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Response of bacteria and phytoplankton from a subtropical front location Southern Ocean to micronutrient amendments *ex-situ*.

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ABSTRACT

The effects of micronutrient amendments such as cobalt (Co), copper (Cu), iron (Fe) and their mixture (Co+Cu+Fe) on bacterial abundance, phytoplankton, bacterial community (BC) composition were examined at a subtropical front (STF) location in the Indian Sector of the Southern Ocean (ISSO) during the austral summer of 2012. From the 15-day on-board experiment it became evident that there was no significant increase in total chlorophyll *a* concentration or phytoplankton cell numbers in micronutrient enriched microcosms (MEM) compared to the control microcosms (CM) with no added micronutrients. Highest bacterial abundance was observed in the Fe-enriched microcosm within 5 days of incubation unlike in other microcosms. Interestingly, significant differences in the BC composition were observed between MEM and CM. In that an increase of ~10 unique phylotypes affiliated with *Psychrobacter* sp, *Prochlorococcus* sp, *Burkholderia* sp, *Cytophaga–flavobactria* cluster, *Roseobacter* sp, *Bacillus* sp and *Vibrio* sp was observed in the MEM. The phylotypes associated with *Cyanobacteria* preferentially responded to the Co and Fe additions, whereas the members of the *Cytophaga–Flavobacteria* cluster responded to the Cu, Fe and mixture amendments. Our results are useful to discern that availability of micronutrients, in particular of Fe, affects the bacterial abundance as well as BC composition, but not the phytoplankton growth/chlorophyll a concentration in the STF location. In addition, micronutrient amendments *ex-situ* appears to lead to predominance of only a few bacterial phylotypes. This shift in bacterial community composition might be due to preferential and/or versatile utilization of exogenously added micronutrients. Phylogenetic diversity of culturable bacterial populations from this sampling location was also assessed using 99 pure bacterial cultures. For this, a select set of biochemical characteristics was examined and numerical profiling was done before subjecting to 16S rRNA sequencing based identification. This effort yielded close to 20 clusters, and representative isolates from each cluster were subjected to molecular taxonomic analyses using 16S rRNA marker gene. From this analysis, ~90% of bacterial isolates were found to be affiliated with *Firmicutes* and ~10% to *Gammaproteobacteria* suggesting preponderance of only a few phylotypes in the deep chlorophyll maximum zone of this STF location.

1. Introduction

The subtropical front (STF) extends almost continuously around the Antarctic (except for the South American sector) at ~40°S. It separates sub-tropical region (STR) from the subantarctic region (SAR) (Orsi et al., 1995), and strongly influences the meridional transport of heat, salt and nutrients between the Southern Ocean and subtropical gyres (Jayne and Marotzke, 2002). The subtropical frontal zone (STFZ) in the Southern Indian Ocean is enclosed by two sharp fronts: the north subtropical front (NSTF) and south subtropical front (SSTF; Belkin, 1988; Kostianoy et al., 2004). The extremely oligotrophic NSTF between 34°S and 35° is characterized by low chlorophyll *a* concentration and high microzooplankton abundance (Jasmine et al., 2009). On the other hand, SSTF is characterized by high biological productivity (Pakhomov and McQuaid, 1996) caused by the mixing of warm

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macronutrient-poor but micronutrient-rich subtropical water (STW) with cool macronutrient-rich yet micronutrient-poor subantarctic surface water (SASW, Boyd et al., 1999). This makes SSTF region an important carbon sink (Currie and Hunter, 1998; Metzl et al., 1999; Metzl, 2009; Ito et al., 2010; Currie et al., 2011; Shadwick et al., 2015). Despite high primary productivity in the STF transfer of biogenic carbon to depth via biological pump is reported to be very low mainly due to efficient remineralization process and/or a high bacterial activity (Blain et al., 2002; Sedwick et al., 2002; Coppola et al., 2005). Jasmine et al., (2009) observed higher abundance of micro-zooplankton and mesozooplankton along with higher biological productivity in the STF. Such a scenario ought to imply an active microbial loop in biogenic carbon sink in the STF region. The microbial loop is a critical part of 'biological carbon pump' and can alter the efficiency of carbon export via remineralization of organic matter back to inorganic phase and/or conversion of organic matter to biomass (Robinson and Ramaiah, 2011). Therefore, it is imperative to elucidate the bacterial diversity as well as factors influencing bacterial growth and community composition in STF region of the Indian Sector of the Southern Ocean (ISSO).

Micronutrients are essential for virtually all organisms, but are extreme1y scarce in the Southern Ocean (Ellwood, 2004, 2008, Ellwood et al., 2005, Croot et al., 2011, Hassler et al., 2012). Dissolved Fe concentration of 0.09 nM (Blain et al., 2002) reported from STF region is quite low. So also are ranges of dissolved-Co (24–49 pM; Castrillejo et al., 2013) and -Cu (0.5–2.5 nM; Boye et al., 2012). Thus, these earlier studies indicate that the macro-nutrients (nitrate, phosphate and silicate) as well as dissolved Fe (<0.09 nM) deficiency impose limitation on phytoplankton growth in the STF region (Indian Sector, Sedwick et al., 2002; Blain et al., 2002). However, effect of micronutrients on bacterial growth and community structure in the Indian Sector of the STF region, are large1y unknown. In this regard, experimental addition of 2.5 nM Fe by Church et al. (2000) led to 2.5 fold increase in bacterial abundance as well as bacterial production in the water sample from STF region (Australian Sector). On the other hand, Hutchins et al. (2001) observed minor iron-mediated changes in bacterial community structure in the subantarctic region.

In this study, we examined the effect of micronutrient amend-ments such as cobalt (Co), copper (Cu) and iron (Fe) on total Chlorophyll *a* concentration, bacterial abundance, phytoplankton, and bacterial community structure by conducting an on-board microcosm experiment. For this, a bulk sample of water was collected from deep chlorophyll maximum (DCM) depth of ~60 m. Further, over 100 randomly chosen pure cultures of bacterial isolates from this depth were brought to the laboratory for biochemical characterization as well as 16S rRNA gene sequen-cing in order to note the diversity of culturable bacteria.

2. Materials and methods

2.1. Enumeration of viable populations of heterotrophic bacteria from sampling site

Seawater nutrient agar (SWNA) medium containing yeast extract 0.3%, peptone 0.5%, agar 1.5% and seawater: distilled water (50:50 V/V) was used to quantify the culturable or 'platable' populations of aerobic, heterotrophic bacterial colony forming units (CFU) from different depths at station-1 (stn-1, latitude: 39° 59′0.975S; longitude: 58° 29′0.919E) shown in Fig. 1. Briefly, 100 μl of the seawater sample was spread plated onto the SWNA. The plates were incubated at 4 °C, 10 °C and 20 °C until visible bacterial colonies were seen on the plates. The bacterial cultures were purified by streaking single isolated colony using sterile microbiological loop onto the fresh SWNA plates. The plates were wrapped, stored at 4 °C, and brought back to the laboratory. Membrane filtration technique was also followed to ensure recov-ery of all possible colony forming units (CFU) of aerobic, hetero-trophic bacteria. Upto 20 ml subsample was filtered through 0.2 μm pore size cellulose acetate nitrate filters (Millipore, USA) and placed on SWNA plates and incubated at 4 °C (for up to 20 days), 10 °C (at least for a week) and 20 °C (for up to four days) before enumerating the final counts of CFU.

2.2. Isolation and biochemical characterization of bacterial cultures

Cultivation-based or culture-dependent approach is important, since ecological role of prokaryotes in natural environments can be assessed only when they are successfully cultivated and character-ized. Sequences of genes cloned directly from environmental DNA are shown to not correspond to the genes of cultured marine taxa (Suzuki et al., 1997). Therefore, we studied cultivable bacterial diversity from stn-1 at the DCM depth (Fig. 1) to note the possible diversity in the DCM from where the bulk sample was drawn for onboard microcosm experiments. For this, well isolated and morphologically distinct colonies were selected, purified and brought to the laboratory. Overall, 105 cultures were isolated and 99 of the finally available isolates were subjected to various morphological and biochemical tests (Table 1). Bacterial cultures were clustered in to groups using numerical profiling based on the results of biochemical characteristics. Up to 3 or 4 representative cultures from each group was taken up for identification on the basis of 16S rRNA gene sequencing.

2.3. Extraction of genomic DNA and amplification of 16S rRNA gene from bacterial cultures

The genomic DNA from the pure cultures was extracted using Genomic DNA isolation kit (Sigma-Aldrich, USA), as per manufac-turer's instructions. Bacterial 16S rRNA gene was amplified by following standard Polymerase chain reaction (PCR) method using universal primer set, 27F (5′–AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACT T-3′) developed by Weisburg et al. (1991). The PCR mixture (50 μl) contained 1μl of extracted DNA (5–50 ng μl^{-1}), 1 μl of each primer at a concentration of 0.5 μM, 25 μl of Ready MixTaq PCR mix (Sigma-Aldrich, USA) [1.5 U Taq DNA polymerase; 10 mM Tris–HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin, 0.2 mM deoxynucleoside triphosphate (dNTP), stabilizers], and 22μl of milliQ water. Then the PCR was carried out with the temperature profile as follows. Initial denaturation step of 3 min at 95 °C, followed by 25 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s. The final extension step was for 10 min at 72 °C. The PCR was performed via Thermocycler (Applied Biosystems, USA). PCR products thus obtained were checked on 1% agarose gel.

2.4. Sequencing and phylogenetic analysis of culturable bacteria

The purified PCR products were then cloned into pCR®4-TOPO vector (Invitrogen, USA) and transformed into *E. coli* TOP-10 compe-tent cells according to manufacturer's instructions. Colony-PCR was used for checking the positive transformants containing appropriate size of DNA insert, using M13–20 forward and M13 reverse primers (TOPO-TA cloning guide, Invitrogen, USA), which corresponded to both sides of the cloning site on the vector. The clones were then sequenced using ABI 31310XL (Applied Biosystems, USA) genetic analyzer housed in our laboratory. The sequences obtained were edited with the software Vecscreen (http://www.ncbi.nlm.nih.gov/tools/vecscreen/), and compared with the NCBI database through BLAST searches (http://blast.ncbi.nlm.nih.gov). In this comparison, sequences of type strains most closely related to the sequences of the isolates were