

Persistence of Fecal Indicator Bacteria associated with Zooplankton in a Tropical Estuary-West Coast of India

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Abstract

In a study carried out during 2014, bacteria associated with zooplankton in the Zuari estuary were 3–4 orders of magnitude higher in abundance than in seawater. The live zooplankton carried much more bacterial load compared to the carcasses and the fecal pellets harbored the highest density of bacteria *ie.* 8×10^{13} CFU cm^{-3} . The diversity of bacteria was higher in live zooplankton and also in seawater. But the activity of the zooplankton-associated bacteria was much higher compared to the free-living ones. Most of the associated bacteria belonged to the genus *Enterobacter*, *Pseudomonas*, *Aeromonas* and *Bacillus*. In growth experiments, *Aeromonas* and *Bacillus* were found to have lower salinity optima than *Enterobacter* (20 psu) and *Vibrio* and *Pseudomonas* (normal seawater salinity). Better growth of bacteria was observed in the medium containing the diatom *Chaetoceros* sp. than *Navicula* sp. Bacterial isolates were also able to survive in oligotrophic conditions and produce optimum biomass in two days at salinity 5 psu but in freshwater, the bacteria took a week's time to attain the optima. At salinities 0– 35, the bacteria survived even for 3 months without nutrient addition, indicating resilience in these bacteria and mechanisms to persist in the estuaries even in adverse conditions.

Keywords Zooplankton, associated bacteria, Zuari estuary, enterobacteria, *Aeromonas*

Introduction

Zooplankton have complex body structures and extensive surface area which provide living microhabitats for bacteria (Jacobsen and Azam, 1984; Lampitt et al., 1990; Turner, 1991). Unlike detritus and marine snow, living zooplankton continuously collect and concentrate food particles onto their mouth parts, some of which are lost due to sloppy feeding and some ingested matter from the gut is eventually released as fecal pellets (Moller and Nielsen, 2001). The dissolved organic matter released from zooplankton makes them 'microbial hotspots' (Smetacek, 1980) as it promotes continuous attachment and colonization and release of pelagic bacteria (Peduzzi and Herndl, 1992; Carman, 1990). Bacterial association is considered to be beneficial for the zooplankton too, since the bacterial scavenging prevents the buildup of debris and thus improves swimming efficiency of the zooplankton (Holland and Hergenrader, 1981). Some attached bacteria are also reported to provide nutrients like essential amino acids and vitamins (Fong and Mann, 1980) and enhance the host digestive abilities by contributing enzymes (Sochard et al., 1979).

In the open oceans, over 95% of the bacteria exist as free-living (Bano et al., 1997), mainly because of fewer particulate organic substrates (Unanue et al., 1992) and comprises mostly the α -*Proteobacteria*. In coastal waters, where particles are more abundant, the contribution of particle-associated bacteria may reach >60% of total bacterial abundance (Garneau et al., 2009). Among the particle-associated bacteria, *Cytophaga*, *Planktomyces*, and the γ -*Proteobacteria* are the key groups (Heidelberg et al., 2002). *Vibrio* spp. comprises a significant portion of the bacterial flora found naturally associated to chitinous marine zooplankton such as the copepods (Choudhury et al., 1989; Carli et al., 1993). Several *Vibrio* spp. are pathogenic for aquatic animals and cause significant losses of commercially important finfish and shellfish (Egidius 1987; Lightner et al. 1992). Chitinous exoskeletons of zooplankton can protect *V. cholerae* from stomach acid when ingested by humans and appear to amplify *V. cholerae* populations by either initiating or exacerbating cholera epidemics mostly in developing countries such as Bangladesh (Huq et al. 1983). But colonization of zooplankton by *Pseudomonas* spp., *E. coli* (Huq et al., 1983; 1995), *Salmonella* spp. (Venkateswaran et al., 1989) and *Aeromonas hydrophila* (Krovacek et al., 1994) is rarely reported.

Though numerous studies have addressed the occurrence of dense bacteria in copepod oral cavities, on appendages, guts, and fecal pellets (Honjo and Roman, 1978; Turner, 1979; Nagasawa et al., 1985; Nagasawa and Nemoto, 1988; Carman, 1994; Delille and Razouls, 1994; Hansen and Bech, 1996; Carman and Dobbs, 1997), little is known about the interactions between the bacteria and their hosts and also their gut bacterial community composition. Because many earlier studies on zooplankton associated bacteria relied on culturing and biochemical tests, they might have missed on

many bacterial phylotypes. In comparison, the use of molecular techniques in identification of the cultured isolates from zooplankton revealed a highly specialized bacterial community that is able to colonize the zooplankton interior and exterior using adhesion mechanisms and mediate biogeochemical processes generally underrepresented in the open ocean (eg. De Corte et al., 2018).

In Indian estuaries, disposal of raw sewage has been particularly rampant (Ramaiah et al., 2007) and one serious consequence of this is the addition of pathogenic micro-organisms such as *Vibrios*, *Salmonella* spp., and *E. coli*O157:H7 (Ramaiah, 2002; Nagvenkar and Ramaiah et al., 2009; Rodrigues et al., 2011; Khandeparker et al., 2015). The main effects of such contamination is the transfer of pathogens up the food chain and deterioration of the quality and safety of seafood and hence the public health. Therefore, it appears crucial to improve our knowledge of the number and types of potentially human- pathogenic bacteria in particle bound and free-living forms in estuarine waters. Recent studies revealed the dominance of γ -*Proteobacteria* which includes potential pathogens from families such as Enterobacteriaceae, Vibrionaceae and Pseudomonadaceae (Khandeparker et al 2017). Another study indicated high abundance of bacteria in the estuarine sediments during monsoon, probably from terrestrial runoff sediments (Gardade and Khandeparker 2017). They found that organic matter profoundly determines the distribution of *Vibrio* spp. and pathogenic bacteria such as *Shigella* spp. in the Zuari estuarine sediments. Potentially virulent strains of *E coli* were also detected in water and mangrove originated foods, supporting high probability of fecal contamination in the coastal waters of Goa (Poharkar et al 2014)

We attempted to study zooplankton associated bacteria in the tropical Zuari estuary, on the central west coast of India, which annually receives enormous runoff suspended particulate matter ranging from 2 to 90 mg L⁻¹ during active southwest monsoon (Shetye et al 1987; Rao et al., 2011). Zuari estuary is used for navigation purpose, finfish and shellfish harvesting, transport of ores, discharge of wastes from households, ships, port- and tourist- related activities. The amount of sewage discharged into the Mandovi-Zuari estuaries is estimated to be 30 million litres per day (Pangare et al 2006). The objectives of this study were to estimate 1) the total, viable and human pathogenic bacteria associated with surfaces and guts of various zooplankton taxa as compared to seawater 2) the activity of zooplankton associated bacteria compared to the free-living bacteria 3) The effect of food substrate and density on the abundance of zooplankton associated bacteria 4) the effect of salinity on the growth optima of some potentially pathogenic bacteria in seawater 5) the survival rates of enteric bacteria at low nutrient levels in seawater under varying salinity conditions.

Materials and methods

Sample collection

The present study was carried out in the Zuari estuary, located at lat. 15° 25' N and long. 73° 59' E along the central west coast of India (Fig. 1). The sampling station chosen was off Dona Paula at the mouth of the estuary, where the depth of water is about 2–4.5 m during high tide. Surface seawater sample was collected into a 100 ml clean plastic bottle and zooplankton samples with a Heron Tranter net (Hydrobios, Germany). Water temperature was measured using a mercury thermometer and salinity by portable handheld refractometer (ATAGO, S/ Mill, Japan). One liter of seawater was collected for chlorophyll *a* and measured by fluorometer (Turner Designs, 10 AU). After collection, samples were immediately transported to the laboratory and processed for plankton and bacterial analyses.

Sample processing

Zooplankton was collected by HT net and various groups/genera were sorted immediately under a stereozoom microscope for isolation of bacteria. The zooplankton community consisted of live as well as dead zooplankton. An amphipod and two copepods *viz.* *Acrocalanus* sp. and *Paracalanus* sp. were selected from the live component. From the dead zooplankton, a fresh carcass of copepod and aged carcasses of chaetognath and euphausiid, were selected for the analysis. The procedure observed for isolation of zooplankton associated bacteria, according to Sochard et al. (1979) is as follows:

a) Surface isolates- One to five zooplankton individuals of each available group were taken in a 2 ml sterile eppendorf tube, washed twice in 1 ml sterile seawater, and the second wash was used to obtain "zooplankton surface isolates" of bacteria.

b) Gut isolates- The zooplankton were drained of all the water used for the second wash and washed for third time and wash water discarded. Another 1 ml of sterile seawater was added and the animals were ground/mashed with the help of a sterile pestle. The homogenized zooplankton tissue was briefly centrifuged at low rpm, allowed to settle down at the bottom of the tube and the supernatant was used for enumeration of various bacterial groups described below.

Total, viable, coliform and fecal coliform bacteria associated with zooplankton and in seawater

Seawater filtered through sterile GF/C filter for free-living bacteria, 200 µl of wash water from 'a' for zooplankton surface- isolates and supernatant from 'b' for zooplankton gut- isolates were fixed with 2% (final concentration) formaldehyde and stored at 4°C until processing. Total bacterial abundance was enumerated by the DAPI (4', 6-Diamidino-2-Phenylindole, Dihydrochloride) method

(Porter and Feig, 1980). At least 20 fields were counted in an epifluorescence microscope (Olympus BX 51) for each sample, and total bacterial numbers were expressed as numbers cm^{-3} .

Similarly, samples were plated on Nutrient agar for total viable counts, Hichrome Coliform Agar for total coliforms, M-FC Agar for fecal coliforms, Xylose lysine deoxycholate agar for *Shigella*, *Proteus* and *Klebsiella*, Improved Salmonella Agar for *Salmonella* and *Escherichia coli*, Thiosulfate citrate bile salts sucrose agar for *Vibrio*, Cetrinide Agar for *Pseudomonas aeruginosa* and Rapid enterococci Agar for fecal streptococci. The culture media used were from HiMedia, Mumbai and except for TCBS, all were prepared in 10% seawater.

Biochemical Characterization and molecular identification using 16S rDNA sequencing of bacterial isolates

Characteristic bacterial colonies from each of the specific media were isolated, purified and subjected to a set of biochemical tests using the KB002 TM HiAssorted Biochemical Test Kit for Gram negative rods (Table 1). This kit allows a convenient way to perform 12 different biochemical tests. These include citrate utilization, lysine decarboxylase, ornithine decarboxylase, urease, phenylalanine deamination, nitrate reduction, H_2S production, glucose, adonitol, lactose, arabinose and sorbitol utilization tests.

From a total of 137 pure cultures of bacteria isolated from the above media, genomic DNA was extracted by the CTAB method (William et al., 2004, Current Protocols in Molecular Microbiology) and quantified with the ThermoScientific™ NanoDrop 1000 spectrophotometer. Agarose gel electrophoresis of the extracted DNA was carried out to check the integrity, gels stained with ethidium bromide and viewed in GelDoc (BioRad). The 1500 bp 16S rRNA gene from all the DNA samples were amplified using the 27F and 1492R universal eubacterial primers. All the PCR amplification products were purified with Aygen Axyprep PCR Clean-up Kit and partially sequenced in a DNA gene sequencer (ABI) by following standard cycle sequencing protocol.

The gene sequences were compared with others in the GenBank databases using the NCBI BLAST (www.ncbi.nlm.nih.gov). All the 16S rDNA sequences (see Table 3 for their accession numbers) of the bacteria identified to be associated with zooplankton and ambient water during this study were submitted to GenBank. Good quality sequences of 16S rDNA of representative organisms were obtained from GenBank and aligned with gene sequence of our isolates using multiple sequence alignment program CLUSTAL X software (Thompson, 1997). The aligned sequences were used to construct a distance matrix, after the generation of 500 bootstrap sets, which was

subsequently used to construct a phylogenetic tree using the neighbor-joining method (Holmes, 2003)

Production rates of free-living and zooplankton associated bacteria by ^3H method

For estimating zooplankton associated bacteria, 5 copepod individuals of *Paracalanus* sp., *Acrocalanus* sp. and *Acartia* sp. were ground separately in sterile seawater and made up to 10 ml in polycarbonate tubes. The tubes were vortexed thoroughly and filtered through 10 μm mesh to remove any zooplankton debris. A blank tube was prepared in the same manner and formalin was added at the beginning of the experiment. For the free-living bacteria, 10 ml of 1.2 μm - filtered seawater was used. Bacterial production (BP) was estimated by measuring the rate of [methyl- ^3H] thymidine incorporation (Fuhrman and Azam 1982) by bacterial cells. All samples were incubated for 90 min with ^3H -thymidine (18000 mCi mmole $^{-1}$, Bhabha Atomic Research Centre, Mumbai) at a final concentration of 10 nM. The reaction was terminated with 2% formalin. The samples were then filtered through 0.22 μm polycarbonate filters, extracted with cold 5% TCA, and rinsed with ethanol. The dried filters were then placed in scintillation vials containing cocktail W (SRL, Mumbai). The samples were then radio-assayed in a Perkin Elmer Wallac 1409 DSA liquid Scintillation Counter.

Tritiated thymidine incorporated (TdR, pM ind h $^{-1}$) was calculated by the formula: $\text{TdR} = (\text{DPMs} - \text{DPMb}) / (\text{SA} \cdot \text{SV} \text{ or } \text{NZ} \cdot \text{T} \cdot 2.22)$. Where, DPMs = disintegrations per minute of the sample on the filter, DPMb = disintegrations per minute (DPM) of the blank on the filter, SA = specific activity (mCi mmol $^{-1}$), SV = seawater sample volume or NZ = number of zooplankton, T = incubation time in hours. Thymidine incorporation rates were converted to bacterial carbon production (BCP) by using a conversion factor of 2×10^{18} cells produced per mole of thymidine incorporated (Iriberry et al., 1990) and a carbon conversion factor of 11 fg C cell $^{-1}$. For comparison with the bacteria per unit volume of sea water, BCP per copepod was converted to per centimeter cube units by using copepod body volume.

Effect of feed type and concentration on copepod associated bacteria

An experiment was conducted to test the hypothesis that the quality and quantity of substrate favor different types of bacterial attachment as indicated by different growth kinetics of bacteria. A consortium of the bacteria isolated from zooplankton was made. An aliquot of this was added to six flasks containing 250 mL sterilized seawater. To the first three flasks, cultured diatom *Chaetoceros* sp. was added in concentrations of 50,000 cells L $^{-1}$, 5000 cells L $^{-1}$ and 1000 cells L $^{-1}$ respectively. Similarly, to the other three flasks was added the diatom *Navicula* sp. in similar concentrations.

Samples from all six flasks were plated everyday on nutrient agar plates to record the bacterial counts (CFU) from 0 day to 8th day.

Growth characteristics of some potentially pathogenic bacteria, under varying salinity

Three different concentrations of aged seawater, viz. salinities 5, 20 and 35 psu were made to mimic estuarine conditions. To these, nutrient broth was added at quarter strength, sterilized and 250 mL was dispensed in 5 sets of sterile flasks. To each set of flasks containing the above solution of 5, 20 and 35 psu, a known volume of culture of the dominant bacterial species isolated from zooplankton in this study, viz. *Aeromonas hydrophila*, *Pseudomonas fulva*, *Enterobacter* sp., *Bacillus pumilus* and *Vibrio* sp. was added. Flasks were plugged with cotton and incubated at room temperature on low shaking condition. Samples were taken on days 0, 1, 4, 7, 10 and 14 and plated on nutrient agar plates. After 24 hrs, CFU were counted.

Survival time of associated bacteria in seawater

A flask containing 250 ml sterile freshwater, and three others with sterile aged seawater at salinities 5, 20 and 35 psu each were inoculated with ~5 dead copepods and kept at room temperature on a shaker at low rpm. Water sample was plated on nutrient agar on days 0, 1, 2, 3, 7, 14, 21, 30, 60 and 90. After 24 hrs incubation at RT, colonies were counted and expressed as CFU mL⁻¹.

Antibiotic susceptibility testing

Antibiotic resistance of few bacterial isolates of Enterobacteriaceae family was determined by the disc diffusion method (Bauer et al., 1996). Isolates W1, W2, W4, W6, S4, S6, S9, and S11 grown overnight at 37°C and adjusted to Mc Farland turbidity 0.5 were spread plated on nutrient agar plates separately and labeled carefully. The plates were allowed to dry. Each isolate was exposed to the following 20 antibiotics on inert flat circular discs of 6 mm diameter (HiMedia Icosa G-I-Minus IC003). The antibiotics used were Imipenem (IPM) 10µg, Ciprofloxacin (CIP) 5µg, Tobramycin (TOB) 10µg, Moxifloxacin (MO) 5µg, Ofloxacin (OF) 5µg, Ceftazidime (CAZ) 30µg, Levofloxacin (LE) 5µg, Norfloxacin (NX) 10µg, Co-Trimoxazole (COT) 25µg, Colistin (CL) 10µg, Nalidixic acid (NA) 30µg, Augmentin (AMC) 30µg, Cefoxitin (CX) 30µg, Gatifloxacin (GAT) 5µg, Gentamicin (GEN) 10µg, Amikacin (AK) 30µg, Aztreonam (AT) 30µg, Ceftriaxone (CTR) 30µg, Cefpodoxime (CPD) 10µg and Nitrofurantoin (NIT) 300µg.

The antibiotic discs were placed over the nutrient agar plates. These media plates were then incubated at room temperature for 24hrs. The plates were observed for zone of inhibition/growth. Zones of inhibition were measured by Zonescale Easy (HiMedia) and scores of susceptibility and resistance were taken according to National Committee for Clinical Laboratory Standards for

antimicrobial susceptibility tests (NCCLS, 2002). The results were used to calculate the Antibiotic resistance Index (ARI) and the Multiple antibiotic resistance (MAR) index for total number of isolates as $ARI = y / nx$, where y is the number of resistant isolates, n is the number of isolates tested and x is a number of antibiotics (Hinton, Linton, 1983) and MAR index = a/b , where a is a number of antibiotics to which the isolates are resistant and b is a total number of antibiotics exposed.

Results

General seawater characteristics

The air temperature was 26°C and the water temperature 28°C at the time of sampling (noon time). Salinity was 20 psu. Chlorophyll *a* was 1.0 mg m⁻³. The phytoplankton community comprised of diatoms, mainly dominated by genera *Chaetoceros*, *Thalassiosira*, *Coscinodiscus*, *Navicula*, *Rhizosolenia* and *Melosira*. Average phytoplankton abundance was 4 x 10³ cells L⁻¹.

Total, viable, coliform and fecal coliform bacteria associated with the zooplankton and in the seawater

Bacterial total numbers as well as the colony forming units did not differ significantly between the surface and gut of the live zooplankton. The total direct counts (TDC) of bacteria varied from 10⁸ to 10⁹ cells cm⁻³ of the zooplankton surface (Fig. 2). The total viable counts (TVC) were 5–13 (x 10⁷ CFU cm⁻³). Fecal coliform number was similar to the total coliforms (TC) and varied from 1–6 (x 10⁶ CFU cm⁻³). The highest counts of all the bacterial types were found in the live copepod *Acrocalanus* sp.

Compared to the live plankton, the total bacteria as well as its subgroups were lower and largely variable in the zooplankton carcasses. TDC varied from 5 x 10⁵ cells cm⁻³ to 2 x 10⁸ cells cm⁻³. TVC varied from 3 x 10⁵ to 1 x 10⁷ cells cm⁻³. TC varied from 5 x 10³ to 3 x 10⁶ cells cm⁻³. Fecal Coliforms (FC) varied from 5 x 10³ to 4 x 10⁶ CFU cm⁻³. The highest counts were obtained in the fresh carcass *i.e* that of the copepod and the lowest was found in the aged carcass *i.e* that of the euphausiid.

In the seawater, the TDC was 5 x 10⁵ CFU cm⁻³, in the sediment it was 7 x 10⁶ CFU cm⁻³ and in the fecal pellet, 8 x 10¹³ CFU cm⁻³. TVC, TC and FC in the water were 1800, 180 and 120 CFU cm⁻³ respectively. In sediment, they were 2 x 10⁵, 3200 and 1840 CFU cm⁻³. In the fecal pellet, the respective counts were 8 x 10¹¹, 4 x 10¹⁰ and 1 x 10¹⁰ CFU cm⁻³. Counts of all bacterial types were the lowest in the water sample and the highest in the fecal pellet.

Composition of associated bacteria from zooplankton

Based on the biochemical profiles (Table 1) of the major bacterial types isolated from various sources on different selective media, ~150 isolates were taken up for identification by 16S gene sequencing. From the good quality sequences that were obtained, 135 isolates of bacteria from all sources were identified. All the sequences obtained are deposited in the GeneBank (Table 2). Comparative 16S rRNA gene sequence analysis affiliated the isolates to diverse phylogenetic groups (Fig 3). A great majority belonged to γ -Proteobacteria (91%), 8% to Firmicutes comprising *Bacillus*, *Oceanobacillus* and *Enterococcus* and 0.73% to Actinobacteria containing an isolate of *Rothia* sp. A total of 45 species of bacteria from 20 genera were found (Fig 4). The genera *Enterobacter* (41.6%), *Pseudomonas* (20.4%), *Aeromonas* (14.6%) and *Bacillus* (6.6%) predominated among others.

Most of the sequenced isolates had 16S rRNA gene sequence similarities of 98 or more and shared numerous phenotypic properties with known bacterial species which are wide-spread in the marine environment. *Enterobacter* represented the largest number of species occurring during this study (Fig. 5). Of its 9 species that were found, *E. cloacae* and *E. ludwigii* were the most frequently occurring ones. *Enterobacter aerogenes*, which is known to be a pathogen, was also found in some samples. Seven species of *Pseudomonas* were found and *P. pseudoalcaligenes* was the most common one. Two isolates of *P. aeruginosa* were also found, one in live amphipod and the other in water sample. Six species of *Aeromonas* were found and *A. caviae* was common. Four isolates of *A. hydrophila*, also an important pathogen were found on surface and gut of the live copepod *Acrocalanus* sp. As seen in Fig. 5, only 22 bacterial species were found in or more than two samples. About 50% species were rare, that were found just once in any one of the samples. The bacterial species diversity was found to be higher in seawater and in live zooplankton, followed by sediment, fecal pellet and was the least in the carcasses (Table 3).

Production rates of free-living and zooplankton associated bacteria by ^3H method

The activity of free-living bacteria in seawater, measured in terms of thymidine incorporation rates was found to be much lower than the zooplankton associated bacteria. The BP was only $0.004 \text{ g C m}^{-3} \text{ d}^{-1}$ in the water sample and it varied between 4.5 and $4.8 \text{ g C m}^{-3} \text{ d}^{-1}$ in the three copepods (Fig. 6). Using this- and the bacterial abundance data, the bacterial growth rates were found to be $5.3\text{--}7.3 \text{ d}^{-1}$ for the copepod associated bacteria and 0.6 d^{-1} for the free-living bacteria. This means that the bacterial population associated with the zooplankton doubles every 4-5 hours in contrast to free-living bacteria which takes more than a day.

Effect of feed type and density on associated bacterial numbers

The initial bacterial load on copepods varied from 1500 to 2000 CFU cm⁻³ on day 0. In all the flasks where *Chaetoceros* sp. was added as the feed, a rapid increase in the counts of bacteria was seen after day 2 especially in flasks with algal concentrations 50,000 cells L⁻¹ and 5000 cells L⁻¹ (Fig. 7). In the flasks where *Navicula* sp. was used as feed, the increase in associated bacterial numbers was much lesser and leveled off after day 6.

Growth characteristics of some zooplankton associated bacteria, under varying salinity

The growth rate as well as growth optima of four bacterial species isolated from zooplankton and inoculated in minimal media varied with salinity. The species tested showed growth optima between days 1–7. From an initial inoculum of 10⁴ CFU ml⁻¹, *Aeromonas hydrophila* multiplied to 10⁷ CFU ml⁻¹ on day 4 at salinity 5 psu (Fig. 8). The cell numbers decreased only after day 7. Similarly, *Bacillus pumilus* showed higher growth at 5 psu. *Pseudomonas fulva* showed good growth at all salinities ranging from 5 to 35. *Enterobacter* sp. grew better at salinity 20 and *Vibrio* sp. clearly showed a preference for salinity 35.

Survival time of associated bacteria in seawater

When dead copepods were introduced in sterile aged seawater at different salinities and also in freshwater and incubated at room temperature for a period of 90 days, the copepod-associated bacteria showed different growth patterns. The associated bacteria grew best at salinity 5, producing the highest biomass of 40 x 10³ CFU ml⁻¹ on day 2 (Fig 9), followed by the bacteria at salinities 20 and 35. Bacteria were slowest to grow in freshwater but produced peak biomass, matching that at salinity 5 on day 7. At salinity 35, the counts remained similar to the initial inoculum even on day 90, compared to the rest where the bacterial numbers were much lesser (100–300 CFU ml⁻¹) at the end of the experiment. The contents of flasks showed lots of degraded remains of zooplankton and dense bacteria associated with it even on day 90 (Plate 1).

Antibiotic susceptibility

The 8 isolates of bacteria tested for antibiotic susceptibility, from water and zooplankton were mostly susceptible to the antibiotics Amikacin, Gentamycin, Tobramycin, Ceftriaxone, Ciprofloxacin, Ofloxacin (5), Levofloxacin (5) Norfloxacin (10), Gatifloxacin (5) and Moxifloxacin (5) (Table 4). The first three are aminoglycosides, Ceftriaxone is a cephalosporin and the rest are fluoroquinolones.

Out of the 20 antibiotics tested, Augmentin- the aminopenicillin is completely ineffective as all of the environmental isolates were completely resistant to it, showing no zone of inhibition. Most of the isolates were also resistant to Aztreonam, Imipenem (both carbapenems), Cefoxitin, Cefpodoxime,

Ceftazidime (cephalosporins) and Nitrofurantoin. Only few isolates were resistant to fluoroquinolones such as Nalidixic acid. Every isolate was resistant to more than one antibiotic, with a MAR index that ranged from 0.35 to 0.5 and which was not very different for the zooplankton associated isolates and the water isolates. The ARI index was 0.05.

Discussion

It is evident from the present study that copepod bodies and fecal pellets are organic rich substrates for bacterial colonization. The highest number of total bacteria *viz* 8×10^{13} cells cm^{-3} was found associated with the fecal pellet, compared to the live zooplankton (10^8 - 10^9 cells cm^{-3}) and the aged carcasses (10^5 - 10^8 cells cm^{-3}). In contrast, the abundance of free-living bacteria in seawater was only 5×10^5 cells cm^{-3} . This is because the bacteria within the gut are relatively less active (Hansen and Bech, 1996) but once egested by zooplankton through fecal pellets, they grow and proliferate within the pellets into the water and pelagic bacteria also colonize the fecal pellets (Jacobsen and Azam, 1984). Higher number of bacteria in the fecal pellet is also related to the higher oxygen concentration and nutrient accessibility in the ambient water compared to within the zooplankton gut (Gowing and Wishner, 1992). The bacteria in the fecal pellet not only play a significant role in degrading the fecal pellets (Gowing and Silver, 1983, Jacobsen and Azam, 1984), but may also be a food source for bacterivorous protozoans in the water column (Kiørboe et al., 2004).

In a previous study, the viable bacteria in the seawater from Zuari estuary were <1% of TDC (Nagvenkar and Ramaiah, 2009). In our study, the TVC was 1–10% of the TDC. Nearly all of the coliforms were of fecal origin and on average one or two orders of magnitude lower than the viable counts in most zooplankton specimens. The FC abundance in the water was 120 CFU cm^{-3} but counts up to 4920 CFU ml^{-1} were recorded in the Zuari estuary during the years 2002–2007 (Rodrigues et al., 2011). In a recent study (Khandeparker et al., 2017) too, a wide range of FC ranging from $<10^1$ – 10^5 ml^{-1} from the surface and bottom waters of the estuary were observed. The low values of FC in our study could be as a result of absence of major polluting sources in the vicinity of the sampling area and also due to attenuation by high intensity of solar radiation (Conan et al., 2008; Troussellier et al., 2004).

As reported by Ruiz et al. (2000), bacteria associated with plankton are the principal contributors of pathogenic bacteria. The association of FC with zooplankton probably confers them with higher survival rates based on glucuronidase activity that was 100 times higher in marine snow compared to ambient coastal water (Muller-Niklas and Herndl, 1992). In our study, the activity of associated bacteria based on rate of tritiated thymidine incorporation was three orders of magnitude higher in

zooplankton associated bacteria than in bacterioplankton. Alldredge and Gotschalk (1990) also found that associated bacteria were growing >3 times faster than free-living bacteria. As Carman (1994) and (Turley 1993) suggest, the enhanced activity of bacteria associated with zooplankton could be stimulated by the higher amounts of labile organic matter by host's excretion. Bouvy et al. (2008) working in Senegal also found that it was the substrate concentration that controlled persistence of FIB in coastal waters. To our knowledge, this is one of the first reports publishing information on the density of FC in varied genera of zooplankton in the coastal waters of India.

During this study, copepods (*Acrocalanus* and *Paracalanus*) amphipod, chaetognath and euphausiid representing the major zooplankton in the coastal marine environment were host to a wide range of bacterial genera such as *Enterobacter* (41.6%), *Pseudomonas* (20.4%), *Aeromonas* (14.6%) and *Bacillus* (6.6%) among others. Similar to the study of Bianchi et al., (1992), the composition of bacteria on zooplankton bodies was very similar to that in seawater which means that the origin of the microbial community associated with zooplankton is from the ambient water. γ -Proteo bacteria was the dominant group of bacteria in the associated fractions as well as in seawater similar to an earlier observation in Zuari estuarine surface sediments (Khandeparker et al 2017). In contrast to our findings, DeLong et al. (1993) found that the bacterial populations associated with the particles differ significantly compared to that in water. They found that γ -Proteobacteria dominated the particle-associated bacteria and α - Proteobacteria among the free-living bacteria. Firmicutes comprised at least 8% in our study even as they were consistently reported in copepod guts by Shoemaker and Moisanter (2017) and suggested to form longterm endobiotic associations.

Although the genus *Enterobacter* which is a commensal of human gut and animals and is ubiquitous in aquatic environments contains more than 20 species, only members of the *Enterobacter cloacae* complex (Ecc) are of clinical significance as nosocomial pathogens (Sanders and Sanders, 1997; Paauw et al., 2008). Ecc comprises 6 species (Mezzatesta et al., 2012) that includes three commonly occurring species viz. *Enterobacter cloacae*, *E. ludwigii* and *E. hormachei* during our study. *E. aerogenes* and *E. cloacae* have been reported as important opportunistic and multi drug resistant bacterial pathogens for humans during the last three decades in hospital wards (Davin-Regli and J Pagès, 2015). *E. aerogenes* was also found in some zooplankton samples but is said to be less virulent than *E. cloacae* (Flynn et al., 1987). In Asia, *E. cloacae* strains harboring metallo- β -lactamases have been described (Huang et al., 2012; Dai et al., 2013; Hamprecht et al., 2013; Jaskulski et al., 2013). Their dissemination is due to presence of enzymes and genes involved in antibiotic degradation and resistance, which help them to colonize in different environments/ hosts

and rapidly and efficiently adapt their metabolism and physiology to external conditions and environmental stresses.

Surprisingly, *Vibrio* which is often associated with many crustacean zooplankton, was rare in our study and could have been because of low salinity in the estuary during monsoon. This was felt to be the reason because in our experiment too, *Vibrio* sp. clearly showed a preference for salinity closer to seawater. Lower rates of their recovery are also because they and many other bacteria enter a viable but nonculturable (VBNC) state under environmental conditions adverse to active cell metabolism and division (Roszak and Colwell 1987). When a large portion of *Vibrio* spp. are VBNC, culture methods significantly underestimate bacterial populations present in a sample (Xu et al., 1982). In countries such as Bangladesh copepods play a major role in the multiplication, survival and potential transmission of *V. cholerae* (Colwell and Huq 1994), exacerbating cholera epidemics (Tamplin et al., 1990). To ascertain the absence of *Vibrio cholera* however would require extensive sampling over larger spatio-temporal scales in this estuary.

Aeromonas showed optimal growth at salinity 5 and their species have been isolated commonly from waters along Italian coasts (Fiorentini et al., 1998). In estuarine habitat, higher abundance of *A. hydrophila* has been found during algal blooms (Hazen and Esch, 1983) and terrestrial input (Maugeri et al., 2004). *Bacillus* on the other hand is abundant in terrestrial environment but *B. pumilus* has increasingly been isolated from marine environments including Kochi waters (Parvathi et al., 2009). In our experiments, *Bacillus* showed good growth at salinity 5 which means it is suited for survival in estuarine habitats.

Tang (2005) has shown the effect of host feeding activities on the abundance of associated bacteria. By feeding on different food, the copepods may introduce different types of organic substrates into their guts and fecal pellets to favor the growth of different bacteria. In our study, the density of the copepod associated bacteria varied with the type of feed present in the medium. The diatom *Chaetoceros* stimulated the highest density of bacteria similar to the study by Tang et al. (2009).

In recent years, there is growing development of resistance of enterobacteria to multiple antibiotics, the most notable among these being resistance to β -lactam group of antibiotics such as cephalosporins and carbapenems (Arrias and Murray, 2009). Our study revealed the presence of multiple antibiotic resistance in all 8 isolates tested. Maximum drug resistance was observed towards, carbapenems, cephalosporins and nitrofurantoin. All the 8 isolates of bacteria were resistant to antibiotic Augmentin which is a combination of Amoxicillin and clavulanate potassium. It was

noted in another study (Singh et al. 2017) that 39% of the isolates in seafood were resistant to Augmentin. The MAR index ranged from 0.3 to 0.5, which indicates that they are from high risk source and possibly fecal contamination. Multiple antibiotic resistance has been observed previously in coastal waters of India (Nair et al., 1992, Maloo et al., 2014) and is a result of antibiotics or other antimicrobials (antiseptics, disinfectants, heavy metals etc.) released in natural ecosystems in concentrations leading to the selective survival of resistant bacteria with multi-drug resistance genes (Matyar et al., 2008). There were similar findings in a study conducted on the beaches of Algeria (Alouache et al. 2012).

Antibiotic resistance in fecal indicator bacteria (FIB) isolated from water has been described since last half century (Smith, 1970). The incidence of human enteric pathogens such as *Escherichia coli* and *Salmonella enterica* in seafood is primarily due to the contamination of water bodies from where the fish are harvested. Though the ARI of 0.05 in the present study does not indicate severe pollution from antibiotics in the study area, the threat from enteric pathogens in seafood is confounding when such bacteria are multi-drug resistant (MDR), the prevalence of which in seafood has been reported in many studies in the recent past (Adesiji et al., 2014).

The sampling point used in the present study in the Zuari estuary is regularly used for harvesting various kinds of finfish and shell fish and also for recreation purposes. Presence of a large bacterial load including fecal coliforms associated with planktonic invertebrates, presence of multi- antibiotic resistance and longer survival times of enterobacteria in wide range of salinity indicates a potential human health risk. Strategies such as curbing of untreated disposal of domestic and commercial sewage, regulation of human settlement, and pollution along the coast are needed to reduce dissemination of pathogens through seawater and vectors such as zooplankton into seafood and to humans.

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Legends to Figures

Fig1. Location of sampling station in the Zuari estuary

Fig2. Variation of different bacterial groups: TDC (Total Direct counts), TVC (Total viable counts), TC (Total coliforms) and FC (Fecal coliforms) on the surface and in gut of live and dead zooplankton collected from the Zuari estuary.

Fig 3. Phylogenetic relationship of bacterial isolates recovered from zooplankton, fecal pellets, sediment and water samples, on the basis of 16S rDNA gene sequences. The branching pattern was generated by the neighbor-joining method. Numbers indicate bootstrap values.

Fig 4. Composition of bacteria genera found associated with zooplankton, fecal pellets, sediment and water in the Zuari estuary during southwest monsoon

Fig 5. Frequency of occurrence of bacterial species in surface and guts of live and carcass of zooplankton, in sediment, zooplankton fecal pellets and in seawater.

Fig 6. Production rates of free-living bacteria and bacteria associated with different copepod genera in the Zuari estuary based on tritiated thymidine incorporation.

Fig 7. Variation in growth rate of copepod- associated bacteria in different concentrations of phytoplankton species.

Fig 8. Variation in growth optima of some bacteria associated with zooplankton in varying salinity conditions.

Fig 9. Survival time of associated bacteria in oligotrophic medium at different salinities.

Plate 1. Scanning electron micrographs of flask contents in survival experiments on day 1(a), day 2-7 (b, c) and 90 days (d -l) wherein dead copepods were inoculated in aged seawater and incubated at room temperature for 90 days.

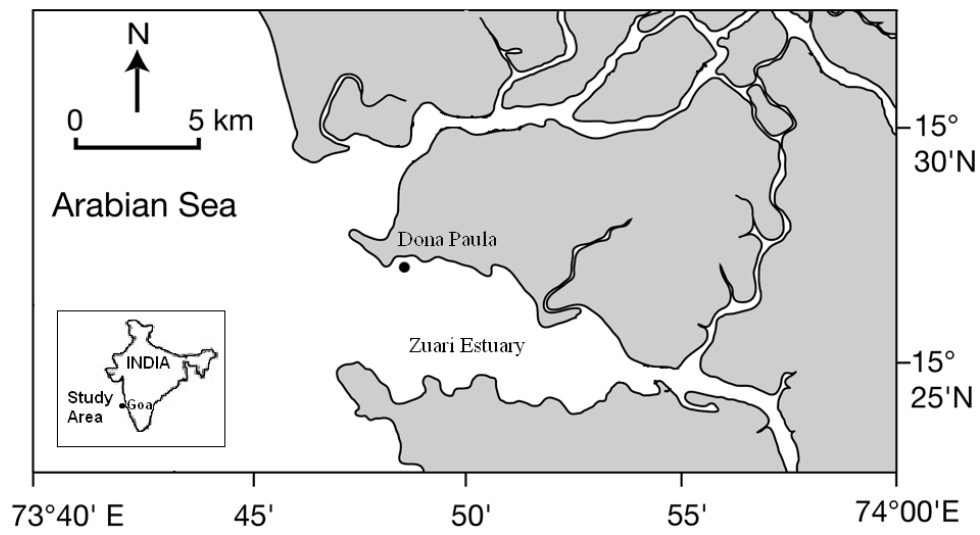


Fig 1.

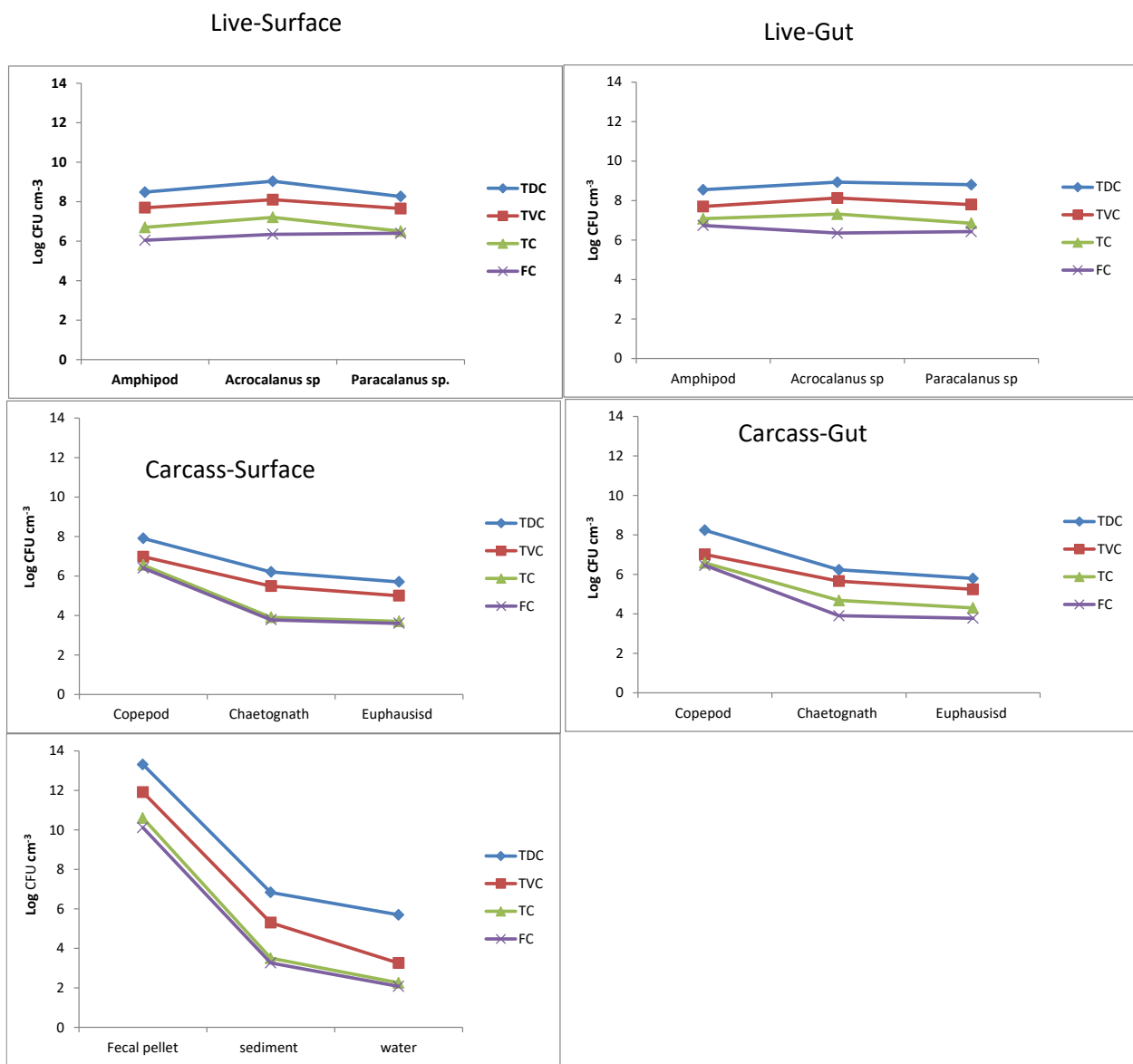


Fig 2

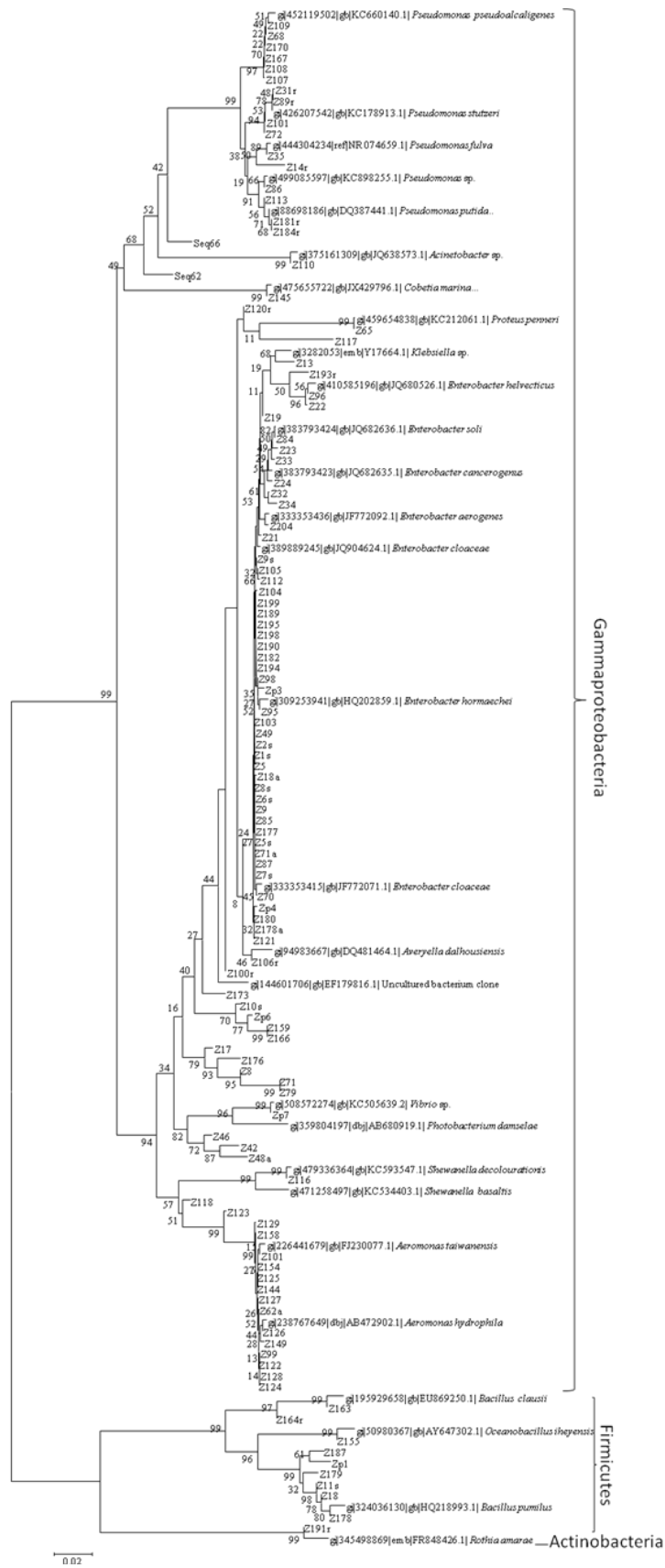


Fig 3.

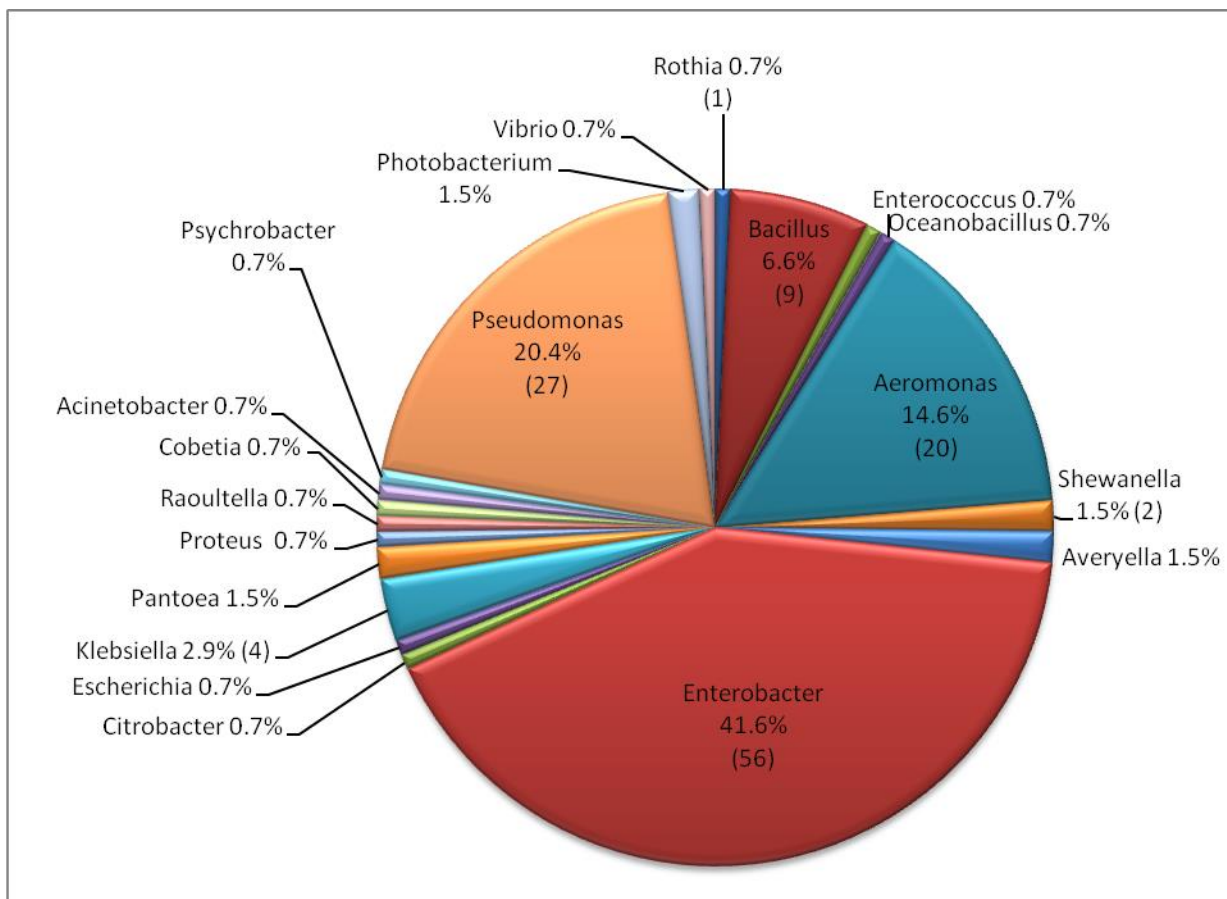


Fig 4.

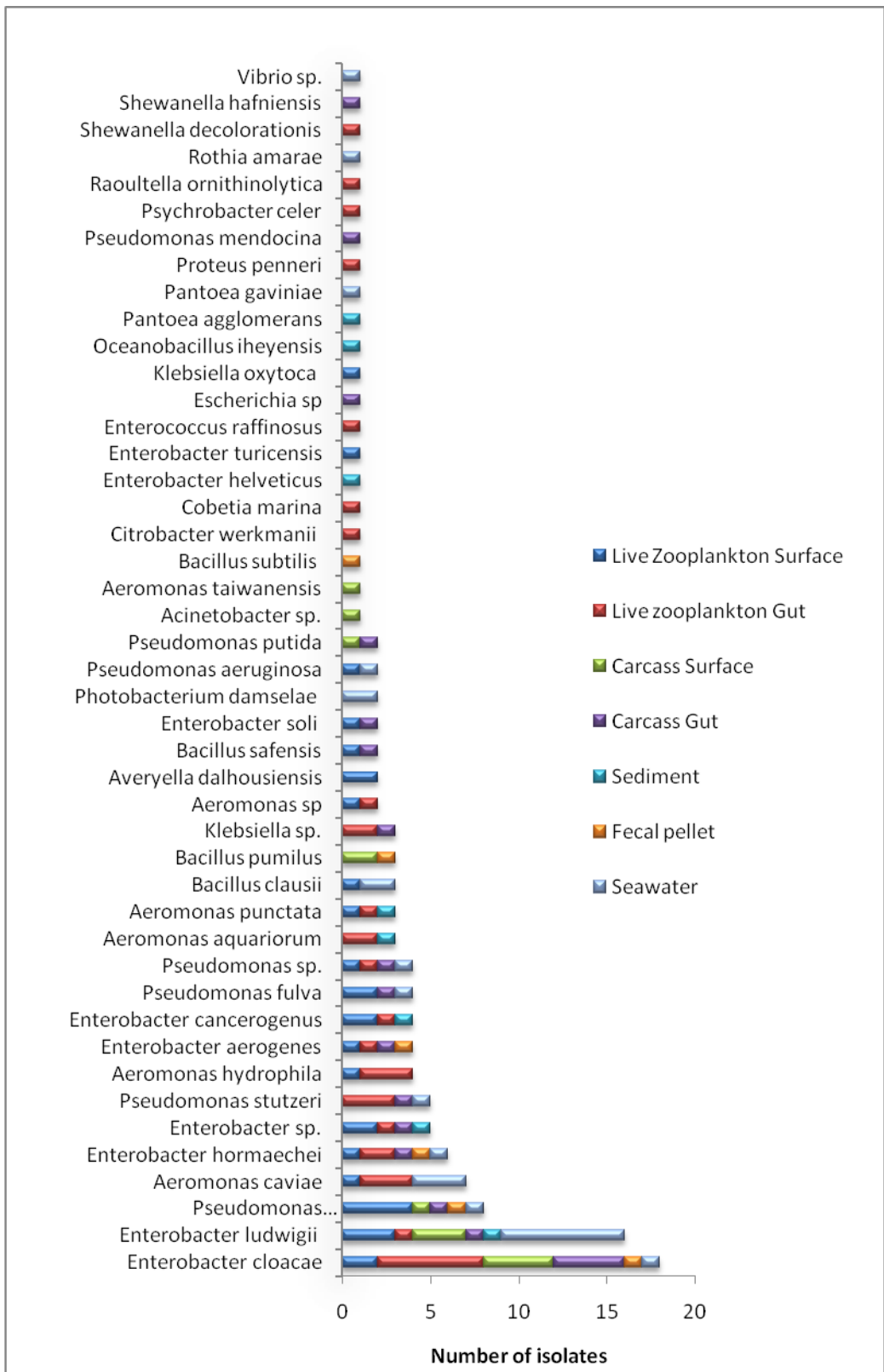


Fig 5.

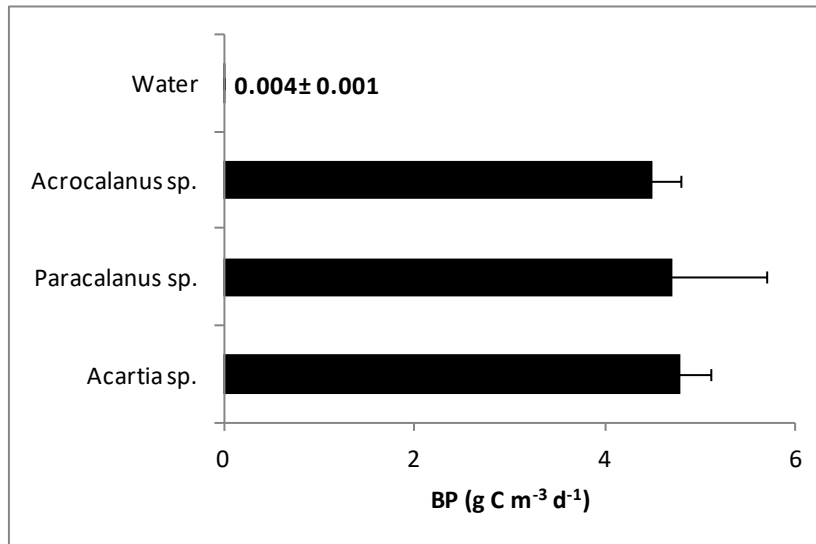


Fig 6.

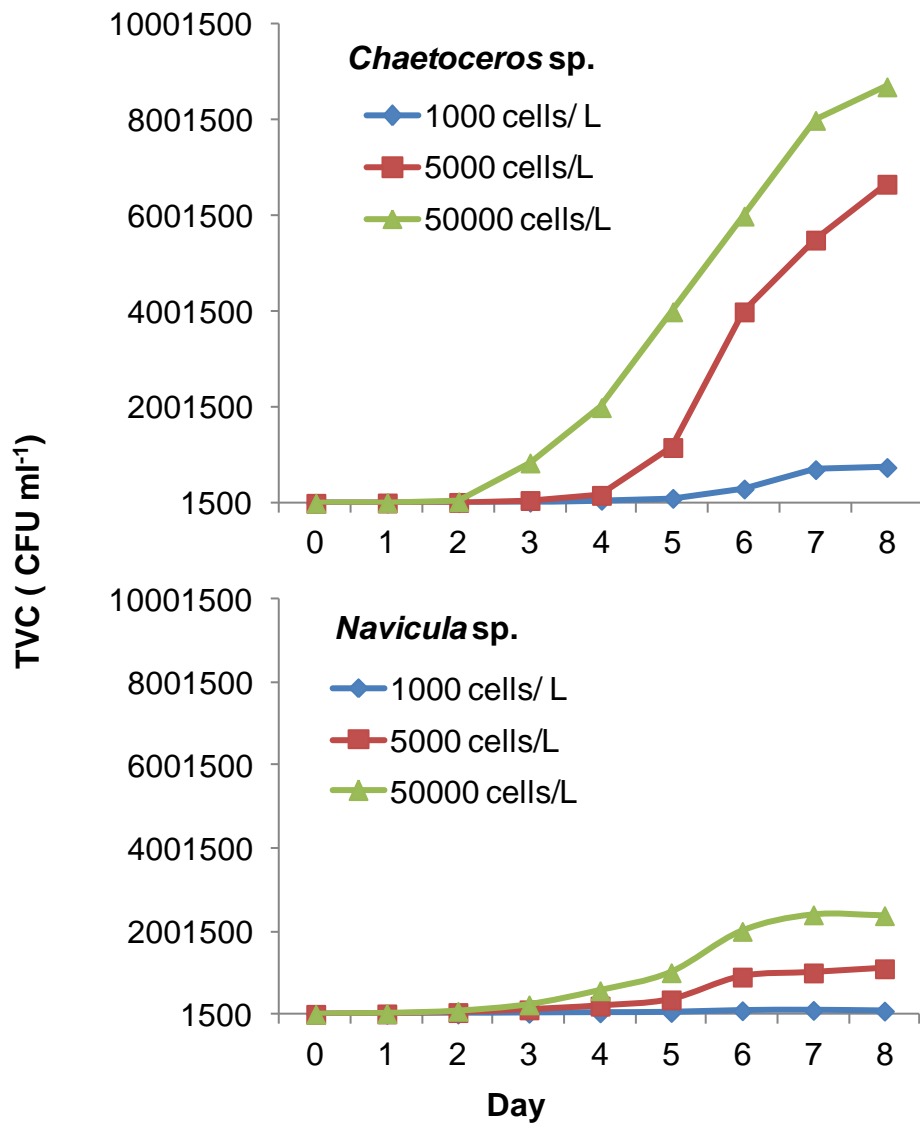


Fig 7.

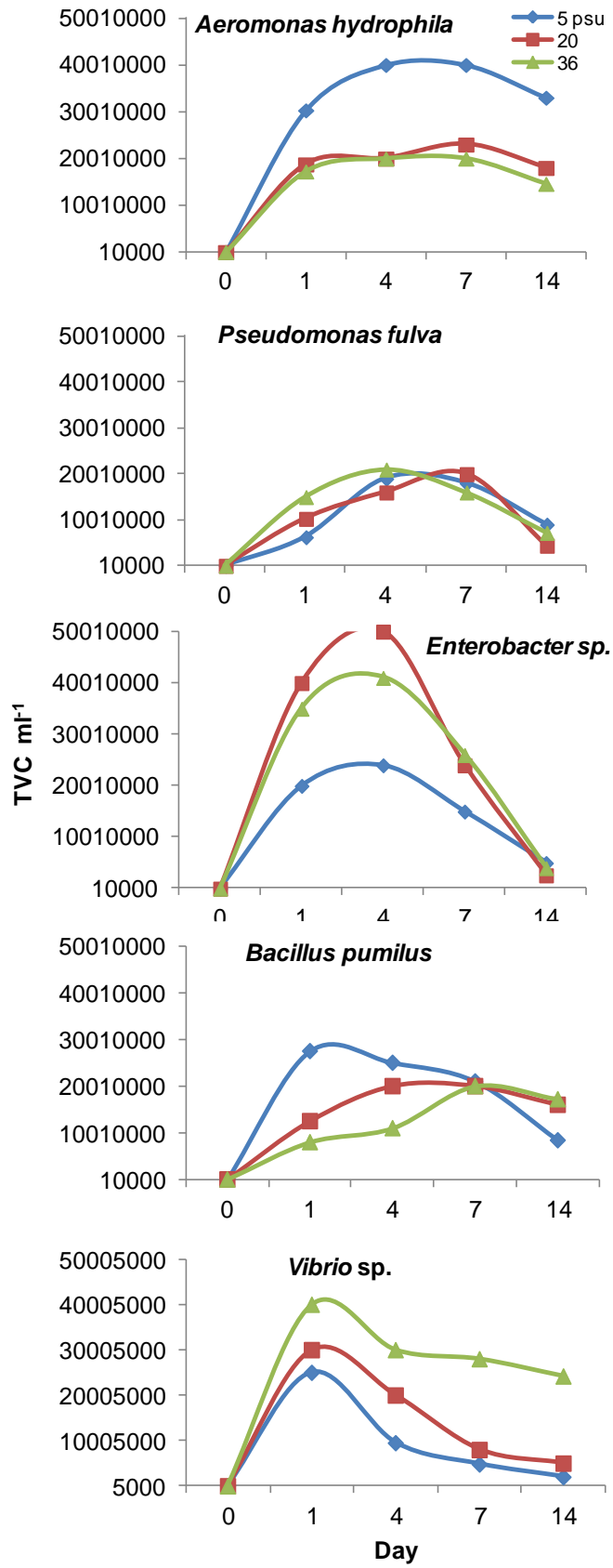


Fig 8.

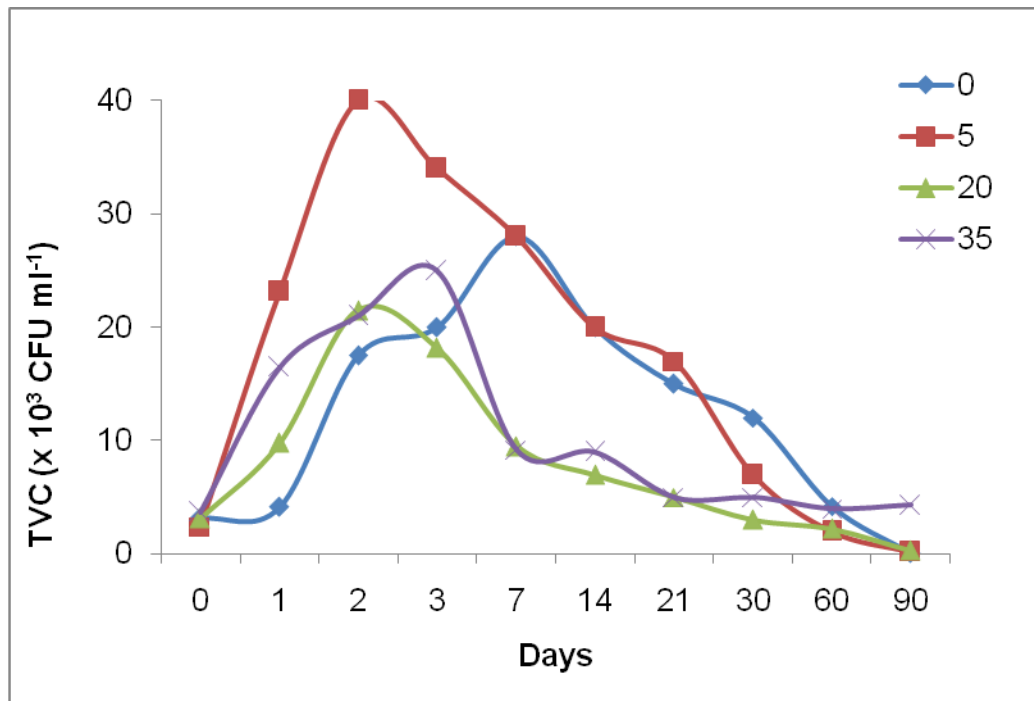


Fig 9.

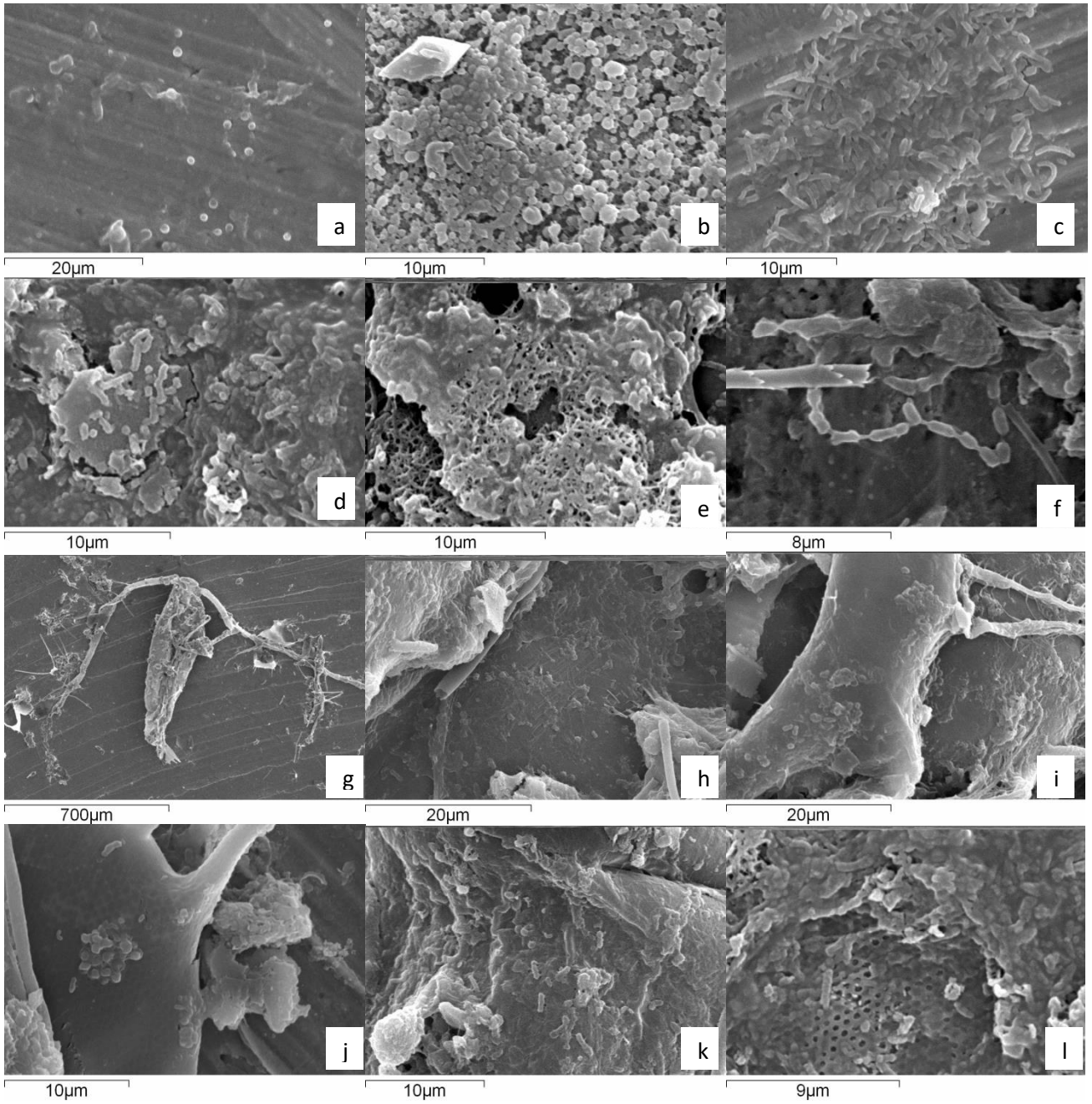


Plate 1.

Table 1. Biochemical profiles of the dominant bacterial genera isolated from zooplankton and seawater

Biochemical tests	<i>Enterobacter</i>	<i>Klebsiella</i>	<i>Vibrio/Aeromonas</i>	<i>Pseudomonas</i>	<i>Bacillus</i>
Gram character	-	-	-	-	+
Motility	+	-	+	+	+
Catalase	+	+	+	+	+
Oxidase	-	-	+	+	+
Indole	-	-/+	+	-	-
Growth at 37 °C	+	+	+	+	+
Citrate utilization	+	+	+	+	-
Lysine	+/-	+/-	+/-	-	-
Ornithine	+	-	+/-	-	-
Urease	-	+	-	-	-
Phenylalanine deaminase	-	-	-	-	-
Nitrate reduction	+	+	+	+	+
H ₂ S production	-	-	-	-	-
Glucose	+	+	+	-	-
Adonitol	-	+	-	-	-
Lactose	+	+	+	-	-
Arabinose	+	+	-	-	+
Sorbitol	+	+	-	-	+

- Negative

+ Positive

Table 2. Distribution of bacterial species associated with live and dead plankton, sediment and in water in the Zuari estuary

Sr. No	Isolate No	Accession No	Description	Accession No of closest match	Identity (%)
Live zooplankton-1) Amphipod surface					
1	Z149	KF835807	<i>Aeromonas punctata</i> , 89a	FJ168769.1	99
2	Z17	KF835729	<i>Averyella dalhousiensis</i> , VTan-2	DQ481464.1	95
3	Z15r	KF835728	<i>Bacillus clausii</i> , 1-8AIAab	FN397450.1	99
4	Z18	KF835731	<i>Bacillus safensis</i> , MBCU6	KC907390.1	99
5	Z18a	KF835732	<i>Enterobacter cloacae</i> , LSRC11	JF772071.1	99
6	Z9s	KF429497	<i>Enterobacter ludwigii</i> , NRCG12	HG003646.1	99
7	Z19	KF835733	<i>Klebsiella oxytoca</i> , NBA-1	KC336083.1	99
8	Z14r	KF835727	<i>Pseudomonas aeruginosa</i> , GVS1	KC893551.1	100
9	Z97	KF835773	<i>Pseudomonas pseudoalcaligenes</i> , PPS	KC660140.1	94
Amphipod gut					
10	Z144	KF835805	<i>Aeromonas aquariorum</i> , S5-18	KC202271.1	99
11	Z10r	KF429498	<i>Citrobacter werkmanii</i> , BF-6	KC489166.1	99
12	Z145	KF835806	<i>Cobetia marina strain</i> , S263-N5	JX429796.1	100
13	Z9	KF429496	<i>Enterobacter cloacae</i> , LSRC11	JF772071.	99
14	Z186r	KF835828	<i>Enterococcus raffinosus</i> , BWKUA11	KC707580.1	99
15	Z13	KF835726	<i>Klebsiella sp.</i> , XC-08	KC787534.1	99
16	Z12r	KF835725	<i>Pseudomonas fulva</i> , AR_PSTS1	HM582882.1	100
17	Z16r	KF835730	<i>Pseudomonas fulva</i> , Pful-1	JQ614019.1	99
18	Z8	KF429494	Uncultured bacterium, 4.5	EF179816.1	95
2) Acrocalanus surface					
19	Z124	KF835799	<i>Aeromonas caviae</i> , JXZ-3	JF496552.1	100
20	Z123	KF835798	<i>Aeromonas hydrophila</i> , 62	AB472902.1	98
21	Z122	KF835797	<i>Aeromonas sp.</i> , AGR/IICT/3	JQ791253.1	100
22	Z173	KF835817	<i>Averyella dalhousiensis</i> , VTan-2	DQ481464.1	97
23	Z34	KF835742	<i>Enterobacter aerogenes</i> , FCC47	JF772092.1	99
24	Z32	KF835740	<i>Enterobacter cancerogenus</i> , KNUC5010	JQ682635.1	98
25	Z33	KF835741	<i>Enterobacter soli</i> , KNUC5006	JQ682636.1	99
26	Z121	KF835796	<i>Enterobacter sp.</i> , 3EAG2	GQ383912.1	99
27	Z35	KF835743	<i>Pseudomonas fulva</i> 12-X	CP002727.1 NR_074659.	99
28	Z36	KF835744	<i>Pseudomonas fulva</i> 12-X, 12-X	1	94
29	Z170	KF835816	<i>Pseudomonas pseudoalcaligenes</i> , PPS	KC660140.1	99
30	Z31r	KF835739	<i>Pseudomonas stutzeri</i> , 1	KF171338.1	100
Acrocalanus gut					
31	Z125	KF835800	<i>Aeromonas aquariorum</i> , S5-18	KC202271.1	99
32	Z129	KF835804	<i>Aeromonas caviae</i> , NCIMB 13016	X60408.2	100
33	Z128	KF835803	<i>Aeromonas caviae</i> , P055	KC252796.1	100

34	Z127	KF835802	<i>Aeromonas hydrophila</i> , B66	JQ040122.1	100
35	Z118	KF835793	<i>Aeromonas hydrophila</i> , KAE23	AB473043.1	95
36	Zp6	KF835843	<i>Aeromonas hydrophila</i> , WA24	KC549805.1	95
37	Z126	KF835801	<i>Aeromonas punctata</i> , tu1	FJ544390.1	99
38	Z5	KF429489 KF42948	<i>Enterobacter cancerogenus</i> , MSA2	HM131221.1	99
39	Z2s	7	<i>Enterobacter cloacae</i> , LSRC11	JF772071.1	99
40	Z3s	KF429488	<i>Enterobacter cloacae</i> , LSRC11	JF772071.1	99
41	Z71a	KF835757	<i>Enterobacter cloacae</i> , LSRC11	JF772071.1	99
42	Z117	KF835792	<i>Klebsiella sp.</i> , AN-4	EU888474.1	95
43	Z71	KF835756	<i>Pseudomonas sp.</i> , 17.4 KSS	HE575917.1	94
44	Z115	KF835790	<i>Pseudomonas stutzeri</i> , TRA27A	JQ782508.1	95
45	Z72	KF835758	<i>Pseudomonas stutzeri</i> , GAPP4	GU396288.1	100
46	Z119	KF835794	<i>Psychrobacter celer</i> , KOPRI24932	EF101550.1	99
47	Z120r	KF835795	<i>Raoultella ornithinolytica</i> , NEHU.FNSRJ.120	JX390624.1	99
48	Z116	KF835791	<i>Shewanella decolorationis</i> , M30	KC593547.1	99
3) Paracalanus surface					
49	Z21	KF835734	<i>Enterobacter cancerogenus</i> , NBRC 105713	AB682281.1	99
50	Z7s	KF429493	<i>Enterobacter cloacae</i> , LSRC11	JF772071.1	99
51	Z106r	KF835782	<i>Enterobacter hormaechei</i> , T-39	HQ202859.1	100
52	Z104	KF835780	<i>Enterobacter ludwigii</i> , IRQBAS1	HG003646.1	99
53	Z105	KF835781	<i>Enterobacter ludwigii</i> , IRQBAS1	HG003646.1	99
54	Z103	KF835779	<i>Enterobacter sp.</i> , 3EAG2	GQ383912.1	99
55	Z22	KF835735	<i>Enterobacter turicensis</i> , LMG 23730	HQ992947.1	98
56	Z107	KF835783	<i>Pseudomonas pseudoalcaligenes</i> , PPS	KC660140.1	99
57	Z108	KF835784	<i>Pseudomonas pseudoalcaligenes</i> , PPS	KC660140.1	99
Paracalanus gut					
58	Z101	KF835777	<i>Aeromonas caviae</i> , W49	KF017367.1	100
59	Z99	KF835775 KF42949	<i>Aeromonas sp.</i> AGR/IICT/3	JQ791253.1	100
60	Z8s	5	<i>Enterobacter cloacae</i> , LSRC11	JF772071.1	99
61	Zp3	KF835840	<i>Enterobacter cloacae</i> , SN19	JQ904624.1	99
62	Z100r	KF835776	<i>Enterobacter hormaechei</i> , T-39	HQ202859.1	99
63	Z25r	KF835738	<i>Enterobacter hormaechei</i> , p9_B03	JQ831744.1	99
64	Z98	KF835774	<i>Enterobacter ludwigii</i> , NRCG12	HG003646.1	99
65	Z65	KF835753	<i>Proteus penneri</i> , Z70	KC212061.1	99
66	Z102	KF835778	<i>Pseudomonas stutzeri</i> , GAPP4	GU396288.1	99
Carcasses-1) Copepod surface					
67	Z68	KF835754	<i>Pseudomonas pseudoalcaligenes</i> , PPS	KC660140.1	99
Copepod gut					

68	Z110	KF835786 KF42949	<i>Acinetobacter sp.</i> CIP 101934	JQ638573.1	99
69	Z5s	0	<i>Enterobacter cloacae</i> , LSRC11	JF772071.1	99
70	Z70	KF835755	<i>Enterobacter cloacae</i> , LSRC11	JF772071.1	99
71	Z111r	KF835787	<i>Enterobacter hormaechei</i> , T-39	HQ202859.1	100
72	Z112	KF835788	<i>Enterobacter ludwigii</i> , IRQBAS1	HG003646.1	99
73	Z109	KF835785	<i>Pseudomonas pseudoalcaligenes</i> , PPS	KC660140.1	99
74	Z113	KF835789	<i>Pseudomonas putida</i> , S16	DQ387441.1	100
2) Chaetognath surface					
75	Z176	KF835818	<i>Aeromonas taiwanensis</i> , A2-50	FJ230077.1	95
76	Z178	KF835820	<i>Bacillus pumilus</i> , TKLW-C5-1	HQ218993.1	100
77	Z179	KF835822	<i>Bacillus pumilus</i> , TKLW-C5-1	HQ218993.1	98
78	Z185r	KF835827	<i>Enterobacter aerogenes</i> , RB22	KC431800.1	99
79	Z177	KF835819	<i>Enterobacter cloacae</i> , LSRC11	JF772071.1	99
80	Z178a	KF835821	<i>Enterobacter cloacae</i> , LSRC11	JF772071.1	99
81	Z182	KF835825	<i>Enterobacter ludwigii</i> , IRQBAS1	HG003646.1	99
82	Z180	KF835823	<i>Enterobacter ludwigii</i> , IRQBAS1	HG003646.1	99
83	Z10s	KF429499	<i>Enterobacter ludwigii</i> , NRCG12	HG003646.1	95
84	Z184r	KF835826	<i>Klebsiella sp.</i> , BEC441	Y17664.1	99
85	Z181r	KF835824	<i>Pseudomonas putida</i> , W30	HG416957.1	99
Chaetognath gut					
86	Z11s	KF835724	<i>Bacillus safensis</i> , ACL32	JX042470.1	99
87	Z7	KF429492	<i>Pseudomonas mendocina</i> , SM5	JX102498.1	97
3) Euphausid surface					
88	Z85	KF835764	<i>Enterobacter cloacae</i> , LSRC11	JF772071.1	99
89	Z87	KF835766	<i>Enterobacter cloacae</i> , LSRC11	JF772071.1	99
90	Z84	KF835763	<i>Enterobacter soli</i> , KNUC5006	JQ682636.1	99
91	Z47	KF835747	<i>Pseudomonas fulva</i> 12-X	CP002727.1	94
92	Z86	KF835765	<i>Pseudomonas sp.</i> , S14(2013)	KC898255.1	100
93	Z48a	KF835749	<i>Shewanella basaltis</i> , VSG818	KC534403.1	97
94	Z48r	KF835748	<i>Shewanella hafniensis</i> , P010	NR_041296.1	98
Euphausid gut					
95	Z90	KF835768	<i>Enterobacter aerogenes</i> , HK 20-1	AY335554.1	93
96	Z49	KF835750	<i>Enterobacter cloacae strain</i> LSRC11	JF772071.1	99
97	Zp4	KF835841	<i>Enterobacter cloacae</i> , SN19	JQ904624.1	99
98	Z50	KF835751	<i>Pseudomonas pseudoalcaligenes</i> PPS	KC660140.1	99
99	Z89r	KF835767	<i>Pseudomonas stutzeri</i> , SW229	KC178913.1	100
Fecal pellet					
100	Z187	KF835829	<i>Bacillus pumilus</i> , 2-1	EU594552.1	98

101	Zp1	KF835839	<i>Bacillus subtilis</i> , SK25.001	KC733848.1	99
102	Z93	KF835769 KF42949	<i>Enterobacter aerogenes</i> , HK 20-1	AY335554.1	93
103	Z6s	1	<i>Enterobacter cloacae</i> , LSRC11	JF772071.1	99
104	Zp5r	KF835842	<i>Enterobacter hormaechei</i> strain T-39	HQ202859.1	99
105	Z94f	KF835770	<i>Ps. pseudoalcaligenes</i> , HPCAQSB18	KC855047.1	99
Sediment					
106	Z154	KF835808	<i>Aeromonas aquariorum</i> , S5-18	KC202271.1	99
107	Z62a	KF835752	<i>Aeromonas punctata</i> , 12 H 339	AB473000.1	99
108	Z24	KF835737	<i>Enterobacter cancerogenus</i> , KNUC5010	JQ682635.1	99
109	Z96	KF835772	<i>Enterobacter helveticus</i> , BM0061	JQ680526.1	99
110	Z95	KF835771	<i>Enterobacter ludwigii</i> , NRCG12	HG003646.1	99
111	Z155	KF835809	<i>Oceanobacillus iheyensis</i> , MSU3110	AY647302.1	99
112	Z23	KF835736	<i>Pantoea agglomerans</i>	EU879089.1	99
Seawater					
113	Z158	KF835810	<i>Aeromonas caviae</i> , W49	KF017367.1	100
114	Z159	KF835811	<i>Aeromonas caviae</i> , W49	KF017367.1	96
115	Z166	KF835813	<i>Aeromonas caviae</i> , W49	KF017367.1	96
116	Z164r	KF835815	<i>Bacillus clausii</i> , 2P04SA 1	EU977787.1	99
117	Z163	KF835812	<i>Bacillus clausii</i> , BG-B15	EU869250.1	99
118	Z75	KF835760	<i>Enterobacter cloacae</i> , MR1	KC999878.1	99
119	Z204	KF835838	<i>Enterobacter hormaechei</i>	AY995561.1	100
120	Z189	KF835830	<i>Enterobacter ludwigii</i> , IRQBAS1	HG003646.1	99
121	Z190	KF835831	<i>Enterobacter ludwigii</i> , IRQBAS1	HG003646.1	99
122	Z194	KF835834	<i>Enterobacter ludwigii</i> , IRQBAS1	HG003646.1	99
123	Z195	KF835835	<i>Enterobacter ludwigii</i> , IRQBAS1	HG003646.1	99
124	Z198	KF835836	<i>Enterobacter ludwigii</i> , IRQBAS1	HG003646.1	99
125	Z199	KF835837	<i>Enterobacter ludwigii</i> , IRQBAS1	HG003646.1	99
126	Z1s	KF417712	<i>Enterobacter ludwigii</i> , NRCG12	HG003646.1	99
127	Z193r	KF835833	<i>Pantoea gaviniae</i> , A18/07	GQ367483.1	99
128	Z46	KF835746	<i>Photobacterium damsela</i> , NBRC 15633	AB680919.1	93
129	Z42	KF835745	<i>Photobacterium damsela</i> , NBRC 15633	AB680919.1 NR_074659.	94
130	Z73r	KF835759	<i>Pseudomonas fulva</i> , 12-X, 12-X	1	100
131	Z167	KF835814	<i>Pseudomonas pseudoalcaligenes</i> , PPS	KC660140.1	99
132	Z79		<i>Pseudomonas sp.</i>	JX193073.2	94

KF835761

133	Z83r	KF835762	<i>Pseudomonas stutzeri</i> , JD4	HF571096.1	99
134	Z191r	KF835832	<i>Rothia amarae</i> , 0410ARD15N_5	FR848426.1	99
135	Zp7	KF835844	<i>Vibrio sp.</i> , Persian 1	KC505639.2	100

Table 3. Species diversity indices of bacteria associated with live and dead plankton, fecal pellet, sediment and from water

	<i>S</i>	<i>d</i>	<i>J'</i>	<i>H'</i>
<u>Live Zooplankton</u>				
amphipod_S ^{\$}	9	3.64	1.00	2.20
amphipod_G [#]	7	3.08	1.00	1.95
Acrocalanus_S	11	4.02	0.99	2.37
Acrocalanus_G	13	4.08	0.96	2.45
Paracalanus_S	7	2.73	0.97	1.89
Paracalanus_G	7	2.73	0.97	1.89
<u>Carcasses</u>				
Copepod_S	3	1.82	1.00	1.10
Copepod_G	4	1.86	0.96	1.33
Chaetognath_S	4	1.44	0.95	1.32
Chaetognath_G	4	2.16	1.00	1.39
Euphausid_S	1	0.00		0.00
Euphausiid_G	9	3.47	0.98	2.16
<u>Sediment</u>	8	3.37	1.00	2.08
<u>Fecal pellet</u>	6	2.79	1.00	1.79
<u>Seawater</u>	14	4.09	0.89	2.36

\$ Surface, # Gut

Table 4. Antibiotic susceptibility tests of some of the isolates from water (W) and from zooplankton (Z). Depending on the zones of growth inhibition of the test bacteria, they are called as resistant (R), inhibited (I) or susceptible (S) and the percentage (%) of the 8 isolates susceptible to the particular antibiotic is given. MAR= Multiple antibiotic resistance and ARI= antibiotic resistance index. “-“ denotes no zone of inhibition.

Antibiotic used (concentration ug)	Class of antibiotic	Zone of growth inhibition (mm)								std zone of inhibition (mm)			
		W1	W2	W4	W6	S4	S6	S9	S11	R	I	S	% R
Amikacin (30)	Aminoglycosides	22	18	20	20	18	16	18	18	14	15-16	17	0
Gentamicin (10)	Aminoglycosides	20	14	20	-	18	16	16	16	12	13-14	15	13
Tobramycin (10)	Aminoglycosides	20	15	20	18	16	18	14	18	<12	13-15	16	0
Augmentin (30)	Aminopenicillins	-	-	-	-	-	-	-	-	<13	14-17	18	100
Aztreonam (30)	Carbapenems	17	12	12	20	18	16	12	14	17	18-20	21	75
Imipenem (10)	Carbapenems	-	-	-	-	9	10	8	8	13	14-15	16	100
Cefoxitin (30)	Cephalosporins	-	10	10	-	22	20	10	20	14	15-17	18	63
Ceftriaxone (30)	Cephalosporins	32	28	30	28	36	24	20	20	19	20-22	23	0
Cefpodoxime (10)	Cephalosporins	14	-	10	18	16	12	12	14	17	18-20	21	88
Ceftazidime (30)	Cephalosporins	16	-	14	-	16	12	10	16	17	18-20	21	100
Nalidixic acid (30)	Fluoroquinolones	20	-	-	-	18	20	14	18	<13	14-18	19	38
Ciprofloxacin (10)	Fluoroquinolones	30	20	30	26	22	22	18	24	15	16-20	21	0
Ofloxacin (5)	Fluoroquinolones	30	20	24	30	24	18	18	24	<12	13-15	16	0
Levofloxacin (5)	Fluoroquinolones	26	22	24	28	26	20	16	22	13	14-16	17	0
Norfloxacin (10)	Fluoroquinolones	-	20	20	28	16	18	20	24	<12	13-16	17	13
Gatifloxacin (5)	Fluoroquinolones	22	20	26	26	20	20	16	22	15	15-18	19	0
Moxifloxacin (5)	Fluoroquinolones	28	18	22	28	22	24	22	18	<20	21-23	>24	25
Nitrofurantoin(300)	Nitrofurans	14	15	14	14	9	8	12	14	14	15-17	17	88
Colistin (10)	Polypeptides	16	13	16	14	14	14	10	14	<11		>14	13
Co-Trimoxazole(25)	Sulphonamides	-	-	20	22	10	10	18	20	15		16	50
MAR		0.4			0.3		0.3		0.3				
		5	0.5	0.4	5	0.3	5	0.4	5				
ARI		0.0											
		5											