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Abundance of sewage-pollution indicator and human pathogenic bacteria in a tropical estuarine complex

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Abstract Studies on abundance and types of various pollution indicator bacterial populations from tropical estuaries are rare. This study was aimed to estimate current levels of pollution indicator as well as many groups of human pathogenic bacteria and their seasonal variations in different locations in Mandovi and Zuari Rivers in the central west coast of India. The sampling covered the estuarine and upstream regions of these rivers representing premonsoon (May 2005), monsoon (September 2006) and post-monsoon (November 2005). Both the abundance and types of autochthonous and allochthonous microbial populations in the near shore environments are affected by land drainages, domestic sewage outfalls and other discharges. The overall ranges (and their mean abundance; no. ml^{-1}) of the monitored groups of bacteria were: total coliforms: 0–29,047 (3,134 ml^{-1}); total streptococci: 3–14,597 (798); total vibrios: 13–42,275 (2,530); *Escherichia coli*: 0–1,333 (123); *Vibrio cholerae*: 0–3,012 (207); *Salmonella* spp: 0–1,646 (90); *Streptococcus faecalis*: 0–613 (88) and *Aeromonas* spp: 0–2,760 (205). In general, abundance of sewage pollution indicator bacteria such as total coliforms and total streptococci was lower than that reported from many other locations worldwide.

Keywords Sewage pollution indicators · Human pathogenic bacteria · Tropical estuaries · Marine environmental contamination · Allochthonous bacteria

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Introduction

Environmental surveys are necessary for understanding and documenting the occurrence and distribution of pollution indicator and human pathogenic bacteria. In order to quantify and understand their relationship with relevant environmental factors, several investigators have examined distribution of these groups of bacteria and certain viruses in coastal waters (Colwell et al. 1977; Marchand 1986; Patti et al. 1987; Piccolomini et al. 1987; Ramaiah and Chandramohan 1993; Ruiz et al. 2000; Ramaiah and De 2003). Mortality and survival rates of fecal contamination indicator *Escherichia coli* in the marine regimes have also been studied (Thom et al. 1992; Darakas 2001). Findings from these studies affirm persistence of allochthonous microflora in the marine environment. Further, different species of bacteria including pathogenic ones (Colwell et al. 1981;

Xu et al. 1982; Huq et al. 1984; McCarthy and Khambaty 1994; McCarthy 1996; Wait and Sobsey 2000; Darakas 2001; Ramaiah et al. 2002a) survive in seawater for one to several weeks. Once introduced into the marine environment, allochthonous pathogens of human health concerns can disperse far and wide to other regions.

Land drainages, domestic sewage outfalls, and other discharges alter the abundance and type of both autochthonous and allochthonous microbial populations in the near shore environments (Colwell et al. 1977; Marchand 1986; Patti et al. 1987; Piccolomini et al. 1987). Further, higher proportions of allochthonous microflora that not only survive but also out-compete native microflora are certain to cause undesirable ecosystem imbalances (Colwell et al. 1981; Huq et al. 1984). An understanding of incidence and distribution of bacterial species, their physiological characteristics including pathogenicity to both plants and animals is important for gaining insights on the presence of harmful microbial communities. In order to assess the importance of microbial pathogens in the marine environment, information on the microbial load in any given ecosystem is mandatory. Once at hand, such data can be used for evolving advisories on controlling or regulating their abundance in any ecological situation. These aspects have been widely investigated and, the important coastal pollution surveillance needs no overemphasis (Fujioka et al. 1997). However, studies on abundance and types of various pollution indicator bacterial populations from tropical estuaries are rare.

This study was carried out to document current levels of pollution indicator bacteria as well as some groups of human pathogenic bacteria in different areas in both the estuaries and offshore waters of Goa on the central west coast of India. The study also focused on understanding the seasonal differences in all microbial groups looked for, so as to provide an annual cycle of microbial load.

Materials and methods

Various sampling locations sampled are shown in Fig. 1. Zuari and Mandovi are the two major rivers of Goa. With the rainfall in excess of 2,500 mm annually (Shankar et al. 2004), these rivers drain enormous quantities of land materials during June–September, the months of intense rains. Apart from this, there are routine releases of urban sewage effluents in the range of 10 million litres per day (Sawkar et al. 2003). Further, ~300 barges move iron-ore daily into the harbor from mining sites upstream of both the rivers for loading ~15–20 ore carriers for export. Thus, the region receives barge-related wastes in addition to the effluents as above. Being an international tourist destination, Goa receives over 1.2 million visitors annually. Including the lower stretches of Mandovi and Zuari, its coastal spaces are of recreational interest.

Sampling was carried out during May 2005 representing pre-monsoon period (March–May), November 2005 (post-monsoon: November–February) and September 2006 (monsoon: June–October). Samples were collected from many locations offshore, in the Marmugao Bay and from upstream stretches of both the rivers. Since the sampling was combined for other biological measurements, samples for microbiological analysis were collected from 22 different sampling sites. Sites 1–3 were located offshore (station depths 16 to 20 m). Sites 4–7, 9–12 and, 18–22 were in Marmugao Bay. Sites 14–16 were in Mandovi and 8, 13 and 24 in Zuari mouth and upstream zone. This sampling was so planned to represent coastal and estuarine zones of Mandovi and Zuari. These locations were chosen to generally represent a seaward gradient in land drainages and other effluents. To avoid contamination from surface micro-layer, water samples were collected from *ca.* 1 m below the surface by using Niskin (5 L capacity, General Oceanics, FL) samplers. One hundred milliliters of sample from each location was collected in to pre-sterilized bottles. Approximately 100 g of sediment samples collected by a van Veen grab at different locations were removed aseptically into polythene bags. Up to 10 ml samples of zooplankton collected using a Heron Tranter net (200 μm mesh sized bolting silk) were taken out for microbiological analyses. All samples were collected with precautions required for microbiological analysis, held on ice in an icebox

and transported to the laboratory for further analysis.

During all the three collections, water and sediment samples were collected from all locations listed above for quantification of different groups of bacteria. Also examined were the zooplankton samples collected from stations 4, 9, 16, 18 and 24. Following groups of bacteria were quantified. Specific media (Hi-Media) used for enumerating are listed against them.

Nutrient Agar	Total viable counts (TVC)
McConkey Agar	Total coliforms (TC)
	Escherichia coli (EC)
Thiosulphate citrate bile salts sucrose (TCBS) Agar	Total vibrios (VLO)
	Vibrio cholerae (VC)
	Vibrio parahaemolyticus (VP)
Hi-Crome *Salmonella* Agar	*Salmonella* spp (SA)
M *Enterococcus* Agar	Total Streptococci (TS)
	Streptococcus faecalis (SF)
Enterococcus confirmatory Agar	Total streptococci (TS)
	Streptococcus faecalis (SF)
Aeromonas Isolation Agar	*Aeromonas* spp (AA)

Most of the media were prepared with the addition of aged seawater to attain a minimum of 15 PSU salinity and, autoclaved. Only TCBS was prepared using deionised water and steam sterilized as required. All plates were prepared 5 days prior to sampling. Aliquots of 0.1 ml and 0.2 ml of water samples were spread plated in triplicate on nutrient agar plates prepared at least 5 days prior to plating. And aliquots of 0.2 ml, 0.3 ml and 0.4 ml of water samples were spread plated in triplicate on all the selective Media mentioned above. These volumes spread on to the medium in 90 mm dia plates were easily absorbed without any trace of liquid. The Seawater nutrient agar (SWNA) plates were incubated at room temperature (26 $\pm$ 0.5°C) and all other plates of specific media were incubated at 37°C at least for 24 to 48 h and final counts of colonies were noted. When very high counts of any specific group of bacteria were observed in any media ca. 24 h incubation, water samples held in a refrigerator were diluted 10 or 100 fold and reexamined as above by spread plating method. In addition to these plate counts, total direct counts (TDC) of bacteria were enumerated from all the water samples collected during November 2005 and September 2006 following Hobbie et al. (1977) using acridine orange as the fluorochrome.

About 1 g wet sediment samples were suitably diluted using saline water blanks to attain 100 or 1,000 fold dilutions. Two duplicate aliquots of 0.1 ml and 0.2 ml supernatants from both the dilutions were spread plated respectively on nutrient agar plates (prepared at least 5 days prior to plating) and selective media and incubated as mentioned above. About 1 g wet zooplankton samples were homogenized in a tissue blender with appropriate volumes of saline water blanks to attain 100 or 1,000 fold dilutions and duplicate aliquots of 0.1 ml and 0.2 ml supernatants plated as done for sediments. Dry weights of all sediment and zooplankton samples used for enumeration of bacterial groups were determined and colony counts expressed on dry weight basis.

To reduce the uncertainties associated with counting the pathogenic bacteria as 'like organisms' for instance *Escherichia coli* like organisms (ECLO), we carried out appropriate biochemical and morphological tests. For this, a total 564 colonies of ECLO [109 isolates], *Vibrio cholerae* like organisms (VCLO; [102 isolates]),*Streptococcus faecalis* like organisms (SFLO; [102]), *Salmonella* like organisms (SALO; [102]) and *Aeromonas* like organisms (AALO; [102]) from different specific media were picked out, purified and characterized (unpublished

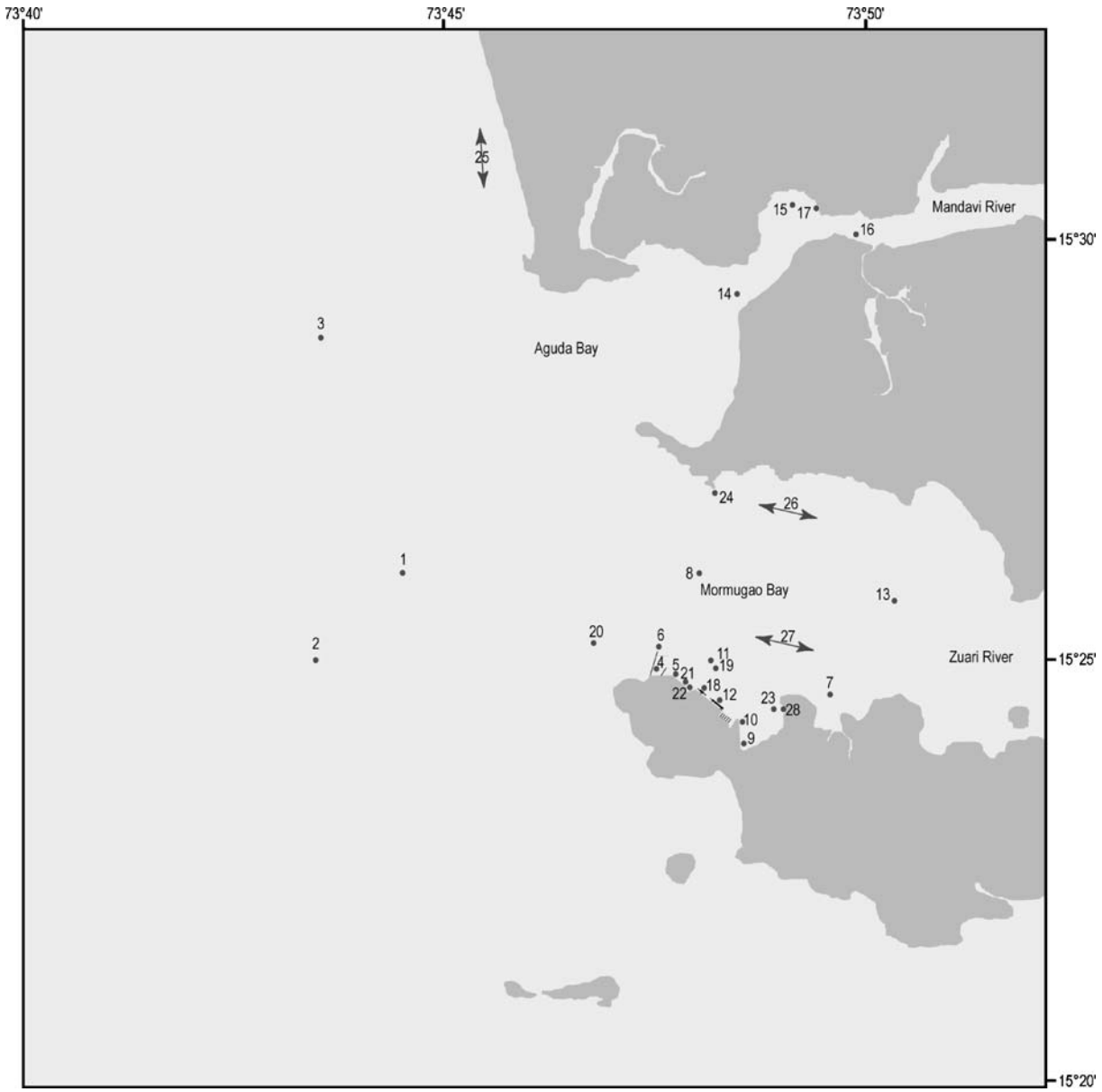

Fig. 1 Map showing sampling locations in the study area of Mandovi–Zuari estuarine complex

results). We find that ∼75% of ECLO are EC and ∼72% each of VCLO, SFLO, SALO and AALO are VC, SF, SA and AA respectively. Using this information, "nearly true" percentages of EC, VC, SF, SA and AA from their 'LO' counts are calculated and presented here.

Results

In brief, the physico chemical characteristics in the study area were as follows. Depths at sampling locations in the study ranged from 3.8 to 20 m, deepening offshore. During May 2005, the

water temperature ranged from 28.0°C to 32.6°C; during November 2005 from 27.6 to 28.9°C and during September 2006 from 27.4°C to 29.5°C. Salinity ranged from 7.3 to 36.3 PSU during September/November. It was quite high (34.7 to 36.7 PSU) during May. Concentrations of NO_3; PO_4 and, SiO_4 respectively were in the range of 0.01–12.57 and 0.01–4.28; 0.05–0.51 and 0.02–1.8 and, 0.02–10.1 and 0.4–19.3 μM during May and November. Chlorophyll concentrations during these two sampling months ranged from 1.22 to 11.45 and, 1.17 to 6.14 mg m^{-3}.

On the basis of media manufacturer's guide and on the knowledge of innumerable previous analyses, typical colony morphology characteristics of different bacterial groups were recognized and initial enumeration of pollution indicator and pathogenic bacteria was completed. Typical colony characteristics of each group are listed below:

McConkey Agar: all colonies grown on this medium counted as total coliforms. Typical, pink 2–3 mm dia colonies counted as *Escherichia coli* like organisms. M *Enterococcus* Agar was used for plating samples collected during May 2005. For later samples *Enterococcus* confirmatory agar was used: all colonies on M *Enterococcus* counted as streptococci. While those typically pink enumerated as *Streptococcus faecalis* like organisms. All colonies on *Enterococcus* confirmatory agar counted as streptococci. Those typically blue, <2 mm dia were enumerated as *Streptococcus faecalis* like organisms. TCBS Agar: all colonies grown on this medium were counted as *Vibrio* like organisms. Sucrose fermenting, yellow, entire, raised <2 mm dia colonies were counted as *Vibrio cholerae* like organisms. Punctate, raised, bluish green, ~1 mm dia colonies were enumerated as *Vibrio parahaemolyticus* like organisms. Hi Crome *Salmonella* Agar: reddish, small, round, convex colonies were counted as *Salmonella* spp (SA). *Aeromonas* Isolation Agar: yellow, entire 2–3 mm dia, glistening, convex colonies were counted as *Aeromonas* spp.

Total and indicator bacterial populations

Total direct counts (TDC) for water samples were the highest during month of November and the least during September (Fig. 2). The mean TDC ranged from 2.5–3.3 [$\times 10^6$] ml^{-1} during November in coastal zone. In Marmugao Bay their number were more (2.6–22.7 [$\times 10^6$] ml^{-1}) during November than during September (1.9–6.0 [$\times 10^3$] ml^{-1}). During November their ranges were 15.9–16.3 [$\times 10^6$] ml^{-1} in Mandovi and, 2.7–9.3 [$\times 10^6$] ml^{-1} in Zuari locations. The TDC were quite low during September at these locations (Fig. 2).

The sampling area covered for this study was quite large. Since we sampled 22 sites each season, the data collected are numerous. Thus, we present here the average abundances of different bacterial populations for each season from water (Fig. 3) and other samples (Figs. 4 and 5). While the differ-

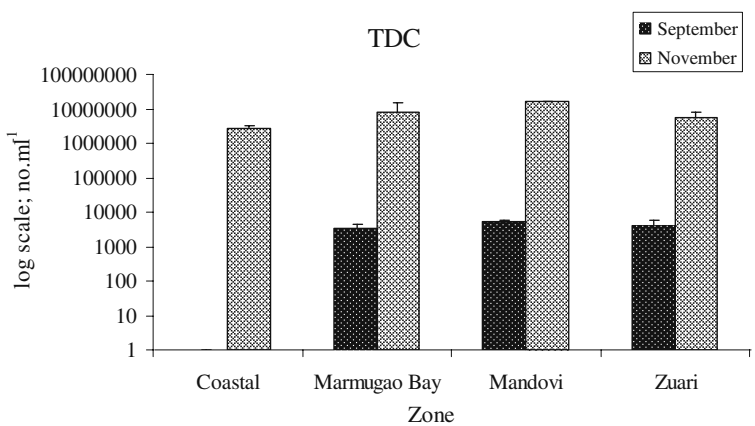

Fig. 2 Total direct counts (*TDC*) of bacteria in water samples collected from Mandovi–Zuari estuarine complex during different seasons

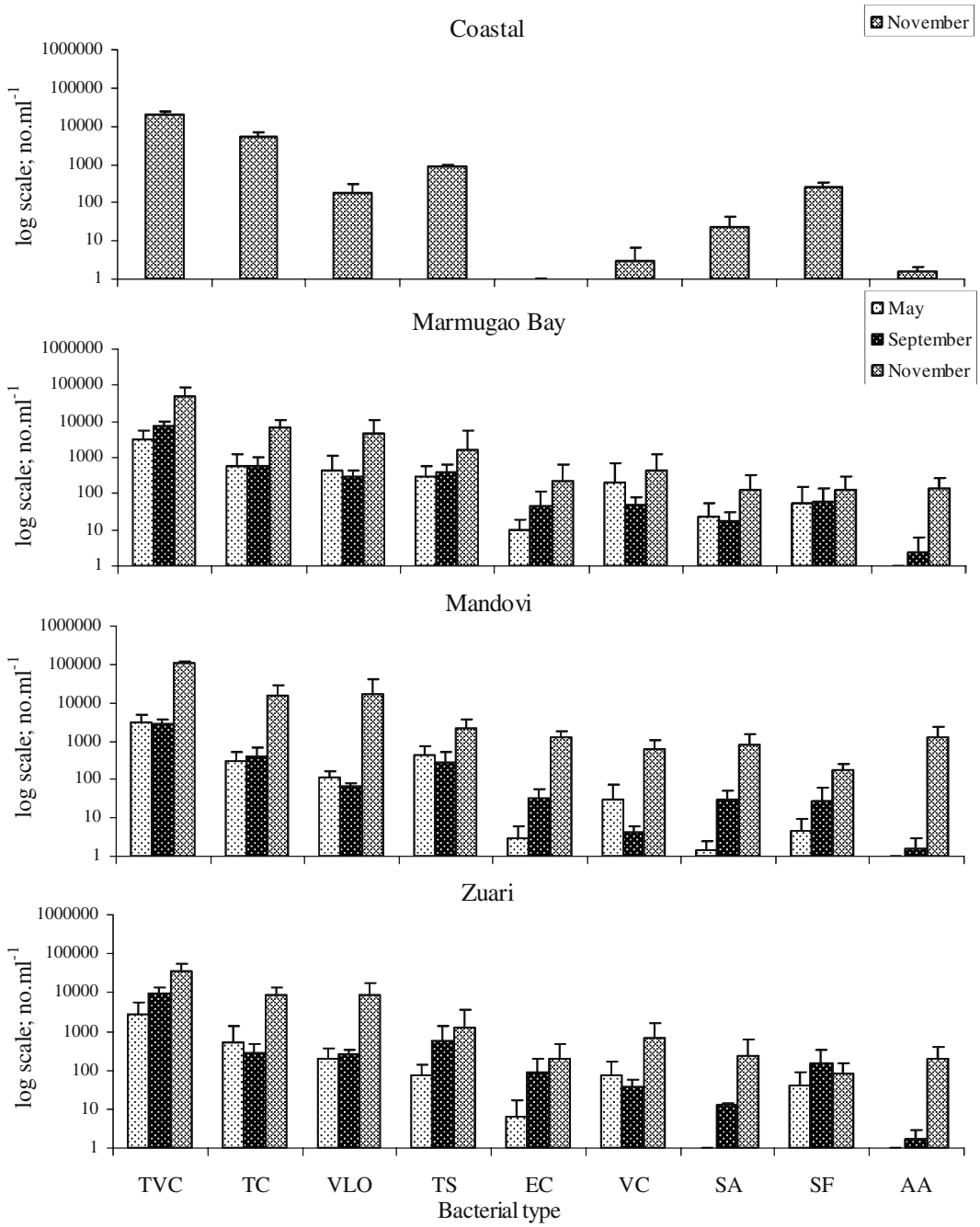

Fig. 3 Indicator and pathogenic bacterial numbers in water samples collected from Mandovi–Zuari estuarine complex during different seasons. *TVC*—total viable counts; *TC*—total coliforms; *VLO*—total vibrios; *TS*—total streptococci; *EC—Escherichia coli*; *VC—Vibrio cholerae*; *SA—Salmonella* spp; *SF—Streptococcus faecalis*; *AA—Aeromonas* spp

ences in their abundances between the sampling locations are statistically insignificant (Table 1), strong signals of seasonal variations could be discerned. In the overall, TVC were more during November (1.6–14.7 [$\times 10^4$] ml^{-1}) in Marmugao Bay than in Zuari (1.6–5.0 [$\times 10^4$] ml^{-1}). Similar

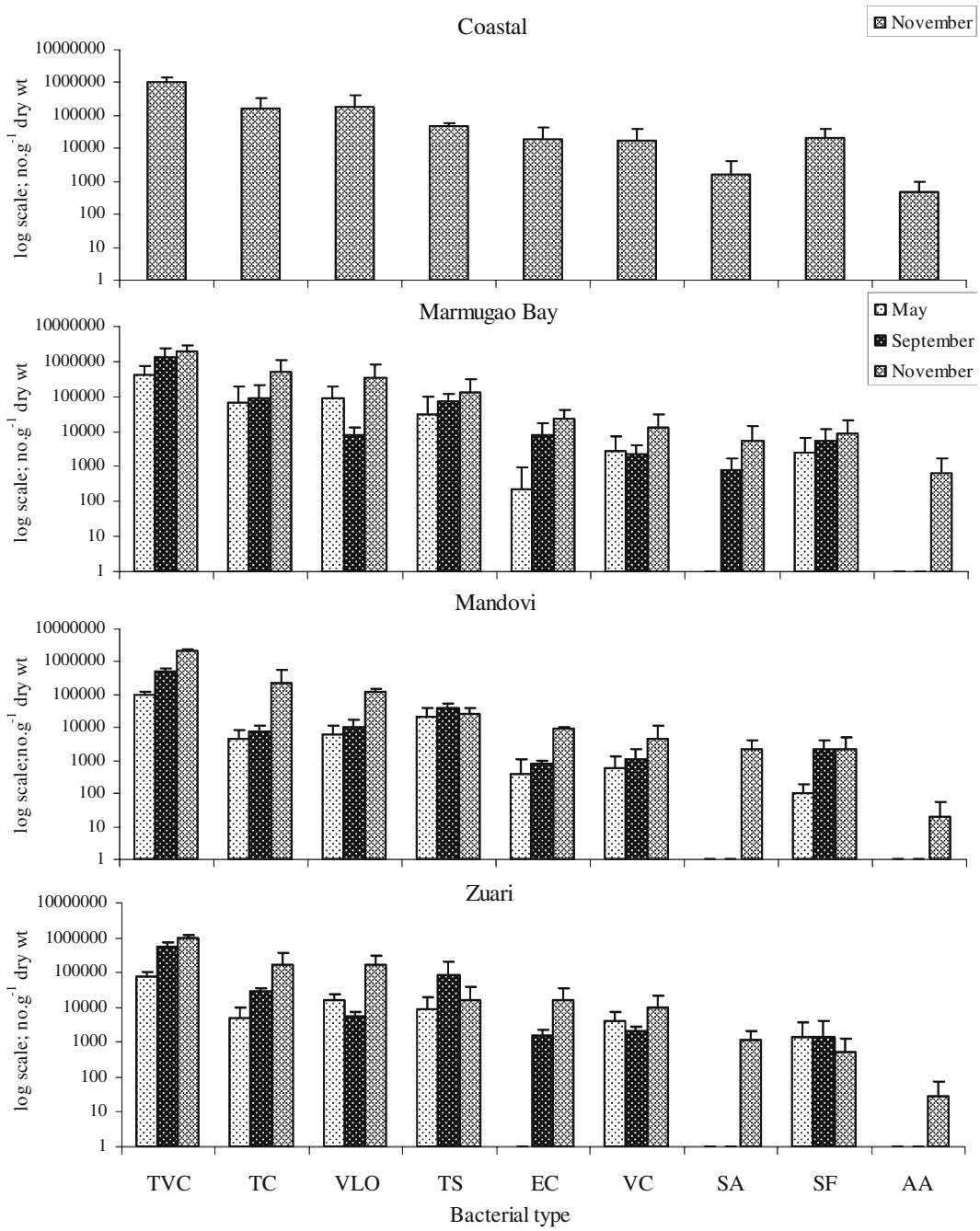

Fig. 4 Indicator and pathogenic bacterial numbers in sediment samples collected from Mandovi–Zuari estuarine complex during different seasons. *TVC*—total viable counts; *TC*—total coliforms; *VLO*—total vibrios; *TS*—total streptococci; *EC—Escherichia coli*; *VC—Vibrio cholerae*; *SA—Salmonella* spp; *SF—Streptococcus faecalis*; *AA—Aeromonas* spp

was the case with the TC and TS (Fig. 3). Owing to rough weather conditions, sampling was not possible from offshore stations 1, 2 and 3 during September 2006. In general, sediment samples had higher TC during November and their counts in increased downstream in both Zuari and Mandovi.

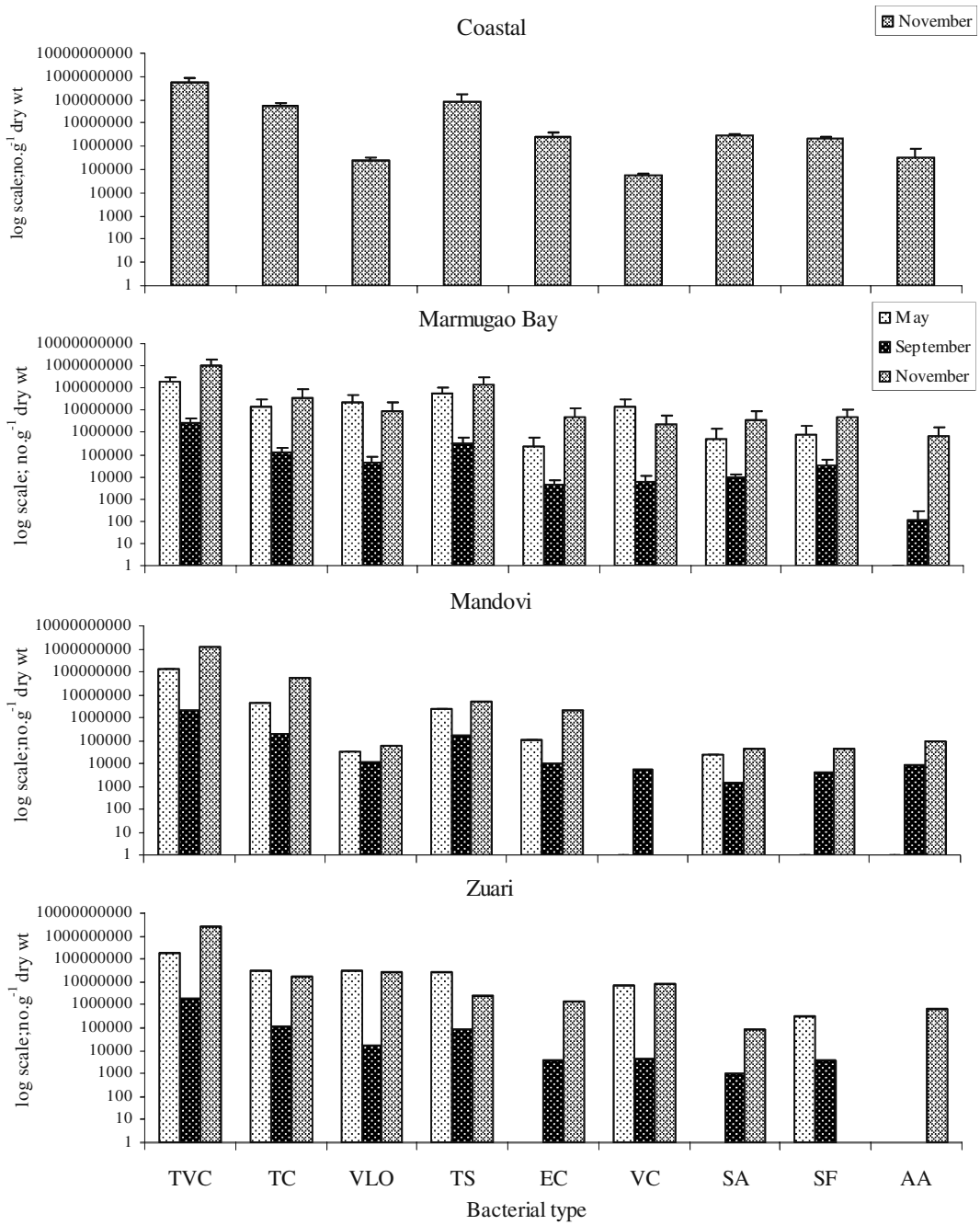

Fig. 5 Indicator and pathogenic bacterial counts from zooplankton samples collected from Mandovi–Zuari estuarine complex during different seasons. Only one zooplankton sample collected from Mandovi and Zuari stations. *TVC*—total viable counts; *TC*—total coliforms; *VLO*—total vibrios; *TS*—total streptococci; *EC*—*Escherichia coli*; *VC*—*Vibrio cholerae*; *SA*—*Salmonella* spp; *SF*—*Streptococcus faecalis*; *AA*—*Aeromonas* spp

Pathogenic bacterial abundance

The abundance of five different types of pathogenic bacteria in water samples during different months is presented in Fig. 3. During November, the counts of *E. coli*, VC and SA were generally more in the entire region. The seafood-borne pathogen, *V. parahaemolyticus* was absent in all

Table 1 Analysis of variance (ANOVA, [$F_{18,2}$]) for different populations of bacteria to distinguish the effect of seasons and locations on their abundance

Bacterial type	ANOVA ($F_{18,2}$)	
	Between seasons	Between locations
TVC	84.5698***	1.1574
TC	46.9021***	1.8224
TS	1.8091	1.1626
VLO	26.1322***	1.7142
VC	8.4839***	1.4905
SA	14.2818***	1.6329
AA	34.0359***	1.0646
EC	11.2339***	1.1771
SF	6.5391*	0.5467

***Significant at $p < 0.0001$; *significant at $p < 0.001$; other values not significant. *TVC*—total viable counts; *TC*—total coliforms; *VLO*—total vibrios; *TS*—total streptococci; *EC—Escherichia coli*; *VC—Vibrio cholerae*; *SA—Salmonella* spp; *SF—Streptococcus faecalis*; *AA—Aeromonas* spp

except two samples (<3 cells ml^{-1}) during November and September. Counts of SF varied widely between seasons. Similar to most other pathogenic groups, the AA counts were high during November. Large variations in the abundance of different pathogenic bacterial types were evident in both sediment and zooplankton samples (Figs. 4 and 5). Counts of EC, VC, SA, SF and AA were in the range of 0 to 1,333 ml^{-1}, 0 to 3,012 ml^{-1}, 0 to 1,646 ml^{-1}, 0 to 613 ml^{-1} and, 0 to 2,760 ml^{-1} respectively.

Discussion

In line with the 'great plate count anomaly' (Staley and Konopka 1985), plate counts of all populations of bacteria enumerated in this study were less than 1 to 0.1% of the TDC. Notwithstanding the deficiencies, the culturable populations of bacteria are useful to obtain insights on the prevalence of certain pathogenic and/or sewage-pollution indicator bacteria.

Sewage contamination of aquatic habitats is detected by enumerating the coliform groups of bacteria (Fujioka 2002). As is universally accepted, higher sewage contamination would lead to increased numbers of coliforms in natural water bodies. Indiscriminate, deliberate, accidental or regular/routine disposals of sewage in most developing countries lead to higher abundance of coliform groups. Ecological surveillance for microbiological analysis is therefore necessary on a continuous basis for realizing the impacts of effluent discharges. Further, as innumerable pathogenic bacteria will constitute the microflora of effluents discharged from domestic, urban, agricultural and certain manufacturing practices, quantifying different groups of pathogenic prokaryotes ought to be part of such surveys. For instance, information on occurrence, abundance and distribution of potent human pathogens, *Vibrio cholerae* (causing cholera in humans), *Vibrio parahaemolyticus* (gastroenteritis), *Salmonella* and *Shigella* spp (typhoid fever; food poisoning), *Streptococcus* spp (meningitis and skin infections) and aeromonads (septicaemic conditions) in aquatic ecosystems may prove useful in public health management.

From a comparative assessment of distribution and abundance of pollution indicator and human pathogenic bacteria in the typically tropical estuaries of Mandovi and Zuari in the central west coast of India, it is inferred that the counts of all the groups are lower than those reported from other regions of the Indian coast (Ramaiah et al. 2004). In general, the highest abundance of all the examined groups was observed during November. However, the fecal coliform counts are lower than those reported from the coastal waters of Hong Kong (Ni and Lin 1986) and the Seine River and its estuary (George et al. 2001) and Mumbai waters (Ramaiah et al. 2004). Apparently, in most coastal locations around the world, the

reported counts of coliforms and/or certain human pathogenic bacteria (APHA 1980; Cabelli 1983; Dufour 1984; Ni and Lin 1986; George et al. 2001; Ramaiah et al. 2004) are more than those observed in this study.

Microbiologists rely on the principle that higher the incidence of sewage indicator bacteria in any environment, higher would be the chances for human pathogenic bacteria to be present (Brock et al. 1994; Fujioka 2002). Further, bacterial metabolism is such that, if a particular group, say *Vibrio cholerae* is the dominant bacterium in the sewage discharges, it can compete and rapidly outgrow the native microflora leading to increased levels of indicator bacteria in natural water bodies. Pathogenic bacteria of human health concern have been studied mostly for their survival in the marine environment (Huq et al. 1984; Byrd and Colwell 1990; Smith et al. 1994; Oliver et al. 1995). It is evident that the abundance of pathogenic bacteria we studied fluctuated widely in the water samples in the study area.

The bacterial load in sediment samples are reported to vary from as low as $9.3 \pm 1.1 \times 10^6$ to as high as $1.9 \pm 1.5 \times 10^8$ g^{-1} dry wt. sediment (Al-Harbi and Uddin 2003). During the present investigation the bacterial load TVC in the sediment was $< 9.3 \pm 1.1 \times 10^7$ g^{-1} dry wt. Higher TC, and their increasing numbers downstream in Zuari and Mandovi, imply cumulative effect of land drainages. Further, both EC and SF were preponderant mostly during November throughout the region. These numbers, particularly in water samples, largely denote recent sewage/land drainage contamination. Such abundance in sediments is indicative of past contamination and, extended survival of these microflora mostly brought in through land drainages. In essence, premonsoonal abundance of both pollution indicator TC and human pathogenic bacteria is an indication of an ability of these allochthonous flora to endure and survive the benthic habitats. The ranges observed in this study are generally similar to those previously reported from the coastal environs of Goa (Ramaiah et al. 2002b).

Results from this study also indicate a strong association of general, indicator and human pathogenic bacteria with zooplankton. Association of general, pollution-indicator and pathogenic bacteria with zooplankton is a common feature (West and Lee 1982; Hansen and Bech 1996; Islam et al. 2001; Signoretto et al. 2004; Dixon 2005). Such association in particular with copepods, the predominant zooplankton community, is reported to enhance the survival of microbial populations (Huq et al. 1984). Also, the molting of chitinous exoskeleton will enhance the availability of organic nutrients for the associated microflora. The fact that fecal pellets contain several species of bacteria including human pathogens (Hansen and Bech 1996) clearly suggests the proliferation of allochthonous microflora in the marine environment. Members of *Vibrio* spp., *Aeromonas* spp., *Escherichia coli, Enterococcus* spp., *Campylobacter* spp.and *Arcobacter* spp. have been reported from zooplankton samples (Dixon 2005).

This comparative assessment of distribution and abundance of pollution indicator and human pathogenic bacteria is helpful to infer that the tropical estuaries of Mandovi and Zuari experience impacts of sewage outfalls. These observations and ensuing inferences of this study are useful for managing effluent outfall into coastal ecosystems. Safeguarding the ecosystem from adding undesirable microbial populations calls for evolving appropriate policies and regulations. Every effort leading to reduction in pollution indicating bacteria and microbes of human health concern has to be promoted and implemented.

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