# MICROBIAL INTERACTIONS ASSOCIATED WITH BIOFILMS ATTACHED TO *TRICHODESMIUM* SPP. AND DETRITAL PARTICLES IN THE OCEAN

By

Laura Robin Hmelo
B.A., Carleton College, 2002

Submitted in partial fulfillment of the requirements for the degree of
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Author

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Joint Program in Oceanography/Applied Ocean Science and Engineering
Massachusetts Institute of Technology
And Woods Hole Oceanographic Institution
May 11, 2010

Certified by

_____

Dr. Benjamin A.S. Van Mooy
Associate Scientist of Marine Chemistry and Geochemistry, WHOI
Thesis Supervisor

Accepted by

_____

Prof. Roger E. Summons
Professor of Earth, Atmospheric, and Planetary Sciences, MIT
Chair, Joint Committee for Chemical Oceanography
Woods Hole Oceanographic Institution

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THESIS ABSTRACT

Quorum sensing (QS) via acylated homoserine lactones (AHLs) was discovered in the ocean, yet little is known about its role in the ocean beyond its involvement in certain symbiotic interactions. The objectives of this thesis were to constrain the chemical stability of AHLs in seawater, explore the production of AHLs in marine particulate environments, and probe selected behaviors which might be controlled by AHL-QS. I established that AHLs are more stable in seawater than previously expected and are likely to accumulate within biofilms. Based on this result, I chose to study AHL-QS in the bacterial communities inhabiting biofilms attached to *Trichodesmium* spp. and detrital (photosynthetically-derived sinking particulate organic carbon, POC) particles. These hot spots of microbial activity are primary sites of interaction between marine primary producers and heterotrophs and crucial components of the biological pump.

Biofilm communities associated with *Trichodesmium thiebautii* colonies in the Sargasso Sea differed considerably from seawater microbial communities. In addition, there was no overlap between the communities associated with tuft and puff colonies. These results suggest that bacterial communities associated with *Trichodesmium* are not random; rather, *Trichodesmium* selects for specific microbial flora. Novel 16S rRNA gene sequences are present both in clone libraries constructed from DNA extracted from colonies of *Trichodesmium* spp. and in culture collections derived from wild and laboratory cultivated *Trichodesmium* spp., supporting the idea that the phycosphere of *Trichodesmium* is a unique microenvironment.

Using high performance liquid chromatography-mass spectrometry, I demonstrated that bacteria isolated from *Trichodesmium* synthesize AHLs. In addition, I detected AHLs in sinking particles collected from a site off of Vancouver Island, Canada. AHLs were subsequently added to laboratory cultures of non-axenic *Trichodesmium* colonies and sinking POC samples. This is the first time AHLs have been detected in POC and indicates that AHL-QS was occurring in POC. Further, I showed that AHLs enhanced certain organic-matter degrading hydrolytic enzyme activities. My results suggest that AHL-QS is a factor regulating biogeochemically relevant enzyme activities on sinking POC and within the biofilms attached to *Trichodesmium* colonies and thereby may impact the timing and magnitude of biogeochemical fluxes in the ocean.

Thesis supervisor: Dr. Benjamin Van Mooy
Title: Associate Scientist, Department of Marine Chemistry and Geochemistry, WHOI

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# CHAPTER 1:

# AN INTRODUCTION TO MARINE BIOFILMS AND QUORUM SENSING

## *Biofilms in the ocean*

Biofilm-associated bacteria are key components in the global cycles of carbon and nitrogen, primarily through their role in the degradation of organic carbon and they are ubiquitous in the marine environment (Decho, 2000). They consist of dense bacterial populations embedded in an exopolysaccharide (EPS) matrix that binds bacteria to each other and to surfaces (Costerton *et al.*, 1995). Although biofilms on suspended particles typically contribute to less than 10% of the total bacterial population in the marine water column, they are at times responsible for 41-99% of extracellular enzyme production (Karner & Herndl, 1992; Smith *et al.*, 1995). Consequently, bacterial biofilms contribute significantly to the remineralization of organic material in the ocean (Decho, 2000).

Biofilm-associated bacterial communities in the marine water column are composed of different taxonomic groups of bacteria than free-living communities (DeLong *et al.*, 1993). DeLong and colleagues (1993) constructed small subunit rRNA gene clone libraries from DNA extracted from particle-attached and free-living bacteria collected in the Santa Barbara Channel off the west coast of the United States. They compared the diversity of rRNA clones from the two communities by restriction fragment length polymorphism (RFLP) analysis. Significantly, they found no overlapping restriction fragments between the two groups of samples. They determined that the planktonic communities were dominated by Alpha- and Gammaproteobacteria while

sinking particles within the same waters were dominated by Gammaproteobacteria (phylogenetically distinct from their free-living counterparts), Cytophaga, and Planctomyces. Since 1993, culture-independent techniques have repeatedly demonstrated that biofilms communities associated with particles and those which are free-living differ significantly in both species diversity and distribution. In addition, they necessarily express a different suite of phenotypes which are required for a surface-attached existence (Costerton *et al.*, 1995; Sauer & Camper, 2001; Whiteley & Greenberg, 2001; Pasmore & Costerton, 2003).

*Quorum sensing*

After planktonic bacteria initially attach to a surface, they proliferate and form clonal microcolonies. In the natural environment, these clonal populations contend with a multispecies community in which they must compete for niche space and nutrients. By necessity, each clonal population living within a biofilm must be capable of engaging in competition and cooperation in order to survive.

Quorum-sensing (QS) is a mechanism by which bacteria coordinate and cooperate with one another (Waters & Bassler, 2005). Gram-negative QS bacteria synthesize and respond to small (< 400 Da), diffusible chemical signals to gauge the density of their clonal population and engage in group-beneficial behaviors at high population density. These 'behaviors' aid in the colonization of a particular niche, the acquisition of nutrients and the expression of collective-defenses (Badri *et al.*, 2009). Specific QS-controlled phenotypes include bioluminescence, the production of antibiotic compounds to ward off

competitors, the production of extracellular hydrolytic enzymes to degrade complex organic substrate and the production of the EPS biofilm matrix.

When a diffusible product (e.g. enzyme or antibiotic) is produced or a biofilm phenotype is expressed (e.g. EPS production), it is advantageous to restrict the behavior until a critical mass of bacteria accumulates (Keller & Surette, 2006). By producing diffusible products in a coordinated fashion, the individual is ensured a greater return on the energetic investment in their production (Vetter *et al.*, 1998). Similarly, members of a mature biofilm are capable of exploiting the benefit of collective defense (provided by the biofilm EPS matrix or antibiotic production) (Keller & Surette, 2006). By limiting the production of energetically expensive chemicals until they can be used most efficiently, QS serves to enhance the survival of the individual and enhance the fitness of the clonal population (Keller & Surette, 2006).

In its most basic form, a quorum sensing system involves a gene that transcribes a signal molecule synthase protein, a chemical signal produced by the signal synthase, and a gene that transcribes a response regulator protein that responds to a signal and is capable of initiating or upregulating transcription of QS-regulated genes (Miller & Bassler, 2001; Waters & Bassler, 2005; Dickschat, 2010). At low population densities, signals are produced by a bacterium at a basal level and they diffuse out of the cell and into the extracellular environment. The bacterium interprets the concentration of the signal molecules as a proxy for the density of like-bacteria. As the concentration of the signals in the extracellular environment reaches a threshold level, they bind to the response regulator which initiates transcription of downstream QS-regulated genes.

Transcription is initiated simultaneously by all the members of the population such that the genes are synchronously expressed.

A variety of classes of bacterial QS signal molecules have been identified. They are all secondary metabolites that are not involved in primary metabolism (Keller & Surette, 2006). Acylated homoserine lactones (AHLs, Eberhard *et al.*, 1981), peptides (Novick & Muir, 1999), quinolones (Pesci *et al.*, 1999), furanosyl-borate diesters (Chen *et al.*, 2002) and gamma-butyrolactones (Onaka *et al.*, 1995) all function as intercellular signaling molecules. AHLs are the most common autoinducers produced by Proteobacteria (Lazdunski *et al.*, 2004), the most abundant bacteria in the oceans (Giovannoni & Rappe, 2000; Rusch *et al.*, 2007).

Thus far, homologues of the LuxI AHL-synthase (encoded by the *luxI* gene) have only been observed in the Alpha-, Beta- and Gammaproteobacteria (Case *et al.*, 2008). As of 2008, 26% of proteobacterial genomes (68 in total) contained *luxI* homologues (Case *et al.*, 2008) although the number of Proteobacteria in culture which produce AHLs is much higher (reviewed in Dickschat, 2010). Recently, several reports indicate that AHL-production might be a more widespread phenomenon. Paggi and co-workers observed putative AHL production in the haloalkaliphilic archaeon *Natronococcus occultus* (Paggi *et al.*, 2003). More recently AHLs have been structurally identified in extracts of a cyanobacterium (Sharif *et al.*, 2008) and a member of the Bacteroidetes (Romero, 2010) although AHL synthase genes (*luxI*) have yet to be identified in these organisms.

Taxonomic specificity of AHLs is conferred by structural variations on the acyl side chain. They can vary in the length and degree of unsaturation of the acyl-side chain and the substitutions on the 3-carbon (Figure 1). AHLs with branched acyl-side chains have recently been identified (Thiel *et al.*, 2009). In addition, aryl-homoserine lactones have been discovered; these molecules are synthesized by LuxI homologues and mediate cell-density dependent processes in the same manner as AHLs (Schaefer *et al.*, 2008).

*Quorum quenching and quorum sensing inhibition*

As QS is a mechanism by which bacteria in a biofilm can coordinate with one another to achieve a competitive behavior, QS can be a target of antagonistic bacteria. AHLs can be degraded by abiotic mechanisms which include base hydrolysis of the lactone ring (Yates *et al.*, 2002) and Claisen-like condensation to tetramic acids (Kaufmann *et al.*, 2005). In addition, AHLs can be biologically degraded by targeted AHL-acylases or AHL-lactonases (Dong & Zhang, 2005). Prior to the work presented in this thesis, quorum quenching by enzymatic means had not yet been demonstrated in marine environments, although it has been observed in terrestrial environments (Wang & Leadbetter, 2005). In addition to enzymatic quorum quenching, antagonistic bacteria and eukaryotes can produce chemicals capable of desensitizing the response regulators of competing bacteria. For example, Dulla and colleagues (2009) observed that bacterial epiphytes associated with the surface of leaves, were capable of prematurely inducing QS by the plant pathogen *Pseudomonas syringae* by producing *P. syringae*'s cognate autoinducer, 3-oxo-C6-HSL. In this manner, the swarming motility of *P. syringae* is suppressed and it

is unable to induce disease symptoms on the leaf surface. In the marine environment, the red algae *Delisea pulchra* has been observed to produce halogenated furanone compounds which interfere with the AHL-sensing response regulator, the LuxR protein, effectively preventing bacteria from settling on its surface (Manefield, 1999).

*Shifting views on the role of QS in biofilms*

The canonical role of QS is that it regulates population-density dependent gene expression; this view emerged from studies of bacteria grown in liquid batch cultures where cell densities exceed $10^6$ cells $mL^{-1}$ and absolute populations are orders magnitude greater. As QS is studied in the context of biofilms, such as on the surface of leaves (Dulla & Lindow, 2008) or within the rhizosphere of plants (Gantner *et al.*, 2006), this prevailing view of QS is coming under scrutiny. Even within the traditional model of QS, AHL concentration is decoupled from cell density because signal production is often positively regulated by the signal molecules themselves (autoinduction) and quorum quenching processes actively consume AHLs as they are produced (Dunn & Stabb, 2007).

"Efficiency-sensing" has been proposed as more appropriate description of the biochemical processes typically referred to as QS (Hense *et al.*, 2007). This term reflects the emerging acceptance that AHL-mediated biochemistry responds not only to population density, but to the spatial distribution or confinement of cells and mass-transfer properties of the environment (e.g. the hydrophobicity of a biofilm matrix) (Redfield, 2002; Hense *et al.*, 2007; Boyer & Wisniewski-Dyé, 2009). It is becoming

clear that QS is not only induced at high population densities typical of liquid cultures but also within small numbers of spatially discrete cells under appropriate physical circumstances which allow signal molecules to accumulate (e.g. Dulla & Lindow, 2008). In fact, under the right conditions, a single cell can be a 'quorum' (Gantner *et al.*, 2006; Carnes *et al.*, 2010). This new view of QS may be particularly meaningful when applied to bacteria embedded in hydrophobic biofilm matrices which restrict the diffusion of signal molecules; if QS can be induced by one cell, or tens of cells, QS may be more widespread in marine biofilms than previously considered.

*A brief history of the study of quorum sensing in the ocean*

The Hawaiian bobtail squid, *Euprymna scolopes*, along with numerous other marine squids and fishes, contains a light organ which is fueled by an extremely dense monoculture of the luminescent bacterium *Vibrio fischeri* (Ruby & Lee, 1998). It was demonstrated that *V. fischeri* regulates the emission of light in a cell-density dependent manner via diffusible 'autoinducer' signals (AHLs) in the process which would become known as 'quorum sensing' (Nealson *et al.*, 1970; Eberhard *et al.*, 1981; Fuqua *et al.*, 1994). The discovery of QS resulted from studies of the cell-density dependent bioluminescence of *V. fischeri* and since then, QS has been observed by numerous bacteria from a wide range of environments.

Although the vast majority of research has explored the role of QS in clinical isolates, numerous QS-capable proteobacteria have been isolated from marine snow (Gram *et al.*, 2002), corals (Tait *et al.*, 2010), sponges (Mohamed *et al.*, 2008) and

dinoflagellates (Wagner-Dobler *et al.*, 2005). In all of these cases, QS isolates have been Alpha- and Gammaproteobacteria. Hence, QS bacteria can be isolated from a wide range of marine environments without significant difficulty. These cultivation efforts remain valuable endeavors as these efforts continue to expand the landscape of known QS-capable genera (e.g. Thalassomonas and Spongiobacter, Mohamed *et al.*, 2008). These isolates will be a valuable resource as scientists continue to explore the role of QS in environmental bacteria.

While the isolation of QS-capable bacteria from marine environments indicates that a genetic capability for QS exists in this environment, it does not provide insight into *in-situ* QS activity. However, the results of several studies that report the presence of AHLs in marine samples imply that QS is occurring in natural marine microbial communities. For example, AHLs were detected in extracts of corals, by both biosensor and mass spectrometry, indicating that AHLs are produced by coral-associated bacteria *in-situ* (Taylor *et al.*, 2004). More recently, AHLs have been identified using sensitive mass spectrometric techniques in extracts of stromatolites (Decho *et al.*, 2009). AHLs have been demonstrated to stimulate the settlement of zoospores of the macroalgae *Ulva intestinalis* (Tait *et al.*, 2005) and *Enteromorpha* (Joint *et al.*, 2002). AHL-QS has also been implicated in large-scale bioluminescence events coincident with algal blooms (Nealson & Hastings, 2006), in the degradation of organic carbon (Gram *et al.*, 2002; Ziervogel & Arnosti, 2008) and in the regulation of algal blooms (Nakashima *et al.*, 2006).

Only a handful of studies have probed the dynamics of QS in natural environments. These studies provide insight into QS behavior in complex, multi-species environments which are difficult to replicate in the laboratory. In 2007, Huang and colleagues demonstrated that biofilms grown in the subtidal zone on petri dishes induced positive results by different biosensors at different stages of maturity (Huang *et al.*, 2007). This study was followed by one which replicated the latter results and provided conclusive mass spectral data confirming that different suites of AHLs were produced at different times (Huang *et al.*, 2008). These results imply that the suite of AHLs produced in a maturing multi-species biofilms is variable over time, which may reflect the succession of microbial taxa during biofilm-development. Decho and colleagues (2009) recently demonstrated that variability in AHL concentrations in natural stromatolites corresponds with diel variability. The variability is coincident with changes in pH within the stromatolite over the twenty-four hour period. They postulated that bacteria might differentially exploit AHLs based on their sensitivity to the alkaline conditions which develop in microbial mats during daylight hours; this would allow them to alternate the induction of particular gene sets on a day/night schedule (Decho *et al.*, 2009). Finally, Valle and colleagues (2004) were able to demonstrate that the addition of AHLs to an industrial wastewater treatment system induced changes in both the microbial community composition and ability of that community to degrade phenol. They concluded that AHL-mediated gene expression influenced the composition and function of the endogenous microbial community.

Together, these three studies offer tantalizing glimpses into QS by bacteria in complex environments. Undoubtedly, we will learn more about these systems in the coming years, but there are many others which remain to be explored. In this thesis, I initiated investigations into QS within two heretofore unexplored environments: the phycosphere of *Trichodesmium* spp. and bacterial communities attached to sinking particulate organic matter. These studies will be discussed further in a subsequent section of this Introduction.

The marine environment presents unique challenges to QS bacteria. First, the aqueous environment is one in which signals can be lost by diffusion very rapidly. Second, seawater is slightly basic (pH 8.2) and AHLs may be rapidly degraded by base-catalyzed hydrolysis (Yates *et al.*, 2002). Finally, in addition to the challenges presented by the abiotic environment, it is also likely that biological quorum quenching is a prevalent threat to QS bacteria.

Some of the physical challenges presented by the marine environment (e.g. diffusion) may be mitigated within biofilms. Within biofilms, the pH is generally lower (by up to 2 pH units) than that of the ambient environment (Vroom *et al.*, 1999; Horswill *et al.*, 2007 and references therein) which will slow the degradation of AHLs by abiotic base-catalyzed hydrolysis. In addition, biofilm structure may physically dramatically limit loss of AHLs by diffusion.

Marine bacteria seem to have evolved strategies to contend with some of the challenges presented to them by their chemical environment. For example, they tend to use long-chain AHLs or AHLs with oxo-substitutions on the 3-carbon of the acyl chain

(Wagner-Dobler *et al.*, 2005; Huang *et al.*, 2008). Compared to short-chain AHLs, long-chain AHLs are more stable molecules in seawater; they degrade more slowly by base-catalyzed hydrolysis (Yates *et al.*, 2002; Hmelo & Van Mooy, 2009). Interestingly, long-chain AHLs are less soluble than short chain AHLs; the logP (octanol-water partition coefficient) of unsubstituted-AHLs of chain lengths $C_4$ to $C_{12}$ varies from -0.11 to 4.9. The hydrophobic chemistry of the biofilm matrix may help to concentrate longer-chain AHL homologues (Horswill *et al.*, 2007 and references therein), suggesting that long-chain AHLs may be more effective within biofilms in comparison to a more aqueous setting. On the other hand, oxo-substituted AHLs are substantially more soluble than their unsubstituted homologues; the oxo-substitution lowers the logP of an unsubstituted-AHL of a given chain length by 1.26 units. Whether long chain unsubstituted AHLs are preferentially utilized within biofilms or if oxo-substituted AHLs are preferentially utilized in more aqueous environments remains to be investigated.

*Analytical approaches to the investigation of QS in natural environments*

Until recently, the occurrence of QS has almost always been inferred by the use of biosensor assays (McLean *et al.*, 1997; Gram *et al.*, 2002; Taylor *et al.*, 2004). In these assays (Steindler & Venturi, 2007; Dickschat, 2010) a mutant QS bacterium deficient in its ability to synthesize AHLs is monitored for an easily observable response (e.g. a color change) when it is exposed to exogenous AHLs. These assays are sensitive to the presence of AHL-like bioactivity and can detect extremely low quantities of AHL (see Table 1, Ravn *et al.*, 2001); however, these assays are subject to false positive and false

negative results (Holden, 1999; Ortori *et al.*, 2007; Steindler & Venturi, 2007; Garcia-Aljaro *et al.*, 2008). In addition, each biosensor can detect only a limited suite of specific AHL molecules (Steindler & Venturi, 2007), they are variably sensitive to those structures which they can detect (e.g. Winson *et al.*, 1995) and they do not provide direct structural information.

Coupling biosensor analysis with thin-layer chromatography affords a degree of structural identification unattainable by use of a biosensor alone (Shaw *et al.*, 1997). This hybrid technique has been used successfully in a number of studies to determine the AHL structures produced by environmental isolates (Gram *et al.*, 2002; Mohamed *et al.*, 2008; Tait *et al.*, 2010). Unfortunately, this technique still has limitations; it is still subject to the detection biases of the biosensor and it does not detect novel AHLs or structures for which authentic standards are not available.

Radioactive assays are among the most sensitive methods by which AHLs can be detected (see Table 1). $C^{14}$-radiolabelled methionine, one of the biosynthetic substrates required for the synthesis of AHLs, is incubated with a sample. The sample is extracted into ethyl acetate, separated by reverse-phase chromatography and then collected into fractions. The radioactivity of these fractions can then be assessed and retention times can be compared to standards. This approach offers the advantage of being unbiased toward particular structures and enables the detection of very low (e.g. femtomole) quantities of AHLs (Schaefer *et al.*, 2001). Since ethyl acetate extractable compounds that incorporate methionine are almost always acyl- or aryl-homoserine lactones produced by the well-characterized AHL-biosynthetic pathway (A. Schaefer, personal

communication) this method may be extremely effective at identifying novel AHLs, particularly if performed in parallel with mass spectrometry or nuclear magnetic resonance spectroscopy. While it has been used successfully to document production of AHLs in mucus from lungs infected with cystic fibrosis (Singh *et al.*, 2000) and the elucidation of the novel signaling compound, *p*-coumaroyl homoserine lactone, from *Rhodopseudomonas palustris* (Schaefer *et al.*, 2008), it has yet to be applied to environmental samples.

Chromatographic techniques coupled with mass spectrometry have been used extensively to identify specific AHL structures present in culture extracts and extracts from environmental samples (summarized in Table 1). Gas chromatography coupled to mass spectrometry (GC-MS) has been used to identify AHLs in extracts of environmental samples and culture extracts (Taylor *et al.*, 2004) . Unfortunately, oxo- and hydroxyl-AHLs are thermally labile and require derivitization prior to analysis by GC (Cataldi *et al.*, 2008).

High performance liquid chromatography (HPLC) is frequently utilized and can be coupled to some of the most sensitive mass spectrometers. HPLC has been coupled to fourier-transform ion-cyclotron-resonance mass spectrometry (FT-ICR-MS, Cataldi *et al.*, 2008), hybrid quadrupole-linear ion trap mass spectrometry (Ortori *et al.*, 2007), and triple-quadrupole mass spectrometry (Decho *et al.*, 2009). Each of these MS techniques provides distinct advantages (summarized in Table 1). FT-ICR-MS can measure mass-to-charge ratios at 1 ppm accuracy which, in combination with chromatographic retention time and/or mass fragmentation data, can allow the assignment of an elemental formula

to an analyte of interest (e.g. Cataldi *et al.*, 2008). Linear-quadrupole ion trap devices are capable of secondary mass spectrometry, enabling fine structural investigations of unknown analytes (e.g. Morin *et al.*, 2003; Ortori *et al.*, 2007). Finally, triple quadrupole devices are extremely sensitive and selective; they are capable of operating in a selected reaction monitoring (SRM) mode which allows quantitative observation of specific ions amidst an extremely complex matrix. While ion-trap MS have limits of detection (LOD) as low as 10 pmol (Morin *et al.*, 2003), no absolute analytical limits of detection have been published for FT-ICR-MS or triple-quadrupole MS devices analyzing AHLs; however, Cataldi et al (2008) determined that FT-ICR-MS is about an order of magnitude more sensitive than ion-trap MS and so we can estimate the LOD of FT-ICR-MS to be around 1 pmol (see Table 1). All HPLC-MS approaches utilize an electrospray-ionization interface, which can cause bias in the efficiency at which AHLs of different chain lengths are ionized (Ortori *et al.*, 2007). These biases can be overcome with the use of standard curves.

*An emerging ecological perspective*

In laboratory cultures, QS is known to regulate extracellular hydrolytic enzyme activity (Miller & Bassler, 2001) and the production of compounds toxic to algae (e.g. prodigiosin, Nakashima *et al.*, 2006), two processes which may directly impact the fate of photosynthetically derived carbon in the ocean. QS has been implicated in the degradation of marine snow in the environment (Gram *et al.*, 2002), although mechanistic studies have yet to be published. It is also becoming clear that QS may be involved in the

recruitment of eukaryotes to living and artificial surfaces in the ocean (Tait *et al.*, 2005; Dobretsov *et al.*, 2007). We might speculate from this that QS is also involved in the recruitment of eukaryotes to the surface of detrital particles as well. In recent years, several reviews have addressed the occurrence of QS in marine habitats (Cicirelli *et al.*, 2008; Dobretsov *et al.*, 2009; Decho *et al.*, 2010; Dickschat, 2010), reflecting a growing awareness that QS is prevalent in marine microbial communities.

*Objectives of this thesis*

QS is an understudied process in the oceans. Despite the extensive progress which has been achieved regarding the biochemistry of QS, very little is known about the basic occurrence in the environment (particularly in the marine environment) and the specific phenotypes controlled by QS in environmental bacteria. Most of the work has been undertaken using clinical isolates. The role of QS in marine bacteria (with the exception of symbiotic *Vibrio* spp.) is practically unknown.

In this thesis, I aimed to extend our knowledge of surface-attached bacterial communities in the ocean and the role which QS may play in those communities. In Chapter 2, I undertook an investigation of the chemistry of AHLs in seawater, seeking to place constraints on where QS is most likely to occur in the marine environment, thus guiding the remaining chapters. The results of Chapter 2 suggested that the bacterial communities associated with the surface of marine phytoplankton and marine snow occur at densities capable of supporting QS behavior.

In Chapters 3 through 5 and subsequent Appendices (1 and 2), I investigated the bacterial communities within biofilms attached to the bloom forming cyanobacterium, *Trichodesmium*, as well as photosynthetically-derived, detrital sinking-particulate organic carbon. In Chapter 3, I analyze a culture collection derived from *Trichodesmium* epibiont communities. Among the results of this study is the observation of QS in *Erythrobacter*, a genus within the alphaproteobacteria in which QS has not yet been observed. In Chapter 4, I employ a culture independent clone library approach to more deeply investigate the structure of the epibiont community associated with *Trichodesmium* spp. from the same field site as the samples used in Chapter 3 (Bermuda Atlantic Time Series station in the Sargasso Sea). I explore another microbial system in Chapter 5, in which I used a novel approach to investigate the role of QS in sinking particulate organic carbon-associated bacteria. The results of this study demonstrate for the first time that QS is involved in the regulation of extracellular enzyme production in sinking particulate organic carbon-attached bacteria. The implications of these results with regards to our understanding of the marine carbon cycle are discussed in detail.

Appendices 1 and 2 are extensions of Chapters 3-5 in which I present the results of pilot studies which further investigate the community structure of *Trichodesmium* spp. and the occurrence and potential functions of QS within those communities. General conclusions and suggestions for future research directions are discussed in the final chapter of this thesis.

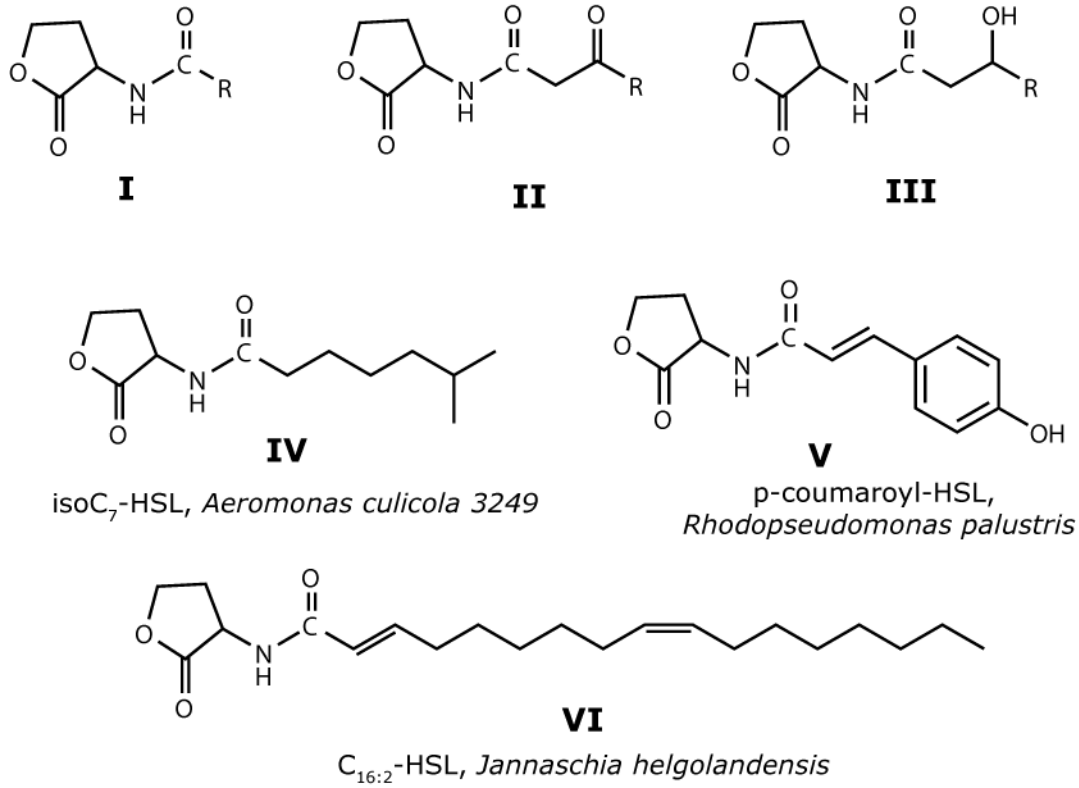


**Figure 1.** Examples of known AHL structures. **I**, **II**, and **III** represent the canonical forms in which **I** is an 'unsubstituted' AHL, II is a '3-oxo' AHL and **III** is a '3-hydroxy' AHL. R is an acyl side chain of length of up to 18 carbons. **IV**, **V**, and **VI** are examples of recently elucidated structural variations. **IV** is an *iso*-$C_7$-HSL produced by *Aeromonas culicola* 3249 (Thiel *et al.*, 2009). **V** is p-coumaroyl-homoserine lactone, an aryl-homoserine lactone, produced by *Rhodopseuodomonas palustris* (Schaefer *et al.*, 2008). **VI** is a $C_{16:2}$-HSL produced by the alphaproteobacterium *Jannaschia helgolandensis* (Thiel *et al.*, 2009).

**Table 1.** Summary of analytical techniques discussed in this chapter. The mass resolution of the various mass spectrometric techniques is indicated. Low mass resolution is nominal mass resolution ('1'). In this dissertation, high mass resolution corresponds to 100,000 at m/z 400. [a]High performance liquid chromatography (HPLC). [b]LCQ, LTQ and TSQ are not acronyms but rather nicknames for the Thermo Finnigan/Thermo LCQ Deca-XP ion trap mass spectrometer, LTQ-Ultra ion trap mass spectrometer, or TSQ triple-quadrupole mass spectrometer, respectively. Samples were introduced to all three mass spectrometers via an electrospray ionization interface. [c]The LOD for FT-ICR-MS is estimated from the LOD from the ion trap mass spectrometer. See text for details. [d]The LOD for the biosensor technique is variable and depends both on the specific biosensor in question and its sensitivity to a given AHL

| Analytical Technique | Acronym | Application | Mass Resolution | Limit of Detection (LOD) | References |
|---|---|---|---|---|---|
| HPLC[a]-Ion trap mass spectrometry | LCQ or LTQ[b] | Molecular ion and mass fragmentation for structure elucidation | low | ~10 pmol | (Morin *et al.*, 2003; Hmelo & Van Mooy, 2009) |
| HPLC- Fourier-transform ion-cyclotron resonance mass spectrometry | FT-ICR-MS | Exact mass of molecular ion for elemental composition for structure elucidation | high | ~1 pmol[c] | (Cataldi *et al.*, 2008) |
| HPLC- Triple quadrupole mass spectrometry | TSQ[b] | Highly selective selected-reaction-monitoring (SRM) mode | low | unknown | (Decho *et al.*, 2009) |
| Gas chromatography-mass spectrometry | GC-MS | Low LOD and mass fragmentation for structure elucidation | low | ~0.1 pmol | (Charlton *et al.*, 2000; T. R. I. Cataldi, G. Bianco, M. Frommberger, Ph. Schmitt-Kopplin, 2004) |
| Biosensor | n/a | Indicates AHL-like bioactivity | n/a | as low as <10 pmol[d] | (McClean *et al.*, 1997; Brelles-Marino & Bedmar, 2001; Ravn *et al.*, 2001; Steindler & Venturi, 2007) |
| Biosensor- Thin layer chromatography | TLC | Adds a degree of compound-specificity when coupled with biosensor overlay | n/a | n/a | (Shaw *et al.*, 1997) |
| Radiolabel assay | n/a | Synthesis rates | n/a | ~90 fmol | (Blosser-Middleton & Gray, 2001; Schaefer *et al.*, 2001) |

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## CHAPTER 2:

# KINETIC CONSTRAINTS ON ACYLATED HOMOSERINE LACTONE-BASED QUORUM SENSING IN MARINE ENVIRONMENTS

Laura Hmelo and Benjamin A. S. Van Mooy

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## ABSTRACT

Quorum sensing (QS) via acylated homoserine lactone (AHLs) was discovered in the ocean, yet AHLs are expected to be very short-lived at seawater pH due to rapid abiotic degradation. Quorum quenching, the enzymatic degradation of AHLs, is also likely. To better understand the potential for QS to regulate behaviors of marine bacteria we investigated the degradation of a variety of AHL molecules in several types of saltwater media. We did this by incubating AHLs and tracking their concentration using high performance liquid chromatography / electrospray-ionization mass-spectrometry (HPLC/ESI-MS). AHL concentrations decreased with time, and degradation rate coefficients were calculated by applying a first-order rate law. The rate of abiotic degradation showed strong dependence on acyl-chain length and the presence of 3-oxo substitutions on the acyl-chain. We found that the rate of abiotic degradation of AHLs in

artificial seawater was much slower than that predicted by an oft-cited equation that takes only pH into account. However, AHLs degraded more rapidly in natural seawater than in artificial seawater, an observation we found to be due to quorum quenching enzyme activity. By applying calculated degradation rates in a simple steady-state calculation, we suggest that despite the observed quorum quenching activity, AHLs are likely to be viable signals in organic particles and in other microbial "hotpsots" in marine environments.

## INTRODUCTION

The first description of bacterial quorum sensing (QS) arose from studies of the marine luminescent bacterium *Vibrio fischeri* which colonizes the light organs of eukaryotic hosts, most famously, the Hawaiian bobtail squid (*Euprymna scalopies*) (Coffey 1967, Eberhard et al. 1981) . *V. fischeri*'s QS system was found to be regulated by acylated homoserine lactones (AHLs), and AHL-based QS has been subsequently identified in numerous isolates of marine Proteobacteria (e.g. Eberhard et al. 1981, Gram et al. 2002, Wagner-Dobler et al. 2005). Since Proteobacteria often dominate marine microbial communities (Giovannoni & Rappe 2000), AHL-based QS has the potential to be widespread in the sea. For example, Miller et al. (2005) invoked QS-induced bacterial luminescence to explain a "milky sea" event where a 15,400 $km^2$ patch of the Arabian Sea was observed by satellite to glow for several days. These authors speculated that the light was produced by a dramatic increase in the local concentration of the luminescent bacterium *V. harveyi* in response to resources supplied by a coincident algal bloom.

In addition to luminescence, AHLs have been shown to regulate a number of potentially ecologically relevant phenotypes; these include siderophore production, hydrolytic enzyme activity and biofilm formation (Miller & Bassler 2001 and refs therein). These behaviors could play an important role in the degradation of sinking organic matter in the ocean, and since AHL-producing bacteria were first isolated from marine snow by Gram et al (2002) QS has been increasing implicated as a potentially significant process in the marine carbon cycle (e.g. Buchan et al. 2005, Ziervogel & Arnosti 2008).

AHLs are not only signals for bacterial communication; they can also mediate the settlement of eukaryotic larvae on bacterial biofilms (Joint et al. 2002, Tait et al. 2005). Larvae have been shown to use the presence or absence of AHLs to determine the suitability of a surface for permanent settlement (Joint et al. 2002, Tait et al. 2005). AHL production in biofilms thus plays a role in the ecology of higher organisms and in the biofouling of submerged surfaces (e.g. shiphulls).

AHLs are active as QS-signals only in their intact, lactone-based form, and the lactone moiety of AHLs is highly susceptible to rapid, base-catalyzed abiotic degradation (Yates et al. 2002). It has been shown that AHLs are very short-lived in aqueous media with pH in the range of seawater (Yates et al. 2002). Furthermore, it appears that AHLs are also degraded enzymatically in natural systems (Delalande et al. 2005, Wang & Leadbetter 2005), a process that has been termed "quorum quenching".

Taxonomic and functional specificity of AHLs is conferred by the length, degree of unsaturation, and presence or absence of oxo- and hydroxyl-substitution on the acyl

chain. Multiple AHLs may be used by the same organism to regulate independent processes (Gonzalez & Marketon 2003 and refs therein). Importantly, the rate of both abiotic degradation and quorum quenching also appears to be affected by these aforementioned variations in AHL structure (Yates et al. 2002, Delalande et al. 2005, Wang & Leadbetter 2005). It is likely, although not demonstrated, that organisms will produce a particular suite of AHLs based on each molecule's stability in a given environment.

In this study, we sought to quantify the rates of abiotic and enzymatic AHL degradation in seawater and to gain a better understanding of the conditions that must be met in order for AHL-based QS to take place in marine environments. To do this, we used high performance liquid chromatography / electrospray-ionization mass-spectrometry (HPLC/ESI-MS) to track the degradation of intact, biologically active AHLs in a number of seawater incubation experiments. Even though QS research owes its provenance to the study of marine bacteria, there have been few previous investigations of the stability of AHLs under natural seawater conditions (Tait et al., 2005).

## METHODS

*A note on nomenclature:* We abbreviate the names of specific AHLs as:(3O)C(*n*)-HSL where *n* refers to length of acyl side chain bound to homoserine lactone (HSL) and 3O indicates the presence of a 3-'oxo'(ketone) group on the acyl-side chain (Fig. 1).

**Incubation design**

Rates of AHL degradation were determined by incubating synthetic AHLs in defined liquid media and tracking their concentrations by HPLC/ESI-MS. Synthetic C4-, 3OC6-, C6-, C7-, 3OC8-, C8- and C12-HSL were purchased from Sigma-Aldrich and individual solutions were made with HPLC-grade methanol to a concentration of 500 μmol $L^{-1}$. A 30 μL aliquot of a standard was added to the bottom of a pre-combusted 40 mL clear glass vial, and the methanol was allowed to evaporate from the bottom of the vial. Next, 30 mL of liquid incubation media (defined below) was added. The vials were then sealed with UV-sterilized PTFE (Teflon) lined caps and incubated on the lab bench. All incubations were conducted at room temperature (approximately 23°C). The final AHL concentration at the start of all experiments was 500 nmol $L^{-1}$; this value is comparable to the range of AHL concentrations observed in culture media and measured in the light organs of *Euprymna scolopes* and *Euprymna morsei* (0.4 – 400 nmol $L^{-1}$) (Kaplan & Greenberg 1985, Schaefer et al. 2002, Burton et al. 2005). All incubations were conducted such that triplicate incubations could be sacrificed at each time point for AHL extraction, analysis, and quantification.

**Extraction and analysis of AHLs**

Incubations were extracted three times with 13mL dichloromethane (Fisher Scientific). Immediately prior to extraction, 10 μL of 500 μmol $L^{-1}$ Z-homoserine lactone (Sigma-Aldrich) was added to the aqueous phase as an internal standard. The combined extracts were passed through a column of combusted sodium sulfate to remove residual water and

then dried under nitrogen. Extracts were transferred in methanol to small glass vials for analysis on a Thermo Finnegan Surveyor HPLC coupled to a Thermo-Finnegan LCQ Deca-XP Mass Spectrometer through an electrospray ionization interface. Separation of AHLs was achieved by running a water-methanol gradient through an Altima HP C18 reverse-phase chromatography column (Agilent; 5 μm, 2.1x150 mm) with guard column. Solvent A was MilliQ water (0.1% formic acid) and solvent B was methanol (0.1% formic acid). The gradient program was as follows: 0-25 min, gradient from 90% A/ 10% B to 100% B; 25-26 min, 100% B; 26-27 min, gradient to 90% A/ 10% B; 27-36min 90% A/ 10% B (column equilibration). Intact AHLs have masses, fragmentation patterns, and chromatographic properties that are distinct from their degradation products (data not shown), and the peak areas of the molecular ions from intact the AHLs (Fig. 1) were integrated and converted to concentration units based on the recovery of the internal standard and comparison to standard curves. Standard curves were prepared daily with a fresh mixture of AHLs, and the standard mixture was reanalyzed after every sixth sample.

**Incubation media**

All liquid incubation media were prepared immediately prior to their use in the AHL degradation incubations. Whole seawater for all incubation experiments was collected in March 2008 from Vineyard Sound, Massachusetts (41$^{o}$ 32' N, 70$^{o}$ 40'W) and was filtered in the field through a coarse (100 μm) mesh filter to remove seaweed and large particulates. Shortly thereafter in the laboratory, the seawater was filtered through a 0.22

μm cellulose acetate bottle top filter (Corning) under 200 mbar vacuum, and the pH determined (see results).

Artificial seawater was prepared using 18MΩ MilliQ water according to the Trace MBL artificial seawater recipe (423 mmol $L^{-1}$ NaCl, 8.27 mmol $L^{-1}$ KCl, 9.27 mmol $L^{-1}$ $CaCl_2$, 22.94 mmol $L^{-1}$ $MgCl_2$, 25.50 mmol $L^{-1}$ $MgSO_4$, 2.14 mmol $L^{-1}$ $NaHCO_3$, 0.23 mmol $L^{-1}$ $SrCl_2$. 0.07 mmol $L^{-1}$ NaF, 0.39 mmol $L^{-1}$ $H_3BO_3$, 0.75 mmol $L^{-1}$ KBr). The artificial seawater was filtered through a 0.22 μm cellulose acetate bottle top filter (Corning) under 200 mbar vacuum, and the pH adjusted to that of Vineyard Sound seawater prior to being used in the degradation experiments by adding small quantities of 1 mol $L^{-1}$ HCl and 1 mol $L^{-1}$ NaOH as required.

To assess the impact of enzymes on the degradation of AHLs in Vineyard Sound seawater, endogenous enzymes were denatured either by treating it with 40 μg $L^{-1}$ proteinase-K (Fisher) and incubating at 37°C for one hour or by boiling the seawater for 10 minutes. The pH of these treated seawater media was also measured and adjusted to pH 7.9.

**Data Analysis**

The decrease in the concentrations of these molecules with time was modeled using the first-order rate law equation:

$$Ln\frac{C}{C_o} = -kt \quad (1)$$

Where, C is the concentration at a given time point, $C_o$ is the concentration at time zero, t is the length of the incubation (hr) and k is the rate coefficient ($hr^{-1}$). Data when plotted in this manner will fall in a straight line if the reaction kinetics are first order; the rate coefficient is the negative slope of the line and is reported ± the standard error of the slope. We estimate that two slopes from two sets of degradation experiments are different from one another when their difference is greater than twice the combined standard error of the two slopes.

Incubations with C6-HSL and 3OC6-HSL were conducted such that they could be sampled at five time points. First order kinetics were established for both C6-HSL (Fig. 2) and 3OC6-HSL. Subsequently, only two time points were measured and used to determine degradation coefficients of the other AHLs.

**RESULTS**

The AHLs C6- and 3OC6-HSL are the archetypal AHLs from vibrios and are common in many other QS Proteobacteria (Miller & Bassler 2001); our study focused primarily on these two molecules. In our incubation experiments, all of which were conducted at a pH of 7.9 (the pH of Vineyard Sound seawater), we found that the concentrations of these

AHLs decreased with time according to first order degradation kinetics in natural and artificial media (Fig. 2). Loss of AHLs by first order kinetics was observed whether the AHL was incubated under abiotic conditions (artificial seawater) or in natural seawater from Vineyard Sound, Massachusetts (filtered through 0.2 μm poresize membrane).

The degradation rate coefficient of C6-HSL in natural seawater was 0.043 ± 0.003 $hr^{-1}$, and this rate is significantly faster than the rate we observed in artificial seawater, which was 0.028 ± 0.001 $hr^{-1}$ (Fig. 3). Likewise, 3OC6-HSL degraded at a rate of 0.116 ± 0.005 $hr^{-1}$ in natural seawater, which was more rapid than the 0.094 ± 0.002 $hr^{-1}$ observed in artificial seawater (Fig. 3). Similar observations were made for 3OC8-HSL (Fig. 3). Regardless of media, the oxo-substituted AHLs (3OC6-and 3OC8-HSL) degraded at least twice as fast as that of C6-HSL.

We pretreated natural seawater with proteinase-K to digest endogenous enzymatic proteins and found that this slowed the degradation of C6-, 3OC6-, and 3OC8-HSL relative to that in untreated filtered seawater (Fig. 3). The degradation rate coefficients in proteinase-K treated natural seawater were 0.032 ± 0.001, 0.102 ± 0.003, 0.102 ± 0.004 $hr^{-1}$ for C6-, 3OC6- and 3OC8-HSL respectively and were indistinguishable from the rates observed in artificial seawater. We performed a similar experiment by heat-treating the natural seawater and the degradation coefficients also decreased to 0.034 ± 0.002, 0.089 ± 0.2, and 0.087 ± 0.01 $hr^{-1}$, respectively.

The degradation rate coefficients of C4-, C7-, and C8-, and C12-HSLs were also measured in natural and artificial seawater. We observed a chain-length dependence amongst straight-chain AHLs such that longer-chain AHLs degrade more slowly than

shorter chain AHLs (Fig. 4). This relationship did not appear to hold for 3-oxo substituted AHLs, although only two molecules of this type were examined.

We assessed the potential impact of abiotic sorption of AHLs to the glass walls of the incubation vials; this was done in incubations of C6-HSL and 3OC6-HSL conducted with different volumes of water but in the same 40 mL vials. By doing this, we affected a 5-fold difference in the surface area to volume ratio of the incubations, but observed no measurable differences in degradation rate coefficients. Thus, surface sorption did not impact the observed rates of AHL degradation and will not be further discussed.

## DISCUSSION

The effect of pH on the abiotic degradation of AHLs in aqueous media (LB media, organic buffers or pure water) is well established: AHLs degrade more rapidly at higher pH (Yates et al. 2002, Delalande et al. 2005). In seawater, with an average pH of 8.2, AHLs are expected to degrade quite rapidly. However, to our knowledge, the degradation rates of AHLs in seawater have yet to be explicitly determined. In this study, we focused our attention on the understanding the effects of both abiotic degradation and quorum quenching enzyme activity on AHL degradation in natural seawater collected from Vineyard Sound, Massachusetts.

In artificial seawater, only abiotic factors influenced the degradation of AHLs. Both unsubstituted and 3-oxo substituted AHLs are highly susceptible to degradation by base-catalyzed abiotic lactonolysis (Yates et al., 2002). In addition, 3-oxo substituted AHLs may degrade via an abiotic Claisen-like rearrangement (Kaufmann et al. 2005).

Accordingly, 3OC6- -HSL degraded significantly faster than C6-HSL in artificial seawater and all natural seawater-based media we tested (Fig. 3). However, we observed that 3OC6-HSL degrades much more slowly in artificial seawater, ($0.094 \pm 0.002$ $hr^{-1}$) than the rate predicted by the pH-dependent formula ($0.26$ $hr^{-1}$) of Schaffer et al (2000), which is often cited (e.g. Wang and Leadbetter, 2005). This suggests that AHL degradation may occur more slowly in saltwater relative to other aqueous media.

In contrast, AHLs in natural seawater are subject to the above abiotic degradation mechanisms as well as attack from quorum quenching enzymes. In natural seawater the degradation rate coefficients of C6-, 3OC6- 3OC8-HSL were 54, 23 and 57% faster respectively than in artificial seawater and we assert that this difference was due to quorum quenching activity. Evidence for this assertion comes from the observation that the denaturation of endogenous proteins in natural seawater decreased the rates of AHL degradation such that they were indistinguishable from artificial seawater, which confirms that at significant fraction of the observed quorum quenching activity was due to free enzymatic proteins.

Our observations of quorum quenching activity in seawater is striking since it suggests that the marine microbiota in Vineyard Sound have invested in enzymes to accelerate the degradation of AHL molecules that are already relatively short-lived in seawater due to abiotic degradation processes. Alternatively, the AHLs may have been degraded by non-specific enzyme activity, which suggests that quorum quenching is not the result of a specific strategy directed at disrupting QS systems but is merely the fortuitous result of entirely unrelated enzyme activity. Our observation of active quorum

quenching in natural seawater outside of biofilms, suggests that this process could place important constraints on the role AHLs in eukaryotic larval recruitment under natural conditions.

In this study, short-chain AHLs degraded more rapidly than long-chain AHLs in both artificial seawater and filtered natural seawater. This result is consistent with previous studies in freshwater media showing that AHLs with shorter acyl chains are shorter-lived (Yates et al. 2002) Longer chain AHLs may be better optimized to function in a seawater medium due to their longer half-life with respect to abiotic degradation. This inference is consistent with numerous reports that AHL-producing marine bacterial isolates often produce long chain AHLs rather than short chain AHLs (Schaefer et al. 2002, Wagner-Dobler et al. 2005). On the other hand, bacteria in marine environments could use AHLs with shorter acyl chains to communicate over relatively shorter distances. Yet organisms using shorter chains AHLs would be required to synthesize them at a greater rate to achieve a given concentration in the environment.

Using our measured rate coefficient for 3OC6-HSL degradation in natural Vineyard Sound seawater as an example, we can make a steady state estimate of the rate of production required to maintain 3OC6-HSL at concentrations sufficient for the induction of bacterial QS systems in marine environments. We attempted to measure AHLs directly in natural seawater from Vineyard Sound, but found that levels were below our analytical limit of detection of approximately 10 pmol $L^{-1}$. QS systems in Proteobacteria are generally not induced until AHL concentrations reach at least 10 nmol $L^{-1}$ (Kaplan & Greenberg 1985), which is three orders of magnitude higher than our

detection limit; this suggests that AHL-regulated behaviors are very unlikely to be active in open seawater. However, if we assume the required concentration of 3OC6-HSL in seawater is indeed 10 nmol $L^{-1}$, then we can calculate the maximum first-order molar degradation rate of this AHL as simply the product of this concentration and the degradation rate coefficient (k) we measured:

$$\textit{Degradation rate} = k[3OC6\text{-}HSL] = 0.11\ hr^{-1} \times 10\ nmol\ L^{-1} \approx 1.1\ nmol\ L^{-1}\ hr^{-1} \quad (3)$$

At steady state, the degradation rate this AHL would be equal to its production rate.

Using the 3OC6-HSL production rate the required density of AHL producing bacterial cells can also be estimated. Fully-induced QS Proteobacteria synthesize AHLs at rates on the order of 1 amol $cell^{-1}$ $hr^{-1}$ (Wang & Leadbetter 2005) and so at steady state, a minimum of $1.1 \times 10^9$ cells $L^{-1}$ would be required in order for production to match our calculated molar degradation rate. While total cell concentrations in the open ocean may reach these concentrations, for monospecific concentrations to approach this level would require truly exceptional environmental conditions. Obviously, the uncertainty in this estimate could be as high as an order of magnitude. Nonetheless, this analysis suggests, as expected, that QS-thresholds are much more likely to occur in marine biofilms, such as those that occur on sinking particles where total bacterial cell concentrations in excess of $10^{11}$ cells $L^{-1}$ are possible. These and other microbial "hotspots" play a significant role in the marine carbon cycle (Azam et al., 1998, and refs therein).

The rates of AHL degradation we determined in this study enabled us to make an estimate of the concentration of bacteria required to induce QS behaviors in natural marine systems. Our data do not preclude a planktonic bacterial origin for the large-scale 'milky sea' reported by Miller et al (2005), but they do put minimum constraints on planktonic population densities that would be required for QS-induced Proteobacterial luminescence events to occur in the open ocean.

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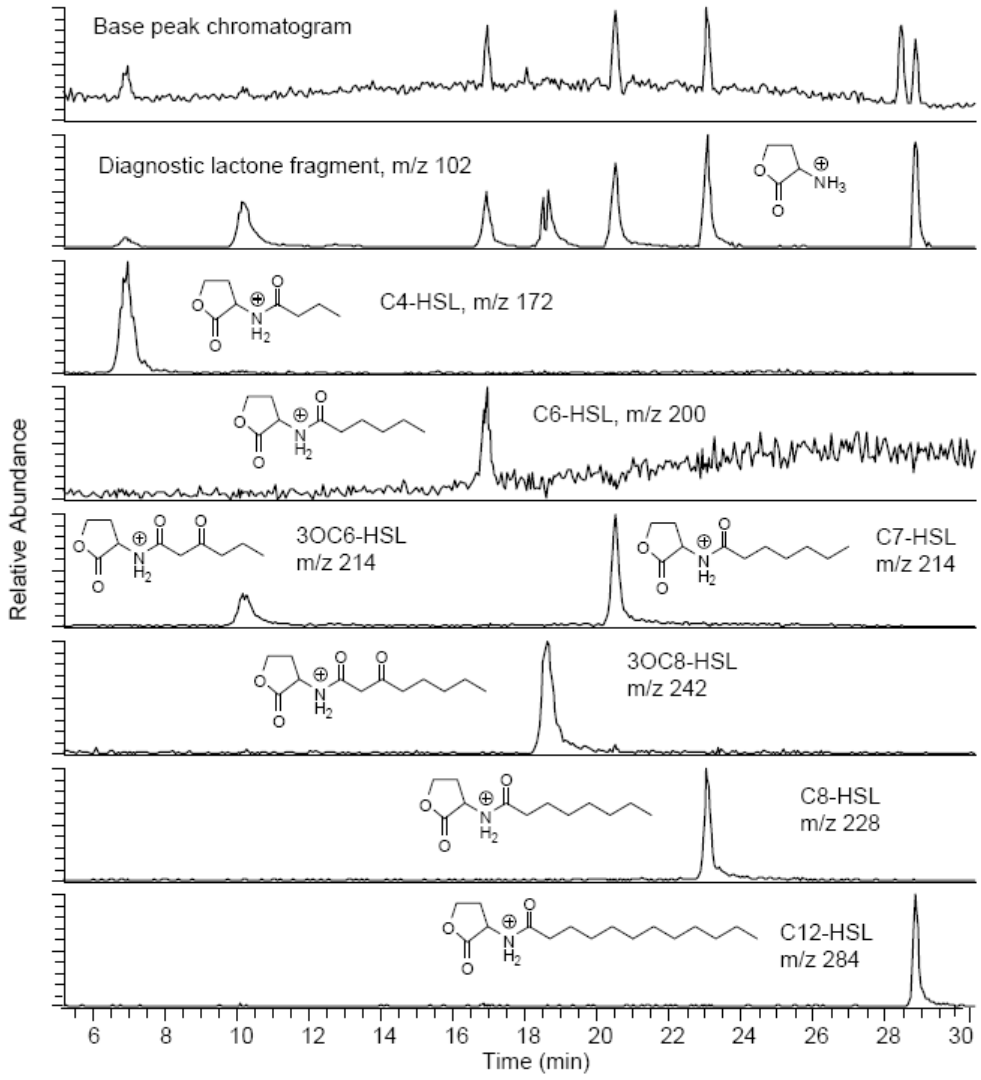


**Figure 1.** Extracted ion chromatograms from HPLC/ESI-MS analysis of AHLs used in this study. Molecular structures of C4-, 3OC6-, C6-, C7-, 3OC8-, C8-, and C12-HSL are inset next to respective the chromatogram . The names of specific AHLs are abbreviated as:(3O)C(n)-HSL where n refers to length of acyl side chain bound to homoserine lactone (HSL) and 3O indicates the presence of a 3-'oxo'(ketone) substituent on the acyl-side chain.

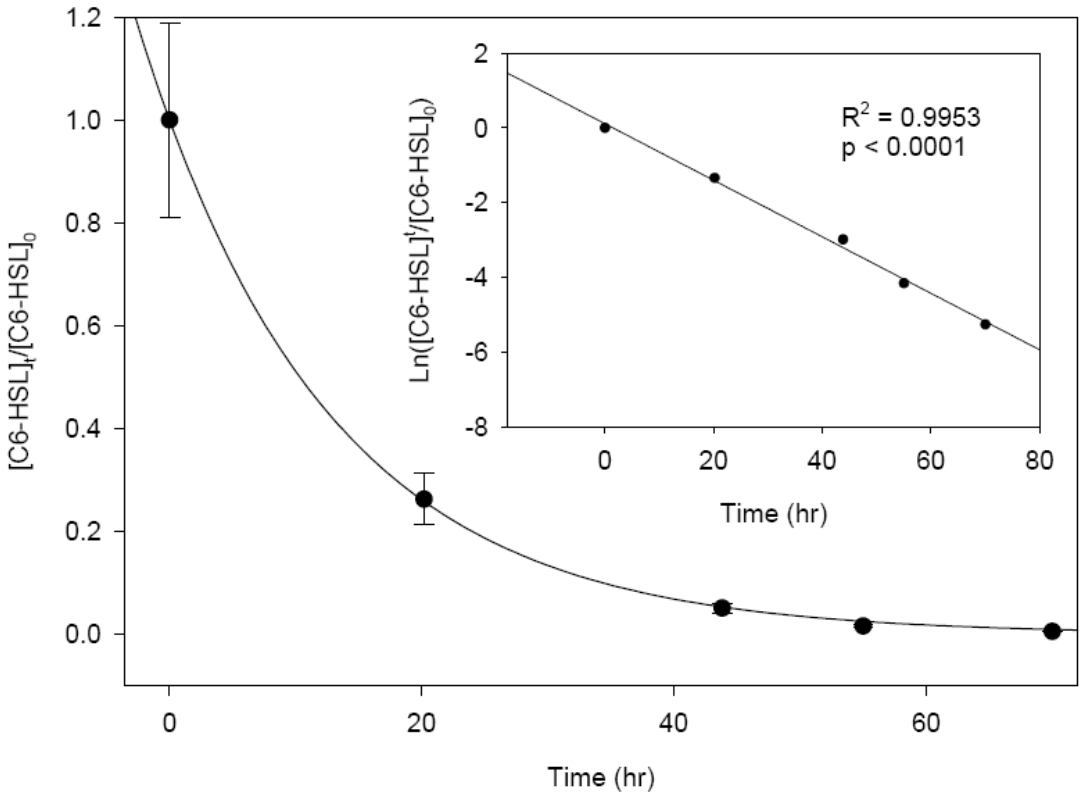


**Figure 2.** Plot of C6-HSL concentration over time during incubation in seawater from Vineyard Sound, Massachusetts. The inset is the natural logarithm transformed data as applied in Equation 1.

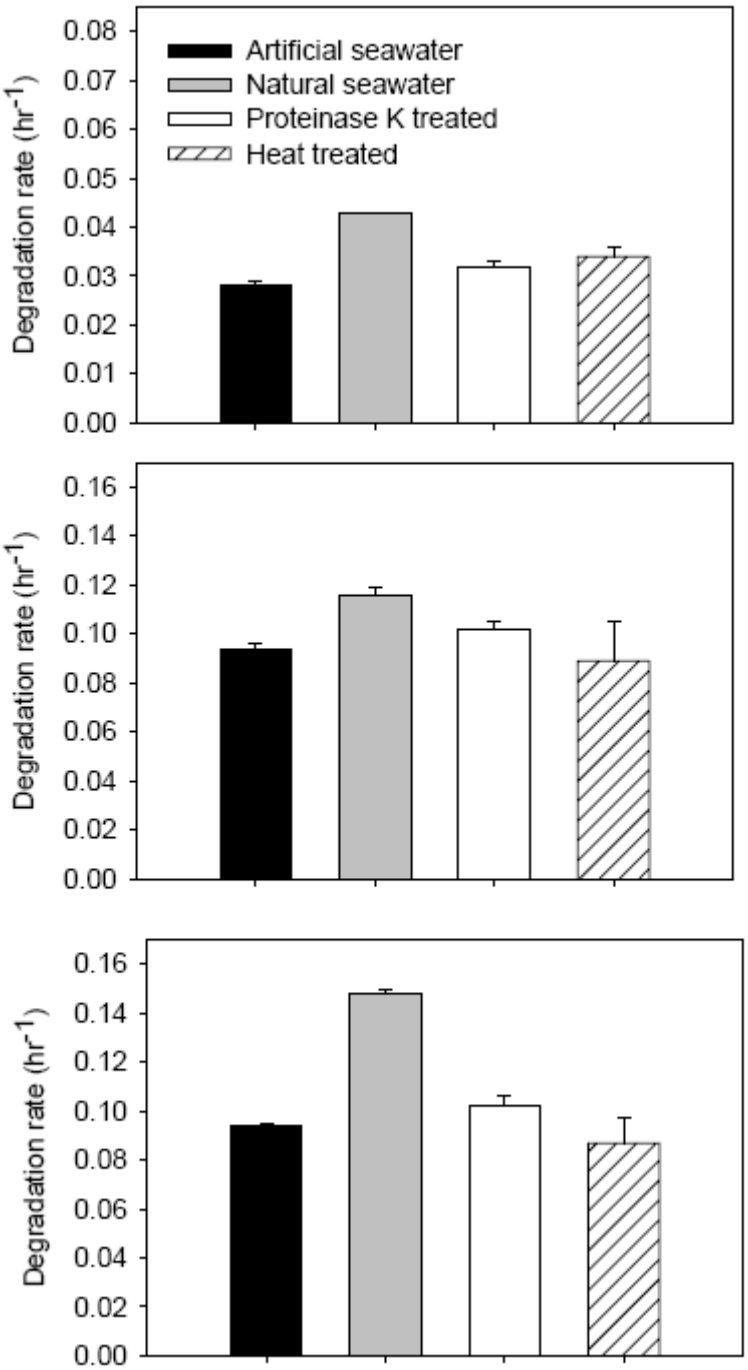


**Figure 3.** Degradation rate coefficients of C6-HSL (top), 3OC6-HSL (middle) and 3OC8-HSL (bottom) in artificial seawater, natural seawater, proteinase K treated natural seawater, and heat-treated natural seawater.

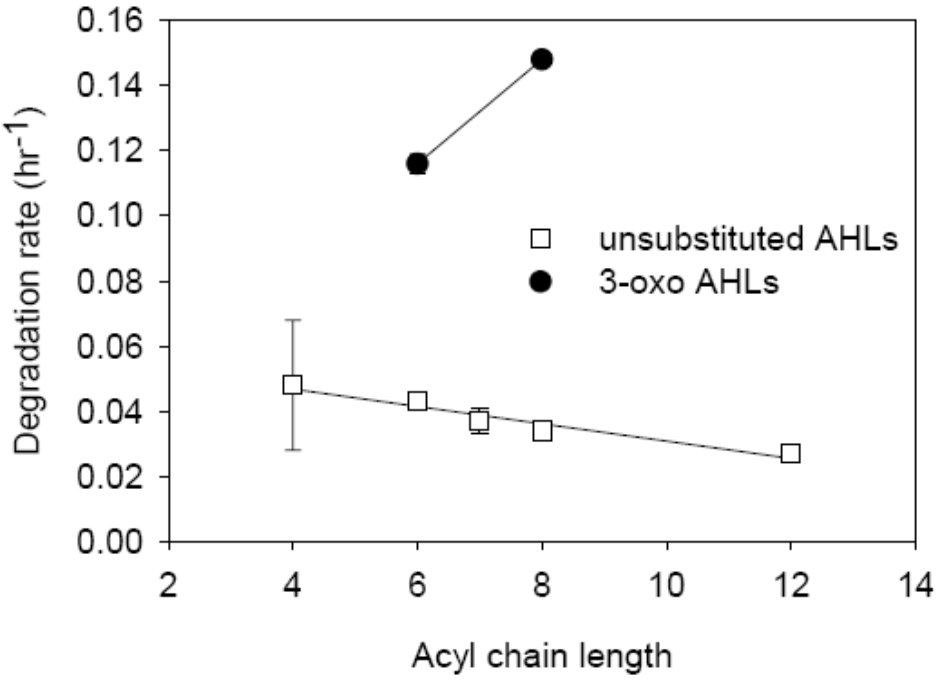


**Figure 4.** Plot of the degradation rate coefficients of various AHLs in 0.22 μm filtered seawater versus the length of the acyl-side chain.

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## CHAPTER 3:

# COMBINED BIOSENSOR AND HPLC-MS APPROACH TO THE ANALYSIS OF ACYLATED HOMOSERINE LACTONE SIGNALS FROM COMPLEX SAMPLE-MATRICES: APPLICATION TO THE ANALYSIS OF AHLS PRODUCED BY EPIBIONTS OF WILD *TRICHODESMIUM* SPP.


## ABSTRACT

*Trichodesmium* spp. are known to harbor dense epibiont populations, a sizeable percentage of which are related to the Proteobacteria. Proteobacteria are known to utilize quorum sensing (QS) under laboratory conditions and the physical and chemical environment of the *Trichodesmium* phycosphere should be conducive to efficient QS mediated by the signal, acylated homoserine lactone (AHL). In this study, 142 cultivars were screened for the production of AHLs by use of a broad-spectrum biosensor as well as two different mass spectrometric methods; a comparison of a commonly used low-sensitivity, low-resolution ion trap device and a high-sensitivity, high-resolution device (Fourier transform-ion cyclotron resonance-mass spectrometry, FT-ICR-MS) clearly showed that the FT-ICR-MS is the most appropriate tool for the analysis of AHLs in complex sample matrices. Eight bacterial isolates, of the genus *Vibrio* and *Erythrobacter* were determined to produce AHLs although this is likely a lower limit on the diversity and proportion of AHL-producing bacteria in the *Trichodesmium* phycosphere. This is the first report of AHL-production by an *Erythrobacter* sp. and highlights the continued

importance of cultivation efforts in expanding the landscape of known-AHL producing genera.

## INTRODUCTION

Quorum sensing (QS) is a cell-to-cell communication mechanism used by bacteria to regulate group-beneficial behaviors. It has been extensively studied by biomedical researchers for the role that it plays in human disease. However, QS was originally discovered in the marine environment (Nealson *et al.*, 1970; Eberhard *et al.*, 1981; Kaplan & Greenberg, 1985), and it is becoming clear that it is involved with important ecological processes and biogeochemical transformations in the ocean (Gram *et al.*, 2002; Buchan *et al.*, 2005; Nealson & Hastings, 2006; Decho *et al.*, 2009; Hmelo, 2010). The study of QS in the ocean has been hindered by the challenge of extracting detectable quantities of labile, low-concentration signal molecules from the complex mixture of biochemicals in seawater.

Marine bacterial communities contain large numbers of Proteobacteria (greater than 50% of total populations (Rusch *et al.*, 2007)). Many of these bacteria use acylated homoserine lactones (AHLs) to mediate QS-regulated responses (Case *et al.*, 2008). AHLs are extremely labile chemicals; they are rapidly degraded by abiotic and biological mechanisms (Yates *et al.*, 2002; Hmelo & Van Mooy, 2009). AHLs are constitutively produced at low levels by bacteria. However, in the absence of other bacteria, they do not accumulate in the extracellular environment due to diffusive loss and abiotic degradation to a biochemically inactive form. As bacteria proliferate in a given

environment, the signal concentration builds up and a bacterium interprets this increase in concentration as an increase in a clonal population density. At a given threshold, the population simultaneously expresses genes under QS control. Often, the QS signal autoregulates its own production ('autoinduction') such that signal production increases dramatically once the extracellular signal concentration is reached (e.g. Fuqua *et al.*, 1996).

The marine environment presents unique challenges to QS organisms. First, the aqueous environment allows for rapid diffusive loss of AHLs. Second, the pH of seawater is relatively basic (pH=8.2) catalyzing rapid base-hydrolysis of the lactone moiety of the AHL (lactonolysis, Yates *et al.*, 2002). Finally, AHL-degrading enzyme activity is present in seawater (Hmelo & Van Mooy, 2009). For these reasons, it should be expected that QS occurs only in specific niches within the ocean.

It has been predicted that a density of $10^9$ cells $L^{-1}$ is the minimum threshold density required for QS bacteria to induce QS-regulated genes in the ocean (Hmelo & Van Mooy, 2009). This density is not uncommon in biofilms; not only do biofilms host high densities of bacteria, but they also offer a physical barrier to prevent the diffusive loss of AHLs into seawater. Within biofilms, AHL concentrations are maintained at significantly higher concentrations (up to four orders of magnitude) than occur in the external environment (Charlton *et al.*, 2000; Fekete *et al.*, 2007). In addition to deterring the diffusive loss of AHLs, the chemical environment within biofilms is typically more acidic than the surrounding environment (Vroom *et al.*, 1999) which will have a stabilizing effect on AHLs by reducing the rate of abiotic base-catalyzed lactonolysis.

Biofilms are present on almost every living, detrital, and artificial surface in the ocean including algae, sinking particulate organic carbon, and man-made structures (Decho, 2000) and QS likely plays a role in the formation and function of these communities.

The detection of AHLs in marine samples is hindered by numerous challenges. AHLs are effective at low concentrations (0.1-400 nM, Kaplan & Greenberg, 1985; Schaefer *et al.*, 2002; Burton *et al.*, 2005) and as already mentioned, they degrade rapidly in aqueous solutions. While high concentrations of AHLs may accumulate in biofilms (far greater than the minimum QS-induction thresholds of 0.1 to 400 nM observed in culture, e.g. Charlton *et al*., 2002) they are likely much more dilute in seawater. Finally, the presence of AHLs can be masked by the presence of the other organic molecules in the natural environment (i.e. the matrix) that occur at or above the concentration of AHLs.

Recently, we constructed clone libraries derived from total *Trichodesmium thiebautii* community DNA in the Sargasso Sea (Hmelo, 2010). We detected a diverse population of bacteria consistent with previous reports suggesting that *Trichodesmium* spp. can host dense bacterial communities (up to $10^8$ cells $mL^{-1}$) (Sheridan *et al.*, 2002). Of the two clone libraries we produced (one derived from puff-morphology colonies and one derived from tuft-morphology colonies), 23 and 12 percent, respectively, were composed of Proteobacteria, many representatives of which are known to utilize QS in the laboratory.

We collected scanning electron micrographs and epifluorescence images of laboratory-cultivated *Trichodesmium* spp. (Figures 1 and 2). These figures reveal that

high concentrations of bacteria organize in discrete clumps on the cell surface of *Trichodesmium*, suggestive of the types of structures produced by clonal populations (e.g. Davies *et al.*, 1998; Riedel *et al.*, 2001). On the basis of these images and the preceding arguments, we posit that the epibiont community associated with *Trichodesmium* spp. is one in which QS is occurring. In order to test this hypothesis, we engaged in an effort to cultivate QS bacteria from *Trichodesmium* spp.

We isolated bacteria from natural populations of *Trichodesmium* collected at sea and surveyed each of them for AHL production using a broad-spectrum biosensor. We confirmed the presence of AHLs detected by the biosensor using sensitive mass spectrometric analysis (specifically, ion trap and Fourier-transform ion-cyclotron mass spectrometry). Our results highlight the advantage of combining microbiological and chemical approaches to the detection of AHLs in natural samples and provide a platform for additional studies of QS amongst *Trichodesmium* hosted bacterial populations. In addition, we suggest that the methods employed in this study are well suited to the analysis of AHLs in other complex marine-derived samples.

## METHODS

### Isolation and cultivation

#### *Isolation of cultivars from fresh* Trichodesmium *colonies*

*Trichodesmium* sp. colonies were collected in September 2008 on a cruise of the R/V *Atlantic Explorer*, at the Bermuda Atlantic Time Series (BATS) station. Colonies were collected from the near surface (approximately the upper 20m) by a hand-held 130 μm

plankton net and individual colonies were gently picked using an inoculating loop. Filters with discrete colonies were frozen at -80ºC for an additional isolation-effort at WHOI. At sea, freshly-picked colonies were sequentially washed in three 0.2 µm-filtered seawater baths. Epibionts were isolated by streaking colonies of *Trichodesmium* onto agar plates made with either a base of Marine Broth (MB) or Vineyard Sound, MA seawater supplemented with 1 g $L^{-1}$ tryptone (SWT). A control plate of each type of media was inoculated with a clean inoculating loop which was passed through the three 0.2 µm filtered water baths. Single colonies of epibionts were picked and re-streaked to purity. Individual epibiont colonies were picked using a sterile inoculating loop and propagated in liquid media (0.2 µm filtered Vineyard Sound seawater amended with 1 g $L^{-1}$ tryptone). 10% DMSO freezer stocks were prepared from overnight dense cultures and stored at -80ºC.

***Isolation of cultivars from frozen* Trichodesmium *colonies***

Isolates were obtained in a separate cultivation effort at the Woods Hole Oceanographic Institution (WHOI). One filter that contained four frozen puff-type colonies was aseptically divided into quarters. Each section of filter was vortexed for 3 minutes in 1.5 ml of sterile Vineyard Sound seawater (VSS) to dislodge bacteria from the filter and then treated by three different methods which are described below.

**(Method 1)** Each piece of filter was stamped onto 3 plates, VSS prepared with 1% agar, SWT prepared with 1% agar, and VSS amended with 1% methanol and 1ug $ml^{-1}$ vitamin

B12 and prepared with 1% agar. Colonies were picked from these plates and streaked to purity on sterile solid media.

**(Method 2)** An aliquot of the VSS which was vortexed with the filter was streaked as a lawn onto one-third of each of the three types of plates described above. Each plate was then streaked to extinction. Colonies were picked from these plates and streaked to purity on clean plates.

**(Method 3)** Each of the wells in the first column of a 24 deep-well plate was inoculated with 100 µl of the VSS used to vortex the filter. Each row contained 900 µl of a different type of media: Row 1- VSS; Row 2- SWT; Row 3- Methylophaga Broth (Vitamin B12, 1 mg $mL^{-1}$, and Methanol, 0.5% in natural seawater diluted to 75% strength with MilliQ water) ; Row 4- mineral salts amended with 1% MeOH and 1 µg $ml^{-1}$ vitamin B12. Samples were serially diluted 10:1 across the rows, with the final (6th) well containing a $10^{-6}$ dilution of the original 1.5 ml seawater-filter particle solution. These were incubated for 2 weeks and then spread onto plates to extinction similarly to Method 2 above. All incubations took place at 28°C. Most isolates eventually were grown on SWT regardless of initial isolation-media in order to yield biomass sufficient for 16S sequencing and cryopreservation. Few isolates grew in liquid media, cultures displaying growth at the liquid interface or in tight clumps were common.

**(Method 4)** Biofilms developed within the wells of the dilution plate inoculated as per Method 3, both along the walls of the wells and at the media-air interface. These biofilms were gently removed and streaked onto sterile solid media corresponding to the

liquid media from which the biofilms were extracted. Colonies were picked from these plates and streaked to purity on sterile solid media.

**Biosensor assay**

*Agrobacterium tumefaciens* NTL4(pZLR4) is a broad-sensitivity biosensor detecting unsubstituted $C_6$-, $C_8$-, $C_{10}$-, $C_{12}$-, $C_{14}$-, all 3-oxo-AHLs, 3-hydroxy-$C_6$-, $C_8$-, and $C_{10}$-AHLs (Farrand *et al.*, 2002). The use of *Agrobacterium* as a biosensor strain for AHLs is described in detail in Farrand et al. (2002). Briefly, soft agar suspensions of the biosensor strain were prepared by adding 100 µL of an overnight culture of *A. tumefaciens NTL4(pZLR4)* to a suspension of 0.5% agarose in water supplemented with 40µg/mL X-Gal (5 Prime, Hamburg, Germany). Soft agar suspensions were overlaid on base plates of 1.5% agar in AB Minimal media (Chilton *et al.*, 1974) supplemented with 0.2% glucose and 40 µg/mL X-Gal. 2.5 µL of an overnight culture of *A. tumefaciens* NTL4, which does not produce any AHL, was spotted onto the overlays as a negative control. As a positive control, 2.5 µL of an overnight culture of *A. tumefaciens NT1(pTiC58)*, which constitutively produces AHLs, was spotted onto the overlays. Control plates in which *A. tumefaciens NTL4(pZLR4)* was not seeded into the soft-agar overlay were prepared and treated in parallel. Overnight cultures of strains to be tested for AHL production were spotted onto the soft agar overlay in 2.5 µL aliquots. At 24 hours, plates were checked for growth and the appearance of a blue spot. The appearance of a blue zone at the site of sample application constitutes a positive response suggesting the presence of AHLs.

**Organic Extraction of AHLs**

Cultured *Trichodesmium* isolates which elicited a positive response by the biosensor assay were grown in 200 mL SWT broth. After 24 hours, cells were removed by filtration; the spent media was acidified to pH 2 with concentrated hydrochloric acid.

Ethyl acetate and dichloromethane are commonly used as extraction solvents for AHLs (reviewed in Fekete *et al.*, 2007). However, ethyl acetate has been shown to be a more effective solvent (Eberhard *et al.*, 1981) and is improved further by slight acidification by 0.1% formic acid (Ravn *et al.*, 2001). Spent media was extracted three times each with 60 mL acidified (0.1% formic acid) ethyl acetate. The combined extracts were passed through a column of combusted sodium sulfate to remove residual water and concentrated by rotory evaporation for analysis by high performance liquid chromatography (HPLC)- mass spectrometry (MS). Analytical controls were performed by extracting clean MilliQ water into ethyl acetate and performing all downstream sampling handling and preparation. All control extracts were free of AHLs.

**HPLC-ESI-MS**

Extracts were transferred in ethyl acetate to glass vials for analysis on either a Surveyor HPLC coupled to a LCQ Deca-XP ion trap MS or a Surveyor HPLC coupled to a LTQ-Ultra ion trap/ 7-T Fourier transform-ion cyclotron resonance MS (FT-ICR-MS, all instruments are Thermo Finnigan/ Thermo). In either case, samples were ionized via an electrospray ionization (ESI) interface. Separation of AHLs was achieved by running a water-acetonitrile gradient through an Altima HP C18 reverse-phase chromatography

column (Agilent; 5 µm, 2.1x150 mm) with guard column. Eluent A was MilliQ water with 0.1% formic acid and eluent B was acetonitrile with 0.1% formic acid. The gradient program was as follows: 0-25 min, gradient from 90% A/ 10% B to 100% B; 25-26 min, 100% B; 26-27 min, gradient to 90% A/ 10% B; 27-36min 90% A/ 10% B (column equilibration).

Electrospray conditions in the LCQ-MS are as follows: The source voltage was set to 4.5 kV, the capillary temperature was set to 275 º C, and the capillary voltage was set to 1.5 V. In the LTQ, the source voltage was set to 4.2 kV, the capillary temperature was set to 265º C, and the capillary voltage was set to 27.5 V. Full Scan experiments were performed in the LCQ-MS and the FT-ICR-MS in the range m/z 160-500. Masses were acquired by the LCQ at low resolution (nominal mass resolution) and at 100,000 (FWHM) at m/z 400 by the FT-ICR-MS. In the LCQ ion trap, secondary mass spectra were collected for parent ions from a comprehensive list of 3-oxo, 3-hydroxy, and fully reduced AHLs ranging from C4 to C16. In contrast, secondary mass spectra were only collected for ions with a nominal m/z of 242, 256, 270, and 284 in the LTQ ion trap. These masses correspond to the nominal masses of AHL-like compounds detected by LCQ-MS. If none of the four parent ions were present, secondary mass spectra were collected for the most intense ion.

**DNA extraction**

DNA was obtained from 1 mL aliquots of overnight cultures of cultivars grown in SWT media. Cells were pelleted by centrifugation and extracted by phenol-extraction

according to a protocol adapted from J. Marmur (1961). Aliquots of DNA (25-100 ng) were added as template to polymerase chain reactions (PCR) for amplification of the SSU rRNA gene sequence. Each PCR consisted of the following: 0.2 mM dNTPs each, 0.5 uM each forward primer 27F (5' AGAGTTTGATCMTGGCTCAG) and reverse primer 1492R (5' TACGGYTACCTTGTTACGACTT) (Invitrogen, Carlsbad, CA), 2 units Paq5000 thermostable proofreading polymerase (Stratagene, La Jolla, CA), for a 20 μL final reaction volume. PCR cycles were as follows: an initial denaturation step of 2 minutes at 94°C; 30 seconds at 94°C, 30 seconds at 55°C, and 90 seconds at 72°C for a total of 30 amplification cycles.

**Sequencing, Taxonomic assignments and Phylogenetic analysis**

End-sequencing of PCR amplicons was performed offsite either by MWG-Operon (Huntsville, AL) or Agencourt Biosciences (Beverly, MA). Raw sequences were aligned via the web-based SINA aligner (http://www.arb-silva.de/aligner/). Aligned sequences were imported into ARB (Ludwig, 2004) (version 07.12.07). The Ribosomal Database Project II Classifier (Cole *et al.*, 2003) was used to assign provisional taxonomic affiliations all sequences. BLASTn searches (Altschul *et al.*, 1997) of the National Center for Biotechnology Information (NCBI) 16S rRNA gene sequence databases were performed to confirm taxonomic placements.

**Scanning electron microscopy (SEM)**

SEM was performed with a JEOL JSM-840 1- 35KV (JEOL, Tokyo, Japan) scanning electron microscope at the Marine Biological Laboratory Central Microscopy Facility (MBL-CMF). Samples were prepared according to standard operating protocol supplied by MBL-CMF. Briefly, colonies of *Trichodesmium erythraeum* strain K-11#131 were fixed with 4% gluteraldehyde in phosphate-buffered saline (PBS), dehydrated in a concentration series of ethanol in PBS (10% to 100%, in 10% steps), critical point dried using a Tousimis Samdri 780A Critical Point Dryer and sputtered with gold in Tousimis Samsputter-2a (Tousimis, Rockville, MD).

**Epifluorescence microcroscopy**

Individual colonies of *T. erythraeum* strain K-11#131 were picked using an inoculating loop and gently placed on a clear glass microscope slide and mixed with a stain cocktail consisting of 5µg/mL FM 4-64 (5µg/mL, Invitrogen), 1µg/mL 4'-6-diamidino-2-phenylindole (DAPI, vendor) and 30µg/mL MitoTracker Green FM (Invitrogen) and immobilized with a poly-L-lysine coated coverslip (Fisher scientific). Cells were visualized using rhodamine, DAPI and acridine orange filter sets.

**RESULTS**

**Microscopy**

Microscope images revealed bacteria growing in dense colonies on Trichodesmium (Figure 1). It should be noted that bacteria are observed in lightly populated patches

(Figure 1, a) as well as in dense, clump-like colonies which protrude from the surface (Figure 1, b). Bacteria appear to associate directly with the *Trichodesmium* surface (Figure 1, a) as well as with the mucilaginous sheath surrounding the colonies (Figure 1, b and c, Figure 2). The occurrence of dense populations of bacteria in "ghost sheaths" visible in Figure 2 is particularly striking and highlights the elevated concentration of epibiotic bacteria in the sheath.

**Phylogenetic analysis**

78 cultivars (excluding the 8 isolated from the control seawater) were isolated from fresh *Trichodesmium* colonies collected at the BATS site (listed in Supplemental Table 1). An additional 63 cultivars were isolated from frozen colonies (listed in Supplemental Table 2). Of the four methods used to isolate epibionts from frozen *Trichodesmium* colonies, methods 1, 3, and 4 were successful. No cultivars were obtained using Method 2. The greatest number of frozen-colony derived cultivars was obtained using Method 1 (46 of 63). Regardless of the method of isolation, almost all of the cultivars were isolated on either VSS (10 total) or SWT (52 total) media. In fact only one cultivar was obtained by using another media type; One cultivar (A137) was isolated using Methylophaga medium by means of Method 4. With respect to Methods 3 and 4, with one exception, only the 1:10 and 1:100 dilutions yielded isolates. A137 was isolated by streaking a 1:1000 dilution onto Methylophaga media.

The epibionts which were isolated from fresh *Trichodesmium* colonies and frozen *Trichodesmium* colonies differed with respect to one another. Of the 78 cultivars isolated

from fresh samples, 83% were related to Proteobacteria and 12% were related to Actinobacteria. In contrast, 79% of the 63 cultivars isolated from the frozen filter were related to Actinobacteria and only 6% were related to Proteobacteria. 8% of cultivars isolated from the frozen filter were related to the Bacteroidetes while no Bacteroidetes were isolated from fresh Trichodesmium colonies. All five of the Bacteroidetes isolated from the frozen *Trichodesmium* colonies were cultivated by Method 3 or 4 on SWT media. For the remainder of the results and discussion, the cultivars obtained from either fresh or frozen colonies will be considered as one population.

These isolates can be assigned to 5 phylogenetic classes; 43% of cultivars (61 individuals) are related to the Actinobacteria, 27% (39 individuals) are related to the Alphaproteobacteria, 24% (34 individuals) are related to the Gammaproteobacteria, 4% (5 individuals) are related to the Flavobacteria, and 2% (3 individuals) are related to the Firmicutes (Figure 2). Of the 79 cultivars isolated on MB or SWT media at sea, all share 97 percent sequence identity (PSI) or greater with their nearest neighbors in GenBank (Table 3). Of the 63 isolates which were cultivated from frozen colonies in the laboratory, all but 12 share 97 PSI or better with their nearest GenBank neighbor. Ten of these 'novel' isolates cluster within the Actinobacteria and differ from their nearest GenBank neighbor by up to 11 PSI. The other two 'novel' isolates are members of the Flavobacteria and Alphaproteobacteria and differ from their nearest GenBank neighbor by 7 and 6 PSI, respectively.

The Alphaproteobacteria are composed of members of two orders, the Sphingomonadales (54%) and the Rhodobacterales (46%). Of the isolates within each of

these orders, 96% can be assigned to either *Erythrobacter* or *Paracoccus*, respectively. The gammaproteobacterial class is more diverse and is composed of 15% Pseudomonadales, 10% Chromatiales, 35% Vibrionales, and 38% Alteromonadales. The 5 Flavobacteria isolates are all within the genus *Vitellibacter* of the Flavobacteriales. All of the Actinobacteria are members of the Actinomycetales and 85% can be assigned to a known genus.

The marine broth negative control plate did display growth of contaminant colonies, albeit very few. In total, 8 cultivars were isolated; four were members of the Firmicutes (genera *Bacillus*), three were members of the Sphingomonadaceae, and one was a member of the Micrococcaceae.

Detailed phylogenetic placements of all cultivars isolated in this study were determined using the neighbor joining algorithm in ARB and are displayed in Figure 3. The nearest neighbors in GenBank are listed in Supplemental Tables 1 and 2.

**Limit of Detection**

The initial survey instrument used in this experiment was the LCQ-MS (ion trap MS). Based on linear calibration from 2 pmol to 1000 pmol injection of standard AHLs, the limit of detection, defined as the concentration at which an integratable peak has a height three times the baseline, is 10 pmol. If we achieve 100 % extraction efficiency, we should be able to detect AHLs present at concentrations of 50 pM or higher in the 200mL cultures grown for this experiment. This corresponds to three orders of magnitude less than the physiologically relevant concentrations of 10 nM and higher which are the

lowest documented concentrations which will induce a QS response (Kaplan & Greenberg, 1985).

**Biosensor hits and AHL analysis**

14 isolates produced a positive response on the *A. tumefaciens* biosensor (Table 1). We tested 12 of these isolates by mass spectrometry. AHLs were detected by LCQ-MS in 7 isolates and using FT-ICR-MS we were able to identify one additional AHL-producing strain (for a total of eight, see Table 1); the specific AHLs detected in each strain are summarized in Table 2. Using LCQ-MS, AHLs were detected in four *Vibrio* strains (A507, A468, A491, and A477) and three *Erythrobacter* sp. strains (A537, A541, and A513). Five of the strains which induced a positive reaction from the biosensor did not produce AHLs detectable by the LCQ-MS methods employed here. The biosensor-positive, AHL-negative (by LCQ) isolates are A463, A484, A492, A072, and A111. These correspond to a *Vibrio* sp., *Alteromonas* sp., *Erythorobacter* sp., *Gordonia* sp., and *Rhodococcus* sp., respectively. Detailed phylogenetic information can be found in Figure 3 and Supplemental Tables 1 and 2.

LCQ analysis revealed that the AHL-producing *Vibrio* spp. produced appreciable quantities (detected as an 'integratable' peak with approximately three times the height of the baseline) of compounds that we tentatively identified as 3-oxo-$C_9$, 3-oxo-$C_{10}$, and 3-oxo-$C_{11}$-HSLs. Standards of 3-oxo-$C_9$, 3-oxo-$C_{10}$, and 3-oxo-$C_{11}$-HSLwere not available at the time of analysis and so the abovementioned compounds were tentatively identified on the basis of their estimated retention time, fragmentation pattern and exact mass as

determined by FT-ICR-MS. The FT-ICR-MS generates masses accurate to 1 ppm which is sufficient to assign an elemental formula; in combination with chromatographic retention data and secondary mass spectra, it is possible to tentatively assign a molecular structure.

We have previously observed that the efficiency with which AHLs are ionized by the electrospray ionization source varies according to the chain length of the acyl chain (also documented by Ortori *et al.*, 2007), thus the relative response factor (RRF) of the analytical method is different for each individual molecule. As such, we were unable to quantify these compounds on the basis of a calibration curve generated using similar AHL for which a standard was available. Appreciable quantities of 3-oxo-$C_8$-HSL were detected in *Vibrio* sp. A468 and A491 and trace quantities (defined as a peak which is less than three times the height of the baseline) were present in A477. No 3-oxo-$C_8$-HSL was detected in cultures of strain A507. To avoid contaminating culture media with trace exogenous AHLs, we did not spike the samples with an internal AHL standard prior to extraction. Thus, we were unable to quantify the AHLs produced by AHL-producing cultivars.

The FT-ICR MS is a more sensitive analytical detector than the LCQ-MS and we were able to indentify two additional AHLs in the *Vibrio* spp. by FT-ICR MS that we did not detect by LCQ. Significant peaks of 3-oxo-$C_{12}$-HSL were detected in all four *Vibrio* spp. and $C_{12}$-HSL was detected in A507, A491, and A497. $C_{12}$-HSL was present in extremely low quantity and a peak was not present. However, the high resolving power

of the FT-ICR-MS allows the identification of the $C_{12}$-HSL molecular ion at the chromatographic retention time at which the $C_{12}$-HSL standard elutes (Figure 3).

The AHL profiles of the *Erythrobacter spp.* A537, A541, and A513 are quite different. Spent media from *Erythrobacter* isolates A537 contained trace quantities of 3-oxo-$C_8$-HSL as determined by LCQ analysis, compared to a standard. LCQ analysis determined that A541 and A513 produced appreciable quantities of $C_{12}$-HSL. When we analyzed these same samples by FT-ICR-MS, we detected appreciable quantities of $C_{13}$- and $C_{14}$-HSL in A541 and A513 as well.

Although no AHLs were detected in the *Vibrio* strain A463 (99 PSI *Vibrio* sp. S1162) by LCQ-MS. FT-ICR-MS revealed a suite of AHLs from 3-oxo-$C_8$- to 3-oxo-$C_{11}$-HSL in the extract. A463 thus produced AHLs, albeit at a lower concentration than the other *Vibrio* strains investigated, assuming extraction efficiencies are identical culture to culture.

## DISCUSSION

In order to address the hypothesis that epibiotic bacteria associated with *Trichodesmium* spp. engage in QS, we isolated 142 cultivars from samples collected at BATS. To make efficient use of analytical resources, it was useful to assay these samples for the presence of AHLs with a rapid and high-throughput method before performing the more laborious chemical analyses necessary to identify specific structural variants of AHL. We used a broadly sensitive biosensor *A. tumefaciens* NTL4(pZLR4) in this initial survey.

In this study, we found that only eight of twelve isolates which induced a positive response by the *Agrobacterium* biosensor actually produced AHLs. Culture media from the "biosensor-positive" isolates was analyzed by MS and in only eight cases, contained AHLs which could be positively identified by comparison to authentic standards or tentatively identified by their relative chromatographic retention time, fragmentation patterns, and exact mass. Four of the "biosensor-positive" isolates did not produce AHLs which could be detected by the methods employed in this study; in fact, biosensors have been previously criticized for producing false positive hits (Holden, 1999; Ortori *et al.*, 2007; Garcia-Aljaro *et al.*, 2008). While the lack of MS-detectable AHLs in four of the culture media-extracts may signal that the biosensor provided a 'false positive', a few other explanations deserve consideration. First, the detection limit of the biosensor used in this study (0.02 pmol, Ravn *et al.*, 2001), is lower than that of the LCQ-MS (10 pmol). Second, the production of AHLs by a given bacterium generally occurs within a relatively short and highly specific portion of the growth phase (e.g. Blosser-Middleton & Gray, 2001); as a result, small differences in the length of incubation leading up to the biosensor assay or chemical analysis may translate into large differences in AHL concentrations. Despite these potential explanations for the discrepancy between the biosensor analysis and chemical analysis, the biosensor result cannot stand alone; chemical analysis is necessary in order to absolutely confirm the presence of AHLs in a sample.

For several reasons, the methods employed in this study provide only a lower limit on the proportion of the epibiont community which may be capable of producing

AHLs. First, as mentioned above, the concentration of AHL is strongly dependent on the growth stage of the cell cultures (Blosser-Middleton & Gray, 2001; Brelles-Marino & Bedmar, 2001 and references therein) but we did not measure AHLs at all stages of the batch culture growth cycle (lag, early and late exponential, and stationary phases). It is reasonable to hypothesize that many more of the isolates related to known AHL-producing genera produce AHL, and if we had sampled them earlier or allowed the cultures to grow longer we might have detected these.

Second, the cultivable bacteria only represent a very small minority of the bacteria associated with *Trichodesmium* colonies. Clone library analysis of wild *Trichodesmium* collected at the same site reveal the presence of a plethora of proteobacterial genera (Hmelo, 2010), some portion of which likely utilize QS as a metabolic strategy. It is well known that clone libraries are very efficient at capturing the abundant taxa in an ecosystem and cultivation efforts are often biased towards detecting low-abundance populations (Pedrós-Alió, 2006). Indeed, we isolated bacterial taxa (e.g. *Vibrio* and *Erythrobacter*) which are typically cultured from marine habitats but are not major components of clone libraries (Giovannoni & Rappe, 2000).

Third, it has been previously demonstrated that many AHL-producing bacteria do not produce AHLs in high-nutrient media and thus may have produced AHLs had we selected different growth conditions (Brelles-Marino & Bedmar, 2001). Some bacteria only produce AHLs given a particular carbon substrate. For example, the methylotroph *Methylobacterium extorquens* AM1 only produces AHLs when grown under methylotrophic conditions (Nieto Penalver *et al.*, 2006). We detected bacteria with

similarity to methylotrophic clades (e.g. members of the Thiotrichales), however, we have not yet tested their ability to produce AHLs under methylotrophic conditions.

Finally, our MS-analyses were directed by our biosensor responses. Biosensors only detect a limited suite of AHL structures and are variably sensitive to those which it can detect (Brelles-Marino & Bedmar, 2001). We chose *A. tumefaciens* NTL4 (pZLR4) because it detects a broad range of AHLs and favors the long-chain varieties which tend to dominate in marine bacteria (Wagner-Dobler *et al.*, 2005; Decho *et al.*, 2009). It will not, however, detect short-chain AHLs or homoserine-lactone derivatives with unusual structures, such as the *p*-coumaroyl homoserine lactone (Schaefer *et al.*, 2008) or branched AHLs (Thiel *et al.*, 2009). Similarly, we used LCQ-MS to select samples to be analyzed by FT-ICR-MS. Thus it is possible that isolates which produce low-abundance AHLs would have been overlooked by LCQ-MS. Indeed, isolate A463 produced AHLs which were detectable by FT-ICR-MS but not by LCQ-MS. Ion-trap MS is a very common tool in the analysis of AHLs from biological samples (Morin *et al.*, 2003; Nieto Penalver *et al.*, 2006); however, our results suggest that this type of instrument may not be the most appropriate tool for the comprehensive analysis of AHLs from extremely complex matrices.

The analysis of AHLs in environmental samples requires a selective and sensitive technique which is capable of detecting AHLs within a complex sample matrix. While the LCQ-MS enables convenient and relatively inexpensive investigation of molecular structures, it can only measure mass-to-charge at nominal mass unit resolution which can be problematic if an analyte of interest is 'buried' in a complex chemical matrix. On the

other hand, the FT-ICR-MS is highly sensitive and selective in addition to measuring mass-to-charge with remarkable accuracy (+/- 1 ppm). We were able to use the FT-ICR-MS to distinguish $C_{12}$-HSL from a background which masked its presence using the nominal mass resolution device (LCQ-MS, Figure 3). FT-ICR-MS has been successfully utilized to identify known and unknown AHLs in complex culture extracts previously (Cataldi *et al.*, 2008) and here we confirmed that, with respect to isolates grown in a seawater-medium, this technique is more sensitive and capable of identifying a wider range of AHLs with respect to the LCQ-MS.

All of the AHLs which were detected in this study were produced by bacteria related to known AHL-producing lineages, namely *Vibrio* spp. (Milton, 2006) and members of the Roseobacter clade (Martens *et al.*, 2007), both members of the Proteobacteria. To our knowledge, this is the first report of bacteria closely related to the *Erythrobacter* genus having been demonstrated to produce AHLs.

Proteobacteria, which contains both *Vibrio* spp. and members of the Roseobacter clade, is the only phylum which contains bacteria known to harbor homologues of the AHL-synthesizing protein, LuxI. Scattered reports of QS by cyanobacteria (Sharif *et al.*, 2008), archaea (Paggi *et al.*, 2003), and a member of the Cytophoga-flavobacterium-bacteroidetes clade (Romero, 2010)) appear in the literature. While Sharif *et al*. (2008) assert that their cyanobacterial culture (*Gleothece* PCC6909) was axenic, AHL production did not begin to increase rapidly until 55 days. QS bacteria with *luxI* homologues whose QS systems have been characterized in the laboratory tend to produce their AHL signals on the order of hours rather than days (e.g. Blosser-Middleton & Gray,

2001). In the absence of the discovery of a *luxI* homologue in *Gleothece*, it is tempting to conclude that these results reflect contamination of the culture by a QS-heterotrophic bacterium after a lengthy incubation period. Biosensor data alone was provided to support the claim of QS in the archaeon *Natronococcus occultus* (Paggi *et al.*, 2003), and as discussed earlier, biosensor data is subject to false positive reports. While the results of Romero (2010) do provide compelling evidence of AHL production by a CFB bacterium, all three of these reports will require independent replication and genetic evidence to convincingly and conclusively prove that QS is not constrained to the Proteobacteria. Interestingly, a complete *luxI/R* circuit is present in the genome of a *Leptospirillum* sp. (phylum, Nitrospirae), however, this bacterium is not in culture and thus its QS system can not be further investigated (Simmons *et al.*, 2008).

The prospect of extending the range of QS is exciting as it would expand the range of potential inter-species and inter-phylum interactions in mixed bacterial communities and provide insight into the evolutionary origins of the QS. In this study, three actinobacterial strains isolated in this study induced a positive response by the biosensor used in this study (A072, A111 and A113). We evaluated culture extracts from two of these strains (A072 and A111) using LCQ-MS but we did not detect any AHLs. Strains A111 and A113 share 99 PSI with a *Rhodococcus* sp. (accession number FJ497702) while strain A072 shares only 92 PSI with its nearest neighbor in GenBank (*Gordonia* sp. FWA92). On the basis of 16S rRNA sequence data, isolate A072 is a novel bacterium which has not yet been characterized. Perhaps future characterization of this bacterium will reveal the ability to produce AHLs; in the absence data other than its

ribomosaml RNA gene sequence, we can not preclude this possibility. Regardless, it may be interesting to pursue a more aggressive characterization of the spent culture media from this isolate in order to identify the chemical responsible for inducing the biosensor.

The AHLs detected in this study (3-oxo-$C_8$ to 3-oxo-$C_{12}$-HSL and $C_{12}$- to $C_{14}$-HSLs) are typical of the AHLs which are often observed in marine environments and in isolates of marine bacteria. Long chain AHLs are often prevalent in marine-derived samples, likely because their long chain length renders them more stable than their short-chain homologues (Hmelo & Van Mooy, 2009). The suites of AHLs detected in this study, namely the 3-oxo-$C_8$ to 3-oxo-$C_{12}$-HSL series in the *Vibrio* spp. and the $C_{12}$- to $C_{14}$-HSL suite in the *Erythrobacter* spp. raise an interesting question about the ecological role of AHLs. Are the individual AHLs deliberately synthesized by the cells, i.e. each by a unique LuxI protein, or are they the bi-products of a single LuxI which is able to interact with a range of acyl-precursors? The latter phenomenon has been documented (Marketon *et al.*, 2002) although the biological (and ecological) relevance has yet to be established (Waters & Bassler, 2005). Promiscuous production of AHLs could interfere in the signaling of neighboring bacterial colonies although co-evolved populations in biofilms may account for such production. Similarly, this promiscuous production could render the 'accidental' AHLs biologically irrelevant in a system which has evolved in their presence. Investigations of AHL-QS in multi-species communities are in their infancy. Existing studies only often consider two bacteria grown in co-culture (Riedel *et*

*al.*, 2001; Dulla & Lindow, 2009). Investigations of QS in biofilms with two or more members will be necessary in order to answer these types of questions in the future.

*Future directions and recommendations*

A crucial piece of data necessary to definitively implicate QS in epibiont interactions will be the detection of an AHL from *Trichodesmium* cultures. This is a formidable task: Although concentrations of AHLs may reach mmol $L^{-1}$ concentrations within a given microcolony, this may not translate into a large number of molecules on the scale of a batch culture of *Trichodesmium* colonies. Indeed, we have invested considerable effort into extracting AHLs from laboratory-derived cultures of *Trichodesmium* spp. without detecting so much as trace amounts of the molecules. It should be noted that all of our initial MS work was performed by LCQ-MS. If we are to detect AHLs in batch cultures or environmental samples of *Trichodesmium*, the results of this study suggest that FT-ICR-MS, as well as triple-quadrupole-MS, another highly sensitive and selective form of mass spectrometry, are more appropriate analytical tools.

Potential improvements and enhancements to the cultivation of QS-bacterium from epibiont communities include the use of creative media designed to target specific organisms which are present in the clone library. These modifications could include amending some of these media with AHLs. We are targeting bacteria which are capable of complex interactions with their communities, and efforts to obtain them in pure culture may be improved by adding exogenous AHLs to the media. This technique has been successfully employed in improving the efficiency of cultivating bacteria from Baltic Sea

seawater (although the addition of cAMP was even more successful) (Bruns *et al.*, 2002). One reason why the addition of AHLs improves cultivation efficiency may be that AHL-QS is involved in a population's resuscitation from lag phase (Bruns *et al.*, 2002 and references therein). Other studies have demonstrated that many difficult-to-cultivate bacteria may need not only signal molecules, but other growth factors from neighboring bacterial populations, such as siderophores (D'Onofrio *et al.*, 2010).

Finally, we can use cultivation-independent approaches to investigate the occurrence of QS using laboratory cultured *Trichodesmium* spp. For example, the effects of AHLs on laboratory-cultured *Trichodesmium* spp. can be gauged by amending cultures with AHLs produced by epibionts and monitoring specific features of the batch culture. (A preliminary experiment of this variety is included in this dissertation in Appendix 2) Alternatively, it may be possible to perform gene expression or protein expression experiments in which the effects of endogenous AHLs on the Trichodesmium community can be assessed.

*Conclusions*

In this study, we attempted to isolate QS-bacteria from *Trichodesmium* in order to begin investigating the role of QS in the cell-cell interactions of epibiotic populations. We showed that *Trichodesmium*-associated *Vibrio* isolates produce AHLs. We also demonstrated for the first time that (*Trichodesmium*-associated) *Erythrobacter* spp. produce AHLs. While we were successful in demonstrating that individual strains of *Trichodesmium*-associated epibionts possess the ability to utilize QS, a few crucial data

points remain to be acquired to definitively make the case that QS occurs in the mixed-epibiont community. The epibiotic bacteria associated with *Trichodesmium* are important for the ecology of *Trichodesmium* and the biogeochemistry of the ocean. Obtaining isolates of these bacteria in culture, will allow us to begin to assess their metabolic strategies and contributions to marine biogeochemical cycling.

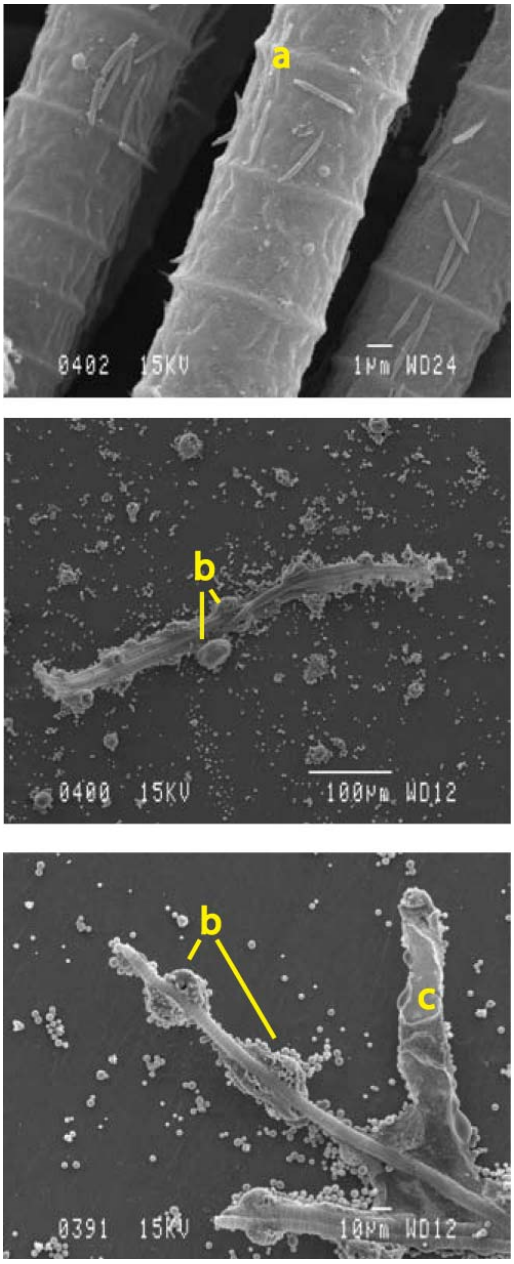


**Figure 1.** SEM images of *T. erythraeum* strain K-11#131. Different areas of the filaments are characterized by light or heavy colonization of bacteria (a or b, respectively). High densities of bacteria tend to cluster in discrete 'clumps' or 'nodes' which extend from the cell surface of *Trichodesmium.* (b). Note the presence of a vacant mucilaginous sheath (c) and the heavy colonization of bacteria within it.

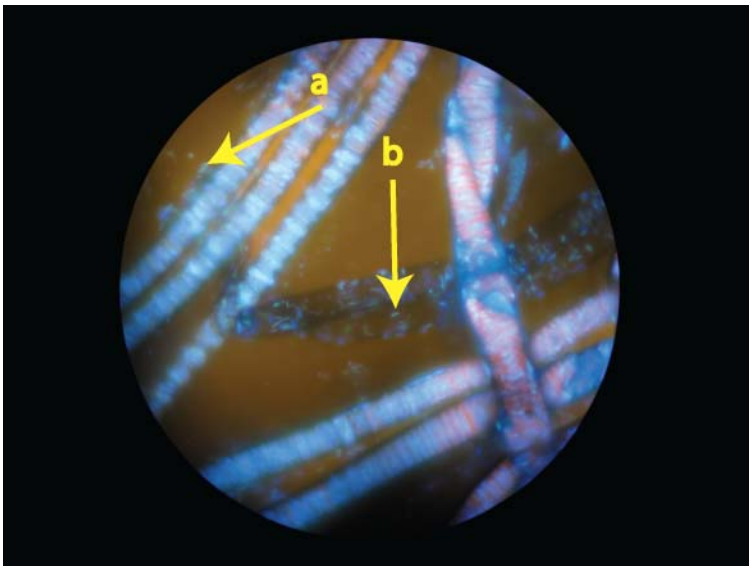


**Figure 2.** Epifluorescense image of K-11#131 stained with DAPI. Methods are described in the text. Note the dense concentrations of bacteria (discrete clusters) associated with the surface of Trichodesmium filaments (a). Of special note in this image is the presence high numbers of bacteria in an empty ("ghost") mucilaginous sheath (b).

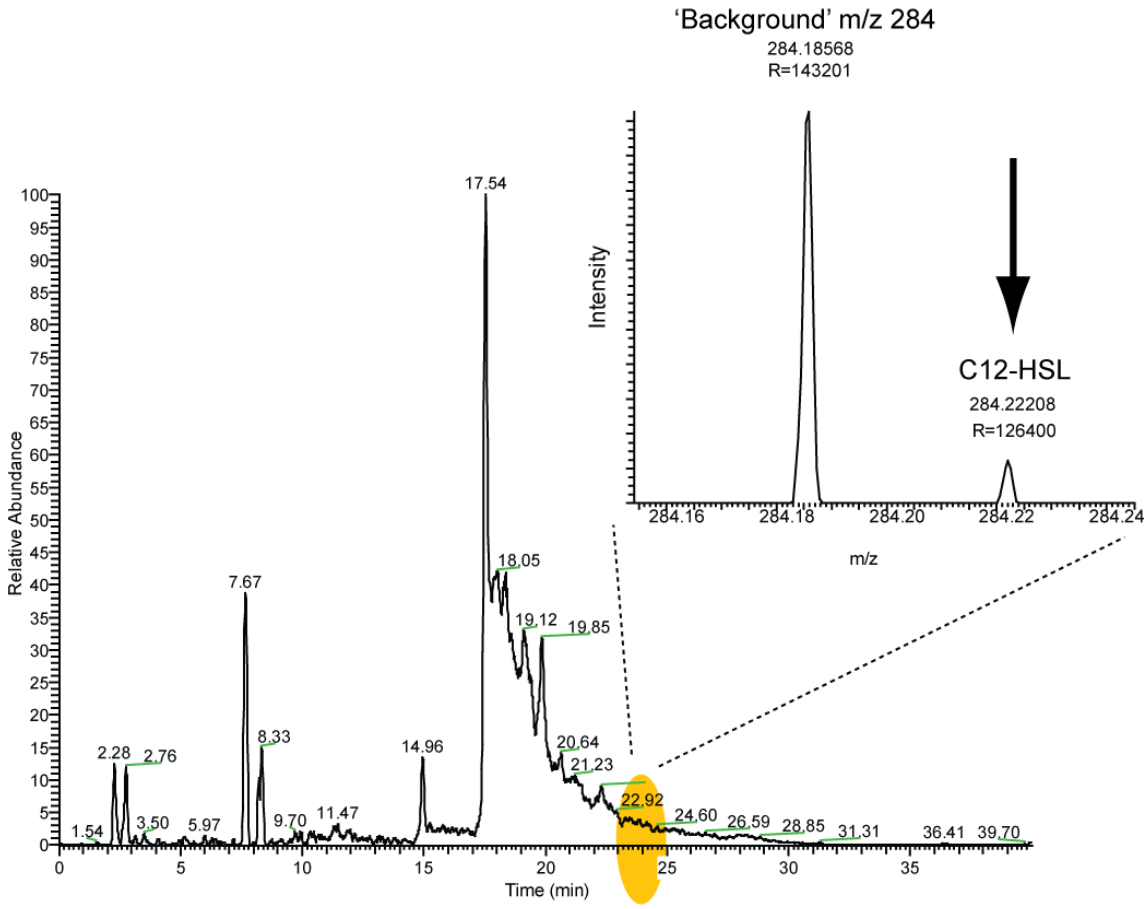


**Figure 3.** Extracted ion chromatogram showing the intensity of ions with a nominal mass-to-charge ratio (m/z) of 284. The inset illustrates the power of high mass resolution to separate the molecular ion of C12-HSL (m/z 284.22208) from the 'background' m/z 284.18568 at the chromatographic retention time of an authentic C12-HSL standard (22.9 minutes, indicated by yellow shading). 'R' indicates the resolution of the mass spectral peak.

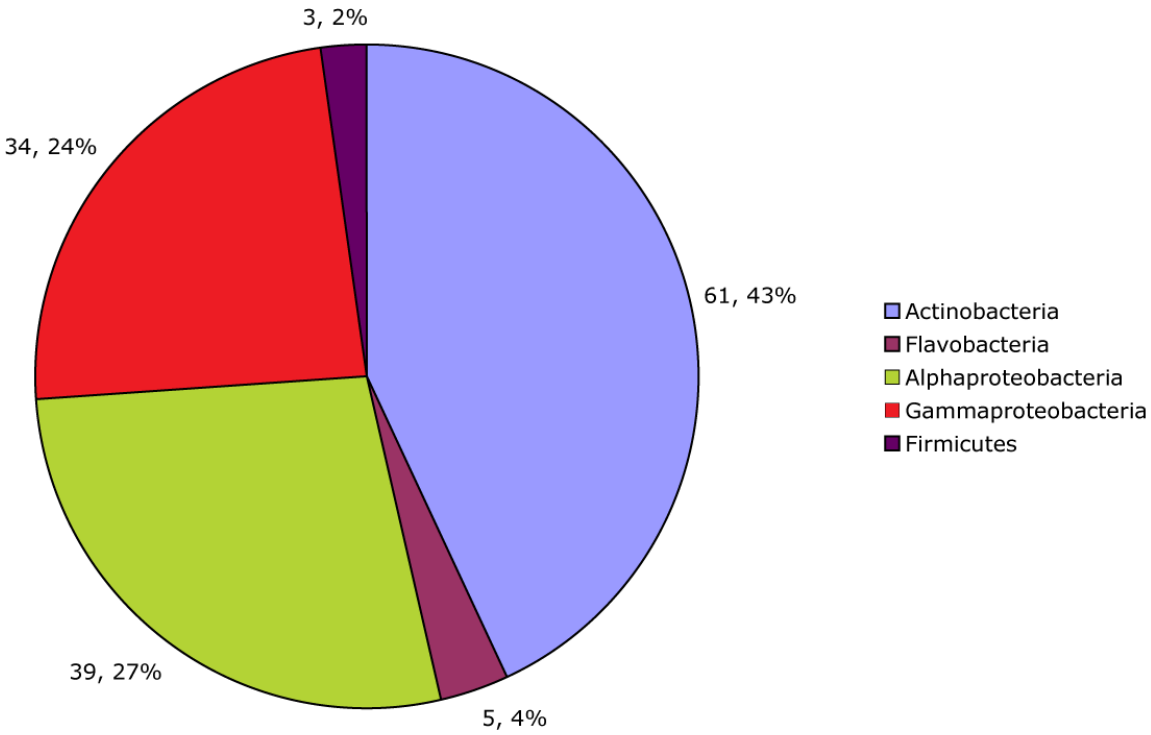


**Figure 4.** Class-level distribution of isolates obtained in this study. Values next to each wedge of the chart indicate the number of isolates in the category, followed by the percentage of the total.

**Figure 5.** Phylogenetic trees detailing the relationships between isolates obtained in this study and other cultured and uncultured organisms deposited in GenBank. Trees were constructed using the Neighbor Joining algorithm in ARB and bootstrapped 1000 times using the neighbor joining algorithm within Phylip (v 3.68). Asterisks indicate 70% or greater bootstrap confidence. Sequences have been organized in one of four trees: (a) Alphaproteobacteria (b) Gammaproteobacteria (c) Actinobacteria (d) Firmicutes and Bacteroidetes.

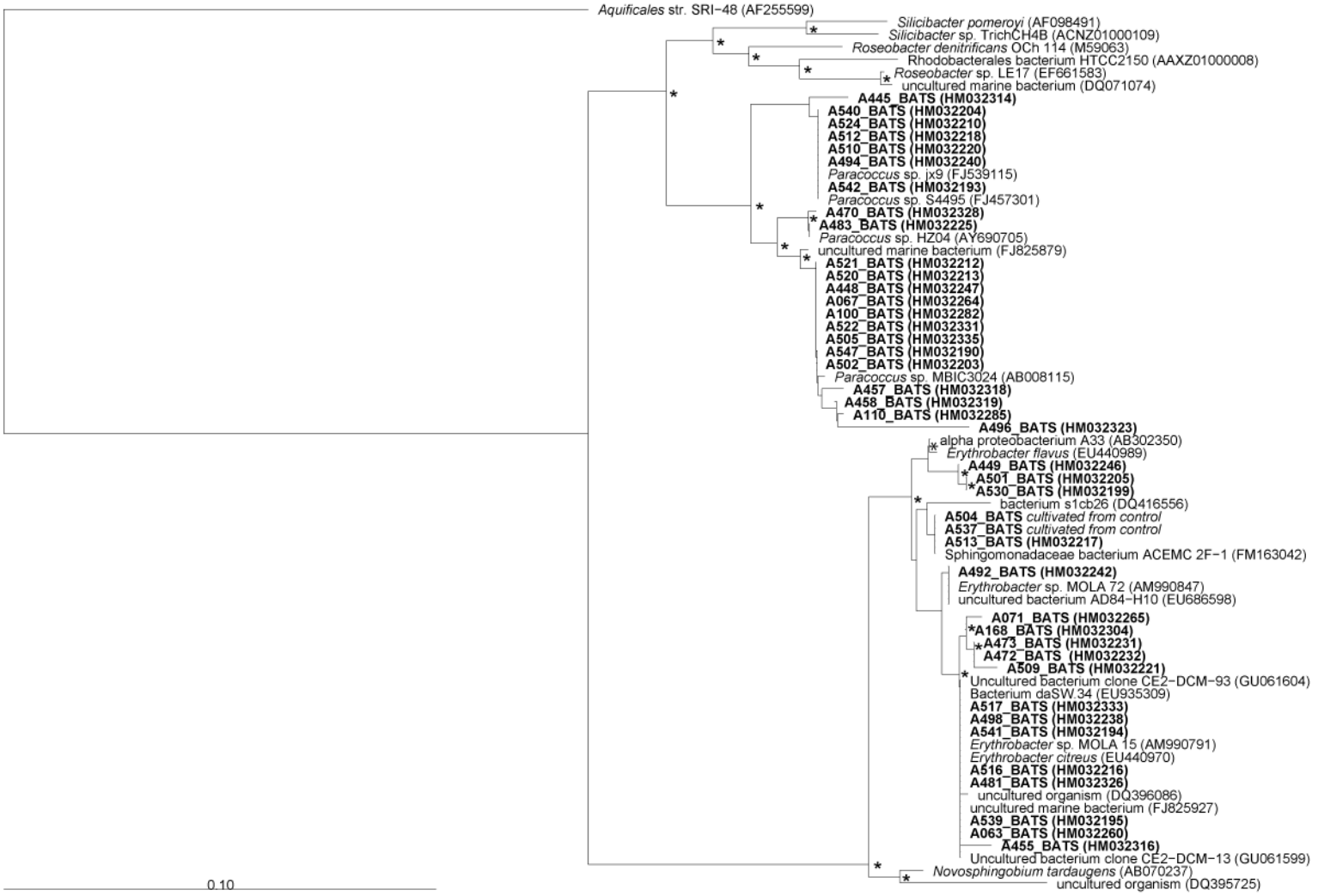
*Aquificales* str. SRI−48 (AF255599)
*Silicibacter pomeroyi* (AF098491)
*Silicibacter* sp. TrichCH4B (ACNZ01000109)
*Roseobacter denitrificans* OCh 114 (M59063)
Rhodobacterales bacterium HTCC2150 (AAXZ01000008)
*Roseobacter* sp. LE17 (EF661583)
uncultured marine bacterium (DQ071074)
**A445_BATS (HM032314)**
**A540_BATS (HM032204)**
**A524_BATS (HM032210)**
**A512_BATS (HM032218)**
**A510_BATS (HM032220)**
**A494_BATS (HM032240)**
*Paracoccus* sp. jx9 (FJ539115)
**A542_BATS (HM032193)**
*Paracoccus* sp. S4495 (FJ457301)
**A470_BATS (HM032328)**
**A483_BATS (HM032225)**
*Paracoccus* sp. HZ04 (AY690705)
uncultured marine bacterium (FJ825879)
**A521_BATS (HM032212)**
**A520_BATS (HM032213)**
**A448_BATS (HM032247)**
**A067_BATS (HM032264)**
**A100_BATS (HM032282)**
**A522_BATS (HM032331)**
**A505_BATS (HM032335)**
**A547_BATS (HM032190)**
**A502_BATS (HM032203)**
*Paracoccus* sp. MBIC3024 (AB008115)
**A457_BATS (HM032318)**
**A458_BATS (HM032319)**
**A110_BATS (HM032285)**
**A496_BATS (HM032323)**
alpha proteobacterium A33 (AB302350)
*Erythrobacter flavus* (EU440989)
**A449_BATS (HM032246)**
**A501_BATS (HM032205)**
**A530_BATS (HM032199)**
bacterium s1cb26 (DQ416556)
**A504_BATS** *cultivated from control*
**A537_BATS** *cultivated from control*
**A513_BATS (HM032217)**
Sphingomonadaceae bacterium ACEMC 2F−1 (FM163042)
**A492_BATS (HM032242)**
*Erythrobacter* sp. MOLA 72 (AM990847)
uncultured bacterium AD84−H10 (EU686598)
**A071_BATS (HM032265)**
**A168_BATS (HM032304)**
**A473_BATS (HM032231)**
**A472_BATS (HM032232)**
**A509_BATS (HM032221)**
Uncultured bacterium clone CE2−DCM−93 (GU061604)
Bacterium daSW.34 (EU935309)
**A517_BATS (HM032333)**
**A498_BATS (HM032238)**
**A541_BATS (HM032194)**
*Erythrobacter* sp. MOLA 15 (AM990791)
*Erythrobacter citreus* (EU440970)
**A516_BATS (HM032216)**
**A481_BATS (HM032326)**
uncultured organism (DQ396086)
uncultured marine bacterium (FJ825927)
**A539_BATS (HM032195)**
**A063_BATS (HM032260)**
**A455_BATS (HM032316)**
Uncultured bacterium clone CE2−DCM−13 (GU061599)
*Novosphingobium tardaugens* (AB070237)
uncultured organism (DQ395725)
0.10

**Figure 5a**

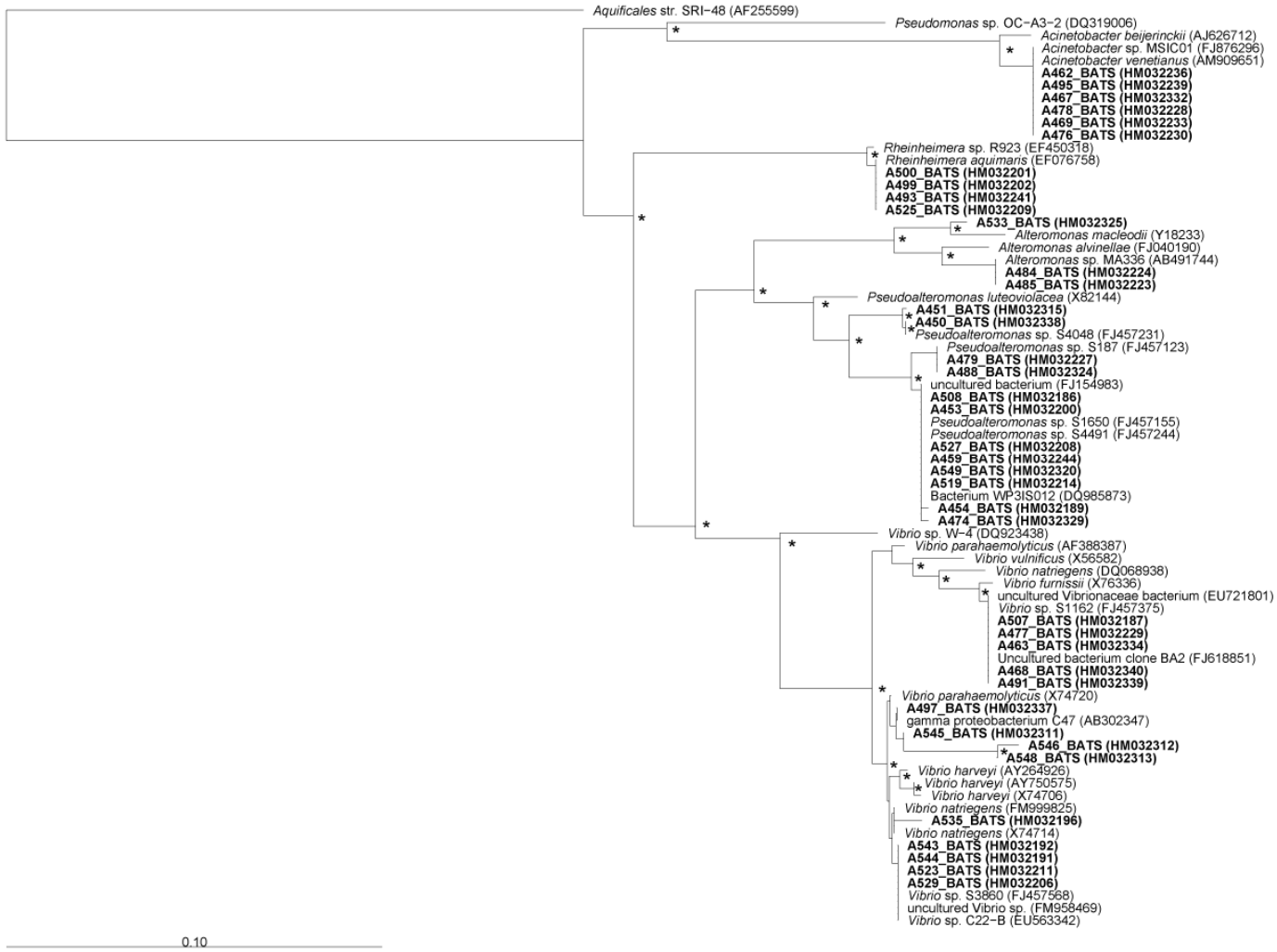
Aquificales str. SRI−48 (AF255599)
Pseudomonas sp. OC−A3−2 (DQ319006)
Acinetobacter beijerinckii (AJ626712)
Acinetobacter sp. MSIC01 (FJ876296)
Acinetobacter venetianus (AM909651)
A462_BATS (HM032236)
A495_BATS (HM032239)
A467_BATS (HM032332)
A478_BATS (HM032228)
A469_BATS (HM032233)
A476_BATS (HM032230)
Rheinheimera sp. R923 (EF450318)
Rheinheimera aquimaris (EF076758)
A500_BATS (HM032201)
A499_BATS (HM032202)
A493_BATS (HM032241)
A525_BATS (HM032209)
A533_BATS (HM032325)
Alteromonas macleodii (Y18233)
Alteromonas alvinellae (FJ040190)
Alteromonas sp. MA336 (AB491744)
A484_BATS (HM032224)
A485_BATS (HM032223)
Pseudoalteromonas luteoviolacea (X82144)
A451_BATS (HM032315)
A450_BATS (HM032338)
Pseudoalteromonas sp. S4048 (FJ457231)
Pseudoalteromonas sp. S187 (FJ457123)
A479_BATS (HM032227)
A488_BATS (HM032324)
uncultured bacterium (FJ154983)
A508_BATS (HM032186)
A453_BATS (HM032200)
Pseudoalteromonas sp. S1650 (FJ457155)
Pseudoalteromonas sp. S4491 (FJ457244)
A527_BATS (HM032208)
A459_BATS (HM032244)
A549_BATS (HM032320)
A519_BATS (HM032214)
Bacterium WP3IS012 (DQ985873)
A454_BATS (HM032189)
A474_BATS (HM032329)
Vibrio sp. W−4 (DQ923438)
Vibrio parahaemolyticus (AF388387)
Vibrio vulnificus (X56582)
Vibrio natriegens (DQ068938)
Vibrio furnissii (X76336)
uncultured Vibrionaceae bacterium (EU721801)
Vibrio sp. S1162 (FJ457375)
A507_BATS (HM032187)
A477_BATS (HM032229)
A463_BATS (HM032334)
Uncultured bacterium clone BA2 (FJ618851)
A468_BATS (HM032340)
A491_BATS (HM032339)
Vibrio parahaemolyticus (X74720)
A497_BATS (HM032337)
gamma proteobacterium C47 (AB302347)
A545_BATS (HM032311)
A546_BATS (HM032312)
A548_BATS (HM032313)
Vibrio harveyi (AY264926)
Vibrio harveyi (AY750575)
Vibrio harveyi (X74706)
Vibrio natriegens (FM999825)
A535_BATS (HM032196)
Vibrio natriegens (X74714)
A543_BATS (HM032192)
A544_BATS (HM032191)
A523_BATS (HM032211)
A529_BATS (HM032206)
Vibrio sp. S3860 (FJ457568)
uncultured Vibrio sp. (FM958469)
Vibrio sp. C22−B (EU563342)
0.10

**Figure 5b**

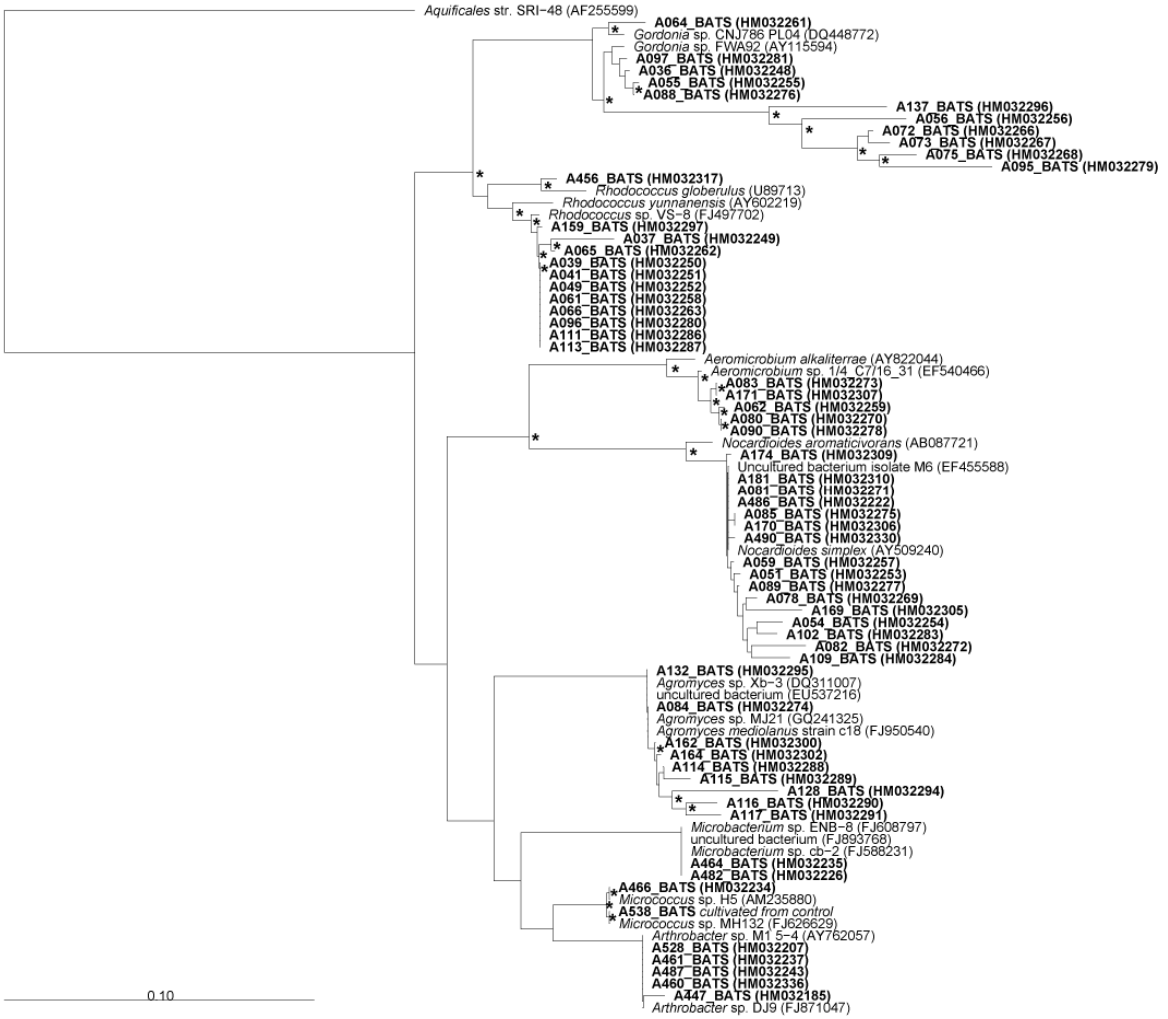
Aquificales str. SRI−48 (AF255599)
A064_BATS (HM032261)
Gordonia sp. CNJ786 PL04 (DQ448772)
Gordonia sp. FWA92 (AY115594)
A097_BATS (HM032281)
A036_BATS (HM032248)
A055_BATS (HM032255)
A088_BATS (HM032276)
A137_BATS (HM032296)
A056_BATS (HM032256)
A072_BATS (HM032266)
A073_BATS (HM032267)
A075_BATS (HM032268)
A095_BATS (HM032279)
A456_BATS (HM032317)
Rhodococcus globerulus (U89713)
Rhodococcus yunnanensis (AY602219)
Rhodococcus sp. VS−8 (FJ497702)
A159_BATS (HM032297)
A037_BATS (HM032249)
A065_BATS (HM032262)
A039_BATS (HM032250)
A041_BATS (HM032251)
A049_BATS (HM032252)
A061_BATS (HM032258)
A066_BATS (HM032263)
A096_BATS (HM032280)
A111_BATS (HM032286)
A113_BATS (HM032287)
Aeromicrobium alkaliterrae (AY822044)
Aeromicrobium sp. 1/4_C7/16_31 (EF540466)
A083_BATS (HM032273)
A171_BATS (HM032307)
A062_BATS (HM032259)
A080_BATS (HM032270)
A090_BATS (HM032278)
Nocardioides aromaticivorans (AB087721)
A174_BATS (HM032309)
Uncultured bacterium isolate M6 (EF455588)
A181_BATS (HM032310)
A081_BATS (HM032271)
A486_BATS (HM032222)
A085_BATS (HM032275)
A170_BATS (HM032306)
A490_BATS (HM032330)
Nocardioides simplex (AY509240)
A059_BATS (HM032257)
A051_BATS (HM032253)
A089_BATS (HM032277)
A078_BATS (HM032269)
A169_BATS (HM032305)
A054_BATS (HM032254)
A102_BATS (HM032283)
A082_BATS (HM032272)
A109_BATS (HM032284)
A132_BATS (HM032295)
Agromyces sp. Xb−3 (DQ311007)
uncultured bacterium (EU537216)
A084_BATS (HM032274)
Agromyces sp. MJ21 (GQ241325)
Agromyces mediolanus strain c18 (FJ950540)
A162_BATS (HM032300)
A164_BATS (HM032302)
A114_BATS (HM032288)
A115_BATS (HM032289)
A128_BATS (HM032294)
A116_BATS (HM032290)
A117_BATS (HM032291)
Microbacterium sp. ENB−8 (FJ608797)
uncultured bacterium (FJ893768)
Microbacterium sp. cb−2 (FJ588231)
A464_BATS (HM032235)
A482_BATS (HM032226)
A466_BATS (HM032234)
Micrococcus sp. H5 (AM235880)
A538_BATS cultivated from control
Micrococcus sp. MH132 (FJ626629)
Arthrobacter sp. M1 5−4 (AY762057)
A528_BATS (HM032207)
A461_BATS (HM032237)
A487_BATS (HM032243)
A460_BATS (HM032336)
A447_BATS (HM032185)
Arthrobacter sp. DJ9 (FJ871047)
0.10

**Figure 5c**

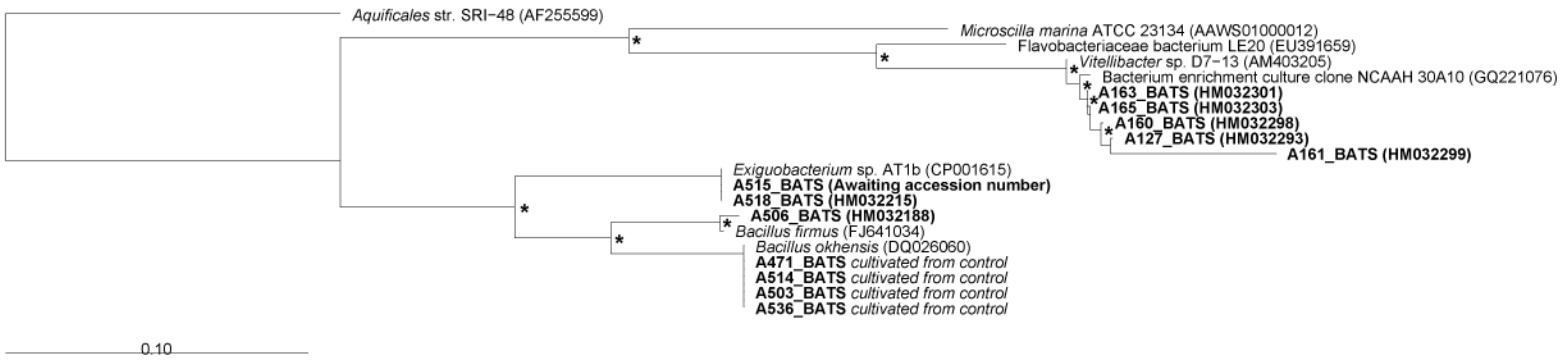
*Aquificales* str. SRI−48 (AF255599)
*Microscilla marina* ATCC 23134 (AAWS01000012)
Flavobacteriaceae bacterium LE20 (EU391659)
*Vitellibacter* sp. D7−13 (AM403205)
Bacterium enrichment culture clone NCAAH 30A10 (GQ221076)
**A163_BATS (HM032301)**
**A165_BATS (HM032303)**
**A160_BATS (HM032298)**
**A127_BATS (HM032293)**
**A161_BATS (HM032299)**
*Exiguobacterium* sp. AT1b (CP001615)
**A515_BATS (Awaiting accession number)**
**A518_BATS (HM032215)**
**A506_BATS (HM032188)**
*Bacillus firmus* (FJ641034)
*Bacillus okhensis* (DQ026060)
**A471_BATS** *cultivated from control*
**A514_BATS** *cultivated from control*
**A503_BATS** *cultivated from control*
**A536_BATS** *cultivated from control*
0.10

**Figure 5d**

| Isolate | AHLs |
|---|---|
| A071 | n.t. |
| A072 | - |
| A111 | - |
| A113 | n.t. |
| A463 | - |
| A468 | + |
| A477 | + |
| A484 | - |
| A492 | - |
| A491 | + |
| A507 | + |
| A513 | + |
| A537 | + |
| A541 | + |

**Table 1.** Biosensor hit table. Isolates listed in this table induced a blue spot on the *A. tumefaciens* NTL4 bioassay which indicates they produce substances with the bioactivity of AHLs. These isolates were subsequently screened by LCQ-MS unless indicated (not-tested, n.t.). A plus sign (+) indicated we detected AHLs by HPLC-MS where as a minus sign (-) indicates that we did not detect AHLs by HPLC-MS.

| Isolate ID | Nearest GenBank match (PSI) | 3OC8 | 3OC9 | 3OC10 | 3OC11 | 3OC12 | C12 | C13 | C14 |
|---|---|---|---|---|---|---|---|---|---|
| A507 | *Vibrio vulnificus* strain MP-4 (100%) | -- | ✓ | ✓ | ✓ | ✓ | trace | -- | -- |
| A468 | *Vibrio vulnificus* strain MP-4 (100%) | ✓ | ✓ | ✓ | ✓ | ✓ | -- | -- | -- |
| A491 | *Vibrio vulnificus* strain MP-4 (99%) | ✓ | ✓ | ✓ | ✓ | ✓ | trace | -- | -- |
| A477 | *Vibrio vulnificus* strain MP-4 (100%) | trace | ✓ | ✓ | ✓ | ✓ | trace | -- | -- |
| A463 | *Vibrio* sp. S1162 (99%) | trace | ✓ | ✓ | ✓ | -- | -- | -- | -- |
| A537 | *Erythrobacter vulgaris* strain 022 2-10 (100%) | trace | -- | -- | -- | -- | -- | -- | -- |
| A541 | *Erythrobacter citreus* strain PR52-9 (100%) | -- | -- | -- | -- | -- | ✓ | ✓ | ✓ |
| A513 | *Erythrobacter vulgaris* strain 022 2-10 (100%) | -- | -- | -- | -- | -- | ✓ | ✓ | ✓ |

**Table 2.** Table of AHLs detected in isolates. Next to each isolate ID is the identity of the nearest GenBank match with the percent sequence identity (PSI) in parentheses. If a peak of approximately three times the height of the baseline is present (considered to be an 'integratable' peak), the compound is marked with a check mark (✓). If the compound is detectable although in a quantity which is less than the latter threshold, the compound is considered 'trace'.

**Supplemental Table 1**. Nearest GenBank neighbors of cultivars isolated at BATS from fresh *Trichodesmium* colonies. Isolates are organized by the particular colony from which they were cultivated.

| Isolate ID | Source colony | Accession no. | Sequence ID of nearest BLAST match | Accession no. (BLAST match) | PSI | Number of bases compared | Bit score | Lineage |
|---|---|---|---|---|---|---|---|---|
| A471 | control, 4 | not assigned | *Bacillus okhensis* strain Kh10-101 16S ribosomal RNA gene | DQ026060 | 100 | 755 | 755 | Bacteria; Firmicutes; Bacillales; Bacillaceae; Bacillus |
| A503 | control, 4 | not assigned | *Bacillus okhensis* strain Kh10-101 16S ribosomal RNA gene | DQ026060 | 99 | 715 | 715 | Bacteria; Firmicutes; Bacillales; Bacillaceae; Bacillus |
| A504 | control, 4 | not assigned | Sphingomonadaceae bacterium ACEMC 2F-1 partial 16S rRNA gene,isolate ACEMC 2F-1 | FM163042 | 100 | 736 | 736 | Bacteria; Proteobacteria; Alphaproteobacteria; Sphingomonadales;Sphingomonadaceae |
| A513 | control, 4 | not assigned | Sphingomonadaceae bacterium ACEMC 2F-1 partial 16S rRNA gene, isolate ACEMC 2F-1 | FM163042 | 100 | 741 | 741 | Bacteria; Proteobacteria; Alphaproteobacteria; Sphingomonadales;Sphingomonadaceae |
| A514 | control, 4 | not assigned | *Bacillus okhensis* strain Kh10-101 16S ribosomal RNA gene | DQ026060 | 99 | 732 | 732 | Bacteria; Firmicutes; Bacillales; Bacillaceae; Bacillus |
| A536 | control, 4 | not assigned | *Bacillus okhensis* strain Kh10-101 16S ribosomal RNA gene | DQ026060 | 100 | 739 | 739 | Bacteria; Firmicutes; Bacillales; Bacillaceae; Bacillus |
| A537 | control, 4 | not assigned | Sphingomonadaceae bacterium ACEMC 2F-1 partial 16S rRNA gene,isolate ACEMC 2F-1 | FM163042 | 100 | 756 | 756 | Bacteria; Proteobacteria; Alphaproteobacteria; Sphingomonadales;Sphingomonadaceae |
| A538 | control, 4 | not assigned | *Micrococcus* sp. MH132 16S ribosomal RNA gene | FJ626629 | 100 | 753 | 753 | Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Micrococcineae; Micrococcaceae; Micrococcus |
| A449 | puff, 5 | HM032246 | *Erythrobacter flavus* strain 2PR56-3 16S ribosomal RNA gene | EU440989 | 98 | 698 | 699 | Bacteria; Proteobacteria; Alphaproteobacteria; Sphingomonadales;Erythrobacteraceae; Erythrobacter |
| A450 | puff, 5 | HM032338 | *Pseudoalteromonas* sp. S4048 16S ribosomal RNA gene | FJ457231 | 100 | 557 | 557 | Bacteria; Proteobacteria; Gammaproteobacteria; Alteromonadales;Pseudoalteromonadaceae; Pseudoalteromonas |
| A472 | puff, 5 | HM032232 | Uncultured marine bacterium clone BM1-4-49 16S ribosomal RNA | FJ825879 | 99 | 715 | 715 | N/A |
| A472 | puff, 5 | HM032232 | *Erythrobacter citreus* strain PR52-9 16S ribosomal RNA gene | EU440970 | 99 | 715 | 715 | Bacteria; Proteobacteria; Alphaproteobacteria; Sphingomonadales;Erythrobacteraceae; Erythrobacter |
| A473 | puff, 5 | HM032231 | Uncultured marine bacterium clone BM1-4-49 16S ribosomal RNA | FJ825879 | 99 | 730 | 730 | N/A |
| A473 | puff, 5 | HM032231 | *Erythrobacter citreus* strain PR52-9 16S ribosomal RNA gene | EU440970 | 99 | 730 | 730 | Bacteria; Proteobacteria; Alphaproteobacteria; Sphingomonadales;Erythrobacteraceae; Erythrobacter |
| A474 | puff, 6 | HM032329 | *Pseudoalteromonas* sp. S1650 16S ribosomal RNA gene | FJ457155 | 99 | 600 | 600 | Bacteria; Proteobacteria; Gammaproteobacteria; Alteromonadales; Pseudoalteromonadaceae; Pseudoalteromonas |
| A476 | puff, 6 | HM032230 | *Acinetobacter* sp. MSIC01 16S ribosomal RNA gene | FJ876296 | 99 | 777 | 778 | Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales; Moraxellaceae; Acinetobacte |
| A477 | puff, 6 | HM032229 | Uncultured Vibrionaceae bacterium clone D004023G01 16S | EU721801 | 100 | 739 | 739 | N/A |
| A477 | puff, 6 | HM032229 | *Vibrio* sp. S1162 16S ribosomal RNA gene | FJ457375 | 100 | 739 | 739 | Bacteria; Proteobacteria; Gammaproteobacteria; Vibrionales;Vibrionaceae; Vibrio |
| A498 | puff, 9 | HM032238 | Uncultured marine bacterium clone BM1-4-49 16S ribosomal RNA | FJ825879 | 99 | 736 | 736 | N/A |
| A498 | puff, 9 | HM032238 | *Erythrobacter citreus* strain PR52-9 16S ribosomal RNA gene | EU440970 | 99 | 736 | 736 | Bacteria; Proteobacteria; Alphaproteobacteria; Sphingomonadales;Erythrobacteraceae; Erythrobacter |
| A499 | puff, 9 | HM032202 | *Rheinheimera* sp. R923 16S small subunit ribosomal RNA gene | EF450318 | 99 | 690 | 690 | Bacteria; Proteobacteria; Gammaproteobacteria; Chromatiales;Chromatiaceae; Rheinheimera |
| A500 | puff, 9 | HM032201 | *Rheinheimera aquimaris* strain SW-369 16S ribosomal RNA gene | EF076758 | 99 | 723 | 725 | Bacteria; Proteobacteria; Gammaproteobacteria; Chromatiales;Chromatiaceae; Rheinheimera |
| A501 | puff, 9 | HM032205 | Alpha proteobacterium A33 gene for 16S rRNA | AB302350 | 99 | 706 | 703 | Bacteria; Proteobacteria; Alphaproteobacteria |
| A502 | puff, 9 | HM032203 | *Paracoccus* sp. MBIC3024 gene for 16S rRNA | AB008115 | 99 | 711 | 711 | Bacteria; Proteobacteria; Alphaproteobacteria; Rhodobacterales;Rhodobacteraceae; Paracoccus |
| A520 | puff, 9 | HM032213 | *Paracoccus* sp. MBIC3024 gene for 16S rRNA | AB008115 | 99 | 737 | 737 | Bacteria; Proteobacteria; Alphaproteobacteria; Rhodobacterales;Rhodobacteraceae; Paracoccus |
| A521 | puff, 9 | HM032212 | *Paracoccus* sp. MBIC3024 gene for 16S rRNA | AB008115 | 99 | 733 | 733 | Bacteria; Proteobacteria; Alphaproteobacteria; Rhodobacterales;Rhodobacteraceae; Paracoccus |

| Isolate ID | Source colony | Accession no. | Sequence ID of nearest BLAST match | Accession no. (BLAST match) | PSI | Number of bases compared | Bit score | Lineage |
|---|---|---|---|---|---|---|---|---|
| A522 | puff, 9 | HM032331 | *Paracoccus* sp. MBIC3024 gene for 16S rRNA | AB008115 | 99 | 889 | 890 | Bacteria; Proteobacteria; Alphaproteobacteria; Rhodobacterales;Rhodobacteraceae; Paracoccus |
| A460 | tuft, 1 | HM032336 | *Arthrobacter* sp. DJ9 16S ribosomal RNA gene | FJ871047 | 100 | 805 | 805 | Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales;Micrococcineae; Micrococcaceae; Arthrobacter |
| A461 | tuft, 1 | HM032237 | *Arthrobacter* sp. DJ9 16S ribosomal RNA gene | FJ871047 | 100 | 723 | 723 | Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales;Micrococcineae; Micrococcaceae; Arthrobacter |
| A462 | tuft, 1 | HM032236 | *Acinetobacter* sp. MSIC01 16S ribosomal RNA gene | FJ876296 | 100 | 722 | 722 | Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales; Moraxellaceae; Acinetobacte |
| A463 | tuft, 1 | HM032334 | Uncultured Vibrionaceae bacterium clone D004023G01 16S | EU721801 | 99 | 808 | 809 | N/A |
| A463 | tuft, 1 | HM032334 | *Vibrio* sp. S1162 16S ribosomal RNA gene | FJ457375 | 99 | 808 | 809 | Bacteria; Proteobacteria; Gammaproteobacteria; Vibrionales;Vibrionaceae; Vibrio |
| A464 | tuft, 1 | HM032235 | Uncultured bacterium clone nbt35b03 16S ribosomal RNA gene | FJ893768 | 99 | 761 | 761 | N/A |
| A464 | tuft, 1 | HM032235 | *Microbacterium* sp. cb-2 16S ribosomal RNA gene | FJ588231 | 99 | 761 | 761 | Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales;Micrococcineae; Microbacteriaceae; Microbacterium |
| A509 | tuft, 10 | HM032221 | Uncultured marine bacterium clone BM1-4-49 16S ribosomal RNA | FJ825879 | 99 | 621 | 621 | N/A |
| A509 | tuft, 10 | HM032221 | *Erythrobacter* sp. MOLA 15 partial 16S rRNA gene | AM990791 | 99 | 621 | 621 | Bacteria; Proteobacteria; Alphaproteobacteria; Sphingomonadales;Erythrobacteraceae; Erythrobacter |
| A510 | tuft, 10 | HM032220 | *Paracoccus* sp. jx9 16S ribosomal RNA gene | FJ539115 | 99 | 690 | 690 | Bacteria; Proteobacteria; Alphaproteobacteria; Rhodobacterales;Rhodobacteraceae; Paracoccus |
| A511 | tuft, 10 | HM032219 | Uncultured marine bacterium clone BM1-4-49 16S ribosomal RNA | FJ825879 | 99 | 644 | 640 | N/A |
| A511 | tuft, 10 | HM032219 | *Erythrobacter* sp. MOLA 15 partial 16S rRNA gene | AM990791 | 99 | 644 | 640 | Bacteria; Proteobacteria; Alphaproteobacteria; Sphingomonadales;Erythrobacteraceae; Erythrobacter |
| A512 | tuft, 10 | HM032218 | *Paracoccus* sp. jx9 16S ribosomal RNA gene | FJ539115 | 100 | 695 | 695 | Bacteria; Proteobacteria; Alphaproteobacteria; Rhodobacterales;Rhodobacteraceae; Paracoccus |
| A516 | tuft, 10 | HM032216 | Uncultured marine bacterium clone BM1-4-49 16S ribosomal RNA gene | FJ825927 | 100 | 730 | 730 | N/A |
| A516 | tuft, 10 | HM032216 | *Erythrobacter citreus* strain PR52-9 16S ribosomal RNA gene | EU440970 | 100 | 730 | 730 | Bacteria; Proteobacteria; Alphaproteobacteria; Sphingomonadales;Erythrobacteraceae; Erythrobacter |
| A517 | tuft, 10 | HM032333 | Uncultured marine bacterium clone BM1-4-49 16S ribosomal RNA | FJ825927 | 100 | 873 | 873 | N/A |
| A517 | tuft, 10 | HM032333 | *Erythrobacter citreus* strain PR52-9 16S ribosomal RNA gene | EU440970 | 100 | 873 | 873 | Bacteria; Proteobacteria; Alphaproteobacteria; Sphingomonadales;Erythrobacteraceae; Erythrobacter |
| A530 | tuft, 10 | HM032199 | Alpha proteobacterium A33 gene for 16S rRNA | AB302350 | 98 | 725 | 727 | Bacteria; Proteobacteria; Alphaproteobacteria. |
| A531 | tuft, 10 | HM032198 | Uncultured marine bacterium clone BM1-4-49 16S ribosomal RNA | FJ825927 | 100 | 574 | 574 | N/A |
| A531 | tuft, 10 | HM032198 | *Erythrobacter* sp. MOLA 15 partial 16S rRNA gene | EU440970 | 100 | 574 | 574 | Bacteria; Proteobacteria; Alphaproteobacteria; Sphingomonadales;Erythrobacteraceae; Erythrobacter |
| A532 | tuft, 10 | HM032327 | Uncultured marine bacterium clone BM1-4-49 16S ribosomal RNA | FJ825927 | 99 | 764 | 764 | N/A |
| A532 | tuft, 10 | HM032327 | *Erythrobacter* sp. MOLA 15 partial 16S rRNA gene | EU440970 | 99 | 764 | 764 | Bacteria; Proteobacteria; Alphaproteobacteria; Sphingomonadales;Erythrobacteraceae; Erythrobacter |
| A533 | tuft, 10 | HM032325 | *Alteromonas macleodii* 16S rRNA gene, strain CH-460 | Y18233 | 97 | 867 | 855 | Bacteria; Proteobacteria; Gammaproteobacteria; Alteromonadales;Alteromonadaceae; Alteromonas |

| Isolate ID | Source colony | Accession no. | Sequence ID of nearest BLAST match | Accession no. (BLAST match) | PSI | Number of bases compared | Bit score | Lineage |
|---|---|---|---|---|---|---|---|---|
| A534 | tuft, 10 | HM032197 | Uncultured marine bacterium clone BM1-4-49 16S ribosomal RNA | FJ825879 | 100 | 586 | 586 | N/A |
| A534 | tuft, 10 | HM032197 | *Erythrobacter* sp. MOLA 15 partial 16S rRNA gene | AM990791 | 100 | 586 | 586 | Bacteria; Proteobacteria; Alphaproteobacteria; Sphingomonadales;Erythrobacteraceae; Erythrobacter |
| A539 | tuft, 10 | HM032195 | Uncultured marine bacterium clone BM1-4-49 16S RNA gene | FJ825879 | 100 | 733 | 733 | N/A |
| A539 | tuft, 10 | HM032195 | *Erythrobacter citreus* strain PR52-9 16S ribosomal RNA gene | EU440970 | 100 | 733 | 733 | Bacteria; Proteobacteria; Alphaproteobacteria; Sphingomonadales;Erythrobacteraceae; Erythrobacter |
| A540 | tuft, 10 | HM032204 | *Paracoccus* sp. S4495 16S ribosomal RNA gene | FJ457301 | 100 | 758 | 758 | Bacteria; Proteobacteria; Alphaproteobacteria; Rhodobacterales;Rhodobacteraceae; Paracoccus |
| A541 | tuft, 10 | HM032194 | Uncultured marine bacterium clone BM1-4-49 16S RNA gene | FJ825927 | 100 | 739 | 739 | N/A |
| A541 | tuft, 10 | HM032194 | *Erythrobacter citreus* strain PR52-9 16S ribosomal RNA gene | EU440970 | 100 | 739 | 739 | Bacteria; Proteobacteria; Alphaproteobacteria; Sphingomonadales;Erythrobacteraceae; Erythrobacter |
| A542 | tuft, 10 | HM032193 | *Paracoccus* sp. S4495 16S ribosomal RNA gene | FJ457301 | 100 | 740 | 740 | Bacteria; Proteobacteria; Alphaproteobacteria; Rhodobacterales;Rhodobacteraceae; Paracoccus |
| A507 | tuft, 11 | HM032187 | Uncultured bacterium clone BA2 16S ribosomal RNA gene | FJ618851 | 100 | 711 | 711 | N/A |
| A507 | tuft, 11 | HM032187 | *Vibrio* sp. S1162 16S ribosomal RNA gene | FJ457375 | 100 | 711 | 711 | Bacteria; Proteobacteria; Gammaproteobacteria; Vibrionales;Vibrionaceae; Vibrio |
| A508 | tuft, 11 | HM032186 | Uncultured bacterium clone AJ 16S ribosomal RNA gene | FJ154983 | 99 | 684 | 684 | N/A |
| A508 | tuft, 11 | HM032186 | *Pseudoalteromonas* sp. OC-A5-12 16S ribosomal RNA gene | DQ319006 | 99 | 684 | 684 | Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales;Pseudomonadaceae; Pseudomonas |
| A515 | tuft, 11 | | *Exiguobacterium* sp. AT1b | CP001615 | 100 | 738 | 738 | Bacteria; Firmicutes; Bacillales; Bacillales Family XII. Incertae;Sedis; Exiguobacterium |
| A518 | tuft, 11 | HM032215 | *Exiguobacterium* sp. AT1b | CP001615 | 100 | 760 | 760 | Bacteria; Firmicutes; Bacillales; Bacillales Family XII. Incertae;Sedis; Exiguobacterium |
| A519 | tuft, 11 | HM032214 | Bacterium WP3ISO12 16S ribosomal RNA gene | DQ985873 | 99 | 732 | 733 | |
| A523 | tuft, 11 | HM032211 | Uncultured *Vibrio* sp. partial 16S rRNA gene, clone HG136 | FM958469 | 100 | 681 | 681 | N/A |
| A523 | tuft, 11 | HM032211 | *Vibrio* sp. S3860 16S ribosomal RNA gene | FJ457568 | 100 | 681 | 681 | Bacteria; Proteobacteria; Gammaproteobacteria; Vibrionales;Vibrionaceae; Vibrio |
| A527 | tuft, 11 | HM032208 | *Pseudoalteromonas* sp. S1650 16S ribosomal RNA gene | FJ457155 | 100 | 716 | 716 | Bacteria; Proteobacteria; Gammaproteobacteria; Alteromonadales; Pseudoalteromonadaceae; Pseudoalteromonas |
| A528 | tuft, 11 | HM032207 | *Arthrobacter* sp. DJ9 16S ribosomal RNA gene | FJ871047 | 100 | 728 | 728 | Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales;Micrococcineae; Micrococcaceae; Arthrobacter |
| A529 | tuft, 11 | HM032206 | Uncultured *Vibrio* sp. partial 16S rRNA gene, clone HG136 | FM958469 | 100 | 724 | 724 | N/A |
| A529 | tuft, 11 | HM032206 | *Vibrio* sp. S3860 16S ribosomal RNA gene | FJ457568 | 100 | 724 | 724 | Bacteria; Proteobacteria; Gammaproteobacteria; Vibrionales;Vibrionaceae; Vibrio |
| A535 | tuft, 11 | HM032196 | *Vibrio natriegens* partial 16S rRNA gene, strain CECT 7466 | FM999825 | 99 | 683 | 683 | Bacteria; Proteobacteria; Gammaproteobacteria; Vibrionales;Vibrionaceae; Vibrio |
| A497 | tuft, 2 | HM032337 | Gamma proteobacterium C47 gene for 16S rRNA | AB302347 | 99 | 858 | 859 | Bacteria; Proteobacteria; Gammaproteobacteria |
| A547 | tuft, 2 | HM032337 | *Paracoccus* sp. MBIC3024 gene for 16S rRNA | AB008115 | 99 | 77 | 707 | Bacteria; Proteobacteria; Alphaproteobacteria; Rhodobacterales;Rhodobacteraceae; Paracoccus |
| A448 | tuft, 3 | HM032247 | *Paracoccus* sp. MBIC3024 gene for 16S rRNA | AB008115 | 99 | 767 | 767 | Bacteria; Proteobacteria; Alphaproteobacteria; Rhodobacterales;Rhodobacteraceae; Paracoccus |
| A466 | tuft, 3 | HM032234 | *Micrococcus* sp. H5 partial 16S rRNA gene, strain H5 | AM235880 | 99 | 601 | 601 | Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Micrococcineae; Micrococcaceae; Micrococcus |

| Isolate ID | Source colony | Accession no. | Sequence ID of nearest BLAST match | Accession no. (BLAST match) | PSI | Number of bases compared | Bit score | Lineage |
|---|---|---|---|---|---|---|---|---|
| A467 | tuft, 3 | HM032332 | *Acinetobacter venetianus* partial 16S rRNA gene, strain ACI555 | AM909651 | 100 | 772 | 772 | Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales;Moraxellaceae; Acinetobacter |
| A468 | tuft, 3 | HM032340 | Uncultured Vibrionaceae bacterium clone D004023G01 16S | EU721801 | 100 | 752 | 752 | N/A |
| A468 | tuft, 3 | HM032340 | *Vibrio* sp. S1162 16S ribosomal RNA gene | FJ457375 | 100 | 752 | 752 | Bacteria; Proteobacteria; Gammaproteobacteria; Vibrionales;Vibrionaceae; Vibrio |
| A469 | tuft, 3 | HM032233 | *Acinetobacter* sp. MSIC01 16S ribosomal RNA gene | FJ876296 | 100 | 741 | 741 | Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales; Moraxellaceae; Acinetobacte |
| A470 | tuft, 3 | HM032328 | *Paracoccus* sp. HZ04 16S ribosomal RNA gene | AY690705 | 99 | 830 | 831 | Bacteria; Proteobacteria; Alphaproteobacteria; Rhodobacterales;Rhodobacteraceae; Paracoccus |
| A453 | tuft, 7 | HM032200 | *Pseudoalteromonas* sp. S1650 16S ribosomal RNA gene | FJ457155 | 100 | 733 | 733 | Bacteria; Proteobacteria; Gammaproteobacteria; Alteromonadales; Pseudoalteromonadaceae; Pseudoalteromonas |
| A454 | tuft, 7 | HM032189 | *Pseudoalteromonas* sp. S4491 16S ribosomal RNA gene | FJ457244 | 99 | 705 | 705 | Bacteria; Proteobacteria; Gammaproteobacteria; Alteromonadales; Pseudoalteromonadaceae; Pseudoalteromonas |
| A478 | tuft, 7 | HM032228 | *Acinetobacter* sp. MSIC01 16S ribosomal RNA gene | FJ876296 | 100 | 743 | 743 | Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales; Moraxellaceae; Acinetobacter |
| A479 | tuft, 7 | HM032227 | *Pseudoalteromonas* sp. S187 16S ribosomal RNA gene | FJ457123 | 100 | 715 | 715 | Bacteria; Proteobacteria; Gammaproteobacteria; Alteromonadales; Pseudoalteromonadaceae; Pseudoalteromonas |
| A481 | tuft, 7 | HM032326 | Uncultured marine bacterium clone BM1-4-49 16S ribosomal RNA gene | FJ825927 | 99 | 812 | 812 | N/A |
| A481 | tuft, 7 | HM032326 | *Erythrobacter citreus* strain PR52-9 16S ribosomal RNA gene | EU440970 | 99 | 812 | 812 | Bacteria; Proteobacteria; Alphaproteobacteria; Sphingomonadales;Erythrobacteraceae; Erythrobacter |
| A482 | tuft, 7 | HM032226 | Uncultured bacterium clone nbt35b03 16S ribosomal RNA gene | FJ893768 | 100 | 722 | 722 | N/A |
| A482 | tuft, 7 | HM032226 | *Microbacterium* sp. ENB-8 16S ribosomal RNA gene | FJ608797 | 100 | 722 | 722 | Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales;Micrococcineae; Microbacteriaceae; Microbacterium |
| A483 | tuft, 7 | HM032225 | *Paracoccus* sp. HZ04 16S ribosomal RNA gene | AY690705 | 100 | 730 | 730 | Bacteria; Proteobacteria; Alphaproteobacteria; Rhodobacterales;Rhodobacteraceae; Paracoccus |
| A484 | tuft, 7 | HM032224 | *Alteromonas* sp. MA336 gene for 16S rRNA | AB491744 | 100 | 717 | 717 | Bacteria; Proteobacteria; Gammaproteobacteria; Alteromonadales;Alteromonadaceae; Alteromonas |
| A485 | tuft, 7 | HM032223 | *Alteromonas* sp. MA336 gene for 16S rRNA | AB491744 | 100 | 725 | 725 | Bacteria; Proteobacteria; Gammaproteobacteria; Alteromonadales;Alteromonadaceae; Alteromonas |
| A486 | tuft, 7 | HM032222 | *Pimelobacter simplex* strain S151 16S ribosomal RNA gene | AY509240 | 100 | 724 | 724 | Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales;Propionibacterineae; Nocardioidaceae; Pimelobacter |
| A487 | tuft, 7 | HM032243 | *Arthrobacter* sp. DJ9 16S ribosomal RNA gene | FJ871047 | 100 | 745 | 745 | Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales;Micrococcineae; Micrococcaceae; Arthrobacter |
| A488 | tuft, 7 | HM032324 | *Pseudoalteromonas* sp. S187 16S ribosomal RNA gene | FJ457123 | 99 | 843 | 843 | Bacteria; Proteobacteria; Gammaproteobacteria; Alteromonadales; Pseudoalteromonadaceae; Pseudoalteromonas |
| A490 | tuft, 7 | HM032330 | *Pimelobacter simplex* strain S151 16S ribosomal RNA gene | AY509240 | 99 | 683 | 683 | Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales;Propionibacterineae; Nocardioidaceae; Pimelobacter |
| A491 | tuft, 7 | HM032339 | Uncultured Vibrionaceae bacterium clone D004023G01 16S | EU721801 | 99 | 730 | 732 | N/A |
| A491 | tuft, 7 | HM032339 | *Vibrio* sp. S1162 16S ribosomal RNA gene | FJ457375 | 99 | 730 | 732 | Bacteria; Proteobacteria; Gammaproteobacteria; Vibrionales;Vibrionaceae; Vibrio |
| A492 | tuft, 7 | HM032242 | Uncultured bacterium AD84-H10 genomic sequence | EU686598 | 100 | 727 | 727 | N/A |
| A492 | tuft, 7 | HM032242 | *Erythrobacter* sp. MOLA 72 partial 16S rRNA gene | AM990847 | 100 | 727 | 727 | Bacteria; Proteobacteria; Alphaproteobacteria; Sphingomonadales;Sphingomonadaceae |

| Isolate ID | Source colony | Accession no. | Sequence ID of nearest BLAST match | Accession no. (BLAST match) | PSI | Number of bases compared | Bit score | Lineage |
|---|---|---|---|---|---|---|---|---|
| A493 | tuft, 7 | HM032241 | *Rheinheimera aquimaris* strain SW-369 16S ribosomal RNA gene | EF076758 | 99 | 772 | 774 | Bacteria; Proteobacteria; Gammaproteobacteria; Chromatiales;Chromatiaceae; Rheinheimera |
| A505 | tuft, 7 | HM032335 | *Paracoccus* sp. MBIC3024 gene for 16S rRNA | AB008115 | 99 | 740 | 741 | Bacteria; Proteobacteria; Alphaproteobacteria; Rhodobacterales;Rhodobacteraceae; Paracoccus |
| A506 | tuft, 7 | HM032188 | *Bacillus firmus* strain IMAUB1032 16S ribosomal RNA gene | FJ641034 | 98 | 712 | 712 | Bacteria; Firmicutes; Bacillales; Bacillaceae; Bacillus |
| A524 | tuft, 7 | HM032210 | *Paracoccus* sp. jx9 16S ribosomal RNA gene | FJ539115 | 100 | 647 | 647 | Bacteria; Proteobacteria; Alphaproteobacteria; Rhodobacterales;Rhodobacteraceae; Paracoccus |
| A525 | tuft, 7 | HM032209 | *Rheinheimera aquimaris* strain SW-369 16S ribosomal RNA gene | EF076758 | 99 | 725 | 727 | Bacteria; Proteobacteria; Gammaproteobacteria; Chromatiales;Chromatiaceae; Rheinheimera |
| A543 | tuft, 7 | HM032192 | Uncultured *Vibrio* sp. partial 16S rRNA gene, clone HG136 | FM958469 | 99 | 713 | 713 | N/A |
| A543 | tuft, 7 | HM032192 | *Vibrio* sp. S3860 16S ribosomal RNA gene | FJ457568 | 99 | 713 | 713 | Bacteria; Proteobacteria; Gammaproteobacteria; Vibrionales;Vibrionaceae; Vibrio |
| A544 | tuft, 7 | HM032191 | *Vibrio* sp. C22-B 16S ribosomal RNA gene | EU563342 | 100 | 725 | 725 | Bacteria; Proteobacteria; Gammaproteobacteria; Vibrionales;Vibrionaceae; Vibrio |
| A459 | tuft, 8 | HM032244 | *Pseudoalteromonas* sp. S4491 16S ribosomal RNA gene | FJ457244 | 99 | 722 | 722 | Bacteria; Proteobacteria; Gammaproteobacteria; Alteromonadales; Pseudoalteromonadaceae; Pseudoalteromonas |
| A494 | tuft, 8 | HM032240 | *Paracoccus* sp. jx9 16S ribosomal RNA gene | FJ539115 | 99 | 716 | 716 | Bacteria; Proteobacteria; Alphaproteobacteria; Rhodobacterales;Rhodobacteraceae; Paracoccus |
| A495 | tuft, 8 | HM032239 | *Acinetobacter venetianus* partial 16S rRNA gene, strain ACI555 | AM909651 | 100 | 671 | 679 | Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales; Moraxellaceae; Acinetobacter |

**Supplemental Table 2**. Nearest GenBank neighbors of cultivars isolated at WHOI from frozen *Trichodesmium* colonies collected at BATS.

| Isolate ID | Source colony | Isolation method | Accession no. | Sequence ID of nearest BLAST match | Accession no. (BLAST match) | PSI | Number of bases compared | Bit score | Lineage |
|---|---|---|---|---|---|---|---|---|---|
| A054 | Frozen filter | Method 1, SWT | HM032254 | *Pimelobacter simplex* strain S151 16S rRNA gene | AY509240 | 97 | 619 | 1061 | Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Propionibacterineae; Nocardioidaceae; Pimelobacter |
| A055 | Frozen filter | Method 1, SWT | HM032255 | *Gordonia* sp. FWA92 16S rRNA gene | AY115594 | 99 | 696 | 1264 | Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Corynebacterineae; Gordoniaceae; Gordonia |
| A059 | Frozen filter | Method 1, SWT | HM032257 | *Pimelobacter simplex* strain S151 16S rRNA gene | AY509240 | 99 | 643 | 1182 | Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Propionibacterineae; Nocardioidaceae; Pimelobacter |
| A061 | Frozen filter | Method 1, SWT | HM032258 | *Rhodococcus* sp. VS-8 16S rRNA gene | FJ497702 | 99 | 745 | 1352 | Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales;Corynebacterineae; Nocardiaceae; Rhodococcus |
| A062 | Frozen filter | Method 1, SWT | HM032259 | *Aeromicrobium* sp. 1/4_C7/16_31 16S rRNA gene | EF540466 | 98 | 764 | 1354 | Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Propionibacterineae; Nocardioidaceae; Aeromicrobium |
| A063 | Frozen filter | Method 1, SWT | HM032260 | Uncultured bacterium clone CE2-DCM-13 16S rRNA gene | GU061599 | 99 | 766 | 1402 | N/A |
| A064 | Frozen filter | Method 1, SWT | HM032261 | *Gordonia* sp. CNJ786 PL04 16S rRNA gene | DQ448772 | 97 | 634 | 1092 | Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Corynebacterineae; Gordoniaceae; Gordonia |
| A065 | Frozen filter | Method 1, SWT | HM032262 | *Rhodococcus* sp. VS-8 16S rRNA gene | FJ497702 | 99 | 720 | 1301 | Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales;Corynebacterineae; Nocardiaceae; Rhodococcus |
| A066 | Frozen filter | Method 1, SWT | HM032263 | *Rhodococcus* sp. VS-8 16S rRNA gene | FJ497702 | 99 | 691 | 1264 | Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales;Corynebacterineae; Nocardiaceae; Rhodococcus |
| A067 | Frozen filter | Method 1, SWT | HM032264 | *Paracoccus* sp. MBIC3024 gene for 16S rRNA | AB008115 | 99 | 655 | 1199 | Bacteria; Proteobacteria; Alphaproteobacteria; Rhodobacterales; Rhodobacteraceae; Paracoccus |
| A071 | Frozen filter | Method 1, SWT | HM032265 | Bacterium daSW.34 16S rRNA gene | EU935309 | 99 | 770 | 1387 | N/A |
| A072 | Frozen filter | Method 1, SWT | HM032266 | *Gordonia* sp. FWA92 16S rRNA gene | AY115594 | 92 | 595 | 854 | Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Corynebacterineae; Gordoniaceae; Gordonia |
| A073 | Frozen filter | Method 1, SWT | HM032267 | *Gordonia* sp. FWA92 16S rRNA gene | AY115594 | 92 | 595 | 843 | Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Corynebacterineae; Gordoniaceae; Gordonia |
| A075 | Frozen filter | Method 1, SWT | HM032268 | *Gordonia* sp. FWA92 16S rRNA gene | AY115594 | 91 | 602 | 828 | Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Corynebacterineae; Gordoniaceae; Gordonia |
| A078 | Frozen filter | Method 1, SWT | HM032269 | *Pimelobacter simplex* strain S151 16S rRNA gene | AY509240 | 98 | 690 | 1208 | Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Propionibacterineae; Nocardioidaceae; Pimelobacter |
| A080 | Frozen filter | Method 1, SWT | HM032270 | *Aeromicrobium* sp. 1/4_C7/16_31 16S rRNA gene | EF540466 | 98 | 743 | 1321 | Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Propionibacterineae; Nocardioidaceae; Aeromicrobium |
| A081 | Frozen filter | Method 1, SWT | HM032271 | *Pimelobacter simplex* strain S151 16S rRNA gene | AY509240 | 99 | 763 | 1402 | Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Propionibacterineae; Nocardioidaceae; Pimelobacter |
| A082 | Frozen filter | Method 1, SWT | HM032272 | *Pimelobacter simplex* strain S151 16S rRNA gene | AY509240 | 97 | 593 | 1002 | Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Propionibacterineae; Nocardioidaceae; Pimelobacter |
| A083 | Frozen filter | Method 1, SWT | HM032273 | *Aeromicrobium* sp. 1/4_C7/16_31 16S rRNA gene | EF540466 | 99 | 772 | 1387 | Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Propionibacterineae; Nocardioidaceae; Aeromicrobium |
| A084 | Frozen filter | Method 1, SWT | HM032274 | *Agromyces* sp. Xb-3 16S rRNA gene | DQ311007 | 99 | 761 | 1393 | Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Micrococcineae; Microbacteriaceae; Agromyces |
| A085 | Frozen filter | Method 1, SWT | HM032275 | *Pimelobacter simplex* strain S151 16S rRNA gene | AY509240 | 99 | 712 | 1304 | Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Propionibacterineae; Nocardioidaceae; Pimelobacter |
| A088 | Frozen filter | Method 1, SWT | HM032276 | *Gordonia* sp. FWA92 16S rRNA gene | AY115594 | 99 | 693 | 1258 | Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Corynebacterineae; Gordoniaceae; Gordonia |
| A089 | Frozen filter | Method 1, SWT | HM032277 | *Pimelobacter simplex* strain S151 16S rRNA gene | AY509240 | 99 | 753 | 1363 | Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Propionibacterineae; Nocardioidaceae; Pimelobacter |

| Isolate ID | Source colony | Isolation method | Accession no. | Sequence ID of nearest BLAST match | Accession no. (BLAST match) | PSI | Number of bases compared | Bit score | Lineage |
|---|---|---|---|---|---|---|---|---|---|
| A090 | Frozen filter | Method 1, SWT | HM032278 | *Aeromicrobium* sp. 1/4_C7/16_31 16S rRNA gene | EF540466 | 98 | 723 | 1284 | Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Propionibacterineae; Nocardioidaceae; Aeromicrobium |
| A095 | Frozen filter | Method 1, SWT | HM032279 | *Gordonia* sp. FWA92 16S rRNA gene | AY115594 | 89 | 577 | 743 | Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Corynebacterineae; Gordoniaceae; Gordonia |
| A096 | Frozen filter | Method 1, SWT | HM032280 | *Rhodococcus* sp. VS-8 16S rRNA gene | FJ497702 | 99 | 749 | 1365 | Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Corynebacterineae; Nocardiaceae; Rhodococcus. |
| A097 | Frozen filter | Method 1, SWT | HM032281 | Gordonia sp. CNJ786 PL04 16S rRNA gene | DQ448772 | 99 | 743 | 1332 | Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Corynebacterineae; Gordoniaceae; Gordonia |
| A100 | Frozen filter | Method 1, SWT | HM032282 | *Paracoccus* sp. MBIC3024 gene for 16S rRNA | AB008115 | 99 | 669 | 1225 | Bacteria; Proteobacteria; Alphaproteobacteria; Rhodobacterales;Rhodobacteraceae; Paracoccus |
| A102 | Frozen filter | Method 1, SWT | HM032283 | *Pimelobacter simplex* strain S151 16S rRNA gene | AY509240 | 97 | 600 | 1013 | Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Propionibacterineae; Nocardioidaceae; Pimelobacter |
| A109 | Frozen filter | Method 1, SWT | HM032284 | *Pimelobacter simplex* strain S151 16S rRNA gene | AY509240 | 98 | 681 | 1190 | Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Propionibacterineae; Nocardioidaceae; Pimelobacter |
| A110 | Frozen filter | Method 1, SWT | HM032285 | *Paracoccus* sp. MBIC3024 gene for 16S rRNA | AB008115 | 98 | 734 | 1310 | Bacteria; Proteobacteria; Alphaproteobacteria; Rhodobacterales;Rhodobacteraceae; Paracoccus |
| A056 | Frozen filter | Method 1, VSS | HM032256 | *Gordonia* sp. FWA92 16S RNA gene | AY115594 | 89 | 548 | 704 | Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Corynebacterineae; Gordoniaceae; Gordonia |
| A036 | Frozen filter | Method 1, VSS | HM032248 | *Gordonia* sp. FWA92 16S rRNA gene | AY115594 | 98 | 693 | 1221 | Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Corynebacterineae; Gordoniaceae; Gordonia |
| A037 | Frozen filter | Method 1, VSS | HM032249 | *Rhodococcus sp.* VS-8 16S rRNA gene | FJ497702 | 96 | 728 | 1208 | Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales;Corynebacterineae; Nocardiaceae; Rhodococcus |
| A039 | Frozen filter | Method 1, VSS | HM032250 | *Rhodococcus sp.* VS-8 16S rRNA gene | FJ497702 | 99 | 749 | 1365 | Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales;Corynebacterineae; Nocardiaceae; Rhodococcus |
| A041 | Frozen filter | Method 1, VSS | HM032251 | *Rhodococcus sp.* VS-8 16S rRNA gene | FJ497702 | 99 | 773 | 1410 | Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales;Corynebacterineae; Nocardiaceae; Rhodococcus |
| A049 | Frozen filter | Method 1, VSS | HM032252 | *Rhodococcus sp*. VS-8 16S rRNA gene | FJ497702 | 99 | 773 | 1415 | Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales;Corynebacterineae; Nocardiaceae; Rhodococcus |
| A051 | Frozen filter | Method 1, VSS | HM032253 | *Pimelobacter simplex* strain S151 16S rRNA gene | AY509240 | 99 | 762 | 1373 | Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Propionibacterineae; Nocardioidaceae; Pimelobacter |
| A111 | Frozen filter | Method 3, VSS | HM032286 | *Rhodococcus* sp. VS-8 16S rRNA gene | FJ497702 | 99 | 746 | 1360 | Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Corynebacterineae; Nocardiaceae; Rhodococcus. |
| A113 | Frozen filter | Method 3, VSS | HM032287 | *Rhodococcus* sp. VS-8 16S rRNA gene | FJ497702 | 99 | 741 | 1356 | Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Corynebacterineae; Nocardiaceae; Rhodococcus. |
| A114 | Frozen filter | Method 3, SWT | HM032288 | *Agromyces* sp. MJ21 16S rRNA gene | GQ241325 | 98 | 649 | 1158 | Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Micrococcineae; Microbacteriaceae; Agromyces |
| A115 | Frozen filter | Method 3, SWT | HM032289 | *Agromyces* sp. MJ21 16S rRNA gene | GQ241325 | 97 | 648 | 1112 | Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Micrococcineae; Microbacteriaceae; Agromyces |
| A116 | Frozen filter | Method 3, SWT | HM032290 | *Agromyces* sp. MJ21 16S rRNA gene | GQ241325 | 97 | 697 | 1208 | Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Micrococcineae; Microbacteriaceae; Agromyces |
| A117 | Frozen filter | Method 3, SWT | HM032291 | *Agromyces* sp. MJ21 16S rRNA gene | GQ241325 | 96 | 649 | 1086 | Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Micrococcineae; Microbacteriaceae; Agromyces |
| A118 | Frozen filter | Method 3, SWT | HM032292 | *Agromyces* sp. MJ21 16S rRNA gene | GQ241325 | 95 | 520 | 828 | Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Micrococcineae; Microbacteriaceae; Agromyces |
| A127 | Frozen filter | Method 4, SWT | HM032293 | Bacterium enrichment culture clone NCAAH 30A10 | GQ221076 | 98 | 769 | 1371 | N/A |
| A127 | Frozen filter | Method 4, SWT | HM032293 | *Vitellibacter* sp. D7-13 partial 16S rRNA gene, isolate D7-1( | AM403205 | 98 | 766 | 1347 | Bacteria; Bacteroidetes; Flavobacteria; Flavobacteriales; Flavobacteriaceae; Vitellibacter |
| A128 | Frozen filter | Method 4, SWT | HM032294 | *Agromyces* sp. MJ21 16S rRNA gene | GQ241325 | 96 | 621 | 1016 | Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Micrococcineae; Microbacteriaceae; Agromyces |
| A132 | Frozen filter | Method 4, SWT | HM032295 | *Agromyces mediolanus* strain c18 16S ribosomal RNA gen | FJ950540 | 100 | 730 | 1349 | Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Micrococcineae; Microbacteriaceae; Agromyces |

| Isolate ID | Source colony | Isolation method | Accession no. | Sequence ID of nearest BLAST match | Accession no. (BLAST match) | PSI | Number of bases compared | Bit score | Lineage |
|---|---|---|---|---|---|---|---|---|---|
| A137 | Frozen filter | Method 4, Methylophaga | HM032296 | *Gordonia* sp. FWA92 16S rRNA gene | AY115594 | 91 | 549 | 758 | Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Corynebacterineae; Gordoniaceae; Gordonia |
| A159 | Frozen filter | Method 3, VSS | HM032297 | *Rhodococcus* sp. VS-8 16S rRNA gene | FJ497702 | 99 | 749 | 1360 | Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Corynebacterineae; Nocardiaceae; Rhodococcus. |
| A160 | Frozen filter | Method 3, SWT | HM032298 | Bacterium enrichment culture clone NCAAH 30A10 | GQ221076 | 99 | 769 | 1384 | N/A |
| A160 | Frozen filter | Method 3, SWT | HM032298 | *Vitellibacter* sp. D7-13 partial 16S rRNA gene, isolate D7-1( | AM403205 | 98 | 769 | 1365 | Bacteria; Bacteroidetes; Flavobacteria; Flavobacteriales; Flavobacteriaceae; Vitellibacter |
| A161 | Frozen filter | Method 3, SWT | HM032299 | Bacterium enrichment culture clone NCAAH 30A10 | GQ221076 | 94 | 592 | 900 | N/A |
| A161 | Frozen filter | Method 3, SWT | HM032299 | *Vitellibacter* sp. D7-13 partial 16S rRNA gene, isolate D7-1( | AM403205 | 93 | 592 | 876 | Bacteria; Bacteroidetes; Flavobacteria; Flavobacteriales; Flavobacteriaceae; Vitellibacter |
| A162 | Frozen filter | Method 3, SWT | HM032300 | *Agromyces* sp. MJ21 16S rRNA gene | GQ241325 | 99 | 701 | 1266 | Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Micrococcineae; Microbacteriaceae; Agromyces |
| A163 | Frozen filter | Method 3, SWT | HM032301 | Bacterium enrichment culture clone NCAAH 30A10 | GQ221076 | 99 | 768 | 1397 | N/A |
| A163 | Frozen filter | Method 3, SWT | HM032301 | *Vitellibacter* sp. D7-13 partial 16S rRNA gene, isolate D7-1( | AM403205 | 99 | 768 | 1378 | Bacteria; Bacteroidetes; Flavobacteria; Flavobacteriales; Flavobacteriaceae; Vitellibacter |
| A164 | Frozen filter | Method 3, SWT | HM032302 | *Agromyces* sp. MJ21 16S rRNA gene | GQ241325 | 99 | 684 | 1229 | Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Micrococcineae; Microbacteriaceae; Agromyces |
| A165 | Frozen filter | Method 3, SWT | HM032303 | Bacterium enrichment culture clone NCAAH 30A10 | GQ221076 | 99 | 772 | 1404 | N/A |
| A165 | Frozen filter | Method 3, SWT | HM032303 | *Vitellibacter* sp. D7-13 partial 16S rRNA gene, isolate D7-1( | AM403205 | 99 | 769 | 1380 | Bacteria; Bacteroidetes; Flavobacteria; Flavobacteriales; Flavobacteriaceae; Vitellibacter |
| A168 | Frozen filter | Method 1, SWT | HM032304 | Uncultured bacterium clone CE2-DCM-93 16S rRNA gene | GU061604 | 99 | 644 | 1158 | N/A |
| A168 | Frozen filter | Method 1, SWT | HM032304 | Bacterium daSW.34 16S rRNA gene | EU935309 | 99 | 644 | 1158 | N/A |
| A169 | Frozen filter | Method 1, SWT | HM032305 | *Pimelobacter simplex* strain S151 16S rRNA gene | AY509240 | 97 | 668 | 1129 | Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Propionibacterineae; Nocardioidaceae; Pimelobacter |
| A170 | Frozen filter | Method 1, SWT | HM032306 | Uncultured bacterium isolate M6 16S rRNA gene | EF455588 | 99 | 687 | 1258 | N/A |
| A171 | Frozen filter | Method 1, SWT | HM032307 | *Aeromicrobium* sp. 1/4_C7/16_31 16S rRNA gene | EF540466 | 99 | 772 | 1399 | Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Propionibacterineae; Nocardioidaceae; Aeromicrobium |
| A172 | Frozen filter | Method 1, SWT | HM032308 | Uncultured organism clone ctg_CGOF202 16S rRNA gene | DQ395725 | 94 | 484 | 745 | N/A |
| A172 | Frozen filter | Method 1, SWT | HM032308 | *Novosphingobium tardaugens* | AB070237 | 94 | 483 | 734 | Bacteria; Proteobacteria; Alphaproteobacteria; Sphingomonadales; Sphingomonadaceae; Novosphingobium |
| A174 | Frozen filter | Method 1, SWT | HM032309 | *Pimelobacter simplex* strain S151 16S rRNA gene | AY509240 | 99 | 723 | 1323 | Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Propionibacterineae; Nocardioidaceae; Pimelobacter |
| A181 | Frozen filter | Method 3, SWT | HM032310 | *Pimelobacter simplex* strain S151 16S rRNA gene | AY509240 | 99 | 756 | 1384 | Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Propionibacterineae; Nocardioidaceae; Pimelobacter |

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# CHAPTER 4:

# *TRICHODESMIUM THIEBAUTII* COLONIES AT THE BERMUDA ATLANTIC TIME SERIES STATION ARE ASSOCIATED WITH DISTINCTIVE EPIBIONT POPULATIONS


## ABSTRACT

Scientific interest in *Trichodesmium* has been fueled by its prominent role in the marine nitrogen cycle, however, it is often overlooked that *Trichodesmium* colonies are only one member of a complex consortia composed of bacteria, microzooplankton, and metazoa. Associated bacteria, in addition to improving the efficiency of nitrogen fixation, may also dictate the longevity of *Trichodesmium* blooms, and the fate of the carbon and nitrogen fixed by *Trichodesmium*. In this manner, the attached bacteria are themselves important players in the marine carbon cycle and of biogeochemical significance in their own right. In this study, we aimed to phylogenetically classify the communities associated with two morphological types of *Trichodesmium* colonies, tufts and puffs. Samples were obtained at the Bermuda Atlantic Time Series (BATS) site in the Sargasso Sea and data was compared with previously published clone libraries constructed from seawater at the same site. Communities associated with *Trichodesmium* tufts and puffs are distinct with respect to seawater and with respect to one another: no overlapping OTUs at the 97 percent similarity threshold were identified between puff and tuft samples, possibly indicating a role for heterotrophic bacteria in determining the colony morphology and

perhaps different ecosystem roles for tufts and puffs in the oceans. Overall, these data suggest that *Trichodesmium* possess an epibiotic microbial community distinctly different from that observed in bacterial picoplankton (interestingly, no archaeal or eukaryotic SSU rRNA gene sequences were detected). Moreover, *Trichodesmium* epibiotic communities display drastically lower diversity than bacterioplankton, supporting the idea that these assemblages are microbial hot spots in marine oligotrophic surface water environments.

## INTRODUCTION

Heterotrophic bacteria colonize the surface of marine eukaryotic algae (Fisher *et al.*, 1998; Sapp *et al.*, 2007) and cyanobacteria (Herbst & Overbeck, 1978; Paerl, 1982; Siddiqui *et al.*, 1992; Nausch, 1996; Sheridan *et al.*, 2002; Stevenson & Waterbury, 2006; Simmons *et al.*, 2008) with very few exceptions. These associations are the subject of numerous studies and in many cases have been found to be quite specific (Paerl & Gallucci, 1985; Fisher *et al.*, 1998; Stevenson & Waterbury, 2006; Tuomainen *et al.*, 2006) and in some cases, symbiotic (Behrens *et al.*, 2008). Bacteria colonize algae throughout their life-cycle and at all bloom stages and this colonization is not considered to be a sign of senescence or disease (Paerl *et al.*, 1989; Sapp *et al.*, 2007). The physical attachment and specific association between healthy algae and bacteria is suggestive that a functional mutualism has evolved between the two sets of organisms.

Scientific interest in the cyanobacterial genera *Trichodesmium* has largely been driven by their significant role as nitrogen-fixers (Capone *et al.*, 1997; Karl *et al.*, 1997).

They are capable of fixing at least 80 Gt N $y^{-1}$ in extremely low-nutrient, oligotrophic marine environments; as such, they are understood to be a major source of fixed carbon and reduced nitrogen to these environments and fuel hotspots of microbial activity in surface waters (Letelier & Karl, 1996; Capone *et al.*, 1997; Carpenter *et al.*, 1997)

The heterotrophic epibionts attached to nitrogen-fixing cyanobacteria may well achieve broad ecological impacts insomuch as they may influence the longevity of blooms (Tuomainen *et al.*, 2006) and the efficiency of nitrogen fixation by contributing to the formation of anoxic microzones within cyanobacterial colonies (Herbst & Overbeck, 1978; Paerl & Pinckney, 1996). It is extraordinarily difficult to isolate *Trichodesmium* spp. in axenic culture, suggesting that they have evolved an essential relationship with their epibionts (Waterbury, 1991).

Without exception, heterotrophic bacteria and, frequently, filamentous cyanobacteria are observed attached to the surface of *Trichodesmium* spp. (Herbst & Overbeck, 1978; Paerl *et al.*, 1989; Siddiqui *et al.*, 1992). Cell densities on *Trichodesmium* are reported to range from $8.2 \times 10^{8}$ cells $ml^{-1}$ (Sheridan *et al.*, 2002) up to $2.6 \times 10^{11}$ cells $ml^{-1}$ (Paerl, 1982) which is 3-5 orders of magnitude more concentrated than planktonic bacteria in seawater. Epibiotic bacteria concentrate more heavily on *Trichodesmium* than on other particles, such as marine snow (Sheridan *et al.*, 2002).

Individual *Trichodesmium* cells typically form multi-cell trichomes which can bundle together in multi-trichome colonies (Carpenter *et al.*, 2004). These colonies tend to be dominated by two morphologic types, a spherical "puff"-type and a fusiform "tuft"-type. Bacteria on tuft-type colonies have been observed to occur in higher densities

(Sheridan *et al.*, 2002) and exhibit higher hydrolytic enzyme activity (Nausch, 1996) relative to those on puff-type colonies, although the diversity of metazoa and microzooplankton associated with puff-type colonies appears to be greater than that associated with tufts (Sheridan *et al.*, 2002). The diversity of the bacterial community associated with *Trichodesmium* colonies and the basis for the discrepancy between microbial activities associated with tufts and puffs has not been examined.

The balance between primary production and heterotrophic remineralization processes is a major process in the ocean's carbon cycle. Attached bacteria consume nitrogen-rich organic exudates (Herbst & Overbeck, 1978) and thereby retain nitrogen fixed by *Trichodesmium* in the euphotic layer of the ocean (Nausch, 1996). Nitrogen fixed by *Trichodesmium* fuels new production responsible for export production, a major vehicle for carbon export into the deep ocean. It has been speculated that in contrast to other types of marine particles or aggregates and despite their impact on export production, more of the *Trichodesmium* biomass is consumed in the ocean's surface layer (Nausch, 1996). Degradation of these colonies is likely mediated by attached bacteria and thus these organisms strongly influence the residence time of fixed nitrogen and carbon in the upper ocean as well as the composition of the material exported into the deep ocean.

Considering the global environmental significance of this specific algal-bacterial association, the role of ubiquitous heterotrophic epibionts associated with *Trichodesmium* spp. has been relatively understudied. Here we investigate the structure of the microbial epibiont community associated with *Trichodesmium* colonies collected in the Sargasso

Sea employing a culture-independent 16S rRNA gene sequence-based clone library survey. In the analysis of these clone libraries, we compare communities associated with two morphologically different types of colonies, tuft-type and puff-type, and consider the ecology which may serve to structure these communities.

## METHODS

### Sample collection

*Trichodesmium* spp. colonies were collected under non-bloom conditions (i.e. no obvious bloom was observed during any time of collection) in September 2008 on a cruise of the R/V *Atlantic Explorer*, at the Bermuda Atlantic Time Series (BATS) station. Aggregates were collected from the near surface (approximately the upper 20 m) by a hand-held 130 μm plankton net and individual colonies were gently picked using an inoculating loop. Colonies were sequentially washed in three times with 0.2 μm-filtered seawater. Individual colonies were preserved for DNA extraction. Colonies were preserved in SET buffer (0.75M Sucrose, 50mM Tris pH 8, 40mM EDTA pH 8) and frozen in liquid $N_2$ at sea and then transferred to a -80°C freezer in Woods Hole.

### DNA extraction and Clone Library construction

Clone libraries of bacterial 16S rRNA gene sequences were constructed from two samples. One sample corresponded to puff-type (PT) colonies, and one sample consisted of tuft-type (TT) colonies. Each sample was a composite of 10 colonies. DNA was obtained by phenol-extraction according to a protocol adapted from Marmur (1961).

Aliquots of community *Trichodesmium* spp. DNA (25-100 ng) were added as template to Polymerase Chain Reactions (PCR) for amplification of the SSU rRNA gene sequence. Each PCR consisted of the following: 0.2 mM dNTPs each, 0.5 uM each forward primer 27F (5' AGAGTTTGATCMTGGCTCAG) and reverse primer 1492R (5' TACGGYTACCTTGTTACGACTT) (Invitrogen, Carlsbad, CA), 2 units "Easy A" thermostable proofreading polymerase (Stratagene, La Jolla, CA), for a 20 µL final reaction volume. PCR cycles were as follows: an initial denaturation step of 2 minutes at 94°C; 30 seconds at 94°C, 30 seconds at 55°C, and 90 seconds at 72°C for a total of 30 amplification cycles. Reconditioning PCR was carried out to reduce heteroduplex formation (Thompson *et al.*, 2002) as follows: initial reaction products were diluted ten-fold, and re-amplified using parameters identical to the above, except that only three thermal cycles were performed. Triplicate PCR reactions were pooled and cloned into pCR 4-TOPO plasmid vectors using a TOPO TA cloning kit (Invitrogen, Carlsbad, CA). From each library 384 clones were picked and clone insert DNA was amplified by PCR consisting of the following: 0.2 mM dNTPs each, 0.5 uM each forward primer M13F (5'GTAAAACGACGGCCAG) and reverse primer M13R (5' CAGGAAACAGCTATGAC) (Invitrogen, Carlsbad, CA), and 1 U Paq5000 DNA polymerase (Stratagene, La Jolla, CA), for a 20 µL final reaction volume. 35 reaction cycles were completed using the same parameters as above. In total, 384 bacterial SSU rRNA gene sequences were sequenced from each library.

**PCR with archaeal and eukaryotic primers**

DNA extracts were screened for the presence of archaeal 16S rRNA genes and eukaryotic 18S rRNA genes. PCR was performed as described above, using Paq500 DNA polymerase, with the following modifications: Archaeal DNA was amplified using Ar20F (5'-TTCCGGTTGATCCYGCCRG) and Ar958R (5'-YCCGGCGTTGAMTCCAATT) primers which were allowed to anneal to DNA at 60 degrees. Eukaryotic DNA was amplified using EukF (5'-AACCTGGTTGATCCTGCCAGT) and EukR (5'-TGATCCTTCTGCAGGTTCACCTAC) primers which were allowed to anneal to DNA at 65 degrees. DNA from a fosmid template clone 4B7 (Group I Crenarchaea) (Stein *et al.*, 1996) and a eukaryotic enrichment produced from seawater collected during HOTS cruise 179 (http://hahana.soest.hawaii.edu/hot/hot_jgofs.html) at Station ALOHA at 25 m were used as positive amplification controls, respectively.

**Sequencing**

Clone inserts were sequenced using primer 27F. End-sequencing was performed offsite either by MWG-Operon (Huntsville, AL) or Agencourt Biosciences (Beverly, MA). 339 high-quality sequences were generated from the TT-colony library and 331 high-quality sequences were generated from the PT-colony library.

Raw sequences were aligned via the web-based SINA aligner (http://www.arb-silva.de/aligner/). Aligned sequences were imported into ARB (Ludwig, 2004) (version 07.12.07). Sequences were dereplicated at 97, 99, and 100 percent similarity threshold

using the "percent similarity with gaps" algorithm within FastGroup II (Yu *et al.*, 2006) (http://biome.sdsu.edu/fastgroup). Unique sequences were submitted to the Bellerophon server (Huber *et al.*, 2004) (http://greengenes.lbl.gov/cgi-bin/nph-bel3_interface.cgi) in order to identify putative chimeras. Sequences submitted to Bellerophon were internally aligned using ClustalW and evaluated with a Huber-Hugenholtz correction and a window size of 200. Sequences identified as chimeric by Bellerophon were confirmed or disregarded with information from the Ribosomal Database Project (RDP II) chimera detection software (Cole *et al.*, 2003) (http://rdp8.cme.msu.edu/cgis/chimera.cgi?su=SSU). Chimeric sequences were excluded from further analyses. Overall, 6 chimeric sequences were excluded from the PT library and one sequence was excluded from the TT library.

**Taxomomic assignments and phylogenetic analyses**

The Ribosomal Database Project II Classifier (Cole *et al.*, 2003) was used to assign provisional taxonomic affiliations to 97 percent similar OTUs. BLASTn searches (Altschul *et al.*, 1997) of the National Center for Biotechnology Information (NCBI) 16S rRNA gene sequence databases were performed to confirm taxonomic placements as many of our clones are relatively novel and thus yielded low bootstrap values in the automatic classifier. The proportions of major taxonomic groups were calculated (Figure 1 a-f) and the BLAST results are available in Supplemental Tables 1 and 2, all BLAST searches were performed with the GenBank database updated January 1, 2010.

Rarefaction, coverage, and richness were all calculated for a 97 percent similarity threshold. Rarefaction results were calculated by Fast Group II (Yu *et al.*, 2006). The coverage of our clone libraries (percentage of actual species richness that we detected in our clone libraries) was calculated according to Good (1953) and Chao (Chao & Yang, 1993). The former estimates coverage related to relatively rare OTUs while the latter estimates coverage based on the abundance of dominant OTUs providing a lower and upper bound on coverage, respectively. Species richness was estimated using the Chao1 nonparametric richness estimator (Chao, 1987) (Table 1), yielding the probable number of OTUs in the sample on the basis of singletons and doubletons detected in the clone library (Chao, 1987). Fast Group II also provided the Shannon-Wiener diversity index which takes into account not only species richness but the evenness of species distribution.

**Nucleotide sequence accession numbers**

All sequences were deposited in GenBank under accession numbers GU725472-GU726134.

**RESULTS**

For comprehensive coverage and richness calculations, we binned sequences at a 97 percent sequence identity (PSI) threshold. Operational taxonomic units (OTUs), richness and diversity data are provided in Table 1 for 97, 99 and 100 percent similar OTUs. The coverage of each library was estimated by two independent measures which rely on the

occurrence of either rare (Good's coverage index) (Good, 1953) or abundant (Chao's coverage index) (Chao, 1987) OTUs. These estimates indicate that the PT library captured 75-95% of actual species richness and the TT library captured a similar range of 68-94% of actual richness. A complementary indicator of probable species richness (chao1) (Chao & Yang, 1993) was calculated and the PT library yielded a probable 49 OTUs and the TT library yielded 32. Due to our library coverage being less than 100%, actual measured richness was lower, 33 for the PT library and 22 for the TT. All of these values are likely low-estimates because we chose to analyze our samples as 97 PSI OTUs, and species richness estimates increase if calculations are considered at 99 or 100 PSI OTUs (see Table 1). The value of the Shannon-Wiener index, which considers the evenness of OTU distribution in addition to the OTU richness, is slightly higher in the PT library (1.9) compared to the TT library (1.6). Rarefaction curves were calculated for each library in order to determine the likelihood that our sampling effort was sufficient to produce an unbiased estimate of species richness as well as to reveal all the OTUs present in the samples (Figure 1). In the case of both libraries, the curves approach an asymptote, or saturation, which indicates that our sampling effort is acceptable and likely to yield reliable richness data.

**Eukaryotes and archaea**

No archaeal 16S or eukaryotic 18S sequences were amplified from either the TT or PT library.

**Cyanobacteria**

Although this study was focused on the epibiont populations associated with *Trichodesmium* spp., we chose a DNA lysis method which effectively lysis a wide range of cells. As a result, we recovered high numbers of cyanobacterial 16S sequences and these provided a valuable insight into the cyanobacterial 'base' of the colonies. Morphologically, the PT library was constructed from 10 colonies of the "puff"-type, and the TT library was constructed from 10 colonies of the "tuft"-type. 99% of the cyanobacterial clones associated with the PT library were related to *Trichodesmium thiebautii* (AF013027) at 99 PSI. The other 1% were most closely related to *T. thiebautii*, at a 95 PSI or higher level. The TT colonies contained a more diverse cyanobacterial population. 69% (150 clones) were related to *T. thiebautii* at a 99 PSI. However, 29% of the clones were most closely related (95 PSI) to an uncultured bacterium (EF630220) sharing 92 PSI with its nearest cultured relative in GenBank, a *Limnothrix*-like species (EF088338). Interestingly, both sequences derive from a *Mycale laxissima* sponge collected off a coral reef near Key Largo, FL (Mohamed *et al.*, 2008). A phylogenetic tree detailing the taxonomic placement of cyanobacterial clone sequences is presented in Figure 4.

**Other epibiotic bacteria**

Non-cyanobacterial sequences in both clone libraries contained were dominated by Alphaproteobacteria and Flavobacteria. The two libraries displayed some class-level similarities, although there was significant heterogeneity at the family-level (Figure 2a).

Flavobacteria composed 47% of the recovered non-cyanobacterial clones from the TT colonies. Sphingobacteria composed 35% of remaining clones, Alphaproteobacteria composed another 12% of clones, and Firmicutes and the candidate phylum SR1 comprised the remaining 6% of the clones. Flavobacteria constituted a similar proportion (46%) of PT non-cyanobacterial clones and Alphaproteobacteria composed 10% of total clones. The PT-colonies contained no Sphingobacteria, Firmicutes, or SR1. Instead, the rest of the PT library consisted of Chloroflexi (25%), Gammaproteobacteria (12%), Verrucomicrobia (5%), Deltaproteobacteria (1%) and unclassified sequences (1%). Phylogenetic trees detailing the relationships between all PT and TT heterotrophic clone sequences are presented in Figures 5 and 6.

**Alphaproteobacteria**

Alphaproteobacteria are represented in both the PT and TT clone libraries. Alphaproteobacteria associated with the TT-colonies were comprised of four families: Phyllobacteriaceae (50%), Rhodospirillaceae (25%), Rhodobacteraceae (17%) and the Aurantimonadaceae (8%). The PT library also contained representatives from the Rhodospirillaceae and Rhodobacteraceae which composed 7% and 66%, respectively, of the total alphaproteobacterial clones. The remainder of the PT library comes from the order Rhizobiales: Rhodobiaceae (7%) and Hyphomicrobiaceae (20%).

**Bacteroidetes**

All 73 flavobacterial clones isolated from the PT library are members of the Flavobacteraceae. In contrast, only 3 of 56 Flavobacteriales clones retrieved from the TT library fall into the Flavobacteraceae. The other 43 are all related to the Cryomorphaceae. In addition to clones related to Flavobacteriales, the TT library also contains 36 clones which classify as Sphingobacteriales.

## DISCUSSION

*Trichodesmium* spp. are notable in several respects. First, they thrive in the most oligotrophic regions of the world's oceans. Second, they fix nitrogen despite lacking the heterocystous cells that typically isolate oxygen-sensitive nitrogen-fixing enzymes from oxygen-producing photosynthetic processes. Finally, they are frequently observed to exist not as individual trichomes, but in colonies of cells which can occur in several distinct morphological varieties and in close association with numerous types of other microbes. The genome sequence of *Trichodesmium erythraeum* IMS101, the only fully sequenced *Trichodesmium* sp., is notable for its low gene coding density and high degree of horizontal gene transfer, both hallmarks of hosts benefitting from a mutualistic relationship (observation, personal communication with T. Mincer).

*Trichodesmium* spp. act as a surface in the upper ocean, host to a variety of bacteria, microzooplankton, and metazoa that would typically not thrive in harsh oligotrophic conditions. Much of the research interest in *Trichodesmium* stems from its role in the nitrogen cycle, however, comparably less work has been devoted to the

*Trichodesmium* colony as a functional unit, a consortial community whose members would be unlikely to thrive in the absence of one another. In this study, we set out to investigate how the bacterial community associated with *Trichodesmium* spp. compares with that of ambient seawater and whether it varies between morphologic varieties.

On the basis of metazoan and microzooplankton data, it has been suggested that tuft and puff colonies form distinct niches for microbial communities (Sheridan *et al.*, 2002). In our study, we were able to test this hypothesis as it relates to bacterial community structure. Although differences in bacterial density (Sheridan *et al.*, 2002), enzyme activity (Nausch, 1996; Stihl *et al.*, 2001) and redox conditions (Paerl *et al.*, 1989) between the two colony types is suggestive of differences in microbial community structure, to our knowledge, this is the first phylogenetic investigation of the community structure of the total microbial epibionts associated with the two morphologic types.

### *Puff and tuft-type* Trichodesmium *colonies are a unique environment for marine microbes*

Each of our samples was aggregated from 10 PT or TT colonies collected at the same time, and thus the clone libraries represent an average community associated with both colony types. We observed much greater species richness associated with our PT colonies. This result mirrors the richer community of microzooplankton and metazoa previously documented in puff-type colonies (Sheridan *et al.*, 2002).

Both types of *Trichodesmium* colonies are host to communities of much lower species richness than is present in ambient seawater. Pommier and colleagues (Pommier

*et al.*, 2007) found that Sargasso Sea seawater collected at 5 m had an estimated richness (chao1) of 150 at the 97 PSI threshold. Using a dataset published by Carlson and colleagues (Carlson *et al.*, 2008) we calculated the estimated richness of Sargasso Sea water collected at the BATS site from 40 m and determined a richness estimate of 68 at the 97 PSI threshold. Both of these values exceed that which we estimated on either PT colonies or TT colonies (49 and 32, respectively).

That the number of species associated with these colonies is lower than in the surrounding seawater suggests that the phycosphere of *Trichodesmium* selects for a specific subgroup of organisms which are not as well supported by ambient seawater. The nutrient and redox conditions of seawater vary quite dramatically from those in the interior of a *Trichodesmium* colony and different metabolic strategies are likely to be required to thrive in either one. In contrast to seawater, the interior of puff- and tuft-type colonies is more reducing (as measured by cellular tetrazolium salt reduction) and becomes anoxic during nighttime hours (Paerl *et al.*, 1989).

The chao1 richness estimate is based on the low-abundance OTUs detected in a population. The high species richness of seawater reflects the great number of low-abundance free-living bacteria which may not actively participating in the biogeochemical cycling of elements in seawater (Pedrós-Alió, 2006). The bacteria which are abundant on *Trichodesmium* are likely originally "seeded" from these low-abundance members of the seawater community (i.e. the ones least likely to be represented in a cultivation-independent analysis of seawater). In fact, when we look for common seawater associated-bacteria in our *Trichodesmium* clone libraries, they are

conspicuously absent. *Trichodesmium* epibionts are also likely to be inherited from mother to daughter cells and transferred between *Trichodesmium* colonies at unknown frequencies.

Typical Sargasso Sea seawater associated species (e.g. SAR11, SAR 86, Prochlorococcus or Synechococcus) were not detected in our libraries (Treusch *et al.*, 2009). The OTUs we recovered cluster more closely with clones and isolates from benthic environments- biofilms associated with sponges and corals (e.g. numerous clone sequences detected in our *Trichodesmium* colony clone libraries are similar to those reported in Mohamed *et al.*, 2008 and Sunagawa *et al.*, 2009). Further, when we compared members of our library with common surface associated or algal associated organisms- for example, specific members of the Roseobacteria clade (Mayali *et al.*, 2008; Wagner-Döbler *et al.*, 2009), including an epibiont isolated from the surface of *Trichodesmium* in the Caribbean Sea (GenBank accession ACNZ01000108), we did not find close relationships. A quarter of clones recovered in the PT library shared 90 PSI or less with nearest neighbors in GenBank. The community associated with the TT-colonies is even more unusual in that 81 percent of clones share 90 PSI or less with their neighbors in GenBank. On the other hand, these communities are composed of only 21% and 9% respectively of OTUs which share 97 PSI or better with their nearest GenBank relatives. These results are similar to those obtained during a study of the bacterial flora associated with another filamentous cyanobacterium, *Nodularia*, which found that the majority of epibiont clones associated with *Nodularia* represented novel taxa (Tuomainen *et al.*, 2006). Our data suggest that the phycosphere associated with *Trichodesmium* and

other cyanobacteria is a novel ecological niche host to unexplored genetic diversity. If the species we observe on *Trichodesmium* indeed originate from the rare-members of the seawater microbial community, it serves to reason they would not be well represented in public databases which are biased towards the most abundant pelagic bacteria or abundant members of more 'traditional' benthic marine surfaces such as corals or sponges.

Archaea and eukaryotes were conspicuously absent from the *Trichodesmium* colonies. Euryarchaea may represent up to 30% of the microbial populations in the upper 100 m of the open ocean water column (Frigaard *et al.*, 2006) and crenarchaea are one of the most abundant types of cell in the ocean when bathy- and mesopelagic populations are considered (Karner *et al.*, 2001) and are important nitrifiers (Nicol & Schleper, 2006). *T. thiebautii* is known to migrate vertically in the water column over the diel cycle (Villareal & Carpenter, 1990) and *Trichodesmium* spp. have been observed at depths of 100 m and greater (Letelier & Karl, 1998; Davis & McGillicuddy Jr, 2006), the edge of the crenarchaeotal range (Karner *et al.*, 2001). It is interesting that such an important nitrifying organism appears not to participate in nitrogen cycling in association with the ocean's most abundant nitrogen fixer. There have been many reports of eukaryotes associated with *Trichodesmium* colonies, however, they may be less tightly associated with the colonies and more easily separated during the sample preparation process. It is important to consider that our samples were collected under non-bloom conditions, and the contribution of archaea and eukaryotes might increase during a bloom event.

*An intercomparison of the epibiotic community associated with PT and TT samples*

We observed that in both our PT samples and TT samples, additional photosynthetic members contributed to the community. In the TT sample, additional photosynthetic members were distantly related to *Limnothrix* (92 PSI). Non-*Trichodesmium* cyanobacterial filaments have been reported in association with *Trichodesmium* colonies numerous times (Paerl *et al.*, 1989; Siddiqui *et al.*, 1992; Dyhrman *et al.*, 2002; Hewson *et al.*, 2009). These filaments have been described as *Phormidium*-like or *Plectonema*-like, although to our knowledge no absolute phylogenetic identification has been ascribed to this group. In one case where a distinction was made between colony morphologies, Sheridan and colleagues (2002) noted that they observed the matrix of puff colonies contained bacteria and microflagellates whereas the matrix of tuft colonies contained bacteria and cyanobacterial filaments (Sheridan *et al.*, 2002). Siddiqui and co-authors (Siddiqui *et al.*, 1992) noted that while they observed contaminating cyanobacterial filaments within both colony types, they were significantly more abundant and more reliably associated with tuft colonies. Thus, it may be common for tuft colonies to host additional cyanobacterial species (Sheridan *et al.*, 2002). In contrast to our TT samples, we detected no cyanobacterial OTUs other than those related to *T. thiebautii* in the PT sample. In fact we detected significant numbers of OTUs related to other possible photosynthetic groups such as the phylum Chloroflexi (Zinder & Dworkin, 2000) and members of the Roseobacter clade of the Alphaproteobacteria, known to be aerobic anoxygenic phototrophs (Buchan *et al.*, 2005).

The role of these additional phototrophs is unclear. They likely benefit from the buoyancy of *Trichodesmium* colonies and contribute to the mucilage layer which defines the colony (Siddiqui *et al.*, 1992). The cyanobacterial filaments have been demonstrated to fix nitrogen (Siddiqui *et al.*, 1992) so it is unlikely the cyanobacteria are benefitting from the rich source of fixed nitrogen in the *Trichodesmium* phycosphere. In addition, while it is difficult to remove heterotrophic epibionts from cultures of *T. erythraeum* or *T. thiebautii*, it does not appear to be difficult to remove the cyanobacteria; in clone libraries of three laboratory cultured, non-axenic *Trichodesmium* strains (IMS101, *T. erythraeum* tuft colony; K-11#131, *T. erythraeum* tuft colony; H9-4A, *T. thiebautii* puff colony), no cyanobacterial OTUs other than those associated with *Trichodesmium* were detected (*Hmelo, Van Mooy and Mincer, Appendix 1*). From these data we might speculate that the relationship between *Trichodesmium* and its heterotrophic epibionts may be mutual where as the relationship between *Trichodesmium* and the 'other' cyanobacteria may be commensal.

It has become apparent that a 'typical' surface associate flora does not exist despite superficial patterns in phylogenetic associations. A large body of literature has been generated by the study of bacteria associated with eukaryotic microalgae. One can broadly generalize (at the class level) that eukaryotic microalgal cultures associate with Alphaproteobacteria, Gammaproteobacteria, and members of the Flavobacteria-Sphingobacteria group (DeLong *et al.*, 1993; Grossart *et al.*, 2005; Sapp *et al.*, 2007). In a recent transcriptomic effort exploring a natural *Trichodesmium* bloom north of Fiji, these same groups, Alphaproteobacteria, Gammaproteobacteria and Bacteroidetes,

comprised roughly a quarter of non-cyanobacterial transcripts recovered during the daytime (Hewson *et al.*, 2009). It is important to note that Hewson and co-workers collected Trichodesmium samples from a mature bloom and determined community composition using a different method than we employed in this study. Considering the limited number of instances in which the epibiont community associated with Trichodesmium has been described, their result serves as an interesting and infrequent point of comparison, so long as the preceding caveat is kept in mind. In contrast to the community composition described in the Hewson et al. study, Zehr (1995) identified Flavobacteria as the dominant members of the *Trichodesmium* epibiont community using fluorescently labeled probes for 16S rRNA genes and notably reported detecting no Alphaproteobacteria.

We detect abundant Alphaproteobacteria in both of our libraries. Despite the aforementioned results by Zehr and colleagues, the presence of Alphaproteobacteria is not surprising as many members of this group are opportunistic colonizers and copiotrophs (e.g. members of the Roseobacter clade) (Buchan *et al.*, 2005; Dang *et al.*, 2008). Members of the Rhodobacterales have been identified as ubiquitous early colonizers of surfaces in the oceans (Dang *et al.*, 2008), and they comprise the majority of the alphaproteobacterial clones associated with the PT sample. It is striking that the Rhodobacterales contribute only a minor proportion of the clones associated with the TT sample.

The TT library is unusual in another regard: no gammaproteobacterial clones were sequenced from the TT library. This result is surprising given that

Gammaproteobacteria are generally very common on marine surfaces, including particles and in particular, on algae (Fisher *et al.*, 1998; Dang & Lovell, 2000; Tuomainen *et al.*, 2006; Sapp *et al.*, 2007; Dang *et al.*, 2008). Gammaproteobacteria were detected in the PT library, although they originate from Thiotrichales rather than *Alteromonas, Marinobacter* or *Pseudoalteromonas* as observed by Hewson and co-workers (Hewson *et al.*, 2009). Although this observation may imply something interesting about the *Trichodesmium* phycosphere with respect to other surfaces, the functional properties of associated microbes remains unknown.

The TT library contained two clones related to the deeply branching candidate division SR1. There are no members of SR1 in cultivation and very few clones from this division have been recovered from marine environments (Davis *et al.*, 2009). On the basis of their ecological distributions and association with bacteria known to be involved in sulfur transformations, it has been suggested that these bacteria are also involved in sulfur transformations in the environment although this assertion is tenuous until more data is available (Harris *et al.*, 2004; Davis *et al.*, 2009).

*Concluding thoughts*

As mentioned earlier, it is difficult to infer function from phylogenetic information alone, but a few generalizations can be inferred from the clone library composition. Many of the taxa present in these clone libraries may be metabolically flexible; that is, they are related to cultivars known to be capable of exploiting a range of redox conditions and carbon substrates. While additional physiological information about associated epibionts

is clearly required to say with certainty that all of the major biogeochemical cycles are represented in *Trichodesmium* colonies, we can speculate that *Trichodesmium*-bacterial communities, in analogy to cyanobacterial microbial mat communities, function as true consortia, in which nitrogen, sulfur, and carbon are cycled within the colony associated community.

Despite distinct colony morphology, both the PT and TT colonies analyzed in this study are built around bundles of *T. thiebautii*. Bacterial density (Sheridan *et al.*, 2002), enzyme activities (Nausch, 1996; Stihl *et al.*, 2001) and redox gradients from the outside to the inside of the colony (Paerl *et al.*, 1989) vary substantially between puff and tuft type colonies which suggests that the two types of colonies can host very different types of microbial communities. The libraries constructed in this study contain no common OTUs at the 97 PSI threshold. Our data are thus consistent with the preceding hypothesis and confirm that bacterial flora associated with PT colonies and TT colonies are quite different.

At this time, interpretation of these results relies to a degree on speculation, particularly since the colony to colony variation within the pooled samples from which our data originates remains unknown. However, such speculation generates new questions and testable hypotheses essential to improving our understanding of the ecological role of the epibiotic bacteria associated with *Trichodesmium* spp. In combination with the results of previous studies discussed earlier in this manuscript (e.g. those of Sheridan et al., 2002) the striking dissimilarity between the PT and TT samples suggest that the colony morphology may be influenced by the relative abundance and

identity of bacterial epibionts associated with the colonies. Alternatively, the mutually exclusive flora associated with either colony type may indicate that the two types of colonies perform different ecosystem functions. Puffs and tufts exhibit different redox gradients (Paerl *et al.*, 1989) and perhaps this difference translates into the recruitment of different bacteria suited to exploit each niche. Just as colony morphology may be influenced by the attached heterotrophic bacteria, colony morphology may influence the photosynthetic members. Relative to tuft-colonies, puffs are associated with greater bacterial density, a physically denser colony "core" (John Waterbury, personal communication), and a greater amount of exopolysaccharide material (John Waterbury, personal communication). These parameters can affect the extent of shading within the colony. The extent of internal shading may in turn influence whether other phototrophs colonize the TT community, as fits our observations (see Figure 3).

Biofilm communities are typically highly structured and efficiently regulated. The bacterial communities associated with *Trichodesmium* compose biofilms, and can be expected to be strongly internally regulated by its constituent members. The community composition is likely to be influenced by bacterial-bacterial interactions such as competition for resources, secondary metabolite production, and cooperation. It is likely that Proteobacteria hosted by *Trichodesmium* use the same types of cooperative strategies employed by other marine biofilm-forming bacteria (e.g. quorum sensing) (Dang *et al.*, 2008).

Bacteria associate with *Trichodesmium* colonies are reported to occur at densities of $10^7$-$10^9$ cells $ml^{-1}$ (Sheridan *et al.*, 2002) which can support cell-cell interactions via

quorum sensing (QS) (Hmelo & Van Mooy, 2009). In fact, QS has been observed in stromatolites, another type of cyanobacteria-heterotrophic bacteria consortia (Decho *et al.*, 2009). In addition to structuring the bacterial epibiont community, QS might play a role in attracting microzooplankton or metazoa to the colonial community. AHL-production in biofilms has been previously observed to attract *Ulva* propagules to seaweed and is hypothesized to act as a chemo-attractant (Tait *et al.*, 2005). Colonization of *Trichodesmium* by QS bacteria may thus affect when the colonies are colonized by eukaryotes and which eukaryotes choose to settle.

The data we present here indicate that *Trichodesmium*-associated bacterial communities are distinct from other marine pelagic and benthic microbial communities. The adaptations which provide these organisms with a competitive advantage in the *Trichodesmium* phycosphere may be associated with novel metabolic pathways, secondary metabolite production, or detoxification strategies. These data provide a phylogenetic basis for investigations of these adaptive strategies, in particular, the cooperative social strategies (QS) of *Trichodesmium*-associated bacterial communities.

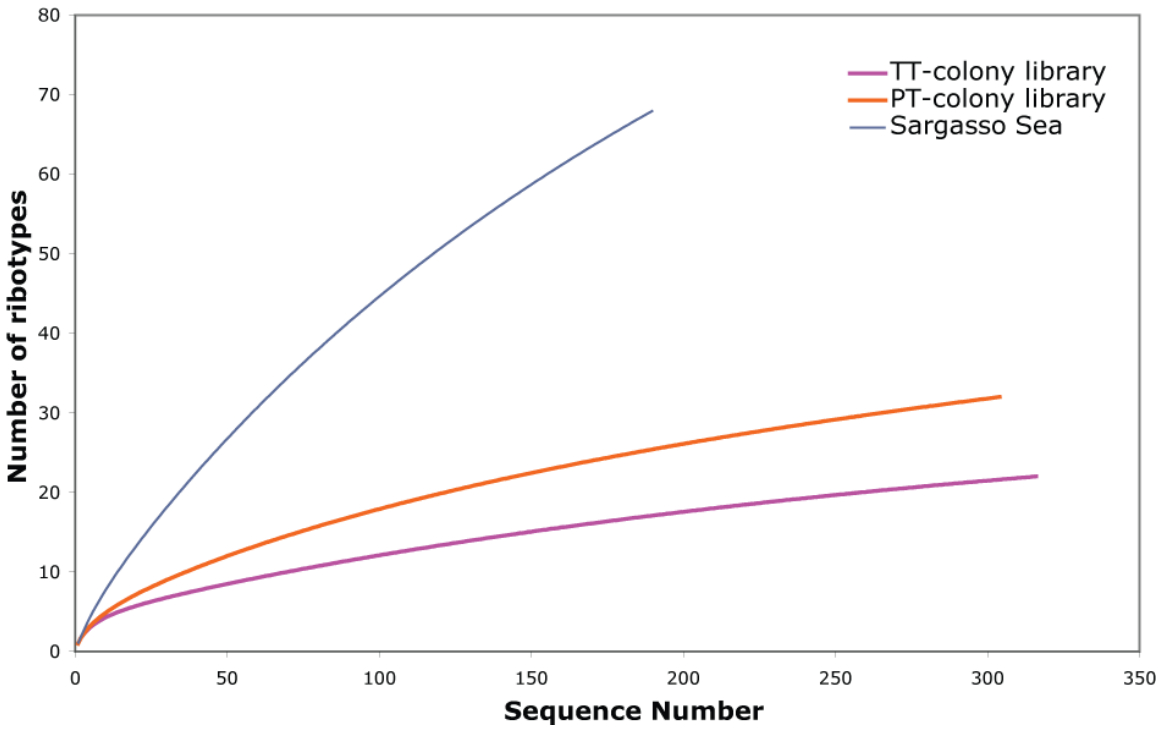



**Figure 1.** Rarefaction curve of PT and TT 16S rDNA clone libraries generated in this study as well as a Sargasso Sea (BATS site, 40 m depth, Carlson *et al.*, 2008) clone library data, summarized in Figure 2.

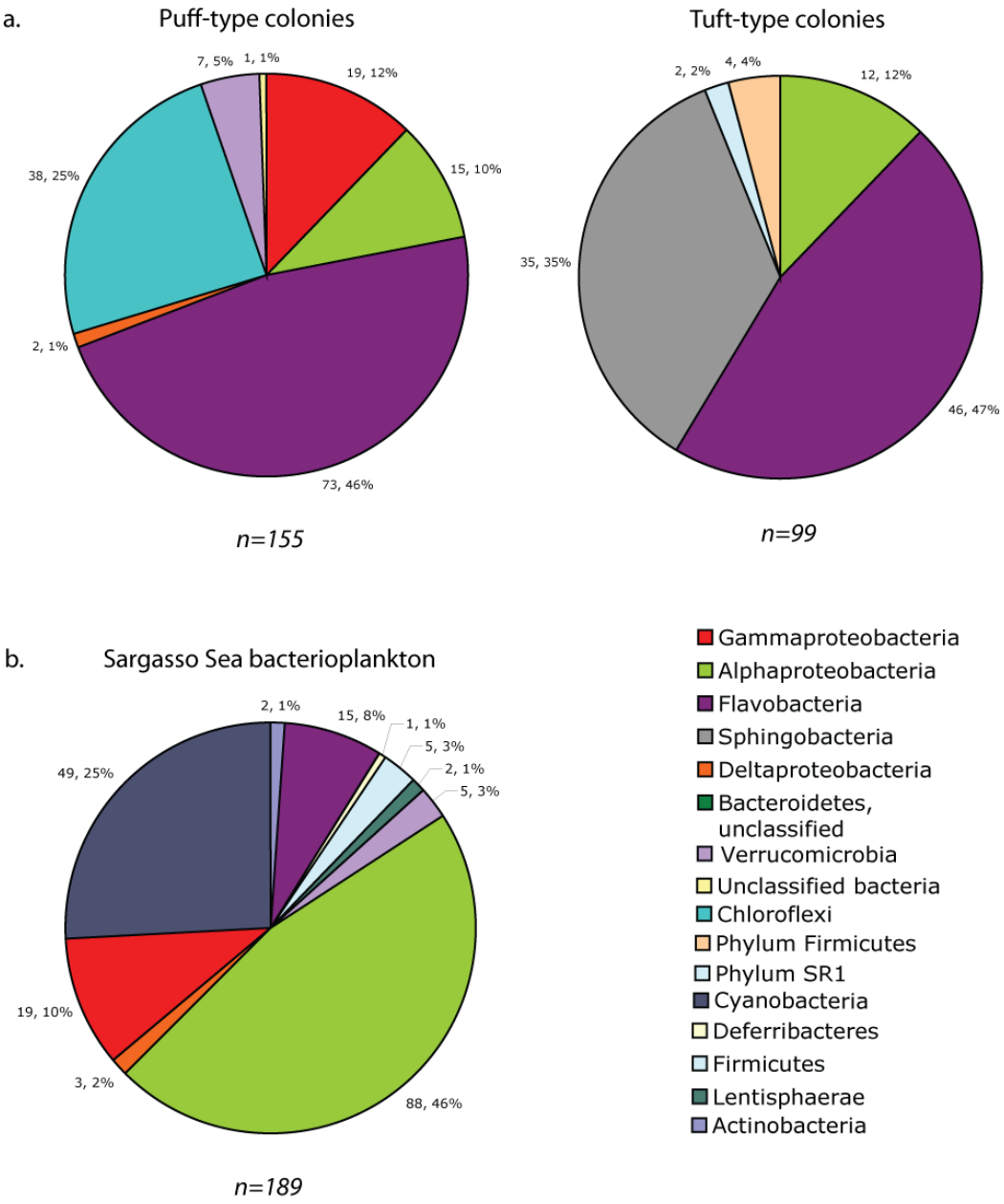
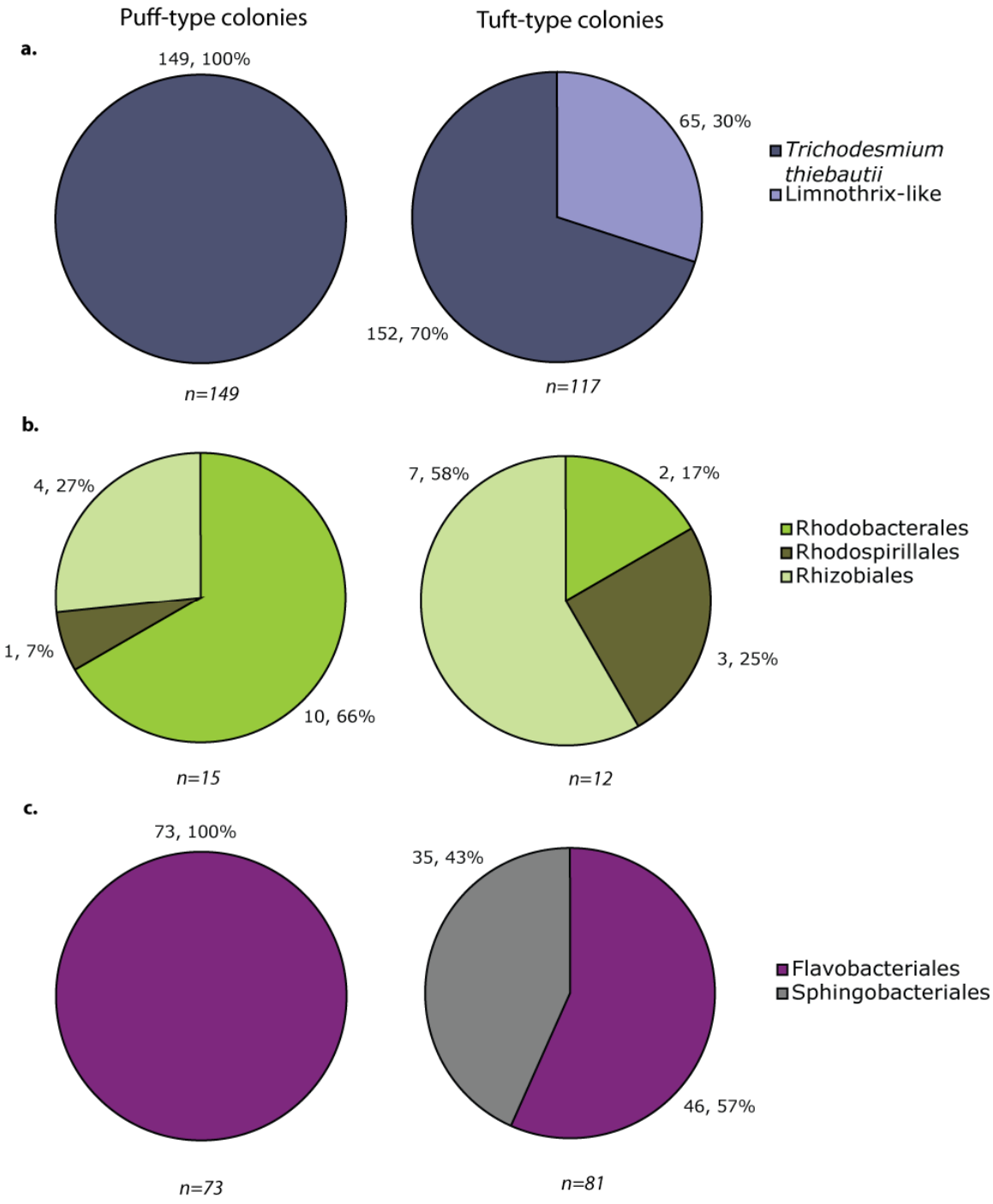



**Figure 2.** Class-level phylogenetic composition of (a) PT and TT ebibiont communities and (b) Sargasso Sea bacterioplankton (for comparison). Values listed adjacent to each 'wedge' of the pie chart provide the number of clones in that category, followed by the percentage of the total non-cyanobacterial clones recovered. The number of non-cyanobacterial clones recovered is listed underneath each chart.

**Figure 3.** Phylogenetic composition of PT and TT epibiont communities (in the left-hand and right-hand column, respectively). (a) Distribution of clones related to Cyanobacteria in PT colonies. Values listed adjacent to each 'wedge' of the pie chart provide the number of clones in that category, followed by the percentage of the total cyanobacterial clones recovered. The number of cyanobacterial clones recovered is listed underneath each chart. (b) Family-level distribution of clones within the class Alphaproteobacteria. (c) Family-level distribution of clones within the class Bacteroidetes. In (b) and (c) values listed adjacent to each 'wedge' of the pie chart provide the number of clones in that category, followed by the percentage of the total non-cyanobacterial clones recovered. The number of non-cyanobacterial clones recovered is listed underneath each chart.


Puff-type colonies
Tuft-type colonies
a.
149, 100%
65, 30%
152, 70%
Trichodesmium thiebautii
Limnothrix-like
n=149
n=117
b.
4, 27%
1, 7%
10, 66%
7, 58%
2, 17%
3, 25%
Rhodobacterales
Rhodospirillales
Rhizobiales
n=15
n=12
c.
73, 100%
35, 43%
46, 57%
Flavobacteriales
Sphingobacteriales
n=73
n=81

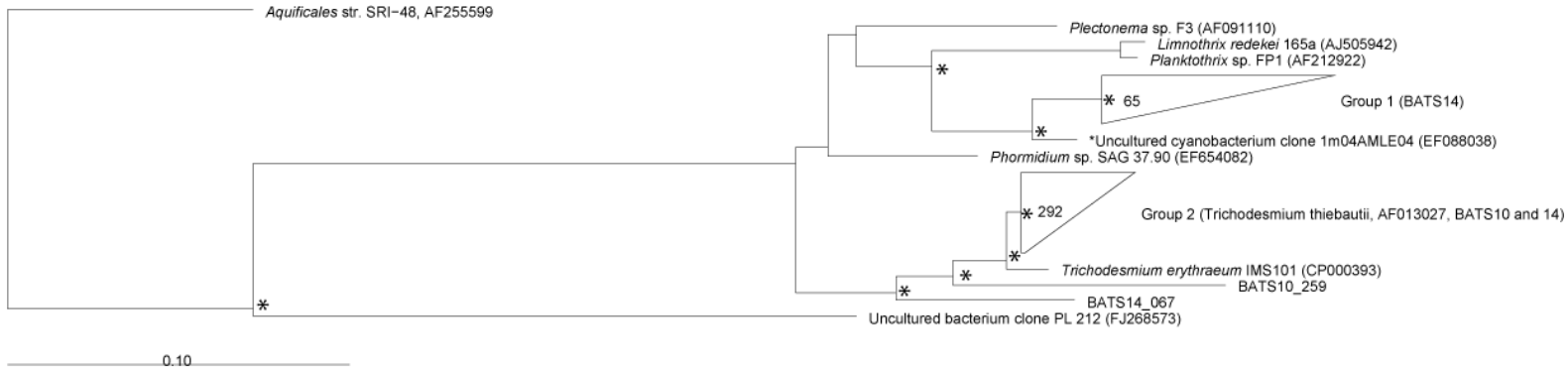



**Figure 4.** Phylogenetic tree containing cyanobacterial sequences recovered from *Trichodesmium* colonies in this study. Thus tree was created using Neighbor Joining algorithm in ARB and bootstrapped 1000 times using Phylip (v 3.68). Asterisks indicate bootstrap values of 60% or better. Sequences generated in this study are indicated by boldface type. Accession numbers are in parentheses following all sequences. BATS10 identifies PT samples and BATS14 identifies TT samples.

**Figure 5.** Phylogenetic tree containing proteobacterial sequences recovered from *Trichodesmium* colonies in this study. Thus tree was created using Neighbor Joining algorithm in ARB and bootstrapped 1000 times using Phylip (v 3.68). Asterisks indicate bootstrap values of 60% or better. Sequences generated in this study are indicated by boldface type. Accession numbers are in parentheses following all sequences. BATS10 identifies PT samples and BATS14 identifies TT samples. All of the sequences collapsed into Group 1 differ by less than 3%.

**Figure 6.** (a) Phylogenetic tree containing sequences which are not categorized as cyanobacterial or proteobacterial (namely, members of Bacteroidetes, Verrucomicrobia, Cloroflexi, Firmicutes, and the candidate phylum SR1) recovered from *Trichodesmium* colonies in this study. Thus tree was created using Neighbor Joining algorithm in ARB and bootstrapped 1000 times using Phylip (v 3.68). Asterisks indicate bootstrap values of 60% or better. Sequences generated in this study are indicated by boldface type. Accession numbers are in parentheses following all sequences. BATS10 identifies PT samples and BATS14 identifies TT samples. Sequences which are collapsed into Groups 1 and 3 differ by less than 3%. Sequences in Group 2 differ by 4.9% and sequences in Group 4 differ by 3.5%. (b) Expanded tree detailing the individual OTUs contained within Group 2. Asterisks indicate bootstrap values of 60% or better. (c) Expanded tree detailing the individual OTUs contained within Group 4. Asterisks indicate bootstrap values of 70% or better.

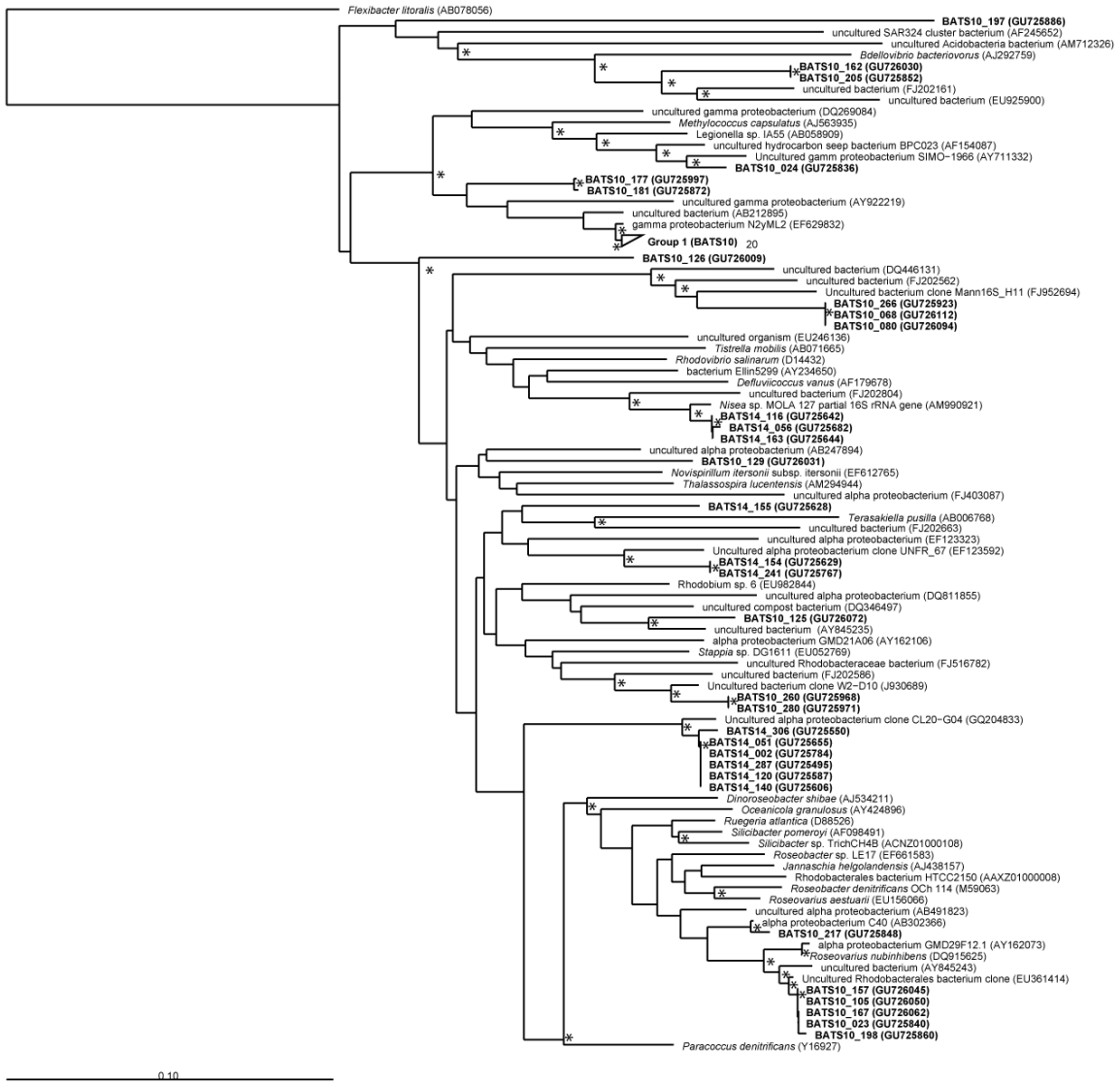

Flexibacter litoralis (AB078056)
BATS10_197 (GU725886)
uncultured SAR324 cluster bacterium (AF245652)
uncultured Acidobacteria bacterium (AM712326)
Bdellovibrio bacteriovorus (AJ292759)
BATS10_162 (GU726030)
BATS10_205 (GU725852)
uncultured bacterium (FJ202161)
uncultured bacterium (EU925900)
uncultured gamma proteobacterium (DQ269084)
Methylococcus capsulatus (AJ563935)
Legionella sp. IA55 (AB058909)
uncultured hydrocarbon seep bacterium BPC023 (AF154087)
Uncultured gamm proteobacterium SIMO−1966 (AY711332)
BATS10_024 (GU725836)
BATS10_177 (GU725997)
BATS10_181 (GU725872)
uncultured gamma proteobacterium (AY922219)
uncultured bacterium (AB212895)
gamma proteobacterium N2yML2 (EF629832)
Group 1 (BATS10)
20
BATS10_126 (GU726009)
uncultured bacterium (DQ446131)
uncultured bacterium (FJ202562)
Uncultured bacterium clone Mann16S_H11 (FJ952694)
BATS10_266 (GU725923)
BATS10_068 (GU726112)
BATS10_080 (GU726094)
uncultured organism (EU246136)
Tistrella mobilis (AB071665)
Rhodovibrio salinarum (D14432)
bacterium Ellin5299 (AY234650)
Defluviicoccus vanus (AF179678)
uncultured bacterium (FJ202804)
Nisea sp. MOLA 127 partial 16S rRNA gene (AM990921)
BATS14_116 (GU725642)
BATS14_056 (GU725682)
BATS14_163 (GU725644)
uncultured alpha proteobacterium (AB247894)
BATS10_129 (GU726031)
Novispirillum itersonii subsp. itersonii (EF612765)
Thalassospira lucentensis (AM294944)
uncultured alpha proteobacterium (FJ403087)
BATS14_155 (GU725628)
Terasakiella pusilla (AB006768)
uncultured bacterium (FJ202663)
uncultured alpha proteobacterium (EF123323)
Uncultured alpha proteobacterium clone UNFR_67 (EF123592)
BATS14_154 (GU725629)
BATS14_241 (GU725767)
Rhodobium sp. 6 (EU982844)
uncultured alpha proteobacterium (DQ811855)
uncultured compost bacterium (DQ346497)
BATS10_125 (GU726072)
uncultured bacterium (AY845235)
alpha proteobacterium GMD21A06 (AY162106)
Stappia sp. DG1611 (EU052769)
uncultured Rhodobacteraceae bacterium (FJ516782)
uncultured bacterium (FJ202586)
Uncultured bacterium clone W2−D10 (J930689)
BATS10_260 (GU725968)
BATS10_280 (GU725971)
Uncultured alpha proteobacterium clone CL20−G04 (GQ204833)
BATS14_306 (GU725550)
BATS14_051 (GU725655)
BATS14_002 (GU725784)
BATS14_287 (GU725495)
BATS14_120 (GU725587)
BATS14_140 (GU725606)
Dinoroseobacter shibae (AJ534211)
Oceanicola granulosus (AY424896)
Ruegeria atlantica (D88526)
Silicibacter pomeroyi (AF098491)
Silicibacter sp. TrichCH4B (ACNZ01000108)
Roseobacter sp. LE17 (EF661583)
Jannaschia helgolandensis (AJ438157)
Rhodobacterales bacterium HTCC2150 (AAXZ01000008)
Roseobacter denitrificans OCh 114 (M59063)
Roseovarius aestuarii (EU156066)
uncultured alpha proteobacterium (AB491823)
alpha proteobacterium C40 (AB302366)
BATS10_217 (GU725848)
alpha proteobacterium GMD29F12.1 (AY162073)
Roseovarius nubinhibens (DQ915625)
uncultured bacterium (AY845243)
Uncultured Rhodobacterales bacterium clone (EU361414)
BATS10_157 (GU726045)
BATS10_105 (GU726050)
BATS10_167 (GU726062)
BATS10_023 (GU725840)
BATS10_198 (GU725860)
Paracoccus denitrificans (Y16927)
0.10

**Figure 5**

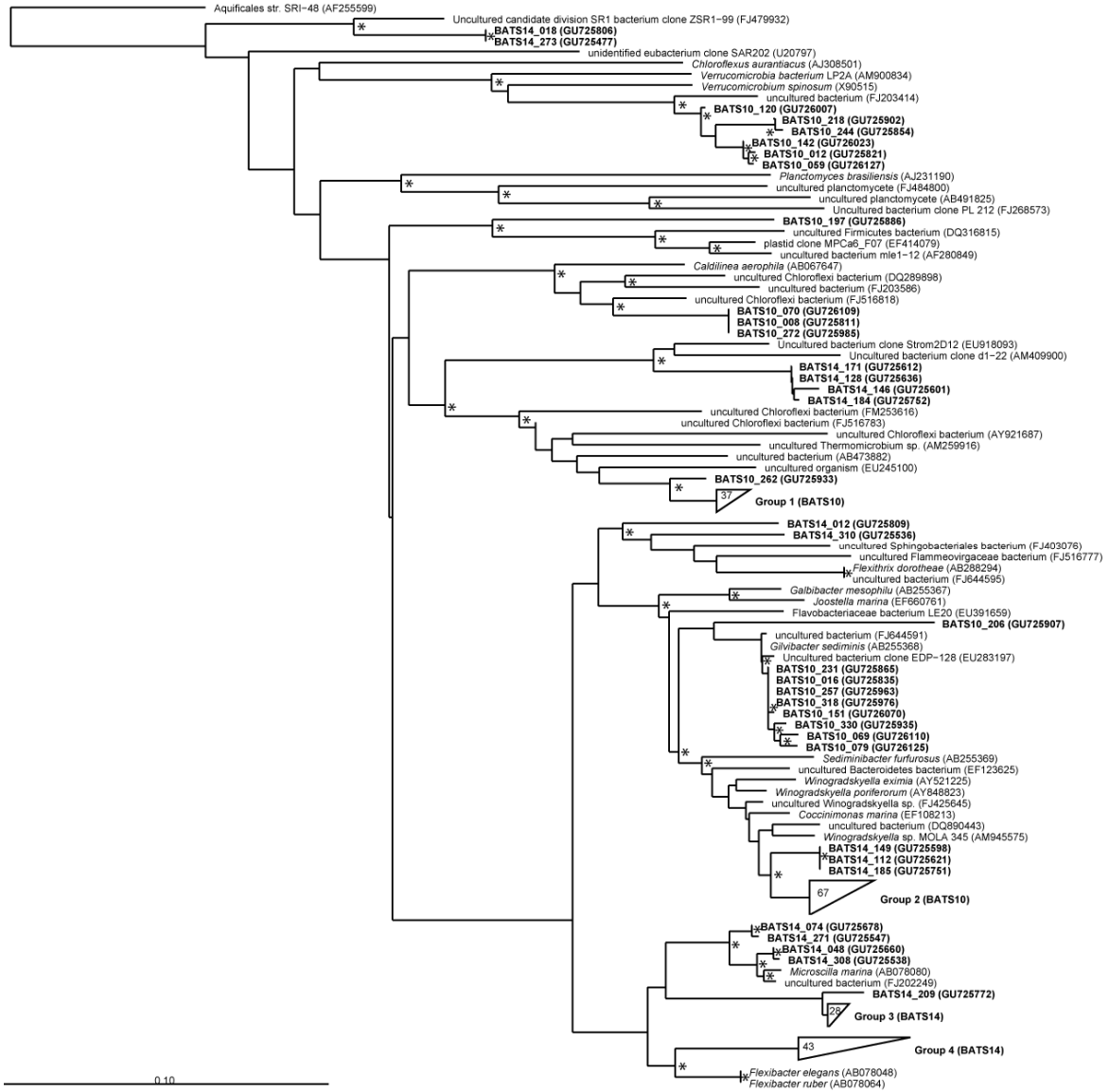

Aquificales str. SRI−48 (AF255599)
Uncultured candidate division SR1 bacterium clone ZSR1−99 (FJ479932)
BATS14_018 (GU725806)
BATS14_273 (GU725477)
unidentified eubacterium clone SAR202 (U20797)
Chloroflexus aurantiacus (AJ308501)
Verrucomicrobia bacterium LP2A (AM900834)
Verrucomicrobium spinosum (X90515)
uncultured bacterium (FJ203414)
BATS10_120 (GU726007)
BATS10_218 (GU725902)
BATS10_244 (GU725854)
BATS10_142 (GU726023)
BATS10_012 (GU725821)
BATS10_059 (GU726127)
Planctomyces brasiliensis (AJ231190)
uncultured planctomycete (FJ484800)
uncultured planctomycete (AB491825)
Uncultured bacterium clone PL 212 (FJ268573)
BATS10_197 (GU725886)
uncultured Firmicutes bacterium (DQ316815)
plastid clone MPCa6_F07 (EF414079)
uncultured bacterium mle1−12 (AF280849)
Caldilinea aerophila (AB067647)
uncultured Chloroflexi bacterium (DQ289898)
uncultured bacterium (FJ203586)
uncultured Chloroflexi bacterium (FJ516818)
BATS10_070 (GU726109)
BATS10_008 (GU725811)
BATS10_272 (GU725985)
Uncultured bacterium clone Strom2D12 (EU918093)
Uncultured bacterium clone d1−22 (AM409900)
BATS14_171 (GU725612)
BATS14_128 (GU725636)
BATS14_146 (GU725601)
BATS14_184 (GU725752)
uncultured Chloroflexi bacterium (FM253616)
uncultured Chloroflexi bacterium (FJ516783)
uncultured Chloroflexi bacterium (AY921687)
uncultured Thermomicrobium sp. (AM259916)
uncultured bacterium (AB473882)
uncultured organism (EU245100)
BATS10_262 (GU725933)
37
Group 1 (BATS10)
BATS14_012 (GU725809)
BATS14_310 (GU725536)
uncultured Sphingobacteriales bacterium (FJ403076)
uncultured Flammeovirgaceae bacterium (FJ516777)
Flexithrix dorotheae (AB288294)
uncultured bacterium (FJ644595)
Galbibacter mesophilu (AB255367)
Joostella marina (EF660761)
Flavobacteriaceae bacterium LE20 (EU391659)
BATS10_206 (GU725907)
uncultured bacterium (FJ644591)
Gilvibacter sediminis (AB255368)
Uncultured bacterium clone EDP−128 (EU283197)
BATS10_231 (GU725865)
BATS10_016 (GU725835)
BATS10_257 (GU725963)
BATS10_318 (GU725976)
BATS10_151 (GU726070)
BATS10_330 (GU725935)
BATS10_069 (GU726110)
BATS10_079 (GU726125)
Sediminibacter furfurosus (AB255369)
uncultured Bacteroidetes bacterium (EF123625)
Winogradskyella eximia (AY521225)
Winogradskyella poriferorum (AY848823)
uncultured Winogradskyella sp. (FJ425645)
Coccinimonas marina (EF108213)
uncultured bacterium (DQ890443)
Winogradskyella sp. MOLA 345 (AM945575)
BATS14_149 (GU725598)
BATS14_112 (GU725621)
BATS14_185 (GU725751)
67
Group 2 (BATS10)
BATS14_074 (GU725678)
BATS14_271 (GU725547)
BATS14_048 (GU725660)
BATS14_308 (GU725538)
Microscilla marina (AB078080)
uncultured bacterium (FJ202249)
BATS14_209 (GU725772)
28
Group 3 (BATS14)
43
Group 4 (BATS14)
Flexibacter elegans (AB078048)
Flexibacter ruber (AB078064)
0.10

**Figure 6**


Aquificales str. SRI−48 (AF255599)
BATS14_012 (GU725809)
uncultured Flammeovirgaceae bacterium (FJ516777)
Flexithrix dorotheae (AB288294)
uncultured bacterium (FJ644595)
BATS14_310 (GU725536)
uncultured Sphingobacteriales bacterium (FJ403076)
Flavobacteriaceae bacterium LE20 (EU391659)
Galbibacter mesophilus (AB255367)
Joostella marina (EF660761)
BATS10_206 (GU725907)
Gilvibacter sediminis (AB255368)
uncultured bacterium (FJ644591)
Uncultured bacterium clone EDP−128 (EU283197)
BATS10_151 (GU726070)
BATS10_016 (GU725835)
BATS10_231 (GU725865)
BATS10_257 (GU725963)
BATS10_318 (GU725976)
BATS10_330 (GU725935)
BATS10_069 (GU726110)
BATS10_079 (GU726125)
Sediminibacter furfurosus (AB255369)
uncultured Bacteroidetes bacterium (EF123625)
uncultured Winogradskyella sp. (FJ425645)
Winogradskyella poriferorum (AY848823)
Cocoinimonas marina (EF108213)
uncultured bacterium (DQ890443)
Winogradskyella eximia (AY521225)
Winogradskyella sp. MOLA 345 (AM945575)
BATS14_149 (GU725598)
BATS14_112 (GU725621)
BATS14_185 (GU725751)
BATS10_299 (GU725965)
BATS10_019 (GU725834)
BATS10_174 (GU726000)
BATS10_139 (GU726085)
BATS10_161 (GU726083)
BATS10_155 (GU726080)
BATS10_170 (GU726055)
BATS10_152 (GU726025)
BATS10_121 (GU726005)
BATS10_271 (GU725986)
BATS10_319 (GU725946)
BATS10_212 (GU725922)
BATS10_011 (GU725837)
BATS10_025 (GU725826)
BATS10_210 (GU725909)
BATS10_320 (GU725945)
BATS10_278 (GU725973)
BATS10_164 (GU726003)
BATS10_116 (GU726020)
BATS10_173 (GU726052)
BATS10_097 (GU726077)
BATS10_308 (GU725956)
BATS10_209 (GU725877)
BATS10_034 (GU725847)
BATS10_211 (GU725908)
BATS10_261 (GU725934)
BATS10_279 (GU725972)
BATS10_175 (GU725999)
BATS10_087 (GU726013)
BATS10_146 (GU726051)
BATS10_140 (GU726076)
BATS10_311 (GU725953)
BATS10_232 (GU725864)
BATS10_268 (GU725931)
BATS10_297 (GU725967)
BATS10_273 (GU725996)
BATS10_145 (GU726008)
BATS10_119 (GU726041)
BATS10_168 (GU726066)
BATS10_098 (GU726081)
BATS10_216 (GU725849)
BATS10_191 (GU725916)
BATS10_200 (GU725905)
BATS10_219 (GU725901)
BATS10_004 (GU725816)
BATS10_022 (GU725813)
BATS10_005 (GU725828)
BATS10_179 (GU725861)
BATS10_239 (GU725882)
BATS10_073 (GU726097)
BATS10_002 (GU725817)
BATS10_237 (GU725884)
BATS10_230 (GU725866)
BATS10_233 (GU725875)
BATS10_255 (GU725891)
BATS10_253 (GU725893)
BATS10_009 (GU725824)
BATS10_317 (GU725947)
BATS10_220 (GU725913)
BATS10_263 (GU725962)
BATS10_057 (GU726106)
BATS10_058 (GU726105)
BATS10_062 (GU726091)
BATS10_083 (GU726089)
BATS10_072 (GU726116)
BATS10_052 (GU726099)
BATS10_064 (GU726130)
0.10

**Figure 6a**


Aquificales str. SRI−48 (AF255599)
Winogradskyella poriferorum (AY848823)
Microscilla marina (AB078080)
Flexibacter elegans (AB078048)
Flexibacter ruber (AB078064)
BATS14_266 (GU725481)
BATS14_105 (GU725562)
BATS14_162 (GU725645)
BATS14_052 (GU725654)
BATS14_220 (GU725740)
BATS14_330 (GU725517)
BATS14_311 (GU725535)
BATS14_107 (GU725560)
BATS14_099 (GU725568)
BATS14_096 (GU725571)
BATS14_166 (GU725582)
BATS14_147 (GU725600)
BATS14_145 (GU725602)
BATS14_093 (GU725613)
BATS14_092 (GU725614)
BATS14_208 (GU725773)
BATS14_023 (GU725785)
BATS14_200 (GU725776)
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BATS14_142 (GU725605)
BATS14_235 (GU725703)
BATS14_059 (GU725667)
BATS14_045 (GU725688)
BATS14_015 (GU725803)
BATS14_244 (GU725731)
BATS14_292 (GU725490)
BATS14_024 (GU725783)
BATS14_218 (GU725742)
BATS14_229 (GU725724)
BATS14_070 (GU725666)
BATS14_098 (GU725569)
BATS14_275 (GU725475)
BATS14_151 (GU725596)
BATS14_236 (GU725694)
BATS14_084 (GU725572)
BATS14_245 (GU725730)
BATS14_297 (GU725485)
BATS14_041 (GU725653)
BATS14_204 (GU725701)
BATS14_285 (GU725498)
BATS14_094 (GU725647)
BATS14_136 (GU725610)
0.10

**Figure 6b**

| | Percent similiarity | no clones analyzed | OTUs | Richness Index Chao1 | Diversity Index Shannon-Wiener |
|---|---|---|---|---|---|
| | 100 | 304 | 88 | 496 | 3.0 |
| PT Library | 99 | 304 | 47 | 127 | 2.2 |
| | 97 | 304 | 33 | 49 | 2.0 |
| | | | | | |
| | 100 | 316 | 48 | 171 | 2.2 |
| TT Library | 99 | 316 | 25 | 39 | 1.8 |
| | 97 | 316 | 22 | 32 | 1.7 |
| | | | | | |
| Sargasso Sea | 100 | 189 | 141 | 547 | 4.8 |
| | 99 | 189 | 98 | 268 | 4.1 |
| | 97 | 189 | 70 | 141 | 3.4 |

**Table 1.** Statistical description of PT, TT, and Sargasso Sea bacterioplankton clone libraries. OTUs, the Chao1 richness index, and the Shannon-Wiener diversity index were all calculated using the FastGroup II website which is described in the Methods. The number of OTUs and the values of the Chao1 and Shannon-Wiener indices are calculated at the 97, 99, and 100 percent sequence identity threshold (PSI). The results of the 97 PSI analysis are described in detail in the text.

**Supplemental Table 1.** Nearest GenBank neighbors of 97 PSI OTUs present within 16S rDNA clone libraries constructed from DNA extracted from tuft-type (TT) *Trichodesmium* colonies. In cases where the nearest neighbor is an uncultivated organism, the nearest cultivated organism is included as well.

**Supplemental Table 2.** Nearest GenBank neighbors of 97 PSI OTUs present within 16S rDNA clone libraries constructed from DNA extracted from puff-type (PT) *Trichodesmium* colonies. In cases where the nearest neighbor is an uncultivated organism, the nearest cultivated organism is included as well

## Supplemental Table 1

| Clone ID *(BATS14_)* | Accession no. | Sequence ID of nearest BLAST match | Accession no. (BLAST match) | PSI | Number of bases compared | Bit score | Number of clones recovered |
|---|---|---|---|---|---|---|---|
| 163 | GU725644 | Uncultured alpha proteobacterium partial 16S rRNA gene, clone bc4.49 | FM896960 | 99 | 431 | 780 | 3 |
| 163 | GU725644 | *Nisaea* sp. MOLA 147 partial 16S rRNA gene, culture collection MOLA:147 | AM990921 | 98 | 431 | 763 | |
| 051 | GU725655 | Uncultured alpha proteobacterium clone CL20-G04 16S ribosomal RNA gene | GQ204833 | 98 | 431 | 758 | 6 |
| 051 | GU725655 | *Paracoccus versutus* strain GW1 16S ribosomal RNA gene | GU111570 | 93 | 433 | 630 | |
| 154 | GU725629 | Uncultured alpha proteobacterium clone UNFR_67 16S ribosomal RNA gene | EF123592 | 95 | 428 | 673 | 2 |
| 155 | GU725628 | *Stappia* sp. DG1611 16S ribosomal RNA gene | EU052769 | 93 | 432 | 638 | 1 |
| 186 | GU725764 | Uncultured cyanobacterium clone 1m04AMLE04 16S ribosomal RNA gene | EF630220 | 96 | 433 | 726 | 64 |
| 186 | GU725764 | *Limnothrix* sp. CENA110 16S ribosomal RNA gene | EF088338 | 92 | 439 | 610 | |
| 087 | GU725633 | *Trichodesmium thiebautii* 16S ribosomal RNA gene | AF013027 | 99 | 433 | 795 | 150 |
| 261 | GU725545 | Uncultured cyanobacterium clone 1m04AMLE04 16S ribosomal RNA gene | EF630220 | 93 | 435 | 632 | 1 |
| 261 | GU725545 | *Limnothrix* sp. CENA110 16S ribosomal RNA gene | EF088338 | 89 | 440 | 532 | |
| 317 | GU725529 | *Trichodesmium thiebautii* 16S ribosomal RNA gene | AF013027 | 97 | 433 | 739 | 1 |
| 217 | GU725743 | *Trichodesmium thiebautii* 16S ribosomal RNA gene | AF013027 | 92 | 436 | 628 | 1 |
| 292 | GU725490 | *Flexibacter ruber* gene for 16S rRNA, strain:IFO 16675 | AB078064 | 87 | 474 | 531 | 1 |
| 204 | GU725701 | *Flexibacter ruber* gene for 16S rRNA, strain:IFO 16675 | AB078064 | 89 | 474 | 592 | 42 |
| 112 | GU725621 | *Winogradskyella* sp. K7-7 16S ribosomal RNA gene | FJ425226 | 93 | 369 | 682 | 2 |
| 149 | GU725598 | *Winogradskyella* sp. K7-7 16S ribosomal RNA gene | FJ425226 | 93 | 474 | 689 | 1 |
| 328 | GU725519 | *Microscilla marina* gene for 16S rRNA, strain:IFO 16560 | AB078080 | 87 | 793 | 893 | 28 |
| 308 | GU725538 | *Microscilla marina* gene for 16S rRNA, strain:IFO 16560 | AB078080 | 96 | 753 | 1221 | 2 |
| 012 | GU725809 | *Flexibacter aggregan* s gene for 16S rRNA, strain: IAM 14894 | AB288294 | 89 | 789 | 953 | 1 |
| 310 | GU725536 | Uncultured Sphingobacteriales bacterium clone MD2.27 | FJ403076 | 86 | 790 | 808 | 1 |
| 209 | GU725772 | *Microscilla marina* gene for 16S rRNA, strain:IFO 16560 | AB078080 | 87 | 735 | 819 | 1 |
| 271 | GU725547 | *Microscilla marina* gene for 16S rRNA, strain:IFO 16560 | AB078080 | 94 | 728 | 1112 | 2 |
| 171 | GU725612 | Uncultured bacterium partial 16S rRNA gene, clone d1-22 | AM409900 | 89 | 738 | 933 | 3 |
| 171 | GU725612 | Uncultured bacterium clone Strom2D12 16S ribosomal RNA gene | EU918093 | 89 | 737 | 931 | |
| 184 | GU725752 | Uncultured bacterium clone Strom2D12 16S ribosomal RNA gene | EU918093 | 89 | 738 | 928 | 1 |
| 273 | GU725477 | Uncultured candidate division SR1 bacterium clone ZSR1-99 | FJ479932 | 90 | 675 | 869 | 2 |

## Supplemental Table 2

| Clone ID *(BATS10_)* | Accession no. | Sequence ID of nearest BLAST match | Accession no. (BLAST match) | PSI | Number of bases compared | Bit score | Number of clones recovered |
|---|---|---|---|---|---|---|---|
| 125 | GU726072 | Uncultured bacterium clone 31P16 16S ribosomal RNA gene | AY845235 | 96 | 700 | 621 | 1 |
| 125 | GU726072 | *Brucella* sp. HJ114 16S ribosomal RNA gene, partial sequence | AY234650 | 92 | 706 | 542 | |
| 217 | GU725848 | Uncultured bacterium clone SHFG540 16S ribosomal RNA gene | FJ203138 | 98 | 627 | 1120 | 1 |
| 217 | GU725848 | Alpha proteobacterium C40 gene for 16S rRNA | AB302366 | 98 | 627 | 1120 | |
| 312 | GU725952 | Uncultured alpha proteobacterium gene for 16S rRNA, clone: pKB7B-1 | AB247894 | 93 | 347 | 641 | 2 |
| 312 | GU725952 | *Aquaspirillum itersonii* subsp. itersonii strain LMG 4337 16S ribosomal RNA gene | EF612765 | 92 | 428 | 614 | |
| 266 | GU725923 | Uncultured bacterium clone Mann16S_H11 16S ribosomal RNA gene | FJ952694 | 92 | 667 | 944 | 3 |
| 266 | GU725923 | Marine alpha proteobacterium RS.Sph.017 16S ribosomal RNA gene | DQ097291 | 91 | 670 | 894 | |
| 126 | GU726009 | *Rhodobium* sp. 6 16S ribosomal RNA gene | EU982844 | 89 | 764 | 961 | 1 |
| 157 | GU726045 | Uncultured Rhodobacterales bacterium clone HF770_42O01 SSU rRNA gene | EU361414 | 98 | 744 | 1315 | 5 |
| 157 | GU726045 | *Roseovarius nubinhibens* strain DSM15170 16S ribosomal RNA gene | DQ915625 | 97 | 744 | 1277 | |
| 260 | GU725968 | Uncultured bacterium clone W2-D10 16S SSU rRNA gene | FJ930689 | 96 | 630 | 1061 | 2 |
| 260 | GU725968 | Alpha proteobacterium A30 gene for 16S rRNA | AB302381 | 94 | 651 | 959 | |
| 124 | GU726028 | Gamma proteobacterium N2yML2 16S ribosomal RNA gene, | EF629832 | 99 | 704 | 1284 | 17 |
| 124 | GU726028 | Gamma proteobacterium KMM 3900 gene for 16S ribosomal RNA | AB500095 | 96 | 752 | 1223 | |
| 177 | GU725997 | Gamma proteobacterium N2yML2 16S ribosomal RNA gene | EF629832 | 92 | 701 | 989 | 1 |
| 024 | GU72583 | Uncultured gamma proteobacterium clone SIMO-1966 16S rRNA gene | AY711332 | 96 | 746 | 1238 | 1 |
| 024 | GU72583 | *Legionella* sp. IA55 gene for 16S rRNA | AB058909 | 91 | 749 | 1024 | |
| 300 | GU725964 | *Trichodesmium thiebautii* 16S ribosomal RNA gene | AF013027 | 99 | 711 | 1310 | 141 |
| 147 | GU726035 | *Trichodesmium thiebautii* 16S ribosomal RNA gene | AF013027 | 99 | 711 | 1293 | 2 |
| 304 | GU725960 | *Trichodesmium thiebautii* 16S ribosomal RNA gene | AF013027 | 95 | 548 | 881 | 1 |
| 184 | GU725887 | *Trichodesmium thiebautii* 16S ribosomal RNA gene | AF013027 | 99 | 562 | 1009 | 1 |
| 330 | GU725935 | Uncultured bacterium clone EDP-128 16S ribosomal RNA gene | EU283197 | 98 | 658 | 1171 | 8 |
| 330 | GU725935 | *Gilvibacter* sp. MOLA 433 partial 16S rRNA gene | AM990700 | 98 | 658 | 1166 | |
| 073 | GU726097 | Uncultured bacterium clone livecontrolB18 16S ribosomal RNA gene | FJ264752 | 93 | 737 | 1068 | 1 |
| 073 | GU726097 | *Coccinimonas marina* strain IMCC1846 16S ribosomal RNA gene | EF108213 | 92 | 732 | 1057 | |
| 052 | GU726099 | Uncultured bacterium clone 654 16S ribosomal RNA gene | FJ223527 | 92 | 670 | 953 | 4 |
| 052 | GU726099 | *Winogradskyella* sp. MOLA 413 partial 16S rRNA gene | AM990680 | 92 | 669 | 937 | |
| 174 | GU726000 | Uncultured bacterium clone pt181 16S ribosomal RNA gene | DQ890443 | 94 | 710 | 1072 | 1 |
| 174 | GU726000 | *Winogradskyella* sp. MOLA 345 partial 16S rRNA gene | AM945575 | 93 | 715 | 1055 | |
| 206 | GU725907 | *Gilvibacter sediminis* gene for 16S rRNA | AB255368 | 99 | 534 | 957 | 1 |
| 253 | GU725893 | Uncultured bacterium clone pt181 16S ribosomal RNA gene | DQ890443 | 94 | 718 | 1081 | 3 |
| 253 | GU725893 | *Winogradskyella* sp. MOLA 345 partial 16S rRNA gene | AM945575 | 93 | 723 | 1064 | |

## Supplemental Table 2 (cont'd)

| Clone ID *(BATS10_)* | Accession no. | Sequence ID of nearest BLAST match | Accession no. (BLAST match) | PSI | Number of bases compared | Bit score | Number of clones recovered |
|---|---|---|---|---|---|---|---|
| 026 | GU725822 | Uncultured bacterium gene for 16S rRNA, partial sequence, clone: SS_04 | AB473882 | 90 | 713 | 944 | 34 |
| 272 | GU725985 | Uncultured Chloroflexi bacterium clone TDNP_Bbc97_69_2_134 16S rRNA gene | FJ516818 | 92 | 724 | 1029 | 3 |
| 262 | GU725933 | Uncultured organism clone MAT-CR-H2-A10 16S ribosomal RNA gene | EU245100 | 88 | 716 | 839 | 1 |
| 162 | GU726030 | Uncultured bacterium clone SGUS1230 16S ribosomal RNA gene | FJ202161 | 92 | 727 | 1035 | 2 |
| 143 | GU726043 | Uncultured bacterium clone PL 212 16S ribosomal RNA gene | FJ268573 | 83 | 565 | 507 | 1 |
| 120 | GU726007 | Uncultured bacterium clone SHFH449 16S ribosomal RNA gene | FJ203414 | 93 | 752 | 1112 | 3 |
| 012 | GU725821 | Uncultured bacterium clone SHFH449 16S ribosomal RNA gene | FJ203414 | 93 | 555 | 819 | 2 |
| 244 | GU725854 | Uncultured bacterium clone SHFH449 16S ribosomal RNA gene | FJ203414 | 93 | 723 | 1053 | 2 |

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# CHAPTER 5:

# BACTERIAL COMMUNICATION REGULATES THE DEGRADATION OF SINKING ORGANIC MATTER IN THE SEA

The ocean's biological pump is a dynamic and complex system that transports carbon from the atmosphere to the deep ocean. A central component of the biological pump is carbon export via sinking particles composed of photosynthetically derived particulate organic carbon (POC) and biominerals. Bacteria colonize these particles and produce enzymes that dissolve and degrade sinking POC[1], thereby acting as one of the major controls on the biological pump[2]. Here we show that bacteria use a cell-cell communication mechanism, quorum sensing (QS), to regulate the activity of organic carbon degrading enzymes on sinking particles. We collected sinking POC from a site of intense primary productivity off Vancouver Island, Canada and found that sinking particles contained acylated homoserine lactones (AHLs), a suite of communication molecules well-known to be produced by bacteria with QS ability[3-5]. In addition, we conducted incubation experiments with the sinking POC and discovered that the addition of exogenous AHLs enhanced the activity of key POC-degrading enzymes to various degrees[6, 7]. Our results suggest that AHL-based QS may play an important role in regulating POC degradation in sinking particles and that variability in QS is a heretofore unrecognized process that affects the strength of biological pump.

Sinking POC flux declines significantly in the upper few hundred meters of the ocean[8, 9]; only a small percentage (~10%) of organic material fixed by photosynthesis is

transferred to the deep ocean where it is removed from the atmosphere for hundreds to thousands of years. The attenuation of sinking POC flux has been the subject of several major scientific research initiatives over the past three decades. It is generally accepted that organic carbon degradation by POC-attached bacteria contributes significantly to flux-attenuation[2]. However, parameterizing the activity of POC-attached bacteria remains a significant obstacle to the development of accurate carbon cycle models, which are needed to predict future trends in the earth's carbon dioxide budget in response to natural and anthropogenic environmental changes [10]. Currently, flux attenuation is represented by empirical[8] or chemical models[11]which do not explicitly incorporate the activities of particle-attached bacteria.

Particle-attached bacterial communities are generally comprised of several bacterial classes, and invariably include significant numbers of Proteobacteria[12]. Many Proteobacteria use QS, mediated by AHLs[13], to synchronize gene expression at the high population densities likely to be encountered in sinking POC (up to $10^9$ cells/mL)[14]. In pure culture, QS has been shown to be involved in the coordination of genes encoding a number of group-beneficial behaviors (e.g. exoenzyme production and biofilm formation)[15]; the ability to synchronize these behaviors may confer ecological and evolutionary advantages (e.g. nutrient acquisition or collective defense)[16].

AHLs are rapidly degraded by biological and abiotic mechanisms but remain effective at very low concentrations[14, 17]. Direct measurements of AHLs in environmental samples require extremely sensitive and selective analytical tools that are only now

becoming available to the oceanographic community. Although bacterial isolates from marine particles have been shown to possess the capacity for QS[3], *in-situ* QS activity has yet to be demonstrated in sinking POC.

In order to test the hypothesis that AHL-QS is occurring in marine particles, we collected sinking POC using a sediment net trap[18] in Clayoquot Sound on the western coast of Vancouver Island, B.C. in July 2009. We chose Clayoquot Sound because of its high fluxes of marine photosynthetically-derived POC in the summer months (approaching 1 mol C per $m^2$ per day, *unpublished data, R. Keil*). During our sampling, the euphotic zone was observed to extend no deeper than 30 m. Sinking POC was collected by traps suspended at 55 m and assayed for AHLs by two independent methods. First, POC was applied to agar plates seeded with the AHL-sensing bacterium *Agrobacterium tumefaciens NTL4(pZLR4)*[19]; the POC induced the distinct response of this biosensor to AHLs (data not shown). This sensitive and robust method clearly indicated that the particles contained chemicals with the bioactivity of AHLs. Second, samples were returned to the laboratory at Woods Hole for chemical analysis by high performance liquid chromatography / electrospray-ionization mass spectrometry (HPLC/ESI-MS). Two AHLs- octanoyl homoserine lactone (C8-HSL) and dodecanoyl homoserine lactone, (C12-HSL)- were structurally identified in the sinking POC on the basis of chromatographic retention time, molecular masses and secondary mass spectra, which were identical to authentic standards (Figure 1). These results confirm that AHLs were present in POC samples and were responsible for the biosensor response. Since AHLs are known to have a rapid turnover time in natural seawater (0.5 to 1.5 $day^{-1}$

depending on the structure)[14], the detection of these compounds in our POC samples indicates in-situ activity. AHLs have been directly detected only a handful of times in natural marine environments (e.g. biofilms coating rocks in intertidal pools[20] and stromatolites[21]); this study represents the first time that AHLs have been detected in sinking POC.

Over two decades ago, Smith and Azam[22] demonstrated that particle-attached bacteria are responsible for intense organic carbon-degrading enzyme activity on sinking POC, which ultimately catalyzes the conversion of sinking POC to suspended POC, dissolved organic carbon (DOC), and ultimately, $CO_2$ via heterotrophic respiration[1]. It has been suggested that coordination of organic carbon degrading enzyme activity is advantageous for POC-attached bacteria[23, 24]. Our observation of AHLs in sinking POC supports this suggestion, and during our cruise we conducted incubation experiments to test whether AHL-QS affected rates of organic carbon degradation. We amended incubations containing samples of sinking POC with one of two AHLs- 3-oxo-hexanoyl homoserine lactone (3-oxo-C6-HSL) and 3-oxo-octanoyl homoserine lactone (3-oxo-C8-HSL)- at either 500nM or 5000nM. At two time points (6 and 24 hours) we measured the effect of these AHL amendments on protease, lipase, phosphatase, chitinase, and α- and β-glucosidase activity based upon the methods employed by Smith and Azam. We then compared the incubations amended with AHLs to controls without AHLs and found clear evidence that AHLs enhanced the level of enzyme activity in some instances.

We observed a strong response to AHL amendments on Day 1 of our sampling program, but samples from Days 2 and 3 displayed diminished responses (corresponding to Traps 1, 2 and 3, respectively). Protease activity was enhanced by more than a factor of two by all AHL treatments in Trap 1, but was enhanced to a lesser extent in Trap 2, and virtually not at all in Trap 3 (Figure 2). Lipase and phosphatase activity were enhanced approximately 3- and 8-fold, respectively, by all AHL treatments in Trap 1 and lipase activity was enhanced by low concentrations of AHL in Trap 3. In comparison, the three glucosidases (chitinase, α-glucosidase, and β-glucosidase) were minimally affected in all three traps (Supp. Fig. 2). Despite the heterogeneity of our samples, these results indicate a biochemical linkage between AHLs and enzyme activity and suggest that the magnitude of this effect may be variable in time and co-regulated by environmental factors, particle properties or microbial flora composition.

The observation that AHLs enhanced the activity of key carbon-degrading enzymes provides strong support for the hypothesis that AHL-based QS regulates sinking POC degradation, and thus POC flux attenuation in the sea. However, an alternative explanation deserves consideration: instead of acting as signalling molecules AHLs could simply have provided carbon and/or nitrogen supplement to particle-attached bacteria and the observed changes in enzyme activity only reflected changes in bacterial nutrition. We discount this latter explanation for the following reasons: First, the amount of carbon we added as AHL was likely to have been 3-4 orders of magnitude lower than the DOC concentration in the interstitial water (~1 to 5 mM) of typical marine aggregates[25] and thus it is unlikely that the small amount of AHLs would stimulate such a dramatic

response. Second, additional AHL in the 5000nM treatments did not systematically enhance the effect induced by the 500nM treatment; this is suggestive of the threshold response characteristic of QS. In consideration of the preceding arguments, we believe that our first explanation best supports our data: AHL-based QS is a factor in regulating the expression of organic carbon-degrading enzymes.

The occurrence of QS in sinking particles validates theoretical model calculations describing the production of diffusible extracellular enzymes by particle-attached bacteria[23]. Vetter and colleagues[23] suggested that coordinated action by microbial consortia or clonal populations would maximize the energetic returns on the production of diffusible enzymes to an individual bacterium. Such coordinated behavior is the hallmark of quorum sensing. There are two implications of QS control of hydrolytic enzyme production in sinking particles. First, by attenuating the downward flux of POC, POC-associated bacteria are acting to retain themselves in productive, particle-rich surface waters to which they are adapted. Second, by preferentially stimulating organic N and P-degrading enzyme activities (protease, lipase, and phosphatase), these same cooperative behaviors may in fact be promoting the retention of major nutrients in surface waters which in turn could support additional primary productivity and bacterial production[1].

If, as our data suggest, AHL-based QS is a control on organic carbon degradation in sinking particles, then AHL-based QS is likely to be a critical link between atmospheric and oceanic reserves of carbon. Specifically, QS could be a factor in

determining the remineralization depth of sinking POC. A recent study has shown that modest changes in the remineralization depth can have a major impact on atmospheric carbon dioxide concentrations[26]. We observed that QS can have as much as an 8-fold impact on enzyme hydrolysis rates and we posit that this could in turn result in significant variability on the magnitude of the export flux of sinking POC from the surface ocean and thereby the depth at which it is degraded. If QS has even a 2-fold effect on the hydrolysis of key biochemical fractions (e.g. protein; Fig. 2) of sinking organic matter, then QS could play an important role in regulating of the depth at which POC is remineralized to $CO_2$. A doubling of the hydrolysis rate of proteins (40% of labile POC)[27] would decrease the "e-folding" depth of remineralization by ≈ 30 m globally whereas a halving of the rate would increase this depth by ≈ 40 m (Supp. Fig. 3). The e-folding depth is defined by convention as the depth at which 63% of sinking POC is remineralized[26]. Kwon and colleagues calculate a change in e-folding depth as modest as 24 m can impact $pCO_2$ by 10-27 ppm[26] which is equivalent to the increase in atmospheric $CO_2$ over the last 5-10 years. Thus, QS has the potential to act as an important buffer of atmospheric $CO_2$.

Future increases in atmospheric $CO_2$ may impact QS because the concomitant decrease in ocean pH would stabilize AHLs; these molecules are rapidly deactivated by base-catalyzed hydrolysis in the range of pH 7 - 8[28]. Remineralization processes within POC cause the pH to be lower inside the aggregate (as low as 7.2[30]) with respect to ambient seawater (pH = 8.2). If seawater pH decreases to 7.7, as is predicted by the end of this century[29], the pH within sinking particles may similarly decline. Lowering the pH

within POC aggregates will decrease the rate of base-catalyzed hydrolysis of AHLs, which could in turn impact the regulation of organic-matter degradation. Ocean acidification has been projected to impact numerous marine biological processes, including the calcification of corals and planktonic algae, however, the impact on heterotrophic processes has received little consideration[29].

QS may serve as a mechanism to translate environmental variability into a biogeochemical response. We suggest that this variability may someday be incorporated into carbon cycle models to capture spatial and temporal variability in POC flux; clearly additional work is required in order to identify the environmental and biological parameters (e.g. particle composition, bacterial community structure) that affected the observed variability in QS-regulated organic matter degradation. Our results show that QS is a heretofore unexplored molecular switch that could play an important role in regulating POC degradation.

**METHODS**

Samples were collected using moored sediment net traps[18] in Clayoquot Sound (49º9.93N, 125º41.556W) off the western coast of Vancouver Island at 55m depth for 24 hours. Samples were collected at a single site over the course of 4 trap deployments (Traps 1,2, 3, and 4) on separate days in June 2009. Upon trap retrieval, samples were processed immediately. Samples to be applied to an AHL-biosensor were centrifuged for 1 minute at 3000 rpm and applied to agar-plates seeded with the biosensing strain *Agrobacterium tumefaciens NTL4(pZLR4)*[19]. Samples to be analyzed by mass spectrometry were acidified to pH 2 and frozen at -20º C until transported back to Woods Hole. These particle samples and supernatants were then thawed and separated by centrifugation. Supernatants were extracted in ethyl acetate containing 0.1% formic acid according to previously published methods[14]. Extracts were analyzed on a Thermo Fisher TSQ triple quadrupole mass spectrometer in selected reaction monitoring mode.

Samples were collected for use in enzyme assays from Traps 1, 2 and 3 and were split into equal 15ths. Endogenous AHLs were removed from particles by gentle rinsing as follows: each aliquot was centrifuged for one minute at 3000rpm and the supernatant was poured off. Next, 10mL 0.2 μm filter-sterilized seawater from 55m was reintroduced to the particles. Particles were gently vortexed, centrifuged, and the supernatant was poured off. This procedure was followed one additional time (three washes total). Particles were resuspended by gently vortexing. We amended triplicate tubes with 500nM and 5000nM 3-oxo-C6-HSL and 3-oxo-C8-HSL. In addition, we conducted triplicate control incubations (particles, no-AHL amendment). The incubations were

sampled at six and twenty-four hour intervals. At each sampling time-point, an aliquot of the incubation was removed and assayed for one of six enzyme activities. Enzyme activities were assayed by observing the hydrolysis product of the commonly used fluorogenic substrates L-leucine-7-amino-4-methylcoumarin (for protease), 4-methylumbelliferyl (MUF)-phosphate (for phosphatase), MUF-N-acetyl-β-D-glucosamine (for chitinase), MUF-butyrate (for lipase), MUF-α-glucopyranoside and MUF-β-glucopyranoside (for α- and β-glucosidases, respectively)[1]. After substrates were added to aliquots of the incubation, the fluorescence was immediately measured by a CytoFluor Series 4000 multi-well plate reader and again after six and twenty-four hours. An enzyme activity rate was calculated assuming that fluorescence increased linearly with time by applying a calibration to this difference.

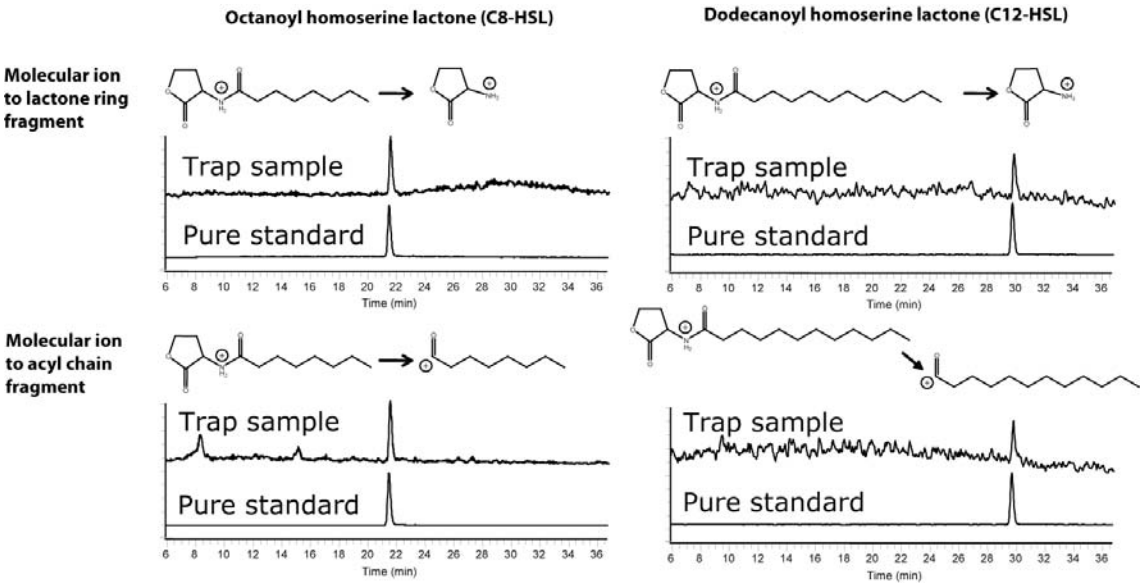
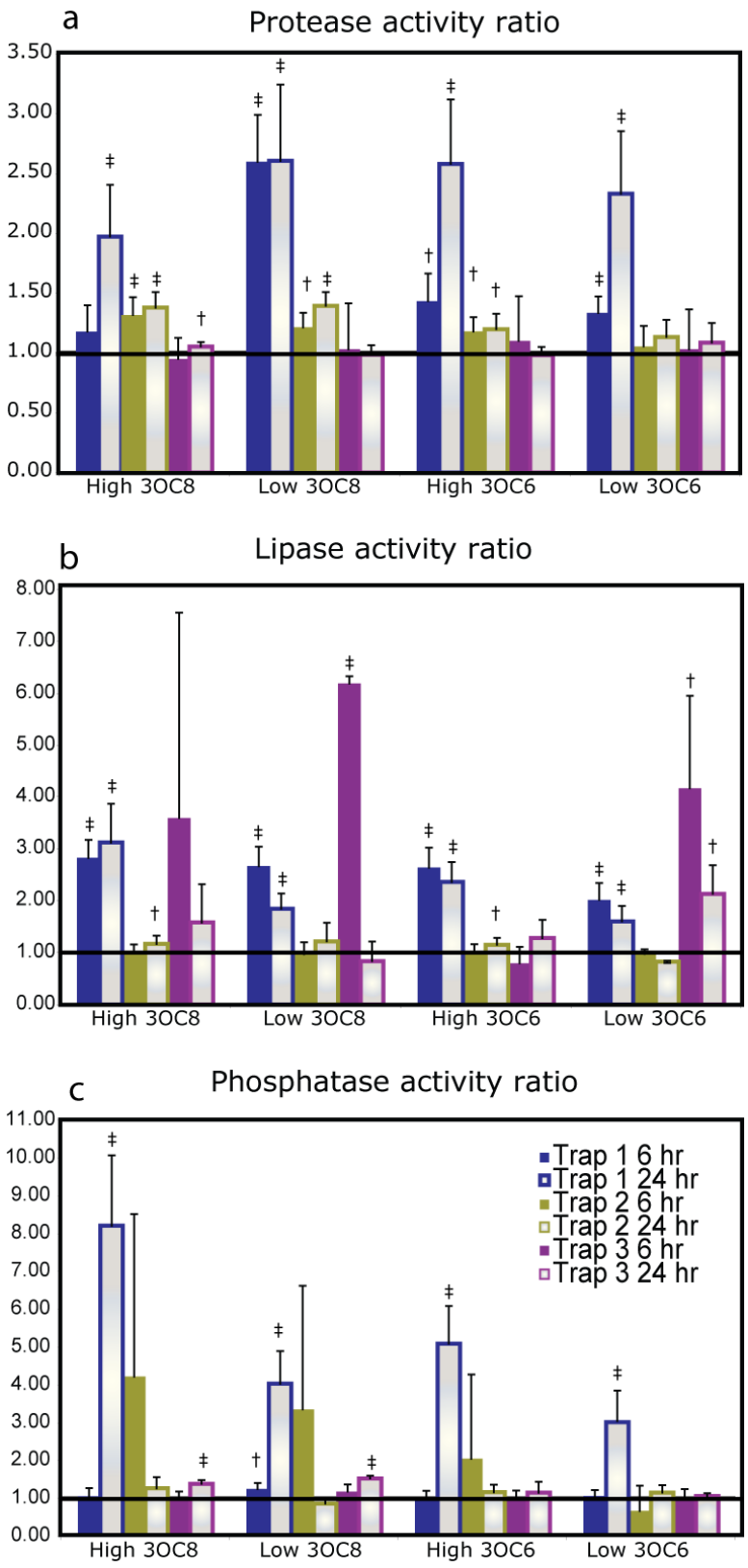



**Figure 1:** HPLC/ESI-MS data showing the presence of endogenous AHLs present in POC from Trap 4. Each set of traces represents a highly diagnostic fragmentation reaction of a C8-HSL (left quadrants) or C12-HSL (right quadrants) to a lactone ring (upper quadrants) or acyl chain (lower quadrants). In each quadrant, upper traces are from our sinking POC samples and lower traces derive from standards.

**Figure 2.** Results of protease (2a), lipase (2b) and phosphatase (2c) assays. Results are presented as ratios of experimental rates relative to unamended-control rates. Error bars are +/- one standard deviation propagated from the error of replicate measurements of treated and control experiments. Daggers (†) indicate 90% confidence or greater that the ratio is greater than one (i.e. the sample is different from the control). Double daggers (‡) indicate 95% confidence or greater that the ratio is greater than one. Note, the y-axis scale varies in 2a, 2b, and 2c.


a
Protease activity ratio
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
High 3OC8
Low 3OC8
High 3OC6
Low 3OC6
b
Lipase activity ratio
8.00
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
High 3OC8
Low 3OC8
High 3OC6
Low 3OC6
c
Phosphatase activity ratio
11.00
10.00
9.00
8.00
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
Trap 1 6 hr
Trap 1 24 hr
Trap 2 6 hr
Trap 2 24 hr
Trap 3 6 hr
Trap 3 24 hr
High 3OC8
Low 3OC8
High 3OC6
Low 3OC6

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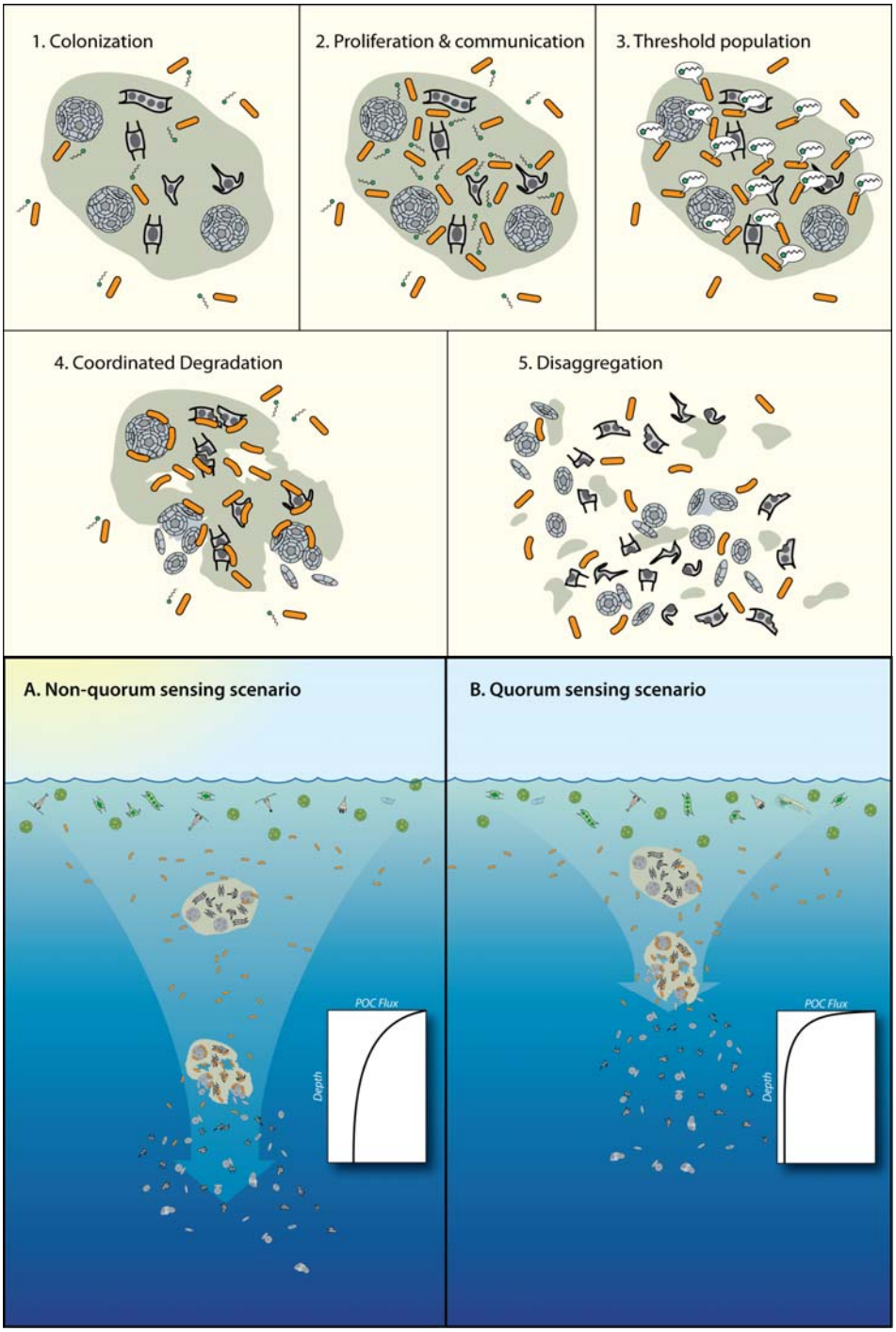
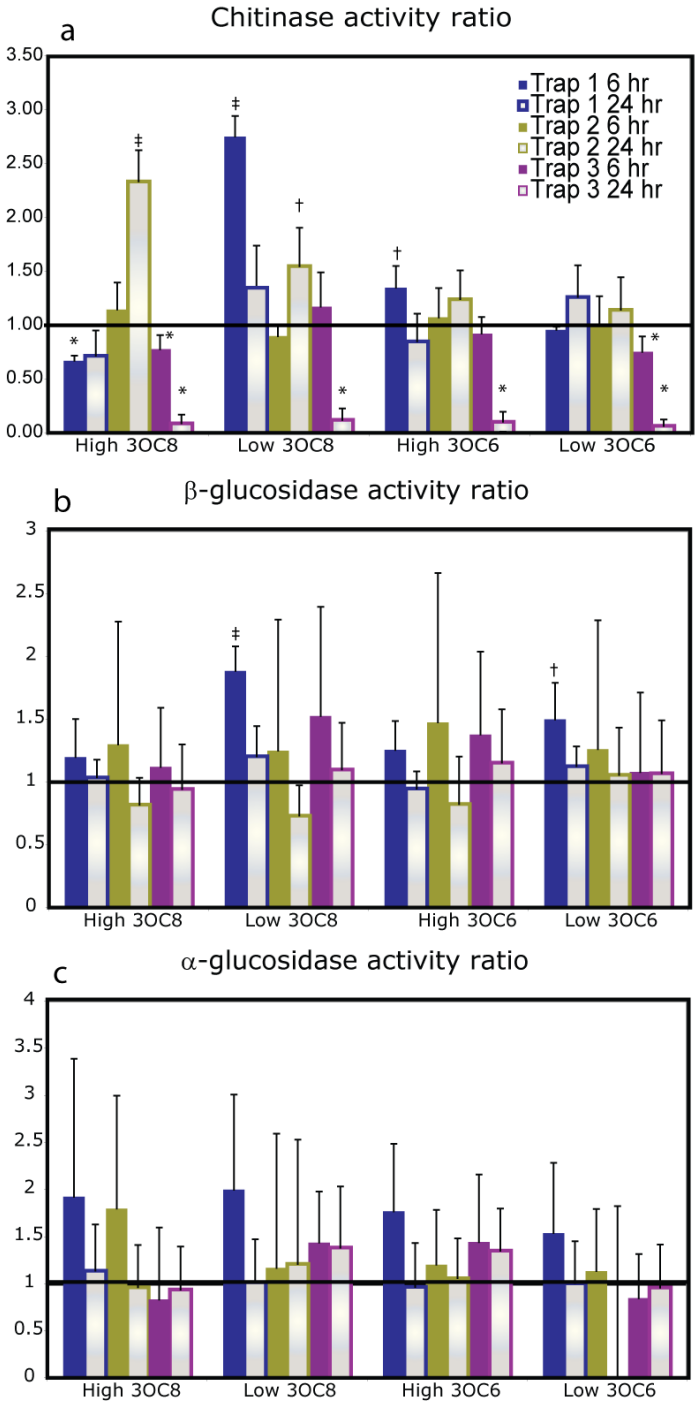
**Supplemental Figure 1:** (Top panel:) Conceptual illustration of QS activity within sinking POC. 1. Bacteria colonize freshly formed sinking POC. 2. Bacteria produce QS signals at a basal rate while they proliferate and clonal populations grow. 3. A particular QS-signal concentration threshold signifies to bacteria that a threshold population has been reached. 4. At this population threshold, bacteria initiate a coordinated expression of hydrolytic enzymes. 5. The production of hydrolytic enzymes leads to the dissolution of sinking POC into smaller suspended POC, DOC, or dissolved $CO_2$.
(Bottom panel:) Conceptual illustration of the potential impact of QS on the flux of sinking POC into the deep ocean. (a) Non-QS scenario where environmental conditions, particle composition, or microbial flora do not support activation of QS-regulated genes (e.g. those for the production of organic-matter degrading enzymes). Enzymes are produced only at a low level and POC is able to sink deep into the water column before it is degraded. (b) QS-scenario where environmental conditions, particle composition, or microbial flora do support QS activity and genes responsible for the synthesis of organic-matter degrading enzymes are activated (up-regulated). QS-regulated enzyme activity catalyzes the degradation of sinking POC to suspended POC, DOC, or $CO_2$ at shallower depths where they may be more rapidly mixed up into contact with surface water by physical mixing.


1. Colonization
2. Proliferation & communication
3. Threshold population
4. Coordinated Degradation
5. Disaggregation
A. Non-quorum sensing scenario
B. Quorum sensing scenario
POC Flux
Depth
POC Flux
Depth

**Supplemental Figure 2:** Results of α- and β-glucosidase activity assays. Results are presented as ratios of experimental rates to unamended-control rates. Error bars are +/- one standard deviation propagated from the error of replicate measurements of treated and control experiments. Daggers (†) indicate 90% confidence or greater that the ratio is greater than one (i.e. the sample is different from the control). Double daggers (‡) indicate 95% confidence or greater that the ratio is greater than one. Asterisks indicate 90% or greater confidence that ratios are less than one which may indicate that chitinase activity was repressed by the AHL additions. Interestingly, a recent report indicates that chitinase activity in *Vibrio harveyi*, a common marine *Vibrio* species, is repressed when its QS genes are activated[1]. Note, the y-axis scale varies in a, b, and c.


a
Chitinase activity ratio
Trap 1 6 hr
Trap 1 24 hr
Trap 2 6 hr
Trap 2 24 hr
Trap 3 6 hr
Trap 3 24 hr
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
High 3OC8
Low 3OC8
High 3OC6
Low 3OC6
b
β-glucosidase activity ratio
3
2.5
2
1.5
1
0.5
0
High 3OC8
Low 3OC8
High 3OC6
Low 3OC6
c
α-glucosidase activity ratio
4
3.5
3
2.5
2
1.5
1
0.5
0
High 3OC8
Low 3OC8
High 3OC6
Low 3OC6

**Supplemental Figure 3:** POC flux versus depth for three scenarios. Each line is a solution to the equation:

$$POC_{total} = POC_{Protein}\, e^{-k_{protien}t} + POC_{Non\text{-}protein}\, e^{-k_{non\text{-}protein}t}$$

for 6 days where $POC_{Protein}$ is the fraction of total sinking POC flux consisting of proteinacous organic matter at 100 m (0.4), $POC_{Non\text{-}protein}$ is the fraction of total sinking POC flux composed of non-proteinacous organic material at 100 m (0.6), $POC_{total}$ is the flux at time 't' (in days), and 'k' is the degradation rate ($day^{-1}$). Time is calculated as:

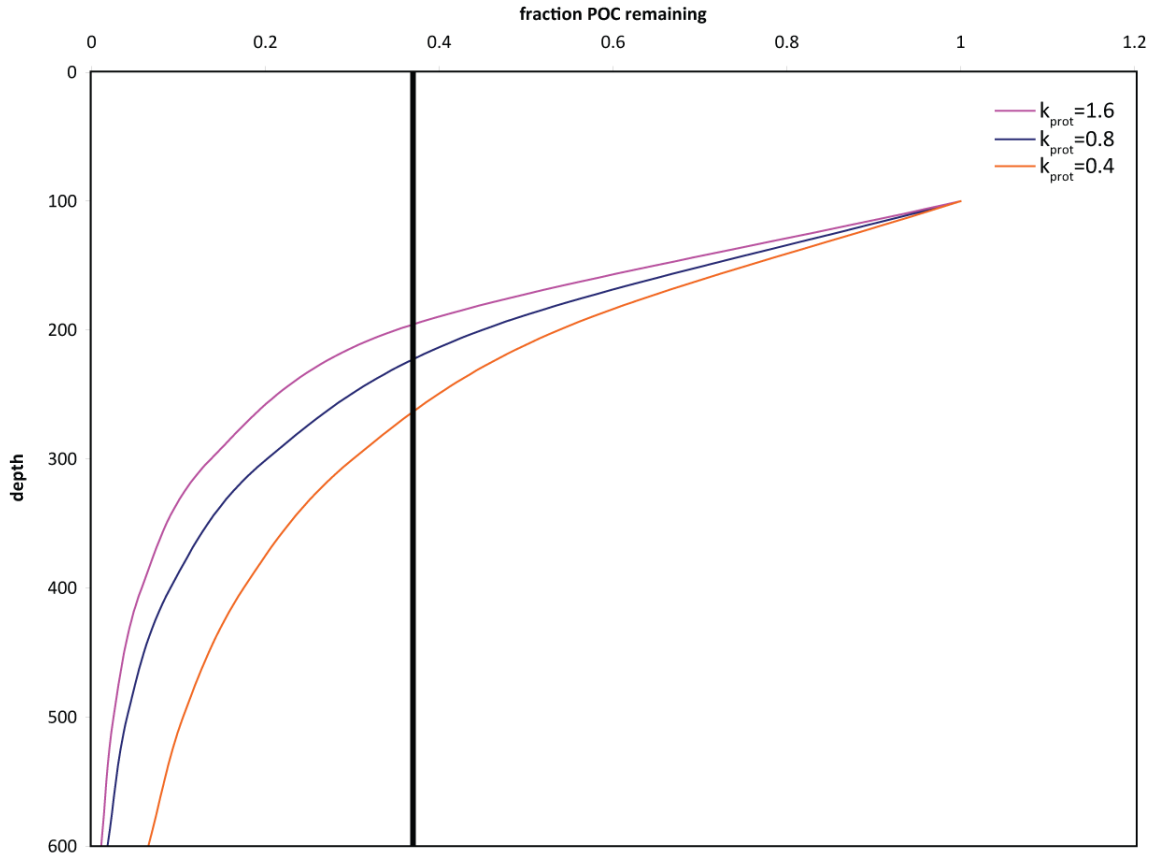
$$t = \text{depth (m)}/\text{sinking rate}$$

where the sinking rate is approximated as 100 m $day^{-1}$. In each curve, the concentration of non-proteinaceous material is calculated with a degradation rate ($k_{non\text{-}prot}$) of 0.8 $day^{-1}$. The degradation rate of protein ($k_{prot}$) varies in the three curves as either, 1.6 $day^{-1}$, 0.8 $day^{-1}$, or 0.4 $day^{-1}$. The rate of 0.8 $day^{-1}$ is a reference rate obtained from Van Mooy et al. (2002)[2], 0.4 $day^{-1}$ represents a scenario where the reference value is halved by a cessation of QS and 1.6 $day^{-1}$ represents a scenario in which the degradation rate is doubled by activation of QS genes. A thick black vertical line is drawn where 63% of $POC_{total}$ has been degraded (the 'e-folding' reference depth; conceptually defined as the sinking rate of POC divided by the degradation rate). This calculation demonstrates that varying the degradation rate of 40% of POC by a factor of 2 can impact the e-folding depth by shoaling it 30 m or deepening it 40 m.

If we can solve these equations for specific depths, then we can assess flux attenuation by fitting a Martin curve[3]:

$$F/F_{100} = z/z_{100}^{-b}$$

where F is POC flux, $F_{100}$ is flux at 100m, z is depth, $z_{100}$ is depth at 100m, and b is a flux attenuation coefficient, we determine that varying the protein degradation rate from .4 $day^{-1}$, 0.8 $day^{-1}$ or 1.6 $day^{-1}$. 0.8 $day^{-1}$ is reflected in an increase of the b value from 1.17, to 1.48, to 1.75, indicative of more rapid flux attenuation (calculation not shown).


fraction POC remaining
0
0.2
0.4
0.6
0.8
1
1.2
depth
0
100
200
300
400
500
600
k prot =1.6
k prot =0.8
k prot =0.4

Supplemental References

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# CHAPTER 6:

# CONCLUSIONS

To varying degrees, bacteria are social organisms that exhibit deliberate and calculated behaviors. They are capable of adjusting and transforming their behavior depending on their neighbors, their mode of existence (attached to a surface or free-living), or their available food substrates. The manner in which bacteria in the ocean interact has profound implications for ecological processes and biogeochemical transformations within the ocean. Through their associations with phytoplankton and detrital carbon, bacteria play an important role in the lifecycle of algal blooms and the variability in the strength of the biological pump.

Although bacteria have been studied for over three hundred years, it was not until thirty years ago that two very remarkable ideas were presented. In 1974, Pomeroy proposed the concept of 'the Microbial Loop' and implicated bacteria as major conduits of carbon and energy in the ocean (Pomeroy, 1974). Around the same time, independent of the latter idea, the concept was introduced that bacterial cells might not be as independent as previous thought but in fact they interact with one another through the production of specific chemicals (Tomasz, 1965; Nealson *et al.*, 1970). Even more recently, it was proposed that specific interactions between bacteria in the microbial loop could shape the biogeochemistry of the ocean (Azam *et al.*, 1994). This Ph.D. dissertation is deeply rooted in these ideas and aimed to deepen our knowledge and understanding of

bacterial interactions in the ocean by elucidating some of the specific bacterial interactions which impact the ocean's carbon cycle.

In this thesis, I examined two types of bacterial interactions, the cell-to-cell communication mechanism dubbed "quorum sensing" and the implied mechanism which structures the unique epibiont community associated with *Trichodesmium* spp. I observed that bacteria actively partner with other bacteria taxa. Whether *Trichodesmium* actively or passively recruits the bacteria with which it exists in a consortial relationship, they are not randomly selected from the abundant members of the pelagic seawater community. In fact, I detected bacteria which have never been observed before. While the metabolic capacity of these bacteria remains unknown, it is likely that they are fulfilling specialized roles in the unique environment of the *Trichodesmium* consortia. Remarkably, these bacteria, which are rare within the pelagic bacterial community, find their way to specific hosts and partnerships.

I observed that bacteria associated with *Trichodesmium* are capable of both quorum sensing (QS) and bioluminescence, a biochemical behavior mediated by QS. Bioluminescence, while regulated by bacterial communication, is in turn thought to play a role in communication with higher organisms. With respect to bacterial bioluminescence, it is thought to assist in dispersion and propagation of bacterial cells through ingestion and egestion from zooplankton. Further, I was able to manipulate the bacteria associated with *Trichodesmium* by providing them with exogenous QS signals and thereby induce them to produce additional enzyme activity.

I similarly manipulated the bacterial communities which associate with particulate organic carbon, one of the main vehicles for carbon flux into the deep sea. The success of this approach suggests that particle-attached bacteria of numerous varieties are sensitive to QS signals and are by implication, social organisms.

The results of this thesis are consistent with the ideas set forth in the first paragraph of this chapter; I showed that bacteria associated with *Trichodesmium* colonies and photosynthetically derived detritus responded to signals (AHLs) which mediate social behavior and that these AHLs impacted phenotypes which are involved in accessing nutrients bound within photosynthetically derived particles. I observed variability of the response of these communities to QS signals. Although I can only speculate as to the environmental factors co-regulating their effectiveness, it is evident that QS may be one mechanism which can translate environmental variability into a biogeochemical response.

In 1994, Farooq Azam and his colleagues wrote: "…a fundamental challenge for [oceanographers in] the future is to understand the ecological interactions among the members of the microbial loop, because such interactions form the bases of the biogeochemical fluxes." As I discussed in Chapter 5, parameterizing the interactions and behavior of bacteria is necessary in order to develop accurate carbon cycle models which are needed to predict future trends in earth's carbon dioxide budget in response to natural and anthropogenic environmental changes. In this thesis, I have contributed to our knowledge of microbial interactions in the ocean and laid groundwork for future investigations into the unexplored aspects of microbial interactions, in general, and QS,

in particular, on the ocean's biogeochemical cycles and the variability inherent within them.

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# APPENDIX I:

# INTER-COMPARISON OF EPIBIONT COMMUNITIES ASSOCIATED WITH LABORATORY-CULTIVATED *TRICHODESMIUM* OBTAINED FROM THE CULTURE COLLECTION AT THE WOODS HOLE OCEANOGRAPHIC INSTITUTION

## INTRODUCTION

*Trichodesmium* spp. are of widespread scientific interest because they are significant players in both the marine carbon cycle as well as the nitrogen cycle (see introduction to Chapter 4). Extensive effort has been devoted to isolating individual strains of these organisms and cultivating them in the laboratory for detailed physiological and ecological studies (Waterbury, 1991). While studies of isolated *Trichodesmium* spp. are certainly valuable, their associated heterotrophic epibionts are rarely considered in parallel.

*Trichodesmium* spp. are notoriously difficult to cultivate axenically. *T. erythraeum* strain IMS101 has been successfully cultivated in axenic culture, however, this strain does not currently exist in axenic culture (J. Waterbury, personal communication). The identities of the epibionts which now associate with *T. erythraeum* strain IMS101, as well as the identity of epibionts on strains which have never been axenic, are currently unknown.

It is well known that bacteria in laboratories can exhibit different traits than their relatives in the 'wild' (Palková, 2004). In addition, it is also demonstrated that

members of biofilms can change over time under laboratory conditions. Considering that these epibionts are essential members of *Trichodesmium* consortia, they likely contribute to some of the physiological traits exhibited by '*Trichodesmium*' in the lab; it is therefore important to deduce the identities of these epibionts. As a complement to a field study of the epibiotic bacteria associated with *Trichodesmium* (Chapters 3 and 4), we examined the epibionts associated with three laboratory-cultivated strains of *Trichodesmium* by cultivation dependent and independent approaches. There were two specific aims associated with this study. First, we wished to examine whether or not the epibionts associated with laboratory strains of *Trichodesmium* are similar to those associated with field populations. Second, we were interested to determine whether or not the bacteria that had 'contaminated' the once-axenic *T. erythraeum* strain IMS101 are similar to or different from those present on other strains of *Trichodesmium* housed within the same culture collection.

## METHODS

### Origin of sample strains

*Trichodesmium* spp. were obtained from the Woods Hole Oceanographic Institution culture collection. *Trichodesmium erythraeum* strain K-11#131 was originally isolated from the S. Pacific (27°41'S 162°02'E) in 2007 (J.B. Waterbury). *T. thiebautii* strain H9-4A was originally isolated from the N. Pacific, Station ALOHA (22°45'N 158°00'W) in 2000 (J.B. Waterbury). *T. erythraeum* strain IMS101 was isolated from the Gulf Stream off of the N. Carolina Coast in 1992 (Prufert-Bebout, 1993).

**DNA extraction and subsequent processing**

DNA was extracted, amplified, cloned and sequenced in the same manner as described in Chapter 4 for PT and TT clone libraries. Biomass extracted for DNA was extracted from 40 mL aliquots of 18 day old cultures of *T. erythraeum* strains K-11#131 and IMS101 and *T. thiebautii* strain H9-4A by gentle filtration onto 5 μm polycarbonate filters.

**RESULTS**

*A note on nomenclature: Trichodesmium* strains utilized in this study will be here forth referred to only by their strain designations (e.g. IMS101, K-11#131, or H9-4A).

After chimeric sequences were removed from the clone library datasets, 91, 108, and 116 high-quality sequences remained in K-11#131, H9-4A, and IMS101 clone libraries, respectively (Table 1). According to Good's coverage calculation, this corresponds to 86%, 83%, and 95% coverage, respectively. This result is mirrored in rarefaction curves (Figure 1) which indicate that our datasets approached rarefaction and are likely to generate a reliable profile of the microbial community on each of these *Trichodesmium* strains. As a result of obtaining coverage of less than 100%, the observed richness in our communities is less than that predicted by the Chao1 estimated richness index. We observed 16, 20, and 12 OTUs at the 97 percent sequence identity (PSI) threshold for K-11#131, H9-4A, and IMS101, respectively (Table 1). This is in contrast to 66, 50, and 25 which are predicted to be the actual richness present in these communities. Despite the

greater predicted richness in H9-4A, K-11#131 is associated with a more diverse epibiotic community (as indicated by the Shannon-Wiener index; Table 1). As indicated, these results are strongly dependent on our choice of the 97 PSI threshold; evaluating our clone libraries at 99 or 100 PSI would lead to higher OTUs, richness and diversity indices (Table 1).

**Archaea and Eukarya**

No archaeal or eukaryotic 16S or 18S small subunit ribosomal DNA was amplified employing conserved primers in the PCR with DNA templates derived from either K-11#131, H9-4A or IMS101 community. DNA from a fosmid template clone 4B7 (Group I Crenarchaea) (Stein *et al.*, 1996) and a eukaryotic enrichment produced from seawater collected during HOTS cruise 179 (http://hahana.soest.hawaii.edu/hot/hot_jgofs.html) at Station ALOHA at 25 m were used as positive amplification controls, respectively; all positive controls amplified.

**Epibiotic bacteria identified within clone libraries**

All of the heterotrophic members of the clone libraries were binned at 97 PSI and assigned to a taxonomic group at a class level (Figure 2). The H9-4A epibiont community is composed of 50% Gammaproteobacteria, 27% Alphaproteobacterial, and 1% Deltaproteobacteria. The remaining 22% are composed about 13%, 4%, and 4% Planctomycetes, Sphingobacteria and Flavobacteria, respectively. At a class level, IMS101 is composed of 41% Alphaproteobacteria, 21% Gammaproteobacteria, and 12%

and 26% Flavobacteria and Sphingobacteria. K-11#131 is 59% Gammaproteobacteria, 24% Alphaproteobacteria, 12% and 5% Flavobacteria and Sphingobacteria.

The Gammaproteobacteria associated with K-11#131 and IMS101 are exclusively associated with the order Alteromonadales. All but six of the K-11#131 Alteromonadales can be assigned with high confidence (greater than 80% bootstrap value according the the RDP II automatic classifier) to the genus *Alteromonas*. All of the IMS101 Alteromondales can be assigned to *Alteromonas* with high confidence. On the other hand, the single clone which clusters near the Alteromonadales in the H9-4A library only does so with 50% confidence. In fact, 95% (21 of 22) of the gammaproteobacterial clones associated with H9-4A are related to the order Thiotrichales with high confidence. Of those, all but one are members of the genus *Methylophaga*. The similarity between the K-11#131 and IMS101 clone libraries, as well as the contrast between these two libraries and H9-4A is evident when the sequences are viewed in a phylogenetic tree (Figure 4).

The Alphaproteobacteria associated with K-11#131 cluster near the order Rhizobiales with low bootstrap confidence (individual sequences ~40-45%). Indeed, these sequences (K-11#131_097, 098, 102, 092, 087, 093, 094, 099, 086, 088, 089, 090, 091, and 100) group together in a phylogenetic tree with long branch lengths separating them from their nearest neighbors (Figure 3). On the other hand, all but five of the Alphaproteobacteria associated with H9-4A are very strongly associated with the Rhodobacterales and can in fact be assigned to the family Rhodobacteraceae with greater than 80% confidence. Nine of the IMS101 clones can be assigned to the Rhodobacterales

(and Rhodobacteraceae) with greater than 80% confidence and a single clone can be assigned to the Caulobacterales with 100% confidence (genus *Maricaulis*, 100% confidence). Four clones within the IMS101 library are distantly related to the Rhodobacterales (IMS101_054, 092, 039, and 064) and cluster together with the five H9-4A clones which did not cluster strongly with the Rhodobacterales (H9-4A_112, 071, 051, 082, and 012; Figure 3).

None of the members of the Bacteroidetes (Flavobacteriales and Sphingobacterales) within the K-11#131 could be assigned to a family with greater than 80% confidence. In fact, only the sequences labeled in Figure 2 as "Flavobacteriales" could be assigned to the Bacteroidetes with such confidence. In may be more appropriate to consider the K-11#131 clone sequences provisionally assigned to Flavobacteriales and Sphingobacterales in Figure 2 and represented in Figure 5 as "unclassified bacteria". In contrast, the Bacteroidetes in the IMS101 and H9-4A clone libraries can be confidently assigned to taxa down to the family level. The two H9-4A Sphingobacterales sequences can be further assigned to the family Flammeovirgaceae as can one clone within the IMS101 library. IMS101 contains four additional Sphingobacterales clones which can be confidently categorized as members of the family Chitinophageaceae. Both Flavobacteriales clone in the H9-4A library can be further categorized as Flavobacteraceae as can the four IMS101 Flavobacteriales clones.

The H9-4A epibionts classified as Planctomycetes (six clones) and Deltaproteobacteria (one clone) in Figure 2 are classified with low bootstrap confidence. While the deltaproteobacterial clone is similar (at 99 PSI) to other sequences in GenBank,

the planctomycetes share only 85 PSI with their nearest relatives. The deltaproteobacterial clone shows high similarity with a clone derived from the burrow wall of a polychaete worm (accession number FJ752802) as well as an isolate of a marine sponge (accession number EU346460)

**Epibiotic bacteria cultivated from *Trichodesmium***

In total, thirty-five bacteria were isolated from laboratory cultivated *Trichodesmium.* Seventeen cultivars are derived from IMS101 and ten and eight are derived from K-11#131 and H9-4A, respectively. Twenty-four of the cultivars are members of the Gammaproteobacteria, ten are members of the Alphaproteobacteria, and one is a member of the Bacteroidetes.

All of the alphaproteobacterial cultivars were isolated from IMS101. Six of the ten are of the genus *Erythrobacter* and four are members of *Maricaulis*. Classifaction of the gammaproteobacterial isolates is less straightforward. All but one of the H9-4A cultivars (A001, A004, A594, A005, A006, A002 and A003) share only 86-88 PSI with their nearest relative in GenBank. When these cultivars are placed in a phylogentic tree with other sequences from this study as well as their nearest relatives in GenBank, they stand out quite dramatically as a cluster with a long branch length (Figure 3). Several cultivars from IMS101 also fall within this cluster (A017, A607, A603, A605, A606 and A608). None of the cultivars from K-11#131 are phylogenetically similar to this cluster. All of the gammaproteobacterial cultivars isolated from K-11#131 are members of the

*Alteromonas*. One additional cultivar was isolated from H9-4A and is a member of the *Methylophaga*.

The one Bacteroidetes isolate was cultivated from IMS101 and is a member of the Flavobacteriales. In contrast to the isolates retrieved from field strains of *Trichodesmium* (Chapter 3), no Actinobacteria were isolated.

**Representation of cultivated strains within clone libraries**

In contrast to the results of our field sampling study (Chapters 3 and 4) in which no cultivars were similar to 97 PSI OTUs represented in clone libraries generated from samples at the same site, in this study, we see some overlap between 16S rRNA gene sequences extracted from cultivars and represented in clone libraries derived from the same cultured strains of *Trichodesmium* as is evident in Figures 3, 4, and 5. For example, cultivar A593_H9-4A clusters very tightly with *Methylophaga* clones retrieved from the H9-4A clone library. Similarly, A604_K-11#131, A599_K-11#131 and A595_K-11#131 cluster tightly with *Alteromonas* clones retrieved from K-11#131 and IMS101 clone libraries. Members of the genus *Maricaulis* are represented in the clone library and cultivars of IMS101. Interestingly, two isolates derived from *Trichodesmium* collected at the BATS site (A484_BATS and A485_BATS; Chapter 3) cluster tightly with the *Alteromonas* isolates and clones from K-11#131 and IMS101 although no *Alteromonas* were present in either clone library derived from samples collected at BATS.

## DISCUSSION

Epibiotic bacteria associated with *Trichodesmium* are postulated to exist in a mutualistic or even symbiotic association in which bacteria might detoxify waste products of *Trichodesmium* (Paerl & Millie, 1996) or contribute essential growth factors (Paerl & Millie, 1996). Many eukaryotic algae have external vitamin requirements, for example, many require an exogenous source of vitamin B12 (Croft *et al.*, 2005). Croft et al. (2005) specifically addressed the B12 requirements of eukaryotic algae, they detected a B12 requirement in eight different algal phyla, and 171 species overall, suggesting that a common role of epiphytic bacteria is the supply of this vitamin. Much less work has been done to address the vitamin B12 requirements of cyanobacteria, but there is limited evidence to suggest that several genera of cyanobacteria do require exogenous sources of this vitamin (Pintner & Provasoli, 1958; Baalen, 1961; Burkholder, 1963). While the genetic pathway for B12 synthesis appears to be intact in the genome of *T. erythraeum* strain IMS101, little is known about B12 requirements of other *Trichodesmium* spp. (T. mincer, personal communication). The goal here is not to argue that the epibionts associated with *Trichodesmium* are supplying vitamin B12 in particular, but to argue that there is ample precedent for the supply of essential growth factors by epibionts to their algal host. No work has been done addressing the exchange of specific metabolites between *Trichodesmium* and its associated epibionts, but it is likely that they do supply *Trichodesmium* with essential growth factors. In addition to possibly supplying metabolites to *Trichodesmium,* attached-bacteria may play a role in iron acquisition (Barbeau *et al.*, 2010) or the maintenance of an appropriate redox environment for

*Trichodesmium* (Paerl *et al.*, 1989). Differences in the community structure should be expected to affect the physiological state and ecological function of the *Trichodesmium* colony.

It is evident from a cursory examination of Figures 3, 4, and 5, that K-11#131 and H9-4A host entirely different microbial populations. In fact, there exist no overlapping OTUs at the 97 PSI threshold. This is perhaps not unexpected; K-11#131 and H9-4A are different species of *Trichodesmium* and they were isolated from different parts of the Pacific Ocean during different years. What is unexpected is that IMS101, which is the same species as K-11#131 but was isolated from a separate ocean basin, would share many of the same 97 PSI OTUs with both K-11#131 and H9-4A. As discussed earlier, IMS101 was formerly axenic and housed within the same culture collections as K-11#131 and H9-4A. It is therefore likely that the 'contaminating' heterotrophs derive not only from K-11#131 and H9-4A, but others within the collection. It would be interesting to compare the epibionts associated with the model organisms, IMS101, from several laboratories in which it is studied. We hypothesize that they will exhibit different epibiont profiles which will reflect the other strains in the same culture collection.

In Chapter 4 of this thesis, two additional clone libraries, derived from aggregated field populations of *T. thiebautii* are described. A broad comparison between these field samples and H9-4A is interesting, especially since the cyanobacteria associated with the PT library are exclusively *T. thiebautii*. 95% of the gammaproteobacterial clones associated with H9-4A are categorized by RDP II as members of the Thiotrichales with very high confidence. Interestingly, the gammaproteobacterial clones associated with the

PT-library also cluster nearest to Thiotrichales, albeit with very low bootstrap confidence. 66% of the PT Alphaproteobacteria are members of the Rhodobacterales while 100% of H9-4A Alphaproteobacteria cluster with the Rhodobacterales. Both libraries contain member of the Flavobacteriales (further classified as Flavobacteraceae). Some major differences do exist; The PT library contains members of the Chloroflexi and Verrucomicrobia and contains no Planctomyces, which are present in the H9-4A library. In addition, even the Thiotrichales-like clones, Rhodobacterales clones, and Flavobacteriales clones differ between the two libraries at finer taxonomic designations. These differences may be important when and if results of studies of H9-4A are extrapolated to field populations of *T. thiebautii.*

The communities associated with these clone libraries are, broadly speaking, similar to other surface associated communities. They are largely composed of Bacteroidetes, Alpha- and Gammaproteobacteria, as well as a few Planctomycees. However, consistent with the results presented in Chapter 4, the *Trichodesmium* spp. strains examined in this study are quite unlike other organisms which are represented in public databases (GenBank, based on a comparison of the 16S rRNA gene). It is extremely exciting, despite the relatively small scale of the cultivation effort undertaken in this study, that some 'novel' organisms are present in the culture collection. With these epibionts in culture, we can evaluate them for novel metabolisms and explore their role in the epibiotic community.

The most unusual 16S sequences among cultivars are associated with H9-4A and IMS101 and are classified as Gammaproteobacteria, although they only share 86-88 PSI

with known gammaproteobacterial 16S rRNA genes. H9-4A also hosts some of the more unusual cloned rRNA genes detected in this study; of particular note are unusual Planctomyces-like sequences.

The epibiont community associated with H9-4A has a number of unusual features, and it will be interesting to investigate some of these features in subsequent studies. One feature of particular note is high percentage of the gammaproteobacterial clones associated with H9-4A which are very similar to cultivated strains of *Methylophaga marina* (99 PSI or better). Fortuitously, an isolate with a 16S rRNA gene identical to these clones and *Methylophaga marina* (accession number X95459) was isolated. While the exchange of organic carbon and fixed nitrogen between cyanobacteria and heterotrophic bacteria is well-documented (Herbst & Overbeck, 1978; Behrens *et al.*, 2008), these exchanges have never been studied between *Trichodesmium* and its epibionts nor has the exchange of specific carbon-compounds been investigated. *Methylophaga* has been isolated in association with eukaryotic algae in the ocean (Janvier *et al.*, 1985; Neufeld *et al.*, 2008). Interest in these bacteria is driven by the possibility that the production of methanol in the ocean may contribute significantly to the atmospheric methanol inventory (Heikes *et al.*, 2002). H9-4A and associated *Methylophaga* may well serve as a model system in which to study the exchange of a specific metabolite between *Trichodesmium* and its epibionts as well as the release of methanol from an algal-bacterial consortia in the ocean. *Methylophaga* species, *M. marina* in particular, may be well suited for such a targeted study because they are only capable of growth on a limited number of organic carbon substrates (Janvier *et al.*, 1985).

Not all of the unusual 16S rRNA gene sequences represented within the clone libraries generated in this study are matched by cultivars in the culture collection (e.g., the Planctomyces-like sequences described above). However, the original consortia from which they derive is in culture. This affords us the possibility of probing the ecological function of these taxa using culture independent means and/or extending our cultivation effort with a more targeted approach.

The presence of numerous novel organisms, both represented in the clone libraries and culture collection, reaffirms the result presented in Chapter 4; *Trichodesmium* spp. are capable of supporting organisms which have not yet been observed in the laboratory. From this result we may infer that *Trichodesmium* offers a unique (unexplored) environment to microbes in pelagic marine environments.

The results of this study highlight the fact that what we call '*Trichodesmium*' is not one organism but it is a consortial community whose structure is highly variable depending on the environmental source of the colony and perhaps the laboratory environment in which it is cultivated; the ecology of the consortial community is likely to depend on its members and thus may also be variable. While experiments with laboratory cultivated strains are essential and have provided much of our insight into the role of *Trichodesmium* in the oceans, when we extrapolate the results of studies performed with IMS101 or H9-4A (or other model organisms) to *T. erythraeum* or *T. thiebautii* in the ocean, the composition of the heterotrophic community should be of greater consideration.

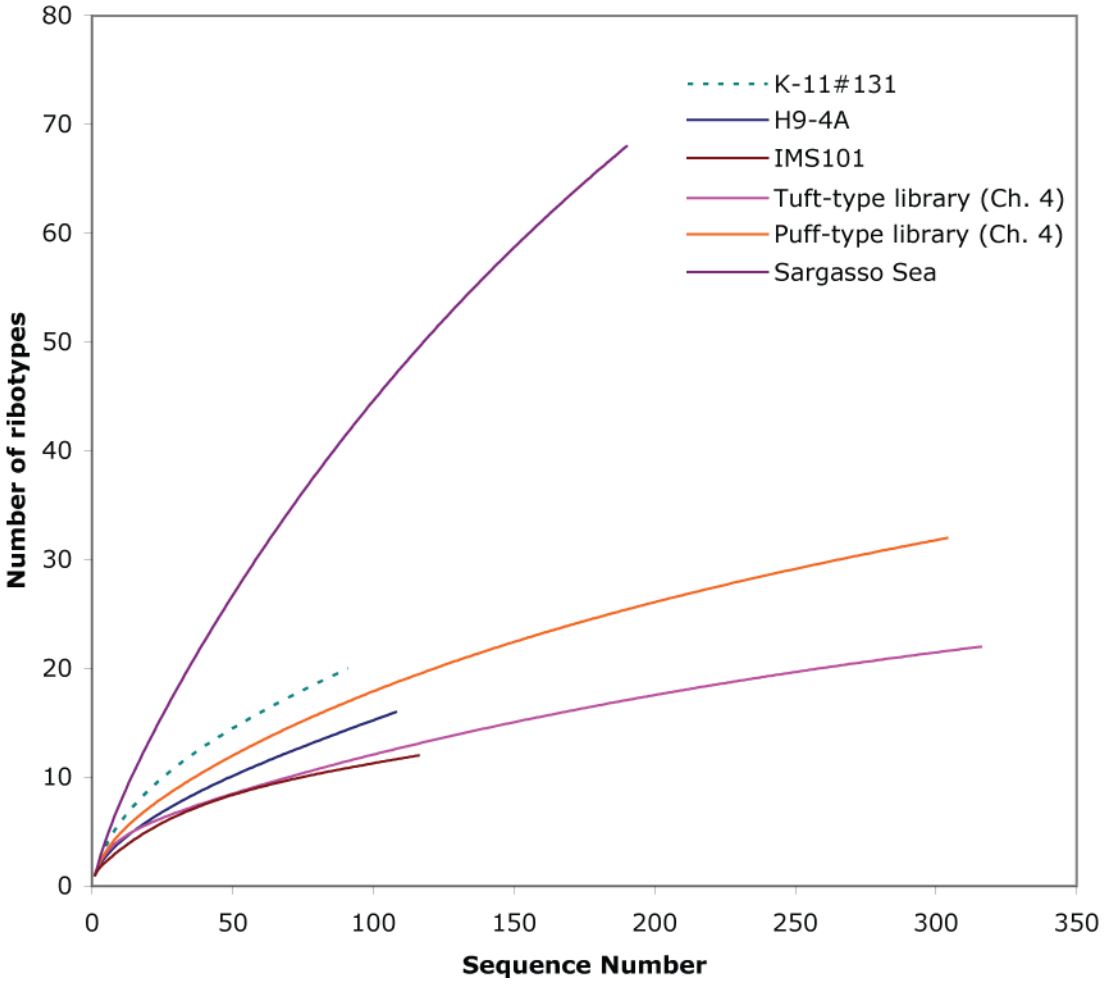



**Figure 1.** Rarefaction curve containing IMS101, K-11#131, and H9-4A as well as datasets discussed in Chapter 4 for reference. Calculations were performed at 97 PSI by the FastGroup II program (97 PSI 'with gaps' algorithm, sequences were manually trimmed in ARB prior to submission to FastGroup II).

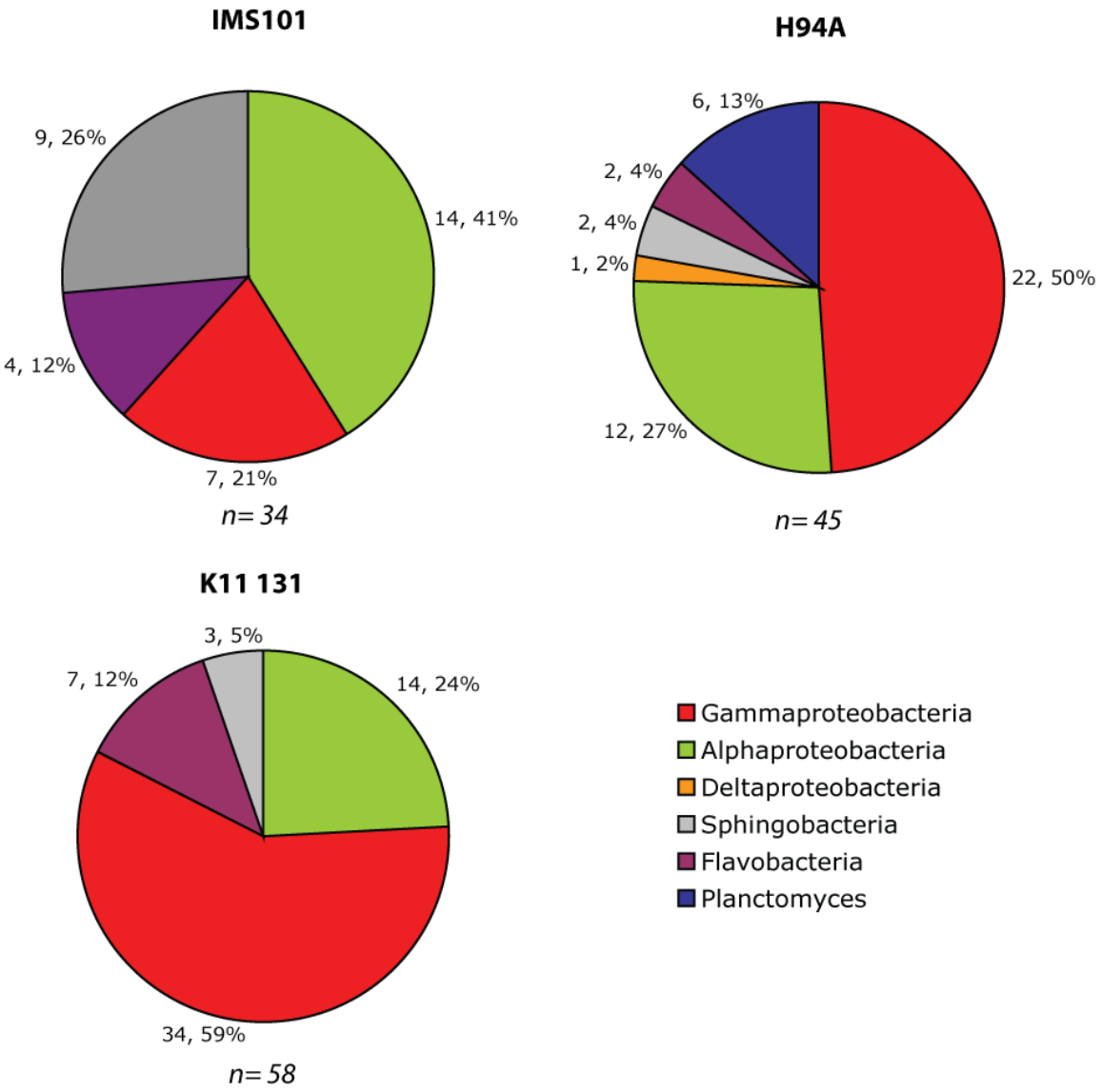



**Figure 2.** Class level comparison of K11 131, H9-4A, and IMS101 at 97 PSI. Values listed adjacent to each 'wedge' of the pie chart provide the number of clones in that category, followed by the percentage of the total non-cyanobacterial clones recovered. The number of non-cyanobacterial clones recovered is listed underneath each chart.

**Figure 3.** Phylogenetic tree containing all alphaproteobacterial clones and isolates generated in this study, as well as reference sequences. This tree was created using Neighbor Joining algorithm in ARB and bootstrapped 1000 times using the neighbor joining algorithm in Phylip. Asterisks indicate bootstrap values of 70% or better. Sequences generated in this study are indicated by boldface type. Accession numbers are in parentheses following all sequences.

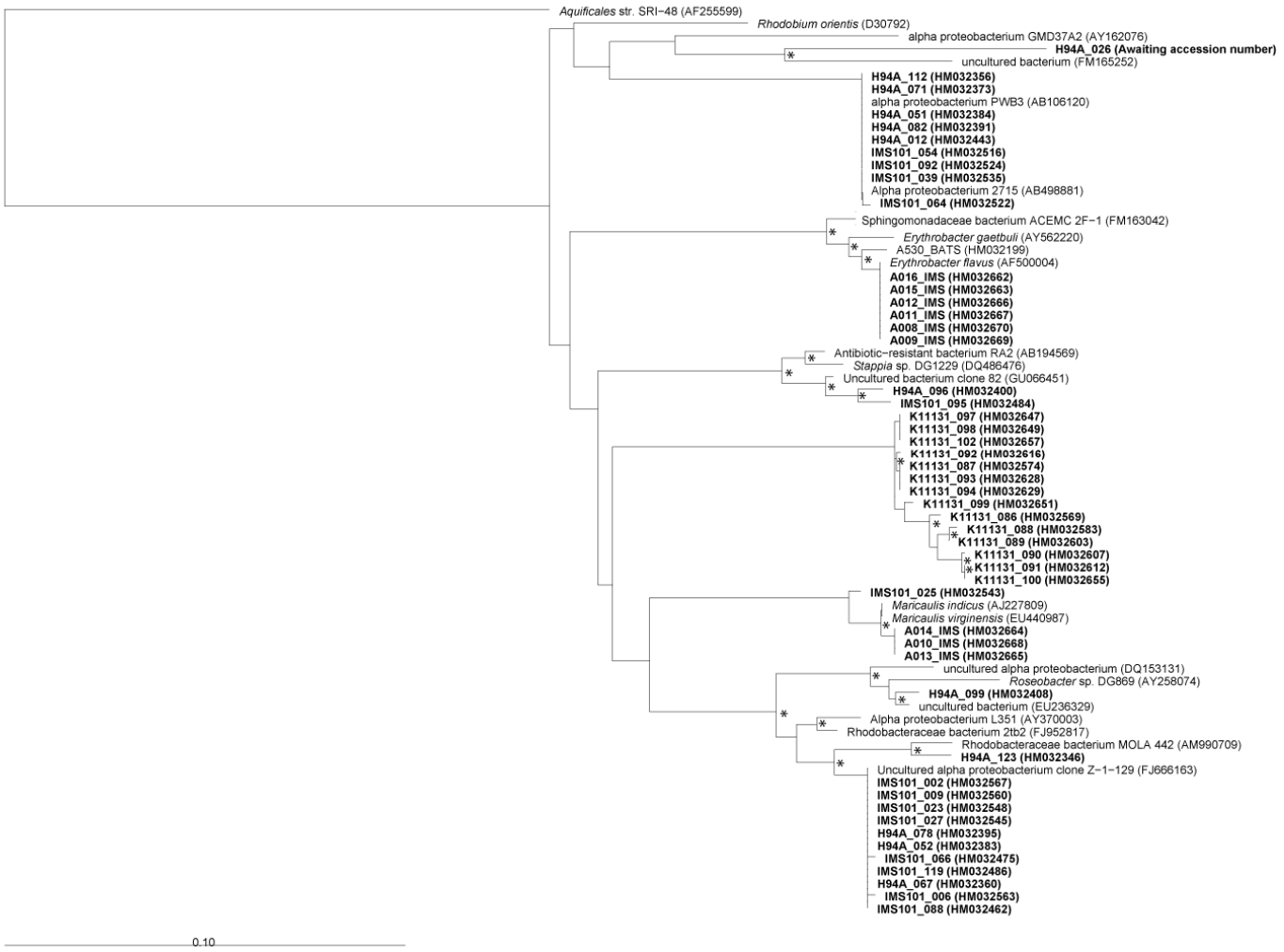

Aquificales str. SRI−48 (AF255599)
Rhodobium orientis (D30792)
alpha proteobacterium GMD37A2 (AY162076)
H94A_026 (Awaiting accession number)
uncultured bacterium (FM165252)
H94A_112 (HM032356)
H94A_071 (HM032373)
alpha proteobacterium PWB3 (AB106120)
H94A_051 (HM032384)
H94A_082 (HM032391)
H94A_012 (HM032443)
IMS101_054 (HM032516)
IMS101_092 (HM032524)
IMS101_039 (HM032535)
Alpha proteobacterium 2715 (AB498881)
IMS101_064 (HM032522)
Sphingomonadaceae bacterium ACEMC 2F−1 (FM163042)
Erythrobacter gaetbuli (AY562220)
A530_BATS (HM032199)
Erythrobacter flavus (AF500004)
A016_IMS (HM032662)
A015_IMS (HM032663)
A012_IMS (HM032666)
A011_IMS (HM032667)
A008_IMS (HM032670)
A009_IMS (HM032669)
Antibiotic−resistant bacterium RA2 (AB194569)
Stappia sp. DG1229 (DQ486476)
Uncultured bacterium clone 82 (GU066451)
H94A_096 (HM032400)
IMS101_095 (HM032484)
K11131_097 (HM032647)
K11131_098 (HM032649)
K11131_102 (HM032657)
K11131_092 (HM032616)
K11131_087 (HM032574)
K11131_093 (HM032628)
K11131_094 (HM032629)
K11131_099 (HM032651)
K11131_086 (HM032569)
K11131_088 (HM032583)
K11131_089 (HM032603)
K11131_090 (HM032607)
K11131_091 (HM032612)
K11131_100 (HM032655)
IMS101_025 (HM032543)
Maricaulis indicus (AJ227809)
Maricaulis virginensis (EU440987)
A014_IMS (HM032664)
A010_IMS (HM032668)
A013_IMS (HM032665)
uncultured alpha proteobacterium (DQ153131)
Roseobacter sp. DG869 (AY258074)
H94A_099 (HM032408)
uncultured bacterium (EU236329)
Alpha proteobacterium L351 (AY370003)
Rhodobacteraceae bacterium 2tb2 (FJ952817)
Rhodobacteraceae bacterium MOLA 442 (AM990709)
H94A_123 (HM032346)
Uncultured alpha proteobacterium clone Z−1−129 (FJ666163)
IMS101_002 (HM032567)
IMS101_009 (HM032560)
IMS101_023 (HM032548)
IMS101_027 (HM032545)
H94A_078 (HM032395)
H94A_052 (HM032383)
IMS101_066 (HM032475)
IMS101_119 (HM032486)
H94A_067 (HM032360)
IMS101_006 (HM032563)
IMS101_088 (HM032462)
0.10

**Figure 4.** Phylogenetic tree containing all gammaproteobacterial clones and isolates generated in this study, as well as reference sequences. This tree was created using Neighbor Joining algorithm in ARB and bootstrapped 1000 times using the neighbor joining algorithm in Phylip. Asterisks indicate bootstrap values of 60% or better. Sequences generated in this study are indicated by boldface type. Accession numbers are in parentheses following all sequences.

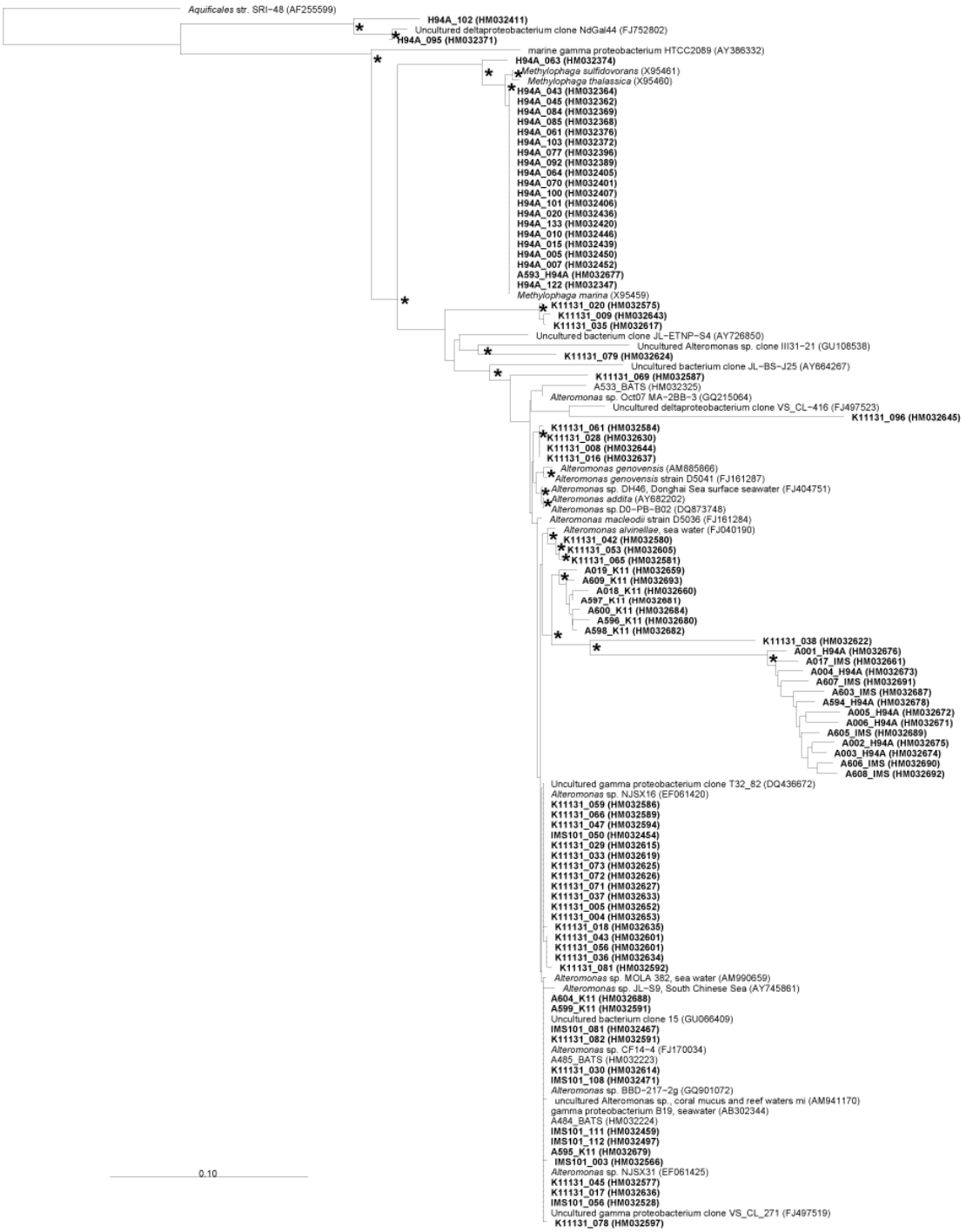
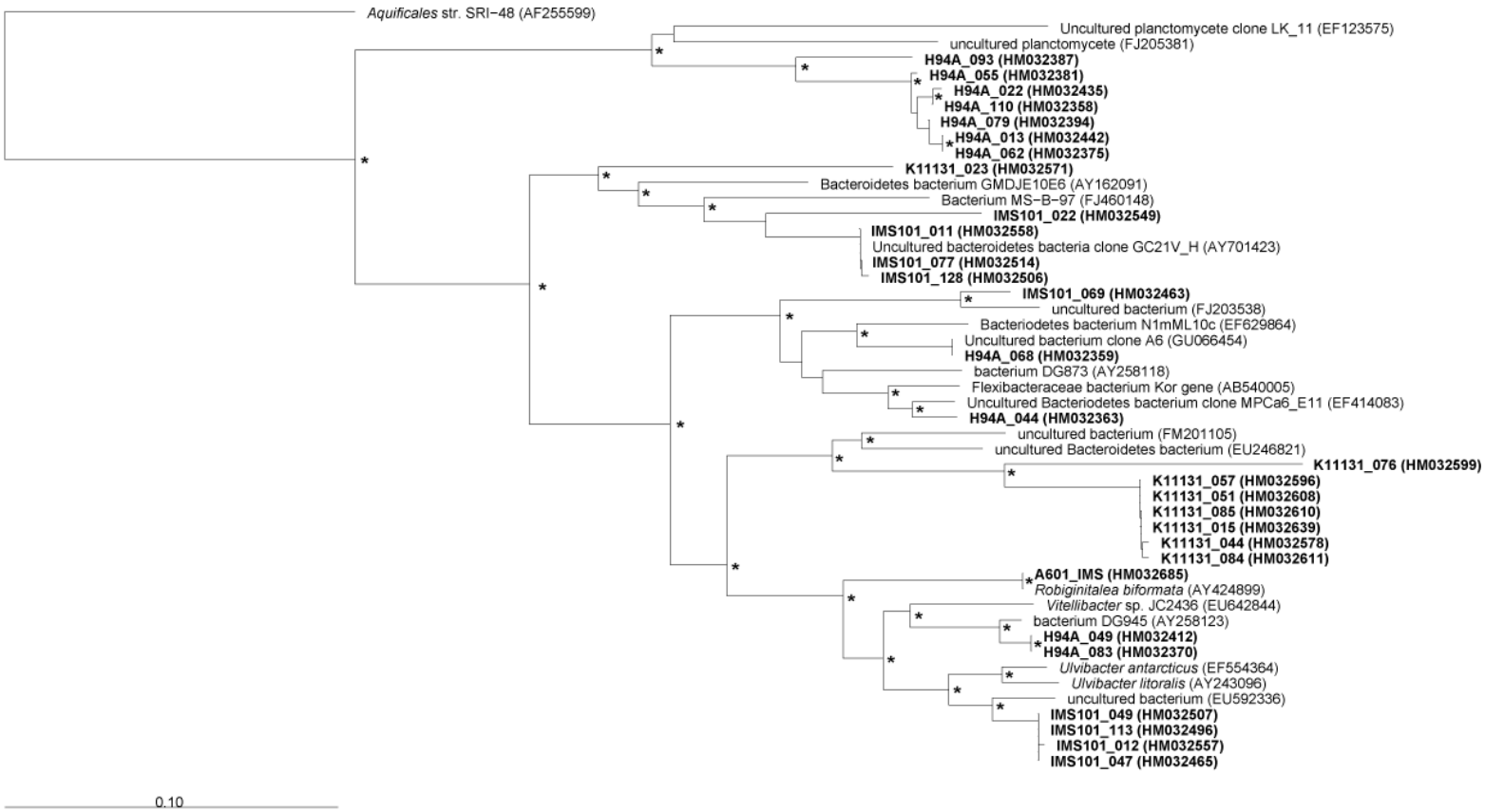

Aquificales str. SRI−48 (AF255599)
H94A_102 (HM032411)
Uncultured deltaproteobacterium clone NdGal44 (FJ752802)
H94A_095 (HM032371)
marine gamma proteobacterium HTCC2089 (AY386332)
H94A_063 (HM032374)
Methylophaga sulfidovorans (X95461)
Methylophaga thalassica (X95460)
H94A_043 (HM032364)
H94A_045 (HM032362)
H94A_084 (HM032369)
H94A_085 (HM032368)
H94A_061 (HM032376)
H94A_103 (HM032372)
H94A_077 (HM032396)
H94A_092 (HM032389)
H94A_064 (HM032405)
H94A_070 (HM032401)
H94A_100 (HM032407)
H94A_101 (HM032406)
H94A_020 (HM032436)
H94A_133 (HM032420)
H94A_010 (HM032446)
H94A_015 (HM032439)
H94A_005 (HM032450)
H94A_007 (HM032452)
A593_H94A (HM032677)
H94A_122 (HM032347)
Methylophaga marina (X95459)
K11131_020 (HM032575)
K11131_009 (HM032643)
K11131_035 (HM032617)
Uncultured bacterium clone JL−ETNP−S4 (AY726850)
Uncultured Alteromonas sp. clone III31−21 (GU108538)
K11131_079 (HM032624)
Uncultured bacterium clone JL−BS−J25 (AY664267)
K11131_069 (HM032587)
A533_BATS (HM032325)
Alteromonas sp. Oct07 MA−2BB−3 (GQ215064)
Uncultured deltaproteobacterium clone VS_CL−416 (FJ497523)
K11131_096 (HM032645)
K11131_061 (HM032584)
K11131_028 (HM032630)
K11131_008 (HM032644)
K11131_016 (HM032637)
Alteromonas genovensis (AM885866)
Alteromonas genovensis strain D5041 (FJ161287)
Alteromonas sp. DH46, Donghai Sea surface seawater (FJ404751)
Alteromonas addita (AY682202)
Alteromonas sp.D0−PB−B02 (DQ873748)
Alteromonas macleodii strain D5036 (FJ161284)
Alteromonas alvinellae, sea water (FJ040190)
K11131_042 (HM032580)
K11131_053 (HM032605)
K11131_065 (HM032581)
A019_K11 (HM032659)
A609_K11 (HM032693)
A018_K11 (HM032660)
A597_K11 (HM032681)
A600_K11 (HM032684)
A596_K11 (HM032680)
A598_K11 (HM032682)
K11131_038 (HM032622)
A001_H94A (HM032676)
A017_IMS (HM032661)
A004_H94A (HM032673)
A607_IMS (HM032691)
A603_IMS (HM032687)
A594_H94A (HM032678)
A005_H94A (HM032672)
A006_H94A (HM032671)
A605_IMS (HM032689)
A002_H94A (HM032675)
A003_H94A (HM032674)
A606_IMS (HM032690)
A608_IMS (HM032692)
Uncultured gamma proteobacterium clone T32_82 (DQ436672)
Alteromonas sp. NJSX16 (EF061420)
K11131_059 (HM032586)
K11131_066 (HM032589)
K11131_047 (HM032594)
IMS101_050 (HM032454)
K11131_029 (HM032615)
K11131_033 (HM032619)
K11131_073 (HM032625)
K11131_072 (HM032626)
K11131_071 (HM032627)
K11131_037 (HM032633)
K11131_005 (HM032652)
K11131_004 (HM032653)
K11131_018 (HM032635)
K11131_043 (HM032601)
K11131_056 (HM032601)
K11131_036 (HM032634)
K11131_081 (HM032592)
Alteromonas sp. MOLA 382, sea water (AM990659)
Alteromonas sp. JL−S9, South Chinese Sea (AY745861)
A604_K11 (HM032688)
A599_K11 (HM032591)
Uncultured bacterium clone 15 (GU066409)
IMS101_081 (HM032467)
K11131_082 (HM032591)
Alteromonas sp. CF14−4 (FJ170034)
A485_BATS (HM032223)
K11131_030 (HM032614)
IMS101_108 (HM032471)
Alteromonas sp. BBD−217−2g (GQ901072)
uncultured Alteromonas sp., coral mucus and reef waters mi (AM941170)
gamma proteobacterium B19, seawater (AB302344)
A484_BATS (HM032224)
IMS101_111 (HM032459)
IMS101_112 (HM032497)
A595_K11 (HM032679)
IMS101_003 (HM032566)
Alteromonas sp. NJSX31 (EF061425)
K11131_045 (HM032577)
K11131_017 (HM032636)
IMS101_056 (HM032528)
Uncultured gamma proteobacterium clone VS_CL_271 (FJ497519)
K11131_078 (HM032597)
0.10

**Figure 5.** Phylogenetic tree containing all clones and isolates not assigned to the proteobacteria generated in this study, as well as reference sequences. This tree was created using Neighbor Joining algorithm in ARB and bootstrapped 1000 times using the neighbor joining algorithm in Phylip. Asterisks indicate bootstrap values of 70% or better. Sequences generated in this study are indicated by boldface type. Accession numbers are in parentheses following all sequences.


*Aquificales* str. SRI−48 (AF255599)
Uncultured planctomycete clone LK_11 (EF123575)
uncultured planctomycete (FJ205381)
**H94A_093 (HM032387)**
**H94A_055 (HM032381)**
**H94A_022 (HM032435)**
**H94A_110 (HM032358)**
**H94A_079 (HM032394)**
**H94A_013 (HM032442)**
**H94A_062 (HM032375)**
**K11131_023 (HM032571)**
Bacteroidetes bacterium GMDJE10E6 (AY162091)
Bacterium MS−B−97 (FJ460148)
**IMS101_022 (HM032549)**
**IMS101_011 (HM032558)**
Uncultured bacteroidetes bacteria clone GC21V_H (AY701423)
**IMS101_077 (HM032514)**
**IMS101_128 (HM032506)**
**IMS101_069 (HM032463)**
uncultured bacterium (FJ203538)
Bacteriodetes bacterium N1mML10c (EF629864)
Uncultured bacterium clone A6 (GU066454)
**H94A_068 (HM032359)**
bacterium DG873 (AY258118)
Flexibacteraceae bacterium Kor gene (AB540005)
Uncultured Bacteriodetes bacterium clone MPCa6_E11 (EF414083)
**H94A_044 (HM032363)**
uncultured bacterium (FM201105)
uncultured Bacteroidetes bacterium (EU246821)
**K11131_076 (HM032599)**
**K11131_057 (HM032596)**
**K11131_051 (HM032608)**
**K11131_085 (HM032610)**
**K11131_015 (HM032639)**
**K11131_044 (HM032578)**
**K11131_084 (HM032611)**
**A601_IMS (HM032685)**
*Robiginitalea biformata* (AY424899)
*Vitellibacter* sp. JC2436 (EU642844)
bacterium DG945 (AY258123)
**H94A_049 (HM032412)**
**H94A_083 (HM032370)**
*Ulvibacter antarcticus* (EF554364)
*Ulvibacter litoralis* (AY243096)
uncultured bacterium (EU592336)
**IMS101_049 (HM032507)**
**IMS101_113 (HM032496)**
**IMS101_012 (HM032557)**
**IMS101_047 (HM032465)**
0.10

| | Percent similiarity | no clones analyzed | OTUs | Richness Index Chao1 | Diversity Insex Shannon-Wiener |
|---|---|---|---|---|---|
| | 100 | 108 | 20 | 118 | 1.7 |
| H9-4A | 99 | 108 | 17 | 78 | 1.6 |
| | 97 | 108 | 16 | 66 | 1.5 |
| | | | | | |
| | 100 | 91 | 36 | 94 | 2.9 |
| K-11#131 | 99 | 91 | 25 | 58 | 2.5 |
| | 97 | 91 | 20 | 50 | 2.2 |
| | | | | | |
| | 100 | 116 | 35 | 427 | 2.1 |
| IMS101 | 99 | 116 | 13 | 31 | 1.2 |
| | 97 | 116 | 12 | 25 | 1.1 |

**Table 1.** Statistical description of K-11#131, IMS101 and H9-4A clone libraries. OTUs, the Chao1 richness index, and the Shannon-Wiener diversity index were all calculated using the FastGroup II website which is described in the Methods. The number of OTUs and the values of the Chao1 and Shannon-Wiener indices are calculated at the 97, 99, and 100 percent sequence identity threshold (PSI). The results of the 97 PSI analysis are described in detail in the text.

**Table 2.** Nearest GenBank neighbors of 97 PSI OTUs present within 16S rDNA clone libraries constructed from DNA extracted from K-11#131 *Trichodesmium* colonies. In cases where the nearest neighbor is an uncultivated organism, the nearest cultivated organism is included as well.

| Clone ID *(K11131_)* | Accession no. | Sequence ID of nearest BLAST match | Accession no. (BLAST match) | PSI | Number of bases compared | Bit score | Number of clones recovered |
|---|---|---|---|---|---|---|---|
| 012 | HM032641 | *Trichodesmium erythraeum* IMS101 | CP000393 | 99 | 539 | 989 | 1 |
| 055 | HM032602 | *Trichodesmium erythraeum* IMS101 | CP000393 | 99 | 539 | 979 | 29 |
| 069 | HM032587 | Uncultured bacterium clone JL-BS-J25 16S ribosomal RNA gene | AY664267 | 90 | 552 | 710 | 1 |
| 086 | HM032569 | Uncultured bacterium clone Mann16S_E05 16S ribosomal RNA gene | FJ952687 | 90 | 743 | 979 | 5 |
| 086 | HM032569 | *Woodsholensia maritima* partial 16S rRNA gene, strain CM251 | AJ578477 | 90 | 691 | 896 | |
| 092 | HM032616 | Uncultured bacterium clone Mann16S_E05 16S ribosomal RNA gene | FJ952687 | 91 | 736 | 1011 | 9 |
| 092 | HM032616 | *Woodsholensia maritima* partial 16S rRNA gene, strain CM251 | AJ578477 | 91 | 690 | 922 | |
| 017 | HM032636 | *Alteromonas* sp. BBD-217-2g 16S ribosomal RNA gene, partial sequence | GQ901072 | 99 | 571 | 1048 | 2 |
| 020 | HM032575 | Uncultured gamma proteobacterium clone JL-ETNP-S4 16S ribosomal RNA gene | AY726850 | 98 | 454 | 793 | 3 |
| 020 | HM032575 | *Alteromonas genovensis* strain D5041 16S ribosomal RNA gene | FJ161287 | 98 | 439 | 771 | |
| 030 | HM032614 | Uncultured bacterium clone 15 16S ribosomal RNA gene | GU066409 | 100 | 571 | 1055 | 20 |
| 038 | HM032622 | *Alteromonas* sp. DH46 16S ribosomal RNA gene, partial sequence | FJ404751 | 90 | 574 | 752 | 1 |
| 043 | HM032579 | *Alteromonas* sp. Oct07-MA-2BB-3 16S ribosomal RNA gene | GQ215064 | 97 | 588 | 989 | 4 |
| 047 | HM032594 | Uncultured gamma proteobacterium clone T32_82 16S ribosomal RNA gene | DQ436672 | 100 | 572 | 1057 | 2 |
| 047 | HM032594 | *Alteromonas* sp. CF14-4 16S ribosomal RNA gene | FJ170034 | 99 | 572 | 1051 | |
| 079 | HM032624 | Uncultured *Alteromonas* sp. clone F3C95 16S ribosomal RNA gene | AY936198 | 93 | 567 | 841 | 1 |
| 096 | HM032645 | *Alteromonas* sp. SKUK MB1007 16S ribosomal RNA gene | EU907922 | 87 | 533 | 597 | |
| 023 | HM032571 | Uncultured Bacteroidetes bacterium clone GC21V_H 16S small subunit ribosomal RNA gene | AY701423 | 92 | 455 | 636 | 1 |
| 076 | HM032599 | Uncultured bacterium partial 16S rRNA gene, clone MBR-8_LF_BF99 | FM201105 | 85 | 548 | 540 | 1 |
| 084 | HM032611 | Uncultured Bacteroidetes bacterium clone Cobs2TisF6 16S ribosomal RNA gene | EU246821 | 86 | 584 | 645 | 6 |
| 096 | HM032645 | Uncultured Bacteroidetes bacterium clone GC21V_H 16S small subunit ribosomal RNA gene | AY701423 | 93 | 412 | 606 | 1 |

**Table 3.** Nearest GenBank neighbors of 97 PSI OTUs present within 16S rDNA clone libraries constructed from DNA extracted from H9-4A *Trichodesmium* colonies. In cases where the nearest neighbor is an uncultivated organism, the nearest cultivated organism is included as well.

| Clone ID *(H94A_)* | Accession no. | Sequence ID of nearest BLAST match | Accession no. (BLAST match) | PSI | Number of bases compared | Bit score | Number of clones recovered |
|---|---|---|---|---|---|---|---|
| 066 | HM032403 | *Trichodesmium thiebautii* 16S ribosomal RNA gene | AF013027 | 99 | 564 | 1037 | 63 |
| 012 | HM032443 | Alpha proteobacterium 2715 gene for 16S rRNA | AB498881 | 100 | 563 | 1040 | 5 |
| 026 | | Uncultured bacterium partial 16S rRNA gene, clone V2tb43 | FM165252 | 90 | 583 | 767 | 1 |
| 026 | | Alpha proteobacterium GMD37A2 small subunit ribosomal RNA gene | AY162076 | 95 | 442 | 688 | |
| 067 | HM032360 | Uncultured alpha proteobacterium clone Z-1-129 16S ribosomal RNA gene | FJ666163 | 100 | 563 | 1040 | 3 |
| 067 | HM032360 | Rhodobacteraceae bacterium 2tb2 16S ribosomal RNA gene | FJ952817 | 97 | 563 | 952 | |
| 096 | HM032400 | Uncultured bacterium clone 82 16S ribosomal RNA gene | GU066451 | 98 | 565 | 985 | 1 |
| 096 | HM032400 | Antibiotic-resistant bacterium RA2 gene for 16S rRNA | AB194569 | 96 | 566 | 929 | |
| 099 | HM032408 | Uncultured bacterium clone Hg5a1F11 16S ribosomal RNA gene | EU236329 | 99 | 563 | 1013 | 1 |
| 099 | HM032408 | Marine sponge bacterium FILTER13C237 16S ribosomal RNA gene | EU346412 | 98 | 563 | 996 | |
| 123 | HM032346 | Rhodobacteraceae bacterium MOLA 442 partial 16S rRNA gene | AM990709 | 98 | 563 | 979 | 1 |
| 015 | HM032439 | *Methylophaga marina* mRNA for 16S ribosomal RNA | X95459 | 100 | 602 | 1112 | 19 |
| 093 | HM032387 | Uncultured planctomycete clone II9E 16S ribosomal RNA gene | FJ205381 | 86 | 471 | 499 | 1 |
| 093 | HM032387 | *Alteromonas* sp. D0-PB-B02 16S ribosomal RNA gene | DQ873748 | 80 | 614 | 438 | |
| 095 | HM032371 | Uncultured delta proteobacterium clone NdGal44 16S ribosomal RNA gene | FJ752802 | 99 | 590 | 1074 | 1 |
| 095 | HM032371 | Marine sponge bacterium LIQUIDdw06F03 16S ribosomal RNA gene | EU346460 | 98 | 579 | 1020 | |
| 102 | HM032411 | Uncultured delta proteobacterium clone NdGal44 16S ribosomal RNA gene | FJ752802 | 93 | 594 | 880 | 1 |
| 102 | HM032411 | Marine sponge bacterium LIQUIDdw06F03 16S ribosomal RNA gene | EU346460 | 93 | 583 | 854 | |
| 068 | HM032359 | Uncultured bacterium clone A6 16S ribosomal RNA gene | GU066454 | 100 | 599 | 1107 | 1 |
| 068 | HM032359 | Bacteroidetes bacterium N1mML10c 16S ribosomal RNA gene | EF629864 | 93 | 603 | 902 | |
| 083 | HM032370 | Bacterium DG945 small subunit ribosomal RNA gene | AY258123 | 97 | 600 | 1016 | 2 |
| 044 | HM032363 | Uncultured Bacteroidetes bacterium clone MPCa6_E11 16S ribosomal RNA gene | EF414083 | 97 | 601 | 1035 | 1 |
| 044 | HM032363 | Flexibacteraceae bacterium Kor gene for 16S rRNA | AB540005 | 95 | 603 | 968 | |
| 062 | HM032375 | Uncultured planctomycete clone LK_11 16S ribosomal RNA gene | EF123575 | 85 | 638 | 640 | 6 |

**Table 4**. Nearest GenBank neighbors of 97 PSI OTUs present within 16S rDNA clone libraries constructed from DNA extracted from IMS101 *Trichodesmium* colonies. In cases where the nearest neighbor is an uncultivated organism, the nearest cultivated organism is included as well.

| Clone ID *(IMS101_)* | Accession no. | Sequence ID of nearest BLAST match | Accession no. (BLAST match) | PSI | Number of bases compared | Bit score | Number of clones recovered |
|---|---|---|---|---|---|---|---|
| 001 | HM032568 | *Trichodesmium erythraeum* IMS101, complete genome | CP000393 | 99 | 573 | 1051 | 1 |
| 008 | HM032561 | *Trichodesmium erythraeum* IMS101, complete genome | CP000393 | 100 | 573 | 1059 | 85 |
| 006 | HM032563 | Uncultured alpha proteobacterium clone Z-1-129 16S ribosomal RNA gene | FJ666163 | 99 | 572 | 1051 | 8 |
| 006 | HM032563 | Rhodobacteraceae bacterium 2tb2 16S ribosomal RNA gene | FJ952817 | 97 | 572 | 963 | |
| 025 | HM032543 | *Maricaulis virginensis* strain PR54-12 16S ribosomal RNA gene | EU440987 | 98 | 572 | 1018 | 1 |
| 092 | HM032524 | Alpha proteobacterium PWB3 gene for 16S rRNA | AB106120 | 100 | 572 | 1057 | 4 |
| 095 | HM032484 | Uncultured bacterium clone 82 16S ribosomal RNA gene | GU066451 | 98 | 574 | 1007 | 1 |
| 095 | HM032484 | Antibiotic-resistant bacterium RA2 gene for 16S rRNA | AB194569 | 96 | 575 | 952 | |
| 081 | HM032467 | *Alteromonas* sp. BBD-217-2g 16S ribosomal RNA gene | GQ901072 | 100 | 605 | 1118 | 5 |
| 111 | HM032459 | Uncultured gamma proteobacterium clone VS_CL-271 16S ribosomal RNA gene | FJ497519 | 100 | 606 | 1120 | 2 |
| 111 | HM032459 | *Alteromonas* sp. CF14-4 16S ribosomal RNA gene | FJ170034 | 100 | 606 | 1120 | |
| 022 | HM032549 | Uncultured Bacteroidetes bacterium clone GC21V_H 16S small subunit ribosomal RNA gene | AY701423 | 92 | 611 | 880 | 1 |
| 022 | HM032549 | Bacterium MS-B-97 16S ribosomal RNA gene | FJ460148 | 89 | 560 | 682 | |
| 049 | HM032507 | Uncultured bacterium clone SSW3Ap 16S ribosomal RNA gene | EU592336 | 94 | 611 | 939 | 4 |
| 049 | HM032507 | Ulvibacter antarcticus strain IMCC3101 16S ribosomal RNA gene | EF554364 | 93 | 608 | 911 | |
| 069 | HM032463 | Uncultured bacterium clone SHFH609 16S ribosomal RNA gene | FJ203538 | 94 | 612 | 952 | 1 |
| 069 | HM032463 | Bacterium DG873 small subunit ribosomal RNA gene | AY258118 | 88 | 622 | 739 | |
| 077 | HM032514 | Uncultured Bacteroidetes bacterium clone GC21V_H 16S small subunit ribosomal RNA gene | AY701423 | 100 | 612 | 1131 | 3 |
| 077 | HM032514 | Bacteroidetes bacterium GMDJE10E6 small subunit ribosomal RNA gene | AY162091 | 92 | 618 | 876 | |

**Table 5**. Nearest GenBank neighbors of cultivars isolated from *T. erythraeum* strain K-11#131 colonies.

| Isolate ID | Accession no. | Sequence ID of nearest BLAST match | Accession no. (BLAST match) | PSI | Number of bases compared | Bit score | Lineage |
|---|---|---|---|---|---|---|---|
| A019_K11 | HM032659 | Gamma proteobacterium B19 gene for 16S rRNA, partial sequence | AB302344 | 98 | 626 | 1090 | Bacteria; Proteobacteria; Gammaproteobacteria; Alteromonadales; Alteromonadaceae; Alteromonas. |
| A018_K11 | HM032660 | *Alteromonas* sp. MOLA 382 partial 16S rRNA gene | AM990659 | 97 | 645 | 1120 | Bacteria; Proteobacteria; Gammaproteobacteria; Alteromonadales; Alteromonadaceae; Alteromonas. |
| A595_K11 | HM032679 | *Alteromonas* sp. NJSX31 16S ribosomal RNA gene | EF061425 | 99 | 734 | 1351 | Bacteria; Proteobacteria; Gammaproteobacteria; Alteromonadales; Alteromonadaceae; Alteromonas. |
| A596_K11 | HM032680 | *Alteromonas alvinellae* strain MED76 16S ribosomal RNA gene | AY136113 | 97 | 717 | 1230 | Bacteria; Proteobacteria; Gammaproteobacteria; Alteromonadales; Alteromonadaceae; Alteromonas. |
| A597_K11 | HM032681 | *Alteromonas alvinellae* strain MED76 16S ribosomal RNA gene | AY136113 | 98 | 651 | 1142 | Bacteria; Proteobacteria; Gammaproteobacteria; Alteromonadales; Alteromonadaceae; Alteromonas. |
| A598_K11 | HM032682 | *Alteromonas alvinellae* strain MED76 16S ribosomal RNA gene | AY136113 | 98 | 638 | 1118 | Bacteria; Proteobacteria; Gammaproteobacteria; Alteromonadales; Alteromonadaceae; Alteromonas. |
| A599_K11 | HM032683 | *Alteromonas* sp. NJSX31 16S ribosomal RNA gene | EF061425 | 99 | 739 | 1360 | Bacteria; Proteobacteria; Gammaproteobacteria; Alteromonadales; Alteromonadaceae; Alteromonas. |
| A600_K11 | HM032684 | *Alteromonas alvinellae* strain MED76 16S ribosomal RNA gene | AY136113 | 97 | 675 | 1170 | Bacteria; Proteobacteria; Gammaproteobacteria; Alteromonadales; Alteromonadaceae; Alteromonas. |
| A604_K11 | HM032688 | *Alteromonas* sp. NJSX31 16S ribosomal RNA gene | EF061425 | 99 | 737 | 1356 | Bacteria; Proteobacteria; Gammaproteobacteria; Alteromonadales; Alteromonadaceae; Alteromonas. |
| A609_K11 | HM032693 | *Alteromonas alvinellae* strain MED76 16S ribosomal RNA gene | AY136113 | 97 | 717 | 1242 | Bacteria; Proteobacteria; Gammaproteobacteria; Alteromonadales; Alteromonadaceae; Alteromonas. |

**Table 6**. Nearest GenBank neighbors of cultivars isolated from *T. thiebautii* strain H9-4A colonies.

| Isolate ID | Accession no. | Sequence ID of nearest BLAST match | Accession no. (BLAST match) | PSI | Number of bases compared | Bit score | Lineage |
|---|---|---|---|---|---|---|---|
| A006_H94A | HM032671 | *Alteromonas* sp. CF14-4 16S ribosomal RNA gene | FJ170034 | 87 | 680 | 758 | Bacteria; Proteobacteria; Gammaproteobacteria; Alteromonadales; Alteromonadaceae; Alteromonas. |
| A005_H94A | HM032672 | *Alteromonas* sp. CF14-4 16S ribosomal RNA gene | FJ170034 | 87 | 621 | 695 | Bacteria; Proteobacteria; Gammaproteobacteria; Alteromonadales; Alteromonadaceae; Alteromonas. |
| A004_H94A | HM032673 | *Alteromonas* sp. CF14-4 16S ribosomal RNA gene | FJ170034 | 88 | 668 | 785 | Bacteria; Proteobacteria; Gammaproteobacteria; Alteromonadales; Alteromonadaceae; Alteromonas. |
| A003_H94A | HM032674 | *Alteromonas* sp. CF14-4 16S ribosomal RNA gene | FJ170034 | 87 | 619 | 697 | Bacteria; Proteobacteria; Gammaproteobacteria; Alteromonadales; Alteromonadaceae; Alteromonas. |
| A002_H94A | HM032675 | *Alteromonas* sp. CF14-4 16S ribosomal RNA gene | FJ170034 | 86 | 689 | 767 | Bacteria; Proteobacteria; Gammaproteobacteria; Alteromonadales; Alteromonadaceae; Alteromonas. |
| A001_H94A | HM032676 | *Alteromonas* sp. CF14-4 16S ribosomal RNA gene | FJ170034 | 88 | 690 | 815 | Bacteria; Proteobacteria; Gammaproteobacteria; Alteromonadales; Alteromonadaceae; Alteromonas. |
| A593_H94A | HM032677 | *Methylophaga marina* mRNA for 16S ribosomal RNA | X95459 | 100 | 690 | 1275 | Bacteria; Proteobacteria; Gammaproteobacteria; Thiotrichales;Piscirickettsiaceae; Methylophaga |
| A594_H94A | HM032678 | *Alteromonas* sp. CF14-4 16S ribosomal RNA gene | FJ170034 | 88 | 629 | 739 | Bacteria; Proteobacteria; Gammaproteobacteria; Alteromonadales; Alteromonadaceae; Alteromonas. |

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**Table 7.** (displayed on next 2 pages) Nearest GenBank neighbors of cultivars isolated from *T. thiebautii* strain IMS101.

| Isolate ID | Accession no. | Sequence ID of nearest BLAST match | Accession no. (BLAST match) | PSI | Number of bases compared | Bit score | Lineage |
|---|---|---|---|---|---|---|---|
| A015_IMS | HM032663 | *Erythrobacter flavus* strain D5033 16S ribosomal RNA gene | FJ161282 | 100 | 714 | 1319 | Bacteria; Proteobacteria; Alphaproteobacteria; Sphingomonadales;Erythrobacteraceae; Erythrobacter |
| A014_IMS | HM032664 | *Maricaulis* sp. DNA for 16S ribosomal RNA, strain MCS26 | AJ227809 | 99 | 745 | 1365 | Bacteria; Proteobacteria; Alphaproteobacteria; Rhodobacterales; Hyphomonadaceae; Maricaulis |
| A013_IMS | HM032665 | *Maricaulis* sp. DNA for 16S ribosomal RNA, strain MCS26 | AJ227809 | 99 | 752 | 1378 | Bacteria; Proteobacteria; Alphaproteobacteria; Rhodobacterales; Hyphomonadaceae; Maricaulis |
| A012_IMS | HM032666 | Bacterium daSW.33 16S ribosomal RNA gene | EU935320 | 100 | 716 | 1323 | Bacteria; Proteobacteria; Alphaproteobacteria; Sphingomonadales;Erythrobacteraceae; Erythrobacter |
| A011_IMS | HM032667 | Bacterium daSW.33 16S ribosomal RNA gene | EU935320 | 100 | 761 | 1406 | Bacteria; Proteobacteria; Alphaproteobacteria; Sphingomonadales;Erythrobacteraceae; Erythrobacter |
| A010_IMS | HM032668 | *Maricaulis* sp. DNA for 16S ribosomal RNA, strain MCS26 | AJ227809 | 99 | 736 | 1360 | Bacteria; Proteobacteria; Alphaproteobacteria; Rhodobacterales; Hyphomonadaceae; Maricaulis |
| A009_IMS | HM032669 | *Erythrobacter flavus* strain D5033 16S ribosomal RNA gene | FJ161282 | 100 | 715 | 1321 | Bacteria; Proteobacteria; Alphaproteobacteria; Sphingomonadales;Erythrobacteraceae; Erythrobacter |
| A008_IMS | HM032670 | Bacterium daSW.33 16S ribosomal RNA gene | EU935320 | 100 | 749 | 1384 | Bacteria; Proteobacteria; Alphaproteobacteria; Sphingomonadales;Erythrobacteraceae; Erythrobacter |
| A601_IMS | HM032685 | *Robiginitalea biformata* strain HTCC2501 16S ribosomal RNA gene | AY424899 | 100 | 725 | 1339 | Bacteria; Bacteroidetes; Flavobacteria; Flavobacteriales;Flavobacteriaceae; Robiginitalea |
| A602_IMS | HM032686 | *Maricaulis virginensis* strain PR54-12 16S ribosomal RNA gene | EU440987 | 99 | 616 | 1122 | Bacteria; Proteobacteria; Alphaproteobacteria; Rhodobacterales; Hyphomonadaceae; Maricaulis |
| A603_IMS | HM032687 | *Alteromonas* sp. CF14-4 16S ribosomal RNA gene | FJ170034 | 87 | 630 | 732 | Bacteria; Proteobacteria; Gammaproteobacteria; Alteromonadales; Alteromonadaceae; Alteromonas. |

| Isolate ID | Accession no. | Sequence ID of nearest BLAST match | Accession no. (BLAST match) | PSI | Number of bases compared | Bit score | Lineage |
|---|---|---|---|---|---|---|---|
| A605_IMS | HM032689 | *Alteromonas* sp. CF14-4 16S ribosomal RNA gene | FJ170034 | 88 | 629 | 739 | Bacteria; Proteobacteria; Gammaproteobacteria; Alteromonadales; Alteromonadaceae; Alteromonas. |
| A606_IMS | HM032690 | *Alteromonas* sp. CF14-4 16S ribosomal RNA gene | FJ170034 | 87 | 612 | 706 | Bacteria; Proteobacteria; Gammaproteobacteria; Alteromonadales; Alteromonadaceae; Alteromonas. |
| A607_IMS | HM032691 | *Alteromonas* sp. CF14-4 16S ribosomal RNA gene | FJ170034 | 88 | 623 | 747 | Bacteria; Proteobacteria; Gammaproteobacteria; Alteromonadales; Alteromonadaceae; Alteromonas. |
| A608_IMS | HM032692 | *Alteromonas* sp. CF14-4 16S ribosomal RNA gene | FJ170034 | 87 | 628 | 721 | Bacteria; Proteobacteria; Gammaproteobacteria; Alteromonadales; Alteromonadaceae; Alteromonas. |
| A017_IMS | HM032661 | *Alteromonas* sp. CF14-4 16S ribosomal RNA gene | FJ170034 | 88 | 618 | 736 | Bacteria; Proteobacteria; Gammaproteobacteria; Alteromonadales; Alteromonadaceae; Alteromonas. |
| A016_IMS | HM032662 | Bacterium daSW.33 16S ribosomal RNA gene | EU935320 | 100 | 731 | 1351 | Bacteria; Proteobacteria; Alphaproteobacteria; Sphingomonadales;Erythrobacteraceae; Erythrobacter |

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# APPENDIX 2:

# BIOLUMINESCENCE OF *TRICHODESMIUM* AND AN INVESTIGATION OF QUORUM SENSING BY MEMBERS OF *TRICHODESMIUM* CONSORTIA

## INTRODUCTION

A fortuitous observation that Trichodesmium colonies produce light measurable by a standard luminometer inspired a large portion of this thesis. In the ocean, bacteria and eukaryotes produce light; however, the light produced by bacteria and eukaryotes is quite different (Hastings, 1995). Eukaryotes produce light in pulses where as bacteria emit light continuously (Haas, 1980). To date, all luminescent bacteria regulate their light production by quorum sensing (QS) (Hastings, 1995). Here, we exploited this property of bacterial bioluminescence in order to identify bacterial QS within the phycosphere of *Trichodesmium*.

Eukaryotic bioluminescence is quite common. Dinoflagellates and ctenophores produce light which is easily observable at night in the wake of a ship or at beaches on summer nights (Hastings, 1995). Bacterial luminescence is less visible to the casual observer but is no less common in the ocean. Bacterial endosymbionts produce light in the light organs of numerous marine animals (Hastings, 1995), most famously, the Hawaiian bobtail squid. They have also been implicated as the source of luminescence in the impressive 'Milky Sea' displays which occur most frequently in the Indian Ocean

(Nealson & Hastings, 2006). Finally, bacterial luminescence has been observed on small spatial scales such as on particulate detritus in the ocean (Andrews *et al.*, 1984).

As mentioned, the only known mechanism by which bacteria produce luminescence is via quorum-sensing regulated pathways (Hastings, 1995). Light is produced as a byproduct of a molecular-oxygen dependent enzyme catalyzed aldehyde reduction (Hastings, 1995). The genes for the requisite substrates and enzymes are part of a gene cassette linked to *luxR* (quorum sensing response regulator) in a QS-regulated operon.

The observation of luminescence on *Trichodesmium* led us to postulate the QS was utilized by epibiont bacteria associated with *Trichodesmium* colonies. QS by algal-associated bacteria has been implicated in the bloom-dynamics of other marine algae (Nakashima *et al.*, 2006) and lacustrine cyanobacteria (Braun & Bachofen, 2004), although not yet in association with *Trichodesmium* or any other marine cyanobacterium. However, QS has been observed to occur within cyanobacterial communities associated with stromatolites, although the role of QS is yet unknown (Decho *et al.*, 2009).

The phycosphere of *Trichodesmium* is colonized by extremely high densities of bacteria (Sheridan *et al.*, 2002). We confirmed this by performing scanning electron microscopy and epifluorence microscopy which are described in detail in Chapter 3 of this thesis (see Chapter 3 Figures 1 and 2). In addition to hosting extremely high densities of bacteria, biofilms (such as those which adhere to *Trichodesmium*) are known to be diffusion limited environments as well as having lower pH than their ambient environment (Horswill *et al.*, 2007). These characteristics are all conducive to efficient

QS. In this study, we investigated QS by epibionts of *Trichodesmium* spp. by observing luminescence, evaluating *Trichodesmium* cultures for the production of the QS signal, acylated homoserine lactone (AHL), and performing enzyme activity assays to evaluate the response of the community to exogenous AHL addition.

## METHODS

### Luminescence

Luminescence measurements were conducted with samples of *Trichodesmium* collected at the Bermuda Atlantic Time Series station in the Sargasso Sea (see Chapter 3 and 4 methods for additional details) as well as numerous laboratory cultivated strains of Trichodesmium. The following strains obtained from the Woods Hole Oceanographic Institution culture collection were evaluated for the production of bioluminescence: *T. erythraeum* strains K11131 and IMS101, *T. thiebautii* H94A . Luminescence was measured using a Turner Biosystems (Sunnyvale, CA) 20/20n luminometer.

### Evaluation of culture extracts for AHLs

Batch cultures of Trichodesmium were extracted with organic solvents and analyzed for the presence of AHLs using the methods documented in Chapter 3 of this thesis. Extracts were evaluated by HPLC-LCQ-MS.

### Enzyme activity assays

Enzyme activity was assayed according to the methods introduced in Chapter 5 of this thesis. Details specific to this experiment will be described here. Experiments were performed on eighteen 10mL two-week old batch cultures of *T. erythraeum* strain K11131. Triplicate cultures were amended with either 500 or 5000 nM 3-oxo-C8-HSL, 500 nM 3-oxo-C12 or 500 nM C12-HSL. 3-oxo-C12-HSL and C12-HSL are not readily soluble in water and so were dissolved in DMSO. Triplicate control incubations to which no AHL was added, as well as control incubations to which only DMSO was added, were performed in parallel to the AHL-amended incubations. Amendments were added in 100 µl aliquots to the 10 mL cultures. Incubations were assayed for enzyme activities at one, six, and twenty-four hours as described in Chapter 5.

## RESULTS

### Luminescence

Bioluminescence of 13 individual tuft-colonies were measured at sea. Based on their luminescence values, they could be divided into a low luminescence group (Group 1) and a high luminescence group (Group 2). Group 1 (n=7) had an average luminescence which was 2.3 times that of seawater (32% standard deviation, Table 1). Group 2 (n=6) displayed an average luminescence which was 5.9 times that of seawater (23% standard deviation, Table 1). 8 puff-colonies were measured. On average, they were 2.1 times as luminous as seawater (17% standard deviation, Table 1). The luminescence of seawater

was practically indistinguishable from the background of the instrument at the time of measurement.

Laboratory strains also display luminescence although due to the high concentrations of individual trichomes in the culture media and the smaller size of the colonies, it was difficult to measure individual colonies. Instead, 1 mL from dense cultures were measured. *T. erythraeum* strain IMS101 produced as much as 12 times the luminescence of the intstrument background; *T. erythraeum* strain K11131 produced as much as 13 times the luminescence of the background and *T. thiebautii* produced 5 times the luminescence of background.

**AHLs**

No AHLs were detected in field or batch cultures of Trichodesmium despite extensive efforts.

**Enzyme Assay experiments**

Although incubations were sampled at one, six and twenty-four hours, only the twenty-four hour results will be discussed here. AHLs did not induce enhanced protease, phosphotase, chitinase, α-glucosidase actity or luminescence.

Enhanced lipase and β-glucosidase activities were observed in response to AHL amendments. 3-oxo-C8-HSL (500 nM and 5000 nM), 3-oxo-C12-HSL and C12-HSL enhanced β-glucosidase activity (Figure 2a). A DMSO-only addition was performed as a control and it enhanced activity of β-glucosidase to the same extent as the 3-oxo-C12-

HSL and C12-HSL amendments. Thus we can not conclude these AHLs had any affect on β-glucosidase activity. However, 3-oxo-C8-HSL enhanced β-glucosidase activity to greater than ten times the activity in the no addition control. The ratio of activity in the amended sample to that of the control is greater than the control by two standard deviations. The effect of the high and low concentration 3-oxo-C8-HSL additions were indistinguishable.

AHL activities had a less dramatic although significant impact on lipase activity. 500nM 3-oxo-C8 HSL activity increased the activity of lipase by about 30% (Figure 2b). While this increase is small compared to the effect induced by 3-oxo-C8-HSL on β-glucosidase activity (13-fold increase), the ratio of activity in the amended sample to that of the control is greater than the control by three standard deviations; we can say with greater than 95% confidence that the AHL amendment was effective. It is interesting to note that the 5000 nM 3-oxo-C8 treatment had no effect on the activity of lipase. 3-oxo-C12-HSL and C12-HSL enhanced lipase activity although DMSO enhanced lipase activity as well; however, the enzyme activity induced by either C12-HSL or 3-oxo-C12-HSL is significantly greater than that induced by DMSO alone (t-test, $p=0.04$ and $p=0.01$, respectively).

## DISCUSSION

The phycosphere of *Trichodesmium* is an ideal environment for QS induction because of the high cell densities which are hosted within a diffusion-limiting EPS matrix. The observation of bacteria-like luminescence emitted from the colonies supported this

hypothesis. We undertook this study in order to evaluate the occurrence of QS in *Trichodesmium* colonies.

We were unable to detect AHLs in organic extracts obtained from either field or laboratory-cultivated *Trichodesmium* samples. The extracts were measured using the LCQ-MS, which as suggested in Chapter 3, may not be sensitive enough to measure very low concentrations of AHLs which are likely to be produced within these colonies.

The extraction of AHLs from Trichodesmium colonies presents special challenges. First, while AHL concentrations in individual clonal colonies of bacteria may be quite high, these individual colonies represent a small component of the biomass within a large batch culture and thus the overall AHL concentration is likely to be low. Second, the polysaccharide matrix which provides the structure of the biofilm is very hydrophobic (Horswill *et al.*, 2007) and likely competes with the organic solvent with respect to its chemical affinity for the AHLs. Finally, the biofilm matrix, if not completely disrupted during the extraction process, may protect clonal communities from the organic solvent thus preventing the extraction of AHLs. In consideration of the special challenges imposed by the structure of Trichodesmium colonies, it would be prudent to use the most sensitive analytical device available. In subsequent experiments, the FT-ICR-MS or triple quadruopole TSQ-MS are likely to achieve greater success.

In Chapter 3, we discovered that several strains of epibiotic bacteria associated with Trichodesmium from BATS produce AHLs. In this experiment, we added three of the AHLs (3-oxo-C8-HSL, C12-HSL and 3-oxo-C12-HSL) produced by BATS epibionts to batch cultures of *T. erythraeum* strain K-11#131 and evaluated the effect these had on

six common fluorogenic enzyme substrates. We detected a significant response in β-glucosidase and lipase activities; we did not detect an effect on the luminescence of the colonies. The upregulation of these two enzymes is interesting when compared to the results presented in Chapter 5 in which similar assays were performed on marine detrital aggregates. In Chapter 5, AHL was shown to upregulate the activity of protease, phosphotase and lipase. Upregulation of those particular enzymes makes ecological sense in the detrital-particulate environment because they serve to retain nitrogen and phosphorus in sunlit waters where they can support additional primary productivity. Here, we supplemented the *Trichodesmium* media with phosphorus and *Trichodesmium* likely supplies the colony with sufficient nitrogen. The enzymes upregulated in the phycosphere of *Trichodesmium* are therefore likely to be engaged in accessing the organic polysaccharide-based matrix.

We observed that the enzyme activities in samples amended with AHLs and those which had no amendments occurred in the relative intensities: lipase> protease> phosphotase> β-glucosidase> α-glucosidase> chitinase. It is interesting that although lipase and β-glucosidase were inducible by AHLs that β-glucosidase is less active than protease and phosphotase. Nausch and colleagues (1996) performed a similar enzyme activity survey on field populations of *Trichodesmium* collected in the Caribbean Sea. They report maximal phosphotase activity, followed by protease and β-glucosidase activity. Chitinase and α-glucosidase activity were very low and lipase activity was not measured. That phosphotase is the most active enzyme in the latter data set likely reflects

the nutrient availability in ambient waters at the time of collection, although this was not discussed.

Bacterial luminescence has been hypothesized to assist in the dispersion and propagation of bacterial species (Andrews *et al.*, 1984). While this explanation may explain why luminescent enteric bacteria associate with faecal pellets, it does not necessarily provide a satisfactory motive for the association of luminescent bacteria with *Trichodesmium* as *Trichodesmium* spp. are not commonly found in the gut contents of zooplankton (e.g. Eberl & Carpenter, 2007).

No eukaryotic or archaeal DNA were amplified from DNA extracts of Trichodesmium (discussed in Chapter 4 and Appendix 1), all observed luminescence must be of bacterial origin. To date, the only known bacterial luminescence mechanisms are regulated by QS. In addition, members of the K11131 consortia responded to the addition of exogenous AHLs by the upregulation of β-glucosidase activity as well as lipase activity. Despite the lack of detection of AHLs, two pieces of evidence support a role for QS in the *Trichodesmium* phycosphere. Further work will be required in order to assess the ecological impact of QS bacteria associated with *Trichodesmiuum* in the 'wild'. However, it is becoming evident that QS has important ecological and/or biogeochemical effects in the ocean and thus it will be excited to find out what role it plays in regulated the lifecycle of this globally important consortial cyanobacterium.

| | sample size | average luminescence | % standard deviation | Average luminescence/ seawater luminescence | % standard deviation |
|---|---|---|---|---|---|
| **Group 1 Tufts** | 7 | 281 | 31 | 2.3 | 32 |
| **Group 2 Tufts** | 6 | 714 | 23 | 5.9 | 24 |
| **Puffs** | 8 | 257 | 15 | 2.1 | 17 |
| **Seawater** | 8 | 121 | 8.3 | n/a | |

**Table 1.** Table of compiled luminescence data collected at BATS.

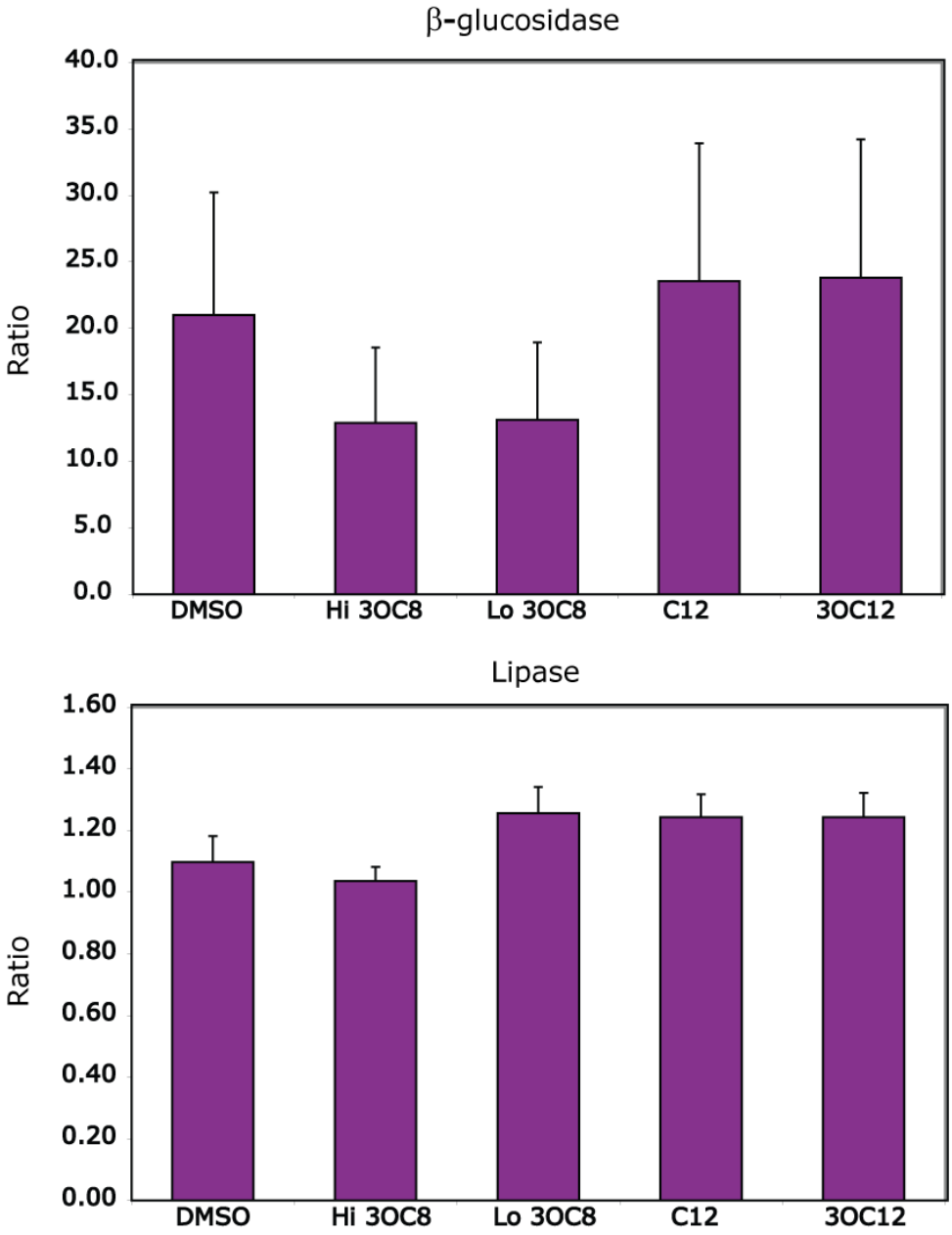


**Figure 1.** Results of β-glucosidase (a) and lipase (b) assays. Results are presented as ratios of experimental rates relative to unamended-control rates. Error bars are one standard deviation propagated from the error of replicate measurements of treated and control experiments. Note, the y-axis scale varies between (a) and (b).

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## APPENDIX 3:

# MEASUREMENT OF THE ACTIVATION ENERGY OF HEXANOYL-HOMOSERINE LACTONE IN ARTIFICIAL SEAWATER

The activation energy of C6-HSL degradation in artificial seawater was calculated from the degradation rate of C6-HSL measured at temperatures of 3°C, 24°C, and 37°C. The degradation rate of C6-HSL was determined as described in Chapter 2 (published as Hmelo & Van Mooy, 2009). The natural log of the rates was plotted against the inverse of the temperature (see Figure 1) and modeled using the Arrhenius equation (Atkins, 1998):

$$\ln k = \ln A - \frac{E_a}{RT} \qquad (1)$$

where k is the turnover rate, A is the Arrhenius constant, $E_a$ is the activation energy for the reaction of hydroxide ions with AHL, R is the universal gas constant, and T is the temperature in Kelvin at which the incubation was conducted.

The degradation rate of C6-HSL increased predictably with temperature (Figure 1). By modeling the data with the Arrhenius equation, we determined that the activation energy of C6-HSL in artificial seawater is 83 kJ/mol. From the activation energy of C6-HSL in artificial seawater, we calculated a $Q_{10}$ of 3.6. $Q_{10}$ is a coefficient is a measure of the change in the rate of a reaction with a 10ºC increase in temperature. Our calculated

$Q_{10}$ value was higher than that found previously in pure water (2.0, data from Yates *et al.*, 2002) indicating a greater temperature sensitivity in seawater.

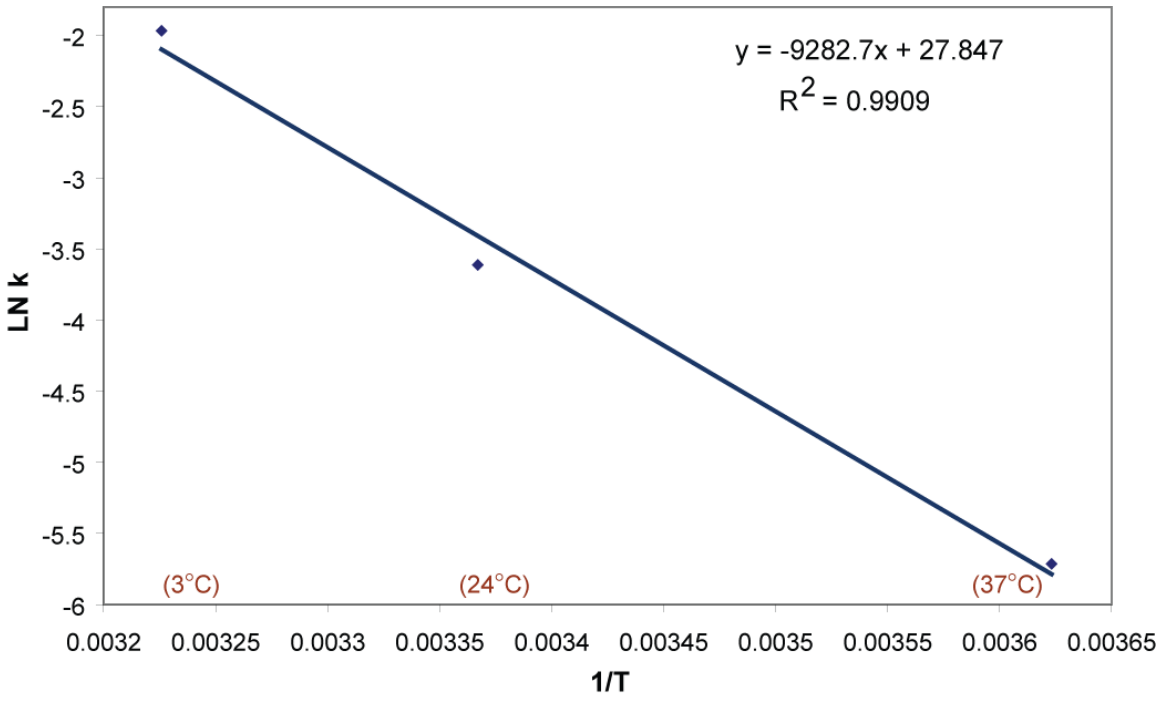


**Figure 1.** Plot of the natural log of the degradation rate of C6-AHL in artificial seawater versus the temperature at which the rate was measured. The slope of the line is equal to $-E_a/R$, see text for details.

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