EXPLORING THE DIVERSITY, HOST-MICROBE EFFECTS, AND FUNCTIONAL PROPERTIES OF DIATOM-ASSOCIATED BACTERIA IN THE OLIGOTROPHIC OCEAN

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DEDICATION

This dissertation is dedicated to my family, especially my mother, Cynthia Giroux, and my grandmother Regina Baker, for always impressing on me the value of education, hard work, and independence.

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ABSTRACT

The ecology of diatoms may be better explained by conceptualizing them as composite organisms consisting of the host cell and its bacterial associates. Attached bacteria have been shown to impact diatom growth, prolong blooms, and trigger the production of algal toxins in relatively eutrophic systems. This research is among the first to explore diatom-bacterial interactions in an oligotrophic system. In previous work, we found that bacterial assemblages attached to individual host cells varied substantially even among closely related hosts. The assemblages could be separated into three distinct groups, irrespective of host cell identity. Instead, the distinct groups were best explained by the diversity of the bacteria in each group. For example, in one group, a single bacterial genus (*Arthrobacter*) occurred to the near-exclusion of any other bacteria on the same host cell, whereas a second group was much more diverse. The presence of markedly different bacteria assemblages led to the present body of work, which examines whether such groups represent persistent diatom-bacterial associations, and how these associations might be affected by abiotic (e.g. nutrients) or biotic (e.g. host-bacteria or bacteria-bacteria interactions) factors. The effect of abiotic stressors was tested in a multi-factor experiment designed to examine changes in the phylogenetic composition of bacteria attached to a *Chaetoceros* diatom. Ribosomal DNA was used to monitor changes in the composition of host-associated bacteria in response to changes in the host's growth induced by manipulations of nutrient concentration and viral infection. *Marinobacter* and *Alteromonas* phylotypes dominated the bacterial consortia attached to the *Chaetoceros* in all treatments, regardless of host growth stage. Nutrient concentration and host growth stage were found to have a statistically significant effect on the phylogenetic composition of the attached bacteria. Additionally, interactions between attached bacteria were found to significantly affect the composition of host-associated bacteria. These results led to an exploration of the effects of perturbations of the relative proportions of bacteria on a xenic diatom host. Multiple strains of *Alteromonas* and *Marinobacter* were isolated from a *Chaetoceros* culture descended from a single isolated *Chaetoceros* cell. Individual bacterial strains were added to three different xenic diatom hosts (the origin host *Chaetoceros* sp. KBDT20, a naïve

Chaetoceros host, and a naïve *Amphiprora* host), to evaluate whether perturbations in their bacterial consortia could affect host growth, carrying capacity, and decline. Additionally, inoculations were repeated in vitamin-rich and vitamin-poor media to evaluate if vitamin concentrations modify the effect of bacterial inoculations on host trajectory. For the naïve *Chaetoceros* host, manipulating the co-cultured bacteria had lasting effects on host trajectory, demonstrating a lack of resilience or resistance of the host-bacteria association to perturbations of the bacterial component. In contrast, manipulating the co-cultured bacteria had minimal effects on the origin *Chaetoceros* host or the more distantly related *Amphiprora* host, implying that host-bacteria associations may vary greatly in their resistance or resilience to perturbation. None of the bacterial strains had a constantly negative or positive impact on all three tested hosts, but the most common significant outcome was a negative effect on the host. Additionally, different strains of the same bacterial genus had different magnitudes of impact on the host. The concentration of vitamins affected the impact of bacterial inoculations. Mutualistic effects were only observed in vitamin-replete media, whereas effects that suggest parasitism or competition were observed in vitamin-deplete media. Finally, a metagenomic analysis focused on the bacteria associated with field-collected, single diatom cells to gain a better understanding of the functional capabilities contributed by bacteria to a diatom-bacterial association in nature. Six diatom cells derived from two of the three distinct groups mentioned above were selected for analysis; three cells were from a group with more diverse bacterial associates, and three from a group dominated by a single bacterial genus. Although the groups were phylogenetically distinct, the general functional capacities of the bacterial consortia did not significantly differ between groups; however, individual genes were significantly enriched in the less diverse group. Although many of the genes previously suggested to be indicative of diatom-bacterial interactions were absent, functional genes associated with enhanced metabolic and colonization capacities were present in these diatom microbiomes. As a whole, diatom-associated bacteria were genetically distinct from all other samples used for comparison, including free-living and microplastic-associated bacteria from the oligotrophic North Pacific Ocean. This suggests that the *Thalassiosira*-associated bacteria in our samples are in some ways functionally distinct from free-living and plastic-associated bacteria from the oligotrophic open ocean, even if

these functions were not previously considered indicative of symbiotic interactions. Overall, the findings of this research suggest that diatom-bacterial interactions are dependent on host growth state as well as both micro- and macronutrients, and advocates for further study of this interaction in oligotrophic systems and xenic cultures.

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Chapter 1: General introduction to diatom bacterial interactions and the environments studied

Diatom health and growth

Diatoms are responsible for nearly 40% of total marine primary productivity; the carbon fixed by oceanic diatoms is equivalent to the organic carbon produced by all of the terrestrial rainforests combined (Nelson et al. 1995). Diatoms not only generate organic carbon from carbon dioxide, but also play a major role in the 'biological pump', wherein nutrients (N, P, Fe, Si, trace metals) are taken up in the euphotic zone and sink to the benthos as biological material incorporated into fecal pellets or marine snow. Nearly half of the sinking organic carbon produced by diatoms is consumed by bacteria in the water column and remineralized into the upper ocean as inorganic nutrients, a pathway referred to as the microbial loop (Azam et al. 1983). Microbial remineralization is essential to maintain major nutrients in this system (Williams and Yentsch 1976; Cole 1982), including silica, which otherwise may limit diatom growth (Bidle et al. 2003).

Diatoms are typically the dominant phytoplankton in well-mixed, high nutrient environments such as upwelling regions, along the coast, and at the edge of sea-ice in polar regions (Armbrust 2009). However, diatoms are also important contributors to sinking organic carbon in the oligotrophic ocean; diatoms are estimated to be responsible for only 2-7% of the total photosynthetic production at station ALOHA (22° 45'N, 158° 00'W); however, they are disproportionately represented in sinking biomass and have been estimated to account for 9-20% of the total organic carbon export (Brzezinski et al. 2011).

The health and productivity of diatoms is strongly influenced by their interactions with other microbes, particularly bacteria. Early studies assumed that bacteria only attach to dead or dying diatoms (Azam et al. 1983), possibly because healthy diatoms are capable of producing antimicrobial agents. It is now accepted that bacteria attach to living, healthy diatoms, and that bacteria may affect diatom metabolism and health (Grossart 2010; Amin et al. 2012a, 2015; Gärdes et al. 2013; Grant et al. 2014). Much of this research has utilized cultures to study marine diatom-bacterial interactions (Kogure et al. 1982; Grossart 1999; Grossart et al. 2005;

Kaczmarska et al. 2005; Grossart and Simon 2007). Few studies of diatom-bacterial interactions have been conducted using native populations of diatoms (Kaczmarska et al. 2005), and only one study involved a simulated bloom in a mesocosm (Smith et al. 1995).

Previous studies of diatom-bacterial interactions have commonly focused on a single mode of a classic symbiotic relationship, e.g. mutualism, commensalism, or parasitism. For example, Croft proposed that bacteria in the muciferous layer of *Thalassiosira pseudonana* provide the diatom vitamin B_{12} and in return gain a secure source of carbon, thereby forming a mutualistic relationship (Croft et al. 2005). Similarly, commensalism has been suggested wherein diatoms are unaffected while bacteria have access to a secure carbon source (Rosowski 1992; Droop 2007). Bacteria have also been shown to act as parasites, producing enzymes that can cause dissolution of the organic matrix on which diatom frustules are built (Bidle and Azam 1999), and some diatoms have been shown to produce antibiotics to ward off bacterial parasites (Grossart 1999). However, bacterial interactions with algal hosts can also be biphasic; shifting from a mutualistic interaction during host growth to a parasitic interaction during host decline(Seyedsayamdost et al. 2011; Wang et al. 2014).

Diatom-bacterial interactions have been shown to affect bloom duration and diatom biomass in mesocosms (Smith et al. 1995), implying that diatom-bacterial interactions may also be important in the open ocean. The effects of diatom-bacterial associations on bloom dynamics, diatom health or survival are likely to influence ocean biogeochemistry, with potential implications for particulate carbon production and carbon sequestration. Different diatoms have very different propensities to aggregate and sink, and diatom-bacterial associations may influence export by influencing the success of different diatom species. Properties of diatom-bacteria associations can also impact human health. For example, direct contact between *Pseudo-nitzschia* host cells and their associated bacteria appears to be required for increased host production of domoic acid (Kobayashi et al. 2009; Guannel et al. 2011), an amino acid that causes amnesiac shellfish poisoning in humans.

Presumably, environmental studies based on bulk or community-level properties have always measured the net result of biological interactions within diatom-bacterial associations, but their conclusions may be biased by a failure to recognize the true complexity of this interaction. A better understanding of diatom-bacteria associations may improve predictions regarding biomass production, degradation, and export. For example, the relative success of different diatom species responding to nutrient inputs may be contingent upon properties of the respective association, not easily predicted from measuring properties of the diatom alone.

Diatom-bacterial symbiosis

For 200 million years, interactions between marine diatoms and bacteria likely influenced the evolutionary trajectory of both (Amin et al. 2012b). Symbiotic relationships in coral (Carlos et al. 2013; Glasl et al. 2016) and sponges (Blanquer et al. 2013; Thomas et al. 2016)developed over a similar timespan. Diatom-microbe interactions in the ocean have become an increasingly important area of study (Olson and Kellogg 2010), because of their ecological importance (Fuhrman et al. 2015), their practical importance in the aquaculture industry (Fukami et al. 1997; Fuentes et al. 2016), and their relevance to human health (Kobayashi et al. 2009; Guannel et al. 2011).

A bacterial cell detects and travels to a source of chemical stimuli, such as a diatom, by directing its ‘walk' in a process called chemotaxis. The general model for chemotaxis is a random walk in which the bacterium travels in a straight line and stops at intervals to ‘tumble' and swim in a new direction, in response to a chemical stimulus. Chemotaxis in aquatic systems differs from those of terrestrial and enteric bacteria in that aquatic bacteria can form clusters relatively quickly. Aquatic bacteria have adapted to find microenvironments that are only tens of micrometers across, such as a detrital particle or a living alga. It is assumed that chemotaxis is prevalent in the open ocean because chemotactic behavior is especially advantageous in systems where there are inhomogeneous and ephemeral distributions of nutrients (Purcell 1977). Performing chemotaxis requires a

large genetic burden (Barbara and Mitchell 2003) with an associated energetic cost. Even though chemotaxis is genetically and energetically expensive, it has been estimated that as much as 60% of marine bacteria possess the ability to be motile, if only for a short amount of time or under specific conditions (Grossart et al. 2001), such as during algal bloom and collapse (Smriga et al. 2016). Overall, motility may be essential for bacteria to associate with diatoms (Ivars-Martínez et al. 2008; Sonnenschein et al. 2012).

Surface attachment also may be essential for bacteria to associate with diatoms (Kaczmarska et al. 2005). Select bacteria are capable of attaching to surfaces in the open ocean (Delong et al. 1993; Mitchell et al. 1995; Blackburn 1998; Grossart et al. 2006; Grossart and Simon 2007); this adaptation may confer an evolutionary advantage in exchange for the genetic load and metabolic cost of expressing genes associated with a surface-attached lifestyle. For example, to attach to a surface a bacterium produces a large extracellular glycolipids and glycoproteins (Desai and Banat 1997) that can be up to 10nm in diameter (Auerbach et al. 2000). Even when there are sufficient nutrients in the water to support growth, some bacteria colonize surfaces and subsequently invest in the production of antibacterial compounds to reduce competition with other species for the same surface (Yan et al. 2002).

Nutrient limitation at the study sites

This study explores diatom-bacterial interactions in two oligotrophic systems where diatoms are not commonly the dominant phytoplankton, a predominantly oligotrophic bay (Kāneʻohe Bay) and the permanently oligotrophic open ocean (Station ALOHA). Although diatoms can sometimes dominate Kāneʻohe Bay following nutrient inputs from storm events (Ringuet and Mackenzie 2005; Hoover and MacKenzie 2009), the system is normally oligotrophic (Droop 2007) and dominated by picoplankton (*Synechococcus*) (Yeo et al. 2013). The dominant phytoplankton of Station ALOHA are also picoplankton (*Prochlorococcus*) (Karl and Church 2014).

Diatoms are most active in the euphotic zone. The depth of the euphotic zone varies in Kāne'ohe bay depending on turbidity, but at Station ALOHA typically extends to 200m, with light levels of 10-100 μE $m^{-2}s^{-1}$ (Cortés et al. 2001). Diatoms are thought to be relatively resilient to changing light levels (Mitrovic et al. 2003), however, their ability to thrive under varying light depends on other factors including salinity and temperature (Miller and Kamykowski 1986). Nutrient requirements are highly variable among diatom species. Table 1 compares nutrient requirements for diatoms to the nutrient concentrations commonly seen at Station ALOHA and Kāne'ohe Bay. Maximum growth rates are also highly variable among diatom species, ranging from 0.2 d^{-1} to 3.3 d^{-1} with an average of 1.5 ± 0.8 d^{-1} (n=67). The most likely cause of this variation is diatom cell volume, which can vary from 13 μm^3 to $7x10^5$ μm^3 (Sarthou et al. 2005). Larger cells have a lower surface to volume ratio than smaller cells of the same morphology, thereby limiting diffusion of nutrients into the larger cells.

A comparison of the range of nutrient concentrations at Station ALOHA and to average Ks values (Table 1) suggests that nitrogen may sometimes limit diatom growth whereas phosphorus and iron are less likely to be limiting. The average concentration of nutrients in Kāne'ohe Bay is significantly higher, but suggests that phosphorous may be limiting for some diatom species. Silica has been hypothesized to be the most important nutrient in maintaining diatom growth (Brzezinski et al. 2011). The average concentration of undissociated monomeric silicic acid ($Si(OH)_4$) in the euphotic zone of the world's ocean is 10 μM (Tréguer and De La Rocha 2013); concentrations at Station ALOHA range from 0.6 to 1.6 μM in surface waters (Brzezinski et al. 2011), and from 3.2 to 46.3 μM in the deep chlorophyll maximum (DCM) (Table 1). Brzezinski et al (2011) suggested that diatoms in this system adapt to low silica levels by adjusting their Si uptake efficiency. In more productive systems, silicic acid concentrations can vary with biological consumption and export. The average concentration of silica at Kāne'ohe Bay is well below the maximum Ks, and some diatom species may be silica limited. At Station ALOHA, biological activity has little effect on the concentration of silicic acid, as it would take between 50-100 days for the populations that utilize silicic acid to cause even a 1 μM difference (Brzezinski et al. 2011). Therefore, the concentration in this system is primarily dependent on the history of the water mass that traverses the station.

Nitrogen, phosphorous, silica, and iron are not the only inorganic nutrients required for diatom growth. Other nutrients seldom measured in open-ocean systems may also be limiting, such as zinc (Zn) (Varela et al. 2011), and cadmium (Cd), which can replace Zn as a cofactor of carbonic anhydrase when Zn is in limiting supply (Lane and Morel 2000).

Vitamin auxotrophy, or the inability to synthesize organic nutrients required for growth, may be relatively common in microalgal species. B-vitamins are important cofactors in multiple metabolic processes and are required to fix CO_2, transfer electrons in oxidation-reduction reactions, and synthesize deoxyribose, fatty acids, carbohydrates, and branched-chain amino acids. Based on algal culture studies, it has been projected that one-fifth of microalgae require external sources of vitamin B_1 (thiamine) and that one in twenty require B_7 (biotin). Nearly half of all microalgae are estimated to be vitamin B_{12} (cobalamin) auxotrophic (Croft et al. 2006), and more specifically, 60% of all cultured haptophytes and heterokonts (Tang et al. 2010) require an external source of B_{12}. Additionally, vitamin B_{12} auxotrophy in diatoms can be inferred from the variant of methionine synthase present in a population; metH requires vitamin B_{12} as a cofactor and metE does not. Two diatom species genomes have been completed, for *Phaeodactylum tricornutum* and *Thalassiosira pseudonana* (Armbrust et al. 2004; Bowler et al. 2008). The B_{12}-independent metE variant was found in the *P. tricornutum* genome but only metH was found in the *T. pseudonana* genome (Croft et al. 2005). From this, we can infer that the growth of *P. tricornutum* does not require external sources of B_{12} to complete methionine synthase, while the growth of *T. pseudonana* is dependent on external sources of B_{12}. Vitamin auxotrophy in the genus *Thalassiosira* is also supported by various studies of the genus in culture (Armbrust et al. 2004; Grant et al. 2014; Durham et al. 2015)

Despite their metabolic importance, B-vitamin concentrations are not often studied because of the complex methods required for measurement. Much of the open ocean has been proposed to be a virtual “vitamin

desert," devoid of the major B-vitamins necessary for growth, while coastal environments are relatively rich in B-vitamins. B-vitamins in the open ocean have generally been found to be below current detection limits (Panzeca et al. 2009; Sañudo-Wilhelmy et al. 2012) with highest concentrations at mid-depths (around 200 and 500m) (Sañudo-Wilhelmy et al. 2012). The detection limit for B_{12} is 0.18 pM, while the half saturation constant for B_{12} in diatoms is between 0.1 and 0.3 pM (Droop 2007). Therefore, until detection levels improve, we are unable to measure when there is insufficient B_{12} for diatom growth. However, enriching the open ocean with vitamin B_{12}, even in picomolar concentrations, has been shown to result in enhanced phytoplankton growth (Sañudo-Wilhelmy et al. 2006; Bertrand et al. 2007; Gobler et al. 2007; Panzeca et al. 2008; Koch et al. 2011), demonstrating the potential importance of B_{12} concentrations in controlling biological production.

Nutrient limitation may be more important than bulk measurements would suggest. The flow of nutrients and wastes around a small organism, such as a diatom or a bacterium, are dictated by viscosity. This limits the rate of diffusion, resulting in a boundary layer microenvironment adjacent to the cell (Lazier and Mann 1989). The boundary layer around the organisms limits nutrient uptake, and nearly half of the total biomass of the oceans are similarly "diffusion limited" (Sheldon et al. 1972). This diffusion limitation may explain the prevalence of algal-bacterial symbiosis in marine systems because bacterial sources of limited nutrients, such as vitamin B_{12} and biologically available iron (Croft et al. 2005; Amin et al. 2009), can provide a localized source of otherwise limiting nutrients.

Diatom diversity and abundance at the sample sites and cultures used in this study

The NPSG is considered to be the largest contiguous biome on Earth; it is an oligotrophic system with anticyclonic circulation from 15ºN to 35ºN and 135ºE and 135ºW. Primary production occurs at relatively high rates, but low biomass is observed in this system due to the efficient recycling of nutrients. The NPSG is a typical two-layer system. The bottom layer is nutrient rich but light limited. The top layer is not light limited but is nutrient poor, with primary productivity supported by nutrient recycling (Karl, 1999). Summer blooms that

occur in this system are enigmatic; the methods for introducing the nutrients required to support an increase in biomass have yet to be revealed. Typical species seen in blooms from June through September and include: *Rizosolenia*, *Richelia*, *Hamiaulus*, *Rizosolenia*, *Trichodesmium*, and *Mastogloia* (Dore et al. 2008).

The diversity and abundance of diatom species at Station ALOHA were extensively discussed in the dissertation of University of Hawaii graduate Dr. Binglin Li. Diatoms in the euphotic zone were investigated using real-time PCR to detect the chloroplast gene rbcL. rbcL gene copies per cell vary greatly among diatom genera (*Pseudo-nitzschia/Nitzschia*: 26 ± 4, *Hemiaulus*: 273 ± 22, *Chaetoceros* 320 ± 44, and *Rhizosolenia* 110 ± 10 gene copies), making it difficult to translate gene copies into absolute cell numbers. However, rbcL gene abundance could be used as a relative measure of the abundance of each diatom genus through time. Li found that *Hemiaulus* and *Rhizosolenia* increased in abundance during the late spring and early summer months while *Chaetoceros* showed no obvious seasonality. The most abundant rbcL phylotypes were *Pseudonitzschia* and *Nitschia*. (Li 2011, Chapter 3). On average, diatom abundance was relatively constant through the upper water column, declining below the mixed layer.

Thalassiosira spp. were the dominant diatoms in the Station ALOHA deep chlorophyll maximum (DCM) sample on which my Master's work was based. I sampled the DCM not only because of its ecological importance but also because previous data indicated that diatoms are typically smaller (relative to higher in the water column) with a relatively high chlorophyll concentration per cell (Scharek et al. 1999). The latter factors are useful for flow cytometry, as described in Baker and Kemp (2014). Marine diatom-bacterial interactions have been studied most often using the bulk properties of diatoms in culture (Kogure et al. 1982; Grossart 1999; Grossart et al. 2005; Kaczmarska et al. 2005; Grossart and Simon 2007). Very few studies have been conducted using native populations of diatoms (Kaczmarska et al. 2005). My Master's work was the first to study diatom-bacterial interactions at the single-cell level and the first to look at this association in the oligotrophic open ocean.

All diatom cultures utilized in this dissertation were gathered from Kāneʻohe Bay by Chris Schvarcz in Spring 2011. Although mostly oligotrophic and supporting picophytoplankton, diatom species become more abundant in response to stream input from storm events (Drupp et al. 2011). Monthly counts of cells >63uM in 2012 documented a shift from dinoflagellate populations in the summer to diatom-dominated populations in the fall and winter. The dominant genus during fall and winter were *Chaetoceros*, followed by *Guardia* and *Thalassiosira*; *Haslea* and *Pleurosigma* species were present but counted less frequently (Hoover et al. 2006). The diatom cultures collected by Chris Schvarcz from Kaneohe Bay were not intended to be fully representative of the system; they include *Amphiprora*, *Chaetoceros*, *Cylindrotheca*, and *Thalassiosira*.

Research Objectives

The past two decades have seen a shift toward a new suite of paradigms based on the assumption that microbes are highly interdependent at a cellular, molecular, and genetic level, and that these interdependencies may control the rates and routes of major biogeochemical cycles. Technological advances (single cell methods, inexpensive sequencing) allow us to examine these interdependencies in fine detail. One class of interdependencies, i.e. symbiosis, is being recast in terms of the shared genetic potential of organisms that live in obligate relationships. This dissertation identifies some of the specific interdependencies of bacteria and their diatom hosts by evaluating the diversity, host-microbe effects, and functional properties of diatom-associated bacteria, as well as interactions among diatom-associated bacteria and between bacteria and the diatom host cell. Both single-cell and population level approaches were employed using natural and cultured populations, as well as environmental manipulations. The major questions addressed by this dissertation are:

- Are bacteria attached to a host at all phases of growth (not just during decline)? (Chapter 1)
- How is the composition of bacteria attached to a diatom host affected by abiotic or biotic factors? (Chapter 1)

- Do perturbations of the composition of attached bacteria have substantial effects on the host in a xenic system? (Chapter 2)
- What are the potential functional contributions of bacteria to diatom-bacterial associations in nature, and are these functions similar to those of bacteria in other systems? (Chapter 3)

The diatoms investigated in all studies in this dissertation are xenic, and this research is among the first (Ellis 2015) to study xenic diatom cultures. Previous research has focused on axenic diatom hosts (Grossart 1999; Gärdes et al. 2012; Sison-Mangus et al. 2014; Amin et al. 2015; Zecher et al. 2015; Han et al. 2016). In culture, diatoms grown without bacteria grow poorly or not at all (Windler et al. 2014; Sison-Mangus et al. 2014; Amin et al. 2015), with irregular frustule morphology, growth rate, and culture stability (Windler et al. 2014). Indeed, poor growth has been used as evidence that diatoms require bacteria to grow normally. Axenic cultures are difficult to maintain, and diatom cultures given repeated applications of antibiotics are seldom completely axenic (Jones et al. 1973; Zecher et al. 2015). For the present research, methods were developed to preserve the attached bacteria of environmental samples (Baker and Kemp 2014, Ch. 3) and no efforts were made to limit or reduce the co-cultured bacteria associated with diatom hosts (Ch. 1 &2). These methodologies were adopted to yield results more representative of natural systems, and experiments, therefore, resemble ecological more than microbiological studies.

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Table 1. The range of observed half saturation constants (Ks) for marine diatoms, as reported in Sarthou et al., 2005. The half saturation constant is the nutrient concentration at which uptake is ½ of the optimal rate. Concentration ranges for Station ALOHA were average values taken at standard depths from 5 to 175 meters. The iron concentrations at Station ALOHA are taken from (Boyle et al., 2005). The concentration ranges for Kāneʻohe Bay are listed from the closest research buoy to the collection (Ringuet and Mackenzie, 2005). All values listed are in µM.

Nutrient	Minimum Ks	Maximum Ks	Average Ks	n	Ave conc. Station ALOHA	Ave conc. Kāneʻohe Bay
N	0.02	10.2	1.6 ± 1.9	35	(3.16 ± 0.54) x10-3	14 ± 0.1
P	0.01	8.9	1.2 ± 2.5	18	(46.39 ± 8.60) x10-3	0.11 ± 0.07
Si	0.2	22	0.2 ± 22	25	1.17 ± 0.05	7.05 ±2.68
Fe	3.5x10-7	1.12x10-3	(3.5 ± 4.4) x 10-4	12	(2.1–7.1) x10-4	

Chapter 2: Response of diatom-associated bacteria to host growth state, nutrient concentrations, and viral host infection in a model system.

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ORIGINALITY-SIGNIFICANCE STATEMENT

For decades, bacteria have been known to attach to the surfaces of aquatic organisms such as phytoplankton cells. In the case of marine diatoms, earlier studies suggested that attached bacteria are associated with the decline of a diatom population, and are uncommon in healthy populations. Recent studies indicate that attached bacteria may be present at other stages of a diatom life cycle, and may engage in symbiotic interactions with their diatom host including mutualism, parasitism, and commensalism. These interactions are proposed to affect diatom health, persistence, production, aggregation, sinking, and other vital ecosystem processes. If so, then the ecological role of marine diatoms may be heavily influenced by their bacterial associates. However, very little is known of the relationship between diatoms and attached bacteria in oligotrophic systems that comprise a majority of the world's oceans. We examined the effect of abiotic and biotic factors on the composition of the bacterial associates of a marine diatom growing under low- and high-nutrient conditions. The composition of attached bacteria was found to depend on nutrient supply, the growth stage of the host, and interactions among attached bacteria. Our results support the hypothesis that attached bacteria are common throughout the growth cycle of the diatom host, and not merely limited to host decline. They suggest that attached bacteria may differ in eutrophic and oligotrophic systems. Finally, they suggest that bacterial competition or cooperation may be important determinants of the composition of attached bacteria. Our experimental results identify important and promising environmental factors for future study of diatom-bacteria symbioses, and by extension environmental factors that may important to bacterial symbioses with other phytoplankton.

SUMMARY

Diatoms are photosynthetic unicellular eukaryotes found ubiquitously in aquatic systems. Frequent physical associations with other microorganisms such as bacteria may influence diatom fitness. The predictability of bacterial-diatom interactions is hypothesized to depend on availability of nutrients as well as the physiological state of the host. Biotic and abiotic factors such as nutrient levels, host growth stage, and host viral infection were manipulated to determine their effect on the ecological succession of bacterial communities associated with a single cell line of *Chaetoceros* sp. KBDT20; this was assessed using the relative abundance of bacterial phylotypes based on 16S rDNA sequences. A single bacterial family, Alteromonadaceae, dominated the attached-bacterial community (84.0%), with the most prevalent phylotypes belonging to the *Alteromonas* and *Marinobacter* genera. The taxa comprising the other 16% of the attached bacterial assemblage include Alphaproteobacteria, Betaproteobacteria, Bacilli, Deltaproteobacteria, other Gammaproteobacteria, and Flavobacteria. Nutrient concentration and host growth stage had a statistically significant effect on the phylogenetic composition of the attached bacteria. We infer that interactions between attached bacteria, as well as the inherent stochasticity mediating contact may also contribute to diatom-bacterial associations.

INTRODUCTION

In marine systems, bacteria are frequently associated with surfaces (Zobell, 1943, 1946; Kriss, 1963) such as the exterior of phytoplankton cells (Amin *et al.*, 2012). Bacteria attached to diatoms are thought to engage the host in symbiotic interactions including mutualism (Croft *et al.*, 2005; Amin and Green, 2009; Gärdes *et al.*, 2012), parasitism (Bidle and Azam, 1999; Mayali and Azam, 2004; S. Amin *et al.*, 2012), and commensalism (Rosowski, 1992; Droop, 2007). Despite having been studied in the laboratory for over 80 years, a clear understanding of diatom-bacterial interactions remains elusive. Some studies suggest that diatoms are rarely associated with bacteria (Droop and Elson, 1966; Kogure *et al.*, 1980; Bratbak and Thingstad, 1985; Graff *et al.*, 2011), and other studies suggest that host-attached bacteria are more abundant during diatom population decline (Waksman and Butler, 1937; Cole, 1982; Azam *et al.*, 1983). Early research on host-associated bacteria relied on culturing (Waksman and Butler, 1937; Droop and Elson, 1966) and enumeration by fluorescence microscopy (Droop and Elson, 1966) to identify host-attached bacteria. One such study indicated that *Flavobacterium*, *Acinetobacteria*, *Pseudomonas*, and *Vibrio* changed in relative proportions over the course of four days (Kogure *et al.*, 1982). Sapp *et* al. (2007) found that extended culturing of diatom cultures (upwards of 1 year) selected for shifts in the community that favored Gammaproteobacteria. Notably, the total number of phylotypes Sapp *et al.* (2007) documented with denaturing gradient gel electrophoresis (18 phylotypes) was low compared to recent studies employing more updated sequencing methods, which have reported 3-10x more operational taxonomic units (OTUs) per diatom isolate (Guannel *et al.*, 2011; Sison-Mangus *et al.*, 2014).

Under some circumstances, nutrient stress on host cells affects bacterial metabolism, which may change the composition of diatom-associated bacteria. Obernosterer and Herndl (1995) found that algal exudate composition from *Chaetoceros affinis* changed with nutrient limitation, subsequently affecting production of bacterial extracellular enzymes. Other studies have demonstrated that surface-attached bacteria can affect release of algal exudates (Bruckner *et al.*, 2011) and formation of transparent exopolymers (Grossart *et al.*, 2006), thereby influencing aggregation and sinking of diatoms (Smith *et al.*, 1995; Gärdes *et al.*, 2011). Nutrient

limitation can shift the metabolism of host-associated bacteria (Obernosterer and Herndl, 1995), possibly limiting glycosidase activity (Chróst, 1991; Obernosterer and Herndl, 1995), which would result in limited bacterial utilization of algal exudate as a nutrient source.

Viruses can greatly affect the health and stability of diatom populations. Phytoplankton viruses are thought to have a specific host range (Nagasaki *et al.*, 2005), contribute to bloom termination (Bratbak *et al.*, 1990), and increase local nutrient concentrations (Brum *et al.*, 2014). Viruses are estimated to be responsible for 10-20% of phytoplankton mortality (Baudoux *et al.*, 2006; Kimmance *et al.*, 2007). The effect of viruses on phytoplankton is influenced by the presence of bacteria. For example, xenic cultures of the diatom *Chaetoceros tenuissimus* are more resistant to viral lysis than axenic diatoms (Kimura and Tomaru, 2014).

Diatom-bacterial interactions are particularly understudied in oligotrophic systems, conditions which comprise a majority of the world's oceans (Baker and Kemp, 2014). Here we examine the response to nutrient concentrations and host growth state of diatom-associated bacteria from an oligotrophic environment. We employed a factorial experimental design using a xenic *Chaetoceros* culture to examine the effects of abiotic (light and nutrient limitation) and biotic (diatom viral infection and bacterial interactions) factors on microbial assemblages attached to diatom cells both independently and concurrently, upon a single dependent variable (bacterial composition). The composition of diatom-attached bacteria was examined at different stages of host growth including initial growth, early exponential growth, late exponential growth, early decline, and a virally induced near-collapse. By assessing the stability and predictability of a diatom microbiome, our study lays the foundation for future work regarding the response of the microbiome to mediators such as nutrient availability and host growth.

RESULTS AND DISCUSSION

Host abundance as a function of nutrient availability

We examined the abundance of *Chaetoceros* sp. KBDT20 in response to high (f/2 medium) and low (f/200 medium) nutrient availability (Guillard and Ryther, 1962; Guillard, 1975). Nutrient concentrations in the high nutrient treatment bottles remained two orders of magnitude higher than in the low nutrient treatment bottles throughout the experiment (Table 1). At all time points, host cells in low nutrient medium were significantly less abundant than the corresponding samples in high nutrient medium (Figure 1). The carrying capacity of the low nutrient treatment was ca. 25% of the cell number found in the high nutrient treatment (Figure 1).

Four distinct phases were observed in each culture: (1) initial growth, (2) early exponential phase (3) late exponential phase, and (4) decline (Figure 1). In addition, a final sample was taken four days after addition of a virus obtained from this host (Figure 1, arrowheads and Figure 2). No initial lag in diatom growth was observed in either nutrient treatment, suggesting that the inoculum contained actively dividing cells. For the low nutrient treatment, cell concentrations during initial growth (day 4, 15-18 cells mL^{-1}), early exponential (day 8-16, 83 cells mL^{-1}), late exponential (day 20-32, 800-850 cells mL^{-1}), and decline (day 32-36, 400-550 cells mL^{-1}) were significantly lower than in the corresponding samples in the high nutrient treatment: initial growth (day 4, 30-60 cells mL^{-1}), early exponential (day 8, 130-160 cells mL^{-1}), late exponential (day 20-28, 6000-6400 cells mL^{-1}), and decline (day 28-32, 3000-4400 cells mL^{-1}) (Figure 1). During the exponential phase, low nutrient concentrations resulted in slower growth (0.19 ± 0.05 cells day^{-1} with a doubling time of 3.62 days) and lower maximum concentrations than high nutrient treatments (0.25±0.09 cells day^{-1} doubling time of 2.72 days) (Figure 1). Following addition of a host-specific virus, the diatom population in each bottle declined to approximately 2-5% of the peak population (Figure 1, final time point).

By comparing the half saturation constants for this group of diatoms to the nutrient concentration of the culture medium, we assessed whether declining diatom abundance was due to nutrient limitation, as measured 8 days after peak diatom abundance. In the high nutrient treatment, macronutrient concentrations (N, P, Si) remained above the half saturation constant for diatoms (Table 1), indicating that macronutrient limitation did not cause diatom decline. Density-dependent factors may have been responsible, such as self-shading from the light source, or the accumulation of diatom or bacterially produced wasted products. In the low nutrient treatment, macronutrient concentrations remained above the average half saturation constants until late exponential growth. Before virus was added, nitrogen concentrations for the replicates dropped to 0.9 and 2.03 µM (average half saturation constant for diatoms is 1.6±1.9 µM) and silica concentrations fell to 1.96 and 1.88 µM (average half saturation constant for diatoms is 3.9±5.0 µM) (Sarthou *et al.*, 2005). Declining growth in the low nutrient treatment may have been caused in part by macronutrient limitation.

The initial concentrations of micronutrients in both high and low nutrient treatments were an order of magnitude higher than the average half-saturation constants and were not measured during the course of this experiment as they were assumed to not be limiting. However this assumption could not be tested directly because detection levels for important micronutrients are orders of magnitude higher than the average half-saturation constants for diatoms. For example, the essential nutrient vitamin B_{12} (cyanocobalamin) has a detection level of 2.9 $x10^{-5}$ µM (Sañudo-Wilhelmy *et al.*, 2012). The half saturation constant for B_{12} in diatoms is between 1 $x10^{-7}$ and 8 $x10^{-7}$ µM (Droop, 2007). The initial concentration of vitamin B_{12} was 3.69 $x10^{-4}$ µM and 3.69 $x10^{-6}$ µM for the high and low nutrient treatment respectively. Even in the low nutrient treatment, the starting concentration of vitamin B_{12} was an order of magnitude higher than in previous nutrient limitation studies of the diatom *Skeletonema costatum* (Droop, 2007). If we assume micronutrients were utilized at relative rates comparable to macronutrients, micronutrients would not have become limiting.

Diatom-associated bacterial abundance and diversity

To investigate whether host-associated bacteria are a hallmark of diatom decline, we enumerated the attached bacteria per diatom during early exponential growth and after virus addition for each treatment (Figure 2) in order to represent the extremes of healthy and growing versus unhealthy and dying cells. The bacterial cell to diatom cell ratio ranged from 0-35, with an overall mean of 8±6. The mean ratio of bacteria to diatom was not significantly different between nutrient treatments in the post-virus counts, and did not change significantly in the low nutrient treatment between the early exponential and post-virus stage. However, the mean ratio of bacteria to diatom was significantly higher in the high nutrient treatment, early exponential stage (Supplemental Table 1), indicating that surface-attached bacteria are typical on actively growing diatoms.

Next, to understand the nature of the diatom-attached bacterial community, 1351 bacterial sequences were obtained from twenty samples (two replicates of two treatments at five time points). Because viral lysis caused drastic changes in a host population, we examined how the introduction of a strain-specific virus affected the diatom microbiome. Host-attached bacteria were diverse in a diatom culture that originated with a single founder diatom cell and its bacterial associates. In total, clustering at 98% similarity resulted in in 83 OTUs, hereafter referred to as phylotypes, which were assigned to 53 unique taxonomic identifications (Genbank KM382465- KM383733). Rarefaction curves were generated using S_{ACE} and S_{Chao1} as richness indicators; a sufficient coverage for each library was confirmed by the richness estimates reaching a stable asymptotic value (Kemp and Aller, 2004). Representative sequences are shown in Figure 3 with recurrence and relative abundance of each phylotype. In our previous work, we found a maximum of 61 bacterial cells attached to a single field-collected diatom cell (mean of 24 bacterial cells per diatom), but a maximum of 11 different bacterial OTUs co-occurring on one diatom cell cells (Baker and Kemp, 2014). This may indicate that some free-living bacteria were carried over during the original isolation. We calculate that micropipette isolation may have resulted in 2-128 free-living bacteria being transferred along with the original host cell (Supplemental materials, *Establishment of diatom cell line*). It is possible that the proportions of phylotypes may have shifted

among either attached or free-living bacteria during the >1 year of culturing; it is also possible that some phylotypes have both attached and free-living populations.

In contrast to our previous work on the attached bacteria of field-collected single diatom cells (Baker and Kemp, 2014), the two major phylotypes associated with *Chaetoceros* sp. KBDT20 were *Alteromonas* and *Marinobacter*, two genera previously reported to be associated with algal hosts including diatoms (Amin *et al.*, 2012). A single bacterial family, Alteromonadaceae, comprised 84.0% of all bacterial sequences; within this family *Alteromonas*, *Aestuariibacter*, *Colwelliaceae*, *Glaciecola*, and *Marinobacter* were the predominant genera (Figure 4). Nine observed phylotypes of *Alteromonas* comprised 30.1% of all bacterial sequences. One *Alteromonas* phylotype was present in every sample (totaling 13.0% of all bacterial sequences) and BLAST analysis revealed its sequence was 99% identical to *Alteromonas macleodii* (ARB identification X82145). However the most prevalent single phylotype was a *Marinobacter*, which made up 24.8% of the bacterial sequences. The closest cultured relatives of this phylotype are the *M. aquaeolei* strain VT8 (99% identical in BLAST), the *M. hydrocarbonoclasticus* strain AK5 (99% identical in BLAST), and *M. lutaoensis* (AF288157). The closest uncultured neighbor was isolated from an octocoral (DQ396166). In total, 10 *Marinobacter* phylotypes contributed 45.8% of the total bacterial sequences.

Alteromonas and *Marinobacter* display flagellar motility (Baumann *et al.*, 1972; Gärdes *et al.*, 2010), a critical feature that mediates initial attachment of bacteria to surfaces and host cells (Sonnenschein *et al.*, 2012). *Alteromonas* is commonly associated with marine surfaces (Dang *et al.*, 2008) including sponges (Wichels *et al.*, 2006), dinoflagellates (Brinkmeyer *et al.*, 2000; Hold *et al.*, 2001) and diatoms (Stewart *et al.*, 1997; Grossart *et al.*, 2005; Guannel *et al.*, 2011). *Marinobacter* is found associated with particles (Grossart and Kiørboe, 2003), dinoflagellates (Hold *et al.*, 2001; Jasti and Sieracki, 2005), corals (Carlos *et al.*, 2013) and diatoms (Grossart *et al.*, 2005; Amin *et al.*, 2009; Gärdes *et al.*, 2011). *Alteromonas* species have been found to have algicidal interactions with diatoms and antibacterial interactions with host associated bacteria. Some members of the

Alteromonadaceae including *Pseudoalteromonas* and *Alteromonas* are known to effectively metabolize the organic matrix of a diatom host, exposing the silica shell to increased dissolution by the surrounding water (Bidle and Azam, 2001). *Alteromonas* species such as *A. colwelliana* have algicidal activity and have been shown to inhibit the growth of *Chaetoceros calcitrans* (Kim *et al.*, 1999), potentially in a host-specific manner (Paul and Pohnert, 2011). Some *Alteromonas* are also capable of producing antimicrobial compounds that affect other host-associated bacteria (Barja *et al.*, 1989). Further investigation of the *Alteromonas* phylotypes observed in this study is needed to determine whether our results may be influenced by algicidal or bactericidal activity.

In contrast, *Marinobacter* species are thought to provide essential vitamins including B_{12} to the diatom host (Croft *et al.*, 2005). Approximately 60% of all culturable haptophytes and heterokonts, including a number of *Chaetoceros* species (King *et al.*, 2011), require an external source of B_{12} (Tang *et al.*, 2010). Host cells may benefit from colonization by vitamin B_{12}-producing bacteria (Croft *et al.*, 2005). Host-associated bacteria may provide other micronutrients as well; for example, *Marinobacter* can provide biologically available iron to the host cell through the production of vibrioferrin (Amin *et al.*, 2012). Attached bacteria may be competitors for micronutrients instead; for example, attached B_{12} auxotrophs may compete with a diatom host for vitamin B_{12} (Bertrand and Allen, 2012; Sañudo-Wilhelmy *et al.*, 2012).

The remaining phylotypes (15.7 %) were identified as: Actinobacteria (2.7%), Alphaproteobacteria (6.3%), Bacilli (0.9%), Betaproteobacteria (0.8%), Flavobacteria (0.1%), and Deltaproteobacteria (0.1%), other Gammaproteobacteria (not including *Alteromonadaceae*) (4.2%), and Verrucomicrobia (0.6%) (Figure 4). Bacterial phylotypes comprising the remaining <0.3% were often singletons (recovered only once during the experiment); the influence of including singletons when analyzing environmental influence on bacterial community composition is discussed further below.

In both nutrient treatments, the number (Figure 5) and relative proportions (Figure 4) of attached bacterial phylotypes differed among host growth stages, a reminder that the attached bacterial assemblage can change over the course of days. The largest number of bacterial phylotypes (15±4) was found during early exponential host growth, for both high and low nutrient treatments. The number of bacterial phylotypes was lower in early exponential and late exponential growth phases for both nutrient treatments. There were no significant differences in the number of bacterial phylotypes recovered in high versus low nutrient treatments (Figure 5). Future studies of native diatom populations conducted over the course of days to weeks should examine the temporal variability of their bacterial assemblages.

Environment influences bacterial community composition

The *Chaetoceros* sp. KBDT20 culture was derived from a single, washed diatom cell together with its co-cultured bacteria. Its cultured descendants were added at 157 diatom cells L^{-1} into relatively large (4.5 L) incubation volumes that were rotated constantly to ensure a uniform distribution of cells. Thus, we had reason to expect that the response of diatoms and bacteria would be uniform across replicate bottles. Two statistical approaches were used to assess bottle effects. A mixed effect model identified significant bottle effects on the relative abundance of bacterial OTUs; whereas *adonis* analysis did not. Comparable bottle effects are not unprecedented. Schäfer *et al.* (2002) examined bacteria associated with a variety of diatom host species, including a *Chaetoceros*. In that study, replicate bottles containing other diatom species had nearly identical 16S rRNA DGGE banding patterns, whereas replicate bottles containing *Chaetoceros* cultures did not (Schäfer et al. 2002, their Figure 1). We speculate that differences we observed between replicate bottles may be due to: (1) differences between bottles in bacterial seed assemblages; (2) stochasticity in the initial growth of diatom cells (3) inherent variability in *Chaetoceros* as a host organism.

To investigate abiotic (nutrient concentrations) as well as biotic (growth stage and a viral-caused collapse) factors that may influence bacterial abundance, we examined differences in the relative abundance of bacterial

OTUs using principal coordinates analysis (PCoA), *adonis*, and a mixed effect model, which differ in the way relative bacterial OTU abundance is calculated. PCoA and *adonis* use a weighted Unifrac distance matrix that incorporates the similarity or relatedness of OTUs and whereas the mixed effect model uses raw OTU abundance.

Taking OTU relatedness into account, PCoA (Figure 6) and *adonis* (Table 2) suggest that nutrient treatment and growth were significant factors in determining bacterial composition. PCoA showed that nutrient treatment had a significant effect on bacterial community composition, as evidenced by clustering of low-nutrient samples (large dashed oval, Figure 6A), which account for 11.1% of the total variation ($p = 0.046$; Table 2). Together, Axis 1 and Axis 2 explain 76.7% of the total variation of bacterial community. The early exponential growth time point in the low nutrient, replicate 2 bottle (Figure 6A, L2 cross at lower left) is an exception to this apparent clustering. Plotting the data without the anomalous point shows similar clustering of low nutrient samples (Figure 6B); the total explained variation decreased by only 2.1%. No significant differences in bacterial composition were found between replicates. Virus addition (13.2% of the total variation, $p=0.016$) and host growth stage (16.6% of the total variation, $p=0.006$) also had significant effects on bacterial composition. Using *adonis* we tested the influence of within-bottle effects, host growth stage, nutrient treatments, and viral-induced decline on bacterial community composition, excluding singletons. Though no single factor tested was statistically significant when considered individually (Table 2), nutrient treatment, viral-induced decline, and host growth stage were significant effects when all factors were examined jointly.

To identify a minimal set of significant covariates ignoring OTU relatedness and excluding singletons, we employed a generalized linear mixed effect model using ANOVA. The generalized linear mixed effect model suggested that the host growth stage was a statistically significant factor, whereas nutrient treatment was not significant. Both fixed and random effects were shown to have significant effects on the relative abundance of host-associated bacterial OTUs (ANOVA, $p < 0.05$). The fixed effect of growth stage was statistically significant,

meaning that OTU abundance across all OTUs changed with growth stage. Among random effects, the degree to which the effect of relative OTU abundance changed randomly over the course of the experiment was significant. Random bottle effect, i.e. the degree to which relative OTU abundance was affected by interactions of the variability of bacterial assemblages as they differ among bottles, was also significant. Random factors not accounted for by a Poisson distribution also significantly affected the relative abundance of bacterial OTUs. Fixed effects that were not significant ($p>0.05$) included the effect of nutrient treatment, meaning OTU abundance across all OTUs did not change with nutrient treatment. Random effects that were not significant include variability associated with an interaction between nutrient treatments, the composition of the bacterial assemblage, and the random effect of growth stage on the relative abundance of bacterial OTUs.

Differences in statistical significance obtained by using or ignoring OTU relatedness in these statistical methods suggest that phylogenetic similarity might be a key factor in response of diatom-associated bacteria to varying nutrient conditions. In addition, abundance of individual bacterial phylotypes may influence bacterial response at different host growth stages. Notably, these insights were obtained only by employing approaches with and without considering OTU relatedness.

Phylogenetic similarity may influence response of diatom-associated bacteria to nutrient availability

Our experimental design uses the typical growth and decline of a batch culture as a means of manipulating diatom health. In addition, we simulated an extreme event, a near collapse of the host population induced by a virus. Because we collected virally-infected samples using 5µm Nucleopore® polycarbonate filters, our samples contained both lysed diatom fragments as well as intact diatom cells. We were not able to separate the bacteria associated with fragments from the bacteria associated with intact diatoms. However, the *adonis* analysis indicated that viral lysis was a significant factor in determining the composition of attached bacteria, and we can at least conclude that a near collapse of the diatom population is associated with changes in the composition of particle-attached bacteria.

PCoA, *adonis,* and a mixed effect model all indicated that growth stage was related to the composition of attached bacteria. In both the high and low nutrient treatments, the total number of bacterial OTUs varied among different diatom growth stages (Figure 5). The mixed effect model indicated that the composition of the bacterial assemblage, excluding singletons, was significantly affected by growth stage. If singletons were included, growth stage became statistically non-significant, although other fixed and random effects remained statistically significant. By definition, bacterial OTUs that occur only once cannot be statistically associated with a specific diatom growth stage; collectively they may represent statistical noise, and if anything might be expected to obscure differences among treatments. However, these irregular colonizers may still play a role in diatom-bacteria interactions that could not be discerned in this study.

Including singletons had little effect on most statistical analyses. Including singletons in the PCoA analysis results in a similar ordination, and only a small decrease in the percent of variance explained by axes 1 and 2, from 76.7% to 73% (Supplementary Figure 2). The results of the *adonis* analysis were relatively unchanged by the inclusion of singletons (Supplementary Table 2). When singletons are included in the input for the generalized linear mixed-effect model, all fixed and random effects remain the same except that the fixed effect of growth stage is no longer statistically significant; this was the sole test in which inclusion of singletons affected the apparent significance of a tested factor.

CONCLUSION

Although a number of studies have considered diatom-associated bacteria under different nutrient concentrations (Obernosterer and Herndl, 1995; Bruckner *et al.*, 2011) or different diatom life stages (Waksman *et al.*, 1933; Waksman and Butler, 1937; Droop and Elson, 1966; Kogure *et al.*, 1982; Schäfer *et al.*, 2002; Sapp *et al.*, 2007), an important question remained unanswered: are attached bacteria a normal part of the existence of diatoms, or are they only present under specific conditions? To answer that question, the

present study examined whether environmental factors affected associations between diatoms and their attached bacteria in a *Chaetoceros*-bacteria-virus model system. In this simplified system, we determined that attached bacteria were found at all host growth stages. The composition of the recurring (i.e. excluding singletons), diatom-associated bacterial assemblage was related to the host's growth stage and to nutrient concentration in its environment. The number of bacteria per diatom cell was significantly higher during nutrient-replete, early exponential growth. We conclude that that attached bacteria are a normal part of a diatom host's existence throughout the growth cycle, and not solely during its decline.

These results also expand previous research suggesting that the attached bacterial community changes throughout a host's growth cycle (Kogure *et al.*, 1982; Sapp *et al.*, 2007). Finding a significant effect of nutrient treatment suggests that the composition of recurring attached bacteria may differ between eutrophic and oligotrophic systems. Bacterial relatedness, nutrient levels, viral host infection, and host growth stage were identified as significant factors determining the phylogenetic composition of diatom-attached bacteria. A generalized linear mixed-effect model also indicated that diatom growth stage influences the composition of attached bacteria. Further, we identified significant bottle effects indicating interactions between bacterial OTUs; i.e. that at least some bacterial OTUs co-vary or vary oppositely. We also observed that a substantial proportion of all OTUs were only found sporadically among the attached bacteria. We are led to speculate that bacterial interactions, either positive or negative, may determine the current composition of bacteria attached to diatom hosts, as that composition changes through the host's life cycle.

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Table 1. The average range of observed half saturation constants (K_s) for marine diatoms, as well as the number of studies reporting K_s (Sarthou *et al.*, 2005). Concentration ranges for Kāneohe Bay were taken from the closest research buoy (Ringuet and Mackenzie, 2005). The average values for the low and high nutrient treatment are listed as well. All values listed are in µM.

Nutrient	Average Ks	n	Range Conc. Kaneohe Bay (n=16)	Average Conc. Low Nutrient	Average Conc. High Nutrient
N	1.6 ± 1.9	35	0.14 ± 0.10	6.75 ± 3.2	855.3 ± 43.1
P	0.24 ± 0.29	14	0.11 ± 0.07	0.36 ± 0.1	39.9 ± 1.9
Si	3.9 ± 5.0	25	7.05 ± 2.68	8.12 ± 1.0	77.5 ± 8.1
Cyanocobalamin	(1.1 ± 2.46) $x10^{-6}$	13	not available	3.69 $x10^{-6}$	3.69 $x10^{-4}$

Table 2. Results of *adonis* analysis, exluding singletons (phylotypes recovered only once in the experiment). The percent of the total variation explained by each environmental condition is denoted as R^2, and statistically significant (*) when the $p< 0.05$. The R^2 and p-value is listed when all experimental conditions are considered concurrently ("all") and when each experimental condition is considered individually ("indiv").

Experimental condition	R^2 (all)	p (all)	R^2 (indiv)	p (indiv)
Nutrient treatment	11.1%	0.046*	9.6%	0.087
Viral induced decline	13.2%	0.016*	3.9%	0.597
Host growth stage	16.6%	0.006*	7.5%	0.193
Bottle effects	5.7%	0.248	4.1%	0.579

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Figure 1. Diatom abundance in response to nutrient availability. Mean abundance and standard deviation calculated from 50-100 cells. High nutrient replicates are filled circles and squares and low nutrient replicates are open up and down triangles. The arrow denotes addition of *Chaetoceros* sp. KBDT20 infective virus to respective cultures.

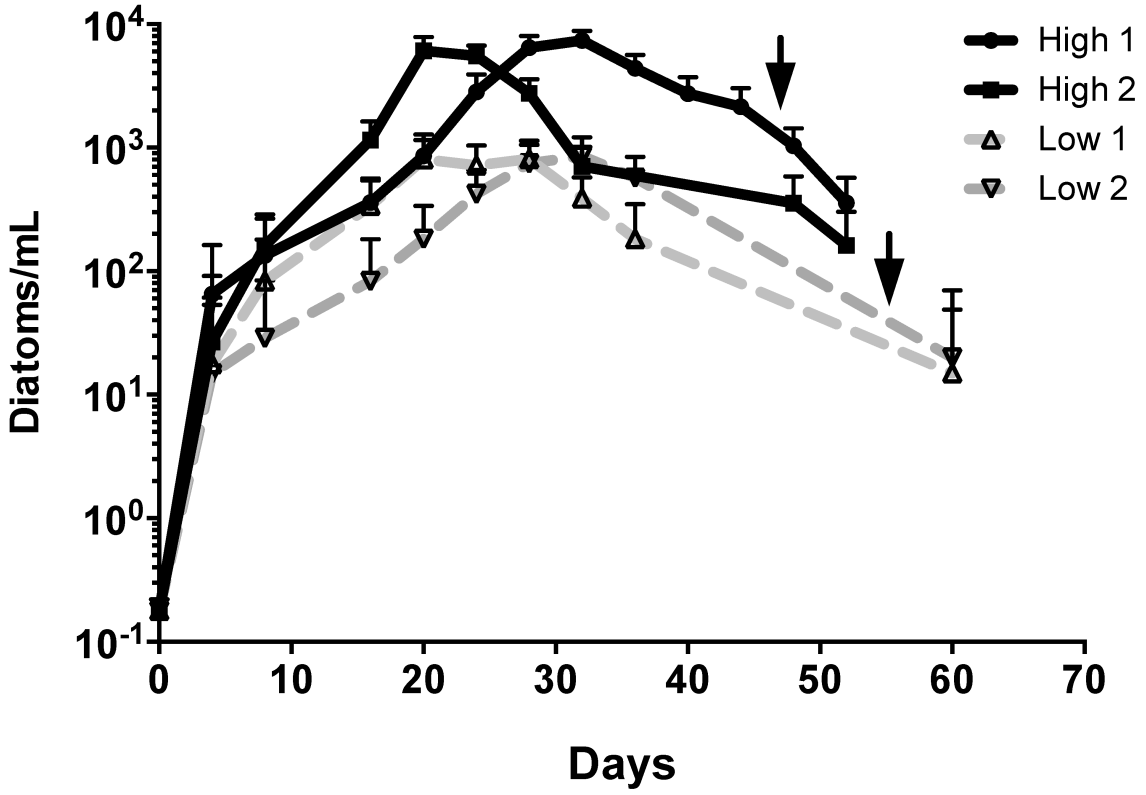


Figure 2. Attached bacteria per diatom during early exponential (light grey) and post viral addition (dark grey). Replicate 1 and 2 are shown for the high and low nutrient treatment. The mean and standard deviation are estimated from 20-32 diatoms.

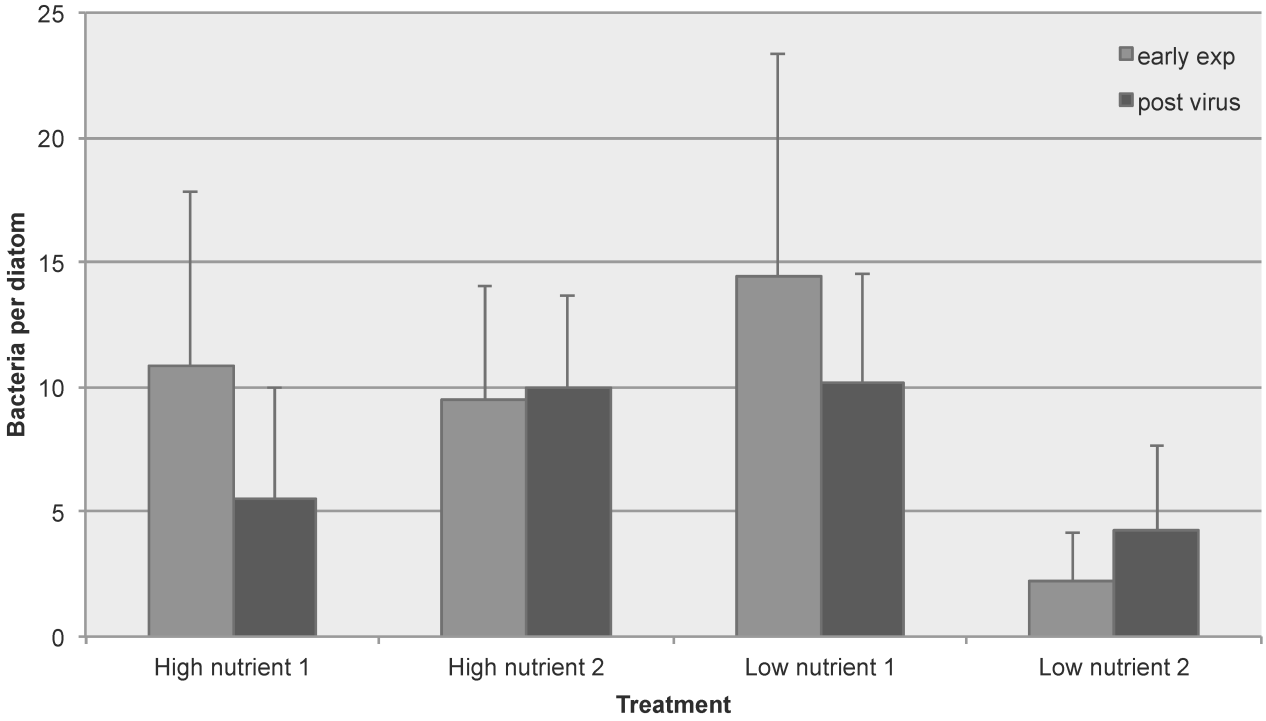


Figure 3. Phylogenetic relationships of observed bacterial phylotypes (class level), constructed using recommendations from a J-model test based on the Akaike Information Criterion (AIC = 27541) and log likelihood (-lnL = 13573) for (GTR) distribution model plus gamma (+G) as the best-fit model. The number of colored symbols at each branch shows the recurrence of a given phylotype. Symbol shape denotes high (circle) versus low (triangle) nutrient treatments. The relative abundance indicated by the size of the symbol. Colors denote phylotype class. The most commonly observed family, the Alteromonadaceae, is found within Gammaproteobacteria.

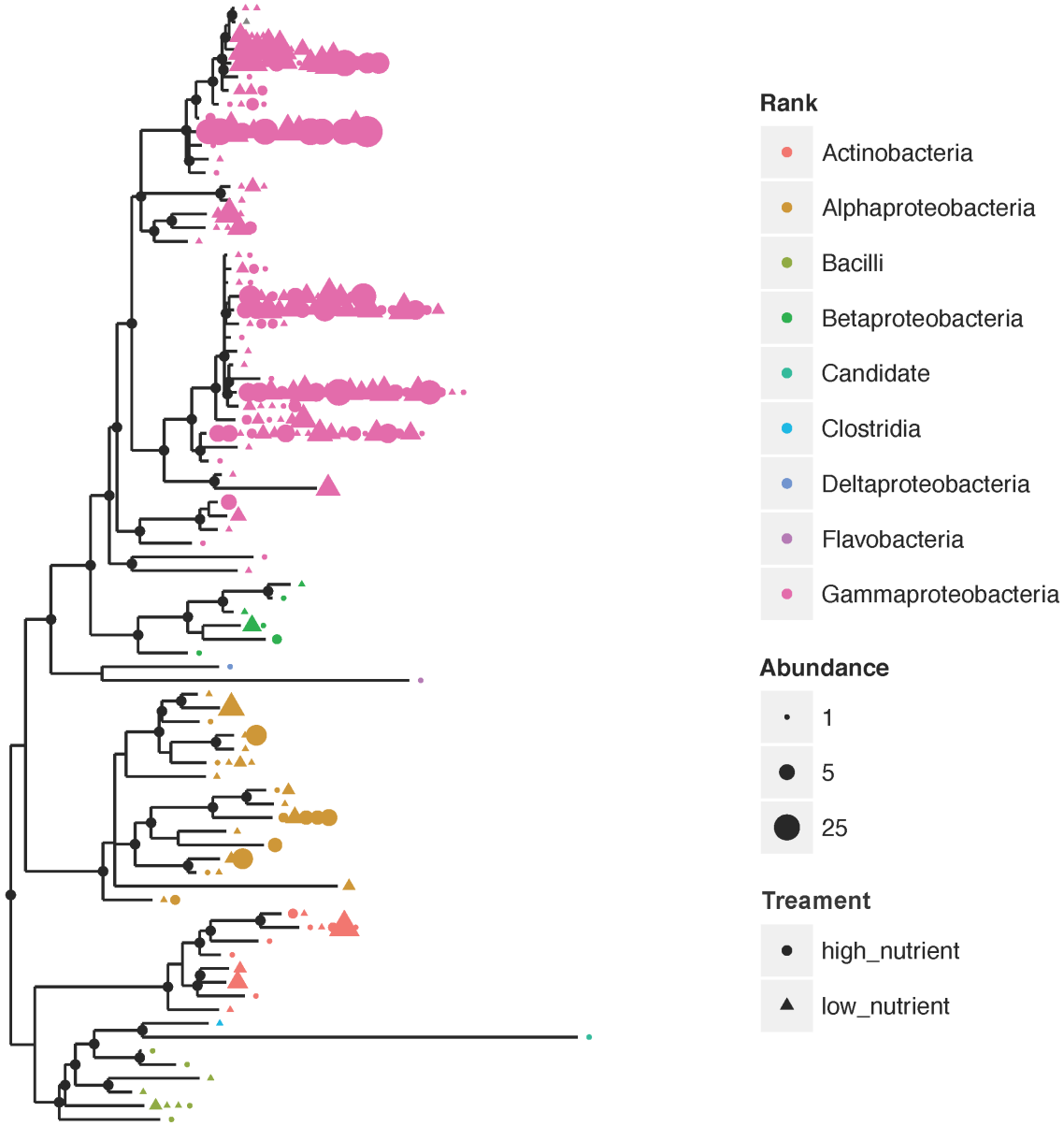


Figure 4. Bacterial OTU relative abundance at each host growth stage in response to nutrient availability. Each OTU was identified to genus level or the most specific available level over the course of host growth (initial, early exponential, late exponential, culture decline, and after viral addition) in high (left) and low (right) nutrient treatments, 2 replicates per treatment.

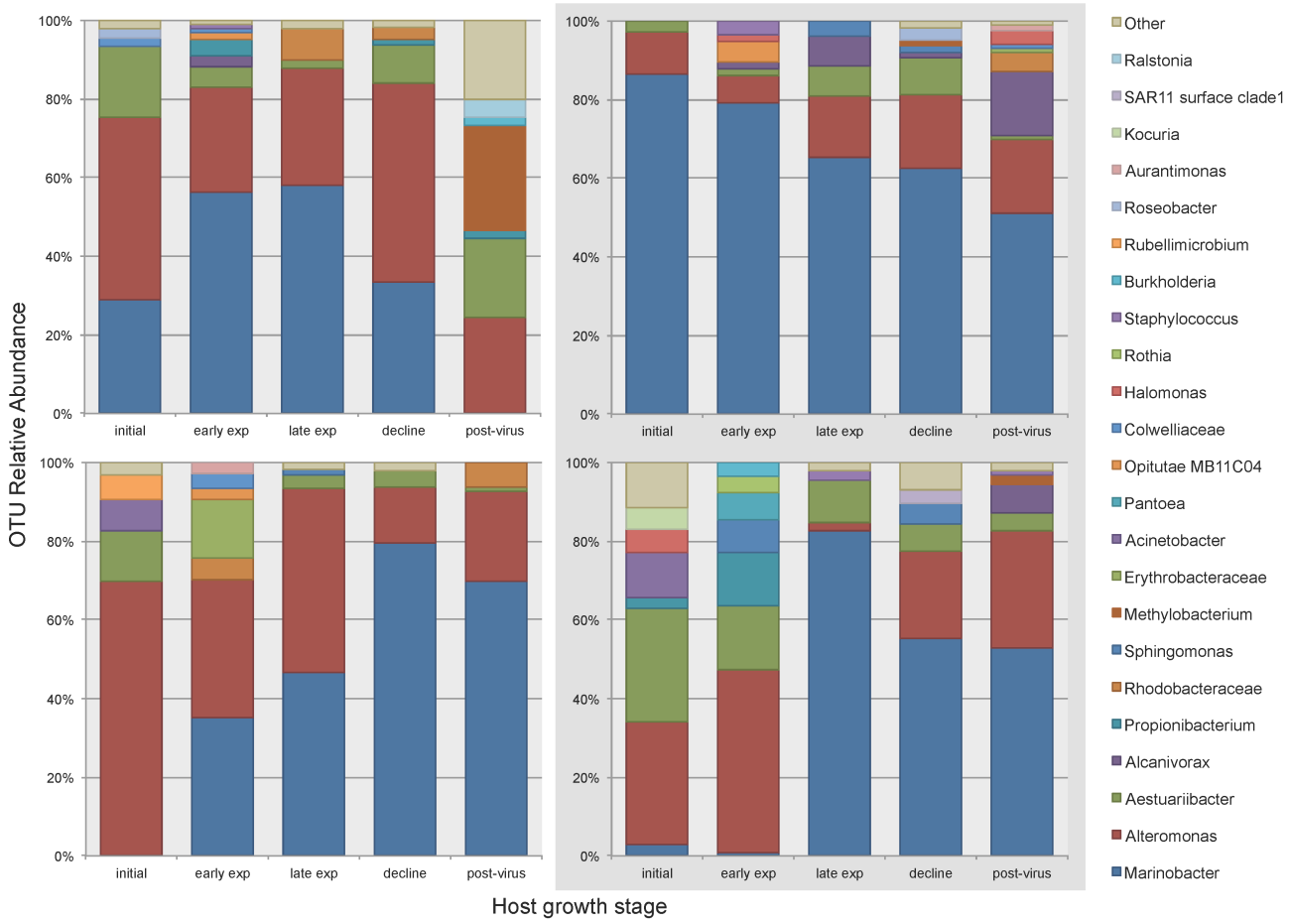


Figure 5. Total number of recurring OTUs observed at each host growth stage, in high (dark grey) and low (light grey) nutrient treatments. The mean and standard errors of replicate bottles are shown.

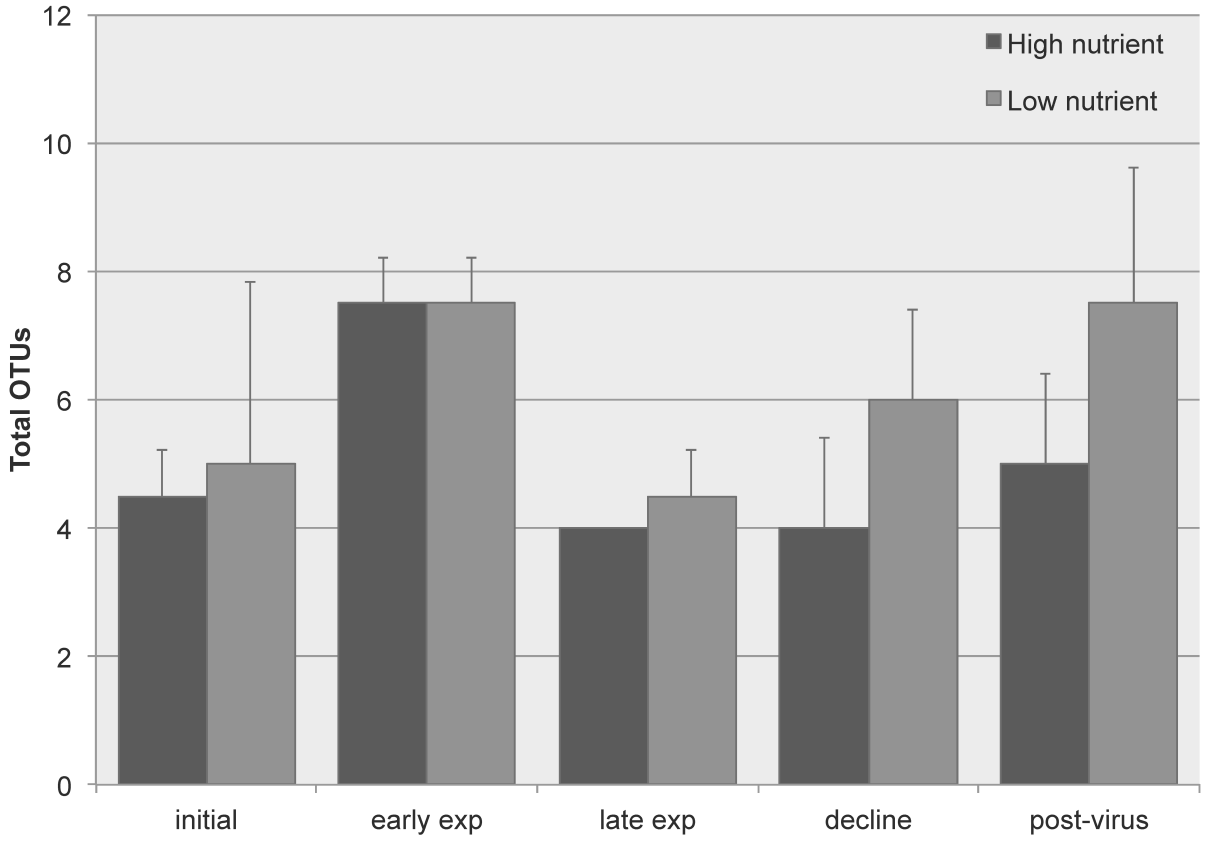
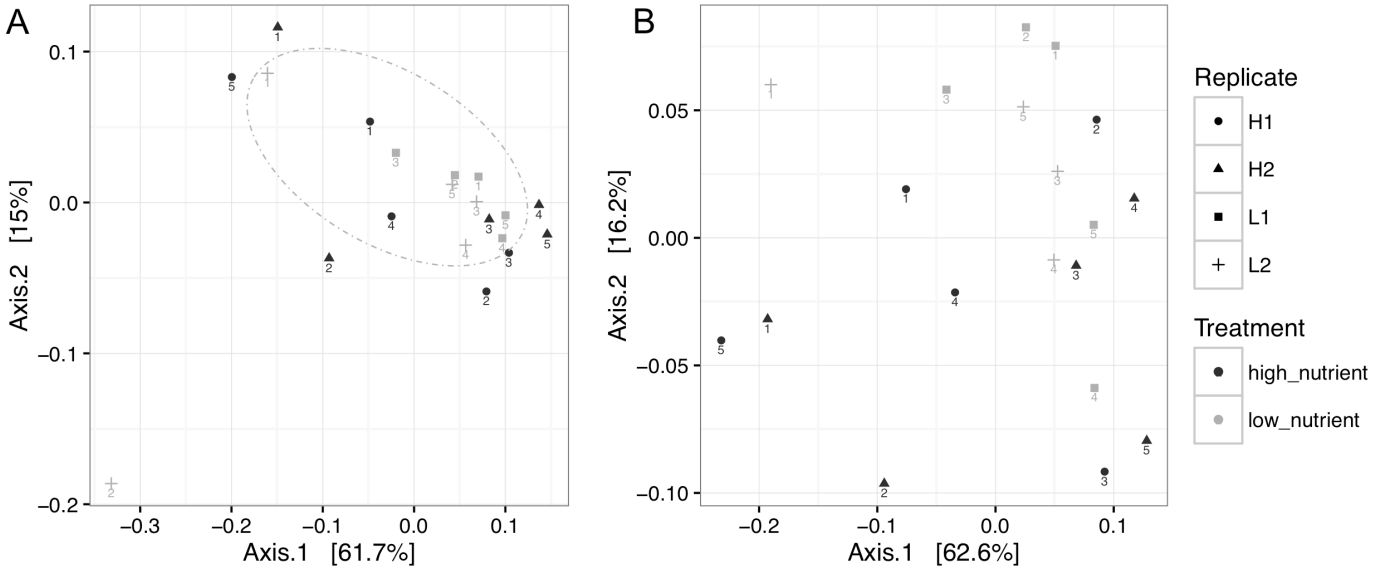


Figure 6. Axis 1 and 2 of the PCoA of all sequences excluding singletons. Replicates are denoted by shapes, high nutrient treatments are dark grey and shown as circles (replicate 1) and triangles (replicate 2) and low nutrient treatments are light grey shown as squares (replicate 1) and crosses (replicate 2). Numbers indicate growth stages: (1) initial growth, (2) early exponential growth, (3) later exponential growth, (4) culture decline; (5) after viral addition. Apparent clustering of low nutrient samples shown in dotted line (top right). An exception to this apparent clustering is the early exponential growth time point in the low nutrient, replicate 2 bottle (6A, L2 cross at lower left). (6B). Re-plot of the same data without the anomalous point. Low nutrient samples remain clustered in the top right quarter. This single point accounted for 2.1% of the total explained variation.



SUPPLEMENTARY MATERIALS

EXPERIMENTAL PROCEDURES

Establishment of diatom cell line

The diatom (*Chaetoceros* sp. KBDT20) was isolated from Kāne'ohe Bay off of the island of O'ahu (21.429°N 202.208°W) on May 8, 2011. Kāne'ohe Bay is typically oligotrophic (average conditions, Table 1) but is intermittently exposed to high nutrients as a consequence of freshwater input from storm events (Drupp *et al.*, 2011). A monoculture was established by micropipette isolation of a single diatom cell and its closely associated microbes. The founder cell was isolated in 2-5 µL of original seawater added to 50 µL of sterile seawater, followed by two additional transfers. With an assumed starting concentration of 2000 bacteria μL^{-1} (Yeo *et al.*, 2013) three serial dilutions of 4 µL would result in 2 free bacteria being transferred. However, bacterial aggregation, loose host associations, or incomplete mixing could lead to incomplete dilution and transfer of a larger number of bacteria.

The diatom was identified by isolating 18S rDNA from the monoculture using the DNeasy Blood and Tissue Kit, (Qiagen®, Hilden, Germany). DNA amplification was performed using Platinum® Taq Polymerase (Invitrogen, Carlsbad, CA, USA) using EukF/EukR primers (DeLong, 1992). The master mix was made following the Platinum® Taq user manual, modified by increasing the concentration of $MgCl_2$ to 2.5 µM. Amplified material was isolated on a 1.3% agarose gel in 0.5X TAE buffer using the exACT Gene™ DNA Low Range Plus Ladder (ThermoFisher Scientific, Waltham, MA, USA), excised, purified using the PureLink™ PCR Purification Kit (Invitrogen®), and sequenced (Advanced Studies of Genomics, Proteomics and Bioinformatics, UH Mānoa). The sequence was submitted to Genbank (KU867951).

The *Chaetoceros sp*. KBDT20 culture was maintained by transfer every 30-60 days into sterile surface seawater (SSW) collected at Station ALOHA (22° 45'N, 158° 00'W) and amended with f/2 media (average conditions listed in Table 1, under average high nutrient concentrations) (Guillard and Ryther, 1962; Guillard, 1975) without antibiotics. Station ALOHA SSW has between 0.02-1.86 µM nitrogen and 0.06-0.2 µM phosphorous, similar to average conditions at Kāne'ohe Bay, but about half of the silica concentration (1.25-

2.24 µM) found in Kāneʻohe Bay. The culture was maintained in 60 mL polycarbonate bottles containing 50 mL of SSW + f/2 under constant light (15 µmol m^{-2} sec^{-1}) and temperature (26 °C).

Isolation of Chaetoceros KBDT20 infective virus

The experimental design included a sequence of diatom growth stages from early exponential to decline. A final stage represented the effects of a virus-induced crash of the diatom population. The diatom virus was isolated from surface seawater collected from Kāneʻohe Bay, Oʻahu Hawaiʻi on June 10, 2011. Approximately 200L of surface seawater was filtered through a 142 mm, 0.8 µm pore-size Isopore (Millipore, Billerica, MA, USA) filter membrane, which was overlaid with a 125 mm GF/C filter. The virus-containing filtrate was concentrated by tangential-flow filtration using the Pellicon 2 Mini system (Millipore) and 30 kDa membranes. The *Chaetoceros* sp. KBDT20 culture was inoculated with a small fraction of the virus concentrate and monitored for signs of lysis. The lytic agent in *Chaetoceros* sp. KBDT20 lysate could be propagated and maintained ability to re-infect healthy culture after 0.2 µm filtration. The virus was further isolated by three consecutive purifications by dilution-to-extinction (Nagasaki and Bratbak 2010).

Transmission electron microscopy of Chaetoceros sp. KBDT20 lytic agent

Approximately 150 mL of host cell lysate was clarified by centrifugation at 5120 RCF for 20 minutes at 4°C, then filtered through a 0.22 µm Sterivex filter (Millipore). The virus-containing filtrate was concentrated using a Centricon Plus-70 (30 kDa; Millipore) ultrafiltration device, and the resulting concentrate was filtered through a 25 mm, 0.1 µm Durapore (Millipore) filter membrane. The 0.1 µm filtrate was then concentrated a second time using an Amicon Ultra-0.5 (30 kDa; Millipore) ultrafiltration device, to a final volume of approximately 100 µL. Samples were negatively stained by diffusing a small drop of the viral concentrate (4 µL) onto a formvar-carbon coated grid for 30 seconds, followed by a ddH_20 rinse addition of 2% uranyl acetate. The sample was analyzed using a Hitachi HT7700 transmission electron microscope (Biological Electron Microscope Facility, UH Mānoa), Supplementary Figure 1. It is possible that bacterial viruses were present as well; the

isolation method did not exclude bacteriophages and we did not attempt to control for bacteriophages in this study.

Factorial experiment

One month prior to the experiment, *Chaetoceros* sp. KBDT20 was shifted to 26° C and 27 µmol m^{-2} sec^{-1} on a light-dark cycle of 12 + 12 hours, to mimic the natural environment. High and low nutrient treatments were prepared as follows. Autoclaved Station ALOHA SSW was cooled to room temperature and decanted into four acid-washed 4.5 L polyurethane bottles. Two bottles were amended with f/2 media ("high nutrient treatment") and two bottles were amended with f/200 media ("low nutrient treatment"). The resultant nutrient concentrations were determined to be relatively high and low by comparison with typical half saturation constants for diatoms (i.e. the nutrient concentration at which uptake is ½ of the optimal rate; Michaelis *et al.*, 1913). All bottles were maintained at 26 °C for 24 hours prior to inoculation.

Each treatment bottle was inoculated with 5 mL of diatom culture at a concentration of 142 ± 33 diatoms mL^{-1}. The inoculum included diatoms, bacteria attached to the diatoms, and any free-living bacteria present in the inoculation volume, all of which originated from the initial isolation of a single founder diatom cell and its microbial associates. Every effort was made to avoid introduction of bacteria during maintenance of the culture and during the experiment.

Sampling schedule

Bottles were rotated continuously at 40 rotations hr^{-1} to prevent diatoms from settling and to ensure a more uniform distribution of host cells. 27 mL samples were taken every 4 days for 60 days. Bottles were mixed gently by inversion before sampling. The sample volume was not replaced, but no more than 9% of the total bottle volume was removed over the course of the experiment. All samples were stored at -20° C before processing. Diatom cell counts were used to define major subsampling time points representing initial growth (after 4 days), early exponential growth (after 8-12 days, depending on the bottle), late exponential growth

(after 16-28 days), and during initial decline (after 24-32 days). Finally, virus was added to each bottle when the diatom abundance had declined to approximately 10% of peak abundance (48-52 days from the start). The multiplicity of infection (MOI, the ratio of virus to host cells) was 4.44±1.45 $x10^{-5}$ MOI for the high nutrient treatment, and 8.20±2.22 $x10^{-4}$ MOI for the low nutrient treatment. A final time point was taken 4 days following addition of virus.

At each major time points, subsamples of the 27 mL sample were analyzed to measure host cell concentrations (1 mL), the number of bacteria per host cell (1 mL), and nutrient concentrations (15 mL); and to identify diatom-associated bacteria (10 mL).

Diatom and bacteria cell counts

For diatom cell counts, a 1 mL subsample from each treatment bottle was fixed in Lugol's iodine to a final concentration of 0.005 µg mL^{-1} and kept at -20° C before processing. The sample was brought to room temperature and placed in a Sedgewick Rafter counting slide mounted on a Zeiss Axio Observer Z1 microscope at 100X total magnification; total diatoms were counted for 50-100 counting cells.

To count the number of bacteria attached per diatom cell, a 1 mL subsample was stained with 0.005µg/mL DAPI and filtered onto a 25mm black 0.2µm Nucleopore™ filter (Sigma-Aldrich, St. Louis, MO, USA). Filters were mounted on a Nikon Eclipse 90 microscope at 1000X total magnification; bacteria per diatom were counted for 20-32 diatoms. The impact of growth stages and nutrient treatment on the number of attached bacteria were evaluated using two tailed tests of log[x+1] transformed counts.

Nutrient concentration

Subsamples for nutrients were defrosted in a 4 °C refrigerator for 48 hours and gently filtered through a 0.2 µm pore-size Nuclepore™ filter to remove particulates. Concurrent analysis of nitrate, nitrite, ammonia, phosphate, and silicate was performed using a SEAL Analytical AA3 HR Nutrient Autoanalyzer (SEAL Analytical, Mequon, Wisconsin) at the SOEST Laboratory for Analytical Biogeochemistry (UH Mānoa, Honolulu HI).

Attached-bacteria community analysis

Subsamples for the identification of diatom-attached bacteria were filtered immediately after collection. The sample was filtered onto a 25 mm diameter, 5 µm pore-size Nuclepore™ filter using a peristaltic pump at a low flow rate. Filters were rinsed with 10 mL of SSW and kept at room temperature for 4 hours in RNAlater® buffer (Qiagen®) to saturate cells before being stored at -20°C until further processing. Before DNA extraction, cells were washed off the filter using the storage solution, in a UV-sterilized laminar fume hood. To remove RNAlater®, cells were pelleted at 1500 rpm for 10 minutes in an Eppendorf Microcentrifuge 5415R (Eppendorf, Hamburg, Germany) at 4°C, and the supernatant removed. 500 µL of buffer ATL (a tissue lysis buffer) was added before pelleting at 1500 rpm for 5 minutes, and the supernatant was again removed. The extraction proceeded as outlined in the DNeasy Blood and Tissue Kit (Qiagen®), which employs a guanidinium-based lysis buffer and adsorption to a silica spin column. DNA amplification was performed using Platinum® Taq Polymerase (Invitrogen, Carlsbad, CA, USA). The master mix was made following the user manual, modified by increasing the concentration of $MgCl_2$ to 2.5 µM. Each sample was amplified in triplicate and pooled for later analysis (Polz and Cavanaugh, 1998). Bacterial 16S rDNA was amplified using primers 895F and 1513R (Weisburg *et al.*, 1991; Hodkinson and Lutzoni, 2010; Baker and Kemp, 2014) with minor modifications to prevent over-amplification (Polz and Cavanaugh, 1998). The 895F primer was used to reduce amplification of non-target 16S rDNA sequences contained in diatom mitochondria and chloroplasts (Baker and Kemp, 2014); it also selects against cyanobacteria. A preliminary test of 10 random samples was amplified using SYBR Green (ThermoFisher Scientific, Waltham, MA, USA) to determine the optimal number of amplification cycles. All samples reached a critical threshold value between 22 and 24 cycles and amplifications in the second cycle was decreased from twelve amplification cycles to four and confirmed by gel electrophoresis. Triplicate amplifications were pooled, run on 1.3% agarose gel in 0.5X TAE buffer using the exACT Gene™ DNA Low Range Plus Ladder (ThermoFisher Scientific, Waltham, MA, USA). Amplifications similar to the 1.9-3.6 ng/µL bands on

the exACT Gene™ ladder were excised and purified using the PureLink™ PCR Purification Kit (Invitrogen) and sequenced (Advanced Studies of Genomics, Proteomics and Bioinformatics, UH Mānoa).

DNA sequence processing and tree building

Chromatograms were viewed, evaluated, and edited using Geneious® software. Sequences were aligned using the Silva INcremental Aligner (SINA) in Silva (Pruesse *et al.*, 2012); sequences below 70% identity in Silva were rejected from further analysis. The SINA alignment was assessed in ARB (Ludwig *et al.*, 2004). Non-target sequences were excluded from further analysis. Suspected contaminants such as sequences with a nearest neighbor from non-marine sources or noted as common laboratory contaminants were also excluded. The resulting sequences were checked for chimeras using UCHIME (Edgar *et al.*, 2011) before further processing. Sequences were clustered into OTU groups at 98% similarity using Mothur (Schloss *et al.*, 2009) as implemented in Qiime version 1.8.0 (Caporaso *et al.*, 2010). Representative sequences for the OTUs were chosen by Qiime and assigned to taxonomic phylotypes based on comparisons to nearest neighbors in the SILVA 111 reference database (Quast *et al.*, 2013). Sequence effort was evaluated using richness estimators (S_{ACE} and S_{CHAO1}) as described in Kemp and Aller (2004); sampling effort was considered exhaustive when richness estimates consistently reached a stable asymptotic value (Kemp and Aller, 2004). Sequences found in all or nearly all samples were identified and OTUs of interest were further evaluated using ARB as well as the basic local alignment search tool (BLAST) (Altschul *et al.*, 1990). Sequences were evaluated by a J-model test to determine the best nucleotide evolution model (Guindon and Gascuel, 2003; Darriba *et al.*, 2012); the chosen model was implemented in PhyML 3.0 (Guindon *et al.*, 2010).

Statistical analysis of the environmental influences on bacterial community composition

The influence of abiotic and biotic factors on bacterial community composition was examined using methods that either account for bacterial relatedness (PCoA and *adonis*) or ignore relatedness (linear modeling), as well as either excluding sequences that occurred only once (only evaluating recurring sequences)

or including sequences that occurred only once in the sampling (hereafter referred to as singletons). The significance of within-bottle effects, host growth stage, nutrient treatment, and viral-induced decline were evaluated using each method.

To evaluate differences in bacterial composition at each timepoint and each replicate, Principle Coordinates Analysis (PCoA) was performed using the weighted Unifrac metric (Lozupone and Knight, 2005), which accounts for phylogenetic distances between samples as well as sample frequency. A PhyML phylogenetic tree, the relative sequence frequencies, and sample nutrient concentration data were input for analyses in R using phyloseq (McMurdie and Holmes, 2013). The influence of environmental factors on bacterial composition was evaluated using *adonis* (Oksanen *et al.*, 2010), a nonparametric method that utilizes the Unifrac weighted distance matrices to perform a permutational multivariate analysis of variance. *Adonis* tests whether variation is attributable to experimental conditions or to uncontrolled covariates; experimental conditions were tested with *adonis* both individually and together with other experimental conditions.

Finally, we employed a generalized linear mixed-effect model created using the R function 'lmer' (R Development Core Team, 2012; Bates *et al.*, 2015), which uses linear algebraic methods for fitting and analyzing mixed models that include both fixed and random effects. This approach accounts for the structure of the experimental design and tests for the effect of different factors on community composition.

In the mixed effect model, data are not reinterpreted through multivariate distances between samples. Instead, OTU abundance is used to evaluate variation in composition; as a result this approach is likely to be more sensitive to detecting differences. The mixed effect model quantifies variation between samples without weighting OTU differences by their phylogenetic distance. The abundance of the OTUs in each sample was modelled as a Poisson-distributed random variable. The effects of different factors (both fixed and random) on community composition were examined. Fixed effects included growth stage and nutrient treatment. Random effects included average relative abundance of each OTU; bottle effects; and the changes in the relative abundance of bacterial OTUs in each bottle, each growth stage, and each nutrient treatment. The effect of variability that did not fit a Poisson distribution was also included in the modeled effects. Each effect in the

model was evaluated by comparing the full model to a model in which that term was removed, using analysis of variance (ANOVA). Effects were removed from the model if $p>0.05$; the remaining effects were considered to be the significant factors in determining the relative abundance of bacterial OTUs.

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SUPPLEMENTARY LIST OF TABLES

Supplementary Table 1. Bacteria per diatom cell based on fluorescence microscopy direct counts. Means and standard deviations are shown. Two tailed t-tests were performed for log[x+1] transformed counts in two nutrient treatments (high and low) and at two growth stages (early exponential and post virus addition).

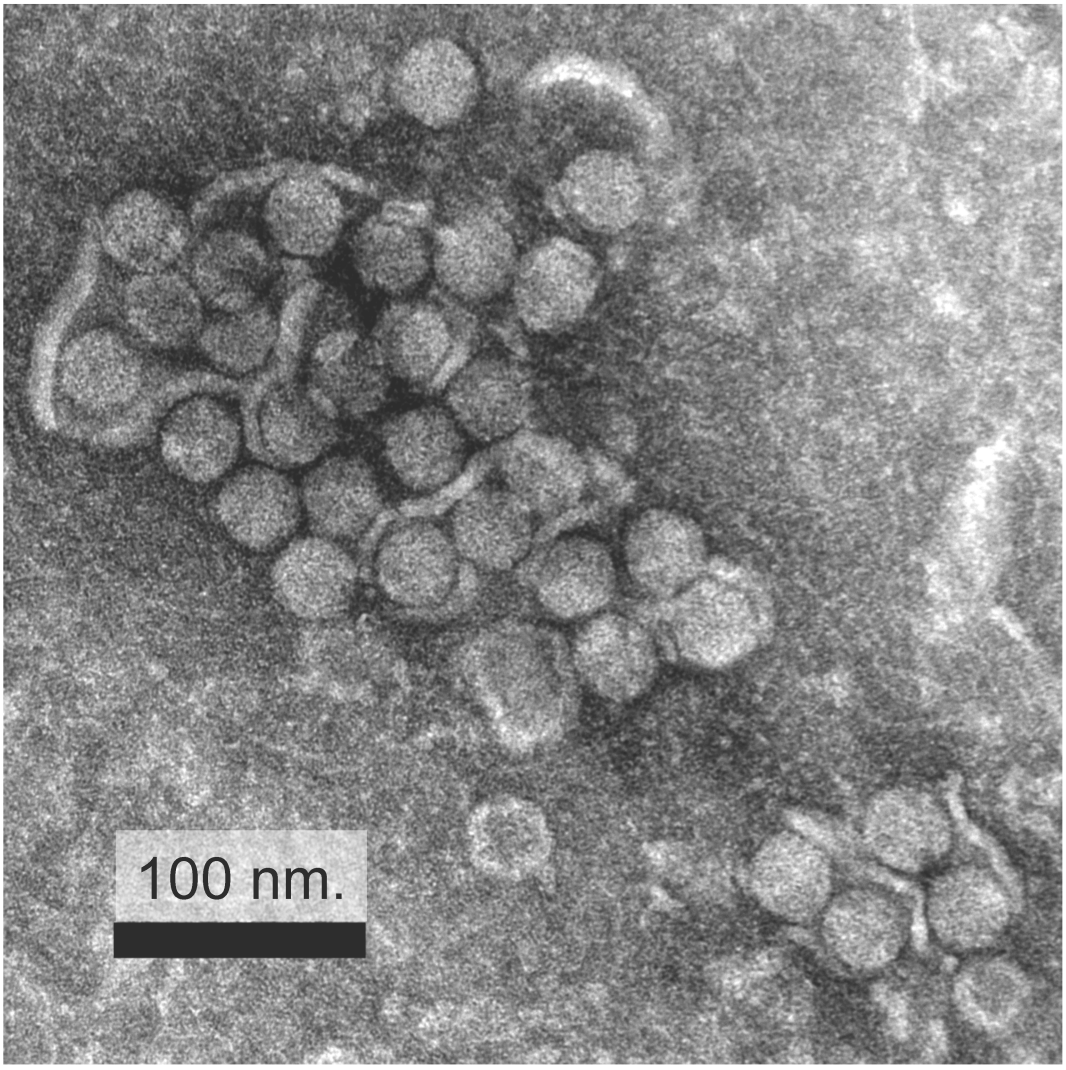
	High nutrient	Low nutrient	ttest, p-value
Early exponential	10±6	8±9	0.001*
Post virus	7±5	7±5	0.884
ttest, p-value	0.004*	0.339	

Supplementary Table 2. Results of *adonis* analysis, including singletons (phylotypes recovered only once in the experiment). The percent of the total variation explained by each environmental condition is denoted as R^2, and is statistically significant (*) at $p < 0.05$. The R^2 and p-value is listed when all experimental conditions are considered concurrently ("all") and when each experimental condition is considered individually ("indiv").

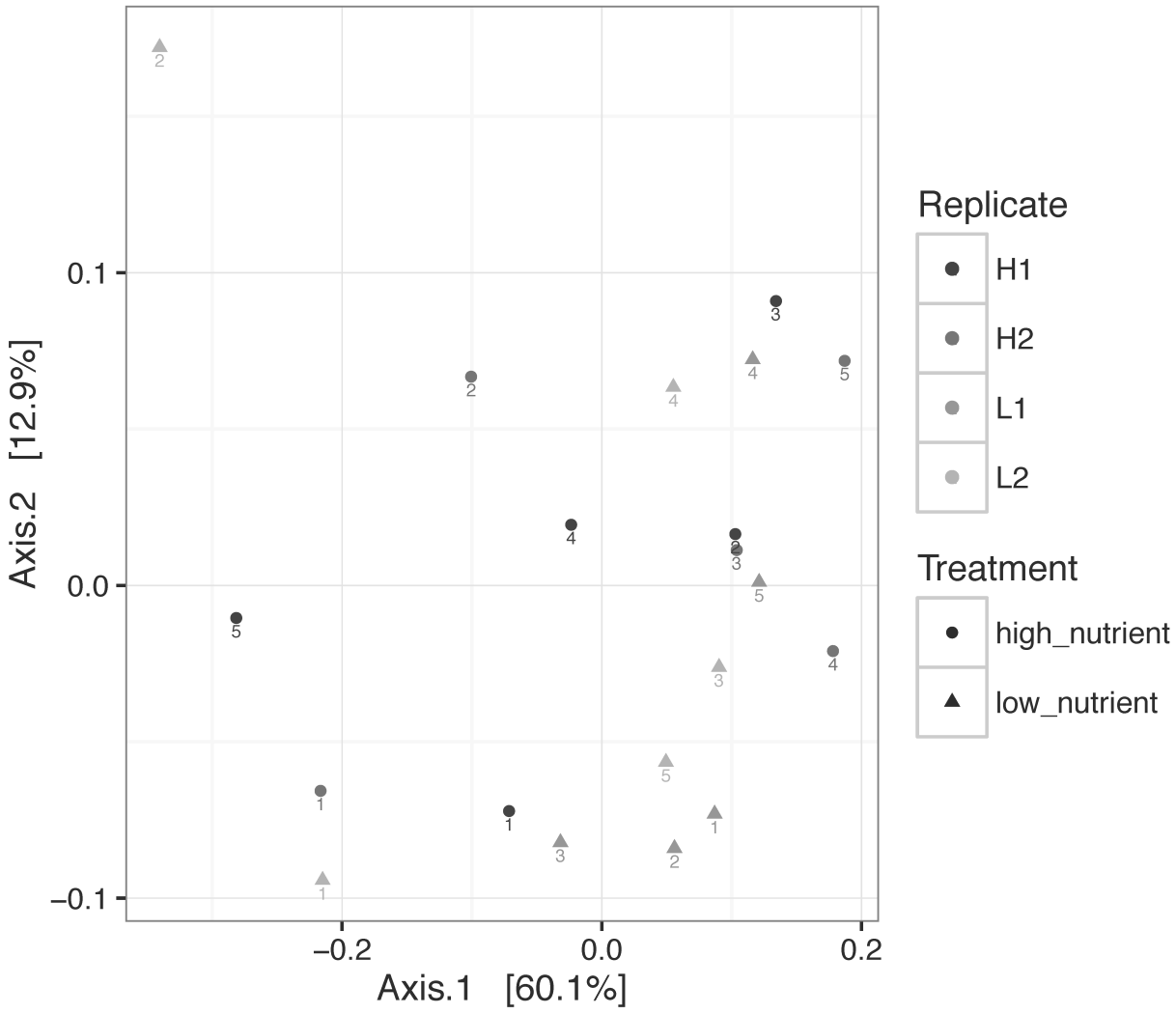
Experimental condition	R^2 (all)	p (all)	R^2 (indiv)	p (indiv)
Nutrient treatment	10.9%	0.035*	9.5%	0.110
Viral induced decline	12.8%	0.016*	7.5%	0.613
Host growth stage	16.2%	0.005*	4.1%	0.183
Bottle effects	5.7%	0.230	4.3%	0.565

SUPPLEMENTARY LIST OF FIGURES

Supplementary Figure 1. Transmission electron micrograph of the putative *Chaetoceros* sp. KBDT20-infecting virus particles. Scale bar equals 100 nm.



Supplementary Figure 2. Axis 1 and 2 of the PCoA of all sequences including singletons. Replicates are denoted by shapes, high nutrient treatments are dark grey and shown as circles (replicate 1) and triangles (replicate 2) and low nutrient treatments are light grey shown as squares (replicate 1) and crosses (replicate 2). Growth stages are indicated by numbers: (1) initial growth, (2) early exponential growth, (3) later exponential growth, (4) culture decline, (5) after viral addition.



Chapter 3: Perturbations of the diatom microbiome affect host health

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ORIGINALITY STATEMENT

For decades, bacteria have been known to attach to the surfaces of aquatic organisms such as phytoplankton cells. These interactions can affect diatom health, persistence, production, aggregation, sinking, and other vital ecosystem processes; therefore, bacterial associates may heavily influence the ecological role of marine diatoms. However, studies of diatom bacterial interactions are often simplified by working with axenic diatom cultures and single bacterial strains, even though efforts to make diatom cells axenic may lead to abnormal host morphology and culture instability. In the present study, inoculating xenic host cells with individual isolates of commonly reported diatom associates (*Marinobacter* and *Alteromonas*) had long-term impacts on host health. The effect of bacterial inoculations on host growth rate, carrying capacity, and decline was evaluated using flow cytometry. Hosts differed significantly and substantially in their reactions to bacterial strains. Inoculations with *Marinobacter* and *Alteromonas* strains did not produce exclusively positive (mutualistic) or negative (parasitic or competition) effects on host cells, as expected from published reports. Instead, the positive or negative effect of bacterial inoculation was modulated by growth in vitamin-replete or vitamin-deficient media, and varied even among closely related, congeneric bacterial strains. Overall, this study demonstrates that the impact of bacteria on diatom health can be studied effectively with an intact diatom microbiome, yielding results that differ from prior work with axenic systems. In addition, this study emphasizes the role micronutrients may play in modulating the impact of diatom-bacterial interactions.

SUMMARY

Diatoms commonly host bacteria. In this study, multiple *Alteromonas* and *Marinobacter* strains were isolated from a *Chaetoceros* diatom to evaluate how bacterial inoculations impact the growth, carrying capacity, and decline of xenic diatom hosts, including: the origin host (*Chaetoceros* sp. KBDT20) and naïve diatom strains (*Chaetoceros* sp. KBDT32 and *Amphiprora* sp. KBDT35). Inoculation with individual bacterial strains occurred under vitamin-replete and vitamin-deficient conditions. Bacterial inoculations affected the trajectory of all diatom strains evaluated; however, the origin *Chaetoceros* and naïve *Amphiprora* were minimally affected, whereas the naïve *Chaetoceros* was more substantially affected. Closely related bacterial strains had differing impacts on host health, but neither genus resulted in exclusively negative impacts (parasitism or competition) or exclusively positive impacts (mutualism). The impact of bacterial inoculation on host cells was more often negative. Vitamin concentrations modulated the impact of bacterial inoculations on naïve hosts; all positive impacts were observed in vitamin-replete media and all negative impacts were observed in vitamin-deficient media. These results suggest that diatom-associated bacteria have differential effects on host cells that cannot be predicted from bacterial or host relatedness and that these effects can be modulated by micronutrient concentrations.

INTRODUCTION

For nearly 200 million years, diatoms and bacteria have coexisted in the marine environment, and their interactions likely influenced the ecological success or failure of both (Amin *et al.*, 2012). Diatoms are the base of the food web in many ecosystems and export a significant amount of carbon from the photic zone (Michaels and Silver, 1988; Brzezinski *et al.*, 2011), even in systems where they are relatively uncommon (Karl *et al.*, 2012). Bacteria can stimulate diatom host growth, affect bloom duration, and induce diatom aggregation (Smith *et al.*, 1995; Gärdes *et al.*, 2011; Amin *et al.*, 2015); understanding the influence of bacteria is, therefore, vital to understanding the ecology of diatoms.

Diatoms are a source of organic nutrients for bacteria (Azam *et al.*, 1983); during all growth stages phytoplankton release organic substances that are primarily composed of carbohydrates (80-90%) (Myklestad, 1995). These exudates attract (Barbara and Mitchell, 2003) and retain bacterial symbionts (Kogure *et al.*, 1982; Rosowski, 1992). In turn, the presence of bacteria can be beneficial, harmful, or neutral to their algal host. Bacteria associated with diatoms can stimulate host cell division (Amin *et al.*, 2015), prevent virus-induced host cell lysis (Kimura and Tomaru, 2014) or provide micronutrients required by the host, such as B-vitamins (Croft *et al.*, 2005) or biologically available iron (Amin *et al.*, 2009, 2012). Surface-resident bacteria can be algicidal (Kim *et al.*, 1999) or conversely, can protect the host from algicidal bacteria (Barja *et al.*, 1989). Diatom-bacterial interactions can also be commensal, where bacteria benefit from being associated with diatoms but diatoms receive neither benefit nor harm (Rosowski, 1992; Droop, 2007).

Two bacteria genera are commonly reported as associates of diatoms in culture: *Alteromonas* and *Marinobacter*. Previous research suggests these genera will have parasitic and mutualistic interactions with a diatom host, respectively. Strains of *Alteromonas* have algicidal interactions with diatoms and antibacterial interactions with diatom-associated bacteria (Nelson and Wear, 2014; Wear *et al.*, 2015); some have been found to digest host setae (Cottrell *et al.*, 2000) or inhibit host growth (Kim *et al.*, 1999). Algicidal effects have

been found to be highly host specific (Paul and Pohnert, 2011), which could lead to an ecological advantage for unaffected hosts and thereby influence the structure of diatom communities. *Alteromonas* capable of producing antibiotics can also harm other host-associated bacteria (Barja *et al.*, 1989), potentially to the detriment of the host if the antibiotics target mutualistic bacteria, or to the benefit of the host if the antibiotics target parasitic bacteria. In contrast, research on *Marinobacter* suggests they form mutualistic associations with diatoms by providing their host with B-vitamins (Stahl and Ullrich, 2016). B-vitamins are essential cofactors in multiple metabolic processes and are necessary micronutrients for many microalgae (Croft *et al.*, 2006). For example, nearly 60% of all cultured haptophytes and heterokonts require an external source of vitamin B_{12} (Tang *et al.*, 2010).

Alleviating vitamin limitation in microalgae results in increased growth rates, but does not affect carrying capacity (Haines and Guillard, 1974); to the best of our knowledge, there is no research on the effect of added vitamins on the rate of decline of microalgae. Field studies of vitamin limited microalgae found that adding vitamin B_{12} stimulated the growth of larger phytoplankton (>2uM), including *Chaetoceros* species (Koch *et al.*, 2011). Moreover, all members of the *Chaetoceros* genus may be vitamin B_{12} auxotrophic (King *et al.*, 2011). In a previous study of *Chaetoceros* cultures, adding vitamin B_{12} to media resulted in increased growth rates when the diatom was grown either xenically or axenically (Ellis, 2015). For B_{12}-auxotrophic diatoms, harboring vitamin B_{12}-producing bacteria may ultimately benefit both host cell and bacteria (Croft *et al.*, 2005).

Previous studies of bacteria-diatom interactions in model systems have been based on the re-introduction of bacteria to cultures of axenic diatom hosts (Grossart, 1999; Gärdes *et al.*, 2012; Sison-Mangus *et al.*, 2014; Amin *et al.*, 2015; Zecher *et al.*, 2015; Han *et al.*, 2016). However, in nature diatoms are commonly found with associated bacteria (Amin et al. 2012a); diatoms deprived of their resident bacteria grow poorly or not at all (Sison-Mangus *et al.*, 2014; Windler *et al.*, 2014; Amin *et al.*, 2015). Growing diatoms axenically can affect frustule morphology, growth rate, and culture stability (Windler *et al.*, 2014). Antibiotics used to create axenic

cultures can be problematic, affecting diatom health and growth directly (D'Costa & Anil 2011, and references therein), and trace concentrations of antibiotics affect subsequent bacterial inoculations (Roose-Amsaleg and Laverman, 2016). Additionally, it is difficult to ensure that a diatom culture is axenic even after repeated applications of antibiotics (Jones *et al.*, 1973; Zecher *et al.*, 2015).

The aim of this study is to seek evidence for parasitic or mutualistic effects of bacteria on diatom hosts, by inoculating common diatom associates (*Alteromonas* and *Marinobacter*) into xenic diatom cultures; i.e. diatoms with a pre-existing microbiome. The use of xenic diatoms is intended to reduce the known deleterious effects of growing diatoms in axenic culture. Three different diatom hosts were tested: the origin host from which the bacterial strains were obtained, a centric diatom (*Chaetoceros* sp. KBDT20); a naïve host of the same genus (*Chaetoceros* sp. KBDT32); and a more distantly related naïve host, a pennate diatom (*Amphiprora* sp. KBDT35). Multiple strains of *Alteromonas* and *Marinobacter* were inoculated into host cultures to evaluate whether strains vary in their positive or negative effects on host growth trajectory. Additionally, this study evaluates whether their impact is modulated by the concentration of vitamins in the culture. Because previous studies suggest that bacteria-derived micronutrients are a key factor affecting diatom hosts, the experiments were repeated in vitamin-replete and vitamin-deficient f/2 media in a fully-factorial experimental design.

RESULTS

Diversity of bacteria isolated from Chaetoceros *sp. KBDT20*

Among all strains previously identified in the *Chaetoceros sp. KBDT20* culture (Baker et al., 2016), only a few were culturable using the chosen methods. A total of 96 bacterial isolates were sequenced. Sequences that were incomplete or contained ambiguous peaks made up 34% of the sequence effort; the remaining 65 sequences from the direct plating method and 61 sequences from the single-cell isolation method were aligned and identified using Silva/SINA. The direct plating isolates were predominantly *Alteromonas* (93%); all other isolates were identified as *Erythrobacteracea* (7%). In contrast, only 30% of bacteria isolated by FACS were

Alteromonas; the majority of bacteria (70%) were identified as *Marinobacter*. Sequences from both methods clustered at 99% similarity using mothur (Schloss *et al.*, 2009) resulted in 14 strains: 7 *Alteromonas*, 6 *Marinobacter*, and 1 *Erythrobacter*.

Bacteria were selected for further analysis based on their similarity to prevalent strains identified in past experiments using *Chaetoceros* sp. KBDT20 (Figure 1). *Marinobacter* strains scs77 and scs85 were similar (99% in BLAST) to strains observed in our previous work (strain KM283562; Baker et al. 2016), to *Marinobacter* isolated from sponges (Esteves *et al.*, 2016), and to *Marinobacter* sp. SA55 and *Marinobacter* sp. SA50 isolated from *Pseudonitzschia multiseries* (Amin *et al.*, 2015). *Alteromonas* strains 2016, 2024, and scs5 also were selected. *Alteromonas* scs5 was closely related to the most prevalent strain isolated in our previous work (strain KM382524; Baker et al. 2016). The remaining *Alteromonas* strains all were closely related (99% similar using mothur), and similar to amplified *Alteromonas* strains observed in our previous work (strain KM383636; Baker et al. 2016). They were also closely related (99% identical in BLAST) to strains that were found to influence the settling of the coral *Pocillopora damicornis* (Tran and Hadfield, 2011) or found in association with the sea sponge *Rhabdastrella globostellata* (Steinert *et al.*, 2014). *Alteromonas* 2016 and 2024 were chosen based on their unique growth physiology in culture; after 24 hours in filtered marine broth, *Alteromonas* 2016 and 2024 formed aggregates but neither formed biofilms on glass or plastic culture vessels. All other bacterial isolates in this study grew evenly suspended in marine broth.

Similarity between bacterial inoculum and bacteria co-cultured with naïve diatoms

Based on 16S rDNA, multiple *Alteromonas* strains exist in the cultures of naïve hosts. Both naïve hosts supported *Alteromonas* strains that were at least 99% similar to the three *Alteromonas* strains (2016, 2024, and scs5) obtained from *Chaetoceros* KBDT20. However, none of the 16S sequences obtained from the naïve hosts were identified as strains of *Marinobacter*.

Bacterial inoculum growth, carrying capacity, and decline

Marinobacter and *Alteromonas* strains had similar growth rates in marine broth, but differed in their carrying capacity, rate of decline, and final concentration at stationary phase (Figure 2). The mean growth rate for all strains was 2.89±0.15 day^{-1}, with no significant difference between genera (p=0.589). *Marinobacter* had a significantly higher carrying capacity than *Alteromonas* (9%; p= 0.0006); *Marinobacter* had a carrying capacity of 2.62±0.18 x 10^4 cells μL^{-1} and *Alteromonas* had a carrying capacity of 2.31±0.05 x 10^4 cells μL^{-1}. *Marinobacter* scs77 required 24 hours to reach carrying capacity, while all other bacteria reached carrying capacity within 18 hours of inoculation. *Marinobacter* declined significantly faster than *Alteromonas* ($p<0.0001$), and all bacteria were at stationary phase after 42 hours of growth. At stationary phase, *Marinobacter* persisted at significantly lower concentrations (48% lower) than *Alteromonas* (p=0.0003). Strains acted as is described for genera for all aforementioned parameters, with the exception of *Alteromonas* scs5. *Alteromonas* scs5 had a significantly faster growth rate (44% faster; p=0.006), but did not significantly differ from other *Alteromonas* or *Marinobacter* strains in carrying capacity, decline, or concentration at stationary phase. All bacterial inoculations were adjusted to account for differences in the stationary phase concentrations of the bacterial isolates.

The influence of vitamins and bacterial inoculations on growth, carrying capacity, and decline of diatoms

If the identity of the diatom is ignored (i.e. all diatoms are evaluated concurrently), the growth and decline of diatoms was only significantly affected by vitamin concentrations ($p<0.01$); bacterial inoculations did not have a significant effect. Additionally, there was no significant interaction between bacterial inoculation and vitamin concentration. Carrying capacity was not affected by either vitamin concentrations or bacterial inoculations.

The influence of vitamins on growth, carrying capacity, and decline of *different diatom hosts*

The addition of vitamins affected the growth and decline rates of diatom controls (diatoms not inoculated with additional bacteria) (Figure 3, compare lines for diatoms with inoculation, to controls). Only *Amphiprora* sp.

KBDT35 responded to the addition of vitamins in a manner consistent with vitamin-limited growth; i.e. a significant increase in growth rate was observed (Figure 3, bottom panels) ($p<0.001$). In contrast, *Chaetoceros* sp. KBDT20 and *Chaetoceros* sp. KBDT32 controls responded to added vitamins with significantly lower growth rates and higher decline rates compared to the no-vitamin treatment ($p<0.003$).

The addition of vitamins resulted in an increased carrying capacity for the *Chaetoceros* sp. KBDT32 control ($p=0.003$), but no difference in carrying capacity for *Chaetoceros* sp. KBDT20. Rather than releasing the diatoms from vitamin limitation, the addition of vitamins appeared to inhibit both *Chaetoceros* cultures. It is possible that the low concentrations required for the *Chaetoceros* species to survive were provided by trace vitamins present in the vitamin-deficient f/2 or produced by the co-cultured bacteria; however, high concentrations of vitamins may have promoted the growth of an antagonistic bacterial population, or triggered a negative response in the co-cultured bacteria or diatom.

The influence of bacterial inoculations on growth, carrying capacity, and decline of different diatom hosts

Only naïve host species were found to significantly differ in how bacterial inoculations impacted the host in either vitamin-replete or vitamin-deficient f/2. The impact of bacterial inoculations on the trajectory of the origin host, *Chaetoceros* sp. KBDT20, was not impacted by the concentration of vitamins in the media. Additionally, only naïve diatom hosts were impacted differently by *Alteromonas* vs. *Marinobacter* inoculations (Figure 3). The effect of bacterial inoculations on diatom growth, carrying capacity, and decline are considered separately in the following sections.

Diatom growth

Inoculating the origin host *Chaetoceros sp.* KBDT20 with bacteria derived from the same diatom culture had minimal impact on its growth. The overall effect of bacterial inoculation, or inoculation with a particular bacterial genus, to *Chaetoceros sp.* KBDT20 was not significantly different from the control. However, some

individual strains did significantly affect growth rate (Table 2A). Specifically, inoculating *Chaetoceros sp.* KBDT20 with *Alteromonas* scs5 or *Marinobacter* scs77 resulted in a significant decrease in the growth rate (Table 2B: Growth, *Chaetoceros sp.* KBDT20, strain), suggesting parasitism or competition between bacteria and host. No significant interactions between bacterial inoculations and vitamin additions were observed at the genus level, or at the strain level (Table 2A).

Bacterial inoculation had the greatest effect on the growth of the naïve host *Chaetoceros* sp. KBDT32, and there were significant differences between the response of *Chaetoceros* sp. KBDT32 to bacterial inoculations in vitamin-deficient vs vitamin-replete f/2 (Table 2A). Inoculation with bacteria resulted in an increased growth rate for *Chaetoceros* sp. KBDT32 in vitamin-replete f/2 (vit), and a decreased growth rate in vitamin-deficient f/2 (novit). The latter occurred both when assessing bacterial inoculations vs. control, and in comparing the two bacterial genera vs. control (Table 2B, *Chaetoceros* sp. KBDT32). At the level of assessing individual bacterial strains vs. control, a significant interaction between strain and vitamin concentrations was observed for all strains (Table 2A, strain x vit; see Table 2B for individual strain information). In vitamin-deficient f/2, inoculation with all bacterial strains resulted in a decrease in the growth rate of *Chaetoceros* sp. KBDT32 (Table 2B, Growth, *Chaetoceros* sp. KBDT32, bac(novit)). In vitamin-replete f/2, inoculation with three of the five bacterial strains resulted in a significant increase in the growth rate of *Chaetoceros* sp. KBDT32 (Table 2B, Growth, *Chaetoceros* sp. KBDT32, bac(vit)). Although there were differences in the extent to which host strains impacted host growth (Table 2A), there was no significant difference between *Alteromonas* strains compared to *Marinobacter* strains (Table 2B, Growth, *Chaetoceros* sp. KBDT32, Alt vs Mar). Overall, bacterial inoculations sometimes resulted in lower growth suggestive of parasitic or competitive interactions, and sometimes higher host growth suggestive of mutualistic interactions with *Chaetoceros* sp. KBDT32. These effects were moderated by the concentration of vitamins, and were not associated solely with either *Alteromonas* or *Marinobacter*.

For the naïve diatom *Amphiprora sp. KBDT35*, bacterial inoculations (without accounting for genus or strain) did not result in a significant response (Table 2B, Growth, *Amphiprora sp.* KBDT35) relative to the control. However, *Amphiprora sp.* KBDT35 inoculated with *Alteromonas* grew significantly slower than those inoculated with *Marinobacter*, but only in vitamin-replete f/2. Thus, *Marinobacter* had a more mutualistic interaction with *Amphiprora sp.* KBDT35 than *Alteromonas*, when grown in vitamin-replete f/2.

Diatom carrying capacity

Perturbing the microbiome of the origin host *Chaetoceros* sp. KBDT20 with its own bacteria resulted in a lower carrying capacity (Table 2B, Carrying Capacity, *Chaetoceros* sp. KBDT20), and this negative effect was not modified by vitamin concentration. There was no significant difference between inoculations with *Alteromonas* vs *Marinobacter*; both resulted in a reduced carrying capacity of the host, which is consistent with a parasitic or competitive interaction with the host. All bacterial strains resulted in a significantly reduced carrying capacity of the origin host except *Alteromonas* 2024, which did not have a significant effect.

The carrying capacity of the naïve host *Chaetoceros sp*. KBDT32 was affected differently by inoculation with *Alteromonas* vs *Marinobacter* (Table 2B, Carrying Capacity, *Chaetoceros* sp. KBDT32); addition of *Alteromonas* resulted in a higher carrying capacity than the addition of *Marinobacter*. At the level of individual *Alteromonas* strains (Table 2B, Carrying Capacity, *Chaetoceros* sp. KBDT32, strains), only *Alteromonas* 2016 and 2024 caused a significant increase in carrying capacity, regardless of vitamin concentration. The increase in carrying capacity is suggestive of a mutualistic interaction between both *Alteromonas* 2016 and 2024 and the naïve host *Chaetoceros sp*. KBDT32.

The carrying capacity of the naïve host *Amphiprora* sp. KBDT35 was impacted by bacterial inoculations whether considered at the genus or strain level, and the impact of bacterial inoculations was affected by vitamin concentrations (Table 2A, Carrying Capacity, *Amphiprora* sp. KBDT35). In vitamin-deficient f/2 the addition of

Marinobacter had a significantly more negative effect on carrying capacity than addition of *Alteromonas* (Table 2B, Carrying Capacity, *Amphiprora* sp. KBDT35, bac(novit)). Four of the five strains resulted in significantly lower carrying capacity in vitamin-deficient f/2 (the exception was *Alteromonas* scs5), suggestive of parasitic or competitive interactions. A different pattern was observed in the vitamin-replete f/2, where addition of one strain (*Marinobacter* scs77) resulted in an increased carrying capacity suggestive of a mutualistic interaction, but the other four strains had no effect on the host.

Diatom decline

Bacterial inoculation resulted in a pronounced decrease in the rate of decline for the naïve host *Chaetoceros* sp. KBDT32 in vitamin-replete f/2 medium; individual strains varied in the magnitude of their effect (Table 2B, Decline, *Chaetoceros* sp. KBDT32). In vitamin-deficient f/2, one *Alteromonas* strain (2024) increased the decline rate of *Chaetoceros* sp. KBDT32, and the other strains had no effect. For the origin host *Chaetoceros* sp. KBDT20, one *Alteromonas* strain (2016) resulted in a decreased rate of decline, and the other bacteria had no effect. None of the bacterial strains affected the rate of decline of the naïve host *Amphiprora sp.* KBDT35. No significant differences were found between the effects of additions of *Alteromonas* vs *Marinobacter*. Overall, the results support a mutualistic interaction between bacteria and naïve host *Chaetoceros* sp. KBDT32, but only under vitamin-replete conditions; little effect on the origin host *Chaetoceros* sp. KBDT20; and no effect on the naïve host *Amphiprora* sp. KBDT35.

Summary of interaction between vitamins and bacterial inoculations on diatom trajectory

The impact of bacterial inoculations on naïve hosts was affected by the concentration of vitamins in the media, most prominently for *Chaetoceros sp.* KBDT32. In vitamin-deficient f/2, inoculation with any bacterial strain decreased the host's growth rate; in vitamin-replete f/2, inoculation with three of the five bacterial strains increased the host's growth rate. Bacterial effects on the rate of decline were also moderated by vitamin concentration. In vitamin-deficient f/2, only one bacterial strain (*Alteromonas* 2024) resulted in an increased

rate of decline, and the rest had no effect; in vitamin-replete f/2, inoculation with any bacterial strain resulted in a slowed rate of decline. The effect of bacterial inoculations on the host's carrying capacity is also modified by vitamin additions, to a greater or lesser extent, but only for the more distantly related naïve host *Amphiprora sp.* KBDT35. Vitamin additions strongly modified the effect bacterial inoculations had on carrying capacity of *Amphiprora* sp. KBDT35. In vitamin-deficient f/2, four of five bacterial strains resulted in a reduced carrying capacity; in vitamin-replete f/2, one bacterial strain (*Marinobacter* scs77) increased the carrying capacity, and the others had no effect.

DISCUSSION

Alteromonas and *Marinobacter* are commonly associated with diatoms (Amin et al 2012); in this study inoculating xenic diatom cultures with multiple strains of *Marinobacter* and *Alteromonas* (Figure 1 and 3) resulted in highly variable impacts on different diatom hosts. The trajectory of the origin host *Chaetoceros* sp. KBDT20 and the naïve host *Amphiprora* sp. KBDT35 were minimally impacted by inoculation with bacteria, whereas the growth and decline of the naïve *Chaetoceros sp*. KBDT32 was impacted by inoculation with all strains (Figure 3 and Table 2B). Although bacterial inoculations had some impact on the growth, carrying capacity, or decline of the diatom hosts (Table 2A) there was no indication of consistent parasitic or mutualistic interactions between the selected bacterial strains and the origin nor the naïve hosts. Overall, the bacterial effects observed in this study were most often detrimental to the host trajectory; however, vitamin-replete f/2 media sometimes resulted in bacterial inoculations having a beneficial effect on host trajectory (Table 2B).

Inoculations of phylogenetically similar bacteria had significantly different effects on host trajectory

The bacteria utilized in this study were derived from a single host cell of *Chaetoceros sp.* KBDT20 and were from closely related bacterial strains, based on 16S rDNA sequence identity. However, they differed in their performance in culture, even between congeneric strains under optimal culture conditions (marine broth at 26°C). For example, *Alteromona*s 2016 grew faster and *Marinobacter* scs77 took the longest to reach carrying

capacity relative to all other strains. Bacterial strains also differed in their physiology; although *Alteromonas* 2016 and *Alteromonas* 2024 were 99% similar to *Alteromonas* scs5 using 16S rDNA, the former two strains consistently formed aggregates. The two genera also differed: *Marinobacter* strains had higher carrying capacity, but declined faster and maintained lower concentrations at stationary phase than *Alteromonas* strains. However, the concentration of all bacteria investigated declined in vitamin-deficient f/2, suggesting that without a diatom host, all strains would be unable to grow in this minimal media.

Cultures of naïve diatoms evaluated in this experiment contained *Alteromonas* sequences similar to the *Alteromonas* strains isolated from *Chaetoceros* sp. KBDT20; similar *Marinobacter* strains were not found. Both the origin culture and the naïve cultures were impacted by the addition of *Alteromonas* and *Marinobacter*. Although the only significant differences between inoculation with *Alteromonas* vs *Marinobacter* occurred for the naïve hosts, they occurred too infrequently to speculate on similarities or differences between the *Alteromona*s present in naïve cultures and the *Alteromonas* strains used as bacterial inoculum. It is possible that similar bacterial strains developed host-specific functional adaptations that would not be detected using 16S rDNA data (Zecher *et al.*, 2015). Therefore, hosts were still referred to as being either native or naïve.

Inoculation with bacterial strains from the same genus had inconsistent impacts on host trajectory

Inoculation of different bacterial strains from the same genus rarely had identical impacts on the trajectory of a diatom host. More often than not, only one strain of a genus significantly impacted the growth, carrying capacity, or decline of a diatom host, while the other strains had no effect. When more than one strain of a genus affected the trajectory of a host, the difference in the magnitude of the effect was commonly near 20% (Table 2B). This finding is not unique. Previous studies using axenic diatoms also found that bacterial strains with nearly identical 16S-rDNA can impact the host differently (Zecher *et al.*, 2015). In the present study, even closely related strains descended from the associates of an individual founder diatom cell can have different effects on a xenic diatom host.

A given bacterial strain could have dissimilar impacts on the trajectory of different diatom hosts. For example, inoculation with *Alteromonas* 2016 significantly decreases the carrying capacity for both *Chaetoceros* sp. KBDT20 and *Amphiprora* sp. KBDT35, but increases the carrying capacity of *Chaetoceros* sp. KBDT32.

In general, bacterially inoculations most often impacted the trajectory of naïve host *Chaetoceros sp*. KBDT32 and had little to no affect on the other diatoms evaluated. The growth and decline of *Chaetoceros sp.* KBDT32 was impacted by all strains (although the strain effect varied between vitamin deficient and vitamin replete media, as discussed below); the growth and decline of *Chaetoceros* sp. KBDT20 was affected by two strains and one strain, respectively, and *Amphiprora* sp. KBDT35 was unaffected. The variability of effects was surprising and demonstrates that manipulations of common diatom-associated bacteria can have different impacts on the origin host as well as on other diatom hosts.

Perturbations of the diatom microbiome were more often detrimental than favorable to the host

Of all significant effects observed upon adding bacteria to host cultures, more than half (9/17) represented a detrimental effect on the host (Table 2B). Based on previous studies, it would be reasonable to expect detrimental effects from adding *Alteromonas* strains, but positive effects from adding *Marinobacter* strains. For example, some *Alteromonas* strains inhibit or kill their diatom host (Kim *et al.*, 1999; Cottrell *et al.*, 2000; Bidle and Azam, 2001; Paul and Pohnert, 2011) or other associated bacteria (Barja *et al.*, 1989). We are not aware of any studies showing detrimental effects of *Marinobacter* on diatom hosts or other bacteria. In our experiment, inoculating *Chaetoceros* cultures with *Alteromonas* or with strain *Marinobacter* scs77 resulted in a suppressed growth rate of the host. This may be indicative of bacterial competition for resources or other unforeseen interactions with co-cultured bacteria or host cells, but to the best of our knowledge this is the first reported negative effect of *Marinobacter* on a diatom host.

When vitamin concentration had a significant effect, all detrimental effects of bacterial inoculations (10) occurred in vitamin-deficient media and all beneficial effects (9) occurred in vitamin-replete media. Collectively, these results suggest that in the presence of sufficient vitamins, added bacteria have a positive impact on diatoms; whereas in low-vitamin conditions, the same bacteria may compete with diatoms.

We hypothesis that this complex set of interactions may be related to transparent exopolymer particles (TEP) production by the host and B-vitamin production by bacteria. Bacterial inoculation into xenic diatom cultures may have resulted in elevated TEP production by diatom hosts (Middelboe *et al.*, 1995; Kahl *et al.*, 2008; Kaeppel *et al.*, 2012). High TEP concentrations would otherwise increase diatom-sticking efficiency, which been associated with diatoms experiencing oxidative stress, lowered photosynthetic quantum efficiency, and the first steps of programed cell death (Kahl *et al.*, 2008). Bacteria are able to consume diatom TEP (Smith *et al.*, 1995). In high-vitamin concentrations bacteria may be more able to consume excess TEP compared to low-vitamin conditions. In low-vitamin conditions, bacterial production of vitamin B_{12} is energetically expensive (Roth *et al.*, 1996). If bacteria are vitamin limited, they may be less able to consume TEP and prevent negative host outcomes. However, transcriptomic or proteomic studies would be needed to resolve this hypothesis.

Vitamin replete media had an unexpected detrimental effect

In addition to the surprising variability in the effects of bacteria on host diatoms, the negative response of *Chaetoceros* cultures to added vitamins was unexpected. The concentration of B-vitamins in vitamin-deficient f/2 was anticipated to be less than or equal to Ks, and was expected to be quickly exhausted. It would be reasonable to expect that vitamin deficiency would result in slower growth and/or lower carrying capacity of diatoms. Bacteria co-cultured with the diatoms could be capable of providing vitamins at a rate sufficient to support diatom growth (Croft *et al.*, 2005); therefore, it would not be surprising to find that additions of bacteria ameliorate the effects of vitamin deficiency. Instead, a higher vitamin concentration was associated with negative impacts on the *Chaetoceros* cultures. It is possible that an overabundance of vitamins favored

the growth of harmful bacteria in these cultures. Negative vitamin effects appear to be alleviated by the addition of some bacterial strains to *Chaetoceros* sp. KBDT32, but *Chaetoceros* sp. KBDT20 was unaffected by the addition of the same strains. Presently we are unsure of the mechanism by which vitamins could decrease diatom growth rate, or how bacteria could alleviate this effect.

Conclusions

Understanding how bacteria impact a host cell is vital to the study of bloom dynamics (Smith *et al.*, 1995; Gärdes *et al.*, 2012; Buchan *et al.*, 2014) and microalgal production in algal biotech industries (Natrah *et al.*, 2014; Fuentes *et al.*, 2016). The concentration of bacteria used in this study is similar to those employed in studies of axenic diatoms (Amin *et al.*, 2015); at this concentration, perturbations of the bacterial assemblage had long-lasting effects on the trajectory of all three xenic diatom cultures evaluated. Inoculation with *Alteromonas* and *Marinobacter* strains produced both positive and negative effects on the host diatoms evaluated, however, effects were not specific to the host, or to the bacterial strain added. Bacterial inoculations most often resulted in negative effects on the host trajectory. When the affect of bacterial inoculations were modified by vitamin concentrations, all negative effects occurred in vitamin-depleted conditions, and all positive effects occurred in vitamin-replete conditions. This would argue against the hypothesis that symbiotic bacteria provide B vitamins to their diatom host. Instead, it suggests that added bacteria competed with the host diatoms when vitamins were scarce, but had positive effects when vitamins were plentiful. The cause of the observed positive effects is unknown, but may be related to bacterial consumption of host TEP.

Co-occurring bacteria may be a major determinant of diatom abundance and diversity in the natural environment. These findings also suggest that (1) bacteria are able to detect and react differently to different co-occurring diatoms and/or (2) diatoms may vary in their susceptibility to either beneficial or detrimental secondary compounds produced by co-cultured bacteria. In either case these interactions may be moderated

by the concentration of vitamins in the environment. Further studies of these possible interactions at the level of transcriptomics or proteomics would resolve these hypotheses. Understanding these complex interactions may be vital to understanding how bacteria contribute to the health, success, or failure of their host.

EXPERIMENTAL PROCEDURES

Diatom culture collection

The diatoms evaluated were the origin host of the bacterial isolates (*Chaetoceros* sp. KBDT20) and two naïve recipient hosts (*Chaetoceros* sp. KBDT32 and *Amphiprora* sp. KBDT35). Naïve hosts had not been intentionally exposed to *Chaetoceros* sp. KBDT20 bacterial isolates previously, although related strains may exist in the naïve host cultures. *Chaetoceros* sp. KBDT20, *Chaetoceros* sp. KBDT32, and *Amphiprora* sp. KBDT35 were collected by Christopher Schvartcz on May 8, 2011 from surface seawater in Kāneʻohe Bay (O`ahu, Hawai`i) at the dock of the Hawaiʻi Institute for Marine Biology. Diatom identity was assessed by microscopy. For each culture, a single founder cell was isolated along with its microbiome in sterile seawater. Sterile seawater was prepared from Station ALOHA (22° 45'N, 158° 00'W) surface seawater filtered using a 0.1 µM Supor membrane AcroPak filter cartridge (Pall Corporation, Port Washington, NY, USA), and autoclaved for 30 minutes per L at 260°C. This single cell inoculum was grown in sterile seawater amended with the f/2 medium kit (NCMA, Boothbay, ME, USA), hereafter referred to as vitamin-replete f/2. If f/2 media is prepared without vitamins, it will be referred to as vitamin-deficient f/2. The estimated vitamin concentrations in f/2 media are discussed in the Supplementary Materials. Cultures were grown at 15 µmol photons m^{-2} sec^{-1} and 26 °C. Every 30-50 days, 0.5 mL of the culture was transferred to 50 mL of fresh vitamin-replete f/2.

Diversity of bacteria present in origin and naïve host cultures

Bacteria co-cultured with each naïve diatom were identified using 16S rDNA. For DNA extraction, diatom cultures and their co-cultured bacteria were grown to late exponential phase before being homogenized by shaking with autoclaved 0.1 mm Zirconia/Silica beads (Biospec, Bartlesville, OK, USA) for 30 seconds at 6.0

speed using a Fast Prep FP120 Cell Disrupter (Qbiogene Inc, Carlsbad, CA, USA). The homogenized cells were then centrifuged for 10 minutes at RCF 15000 g at 4°C using a Sorvall Primo R centrifuge (Thermo Fisher Scientific). The supernatant was removed, and the pellet was extracted as outlined in the DNeasy Blood and Tissue Kit, (Qiagen®, Hilden, Germany). DNA was amplified using Taq Polymerase (Invitrogen, Carlsbad, CA, USA) using the recommended protocol with the exception of higher concentrations of $MgCl_2$ (2.5 µM), and V4 primers (Caporaso *et al.*, 2012). Amplifications were run on a 1.3% agarose gel in 0.5X TAE buffer to confirm amplification and adequate DNA concentrations (Invitrogen). Amplifications were cleaned and normalized using the SequalPrep™ Normalization Plate Kit (ThermoFisher Scientific, Waltham, MA, USA) and sequencing was performed using 500 bp paired-end MiSeq (Illumina, San Diego, CA, USA). Sequences were aligned and classified using the Silva INcremental Aligner (SINA) in Silva (Pruesse *et al.*, 2012); sequences below 70% identity in Silva were rejected from further analysis. Strains were determined at 99% relatedness using Mothur (Schloss *et al.*, 2009) as implemented in Qiime (Caporaso *et al.*, 2010). Sequences closely related to *Alteromonas* and *Mariobacter* (*Alteromonadaceae*) were submitted to Genbank (PRJNA382430).

Isolation and identification of host-associated bacteria

Chaetoceros sp. KBDT20 associated bacteria were isolated using Difco™ Marine Agar 2216 (BD Biosciences, East Rutherford, NJ, USA) and grown in the dark at 26°C for 24 hours. Initial isolation was performed by spreading 1µL of diatom culture onto marine agar and incubating at 26°C in the dark for 24 hours. Ninety-six bacterial colonies were isolated using the T-streak method repeated in triplicate with the intent to isolate a single bacterial strain. Isolated colonies were grown in Difco™ Marine Broth 2216 (BD Biosciences, East Rutherford, NJ, USA) at 26°C for 24 hours before being preserved in glycerol (final concentration 30%) and stored at -80°C.

In an effort to obtain additional strains, host-associated bacteria also were isolated by fluorescence-activated cell sorting (FACS) of *Chaetoceros* sp. KBDT20 cells. Individual host cells were sorted onto marine agar using the BD Influx™ flow cytometer (BD Biosciences, East Rutherford, NJ, USA). The sheath fluid was 0.2 µM filtered and

autoclaved surface seawater from Station ALOHA; the influx sheath tank was UVC sterilized for 12 h before adding sheath fluid and for two hours after adding sheath fluid. Cell sorting was performed at 6.0 psi sheath fluid pressure and 5.5 psi sample pressure using a 150 µM nozzle. Samples were triggered on 488-RED at a level of 26 and cells were gated using forward scatter ($10^{1/2}$ - $10^{2.5}$) and 488-RED (10^{2} - $10^{3.5}$). Single cell sorting was visually confirmed by microscopy before sorting into a 96 well culture plate containing marine agar. Contamination was assessed using sterile seawater containing fluorescent beads sorted into an additional 96 well culture plate. Plates were incubated in the dark at 26°C and evaluated every 24 hours for 5 days. Colonies were selected from twenty wells based on variations in colony morphology and time until first colony formation; selected colonies were T-streaked on marine agar. From the twenty T-streaked colonies, 2-10 colonies were selected from each to maximize the diversity of culture morphologies, resulting in 96 isolates. These 96 isolates were purified using three iterations of the streak plate method as described above.

Bacterial isolates were confirmed to include a single phylotype by 16S rDNA sequencing. Bacterial DNA was isolated from colonies cultured in marine broth for 24 hours. 1µL of culture was diluted in 50 mL of sterile PCR clean water (Qiagen®, Hilden, Germany) before lysing using one cycle of freeze-thaw. Bacterial 16S rDNA was sequenced and evaluated as described in Baker et al., 2016.

Cultures containing a single bacterial isolate were aligned and similarity was calculated. Representatives of five distinct strains were selected for further investigation based on their relationship to prevalent strains in past experiments (Baker et al. 2016) and sequences were submitted to Genbank (KY921850- KY921854). Similarity to previously isolated strains was also performed using the Basic Local Alignment Search Tool (BLAST) excluding uncultured organisms (Altschul *et al.*, 1990). These sequences were aligned using Silva/SINA (Pruesse *et al.*, 2012) and a tree was constructed using PhyML (Guindon *et al.*, 2010). Strains were named based on the isolation method; 20XX isolates were obtained using the plating method, and scsXX isolates using the single cell FACS method.

Preparation and evaluation of bacterial inoculum

In low nutrient marine environments bacteria rarely grow exponentially and are most often found in stationary phase (Kolter *et al.*, 1993). During stationary phase, bacteria may alter their metabolic pathways and express stress-response genes (Finkel, 2006). In an attempt to better represent a natural system, bacteria in this experiment were grown to stationary phase prior to being added to diatom cultures.

To acquire the dense bacterial concentrations required for inoculations, bacterial strains were grown in Marine Broth. After 48 hours, bacteria were pelleted for 10 minutes at RCF 7500 x g at 4°C using a Sorvall Primo R centrifuge (Thermo Fisher Scientific), the supernatant was removed, and the pellet was resuspended twice in vitamin-deficient f/2, to remove trace amounts of marine broth. Subsamples were plated on marine agar to confirm the survival of bacteria.

The growth, carrying capacity (i.e. the peak concentration), and rate of decline of bacteria grown in Marine Broth and resuspended in f/2 were estimated from cell counts taken from cultures of bacterial inoculum grown in triplicate. Glycerol preserved bacteria were grown in autoclaved marine broth that was 0.2 µM filtered to remove particulates (Corning Inc., Corning, NY, USA). Bacterial cells were collected in 3% formaldehyde every 2-10 hours during the 48-hour incubation in Marine Broth and after 24-hour incubation in vitamin-deficient f/2. Cells were diluted 1:100, stained using SYBR® (Thermo Fisher Scientific) to a concentration of 1X, and counted using an Attune flow cytometer (Thermo Fisher Scientific). The growth rate, carrying capacity, and rate of decline were determined as described below for diatom cultures. The resulting rates and concentrations were compared for genera and strain using lsmeans and one-way ANOVA using Companion to Applied Regression (car) in R (Fox *et al.*, 2015; Lenth, 2016). Differences in the time required for different genera or strains to reach carrying capacity were also noted.

The potential for bacterial competition was evaluated for each strain. Bacteria were grown in marine broth at 26°C. After 24 hours, high-density cultures of individual strains were spread on marine agar plates. After culture media had absorbed to the agar plate, 3 µL of each bacterial strain evaluated was spotted and left to absorb. This was repeated in triplicate for each strain. Competition was determined by observation of bacterial growth patterns: bacteria capable of competition form colonies visibly separated from one another, otherwise bacteria are assumed to not be competing with one another to colonize a surface.

Bacterial and diatom culture preparation

Bacteria and diatoms were prepared separately and counted using an Attune flow cytometer before being inoculated into new vitamin-replete and vitamin-deficient media. Replicates of each bacterial strain and negative controls were prepared as described above. Following the 24-hour incubation in vitamin-deficient f/2, bacterial cultures and negative controls stained using 1X SYBR®; the threshold was set on BL1 (Excitation 488nm, Emission filter 530/30nm) at 10,000 and a voltage of 3,300 and bacteria were gated at BL1-A ($10^{4.5}$-$10^{7.5}$) and SSC-A (10^{1}-$10^{4.5}$). Diatoms were grown to mid-exponential phase and counted using a threshold on BL3 (Emission filter >640 nm) at 10,000 and a voltage of 2,550. Cells were gated using BL3-H ($10^{5.5}$-$10^{6.5}$) and VL2-H ($10^{2.5}$-$10^{4.5}$). Diatom cultures were then diluted to a concentration of 1-10 diatom cells μL^{-1} and bacterial cultures were diluted to a concentration of 50-100 bacterial cells μL^{-1} in 50 mL of vitamin-replete f/2 and vitamin-deficient f/2. The inoculation volume of the negative control was the average inoculation volume of the corresponding bacteria-containing medium for each host cell.

Measuring and analyzing the effect of vitamins and bacterial inoculations on host cells

The effects of vitamin concentrations and bacterial inoculations were evaluated using cell counts of host cells. Similar analyses of co-cultured free-living bacterial concentrations were also performed and can be found in Supplementary Material (*Growth of free-living bacteria* and *Supplementary Figure 1*). Initial counts of diatoms were taken within 2 hours after they were added to new media. These counts were repeated 19-26 hours after

the initial inoculation and then every 48-72 hours to follow population abundance over time. Analyses were performed on natural log-transformed count data for each replicate and timepoint. The carrying capacity of was evaluated for all diatom cultures, which was was determined as the average of the three highest concurrent counts for each replicate.

The effect of vitamin concentration and bacterial inoculations on diatom growth rate, carrying capacity, and decline was evaluated for diatoms (inclusive of all diatoms tested), as well as for each diatom host strain. Growth and decline was modeled using the Churchill equation (Membré et al. 1997, Eqn 3) using non-linear least squares (nlr) as implemented in R. The Churchill equation is:

$$\ln(N)=((1/K_1)e^{-\lambda 1*t)=(} + (1/K_2)\, e^{\lambda 2*t})^{-1}$$

λ_1 is the exponential rate of increase during log-phase growth, hereafter referred to as growth rate; K_1 and K_2 are constants that account for the initial density and peak density respectively; λ_2 is the exponential rate of decline after the culture reaches carrying capacity, hereafter referred to as the rate of decline; t is time in days. Estimators of carrying capacity, exponential growth rate (λ_1), and decline rate (λ_2) were derived for each replicate. The effects of (1) vitamin addition and (2) bacterial inoculation on these three parameters were evaluated using two-way factorial ANOVA as described above. Vitamin addition was evaluated as a dichotomous variable (with or without added vitamins). The effect of bacterial inoculation was evaluated at three levels of phylogenetic detail: (1) testing for any significant effects of bacterial inoculations vs no bacterial inoculation (=negative control); (2) testing for any significant effects of *Marinobacter* vs. *Alteromonas* vs. no bacterial inoculation; (3) testing for any significant effects of inoculating five different bacterial strains, vs no bacterial inoculation. Factors were considered significant if $p<0.05$.

If significant effects were found in these general-level ANOVAs, the analysis proceeded to test the effects of inoculation at the level of a single bacterial genus or strain. Responses to inoculation with different bacterial genera were partitioned to investigate (1) *Marinobacte*r vs. control (2) *Alteromonas* vs. control, and (3) *Alteromonas* vs. *Marinobacter*. Inoculations with different bacterial strains were partitioned into five different tests, where each strain was tested against the control. If the interaction term was significant (bacteria*vitamin), one-way ANOVAs were performed on the effect of bacterial inoculation (1) in vitamin-replete f/2 , and (2) in vitamin-deficient f/2. These results are presented as a heatmap of the significant effects ($p<0.05$) as well as their magnitude. The magnitude is calculated as the percent difference between the effector (vitamin-deficient conditions or bacterial inoculations) and the default conditions (i.e. vitamin-replete conditions or no bacterial inoculations, respectively). All significant results were examined to investigate possible symbiotic interactions. Possible indicators of parasitism and/or bacterial competition with the diatom host include slower growth rate, lower carrying capacity, or faster rates of decline of the host diatom compared to controls. Possible indicators of mutualism include faster growth rate, higher carrying capacity, or slower rates of decline of the host diatom compared to controls.

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Table 1. Nutrient concentrations for marine diatoms under varying nutrient regimes. The range of observed half saturation constants (Ks) for marine diatoms (Sarthou et al. 2005) for nitrogen, phosphorous, and silica. The Ks for vitamin B12 is based on studies of cultured algae (Droop 2007). The half saturation constant is the nutrient concentration at which uptake is ½ of the optimal rate. Concentration ranges for Station ALOHA were average and standard errors taken at the surface (from 5-25 m) from 1988-2015. Nutrient concentrations of f/2 are taken from Guillard and Ryther 1962. All values listed are in µM.

Nutrient	Average Ks	n	Range Conc. Station ALOHA	Conc. f/2
Nitrogen	1.6 ± 1.9	35	$(3.16 \pm 0.64) \times 10^{-3}$	882
Phosphorous	0.24 ± 0.29	18	$(46.29 \pm 8.60) \times 10^{-3}$	36.2
Silica	3.9 ± 5.0	25	1.17 ± 0.05	106
Vitamin B_{12}	$(1.17 \pm 0.77) \times 10^{-6}$	13	$<0.6 \times 10^{-13}$	3.69×10^{-4}

Table2. (**A**) Effect of vitamin concentration and bacterial inoculations evaluated by two-way factorial ANOVA. The effect of bacterial inoculations was investigated at the level of inoculation or control (bac); genus Marinobacter, Alteromonas or control (gen); and five different strains or the control (strain). The effects of vitamin level in the two way ANOVA are listed as Vitamin(bacteria), Vitamin (genera), or Vitamin (strain), and the interaction term is listed with an "X".

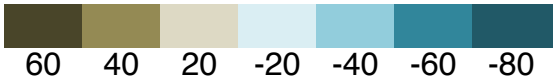
	Diatom	Bacteria	Genera	Strain	Vitamin (bacteria)	Vitamin (genera)	Vitamin (Strain)	Bacteria X Vitamin	Genera X Vitamin	Strain X Vitamin
Growth	*Chaetoceros* sp. KBDT20			*	***	***	***			
	Chaetoceros sp. KBDT32	*	*		***	***	***	***	***	***
	Amphiprora sp. KBDT35				**	***	***		*	*
Carrying capacity	*Chaetoceros* sp. KBDT20	***	**	**	**	**	**			
	Chaetoceros sp. KBDT32		*	*	*	*	*			
	Amphiprora sp. KBDT35		**	***	**	***	***	*	***	***
Decline	*Chaetoceros* sp. KBDT20	*	**	*						
	Chaetoceros sp. KBDT32	***	***	***	***	***	***	***	***	***
	Amphiprora sp. KBDT35				***	***	***			

(**B**) The effects of bacterial inoculations and vitamin concentrations on the growth, carrying capacity, and decline of each diatom, evaluated with two-way ANOVA (bac) and one-way ANOVA (bac (vit) and bac (novit)). The effect of bacterial inoculations was investigated at the level of inoculation or control (bac); Alteromonas vs control (Alteromonas), Marinobacter vs control (Marinobacter), Alteromonas vs Marinobacter (Alt vs Mar); and five different strains individually tested against the control (strain). The effects of bacterial inoculation in the presence or absence of added vitamins are denoted with a (): cultures grown in vitamin replete f/2 (bac (vit)); cultures grown in vitamin deficient f/2 (bac (novit)). The effects of bacterial inoculation at the level of bacterial addition vs control (bac), Marinobacter vs Alteromonas (genus), or individual strains (strain) are identified by

the column name. The magnitude of the effect (percent difference) is only given for significant effects. (A&B)

Significance is listed with asterisks, with * == 0.05>p>0.01, ** == 0.01>p>0.001, and *** == 0.001>p.

			Bac	Genera			Strain				
	Diatom	effect	Bacteria	*Alteromonas*	*Marinobacter*	*Alt vs Mar*	*Alteromonas* 2016	*Alteromonas* 2024	*Alteromonas* scs5	*Marinobacter* scs77	*Marinobacter* scs85
GROWTH	*Chaetoceros sp. KBDT20*	bac							*	*	
		bac (vit)									
		bac (novit)									
	Chaetoceros sp.KBDT32	bac	*	*							
		bac (vit)	**	*	**				*	*	*
		bac (novit)	***	***	**		*	**	*	**	*
	Amphiprora sp. KBDT35	bac									
		bac (vit)				*					
		bac (novit)									
CARRYING CAPACITY	*Chaetoceros sp. KBDT20*	bac	***	**	**		*		**	***	*
		bac (vit)									
		bac (novit)									
	Chaetoceros sp.KBDT32	bac		**		***	***	**			
		bac (vit)									
		bac (novit)									
	Amphiprora sp. KBDT35	bac			***	**	**			**	***
		bac (vit)								*	
		bac (novit)	*	*	***	***	**	*		***	**
DECLINE	*Chaetoceros sp. KBDT20*	bac	*	**			**				
		bac (vit)									
		bac (novit)									
	Chaetoceros sp.KBDT32	bac	***	***	***		**	**	***	***	**
		bac (vit)	***	***	***		**	**	**	**	*
		bac (novit)						*			
	Amphiprora sp. KBDT35	bac									
		bac (vit)									
		bac (novit)									



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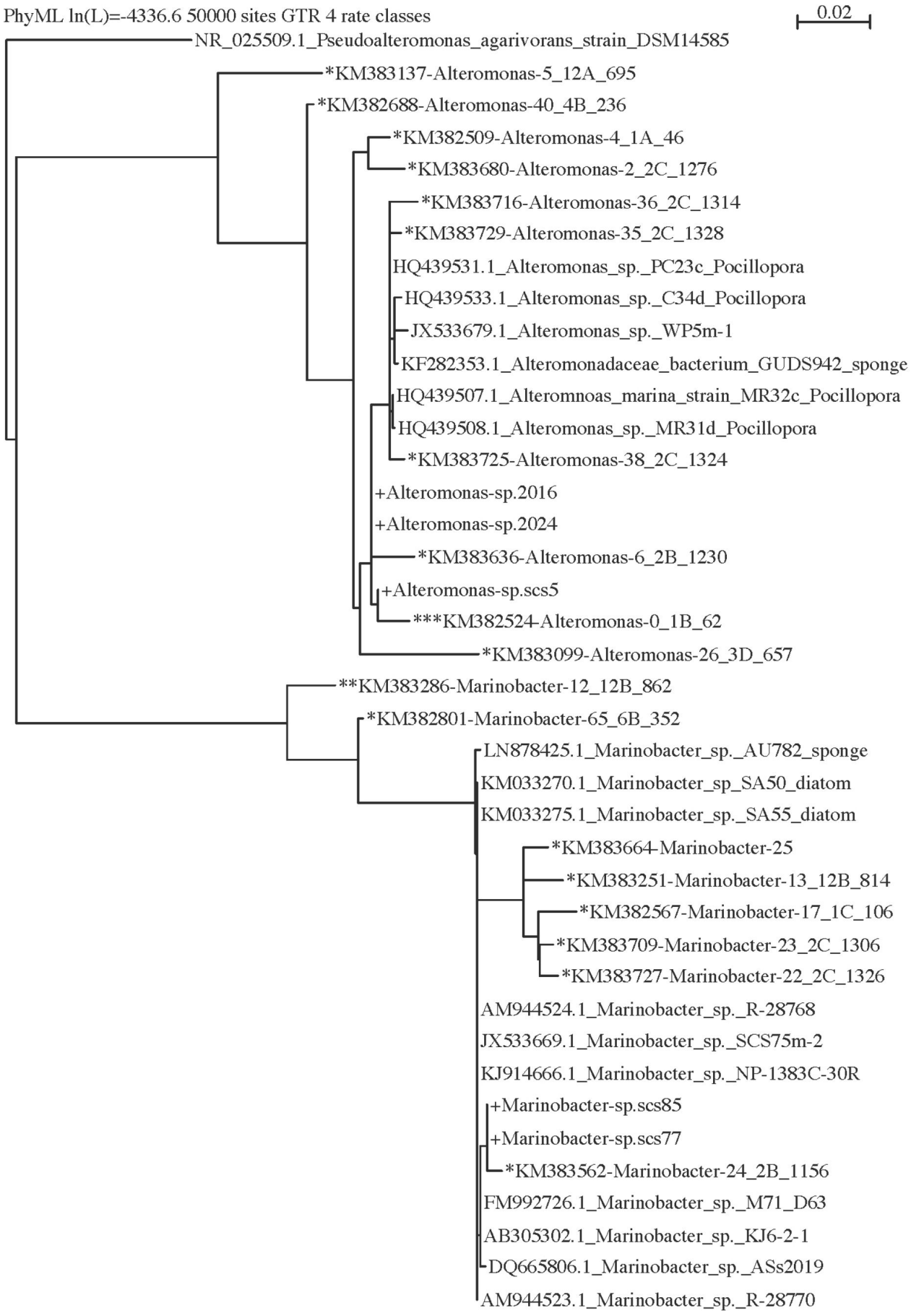
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*KM382509-Alteromonas-4_1A_46
*KM383680-Alteromonas-2_2C_1276
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JX533679.1_Alteromonas_sp._WP5m-1
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HQ439508.1_Alteromonas_sp._MR31d_Pocillopora
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+Alteromonas-sp.2016
+Alteromonas-sp.2024
*KM383636-Alteromonas-6_2B_1230
+Alteromonas-sp.scs5
***KM382524-Alteromonas-0_1B_62
*KM383099-Alteromonas-26_3D_657
**KM383286-Marinobacter-12_12B_862
*KM382801-Marinobacter-65_6B_352
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*KM383251-Marinobacter-13_12B_814
*KM382567-Marinobacter-17_1C_106
*KM383709-Marinobacter-23_2C_1306
*KM383727-Marinobacter-22_2C_1326
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Figure 2. Cell counts of bacterial isolates used to determine the rate of growth and decline. This was performed for bacteria grown in marine broth (0-48 h) and in vitamin deficient f/2 (48-72 h). Transfer to new media at 48 hr denoted by arrow. Error shown is standard error.

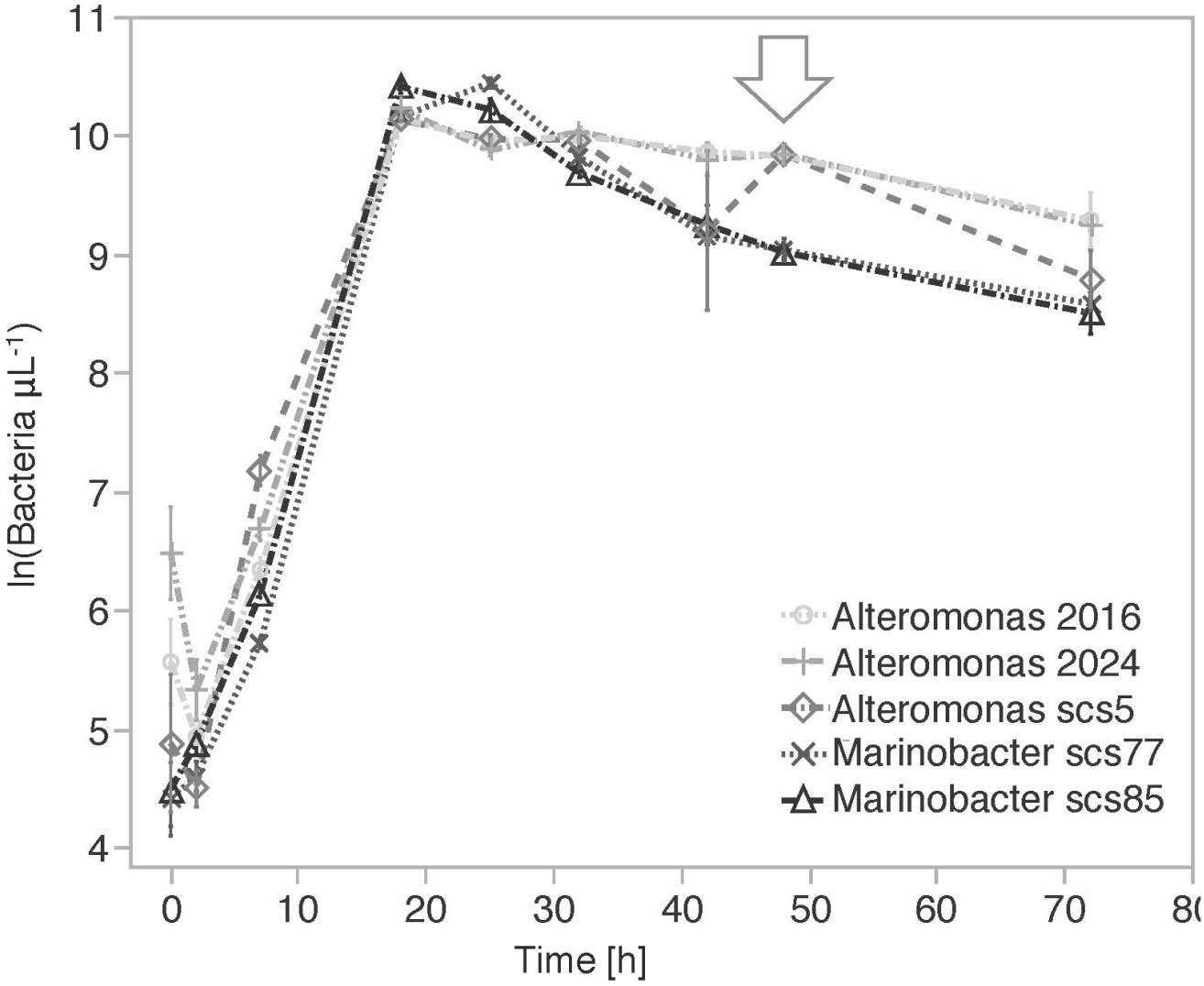
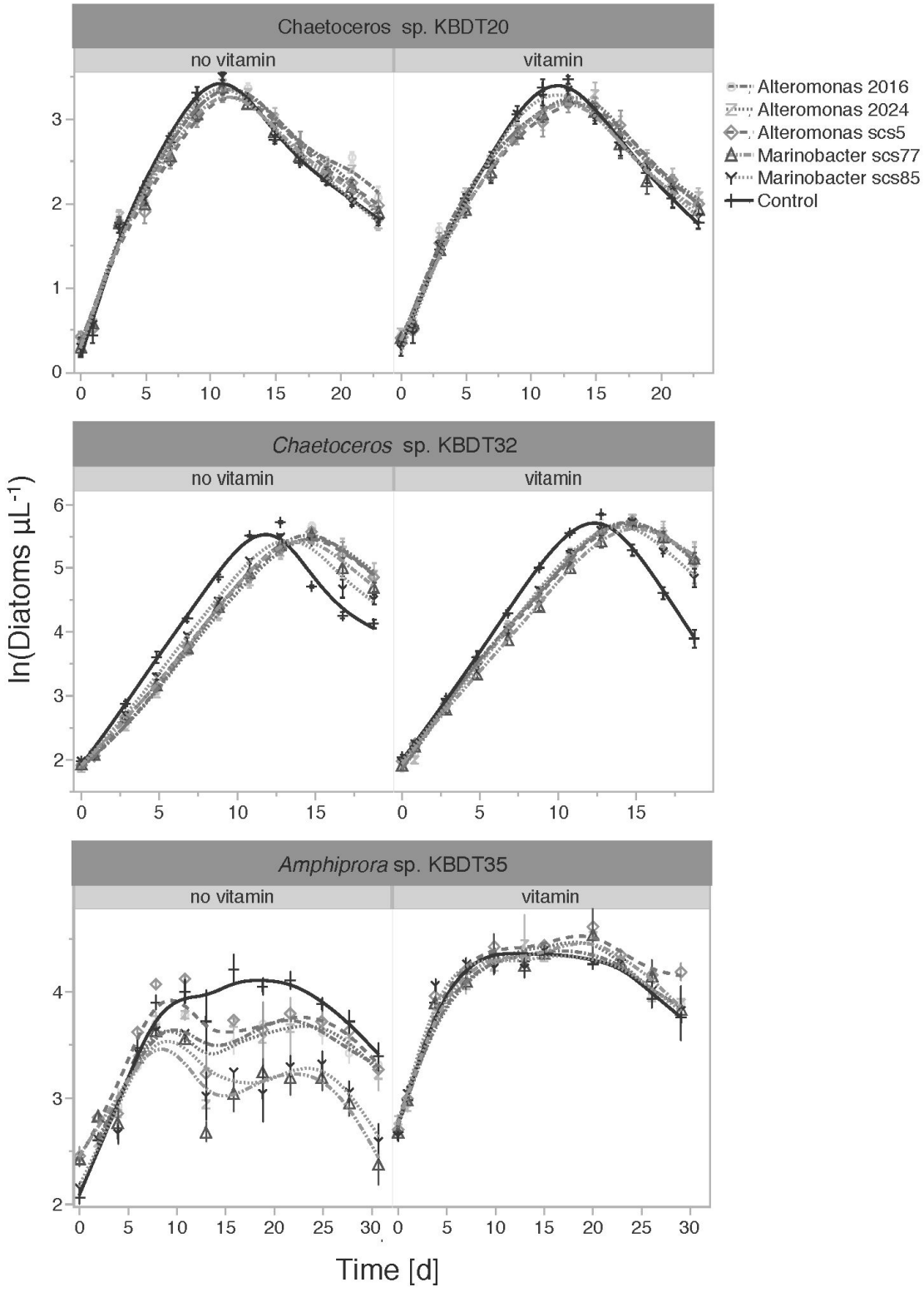


Figure 3. Growth of each diatom species in vitamin deficient f/2 (no vitamin) and vitamin replete f/2 (vitamin). Diatoms were growth in the presence of bacteria (dotted lines) or no inoculation (control, + and black solid line). Error is shown as standard error.



SUPPLEMENTARY MATERIALS

Growth of free-living bacteria in co-culture

The impact of host identity and vitamin concentrations on the carrying capacity of free-living bacteria was evaluated using two-way factorial ANOVA, employing R packages lsmeans and car. Similarly, for each host cell, the effects of vitamin concentrations and bacterial inoculation on the carrying capacity of free-living bacteria were evaluated using two-way factorial ANOVA. Differences were found to be significant if $p<0.05$.

Growth of free-living bacteria in some diatom co-cultures effected by host and vitamin concentrations

Because xenic diatom cultures contain both attached and free-living bacteria, some co-cultured bacteria were transferred when diatom cultures are diluted into new media. This high background of free-living bacteria in the diatom cultures, coupled with the error associated with our counting method, prevented an accurate count of the bacterial inoculum at the time of the first free-living bacterial count. The estimated concentration of free-living bacteria transferred with the diatom host at the start of the experiment was 79 ± 21 cells μL^{-1} transferred with *Chaetoceros* sp. KBDT20 cultures, 48 ± 12 cells μL^{-1} transferred with *Chaetoceros* sp. KBDT32 cultures, and 100 ± 29 cells μL^{-1} transferred with *Amphiprora* sp. KBDT35 cultures. Bacterial inoculum was diluted to add 50-100 bacteria per μL^{-1} to the xenic culture; however, the between-replicate variation in counts of free-living bacteria was similar to the concentration of the bacterial inoculum, precluding measurements of the initial bacterial inoculum (data not shown). Inoculations had no discernable effect on the maximum concentration of free-living bacteria in each culture, compared to negative controls ($p>0.05$).

For most cultures, the concentration of free-living bacteria increased as the host cell declined (Figure 3). The one exception to this trend is *Chaetoceros* sp. KBDT32 grown in vitamin-replete f/2 (Figure 3, center right panel); the concentration of free-living bacteria decreased as *Chaetoceros* sp. KBDT32 decreased. In all other

diatom cultures, the concentration of free-living bacteria increased during diatom decline at a rate of approximately 90-250 cells μL^{-1} day^{-1} (Figure 3, remaining panels).

The concentration of vitamins in the f/2 media affected the peak densities of free-living bacteria in *Chaetoceros* sp. KBDT32 (Figure 3, center panels) and *Amphiprora* sp. KBDT35 (Figure 3, bottom panels) cultures ($p<0.0001$), but not in *Chaetoceros* sp. KBDT20 (Figure 3, top panels)($p>0.05$). *Chaetoceros* sp. KBDT32 grown in vitamin-deficient f/2 contained 171% more free-living bacteria at carrying capacity than those grown in vitamin-replete f/2. *Amphiprora* sp. KBDT35 grown in vitamin-deficient f/2 contained 35% fewer free-living bacteria at carrying capacity than those grown in vitamin-replete f/2. The abundance of free-living bacteria at carrying capacity was significantly different among the three host diatoms ($p<0.0001$). *Chaetoceros* sp. KBDT20 supported the highest abundance of free-living bacteria ($2.86\pm0.06 \times 10^3$ cells μL^{-1}), followed by *Chaetoceros* sp. KBDT32 ($1.73 \pm 0.98 \times 10^3$ cells μL^{-1}), and the lowest abundance of free-living bacteria was hosted by *Amphiprora* sp. KBDT35 ($1.32\pm0.07 \times 10^3$ cells μL^{-1}).

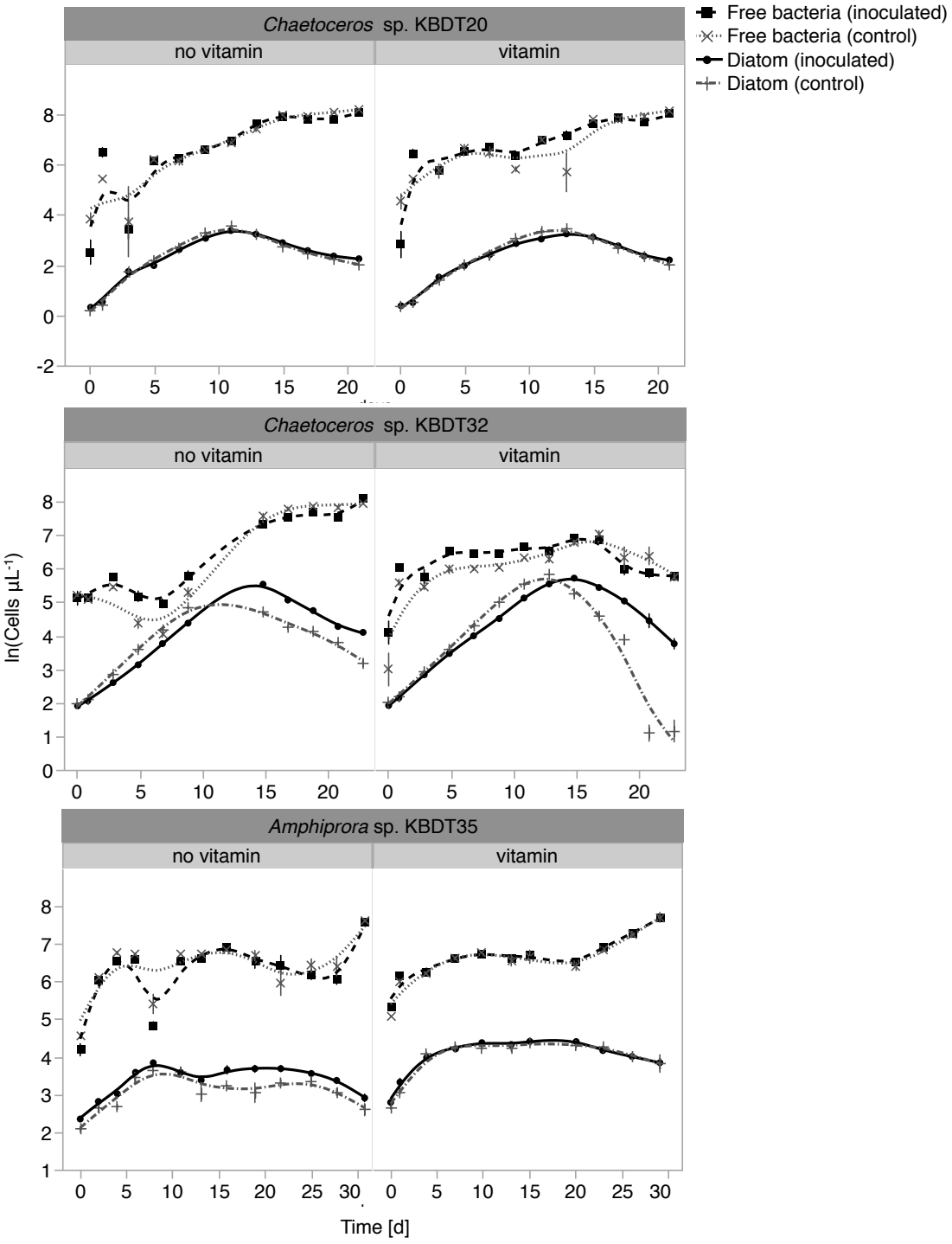
Vitamin concentrations in vitamin-replete and vitamin-deficient f/2

Experiments were performed in both vitamin-replete and vitamin-deficient f/2. The source of sterilized surface seawater was from Station ALOHA cruise HOT 277 (October 12-16, 2015). The average concentrations of macronutrients for the month of October are well below the average diatom half saturation constant (Ks) (the nutrient concentration at which uptake is ½ of the optimal rate) (Table 1). The average vitamin concentrations at Station ALOHA are unknown. The current detection level for vitamin B_{12} is 29 pM (Sañudo-Wilhelmy *et al.*, 2012). An unpublished report on the vitamin concentrations at Station ALOHA in Fall 2012 documents B_1 at <20pM, B7<5pM, and B_{12}<6pM (pers. comm. D. del Valle). The concentration of B_{12} at Station ALOHA may be near the average half saturation constant for diatoms (1.17±0.77 pM). However, B-vitamins are not heat stable. For example, vitamin B_{12} in serum is significantly degraded after autoclaving for 30 minutes (Orrell and Caswell, 1972), the minimum amount of time surface seawater was sterilized. Therefore, although diatoms could utilize

B-vitamins at the low concentrations present in the seawater used to prepare f/2, the residual B_{12} concentration following autoclaving would be much lower. In vitamin-replete f/2, vitamins are added after media is autoclaved and brought to room temperature; the final concentrations are approximately 2.96×10^5 pM vitamin B_1, 2.05×10^3 pM vitamin B_7, and 3.69×10^8 pM vitamin B_{12}.

SUPPLEMENTARY LIST OF FIGURES

Figure 1. Growth of free-living bacteria (square and x) and diatoms (circle and cross) in vitamin deficient f/2 (no vitamin) and vitamin replete f/2 (vitamin). The presence of bacterial inocula (black lines) and without bacterial inocula (grey lines) are also listed. Error is shown as standard error.



Chapter 4: Comparative functional metagenomic profiling of bacterial communities associated with single diatom cells from the oligotrophic open ocean.

Lydia J. Baker[1], Paul F. Kemp[1,2]

1. University of Hawaii Manoa, 2. University of Colorado Boulder

ABSTRACT

Microalgae, such as diatoms, are often found with attached bacteria that may be mutualistic, parasitic, or commensal; these interactions may result in enhanced host growth, prolonged blooms, or the production of algal toxins. Diatoms and their microbiome were collected from the oligotrophic open ocean at Station ALOHA (22° 45'N, 158° 00'W). A subset of genes previously implicated as being involved in diatom-bacterial symbiosis or bacteria-bacteria interactions were annotated as a part of diatom-attached bacterial metagenomes. With respect to general functional categories, diatom-associated bacterial metagenomes were significantly different relative to metagenomes for bacterioplankton and particle-associated bacteria from a similar environment (the North Pacific Subtropical Gyre). Additionally, individual functional genes were enriched in diatom-associated bacteria relative to the other metagenomes; these genes were associated with rapid bacterial growth, which may be beneficial in the carbon-rich environment of the host phycosphere. Comparison of the functional capacity of diverse vs. less-diverse diatom-attached bacterial assemblages revealed that many genes were significantly enriched in the less-diverse assemblage, and these were also associated with rapid bacterial growth. The absence of genes that are suggestive of a benefit or harm to the host suggests bacteria have a commensal relationship with their diatom host. These results serve as a starting point for further investigations of the functional basis of diatom-bacterial interactions, and more broadly, interactions between eukaryotic algae and bacteria in the oligotrophic open ocean.

INTRODUCTION

Diatoms are a vital part of the oceanic carbon cycle; diatoms perform nearly 40% of total marine primary productivity and 9-20% of total organic carbon export in oligotrophic systems (Nelson et al. 1995; Brzezinski et al. 2011). The productivity, health, and stability of diatoms can be profoundly affected by interactions with attached bacteria (Amin et al. 2012a). For example, diatom-bacterial interactions have been shown to increase host biomass and prolong bloom duration (Smith et al. 1995) or cause host-specific lysis (Paul and Pohnert 2011). However, individual diatom cells can host multiple bacteria (Baker and Kemp 2014), which may result in parasitic (Bidle and Azam 1999; Mayali and Azam 2004), mutualistic (Croft et al. 2005; Amin et al. 2009; Gärdes et al. 2012), and commensal (Rosowski 1992) bacteria occurring on the same host. It is also possible for a bacterial strain to have biphasic interactions with a host cell; bacteria may have mutualistic interactions during host growth and then shift to parasitic interactions during host decline (Seyedsayamdost et al. 2011; Wang et al. 2014). Attached bacteria have also been found to interact with one another (Baker et al. 2016) which may result in competitive (Barja et al. 1989) or complementary (Mori et al. 2016) bacteria-bacteria interactions. Such interactions may impact the host as well, for example by eliminating parasitic or cultivating mutualistic associates.

Several previously studied bacterial functional genes can be used as indictors of how bacteria interact with a host cell. Bacterial functional genes that may be indicative of parasitic interactions include those involved in host cell degradation (Cottrell et al. 2000; Bidle and Azam 2001; LeCleir et al. 2004; Xiao et al. 2005) or lysis (Li et al. 2016). Functional genes related to bacterial production of nutrients may be indicative of mutualistic interactions. For example, bacteria are thought to provide their host with B-vitamins (Croft et al. 2005), biologically available iron (Amin et al. 2009), and ammonium (Suleiman et al. 2016). As much of the ocean is B-vitamin, iron, and/or nitrogen limited (Sañudo-Wilhelmy et al. 2012; Moore et al. 2013) hosting bacteria capable of providing limited nutrients would greatly benefit a diatom host. Bacterial functional genes have also

been found to promote the growth of a diatom host through the production of indole-3-acetic acid (IAA) from both endogenous and host-derived tryptophan (Amin et al. 2015).

Commensal interactions are challenging to prove using bacterial functional genes because it is difficult to demonstrate that the attached bacteria are neither harming nor benefiting the host. For example, many studies have suggested bacteria utilize diatom exudates as a carbon source (Rosowski 1992; Gurung et al. 1999) see also a discussion in Amin et al. (Amin et al. 2012b). One might assume utilization of exudates is inconsequential to the host, but beneficial to bacteria. However, it may be detrimental to a diatom, as bacterial association with diatoms in culture has been found to enhance diatom stickiness, aggregation and self-shading, and ultimately leads to a loss of buoyancy (Kahl et al. 2008; Gärdes 2010; Bruckner et al. 2011). Whether diatoms grown in the natural environment (i.e. in conditions that are less concentrated and homogenous) would react similarly is unknown. Bacterial associations also have been shown to impact diatom host cells in positive ways that may be genetically based but have not been linked to specific genes. For example, attached bacteria have been found to prevent viral lysis of the host cell (Kimura and Tomaru 2014). Many studies have also recognized that diatom cultures grown without resident bacteria grow poorly or not at all (Windler et al. 2014; Sison-Mangus et al. 2014; Amin et al. 2015), with atypical frustule morphology, as well as slower and more unstable growth (Windler et al. 2014).

More broadly, diatom-associated bacteria may have functional attributes characteristic of a host-associated or surface-associated lifestyle, irrespective of interactions with the host. Particle-associated bacteria are thought to be copiotrophic, have higher rates of activity than bacterioplankton, and have a generally larger cell size (Alldredge et al. 1986; Grossart et al. 2003; Grossart and Tang 2007; Allen et al. 2013). In addition, relative to bacterioplankton, particle-associated metagenomes are enriched for functions involved in “social” interactions, including: biofilm formation, bacteria-bacteria communication, genetic exchange, and antibiotic resistance (Ganesh et al. 2014). Specific qualities of particle-associated bacteria may be a prerequisite to diatom

colonization, namely motility (Ivars-Martínez et al. 2008; Sonnenschein et al. 2012) and attachment (Kaczmarska et al. 2005). To the best of our knowledge, bacterial functional genes suggested to be indicators of parasitism or mutualism have not been shown to be required for colonization of diatoms.

Prior studies emphasizing the bulk properties of axenic, single-species diatom cultures and their interaction with a single bacterial strain have provided valuable insight into some functional aspects of diatom-bacterial interactions; including a better understanding of bacterial metabolism (Chróst 1991; Obernosterer and Herndl 1995) and the role of interspecies signaling molecules (Amin et al. 2015). However, bacteria often found associated with diatoms in culture (notably *Marinobacter* and *Alteromonas*) are typically copiotrophs, and may not be representative of the bacteria associated with diatoms in oligotrophic systems (Baker and Kemp 2014). Additionally, an emphasis on population-based culture studies may obscure interactions that take place at the scale of individual host cells, or in unmanipulated natural systems.

In the present study we characterize the functional capacity of bacteria associated with single diatom host cells isolated from the oligotrophic open ocean, relative to bacterioplankton and particle-associated bacteria from a similar environment (the North Pacific). The phylogenetic compositions of bacteria associated with particles (Delong et al. 1993; Ghiglione et al. 2009; Bryant et al. 2016) and diatoms (Grossart 1999; Grossart and Tang 2007) are distinct from that of bacterioplankton; we ask whether such phylogenetic differences are accompanied by differences in functional capacity. We also assess whether functional differences are consistent with previously identified beneficial or harmful effects of bacteria, and whether functional capacity differs between high and low diversity bacterial assemblages.

In a previous study of the 16S rDNA of bacteria attached to single algal host cells (Baker and Kemp 2014), host cells were clustered into three distinct groups based on the phylogenetic similarities between their bacterial assemblages. The bacterial associates in one group were dominated by α-, β-, and γ–Proteobacteria (Group 1),

while the bacterial genus *Arthrobacter* (phylum Actinobacteria) dominated another (Group 2); this suggested the existence of phylogenetically distinct assemblages of bacteria associated with diatoms (Baker and Kemp 2014). Differences in gene prevalence between high diversity assemblages (Group 1) and low diversity assemblages (Group 2) may reveal functional differences in host colonization and diatom-bacterial interactions.

Our data provides some of the first insights into the functional capacities of bacteria associated with a single diatom cell, as well as the nature of symbiotic interactions between attached bacteria and a diatom host. Previous studies have explored the phylogenetic composition of bacteria associated with phytoplankton populations in a natural environment (Schäfer et al. 2002; Kaczmarska et al. 2005; Amin et al. 2012b), and the phylogenetic composition of bacteria isolated from a single cell (Yoon et al. 2011; Martinez-Garcia et al. 2012). One previous study has explored the functional capacity of a single bacterium isolated from a *Thalassiosira* (Gärdes et al. 2010). To the best of our knowledge this will be the first study to compare the functional capacities of bacterial consortia associated with single cells, and bacterial consortia from the natural environment.

METHODS

Collecting diatom hosts and their bacterial associates

Samples were collected and processed as described previously (Baker and Kemp 2014). Briefly, samples were collected from the deep chlorophyll maximum of the oligotrophic waters at Station ALOHA (22° 45'N, 158° 00'W) using methods designed to retain diatom-associated bacteria. Preserved samples were sent to the Bigelow Laboratory Single Cell Genomics Center (SCGC) for fluorescently activated cell sorting (FACS) and subsequent whole-genome amplification by multiple displacement amplification (MDA). Following MDA, all FACS wells were tested with a real-time PCR screening for eukaryotic 18S rDNA. Of the 40 host cells isolated by FACS, 26 were *Thalassiosira* spp diatoms. Concurrently with the sample from which single cells were isolated, bulk samples of particle-associated bacteria (retained on 5 µm filter) and bacterioplankton (retained on 2 µm

filter) were collected. The compositions of the bacterial consortia were determined by 16S rDNA sequencing for each of the 40 single-cell isolates as well as the bulk samples. Host cells divided into three groups based on their bacteria consortia using principle coordinates analysis (PCoA) and environmental clustering analysis (ECA), with *Thalassiosira* found in all three groups (Baker and Kemp 2014). Group 1 contained the most diverse bacterial consortia as well as the bulk samples. Group 2 contained the least diverse bacterial consortia and was dominated almost entirely by phylotypes of the genus *Arthrobacter*. Three *Thalassiosira* host cells from each of Groups 1 and 2 were selected for further investigation of the functional capacity of their associated bacteria.

Metagenomic sequencing and bioinformatics

Indexed libraries derived from the six host cells and their bacterial associates were sequenced at the University of Georgia's Georgia Genomics Facility (GGF) using MiSeq PE300 (Illumina, San Diego, CA). With an estimated diatom genome size of 34 MBp, and bacterial genome sizes of 1-2 MBp (Raes et al. 2007) and 5-25 bacteria per cell (Armbrust et al. 2004; Baker and Kemp 2014), sequence coverage for the diatom and its associated microbiome was estimated at 150X coverage per library, as recommended by the University of Georgia's Quantitative Biology Consulting Group (Walt Lorenz, Lead Consultant, personal communication). GGF also utilized Trimmomatic (Bolger et al. 2014) to remove adapters and quality trim. Trimmed reads with lengths below a 50 base threshold were discarded. Unaligned sequences were uploaded to MG-RAST version 4.0 (Meyer et al. 2008) (mgs 540192, 540195, 540198, 540201, 540204, 540207).

Annotation of bacterial genes from diatom-associated bacteria and comparison metagenomes

Annotation of bacterial functional genes was performed using MG-RAST using the Kyoto Encyclopedia of Genes and Genomes (KEGG) (Ogata et al. 1999; Kanehisa et al. 2016). Annotation in KEGG is performed within a hierarchy; individual genes are placed within functional pathways, and those pathways are placed within general functional categories (Environmental Information Processing, Genetic Information Processing, Metabolism, Cellular Processes, Human Disease, and Organismal Systems). Functional genes within KEGG are

also assigned taxonomy. Because of the shared ancestry of cyanobacterial and chloroplast genes, cyanobacterial functional genes as annotated by KEGG were excluded from bacterial analysis in this study. Diatom functional genes annotated by KEGG were also excluded. Further analysis was thus limited to non-cyanobacterial, bacterial functional genes in an attempt to isolate host-associated bacterial functional capacity. This metagenome will be referred to as *Thalassiosira*-associated or the "T" sample set (individual metagenomes are designated T1-T6). For all samples annotated in MG-RAST, the parameters were set at an e-value of 5, percentage identity of 60%, minimum length of 15, and minimum abundance of 1.

Using the MG-RAST database, the prevalence of functional genes from *Thalassiosira*-associated samples was compared to metagenomes sequenced from samples collected in the oligotrophic Northern Pacific Ocean. The comparison data included bacterioplankton samples taken from the North Pacific Ocean as part of the Center for Microbial Oceanography: Research and Education cruise "Biological Oceanography of the Upper Ocean: Latitudinal Assessment" (C-MORE BULA), hereafter referred to as the North Pacific sample set ("N", four samples designated S_35162, S_35163, S_35171, S_35179, hereafter designated as N1-N4). The North Pacific samples were collected from the surface waters along a transect from the Pacific North Equatorial to the North Pacific Subtropical Gyre; samples were size fractionated at 5.0 and 0.2 µm, and sequenced using pyrosequencing. A second comparison data set (DeLong et al. 2006; Martinez et al. 2010) included bacterioplankton collected in the photic zone at Station ALOHA (sample set "A"; depths 10m, 70m, 130m, 200m designated as A1-A4); these samples were collected using size fractionation between a Whatman glass microfiber filter and a 0.2 µm filter, and sequenced using pyrosequencing. Data on bacterial functional genes (excluding cyanobacteria) were downloaded for N, A, and T data sets from MG-RAST; the parameters were set at an e-value of 5, percentage identity of 60%, minimum length of 15, and minimum abundance of 1. The resulting hits per gene were normalized by dividing by the total number of hits to the KO functional database for each sample. Samples were also compared to microbial communities present on plastic debris (Plastic or "P") as well as bacterioplankton collected from the surface waters by the Hawaii Ocean Time-series program

(HOT or "H"). The P and H sample sets were compared in a previous study (Bryant et al. 2016). P metagenomes included both *Bryozoa* as well as bacterial functional sequences; therefore, the attached bacteria from this study may have been attached to plastic, or to a biological surface such as *Bryozoa*. Both P and H samples were limited to bacterial DNA based on GC content, and annotated using KEGG. Hits per gene were normalized to the total functional genes hits for each sample and were compared to our *Thalassiosira*-associated, non-cyanobacterial, bacterial functional genes annotated in KEGG and downloaded through MG-RAST. The normalized hits per gene will hereafter be referred to as the percentage of annotated reads. The sequence depth for each sample set was evaluated by comparing the average total number of functional gene hits per sample set metagenome to the average for the *Thalassiosira*-associated sample set. The relative sequence effort of genes used in the analysis of non-cyanobacterial bacterial functional genes was similarly evaluated by comparing the total number of non-cyanobacterial functional genes per sample. This analysis was repeated to compare sequence depth and sequence effort of genes used in analysis for Group 1 and Group 2.

Analysis of bacterial functional genes

Subsequent analysis of differences between the functional categories and individual genes within sample sets were limited to those that were present in the *Thalassiosira*-associated sample set. Differences between diatom-associated (T) and comparison bacterial metagenomes (P, N, H, and A) were investigated using multivariate analysis (PERMANOVA) to explore differences in individual KEGG functional categories, and univariate analysis (general linear modeling (GLM)) to investigate the differences in the percentage annotated reads of individual genes.

Differences in the general functional capacity of sample sets were evaluated for individual KEGG general functional categories, including: Environmental Information Processing, Genetic Information Processing, and Metabolism. Differences between categories were evaluated at the KEGG hierarchical level of pathway, by summing the percentage of annotated reads for genes within that pathway for each category. Differences

between samples sets were evaluated using PERMANOVA in the R package vegan (Oksanen et al. 2010), which evaluates whether variation is attributable to the sample condition or to uncontrolled variance. This analysis compared (1) *Thalassiosira*-associated bacterial samples (T) to each of the other sample sets individually (P, H, N, and A); (2) all bacterioplankton samples (H, N, A) to all particle-associated samples (P, T); (3) *Thalassiosira*-associated bacterial samples (T) to all other samples concurrently (P, H, N, A); and (4) samples from Group 1 to Group 2. Sample groups were considered significantly different if $p<0.05$; the total variation explained by differences between sample sets (R^2) and the number of KEGG pathways within each category (n) are given for each test.

This analysis also investigated differences in individual diatom-associated bacterial functional genes relative to the other sample sets evaluated. Functional genes that were significantly enriched in the *Thalassiosira*-associated bacterial samples relative to other sample sets are potentially related to some aspect of bacterial symbiosis with the host cell. Conversely, those that are underrepresented in *Thalassiosira*-associated libraries relative to the other sample sets may indicate genes that are not necessary for bacterial symbiosis with a diatom host, or that are specifically associated with properties of the comparison samples, e.g. particle attachment or free-living lifestyles. The relative proportions of functional genes in Groups 1 and 2 were also compared to test for significant differences between these groups; this analysis was limited to genes that were greater than 0.0001% of the annotated reads in more than one sample. Samples were considered significantly different if $p<0.05$ for a negative binomial GLM; the false discovery rate was controlled by first adjusting p-values as in Benjamini & Hochberg (Benjamini and Hochberg 1995) so that less than 5% of significant tests are likely to be incorrectly identified. Using a GLM, the random component is the number of gene hits per sample and the systematic component is the origin of the sample (i.e. T, P, N, H, A; or Group1, Group 2), offset by the log of the sequence effort. For each gene, the average percent of annotated reads for each sample set was compared to the *Thalassiosira*-associated bacterial sample sets; genes that have a greater average percent of annotated reads in the *Thalassiosira*-associated bacterial sample sets were considered enriched and those with

a lower average percent of annotated reads were considered underrepresented. A similar approach was used to compare gene enrichment or depletion in Group 1 vs Group 2, within the *Thalassiosira* samples. Genes that are significantly enriched or underrepresented will be discussed in the context of their position in a KEGG pathway.

RESULTS

Sequence quality and effort of bacterial metagenome data sets

Submission of raw sequences to MG-RAST resulted in removal of the majority of the sequences from further analysis (60-80%) due to identification as artificial duplicate reads by MG-RAST. However, all samples had at least $6x10^5$ sequences remaining for processing, with an average of $14 \pm 9 x10^5$ sequences per sample. On average, the number of usable, post quality control sequences in the *Thalassiosira*-associated data was 10-200X greater than the comparison data sets also processed through MG-RAST (North Pacific and ALOHA). MG-RAST quality control resulted in a decrease in mean sequence length for all *Thalassiosira*-associated samples except T5, and an increase in the GC percent for all *Thalassiosira*-associated samples except T3 and T6. The number of sequences and sequence length in the *Thalassiosira* samples were more comparable to the North Pacific sample set, even though both ALOHA and North Pacific sample sets were generated using pyrosequencing, which has greatly reduced sequence yield relative to Illumina methods. Although pyrosequencing produces longer reads and fewer reads per run, previous studies comparing the technology suggest that the relative abundance of genes found using the two methods are comparable (Luo et al. 2012)

Although a single diatom cell and its microbial consortium provided the starting material for each of the six *Thalassiosira*-associated metagenomes, the amplification and sequencing results varied greatly. Based on the expected size of the *Thalassiosira* genome (34 Mb) and marine bacteria (1-2 Mb), and assuming a minimum of one bacterium per phylotype previously documented (Baker and Kemp 2014), bacterial DNA was estimated to make up a minimum of 6% of the total DNA present, and therefore of the total resulting sequences. For all

samples, the percentage of sequence that estimated by MG-RAST to be bacterial in origin was higher than the minimum expected (Table 2: comparing Est % Bac DNA to % Bac DNA).

The sequencing depth of the samples used for comparison varied greatly. Using the average number of functional gene hits for each metagenome as a metric of sequence effort, the HOT and Plastics sample sets had greater effort than the *Thalassiosira*-associated bacterial samples by an average of 4X and 10X, respectively. However, the North Pacific and ALOHA sample sets had 4X and 40X less effort, respectively. If the metric for sequence effort is limited to the total gene hits of the non-cyanobacterial bacterial genes used in the GLMs, then HOT and Plastic samples only have 3X and 4X greater effort, and North Pacific and ALOHA samples have 6X and 60X less effort, relative to the *Thalassiosira*-associated bacterial metagenome (data not shown). Although these datasets are representative of larger communities and are not intended to be a full representation of the functional capacity of the bacteria present in this system, they have comparable sequence effort to single-cell *Thalassiosira* isolates.

The sequence efforts for Group 1 and Group 2 are nearly equal. However, Group 1 has nearly 3X greater sequence effort of non-cyanobacterial bacterial functional genes than Group 2. This may indicate not only that a more diverse consortium of bacteria was present in Group 1, but that a greater total number of non-cyanobacterial cells were present.

Functional categories are distinct in Thalassiosira-associated metagenomes

An evaluation of the variance in composition of bacterial functional pathways within each of the general KEGG Ortholog categories (Genetic Information Processing, Metabolism, and Environmental Information Processing) indicated that the *Thalassiosira*-associated bacterial sample set was significantly different from all other sample sets, whether sample sets are evaluated individually or concurrently (Table 3). When particle-associated libraries (P, T) were compared to bacterioplankton sample sets (H, N, A), the general functions for particle-

associated were significantly different from the bacterioplankton. Finally, although previous analysis using 16S rDNA revealed phylogenetic differences between Group 1 and Group 2, none of the KEGG categories evaluated for Group 1 and Group 2 demonstrated a significant difference between the two groups. This suggests that Group 1 and 2 have similar capacities for Genetic Information Processing, Metabolic, and Environmental Information Processing.

Bacterial genes enriched in Thalassiosira-associated metagenomes are involved in metabolism and DNA repair

Most genes in the *Thalassiosira*-associated bacterial metagenomes were present in at least one metagenome used for comparison (P, H, N, or A). The exceptions were functional genes for a D-alanine transfer protein that is part of a *Staphylococcus aureus* infection pathway (ko05150; dltD), and pseudouridylate synthase that is part of a pyrimidine metabolism pathway (ko00240) (Supplementary Table 1). However, pseudouridylate synthase is a likely candidate for horizontal gene transfer from an endosymbiosis event, which would explained its absence from the free-bacterial samples and presence in the *Thalassiosira*-associated bacteria in sample T6 (Ball et al. 2013).

Relative to all other sample sets evaluated, three ribosomal genes (ko03010;RP-S13, -S19, and -S8), as well as genes involved in oxidative phosphorylation (ko00190; *nuoACGHKM*), purine metabolism (ko00230; *nrdAE*, *nrdBF*), and galactose metabolism (ko00052; *lacZ*) were significantly enriched in the *Thalassiosira*-associated bacterial samples (Table 4), and were found in all other sample sets. Because these genes are significantly enriched in most of the *Thalassiosira*-associated samples relative to all other sample sets, it is likely that they are vital to establishing and/or maintaining bacterial associations with diatoms.

Genes that were significantly enriched in *Thalassiosira*-associated samples relative to those found on particles include the oxidative phosphorylation gene *atpD*, and the purine metabolism gene *purCD*. Genes that were significantly enriched in *Thalassiosira*-associated samples relative to bacterioplankton samples include part of a

nucleotide base excision repair pathway (ko03410; *nei*); genes involved with metabolism, such as alanine, aspartate, and glutamate metabolism (ko00250; *ansAB*, *glsA*), and pyrimidine metabolism (ko00240; *pyrD*); genes involved in biosynthesis of phenylalanine, tyrosine and tryptophan (00400; *trpEG*, *trpF*); and siderophore nonribosomal peptide biosynthesis genes (ko01053; *dhbF*) (Table 4).

Cell signaling, translation, and metabolism genes differ between phylogenetically distinct Thalassiosira *groups*

There were significant differences in the relative proportion of individual functional genes between Group 1 and Group 2; however, all enriched functional genes were enriched in Group 2 relative to Group 1. These included genes that are part of cellular transport (ko04146; ACSL, *fadD*) and membrane transport (ko03070; *secY*), as well as genes involved in genetic information processing (ko03020 and ko03010), and metabolic pathways (ko00250, ko00010), including oxidative phosphorylation (ko00190; *coxA*, *nuoJ*, *nuoL*). The *tuf* gene, part of a plant-pathogen interaction pathway (ko04626), was also enriched.

Thalassiosira-associated bacterial functional genes are most often significantly underrepresented relative to other sample sets

In the *Thalassiosira*-associated bacterial sample set, only about 5% of bacterial functional genes were enriched, while 54% were underrepresented (having lower average percent of annotated reads) relative to all other samples tested. Functional genes that were underrepresented were involved in all 6 general KEGG functional categories (Supplementary Table 2). For example, for numerous carbohydrate metabolism pathways, all observed genes are underrepresented in the *Thalassiosira*-associated bacterial sample set relative to all other sample sets evaluated. This included genes involved in glycolysis (ko00010), citrate cycle (ko00020), the pentose phosphate pathway (ko00030), and ascorbate and aldarate metabolism (ko00053). The only KEGG pathway that had nearly as many genes enriched as were underrepresented is oxidative phosphorylation (ko00190); 9 genes were enriched and 9 genes were underrepresented, relative to other sample sets.

Genes implicated in symbiosis found in Thalassiosira-associated bacterial samples

Bacterial genes previously reported from bacteria-diatom associations include genes related to initiating contact, providing nutrients, conveying resistance to host defenses, or involved in production of antibiotics (Supplementary Table 3). Initiating contact with a host cell may involve motility (*flgD*) and host signaling (K08303). Quorum sensing requires the transport of bacterial infochemicals, which can be performed using bacterial secretion systems (ko03070), ABC transporters (ko02010), and/or a two-component signal transduction system (ko02020). Only one gene involved in the signal transduction, ubiquinol-cytochrome c reductase cytochrome b subunit (ko02020*;* CYTB, *petB*), was found in all samples and was significantly enriched in *Thalassiosira*-associated bacterial sample sets relative to particle-associated (P) and bacterioplankton (H and N) sample sets. All *Thalassiosir*a-associated samples except T3 were observed to have genes involved in environmental information processing using bacterial secretion systems (ko03070). Potential biofilm-related genes (*rpoS*, *thiG*, and vibriolysin) were observed primarily in Group 2 samples; only one sample from Group 1 (T3) was observed to have a potential biofilm-related gene (*thiG*). Once attached to a cell, a bacterial cell may have to resist host cell defenses (*dltD*, *ptrB*, vibriolysin), or the defenses of other bacteria (*mdtB*, and dimethylaniline monooxygenase). Only one of these genes (vibriolysin) was enriched in our *Thalassiosira*-associated samples relative to the particle-associated and bacterioplankton sample sets. Once associated with a host cell, bacteria may provide the diatom with macronutrients (nitrogen) or and micronutrients (e.g. B-vitamins). Genes that would provide limiting nutrients to the host were only observed in some *Thalassiosira*-associated bacterial samples, which is surprising as they originated from an oligotrophic system in which diatoms may be more reliant on their bacterial associates (Baker and Kemp 2014).

DISCUSSION

To our knowledge, this is the first evaluation of the potential functional capacities of bacteria associated with single diatom cells isolated from a field sample. It is also the first study of the functional capacity of the bacterial consortia associated with diatoms collected in the oligotrophic open ocean. These two qualities may explain why the *Thalassiosira*-associated bacteria in this study differed from the taxa commonly reported as diatom associates (Amin et al. 2012a; Baker and Kemp 2014). Regardless of phylogenetic differences, a *priori*, we expected to find bacterial functional genes indicative of diatom-bacterial symbiosis in our sample that were similar to genes observed in previous whole-community studies of symbiosis in other environments. Moreover, we expected these functional genes would be enriched in diatom-associated bacteria relative to bacterioplankton. However, we did not observe many of the bacterial functional genes previously suggested as indicators of parasitism or mutualism, and those we observed were not found in all samples. Genes involved in a surface-attached lifestyle, such as motility, forming attachment structures, and bacterial competition, were significantly enriched in at least some *Thalassiosira*-associated bacterial samples although they were not found in all samples.

The general functional capacities of *Thalassiosira*-associated bacteria were found to be significantly different relative to both bacterioplankton and particle-associated bacterial sample sets, despite that the comparison samples were obtained from similar environments. Relative to the comparison samples, *Thalassiosira*-associated bacterial metagenomes have a more narrow capacity for carbohydrate metabolism, as well as amino acid and nucleotide metabolism. Additionally, PERMANOVA analysis showed that the general functions represented in the particle-associated sample sets were significantly different from free-living sample sets. Although particle-associated and free-living bacteria have been found to be taxonomically distinct (Delong et al. 1993; Ganesh et al. 2014; Rieck et al. 2015), these differences may not lead to significant differences in function if different taxa are functionally redundant. For example, previous studies of metabolism of specific carbohydrate species have suggested that there are no significant differences between particle-associated and

bacterioplankton species (Worm et al. 2001; D'Ambrosio et al. 2014), despite their taxonomic differences. However, some studies suggest that functional redundancy is lower than previously posited (Fuhrman et al. 2006; Leflaive et al. 2008). This is supported by the present study, as we found the broader functional capacities of particle-associated bacteria are distinct from those of bacterioplankton.

Functional genes that were enriched in *Thalassiosira*-associated bacterial samples relative to the other sample sets suggest that the attached bacteria were adapted to rapid consumption of diatom exudates and enhanced growth rate. *Thalassiosira*-associated bacterial samples were significantly enriched in multiple oxidative phosphorylation genes; increased activity in this pathway has been observed in response to an increase in photosynthetic activity (Aylward et al. 2015; Viviani and Church 2017). Surface-attached bacteria have also been found to have higher metabolic activity (Unanue et al. 1998; Grossart and Tang 2007). Diatom-attached bacteria may also be capable of an increased growth rates relative to the other sample metagenomes evaluated; this was indicated by the enrichment of genes related to purine metabolism and the production of ribosomes (Maitra and Dill 2015). Genes in this pathway perform DNA synthesis and repair and were observed in all samples evaluated and were enriched in the *Thalassiosira*-associated metagenomes.

A number of bacterial functional genes that were expected to be present and possibly enriched in our *Thalassiosira*-associated bacterial samples were not found. These include genes that would complete the flagellar assembly pathway (ko02040), which would confirm motility, and the porphyrin pathway (ko00860), which would confirm vitamin B_{12} production. These functions were hypothesized to be essential to establishing and maintaining symbiosis, respectively (Croft et al. 2005; Sonnenschein et al. 2012). One possible hypothesis is that that the bacterial microbiomes of individual diatom cells are not self-contained, fully functional communities. Mutualistic or parasitic interactions might still occur through the collective properties of hosts and bacteria on larger scales. Alternatively, bacterial functions that were previously reported as suggestive of symbiotic interactions may have been associated with temporary residents or loosely associated bacteria,

neither of which would have been captured in this study. Indeed, very little information is available regarding such temporary associations (but cf. Smriga et al. 2016).

Although we found no direct evidence that the diatom-associated bacteria in this study are capable of B_{12} production, *Thalassiosira*-associated bacteria and the *Thalassiosira* host cell are likely influenced by vitamin B_{12} concentrations. The bacterial growth genes *nrdA* and *nrdB* are regulated by the coenzyme vitamin B_{12} (Borovok et al. 2006; Bankevich et al. 2012). In addition, previous research suggests *Thalassiosira* require external sources of vitamin B_{12} (Armbrust et al. 2004; Grant et al. 2014; Durham et al. 2015). Because both the host and the bacteria are affected by the concentration of vitamin B_{12}, we can speculate that the pathway to produce this molecule was present but was not fully captured, or that B_{12} was provided by an external and/or ephemerally present bacterial source.

Individual diatom-associated bacterial metagenomes were enriched for genes that would suggest parasitism or mutualism for a subset of interactions; however, full pathways were not discovered. Enrichment for galactosidase (T5) and *dltD* (T4) would indicate parasitism. Galactosidase activity has been previously documented in the agrolytic bacteria *Pseudoalteromonas agraivorans* (Romanenko et al. 2003); however, it may also play a role in bacteria attachment (Delille and Razouls 1994). In bacteria the *dlt* operon may play a role in protecting against host defense peptides, such as defensins and protegins (Peschel et al. 1999) and may be indicative of a pathogenic or commensal interaction with the host cell. In addition, genes involved in riboflavin (T6) and tryptophan production (T5 and T6) suggest mutualistic interactions. Riboflavin is a co-factor for multiple flavoprotein-mediated redox reactions (De Colibus and Mattevi 2006), may be a factor in quorum sensing (Rajamani et al. 2008), and may act as a precursor to bioavailable cobalamin (vitamin B_{12}) (Helliwell et al. 2016). The presence of tryptophan is less indicative of mutualism, as tryptophan and its derivatives are common signaling molecules for both diatoms and bacteria (Amin et al. 2015); this suggests increased bacteria-to-bacteria or possibly bacteria-to-host communication. Interestingly, the infochemical idole-3-acidic acid (IAA)

is a tryptophan derivative and has been previously found to increase the growth rate of a diatom host (Amin et al. 2015). However, the genes involved in in IAA production are absent from the MG-RAST KEGG Ortholog database, preventing us from assessing whether this pathway was present in our samples.

Not all significantly enriched genes were suggestive of symbiotic interactions. Other genes that were only enriched relative to bacterioplankton samples include *nei* (base excision repair ko03410), *anasB* and *glsA* (alanine, asparatate, and glutamate metabolism ko00250), *dhbF* (biosynthesis of nonribosomal peptides), *pyrD* (purine metabolism ko00230). Future studies may discover a role for these genes in the context of host-associated bacteria.

Although the general functional pathways (Genetic Information Processing, Metabolism, and Environmental Information Processing) were not significantly different between Groups 1 and 2, individual genes were enriched in the less phylogenetically diverse Group 2 relative to the more diverse Group 1. Genes in the RNA polymerase pathway (ko03020), ribosomal genes (ko03010), and metabolism (ko00250; ko00010; ko00190) were enriched by a factor of greater than 50. This may be indicative of greater growth and metabolic capacities of bacteria associated with Group 2 relative to Group 1. The *tuf* gene was also enriched in Group2; *tuf* is involved with protein folding and renaturation in response to cellular stressors (Caldas et al. 1998), as well as being an infochemical sensed by higher eukaryotes as part of pathogen-associated molecular patterns (PAMPs) (Zipfel et al. 2006). If diatoms can sense the *tuf* product, it did not preclude colonization of the host cells in our samples. Enrichment of these genes suggest Group 2 may be better at colonizing and utilizing diatom exudates; whether these functional capacitates constitutes a competitive advantage requires further study of these genes and their role in diatom-bacterial associations.

In the absence of a clear benefit to the host diatom (e.g. evidence for bacterial B_{12} production), our data support a conclusion that *Thalassiosira*-associated bacteria are acting as commensals, utilizing diatoms as a

source of organic nutrients without providing a similar resource to the host cell. Individual cells were enriched for single functional genes that could indicate parasitism or mutualism; however, these same genes have multiple functions and may have no effect on the host cell. Bacterial attachment may have consequences to the host that are not readily traced back to bacterial genes. For example, bacterial presence has been found to deter viral infection, maintain frustule morphology, and stabilize growth (Kimura and Tomaru 2014; Windler et al. 2014); these effects undoubtedly have a genetic basis, but may not be traceable to specific indicator genes..

The *Thalassiosira*-associated bacteria in our samples are functionally distinct from free-living and particle-associated bacteria from the oligotrophic open ocean, even if these functions have not been considered indicative of symbiotic interactions. A total of 30 functional genes from 16 different KEGG pathways were significantly enriched in our samples relative to the comparison sample sets. An additional 34 genes from 28 different KEGG pathways were found in our samples but not found in one or more of the comparison sample sets; this included two genes that were not present in any of the comparison sample sets. Moreover, this analysis identified 16 individual functional genes that differed when comparing a depauperate versus a diverse bacterial consortium, both capable of colonizing diatom cells. Although the functions of the variable genes found throughout this analysis are multifarious, their prevalence in this analysis argues their importance in further investigations of diatom-bacterial interactions in the natural environment, especially the oligotrophic open ocean.

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LIST OF TABLES

Table 1. Quality control statistics taken for each sample downloaded from MGRAST, with the sample names used in this analysis (Sample) and in Baker and Kemp (2014) (Previous sample name). Columns marked "Upload" are the parameter before quality control, and columns marked Post QC are after MG-RAST quality control was performed. Base pair (bp) and percent (%) units are indicated.

Sample	Previous sample name	Upload: Sequences Count (10^4)	Artificial Duplicate Reads (%)	Post QC: Sequences Count (10^4)	Upload: Mean GC percent (%)	Post QC: Mean GC percent (%)	Mean Sequence Length (bp)	Mean Sequence Length (bp)
T1	e01	777	0.88	57	26 ± 6	29 ± 10	299 ± 13	213 ± 91
T2	j19	1025	0.83	123	31 ± 11	40 ± 13	298 ± 15	222 ± 84
T3	o16	889	0.87	62	33 ± 7	33 ± 8	299 ± 13	222 ± 90
T4	co5	807	0.83	95	30 ± 9	37 ± 12	299 ± 12	228 ± 84
T5	c17	828	0.64	215	47 ± 11	47 ± 11	299 ± 12	229 ± 81
T6	g13	1019	0.61	293	48 ± 9	48 ± 9	299 ± 13	227 ± 80
A1	ALOHA 10m	0.78	0.00	0.78	49 ± 10	49 ± 10	955 ± 151	955 ± 151
A2	ALOHA 70m	1.10	0.00	1.10	49 ± 9	49 ± 9	985 ± 211	985 ± 211
A3	ALOHA 130m	0.68	0.00	0.68	53 ± 12	53 ± 12	896 ± 216	896 ± 216
A4	ALOHA 200m	0.83	0.00	0.83	51 ± 10	51 ± 10	946 ± 150	946 ± 150
N1	S_35162	7.5	0.18	5.8	53 ± 13	54 ± 12	212 ± 64	223 ± 54
N2	S_35163	28.3	0.09	23.5	49 ± 14	50 ± 14	228 ± 60	242 ± 41
N3	S_35171	6.1	0.06	5.3	43 ± 13	43 ± 13	235 ± 57	247 ± 41
N4	S_35179	22.7	0.07	19.3	44 ± 14	45 ± 14	229 ± 60	242 ± 42

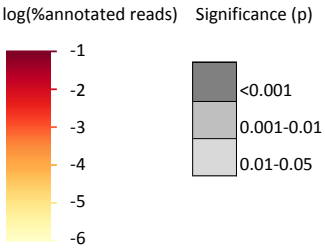
Table 2. The 16S rDNA phylotypes of the *Thalassiosira*-associated bacteria. Phylotypes in parentheses are not verified to be from marine origins. The Diatom column gives the closest species based on 18S rDNA identity. The estimated percentage of total sequences identified as bacterial, archaeal, or eukaryotic, as well as the % of non-cyanobacteria bacterial (%no Cy DNA) and % of cyanobacterial genes (%Cy DNA) are shown for all samples from the MGRAST KEGG database. The average and standard deviation for ALOHA and North Pacific samples is also given.

Sample	Bacteria	Diatom	Group	Est. % Bac DNA	% Bac DNA	% Euk DNA	%Arc DNA	%No Cy DNA	%Cy DNA
T1	*Kocuria, Sphingomonas, Brachybacterium, (Propionibacterium)*	*antarctica*	1	11.8	66.3	33.6	0.1	52.1	14.3
T2	*Bosea, Phyllobacterium, Synergistacea (Streptococcus)*	*punctigera*	1	11.8	32.5	67.4	0.0	32.1	0.4
T3	*Bradyrhizobium, Caulobacteraceae (2), Delftia, (Propionibacterium)*	*antarctica*	1	14.7	85.3	9.6	5.1	84.3	1.0
T4	*Arthrobacter (4) (Lactococcus)*	*punctigera*	2	14.7	30.5	69.4	0.1	19.5	10.9
T5	*Arthrobacter(4)*	*punctigera*	2	11.8	45.2	54.7	0.1	14.5	33.2
T6	*Arthrobacter, Perebacter*	*punctigera*	2	6	10.4	89.2	0.3	7.3	3.1
ALOHA	--	--	--	--	90.0±1.8	4.4±0.5	5.7±1.7	83.0±2.0	6.9±2.6
North Pacific	--	--	--	--	80.5±18.6	18.0±18.8	1.5±0.4	64.3±10.2	16.2±9.5

Table 3. The R^2 and p-values for the evaluation of variance in KEGG functional pathways from most genereal catagories in KO.(Genetic Information Processing, Metabolism, and Environmental Information Processing) using PERMANOVA. The n is the average and standard deviation for the number of KEGG pathways evaluated for each sample set within the KEGG functional catagory. *Thalassiosira*-associated samples were compared to Plastic, HOT, North Pacific, and ALOHA sample sets. Other comparisons shown include: Free-living vs. particle-associated samples (Free-living); *Thalassiosira*-associated samples vs. all other samples (All); and group 1 vs. 2 samples (Group 1 vs. 2).

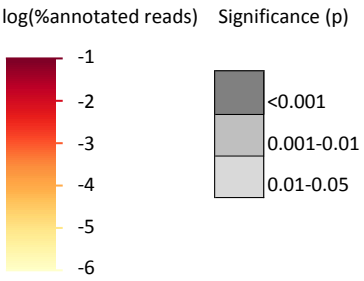
Sample set	**Genetic Information Processing**			**Metabolism**			**Environmental Information Processing**		
	n	R^2	p	n	R^2	p	n	R^2	p
Plastic	11 ± 0	0.60	0.001*	52 ± 1	0.43	0.001*	6 ± 0	0.52	0.001*
HOT	11 ± 0	0.58	0.001*	50 ± 1	0.43	0.001*	6 ± 0	0.55	0.001*
North Pacific	9 ± 2	0.39	0.018*	45 ± 4	0.27	0.009*	6 ± 0	0.36	0.02*
ALOHA	8 ± 1	0.36	0.018*	40 ± 2	0.30	0.008*	3 ± 2	0.35	0.015*
All	10 ± 1	0.50	0.001*	49 ± 4	0.35	0.001*	6 ± 0	0.48	0.001*
Free-living	(F) 10 ± 1 (P) 9 ± 3	0.14	0.001*	(F) 47 ± 4 (P) 41 ± 16	0.13	0.001*	(F) 6 ± 0 (P) 5 ± 2	0.14	0.001*
Group 1 vs. 2	(1) 4 ± 3 (2) 7 ± 3	0.26	0.3	(1) 12 ± 9 (2) 30 ±12	0.08	1	(1) 3 ± 2 (2) 3 ± 2	0.17	0.7
Thalassiosira	6 ± 3	--	--	21 ± 13	--	--	6 ± 3	--	--
Total	11	--	--	54	--	--	6	--	--

Table 4. Gene counts normalized to sequence effort are listed in the heatmaps below, which show bacterial genes in each of the *Thlassiosira*-associated bacterial samples (T1-T6), as well as average values for the *Thlassiosira* samples (AveT) and for other samples tested (P = Plastics, H= HOT, N = North Pacific, and A = ALOHA). Only samples that are significantly enriched in T samples relative to others are listed; the significance level is indicated in columns T>P, T>H, T>N, T>A.



Pathway	Function	T1	T2	T3	T4	T5	T6	Ave T	Ave P	Ave H	Ave N	Ave A	T>P	T>H	T>N	T>A
Flagellar assembly (ko02040)	flgD; flagellar basal-body rod modification protein FlgD															
Peroxisome (ko04146)	ACSL, fadD; long-chain acyl-CoA synthetase [EC:6.2.1.3]															
Two-component system (ko02020)	CYTB, petB; ubiquinol-cytochrome c reductase cytochrome b subunit															
Base excision repair (ko03410)	nei; endonuclease VIII [EC:3.2.2.- 4.2.99.18]															
RNA polymerase (ko03020)	rpoB; DNA-directed RNA polymerase subunit beta [EC:2.7.7.6]															
Ribosome (ko03010)	RP-S13, rpsM; small subunit ribosomal protein S13															
	RP-S19, rpsS; small subunit ribosomal protein S19															
	RP-S8, rpsH; small subunit ribosomal protein S8															
Vibrio cholerae infection (ko05110)	E3.4.24.25; vibriolysin [EC:3.4.24.25]															
Alanine, aspartate and glutamate metabolism (ko00250)	E3.5.1.1, ansA, ansB; L-asparaginase [EC:3.5.1.1]															
	glsA, GLS; glutaminase [EC:3.5.1.2]															
Phenylalanine, tyrosine and tryptophan biosynthesis (ko00400)	trpEG; anthranilate synthase [EC:4.1.3.27]															
	trpF; phosphoribosylanthranilate isomerase [EC:5.3.1.24]															
Galactose metabolism (ko00052)	lacZ; beta-galactosidase [EC:3.2.1.23]															
Oxidative phosphorylation (ko00190)	ATPF1B, atpD; F-type H+-transporting ATPase subunit beta [EC:3.6.3.14]															
	nuoA; NADH-quinone oxidoreductase subunit A [EC:1.6.5.3]															
	nuoC; NADH-quinone oxidoreductase subunit C [EC:1.6.5.3]															
	nuoD; NADH-quinone oxidoreductase subunit D [EC:1.6.5.3]															
	nuoG; NADH-quinone oxidoreductase subunit G [EC:1.6.5.3]															
	nuoH; NADH-quinone oxidoreductase subunit H [EC:1.6.5.3]															
	nuoK; NADH-quinone oxidoreductase subunit K [EC:1.6.5.3]															
	nuoL; NADH-quinone oxidoreductase subunit L [EC:1.6.5.3]															
	nuoM; NADH-quinone oxidoreductase subunit M [EC:1.6.5.3]															
Riboflavin metabolism (ko00740)	ribB, RIB3; 3,4-dihydroxy 2-butanone 4-phosphate synthase [EC:4.1.99.12]															
Glutathione metabolism (ko00480)	RRM1; ribonucleoside-diphosphate reductase subunit M1 [EC:1.17.4.1]															
Biosynthesis of siderophore nonribosomal peptides (ko01053)	dhbF; nonribosomal peptide synthetase DhbF															
Purine metabolism(ko00230)	nrdA, nrdE; ribonucleoside-diphosphate reductase alpha chain [EC:1.17.4.1]															
	nrdB, nrdF; ribonucleoside-diphosphate reductase beta chain [EC:1.17.4.1]															
Purine metabolism (ko00230)	purCD; fusion protein PurCD [EC:6.3.2.6 6.3.4.13]															
	pyrD; dihydroorotate dehydrogenase (fumarate) [EC:1.3.98.1]															

Table 5. Genes that are significantly more enriched in one group over another are represented in the heatmap below. T1-T3 were in Group 1, and T4-T6 were in Group 2. Genes were evaluated if they were present in more than one sample. All genes are significantly enriched in Group 2 relative to Group 1. The p-value for each test is listed under Group and the enrichment factor (Group2/Group1) is listed under Enrich.



KEGG pathway	Function	T1	T2	T3	T4	T5	T6	Group	Enrich
Peroxisome (ko04146)	ACSL, fadD; long-chain acyl-CoA synthetase [EC:6.2.1.3]								--
Bacterial secretion system (ko03070)	secY; preprotein translocase subunit SecY								3
RNA degradation (ko03018)	groEL, HSPD1; chaperonin GroEL								1
RNA polymerase (ko03020)	rpoB; DNA-directed RNA polymerase subunit beta [EC:2.7.7.6]								1735
Ribosome (ko03010)	RP-L13, rplM; large subunit ribosomal protein L13								21
	RP-L27, rpmA; large subunit ribosomal protein L27								3211
	RP-L5, rplE; large subunit ribosomal protein L5								3
	RP-S11, rpsK; small subunit ribosomal protein S11								3
	RP-S17, rpsQ; small subunit ribosomal protein S17								256
	RP-S8, rpsH; small subunit ribosomal protein S8								6
Alanine, aspartate, glutamate metabolism (ko00250)	glsA, GLS; glutaminase [EC:3.5.1.2]								985
Glycolysis/Gluconeogenesis (ko00010)	FBP, fbp; fructose-1,6-bisphosphatase I [EC:3.1.3.11]								67
Oxidative phosphorylation (ko00190)	coxA; cytochrome c oxidase subunit I [EC:1.9.3.1]								106
	nuoJ; NADH-quinone oxidoreductase subunit J [EC:1.6.5.3]								152
	nuoL; NADH-quinone oxidoreductase subunit L [EC:1.6.5.3]								70
Plant-pathogen interaction (ko04626)	tuf, TUFM; elongation factor Tu								83

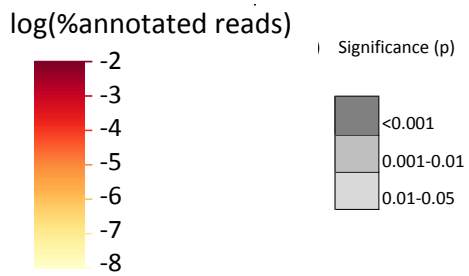
SUPPLEMENTARY MATERIALS

SUPPLEMENTARY LIST OF TABLES

Supplementary Table 1. Genes that were observed in the *Thalassiosira*-associated bacterial samples (T) but not observed in one or more comparison libraries (P= Plastics, H= HOT, N = North Pacific, ALOHA=A). If present, the total gene hits from each sample divided by sequence effort are given as a measure of relative abundance.

Level 1	Level 2	Level 3	Function	P	H	N	A	T
Cellular Processes	Cell growth and death	04115 p53 signaling pathway	RRM2; ribonucleoside-diphosphate reductase subunit M2 [EC:1.17.4.1]	1E-06	0	6E-05	8E-05	3E-06
Environmental Information Processing	Membrane Transport	02010 ABC transporters	cmpD; bicarbonate transport system ATP-binding protein [EC:3.6.3.-]	4E-06	0	0.0007	0.0006	7E-06
		03070 Bacterial secretion system	gspJ; general secretion pathway protein J	3E-06	2E-06	2E-05	0	3E-06
			gspL; general secretion pathway protein L	3E-06	1E-06	0	0	3E-06
	Signal transduction	02020 Two-component system	mdtB; RND superfamily, multidrug transport protein MdtB	1E-06	4E-07	0	0	1E-06
			rcsC; two-component system, NarL family, capsular synthesis sensor histidine kinase RcsC [EC:2.7.13.3]	7E-06	6E-07	2E-05	0	1E-06
Genetic Information Processing	Folding, sorting and degradation	04122 Sulfur relay system	moaC; molybdenum cofactor biosynthesis protein C	0	0	0.0003	0.0002	4E-06
	Translation	03010 Ribosome	RP-L35, rpmI; large subunit ribosomal protein L35	6E-05	0.0002	0.0003	0	2E-05
Human Diseases	Infectious diseases	05110 Vibrio cholerae infection	E3.4.24.25; vibriolysin [EC:3.4.24.25]	9E-06	7E-07	0	0	0.0002
		05111 Vibrio cholerae pathogenic cycle	SIG2, rpoS; RNA polymerase nonessential primary-like sigma factor	0.0007	0.0007	3E-05	0	8E-06
		05120 Epithelial cell signaling in Helicobacter pylori infection	K08303; putative protease [EC:3.4.-.-]	0.0003	2E-05	9E-06	0	1E-06
		05150 Staphylococcus aureus infection	dltD; D-alanine transfer protein	0	0	0	0	3E-06
Metabolism	Amino acid metabolism	00250 Alanine, aspartate and glutamate metabolism	E2.4.2.14, purF; amidophosphoribosyltransferase [EC:2.4.2.14]	0	0	0.0016	0.0014	1E-05
			glsA, GLS; glutaminase [EC:3.5.1.2]	0.0004	3E-05	2E-05	0	0.0011
		00270 Cysteine and methionine metabolism	mtnB; methylthioribulose-1-phosphate dehydratase [EC:4.2.1.109]	2E-05	5E-06	9E-06	0	1E-06
		00300 Lysine biosynthesis	E2.3.3.14; homocitrate synthase [EC:2.3.3.14]	0.0001	3E-05	9E-06	0	1E-06
			dat; D-alanine transaminase [EC:2.6.1.21]	0.0002	0.0001	0.0001	0	4E-06
			E4.1.1.18, ldcC, cadA; lysine decarboxylase [EC:4.1.1.18]	1E-05	0.0006	0	8E-05	1E-06
		00330 Arginine and proline metabolism	E4.1.1.19A, adi; arginine decarboxylase [EC:4.1.1.19]	4E-05	2E-06	0	0	1E-06
			nos; nitric-oxide synthase, bacterial [EC:1.14.13.39]	2E-05	4E-06	7E-05	0	1E-06
		00340 Histidine metabolism	E2.1.1.-; [EC:2.1.1.-]	0	0	0.0002	0.0004	0.0003
		00350 Tyrosine metabolism	FAHD1; acylpyruvate hydrolase [EC:3.7.1.5]	5E-07	2E-06	2E-05	0	1E-06
		00360 Phenylalanine metabolism	E6.2.1.12; 4-coumarate--CoA ligase [EC:6.2.1.12]	4E-06	2E-06	0	0	5E-06
	Carbohydrate metabolism	00052 Galactose metabolism	gal; D-galactose 1-dehydrogenase [EC:1.1.1.48]	3E-05	2E-07	2E-05	0	1E-06
		00053 Ascorbate and aldarate metabolism	MIOX; inositol oxygenase [EC:1.13.99.1]	2E-05	2E-06	4E-05	0	1E-06
		00500 Starch and sucrose metabolism	PGM2L1; glucose-1,6-bisphosphate synthase [EC:2.7.1.106]	0	0	9E-06	0.0002	1E-06
		00660 C5-Branched dibasic acid metabolism	E5.4.99.1; methylaspartate mutase [EC:5.4.99.1]	7E-07	4E-07	0	0	1E-06
	Energy metabolism	00680 Methane metabolism	E1.14.13.8; dimethylaniline monooxygenase (N-oxide forming) [EC:1.14.13.8]	3E-05	4E-06	0.0002	0	3E-06
		00680 Methane metabolism	frmB, ESD, fghA; S-formylglutathione hydrolase [EC:3.1.2.12]	0.0003	3E-05	0.0002	0	3E-06
	Lipid metabolism	00591 Linoleic acid metabolism	E1.14.19.3; linoleoyl-CoA desaturase [EC:1.14.19.3]	0.0002	8E-06	3E-05	0	1E-06
	Metabolism of cofactors and vitamins	00790 Folate biosynthesis	pabAB; para-aminobenzoate synthetase [EC:2.6.1.85]	2E-05	5E-06	0	0.0002	1E-06
	Metabolism of other amino acids	00480 Glutathione metabolism	RRM1; ribonucleoside-diphosphate reductase subunit M1 [EC:1.17.4.1]	2E-06	2E-06	0.0001	0	1E-05
	Metabolism of terpenoids and polyketides	01053 Biosynthesis of siderophore group nonribosomal peptides	dhbF; nonribosomal peptide synthetase DhbF	2E-05	2E-06	0	0	3E-05
	Nucleotide metabolism	00240 Pyrimidine metabolism	E4.2.1.70; pseudouridylate synthase [EC:4.2.1.70]	0	0	0	0	2E-05

Supplementary Table 2. Genes that are depleted in T samples relative to others are listed. Bacterial genes in each of the *Thlassiosira*-associated bacterial samples (T1-T6), as well as average values for the *Thlassiosira* samples (AveT) and for other samples tested (P = Plastics, H= HOT, N = North Pacific, and A = ALOHA) are shown, as well as the significance level is indicated in columns T>P, T>H, T>N, T>A. This was found for (A) Cellular Processes (B) Environmental Information Processes (C) Genetic Information Processing (D) Human Disease (D) Organismal Systems (E) Metabolism.



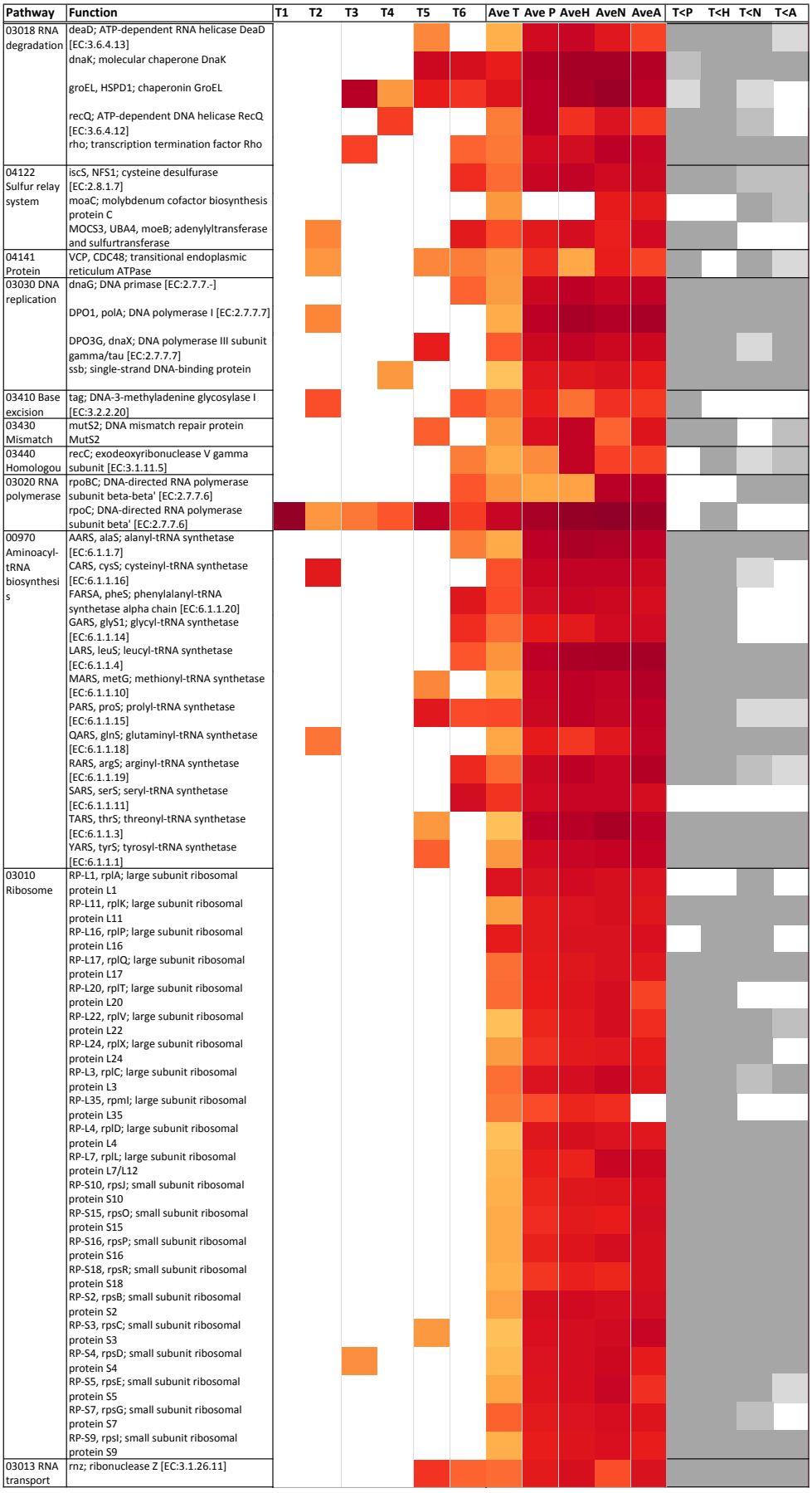
A.

Pathway	Function	T1	T2	T3	T4	T5	T6	Ave T	Ave P	AveH	AveN	AveA	T<P	T<H	T<N	T<A
04112 Cell cycle - Caulobacter	clpP, CLPP; ATP-dependent Clp protease, protease subunit [EC:3.4.21.92]															
	dnaB; replicative DNA helicase [EC:3.6.4.12]															
	ftsZ; cell division protein FtsZ															
	lon; ATP-dependent Lon protease [EC:3.4.21.53]															
04113 Meiosis - yeast	E4.6.1.1; adenylate cyclase [EC:4.6.1.1]															
04115 p53 signaling pathway	RRM2; ribonucleoside-diphosphate reductase subunit M2 [EC:1.17.4.1]															
04146 Peroxisome	E2.7.1.36, MVK, mvaK1; mevalonate kinase [EC:2.7.1.36]															
	E3.6.1.22, NUDT12, nudC; NAD+ diphosphatase [EC:3.6.1.22]															
	IDH1, IDH2, icd; isocitrate dehydrogenase [EC:1.1.1.42]															
	SOD2; superoxide dismutase, Fe-Mn family [EC:1.15.1.1]															

B.

Pathway	Function	T1	T2	T3	T4	T5	T6	Ave T	Ave P	AveH	AveN	AveA	T<P	T<H	T<N	T<A
02010 ABC transporters	ABC.FEV.S; iron complex transport system substrate-binding protein ABC.PE.A; peptide/nickel transport system ATP-binding protein cbiO; cobalt/nickel transport system ATP-binding protein cmpD; bicarbonate transport system ATP-binding protein [EC:3.6.3.-] msbA; ATP-binding cassette, subfamily B, bacterial MsbA [EC:3.6.3.-]															
03070 Bacterial secretion system	secY; preprotein translocase subunit SecY															
02020 Two-component system	narG; nitrate reductase 1, alpha subunit [EC:1.7.99.4] rcsC; two-component system, NarL family, capsular synthesis sensor histidine kinase															
04066 HIF-1 signaling pathway	ENO, eno; enolase [EC:4.2.1.11] PDHB, pdhB; pyruvate dehydrogenase E1 component subunit beta [EC:1.2.4.1]															
04070 Phosphatidylinositol	E3.1.3.25, IMPA, suhB; myo-inositol-1(or 4)-monophosphatase [EC:3.1.3.25]															
04151 PI3K-Akt signaling pathway	htpG, HSP90A; molecular chaperone HtpG															

C.


Pathway
Function
T1
T2
T3
T4
T5
T6
Ave T
Ave P
AveH
AveN
AveA
T<P
T<H
T<N
T<A
03018 RNA degradation
deaD; ATP-dependent RNA helicase DeaD [EC:3.6.4.13]
dnaK; molecular chaperone DnaK
groEL, HSPD1; chaperonin GroEL
recQ; ATP-dependent DNA helicase RecQ [EC:3.6.4.12]
rho; transcription termination factor Rho
04122 Sulfur relay system
iscS, NFS1; cysteine desulfurase [EC:2.8.1.7]
moaC; molybdenum cofactor biosynthesis protein C
MOCS3, UBA4, moeB; adenylyltransferase and sulfurtransferase
04141 Protein
VCP, CDC48; transitional endoplasmic reticulum ATPase
03030 DNA replication
dnaG; DNA primase [EC:2.7.7.-]
DPO1, polA; DNA polymerase I [EC:2.7.7.7]
DPO3G, dnaX; DNA polymerase III subunit gamma/tau [EC:2.7.7.7]
ssb; single-strand DNA-binding protein
03410 Base excision
tag; DNA-3-methyladenine glycosylase I [EC:3.2.2.20]
03430 Mismatch
mutS2; DNA mismatch repair protein MutS2
03440 Homologou
recC; exodeoxyribonuclease V gamma subunit [EC:3.1.11.5]
03020 RNA polymerase
rpoBC; DNA-directed RNA polymerase subunit beta-beta' [EC:2.7.7.6]
rpoC; DNA-directed RNA polymerase subunit beta' [EC:2.7.7.6]
00970 Aminoacyl-tRNA biosynthesis
AARS, alaS; alanyl-tRNA synthetase [EC:6.1.1.7]
CARS, cysS; cysteinyl-tRNA synthetase [EC:6.1.1.16]
FARSA, pheS; phenylalanyl-tRNA synthetase alpha chain [EC:6.1.1.20]
GARS, glyS1; glycyl-tRNA synthetase [EC:6.1.1.14]
LARS, leuS; leucyl-tRNA synthetase [EC:6.1.1.4]
MARS, metG; methionyl-tRNA synthetase [EC:6.1.1.10]
PARS, proS; prolyl-tRNA synthetase [EC:6.1.1.15]
QARS, glnS; glutaminyl-tRNA synthetase [EC:6.1.1.18]
RARS, argS; arginyl-tRNA synthetase [EC:6.1.1.19]
SARS, serS; seryl-tRNA synthetase [EC:6.1.1.11]
TARS, thrS; threonyl-tRNA synthetase [EC:6.1.1.3]
YARS, tyrS; tyrosyl-tRNA synthetase [EC:6.1.1.1]
03010 Ribosome
RP-L1, rplA; large subunit ribosomal protein L1
RP-L11, rplK; large subunit ribosomal protein L11
RP-L16, rplP; large subunit ribosomal protein L16
RP-L17, rplQ; large subunit ribosomal protein L17
RP-L20, rplT; large subunit ribosomal protein L20
RP-L22, rplV; large subunit ribosomal protein L22
RP-L24, rplX; large subunit ribosomal protein L24
RP-L3, rplC; large subunit ribosomal protein L3
RP-L35, rpmI; large subunit ribosomal protein L35
RP-L4, rplD; large subunit ribosomal protein L4
RP-L7, rplL; large subunit ribosomal protein L7/L12
RP-S10, rpsJ; small subunit ribosomal protein S10
RP-S15, rpsO; small subunit ribosomal protein S15
RP-S16, rpsP; small subunit ribosomal protein S16
RP-S18, rpsR; small subunit ribosomal protein S18
RP-S2, rpsB; small subunit ribosomal protein S2
RP-S3, rpsC; small subunit ribosomal protein S3
RP-S4, rpsD; small subunit ribosomal protein S4
RP-S5, rpsE; small subunit ribosomal protein S5
RP-S7, rpsG; small subunit ribosomal protein S7
RP-S9, rpsI; small subunit ribosomal protein S9
03013 RNA transport
rnz; ribonuclease Z [EC:3.1.26.11]

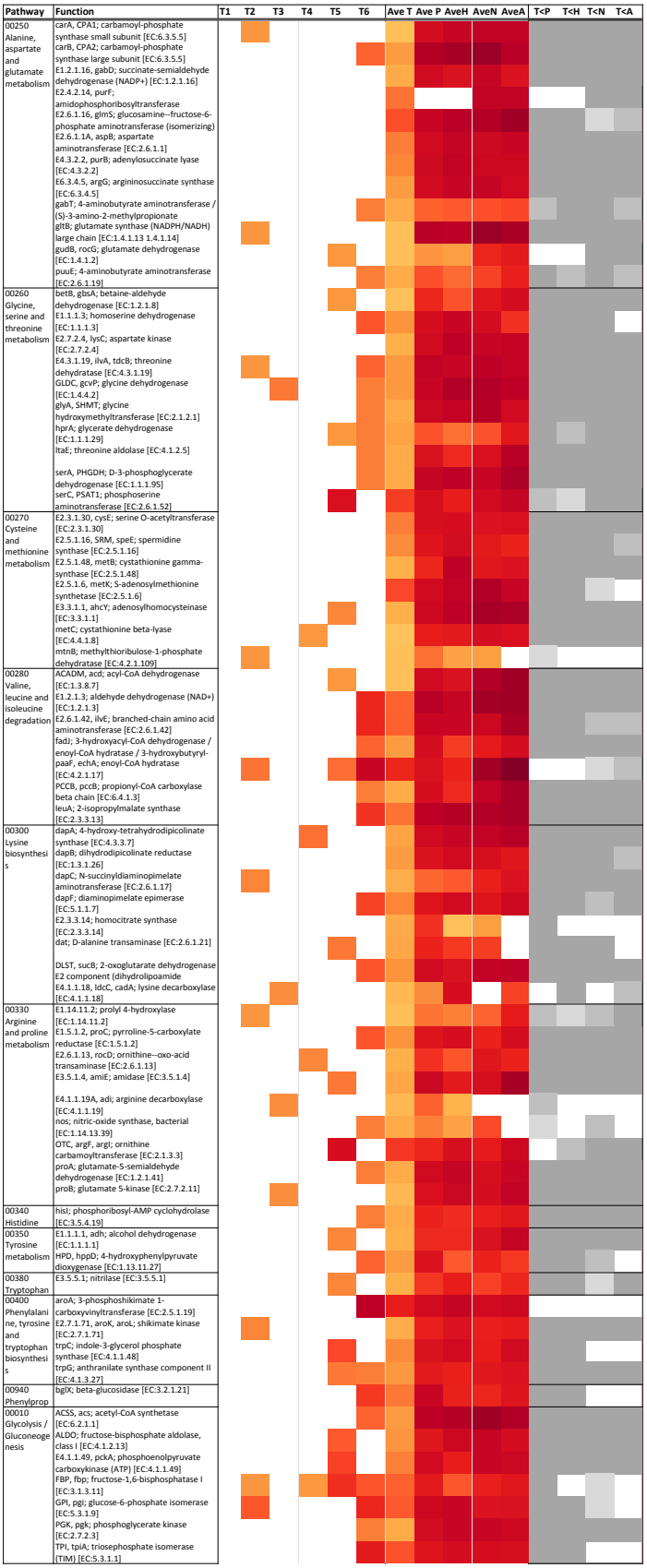
D.

Pathway	Function	T1	T2	T3	T4	T5	T6	Ave T	Ave P	AveH	AveN	AveA	T<P	T<H	T<N	T<A
05200 Pathways in	E4.2.1.2B, fumC; fumarate hydratase, class II [EC:4.2.1.2]															
05203 Viral carcinogene	PK, pyk; pyruvate kinase [EC:2.7.1.40]															
05204 Chemical	frmA, ADH5, adhC; S-(hydroxymethyl)glutathione															
05111 Vibrio	SIG2, rpoS; RNA polymerase nonessential primary-like sigma factor															
05120 Epithelial	K08303; putative protease [EC:3.4.-.-]															
05142 Chagas	ptrB; oligopeptidase B [EC:3.4.21.83]															

E.

Pathway	Function	T1	T2	T3	T4	T5	T6	Ave T	Ave P	AveH	AveN	AveA	T<P	T<H	T<N	T<A
04626 Plant-pathogen	tuf, TUFM; elongation factor Tu															

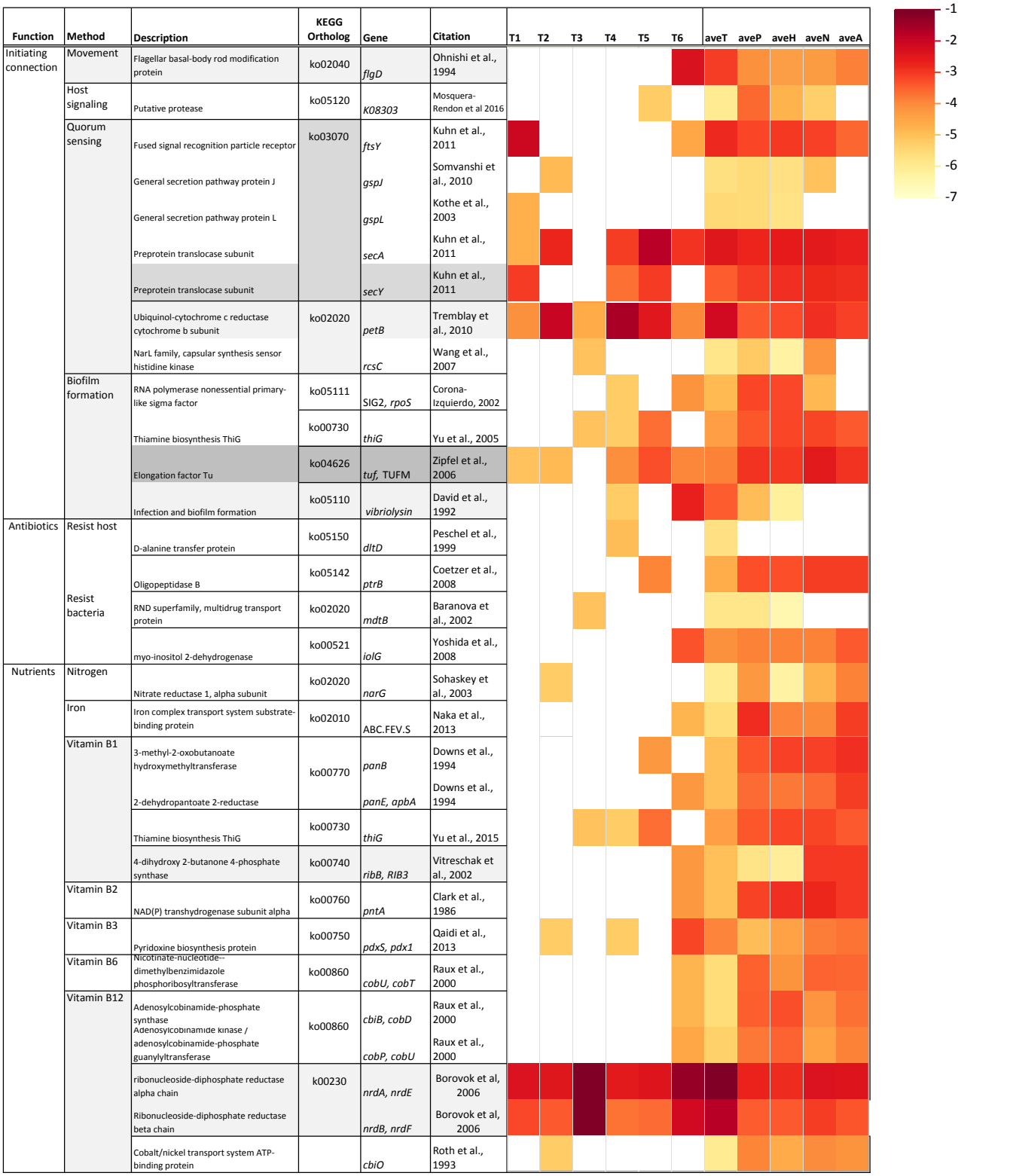
F.


Pathway
Function
T1
T2
T3
T4
T5
T6
Ave T
Ave P
AveH
AveN
AveA
T<P
T<H
T<N
T<A
00250 Alanine, aspartate and glutamate metabolism
carA, CPA1; carbamoyl-phosphate synthase small subunit [EC:6.3.5.5]
carB, CPA2; carbamoyl-phosphate synthase large subunit [EC:6.3.5.5]
E1.2.1.16, gabD; succinate-semialdehyde dehydrogenase (NADP+) [EC:1.2.1.16]
E2.4.2.14, purF; amidophosphoribosyltransferase
E2.6.1.16, glmS; glucosamine--fructose-6-phosphate aminotransferase (isomerizing)
E2.6.1.1A, aspB; aspartate aminotransferase [EC:2.6.1.1]
E4.3.2.2, purB; adenylosuccinate lyase [EC:4.3.2.2]
E6.3.4.5, argG; argininosuccinate synthase [EC:6.3.4.5]
gabT; 4-aminobutyrate aminotransferase / (S)-3-amino-2-methylpropionate
gltB; glutamate synthase (NADPH/NADH) large chain [EC:1.4.1.13 1.4.1.14]
gudB, rocG; glutamate dehydrogenase [EC:1.4.1.2]
puuE; 4-aminobutyrate aminotransferase [EC:2.6.1.19]
00260 Glycine, serine and threonine metabolism
betB, gbsA; betaine-aldehyde dehydrogenase [EC:1.2.1.8]
E1.1.1.3; homoserine dehydrogenase [EC:1.1.1.3]
E2.7.2.4, lysC; aspartate kinase [EC:2.7.2.4]
E4.3.1.19, ilvA, tdcB; threonine dehydratase [EC:4.3.1.19]
GLDC, gcvP; glycine dehydrogenase [EC:1.4.4.2]
glyA, SHMT; glycine hydroxymethyltransferase [EC:2.1.2.1]
hprA; glycerate dehydrogenase [EC:1.1.1.29]
ltaE; threonine aldolase [EC:4.1.2.5]
serA, PHGDH; D-3-phosphoglycerate dehydrogenase [EC:1.1.1.95]
serC, PSAT1; phosphoserine aminotransferase [EC:2.6.1.52]
00270 Cysteine and methionine metabolism
E2.3.1.30, cysE; serine O-acetyltransferase [EC:2.3.1.30]
E2.5.1.16, SRM, speE; spermidine synthase [EC:2.5.1.16]
E2.5.1.48, metB; cystathionine gamma-synthase [EC:2.5.1.48]
E2.5.1.6, metK; S-adenosylmethionine synthetase [EC:2.5.1.6]
E3.1.1.1, ahcY; adenosylhomocysteinase [EC:3.3.1.1]
metC; cystathionine beta-lyase [EC:4.4.1.8]
mtnB; methylthioribulose-1-phosphate dehydratase [EC:4.2.1.109]
00280 Valine, leucine and isoleucine degradation
ACADM, acd; acyl-CoA dehydrogenase [EC:1.3.8.7]
E1.2.1.3; aldehyde dehydrogenase (NAD+) [EC:1.2.1.3]
E2.6.1.42, ilvE; branched-chain amino acid aminotransferase [EC:2.6.1.42]
fadJ; 3-hydroxyacyl-CoA dehydrogenase / enoyl-CoA hydratase / 3-hydroxybutyryl-paaF, echA; enoyl-CoA hydratase [EC:4.2.1.17]
PCCB, pccB; propionyl-CoA carboxylase beta chain [EC:6.4.1.3]
leuA; 2-isopropylmalate synthase [EC:2.3.3.13]
00300 Lysine biosynthesis
dapA; 4-hydroxy-tetrahydrodipicolinate synthase [EC:4.3.3.7]
dapB; dihydrodipicolinate reductase [EC:1.3.1.26]
dapC; N-succinyldiaminopimelate aminotransferase [EC:2.6.1.17]
dapF; diaminopimelate epimerase [EC:5.1.1.7]
E2.3.3.14; homocitrate synthase [EC:2.3.3.14]
dat; D-alanine transaminase [EC:2.6.1.21]
DLST, sucB; 2-oxoglutarate dehydrogenase E2 component (dihydrolipoamide
E4.1.1.18, ldcC, cadA; lysine decarboxylase [EC:4.1.1.18]
00330 Arginine and proline metabolism
E1.14.11.2; prolyl 4-hydroxylase [EC:1.14.11.2]
E1.5.1.2, proC; pyrroline-5-carboxylate reductase [EC:1.5.1.2]
E2.6.1.13, rocD; ornithine--oxo-acid transaminase [EC:2.6.1.13]
E3.5.1.4, amiE; amidase [EC:3.5.1.4]
E4.1.1.19A, adi; arginine decarboxylase [EC:4.1.1.19]
nos; nitric-oxide synthase, bacterial [EC:1.14.13.39]
OTC, argF, argI; ornithine carbamoyltransferase [EC:2.1.3.3]
proA; glutamate-5-semialdehyde dehydrogenase [EC:1.2.1.41]
proB; glutamate 5-kinase [EC:2.7.2.11]
00340 Histidine
hisI; phosphoribosyl-AMP cyclohydrolase [EC:3.5.4.19]
00350 Tyrosine metabolism
E1.1.1.1, adh; alcohol dehydrogenase [EC:1.1.1.1]
HPD, hppD; 4-hydroxyphenylpyruvate dioxygenase [EC:1.13.11.27]
00380 Tryptophan
E3.5.5.1; nitrilase [EC:3.5.5.1]
00400 Phenylalanine, tyrosine and tryptophan biosynthesis
aroA; 3-phosphoshikimate 1-carboxyvinyltransferase [EC:2.5.1.19]
E2.7.1.71, aroK, aroL; shikimate kinase [EC:2.7.1.71]
trpC; indole-3-glycerol phosphate synthase [EC:4.1.1.48]
trpG; anthranilate synthase component II [EC:4.1.3.27]
00940 Phenylprop
bglX; beta-glucosidase [EC:3.2.1.21]
00010 Glycolysis / Gluconeogenesis
ACSS, acs; acetyl-CoA synthetase [EC:6.2.1.1]
ALDO; fructose-bisphosphate aldolase, class I [EC:4.1.2.13]
E4.1.1.49, pckA; phosphoenolpyruvate carboxykinase (ATP) [EC:4.1.1.49]
FBP, fbp; fructose-1,6-bisphosphatase I [EC:3.1.3.11]
GPI, pgi; glucose-6-phosphate isomerase [EC:5.3.1.9]
PGK, pgk; phosphoglycerate kinase [EC:2.7.2.3]
TPI, tpiA; triosephosphate isomerase (TIM) [EC:5.3.1.1]


Pathway
Function
T1
T2
T3
T4
T5
T6
Ave T
Ave P
AveH
AveN
AveA
T<P
T<H
T<N
T<A
00020 Citrate cycle (TCA cycle)
PC, pyc; pyruvate carboxylase [EC:6.4.1.1]
sucC; succinyl-CoA synthetase beta subunit [EC:6.2.1.5]
00030 Pentose phosphate pathway
E2.2.1.1, tktA, tktB; transketolase [EC:2.2.1.1]
E2.2.1.2, talA, talB; transaldolase [EC:2.2.1.2]
G6PD, zwf; glucose-6-phosphate 1-dehydrogenase [EC:1.1.1.49]
rbsK, RBKS; ribokinase [EC:2.7.1.15]
rpe, RPE; ribulose-phosphate 3-epimerase [EC:5.1.3.1]
00040 Pentose and glucuronate
E2.7.1.17; xylulokinase [EC:2.7.1.17]
UGDH, ugd; UDPglucose 6-dehydrogenase [EC:1.1.1.22]
00052 Galactose metabolism
gal; D-galactose 1-dehydrogenase [EC:1.1.1.48]
galE, GALE; UDP-glucose 4-epimerase [EC:5.1.3.2]
00053 Ascorbate
MIOX; inositol oxygenase [EC:1.13.99.1]
00500 Starch and
PGM2L1; glucose-1,6-bisphosphate synthase [EC:2.7.1.106]
00520 Amino
murA; UDP-N-acetylglucosamine 1-carboxyvinyltransferase [EC:2.5.1.7]
00620 Pyruvate metabolism
E2.3.3.9, aceB, glcB; malate synthase [EC:2.3.3.9]
ppc; phosphoenolpyruvate carboxylase [EC:4.1.1.31]
ppdK; pyruvate,orthophosphate dikinase [EC:2.7.9.1]
pps, ppsA; pyruvate, water dikinase [EC:2.7.9.2]
00630 Glyoxylate and dicarboxylat
E1.2.1.2A; formate dehydrogenase, alpha subunit [EC:1.2.1.2]
gyaR; glyoxylate reductase [EC:1.1.1.26]
00190 Oxidative phosphorylation
ATPF0A, atpB; F-type H+-transporting ATPase subunit a [EC:3.6.3.14]
ATPF0C, atpE; F-type H+-transporting ATPase subunit c [EC:3.6.3.14]
ATPF1B, atpD; F-type H+-transporting ATPase subunit beta [EC:3.6.3.14]
COX11; cytochrome c oxidase assembly protein subunit 11
coxA; cytochrome c oxidase subunit I [EC:1.9.3.1]
coxC; cytochrome c oxidase subunit III [EC:1.9.3.1]
nuoB; NADH-quinone oxidoreductase subunit B [EC:1.6.5.3]
nuoN; NADH-quinone oxidoreductase subunit N [EC:1.6.5.3]
ppa; inorganic pyrophosphatase [EC:3.6.1.1]
00680 Methane metabolism
E1.14.13.8; dimethylaniline monooxygenase (N-oxide forming)
E1.5.1.20, metF; methylenetetrahydrofolate reductase
frmB, ESD, fghA; S-formylglutathione hydrolase [EC:3.1.2.12]
00720 Carbon
fhs; formate--tetrahydrofolate ligase [EC:6.3.4.3]
00920 Sulfur
cysC; adenylylsulfate kinase [EC:2.7.1.25]
00061 Fatty acid biosynthesis
fabB; 3-oxoacyl-[acyl-carrier-protein] synthase I [EC:2.3.1.41]
fabF; 3-oxoacyl-[acyl-carrier-protein] synthase II [EC:2.3.1.179]
fabG; 3-oxoacyl-[acyl-carrier protein] reductase [EC:1.1.1.100]
fabI; enoyl-[acyl-carrier protein] reductase I [EC:1.3.1.9 1.3.1.10]
fabK; enoyl-[acyl-carrier protein] reductase II [EC:1.3.1.-]
00561 Glycerolipid
E2.7.1.30, glpK; glycerol kinase [EC:2.7.1.30]
00564 Glycerophо
E3.1.4.46, glpQ, ugpQ; glycerophosphoryl diester phosphodiesterase [EC:3.1.4.46]
00590 Arachidonic
E1.11.1.9; glutathione peroxidase [EC:1.11.1.9]
00591 Linoleic acid
E1.14.19.3; linoleoyl-CoA desaturase [EC:1.14.19.3]
00130 Ubiquinone and other terpenoid-
ubiB, aarF; ubiquinone biosynthesis protein
ubiE; ubiquinone/menaquinone biosynthesis methyltransferase
00730 Thiamine
thiG; thiamine biosynthesis ThiG
00740 Riboflavin
ribB, RIB3; 3,4-dihydroxy 2-butanone 4-phosphate synthase [EC:4.1.99.12]
00760 Nicotinate
pntA; NAD(P) transhydrogenase subunit alpha [EC:1.6.1.2]
00770 Pantothenate and CoA biosynthesi
panB; 3-methyl-2-oxobutanoate hydroxymethyltransferase [EC:2.1.2.11]
panE, apbA; 2-dehydropantoate 2-reductase [EC:1.1.1.169]
00780 Biotin
bioA; adenosylmethionine-8-amino-7-oxononanoate aminotransferase
00790 Folate
pabAB; para-aminobenzoate synthetase [EC:2.6.1.85]
00830 Retinol
RETSAT; all-trans-retinol 13,14-reductase [EC:1.3.99.23]
00860 Porphyrin and chlorophyll metabolism
cbiB, cobD; adenosylcobinamide-phosphate synthase [EC:6.3.1.10]
cobP, cobU; adenosylcobinamide kinase / adenosylcobinamide-phosphate
cysG; uroporphyrin-III C-methyltransferase / precorrin-2
E2.4.2.21, cobU, cobT; nicotinate-nucleotide--dimethylbenzimidazole
hemB, ALAD; porphobilinogen synthase [EC:4.2.1.24]
hemE, UROD; uroporphyrinogen decarboxylase [EC:4.1.1.37]
hemF, CPOX; coproporphyrinogen III oxidase [EC:1.3.3.3]
00450 Selenocom
E1.8.1.9, trxB; thioredoxin reductase (NADPH) [EC:1.8.1.9]
00480 Glutathione metabolism
CARP, pepA, leucyl aminopeptidase [EC:3.4.11.1]
E1.8.1.7, GSR, gor; glutathione reductase (NADPH) [EC:1.8.1.7]
pepN; aminopeptidase N [EC:3.4.11.2]
RRM1; ribonucleoside-diphosphate reductase subunit M1 [EC:1.17.4.1]
00230 Purine metabolism
E2.4.2.7, apt; adenine phosphoribosyltransferase [EC:2.4.2.7]
E2.7.4.3, adk; adenylate kinase [EC:2.7.4.3]
E2.7.4.8, gmk; guanylate kinase [EC:2.7.4.8]
E6.3.5.2, guaA; GMP synthase (glutamine-hydrolysing) [EC:6.3.5.2]
E6.3.5.3, purL; phosphoribosylformylglycinamidine
purC; phosphoribosylaminoimidazole-succinocarboxamide synthase [EC:6.3.2.6]
purCD; fusion protein PurCD [EC:6.3.2.6 6.3.4.13]
xdhA; xanthine dehydrogenase small subunit [EC:1.17.1.4]
00240 Pyrimidine metabolism
comEB; dCMP deaminase [EC:3.5.4.12]
pyrE; orotate phosphoribosyltransferase [EC:2.4.2.10]
pyrF; orotidine-5'-phosphate decarboxylase [EC:4.1.1.23]
upp, UPRT; uracil phosphoribosyltransferase [EC:2.4.2.9]
00621 Dioxin
E1.14.13.1; salicylate hydroxylase [EC:1.14.13.1]

Supplementary Table 3. Bacterial functional genes that may be essential to diatom-bacterial symbiosis. Genes enriched in previous analysis relative to other sample sets are highlighted in light grey, and in darker grey for comparisons of Group 1 vs Group 2.

Function	Method	Description	KEGG Ortholog	Gene	Citation
Initiating connection	Movement	Flagellar basal-body rod modification protein	ko02040	*flgD*	Ohnishi et al., 1994
	Host signaling	Putative protease	ko05120	*K08303*	Mosquera-Rendon et al 2016
	Quorum sensing	Fused signal recognition particle receptor	ko03070	*ftsY*	Kuhn et al., 2011
		General secretion pathway protein J		*gspJ*	Somvanshi et al., 2010
		General secretion pathway protein L		*gspL*	Kothe et al., 2003
		Preprotein translocase subunit		*secA*	Kuhn et al., 2011
		Preprotein translocase subunit		*secY*	Kuhn et al., 2011
		Ubiquinol-cytochrome c reductase cytochrome b subunit	ko02020	*petB*	Tremblay et al., 2010
		NarL family, capsular synthesis sensor histidine kinase		*rcsC*	Wang et al., 2007
	Biofilm formation	RNA polymerase nonessential primary-like sigma factor	ko05111	SIG2, *rpoS*	Corona-Izquierdo, 2002
		Thiamine biosynthesis ThiG	ko00730	*thiG*	Yu et al., 2005
		Elongation factor Tu	ko04626	*tuf*, TUFM	Zipfel et al., 2006
		Infection and biofilm formation	ko05110	*vibriolysin*	David et al., 1992
Antibiotics	Resist host	D-alanine transfer protein	ko05150	*dltD*	Peschel et al., 1999
	Resist bacteria	Oligopeptidase B	ko05142	*ptrB*	Coetzer et al., 2008
		RND superfamily, multidrug transport protein	ko02020	*mdtB*	Baranova et al., 2002
		myo-inositol 2-dehydrogenase	ko00521	*iolG*	Yoshida et al., 2008
Nutrients	Nitrogen	Nitrate reductase 1, alpha subunit	ko02020	*narG*	Sohaskey et al., 2003
	Iron	Iron complex transport system substrate-binding protein	ko02010	ABC.FEV.S	Naka et al., 2013
	Vitamin B1	3-methyl-2-oxobutanoate hydroxymethyltransferase	ko00770	*panB*	Downs et al., 1994
		2-dehydropantoate 2-reductase		*panE, apbA*	Downs et al., 1994
		Thiamine biosynthesis ThiG	ko00730	*thiG*	Yu et al., 2015
		4-dihydroxy 2-butanone 4-phosphate synthase	ko00740	*ribB, RIB3*	Vitreschak et al., 2002
	Vitamin B2	NAD(P) transhydrogenase subunit alpha	ko00760	*pntA*	Clark et al., 1986
	Vitamin B3	Pyridoxine biosynthesis protein	ko00750	*pdxS, pdx1*	Qaidi et al., 2013
	Vitamin B6	Nicotinate-nucleotide--dimethylbenzimidazole phosphoribosyltransferase	ko00860	*cobU, cobT*	Raux et al., 2000
	Vitamin B12	Adenosylcobinamide-phosphate synthase	ko00860	*cbiB, cobD*	Raux et al., 2000
		Adenosylcobinamide kinase / adenosylcobinamide-phosphate guanylyltransferase		*cobP, cobU*	Raux et al., 2000
		ribonucleoside-diphosphate reductase alpha chain	k00230	*nrdA, nrdE*	Borovok et al, 2006
		Ribonucleoside-diphosphate reductase beta chain		*nrdB, nrdF*	Borovok et al, 2006
		Cobalt/nickel transport system ATP-binding protein		*cbiO*	Roth et al., 1993



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CHAPTER 5: Conclusions, key findings, and future directions

This dissertation focuses on diatom-bacterial interactions in the oligotrophic North Pacific Subtropical Gyre, and investigates how the composition and impact of diatom-associated bacteria are effected by nutrient concentrations. The findings and conclusions in this dissertation often differ from the published literature on diatom-bacterial interactions. This may be due in part to the differences in the approaches taken in this study, which emphasized the use of conditions that were more representative of the natural environment. Chapter 1 and 2 utilize a xenic model system that was originally collected from the oligotrophic nearshore. Previously reported culture-based research has often employed axenic hosts. Although this complicates the experimental setup (enough negative controls, replicates, etc), and adds possible confounding factors (bacteria-bacteria interactions), overall the results may be more indicative of those seen in a natural environment.

My Master's thesis and Chapter 3 explores samples taken directly from the oligotrophic open ocean whereas much of the previous research has explored eutrophic systems that are dominated by the diatom in study. Although diatoms are not as conspicuously abundant in the oligotrophic ocean as they are in coastal waters (e.g. coastal diatom blooms), they are disproportionately important in carbon export and sequestration. The present focus on the oligotrophic ocean was stimulated by previous research in coastal waters showing that host-associated bacteria can strongly influence diatom growth and death. If host-associated bacteria have a similarly strong impact on host processes in oligotrophic systems, they may profoundly influence global carbon production and sequestration. These fundamental differences in approach are likely causes of some of the differences in my results and conclusions.

In the following sections, I will return to the questions posed in the Introduction; discuss how the results of the present work should alter our view of diatom-microbe interactions; and summarize the most interesting and challenging questions that remain, as well as questions generated by this dissertation research.

Diatoms host a bacterial consortium under a variety of environmental conditions

Early work suggested that bacteria were associated with algal hosts primarily during the decline of the host, and emphasized the importance of bacteria in the process of remineralization (Waksman *et al.*, 1933; Cole, 1982; Azam *et al.*, 1983). Newer literature documents regular association of bacteria with diatoms (Amin et al. 2012, and references therein). This dissertation further establishes that bacterial consortia are attached to diatoms at all stages of diatom growth, and that these consortia are affected by host growth stage and nutrient concentrations (Chapter 1; Baker et al. 2016). Overall, the total number of bacteria attached to a diatom remained stable in a batch culture (Chapter 1; Baker et al. 2016). The exception was under high nutrient conditions during early exponential growth of the diatom host (i.e. similar to a diatom bloom), where the number of bacteria per host cell was higher than in a low nutrient treatment with slow diatom growth rates. This result is in striking contrast to a study of bacteria attached to diatoms during bloom conditions in an upwelling system, which reported very few to no attached bacteria (Graff *et al.*, 2011). The conflicting results suggest that comparative studies are needed in different ecosystems and under contrasting environmental conditions.

The composition of host-associated bacteria changed rapidly in relative abundance in response to abiotic and biotic stimuli, in as few as four days (Chapter 1; Baker et al. 2016). This implies that bacterial composition is not static; it may even be highly plastic and dependent on the current environmental conditions. Extended sampling periods may be required to fully capture the diversity of bacteria associated with a diatom host. Experiments in this dissertation research lasted for up to 60 days; further research would be needed to understand temporal variability over shorter or longer timescales (i.e. hours or months).

The composition of diatom-associated bacteria was sensitive to the concentration of nutrients in the media. This complements our prior research showing that the composition of diatom-attached bacteria in an

oligotrophic sample differed from previous reports of eutrophic systems (Baker and Kemp, 2014). Much of the previous work in diatom-bacterial associations has overlooked oligotrophic systems. Arguably, oligotrophic systems may require diatoms to be more dependent on their bacterial symbionts for necessary nutrients, such at B-vitamins (Croft *et al.*, 2005), nitrogen (Suleiman *et al.*, 2016), and biologically available iron (Amin *et al.*, 2009). I will return to this point in my discussion of Chapter 3.

This study demonstrated that the composition of attached bacterial can shift in response to host growth and decline as well as in response to host cell lysis (Chapter 1, Baker et al. 2016). This agrees with prior research showing that bacterial composition shifts in response to bloom and bust conditions (Buchan et al. 2014, and references therein). In the present study, the chosen methods captured both intact and lysed particulate matter derived from diatom hosts. Further study of how attached bacterial populations respond to host lysis would benefit from separating out the live and particulate matter. Additionally, being able to track how the free-living bacteria responds to similar stimuli would facilitate exploration of how these populations are influenced by growth with a diatom host.

Within a culture, bacteria influence the attached bacterial consortia as well as the host trajectory

Marine bacteria have been shown to compete for resources and space (Barja *et al.*, 1989; Yan *et al.*, 2002; Hibbing *et al.*, 2010), suggesting that competition may influence which bacteria are able to colonize a diatom host. Interestingly, bacterial interactions significantly impacted the composition of host-associated bacteria (Chapter 1; Baker et al. 2016, from general linear modeling results). In surface colonization competition studies using unidentified isolates derived from the diatom host culture, none of the strains competed for space with other strains on a high nutrient surface (marine agar). However, I did not attempt an exhaustive study of all bacteria associated with the host diatom; similar studies with less dominant bacterial genera may still result in some phylotypes found to be capable of competing with the other isolates. It is also possible that the

competition is less direct (e.g. metabolic interference, quorum sensing affects on nonspecific bacteria), or that interactions may have been the result of positive interactions between attached bacteria (co-metabolism, etc).

Diatoms grown with their bacterial consortia are impacted by the addition of bacterial inoculum to the co-culture. In Chapter 2, adding *Marinobacter* or *Alteromonas* strains had long-term effects on the host culture's trajectory (growth, carrying capacity, and decline). Surprisingly, *Alteromonas* did not demonstrate consistently parasitic host interactions (Kim *et al.*, 1999; Cottrell *et al.*, 2000) and *Marinobacter* did not demonstrate consistently mutualistic host interactions (S. a Amin *et al.*, 2012) as predicted from previous research. Instead, inoculations with *Marinobacter* strains had a similarly negative impact on host growth and decline as inoculations with *Alteromonas*. Other studies of diatom-bacterial interactions using a diatom-*Marinobacter* model were unable to substantiate a mutualistic relationship through the analysis of proteasome of the bacteria and the diatom (Stahl and Ullrich, 2016). It is possible that only some *Marinobacter* are inclined towards mutualism, or that previously reported potential mutualistic effects were not enough to result in significant differences in the host's growth trajectory.

Host diatoms are impacted differently by the addition of bacterial inocula. Inoculation with bacteria had very little impact on the origin host and a more distantly related naïve host, and the greatest impact on a naïve host of the same genus as the origin host. This occurred even though the naïve host supported *Alteromonas* phylotypes similar to those used in the bacterial inocula. Although multiple host cells support similar phylotypes (S. Amin *et al.*, 2012), similar phylotypes may differ in their interactions with their host. Additionally, similar phylotypes isolated from the same host cell affected the growth, carrying capacity, and decline of host cells to different extents, again suggesting that similar phylotypes can differ in their interaction with host cells. A logical next step for phylogeny-based studies of the microbiomes of host cells is to examine differences in functional capacities, even of closely related bacteria as was performed in Chapter 3.

High vitamin concentrations mediates the effect of bacterial inoculations on naïve host cells

Vitamin concentration in the culture medium did not always modify the effects of bacterial additions (Chapter 2); however, when vitamin concentrations did have an effect, all significantly negative effects of bacterial additions were observed in vitamin deplete media, and all positive effects of bacterial additions were observed in vitamin replete media. This was most evident for the growth and decline of the closely related naïve host, where the impact of bacterial inoculation shifts from significantly positive to significantly negative for 3 of the 5 bacterial strains evaluated (Chapter 2, Table 2B, diatom KBDT32), depending on vitamin concentration in the media. Previous studies of bacterial-host interactions have shown that the impact of adding bacteria can be modified by the degree of host cell stress (Seyedsayamdost *et al.*, 2011). It is possible that in the presence of added vitamins, the bacteria can more efficiently consume diatom exudates. Because increased concentrations of diatom exudates has been linked to multiple negative outcomes for the host cell (Kahl *et al.*, 2008), an increased capacity to consume these exudates may lead to increased host health. These results suggest that the bacterial relationship with a host cell may depend upon host and/or environmental stressors. Exploring possible triggers for shifts in the bacteria-host relationship, and examining the specific mechanisms of interaction would be an exciting next step.

Phylogenetically distinguishable diatom-attached bacterial consortia have similar but not identical functional capacities

Diatom-associated bacterial consortia were previously divided into three groups based on phylogenetic differences (Baker and Kemp, 2014). Analysis of the general functional pathways did not support a conclusion that broad functional differences exist between these groups (Chapter 3). However, significant differences were observed in the frequency of occurrence of some individual functional genes that suggest Group 2, dominated by *Arthrobacte*r, may have greater growth and metabolic capacities than Group 1, the more diverse group. It is possible that the dominance of *Arthrobacter* in Group 2 may result from rapid growth after colonization; i.e. *Arthrobacter* may dominate by virtue of outgrowing other colonists. Additional studies would

be required to test this interesting possibility, and to assess whether broad categories of microbiomes exist with different functional capacities, different interactions with their hosts, and/or different propensities to colonize a host.

Thalassiosira-*associated bacteria isolated from the oligotrophic open ocean are commensal symbionts*

Bacteria attached to *Thalassiosira* appear to have some adaptations to relatively rapid consumption of carbon (such as diatom exudates) and an enhanced growth rate, relative to the functional capacities of bacterioplankton and plastic-associated bacteria. This was particularly evident in Group 2, dominated by *Arthrobacter*. Although parasitism or mutualism have been suggested in published studies of diatoms and bacteria in culture, genetic indicators for either parasitism or mutualism were generally absent in the *Thalassiosira*-associated bacterial metagenome. Genes that were involved in establishing an association with the host cell (such as motility, quorum sensing, and making an attachment structure) were present, but did not include all genes from that pathway.

A priori, we had speculated that our diatom host might be more dependent on its bacterial associates for limited nutrients, such as vitamin B_{12} and biologically available iron (Croft *et al.*, 2006; Amin and Green, 2009), in an oligotrophic system. It is possible that the amplification (MDA) and sequencing (400X MiSeq) methods may have resulted in underrepresentation of some genes. In addition, it is possible that the functional genes that were detected in this study have some mutualistic or parasitic function that has yet to be discovered. Within the KEGG database, individual genes are often assigned to and function in multiple pathways. With previous culture-based studies focusing on axenic and eutrophic systems, it is possible genes that are important to xenic or oligotrophic samples are poorly represented in the KEGG database. Utilizing xenic and/or oligotrophic samples holds great promise for learning further functions of previously identified genes and/or discovering new genes important to diatom-bacterial interactions.

It is also possible that this single "snap shot" of diatom microbiomes did not contain genes that were indicative of mutualism or parasitism. However, the bacteria attached to a diatom are not a static population (Chapter 1); moreover, even if bacterial taxa (detected using 16S rDNA) do not noticeably change, there is a possibility that the host cell will be impacted differently by different strains of the same phylotype colonizing the host's surface. This implies that future research on diatom-bacterial interactions should not only focus on observations of functions (through transcriptomics or proteomics), but should occur over the course of over a time course long enough to observe changes in this interaction.

Key findings and future directions

Overall, I found that diatoms affect and are affected by their associated bacteria, but that previously reported modes of diatom-bacterial interactions were mostly absent from my single-cell analyses. Diatom-bacterial interactions are dynamic (Chapter 1) and responsive to external (environmental) (Chapter 1) as well as internal (host or bacterial) (Chapter 1 & 2) cues. On single cells, the interaction between diatoms and bacteria was most likely commensal (Chapter 3). This implies that if mutualistic or parasitic bacteria occur on diatoms, they are not always present on single cells. However, these results do not preclude finding such pathways using population-level studies or even a greater number of single-cells explored during a longer time period (weeks-months).

This dissertation also suggests that the composition of bacteria attached to host cells is responsive to the concentration of macronutrients (Chapter 1) and that the impact of bacteria on a host cell is responsive to micronutrients (Chapter 2). Additionally, different hosts are impacted differently by inoculation with a single bacterial strain (Chapter 2). This suggests that the genes that were previously thought indicative of symbiotic interactions may only be expressed under specific environmental conditions and may only impact a subset of responsive diatom hosts. This advocates for further study of diatom-bacterial interaction in nutrient limited systems. Moreover, further study of bacteria and diatoms in oligotrophic systems may lead to the discovery of

genes that have functions similar to those previously researched (nutrient production or algicidal pathways) or novel functions involved in the interaction between diatoms and their attached bacteria.

In closing, this dissertation was an initial exploration of the relationship between bacteria and diatoms in an oligotrophic system, or in xenic cultures. I hope it has impressed upon the reader the role that nutrients play in determining bacterial associations as well as the importance of exploring the bacterial consortia (as well as individual strains). I look forward to future exploration of microbe-microbe symbiosis in oligotrophic systems and xenic cultures, as the importance of this interaction becomes an increasingly intriguing and accessible area of study to oceanographers around the world.

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