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Denaturing gradient gel electrophoresis profiling of bacterial communities composition in Arabian Sea

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Abstract

Denaturing gradient gel electrophoresis (DGGE) was used to elucidate spatial and temporal variations in bacterial community composition (BCC) from four locations along the central west coast of India. DNA extracts from 36 water samples collected from surface, mid-depth (~10 m) and close to bottom (~20 m) during premonsoon, postmonsoon, monsoon were analyzed by PCR for amplifying variable region of 16S rRNA gene and subsequently through DGGE. Prominent bands were excised, cloned and sequenced indicated the preponderance of gammaproteobacteria, bacteroidetes and cyanobacteria. Non-metric dimensional scaling of the DGGE gels indicated that the spatial variations in BCC were prominent among the sampling locations. Temporal variations in the BCC appear to be influenced by monsoonal processes. The canonical correspondence analyses suggest that the concentration of chlorophyll *a* and nitrate are two important environmental factors for both spatial and temporal variations in BCC. Chlorophyll *a* seems to be impart a top-down control of BCC while nitrate, the bottom-up control. Our results also suggest that BCC can vary over a small geographic distance in highly dynamic, seasonally predisposed tropical coastal waters.

Key words

Arabian sea, Bacterial community, DGGE, Spatio-temporal variations, Central west-coast of India

Introduction

The bacteria play a major role in carbon dynamics of marine ecosystems and, the importance of heterotrophic bacteria in marine ecosystem functioning is very well recognized (Azam *et al.*, 1983). They are a critical component of marine ecosystems, with high abundance and biomass, processing more than one half of the total primary production, regenerating nutrients, and interacting widely with other organisms (Azam, 1998; Fuhrman, 2002). Their biomass is often comparable to that of the phytoplankton, in the euphotic zone (Cho and Azam, 1990; Mitchell *et al.*, 1989; Simon *et al.*, 1992).

Despite such well-documented importance in marine biogeochemistry, analytical difficulties and inabilities to distinguish bacterial species based on morphology in complex communities and uncertainties in culture-based investigations to distinguish the species that comprise a given community make heterotrophic bacteria to fall into a 'black box' (Fuhrman and Hagstrom, 2008).

Denaturing gradient gel-electrophoressis (DGGE) based fingerprinting helps estimate the numbers of dominant phylotype in a given sample (Muyzer *et al.*, 1993). Very diverse bacterial assemblages such as those in the soils present many bands (Muyzer and Smalla, 1998). Direct amplification and analysis of 16S rRNA genes by DGGE have been carried out to examine the predominant sequences in mixed PCR products amplified from environmental samples such as, activated sludge (Boon *et al.*, 2002), river waters (Sekiguchi *et al.*, 2002) and soil (Nakatsu *et al.*, 2000) samples. Simpler communities, such as microbial mats or enrichment cultures, have far less number of bands. Using this technique, only a few (5-14) bands in freshwater lakes to a moderate (20-35) bands in marine waters have also been reported (Schauer *et al.*, 2000).

During summer monsoon, the central west coast of India is a region where seasonal variability in biological processes is pronounced (Shankar and Shetye, 1999). For instance, intense upwelling of nitrate rich deeper waters and, enormous volumes of land drainages during the peak monsoon period (July-August) enhance chlorophyll productivity that often lead to depletion of dissolved oxygen and increased denitrification excessive sulphate

reduction along the entire near-shore water column in this region (Naqvi , 2001). With atmospheric temperatures of 34-37°C (during summer: April-June and rainy season: July-September), and 25-29°C (during 'winter': December-February), the near-shore sea surface temperatures remain warmer (>23 to ~29°C) throughout the year. While the impact of these events on autotrophic community is variously reported (Madhupratap *et al.*, 1996), there is a lack of information on how bacterial assemblages vary during and after the monsoonal span. Bacterial abundance and production are reported to be low during Northeast Monsoon (December-February), moderate during Spring Intermonsoon (March-May) and, higher during Summer Monsoon (June-September) in response to coastal upwelling in particular (Ducklow, 2001; Ramaiah *et al.*, 1996).

DGGE profiling was done to evaluate the significance of certain environmental variables on BCC and, to compare spatio-temporal variations in BCC of four ecologically diverse locations in the central west-coast of India.

Materials and Methods

Sampling: Four locations along the central west coast of India (Fig. 1) were chosen for this study. Sampling was carried out during three distinct periods *viz.*, premonsoon (Pr; Apr-May 2005), post monsoon (Po; Dec 2005-Jan 2006) and monsoon (Mo; Sept–Oct 2006) off Ratnagiri (Lat 73°20'E 18°25'N, Mormugao 73°70'E 15°41'N, Karwar 74°03'E 15°02'N and Bhatkal 74°28'E 13° 88'N). Water samples were collected from ca 1 m (to avoid surface microlayer contamination), mid-depth (~10 m) and close to bottom on the 20 m depth contour. Niskin samplers (General Oceanics, Fl, USA) were used for collection of samples from these discrete depths. To avoid large scale influences of anthropogenic activities in the inter-tidal region, we chose to collect samples at 20 m depth contour.

Seawater temperature, salinity, pH, dissolved oxygen, chlorophyll *a* and nutrients (nitrite, nitrate, phosphate and silicate) were measured by standard methods described in Parsons *et al.* (1984).

For total bacterial count (TBC) enumeration, subsamples of 25 ml were preserved with buffered formaldehyde (2% final concentration) and stored at 4°C in the dark until taken up for analysis, usually within a fortnight of sampling. The preserved seawater samples were incubated with 4'6'-diamidino-2-phenylindole (DAPI; 1 µg ml^{-1}, final concentration) and filtered onto black 0.2 µm-pore-size polycarbonate membrane filters (Millipore, U.S.A). Epifluorescence microscopic counts were made using a Nikon (Eclipse e400, Japan). Minimum of 10 microscopic fields were counted from each sample to obtain a reliable averaging of TBC.

Extraction of DNA: Duplicates of two water samples (~2 lit) were filtered through Sterivex cartridges fitted with 0.22 µm pore sized membrane filter (Millipore, USA) to retain microbial cells for subsequent DNA extraction. The cartridges were filled with 1.8 ml buffer (50 mM Tris pH 8.3, 40 mM EDTA and 0.75 M sucrose), sealed and, frozen on dry ice. They were then transported to the laboratory and stored at -80°C until processed. The DNA extraction and purification were carried out as essentially described by Ferrari and Hollibaugh (1999). In all, a total of 72 extractions were made and, the DNA from replicate samples was pooled. The integrity of all 36 environmental DNA was checked on 0.7% agarose Gel.

DGGE-PCR: DGGE specific primers, 357F-GC(5´-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGC ACGGGGGGCCCTACGGGAGGCAGCAG-3´) and 518R (5´-ATTACCGCGGCTGCTGG-3´) developed by Muyzer *et al.*(1993), were used for amplifying the V3-variable region of 16S rRNA gene. Polymerase chain reaction (PCR) was performed on all the 36, pooled extracts. The DGGE-PCR was carried out with the temperature profile as follows-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 constant annealing temperature of 55°C. The final extension step was for 10 min at 72°C. In all, only 25 cycles of PCR were allowed to avoid excessive amplification of dominant fragments. However, to maximize the detection of as many 'species' as possible, at least six different PCR reactions were carried out for each sample. Replicate PCR were products pooled and checked on 2% agarose gel.

DGGE analysis: DGGE was carried out on PCR products obtained as above using the D-Code universal mutation detection system as per the manufacturer's instructions (Bio-Rad, USA). Fifty microliter each of the PCR products was loaded in to each lane of the DGGE gel. The products were resolved on 10% (w/v) polyacrylamide gels containing a 40 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 UV trans-illuminator. In all, 6 DGGE gels, each with 12 DNA samples were run such that the spatial as well as seasonal differences could be delineated.

Statistical analysis of DGGE: Quantity-one software (version 4.65, Bio-Rad, USA) was used to determine the intensity and relative position of each band. For this analysis, each DGGE band was assumed as operational taxonomic unit (OTU) or phylotype. Bands were detected using the band-searching algorithm of the software which takes care of background subtraction. Since we could not quantify the DNA concentration in the PCR products loaded per sample, the percentage intensity of bands in each lane was calculated as done by Riemann *et al.* (1999) and used to correlate the intensity of the whole lane for a realistic enumeration of OTUs. Analysis of band patterns was performed using 1% position tolerance. Gels were checked visually too for ensuring the number of bands.

Non-metric multidimensional scaling (NMDS) was performed using PRIMER 5.0 software (Clarke and Warwick, 1994), to analyze the spatial and temporal changes in BCC. For NMDS, band intensity data were transformed into presence (1) and absence (0) and,

Bray- Curtis similarity (equivalent to Sorenson coefficient) was calculated.

Canonical correspondence analysis (CCA) was done for each DGGE gel separately and, the ordination plot including DGGE samples (locations) and environmental variables were used to explain the data. It was used to analyze the effect of environmental factors on BCC using Multivariate Statistical Package (MVSP v3.1) software.

Excision and sequencing of DGGE bands: To elucidate the sequences of most prominent and, common OTUs from different depths and locations, 31 bands were excised from three gels and re-amplified using primers 357F (without GC clamp) and 518 R. The PCR products were sequenced on ABI 31310XL genetic analyzer (Applied Biosystems, USA). Since some of the PCR products did not yield reliable quality of sequences, they were cloned in to pGEMT-easy vector (Promega, USA) according to the manufacture's instructions and sequenced.

Nucleotide Sequence accession no. : All the sequences obtained in this study are deposited in Genbank database under accession no GQ860964 to GQ860994.

Results and Discussion

DGGE profiling is an attractive and dependable alternative despite some inherent uncertainties (Fuhrman and Hagstrom, 2008). Being faster, less-expensive and less labor-intensive than sequencing, it allows highly replicated analysis of bacterial communities (Muyzer *et al.,* 1993). The PCR-DGGE is particularly suited to fingerprint the spatial and temporal differences in bacterial communities (Schauer *et al.,* 2000).

Season-wise or, location-wise differences between the temperature, pH and salinity were minimal. Concentrations of silicate (SiO_4) and nitrate (NO_3) varied widely. Chlorophyll *a* ranged between 1.91 and 5.13 mg m^{-3} (Fig. 2). TBC were higher as during monsoon (12 to 30 $X10^5$ cells ml^{-1}) than those observed during pre-monsoon (2.6 to12.2 $X10^5$ cells ml^{-1}) and post-monsoon (1.8 to 9.1 $X10^5$ cells ml^{-1}). The differences in TBC were significant both between seasons ($p<0.0001$) and locations ($p<0.043$) than those between depths ($p>0.196$). The number of OTUs from different locations differed both depth as well as season-wise.

Taxonomic description of DGGE bands: Total 31 bands that were prominent in the gel photographs, whose positions are shown in Fig. 3, were excised and sequenced. All the sequences obtained are deposited in Genbank Data base under accession no GQ860964 to GQ860994. It was clear from the sequence analyses (Table 1) that the phylotypes profiled through DGGE were related to proteobacteria (bands 1, 3, 5, 6, 12, 13, 15, 17, 19, 20-23, 28, 30 and 31), cyanobacteria (10, 11, 14, 16, 18, 24), bacteroidetes (2, 4, 7- 9, 26 and 27) and actinobacteria (25). Band 27 belonged to unclassified bacteria. Of the 31 total bands, only 13 and 15 appeared to indicate co-migration of 16S rDNA of two different species.

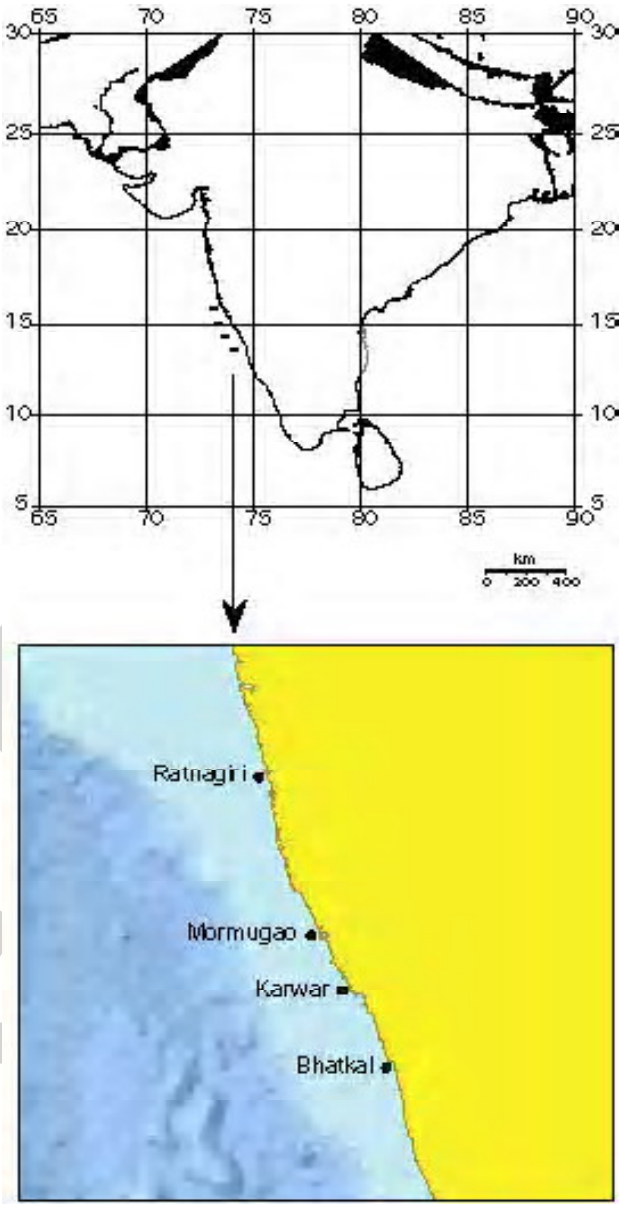

Fig. 1: Sampling sites in the central west coast of India

Our study is useful to recognize that gammaproteobacteria, bacteroidetes, cynobacteria and actinobacteria are the substantial part of bacterial community in the coastal water along the central west coast of India. These bacterial groups are reported for their ubiquitous presence in different oceanic environments (Pommier *et al.*, 2005). Gammaproteobacteria is a well known group in the marine coastal waters. The members of this group have been shown to capture light energy in marine environment (Beja *et al.,* 2000). Using DGGE, bacteroidetes assemblages has been shown to follow seasonal cycles in the Mediterranean sea (Schauer *et al.*, 2003). Bacteroidetes are considered to be actively involved in degradation of organic matter and they have been reported to occur during the algal blooms (Riemann *et al.*, 2000). Actinobacteria are also a major group contributing in bacterial production in coastal waters (Fuhrman and

Table - 1: Homology of sequences of DGGE bands obtained from water samples collected off central west coast of India (See Fig. 3 for position of the bands)

DGGE Band No.	Accession No.	Taxonomic position	Most closely related sequence	Similarity (%)	Accession no. of related sequence
1	GQ860964	Unclassified Bacteria	Uncultured marine bacterium isolate DGGE gel band 0114617(7)T7	99	EF433334
2	GQ860965	Flavobacteria	Uncultured *Flavobacterium* sp. clone	92	FJ744798
3	GQ860966	Gammaproteobacteria	Uncultured bacterium clone 1C227523	100	EU799860
4	GQ860967	Flavobacteria	Uncultured bacterium clone W9-A06	98	FJ930875
5	GQ860968	Gammaproteobacteria	Uncultured gammaproteobacterium clone 916-C22	98	EU315547
6	GQ860969	Gammaproteobacteria	Uncultured gammaproteobacterium isolate DGGE gel band 0130908(22)T7 16S ribosomal RNA gene, partial sequence	100	EF433349
7	GQ860970	Flavobacteria	Uncultured bacterium clone associate with coral *Pocillopora meandrina*	100	FJ497119
8	GQ860971	Flavobacteria	Psychroserpens sp. MEBiC05023	98	EU581702
9	GQ860972	Flavobacteria	Uncultured bacterium clone W3-H08	98	FJ930762
10	GQ860973	Cyanobacteria	Uncultured bacterium clone 4S_2g10	100	FJ382194
11	GQ860974	Cyanobacteria	Uncultured bacterium isolate JH10_C20 16S ribosomal RNA gene	97	AY568777
12	GQ860975	Gammaproteobacteria	Uncultured gamma proteobacterium clone HF70_25F19 small subunitribosomal RNA gene, partial sequence	98	EU361659
13	GQ860976	Gammaproteobacteria	Uncultured bacterium clone VH-FL8-28 16S ribosomal RNA gene, from Vicroria Harbour	99	EF379714
14	GQ860977	Cyanobacteria	Uncultured cyanobacterium clone	98	EU980278
15	GQ860978	Deltaproteobacteria	*Bacteriovorax* sp. NF1 16S ribosomal RNA gene, partial sequence	94	EF092441
16	GQ860979	Cyanobacteria	Uncultured *Synechococcus* sp. clone	100	FJ718237
17	GQ860980	Gammaproteobacteria	Uncultured gamma proteobacterium clone GOM_WB8-85 16S ribosomal RNA	99	GQ250617
18	GQ860981	Cyanobacteria	Uncultured *Synechococcus* sp.	98	FJ999601
19	GQ860982	Gammaproteobacteria	Uncultured gamma proteobacterium clone SHWH_night1_16	100	FJ744967
20	GQ860983	Gammaproteobacteria	Uncultured gamma proteobacterium clone GOM_WB8-85	99	GQ250617
21	GQ860984	Gammaproteobacteria	Uncultured gamma proteobacterium clone b1pl2F05 16S ribosomal RNA	99	EF092639
22	GQ860985	Gammaproteobacteria	*Psychrobacter* sp. CS611037 16S	98	GQ200539
23	GQ860986	Gammaproteobacteria	Uncultured bacterium clone H10P1WR 16S ribosomal RNA gene	97	FJ156718
24	GQ860987	Cyanobacteria	Uncultured cyanobacterium clone	97	FJ844170
25	GQ860988	Actinobacteria	Uncultured actinobacterium clone GOM_WB8-9	99	GQ250624
26	GQ860989	Flavobacteria	Uncultured bacterium clone W9-A06	100	FJ930875
27	GQ860990	Flavobacteria	Uncultured marine bacterium clone C53 16S	100	EU010202
28	GQ860991	Gammaproteobacteria	Uncultured bacterium clone BBD-Oct07-water-94 16S ribosomal RNA	100	GQ215179
29	GQ860992	Unclassified Bacteria	Uncultured bacterium clone 6C232172 16S ribosomal RNA gene, partial	100	EU804270
30	GQ860993	Gammaproteobacteria	*Serratia* sp. AN4	96	FJ230836
31	GQ860994	Gammaproteobacteria	Uncultured gammaproteobacterium isolate DGGE gel band 0130908(22)	100	EF433349

Hagstrom, 2008). The role of this group in marine water is not clear however their diversity decrease with the distance increase from coast (Okazaki and Okami, 1972). Alphaproteobacteria are also a major component of BCC (Pommier *et al.,* 2005; Riemann *et al.,* 1999) but we did not find it in total 31 bands sequenced in this study. This can happen because we did not sequence all prominent bands.

Spatial and temporal variations in bacterial communities: The surface water samples showed higher variations during the monsoon. The banding pattern and the number of OTUs in the surface samples off Ratnagiri, Marmugao and Karwar during monsoon samples were quite similar (Fig. 3). Then the overall, it is apparent that the BCC did differ spatially though it was quite similar in samples off Ratnagiri and Mormugao and those from off Karwar and Bhatkal. From the NMDS plots of the DGGE profiles, it is indicative that differences in BCC are quite low between locations. The NMDS plots also brought out the differences in the BCC during different seasons with higher variation during monsoon. With higher stress values of 0.14 to 0.18, most surface samples collected during monsoon aligned in to two groups together (Fig. 3a) and, those from mid depth (Fig. 3b) in to three groups while all those from bottom (Fig. 3c) samples grouped together. Apparently, the temporal

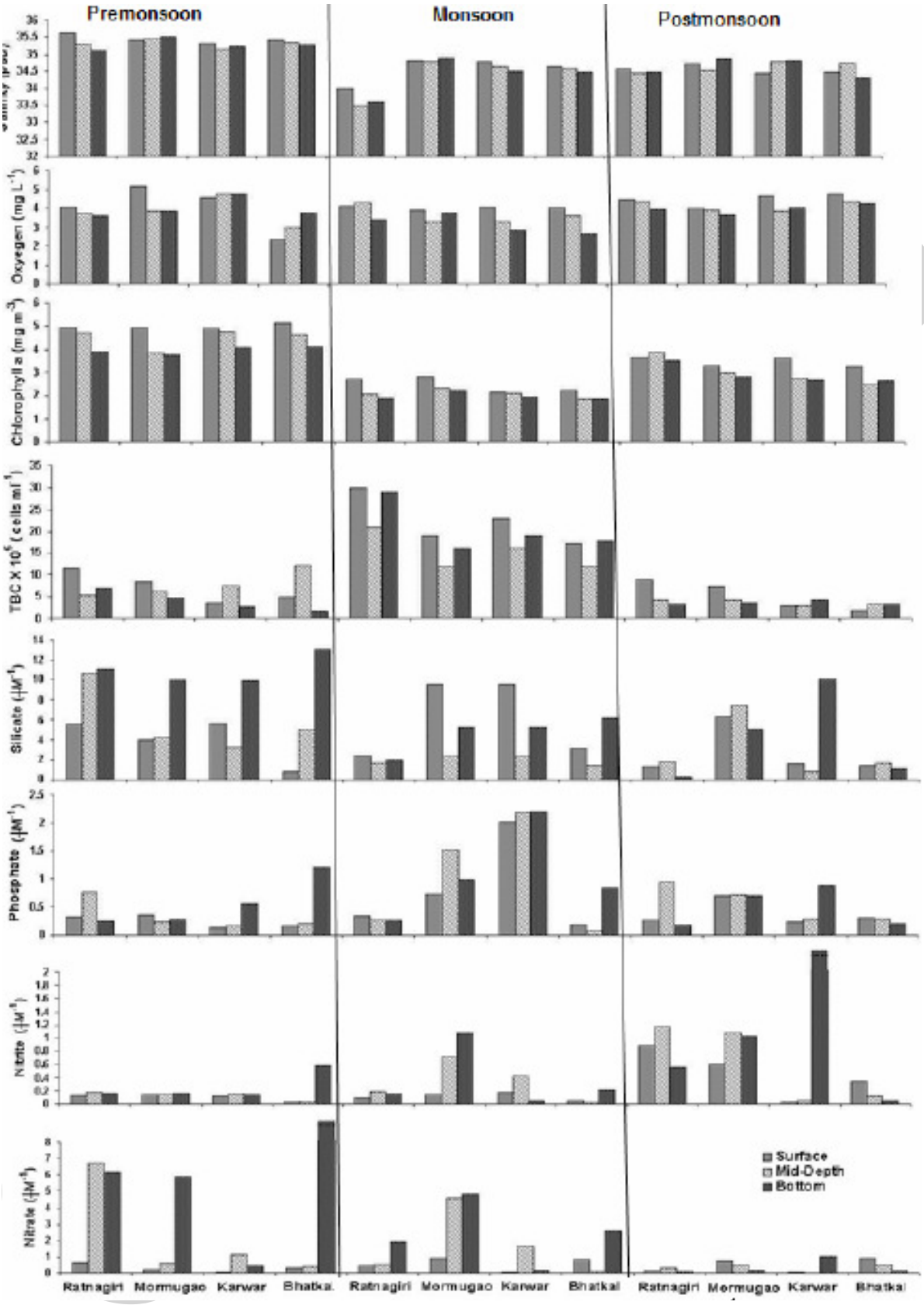

Fig. 2: Season and location-wise variations in salinity, nitrate, nitrite, phosphate, silicate, dissolved oxyegen, chlorophyll *a*, and total bacteria counts

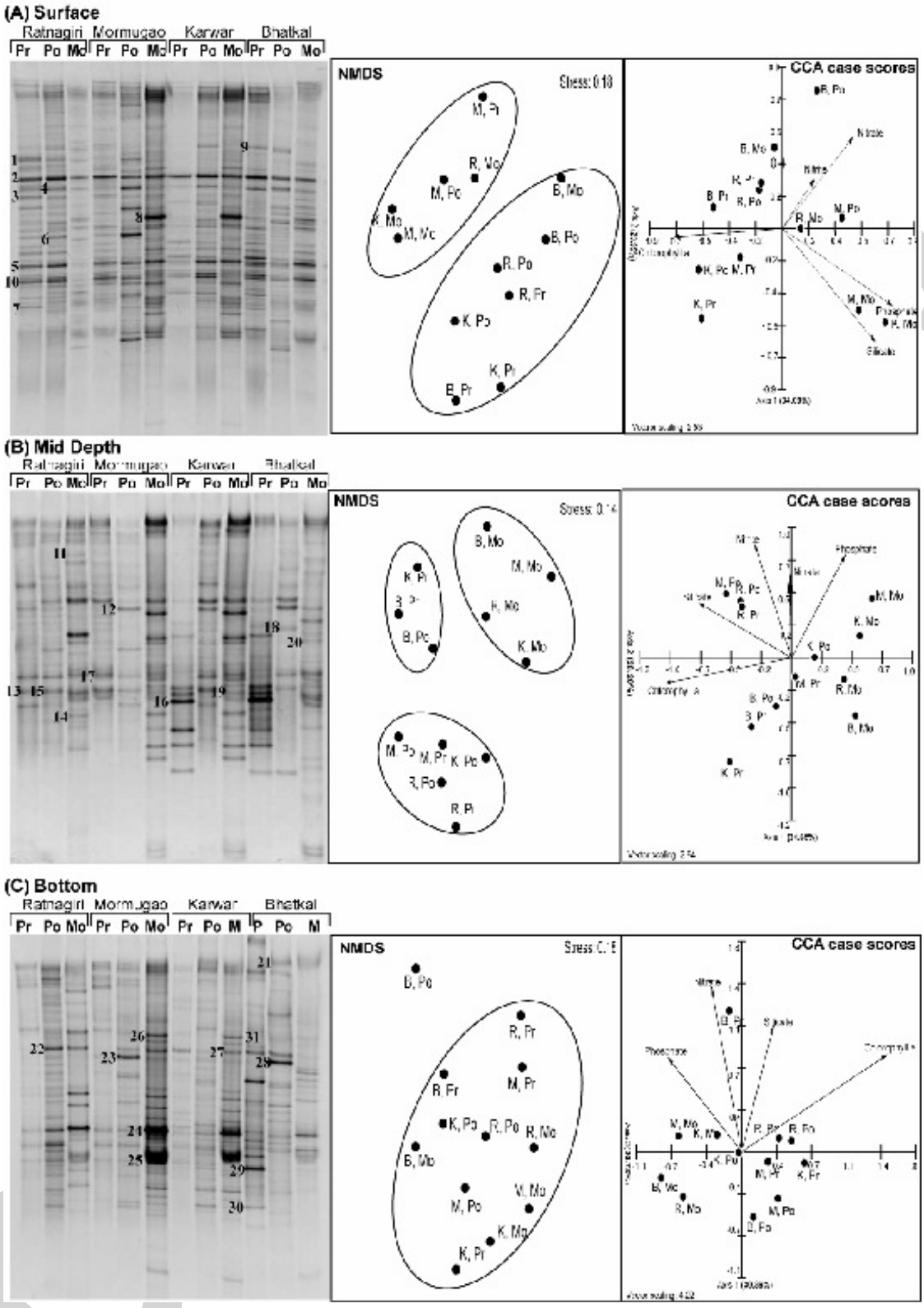

Fig. 3: Saptial and temporal variations in bacterial communities deciphered through DGGE patterns Samples were collected during premonsoon (Pr), postmonsoon (Po) and monsoon (Mo) off Ratnagiri (R), Murmogao (M), Karwar (K) and Bhatkal (B) from surface (A), mid-depth (10 m; B) and bottom (20 m; C). Results from non-metric dimensional scaling (NMDS) and canonical correspondence analysis (CCA) are also included for each gel. The percentages of variations explained through CCA by each axis are in parentheses. Very small vectors are not shown

variation in BCC was the largest in samples from surface and mid-depth at all locations implying the effect of monsoon.

Correlation between phylotypes and environmental variables: The CCA analysis was done on each DGGE gel to explain the environmental factors driving the spatial and temporal variation in BCC (Fig. 2). It brought out that the chl *a* and NO_3, among the five independent explanatory variables chosen (NO_3, NO_2, PO_4, SiO_4 and chl *a*), were responsible for spatial variation in BCC during premonsoon and monsoon (Fig. 3). Other environmental parameters (depth, pH, salinity *etc.*) with high (>20) inflation ratio were not included for the CCA analysis. All the independent variables indicated strong correlation (>88%) with the phylotypes in each lane. Seasonal variations of BCC in the surface and mid-depth samples were explained largely by chl *a* concentrations (Fig. 3a,b). The CCA analysis also indicated increasing correlation of NO_3 with increasing depth. Axis 1 explained 34.09, 34.65 and 30.36% of variation in BCC from surface, mid-depth and bottom waters respectively (Fig. 3).

Bacterial diversity of any place is affected by biogeochemical, anthropogenic factors plus the biogeography of Bacteria (Park and Shin, 2007; Van der *et al.,* 2007) and, by both top-down control and bottom-up controls. In the top-down control, grazers (protozoan, metazoan and dinoflagellate) keep check on bacterial population by selecting the particular size- or actively growing- fraction of bacteria (Adrian and Schneider-Olt, 1999; Bird and Kalff ,1984). In the bottom-up control, availability of quality and quantity of resources regulate the bacterial population dynamics (Grover, 2000; Lebaron *et al.*, 1999). The chl *a* has been used widely as a reference for relating the marine bacterial populations and their spatio- temporal variations (Pinhassi and Hagstrom, 2000). Also, bacterial populations are observed to be affected by the source of organic matter (Crump *et al.,* 2003). Since chl *a* was >1 mg l^{-1} at all locations and depths, it is unlikely that the organic resources were limiting to bacterial processes. In our analysis, the significant correlation observed between the BCC and chl *a* in surface waters and mid-depth waters could be taken to suggest that the bacterial diversity and population sizes are regulated mostly by top-down control rather than by primary productivity. Furthermore, intense bacterivory has been reported previously from coastal waters (Gasol *et al.*, 2002). From our CCA analysis, it is evident that nitrate was a more significant variable explaining the variation of BCC during monsoon than could chl *a* do. Also, the TBC were higher (12 to 30 [X 10^5] cells ml^{-1}) at all locations during monsoon. All these inferences suggest bottom up effect of nitrate on BCC. Ducklow *et al.* (2001) had also shown ~2-fold increase in bacterial secondary production during monsoon. Also, higher numbers of OTUs indicate that diverse groups of bacteria proliferate in the presence of higher organic matter as a consequence increased primary production due to elevated NO_3 concentrations.

Apart from recognizing the gross structure of bacterial assemblages along the central west coast of India, it is possible from this study to suggest that chlorophyll, nitrate, seasonal shifts influence the bacterial diversity pattern. From the DGGE profiles, it is apparent that low mixing due to calmer sea conditions during pre-monsoon leading to stratification and, complete mixing during monsoon leading to well mixed water column contribute to seasonal and vertical differences in the bacterial assemblages. The results are also useful for discerning the effect of seasonal shifts on the variations of bacterial assemblages. Further, the low or insignificant changes in the BCC depth-wise are suggestive that bacterial species in these waters are homogeneously distributed vertically at all the sampling locations.

In summary, the DGGE analysis of BCC along the central west coast of India illustrates spatial variations within the small geographic distances. While concentrations of chlorophyll *a* and nitrate are responsible for affecting the BCC, the temporal variation in BCC is caused mainly by the onset and, prevalence of monsoon. The gammaproteobacteria, bacteroidetes and cyanobaterial species seem to be abundant in these coastal regions. Incidentally, both top-down and bottom-up control of BCC seem to be operating in the study area with monsoon facilitating the bottom-up control.

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