The Role of Phylogeny in Associations Between Marine Phytoplankton and Heterotrophic Bacteria

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Thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Marine Science and Conservation in the Graduate School of Duke University

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Abstract

Empirical studies show coupling between phytoplankton and heterotrophic bacterioplankton across marine ecosystems through correlations between bacterial cell abundances and bulk phytoplankton measurements of chlorophyll-α or primary production. However, it is still not known whether associations between heterotrophic and autotrophic microbes are mediated solely through bulk carbon pools or if specific cellular interactions are involved. We examined the coupling of heterotrophic bacteria and specific phytoplankton populations in a dynamic, coastal ecosystem and explored the phylogenetic diversity of heterotrophic bacteria associated with a model phytoplankton taxon. Using flow cytometry to count distinct microbial groups, we found heterotrophic bacteria and phytoplankton abundances exhibit similar seasonal dynamics at the coastal sampling site, indicative of close coupling between these organisms. Heterotrophic bacterial abundances were more tightly correlated with small eukaryotic phytoplankton than either cyanobacteria group, Prochlorococcus or Synechococcus. Given our finding that associations between bacteria and eukaryotic phytoplankton drive ecologically important heterotroph-phytoplankton coupling, we examined the community composition of heterotrophic bacteria in culture with a model diatom, Thalassiosira rotula using 16S rRNA gene sequencing. Results indicate that strains of T. rotula support unique heterotrophic bacterial taxa but that all T. rotula also
associate with a common set of bacterial phylotypes, comprised of members of the Alphaproteobacteria. Characterizing the phylogenetic associations between heterotrophic bacteria and phytoplankton is essential for identifying factors shaping phytoplankton-bacteria interactions and their role in organic matter processing, trophic dynamics and biogeochemical cycles in marine ecosystems.
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1. Introduction

The total biomass, activity, taxonomic and functional diversity of microbes exceeds that of all other organisms in the ocean (Pomeroy et al. 2007). Coexistence of diverse microbial taxa and the coupling of primary production with heterotrophic biomass and respiration suggest members of microbial plankton are dependent on one another. Marine phytoplankton, responsible for 50% of global primary production (Field et al. 1998), are linked to heterotrophic bacteria via the microbial loop (Azam et al. 1983; Pomeroy et al. 2007). Through the microbial loop, particulate and dissolved organic matter (POM/DOM) produced by organisms, such as phytoplankton, is recycled into inorganic nutrients, decomposed to recalcitrant forms or assimilated into bacterial biomass that may be transferred to higher trophic levels. Historically, interactions between phytoplankton and heterotrophic bacteria were described based on positive correlations between chlorophyll-α and prokaryotic cell counts (Bird and Kalff 1984; Fuhrman et al. 1980). More recently, ecological network analyses of co-occurrence and co-variation of specific heterotrophic and autotrophic phylogenetic groups observed in environmental time-series data yield predicted interactions between phytoplankton and bacteria taxa although the mechanisms underlying these predicted interactions cannot be identified (Chow et al. 2013; Steele et al. 2011). In addition to interaction networks, whole community characterizations through metagenomics and metatranscriptomics suggest microbial groups exhibit cross-species associations mediated by chemical signaling or coordinated gene expression, potentially enabling coupling of metabolic
activities between taxa and trophic levels (Ottesen et al. 2013; Paul et al. 2013; Teplitski et al. 2004; Vanelslander et al. 2012). Despite their importance in the cycling of carbon and energy in ocean ecosystems, little is known about how marine microbes interact and respond at the level of populations or individual cells.

The importance of interspecies interactions for stimulating growth and reproduction of diverse microbial groups has been shown with co-culturing experiments (Mayali et al. 2008; Morris et al. 2008; Xie et al. 2013) and DNA-based studies of microbial community structure and activity (Kazamia et al. 2012; Martinez-Garcia et al. 2011; Tada et al. 2011; Wemheuer et al. 2013). The interactions between bacteria and phytoplankton occur along a spectrum from neutral (the presence of one organism has no effect on the viability of another organism in the same location) to facultative (interactions between organisms are not exclusive and vary in space/time) to tightly coevolved associations with high phylogenetic specificity (the organisms cannot survive in the absence/presence of their partner, symbiont, or antagonist). The interaction spectrum also spans a variety of outcomes from beneficial (mutualism) to neutral (commensalism) to negative (e.g. predation, parasitism, or competition) (Table 1). Many mechanisms dictate the outcome of interactions including direct physical contact, exchange of diffusible metabolites, cross-feeding and production of allelopathic compounds (Croft et al. 2005; Kazamia et al. 2012; Malfatti and Azam 2009; Matz et al. 2008; Ribalet et al. 2008). The nature of any interaction, however, can change depending
on the environmental context and the physiological status of the interacting organisms (Fouilland et al. 2013; Grossart 1999). For example, phytoplankton release more photosynthate in nutrient-limited compared to replete growth. The ability of heterotrophic bacteria to efficiently utilize the increased supply of organic material, however, depends on the stoichiometric ratio of available nutrients (Obernosterer and Herndl 1995). This research will describe the coupling of phytoplankton and heterotrophic bacteria in a dynamic, coastal ecosystem and explore the phylogenetic associations between heterotrophic bacteria and a model microalga.

1.1 To examine coupling of phytoplankton and heterotrophic bacteria in a dynamic, coastal ocean ecosystem.

In aquatic microbial ecology, “coupling” is used to describe the correlation of heterotrophic bacterial abundance and activity with primary production and autotrophic biomass. The phytoplankton-heterotroph linkage is attributed to heterotrophic metabolism of phytoplankton exudates and phytoplankton-derived organic matter released by grazing and viral lysis, as well as symbiotic relationships [as reviewed in Table 1] (Baines and Pace 1991; Cole 1982; Fouilland et al. 2013; Fuhrman 1999; Strom et al. 1997). Coupling influences the balance between primary production and respiration that determines of the state of an ecosystem as net autotrophic or heterotrophic. If the carbon required for growth and respiration of heterotrophic bacteria exceeds in situ phytoplankton carbon fixation, than the system becomes net heterotrophic and bacteria must meet their carbon demand with allochthonous sources
(Del Giorgio et al. 1997). Furthermore, the strength of phytoplankton-bacteria coupling impacts rates of carbon fixation, export and sequestration in the deep ocean, and transfer to higher trophic levels by influencing the amount of phytoplankton-derived carbon directed through the microbial loop. Thus, coupling has implications for global carbon cycling. Most studies analyzing the coupling of phytoplankton and heterotrophic bacteria examine bulk community metrics at ocean-basin scales using coarse temporal resolution (Bird and Kalff 1984; Landry et al. 1996; Li et al. 1992; Morán et al. 2002).

Phytoplankton and heterotrophic bacteria, however, exhibit distinct patterns of abundance and growth at multiple time and space scales (Ducklow et al. 2012; Jiao et al. 2005; Lefort and Gasol 2014; Thomas et al. 2010; Worden et al. 2004). Changes in the structure and abundance of the phytoplankton community as well as the availability and quality of DOM produced influence the degree of coupling observed with heterotrophic bacteria. We are interested in constraining factors regulating coupling in dynamic environments with allochthonous organic matter inputs, turbulent mixing, tidal forcing and variability in the abundance and production of members of the microbial community.

Here we quantify microbial cell abundance using flow cytometry which allows identification of multiple groups of organisms including total prokaryotes (assumed to be largely heterotrophic bacteria), small eukaryotic phytoplankton, as well as two genera of marine cyanobacteria, *Prochlorococcus* and *Synechococcus*, based on their
distinct photosynthetic pigments (Marie et al. 1997). Thus, flow cytometry permits both a quantitative and qualitative understanding of autotroph-heterotroph coupling in the microbial community. Characterizing coupling at a single geographic encompasses small-scale heterogeneity in DOM, environmental conditions, and microbial activity that may not be reflected in investigations with broad temporal and spatial resolution. This research will examine the coupling of heterotrophic bacterioplankton and small phytoplankton at a temperate, coastal ocean site with strong seasonality in environmental conditions to (1) determine the strength of coupling across seasonal cycles and to (2) determine if members of the phytoplankton contribute equally to the strength of coupling observed.

1.2 To determine the phylogenetic specificity of algal-bacteria associations.

While measurements of extracellular photosynthate release by phytoplankton in the field range from 1-70% of fixed carbon (Baines & Pace, 1991), the presence of bacteria in the vicinity of phytoplankton can influence the net-release of algal DOM and formation of transparent exopolymer particles, important for the aggregation and export of phytoplankton-derived carbon out of the euphotic zone (Gardes et al. 2012; Grossart and Simon 2007). Additionally, the quantity and quality of phytoplankton-derived organic matter likely shapes the abundance and community structure of phytoplankton-associated bacteria (Landa et al. 2013; Teeling et al. 2012). A growing body of evidence suggests there is taxonomic specificity in phytoplankton-bacteria interactions (Foster
and Zehr 2006; Jasti et al. 2005; Sapp et al. 2007). In natural and induced phytoplankton blooms, the overall bacterial community structure exhibits changes linked to algal-derived organic matter and the growth of distinct bacterial populations is stimulated, especially members of the Bacteroidetes, Gammaproteobacteria, and Alphaproteobacteria (Pinhasi et al. 2004; Tada et al. 2011; Teeling et al. 2012). Further, community diversity is often reduced during these blooms indicating that algal substrates or interactions with specific phytoplankton taxa select for specific bacterioplankton (Wemheuer et al. 2013). Moreover, algal cultures often support bacterial communities that are essential for growth; however, the culture conditions and culture media may also select for specific bacteria. For example, the bacterial assemblages from *Alexandrium* spp. cultures isolated from diverse geographic regions are closely related and dissimilar to the bacterial assemblages of other phytoplankton taxa (Jasti et al. 2005). Additionally, Sapp et al. (2007) found that bacterial communities associated with diatoms maintained for extended time in culture were dissimilar to both the *in situ* community at the time of isolation and the communities associated with freshly-isolated microalgae. This suggests that the bacterial community associated with phytoplankton is species-specific rather than a stochastic assembly of bacteria from the seawater community present at the time of isolation. The existing literature on phytoplankton-bacteria interactions raises the following questions: do algae exert selective pressure on their associated bacterial community, what mechanisms “select”
for particular associations and at what level of taxonomic classification do algal-bacterial associations exhibit specificity?
Table 1: Description of symbiotic relationships between phytoplankton and bacteria. Symbols represent [+ ] positive, [0] neutral, or [-] negative effect of the relationship on the respective organism(s). Symbols do not account for costs/benefits of the described activities that are not directly related to the symbiotic association.

<table>
<thead>
<tr>
<th>Interaction type</th>
<th>Phytoplankton</th>
<th>Bacteria</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Commensalism</strong></td>
<td>Reduction of oxidative stress [+ ]</td>
<td>Detoxification of reactive oxygen species (e.g. catalase and superoxide dismutase activity) [0]</td>
<td>(Morris et al. 2008)</td>
</tr>
<tr>
<td><strong>Mutualism/ Parasitism/ Commensalism</strong></td>
<td>Obtain growth-limiting iron [+ ]</td>
<td>Produce iron chelating siderophore [0/-] and obtain algal-derived dissolved organic matter [+ ]</td>
<td>(Amin et al. 2009; Keshtacher-Liebso et al. 1995)</td>
</tr>
<tr>
<td><strong>Commensalism</strong></td>
<td>DMSP produced as osmolyte and antioxidant [0]</td>
<td>DMSP encountered in environment is metabolized as carbon and sulfur source [+ facultative]</td>
<td>(Ledyard et al. 1993; Malmstrom et al. 2004; Miller et al. 2004; Sunda et al. 2002; Yoch 2002)</td>
</tr>
<tr>
<td><strong>Mutualism/ Commensalism</strong></td>
<td>Extracellular release of organic matter [0], nutrient acquisition [+ ]</td>
<td>Organic matter acquisition [+ ] and nutrient remineralization [0]</td>
<td>(Azam et al. 1983; Grossart 1999)</td>
</tr>
</tbody>
</table>
Determining the selective pressure phytoplankton exert on bacterial communities is difficult in the marine environment given the diversity of microbial communities and the potential confounding effects of food web dynamics and abiotic processes on community structure. The selection process can be more readily observed in the laboratory by examining the composition of the bacterial community associated with phytoplankton cultures. Interactions with algae might select for particular bacterial

<table>
<thead>
<tr>
<th>Mutualism/Amensalism/Antagonism</th>
<th>Polyunsaturated aldehyde (PUA) production for defense against protistan [0] or bacterial predators [+ ] and selection for beneficial bacterial associates [+ ]</th>
<th>Strain specific growth inhibition [-], stimulation [+], or resistance [0] to PUAs</th>
<th>(Ribalet et al. 2008; Ribalet et al. 2009)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antagonism</td>
<td>Production of compounds that disrupt bacterial quorum sensing signals [+ ]</td>
<td>Quorum sensing disruption [-] and potentially decreased ability to associate with algae [-]</td>
<td>(Teplitski et al. 2004)</td>
</tr>
<tr>
<td>Antagonism</td>
<td>Antibacterial production for defense [+ ]</td>
<td>Growth inhibition [-]</td>
<td>(Desbois et al. 2009; Geng et al. 2008)</td>
</tr>
<tr>
<td>Parasitism</td>
<td>Cell lysis [- ]</td>
<td>Release algalytic compounds to gain access to organic matter from lysed algal cells [+ ]</td>
<td>(Amaro et al. 2005; Doucette et al. 1999; Mayali and Azam 2004)</td>
</tr>
<tr>
<td>Competition</td>
<td>Lack of inorganic nutrients necessary for growth [- ]</td>
<td>Lack of inorganic nutrients necessary for growth [- ]</td>
<td>(Kirchman 1994; Obernosterer and Herndl 1995)</td>
</tr>
</tbody>
</table>
populations in cultures maintained over long time spans. Conditions in algal culture media support photoautotrophic or heterotrophic bacterial metabolisms but competition for light and nutrients and the ample algal-derived organic carbon likely favor heterotrophic phylotypes. Members of the Roseobacter, Alteromonadaceae, and Bacteroidetes are commonly identified in laboratory cultures as well as natural phytoplankton blooms, suggesting these taxonomic groups are selected for by interactions with phytoplankton (Jasti et al. 2005; Pinhassi et al. 2004; Schäfer et al. 2002; Sison-Mangus et al. 2014; Tada et al. 2011). Conflicting results have been reported about the stability of bacterial community composition over time in culture with phytoplankton suggesting interactions between organisms, contamination, and/or environmental conditions may alter the bacterial assemblage during routine maintenance in culture (Landa et al. 2013; Schäfer et al. 2002). Changes in quantity and composition of phytoplankton-derived organic matter in exponential versus stationary phase cultures also alter the co-occurring bacterial community because of selective resource use by specific bacterial clades (Barofsky et al. 2009). Overlap in functional traits across broad taxonomic groups, however, may permit diverse bacterial phylotypes to form associations with algae or utilize a variety of algal substrates. If phytoplankton-associated bacteria occupy broad niches such as “carbon degradation”, then a range of bacterial taxa could fill this niche and the bacterial community composition in algal cultures would reflect stochastic assembly. However, if there are highly specific
interactions involving chemical signaling or metabolic requirements, the bacterial community would exhibit non-random assembly.

Bacterial community structure is likely determined, in part, by the photosynthetic exudates and metabolic requirements of specific microalgal taxa but phytoplankton are also affected by heterotrophic taxa (Xie et al. 2013). Many phytoplankton are difficult to establish and maintain as axenic cultures indicating that associations with heterotrophic organisms are vital for phytoplankton growth and survival. For example, the cyanobacterium, *Prochlorococcus*, requires "helper" bacteria to remove reactive oxygen species in order to grow robustly at low cell concentrations (Morris et al. 2008). While there is evidence that specific bacteria may differentially affect the growth and physiology of microalgal species, it is not known at what level of taxonomic resolution these processes are acting (Giroldo et al. 2007; Grossart and Simon 2007). Studies have demonstrated incongruent effects of the same strain of heterotrophic bacterium on distinct phytoplankton species or ecotypes (e.g., (Grossart 1999; Seyedsayamdost et al. 2011b; Sher et al. 2011)). These results suggest divergent genome evolution among ecotypes reflects interactions with other organisms. Morris and colleagues (2012) proposed the Black Queen Hypothesis, which predicts functional gene loss can afford a selective advantage by conserving an organism’s limiting resources, provided the lost function is retained through interactions with “helper” organisms. For example, many algal lineages lack functional genes for vitamin B12
synthesis and depend on heterotrophic bacteria to supply this growth-limiting vitamin (Helliwell et al. 2011; Kazamia et al. 2012; Wagner-Dobler et al. 2009). The Black Queen Hypothesis suggests some phytoplankton-bacteria associations are not solely based on bulk pools of DOM but specific metabolism, which may very between or within members of the microbial community. The phylogenetic specificity of associations between heterotrophic bacteria and individual strains of eukaryotic microalgae within the same species has not been extensively explored.

This research will examine selection of the bacterial assemblage associated with strains of a diatom taxa maintained in laboratory culture. The aim is to determine if there is a common diatom-associated bacterial assemblage across strains of the same phytoplankton or if the bacterial community in diatom culture is strain-specific. Diatoms (Bacillariophyceae) were chosen as a model system because they are ubiquitous and phylogenetically diverse eukaryotic microalgae that account for 40% of total marine primary productivity and up to 90% in some coastal regions (Nelson 1995, Raven & Falkowski 1999). Diatoms also play an important role in the biological carbon pump because their characteristic silica frustules cause them to sink rapidly when they die, exporting cellular carbon to the deep ocean (Falkowski et al. 1998; Richardson and Jackson 2007; Sarthou et al. 2005; Smetacek 1999). In addition to their importance in global carbon cycling, they are also known to form close associations with heterotrophic
bacteria, primarily members of the *Proteobacteria* and *Bacteroidetes* phyla (Amin et al. 2012b).

The model diatom we are employing in this study, *Thalassiosira rotula*, has three lineages based on the rDNA internal transcribed spacer region I (ITSI). These lineages exhibit characteristic geographic distributions and abundance profiles in the field, as well as consistent physiology and genome size within a lineage suggesting this genetic subdivision is ecologically relevant (Whittaker et al. 2012). Since phytoplankton-bacteria interactions are known to play an important role in microbial ecology, we hypothesize that heterotrophic bacteria may differentially associate with genetically distinct strains of *T. rotula*. If diatom genetic differences correspond to distinct physiology or nutritional requirements, the same heterotrophic bacterium could form a mutualistic interaction with one lineage and a neutral or antagonistic interaction with others.

We hypothesize that exponentially growing diatoms represent a specialized ecological niche where only a subset of seawater bacterial communities can exist. Thus, we predict that diatoms in culture select for a specific bacterial assemblage composed of a limited subset of phylotypes co-existing with the diatom in seawater at the time of isolation. Mutualistic bacteria likely consume diatom-derived organic matter and remineralize nutrients or synthesize metabolites that support diatom growth. If we observe similar bacterial communities in distinct *T. rotula* strains then this diatom and/or the culture conditions exert a strong pressure on the bacterial community. However, if
the bacterial community does not converge on the same phylogenetic composition, it is possible that functional redundancy permits a broad suite of bacterial groups to fill diatom-associated niches or that strain-level variation within the diatom *T. rotula* exerts selective pressure on the bacterial community. Results from simplified model systems, like the one described here, are essential for identifying factors important in shaping phytoplankton-bacteria interactions and their resultant ecosystem effects.
2. Materials and methods

2.1 Phytoplankton-heterotrophic bacteria coupling in a dynamic, coastal ecosystem

2.1.1 Study Site

Water samples were collected weekly as part of the Pivers Island Coastal Observatory (PICO) Time Series in Beaufort, North Carolina, USA. This site (34°71.81’N, 76°67.08’W) is located at the Duke University Marine Lab on Pivers Island in the Beaufort Inlet. Time series measurements were conducted as previously described (Johnson et al. 2013; Yung et al. 2014). Briefly, water was sampled from a depth of 1m at 10:30AM local time using a Niskin bottle. Chlorophyll pigment samples were extracted in 100% methanol and measured fluorometrically using a calibrated Turner 10-AU fluorometer. Samples for flow cytometric counts of heterotrophic bacteria and phytoplankton were fixed with 0.25% gluteraldehyde (final concentration) and stored at -80°C. The following parameters were also measured or calculated: dissolved oxygen, turbidity, nutrients (NO₂, NO₃, PO₄, SiOH₄), salinity, pH, dissolved organic carbon, dissolved inorganic carbon, secchi depth, tidal height, wind speed, and incoming no-sky solar radiation.

2.1.2 Flow Cytometry

Phytoplankton and bacterioplankton were enumerated using a FACS Calibur flow cytometer (BD Biosciences) modified with a syringe pump for quantitative sample delivery (Johnson et al. 2010) and analyzed with FlowJo (TreeStar Inc.). Cells were
excited with 488nm excitation (15mW Ar laser) and forward scatter (<15°), side scatter (90°), green fluorescence (530±30 nm), orange fluorescence (585±42 nm), and red fluorescence (>670 nm) emissions were measured. Population mean properties (scatter and fluorescence) were normalized to 1.0 or 2.0 µm yellow green polystyrene beads (Polysciences YG) following standard population gating schemes (Olson et al. 1989). The three photoautotrophic groups were distinguished according to their positions in plots of red fluorescence vs. 90° light scatter (SSC) and orange fluorescence vs. SSC. Picoeukaryotes were identified by their large size and high red fluorescence. Bacterioplankton were quantified by staining the samples with SYBR Green-I as previously described (Marie et al. 1997). Samples for enumeration of phytoplankton were run separately from those for non-photosynthetic bacterioplankton.

### 2.2 Associations between heterotrophic bacteria and a model microalga

#### 2.2.1 Cultivation of phytoplankton

The phytoplankton strains used in this study are listed in Table 2. All cultures were maintained in f/2+Si medium (Guillard 1975; Guillard and Ryther 1962) or an enriched artificial sea water medium (modASP-M) prepared as described by Goldman and McCarthy (1978) but modified by the addition of f/2 trace metals, vitamins, and macronutrients. Phytoplankton were cultured at 20°C under continuous light (100µE m⁻² s⁻¹). Axenic and xenic strains of the marine diatom *Thalassiosira rotula* CCMP3264, FR3.012 (xenic only), JPNTR18, and WPF8 (xenic only) were obtained from Dr. Tatiana
Rynearson (University of Rhode Island). The axenic strain, *T. rotula* CCMP1647, was obtained from the Provasoli-Guillard National Center for Marine Algae and Microbiota.

**Table 2: Strains of Thalassiosira rotula used in this study.**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Lineage</th>
<th>Geographic origin</th>
<th>Date isolated</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCMP1647*</td>
<td>3</td>
<td>Gulf of Naples, Italy 40.49°N, 14.15°E</td>
<td>2008</td>
</tr>
<tr>
<td>CCMP3264*</td>
<td>3</td>
<td>Gulf of Naples, Italy 40.49°N, 14.15°E</td>
<td>1993</td>
</tr>
<tr>
<td>FR3.012</td>
<td>3</td>
<td>English Channel, France 48.73° N, 3.98° W</td>
<td>2010</td>
</tr>
<tr>
<td>JPNTR18*</td>
<td>3</td>
<td>Seto Inland Sea, Japan 34.16°N, 133.33°E</td>
<td>2007</td>
</tr>
<tr>
<td>WPF8</td>
<td>1</td>
<td>Puget Sound, USA 47.74°N, 122.42°W</td>
<td>2007</td>
</tr>
</tbody>
</table>

*indicates axenic cultures are available

**2.2.2 Analysis of total bacterial community associated with T. rotula cultures**

Cultures of *T. rotula* were maintained mid-exponential phase for at least 10 generations, vortexed briefly and allowed to settle for 2 hours. After algal cultures were mostly settled on the bottom of the tube, the supernatant was filtered through 5.0µm membrane filters (Whatman) to remove remaining algal cells. Filtrate was directly collected on a 0.2µm polyethersulfone membrane filter (Pall Supor-200; Gelman) and stored in a cryovial at -80°C until DNA extraction. All filtrations were performed with sterilized filtration equipment.
2.2.3 Analysis of seawater bacterial community during *T. rotula* bloom

Seawater from a *T. rotula* bloom in November, 2013 in Narragansett Bay, RI (41.53°N, 71.38°W) was collected by filling an autoclaved, opaque 1L Nalgene bottle at the surface. Seawater bacteria were collected on a 0.2µm polyethersulfone membrane filter (Pall Supor-200; Gelman) using vacuum filtration and stored in a cryovial at -80°C for DNA analysis of the bacterial community.

2.2.4 Heterotrophic bacteria isolation and identification

Heterotrophic bacteria were isolated from exponential phase, xenic *T. rotula* cultures and seawater collected during a *T. rotula* bloom (as for the whole-community comparisons). Aliquots from mid-exponential phase *T. rotula* cultures or seawater were plated on f/2 agar plates supplemented with 0.1g L⁻¹ yeast extract (f/2+YE). Plates were incubated in the dark at room temperature for two weeks. Pure isolates were obtained by triplicate streaking on f/2+YE agar plates and grown in f/2+YE liquid medium at room temperature for 48 hours. Stock cultures of the bacterial isolates were stored in glycerol solution (net 35% glycerol (v/v), 0.05M MgSO₄, 12.5mM Tris pH 8.0) at -80°C. Bacterial isolates were identified using 16S rRNA gene sequencing. DNA was extracted using Lyse-N-Go™ reagent (Thermo Scientific) and stored at -20°C. The 16S rRNA gene was amplified using primers 27F-mod (Vergin et al. 1998) and 1492R (Lane et al., 1991) and sequenced on the ABI 3730XL platform at the DukeIGSP Genome Sequencing and Analysis Core Resource using primer 1492R. Sequences were manually edited in
Sequencher (Gene Codes Corporation) and aligned automatically using the Silva Incremental Aligner (SINA) (Pruesse et al. 2012). Taxonomy was assigned using the RDP Naive Bayesian rRNA Classifier, Version 2.6 (Wang et al. 2007). The phylogenetic tree was created using PhyML (Guindon and Gascuel 2003) and visualized using the Interactive Tree of Life tool (Letunic and Bork 2007).

2.2.5 Total bacterial community DNA sequencing and analysis

The Purgene kit (Qiagen) was used to extract DNA from filters to construct 16S rRNA gene libraries of the bacterial communities in T. rotula cultures and seawater. The manufacturer’s protocol was modified by the addition of three 30-second bead beating steps at 4°C using 0.1mm zirconium beads alternated with 30-second incubations on ice. 16S rRNA gene amplicons for dual index sequencing were obtained using the universal bacterial and archaeal primers 926F (Lane et al. 1985) and 1392R (Lane et al. 1991) with added adapter, index, pad, and link sequences for the MiSeq platform (Table 3) as described in by Kozich and colleagues (2013). PCR amplification was carried out in a total volume of 20µL containing 20ng of template DNA, 200µM dNTPs, 2mM MgCl₂, 0.5µM primers, 0.4 U of Q5 High-Fidelity DNA Polymerase (New England Biolabs). Amplification conditions were: hotstart at 98 °C for 30s, followed by 32 cycles of 98 °C for 10s, 58 °C for 30s, and 72 °C for 30s, with a final extension at 72 °C for 2min. Amplicons were gel purified using the QIAquick Gel Extraction Kit (Qiagen). Amplicon libraries were pooled in equal amounts for subsequent paired-end 2x 300 base pair
sequencing on the MiSeq platform (Illumina). Library analysis was performed using QIIME (Caporaso et al. 2010). Sequences were quality filtered based on a Phred quality score of 20, representing an error rate of 1 in 100 with a corresponding call accuracy of 99%. Operational taxonomic units (OTUs) were assigned using USEARCH with a threshold of 97% sequence identity and chimeras were removed (Edgar 2010). Representative sequences were selected as the most abundant sequence from each OTU. These representative sequences were then classified taxonomically using the Ribosomal Database Project (RDP) classifier (Wang et al. 2007) against the RDP core set using a 80% confidence threshold for taxonomic assignment (Cole et al. 2009). Chloroplast sequences and singletons were removed prior to further analysis. Data were rarified at 20,000 sequences per sample to calculate alpha and beta diversity indices. Differences in community composition within and between strains of *T. rotula* were calculated using the unweighted (sensitive to rare taxa) UniFrac metric (Lozupone and Knight 2005) and compared with Bray-Curtis distance and principal coordinate analysis (PCoA). UniFrac significance tests were used to test the statistical significance of bacterial community similarity between all strains of *T. rotula* and the *T. rotula* bloom seawater sample. All p-values were corrected for multiple comparisons using the Bonferroni correction. To test for evidence of a common bacterial community associated with all *T. rotula* strains, we used the “compute_core_microbiome” function in QIIME, which identifies OTUs shared among samples.
**Table 3: Illumina MiSeq forward and reverse primer sequences**

<table>
<thead>
<tr>
<th>Name</th>
<th>Illumina portion</th>
<th>Barcode</th>
<th>Pad</th>
<th>Link</th>
<th>16S</th>
</tr>
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<td></td>
<td></td>
<td></td>
<td></td>
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<td>TATGGTAATT</td>
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<td>CCTACGGGNGGCWSCAG</td>
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<tr>
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<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
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3. Phytoplankton-heterotrophic bacteria coupling in a dynamic, coastal ocean ecosystem

Coupling between heterotrophic bacteria and phytoplankton is evident from measurements of bulk community parameters, including cell abundance, biomass, growth rates, production (primary and secondary), and extracellular enzyme activities. Further evidence for this coupling comes from physiological and genomic data indicating bacterial transport and metabolism of phytoplankton-derived organic matter (Chrost and Faust 1983; Romera-Castillo et al. 2011; Teeling et al. 2012). Yet, the extent to which heterotrophic bacteria depend on phytoplankton-derived organic carbon is still unknown (Fouilland and Mostajir 2010; Morán and Alonso-Sáez 2011). Heterotroph-phytoplankton coupling may be more important than recognized for specific groups because of unequal contribution to the overall microbial community biomass and activity (Campbell et al. 2011; Hunt et al. 2013; Llewellyn et al. 2005; Marañón et al. 2003; Uitz et al. 2010). However, these relationships are often obscured by bulk community parameters that may not fully capture associations at sufficient spatial, temporal, or taxonomic resolution.

For example, estimates of biomass or primary production derived from measures of chlorophyll-α are poorly constrained because cellular photosynthetic pigment content can vary more than two-fold among cells of the same population (Geider et al. 1997; Geider 1987; Henriksen et al. 2002; Llewellyn et al. 2005; Stramski et al. 2002). Variability
is attributed to physiological response to environmental factors such as light and nutrients (Laws et al. 1983; Owens et al. 1980). Changes in the ratio of phytoplankton size classes may also contribute to inconsistency between measures of chlorophyll-a and cell abundance (Li 2002). Research on the contribution of specific phytoplankton to the total autotrophic biomass and primary production is especially important for understanding the mechanisms of phytoplankton-heterotroph coupling which may depend on taxa-specific interactions (Pinhassi et al. 2004). Flow cytometry provides a robust quantification of phytoplankton cells using light scatter and auto-fluorescent pigments. Flow cytometry can also differentiate Prochlorococcus, Synechococcus, eukaryotic phytoplankton, as well as heterotrophic bacteria, on the basis of cell size and unique pigments signatures or appearance with DNA stains, providing details of microbial community structure.

Thus, flow cytometry can be used to identify groups of phytoplankton that may have distinct effects on heterotrophic bacterioplankton. Coupling between heterotrophic bacteria and phytoplankton may be disrupted when bacterial carbon demand is met by mechanisms such as sloppy feeding, viral lysis, and allochthonous inputs of DOM rather than extracellular release of photosynthate (Jumars et al. 1989; Nagata 2008). Additionally, physical processes in coastal ecosystems, such as seasonal stratification, turbulent mixing and tidal cycles, can induce variations in growth and abundance of planktonic organisms that may result in decoupling (Cermeño et al. 2005). These
environmental fluctuations exert transient pressure on plankton community dynamics on time scales of hours to days; yet, many previous studies demonstrate phytoplankton-bacteria coupling through correlations using seasonal or annual sampling. It is unclear how variations in the biotic and abiotic environment influence phytoplankton-bacteria coupling on shorter time-scales.

This research will investigate the strength of coupling among phytoplankton and non-photosynthetic bacterioplankton (comprising both bacteria and archaea) through a weekly time-series at a single geographic location with strong seasonal patterns in environmental variables.

3.1 Results and discussion

Small phytoplankton and bacterioplankton abundances at the study site were measured weekly, from January 2012 through August 2013, using flow cytometry. A suite of environmental variables were measured concurrently with flow cytometric sampling, as described previously (Johnson et al. 2013). All phytoplankton groups analyzed were present throughout the entire sampling period, in contrast to some coastal sites where Prochlorococcus or Synechococcus are not detected for parts of the year (Jiao et al. 2005; Worden et al. 2004). Synechococcus made the greatest average contribution, 54.6 ± 7%, to the total phytoplankton cell counts (Table 3). The abundance of small eukaryotic phytoplankton, including the Chlorophyta, Stramenopiles, Alveolates, Haptophyta and Cryptophyta (Moon-van der Staay et al. 2001) was the least
variable while the number of observed *Prochlorococcus* cells varied by more than two orders of magnitude (Figure 1). Heterotrophic bacterial abundance exceeded the total phytoplankton abundance by more than an order of magnitude for the majority of the study (Figure 1). Seasonal patterns were observed in the relative contribution of the three autotrophic groups to the total phytoplankton community: picoeukaryotes contributed most to the total community during winter months (December and January) while *Prochlorococcus* and *Synechococcus* both peaked in the summer (Figure 1). The difference in abundance of the three classes of phytoplankton suggests their contribution to primary production and the labile DOM pool may vary seasonally.

We also observed a weak correlation between chlorophyll-\(a\), a proxy for primary production, and total phytoplankton, which may be related to changes in size structure of the phytoplankton community (Marañón et al. 2001) (Figure 3). Although chlorophyll-\(a\) measurements showed seasonality with the highest measurements between August and October (Figure 2), the weak positive relationship between phytoplankton cell counts and chlorophyll-\(a\) reiterates that cell abundance is not directly related to physiological parameters such as primary production or quantity/quality of extracellular photosynthate. On a per cell basis, large phytoplankton contribute disproportionately to primary productivity and biomass compared to picoeukaryotes and cyanobacteria which generally dominate the phytoplankton community in relative abundance (Joint et al. 1986). We have not included data quantifying large eukaryotic
phytoplankton because they occur in very low abundance at the study site, though their importance in primary production, DOM, and interactions with heterotrophic bacteria should not be discounted. Even the small eukaryotic phytoplankton we measured exhibit higher growth rates, cellular carbon and chlorophyll content than cyanobacteria, and could explain the decoupling of phytoplankton cellular abundances from chlorophyll-\(a\) we observed (Worden et al. 2004).

Heterotrophic bacteria were also weakly correlated with chlorophyll-\(a\) despite having yearly maxima and minima occurring in the same seasons as those of chlorophyll-\(a\) (Figure 4). In contrast, there was a strong positive relationship (\(R^2 = 0.454\)) between heterotrophic bacteria and total phytoplankton (Figure 4; Table 3). Examining relationships between heterotrophic bacteria and specific phytoplankton groups indicated that the correlation with picoeukaryotes was the strongest (\(R^2 = 0.329\)) (Table 3). Research investigating the dependence of heterotrophic bacteria on phytoplankton suggests that the strength of coupling varies as a function of ecosystem characteristics, where coupling is the strongest in systems where heterotrophic bacteria are carbon limited (Li et al. 2004; Morán et al. 2002). We hypothesize that the association between heterotrophic bacteria and small eukaryotic phytoplankton is based on labile photosynthetic exudates provisioning bacteria with carbon. Studies investigating the extracellular release of dissolved organic carbon by specific taxa or size classes of marine phytoplankton find high extracellular photosynthate release in the small phytoplankton
compared to large eukaryotic phytoplankton or cyanobacteria (Malinsky-Rushansky and Legrand 1996; Teira et al. 2001). The composition and quantity of photosynthetic extracellular release also varies between eukaryotic phytoplankton and cyanobacteria (Biersmith and Benner 1998; Sarmento and Gasol 2012). Interactions with cyanobacteria should not be discounted, however, because some heterotrophic bacteria also exhibit chemotaxis towards the extracellular products of *Prochlorococcus* and *Synechococcus* and preferentially metabolize substrates of cyanobacterial origin (Nelson and Carlson 2012; Sarmento and Gasol 2012; Seymour et al. 2010). Further investigations of interactions among the microbial community with respect to phylogeny and between individual cells will be critical for resolving the specific mechanisms underlying phytoplankton-bacteria coupling.

The extent of coupling with heterotrophic bacteria determines how much phytoplankton primary production is exported versus recycled in the upper ocean via the microbial loop or transferred to higher trophic levels. Here we show tight coupling of heterotrophic bacteria and phytoplankton cell abundances in a dynamic, coastal environment for weekly samples. Additionally, we find unequal contribution to the observed coupling with heterotrophic bacteria by specific groups of small phytoplankton. Understanding phytoplankton community dynamics is important because phytoplankton sizes classes contribute unequally to total photoautotrophic biomass, primary production and carbon export. Changes in global climate, especially
increasing temperatures, may lead to dominance of small phytoplankton (Morán et al. 2010) and higher rates of heterotrophic bacterial metabolism (Hoppe et al. 2008; Wohlers et al. 2009). Constraining patterns and drivers of phytoplankton and heterotrophic bacteria abundances at multiple scales allows better predictions about the roles of specific microbes in ocean biogeochemical cycles and marine food webs in a changing environment. This research suggests an important role for eukaryotic phytoplankton in interactions with heterotrophic bacteria. Consequently, the next chapter will use laboratory studies to explore associations between heterotrophic bacteria and a model eukaryotic phytoplankter (Thalassiosira rotula).
Figure 1: Cellular abundances of *Prochlorococcus* (triangles), small eukaryotic phytoplankton (diamonds), *Synechococcus* (circles), and heterotrophic bacteria (squares) from weekly sampling of the PICO time-series station in the Beaufort Inlet, NC USA from January 2012 to August 2013
Figure 2: Seasonal patterns of total phytoplankton abundance (crosses) and chlorophyll-α (squares) from January 2012 to August 2013 at the PICO time-series station
Figure 3: Relationship between total phytoplankton abundance and chlorophyll-\(a\) measured weekly at the PICO time-series station from January 2012 to August 2013
Figure 4: Relationships between abundance of (a) Prochlorococcus (b) small eukaryotic phytoplankton (c) Synechococcus (d) total phytoplankton (e) chlorophyll-a compared to heterotrophic bacteria from weekly measurements at the PICO timeseries station from January 2012 to August 2013
Table 4: Maximum and mean concentrations of heterotrophic bacteria, picophytoplankton groups and chlorophyll-\(a\) sampled weekly from January 2012 to August 2013 at the PICO time-series station

<table>
<thead>
<tr>
<th></th>
<th>Heterotrophic bacteria (cells ml(^{-1}))</th>
<th>Pico-eukaryotic phytoplankton (cells ml(^{-1}))</th>
<th>Prochlorococcus (cells ml(^{-1}))</th>
<th>Synechococcus (cells ml(^{-1}))</th>
<th>Total picophytoplankton (cells ml(^{-1}))</th>
<th>Chlorophyll (-a) ((\mu\mu g) L(^{-1}))</th>
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<tr>
<td>Maximum</td>
<td>5621782</td>
<td>139835</td>
<td>296154</td>
<td>235355</td>
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<tr>
<td>Mean</td>
<td>2734106</td>
<td>37713</td>
<td>26298</td>
<td>84584</td>
<td>148595</td>
<td>5.0</td>
</tr>
<tr>
<td>Standard error of mean</td>
<td>81301</td>
<td>1806</td>
<td>3771</td>
<td>4787</td>
<td>7895</td>
<td>0.22</td>
</tr>
<tr>
<td>Average contribution to total picophytoplankton</td>
<td>--</td>
<td>25.4 ± 6%</td>
<td>19.9 ± 16%</td>
<td>54.6 ± 7%</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Correlation with heterotrophic bacteria ((R^2))</td>
<td>--</td>
<td>0.33</td>
<td>0.28</td>
<td>0.23</td>
<td>0.45</td>
<td>0.14</td>
</tr>
</tbody>
</table>
4. Associations between heterotrophic bacteria and a model microalga

4.1.1 Phylogenetic specificity of algal-bacteria associations

The aim of this study was to investigate the taxonomic specificity of heterotrophic bacteria for five strains of the diatom species, Thalassiosira rotula. The T. rotula strains were isolated from diverse geographic locations, have been maintained in culture for differing lengths of time, and represent two distinct genetic lineages based on ITS1 sequence (Table 2; (Whittaker et al. 2012)). Previously, no significant difference in physiology or growth rate in axenic versus xenic cultures of these strains of T. rotula was observed (Whittaker et al. 2012) indicating they may not depend on interactions with heterotrophic bacteria for normal growth. However, the composition and phylogenetic specificity of heterotrophic bacteria in cultured T. rotula strains has not been previously described. In this study we take two approaches to examining the community structure of heterotrophic bacteria that are found in co-culture with the diatom T. rotula: culture-based and culture-independent. Culture-based studies will allow us develop an isolate library that can be used in the future to test the effect of specific heterotrophs on the growth of T. rotula. However, culture-dependent investigations do not accurately capture bacterial community diversity due to limitations in our ability to culture all bacteria (Amann et al. 1995). In contrast, culture-independent studies afford a more complete characterization of the bacterioplankton community present, but do not allow us to explore specific phytoplankton-heterotrophic bacteria associations with
experimental manipulations. The combination of these two approaches should yield a more complete picture of the bacteria found in co-culture with distinct strains of *T. rotula*.

### 4.1.2 Results and Discussion

We identified 102 heterotrophic bacterial isolates from *T. rotula* cultures and seawater collected during a *T. rotula* bloom based on partial 16S rRNA gene sequencing. Most cultured isolates were Alphaproteobacteria (40.2%) or Gammaproteobacteria (47.1%) (Figure 5) which are often the dominant classes found in association with phytoplankton (Jasti et al. 2005; Pinhassi et al. 2004; Sapp et al. 2007; Sison-Mangus et al. 2014). Within the Gammaproteobacteria, most sequences have highest homology with *Marinobacter* and *Alteromonas* genera and the largest clade within the Alphaproteobacteria is identified as *Maricaulis* spp. (Figure 5). Members of the *Marinobacter* associate with phytoplankton through a carbon-for-iron mutualism where the bacteria produce siderophores that provision phytoplankton with iron in exchange for phytoplankton DOM (Amin et al. 2012a; Amin et al. 2009) and they also promote aggregate formation in diatoms (Gardes et al. 2011; Gardes et al. 2012; Kaeppel et al. 2011). Our findings indicate that associations between *T. rotula* and Proteobacteria are common or that certain members of the Proteobacteria are well adapted to growth in culture conditions.
Only a minor fraction of the culturable bacteria sequences are in the phyla Bacteriodetes (8.8%) or Actinobacteria (3.9%), and of those, most came from the seawater sample. Actinobacteria, in particular, are rarely found in phylogenetic studies of phytoplankton-associated bacteria (Sapp et al. 2007; Sison-Mangus et al. 2014). It is possible that laboratory conditions or negative interactions with phytoplankton or phytoplankton-associated bacteria select against members of this phyla in culture. Overall, the culturable heterotrophic bacteria isolated from the seawater were significantly different than isolates from \textit{T. rotula} cultures (P < 0.01) based on pair-wise comparisons using the unweighted UniFrac algorithm. This finding suggests that culture conditions and co-existence with a eukaryotic microalgae support different heterotrophic bacteria than those present in a seawater assemblage.

While the observed difference between heterotrophic bacteria from a \textit{T. rotula} bloom and \textit{T. rotula} cultures may be expected, we also examined whether the isolated bacterial phylotypes differed between three strains of \textit{T. rotula}: CCMP3264, JPNTR18, and WPF8. Isolates from strain JPNTR18 were marginally different from strain WPF8 (P = 0.06) but isolates from CCMP3264 were not significantly different from the other strains (P = 0.99, 0.12 respectively). JPNTR18 and WPF8 represent members of \textit{T. rotula} genetic lineages 3 and 1 respectively. Unique culturable bacteria found in association with these \textit{T. rotula} strains could result from fine-scale genetic differentiation in metabolic genes, requiring association with different heterotrophs. Sher and colleagues
(2011) made a similar observation in strains of *Prochlorococcus*, where different *Prochlorococcus* ecotypes harbored unique beneficial, growth-promoting heterotrophic bacterial taxa in culture. The similarity between the bacterial assemblage in culture with CCMP3264, JPNTR18 and WPF8 indicates that there may be heterotrophic taxa shared by all *T. rotula* or more broadly, diatoms. For example, Schäfer and colleagues (2002) found that all diatom cultures harbor at least one type of Alphaproteobacteria and one member of the Bacteroidetes.

In contrast, the observed genetic diversity of heterotrophic bacteria associated with individual *T. rotula* cultures may reflect stochastic maintenance of bacteria that occupy diatom-associated or culture-associated niches. If the niches are broad functional categories such as carbon-degradation or reactive oxygen scavenging, it is possible that many bacterial phylotypes could occupy those niches. However, only a random of subset all possible bacterial taxa are likely represented in culture due to competition or reduction in bacterial diversity during isolation of the diatom (Langenheder and Székely 2011; Lee et al. 2013). Functional redundancy among bacterial taxa could also permit similar phylotypes or guilds to co-exist for the purpose of maintaining metabolic processes that are important for *T. rotula* (Burke et al. 2011a; Burke et al. 2011b). Future studies testing the reproducibility of the heterotrophic bacterial community from multiple cultures of the same strain of *T. rotula* will help validate the specificity and functional niches of phytoplankton-associated bacteria. Overall, these findings suggest
that genetic lineages of *T. rotula* may associate with unique heterotrophic bacterial taxa while also sharing a common set of bacteria that occupy a phytoplankton-associated niche; this niche may or may not be specific diatoms or more exclusively to *T. rotula*. Examining the heterotrophic bacteria assemblages using culture-independent, high-throughput DNA sequencing provides further resolution of the phylogenetic signature of bacteria in culture with specific strains of *T. rotula*.

After chimeric sequences were removed, the culture-independent 16S rRNA gene libraries, generated by the Illumina MiSeq platform, from heterotrophic bacteria associated with *T. rotula* ranged from 20,842 to 57,879 reads (Table 4). All samples were rarified to an equal depth of 20,000 sequences per sample (Figure 6), OTUs were assigned based on a 97% similarity threshold. Libraries were predominately composed of operational taxonomic units (OTUs) from the phylum Proteobacteria (65.6%), especially Alphaproteobacteria (58.2%) and Gammaproteobacteria (7.2%) (percentages indicate relative contribution to the total number of reads). This finding is comparable to the 16S rRNA gene libraries from the cultured bacterial isolates. In contrast, the phylum Bacteroidetes (32.1%), in particular members of the class Flavobacteria (26.0%), was also abundant in the culture-independent samples (Figure 7). Flavobacteria are known to associate with eukaryotic microalgae in phytoplankton blooms and in culture, especially particles and aggregates comprised of phytoplankton cells (Grossart et al. 2005; Pinhassi
et al. 2004; Riemann et al. 2000). Their absence from the cultured isolate 16S gene library likely reflects a bias in the culturing technique (Alonso et al. 2007).

Thus, culture-independent approaches effectively capture more of the bacterial community diversity than culture-based methods, through both increased sequencing depth and an absence of selection for culturable organisms. As expected, the seawater sample had the highest taxonomic richness of all samples (Table 4). Of the bacterial communities in culture, T. rotula strain JPNTR18 and CCMP3264 host the greatest taxonomic diversity (Table 4; Appendix A). Beta diversity analyses using unweighted UniFrac significance indicate that the heterotrophic bacterial community composition of the T. rotula bloom sample is significantly different from all T. rotula culture samples (Table 5; Figure 8). However, the bacterial community from strains of T. rotula representing two genetic lineages showed no clear distinction between lineages.

Statistical comparisons of the community diversity between T. rotula cultures revealed that the bacterial community associated with strain JPNTR18 is significantly different from strains CCMP3264 and FR3.012, although the latter two strains are not significantly different from each other (Table 5). These strains all belong to genetic lineage 3, suggesting factors other than genetic differentiation based on ITS1 sequence may define the diatom-associated bacterial community. Factors including composition of source community, length of time in culture, strain-specific genome content, gene expression
and physiological state of the diatom, that may shape the phylogeny of bacterial associations with different strains of *T. rotula* need to be further explored.

With the observation of some differences in bacterial community composition between strains of *T. rotula* (Figure 8), we also asked whether there is a set of bacterial taxa that commonly associates with all strains of the diatom. We found no OTUs observed in all five cultures and the *T. rotula* bloom sample. Analysis of OTUs shared across at least four of the six total samples, revealed only six common OTUs. All OTUs were members of the Alphaproteobacteria and most OTUs were unclassified at the genus level (Table 6). Notable taxa found in the majority of samples were of the genera *Marivita* and *Phaeobacter*. *Marivita spp.* were also isolated from *T. rotula* cultures and are potential candidates for future experimental studies examining mechanisms of interactions between *T. rotula* and heterotrophic bacteria. *Phaeobacter spp.* fall within the Roseobacter clade, a dominant group that comprises 20 to 30% of bacteria in marine surface waters (Buchan et al. 2005). Members of the genus *Phaeobacter* are recognized for opportunistic symbioses with eukaryotic microalgae mediated by secondary metabolite production and the ability to sense and degrade algal-produced DMSP (Miller and Belas 2004; Thiel et al. 2010). Many *Phaeobacter spp.* synthesize antibiotics, such as tropodithietic acid (TDA), that function to protect host microalgae from pathogenic heterotrophic bacteria (Cude et al. 2012; Geng et al. 2008). They also produce auxins that promote algal growth as well as small, algalytic molecules called roseobacticides, that
are regulated by chemical signaling molecules produced by senescing algal cells and the
Phaeobacter population (Berger et al. 2011; Seyedsayamdost et al. 2011a; Seyedsayamdost et al. 2011b). We hypothesize the Alphaproteobacteria common to T. rotula cultures may engage in symbiotic associations with the diatom that are mediated by secondary metabolites, as described above. Further research is needed to understand the physiological effects of associations between T. rotula and their common heterotrophic bacterial partners and the environmental and metabolic factors underlying these associations. By characterizing interactions between phytoplankton and heterotrophic bacteria, we can better understand the dynamics of phytoplankton and bacterioplankton community composition in marine ecosystems and ultimately determine how microbial community structure influences ecological and biogeochemical processes in the ocean.
Figure 5: Phylogenetic tree of partial 16S rRNA gene sequences from heterotrophic bacterial isolates from seawater collected within a *Thalassiosira rotula* bloom in Narragansett Bay, RI and cultures of three *T. rotula* strains: CCMP3264, JPNTR18 and WPF8 (colored squares outside leaves correspond to culture source: red = CCMP3264, yellow = JPNTR18, blue = WPF8). Leaf labels indicate source of isolate (culture or bloom seawater) and closest genus homology in the RDP database. Orange circles indicate a bootstrap percentage greater than 80% at that node.
Table 5: Alpha-diversity indices for 16S rRNA gene libraries from each sample

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<th>Sample ID</th>
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<th>OTUs</th>
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<th>Shannon index</th>
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<td>40178</td>
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<td>957</td>
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</tr>
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<td>FR3.012</td>
<td>33144</td>
<td>65</td>
<td>78</td>
<td>2.49</td>
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<td>CCMP3264</td>
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<td>112</td>
<td>133</td>
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<tr>
<td>JPNTR18</td>
<td>26752</td>
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<td>181</td>
<td>3.52</td>
</tr>
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<td>WPF8</td>
<td>25331</td>
<td>63</td>
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<td>3.13</td>
</tr>
</tbody>
</table>

Figure 6: Rarefaction collection curves of the number of bacterial and archaeal OTUs based on clustering sequences at 97% similarity versus the number of sequences sampled in each 16S rRNA gene library from T. rotula cultures and seawater
Figure 7: Taxonomic summary of key members of the bacterial communities from *T. rotula* cultures and *T. rotula* bloom seawater at the phylum level. Only the six most abundant phyla are shown.
Table 6: Significance tests* of community dissimilarity between all pairs of samples using the unweighted UniFrac beta diversity metric. Strains labeled in blue are from lineage 3; the red labeled strain is from lineage 1.

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>CCMP1647</th>
<th>CCMP3264</th>
<th>FR3.012</th>
<th>JPNTR18</th>
<th>WPF8</th>
<th>T. rotula bloom</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCMP1647</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td></td>
<td>≤0.001</td>
</tr>
<tr>
<td>CCMP3264</td>
<td></td>
<td>0.15</td>
<td>≤0.001</td>
<td>1</td>
<td></td>
<td>≤0.001</td>
</tr>
<tr>
<td>FR3.012</td>
<td></td>
<td></td>
<td>≤0.001</td>
<td>1</td>
<td></td>
<td>≤0.001</td>
</tr>
<tr>
<td>JPNTR18</td>
<td></td>
<td></td>
<td></td>
<td>0.15</td>
<td></td>
<td>≤0.001</td>
</tr>
<tr>
<td>WPF8</td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>T. rotula bloom</td>
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</tr>
</tbody>
</table>

*Yellow highlighted cells are significantly different (Bonferroni corrected p-value < 0.01)
Figure 8: Principal coordinate analysis (PCoA) of bacterial communities from *T. rotula* cultures CCMP1647 (red), CCMP3264 (blue), FR3.012 (orange), JPNTR18 (green), WPF8 (yellow), and seawater collected from a *T. rotula* bloom (purple). Comparisons are based on Bray-Curtis distance.
Table 7: Taxonomic summary of OTUs shared* between at least four of six *T. rotula* cultures and *T. rotula* bloom seawater samples.

<table>
<thead>
<tr>
<th>Taxon</th>
<th><em>T. rotula</em> bloom</th>
<th>CCMP1647</th>
<th>CCMP3264</th>
<th>FR3.012</th>
<th>JPNTR18</th>
<th>WPF8</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Marivita</em> spp.</td>
<td>Green</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Phaeobacter</em> spp.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unclassified Rhodobacteriales</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unclassified Pelagibacteraceae</td>
<td>Green</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unclassified Sphingomonadales</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unclassified Alphaproteobacteria</td>
<td></td>
<td></td>
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</tbody>
</table>

*Green cells indicate presence of OTU in sample.
5. Summary and future directions

In summary, we examined the taxonomic characteristics of phytoplankton-heterotrophic bacteria associations in a dynamic, coastal ocean environment and in controlled, laboratory cultures. Our findings indicate close coupling of heterotrophic bacterioplankton and small phytoplankton abundances across seasons at a coastal ocean site. The coastal time-series data support classic observations of phytoplankton-bacteria coupling based on bulk community parameters of cell abundances and chlorophyll-\(a\) at ocean-basin scales (Bird and Kalff 1984; Landry et al. 1996; Li et al. 1992; Morán et al. 2002). We demonstrate that heterotrophic bacteria are more tightly correlated with phytoplankton cell abundances than chlorophyll-\(a\) and the ability of this study to differentiate groups of phytoplankton allowed us to constrain which constituents of the phytoplankton are mostly likely to interact with bacteria. Throughout seasonal cycles, heterotrophic bacteria were more closely associated with small eukaryotic phytoplankton than the cyanobacterial groups, \textit{Prochlorococcus} or \textit{Synechococcus}. Given our finding that associations between bacteria and eukaryotic phytoplankton drive ecologically important heterotroph-phytoplankton coupling, we then examined the community composition of heterotrophic bacteria in culture with a model diatom, \textit{Thalassiosira rotula} to determine if specific taxa of heterotrophic bacteria more commonly interact with phytoplankton.
We asked whether strains of *T. rotula* that were isolated from diverse geographic locations in different years, representing two distinct genetic lineages, support unique bacterial communities. The culturable heterotrophic bacteria communities associated with *T. rotula* were phylogenetically distinct from the seawater bacteria community collected during a *T. rotula* bloom suggesting that diatom cultures select for specific bacterial taxa. Furthermore, we found evidence that genetically distinct lineages of *T. rotula* support unique heterotrophic bacterial taxa but that all *T. rotula* may associate with a common set of bacterial phylotypes. For example, members of the Alphaproteobacteria appear to associate closely with all strains of *T. rotula*, in particular, *Marinobacter* and *Phaeobacter spp*. While we provide support for taxonomic specificity in phytoplankton-heterotrophic bacteria associations, the mechanisms structuring bacterial communities in *T. rotula* cultures and their effects on the growth and physiology of these organisms are not known.

The research presented is a snapshot of the heterotrophic bacterial community diversity in *T. rotula* cultures and does not capture dynamics that may exist in the diatom-associated bacterial community. In the future, stability of the heterotrophic bacterial community composition associated with *T. rotula* cultures should be investigated with respect to maintenance in culture for defined periods of time, addition of foreign or “contaminating” bacteria, and physiological state of the diatom to
determine factors that may alter the composition or structure of the phytoplankton-associated bacterial assemblage.

Future research will utilize culturable heterotrophic bacteria isolated in this study to explore the physiological effects of individual heterotrophic bacteria taxa on axenic strains of *T. rotula*. We observed that cultures of *T. rotula* do not grow well in the absence of heterotrophic bacteria, but we do not know which bacteria are responsible for promoting diatom growth or if any heterotrophic bacteria taxa within a broad suite of functionally similar organisms contribute to enhanced growth. By adding individual heterotrophic taxa to axenic *T. rotula* cultures, we can determine which bacterial taxa benefit the diatom by stimulating growth rate or delaying the onset of stationary phase (Grossart 1999; Sher et al. 2011; Sison-Mangus et al. 2014). The mechanisms by which those heterotrophic bacteria support their diatom hosts should then be explored. The question of whether diatom-bacteria relationships are mediated by passive nutrient recycling or are highly regulated by signaling and specific interactions needs to be addressed.

With passive nutrient recycling, there is no direct interaction between phytoplankton and bacteria. If the relationship is symbiotic, however, the heterotrophic bacteria and diatom must have a way to recognize each other to engage in targeted nutrient exchange, and thus coordinate metabolisms. Molecular techniques such as transcriptomics can elucidate molecular signals indicative of symbiotic relationships.
Future research could examine gene expression of model heterotrophic bacteria and *T. rotula*, identified as putatively mutualistic in co-culture studies, to detect transcriptional patterns characterizing beneficial algal-bacterial interactions. Symbioses between heterotrophic bacteria and phytoplankton are often facilitated by cofactor production (e.g. vitamin B₁₂ and siderophores) or detoxification (Croft et al. 2005; Grossart 1999; Kazamia et al. 2012; Morris et al. 2008) and potentially initiated by cell-to-cell signaling (Seyedsayamdost et al. 2011b). Unregulated gene expression related to synthesis of secondary metabolites and signaling molecules in co-cultures of *T. rotula* and a heterotrophic bacterium compared to expression in pure cultures would suggest mutualistic relationships between the bacteria and *T. rotula*. Additional research will characterize the molecular mechanisms underlying phytoplankton-bacteria associations to determine if the taxonomic specificity we observed corresponds to regulated symbioses or passive nutrient and carbon exchange.

In order to understand the ecology of marine microbes and predict the influence of microbial interactions on ocean ecosystems, the taxonomic specificity and physiological effects of heterotrophic bacteria-phytoplankton associations need to be better characterized. Here we show how observations from environmental time-series and phylogenetic characterizations can serve as guides for developing laboratory-based experimental studies. By understanding how associations between phytoplankton and bacteria are formed and maintained under controlled culture conditions, we ask more
informed questions about natural microbial interactions and their importance in the marine environment.
References


Langenheder, S. & A. J. Székely, 2011. Species sorting and neutral processes are both important during the initial assembly of bacterial communities. The ISME journal 5(7):1086-1094.


