Nitrogen-Fixing and Nitrifying Symbioses in the Marine Environment

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1 Introduction

The term "symbioses" was first defined loosely by De Bary (1879) as two or more differently named organisms living together. Although symbiotic interactions are ubiquitous in nature, few of the marine planktonic systems have been well characterized, and comparatively less is known of the functional role of the symbiont for the host and vice versa. Many of the planktonic symbioses are between eukaryotic hosts and cyanobacterial symbionts, or cyanobionts. Cyanobacteria are photosynthetic, and many are capable of nitrogen (N_2) fixation, thus often it is presumed that the cyanobacterial partner functions as a carbon and/or nitrogen source for the host. In the parallel system involving sponges, often the microbial symbionts are a diverse assemblage of heterotrophs, lithotrophs, and phototrophs. One unifying character in sponge-microbe system is the exchange of nitrogen.

Nutrients are often the key limiting factors to primary production in the tropical seas, and symbioses are frequently observed in these types of oligotrophic habitats. From his many microscopy observations in the open ocean, Norris (1967) speculated that a considerable part of the biota were involved at one time or another in a consortium, either temporary or more permanent. Forming a symbiotic association might then be considered an ecological adaptation to life in the oligotrophic ocean.

Compared to their terrestrial counterparts (see Rai et al. 2000), marine symbiotic systems are greatly under-sampled, and thus the many intricacies of these unique relationships remain largely unresolved. Difficulties in isolating and identifying these symbioses have been the primary problems in attaining useful information about them. Without epifluorescence microscopy most of these associations would go unnoticed.

With blue and green excitation, however, the cyanobionts exhibit fluorescence patterns distinct from their photosynthetic (diatom) and heterotrophic (dinoflagellate) partners (Fig. 1). In sponge-microbe associations, the symbionts are more difficult to identify by standard microscopy due to the complexity of the mixed assemblage. Nitrifying bacteria have also been reported in close association with other invertebrates, particularly seep and hydrothermal vent bivalves (clams) and tube worms associated with whale falls (i.e. Deming et al. 1997), however, here we review in greatest detail only the planktonic and sponges symbioses.

For the purpose of this text on symbioses as they relate to the marine nitrogen cycle, we will first emphasize the more common open ocean diatom-diazotrophic associations (DDAs), then summarize the recent advances in our understanding of sponge-nitrifying microbial associations.

2 Diatom-diazotrophic associations

2.1 Hosts and cyanobionts.

Some of the earliest reports of planktonic symbiosis describe the association of a heterocystous cyanobacterium, *Richelia intracellularis*, with various diatoms, including *Rhizosolenia* (Ostenfeld and Schmidt, 1901), *Hemiaulus* spp. and *Guinardia cylindrus* (Taylor, 1982; Sundström, 1984; Villareal, 1992). Up to 13 different species of *Rhizosolenia* have been reported with *Richelia* symbionts; however some authors (Sournia, 1970; Sundström 1984) questioned the host identity, argued that many were misidentified, and proposed that most of the *Rhizosolenia* species were just varieties of *R. clevei*.

Two of the most common *Hemiaulus* species reported with symbiotic *R. intracellularis* are *H. hauckii* and *H. membranaceus*. A third symbioses, occurs between *Richelia* and *H. sinensis*. In *Hemiaulus* host diatoms, it is not known where the symbiotic *Richelia* reside, whereas in *Rhizosolenia* spp. hosts, the *Richelia* remains as an extracellular endosymbiont residing between the plasmalemma and silica wall of the diatom host (Taylor, 1982; Villareal, 1990, Janson et al. 1995). In *Hemiaulus* diatoms, typically there are two trichomes (series of cells comprised of a few vegetative cells and one terminal heterocyst) per host cell, and in *Rhizosolenia* species occasionally 1-32 *Richelia* trichomes have been observed (Sundström, 1984; Villareal 1990).

Lemmerman (1905) was one of the first to depict the unique association of another heterocystous cyanobacterium, *Calothrix rhizosoleniae*, attached to the spines of a *Chaetocoeros compressus* diatom. Norris (1961) noted that the cyanobionts only attach transversely to the intercellular spaces of the diatom with the heterocyst closest to the host diatom. Others report the same symbiont as *R. intracellularis* (Karsten, 1907; Norris 1961; Janson et al. 1999; Gómez et al. 2005), thus there is conflicting taxonomy. For simplicity, here we report the symbionts attached to *Chaetocoeros* diatoms as a *Calothrix*. There have been a few other reports of a *Richelia* symbiont growing epiphytically on the spines of *Bacteriastrum* diatoms (Villareal, 1992; Rai et al. 2000; Carpenter and Foster, 2000; Carpenter 2002).

A few other symbioses have been described between a heterocystous cyanobacterium of similar morphology to *Anabaena* and *Nostoc* cells residing with *Coscinodiscus* and *Roperia tessellata* diatoms, respectively (Taylor, 1982; Villareal, 1992). Interestingly, Carpenter (2002; Plate IIb) found the cyanobionts of a

Coscinodiscus diatom collected near Zanzibar similar in cell morphology and diameter to a *Synechocystis* sp.

Recently, Carpenter and Janson (2000) also reported that the open-ocean chain forming diatom, *Climacodium frauendulum,* typically contains numerous coccoid cyanobacteria (Fig. 1). A similar sized cyanobiont has been described in the freshwater diatom, *Rhopalodia gibba* (Prechtl et al. 2004). The *R. gibba* diatoms are unique since the cyanobionts reside intracellularly and the diatoms have the capacity to fix nitrogen (Floener and Bothe, 1980). It is likely that the cyanobiont partners are fixing the nitrogen since all eukaryotes lack the nitrogenase enzyme (enzyme required for N_2 fixation).

2.2 Cultivation, transmission and cell divisions.

Few have attempted to isolate and cultivate these consortia, and to the best of our knowledge only T. Villareal (1989) was successful for several months in culturing a *Rhizosolenia-Richelia* symbioses. The division cycles of the host *Rhizosolenia* and *Richelia* were asynchronous in culture, and as such several asymbiotic hosts were observed (Villareal, 1989). Transmission from host to daughter cell is typically vertical, however asymbiotic hosts and free-living *Richelia* observed in the field and culture suggest horizontal transmission as well.

Taylor (1982) described the details of the vertical transmission in the *Rhizosolenia-Richelia* symbioses when he observed several *Richelia* cells migrating to opposite valves of host cells prior to host division. Villareal (1989) estimated the trichome migration at approximately 5 $\mu m\ s^{-1}$ and to be independent of host cytoplasmic streaming. The details of symbiont transfer in *Hemiaulus* and *Chaetoceros* have not been clearly described. Gómez et al. (2005) observed free trichomes of *Richelia* (note Gómez

et al. (2005) identify symbionts of *Chaetoceros* as *Richelia*) and suggested that the free filaments, which originate from *Rhizosolenia* (*clevei*) diatoms, colonize senescent *Chaetoceros compressus* diatoms and subsequently spread out after replication. This speculation is not supported by evidence presented by Janson et al. (1999) and Foster and Zehr (2006), which showed high sequence divergence between the various symbionts of the different host diatoms, and thus a high degree of host specificity, or in other words, each *Richelia/Calothrix* strain is specific to one host genus (section 2.3).

In Fall 2004, several chains of *Chaetoceros compressus* chains were hand-picked from the subtropical Pacific (station ALOHA) that had several symbiotic *Calothrix* cells attached to the host diatoms spines (Foster and Zehr, unpubl.). We were successful in culturing the symbiont, and the isolate, *Calothrix* SC01, has been maintained free living (without the diatoms) in nitrogen-deplete media and has been subject to a few experiments, including a phylogenetic study of the precursor gene for the nitrogenase (*nifH*) enzyme (Foster and Zehr 2006) and several acetylene reduction (AR) assays (proxy for N_2 fixation).

2.3 Specificity and symbiont phylogenetic diversity.

In contrast to the co-occurring and free-living cyanobacteria that reside in the open ocean, there are far fewer studies on the phylogentic diversity of the symbiotic *Richelia/Calothrix* and the other open ocean consortias. Difficulties in collection, isolation, and separation from the other phytoplankton populations, have been the primary obstacles. However recent studies (Foster and Zehr, 2006; Janson et al., 1999) using single-cell approaches were successful with molecular genetic analyses and allowed sequence data to be matched back to particular populations. Subsequently, the

sequence data has been directly applicable to other assays (i.e. Quantitative PCR), which estimate cell densities for target phylotype (*Richelia* associated with *Rhizosolenia*) using gene copy abundances (see below, section 2.5).

Janson et al. (1999) was first to report on the high host specificity of the *Richelia* symbionts for four of the DDAs. In their study, the *hetR* gene, a gene that functions in heterocyst and akinete differentiation (Buikema and Haselkorn, 1991; Leganés et al. 1994), was amplified from individual host samples containing several filaments of *Richelia* associated with *Rhizosolenia clevei*, *H. hauckii*, *H. membranaceus*, and *Chaetoceros* sp. The symbiotic specimens were collected from two cruises, one in the Caribbean Sea and one in the South Pacific Ocean. Janson et al. (1999) inferred a high degree of host-symbiont specificity since the symbiont sequences from the different host genera were highly divergent (sequence similarity <85%). In addition, the *hetR* nucleotide sequences derived from *Richelia* symbionts associated with *H. membranaceus* sampled in the Atlantic and Pacific Oceans were nearly identical (98.9% identical), suggesting genetic relatedness was not dependent on geographical location (Janson et al. 1999).

A second phylogenetic investigation of the same DDAs by Foster and Zehr (2006) corroborated the results of Janson et al. (1999) for the *hetR* gene and analyzed the phylogenetic diversity of two additional genes, *nifH* and 16S rRNA. NifH is a functional gene marker, and encodes the iron subunit of dinitrogenase reductase, the enzyme responsible for N_2 fixation. In this later study, sequence identity was highest (98.2%) amongst the16S rRNA sequences, and more divergent for the *hetR* (83.8%) and *nifH* (91.1%) sequences. This study also identified three previously unidentified

heterocystous-like *nifH* sequence groups, which were recently reported from station ALOHA, het-1, het-2, and het-3 (Church et al. 2005; Zehr et al. 2007), as the *Richelia* associated with *Rhizosolenia clevei, H. hauckii,* and *Calothrix* symbiont of *Chaetoceros* sp., respectively.

In addition, Foster and Zehr (2006) found a parallel divergence in the *nifH* sequences as Janson et al. (1999) reported for *hetR* sequences. In the study by Janson et al. (1999), they found that the *hetR* sequence associated with a *Richelia-H. hauckii* was different than the *hetR* sequence of a *Richelia* associated with a *H. membranaceus.* Thus, the specificity was on a host species level. A similar pattern resulted in the *nifH* phylogenetic data presented by Foster and Zehr (2006), which suggested the further delineation of the het-2 group into het-2A and het-2B, to represent *Richelia* associated with *H. hauckii* and *H. membranaceus*, respectively. The same divergence may occur within the *Rhizosolenia* sp. hosts, however it has not been investigated.

There have been a few phylogenetic studies of other planktonic symbioses other than the *Richelia*-Diatom symbioses. These however use a 16S rRNA phylogeny and rely on the high similarity between the resultant sequences to known nitrogen fixers as potential evidence for diazotrophy rather than look for a nitrogen-fixing gene (i.e.*nifH*) directly. A few are briefly reviewed here.

Carpenter and Janson (2000) reported the 16S rRNA phylogeny of the cyanobacterial symbionts that reside within the diatom, *Climacodium frauendulum*. They found a high sequence identity (>98%) between the cyanobiont 16S rRNA sequences and a 16S rRNA sequence derived from the unicellular diazotroph, *Cyanothece* sp. ATCC 51142. A similar 16S rRNA sequence was retrieved from the freshwater diatom,

Rhopalodia gibba (Prechtl et al. 2004). NifD gene sequences were also amplified from *R. gibba,* which were closely related to *Cyanothece* ATCC 51142 *nifD* sequences. In another 16S rRNA study by Foster et al. (2006) several sequences similar to16S rRNA sequences of *Cyanothece* sp. 51142 were recovered from a single *Histiones* (Dinoflagellate) sp. host containing symbiotic cells similar in morphology and cell diameter to *Cyanothece*. In the open ocean, *Crocosphaera watsonii*, which is similar in cell diameter size (3-5μm) and physiology (i.e. temporal segregation of N_2 fixation) to *Cyanothece* ATCC 51142, is a common cell type, and is likely the cyanobionts for many of the above-mentioned symbioses between a marine eukaryote and a coccoid cyanobacterium (Foster, pers. obs.).

2.4 Host-symbiont Interactions

In symbiotic systems, like these where the association appears quite intimate or the symbiont population occupies a majority of the host cell volume, the relationship is assumed necessary (Douglas, 1998) and/or beneficial. The benefit of the DDA relationships is not fully understood nor characterized, and because N_2 fixation has been measured when the DDAs are present, it is presumed that some of the nitrogen fixed by the symbiont is transferred to the host diatom. To date, there are only a few studies that have attempted to understand the nature of the symbiosis between the *Richelia* symbiont and the host diatom.

In a micro-autography study, field collected *Rhizosolenia-Richelia* symbioses were incubated with ^{14}C-labelled bi-carbonate. Higher density of silver grains localized on the symbiotic *Richelia* trichomes than on the host *Rhizosolenia* filaments, suggesting that the *Richelia* were actively photosynthesizing and the host diatom were inactive

(Weare et al. 1974). An equally plausible explanation for less silver grains on the *Rhizosolenia* host is that some of the fixed and labeled photosynthetic products were transferred to the host from the symbiont. Similar scenarios of carbon, and nitrogen transfer, are well documented in terrestrial symbioses with heterocystous symbionts, i.e. *Azolla-Anabaena*, Lichen-*Nostoc* symbioses (Rai et al. 2000). Weare et al. (1974) also speculated that the metabolically active host diatoms act as a source of inorganic nutrients, i.e.phosphate, for their symbionts.

In culture, Villareal (1990) measured growth and nitrogenase activity (acetylene reduction) in the *Rhizosolenia-Richelia* symbioses, and demonstrated light saturation kinetics in both activities. In addition, he demonstrated preliminary evidence for excretion of fixed nitrogen to the surrounding medium (details described in Villareal, 1990), and suggested the extracellular location of the symbiont (between the frustule and the plasmalemma) is mechanistic for nutrient transfer to the medium. Further elucidation of host-symbiont interactions, transfer, and benefit/cost of the relationship is a challenging, yet warranted subject for future investigation.

Janson et al. (1995) also verified the previous work of others (Taylor, 1982; Villareal, 1990) that the *Richelia* cyanobionts were always located outside of the host cytoplasm. Using immuno-cytochemistry coupled with transmission electron microscopy (TEM), Janson et al. (1995) demonstrated the localization of anti-bodies to nitrogenase and Ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO), in the heterocyst and vegetative cells, respectively, of the *Richelia* symbionts of *Rhizosolenia clevei*. They suggested that ammonia assimilation was potentially repressed in the *Richelia* cells, since they found low localization of the ammonia assimilation enzyme, glutamine synthetase

(GS). The immunogold assay does not indicate the activity of a particular enzyme rather it only shows presence of the enzymes when the cells were fixed. Thus the results from Janson et al. (1999) only suggests *Richelia* symbionts have the potential to function as nitrogen and/or carbon sources for their respective hosts.

Although the *Calothrix* cyanobionts of *Chaetoceros* are attached to the diatom spines, the cyanobionts are only located at the intercellular spaces and attached transversely at the heterocyst (Norris, 1961), which could be seen as a morphological adaptation or mechanism for nitrogen transfer. After several months in isolation without the *Chaetoceros* host, our cultured isolate, *Calothrix* SC01, started to change its cell character. For example, the trichome length extended, intercalary heterocysts were observed, and several trichomes appeared to branch. We interpreted such changes in the symbiont trichome and cell integrity as due to loss of control by the diatom host over the symbiont since the symbiont is in a free-living state. These latter observations also suggest that the free-living trichome potentially looks different than that which is observed when it lives symbiotically, and thus could be easily misidentified/overlooked in the field.

Others (Villareal, 1989; Kimor et al. 1978; Foster et al. 2007) have reported that vegetative cells degrade first, and often the heterocysts are the last part of the *Richelia* trichome to remain in a host diatom, which could also suggest host control or some sort of cell signaling between host and symbiont. Gómez et al. (2005) observed *Calothrix* symbionts associated with *Chaetoceros* diatoms which lacked chloroplasts suggesting that the host diatoms were senescent; quite possibly though the symbiotic *Calothrix* act as a source of fixed carbon to their hosts. Transfer of fixed nitrogen and/or carbon remains

undocumented in all the DDAs; it has only been inferred from growth of the *Rhizosolenia-Richelia* symbioses in N-free media in culture (Villareal, 1989).

2.5 Geographical Distribution and cell abundances

Generally speaking, *R. intracellularis* associated with *Hemiaulus* spp. have higher abundances in the Atlantic Ocean (Villareal, 1991, 1994; Carpenter et al. 1999; Foster et al. 2007), and *R. intracellularis* associated with *Rhizosolenia* sp. are more commonly reported from the North Pacific central gyre (Mague et al. 1974; Venrick 1974; Ferrario, 1995; Wilson et al., *in press*). Villareal (1994) reported the occurrence of *Hemiaulus-Richelia* symbiosis was 5-254 times more abundant in the North Atlantic, Caribbean Sea, and Bahama Islands than the *Rhizosolenia-Richelia* symbiosis. There are no observations of the *Chaetoceros-Calothrix* symbioses in the subtropical and tropical Atlantic Ocean, and some have suggested its geographical limitation to the Pacific and Indian Ocean Basins, however, we cannot discount that few have actually looked. Others have reported the DDAs distribution in the Indian Ocean (Norris, 1961), the Red Sea (Kimor et al. 1992), and more recently in the Eastern Mediterranean Sea (Bar-Zeev et al., submitted.), and the western China Seas (Gómez et al. 2005), which make *Richelia* and *Calothrix* the most widespread marine heterocystous cyanobacteria described.

There are a few exceptions where the DDAs are capable of penetrating coastal waters. For instance, Kimor et al. (1978) reported an "unusual occurrence of *Richelia* associated with *Hemiaulus membranaceus*" off the coast of California and one report of *Richelia* associated with *H. hauckii* and *H. membranaceus* in waters off Hawaii (Heniboekl, 1986). Recently, White et al. (2007) observed high abundances (100 L^{-1}) of the *Rhizosolenia-Richelia* symbioses in the Gulf of California. *Hemiaulus-Richelia* also

occurs at Carrie Bow Cay, Belize (Villareal 1994), and off the coast of Texas (Villareal, pers. comm.).

Most heterocystous cyanobacteria dominate brackish and freshwater environments where they occur in the plankton and the benthos as free-living and are seldom found in the open ocean. Few report *Richelia* and *Calothrix* as free-living (Gómez et al. 2005; White et al. 2007), thus both are the exception and have made their successful transition to the open ocean as symbionts.

Some of the highest numbers for the *Hemiaulus-Richelia* symbioses were reported in the western tropical North Atlantic (WTNA). Carpenter et al. (1999) observed an extensive bloom off the NE coast of South America in autumn of 1996. They reported cell densities from 10^2 to 10^6 *Richelia* L^{-1}. Recently, in the same vicinity as the study of Carpenter et al. (1999), Foster et al. (2007) reported extremely high *nifH* gene copy ($>10^5$ copies L^{-1}) abundances (proxy for cell abundances) for *Richelia* associated *H. hauckii* and *Rhizosolenia clevei*. In addition, they found within the plume waters of the Amazon River runoff a positive correlation between salinity and the abundance of the *H. hauckii-Richelia* abundance (Foster et al. 2007).

Church et al. (2005) were first to report the *nifH* gene expression for the het-1 and het-2 groups, which were later identified as *Rhizosolenia-Richelia* and *Hemiaulus-Richelia* symbioses (Foster and Zehr, 2006) from Station ALOHA in the N. Pacific Ocean. They found *nifH* expression for the het-1 group (*Rhizosolenia-Richelia* symbiosis) increased dramatically (10^2 to 10^6 *nifH* cDNA copies L^{-1}) in the early morning (04:00-06:00) and gradually declined throughout the late morning and evening. In addition, they detected *nifH* expression for the het-1 group (*Rhizosolenia-Richelia*

symbiosis) down to 200 m at midday and midnight, indicating a very active DDA population throughout the water column during day and night periods.

The abundance reported by Venrick (1974) and Mague et al. (1974, 1977) in the North Pacific Central Gyre were limited to estimates of the *Rhizoslenia-Richelia* symbioses. An interesting oversight reported in these studies was that the diatom *H. hauckii* was also present. In fact abundance during the Fall 1969 were 250 cells ml^{-1} (Venrick, 1974) and Mague et al. (1974) recorded 4000 cells L^{-1}. These abundances for *H. hauckii* were not reported as symbiotic; the *Richelia* in a *H. hauckii* is extremely inconspicuous with light microscopy (Fig. 1), and it is likely that it was overlooked. Venrick (1974) noted that in winter months (Nov.-Feb.) the *Rhizosolenia-Richelia* densities were low, ~60 cells L^{-1}, and reached 10^3-10^4 cells L^{-1} during summer (June-Sept.). Mague et al. (1974) observed a subsurface maximum in the abundance *Rhizosolenia-Richelia* symbioses (60-80 cells L^{-1}) at 40m in the N. Pacific Gyre. It was also the depth of maximum acetylene reduction (see below, section 2.6).

2.6 Nitrogen fixation

Although there have been observations of large and expansive blooms of DDAs (Villareal, 1994; Carpenter et al. 1999), there have been relatively few reports of the N_2 fixation or contribution of the DDAs to the global marine nitrogen budget. This is largely due to the difficulty of collection of the symbioses without compromising the integrity of the symbiotic complex and thus the physiological measures. A limited number of field studies (Mague et al. 1974, 1977; Venrick 1974; Carpenter et al. 1999; Villareal, 1991; White et al. 2007; Bar-Zeev et al. submitted), and fewer culture experiments (Villareal, 1989; 1990) represent the only physiological measures of N_2 fixation by the DDAs.

Venrick (1974) measured primary production rather than N_2 fixation, and recorded the highest carbon fixation rates (154.4 mg C m^{-2}) during a summer bloom (average abundance 1.5 x 10^7 filaments m^{-2}) of *Rhizosolenia-Richelia* in the North Pacific central gyre. The carbon fixation rate represents the carbon fixed by both host diatom (*Rhizosolenia*) and symbiont (*Richelia*) since both are photosynthetic. She then used the average cell specific N_2 fixation rates reported by Mague et al. (1974) to estimate the range (6.2-12.5 mg N m^{-2}) in daily N_2 fixation rate by *Rhizosolenia-Richelia*. Venrick (1974) extrapolated that 30-60% of excess productivity in the North Pacific central gyre was accounted for by the presence of a *Rhizosolenia-Richelia* bloom. Recently, a similar hypothetical estimate of N_2 fixation was provided by Foster et al. (2007), where they found that a dense population of *H. hauckii* with symbiotic *Richelia* accounted for 89-100% of the N_2 fixation (8.1 x 10^5-7.5 x 10^6 fmol N L^{-1} d^{-1}) in the WTNA. Villareal (1991) did a similar calculation and noted that only 100 cells L^{-1} of the *Hemiaulus-Richelia* symbioses could provide 15% of the entire N_2 fixation. Although these calculations are a crude means of estimating the rate of N_2 fixation and have obvious bias, they do highlight the potential significant influence of DDAs on the nutrient and energy budgets of phytoplankton in the oligotrophic environments.

Mague et al. (1974) reported low (0.024-0.643 μg N mg N^{-1} h^{-1}), but comparable rates of N_2 fixation by *Rhizosolenia-Richelia* to free-living *Trichodesmium*. During the DDA bloom observed by Carpenter et al. (1999) in the WTNA, the *Hemiaulus-Richelia* added an average of 45 mg N m^{-2} d^{-1} to the water column, which far exceeded estimates of new nitrogen flux from below the euphotic. Most recently, White et al. (2007) estimated that N_2 fixation by *Richelia* associated with *Rhizosolenia*, and to a lesser extent

by *Trichodesmium*, supplied 35-48% of the phytoplankton-based nitrogen demand in the central and eastern basins of the Gulf of California. Rates of N_2 fixation were recently estimated by the acetylene reduction technique on bulk water in the Eastern Mediterranean Sea. The highest rates of N_2 fixation (0.4-3.05 nmol N L^{-1} d^{-1}) were recorded in the summer and ~40-70% of the total N_2 fixation was attributed to populations of symbiotic *R. intracellularis* (Bar-Zeev et al., submitted). Besides the laboratory data of Villareal (1990; 1992), the above-mentioned studies represent the few data for N_2 fixation by the DDAs and each demonstrate the obvious ecological importance of these diazotrophic populations.

The earlier works by Mague et al. (1974; 1977), Venrick (1974), and Kimor (1978) attempted to define some of the environmental factors and conditions that control the N_2 fixation activity and distribution of DDA populations. Some have argued that distribution and activity is largely controlled by latitude, temperature, nutrients (i.e. iron, phosphorous), and wind stress for the other co-occurring cyanobacteria.

Kimor (1978) observed that the unusual occurrence of the symbiotic *H. membranceus* off the coast of Southern California occurred during an unusually warm period (18.5 ºC) for that geographical region and season. Later, in Carrie Bow Cay, Belize, Villareal (1994) reported 98% of *Hemiaulus* sp. examined contained *Richelia*, and that symbiotic *Hemiaulus* were present as far north as 31 ºN in the Pacific, further evidence that the geographical range of these symbioses is capable of penetrating cooler and more subtropical boundaries.

In her 9-year field study in the North Pacific Central Gyre, Venrick (1974) reported that for most of the years *Richelia* associated with *Rhizosolenia* were relatively

low (0.1-1 *Richelia* cell ml^{-1}) in abundance, however, in summer months when the upper water column stratified and nutrients were measurably low, symbiotic populations increased 1-2 orders of magnitude. Environmental parameters, i.e. nutrient concentrations, during bloom and non-bloom summers in the upper 45m were indistinguishable, suggesting little evidence for a condition to initiate and perpetuate the blooms. Venrick (1974) proposed that the blooms were a localized phenomena occurring independently at various locations within the central Pacific Ocean basin. Similarly higher rates of N_2 fixation in the eastern Mediterranean Sea were recorded during peak stratification (Bar-Zeev, submitted), thus it seems water column dynamics plays an important role in bloom formation and sustenance.

Gómez et al. (2005) observed the *Chaetoceros-Richelia* (note these authors use an alternative nomenclature) symbioses was restricted to the transition zones between the slope waters and the Kuroshio Current in the Western Pacific Ocean. They proposed that their distribution was related to local mixing of the Kuroshio Current with the coastal waters, where *Chaetoceros* is a dominant member of the neritic phytoplankton population.

Mague et al. (1974) found that highest biological fixation occurred in the summer months in the North Pacific Central Gyre when resident populations of the *Rhizosolenia-Richelia* symbioses began to increase. The surface waters were stratified and concentrations of phosphate and nitrate were undetectable. Enrichments of 0.5 to 5μM orthophosphate to samples containing *Rhizosolenia-Richelia* concentrates increased acetylene reduction, but when concentrations >5μM were added, activity decreased, and at 50 μM amendments the rate was equivalent to the initial. These results suggested that

to some extent the symbioses were P limited. To date, there have been no other nutrient manipulation experiments. Since the nitrogenase enzyme complex has a high iron requirement, an interesting and open question would be the effect of increased iron on N_2 fixation rates.

Typically, diatoms thrive in colder waters with high nitrate concentrations. Thus it seems that the DDAs have gained a successful existence into the warm oligotrophic waters of tropical and subtropical seas by their symbiotic partners. There have been few observations or investigations for the presence of the DDAs in higher nutrient environments, i.e. rivers, estuaries, or in regions of intense upwelling. Abundance for two of the three DDA groups (het-1 & het-2) was recently reported in the Amazon River plume in the WTNA (Foster et al. 2007), where elevated nutrients were measured. Cell abundances (5-120 cells L^{-1}) for symbiotic *Hemiaulus* and *Rhizosolenia* populations were recorded within lower salinity waters of the Orinoco River (Corredor, pers. comm). The same DDAs were detected near the Congo River plume in the eastern tropical north Atlantic (ETNA) (Foster, unpubl.). Combined, these observations suggest that in the Atlantic Ocean, fluvial inputs play an important role in the distribution of the DDAs. Hypothesized, but not yet investigated, is that rivers are the source of free-living *Richelia* populations, since most heterocystous cyanobacteria dominate in brackish and estuarine waters.

These earlier and more recent field measures all demonstrate the importance of these DDAs on the local conditions, however, in most experiments the collection of samples used towed nets, and thus are rather disruptive. Two experiments by Mague et al. (1974, 1977) found that preparing samples by concentration caused a significant (17-

29%) reduction in acetylene reduction activity. It seems that more attention or creative sampling schemes need to be developed to accurately measure the N_2 (and likely carbon) fixation by these DDAs. Studies similar to those presented by Zehr et al. (2007) and Needoba et al. (2007), which combine $^{15}N_2$ uptake rates with quantitative PCR approaches for the target diazotrophs are a plausible alternative since assays are run on bulk water.

2.7 Implications

A recent model presented by Deutsche et al. (2007) estimates global N_2 fixation by applying an oceanic circulation model to the relative changes of nitrate and phosphate concentrations in the surface ocean. Their model predicts N_2 fixation in all the regions of the world's oceans where these DDAs occur and have been reported. A major shortcoming noted in the model was, "diazotrophs with both a high biomass N:P and an unusually high export efficiency, should they be found, would be underestimated by our approach." The DDAs have extremely high vertical fluxes (Schaerek et al. 1999a, 1999b), and represent an excellent example of a population that would be likely overlooked in this type of model.

DDAs are among the most unique phytoplankter populations because they have a dual function. Large and expansive blooms contribute directly to the vertical flux of organic matter to the deep sea (Schaerek et al. 1999a, 1999b), all the while being widespread and sometimes patchy in distribution in the euphotic zone where they provide fixed N to the co-occurring non-diazotrophic phytoplankton population. Although controversial and limited in direct scientific evidence, we assume that a majority of the carbon and presumably fixed nitrogen associated with the DDAs does in fact fall out

below the euphotic into the mesopelagic. Thus the DDAs represent an important link in the biogeochemical cycling of both carbon and nitrogen in the World's oceans, and yet when compared to other larger diazotrophs, i.e. *Trichodesmium*, DDAs are under-represented in nutrient budgets and far under-sampled.

3. Sponge-nitrifier Associations

3.1. Sponge-Microbe specificity and phylogenetic diversity

Sponges act as filter feeders capable of circulating thousands of liters of seawater through their osculum per day while feeding on organic particles and microorganisms from the water column (Vogel, 1977; Pile, 1997). Some sponges, primarily those in the class *Demospongia* (Vacelot and Donadey, 1977), are populated by microbial symbionts, mostly extracellular, that are able to avoid phagocytosis and digestion while residing in the sponge mesohyl matrix. The microbial density within the host biomass can far exceed that of seawater, reaching concentrations up to 10^{10} bacteria per gram of sponge wet weight (Hentschel et al. 2006). For organisms that can avoid digestion, the host provides a favorable microbial habitat due to increased nutrient availability from the active pumping of seawater and release of ammonia, urea, and organic carbon as by-products (e.g. Davey et al. 2002).

Microscopy and molecular genetic techniques have demonstrated that a single sponge often contains a very diverse microbial assemblage including bacteria (Hentschel et al. 2002), archaea (Preston et al. 1996; Margot et al. 2002) and algae (Wilkinson and Fay, 1979; Usher et al. 2004). These 16S ribosomal RNA surveys have detected microorganisms similar to known heterotrophs, photoautotrophs, and chemolithoautotrophs. The nitrogen transformations attributed to these groups include N_2

fixation (e.g. Wilkinson and Fay, 1979), ammonia oxidation (e.g. Hallam et al. 2006a,b), nitrite oxidation (e.g. Hentschel et al. 2002), and nitrogen assimilation (e.g. Davy et al. 2002). The phylogenetic diversity and species richness of the symbiont population found within a single host (Webster, 2001; Hentschel et al. 2002; Taylor, 2004; Hill et al. 2006) are unlike most known marine invertebrate-microbe symbioses which have comparatively low symbiont diversity (Steinert et al. 2000). Nonetheless, the diversity of the sponge-microbe associations are often referred to as host specific. The bacterial symbionts appear to be distinct from the free-living bacterial populations in seawater and seemingly uniform bacterial populations have been detected in many geographically distant sponge species (Hentschel et al. 2002; Hill et al. 2006).

The diversity maintained in the sponge association may be partially attributed to the occurrence of asexual reproduction in sponges, allowing the establishment of a close association without requiring immediate incorporation of microbial cells into the germ line. Usher et al. (2005) reported that germ line incorporation does occur in at least some species and that cyanobacteria could be detected in both the egg and the sperm of *Chondrilla australiensis*. Sharp et al (2007) further demonstrated that phylogenetically diverse, yet sponge specific, microbial lineages including bacteria and archaea could be found in *Corticium sp.* embryos. The stability of these complex associations over evolutionary time scales has only begun to be explored.

In summary, sponges form symbiotic associations with phylogenetically and metabolically diverse microbes. Although there is very limited evidence to document the direct benefits of the symbiosis, the microbes are thought to receive increased nitrogen for growth and the host to benefit from the removal of potentially toxic metabolites, i.e.

ammonia and urea (Davey et al. 2002). The metabolic versatility of these abundant and diverse microbial assemblages in combination with the increased flow rate provided by the filter feeding host creates a bioreactor that can have a large impact on the carbon and nitrogen cycles of a marine habitat.

3.2 Nitrification in Sponges

The process of nitrification in marine sponges was first described by Corredor et al (1988) by measuring the concentration of nitrate released by the coral reef sponges *Chondrilla nucula* and *Anthosigmella varians*. Multiple investigators have re-confirmed the release of nitrate from symbiont containing sponges (Pile, 1996; Diaz and Ward, 1997; Scheffers et al. 2004) although the direct linkage of nitrate release to chemolithoautotrophy has not been established. It is assumed that ammonia released as a by-product of host metabolism is oxidized by microorganisms living within the sponge mesohyl matrix. Corredor et al (1988) observed that the rate of nitrification was not equivalent for all host species and that it varied with symbiont composition. For example, *C. nucula*, a cyanobacteria containing sponge, released nitrate 200 times faster than *A. varians*, a zooxanthellae containing sponge. This difference in nitrate release may be driven by uncharacterized differences in the non-cyanobacterial symbionts but the difference was generally attributed to bacterial symbioses. Similarly, Diaz and Ward (1997) found higher nitrification rates for sponge associated with cyanobionts, than non-cyanobacterial containing sponges. They found that in *Oligoceras violacea*, nitrite was primarily released, while in the other two species (*Chondrilla nucula* and *Pseudaxinella zeai*) high concentrations of nitrate were released. This difference was attributed to an uncoupling of ammonia oxidation and nitrite oxidation in *O. violacea* but the

composition of nitrifier symbionts was not examined. The potential nitrification rates for the sponge symbiont assemblages (up to 2650 nmol g^{-1} h^{-1}) were the greatest weight specific rates that have been reported and areal corrected rates were as much as four orders of magnitude greater than rates reported in coastal sediments (Diaz and Ward, 1997). In Curacao coral reefs, NOx efflux rates from cavities containing sponges were measured as 1.02-9.77 mmol m^{-2} d^{-1} (Scheffers et al. 2004); and 1.9 m^{-2} d^{-1} (van Duyl et al. 2006). These findings suggest that sponge-nitrifier assemblages can be responsible for a large input of oxidized nitrogen to habitats where these associations abound. Marine sponges are unlikely to play a large role in the global nitrogen cycle but in local habitats such as tropical coral reefs, where sponges are both abundant and diverse (Diaz and Rutzler, 2001), their activities could potentially play an important role in controlling the budget of ammonia and NOx (Diaz and Ward, 1997; Scheffers et al. 2004; van Duyl et al. 2006). Thus, the reported decline of sponge biomass (Wulff, 2006) could alter nitrogen cycling in these oligotrophic habitats.

3.3 Genomic studies of nitrifying symbioses

Although molecular analyses have revealed diverse populations of bacteria in sponges (Webster, 2001; Hentschel et al. 2002; Taylor, 2004; Hill et al. 2006), a single archaeal group was found to dominate the marine sponge *Axinella mexicana* (Preston et al. 1996). This symbiont, *Cenarchaeum symbiosum*, is extremely abundant and can account for up to 65% of the total microbial biomass found within the host. When this association was first identified, the metabolic activity of *C. symbiosum* remained a mystery. Molecular phylogenetics identified *C. symbiosium* as a member of the

planktonic, marine nonthermophilic *Crenarchaeota*. This group of archaea is widely distributed in the marine environment (DeLong et al. 1992; Fuhrman et al. 1991), is estimated to account for up to 20% of the oceans total picoplankton (Karner, et al. 2001) and isotopic analyses suggest that it is capable of autotrophic growth (Pearson et al. 2001; Ingalls et al 2006).

Metagenomic analyses conducted in the Sargasso Sea identified a gene sequence similar to bacterial *ammonia monooxygenase* (*amoA*) on a genome scaffold that also contained an crenarchaeotal ribosomal gene. This finding initially implicated the oxidation of ammonia as a chemolithoautotrophic metabolism associated with Archaea (Venter et al. 2004). This finding was rapidly followed by cultivation and characterization of *Nitrosopulmilus maritimus* (Konneke et al. 2005), unequivocally demonstrating the oxidation of ammonia to nitrate as an archaeal process and linking this transformation to some members of this abundant marine group. Ammonia oxidation by *Crenarchaeota* is now thought to be ecologically important and widely distributed in marine, freshwater, and terrestrial environments (Francis et al. 2005; Schleper et al. 2005; Treusch et al. 2005; Wuchter et al 2006; Cavicchioli et al. 2007), a process previously attributed only to bacteria.

Although *C. symbiosum* remains uncultivated, its abundance within *Axinella mexicana* allowed the use of genomic approaches to characterize its metabolic potential (Schleper et al. 1998; Hallam et al. 2006a; Hallam et al. 2006b). These analyses revealed two abundant *C. symposium* symbiont populations co-inhabiting the host. The gene content, order, and orientation for these sympatric populations suggests very little recombination in the evolution of these strains. Localized regions of sequence variation

reveal a limited number of genes under strong selective pressure worthy of additional investigation and a subset of candidate genes likely involved in the symbiosis. The genome was "remarkably distinct from those of other known *Archaea*" and contained a large number of genes most similar to marine environmental sequences thought to be from free-living planktonic *Crenarchaeota* (Hallam et al. 2006b). Future comparisons between the genomes of free-living *Crenarchaeota* and *C. symbiosum* will lead to an increased understanding of the symbiosis.

More importantly, the genomic analyses enabled by the enrichment of *C. symbiosum* in the tissue of *Axinella* provide an opportunity to learn about the closely related, planktonic *Crenarchaeota*. This group is now predicted to be the dominant marine nitrifiers, but eluded cultivation and characterization for many years. *Axinella*'s two symbiont genomes contain many of the genes required for autotrophy and appear to assimilate carbon using a modified 3-hydroxypropionate cycle. There is evidence of a partial oxidative tricarboxylic acid cycle for mixotrophic growth (Hallam et al. 2006a). Most of the genes encoding proteins involved in chemolithotrophic ammonia oxidation have been identified (including *ammonia monooxygenase*, *ammonia permease*, and urease) but a homologue for *hydroxylamine oxidoreductase*, which encodes a key enzyme involved in energy production from ammonia oxidation, has not yet been identified (Hallam et al. 2006b). This either suggests that the symbiont may utilize novel enzymatic reactions for the oxidation of ammonia or it requires re-evaluation of its role as a nitrifier. In either case, the elucidation of this pathway in *C. symbiosum* requires additional investigation and may shed light on alternative mechanisms for ammonia oxidation in both free-living and symbiotic taxa.

3.4 Implications

Sponge-nitrifier associations appear to play a quantitatively significant role in the nitrogen budget of localized marine environments such as tropical coral reefs. The importance of these associations is not limited to their biogeochemical impact in the environment but also extends to their use as a model system for laboratory analyses. The symbiotic association of *A. mexicana* and *C. symbiosum* allows us an alternative method of studying the abundant, but cultivation-resistant, free-living *Crenarchaeota* picoplankton. Further exploration of the *C. symbiosum* genome may help to uncover a new pathway for ammonia oxidation and factors regulating the newly discovered and globally significant archaeal ammonia oxidizers.

4. Other Relevant Symbioses

4.1 Diazotrophs in the copepod gut

An interesting and non-traditional example of symbioses, which has received relatively little attention are zooplankters with associated anaerobic diazotrophs (Zehr et al. 1998; Braun et al. 1999). Two independent studies revealed that planktonic copepods are associated with microorganisms, which possess *nifH* sequences phylogenetically related to strictly anaerobic sulfate reducers and clostridia (Cluster III *nifH* sequences) (Zehr et al. 1998; Braun et al. 1999). In the study by Braun et al. (1999), they also detected ethylene production during acetylene reduction assays on sorted copepods, indicating an active diazotrophic community. In the study by Zehr et al. (1998), the zooplankton derived *nifH* sequences were not recovered or similar to any of the sequences derived from parallel bulk water samples, suggesting that the nitrogen-fixing

species were likely gut-associated and not associated with the copepod's skeleton. Thus, the invertebrate gut may provide an unexpected refuge of suitable conditions for anaerobic N_2 fixation. And furthermore, considering that copepods are amongst the most abundant grazers in the world's oceans, the presence of N_2 fixing microflora associated with their guts is potentially another underrepresented source of nitrogen to the oceans (Zehr et al. 1998).

4.2 Shipworm-bacterial associations

Nitrogen fixation has also been reported from marine shipworms. Shipworms are bivalves, which live attached to wooden ships, in which they bore holes in the hulls of ships, and thus have a diet of wood alone. Cellulose is the principal component of wood, and is indigestible to animals. Certain bacterial species, however, contain the necessary enzymes to break down cellulose, and shipworms are often reported with gut associated bacterial symbionts.

In an early study by Carpenter and Culliney (1975), a bacterium was isolated from the gut of a Sargasso Sea shipworm, *Teredora malleolus*. Under anaerobic conditions and growth in a liquefying cellulose medium, significantly high rates of N_2 fixation rates (up to 1.5 micrograms of nitrogen per milligram dry weight per hour) were recorded. Similarly high rates were also measured in three other coastal shipworms. In a later study, a novel bacterium was also isolated from 6 species of teredinid bivalves (shipworms). Similar to the earlier study, the novel isolate was capable of digesting cellulose and fixing nitrogen (Waterbury et al. 1983). Both studies showed that N_2 fixation associated with the shipworms was significant and suggested that similar symbioses might occur in other organisms that ingest terrestrial plant material (Carpenter

and Culliney 1975). Again, shipworm-bacterial consortiums represent another understudied symbiotic association related to the nitrogen cycle. It should be noted that there are a few more recent studies on the diversity of bacteria associated with shipworms (refer to Sipe et al. 2000, Distal et al. 2002, Luyten et al. 2006), these however will not be reviewed.

4.3 Ascidian-Prochloron symbioses.

There are several didemnids (ascidians) that have been reported with symbiotic *Prochloron* cells. *Prochloron*, a genus of photosynthetic prokaryotes, are found in the marine environment as free-living and also associated with marine invertebrates. The primary role of the *Prochloron* symbionts has been to transfer organic carbon to their respective Ascidian hosts. There is however, some evidence that the nitrogen is also transferred from symbiont to host (Paerl 1984). Others have investigated nitrogen budgets in ascidian-*Prochloron* colonies and have suggested that the host ascidian and symbiotic *Prochloron* efficiently recycle the nitrogen within the colony (Koike et al., 1993), and thereby act more similar to a nitrogen trap (see below, section 4.4.). In nutrient poor environments where the ascidian colonies thrive, an efficient means of recycling of nitrogen and is probably essential for their survival.

4.4 The sponge-phototroph nitrogen trap

The coral-zooxanthellae association is a frequently cited example of a successful symbiotic relationship that forms the foundation for a diverse and ecologically important habitat within tropical, oligotrophic environments. This association is successful due to its ability to tightly recycle nitrogen and carbon. The coupling between an endosymbiotic phototroph and its filter feeding invertebrate host, acts as a nutrient and particle trap

(Cook, 1983; Rahav et al. 1989; Hinrichsen, 1997; Wild et al. 2004). Sponges are abundant in many oligotrophic environments (Wilkinson, 1983). For example, in the coral reef environment, estimates for the percent areal coverage by sponges are as high as 24% of high light, hard substrates and 54% of low light, rubble substrates (Diaz and Rutzler, 2001). Just like corals, sponges are known to specifically associate with certain phototroph symbionts (Wilkinson and Fay, 1979; Usher et al. 2004).

Recent stable isotopic evidence demonstrated that sponge DIN can be used by symbiotic algae and is sufficient to remove nitrogen growth limitation for the phototrophs (Davy et al. 2002). The system is therefore analogous to the coral-zooxanthellae. It had already been demonstrated that cyanobacteria could provide photosynthetically derived organics to the sponge and was capable of supplying the majority of the host's energy requirement (Cheshire et al. 1997). Interestingly, Trautman et al. (2000) observed that sponge-phototroph symbioses are often abundant in areas where corals are scarce, suggesting some degree of competition between these associations in the tropical oligotrophic environment or different responses to environmental conditions such as particle loading. Coral reefs are currently experiencing a sharp global decline and frequent bleaching of the symbiotic phototrophs (Hinrichsen, 1997). It is therefore necessary to understand if a similar global decline is also occurring for the sponge-phototroph nitrogen trap (Wulff, 2006). If it is, what will be the impact of this decline on metazoan biodiversity in the oligotrophic environment and how does this decline relate to coupling of host and symbiont? This association may take on an altered role in the rapidly changing reef environment and is an important system for further study.

5. Future outlook and perspectives

One of the largest obstacles in determining the overall importance of symbiotic associations to the global cycling of nitrogen is the lack of consistent rate measurements. For example, ranges in DDA abundances and N_2 fixation rates from a variety of studies around the world have been reported, however few of the studies measuring DDA N_2 fixation utilized the same means for rate normalization, i.e. biomass, cells, volume. This makes it quite difficult to estimate an overall contribution of these populations to the tropical and subtropical oceanic nitrogen budgets.

It seems that more attention or creative sampling schemes need to be developed to accurately measure the nitrogen (and likely carbon) cycling in the context of these planktonic and invertebrate associations. Studies similar to those presented by Zehr et al. (2007) and Needoba et al. (2007), which combine ^{15}N isotope rate measurements with quantitative PCR approaches provide a promising path forward. While molecular and microscopic characterization of nitrogen symbioses has helped to elucidate the diversity and distribution of symbioses, future work should target a consistent approach to relate these to their biogeochemical importance.

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Figure 1.

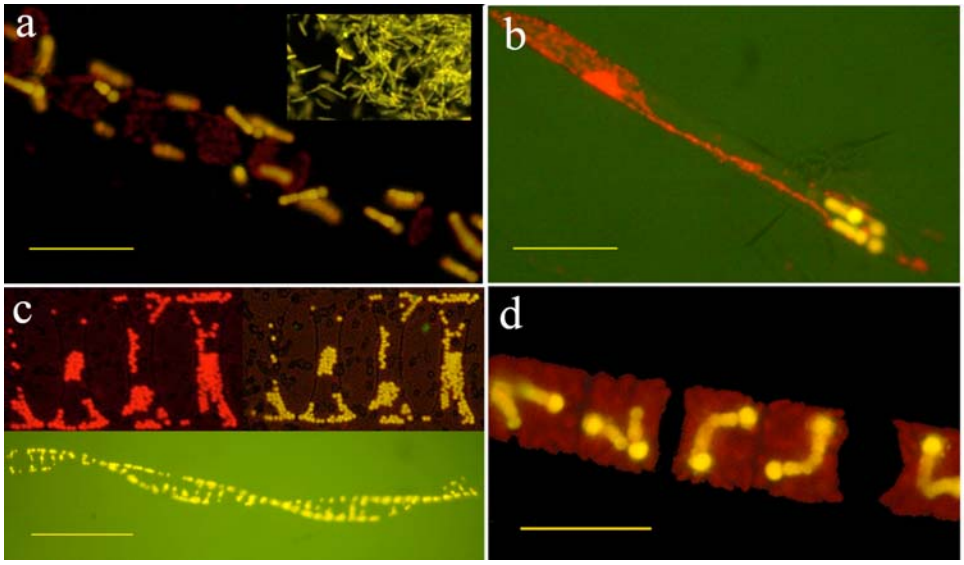

Figure 1. Epi-fluorescent micrographs of the better-studied Diatom-diazotrophic associations (DDAs) common to the World's oligotrophic Oceanic Basins. a) Blue excitation of a *Chaetoceros compressus* chain with attached heterocystous cyanobiont, *Calothrix rhizosoleniae*; inset is *Calothrix* SC01 isolate of Foster and Zehr. **b**) A *Rhizosolenia clevei* filament with several *Richelia intracellularis* trichomes at its apical end. Note the chloroplast of host diatom fluoresces red, while cyanobiont yellowish-orange under blue light. **c**) From top to bottom: Green and blue excitation of a diatom host, *Climacodium frauenfeldianum* with intracellular coccoid cyanobionts. Bottom is a blue excitation of symbioses in free-floating form. **d**) A chain of *Hemiaulus membranaceus* diatoms with pairs of *Richelia intracellularis* symbionts in each host. Scale bar 20 μm. All photographs from Foster except 1d was provided by D.A. Caron.