

Response of Polar front phytoplankton and bacterial community to micronutrient amendments

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Abstract

In a shipboard microcosm experiment we manipulated cobalt (Co), copper (Cu), iron (Fe), and Fe + Co availability in surface water samples collected during austral summer of 2013 from a Polar front (PF) location in the Indian Sector of Southern Ocean, to examine the responses of phytoplankton as well as bacterial community (BC) to these micronutrient amendments. Total chlorophyll *a* (Chl *a*) concentration increased significantly ($P < 0.01$) in all micronutrient- enriched microcosms (MEM), with the incubation period unlike in the control microcosms (CM). Bacterial abundance in Co, Fe and Fe + Co-enriched microcosms increased 1.5 fold within 5 days of incubation, unlike in other microcosms. Despite significant increase in Chl *a*, the DGGE banding pattern reflects minor differences in the BC. However, there were significant differences in relative abundance (i.e band intensity) of two most responsive phylotypes identified as *Rhodospirillales* and *Rosebacter* sp of *Alphaproteobacteria* group between MEM and CM. Strong correlation was not discernable between Chl *a* concentration and bacterial abundance as well as BC structure. Apparently autotrophic processes in this region are not only limited by the availability of Fe, but also Co and Cu.

Keywords: Southern Ocean, Polar front, micronutrients, microcosm, phytoplankton, HPLC, bacterial community, DGGE

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1. Introduction

A third of the 108 elements are known to have biological functions (Brantley et al. 2001). Of these elements, the trace metals/micronutrients, such as Mn, Fe, Co, Ni, Cu, Zn, and Cd are found specifically as cofactors or as part of cofactors in enzymes and as structural entities in various proteins (Butler, 1998). In the marine ecosystem, trace metals are required in various processes such as photosynthesis (Morel et al. 1991), carbon fixation (Reinfelder et al. 2000), and in various biogeochemical processes (Morel et al. 2003, Morel and Price, 2003). However, these trace metals/micronutrients are present in very low concentrations in the open ocean (Ellwood, 2004, 2008; Ellwood et al. 2005; Croot et al. 2011; Hassler et al. 2012). They readily react with organic compounds and organic ligands (Ellwood, 2004, 2008; Ellwood et al. 2005; Lohan et al. 2005). As a result their bioavailability to sustain phytoplankton is reduced. The effect of trace metal availability on primary productivity/phytoplankton growth, community composition and carbon sequestration has been reported earlier (Morel et al. 1991; Morel et al. 1994; Sunda and Huntsman, 1995; Coale et al. 1996; Boyd et al. 2000; Buitenhuis et al. 2003; Saito and Goepfert, 2008; Saito et al. 2008). However, the impact of these dynamics on bacterial community (BC) structure is not that well understood.

Heterotrophic bacteria are critical part of 'biological carbon pump' and influence the efficiency of carbon export via mineralization of organic matter back to inorganic phase and/or conversion of organic matter to biomass (Robinson and Ramaiah, 2011). It has been shown that trace metal limitation (particularly Fe) leads to the lower electron transport chain (ETC) activity and reduce carbon growth efficiency in marine bacteria (Trotell et al. 1996). As a result, bacteria mineralize a large proportion of ingested carbon at the expense of biomass production (Kirchman et al. 2003). This retards the carbon export to deeper depth from the surface of the ocean. The high surface area to volume ratio of bacteria and their ability to produce the organic ligands make them well suited to effectively scavenge trace elements (Azam et al. 1983; Granger and Price, 1999). However, despite of all these adaptations information on growth and activity of bacteria in Southern Ocean regions are limited. Understanding factors controlling bacterial growth, activity and community composition can provide insights on the degree to which they can mediate organic carbon fluxes in the Southern Ocean.

Earlier studies reported iron limitation (Pakulski et al. 1996), dissolved organic matter (DOM)/dissolved organic carbon (DOC) limitation (Hall and Safi, 2001; Oliver et al. 2004) and co-limitation by iron and DOM together (Church et al. 2000) as limiting factors of bacterial growth and activity in Southern

Ocean regions. Generally, most of these studies focused on bacterial abundance and activity. However, only fewer studies reported response of BC to iron (Hutchins et al. 2001, Thiele et al. 2012) and organic matter (Simon et al. 2012) addition in Southern Ocean regions. Particularly, the effect of other micronutrients Co and Cu, which share several properties with Fe in limiting the growth of phytoplankton and/or in mediating their community structure (Morel et al. 1994; Sunda and Huntsman, 1995; Buitenhuis et al. 2003; Saito and Goepfert, 2008; Saito et al. 2008), on BC in the Indian sector of the Southern Ocean, remains to be deciphered. In this study we manipulated cobalt (Co), copper (Cu), and iron (Fe) availability in the samples collected from polar front of Indian sector of the Southern Ocean, in order to examine their impact on total chlorophyll *a* (Chl *a*), phytoplankton community, bacterial abundance as well as community structure. Specifically, we intended to study the coupling between phytoplankton growth and BC structure under these micronutrient amendments.

2. Materials and methods

2.1 Microcosm experimental setup

A microcosm experiment was conducted in a Polar front (PF) location in the Indian Sector of the Southern Ocean [Latitude: 56°35'.235S; Longitude: 57° 40'.342E] (Figure 1), on-board ORV Sagar Nidhi at 10.00 AM on February 5, 2013. For this experiment, 120 liters of unfiltered surface seawater was collected using an acid cleaned polyethylene bucket. In the present study unfiltered seawater samples were used to perform the microcosm experiments in order to avoid diversion from natural conditions as has been suggested by Banse, (1991), Buma et al (1991), and Scharek et al (1997). The seawater was distributed (4.5L per flask) to pre-cleaned flasks of 5 liter capacity. Prior to dispensing seawater into them, the flasks were sequentially cleaned with detergent, 50% v/v nitric acid (ACS grade, Sigma-Aldrich, India), 10% v/v hydrochloric acid (ACS grade, Sigma-Aldrich, India). The flasks were allowed to retain 10% hydrochloric acid rinse for 1 week before being rinsed and filled with ultrapure water (Type-I grade) one day before the experiment. This ultrapure water was discarded and flasks were finally rinsed with the sample seawater. Trace metals as chloride salts obtained from Sigma-Aldrich, India were dissolved in ACS grade 0.01 N HCl, pH ~ 1.0. After filling with sample seawater, 1 ml of concentrated trace metal solutions viz, Co, Cu, Fe, and Fe + Co were added separately to the flasks so as to attain a final concentration of 10 nM L⁻¹. The concentration of trace metals for the amendment experiment was chosen based on published literature. The reported range of trace metals in microcosm experiments range from 0.5 to 10 nM (Coale 1991, Scharek et al 1997, Church et al 2000, Kirchman et

al 2000, Olson et al 2000, Hutichins et al 2001, 2002, Becquevort et al 2007, Eldridge et al 2007, Ramaiah et al submitted). However, in the bottle incubation experiments, part of trace metal added become complexed by the dissolved organic matter (Ellwood, 2004, 2008; Ellwood et al. 2005; Lohan et al. 2005) and a portion of added trace metals will probably be adsorbed to the walls of the bottle, and become unavailable for phytoplankton (Sunda, 1988, Coale, 1991, Scharek et al 1997). To ensure sufficient concentration of bio-available/bio-assessable trace metals during the 15 day long incubation experiment 10 nM concentration of trace metals was added.

All the flasks were incubated in the shipboard refrigerated cold room at $4\text{ C}^{\circ} \pm 1$. To avoid any trace metal contamination the inner walls of the cold room were covered with the thin plastic sheets prior to the experiment. A total of 15 flasks including 12 treatments (three flasks per treatment or micronutrient amendment) and 3 controls were exposed to a light intensity of $150\ \mu\text{mol photons m}^{-2}\ \text{s}^{-1}$ (under cool fluorescent light) under 16: 8h light: dark cycle. Microcosms were maintained at light intensities of $150\text{-}160\ \mu\text{mol photons m}^{-2}\ \text{s}^{-1}$ to mimic the *In-situ* PAR $\sim 170\ \mu\text{mol photons m}^{-2}\ \text{s}^{-1}$ (Gandhi et al 2012). From these a set of five flasks (one control and four treatments) were removed for collecting samples for total Chl *a*, phytoplankton pigments, bacterial abundance, and bacterial community analysis at 5 days interval, until 15th day. To evaluate the direct effect of micronutrient amendments on microbial assemblages a set of five flasks, one dark control and four others Dark-Co (DCo), dark-Cu (DCu), dark-Fe (DFe), and dark-Fe + Co (DFe+Co), were incubated at $4\text{ C}^{\circ} \pm 2$ under complete dark conditions and were analysed only after 15 days of incubation.

2.2 In-situ trace metal concentrations and physical characteristics.

The range of dissolved- Fe and -Co reported earlier in the Indian Sector of Southern Ocean are 0.02 - 0.09 nM (Planquette et al. 2007) and 24 - 49 pM (Castrillejo et al. 2013), respectively. The range of dissolved Cu is 0.5 - 2.5 nM (Boye et al. 2012). Sea surface temperature (SST), sea surface salinity (SSS), mixed layer depth and photo-synthetically active radiation (PAR) reported by Gandhi et al (2012) at this station respectively are $2.5\text{ }^{\circ}\text{C}$, 33.8 psu, 65 m and $170\ \mu\text{mol photons m}^{-2}\ \text{s}^{-1}$.

2.3 Dissolved nutrients, organic and inorganic carbon measurements

For dissolved nutrient analysis 100 ml sample (in duplicate) from each microcosm was filtered through $0.22\ \mu\text{m}$ pore size membrane filter (Millipore, USA) and stored frozen at $-80\text{ }^{\circ}\text{C}$, until analysed. The nutrients (nitrate, nitrite, silicate, and phosphate) were measured using Skalar autoanalyser (Skalar Analytical, The Netherlands) setup back at the NIO, Goa, following standard methods (Grasshoff et al.

1983). For analyzing dissolved-organic carbon (DOC) and -inorganic carbon (DIC) two sub-samples of 100ml from each microcosm was filtered through pre-ashed GF/F filter paper (Whatman, USA), collected in acid cleaned high density polyethylene bottles, and stored at -80 °C, until analysed. DOC and DIC was measured using Liquid TOC analyser (Liqui TOC II, Elementar HANAU, Germany) as per manufacturer's protocol.

2.4 Pigment analysis and phytoplankton counts

For estimating total Chl *a* as well as accessory pigments, two sub-samples of 1 liter each from each flask was filtered through GF/F filter paper (Whatman, USA). The filter paper was stored at -80 °C, until pigment extraction was performed back home in the laboratory. The pigments were extracted in 3 ml of 100% acetone. To minimize pigments degradation extraction was performed within 5 minutes at 4°C under low light conditions. The extract was passed through a Teflon syringe cartridge (Millipore, USA) having a glass fiber pre-acrodisc filter (pore size 0.45 µm) to remove the cellular debris. The clear extract was collected in a 5 ml glass vial and placed directly into the temperature controlled (5°C) auto-sampler tray for HPLC analysis. The pigments were separated in a HPLC system (Agilent Technologies, USA) following a modified method of Van Heukelem (2002) as described in detail by Roy et al. (2006). Briefly, Eclipse XDB C8 (4.6 150 mm) column was maintained at 60 °C. Elution at 1.1 ml min⁻¹ was performed using a linear gradient program over 22 min with 5/95% and 95/5% of solvents B/A, where solvent B was methanol and solvent A was (70:30) methanol and 1 M ammonium acetate (pH 7.2). An isocratic hold at 95% solvent B between 22 to 27 min was used for the eluting α or β -carotene. The eluting pigments were detected at 450 and 665 nm (excitation and emission) by the diode array detector. All chemicals used were of HPLC-grade, procured from E. Merck (Germany). For enumerating phytoplankton counts subsamples of 200 ml were fixed with few drops of acidified Lugol's iodine. The phytoplankton in initial sample and that from day-15 were counted and identified as described earlier by (Naik et al. 2011). Three replicates of 1 ml concentrate (200ml reduced to 10 ml) were pipette into Sedgewick rafter chamber and examined under an Olympus inverted microscope at $\times 100$ and $\times 400$ magnifications. Dinoflagellates were identified using the keys provided by Tomas (1997), and Horner (2002).

2.5 Enumeration of total bacterial cells

Total bacterial counts (TBC) were made using a modified method of Porter and Feig (1980). In brief, subsamples of 50 ml (in duplicates) were fixed with buffered formaldehyde (2% final concentration) and

stored at 4°C in the dark until taken up for analysis. The samples were analysed within one week after reaching NIO, Goa. One to five ml aliquots of these samples were incubated with 4'6-diamidino-2-phenylindole (DAPI, 20µl of 1µg ml⁻¹ working solution per ml) and filtered onto black 0.22 µm-pore-size polycarbonate membrane filters (Millipore, USA). Epifluorescence microscopic counts were made using Olympus BX-51 microscope with the help of Image Pro-plus software (Media Cybernetics, USA). TBC were made from minimum of 20 randomly chosen microscopic fields from each sample to obtain a reliable mean.

2.6 DNA extraction

For extraction of DNA, 2.0 liters sub-sample from each microcosm was passed through a sterivex cartridge fitted with 0.22 µm pore size membrane filter (Millipore, USA) at 60 ml min⁻¹ using a peristaltic pump. Sterivex cartridge was filled with 1.8 ml of lysis buffer (50 mM Tris pH 8.3, 40 mM EDTA and 0.75 M sucrose), sealed and, stored frozen at -80°C until nucleic acid extraction was done in the laboratory following the modified method of Ferrari and Hollibaugh (1999). Following the extraction the DNA was collected and suspended in 40µl TE buffer. Finally, DNA integrity was checked by agarose (0.8 %) gel electrophoresis and stored at -80°C until taken up for further analyses.

2.7 PCR amplification

DNA extracted from all microcosms was subjected to DGGE-PCR, using the method described in Singh and Ramaiah (2011). In brief, V3 region of bacterial 16S rRNA gene was amplified, using DGGE specific primers sets, 357F-GC (5'-GCCCCGCCGCGCGGGCGGGGCGGGGCAC

GGGGGGCCCTACGGGAGGCAGAG-3') and 518R (5'-ATTACCGCGGCTGCTGG-3') (Muyzer et al. 1993), in the thermocycler (Applied Biosystems, USA). The PCR mixture (50 µl) contained 1µl of extracted DNA (5-50 ng µl⁻¹), 1 µl of each primer at a concentration of 0.5µM, 25µl of ReadyMix Taq PCR mix (Sigma-Aldrich, USA) [1.5 U Taq polymerase; 10mM Tris-HCl, 50mM KCl, 1.5mM MgCl₂, 0.001% gelatin, 0.2mM deoxynucleoside triphosphate (dNTP), stabilizers], and 22µl of milliQ water. The DGGE-PCR was carried out with the following temperature profile: an initial denaturation step of 3 min at 95°C, followed by 10 cycles of 94°C for 30 sec, 65-55°C for 30 sec (decrease by 1°C after every cycle) and 72°C for 30 sec. Additional 15 cycles were allowed at the 55°C annealing temperature. The final extension step was for 10 min at 72°C. To maximize the detection of as many different 'species' as possible, at least three different PCR reactions were carried out for each sample. These triplicate PCR products thus obtained were pooled before performing DGGE.

2.8 DGGE analysis

In order to examine the effect of micronutrient amendments on the bacterial community structure, detection of DGGE bands was carried out using the D-Code universal mutation detection system (Bio-Rad, USA) using the method described by Jain et al (2014). In brief, equal concentration of DNA per sample (~1.5 µg per lane) was loaded on the DGGE gel. The PCR products were resolved on 10% (w/v) polyacrylamide gel containing 30% to 60% denaturing gradient of urea and formamide for 16 hr at 60 °C at a constant voltage of 60 V in 1X TAE (40 mM tris– HCl, 20 mM acetic acid, 1 mM EDTA). The gels were stained for 30 min in 1X TAE buffer containing ethidium bromide (0.5 µg ml⁻¹), washed for 15 min and, visualized in a Gel-Doc system (Bio-Rad, USA) using Quantity One software (Bio-Rad, USA).

2.9 Quantitative analysis of DGGE bands

Digitized DGGE gel images were analyzed with Quantity-one software (version 4.65, Bio-Rad, USA). Using rolling disk subtraction logic the software carries out a density profile analysis for each lane, detects the bands, and calculates the relative contribution of each band to the total lane intensity. Each DGGE band was counted as an operational taxonomic unit (OTU). The bands occupying the same position in different lanes of the gel were matched with matching algorithm of the software at < 1% tolerance level. Gels were cross checked visually as well for the number of bands per lane. An intensity matrix was constructed based on the relative contribution of each band to the total intensity of the lane. Univariate analyses were performed with PRIMER v6 software package [Plymouth Marine Laboratory, UK]. Total numbers of DGGE bands were used to calculate the OTU richness (S) in each sample. The relative intensity of each band (P_i) was used to calculate (a) Shannon diversity index (H') and (b) Pielou's equitability index (J') to describe the possible changes in the dominances among DGGE-OTUs according to the following formulae:

$$H' = - \sum_{i=1}^S P_i (\ln P_i) \quad (a)$$

$$J' = \frac{H'}{\ln S} \quad (b)$$

Where S is the total number of OTUs and P_i is the relative intensity of the i^{th} OTU.

2.10 Excision, sequencing and phylogenetic analysis of DGGE bands

The excision and sequencing of DGGE bands were performed using the modified method of Singh and Ramaiah, (2011). In brief, a total of 12 bands at different position in the gel were excised using a sterile scalpel and re-amplified using primers 357F (without GC clamp) and 518 R. The purified PCR products were then cloned into pCR[®]4-TOPO vector (Invitrogen, USA) and transformed into *E. coli* TOP-10 competent cells according to manufacturer's instructions. The clones were then sequenced using BigDye[®] Terminator v3.1 cycle sequencing using ABI 3130_{XL} genetic analyzer (Applied Biosystems, USA). The sequences obtained were compared with NCBI database sequences using BLAST algorithm. Phylogenetic tree of 12 DGGE band sequences was constructed to visualize the relationship and affiliations with the closest relative sequences from NCBI database. The tree was constructed in MEGA-5 software using maximum composite likelihood as substitution model and neighbor-joining as statistical method.

2.11 Nucleotide sequence accession numbers

All 12 band sequences were non chimeric (<http://decipher.cce.wisc.edu/>) and were submitted to the NCBI GeneBank database. The accession numbers obtained for the DGGE bands are KF951485 to KF951496.

3. Results

3.1 Nutrient concentrations

The concentrations of nitrate and silicate increased with the incubation period during the experiment (Table-1). After 5 days of incubation, both nitrate and silicate were higher in micronutrient enriched microcosms (MEM) than those in the control microcosm (CM). Subsequently, nitrate and silicate reduction in MEM compared to the CM during incubation period were not statistically significant.

3.2 Dissolved –organic and –inorganic carbon concentrations

DOC concentration in Co (student's *t* test, $T = 5.4$, $P < 0.01$), Cu ($T = 3.18$, $P < 0.05$), Fe ($T = 2.2$, $P < 0.1$), and Fe + Co ($T = 4.4$, $P < 0.05$) enriched microcosms were statistically lower than that of the CM (Table 2). Interestingly, DOC increased with the incubation period in MEM and CM while DIC concentration decreased with the incubation only in MEM but not in the CM (Table 2). However, higher DIC concentration was observed in the Fe enrichment by day15.

3.3 Total Chl *a*, accessory pigments and phytoplankton counts

Total Chl *a* increased significantly ($T = 3.42$, $P < 0.01$) in MEM with the incubation period, unlike in the CM (Figure 2A). There was no or little accumulation of Chl *a* in the Cu and Fe amended microcosm in the first 5 days of incubation. Following this lag period dramatic increase in Chl *a* was observed for both Cu and Fe amended microcosms. At the end of incubation Chl *a* accumulation was higher in Cu and Co amended microcosms compared to the Fe enriched microcosms. Total Chl *a* concentration in all microcosms incubated under complete dark were significantly ($T = 5.21$, $P < 0.01$) lower than those subjected to light: dark cycles (data not shown). The results of HPLC analysis of major accessory pigments is shown in Table 3. The concentrations of taxon-specific accessory pigments such as 19'-butanoyloxyfucoxanthin (19-but, $T = 2.12$, $P < 0.05$), 19'-hexanoyloxyfucoxanthin (19-hex, $T = 2.81$, $P < 0.05$), Fucoxanthin (fuco, $T = 2.22$, $P < 0.05$) and Divinyl Chl *a* (DV Chl *a*, $T = 2.51$, $P < 0.05$) were significantly higher in the MEM than that of CM. In order to avoid quantitative variations and to reveal taxonomic variations only the pigment concentrations were normalized to Chl *a* (Figure 2B and C). The 19-hex: Chl *a* ratio decreased significantly from 10th to 15th day of incubation in Co ($T = 5.7$, $P < 0.001$), Fe ($T = 7.8$, $P < 0.001$) and Fe + Co ($T = 2.9$, $P < 0.05$), except in CM and Cu amended microcosm (Figure 2B). Conversely, fuco: Chl *a* ratio increased with the incubation period in most of the microcosms, except in Cu enriched microcosm which showed the least increase (Figure 2C). Interestingly, 19-hex: fuco ratio in all the microcosms decrease gradually with the incubation but this decrease was more pronounced in the Co, Fe, and Fe+Co compared to the CM and Cu enriched microcosms (Figure 2D). In general the diatom cell counts in CM and MEM by day 15 were less than those in the initial sample (Table-4). Interestingly, the number of diatoms was least in the Fe-amendment followed by Cu amended microcosms.

3.4 Bacterial abundance

In Fe, Co, and Fe + Co amended microcosms bacterial abundance increased ~ 2 fold in the 5 days of incubation. Following this rapid increase, the abundance decreased in Fe, Co, and Fe + Co with the incubation period (Figure 3). In dark incubated microcosms, including dark-control (DC), and dark-Cu (DCu), bacterial abundance was higher than that of the microcosms exposed to light: dark cycle, after 15 days of incubation (Figure 3). Interestingly, bacterial abundance in dark-Co (DCo) and dark-Fe (DFe) was almost half of the DC, but comparable to that of control, Co and Fe enriched microcosm.

3.5 Bacterial community response to micronutrient amendments

The OTU richness, Shannon diversity index (H') and Pielou's evenness index (J') decrease with the incubation period in all microcosms (Table 5). DGGE banding pattern reflects minor differences in the BC, in terms of dominance and/or decrease of unique phylotypes, among MEM and CM (Figure 4A). However, significant increase in relative abundance (i.e band intensity) of ubiquitous phylotypes was found in the MEM compared to the CM (Figure 4B). Since equal amount of DNA $\sim 1500\text{ng } \mu\text{l}^{-1}$ was added to each lane, the increased band intensity ought to indicate positive response of phylotypes/OTUs to micronutrient enrichment. Particularly, band-7 (B-7) and band-12 (B-12) showed maximum response/increase in the intensity compared to the other bands in MEM (Figure 4B). The intensity of B-7 decreased, while the intensity of B-12 increased in all dark incubated microcosms compared to those subjected to light: dark cycle, after 15 days of incubation (Figure 4B).

3.6 Phylogenetic analysis of DGGE bands

In total, 12 DGGE bands were excised, cloned and sequenced. Of these four sequences were affiliated to *Alphaproteobacteria*, three to *Gammaproteobacteria*, two to *Deltaproteobacteria*, and one each to *Cyanobacteria*, *Bacteroidetes*, and Chloroplast (Table 6). Based on DGGE band sequences a phylogenetic tree was constructed to compare their affiliation to the sequences in NCBI database. (Figure 5). Most of the sequences matched very well ($\sim 99\%$ similarity) with the uncultured marine bacteria reported from Southern Ocean, South Sea, North Atlantic Ocean, South and Equatorial Pacific Ocean, (Figure 5, Table-6). The bacterial community in the initial sample is dominated by members of *Alphaproteobacteria* and *Gammaproteobacteria*. The two most responsive phylotypes B-7 and B-12 in the MEM, were identified as *Rhodospirillales* and *Rosebacter* sp of *Alphaproteobacteria* group, respectively.

4. Discussion

The increased concentration of nitrate and silicate in the microcosms with the incubation period indicates microbially-mediated recycling of organic matter/organic nitrogen /dead/decomposing diatoms present in the initial sample (Bidle and Azam 2001; Sanders et al. 2007). The higher concentration of nitrate and silicate in MEM than that of CM after 5 days of incubation, suggests rapid or increased mineralization of organic matter and/or nutrient regeneration via microbial loop in presences of these micronutrients (Becquevort et al. 2007). DOC (Ogawa et al. 1999) and DIC (Sabine et al. 2002) concentration in the initial sample is comparable to the earlier reported values in the Southern Ocean.

The lower DOC concentration in MEM than that of CM indicate its utilization by bacterial population in presences of these micronutrient enrichments. DOC accumulation in microcosms during extended incubation could be either due to refractory DOC (Legendre and Le Fevre, 1995) and/or reduction in DOC utilization due to decreased bacterial abundance.

There is substantial evidence that Fe availability in the Southern Ocean water limits the phytoplankton growth/primary production/Chl *a* concentration (Martin et al. 1990; Pakulski et al. 1996; Scharek et al. 1997; Timmermans et al. 2001, de Baar et al. 2005; Smetacek et al. 2012). However, the effect of Co and Cu on phytoplankton growth in Southern Ocean is not that well understood (Hassler et al. 2012, Moore et al. 2013). Our results demonstrate that phytoplankton growth/primary production/Chl *a* synthesis in PF region was not only limited by the availability of Fe but also by Co and Cu. Previous bottle incubation studies reported that the effects of Co and Cu addition on phytoplankton growth are less substantial than those for Fe (Coale, 1991; Crawford et al. 2003; Franck et al. 2003; Saito et al. 2005). In this regard our results are noteworthy, wherein we observed higher Chl *a* in Cu and Co added microcosms compared to the Fe enriched microcosms, at the end of incubation period. This could be due to the adaptive strategies employed by phytoplankton, variation in phytoplankton community structure, and/or decrease in grazing activities (Coale 1991). Biswas et al (2013), reported that increased availability of Cu enhances Fe acquisition capabilities as well as Chl *a* concentration in the phytoplankton. Under Fe limited conditions phytoplankton use Cu-containing plastocyanin instead of Fe-rich cytochrome in their photosynthetic pathways (Peers et al. 2005; Peers and Price, 2006). The positive effect of Co addition (3 to 8 nM L⁻¹) on phytoplankton growth had been reported earlier (Graneli and Risinger, 1994; Mitrovic et al. 2004). Co addition can benefit phytoplankton directly via substituting/replacing Zn from carbonic anhydrase enzyme (Price & Morel 1990), and indirectly by acting as substrate for vitamin B₁₂ synthesis by microorganisms (Panzeca et al. 2008).

The taxon-specificity of major accessory pigments found in our samples is described as follows. Chl *a* is the marker of total autotrophic biomass. The 19-hex and 19-but characterizes prymnesiophytes and pelagophytes, respectively, while fuco is mainly a diatom marker (Wright and Jeffrey, 1987; Wright et al. 1991). The increased concentration of 19-hex, 19-but and fuco indicate proliferation of prymnesiophytes, pelagophytes, and diatoms in the MEM, as reported earlier in Southern Ocean iron experiment (SOFeX-North) by Coale et al (2004). Notably diatoms were only a minor portion of the community at the end of the incubation period as compared to the initial sample. Therefore, decrease in 19-hex: Chl *a* and 19-hex: fuco ratio with the concurrent increase in fuco: Chl *a* ratio in MEM during

later part of the experiment could not be accounted for the increase in diatom counts. The reason for this is not clear, however, significant levels of fucoxanthin were found in Prymnesiophyceae (such as *Phaeocystis*), Chrysophyceae and Raphidophyceae (Jeffrey et al., 1997). Bertrand et al (2011) found significant decrease in 19-Hex: Fuco ratio in Fe-amended incubation experiment performed at Ross Sea and attributed this to the increased production of fucoxanthin in *Phaeocystis* sp (Prymnesiophyceae) under elevated Fe concentration. DiTullio et al (2007) reported 10-fold decrease in 19-Hex: Fuco ratio in *Phaeocystis* after addition of 1nM Fe. The decrease in number of diatoms in Cu enriched microcosms could be due to the toxicity of Cu and hindrance of silicic acid uptake by diatoms (Rueter et al. 1981). Taken together, these results suggest that the micronutrient induced growth of nanoflagellates (prymnesiophytes and pelagophytes) better than that of diatoms. These findings corroborate with the nutrient data wherein silicate uptake in MEM compared to CM was not statistically significant.

The 1.5 fold increase in bacterial abundance in Fe, Co, and Fe + Co amended microcosms within 5 days of incubation, suggests that bacterial growth in the PF is generally limited by Fe, Co and/or co-limited by both. This could be either directly or through Fe and Co-induced changes in the quality of organic matter produced by phytoplankton. However, in the Fe amended microcosm no significant increase in the Chl *a* concentration was observed in the first 5 days of incubation (Figure 2A). Therefore, it appears that Fe addition directly induced bacterial growth in the microcosm, as reported earlier Pakulski et al (1996), Cochlan, (2001) and Hutchins et al (2001). On the other hand, direct effect of cobalt addition on marine heterotrophic bacterial growth/abundance is not clear (Antony et al. 2011), except for some marine cyanobacteria including members of genera *Synechococcus* and *Prochlorococcus* which are reported to have specific requirement of Co for their growth (Sunda and Huntsman, 1995; Saito et al. 2002). It appears that phytoplankton utilize microbially produced vitamin B₁₂ for their growth (Panzeca et al. 2008), as evident from the increased Chl *a* accumulation in Co and Fe + Co amended microcosms after 5 days of incubation, and in return produce more organic matter that support higher bacterial growth as evidenced by day 5. Gradual decrease of bacterial abundance with the incubation period in microcosms could be due to the depletion of labile organic matter (Becquevort et al. 2007), refractory organic matter (Legendre and Le Fevre, 1995), bacteria-phytoplankton competition for mineral nutrients (Thingstad et al. 1997) and due to efficient cropping down of increased bacterial abundance by grazers (Thiele et al. 2012). The increased bacterial abundance in DC, DCu, and DFe + Co after 15 days of incubation suggests uncoupling between light driven primary production and bacterial growth and the ability of heterotrophic bacteria to benefit from the leachates due to phytoplankton exudations from

senescence, moribund cells, dead phyto and finally by decomposing the cell mass. The decreased bacterial abundance in DCo and DFe suggests that bacteria (under complete dark condition) decompose/utilize the organic carbon derived from dying phytoplankton faster in presence of these trace metals, and by 15th day their growth become limited, whereas in absence of micronutrients and/or presence of Cu delayed bacterial response might have resulted in the increased bacterial abundance in the DC, and DCu at 15th day. We observed similar response in bacterial abundance in microcosm experiments conducted in the sub-tropical front of the Indian Sector of Southern Ocean (Ramaiah et al submitted).

The decrease in OTU richness, Shannon diversity index (H') and Pielou's evenness index (J') with the incubation period (Table 4), suggests that there is enrichment of specific phylotypes with the increased incubation. Bacterial diversity in aquatic systems is maintained by several bottom-up and/or top-down factors. Resource competition as well as access to bio-available organic carbon often limits bacterial growth (Kirchman and Rich, 1997; Church et al. 2000, Van Wambeke et al. 2002). The decreased diversity observed in our experiment might be due to the factors such as bottom-up control. Since, unfiltered seawater was used to perform the experiment; the possibility of top-down control on bacterial diversity cannot be ruled out. The minor differences in BC observed between MEM and CM implies that heterotrophic bacteria in the PF location are well adapted towards the fluctuations in micronutrient concentration and quantity as well as quality of organic matter. Our results are similar to those of Hutchins et al (2001) and Arrieta et al (2004). Hutchins et al (2001) reported that the bacterial community composition in the subarctic Pacific, the Southern Ocean, and the California coastal upwelling region scarcely changed after iron infusions, although their abundance and production increased. Arrieta et al (2004) also reported relatively stable bacterial community composition inside and outside the iron-enriched patch, during in situ iron-fertilization experiment carried out in the Southern Ocean.

Bacterial community in the initial water sample collected for the microcosm experiment was dominated by clades of *Alphaproteobacteria* and *Gammaproteobacteria*. Earlier studies on bacterial communities revealed that *Alphaproteobacteria*, *Gammaproteobacteria*, *Flavobacteria* and distinct subclusters of these groups are the major phylogenetic lineages found in the Southern Ocean (Simon et al. 1999; Abell and Bowman, 2005; West et al. 2008; Manganelli et al. 2009; Straza et al. 2010; Ducklow et al. 2011). The responses to micronutrient amendments differ among phylotypes since only two phylotypes, *Rhodospirillales* and *Roseobacter* showed the maximum response in terms of increased band intensity.

Eldridge et al (2007) and Kataoka et al (2009) proposed that groups of bacteria within a microbial community respond differently to micronutrient alterations. This differential response of phylotypes occurs because changing the dynamics of a single resource will likely affect coexistence patterns, as that resource may be limiting to some species, causing population increases and driving down other resources that may be limiting for other species (Eldridge et al. 2007).

The increased abundance (in terms of band intensity) of *Rhodospirillales* with the incubation period was more prominently seen in the MEM than those in the CM, exposed to the light: dark cycle. Conversely, the abundance of *Rhodospirillales* decreased in all microcosms incubated under complete dark condition. This suggests that the abundance of *Rhodospirillales* were linked to the conditions associated with the phytoplankton bloom induced by micronutrient enrichment during the experiment. Recently, Landa et al (2013) reported a significant increase in the percentage of sequences affiliated with *Rhodospirillales* in the seawater microcosm amended with the diatom-derived dissolved organic matter (DOM). Howard et al (2011) noted the presence of marine *Rhodospirillales* over the course of an induced phytoplankton bloom in Gulf of Mexico seawater microcosms. The decreased abundance of *Roseobacter*, and other phylotypes (SAR-11, SAR-86, SAR-324, and *Flavobacteria*) with the increased Chl *a* accumulation, suggest their negative response to the bloom conditions and/or shift in the phytoplankton community. The actual reason for this is not clear, however, Kataoka et al (2009) reported the disappearance of bands affiliated with *Roseobacter* in an iron enriched patch during peak Chl *a* level. We speculate that the disappearance of these phylotypes might have contributed to the observed decline in bacterial abundance in MEM with the incubation period. *Roseobacter* clade is reported to preferentially use phytoplankton products (Moran et al. 2002; Grossart et al. 2005; Howard et al. 2006). The increased abundance (in terms of band intensity) of *Roseobacter* observed in the all dark incubated microcosms indicates that they are utilizing the exudations from phytoplankton senescence, moribund cells, and dead phytoplankton.

4. Conclusions

Our results are useful to discern that phytoplankton growth in polar front of the Indian Sector of the Southern Ocean was not only limited by the availability of Fe but also by Co and Cu. Nanoflagellates proliferate better than diatoms in the MEM during the incubation period. The increase in Chl *a* concentration is not well reflected in bacterial abundance as well as BC structure, during the 15 day

experiment. We suggest this uncoupling to be due to competitive exclusion of certain bacterial phylotypes and/or of phytoplankton due to competition for micronutrients in particular Fe and Co.

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Tables

Table 1. Temporal variations in the major macronutrient concentration during microcosm experiment in control microcosms (CM) and micronutrient enriched microcosms (MEM).

Nutrients	Treatment	Nutrient concentration ($\mu\text{M L}^{-1}$)				
		Day-0	Day-5	Day-10	Day-15	Dark incubation Day-15
Nitrate	Control	12.78 \pm 1.1	11.74 \pm 1.25	19.57 \pm 0.9	24.9 \pm 1.47	20.54 \pm 1.29
	Co		*15.56 \pm 1.45	18.70 \pm 1.65	21.03 \pm 1.57	27.96 \pm 1.01
	Cu		*18.35 \pm 1.07	22.62 \pm 0.98	24.98 \pm 1.36	23.15 \pm 1.8
	Fe		*14.5 \pm 0.8	16.8 \pm 1.23	19.08 \pm 1.02	20.98 \pm 1.78
	Fe+Co		*16.32 \pm 0.97	13.77 \pm 1.39	21.16 \pm 1.24	24.82 \pm 1.03
Nitrite	Control	0.51 \pm 0.05	0.32 \pm 0.054	0.4 \pm 0.057	0.48 \pm 0.062	0.5 \pm 0.087
	Co		0.39 \pm 0.058	0.56 \pm 0.096	0.6 \pm 0.044	0.56 \pm 0.056
	Cu		0.37 \pm 0.035	0.58 \pm 0.031	0.56 \pm 0.023	0.31 \pm 0.027
	Fe		0.36 \pm 0.031	0.45 \pm 0.025	0.4 \pm 0.036	0.49 \pm 0.023
	Fe+Co		0.38 \pm 0.026	0.45 \pm 0.028	0.66 \pm 0.034	0.51 \pm 0.039
Phosphate	Control	2.42 \pm 0.20	1.91 \pm 0.25	2.34 \pm 0.34	3.09 \pm 0.21	2.73 \pm 0.31
	Co		2.07 \pm 0.19	2.72 \pm 0.37	2.89 \pm 0.29	3.44 \pm 0.35
	Cu		1.87 \pm 0.2	2.93 \pm 0.31	3.19 \pm 0.35	2.11 \pm 0.22
	Fe		1.75 \pm 0.18	2.17 \pm 0.15	2.52 \pm 0.23	2.92 \pm 0.36
	Fe+Co		1.97 \pm 0.19	1.93 \pm 0.2	3.13 \pm 0.31	3.02 \pm 0.38
Silicate	Control	14.54 \pm 0.98	11.45 \pm 0.51	17.58 \pm 0.68	21.14 \pm 0.41	20.03 \pm 0.47
	Co		*14.46 \pm 0.87	16.72 \pm 0.99	20.77 \pm 1.2	24.55 \pm 1.34
	Cu		*14.17 \pm 0.93	19.03 \pm 1.1	22.71 \pm 1.35	16.38 \pm 0.85
	Fe		*11.98 \pm 0.73	14.99 \pm 0.86	17.04 \pm 1.2	20.5 \pm 0.99
	Fe+Co		*13.7 \pm 0.51	11.28 \pm 0.69	20.03 \pm 1.1	22.89 \pm 1.23

*represents those values which are higher than that of the corresponding control

Table 2. Dissolved-organic and –inorganic carbon (μM) concentration in control microcosm (CM) and micronutrient enriched microcosm (MEM) over the incubation period. .

Dissolved organic carbon (DOC, $\mu\text{M/L}$) concentration					
Treatments	Day-0	Day-5	Day-10	Day-15	Dark-day-15
Control	36.98 \pm 0.75	37.54 \pm 0.73	38.67 \pm 0.62	40.83 \pm 0.79	29.63 \pm 0.24
Co		30.87 \pm 0.32	30.04 \pm 0.46	31.54 \pm 0.34	30.54 \pm 0.23
Cu		33.2 \pm 0.59	33.69 \pm 0.69	34.77 \pm 0.87	28.80 \pm 0.35
Fe		30.21 \pm 0.54	33.53 \pm 0.64	36.85 \pm 0.59	38.01 \pm 0.25
Fe + Co		30.37 \pm 0.68	31.20 \pm 0.59	32.78 \pm 0.77	36.27 \pm 0.36

Dissolved inorganic carbon (DIC, $\mu\text{M/L}$) concentration					
Treatments	Day-0	Day-5	Day-10	Day-15	Dark-day-15
Control	2980 \pm 20	2971 \pm 18	2974 \pm 13	2998 \pm 10	2656 \pm 17
Co		2244 \pm 19.5	2161 \pm 20	2002 \pm 21	2520 \pm 16
Cu		2341 \pm 8	2171 \pm 12	2047 \pm 16	2483 \pm 15
Fe		2362 \pm 19	2115 \pm 14	2640 \pm 18	2481 \pm 11
Mix		2241 \pm 6	2177 \pm 24	2082 \pm 9	2590 \pm 13

Table 3. Variation in concentrations of taxon-specific phytoplankton pigments (ng L⁻¹), in CM and MEM, at different incubation period. Values in bold are higher than that found initially as well as those in the controls at corresponding incubation duration.

Pigments	Treatments	Pigment concentration (ng L ⁻¹)			
		Day-0	Day-5	Day-10	Day-15
19-but	Control	3 ± 0.543	5 ± 0.65	3 ± 0.87	0
	Co		9 ± 0.56	9 ± .84	0
	Cu		4 ± 0.54	5 ± 0.36	6 ± 0.69
	Fe		6 ± 0.32	8 ± 0.48	3 ± 0.654
	Fe+Co		8 ± 0.90	6 ± 0.25	6 ± 0.58
Fuco	Control	28 ± 1.2	16 ± 0.9	19 ± 2.0	22 ± 1.5
	Co		32 ± 0.84	45 ± 1.56	65 ± 2.0
	Cu		13 ± 1.20	36 ± 1.12	46 ± 1.1
	Fe		18 ± 0.92	52 ± 1.45	35 ± 0.8
	Fe+Co		33 ± 1.321	50 ± 1.8	56 ± 1.57
19-hex	Control	27 ± 0.874	29 ± 1.365	30 ± 1.23	26 ± 0.45
	Co		48 ± 1.46	58 ± 2.0	40 ± 1.34
	Cu		26 ± 0.78	49 ± 1.54	65 ± 1.25
	Fe		33 ± 1.45	63 ± 1.36	26 ± 1.65
	Fe+Co		52 ± 1.92	57 ± 1.61	51 ± 1.23
DV Chl a	Control	51 ± 1.19	48 ± 0.732	36 ± 0.685	0
	Co		70 ± 1.235	87 ± 2.01	0
	Cu		44 ± 0.87	87 ± 1.87	0
	Fe		56 ± 1.54	97 ± 1.974	0
	Fe+Co		78 ± 1.43	0	0

Table 4. Phytoplankton counts per liter during the incubation period in the initial sample and at the end of the incubation in the CM and MEM.

Incubation period		Counts per liter	
		Diatoms	Dinoflagellates
Day-0	Control-0	22500	3000
Day-15	Control-15	7500	3500
	Co-15	7000	2500
	Cu-15	500	1000
	Fe-15	100	2000
	Fe+Co-15	4500	1500

Table 5. DGGE banding pattern based OTUs richness (*S*), Shannon diversity index (*H'*) and Pielou's evenness index (*J'*) of bacterial communities in the initial sample (Day-0), and in the microcosms at different incubation periods.

Parameters	Amendments	Incubation time (days)				
		Day-0	Day-5	Day-10	Day-15	Dark/day-15
<i>S</i>	Control	9	9	6	7	6
	Co		10	7	5	7
	Cu		10	9	6	6
	Fe		9	9	7	6
	Fe+Co		10	10	8	6
<i>H'</i>	Control	1.92	1.91	1.57	1.53	1.42
	Co		1.91	1.50	0.80	1.65
	Cu		2.09	1.63	1.24	1.26
	Fe		1.55	1.55	1.53	1.51
	Fe+Co		2.02	1.54	1.37	1.57
<i>J'</i>	Control	0.87	0.87	0.87	0.78	0.79
	Co		0.83	0.77	0.50	0.85
	Cu		0.91	0.74	0.69	0.70
	Fe		0.70	0.70	0.78	0.84
	Fe+Co		0.87	0.67	0.65	0.87

Table 6. Sequence analyses of DGGE bands. Note that `B` stands for the DGGE band.

Band no.	Accession no.	Phylogenetic affiliation	Closest relative	Sim (%)	Environmental description
B1	KF951485	<i>Gammaproteobacteria</i>	Uncultured SAR86 cluster bacterium clone C146100249 (JX530423.1)	100	Derived from the Southern ocean iron fertilization experiment (LOHAFEX)
B2	KF951486	<i>Gammaproteobacteria</i>	<i>Psychrobacter</i> sp. SC35 (EU939718.1)	99	Tidal flat sediment South Sea Korea
B3	KF951487	<i>Deltaproteobacteria</i>	Uncultured SAR324 cluster bacterium clone C146500019 (JX531039.1)	99	Derived from the Southern ocean iron fertilization experiment (LOHAFEX)
B4	KF951488	<i>Deltaproteobacteria</i>	Uncultured marine microorganism clone HOT157_350m92 (JN166364.1)	99	Seawater from subtropical north pacific ocean
B5	KF951489	<i>Alphaproteobacteria</i>	Uncultured bacterium clone 20162U88 (EU237468.1)	99	Subsurface seawater North Atlantic Ocean microbial assemblages
B6	KF951491	<i>Cyanobacteria</i>	Uncultured <i>Prochlorococcus</i> sp. Clone BIOS04_GYR_180m76 (HQ233034.1)	100	High nutrient low chlorophyll waters in the South and Equatorial Pacific Ocean
B7	KF951492	<i>Alphaproteobacteria</i>	Uncultured <i>Rhodospirillales</i> bacterium clone 0306_MLB67 (JQ515099.1)	100	Seawater Media Luna reef in La Parguera, Puerto Rico
B8	KF951493	<i>Bacterioidetes</i>	Uncultured <i>Flavobacteriaceae</i> bacterium clone C139001323 (JX527430.1)	97	Derived from the Southern ocean iron fertilization experiment (LOHAFEX)
B9	KF951494	<i>Chloroplast</i>	<i>Chaetoceros socialis</i> strain NOZ (FJ159135.1)	99	Chloroplast of <i>Chaetoceros socialis</i>
B10	KF951490	<i>Gammaproteobacteria</i>	Uncultured marine bacterium clone SHFB429 (GU234854.1)	98	Surface coastal seawater from Antarctica Peninsula
B11	KF951495	<i>Alphaproteobacteria</i>	Uncultured SAR11 cluster alpha proteobacterium clone C146300252 (JX530856.1)	99	Derived from the Southern ocean iron fertilization experiment (LOHAFEX)
B12	KF951496	<i>Alphaproteobacteria</i>	Uncultured <i>Roseobacter</i> sp. clone C146Chl417 (JX530172.1)	99	Derived from the Southern ocean iron fertilization experiment (LOHAFEX)

Figures

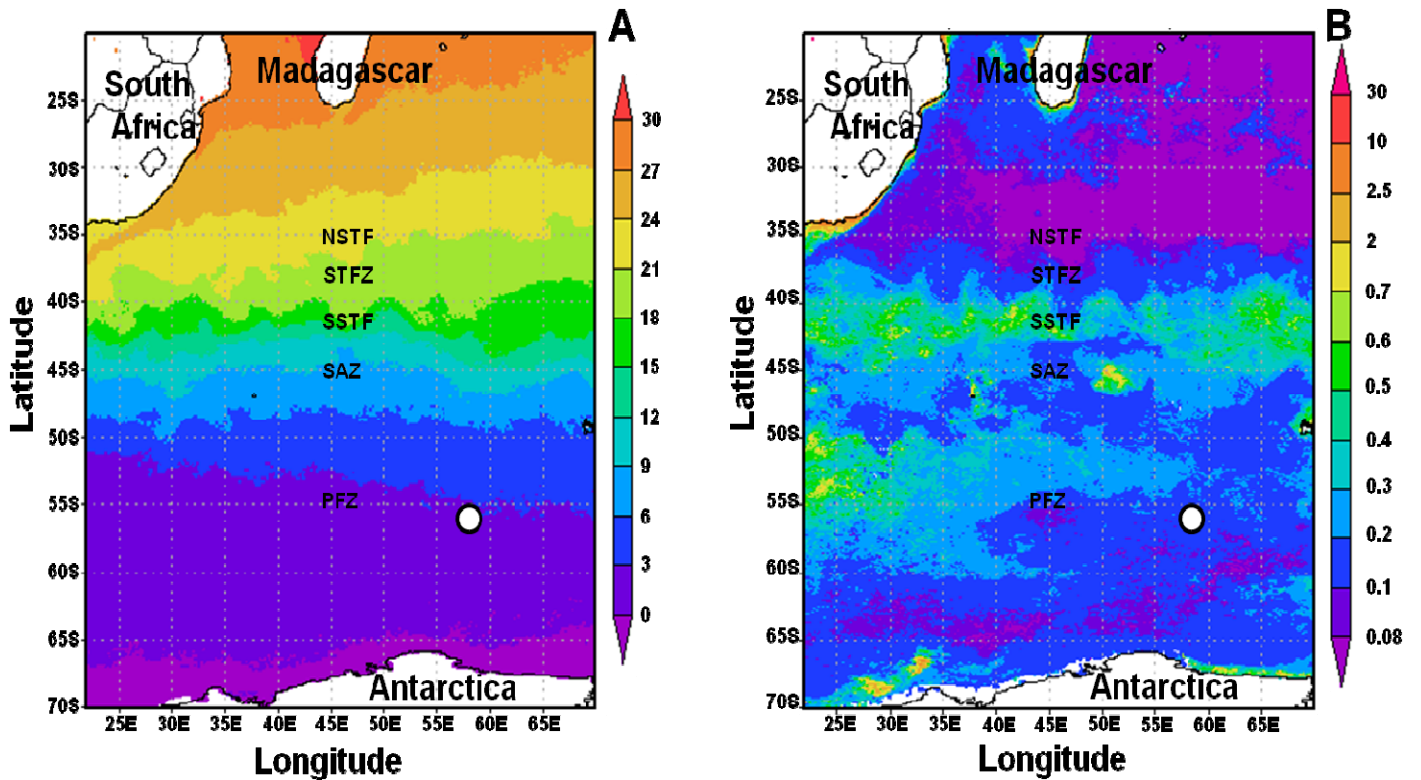


Figure 1. Monthly mean (A) sea surface temperature (SST) and (B) chlorophyll *a* (Chl *a*) concentration obtained from MODIS-aqua for the period of Jan to March 2013 is plotted with the sampling location (shown as white circle). The fronts and frontal zones at 45 °E longitude are marked from Anilkumar et al (2005, 2006, 2007). NSTF, North subtropical front; STFZ, Subtropical frontal zone; SSTF, South subtropical front; SAZ, Sub-antractic zone; PFZ, Polar frontal zone.

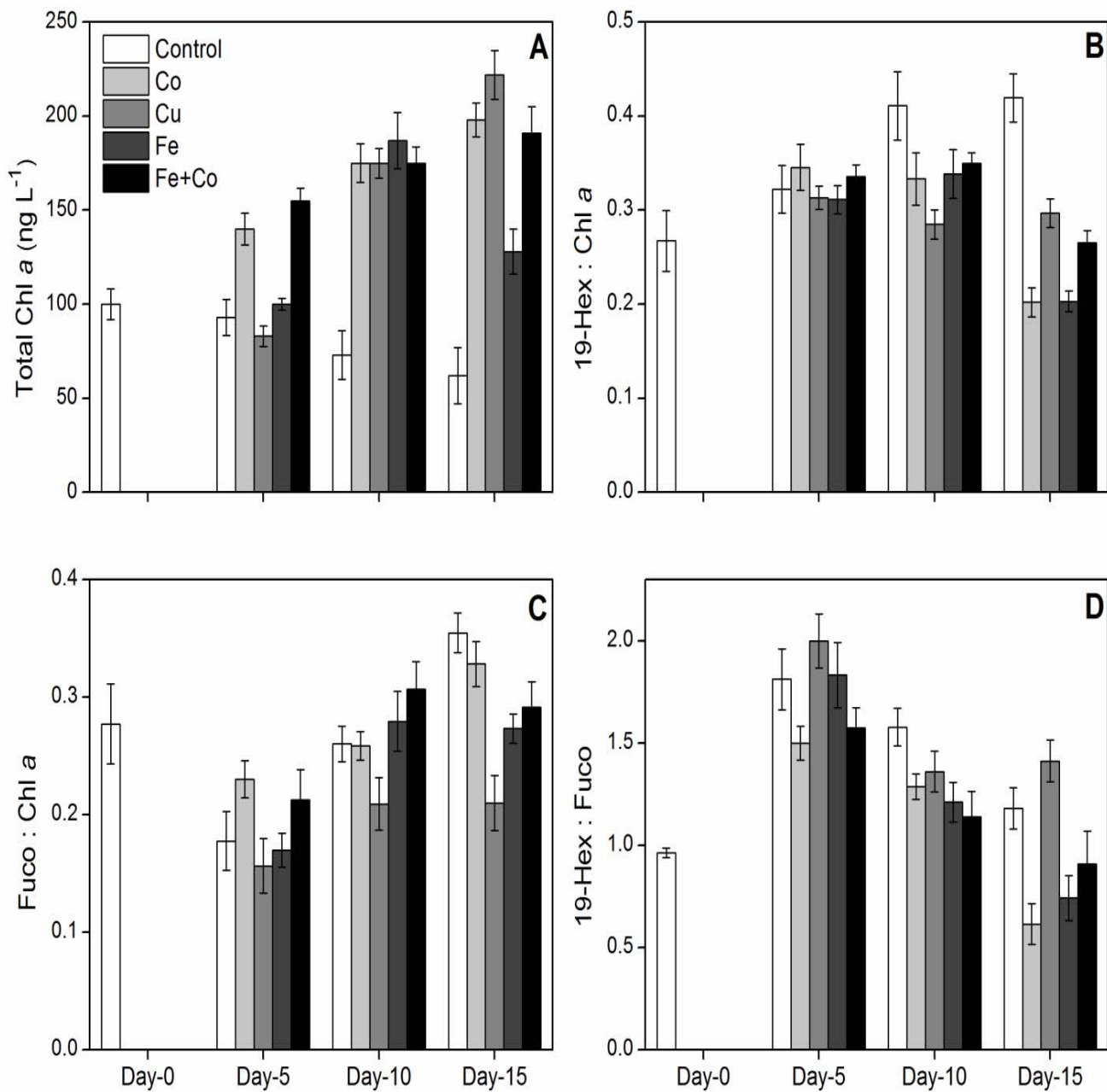


Figure 2. (A) Total chlorophyll *a* (Chl *a*) concentration, (B) 19-hex: Chl *a*, (C) fuco: Chl *a* and (D) 19-hex: fuco ratio in all six treatments over the 15 day incubation period. Treatments include unamended control, and 10 nM of Co, Cu, Fe, and Fe+Co.

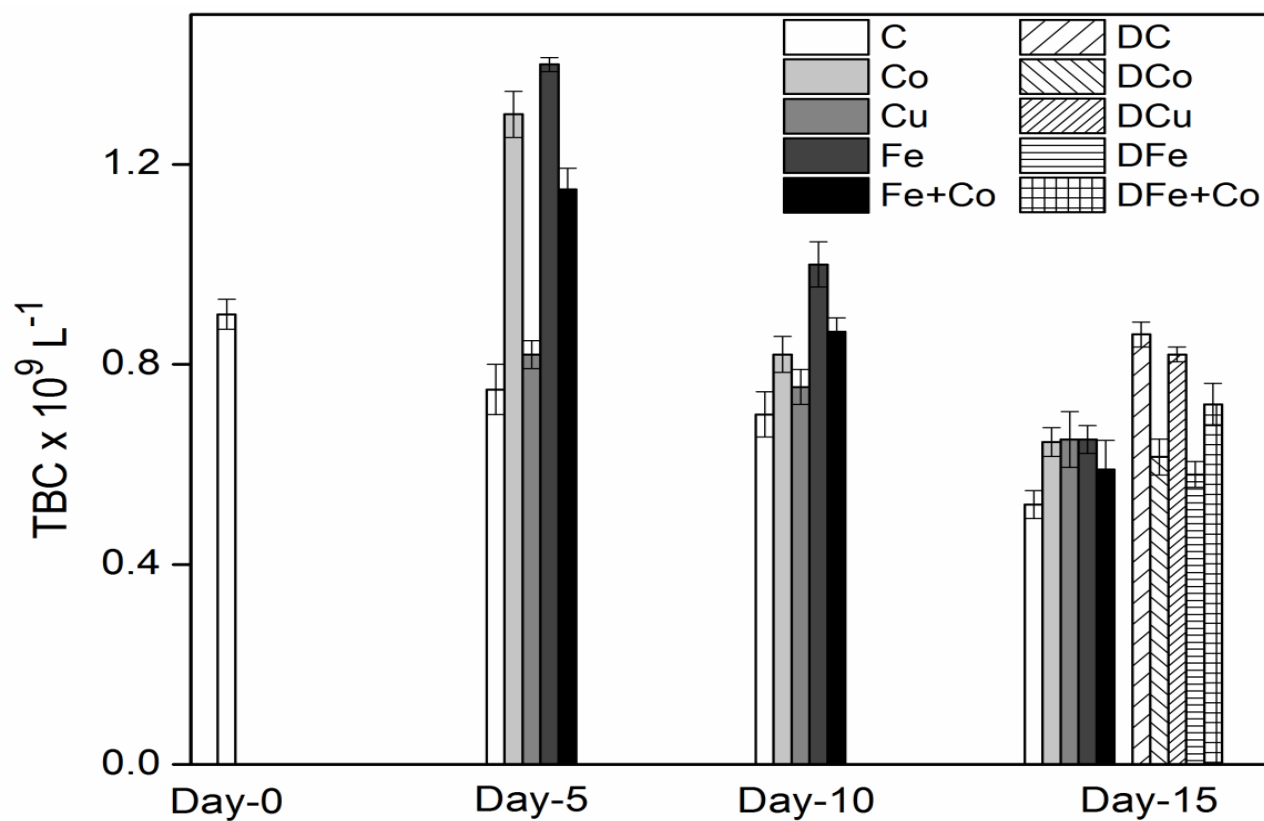


Figure 3. Variation of total bacterial counts over a period of 15 days in the microcosm experiment conducted at the Polar front. DC, DCo, DCu, DFe, and DFe+Co, represent the microcosms incubated under complete dark conditions.

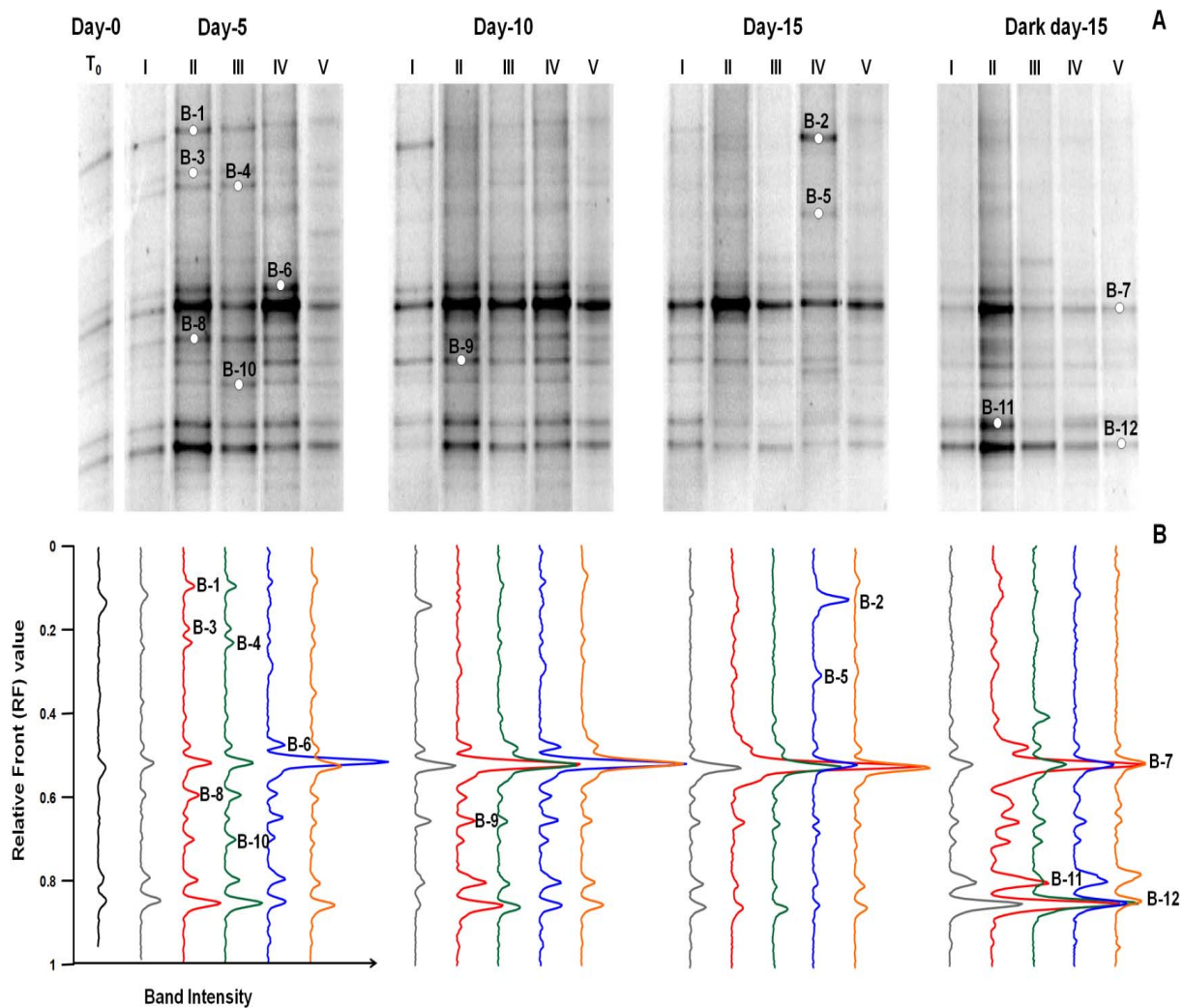


Figure 4. (A) The banding patterns on DGGE gel shown to represent the change in bacterial community between CM and MEM over the incubation period. (B) Plots of relative front (RF) versus band intensity derived from the DGGE gel (shown above) at different incubation period. The I, II, III, IV, and V represents unamended control, Co, Cu, Fe, and Fe + Co enrichment, respectively. Excised bands for sequencing purpose are shown by a circle on them. See methods for sampling details. Note that band-7 (B-7) and band-12 (B-12) showed maximum response/increase in the intensity compared to the other bands in MEM. The intensity of B-7 decreased, while the intensity of B-12 increased in all dark incubated microcosms compared to those subjected to light: dark cycle at 15th day. It should be noted that our DGGE profile represents both free-living and particle associated bacterial communities during the incubation period.

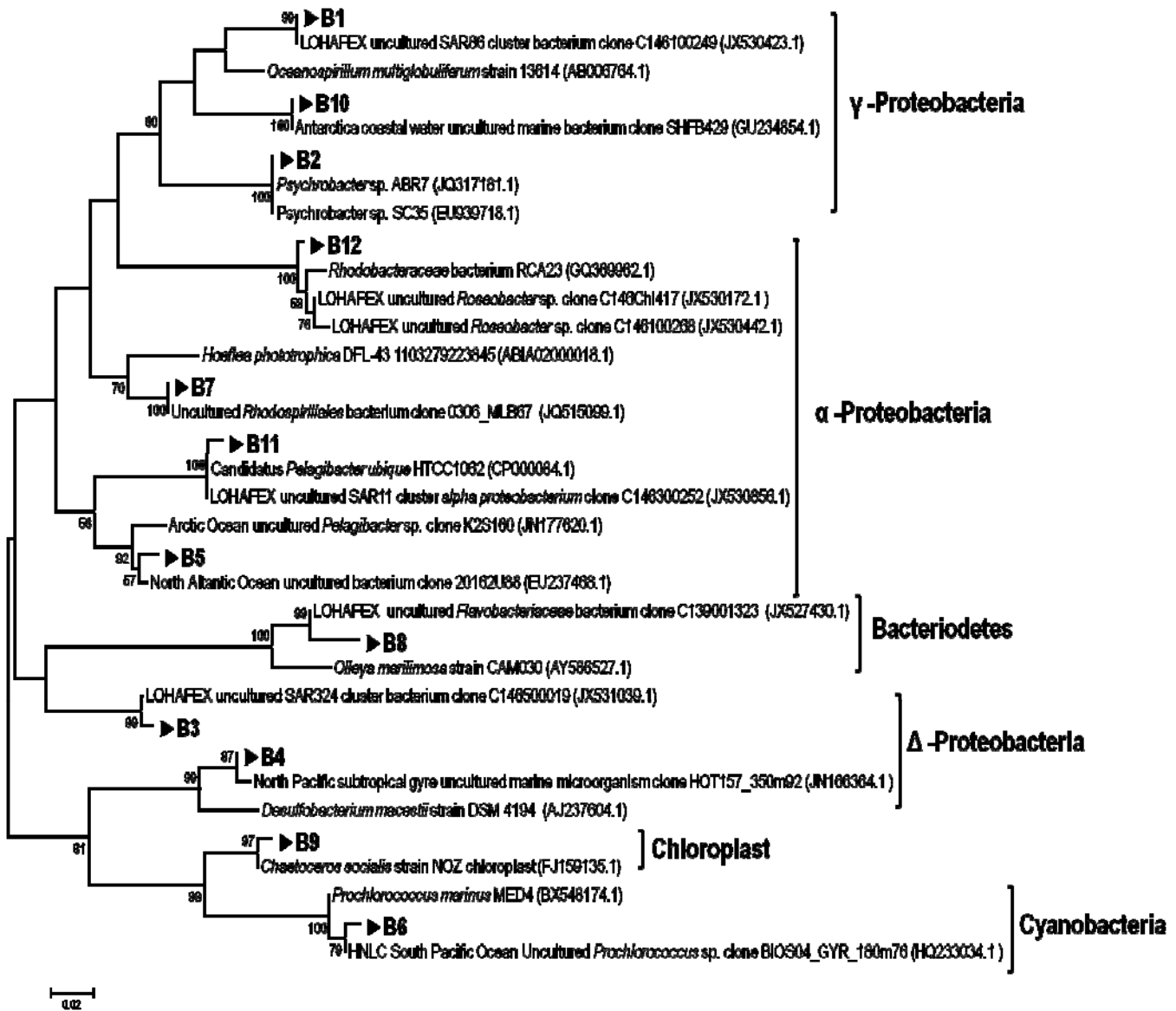


Figure 5. Phylogenetic tree (maximum likelihood) of V3 region of bacterial 16S rRNA gene amplified using DGGE specific primers, showing the affiliation with database sequences. Bootstrap values > 50% are given at the nodes (calculated with the neighbour-joining method). The scale bars indicate the percentage of the sequence divergence.