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The heterotrophic bacterial response during a mesoscale iron enrichment experiment (IronEx II) in the eastern equatorial Pacific Ocean

Abstract—The response of the heterotrophic bacterial community to iron addition was determined during the mesoscale iron-enrichment experiment conducted in the eastern equatorial Pacific during May–June 1995 (IronEx II). Bacterial abundance and ³H-leucine incorporation rates were measured for samples collected from the middle of the mixed layer (15 m) over the course of the iron-induced phytoplankton bloom and its decline. Bacterial abundance and productivity increased 1.7- and threefold, respectively, compared to un-enriched waters. Specific growth rates of heterotrophic bacteria increased three- to fourfold. These results demonstrate that iron addition to this high-nitrate, low-chlorophyll region affects both autotrophic and heterotrophic microorganisms and that bacterial carbon demand can be potentially met by the fivefold increase in photosynthetic productivity in the mixed layer.

Over the last decade, considerable effort has been directed toward understanding the relationship between phytoplankton productivity and the availability of iron, particularly as the reason for the lack of significant autotrophic growth in high-nitrate, low-chlorophyll (HNLC) regions of the open ocean. The results of iron enrichment incubation experiments conducted in the subarctic North Pacific (e.g., Martin and Fitzwater 1988), equatorial Pacific Ocean (e.g., Martin et al. 1991) and the Southern Ocean (e.g., de Baar et al. 1990), as well as in situ Fe enrichment experiments conducted in the equatorial Pacific (e.g., Coale et al. 1996) and the Polar Front

in the south of Australia (Boyd et al. 2000), strongly support the idea that phytoplankton growth in HNLC areas are limited, at least in part, by the availability of Fe. These studies have focused primarily on the phytoplankton response to an alleviation of Fe deficiency, presumably because of the crucial role of Fe in photosynthesis, chlorophyll synthesis, and nitrate assimilation. Heterotrophic bacteria have been less studied despite their numerical dominance in oligotrophic waters (e.g., Fukuda et al. 1998) and their importance in the cycling of carbon and nutrients, including iron (Tortell et al. 1996). The few studies examining iron limitation of heterotrophic bacterial communities have reached contradictory conclusions, and the relative importance of iron versus dissolved organic matter in controlling the rates of bacterial growth in HNLC regions is still unclear.

Previous incubation experiments in Fe-depleted regions have suggested that Fe enrichment may affect heterotrophic bacteria, either directly by the alleviation of Fe limitation or indirectly through the effects of enhanced phytoplankton growth and the increased supply of dissolved organic matter suitable for bacterial utilization. During experiments conducted in the coastal Southern Ocean (Gerlache Strait), Pakulski et al. (1996) found that Fe enrichment increased both heterotrophic bacteria abundance and cell-specific growth rates. These experiments, conducted in the dark and in the absence of phytoplankton and bacterivores, suggest a direct stimulation of heterotrophic bacterial growth by Fe enrich-

ment. Church et al. (2000) conducted similar Fe enrichment experiments in the open ocean regions of the Southern Ocean from south of Tasmania to the Antarctic Polar Front but found that neither bacterial growth nor abundance increased with Fe amendments. However, these authors did observe significant increases in the rates of heterotrophic bacterial growth to combined additions of dissolved organic carbon (DOC) and Fe, relative to DOC additions alone. Similar results were found by Kirchman et al. (2000) during incubation experiments of low-Fe surface waters off central California: Fe additions alone had no effect on heterotrophic bacteria production, but Fe did enhance bacterial growth rates 5- to 10-fold when added together with glucose.

Increases in bacterial abundance have also been reported during Fe incubation experiments conducted in the equatorial Pacific Ocean (Price et al. 1994), Californian coastal upwelling regimes (e.g., Hutchins et al. 1998) and when Fe availability has been artificially regulated by the use of the fungal siderophore desferrioxime B (e.g., Wells 1999). However, these studies did not attempt to separate heterotrophic bacterial and autotrophic responses to Fe availability; therefore, the reported enhancements in bacterial growth were likely tied to increased phytoplankton-derived carbon supplies from Fe-induced phytoplankton growth, rather than an increase in Fe availability to bacteria.

Bottle confinement during such long-term incubation experiments alters both the mortality rate of autotrophic and heterotrophic microorganisms and the supply rate of substrates (including both dissolved organic and inorganic macronutrients and micronutrients) essential for their growth. Consequently, the extrapolation of such small-scale experiments as accurate representations of the actual in situ response to Fe addition have been strongly criticized. Mesoscale Fe enrichments have now been conducted in two different HNLC regions to examine the geochemical, physiological and ecological responses to Fe enrichment without significantly changing these supply and loss factors, thus avoiding many of the limitations inherent in bottle experiments and their interpretation. The goal of the present paper is to quantify the growth of heterotrophic bacteria over the course of the IronEx II mesoscale Fe enrichment experiment conducted in the eastern equatorial Pacific in 1995 (Coale et al. 1996). This is the first report documenting the abundance and production of heterotrophic bacteria to in situ Fe fertilization of the equatorial Pacific—a HNLC area where bacteria are typically a major contributor to the biomass (e.g., Ducklow et al. 1995).

Materials and methods—The IronEx II experiment was initiated on 29 May 1995 (day 0) at 4.6°S, 105°W in a region of high nitrate (ca. 10 μM) and low chlorophyll *a* (Chl *a*, 0.15–0.20 $\mu\text{g L}^{-1}$) concentrations. Iron as acidic iron sulfate; Fe (II), was injected, together with the inert tracer sulfur hexafluoride (SF_6), into a 72-km² grid to produce an initial Fe concentration of 2 nM, a 40-fold increase from the 0.05 nM ambient Fe concentration. Subsequent infusions yielding ca. 1 nM Fe were performed on days 3 and 7. The patch was followed using instrumented buoys and the distribution of SF_6 as it drifted SSW at ca. 2.8 km h⁻¹; stations were

generally sampled inside and outside (control) of the Fe-enriched patch. Samples were collected near dawn (0500–0700 h local time) from a depth of 15 m—the middle of the mixed layer increasing from 25 to 50 m over the first 14 d. Details of the methodology of IronEx II and complete patch behavior are presented in Coale et al. (1996).

Bacterial biomass and activity: Samples for Chl *a* concentration, and bacterial abundance and productivity were collected using 30-L trace metal-free Go-Flo bottles mounted on an instrumented rosette. Samples were collected in acid-washed, trace metal-clean 2-L polycarbonate bottles while wearing polyethylene gloves. All subsequent sample manipulation was carried out in a radioisotope van under reduced light conditions. Within 1 h of collection, subsamples for bacteria abundance were fixed with borate-buffered filtered (0.2 μm) formalin (2% final concentration) and stored at 4°C in the dark. Within 1–2 d, duplicate 5-ml subsamples were stained with acridine orange, filtered onto black Nuclepore polycarbonate filters (0.2- μm pore size), and mounted on slides in Cargille Type A immersion oil (Hobbie et al. 1977). Prepared slides were stored frozen until enumeration ashore using epifluorescence microscopy. At least 200 cells or 10 fields were counted in each of the duplicate filters per sample using an Olympus Vanox T, AH-2 microscope (final magnification $\times 1,000$). Reported bacterial abundance values include *Prochlorococcus*, which cannot be distinguished from heterotrophic bacteria in acridine orange-stained samples. *Prochlorococcus* comprise about 20% of the total bacterial abundance in the central (Binder et al. 1996) and eastern equatorial Pacific, but decreased in the Fe-fertilized patch to approximately 10% over the course of the experiment (Cavender-Bares unpubl. data).

Bacterial biomass was calculated directly from bacterial abundance using the conversion factor of 5.9 fg C cell⁻¹ determined for oceanic bacterial assemblages from the equatorial Pacific (Fukuda et al. 1998). This value is lower than the average carbon content of oceanic samples (12.4 ± 6.3 fg cell⁻¹) reported in their study, and much lower than the coastal bacterial carbon content of 20 fg C cell⁻¹ (Lee and Fuhrman 1987) widely used in the literature. Therefore, the bacteria biomass estimates reported in the present study should be considered as conservative and may be further underestimated when the cells begin to grow more rapidly. Previous work has demonstrated that freshwater bacteria become larger under nutritionally replete conditions compared to depleted conditions (Nagata and Watanabe 1990). Although the calculated rates of bacterial production and growth in the present study are not affected by changes in biomass (*see below*), the contribution of heterotrophic bacterial biomass to total planktonic community biomass may be underestimated and subject to error as the bloom developed during IronEx II.

Bacterial production was estimated from the incorporation rate of ³H-leucine (159 Ci mmol⁻¹, Amersham) according to a modified procedure of Simon and Azam (1989). Each rate estimate is based on triplicate 10-ml subsamples and triplicate trichloroacetic acid (TCA)-killed controls as blanks. Final ³H-leucine concentration was 20.1 nM. Samples were incubated in the dark for 1–2 h in sterile polystyrene test tubes at the in situ temperature. Incubations were terminated

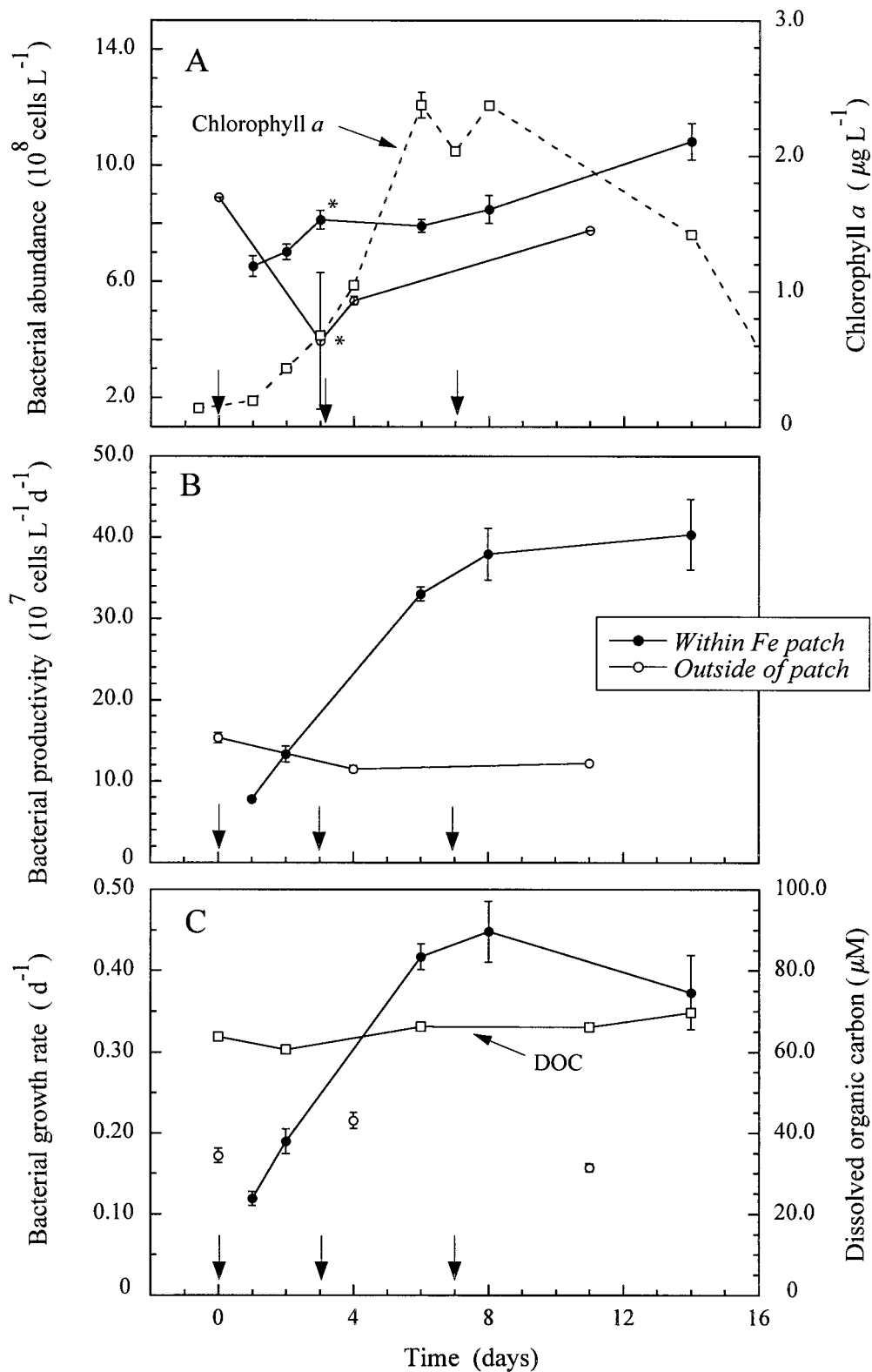


Fig. 1. (A) Phytoplankton Chl *a* and mean bacterial abundance in the Fe-fertilized patch and in ambient "control" seawater during IronEx II, patch-1. Day 3 values (indicated by asterisks) are averages of either two outside or two inside stations during transect of the Fe patch. (B) Average bacterial productivity (cell production rate) in the Fe-fertilized patch and in ambient control seawater. (C) Specific growth rate (bacterial productivity/biomass) of bacteria and dissolved organic carbon concentration (DOC) in the Fe-fertilized patch and in ambient control seawater. Error bars indicate ± 1 SD (error propagation determined by the quadratic sum of errors in Fig. 1C); where error bars are not shown, errors are smaller than symbols. Arrows along the abscissa indicate the days of Fe infusion, and all samples are from a depth of 15 m.

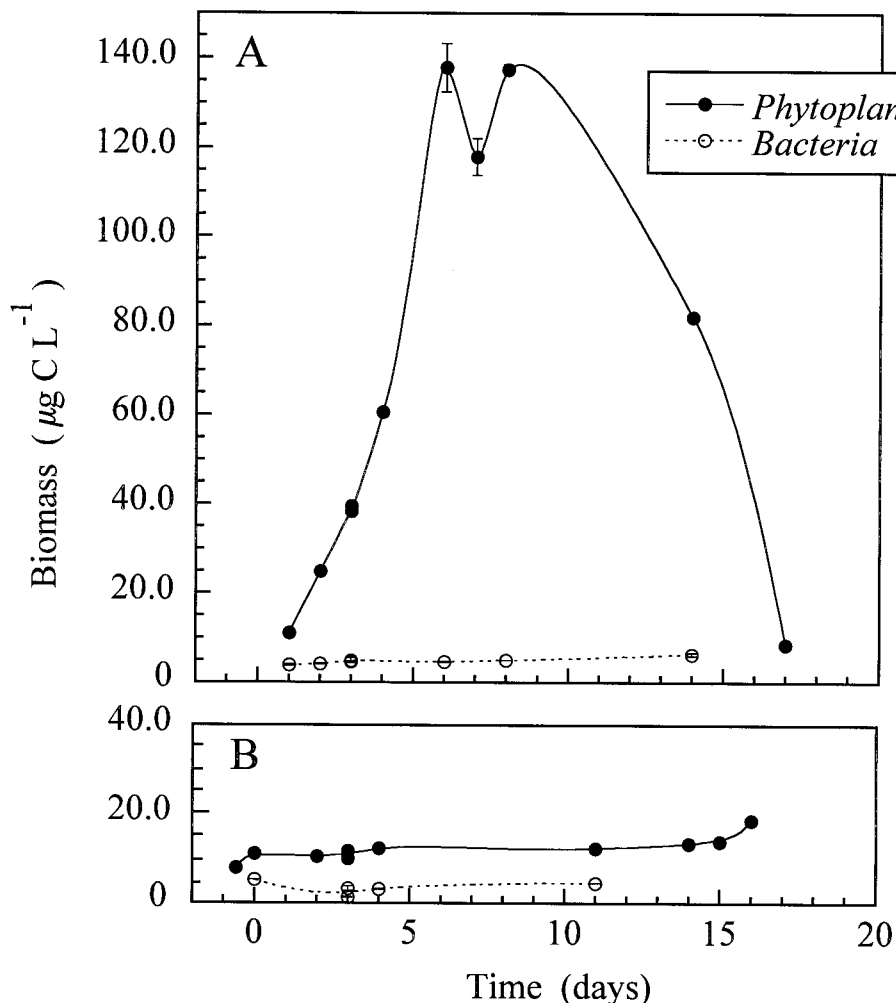


Fig. 2. Phytoplankton and bacterial carbon biomass in the (A) Fe-fertilized patch and (B) non-fertilized "control" waters as a function of time. Error bars indicate ± 1 SD; where error bars are not shown, errors are smaller than symbols. All samples from a depth of 15 m.

by adding ice-cold TCA (5% final conc.), filtering through 0.22- μm Millipore cellulose acetate filters, and rinsing twice with 3–4 ml ice-cold 5% TCA. After dissolving the filters in 1 ml ethyl acetate, 7 ml of Ecoscint A scintillation fluor was added, and the samples were radioassayed. Bacterial production rates were calculated assuming a ^3H -leucine conversion factor of 1.15×10^{17} cells mol^{-1} leucine incorporated (as discussed by Kirchman et al. 1995). This factor is the mean of open ocean studies and is slightly lower than the factor employed by Simon and Azam (1989), who assumed that isotopic dilution is twofold (1.5×10^{17} cells mol^{-1}); hence, the productivity estimates reported here should be considered conservative. Activities of killed controls (samples with 5% TCA added prior to ^3H -leucine addition) were $<4\%$ of live samples and were subtracted from live samples in the calculation of production rates. Net bacterial growth rate (μ), in divisions per day, was calculated by dividing the bacterial cell production rate by the abundance of bacteria determined at the beginning of an incubation period.

Samples for phytoplankton Chl *a* were collected from the

same Go-Flo bottles used for bacterial measurements. Duplicate samples (0.3–0.5 L) were filtered onto uncombusted Whatman® filters, extracted for 24 h in 90% acetone (-20°C), and analyzed for Chl *a* and phaeopigments by in vitro fluorometry using a Turner Designs Model 10-AU fluorometer (Parsons et al. 1984) calibrated using extracts of spinach Chl *a* (Sigma-Aldrich). Phytoplankton carbon biomass was estimated from phaeopigment-corrected Chl *a* using the C:Chl *a* ratio of 58 (weight basis; Eppley et al. 1992). Primary productivity was determined using the ^{14}C technique and on-deck simulated in situ 24-h incubations. Details of the method are discussed elsewhere (Barber et al. 1996). The primary productivity rates in the present study were integrated throughout the mixed layer, and the average mixed layer integrated rates are reported.

Results and discussion—The fertilization of a "patch" of the surface waters of the eastern equatorial Pacific Ocean with small quantities of Fe resulted in a massive bloom of phytoplankton, but a minimal increase (ca. 1.7-fold) in heterotrophic bacterial abundance from the depth studied (15

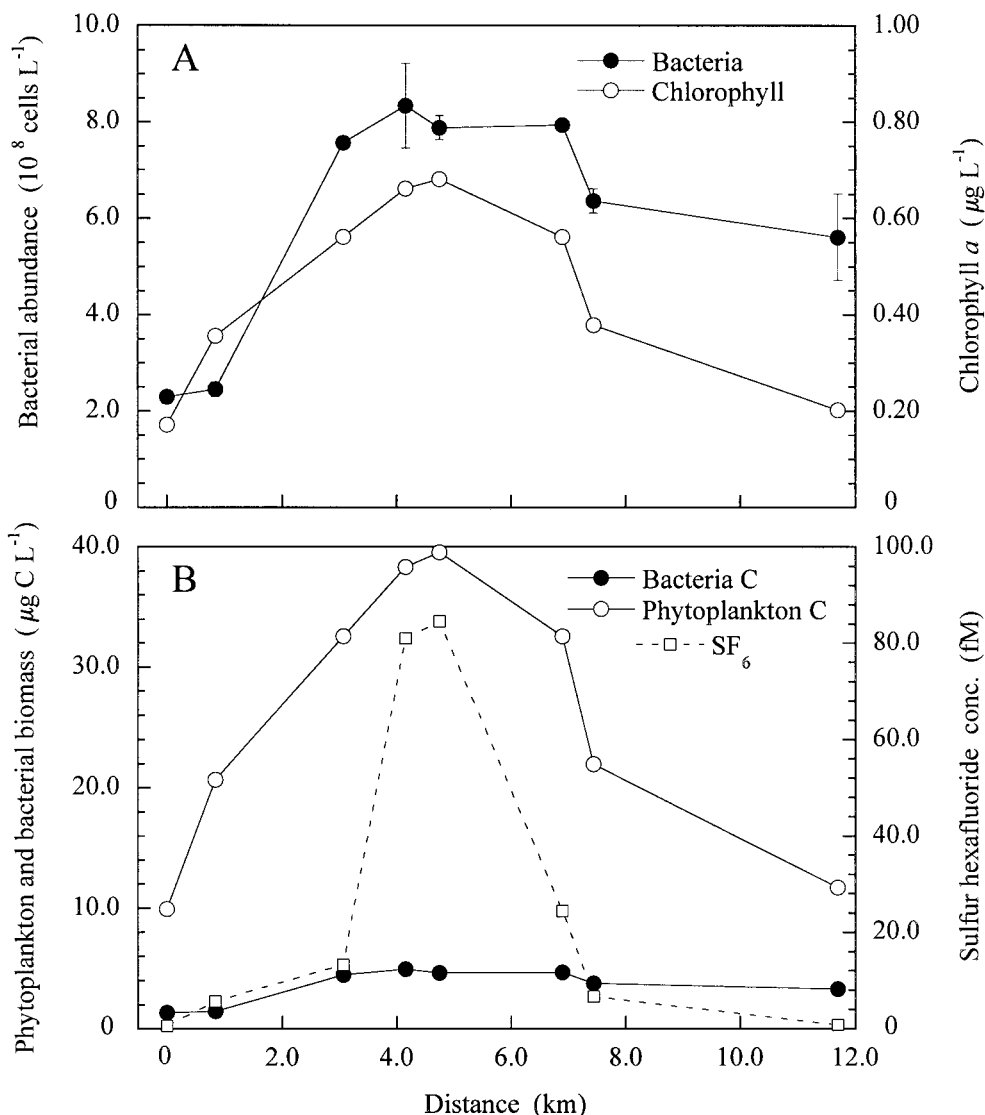


Fig. 3. (A) Bacterial abundance and Chl a concentration from a depth of 15 m along a 12-km transect of the Fe-enriched patch-1 conducted on 1 June 99 (day 3). (B) Phytoplankton biomass and bacterial biomass converted to carbon; dashed line is the concentration of inert chemical tracer sulfur hexafluoride (SF₆), which was mixed in a constant ratio with iron on day 0. Error bars for bacterial abundance indicate ± 1 SD; where error bars are not shown, errors are smaller than symbols. Sampling along transect started at 4°55.09'S, 106°14.99'W and ended 5 h later at 4°52.00'S, 106°2.88'W.

m; Fig. 1A). The phytoplankton bloom, as indicated by the 17-fold increase in Chl a during the first 8 d, was composed primarily of large pennate diatoms (Coale et al. 1996). The unfertilized waters outside of the patch (control) remained relatively unchanged, with low Chl a concentrations consistent with surface values reported previously in the eastern equatorial Pacific (e.g., Chavez et al. 1996). Coincident with the increase in autotrophic biomass, heterotrophic bacterial abundance increased slightly from 6.5×10^8 cells L^{-1} on day 1 to 7.9 and 8.5×10^8 cells L^{-1} at the height of the bloom on days 6 and 8, respectively, and continued to increase to a maximal value of 10.8×10^8 cells L^{-1} (day 14) as the phytoplankton bloom rapidly declined (Fig. 1A). Bac-

teria in the control waters were variable ($6.5 \pm 2.2 \times 10^8$ cells L^{-1}), but their concentrations are consistent with estimates of heterotrophic bacteria reported for equatorial Pacific waters determined further west at 140°W. Ducklow et al. (1995) report abundance estimates, determined from epifluorescence microscopy, ranging from 6 to 8×10^8 cells L^{-1} for the upper 100 m, and Binder et al. (1996) reported mean surface concentrations (0–30 m) of approximately 7×10^8 cells L^{-1} using flow cytometry. Although both phytoplankton and bacterial biomass increased as a result of the Fe enrichment, it is readily apparent that the increase in autotrophic biomass is substantially greater than in bacterial biomass (Fig. 2A). Conversion of Chl a concentration and

bacterial abundance to carbon biomass clearly demonstrates this difference; the increase in phytoplankton carbon is approximately 17-fold compared to the 1.7-fold increase in bacterial carbon within the patch. Outside of the Fe patch (Fig. 2B), the average phytoplankton carbon concentration was triple that of the heterotrophic bacterial carbon concentration. In this simple comparison of heterotrophic and autotrophic carbon, it is assumed that the C:Chl *a* ratio of the Fe-induced phytoplankton bloom remains constant. However this ratio can vary dramatically according to phytoplankton species and their degree of Fe sufficiency (e.g., Sunda and Huntsman 1995). In a detailed study of the microplankton dynamics during IronEx II, Landry et al. (2000a) have demonstrated that as the bloom peaked, the community C:Chl *a* ratio decreased to about half of its original value.

The development of enhanced bacterial and phytoplankton biomass within the patch relative to the unfertilized waters was also apparent during a 12-km transect of the Fe- and SF₆-infused patch on 1 June (day 3) immediately before the second Fe infusion. Maximal concentrations of Chl *a* and bacteria were located at the highest concentrations of SF₆ in the center of the patch, and bacterial and phytoplankton biomass covaried ($P < 0.02$) during the sampling of surface (15 m) water across the patch (Fig. 3A). Conversion of bacteria abundance and Chl *a* concentration to carbon biomass demonstrates that, both outside and inside the Fe patch, the bacterial contribution to the total (phytoplankton + bacteria) carbon biomass is relatively small. Although absolute bacterial abundance increased at the center of the Fe patch, the relative bacterial contribution decreases from <20% of the total C in the outside waters to ca. 12% at the center of the patch (Fig. 3B).

Increases in bacterial cell abundance have been reported previously in Fe-amended bottle experiments from the equatorial Pacific (ca. threefold increase relative to controls; Price et al. 1994), Gerlache Strait, Antarctica (ca. twofold increase; Pakulski et al. 1996), and coastal California (1.6-fold increase; Hutchins et al. 1998) but remained relatively stable throughout the 13-d SOIREE mesoscale Fe experiment in the Southern Ocean (Hall and Safi in press). As both Price et al. (1994) and Hutchins et al. (1998) have argued previously, such increases in bacterial abundance after Fe addition do not imply that heterotrophic bacteria are Fe-limited in situ, but rather that the increases in cell abundance are likely the indirect result of higher DOC concentrations caused by increased phytoplankton production. During IronEx II, the concentration of DOC increased by >10% from ca. 62 to 70 μM over the first 2 weeks (Fig. 1C), despite losses that would be expected from any enhanced bacterial production fueled by phytoplankton-derived DOC.

During the first 8 d of IronEx II, bacterial productivity within the patch was correlated with the Chl *a* concentrations ($P < 0.02$) and primary productivity rates ($P < 0.01$) determined from a depth of 15 m and with their average integrated mixed layer values. Bacterial production rates increased fivefold from day 1 to day 14, the final day of bacterial sampling (Fig. 1B; Table 1), and this rate is triple that of the mean rate determined for the unenriched waters. Average mixed layer primary productivity also increased within the Fe-enriched patch by ca. fivefold compared to the

Table 1. Summary of bacterial productivity (BP), average particulate primary productivity (PP), and the ratio of bacterial carbon demand (BCD) to primary productivity during the 2-week monitoring of IronEx II. BP was converted from cell production rates (Fig. 1B) by assuming 20 fg C cell⁻¹. BCD is based on a bacterial growth efficiency of 22% (see text for details).

Day	Production rate ($\mu\text{g C L}^{-1} \text{d}^{-1}$)		BCD/PP ^b (%)
	Bacterial ^{a,b}	Primary ^c	
Fe-enriched patch			
1	1.56 ± 0.07	19.3	36.6
2	2.66 ± 0.19	29.1	41.5
6	6.61 ± 0.18	54.4	55.2
8	7.59 ± 0.64	59.8	57.6
14	8.06 ± 0.87	19.6	187
Unenriched "control" waters			
0	3.06 ± 0.12	12.4	112
4	2.30 ± 0.08	12.6	83.0
11	2.44 ± 0.03	15.9	69.6

^a Rates determined from 15-m samples.

^b Values divided by 3.4 if 5.9 fg C cell⁻¹ is used to convert bacterial cell production rates.

^c Mixed layer-integrated average rates.

relatively constant primary production rates outside of the patch (Table 1). The bacterial carbon demand (BCD), estimated by dividing bacterial productivity by a growth efficiency of 22% (median value of oceanic values from del Giorgio and Cole 1998), averaged 88% of the particulate primary production rates in the control waters and was consistently greater than the ratio of BCD to primary productivity within the Fe patch during the period of phytoplankton bloom development (mean of days 1 to 8 = 48%; Table 1). Quantifying the flux of carbon into bacteria is dependent on the accuracy of growth efficiency, which, in this case, is assumed to be constant and independent of growth rate, nutrient limitation, or carbon source. However, even with this unlikely assumption of constancy, it appears that any degree of organic carbon limitation that bacteria may have been experiencing in this region decreased with Fe enrichment, either as a result of enhancement of phytoplankton production or of increased efficiency of bacterial carbon metabolism. Such an increase in bacterial growth efficiency under Fe-sufficient conditions has been demonstrated previously for some marine bacterial strains isolated from the Sargasso Sea and the Gulf of Mexico (Tortell et al. 1996).

The specific growth rates of heterotrophic bacteria, calculated as rates of bacterial cell production normalized to abundance, averaged $0.181 \pm 0.030 \text{ d}^{-1}$ ($n = 3$ stations) in the unenriched waters and are typical of rates reported previously in the equatorial Pacific during non-El Niño years (mean $\mu = 0.163 \pm 0.035 \text{ d}^{-1}$; Kirchman et al. 1995). Bacteria in the Fe-enriched patch exhibited a fourfold increase in their specific growth from $0.119 \pm 0.008 \text{ d}^{-1}$ on day 1 to $0.448 \pm 0.038 \text{ d}^{-1}$ on day 8, and this maximal rate was 2.5-fold greater than the average rate measured in the unenriched waters (Fig. 1C). This enhancement of cell-normalized production contrasts with the decrease reported by Price et al. (1994) for Fe-amended natural assemblages from their equa-

torial Pacific bottle experiments and the decrease or constancy of growth rates estimated similarly during Fe-amended Antarctic samples (Pakulski et al. 1996). However as seen in the present study, bacterial specific growth increased by fourfold during the SOIREE in situ Fe enrichment experiment, whereas bacterial abundance changed little (Boyd et al. 2000; Hall and Safi in press).

Although the present study cannot demonstrate a direct stimulatory response of heterotrophic bacteria to Fe enrichment, the increased specific growth of bacteria results in less accretion of in situ cell abundance relative to enhancement of their productivity because of natural losses of cells due to increased grazing by microzooplankton (Coale et al. 1996), potential lysis by bacteriophages (e.g., Cochlan et al. 1993), and sinking by attachment to phytoplankton (e.g., Smith et al. 1995). Although the losses from lysis and sinking were not measured during IronEx II, they normally increase in association with enhanced growth of phytoplankton. If one assumes that the grazing rates of *Prochlorococcus* and heterotrophic bacteria are similar, then the losses due to bacterivory can be estimated from the mortality rates of *Prochlorococcus* derived from either cell abundance data (Mann and Chisholm 2000) or dilution experiments (Landry et al. 2000b). Both estimates yielded similar results and indicated that the rates of *Prochlorococcus* mortality in the Fe patch were double those rates measured in the unenriched waters. As pointed out by Mann and Chisholm, this increase in mortality is not surprising because both the growth and grazing rate of the heterotrophic protists that feed on picoplankton can be Fe-limited as well (Chase and Price 1997).

During IronEx II there was a strong draw down of ca. 5 μM of nitrate, and an 14-fold increase in the absolute uptake rates of nitrate as the phytoplankton bloom developed during the first week (Coale et al. 1996; Cochlan and Kudela in prep.). The contribution of heterotrophic bacteria to this increase in nitrate utilization can be estimated from bacterial carbon production rates assuming a C:N ratio of 5.7 (1.2 fg N cell⁻¹; Fukuda et al. 1998) and correcting for the 67% efficiency of the filters used in the nitrogen uptake experiments to retain bacteria. With these assumptions, the bacterial contribution to the measured nitrate uptake rates is insignificant and averaged only about 1% during the 8 d of bloom development following Fe enrichment. Even outside of the patch, where bacterial biomass was relatively higher, or during the final sampling period (day 14) when bacterial biomass and productivity were maximal, the contributions of bacteria to nitrate uptake were minimal (<5%). These results strongly suggest heterotrophic bacteria were not responsible for any of the enhanced nitrate utilization observed during IronEx II.

Although size-fractionated Fe uptake experiments have demonstrated that heterotrophic bacteria can account for approximately 50–70% (Tortell et al. 1996; Maldonado and Price 1999) of the total Fe uptake in oligotrophic North Pacific waters, the present results of enhanced bacterial productivity and growth are not strong evidence for direct bacterial Fe limitation prior to Fe infusion, such as has been reported for the coastal Southern Ocean (Pakulski et al. 1996), or even indirect bacterial Fe limitation, such as the reduced carbon growth efficiencies reported for Fe-deficient

laboratory cultures (Tortell et al. 1996). Heterotrophic bacteria, with their high surface-to-volume ratio and ability to produce siderophores (e.g., Granger and Price 1999), are probably very efficient competitors for Fe acquisition even in the picomolar Fe environments common to HNLC regions. However, because of their large Fe requirements, these organisms still may be Fe-limited in situ, resulting not only in reduced bacterial growth, but also a reduction in the carbon growth efficiency of both the metabolism of the bacteria (Tortell et al. 1996) and their protozoan consumers (Chase and Price 1997).

During IronEx II, the increase in bacterial specific growth rate coincident with the phytoplankton bloom demonstrates a strong coupling between phytoplankton and bacteria and suggests that the heterotrophic bacteria either responded directly to the increased availability of Fe for uptake or, alternatively, to the increased availability of phytoplankton-derived carbon supplies. These findings, although based on a single depth of sampling, clearly demonstrate that supplementation of a HNLC region with Fe contributes to enhanced heterotrophic bacterial growth and net accumulation of biomass. Given the importance of bacteria-mediated carbon cycling in the open ocean, the biogeochemical consequences resulting from such spatiotemporal increases in bacterial growth must be considered if anthropogenic Fe fertilization is to be considered as a viable tool to increase primary productivity in the sea, to remove carbon dioxide from the atmosphere, or both.

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