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Advancing Development of Porphyra umbilicalis as a Red Algal Model System and Aquaculture Crop

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ADVANCING DEVELOPMENT OF *PORPHYRA UMBILICALIS* AS A RED ALGAL MODEL SYSTEM AND AQUACULTURE CROP

by

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Thesis advisor: Dr. Susan H. Brawley

An Abstract of the Thesis Presented
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The marine red alga *Porphyra umbilicalis* (Rhodophyta, Bangiaceae) has ideal traits to allow it to become a model organism, including its economic value, reproduction in the northwestern Atlantic through asexual neutral spores (NS), and availability of fully-sequenced nuclear and organelle genomes. Research on the bacterial component of the *Porphyra* microbiome is ongoing. To advance model organism development and support microbial studies, data on natural reproductive trends and early embryonic development are needed, along with a system for genetic transformation, and ways of visualizing the attached microbial community. To meet these needs, two years of phenological data were analyzed, revealing seasonal reproductive trends and some location-based effects. Early development of *P. umbilicalis* from neutral spores is mostly linear. Blades bearing neutral spores were frozen (-20 ºC) for 4 weeks to determine if freezing influences early development. Freezing had minor effects on early development compared to the rate and pattern of germination from NS in untreated controls. Attempted genetic transformation of *P. umbilicalis* using biolistics was successful, but whether it was a stable transformation is unknown; hygromycin B was demonstrated to be an

effective antibiotic to use in selection of transformants. Use of scanning electron microscopy (SEM) revealed that microbial communities on the surface of *P. umbilicalis* are often diverse, complex, and vary between different groups of specimens. Together, these studies support continuing development of *P. umbilicalis* as a model organism and a valuable aquaculture crop.

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CHAPTER 1

INTRODUCTION

The red alga *Porphyra umbilicalis* is part of an ancient eukaryotic lineage (Butterfield, 1990), a wild-harvested human food that is a promising aquacultural food crop (Rhatigan, 2009), is available as a fully sequenced nuclear genome (Prochnik et al., 2017), and a macroalgal model for studies of the macroalgal microbiome (Miranda et al. 2013; Kim et al., 2016). The research presented here sought to further development of *P. umbilicalis* as a model organism in these areas by accomplishing the following goals: 1) determining the reproductive seasonality of *P. umbilicalis* at three sites in Maine, 2) testing the effects of frozen storage of blades on development of embryos developing from asexual spores, 3) developing a system for genetic transformation using particle bombardment, and 4) supporting studies of this alga's microbiome by documentation of the coverage and microarchitecture of bacteria and other microbes on the blade of *P. umbilicalis* with scanning electron microscopy.

Systematics of the Rhodophyta

Marine algae in the genus *Porphyra* are members of the family Bangiaceae, in the class Bangiophyceae of the phylum Rhodophyta (Sutherland et al., 2011). The rhodophytes evolved from (a) primary endosymbiotic event(s) in which a free-living cyanobacterium was engulfed by a heterotrophic eukaryote; the cyanobacterium was not digested and evolved into the chloroplast of the red algal cell (Keeling, 2010; Knoll, 2011; Burki et al., 2016, Graham et al., 2016, Hug et al., 2016;). The Archaeplastida supergroup includes glaucophytes and the red and green algal lineages, but the sister group of the red algae within this lineage is debated. Earlier phylogenetic studies

indicated that glaucophytes diverged from the Archaeplastida ancestor before divergence of red and green algae, which have been considered to be sister lineages (Yoon et al., 2006; Qiu et al., 2015). Recent research, however, has challenged this placement (Burki et al., 2016). Newly available genomic sequences for 4 centrohelids (phylum Sarcodina) were used to assemble an updated eukaryotic phylogenetic tree. This analysis indicated that the rhodophytes had diverged before green algae and glaucophytes, tentatively placing the latter two groups as sister lineages. This is an active, continuing area of research.

Fossil evidence confirms that red algae in the Bangiophyceae are ancient organisms. A 1.2 Ga fossil of *Bangiomorpha pubescens* (Butterfield, 1990) has a remarkable similarity to an extant bangiophyte, *Bangia atropurpurea*. This fossil is the oldest taxonomically-resolved multicellular eukaryote known. Recently, even older (1.6 Ga) multicellular red algal fossils were discovered (Bengtsson et al., 2017). These could be bangiophyte sporophytes, but they could also belong to one of two other rhodophyte classes that also have pit plugs. Another fossil bangiophyte, *Paleoconchocelis starmachii*, was dated to 425 Myr (Campbell, 1980), and closely resembles a modern *Porphyra* sporophyte ("conchocelis"). The combination of this fossil evidence and the new support for the early divergence of the rhodophytes make studies of *Porphyra* and related species especially relevant to understanding eukaryotic evolution and development.

Porphyra umbilicalis

Porphyra umbilicalis Kützing is found on rocky substrates in the high-mid intertidal zone throughout its range in the North Atlantic (Fig. 1.1, Brodie et al., 2008; Teasdale and Klein, 2010; Guiry and Guiry, 2015). Populations of *P. umbilicalis* in the eastern Atlantic in Europe and England are known to reproduce both sexually and asexually (Brodie and Irvine, 2003). Sexual *P. umbilicalis* exhibits the typical bangiophyte alternation of generations (Drew, 1949; Graham et al., 2016). The haploid gametophytic phase is a monostromatic, foliose thallus, usually with separate male and female individuals. After fertilization, zygotospores settle and grow into the filamentous sporophyte (2N), and they burrow and grow within calcareous substrates such as mollusc shells (Fig. 2A). The sporophyte was originally classified as a separate species, *Conchocelis rosea*, until Kathleen Drew Baker demonstrated that it was the sporophyte of *P. umbilicalis* (Drew, 1949). This discovery revolutionized aquaculture of Japanese nori (*Pyropia yezoensis*), creating an industry that is worth 1.5 billion USD today (www.fao.org). *Porphyra umbilicalis* in the western Atlantic, however, is only known to reproduce asexually (Fig. 2B, Blouin, 2007; Blouin & Brawley, 2012). Cells at the blade margin develop into neutral sporangia, which divide mitotically to form packets of asexual neutral spores surrounded by mucilage. Rewetting of dried thalli causes release of neutral spores into the seawater. These spores settle and germinate, growing directly into new gametophytes; no sporophyte seems to be present in the northwestern Atlantic portion of the Atlantic metapopulation of *P. umbilicalis*. This is an advantageous trait for development of this alga as an aquaculture crop, because economically-costly culture of the sporophytic phase is unnecessary (Blouin et al., 2011).

Figure 1.1 *Porphyra umbilicalis*

A. Photograph of wild *P. umbilicalis* at low tide at Dark Harbour, New Brunswick (Compliments of S. Brawley). The monostromatic thallus grows as a foliose blade, often drying to the rocks during low tide. Upon resubmergence at high tide it will quickly rehydrate. B. Cultured *P. umbilicalis* strung into lines.

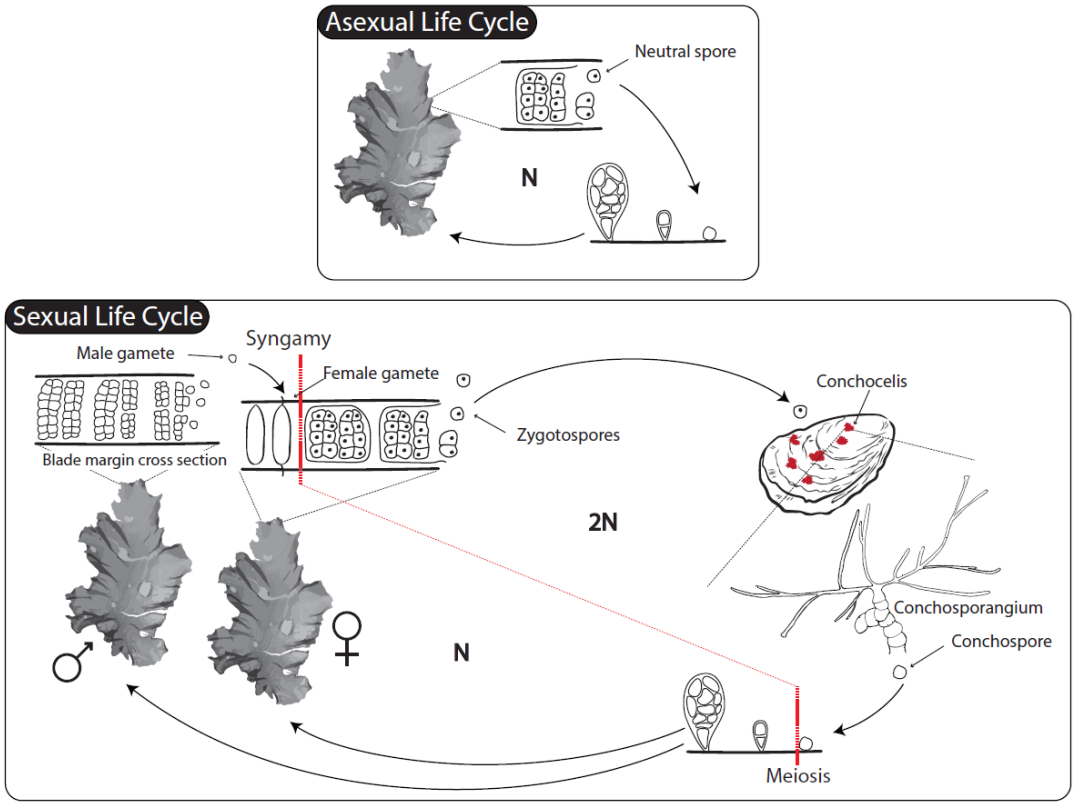

Figure 1.2 *Porphyra umbilicalis* reproductive cycle

Sexual life cycle of *P. umbilicalis* is shown in the bottom panel. Foliose gametophytes are monostromatic and dioecious. After fertilization, zygotospores settle onto calcareous substrates and grow into the filamentous sporophyte ("conchocelis"). Diploid sporophytes release conchospores that settle and divide meiotically, producing new haploid gametophytes. Asexual life cycle of *P. umbilicalis* gametophytes is shown in the top panel. Cells at the edge of the blade margin differentiate into neutral spores via mitosis. Neutral spores are released from the degraded margin, settle, and grow directly into new gametophytes.

Porphyra umbilicalis is affected by many stressors in the intertidal zone, such as desiccation, high light, extreme and rapid changes of temperature, and salinity changes, but it is well-adapted to these conditions. Gametophytes can lose most of their water content every six hours with the tidal cycle, but recover quickly upon resubmergence by the incoming tide. Carotenoids and mycosporine-like amino acids protect the photosystems from excessive visible and UV radiation (Korbee et al., 2005; Blouin et al., 2011; Yang et al., 2014). This alga can withstand a broad seasonal range (-20 to 25 °C) of aerial temperatures at low tide (Lüning, 1990; Blouin et al., 2007) and salinities up to 6 times that of full seawater (Wiencke and Läuchli, 1980). These tolerances make *P. umbilicalis* an ideal aquacultural candidate, because nets with seeded germlings can be frozen for extended periods before outgrowth on farms (as in *P. yezoensis*, Tseng, 1981; Fei et al., 1999; Sahoo and Yarish, 2005).

Years of research have demonstrated that algal morphology, development, and in some cases, stress tolerance, are influenced by the associated microbial epiphytes (Fries, 1975; Provasoli and Pintner, 1980; Morris et al., 2011; Helliwell et al., 2016). Research on the *P. umbilicalis* microbiome is an active subject (Miranda et al., 2013, Kim et al., 2016), but the community associations and microarchitectures of bacteria on the blade are unknown. Understanding community organization is an important facet of this research that needs more investigation. The availability of the assembled nuclear genome (Prochnik et al., 2017) provides another avenue for continuing studies of the effect of bacterial communities on this alga. Some algae that depend on bacterial "helpers" lack some genes or synthetic pathways that epiphytic bacteria provide to them (Morris et al., 2011; Helliwell et al., 2016). Studies of gene function in *P. umbilicalis*, however, are

greatly inhibited by a lack of a genetic transformation system. These systems have been established in several model algal species (reviewed in Qin, 2012), and allow researchers to insert or delete genes of interest. Development of a transformation system in *P. umbilicalis* would advance understanding of the biology of this bangiophyte.

***Porphyra umbilicalis* as a model organism**

Porphyra umbilicalis has ideal traits for becoming a true model organism, due to its easy culture via neutral spores, and a fully-sequenced genome. Continuing studies of its microbiome and interest in genetic function make *P. umbilicalis* the subject of important ecological and evolutionary research, and its economic value make it a commercial aquaculture candidate. My primary goal for this thesis was to continue to develop *P. umbilicalis* as a model organism by investigating its seasonal reproduction and development, establishing a foundation for genetic transformation, and contributing to research on host-microbiome interactions. In Chapter 2, I describe my findings from analysis of two years of phenological survey data on *P. umbilicalis* at three locations in Maine, and pair this with results from my study of the effects of freezing on the development of early germling development. Together these studies contribute to a continuing effort to commercialize *P. umbilicalis* aquaculture in the US. In Chapter 3, I describe the process by which I attempted to transform *P. umbilicalis* using particle bombardment, and the development of an antibiotic selection protocol for this species. In Chapter 4, I present a study of microbial communities on the surfaces of both wild-collected and lab-cultured *P. umbilicalis*, as revealed by imaging with scanning electron microscopy. Chapter 5 contains my final conclusions and my suggestions for further research involving *Porphyra umbilicalis*.

CHAPTER 2

PHENOLOGICAL SURVEY OF WILD *PORPHYRA UMBILICALIS* IN MAINE AND EFFECTS OF COLD STORAGE ON DEVELOPMENT OF YOUNG SPORELINGS

Introduction

Japanese nori (*Pyropia yezoensis)* is valued at 1.5 billion USD per year (www.fao.org), making it the most valuable algal crop in the world. Development of a native *Porphyra* species in the United States is of particular interest because demand for nori and related foods is increasing (Wells et al., 2016). In New England, the red alga *Porphyra umbilicalis* (laver) is a candidate for expanding sea vegetable markets in the US, because laver has a long history of wild harvest across the north Atlantic as human food (Rhatigan, 2011), and *P. umbilicalis* has several advantageous traits for aquaculture. In the northwestern Atlantic, unlike the northeastern Atlantic, laver reproduces only asexually through the production of neutral spores from the blade margin. Neutral spores settle on rocks and grow directly into new blades (Blouin et al., 2007; Blouin et al., 2011). The typical alternation of generations in *Porphyra* sensu lato (Sutherland et al., 2011) between a filamentous sporophyte (2N) and a blade-forming gametophyte (N) through sexual reproduction appears to be absent in *P. umbilicalis* in the northwestern Atlantic (Blouin et al., 2011). This gives *P. umbilicalis* an advantage over other species for aquacultural development because expensive culture of a conchocelis phase is not required (Blouin, 2006; Blouin et al., 2007). In addition, Maine's growing aquaculture industry would benefit from the development of integrated multitrophic aquaculture

systems (IMTA), and laver could be a useful component that would be fertilized by animal wastes, consequently serving as a value-added product that performs bioremediation (Chopin and Sawhney, 2009). *Porphyra umbilicalis* outperforms other New England *Porphyra sensu lato* in terms of nitrogen uptake (Yarish et al., 1998, Carmona et al., 2005; Kim et al., 2007). In these studies, *P. umbilicalis* consistently has the highest rate of ammonia and nitrate uptake of the tested species. This, coupled with neutral spore production, makes *P. umbilicalis* a strong candidate for aquaculture in Maine. Development of seed stock storage and harvesting techniques, and documentation of the reproductive phenology of this species, remain critical next steps to bringing this aquaculture crop to a commercial level in the US.

In the nori aquaculture industry, nets seeded with spores are frozen as a common storage practice (Tseng, 1981; Fei et al., 1999; Sahoo and Yarish, 2005). Freezing live plants bearing neutral spores would extend the viability of seed stocks and possibly provide a benefit over frozen nets, because plants could be stored as soon as spores are produced, eliminating the labor and space required for storing nets. Green and Neefus (2014) demonstrated that *P. umbilicalis* blades (~5.0 mg) could be frozen at -20°C and -80°C for 1, 3, 6, or 12 months with almost no effect on growth when plants were thawed and returned to normal culture conditions. The effects of low temperature storage on tissues bearing neutral spores, and the subsequent growth and development of these spores, however, was not examined.

Porphyra and *Pyropia* conchospores typically follow set patterns of development while undergoing meiosis to produce the first four cells of the blade (see Fig 1.1, Chapter 1). Some species, such as *P. haitanensis*, *P. yezoensis*, and *P. oligospermata*, essentially form a filament of four cells with periclinal divisions of each of the first daughter cells (Ohme and Miura, 1988; Wang et al., 2010; Yan et al., 2010). Other species, such as *P. katadai var. hemiphylla* and *P. purpurea* undergo an anticlinal division of the upper cell of the 2-cell stage, whereas the bottom cell divides periclinally (Mitman and van der Meer, 1994; Wang et al., 2010). In this study, I refer to periclinal divison as "linear" (Yan et al., 2010) and anticlinal division as "bilateral" (Wang et al., 2010).

Wang et al. (2010) found that higher percentages of sporelings of *P. katadai var. hemiphylla* developed as filaments through linear divisions in experimental groups subjected to higher temperatures than those maintained at standard culture temperatures for this species. The pattern of early development from conchospores in *P. umbilicalis* in the northeastern Atlantic appears to be unknown. I wanted to determine what the pattern of early development of germinating neutral spores is in *P. umbilicalis* from Maine, because this could affect selection for cultivars of different shapes, and will contribute to understanding of the Atlantic metapopulation when European phycologists document the division pattern in germinating conchospores.

Understanding the effects of different environmental factors on development of young sporelings is of importance to aquaculture development. Blouin et al. (2007) demonstrated that the spore release of *P. umbilicalis* was highest during the winter months, when freezing of blades at low tide is common, but since this study took place at only one site in Maine and across only one year, additional phenological study is needed.

These investigators also showed that *P. umbilicalis* readily reproduces in water temperatures of approximately 10-12ºC. Establishing a seasonal reproductive cycle across multiple years for wild *P. umbilicalis* would give further insight into seasonal effects of temperature on *P. umbilicalis*, as well as provide useful information to aquaculturists on when to collect fertile blades from different regions of the Maine coast for seed stock.

Recently a *P. umbilicalis* strain with excellent aquaculture potential was identified. This strain was produced from wild stock collected in Lubec, ME in 2015 (strain "CCAR. P.um.2", hereafter as "Pum2"). In the first study, I tracked the daily development of *P. umbilicalis* neutral spores released from Pum2, including both early division patterns and germination rates. In a follow-up study, I tracked the same parameters for spores released from Pum2 blades that were stored at -20 ºC for 6 weeks to observe the effects of freezing on spore development. I also analyzed two years of *P. umbilicalis* phenological data collected from three sites across Maine to evaluate monthly reproductive trends and determine the effects of site and season on *P. umbilicalis* reproduction in Maine.

Finally, *P. umbilicalis* must have specific culture conditions to grow normally and produce large numbers of neutral spores. Here I define those conditions based on my work and earlier studies in the Brawley laboratory, because many biologists need this information following the recent sequencing of the nuclear genome of *P. umbilicalis* (Prochnik et al., 2017). Absent this understanding, several culture collections (e.g., SAMS, Scotland; UTEX, USA) have lost the P.um1 accession that was provided to them by the genome project in 2013 (Brawley, pers. comm.).

Methods

Phenological survey of *P. umbilicalis* in Maine

A bimonthly phenological survey was conducted by members of the Brawley lab from July 2014 to May 2016. Three main sites at Lubec (44°51'24.9"N, 66°59'04.0"W), Schoodic Point (44°22'04.8"N, 68°03'31.3"W), and Pemaquid Point (43°55'30.4", N 69°29'35.5"W) were used, and each site included two sub-sites 0.5-1.5 km apart to evaluate local variability. Carrying Place Cove Rd. (44°48'16.2"N, 66°58'55.8"W) and Quoddy Head Lighthouse (44°48'55.0"N, 66°57'02.1"W) were part of the greater Lubec site; Schoodic Point (44°20'58.0"N, 68°02'45.8"W) and Blueberry Hill (44 ° 20′ 20.39″N, 68 ° 2′ 42.03″ W) were part of the greater Schoodic Point site; and Pemaquid Lighthouse (43°50'13.9"N, 69°30'22.6"W) and Chamberlain (43°53'06.2"N, 69°28'31.3"W) were part of the greater Pemaquid Point site (Fig. 2.1).

Approximately thirty *P. umbilicalis* specimens were collected at each sub-site at randomly selected positions along a 60 m transect at low tide, and returned to the laboratory in plastic bags. Occasionally, a subsite had so few *P. umbilicalis* individuals in a particular season that n=30 was not achievable through random sampling. The reproductive state of each plant was determined by excising a 1cm x 1cm piece of blade margin and examining it using light microscopy. Plants were designated as either vegetative (VEG, no neutral spores, Fig. 2.5, A), subdifferentiated (SUB, margins with immature neutral sporangia, Fig. 2.5, B), or as reproductively mature (NS, having/releasing mature neutral spores Fig. 2.5, C). The SUB category was added for Year 2.

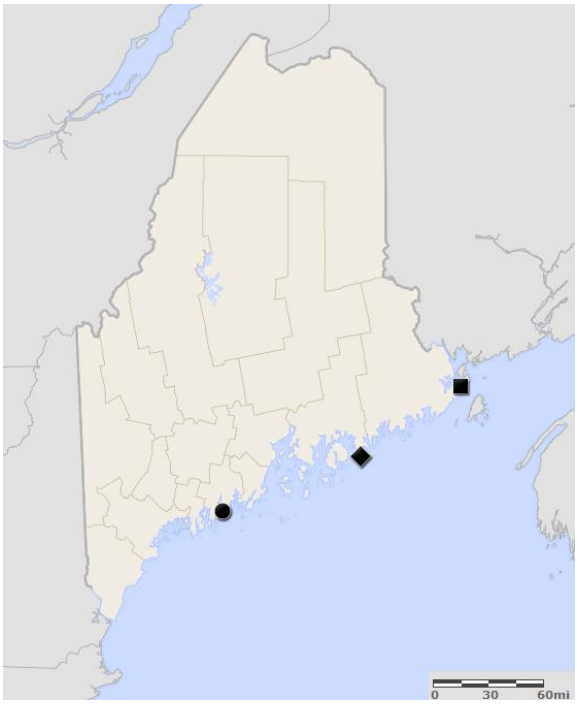

Figure 2.1 Locations of collection of phenological data for *P. umbilicalis* in Maine

The black circle marks the Pemaquid area (subsites Pemaquid Lighthouse and Chamberlain); the black diamond, the greater Schoodic area (subsites Schoodic Point and Blueberry Hill); and the black square, the Lubec area (subsites Quoddy Head Lighthouse and Carrying Place Cove). Base map provided by Maine GIS (http://www.maine.gov/megis/maps/)

Subsite descriptions

The overall sites and their corresponding subsites had unique features. At the Blueberry Hill subsite, where the previous phenology study was conducted, the intertidal zone is composed of large cobble and granite benches (Fig 2.2, A-B). *Porphyra umbilicalis* is common in the mid-zone boulder field, where storm waves often turn the smooth cobbles, removing epiphytes. The Schoodic Point subsite consists primarily of granite benches, but some *Fucus*-covered boulder field occurred near one transect, and this site is also exposed (Fig 2.2 C-D). Both subsites at Lubec are foggy in warmer weather, especially the Quoddy Head Lighthouse; they tend to be cooler and foggier than

Schoodic and Pemaquid overall (Fig 2.3, A-C). The Quoddy Head Lighthouse subsite of Lubec is an exposed cliff with base cobble; here, *P. umbilicalis* is prominent except in the hottest part of summer (Fig 2.3, A-B). *Palmaria palmata* and *Fucus* spp. make up much of the macroalgal biomass above the kelp zone at Quoddy Head. The sister Lubec subsite at Carrying Place Cove is more sheltered and consists of a wider intertidal zone, primarily dominated by *A. nodosum* in the midzone, but supporting kelps, *P. palmata*, and others in lower regions of the shore. The Pemaquid region was the southernmost site, and both subsites supported larger numbers of the gastropod *Littorina littorea* and the low intertidal red alga *Chondrus crispus*, when compared to Lubec and Schoodic (Fig 2.4, A-C). At Chamberlain, large granite benches, and some boulders and cobbles form the substratum, and masses of *P. umbilicalis* can be found growing on them in the upper intertidal zone (Fig 2.4, A-B). At Pemaquid Lighthouse (Fig 2.4, C) the intertidal zone is composed of solid granite, and slopes sharply downward towards the strong incoming waves. The *P. umbilicalis* transect is high in the intertidal zone, in a fissure within the bench.

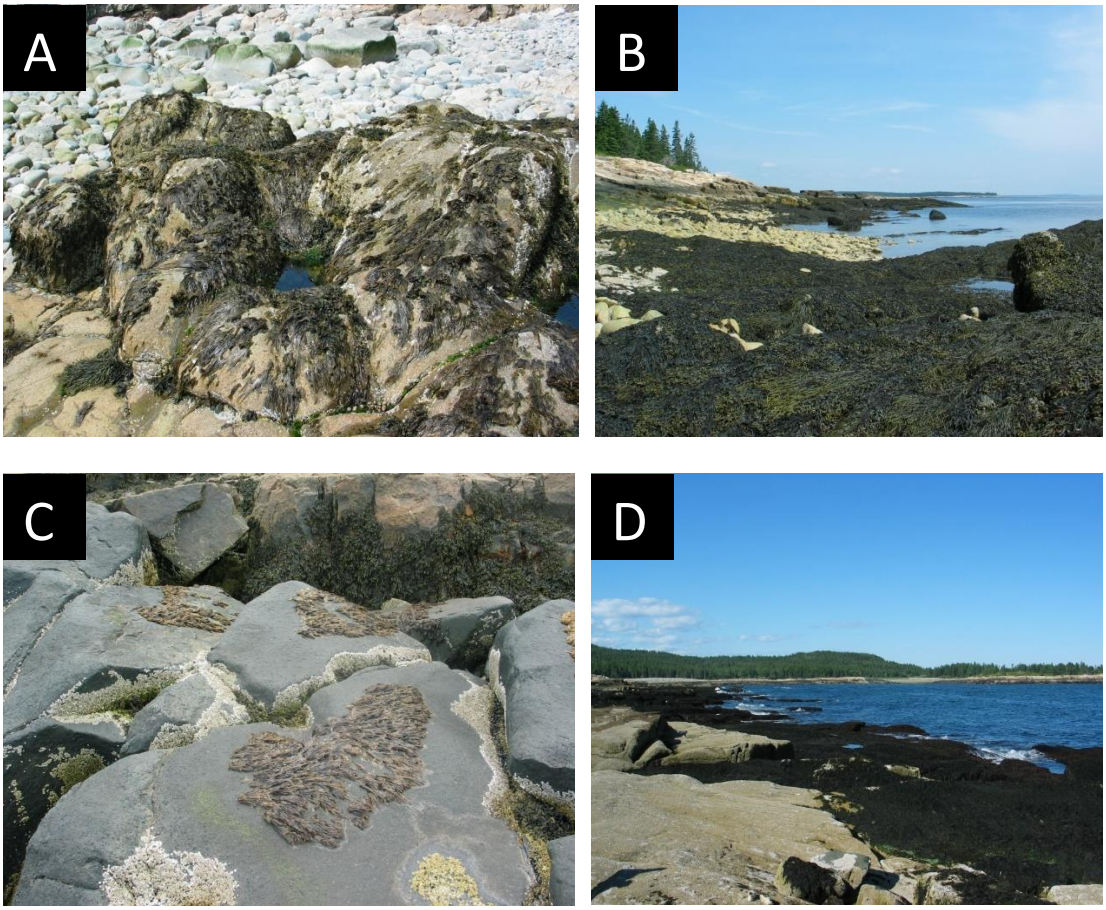

Figure 2.2 Schoodic subsite photos

Schoodic subsite locations Blueberry Hill (A-B) and Schoodic Point (C-D). Photos A and C feature *P. umbilicalis* growing on rocky substrate in their respective locations, while B and D show overall views of each subsite.

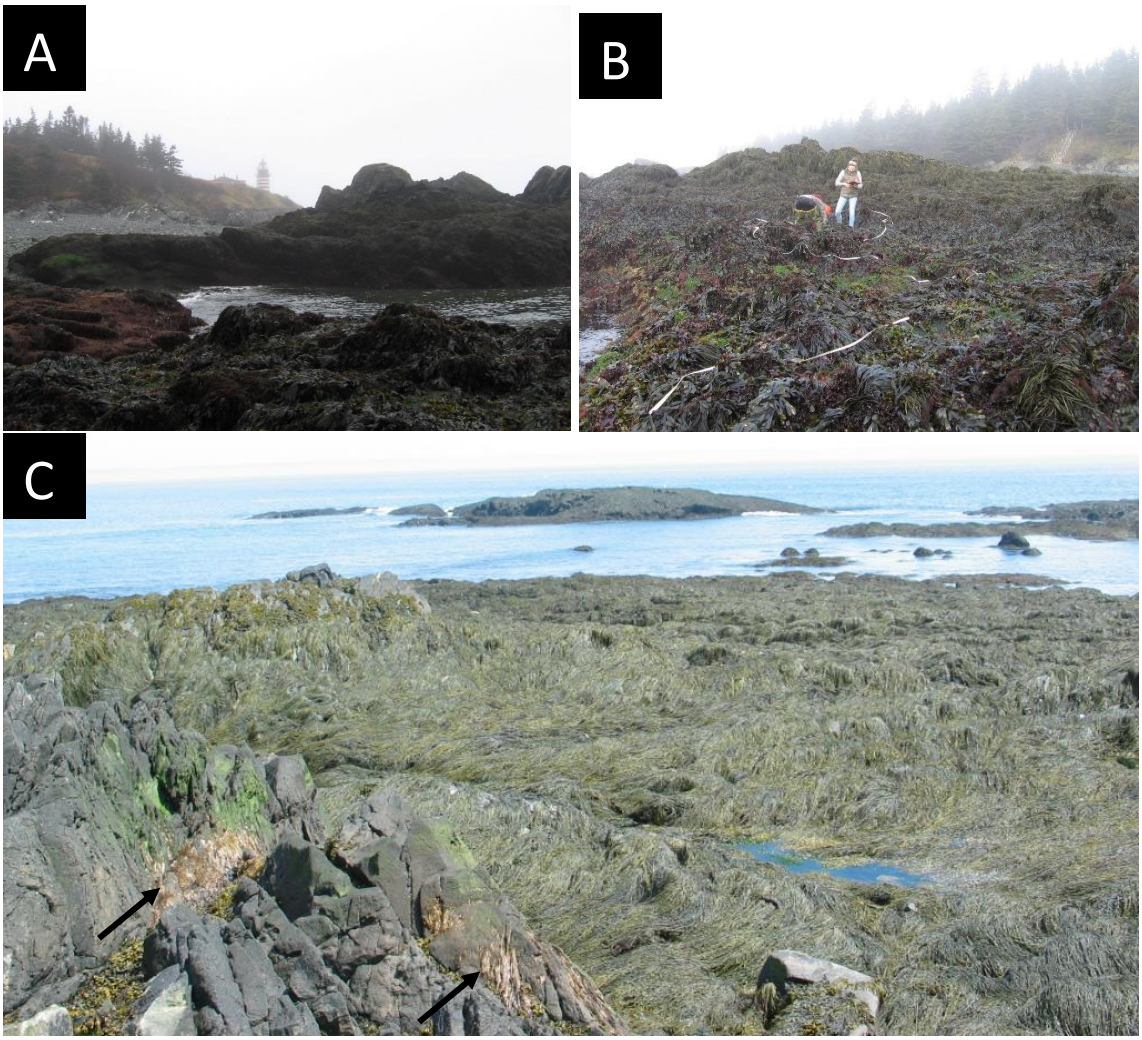

Figure 2.3 Lubec subsite photos

Lubec subsite locations Pemaquid Lighthoue (A-B) and Carrying Place Cove (C). Panel A shows the overall view of Pemaquid Lighthouse, and B shows a close-up of one of the sampling transects. In C, an overall view of Carrying Place Cove is featured, with *P. umbilicalis* growing in the lower right corner (black arrows) and with the large amount of *Ascophyllum nodosum* present in this semi-sheltered site apparent.

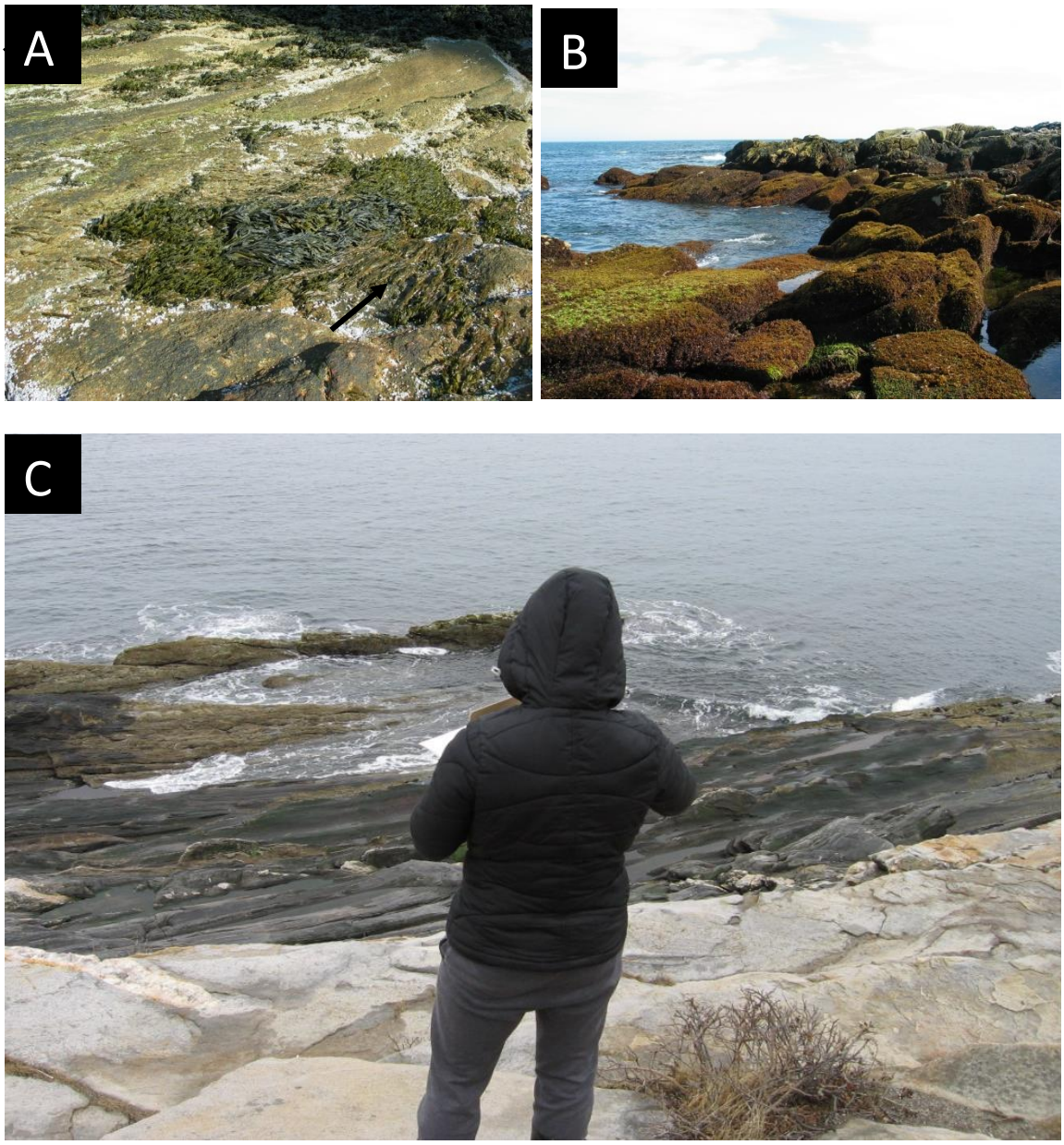

Figure 2.4 Pemaquid subsite photos

Pemaquid subsite locations Chamberlain (A-B) and Pemaquid Lighthouse (C). *Porphyra umbilicalis* can be seen in the bottom right in panel A (black arrow). The Chamberlain site with its well-developed *Chondrus* zone in the lower intertidal zone is featured in B. In C, an overview of the Pemaquid Lighthouse site is shown. The *P. umbilicalis* transect is to the right of this photograph.

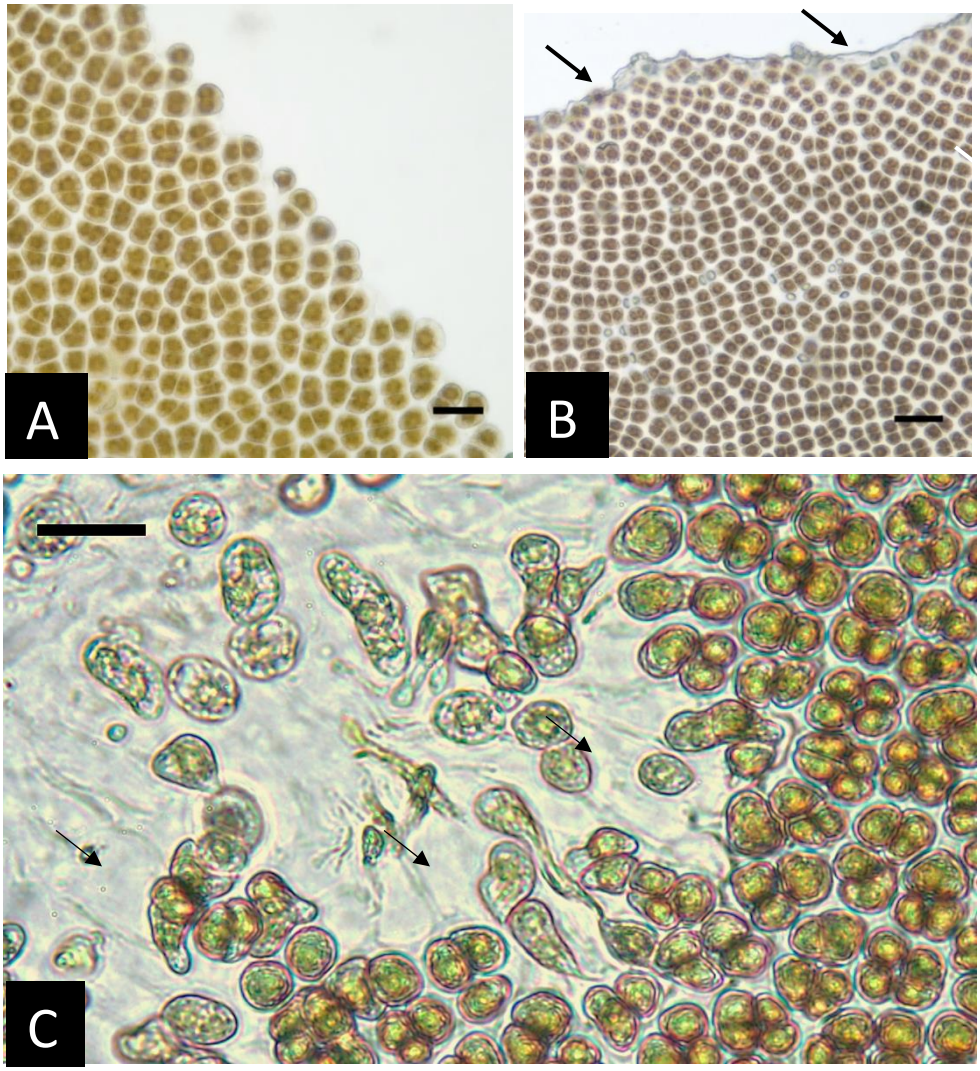

Figure 2.5 Reproductive stages of the *P. umbilicalis* blade margin

Representative photos of three reproductive stages of *P. umbilicalis* observed during phenological survey. **A.** Vegetative blade margin (VEG). Cells are single or in pairs, with a vegetative greenish color. Scale bar = 50 µm **B**. Subdifferentiated blade margin (SUB). Neutral spore packets are forming but are not mature. Scalloped blade margin indicates possible amphipod grazing (black arrows). Scale bar = 50 µm **C**. Mature neutral spore margin with neutral spores that are being released from the blade (NS, arrows). Scale bar = 30 µm

Culturing blades for neutral spore production

Neutral spores obtained from cultured *P. umbilicalis* were seeded onto 400 µm glass beads (Polysciences, Fig 2.6, A, B) and grown to reproductively mature adults. Three plastic cylinders (3L), each holding two plants (n=6 plants), were set up to produce spores. Blades ~ 5 cm long were clipped to the sides of each cylinder using plastic aquarium clips (Ocean Nutrition, Newark, CA, Fig. 2.6, C). Maintaining normal polarity of the blade by tethering it by its holdfast appears especially helpful compared to tumble cultures of blades, which begin to float at a vessel's surface and deteriorate as they get larger (Fig. 2.6, D). Cylinders were filled with sterile seawater enriched with West-McBride nutrient solution (Andersen et al., 2005), 10 ml stock enrichment/L seawater. This nutrient solution is a variant of Provasoli's enriched seawater (PES) and supplies more iron than PES. Germanium dioxide (250 mg/L culture medium) was used to prevent nuisance diatom growth (Stein, 1973). Blades were grown under a 10:14 L: D cycle, 60-80 µmol photons m^{-2} s^{-1}, at 12 °C with vigorous bubbling. Under these conditions, NS were released after ~ 3 mo, but can be forced (Brawley, pers comm.) to times as short as 62 days (Fig 2.6, E).

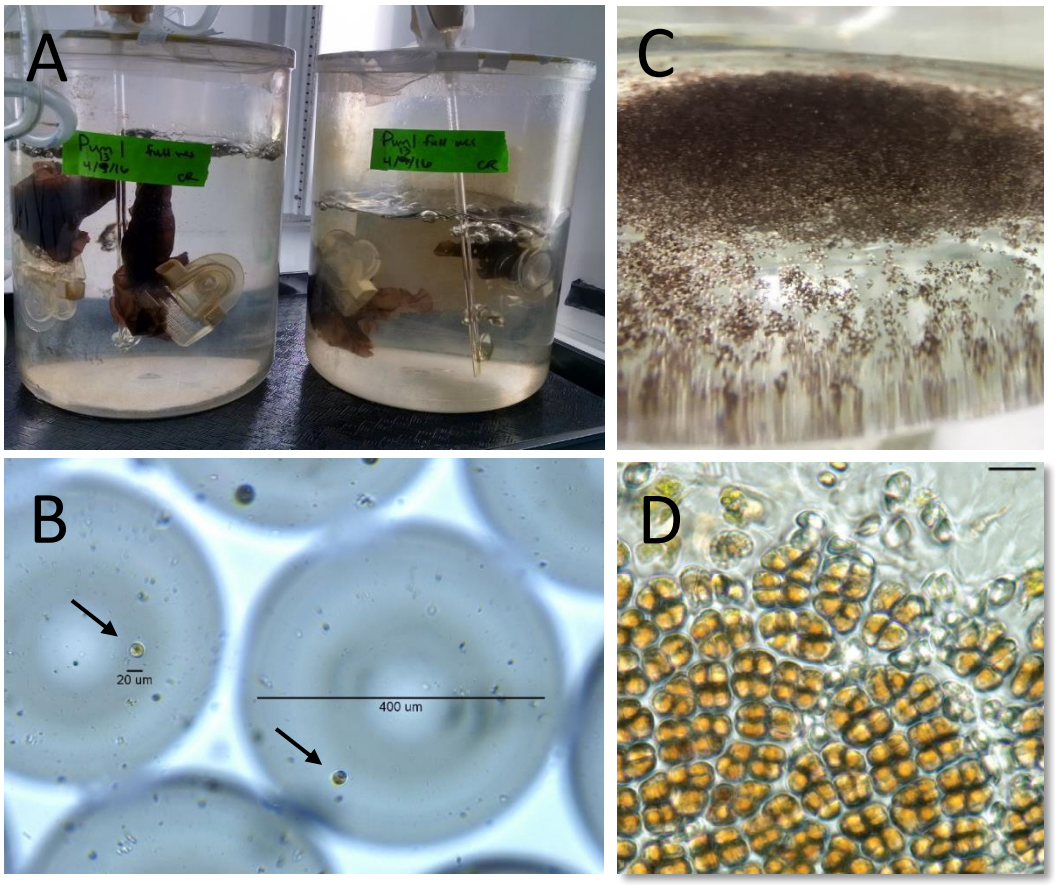

Figure 2.6 New technology for lab culture of *P. umbilicalis*

A. Plants grown for spore production. Adult blades of ~5 cm in length are clipped to the inside of a 3 L plastic cylinder and grown with vigorous aeration to produce spores. Plants pictured are approximately 15 cm long. Plants that are not clipped float and become curled, making them unsuitable for spore production. B. Neutral spores (arrows) of *P. umbilicalis* are seeded onto glass beads (~400 µm diameter). C. Juveniles after approximately 1 month of growth. When sporelings are visible at a few mm of length on beads, they are placed into culture in 2 L flasks or 4 L bottles and cultured as described. D. Neutral spores being released from ripe blade margins.

Obtaining neutral spores for development experiments

Four plants with large NS margins were selected for the research on NS development. The NS-rich margins from half of each plant were removed to 1.5 cm depth, and cut into pieces of 3 cm length. The remaining whole plant tissues were blotted with Kimwipes, double-bagged in Ziploc freezer bags, and placed at -20°C for the second half of the experimentThe removed NS margins were dragged through 0.5% seawater agar in petri dishes, to remove any adherent diatoms or cyanobacteria (Tatewaki and Provasoli, 1964). Then, each cleaned piece was rinsed in sterile seawater to remove agarose, then placed in a sterile 100 ml beaker and allowed to partially dry until rubbery in consistency.

When NS margin pieces felt "rubbery", 10 ml of sterile seawater containing ¼ West-McBride nutrient solution were added to them. The seawater solutions were vigorously mixed by hand to promote release of neutral spores, with not more than 10 min between each agitation. After agitation, each spore solution was poured through a 20 µm mesh (Nitex, Wildlife Supply, Yulee, FL) to remove the blade and any small pieces of tissue that separated from the margin during agitation. Neutral spores are approximately 10 µm in diameter, so a 20 µm mesh is appropriate for filtering them.

Spore growth conditions

After filtration, approximately 2 ml of each spore solution was added to three 30 mm sterile petri dishes with 2 mm grids, each with 8 ml of sterile seawater containing ¼ West-McBride stock enrichment solution and 0.75 mg/L germanium dioxide. Spores were added to three petri dishes from each of the four donor plants, for a total of n=12 dishes. Medium was gently rotated, side to side, to prevent spores from settling in one dense clump.

One dish from each plant was then placed into a black Plexiglas lightbox (TAP Plastics, San Leandro, CA) that I made (Fig. 2.7), with a total of three light boxes used. Each box was placed inside a growth chamber (Percival Scientific, Perry, IA, model I3GLLVLC8) with one box on each shelf in the chamber. Each box was placed next to its own T8 12" light strip that provided ~ 60 μmol photons m^{-2} s^{-1} along the length of the box. The purpose of the light box was to produce a unidirectional light gradient to encourage horizontal growth of germinating NS, which enabled assessment of developmental patterns.

Spore development observations

Spores were allowed 2 days to adhere to the dish, and, afterwards, observation of spore development began. Forty spores were selected from within a 1x 4 grid at the center of each dish (10 spores per square, 16 mm^2 total area) and were assigned numbers. These individuals were observed daily over a 10-day period, and every 5^{th} individual was photographed daily. The germination status and cell division pattern of each individual was noted. If a sporeling died before day 5, a new ungerminated or germinated individual was selected to replace it. After day 5, no replacements were made.

Frozen spore experiments

After approximately 6 weeks of storage at -20 °C, blades were thawed over the course of ~20 minutes at 4°C in their bags. Spores were immediately released as above.

Figure 2.7 Light box used for spore development experiments

Light boxes were designed to hold 4 x 30 mm petri dishes. A T8 light strip was placed directly in front of the light box to provide a unidirectional light source for embryonic growth. Three light boxes, each with one dish from each donor plant, were used in both frozen and control spore experiments. Dishes from different parents were arranged randomly with respect to position in the boxes.

Spore development experiments

Of the 960 total sporelings observed daily, 181 were eliminated before analysis. These germlings had been scored inconsistently; for example, a sporeling that was determined to be 2-celled at one point of observation, then 1-celled the following day. A series of one-way ANOVAs were used to determine whether donor plant and experimental treatment influenced key developmental stages at three time-points (days 1, 5, and 10). A group of 383 control sporelings was compared to 396 -20 ºC stored sporelings for this analysis.

Multinomial linear regression was then used to determine the effect of donor parent and frozen storage on the final developmental outcomes. Each sporeling was assigned a final developmental "path" based on the changing patterns of cell division. An additional 86 sporelings were eliminated from this analysis because their full patterns of development could not be observed, resulting in a final comparison of n = 349 control sporelings to n = 344 -20 ºC stored sporelings. A very large number of possible unique combinations of developmental patterns occurred at later embryonic stages; therefore, statistical analysis of patterns in this study was limited to the appearance of the first bilateral division (i.e., the 4-5 celled embryo).

Statistical analyses

Both the phenology data and the spore development data were analyzed using logistic regression models. R statistical software was used to conduct these analyses, and the functions *lm* or *multinom* were used to run the models. The regression models determined the effects of experimental parameters on outcomes by analyzing the relationship between a baseline outcome and all other outcomes. These relationships were

then compared between different experimental parameters, using a baseline for each independent variable and comparing the other variables to this baseline. For example, in the phenology data, the relationship between the baseline VEG and the outcome NS is compared for all sites to the Blueberry Hill baseline site. In R, the model produces a set of coefficients that explain the relationship between the outcomes for each independent variable examined. A Wald z-test (command *coeftest*) can be performed on the model coefficients to determine if the relationship is significantly different from the baseline. All subsequent *p*-values given for the phenology and developmental models were determined by this test.

Results

Phenological survey – differences by date

Mean spore production was highest in November, January, and March for both years (Fig 2.8, A-1, A-2). Analysis with binomial logistic regression (BLR) compared the relationship between VEG and NS for each month to the January baseline VEG-NS relationship. The BLR indicated that the VEG-NS relationship is significantly different for all months except March ($p < 0.001$ all samples, Table 2.1). Due to a data storage error, recorded data for Blueberry Hill, Schoodic Point, and Chamberlain were lost for September 2014. Dried herbarium samples from these sites were reconstituted in seawater and analyzed, resulting in an n = 3 samples for each of these sites during this month instead of the ~30 samples that were originally collected.

In Year 2, the subdifferentiated (SUB) category was added to the analysis. The subdifferentiatied region of the *P. umbilicalis* blade contains immature neutral sporangia that have not finished dividing to produce and release neutral spores. In many cases, blades at the edge of the peak season of production had margins that were SUB stage, not NS stage. The blade margins had small scalloped edges, indicative of amphipod grazing (Fig. 2.5, B). The addition of this category gave further information about the transition from winter NS-bearing tissues to VEG tissues in the spring and summer. SUB tissues were found to dominate in May, between the March NS peak and the VEG July peak. Otherwise, the shift from VEG to NS was similar to that of Year 1.

Analysis with multinomial logistic regression (MLR) compared the Year 2 relationships between VEG and SUB plants, and VEG and NS plants for each month to the same January baseline relationships. The MLR determined the VEG-SUB relationship differed significantly from the January baseline trend in July and September, when VEG tissues are very high ($p = 0.001$) (Table 2.2). This analysis also determined that the VEG-NS relationship was significantly different from January in July, September, and May ($p = 1.26 \times 10^{-15}$, $p = 2.20 \times 10^{-16}$, $p = 0.02$, respectively).

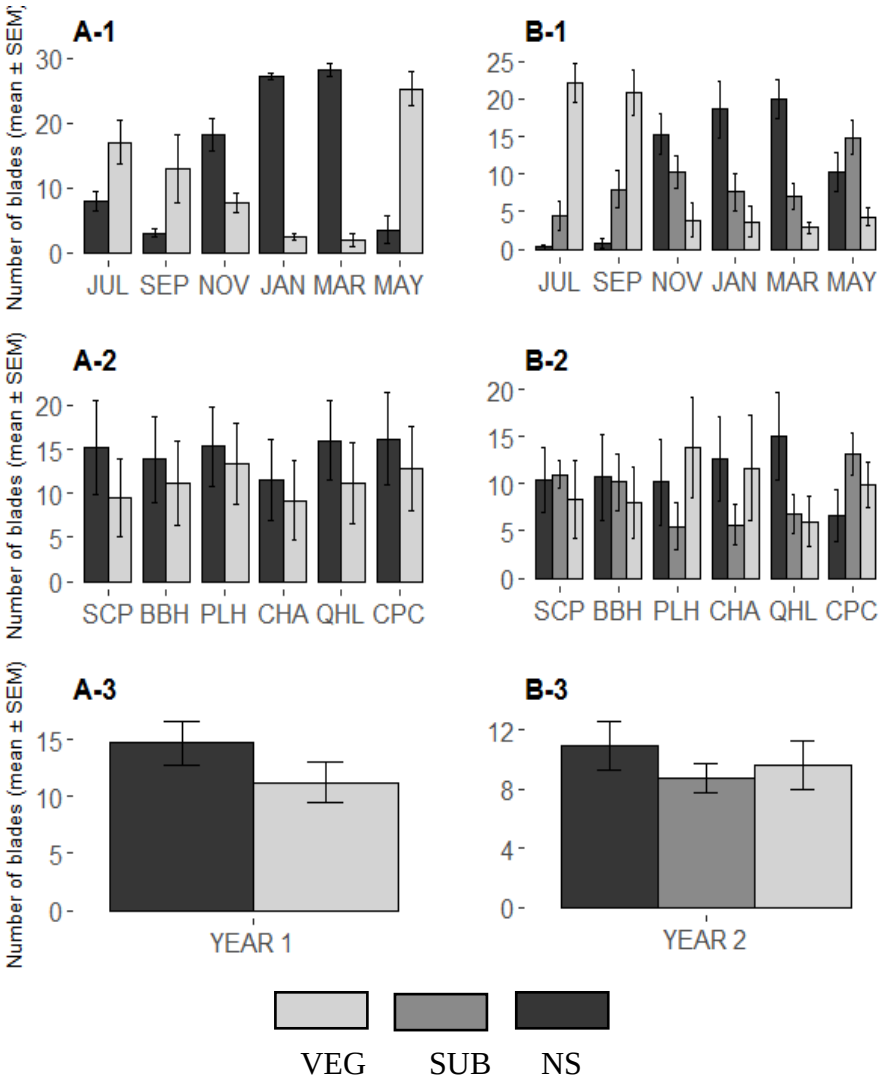

Figure 2.8 Representative means (± SEM) for phenological data

Year 1 data represented in column A, Year 2 in column B. (A-1, B-1) Means of reproductive stages across all sites for each sampling month. A clear trend of higher NS production in winter and spring (November- March) emerged for both sampling years. (A-2, B-2) Means of tissues types across all months for each site. Trends fluctuated across each site between the two sampling years. (A-3, B-3) Yearly trends for reproductive stage averaged across the entire year and all sites. More plants were found across both years to have NS than to be VEG or SUB.

Table 2.1 Binomial logistic regression table for Year 1 phenology

	NS	
	Coeff.	**S.E.**
Mar	0.2775	0.4184
May	**-4.4779**	**0.3658 *** **
July	**-3.2228**	**0.3314 *** **
Sept	**-4.0944**	**0.3962 *** **
Nov	**-1.6511**	**0.3331 *** **
SCP	0.3734	0.3207
CHA	-0.0580	0.3502
PLH	0.3000	0.3100
CPC	0.4067	0.3094
QHL	**0.6280**	**0.3231 ***

Model coefficients and standard errors (S.E) from BLR for year 1 phenology data. NS column represents VEG-NS comparisons. First block of rows represents months March (Mar), May, July, September (Sept) and November (Nov), each compared to January. Second block of rows represents sites Schoodic Point (SCP), Chamberlain (CHA), Pemaquid Lighthouse (PQL), Carrying Place Cove (CPC) and Quoddy Head Lighthouse (QHL), each compared to Blueberry Hill. Model built using base R statistical software. Significances were determined by Wald z-test on model coefficients. Significant values are in boldface. Significance codes: p = 0 ***, p = 0.01 *

Table 2.2 Multinomial logistic regression table for Year 2 phenology

	SUB		NS	
	Coeff.	**S.E.**	**Coeff.**	**S.E.**
Mar	0.1618	0.3939	0.3363	0.3553
May	0.5330	0.3503	-0.8162	**0.3388** *
July	-2.4763	**0.3469** ***	-6.0735	**0.7593** ***
Sept	-1.8426	**0.3229** ***	-5.1400	**0.5265** ***
Nov	0.2683	0.3634	-0.2259	0.3371
SCP	0.1427	0.3173	0.0456	0.3663
CHA	-1.0915	**0.3344** **	-0.3463	0.3534
PLH	-1.4980	**0.3306** ***	-1.0396	**0.3454** **
CPC	-0.1119	0.3060	-0.9710	**0.3633** **
QHL	0.1325	0.3487	1.0777	**0.3912** **

Model coefficients and standard errors (S.E) from MLR for year 2 phenology data. SUB column represents VEG-SUB comparisons, NS column represents VEG-NS comparisons. First block of rows represents months March (Mar), May, July, September (Sept) and November (Nov), each compared to January. Second block of rows represents sites Schoodic Point (SCP), Chamberlain (CHA), Pemaquid Lighthouse (PQL), Carrying Place Cove (CPC) and Quoddy Head Lighthouse (QHL), each compared to Blueberry Hill. Model built using R statistical software using package *nnet*. Significances were determined by Wald z-test on model coefficients. Significance codes: p = 0 ***, p = 0.001 **, p = 0.01 *

Phenological survey, differences by site

There were some differences in the overall reproductive trends between sites (Fig 2.8, B-1, B-2). In Year 1, the VEG-NS relationship at Quoddy Head Lighthouse was slightly different from the Blueberry Hill baseline VEG-NS relationship ($p = 0.052$), having lower amount of VEG plants compared to NS. (Table 2.1). In Year 2, Pemaquid Lighthouse differed the most from Blueberry Hill, having more VEG plants compared to both NS and SUB ($p = 5.88 \times 10^{-6}$; $p = 0.003$, respectively). The VEG-NS relationship at Quoddy Head Lighthouse was again significantly different from the Blueberry Hill VEG-NS relationship ($p = 0.006$) because Quoddy Head Lighthouse had more NS plants compared to VEG. Several other sites also differed from the baseline in terms of VEG-SUB and VEG-NS relationships (Table 2.2). Aside from Quoddy Head Lighthouse having a higher proportion of NS plants for both sampling years, no other consistent trends were observed. The overall annual trend for both years showed NS as being the most common reproductive stage (Fig 2.8, C-1, C-2).

Amphipod collection

Because we observed grazing on *P. umbilicalis* collected during the phenology survey, we collected amphipods from *P. umbilicalis* in October 2016 to try and identify what species were present. Amphipods collected from *P. umbilicalis* at this time were identified as *Apohyale prevostii* (Milne-Edwards, 1850), formerly called *Hyale nilssoni*.

Spore development patterns

Sporelings followed several different developmental paths across the 10 days of the study (Figs. 2.9-2.10, Table 2.3). Nearly all spores germinated and reached the two-celled stage (Fig. 2.10, A). Most sporelings continued linear division to the three-celled stage, but ~10 % of all sporelings in both the -20 ºC and control groups exhibited bilateral division of the upper cell (Fig. 2.10, B1). Linear, three-celled sporelings exhibited a variety of subsequent division patterns, including some instances of double divisions in both linear and bilateral directions (Fig. 2.10, E-F), the most common being a single division producing a linear 4-celled sporeling (Fig. 2.10, E1). This outcome represented ~ 25% of -20 ºC stored sporeling development and ~32% of control sporeling development.

There were many sporelings that did not reach the four-celled stage. In both the -20 ºC and control groups, ~18% exhibited only linear 3-celled development by the end of the study (Fig. 2.10, B2). These sporelings were healthy and growing properly, but did not achieve development to the 4-celled stage by the 10-day cutoff. Some sporelings grew slowly, and, by the end of the 10-day observation period, were either ungerminated, germinated with no additional growth, or had only reached the 2-cell stage. Approximately 15% of control sporelings and 18% of -20 ºC stored ones fit into these categories. Numbers of dead sporelings were low, representing ~1 % of control sporelings and ~2 % of -20 ºC stored ones. A small group of ~2 % of both -20 ºC and control sporelings grew in a “rhizomatous” pattern (Fig. 2.7, D), where a long rhizoid was produced but the single celled-embryo did not divide.

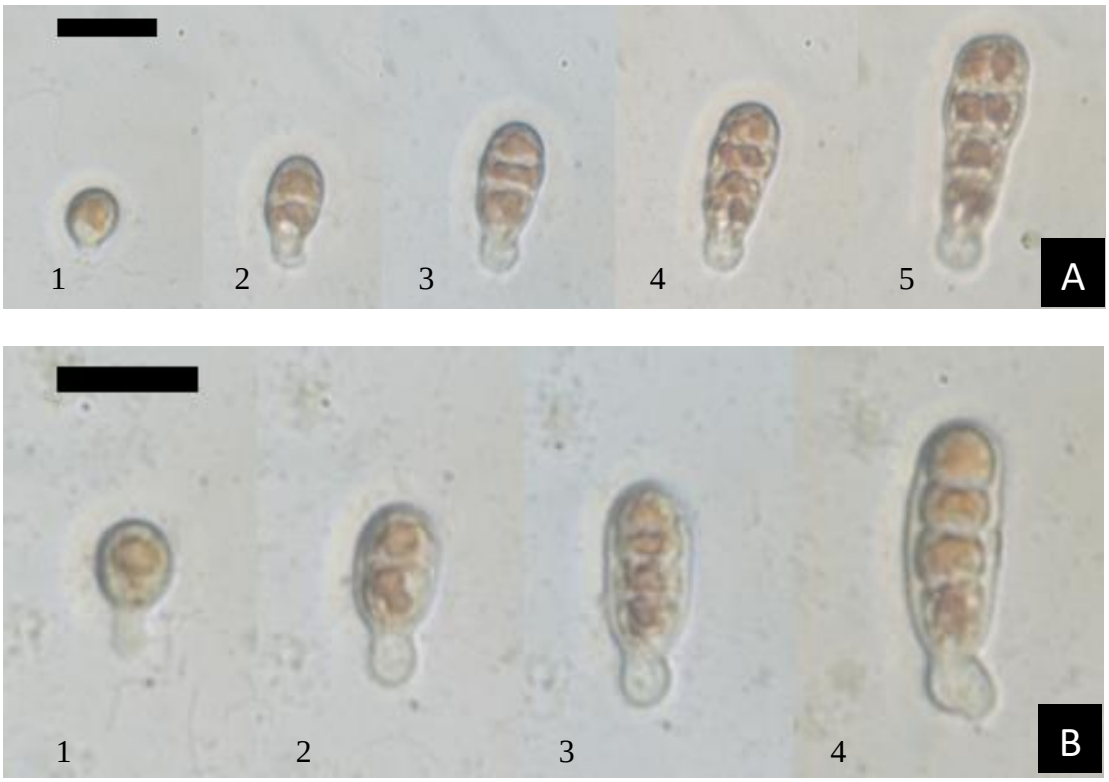

Figure 2.9 Examples of embryonic development from NS

A. Sporeling exhibiting multiple bilateral and linear divisions. A1. Sporeling on first day of observation. A2. Sporeling has completed linear division on day 5 and has two in-line cells. A3. Sporeling has completed another linear division on day 7, forming a 3-celled linear sporeling. A4. On day 8, sporeling begins bilateral division of upper two cells. The basal cell is diving linearly at the same time. A5. The 6-celled embryo (See Fig. 2.7, path F4). B. Sporeling exhibiting multiple linear divisions. B1. Germination is complete on day 2. B2. By day 5, the embryo has completed linear divison resulting in two in-line cells. B3. On day 7, sporeling has begun linear division of both upper and lower cells. Note the chloroplasts have fully divided but the cell walls have not completely split. B4. Completed linear divisions resulting in a linear 4-celled sporeling. Linear sporelings were the most common type of pattern at the 4-celled stage (See Fig. 2.7, path F1). Scale bars = 10 µm

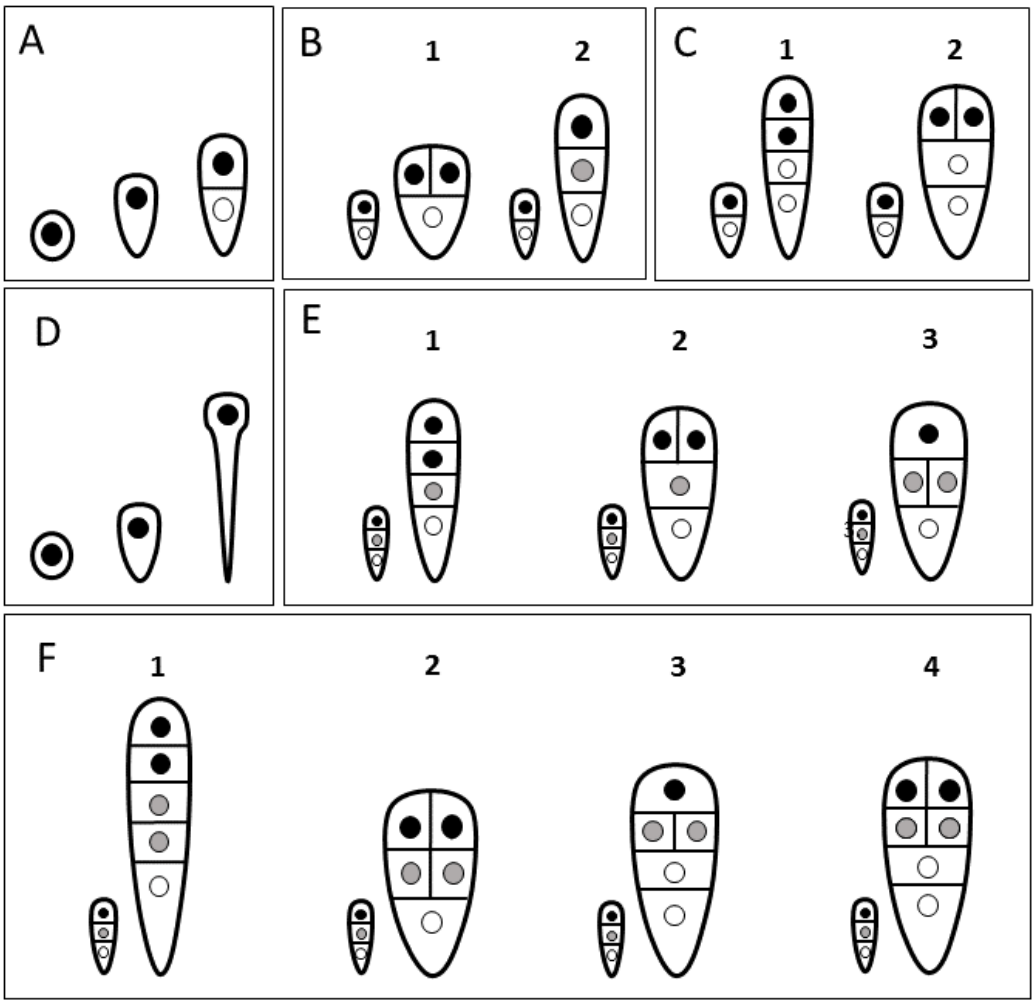

Figure 2.10 *P. umbilicalis* sporeling division patterns

Panel (A) represents the germination pattern followed by nearly all spores. A single-celled spore germinates and divides along a horizontal plane into a sporeling with two in-line cells. In panels B-F the small graphic on the left represents the sporeling pre-division, and the larger graphic represents the sporeling post-divisoin. Two celled sporelings may undergo bilateral division (B1) or continue with linear division (B2). Both cells may also divide simultaneously (C), producing either a 4-celled linear sporeling (C1) or a sporeling with an upper bilateral divison and a lower linear division (C2). Single-celled sporelings may also produce long rhizoids instead of dividing (D). Linear 3-celled sporelings may divide again along a to produce a linear 4-celled sporeling (E1, most common outcome) or along a vertical plane to produce a bilateral 4-celled sporeling (E2, E3). Linear three-celled sporelings may also have double divisions that produce a linear 5-celled sporeling (F1), or a bilateral one (F2). Finally, these sporelings may undergo a combination of both linear and bilateral division, though these patterns are rare (F3, F4). Diagrams not to scale.

Table 2.3 Developmental outcomes of sporelings

CONTROL	**R**	**U**	**G**	**A**	**B1**	**B2**	**C1**	**C2**	**E1**	**E2/E3**	**F2**	**F1**	**F3**	**F4**	**D**
Donor A	1	1	0	1	13	10	8	7	32	0	1	0	3	1	2
Donor B	2	0	4	9	6	25	9	4	21	1	0	1	5	1	1
Donor C	2	0	0	7	6	14	11	1	33	4	1	1	2	1	1
Donor D	0	2	2	28	8	14	10	2	25	3	1	0	0	0	1
Total	5	3	6	45	33	63	38	14	111	8	3	2	10	3	5
% total control	1.43	0.86	1.72	12.89	9.46	18.05	10.89	4.01	31.81	2.29	0.86	0.57	2.87	0.86	1.43

-20 ºC storage	**R**	**U**	**G**	**A**	**B1**	**B2**	**C1**	**C2**	**E1**	**E2/E3**	**F2**	**F1**	**F3**	**F4**	**D**
Donor A	1	0	4	8	15	13	8	5	15	5	1	3	0	3	5
Donor B	2	1	1	8	1	16	18	2	35	2	2	0	0	0	2
Donor C	3	1	0	15	10	19	12	1	22	0	1	2	1	0	0
Donor D	1	3	8	13	13	12	5	1	17	3	1	0	1	2	1
Total	7	5	13	44	39	60	43	9	89	10	5	5	2	5	8
% total -20 ºC	2.03	1.45	3.78	12.79	11.34	17.44	12.50	2.62	25.87	2.91	1.45	1.45	0.58	1.45	2.33

Raw total of spores from donor plants A, B, C, and D for both the control group (upper block, total n = 349) and the -20 ºC storage experimental group (lower block, total n = 344). “Total” rows represent the total n of sporelings from each donor plant combined, and “% total” rows represent the percentage of that outcome out of the total for the respective treatment group. Columns represent final developmental outcomes, which correspond to the patterns displayed in Fig. 2.10, A-E, R=rhizomatous, U = ungerminated, G= germinated, D = dead.

Effect of donor plant and cold storage on developmental outcomes

Multinomial logistic regression (MLR) was used to test whether donor plant and treatment (control or -20 ºC storage) influenced the relationships between developmental outcomes, using developmental pattern E1 (Fig. 2.10) as a baseline (Table 2.4). Donor plant D had significantly higher numbers of spores that only reached the 2-celled stage (Fig. 2.10 A) compared to the other donor plants over the 10-day period of observation ($p = 0.001$). Donor plant D did not produce any sporelings that exhibited the F1 developmental pattern, which the model determined was significant ($p = 2.26 \times 10^{-16}$), but the number of plants in this category was low overall (~1.0%). Plant A had greater numbers of sporelings that exhibited bilateral division in the 3-cell stage (Fig. 2.10, B1) compared to plant B ($p = 0.001$) and greater numbers of sporelings with bilateral division in the 4-celled stage (Fig. 2.10, E2, E3) compared to plant C ($p = 0.01$).

One of the major goals of this study was to see if freezing influenced the growth and development of neutral spores. Interestingly, the only significant difference was found for the number of spores that germinated, but did not continue to grow or divide. The frozen storage group had more of these sporelings compared to the control group. However, such sporelings made up ~2% of the total experimental results, so the treatment had only a small inhibitory effect ($p = 0.03$).

Differences at key developmental stages

One-way Analysis of Variance (ANOVA) was used to determine whether - 20 ºC storage influenced sporelings at key stages of development on days 1, 5, and 10. No significant effects were found for any of the stages or time points examined. (Table 2.5)

Table 2.4 Multinomial logistic regression table for final spore patterns

	Rhizo.		Ungerm.		Germ.		A		B1		B2		E2/E3	
	Coeff.	**S.E.**	**Coeff.**	**S.E.**	**Coeff.**	**S.E.**	**Coeff.**	**S.E.**	**Coeff.**	**S.E.**	**Coeff.**	**S.E.**	**Coeff.**	**S.E.**
Donor B	0.5220	0.8890	-0.1742	1.4285	0.0505	0.7018	0.4615	0.4575	**-1.5604**	**0.4671*** **	0.4033	0.3273	-0.8689	0.5379
Donor C	0.7605	0.8610	-0.1568	1.4286	-13.1857	337.6507	0.7371	0.4430	-0.7161	0.3718	0.2041	0.3367	**-1.9497**	**0.7894 ***
Donor D	-0.5404	1.2500	1.7848	1.1179	1.1086	0.6326	**1.6495**	**0.4258 *** **	-0.1441	0.3597	0.2481	0.3570	-1.2915	0.6804
Frozen	0.5220	0.6040	0.8589	0.7502	**1.0933**	**0.5197 ***	0.2744	0.2606	0.4121	0.2797	0.1736	0.2305	-0.2469	0.4545

	C1		C2		F2		F1		F3		F4		Dead	
	Coeff.	**S.E.**	**Coeff.**	**S.E.**	**Coeff.**	**S.E.**	**Coeff.**	**S.E.**	**Coeff.**	**S.E.**	**Coeff.**	**S.E.**	**Coeff.**	**S.E.**
Donor B	0.3489	0.3729	-0.6849	0.7571	-0.1727	1.0204	-1.2716	1.1730	0.3323	0.7594	-1.5601	1.1362	-1.0210	0.7188
Donor C	0.2063	0.3818	-0.3796	0.7001	-0.1550	1.0205	-0.1556	0.8423	-0.1596	0.8429	-1.5426	1.1364	-2.1021	1.0880
Donor D	0.0735	0.4184	0.3311	0.6434	0.1692	1.0247	**-13.9119**	**0.0000 *** **	-1.0699	1.1763	-0.5253	0.8943	-1.0897	0.8313
Frozen	0.3388	0.2649	0.4821	0.4974	0.7464	0.7457	1.0738	0.8505	-1.4272	0.7885	0.7441	0.7476	0.6741	0.5914

Coefficients and standard errors (S.E.) from the MLR conducted on the spore development data. Columns represent final development patterns, which correspond to the patterns displayed in Fig. 2.10, A-E, R=rhizomatous, U = ungerminated, G= germinated, D = dead. First block of rows represents Donor plants B, C, and D, each compared to plant A. Final row represents frozen spores compared to unfrozen ones. Model built using R statistical software using package *nnet*. Significances determined by Wald z-test on model coefficients. Significant values are bolded. Significance codes: p = 0 ***, p = 0.001 **, p = 0.01 *

Table 2.5 One-way ANOVA tables for key developmental stages

Day 1	**DF**	**Sum Sq.**	**Mean Sq.**	**F**	**P**
u - Treatment	1	703.1	703.1	2.749	0.148
u - Residuals	6	1534.7	255.8		
g - Treatment	1	480.5	480.5	2.009	0.206
g - Residuals	6	1435	239.2		
Day 5	**DF**	**Sum Sq.**	**Mean Sq.**	**F**	**P**
2 - Treatment	1	72	72	1.442	0.275
2 - Residuals	6	299	49.92		
Day 10	**DF**	**Sum Sq.**	**Mean Sq.**	**F**	**P**
3 - Treatment	1	21.13	21.13	0.631	0.457
3 - Residuals	6	200.75	33.46		
4 - Treatment	1	78.12	78.12	2.173	0.191
4 - Residuals	6	215.75	35.96		

One-way ANOVAs were used to determine whether the -20 ºC storage (Treatment) had a significant effect on key developmental stages on days 1, 5, and 10 of the study. No relationships were found to be significant. Development stages: u = ungerminated, g = germinated, 2 = 2-celled, 3 = linear 3-celled, 4 = linear 4-celled.

Discussion

The results from the phenological survey of *P. umbilicalis* in Maine indicate that season has the most influence on reproduction in this alga. Plants are reproductive from fall through the early spring, when ample nutrients and cold temperatures support neutral spore production. During the summer months, depleted nutrients and high temperatures cause plants to die back and become mostly vegetative. Blouin et al. (2007) found that *P. umbilicalis* collected from Blueberry Hill in 2006 were reproductive throughout the year except in summer, and that spore release was highest from fall to spring. In the present study, we observed many blades in the spring and fall with subdifferentiated margins, not NS-rich margins. The presence of broken, scalloped blade edges, staged as SUB,

indicated that grazers appeared to restrict the environmentally determined reproductive season to a smaller period of achieved annual reproduction. We observed *Apohyale prevostii*, gammarid amphipods, on wild *P. umbilicalis* collected in October 2016. From the viewpoint of this study, the time when aquaculturists could collect wild seed stock might be constrained by these grazers. McBane & Croker (1983) reported that *A. prevostii* (as *H. nilssoni*) often grazed *Vertebrata lanosa* (as *Polysiphonia lanosa*) and other ephemeral algae on rocky shores of New England. The investigators also reported that *A. prevostii* in lab tests frequently swam to *P. umbilicalis*, and chose *P. umbilicalis* only slightly less than *V. lanosa*. The investigators did not examine whether amphipods consumed *P. umbilicalis* tissue, but based on the grazing evidence from the *P. umbilicalis* phenology study, this is a strong possibility. Determining the effect of the nutritious spore diet from *P. umbilicalis* on the biology of this amphipod (and possibly other species) would be an important direction for a future study.

Analysis of regression coefficients indicated that site did have some influences on the observed reproductive outcomes. The Quoddy Head Lighthouse site had more plants with NS compared to VEG for both sampling years compared to Blueberry Hill, and had the highest overall NS for Year 2. *Porphyra umbilicalis* at this site is highly exposed to incoming wave action, something that appears to benefit reproduction. In laboratory conditions, vigorous bubbling of plants is necessary to induce neutral spore production, so the influence of heavy wave action on the plants of Quoddy Head Lighthouse may result in their increased production of neutral spores. Overall, the phenology study expands on earlier phenological work by including additional samples and study sites, and provides a clear demonstration of annual reproductive trends in *P. umbilicalis*.

The most significant influence on embryonic division patterns was found between individual donor plants. Plant A appeared to produce more bilateral development in the 3- and 4-cell stages, and donor plant D appeared to grow more slowly than the other plants, having significantly more sporelings remaining at the 2-cell stage on the final day of observation. These categories combined to make up ~26% of the total outcomes, so these may be significant effects for aquacultural applications. Wang et al. (2010) showed that adult plants tended to retain their defining shape from their sporeling stages. Initially this was not considered relevant to my study, because I chose plants of similar shape and size. A next step would be to culture the young sporeling with known developmental patterns to mature size and do comparisons of their final shapes. A next generation of spores could then be collected from these adults, and another comparison done on the young sporelings produced.

Ohme and Miura (1988), Wang, (2010), and Yan et al. (2010) showed that early development in *P. oligospermata*, *P. haitanensis* and *P. yezoensis* is linear, and produces a linear adult plant. Mitman and van der Meer, (1994) observed that *P. purpurea* sporelings had early bilateral division. In the case of *P. purpurea*, some of this bilateral development may be because the adult gametophyte is bilaterally divided into male and female halves, so early differentiation of the blade occurs. The *Porphyra* and *Pyropia* species examined in these studies were derived from conchocelis cultures, not asexual spores. Development in asexual *P. umbilicalis* seems to trend towards linear development, but a significant portion of sporelings had early bilateral development. A comparison between western Atlantic asexual *P. umbilicalis* and conchospores from sporophytes in the northeastern Atlantic *P. umbilicalis* would be useful.

Overall, it is surprising that there are differences in developmental pattern of NS from a single parent, because meiosis did not produce these parents and all NS should be the same. This opens the possibility, as well, that other, microbiotic influences (e.g., particular bacteria near some but not all NS) would affect pattern of development and/or rate of development.

Interestingly, - 20 ºC storage only showed a significant effect in the number of sporelings that germinated but did not divide. The small number of sporelings affected by this difference from controls, however, means that it is not a negative indicator for storage of frozen seedstock for aquaculture. This promising result indicates that short periods of freezing may have little effect on spore viability when reproductively mature *P. umbilicalis* blades are frozen for storage. Now, the storage space for frozen seedstock vs already seeded nets should be compared as part of establishing freezing of ripe blades bearing neutral spores as a standard industry technique. Future studies could freeze blades for longer periods of time and examine the effect this has on sporeling development.

Sporelings that had unclear developmental paths made up a notable amount of the observed individuals. Sporelings would sometimes grow in a twisted form, or perpendicular to the field of view, preventing clear visualization of their development patterns. Investigators who studied developmental patterns in other *Porphyra* and *Pyropia* species made use of color mutants to aid in differentiation between cell division patterns. Color mutants are currently unavailable for asexual *P. umbilicalis*. Mitman and van der Meer, (1994) observed that use of nitrosoguanadine produced some color mutants in *P. purpurea*, so this method might be applied to *P. umbilicalis* to produce color

mutants. Due to the asexual nature of *P. umbilicalis* reproduction, however, continuation of genetic color mutants could pose a challenge because mutagenized neutral spores could grow into single color-mutant blades, with no contrasting wild-type cells. Use of chlorophyll *a* fluorescence or a destructive assay of sporelings at different time points could be used to improve visualization of development.

It should be noted that the analyses of both the phenology and developmental data using multinomial regression present some limitations. Because all comparisons involve choosing a baseline as an independent variable, not all possible combinations of comparisons are performed. For example, all sites in the phenological survey are compared to the Blueberry Hill baseline, but no other inter-site comparisons were made. To determine the sensitivity of these analyses, I changed the baseline month and site in alternative phenological models, and changed the baseline developmental outcome in the developmental study. I found that some other site-site or month-month comparisons were significant in the phenology data, but that changing the developmental outcome baseline for the developmental dataset did not have much effect. Using regression models with a baseline comparison is useful for preliminary analysis, but because alternative comparisons between independent variable parameters are not possible, an alternative analysis is planned soon.

The studies presented here will benefit researchers interested in conducting experimental studies in *P. umbilicalis* by providing detailed methods for laboratory culture, and a guide to basic early development of sporelings. Applied scientists interested in commercial aquaculture can use our findings of *P. umbilicalis* seasonal reproduction in Maine to further commercial aquaculture development in this species.

CHAPTER 3

GENETIC TRANSFORMATION OF *PORPHYRA UMBILICALIS*

Introduction

Genetic transformation is one of the most valuable tools for understanding gene function. Through a variety of methods, foreign DNA constructs are introduced into cells, remaining there for hours or days (transient transformation) or becoming fully incorporated into the genome (stable transformation). More than 20 algal species have been transformed using delivery systems that include glass needles, electroporation, transfection with *Agrobacterium*, and biolistic particle bombardment (reviewed in Qin et al., 2012). More recently, CRISPR technology was successfully applied to transform the diatom *Phaeodactylum* (Nymark et al., 2016), and similar efforts are being made in other microalgae (Shin et al., 2016). Out of the many microalgal species with available transformation protocols, the green flagellate *Chlamydomonas* is the most widely used algal model system, and much of our knowledge of its biology is dependent upon extensive transformation studies (Boynton et al., 1988; Kindle, 1990; Grossman, 2000; Jinkerson and Jonas, 2015). Interest in using microalgae for biofuel production has also motivated transformation efforts (Radakovits et al., 2010; Kilian et al., 2011). A few macroalgae were transformed, including *Pyropia yezoensis* (stable), *Saccharina japonica* (stable), and *Ulva lactuca* (transient) (Huang et al., 1996; Qin et al., 1999; Fukuda et al., 2008; Mikami, 2013; Uji et al., 2014), but transformation of macroalgae proved more challenging than that of microalgae, and few successes are documented (Qin et al., 2012; Mikami, 2013; Lin and Quin, 2014).

Codon optimization and promoter selection are two critical steps for improving transformation success in algae. Viral promoters such as the Cauliflower Mosaic Virus 35s promoter (CaMV-35s) were used to transform higher plants and other organisms, and sometimes increased expression of target microalgal genes (Santos et al., 2013; Lin and Quin, 2014). Endogenous promoters support much higher levels of transcription, especially in species where heterologous promoters were unsuccessful (Fukuda et al., 2008). Codon optimization of target genes is important because differences in the genetic code used by different organisms (Alberts et al., 2014) means that translation of a mRNA can occur by different codons for the same amino acids in different groups of organisms (Bulmer, 1988; Higgs and Ran, 2008). The nuclear genomes of many microalgae and a few macroalgae are now available, including that of the red macroalga *Pyropia yezoensis* (Nakamura et al., 2013). The availability of sequenced genomes provides a wealth of important information necessary to produce codon-optimized genes and to locate promoters best suited for supporting transcription of particular genes (Qin et al., 2012; Mikami, 2013; Lin and Quin, 2014).

Both transient and stable transformation were achieved in *P. yezoensis* after years of study by a Japanese research group at Hokkaido University (Fukuda et al., 2008; Takahashi et al., 2010; Uji et al., 2009; Uji et al., 2012; Hirata et al., 2014; Uji et al., 2013). These investigators determined that the CaMV-35s promoter (part of the commercial pBI221 plasmid vector) was insufficient to achieve expression of the codon-optimized β-glucuronidase (GUS) system in *P. yezoensis*, and instead achieved GUS expression using an endogenous GAPDH promoter (Fukuda et al., 2008). In addition, codon-optimization was found to be necessary to get efficient GUS and selectable marker

expression, because the genome of *P. yezoensis* has high G + C-content (Fukuda et al., 2008; Hirata et al., 2014; Uji et al., 2013) compared to the A + T bias found in *E. coli*. With these important parameters met, the group achieved several successful transformations of *P. yezoensis*, and some of these were stable transformations.

The availability of a complete sequence for the nuclear genome of *P. umbilicalis* (Prochnik et al., 2017) and the close relationship between this alga and *P. yezoensis* prompted my attempt to develop a successful genetic transformation system. This research was proposed in collaboration with Prof. Hiroyuki Mizuta and Dr. Toshiki Uji of Hokkaido University in Hakodate, Japan, as part of an East Asia and Pacific Summer Institute (EAPSI) fellowship sponsored by the NSF and the Japan Society for the Promotion of Science. Dr. Uji and his colleagues had previously used biolistic particle bombardment to achieve stable transformation of *P. yezoensis.* In this method, plasmids are purified and mixed with microscopic gold particles, to which they adhere, and these are ejected at high speed into the subject tissue using biolistic technology (Sanford, 2000). Particle bombardment was applied successfully in *P. yezoensis* to achieve ~2.3 % transformation efficiency (i.e., 700 cells per 1 cm diameter disc of *P. yezoensis* blade that contained ~ 3×10^5 cells, Fukuda et al., 2008; Takahashi et al., 2010).

The goals of my project were to establish a protocol for stable transformation in *P. umbilicalis* using pre-prepared plasmids, which testing the efficiency of the antibiotic hygromycin B previously used by Dr. Uji's team to select transformants. Hygromycin B interferes with translocation, effectively stopping transcription (Lackie, 2015). In the event that a stable transformation system was established, the system would be tested further by creating an overexpression study of heat shock factor (HSF) in the tissue.

HSFs are highly conserved transcription factors (TFs) that are found across all domains of life. There is some variation in sequences between organisms, but the DNA binding domain remains highly conserved (Riechmann et al., 2000; Zhou et al., 2011). The primary function of HSFs is to induce heat shock protein (HSP) expression, which prevents misfolding and denaturation of proteins under conditions such as high heat ((Lis J and Wu C, 1992; Wu, 1995; Lin et al., 2001). Several studies of HSP expression and function were conducted in *Pyropia* (Zhou et al., 2011; Choi et al., 2012; Choi et al., 2015), but the expression of HSF genes remains largely unexplored in macroalgae. Because HSFs are highly conserved and control the system of HSP expression, they are ideal targets for testing a protocol for stable genetic transformation in *P. umbilicalis*.

Here, I demonstrate successful transformation of *P. umbilicalis* using particle bombardment under several different conditions. I also determined the sensitivity of *P. umbilicalis* to the antibiotic hygromycin B.

Methods

Plasmid construction

The PyAct1-PyGus plasmid was used for GUS expression in *P. umbilicalis*. This plasmid was previously constructed from an *E. coli* pBI221 backbone containing the CaMV-35s promoter upstream of the bacterial GUS gene. The bacterial GUS gene was removed and replaced with an artificial GUS gene codon-optimized for use in *Pyropia* (PyGUS) to create p35s-PyGUS (Fukuda et al., 2008). Takahashi et al. (2010) later removed the CaMV-35s promoter and replaced it with the *P. yezoensis* Actin1 promoter (PyAct1) to create PyAct1-PyGus for GUS expression in *Pyropia*.

Culture conditions

Samples for particle bombardment were taken from neutral spore margins of cultured *P. umbilicalis*, which was isolated in 2015 from a wild blade collected in Lubec, ME (strain "CCAR. P.um.2", hereafter as "Pum2"). To produce neutral spores, plants were grown in 3 L transparent plastic cylinders (n=2 plants/cylinder) in sterile seawater with full-strength West-McBride nutrient solution (Andersen et al., 2005, 10 ml stock enrichment/L seawater) and 250 mg/L germanium dioxide (Stein, 1973) to prevent nuisance diatom growth. Plants were grown under conditions of 10:14 L:D cycle, 60-80 µmol photons m^{-2} s^{-1}, at 12 °C with vigorous bubbling to produce good growth and development of a reproductive (NS) margin.

Tissue preparation

Because of the promising result of a preliminary 5-day sulfatase treatment (see Appendix A), a full trial of particle bombardment on sulfatase-treated tissues was used. I incubated sections of *P. umbilicalis* neutral spore margins, measuring 1 cm across the margin edge and 1 cm deep into the blade (final sections: 1cm x 1cm) for 1, 5, or 10 days in sulfatase from *Helix pomatia* (S9626, Sigma-Aldrich, St. Louis) under standard culture conditions (n=4 tissue sections each). A group of freshly-cut, marginal sections with no sulfatase treatment was used as a control (n=4).

I also conducted a second bombardment on newly released neutral spores, which did not incorporate sulfatase pre-treatment because neutral spores do not have cell walls. For this trial, reproductive blade margins from whole plants (cultured as above) were removed and shredded with forceps to produce a loose slurry of spores and tissue. Large

ripe packets of neutral spores were also squeezed from blade margins and added to this mixture to provide as many naked NS as possible (referred to as "NS treatment" hereafter). Approximately 1 ml of this solution was placed in a 35 mm petri dish containing a layer of 2.0 % agarose (Seachem LE, Madison, GA) with a 0.5 cm central divet for the spores (n=4 aliquots).

Particle preparation and bombardment

The Particle Delivery System – 1000/He (PDS-1000/He, Bio-RAD, Hercules, CA) was used to deliver PyAct1-PyGUS into the *P. umbilicalis* tissues. Plasmids were prepared following Uji et al. (2013). A stock solution of 0.6 µm gold particles (Bio-RAD, 60 mg/ml in 50% glycerol) was vortexed in a microcentrifuge tube for 3 min to break up particle aggregations. This was then diluted by adding 200 µl of stock to 600 µl of 50% glycerol in Milli-Q deionized H_2O (EMD-Millipore, Darmstadt, Germany). The diluted gold solution was vortexed, and 40.0 µl was added to 20.0 µl of plasmid (1 µg/µl). The tube was vortexed, and 100 µl of 2.5 M $CaCl_2$ and 40.0 µl of 0.1M spermidine (S2626, Sigma, St Louis, MO) were added to the solution and vortexed. After 2-3 min of vortexing, the solution stood for 5 min at room temperature, and was quick-spun at 5000 rpm in a microfuge (Eppendorf 5145). The supernatant was removed, and 140 µl of 70% reagent-grade ethanol (EtOH) was added and quickly removed by pipetting to wash the particles. Immediately, 140 µl of absolute EtOH (Pharmco) was added and removed again using the same method. Finally, 15.0 µl of absolute EtOH was added to the pellet, and the tube flicked and vortexed to break aggregation. From this final solution, 3.3 µl were withdrawn and spread across a plastic macrocarrier and allowed to dry. This produced enough solution of particles for four macrocarriers (#1652335**,** Bio-RAD).

The tissue sections from the sulfatase treatment groups were placed on 2 cm x 2 cm sections of seawater-dampened filter paper, and blotted with light pressure using a Kimwipe. This ensured that the tissue remained affixed to the target tray and was not displaced within the chamber when the system fired. For the NS treatment, excess seawater was carefully blotted from the dishes using Kimwipes. All treatment groups were bombarded at 1550 psi helium pressure at 6.0 cm distance with a 28" Hg vacuum inside the firing chamber.

GUS staining

Following particle bombardment, *P. umbilicalis* tissues were recovered under standard culture conditions. After two days, tissue sections were placed into 1.5 ml microcentrifuge tubes and incubated in 100-200 µl of 5-bromo-4-chloro-3-indolylglucuronide (X-Gluc) staining solution as specified in Uji et al. (2013). The solution included 2.0 mM X-gluc, 5.0 mM potassium ferricyanide, 5.0 mM potassium ferrocyanide, 0.5% Triton X-100, 1.5 M D-sorbitol and 50.0 mM sodium phosphate buffer (product numbers RES1177B, 702587, 455989, T8787, S1876, S-9763, Sigma, pH 7.0). In the neutral spore trials, a disc of agarose with the settled spores was excised from the dish, trimmed, and placed into a 96-well culture plate with 200 µl of X-Gluc solution (as above). Tissues were incubated in this solution at 37 °C overnight in a Fisher Isotemp culture chamber (model 6842, in darkness) with slow shaking on a New Brunswick Scientific gyrotory shaker (model G2, 100 rpm), then placed onto slides and examined at a light microscope. Transformed regions showed intense blue staining due to GUS expression (Fig. 3.1).

Hygromycin B resistance test

To determine the effect of hygromycin B on *P. umbilicalis*, neutral spores were released from cultured strain Pum2 (Lubec, Maine) in 10 ml of sterile seawater. Two ml of spore solution were added to 10.0 ml plastic petri dishes containing sterile seawater with hygromycin B, for final concentrations of 0.5 mg/ml, 1.0 mg/ml or 2.0 mg/ml hygromycin B (10 ml final volume). Dishes containing no hygromycin B were used as controls. A total of n=3 dishes per treatment were used. Final spore density was ~ 1 x 10^3 cells/ml. Spores were grown in standard culture conditions (10:14 L:D cycle, 60-80 µmol photons m^{-2} s^{-1}, at 12°C, ¼ West-McBride enrichment). After a 2 d culture period, 50 spores were chosen randomly for observation. Development stage and number of dead individuals were recorded. These individuals were observed a total of 5 times (every 2-3 days) using an inverted microscope (Zeiss IM-35) over a total incubation time of approximately 14 days (Uji et al., 2013).

HSF sequence confirmation

To create an overexpression study of HSF in *P. umbilicalis*, a HSF sequence was needed. The *P. umbilicalis* project's genome assembly was searched for sequences matching heat stress transcription factors (HSTFs) found in *Arabidopsis thaliana*. Although *A. thaliana* is a higher plant, the conservation of the DNA-binding domain sequence allowed for identification of HSF genes in *P. umbilicalis* that closely matched those of *A. thaliana* (A-1a, P41151, e-value = $3e^{-38}$; HSTF A-1d, Q9LQM7, e-value= $3e^{-39}$; HSTF A-9, Q9LVW2, e-value= $4e^{-27}$, www.uniprot.org). This search was followed by a blastp (NCBI/NIH, http://blast.ncbi.nlm.nih.gov) against sequences of two red algae, *Chondrus crispus* and *Galdieria sulphuraria* (CDF34110.1, e-value=$1e^{-69}$, EME27275.1, e-value=$4e^{-61}$). The high fidelity of these matches indicated that the target HSF sequence in *P. umbilicalis* was a likely match and a good choice for a target sequence for stable genetic transformation.

cDNA generation

To produce cDNA of the HSF sequence for cloning, two large Pum1 blades (approx. 18 cm) were divided into sections (n=8 sections/plant) after removing the reproductive margins and holdfast. Tissue sections were placed in sterile petri dishes with sterile SW, and sealed with parafilm. After a 24 h recovery period at 12 C, tissue sections were divided into four groups (n=2 sections per plant, per group), a control group at 12 C (no heat shock [HS]) and 1, 3, and 6-h experimental HS groups. The HS experimental groups were placed in culture at 25 ºC and incubated for 1, 3, and 6 h, respectively, to induce HSF expression (Xu et al., 2014; Sun et al., 2015). After the indicated incubation periods, samples were removed from petri dishes, wrapped in aluminum foil, and quickly

dropped into liquid nitrogen (LN_2) to preserve expressed mRNAs. Samples were stored overnight at -80°C, and ground using a mortar and pestle. Samples were kept cold with LN_2 when not in -80°C storage, and ground tissue was quickly frozen again after processing. Extraction of RNA was done with an RNeasy Plant Mini Kit (Qiagen) following the manufacturer's instructions; 30 µl of RNA was eluted in RNase free water. The qScript cDNA SuperMix kit (Quanta) was used to generate cDNA according to the manufacturer's instructions. Products were run on a 1.5% agarose gel to confirm the presence of cDNA and products were amplified and confirmed using qPCR.

PCR conditions

To produce DNA for cloning, PCR was done on the cDNA produced as described using a GeneAmp 9700 thermocycler (ThermoFisher, USA). The initial denaturation step was 98 ºC for 10 s, followed by 38 repetitions of 98 ºC for 10 s, annealing at 55 ºC for 5 s, and extension at 72 ºC for 2 min. Per reaction, 100 ng of cDNA were used with LATaq (TaKaRa, Japan).

Results

Particle bombardment

GUS expression was visible in both sulfatase and NS treatments (Fig 3.1, Table 3.1). Numbers of GUS-expressing cells were lower in the 5-d sulfatase treatments (M = 4.50, SD = 3.11) and 10-d sulfatase treatments (M = 3.50 SD = 4.36) compared to the NS treatment (M=10.00, SD = 6.00). Controls (no sulfatase) lacked GUS-positive staining (as expected). In the sulfatase treatments, polysaccharide fragments from the blade margins appeared in petri dishes during the treatment period. Some of the spores in the NS treatment appeared to have lysed during the X-Gluc incubation (Fig 3.1 D). A one-

way analysis of variance (ANOVA) determined that treatment (including both sulfatase-treated tissue and NS treatment) did affect the transformation efficiency (Table 3.2, $F_{4, 15} = 4.9$, $p = 0.01$). A Tukey test indicated that the transformation efficiency was significantly different between the NS treatment compared to both the control and 1-d sulfatase treatment ($p = 0.01$ for both), but that no other relationships were significant. Overall, the transformation efficiency observed in *P. umbilicalis* across all treatments was low compared to efficiencies achieved in *P. yezoensis.* A 1 cm x 1 cm section of *P. umbilicalis* tissue has ~3.7×10^5 cells, making the transformation efficiency less than 0.00003 %.

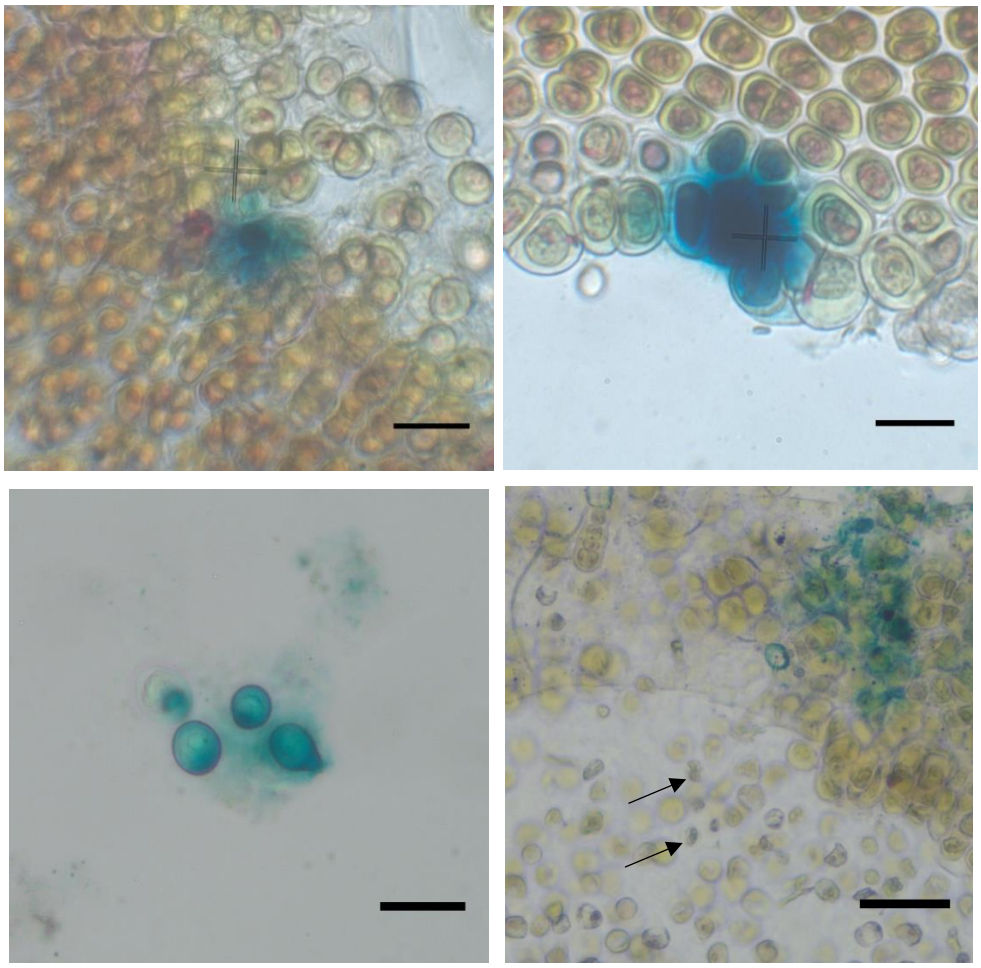

Figure 3.1 GUS expression of *P. umbilicalis* under different treatment conditions

A. GUS-expressing neutral spore at the edge of a neutral spore margin (5-day sulfatase treatment). There were few other transformed regions nearby. B**.** Transformed vegetative cell on the edge of reproductive margin section (10-day sulfatase treatment). C**.** GUS-expressing germlings. GUS expression is clearly leaky in this image, especially in the germling furthest to the left. (fully exposed spore trial). D. GUS-expressing neutral spores still partially bound to adult tissue (right). A number of fully released spores that have lysed can be seen in the lower half of the micrograph, indicated by black arrows. Scale = 30 µm.

Table 3.1 Means and standard deviations for particle bombardment outcomes

Treatment	N (samples)	Mean	S.D.
Control	4	0.00	0.00
1-d sulfatase	4	0.50	1.00
5-d sulfatase	4	4.50	3.11
10-d sulfatase	4	3.50	4.36
NS treatment	4	10.00	6.00

Table 3.2 One-way ANOVA for treatment effect on transformation efficiency

	DF	Sum Sq.	Mean Sq.	F	P
Treatment	4.0	257.0	64.3	4.9	0.01*
Residuals	15.0	197.0	13.1		

Hygromycin resistance test

Hygromycin B had a clear effect on *P. umbilicalis* neutral spores (Fig. 3.2). By day 5 of observation (~14 days after the start of treatment) higher numbers of dead spores were found in the hygromycin B treated samples compared to controls (Table 3.3). Comparing outcomes for the 50 spores/dish (n=3 dishes, all groups) that were tracked daily, in the control group, an average of 3.7 spores (SE ± 1.2) had died. By comparison, an average of 14.34 (SE ± 2.34) died in the 0.5 mg/ml treatments, 28.34 (SE ± 3.84) died in the 1.0 mg/ml treatment, and 37.34 (SE ± 3.34) died 2.0 mg/ml treatments, respectively. A Shapiro-Wilk test indicated these data were non-normal ($p = 0.02$) so a Kruskal-Wallis test was used to analyze the data. This test determined that hygromycin B level had a significant effect on the number of dead spores ($p = 0.02$), and a Dunn test

with Bonferroni correction was used and produced pairwise significance values. The Dunn test indicated that the 2.0 mg/ml treatment was significantly different from the control group (p = 0.03), and no other relationships were found to be significant.

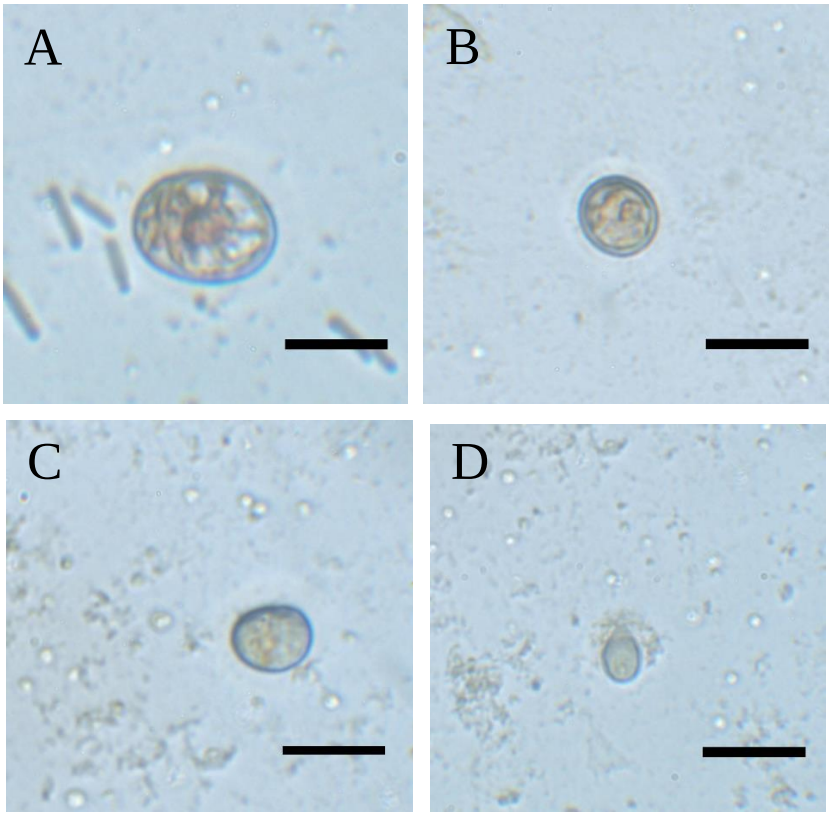

Figure 3.2 Germling condition on day 14 of 14-d hygromycin B experiment

A. Healthy algal spore; the red stellate chloroplast is evident. B. Healthy spore in the 0.5 mg/ml hygromycin B treatment. C. Dead spore from the 2.0 mg/ml treatment group; ~75% of spores in this treatment had died by the final day of observation. D. Dead spore from the 2.0 mg/ml treatment group. In both C and D, numerous bits of dead algal debris were spread throughout the medium. Scale bars = 30 µm.

Table 3.3 Means and S.E. of dead germlings in hygromycin B treatment

Hygromycin	n	Mean	S.E.
Control	3	3.66	1.21
0.5 mg/ml	3	14.34	2.34
1.0 mg/ml	3	28.34	3.84
2.0 mg/ml	3	37.34	3.34

HSF overexpression assay

Multiple rounds of PCR failed to amplify the *P. umbilicalis* HSF sequence from cDNA generated from heat shock treatments. Initially, a PCR using the primers NdeI-PuHSF-F1 and SpeI-PuHSF-R1 was performed. This primer set targets a *Nde*I restriction site 3 bp upstream of the ATG start codon and an *Spe*I site just before the stop codon. The results from PCR produced indistinct, blurred bands (data not shown). A second PCR used two primer sets, Pum-HSF-F1 and Pum-HSF-R1 to target the first exon, and Pum-HSF-F2 and Pum-HSF-R2 to target the second exon. The goal was to amplify this section of the gene as two products, and upon successful product recovery, ligate the fragments and clone them into the expression plasmid. However, results from this PCR also resulted in low product and blurred bands. A third attempt to recover the entire HSF open reading frame by PCR was made, again using two primer sets to amplify this region in two separate parts. Primer set PuHSF-ORF-F1 and Pu-HSF-ORF-R2 began at the first ATG codon and ended at an *Sph*I site approximately 1240 bp from the start. The second primer set Pu-HSF-ORF-F2 and Pu-HSF-ORF-R1 began downstream of the *Sph*I site and ended at 2520 bp, at the final stop codon of the ORF. The results from this third PCR were similar to my previous trials, and I concluded that the cDNA concentration was too low to produce enough PCR product for cloning. A second cDNA production and

purification was done using the methods described above, and cDNA was recovered using a TaKaRa PrimeScript II First Strand kit, and PCR using the ORF primer sets was conducted. Repeated PCRs on the second cDNA product produced results like those of the first group, so no further progress could be made toward a HSF overexpression assay. A complete list of primers can be found in Table 3.4.

Table 3.4 *P.umbilicalis* HSF primers

Gene ID	Primer ID	Sequence
P. umbilicalis HSF	NdeI-PuHSF-F1	5'-GGAATTCCATATGATGTCCACTAAAAACTCCTC-3'
"	SpeI-PuHSF-R1	5'-GACTAGTTCACACTGTTTTCCGCAGAG-3'
"	Pum-HSF-F1	5'-ACGACACAGCGCCTCTTTAC-3'
"	Pum-HSF-R1	5'-CAAACGTCCAGCGATCAGG-3'
"	Pum-HSF-F2	5'-AAAGCCGCTCAACTACATGG-3'
"	Pum-HSF-R2	5'-CTCACCATCATTTGGCGCAT-3'
"	PuHSF-ORF-F1	5'-ATGTCCACTAAAAACTCCTCGAC-3'
"	PuHSF-ORF-R1	5'-TCACACTGTTTTCCGCAGAG-3'
"	PuHSF-ORF-F2	5'-GCATGCGTTAAGGGTGTGG-3'
"	PuHSF-ORF-R2	5'-GCATGCATTCAAAATGCATG-3'

Discussion

This study made significant progress towards creating a transformation system for *P. umbilicalis*. The hygromycin B assay indicated that this antibiotic inhibits *P. umbilicalis* development, although it did not completely kill all *P. umbilicalis* spores. These results differ from those in *Pyropia* where 100 % of germlings had died at the end of a 2-week hygromycin B treatment at all concentrations studied (Uji et al., 2013). The fact that ~75 % of germlings in the 2.0 mg/ml treatment died is promising, but hygromycin B may need to be applied at a higher concentration to select transformants. An additional ~20 % of spores remained ungerminated in this treatment, compared to ~3.0 % in the control group. These spores were alive, but slow growing, and it is likely that the high level of hygromycin B in the 2.0 mg/ml treatment caused inhibition of growth. Neutral spores successfully transformed with a plasmid containing the *Aph7* hygromycin B resistance gene would continue to grow and divide during hygromycin B treatment, making it easy to distinguish them from non-transformed spores that remained as single cells or died.

The results from the particle bombardment trials emphasize the difficulty of achieving successful genetic transformation in macroalgae. Even when tissues were treated with sulfatase or a solution of NS were used, genetic transformation efficiency remained low compared to the efficiency of 700 cells per 1 cm diameter tissue disc achieved in *P. yezoensis* (Fukuda et al., 2008; Mikami et al., 2009; Uji et al., 2013). This is also interesting in the context of the thicker cell wall in *P umbilicalis* compared to *P. yezoensis*, and may be an evolutionary consequence of their positions in the intertidal zone. *Porphyra umbilicalis* occupies the mid to high zones in the Atlantic, whereas *P.*

yezoensis occurs in the low intertidal zone of Japanese shores. ANOVA indicated that the type of treatment had some effect on the transformation efficiency; in particular, transformation of NS was significantly more successful than control or 1-day sulfatase treated whole blade margins bearing neutral spores. However, the NS treatment did not produce significantly more transformed regions than the 5-day and 10-day sulfatase treatments.

One consideration is that the concentration of transformed regions in the NS treatment was much higher compared to using whole blade margins, because of the small amount of spore slurry used. However, one downside of this treatment was that many of the fully exposed spores seemed to lyse during the treatment unless they were still partially bound to the blade margin. It is possible that some of these spores had been transformed, but that any GUS expression had leaked out and dispersed when the spores lysed. Further attempts to expose and concentrate spores might yield better results, particularly if the osmotic conditions required to prevent lysis of spores can be maintained. When a suitable selectable marker is found, such as an antibiotic resistance gene, it would be possible to select even small numbers of transformants from the agarose dishes because the spores in these treatments were highly concentrated. These could then be grown to adult size, and stimulated to produce neutral spores which would carry the desired mutation if they had been stably transformed. In this way, it would be possible to grow high levels of transformants from a small number of transformed spores.

Codon optimization and promoter selection are critical to achieving successful genetic transformation. Codon optimization of the GUS genes is likely sufficient because *P. yezoensis* and *P. umbilicalis* are both G + C-rich (Nakamura et al., 2013; Brawley et al., submitted), but promoter selection may need improvement. Further analysis of *P. umbilicalis* actin genes using RT-PCR would be beneficial in determining whether this promoter or another is best suited for expression of GUS. Fukuda et al. (2008) used a GAPDH promoter with success in *Pyropia*, so this is a possible alternative to Act1.

Repeated attempts to amplify the HSF gene with PCR were unsuccessful. This suggests that the high G + C-content requires longer primers or higher primer concentration and/or that some sequence is inaccurate. If the HSF sequence can be optimized and successfully cloned, transformed *P. umbilicalis* could be selected using a concentrated NS solution as described above, and the HSF expression test could be conducted on germlings from this experiment.

Overall, this study has laid a foundation for developing a system of stable genetic transformation in *P. umbilicalis*. With the availability of a fully-sequenced genome, it will also be possible to develop additional promoters for improved expression of GUS and other genes in *P. umbilicalis*. These techniques provide a valuable tool for any scientist interested in conducting genomic studies in this important model organism, and the particle bombardment protocol developed here will also be applicable to CRISPR development, because biolistics were used for CRISPR studies of the diatom *Phaeodactylum* (Nymark et al., 2016).

CHAPTER 4

USE OF SCANNING ELECTRON MICROSCOPY (SEM) TO VISUALIZE INTERACTIONS BETWEEN *PORPHYRA UMBILICALIS* AND ITS MICROBIOME

Introduction

Bacteria and algae have many interactions (reviewed in Goecke et al., 2010, Goeke et al., 2013). Some bacteria feed on algal cell walls for their carbon source, including some pathogens (Barbeyron et al., 2001; Egan et al., 2001; Uppalapati et al., 2001; Kim et al., 2016), but many provide benefits to their host algae (reviewed by Miranda et al., 2013). Many freshwater and marine eukaryotic algae use vitamin B_{12}-dependent methionine synthase, but only prokaryotes synthesize this vitamin (Croft et al., 2005; Kazamia et al., 2012, Helliwell et al., 2016). The cyanobacterium *Prochlorococcus* depends on bacterial associates to detoxify the hydrogen peroxide (H_2O_2) that forms from photooxidation of dissolved organic carbon in oceanic habitats at shallow depths (Morris et al., 2011). In both examples, the algae do not have the required genes to accomplish what is done for them by their bacterial "helpers".

Bacteria also affect algal morphology and development. Fries (1975) demonstrated that the green alga *Ulva linza* (as *Enteromorpha*) lost its normal morphology after one year of axenic (bacteria-free) culture. Enrichment of seawater with vitamins did not restore the lost morphology, and the alga persisted in a filamentous state for years afterwards. Provasoli and Pintner (1980) later produced a foundational study in this field examining the effects of different bacterial mixtures on *Ulva lactuca* grown in axenic conditions for 15+ years. From the beginning of axenic culture, this *Ulva* strain

exhibited abnormal growth and morphology. Bacterial mixtures and filtrates taken from normal *Ulva* and "Enteromorpha" cultures produced ribbon-like growth from the axenic *Ulva* strain, and readdition of different, single bacterial colonies produced different degrees of recovery, assayed by morphological changes. Filtrates from the bacterial cultures had no effect on growth, indicating the necessity for bacteria to grow with the algae to influence their morphology, possibly in order to induce the bacteria to produce the growth regulator. A later study of *M. oxyspermum* demonstrated that only specific bacterial cultures could rescue normal morphology (Matsuo et al., 2005) and so could culture medium that had a *Cytophaga*-like bacterium growing in it. A plant growth regulator (PGR), termed "thallusin", was isolated from culture of a single bacterium obtained from the surface of *M. oxyspermum*, and thallusin had significant restorative effects on the abnormal growth displayed by this alga under axenic conditions. When conchocelis was cultured axenically in the red alga *Pyropia yezoensis*, conchospores differentiated normally but as they germinated into tiny blades, development became disorganized, and abnormal blades became callus-like under axenic conditions (Yamazaki et al., 1998). Normal growth of axenic *P. yezoensis* gametophytic protoplasts was mostly restored after 6 weeks of co-culture with specific bacterial isolates (Fukui et al., 2014), indicating the importance of bacteria to early algal development.

Despite the observed importance of bacteria to most algae at all life stages, little is known about their physical associations and community structures on the macroalgal surface. Many studies demonstrate that bacterial populations on host algae are highly diverse (Burke et al., 2011; Spoerner et al., 2012; Miranda et al., 2013; Fukui et al., 2014), and that direct contact with the host may sometimes be essential for algal survival

(Provasoli and Pintner, 1980). Because of *Porphyra*'s ancient origins as a member of the Bangiaceae (1.2 Ga *Bangiomorpha* fossil, Butterfield, 1990), the relationships between *P. umbilicalis* and its microbial communities have evolved over long periods of time, and some of these interactions may be quite specific.

Scanning electron microscopy (SEM) is a tool for understanding the physical relationships between microbes and their host organisms. A SEM scans the surface of metal-coated specimens and produces a three-dimensional image of fine structures (Alberts et al., 2014). SEM was used to image microbial communities on both microalgae (Kaczmarska et al., 2005) and macroalgae (Cundell et al., 1977; Goecke et al., 2010; Goecke et al., 2012). In *Pyropia*, SEM helped elucidate interactions between host and parasites (Uppalapati et al., 2001) and the development of reproductive structures (Mei et al., 2005; Yang et al., 2012). To my knowledge, no SEM studies of the microbial community of *Porphyra* are available. Conventional light microscope techniques rarely resolve these communities adequately, although DAPI staining can confirm the presence or absence of bacteria along the algal surface, and FISH techniques can resolve spatial relationships if the bacteria are taxonomically resolved to allow FISH labels to be developed. Often, however, structural information is needed when such taxonomic information is unavailable, and SEM techniques are ideal to reveal structural interactions at high magnification and resolution.

In this study, I used SEM to visualize the location and community structure of microbes on the *P. umbilicalis* blade, and I made comparisons between the microbial component of the holdfast and blade margin using both wild-collected and unialgal cultures of the host. I also used SEM to determine whether bacteria were present on neutral spores, as they are being released from blades, and to determine whether there were differences between wild and cultured specimens.

Methods

Wild specimen collection

SEM was conducted on both wild- and cultured specimens. Wild *P. umbilicalis* (n= 6 individuals) were haphazardly collected from the high intertidal zone at Schoodic Point, Acadia National Park, in late December 2016 (under permit ACAD-2017-SCI-0006) for SEM (n = 6 plants). Temperature was approximately -10 ºC, with full sun. Whole plants were carefully scraped from the rock face using a sterile razor blade, placed in sterile, autoclaved foil packets, and returned to the lab on ice. One piece of reproductive margin (ranging from 0.5 mm^2 to 1 cm^2) and the holdfast were removed from each plant with sterile scissors and forceps, and placed immediately into fixative (see below)

Preparation of cultured *P. umbilicalis*

Neutral-spore margins of cultured *P. umbilicalis* (strain Pum2, see Chapter 2) were isolated from a wild blade in Lubec, ME in May 2015 and were used in SEM. To produce neutral spores, plants were previously grown in 3 L plastic cylinders (n=2 plants per cylinder) in sterile seawater with full-strength West-McBride nutrient solution (Andersen et al., 2005, 10 ml/L) and 250 mg/L germanium dioxide to prevent nuisance diatom growth. Plants were grown under conditions described in Chapter 2.

Blade margins from two groups of these lab grown specimens were used for SEM. In the first group, a single plant bearing NS, ~15 cm long, was selected, and blade margins were removed using the same methods as for wild specimens. The second group of cultured specimens was of similar size, with large neutral spore margins from three plants. Large pieces of spore margin (~5 cm long x 1 cm deep) from each plant were removed and placed into sterile Petri dishes with sterile seawater. Areas with large pockets of mature neutral spores were cut into smaller pieces (0.5 mm^2 to 1 cm^2) with sterile tools and placed into fixative.

Tissue processing for SEM

Fixation procedures were similar to those of Babuka and Pueschel (1998). All specimens were fixed in 5% glutaraldehyde, 0.1 M sodium cacodylate buffer (pH 7.0), and 0.2 M sucrose for 2 h on ice. Specimens were rinsed 3 x for 15 min in the same buffer without sucrose, then post-fixed in 1% OsO_4 and 0.07 M sodium cacodylate for 1 h on ice. Specimens were rinsed again in the same buffer as before (3 x, 15 min each). Tissues were dehydrated in a graded ethanol series (7 min each step, 30 %, 50 %, 70 % [x

2], 80 %, 85 %, 90 %, 100 %[x 4]). A small portion of one blade margin from a cultured individual was freeze-fractured using liquid N_2 by cooling the specimen and cracking it with a cooled razor blade. Tissues were processed in a Tousimis-Samdri critical-point dryer until no trace of ethanol remained. Specimens were mounted on stubs using double-sided carbon tape and sputter-coated with gold-palladium at 0.08 mBar, with 40 mA of current for 70 s to give a final coating that was 27 nm thick (Cressington 108 Auto Sputter Coater) before imaging with an AMRAY-1820 SEM (AMRAY Inc., MA, USA). Final images were analyzed in ImageJ (www.nih.gov/).

Results

Basic host structure

Multiple regions of the *Porphyra* blade were examined (Fig. 4.1). The cells in the monstromatic *P. umbilicalis* blade ranged from 20-30 µm long (Fig. 4.2A). In many cases, it was possible to observe a cell wall ~ 2-3 µm thick surrounding the adult cells (Fig. 4.3). Neutral spores were typically ovoid spheres of approximately 10 µm diameter (Fig. 4.2B). A mucilage with a spongy appearance could be seen coating all neutral spores (Fig. 4.4).

Variability in microbiome

Microbial communities had different structures on the holdfast and blade margin tissues from wild-collected *P. umbilicalis* (Figs. 4.5-4.8). Holdfast communities were complex, diverse, and covered nearly all the holdfast within 200-500 µm of the main attachment point (Figs. 4.5-4.6). Microbial abundance decreased on parts of the blade further from the holdfast and became patchy. Mounds of spherical unicellular organisms were observed, ranging in size from 45-100 µm. The large spherical cells in Figs. 4.9-

4.12 appear to be members of the cyanobacterial family Chroococcaceae (Humm and Wicks, 1980). Endospores appear to occur within many of these cells. In Fig 4.9 a ruptured colony is visible. Numerous bacilli and cocci colonized areas near these putative cyanobacteria. Diatoms and a possible non-geniculate coralline alga (*Leptophyllum*-like) were also observed (not shown).

Morphologically diverse bacteria occurred in all regions of the holdfast. Filamentous and chain-forming bacteria were ubiquitous on all specimens, and were often surrounded by or covered with bacilli and cocci. Some of these also appeared to be damaging the algal tissue (Fig. 4.13). Holdfast communities appeared to be made of many layers of these different organisms.

In contrast, microbial communities at the blade margin were sparser than those found on holdfasts, although diverse patches of microbes were observed along the blade margin and the adjacent portions of the blade (Figs. 4.7-4.8). Community patchiness differed between and within specimens. Some areas of the blade margin were virtually empty of microbes (Fig. 4.7), but others contained dense clusters of microbes that appeared similar to the microbes on holdfast communities (Figs. 4.8-4.9). Overall, composition of holdfast communities appeared to be more diverse than those of blade margins, particularly in terms of putative eukaryotic organisms. Taxonomic diversity of such communities is under investigation with hypervariable regions of the 16S rDNA in the Brawley lab (pers. comm. to CR).

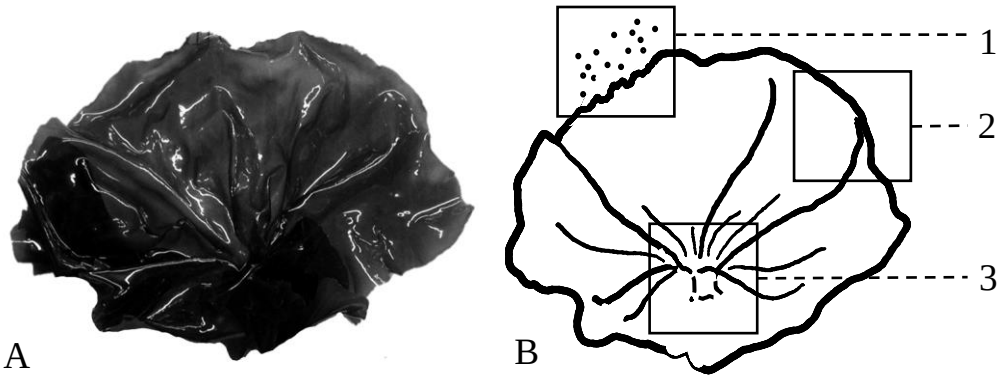

Figure 4.1 Schematic diagram of a *P. umbilicalis* blade

A. Photograph of cultured *P. umbilicalis* specimen. B. Diagram of *P. umbilicalis* based on photographed blade. Box 1: tattered blade margin edge releasing neutral spores (small circles, corresponds to Figs 2B, 4, 7, 8, 16, 17, and 18). Box 2: intact blade margin and surrounding area (corresponds to Figs 2A, 3, 10, 14, and 15) Box 3 represents the holdfast (dashed line) underneath the blade, the attachment point for the alga onto the substrate (hidden in photograph, corresponds to Figs 5, 6, 11, and 13). Not to scale.

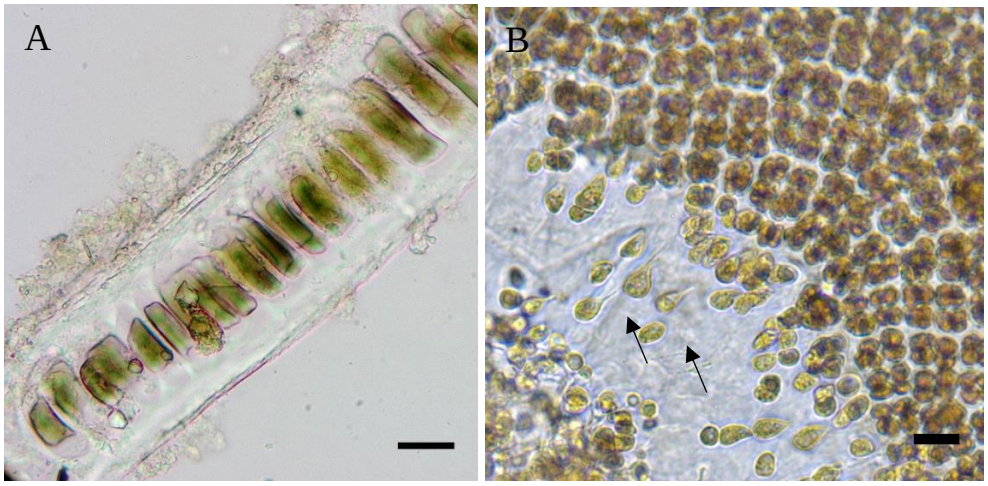

Figure 4.2 Light micrographs of adult *P. umbilicalis* cells

In A, a hand-cut cross-section of the monostromatic *P. umbilicalis* thallus shows the pigmented, large chloroplasts. The cell wall is composed of a thick layer of sulfated polysaccharides. In B, neutral spores are seen separating from the edge of the blade margin (arrows). Scale bars = 30 µm.

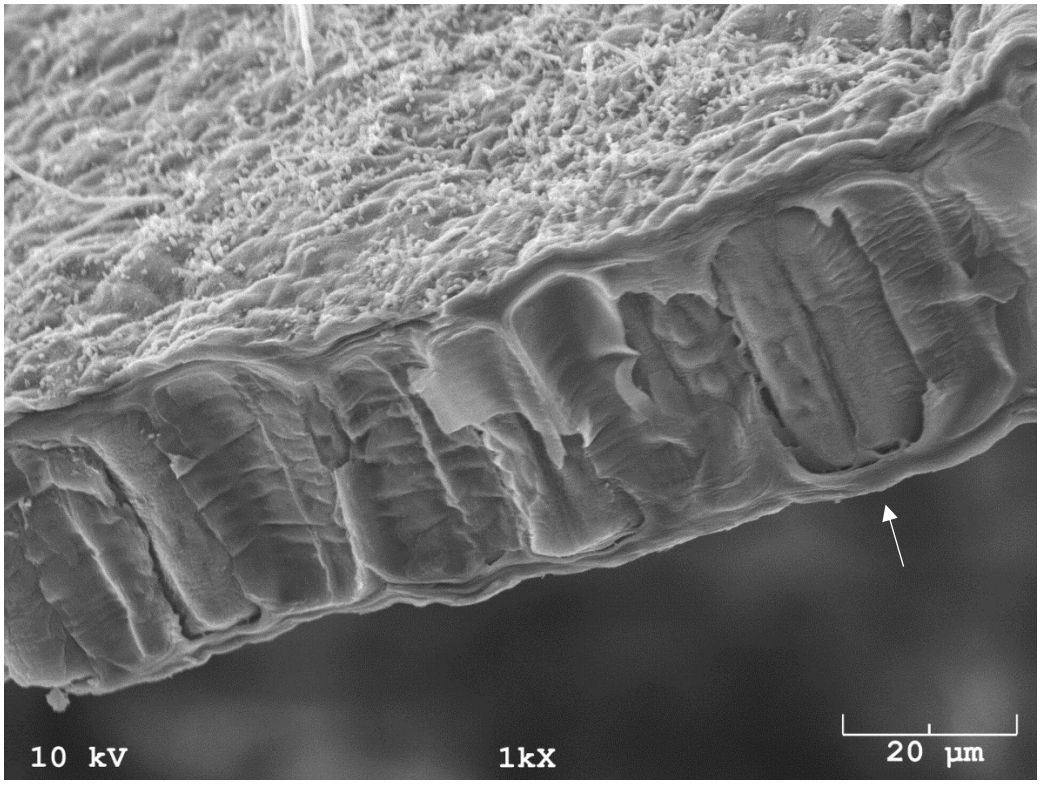

Figure 4.3 SEM micrograph of adult cultured *P. umbilicalis*

Adult cells were only exposed along edges that had been cut or freeze-fractured (shown). White arrow indicates recently dividing cell. A diverse lawn of bacteria is growing on the outer surface of the thallus in the upper portion of the image.

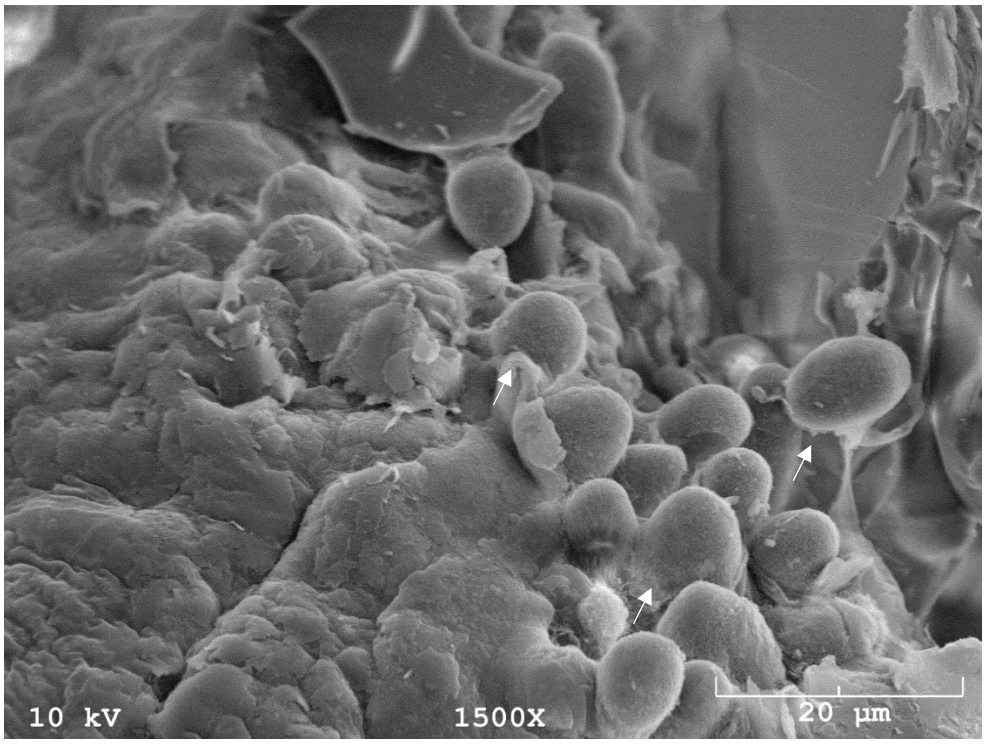

Figure 4.4 Wild *P. umbilicalis* neutral spores

Neutral spores (arrows) as they are being released from thick mucilage at the edge of the blade. Neutral spores were smaller than vegetative cells, and were usually covered with mucilage with a distinctive spongy appearance. The large flat object in the upper right corner appears to be debris embedded in blade margin mucilage. Note the apparent absence of bacterial colonies on the edge of this blade margin.

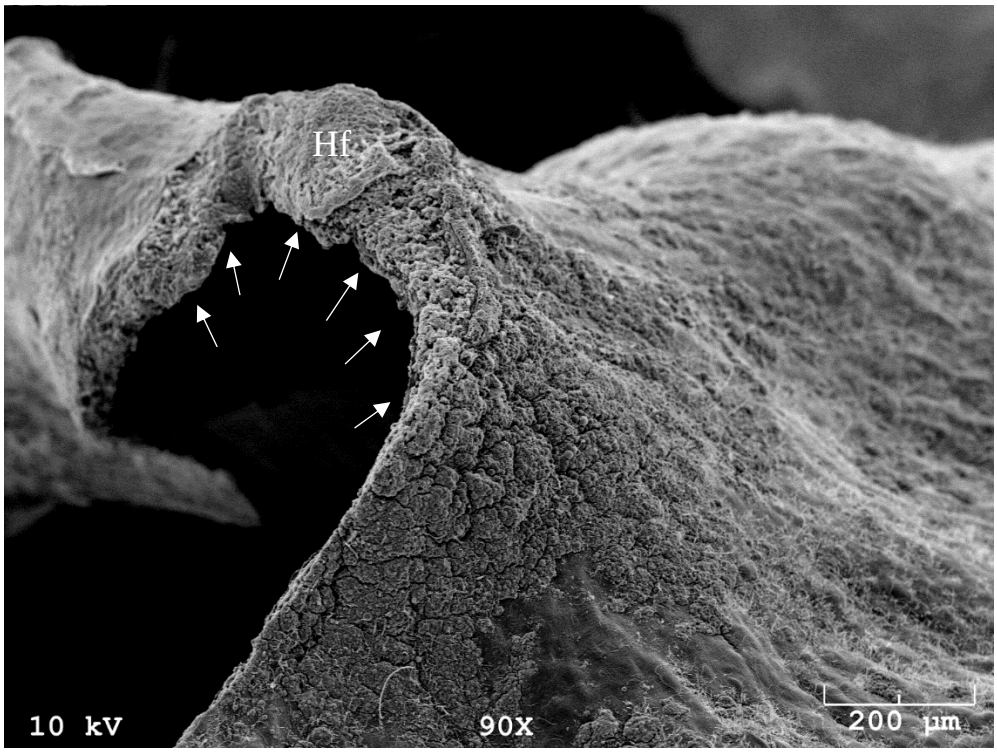

Figure 4.5 Overall holdfast community on a wild *P. umbilicalis* specimen

In this image, the main holdfast disc (Hf) and surrounding edge (marked with white arrows) are observed. These are the main attachment points for the alga, and they make direct contact with the rock at all times.

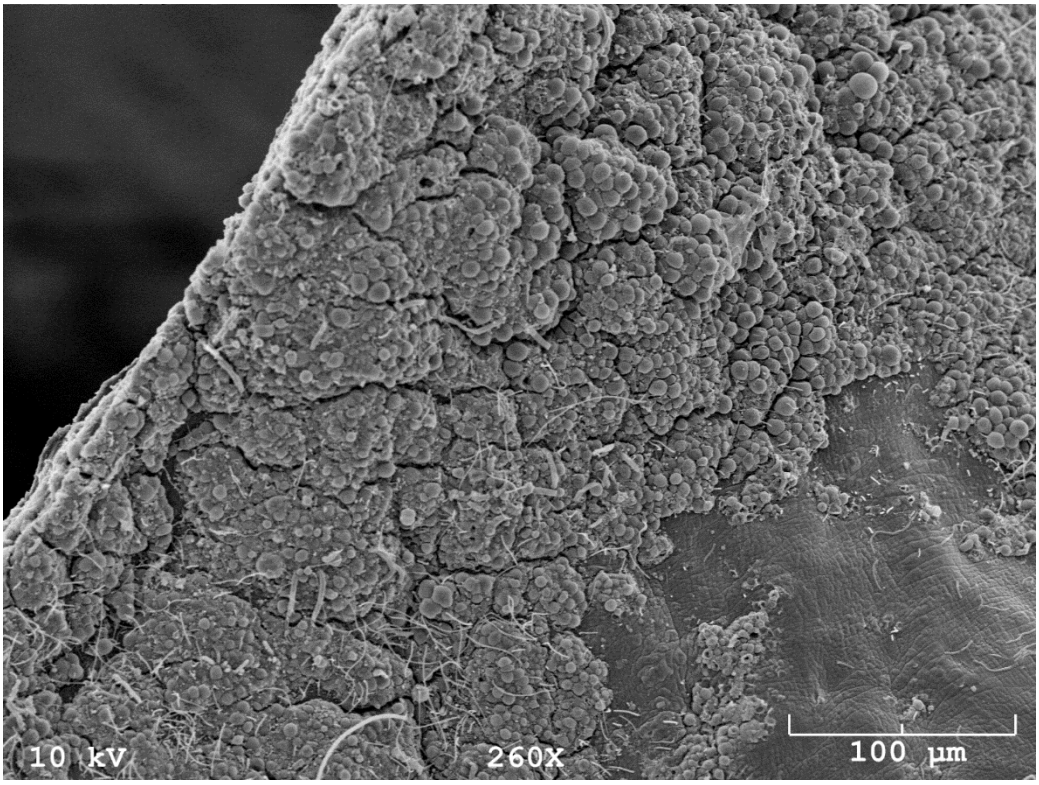

Figure 4.6 Holdfast community on the wild *P. umbilicalis* specimen from Fig. 3

At higher magnification, extensive aggregations of spherical organisms occur on the regions closest to the holdfast. Filamentous bacteria are evident on portions of the blade, but some regions of the blade surface were relatively clear of epiphytes. In the bottom, right, the community becomes patchier in areas further away from the holdfast edge.

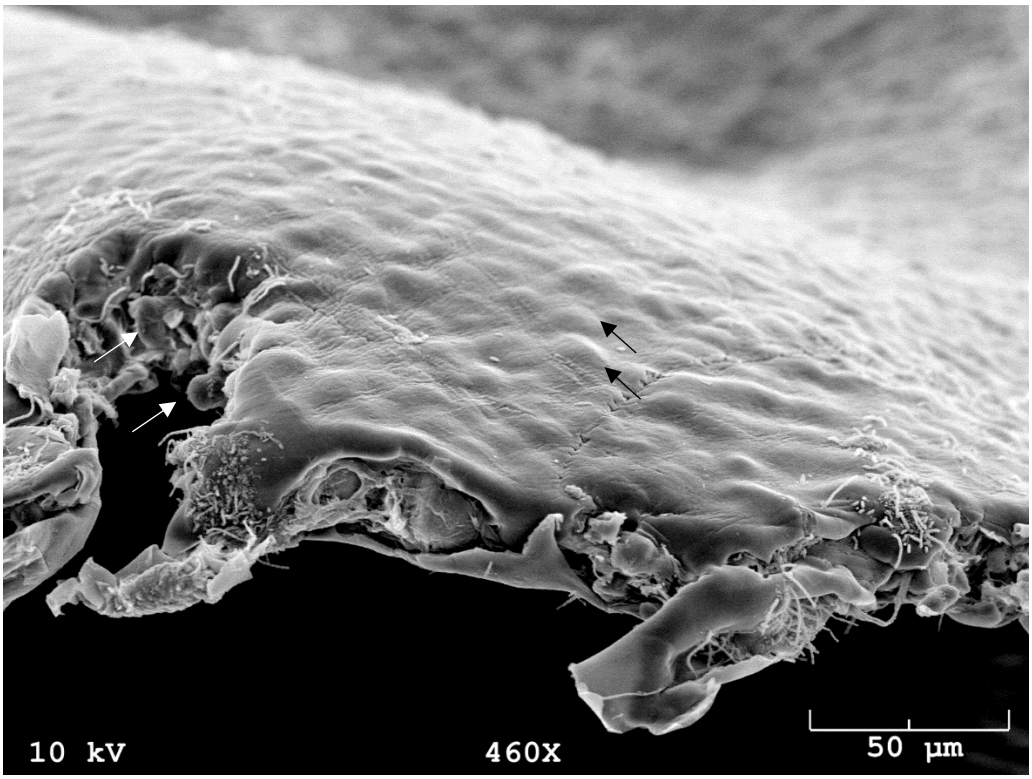

Figure 4.7 Sparse microbial community on blade margin of wild *P. umbilicalis*

This image shows a view directly into the edge of the blade margin, which is tearing open as neutral spores are released. The bacterial community was sparse on this part of the blade margin, though some small colonies are visible on the protruding edge fragments. Note the tattered edges of the margin and the neutral spores being released on the left of the image (white arrows). The thallus in this region has an overall bumpy appearance caused by bulging, mature neutral sporangia (black arrows).

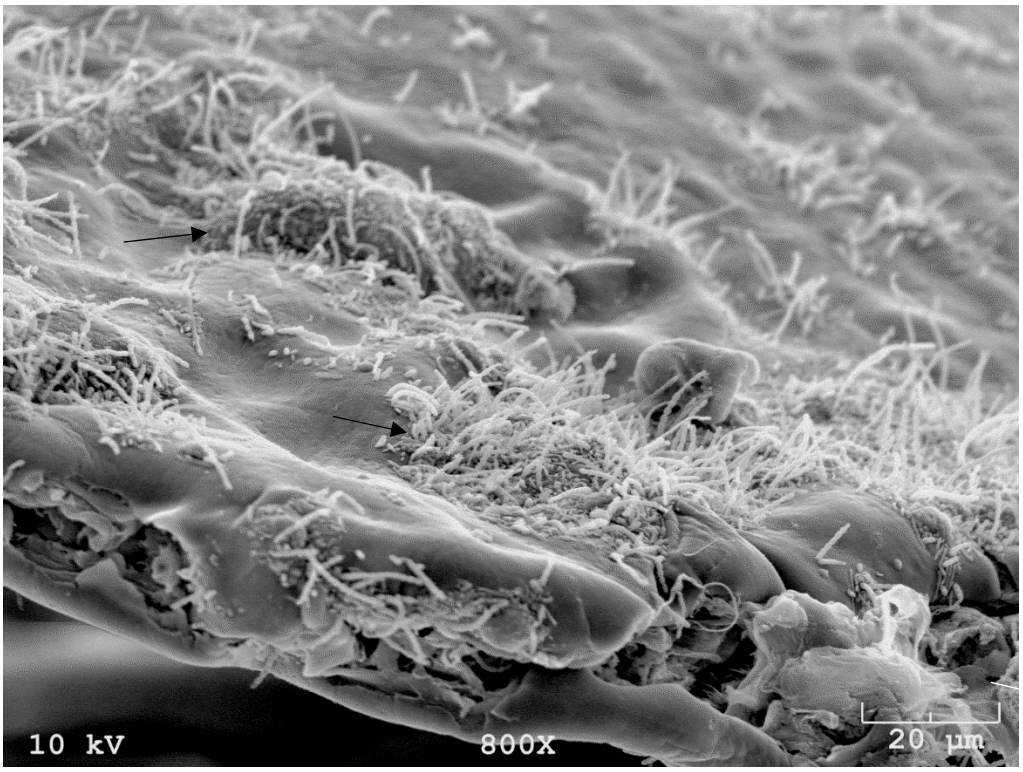

Figure 4.8 Diverse microbial community on blade margin of wild *P. umbilicalis*

This image was taken from a different region of the blade margin from the plant in Fig. 7. This image provides another direct view into the edge of the torn blade margin. In this region, dense colonies of rod-shaped, chain-forming, and filamentous bacteria can be seen growing directly on and near the blade margin opening (black arrows). A neutral spore is in the process of releasing on the right side of the photo (white arrow).

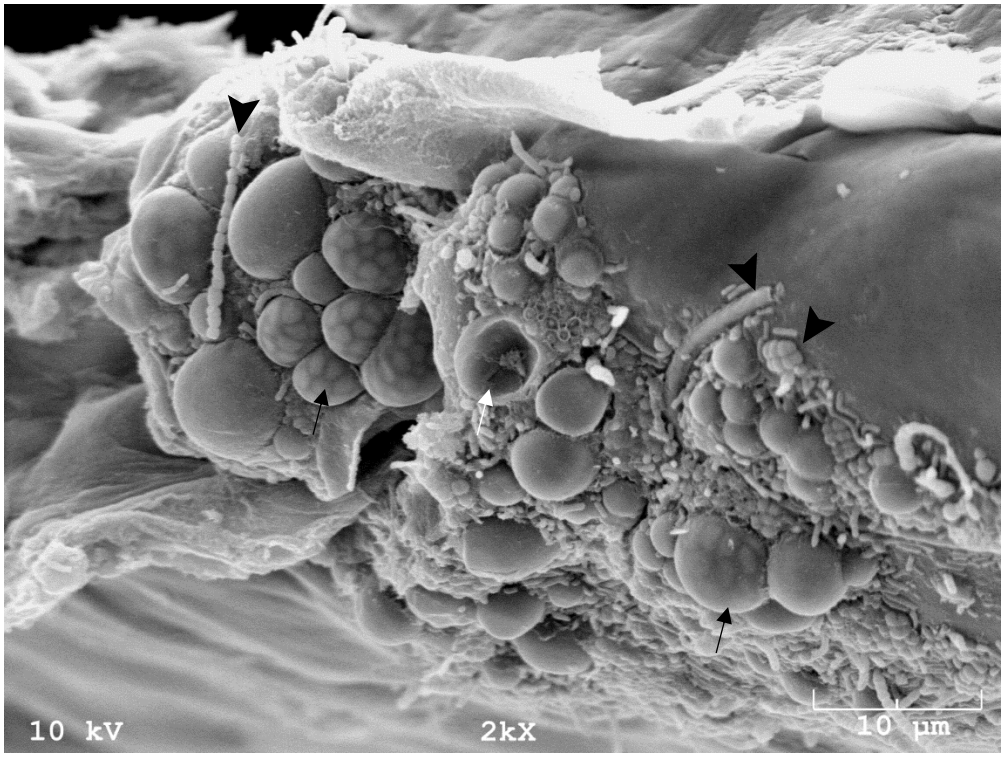

Figure 4.9 Blade margin community of wild *P. umbilicalis*

Cyanobacteria from the family Chroococcaceae on blade margin of wild *P. umbilicalis*. Black arrows indicate internal endospores. One of the large colonies has ruptured and appears to have lost endospores (white arrow). Chain-forming bacteria, bacilli, and sarcinic cocci can be seen surrounding these larger organisms (black arrowheads).

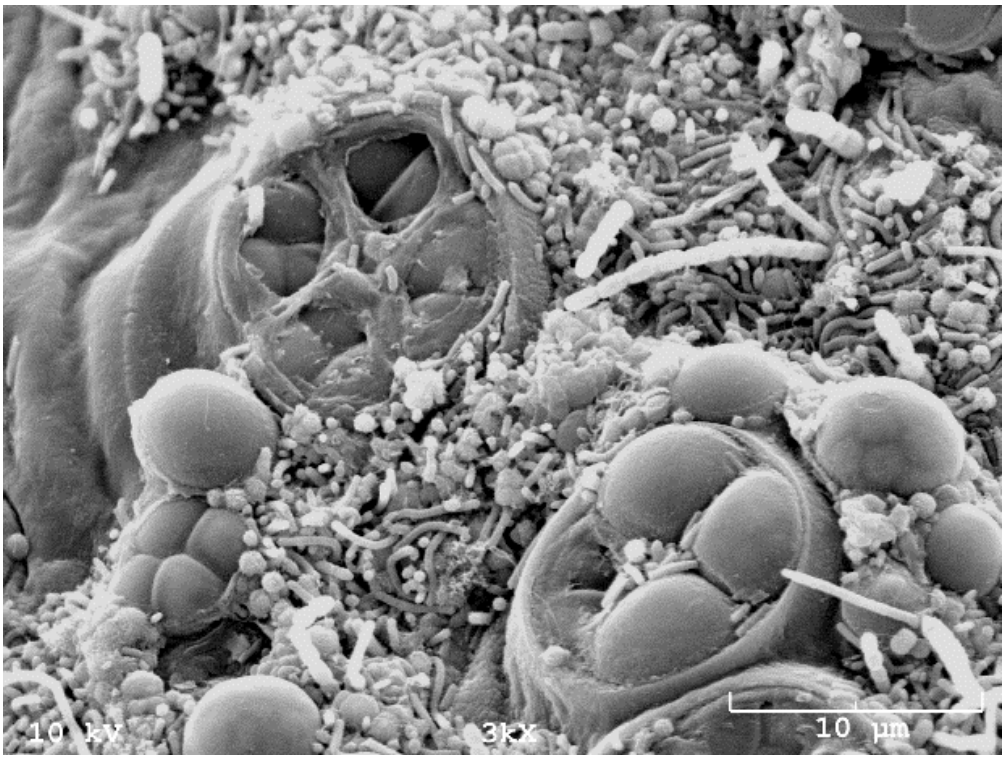

Figure 4.10 Putative cyanobacteria on wild *P. umbilicalis* blade margin

Endospores of this cyanobacterium appear to divide after settling, leaving a 2 x 2 “grid” of cells. A dense community of prokaryotic bacilli and cocci can be seen growing around these ruptured cells, revealing the endospores..

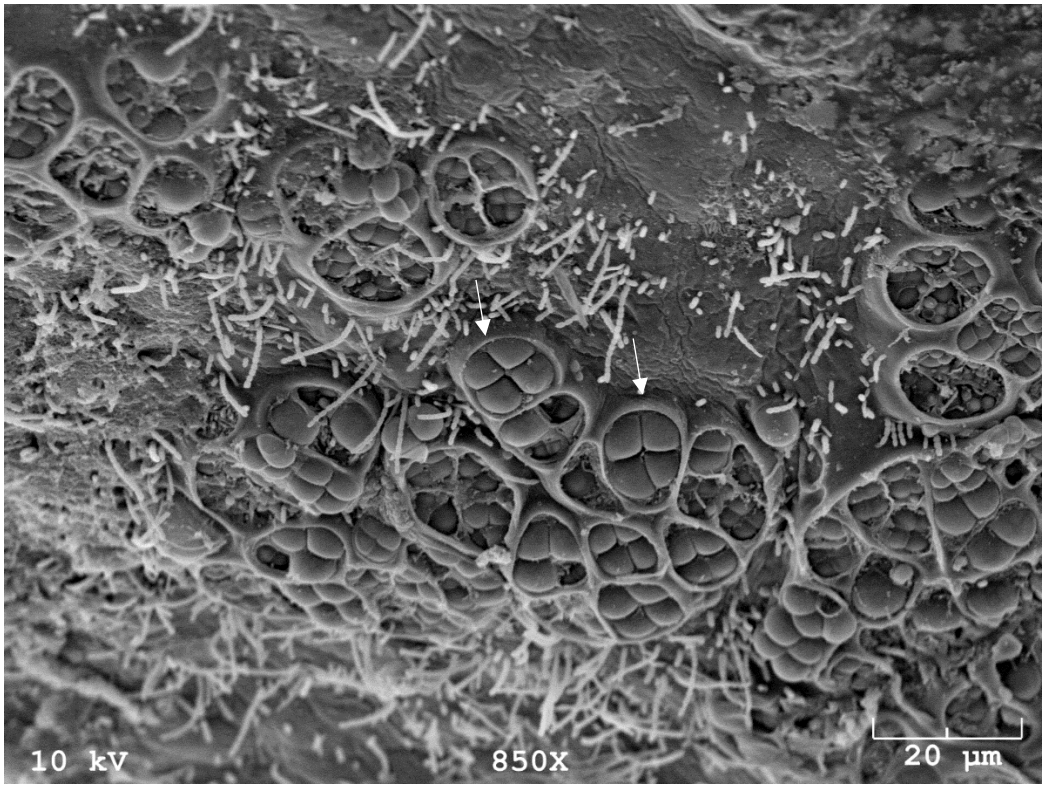

Figure 4.11 Putative cyanobacteria on wild *P. umbilicalis*

These were found growing on a flat part of the thallus near the holdfast attachment point. Ruptured colonies with putative exposed endospores (arrows) could be found growing in colonies (as shown) or singly on the holdfast or blade margin regions.

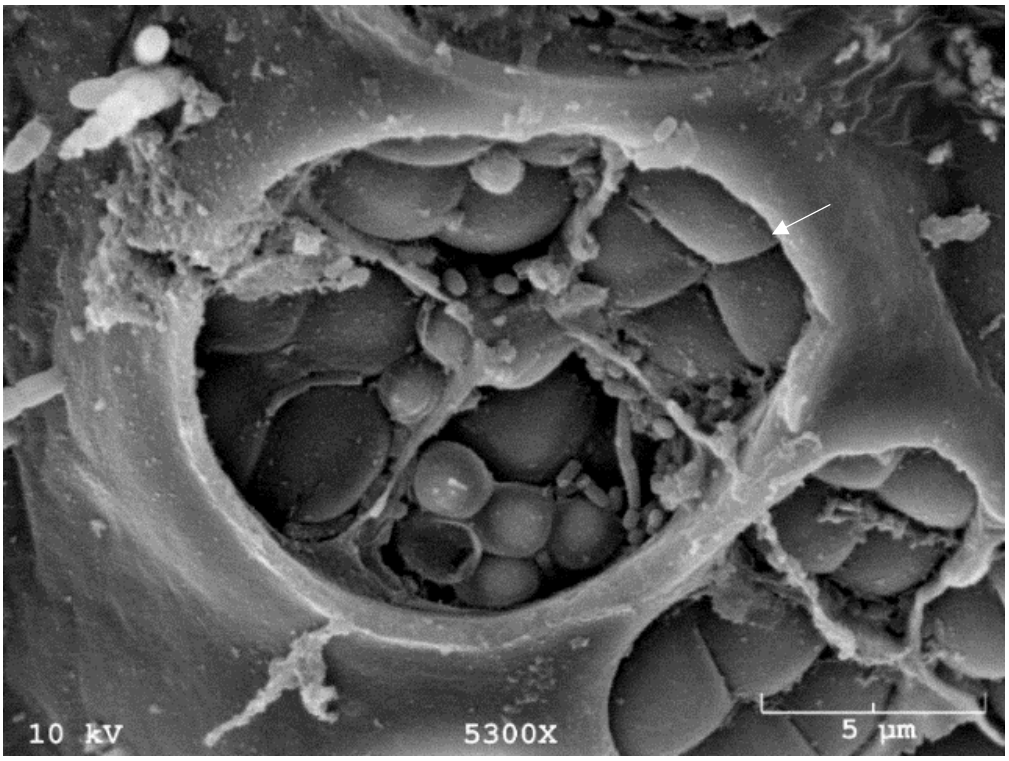

Figure 4.12 Magnified view of putative cyanobacteria

An enlarged view of exposed endospores Fig. 11 shows smaller bacteria colonizing the inner surface of this structure. White arrow indicates a smaller 2 x 2 “grid” of cells that appears to be the result of a larger cell dividing.

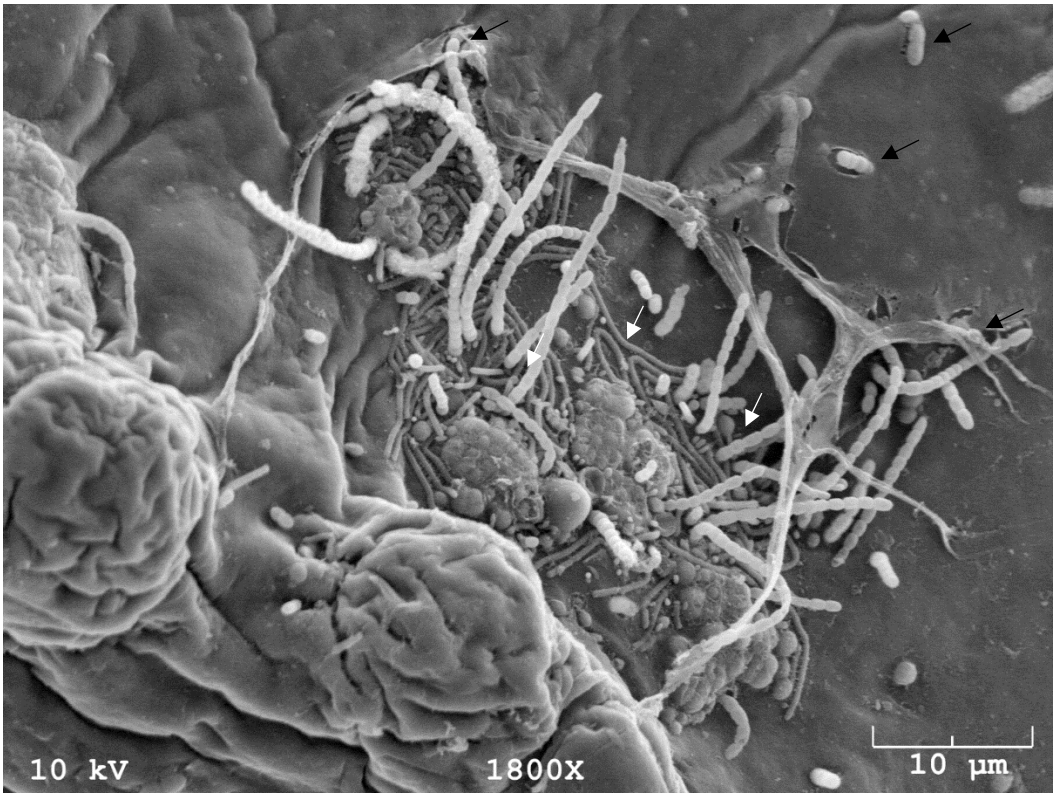

Figure 4.13 Bacteria on wild *P. umbilicalis*

This image was taken on a flat region near the holdfast of the alga. Bacterial chains (black arrows) appear to be embedded within the outer polysaccharide layer of the alga, and smaller rod-shaped bacteria and cocci appear to have colonized the exposed surface (white arrows).

Community differences between cultured and wild *P. umbilicalis*

Noticeable differences between the microbial community of wild and cultured *P. umbilicalis* (strain Pum2) were also observed (Figs. 4.14 – 4.15). Cultured blades had abundant rod-shaped bacteria measuring ~1.0 µm in length. These bacteria were found across the entire blade surface, but most densely covered an area of the blade that had a small lesion (Fig. 4.14). A large filamentous cyanobacterium also occurred on the surface of Pum2 (Fig. 4.15). Filaments were covered with clusters of smaller bacteria in some areas. Undulating or helical bacteria measuring ~15 µm were also observed on one of the cultured specimens in association with the common rod-shaped bacteria (Fig. 4.15). These bacteria were largely absent from wild individuals. Overall, microbes were abundant on cultured hosts, but their diversity appeared to be smaller than that of wild blades.

Some differences between the first and second groups of cultured specimens were also observed. A small sarcinic bacterium approximately 0.5 µm in diameter that was not seen on the first specimen was observed on the second group of specimens. In addition, the undulating or helical bacteria from the first specimen were not observed in the second group of cultured specimens. The abundant bacilli described above were present on both groups of specimens. In general, the two groups of cultured specimens appeared to have similar microbiomes from a morphological perspective.

Bacteria-spore interactions

Bacteria adhered to the neutral spore surface in both wild collected and lab grown specimens (Figs. 4.16-4.18). Interestingly, these bacteria were quite similar in appearance; they were usually rod-shaped, ranging in size from 0.5 – 1.0 µm in length. Most neutral spores with bacteria had just one or two cells on them, and some bacteria could be seen embedded within the thick mucilage of the spore surface. Spores from blade margins with large numbers of microbes did not appear to have higher numbers of bacteria on them compared to spores coming from margins with relatively few attached bacteria. Accurate estimates of the number of spores with bacteria were impossible to obtain, however, because most of the regions bearing spores had high electron emission, and tended to have bright, distorted images.

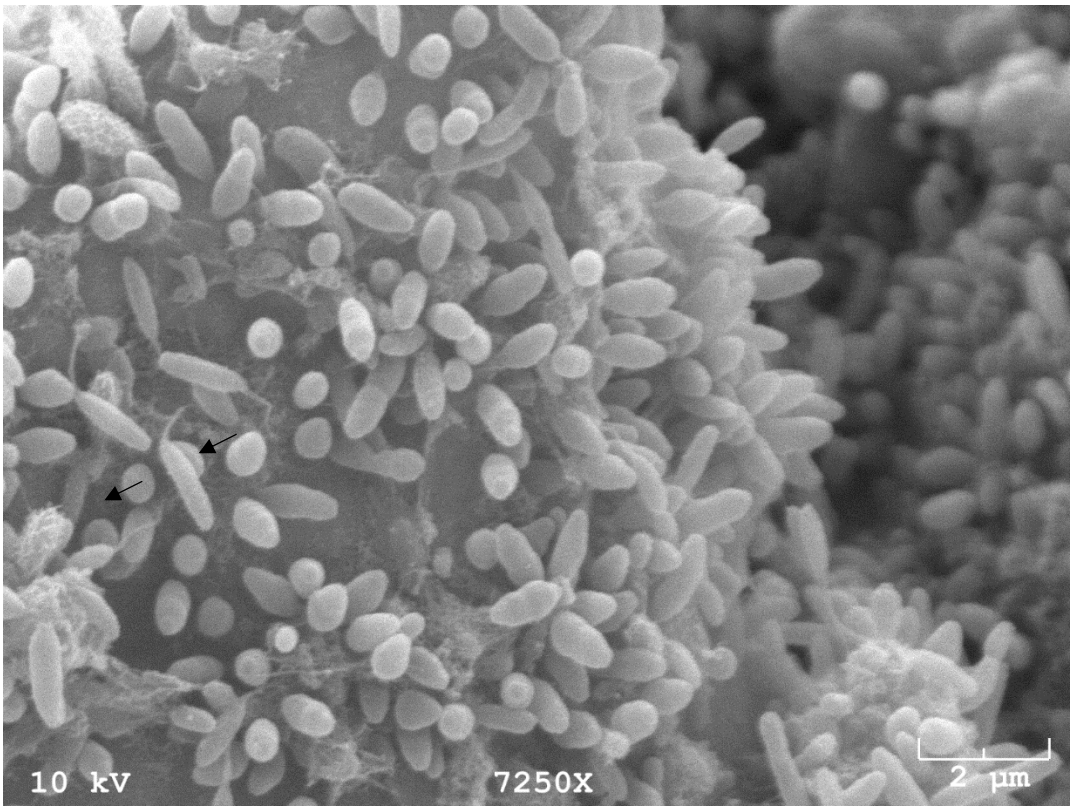

Figure 4.14 Common rod-shaped bacteria on the surface of cultured *P. umbilicalis*

This image was taken on the edge of a small lesion that had appeared near the edge of the blade margin. These bacteria were common across the entire surface of cultured specimens, but were especially dense on this lesion. These organisms appear to have possible attachment stalks (black arrows) by which they adhere to the algal surface.

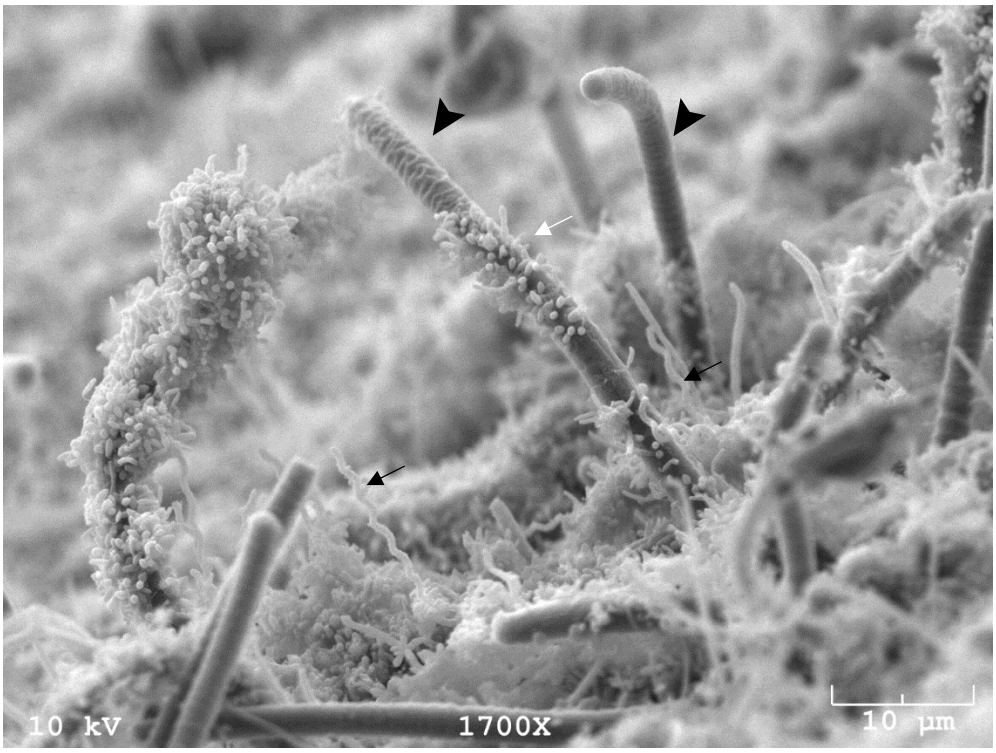

Figure 4.15 Filamentous cyanobacterium on cultured *P. umbilicalis*

These cyanobacteria (arrowheads) were common on cultured specimens. The common rod-shaped bacterium with possible attachment stalk can be seen growing on the cyanobacterium surface (white arrow). A dense lawn of smaller bacteria, including undulating or helical bacteria (black arrows), surrounds the larger cyanobacteria. This image was taken near the blade margin of this cultured specimen.

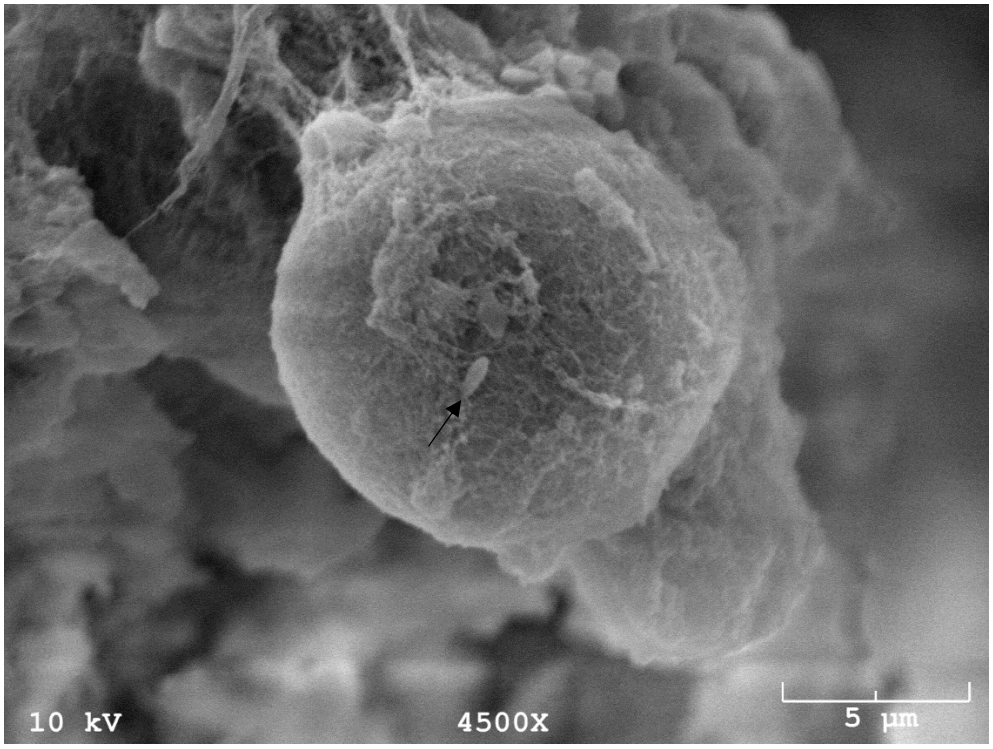

Figure 4.16 Bacterium on neutral spore from cultured *P. umbilicalis*

Arrow indicates bacterium. Note the dense coating of mucilage with a spongy appearance on neutral spore surface.

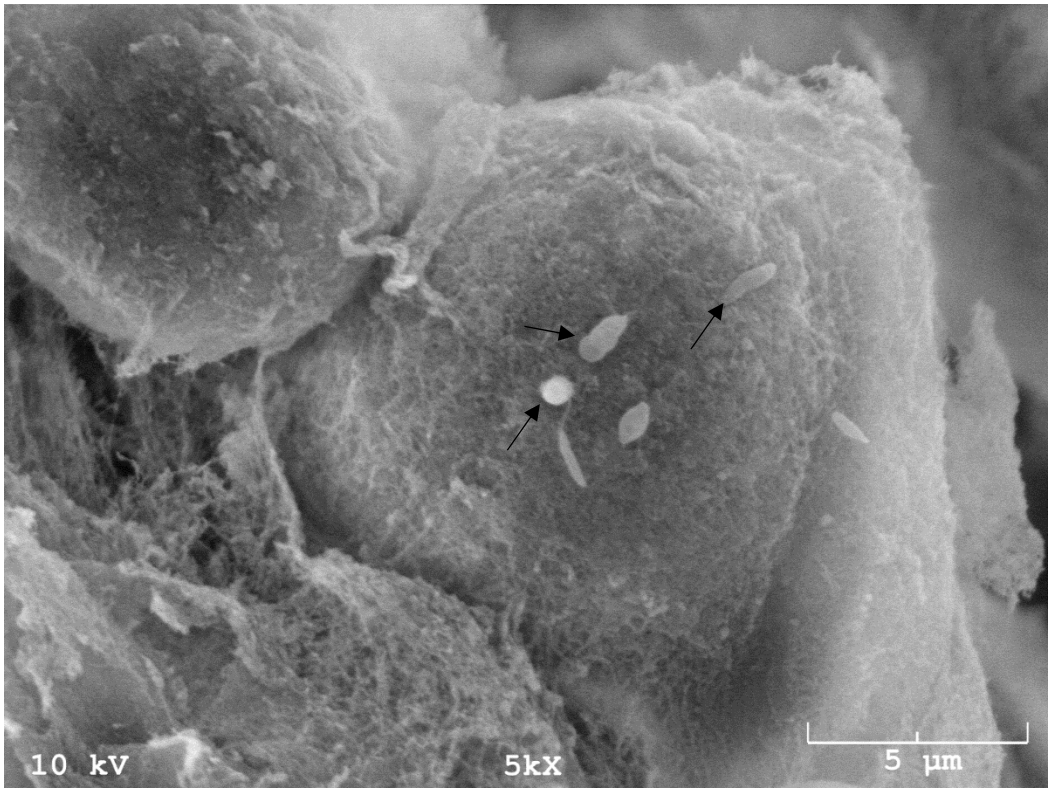

Figure 4.17 Multiple bacteria on neutral spores from cultured *P. umbilicalis*

Here, multiple bacteria on the surface of a neutral spore ejected from a cultured blade of *P. umbilicalis*.

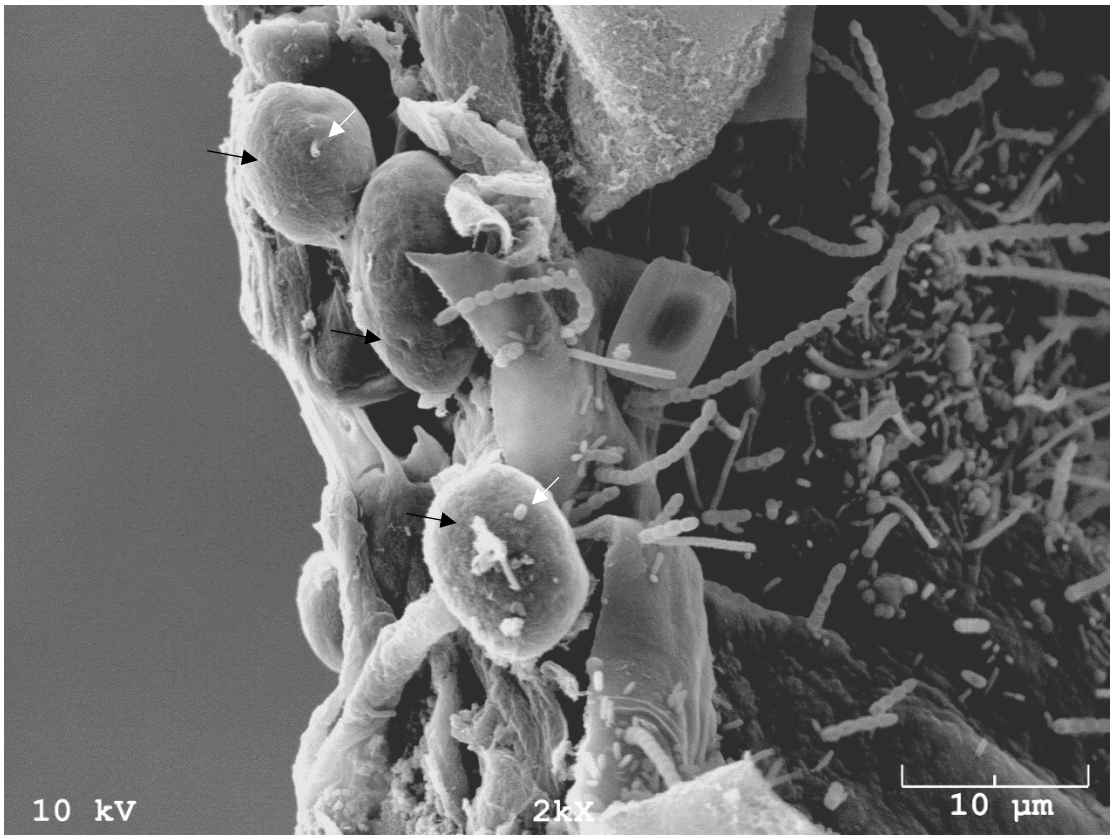

Figure 4.18 Neutral spores releasing from a wild *P. umbilicalis* blade margin

This view is pointing directly into the blade margin, where three neutral spores (black arrows) can be seen. White arrows indicate possible bacteria on spore surfaces. Note the proximity of a diverse community of bacteria growing near the edge where neutral spores are being released.

Discussion

This study demonstrates a diverse and frequently abundant microbiome on the surface of the *P. umbilicalis* blade. Many different bacteria and eukaryotic organisms were observed on wild algal tissue. Cyanobacteria from the family *Chroococcaceae* were seen on both the holdfast and blade margin regions of wild *P. umbilicalis.* These cyanobacteria appeared to produce large clusters of endospores, which settled and grew in a tetradic formation on the *P. umbilicalis* surface after colonial rupture. The *P. umbilicalis* cell wall (gametophyte) is composed of complex polysaccharides, primarily mannans, porphyrans, and agarobiose (Frei and Preston, 1961; Ficko-Blean et al., 2015). It is therefore likely that some of these associates are unique to *P. umbilicalis* because they would require specific forms of enzymes such as porphyranases to break down the *P. umbilicalis* cell wall (Goecke et al., 2010; Kim et al. 2016). Members of the bacterial phyla Bacteroidetes, Planctomycetes, and Proteobacteria are of interest because many possess enzymes required to break down red algal polysaccharides. Members of these phyla occur in association with *Porphyra* (Hehemann et al., 2012; Miranda et al. 2013; Kim et al., 2016).

Of interest is the contrast between the bacteria-dense holdfast region and the more sparsely populated blade margin of wild *P. umbilicalis*. Staufenberger et al. (2008) found that bacterial community diversity was higher on the holdfast regions of the kelp *Saccharina latissima.* Cundell et al. (1977) also observed differences in bacterial populations on different parts of *Ascophyllum nodosum*. The investigators found that the holdfast and tips of *A. nodosum* had few bacteria compared to the regions near nodes. They concluded this was due to high tannin production in the holdfast and tip regions,

preventing colonization by many bacteria. Other brown algae such as *Fucus* can shed an outer cuticle to remove fouling organisms (Moss, 1982; Rickert et al., 2016). I observed the holdfast region of wild *P. umbilicalis* to have the highest density of both prokaryotic and putative cyanobacteria, and although the blade margin's microbes were sparser overall, I observed patches of bacteria and putative cyanobacteria right up to the blade edge. *Porphyra* species do not produce tannins, and I did not observe shedding of the outer cell wall on my specimens, so other factors may contribute to differences between microbial communities of the holdfast and blade margin.

One explanation is that *P. umbilicalis* is a high-mid intertidal species that is exposed to long periods of desiccation during low tide. During this period, the algal blades lie prone upon bare rock faces, and the exposed surfaces may lose up to 90% of their water content (Blouin et al., 2011). Some bacteria may be unable to survive this harsh desiccation, and are eliminated. The holdfast region of *P. umbilicalis* can be densely foliose, and is less likely to dry out during exposure. This would support a higher diversity of both bacteria and fouling eukaryotes in the holdfast region, and provide shelter for grazers such as amphipods. Organisms growing on the rock surface may also grow onto the algal holdfast, explaining the encrusting nature of this community. The blade surface is subjected to more wave motion and turbulence than the holdfast surface, which may prevent some organisms from colonizing there. In the future, I would image rock scrapings of the area immediately next to the holdfast and compare these communities to ones attached to the alga. In addition, filtered seawater samples should be imaged. This would provide valuable information to accompany DNA-based taxonomic studies about which kinds of microbes may be unique to the *P. umbilicalis* surface.

Wild *P. umbilicalis* also appeared to have much higher microbial diversity than cultured specimens. Cultured Pum2 specimens were never treated with antibiotics, but drift during culture changes and production of a new generation may have led to loss of some organisms. Cultured Pum2 was also collected during May, while the wild specimens I examined were collected in December. Differences in the microbiome during these two seasons may have affected the observed bacteria. Another collection of wild specimens in May could provide a better comparison between these two groups.

The discovery of bacteria adhering to neutral spores of *P. umbilicalis* is one of the most interesting outcomes of this study. Spores had bacterial associates regardless of whether they were from areas of the blade margin with large bacterial communities or bare patches, or whether they were cultured or wild-collected. Similar observations were made in *Fucus* (Goecke et al., 2012) where oogonia in the process of being released from the conceptacle pore were covered by bacteria. Because bacteria are important to algae during their early life stages (Yamazaki et al., 1998, Fukui et al., 2014) it is possible that the bacteria observed on *P. umbilicalis* neutral spores are part of an essential group needed for development. The observation that bacteria were present on all samples is quite interesting, particularly because they appear to be similar rod-shaped bacteria (0.5 – 1.0 µm). Staufenberger et al. (2008) found that bacterial communities on *S. latissima* were most similar on the youngest parts of the alga, such as the meristem, and that they differed the most on the oldest parts such as the holdfast. This may indicate a core microbiome of bacteria important to young algal spores and developing germlings. A further investigation would involve isolating freshly-released neutral spores from both wild and lab cultures, with attempts to culture any bacteria adhered to them. If colonies

formed, bacteria could be identified with 16S rDNA sequencing (http://rdp.cme.msu.edu/), and a reconstitution of the bacteria onto axenic neutral spores could be conducted. This could determine whether the bacteria observed on neutral spores in SEM photos are important for early algal development. These methods could also be used to identify the bacteria that appear to be breaking down the algal thallus.

The intricacies of the relationships between bacteria and algae are still being determined. This study revealed differences in diversity and the structural architecture of the microbial community between the holdfast and blade margin of wild *P. umbilicalis*. I have demonstrated that bacteria are present on neutral spores as they are released from the blade, something that may be critical to understanding how bacteria affect algal germlings. The type of analysis presented here supports sequence based studies of the microbiome of this important organism.

CHAPTER 5

CONCLUDING REMARKS

This research contributes to development of *P. umbilicalis* as an aquacultural crop and model organism on several fronts. The phenological survey confirmed the peak times of year for *P. umbilicalis* reproduction, and I discovered the interesting issue of amphipod grazing and its impact on the reproductive season. Identification of one *Porphyra*-associated amphipod, *Apohyale prevostii*, which has been known to graze on other algae in the New England intertidal zone (McBane and Croker, 1983), is important to investigating this phenomenon further. A full survey of amphipod presence in *P. umbilicalis* in different locations in Maine should be conducted throughout the year to determine the diversity of amphipods that may be spore-feeders and to determine how this affects the amphipods' own biology. A further complement to this survey would be to collect amphipods and culture them in the laboratory. After a short starvation period, amphipods could be released into cultures with *P. umbilicalis* bearing mature neutral spores, and then analyze their fecal pellets and/or gut contents for living and dead NS after a set period of grazing. Examining preference of *A. prevostii* for vegetative *P. umbilicalis* compared to *P. umbilicalis* producing neutral spores would also be interesting.

Analysis of spore development showed that germlings from asexual neutral spores do not appear to follow set paths of division. Most these germlings followed a linear division pattern, but there were many bilateral paths as well. Additional research should include isolating and tracking germlings whose early division patterns are known, and determining how this affects their adult shape. Wang et al. (2010) concluded that adult

plants of different *Porphyra* and *Pyropia* species tend to retain the basic shape they displayed as germlings. Because the Wang et al. (2010) plants were all from sexually derived conchospores, the relationship between germling and adult shapes should be investigated in asexual *P. umbilicalis.* If adult shape is dependent on early cell division of germlings, it would be possible to select plants with different shapes for aquaculture, after checking that it is a stable trait in the following generation of plants.

In Chapter 3, I presented results from my attempted genetic transformation in *P. umbilicalis.* This is the first successful demonstration of genetic transformation using biolistics in this alga. Completing development of this system would help advance studies of *P. umbilicalis* in a variety of ways. Investigation of the *P. umbilicalis* asexual pathway could be conducted through genetic transformation assays. Blouin (2010) reported that some cell wall genes are expressed in asexual tissues taken from *P. umbilicalis*, and that these may be important for asexual reproductive pathways. *Pyropia yezoensis* gametophytes that formed archeospores were found to have expression of two novel genes (Kitade, et al., 2008), and a cell wall protein from this study was similar to one investigated by Blouin (2010). If these gene sequences could be isolated and amplified, it would be possible to create an expression assay for understanding their functions more clearly.

Development of a consistent transformation protocol, however, needs further work. I hypothesize that the obstruction of *P. umbilicalis* cells' thick cell walls plays a role in the low transformation efficiency that I achieved. Fully-exposed "naked" spores had a higher transformation efficiency compared to spores that were still bound within adult blades. An improved method to release neutral spores or to make protoplasts and

concentrate them in a small area may improve transformation efficiency further. Increasing the particle size to 1 µm may also improve efficiency, but this might also increase damage to the NS, because they are only ~10 µm in diameter. The addition of a native *P. umbilicalis* promoter may be another step in improving transformation efficiency in this alga. The *Pyropia* GAPDH promoter was used with success in *P. yezoensis* (Fukuda et al., 2008) so the possibility of using a *P. umbilicalis* GAPDH promoter should be considered. The availability of the *P. umbilicalis* genome will provide the information needed to identify and target promoters of interest in this alga.

The study I presented in Chapter 4 revealed the complex and diverse interactions between *P. umbilicalis* and its microbiome. It was clear that differences exist between wild-collected and lab-cultured specimens, and that different parts of the alga had more microbial epiphytes than others. One major gap in this study is the limited ability to identify any of the organisms present on the algal surface. Therefore, I think this type of SEM work is best suited for a reconstitution study, where specific bacterial isolates are added to axenic *P. umbilicalis* spores or germlings. This would enable pairing of the observed microbia to an identification produced from 16S rDNA sequencing, making it possible to further study a known bacterium's affinities for different areas of the blade.

A reconstitution study, or any future SEM work in *P. umbilicalis*, would benefit from companion work with brightfield light microscopy and transmission electron microscopy (TEM). It is possible that some of the microbes I observed on the surface of *P. umbilicalis* were burrowing into the thallus, but this cannot be confirmed because it is impossible to see below the surface with SEM. TEM would allow these regions to be studied better. It would also be prudent to section and image neutral spores using TEM, to visualize how bacteria are attach to the spore surface. The use of TEM to complement the neutral spore SEMs I produced in lab-cultured tissue is currently underway.

Overall, these studies have made significant contributions to advancing *P. umbilicalis* as a model organism and as an aquacultural crop. The information provided by these studies will benefit researchers interested in the *P. umbilicalis* genome project and microbial studies, and those interested in commercial aquacultural development.

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APPENDIX

ESTABLISHMENT OF BOMBARDMENT CONDITIONS

For the first rounds of bombardment of vegetative *P. umbilicalis* blade margins, 650 and 1100 psi were used. When minimal transformation (0-2 regions per section) was observed, firing pressure was increased to 1550 psi. It became apparent after several more trials on untreated tissue at 650, 1100 and 1550 psi that the thick outer portion of the *Porphyra*n cell wall of vegetative *P. umbilicalis* was tough and appeared to impede penetration. Following this discovery, blade margins producing neutral spores were used to attempt to overcome this issue, because the polysaccharide layer was looser, and neutral spores are naked (Blouin et al., 2011). When a similar set of trials failed to increase the number of transformed regions, treatments to weaken the tissue surrounding the neutral spores were explored. Uji et al. (2016) showed that the plant hormone 1-aminocyclopropane-1-carboxylic acid (ACC) weakened the cell walls of *P. yezoensis*, so this was used to produce the same effect in *P. umbilicalis*. The use of sulfatase was also tried because this enzyme is found in the guts of animals that eat algae and plants, and because the *P. umbilicalis* cell wall contains a variety of sulfated polysaccharides (Popper et al., 2011; Kim et al., 2016).

For these trials, tissues bearing neutral spores were incubated in sulfatase for 5 days under standard conditions or ACC for 12 days. Bombardment of sulfatase tissues was at 650 psi (n=1) and 1550 psi (n=1), and ACC tissues at 1550 psi (n=3) and 650 psi (n=1) at 6 cm distance. The sulfatase-treated tissue bombarded at 1550 psi had 37 transformed regions in total, a large increase from the 2-3 transformed regions previously observed. The 650 psi bombardment on the remaining sulfatase-treated tissue did not

result in any transformation. ACC treated tissues bombarded at 1550 psi had 17, 2 and 21 transformed regions, whereas the tissue bombarded at 650 psi had 1 transformed region. Because the yield from the sulfatase treatment was so high, and because the ACC treatment took nearly two weeks, I decided to pursue additional sulfatase treatments to elucidate whether this treatment was truly effective at increasing transformation efficiency.

BIOGRAPHY OF THE AUTHOR

Charlotte Royer was born on August 8, 1989 in Cincinnati, Ohio. She grew up in Hamilton, OH, and used to spend hours exploring the creek and woods behind her house. There she collected fossils, used plant guides to identify interesting flowers, caught butterflies, and called to the barred owls at night as they flew across the yard. She often attributes this time spent outside as building the foundation for her interest in natural science.

In college, Charlotte's love of animals led her to study zoology, and she considered being an ornithologist or ethologist for a time. However, her growing interest in molecular biology, plants, and marine organisms led her to contact Dr. Susan Brawley at the University of Maine, where she was accepted as a Master's student. Charlotte has learned a thing or two about algae during her time at UMaine, and she finds them to be fascinating and organisms. Wherever she ends up in the future, she hopes she will be able to continue working with them. She is a candidate for the Master of Science in Marine Biology from the University of Maine in May 2017.