# IRON LIMITATION AND THE ROLE OF SIDEROPHORES IN MARINE *SYNECHOCOCCUS*

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## Abstract

Marine cyanobacteria in the genus *Synechococcus* are widely distributed and contribute significantly to global primary productivity. In many parts of the ocean their growth is limited by a lack of iron, an essential nutrient that is virtually insoluble in seawater. To overcome this, *Synechococcus* have evolved a number of strategies to acquire iron. Gene distribution, metagenomics and a novel immunological flow cytometry assay in the Costa Rica Upwelling Dome were used to estimate the importance of Fe stress. Genomic and metagenomic measures suggest that iron limitation is, paradoxically, more severe in coastal and upwelling areas than in the open ocean, where iron is less abundant. A serological assay found significant differences in the vertical distribution of the Fe stress protein IdiA over just a few meters.

Despite average surface ocean iron concentrations of just 0.07 nM, most marine oligotrophic cyanobacteria lack iron-binding siderophores that are present in many heterotrophic marine bacteria. Siderophores are widely distributed in the surface ocean and compose an important portion of the pool of natural ligands that bind >99% of all soluble Fe. In bottle incubations from the Sargasso Sea we found the addition of Fe complexed to an excess of the siderophore desferrioxamine B (DFB) limited *Synechococcus* growth and stimulated the growth of heterotrophic bacteria in a concentration dependent manner. Laboratory work revealed that excess DFB decreased *Synechococcus* growth beyond Fe-limited controls at concentrations as low as 20-40 nM. The inhibition was aggravated by light but it could be reversed by the addition of Fe. The DFB inhibition could not be explained by thermodynamic or kinetic models of Fe’ or co-limitation with other metals. DFB may interact with some aspect of cellular physiology to directly inhibit cyanobacterial growth.

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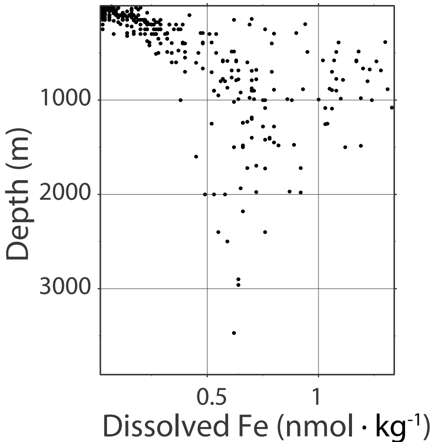
# Chapter 1

# Introduction

Marine cyanobacteria have changed the composition earth's surface more than probably any other group of organisms (Kasting and Siefert, 2002). The evolution of oxygenic photosynthesis in the ancestors of cyanobacteria 2.7Ga altered the chemistry of sulfur and iron (Fe) the ocean and within 400-500 million years oxygenated our atmosphere, paving the way for all eukaryotic life (Canfield et al., 2000; Kump, 2008). Cyanobacteria continue to influence the composition of our atmosphere, consuming carbon dioxide implicated in anthropogenic climate change. Their initial oxygenation of the earth's oceans dramatically decreased the availability of some metals cyanobacteria had come to rely on as cofactors, in particular Fe (Saito et al., 2003).

Although Fe is the fourth most abundant element in the earth's crust (Lide, 2009) it is virtually insoluble in oxygenated seawater, average dissolved Fe concentrations in the surface ocean are just 0.07nM, Figure 1A (Johnson et al., 1997). The residence time of Fe in the ocean on the order of 300-500 years—short by oceanic timescales—less than the transit time of a parcel of water due to thermohaline circulation (Bruland et al., 1994). The short residence time means that Fe concentration is controlled primarily by the Fe supply rate. Fe enters the ocean from wind-driven continental dust, the flux of aeolian Fe to the oceans is estimated at 32 Tg $yr^{1}$, (Duce and Tindale, 1991); rivers and glaciers transport additional Fe that can be locally important (Poulton and Raiswell, 2002). Supply is higher in coastal areas and the subtropical North Atlantic, which receives dust from the Saharan Desert (Jickells et al., 2005), increasing surface Fe concentrations above those found in the subtropical north Pacific, Figure 1B (Wu and Boyle, 2002). The element has a nutrient-like profile in the water column, concentrations of total Fe are

A. Dissolved iron concentrations by depth




B. Dissolved surface iron concentrations

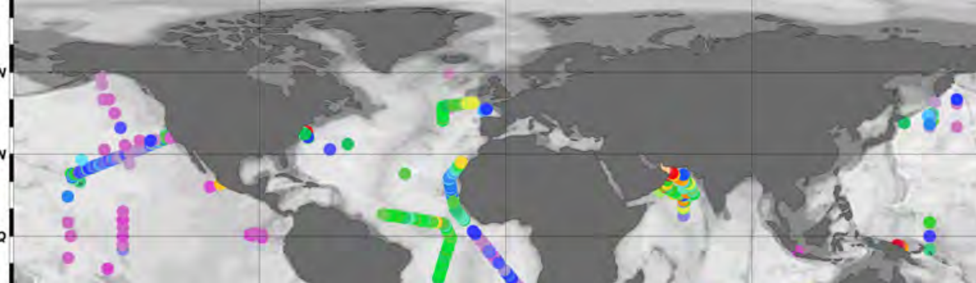
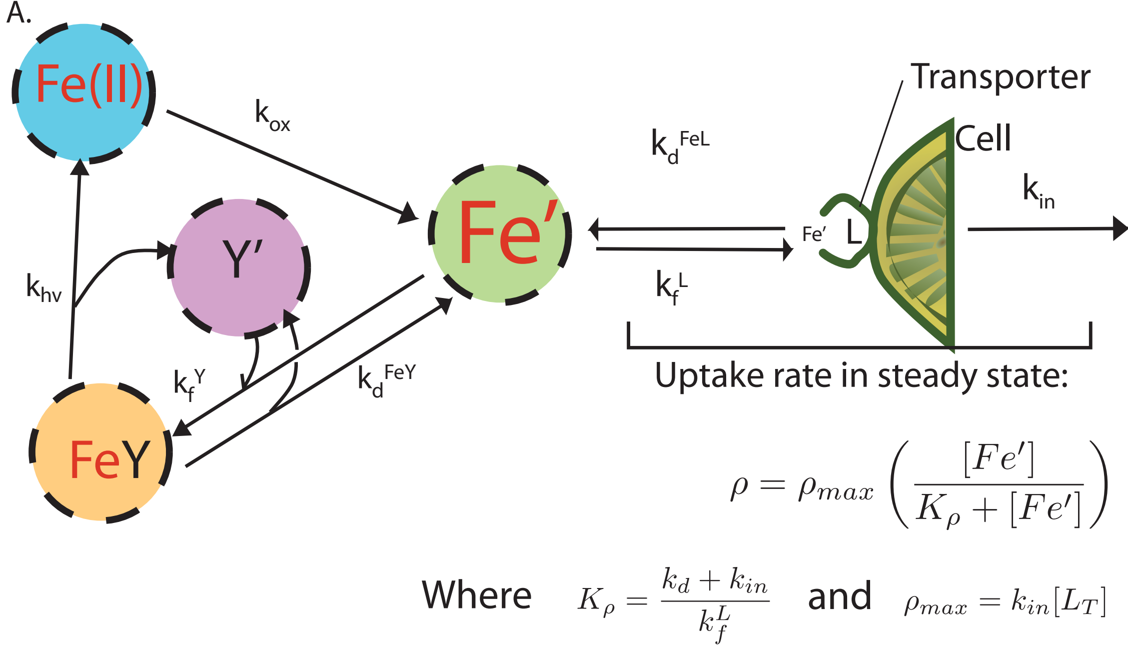



Figure 1 Measured concentrations of Fe in the ocean. A. The concentration of Fe by depth from samples in the in the Pacific and the north Atlantic, the dataset is from Johnson et al. (1997). B. A compilation of surface (<50 m) dissolved iron measurements from data aggregated in (Parekh et al., 2005).

depleted in the photic zone by biological demand from phytoplankton and bacteria (Pilson, 1998).

The biological and chemical reactions in the photic zone make Fe chemistry complex. Fe is present as colloids and in several soluble forms including in complexes with organic and inorganic ligands (Wu et al., 2001; Wu and Boyle, 2002; Hudson, 2005). Soluble Fe(III) forms weak complexes with $OH^-$ and $Cl^-$ and stronger complexes with a number of organic molecules. These organic ligands include components released during cell lysis, humic and fluvic acids in coastal areas, and siderophores (Fe-binding molecules secreted by microbes). Measurements in the Atlantic and Pacific revealed that ~99% of the dissolved Fe pool is bound to strong ligands (Rue and Bruland, 1995; Witter and Luther, 1998). Although the ferric form predominates in the ocean, Fe (II) is abundant in anoxic basins like the Black Sea, approaching micromolar concentrations (Yemenicioglu et al., 2006). In oxygenated water Fe(II) is produced by the photochemical reduction of Fe(III) (Moffett, 2001). Fe(II) concentrations are higher in coastal water where dissolved organic matter enhances photochemical reduction or in cold water where the re-oxidation of Fe(II) is slow (Moffett, 2001). The speciation and redox chemistry of Fe control the amount of Fe available for uptake by phytoplankton.

Biological uptake of Fe is explained well by the free ion model, Figure 2 (Sunda and Guilliard, 1976; Morel et al., 1993; Hudson, 2005). The model's premise is that an equilibrium exists between free metal ions in solution and metal ions bound to a cell's transport enzymes. In this model the metal shifts between being bound to the ligand, existing in the free state and being bound to the cell's transporter; the concentration of




B.

| Symbol | Variable | Units |
|---|---|---|
| $K_\rho$ | Half-saturation constant, instantaneous uptake | $mol \cdot vol^{-1}$ |
| $\rho$ | Instantaneous uptake rate | $mol \cdot cell^{-1} \cdot s^{-1}$ |
| $\rho_{max}$ | Maximal instantaneous uptake rate | $mol \cdot cell^{-1} \cdot s^{-1}$ |
| $k_{ox}$ | Rate constant, $Fe^{2+}$ oxidation | $s^{-1}$ |
| $k_{hv}$ | Rate constant, photoreduction of FeY | $s^{-1}$ |
| $k_d^{FeY}$ | Rate constant, FeY disassociation | $s^{-1}$ |
| $k_f^Y$ | Rate constant, Fe complexation by Y | $mol^{-1}s^{-1}$ |
| $k_f^L$ | Rate constant, Fe complexation by L | $mol^{-1}s^{-1}$ |
| $k_d^{FeL}$ | Rate constant for FeL disassociation | $s^{-1}$ |
| $k_{in}$ | Rate constant, Fe cellular import from FeL | $s^{-1}$ |

Figure 2 A kinetic free ion model of Fe transport in the presence of a photolabile ligand, modified from Hudson and Morel (1990). Most Fe is bound to the ligand Y. Fe is released from the ligand by thermal disassociation and by photoreduction. The Disassociation of Fe(III)’ (denoted as Fe’ for simplicity) and the oxidation of Fe(II)’ to Fe’ provide a pool of bioavailable Fe. The Fe’ pool is bound by the cell’s transport ligand and imported into the cell. When the system is buffered by an excess of Y, the disassociation of Fe’ from the complex is greater than the collective uptake rate of cells and Fe’ remains constant. Under these conditions the uptake rate of Fe’ is defined by the Michaelis–Menten type equation above.

free ion controls the uptake rate. The free ion model explains the uptake of both toxic and nutrient metals in many phytoplankton (Hudson, 2005). The model has been used to describe Fe transport in the diatom *Thalassiosira weissflogii* and the cocolithophore *Pleurochryis carterae* (Hudson and Morel, 1990). That work developed a conceptual and mathematical model for Fe uptake in phytoplankton. Although the model has been altered to incorporate new findings about Fe reduction in diatoms and the cyanobacterium *Lyngbya majuscula* (Shaked et al., 2005; Salmon et al., 2006; Morel et al., 2008) it provides an important framework for understanding Fe uptake.

Discoveries over the last twenty-five years have revealed that Fe limits the primary productivity of marine phytoplankton in areas as geographically varied as high nutrient low chlorophyll (HNLC) areas of the equatorial Pacific, the Southern Ocean and even coastal upwelling areas off Peru and California (Anderson and Morel, 1982; Brand et al., 1983; Martin and Fitzwater, 1988; Martin et al., 1991; Martin et al., 1994; Coale et al., 1996; Hutchins et al., 1998; Boyd and Law, 2001; Hutchins et al., 2002). Shipboard incubations (Martin et al., 1991) and large-scale Fe addition experiments at sea provided direct environmental evidence of Fe limitation. The first of these large-scale experiments was IronEx-I in 1993, the experiment spread 445 kg of Fe over a 64 $km^2$ patch of the equatorial Pacific and observed chemical and biological response to the addition (Martin et al., 1994). It was followed up in by IronEx-II in 1995, and a series of mesoscale Fe addition experiments in the Southern Ocean (SOIREE, EISENEX, SOFEX North, SOFEX South) (Boyd and Law, 2001; Boyd et al., 2000; Coale et al., 2004; Buesseler et al., 2004) and the subarctic North Pacific (SEEDS, SERIES) (Tsuda et al., 2003; Boyd et

al., 2004). These experiments were important in confirming Fe limitation but they also generated a wealth of data about the influence of Fe on primary production, carbon export and phytoplankton community composition. The collective significance of this work was recently reviewed by Boyd *et al.* (2007) and de Baar *et al.*(2005). Both field and laboratory experiments have revealed that Fe limitation is an important nutrient that limits primary productivity.

## *Synechococcus and Fe limitation*

Phytoplankton in the genus *Synechococcus* are abundant and widely distributed across the world's oceans (Zwirglmaier et al., 2008; Waterbury et al., 1986), including upwelling and oligotrophic regions where Fe limitation has been observed. (Saito et al., 2005; Zwirglmaier et al., 2008). However, when the genome of the first marine *Synechococcus* strain (WH8102) was published it revealed that the strain lacked most known Fe stress genes (Palenik et al., 2003). This finding raised a number of questions. Does *Synechococcus* sp. WH8102 use Fe acquisition strategies that have yet to be identified? Did Fe limitation influence the evolution of genetic clades in *Synechococcus* (Rocap et al., 2002)? Have we simply overestimated the importance of Fe stress in the open ocean?

In the second chapter of this thesis I report the distribution of Fe stress genes in 28 genomes of *Synechococcus*, *Prochlorococcus* and *Synechocystis* and in the Global Ocean Survey metagenomic data set. There do appear to be differences in the distribution of Fe stress genes and there is a general trend of more Fe stress genes in strains or

samples from coastal and upwelling regions. Although counterintuitive, this is consistent with findings of Fe limitation in coastal and upwelling areas (Hutchins et al., 2002; Hutchins et al., 2002; Fiedler, 2002). To examine Fe stress at a finer scale, I developed a technique that coupled flow cytometry with intracellular labeling of an Fe stress protein, allowing the detection of Fe stress in marine *Synechococcus* clades 3 and 5 (Rocap et al., 2003). The method was tested in the Costa Rica Upwelling Dome revealing that *Synechococcus* with different Fe stress levels or serotypes were separated by just a few meters of depth (Chapter 2). The work suggests the genetic capacity to respond to Fe stress varies among strains and may influence the community composition of marine *Synechococcus*.

Fe stress proteins are regulated by monitoring the concentration of Fe(II) in the cytoplasm. The ferric uptake regulator (Fur) is the protein responsible for sensing Fe(II) and it acts as a DNA binding repressor in many bacteria (Bagg and Neilands, 1987; Andrews et al., 2003). There is evidence it acts as an autoregulator (Andrews et al., 1993) and in some pathogenic bacteria it even acts as an activator, both directly (Delany et al., 2004) and through interactions with ncRNAs (Masse and Arguin, 2005). Fur is a homodimer approximately 19 kDa in size, each subunit contains a histidine-rich Fe(II) binding site and a DNA pocket that binds a consensus DNA sequence (the Fe box) -35 to -10 bp upstream, typically repressing transcription (Andrews et al., 2003). The recruitment of Fur to the Fe box can attract additional Fur dimers to divergent sites further upstream from the Fe box. When the concentration of Fe(II) in the cytoplasm drops the Fe(II) cofactor disassociates from Fur and the repressor diffuses from the Fe

box. Fur also binds the divalent cations Co (II) and Mn(II) weakly (Bagg and Neilands, 1987) and homologs of Fur regulate other metals including Mn(II) (Bellini and Hemmings, 2006) and Zn(II) (Gaballa and Helmann, 1998).

It is not yet clear how significant ncRNA's are in the regulation of cyanobacterial Fe stress, but several ncRNA have been identified that interact with the Fur system including a Fur cis-antisense RNA (Hernandez et al., 2006) and isrR, a small RNA that influences the regulation of the photosystem protein IsiA (Kunert et al., 2003; Duhring et al., 2006). A group of small RNA's termed cyanobacterial functional RNA (Yfr) were identified in *Prochlorococcus* and *Synechococcus* and many of the Yfr genes are differentially expressed under stress conditions (Axmann et al., 2005; Steglich et al., 2008). In *E. coli* there is a more complete model for post-transcriptional regulation, the bacterium decreases the production of Fe-containing proteins using the ncRNA RyhB. Under replete conditions Fur represses RyhB, but under Fe limitation RyhB is expressed, it binds to the transcripts if Fe containing genes and recruits the RNA degradosome complex to degrade itself and the targeted mRNA (Massé et al., 2007). Such a universal system for regulating Fe containing genes has yet to be found in cyanobacteria but it is becoming evident that ncRNA's play a role in the regulation of the Fe stress response.

Fe enters the cyanobacterial cell by crossing the outer membrane into the periplasm, and then the inner membrane into the cytoplasm. Transport across the outer membrane can be achieved by passive diffusion through porins or active, energy-dependent transport. In gram-negative bacteria the outer membrane is semi-porous so energy needed for active transport is transferred from ATPases on the inner membrane to

transporters on the outer membrane by the TonB-ExbB-ExbD system. In *E. coli* the TonB system powers a number of Fe-ligand import systems including FebA, FecA, FhuA (Andrews et al., 2003). Sequenced isolates from *Synechococcus* and *Prochlorococcus*, all lack the TonB system, based on Pfam assignment (Bateman et al., 2002) and Blast homology (Altschul et al., 1997). This lack of TonB suggests a different strategy for Fe import in marine picocynaobacteria. Rather than having a cell surface lined with high affinity receptors, picocyanobacteria may rely on passive transport into the periplasmic space. While it seems counterintuitive that cells in low nutrient environments would dispense with high affinity transport systems, replacing high affinity systems with more porins may have advantages. A cell surface covered with porins could act as a net allowing the diffusion of ions in while retaining the substrate binding components of ATP binding cassette (ABC) type transporters. A three-dimensional matrix of substrate binding proteins in the periplasm may potentially increase the number of receptors available for binding. It is surprising that the essential first step in Fe transport is unknown; the outer membrane transport system determines how quickly Fe can be taken up and even what forms of Fe are bioavailable, making it an important piece in the Fe acquisition puzzle.

More is known about the cytoplasmic transport of Fe in cyanobacteria. Fe(III) is thought to be transported by an ABC type transporter. The periplasmic component of this system is the Fe deficiency induced protein A (IdiA, known variously as FutA, HitA or SfuA) (Michel et al., 1996; Webb et al., 2001). IdiA is expressed in response to Fe limitation in marine *Synechococcus* (Webb et al., 2001; Rivers et al., 2009) and in

response to Fe limitation oxidative stress or limitation by Mn or Fe in freshwater *Synechococcus* (Michel et al., 1996; Michel et al., 1998; Yousef et al., 2003). It is also widely distributed, the three proteins in the Fe(III) transport system (IdiA, FutB and FutC) are found in all sequenced *Synechococcus* and *Prochlorococcus* genomes (Rivers et al., 2009). In some freshwater cyanobacteria IdiA appears to play an additional role protecting photosystem II (Michel et al., 1998; Exss-Sonne et al., 2000; Lax et al., 2007). *Synechococcus* sp. PCC6301 and *Synechocystis* PCC6803 have paralogous copies of IdiA that go the thylakoid or the periplasm (Michel et al., 2001; Tolle et al., 2002) suggesting the protein participates in both photosystem II protection and transport. The designation may be somewhat plastic however, because inactivating of the periplasmic copy if IdiA in *Synechocystis* sp. PCC6803 results in the cytoplasmic copy being rerouted to the periplasm, partially compensating for the loss. IdiA is an important photosystem and Fe(III) transport gene in cyanobacteria.

Free Fe(III) appears to be the predominant form of Fe transported into the cell by marine *Synechococcus* and *Prochlorococcus* however some evidence exists for Fe(II) and ligand-bound Fe(III) transport. FeoB is a cytoplasmic membrane bound ferrous Fe transporter (Kammler et al., 1993) found in *Synechocystis* sp. 6803 (Katoh et al., 2001) and three coastal marine *Synechococcus* spp. (Palenik et al., 2006; Rivers et al., 2009). There is evidence that some marine *Synechococcus* transport Fe-ligand complexes directly into the cell. The production of siderophores has been reported in *Synechococcus* spp. PCC 7002 and WH8101 (Wilhelm and Trick, 1994; Ito and Butler, 2005), two strains isolated from eutrophic environments (Van Baalen, 1962; Waterbury et al., 1986).

However, sequenced marine *Synechococcus* and *Prochlorococcus* strains the lack of a TonB system required by ligand-siderophore transporters, this suggests that the active transport of siderophores, and by extension siderophore production, may be uncommon in marine picocyanobacteria.

Once Fe enters the cell it needs to be incorporated into proteins or stored. Fe storage proteins help keep Fe(II) concentrations low in the cytosol, preventing oxidative damage from hydroxyl radical formation (Andrews, 1998). These storage proteins also act as reservoirs allowing the cell to survive Fe starvation and use transient Fe pulses by storing excess Fe during periods of "luxury uptake". Genes for the Fe storage proteins ferritin and bacterioferritin are both found in marine *Synechococcus* (Rivers et al., 2009). Ferritin and bacterioferritin are polymeric proteins that form a 24-mer complex capable of storing 2000~3000 atoms of Fe. The Fe is stored as oxidized ferrihydrite or amorphous hydroxyapatite (Andrews, 1998). The oxidation required to store the Fe is catalyzed by a ferrioxidase center located on each subunit. Bacterioferritins are functionally similar to ferritins but contain 12 heme moieties per complex. DpsA is another polymeric Fe storage protein termed a "mini-ferritin, but the monomeric form also binds DNA under stress conditions preventing oxidative damage (Andrews et al., 2003). A DpsA protein isolated from the marine cyanobacterium *Trichodesmium erythraeum* had the ability to bind both Fe and DNA (Castruita et al., 2006). Marine cyanobacteria vary in their complement of Fe storage proteins; all sequenced *Prochlorococcus* contain ferritin and/or bacterioferritin, while about half of the sequenced marine *Synechococcus* contain bacterioferritin. Even Fur itself has been implicated in Fe storage, the protein is found in

very high copy number (2500-10 000 per cell) in *Vibrio cholerae* and *E. coli* and may buffer Fe(II). Fe storage is important cellular strategy for using transient increases in Fe and managing the oxidative effects of too much or too little intracellular Fe.

Large numbers of genes are differentially regulated in response to Fe limitation based on observations from microarray experiments in cyanobacteria (Singh et al., 2003) (Thompson, pers. com. 2009). While some of these genes are directly involved in Fe stress, the function of other genes is unknown. The gene products of some differentially regulated genes contain Fe and are down-regulated or substituted out for non-Fe containing proteins, presumably in an effort to reduce the Fe quota. The replacement of ferredoxin by flavodoxin was one of the first substitutions discovered (Entsch and Smillie, 1972). Ferredoxin is an abundant [2Fe-2S] containing soluble electron carrier that shuttles electrons between photosystem I and the ferredoxin NADP+ oxidoreductase (FNR) (Bottin and Lagoutte, 1992). The substitution has been observed and used as a diagnostic of Fe stress in prokaryotic and eukaryotic phytoplankton (LaRoche et al., 1996; Erdner and Anderson, 1999; Erdner et al., 1999). Flavodoxin is an interesting example of quota reduction in cyanobacteria but it appears to be just one part of a larger global gene regulation response to Fe stress.

The genes involved in Fe acquisition and Fe stress alleviation can be grouped into four categories: acquisition, storage, quota reduction and resistance to oxidative stress. Each category appears to be important to some marine *Synechococcus* spp. although there is considerable diversity between strains on the number and type of Fe stress genes

found. More work is needed to answer the basic question of how *Synechococcus* thrive in such low Fe environments.

## *Siderophores*

When I was developing the Fe biostress assay I began to use the siderophore desferrioxamine B (DFB) cause iron limitation by binding Fe, inducing IdiA expression in cultures of *Synechococcus*. During these experiments cultures behaved differently than cultures that had been limited simply by removing Fe. Rapid bleaching occurred and the expression of IdiA was not consistent, cells would not pellet—in short I observed a number of small but reoccurring differences. I followed up with additional experiments to characterize the effect of the siderophore, eventually expanding it into the work presented in Chapter 3. That chapter examines the physiological effects of hydroxamate siderophores on *Synechococcus* and on a natural community of phytoplankton, finding that some physiological effects of the siderophore do not appear to be attributable to Fe binding alone.

There is increasing evidence that siderophores are significant in the marine environment. Marine siderophores are known to be produced by diverse types of oceanic bacteria including α- and γ-proteobacteria (Vraspir and Butler, 2009) and two strains of coastal *Synechococcus* (Ito and Butler, 2005; Wilhelm and Trick, 1994). Recent work on the distribution of the siderophores desferrioxamine E and G in an Atlantic transect found that these two siderophores alone are were widely distributed at concentrations of 3-20 pM, representing 0.2-4.6% of the total <0.2 μm Fe pool (Mawji et al., 2008). A simple enrichment of coastal seawater led to the production of detectable amounts of 3

hydroxamate siderophores and 4 amphibactin siderophores (Gledhill et al., 2004). Our current knowledge of siderophore structure is largely limited by the bacterial strains we can isolate or enrich. Abundant but uncultured bacteria are likely to produce additional siderophores.

Siderophores bear some similarity to naturally occurring, chemically quantified Fe-binding ligands. Seawater contains Fe-binding ligands that have been operationally defined in some studies as belonging to two classes based on their conditional stability constants (Wu and Luther, 1995; Wu and Luther, 1995; Rue and Bruland, 1995). A higher affinity ligand, $L_1$ is present predominantly in the photic zone (~0.44nM), while the weaker $L_2$ class is present throughout the water column at a higher concentration (~1.5nM) (Rue and Bruland, 1995). In the subtropical North Pacific as much as 99.97% of the dissolved Fe pool was bound to $L_1$ type ligands (Rue and Bruland, 1997) and mesoscale Fe addition increased the concentrations of these Fe binding ligands during IronEx-II (Rue and Bruland, 1996) Competitive ligand exchange has revealed that the $L_1$ ligand class has conditional stability constants similar to those of siderophores (Witter et al., 2000; Macrellis et al., 2001). The chemical similarities and the detection of siderophores in the marine environment suggest that some fraction of the organic ligand pool is comprised of siderophores.

Marine siderophores fall into several structural classes based on and binding moieties and hydrophobicity. The three predominant binding moieties are the hydroxamates O OH N, chatecholates HO OH and α-hydroxy carboxylates, O OH ;

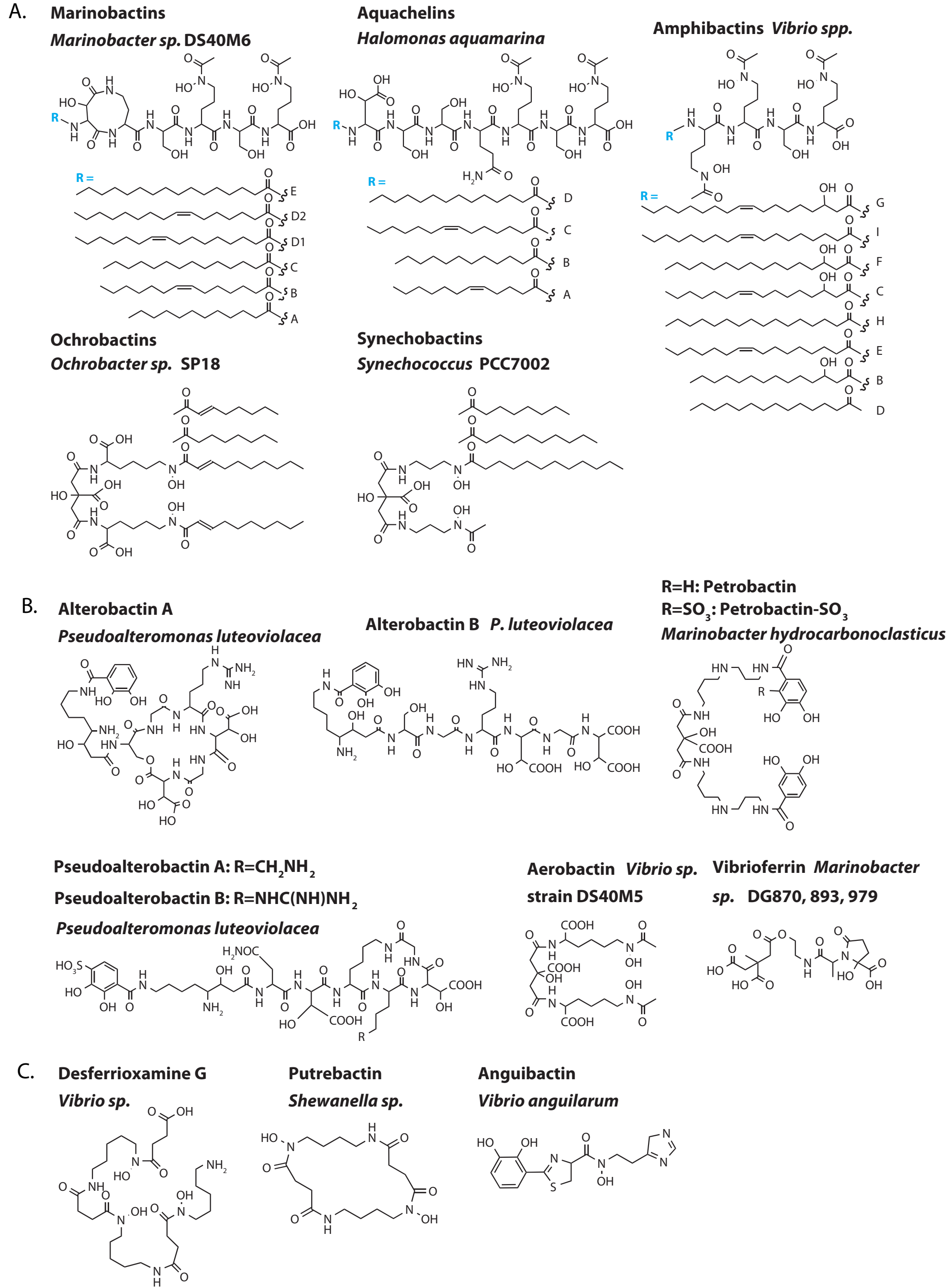



Figure 3 Siderophores produced by marine cyanobacteria. A. Amphiphilic marine siderophores that have been identified include marinobactins (Martinez et al. 2000) aquachelins (Martinez et al. 2000), amphibactins (Martinez et al. 2003), ochrobactins (Martin et al. 2006) and Synechobactins (Ito and Butler 2005). B. Marine siderophores with photolabile $\alpha$-hydroxy carboxalte groups: alterobactins, pseudoalterobactins (Kanoh et al. 2003), aerobactin (Haygood et al. 2003), petrobactin (Barbeau et al 2002), petrobactin-SO3 (Hickford et al. 2004) and vibrioferrin (Amin et al. 2007) C. The hydroxamate siderophores desferrioxamine G (Martinez et al 2001) and putrebactin (Ledyard and Butler 1997), and the mixed class siderophore anguibactin (Lorenzo et al. 2004). Reprinted with permission from Vraspir and Butler (2009).

hydroxamates are the most photo-stable (Barbeau et al., 2003). A siderophore can use any combination of the binding moieties to coordinate Fe(III) octahedrally, Figure 3 (Vraspir and Butler, 2009) A number of marine siderophores are amphiphilic, containing fatty acid tails, some examples include aquachelins, marinobactins, ochrobactins and synechobactins. (Vraspir and Butler, 2009). This tail can tether the siderophore to the cell surface, preventing loss by diffusion (Xu et al., 2002; Martinez et al., 2003; Martin et al., 2006). We are just beginning to understand the role of siderophores in marine environment, but the structural diversity, abundance and distribution imply that these molecules are ecologically important.

* * *

This thesis examines the response of Marine *Synechococcus* to Fe limitation and biological Fe-binding molecules, attempting to interpret findings in an ecological context. I use physiological, molecular and comparative genomic and metagenomic techniques in both laboratory and field populations of *Synechococcus*. This mixture of approaches provides a fuller understanding of the Fe stress response in *Synechococcus*. I hope this work adds to a growing body of research on the importance of Fe and siderophores in the ocean.

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# Chapter 2

# Iron stress genes in marine *Synechococcus* and the development of a flow cytometric iron stress assay

Rivers, A.R., Jakuba, R.W., and Webb, E.A. (2009) Iron stress genes in marine *Synechococcus* and the development of a flow cytometric iron stress assay. *Environmental Microbiology* **11**: 382–396.

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# Iron stress genes in marine *Synechococcus* and the development of a flow cytometric iron stress assay

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## Summary

**Marine *Synechococcus* are frequently found in environments where iron (Fe) is a limiting nutrient. To understand their capacity to respond to Fe stress, we screened picoplankton genomes and the Global Ocean Survey metagenome for known Fe stress genes. Many open ocean strains of *Synechococcus* lack most known genes for Fe stress, while coastal and upwelling strains contain many, suggesting that maintaining multiple Fe limitation compensation strategies is not a selective advantage in the open ocean. All genomes contained iron deficiency-induced protein A (IdiA) and its complementary $Fe^{3+}$ transport proteins. The ubiquity of IdiA was exploited to develop an *in situ* Fe stress bioassay based on immunolabelling and flow cytometry. As a test of field applicability, we used the assay on natural *Synechococcus* populations from one station in the Costa Rica Upwelling Dome where total Fe ranged from <0.08 to 0.14 nM in the upper water column. The bioassay found Fe stress in 5–54% of the population. Based on our findings, we believe that when reactive strains are present this assay can reveal environmental and clade-specific differences in the response of *Synechococcus* to Fe stress.**

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## Introduction

The picoplankter *Synechococcus* is abundant and ecologically important in regions ranging from ocean gyres to upwelling zones where iron (Fe) can limit growth and primary production (Coale *et al*., 1996; Field *et al*., 1998; Hutchins *et al*., 1998; Behrenfeld and Kolber, 1999). Despite its abundance, little is known about how *Synechococcus* acquires Fe from the environment, or the relative importance of the genes involved in Fe stress – even the mechanism that first transports Fe through the outer membrane is undefined (Webb *et al*., 2001). As Fe is a cofactor in many photosynthetic and nitrogen metabolism enzymes (Dean *et al*., 1993; Geider and La Roche, 1994; Lin and Stewart, 1998), it is a key element with the potential to both directly and indirectly control primary production by some of the earth's most abundant phototrophs.

Cyanobacteria can cope with Fe limitation by using high-affinity transporters to acquire more Fe (Katoh *et al*., 2001), storing Fe when it is abundant (Keren *et al*., 2004), substituting flavodoxin for ferredoxin to reduce Fe quota (Leonhardt and Straus, 1992), and reducing oxidative damage that results from Fe deficiency (Park *et al*., 1999; Michel *et al*., 2003). *Synechococcus* and many other bacteria use the ferric uptake regulator (Fur) to sense intracellular $Fe^{2+}$ pools and regulate Fe stress genes accordingly (Bagg and Neilands, 1987; Angerer *et al*., 1992; Ghassemian and Straus, 1996; Andrews *et al*., 2003). The breadth of compensation responses found in cyanobacteria underscores the element's importance to their physiology.

A key response to Fe limitation in marine and freshwater *Synechococcus* is the upregulation of the gene *idiA* (Michel *et al*., 1996; Webb *et al*., 2001). The IdiA family of proteins has been the focus of previous Fe stress research beginning with the identification of the IdiA homologue SfuA in the heterotroph *Serratia marcescens* (Angerer *et al*., 1990). Immunoblotting of IdiA has confirmed its expression under Fe limitation in various marine cyanobacteria, including *Trichodesmium* sp. IMS 101, *Crocosphaera* sp. WH8501 and *Synechococcus* spp. WH8103 and WH7803 (Webb *et al*., 2001). The induced IdiA protein has been detected in association with outer membrane preparations from marine *Synechococcus* sp. WH7803 suggesting a role in transport (Webb *et al*.,

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2001). In some freshwater strains, IdiA has a dual role, both performing cellular Fe transport and protecting photosystem II during Fe stress (Fulda *et al.*, 2000; Tolle *et al.*, 2002). *Synechocystis* sp. PCC6803 has two paralogous genes, *slr0513* and *slr1295*, which encode proteins found in the thylakoids and in the periplasmic space respectively (Fulda *et al.*, 2000; Tolle *et al.*, 2002), and in *Synechococcus* strains PCC6301 and PCC7942 these IdiA proteins are found in both locations and are expressed under Fe and Mn limitation (Michel *et al.*, 1996; 1998; Michel and Pistorius, 2004). Microarray data with *Synechocystis* sp. PCC6803 showed that in Fe-stressed conditions the *idiA* paralogue associated with transport (*slr0513*) was the 9th most induced gene, while the thylakoid-associated IdiA homologue (*slr1295*) was not upregulated (Singh *et al.*, 2003). In contrast to freshwater strains, the role of IdiA in marine *Synechococcus* has not been definitively shown, but genomic and physiological data suggest a primary role in Fe transport.

Here we present data showing the presence of defined Fe stress genes in the genomes of *Synechococcus* and *Prochlorococcus* and in the Global Ocean Survey (GOS) metagenome (Rusch *et al.*, 2007), which indicate that genes for $Fe^{3+}$ transport and DNA protection are prevalent in *Synechococcus*, and that there are differences in the complement of Fe stress genes found in coastal and open ocean *Synechococcus*. The environmental and genomic prevalence of IdiA support its value as a diagnostic marker of Fe stress in *Synechococcus* and led us to develop an IdiA-specific antiserum for quantitative detection of Fe stress in single cells using immunofluorescence and flow cytometry. This bioassay was used to measure the timing of IdiA expression in the laboratory and to detect Fe stress in natural *Synechococcus* populations from a station in the Costa Rica Upwelling Dome (CRD).

## Results

### *Genomic and metagenomic survey*

The genomes of 28 unicellular cyanobacteria, including *Prochlorococcus*, *Synechococcus* and *Synechocystis*, were screened for genes homologous to Fe stress genes defined from the literature cited below (Table 1). Homology was determined by blast search, gene alignment and the construction of maximum likelihood trees with previous literature as a guide. This survey found many differences in the abundance and distribution of Fe stress genes within the cyanobacterial picoplankton. The three genes predicted to form a periplasmic binding protein-dependent ABC transporter for $Fe^{3+}$ (*idiA*, *futB* and *futC*) (Katoh *et al.*, 2001) co-occur in every genome, suggesting a common function and method of Fe acquisition. Three strains of marine *Synechococcus* (RS9917, WH7805 and RCC307) contain two copies of *idiA*, suggesting possible roles for the paralogous proteins in Fe transport and Fe stress protection, as in *Synechocystis* sp. PCC6803 and *Synechococcus* sp. PCC6301 (Michel *et al.*, 2001; Tolle *et al.*, 2002). The capacity for $Fe^{2+}$ acquisition encoded by *feoB* (Kammler *et al.*, 1993) is distributed more sporadically than $Fe^{3+}$ genes, being found only in coastal *Synechococcus* spp. WH5701, RS9917 and CC9311. In *Prochlorococcus* and some *Synechococcus* strains, iron storage under replete conditions is thought to be accomplished with ferritin and/or bacterioferritin, a 24-mer protein capable of storing 2000–3000 Fe atoms by oxidizing Fe to ferrihiydrite or amorphous ferric phosphate (Andrews *et al.*, 1993; Keren *et al.*, 2004). The distribution of these Fe storage genes is complex; for example, *Synechococcus* appears to primarily use bacterioferritin while *Prochlorococcus* typically use ferritin, although some strains have both storage systems and others have neither. Many cyanobacteria reduce their Fe quota by substituting flavodoxin for ferredoxin, which uses a flavin mononucleotide cofactor rather than the Fe-containing Fe-S cluster (Bottin and Lagoutte, 1992; La Roche *et al.*, 1995; Erdner *et al.*, 1999). Flavodoxin, encoded by *isiB*, is present in all *Prochlorococcus* but unexpectedly absent in 64% of the marine *Synechococcus* genomes.

Iron deficiency reduces the efficiency of the photosystem, generating free radicals during photosynthesis that damage DNA. This is countered by the Fe and oxidative stress-induced protein DpsA (Park *et al.*, 1999; Michel *et al.*, 2003), present in 7 of 11 marine *Synechococcus* genomes but largely absent from the *Prochlorococcus* genomes. The gene encoding IsiA, a photosystem I-associated stress protein (Burnap *et al.*, 1993; Park *et al.*, 1999; Michel and Pistorius, 2004; Singh and Sherman, 2007), is found in freshwater *Synechococcus* and *Synechocystis*. We found an *isiA* homologue in some coastal marine *Synechococcus* strains; these homologues form a monophyletic cluster distinct from freshwater *isiA*, *Prochlorococcus pcb* genes (which bind light-harvesting chlorophyll), and the *pcbC* cluster found in other cyanobacteria (phylogenetic data not shown) (Chen *et al.*, 2005). These putative *isiA* genes may be involved in Fe stress but their function must be determined experimentally. The *pcb* genes in *Prochlorococcus* were not categorized because Fe stress-induced *pcb* genes are paralogous and interspersed with constitutive *pcb* genes, making it difficult to phylogenetically infer their role in Fe stress (Bibby *et al.*, 2003).

The metagenomes of open ocean and coastal sites in the GOS were searched to estimate the abundance of Fe stress genes in the environment. To estimate the number of Fe stress genes per genome, the genes with best hits to known *Synechococcus* genes were tallied from open ocean and coastal environments (Fig. 1). These tallied

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**Table 1.** The presence of homologues to Fe stress genes in the genomes of *Prochlorococcus*, *Synechococcus* and *Synechocystis*, indicated by GenBank GeneInfo number.

| | Marine *Synechococcus* | | | | Transport | | | | Storage | | | Oxidation | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Environment[a] | Core genome subcluster[b] | Clade[c] | Genome | Reference or origin[d] | *idiA* | *futB* | *futC* | *feoB* | Bacterio ferritin (*bfr*) | Ferritin (*ftn*) | Substitution *isiB* | *dpsA* | *isiA* |
| Open ocean | – | – | *Prochlorococcus marinus* str. MIT 9303 | Kettler *et al.* (2007) | 124023741 | 124022417 | 124023467 | – | 124023469 | 124023474 | 124023113 | 124024645 | – |
| Open ocean | – | – | *P. marinus* str. MIT 9313 | Rocap *et al.* (2003) | 33862560 | 33863555 | 33862774 | – | 33862772 | 33862768 | 33863073 | – | – |
| Open ocean | – | – | *P. marinus* str. NATL2A | Kettler *et al.* (2007) | 72382613 | 72383658 | 72382051 | – | 72382052 | – | 72382633 | – | – |
| Open ocean | – | – | *P. marinus* str. NATL1A | Kettler *et al.* (2007) | 124026324 | 124025257 | 124025550 | – | 124025551 | – | 124026355 | – | – |
| Open ocean | – | – | *P. marinus* str. MIT 9211 | Kettler *et al.* (2007) | 84517675 | 84518462 | 84517978 | – | 84517979 | – | 84517776 | – | – |
| Open ocean | – | – | *P. marinus* str. MIT 9312 | Coleman *et al.* (2006) | 78779644 | 78778875 | 78779195 | – | – | 78779196 | 78779655 | – | – |
| Open ocean | – | – | *P. marinus* ssp. pastoris str. CCMP1986 (MED4) | Rocap *et al.* (2003) | 33861720 | 33861046 | 33861360 | – | – | 33861361 | 33861727 | – | – |
| Open ocean | – | – | *P. marinus* ssp. marinus str. CCMP1375 (SS120) | Dufresne *et al.* (2003) | 33240721 | 33239939 | 33240331 | – | – | 33240332 | 33238142 | – | – |
| Open ocean | – | – | *P. marinus* str. MIT 9515 | Kettler *et al.* (2007) | 123966564 | 123965788 | 123966013 | – | – | 123966012 | 123966576 | – | – |
| Open ocean | – | – | *P. marinus* str. AS9601 | Kettler *et al.* (2007) | 123968885 | 123968082 | 123968400 | – | – | 123968401 | 123968897 | – | – |
| Open ocean | – | – | *P. marinus* sp. MIT 9215 | Kettler *et al.* (2007) | 157413708 | 157412906 | 157413233 | – | – | 157413234 | 157413720 | – | – |
| Open ocean | – | – | *P. marinus* str. MIT 9301 | Kettler *et al.* (2007) | 126696699 | 126695854 | 126696202 | – | – | 126696203 | 126696711 | – | – |
| Coastal | 5.1B | I | *Synechococcus* sp. CC9311 | Palenik *et al.* (2006) | 113955347 | 113954851 | 113953249 | 113954844 | 113953047<br>113954205 | 113953838 | 113954107 | 113955587 | 113955436[g] |
| Coastal | 5.1B | VIII | *Synechococcus* sp. RS9917 | Moore/JCVI,<br>Fuller *et al.* (2003) | 87124186<br>87123797 | 87123796 | 87123962 | 86167757 | 87124189 | 87124179 | – | 87123421 | – |
| Coastal | 5.2 | – | *Synechococcus* sp. WH 5701 | Moore/JCVI | 87302659 | 87302660 | 87302447 | 87302572 | 87302532 | 87302495 | – | 87300556 | – |
| Coastal | 5.1A | IV | *Synechococcus* sp. CC9902 | JGI/DOE | 78185569 | 78185259 | 78184453 | – | 78185570 | – | 78185574 | – | 78184578[g] |
| Coastal | 5.1A | IV | *Synechococcus* sp. BL107 | Moore/JCVI | 116072152 | 116072862 | 116070883 | – | 116072151 | 116072146 | – | | 116070747[g] |
| Intermediate | 5.1A | II | *Synechococcus* sp. CC9605 | Palenik/JGI/DOE,<br>Toledo *et al.* (1999) | 78213103 | 78212219 | 78212220 | – | 78213104 | – | 78213109<br>78214087 | – | 78213115[g] |
| Coastal | 5.1B | IX | *Synechococcus* sp. RS9916 | Moore/JCVI,<br>Fuller *et al.* (2003) | 116073409<br>116074588 | 116073410 | 116075374 | – | 116074582 | – | – | 116074181 | – |
| Open ocean | 5.1B | VI | *Synechococcus* sp. WH 7805 | Moore/JCVI | 88807643<br>88808400 | 88807642 | 88807742 | – | 88808394 | – | – | 88809465 | – |
| Open ocean | 5.1B | V | *Synechococcus* sp. WH 7803 | Genoscope | 148238336 | 148240145 | 148239029 | – | – | – | – | 148240796 | – |
| Open ocean | 5.3 | – | *Synechococcus* sp. RCC307 | Genoscope | 148242733<br>148242732 | 148242734 | 148242735 | – | – | – | – | 148243539 | – |
| Open ocean | 5.1A | III | *Synechococcus* sp. WH 8102 | Palenik *et al.* (2003) | 33866329 | 33866330 | 33866078 | – | – | – | – | – | – |
| Freshwater | – | – | *Synechococcus elongatus* PCC 6301 | Sugita *et al.* (2007) | 56750155<br>56751929[e] | 56750157 | 56750158 | – | – | – | 56752535 | 56751933 | 56750011 |
| Freshwater | – | – | *S. elongatus* PCC 7942 | JGI/DOE | 14331111 | 81300216 | 81300215 | – | – | – | 81300350 | 81300980 | 81300351 |
| Freshwater | – | – | *Synechocystis* sp. PCC 6803 | Kaneko *et al.* (1996) | 16329434<br>16331793 | 16331231 | 16330805 | 16329800 | 16329907[f]<br>16332097 | – | 16330539 | – | 16330540 |
| Freshwater | – | – | *Synechococcus* sp. JA-2-3B′a(2-13) | TIGR | 86610096 | 86610098 | 86607691 | – | – | – | 86609709 | 86608902 | – |
| Freshwater | – | – | *Synechococcus* sp. JA-3-3Ab | TIGR | 86606749 | 86606751 | 86607396 | – | – | – | 86607220 | 86606482<br>86607086 | – |

**a.** Designation based on isolation location and information in reference column.
**b.** Subcluster designation from Dufresne and colleagues (2008).
**c.** Clade designation from Fuller and colleagues (2003), Ahlgren and Rocap (2006) and Dufresne and colleagues (2008); RCC307 was formerly in clade X.
**d.** Moore, Gordon and Betty Moore Foundation; DOE, Department of Energy, USA; JGI, Joint Genome Institute; TIGR, The Institute for Genomic Research; JCVI, J. Craig Venter Institute.
**e.** gil56751929 is equivalent to gil56686888 and gil2125893.
**f.** The Bfr designation is not based on the presence of glutamate position 60 (Andrews 1998) as normal, it is an exception from Andrews (1998).
**g.** These strains form a separate monophyletic group between IsiA and Pcb proteins; their role in Fe stress is not known.

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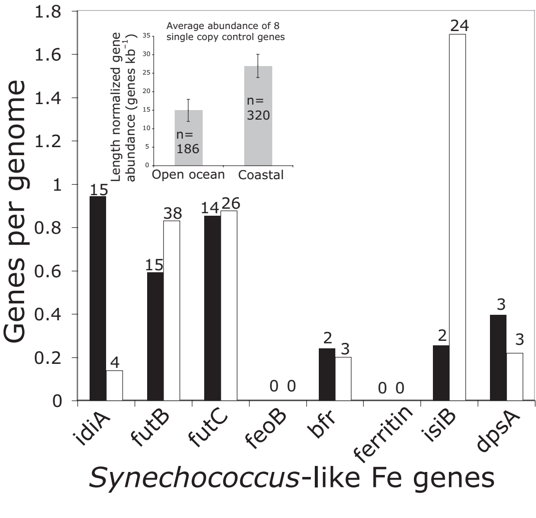


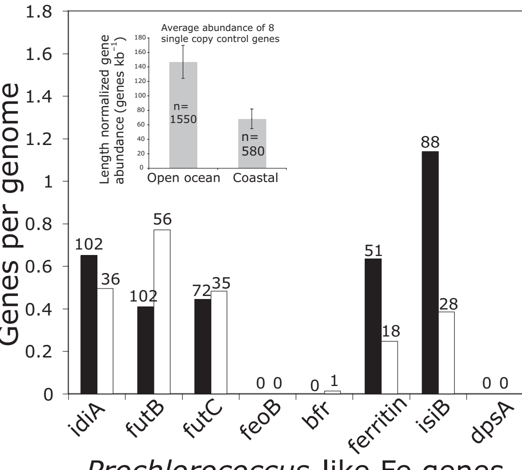


**Fig. 1.** The abundance of Fe stress genes from coastal and open ocean sites in GOS metagenome with best hits to known *Synechococcus* and *Prochlorococcus* genes. Gene abundance is reported in genes per genome. This was estimated by dividing the normalized number of GOS reads recruited for a particular gene by the mean normalized abundance of eight single-copy control genes. Both the control and the Fe genes were length-normalized using the average length of the query gene. The number on the top of each bar represents the total number of GOS reads that correspond to the gene. The inset graph reports the normalized abundance and standard error of the control genes.

genes were first normalized by length, then divided by the mean normalized abundance of the eight single-copy control genes used in Martiny and colleagues (2006). The most abundant open ocean *Synechococcus* Fe stress genes were the genes *idiA*, *futB* and *futC* at abundances between 0.6 and 1 copy per genome. Genes with best hits to bacterioferritin, flavodoxin and *dpsA* were found in 0.2–0.4 copy per genome, and *feoB* and ferritin were not found. Coastal *Synechococcus* showed a similar pattern with two exceptions: *isiB* was more abundant, found in 1.7 gene per genome, and *idiA* was less abundant, found in less than 0.2 gene per genome. Extending this analysis to genes found in the GOS data with best hits to *Prochlorococcus*, we found a more uniform distribution. Among these genes, open ocean sites have 0.4–0.6 copy of *idiA*, *futB* and *futC*, 0.6 copy of ferritin and 1.2 copy of *isiB*: coastal sites have 0.5–0.8 copy of *idiA*, *futB* and *futC*, and 0.2–0.4 copy of ferritin and *isiB*. *Prochlorococcus* genes from all sites have less than 0.1 copy of bacterioferritin and no copies of *feoB*, or *dpsA*. It is important to note that this analysis provides only an initial estimate of relative gene presence in these regions as the metagenomic data sampled a small number of *Prochlorococcus* genomes and even fewer *Synechococcus* genomes (Rusch *et al.*, 2007).

*Generation and testing of an IdiA antiserum*

To develop an Fe stress assay focused on IdiA expression, we generated an antiserum against recombinant IdiA from *Synechococcus* sp. WH8102 expressed in *Escherichia coli* as described in *Experimental procedures*. The removal of a predicted *Synechococcus* signal sequence allowed for the expression of histidine-tagged IdiA in the host strain *E. coli* strain EA38 (Fig. S1). Once an antiserum had been produced against this protein, the reactivity of the antibody was determined by immunoblotting against a cell-free extract from *Synechococcus* sp. WH8102. We confirmed that the antiserum was effective for Western blot detection of IdiA using a titer of 1:175 000 without any detectable background bands (data not shown).

Further experiments with other *Synechococcus* strains determined that the antiserum was highly specific, as it reacted only with *Synechococcus* from clades III and V and with *Synechococcus* sp. WH8101 from clade VIII (Fig. 2) (Rocap *et al.*, 2002; Ahlgren and Rocap, 2006). The antiserum was not cross-reactive with other *Synechococcus* clades or *Prochlorococcus marinus* sp. MED4 (data not shown). Non-specific banding typically did not occur within clades III and V except for a faint lower-molecular-weight band in *Synechococcus* spp. WH7803 and WH8103 that co-occurred with the IdiA band. Once the cross reactivity was determined, it was necessary to test whether IdiA accumulates just in response to Fe limitation and/or in response to more general oxidative stress as well. Middle to late log phase *Synechococcus* sp. WH7803 was exposed to 3 mM hydrogen peroxide or 100 nM of the herbicide methyl viologen, and IdiA accumulation was measured by densi-

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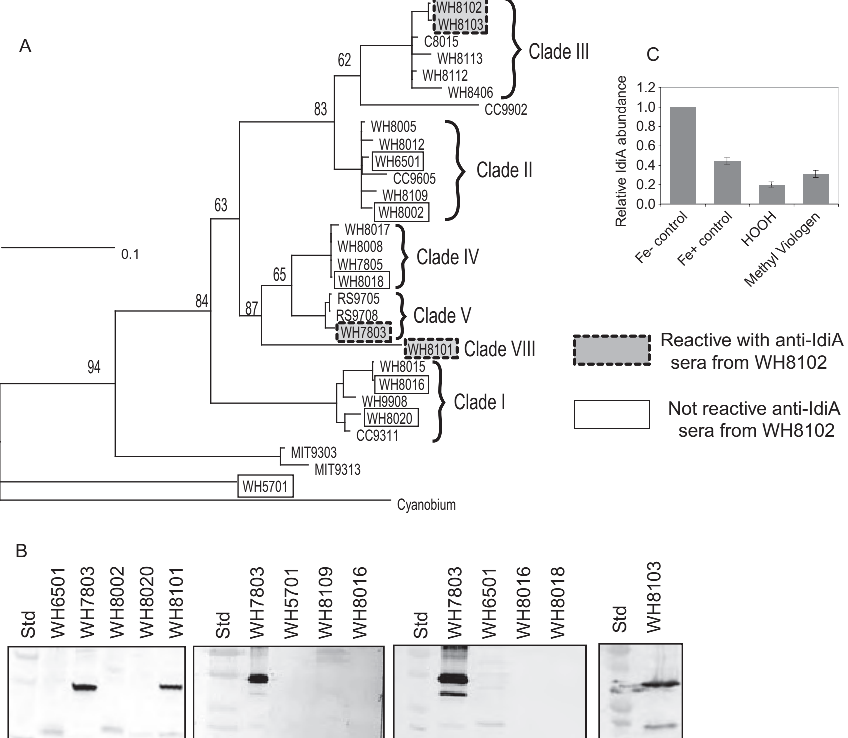


**Fig. 2.** The cross-reactivity of antiserum raised against recombinant IdiA from *Synechococcus* sp. WH8102. Cross-reactivity is shown in a phylogenetic context using a maximum likelihood tree of the ITS sequences (A) (Rocap *et al.*, 2002) and with immunoblots (B); numbers represent quartet puzzling scores. Strains from clades III and V are cross-reactive as is *Synechococcus* sp. WH8101 from clade VIII. A faint lower-molecular-weight band was common for *Synechococcus* sp. WH7803, WH8103 and WH8101 but its presence always coincided with IdiA expression; its intensity varies because of differences in development time.
C. IdiA does not accumulate in response to general oxidative stress. Relative IdiA concentration measured by densitometry from a Western blot for oxidatively stressed cells compared with cells grown in Fe-replete or -deficient conditions. Cells were harvested in late log phase. Three biological replicates were used for all conditions except Fe+ where a representative biological sample grown in Fe-limited 5 nM Fe media was used.

tometry on a Western blot. IdiA expression was compared with Fe-replete cultures and a Fe-limited positive control (Fig. 2C). Both oxidative treatments had slightly less IdiA than the Fe-replete culture and 20–30% of the IdiA in Fe-limited positive control, suggesting that oxidative stress does not lead to IdiA accumulation.

### *Temporal and environmental expression of IdiA*

An assay was developed to label IdiA in individual cells. The assay requires heating then fixing the cells prior to storage. The cell wall is then permeablized and IdiA is labelled with a primary antibody, then a secondary antibody conjugated to a fluorophore for detection by flow cytometry. To test the assay and determine the timing of IdiA expression, the protein was measured in batch cultures of *Synechococcus* sp. WH7803 by whole-cell labelling and spot-checked with Western blotting (Fig. 3). Fresh Fe+ (Fe-amended) and Fe– (Fe-omitted) media was inoculated with a culture transitioning into Fe limitation (cell density $1.2 \times 10^7$ cells $ml^{-1}$, $k = 0.0185\ h^{-1}$, from regression of log-transformed fluorescence, $R^2 = 0.99$). The Fe+ culture contained 20 μM Fe while the Fe– culture contained only the Fe present in the coastal seawater used to prepare the media. To control for differences in fixation and labelling, IdiA expression is reported as the fluorescence of cells treated with IdiA antiserum divided by the fluorescence of those treated with pre-immune serum. The first two measurements of IdiA expression in Fe+ and Fe– cultures, at 27 and 39 h, had a high fluorescence ratio of ~8. This IdiA persisted from the inoculating culture for several days after the initial transfer, then between 39 and 90 h the IdiA expression level dropped to the baseline value of approximately 3.5 for both Fe+ and Fe– cultures (Fig. 3B). By 114 h the expression of IdiA in Fe– cultures had increased to an expression ratio of

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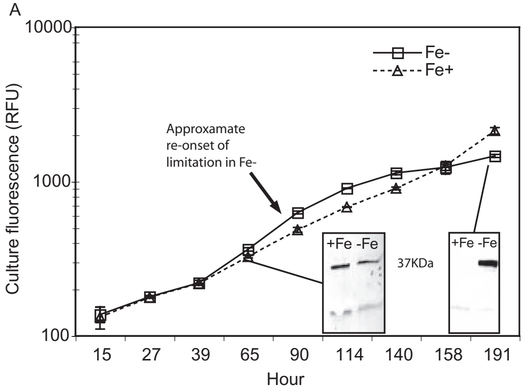


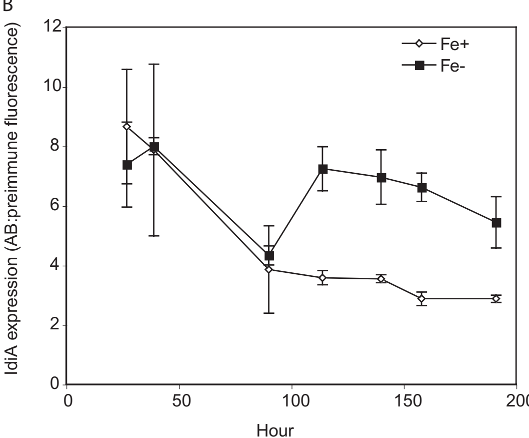


**Fig. 3.** A. The growth of *Synechococcus* sp. WH7803 represented by the orange fluorescence of cultures in Fe-replete and Fe-limited conditions and IdiA expression at two time points quantified by immunoblotting. The increase in growth rate for the Fe– culture between 39 and 90 h may be an experimental artefact.
B. The expression of IdiA in cultures inoculated from Fe-limited media into Fe+ medium containing 20 μM Fe or Fe– media containing only ambient Fe in the coastal seawater used to prepare the medium. The IdiA expression is reported as the green fluorescence per cell for IdiA antiserum-treated cells normalized to the green fluorescence of the pre-immune control measured by flow cytometry. Error bars in (A) and (B) represent standard error; in (A), standard error was generally smaller than the symbols.

~7, while the Fe-replete cells remained at the baseline expression ratio of ~3.5. Immunoblotting at the end of the experiment confirmed this persistent differential expression. This expression pattern agrees with previous Western blotting work on the timing of IdiA expression (Webb *et al*., 2001).

The assay was also tested on a field sample collected from the CRD, a cyclonic upwelling feature (Fiedler, 2002) with a strong seasonal *Synechococcus* bloom that may be limited by Fe (Franck *et al*., 2003) (Fig. 4). Station 13 near the middle of the dome (09°30′N, 92°19′W), was sampled on 25 July 2005 at 12:15 UTC (dawn). The station's thermocline, halocline and chlorophyll maximum were all at 15 m. The Fe concentration at station 13 was extremely low, ranging from 0.08 to 0.14 nM in the upper 80 m. *Synechococcus* were abundant, reaching a density of $9 \times 10^5$ cells $ml^{-1}$ at the surface and declining quickly below the thermocline. Cells from 8, 15, 25 and 40 m were subjected to the assay. Among these samples, 5–54% of the population had a significantly greater fluorescence ($P < 0.05$, using probability binning) after treatment with the anti-IdiA antiserum compared with treatment with preimmune serum alone. The proportion expressing IdiA and the expression ratio (antiserum-treated fluorescence over pre-immune serum-treated fluorescence, corresponding to specific immunological detection of IdiA) mirrored changes in Fe concentration directly. The histograms of labelling at each depth reveal that the community is finely structured in regard to its IdiA expression and/or reactivity. For example, at 8 m there appear to be two subpopulations of cells with cross-reactive IdiA. Just 7 m farther down at the 15 m thermocline, cross-reactive IdiA appears not to be expressed by any part of the population. Below the thermocline at 25 m the community resembles the 8 m assemblages.

## Discussion

### *Genomes and metagenomics*

There is considerable variation in the Fe stress genes found in sequenced marine *Synechococcus* and *Prochlorococcus* genomes. Interestingly, the genomes of open ocean *Synechococcus* spp. WH7803 and RCC307 have only *idiA*, *futB*, *futC* and *dpsA* while *Synechococcus* sp. WH8102 lacks even *dpsA*. In contrast, the genomes of coastal strains (e.g. *Synechococcus* spp. CC9311, CC9902, WH5701, BL107 and RS9917) contain most of the known Fe stress genes present in other cyanobacteria. None of the sequenced open ocean strains (*Synechococcus* spp. WH7803, WH7805, RCC307 or WH8102) are from regions of predicted Fe limitation (Wu *et al*., 2000; 2001); therefore, our open ocean strains may not be universally representative. However, this pattern contrasts sharply with *Prochlorococcus*, where every sequenced strain – isolated from a Fe-limited regime or not – contains Fe storage and substitution genes. The Fe stress gene distribution in *Prochlorococcus* more closely resembles that of coastal rather than open ocean *Synechococcus*. Coastal Fe limitation has been observed in areas including the California Current and the Peru Upwelling (Hutchins *et al*., 1998; 2002; Bruland *et al*., 2001), suggesting that Fe limitation exerts the most selective pressure on *Synechococcus* near the coast or in upwelling zones rather than in oligotrophic regions. This trend was

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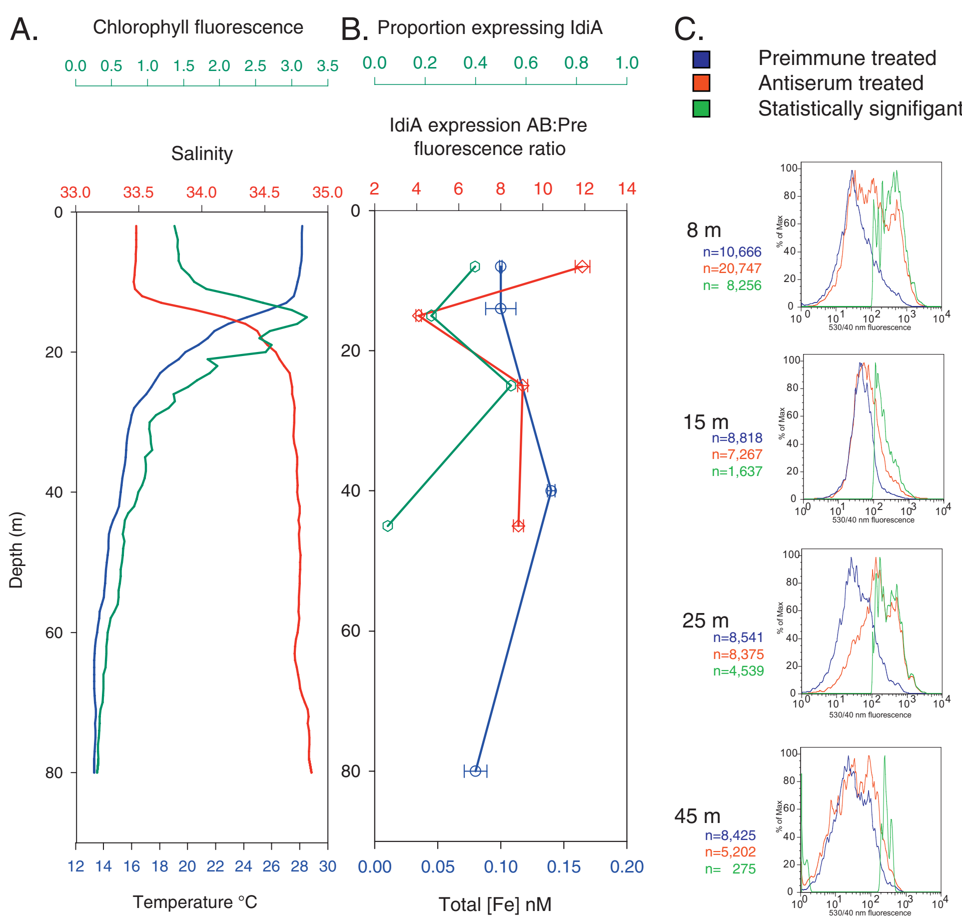


**Fig. 4.** IdiA expression in a natural *Synechococcus* population from the CRD.
A. The thermocline and halocline occur at depth of 15 m and the chlorophyll maximum coincides with the thermocline.
B. Fe concentration is very low ranging from < 0.08 to 0.14 nM; at 8 and 14 m Fe was below the daily detection limit of 0.08 nM; error bars represent standard deviation. IdiA expression is reported by two metrics. In red the proportion of the population that is statistically distinct from the pre-immune treated population at $P < 0.05$ is reported. The green line reports the ratio of flurophore fluorescence for cells treated with the antiserum to cells treated with the pre-immune control serum error bars represent standard error.
C. The histograms of fluorophore fluorescence at four depths. Each line is normalized so its highest point is equal to 100%; blue represents the pre-immune sample, red the antiserum-treated sample and green portion of the antiserum-treated population with fluorescence statistically distinct from the pre-immune control.

seen more broadly for all metal transporters in a comparison of *Synechococcus* spp. CC9311 and WH8102 (Palenik *et al*., 2006). It is important to note that *Synechococcus* is frequently more abundant in coastal settings if not dominant when compared with *Prochlorococcus* (Zwirglmaier *et al*., 2008), so these different trends in the genomes could also be related to spatial niche partitioning as well. More genomic data will likely help answer these questions.

The GOS data reveal a similar but subtler pattern in the distribution of Fe stress genes from coastal and open ocean environments. Most open ocean *Synechococcus* possess homologues to genes for $Fe^{3+}$ transport, while a smaller number possess homologues to genes for iron storage, flavodoxin substitution, and the stress gene *dpsA*. This smaller proportion possessing these additional genes may be adapted to transient Fe limitation. A major difference between coastal and open ocean *Synechococcus* is the abundance of *isiB* in coastal *Synechococcus.* The capacity to intermittently reduce the cellular Fe quota may reflect more rapidly fluctuating levels of Fe in coastal areas. One unexpected result in these data is the low abundance of *idiA* in coastal *Synechococcus* despite the presence of *futB* and *futC* at frequencies

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similar to those in open ocean *Synechococcus*. This contrasts with the genomic data on *idiA* and may indicate that the Fe stress assay described in this paper will be of more limited use in coastal environments. The genomic and metagenomic data for *Prochlorococcus* show a similar and more uniform complement of Fe stress genes, with homologues to the $Fe^{3+}$ transport genes, ferritin and flavodoxin. The number of *Synechococcus* genes in the GOS is relatively small, in part because the collection procedure removed phytoplankton larger than 0.8 μm (Rusch *et al*., 2007). The accurate assignment of genes to a genus is also dependent on having representative samples of *Synechococcus* and *Prochlorococcus* genes in the reference database (nr), which is an important caveat of this analysis. Despite its limitations, the GOS metagenome still provides a provisional estimate of the importance of Fe stress genes in the genomes of the picoplankton.

In this work, the environment where *Synechococcus* genes or strains were found has been crudely divided into 'coastal' and 'open ocean', but in reality each environment has complex chemistry and physics that influence which *Synechococcus* strain is dominant at a given time. Yet, this crude measure does appear to explain some of the differences in the genomes and metagenome. For example, many physiological differences have been explained by habitat specialization within clades and ecotypes, including light and temperature adaptation in *Prochlorococcus* (Moore *et al*., 1998; Moore and Chisholm, 1999; Johnson *et al*., 2006). Genomic comparison in *Prochlorococcus* showed that some niche partitioning (e.g. for light) occurred earlier in evolution than adaptation to particular nutrients (Kettler *et al*., 2007). In *Synechococcus*, work on the core genomes has grouped many strains into two subclusters, Subcluster 5.1A, containing primarily clades II, III and IV, is characterized as being adapted to stable environments, and Subcluster 5.2B (clades I, V, VI, VIII and IX) is characterized as being more adapted to variable environments (Dufresne *et al*., 2008). The presence/absence pattern of Fe stress genes in the genomes does not appear to correlate with the subclusters, suggesting that specialization to Fe stress occurred after the more general divergence between two types of core genomes. The pattern of Fe genes is better explained by the clade-specific community structure observed in sampling across ocean biomes (Zwirglmaier *et al*., 2008). Clades I/IV are found in temperate coastal and continental shelf areas while clade II is a tropical/subtropical counterpart to clades I/IV. These three clades include four of the top six genomes ranked by Fe gene abundance. In contrast, most genomes from the oligotrophic clade III and the more generalist clades V, VI and VII were from open ocean sites and had fewer Fe stress genes.

*Labelling assay*

Both genomic and metagenomic data point to IdiA as a good candidate for a Fe stress marker particularly in the open ocean. We generated a marine *Synechococcus*-specific IdiA antiserum to detect Fe stress in single cells using flow cytometry. The antibody labelling of intracellular proteins followed by flow cytometry is routinely used for eukaryotic cells but has not been widely used in bacteria (Jacobberger *et al*., 1986; Koester and Bolton, 2000). Intracellular proteins from cyanobacteria have been labelled, but the results have not been measured quantitatively with flow cytometry (Currin *et al*., 1990; Scanlan *et al*., 1997; Lin *et al*., 1998; Collier and Campbell, 1999; Toledo *et al*., 1999; Berman-Frank *et al*., 2001); conversely, flow cytometry has been used to measure surface protein abundance in *Synechococcus*, but has not been extended to measurements of intracellular proteins (Campbell, 1988; 1993; Campbell and Iturriaga, 1988). Coupling labelling with flow cytometry is an attractive tool for measuring cellular stress markers because it is quantitative and it rapidly provides expression estimates for thousands of individual cells. This approach is also of great value when studying field populations because it uses a small amount of biomass, approximately $10^4$ cells, and still generates data that can be analysed statistically.

The whole-cell labelling assay we developed is able to detect IdiA expression in both laboratory cultures and field populations where reactive clades are present. The sensitivity and large sample size of the flow cytometer makes it possible to differentiate two populations with slight differences in IdiA expression using probability binning (Roederer *et al*., 2001). The assay is also able to detect IdiA expression when only a small portion of the population is cross-reactive. One major limitation is that the assay cannot distinguish non-reactive cells from Fe-replete cells. As currently developed, this method is a sensitive measure of Fe stress within clades III, V and VIII of marine *Synechococcus*. Data on the global abundance *Synechococcus* clades indicate that cross-reactive clade III is abundant in oligotrophic regions (Zwirglmaier *et al*., 2008). With additional antisera, the assay could also be expanded to IdiA in more clades of *Synechococcus* or other genes altogether.

*Temporal and environmental expression*

*Synechococcus* sp. WH7803 responds *in vivo* to both the onset and alleviation of Fe stress by changing IdiA concentrations over 5 days with major changes in expression occurring over 1–2 days. This coincides with the timing and duration of dust pulses (Betzer *et al*., 1988) that are an important source of Fe in the open ocean (Jickells *et al*., 2005). The 1–2 day timescale for IdiA expression

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also acts as a biological averaging of the Fe stress response that may be useful in the field because it damps shorter-scale (e.g. diel) fluctuations in expression (Armbrust *et al*., 1989; Binder, 2000). The regulation of IdiA is pronounced and occurs on a measurable, biologically relevant timescale, making it an informative marker of Fe stress in the environment.

The method was tested in the CRD, an area of seasonal cyclonic upwelling with high phytoplankton biomass (Fiedler, 2002). The upwelling is unique because it is dominated by high concentrations of *Synechococcus*, rather than eukaryotic phytoplankton, with densities of *Synechococcus* in the surface of the CRD reaching $1.8 \times 10^6$ cells $ml^{-1}$ (Saito *et al*., 2005). These *Synechococcus* populations may potentially be limited by Fe (Franck *et al*., 2003).

When our field samples were collected, station 13 in the CRD (Fig. 4) had the characteristic shallow thermocline and large chlorophyll maximum described before (Fiedler, 2002). The extremely low levels of Fe observed (< 0.08–0.14 nM) are similar to those previously measured at the CRD (Franck *et al*., 2003). In this past study, the addition of Fe seemed to relieve Fe limitation in the microplankton community at the CRD, as evidenced by an almost threefold increase in total cell number and increases in the maximum potential uptake rates of silicic acid and $NO_3^-$ (Franck *et al*., 2003). The strong IdiA expression by *Synechococcus* just above and below the thermocline is consistent with these previous results and also supports the supposition that Fe may be a limiting nutrient to the picoplankton as well as the microplankton in this region. The decrease in IdiA detected at the chlorophyll maximum is likely the result of a non-reactive clade dominating at this depth. As can be seen in the 8 m histogram, the assay is also able to distinguish subpopulations with different IdiA expression/reactivity, although we are unable to attribute this difference to specific clades. However, the finding of physiological Fe stress in the field population is consistent with findings that upwelling regions both in California (Bruland *et al*., 2001) and Peru are also Fe-limited (Hutchins *et al*., 2002). This, combined with the observation that *Synechococcus* strains from coastal regions have more Fe stress genes, supports the idea that *Synechococcus*, and perhaps phytoplankton in general, are more Fe-stressed in coastal and upwelling regions than they are in the open ocean.

### *Future directions*

Looking at the genomic and metagenomic distribution of Fe stress genes has raised some interesting questions about the nature of Fe stress. It appears that *Synechococcus* from coastal and upwelling environments possess a wider range of genes to cope with Fe stress, while open ocean *Synechococcus* contain more putative $Fe^{3+}$ transport genes and *dpsA* homologues. Adaptation to Fe stress may be an important trait that distinguishes marine *Synechococcus* strains from each other and *Prochlorococcus*. The IdiA expression assay could be used to test whether *Synechococcus* endures different levels of Fe stress in coastal, upwelling and open ocean environments. With additional antisera for different clades of *Synechococcus*, the IdiA expression assay may potentially allow us not only to detect Fe stress but also to sort stressed cells using flow cytometry and to identify them by molecular approaches such as quantitative PCR of the internal transcribed spacer (ITS) region (Rocap *et al*., 2004; Zinser *et al*., 2006). We can then look for intrageneric differences in Fe stress, and perhaps definitively determine if Fe stress is an important physiological distinction between the clades of marine *Synechococcus*.

## Experimental procedures

### *Genomic analysis*

Genes involved in compensating for Fe stress were selected based on annotation in genomes or citations in literature. One or more amino acid sequences of these genes were used to perform BLASTP (Altschul *et al*., 1997) searches of the National Center for Biotechnology Information (NCBI) nr protein database using the BLOSUM 64 amino acid matrix, filtering low-complexity sequences out of the search but using those sequences to calculate final expect values. From the BLAST search a minimum evolution tree was constructed in the NCBI web interface as an estimate of the genetic similarity. The node containing the gene of interest in sequenced *Synechococcus*, *Synechocystis* and *Prochlorococcus* genomes was selected and genes in the subtree beyond the node were saved. These genes were aligned and maximum likelihood trees were constructed with Tree-Puzzle version 5.2 (Schmidt *et al*., 2002) using quartet sampling and neighbor joining based on the WAG model of substitution; branches were verified by quartet puzzling. The determination of homology was made based on the maximum likelihood tree and the gene predictions in the genome annotations; the distinction between bacterioferritin and ferritin was made based on the presence of an essential glutamic acid at residue 60 (Andrews, 1998).

### *Metagenomic analysis of iron stress genes*

The abundance of Fe stress genes in the GOS metagenomic data set was measured using a two-step BLAST (version 2.2.15) (Altschul *et al*., 1997) approach modified from Martiny and colleagues (2006). Only GOS sites designated as ‘open ocean’ or ‘coastal’ in the original GOS survey were included (Rusch *et al*., 2007). The set of DNA query sequences for a particular gene was composed of all homologues of that gene found in the sequenced genomes of *Synechococcus*, *Synechocystis* and *Prochlorococcus.* All unique reads recruited with an expect value $< 10^{-10}$ were kept. These reads were

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compared against the NCBI's nr database using BLASTX. The best hit based on bit score was retained (ties were broken by *E*-value). The best hits were tallied only if their gene annotation matched the query gene used to recruit the GOS read. The number of hits observed for a particular gene was normalized to the mean length of each query gene in kilobases. In addition to the selected Fe stress genes, eight single-copy control genes used by Martiny and colleagues (2006) were also tallied, then the length-normalized tally was used to express the abundance of Fe stress genes in terms of genes per genome.

### Synechococcus *cultures*

*Synechococcus* strains (Table 2) representative of six different clades defined by ribosomal ITS regions (Rocap *et al*., 2002) were grown in batch culture. Cells were grown in SN media modified by the substitution of ferric ammonium citrate for ferric citrate and the omission of cyanocobalamin (Waterbury *et al*., 1986) or in Fe-deficient SN media where ferric ammonium citrate was replaced with an equimolar amount of sodium citrate as appropriate. Reagents added to media were all purchased from Sigma (St Louis, MO), with the exception of $Na_2$-EDTA that was purchased from Fisher Scientific (Waltham, MA). The seawater base for the SN media was collected from Vineyard Sound, Woods Hole, MA, then 10 μm-, 5 μm- and 0.7 μm-filtered and stored in the dark until use. To determine antiserum cross-reactivity, 50 ml cultures were grown in 125 ml glass flasks under 15 $\mu E\ m^2\ s^{-1}$ of constant light. Culture growth was monitored by measuring phycoerythrin fluorescence in a TD700 Fluorometer (Turner Designs, Sunnyvale, CA) after aseptically transferring cultures into sterile acid-washed 25 mm glass tubes (Kimble/Kontes, Vineland, NJ). To determine if IdiA was induced under oxidative stress conditions 300 ml of *Synechococcus* sp. WH7803 was grown in 20 $\mu E\ m^2\ s^{-1}$ of constant light at 22°C to late log phase in an acid washed polycarbonate bottle. The culture was divided into nine 28 ml volumes. Three tubes were treated with methyl viologen (final concentration, 100 nM); three received 3 mM hydrogen peroxide for 1 h followed by the addition of 98 units of catalase; and three served as Fe-replete controls. Cells were tracked by flow cytometry for 40 h then harvested for Western blotting.

### *Generation of recombinant* Synechococcus *WH8102 IdiA*

Purified *Synechococcus* WH8102 IdiA was obtained using the PET histidine-tagging system marketed by Novagen (Madison, WI). Using the sequence from the WH8102 genome, PCR primers were designed to clone the *idiA* gene with the putative signal sequence removed and 5′ NdeI and 3′ BamHI sites incorporated into the amplified product (primer sequences: IdiA NdeI 5′-GGAGACAACCAGCCATATGG GCGTCTACTC-3′ and IdiA BamHI 5′-GACGACGGGA TCCTGCCAACCGTTGGCGGCCATCAGCTCGAGCG-3′). The PCR was performed using *Pfu* polymerase (Stratagene, La Jolla, CA) in a ABI2400 PCR machine (Applied Biosystems, Foster City, CA) with the following reaction conditions: 94°C for 5 min, then 25 cycles of 94°C for 1 min, 62°C for 1 min and 72°C for 4 min, and a final dwell of 72° for 10 min. The amplified product was visualized via agarose gel electrophoresis and purified from the gel using the Qiaquick Gel Extraction kit (Qiagen – Valencia, CA). The purified product was ligated into plasmid pET16b (pre-digested with NdeI and BamHI) and transformed into *E. coli* strain *E. coli* BL21 λΔE3 (F–, *omp*T, *hsd*$S_B(r_{B^-}, m_{B^-})$, *dcm*, *gal*, λ(DE3)). Putative clones were screened using restriction enzymes and the plasmid with the correct banding pattern was saved at –80°C as strain EA38. The recombinant IdiA protein was expressed from strain EA38 and visualized (Fig. S1) as described in Webb and Downs (1997).

The *Synechococcus* WH8102 IdiA-expressing extracts from strain EA38 were centrifuged at 11 300 *g* in a Beckman JA-20 rotor for 30 min to separate the soluble and insoluble fractions. As the insoluble fraction contained the recombinant IdiA, the inclusion body purification protocol described in the Novagen PET manual was performed. Once ~90% pure IdiA was obtained it was verified using LS-MS by M.A. Saito (data not shown). This partially purified IdiA was subjected to SDS-PAGE using a 12% polyacrylamide gel; the IdiA band was cut out and sent to Strategic Biosolutions (Windham, ME) for generation of a rabbit anti-*Synechococcus* WH8102 IdiA polyclonal antiserum.

**Table 2.** Synechococcus strains used in antiserum cross-reactivity experiments.

| Strain | Clade[a] | PUB/PEB[b] | Sea | Latitude | Longitude | Isolated by: | Date |
|---|---|---|---|---|---|---|---|
| WH8020 | I | 0.78 | Sargasso Sea | 38.67 | −69.57 | J. Waterbury | June 1980 |
| WH6501 | II | 0.43 | Tropical Atlantic | 8.73 | −50.83 | R. Guillard | June 1965 |
| WH8002 | II | 0.48 | Gulf of Mexico | 19.75 | 92.42 | L. Brand | April 1980 |
| WH8109 | II | 0.89 | Sargasso Sea | 39.48 | −70.47 | L. Brand | June 1981 |
| WH8102 | III | 2.06 | Sargasso Sea | 22.50 | −65.60 | J. Waterbury | March 1981 |
| WH8103 | III | 2.40 | Sargasso Sea | 28.50 | −67.40 | J. Waterbury | March 1981 |
| WH5701 | Marine B | NA | Long Island Sound | – | – | R. Guillard | 1957 |
| WH8101 | VIII | NA | Woods Hole | 41.52 | −70.67 | F. Valois | June 1981 |
| WH7803 | V | 0.39 | Sargasso Sea | 33.75 | −67.50 | L. Brand | July 1978 |
| WH8016 | VI | 0.40 | Woods Hole | 41.52 | −70.67 | F. Valois | June 1980 |
| WH8018 | VI | No PUB | Woods Hole | 41.52 | −70.67 | F. Valois | June 1980 |

**a.** Clade data from Rocap and colleagues (2002) and Ahlgren and Rocap (2006), all other data from Waterbury and colleagues (1986).
**b.** PUB/PEB is the ratio of the pigments phycourobilin to phycoerythrobilin expressed as absorbance at 495 and 545 nm respectively.

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### *Western blots*

For each sample, 5–10 μg of protein extract per lane was either loaded onto a 12.5% Hepes-buffered acrylamide gel (Pierce, Rockford, IL) and run for 45 min at 120 V, or loaded onto a 12.5% Tris-buffered acrylamide gel and run for 35 min at 200 V in a MiniProtean III gel box (Bio-Rad, Hercules, CA). Gels were either transferred by electrophoresis to 0.45 μm nitrocellulose membrane (Bio-Rad, Hercules, CA) for Western blotting or silver-stained (Owl Scientific, Portsmouth, NH) to confirm the presence of bulk protein. In each strain, Fe-replete and Fe-deficient cultures were tested for reaction to the immune serum of rabbit anti-WH8102 IdiA and to the pre-immune serum from the same rabbit. All blotting was done with an amplified alkaline phosphatase blotting kit (Bio-Rad, Hercules, CA) according to the manufacturer's directions, except for the oxidative stress experiments, which used a chemiluminescent blotting kit (Pierce, Rockford, IL).

### *Whole cell labelling*

For whole-cell labelling, cultures of WH7803 were grown in triplicate at 15 $\mu E\ m^{-2}\ s^{-1}$ of constant light intensity in the modified SN media described above, in acid-cleaned 500 ml glass flasks with a culture volume of 250 ml. Growth was monitored by fluorescence as described above and samples for total protein extraction and Western blotting were taken at 2.5 and 7.5 days. Whole-cell labelling samples were taken approximately daily (Fig. S2).

For whole cell labelling, 1 ml of cells varying in concentration from $\sim 1 \times 10^4$ to $\sim 1 \times 10^7$ cells $ml^{-1}$ (depending on growth phase and experiment) was placed in a 1.5 ml centrifuge tube and heat-bleached for 1 h at 65°C to reduce endogenous phycobiliprotein fluorescence (Scanlan *et al.*, 1997). The bleached cells were fixed in seawater media at 4°C with the addition of 0.25 vol. of 4% paraformaldehyde (Harlow and Lane, 1999), incubated overnight (~12 h) at 4°C in a 1% (final concentration) solution of paraformaldehyde, and pelleted and re-suspended in 1× phosphate-buffered saline (PBS) (Harlow and Lane, 1999). Just prior to labelling, the cells were transferred into 96-well Multicsreen plates with a 0.45 μm polycarbonate filter (Millipore, Billerica, MA), which allowed for rapid simultaneous fixation and labelling (Fig. S2). The cell wall was permeabilized using the procedure of Currin and colleagues (1990) except that cells were incubated at 37°C and the lysozyme concentration was 0.5 mg $ml^{-1}$. Permeabilized cells were washed twice for 5 min with a wash solution of 0.025% Triton X-100 in PBS. The cell membrane was permeabilized for 1 h at 25°C with permeabilizing solution (0.5% Triton X-100, 50 mM glycine in PBS) and washed twice with wash solution.

After membrane permeabilization, a 1:100 strength solution of anti-WH8102 IdiA antiserum or pre-immune serum was added as appropriate. The serum was diluted in a 0.22 μm-filtered solution containing 3% bovine serum albumen, 0.025% Triton X-100 and 0.02% sodium azide in PBS. The cells were incubated in the serum solution at 25°C for 1 h then washed three times with wash solution. A 1:200 dilution of Alexaflour 488 fluorochrome tagged goat anti-rabbit antiserum (Molecular Probes, Eugene, OR) was prepared as described for the primary antiserum. The cells were incubated in 75 μl of secondary antiserum solution in the dark at 25°C for 1 h. After this final step the cells were washed 2× with wash solution and once with PBS, then re-suspended in PBS. Cells were transferred out of the plates just prior to analysis with the flow cytometer.

### *Field sampling*

Approximately 800 ml of seawater was collected from station 13 at 8, 15, 25 and 45 m depth aboard the R/V Knorr cruise 182-5 and filtered onto a 25 mm, 0.45 μm polycarbonate filter using a peristaltic pump. The filter was placed in a 1.5 ml tube with 1 ml of 0.2 μm filtered seawater and agitated. The re-suspended cells were fixed and labelled as described in the previous section except that a 1:100 dilution of the secondary antibody was used and cells were incubated in 25 μl of primary and secondary antiserum.

### *Fe measurements*

All samples were collected using trace metal clean techniques. Samples were filtered through acid-cleaned 0.4 μm polycarbonate filters into rigorously acid-cleaned low-density polyethylene bottles. After filtration, samples were acidified to approximately pH 2 by the addition of 2 ml of concentrated HCl (Seastar) per litre of seawater. Total dissolved Fe concentration was measured using isotope dilution and magnesium hydroxide pre-concentration followed by analysis with inductively coupled plasma mass spectrometry (ICP-MS) after Wu and Boyle (1998) and Saito and Schneider (2006). Acid-washed 15 ml polypropylene centrifuge tubes (Globe Scientific Inc.) were rinsed once with sample and then filled with 13.5 ml of sample (exact volume determined gravimetrically). Samples were then spiked with a $^{57}Fe$ spike (> 95% as $^{57}Fe$, Cambridge Isotope Laboratories) and allowed to equilibrate overnight. The following day, 125 μl of 11 M ammonium hydroxide (Seastar) was added to each tube. After 90 s, the tube was inverted and after an additional 90 s, tubes were centrifuged for 3 min at 3000 *g* (3861 r.p.m.) using a swinging bucket centrifuge (Eppendorf). The majority of the supernatant was decanted carefully and then tubes were re-spun for 3 min to form a firm pellet and the remaining supernatant was shaken out. Pellets were stored dry until the day of analysis (no longer than a few days). Pellets were dissolved on the day of ICP-MS analysis using 0.5–1.5 ml of 5% nitric acid (Seastar). ICP-MS measurements were made using a Finnigan ELEMENT2 in medium resolution mode. The reported values have had the procedural blank subtracted. To measure the procedural blank, 1 ml of low-Fe surface seawater was treated as the samples but calculations were performed as though it was a 13.5 ml sample (Fe contribution from the 1 ml was subtracted from blank value). The average blank value was 0.08 nM and the average detection limit was 0.08 nM. When values fell below the daily detection limit, they are reported as less than daily detection limit.

### *Flow cytometry*

Flow cytometry on the laboratory experiments was performed on FACSCalibur flow cytometer (Becton, Dickinson, Franklin

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Lakes, NJ) using a 488 nm argon laser as an excitation source. The Alexafluor 488 signal of the population was measured from the mean fluorescence in the 530/30 nm channel. Cells were diluted as necessary in the same PBS buffer so that fluorophores would be exposed to the same pH. The field sample was measured using a Cytopeia Influx flow cytometer (Seattle, WA) using a 488 nm laser. Labelling was measured in the 530/40 nm channel.

Post-collection analysis of the fluorescent intensity and associated statistics were calculated using FlowJo for Macintosh version 8 (Tree Star, Ashland, OR). Populations were gated by chlorophyll and forward scatter. The mean of the 530/30 nm channel was calculated for the pre-immune and immune treatments of each biological replicate. In the laboratory samples the antibody-treated mean fluorescence was divided by the pre-immune serum-treated fluorescence for each biological replicate. As a summary statistic, the ratio and standard error were calculated from the ratios by bootstrap estimation ($n = 50$) in Stata 9.1 (Statacorp, College Station, TX). In the field sample, probability binning (Roederer *et al*., 2001) was used to gate the portion of the cell population with > 95% chance of being distinct from the pre-immune control population. The method was also used to identify the population with > 95% probability of being part of the control population.

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## Supporting information

Additional Supporting Information may be found in the online version of this article:

**Fig. S1.** Heterologous expression and partial purification of histidine-tagged *Synechococcus* sp. WH8102 IdiA. (A) Comparison of banding between extracts of *E. coli* strains containing plasmids with or without IdiA insert: non-induced pET16b (1), induced pET16b (2), pET16b-IdiA1 (3) and pET16b-IdiA2 (4). (B) Partial purification of his-IdiA cloned into *E. coli.* Shown are: molecular weight standard (1), column flow through (2), wash flow through (3), and eluted protein (4). Arrows denote IdiA.

**Fig. S2.** The procedure for labeling cells. Samples were taken and heat bleached for 1 hr at 65°C to remove endogenous phycobiliprotein fluorescence, then fixed with 1% paraformaldehyde at 4°C overnight, rinsed and stored at 4°C. The pre-immune control samples and their matched experimental samples are drawn from the same tube to minimize fixation and cell density differences. The permeabilization and labeling are done simultaneously in 96-well plates to further reduce variation in labeling.

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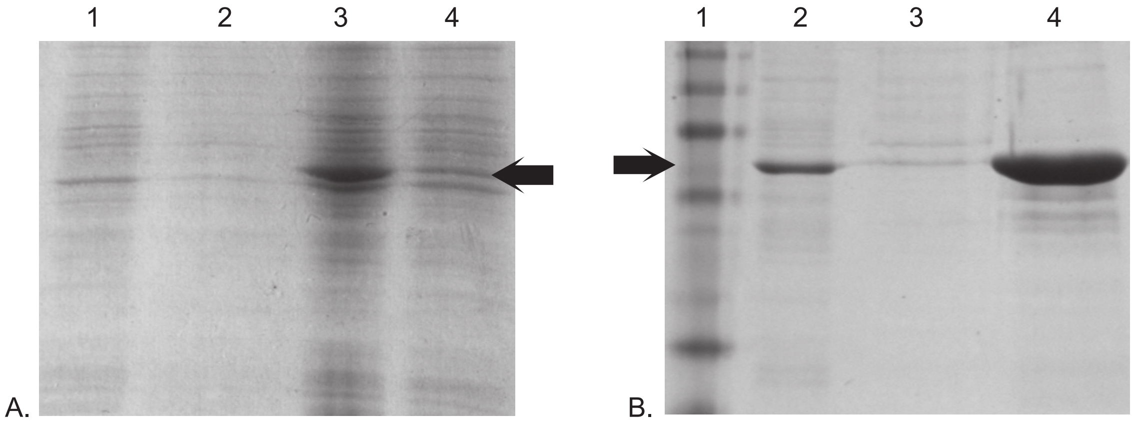
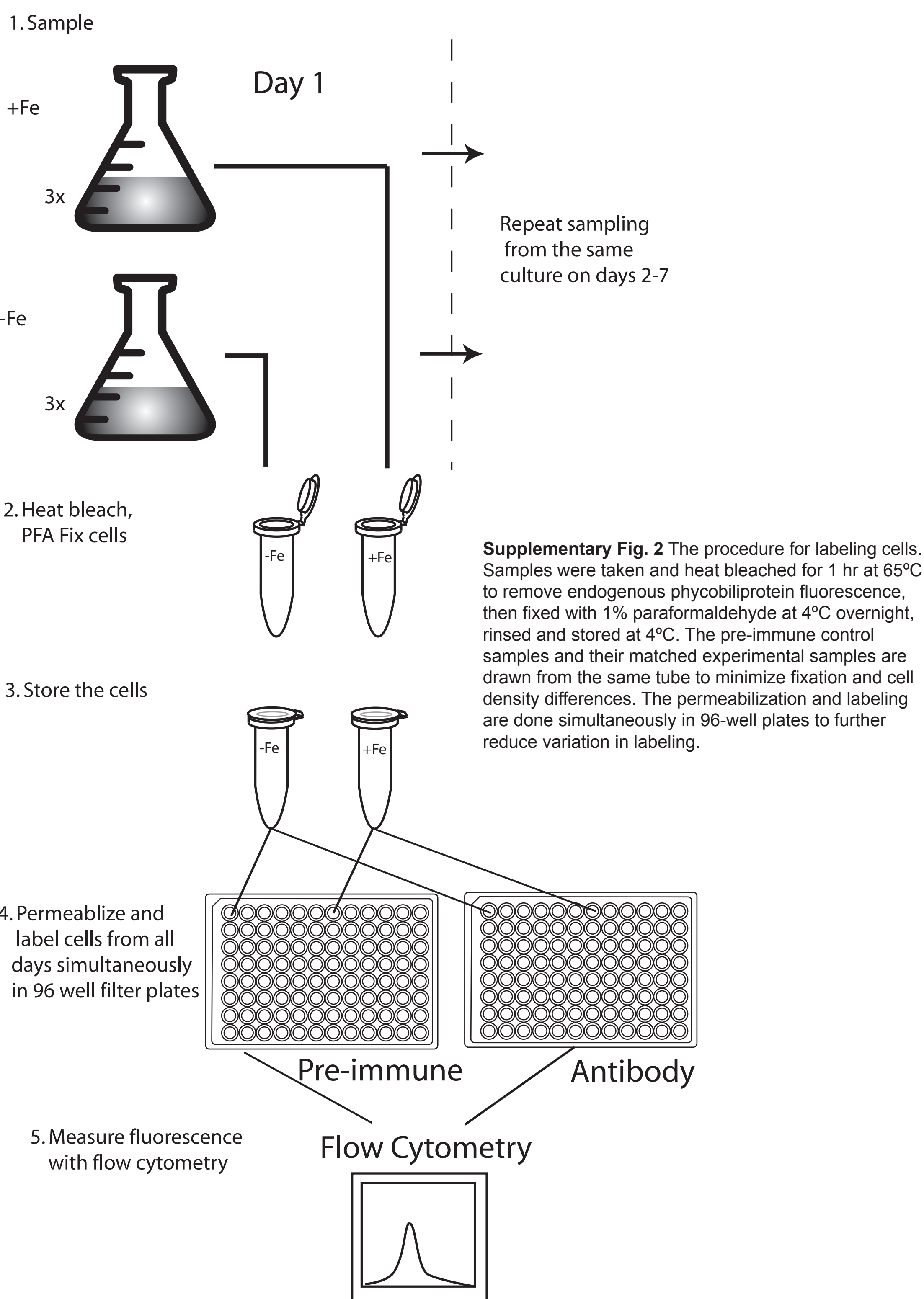
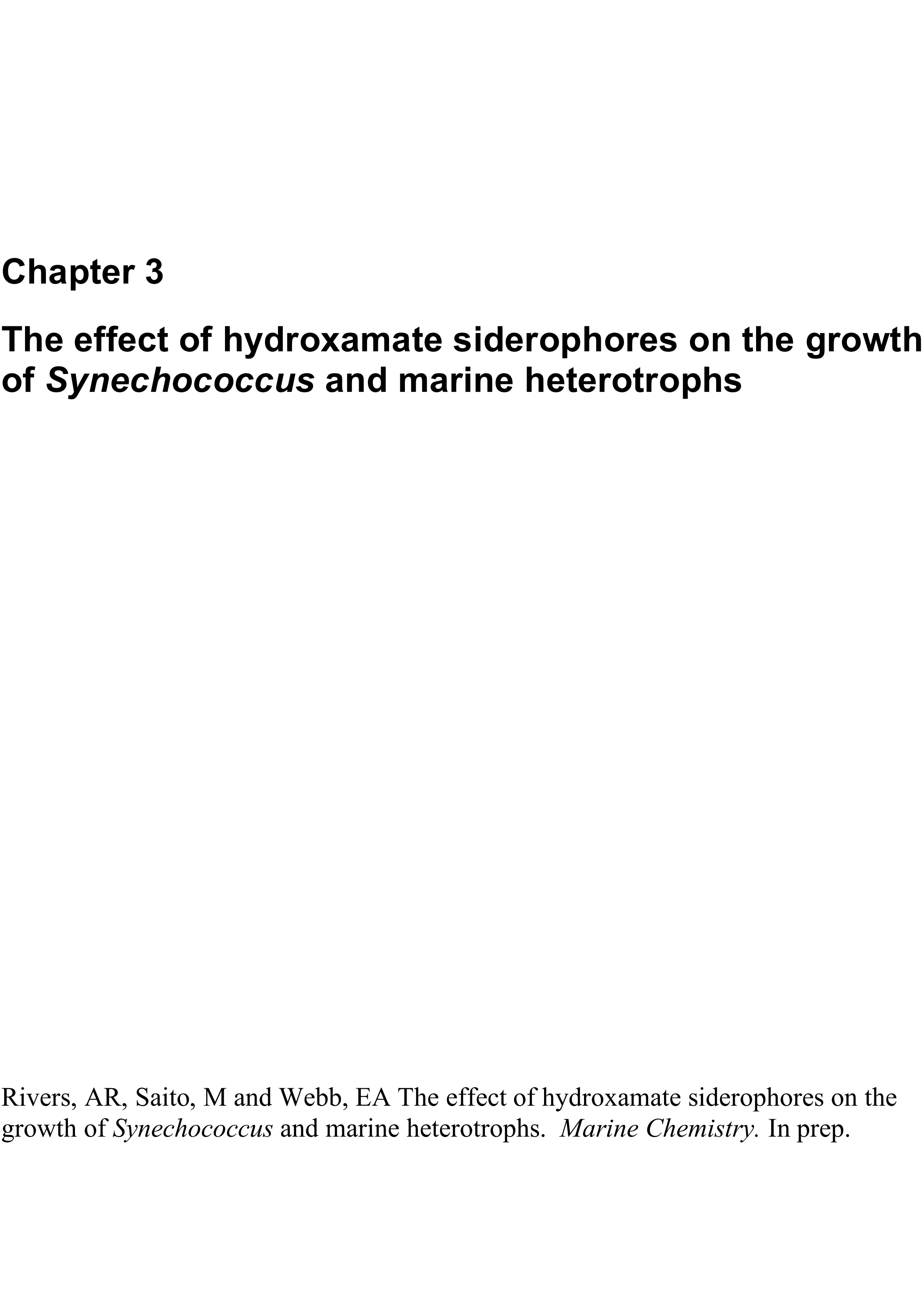



**Supplementary Fig. 1** Heterologous expression and partial purification of histidine-tagged *Synechococcus* sp. WH8102 IdiA. (A) Comparison of banding between extracts of *E. coli* strains with or without IdiA insert: non-induced pet16b (1), induced pet16b (2), pet16b-IdiA1 (3) and pet16b-IdiA2 (4). (B) Partial purification of his-IdiA cloned into *E. coli.* Shown are: molecular weight standard (1), column flow through (2), wash flow through (3), and eluted protein (4). Arrows denote IdiA.




**Supplementary Fig. 2** The procedure for labeling cells. Samples were taken and heat bleached for 1 hr at 65ºC to remove endogenous phycobiliprotein fluorescence, then fixed with 1% paraformaldehyde at 4ºC overnight, rinsed and stored at 4ºC. The pre-immune control samples and their matched experimental samples are drawn from the same tube to minimize fixation and cell density differences. The permeabilization and labeling are done simultaneously in 96-well plates to further reduce variation in labeling.



# Chapter 3

# The effect of hydroxamate siderophores on the growth of *Synechococcus* and marine heterotrophs

Rivers, AR, Saito, M and Webb, EA The effect of hydroxamate siderophores on the growth of *Synechococcus* and marine heterotrophs. *Marine Chemistry.* In prep.

## Abstract

Siderophores are iron-binding molecules synthesized by a wide range of bacteria and fungi in iron-limited environments ranging from oceans to animal hosts. In the oligotrophic ocean many heterotrophic bacteria produce siderophores but oligotrophic strains of *Synechococcus* of *Prochlorococcus* are not known to produce siderophores, despite average surface ocean Fe concentrations of just 0.07 nM. To understand the physiological effects of siderophores and the bioavailability of Fe bound to siderophores, we examined the influence desferrioxamine B (DFB) on a natural plankton population and two strains of *Synechococcus*. In experiments with natural phytoplankton populations from the Sargasso Sea, DFB inhibited the growth of *Synechococcus* in a concentration-dependent manner at as little as 20-40 nM while simultaneously stimulating the growth of heterotrophic bacteria. The severity of the inhibition of *Synechococcus* could not be attributed to decreases total or in free Fe. The growth inhibition is reversible but its severity is increased by light. The hydroxamate siderophore Ferrichrome also inhibited *Synechococcus* suggesting the effect may extend to other hydroxamate siderophores. The bacteriostatic effect DFB at such low concentrations raises the possibility that siderophores may serve some antibiotic function the environment.

## Introduction

Siderophores are biogenic molecules that bind Fe(III) with extremely high stability. These molecules are used for iron scavenging by free-living and pathogenic bacteria and fungi (Miethke and Marahiel, 2007; Neilands and Leong, 1986) and there is emerging evidence that siderophores are ecologically important in the oceans. The hydroxamate siderophores ferrioxamine G and ferrioxamine E are widely distributed in the Atlantic at concentrations of 3-20 pM representing as much as 4.6% of the soluble Fe pool (Mawji et al., 2008). Ferrioxamine G, E and other siderophores may represent an important portion of the Fe ligands that collectively bind ~99% of the Fe in the ocean (Rue and Bruland, 1995; Witter et al., 2000).

The bioavailability of siderophores varies in different marine phytoplankton and bacterioplankton. The eukaryotic diatom *Thalassiosira oceanica* can take up iron bound to DFB by reducing the ligand complex (Maldonado and Price, 2001). In laboratory experiments this Fe uptake supported growth, but surprisingly growth rates of *T. oceanica* decreased as the concentration of the bioavailable FeDFB complex increased (Maldonado and Price, 2001). Another diatom *Ditylum brightwellii* has also been shown grow on Fe complexed to DFB (Naito et al., 2008). Other eukaryotic phytoplankton were unable to grow on DFB bound Fe including isolates the groups Raphidophyceae, Dinophyta, Dinophyta, Haptophyta and Chlorophyta when 2 µM Fe was supplied pre-complexed to 20 µM DFB (Naito et al., 2008). The chlorophyte *Chlorella vulgaris* was able to take up Fe from DFB but its ability to grow on Fe was not assessed (Allnutt and

Bonner, 1987). Less is known about what forms of Fe are bio-available to cyanobacteria. A culture of *Synechococcus* sp. CCMP1334 (equivalent to *Synechococcus* sp. WH7803) was reported to take up Fe from DFB, but growth was not measured (Hutchins et al., 1999). Siderophore bound Fe appears to be most bioavailable to heterotrophic marine proteobacteria, in one experiment every one of the seven heterotrophic strains tested used the FeDFB complex for Fe uptake and growth (Granger and Price, 1999).

Siderophores are produced by many marine bacteria including, heterotrophic bacteria in the genera *Marinobacter* (Martinez and Butler, 2007), *Halomonas* (Martinez et al., 2000), *Vibrio* (Martinez et al., 2001) , *Alteromonas* (Reid and Butler, 1991) *Pseudoalteromonas* (Kanoh et al., 2003), and *Roseobacter* (Krey, 2008). Siderophores were produced by 40% of heterotrophic bacteria in a culture library of isolates from the eastern subtropical Pacific (Krey, 2008). The coastal marine cyanobacteria *Synechococcus* spp. PCC7002 and WH8101 also produce siderophores, however these compounds have yet to be found in oligotrophic cyanobacteria. The system that provides the energy needed to actively transport siderophore-bound Fe (TonB-ExbD-ExbD) has yet to be identified in any of the *Synechococcus* or *Prochlorococcus* genomes, suggesting the siderophore secretion is uncommon in oligotrophic picocyanobacteria. It is unclear why siderophore production is more common in heterotrophic bacteria than cyanobacteria from the open ocean.

The ecological role of siderophores in the open ocean may differ from other environments. Cell densities in the open ocean are low relative to coastal and terrestrial environments, but the open ocean also contains particles where cell densities can be

higher. In a dense biofilm, secreting Fe scavenging molecules is likely to benefit the group but the energetic cost of secreting siderophores is higher in more diffuse environments. A model of siderophore secretion in the ocean suggests that the process is only effective when high densities of cells collectively secrete a siderophore. (Völker and Wolf-Gladrow, 1999). The collective process is rendered less effective by siderophore piracy from unrelated bacteria (Stintzi et al., 2000) or genetically related cheaters who take up siderophores without producing them (Harrison et al., 2007), although this is less of a problem in dilute environments (Greig and Travisano, 2004). Some marine strains overcome diffusion losses by producing amphiphilic siderophores that attach to the cell membrane preventing loss by diffusion (Martinez and Butler, 2007). Despite their energetic costs, soluble siderophores appear to be widely distributed in the marine environment.

In this work, we examined DFB's effect on the globally important marine autotroph *Synechococcus* (Partensky et al., 1999; Zwirglmaier et al., 2008), and on a natural assemblage of phytoplankton and bacterioplankton from the Sargasso Sea. DFB reduced the growth rate of *Synechococcus* and, at higher concentrations, even arrested growth and decreased *in vivo* fluorescence. Light aggravated the effect, decreasing the inhibitory concentration to as little as 20-40 nM. The growth inhibition did not correspond to total Fe or modeled thermodynamic or kinetic estimates of Fe' (free Fe, the sum of all dissolved, non-organically complexed Fe(III)). This work suggests that the effect of hydroxamate siderophores on plankton is complex and may vary greatly between phytoplankton and bacterioplankton.

# Materials and Methods

## General Culture

SNAX medium was the base medium used for maintaining *Synechococcus* and performing experiments (Waterbury et al., 1986). Unless otherwise noted the medium was modified by the omission of cyanocobalamin and an increase in EDTA concentration to 20 μM, the concentrations of the nutrients and ligands in all experimental media are given in Table 1. The seawater base for all media was Sargasso seawater, 0.2 μm filtered and stored in acid-washed polyethylene bottles. Prior to preparing media, the seawater was re-filtered and diluted to 75% with ultra-pure water. Total Fe in the media was not quantified. All cultures were grown at 24ºC in continuous light. Nutrients used in the preparation of media were SigmaUltra grade (Sigma, St. Louis, MO) except for the ACS grade $Na_2$-EDTA, which was from Fisher Scientific (Waltham, MA). Macronutrient stocks were purified with a chelex resin column washed for trace media preparation (Sigma, St. Louis, MO) (Price et al., 1988). All DFB used was in the form of Desferrioxamine B mesylate salt (catalog # D9533, Sigma, St. Louis, MO). The pH of Fe- ligand solutions was adjusted with trace metals analysis grade HCl (Mallinckrodt Baker Phillipsburg, NJ), and NaOH.(Fluka, Milwaukee, WI).

## Field studies

Water was collected near the Bermuda Atlantic Time Series site (31º 35.16 N, 64º 11.18 W) using the sampling rosette aboard the R/V Atlantic Explorer. The sample was

Table 1 The log molarity of nutrients and ligands added to experimental media

| | | | | Additions | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Macronutrients | | | Trace Metals | | | | | Ligands | | | |
| Media | | Experimentsa | Seawater base | NO3 | NH4 | PO4 | Mn | Zn | Mo | Co | Fe | EDTA | DFB | Ferri-chrome | EDDHA |
| 1 | SNAX Low EDTA FeDFB 2:5 | 1 | 75% Sargasso SW | -3.00 | -4.00 | -5.05 | -6.15 | -7.12 | -6.79 | -8.07 | -5.70 | -5.70 | -5.30 | | |
| 2 | SNAX Low EDTA FeDFB 0.67:1.7 | 1 | 75% Sargasso SW | -3.00 | -4.00 | -5.05 | -6.15 | -7.12 | -6.79 | -8.07 | -6.17 | -5.70 | -5.78 | | |
| 3 | SNAX Low EDTA FeDFB 0.2:0.5 | 1 | 75% Sargasso SW | -3.00 | -4.00 | -5.05 | -6.15 | -7.12 | -6.79 | -8.07 | -6.70 | -5.70 | -6.30 | | |
| 4 | SNAX Low EDTA FeDFB 0.067:0.17 | 1 | 75% Sargasso SW | -3.00 | -4.00 | -5.05 | -6.15 | -7.12 | -6.79 | -8.07 | -7.17 | -5.70 | -6.78 | | |
| 5 | SNAX Low EDTA FeDFB 0.02:0.05 | 1 | 75% Sargasso SW | -3.00 | -4.00 | -5.05 | -6.15 | -7.12 | -6.79 | -8.07 | -7.70 | -5.70 | -7.30 | | |
| 6 | SNAX Hi EDTA +Fe 2 | 2,4 | 75% Sargasso SW | -3.00 | -4.00 | -5.05 | -6.15 | -7.12 | -6.79 | -8.07 | -5.70 | -4.70 | | | |
| 7 | SNAX Hi EDTA -Fe | 2,4 | 75% Sargasso SW | -3.00 | -4.00 | -5.05 | -6.15 | -7.12 | -6.79 | -8.07 | | -4.70 | | | |
| 8 | SNAX Hi EDTA FeDFB 2:4 | 2,3,4 | 75% Sargasso SW | -3.00 | -4.00 | -5.05 | -6.15 | -7.12 | -6.79 | -8.07 | -5.70 | -4.70 | -5.40 | | |
| 9 | SNAX Hi EDTA FeDFB 0.02:0.04 | 2 | 75% Sargasso SW | -3.00 | -4.00 | -5.05 | -6.15 | -7.12 | -6.79 | -8.07 | -7.70 | -4.70 | -7.40 | | |
| 10 | SNAX Hi EDTA FeFerrichlome 2:4 | 2 | 75% Sargasso SW | -3.00 | -4.00 | -5.05 | -6.15 | -7.12 | -6.79 | -8.07 | -5.70 | -4.70 | | -5.40 | |
| 11 | SNAX Hi EDTA FeDFB 2:1 | 3 | 75% Sargasso SW | -3.00 | -4.00 | -5.05 | -6.15 | -7.12 | -6.79 | -8.07 | -5.70 | -4.70 | -6.00 | | |
| 12 | SNAX Hi EDTA FeDFB 0.2:0.4 | 3 | 75% Sargasso SW | -3.00 | -4.00 | -5.05 | -6.15 | -7.12 | -6.79 | -8.07 | -6.70 | -4.70 | -6.40 | | |
| 13 | SNAX Hi EDTA FeDFB 0.2:0.1 | 3 | 75% Sargasso SW | -3.00 | -4.00 | -5.05 | -6.15 | -7.12 | -6.79 | -8.07 | -6.70 | -4.70 | -7.00 | | |
| 14 | Botte amend FeEDTA | 5 | Fresh Sargasso SW | | | | | | | | -6.10 | -6.10 | | | |
| 15 | Bottle amend FeDFB 0.4:0.8 | 5 | Fresh Sargasso SW | | | | | | | | -6.40 | | -6.10 | | |
| 16 | Bottle amend FeDFB 0.04:0.08 | 5 | Fresh Sargasso SW | | | | | | | | -7.40 | | -7.10 | | |
| 17 | Bottle amend FeDFB 0.012:0.024 | 5 | Fresh Sargasso SW | | | | | | | | -7.92 | | -7.62 | | |
| 18 | Bottle amend FeDFB 0.004:0.008 | 5 | Fresh Sargasso SW | | | | | | | | -8.40 | | -8.10 | | |
| 19 | Bottle amend EDDHA no Fe | 5 | Fresh Sargasso SW | | | | | | | | | | | | -6.10 |

a. Experiments

1. Low light growth rate inhibition (Fig. 1)
2. Light titration experiment (Fig. 2)
3. Total/Bound/Unbound DFB experiment (Fig. 3)
4. DFB rescue experiment (Fig. 4)
5. Sargasso bottle incubations (Fig. 5)

collected at 15:40 UTC on 7 October 2008 from 80 m depth. Collected water was transferred into microwave Tyndalized, acid-washed 125 mL polycarbonate bottles. Triplicate bottles were amended with: pH 8.0 FeDFB complexed at concentrations of 400:800, 40:80, 12:24 or 4:8 nM; or 800 nM ethylenediamine-N,N'-bis(2-hydroxyphenylacetic acid) (EDDHA) without Fe; or 800 nM FeEDTA complex pH8.0. As a control one set of bottles was not amended. Samples were placed in a deckboard incubator maintained at ambient surface seawater temperature and shaded to 3% of surface light intensity with blue lighting gels and window screen. Samples were collected for flow cytometry approximately every 12 hours for 96 hours and preserved by incubating cells with 0.125% glutaraldehyde in the dark for 10 minutes at room temperature then freezing them in liquid nitrogen. Preserved cells were counted and characterized using an Influx cytometer (Cytopia-BD, Seattle, WA). All size and fluorescence side scatter and forward scatter readings were normalized to fluorescent 2 μM polystyrene beads (Polysciences, Warrington , PA) (Olson et al., 1985; Cavender-Bares et al., 1999).

### *Low light growth rate*

*Synechococcus* sp. WH7803 was grown at 10 μE $m^{-2}$ $s^{-1}$ continuous light in SNAX -Fe medium containing 2μM EDTA. The medium was amended with varied concentrations (2, 0.67, 0.2, 0.067, 0.02 μM) of ferric citrate precomplexed to an excess of DFB in a 2:5 ratio at pH 8.0, Table 1. The different media were inoculated with washed cells and 250 μL was dispensed into 8 replicate wells of a 96 well plate. The inoculum was washed by twice centrifuging the culture at 8000 RCF and resuspending it

in SNAX -Fe. Growth rate was measured by the change in *in vivo* phycoerytherin fluorescence.

### *Combined light and DFB titrations*

Modified SNAX -Fe, was amended with: 2 µM FeEDTA pH 8, or 2:4 µM Fe:DFB pH 8, or 0.02 0.04µM Fe:DFB pH 8 or 2:4 µM Fe:Ferrichrome, pH 8. 25 mL of medium was dispensed into acid-washed polycarbonate centrifuge tubes. Each treatment had three biological replicates. To inoculate each culture a mid-log phase, light acclimated culture was vacuum filtered into a sterile, acid-washed 0.45µm polycarbonate membrane held in an acid-washed, autoclaved glass filter frit. The cells were washed with sterile 75% Sargasso seawater, then the filter was aseptically transferred to a sterile acid-washed polycarbonate tube and cells were resuspended in ~20 mL of sterile 75% Sargasso seawater. 1.0 ml of the cell suspension was added to each tube. Growth was monitored by *in vivo* fluorescence using a TD700 fluorometer (Turner Instruments, Sunnyvale, CA) with a phycoerytherin filter set (excitation 544/10 nm, emission 577 nm long pass). Cells were grown at 10, 40 or 70 µE $m^{-2}$ $s^{-1}$ continuous light, as indicated.

### *Western blotting*

At the end of the culture experiment described above, cells of *Synechococcus* sp. WH7803 were filtered onto a 0.45 µm polycarbonate membrane filter and frozen at -20ºC. Protein was extracted by adding 65 µL of Bugbuster protein extraction reagent (EMD Chemicals, Gibbstown, NJ) and vortexing for 1 min. Samples were incubated at room temperature for 10 minutes then sonicated in 1.5 ml polypropylene tubes for 6 minutes (30 seconds on/30 seconds off) on high power at 0ºC in a BioRuptor sonic bath

(Diagenode, Sparta, NJ). Filters were pinched in the top of the tube and the tube was centrifuged at 12,000 × g for 3 minutes. The supernatant was transferred and was stored at 4ºC.

The protein was quantified using 5 μL of the protein extract and a Micro BCA protein quantification kit (Pierce, Rockford, IL) according to the manufacturer's instructions. 1 μg of protein extract from each culture was separated on pre-cast 4-20% SDS-PAGE gels (Pierce, Rockford, IL) and transferred onto a nitrocellulose membrane using the Mini Protean III System (Bio-Rad Hercules , CA). Western blotting was done using polyclonal rabbit antisera raised against recombinant IdiA from *Synechococcus* sp. WH8102 (Rivers et al., 2009). IdiA was detected with Super Signal western blotting kit (Pierce, Rockford, IL).

### Metal addition and cellular recovery

*Synechococcus* sp. WH8102 was grown in 40μE $m^{-2}$ $s^{-1}$ continuous light. Late log phase culture was filtered and resuspended as described previously in modified SNAX medium deficient in Fe and Mn. Fe in the form of Fe EDTA (2 μM) pH8, FeDFB (2:4 μM) pH 8 was added to cultures as appropriate or omitted in the -Fe control. After 144 hours ferric citrate was added to selected cultures to raise the Fe concentration by 3 μM and saturate unbound DFB. To test the hypothesis that DFB was catalyzing the oxidation of Mn(II) and binding Mn(III) (Duckworth and Sposito, 2005), $MnCl_2$ was added to raise the final Mn concentration by 3 μM. Cultures were grown in triplicate in acid-washed glass tubes and growth was monitored by fluorescence as described previously.

### *Chemical modeling*

To understand the effect of DFB on Fe chemistry in the media both thermodynamic and kinetic modeling was used and the results were compared. All thermodynamic speciation calculations were made with the chemical modeling software PHREEQC for Windows version 2.15 (Post, 2008; Parkhurst and Appelo, 1999). The chemical species modeled are listed in the footnote in Table 2. The MINTEQ database was used and data on DFB reactions were added from Morel and Hering (1993). Fe speciation was modeled without redox reactions.

The system was also modeled kinetically using the ODE23s package in Matlab version 7.4 (Mathworks, Natick, MA). Kinetic modeling was important because the dissociation of DFB and EDTA occur slowly. The conditional kinetic rate constants and biological constants used are presented in Table 3. The ordinary differential equations defining the system are presented in Table 4. Kinetic rate constants for biological Fe(III)' uptake are from the diatom *Thalassiosira weissflogii*.

## Results

### *The effect of desferrioxamine B on an Atlantic microbial population*

To test the effect of desferrioxamine B on a natural microbial population, seawater was collected near Bermuda and incubated for 4 days with several Fe-ligand complexes, Figure 1. The addition of 800 nM of FeEDTA, stimulated the growth of *Synechococcus* cells over the unamended control suggesting some degree of Fe limitation, although EDTA could have altered the speciation of other metals as well. Fe

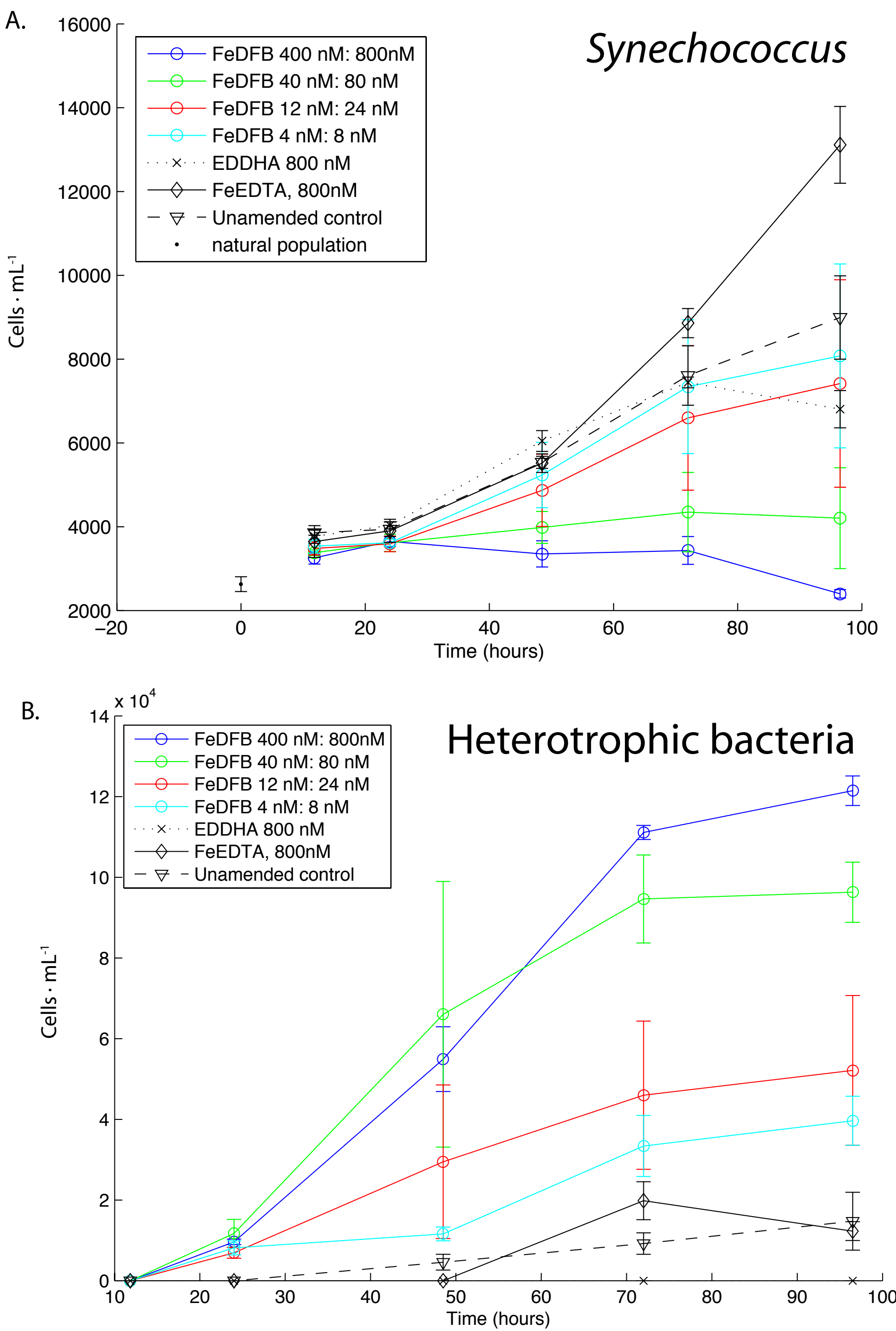



Figure 1 The response of a natural seawater sample to amendments with different forms of complexed Fe or ligand. Water was collected from 80 m depth in the Sargasso Sea and incubated in a deckboard incubator for 96 hours at 3% of surface irradiance. Bottles were amended with four different concentrations of FeDFB complex, the aminocarboxylate Fe ligand EDDHA, 800 nM FeEDTA or left unamended. A. The growth of *Synechococcus* measured by flow cytometry in the presence of different ligands. B. The growth of putative heterotrophic bacteria. In cases where the value is at the origin no gate as drawn because there was not a discernible population.

complexed to DFB in a 1:2 ratio was added to seawater at concentrations of 400:800 nM, 40:80 nM, 12:24 nM, 4:8 nM FeDFB. The lowest concentrations of FeDFB only slightly retarded growth, but at the two higher concentrations (40:80 nM and 400:800 nM FeDFB) growth was significantly inhibited; in all four treatments growth decreased with increasing DFB concentration. For comparison the synthetic ligand EDDHA was added without additional Fe. This ligand forms strong (Yunta et al., 2003) but photolabile Fe complexes and is known to induce Fe limitation in *Synechococcus* (Chadd et al., 1996). Growth in the presence of EDDHA was similar to the unamended control. Chlorophyll fluorescence per cell measured by flow cytometry declined in the *Synechococcus* and *Prochlorococcus* populations regardless of the treatment, possibly because of increased light irradiance in the shipboard incubators. This bleaching made it difficult to accurately count *Prochlorococcus* and for this reason their growth is not presented. The presence of FeDFB complexes significantly stimulated the growth of non-chlorophyll containing bacterioplankton in a concentration-dependent manner. The stimulation was not observed in the unamended control or after the addition of FeEDTA or the synthetic chelator EDDHA.

### *DFB induced growth inhibition in Synechococcus*

The effects of DFB on *Synechococcus* were investigated in greater detail through a series of laboratory culture experiments. To determine if DFB inhibited growth in axenic cultures, *Synechococcus* sp. WH7803 was grown at low light intensity (10 µE $m^{-2}$ $s^{-1}$) with varied concentrations of Fe (2, 0.67, 0.2, 0.067, 0.02 µM) complexed to DFB in a 2:5 ratio (Figure 2). DFB inhibited the growth rate linearly in a concentration

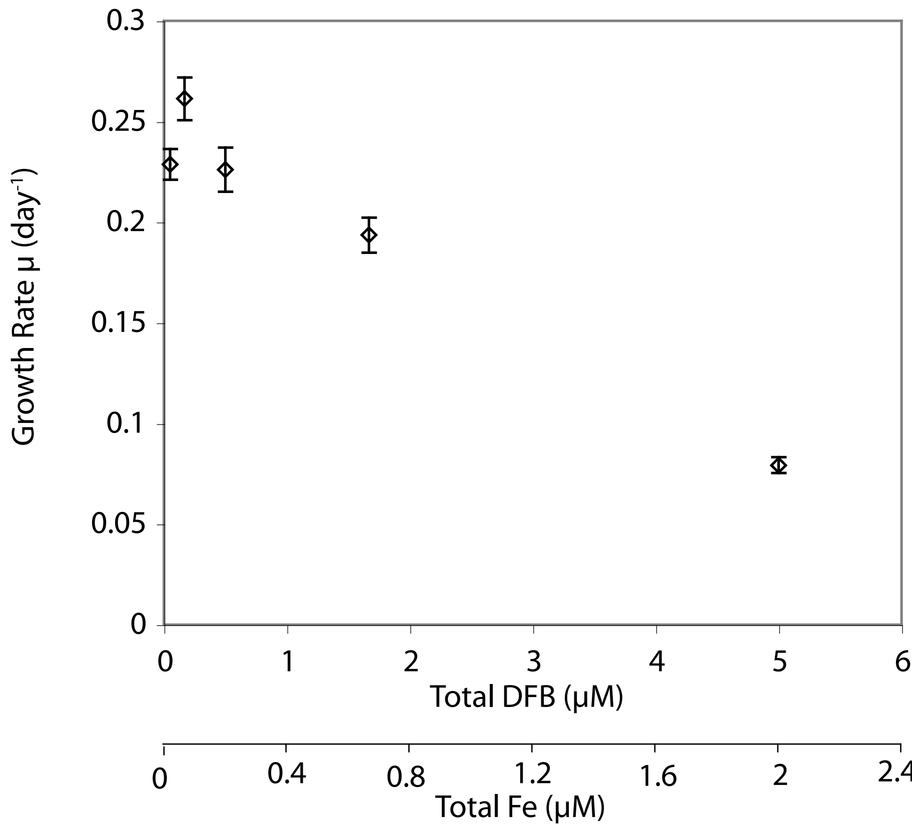



Figure 2 The growth rates of *Synechococcus* sp. WH7803 at constant low light (10 μE $m^{-2}$ $s^{-1}$) in the presence of varied amounts of Fe precomplexed to DFB at a fixed 2:5 ratio. The total amount of EDTA was 2 μM at all concentrations.

dependent manner. The results show that growth rate is negatively correlated with total Fe, total DFB and modeled values of FeDFB and unbound DFB. Modeled values of Fe' were not able to explain the decrease in growth rate (discussed later).

### ***Light and growth inhibition by two hydroxamate siderophores***

At higher light intensities, DFB was a more potent growth inhibitor (Figure 3, Supplementary Figure 1), causing complete arrest of growth and bleaching rather than just slowing growth rate as in Figure 2. Cells of *Synechococcus* sp. WH7803 were grown at 70 µE $m^{-2}$ $s^{-1}$ with Fe, without Fe, and at high (2:4 µM) and low concentrations (0.02:0.04 µM) of FeDFB at a fixed ratio of 1:2 (Figure 3A). The high DFB culture declined while the low DFB culture grew at the same rate and reached the same yield as the -Fe culture. This same effect occurs in *Synechococcus* sp. WH8102 at light levels down to 40 µE $m^{-2}$ $s^{-1}$ (Figure 3D). The growth rates for *Synechococcus* sp. WH7803 grown at 10 µE $m^{-2}$ $s^{-1}$ under high and low DFB concentrations (Figure 3E) differ by less would be expected from Figure 2, this is likely due to differences in culturing; the EDTA concentration was higher, cells were grown in tubes rather than microwell plates and the work was done in separate laboratories. At the lowest light level high DFB treated cultures did not initially decline and in *Synechococcus* sp. WH8102 growth initially increased, but began to decline before it did in the  -Fe and the low FeDFB treatments. In panels A-B, cells were also grown in the presence of Fe:Ferichrome at a concentration of 2:4 µM.  The inhibitory effect of this other hydroxamate siderophore closely matched the inhibitory effect of DFB. The decline of *in vivo* culture fluorescence in high DFB cultures occurred at all but the lowest light level.

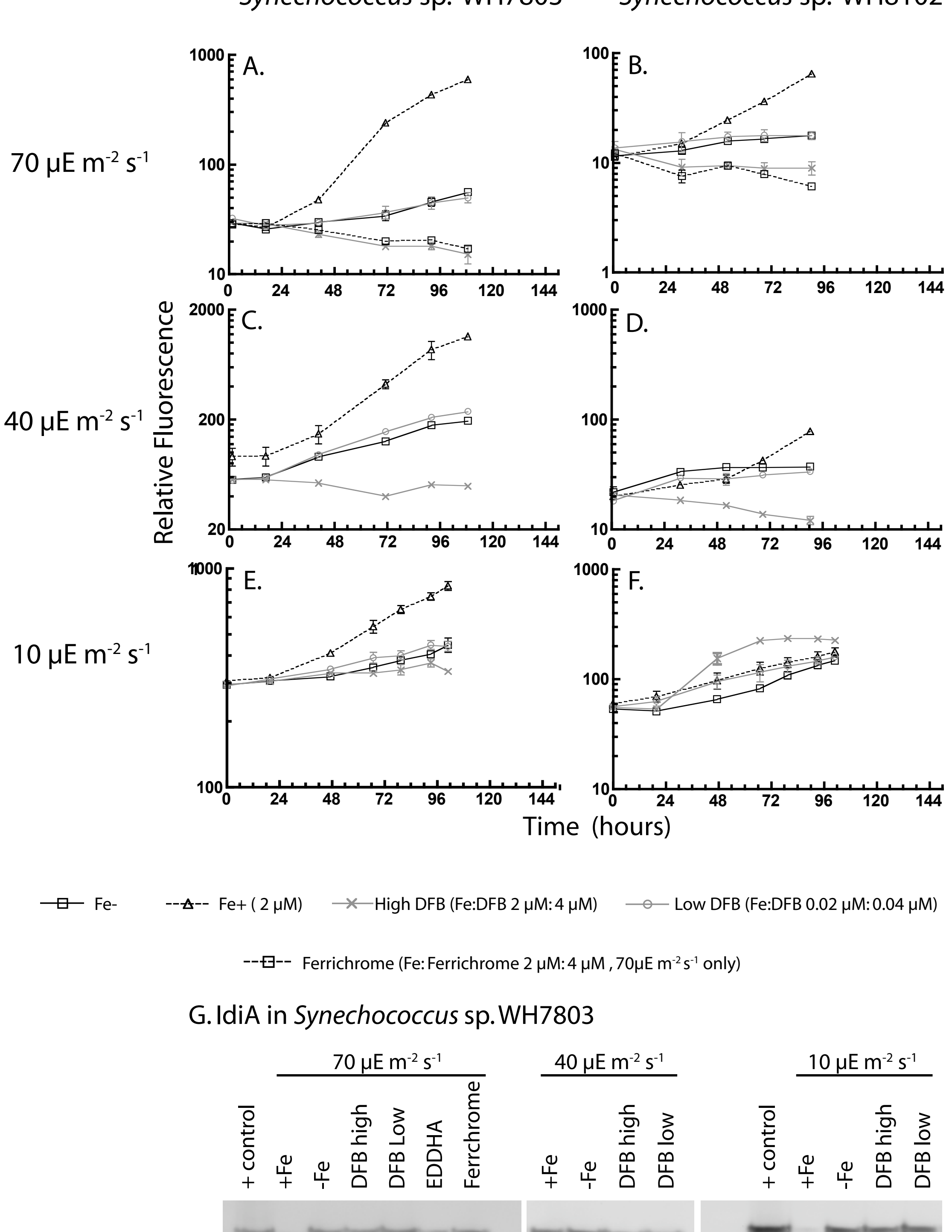
At the end of the growth experiment, cells of *Synechococcus* sp.WH7803 were harvested to detect the abundance of the iron stress protein IdiA by quantitative immunoblotting, Figure 3G. IdiA accumulated under Fe limitation in marine *Synechococcus* (Webb et al., 2001; Rivers et al., 2009). IdiA was less abundant in the +Fe cultures, although in the 40 μE $m^{-2}$ $s^{-1}$ treatment IdiA was present in quantities similar to -Fe cultures, likely because the culture was nearing stationary phase. IdiA expression was high in all -Fe, high DFB, low DFB, Ferrichrome or EDDHA treatments (for simplicity growth curves were omitted for EDDHA). Expression was not higher in either FeDFB treatment than it was in the -Fe treatment. Fe stress did not appear to be greater in either of the DFB treatments, although it is possible that IdiA expression was already at its maximal level.

### *Recovery of growth rate after saturating Fe addition*

DFB at concentrations of ~20-40 nM more than Fe appear to cause characteristic growth inhibition and cellular bleaching at all but very low light levels. In order to determine if this stress is reversible, *Synechococcus* sp. WH7803 was grown in the presence of 2:4 μM FeDFB, Figure 4. Approximately 2 days after *in vivo* fluorescence began to decline, ferric citrate was added raising the Fe concentration by 3 μM, enough to saturate the unbound DFB and provide 1 μM of Fe for cellular growth. This addition allowed the cells to resume growth, indicating that the effect is reversible.

DFB has been reported to catalyze the oxidation of Mn(II) to Mn(III) (Duckworth and Sposito, 2005) and tightly bind Mn(III). To test the theory that DFB was causing Mn co-limitation, Mn(II) was added to raise the Mn concentration by 3μM, Figure 4. The

Figure 3 The growth of *Synechococcus* spp. WH7803 and WH8102 at different Fe and DFB concentrations and varied light intensities. Growth was measured by relative phycoerytherin fluorescence. In each panel +Fe and Fe cultures serve as controls. All FeDFB complexes were formed prior to addition at a 1:2 ratio. In A-B the cells were also grown in the presence of 2μM Fe pre-complexed to 4 μM of the hydroxamate siderophore Ferrichrome. G) The presence of the iron stress protein IdiA from extracts of *Synechococcus* sp. WH7803. Each lane contains 1 μg of total protein extract taken at the end of the growth curves. For simplicity representative bolts are shown. In all plots the positive control was 1 μg of an extract of *Synechococcus* sp. WH7803 grown at 5 nM total Fe and known to contain IdiA. The 10 and 40 μE $m^{-2}$ $s^{-1}$ bands were run on the same blot and share a positive control, the 70 μE $m^{-2}$ $s^{-1}$ was run on a separate blot.



Synechococcus sp. WH7803
Synechococcus sp. WH8102
70 μE m-2 s-1
40 μE m-2 s-1
10 μE m-2 s-1
A.
B.
C.
D.
E.
F.
Relative Fluorescence
Time (hours)
Fe-
Fe+ ( 2 μM)
High DFB (Fe:DFB 2 μM: 4 μM)
Low DFB (Fe:DFB 0.02 μM: 0.04 μM)
Ferrichrome (Fe: Ferrichrome 2 μM: 4 μM , 70μE m-2 s-1 only)
G. IdiA in Synechococcus sp. WH7803
+ control
+Fe
-Fe
DFB high
DFB Low
EDDHA
Ferrchrome
DFB low

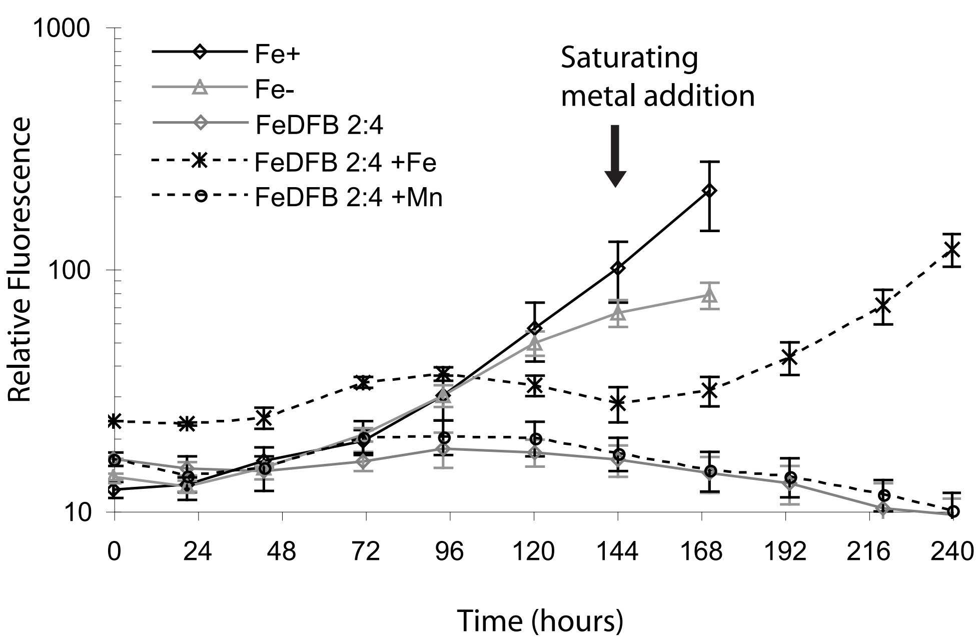



Figure 4 Reversing of the inhibitory effect of DFB. Cultures of *Synechococcus* sp. WH8102 were grown at 40 μE $m^{-2}$ $s^{-1}$ with 2μM Fe, without added Fe or with 2 μM Fe complexed to an excess of DFB (4μM). At Hour 144, ferric citrate or Mn(II) was added to increase the concentration by 3μM in selected FeDFB treatments.

Mn(II) addition did not rescue the cultures and the addition of Fe and Mn (data not shown) did not improve the growth rate over the Fe amended culture. This suggests that the effect is not related to extracellular Mn co-limitation and that Fe must be replaced for recovery even if unbound DFB has presumably been complexed to some degree by other metals.

### *Kinetic and thermodynamic models of Fe speciation and uptake*

The concentration of Fe' was modeled in an attempt to understand how DFB influenced Fe speciation in the previous experiments. Fe' was modeled both kinetically and thermodynamically in an attempt to place boundaries on the range of possible Fe' values. The thermodynamic modeling assumed equilibrium was reached between all the species in seawater, and that the uptake by phytoplankton had a negligible effect on the Fe' (Table 2). Under these assumptions the log Fe' = -16.9 when the Fe:DFB ratio was 1:2, regardless of the concentration. This is a very low Fe' concentration, but the assumptions of the thermodynamic modeling may not hold for a system with two ligands and biological uptake.

To account for biological uptake and ligand competition, the system was modeled kinetically to represent the formation and dissociation and photo-reduction of the ligands DFB and EDTA in the presence of biological uptake, Figure 5. Cellular uptake was modeled using Fe(III)' uptake parameters from the diatom *T. weissflogii* (Hudson and Morel, 1990), because rate constant data was not available for *Synechococcus* or *Prochlorococcus*. *T. oceanica* has been shown to take up Fe bound to DFB (Maldonado and Price, 2001) but this should not have effected the modeling results because only

Table 2 Thermodynamically predicted log molarity of Fe', ligand and Fe-ligand complexes

| | Media | Experiments[a] | Seawater base | Thermodynamically Calculated values[b] | | | |
|---|---|---|---|---|---|---|---|
| | | | | unbound DFB | FeDFB | Fe' | FeEDTA |
| 1 | SNAX Low EDTA FeDFB 2:5 | 1 | 75% Sargasso SW | -5.5 | -5.7 | -17.1 | -15.9 |
| 2 | SNAX Low EDTA FeDFB 0.67:1.7 | 1 | 75% Sargasso SW | -6.0 | -6.2 | -17.1 | -15.9 |
| 3 | SNAX Low EDTA FeDFB 0.2:0.5 | 1 | 75% Sargasso SW | -6.5 | -6.7 | -17.1 | -15.9 |
| 4 | SNAX Low EDTA FeDFB 0.067:0.17 | 1 | 75% Sargasso SW | -7.0 | -7.2 | -17.1 | -15.9 |
| 5 | SNAX Low EDTA FeDFB 0.02:0.05 | 1 | 75% Sargasso SW | -7.5 | -7.7 | -17.1 | -15.9 |
| 6 | SNAX Hi EDTA +Fe 2 | 2,4 | 75% Sargasso SW | | | -7.9 | -5.7 |
| 7 | SNAX Hi EDTA -Fe | 2,4 | 75% Sargasso SW | - | - | - | - |
| 8 | SNAX Hi EDTA FeDFB 2:4 | 2,3,4 | 75% Sargasso SW | -5.7 | -5.7 | -16.9 | -14.6 |
| 9 | SNAX Hi EDTA FeDFB 0.02:0.04 | 2 | 75% Sargasso SW | -7.7 | -7.7 | -16.9 | -14.6 |
| 10 | SNAX Hi EDTA FeFerrichlome 2:4 | 2 | 75% Sargasso SW | - | - | - | - |
| 11 | SNAX Hi EDTA FeDFB 2:1 | 3 | 75% Sargasso SW | -14.6 | -6.0 | -8.3 | -6.0 |
| 12 | SNAX Hi EDTA FeDFB 0.2:0.4 | 3 | 75% Sargasso SW | -6.7 | -6.7 | -16.9 | -14.6 |
| 13 | SNAX Hi EDTA FeDFB 0.2:0.1 | 3 | 75% Sargasso SW | -14.6 | -7.0 | -9.3 | -7.0 |
| 14 | Botte amend FeEDTA | 5 | Fresh Sargasso SW | | | -6.6 | -6.3 |
| 15 | Bottle amend FeDFB 0.4:0.8 | 5 | Fresh Sargasso SW | -6.4 | -6.4 | -16.9 | |
| 16 | Bottle amend FeDFB 0.04:0.08 | 5 | Fresh Sargasso SW | -7.4 | -7.4 | -16.9 | |
| 17 | Bottle amend FeDFB 0.012:0.024 | 5 | Fresh Sargasso SW | -7.9 | -7.9 | -16.9 | |
| 18 | Bottle amend FeDFB 0.004:0.008 | 5 | Fresh Sargasso SW | -8.4 | -8.4 | -16.9 | |
| 19 | Bottle amend EDDHA no Fe | 5 | Fresh Sargasso SW | - | - | - | - |

a. Experiments

1. Low light growth rate inhibition (Fig. 1)
2. Light titration experiment (Fig. 2)
3. Total/Bound/Unbound DFB experiment (Fig. 3)
4. DFB rescue experiment (Fig. 4)
5. Sargasso bottle incubations (Fig. 5)

b. Calculated in PHREEQC for Windows assuming pH 8.1, pe 4, 25ºC, Species in the model were:
Na+, Cl-, K+, NO3-, NH4+, SO4-2,PO4-2,HCO3-, Mg+2, Ca+2, Mn+2, Zn+2, Fe+3, EDTA, DFB, (dashed columns not calculated)

Table 3 Constants used in the kinetic modeling of Fe speciation

| Symbol | description | Value | Symbol | Reference |
|---|---|---|---|---|
| $k_d^{FeEDTA}$ | Rate constant, FeEDTA dissociation | $1 \times 10^{-6}$ | $s^{-1}$ | Hudson and Morel (1990) |
| $k_f^{FeEDTA}$ | Rate constant, FeEDTA formation | 20 | $mol^{-1}s^{-1}$ | Hudson and Morel (1990) |
| $k_{h\nu}^{FeEDTA}$ | Rate constant, FeEDTA photoreduction | $4.4 \times 10^{-7}$ | $s^{-1}$ | Sunda and Huntsman (2003) |
| $k_d^{FeDFB}$ | Rate constant, FeDFB dissociation | $1.5 \times 10^{-6}$ | $s^{-1}$ | Witter *et al.* (2000) |
| $k_f^{FeDFB}$ | Rate constant, FeDFB formation | $1.9 \times 10^{6}$ | $mol^{-1}s^{-1}$ | Witter *et al.* (2000) |
| $k_{ox}^{Fe^{2+}}$ | Rate constant, $Fe^{2+}$ oxidation | $6.2 \times 10^{-3}$ | $s^{-1}$ | Sunda and Huntsman (2003) |
| $k_f^{FeL}$ | Rate constant, FeL formation | $9 \times 10^{5}$ | $mol^{-1}s^{-1}$ | Hudson and Morel (1990) |
| $k_d^{FeL}$ | Rate constant, FeL dissociation | $2 \times 10^{-4}$ | $s^{-1}$ | Hudson and Morel (1990) |
| $k_{in}$ | Rate constant, Fe cell import from FeL | $2 \times 10^{-3}$ | $s^{-1}$ | Hudson and Morel (1990) |
| $L_{cell}$ | Number of ligands L per cell | $1.7 \times 10^{-17}$ | $mol\ cell^{-1}$ | Hudson and Morel (1990) |

a. All chemical rate constants are conditional for seawater
b. Biological constants measured with Fe replete cultures of *Thalassiosira weissflogii*

Table 4 Ordinary differential equations used in kinetic modeling

$$\frac{d[\mathrm{Fe}']}{dt} = k_d^{FeL}[\mathrm{FeL}] - k_f^{FeL}[\mathrm{Fe}'][\mathrm{L}] + k_d^{FeDFB}[\mathrm{FeDFB}] - k_f^{FeDFB}[\mathrm{Fe}'][\mathrm{DFB}]\ldots \quad (1)$$

$$+ k_d^{FeEDTA}[\mathrm{FeEDTA}] - k_f^{EDTA}[\mathrm{Fe}'][\mathrm{EDTA}] + k_{ox}^{Fe^{2+}}[\mathrm{Fe}^{2+}] \quad (2)$$

$$\frac{d[\mathrm{FeEDTA}]}{dt} = k_f^{FeEDTA}[\mathrm{Fe}'][\mathrm{EDTA}] - k_d^{FeEDTA}[\mathrm{FeEDTA}] - k_{h\nu}^{FeEDTA}[\mathrm{FeEDTA}] \quad (3)$$

$$\frac{d[\mathrm{EDTA}]}{dt} = k_d^{FeEDTA}[\mathrm{FeEDTA}] - k_f^{FeEDTA}[\mathrm{Fe}'][\mathrm{EDTA}] + k_{h\nu}^{FeEDTA}[\mathrm{FeEDTA}] \quad (4)$$

$$\frac{d[\mathrm{FeDFB}]}{dt} = k_f^{FeDFB}[\mathrm{Fe}'][\mathrm{DFB}] - k_d^{FeDFB}[\mathrm{FeDFB}] \quad (5)$$

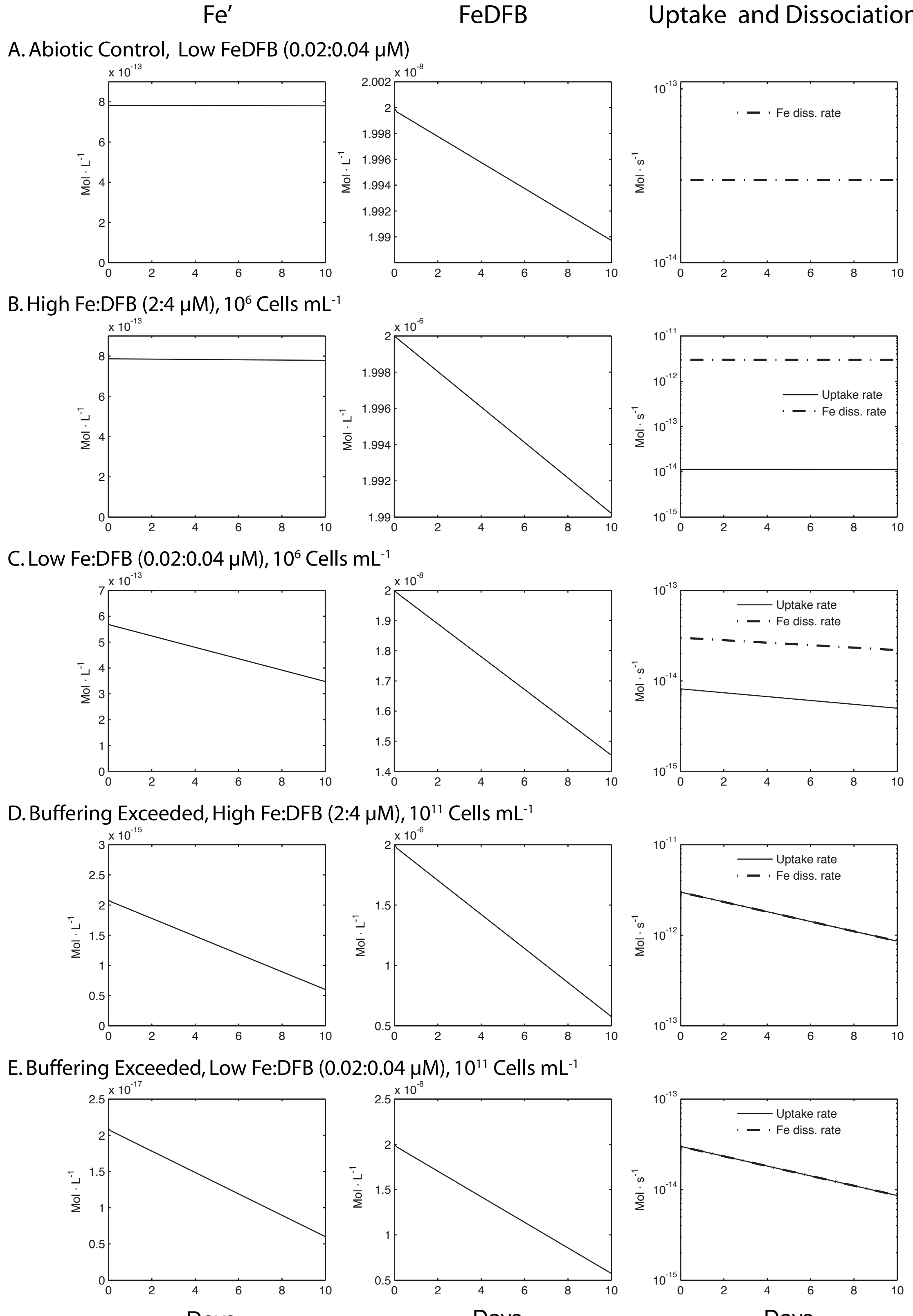
$$\frac{d[\mathrm{DFB}]}{dt} = k_d^{FeDFB}[\mathrm{FeDFB}] - k_f^{FeDFB}[\mathrm{Fe}'][\mathrm{DFB}] \quad (6)$$

$$\frac{d[\mathrm{FeL}]}{dt} = k_f^{FeL}[\mathrm{Fe}'][\mathrm{L}] - k_d^{FeL}[\mathrm{FeL}] - k_{in}[\mathrm{FeL}] \quad (7)$$

$$\frac{d[\mathrm{Fe}^{2+}]}{dt} = k_{h\nu}^{FeEDTA}[\mathrm{FeEDTA}] - k_{ox}^{Fe^{2+}}[\mathrm{Fe}^{2+}] \quad (8)$$

$$\frac{d[\mathrm{cellular}]}{dt} = k_{in}[\mathrm{FeL}] \quad (9)$$

Figure 5 Kinetic modeling of Fe speciation between DFB, EDTA and cellular uptake. The concentration of Fe' and Fe bound to ligands was modeled as was the cumulative uptake rate for the cells and the cumulative disassociation rate for both ligands. The constants and equations for the model are provided in Tables 3 and 4. Kinetic uptake constants are for Fe(III)' are from the diatom *T. weissflogii*. The cell concentration was held constant in each simulation. A. The ligand system alone was modeled. B-C. The high and low FeDFB concentrations used in experiments were modeled. $1\times10^{6}$ cells $mL^{-1}$ was chosen because this represents an extremely dense culture of *T. weissflogii* D-E. In these conditions the cell density was set artificially high to examine the effect of exceeding DFB's buffering capacity. Once the system became unbuffered Fe' correlated with FeDFB.


Fe'
FeDFB
Uptake and Dissociation
A. Abiotic Control, Low FeDFB (0.02:0.04 μM)
B. High Fe:DFB (2:4 μM), 10^6 Cells mL^-1
C. Low Fe:DFB (0.02:0.04 μM), 10^6 Cells mL^-1
D. Buffering Exceeded, High Fe:DFB (2:4 μM), 10^11 Cells mL^-1
E. Buffering Exceeded, Low Fe:DFB (0.02:0.04 μM), 10^11 Cells mL^-1
Mol · L^-1
Mol · s^-1
Uptake rate
Fe diss. rate
Days
Days
Days

Fe(III)' rate constants were used. The combined physical and biological parameters were modeled using the constants and equations in Tables 3 and 4. The abiotic chemical equilibration between FeDFB and EDTA revealed that the over the time span of the growth experiments EDTA bound Fe was not a significant source of Fe to the Fe' pool. The predicted Fe' in the abiotic run of the kinetic model was quite low, (~$8\times10^{-13}$ M) but still much higher than the concentration predicted by thermodynamic modeling.

Under buffered conditions Fe' should remain fairly constant. The buffering capacity of the system was tested in two ways, first the Fe' concentration was calculated in the high and low FeDFB conditions and compared to the abiotic conditions. In both the abiotic and high FeDFB condition Fe' remained constant at ~$8\times10^{-13}$ M, and in the low FeDFB condition it averaged ~$5\times10^{-13}$ M, decreasing slightly over time. The supply rate of the ligand was compared to the uptake rate by phytoplankton as a second means of assessing the buffering capacity. The rates of FeDFB and FeEDTA disassociation and Fe(II) oxidation were summed and this combined rate was compared to the instantaneous uptake rate of cells (the derivative of intracellular concentration with respect to time). In both the high and low FeDFB conditions the rate of uptake was considerably less than the disassociation rate, suggesting that the FeDFB ligand was sufficient for the low rate of cellular uptake that was occurring.

The maximum yields reported for *T. weissflogii* are around $10^{-5}$-$10^{-6}$ so it appears DFB can buffer a dense culture, although it does so at an [Fe'] that is insufficient for appreciable growth. In the model it took between ~ $10^{10}$-$10^{11}$ cells mL$^{-1}$ to exceed the buffering capacity of FeDFB. This effect is shown in Figure 5. Under these "blown

buffer” conditions Fe’ was proportional to the initial concentration of the FeDFB complex: Fe’ equaled ~$1\times10^{-15}$ M in low FeDFB or ~$1\times10^{-17}$ M in high FeDFB and the uptake rate matched the disassociation rate.

# Discussion

## *The bioavailability of siderophore bound Fe to Synechococcus*

Siderophore bound Fe is bioavailable if a cell both can take up Fe from the complex and use that Fe for growth. Our experiments did not measure Fe uptake from the siderophore complex but instead focused on initial growth. A previous study measured Fe uptake by axenic cultures of *Synechococcus* sp. CCMP1334 (equivalent to *Synechococcus* sp. WH7803) over 2d, finding that the cyanobacterium was able to take up Fe bound to DFB (Hutchins et al., 1999). Several other field studies that found DFB limited the short term Fe uptake by medium (2-5 μm) and large (<5μm) phytoplankton and to a lesser degree limited uptake by the total planktonic community (phytoplankton and heterotrophic microorganisms) (Wells et al., 1994; Wells, 1999; Wells and Trick, 2004). Our results indicate *Synechococcus* is not able to grow well in the presence of DFB bound Fe and unbound DFB. At most light levels and DFB concentrations the cells did not grow. The only growth observed was at very low light levels, and it was not a true measure of bioavailibiltiy because the culture was not transferred multiple times, beyond the point when all stored Fe was exhausted. Even at the lowest DFB concentrations, growth looked similar to the Fe- treatment, suggesting DFB was at best, not very bioavailable. It is remarkable that *Synechococcus* can take up DFB bound Fe but cannot

use it for growth  This suggests that Fe in FeDFB complex is not utilized once transported, or alternatively that the ligand DFB interferes with growth in some way.

## ***Desferrioxamine induced growth inhibition in Synechococcus***

In both field incubations and laboratory experiments DFB consistently inhibited the growth of *Synechococcus* but the cause of this limitation is unclear. The most parsimonious explanation is that adding more FeDFB decreased the amount of Fe', but chemical modeling found no evidence of this. The addition of purely uncomplexed DFB decreases the Fe' concentration, however in all the experiments presented here DFB exceeded Fe by a fixed ratio. At chemical equilibrium, this would mean that the Fe' was constant at all additions (Table 2). However, in a dynamic biological system, uptake could have altered Fe'; to address this possibility the system was also modeled kinetically (Table 3, 4, Figure 5).  The kinetic modeling of DFB suggests that DFB has a large buffering capacity in part because it holds Fe' at such low concentrations (discussed later). This buffering acts to keep the [Fe'] fairly constant at different concentrations of FeDFB. Even if the buffering capacity of DFB were exceeded, additional FeDFB would increase, not decrease Fe', so extracellular decreases in Fe' cannot explain the growth limitation observed. Both kinetic and thermodynamic modeling of Fe' in the medium cannot explain the growth inhibition caused by DFB.

An experiment with the diatom *T. oceanica* found the same sort of concentration-dependent growth inhibition that we observed in *Synechococcus*.  *T. oceanica* differs from *Synechococcus* because it has the ability to take up siderophore DFB bound Fe by means of cell surface reductases (Maldonado and Price, 2001).  FeDFB is bioavailable to

*T. oceanica,* but its growth rate was inhibited when increasing amounts of Fe:DFB were added at a fixed ratio. Using thermodynamic modeling, Maldonado and Price (2001) estimated that Fe' was constant at all concentrations. It appears the inhibitory effect of DFB occurs in both eukaryotic and prokaryotic phytoplankton although *Synechococcus* appears to be more sensitive to growth inhibition.

DFB has recently been shown to bind Co(III) and Mn(III) with high affinity (Duckworth and Sposito, 2005; ). To determine if DFB induced metal co-limitation, cells were cultured in DFB then either Fe(III) or Mn(II) was added to see if growth could be recovered. Although Mn(II) is the predominate form of Mn in seawater, DFB can catalize Mn oxidation under some conditions (Duckworth and Sposito, 2005; Duckworth et al., 2009). Mn is present at nanomolar concentrations in the ocean and does not normally limit growth (Bruland et al., 1994). By contrast, Co is found at picomolar concentrations and can limit phytoplankton (Saito and Moffett, 2001). The speciation of Co is complex, it exists predominantly in the Co(II) state but Co(III) ligands are found in the ocean (Saito et al., 2005). In the rescue experiment (Figure 4) the addition of Fe alone was able to revive cells limited by DFB while the addition of Mn(II) was not. This suggests that Fe is the primary metal involved in growth limitation by DFB. It also indicated that unbound DFB does not kill cells but rather has a bacteriostatic effect that can be alleviated by complexing all unbound DFB and replenishing Fe.

The physiological effects of siderophores were examined further by looking at the influence of light on the growth of *Synechococcus*. When cells were grown at light levels above 10 $\mu E\ m^{-2}\ s^{-1}$ higher concentrations of DFB inhibited growth more acutely and

caused bleaching. In one sense this is surprising, higher light levels typically require lower Fe quotas (Sunda and Huntsman, 1997) so under Fe limitation strains grown in high light should have an advantage, but they do not. Under Fe limitation *Synechococcus* may experience increased oxidative stress and in fact, some Fe stress proteins are known to be expressed under oxidative stress (Exss-Sonne et al., 2000; Yousef et al., 2003; Dwivedi et al., 1997). DFB has been shown to convert superoxide into long- lived nitroxide radicals potentially making increasing oxidative stress (Davies et al., 1987). However, an experiment using superoxide dismutase to decrease superoxide or xanthine and xanthine oxidase to generate superoxide, found no significant differences in the DFB growth effect (data not shown). The decline in growth rate with increasing light intensity is  ihe opposite of what is seen in both Fe replete and Fe limited cultures, this suggests the inhibitory effect of DFB is distinct from natural Fe starvation.

An alternative explanation of the DFB effect is that above some threshold concentration the unbound ligand begins to interfere with Fe(III)-containing proteins. Intracellular proteins that contain Fe(III) are a possible target but the potential mechanism of entry into the cell is unclear; *Synechococcus* and *Prochlorococcus* can take up organic carbon (Mary et al., 2008; Moore et al., 2005; Paoli et al., 2008). There is also evidence showing *Prochlorococcus* sp. MED4 has a high sensitivity to the antibiotics ciprofloxacin and rifampcin, suggesting that it may transport small molecules more readily than other gram negative bacteria (Osborne, pers. comm. 2009). Full growth inhibition by DFB occurs at concentrations below the minimum inhibitory concentrations of these antibiotics. In mammals, intracellular DFB inhibits ribonucleotide reductase, arresting

cells in S-phase (Nyholm et al., 1993). Cyanobacteria use a vitamin $B_{12}$-containing ribonucleotide reductase (Gleason and Olszewski, 2002) and S-phase arrest did not occur in *Synechococcus* sp. WH8102 (data not shown). However, *Synechococcus* possesses Fe-containing photosystem proteins that present additional targets for inactivation by intracellular DFB. Even if DFB did not enter the cytoplasm it may still disrupt Fe-containing proteins. Unbound DFB has a mass of 560.69 daltons so it could potentially pass through porins and interfere with Fe(III)-containing proteins in the periplasm, cytoplasmic membrane or outer membrane.

### *The stimulation of heterotrophic bacteria by desferrioxamine B*

In bottle incubations DFB inhibited the growth of *Synechococcus* while simultaneously stimulating the growth of a group of heterotrophic bacteria in a concentration-dependent manner. The difference between the two populations is pronounced and it suggests heterotrophic bacteria may benefit from siderophores at the expense of cyanobacteria. This is consistent with work in six strains of marine heterotrophs showing that siderophore-bound Fe is taken up and that the addition of DFB can increase growth rates in the presence of the EDTA bound Fe (Granger and Price, 1999). There are several plausible explanations for enhanced growth in heterotrophic bacteria. It is possible FeDFB provided Fe needed for heterotrophic growth, although the addition of FeEDTA did not stimulate growth. It is also possible that DFB was used as a carbon source by the heterotrophic bacteria in our bottle incubations. Since structurally similar ferrioxamine E and G are present in the ocean (Mawji et al., 2008), the

consumption of siderophores is plausible. Alternatively, DFB may have indirectly stimulated heterotroph growth. Both nutrient stress and the transition into stationary phase have been shown to increase the production of dissolved organic material (DOM) in phytoplankton. (Nagata, 2000; Myklestad et al., 1989; Myklestad, 1995; Puddu et al., 2003). Phytoplankton inhibited by DFB could have released additional DOM into the medium, providing polysaccharides and amino acids for the heterotrophs.

## *Chemical kinetics*

This is, to our knowledge, the first attempt to model what happens when the buffering capacity of a metal ligand is exceeded by phytoplankton uptake, and it reveals some surprising results about buffering by strong ligands. It is surprising that DFB can effectively buffer Fe. DFB has an forward rate constant ($k_f$ =1.9×10$^6$ M$^{-1}$ s$^{-1}$) that is four orders of magnitude greater than EDTA ($k_f$ =20 M$^{-1}$ s$^{-1}$), but the dissociation constants for the two ligands are quite similar ($k_d$ of 1.5×10$^{-6}$ s$^{-1}$ and 1×10$^{-6}$ s$^{-1}$ respectively) (Witter et al., 2000; Hudson and Morel, 1990). This means the rate DFB and EDTA can add Fe' to the Fe pool is similar at a given complex concentration. However, FeDFB forms more quickly than FeEDTA reducing the concentration of Fe'; this reduces the uptake rate of phytoplankton because uptake is dependent on [Fe']. This dependency can be seen in the simplified rate equation $\frac{d[Fe_{\mathrm{cell}}]}{dt} = k_{Uptake}[Fe'][cells]$. DFB's strong binding slows the rate of cellular uptake, making it harder to exceed the buffering capacity of DFB. The distinction between a "blown buffer" limiting growth rate and a strong buffer like DFB limiting growth rate is subtle. Under a "blown buffer" scenario, biological uptake outpaces chemical disassociation, this drives the Fe' concentration below what is

found in a purely chemical system. In contrast, DFB chemically buffers Fe' to such a low level, even in the absence of cells ($8\times10^{-13}$ M), that the rate of cellular uptake is extremely low, and in fact it is insufficient for growth. At uptake rates that low, even the relatively slow DFB ligand can keep up with demand.

The uptake rates used to model Fe' are not specific to *Synechococcus* so it is possible that cyanobacteria could exceed the buffering capacity of the system. The first way a cyanobacterium could exceed the buffering capacity of DFB is to increase the total number of receptors competing for Fe'. This can be done by increasing cell concentration or by increasing the number of receptors per cell (as in Figure 5D-E), although there are physical limits to both. Alternatively *Synechococcus* may have significantly different kinetic rate constants than diatoms. Diatoms and have optimized their Fe transport ligand to values approaching the diffusion controlled limit for enzymatic efficiency. Estimates of $K_\rho \cdot \rho_{\max} / L_{cell}$ (equivalent to $k_{cat} / K_m$, the measure of enzymatic efficiency) for the diatoms *Pleurochrysis carterae* and *T. weissflogii* equal $9.7\times10^8$ and $9.4\times10^8$ $M^{-1}$ $s^{-1}$ respectively, calculated from data in Hudson and Morel (1990). It is not possible for cyanobacteria to improve on the efficiency of transport but their rate constants could be different, lowering their half saturation constant for instantaneous Fe uptake ($K_\rho$). This alteration would make them better able to take up Fe' at extremely low Fe' concentrations. Lowering the $k_d^{FeY}$ and the $k_{in}$ rate constants or increasing $k_f^{FeY}$ would decrease $K_\rho$, potentially increasing the uptake rate at very low Fe concentrations to the point where the buffering capacity of DFB is exceeded. From kinetic modeling the ligand

system appears to be in equilibrium, but even if the buffering capacity of DFB was exceeded, a decrease in the Fe' concentration cannot explain growth inhibition by DFB.

### *Future directions*

Hydroxamate siderophores have the ability to inhibit the growth of *Synechococcus* at low concentrations while simultaneously enhancing the growth of heterotrophic marine bacteria. The inhibitory effect raises interesting questions about the role of siderophores in the marine environment. What is the mechanism of growth inhibition in *Synechococcus*? Do siderophores represent a form of chemical competition between microbes? It is conceivable that bacteria produce siderophores, in part, to inhibit the growth of other bacteria or cyanobacteria in addition to acquiring Fe. Before any potential bacterial interactions can be investigated in detail, it is necessary to understand the mechanism of siderophore inhibition in *Synechococcus.*

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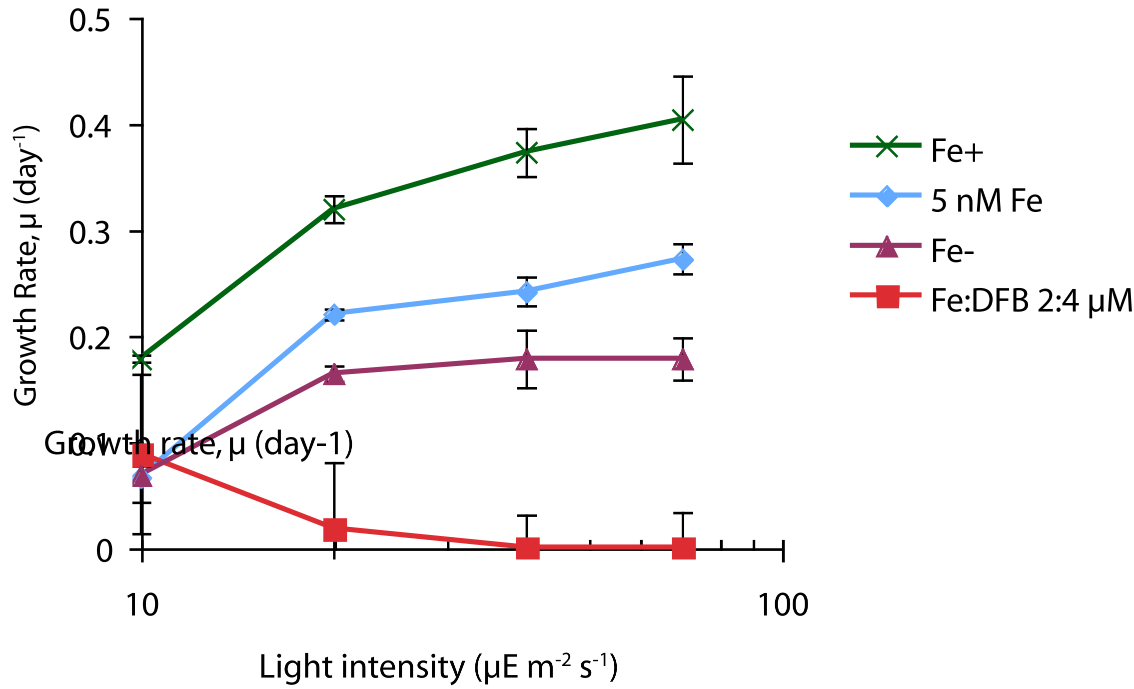


Supplemental Figure 1 The growth rates of *Synechococcus* sp. WH7803 photo-acclimated then grown in constant light at 10, 20, 40 or 70 μE $m^{-2}$ $s^{-1}$. Each line represents decreasing concentrations of total Fe in the media or media containing 2:4 μM FeDFB. The growth rates were calculated from *in vivo* phycoerytherin fluorescence. Error bars represent standard error of 3 biological replicates.

# Chapter 4
# Conclusions and future directions

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The work presented here has begun to reveal how marine *Synechococcus* adapted their gene content and physiology in response to Fe limitation induced by low Fe concentrations or the presence of Fe binding siderophores. This work builds on previous physiological and chemical work describing Fe acquisition by applying molecular and genetic methods to answer questions about adaptation of *Synechococcus* to Fe limitation.

### *Fe limitation in marine Synechococcus*

*Prochlorococcus* have a uniform Fe stress gene distribution while *Synechococcus* strains can vary in their complement of Fe stress genes. The variation in *Synechococcus* suggests that Fe may play a role in the differentiation of strains and clades. Within *Synechococcus*; Fe stress genes are more prevalent in coastal strains of *Synechococcus*. The geographical trend was also observed in the GOS metagenomic dataset. To understand how different clades of *Synechococcus* respond to Fe stress, I developed an imunocytochemical flow cytometry method to detect the expression of the iron stress protein IdiA in cells. The assay detected Fe stress in the Costa Rica Upwelling dome and revealed that different population of *Synechococcus* with distinct Fe stress levels or IdiA serotype inhabit water separated vertically by just a few meters.

Despite higher Fe concentrations, Fe limitation may be a stronger selective force in coastal and upwelling environments than it is in the open ocean. The findings agree with chemical and biological observations finding that coastal and upwelling areas experience Fe limitation (Hutchins et al., 1998; Hutchins et al., 2002; Fiedler, 2002). The trend seen in Chapter 2 also agrees with the observation that transport genes for other

metals are prevalent in coastal *Synechococcus* sp. CC9311 (Palenik et al., 2006). It appears that Fe stress responses are more important under changing nutrient conditions or in areas where other nutrients increase transiently by mixing or upwelling. It may be that there is little ecological or evolutionary value in optimizing the cell for Fe limitation in an environment where nitrogen or phosphate are also likely to limit growth. Of course it is also possible that we have yet to discover a key Fe transport system used by oligotrophic cyanobacteria.

More work needs to be done to determine the effect of Fe limitation on the structure of *Synechococcus* communities. The assay described in Chapter 2 could be expanded into a general *Synechococcus* Fe stress assay with the production of a less specific IdiA antiserum developed raised against native IdiA. A less specific antiserum could serve as a broad screening tool but it would also allow subpopulations that were particularly sensitive or resistant to Fe limitation to be identified by sorting and sequencing their internally transcribed spacer region. Work is also needed to determine physiological differences in Fe acquisition by measuring the quotas, uptake rates and half saturation constants for marine *Synechococcus* strains. This could provide direct evidence of differences in Fe stress response and provide invaluable data for comparing and modeling phytoplankton growth.

## *Siderophores and Synechococcus*

Siderophores have been extensively studied in a pathogenic context but the bioavailibity and physiological effects of these complexes to phytoplankton and bacterioplankton is just beginning to be explained (Maldonado and Price, 2001; Granger

and Price, 1999; Hutchins et al., 1999; Wells and Trick, 2004). In the third chapter I look ed at the effects of a hydroxamate siderophore on a natural assemblage of plankton, revealing that Fe bound to an excess of DFB stimulates the growth of heterotrophic bacteria while simultaneously limiting the growth of *Synechococcus* in a dose dependent manner. Additional laboratory experiments with *Synechococcus* revealed that DFB could inhibit the growth of *Synechococcus* beyond what is observed in Fe deficient media. This effect occurred at concentrations as low as 20-40 nM. The effect was reversible by Fe addition, however it was exacerbated at increasing light intensities. The growth inhibition by DFB cannot be explained by extracellular decreases in Fe' or total Fe. The possibility that DFB induced co-limitation by completing Mn(III) or Co(III) was examined using Fe and Mn additions, but no evidence for this explanation was found. One possible explanation for the growth inhibition is the hypothesis that DFB interferes with Fe(III) containing proteins in the cytoplasm, cytoplasmic membrane or periplasm.

My work and work by others suggest that siderophore use may not be a common strategy for oligotrophic picocyanobacteria. Siderophore production has been observed *Synechococcus* spp. PCC7002 and WH8101 isolated from shorelines (Van Baalen, 1962; Waterbury et al., 1986; Vraspir and Butler, 2009), but siderophore production has not been observed in oligotrophic strains and all sequenced *Synechococcus* strains lack the TonB energy system used by siderophore transporters. One report exists of *Synechococcus* sp CCMP1334 taking up Fe bound to DFB but other experiments with field populations have shown little Fe uptake (Wells et al., 1994; Wells, 1999; Wells and Trick, 2004). From my work it appears DFB, and potentially hydroxamate siderophores

in general, inhibit the growth of oligotrophic *Synechococcus* but benefit some heterotrophic marine bacteria.

The kinetic modeling of the DFB-EDTA ligand system in Chapter 3 is, to my knowledge, the first attempt to model the “blown buffer” problem in marine chemistry that occurs when cellular uptake exceeds the buffering capacity of a metal ligand. The modeling reveals that strong ligands can have high buffering capacities by reducing Fe’ and indirectly limiting cellular uptake. It also reveals the capacity cells have to lower the Fe’ to extrealmy low levels under Fe stress. Finally it reveals that thermodynamic estimates of Fe’ are not suitable for systems with more than one ligand. These observations provide a framework for designing culture experiments with valid estimates Fe’.

The growth inhibition by siderophores raises interesting questions about how DFB interacts with the cyanobacterial cell. Future work with siderophores should attempt to answer this mechanistic question. Experiments looking for DFB uptake by radiolabeled DFB could provide direct evidence of any intracellular effect of DFB. Uptake by *Synechococcus* would raise a number of interesting questions about the role of siderophores in chemical competition and the possibility that *Synechococcus* “pirate” siderophores. The effects of siderophores on co-cultures of *Synechococcus* and heterotrophic bacteria is another interesting avenue of future research. Does siderophore induced stress in phytoplankton benefit heterotrophic bacteria in ways that siderophores alone do not? Siderophores may benefit heterotrophic bacteria in multiple ways. Very little is known about how bacteria and cyanobacteria interact with siderophores, further

research may reveal that siderophores have additional functions in the environment, possibly even serving a role as antibiotics.

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