Ion Trap (Esquire 3000) mass spectrometer, which gives monoisotopic resolution throughout the mass range of 3000 amu. The method is linear over a wide concentration range with a correlation coefficient greater than 0.996. The analytical protocol based on MS/MS has successfully been employed by us for analysis of camptothecin in crude extracts obtained from plant sources, fermentation broths and mycelia cultured in the laboratory. The assay method is sensitive and selective, allows minimal matrix interferences and requires a simple sample clean-up procedure. The experimental results obtained by us during the fermentation experiments for the production of camptothecin demonstrate the usefulness of the assay in monitoring the metabolic flux of CPT in endophytes residing in the plants.

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Microbial genetic resource mapping of East Calcutta wetlands

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East Calcutta Wetland (ECW) is the world's largest natural treatment plant for solid and soluble waste, where bioremediation and biodegradation of complex compounds is mainly based on microbial activity and is recognized as a potential source of bacteria of biotechnological impact. Here we discuss the microbial resource mapping of this important Ramsar site. Culture-independent analysis of the microbial population from soil and water through community DNA isolation, amplification, cloning and partial sequencing (320 clones) of 16Sr RNA gene sequence and finally secondary structure-based phylogenetic analysis of the novel sequences (292 GenBank submissions) using ARB software indicated the presence of microorganisms from 12 different main bacterial phyla, thus revealing the rich natural microbial resource at ECW.

Keywords: ARB software, bioremediation, East Calcutta wetlands, microbial biodiversity, phylogenetic analysis.

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BIODIVERSITY screening is an active area of research as it provides inexhaustible data which can be used for the purpose of developing products for the pharmaceutical, agricultural, chemical processing and industrial markets[1]. Knowledge on the biodiversity of crucial ecological areas would yield a better understanding of their community composition, spatial relationship among organisms, nature of the operating biochemical cycles and the mechanism of the existing energy balance[1]. These would provide a framework for the conservation and sustainable development of ecological hotspots and give us insights of how nature solves the challenging problem of energy recycling: a knowledge that might be used for commercial utilization. Patterns in the spatial distribution of organisms provide important information about the mechanisms that regulate diversity of life and complexity of ecosystem[2]. The complexity of these communities is not only intriguing but also presents a challenge to biotechnology[3].

India has about 10% of the world's biodiversity wealth, but its share in the total world trade based on biodiversity according to the Exim Bank Report (1997) is negligible. The East Calcutta Wetland (ECW) happens to be the largest open waste dumping ground and natural waste recycling system and also a Ramsar site. It lies to the east of Calcutta, covering an area of 12,500 ha. This wetland lies approximately between 22°25′–22°40′ lat. N and 88°20′–88°35′ long. E. Climate in this part of the country is mainly tropical and average rainfall is 1650 mm – 90% from June to October. Thus this area would behave as an incubator for microbial growth.

The wetland bears the oldest tradition of resource recovery from the city's waste, besides being the largest such system in the world. Since the beginning of the last century, various forms of agri and pisci culture have been practised in the region. It is renowned not simply as the sensitive ecosystem of biodiversity value, but more specifically as an example of best practice in the 'wise use' of wetlands (from 1879 onwards) for garbage farming (2500 metric ton solid waste dumped every day), sewage-treated fisheries (bheri) and sewage farming. In addition, organic fertilizer produced by processing the solid waste from this area is found to be commercially important. But little scientific attention has hitherto been given to the ECW, a unique agro-ecosystem that had sustained the world's largest practice of integrated resource recovery for more than 70 years now[4,5]. As the entire bio-remediation at ECW is microbe-based, it is expected to yield a rich source of biotechnologically important microbes.

Soil is a complex and dynamic environment in which biological activity is mostly governed by microorganisms. The beneficial effects of soil microorganisms are manifold and range from nitrogen fixation and organic matter decomposition to breakdown of metabolic by-products and agrochemicals, enhancing the bioavailability of nitrates, sulphates, phosphates and essential metals[6]. It has been proposed by different groups that about 96 to 99% of the microbes are non-cultivable under standard laboratory conditions[7,8]. Thus the traditional cultivation-based approach for microbial screening gives a biased picture of only 1 to 4% of the entire community. In fact, most of the species in many environments have never been described[9], and this will not be possible until new culture technologies are developed[3]. A molecular analysis of 16Sr RNA gene sequence is now central to studies examining the diversity of microorganisms in the environment[10]. Thus isolation of total DNA (community DNA) from the natural environment has become a useful tool for studying the microbial profile of ecologically important niches for both cultivable and as yet uncultivable bacteria[11].

The objective of this study was to identify the different microbial communities living in the ECW (commonly called Dhapa), with a view to resource-map the area for exploitable biology. The community profile at Dhapa would eventually help in understanding the different metabolic cycles involved in the bioremediation processes operating there. Screening of these areas would most likely reveal a rich source of biotechnologically important bacteria.

Soil was collected from wetlands in the dumping grounds of Dhapa using sterile hollow metal rods of 14.5 cm length. The soil was entrapped in the hollow space and was immediately sealed inside polypropylene packets. The soil retained its moisture and was used fruitfully for community DNA isolation each time it was implemented.

Water from sewage as well as bheri (shallow wastewater-fed fish ponds) was collected in sterile jars from 1.5 inches below the surface. They were sealed immediately.

A modified indirect method of DNA isolation and purification was followed[1]. About 8 ml buffer A [50 mM Tris, pH 8.3; 200 mM NaCl; 5 mM EDTA; 0.05% Triton X 100] was used to resuspend 4 g (wet weight) of soil. It was mixed gently for 30 min for resuspending cells present in the soil. The suspension was centrifuged at 100 *g* for 5 min and the slurry (supernatant) was taken into another tube. It was centrifuged at 10,000 *g* for 10 min. The supernatant was discarded and the pellet was resuspended in the same buffer, mixed and centrifuged at 10,000 *g* for 10 min. This was done to wash out humic acid adhering to the cells. This process was repeated until the supernatant had no traces of brown colour because of eluted humic acid. Then the pellet was resuspended in the same buffer without Triton X 100 (buffer B) and washed in similar manner as in the previous case, thrice. The pellet was resuspended in 0.125 M EDTA, pH 8.0 and treated as in the previous case. The pellet was resuspended in TE buffer (buffer C) [10 mM Tris, pH 8.0 and 0.1 mM EDTA] and treated as in previous step, thrice. All these steps ensured removal of co-eluting inhibitors that adhere to the cells, mainly humic acid. The pellet was finally resuspended in 800 µl lysis buffer consisting of 10% sucrose; 0.7 M NaCl; 40 mM EDTA and 50 mM Tris, pH 8.3. After addition of lysozyme (1 mg/ml), the suspension was subjected to three cycles of freeze–thawing (–70°C and +65°C). To

the lysate 50 µg/ml proteinase K and 1% SDS were added and the lysate was incubated at 55°C for 60 min. Next 1% CTAB was added and the lysate was incubated further at 55°C for 30 min. The lysate was centrifuged at 10,000 *g* for 10 min. The supernatant was extracted with phenol : chloroform thrice and chloroform thrice. The aqueous layer was precipitated with 0.1 vol 3 M sodium acetate and 2.5 vol ethanol. The pellet was resuspended in 100 µl of sterile triple-distilled water. The isolated community DNA was subjected to precipitation following incubation for 2 h at room temperature with 16.7% polyethylene glycol (PEG) 8000 and 330 mM NaCl. The pellet was resuspended in sterile distilled water and treated with acid-washed polyvinylpolypyrrolidone (PVPP) at 37°C for 30 min. The sample was centrifuged at 10,000 *g* for 10 min and the supernatant was filtered through a 0.45 µm filter to remove trace amounts of PVPP. The filtrate was precipitated and purified DNA was obtained. This method gave lesser yield compared to the direct method, but the DNA had lower amount of co-eluted humic substances[12].

The DNA was used in 1 : 50 and 1 : 100 dilutions for PCR[13]. The DNA extracted from water was amplifiable after the PEG–NaCl precipitation and did not need treatment with acid-washed PVPP. Moreover, for DNA isolated from bheri water no further dilution of template was needed.

PCR was performed in a Genecycler (BioRad). Degenerate universal primers 5′-TGA CTG ACT GAG TGC CAG CMG CCG CGG-3′ and 5′-TGA CTG ACT GAG AGC TCT ACC TTG TTA CGM YTT-3′, M = A/C, Y = C/T were used[14] for amplification of 16Sr RNA gene sequence fragment (1050 nt) with the touch-up program mentioned elsewhere[1]. Bacterial primers[3,15,16] 5′-AGA GTT TGA TCA TGG CTC-3′ and 5′-CTA GCG ATT CCG ACT TCA-3′ were also used for amplification of 16Sr RNA gene sequence (1300 nt). After denaturation for 2 min at 92°C, 40 cycles of 1 min at 92°C, 1 min at 50°C and 2 min at 72°C were performed. The reaction mixture (50 µl) consisted of 1 to 6 µl template (up to 500 ng depending upon the concentration of co-eluted inhibitor), 5 µl of 10 × PCR buffer [10 mM Tris-HCl (pH 9.0), 1.5 mM $MgCl_2$, 50 mM KCl, 0.01% gelatin], 250 µM deoxyribonucleotide triphosphate, 3U Taq polymerase, and 0.7 µM of each primer. The PCR product was analysed on a 1.5% agarose gel.

PCR products were purified using High Pure PCR Product Purification kit from Roche, according to the manufacturer's protocol. Wherever needed, the purified PCR product was concentrated by vacuum evaporation (Heto Leopard – speed vacuum concentrator). The purified products were ligated into the pGEM-T vector according to the manufacturer's protocol (pGEM-T Easy Vector System I Promega, Madison, USA) and transformed into *Escherichia coli* JM109 using TSS method of transformation[17]. Monitoring gel retardation of the isolated plasmid, the positive clones were screened (Figure 1). Sequencing grade plasmid was isolated according to the mini alkaline lysis method, as specified by the manufacturers (ABI-Perkin Elmer). Partial sequencing of the clones was done using the standard M13 primers in an ABI-automated DNA sequencer. The sequences were subjected to on-line Blast analysis (http://www.ncbi.nlm.nih.gov/blast) and chimera check (http://rdp.cme.msu.edu/html/analyses.html). Novel sequences were submitted to GenBank (Dpcom clones).

The sequences were analysed using the ARB software package (Linux beta version 030822) and the ARB database ssu_jan03 (Department of Microbiology, Technical University of Munich, Munich, Germany [http://www.arb-home.de]). The non-chimeric novel 16Sr RNA gene sequence fragments were added to the database. Using the ARB sequence editor with the fast_aligner, the clone sequences were automatically aligned. The alignments were refined manually by visual inspection. Applying the ARB parsimony tool, phylogenetic analysis based on 16Sr RNA gene sequence was performed. ARB parsimony is the only program where a partial sequence can be aligned with a complete sequence. The number of clones was plotted against the number of operational taxonomic units (OTUs) to understand the compositional pattern of the microbial community at ECW. It represents the rarefaction curve of the number of OTUs in the 16Sr RNA library constructed with standard amplification protocols.

The novel sequences (292) obtained in this study are available in GenBank under accession numbers AF513607-AF513618, AF514783-AF514791, AF515616-AF515619, AF521024-AF521036, AF537610-AF537619, AY144584-AY144586, AY150565-AY150569, AY304012-AY304032, AY304522-AY304535, AY305382, AY311785-AY311820, AY316202, AY371262-AY371269, AY373325-AY373336, AY375566-AY375567, AY386235-AY386238, AY398618-AY398622, AY422189-AY422192, AY422814-AY422820, AY423562-AY423566, AY423567-AY423580, AY427824-AY427826, AY429445-AY429456, AY434017-AY434031, AY453226-AY453275, AY664637-AY664659.

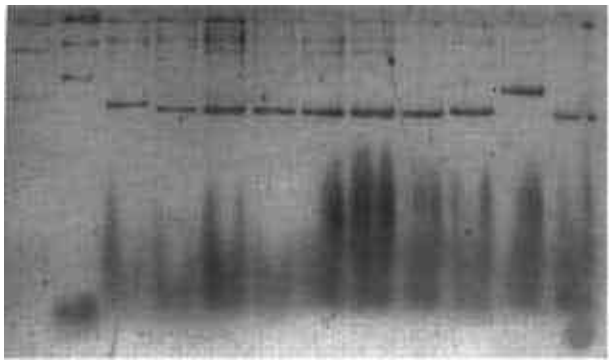

Figure 1. Inverted image of ethidium bromide-stained 1% agarose gel showing screening of clones using gel retardation. From left to right the lanes are as follows: Lane 1, Plasmid 3.5 kb (marker); lane 2, Plasmid 4.4 kb (marker); lanes 3–12, Clones to be screened [only lane 11 has plasmid with an insert (plasmid 4.06 kb), while the rest are self-ligated pGEMT vectors (plasmid 3kb)].

As early as 1978, it had been identified that reuse of sewage in fish culture, algal and aquatic plant production and energy production is to be viewed as a promising technology that radically changes the context of urban sanitation[4]. This has been practised at ECW for more than 70 years now[4,5]. But little scientific and technical attention has hitherto been given to the ECW. These natural wetlands have been modified into clusters of shallow, flat-bottom ponds (bheri) that grow fish on sewage. Pond effluent is then used for irrigation. The bheris serve as the world's largest fisheries utilizing raw sewage as input water and in turn purifying it by more than 96% on coliform count. It produces one-third of the cities fish requirement utilizing one-third of the cities raw liquid sewage. However, the practice is largely retained as an oral tradition of local farmers[4].

ECW being the largest such natural recycling centre, it is expected to be a rich source of bioremediants. This study was an attempt to resource-map the area for microbial community. A culture-independent approach was adopted to obtain a true picture of the microbial diversity of these areas. For convenience, the ECW was divided into various sites with different functions or practices that are being carried out there. A modified indirect lysis method for DNA extraction was used. It gave lesser yield of DNA, but ensured minimal co-eluted inhibitors. The community DNA obtained from both the soil and water was of high molecular weight (result not shown). The community DNA from water was of sufficient purity to be used for PCR amplification in high template concentration, but those from soil had to be diluted 50 times to get the required amplification. Irrespective of the type of sample (different types of soil or water), this method of isolation worked well with minor modifications (results not shown).

ARB is the only software for secondary structure-based phylogenetic analysis. This ensures that the prediction of the putative position of the novel sequence is more precise than when using the primary structure-based prediction. ARB has a database of 50,000 already aligned complete 16Sr RNA gene sequences. The ARB parsimony program, though less sensitive than the maximum likelihood method, is the only one of its kind which allows comparison of a partial sequence with a complete sequence, thus ensuring better prediction of phylogenetic position even with partial sequence data. The novel non-chimeric sequences of the clones when placed in the phylogenetic tree were found to be distributed throughout the bacterial domain (Figures 2 and 3). There were 24 sequences in the phylum *b*–*g*-Proteobacteria, 56 sequences in *a*-Proteobacteria, one sequence in *e*-Proteobacteria, five sequences in Bacteroidetes, 75 sequences in Cyanobacteria, 42 sequences in *d*-Proteobacteria, 12 sequences under one uncultured clus-

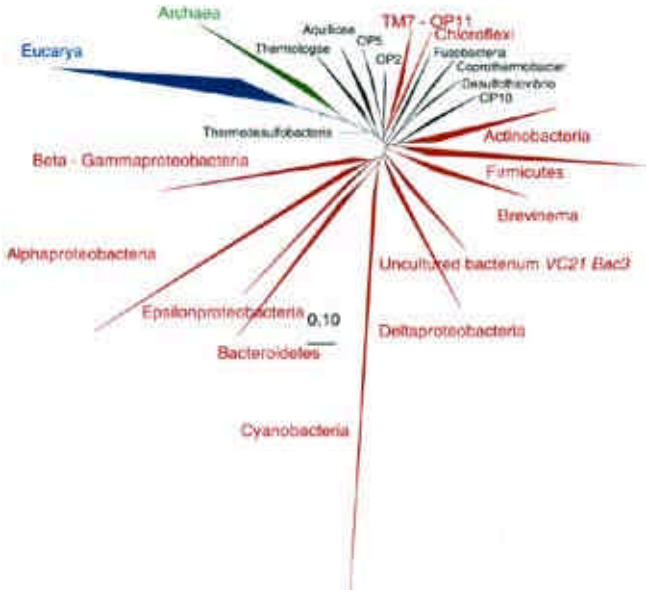

Figure 2. Unrooted tree highlighting phyla with Dpcom clones (in red). All clones were distributed among the 12 different phyla of domain bacteria. Branch length is a true representation of the extent of diversity within a phylum. Scale bar represents the actual distance among the members of each phylum.

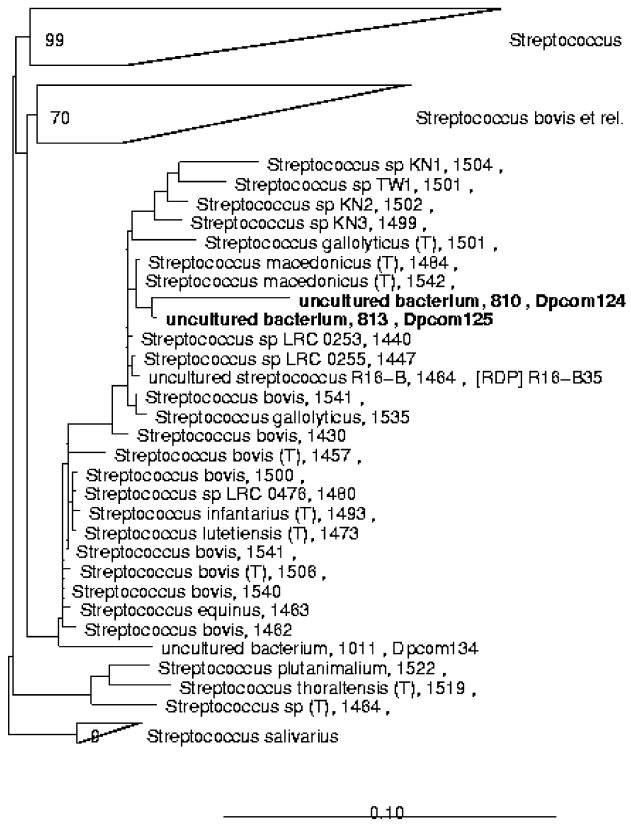

Figure 3. Subtree of phyla Fermicutes showing two clones (Dpcom 124 and Dpcom 125) which are closest to *Streptococcus macedonicus* (antimicrobial agent, e.g. bacteriocin as well as protease-producing strain). Scale bar represents distance of the clones with the cultivable neighbour. Numerical values beside the organism names represent the length of the sequence under investigation.

Table 1. Distribution of clones from different sites of ECW among the 12 bacterial phyla. Site-a represents an old solid dumping ground that has been converted to cultivable land. Site-b represents the oldest solid dumping ground where no waste has been dumped for more than 60 years. These areas have been covered with thick vegetation (mostly trees simulating forest ecosystem). Site-c represents the shallow flat-bottom wastewater-fed fisheries (bheri). Site-d represents the raw liquid sewage canal that drains into the bheri

ECW site	*b–g*-Proteo-bacteria	*a*-Proteo-bacteria	*e*-Proteo-bacteria	Bactero-idetes	Cyano-bacteria	*d*-Proteo-bacteria	Uncul-tured	Brevi-nema	Fermi-cutes	Actino-bacteria	Chloro-flexi	TM7-OP11
Site-a	+	+			+				+	+		
Site-b		+			+							
Site-c	+	+	+	+	+	+	+	+	+	+	+	+
Site-d	+	+			+	+			+			

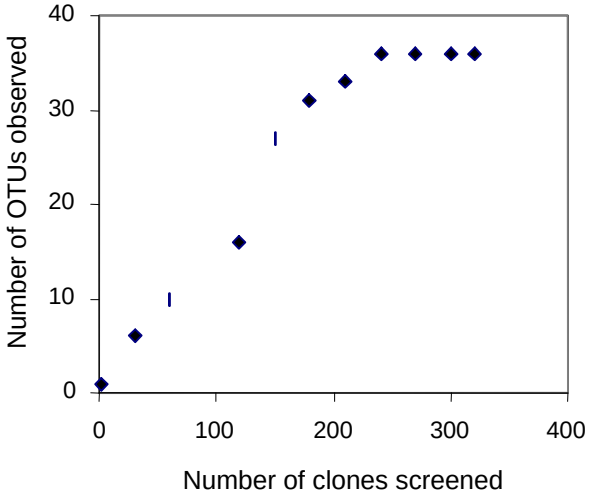

Figure 4. Compositional pattern of microbial population at ECW. Rarefaction curve of the number of OTUs in the 16Sr RNA gene sequence library constructed with standard amplification protocol as mentioned in the text. The pattern shows saturation of species diversity in this study.

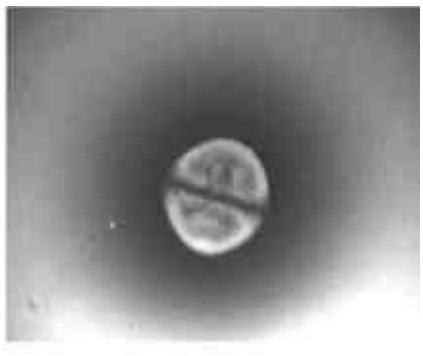

Figure 5. Transmission electron micrograph of novel protease-producing diplococci showing deposition of electron-dense material. Microbes were grown in the presence of saturating concentration of Ag^{+} in liquid media. The image is under negative staining with 1% phosphotungstic acid. Imaging was done under 120 KV transmission mode.

ter, while one sequence within another uncultured cluster TM7-OP11, two sequences in Brevinema, 26 sequences in Fermicutes, 24 sequences in Actinobacteria, while 11 of them in Chloroflexi. Most of the sequences were obtained using the universal primer, while 10 from Cyanobacteria, five from *a*-Proteobacteria, and 15 from *d*-Proteobacteria were obtained using bacterial primers. There was variation in population at different sites of ECW indicating the underlying bioremedial activity operating there. Cyanobacteria and *a*-Proteobacteria-like clones are found in all sites, while members from all 12 phyla were present in the bheri, pointing towards the vide diversity that is present in the bheri (Table 1). This is at par with the microscopic examination of the bheri water (data not shown).

The presence of Actinobacteria and Fermicutes indicates the possibility of nitrophenol, nitroaromatic compound, pesticide and herbicide degradation. This is expected in a dumping ground which receives solid and liquid waste from the entire city and also with the ongoing agricultural activity. These microbes are also involved in decomposition and humus formation. The Proteobacteria present at the ECW would be involved in bioremediation of heavy metals from solid as well as liquid sources. They would cause degradation and recycling of woody tissue of plants and biodegradation of oil-contaminated soil and toxic compounds. They are involved in nitrogen fixation along with the Cyanobacteria in the bheri. They would also be involved in metal-bioremediation.

The fact that only bacterial clones were found does not mean that no Archaea or lower Eukarya are present. But the method used for community DNA isolation may be better for bacterial DNA isolation or might reveal only microbes that are in majority. Compositional pattern of microbial population was seen to reach a saturation on plotting the number of clones against the number of OTUs observed (Figure 4). Novel sequences falling within Proteobacteria, Fermicutes and Actinobacteria indicate the probable presence of metal-accumulating[18], oil-degrading, antimicrobial compound-producing as well as enzyme-producing bacteria[19]. Thus attempts were made to isolate metal-accumulating bacteria from ECW using growth media for Proteobacteria and Fermicutes. Twenty bacterial isolates thus obtained were found to grow in the presence of different concentrations of various heavy metals like aluminium(III), copper(II), nickel(II), cobalt(II), iron(II), chromium(III), lead(II), silver(I) and cadmium(II). Energy-dispersive X-ray fluorescence and transmission elec-

tron microscopy reveals the accumulation of electron-dense metals within the cells, thus confirming their metal-accumulating ability (Figure 5). Molecular characterizations of the isolates are currently underway [GenBank DQ256262 – DQ256264].

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Do gas seepage sites support distinct macrofaunal community? – an observation in the tropical shelf region of Goa, Arabian Sea, India

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We examined the macrofaunal community in the shelf region (depth 15–50 m) of Goa, Arabian Sea, India, at seven sites in the gas seep and seven sites in non-seep areas during March 2005. A total of 56 species were recorded, among which 21 were found in the seep sites, 12 in the non-seep sites and 23 species were common. The faunal community differed between these two areas and community indices were relatively higher in seep sites, whereas the population density was higher in non-seep sites. Such difference is largely due to relatively high organic carbon and clay content in the seep sites. Polychaetes, *Heteromastus similis*, *Pulliella armata* and *Capitella capitata* belonging to family Capitellidae, were found only at all the seep sites and can be considered as good indicator species.

Keywords: Goa coast, indicator species, macrofauna, seep areas.

DISCOVERIES of gas seepage areas and hydrothermal vents in the ocean floor and the associated fauna have fascinated marine biologists all over the world due to the unique

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