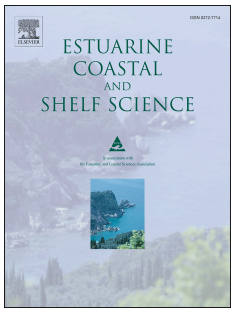

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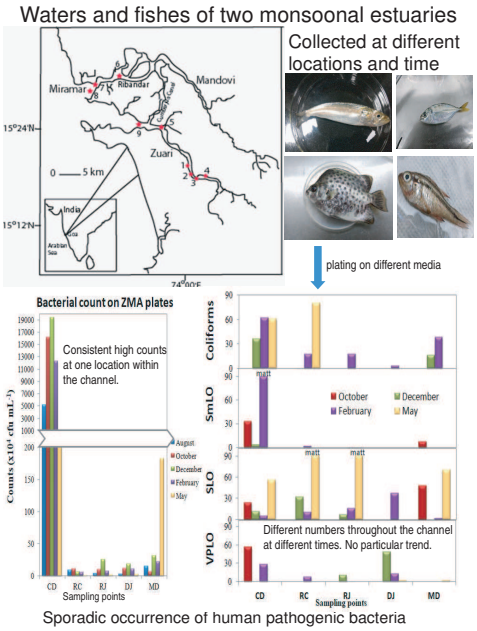

Do spatial differences account for the variation in abundance of human pathogenic bacteria in waters and fishes of the monsoonal estuary?

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Abstract

Spatiotemporal variation of human pathogenic bacteria was observed in the surface waters of a monsoonal estuary and these bacteria were also found to be associated with the native fish of two monsoonal estuaries. The temporal variation was in accordance with any monsoonal estuary. Also, the spatial variation in physicochemical parameters and bacterial counts was very evident at any given time. For instance, one sampling location in the estuary consistently had 10^3 fold higher bacterial counts than the rest of the locations within the same estuary. Coliforms, *Salmonella*-like (SmLO) organisms, *Shigella*-like (SLO) organisms, *Vibrio parahaemolyticus*-like (VPLO) organisms and *Vibrio cholerae*-like (VCLO) organisms occurred at many locations, but the abundance of each group varied differently with time at different locations in the estuary. Coliforms, unlike others, showed an increase in abundance from post-monsoon to pre-monsoon season. VCLO were the most abundant amongst all. Not all the pathogenic bacteria were widespread throughout the channel indicative of dominance in spatial variation over seasonal in this estuary. Most of the fish samples showed similar trend of bacterial numbers as that in surrounding waters, except a few. Presence of sewage pollution indicator bacteria in the fish indicated that fish served as reservoirs of these bacteria. Thermal death time of randomly picked fish-associated isolates was 30 min at 80^o C and these could survive freezing temperature for 24 h and produce siderophore which can account for their pathogenic nature. The monsoonal estuaries thus render survival ground of such bacteria.

Keywords

Monsoonal estuary, Zuari, Mandovi, pathogenic bacteria, spatiotemporal variation, fish

1. Introduction

Estuaries are the water bodies that link the rivers to the sea and are dynamic due to the transition between the fresh and marine waters. Flora and fauna present in estuaries are subjected to recurring changes in physicochemical parameters such as temperature, salinity, nutrients and sporadic changes due to anthropogenic activities or storms and hurricanes (Grimes, 1991; Fries et al., 2007; Malham et al., 2014). Anthropogenic activities affect the community structure of metabolically active microorganisms in the estuaries, which in turn results in stimulating the functioning of the estuarine ecosystem (Row, 1981; Shetye et al., 1995; Fulke et al. 2019; Udyavara et al., 2019). The microorganisms present there are either autochthonous or introduced through human intervention. The seasonal and aperiodic changes in the habitat of microorganisms confer resistance to these organisms to survive a wide range of temperature or salinity or nutrients (Grimes, 1991). The health of the estuary is determined by the autochthonous material like suspended particles as well as by the fates and fluxes of extraneous material received anthropogenically (Joseph, 2002). Moreover, the introduction of human pathogenic bacteria into estuarine waters may turn these waters into public health concern by hampering fitness due to recreational activities in polluted waters as well as due to ingestion of contaminated seafood. Hence it is necessary to regularly monitor the health of these waters, and this can be done by detecting the presence of indicator organisms such as faecal coliforms (Ferguson et al., 1996; Udyavara et al. 2019).

Monsoonal estuaries are characterized by tidal control of water level at the downstream region throughout the year and that by the run-off at the upstream region during the monsoon. Microorganisms in such estuaries are therefore under the influence of not only the allochthonous materials that add in through run-off but also the tides, i.e. the spring and neap tides which

represent enhanced and weakened tidal elevation amplitude and tidal currents respectively. (Khandeparker et al., 2017). Whether occurrence and survival of such microorganisms during the times other than spring and neap tides is influenced temporally, i.e. by seasons or spatially, i.e. by geographical location which in turn is influenced by adjoining human activities is not known. The present study was carried out to attend to this problem.

Zuari estuary, located in Goa, was selected for this study. The geochemical as well as the physical processes along the entire channel of the Zuari estuary, dictates this estuary to be a typical monsoonal estuary along the west coast of India (Mesquita and Kaisary, 2007; Sardessai and Sundar, 2007; Shetye et al., 2007a; Manoj and Unnikrishnan, 2009; Rao et al., 2011; Subha Anand et al., 2014a, b; Rao and Chakraborty, 2016). Hence the oceanographic processes in this estuary differ significantly in the monsoon from that during the post-monsoon and pre-monsoon (Shetye et al., 2007b).

There has been disposal of sewage in these waters due to nearby human settlement and other anthropogenic activities too (Ramaiah et al., 2007). Transportation activities lead to constructing jetties at the banks and dredging of the channel for movement of ferries across the waters. All these activities cause a disturbance in the levels of micro- and macronutrients and thus to the flora (Pednekar et al., 2012). In addition to all these, the Zuari estuary has a vital function in the economy of the mining industry in Goa by providing a relatively budgeted and affordable means of ore transport. Along the estuary, there are small jetties and wharfs created for the same purpose as well as for local transport thereby resulting in environmental dwindling and modifications in the marine environment as these sites are augmented with heavy metals during these processes (Shetye et al., 2007c; Row, 1981). All these bring about dynamic responses of microorganisms (Khandeparker et al., 2015). Therefore, understanding temporal and spatial

variation in microbial community structure is significant to understand the impact of anthropogenic inputs and to maintain these microbial sources for processing of various anthropogenic inputs to bring their toxic levels to withstand-able levels (Joseph, 2002; Fulke et al. 2019).

Apart from the investigations made during 2002-2003 by Rodrigues et al. (2011) and in 2011 by Khandeparker et al. (2017), there are no reports on the existence of human pathogenic bacteria in the channel of the Zuari estuary. Studies relating to these bacteria have been restricted to the mouth of the estuary by Khandeparker et al. (2015), Rodrigues et al. (2011), Nagvenkar and Ramaiah (2009), and Ramaiah et al. (2007). Since the presence of human pathogenic bacteria indicates sewage contamination, exploiting these estuarine waters for fishing and recreational activities lies ambiguous (Lipp et al., 2001).

In addition to Zuari estuary, Mandovi estuary also serves as a lifeline of the state of Goa because of its extensive use for fishing and other activities which include transportation as well as waste dumping (Shetye et al., 2007d). Investigating the health of the waters of this estuary as well as the fish within, is important for the well-being of adjacent human settlement that is dependent on this fish as their staple food. In tropical conditions, apart from transmission through contaminated water, infections are transmitted by consuming uncooked or undercooked contaminated seafood (Rodrigues et al., 2011). To gain proper knowledge of ecology and dispersal of pathogens in the food chain, it is obligatory to study the prevalence of pathogens in fish.

The first objective of the present study was to understand the distribution of sewage pollution indicator bacteria in an estuary (spatial variation) with respect to time (temporal variation) and

statistically examine the influence of the type of variation that governs the occurrence of these bacteria. The second objective was to correlate the pathogenic bacterial load from waters in the estuaries with the fish from the same habitat. We assume that the fish may serve as reservoirs of these bacteria. This study will thus provide an insight into the dominance of the type of variation that governs human pathogenic bacterial population in the monsoonal estuary and also the relation between the source (region) of the estuarine fish and its associated microbial flora pathogenic to humans.

2. Materials and Methods

2.1. Seawater samples

2.1.1. Study area

Five different sampling points along the Zuari estuary were chosen for the present study (**Fig. 1**). Four of these points were selected based on their proximity to dockyard and jetty. These were Chowgule Dock (CD), Rassaim Clean (RC), Rassaim Jetty (RJ) and Durbhat Jetty (DJ). CD is named so in this study as it was next to the Rassaim shipyard of Chowgule & Company Private Limited which possesses ship building region of 15000 sq. m. along the bank of the estuary. DJ and RJ were located at the two banks opposite to each other and these are the sites of ferry transportation across the estuary. RC point lied close to RJ point but its waters looked clearer, i.e. less turbid during monsoon than the rest of the places and hence was chosen for comparison. The fifth point was named as Madkai Dock (MD) as it was at Madkai region of the Zuari estuary, around 14 km upstream from its mouth and in contact with Cumbharjua Canal. At this point, gas pipeline by Gas Authority of India Pvt. Ltd (GAIL pipeline) is constructed across the estuary. Construction of this pipeline led to de-forestation thereby causing surface sediment run-off from

the hill into the low-lying water. MD is approximately >10 km apart from RJ and other points. Thus all sampling locations were either in the vicinity of shipbuilding activities, or iron and other mineral ore transport and ferry transportation.

2.1.2. Sampling time

Bi-monthly sampling was carried out from August 2016 to May 2017 representing different seasons viz., monsoon, post-monsoon and pre-monsoon. Monsoon sampling was carried out on 14th August 2016. Samples that were taken on 26th October 2016, 28th December 2016 and 1st February 2017 represented the post-monsoon samples. Pre-monsoon sample was taken on 8th May 2017. Samples were collected either towards the onset of or midway of the high tide.

2.1.3. Sample collection

Surface water was collected using a clean plastic bucket. The samples were immediately fixed for dissolved oxygen (DO) analysis, chlorophyll estimation, and stored separately for other analyses. For analysis of DO, water samples were collected in 300 mL glass stoppered bottles avoiding air bubbles and immediately fixed using Winklers A and Winklers B. For chlorophyll a estimation, water was transferred to clean 2 L bottles and 7 – 8 drops of $MgCO_3$ were added. Water samples (~ 150 ml) for nutrients and microbiological analysis were collected in air-tight plastic bottles that were rinsed with methanol. All these were carried to the laboratory in an ice box.

2.1.4. Analysis of physicochemical parameters

2.1.4.1. Temperature, salinity, pH: Temperature was measured during the collection of water sample using a thermometer. Salinity and pH of the samples were checked by the refractometer (RHS-10ATC) and pH meter respectively in the laboratory.

2.1.4.2. Dissolved oxygen: This was carried out by Winkler's method (Strickland and Parsons, 1972) of standard iodometric titration. One mL of concentrated sulphuric acid was added in 300 mL of pre-fixed glass stoppered bottles to dissolve the precipitate, by continuous shaking and was titrated against 0.01 N sodium thiosulphate using starch as indicator.

2.1.4.3. Nutrients (nitrates and phosphates): Nitrate concentration was determined using the spectrophotometric method of Howse (1997) wherein 10 mL of water sample was added in standard volumetric flasks and a pinch of zinc dust was added with 80 mL of distilled water. Later 1mL of sulfanilamide solution (1 %) was added, mixed and after 3-4 min, one mL of a coupling reagent, N-(1-naphthyl)-ethylene-diamine dihydrochloride (NED) (0.1 %) was added that produced pink color complex. This colour change was measured at 543 nm. A standard curve was prepared using sodium nitrate (0.5-5 µM). Phosphate analysis was carried out using Murphy and Riley (1962) method, wherein formation and reduction of blue colored phosphomolybdic acid occurs due to the acidic solution, containing sulphuric acid, ascorbic acid, ammonium molybdate and potassium antimonyl tartrate solution. Calibrated flasks treated with concentrated sulphuric acid were used. Eight mL of reagent mix was added to 40 mL of the water sample and final volume was made to 50 mL by diluting it with distilled water. Optical density was measured at 882 nm within 30 min after the experimentation. A standard curve was performed using potassium dihydrogen orthophosphate (0.5-5 µM).

2.1.4.4. Chlorophyll a: This estimation was carried out using Parsons et al. (1984) method. Eight hundred mL of water samples were filtered through GF/F filter paper (Whatman, 47mm). The filter paper retaining the phytoplankton was then placed in 15 mL centrifuge tube to be processed immediately or stored at -20°C until further processing. Processing involved the following steps. The centrifuge tube was covered with foil, and 10 mL of 90 % acetone was added to it. The filter

paper was crushed using glass rod. The samples were incubated for 24 h in refrigerator. The volume was made up to 15 mL with 90 % acetone. Centrifugation of the mixture for 10 min at 5,000 rpm was carried out. OD was recorded at 630, 663, 645 and 750 nm using 90 % acetone as blank.

2.1.5. Total viable count

Total bacterial abundance was enumerated by spread plate technique on Zobell Marine Agar 2216 (ZMA). Sample dilutions up to 10^{-6} of CD, RC, RJ, DJ, and MD were prepared using sterile seawater, and 0.1 mL of dilution was spread plated on ZMA. Plates were incubated for 24 h at room temperature. The colonies obtained were counted and expressed in terms of colony forming units per mL (cfu mL^{-1}).

2.1.6. Enumeration of pathogens

Sewage pollution indicator bacterial populations from the chosen sampling sites were enumerated by the spread-plate technique on selective media. Sample dilutions up to 10^{-3} were prepared using sterile sea water and 0.1 mL of each dilution was spread plated on selective media such as Mac Conkey's agar for coliforms, Salmonella Shigella agar for *Salmonella* spp and *Shigella* spp, and Thiosulfate Citrate Bile salts Sucrose agar for *Vibrio* spp. Plates were incubated at 37^{o} C for 24 h, and then the colony counts were noted.

2.2. Fish samples

These were collected from Zuari as well as Mandovi estuary at different times. Fish from Zuari estuary were collected using fishing rod during December 2016 and May 2017 (near DJ sampling location) and by trawling during November 2017 (at the mouth of Zuari estuary) (Fig. 1). Fish from Mandovi estuary were collected between August 2017 to January 2018 using

fishing rod near Ribandar jetty, Panaji jetty and by trawling near Miramar beach. Details of these samples are given in **Table 1**. Seawater from Mandovi estuary was also collected along with fish to enumerate the probable pathogens. Fish samples in a zip-lock bag and seawater were placed in ice-box and carried to the laboratory.

2.2.1. Enumeration of pathogens

Each fish sample was washed with sterile seawater. The surface was swabbed using a sterile cotton swab and placed in a tube containing sterile sea water. Using dissecting scissors and forceps, fish was dissected and approximately $1cm^2$ piece of the gut, was removed and placed separately in tubes containing sterile seawater. Sewage pollution indicator bacterial populations from the chosen sampling sites were enumerated by spread plate technique on selective media as mentioned above for the estuarine water samples.

2.2.2. Stability of randomly selected bacterial cultures obtained from fish

Four isolates were picked at random for these studies. These were SLO from the surface of *Ilisha megaloptera* (isolate 1), VCLO obtained from seawater and surface of *Ilisha megaloptera* (isolates 2 and 3), and coliform from the gut of *Sillago sihama* (isolate 4). The details of these are mentioned as **Supplementary Table 1**.

2.2.2.1. Temperature and Time

A loopful of the four isolates were inoculated in 1:5 strength Zobell Marine Broth separately and kept on a shaker at 120 rpm for 24 h. An aliquot of 350 µl of the broth was dispensed in Eppendorf tubes under sterile conditions and was centrifuged at 8000 rpm for 5 min. The supernatant was discarded, and the pellet was subjected to a range of different

temperatures of 50° C, 80° C, boiling water temperature using a water bath and at freezing temperature in a freezer (-4° C freezer) for different time period (10 min, 20 min and 30 min). Plating was carried out at the end of each incubation period. Stability at freezing temperature was examined by incubation for 24 h at that temperature. Zero-hour plating was done before subjecting the pellet to different temperatures (control).

For the plating after every time interval, the Eppendorf tubes were removed from the respective water baths, and dilutions up to 10^{-3} were prepared using sterile sea water. The last dilution was plated out on ZMA. Plates were stored at room temperature for 24 h and checked for growth.

2.2.2.2. Thermal Death Time

This was carried out for all the above 4 isolates at 80° C. The procedure followed was similar as above except for the modification that instead of varying temperature, the incubation time was varying. Samples (after dilutions) were plated at the end of each incubation period of 10 min, 20 min, 30 min, 40 min, 60 min and 120 min and the plates were incubated at room temperature for 24 h.

2.2.3. Siderophore production

These four isolates were also checked for the production of siderophore by spot inoculating on Chrome Azurol S (CAS) agar and incubating at 37° C for 24-72 h. Plates were checked for yellow-orange colour halos around the colonies showing siderophore production.

2.4. Statistical analysis The viable count of bimonthly sampling and its physicochemical parameters were analyzed by two-way ANOVA using Microsoft Office Excel 2007. This was

carried out separately for each pathogenic bacterial group also. Correlation between viable count and other parameters was also examined. Principal Component Analysis was carried out using Past 3.14 after log transformation of the data set.

3. Results

3.1. Physicochemical parameters

There were differences in physicochemical parameters between all the sampling locations at any given time. The pH during August 2016 ranged from 6.73 - 7.12, during October from 7.81 - 8.20, during December 2016 from 7.54 - 7.82, during February 2017 from 7.61 - 7.78, during May 2017 from 7.73 - 7.87 (**Fig. 2a**). pH was highest during October (post-monsoon) and lowest during August (monsoon). It was overall lowest at the CD as compared to other locations except during October. Salinity was highest during May 2017 (pre-monsoon) and lowest (0 psu) at all sampling points during August 2016 (monsoon). It ranged from 3-15 psu during October 2016, 17-26 psu during December 2016, 6-29 psu during February 2017 and 32-36 psu during May 2017 (**Fig. 2b**). Salinity was also the lowest at CD point at all the times. Temperature was the lowest during December (post-monsoon: winter) and highest during October (post-monsoon) except at MD. At MD the maximum temperature of all the locations was found in May (pre-monsoon). The temperature ranged from 28.3-29.6° C during August 2016, 28.9-31.2° C during October 2016, 26.6-27.9° C during December 2016, 26-30° C during February 2017 and 28-32° C during May 2017 (**Fig. 2c**). Dissolved oxygen was the lowest during May and highest during October for all locations except MD. Here DO was highest during December. Dissolved oxygen ranged from 0.85-0.90 mg mL^{-1} during August 2016, 0.94-1.11 mg mL^{-1} during October 2016, 0.83-1.10 mg mL^{-1} during December 2016, 0.57-0.69 mg mL^{-1} during February 2017 and 0.22-0.39 mg mL^{-1} during May 2017 (**Fig. 2d**). Nitrates ranged from 0.11-2.14 µM during August

2016, 0.05-0.45 µM during October 2016, 0.52-2.01 µM during December 2016, 0.11-0.79 µM during February 2017 and 0.79-1.65 µM during May 2017 (**Fig. 2e**). Phosphates ranged from 0.57-2.83 µM during August 2016, 0.36-0.41 µM during October 2016, 0.37-0.51 µM during December 2016, 1.94-6.84 µM during February 2017 and 0.73-2.68 µM during May 2017 (**Fig. 2f**). High levels of phosphates were seen during February at all locations with CD outcompeting others. Chlorophyll a concentration was the lowest during October and highest during August for all locations and during May also for DJ only. The chlorophyll a concentration varied from 0.52-1.48 mg m^{-3} during August 2016, 0.12-0.27 mg m^{-3} during October 2016, 0.13-0.39 mg m^{-3} during December 2016, 0.25-0.55 mg m^{-3} during February 2017 and 0.26-0.85 mg m^{-3} during May 2017 (**Fig. 2g**).

3.2. Total viable count

The highest counts were observed at CD (**Fig. 3a**). The counts here ranged from 40 to 194×10^6 cfu mL^{-1}. The bacterial counts in the other sampling areas ranged from 5.74- 11.2×10^4 cfu mL^{-1} at RC, 0.2-7.70×10^4 cfu mL^{-1} at RJ, 3.22- 18.44×10^4 cfu mL^{-1} at DJ and 6.46- 31.63×10^4 cfu mL^{-1} at MD. The lowest count during August was seen at DJ, during October has been observed in MD, during December and February at RC and during May at RJ. The highest counts apart from the CD point were seen at MD during August, at DJ during October, at MD again during December, February and May. Thus, after CD, MD was the next highly populated location during all the sampling periods except October.

Two-way ANOVA of the entire data set showed significant differences in mean between locations i.e. the relationship between overall bacterial abundance with physicochemical parameters changes with locations (P 1.50013E-06) but does not change significantly with

seasons (P 0.8) (**Table 2**). However, significant interaction effects indicated that seasons also have some effect on the relationship between various physicochemical parameters and bacterial abundance at different locations (P 6.72012E-19). Nevertheless, the changes in bacterial counts can be accounted more by spatial than seasonal variation. The bacterial counts at CD and DJ showed significantly negative correlation with chlorophyll a (r -0.92 and -0.7 respectively) and positive correlation with DO (r 0.97 and 0.77 respectively). In contrast to that, at MD the counts showed significantly negative correlation with DO (r -0.84) and positive correlation with salinity (r 0.79). A significant correlation between the counts and nitrates was seen at MD and between the counts and both the nutrients was seen at DJ. All significant correlations are presented in **Table 3**.

PCA analysis (**Fig. 4a**) showed that the physicochemical parameters influenced PC1 whereas bacterial abundance influenced PC2. PC1 and PC2 axes (eigenvalues of 1.566 and 0.632 respectively) explained 55.8 and 22.5 %, respectively of the total variance of the relation between bacterial abundance and physicochemical parameters and the month-wise sampling locations. All the physicochemical parameters didn't seem to correlate with the abundance. The PCA biplots showed that CD was distinct from the rest. Also, RJ, RC and DJ samples during August (monsoon), February and May (pre-monsoon) clustered together whereas October and December (post-monsoon) clustered separately. This holds true for MD samples as well. Thus post-monsoon samples were clearly separated from pre-monsoon and monsoon samples indicating a possible influence of the seasons on physicochemical parameter and not on the abundance.

3.3. Enumeration of pathogens in seawater

Enumeration of pathogenic organisms revealed their presence during all the sampling times. These were grouped as coliforms, *Salmonella*-like (SmLO) organisms, *Shigella*-like (SLO) organisms, *Vibrio parahaemolyticus*-like (VPLO) organisms and *Vibrio cholerae*-like (VCLO) organisms based on their colony morphological characteristics on selective media as given in **Supplementary Table 2.** Since the August sample involved enrichment followed by plating on selective media, no counts could be measured. However, after streaking, the presence of coliforms was noted from RJ and DJ samples, SmLO from RJ and MD samples, and VCLO from DJ and RC samples. No SLO and VPLO were obtained from August samples.

Highest coliform counts of 80±6 cfu mL^{-1} were observed during May 2017 at RC, followed by 62±12 and 61±4 cfu mL^{-1} at CD during February and May 2017 respectively, 38±6 cfu mL^{-1} at MD during February 2017 and 36 cfu mL^{-1} at CD during December 2017 (**Fig. 3b**). Thus, overall CD had the highest coliform counts at all times. With respect to SmLO, matt growth was seen at CD during February 2017 followed by 33±11 cfu mL^{-1} during October 2016 and 4±1 cfu mL^{-1} during December 2016. MD point also displayed a count of 8±2 cfu mL^{-1} during October 2016. SmLO counts of 2 cfu mL^{-1} were observed at RC during February 2017, and 1 cfu mL^{-1} were observed at RJ during February and May 2017 (**Fig. 3c**). No SmLO colonies were seen rest of the times and rest of the points. SLO were the highest during May 2017 at MD (70±22 cfu mL^{-1}), CD (56±15 cfu mL^{-1}) and matt growth at RC and RJ (**Fig. 3d**). MD also witnessed high SLO of 48±14 cfu mL^{-1} during October 2016. Overall SLO were seen at all points during February 2017. VCLO were the highest amongst the pathogenic organisms tested at all times (**Fig. 3e**). The highest number of 325 cfu mL^{-1} was seen at MD and DJ during December 2016 and February 2017 respectively. This was followed by 312±36 and 276±52 cfu mL^{-1} at CD and RC respectively during December 2017, 116±26 and 93±6 cfu mL^{-1} at CD and MD respectively

during February 2017. Thus VCLO were the highest during December 2016 followed by February 2017. VCLO were also encountered during October 2016 with high numbers of 167±25 cfu mL^{-1} at MD. Similarly, these were also seen to be maximum during May 2017 at MD as observed by the matt growth. As compared to VCLO, VPLO were low in numbers during all times with highest numbers of 56±18 cfu mL^{-1} at CD during October 2016 followed by 48±2 cfu mL^{-1} at DJ during December 2016 (**Fig. 3f**). MD point did not show any VPLO colonies at all times except during May 2017 with a count as low as 2 cfu mL^{-1}. These were more prevalent at DJ than other points.

The two-way ANOVA of each bacterial group with physicochemical parameters showed varied results for each group (**Table 2**). Season-wise variation was seen significantly in case of coliforms (P 0.014) and SLO (P 2.66124E-05) only. Post-hoc pairwise comparison using Bonferroni correction (0.083) showed that coliform abundance is significantly different between October and February samples (P 0.036), and SLO abundance is significantly different between December and May samples (P 0.059). Location-wise variation was not observed for any group ($P > 0.05$). Interaction effects were seen in case of coliforms, SmLO, VCLO and VPLO. Thus, abundance of coliforms showed seasonal variation (main effect) and also some effect of location (interaction effect). Abundance of SmLO, VCLO and VPLO demonstrated effect of parameters that did not show dominance of either seasonal or spatial variation alone (i.e. no significant main effect) but were influenced by both seasonal and spatial variation together (interaction effect).

The PCA analysis of each group of pathogenic bacteria against physicochemical parameters produced different biplots for each group (**Fig. 4b-f**). All these biplots showed clear partitioning of October samples from the rest along PC1. In addition to this, PCA biplot of SmLO abundance showed partitioning of February samples from the rest along PC2. PCA biplot of SLO abundance

showed partitioning of most of the May samples (except DJ May) from February and December along the PC2. PCA biplot of VCLO showed partitioning of May samples from the rest (except RJ Dec & RJ Feb) along the PC2 and that of VPLO showed partitioning of February samples from December and May along the PC2. In case of coliforms, parameters such as phosphates, nitrates and temperature contributed to the PC1 which accounted for 36.7 % of the variance in the data (eigen value 2.94) and DO, pH, salinity and chl a contributed to PC2 which accounted for 22.1 % of the total variance (eigen value 1.77). In case of SmLO, PC1, which accounted for 36.4 % of the variance in the data (eigen value 2.91) strongly correlated with phosphates, and PC2, which accounted for 25.2 % of the variance in the data (eigen value 2.02) correlated with salinity, nitrates, chl a and DO. Similar correlation of physicochemical parameters to the two components was also observed in case of biplot of SLO in which PC1 and PC2 accounted for 37.8 % and 20.7 % of the variance in the data, respectively. Phosphates, temperature and pH contributed significantly to PC1 of VCLO and VPLO biplots (accounting for 35.4 and 36.6 % variance, 2.83 and 2.93 eigen values, respectively) and nitrates, salinity, DO and chl a contributed to PC2 (accounting for 18.4 and 18.7 % variance, 1.47 and 1.50 eigen values, respectively).

3.4. Enumeration of pathogens in fish

Human pathogenic bacteria were found to be present after plating the fish samples on selective media. However, not all samples showed their presence. Matt growth was seen on ZMA by *Valamugil cunnesius* from DJ during May 2017 and *Sardinella longiceps* from Panaji Jetty during December 2017. The *Valamugil cunnesius* sample of December 2016 also showed very high but countable numbers (130.3×10^6 in the gut and 159.3×10^6 cfu mL^{-1}on the surface). Thus the total viable count was much higher in the surface than in the gut during December

2016, but the selective media showed the opposite scenario. Gut showed more numbers of organisms than the surface. This was mostly seen for all the samples except for *Vibrio*-like organisms in *Scatophagus argus*, *Escualosa thorracuta*, *Sardinella longiceps* from Panaji Jetty and *Ilisha megaloptera* from Zuari estuary; and SLO in *Valamugil cunnesius* from DJ, *Ilisha megaloptera* from Zuari and *Sardinella longiceps* from Panaji Jetty and Ribandar; and coliforms from *Sardinella longiceps* from Panaji Jetty (**Fig. 5**).

Five out of 18 fish samples showed no *Vibrio*-like organisms in the gut, and 8 out of 18 showed none on the surface. No SLO were found in 5 out of 18 samples. SmLO were observed in only five samples viz., *Scatophagus argus* from Panaji Jetty, *Escualosa thorracuta* from Miramar beach, *Sillago sihama*, *Ilisha megaloptera* from Zuari estuary and *Sardinella longiceps* from Ribandar. No coliforms were found in 6 out of the 18 samples. No growth was observed in any media after plating surface swab as well as the gut of *Sepiella inermis* and *Gerres oyena* from Zuari.

The seawater collected along with fish at Mandovi estuary showed higher bacterial numbers than the surface and gut of respective fish on ZMA while the opposite was observed on a few selective media.

3.5. Stability of the bacterial isolates obtained from fish

All the isolates showed growth up to the first 30 min of incubation at all the temperatures tested (**Supplementary Table 3**). However, the growth was reduced within 20 min at 100^{o} C of the three isolates 2-4 (VCLO and coliform) and within 10 min at 80^{o} and 100^{o} C of the isolate 1 (SLO). The thermal death time at 80^{o} C is 30 min for isolate 1 and 40 min for isolates 2-4. When

all the four isolates were kept at freezing temperature for 24 h, they showed matt growth after plating.

3.6. Siderophore production by the bacterial isolates obtained from fish

All the four isolates produced yellow halos around the colonies on blue CAS agar plates indicating positive production of siderophores (**Supplementary Fig. 1**).

4. Discussion

4.1. Temporal variation

Variation in microbial load did not show any particular trend. Overall, the nutrients were high in August (monsoon) at all locations throughout the channel (except for low nitrates in DJ and MD). This corresponded to high chlorophyll a during this time. The DO levels were also towards higher range, the highest seen in October. All these were indicative of upwelling conditions which bring up cold nutrient-rich waters from bottom to surface, thus increasing productivity (Sarma et al., 2001). Southwest monsoon season is the time when upwelling of water occurs (Sardessai and Sundar, 2007). In addition to that run-off during monsoon also adds nitrates to the waters (Sardessai and Sundar, 2007). Also, the pH was lowest at this time, and the salinity too was zero indicating fresh water influx (Manoj and Unnikrishnan, 2009). Average pH towards lower range is associated with low salinity waters in Zuari estuarine system during monsoon (Sarma et al., 2001).

October was characterized by slight increase in pH and salinity as well as high counts of bacteria on ZMA. The temperature and DO also were found to be the highest. However, the decrease in the concentration of nutrients, followed by a decrease in chlorophyll a values could probably be attributed to subsidence of blooming conditions that might have prevailed at the end of the

southwest monsoon (Prabhu Matondkar et al., 2007). This explains the increase in microbial load as microorganisms are important in decomposition processes during the decay of the bloom (Buchan et al., 2014). However, since bi-monthly samples were collected, the probable bloom was missed out in the present study.

December was characterized by still higher microbial counts and lowest temperature amongst all sampling times. There was further increase in salinity but decrease in pH. Nutrients increased, but this did not correspond with the chlorophyll a. There was only a slight increase in chlorophyll a. The increase in nutrients corresponded to earlier observations of the increase in nitrates during northeast monsoon, i.e. November-December (Shenoy and Patil, 2003).

February witnessed an increase in temperature. There was a slight decrease in salinity at most sampling points except MD where salinity was 5 psu more than the rest of the places. A day prior to this sampling was witnessed by spring tide, which is responsible for salinity higher than the usual high tides and also salinity intrusion (Haddout et al., 2018; Shetye et al., 2007c). The effect of salinity intrusion might have subsided by the time the seawater was sampled at all the locations leaving its effect only at the seaward station i.e. MD. Amongst all the places, salinity at CD was 6 psu which could be due to a sudden local discharge of fresh water. The phosphates here were also very high as compared to other locations providing evidence that there may be a local discharge of fresh water rich in organic wastes, thus lowering salinity at this location (Pradhan and Shirodkar, 2009). However, the nitrates were very low and so was the chlorophyll a at this point.

The pre-monsoon sampling of May was characterized by high salinity as commonly seen during this season (Shetye et al., 2007a). DO was lowest at this time which frequently reflects

increasing microbial activity (Spietz et al., 2015). Usually, pre-monsoon season is characterized by high chlorophyll a due to phytoplankton bloom followed by low DO due to microbial decomposition processes after the subsidence of the bloom.

Each pathogenic bacterial group displayed different pattern of variability in counts with the changing season. Coliforms showed increase in numbers from post-monsoon to pre-monsoon. SLO also illustrated seasonal variation as seen by ANOVA. The others showed varying pattern. Such temporal variation of coliforms and SLO was evident in PCA biplots as well. PCA biplots of other bacterial groups demonstrated remarkable clustering of October samples of all locations but not very striking clustering of other seasons.

4.2. Spatial variation

Spatial variation in bacterial abundance was evident by the remarkably high numbers of bacterial counts at CD only, as compared to other points. These were almost 10^3 times higher than at the other places. Such numbers were much higher than the counts reported by Rodrigues et al. (2011) during their entire study in the Zuari estuary. The CD point lies next to the Rassaim shipyard of Chowgule & Company Private Limited and hence subjected to drainage related to shipbuilding as well as due to domestic activities owing to the human settlement nearby. The second most bacterially populated point was MD which was around 14 km upstream from the mouth, 4-5 km downstream of CD and near the gas pipeline.

RC being upstream to CD may make one believe that the highly populated CD waters won't spread towards RC, maintaining that point clean. However, in reality, RC which was closer to RJ demonstrated high bacterial load occasionally including some of the human pathogenic bacteria tested. The waters from the mouth of the estuary may extend upstream during high tide and to a

greater extent during spring tide (Rajaneesh and Mitbavkar 2013; Unnikrishnan and Manoj, 2007). During the dry season, i.e. after the withdrawal of monsoon, the flow in the entire estuarine channel is influenced by the tide at the mouth of the channel, and the waters thus remain mixed (Shetye et al., 2007d). Moreover, apart from the times dealing with the tidal cycle, the circulation in the well-mixed estuary consists of downstream-directed surface fresh waters and upstream-directed bottom saline waters (Unnikrishnan and Manoj, 2007). The well-mixed waters in the channel will, therefore, extend the pathogens from one place to another.

Further evidence of spatial variation in bacterial abundance was provided by the coliform counts. These were highest at the CD most of the times followed by MD. Their numbers in the present study were similar to the numbers observed during premonsoon season by Khandeparker et al. (2017) but much lower than the numbers reported by the same study during other seasons and also lower than the numbers reported by Khandeparker et al. (2015). The former study (Khandeparker et al., 2017) referred to different sites in the Zuari estuary while the latter (Khandeparker et al., 2015) referred only at the mouth of the Zuari estuary. The variation in coliform abundance in the present study as compared to the others could be attributed to the different anthropogenic processes prevailing, since the coliform counts depend on anthropogenic disturbances from the adjacent shoreline, influenced by not only the time sampled, but also on the location (Kirby-Smith and White, 2006). SmLO and VCLO also demonstrated spatial variation. The SmLO were most abundant at CD whereas VCLO were most abundant at MD. The abundance of SmLO detected in CD was significantly higher than those stated from the Cochin estuary (Hatha et al., 2004). *Vibrio* sp. are mostly considered as opportunistic organisms and Vibrios are known to adapt well to particle associated lifestyle (Pernthaler and Amann, 2005). The dominant bacterium, VCLO was found to be present everywhere in all locations, but

the incidence level was higher than those reported by Khandeparker et al. (2015) at the mouth of the Zuari estuary and at par with Khandeparker et al. (2017) in the surface waters at different locations in the same estuary.

Variations were also seen in physicochemical parameters that interact with biological components bringing about a diverse ecosystem (Clark and Cripe, 1993). High nitrates at DJ coincided with high VCLO numbers in December and high chlorophyll a values during May. The sudden boost in phosphates during February at CD also coincided with very high numbers of SmLO and coliforms (also evident in PCA biplot), indicating high discharge of contaminated matter at that location. VCLO numbers in February were lower than December at CD, RC and MD while higher than December at RJ and DJ. VPLO also displayed different trend in numbers with varying season at different locations. SLO too showed the different trend at different locations except for the common feature of the highest load during pre-monsoon season (May). SmLO were not detected at many locations at different times and hence could not deduce any pattern. Although spatial variation was evident in case of total viable count during ANOVA, none of the bacterial groups demonstrated the same significantly. However, indirect effect of locations was seen due to interaction effects in ANOVA. The physical characteristics of estuarine seawater depend not only on the season in which it is collected but also on the geographical location, and different geographical locations will demonstrate different values due to differing anthropogenic activities, thus eliciting different response in bacterial counts (Fulke et al., 2019). The outcome of temporal variation on coliforms and SLO only, and not on other bacterial groups, signifies the dominance of spatial variation over temporal. This indicated that local anthropogenic factors were more influential and disparaging the seasonal changes in parameters.

4.3. Influence on fish-associated pathogenic microbial flora

Increasing incidence of human bacterial pathogens in marine environment and their spread through the seafood worldwide (Thompson et al., 2005; Novotny et al., 2004), and the evidence of pathogenic bacteria in the waters of the Zuari estuary during most of the times in the present study, led the analysis of the pathogens associated with fish from the same waters. The trend of types of bacteria found on fish was similar to that seen in the surrounding DJ waters at that time except for SLO. The SLO were detected in fish and not in waters. However, SLO were detected in high numbers in waters from the other sampling locations during the same time. Thus it implies that the spread of SLO was through contaminated fish from one region to the other, thus acting as a carrier and vector of pathogens. The VPLO counts also, detected on the fish surface, were double than that found in the waters and, those detected in the fish gut were about 10 times more than in the surrounding waters. In order to check if this phenomenon is observed in other monsoonal estuaries, fish from a different monsoonal estuary were collected to examine the pathogenic bacterial load on them vis-à-vis surrounding seawater. Hence fish from Mandovi estuary were collected during 2017-18 and their examination also showed a higher bacterial load in gut and surface than the surrounding waters at most instances.

Randomly selected isolates, when checked for their stability, showed that cooking time of 40 min at 80^{o} C or 30 min for 100^{o} C would be sufficient to get rid of these organisms from the fish. These temperatures were selected because Indian cuisine subjects the food to mostly 80-100^{o} C or higher (baking/frying). Stability was examined to check the resistance of these organisms to high temperatures to determine if fish from these areas can be consumed. The fish forage or take refuge in estuarine areas and thus get exposed to pathogens. Fish contain bacteria as their normal flora and because of continuous exposure to contaminated water, their skin and gills show

bacterial colonisation (Novoslavskij et al., 2016). Also, contaminated feed or water affects their gut. A weakened immune system may lead to contamination of fish muscle (Aguirre-Guzmán et al., 2012).

Fishes are always frozen for storage. Stability at freezing temperature showed no negative effect on the isolates. Freezing causes log reduction in cells of a few pathogens but not all, and usually, cells remain viable and grow once brought back to room temperature (Gao et al., 2007). Moreover, the siderophore-producing ability of the four isolates justified their pathogenicity since siderophores are one of the virulence factors in Gram negative bacteria (Holden and Bachman, 2015). Hence such fish need to be well cooked before consumption.

4.4. Conclusion

There is a lot of spatiotemporal variability in the microbial population and the physicochemical parameters in the estuary. In spite of proximity to areas of high bacterial load and propagation of tides throughout the channel, not all the pathogenic bacteria were prevalent in the entire area covered in this study. The variability in bacterial counts could therefore be accounted for more by spatial rather than seasonal scale. The estuarine waters as well as the fish dwelling there are a potential ground for human pathogenic bacteria. These may spread to different areas in the estuarine waters via hitchhiking on fish as perceived by their occurrence on fish. The fish serve as vectors of such bacteria transporting them to different locations in the estuary. As a consequence there seem to be dominance of spatial variation over temporal in the abundance of these bacteria in the estuarine waters.

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Table no. 1 Details of the fish samples collected during this study.

Sample	Place	Date	Scientific name
Sample 1	Durbhat Jetty	28-Dec-2016	*Valamugil cunnesius*
Sample 2	Durbhat Jetty	8-May-2017	*Valamugil cunnesius*
Sample 3	Ribandar	1-Aug-2017	*Escualosa thorracuta*
Sample 4	Ribandar	17-Aug-2017	*Lutjanus indicus*
Sample 5	Panaji Jetty	6-Sept-2017	*Scatophagus argus*
Sample 6	Miramar Beach	5-Oct-2017	*Escualosa thorracuta*
Samples 7-8	Panaji Jetty	23-Oct-2017	*Escualosa thorracuta*
Samples 9-16	Zuari Estuary	15-Nov- 2017	*7- Sillago sihama* *8- Ilisha megaloptera* *9- Pelates quadrilineatus* *10- Mene maculata* *11- Caranx* sp. 12- *Sepiella inermis* *13- Gerres oyena* *14- Thryssa mystax*
Sample 17	Panaji Jetty	9-Dec-2017	*Sardinella longiceps*
Sample 18	Ribandar	4-Jan-2018	*Sardinella longiceps*

Table 2. Two-way ANOVA for bacterial abundance and physicochemical parameters. Significant F values are represented as * (P<0.05), ** (P<0.01) and * (P<0.001).**

Bacterial type	Between seasons			Between locations			Interaction effect		
	df	MS	F	df	MS	F	df	MS	F
TVC	3	1565133	0.318	4	14838159	9.251***	28	14869404	9.271***
Coliforms	3	278.68	3.662*	4	73.256	0.926	28	146.68	1.850*
SmLO	3	48.43	0.802	4	40.222	0.722	28	117.32	2.106*
SLO	3	634.37	8.710***	4	61.637	0.420	28	54.39	0.370
VCLO	3	2795.76	1.313	4	3189.47	1.619	28	3173.56	1.611*
VPLO	3	14.163	0.343	4	17.623	0.404	28	59.276	1.359*

Table 3. Significant correlation coefficients between viable bacterial counts and other parameters of location-wise correlation analysis of the viable counts with chlorophyll a concentration and various physicochemical parameters. Positive r values are marked in bold.

Sampling site	Parameter (n)	significant r values between n and viable count
CD	Chlorophyll a	-0.915
	Dissolved oxygen	**0.972**
	Salinity	-0.709
RC	pH	**0.851**
DJ	Chlorophyll a	-0.693
	Nitrates	-0.752
	Phosphates	-0.724
	Dissolved oxygen	**0.771**
MD	Nitrates	**0.699**
	Dissolved oxygen	-0.843
	Temperature	**0.832**
	Salinity	**0.792**

Legends to figures

Fig 1. Map showing sampling locations (red stars) in Zuari and Mandovi estuaries

Fig. 2. Physicochemical characteristics of the surface waters at the sampling points during various seasons.

Fig. 3. Abundance of various bacteria in surface waters during different seasons at four sampling locations in Zuari estuary.

Fig. 4. PCA of bacterial numbers and physicochemical parameters at various sampling locations and time (a) TVC (b) Coliforms (c) SmLO (d) SLO (e) VCLO and (f) VPL.

Fig. 5. Viable count of human pathogenic bacteria on fish gut and surface in the present study. Viable count of the first 2 samples i.e. *Valamugil cunnesius* are in terms of 10^6 cfu mL^{-1} and the rest in 10^5 cfu mL^{-1}.

Fig. 1

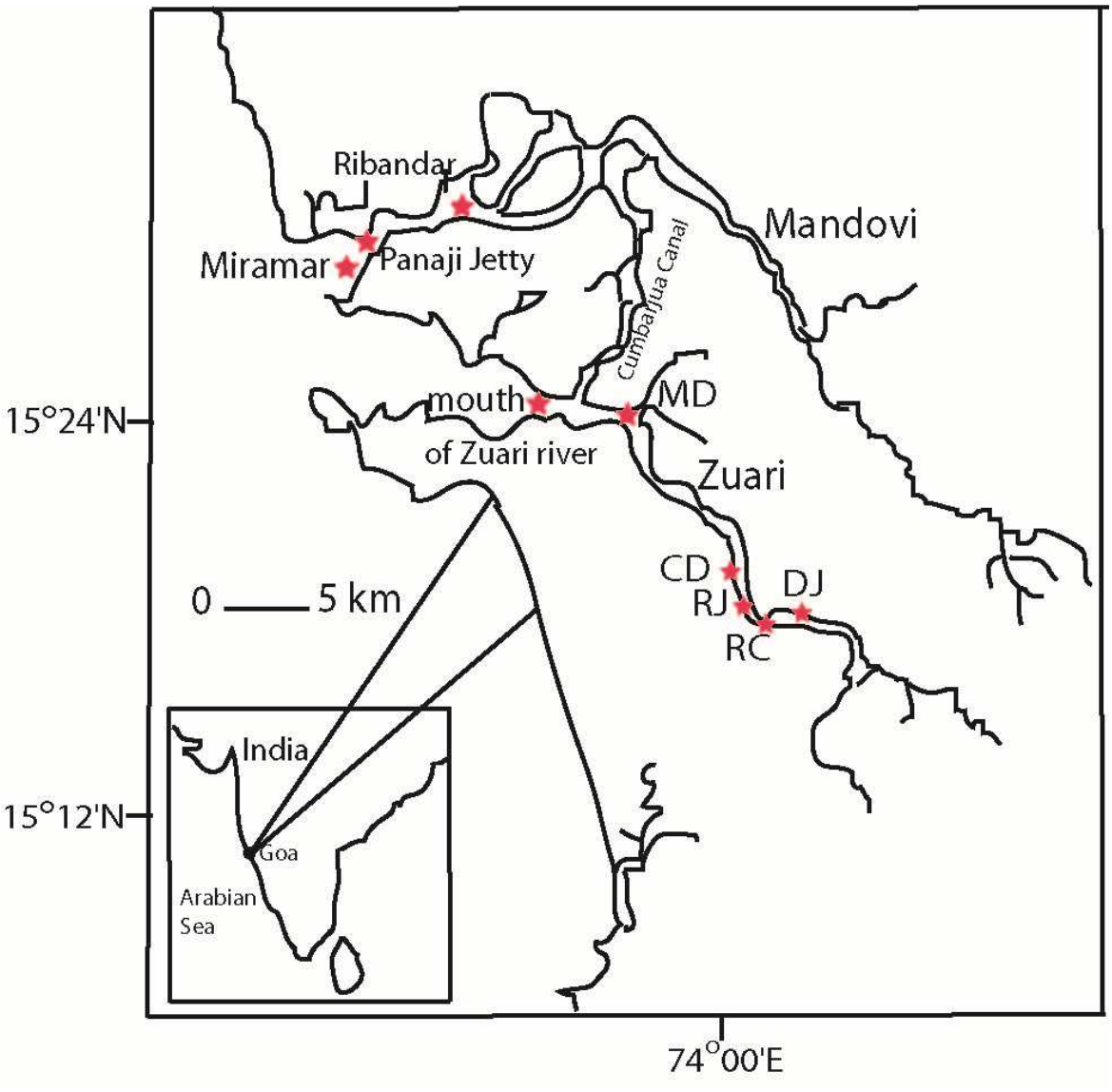

Ribandar
Miramar
Panaji Jetty
Mandovi
Cumbarjua Canal
mouth
of Zuari river
MD
Zuari
CD
DJ
RJ
RC
0 5 km
India
Goa
Arabian
Sea
15°24'N
15°12'N
74°00'E

Fig. 2

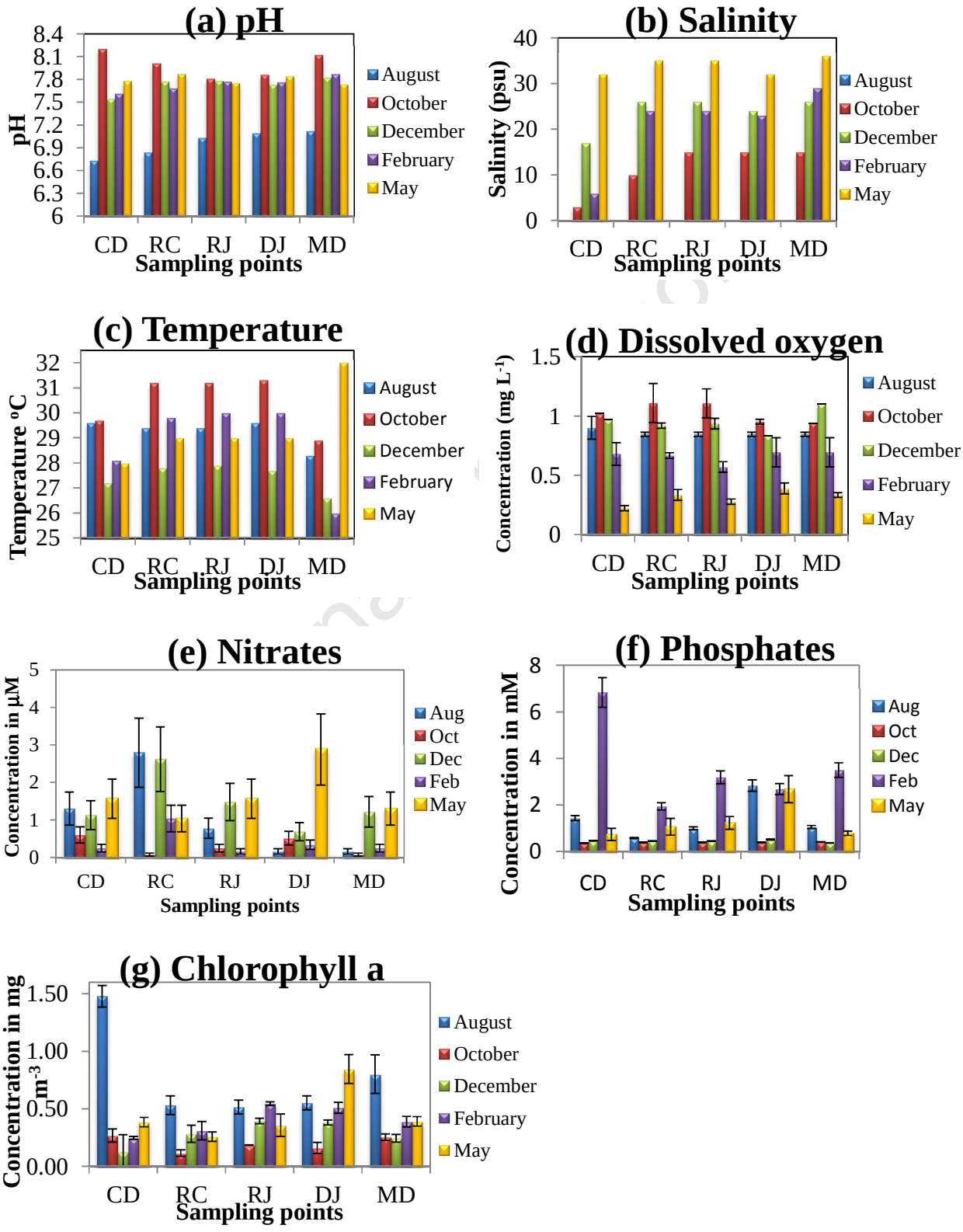

(a) pH
pH
8.4
8.1
7.8
7.5
7.2
6.9
6.6
6.3
6
CD RC RJ DJ MD
Sampling points
August
October
December
February
May
(b) Salinity
Salinity (psu)
40
30
20
10
0
CD RC RJ DJ MD
Sampling points
August
October
December
February
May
(c) Temperature
Temperature °C
32
31
30
29
28
27
26
25
CD RC RJ DJ MD
Sampling points
August
October
December
February
May
(d) Dissolved oxygen
Concentration (mg L-1)
1.5
1
0.5
0
CD RC RJ DJ MD
Sampling points
August
October
December
February
May
(e) Nitrates
Concentration in µM
5
4
3
2
1
0
CD RC RJ DJ MD
Sampling points
Aug
Oct
Dec
Feb
May
(f) Phosphates
Concentration in mM
8
6
4
2
0
CD RC RJ DJ MD
Sampling points
Aug
Oct
Dec
Feb
May
(g) Chlorophyll a
Concentration in mg m-3
1.50
1.00
0.50
0.00
CD RC RJ DJ MD
Sampling points
August
October
December
February
May

Fig. 3

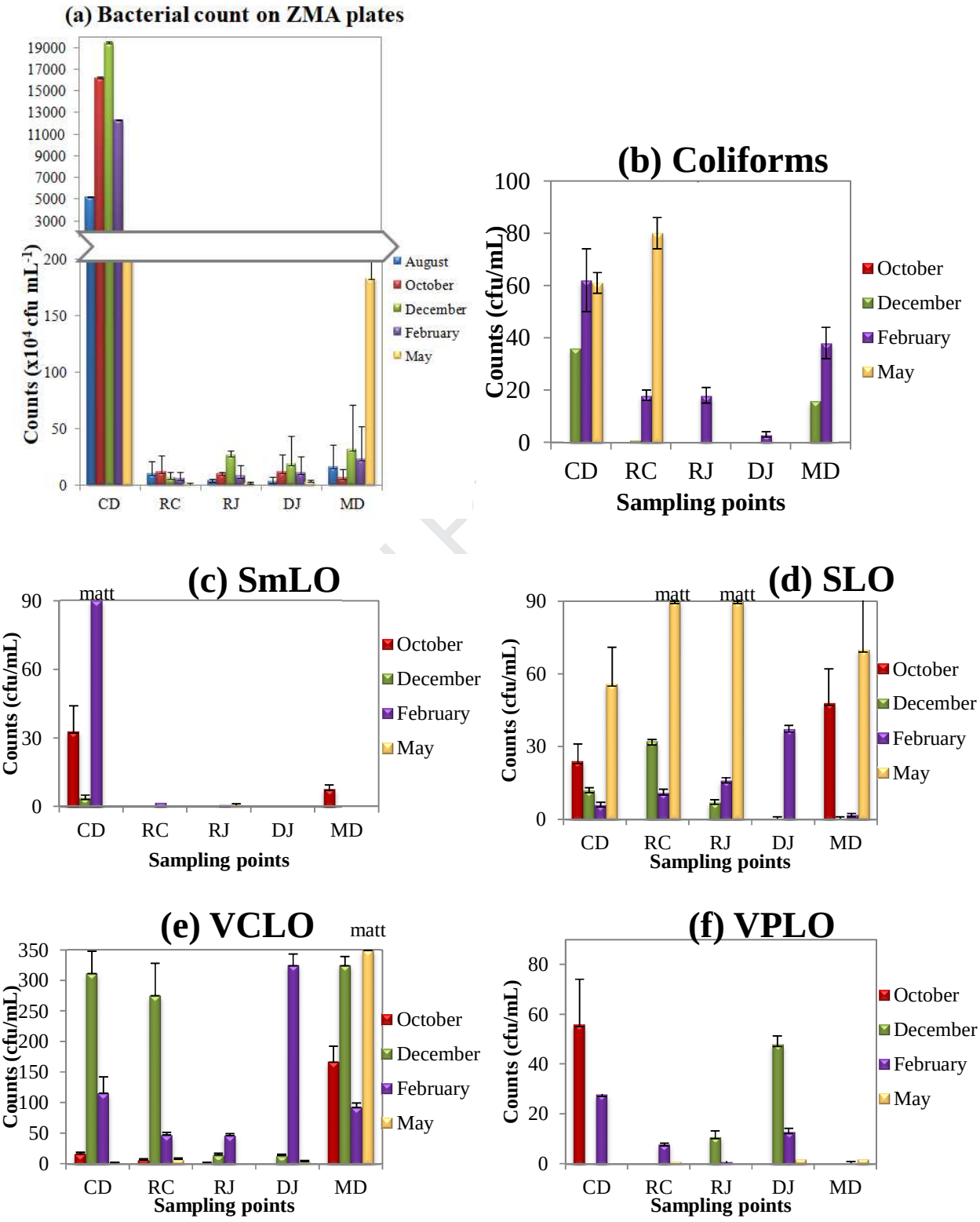

(a) Bacterial count on ZMA plates
Counts (x10⁴ cfu mL⁻¹)
19000
17000
15000
13000
11000
9000
7000
5000
3000
200
150
100
50
0
CD
RC
RJ
DJ
MD
August
October
December
February
May
(b) Coliforms
Counts (cfu/mL)
100
80
60
40
20
0
CD
RC
RJ
DJ
MD
Sampling points
October
December
February
May
(c) SmLO
matt
Counts (cfu/mL)
90
60
30
0
CD
RC
RJ
DJ
MD
Sampling points
October
December
February
May
(d) SLO
matt
matt
Counts (cfu/mL)
90
60
30
0
CD
RC
RJ
DJ
MD
Sampling points
October
December
February
May
(e) VCLO
matt
Counts (cfu/mL)
350
300
250
200
150
100
50
0
CD
RC
RJ
DJ
MD
Sampling points
October
December
February
May
(f) VPLO
Counts (cfu/mL)
80
60
40
20
0
CD
RC
RJ
DJ
MD
Sampling points
October
December
February
May

Fig. 4

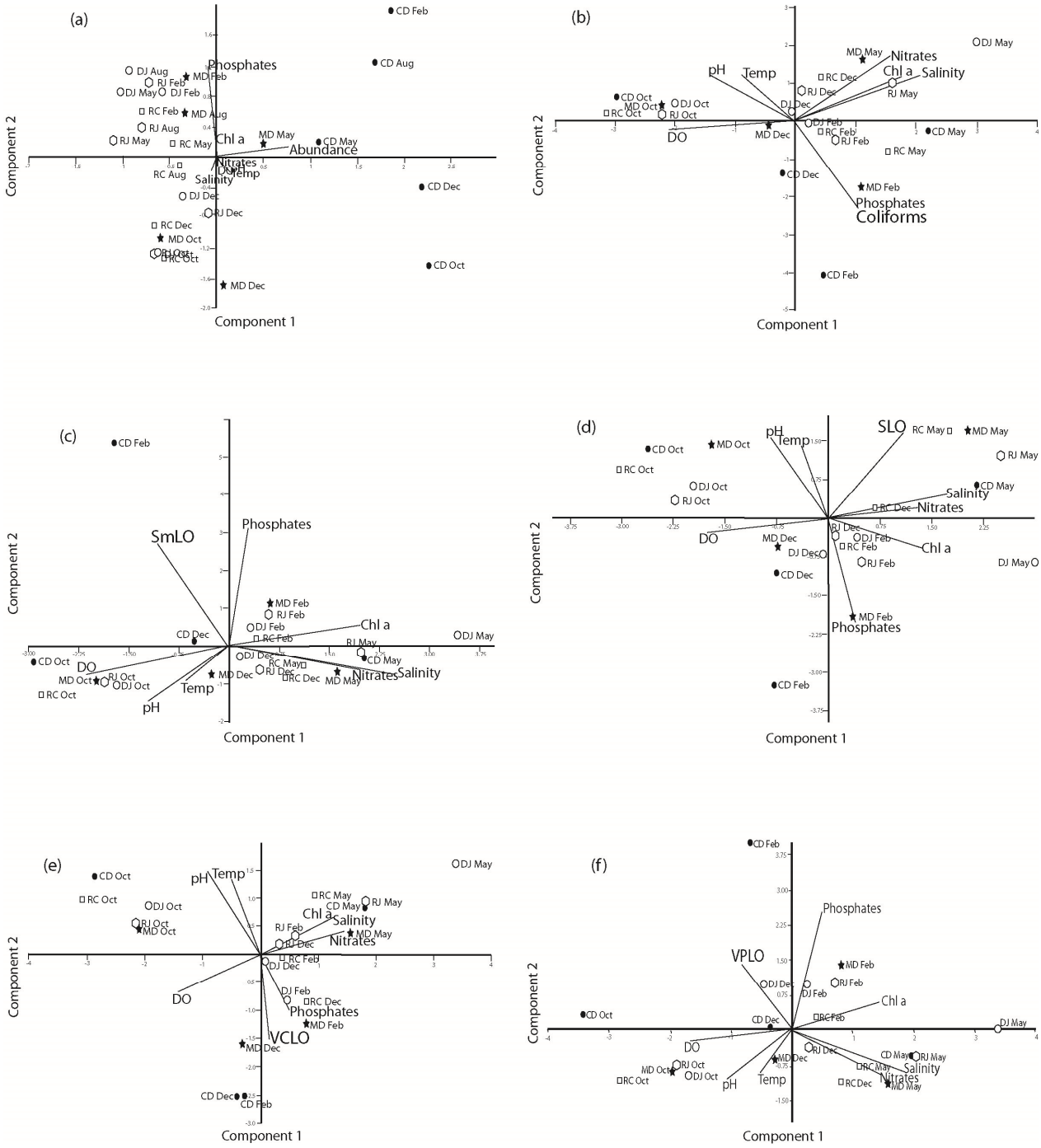
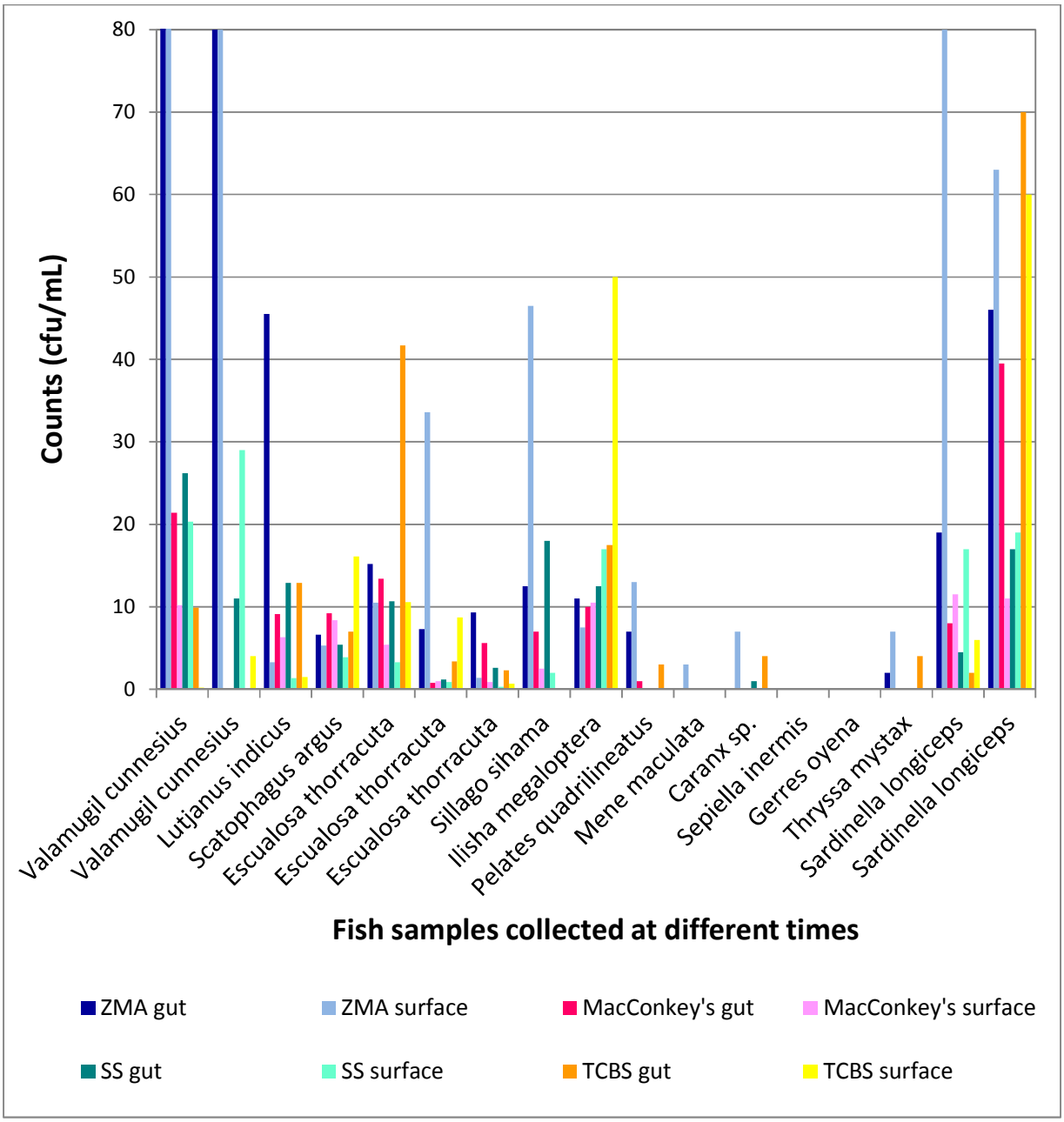

(a)
Component 2
Component 1
CD Feb
CD Aug
DJ Aug
RJ Feb
MD Feb
DJ May
DJ Feb
Phosphates
RC Feb
MD Aug
RJ Aug
RJ May
RC May
Chl a
MD May
CD May
Abundance
Nitrates
DO
Temp
RC Aug
Salinity
CD Dec
DJ Dec
RJ Dec
RC Dec
MD Oct
RC Oct
CD Oct
MD Dec
(b)
Component 2
Component 1
DJ May
MD May
Nitrates
pH
Temp
Chl a
Salinity
RC Dec
RJ Dec
RJ May
CD Oct
MD Oct
DJ Oct
RC Oct
RJ Oct
DJ Dec
DO
RC Feb
CD May
MD Dec
RJ Feb
RC May
CD Dec
MD Feb
Phosphates
Coliforms
CD Feb
(c)
Component 2
Component 1
CD Feb
SmLO
Phosphates
MD Feb
RJ Feb
DJ Feb
Chl a
RC Feb
RJ May
DJ May
CD Dec
DJ Dec
RC May
CD May
CD Oct
DO
RJ Dec
RC Dec
MD May
RJ Oct
MD Oct
DJ Oct
Nitrates
Salinity
Temp
MD Dec
RC Oct
pH
(d)
Component 2
Component 1
pH
Temp
SLO
RC May
MD May
RJ May
CD Oct
MD Oct
RC Oct
DJ Oct
RJ Oct
CD May
Salinity
RC Dec
Nitrates
RJ Dec
DO
MD Dec
DJ Feb
RC Feb
Chl a
DJ Dec
RJ Feb
DJ May
CD Dec
MD Feb
Phosphates
CD Feb
(e)
Component 2
Component 1
DJ May
CD Oct
pH
Temp
RC Oct
RC May
CD May
RJ May
DJ Oct
Chl a
Salinity
RJ Oct
MD Oct
RJ Feb
MD May
Nitrates
RJ Dec
RC Feb
DJ Dec
DO
DJ Feb
RC Dec
Phosphates
MD Feb
VCLO
MD Dec
CD Dec
CD Feb
(f)
Component 2
Component 1
CD Feb
Phosphates
VPLO
MD Feb
DJ Dec
RJ Feb
DJ Feb
Chl a
CD Oct
CD Dec
RC Feb
DJ May
DO
RJ Dec
MD Dec
CD May
RJ May
MD Oct
RJ Oct
RC May
Salinity
RC Oct
DJ Oct
Nitrates
pH
Temp
RC Dec
MD May

Fig. 5


80
70
60
50
40
30
20
10
0
Counts (cfu/mL)
Valamugil cunnesius
Valamugil cunnesius
Lutjanus indicus
Scatophagus argus
Escualosa thorracuta
Escualosa thorracuta
Escualosa thorracuta
Sillago sihama
Ilisha megaloptera
Pelates quadrilineatus
Mene maculata
Caranx sp.
Sepiella inermis
Gerres oyena
Thryssa mystax
Sardinella longiceps
Sardinella longiceps
Fish samples collected at different times
ZMA gut
ZMA surface
MacConkey's gut
MacConkey's surface
SS gut
SS surface
TCBS gut
TCBS surface

Supplementary Table 1. Details of the isolates selected for stability experiments.

Isolate	# 1	# 2	# 3	# 4
Type	*Shigella*-like (SLO)	*Vibrio cholerae*- like (VCLO)	VCLO	Coliform
Source	Surface of *Ilisha megaloptera*	Seawater	Surface of *Ilisha megaloptera*	Gut of *Sillago sihama*
Colony Size	1 mm	pinpoint	1 mm	1 mm
Colony Shape	Circular	Circular	Circular	Circular
Colony Color	Colourless	Yellow	Yellow	Pink
Colony Margin	Entire	Entire	Entire	Entire
Colony Elevation	Flat	Flat	Raised	Flat
Colony Consistency	Butyrous	Butyrous	Butyrous	Butyrous
Gram Character	Gram negative	Gram negative	Gram negative	Gram negative

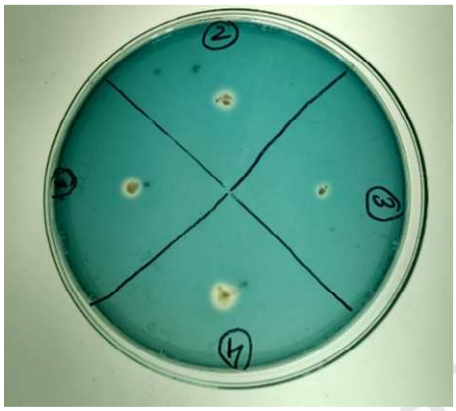
Supplementary Table 3. Colony characteristics and Gram character of representative group of probable pathogenic bacteria as seen on selective media.

Colony Characteristics	*E.coli*-like (coliforms)	*Enterobacter*-like (coliforms)	*Klebsiella*-like (coliforms)	*Salmonella*-like (SmLO)	*Shigella*-like (SLO)	*Vibrio parahaemolyticus*-like (VPLO)	*Vibrio cholerae*-like (VCLO)
Size	1-2 mm	1-2 mm	1-2 mm	1 mm	Pinpoint	1-2 mm	1-2 mm
Shape	Circular	Circular	Circular	Circular	Circular	Circular	Circular
Elevation	Raised	Raised	Raised	Raised	Flat	Raised	Raised
Color	Pink	Light pink	Light pink	Cream with black dot	Colorless	Green	Yellow
Consistency	Butyrous	Butyrous	Mucoid	Dry	Butyrous	Butyrous	Butyrous
Margin	Complete	Complete	Complete	Complete	Complete	Complete	Complete
Opacity	Opaque	Opaque	Opaque	Translucent	Transparent	Opaque	Opaque
Gram character	Gram negative	Gram negative	Gram negative	Gram negative	Gram negative	Gram negative	Gram Negative

Supplementary Table 3. Stability of the four isolates at different temperature. (Key: +++ heavy growth, ++ moderate growth, + very less growth, - no growth)

Isolates	Temperature	0 min	10 min	20 min	30 min	40 min	60 min	120 min
Isolate 1	50° C	+++	+++	+++	+++			
	80° C	+++	++	+	-	-	-	-
	100° C	+++	++	+	-			
Isolate 2	50°C	+++	+++	+++	+++			
	80°C	+++	+++	+++	++	-	-	-
	100°C	+++	+++	++	+			
Isolate 3	50°C	+++	+++	+++	++			
	80°C	+++	+++	+++	++	-	-	-
	100°C	+++	+++	++	+			
Isolate 4	50°C	+++	+++	++	++			
	80°C	+++	+++	++	+	-	-	-
	100°C	+++	+++	+	+			

Supplementary Fig. 1 Fish-associated bacterial isolates showing positive siderophore production on blue CAS agar plates.



Highlights

- Abundance of human pathogenic bacteria in the monsoonal estuary display spatiotemporal variation.
- Not all the pathogenic bacteria were widespread throughout the estuarine channel.
- Influence of spatial variation was more significant than that of the temporal variation.
- The human pathogenic bacteria were also associated with the fish tissue and gut.
- Bacteria isolated from fishes produced siderophores.
- Bacteria isolated from fishes withstood high temperatures for 30 min and freezing temperature for 24 h.
- Thus, estuarine waters and fishes were a potential ground of human pathogenic bacteria.

Declaration of interests

☐✓ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐X The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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