To the Graduate Council:

I am submitting herewith a dissertation written by Melanie Lynn Eldridge entitled "The effects of Fe on plankton in HNLC regions of the world's oceans." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Microbiology.

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We have read this dissertation

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(Original signatures are on file with official student records)

The effects of Fe on plankton in HNLC regions of the world's oceans.

A Dissertation

Presented for the

Doctor of Philosophy

Degree

The University of Tennessee, Knoxville

Melanie Lynn Eldridge

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Dedication

This work is dedicated to my husband Brent and Nomis. I could never have made it through this without them.

Acknowledgements

I would like to thank Brent for having the stamina to deal with all my moods throughout the process, and for constant support in any endeavor I attempt. I would also like to thank Nomis for constant input during the preparation of this document and for helping me make it through my preliminary exam (both times), and to my mother Karen Kelley for always being there for me during my meltdowns. I owe this to you guys.

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Abstract

Ecosystem-scale productivity in many marine environments has been demonstrated over the last 15 years to be regulated by the biological availability of iron (Fe). Increasingly, more attention has been focused on coastal upwelling regions, like those off the coasts of California and Peru, since these areas are responsible for a disproportionately high contribution to new global production. A mosaic of nutrient gradients exists in upwelling regions, making them ideal sites for studies of nutrient affects on aquatic microbial communities. Paradoxically, both the California and Peruvian upwelling regions have also been shown to be Fe-limited. The present studies took place in the Peruvian upwelling region to demonstrate that alterations in Fe concentrations influence both the productivity and community structure of natural populations of plankton, and that these alterations have significant implications for biogeochemical cycling and the 'biological carbon pump'.

The work within this dissertation has demonstrated that phytoplankton in the Peruvian upwelling region are limited by Fe. More importantly, this work has shown that phytoplankton community structure is influenced by Fe concentration. Because dissolved organic matter produced by phytoplankton is used by bacteria and correlations have been drawn between bacterial diversity and ability to consume diverse carbon substrates, the effects of iron concentration on bacterial diversity was also assessed. I have examined how the bacterial community

responds to changes in iron concentration using both a natural iron gradient (a nearshore to offshore marine transect over the coastal shelf) and controlled shipboard experiments. I have determined that iron additions in this area increased bacterial diversity in on-deck bottle incubations. Samples collected *in situ* further demonstrated that iron and other factors cause changes in bacterial diversity, which highlights the complexity of interactions between bacteria and the physical characteristics of the upwelling region. These results are the first to show that bacterial diversity in marine systems is influenced by iron availability.

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Part I

Literature Review

BACKGROUND

i.) The biological carbon pump

Globally the oceans cover over 70% of the earth's surface (Costanza et al. 1997) and act as a primary habitat for photosynthetic organisms, which convert CO_2 into organic matter that may subsequently be used for energy. Photosynthetic microorganisms have long been recognized as the base of the food chain in ocean systems (e.g. Ryther 1969). Globally marine phytoplankton carry out as much photosynthesis as all the plants on land, contributing *ca* 50% of global primary production (Field et al. 1998). In fact, it has been suggested that excess phytoplankton-mediated primary productivity was responsible for the rapid glacial-interglacial changes in atmospheric CO_2 content over geological time scales (Martin 1990a). It was not until the early 1980s, however, that the microbial loop (the flux of carbon through bacteria) was recognized as an important path for carbon in ocean systems (Azam et al. 1983).

It is now known that bacterial activity may be incorporated into marine food webs in at least two ways. Bacteria may act as 'links' for organic carbon by assimilating photosynthetic products and subsequently producing new bacteria, which may be eaten by larger microorganisms transfering the carbon to the next trophic level. Also, they may act as 'sinks' for organic carbon by performing respiration, resulting in a loss of CO_2 back to the atmosphere. One of the most important realizations to come from this is that bacterial respiration decreases the

efficiency of the 'biological carbon pump', through which CO_2 is fixed from the atmosphere by phytoplankton and transported to the deep ocean as sinking particulate organic matter (del Giorgio & Duarte 2002, Neuer et al. 2002) (Figure 1, all figures and tables for each section can be found at the end of that section). It is estimated that bacterial respiration equals or exceeds primary production in vast stretches of the oligotrophic (unproductive) open ocean (del Giorgio et al. 1997). In more productive (eutrophic) ecosystems, primary production (P) typically exceeds respiration (R), though the ratio of P / R is extremely variable, allowing the oceans to act as net sinks for atmospheric CO_2 (Duarte & Augusti 1998).

ii.) Iron in the oceans

Prior to the late 1970s, studies conducted on seawater suffered from the under appreciated possibility of contamination with metals. Even as far back as the 1930s, scientists recognized that factors other than phosphate, nitrate, light, and temperature could regulate phytoplankton growth (Gran 1931). In fact, it was concluded that Fe may be one of the factors affecting phytoplankton growth (Hart 1941). Papers of this kind were discounted for decades due to the amount of Fe contamination thought to be present. As such, some landmark papers (e.g. Redfield 1958) did not consider the influence of trace metals in the ocean because previous estimates of their concentrations were high due to contamination. Since trace metals are required in only scant amounts by marine

microorganisms, any mishandling of samples could result in a greater concentration of metal in the collected sample than was present in ambient seawater (Bruland et al. 1979). Bruland et al. (1979) developed intricate cleaning protocols (termed trace metal – clean techniques), which allow scientists to accurately measure trace metals, which we now know persist at only nanograms per liter concentrations in seawater. These techniques have allowed for an explosion of research examining the role of trace metals in microbial metabolism, of which the most well studied is Fe. In general, these techniques address the use of clean materials for research (e.g., while metals can be removed by acid leaching from polycarbonate and Teflon, glass can never be made truly metal clean), improved sampling techniques (the development of clean sampling systems for marine surface waters) and improved sampling processing protocols (e.g., the use of Class – 100 HEPA filter units to provide dust free air during on-ship and laboratory handling of samples).

Concurrent to the above ideas, the application of these techniques to bottle incubation experiments (sometimes termed micro- or mesocosms) allowed scientists to study the influence of nutrients on planktonic microorganisms in a controlled environment while using seawater as a growth medium. Shipboard incubation experiments became critical to understanding the factors that limit primary production in the world's ocean. Martin & Fitzwater (1988) and Martin & Gordon (1988) used these approaches to present evidence that phytoplankton in the open ocean are Fe-limited, which renewed a debate over what actually limits

primary production in the sea, as well as how one determines this is a limiting factor (see Banse 1990, Martin et al. 1990, Banse 1991, Martin et al. 1991a). Martin ultimately legitimized his theory with a rigorous evaluation of bottle incubation experiments in several high-nutrient, low-chlorophyll (HNLC) regions of the world's oceans (Martin et al. 1990, Martin et al. 1991b) and planned a mesoscale Fe fertilization experiment, which was carried out posthumously by his colleagues (Martin et al. 1994). The ideas of JH Martin and colleagues led to the theory that if the ocean were fertilized with large quantities of Fe there would be an increase in phytoplankton productivity and CO_2 drawdown from the atmosphere, which would mitigate the greenhouse effect (Martin 1990a). Mesoscale Fe fertilization experiments have now conclusively shown that phytoplankton in vast stretches of open ocean (covering approximately 30% of the world's oceans) are limited by Fe (Boyd et al. 2000, Behrenfeld & Kolber 1999, Martin et al. 1994, Coale et al. 1996). More recently, upwelling regions off the west coasts of North and South America have been discovered to be Fe-limited (Hutchins et al. 1998, Hutchins et al. 2002, Firme et al. 2003, Eldridge et al. 2004a).

Bacteria in these regions have been the subject of much less research than phytoplankton and the studies that have been conducted disagree on the response of the bacterial community to Fe addition. Some studies have shown that Fe addition stimulates bacterial abundance, productivity, or growth rate in high nutrient – low chlorophyll (HNLC) waters of the world's oceans (Paluski et

al. 1996, Cochlan 2001), while others have concluded that Fe addition has no direct effect on the bacterial community (Church et al. 2000, Kirchman et al. 2000). These studies examined either total bacterial changes or total heterotrophic bacterial changes, but only one study to date has monitored the effects of Fe on bacterial diversity in HNLC regions (Hutchins et al. 2001). Hutchins et al. (2001) found little to no change in bacterial diversity with Fe addition in three Fe-limited regimes (discussed later).

Understanding the factors that drive bacterial diversity in the ocean is critical to understanding the 'biological carbon pump', as bacteria have a great potential to decrease carbon export to deep waters and to shunt significant quantities of CO_2 back into the atmosphere. Cottrell and Kirchman (2000) have shown that different bacteria selectively consume different forms of carbon, and that no group was able to utilize all of the forms of carbon in the DOM (dissolved organic matter) pool. As such they concluded that a diverse assemblage of bacteria is essential for complete degradation of all marine DOM. Therefore, as bacterial diversity increases, the community may be better equipped to consume carbon, making studies of the interactions of bacteria with nutrients critical to understanding the 'biological carbon pump'.

iii.) HNLC and upwelling regions

HNLC regions have been the subject of significant research during the last decade as there are excess concentrations of macronutrients (N, P, Si) relative

to the biomass of phytoplankton (as estimated by chlorophyll). It is in these regions that Martin and others have proposed Fe limits system production (e.g., Martin et al. 1994). Typical nutrient profiles in these regions contain dissolved nitrate, phosphate, and silicate concentrations in the micromolar range, while dissolved Fe concentrations in HNLC environments typically occur in the picomolar range. Prior to the Southern Ocean Iron Enrichment Experiment (SOIREE), a mesoscale Fe fertilization experiment, concentrations of dissolved nutrients in the upper mixed layer were 25 μM nitrate, 1.5 μM phosphate, 10 μM silicate, and 80 pM Fe (Boyd et al. 2000). Hutchins et al. (2002) measured similar concentrations in the Humboldt Current and Peruvian upwelling regions of the equatorial Pacific (8.9 and 16.3 μM nitrate, 0.63 and 1.2 μM phosphate, 5.6 and 7.8 μM silicate, 100 and 80 pM Fe, for the Humboldt Current and Peruvian upwelling, respectively). HNLC conditions occur in remote, offshore areas of the subarctic north Pacific, subtropical equatorial Pacific, and Southern Ocean, and have now been the sites of intensive study and mesoscale Fe fertilization experiments.

It is now known that some coastal upwelling regions, such as those that occur off the coasts of the Americas and Africa, may contain the same nutrient profiles as open-ocean HNLC regions. Phytoplankton in areas off the coast of California and Peru have been shown to be limited by Fe (Eldridge et al. 2004a, Firme et al. 2003, Hutchins et al. 2002, Hutchins et al. 1998). The interactions of the physical characteristics of each region and the metabolism of the planktonic

organisms results in classic HNLC conditions. Each region is characterized by a fairly shallow continental shelf, with a steep shelf break, and a rapid decline in depth offshore. Winds drive surface water by Ekman transport from nearshore out to sea, and this removal of nearshore water requires some form of compensatory flow to the surface. Deep-circulating water hits the steep continental shelf break and flows rapidly to the surface (Brink 1983). Deep water is characterized by higher nutrient concentrations than surface water (Brink 1983) which, when transvected offshore, creates a nutrient gradient profile. The Peruvian upwelling region is of particular interest because of its year-round upwelling (Longhurst 1998) (versus seasonal upwelling off the coast of California) and near absence of other sources of nutrients (e.g. riverine input) (Brink 1983). Bruland et al. (2004) measured nitrate, phosphate, silicate, and Fe during a nearshore to offshore transect in the Peruvian upwelling region and found this gradient to occur in our sampling region (Figure 2). HNLC conditions arise in areas of upwelling because Fe (with its low concentration and absolute requirement by all organisms) is rapidly depleted in offshore areas.

COMMUNITY ANALYSIS

i.) Flow cytometry

Flow cytometry is a method that allows scientists to quickly enumerate different groups of phytoplankton based on size, density, and pigment content.

Most flow cytometers contain three basic parts: a pressurized fluid injection system (that focuses the sample into a narrow beam such that only single particles pass through), an illumination source (that emits light onto each particle, exciting fluorescent molecules), and a detection system (that records properties of each particle as 'events') (Collier & Campbell 1999) (Figure 3). Samples are injected into the flow cytometer (along with internal standard beads) and focused so that the particles individually pass through the light and detection systems. Pigments within phytoplankton are excited by the laser light and emission data (its fluorescence and how it scatters light) is recorded for each particle. Light scatter is divided into two components: forward angle light scatter (FALS) and side scatter (SSC), each of which is influenced by different properties of the particles. FALS is influenced primarily by particle size, while SSC is influenced by not only the particle's size but also by its refractive index and internal cell structure (Campbell 2001).

Standard photomultiplier tube detectors in current systems are capable of detecting four ranges of fluorescence, termed FL1 – FL4. Green fluorescence in the range of 515 – 545 nm (FL1) can be used to detect nucleic acid stains (e.g. SYBR green I from Molecular Probes). Orange fluorescence in the range of 564 – 606 nm (FL2) is used to detect phycoerythrin of Cyanobacteria and Cryptomonad algae. Long-pass red fluorescence, with wavelengths greater than 670 nm (FL3), is used to detect the major photosynthetic pigment found in phytoplankton, Chlorophyll *a* (Chl *a*). Narrow band-pass red fluorescence (653 –

669 nm) is used to detect phycocyanin of Cyanobacteria and Cryptomonad algae. The properties of each particle are recorded by a computer and analyzed with software that allows the user to place each particle into defined groups based on size versus fluorescence signature. A complex algorithm determines the size of each particle based on FALS (Salzman et al. 1990). In total, single cell analysis combined with the use of multiple fluorescence and scatter parameters allows scientists to identify and enumerate the abundance of cells within each of a series of subgroups.

Advantages

Speed - With flow cytometry thousands of cells are analyzed every second, allowing scientists to quickly gather tens of thousands of data points. Epifluorescence microscopy (including fluorescence in situ hybridization, FISH) counting of bacteria is limited by the number of cells counted, generally only a few hundred cells, while flow cytometry allows for a much more statistically sound sampling of the community in a fraction of the time (Campbell 2001). Although ongoing efforts are seeking to merge these technologies (FISH by flow cytometry) there are still technical hurdles which have not been overcome.

Sensitivity - Trousselier et al. (1993) reported accurate counting of a culture with as few as 10 cells mL^{-1}. While this lower limit is unrealistic for field-based studies, as few as 10^2 - 10^3 cells mL^{-1}can be confidently counted with flow cytometry,

which is 10 – 100x more sensitive than microscopy (Collier & Campbell 1999, Campbell 2001). Moreover, flow cytometers are capable of detecting dimly fluorescent phytoplankton such as *Prochlorococcus* spp. (the most abundant photosynthetic organism in the ocean), which may be too dim for detection with epifluorescence microscopy (Partensky et al. 1999, Olson et al. 1993, Chisholm et al. 1988).

Precision - The amount of variation observed for epifluorescence microscopy is dependent upon several factors (e.g. field of view) and estimated abundance values may vary by as much as 10% (Kirchman et al. 1982). With flow cytometry, the amount of variation observed for the repeated detection of standard beads can be as low as 1% (Olson et al. 1993) and for routine use typical values of 1.4% variation for forward light scatter and 1.9% for fluorescence may be obtained (Frankel et al. 1990).

Sorting capability - With flow cytometry it is possible to capture only those cells that have user-defined characteristics for further analysis (e.g. HPLC analysis of photosynthetic pigments) (Olson et al. 1993, Mackey et al. 2002). For example, with the FACSCalibur flow cytometer, individual cyanobacterial cells with properties similar to *Synechococcus* spp. could be collected to later determine that these were in fact *Synechococcus* spp. or to determine which species were present.

Real-time analysis - Flow cytometers, such as the one used in this study, are routinely used in ship-based experiments to gather data on cellular abundance of phytoplanktonic groups (e.g. DiTullio et al. 1993, Li 1994). Thus, it is possible to redesign experiments based on data gathered at sea (Campbell 2001), which allow scientists to maximize the time spent on ship. In addition, by performing analyses at sea, sample transport back to the lab for analysis becomes unnecessary, as does fixation of samples with hazardous substances such as glutaraldehyde (used to preserve samples for epifluorescence microscopy counting). Furthermore, by analyzing samples on ship, the error associated with the preservation of large cells can be eliminated (see Lepesteur et al. 1993).

Common uses

Flow cytometry has been used in hundreds of studies, including many in marine systems, to examine the abundance and diversity of aquatic plankton populations. In fact, 302 papers that used flow cytometry on samples from the ocean were published in the years between 1989 and 1999, of which, 77% examined phytoplanktonic populations (Legendre et al. 2001). This method has previously been used to assess the effect of Fe on the phytoplankton community in the tropical north Pacific (e.g. DiTullio et al. 1993), the south Pacific (e.g. DiTullio et al. 2003) and the Southern Oceans (e.g. van Leeuwe et al. 1998). DiTullio et al. (1993) found very little change in phytoplankton community

composition when Fe alone was added, but noted dramatic changes after the addition of Fe in combination with added nitrate, phosphate, and silicate. DiTullio et al. (2003) noted different abundance patterns for each of the four groups of organisms examined with flow cytometry during a transect in the southern Pacific Ocean, though no attempt was made to relate these abundances to Fe concentration. Brown et al. (2003) have used flow cytometry to monitor several planktonic groups along a transect in the equatorial Pacific, demonstrating that community structure and distributions of individual groups are correlated with the physical structure of the equatorial Pacific (something we have also independently determined for bacteria in the Peruvian upwelling region). In addition, this technique led to the discovery of free-living marine *Prochlorococcus* spp. (Chisholm et al. 1988), the most abundant photosynthetic organism in the ocean (Landry & Kirchman 2002). While this method was traditionally used for phytoplankton, due to their autofluorescent properties, increasingly scientists are examining bacterial populations as well. Most studies that examine bacterial populations with flow cytometry limit their analysis to three groups: *Prochlorococcus* spp., *Synechococcus* spp., and total heterotrophic bacteria (DiTullio et al. 2003). These two genera of Cyanobacteria are easily distinguishable because they contain different signature pigments, while the heterotrophic bacteria can be examined through staining with different fluorescent DNA-binding dyes.

ii.) Fast repetitive rate fluorometry

The fast repetitive rate fluorometry (FRRF) technique is used to determine the photochemical quantum efficiency of photosynthetic reaction centers in phytoplankton, which can be equated to overall photosynthetic health of the population and is calculated as the ratio of variable fluorescence to maximal fluorescence given off from Photosystem II (PSII) in response to pulses of light (Kolber et al. 1998). Dark-adapted samples are subjected to a series of short light pulses using a cylinder of blue-light LEDs, of which the intensity, duration, and length between them are controlled (Kolber et al. 1998). A series of filters collects fluorescence readings from the bottom of the sample and records data to a computer, which is equipped with FAST^{tracka} FRRF post-processing program version 1.4 (Chelsea Instruments, Ltd.). Two parameters (out of several possible) are calculated for each sample: minimal (or baseline) fluorescence yield (F_0, which occurs when all the photosystems are in an 'open,' oxidized state) and maximal fluorescence yield (F_m, which occurs when all of the photosystems are in a 'closed,' reduced state) (Kolber et al. 1998, Kolber & Falkowski 1993). To infer photosynthetic efficiency (F_v / F_m), F_v is calculated from F_0 and F_m by:

$$F_v = F_m - F_0 \quad (1).$$

Advantages

<u>*Speed*</u> - Measurements of photosynthetic efficiency are performed on ship, and after initial dark-adaptation, samples may be analyzed, each in a few

microseconds with results available relatively quickly (Kolber et al. 1998). Furthermore, measurements of variable fluorescence are non-destructive to cells allowing for their continued use in experiments.

Real-time analysis - Because data is generated on-ship, experimental design, cruise path, and station location can all be altered based on the measurement of photosynthetic efficiency, to maximize the time on ship. Fast repetitive rate fluorometers may even be hooked in-line with a continuous surface water pumping system (Kolber & Falkowski 1993).

Multiple-parameter analysis - By varying the duration, intensity, and interval of light flashlets at least six key photosynthetic parameters are calculable for inclusion in photosynthesis models: functional (i.e. photochemically effective) absorption cross-section of PSII (σ_{PSII}), minimal fluorescence yield (F_0), maximal fluorescence yield (F_m), the extent of energy transfer between PSII reaction centers (p), maximum quantum efficiency of photosynthetic reaction centers (F_v / F_m), and rates of electron transport within PSII (Babin et al. 1996, Kolber et al. 1998).

Precision - The measurement of active fluorescence derives solely from photosynthetic organisms (with the possible exception of pheopigments, which

typically occur at the bottom of the euphotic zone), therefore the measurements are unquestionably related to gross photosynthesis (Kolber & Falkowski 1993).

Common uses

As stated in Laney (2003), fast repetitive rate fluorometry has become indispensable in the field of aquatic microbial ecology. It has been used to confirm that phytoplankton in the equatorial Pacific were Fe-limited, during the IronExII (Behrenfeld et al. 1996), SOIREE (Boyd & Abraham 2001), and EisenEx (Gervais et al. 2002) mesoscale Fe fertilization experiments. Typical values of F_v / F_m in healthy, nutrient-rich cultures of phytoplankton routinely approach 0.65 (Kolber et al. 1988) (Table 1). Berman-Frank et al. (2001) found that in *Trichodesmium* spp. (the genus of organisms responsible for a large percentage of nitrogen fixation in the ocean) Fe-replete cultures had values of 0.54, while Fe-limited cultures had values measured at 0.25, demonstrating that these organisms have decreased photosynthetic efficiency with nutrient limitation. In the sea, it has been noted F_v / F_m decreases with vertical stratification and nitrate limitation (Moore et al. 2003) in coastal waters and increases upon addition of Fe to open ocean Fe-limited waters (Gervais et al. 2002, Boyd & Abraham 2001, Behrenfeld et al. 1996). As a result of these and other studies, this parameter is widely accepted as an accurate measure of photosynthetic efficiency and as a proxy for demonstrating limitation by nutrients.

iii.) Chlorophyll a

Measurement of Chlorophyll *a* using the protocol of Welschmeyer (1994) includes filtration of water to collect cells, followed by extraction in 90% acetone overnight, measurement of a single wavelength fluorescence emission, and a simple mathematical conversion.

Advantages

Discrimination against other pigments - Measurement of photosynthetic pigments with the protocol of Welschmeyer (1994) has been optimized to achieve maximum Chl *a* fluorescence, while minimizing or eliminating fluorescence from other pigments (Chl *b*, phytin *a*, and phytin *b*).

Ease - After extraction in acetone overnight, this method is reduced to a single measurement (at one wavelength) and one conversion calculation. Previous groups have attempted to isolate Chl *a* fluorescence from other pigments but these methods were less desirable due to the need for multiple wavelength readings for each sample (e.g. Loftus & Carpenter 1971).

Compatibility - All Turner-designs fluorometers (standard) with adequate blue excitation energy will have similar wavelengths of peak energy in the blue (436 nm) spectra, though certain lamps and fluorometers are superior in their ability to lessen the impact of pheopigments (Welschmeyer 1994).

Sensitivity - Methods used in the protocol of Welschmeyer (1994) are applicable to samples with chlorophyll concentrations as low as the nanogram per milliliter amount. Studies in oligotrophic regions, prior to the introduction of this method, were hampered by a lack of methods sufficiently sensitive to measure Chl *a* without simultaneously measuring Chl *b*.

Common uses

The amount of Chlorophyll *a* present in samples has been routinely used as a gross indicator of the living photosynthetically active phytoplankton biomass (Sakshaug et al. 1997), and has been performed in every mesoscale Fe fertilization experiment to date. Boyd (2002) compared Chl *a* values in two of these experiments SOIREE and IronExII, finding similar concentrations of Chl *a* upon addition of Fe (mean 1.8 µg / L and maximum 3 µg / L for SOIREE, mean 2.4 µg / L and maximum near 4 µg / L for IronExII), but different lengths of time required to achieve peak concentration (Table 1). Chl *a* values increased by a factor of 10 during IronExII and by a factor of 6 in SOIREE when each was sampled prior to Fe addition and during the ensuing bloom of phytoplankton (Boyd 2002).

iv.) T-RFLP analysis

As with bacteria from other environments, most marine bacteria are not easily grown in the lab. It has been estimated that less than 1% of marine bacteria are culturable with standard media (Jannasch & Jones 1959, Lee & Fuhrman 1991). In the last two decades, molecular methods have been developed that have allowed scientists to examine microbial communities without the need for culture. The clone and sequence method, while a powerful approach to analyze microbial communities due to genetic sequences obtained from non-cultured members, requires that a sufficient number of clones be analyzed to ensure complete sampling of operational taxonomic units (OTUs) (Kemp & Aller 2004), necessitating costly amounts of sequencing, which can also be very slow and laborious (Fuhrman et al. 2002). Restriction fragment length polymorpishm (RFLP) analysis of clone libraries, while allowing scientists to decrease the total numbers of clones sequenced may cause related sequences to be construed as identical and therefore underestimate the true amount of diversity in libraries (though this is also a concern for terminal restriction fragment length polymorphism (T-RFLP) analysis). Because of these limitations, newer methods for community analysis may be more amenable to community profiling than is cloning.

T-RFLP vs. DGGE

Community profiling of naturally occurring bacteria in the ocean is still in its infancy (Fuhrman & Campbell 1998), though many methods exist to track changes in microbial populations across diverse environmental or experimental parameters. The two most popular methods used by marine microbial ecologists are denaturing gradient gel electrophoresis (DGGE) and terminal restriction fragment length polymorphism (T-RFLP) analysis, both of which are PCR-based (and therefore subject to all of the inherent biases associated with PCR, Wintzingerode et al 1997). A detailed explanation of T-RFLP methods and principles may be found in Part III of this dissertation. Briefly, samples are subjected to PCR amplification (with fluorescently labeled primers), restriction digested, and terminal fragments are sized with capillary electrophoresis systems (Moeseneder et al. 1999) (see Figure 4 of Part I and Figure 2 of Part IV). With DGGE, samples are PCR amplified (utilizing a 40-mer GC-clamp on one primer), electrophoresed on a polyacrylamide gel (with a known gradient of denaturant running vertically down the gel), and band intensity quantified with band-finding algorithms (Schäfer & Muyzer 2001). T-RFLP is coming to the forefront of community profiling techniques, given the increase in the number of papers in the last few years and new computer algorithms being generated for the analysis of T-RFLP data (e.g. TAP-TRFLP from RDP, T-RFLP Operation Results Analysis Software Tool, and the Phylogenetic Analysis Tool).

In a comparison between optimized methods for T-RFLP and DGGE, Moeseneder et al. (1999) found that the detection limit with T-RFLP is approximately 1 fg of DNA, while that for DGGE is 20 pg, making T-RFLP advantageous for the detection of less abundant OTUs. This issue arises from the detection systems used with each method. Typically DGGE gels are stained with various DNA-binding stains (e.g. Sybr green, ethidium bromide), with intensity determined through analysis of photographs of gels (Schäfer & Muyzer 2001), whereas T-RFLP products are analyzed by fluorescence detection using fully automated laser-detection systems already commonplace in DNA sequence analysis (Moeseneder et al. 1999).

Another problem with DGGE is that although the ability to sequence bands from DGGE gels seems an advantage over T-RFLP, sequences that are closely spaced are not amenable to direct sequencing and require reamplification or cloning to obtain single sequence products. It is often necessary to re-run excised and reamplified bands of interest alongside whole community samples to ensure a single sequence has been obtained prior to sequencing, though this does not always guarantee single sequence capture (Schäfer & Muyzer 2001). Also, with DGGE it is often necessary to create standards applicable to the particular system of interest, so that comparison of samples run on different gels is possible, while attempting to overcome gel-to-gel variation (Schäfer & Muyzer 2001).

T-RFLP vs. ARISA

Community profiling with Automated method of Ribosomal Intergenic Spacer region Analysis (ARISA), in which the length of the internal spacer region between 16S rRNA and 23S rRNA genes is used to profile communities suffers from the fact that peaks in an electropherogram (designated OTUs) may contain more than one species. ARISA patterns may yield similar lengths for genera or species that are completely unrelated (Fisher & Triplett 1999). With T-RFLP, because the position of restriction sites is related to the 16S rDNA sequence for each fragment, organisms that produce peaks of identical size are more likely to be phylogenetically related. Moreover, with ARISA, Fisher and Triplett (1999) found differences in sizes for multiple copies of the 16S-23S internal spacer region within a single organism to be greater than 250 bp, a fact confirmed by Boyer et al. (2001). In addition, though ARISA PCR products may be sequenced, difficulty in assigning lengths of PCR fragments to lengths in the ARISA profile exist for fragments that are longer than approximately 600 bp. Sequencing fragments from each end would be necessary to determine the full size of these PCR products and for the largest sized products this may still be insufficient to determine the entire fragment length.

RESEARCH OBJECTIVES

I hypothesize that in a system where primary production is Fe-limited, changes in ambient Fe concentrations will result in a change in the diversity of both phytoplankton and heterotrophic bacteria. This is of particular interest because it is thought that primary production in large parts of the ocean is limited by Fe availability (e.g. Boyd et al. 2000). Phytoplankton produce most of the dissolved organic matter in the sea, which varies in amount and chemical composition with each organism (Biersmith & Benner 1998, Biddanda & Benner 1997). In fact, the organic matter content of phytoplankton has been shown to vary based on nutrient content (Myklestad & Haug 1972, Myklestad 1977). Since individual bacteria are equipped to use different forms of dissolved organic matter (Cottrell & Kirchman 2000) it is thought that bacterial diversity should be influenced, albeit indirectly, by Fe concentration. With only one known study available, we remain unsure whether bacterial diversity changes in response to Fe concentration. Testing these questions includes two phases of research:

Hypothesis 1. The Fe concentration in surface waters of the Peruvian upwelling affects phytoplankton community composition.

Hypothesis 2. The Fe concentration in surface waters of the Peruvian upwelling affects bacterial community composition.

To investigate each of these hypotheses (that is, to reject the nulls for each), gradients of Fe (both artificial and natural) were monitored for changes in community composition. Community composition has been examined through the use of both traditional methods (for phytoplankton community analysis) and newer molecular methods (for bacterial community analysis).

APPENDIX (PART I)

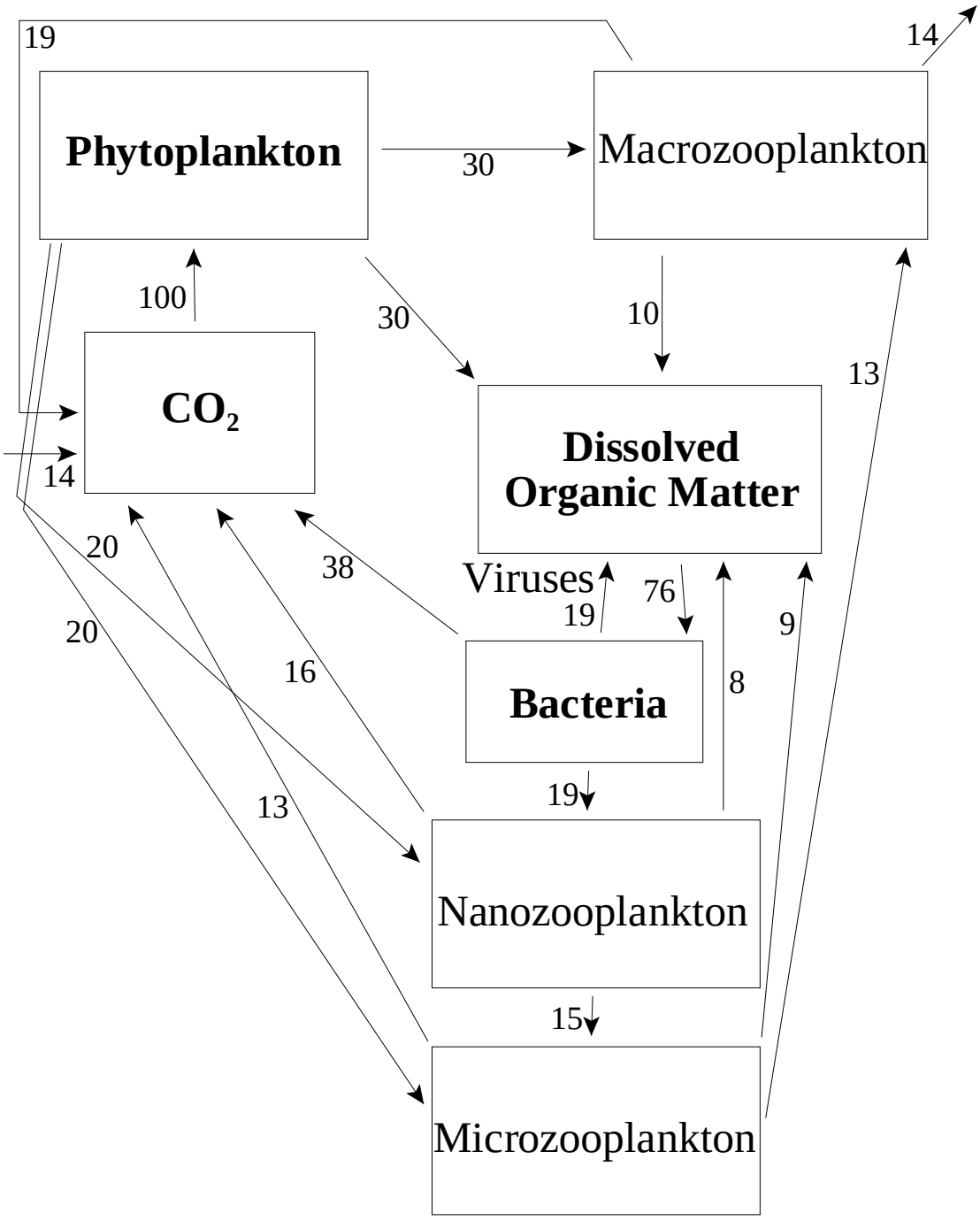

Figure 1. Hypothetical carbon-flux model of marine food webs.
Depicted is a hypothetical model of how carbon is transferred among different trophic levels in marine systems. The microbial loop is shown, indicating that a substantial portion of dissolved organic matter from phytoplankton is respired by bacteria as CO_2. Figure is recreated from Fuhrman (1992).

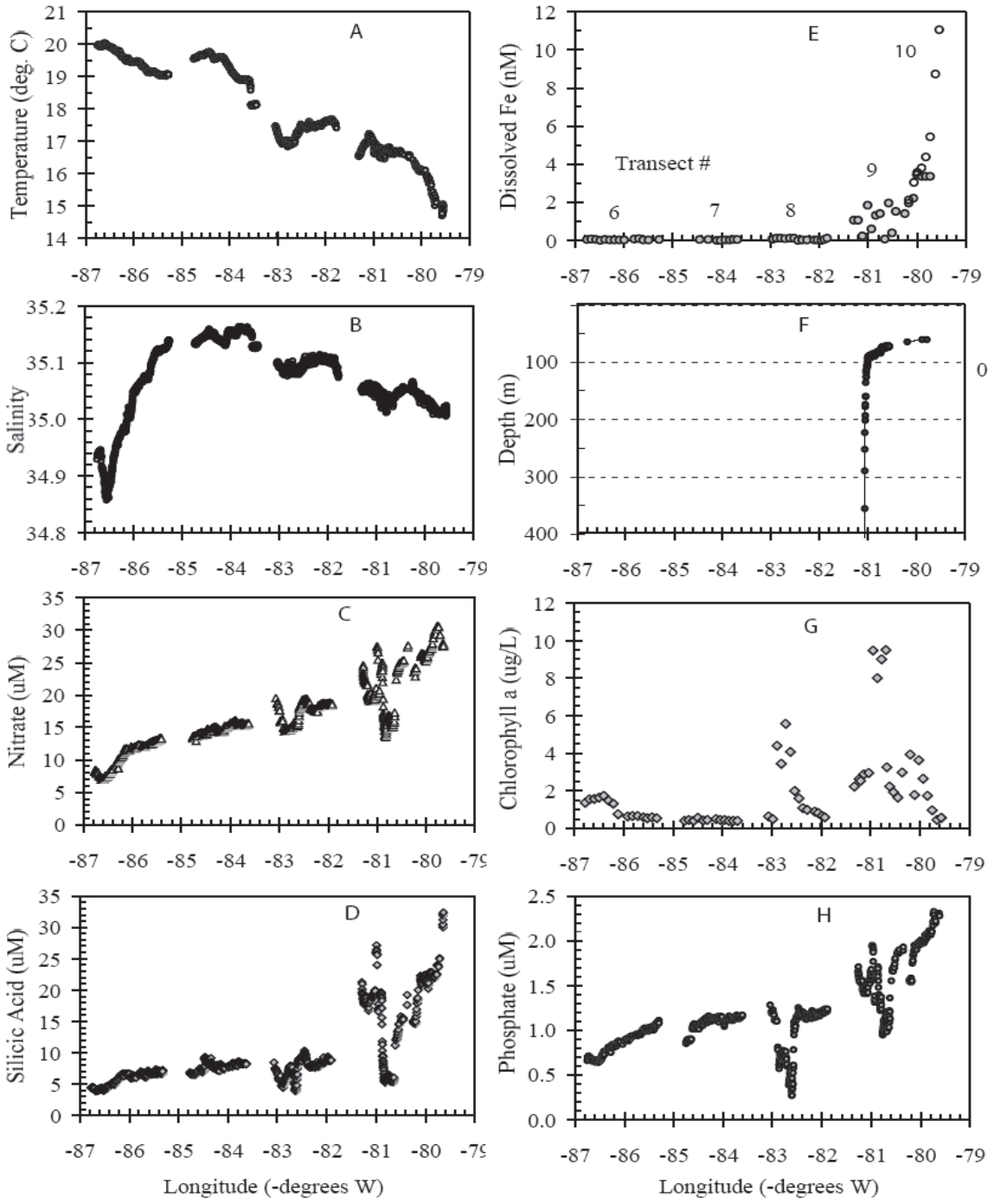

Figure 2. Profiles of conditions during transects in the Peruvian upwelling regime. Shown are the nutrient, salinity, temperature, depth, and Chlorophyll *a* profiles for stations along the cruise transects in the Peruvian upwelling. Typical gradient-like conditions exist in this nearshore to offshore sampling area. Figure courtesy of Dr. KW Bruland (Bruland et al. 2004).

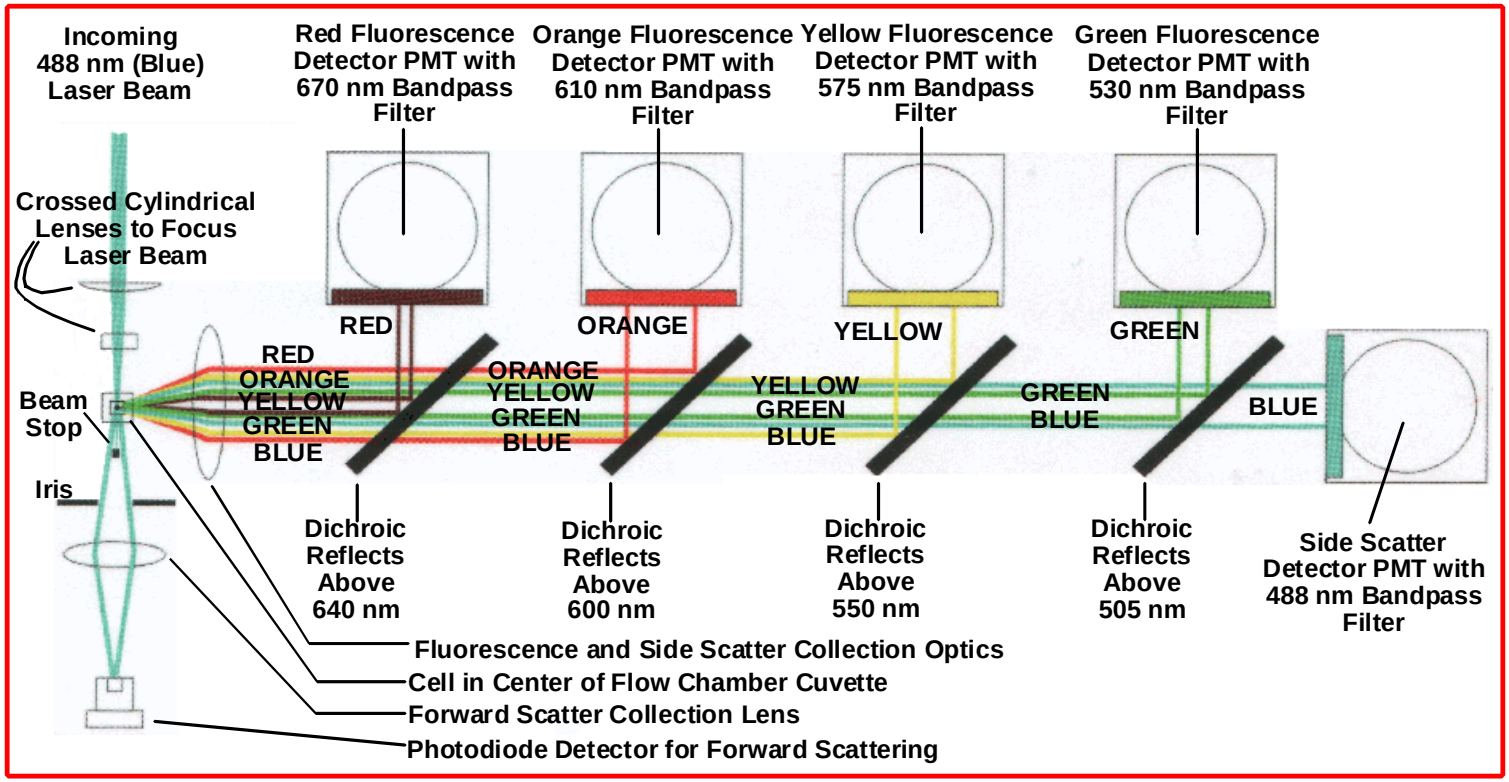

Figure 3. Schematic of the optical sensor of a fluorescence flow cytometer.
Depicted is the optical system of a fluorescence-based flow cytometer, showing a series filters and a photodiode used to collect fluorescence and light scattering properties of each particle. Figure from Shapiro (2003).

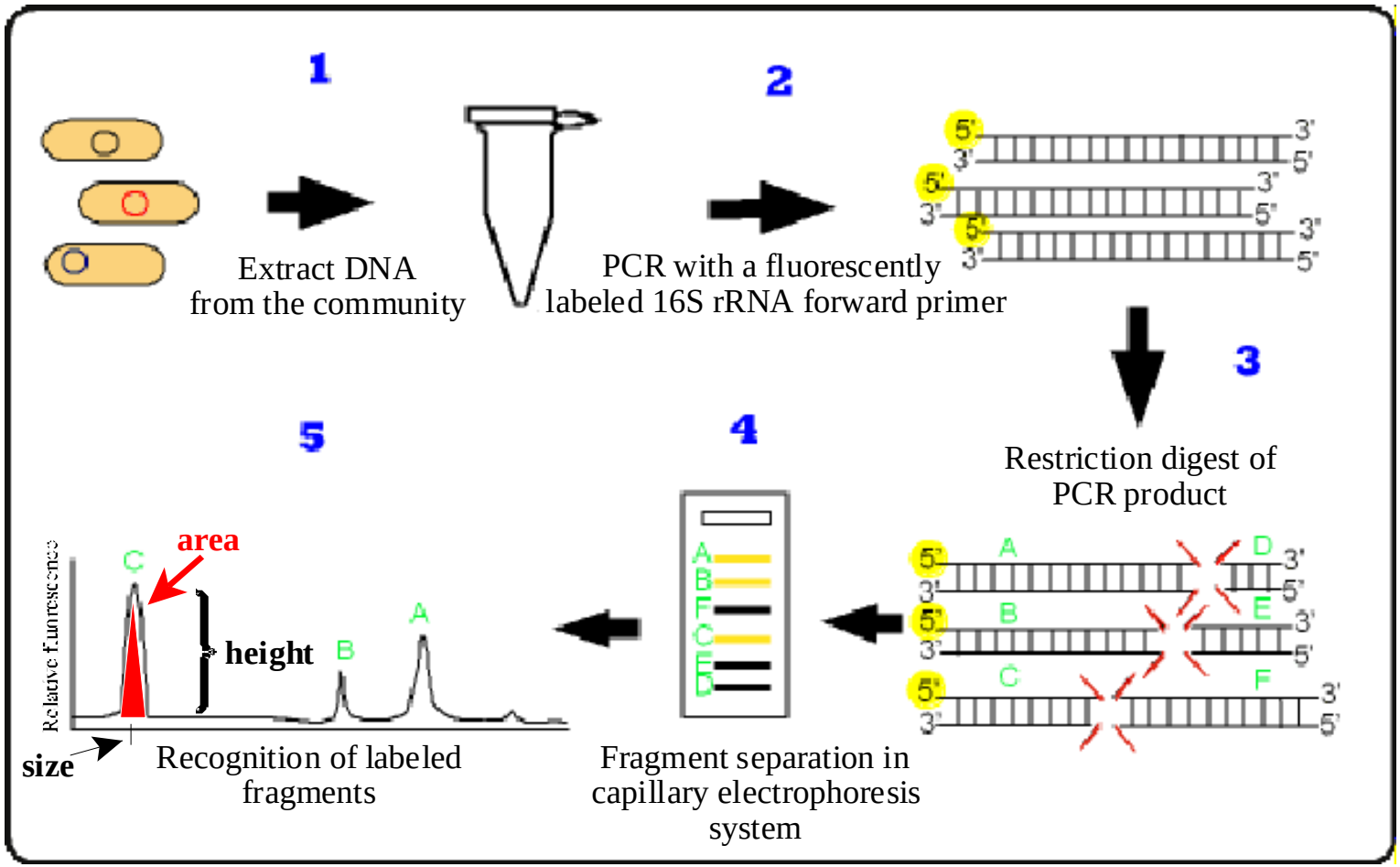

Figure 4. T-RFLP procedures schematic.
Shown are the methods for terminal restriction fragment generation. Indications of peak size, height, and area are also shown. Figure adapted from Cole et al. (2003).

Table 1. A comparison between IronExII and SOIREE mesoscale Fe fertilization experiments. Shown are typical Chl *a* (used to infer phytoplankton biomass production) and F_v F_m^{-1} values (used to infer photosynthetic efficiency) in HNLC waters before and after mesoscale Fe addition. Data adapted from Boyd (2002).

Property	IronExII (Initial)	SOIREE (Initial)	IronExII (bloom)	SOIREE (bloom)
Temperature (oC)	>23	2.0	>23	2.5
Nitrate (μM)	10.0	25.0	5.0	22.0
Silicic acid (μM)	5.0	10.0	<1.0	>6.0
Dissolved Fe (pM)	20	80	800-1000	1000
Chlorophyll (μg L-1)	0.20	0.25	>2.0	>1.5
Algal community structure	Pico-dominated	Pico-dominated	Diatom-dominated	Diatom-dominated
Fv Fm-1	0.24	0.22	0.57	0.65

Part II

Response of the Marine Phytoplankton Community to a Manipulation of Bioavailable Iron in HNLC Waters of the Subtropical Pacific Ocean

This section was published in the journal Aquatic Microbial Ecology in April 2004, under the same name by the authors Melanie L. Eldridge, Charles G. Trick, Mel B. Alm, Giacomo R. DiTullio, Eden L. Rue, Kenneth W. Bruland, David A. Hutchins, and Steven W. Wilhelm:

Eldridge ML, Trick CG, Alm MB, DiTullio GR, Rue EL, Bruland KW, Hutchins DA, Wilhelm SW (2004) Response of the marine phytoplankton community to a manipulation of bioavailable iron in HNLC waters of the subtropical Pacific Ocean. Aquat Microb Ecol 34: 79-91.

My use of “we” in this chapter refers to my co-authors and myself. My primary contributions to this paper were: (1) co-design and implementation of incubation experiments, (2) extracted Chlorophyll sampling and measurements, (3) extensive editing of the manuscript, and (4) preparation of responses to reviewers.

INTRODUCTION

Ecosystem scale Fe fertilization experiments have provided conclusive evidence that the availability of Fe to phytoplankton in open-ocean high nutrient low chlorophyll (HNLC) environments can limit primary production (Martin et al. 1994, Coale et al. 1996, Boyd et al. 2000). Moreover, on-deck bottle incubation

experiments have demonstrated that Fe-limited HNLC conditions can also occur in coastal environments, when low riverine and aeolian Fe inputs are combined with high levels of upwelled nutrients (Hutchins & Bruland 1998, Hutchins et al. 1998, Bruland et al. 2001, Firme et al. 2003). Recently, Hutchins et al. (2002) have demonstrated similar Fe-limiting conditions at two stations in the subtropical equatorial eastern Pacific Ocean off the coast of Peru. Although the upwelling regions along the western coasts of the temperate continents cover only a small percentage of the ocean's surface, they make a significant contribution to global biogeochemical cycles (Chavez 1995, Lluch-Cota 2000). It has been estimated that coastal upwelling areas support about 10% (~ 0.8 Pg C yr^{-1}) of the total global new production (Chavez & Toggweiler 1995). The Humboldt Current (which runs northward along the western coast of South America) is extremely productive; 19.9 g C m^{-2} d^{-1} are fixed by photosynthesis in the south and central fishing areas, and 9.3 g C m^{-2} d^{-1} in the Antofagasta upwelling to the south (Daneri et al. 2000). Recent estimates suggest that the Humboldt Current produces 7 million tonnes in annual fish catch (Daneri et al. 2000).

In pelagic HNLC regions of the equatorial Pacific, picoplanktonic cyanobacteria such as *Synechococcus* and *Prochlorococcus* are the most abundant photosynthetic organisms in the water column (Chavez et al. 1991, Fogg 1995). Although no direct evidence exists to account for their success, their ability to alter cellular Fe quotas, their small size, and their ability to use high-affinity Fe acquisition mechanisms may allow for their persistence in Fe-

limited HNLC environments (Brand 1991, Wilhelm & Trick 1994, 1995, Hutchins et al. 1999b). These studies, mostly in lab settings, suggest that these cyanobacteria assume an alternate physiology that allows them to persist when Fe availability may be growth limiting. In field studies where HNLC environments have been fertilized with Fe a shift in species composition is usually observed, with large diatoms replacing small prokaryotic phototrophs such as *Synechococcus* (Coale et al. 1996, de Baar & Boyd 2000).

In parallel to Fe fertilization studies, Fe "removal" studies have evolved where the concentration of bioavailable Fe is reduced by the addition of a xenobiotic chelating agent. Desferrioxamine B (DFB, sold commercially as Desferal®) is a commercially available aposiderophore produced by the terrestrial actinomycete *Streptomyces pilosus* (Winkelmann 1991). DFB has been demonstrated to effectively reduce the available Fe to most members of the phytoplankton community (Wells et al. 1994, Wells 1999, Hutchins et al. 1999a, Timmermans et al. 2001). However, some evidence suggests that a diatom isolate (Soria-Dengg & Horstmann 1995, Hutchins et al. 1999a) and phytoplankton communities in the Subarctic Pacific (Maldonado and Price 1999) can access at least small amounts of DFB-bound Fe, (although probably not enough Fe to satisfy cellular requirements, Hutchins et al. 1999a). As well, Martinez et al. (2001) have recently demonstrated the production of desferrioxamine G (a close structural relative to DFB) by a marine *Vibrio* sp.

isolated from an invertebrate larva, suggesting that there may also be natural sources of related compounds in marine systems.

We present a series of experiments designed to elucidate the *in situ* Fe status of the major groups of the natural phytoplankton community in a subtropical Pacific HNLC upwelling region. In an attempt to infer the Fe-status of the population without perturbing it, we have combined the Fe addition and "removal" experiments described above to manipulate the concentration of bioavailable Fe both above and below the ambient concentration. Moreover, we have done this in the presence of the naturally occurring Fe-binding ligands using native phytoplankton populations. As such, we can observe the results of the control population in reference to both increases and decreases in Fe availability. Our overall goal was to distinguish between Fe-replete (where growth is limited by something other than Fe), Fe-stressed (where growth is restricted by Fe availability but can be further constrained by lowering available Fe) and Fe-starved (where growth cannot be further restricted by more severe Fe-limitation) populations within these natural communities. The results confirm that phytoplankton in the Humboldt Current and Peruvian upwelling can experience Fe limitation, and demonstrate that the abundance and cellular physiology of different populations within the same phytoplankton community respond in distinct ways to alterations of Fe availability.

MATERIALS AND METHODS

i.) Sample collection

Water samples for incubation experiments were collected off the coast of Peru in September 2000 (Figure 1, for station characteristics see Table 1, Figures and Tables are located in the appendix for Part II). Stations were chosen based on the concentration of dissolved Fe in surface waters. All stations had ambient concentrations of < 100 pM, which we considered to represent Fe-limiting or near-limiting conditions. Surface seawater (~ 7 m depth) was collected with a clean surface pump system (Bruland et al. 2001) using an all PTFE Teflon™ diaphragm pump (Osmonics Bruiser™) and PFA Teflon™ tubing with a PVC "fish" deployed off the side of the ship outside of the wake. Water was collected directly into a dedicated 50 - L acid-cleaned polyethylene carboy for homogenization. Once mixed, seawater was dispensed into 1.1 - L acid-cleaned polycarbonate bottles. All water sampling was carried out under class-100 clean conditions.

ii.) Manipulations of available Fe

Fe availability to phytoplankton in this study was decreased by the addition of DFB. Rue and Bruland (1995) have estimated the conditional stability constant of Fe(III) with Desferal to be $10^{16.5}$ M^{-1} (with respect to Fe(III)'). A 10 nM addition of Desferal, if in equilibrium with Fe(III), would result in a ratio of [Fe(III)-

Desferal] to [Fe(III)'] of approximately $10^{8.5}$ and result in extremely low equilibrium concentrations of Fe(III)'. Moreover, as discussed in previous work (Rue & Bruland 1997), it is likely that photochemical activity will increase the dissolution rates of naturally occurring Fe – ligand complexes, leading to an increased formation rate of photostable Fe – Desferal complexes (Barbeau et al. 2003).

DFB was purchased from the Sigma Chemical Company and dissolved into sterile, Chelex-100 treated water (Price et al. 1989) from a Millipore BioCell water purification system. Aliquots of the DFB stock were added to sample bottles to generate a replicate series of DFB concentrations of 1 to 10 nM DFB. In parallel, +Fe bottles were amended with $FeCl_3$ (dissolved in 0.01 M Ultrex HCl) to final concentrations ranging from 0.5 to 5.0 nM added Fe. In combination with control bottles (bottles with no DFB or Fe added) this resulted in concentrations of Fe ranging from 10 nM < ambient (+10 nM DFB) to 5.0 nM > ambient (+5.0 nM Fe). Sealed bottles were placed in on-deck, flowing seawater incubators (temperature ranged from 16.3 – 20.3°C across stations) lined with a 3.2 mm-thick sheet of spectrum-correcting blue Plexiglas® to simulate the light intensity and spectral quality of the 50% incident depth in the water column (Laws et al. 1990, Wells 1999). All subsampling occurred after sunset to reduce light exposure effects, and to guarantee a full solar day exposure per 24 h period.

For the first station subsamples were collected daily (up to 96 h) from the bottles under class–100 conditions. For subsequent stations samples were collected only at the 72 h time point in order to reduce the potential for

contamination during subsampling and the influence of bottle effects that were observed after this time point. Total chlorophyll was determined from 50 mL samples collected on 0.2 µm pore-size polycarbonate filters (Osmonics) after extraction (~ 24 h) in 90% acetone. Chlorophyll (> 0.2 µm (total) and > 5.0 µm) was quantified with a Turner designs 10-AU fluorometer using the non-acidification protocol of Welschmeyer (1994).

iii.) Flow cytometry

Phytoplankton communities were analyzed immediately upon sampling (without initial preservation) using a Becton Dickinson FACSCalibur flow cytometer (which handles particles up to 180 µm) equipped with a 15 mW argon laser (488 nm excitation) and CellQuest analysis software. To normalize the spectrum of cell responses, the software was calibrated using 1, 2, 4, 10, 16-µm non-fluorescent beads for cell-size based calibration, 10-µm fluorescent beads and lab phytoplankton cultures to standardized size and fluorescence corresponding to chlorophyll (660 – 700 nm) and phycoerythrin (530 – 630 nm). Fluorescence and cell light-scattering properties were used in a two-dimensional analysis to distinguish phytoplankton groups (Frankel et al. 1990).

iv.) Fast repetition rate fluorometry

Photosynthetic efficiency (the ratio of variable fluorescence to maximal fluorescence, F_v/F_m) was measured by analyzing 50 mL aliquots of samples in

the dark chamber of the FAST^{tracka} fast repetition rate fluorometer (FRRF; Chelsea Instruments Ltd.). All samples were analyzed ~ 2 hrs after local sunset to avoid diel periodicity effects, and were dark-adapted at room temperature for at least 15 min prior to analysis. Data was analyzed using the FAST^{tracka} FRRF post-processing program version 1.4 (Chelsea Instruments, Ltd.).

v.) Nutrient and Fe measurements

Combined nitrogen (NO_3^- + NO_2^-), phosphate and silicic acid concentrations were measured on a Lachat Quick Chem 8000™ Flow Injection Analysis system using standard methods (Parsons et al. 1984). Total dissolved Fe was measured electrochemically on board the ship by competitive ligand equilibration (CLE) - followed by adsorptive cathodic stripping voltammetry (ACSV) with salicylaldoxime (SA) as the competing ligand (Rue & Bruland 1995). Dissolved (< 0.2 µm) Fe was measured by acidifying the sample (pH 1.7), followed by briefly microwaving and, once cooled to room temperature, applying the ACSV technique (Bruland et al. 2001).

vi.) Fe assimilation measurements

To demonstrate that DFB inhibited Fe uptake in the natural microbial community, we carried out an Fe assimilation experiment at Bio-7. Assimilation rates were monitored through the addition of ^{55}Fe (as $^{55}FeCl_3$ in 0.5 M HCl, *ca* 42 mCi mg^{-1}, from New England Nuclear, Boston MA) to water samples. Cleanly

collected water was dispensed into acid-clean 1.2 - L polycarbonate bottles and amended with ^{55}Fe to a final concentration of 2 nM. Samples were allowed to incubate for 48 h in the on-deck incubators, and were terminated by the collection of cells onto 0.2, 1.0 or 8.0 μm filters and the removal of extracellular ^{55}Fe with the titanium – citrate – EDTA wash (3 minutes total exposure) of Hudson and Morel (1989). Incorporated Fe was subsequently determined by scintillation counting and normalized to the volume filtered for each size class.

RESULTS

i.) Station description

The four stations occupied during these experiments all displayed classic HNLC characteristics (Table 1). High concentrations of NO_3^- + NO_2^- (11 – 24 μM), phosphate (1.2 – 1.5 μM) and silicate (6 – 15 μM) were accompanied by low concentrations of dissolved Fe (60 – 100 pM) and chlorophyll (0.7 – 1.5 μg L^{-1}).

ii.) Total community response to Fe additions and removal

Increases in total Fe concentrations lead to dramatic increases in phytoplankton biomass, as estimated by total chlorophyll (Figure 2). Averaged across all stations, the total chlorophyll in the +Fe bottles was 218% (± 62%) of the concentrations reached in the control bottles. Similarly, the +DFB bottles showed depressions in total chlorophyll (47 ± 15%). These results imply that both

the addition and removal of Fe led to changes in the physiology or abundance of the cells, in the overall phytoplankton community structure, or in all of these parameters. Changes in the physiology of the phytoplankton are seen in the F_v/F_m data from Figure 3. Photosynthetic efficiency was increased in all samples (113 – 210% of control values) where Fe was added, and remained low or decreased slightly (83 – 103% of control values) when DFB was added. These results suggest that the entire phytoplankton community at each station existed in a state of Fe-stress and that while Fe addition alleviated constraints on growth, the community could be pushed into further physiological stress by the addition of DFB. Interestingly, it does not appear that the Fe requirements of the phytoplankton communities were saturated by our additions, as neither the F_v/F_m nor the chlorophyll values plateau in our +Fe treatments.

Nutrient assimilation data from three of the stations (measurements were not made at Bio-4) also suggest that availability of Fe constrained the assimilation of macronutrients by plankton populations at Stations Bio-5 and Bio-6 (Figure 4). The residual combined nitrogen (NO_3^- + NO_2^-) concentration in +Fe bottles at Station Bio-5 was significantly less (paired T-test, $p < 0.05$) than the unamended control for both the 1.5 nM and 5 nM treatments. More striking were the results of the DFB additions at stations Bio-5 and Bio-6 that resulted in a repression of drawdown of these nutrients. Considering the Fe and DFB addition data together, the overall trend supports the role of Fe as a limiting agent at these two stations.

iii.) Response of specific groups to alterations in Fe availability

To examine how alterations in Fe availability may affect population diversity, we examined the community composition by flow cytometry (Figure 5). Figure 5a demonstrates the predicted response of different phytoplankton populations that are Fe-replete (a), Fe-stressed (b) or Fe-starved (c) (discussed below). Of the eight separate populations that could be identified with the flow cytometer (based on differences in cell size, density and pigment composition), three populations of larger cells were not considered for analysis due to low (< 200 mL^{-1}) cell densities which would cause statistical uncertainty. The five remaining populations represented cyanobacteria, picoeukaryotes (2-5 μm), small eukaryotes (5-10 μm), eukaryotes (10-20 μm) and large eukaryotes (> 20 μm). Large particles that demonstrated no autofluorescence were also not considered. While there are inherit limitations to these groupings (see Discussion) they do provide an operational approach to rapidly examining community structure.

Results from station Bio-4 provided clear evidence that different populations were responding differently to alterations in Fe-availability (Figure 5). The three largest eukaryotic groups all demonstrated increases in cell density with the addition of Fe, but no significant alterations in cell abundance or relative chlorophyll $cell^{-1}$ with the addition of DFB. While the cyanobacteria also demonstrated an increase in cell density in the +Fe treatments, additions of DFB

led to suppression in relative cell density. Perhaps the most striking response was that of the picoeukaryotic phytoplankton, which showed no change in the Fe addition experiments but increased markedly in the plus DFB treatments. DFB addition did lead to decreases in the estimates of the relative chlorophyll $cell^{-1}$ in all these populations in the Bio-4 incubations, including the picoeukaryotes (Figure 6). This change in the picoeukaryotes was also accompanied by a change in the mean cell-size of this population, which decreased over a 4-fold range from the +Fe to the +DFB bottles (Figure 7). Other phytoplankton groups showed no dramatic changes in cell size among treatments.

iv.) Influence of DFB on ^{55}Fe uptake

Since some data suggest that DFB bound Fe is available to phytoplankton (e.g., Maldonado & Price 1999), we felt it prudent to demonstrate that DFB inhibited Fe uptake in this region. Increasing concentrations of DFB added to bottles effectively limited the Fe uptake ability of phytoplankton in each of three different size classes (> 0.2 μm, > 1.0 μm, > 8.0 μm) in our uptake experiments (Figure 8). In all size classes, rates of assimilation decreased ~ 3-fold at concentrations higher than 5 nM. In previous studies the availability of ^{55}Fe-DFB to a marine plankton was demonstrated after only 4 h (Maldonado & Price 1999, Maldonado & Price 2001) – as such our 48 h incubation period confirms that the planktonic community had a reduced ability to assimilate ^{55}Fe-DFB, and was not able to activate any potential high-affinity transport systems (*e.g.*, siderophore

mediated pathways) or enzymes (*e.g.,* surface reductases) that would allow the cells to assimilate the Fe from the DFB. That said, it should also be noted that longer incubation periods (required for time consistency with the grow outs) may lead to underestimates of Fe assimilation (*e.g.,* due to isotopic dilution, biphasic assimilation, etc.). We have attempted to decrease the impact of this on the data by expressing the results as total ^{55}Fe-assimilated over the incubation period.

DISCUSSION

Fe addition experiments have demonstrated that production by phytoplankton along the western coast of Peru can be limited by the availability of Fe (Hutchins et al. 2002), but these experiments did not elucidate the degree to which these populations may be limited. The basic approach of our study was to infer the *in situ* status of the phytoplankton community from the impacts of both increases and decreases in Fe availability. This process allows for a determination of the status of the *in situ* community as an interpolation of the data.

There are several important conclusions that can be drawn from this work. Firstly, this study confirms the findings of Hutchins et al. (2002) that the phytoplankton community in the Peruvian upwelling can be Fe-limited in a manner analogous to previously studied communities in the coastal upwelling systems of California (Hutchins & Bruland 1998; Bruland et al. 2001). Secondly,

this work confirms that DFB can be used to reduce the biological availability of Fe to the total community of phytoplankton in bulk analyses. Finally, it demonstrates how a xenosiderophore can be employed in conjunction with Fe addition experiments to distinguish between populations of cells that are Fe-starved, Fe-stressed and Fe-replete. The use of shipboard flow cytometry in conjunction with *in situ* dissolved Fe measurements also allows us to draw two important conclusions regarding phytoplankton physiology. Most phytoplankton demonstrated dramatic changes in chlorophyll cell^{-1} with changes in bioavailable Fe, and picoeukaryotes became more successful when Fe levels were decreased with DFB, most likely via miniaturization. These conclusions are discussed in more detail below.

The most common analysis of phytoplankton response to fertilization in both bottle amendment (Martin & Gordon 1988, Hutchins & Bruland 1998) and mesoscale fertilization (Coale et al. 1996, Boyd et al. 2000) experiments has been a comparison of the total chlorophyll in the control vs. treatment populations. Although growth rate is strictly defined as the changes in cell number over time (Madigan et al. 2000), water column or sample chlorophyll is often used as a proxy for autotrophic community biomass. In this study we demonstrate that chlorophyll concentrations increase in all the added Fe amendments and decrease in all the added DFB amendments relative to our unamended control bottles. One parameter that is often overlooked is the changes that can occur in individual cells within the community that may skew

the results of “bulk” measurements. It has been shown in several lab studies that populations of both cyanobacteria (Wilhelm & Trick 1995, Wilhelm et al. 1996) and eukaryotic phytoplankton (Muggli et al. 1996) decrease their relative chlorophyll cell^{-1} when they are moved from Fe-replete to Fe-deficient culture conditions. In cyanobacteria, this change in chlorophyll cell^{-1} can be 2-fold or greater (Wilhelm et al. 1996). For eukaryotes, the diatom *Actinocyclus* sp. changes chlorophyll cell^{-1} ~ 1.9 – 3.2 fold (Muggli et al. 1996), while the diatom *Phaeodactylum tricornutum* decreases chlorophyll cell·volume^{-1} by 50% under Fe-deficient conditions (Kudo et al. 2000). In the current study, results from the flow cytometric analyses clearly demonstrate that the chlorophyll cell^{-1} in the individual groups of phytoplankton were altered by changes in Fe availability, with cells in +Fe conditions containing 25 – 200% more chlorophyll than cells in the +DFB bottles. However, the flow cytometry data confirm that alterations in cell abundance for each of these populations also occurred (although in the case of picoeukaryotes, not as would have been predicted). Taken together, the results suggest that while bulk chlorophyll measurements remain a valid method for the determination of bulk effects on phytoplankton populations, alterations at the cell level may exaggerate these results and other factors such as light and N-availability may further exacerbate the differences.

One of the best indicators of the status of an entire phytoplankton community may be the photosynthetic efficiency (Behrenfeld et al. 1996). As with the total chlorophyll, the F_v/F_m increased (to as much as 200% of the control) with

increasing Fe availability. In incubations carried out at stations Bio-5, Bio-6 and Bio-7 these increases were predictably hyperbolic with changes in Fe concentration. Increases in F_v/F_m at Station Bio-4 also occurred, but due to the limited number of treatments the resolution of the relationship of the change to Fe availability was markedly reduced. In all though, these results suggest that cellular physiology was limited at the photosynthetic level (at least in part) due to the availability of Fe.

The most significant result from this study is that it provides us with a novel approach to gauge the *in situ* status of the natural phytoplankton population in terms of Fe availability. In their study of the California HNLC coastal upwelling, Hutchins et al. (1998) described four stages of Fe-limitation for an entire community. This classification was based primarily on H_2SiO_3:NO_3 drawdown ratios and permitted the identification of four distinct classifications for surface waters in the region, ranging from Fe-replete to severely Fe-limited. In our approach, we are not yet able to quantitatively characterize the degree of Fe-limitation of the entire population. However, while there are obvious concerns with bottle effects in on-deck amendment experiments and effects of changes of cellular physiology, the results achieved in this study clearly show that there is at least an overall effect of Fe availability on the populations, and that the phytoplankton in these regions are in a state of Fe-stress.

Diatoms have most consistently been the organisms that proliferate in both on-deck (Martin & Gordon 1988, Hutchins et al. 1998) and mesoscale Fe

addition (Coale et al. 1996, Boyd et al. 2000) experiments. In the Bio-4 incubations, eukaryotic cells > 5 μm increased ~ 1.5 to 2.5 fold in abundance in the +Fe treatments. While the abundance of cells in each of these populations changed significantly, the contribution to total chlorophyll remained relatively static for each group but the 5-10 μm cells. Cyanobacteria also increased in both abundance (~ 2.5 fold) and contribution to total chlorophyll (from a low of 0.5% to a high of > 4.0%) in the +Fe bottles. However, unlike the larger eukaryotes, these cells demonstrated decreases in both abundance and contribution to total chlorophyll with the addition of DFB. Cyanobacteria have been demonstrated to produce siderophores during periods of Fe stress (Wilhelm & Trick 1994, Wilhelm 1995) and to use these compounds to increase their Fe-assimilation during these periods (Trick & Wilhelm 1995, Wilhelm et al. 1998). It was therefore hypothesized that this population should more effectively assimilate Fe from the DFB treatments due to a ligand-ligand exchange of Fe (between the DFB and the siderophores). However, as with the previous work of Wells et al. (1994) the current study suggests that Fe complexed to this xenosiderophore (DFB) is not available to *Synechococcus* spp. from this region. The results here also suggest that the cyanobacterial populations in this region are Fe-stressed, but not Fe-starved, as the addition of DFB could force them into a state of greater limitation. This is in contrast to the three eukaryotic populations, which demonstrated an Fe-starved response to our treatments.

The picoeukaryotic organisms present the most striking results in this study. In HNLC regimes of the Southern Ocean, Boyd et al. (2000) found that picoeukaryotes were a dominant component of the system, and initially began to proliferate upon addition of Fe. However, after several days these cells decreased in abundance in the fertilized patch and were replaced by larger eukaryotic phytoplankton. Given the temporal resolution of our study, we were unable to detect any initial proliferation by the picoeukaryotes in the +Fe treatments. Surprisingly though, the picoeukaryotes abundance in the +DFB bottles suggests the cells were able to proliferate under these conditions. However, if one examines the chlorophyll cell^{-1} and mean cell size data for this population across the range of treatments, it demonstrates that there was a marked decrease in both parameters. While there is a possibility that changes in Fe availability alter the competitive interactions between picoeukaryotes and the larger eukaryotes for macronutrients (*e.g.,* NO_3^-), distinct physiological changes in the picoeukaryotes suggest that, in the case of DFB additions, the increase in cell abundance is a direct consequence of decreased Fe-availability. Although the picoeukaryotic phytoplankton appear to at best assimilate the DFB – Fe at a reduced rate (Figure 8), these cells can alter their physiology (chlorophyll quota and apparently cellular Fe quota) to maintain their growth rate.

Responses to nutrient limitation by aquatic organisms are numerous. In the case of Fe, research has commonly focused on changes in cell quota (Sunda et al. 1991, Wilhelm 1995) or the activation of high-affinity transporters (Wilhelm

& Trick 1994) as the mechanism by which cells compensate for nutrient limitation. One classic response to nutrient stress that is often overlooked in the case of Fe is changes to cell size. Microbial miniaturization has been shown to occur during periods of stress to increase the surface to volume ratio of cells (Morita 1975). This phenomenon has been observed in diatom communities where significant reductions in volumes of *Nitzschia* occurred in the California HNLC system (Hutchins et al 1998). In theory, this increases the amount of surface receptor a cell has (a function of the total surface area) in respect to the requirement of the cell for the nutrient element (an approximate function of the cell volume). As such, individual cells can increase their ability to scavenge limiting nutrients from the environment without expending energy on transport systems (Chisholm 1992).

In laboratory studies, Fe-stressed *Anacystis nidulans* R2 *(a Synechococcus* sp.) decreases its cell size to between 1/2 and 1/3 of the length of Fe-replete cells (Sherman & Sherman 1983). The filamentous cyanobacterium *Anabaena flos-aquae* carries out similar size changes as well as pronounced changes in filament coiling when maintained in Fe-deficient medium (Gorham et al. 1964). In this particular study, the picoeukaryotic community decreased ~ 4-fold in size. This would result in a proportionate change in the surface area to volume ratio (given that the surface area to volume ratio is altered as 6/d, where d is the diameter of the cell). It is important to point out though that there is an inherent limitation to the miniaturization process- cells can only get so small and

still be a complete functioning entity with all metabolic systems intact (Raven 1987). The fact that minimum picoeukaryote cell size was reached at the 1 nM DFB addition, and that they didn't get any smaller at the 4 nM DFB addition (Fig 7), suggests they may have reached their minimum size rather quickly. While we cannot rule out that a separate species of smaller picoeukaryotes bloomed in our +DFB treatments, the absence of a detectable seed population at the beginning of the experiment for this small size group (data not shown) strongly supports miniaturization.

Previous studies have suggested that Fe fertilization resulted in a shift in species from smaller picoplankton to larger eukaryotic phytoplankton. In those studies, a direct correlation between an alleviation of Fe-limitation and production by larger diatoms could be attributed to the relationship between cell size and nutrient acquisition (Hutchins et al. 1998).

In the HNLC waters of this region, Hutchins and colleagues (2002) demonstrated that small diatoms, coccolithophorids and *Phaeocystis* spp. proliferated in the Fe addition regimes. At stations Bio-5 and Bio-6, changes in the Si:N drawdown were small but significant ($P < 0.05$) between 5 nM Fe-enriched (Bio-5 Si:N = 0.248 ± 0.010; Bio-6 Si:N = 0.288 ± 0.019), 5 nM DFB added (Bio-5 Si:N = 0.332 ± 0.035; Bio-6 Si:N = 0.117 ± 0.075) and the controls (Bio-5 = 0.266 ± 0.033; Bio-6 Si:N = 0.238 ± 0.093) resulting in opposing trends: decreasing Si:N with increasing Fe at Bio-5 and increasing Si:N with increasing Fe at Bio-6. Our data from Bio-7 suggest that no differences in silicic acid

utilization occurred between treatments. The Bio-6 results concur with the Si:N drawdown ratios observed for Fe-enriched communities at two other locations in this region (Hutchins et al. 2002); we hypothesize the differences at Bio-5 are most likely due to differences in initial (and thus final) populations. At Bio-7, combined-N drawdown increased ~ 177% with the shift in Fe availabilities, with the results of independent bottles being significantly different from the control at $p < 0.10$ (3 nM DFB, $p = 0.09$; 5 nM DFB, $p = 0.06$; 10 nM DFB, $p = 0.08$). Interestingly enough, no significant differences ($p < 0.10$) were seen between the controls and any treatments with regard to Si concentrations. While part of this result is probably a manifestation of the observed species shift in the system, it may also be a result of alterations in cellular nitrate scavenging abilities within populations as limitation of nitrate reductase activity (due to enzymatic Fe-limitation; Raven 1988, Price et al. 1991, Price et al. 1994) was alleviated. Regardless of the factors causing this shift, the results call into question the universal applicability of Si:N drawdown ratios as indicators of community Fe stress; while there is an effect at each station, the degree of that response appears in this case to be somewhat community dependent.

CONCLUSIONS

Our results here confirm Fe-limitation in upwelling regimes, and demonstrate that the degree of Fe-limitation is not only spatial in nature, but can

be group related within a given phytoplankton community. These results further demonstrate that cellular miniaturization is a response used by members of the phytoplankton community (in this case picoeukaryotic algae) to compensate for growth-limiting levels of Fe. Our experiments also confirm that DFB can be used to effectively limit Fe availability to these marine plankton communities. The application of the titration approaches described in this paper to other HNLC regions should provide insight into the group specific requirements for Fe in other HNLC environments, ultimately permitting for a quantifiable approach to resolving the degree of Fe-limitation of phytoplankton in HNLC marine systems.

Ecosystem scale Fe-fertilization proposals cite the potential for Fe fertilization to enhance the export of carbon from the atmosphere to the deep ocean *via* a combined increase in photosynthesis and enhanced export of biomass (in terms of sinking phytoplankton) from surface waters to the deep ocean. The alterations in the phytoplankton community that would allow for this are critical, as small picoplankton (*e.g.*, *Synechococcus* and *Prochlorococcus* spp.) do not play a significant role in export production in marine systems (Boyd & Newton 1999). This is in part due to their small size and higher buoyancy, but may in part also be due to their enhanced susceptibility to mortality mechanisms (*e.g.*, viral lysis) that can cause increased turnover of carbon as DOC (Suttle & Chan 1994, Wilhelm & Suttle 1999). Estimates of CO_2 sequestration are generally based on a Redfield drawdown of NO_3^- by the plankton community relative to the Fe added during the fertilization process (Martin 1990b). It

appears from our data that models of the effects of Fe fertilization will also need to consider changes in species composition, cell size and N-utilization thus emphasizing the need for realistic, experimentally-derived, taxon-specific assays in future models.

ACKNOWLEDGEMENTS

We thank Jenn Conn, Jennifer Maucher, Johanna Rinta-Kanto, Geoff Smith, Richard Weaver and the captain and crew of the RV *Melville* for assistance in collection of samples and three anonymous reviewers for their comments. This work was supported by grants from the National Science Foundation to SWW (OCE-9977040 & OCE-0002968), DAH (OCE-9811062), GRD (OCE-9907931), KWB (OCE–9811114), a University of Tennessee SARIF award to SWW, and funds from the Natural Sciences and Engineering Research Council of Canada, and The Center for Environmental BioInorganic Chemistry (CEBIC, Princeton) to CGT.

APPENDIX (PART II)

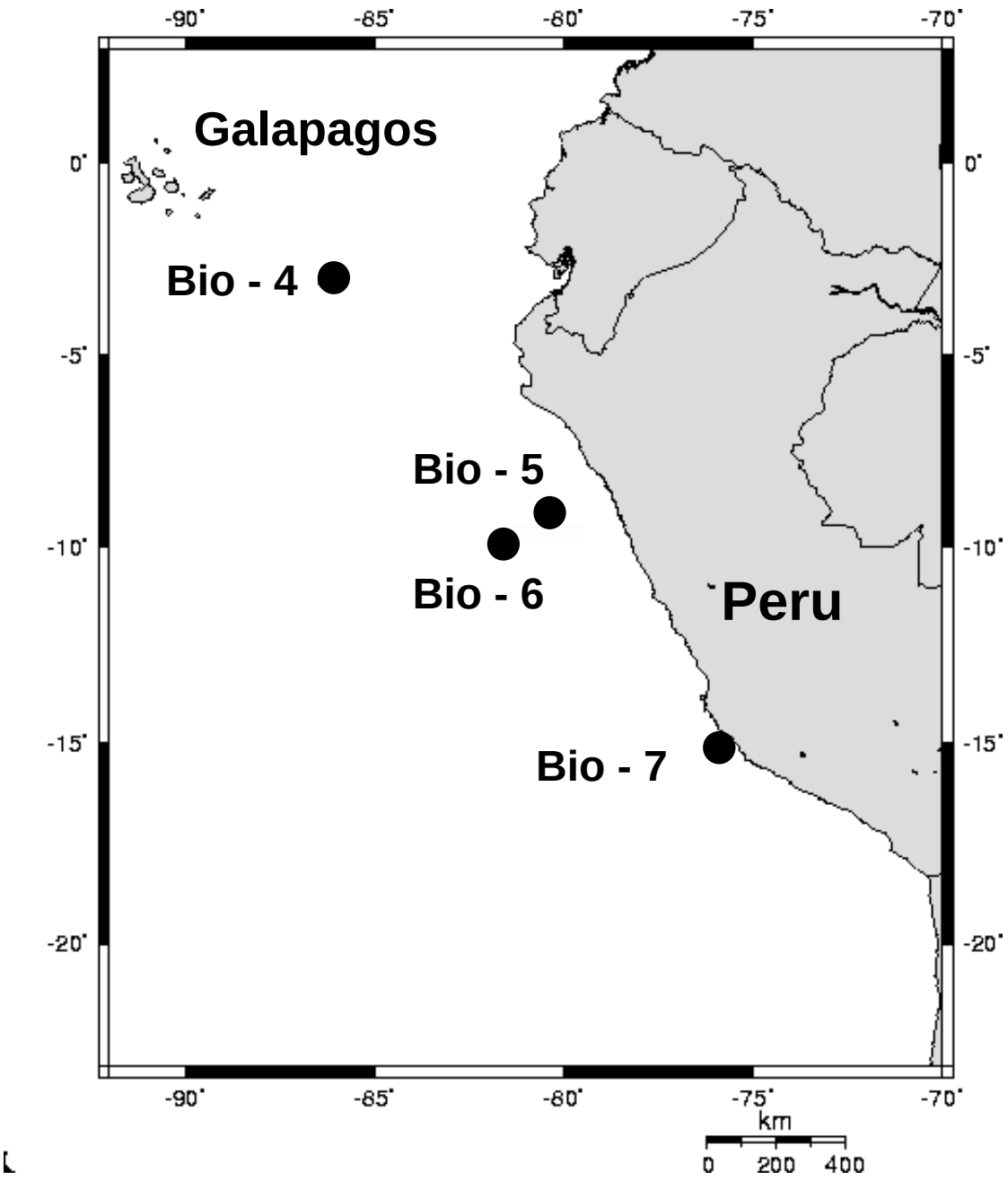

Figure 1. Location of stations in the subtropical Equatorial Pacific Ocean.

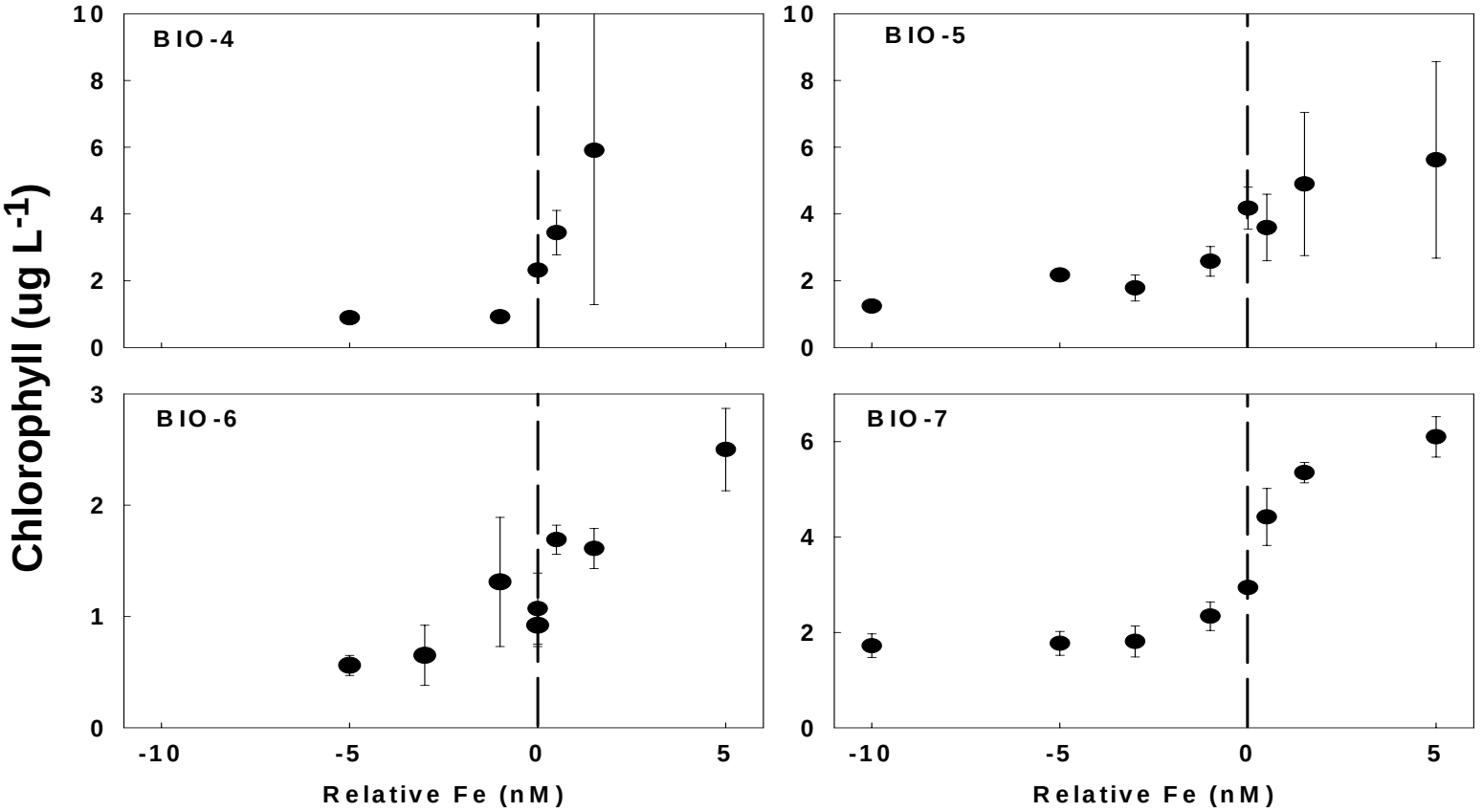

Figure 2. Phytoplankton biomass.

Phytoplankton biomass in on-deck incubations as estimated by total chlorophyll *a* (> 0.2 µm) after a 72 h incubation. Results are the means and error of either duplicate (Bio-4, ± range) or triplicate (Bio-5, Bio-6, Bio-7, ± SD) incubations and are presented in respect to the ambient concentration (dashed line, where relative Fe = 0).

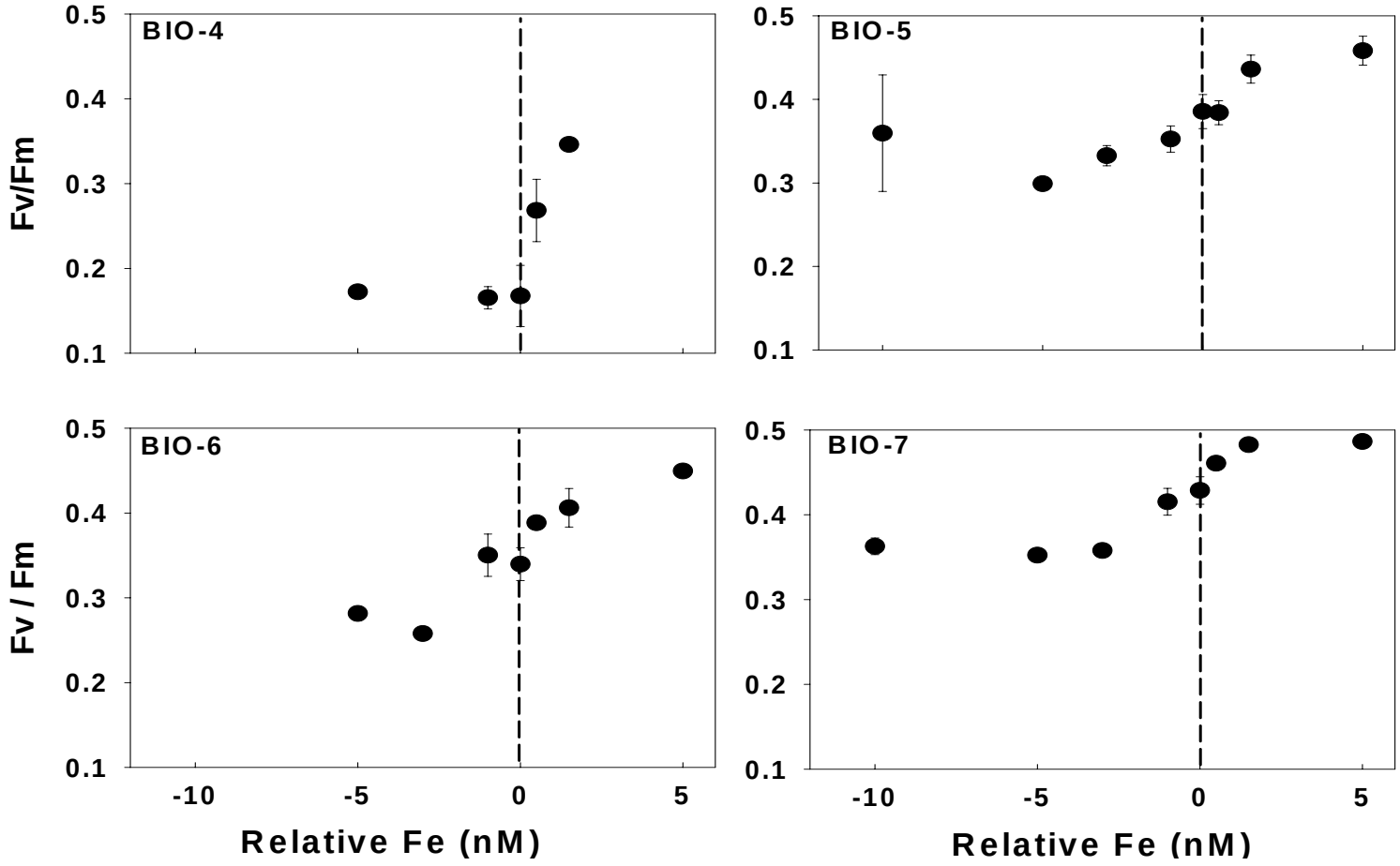

Figure 3. Photosynthetic efficiency.
Photosynthetic efficiency (F_v/F_m) of populations from on-deck incubations (as per Figure 2) as estimated by Fast repetition rate fluorometry after 72 h incubation.

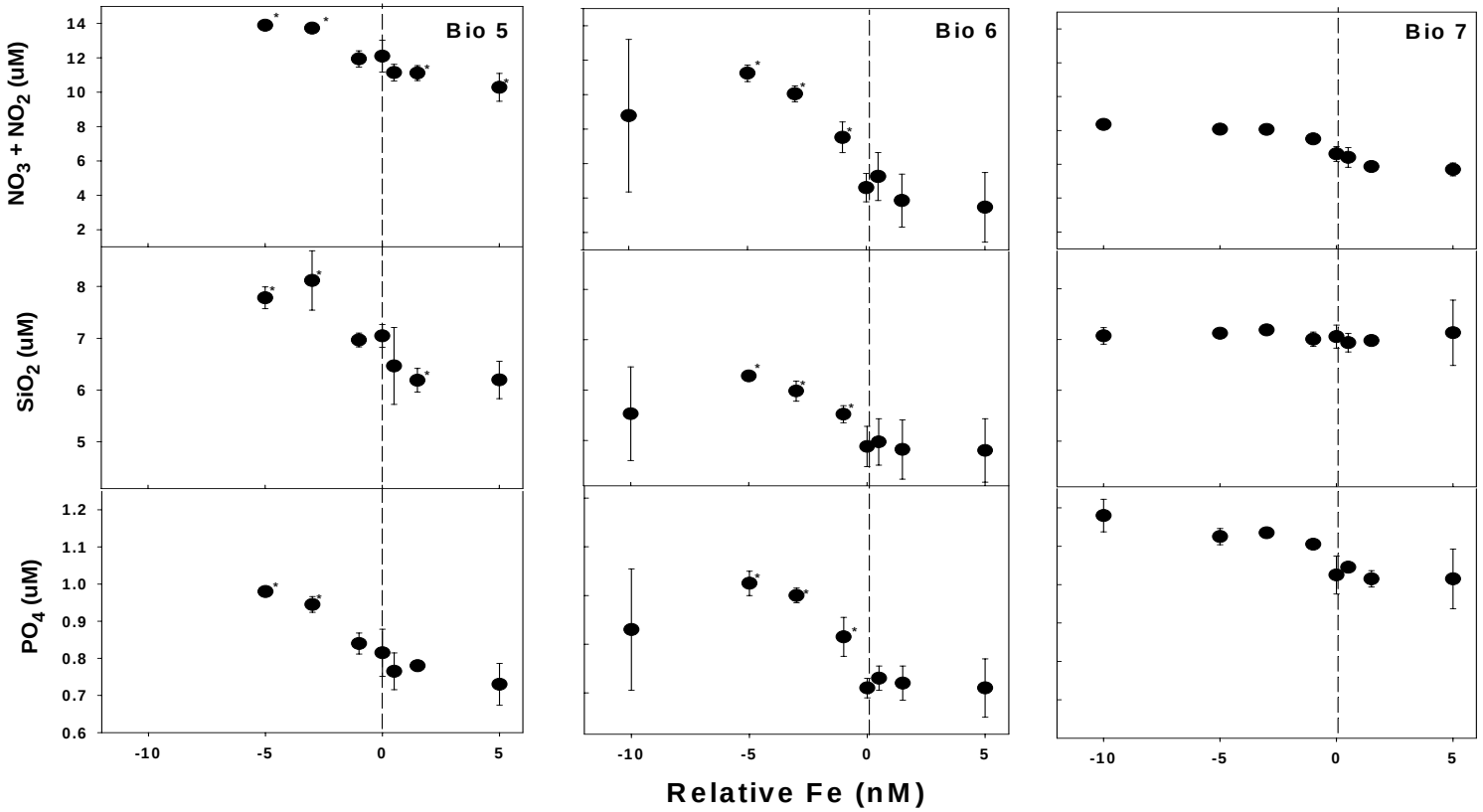

Figure 4. Nutrient concentrations at 72 h.

Nutrient concentrations in samples at the termination of the incubations after 72 h incubation. Results are the means of triplicate incubations and presented in respect to the relative ambient concentration (where relative Fe = 0). Nutrient samples for Bio-4 were contaminated and thus are not presented. Samples statistically different from the control ($P < 0.05$) are denoted with *. The significance of some treatments at greater p-values is discussed in the text.

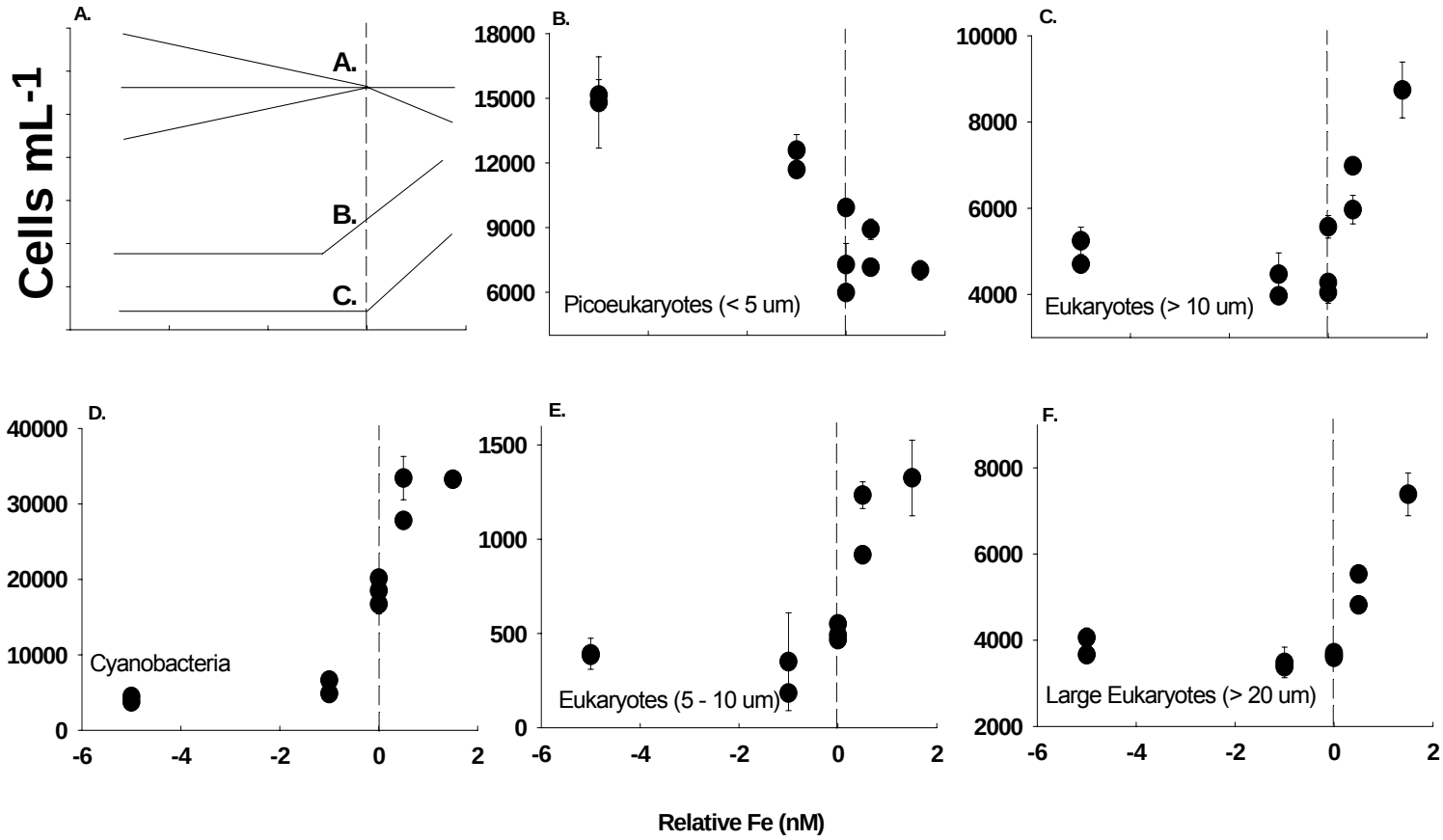

Figure 5. Flow cytometry of Bio-4.

Cell density of different groups in on-deck incubations from Bio-4 as estimated by flow cytometry after 72 h (including a third control bottle). A. Predicted response of populations in conditions of a) Fe-sufficient, b) Fe-stressed, and c) Fe starvation. These hypothetical responses were predicted *a priori* to account for all potential responses. B-F. The five major groups of plankton are presented in respect to the relative Fe concentration.

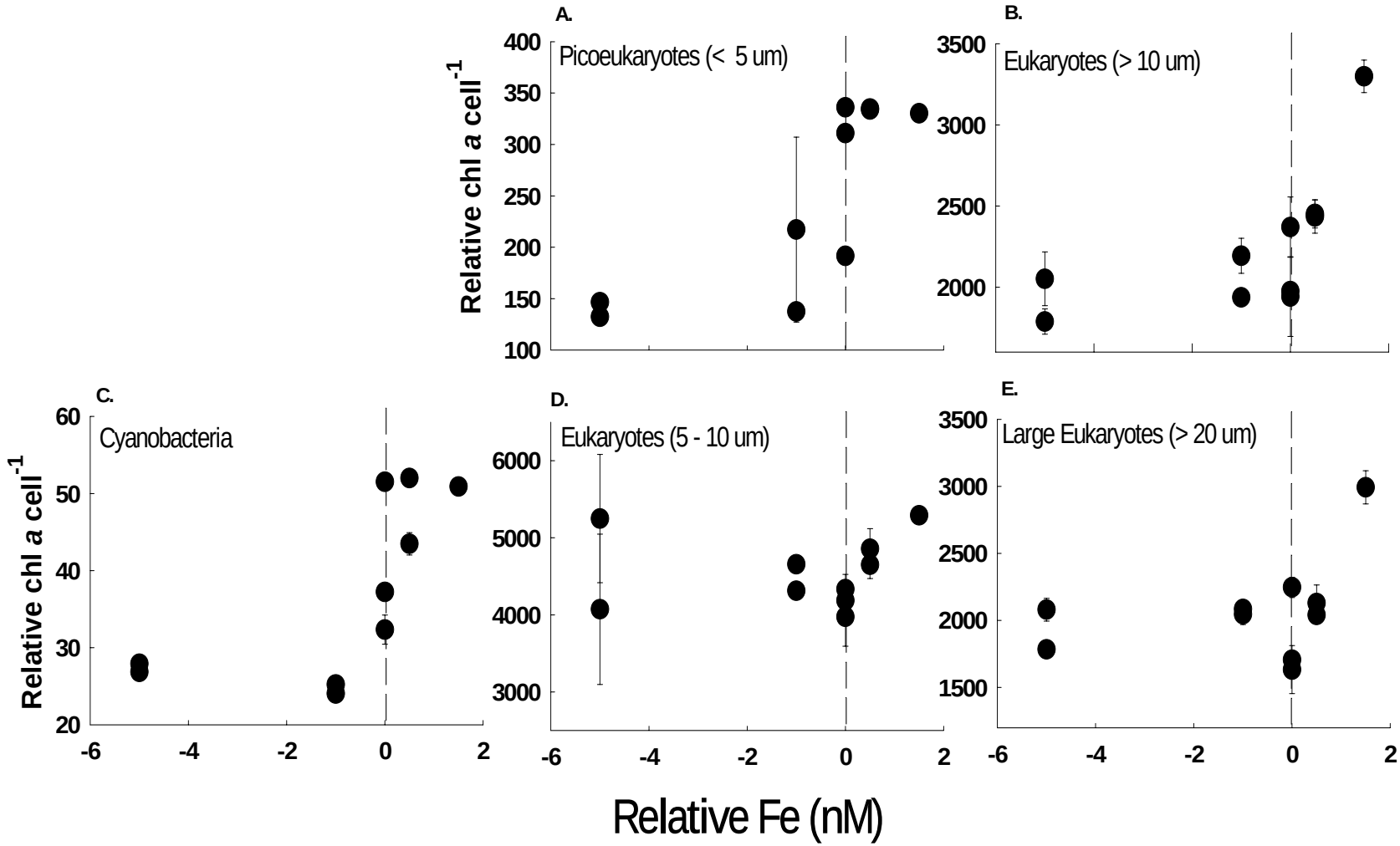

Figure 6. Relative chlorophyll *a* per cell.

Relative chlorophyll *a* cell^{-1} in on-deck incubations from Bio-4 as estimated by flow cytometry after 72 h (including a third control bottle). The five major groups of plankton are presented in respect to the relative Fe concentration.

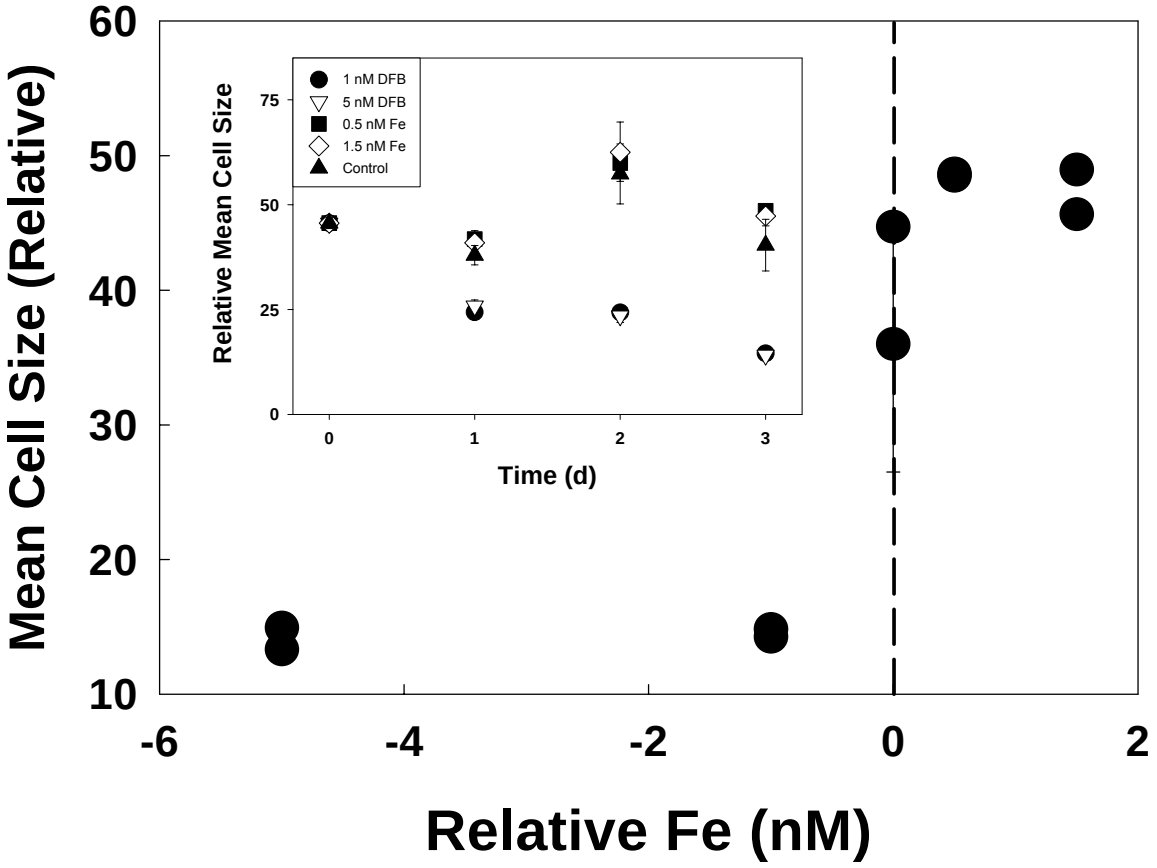

Figure 7. Mean cell size of picoeukaryotes across Fe treatments.
Mean cell size of picoeukaryotes in Bio-4 samples as estimated by flow cytometry after 72 h. Mean size of the population changed four-fold over the range of experimental Fe availabilities. Inset to the figure are daily estimates of picoeukaryotic size collected every 24 h over the 72 h period of the Bio-4 experiment.

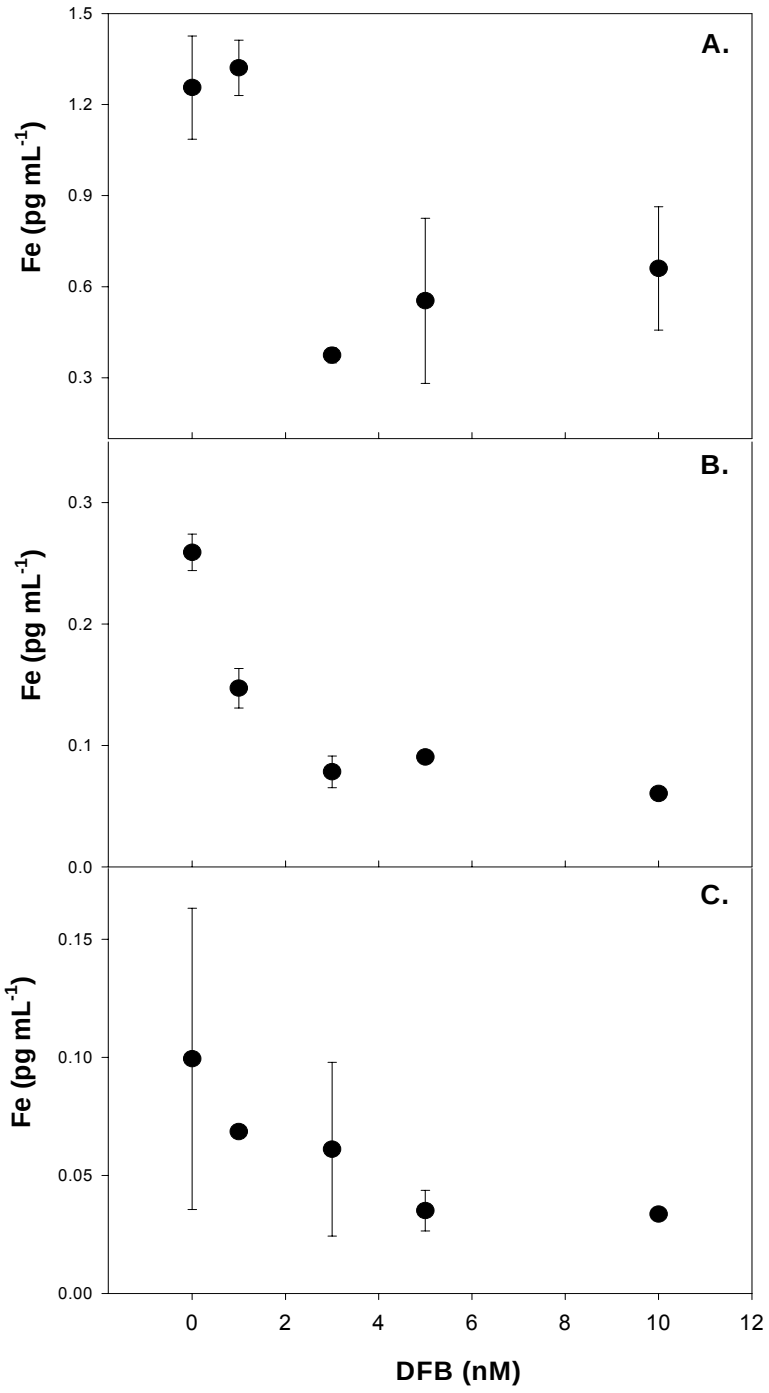

Figure 8. ^{55}Fe uptake.
Fe uptake (as ^{55}Fe) across increasing concentrations of DFB at Bio-7 after 48 h. Mean estimates (± range, n = 2). A. > 0.2 µm fraction. B. > 1.0 µm. C. > 8.0 µm.

Table 1. Station characteristics.
Nutrient concentrations are in μM except for Fe, which is in nM.

	Location	Total Chl *a*	Salinity	Temperature	Nutrient Concentrations			
		($\mu g\ L^{-1}$)	(psu)	(°C)	$NO_3^- + NO_2^-$	PO_4	SiO_2	Fe
Bio-4	86.30° W, 3.10° S	1.5	35.0	19.6	24	1.5	15	0.06
Bio-5	80.47° W, 9.16° S	1.3	35.1	18.3	17.3	1.16	6.9	0.1
Bio-6	81.57° W, 9.81° S	0.7	35.2	18.8	14 -15	1.2-1.3	6.0	0.1
Bio-7	76.02° W, 15.23° S	1.2	34.8	15.8	11-12	1.5	10-12	0.08

Part III

Alterations in Bacterial Community Structure in the Eastern Subtropical Pacific Ocean in Response to Changes in Iron Assessed by Molecular Methods

This part was submitted to the journal Aquatic Microbial Ecology in March 2004, under the same name by the authors Melanie L. Eldridge, Marc W. Cadotte, Steven W. Wilhelm.

Eldridge ML, Cadotte MW, Wilhelm SW (2004) Alterations in bacterial community structure in the Eastern subtropical Pacific Ocean in response to changes in iron assessed by molecular methods. Aquat Microb Ecol (*submitted*).

My use of “we” in this chapter refers to my co-authors and myself. My primary contributions to this paper were: all phases of experimentation, data analysis, and writing of the manuscript, with the exception of part of the statistical analysis of T-RFLP data and writing of the corresponding section, which was performed by Marc Cadotte or both Marc Cadotte and myself. All of the authors participated extensively in the editing of this manuscript.

INTRODUCTION

Global change is a real phenomenon, affecting ecosystems from all parts of the world (e.g., Quayle et al. 2002, Verburg et al. 2003). Due to concerns about anthropogenic CO_2 loads, researchers have been working to determine if we can feasibly manipulate the export of CO_2 from the atmosphere to the deep ocean to limit the effect of CO_2 on climate change. Stimulation of primary

production has been considered to be one way to increase the flow of CO_2 from the atmosphere to the deep ocean (Martin 1990a). In high nutrient – low chlorophyll (HNLC) regions of the ocean, scientists have convincingly determined that phytoplankton are limited by Fe (Boyd et al. 2000, Behrenfeld & Kolber 1999, Martin et al. 1994, Coale et al. 1996), demonstrating the key role that Fe plays in ocean systems. While the role that phytoplankton play in carbon sequestration and export is becoming clearer, the impact of the bacterial community on these processes has not been thoroughly investigated. Bacteria may also be critical players in biogeochemical cycling of Fe and also affect the 'biological carbon pump' (reviewed by del Giorgio & Duarte 2002). Only a small part of the dissolved organic carbon (DOC) produced by primary production in the euphotic zone is carried to the deep ocean, with the majority of carbon being utilized for bacterial respiration (Ducklow 1995). In addition, some bacteria are better equipped to out-compete phytoplankton for limiting amounts of Fe because they are capable of the production and utilization of high-affinity Fe-scavenging systems, such as siderophore production (Wilhelm 1995, Butler 1998).

Although the addition of Fe stimulates bacterial growth in the open ocean (Cochlan 2001, Hutchins et al. 2001, Paluski et al. 1996), it is not clear whether the stimulation is a direct effect of Fe itself or an indirect effect through stimulation of phytoplankton and subsequent release of DOC. Paluski et al. (1996) demonstrated a two-fold increase in both heterotrophic bacterial abundance and growth rates in response to Fe addition in dark-incubated

Gerlache Strait (Southern Ocean) samples, indicating that Fe addition limited the heterotrophs in this system. Similarly, Cochlan (2001) found a 1.7-fold increase in bacterial abundance in response to Fe addition during the mesoscale Fe fertilization experiment, IronExII. In contrast, Church et al. (2000) found no increase in bacterial abundance or growth rate in response to Fe alone, but did see an increase in growth rate with the addition of both DOC and Fe. While these studies were focused on the bacterial abundance, they did not examine bacterial community structure changes in response to Fe. Hutchins et al. (2001), however, found that the addition of Fe, in three HNLC regions, while resulting in increases in bacterial abundance and production, resulted in only minor changes to bacterial community composition despite dramatic changes in the phytoplankton community. Hutchins et al. (2001) represents the only examination, prior to the present study, that has examined changes in bacterial community structure changes with Fe addition in HNLC regions.

In the current study we have focused on the bacterial community structure in HNLC waters of the Peruvian upwelling region in response to Fe addition and removal to determine whether bacterial community structure and diversity is affected by Fe concentration. Fe reduction was achieved through the addition of desferrioxamine B (DFB), which has been previously demonstrated to markedly reduce Fe availability to this *in situ* community (Eldridge et al. 2004a).

The method we use to examine pelagic communities employs within bottle incubations. On-deck incubations are a powerful way to study the outcome of

varying experimental parameters on a population and have been used to demonstrate the effects of nutrient additions to ocean surface water (e.g., Martin & Gordon 1988, Hutchins & Bruland 1998), including the role of iron in the limitation of phytoplankton growth in this region (Hutchins et al. 2002, Eldridge et al. 2004a).

One method of whole community molecular analysis that is gaining wider acceptance is Terminal Restriction Fragment Length Polymorphism (T-RFLP) analysis (Liu et al. 1997, Luklow et al. 2000). Combining the analytical power of RFLP with the precise detection of end-fragment sizing using fluorescent tags, this technique allows scientists to obtain a picture of community diversity and to track changes across experimental parameters. Whole communities may be assessed inexpensively and much quicker than with standard cloning methods (Moeseneder et al. 1999). With clone libraries a large number of clones must be analyzed to be confident that an accurate picture of the community has been obtained (Kemp & Aller 2004).

T-RFLP has been widely used on environmental samples (Liu et al. 1997, Clement et al. 1998, Osborn et al. 2000 as examples), including many from marine systems (Moeseneder et al. 1999, van der Maarel et al. 1998, Hutchins et al. 2001). It has also been employed during enrichment culture experiments (Chin et al. 1999, Hutchins et al. 2001). Though most studies target the 16S rRNA gene, no commonality exists regarding the choice of primers, region of the 16S gene targeted, or starting template concentration during PCR. More

importantly, no method to analyze the massive amounts of data generated from the T-RFLP process has been chosen as a consensus. One of the most common approaches is principal component analysis (Clement et al. 1998, Dollhopf et al. 2001, Kaplan et al. 2001, Franklin et al. 2001) as it allows researchers to tease apart relationships between samples with extremely large and complex data sets (Legendre & Legendre 1998).

T-RFLP analysis is similar to RFLP analysis, in that PCR amplified DNA is restriction digested (typically with a tetrameric restriction enzyme) and the resulting fragment pattern is examined (Clement et al. 1998, Moeseneder et al. 1999). With community profiling, however, a complex system may yield too many fragments for convenient analysis. For T-RFLP, one of the PCR primers was labeled with a fluorescent molecule, such that only the 5' (terminal) fragments were visualized (Clement et al. 1998, Moeseneder et al. 1999). The entire set of fragments can be analyzed by automated capillary electrophoresis, with only the labeled fragments detected by the laser detection system. With the utilization of T-RFLP to examine the whole community and a combination of the addition and removal of Fe to the population, a much more powerful method to detect community responses is achieved.

MATERIALS AND METHODS

i.) Sampling and iron amendments

A 50 L surface seawater sample was collected off the coast of Peru (latitude 86.30° W, longitude 3.10° S, September 1998) from the upper mixed layer of the photic zone (~ 7 m depth), into a dedicated acid-cleaned polyethylene, mixing carboy during a ship's transect in the equatorial Pacific Ocean. To prevent trace metal contamination, water was gathered through an all Teflon™ pumping system (Bruland et al. 2001) using a PTFE Teflon ™ diaphragm pump (Osmonics Bruiser ™) and PFA Teflon ™ tubing with a PVC "fish" hanging off the ship to collect water outside of the ship's wake. Water was pumped into a positively pressurized clean room that was constantly flushed with HEPA filtered air. Seawater (2.7 L) was directly dispensed into each of ten acid-cleaned bottles.

Fe and DFB stocks were made according to the protocol of Eldridge et al. (2004), which includes dissolving DFB in Chelex-100 treated water (Price et al. 1989) from a Millipore BioCell water purification system and dissolving Fe (as $FeCl_3$) in 0.01 M Ultrex HCl. Amendments were made to generate a range of Fe concentrations, in duplicate, from 5.0 nM Fe below ambient concentrations (with DFB stocks) to 1.5 nM Fe above ambient concentrations (with $FeCl_3$ stocks). Control bottles, also in duplicate, received no additions

These bottles were then sealed with Parafilm to prevent seawater intrusion and incubated in an on-deck shipboard incubator made of Plexiglas™ that attenuates natural sunlight to mimic spectral conditions at a 50% spectral irradiance (Laws et al. 1990, Wells 1999). To match *in situ* temperature conditions, seawater was continuously passed over the bottles by pumping surface water through the incubator. Incubations continued for 72 hours, with sampling for flow cytometry, chlorophyll, and Fv/Fm (photosynthetic efficiency) every 24 hours, as previously reported in Eldridge et al. (2004a). Then the remaining water was filtered through 25 mm GF/F filters and frozen for transport.

ii.) DNA extraction

Each filter was aseptically cut into slices and placed in 50 mL polypropylene tubes for DNA extraction. DNA isolation was a modification of the classical phenol-chloroform extraction (Maniatus et al. 1982). For extraction, 15 mL of STE buffer (pH 8.0) (10 mM Tris-Cl, pH 8.0, 0.1 M NaCl, 1 mM EDTA, pH 8.0) was added to the tubes with lysozyme to a final concentration of 3 mg / mL. The filters were homogenized to loosen cell debris and the tubes were incubated with shaking (200 rpm) at 37°C for 2 h. Sodium dodecyl sulphate (SDS, 1.5 mL of 10% w/v, pH 7.2) was subsequently added and the tubes incubated for 1 h at 37°C. Samples were then subjected to one round of a freeze-thaw cycle (95°C 15 min, -80°C 15 min, 37°C 15 min). The tubes were centrifuged for 7 min at 7000 rpm to pellet the filters and then 7.5 mL of equilibrated phenol was added to

each tube, which was then mixed thoroughly and incubated at room temperature for 5 min. Chloroform : isoamyl alcohol (24:1, 7.5 mL) was added, the tubes were mixed, and then were incubated at room temperature for 5 min. The tubes were finally centrifuged for 10 min at 7000 rpm and the aqueous phase was removed. This aqueous phase (top layer) was transferred to a 48-mL FEP Oak Ridge tube (Nalgene) for a second chloroform : isoamyl alcohol purification. Two volumes of 100% ethanol, a $1/10^{th}$ volume of ammonium acetate (10M) was added and the tubes incubated overnight at -20°C to precipitate DNA, which was harvested from tubes by centrifugation (25 min at 4°C and 10,000 rpm). Air dried DNA was resuspended in 200 μL of 0.5X TE (pH 8.0) (10 mM Tris-Cl, pH 8.0, 1 mM EDTA, pH 8.0) and quantitated spectrophotomerically (Sambrook & Russell 2001).

iii.) PCR amplification

PCR was performed on each sample in triplicate, using universal eubacterial primers 46F (GCYTAACACATGCAAGTCGA) (Kaplan et al. 2001) and 519R (TTATTACCGCGGCKGCTG) (Lane 1991, W. Jeffrey, *pers. comm.*), which amplify a fragment of the 16S rDNA gene (using the *Escherichia coli* numbering system) approximately 473 bases long. The primer pair 46F and 536R (nearly identical to 519R of Lane 1991) listed in Kaplan et al. (2001) has been demonstrated to hybridize with 90% of 1,500 bacterial sequences tested (90% for 46F and 99% for 519R). PCR products were produced using either a 5-carboxyfluorescein (6-FAM) labeled forward primer (T-RFLP samples) or an

unlabeled forward primer (cloned samples). PCR amplification and analysis continued for only one set of duplicate bottles due to constraints on the number of samples that could be processed and because preliminary analysis of duplicates demonstrated good agreement between the replicate samples (Figure 1).

PCR reactions (50 μL) were prepared in EasyStart tubes (Molecular BioProducts) according to the manufacturers instructions and contained 25 ng template DNA, 40 ng each primer (Sigma), 2.5 U Taq DNA polymerase (Promega), 1X PCR Buffer, 0.2 mM dNTP Mix, 2 mM $MgCl_2$ (Molecular BioProducts). Cycling conditions consisted of an initial denaturation step at 94°C for 5 minutes, touchdown cycling for 10 cycles by 94°C for 30 sec, 65°C for 30 sec (minus 1°C per cycle), 72°C for 30 sec, then normal cycling for 25 cycles by 94°C for 30 sec, 55°C for 30 sec, 72°C for 30 sec, then a final extension for 7 minutes at 72°C. This approach should yield reaction products that are sub-plateau given that only 25 ng of starting template and 35 cycles were used (Trotha et al. 2002). In addition, since the starting template comes from a mixed population of organisms, it is believed that initial concentrations of each DNA would be much lower and should maintain sub-plateau conditions. Appropriate sized fragments (~473 bp) were excised from an agarose gel (1%) and DNA extracted using a Qiaquick Gel Extraction Kit (Qiagen) according to the manufacturer's protocol, with the exception that the resuspension volume of

buffer EB was 40 μL. These products were subsequently used for T-RFLP analysis.

iv.) T-RFLP analysis

T-RFLP analysis of purified samples was carried out for all samples. Restriction endonuclease digests were performed by digesting separate 8 μL aliquots of the gel-extracted samples with each *Alu*I, *Hha*I, and *Rsa*I (0.25 U / μL final concentration, New England Bioproducts), 1X reaction buffer (New England Bioproducts), and in the case of *Hha*I, 1X bovine serum albumin. Enzymes were chosen based on examination of hypothetical restriction sites (Cole et al. 2003) and on the commonality of each enzyme amongst previously published studies (Moeseneder et al. 1999, Dunbar et al. 2000, Osborn et al. 2000, Dollhopf et al. 2001). The reactions were allowed to incubate at 37°C overnight (approximately 16 h). Digested DNA was purified using a MinElute Reaction Clean-Up Kit (Qiagen) according to the manufacturer's protocol. Formamide (18 μL) (Fisher) and GENESCAN ROX-500 size standards (0.75 μL, Applied Biosystems) were added to the DNA purification mixture, which was then denatured at 95°C for 5 min and chilled on ice for 2 min. These samples were resolved in an ABI-310 Genetic Analyzer using GENESCAN Analysis software, 30 min reaction time, and otherwise default electrophoresis parameters. Electropherograms, as well as tables of peak size, area, and height, were generated for each bottle, in triplicate. Local southern size calling was used to assign peak sizes because previous

reports (Osborn et al. 2000, Kaplan & Kitts 2003) have demonstrated that this method produces peaks more close in size to actual T-RF length than the other size calling methods (global southern, cubic spline, 2nd order least squares, 3rd order least squares). An independent comparison of these size calling methods for our samples produced fragments of the same size with all methods (data not shown).

v.) Statistical analysis of T-RFLP data

Peak size and area data for each sample were transformed for statistical analysis. Transformations were made based on triplicate samples using a method similar to Dunbar et al. (2001), in an effort to minimize noise generated during the processing of T-RFs. Size values for all triplicates within a treatment and digest were aligned and any peaks not occurring in all three profiles were removed. Values for three replicates were averaged and rounded to the nearest integer. Initial examination was performed with size data only and consisted of calculation of Jaccard's coefficient of similarity (for an estimation of similarity between treatments by considering if T-RFs are present and how many T-RFs are common between treatments) (Liu et al. 1997) and Agglomerative single linkage Euclidean distances (a clustering technique used for measurement of which treatments are most similar / dissimilar) (Townend 2002), using Community Analysis Package (CAP) version 1.2 (Pisces Conservation LTD, Lymington, UK). Jaccard's coefficients were calculated by determining the

number of peaks in common between two samples and the number of peaks found only in each sample. The calculation of Jaccard's coefficient was made as follows:

$$S_j = \frac{c}{a + b + c} \quad (1)$$

in which, with two samples A and B, *a* is the number of operational taxonomic units (OTUs) in A but not in B, *b* is the number of OTUs in B but not in A, and *c* is the number of OTUs in common between A and B. Both peak heights (Moeseneder et al. 1999, Dunbar et al. 2000, Osborn et al. 2000, Dollhopf et al. 2001) and peak areas (Buchan et al. 2002, Horz et al. 2000, Kaplan et al. 2001, Trotha et al. 2002) have been used to estimate the abundance of each T-RF, but since T-RFLP is subject to all of the inherent biases associated with PCR (Wintzingerode et al. 1997), we used correlation analysis to determine how precise area estimates were among the triplicates. Pearson's correlation coefficients, calculated using SYSTAT 10 (SPSS Inc. Chicago, IL, USA), were calculated for replicate peak areas within a treatment and digest, then transformed triplicate size values were each reassigned their corresponding peak areas. Principal component analysis (PCA) was performed on all three digests combined and on individual digests (Community Analysis Package v. 2.1), shown by plots of the first two principal components. To determine whether or not diversity changed across the treatments, we calculated the Simpson's index of

diversity for each sample, by first calculating the probability that any two individuals are the same species:

$$\lambda = \sum p_i^2 \tag{2}$$

where p_i = the relative frequency of a species, *i*, within the community, then Simpson's D = 1 – λ. Next, the Shannon-Weiner index of diversity (H') was calculated for each sample, by

$$H' = -\sum_{i=1}^{S} p_i \ln p_i \tag{3}$$

where p_i is the proportion of the abundance of the i^{th} species expressed as a proportion of the total coverage. These diversity indices were calculated using the area values for each triplicate within a digest. An analysis of variance (ANOVA) among treatments within enzyme digests was calculated for each diversity index using SYSTAT 10.

We wanted a measure of the strength of the effect Fe levels had on community composition. Mantel's test correlates the distance matrix of dissimilarities among bacterial communities (composition and abundance) to the distance matrix of Fe concentrations. The distance matrices were constructed using the Sørensen distance measures (McCune et al. 2002). Mantel's Test produces an r-value, which is analogous to a Pearson correlation coefficient, as well as a Z statistic used for significance testing (McCune et al. 2002). To

determine significance we compared the observe Z statistic to a distribution created from random Monte Carlo permutations, with 1000 randomizations. This test was done using PC-ORD for Windows (MjM Software, Gleneden Beach, Oregon, USA).

vi.) Cloning, sequencing and phylogenetic analysis

Gel-purified non-labeled PCR products (for the 1.5 nM Fe, control, and 5.0 nM DFB treatments) were cloned using a TOPO-TA cloning kit (Invitrogen) according to the manufacturer's protocol with the exception that ligation reactions were allowed to incubate at 14°C overnight. Minipreps (20-30 colonies per sample) were performed with Wizard Miniprep DNA Purification kit (Promega). Sequences were aligned in BioEdit Sequence Alignment Editor (Hall 1999), along with sequences of known organisms (Cole et al. 2003, Benson et al. 2003). Previously published sequences were truncated to include only sites obtained in this study (approximately 473 bases, depending on sequence length heterogeneity). A ClustalW alignment (Thompson et al. 1994) was generated within BioEdit and then an UPGMA (unweighted pair group with mathematical averages) dendogram was made using Molecular Evolutionary Genetics Analysis package (MEGA v. 2.1, Kumar et al. 2001), with distances calculated using a Kimura2 model and bootstrapping 5000 permutations.

vii.) Taxonomic identification of T-RFLP output

A recently published paper by Kent et al. (2003), explores the use of the Phylogenetic Analysis Tool (PAT), an online program used to assign taxonomic identity to fragments within a T-RFLP digest. We have used this tool to generate taxonomic information about the communities found in our treated bottles. A database was made using the online Microbial Community Analysis (MiCA) website (http://hermes.campus.uidaho.edu/), which allowed us to submit forward primer information, perform an *in silico* PCR, and digest retrieved fragments. The output contained a large database of T-RFs that we may obtain from natural samples. This database was uploaded into the PAT website with Genescan output files from each of our digests and the matching algorithm was performed within PAT to determine which of the possible species occurred in our digests. Default bin sizes were used; 1.0 bp for fragments <200 bp, 1.5 bp for fragments with sizes 201-400 bp, 4.0 bp for fragments 401-600 bp, and 5.0 bp for fragments 601-800 bp. The output was analyzed to determine the pattern of occurrence of each OTU, which were operationally defined as unique T-RF patterns, using the same bin size tolerances as that for PAT. Also, clone T-RF patterns, which were predicted based on sequence, were compared to the PAT results.

RESULTS

i.) PCR amplification

Bands of DNA within gels appeared as doublets. Previous studies have found that the size of the 16S gene is not consistent for all bacteria (Suzuki et al. 1998, Tiirola et al. 2003). The primers chosen for this study, by targeting the 5' end of the 16S gene, include three hypervariable regions in which length heterogeneities have been shown to cause greater than 50 base pair differences in size (Suzuki et al. 1998). Tiirola et al. (2003), using primers targeted to a similar region of the 16S gene, found that sizes of the gene varied by as much as 98 base pairs. Sequences deposited in GenBank (Benson et al. 2003) were examined for probable sizes generated with the primer set used in this study. Greater than 50 bp differences were noted between different genera of bacteria (data not shown). Therefore, doublets visualized within gels were acceptable and when fragments were excised for gel purification, the entire doublet was removed as one slice.

ii.) Community analysis – species richness

Initial analysis of T-RFLP data was made using a presence - absence approach, in which only size data was considered, so that known problems with the T-RFLP technique could be avoided (Moeseneder et al. 1999, Osborn et al. 2000). Jaccard's coefficient of similarity (S_j) was calculated between treatments

to examine how similar each treatment was to the others (Table 1). If Fe is a significant factor affecting community structure, then +Fe treatments should be more similar to each other and +DFB treatments should be more similar to each other, but the +Fe samples should be dissimilar to the +DFB samples. Treatments within the *Alu*I digest had similarity values averaging 0.370 ± 0.095, while *Hha*I and *Rsa*I had similarity values on average 0.470 ± 0.099 and 0.403 ± 0.135, respectively.

Two values that stand out are the comparison between 0.5 nM Fe and 1.5 nM Fe in the *Hha*I treatment (66.67% similar) and that between 5.0 nM DFB and 1.0 nM DFB in the *Rsa*I treatment (73.33% similar). These values indicate that in the *Hha*I digest, the two +Fe samples were the most similar and that in the *Rsa*I digest the two +DFB samples were the most similar. Though the values were lower in the *Alu*I digest, the highest value achieved was the comparison between the two +DFB samples (50.00%), similar to the *Rsa*I digestions. In addition, as predicted, the lowest similarity values occur when comparing +Fe treatments to +DFB treatments; in the *Alu*I digest the lowest value obtained was found when comparing 1.5 nM Fe to 5.0 nM DFB and in the *Hha*I and *Rsa*I digests the lowest values occurred when comparing 0.5 nM Fe to 5.0 nM DFB. Interestingly, with *Alu*I digestion very low similarity values are seen when comparing the control to 1.5 nM Fe and 5.0 nM DFB, indicating that neither of these samples is very similar to the control.

Similarly, Agglomerative single linkage Euclidean distances were calculated to show relatedness among the treatments. In the *Rsa*I and *Alu*I digests, the +DFB treatments are most similar among all the samples (data not shown), however in the *Hha*I digest the +Fe treatments are the most similar, with the +DFB samples instead being equally dissimilar to all others (Figure 2). In the *Alu*I and *Rsa*I digested samples, the +Fe treatments are unlike the others, with the 1.5 nM Fe treatment being the most dissimilar. However with *Hha*I digestion, the DFB treatments are the most dissimilar, with both being equally different from the rest (Figure 2). While it appears that one enzyme alone was unable to determine exact relationships between samples with confidence, taken together it appears that the community in +Fe treatments is different from that which occurs in +DFB treatments.

iii.) Community analysis – diversity

Many studies that report using T-RFLP analysis endeavor some form of replication among samples (for example, Dunbar et al. 2001, Clement et al. 1998, Kaplan et al. 2001, Hutchins et al. 2001). While these studies attempt to minimize inconsistencies with the technique (such as variation in fragments amplified with PCR or variation in fragments achieved after restriction digestion), true replication is not typically achieved because most groups pool either multiple amplification reactions prior to digestion or perform replication only by digesting multiple aliquots of the same amplification reaction. In this study an attempt was

made to approach true replication within a treatment, though admittedly this would only be achieved through the examination of our duplicate treatment bottles. Three separate PCR amplifications from each of five bottles were performed, as well as separate gel extractions, restriction digests, and digest purifications, plus each sample was run in a separate tube through the Genetic Analyzer and statistical analysis was performed maintaining triplicates as separate. As such, a total of 9 profiles were generated per treatment (bottle). Transformations were made to aligned tabular raw data to remove data points that did not appear in all three replicates. In this way, variations in PCR amplifications and the T-RFLP process in general could be minimized while differences between replicates could still be determined. Pearson's correlation coefficients were calculated to determine if area values were consistent between replicates. Coefficients demonstrated a high degree of correlation between replicates ($r = 0.85\text{-}0.99$, $P < 0.0001$) (*Hha*I Pearson values given in Table 2).

In this way, we have determined that with true replication, not only is pooling of samples unnecessary, but that our method is highly reproducible. We thought it may also be interesting to use the area values to make inferences concerning the abundance of the OTUs within the community. Principal component analysis was performed using size and area values, by combining data from all three digests. Interestingly, PC1 separated each of the samples based on enzyme treatment, but PC2 could only separate the samples digested with *Alu*I and not those digested with *Hha*I or *Rsa*I. Initially combining all the data

from each digest was performed because previous studies have found that combined data yielded better results (Clement et al. 1998, Franklin et al. 2001). Dollhopf et al. (2001), however, found that a combination of all the data yielded PCA ordinations that were too complex to interpret. More than three factors were required to explain much of the variation observed with our combined data set and samples separated based only on enzyme digest; therefore it became necessary to examine each restriction digest separately.

For *Alu*I digested samples, 66% of the variation in samples was explained by the first two principal components (41% and 25%, respectively). The first principal component separates both the control samples and 1.5 nM Fe samples independently from all others. The second principal component separates both the 1.5 nM Fe samples and 5.0 nM DFB samples from all others, but failed to distinguish between the control, 0.5 nM Fe, and 1.0 nM DFB samples. Figure 3 shows that for samples digested with *Hha*I, 89% of variation was explained with the first two principal components (48% and 41%, respectively). PC1 separates the +Fe treatments from the +DFB treatments, while the control samples seem to fall somewhere in the middle. PC2 separates both the control samples and the 5.0 nM DFB samples independently from all others. For samples digested with *Rsa*I, 81% of variation is explained in the first two principal components (56% and 25%). PC1 separates the +Fe treatments from the +DFB treatments, and also from the control. It separates 1.5 nM Fe samples from 0.5 nM Fe samples, but failed to separate the 1.0 nM DFB and 5.0 nM DFB samples from each other.

Overall, each PCA separated the +Fe treatments from the +DFB treatments, suggesting that differences occurred between samples based on Fe. In addition, in each plot at least one principal component separated 1.5 nM Fe samples from 0.5 nM Fe samples. Plus, control samples are distinguishably different from the other samples in all PCA plots, sometimes occurring intermediate between +Fe and +DFB samples (data not shown) and other times occurring as isolated groups (Figure 3).

Results of Mantel's test, examining the correlation between the dissimilarity matrix of bacteria species composition and abundance and the dissimilarity matrix of Fe concentrations, reveal that the Fe gradient was important for explaining the differences in community composition among treatments. There was a significant effect associated with the Fe gradient for each enzyme (*AluI*: $r = 0.756$, $P < 0.001$; *HhaI*: $r = 0.621$, $P < 0.001$; *RsaI*: $r = 0.621$, $P < 0.001$).

While the above tests demonstrated that there was an overall effect of changes in Fe availability on the community, they do not reflect on whether there were changes in community diversity. To determine whether diversity varied, both Simpson's and Shannon-Weiner diversity indices were calculated, and then ANOVAs were performed to determine if diversity changed across the treatments. The results are presented in Table 3, Figure 4, and Figure 5. Interestingly, though not all differences are significant, the same trend is apparent for each enzyme with both estimates of diversity. As Fe is added,

community diversity seems to increase relative to controls and as DFB is added, diversity seems to decrease, especially for the 5.0 nM DFB addition.

iv.) Sequence analysis

Sequences fell into the classes alpha and gamma Proteobacteria, Cyanobacteria, and Cytophaga-Flavobacterium-Bacteroides (CFB). Within the Proteobacteria, six sequences similar to gamma Proteobacteria and 16 sequences similar to alpha Proteobacteria were found. While six sequences similar to Cyanobacteria in the genus *Synechococcus* and 12 sequences similar to CFB group bacteria were also found.

Sequences within the gamma subdivision of the Proteobacteria aligned mainly with *Pseudoalteromonas* spp. and *Alteromonas* spp. (five of six clones), while one clone was similar to a set of two sequences from endosymbionts found within a marine sponge *Halichondria panicea* (Althoff et al. 1998). Of the 15 clones that clustered with alpha proteobacteria, seven were similar to *Roseobacter* spp., two clustered with *Sulfitobacter* sp., one was similar to the *Azospirillum-Rhodospirillum* group, four clones were similar to a sequence isolated from the Atlantic Ocean near Cape Hatteras at a depth of 10 m (Field et al. 1997), and one clone did not cluster tightly with any of the previously sequenced alpha proteobacteria, but had the greatest similarity to an uncultured proteobacterium EBAC40E09 (Beja et al. 2000) at 94% similarity. Not surprisingly, all six Cyanobacteria sequences isolated were found to be similar to

organisms within the genus *Synechococcus*, known to be ubiquitous among marine isolates (Waterbury et al. 1986). Clones that aligned with CFB group were found to cluster mainly with sequences that were isolated from natural systems. One clone was found to be identical to *Tenacibaculum mesophilum*, a newly reclassified marine genus (M Suzuki et al. 2001).

Though a low total number of clones were sequenced from each of the three libraries and therefore this was not exhaustive enough to reliably say that all species were sampled (Kemp & Aller 2004), some interesting trends are present. Alpha Proteobacteria were numerically dominant (though not overwhelmingly so) in each of the three libraries, with clones representative of *Roseobacter* spp. that are very commonly found in marine systems (Allgaier et al. 2003, Cottrell & Kirchman 2000) appearing in both +Fe and +DFB libraries. Both gamma Proteobacteria and CFB group bacteria were also numerous in each library. Cottrell and Kirchman (2000), using both fluorescence in situ hybridization (FISH) and cloning found that in most samples, dominant groups were alpha and gamma Proteobacteria and CFB group bacteria, with beta Proteobacteria making up only minute portions of each sample, though percentages varied. Giovannoni and Rappé (2000) determined that beta Proteobacteria were not commonly found in marine systems. Interestingly, no beta proteobacteria were found in our libraries (although this does not preclude their existence in samples).

v.) Taxonomic identification of T-RFs

Given the default bin size tolerances used in this study, PAT was able to identify 88-55% of the peaks within each digest (Table 4), yielding 57 unique OTUs. Only 16 (28.1%) were identified to the genus level, due to multiple sequences yielding the same T-RF pattern and to identified T-RFs not being classified down to the genus or species level within RDP II (Cole et al. 2003), but nearly all of the identified OTUs have members of the same genus isolated from marine systems (data not shown). Class-wide taxonomic affiliation was more easily discernible for each identified T-RF, with alpha Proteobacteria again numerically dominating all samples (data not shown). Many T-RFs were identified as gamma Proteobacteria and CFB group bacteria, but there were also assignments made of beta, delta, and epsilon Proteobacteria, Firmicutes-Clostridia, Actinobacteria, and Cyanobacteria. Interestingly, of the 16 unique OTUs that occurred in only one sample, 11 were found in the 1.5 nM Fe treated sample (19.3% of the total OTUs) and only three were in the 1.0 nM DFB sample (5.3%), with none occurring in 5.0 nM DFB treated samples. Of the 15 unique T-RFs that occurred in two samples, seven occurred in +Fe samples (12.3%) while only four occurred in +DFB samples (7.0%). In addition according to the PAT, only 8 identified T-RFs (14%) occurred in all five samples.

Restriction maps of sequences obtained through cloning were compared to PAT results, but the PAT program was only able to assign 64% of the clones. Of the clones that were assigned, very few were identified to the genus level, but

86% were accurately identified to the class level. Exceptions include two assignments (10%) that could be made to either one class or another (in each case the actual class to which the sequence belonged was one of the two) and rarely absolute mistaken identifications (5%).

DISCUSSION

i.) Community analysis – species richness

Prokaryotic organisms are estimated to produce 1.7 x 10^{30} new cells globally per year, with the greatest new production occurring in the open ocean (Whitman et al. 1998). The total amount of carbon in prokaryotic organisms is estimated to be 60-100% as much as is in all plants (Whitman et al. 1998). Yet relatively little attention is paid to the way in which these microorganisms may influence biogeochemical cycling, relative to the amount of attention paid to the influence of photosynthetic organisms. Mean values for number of prokaryotes in the upper 200 m of the ocean and along the continental shelf estimate their numbers to be approximately 5 x 10^5 cells / mL of which only approximately 8 % are autotrophic prokaryotes (Whitman et al. 1998). We have examined the influence that Fe has on the eubacterial community through the use of an Fe titration experiment and a relatively new molecular technique, T-RFLP. The statistical analysis of this data clearly shows that Fe is affecting community structure, with multiple statistics confirming these results.

Our results show that either the +Fe samples are the most similar to each other or the +DFB samples are the most similar to each other, while the control samples have intermediate similarity with reference to +Fe and +DFB samples. These results support a predicted pattern that Fe controls community structure and that bacterial communities change across the gradient of Fe. The concordance between the two tests used (Jaccard's similarity and Agglomerative single linkage Euclidean distances) reinforces the confidence of the results and demonstrates that T-RFLP is useful for examination of communities within bottle incubations.

ii.) Community analysis – diversity

With a growing number of T-RFLP studies pooling amplification reactions prior to restriction digestion, it was important to examine if this method was reproducible. Pearson's correlation coefficient calculations revealed that the replicates were strongly positively correlated (Table 2) indicating that pooling of samples was unnecessary. When samples are pooled, there is no way to judge the degree of reproducibility of a PCR. In addition, if a single PCR (out of three identically created PCRs) is contaminated with unknown DNA that amplifies with high efficiency one would not know that this OTU is not from the original sample. Studies that employ the peak-removing transformation of the data (Dunbar et al. 2001, and the present study) reveal that the pooling of samples prior to digestion is unnecessary.

Principal component analysis was used to visually compare the relationship among samples based on species abundances (using peak size). For all three digests, the +Fe samples are distinguishable from the +DFB samples. In addition, along some principal components it seems that communities within control, 0.5 nM Fe, and 1.0 nM DFB bottles were more similar to each other than to the two most extreme Fe treatment communities, supporting the results based on species richness measures. Further, the results of Mantel tests reveal that these compositional differences are highly correlated with the Fe manipulations.

The role that heterotrophic bacteria play in biogeochemical cycling remains unclear. While some studies have examined the bacterial population as a whole in response to Fe limitation, virtually no one has attempted to examine diversity changes within the bacterial community in response to Fe. Hutchins et al. (2001) sampled water from three HNLC regions for Fe addition experiments, monitoring community diversity through DGGE and, in two of three cases, T-RFLP. As Fe was added very little change was observed in bacterial community structure (Hutchins et al. 2001). In some of these same areas, previous studies have determined that the addition of Fe has little effect on bacterial production or abundance (Church et al. 2000, Kirchman et al. 2000), though results from other studies that contradict this remain (Paluski et al. 1996). In waters of the equatorial Pacific, however, Cochlan (2001) demonstrated that addition of Fe to bacterial communities resulted in an increase in both abundance and productivity

of bacteria. Using two different diversity indices, we have determined that as Fe is added, bacterial diversity in the Peruvian upwelling region of the equatorial Pacific increases (and decreases as DFB is added). Since it has also been demonstrated that bacteria in this region may account for up to 70% of the biomass (Ducklow 1995), our results indicate that these bacteria are potentially critical players in biogeochemical cycling. Bacteria are able to effectively compete with phytoplankton for Fe due to their small size and subsequent increased surface-area to volume ratio (Morita 1975), or in some cases by employing high-affinity Fe uptake mechanisms (Wilhelm 1995, Butler 1998) that more efficiently scavenge this limiting resource. As well, different bacteria have been shown to consume different forms of dissolved organic carbon (Cottrell & Kirchman 2000) and are differentially able to take up Fe bound to natural ligands (Weaver et al. 2003), so that when many types of bacteria are present, the potential to short-circuit the 'biological carbon pump' is increased through the ability of the bacterial population to response in a varied way to limiting resources.

iii.) Sequence analysis

Since communities within the most extreme treatments were the most different from control samples, only 1.5 nM Fe, control, and 5.0 nM DFB samples were cloned. Of the 40 clones sequenced, none were unusual for marine systems. With sequencing of only a limited number of clones, judgments about

frequency within the library or whether we have obtained a representative sampling of sequences cannot be made (Kemp & Aller 2004). Doublets in our gels were confirmed through sequence analysis, in which cloned sequences varied size by as much as 50 bp. Several clones from our study were very similar or identical to two sequences obtained from the Red and Mediterranean Seas, RS9915 (AY172825) and Minos02-C2C2 (AY172809), which are taxonomically positioned within the third clade of *Synechococcus* and that appear non-motile, even though nearly all clade III *Synechococcus* are motile (Fuller et al. 2003). Clade designations were previously made by Rocap et al. (2002), and have been extended by Fuller et al. (2003). Two other clones were very similar or matched identically a clone isolated by MT Suzuki et al. (2001) off the coast of California. All aligned closely, or matched identically, with sequences obtained from marine environmental samples, some of which remain uncultured.

iv.) Taxonomic identification with PAT

Assignment of species names by the PAT program to T-RFs within our digests was fairly consistent with our sequence data. Alpha proteobacteria sequences, which were numerically dominant in PAT output, were found in all three clone libraries generated from our samples (1.5 nM Fe, control, 1.0 nM DFB). A clone (OM42) first isolated from coastal Atlantic waters (Rappé et al. 1997) was assigned to one of the OTUs in our analysis. Hutchins et al. (2001) also found this same isolate in +Fe and control samples from each of the three

HNLC waters examined, indicating its ubiquity amongst marine systems. Interestingly, this isolate was found in both +Fe and +DFB treated samples. Many T-RFs were identified as environmentally isolated clones from aquatic systems that we were unable to isolate with cloning and sequencing, though a more exhaustive number of sequences would be needed to ensure sampling of all bacteria present. Since, we are interested in community-wide responses to iron amendments, only preliminary sequencing to confirm that we had appropriate PCR products was performed.

The PAT program may be limited by the database chosen for analysis. Sequences deposited within a database may be overwhelmingly skewed toward genera not found in marine samples. However, genera that occur in marine systems are not always particular to aquatic environments therefore if a large number of sequences are present in the database chosen, many of the same genera should be present. Careful screening of the output files allows a researcher to select sequences that may be particular to typical environmental samples. Operator error may, however, lead to incorrect identification when multiple assignments have been made to that same OTU.

The percentage of peaks left unmatched decreases with increasing bin size tolerance because a wider size range of fragment lengths are assigned to each T-RF. Because the communities in our experiments were fairly complex (11-33 peaks for each digested sample) and only three restriction enzymes were used, bin sizes were left as default for the PAT analysis. Increasing bin size

tolerance to twice the defaults (not an impractical increase) led to a dramatic decline in percent-unmatched peaks for most samples (from 27% on average to 13%) but it led to an even more dramatic increase in the number of OTU identifications in the output (1,664 identities with default bin sizes versus 7,752 identities with larger bin sizes). Being able to manage the output was considered paramount to identifying every peak. It is recommended that future PAT users, to increase the specificity of taxonomic identification, utilize more than three restriction digests in combination with larger bin sizes.

CONCLUSIONS

The results of this study should be viewed in light of our previous study (Eldridge et al. 2004a) in which it was demonstrated that the phytoplankton community structure changed in response to the addition of Fe. Total chlorophyll *a* and photosynthetic efficiency (Fv / Fm) increased in +Fe samples, as did cell numbers for three classes of larger eukaryotes. The effects of Fe limitation on the marine bacterial community are clear, but what remains questionable is whether this is a direct or an indirect effect. Kirchman et al. (2000) have demonstrated that carbon, not Fe, limit bacteria in low-Fe waters off the California coast, though they do see that with an addition of DOC alone Fe quickly becomes limiting. In contrast, Paluski et al. (1996) have shown that Fe specifically limits light-independent production (assumingly heterotrophic bacteria) in Southern Ocean

waters by conducting their experiments in the dark. While we are unable to determine whether Fe actually stimulated bacterial diversity increases or whether the phytoplankton's increased productivity drives bacterial diversity change, we feel that this holistic approach better represents a natural system. Any natural or anthropogenic inputs of Fe to marine systems would certainly affect phytoplankton, bacteria, and their interactions.

Community profiling has great potential to determine what factors affect microbial populations. With traditional cloning, it is required to sequence a very large number of clones in order to be certain that an accurate picture of the community is obtained (Kemp & Aller 2004). Studies should be viewed with caution if they use cloning without some statistical measure (such as species accumulation curves) to judge whether total diversity has been sampled. Profiling methods such as DGGE and T-RFLP are able to examine total community responses to varying experimental conditions in a much quicker and inexpensive fashion compared to cloning methods. Combining this with titrations of nutrients, a very powerful technique for examining community structure is achieved. We have demonstrated that this method is applicable to bottle incubations, which are commonplace now in the field of oceanic microbial ecology. Plus, the utility of the technique remains to be seen as the eventual goal of accurately assigning species names to T-RFs has nearly become achieved.

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APPENDIX (PART III)

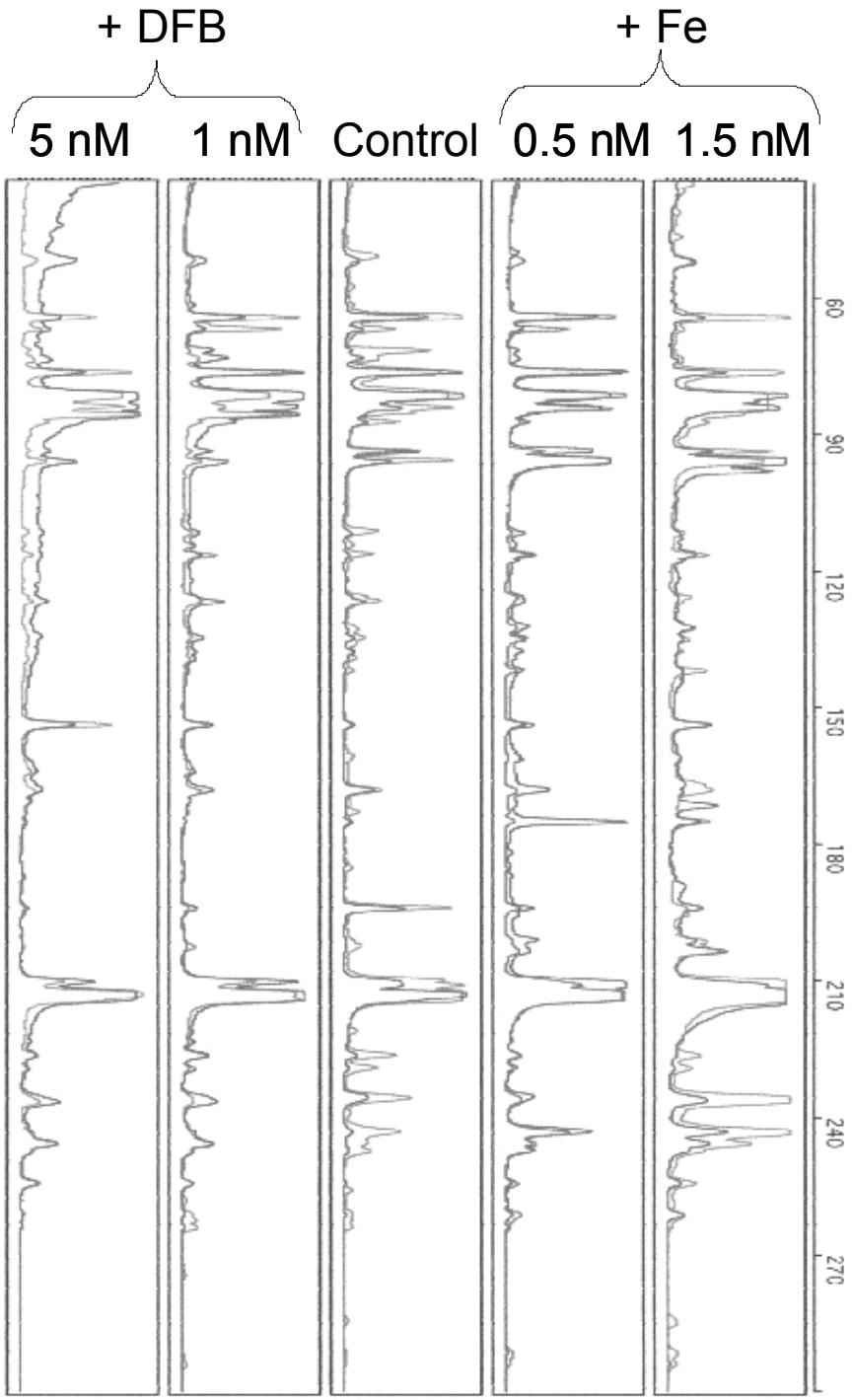

Figure 1. T-RFLP profile of duplicate bottles.

T-RFLP profiles of preliminary samples from duplicate bottles. Samples were shown to replicate one another faithfully; therefore all subsequent analysis was performed with a single set of bottles.

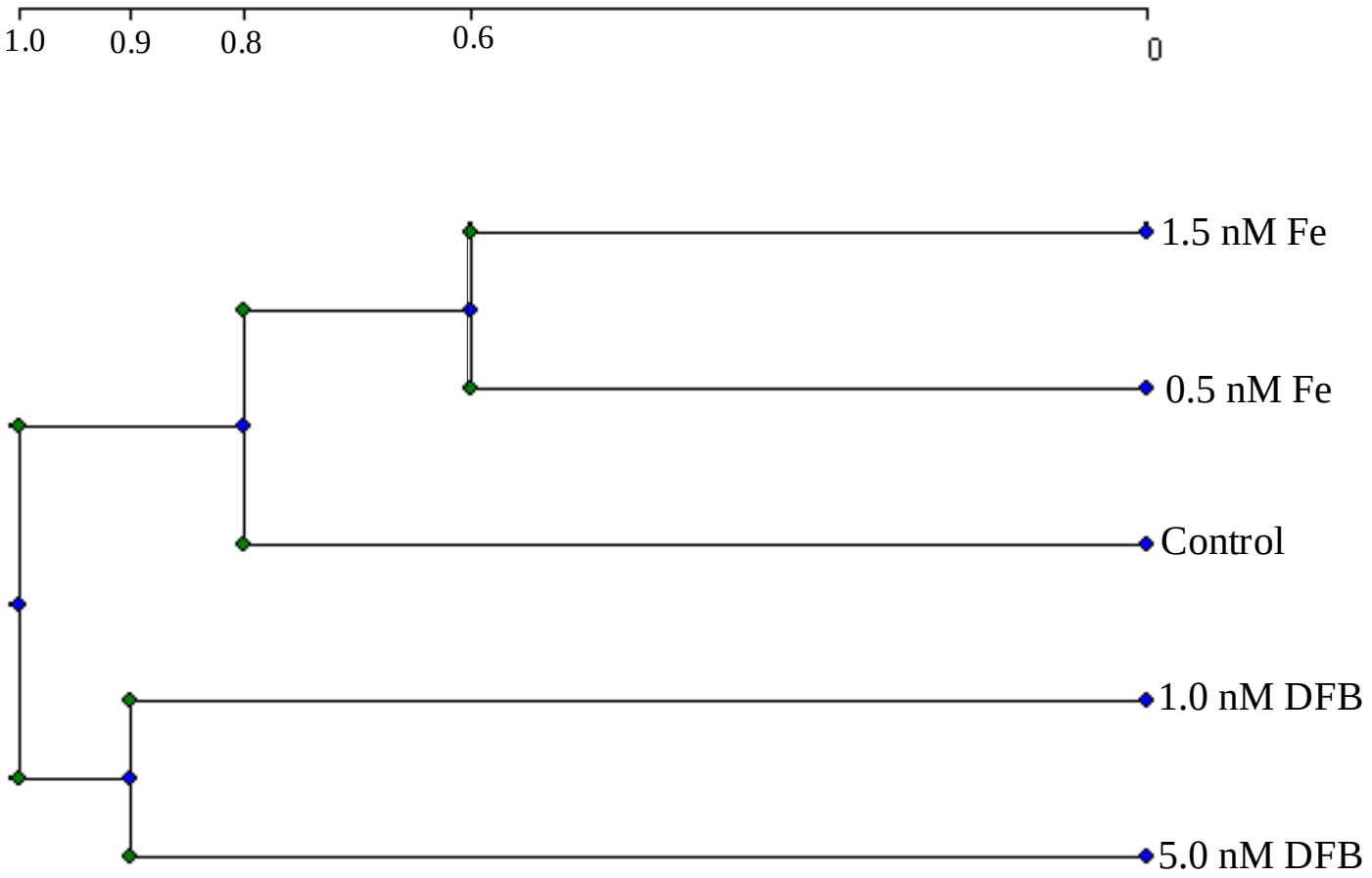

Figure 2. Agglomerative single linkage Euclidean distances dendogram of *Hha*I digested samples.
Fe addition samples are the most similar, with DFB addition samples being equally dissimilar from the others and the control intermediate in similarity.

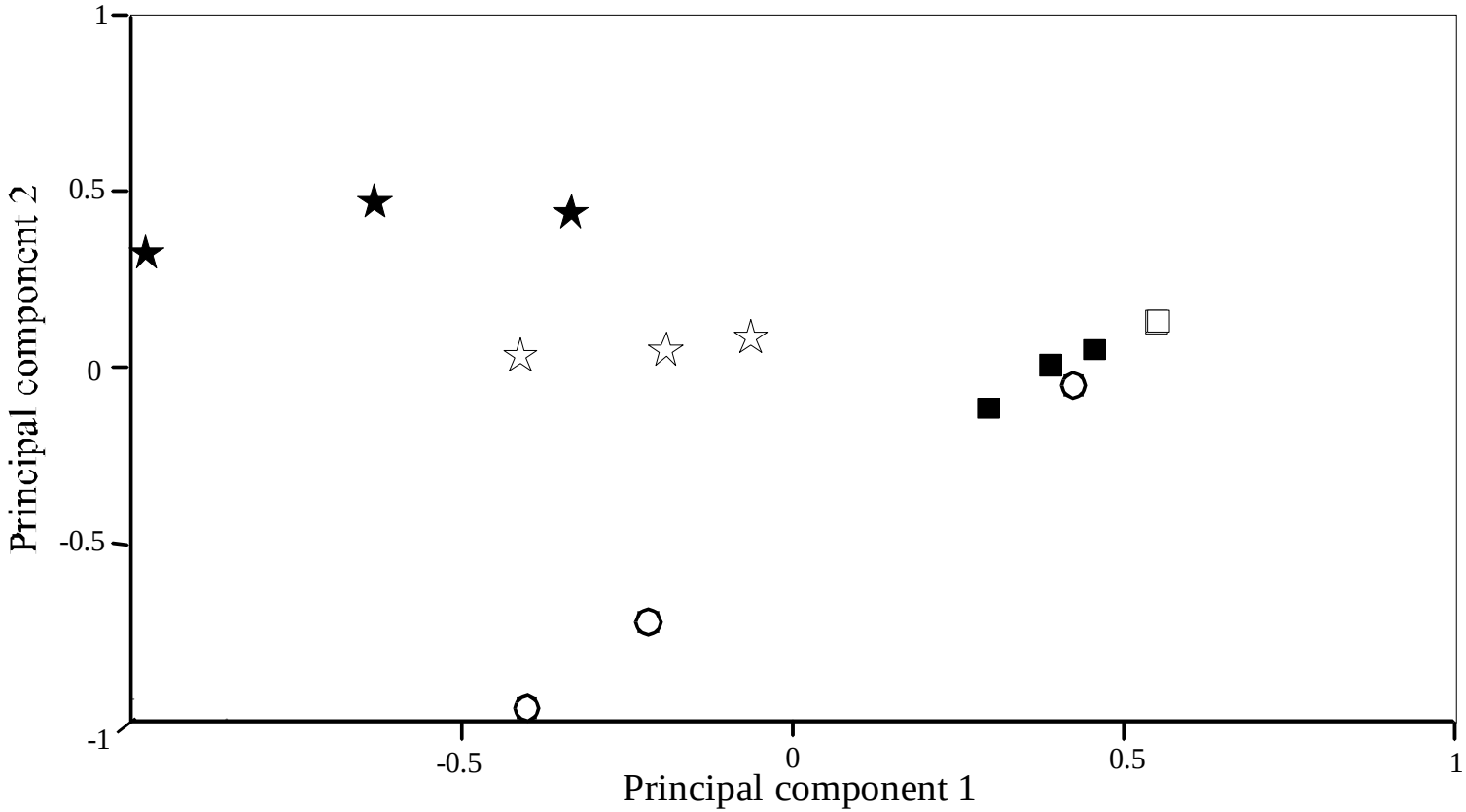

Figure 3. PCA of *Hha*I digested samples.

Principal component analysis of *Hha*I digested samples. Plot of the first two principal components, in which PC1 explains 48% of the variation observed and PC2 explains 41% of the variation observed. Closed squares are 1.5 nM Fe samples, open squares are 0.5 nM Fe samples, open circles are control samples, open stars are 1.0 nM DFB samples, and closed stars are 5.0 nM DFB samples.

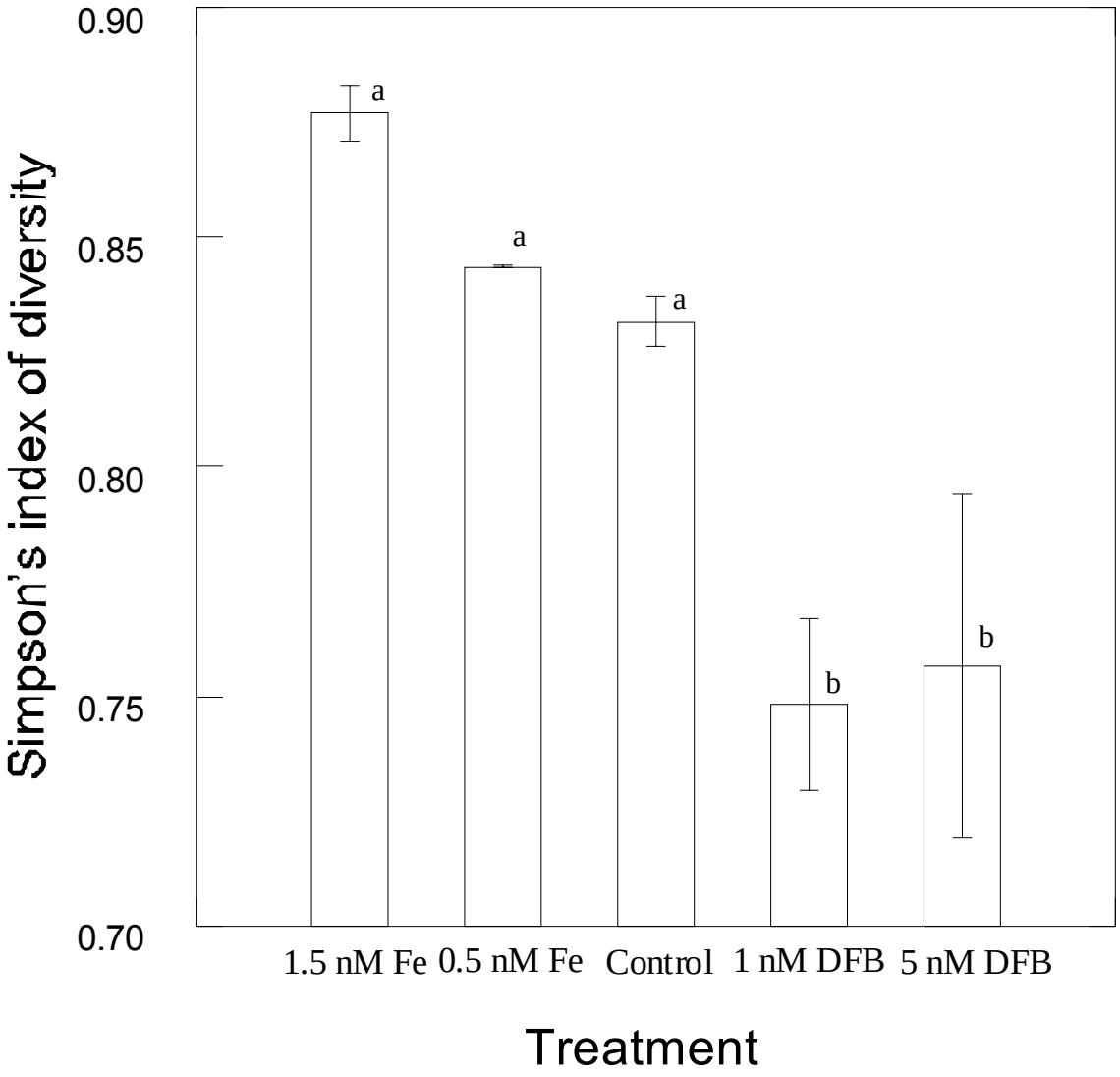

Figure 4. Simpson's index of diversity for *Rsa*I digested samples. Lowercase letters indicate significant differences ($P < 0.05$) from Bonferroni post hoc analyses.

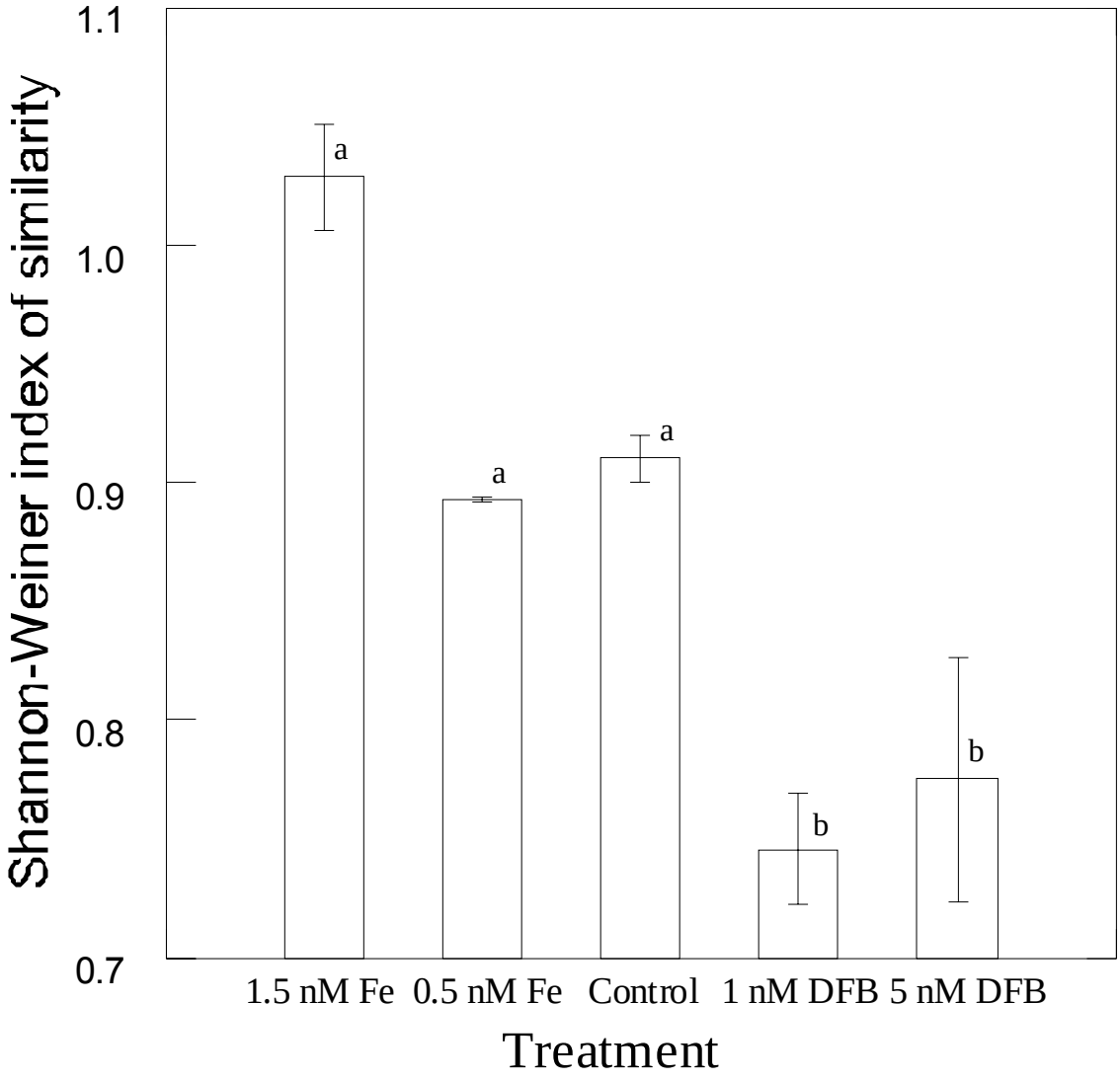

Figure 5. Shannon-Weiner index of diversity for *Rsa*I digested samples. Lowercase letters indicate significant differences ($P < 0.05$) from Bonferroni post hoc analyses.

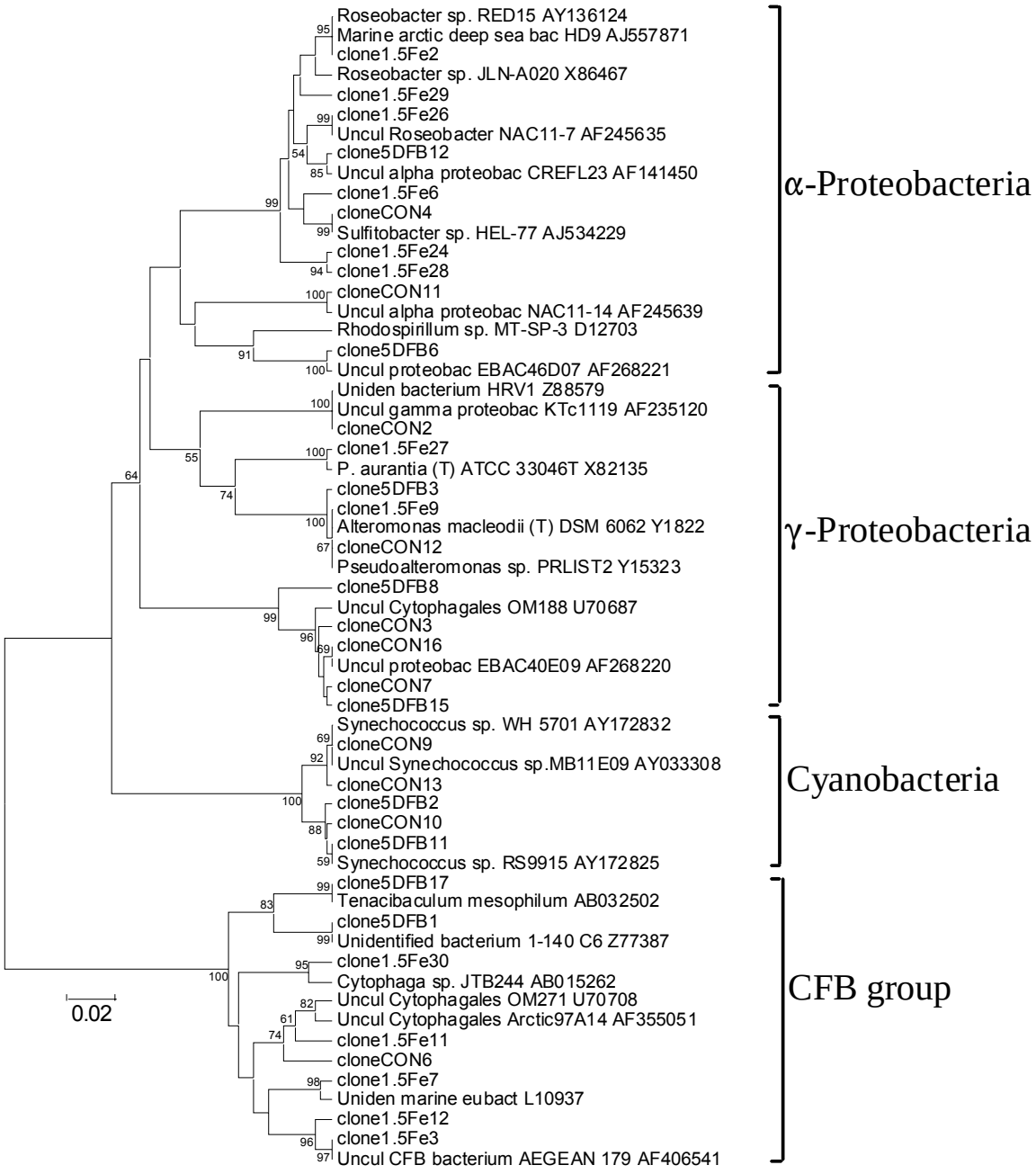

Figure 6. Phylogenetic dendogram of cloned sequences.
UPMGA dendogram of cloned sequences with published sequences added (isolate numbers are given as well as GenBank ascession numbers).

Table 1. Jaccard's coefficient of similarity between treatments for all enzymes. Similarity values were highest in each digest for comparisons between either +Fe samples or +DFB samples and typically lowest for comparisons between +Fe and +DFB samples.

	1.5 nM Fe	0.5 nM Fe	Control	1.0 nM DFB
*Rsa*I				
0.5 nM Fe	0.391			
Control	0.400	0.471		
1.0 nM DFB	0.320	0.278	0.444	
5.0 nM DFB	0.346	0.250	0.400	0.733
*Hha*I				
0.5 nM Fe	0.667			
Control	0.474	0.500		
1.0 nM DFB	0.478	0.364	0.474	
5.0 nM DFB	0.435	0.318	0.421	0.571
*Alu*I				
0.5 nM Fe	0.475			
Control	0.250	0.412		
1.0 nM DFB	0.333	0.400	0.452	
5.0 nM DFB	0.231	0.367	0.276	0.500

Table 2. Pearson's correlation coefficients for *Hha*I digested samples. Estimating the similarity between replicates within a treatment. P values for all calculations were $P < 0.0001$.

*Hha*I	1.5 nM Fe 1	1.5 nM Fe 2
1.5 nM Fe 2	0.990	
1.5 nM Fe 3	0.972	0.985
	0.5 nM Fe 1	0.5 nM Fe 2
0.5 nM Fe 2	0.992	
0.5 nM Fe 3	0.958	0.985
	Control 1	Control 2
Control 2	0.998	
Control 3	0.988	0.995
	1.0 nM DFB 1	1.0 nM DFB 2
1.0 nM DFB 2	0.997	
1.0 nM DFB 3	0.994	0.993
	5.0 nM DFB 1	5.0 nM DFB 2
5.0 nM DFB 2	0.970	
5.0 nM DFB 3	0.982	0.998

Table 3. Simpson's index of diversity and Shannon-Weiner index of diversity for each enzyme. Bonfarroni trends are indicated (P < 0.05), with italics indicating values that were not statistically different.

Source	SS	DF	F ratio	P value	Bonferroni trends
Simpson's D - *Alu*I					
Treatment	0.027	4	4.958	0.018	*1.5Fe*=0.5Fe=control=*1DFB*>5DFB
Error	0.013	10			
Shannon-Weiner - *Alu*I					
Treatment	0.211	4	15.696	0.000	1.5Fe=0.5Fe=control=1DFB>5DFB
Error	0.034	10			
Simpson's D - *Hha*I					
Treatment	0.166	4	78.179	0.000	1.5Fe=0.5Fe>1DFB>control=5DFB
Error	0.005	10			
Shannon-Weiner - *Hha*I					
Treatment	0.524	4	124.500	0.000	1.5Fe=0.5Fe>*control*=5DFB>*1DFB*
Error	0.011	10			
Simpson's D - *Rsa*I					
Treatment	0.038	4	15.307	0.000	1.5Fe=0.5Fe=control>1DFB=5DFB
Error	0.006	10			
Shannon-Weiner - *Rsa*I					
Treatment	0.155	4	29.807	0.000	1.5Fe>0.5Fe=control>1DFB=5DFB
error	0.013	10			

Table 4. Percentage of T-RFs matched with PAT.
Shown is the percentage of peaks within each digest that were identified with the Phylogenetic Analysis Tool. Default bin sizes were used in an effort to narrow the choices for peak identity.

Sample ID	*Alu*I % peaks matched	*Hha*I % peaks matched	*Rsa*I % peaks matched
1.5 nM Fe	66.7	88.2	71.4
0.5 nM Fe	65.4	84.6	83.3
Control	54.6	72.7	71.4
1.0 nM DFB	65.2	70.6	83.3
5.0 nM DFB	73.3	68.8	78.6

Part IV

Bacterial Community Composition Across a Nearshore to Offshore Gradient of Nutrients in the Northern Peruvian Upwelling Regime

INTRODUCTION

The upwelling region of the Pacific coast of central Peru comprises a fairly wide (~125 km) and shallow (~50 m) continental shelf along with a steep shelf break that rapidly drops to a depth of 3.6 km (Wessel & Smith 1995). Water from the deep ocean is transvected upward until it reaches the shelf, whereupon it causes introduction of fresh nutrients. This deep water is rich in nutrients, such as silicate, nitrate, phosphate, and Fe (at least partially due to reduced levels of oxygen at depth) (Bruland et al. 2004), making these areas of extremely high productivity (Chavez & Toggweiler 1995). It is estimated that coastal upwellings, while accounting for only a small fraction of the surface area of the world's oceans (Ryther 1969), are responsible for a disproportionately large amount (11%) of global new production (Chavez & Toggweiler 1995).

Recent research has demonstrated that phytoplankton primary production in these regions may be limited by the biological availability of Fe. It has been shown that Fe limits primary production in upwelling regions off the coast of California (Hutchins et al. 1998, Firme et al. 2003), and more recently in the subtropical Pacific Ocean upwelling along the coast of Peru (Hutchins et al. 2002, Eldridge et al. 2004a). The California upwelling receives seasonal inputs of riverine runoff, which adds nutrients and alleviates Fe limitation sporadically (Hutchins et al. 1998). In contrast the Peruvian upwelling borders the Atacama Desert and as such receives only negligible surface runoff (Scheidegger &

Krissek 1981). Subsequently the introduction of new Fe to surface waters in this region occurs primarily through upwelling (Hutchins et al. 2002).

Bacterial community structure and diversity in the Peruvian upwelling has been shown to be influenced by Fe availability in bottle incubation experiments (Eldridge et al. 2004b), however the degree to which this is true for bacteria *in situ* remains unknown. High concentrations of macronutrients occur in areas of the upwelling close to shore and as surface waters transvects offshore a predictable decline in nutrients should be seen (Toggweiler & Carson 1994). In a recent paper, Bruland et al. (2004) measured concentrations of silicic acid, phosphate, nitrate, Fe, and chlorophyll during nearshore to offshore transects and found a decline in these nutrient concentrations with increasing distance from shore, with the exception of a section through the longitudes 79.1 – 79.4^{o} W. Areas of the transect that were near to shore had high concentrations of all nutrients, while offshore regions comprised lower concentrations, especially for Fe which in some areas was estimated at less than 100 pM (Bruland et al. 2004).

Previous studies that have examined the bacterial community across transects in high and low nutrient regions typically separate the bacterial community into only three components (heterotrophic bacteria, *Prochlorococcus* spp., and *Synechococcus* spp.) (Brown et al. 2003, Landry & Kirchman 2002, Cavender-Bares et al. 2001) whereas some only examine total heterotrophic bacterial or total eubacterial abundance changes (Zubkov et al. 2001, Uye et al. 1999). Landry and Kirchman (2002) have shown that the abundances of

heterotrophic bacteria, *Prochlorococcus* spp., and *Synechococcus* spp. all increase in HNLC regions. Brown et al. (2003) have shown that heterotrophic bacterial abundances show a distribution similar to *Prochlorococcus* spp., but did not directly equate this to nutrient content. On the other hand, Uye et al. (1999) have demonstrated that total bacterial abundance decreases in samples collected during a nearshore to offshore transect that followed the pattern of eutrophic - mesotrophic - oligotrophic conditions. While it is important to consider total bacterial abundance as one examines regimes of different nutrient concentration, it is equally important to examine nutrient concentrations with respect to changes in the presence or absence of individual groups of bacteria to discover how nutrients may influence bacterial diversity.

It has been demonstrated over the past several years that bacteria have the potential to short circuit the 'biological carbon pump' by respiring carbon (as reviewed by del Giorgio & Duarte 2002). Furthermore, Cottrell and Kirchman (2000) have found that bacteria are able to breakdown different size classes and types of dissolved organic matter (DOM), with no one group dominating the population for each carbon compound tested. They concluded that a complex network of bacteria were needed to fully breakdown DOM, which suggests that changes in bacterial diversity may occur during Fe enrichment studies due to alterations in DOM production by Fe-stimulated primary producers. Unfortunately, the mesoscale Fe fertilization events (e.g. Boyd et al. 2000) that are needed to study this phenomena *in situ* are expensive and in many cases

inconvenient to sample. However, since natural gradients of Fe exist, researchers have the unique opportunity to study bacterial community structure across a mosaic of conditions in a natural setting.

Monitoring diversity across large numbers of stations during long transects requires some measure to estimate the diversity of organisms in each sample. Determining changes in bacterial community composition by a shotgun cloning / sequencing approach would be prohibitive due to the large number of sequences required (Kemp & Aller 2004). Terminal restriction fragment length polymorphism (T-RFLP) analysis has been widely used to examine bacterial diversity (e.g. Moeseneder et al. 1999) and recently used to examine changes in bacterial diversity in this region in response to changes in the biological availability of Fe (Eldridge et al. 2004b). Here we present estimates of bacterial richness in samples collected along a nearshore to offshore transect in the subtropical southeastern Pacific Ocean. Changes in bacterial community structure were contrasted with significant changes in NO_3 and Fe concentrations associated with the distance from shore and from the continental shelf break, so that we could determine how these factors ultimately influenced the bacterial community.

MATERIALS AND METHODS

i.) Sampling, DNA extraction, PCR

Twenty separate 50 L surface seawater samples (~ 5 m depth) were collected directly from the clean water sampling system on the R/V *Melville* off the coast of Peru into a dedicated acid-cleaned polyethylene carboy during a ship transect in the southern equatorial Pacific Ocean on September 14 (Transect 11) and 15 (Transect 12), 2000. Samples were collected hourly for this study and included Transect 11 (T11): 2 pm – 5 pm, 7 pm – 10 pm, and Transect 12 (T12): 11 am – 10 pm (see Figure 1 for collection locations) as previously described by Bruland et al. (2004). Water was filtered through two 142 mm GF/F filters (Whatmann) using a pressurized manifold filtration system. The filters were immediately frozen at –20^{o}C for transport back to the lab. DNA was extracted from filters according to the protocol described in Eldridge et al. (2004b). Briefly, filters were subjected to treatment with lysozyme and SDS, incubated for 3 hours at 37^{o}C, extracted with phenol : chloroform : isoamyl alcohol (38:37:1) and DNA subsequently ethanol precipitated and resuspensed in 0.5X TE (10 mM Tris-Cl, pH 8.0, 1 mM EDTA, pH 8.0).

To examine community change across the transect, PCR was performed on each sample using the following conditions: 12 ng template DNA, 20 ng each primer (Sigma), 2.5 U Taq DNA polymerase (Promega), 1X PCR Buffer, 0.2 mM dNTP Mix, 2 mM $MgCl_2$ in EasyStart tubes (Molecular BioProducts) with a

reaction volume of 50 μL. Universal eubacterial primers 46F (GCYTAACACATGCAAGTCGA) (Kaplan et al. 2001) and a modified 519R (TTATTACCGCGGCKGCTG) (Lane 1991, W. Jeffrey, *pers. comm.)* were used. This pair, which targets the 5' end of the 16S rRNA gene including three hypervariable regions, hybridizes with *ca*. 90% of the bacterial sequences tested (Kaplan et al. 2001). For T-RFLP, a 5-carboxyfluorescein-labeled 46F primer was used. Cycling conditions have been previously reported (Eldridge et al. 2004b). DNA was purified from agarose gels (1%) using Qiaquick Gel Extraction Kit (Qiagen) according to the manufacturers protocol with the exception that DNA was resuspended in 40 μl of buffer EB (Qiagen).

ii.) TRF generation

Gel-purified PCR amplicons for each sample were restriction digested with *Mnl*I or *Dde*I restriction enzyme (New England Bioproducts) (0.25 U / μL) and 1X reaction buffer in a 30 μL reaction volume, (*Mnl*I reactions were supplemented with 1X bovine serum albumin, according to the manufacturer's protocol). Reactions were allowed to incubate for 6 hours at 37^{o}C, then two volumes of cold 95% ethanol was added to each and they were placed at –80^{o}C for 1.5 hours. The samples were centrifuged at 12,000 x g (4^{o} C) for 15 min, ethanol was removed, and the sample rewashed in cold 70% ethanol. Centrifugation was repeated and pellets were left to dry under vacuum for 1 h, prior to DNA resuspension in 10 μL of sterile, Milli-Q Biocel (Millipore) processed water.

Formamide (18 μL) (Fisher) and GENESCAN ROX-500 size standards (0.75 μL, Applied Biosystems) were added to the approximately 8 μL of DNA, which was then denatured at 95°C for 5 min and chilled on ice for 2 min. Samples were processed with an ABI-310 Genetic Analyzer using GENESCAN Analysis software, 30 min reaction time, and otherwise default electrophoresis parameters. Electropherograms, as well as tables of peak size, area, and height, were generated for each sample, using local southern size calling to assign peak sizes. See Figure 2 for sample electropherogram output.

iii.) Statistical analysis of T-RFLP data

Size values of each peak from tabular T-RF data for all samples within a digest were aligned in a method similar to the one used for replicate samples in Eldridge et al. (2004b). Size values were averaged across all samples and rounded to the nearest integer. Each transformed profile consisted of a set of integer values corresponding to peaks that appeared in the original profile. Due to the large sample size (20 samples, with two enzymatic digests for each), independent triplication within digests as in Eldridge et al. (2004b) was not possible. Instead of using area data, only size values were used because the lack of replication made it impossible to calculate confidence of the area values. In using size values, we employed a presence-absence approach to examine the species richness of the community. A binary matrix was created using transformed size values, which was then subjected to Agglomerative single

linkage Euclidean distances calculations and Jaccard's coefficient of similarity calculation, which measure how similar samples are to one another (Liu et al. 1997, Townend 2002). Statistics were process using the Community Analysis Package (CAP) version 1.2 (Pisces Conservation LTD, Lymington, UK). The calculation of Jaccard's coefficient was made as follows:

$$S_j = \frac{c}{a + b + c} \quad (1)$$

in which, for samples A and B, *a* is the number of operational taxonomic units (OTUs) in A but not in B, *b* is the number of OTUs in B but not in A, and *c* is the number of OTUs in common between A and B.

Detrended correspondence analysis (DCA) (CAP v. 1.2), which is similar to PCA but superior for binary data, was used to generate plots of samples with distances indicative of relative differences and variation explained by each axis (Hill 1979, Hill and Gauch 1980). The version of DCA used adapts the original version of DECORANA (Hill 1979) to include not only the required detrending (Hill and Gauch 1980), but also other corrections (Oksanen and Minchin 1997).

Mantel's test correlates the distance matrix of dissimilarities among bacterial communities to the distance matrix of independent variables, and was used to measure of the strength of the effect that independent variables had on community composition. Distance matrices were constructed using the Sørensen distance measures (McCune et al. 2002). Mantel's Test produces an r-value,

which is analogous to a Pearson correlation coefficient, as well as a Z statistic used for significance testing (McCune et al. 2002). To determine significance we compared the observed Z statistic to a distribution created from random Monte Carlo permutations, with 1000 randomizations. This test was done using PC-ORD for Windows (MjM Software, Gleneden Beach, Oregon, USA). Mantel's test was carried out using the matrix of species richness (for each enzyme) against four types of matrices: 1) all independent factors used in this analyses; 2) the entire regression model; 3) each factor identified by regression analyses, with Bonferroni corrected probabilities; and 4) the distance from shore as a surrogate for other factors.

iv). Regression analysis

We performed multiple regression analyses to determine if measurements of Fe and nitrate concentrations, as well as time of day in which samples were taken, distance from shore (km) or distance from the continental shelf (km) were predictors for species richness. Species richness was determined for both enzymes. Variables in the regression model were selected using Mallow's Cp. In each iteration, several diagnostic statistics were calculated to ensure that individual observations were not having unbalanced effects, independent variables were not correlated with one another, and that variables were not inflating variance. These statistics included: correlation matrices, variance inflation, Cook's D, Studentized residuals, DFFITS, and DFBETAS (Kutner et al.

1996). We found that Fe concentration and distance from shore were strongly correlated (Fig. 3). Subsequently, we removed distance from shore from this analysis, as Fe has a proposed mechanistic affect on the bacterial community. No observations were found to violate any of the diagnostic statistics.

RESULTS

i.) Agglomerative single linkage Euclidean distances

Agglomerative single linkage Euclidean distances were calculated to determine how related were samples across the transect. If nutrient concentrations influence community structure, samples with similar nutrient levels should cluster together. Dendograms indicate a clustering of samples from T12; *Dde*I digested samples show close similarity for samples T12: 1 pm – 7 pm (Figure 4), while *Mnl*I digested samples from samples T12: 2 pm – 8 pm cluster together (Figure 5), indicating that of all the samples this large set is among the most related. With the *Dde*I digestions, another small group of samples also cluster together (T12: 8 pm – 10 pm) that appear closely related to the aforementioned cluster. One sample, T11: 2 pm, which was collected from the point closest to shore, seem unrelated to all other samples regardless of the enzyme chosen.

ii.) Jaccard's coefficient of similarity

Jaccard's coefficient of similarity was calculated to independently determine which samples were the most related. Again, a cluster of high similarity values is found around T12: 1 pm – 7 pm for *Dde*I digested samples and T12: 2 pm – 8 pm for the *Mnl*I digest. The overall highest similarity scores occur for T12 late afternoon samples. Low similarity values occur in comparisons of single samples to all other samples; for example, again T11: 2 pm shows very low similarity to all other samples. Other low similarity values occur in the comparison between T11: 2 pm – 5pm and T12: 8 pm – 10 pm for the *Dde*I digest.

iii.) Detrended correspondence analysis

DCA resolved samples from the *Dde*I digest on each axis in different fashions (Figure 6). On axis 1 correspondence analysis distributed samples T11: 2 pm – 10 pm from right to left across the graph, separating them according to distance from shore, indicating OTU distributions within each sample varied with distance from shore. While axis 2 separated samples based on side of the continental slope from which they were taken; the samples form two loose groups, T11: 2 pm – 10 pm (samples closest to shore) forming group one, and T12: 1 pm – 10 pm (samples offshore, past shelf break) forming the second group. This indicates that community composition is different for nearshore and

offshore samples, and shows that for nearshore samples the difference occurs gradually.

DCA of *Mnl*I digested samples is not as clear as that with *Dde*I digested samples (Figure 7), although the same group of samples form a cluster, T12: 2 pm – 8 pm. These samples form a group in both digests with Agglomerative single linkage Euclidean distances dendograms, in Jaccard's coefficient of similarity calculations, and with both DCAs. The DCA of *Mnl*I digested samples also demonstrates nearshore and offshore groupings. One group contains the samples obtained closest to shore and on the continental shelf (T11: 2 pm – 8 pm) while the second group represents samples collected further offshore, past the continental shelf break (T12: 2 pm – 8 pm). While this indicates variation in the samples based on side of the continental slope from which they were taken, there is no ability to resolve samples based on distance from shore with *Mnl*I digestion.

iv.) Regression analysis

To determine the factors influencing community structure, we ran the multiple regression model for each enzyme, including all independent variables (excluding distance from shore –see methods). For species richness based on *Dde*I, we used Mallow's Cp as the selection criteria, and found the best model to included Fe concentration, time of day sample was taken (24 hr clock), and

nitrate concentration, with Cp = 3.002 and R^2 = 0.585 (Table 1). The resulting model was found to be:

Richness (*Dde*I) = -53.601 – 1.878(hr) + 12.514(Fe) + 6.943(N)

We ran an identical model for the second measure of species richness, *Mnl*I. Mallow's Cp identified the best model as nitrate concentration, time and distance from the continental shelf, with Cp = 3.025 and R^2 = 0.293 (Table 1). The resulting model was found to be:

Richness (*Mnl*I) = -1.843 – 2.485(hr) + 6.212(N) + 0.209(Distance to shelf)

v.) Mantel's test

The results of the Mantel's tests revealed that many of the factors measured were correlated with changes in bacterial community composition (Table 2). For both the enzymes used in these analyses, correlation models including all possible variables and the variables selected from the regression models, were highly correlated with changes in community composition (Table 2). In examining individual variables, Fe actually had the weakest relationship with community composition, while time of sampling was the strongest (Table 2).

DISCUSSION

i.) Community richness – similarity measures

Very little attention has been paid to the contribution of prokaryotic organisms to biogeochemical cycling in comparison to phytoplankton, but it is clear that they have the potential to short-circuit the 'biological carbon pump' through respiration of carbon compounds produced during primary production (del Giorgio & Duarte 2002). The total amount of prokaryotic carbon equals approximately 60-100% of that found in all plants (Whitman et al. 1998) and as such their role in pelagic carbon cycles is potentially great. As a first step to understanding the role of bacterial community diversity in the functioning of HNLC waters, we have attempted to compare changes in diversity with surface water chemistry on a nearshore to offshore gradient. Clustering of samples within our similarity measures, while not making any assertions about species abundance, is indicative of the presence of similar OTUs at each station.

Transect 12 samples (offshore stations) cluster in both Agglomerative single linkage Euclidean distances calculations and Jaccard's coefficient of similarity calculations, the only difference being that with each of the two enzymes the group that clusters is only slightly skewed. In fact, each enzyme behaved alike for both measures of similarity. Three of the lowest similarity values in Jaccard's calculations were T11: 2 pm, T12: 12 pm, T12: 10 pm, not surprisingly two of these are the first and last samples along the transects and so

are estimated to be 240 km apart. The sample T11: 2 pm is the least similar to all the other samples in the Agglomerative single linkage Euclidean distances dendograms for both digests. In our previous study (Eldridge et al. 2004b), samples that clustered together with each of these two similarity measures were determined to have similar levels of bacterial diversity with both Shannon-Weiner and Simpson's indices of diversity (which considered not only presence or absence of OTUs but also the area in the electropherogram used as a measure of abundance). Both results contrast to those of Hutchins et al. (2001) in which very little change in diversity was seen with Fe addition to bottle incubations in three HNLC regions.

ii.) Community richness – correspondence analysis

PCA has been used in a number of studies to analyze the large quantities of data generated with T-RFLP (Clement et al. 1998, Dollhopf et al. 2001, Franklin et al. 2001, Kaplan et al. 2001, Eldridge et al. 2004b), but DCA proved to be superior over PCA for our data analysis since only the present or absence of OTUs were considered. As the results given from each analysis are similar, samples analyzed with DCA should be comparable to samples analyzed with PCA. The current study demonstrated that distance from shore and side of shelf break for each sample were important. *Dde*I generated T-RFLPs also demonstrated that samples in transect 11 separated based on distance from shore. While this specific analysis does not include nutrient concentrations, it

demonstrates that the presence of this gradient may functionally influence bacterial diversity.

iii.) Community richness – regression analysis

These regression models, based on richness data from different enzyme digests, gave slightly different results. The model using *Dde*I richness as the dependent variable explained more of the variation (R^2 = 0.585) than the others. This model found shows that richness is affected by Fe and N concentrations, and the time of day when samples were taken. Figure 8 shows individual relationships between richness and these predictor variables. Here richness is positively related to Fe and N concentrations and negatively related to time of day.

The second model, using richness from the *Mnl*I enzyme, explained less than 50% of the variation in species richness, indicating that there were unmeasured sources of variation. The overall model was not significant (P = 0.170), and plots of the effects of individual predictor variables on richness contained much more scatter (not shown).

Both of these regression models highlight the fact that Fe, although important to bacterial richness, was not the strongest factor influencing richness. There is no doubt that the factors controlling bacterial richness are complex, as much unexplained variation remains. Likely factors influencing these patterns were currents, transporting biotic communities as well as abiotic resources,

localized patchiness in upwelling and resource dynamics, and species interactions. We investigated this last factor, species interactions (at least among bacteria), by exploring how changes in community composition were correlated with the abiotic factors using Mantel's tests. Other factors, including DOM supply and the synergies between Fe and N-limitation (in that Fe is required for N-assimilation enzymes, Falkowski et al. 1998) are outside the scope of the current study.

iv.) Community composition – Mantel's tests

Bacterial community composition appeared to be correlated with a number of measured variables (Table 2), highlighting the complex relationship between bacterial richness, community composition and abiotic influences. We found that Fe was not as highly correlated with community composition changes as some other factors, most notably the time of day when samples were taken. Interestingly, distance from shore proved to be a very efficacious surrogate for exploring community composition change (Table 2). Distance from shore likely encapsulated many the individual effects of the differing abiotic influences.

CONCLUSIONS

The Peruvian upwelling region, with its persistent upwelling and subsequent gradient of nutrient concentrations, provides an ideal circumstance in

which to study the effects of various nutrients on bacterial diversity. This region is one of the most productive in the world (Chavez & Toggweiler 1995), therefore any factors that affect the bacterial community could subsequently affect biogeochemical cycling and global productivity.

Many studies have shown that bacterial abundance increases with increasing Fe concentration (Price et al. 1994, Paluski et al. 1996, Hutchins et al. 1998, Cochlan 2001), while others suggest this group may be held in check due to the activity of grazers and viruses (Price et al. 1994, Poorvin et al. 2004). What we have shown is that bacterial diversity also increases with increasing Fe concentration, though other factors have an influence as well. Our experimental design, while allowing for an examination of the effects of ambient Fe on bacterioplankton as in previous bottle studies (Eldridge et al. 2004b), also incorporates other naturally variable parameters, as we wanted to decipher the controls on microbial community structure in this region. While not surprisingly it appears that multiple factors influence bacterial diversity, we do demonstrate that Fe was an important factor affecting microbial diversity (with all statistical measures), just as it was in our bottle incubation experiments (Eldridge et al. 2004b). In both cases, increased Fe led to increased diversity and decreased Fe led to decreased diversity.

Scientists disagree on whether Fe limitation of bacteria in HNLC regions of the world's oceans is a direct effect of the iron itself or an indirect effect of DOM produced by phytoplankton (Paluski et al. 1996, Kirchman et al. 2000). We

have shown that Fe affects bacterial community structure and that more than one factor is needed to explain diversity change in our samples. Concurrent with this study, Hutchins et al. (2002) found that phytoplankton in the Peruvian upwelling region are limited by Fe. They also found that phytoplankton communities within Fe-addition bottles were different from initial samples and control bottles. Since Fe and chlorophyll concentrations correlate well in this region (Bruland et al. 2004) it is easy to hypothesize that DOM production by phytoplankton also correlates with Fe concentrations, thereby providing a link between bacterial diversity (and abundance) to Fe levels.

We conclude from this work that T-RFLP is a useful tool to examine the relationship between diversity and nutrient concentrations. What remains apparent is that some technical issues (i.e., the importance of enzyme choice) require further examination. In the current study, *Dde*I digestion seems much more sensitive to relationships amongst samples with each of the statistical measures, but that this could be enhanced by surveying more enzymes (e.g. Eldridge et al. 2004b). The utility of conducting multiple single enzyme digests has been documented for the T-RFLP technique (Clement et al. 1998), as is the importance of enzyme selection (Engebretson & Moyer 2003). As such enzyme choice may have influenced the analysis of our results, as demonstrated by lower sensitivity to relationships determined with *Mnl*I. The *Dde*I restriction enzyme has been previously evaluated yielding the second highest number of peaks in the range 50 - 500 bp (the size range use in the current study) (Engebretson &

Moyer 2003), indicating that it should be effective at providing an accurate picture of diversity. Despite the lower resolution obtained with certain restriction enzymes, the overall pattern is clear: Fe affects bacterial diversity in a positive fashion in bottle incubations (Eldridge et al. 2004b) and species richness in the environment for waters of the Peruvian upwelling.

ACKNOWLEDGEMENTS

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APPENDIX (PART IV)

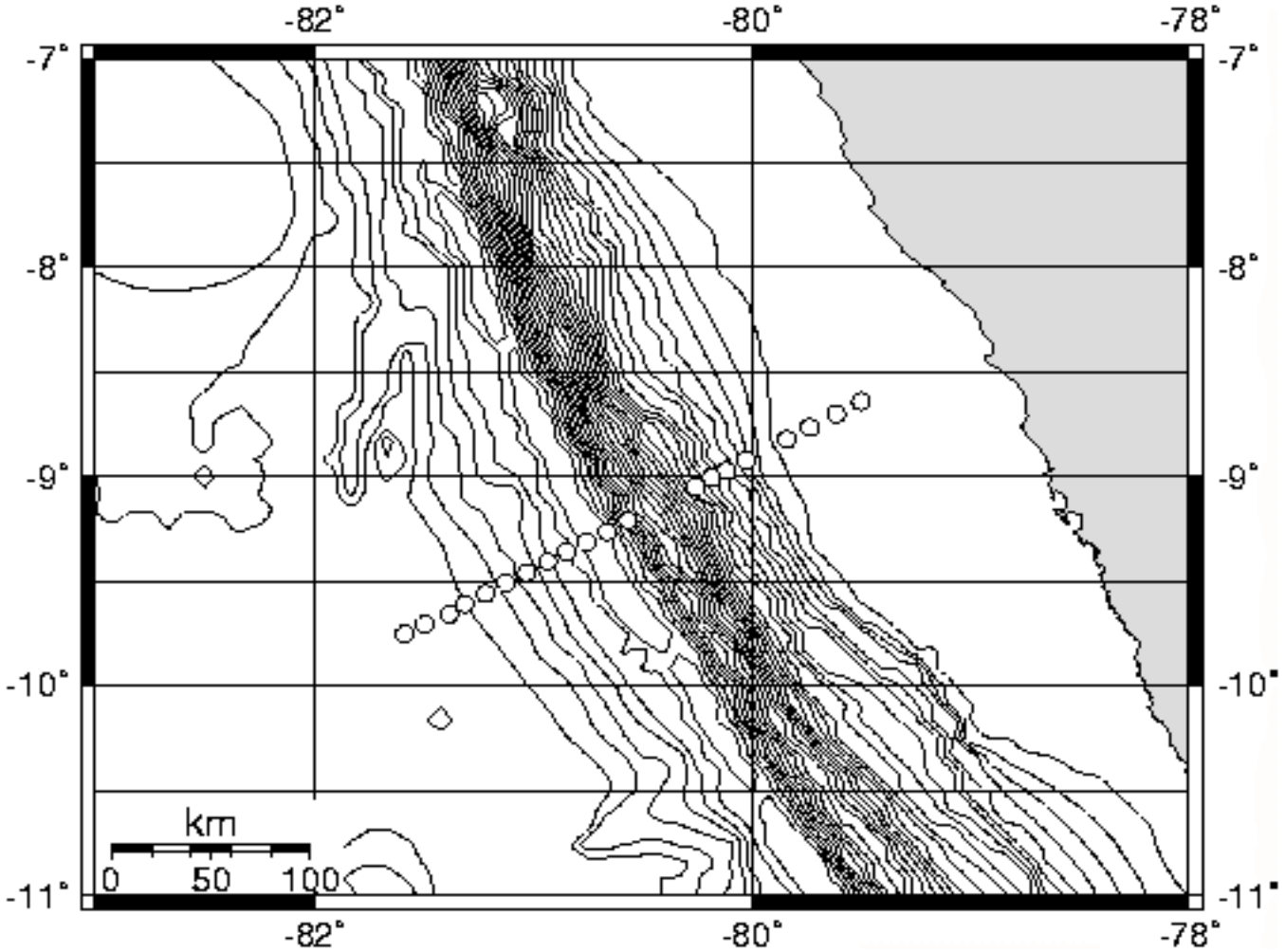

Figure 1. Map showing locations of stations (Wessel & Smith 1995).

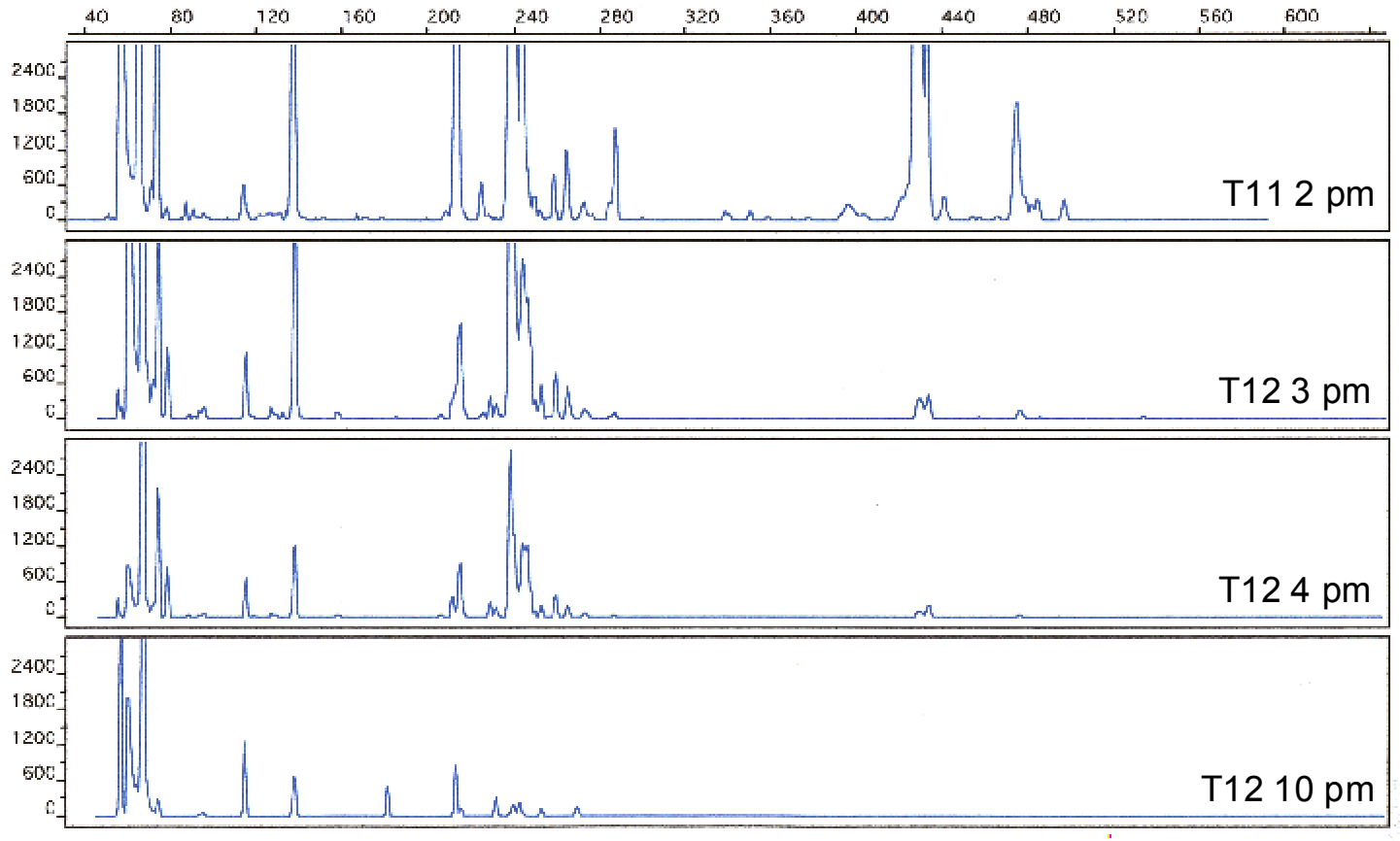

Figure 2. Sample electropherogram output.
Shown are electropherograms for DdeI digested samples T11 2 pm, T12 3 pm, T 12 4 pm, T12 10 pm. Transect samples T11 2 pm and T12 10 pm are distinguishably different (occurring at the first and last points of sampling), while T12 3 pm and T12 4 pm are similar and occur only one hour apart.

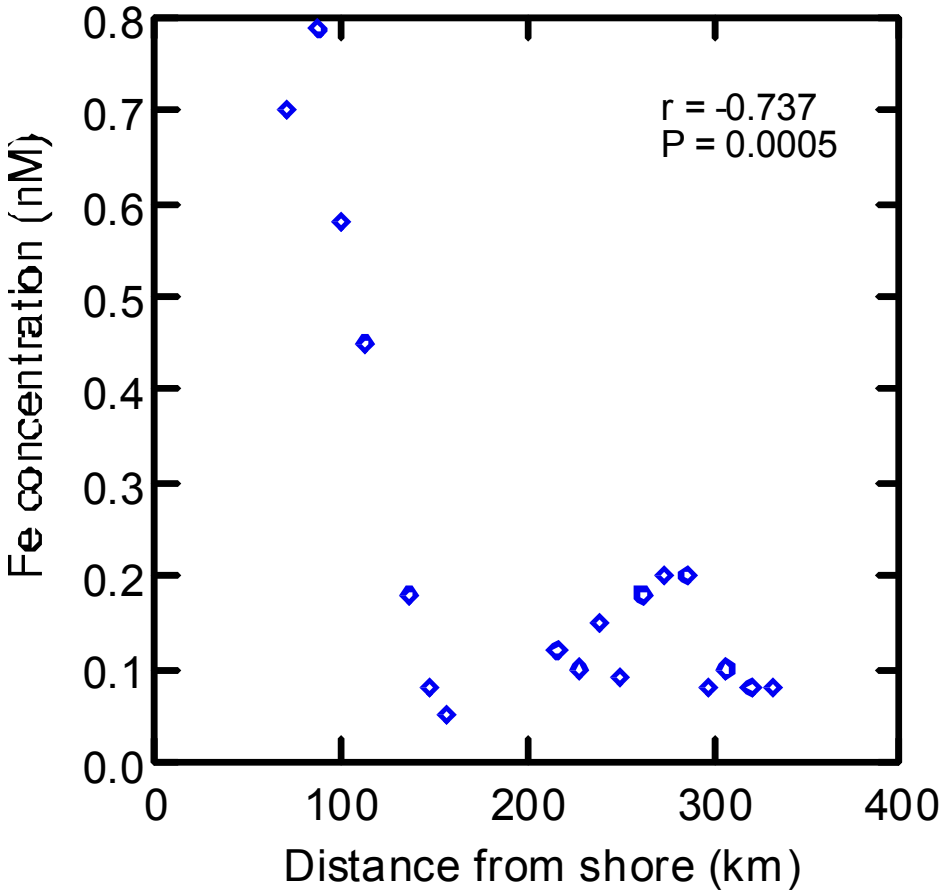

Figure 3. Correlations among iron concentrations and distance from shore. Only iron concentration was used in the regression models.

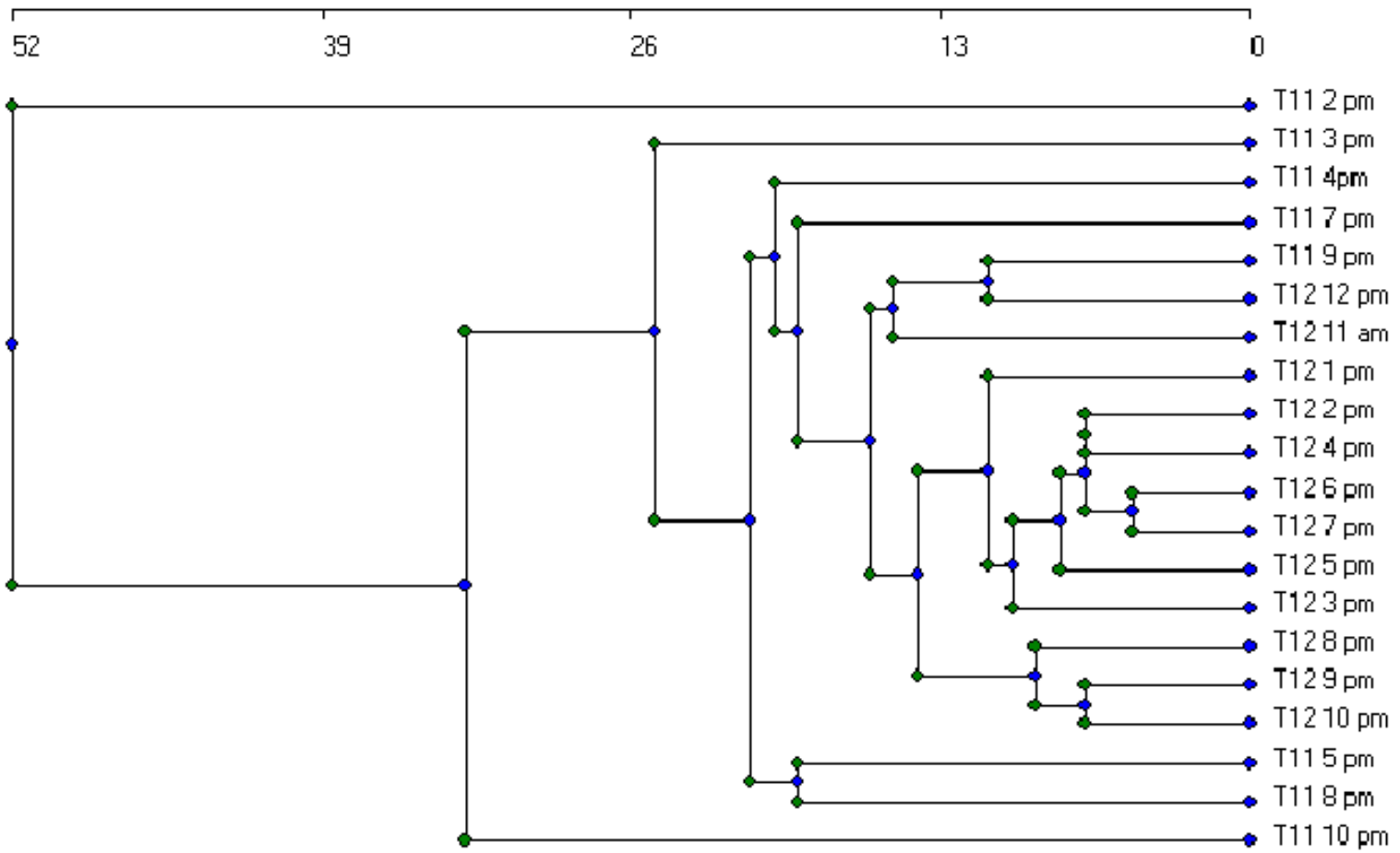

Figure 4. Agglomerative single linkage euclidean distance – *Dde*I digest.
Agglomerative single linkage Euclidean distances calculations show a distinct group of offshore samples.

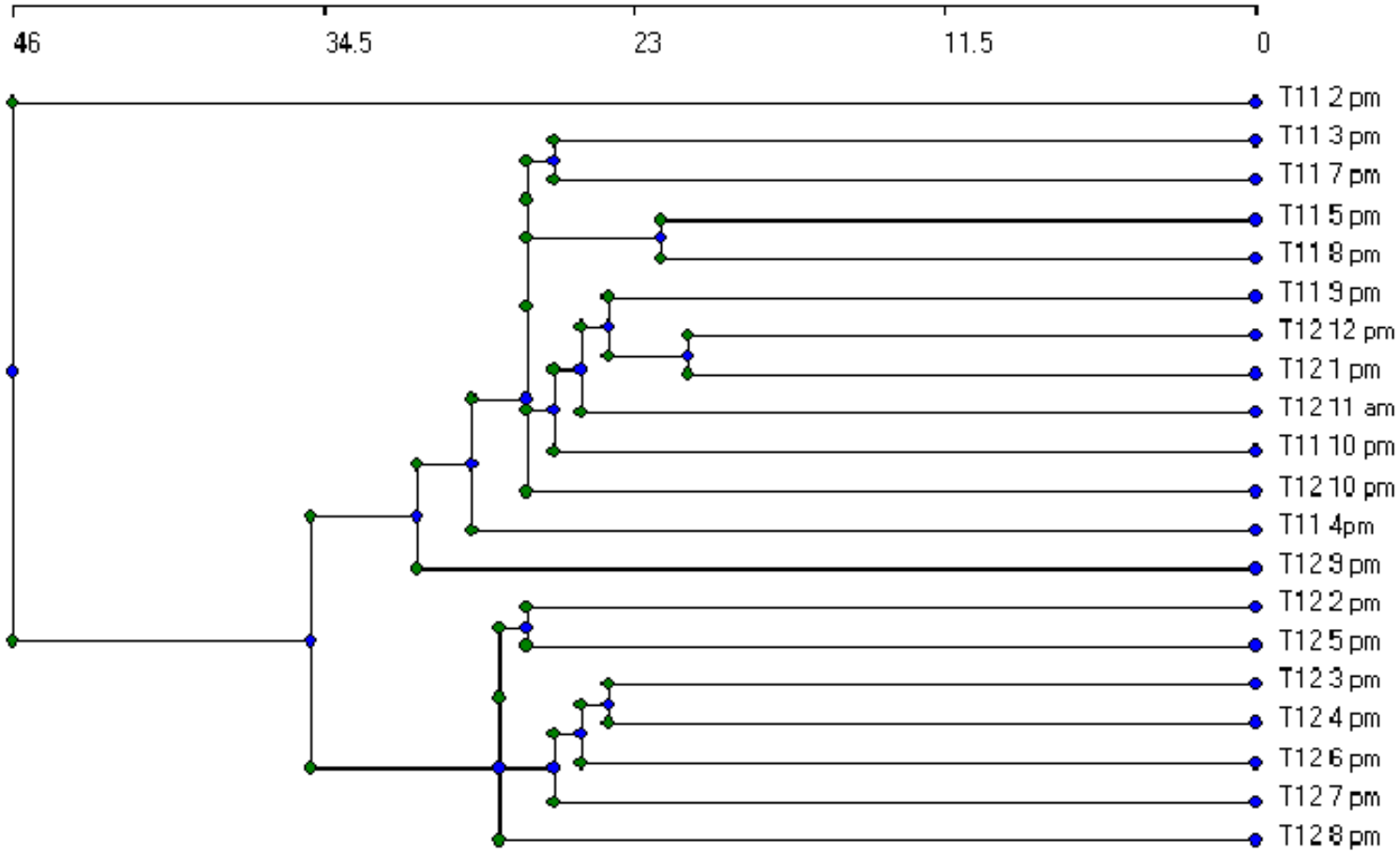

Figure 5. Agglomerative single linkage euclidean distance – *Mnl*I digest.
Agglomerative single linkage Euclidean distances calculations indicate two groupings; one of offshore samples and another of nearshore samples.

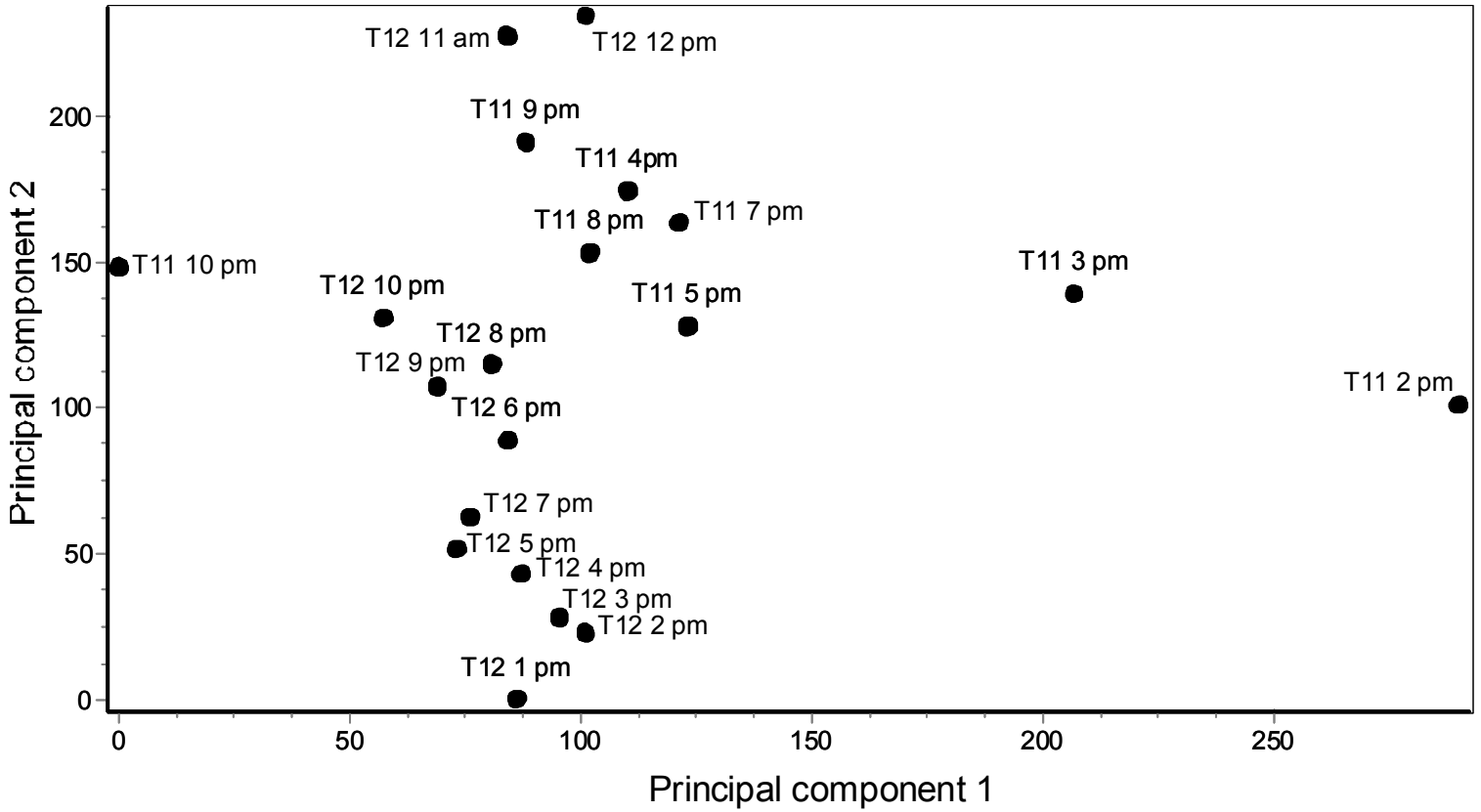

Figure 6. DCA of *Dde*I digested samples.

DCA demonstrates that samples form groups based on two factors: distance from shore and side of the continental shelf break from which the samples were taken. Nearshore samples form one group, as do offshore samples, but for nearshore samples separation is also occurring based on distance from shore. This indicates a gradual shift in community composition for nearshore samples.

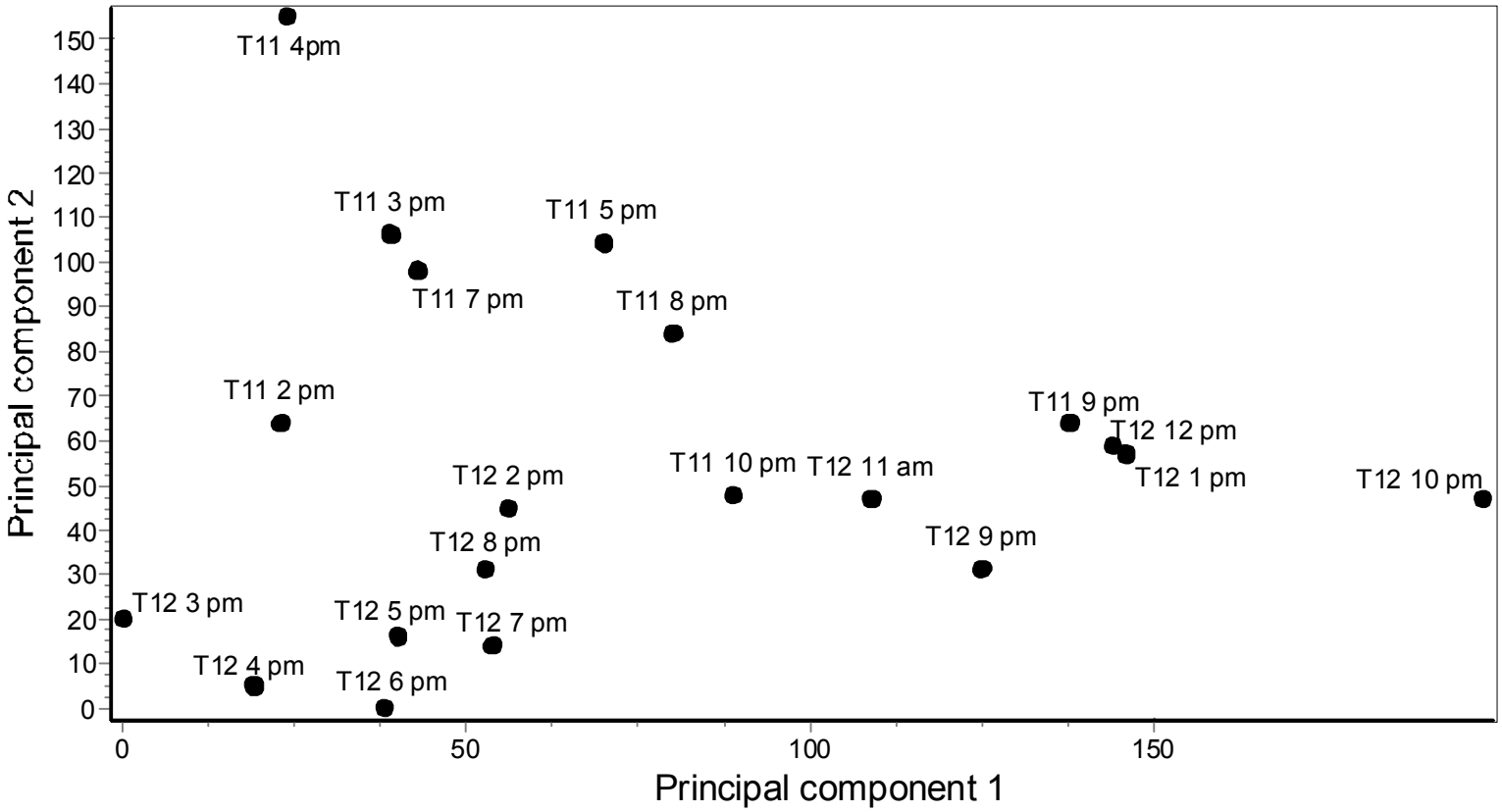

Figure 7. DCA of *Mnl*I digested samples.
DCA of *Mnl*I digested samples was not as clear as that for *Dde*I digested samples, but the consistent grouping of samples according to whether they occur nearshore or offshore is preserved.

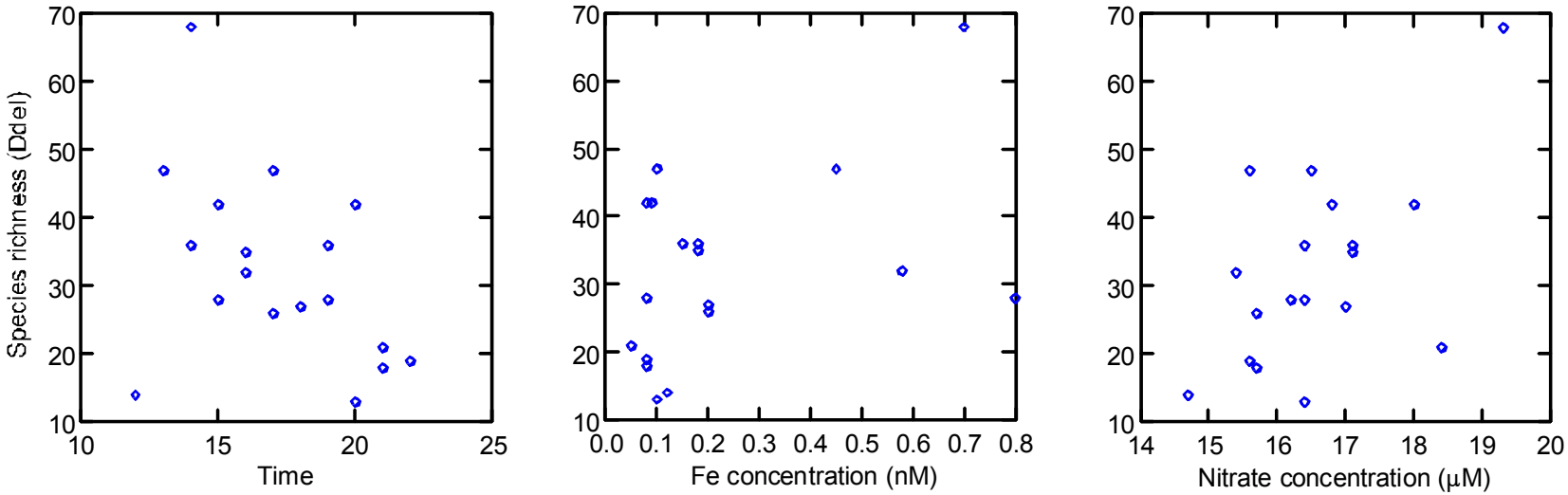

Figure 8. Scatterplots of richness versus predictor variables.
Scatterplots showing the relationships between bacterial richness and the three factors (Time of day, Fe and N concentrations) from the *Dde*l regression model.

Table 1. ANOVA table of the final regression models for both enzymes. Model variables include N (nitrate concentration) and distance from shore.

Source	DF	SS	F-value	P
Species richness *Dde*I				
Model (Time, Fe, N)	3	1901.078	6.57	0.0053
Error	14	1349.422		
Corrected total	17	3250.500		
Species richness *Mnl*I				
Model (Time, N, Dist slope)	3	1557.385	1.94	0.170
Error	14	3755.060		
Corrected total	17	5312.444		

Table 2. Results from Mantel's tests.

Results from Mantel's test, testing the correlation between species composition and independent factor dissimilarity matrices. Asterisks refer to significance levels from Bonferroni corrections for the three independent factors from the regression model: ns = non-significant * $P \leq 0.05$ and ** $P \leq 0.01$.

Enzyme	Model	Factor (s)	Correlation coefficient (r)	P
*Dde*I	All Factors	Fe, N, Time, Dist to shore and shelf	0.6598	0.001
	Regression	Fe, N and Time	0.4603	0.001
	Regression	Fe	0.3186	0.019^{ns}
		N	0.3479	0.015*
		Time	0.3297	0.005**
	Proxy	Distance to shore	0.6289	0.001
*Mnl*I	All Factors	Fe, N, Time, Dist to shore and shelf	0.4108	0.003
	Regression	Fe, N and Time	0.3285	0.007
	Regression	N	0.2234	0.072^{ns}
		Dist to slope	0.2969	0.017*
		Time	0.2627	0.013*
	Iron	Fe	0.2297	0.024
	Proxy	Distance to shore	0.3266	0.008

Part V

Conclusions

The potential role of Fe as a growth-limiting nutrient for phytoplankton in the ocean has been a topic of interest for over half a century (Hart 1941). Popularized in the 1980s, this theory has recently been proven for vast stretches of the open ocean (e.g. Boyd et al. 2000, Coale et al. 1996). More recently, phytoplankton in upwelling regions off the coasts of California and Peru have also been demonstrated to be Fe-limited (Hutchins et al. 1998, Firme et al. 2003, Hutchins et al. 2002, Eldridge et al. 2004a). The research in this dissertation has shown, using flow cytometry, photosynthetic efficiency estimations, and Chl *a* concentrations along a micronutrient gradient, that phytoplankton at four sites of the Peruvian upwelling were influenced by Fe concentration. Moreover, distinct changes in phytoplankton community structure were shown to occur with varying Fe concentration. This has allowed me to reject the null hypothesis that Fe concentration has no effect on phytoplankton community structure and accept that Fe does affect the community structure of phytoplankton.

Altered phytoplankton diversity has important implications for the bacterial community as different types of phytoplankton have been shown to produce dissolved organic matter which differs in both bulk total and composition (Biersmith & Benner 1998, Biddanda & Benner 1997). Since the supply of DOM (and indirectly Fe concentration) may influence bacterial community structure, the bacterial diversity was also studied. Bacteria in the upper water column are able to utilize the products of photosynthesis for energy and by performing respiration; carbon (as CO_2) is shunted from the ocean back to the atmosphere,

decreasing the effectiveness of the ‘biological carbon pump’. Moreover, since it has been suggested that a more diverse assemblage of bacteria are better able to breakdown the products of photosynthesis (Cottrell & Kirchman 2000), bacterial diversity changes have a great potential to influence global productivity.

It was determined that bacterial diversity in bottle incubations increases with Fe concentration, though whether this was a direct or an indirect effect of the added Fe remains an unknown. In addition, it has been determined that in a natural gradient of nutrient concentrations, bacterial diversity is influenced by Fe concentration, however as with any study in an unperturbed system, many factors are involving in shaping the structure of these communities. In our model it was determined that other factors besides Fe also have an effect, though whether this undetermined factor is dissolved organic matter or synergies between nutrients (e.g., the dependence of nitrogen transporters on Fe) remains to be seen.

Although Fe fertilization continues to receive attention as a viable method to decrease the concentration of CO_2 in the atmosphere (Song 2003), more research is required to determine the complex and long-term effects on the microbial community. Most studies in which seawater is amended with Fe focus on phytoplankton because of their obvious role in carbon sequestration, but relatively little attention is paid to the response of bacteria in these experiments. This data has shown that bacterial diversity increases with added Fe, which could decrease the effectiveness of the ‘biological carbon pump’ and negate the

effects of Fe fertilization. As well, since marine upwellings are extremely productive in terms of both primary productivity (11% of total global production) (Chavez & Toggweiler 1995) and fish catch per year (e.g. 18 - 20% of the annual global fish harvest occurs in the Peruvian upwelling region, Sherman & Tang 1999), any possible sources of decreased productivity in the ocean are important to consider for global resource management.

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Vita

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