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Fe and C co-limitation of heterotrophic bacteria in the naturally fertilized region off Kerguelen Islands

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Abstract

It has univocally been shown that iron (Fe) is the primary limiting nutrient for phytoplankton metabolism in High Nutrient Low Chlorophyll (HNLC) oceans, yet, the question of how this trace metal affects heterotrophic microbial activity is far less understood. We investigated the role of Fe for bacterial heterotrophic production and growth at three contrasting sites in the naturally Fe-fertilized region east of Kerguelen Islands and at one site in HNLC waters during the KEOPS2 (Kerguelen Ocean and Plateau Compared Study 2) cruise in spring 2011. We performed dark incubations of natural microbial communities amended either with iron (Fe, as $FeCl_3$), or carbon (C, as trace-metal clean glucose), or a combination of both, and followed bacterial abundance and heterotrophic production for up to 7 days. Our results show that single and combined additions of Fe and C stimulated bulk and cell-specific bacterial production at all sites, while bacterial growth was enhanced only in two out of four occasions. The extent of stimulation of bulk bacterial heterotrophic production by single Fe or C additions increased with increasing in situ bacterial Fe uptake rates in the surface mixed layer. Our results provide evidence that both Fe and C are present at limiting concentrations for bacterial heterotrophic activity, in HNLC and fertilized regions, in spring. The observation that the extent of stimulation by both elements was related to Fe-uptake rates highlights the tight interaction between the C- and Fe-cycles through bacterial heterotrophic metabolism in the Southern Ocean.

1 Introduction

Iron (Fe) is an essential element for biological activity, but present at trace amounts in the ocean. The role of Fe as a limiting nutrient was extensively studied in High Nutrient Low Chlorophyll (HNLC) oceans with focus on phytoplankton productivity and growth. Mesoscale fertilization experiments (see review by Boyd et al., 2007) and investigations in naturally Fe fertilized regions (Blain et al., 2007; Pollard et al., 2009) have

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conclusively shown that Fe controls primary productivity and the drawdown of carbon dioxide (CO_2) in large areas of the global ocean. Phytoplankton primary production is intimately linked to heterotrophic bacterial activity in different ways. First, heterotrophic bacteria are potential competitors for the access to limiting nutrients, such as Fe in the HNLC ocean (Maldonado and Price, 1999; Boyd et al., 2012), and second, bacteria remineralize a substantial fraction of phytoplankton-derived dissolved organic matter (DOM). Through these processes bacteria contribute to the extent and fate of primary production. However, up to date, relatively little attention was paid to the effects of Fe limitation on heterotrophic bacteria, and the potential consequences on the tight coupling between production and remineralization of organic matter.

Heterotrophic bacteria responded to variable extents to Fe addition in mesoscale fertilization experiments and in natural fertilization studies (see for overview Christaki et al., 2014). Whether the increase in bacterial abundance and production in the Fe-fertilized patches was induced directly by Fe, or indirectly by the enhanced DOM production by phytoplankton, or a by combination of both was difficult to conclude from these observations. Only a few studies have examined the potential role of Fe as limiting factor for heterotrophic bacteria experimentally, reporting contrasting results. While Fe addition alone did not lead to enhanced bacterial production and growth in HNLC areas such as the coastal Californian Pacific (Kirchman et al., 2000) and different frontal zones south of Tasmania (Church et al., 2000), bacterial activity increased upon Fe-addition in the Gerlache Strait (Pakulski et al., 1996) and the Ross Sea (Bertrand et al., 2011). The variable responses of heterotrophic bacteria than phytoplankton to additions of Fe in different oceanic environments suggests a more complex interplay between Fe and bacterial metabolism, which could in part be driven by the availability of DOM.

The Kerguelen Ocean and Plateau compared Study 2 (KEOPS2) provided access to naturally Fe fertilized sites above the Kerguelen Plateau and in offshore waters south and north of the Polar Front, each with distinct hydrodynamic and geochemical properties (Park et al., 2014). As a consequence, concentrations of dissolved iron (DFe,

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Queroué et al., 2014), the extent and age of the phytoplankton blooms induced by Fe fertilization (Lasbleiz et al., 2014), and the bacterial responses (Christaki et al., 2014) were variable across sites. The objective of the present study was to examine the role of Fe and C as limiting elements for bacterial heterotrophic activity to better understand the bacterial response to Fe fertilization of the Southern Ocean.

2 Material and methods

2.1 Experimental design

The effect of iron (Fe) and organic carbon (C) additions on bacterial heterotrophic production and growth were determined at three stations located in the naturally Fe-fertilized region east of Kerguelen Island (Stations E-4W, E-3 and E-5) and at the reference station R2 in HNLC waters west of Kerguelen Island (Table 1; a figure is provided in Blain et al., 2014). At all stations, seawater was collected with 10 L Teflon-lined Niskin-1010X bottles mounted on a 1018 rosette system adapted for trace metal clean work (Bowie et al., 2014). Sampling depths (surface mixed layer) were 40 m at Stations R2 and E-4W, 37 m at Station E-3 and 25 m at Station E-5. The Niskin bottles were transferred to a trace-metal clean container, where 2 L polycarbonate (PC) bottles were filled with unfiltered seawater. The 2 L PC bottles were transferred to a trace metal clean lab and 300 mL of seawater was dispatched to 12 × 500 mL PC bottles. All PC bottles were soaked in HCl (10 %) and thoroughly rinsed with Milli Q water before use. Besides the control that consisted in unamended seawater, the following 3 treatments were prepared: seawater + Fe, seawater + C, and seawater + Fe + C. Triplicate incubations were done for all treatments and the control. Iron was added as $FeCl_3$ (final concentration of $FeCl_3$ 1 nM), and C was added as trace-metal clean glucose (final concentration 10 µM). To eliminate trace metal contamination, the working solution of glucose was passed over a Chelex 100 ion exchange resin (Bio-rad). The incubations were done in the dark in a temperature-controlled lab at in situ temperature. For sub-

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sampling, incubation bottles were transferred to the trace-metal clean lab and opened under a laminar flow hood (ISO class 5). Subsamples for bacterial abundance and production were taken at Day 0 (T_0), Day 2 (T_2), Day 4–5 (T_4) at all sites and at Day 7 (T_7) also at Station R2.

2.2 Enumeration of heterotrophic bacteria

For bacterial abundance, 1.8 mL subsamples were fixed with formaldehyde (2 % final concentration), stored in the dark for 20 min and then shock-frozen in liquid nitrogen. The samples were stored at −80 °C until analyses by flow cytometry. Counts were made using a FACSCalibur flow cytometer (BD20 Biosciences) equipped with an air-cooled laser, providing 15 mW at 488 nm with the standard filter set-up. Heterotrophic bacteria were stained with SYBRGreen I, and determined by flow cytometry as described in detail in Obernosterer et al. (2008).

2.3 Bacterial heterotrophic production

Bacterial production was estimated by [^{3}H] leucine incorporation applying the centrifugation method (Smith and Azam, 1992) as described in Obernosterer et al. (2008). Briefly, 1.5 mL samples were incubated with a mixture of [3,4,5-^{3}H(N)] leucine (Perkin Elmer, 144 Ci mmol^{-1}; 7 nM final concentration) and nonradioactive leucine (13 nM final concentration). Controls were fixed with Trichloracetic Acid (TCA; Sigma) at a final concentration of 5 %. Samples were incubated for 2–3 h under the same conditions as the cultures as described above. Incubations were terminated with TCA (5 % final concentration). The radioactivity incorporated into bacterial cells was measured in a Tricarb® scintillation counter.

2.4 Dissolved organic carbon analyses

In situ samples for dissolved organic carbon (DOC) analyses were taken with Teflon-lined Niskin-1010X bottles adapted for trace metal clean work (Bowie et al., 2014).

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Seawater was filtered through 2 combusted GF/F (0.7 µm nominal pore size, Whatman) filters and 15 mL of the filtrate was stored acidified (H_3PO_4, final pH = 2) in combusted 20 mL glass ampoules at room temperature in the dark. DOC concentrations were measured on a Shimadzu TOC-VCP analyzer with a Pt catalyst at 680 °C (Benner and Strom, 1993) as detailed in Tremblay et al. (2014).

3 Results

3.1 Environmental setting of the study sites

The offshore waters east of Kerguelen Islands represent a region with intense mesoscale activity (Park et al., 2014) that is reflected in variable physical and biological characteristics of the stations chosen for the present study (Table 1). The three stations within Fe-fertilized waters, all located south of the Polar Front, revealed considerable variability in concentrations of DFe (up to 5.8-fold in the mixed layer; Queroué et al., 2014) and Chl *a* (up to 2.2-fold; Lasbleiz et al., 2014), while bacterial abundance and heterotrophic production were more similar among these sites (Christaki et al., 2014). The reference site R2 in HNLC waters is located west of Kerguelen Island, and concentrations of DFe, Chl *a*, bacterial abundance and production were substantially lower than those in surface waters of the Fe-fertilized stations (Table 1). Among the parameters of interest to the present study, only concentrations of DOC did not differ between fertilized and non-fertilized sites. This is most likely due to the rapid consumption of phytoplankton-derived DOM in Fe-fertilized waters, as reflected in the marked enhancement of bacterial heterotrophic production (by up to a factor of 11) (Table 1).

3.2 Bacterial responses to Fe and C additions

Single and combined additions of Fe and C significantly stimulated bulk and cell-specific production at all stations, but the temporal patterns and the extent of stimulation varied among experiments (Fig. 1). By contrast, Fe and C additions lead to

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increased bacterial abundance only at 2 out of the 4 stations (E-4W and E-5). At the HNLC-site R2, single additions of Fe and C stimulated bulk and cell-specific production at T_7 (by 1.4 to 1.8-fold), and the combined addition of Fe and C resulted in an enhancement of 1.9 as compared to the unamended control (Fig. 1a–c). By contrast, bacterial abundance was not significantly different in any of the treatments. At Station E-4W, the most contrasted station to the reference site with respect to Chl *a* and in situ bacterial abundance and production, the combined addition of Fe and C rapidly stimulated bulk and cell-specific production at T_2 (by 1.5-fold), and this enhancement was maintained at T_4 (by 1.6-fold) (Fig. 1e and f). Additions of Fe and C alone resulted in enhanced bacterial abundance, bulk and cell-specific production only at T_4 (by 1.2- to 1.7-fold). Stations E-3 and E-5 have close geographical position and were sampled in a quasi-Lagrangian manner with a 17 days time interval. The pattern observed at these two stations was strikingly similar, but the extent of the response decreased from E-3 (Fig. 1g–i) to E-5 (Fig. 1j–l). A pronounced response in bulk and cell-specific production was observed to single and combined additions at T_2 (by 1.7–2.2-fold at Station E-3 and by 1.3–1.5-fold at Station E-5). At the end of the experiment, the enhancement of these parameters was detectable in the C and the combined C and Fe additions, but not in the Fe-amended treatment.

3.3 Linking the extent of stimulation to in situ bacterial Fe uptake

To further explore the variable extent of stimulation of bacterial production by single Fe and C additions among stations, we determined the respective maximum ratio of bacterial production in the Fe- and C-amended to the control treatments for each experiment. Single additions of both Fe and C resulted in the most pronounced responses at Station E-3 (1.9- and 2.0-fold, respectively), followed by Stations E-4W (1.7-fold for Fe and C) and E-5 (1.50-fold for Fe and C). The maximum extent of stimulation at Station R2 was overall in the range of those observed in Fe-fertilized waters, but the difference in the response to C (1.7-fold) and to Fe (1.4-fold) was most pronounced at this site. Interestingly, the maximum extent of stimulation of bacterial heterotrophic production by

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single additions of Fe and C was positively related to in situ bacterial Fe uptake rates as determined by 24 h incubations of the microbial community with ^{55}Fe (Fourquez et al., 2014), and this relationship is particularly well established across the Fe-fertilized sites (Fig. 2). A similar relation was observed for cell-specific production rates (data not shown). The maximum extent of stimulation was also positively related to in situ DFe concentrations, but not to in situ Chl *a* or bacterial abundance and production.

4 Discussion

4.1 Resource co-limitation

The concept of resource limitation has shifted over the past decades from the theory that a single nutrient limits growth at a given time (Liebig's law of the minimum) to the recognition that co-limitation by multiple resources frequently occurs in the ocean (Arrigo, 2005; Saito et al., 2008; Harpole et al., 2011). Based on theoretical considerations, different types of nutrient co-limitation of phytoplankton were proposed (Arrigo, 2005; Saito et al., 2008). We refer here briefly to two types of resource co-limitation where in one case either two or more nutrients are present at concentrations too low for microbial uptake, and in the second case the enhanced concentrations of one limiting resource may facilitate the uptake of another resource (Arrigo, 2005; Saito et al., 2008). In addition, microbial taxa could each be limited by different nutrients due to their specific strategies to access a limiting resource and this feature could further add to the observation of co-limitation of a diverse microbial community (Sebastián and Gasol, 2013).

How do these concepts apply to the possible co-limitation of heterotrophic bacteria by Fe and C? Determining the limiting concentrations of these resources is difficult. Despite the high accuracy of chemical analytical methods, the bulk concentrations of DFe and DOC do not provide information on the biologically available fractions. A simple comparison between the bacterial Fe quota in Fe limited cultures (9 µmol Fe (mol C)$^{-1}$;

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Tortell et al., 1996) and in the KEOPS study region (4–8 µmol Fe (mol C)$^{-1}$; Fourquez et al., 2014) and the ratio DFe : DOC (range 3–7 × 10^{-6} µmol DFe (mol DOC)$^{-1}$; Table 1) clearly identifies Fe as a potentially limiting resource for heterotrophic bacteria. Even though DOC is present in the micro-molar range, the fraction of this bulk DOC that is biologically available is much smaller, in particular under non-bloom conditions in the Southern Ocean. This is due to the permanent upwelling that transports highly refractory DOM from the deep ocean to the surface. As a consequence of the upwelling and the concurrent low phytoplankton primary production, DOC concentrations in the Southern Ocean are the lowest in surface waters of the global ocean (Hansell, 2013). These basic considerations help to understand the positive response of heterotrophic bacteria to the addition of biologically labile forms of both Fe and C.

An exciting finding was the relationship between the extent of Fe- and C-stimulation of bulk and cell-specific bacterial production and in situ bacterial Fe-uptake rates. These relationships highlight the close coupling between C and Fe for bacterial heterotrophic metabolism, and they further support the idea that the addition of either of these elements facilitates the utilization of the other limiting element (Arrigo, 2005; Saito et al., 2008). The response to single C amendment increased at higher in situ bacterial Fe uptake rates and DFe concentrations, which could indicate that a larger fraction of the added glucose can be utilized under these conditions. The enhanced response to single Fe addition at higher in situ bacterial Fe uptake rates could indicate the processing of a larger fraction of the DOM present in situ or the utilization of DOM with a higher efficiency. A synergistic effect, such as the increase in Fe bioavailability by compounds released by phytoplankton could also be considered (Hassler et al., 2011). Within the bacterial cell, the Fe and C cycles are tightly linked in the tricarboxylic acid (TCA) cycle and the electron transport system of the respiratory chain, as these key pathways of the cellular carbon metabolism harbor several Fe-containing enzymes. The limitation by Fe can therefore affect the quantity of organic compounds processed by the bacterial cell, and likely also the bacterial growth efficiency (Tortell et al., 1996). Indeed, reduced bacterial growth and respiration under Fe-limited conditions were re-

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cently associated to the changes in the expression of Fe-containing enzymes, and to the induction of the glyoxylate shunt, a bypass of the TCA cycle, that has important consequences on the fate of organic carbon processed by the bacterial cell (Fourquez et al., 2014; Beier et al., 2014).

The complexity of natural sources of Fe and organic matter and the large differences in concentrations in the ocean require specific metabolic properties such as siderophore production and high affinity uptake systems for Fe, and enzyme machineries for the cleavage of complex organic carbon compounds. These metabolic capabilities were shown to be associated with specific taxa (Cottrell and Kirchman, 2000; Bauer et al., 2006; Eldridge et al., 2007; Toulza et al., 2012). Thus, it is conceivable to argue that the extent of limitation depends on the metabolic capabilities of the members within the community, and that the bulk response to either addition might be driven by specific taxa.

It was, however, surprising to note that the combined addition of Fe and C did not stimulate bacterial heterotrophic production to a larger extent than the single additions. Besides Fe and C, temperature, ranging between 3–4 °C in the present study, could have limited bacterial heterotrophic activity in surface waters of the study region. We propose that temperature set an upper limit to the potential response to Fe and C additions. Similar experiments performed at higher temperatures (9–14 °C; Church et al., 2000; Kirchman et al., 2000) observed stimulations of bacterial heterotrophic activity several fold higher than in the present study, which supports the idea of the additional control by temperature.

4.2 Spatial and temporal variability in Fe-limitation

Our results from the naturally Fe-fertilized region off Kerguelen Islands add to incubation experiments performed in prominent HNLC regions of the Southern Ocean (Church et al., 2000; Hutchins et al., 2001) and the Pacific Ocean (Hutchins et al., 2001; Kirchman et al., 2000; Kuparinen et al., 2011; Price et al., 1994), and also in high-nutrient waters off Antarctica such as the Gerlache Strait (Pakulski et al., 1996)

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and the Ross Sea (Bertrand et al., 2011) (see Table 2 for an overview). These previous studies reveal an interesting pattern that appears to be set, in part, by the dark or light incubation regime. While in dark incubations the addition of Fe alone had an effect on bacterial metabolism only in some locations, Fe amendments lead to enhanced bacterial production and growth in all incubations performed in the light and in the presence of autotrophic members of the microbial community. This suggests that the stimulation of phytoplankton by Fe and the associated release of DOM could relieve the organic carbon limitation for heterotrophic bacteria. Taken together, these results point to a strong coupling between organic carbon and Fe in controlling bacterial heterotrophic metabolism in HNLC oceans.

While Fe was clearly identified as a limiting nutrient for bacterial heterotrophic activity in the present study, the addition of Fe alone did not stimulate bacterial growth along a transect south of Tasmania to the Antarctic Polar Front (Church et al., 2000). These contrasting findings could suggest that bacterial resource limitation in the Southern Ocean varies among water masses with distinct hydrographic and chemical properties. Besides this possible spatial heterogeneity, the role of Fe as limiting nutrient could vary with season. In this case, Fe limitation would be more pronounced in early spring, as demonstrated in the present study, than in late summer (Church et al., 2000). We consider two possible underlying mechanisms to explain this seasonal pattern. First, heterotrophic bacteria and phytoplankton are competing for Fe acquisition (Fourquez et al., 2014). In spring, the phytoplankton community is dominated by small, fast growing cells that outcompete heterotrophic bacteria for Fe acquisition, whereas in summer, the lower primary production by less competitive large diatoms could result in a reduction of Fe limitation for heterotrophic bacteria. Alternatively or concomitantly, Fe limitation could be reduced in summer compared to spring due to an overall increased Fe availability resulting from enhanced Fe regeneration mediated by biological activity (Bowie et al., 2014). This could thereby relieve in part the limitation by this micronutrient for the summer bacterial community. The idea of seasonal changes in resource limitation is further supported by the higher bacterial Fe quota and cell-specific Fe uptake

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rates in spring than in summer, that point to enhanced bacterial Fe requirements early in the season (Fourquez et al., 2014). Thus, resource supply and biological interactions determine both the extent of Fe-limitation of heterotrophic bacteria, with possible important feedbacks on the Fe- and C-cycles in the HNLC Southern Ocean.

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Table 1. Date, location and environmental characteristics of the study sites. All values are mean ±SD of the mixed layer (ML).

Station	R2	E-4W	E-3	E-5
Date	27 Oct	11 Nov	3 Nov	19 Nov
Latitude S	50°38.95′	48°76.63′	48°70.22′	48°24.69′
Longitude E	66°69.27′	71°42.98′	71°96.68′	71°53.99′
Temperature ML (°C)	2.1	2.5	3.1	3.2
Z_{ML} (m)	105 ± 15	61 ± 11	38 ± 9	46 ± 13
DFe (nM)[a]	0.13 ± 0.05	0.17 ± 0.03	0.35 ± 0.05	0.06[d]
DOC (µM)	48 + 0.4	49 ± 0.4	49 ± 1	48 ± 0.3
Chl *a* ($\mu g\,L^{-1}$)[b]	0.3 ± 0.1	1.3 ± 0.1	0.6 ± 0.1	1.2 ± 0.1
Bacterial Abundance ($\times 10^8$ cells L^{-1})[c]	2.7 ± 0.3	6.0 ± 0.1	5.1[d]	4.6 ± 0.0
Bacterial heterotrophic production ($ng\,C\,L^{-1}\,h^{-1}$)[c]	2.6 ± 0.5	29.1 ± 3.9	24.9 ± 1.7	27.4 ± 1.3

[a] From Queroué et al. (2014). [b] From Lasbleiz et al. (2014). [c] From Christaki et al. (2014). [d] Only one data point available for the Z_{ML}.

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Table 2. Results from Fe- and C-enrichment experiments in the Southern Ocean and the Pacific Ocean.

Region	Treatment			Experimental Description	Study
	+ Fe	+ C	+ Fe + C		
Dark Incubations					
Southern Ocean					
Naturally Fe fertilized region off Kerguelen	+	+	+	Whole seawater 1 nM Fe 10 µM glucose, BA, Leu	Present study
Ross Sea	+	nd	nd	< 0.65 µm size fraction 1 nM Fe, BA, Leu	Bertrand et al. (2011)
Subtropical Front	–	+	+	Whole seawater 2.5 nM Fe 1–10 µM glucose BA, Leu, Tdr	Church et al. (2000)
Subantarctic Zone	–	+	+		
Subantarctic Front	–	+	+		
Antarctic Polar Front	–	–	–		
Gerlache Strait	+	nd	nd	< 0.8 µm size fraction 3.8 nM Fe, BA, Leu	Pakulski et al. (1996)
Pacific Ocean					
Coastal California				Whole seawater	Kirchman et al. (2000)
1.1 nM Fe in situ	–	+	+	2 nM Fe	
0.2 nM Fe in situ	–	–	+	1 µM glucose	
0.06 nM Fe in situ	–	–	+	BA, Leu	
Light Incubations					
Southern Ocean					
Ross Sea	+	nd	nd	Whole seawater 20 % surface irradiance 1 nM Fe, BA	Bertrand et al. (2011)
Subantarctic 46.5° S 142.0° E	+	nd	nd	Whole seawater 40 % surface irradiance 1.9 nM Fe, Leu	Hutchins et al. (2001)
Naturally Fe fertilized region off Kerguelen	+	nd	nd	Whole seawater 50 % surface irradiance 1 nM Fe, BA, Leu	Obernosterer et al. (2008)
Pacific Ocean					
South Pacific OUT patch SAGE Experiment	+	–	+	Whole seawater 40 % surface irradiance 4.9 nM Fe 6 µM sucrose, Tdr	Kuparinen et al. (2011)
Subarctic Pacific 50.0° N 145.0° W	+	nd	nd	Whole seawater 40 % surface irradiance 3.2 nM Fe, BA, Leu	Hutchins et al. (2001)
Californian Coastal Upwelling	+	nd	nd	Whole seawater 40 % surface irradiance 2.5 nM Fe, BA, Leu	Hutchins et al. (2001)
Equatorial Pacific Ocean Transect 10° N 150° W–20° S 135° W	+	nd	nd	Whole seawater On-deck incubation 1 nM Fe BA, Leu	Price et al. (1994)

A positive or negative response of heterotrophic bacteria is indicated by "+" and "–" symbols, respectively; nd – not determined. Final concentrations of added Fe and C in the incubation experiments are given. Fe was added as $FeCl_3$. BA refers to Bacterial Abundance. Leu and Tdr refer to ^{3}[H] leucine and ^{3}[H] thymidine as tracers for bacterial heterotrophic production, respectively.

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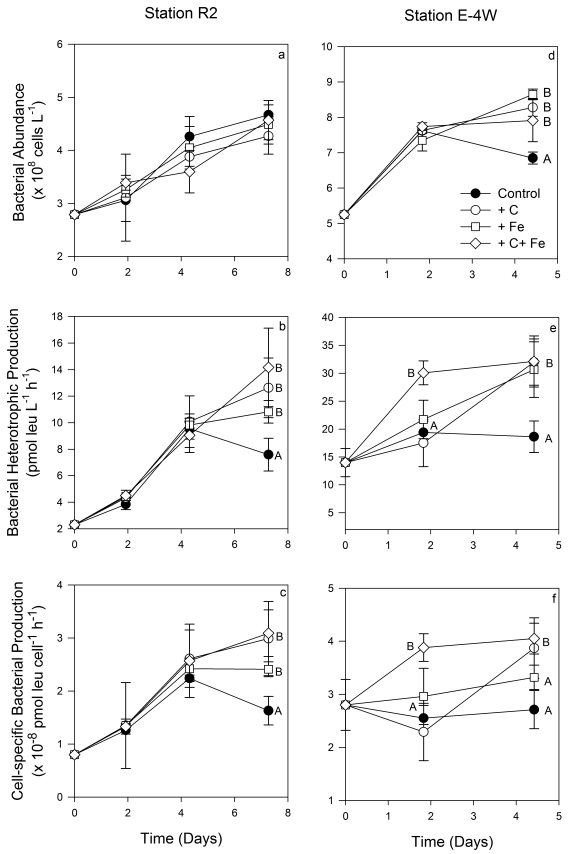

Figure 1. Changes in bacterial abundance (upper panels), bacterial heterotrophic production (middle panels) and cell-specific bacterial production (lower panels) in the control, C-amended, Fe-amended, and both C- and Fe-amended treatments over time. Treatments with the same letters are not statistically different, while different letters indicate treatments that are statistically different (Student's t test; $p < 0.05$).

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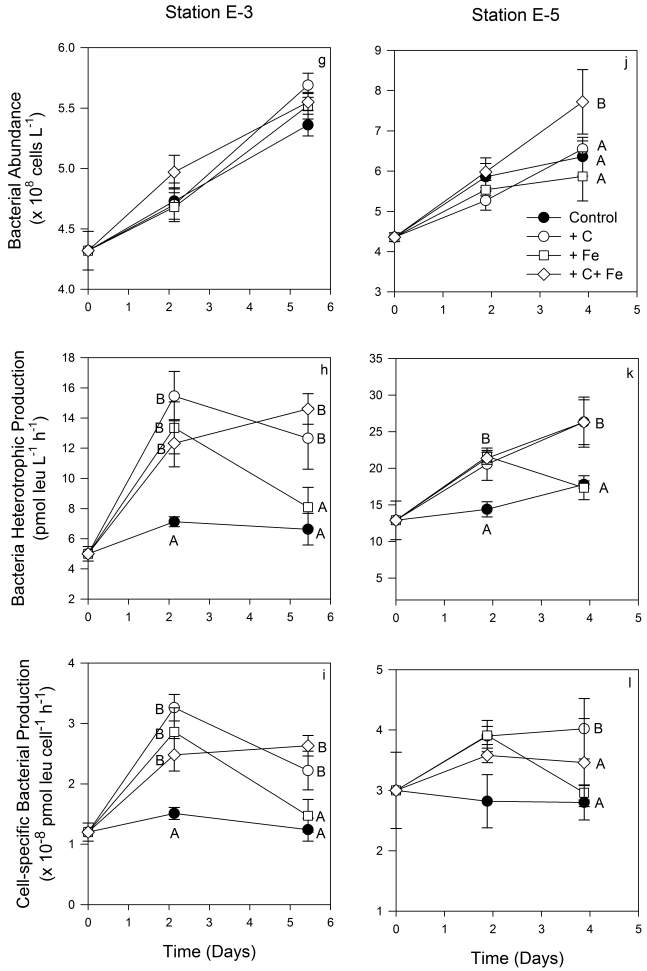

Figure 1. Continued.

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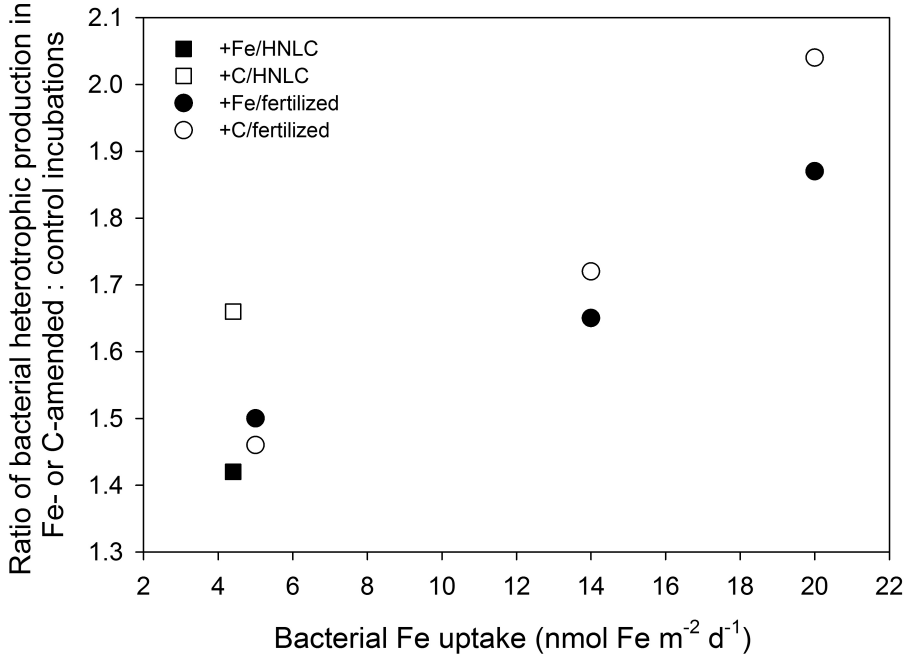

Figure 2. Relationship between the maximum extent of stimulation of bacterial heterotrophic production by Fe- or C-addition and in situ bacterial Fe uptake rates as determined by 24 h incubations of the microbial community with ^{55}Fe (Fourquez et al., 2014).

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