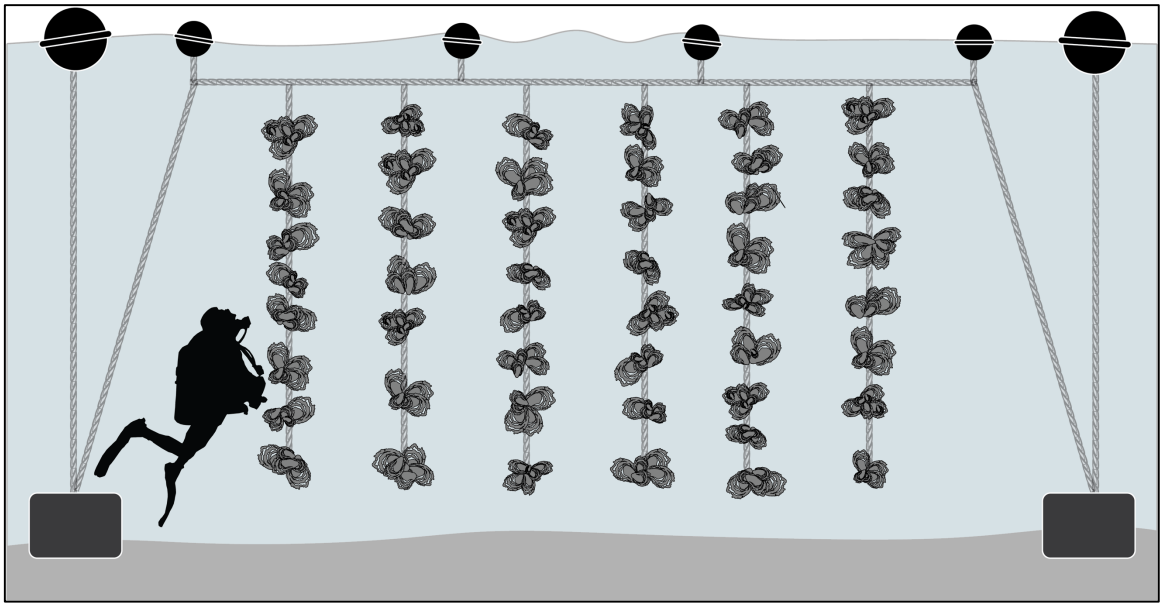

Figure 7. Longline subtidal oyster farm infrastructure.

Unfortunately, there is not a continuous water quality monitoring asset within the Triangle itself, which is situated at the mouth of an estuary and the outfall of the Umpqua River, a moderately large river that drains 12,103 km^2 of western Oregon (Fig. 6; Wallick et al. 2011). Moreover, the jetty walls of the Triangle are expected to limit the complete exchange of water and salinity of the water within the Triangle during every tidal cycle. Therefore, to account for longer-term water overturn and evaluate the relationship between *D. vexillum* cover and salinity, we used the Umpqua River output over a longer time frame—the 15-day average prior to the survey date—as a proxy for salinity (USGS Station #14321000; U.S. Geological Survey 2018). HOBO® (Onset Computer Corporation, Massachussets) probes collected temperature and salinity data from January 2012 to January 2013 in the Triangle. These data were inversely related to Umpqua River

output during the same time frame, and we thus found the 15-day average output as a sufficient salinity proxy. Further, the temperature fluctuations recorded in the Triangle during the HOBO® probe deployment period were comparable to the sea surface temperatures recorded further offshore at NOAA Buoy 139 (National Data Buoy Center 2017) within the same period (Rumrill et al. 2014); therefore, we used the NOAA data to calculate the 15-day average temperature recorded prior to the survey date, and used these values for the analyses below as a proxy for temperature within the Triangle.

We used RStudio (v. 1.1.414; RStudio Team 2018) for all statistical analyses. First, we performed two-sample t-tests to compare the fall and spring averages of total length of line covered (m), proportion of line covered (%), colony abundance, and pre-survey 15-day average Umpqua River discharge (m^3s^{-1}) and offshore sea surface temperature (ºC; Table 3). We then visualized total line covered and proportion of line covered, and abundance over time, as well as vertical colony distribution using the depth and cover distance data in point and whisker plots. Several of the variables I sought to fit to a linear model (survey means of: length of line covered [m; n = 10], proportion of line covered [%; n = 10], and colony length [m; n = 10]) were not normally distributed, nor were their residuals. Therefore, I used R package "TeachingDemos" to Box-Cox transform the data:

$$f(y) = (y^{\lambda}-1)/ \lambda \qquad \text{(Box and Cox 1964)}$$

where y is the dependent variable and λ is the transformation parameter that best normalizes the dependent variable's distribution. I regressed these normalized data against the salinity and temperature proxies, as well as season, and summarized these results with plots and summary tables.

Results

Seasonal trends

The overall average *Didemnum vexillum* colony abundances between spring and fall were not significantly different ($p = 0.8$). However, there were significant differences between the overall spring and fall averages for: length of line covered (m; $p = 0.0009$), proportion of line covered (%; $p = 0.03$, Umpqua River discharge (m^3s^{-1}; $p = 0.008$), and sea surface temperature (ºC; $p = 0.05$; Table 3).

Table 3. Two-sample t-tests between average seasonal measurements.

	spring $\bar{x}$	**fall $\bar{x}$**	***t***	***df***	***p***
length of line covered (m)	3.85	6.12	3.41	135	0.0009*
proportion of line covered (%)	18.9	26.6	2.18	161	0.03*
abundance (colonies per line)	6.74	6.51	0.309	150	0.8
pre-survey 15-day average Umpqua River discharge (m^3s^{-1})	292	41.1	4.70	4	0.02*
pre-survey 15-day average sea surface temperature (ºC)	11.4	14.3	2.38	4	0.05*

*p-values significant at the $\alpha < 0.05$ level

Across the biannual surveys conducted, the measured average *D. vexillum* cover per oyster longline (m) was lowest in May 2011 (4.02 ± 1.57 [mean ± SE]) and greatest in October 2012 (12.9 ± 1.89; Fig. 8a). *D. vexillum* cover was generally greater in fall than spring (Table 3). However, the differences in cover across consecutive surveys were only significant between May 2012, October 2012, and May 2013 (3.58 ± 0.81, 12.9 ± 1.89, and 3.98 ± 0.76 m, respectively) and May and October 2014 (4.16 ± 0.69 and 6.77 ± 1.01 m; note that we did not conduct a survey in May 2015). Cover in the spring returned to statistically similar levels each year from May 2011 to May 2016 (4.08 ± 0.88 m). Conversely, fall cover was more sporadic, with cover nearly tripling between October

2011 (3.85 ± 1.06 m) and 2012, followed by significantly less cover in October 2013 (5.56 ± 0.95 m), 2014, and 2015 (3.90 ±0.71 m; Fig. 8a).

The mean *D. vexillum* percent cover per line followed a similar pattern to the cover data. Lines were proportionally more covered in the fall than the spring, with one notable exception: percent cover decreased, though not significantly, from May 2011 (25.9 ± 10.0%) to October 2011 (16.0 ± 4.2%; Fig. 8b). In May 2012, the percent cover was lowest measured across all surveys (13.3 ± 2.6%), followed by the greatest—and significantly different—percent cover measured in October 2012 (52.8 ± 8.0%). The May 2013 and 2014 surveys (17.4 ± 3.1 and 16.7 ± 2.5%, respectively) both had lower proportional cover than their successive October 2013 and 2014 surveys (25.3 ± 4.1 and 28.9 ± 4.1 %, respectively); percent cover did not differ between the October 2015 and May 2016 surveys (18.5 ± 3.3 and 17.4 ± 3.4 %, respectively). As with cover, percent cover did not differ between the five spring surveys, though it did for the five fall surveys (Fig. 8a, b).

The average *D. vexillum* abundance ranged from a minimum of 2.29 ± 0.58 colonies per line in October 2011 to a maximum of 10.9 ± 1.80 colonies per line in May 2014 (Fig. 8c). The minimum was not significantly different from the abundance during the May 2011 survey (3.09 ± 0.79 colonies per line), and the maximum did not differ from the abundance measured in October 2014's survey (8.41 ± 0.87 colonies per line). However, abundance did increase from October 2011 to May 2012 (7.50 ± 0.84 colonies per line), as well as from May 2013 to October 2013 (4.90 ± 0.79 and 7.29 ± 1.01 colonies per line, respectively), and October 2013 to May 2014. Abundance did not change between October 2015 and May 2016 (6.04 ± 0.80 and 5.81 ± 1.00 colonies per

line, respectively), but did decrease between October 2012 (8.50 ± 1.20 colonies per line) and May 2013 (Fig. 8c).

D. vexillum did not grow well toward the surface-oriented portions of the longlines. The mean cover of *D. vexillum* colonies at 0-2.5 m depth did not exceed 20%, with the exception of the October 2012 survey, in which cover exceeded 20% as shallow as 0.5-1.0 m (Fig. 9). Similarly, mean colony cover generally did not exceed 20% at depths past 7.5 m (but see fall 2012, where cover was 12.3 ± 12.3% at 7.5-8.0 m depth). On average, *D. vexillum* covered the greatest proportion of line between 5.0-5.5 m depth in both the spring (40.9 ± 17.7%) and fall (42.1 ± 15.9%). Broadly, the colonies covered the greatest proportion of line between ~4.5-6.5 m depth. The shapes of the distributions were considerably more normal in the fall surveys compared to those in the spring; the centers of these distributions are depicted in Fig. 8b.

The percent cover of *D. vexillum* colonies on the lines peaked at depths of 5.0-5.5 m in spring 2011 (49.3 ± 9.6%), 5.5-6.0 m in fall 2011 (27.1 ± 8.8%), and 5.5-6.0 m in spring 2012 (37.9 ± 9.3%). In fall 2012, maximum percent cover occurred at 5.0-5.5 m depth (54.9 ± 7.1%), but percent cover also exceeded 50% at a range of depths (1.5-6.0 m). Further, in spring 2013 *D. vexillum* covered the greatest proportion of line at depths of 5.5-6.0 m (43.5 ± 7.8%), while it peaked at 5.0-5.5 m in fall 2013 (48.0 ± 5.86 m), and 4.5-5.0 m in spring 2014 (37.6 ± 5.15 m). In similarity to the fall 2012 survey, in fall 2014 maximum percent cover occurred at 5.0-5.5 m depth (54.6 ± 6.7%), but neared or exceeded 50% at depths of 3.0-6.0 m. Maximum percent cover occurred at the deepest point in the fall 2015 survey (6.5-7.0 m; 36.3 ± 4.8%). Finally, percent cover peaked at depths of 5.5-6.0 m in spring 2016 (45.7 ± 9.9%; Fig. 9).

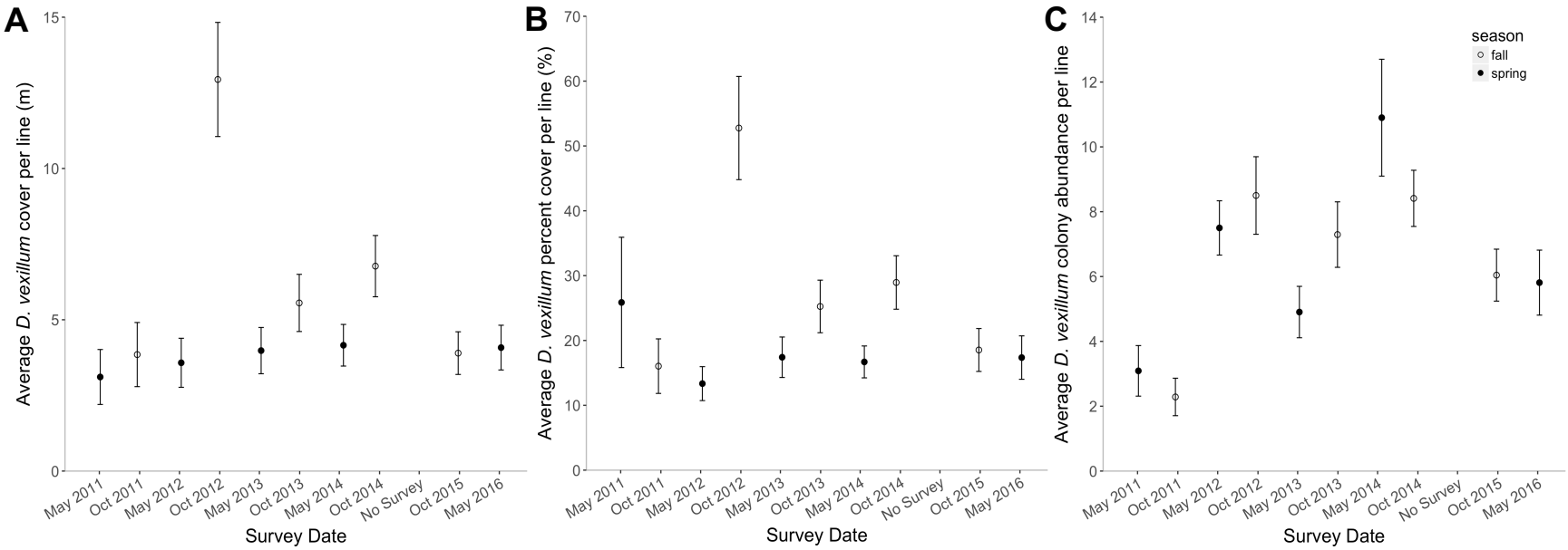

Figure 8. Mean *D. vexillum* **A)** total cover (m), **B)** percent cover (%), and **C)** colony abundance per oyster longline for biannual Triangle surveys conducted from May 2011 to May 2016. Space between points is proportional to time between surveys. No survey occurred in May 2015. Error bars are ± 1 SE (propagated).

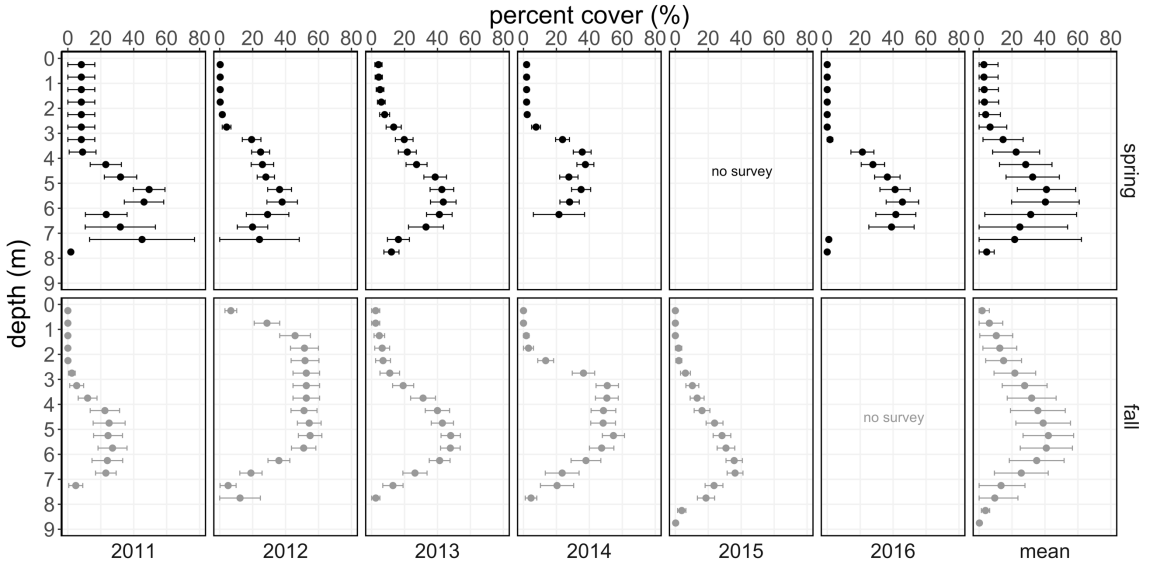

Figure 8. Seasonal survey profile of percent *D. vexillum* colony cover distributed along subtidal oyster longlines. Points represent 0.5 m sections of line (e.g., a point at 6.25 is the mean percent cover at 6-6.5 m depth). No survey occurred in spring 2015 or fall 2016. Error bars are ± 1 SE (spring and fall means propagated).

Linear regression models

Optimal normalization factors (λ), as well as kurtosis and skewness values for transformed data are reported in Table 4.

Table 4. Box-Cox transformation factors (λ) for tested dependent variables, and skewness and kurtosis values of the normalized values.

	λ	**skewness**	**kurtosis**
length of line covered (m)	-5	0.151	2.13
proportion of line covered (%)	-1.5	0.081	2.01

We did not use a linear regression analysis on the overall abundance of *D. vexillum* colonies because they were not significantly different between the fall and spring. Umpqua River discharge predicted variance in the dependent variables of average *D. vexillum* cover per line ($r^2 = 0.412$), though it was not a good predictor of variance in

percent *D. vexillum* cover per line ($r^2 = 0.147$; Fig. 10a-b). The slope from the length of line covered versus Umpqua River discharge model was significantly different than 0 ($p = 0.046$; Fig. 10a), but the slopes for the other two models were not ($p_{\text{percent cover}} = 0.274$; Fig. 10b). Sea surface temperature did not predict the variance in cover per line or percent cover per line ($r^2_{\text{cover}} = 0.081$; $r^2_{\text{percent cover}} = 0.001$), nor were the slopes from these linear regression models significant ($p_{\text{cover}} = 0.427$; $p_{\text{percent cover}} = 0.939$; Table 5). No multiple linear regression combination of discharge, temperature, and season yielded a model with a significant slope or could explain the variances of cover, percent cover, or colony length (Table 5). There were no significant serial correlations within any of these models.

Table 5. Summary of simple and multiple linear regression statistics for **A)** Box-Cox transformed cover (m), **B)** Box-Cox transformed percent cover (%), and **C)** Box-Cox transformed colony length (m).

	F	***df***	r^2	***p***
A) cover				
river discharge[§]	5.60	1,8	0.412	0.046*
temperature[†]	0.701	1,8	0.081	0.427
river discharge + temperature	2.68	2,7	0.272	0.137
river discharge + season	1.73	2,7	0.196	0.259
temperature + season	1.49	2,7	0.099	0.289
river discharge + temperature + season	1.62	3,6	0.171	0.282
B) percent cover				
river discharge[§]	1.38	1,8	0.147	0.274
temperature[†]	0.006	1,8	0.001	0.939
river discharge + temperature	0.988	2,7	-0.003	0.419
river discharge + season	0.805	2,7	-0.045	0.485
temperature + season	1.38	2,7	0.078	0.312
river discharge + temperature + season	0.906	3,6	-0.032	0.492

*p-values significant at the $\alpha < 0.05$ level; [§]prior 15-day average Umpqua River discharge (m^3s^{-1}), [†]prior 15-day average temperature (ºC) recorded at NOAA Buoy 1

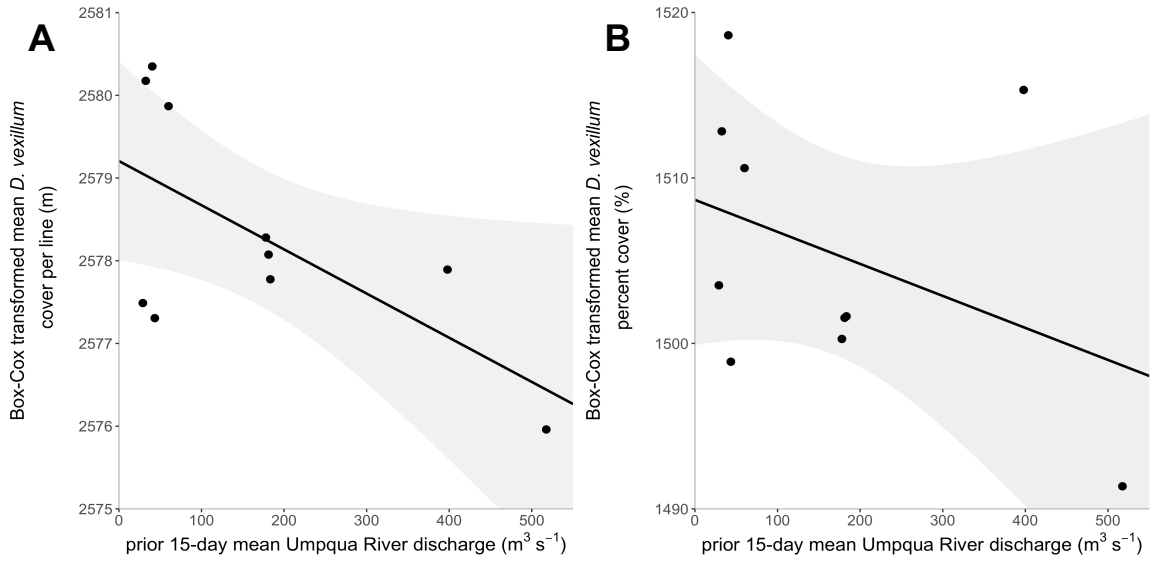

Figure 9. Linear regression models of the prior 15-day average Umpqua River discharge (m^3s^{-1}, a proxy for salinity) and average **A)** total *D. vex* cover per line (m; $\lambda = -5.00$; $r^2 = 0.412$; $p = 0.046$) and **B)** percent *D. vex* cover per line (%; $\lambda = -1.50$; $r^2 = 0.147$; $p = 0.274$Grey area represents regression 95% confidence interval. Each point is a seasonal survey average. Dependent variable values are Box-Cox normalized, where $f(y) = (y^{\lambda} - 1) / \lambda$.

Discussion

Subtidal survey data of *Didemnum vexillum* on oyster longlines in Winchester Bay, OR, revealed a population with erratic fluctuations in all parameters measured. Average *D. vexillum* colony cover per line (m), percent cover per line (%), and abundance per line as measured in biannual surveys from May 2011 to May 2016 (Fig. 8a-c) were variable. Our analyses showed that this variation is seasonal, and that the average colony cover per line, percent cover per line, and size are all significantly greater in the fall than the spring (Table 1). Percent colony cover was greatest at depths of ~4.5-6.5 m depth (Fig. 9). The mean Umpqua River discharge (m^3s^{-1}) and sea surface temperature (ºC) of the 15 days prior to each survey—the respective proxies for salinity and temperature trends within the Triangle in absentia of those data—were also significantly different between fall and spring. Discharge was significantly greater in the

spring (meaning that salinity was lower), while temperature was significantly lower. Based on this evidence, we found no support for our *a priori* null hypothesis that *D. vexillum* colony cover is the same across seasons. Notably, the abundance of colonies did not differ between fall and spring (Table 1), demonstrating that this Oregon *D. vexillum* population experiences seasonal regression in size but not total colony death. This finding aligns with what has been reported for a population in New England's Georges Bank (Valentine et al. 2007b).

Because we found that there is a weak, but significant ($r^2 = 0.412$; $p = 0.046$), linear relationship between freshwater output (salinity) and the transformed mean *D. vexillum* cover per line (m; Fig. 10a), salinity is likely one of the key environmental factors driving *D. vexillum*'s regression between fall and spring. Salinity is a well-established control for *D. vexillum* and other colonial ascidians in the literature (Brunetti et al. 1980; Valentine et al. 2007b; Bullard and Whitlatch 2009; Epelbaum et al. 2009a; Gröner et al. 2011). Conversely, we found no relationship between salinity and other measurements of *D. vexillum* growth (e.g., percent cover; Fig. 10b), nor did temperature explain any variation in of *D. vexillum* cover as measured (Table 5). Multiple linear regression models combining the Umpqua River discharge, sea surface temperature, and season were also unsuccessful in explaining the variances in mean *D. vexillum* cover and percent cover.

It is possible that the available proxies for salinity and temperature in this analysis were tenuous on account of how the Triangle survey area could potentially limit water exchange. Further, sea surface temperature data may not be a good metric for organisms that exhibit a clear depth preference; vertical temperature profiles of the water column

through time would be more appropriate. Since *D. vexillum* salinity-driven mortality has been shown to be duration-dependent, not intensity-dependent (Gröner et al. 2011), the time frame for which I evaluated freshwater discharge may have been too long or too short, and could in part explain the lack of a clear relationship. It is also possible that another environmental factor that we did not account for (e.g., food quality and availability, ocean hydrodynamics; Bates 2005) more strongly describes the observed trends in *D. vexillum* cover. For example, Grosberg (1988) demonstrated that food availability directly impacted egg production rates in another colonial tunicate, *Botryllus schlosseri*. The growth rate of *B. schlosseri* is lower in the laboratory than the field, which previous studies attribute to the frequency of food delivery and diversity of food types (Brunetti and Copello 1978; Chadwick-Furman and Weissman 1995). Food therefore may too explain *D. vexillum*'s population dynamics, especially considering the substantial impact coastal upwelling has on nutrient availability to Oregon's coastal habitats (Barth et al. 2007).

Despite the lack of a strong linear correlation between salinity and mean *D. vexillum* percent cover and colony length, the survey profiles of *D. vexillum*'s mean proportional distribution along subtidal oyster longlines (Fig. 9) lend additional evidence of growth being salinity-driven. Tidal fluctuation and freshwater input induce a stratified water column in estuarine and near-coast habitats (Simpson et al. 1990), wherein a horizontal gradient of fresher water forms at the water's surface. The general lack of *D. vexillum* cover in the upper portions of the water column indicates its aversion to less saline conditions. While *D. vexillum* observed during the October 2012 survey exhibited growth near the top of the water column, there were other surveys for which the mean 15-

day pre-survey Umpqua River output was lower (e.g., October 2014 and 2015). The profiles of the October 2014 and 2015 surveys follow a general trend where percent cover is concentrated within a narrower, deeper band—indicating growth near the surface in October 2012 was the anomaly. While the findings presented here do not match clear descriptions of salinity- and temperature-driven fluctuations of growth in *D. vexillum* and other colonial tunicates (McCarthy et al. 2007; Valentine 2009; Fletcher et al. 2013a), it is known that *D. vexillum* exhibits measurable interregional and interpopulation variation in behavior and sensitivity to environmental conditions. Such variation is apparent across seasons within the present dataset. Indeed, it is likely that the population widely fluctuates within the months between the surveys—particularly between late June and October, when environmental factors are most favorable to *D. vexillum* reproduction and population growth. The methods performed in these surveys only capture a snapshot of the population through time. Only more frequent sampling would elucidate the nuances of this population's growth and recession; these ten survey snapshots nevertheless provide valuable insight to this *D. vexillum* population.

Despite finding that the *D. vexillum* population varied significantly throughout the duration of this study, perhaps our most interesting finding is that both cover per line and percent cover in May 2016 did not differ from than that of May 2011. This observation is critical because it shows that the threat of *D. vexillum*'s invasion in the Triangle may not be as severe relative to what has been reported and forecasted for other systems. To date, most literature on the invasion ecology of *D. vexillum* has emphasized the extensive threats the species poses to quickly taking over nearshore ecosystems and aquaculture operations (e.g., Bullard et al. 2007; Daley and Scavia 2008). However, the fact that we

found no net growth of *D. vexillum* colonies during the study period at this site in Oregon does not mean it does not pose a future risk. The existing colonies maintain the potential to spread and grow, should a future environmental condition trigger a significant outbreak from this population's current foothold. In addition, our time series also identified that there were significantly fewer, but larger, colonies in May 2011 compared to May 2016's more abundant, but smaller colonies (Fig. 8c, d). As of July 2017, *D. vexillum* still exists in the Triangle (pers. obs.), though it had receded from the jetty rocks it reportedly colonized in earlier surveys (Rumrill et al. 2014). This finding, however, may also reflect some variability in the divers' execution of measuring and counting colonies, as a different team performed each survey.

The broad population decline between October 2012 and May 2016 in Winchester Bay parallels that of the recent decline in Oregon's second known *D. vexillum* population in Charleston, 36 km south of the Triangle. Divers conducted *D. vexillum* presence/absence surveys of the Charleston Marina within the same general time frame of this study. Upon encountering *D. vexillum* in the Charleston Marina, divers removed the colonies and dropped them into the soft sediment substrate. They did not attempt such eradication methods in the Triangle, as doing so could have adversely impacted Umpqua Aquaculture's apparatus. During the most recent survey in January 2017, the divers found relatively few colonies (pers. obs.). Since that survey, this author and other scientists located at the Oregon Institute of Marine Biology have yet to find *D. vexillum* colonies in the Charleston Marina. With caution, we can state that some confluence of environmental variables unfavorable to *D. vexillum* and manual removal of found colonies via SCUBA seem to have, at the very least, resulted in significant progress toward eradication, or at

least ecologically significant slowing of Oregon's *D. vexillum* population in Charleston Harbor.

Curiously, populations of *D. vexillum* on the North American west coast are now retained within protected harbors or other artificial structures. This observation contrasts the east coast, where *D. vexillum* has carpeted 50-90% of a 230 km^2 area of the Georges Bank benthos (Valentine et al. 2007b). There are numerous reasons for which this dichotomy may occur, including localized genetic adaptations to tolerate extreme temperature (Grosholz 2001) and salinity (Renborg et al. 2014), or large-scale oceanographic phenomena (e.g., coastal upwelling, which drives temperature and nutrient availability). Predation on *D. vexillum* may also play a role, but this interaction is still poorly studied. Simkanin et al. (2013) showed that the predation of another invasive colonial tunicate, *Botrylloides violaceus*, by native predators limits its spread from marinas to nearby rocky reefs in British Columbia. Further, Forrest and colleagues (2013) provided compelling evidence for predatory biotic resistance to *D. vexillum*'s establishment in cobble habitats of New Zeland. However, *D. vexillum* is chemically adapted to resist predation, and another experiment proved its control via predation unsuccessful (Carman 2009). As *D. vexillum* has exhibited high reproductive plasticity elsewhere (Ordóñez et al. 2015) and environmental conditions on the west coast allow for year-round survival and—potentially—reproduction, it is possible that west coast *D. vexillum* populations may eventually establish reproductively viable populations in more natural habitats. Moreover, *D. vexillum*'s demonstrated ability to raft on eelgrass blades (Carman et al. 2014) and reproduce while fragmented in suspension (Morris and Carman 2012), justifies prioritizing its regular monitoring in Oregon and along the west coast.

Conclusions

The present study is: 1) the first to report seasonal trends and fluctuations of a *D. vexillum* population in Oregon; and 2) the longest-term survey of a *D. vexillum* population in the literature to date. We found that the population of *D. vexillum* in the Triangle exhibited extensive fluctuation in colony cover from May 2011 to May 2016, especially between fall and spring, and that this fluctuation can be in part explained by the mean Umpqua River discharge rate for the 15 days preceding the survey. These data do not affirm other studies' findings that temperature is significantly related to *D. vexillum* growth. It remains unclear what other environmental factors primarily drive the trends observed here. We have documented an ultimately net zero change in *D. vexillum* cover in the Triangle between the first and last surveys in a 5-year period.

Given that changes in freshwater output significantly impacted *D. vexillum* cover, warm winters with low snowpack—a current (February 2018) occurrence in Oregon—may forecast a pulse of *D. vexillum* growth in the Triangle. As climate change continues its warming encroach and such winters become more frequent (Mote et al. 2005; Sproles et al. 2013), environmental conditions may tilt favorably toward *D. vexillum* in Oregon and globally. A significant expansion of this ascidian's populations may be costly to aquaculture operations, as well as their products. While others have studied how *D. vexillum* impacts other bivalves it fouls, such impacts have yet to be explored for the Pacific oysters grown in the Triangle, *Crassostrea gigas*. We recommend continued monitoring of the two *D. vexillum* populations in Oregon, as well as determining empirically what—if any—impact its increased fouling may have on the condition of the oysters ultimately produced.

CHAPTER III

EFFECTS OF *BOTRYLLOIDES VIOLACEUS* BIOFOULING ON *CRASSOSTREA GIGAS* AND *MYTILUS TROSSULUS* IN OREGON AND WASHINGTON

Introduction

Epibiotic relationships are established when one living organism, the epibiont, settles on and colonizes another living substrate, the host basibiont. Epibiosis, while highly variable, is predominantly facultative and opportunistic (Wahl and Mark 1999). Basibionts suffer numerous consequences from epibiotic relationships, including increased weight, decreased buoyancy and mobility, and a direct competition for nutrients and oxygen with the epibiont (Wahl 1989; Ferguson et al. 2013). An epibiont may, however, serve a mutualistic role by protecting its basibiont (Vance 1978; Wahl et al. 1997)—be it from predation through camouflage (Laudien and Wahl 2004), or desiccation via insulation (Penhale and Smith 1977). Though the adverse effects frequently overshadow the positive (Harder 2008), the ecological costs for a basibiont are dependent on the context and intensity of the relationship with its epibiont.

Biofouling occurs when an organism colonizes a living or dead solid substrate (Wahl 1989). Fouling organisms present a unique problem to marine aquaculture operations, as they often impact both the infrastructure and the consumable product. Bivalves grown using subtidal longline methods—including the oyster *Crassostrea gigas* (Thunberg 1793) and the mussel *Mytilus trossulus* (Gould 1850)—are archetype basibionts because they are sessile and possess a shell whose surface area is large relative to prospective colonizers (Wahl and Mark 1999). In 2010, over 12.3 million tons of bivalves were raised in aquaculture globally (Pauly and Zeller 2016). In 2013, over 101

million dollars' (USD) worth of mussels and oysters were produced in Oregon and Washington alone, accounting for nearly 31% of all mollusc sales in the United States that year (USDA 2014). These organisms are clearly both critical to the coastal Pacific Northwest economy, as well as potentially vulnerable to the impacts of biofouling.

Invasive colonial tunicates are among the most pervasive biofouling agents in aquaculture (Carver et al. 2006; Bullard and Carman 2009; Watson et al. 2009). *Botrylloides violaceus* (Oka 1927), endemic to Japan (Berrill 1950), is one such tunicate. The first confirmed appearance of *B. violaceus* on the United States West Coast occurred in the 1970s (Fay and Vallee 1979; Berman et al. 1992), though it may have been sighted and misidentified as another *Botrylloides* spp. as early as 1945 (Van Name 1945). Now, *B. violaceus* is a cosmopolitan organism, and has infiltrated shellfish aquaculture operations along the east coast (Ramsay et al. 2008; Carman et al. 2010; Arens et al. 2011) and west coast (Carver et al. 2006; pers. obs.) of North America. *B. violaceus* can overgrow or otherwise outcompete native benthic organisms for space, posing threat to the community's assemblage and diversity (Dijkstra et al. 2007a; Dijkstra and Harris 2009). Other studies have suggested that *Botrylloides* spp. may outcompete resident filter feeders for food (Zajac et al. 1989), though the filtering capacity of colonial ascidians is highly variable and difficult to determine. Though anecdotal remarks suggest that *B. violaceus* has no direct impact on the growth of cultured mussels (Carver et al. 2006), no empirical evidence of such exists in the literature to date.

While the ecological consequences to *B. violaceus*'s biofouling are unknown, shellfish growers in Washington and Oregon do indicate that invasive ascidians are a nuisance due to the extra labor costs incurred by needing to clean the final products

before sending them to market (Gordon King and Sharon Chudy, pers. comm.). Approximately 77% of the aforementioned 101 million USD of revenue from mussel and oyster aquaculture in Oregon and Washington is attributed to production costs (Washington Sea Grant 2015), and conservative estimates put the expense of biofouling at 5-10% of production costs (Fitridge et al. 2012).

The objective of this study is to determine what, if any, impact *B. violaceus* has on the growth, condition index, and organic composition of the meat of two bivalves commonly grown using longline aquaculture methods. I hypothesized that *B. violaceus* would significantly slow the growth, deplete the condition, and alter the organic composition of the meat of its basibionts *C. gigas* and *M. trossulus*. To test these hypotheses, I deployed experimental lines of *C. gigas* and *M. trossulus* with varying levels of fouling cover in the Inner Boat Basin of the Charleston Marina. After four months, I evaluated the effects of fouling on the individual using several morphometric measurements. Though mussels are not currently actively farmed in Oregon, the location of the present study, they are grown in Washington; therefore, results of this investigation are of regional relevance to shellfish growers in the Pacific Northwest who are concerned with both the economic and potential ecological effects of *B. violaceus* overgrowth.

Methods

Study Site

This experiment occurred on boat slips I-81 and I-83 in the Inner Boat Basin (IBB) of the Charleston Marina in Oregon (Figure 11, 43°20'47.3"N 124°19'39.4"W). I selected these docks because *Botrylloides violaceus* is well-known to grow there profusely, including on live *Mytilus trossulus* mussels (pers. obs.). Further, I chose an

area away from recreational crabbing hotspots to avoid the lines being pulled up or tampered with by a curious crabbers. These docks are near the mouth of the Coos Estuary, but a large sand and rock breakwater protects them from strong tidal currents and wave action during storms (Marshall et al. 2006). The fouling community in the Charleston Marina is diverse, a result of high traffic from recreational and commercial boats.

Specimen Collection

For the sake of time and simplicity, I chose bivalve individuals that would reach market size by the end of the four-month experiment rather than seeking bivalves of multiple size classes. Qualman Oyster Farms, located in the South Slough Estuary (Fig. 11; 43°20'07.9"N 124°19'08.0"W), grew and supplied the *Crassostrea gigas* (hereafter 'oysters'; n = 135, 44.0 ± 7.56 mm [$\overline{x}$ ± SD]) used in this study. Qualman Oyster Farms uses intertidal stake oyster aquaculture methods to grow oysters from spat to market. Presently, *Mytillus* spp. are not cultured for commercial sale in the Coos Estuary, so are not available as sub-adults locally, but are commonly raised in California and Washington; I therefore collected *M. trossulus* (hereafter 'mussels'; n = 150, 45.0 ± 2.83 mm [$\overline{x}$ ± SD]) from boat slip I-85 in the IBB. I cleaned the live, single mussels and oysters of epibionts and stored them in an aerated raw water flow-through sea table at the Oregon Institute of Marine Biology (OIMB) in Charleston, OR for a week prior to deployment. While mussels and oysters grown in a longline setting are traditionally grown in clumps, this experimental design removed the potential interaction of intraspecifics—thereby isolating the bivalve-*B. violaceus* relationship.

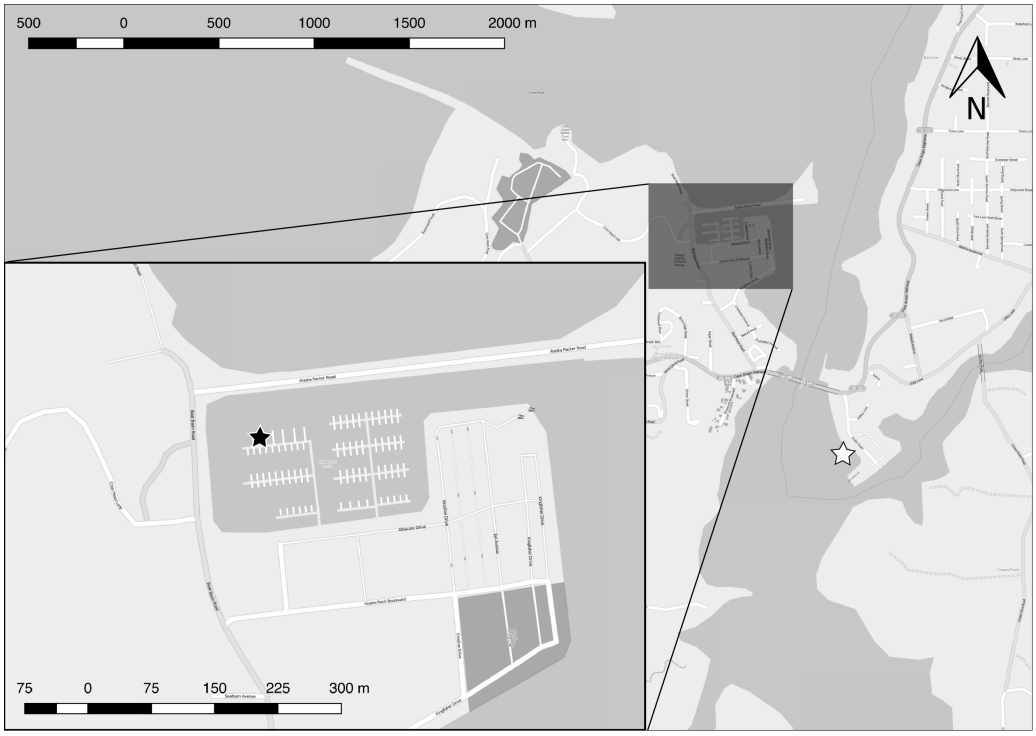

Figure 10. Map of study area in the Charleston Marina, Oregon, depicting ★ initial mussel collection site and experimental line deployment and ☆ initial oyster collection site, Qualman Oyster Farms. Figure rendered in QGIS (QGIS Development Team 2018).

Experimental Setup

Oysters

On 13 September 2017, I randomly sorted the oysters into groups (n =15 groups, n = 9 individuals per group). Using a VWR® digital caliper and E-Series Balance, I measured the length (mm), width (mm), and wet weight (g), respectively, of each individual and ensured equal variances of these measurements across groups. Next, I used Splash Zone® 2-Part Epoxy Compound to attach the oysters (n = 9) to 50 cm pieces of 0.635 cm-diameter PVC pipe (n =15). Recording the placement of oysters on the pipe (Fig. 12a) allowed me to track each individual through the experiment's entirety. After the epoxy cured overnight in the sea table, I randomly assigned each oyster-seeded pipe one of three treatments (n = 5 replicates per treatment): "control," in which I regularly

removed epibionts; "ambient," in which fouling was left unchecked; and "*Botrylloides*," in which I seeded the oysters with *B. violaceus* colonies. I used Gorilla® Super Glue Gel to facilitate initial colony attachment of colonies to the treatment oysters. The tunicate colonies established their own attachment to the treatment oysters in approximately 48 hours.

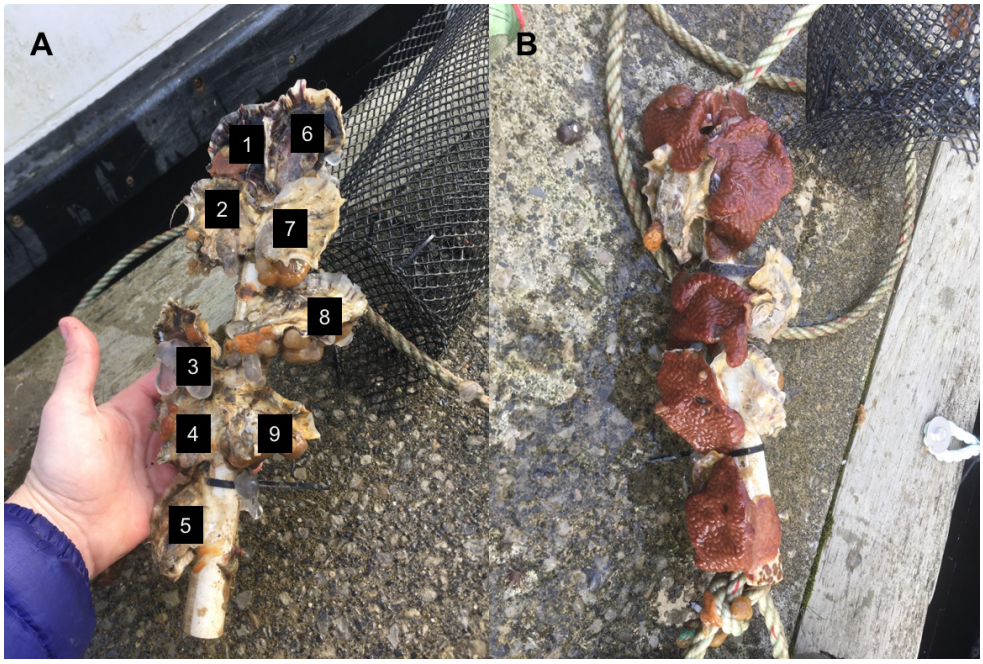

Figure 11. Photos of oyster lines with **A)** ambient and **B)** *Botrylloides* fouling treatments and plastic mesh cages on the side. Oysters were positioned and numbered on each line as shown in **A)**.

To deploy the oyster lines, on 15 September 2017 I tied a piece of line to a brick to act as a weight, then threaded the line through the PVC pipe. I bolted all 15 lines to dock I-81 in the Inner Boat Basin of the Charleston Marina (Fig. 11, 43°20'47.3"N 124°19'39.4"W) so that each line was hung 60cm away from its neighbor and the oysters were submerged 2.5m in the water column (Fig. 13). Every two weeks, I checked the "*Botrylloides*" treatment oysters to ensure they were still fouled. If the *B. violaceus* colonies had receded (Fig. 13B), I attached new colonies freshly transplanted from

nearby boat slips. The objective of this methodology was to ensure a 'worst case scenario' fouling response. I measured the length (mm) and width (mm) of each individual approximately every 30 days for 120 days. After the first 30 days oyster mortality was low, but some had been preyed upon, so I applied predator exclusion cages to the lines. On 13 January 2018, I collected the oysters from the lines and put them an aerated flow-through sea table filled with 5µm-filtered sea water for 48 hours to allow them to clear their gut contents before processing.

Mussels

I collected the mussels on 25 August 2017, then randomly sorted them into 15 groups (n = 10 individuals per group) and measured them as aforementioned. Again using Gorilla® Super Glue Gel, I encouraged mussel attachment to similarly-sized PVC pipes by gluing their byssal threads to the pipe. Because mussels can migrate—albeit limited—over their position on the pipe, I did not track individuals through the entirety of the experiment. During the first attempt at this procedure I labelled each individual mussel using a tag and glue (Betterbee® queen bee marking kit and Kiss® nail glue), which would have allowed the identification of individuals even if they moved on the pipe. Unfortunately, within 50 days of initial deployment (27 August – 10 October 2017), the majority of the mussels perished from predation, prompting a swift reboot during which I did not have sufficient time to tag the new mussels. After I attached the new mussels to the pipes, I assigned each one of the three treatments and attached *B. violaceus* colonies to the "*Botrylloides*" treatment as stated above.

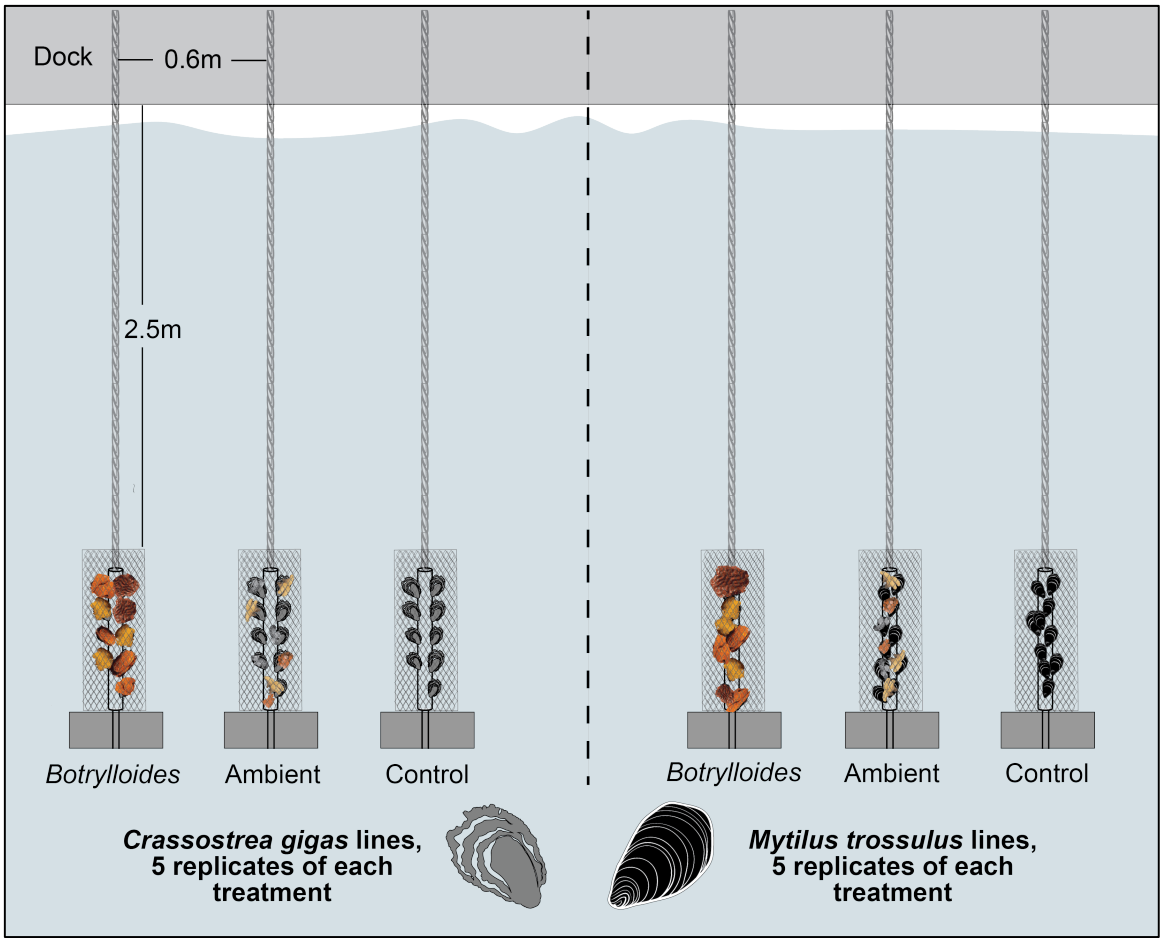

Figure 12. Schematic of field experiment. Lines are spaced 0.6 m apart and the cages are 2.5 m deep in the water column.

Following the oyster deployment procedure, on 16 October 2017, I again hung the mussel lines on slip I-83—this time, with predator exclusion cages to prevent mortality (Fig. 13). Whenever I checked on the oyster lines to ensure *B. violaceus* colony cover, I also checked the mussel lines for the "*Botrylloides*" treatment. I measured the length (mm) of the mussels at 33, 83, and 121 days post-deployment. The notable break from the oyster procedure in measurement frequency is a response to the low growth observed in the first 33 days; I anticipated that taking measurements less frequently would be a more efficient use of time. At the time of each measurement, I noted the mussel mortality per line. On 18 March 2018 I retrieved the mussels from the IBB and allowed them to

clear their guts for 48 hours in an aerated, 5µm-filtered flow-through sea table at OIMB prior to processing.

Bivalve Processing

The majority of this procedure applies to both oysters and mussels, though note that I processed them on different dates (15 January and 20 March 2018, respectively). I cleaned individual bivalves of any epibionts, then recorded its length, width (and in the case of mussels, thickness), and wet weight of each. I opened the two valves and excavated the soft tissue from the shell and carefully separated it into two parts: gut and gonad, and other tissue (mantle, adductor muscle, foot, and gills. I gently dabbed the bivalve tissue samples with a Kimwipe® to remove excess water and recorded the mass of both parts before placing them into labeled 20mL glass scintillation vials. After freezing the samples at -80ºC for a minimum of four hours, I dried them in a Labconco® 2.5-L FreeZone freeze drier at -50ºC for at least 48 hours. I recorded the dry weight (g) of the divided tissue using a VWR® A-Series Balance, homogenized the respective parts into a fine powder using a stainless steel mortar and pestle, and then returned the powder to the scintillation vials in the -80ºC freezer until analysis for organic content. Finally, I recorded the dry mass (g) of the empty shells. Doing so allowed me to calculate the condition indices of both the mussels and the oysters:

$$\text{Mussel Condition Index} = \frac{\text{wet soft tissue weight (g)}}{\text{wet soft tissue weight (g) + shell weight (g)}} \quad \text{(Cartier et al. 2004)}$$

$$\text{Oyster Condition Index} = \frac{\text{total dry tissue weight (g)}}{\text{internal shell cavity volume (ml)}} \times 100 \quad \text{(Hopkins 1949)}$$

I determined the internal shell cavity volume of an oyster by subtracting its dry shell weight from its live and intact wet weight (where the oyster meat and liquors density is 1g cm^{-3}; Lawrence and Scott 1982).

Organic Composition Analyses

Ash Free Dry Weight

To determine the proximal content of the bivalve tissues, I recorded the mass (g) of a small aluminum weigh boat, added approximately ~20g of freeze dried and homogenized tissue to the boat, and recorded their combined mass. I prepared three replicates for each tissue type (n = 4; mussel gut and gonad, mussel other tissue, oyster gut and gonad, and oyster other tissue) within each treatment group (n = 3; control, ambient, *Botrylloides*) for a total of 36 samples. I cooked the samples in a muffle furnace at 450ºC for four hours. Once the samples cooled for 24 hours to room temperature, I recorded the ash free dry weight (AFDW) of the material remaining in the weigh boat and calculated the percent organic content:

$$\% \text{ Organic Content} = \frac{\text{dry sample weight (g)- AFDW (g)}}{\text{dry sample weight (g)}} \times 100\%$$

Carbohydrate

I adapted DuBois et al.'s (1956) protocol for colorimetric carbohydrate analysis for this experiment. Briefly, I used D-glycogen for a carbohydrate standard. From a 0.10mg ml^{-1} stock solution of D-glycogen, with which I prepared a five standard dilution series ranging from 20-200µg ml^{-1}. To prepare the samples, I weighed out ~7 and ~15mg of freeze dried, homogenized oyster and mussel tissue, respectively. I added each tissue aliquot to a 15mL test tube, then digested the tissue with 5mL of a 5% trichloroacetic acid solution in a 60ºC water bath for 60 minutes. After the digests cooled to room temperature, I homogenized them with a vortex. I transferred a respective 200µL, 500µL, and 2mL of the oyster tissue digest, mussel tissue digest, and standard to separate 10mL test tubes. Then, I added 0.5mL of a 5% phenol solution and 5mL of concentrated

sulfuric acid. Once cool, I carefully vortexed the test tubes, distributed the solutions into disposable acrylic cuvettes, and measured the absorbency at 490nm with a Spectronic® 20 Genesys™ spectrophotometer. I tested all samples in triplicate. Using the linear equation derived from the standard curve, I determined the mass (mg) and proportion (mg of carbohydrate : mg of original sample digested; %) of carbohydrate (mg) in the sample.

Protein

I analyzed protein content using a modification of Bradford (1976). Briefly, I transferred ~10mg of freeze dried and homogenized tissue to a 10mL test tube, then added 5mL of 1N NaOH to each sample. I vortexed each test tube before allowing the samples to digest for 24 hours. The next day, I reconstituted bovine albumin serum (BSA) with a 0.15M NaCl solution to a concentration of 0.5mg BSA ml^{-1}. With that stock I prepared a six-sample dilution series ranging from 5-30µg ml^{-1}, using 0.15M NaCl solution as the solvent. I transferred a 100µL aliquot of each standard solution into three 10mL test tubes each, to which I added 1mL of filtered Bradford reagent dye; I repeated this step for the sample digests, but instead used a 20µL aliquot rather than 100µL. After allowing the aliquot-dye solution to incubate for 5-45 minutes, I used the same spectrophotometer the measure the absorbency of the samples at 595nm. I determined the mass (mg) and proportion (mg of protein : mg of original sample digested; %) of protein in the sample from the standard curve's linear equation.

Lipid

I analyzed total lipid using a protocol modified from Heissenberger et al. (2010). For each sample, I placed ~20mg of freeze dried tissue into a 10mL centrifuge tube, added 3mL of a 2 $CHCl_3$: 1 MeOH solution, and sealed the mixture with N_2 gas.

Following a short vortex, I sonicated the samples in an ice bath for ten minutes, added 0.75mL of a 0.9% NaCl solution, sealed the solution under N_2 and vortexed it again, then placed it in a centrifuge at 3000rpm for five minutes. To transfer the bottom $CHCl_3$ lipid extract layer into an 8mL scintillation vial, I used a double Pasteur pipette technique. I added an additional 2mL of $CHCl_3$ to the centrifuge tube and repeated the vortex, sonication, centrifuge, and double pipette steps. Once the lipid extract layer in the scintillation vial evaporated down to 1.5mL under N_2 gas, I transferred 1mL of it to a fresh 10mL centrifuge tube and finished extracting the fatty acids for future analysis. Using simple gravimetry, I determined the mass of the lipids in the extract: I transferred 80µL of the extract into two tins pre-weighed on a Mettler Toledo XPR2U microbalance, allowed the liquid to evaporate overnight, and re-weighed the tins. The difference in weight was total lipid weight (mg), with which I could calculate the proportion (%) of lipids in the original sample tissue. I tested n = 5 organisms each from the control and *Botrylloides* fouling treatments.

Statistical Analyses

I used RStudio (v. 1.1.414; RStudio Team 2018) for all statistical analyses. Using simple point and whisker plots, I visualized bivalve size by treatment and mussel mortality over the four-month study period. To observe the condition indices in more detail, I plotted them as box and whisker plots—where the whiskers are the interquartile range (IQR). I also used point and whisker plots to depict the proportions of total carbohydrates, proteins, and lipids in the 1) gut and gonad and 2) other tissue for oysters and mussels across treatments. Coupled with the knowledge of those masses relative to the total tissue dry mass, I calculated the overall nutrient (i.e., carbohydrate, protein, and

lipid) composition of both bivalves. I used a nested ANOVA design to evaluate whether or not fouling treatment explained any variance in the above observations, where line number (1-15) was nested inside each of the three fouling treatments. In some cases, bivalve mortality and subsequent low subreplicate abundance (< 5) on some lines rendered them unusable as a replicate within the nested design. Thus, for the oyster nested ANOVA analyses I had n = 4, 5, and 4 line replicates for treatments control, ambient, *Botrylloides*, respectively; following the same respective order, I used n = 4, 4, and 5 line replicates for the mussel nested ANOVAs. The data did not violate the assumptions of these analyses and thus did not require any transformation.

Results

Bivalve Growth and Condition

At each checkpoint, *B. violaceus* covered 50% or more of the *Botrylloides*-fouled bivalves, and this coverage was generally concentrated around the ventral portions of the shells. The fouling treatment (control, ambient, *Botrylloides*) did not significantly influence the final lengths (mm; $p = 0.285$) or masses (g; $p = 0.741$) of the oysters (*Crassostrea gigas*), nor did it explain the individual oysters' change in length ($p = 0.612$) or wet weight ($p = 0.646$; Table 6). Likewise, the fouling treatment did not significantly impact the end lengths or wet weights of the mussels (*Mytilus trossulus*; $p = 0.085$ and 0.076, respectively). Because I did not track the mussel to the individual, I was not able to report their change in length and wet weight. While the mean oyster length increased by nearly 30mm—and significantly—over the course of the experiment (Fig. 14a), the mussels did not increase their length significantly (Fig. 14b). Moreover, the oysters and mussels fouled ambiently or with *Botrylloides* were not significantly different

in length compared to their respective controls at any day on which I took measurements (Fig. 14a, b).

Table 6. Summary of nested ANOVAs, where four or five replicate lines are nested inside of one of three fouling treatments for both **A)** *C. gigas* and **B)** *M. trossulus*.

	F	***df***	***p***
A) *Crassostrea gigas*			
change in length (mm)	0.515	2,10	0.612
change in mass (g)	0.457	2,10	0.646
end length (mm)	1.43	2,10	0.285
end wet weight (g)	0.309	2,10	0.741
Hopkin's condition index (g mL^{-1}*100)	1.36	2,10	0.301
carbohydrate (%) – divided tissue†	14.0	5,62	<0.001*
carbohydrate (%) – whole tissue	0.294	2,10	0.752
protein (%) – divided tissue	16.6	5,62	<0.001*
protein (%) – whole	1.07	2,10	0.378
lipid (%) – divided tissue	19.0	3,8	<0.001*
lipid (%) – whole	0.020	1,8	0.892
B) *Mytilus trossulus*			
end length (mm)	3.20	2,10	0.085
end wet weight (g)	3.38	2,10	0.076
condition index	0.214	2,10	0.811
carbohydrate (%) – divided tissue†	1.29	5,70	0.277
carbohydrate (%) – whole	1.11	2,12	0.360
protein (%) – divided tissue	4.15	5,70	0.002*
protein (%) – whole	0.075	2,12	0.930
lipid (%) – divided tissue	10.8	3,9	0.002*
lipid (%) – whole	2.53	1,8	0.150

*p-values significant at the α <0.05 level; † "gut and gonad" and "other tissue"

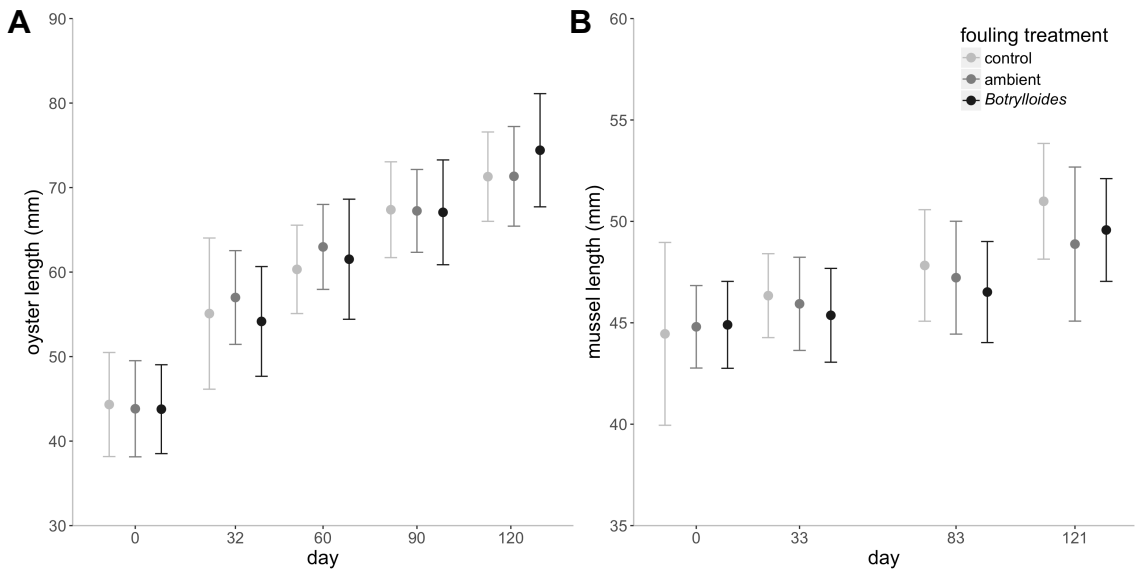

Figure 13. Lengths (mm) of **A)** *C. gigas* and **B)** *M. trossulus* throughout the experiment. Error bars are ± 1 SE (propagated).

There was also no apparent effect of fouling treatment on oyster and mussel condition indices (nested ANOVAs $p = 0.301$ and 0.811, respectively; Table 6). The *Botrylloides*-fouled oysters' Hopkin's condition index (g mL^{-1}*100) was slightly lower than that of oysters in the control treatment group (interquartile range [IQR] = 5.81-6.31 and 6.42-6.73, respectively), though not significantly (Fig. 15a). And though the median *Botrylloides*-treated mussels' condition index was slightly higher than that of the other treatments, their IQR fell within the control mussels' IQR (Fig. 15b).

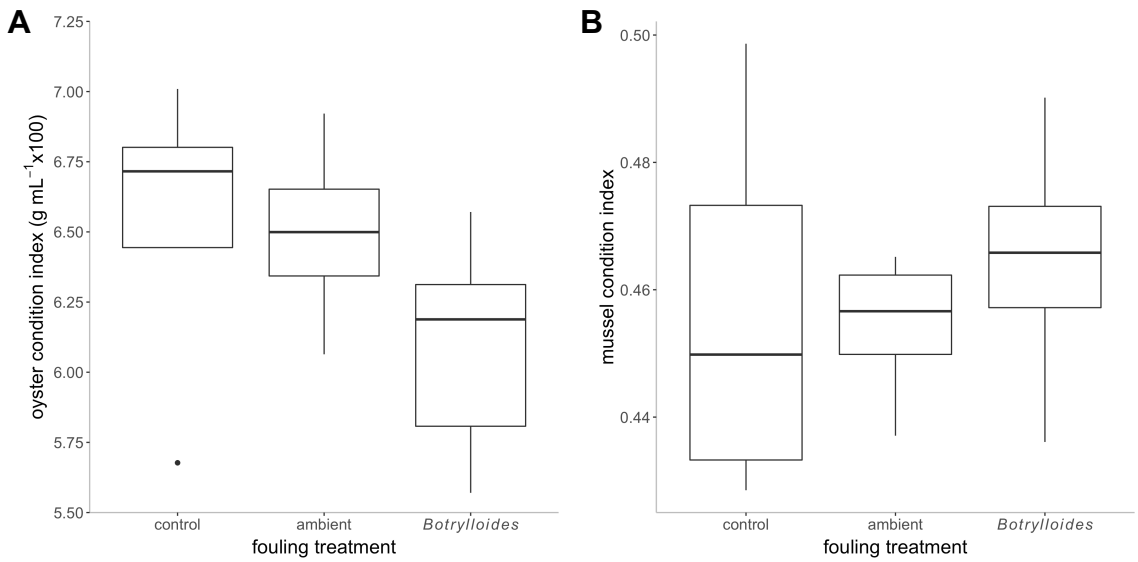

Figure 14. Condition indices for **A)** *C. gigas* and **B)** *M. trossulus* by fouling treatment. Middle bar of each box corresponds to median; upper and lower box limits represent the first and third quartiles, respectively, while the whiskers indicate IQR and single points are outliers.

Approximately half of all mussels in the *Botrylloides* and control fouling treatments survived the experiment (0.52 ± .04 and 0.54 ± 0.07 proportion survivorship, respectively), while the ambient fouling lines experienced significantly less mortality (0.64 ± 0.02 proportion survivorship). Survivorship was not otherwise significantly different between treatments at days 33 and 83, and mortality was greatest between days 83 and 121 (Fig. 16). Predation was not—at least visibly—the cause of mortality (i.e., no shells were crushed or pried open). No oysters died after the addition of the predator exclusion cages.

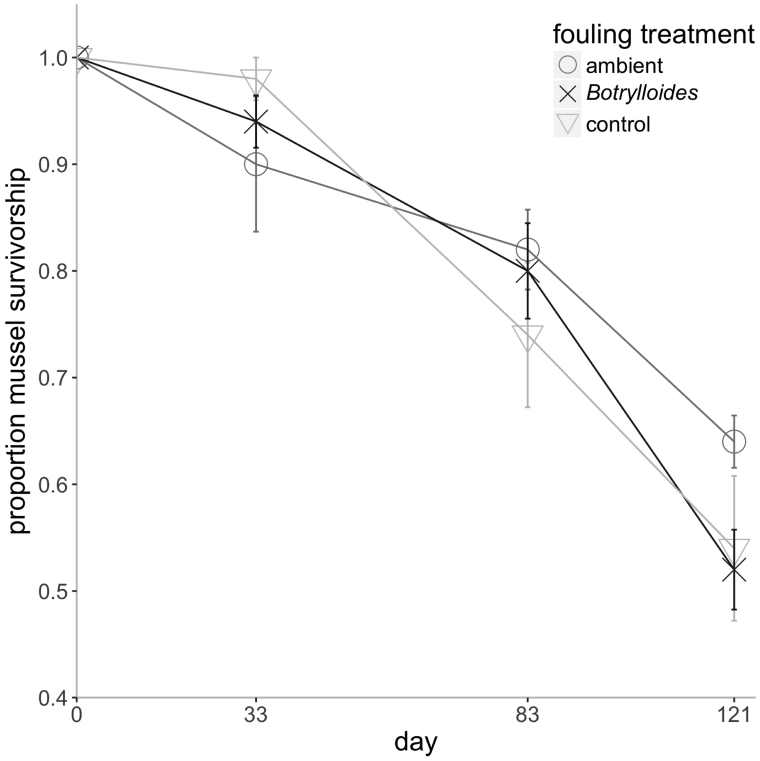

Figure 15. Survivorship of mussels by fouling treatment throughout the study. Error bars are ± 1 SE.

Bivalve Tissue Composition

Organic materials composed 86.6 ± 0.78% of the total oyster tissue mass and 91.9 ± 0.27% (mean ± SD) of the total mussel tissue mass. The sum of the organic constituents accounted for ~40% and ~60% of the organic tissue mass for oysters and mussels, respectively. The carbohydrate content of oyster gut and gonad tissue was approximately double than that of the rest of its soft tissue (~10 and ~5% by mass, respectively; Fig. 17a). Though these measurements were significantly different ($p < 0.001$), fouling treatment had no effect on the proximal composition of each tissue type. Similarly, treatment had no effect on lipids within the two tissue types, though oyster gut and gonad contained significantly more lipids by mass (~15%) than its other tissues (~10%; $p < 0.001$; Fig. 17a). Fouling treatment did, however, impact the protein composition of

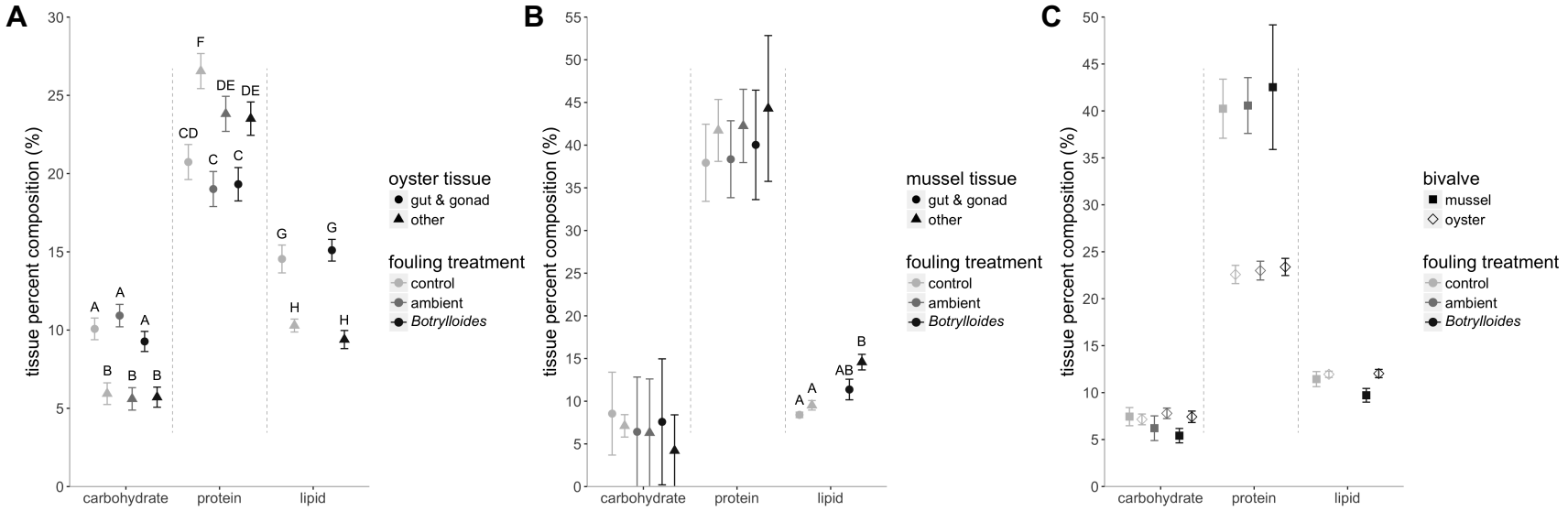

Figure 16. Proportional composition of carbohydrates, proteins, and lipids (% of dry mass) for **A)** *C. gigas* and **B)** *M. trossulus* separated into 'gut and gonad' and 'other' tissues. Combined, whole-body tissue compositions reported in **C)**. Letters are nested ANOVA post-hoc Tukey labels of significance for values within the same nutrient assay, or between the dashed lines, and are not repeated within plots for convenience—e.g., in **A)** 'A' and 'B' are significantly different from each other, though are not to be directly compared with 'G' and 'H', which are also significantly different from each other. Error bars are ± 1 SE.

'other' oyster tissue: the control treatment had a significantly greater protein composition (26.5 ± 2.6%) than the ambient (23.2 ± 3.1%) or *Botrylloides* (23.5 ± 2.4%) treatments. The control oysters' gut and gonad protein composition was slightly less than that the latter two values (20.8 ± 4.1%), and slightly greater than the gut and gonad tissue of ambiently- (19.0 ± 2.4%) and *Botrylloides*-fouled (19.3 ± 2.8%) oysters—though it was not significantly different from either of these two groups (Fig. 17a).

A different pattern emerged for mussels. The carbohydrate content proportion by mass was not significantly different between their gut and gonad tissue and the rest of their soft tissues ($p = 0.277$; Fig. 17b). While the nested ANOVA did reveal a significant difference within the mussel protein proportions (Table 6), fouling treatment had no effect (Fig. 17b). Further, the 'other' tissue from mussels in the *Botrylloides* treatment group contained a significantly greater proportion of lipids (14.6 ± 0.9%) than the control group (9.5 ± 0.6%). No significant difference occurred in the lipid content of mussel gut and gonad tissue (Fig. 17b).

Overall, mussels and oysters contained approximately the same proportion of carbohydrates (~5-7%) and lipids (~10-12%) by mass (Fig. 17c). Mussels did, however, contain proportionally twice as much protein as oysters (~40 and ~22%, respectively). Fouling treatment did not significantly impact the proportional content of carbohydrates ($p = 0.752$), proteins ($p = 0.378$), or lipids ($p = 0.892$) in oysters nor mussels ($p = 0.360$ 0.930, and 0.150, respectively; Fig. 17c).

Discussion

During the independent four-month study periods, the epibiont *Botrylloides violaceus* did not significantly inhibit the growth (measured by length [mm] and mass

[g]) of the oysters (*Crassostrea gigas*) and mussels (*Mytilus trossulus*) it fouled (Table 6; Fig. 14a, b); this invasive colonial tunicate did not depress the condition indices or alter the organic nutrient composition of the overall tissue of these bivalves, either (Fig. 15a, b; Fig. 17c). These findings refuted my *a priori* hypotheses. Therefore, despite the recognized threat that *B. violaceus* and other associated invasive colonial tunicates (e.g., *Didemnum vexillum*) pose to global shellfish aquaculture operations (Carver et al. 2006; Daniel and Therriault 2007; Valentine et al. 2007b; Fitridge et al. 2012), there remains a dearth of empirical evidence for the notion that *B. violaceus* directly impacts shellfish productivity. Rather, the data in the present study support mussel and oyster growers' observations—the threat of colonial tunicate biofouling is primarily to production costs, not the bivalves themselves.

A mass mortality event occurred in the first mussel experiment attempt in August 2017, which caused me to reset the experiment. This mortality was likely due to sea stars and crabs capitalizing on what was quite literally low-hanging fruit, evidenced by frequent personal observations of those organisms preying on the IBB fouling community, as well as crushed shells and the disappearance of shells from the lines altogether. Notably, I observed that the *Botrylloides*-fouled treatment lines incurred less predation than the control and ambient treatment lines. I did not quantify this observation at the time, but it does provide anecdotal support for the idea that *B. violaceus*, as an epibiont, may deter predator from consuming the organisms it fouls (Laudien and Wahl 2004; Epelbaum et al. 2009b). This observation may warrant further study designed to quantify whether *B. violaceus* fouling acts as a predation deterrent.

Adding exclusion cages proved successful in preventing predation in both mussels during the second experimental attempt and oysters. The mussels still incurred considerable mortality—though this mortality was not due to fouling treatment, as the control and *Botrylloides* treatment groups' survivorships were not significantly different at the end of the mussel experiment (Fig. 16). The low mussel survivorship is nevertheless perplexing, as *M. trossulus* grows abundantly elsewhere in the IBB, including on the dock slips at which the experiment took place. It is possible that in a longer study period the difference in control- and *Botrylloides*-treated mussel survivorship may become more pronounced, given that after 121 days the ambient treatment group survivorship was significantly greater than the other two treatments (Fig. 16).

The bivalves that did survive the four months in the IBB did not exhibit significantly different growths by treatment (Fig. 14a, b). At the field experiment's terminus, the oysters had nearly reached the market size of ~76mm (Calvo et al. 1999). The *C. gigas* growth rate (~30mm over four months) was comparable to what has been reported in the literature for this organism globally (Cotter et al. 2010), as was its condition index (Brown and Hartwick 1988; Fig. 14a; Fig. 15a). Conversely, the mussels did not exhibit significant growth, falling ~5mm short of reaching their ~55-60mm market size (Mallet and Carver 1995; Fig. 15b). The condition indices of *M. trossulus* in the present study were slightly lower (~0.1; Fig. 15b) than the condition indices of *M. trossulus* in Atlantic Canada (Hellou and Law 2003; Cartier et al. 2004). My mussels did, however, grow at a comparable rate to *M. trossulus* of the same starting size class (~45mm) from Nova Scotia (~38µm/day; Mallet and Carver 1995). Unfortunately, few

comparable adult *M. trossulus* and *C. gigas* growth and condition index data are available on the North American West Coast for direct regional comparison. Regardless, the similarities between the mussel and oyster aquaculture data that are available and the data presented here suggest that this experimental design successfully mimicked longline aquaculture enough for the results to be applicable on a larger scale.

B. violaceus did not significantly impact the condition indices of its bivalve basibionts; this finding is concurrent with Fletcher et al.'s (2013b) conclusion that *D. vexillum* had no impact on the condition index or growth of farmed *Perna canaliculus*. Those authors did report, though, that *D. vexillum* decreased the density of *P. canaliculus* via displacement. Indeed, this pattern might suggest that these bivalves, who commonly fill the intraspecific ecological role of both epibiont and basibiont (and sometimes concurrently), are in fact well-adapted to dealing with epibiosis. On the other hand, my findings contradict those of Auker (2010), who showed that *D. vexillum* negatively influenced several *M. edulis* parameters, including growth and condition index. It is thus unlikely that *B. violaceus* inhibited *C. gigas*' and *M. trossulus*' feeding in the way that Auker (2010) suggested *D. vexillum* did to *M. edulis*. The organic nutrient composition data support this inference.

The overall mussel protein, carbohydrate, and lipid content (% by dry mass) values I observed were comparable to those published for *M. trossulus* collected during the same time of year at Yaquina Bay, OR (Kreeger 1993). There were again no regional organic composition data with which I could compare my oyster observations, but they aligned with the organic nutrient composition of other published profiles of *C. gigas* (e.g., Dridi et al. 2007; Pogoda et al. 2013). Importantly, fouling treatment did not

significantly impact the overall nutritional content of the mussels and oysters grown during this experiment (Fig. 17c). Fouling did depress the protein content (% by dry mass) of oyster 'other' tissue; but, because there was no significant difference between the ambient and *Botrylloides* fouling treatments, this impact cannot be attributed to *B. violaceus* alone (Fig. 17a). Somewhat counterintuitively, the 'other' tissue from *Botyrlloides*-fouled mussels had a significantly higher lipid content than the control (Fig. 17b). While not impossible that *B. violaceus* provided some trophic benefit to its mussel basibiont, a more likely explanation for this finding is the low sample size for this assay (n = 5). Future analyses of the fatty acid data from the extractions I performed on these samples may tease out the nuances of this finding.

Notably, in the aforementioned *C. gigas* and *M. trossulus* growth, condition index, and tissue organic composition literature, the authors consistently found significant differences in these parameters across seasons (Cotter et al. 2010; Mallet and Carver 1995; Hellou and Law 2003; Cartier et al. 2004; Kreeger 1993; Dridi et al. 2007; Pogoda et al. 2013). Coupling those observations with the knowledge that *B. violaceus* also exhibits significant seasonal fluctuations in growth (Carver et al. 2006; Dorning 2017b) begs the question: does *B. violaceus* have negative impacts on the *M. trossulus* and *C. gigas* at any point throughout the year? The present study offers only a snapshot into the impacts of *B. violaceus* on these basibionts; a year-long field experiment with multiple bivalve class sizes would aid in a more comprehensive evaluation of these invasive tunicate-commercial bivalve relationships.

Conclusions

This study contributes the following to the literature: 1) the first empirical data demonstrating that the invasive colonial tunicate *B. violaceus* has no impact on the growth, condition, or organic composition of the oyster *C. gigas* and the mussel *M. trossulus* grown in a longline aquaculture-like setting; and 2) to my knowledge, documentation of the protein, carbohydrate, and lipid content of *C. gigas* in Oregon for the first time, and of *M. trossulus* in Oregon for the first time since 1993. I report the former findings with cautious optimism, as the scope of this project was narrow—focusing only on one size class of both oysters and mussels. These data affirm the observations of mussel and oyster growers who claim that *B. violaceus* and other invasive colonial tunicates do not impact the bivalves directly, but are rather a threat to the aquaculture operations' infrastructure. I recommend that invasive tunicate-bivalve epibiont-basibiont relationships continue to be investigated, especially considering the erratic fluctuations some invasive tunicate populations exhibit (Chapter II, this thesis), and that many aspects of continued global change are predicted to favor invasive species.

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