Examining the Microbiome of Porphyra Umbilicalis in the North Atlantic

Margaret Aydlett
University of Maine, m.r.aydlett@gmail.com

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EXAMINING THE MICROBIOME OF *PORPHYRA UMBILICALIS*

IN THE NORTH ATLANTIC

by

Margaret Aydlett

A Thesis Submitted in Partial Fulfillment of the Requirements for a Degree with Honors (Marine Science)

The Honors College
University of Maine

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Advisory Committee:
Susan H. Brawley, Advisor, Professor, School of Marine Sciences, University of Maine
Mark Haggerty, Rezendes Preceptor for Civic Engagement, The Honors College, University of Maine
Lee Karp-Boss, Associate Professor, School of Marine Science, University of Maine
John Singer, Professor of Microbiology, Department of Molecular and Biomedical Sciences, University of Maine
Mark Wells, Professor, School of Marine Science, University of Maine
ABSTRACT

Marine macroalgae host a diverse microbiota. Bacteria are the most prominent group, and relationships between the algae and bacteria are complex and dynamic. The goal of this project was to examine the distribution and ASV diversity of Bacteria associated with *Porphyra umbilicalis* with special focus on some isolates, including studies of their temperature dependence and consideration of how they may affect *Porphyra*. Previous studies showed that some bacteria are required for normal algal morphology and growth. *Porphyra umbilicalis* is an abundant red macroalga found in the intertidal zone and is an important food for invertebrates. Because of its significance in intertidal communities across the North Atlantic rocky shore, it is important to understand its microbial associations.

The biodiversity of the microbiota of *P. umbilicalis* samples collected from four trans-Atlantic locations in the winter of 2016 were compared using amplicon sequence variants (ASVs) derived following DNA extraction from sequencing of the V4 hypervariable region of the 16S rDNA. Bacteria were isolated from germlings of *P. umbilicalis* spread on agar plates. PCR of nearly full length 16S rDNA gene sequences identified these bacteria. Temperature optima for growth of six of the 21 isolates were examined. The microbiota of *P. umbilicalis* differed significantly across all four locations and regional specificity was found on the thallus between bacteria on the holdfast versus blade margin of bacterial communities on the plants. Growth experiments showed variability in the effects of temperature on growth of different isolates.
ACKNOWLEDGEMENTS

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CHAPTER ONE

INTRODUCTION

Some macroalgae have extensive biogeographic ranges and play important roles in coastal ecosystems as food, primary producers, and/or habitat-forming niches for intertidal organisms (Graham et al., 2016). Along with the ecological importance of macroalgae, some are economically important as food, in medicine, and for economically important hydrocolloids such as agar and carrageenan in some red algae (Graham et al., 2016). Red algae have the highest protein content among current marine aquaculture crops and make up a major portion, in value and biomass, of the worldwide seaweed market (Wells et al., 2017). The market value of nori (*Pyropia*) and laver (*Porphyra*), which are related red algae in the Bangiales, is ~ $1.3 billion (FAO, 2016). Most of this commercial value is nori aquaculture, but wild harvest and human consumption of *Porphyra* have a long history across the North Atlantic (Rhatigan, 2011).

Red algae, known as rhodophytes, form the monophyletic phylum Rhodophyta (Ragan et al., 1994; Yoon et al., 2006). They are an ancient evolutionary lineage that dates back at least 1 billion years and includes the oldest taxonomically resolved multicellular eukaryotic fossil (*Bangiomorpha*; Butterfield et al., 1990; Gibson et al., 2017). A recent major revision reclassified many species of *Porphyra* into *Pyropia* (Sutherland et al., 2011), but four Atlantic species are still classified as *Porphyra*. Indicative traits of the phylum Rhodophyta include lack of centrioles and flagella, plastids enclosed by two membranes, and the accessory photosynthetic pigments
phycoerythrin, allophycocyanin, and phycocyanin (Graham et al., 2016). The seven classes of red algae are Bangiophyceae (e.g. Porphyra, Pyropia), Compsopogonophyceae, Cyanidophyceae, Florideophyceae, Porphyridiophyceae, Rhodellophyceae, and Stylonematophyceae (Yoon et al., 2016; Muñoz-Gomez et al., 2017).

*Porphyra umbilicalis* is one of the most common of the *Porphyra* species found in the North West Atlantic and can be distinguished from other species in the genus by molecular techniques (Villalard-Bohsack, 2003; Sutherland, 2011). Normally, *Porphyra* and *Pyropia* have a heteromorphic life cycle that alternates between a blade (gametophytic phase) and a boring, filamentous sporophytic phase, but North West Atlantic *P. umbilicalis* lacks this alternation of generations and directly recycles blades from asexual neutral spores that form on blade margins (Blouin et al., 2010). The thallus of the *P. umbilicalis* blade is a relatively simple one, consisting of a holdfast region and a vegetative region with cell walls composed of porphyran (Brawley et al., 2017). The function and physiology of the holdfast and blade vary significantly. The holdfast anchors the individual to the rock, while the blade is where photosynthesis, rapid cell division, and neutral spore formation occurs (Royer et al., 2018).

The strong relationships between bacteria and seaweeds have been studied since the mid-twentieth century. Many studies found that axenic cultures of green and brown macroalgae grew slowly or exhibited abnormal morphogenesis (Provasoli & Pintner, 1964 & 1980; Pedersen, 1968; Kingman & Moore, 1982; Saga et al., 1982; Matsuo et al., 2015; Ghaderiardakani et al., 2017; Weiss et al., 2017). Fries (1964) first recognized this dependency in red algae when working with *Polysiphonia urceolata*. More recently,
similar findings were made in other red algal species like *Pyropia yezoensis* (Yamazaki et al., 1998, as *Porphyra yezoensis*). These investigations into the function of the microbial communities on, and around, marine macroalgae confirm the importance of developing a full understanding of the microbiome of the ecologically and economically important macroalga *Porphyra umbilicalis*.

Here, I study several aspects of the microbiome of *Porphyra umbilicalis* (Bangiophyceae, Bangiales, Bangiaceae), which is wild-harvested in the Atlantic for human consumption (Rhatigan, 2011). The goal of the research is to develop an understanding of the *P. umbilicalis* microbiome through three interconnected projects. First, I compared the microbiota of *P. umbilicalis* collected from four locations in the North Atlantic to determine whether the microbiome is different in these locations. Secondly, I isolated and identified bacteria isolated from neutral spores. Lastly, I determined growth responses of a subset of those bacteria to different temperatures. This knowledge can be applied in conjunction with other studies to better understand the importance of the microbiome to *P. umbilicalis*. Determining latitudinal differences of the microbiota and temperature responses of bacteria present within that microbiome could give insight into future population changes related to changing climate.
CHAPTER TWO

EXAMINING THE MICROBIOME OF *PORPHYRA UMBILICALIS*

Introduction

Marine macroalgae evolved in a world rich in microbes. They host a wide array of microbial organisms, bacteria being the most prominent group. Algal relationships with bacteria are complex and dynamic, and there is evidence that these interactions may be species-specific as well as specific to certain regions of the algal body. Previous studies show that some of these bacteria affect macroalgal growth rates and development.

Yamazaki et al. (1998) reported that the nori (*Pyropia yezoensis*) sporophytes were normal in axenic culture, but germinating spores from the sporophyte formed a disorganized callus rather than a blade. Bacteria were required for normal blade growth. It is known through reconstitution experiments with bacteria on axenic algal germlings that the normal morphologies of *Ulva intestinalis* and *Ulva mutabilis* are completely dependent on particular bacteria (Ghaderiardakani et al., 2017).

Many bacteria are epiphytic colonizers of the thallus surface, and the microbial community of a particular alga differs from the microbiome recovered from the surrounding seawater as well as the microbiome of taxonomically different sympatric species and is dependent on seasonal and spatial variation (Cundell et al., 1977; Lachnit et al., 2009; Quigley et al., 2018). Density and diversity of bacterial species differ among parts of the algal body, suggesting that the macroalgae are providing different specific niches for bacterial growth. Lachnit et al. (2009) showed that the bacterial associations
between *Ulva* spp. from different geographic origins were more similar than two other genera from the same geographical environment; however, Hanyuda et al. (2018) note that *U. australis* is likely an European invasive in Australia, potentially complicating Lachnit’s interpretation. Common epiphytic bacteria on algae belong to Proteobacteria (Alphaproteobacteria and Gammaproteobacteria), Bacteroidetes, Planctomycetes, and Cyanobacteria; however, such similarities do not extend to lower taxonomic levels (e.g., family, genus, Egan et al., 2013). Additionally, it should be noted that different phyla of algae show different proportions of these bacterial phyla. Although this could be due to limitations of data sets, it poses interesting considerations for definition of “core” groups of epiphytes. Additionally, functionally different algae (e.g. from different phyla) might not be expected to have a similar core.

Here, my primary goal is to determine the differentiation of bacteria associated with the blade and holdfast regions of *P. umbilicalis* across four locations in the North Atlantic using Minimum Entropy Decomposition (MED) analysis. MED is a computationally efficient means of organizing marker gene datasets into taxa and it does not require clustering techniques, but determines differences in sequence by entropy analysis (Eren et al., 2015). Identifying bacteria by amplicon sequence variants (ASVs) provides more ecologically significant resolution than the older clustering techniques that produced operational taxonomic units (OTUs; Eren et al., 2013; Needham et al., 2017). With ASVs, functional differences in environmental niches undetectable with standard OTUs can be identified (Eren et al., 2013; Needham et al., 2017). This study aims to answer the following questions: (1) Do bacterial communities on the *Porphyra umbilicalis* holdfasts differ significantly from those on the blades? (2) Does the *P.*
*Porphyra umbilicalis* microbiome have a recognizable core group of bacteria across all four collection locations?

**Methods**

**Collection**

Samples of *Porphyra umbilicalis* were collected in the winter months of 2016 from four locations on two separate days. Collection sites were Schoodic Point, ME (Permit #ACAD-2018-SCI-0003); Amorosa, Portugal; Newport, MA; and Minehead, United Kingdom (Table 1, Figure 1). During each collection by the Brawley lab team, including me at Schoodic, holdfast and blade margin tissue samples were collected from three plants in each of two transects, selected with random numbers, for a total sample of six plants per day. Tissues were rinsed with sterile seawater in the field, placed in sterile foil packets with labels, and stored on ice until flash freezing in the lab. Seawater and substratum samples were also collected.

<table>
<thead>
<tr>
<th>Collection Site</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Transect A</th>
<th>Transect B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amorosa, Portugal</td>
<td>02/07/16</td>
<td>02/09/16</td>
<td>N41.38.621°, W008.49.476’ to N41.38.608°, W008.49.471’</td>
<td>N41.38.574°, W008.49.454’ to N41.38.565°, W008.49.455’</td>
</tr>
<tr>
<td>Minehead, United Kingdom</td>
<td>01/21/16</td>
<td>01/22/16</td>
<td>N51.10.994°, W003.23.093’ to N51.10.999°, W003.23.109’</td>
<td>N51.10.990°, W003.23.116’ to N51.10.987°, W003.23.149</td>
</tr>
<tr>
<td>Newport, Rhode Island</td>
<td>01/11/16</td>
<td>02/10/16</td>
<td>N41.45.135°, W71.35.723’ to N41.45.142°, W71.35.694’</td>
<td>N41.45.075°, W71.35.639’ to N41.45.057°, W71.35.645’</td>
</tr>
<tr>
<td>Schoodic, Maine</td>
<td>01/22/16</td>
<td>02/21/16</td>
<td>N44.33.309°, W68.05.839’ to N44.33.321°, W68.05.836’</td>
<td>N44.33.395°, W68.05.811’ to N44.33.409°, W68.05.804’</td>
</tr>
</tbody>
</table>
**Table 1.** Collection dates and locations of sites.

**Figure 1.** Map showing four sampling sites in the North Atlantic. A = Schoodic, Maine. B = Newport, Rhode Island. C = Minehead, United Kingdom. D = Amorosa, Portugal.

**Processing**

Tissues were flash-frozen in the lab with liquid nitrogen and stored at -80°C until lyophilization. A portion (0.010 - 0.020 g dry wt) of each tissue sample was pulverized using a Geno/Grinder (SpexSamplePrep, Metuchen, NJ; 2 min, 1600 strokes/min, with 2.4 mm zirconium beads) and used for DNA isolation and sequencing. I extracted DNA using the Qiagen DNeasy Plant MiniKit protocol (Germantown, MD). Samples were sent by overnight courier on dry ice to the Marine Biological Laboratory (Woods Hole, MA). The V4 hypervariable region of the 16S rDNA was amplified at the Marine Biological
Laboratory’s sequencing center with PCR. After the products were cleaned, quantified, and pooled, they were sequenced on an Illumina MiSeq (v. 3 sequencing kit and protocol) at the Marine Biological Laboratory in Woods Hole, Massachusetts, and MED analysis provided by the sequencing center. Sequence files were provided to me for analysis.

**Statistical Analysis**

Sequences were normalized and analyzed in RStudio (v. 3.4.4). A Morisita-Horn NMDS, pairwise Adonis, and ANOVA were used to test for statistical difference of the microbiomes between sites and tissue types (RStudio v. 3.4.4., package: vegan). The sequencing at MBL unexpectedly resulted in a high number of mt V4 sequences and sequences that were not assigned to Domain (i.e., Bacteria, Archaea, or Eukarya). Such sequences were removed from each sample. In order to balance normalization to a common number of sequences/sample that would retain many of the sample’s ASVs while avoiding uneven elimination of replicates of particular sample types, Mr. Kyle Capistrant-Fossa advised retention of 84 of the 140 samples while normalizing them randomly without replacement to 1000 sequences/sample. Code for this can be found at https://github.com/kacf24/GreaterNA/blob/master/Biological/normalization.R (Capistrant-Fossa, 2019). A stacked bar plot was created to show the percent abundance of each phylum of Bacteria in the blades and holdfasts from each location. A core group of bacteria was identified by requiring that an ASV be present in 75% of all holdfast samples (= holdfast core) or all blade samples (= blade core).

**Results**

The MED analysis identified discrete ASVs, which I then used to compare the microbiota associated with samples from each location. The most abundant phylum
across all locations was Proteobacteria, followed by Bacteriodetes, Cyanobacteria, Actinobacteria, and Planctomycetes (Figure 2). Across all locations, the percent abundance of Cyanobacteria was higher in the holdfast region than in the blade region. A core group of bacteria was identified for both the blades and holdfasts (Table 2); the most common genus in the core groups was *Granulosicoccus*. An NMDS using a Morisita-Horn distance matrix demonstrated that the microbiota of Amorosa and Minehead show some similarities (Figure 3). However, pairwise adonis found that the microbiome of each location differed significantly (Table 3). An ANOVA confirmed that site, latitude, and tissue type all had a significant effect on microbiomes (Table 4).
**Figure 2.** Stacked bar chart for percent abundance of each phylum across locations (Figure 1) and tissue (B = blade, H = holdfast).
Table 2. Classification of the core bacteria found for *P. umbilicalis* blades and holdfasts based on presence in 75% of samples.

<table>
<thead>
<tr>
<th>Domain</th>
<th>Phylum</th>
<th>Class</th>
<th>Order</th>
<th>Family</th>
<th>Genus</th>
<th>Count</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blade</td>
<td>Bacteria</td>
<td>Actinobacteria</td>
<td>Acidimicrobia</td>
<td>Acidimicrobiaceae</td>
<td>Ilumatobacter</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Proteobacteria</td>
<td>Alphaproteobacteria</td>
<td>Parvularculales</td>
<td>Parvularculaceae</td>
<td>undescribed</td>
<td>2</td>
</tr>
<tr>
<td>Holdfast</td>
<td>Bacteria</td>
<td>Actinobacteria</td>
<td>Acidimicrobia</td>
<td>Acidimicrobiaceae</td>
<td>Ilumatobacter</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Proteobacteria</td>
<td>Alphaproteobacteria</td>
<td>Parvularculales</td>
<td>Parvularculaceae</td>
<td>undescribed</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Gammaproteobacteria</td>
<td>Chromatiales</td>
<td>Granulosicoccaceae</td>
<td>5</td>
</tr>
</tbody>
</table>

Figure 3. Morisita-Horn NMDS for Amorosa, Minehead, Newport, and Schoodic *P. umbilicalis* samples.
Table 3. Pairwise Adonis showing the level of significant difference between microbiomes of each location.

<table>
<thead>
<tr>
<th>Comparison</th>
<th>R(^2)</th>
<th>P</th>
<th>P (adjusted)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amorosa vs Minehead</td>
<td>0.1004329</td>
<td>0.001</td>
<td>0.006</td>
</tr>
<tr>
<td>Amorosa vs Newport</td>
<td>0.2149782</td>
<td>0.001</td>
<td>0.006</td>
</tr>
<tr>
<td>Amorosa vs Schoodic</td>
<td>0.3998583</td>
<td>0.001</td>
<td>0.006</td>
</tr>
<tr>
<td>Minehead vs Newport</td>
<td>0.1794938</td>
<td>0.001</td>
<td>0.006</td>
</tr>
<tr>
<td>Minehead vs Schoodic</td>
<td>0.3721123</td>
<td>0.001</td>
<td>0.006</td>
</tr>
<tr>
<td>Newport vs Schoodic</td>
<td>0.3964891</td>
<td>0.001</td>
<td>0.006</td>
</tr>
</tbody>
</table>

Table 4. ANOVA showing the level of significance of the effect of site, tissue type, and latitude on microbiomes.

<table>
<thead>
<tr>
<th>Factor</th>
<th>R(^2)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Site</td>
<td>0.38025</td>
<td>0.001 ***</td>
</tr>
<tr>
<td>Latitude</td>
<td>0.01884</td>
<td>0.008 **</td>
</tr>
<tr>
<td>Tissue Type</td>
<td>0.02830</td>
<td>0.001 ***</td>
</tr>
</tbody>
</table>

Discussion

Not only do macroalgae have significant roles in coastal ecosystems, they are also used commercially as feed, biofuel, and in biotechnology. Without consideration of microbial relationships, the function of macroalgae in ecological and industrial settings cannot be fully understood. Examining the distribution and ASV diversity of algal-associated microbiomes and determining how these microbiomes may help algae
withstand the changing conditions of the intertidal zone, and the ocean in general, is important to maintain our coastal ecosystems in the face of global change. There are several reasons why the interactions between bacteria and macroalgae persist such as bacterial dependencies for growth, development, supply of nutrients, and protection (Dubilier et al., 2008). Because of such close relationships between macroalgae and the bacteria with which it associates, it is suggested that the system forms a holobiont, like that of corals (Egan et al., 2013). This holobiont system is likely to experience functional loss in the event of environmental stresses. Individual aspects of these relationships vary significantly among algal and bacteria species.

This study determined that the microbiota of *Porphyra umbilicalis* varied among different locations. Water composition, temperature, salinity, and currents can contribute to the diversity of the microbial community on the algae and in the surrounding water (Weigel and Pfister, 2019). Considering the unique climate and water movement of each sampling site, the differences among microbial communities make sense, but this sequencing run needs to be repeated to provide a higher number of resolved sequences/sample, allowing normalization at a higher level of sequences before consideration of environmental effects on differences in the microbiomes of *P. umbilicalis* across the four sites.

There are many phyla of bacteria represented in the microbiome of *Porphyra umbilicalis* from Amorosa, Minehead, Newport, and Schoodic. Over 90% of the ASVs at any location were Proteobacteria, Bacteroidetes, Cyanobacteria, Actinobacteria, or Planctomycetes. Although there is not extensive research on the magnitude of impact that each of these phyla has on *P. umbilicalis*, many studies show the importance of
individual bacterial species to the development and resilience of macroalgae. For example, cyanobacterial pseudocobalamin can be remodeled by *Porphyra* as functional vitamin B$_{12}$ (Helliwell et al., 2016; Brawley et al., 2017). Additionally, many Bacteriodetes and Proteobacteria are able to digest algal cell walls (Hehemann et al., 2010; Thomas et al. 2012), and among these are ones that are established as symbionts of macroalgae (Miranda et al., 2013).

The microbial community associated with the holdfast is different than the community of the blade margin. Regional differentiation, of bacteria, throughout the plant, was recognized as early as the late 1980s (Polne-Fuller & Gibor, 1984). More recently, sequencing of the 16 S rRNA gene further demonstrates the distinction in richness and diversity of bacteria on algae, including *P. umbilicalis* (Miranda et al., 2013; Kim et al., 2016; Quigley et al., 2018). The holdfast and blade margin are specialized parts of the plant. The primary function of the holdfast, composed of thousands of rhizoid cells, is to attach the blade to the substratum. Thick coverage of epiphytic bacteria is indicative of basal holdfast regions, while the blade margin typically has spottier coverage (Royer et al., 2018). The blade margin consists of a central vegetative region and a distal vegetative region. The distal vegetative region is where cell wall divisions take place and produce neutral spores. Bacterial composition of the holdfasts and blade margin differ in both ASV diversity and overall abundance.

A core group of bacteria was identified for both the blade and holdfast regions. The core group of the blades consisted of six species of bacteria while the core group of the holdfasts consisted of nine species; for both tissue types, the core groups represented *Ilumatobacter*, *Granulosicoccus*, and unidentified genera of *Parvularculaceae*. 
*Ilumatobacter* is known to be a gram-positive bacterium that is aerobic, non-motile, and rod-shaped (Matsumoto et al., 2013). The most abundant ASV of the microbiome was *Granulosicoccus*, which is a Gammaproteobacteria that has been previously characterized as chemoheterotrophic and capable of reducing nitrate to nitrite (Baek et al., 2014). The presence and abundance of *Granulosicoccus* could contribute to the high rate of nitrate uptake that has been found in *P. umbilicalis* (Carmona et al., 2006; Kim et al., 2007). The Parvularculaceae isolates belonged to an unidentified genus; however, members of this family have demonstrated unique traits such as possessing a rhodopsin that function as a light-driven inward H⁺ pump, inducing a more relaxed chromophore structure (Keiichi et al., 2016), and high thermotolerance (Arun et al., 2009). Because of their close physical relationship, it is likely that these traits of Parvularculaceae affect the macroalgae, but the means by which they do so are unclear.
CHAPTER THREE

ISOLATION AND IDENTIFICATION OF BACTERIA FROM NEUTRAL SPORE GERMLINGS OF *PORPHYRA UMBILICALIS*

Introduction

*Porphyra umbilicalis* has a complex life history in which it reproduces through a biphasic alternation of generations in the North East Atlantic (Figure 4-a), but only through asexual neutral spore production in blades in the North West Atlantic (Figure 4-b; Blouin et al., 2010). Sexual reproduction involves fertilization of carpogonia (egg cells) on female blades by spermatia released into seawater from male blades. The zygote then divides to form 16 exact genetic copies of the zygote and these diploid “zygotospores” are released and bore into calcareous substrata, such as mollusc shells, as the sporophyte forms (Blouin et al., 2007, 2011; Blouin & Brawley, 2012; Eriksen et al., 2016). The sporophyte matures to produce conchospores, which are released into the seawater and germinate to form blades. In asexual reproduction, neutral spores that mature directly into blades are released from the blade margin.

Surface colonizing bacteria are known as epiphytic. Bacteria benefit from being surface colonizers in many ways. Lin et al. (2018) suggest that capacity to utilize alginate in *Macrocystis* kelp forests is widespread across a diverse group of bacteria they isolated. Some bacteria break down porphyran, the major polysaccharide found in cell walls of bangiophyte red algae (Yoshimura et al., 2005; Hehemann et al. 2012). Questions remain
about the microbial communities of macroalgae at early life stages, particularly why neutral spores are a favorable bacterial host. Macroalgae also benefit from the bacteria that they host. Many bacteria produce antibiotics that could play a role in defending the host from unwanted microbes (Dopazo et al., 1988; Egan et al., 2013). Additionally, Amin et al. (2015) found complex infochemical signaling between phytoplankton and their epiphytic bacteria, in which Sulfitobacter secretes a hormone, indoleacetic acid, that promotes diatom cell division to increase population growth. The benefits of bacterial relationships to phytoplankton and macroalgae, at any stage of life, likely extend well beyond what is known today.

It is important to identify the bacteria specific to the microscopic stages of the life history along with the macroscopic stages to determine if the microbiome differs significantly and develop an understanding of how the bacteria that are present benefit the alga. Here, my goal is to isolate bacteria directly from germlings (Royer et al., 2018) produced from Porphyra umbilicalis neutral spores washed with sterile seawater to identify strains that are important in early life stages.
Methods

Collection

Reproductive *Porphyra umbilicalis* blades were collected from the intertidal zone in Acadia National Park on Schoodic Point at the same location used for biodiversity studies (ME) once on 11 November 2018 (Permit #ACAD-2018-SCI-0006) and a second time on 15 January 2019 (Permit #ACAD-2019-SCI-006). The habitat was a rocky intertidal zone with abundant *Porphyra* and *Fucus* individuals. The samples were immediately placed in storage bags and kept on ice for transport back to the laboratory.

The blades collected in November were split up and dried by either hanging or blotting with a paper towel. Small portions of the blades were rinsed and pulled through 2% agar gel to remove some of the epiphytic bacteria (Andersen, 2005). Slightly different
cleansing procedures were used for the blades collected in January. Blades that were collected in January were dried only by using the blotting method. To clean the blades, small portions were cut, briefly put in a Betadine bath to kill invertebrate larvae and eukaryotic epiphytes (Andersen, 2005), and rinsed. After cleaning, the same procedure was followed for the samples from both collection dates.

Three cleaned portions of the blade margin from each plant were placed in small, sterile petri dishes with sterile, filtered seawater, sealed, and cultured at 12°C (12L:12D). After 24 hours, the portions of blade had released neutral spores and were removed from the petri dishes. Neutral spores were collected in sterile beakers and washed 3-5x with sterile seawater before plating them in new petri dishes and culturing them at 12°C, 12:12 (L:D), 100 µmol m⁻² s⁻¹ photons in Percival incubators (Percival Scientific, Boone, IA). The dishes were re-sealed while the germlings grew.

**Isolation and Subculture**

When the germlings produced from neutral spores reached a size of four to six cells, individuals from each dish were picked up on sterile loops and smeared on agar plates to capture bacteria associated with germlings. Plates were incubated at room temperature until bacterial colonies were visible. Each plate contained colonies of many colors, textures and sizes (Figure 5). Colonies of interest were isolated to pure cultures by resuspension in tubes of marine broth 2216 (Atlas, 2004), followed by streaking a loop of medium with bacteria onto agar plates (Figure 6). I isolated 23 colonies. To make sure the cultures were pure, they were grown in 2216 marine broth and then streaked on plates a second time (Andersen, 2005). Each isolate was then cultured overnight in 2 mL 2216
marine broth. Portions of each broth were set aside for cryopreservation and DNA isolation.

**Figure 5.** Example of bacterial diversity from a germling streaked on agar plate with corresponding bacterial isolate numbers. The germling was from an air-dried *P. umbilicalis* plant collected in November 2018. (9 = *Pseudoalteromonas*, 10 = *Colwellia*, 11 = an undescribed *Flavobacteriaceae*, 12 = *Sulfitobacter*)

**Figure 6.** Example of pure culture, identified later as *Pseudoalteromonas*, isolated from germling-streaked agar plate. Bacteria isolated from an air-dried blade collected in November 2018.

The bacteria were cryopreserved using 0.5 mL of the inoculated broth and 0.5 mL of a 60% glycerol, 40% seawater solution. Three samples of each isolate were preserved by two different methods; two were vortexed lightly and placed in an -80°C ultracold
freezer, and one was vortexed and flash-frozen using liquid nitrogen before storage in an -80°C ultracold. The remaining 0.5 mL of sample was spun down and the pellet was saved for DNA extraction, done by the manufacturer’s instructions with the Qiagen DNeasy Plant MiniKit and protocol.

PCR amplifications of 16S rDNA were performed using a UEB (Universal Eubacteria) primer set: 27F (5’-AGA GTT TGA TCM TGG CTC AG- 3’) and 1525R (5’---AAG GAG GTG WTC CAR CC- 3’). The PCR mixture consisted of 5x Pfusion GC Buffer, 10 mM dNTPs, 10 µM forward and reverse primer, and Pfusion DNA polymerase. PCR was performed in a Bio-Rad S100 Thermal Cycler (Hercules, CA) by an initial 98°C for 30 s, followed by 34 cycles at 98°C for 10s (denatures DNA), 59°C for 30s (primers anneal to strands of DNA), 72°C for 45s (extension, synthesis of DNA), and a final extension at 72°C for 5 min. PCR products were purified using the QiaQuick DNA Extraction protocol. Cleaned PCR products were sequenced at the University of Maine DNA Sequencing Facility.

Identification

The 16S rDNA gene sequences obtained from each sample were edited and aligned using the Sequencher program (Sequencher version 5.4.6 DNA sequence analysis software, Gene Codes Corporation, Ann Arbor, MI). Once the forward and reverse sequences were combined to create contigs, the consensus sequence of each isolate was used in blastn at www.blast.ncbi.nlm.nih.gov as well as submitted to the Ribosomal Database Project (https://rdp.cme.msu.edu) in order to identify the isolates. Consensus sequences were used, except for two isolates. In those cases, the best sequence from the forward or reverse reaction was used for identification.
**Gram Staining and Photomicroscopy**

Samples of the isolates were Gram stained to identify their morphological type (Umbreit, 1962; p. 27-28). A sterile loop was used to smear some sample on the slide. The sample was heat-fixed to the slide over an open flame. Two drops of crystal violet stain were applied for 1 min before rinsing and blotting with bibulous paper. The same protocol was followed for Gram’s iodine staining. The slide was then decolorized with 95% ethanol with constant flow from the wash bottle for 40s, rinsed with distilled water, and then dried again. Lastly, two drops of safranin counterstain were added for one min before a final rinse and blot between bibulous paper. The samples were photographed with a 100 x oil immersion lens on a Nikon Alphaphot-2 microscope and 64 Mp Shifting Pixel Spot Flex camera (Figure 4 – images of stained samples that were not used in growth experiment).

**Results**

Isolates from germlings developing from neutral spores represented only two phyla (Bacteroidetes and Proteobacteria) and three classes (Gammaproteobacteria, Alphaproteobacteria, and Flavobacteria; Table 5). Only two of the bacteria that were isolated from the *Porphyra umbilicalis* neutral spores were present in the microbial communities of the samples from Schoodic, Newport, Amorosa, or Minehead; the two bacteria that overlapped were *Sulfitobacter* and *Zobellia*. *Sulfitobacter* was found on only the Minehead holdfast, while *Zobellia* was found on both the Minehead blade and holdfast as well as the Newport holdfast.
Table 5. Classification as determined by submission of 16S rDNA sequences to the Ribosomal Database Project of the 21 bacteria isolated from *P. umbilicalis* germlings. Bacteria that were present in the microbial biodiversity samples (Chapter 2) are denoted by (*).

<table>
<thead>
<tr>
<th>Kingdom</th>
<th>Phylum</th>
<th>Class</th>
<th>Order</th>
<th>Family</th>
<th>Genus</th>
<th>Count</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacteria</td>
<td>Bacteroidetes</td>
<td>Flavobacteria</td>
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<td>Flavobacteriaceae</td>
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<td></td>
<td></td>
<td></td>
<td>Zobella</td>
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<td></td>
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<td>undescribed genus</td>
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<td></td>
<td></td>
<td></td>
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<td>Sulfitobacter</td>
<td>1*</td>
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<tr>
<td>Gammaproteobacteria</td>
<td>Alteromondales</td>
<td>Colwelliaceae</td>
<td>Pseudoalteromonadaceae</td>
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<td></td>
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<td>Shewanella</td>
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<td>Oceanospirillales</td>
<td>Oceanospirillaceae</td>
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<td>Vibrionales</td>
<td>Vibrionaceae</td>
<td>Vibrio</td>
<td>2</td>
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</table>

Discussion

The growth and development of many marine macroalgae are dependent on the presence of bacteria. Mature *P. umbilicalis* has a core group of epiphytic bacteria, but it is unclear if germlings have a core group or if this core group overlaps with that of the mature plants. None of the bacteria that were isolated from germlings overlap with the core group of bacteria that was found in mature plants; this could be attributed to the fact that these bacteria were isolated from only a small subset of *P. umbilicalis* from a small transect at only one location. Alternatively, the difference could represent developmental, biochemical and/or physiological differences between germlings and adults or indicate that bacterial potentially critical to growth and development are more easily isolated from germlings. Considering the regional specialization present in *P. umbilicalis* plants, it was expected that the bacteria isolated from the neutral spores to be more like those in the
blade margin than in the holdfast. However, ASVs that were similar among germlings and trans-Atlantic sites were not specific to either region of plant. The two bacteria isolated from germlings that are also found in the microbiome of mature plants, *Sulfitobacter* and *Zobellia*, both have unique characteristics that may benefit the mature plants as well as germlings.

*Sulfitobacter* has been observed to secrete IAA, a well-studied plant hormone, and it stimulates diatom cell division (Amin et al., 2015). The discovery of such a specific interaction as infochemical signalling raises many questions about just how important bacteria are to macroalgae during microscopic life stages. Mature *P. umbilicalis* as well as germlings could also rely on IAA from *Sulfitobacter* for some aspects of early development and growth. Species of *Zobellia* are studied extensively because of their unique agarolytic and nitrate reducing characteristics. *Zobellia galactanivorans* has a complex enzyme system that enables it to catalyze degradation of polysaccharides, which has commercial significance (Hehemann et al., 2012).

Overall, the bacteria isolated from the neutral spores were a diverse set of Alphaproteobacteria, Gammaproteobacteria, and Flavobacteria. It is unclear how each bacterium benefits the young germlings, if at all. Some of the isolates have previously been studied more extensively for their unique qualities. *Tenacibaculum* is a catalase positive microorganism (Suzuki et al., 2001), and thus could likely enable the algae to detoxify low amounts of hydrogen peroxide in the water. *Shewanella* produces omega-3 fatty acids that could be beneficial to macroalgae (Yazawa, 1996). A handful of the bacteria may not be symbionts to the neutral spores, and instead be attracted to the algae for other reasons (Egan et al., 2017). *Aquimarina* is an agarolytic microorganism that has
previously been isolated from *Porphyra haitanensis* in China (Lin et al., 2012); in this case, *P. umbilicalis* may not benefit from the presence of this bacteria, but *Aquimarina* benefits by having a constant food source.
CHAPTER FOUR

GROWTH EXPERIMENTS

Introduction

Bacteria play an important role in macroalgal development. It is important to understand the general physiological response of bacteria to environmental stresses. Focus on the influence of temperature and other environmental factors of bacterial physiology has increased in recent years. Considering the influence bacterial communities have on the expansion of species ranges, understanding bacterial responses to changing environments is important. As water temperatures change, the physiology of organisms, including bacteria change too. A common measurement of these changes is $Q_{10}$, the measure of sensitivity of enzymatic reaction rates to changes in temperature. This concept can be applied to bacterial growth; as temperature increases, so does the rate of division.

The optimum growth temperature of bacteria varies significantly (Ratkowsky et al., 1982). Some species of marine bacteria found in arctic or sub-arctic environments, known as psychrophiles, have enzymes adapted to lower optimum growth temperatures (Ratkowsky et al., 1982). Considering these lower optimal growth temperatures and the importance of bacteria to algal development, warming oceans may pose a serious threat to extensive ranges of sub-Arctic or Arctic marine macroalgae if they depend upon bacteria that are psychrophiles. Other environmental factors have an influence on
bacterial growth and function as well. Some species of bacteria are much more tolerant to low pH than others (Russell et al., 1979) and changes in pH affect functional abilities (Sinha et al., 2019). Heavy metal concentrations also have an influence of bacterial growth (Dell’Anno et al., 2002; Sinha et al., 2019). Additionally, Kirchman and Rich (1997) found that the concentration of dissolved organic matter in the equatorial Pacific was a primary limiting factor of bacterial production and growth. Although bacteria can grow in diverse, and sometimes extreme, environments there is a lot of variability in their preferred niches.

Here, my primary goal is to understand responses of bacteria isolated from *P. umbilicalis* germlings to different temperatures. Having been isolated directly from germlings, these bacteria may be essential to the early life stages of *Porphyra* and may remain important as the algae mature. Thus, as the marine environment continues to change, it is essential to understand how vital organisms react to temperature changes. I expected that the growth of the bacteria will be higher in warmer temperatures, and that some bacterial isolates will grow more quickly than others exposed to the same temperature, which could cause an imbalance in the microbiome.

**Methods**

**Growth Experiment**

Six of the bacterial isolates from germlings were used in a growth experiment to test the effect of temperature (*Vibrio* - #1, *Pseudoalteromonas* - #9, *Sulfitobacter* - #12, *Zobellia* - #18 - identified as *Maribacter* when shorter V4 regions were used for strain ID in the biodiversity studies [H. Morrison, pers comm. to M. Aydlett, May 2019], *Aquimarina* - #20, and *Marinomonas* - #23). The experiment was completed in
Percival incubators set at 5°C, 10°C, 15°C, and 20°C. For each isolate, triplicate flasks with 30 mL of 2216 marine broth, pH 7.8, were inoculated with 1 mL of bacteria when the stock reached an absorbance of 0.05 (OD of 1 = ~5x10^8 cells/mL) and placed in each chamber.

**Data Collection**

Growth of the cultures was monitored approximately every 12 hours over 478 hours (~20 days), recording absorbance (600 nm) with a Gilford Stasar III Spectrophotometer (Gilford Instrument Laboratories Inc., Oberlin, OH); the spectrophotometer is a single beam, micro-sample Visible spectrophotometer with a thermo sipper cell. The spectrophotometer was calibrated before each set of measurements with a blank subset of sterile 2216 marine broth. Samples with absorbance of more than 2.300 were diluted to half concentration for measurement. The pH was monitored every 24 hours using pH strips (VWR, pH-Test 4.5-10.0, Radnor, PA).

**Gram Staining and Photomicroscopy**

After 230 h of growth, a sample of each bacterial isolate from the 15°C chamber was collected to be Gram stained for closer inspection; the same Gram staining protocol used for the bacteria isolated from germlings was followed (Umbreit, 1962; p. 27-28). The samples were photographed using a 100x lens on a Nikon Alphaphot-2 microscope and 64 Mp Shifting Pixel Spot Flex camera.

**Statistical Analysis**
Statistical analysis of growth data was completed in RStudio (v. 3.4.4; package: ggplot2). An ANOVA was used to determine significance of temperature and bacterial isolate.

**Results**

Chamber temperature and genus of bacteria both had a significant effect on growth (Table 6). With exception of *Aquimarina*, all isolates showed growth at each temperature. *Aquimarina* only began to grow in the 10°C chamber in the last 5 days of the 20 day trial. At all four temperatures, *Psuedoalteromonas* grew to the highest peak absorbance (Figure 7). For *Vibrio* and *Pseudoalteromonas*, the highest growth occurred at 15°C and 20°C (Figure 8). Each isolate developed a distinct color during growth (Figure 9) and there was an evident change in hue as the amount of growth increased or decreased (Figure 10). Photos taken from the gram stained samples showed morphological differences among bacteria (Figure 11).

**Table 6.** Two-way ANOVA showing significance of bacteria type and chamber temperature on growth.

<table>
<thead>
<tr>
<th>Factor</th>
<th>Mean Square</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature</td>
<td>6.78</td>
<td>0.00219 **</td>
</tr>
<tr>
<td>Sample ID</td>
<td>223.97</td>
<td>&lt; 2e-16 ***</td>
</tr>
</tbody>
</table>
Figure 7. Measured absorbance over time faceted by temperature for each bacterial isolate. Gray shading indicates 95% confidence interval. Cell density inferred by OD of 1.0 = ~5x10^8 cells/mL.
Figure 8. Measured absorbance over time faceted by isolate for each temperature. Gray shading indicates 95% confidence interval. Cell density inferred by OD of 1 = ~5x10⁸ cells/mL.
Figure 9. Image showing one replicate for each of the six bacteria growing in the 20°C chamber (t = 139). (*Vibrio* - #1, *Pseudoalteromonas* - #9, *Sulfitobacter* - #12, *Zobellia* - #18 - identified as *Maribacter* when shorter V4 regions are used for strain ID in the biodiversity studies, *Aquimarina* - #20, and *Marinomonas* - #23)

Figure 10. Image showing one replicate of isolate #23 (*Marinimonas*) from each temperature chamber (t = 163).
Figure 11. Images of the stained samples of four of the bacteria grown in the 15°C chamber. Scale bar represents 10 um.

Discussion

Coastal macroalgae are dependent on an abundance of bacteria. As the effects of climate change continue to impact the world’s oceans, the biogeographic ranges of many macroalgal species are changing. Understanding the bacterial-algal interactions of the biota in very dynamic coastal ecosystems may give insight into how algal species ranges may shift in response to bacterial availability.

Of the twelve different types of bacteria isolated from the neutral spores, six were used in the growth experiments. Each of the six isolates has a unique characteristic that gave reason for use in the growth experiments. *Pseudoalteromonas* (gram-negative) has
antimicrobial and antifouling properties that may help protect the *Porphyra* from unwanted grazers and microbes (Bowman, 2007), but some *Pseudoalteromonas* also kill some algae; this bacterium has a lot of enzymes to digest cell walls of *Porphyra umbilicalis*. *Sulfitobacter* (gram-negative) is a demonstrated symbiont in multiple algal species (e.g. Amin et al., 2015; Ghaderiardakani et al., 2017). A species of *Zobellia* (gram-negative), *Zobellia galactanivorans*, is agarolytic due to a complex enzyme system that enables it to degrade polysaccharides (Hehemann et al., 2012; Ficko-Blean et al., 2017). Some *Aquimarina* (gram-negative) are opportunistic pathogens of seaweeds (Egan et al., 2017), but other *Aquamarina* are non-pathogenic. Some species of *Marinomonas* (gram-negative) produce antibiotics (Wang et al., 2016). These bacteria each contribute a unique trait to the algal-microbial system. The combined traits of some of these bacteria likely provide defense against pathogens and support normal growth and development.

There were significant differences in growth among different isolates across the four temperatures. What causes their differences in growth is unknown, but this brings about interesting questions about growth in the presence of other bacteria. The bacterial growth may be dependent on the presence or absence of other species. The growth curves of *Vibrio* and *Pseudoalteromonas* follow very similar trends at each temperature. *Marinomonas* also follows a similar trend, but with a lower amount of growth. All three of these isolates are Gammaproteobacteria, so this may be a reason for their similarities. *Zobellia* and *Sulfitobacter* also follow a similar growth trend to one another, with the exception of *Zobellia* at 5°C. Unlike the other isolates that had similar growth patterns, these two bacteria do not belong to the same phylum.
Chamber temperature were chosen based on literature and seasonal water temperatures at Schoodic Point in 2016. It is possible that bacteria isolated from germlings during a different season would be entirely different bacteria. Seasonal shifts in bacterial communities are important when considering the effects of climate change and seasonal differences. In a data set collected over several years, Lachnit et al. (2011) found recurring, specific bacterial communities in summer and winter in three different species. The reproductive peak of most macroalgae was winter into late spring (Lachnit et al., 2011). If the occurrence and distribution of these bacterial communities are primarily dependent on temperature, warming in the oceans might cause developmental and reproductive problems for the macroalgae during winter months. Considering the importance of bacterial functions in species-specific relationships, we must think about the implications of germling development if these bacteria were no longer present in intertidal communities.
CHAPTER FIVE

FUTURE WORK

The results of this research are important to understanding the microbiota of *Porphyra umbilicalis*. However, they also raise many more questions. Considering the close relationship between microbiota and healthy growth and development, reconstitution experiments would be beneficial. Some of the bacteria that were isolated from germlings may be an essential part of a core group of epiphytes for early life stages of *Porphyra*. Germlings may benefit from antibiotic resistance, cell signaling, and many other things that are provided by these bacteria.

An experiment in which bacteria are isolated from the germlings of *P. umbilicalis* plants from different locations would help determine if the bacteria that were isolated from Maine germlings are part of a larger core group of bacteria for germlings. Considering the microbiomes of the *P. umbilicalis* from each of the four north Atlantic locations were significantly different, it would be interesting to know if the microbiomes of germlings produced from neutral spores are also significantly different. Additionally, isolating bacteria from germlings during different seasons would also be interesting because the diversity and abundance of bacteria in nature in Maine is different seasonally (Miranda et al., 2013).

Many bacteria produce antibiotics. While this may help the algae deter unwanted microbes, it may also act as a deterrent for wanted microbes. As water temperatures change, the relative concentrations of bacteria may also change. Will changes in relative
concentrations of antibiotic producing bacteria affect the availability of other bacteria to

*P. umbilicalis* germlings? Would a decrease in the relative abundance of a bacteria with a high antimicrobial property result in a decline of health because of more unwanted microbes? Experiments that examine the growth of bacteria in the presence of other genera of bacteria would help answer this question. An experiment using real-time PCR to monitor the growth of multiple species of bacteria growing together in culture at varying temperatures would help determine if there are distinct interactions between bacteria at certain temperatures.

Although *Granulosicoccus* is cited in many studies as being the most abundant ASV (or OTU) in algal microbiomes, little is known about its physiology and traits. Experiments that examine the transcriptomic responses of algal hosts to *Granulosicoccus* would be helpful to develop further understanding about its role in marine ecosystems, specifically in macroalgal microbiomes.
LITERATURE CITED


four species of *Porphyra* (Bangiales, Rhodophyta) native to the New England coast.

*Journal of Applied Phycology* 19(5), 431.


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AUTHOR’S BIOGRAPHY

Margaret Aydlett was born and raised in Virginia Beach, Virginia. She attended and graduated from Tallwood High School in 2016. She decided to continue her education and pursue her interests in the ocean as a student of the School of Marine Sciences at the University of Maine. Maggie was a member of many clubs including Marine Science Club, Maine Outing Club, Ultimate Frisbee Club, Celtic Dance Club, and the Women’s Rugby Club of which she led the team as captain and president. She dedicated much of her time to both the University of Maine community and the Orono community through volunteering and coaching youth basketball.

Following graduation, Maggie plans to spend time abroad working on organic farms as a WWOOF volunteer before joining the gainfully employed. She hopes to continue her career as a marine scientist focused on seaweed aquaculture and science education.