

# The response of bacterial groups to changes in available iron in the Eastern subtropical Pacific Ocean

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Received 26 September 2006; received in revised form 8 February 2007; accepted 14 February 2007

## Abstract

While it has been shown that phytoplankton productivity and community structure are influenced by the availability of Fe in several high nutrient–low chlorophyll (HNLC) regions of the world's oceans, the influence of Fe on the bacterial community remains unresolved. Therefore, we sampled water from the Peruvian upwelling region of the equatorial Pacific Ocean and examined how bacterial community structure changes with Fe additions (1.5 nM, 0.5 nM above ambient) and sequestration, which was accomplished by additions of the fungal siderophore desferrioxamine B (DFB) (1.0 nM, 5.0 nM). We hypothesized that either 1) the bacterial communities are generally Fe-limited and thus show positive responses to Fe addition; or 2) that bacteria form the equivalent of response groups and show a limited number of responses to Fe addition; or else 3) that the bacterial communities show no response to Fe addition. Using Terminal Restriction Fragment Length Polymorphism analysis, we found that the eubacterial community changed in response to Fe. Whereas the overall community shows little abundance and richness responses to Fe availability, bacteria can be arranged into response groups showing divergent responses to Fe addition. With validated cluster analysis, we found that the bacterial community consisted of four response groups. One group showed strong positive responses to increasing Fe availability, while another group showed strong negative responses. The abundance patterns of the final two groups showed no response to alterations in Fe availability, although one persisted at a high abundances and the other a low abundance. These results reveal that it may be difficult to describe a singular bacterial community response to changes in Fe availability, and that understanding the influence of Fe on bacteria dynamics may require an understanding of the different responses of individual sub-groups of bacteria within the microbial community.

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**Keywords:** Bacteria; Diversity; 'Equatorial Pacific'; HNLC; Iron; T-RFLP

## 1. Introduction

Natural systems contain a multitude of coexisting species, yet these species likely exhibit a finite set of

responses to environmental perturbations. Thus, similarly behaving species can be combined into discrete response groups (e.g., Naeem and Li, 1997; Tilman et al., 1997). Understanding community responses to perturbations necessitates knowledge on the nature and diversity of the response groups present. For example, a community with a diverse array of response groups is more likely to maintain function in the presence of an environmental perturbation compared to less diverse communities

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(Tilman et al., 1997). This is because under one environmental regime a limited number of species or response groups likely dominate a community, but under a second regime, a different group of species comes to dominate. Further, knowing how species are distributed among response groups gives biologists the ability to predict how community function will be affected by perturbations (Yachi and Loreau, 1999). To-date ecologists have a limited understanding of bacterial community function and much less concerning the potential response groups that bacterial species belong to. Only recently have biologists begun to explore the nature and importance of response groups for aquatic microorganisms (Weithoff, 2003; Wohl et al., 2004). One area where this may be especially relevant is during Fe enrichment in marine surface waters where Fe availability constrains primary production.

Fe enrichment is an ecologically important issue, because in high nutrient–low chlorophyll (HNLC) regions of the ocean, scientists have convincingly demonstrated that Fe availability can limit phytoplankton productivity (Martin et al., 1994; Coale et al., 1996; Behrenfeld and Kolber, 1999; Boyd et al., 2000), reinforcing the idea that Fe plays a key role in ocean biogeochemical systems. Whereas the role phytoplankton play in carbon sequestration and export has become increasingly clearer, the role of bacteria on these dynamics is not well understood. Bacteria are a critical part of the biogeochemical cycling of Fe and the ‘biological carbon pump’ (see del Giorgio and Duarte, 2002). Only a small part of the dissolved organic carbon (DOC) produced by primary production in the euphotic zone is exported to the deep ocean, with the majority of carbon being consumed during bacterial respiration (Ducklow, 1995). In addition, some bacteria 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 and Trick, 1994; Hutchins, 1995; Butler, 1998).

Although the addition of Fe has been shown to stimulate bacterial growth in the open ocean (Pakulski et al., 1996; Cochlan, 2001; Hutchins et al., 2001), it is not clear whether the stimulation is a direct effect of Fe itself or an indirect effect through the stimulation of primary production and subsequent increase in released DOC. While several studies reveal increases in bacterial abundance with Fe addition (Pakulski et al., 1996; Cochlan, 2001; Hutchins et al., 2001), 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. Generally, studies focus on bacterial abundance,

as opposed to changes in bacterial community structure. Hutchins et al. (2001) found that the addition of Fe resulted in increases in bacterial abundance and production, but showed only minor changes to bacterial community composition despite dramatic changes in the phytoplankton community.

In the current study we experimentally manipulated Fe availability in water samples from HNLC waters of the Peruvian upwelling region to see if bacterial community composition, abundance and richness are affected by Fe. Specifically we wanted to determine whether the bacterial community responded uniformly, or if different response groups dominated under differing Fe concentrations. We also hypothesized that bacterial populations are Fe-limited (whether directly or indirectly), meaning that Fe additions would increase bacterial abundance; and that Fe reduction (*via* chemical sequestration) would decrease species richness through reduced population size and increased local extinctions. We focused on the response of the bacterial community in HNLC waters of the Peruvian upwelling region to Fe additions and reduction. 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., 2004) as well as others (*e.g.*, McKay et al., 2005; Mioni et al., 2005).

One method of whole community molecular analysis that has gained wide acceptance is Terminal Restriction Fragment Length Polymorphism (T-RFLP) analysis (Liu et al., 1997; Lukow et al., 2000; Marsh, 2005). T-RFLP has the advantage of being relatively inexpensive compared to clone library construction, in which a large number of clones must be analyzed to be confident that an accurate picture of the community has been obtained (Kemp and Aller, 2004) and it 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 (van der Maarel et al., 1998; Moeseneder et al., 1999; Denaro et al., 2005). It has also been successfully used during enrichment culture experiments (Chin et al., 1999; Hutchins et al., 2001). We have used this method, in combination with established mesocosm approaches (Eldridge et al., 2004) to provide insight into the above problems.

## 2. Materials and methods

### 2.1. Sampling and iron amendments

Water samples were collected at four locations off the coast of Peru (Eldridge et al., 2004) for experiments to examine influences of Fe on bacterial abundance. To

determine impacts on eubacterial community structure, at one of these sites, a 50 L surface seawater sample was collected (latitude 86.30° W, longitude 3.10° S, September 2000) 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 utilizing established trace metal-clean techniques (Bruland et al., 2001). Seawater was directly dispensed into replicate acid-cleaned polycarbonate bottles (2.4 L) for all experiments.

Fe and DFB stocks were made according to the protocol of Eldridge et al. (2004). Amendments were made to generate a range of Fe concentrations from

5.0 nM Fe below ambient concentrations (with DFB stocks) to 1.5 nM Fe above ambient concentrations (with FeCl<sub>3</sub> stocks) in the community structure experiment. In the experiments to examine bacterial abundance changes, concentrations were varied based on the real time results of previous experiments. Control mesocosms received no additions. All treatments were run in duplicate for the community structure experiment and triplicate for the subsequent abundance effects experiments.

Mesocosms were then incubated in a flowing seawater, on-deck shipboard incubator made of Plexiglas™ that attenuates natural sunlight to mimic spectral conditions at a 50% spectral irradiance (Laws et al.,

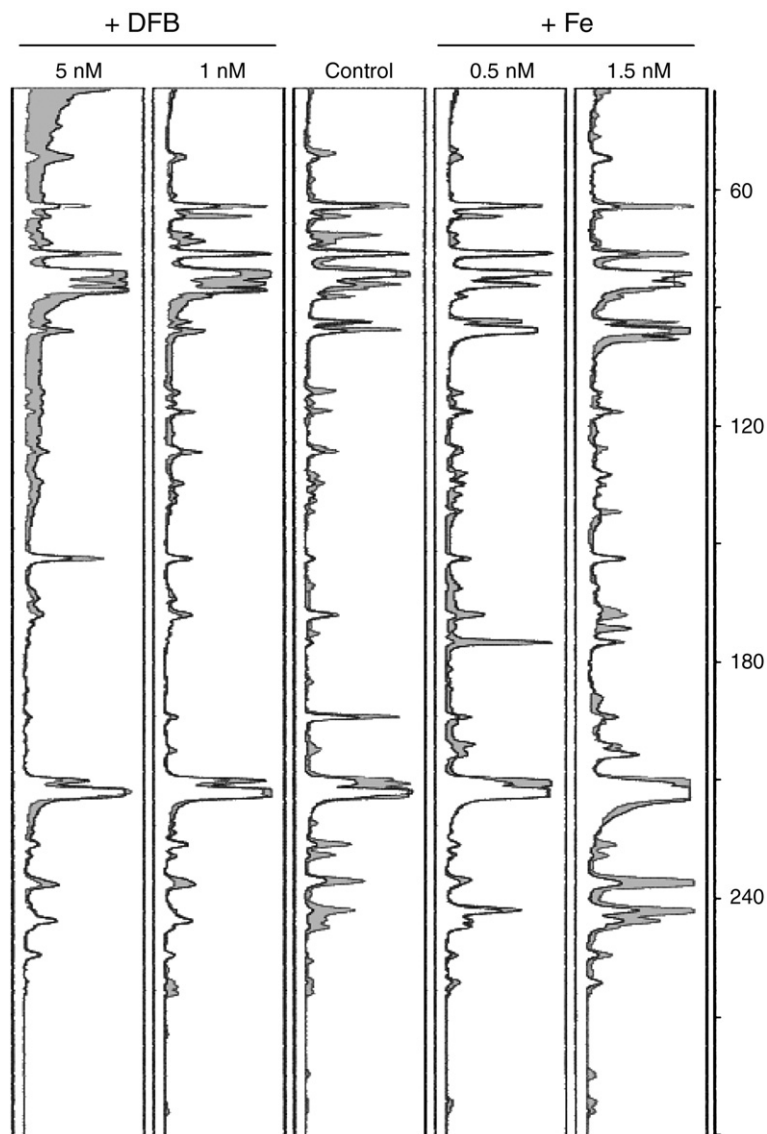


Fig. 1. T-RFLP profiles of samples from independent, duplicate mesocosms. The results demonstrate a high degree of reproducibility between independent samples given the same treatments.

1990; Wells, 1999). Incubations continued for 72 h, were subsampled (5 mL) for bacterial abundances estimates, then the remaining water was filtered through 25-mm diameter, 0.2- $\mu\text{m}$  nominal pore-size polycarbonate filters (Millipore), which were frozen for transport. DNA was extracted according to a standard phenol–chloroform extraction procedure (e.g., Sambrook and Russell, 2001). Bacteria for abundance measurements were enumerated in acridine orange-treated water samples (2 mL) collected on 25-mm diameter, 0.2- $\mu\text{m}$  nominal pore-size polycarbonate filters (Millipore GTBP) (Hobbie et al., 1977).

## 2.2. Community structure analysis

For community structure analysis, PCR was performed on DNA extracted from each bottle, using the universal eubacterial primers 46F (6FAM-GCYTAA-CACATGCAAGTCGA) (Kaplan et al., 2001) and 519R (TTATTACCGCGGCKGCTG) (Lane, 1991, W. Jeffrey, pers. comm.). 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  $\sim 90\%$  of 1500 bacterial sequences tested (90% for 46F and 99% for 536R). Reactions (50  $\mu\text{L}$ ) were prepared in EasyStart tubes (Molecular BioProducts) according to the manufacturer's instructions and contained 25 ng template DNA, 40 ng each primer (Sigma), 2.5 units of Taq DNA polymerase (Promega), 0.2 mM dNTPs, 2 mM  $\text{MgCl}_2$ . Cycling conditions consisted of an initial denaturation step at 94  $^\circ\text{C}$  for 5 min, touchdown cycling for 20 cycles by 94  $^\circ\text{C}$  for 30 ns, 65  $^\circ\text{C}$  for 30 s (minus 1  $^\circ\text{C}$  per cycle), 72  $^\circ\text{C}$  for 30 s, then cycling for 15 cycles by 94  $^\circ\text{C}$  for 30 s, 55  $^\circ\text{C}$  for 30 s, 72  $^\circ\text{C}$  for 30 s, and a final extension for 7 min at 72  $^\circ\text{C}$ .

Restriction endonuclease digests were performed on gel-purified samples (using a Qiagen Qiaquick Gel Extraction Kit) by digestion with *DdeI* (0.25 U /  $\mu\text{L}$  final concentration, New England Bioproducts) overnight at 37  $^\circ\text{C}$ . Digested DNA was purified using a MinElute Reaction Clean-Up Kit (Qiagen). Formamide (18  $\mu\text{L}$ ) (Fisher) and GENESCAN ROX-500 size standards (0.75  $\mu\text{L}$ , Applied Biosystems) were added to the DNA purification mixture, which was then resolved in an ABI-310 Genetic Analyzer using GENESCAN 3.1 analysis software. Electropherograms, as well as tables of peak size, area, and height, were generated for each bottle using local southern size calling (GENESCAN 3.1) (Fig. 1).

Peak size and area data for each sample were transformed for statistical analysis, in an effort to minimize noise generated during the processing of T-RFs, using a method similar to Dunbar et al. (2001), with the exception that no peaks were removed. We examined richness and abundance patterns of the bacterial community as a whole, by correlating abundance and richness with Fe concentrations. However, these types of analyses do not allow us to understand how different species actually respond to changes in Fe availability. Unfortunately, the genetic methods employed do not yet allow us to confidently identify individual species or their ecologies (Kaplan and Kitts, 2003). Therefore we classed operational taxonomic units (OTUs) according to their presence across treatments, and we examined if different groups of species had diverse ecological responses to Fe availability. The use of ordination techniques, likely represents the best approach to classifying microorganisms into response groups (Weithoff, 2003). We therefore used principle components analysis to create orthogonal variables

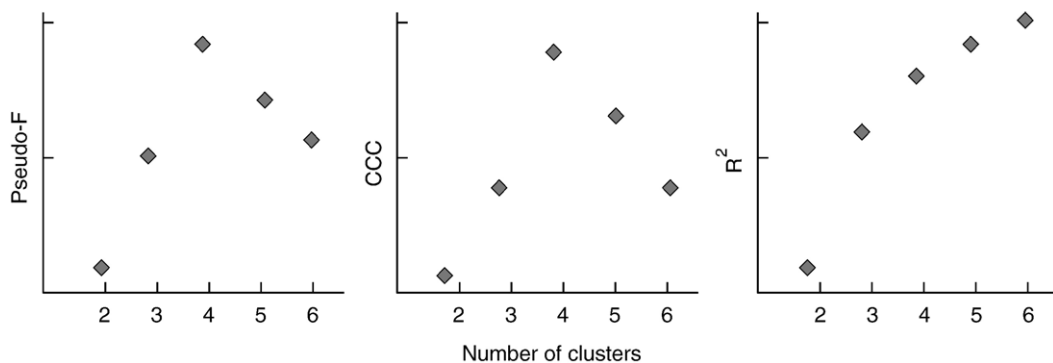


Fig. 2. Diagnostics for determining the number of clusters. With the first two measures, pseudo- $F$  and the cubic clustering criterion (CCC), peak values indicate optimal number of clusters. A plateau in the  $R^2$  value also indicates optimal number of clusters. These measures indicate four clusters as the optimal.

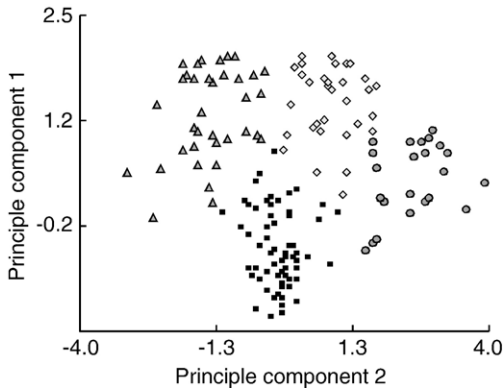


Fig. 3. Bacterial OTU clusters in principle component space. The symbols simply refer to OTU membership in one of four response clusters. Axes 1 and 2 were the only ones with Eigen values  $>0.70$ .

representing presence–absence patterns. We selected the two components that had Eigen values greater than 0.7. Component scores were multiplied by these Eigen values in order to weight according to strength of variance explanation. These scores were used in the SAS fastclus procedure (SAS 9.1, SAS Institute Inc., Cary, NC, USA) to determine if there were distinct

bacterial groups. Three statistics were used for determining the correct number of groups: pseudo- $F$ , CCC, and  $R^2$ . The number of groups are determined by the peak value of the first two measures and confirmed by a plateau in the third (Johnson, 1998). As determined by all three methods, Fig. 2 reveals that four is the optimal number of groups in the data. We cross-validated these groups using discriminant analysis with jackknife errors. This validation revealed that these groups correctly classified  $>95\%$  of OTUs. The clusters are shown in Fig. 3. The abundance and richness of these groups were then correlated with Fe concentration.

### 2.3. 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) in an effort to confirm that amplification products were typical of marine systems. Minipreps (20–30 colonies per sample) were performed with a Wizard Miniprep DNA Purification kit (Promega). Sequencing was completed at the University of Tennessee's Molecular

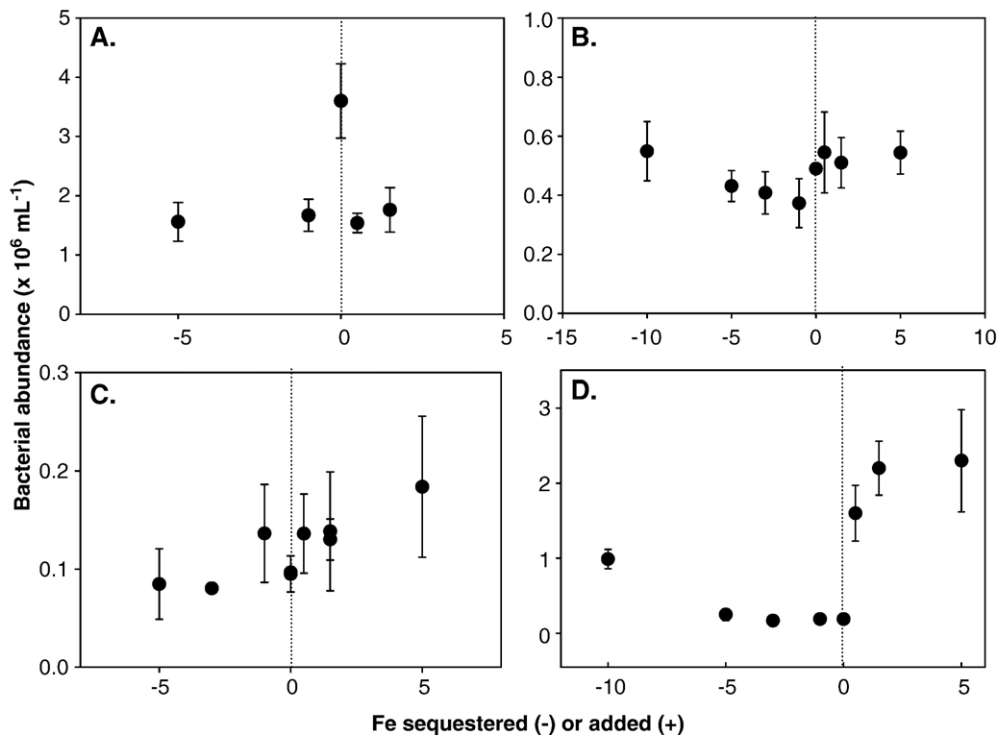


Fig. 4. Bacterial abundance (at 72 h) in Fe-amended/Fe-sequestered (+DFB represented as — Fe) mesocosms. Results are presented for communities at stations Bio-4 (A), Bio-5 (B), Bio-6 (C) and Bio-7 (D). Data are the means  $\pm$  error of independent duplicate (Bio-4,  $\pm$  range) or triplicate (Bio-5, Bio-6, Bio-7,  $\pm$  SD) incubations and are presented in respect to the ambient iron concentration (dashed line, where relative Fe = 0 and negative Fe was achieved through the addition of an equimolar amount of DFB). The response of the phytoplankton community from these experiments has been previously reported (Eldridge et al., 2004).

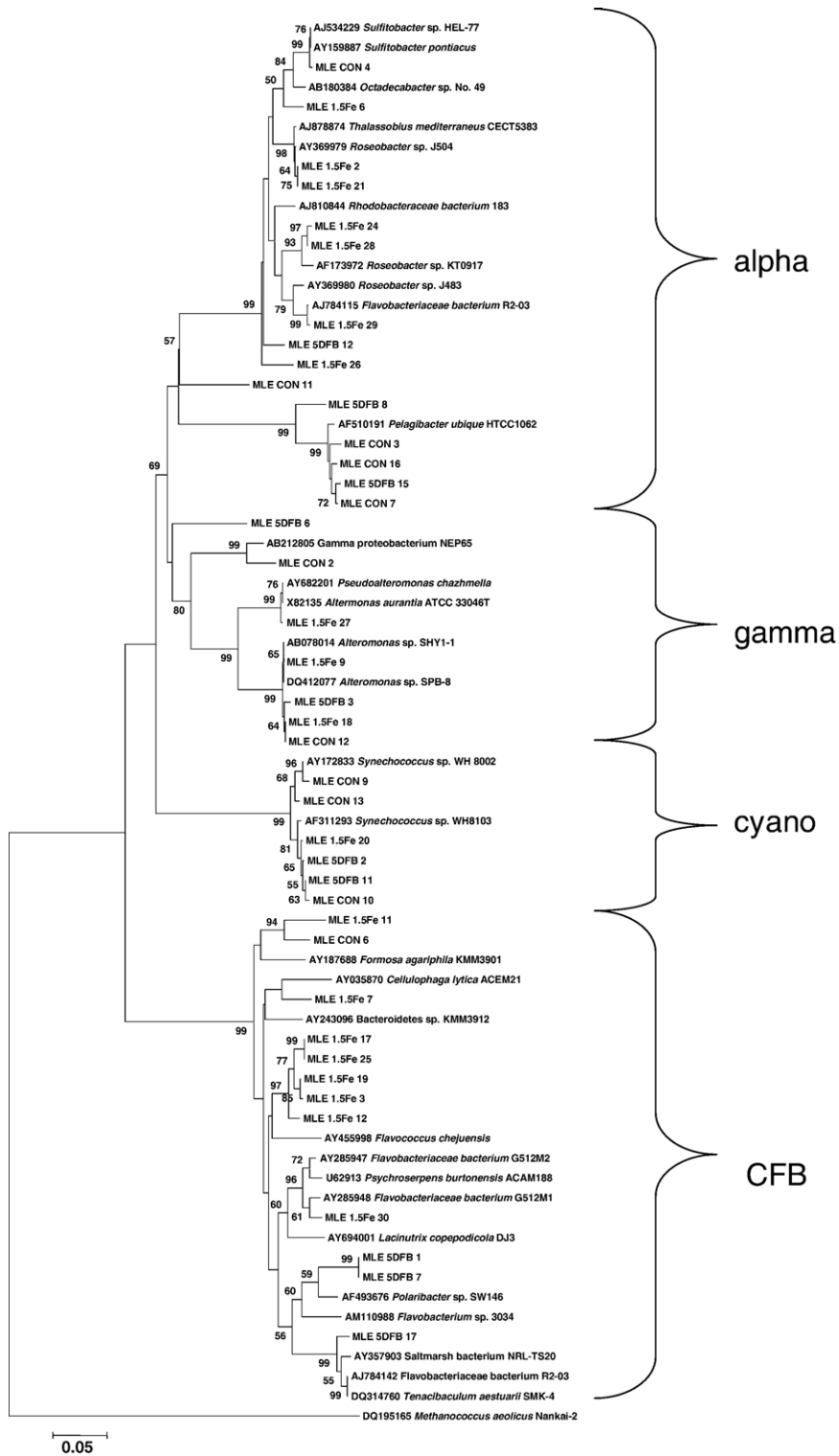


Fig. 5. Phylogenetic analysis (Neighbour-joining dendrogram) of cloned sequences from this study and related published sequences (given with GenBank accession numbers). Sequences from this study are denoted with MLE and the treatment (+1.5 nm Fe, control — CON or +5 nM DFB) they arose from. Class-wide taxonomic divisions are indicated. Bootstrap values (>50) are given as a percentage of 5000 iterations.

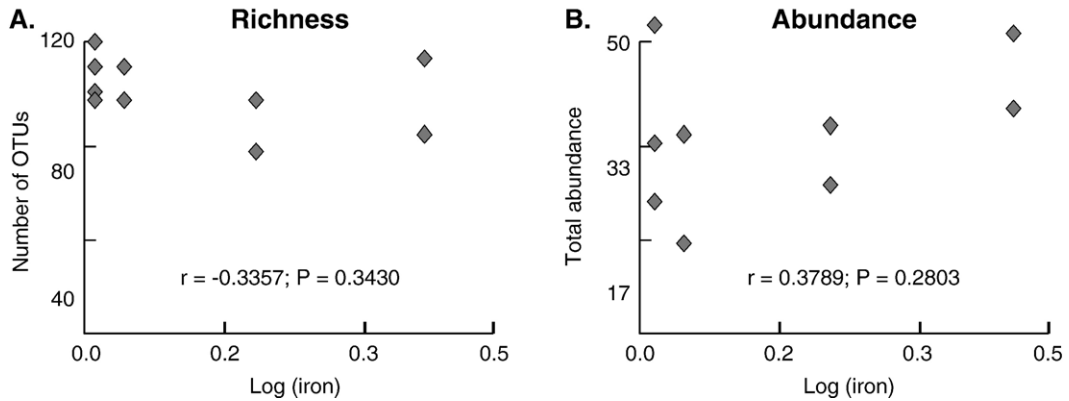


Fig. 6. The relationship between overall OTU A) richness and B) abundance ( $\times 10^4$ ). Neither measure is significantly correlated with Fe concentration. *P* values represent Bonferroni-corrected tests that the Pearson product-moment correlation coefficient is different than 0.

Biology Resource Facility. Sequences were aligned in BioEdit (Hall, 1999) using the ClustalW alignment program (Thompson et al., 1994) and manually checked. Phylogeny was examined with a Neighbor-

joining dendrogram using Molecular Evolutionary Genetics Analysis package (MEGA v. 3.1, Kumar et al., 2004), with 5000 bootstrap permutations. Sequences determined during this study have been

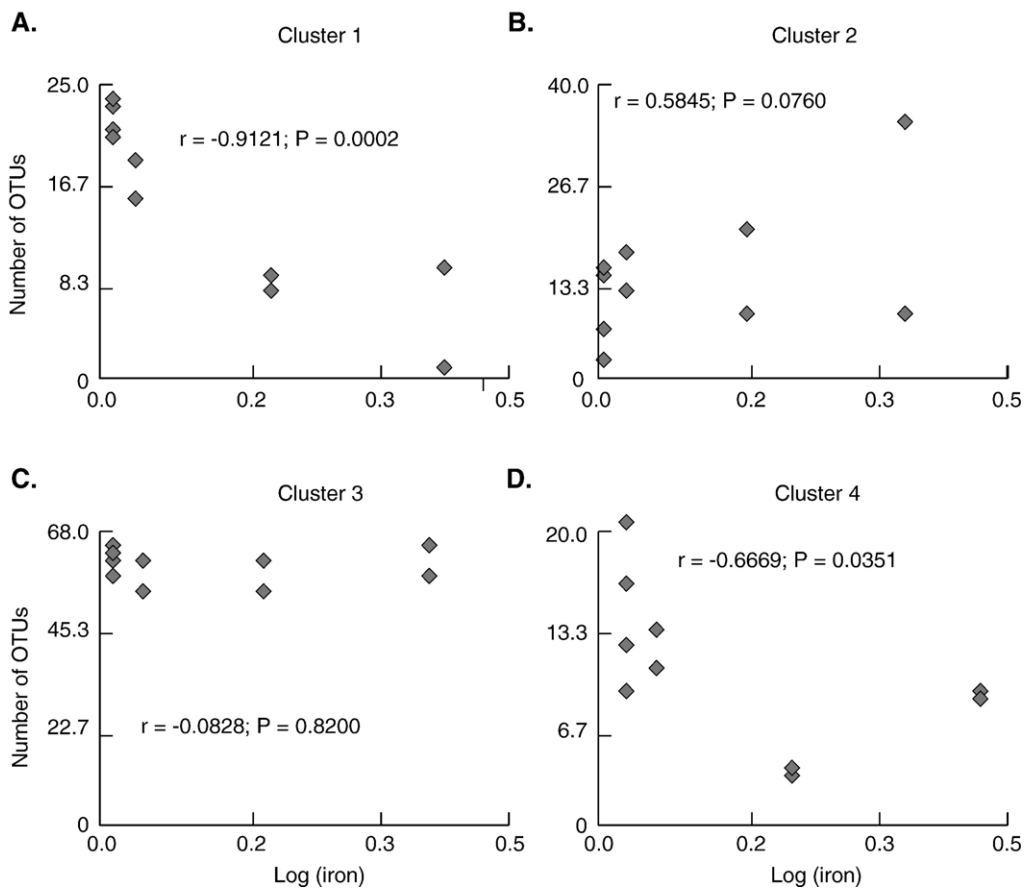


Fig. 7. OTU richness correlated with Fe concentration within the four clusters shown in Fig. 2. Individual clusters of OTUs reveal differing responses to Fe addition. *P* values represent Bonferroni-corrected tests that the Pearson product-moment correlation coefficient is different than 0.

placed in GenBank, and assigned accession numbers DQ975305–DQ975344.

### 3. Results

#### 3.1. Bacterial abundance analysis

Bacterial abundance was determined in four separate Fe-amendment and sequestration experiments during this study. At the first station, bacterial abundance appeared repressed by both the addition and sequestration of Fe in replicated experiments (Fig. 4A). In the second experiment there was no effect of either added Fe or DFB in the mesocosms (Fig. 4B). In the final two experiments (Fig. 4C and D), additions of Fe to the mesocosms resulted in an increase in bacterial abundance at the end of the experiments. Additions of DFB had no effect on the abundance of bacteria, suggesting that Fe concentrations were already sufficiently low so as to constrain the population (*vis a vis* Eldridge et al., 2004).

#### 3.2. Sequence analysis

Clones from three mesocosms (1.5 nM Fe, control, 5.0 nM DFB) were sequenced to determine if the 16S gene sequences obtained were typical of marine systems and if they represented a large diversity of bacteria. Sequences were shown to closely align or exactly match isolates from marine systems (Fig. 5), as was expected. Clones were obtained from two different types of Proteobacteria (alpha and gamma), Cyanobacteria, and CFB group bacteria, covering a diverse range of phylogenies.

#### 3.3. Community structure analysis

Overall, Spearman's correlation coefficients revealed that neither richness nor abundance showed a significant relationship with Fe treatment (Fig. 6) (confirmed with bacterial abundance counts, Fig. 4), which would lead investigators to the conclusion that bacteria do not

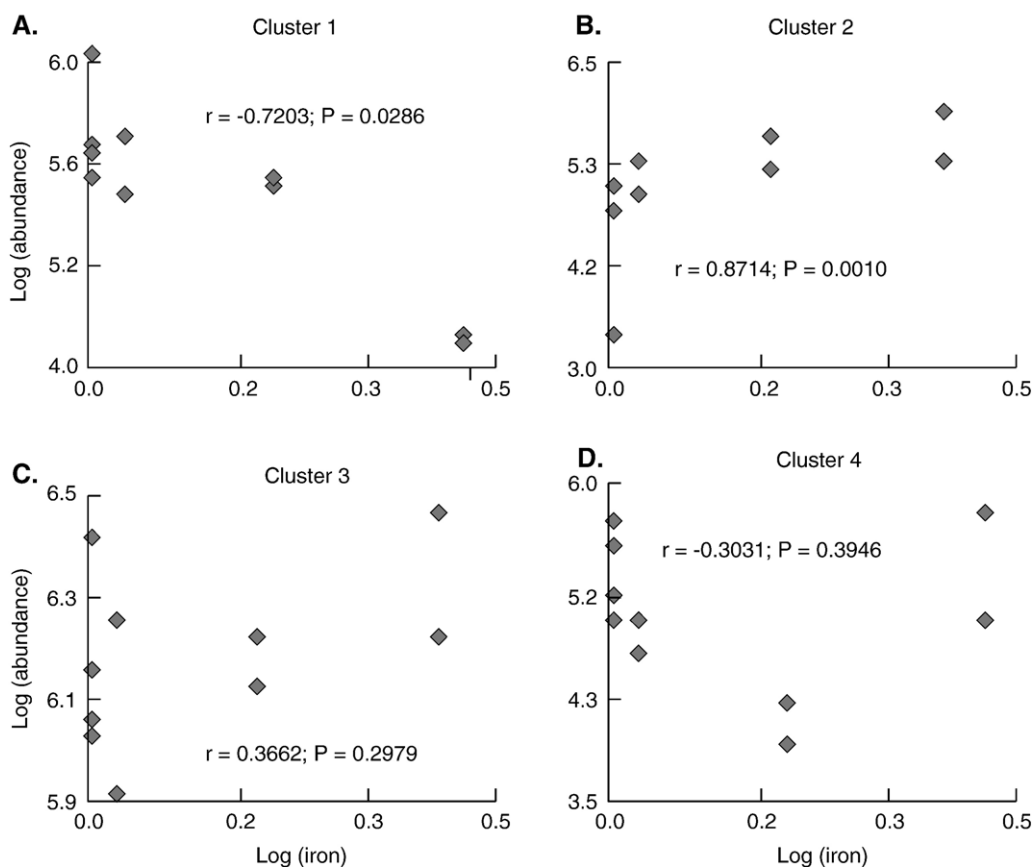


Fig. 8. OTU abundance correlated with Fe concentration within the four clusters shown in Fig. 2. Individual clusters of OTUs reveal differing responses to Fe addition. *P* values represent Bonferroni-corrected tests that the Pearson product-moment correlation coefficient is different than 0.

respond to Fe addition. However, as mentioned earlier, bacterial OTUs were clustered into four response groups. The responses of these groups are shown in Figs. 7 and 8. The richness of OTUs within Cluster 1 showed a decreasing relationship with Fe (Spearman =  $-0.91$ ;  $P=0.0002$ , Fig. 7) as did abundance (Spearman =  $-0.72$ ;  $P=0.029$ , Fig. 8). Therefore, species represented by Cluster 1 seem to be adversely affected by increasing Fe concentrations.

For Cluster 2, both richness and abundance showed a positive relationship with Fe (Spearman =  $0.87$ ;  $P=0.001$  and Spearman =  $0.58$ ;  $P=0.076$ , respectively, Figs. 7 and 8), and therefore responded positively to Fe perturbation. Cluster 3 showed no relationship with Fe treatment, but it was determined that Cluster 3 contained OTUs that had high abundance and richness across all Fe treatments (Figs. 7 and 8). Whereas, Cluster 4, which also showed no overall abundance change with Fe, consisted of OTUs that had low abundance with Fe (Fig. 8). This cluster also showed a significant decline in richness with increasing Fe (Spearman =  $-0.67$ ;  $P=0.035$ ), which means that while some OTUs within cluster 4 went extinct with Fe additions, other OTUs must have showed positive growth responses.

#### 4. Discussion

A number of studies have found that bacterial abundance increases with Fe additions (Pakulski et al., 1996; Cochlan, 2001; Hutchins et al., 2001) while composition and richness change little (Hutchins et al., 2001). Similar to other studies, with both our bacterial counts and with T-RFLP analysis, our results revealed that overall abundance and richness did not change with Fe availability. However, we do demonstrate that bacteria can be placed into response groups that show a limited number of ecological responses. With this approach, we found that one group responded positively to Fe additions, another responded negatively, and two others did not show any strong response, but maintained differing abundances.

Upon examination of individual clusters, rather than the population as a whole, it was demonstrated that the bacterial community in our mesocosms underwent changes with Fe concentration. Identifiable bacterial groups within the population demonstrated a relationship to Fe concentration (whether positive or negative). Analogous to our previous paper (Eldridge et al., 2004), this method provides a mechanism to gain insight into the *in situ* Fe status of the bacterial population. It is apparent that bacteria within Cluster 2 have a need for Fe that is not being met by the ambient Fe concentration;

they show a positive relationship to the Fe additions to this system. Bacteria within Clusters 3 and 4 are in an Fe replete state demonstrated by their lack of response to changes in Fe. Cluster 1 bacteria illustrate a response reminiscent of the picoeukaryotes in Eldridge et al. (2004) in that they show a negative relationship to Fe concentration, increasing in abundance (and richness) with decreasing Fe concentration.

It seems quite logical that groups of bacteria within this (or any) microbial community would respond differently to alterations in resources. Changing the dynamics of a single resource will likely affect coexistence patterns as that resource may be limiting to some species, causing population increases and driving down other resources that may be limiting for other species (Hutchinson, 1961; Chase and Leibold, 2003). Historically, bacteria have been treated as a single “black box”, therefore, the fact that other studies found overall abundance increases is not contrary to our results where different groups within the community had varied responses, and that understanding these responses are key to understanding the bacterial processes associated with Fe additions. Indeed, it does suggest that the responses of sub-groups of the community are significant enough to be detected at an entire community level.

The role that heterotrophic bacteria play in the biogeochemical cycling of Fe remains unclear. While some studies have examined the bacterial community 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 changes in community diversity through DGGE and, in two of three cases, T-RFLP. Fe addition in these experiments resulted in very little observable change 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 contradictory results exist (Pakulski 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. In our study, we have demonstrated that as Fe is added to samples from the Peruvian upwelling region of the equatorial Pacific, abundance and richness responses occurred within distinct groups of the community. 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, since different bacteria have been shown to consume various forms of dissolved organic carbon (Cottrell and Kirchman, 2000) and are differentially able to take up Fe bound to natural ligands (Weaver et al., 2003; Poorvin et al., 2004), changes in the bacterial community may lead to increases carbon utilization, short-circuiting the ‘biological carbon pump’ (Tortell et al., 1996).

One unfortunate realization from this work has been our inability to more closely assign microbial identities to peaks within the electropherograms. Although some groups (e.g., the cyanobacteria) which have been shown to respond to changes in ambient Fe concentration (Eldridge et al., 2004) should be resolvable in the current study, variation in the expected size of each peak based on sequence and that based on electropherogram output (often plus or minus 3–5 bases) resulted in an impossible assignment of organisms to peaks. In addition, upon *in silico* digestion, all cyanobacterial sequences obtained should yield the same sized peak and therefore are additionally not resolvable. Hopefully further work, using newly evolving techniques, may allow us to assign taxonomic identities (either individual or group) to peaks.

## 5. Conclusions

The results of this study should be viewed in light of our companion study (Eldridge et al., 2004), which 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. In spite of varying effects of these treatments on bacterial abundance, the effects of Fe limitation on the marine bacterial community structure are clear although there are questions as to whether these are direct or indirect effects. Kirchman et al. (2000) have demonstrated that carbon, not Fe, limited bacteria in low-Fe waters off the California coast, though they do see that with an addition of DOC alone Fe quickly becomes limiting. While Pakulski et al. (1996) have shown that Fe specifically limits light-independent production (assumably heterotrophic bacteria) in Southern Ocean waters. While we are unable to determine whether Fe itself actually stimulated bacterial

community changes or whether the phytoplankton’s increased productivity drives bacterial diversity change, we feel that this holistic approach better represents a natural system.

## Acknowledgements

We would like to thank G. Smith, J.M. Rinta-Kanto, K.W. Bruland, and the captain and crew of the R/V *Melville* for assisting in collection of samples. We would also like to thank C. Nomis for constant input during the preparation of this manuscript and W.H. Jeffrey and S. Durkin for advice on the use of T-RFLP. This work was supported by grants from the National Science Foundation to S.W. Wilhelm (OCE-9977040 and OCE-0002968). Access to ship time was generously provided by K.W. Bruland and D.A. Hutchins through National Science Foundation support (OCE-9811114).[SS]

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