Microbial community response during the iron fertilization experiment LOHAFEX

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Abstract

Iron fertilization experiments in high nutrient–low chlorophyll areas are known to induce phytoplankton blooms. However, little is known about the response of the microbial community upon iron fertilization. As part of the LOHAFEX experiment in the Southern Atlantic Ocean, *Bacteria* and *Archaea* were monitored within and outside of an induced bloom, dominated by *Phaeocystis*-like nanoplankton, during the 38 days of the experiment. The microbial production increased 1.6-fold (thymidine uptake) and 2.1-fold (leucine uptake) while total cell numbers increased only slightly over the course of the experiment. 454 Tag-pyrosequencing of partial 16S rRNA genes and catalyzed reporter deposition fluorescence *in situ* hybridization (CARD FISH) showed that the composition and abundance of the bacterial and archaeal community in the iron fertilized water body was remarkably constant without developing typical bloom-related succession patterns. Members of groups usually found in phytoplankton blooms, like *Roseobacter* and *Gammaproteobacteria*, showed no or only minor response towards the bloom. However, sequence numbers and total cell numbers of the SAR11 and SAR86 clade increased slightly but significantly towards the end of the experiment. It seems that although microbial productivity was enhanced within the fertilized area, a succession – like response of the microbial community upon the algal bloom was averted by highly effective grazing. Only small-celled members like the SAR11 and SAR86 clades could possibly escape the grazing pressure, explaining a net-increase of those clades in numbers.
Introduction

Vast areas of the ocean feature low concentrations of chlorophyll a despite their high concentrations of nutrients. These so-called high nutrient–low chlorophyll (HNLC) areas cover 25 – 30% of the world oceans, mainly in the northern and equatorial Pacific and the Southern Ocean. Within these regions bioavailable iron was found to be the limiting factor for algal growth (36). Since the early 1990s, several iron fertilization experiments have been conducted in HNLC areas, showing that phytoplankton blooms can be induced (37). Most of these blooms consisted of diatoms (5), but in silicate depleted waters (e.g. during SOFeX-North) the blooms consisted mostly of Phaeocystis-like nanoplanクトン encompassing prymnesiophytes, pelagophytes and dinoflagellates (5). The enhanced primary production within such blooms triggers the biological carbon pump and may lead to a transport of biomass into the deep ocean (10, 56). Heterotrophic nanoplanクトン is potentially counteracting the net deposition of fixed carbon by mineralization of algal biomass, resulting in an immediate release of carbon dioxide back to the atmosphere.

However, not much is known about the direct and indirect effects of iron fertilization on the Archaea and Bacteria. For those iron can also be a limiting nutrient and thus its addition might have a direct effect on their growth. For example, heterotrophic bacteria accounted for 20-45% of the biological iron uptake in the Subarctic Pacific Ocean (60). Alternatively, microorganisms might be indirectly affected by the increased primary production of the algal blooms induced by iron fertilization (6, 21). Besides such a bottom-up control by nutrient and substrate availability, the microbial community will also be top-down controlled by viral lysis and protozoan grazing (13, 45, 46). Viruses are highly specific and change the microbial community by infecting discrete abundant
members (59) while heterotrophic predators rather feed on microorganisms within a particular size range (45). Both bottom up and top down control mechanisms need to be considered in interpreting microbial successions during iron fertilizations.

In an earlier study, (62) the microbial succession had been observed during a phytoplankton bloom induced by natural iron fertilization on the Kerguelen plateau, where a natural inflow of iron rich deep waters led to a major bloom. Using 16S rRNA clone libraries and single strand conformation polymorphism (SSCP), a community structure consisting mainly of *Roseobacter* NAC11-7 (*Alphaproteobacteria*), SAR92 (*Gammaproteobacteria*) and Agg58 (*Bacteroidetes*) was found in the naturally fertilized area. This is supported by the finding of major parts of the bacterial production assigned to high nucleic acid containing bacteria (HNA; 34). Outside the fertilized area, the SAR11 clade (*Alphaproteobacteria*) and *Polaribacter* (*Bacteroidetes*) dominated the microbial community (62). In contrast, no major changes in the microbial community composition of the fertilized patch were found by terminal restriction fragment length polymorphism analyses (T-RFLP) during the iron fertilization experiment EisenEx (‘Eisen’ is German for iron, ‘Ex’ for experiment), although a few days after each of the three subsequent iron additions to the water the microbial production was 2- to 3-fold increased (3).

Since all artificial iron fertilization experiments had so far focused on the onset of the phytoplankton bloom, the iron fertilization experiment LOHAFEX (‘loha’ is Indian for ‘iron’; FEX for Fertilization EXperiment) aimed at following the phytoplankton bloom in an iron fertilized eddy until its senescence and decay, thereby closing a gap in our understanding of carbon transport. This study also allowed for more continuous
monitoring of changes in the microbial diversity and community composition. The hypothesis tested in this study was that succession patterns of bacterial and archaeal populations would occur that are caused either directly or indirectly by the iron fertilization. Monitoring of the microbial community composition during the iron fertilization experiment LOHAFEX was done using 16S rRNA gene Tag-pyrosequencing and catalyzed reporter deposition fluorescence in situ hybridization (CARD FISH) techniques and semi-automated microscopy.
Material & Methods

Iron fertilization

The iron fertilization experiment was done during RV Polarstern cruise ANT XXV/3 (12th January till 6th March, 2009). A cold core eddy in the area between 47°12' S/ 49°12' and 14°00' W /16°24'' W (FIG 1) along the polar front was chosen based on satellite imagery and in situ measurements of physical and chemical parameters (Martin et al. submitted) for the iron fertilization experiment. In two fertilization steps, an acidified iron (II) sulfate solution was released into the water at days 0 (27th January, 2009) and 18 (14th February, 2009) of the experiment. We used drifter buoys, determined the photosynthetic efficiency index ($F_v/F_m$) of the surface water with a fast repetition rate fluorometer (FRRF), measured online the inert tracer gas SF$_6$ (sulfur hexafluoride) concentration which was added with the iron solution to the water, to monitor the movement of the fertilized patch. At a later stage of the experiment when enough biomass had accumulated, chlorophyll a concentrations were measured to determine the location of the fertilised patch and to define “IN” (fertilised) and “OUT” (non-fertilised) stations.

Sampling

Samples from different depth of the wind mixed layer (WML; surface to ~80 m depth in steps of 10 m) were taken inside the fertilized patch on days -1, 5, 9, 14, 18, 22, 27 and 36 (IN stations), while OUT station samples were taken arbitrarily outside the fertilized area but inside the eddy on days -1, 4, 8, 16, 23, 26 and 35 (Figure FIG 1). On days -1, 9, 16 (OUT), 18 and 36 additional samples were taken at 100 m, 300 m and 500 m (except for day 18) depth. Water was collected using 12 L Niskin bottles on a rosette. Depending on the cell abundance, duplicates of 20 ml to 100 ml of water were fixed with 1% of
formaldehyde final concentration and filtered on 0.22 µm pore size polycarbonate filters (Millipore, Eschborn, Germany) for CARD-FISH analyses. These samples were stored at -20°C. In addition, samples were taken for DNA analysis and 454 Tag-pyrosequencing on days -1, 9, 18, 36 by filtering 90 L, 85 L, 67 L and 85 L of 5 µm pre-filtered water on 0.22 µm pore size cellulose acetate filters (Sartorius, Göttingen, Germany) which were subsequently stored at -80°C.

**Bacterial production**

Bacterial production rates were estimated from the measurements of methyl-³H-thymidine (specific activity 18,000 mCi mM⁻¹; Amersham Corp., Amersham, England) incorporation rates (TdR) following the method described by Fuhrman and Azam (17). Samples were taken from 5, 10, 20 and 50 m depth in three replicates of 20 ml from each depth. An aliquot of 100 µl 60 nM working solution of ³H-thymidine was added to each tube and incubated in the dark at 4-5°C for 3 h. The thymidine uptake was stopped by the addition of 300 µl formaldehyde. Cells were collected on 0.22 µm cellulose acetate filters (Millipore India Ltd, Bangalore, India) and rinsed three times with cold trichloroacetic acid (10 % w/v) and once with ethanol (96 %, v/v; final rinse). The filters were stored individually in 8 ml scintillation vials in moisture free condition and 5 ml scintillation cocktail Ultima Golds RX (PerkinElmer, Waltham, USA) was added a day prior to radioassaying in a liquid Scintillation Counter (Perkin Elmer, Waltham, USA) onboard RV Polarstern. The amount of incorporated tritiated thymidine (TdR, pM l⁻¹ h⁻¹) was calculated using the formula given by Fuhrman and Azam (17).

The quantification of [4,5-³H] leucine incorporation was done by following the protocol of Kirchman (23). [4,5-³H] leucine (specific activity 50,000 mCi mM⁻¹; Amersham
Corp., Amersham, England) was added to 2 ml aliquots of water sampled at 5, 10, 20 and 50 m depth to a final concentration of 0.5 nM and incubated in the dark at 4-5°C for 1.5 h. At the end of the incubation TCA was added (5% final concentration). The tubes were then centrifuged at 16,000 rpm for 10 min. and then the supernatant was discarded. Before adding the scintillation cocktail for radio-assaying, all tubes were stored at 4°C for 48 h to reduce the moisture content. The amount of incorporated tritiated leucine (pM l⁻¹ h⁻¹) was calculated following the protocol of Kirchman (24).

### 454 Tag-pyrosequencing

For 454 Tag-pyrosequencing we extracted DNA from the <5 µm fraction of IN station samples on days -1, 9, 18 and 36 according to Zhou et al. (64) combined with an initial FastPrep step (MPBiomedicals, Illkirch Cedex, France) to destroy the filter. After DNA extraction, a PCR was done using the primer pair Bakt341/ Bakt805_W (19; TAB. 1). The amplicon was purified using QIAquick PCR purification kit (Qiagen, Hilden, Germany). 454 Tag-pyrosequencing of 16S rRNA amplicons was done by LGC genomics GmbH (Berlin, Germany) using Roche/454 GS FLX Titanium technology. The retrieved sequence reads had an average read length of 422 ± 52 base pairs and were classified after quality check using a BLAST-based search against the SILVA database release 108 (50, 57). Chao1, Simpson and Shannon indexes were calculated on genus level using the R standard library including the vegan suite (R Development Core Team, 2011).

### CARD FISH

To investigate the changes in the microbial community, CARD FISH was done on samples from the wind mixed layer at depth between 30 m and 60 m, depending on
station, as well as from 100 m, 300 m and 500 m depth using 11 oligonucleotide probes in a nested approach (TAB. 1). Since the WML is a homogenous water body, samples from different depth were used as duplicates. CARD FISH was done according to A. Pernthaler modified by S. Thiele (44, 58). Shortly, after embedding of filters with agarose (0.1%) and lysozyme treatment for cell permeabilization, the hybridization was done using a probe (50 ng µl\(^{-1}\)) to hybridization buffer mix of 1:100 for 2 h. Signal amplification was done using a 5-(and 6-)carboxyfluorescein labelled tyramide (1 mg ml\(^{-1}\)) to amplification buffer mix of 1:100 for 45 minutes. Both hybridization and amplification of the filters were done on glass slides in humidity chambers. All filters were 4',6-diamidino-2-phenylindole (DAPI) stained and counted manually (minimum of 1,000 DAPI signals) or using an automatic counting machine based on an epifluorescence microscope (Zeiss Axioplan II, Carl Zeiss AG, Jena, Germany; 39). The semi-automatic mode was used to count at least 2,000 DAPI signals of a size between 8 and 200 pixels with an S/N ratio of 12. A linear regression between three data sets of all stations did not show significant differences between semi-automated and manual counts (R\(^2\)=0.92; p<0.001, n=110). Thus, semi-automatic counting was used for all probes besides EUB I-III, SAR11 441, POL740 and FORM181A. The total cell counts (TCC) were calculated as an average from all DAPI counts per station and depth.

**Probe design**

A new probe, ROCT1004, targeting the *Roseobacter* clade OCT was designed using the ARB tool ProbeDesign (26; Table 1). Two helper probes (Table 1) increase the target site accessibility (16). ROCT1004 currently covers 74% of the OCT clade when analyzed against SILVA release 108 (50). This comparison also indicated 108 outgroup hits mainly
within the *Antarcobacter* clade of the *Rhodobacteraceae*. Since members of this clade are found in hypersaline lakes (26), false-positives are unlikely to be detected within our study. The probe was used in an equimolar mix with helpers at a formamide concentration of 20%.

**Statistics**

Different ANOVA tests were used to evaluate the changes in cell abundance inside and outside the fertilized patch over time. If the normality tests failed, a non-parametric ANOVA on ranks or ANOVA on measurements was performed using the multiple comparison method recommended for every single case by SigmaStat 3.5 (Statcon, Witzenhausen, Germany). The effects of time and location (IN vs. OUT) and their interactions were examined using linear (time or location) and quadratic regressions (time and location). We used linear models as suggested by permutation tests of $R^2$ (30). Generally no interactions between time and patch location were detected. Statistical calculations were implemented with the R standard library including the vegan suite (R Development Core Team, 2011).
Results

Induction of a phytoplankton bloom

The addition of acidified iron (II) sulphate solution to a clock-wise rotating cold core eddy at days 0 and 18 raised the iron concentration in the upper 30-40 m of the water column to 0.3-1.5 nM (L. M. Laglera, personal communication). This is only slightly higher compared to concentrations between 0.1-1.0 nM reported for iron depleted waters in the Southern Ocean (9). In response to the fertilization, a nearly circular phytoplankton bloom of a size of ~300 km² was induced (FIG 1). The chlorophyll a concentration increased from 0.5 µg l⁻¹ before fertilization to a maximum of 1.25 µg l⁻¹ at day 23 (M. Gauns, personal communication). The temperature in the eddy was increasing slightly from 6.5° C - 7.5° C during the course of the experiment. The fertilized patch circled twice with the eddy in the first two weeks of the experiment, before it moved south. The waters in the eddy were limited in silicic acid (<2 µM). Consequently, the bloom mainly consisted of Prymnesiophytes, and not of diatoms.

Microbial cell numbers and production

Total cell numbers (FIG 2; Supplementary figure 1) inside the fertilized patch increased slightly but significantly from 1.1 x 10⁶ at day -1 to 1.4 x 10⁶ cells ml⁻¹ at day 18 (R²=0.053; p=3.73 x10⁻⁴), and were at that date significantly higher compared to 1.1 x 10⁶ cells ml⁻¹ in the corresponding OUT stations at day 16 (R²=0.141; p=0.017). The low values at day 8 (OUT) might result from errors in eddy determination and thus samples might be from a different water body and should be treated carefully.

The microbial production measured by leucine and thymidine uptake inside the fertilized patch increased from day 5 onwards, while no significant increase was measured outside...
the fertilized patch (FIG. 2). Thymidine uptake rates increased significantly (p=0.002) 1.6
fold from about 15 pM l\(^{-1}\) h\(^{-1}\) on day 18, compared to 15 pM l\(^{-1}\) h\(^{-1}\) on day
16 (OUT), and then decreased again beginning on day 27. Leucine uptake rates doubled
from 27 pM l\(^{-1}\) h\(^{-1}\) on day 5 to 57 pM l\(^{-1}\) h\(^{-1}\) on day 18 (p=<0.001), compared to 31 pM l\(^{-1}\)
h\(^{-1}\) outside the fertilized patch on day 16.

**Bacterial diversity and community composition**

Community richness based on Chao1-values decreased from day-1 (126.0 ± 12.4) to day
9 (111.7 ± 3.4) and increased subsequently towards day 18 (134.9 ± 17.0) and day 36
(157.7 ± 18.7). However, these minute changes in the diversity of the community were
not reflected by the Shannon and Simpson indices. The Shannon index ranged from 3.26
(day 18) to 3.39 (day 36), while the Simpson index ranged from 0.936 (day 18) to 0.944
(day 9).

Within the *Alphaproteobacteria*, the *Roseobacter* clade, including the clades DC5-80-3
and OCT, and the SAR11 clade, in particular the surface 1 clade, were dominant with
together >80% of the sequences (FIG 3). The fraction of SAR11 clade sequences
increased from 49% to 67% and 62% of alphaproteobacterial sequences from day 9 to
day 18 and 36, respectively, while the *Roseobacter* clade sequences decreased from 35%
to 23% and 25% at the same days. The *Gammaproteobacteria* were dominated by the
SAR86 clade and *Alteromonadales* (including the OM60/NOR5 and the SAR92 clade;
FIG 3). An initial decrease of SAR86 clade sequences was followed by an increase from
32% to 43% and 47% at days 9, 18, and 36, respectively. This relative increase was
mirrored by a decrease of *Alteromonadales* sequences from 35% to 29% and 25% at the
same days. The diversity within the *Bacteroidetes* community was rather constant. It was
dominated by Flavobacteriales including the slightly increasing Formosa and the slightly decreasing DE2/NS5 clade (FIG 3). Polaribacter sequences made up less than 3% of the Bacteroidetes sequences.

Abundances of key bacterial clades

Since the 454 tag-pyrosequencing did not show significant changes in microbial community during the course of the experiment, and given the uncertainties in reflecting the abundances of microbial clades (65), specific oligonucleotide probes (Table 1) were selected to quantify representative phylogenetic groups by CARD-FISH in the iron fertilized patch and the surrounding waters. We used the probe mix EUB I-III and CREN554 to determine the abundance of Bacteria and Crenarchaeota. In the WML Bacteria made up $8.7 \times 10^5 - 1.3 \times 10^6$ cells ml$^{-1}$ in the fertilized patch and were significantly higher around day 18 than in the comparable OUT station at day 16 ($R^2=0.081; p=0.033$). Crenarchaeal numbers did not exceed $4.8 \times 10^4$ cells ml$^{-1}$ and did not differ significantly between IN and OUT stations. Both probes together targeted ~90% of the DAPI counts with the exception of day 26 OUT and day 36 IN, where only 78% and 71% were covered.

The SAR11 clade, as detected by probe SAR11-441 (FIG 4A), was the most abundant group accounting frequently for more than 50% of total cell counts. It increased significantly within the fertilized patch from $5.5 \times 10^5$ cells ml$^{-1}$ to $7.7 \times 10^5$ cells ml$^{-1}$ at day 18 ($R^2=0.15; p=0.005$), staying on this level until the end of the experiment. Cell numbers of SAR11 were generally higher inside the fertilized patch compared to the corresponding OUT stations ($R^2=0.09; p=0.016$). Outside the patch a slight increase was
found at the beginning of the experiment, but cell numbers reached a lower maximum of
6.4 x 10^5 cells ml\(^{-1}\) on day 5 compared to the IN stations.

Cell numbers of the *Roseobacter* clade did not change throughout the course of the
experiment in both IN (around 1.0 x 10^5 cells ml\(^{-1}\)) and OUT stations (around 8.5 x 10^4
cells ml\(^{-1}\); probe ROS537; FIG. 4B). The IN counts were significantly higher than the
OUT counts (R\(^2\)=0.31; p=7.7 x 10^{-5} cells ml\(^{-1}\)). Within the *Roseobacter* clade, the OCT
clade was also significantly higher on the IN stations compared to outside of the patch
(probe ROCT1004; FIG. 4C; R\(^2\)=0.1; p=0.022), while the *Roseobacter* DC5-80-3 clade
showed no significant changes (probe RCA1000; FIG 4D; R\(^2\)<0.01; p=0.321).

The total cell numbers of *Bacteroidetes* showed a significant difference between the IN
and OUT stations between days 8 and 27 (probe CF319a; FIG 4E; R\(^2\)=0.13; p=8.5 x 10^{-3}).
Inside the fertilized patch the highest cell numbers were found on day 18 (3.1 x 10^5 cells
ml\(^{-1}\)). This maximum was followed by a decrease in cell numbers towards the end of the
experiment. In the OUT sample of day 4 cell numbers were as high as at the IN station at
day 5 (2.5 x 10^5 cells ml\(^{-1}\)), but decreased to 2.0 x 10^5 cells ml\(^{-1}\) and below. From day 16
on bacteroidetal numbers remained stable on all the OUT stations (1.8 x 10^5 cells ml\(^{-1}\)).

These abundance patterns were not followed by *Polaribacter* (probe POL740; FIG 4F)
which showed cell numbers around 4 x 10^4 cells ml\(^{-1}\) both inside and out of the fertilized
patch over the entire course of the experiment. *Formosa* (probe FORM181A) was found
in low abundances around 2.4 x 10^4 cells ml\(^{-1}\). The DE2/NS5 clade (probe CF6-1267)
was detected at numbers between 1.1 x 10^4 cells ml\(^{-1}\), and 3.1 x 10^4 cells ml\(^{-1}\).

Similar to the abundance of *Alphaproteobacteria* and *Bacteroidetes*, the abundance of
*Gammaproteobacteria* as assessed by probe GAM42a were between 7.0 x 10^4 cells ml\(^{-1}\).
and $1.5 \times 10^5$ cells ml$^{-1}$ (FIG 4G). The dominant group within the \textit{Gammaproteobacteria} class was the SAR86 clade (probe SAR86-1245; FIG 4H), accounting for 60% – 100% of the gammaproteobacterial cell counts. Significant changes were neither found during the progression of the iron fertilization, nor between IN and OUT samples.

**Depth profiles**

Bacterial abundances beneath the WML were quantified at 5 stations at depths of 100 m, 300 m, and 500 m (FIG 5). A decrease in bacterial cell numbers on all taxonomic levels of in average 73% was found from 100 m depth to 300 m depth, followed by a minor decrease of in average 25% towards 500 m. The major decrease of total cell numbers was mainly due to decrease of the numerically dominant SAR11 clade from an average of $6.6 \times 10^5$ cells ml$^{-1}$ in the WML to an average of $3.3 \times 10^4$ cells ml$^{-1}$ at 500 m. \textit{Bacteroidetes} decreased less at days -1 and 36 and subsequently showed a significantly (p=0.004) higher absolute abundance at 300 m depth. While bacterial cell numbers decreased, \textit{Crenarchaea} increased in average 2.1-fold between 100 m ($2.5 \times 10^4$ cells ml$^{-1}$) and 300 m ($5.2 \times 10^4$ cells ml$^{-1}$) depth on all days and were stable towards 500 m depth.
Discussion

In contrast to most other iron fertilization experiments (3, 28, 43), the total cell numbers of bacterioplankton and of the major clades were rather constant throughout the LOHAFEX experiment, although a positive correlation of chlorophyll a with bacterial production implied active growth of the bacterial community (Thy: p=0.033; Leu: p=0.003). This is unlike the recently described dynamic succession of mostly bacteroidetal and gammbacterial genera after a spring phytoplankton bloom in the North Sea (50). Instead we observed a rather subtle change in numbers for the individual clades. Assumingly these changes are comparable to coastal late spring / summer blooms, rather than spring blooms. The reaction of grazers to spring blooms is rather slow, compared to summer blooms, as suggested by Wiltshire and Manly (63).

For SAR11 and Roseobacter, and to a lesser extent for Bacteroidetes, a stimulating effect during the iron fertilization on the cell numbers could be detected. SAR11 is usually dominating in oligotrophic waters outside algal blooms (40, 62), but is also highly abundant and productive in coastal and open ocean waters (31, 32). This clade increased significantly inside the fertilized patch from day 18 of the experiment on and showed high numbers until the end of the bloom. SAR11 uses low molecular weight substrates (18, 52), which might have been more abundant in the later phase of the bloom providing an advantage in resource competition for this group. In addition, SAR11 might escape protist grazing due to its small cell size.

Roseobacter as bacterial generalists are known to accompany and benefit from phytoplankton blooms (4, 54, 66) and attach often to particles and dinoflagellates (53). Inside the LOHAFEX bloom significantly higher cell numbers were found compared to
the OUT stations. However, the *Roseobacter* abundance was lower in the LOHAFEX bloom compared to other phytoplankton blooms before (66). This might be due to relatively low chlorophyll a concentrations indicating a rather weak phytoplankton bloom. Microscopic observations already on board showed that the *Roseobacter* cells, including cells from the OCT and DC5-80-3 clade, were always >1 µm in diameter. This is within the size range of potential grazers and we could occasionally find them inside larger flagellate cells (Supplementary figure 2). Therefore, we assume that the grazer community exerts a strict top-down control on *Roseobacter*, keeping their cell numbers fairly constant.

Most marine *Gammaproteobacteria*, besides members of the SAR86 clade, are r-strategists that react fast on sudden nutrient availability (51). They are also rather susceptible to enhanced mortality by grazing or viral lysis (45). Since the bacterial production was increased during the experiment, but no increase of *Gammaproteobacteria* was found, a mechanism to control the gammaproteobacterial numbers similar to the *Roseobacter* clades can be assumed. Only SAR86 cells which are about as small as SAR11 might have escaped this grazing. SAR86 is known to be abundant in oligotrophic waters (38, 44). In comparison to SAR11, SAR86 is specialized on lipid degradation (11). Thus, SAR86 might have profited from grazing pressure on potential competitors for this substrate, such as other *Gammaproteobacteria* or *Bacteroidetes*. In addition sloppy feeding from those grazers might have led to a release of fatty acids, providing more resources to SAR86.

*Bacteroidetes* have been reported to be highly abundant in *Phaeocystis* blooms in the Southern Ocean (1, 55). Marine members of the *Bacteroidetes* utilize higher molecular
weight carbon sources, such as polysaccharides (20) and proteins (7, 48, 49), and are often present in high numbers during and after algal blooms (27, 49). During LOHAFEX the *Bacteroidetes* were present in fairly high numbers (~20% of all cells) compared to late summer situations with similar water temperature and chlorophyll a concentrations in arctic regions (19, 54). They decreased with the onset of the phytoplankton bloom after the first fertilization campaign, but also at the OUT stations suggesting depletion of substrate originating from a pre-LOHAFEX phytoplankton bloom and again grazing by protozoa (57). The onset of the decay of the LOHAFEX induced *Prymnesiophytes* might have increased the substrate availability and enabled *Bacteroidetes* to slightly increase in numbers.

All bacterial groups examined decreased with depth at any time point during the course of the experiment. Similar to the WML the deeper layers showed no clear change in community composition within the respective layer. There was no accumulation of cells in deeper layers, as one would expect from a sinking and decaying algae bloom (14, 56). Only for the *Bacteroidetes* a slightly higher cell abundance could be monitored at 300 m depth on days -1 and 36 compared to days 14, 18 (both IN) and 16 (OUT). This higher numbers might result from *Bacteroidetes* cells attached to marine snow particles from the hypothesized bloom prior to LOHAFEX (day -1) and the LOHAFEX bloom (day 36). Such an accumulation of marine snow particles, measured as particulate organic carbon (POC), has been found in previous iron fertilization experiments. During the EIFEX iron fertilization experiment POC accumulated in deeper water layer inside the patch at the late phase of the bloom as well as outside the patch as a result of slower sinking particles from natural blooms which occurred prior to the experiment (56).
The analysis of iron fertilization experiments relies strongly on the exact localization of IN and OUT stations, since only the former belong to a homogeneous water mass with a common history. Therefore, only data from these stations can be compared to each other. In turn, the OUT stations might be placed not only outside the fertilized patch, but also outside the eddy, belonging to entirely different water masses originating, e.g., from the Southern Atlantic gyre. Along those lines, the large deviation in cell numbers on day 8 of LOHAFEX compared to other OUT stations might be due to an uncertainty in the determination of the eddy and therefore this station should be treated cautiously. This is also true for day 26 even though total cell counts and CARD FISH counts are similar to the preceding and following days.

In general, our findings are similar to findings from the EisenEx experiment where no changes in the microbial community were found by T-RFLP analyses (3). During EisenEx, SOIREE and IronEx II hints for a negative correlation between bacterial abundance and the abundance of heterotrophic nanoflagellates (HNFs) were found, giving rise to the assumption of a top-down controlled system by bacteriovorous grazers (3, 6, 21). Most likely in LOHAFEX an effective grazer population had established during a preceding austral summer bloom which responded directly to the increasing microbial cell numbers after the iron fertilization. On board flow cytometry and microscopic counting showed a nanoplankton abundance of ~10,000 cells ml⁻¹ (S. Thiele, I. Schulz and B. M. Fuchs, unpublished data), compared to an average of ~1000 cells ml⁻¹ in open ocean waters of the Southern Gyre (68). Assuming that most nanoplankton cells are mixotroph (15), this would result in a predator/prey ratio of ~1:100 during LOHAFEX which is 10-fold higher than the commonly observed ratio of ~1:1000 (13).
This well established community of nanoflagellates might have exerted a high grazing pressure onto the microbial community and keeping cell numbers more or less constant despite increased bacterial production. The control of the microbial community by grazers is congruent with findings from SOFeX North, where a bloom of nanoflagellates was established in silicate depleted waters (5). Furthermore, the typical grazing range of HNFs is between 1 – 3 µm cell length (45). During LOHAFEX the cell sizes of *Roseobacter* (length ~1.6 µm; n=10), *Gammaproteobacteria* (length ~1.5 µm; n=10) and *Bacteroidetes* (length ~1.0 µm; n=10) were well within this edible size range. The tiny SAR11 (length ~0.4 µm; n=10) and the similar sized SAR86 would escape the HNF grazing, explaining why they showed slight increases. These preliminary observations have to be tested in future grazing experiments during similar summer blooms with strong grazer communities.

The overall aim of LOHAFEX was to investigate the sequestration of a phytoplankton bloom induced by iron fertilization. As a consequence of the phytoplankton-bacterioplankton coupling we hypothesized that algal exudates and decaying algal biomass would result in a dynamic bacterial succession of distinct genera (57). Based on our results indicating a general lack of a substrate-driven succession or direct effects of iron fertilization on the bacterioplankton composition we speculate that - similar to the tight top-down control of the LOHAFEX phytoplankton bloom - any changes on the bacterioplankton abundance and composition were tightly controlled by flagellate grazing. In retrospect, the late summer situation, in which the LOHAFEX experiment was conducted, was characterized by a well-balanced microbial loop. Extra nutrient pulses as
induced by the iron-fertilization were effectively processed through the microbial food web.
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Tables

TAB. 1: List of oligonucleotide probes and primers used. a Probe target position on *E. coli* 16S rRNA according to (16), b Formamide concentration in the CARD FISH hybridization buffer, c Used in the mix EUB I-III, d Used in a used with the unlabeled competitor BETA42a.

Figure Legends

FIG. 1: Map of the fertilized area. The chlorophyll a picture shows the LOHAFEX bloom (encircled). Stations and days of both the IN – (black) and OUT – Stations (white) are shown in the small map. The X marks day -1, the beginning of the experiment.

FIG. 2: Total cell counts calculated from DAPI counts after CARD FISH (A) with error bars showing standard deviation of all counts. Microbial production measured as Thymidine (Thy) and Leucine (Leu) uptake rates (B) with error bars showing standard deviation of all measurements. The * marks sampling points for 454 tag-pyrosequencing.

FIG. 3: Results from 454 Tag-pyrosequencing of the WML from stations on days -1 (Control), 9 (IN), 18 (IN) and 36 (IN). *Alphaproteobacteria* (A), *Gammaproteobacteria* (B) and *Bacteroidetes* (C) are separately shown, while percentage of each group of the total sequences is stated above the columns. For simplicity reasons only clades with more than 3% of group sequences on at least
one of the days are shown. All clades below that threshold are summed up into the Others section.

FIG. 4: Absolute cell numbers calculated from total cell numbers (FIG. 2) and abundances retrieved in triplicates from CARD FISH analyzes of 3 independent filters. For simplification error bars show the standard error of the CARD FISH triplicates. Absolute cell numbers of IN (black, straight lines) and OUT (white, dotted lines) stations are shown for SAR11 (A), Roseobacter (B), the Roseobacter subgroups clade OCT (C) and clade DC5-80-3 (D), Bacteroidetes (E) and the subgroup Polaribacter (F) and Gammaproteobacteria (G) with the subgroup SAR86 (H). We used a different scale for the SAR11 clade numbers. The days of iron fertilization events are marked with asterisks (*).

FIG. 5: Depth profiles of the total cell numbers of the first 500 m of the water column. Days -1, the IN stations days 9, 18 and 36 and the OUT station day 16 where investigated additionally to the WML at depth of 100 m, 300 m and 500 m. Missing values (*) for SAR11 at days 14 and 16 (OUT) are due to limited sample quantities. In addition no sampling was done at 500 m on day 18 (#). Error bars are omitted for simplicity.

**Supplementary**

Supplementary figure 1: Table of total cell counts (TCC) and total cell numbers for all probes (beside ALF968 and CF6-1267) and all days.
Supplementary figure 2: Pictures of heterotrophic nanoflagellates with ingested cells of *Alteromonas* (A+B; probe ALT1413 (Eilers et al., 2000), *Roseobacter* (C+D) and *Bacteroidetes* (E+F) from inside the patch at different stations. Ingested cells are marked with arrows. The scale bars mark 10 µm.
<table>
<thead>
<tr>
<th>Target organism</th>
<th>Sequence (5'→ 3')</th>
<th>E. coli position</th>
<th>FA (%)</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>EUB 338c Most Bacteria</td>
<td>GCTGCCTCCCGTAGGAGT</td>
<td>338-355</td>
<td>35</td>
<td>(2)</td>
</tr>
<tr>
<td>EUB II Planctomycetales</td>
<td>GCAGCCACCGGTAGGTT</td>
<td>338-355</td>
<td>35</td>
<td>(8)</td>
</tr>
<tr>
<td>EUB HF Verrucomicrobiales</td>
<td>GCTGCCACCGGTAGGTT</td>
<td>338-355</td>
<td>35</td>
<td>(8)</td>
</tr>
<tr>
<td>NON338 Control</td>
<td>ACTCTACGGGAGGAGC</td>
<td>338-355</td>
<td>35</td>
<td>(84)</td>
</tr>
<tr>
<td>CREN554 Thaumarchaeota</td>
<td>TTAGGCCCAATAATCMTCT</td>
<td>554-573</td>
<td>8</td>
<td>(39)</td>
</tr>
<tr>
<td>ALF968 Alphaproteobacteria except Rickettsiales</td>
<td>GGTAAAGGTCCTCGGGATT</td>
<td>968-985</td>
<td>35</td>
<td>(41)</td>
</tr>
<tr>
<td>SAR11-441 SAR11 clade</td>
<td>AAAAAATACAGTCATTCTCCTCCCCGAC</td>
<td>441-463</td>
<td>25</td>
<td>(52)</td>
</tr>
<tr>
<td>RO5537 Roseobacter, SARR3</td>
<td>CAACGCTAACCCCTCCC</td>
<td>537-553</td>
<td>35</td>
<td>(12)</td>
</tr>
<tr>
<td>RCA1000 Roseobacter clade DC5-80-3 (former RCA)</td>
<td>ATCTCTGTTAGTAGCACA</td>
<td>1000-1017</td>
<td>20</td>
<td>(57)</td>
</tr>
<tr>
<td>RCA1000-comp Competitor to RCA1000</td>
<td>ATCTCTGTTAGTAGCACA</td>
<td>1000-1017</td>
<td>20</td>
<td>(57)</td>
</tr>
<tr>
<td>RCA1000-h3 Helper to RCA1000</td>
<td>CGTCCCCGAAGGAACTACCC</td>
<td>1018-1038</td>
<td>20</td>
<td>(57)</td>
</tr>
<tr>
<td>RCA1000-h5 Helper to RCA1000</td>
<td>GGATGTAAGGTTGGTGGG</td>
<td>979-999</td>
<td>20</td>
<td>(57)</td>
</tr>
<tr>
<td>ROCT1004 Roseobacter clade OCT cluster</td>
<td>CTCCATCTTCGGAGC</td>
<td>1004-1021</td>
<td>20</td>
<td>This study</td>
</tr>
<tr>
<td>ROCT1004-h3 Helper to ROCT1004</td>
<td>CTCCATCTCCTCGGAC</td>
<td>1022-1042</td>
<td>20</td>
<td>This study</td>
</tr>
<tr>
<td>ROCT1004-h5 Helper to ROCT1004</td>
<td>GAGGAGTAGGAGGAGG</td>
<td>984-1004</td>
<td>20</td>
<td>This study</td>
</tr>
<tr>
<td>GAM422a Gammaproteobacteria</td>
<td>GCCCTCCCCACATCGTT</td>
<td>1027-1043</td>
<td>35</td>
<td>(35)</td>
</tr>
<tr>
<td>SAR86-1245 SAR86 clade</td>
<td>TTAGCCCTCCGTTAT</td>
<td>1245-1262</td>
<td>35</td>
<td>(67)</td>
</tr>
<tr>
<td>SAR86-1245-h3 Helper to SAR86 1245</td>
<td>GGTATGGCACCACCTCGGCG</td>
<td>1263-1283</td>
<td>35</td>
<td>(67)</td>
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<tr>
<td>SAR86-1245-h5 Helper to SAR86 1245</td>
<td>CATTTCGACCTCGGTGAGT</td>
<td>1222-1242</td>
<td>35</td>
<td>(67)</td>
</tr>
<tr>
<td>CF319a most Flavobacteria, some Bacteroidetes, some Spingobacteria</td>
<td>TGGTCCGTGTCCTCAGTAC</td>
<td>319-336</td>
<td>35</td>
<td>(34)</td>
</tr>
<tr>
<td>CF6-1267 marine Flavobacteria - DE2 clade (incl. NS5)</td>
<td>GAAGATTGCCCTCTCCTC</td>
<td>1267-1284</td>
<td>35</td>
<td>(25)</td>
</tr>
<tr>
<td>Primer Code</td>
<td>Organism</td>
<td>Description</td>
<td>Sequence</td>
<td>Length</td>
</tr>
<tr>
<td>-------------</td>
<td>----------</td>
<td>-------------</td>
<td>----------</td>
<td>--------</td>
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<tr>
<td>FORM181A</td>
<td>Formosa related clade A</td>
<td>GATGCCACTCTAAGAGAC</td>
<td>181-199</td>
<td>25</td>
</tr>
<tr>
<td>FORM181B</td>
<td>Competitor to FORM181A</td>
<td>GATGCCACTCTTAGAGAC</td>
<td>181-199</td>
<td>35</td>
</tr>
<tr>
<td>POL740</td>
<td>Polaribacter</td>
<td>CCC TCAGGGTCAGTACATACG T</td>
<td>740-761</td>
<td>35</td>
</tr>
<tr>
<td>BAKT_341_F</td>
<td>Primer Bacteria</td>
<td>CCTACGGGNGGCGWCGAG</td>
<td>341-358</td>
<td>-</td>
</tr>
<tr>
<td>BAKT_805_R</td>
<td>Primer Bacteria</td>
<td>GACTACHVGGGTATCTAATCC</td>
<td>805-785</td>
<td>-</td>
</tr>
</tbody>
</table>
FIG 4. Absolute cell numbers calculated from total cell numbers (FIG. 2) and abundances retrieved as triplicates from CARD-FISH analyses of 3 independent filters. Per amplification error bars show the standard error of the CARD-FISH triplicates. Absolute cell numbers of H (black, straight line) and COT (white, dotted line) stations are shown for SAR11 (A), Roseobacter (B), the Roseobacter subgroup clade OCT (C) and clade DC1-50-2 (D), Bacteroides (E) and the subgroup Polaribacter (F) and Gammaproteobacteria (G) with the subgroup SAR16 (H). We used a different scale for the SAR11 clade numbers. The days of spore stimulation events are marked with asterisks (*)