Bacterioplankton diversity and pollution levels in the estuarine regions of Chaliyar and Anjarakkandi rivers, Kerala, India

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Short title: Bacterioplankton diversity comparison in estuarine areas

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24   **Abstract**

25   Rivers and the estuarine regions play crucial roles in the environment by controlling the
 26   nutrient cycling, waste disposal, and also in supporting biodiversity. However, the estuarine
 27   regions of rivers are highly susceptible to disruptive changes by anthropogenic pollutants and
 28   eutrophication. Bacterioplankton diversity is the best indicators of the pollution level and
 29   eutrophication status of the rivers. The present study evaluated the eutrophication status and
 30   bacterioplankton diversity of the estuarine regions of Chaliyar and the Anjarakkandi
 31   originated from the Western Ghats. The present study observed the presence of increased
 32   incidence of bacterioplankton comprising of proteobacteria, actinobacteria and cyanobacteria
 33   in the estuarine regions of Anjarakkandi river compared to that of Chaliyar river. Further, the
 34   percentage incidence of faecal bacteria including *Bacillus subtilis* and *E. coli*, were also
 35   found to be in high in the Anjarakkandi river; in corroborating with this, the diversity of
 36   nitrogen metabolizing bacteria was also higher in the estuarine samples of Anjarakkandi
 37   river. Corroborating with this, the levels of water nutrients including nitrates, nitrites and
 38   phosphates were high in the Anjarakkandi compared to the Chaliyar river. Together, it is
 39   possible that there might be increased faecal contamination and subsequent increased
 40   eutrophication in the estuarine regions of Anjarakkandi river. Altogether, the study concludes
 41   that the Anjarakkandi river may be more polluted and which is partly contributed by faecal
 42   contaminants and also responsible for the change in the bacterioplankton community of the
 43   estuaries.

44   **Keywords:** Bacterioplankton; Water quality; Diversity; Estuary; Chaliyar river;
 45   Anjarakkandi river

46

47 **1. Introduction**

48 Indian sub-continent has a wide network of aquatic systems including small streams, rivers,
 49 lakes, marine waters and estuaries (Khadse et al. 2012; Khadse et al. 2010). Among these,
 50 several important rivers are being originated from the Western Ghats, which are important
 51 water resources in southern India (Ramachandra et al. 2018). However, the pollution levels of
 52 these south Indian rivers are increasing every year and which is most affected in their estuarine
 53 regions (Gopal et al. 2018). Estuaries are one among the most urbanised ecosystem and
 54 greatly facing environmental pressure by anthropogenic activities includes habitat loss, algal
 55 blooms, anoxia, and contamination by sewage, heavy metals and other organic and inorganic
 56 pollutants (Bashir et al. 2020). They are also considered as the highly productive and diverse
 57 ecosystem, and possess unique microbial stress indicators which are vulnerable to
 58 anthropogenic contaminants (Duarte et al. 2017; Julian and Osborne 2018). Darmadam and
 59 Chaliyum are estuarine regions of Anjarakkandy and Chaliyar river respectively which are
 60 flowing through both rural and urban banks constitute anthropogenic contamination.

61 In usual scenario shows a ubiquitous distribution of bacterioplankton around the globe
 62 because of their small size, great abundance and easy dispersal. But some marine
 63 bacterioplankton possess geographical distribution pattern like macroscopic animals possess
 64 (Liu et al. 2015). Evaluating the distribution pattern of both particle associated and free living
 65 bacterioplankton are of higher significance, as the functioning of these bacteria remain key
 66 determinants of nutrient concentrations and fluxes by fixing carbon and nitrogen, assimilating
 67 and remineralizing dissolved and particulate organic matter in the ecosystem (Gómez-
 68 Consarnau et al. 2019). Hence, the diversity of bacterioplankton present in the aquatic
 69 environment can be an indicative of the quality of water and level of pollution in the water-
 70 body (Salloto et al. 2012; Zhao et al. 2020). Several methods are being utilized for the

71 analysis of bacterioplankton; among these the predominant are culture independent methods
72 (Li et al. 2019).

73 In recent years, microbial ecologists have effective ways to characterise microbial
74 communities have been created. Numerous studies have indicated taxonomic variability in
75 both time and space in the genetic diversity of bacterioplankton communities. However,
76 because this data is focused largely on 16S rRNA gene sequences, the physiological
77 characteristics and ecological functions of the genotypes identified remain largely unknown.
78 The use of high performance sequencing technologies has made way for the study of the
79 microbial group structure using amplicon sequencing and metagenomics. Hence, the present
80 evaluated the bacterioplankton diversity in the estuarine regions of two selected rivers of
81 Kerala employing high throughput pyro-sequencing techniques of 16s rRNA gene for culture
82 independent bacterioplankton.

83 **2. Materials and Methods**

84 ***2.1 Study area and Sampling***

85 Chaliyom and Darmadam are Estuarine regions of Chaliyar and Anjarakkandi river
86 respectively. Water samples were obtained from three stations of each site. Samples (post
87 monsoon) were collected from 0.5m below the air -water interface using depth sampler. The
88 collected samples were stored in a dark container and kept in 4°C immediately after the
89 collection. Physico chemical parameters like Temperature (T), pH, Salinity, Total Dissolved
90 Solids(TDS), Conductivity were measured using water analyser(EUTECH PC 450) and
91 Dissolved oxygen(DO), Biochemical oxygen demand(BOD), Chemical oxygen demand(
92 COD), of samples of each stations were measured using standard methods(APHA 2005).

93 The samples from there stations were pooled and filtered through 3.0-µm-pore-size and 0.2
94 µm-pore-size membrane filters to collect particle-associated (PA) and free-living (FL)

bacterioplankton, respectively and stored in - 20°C deep freezer after filtration. The remaining sample of water from each station of two sites were used for nutrient analysis. The filters were subjected to DNA extraction

2.2 Nutrient Analysis

Both organic and inorganic nutrients were measured by following the standard methods for the examination of water and waste water. Total hardness, Calcium, Magnesium were measured by EDTA titration method. Chloride (Argentometric method), Iron (Phenanthroline method), Nitrate (Phenol- disulphonic acid method), Sulphate (Turbidity method), Ammonium (Nessler's method), Phosphate (Stannous chloride method), Particulate organic carbon (POC) and Dissolved organic carbon (DOC) were found using Ash method (APHA 2005).

2.3 DNA Extraction and PCR amplification

DNA isolation from frozen filter were done using the EXpure Microbial DNA isolation kit (Bogart Bio Stores Pvt Ltd) by following manufacturer's instructions. We used lysis buffer to homogenise the cell. After the extraction DNA concentrations were measured by Qubit 3.0 and the DNA samples were stored at -20°C until further use.

16S rRNA genes were PCR amplified from extracted DNAs using primers that were designed specifically for 454 high throughput pyrosequencing. The forward primer was 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and the reverse primer was 1492R (5'-AAGGAGGTGATCCAGCCGCA-3'). Composition of the Taq Master Mix consists of Taq DNA polymerase supplied in 2X Taq buffer, 0.4mM dNTPs, 3.2mM MgCl₂ and 0.02% bromophenol blue. A 25 µL reaction solution contains 5 µL of isolated DNA, 1.5 µL of Forward Primer and Reverse Primer, 5 µL of deionized water, and 12 µL of Taq Master Mix

This PCR amplification consist of 25 cycles with initial 2 minutes denaturation(95°C) and final 10 minute extension (72°C). Each cycle had Denaturation (95°C 30 sec) Annealing (60°C 30 sec) Extension(72°C2min). Unincorporated PCR primers and dNTPs from PCR products removed by using Montage PCR Clean up kit. The quality, quantity (App.100ng/μl) and formulation of the PCR product was checked using Qubit Fluorometer 3.0.

2.4 Sequencing annotation and library coverage calculation

The pyrotag sequences obtained for each of the sample were sorted and low quality sequences were removed as per the previously described protocols. Further processing and taxonomic identification upto species level was done using BLASTn program based on the 16S rRNA sequence similarity as per the protocols described by Mou et al. (2013).

2.5 Statistical analysis

The values were expressed as mean± SD of eight different sampling sites from both the estuarine regions. The statistical analysis used paired t-test for comparison of nutrient quality of water samples from both the stations and a variation of $p < 0.05$ is considered statistically significant.

3. Results

3.1 Nutrient composition of the estuaries

Changes in the various physical and chemical parameter along with the biological aspects of water quality from the estuarine regions of Anjarakkandi and Chaliyar river are listed in the Table 1. As shown in the table, there was limited variation in the temperature of water present in both the estuarine regions; however, the Anjarakkandi river had significantly reduced levels of dissolved oxygen content. Concomitantly, there observed an increased level of

nitrate, ammonium, phosphate and sulphate. Further, there observed significantly elevated BOD and COD values in these samples (Table 1).

3.2 Bacterioplankton diversity of the estuaries

The diversity of bacterioplankton in the samples is given in table 2 and 3. The kingdom level analysis indicated the presence of both particle-associated and free living bacterioplanktons. In the estuarine regions of Anjarakkandi river the presence of bacterial diversity was significantly higher than those present in the estuarine regions of Chaliyar river (Table 1). Further, the phylum level analysis indicated the diversity of proteobacteria (67.07%) and firmicutes (17.24%) in the estuaries of Anjarakkandi river. On contrary, the diversity of proteobacteria and firmicutes in the Chaliyar river range was 46.35 and 31.51%, which was significantly lower than that of the Anjarakkandi river. In the species level identification, the faecal bacteria *Bacillus subtilis* was much higher in the Anjarakkandi river estuaries with 16.19%; the same in Chaliyar river was 10.07% (Supplementary material 1).

Discussion

Coastal waters, especially the estuarine regions are at a higher threat of eutrophication by various agricultural and other anthropogenic activities (Dai et al. 2017). Further, the report has also indicated that the bacterioplankton community structure is also changes in relation with the eutrophication status of the water body. The present study observed significantly higher number of proteobacteria, Actinobacteria, and Cyanobacteria in the estuarine waters of Anjarakkandi river, Dharmadam. Previous studies have indicated that the bacterioplankton belonging to the proteobacteria, Actinobacteria, and Cyanobacteria predominates in the eutrophic waters compared to other bacterial groups (Kiersztyn et al. 2019). The increased diversity of these bacteria correlates with the increased level of nitrogenous compounds present in the Anjarakkandi river and possibly an indicative of the eutrophication status of the

estuary. Further, there observed significant elevation in the firmicutes diversity; these bacteria are present in the gut of humans and often indicates the faecal contamination in the water (Paruch et al. 2019a; Paruch et al. 2019b). Apart from that, there observed the presence of various nitrogen metabolizing bacteria including the roseobacter, Nitratiruptor and sulfitobacter. The Roseobacter is one the predominant bacterioplankton which is involved in the degradation various organic matter (Pohlner et al. 2019). Likewise, nitratiruptor is another nitrite reducing bacteria, which is active in the eutrophic waters (Nakagawa et al. 2005). It is thus possible that the increased occurrence of these bacterioplanktons in the Anjarakkandi river possibly indicate increased faecal contamination, subsequent increase in nitrogenous compounds and subsequent eutrophication.

DECLARATIONS

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Conflicts of interest

The authors declare no conflict of interest in the present study.

Availability of data and material

The data will be made available on valid request.

Authors' contributions

Jisha Jacob was contributed to the design, analysis of result, manuscript draft preparation, and statistical analysis. Nikhitha D conducted the experiments and carried out analysis and

prepared the initial draft. Arunaksharan Narayanankutty contributed to the analysis of data and final manuscript editing. All authors have approved submission to the journal.

Ethics approval

The study doesn't involve any animal/ human subjects.

Consent for publication

The study doesn't use any third party material.

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Reference

- Bashir I, Lone FA, Bhat RA, Mir SA, Dar ZA, Dar SA (2020) Concerns and Threats of Contamination on Aquatic Ecosystems Bioremediation and Biotechnology:1-26
doi:10.1007/978-3-030-35691-0_1
- Dai W, Zhang J, Tu Q, Deng Y, Qiu Q, Xiong J (2017) Bacterioplankton assembly and interspecies interaction indicating increasing coastal eutrophication Chemosphere 177:317-325
- Duarte IA, Reis-Santos P, França S, Cabral H, Fonseca VF (2017) Biomarker responses to environmental contamination in estuaries: A comparative multi-taxa approach Aquat Toxicol 189:31-41

205 Gómez-Consarnau L, Needham DM, Weber PK, Fuhrman JA, Mayali X (2019) Influence of
206 Light on Particulate Organic Matter Utilization by Attached and Free-Living Marine
207 Bacteria *Frontiers in Microbiology* 10 doi:10.3389/fmicb.2019.01204

208 Gopal V, Shanmugasundaram A, Nithya B, Magesh NS, Jayaprakash M (2018) Water quality
209 of the Uppanar estuary, Southern India: Implications on the level of dissolved
210 nutrients and trace elements *Mar Pollut Bull* 130:279-286

211 Julian P, 2nd, Osborne TZ (2018) From lake to estuary, the tale of two waters: a study of
212 aquatic continuum biogeochemistry *Environ Monit Assess* 190:017-6455

213 Khadse GK, Labhasetwar PK, Wate SR (2012) Water resource management: an Indian
214 perspective *J Environ Sci Eng* 54:577-591

215 Khadse GK, Talkhande AV, Andey SP, Kelkar PS (2010) Water resources development and
216 management: an experience in rural hilly area *J Environ Sci Eng* 52:67-74

217 Kiersztyn B, Chróst R, Kaliński T, Siuda W, Bukowska A, Kowalczyk G, Grabowska K
218 (2019) Structural and functional microbial diversity along a eutrophication gradient of
219 interconnected lakes undergoing anthropopressure *Scientific Reports* 9:11144
220 doi:10.1038/s41598-019-47577-8

221 Li A-Z, Han X-B, Zhang M-X, Zhou Y, Chen M, Yao Q, Zhu H-H (2019) Culture-
222 Dependent and -Independent Analyses Reveal the Diversity, Structure, and Assembly
223 Mechanism of Benthic Bacterial Community in the Ross Sea, Antarctica *Frontiers in*
224 *Microbiology* 10 doi:10.3389/fmicb.2019.02523

225 Liu L, Yang J, Yu Z, Wilkinson DM (2015) The biogeography of abundant and rare
226 bacterioplankton in the lakes and reservoirs of China *ISME J* 9:2068-2077
227 doi:10.1038/ismej.2015.29

228 Mou X, Jacob J, Lu X, Robbins S, Sun S, Ortiz JD (2013) Diversity and distribution of free-
229 living and particle-associated bacterioplankton in Sandusky Bay and adjacent waters

230 of Lake Erie Western Basin Journal of Great Lakes Research 39:352-357
231 doi:<https://doi.org/10.1016/j.jglr.2013.03.014>

232 Nakagawa S, Takai K, Inagaki F, Horikoshi K, Sako Y (2005) *Nitratiruptor tergarcus* gen.
233 nov., sp. nov. and *Nitratifactor salsuginis* gen. nov., sp. nov., nitrate-reducing
234 chemolithoautotrophs of the epsilon-Proteobacteria isolated from a deep-sea
235 hydrothermal system in the Mid-Okinawa Trough Int J Syst Evol Microbiol 55:925-
236 933

237 Paruch L, Paruch AM, Eiken HG, Sørheim R (2019a) Aquatic microbial diversity associated
238 with faecal pollution of Norwegian waterbodies characterized by 16S rRNA gene
239 amplicon deep sequencing Microbial Biotechnology 12:1487-1491
240 doi:<https://doi.org/10.1111/1751-7915.13461>

241 Paruch L, Paruch AM, Eiken HG, Sørheim R (2019b) Faecal pollution affects abundance and
242 diversity of aquatic microbial community in anthropo-zoogenically influenced lotic
243 ecosystems Scientific Reports 9:19469 doi:10.1038/s41598-019-56058-x

244 Pohlner M, Dlugosch L, Wemheuer B, Mills H, Engelen B, Reese BK (2019) The Majority of
245 Active Rhodobacteraceae in Marine Sediments Belong to Uncultured Genera: A
246 Molecular Approach to Link Their Distribution to Environmental Conditions
247 Frontiers in Microbiology 10 doi:10.3389/fmicb.2019.00659

248 Ramachandra TV, Vinay S, Bharath S, Shashishankar A (2018) Eco-Hydrological Footprint
249 of a River Basin in Western Ghats Yale J Biol Med 91:431-444

250 Salloto GRB et al. (2012) Pollution impacts on bacterioplankton diversity in a tropical urban
251 coastal lagoon system PloS one 7:e51175-e51175 doi:10.1371/journal.pone.0051175

252 Zhao W et al. (2020) Bacterioplankton community variation in Bohai Bay (China) is
253 explained by joint effects of environmental and spatial factors MicrobiologyOpen
254 9:e997 doi:<https://doi.org/10.1002/mbo3.997>

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258 **Figure Legends**

259 Figure 1. The site map of estuarine regions of Chaliyar river (a) and Anjarakkandi river (b).

260

Table 1. The nutrient composition of the estuarine regions of Chaliyar and Anjarakkandi river, Kerala, India.

Parameter	Chaliyar	Anjarakkandi
Temperature(°c)	31.0± 0.0	31.0± 0.00
pH	7.57 ± 0.02	7.72± 0.02*
Salinity (ppt)	10.60 ± 0.00	13.67± 0.06***
TDS (g/l)	9.94 ± 0.00	12.63± 0.06***
Conductivity (ms/cm)	13.95 ± 0.06	22.06± 0.07**
Total Hardness (mg/ml)	1548.33 ± 2.89	2150.33±0.058***
DO (mg/l)	6.35 ± 0.03	3.82± 0.04**
BOD (mg/l)	2.07 ± 0.01	8.87± 0.21**
COD (mg/l)	39.10 ± 0.17	77.83± 0.81***
Calcium (mg/l)	100.00 ± 0.00	158.33± 1.53*
Magnesium (mg/l)	320.80 ± 3.82	426.00± 1.73**
Chloride (mg/l)	5998.44 ± 0.49	8463.41± 0.13***
Iron (ppm)	1.42 ± 0.10	0.50± 0.00*
Nitrate (ppm)	0.29 ± 0.03	0.46 ± 0.00*
Ammonium (ppm)	0.31 ± 0.01	0.43± 0.00*
Sulfate (ppm)	139.62 ± 0.06	186.62± 0.04*
Phosphate(ppm)	0.03 ± 0.01	0.02± 0.00
POC (%)	0.21 ± 0.02	2.12± 0.00***
DOC (%)	0.14 ± 0.01	0.39± 0.01**

Table 2. Changes in the diversity of particle-associated and free living bacterioplankton in the estuarine regions of Anjarakkandi and Chaliyar river of Kerala, India

	Kingdom level			Phylum level		
	Bacteria	Archaea	Eukaryota	Proteobacteria	Firmicutes	Euryarchaeota
Anjarakkandi- PA	1768	217	157	1067	572	181
Anjarakkandi- FL	1027	78	77	778	200	71
Chaliyar- PA	744	84	65	492	186	73
Chaliyar- FL	321	61	21	178	121	13

PA- Particle-associated; FL- Free living

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Table 3 Percentage abundance of different bacterioplankton in both Anjarakkandi and Chaliyar river, Kerala, India.

		Anjarakkandi	Chaliyar
Phylum	Proteobacteria	67.07	46.35
	Firmicutes	17.24	31.51
	Euryarchaeota	6.12	10.68
	Chordata	3.45	3.39
Species	Marivivens sp. JLT3646	11.97	10.53
	Sulfitobacter sp. AM1-D1	11.97	3.2
	Bacillus subtilis	16.19	10.07

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