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Bacterioplankton diversity in the estuarine regions of two peninsular rivers: A metagenomic approach

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ABSTRACT

Objective: In this present study we evaluated the bacterioplankton diversity employing a culture independent high throughput 16srRNA pyro-sequencing technique in two selected estuarine regions that belongs to two peninsular rivers on which the people living in the area heavily depend for their water needs. In spite of several advantages to metagenome based bacterioplankton diversity analysis, no such studies have been carried out in the region so far.

Methods: Samples were collected from three different sites for each estuaries. A comparative study on physico-chemical analysis was done on water samples from the estuaries using standard analytical methods. High-throughput sequencing reads obtained from metagenomic sequencing were classified into a taxonomic category using the NCBI taxonomy reference database.

Result: In the study we observed higher percentage composition of bacterioplanktons appertaining to the actinobacteria, cyanobacteria, and proteobacteria groups which can be attributed to higher levels of water nutrients such as nitrates, nitrites and phosphates. Firmicutes identified in the samples indicates the faecal contamination in the water. Apart from these numerous nitrogen-metabolizing bacteria, such as Roseobacter, Nitratiruptor, and Sulfitobacter, were present, as observed. One of the most common bacterioplankton species that is responsible for the breakdown of different types of organic matter is the Roseobacter. Similarly, nitratiruptor is a type of bacteria that reduces nitrite and can break down nitrate in water. Sulfitobacter and Pseudoruegeria are other two strains observed in both the Chaliyam and Dharmadam estuarine regions. It is reported that the Sulfitobacter strains are predominantly distributed in the areas of oil polluted marine environments;it has been associated with hydrocarbon removal from seawater.

Conclusion: Most of the diverse bacterioplanktons reported are capable of utilizing the dissolved organic matter for their growth and functioning. Isolation of such bacterial communities is having great significance in the aspects of waste water treatment and pollution abatement. We did not observe any major difference in diversity of particle associated bacterioplanktons in the two estuaries investigated. However, there was a significant variation observed in the diversity of free living bacterioplankton attributed to changes in physio-chemical parameters. Overall, metagenomics is a more effective and insightful way to examine microbial populations than standard culture techniques.

1. Introduction

Diverse aquatic systems, comprising little streams, rivers, lakes, sea waters, and estuaries, are found throughout the Indian subcontinent. Among these, the Western Ghats, a significant supply of water for southern India, are the source of several significant rivers. (Khadse et al.,

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2010; Khadse et al., 2012; Ramachandra et al., 2018). As they regulate the nitrogen cycle and the disposal of trash, rivers and estuarine environments are essential to the maintenance of biodiversity. These rivers' estuarine regions are extremely productive, have a variety of habitats, and have distinct microbial populations.(Duarte et al., 2017; Julian and Osborne, 2018). Bacterioplanktons are free floating and play a significant part in functioning of global ecosystem through biogeochemical cycle, Primary producton and food web dynamics.These microbial communities are important in the regulation of particulate and dissolved organic carbon and thereby regulating the various mineral and nutrient cycling (Lønborg et al., 2019; Molina and Fernández, 2020, Mou et al., 2013).. Applications in industry and commerce include the targeted isolation of specific bacterial strains and the diversity study of both particle-associated and free-living bacterioplankton (Soares et al., 2018; Mou et al., 2015).

Analysing the distribution pattern of both free-living and particle-associated bacterioplankton is more important because the way these bacteria function continues to be a major factor in determining nutrient fluxes and concentrations because they fix carbon and nitrogen and assimilate and remineralize particulate and dissolved organic matter in the ecosystem. (Gómez-Consarnau et al., 2019; Mou et al., 2013). Consequently, the variety of bacterioplankton seen in aquatic environments can serve as an indication for the water's quality and degree of contamination in the body of water. (Salloto et al., 2012; Zhao et al., 2020). A variety of methods are used to analyse bacterioplankton, with culture-independent techniques being the most often used. (Li et al., 2019; Salloto et al., 2012, Robbins et al., 2011).

Microbial ecologists have identified more efficient techniques for analyzing microbial populations in recent times. The application of advanced sequencing technologies has enabled the examination of microbial group structures through amplicon sequencing and metagenomics. (Abia et al., 2018). The current study assessed the variety of bacterioplankton in the estuarine areas of two adjacent rivers, Chaliyar and Anjarakkandy, which originate in the Western Ghats of southern India, Kerala. Little was known about bacterioplankton diversity of the proposed water bodies, including those that are particle associated or free-living, despite their importance in maintaining the ecosystems. In order to analyse the bacterial makeup, the current work used high throughput pyro-sequencing techniques of the 16 s rRNA gene. To obtain a better understanding of how environmental circumstances affect the taxonomic composition and diversity of bacteria, a thorough physio-chemical examination of the water samples was conducted. It is crucial to study the diversity of bacterioplankton because it provides the answers to many questions about the health, resilience, and sustainability of our planet's essential water supplies as well as the intricate web of interactions that shape aquatic ecosystems.

2. Methods

2.1. Site and sampling

Water samples (Post monsoon) were collected from the esturine regions (Chaliyum and Darmadom) of Chaliyar and Anjarakandi River (Fig. 5). The sites were split up into three stations, and samples were collected from 0.5 m below the air–water interface using a depth sampler. As soon as the samples were collected, they were placed in a dark carboy and preserved at 4 °C. In the site Physico-chemical parameters like Temperature (T), pH, salinity, Total Dissolved Solids (TDS), Conductivity were measured (EUTECH PC 450). Using accepted techniques, the dissolved oxygen (DO), biochemical oxygen demand (BOD), and chemical oxygen demand (COD) of each station's samples were determined. (APHA 2005).

After the samples from three stations were combined, they were filtered via membrane filters with pore sizes of 3.0 μm and 0.2 μm to separate the particle-associated (PA) and free-living (FL) bacterioplankton, respectively. The filtered samples were then kept in a deep freezer at −20 °C. For the nutrient analysis, the residual water sample from each of the three stations was utilised. The filters were subjected to DNA extraction.

2.2. Nutrient analysis

Conventional methods have been employed to analyse water's organic and inorganic nutrients. Using the EDTA titration method, total hardness, calcium, and magnesium were assessed. Ash method was used to determine the particulate organic carbon (POC) and dissolved organic carbon (DOC). Stannous chloride method was used for phosphate, ammonium was determined by Nesseler's method, sulphate by Turbidity method, nitrate was done by Phenol-disulphonic acid method, iron by Phenanthroline method, finally Chloride by Argentometric method. (APHA 2005).

2.3. DNA extraction and PCR amplification

DNA isolation from frozen filter was done using the EXpure Microbial DNA isolation kit (BogarBio Bee stores Pvt Ltd) by following manufactures instructions. The cells grown as monolayer were lysed by suspending 1–3 colonies in0.45 mL of lysis buffer and subsequent pipetting; RNAase and 0.25 mL of neutralization buffer were and incubated at 65 °C for 30 min.The tubes were centrifuged for 20 min (12,000 g)in a refrigerated centrifuge. The viscous supernatant was collected and 0.6 mL of binding buffer was added and again incubated at 25 °C (5 min). The contents were transferred in to a spin column and centrifuged at 12,000 g (2 min). The bound DNA material was washed using washing buffer II and the DNA was collected by elution buffer.

DNA with 16S rRNA genes were obtained via the DNA extraction procedure, and these were then amplified by PCR using primers designed especially for 454 high-throughput pyrosequencing. 27F (5′AGAGTTTGATCMTGGCTCAG3′) was the forward primer, while 1492R (5′AAGGAGGTGATCCAGCCGCA3′) was the reverse primer.. The Taq Master Mix is made up of 0.02 % bromophenol blue, 3.2 mM MgCl2, 0.4 mM dNTPs, and TaqDNA polymerase in 2X Taq buffer. A 25 μL reaction solution is made up of 12 μL of Taq Master Mix, 5 μL of deionized water, 1.5 μL of forward and reverse primers, and 5 μL of extracted DNA..The 25 cycles of this PCR amplification include a 2-minute denaturation step at 95 °C and a 10-minute extension step at 72 °C. Denaturation (95 °C) and Annealing (60 °C) and Extension (72 °C) were the three steps in each cycle. Each cycle had Denaturation (95 °C, 30 s) Annealing (60 °C, 30 s) Extension (72 °C 2 min) (Kikani et al., 2017). The Montage PCR Clean-Up kit was employed to eliminate unincorporated PCR primers and dNTPs from the PCR products. Subsequently, the PCR product was assessed for quality and quantity, yielding an approximate concentration of 100 ng/μl, and its formulation was verified using the Qubit Fluorometer 3.0.

2.4. Sequencing and bioinformatics analysis

The sequencing process was carried out utilising a 1 μg DNA template. AMPure XP bead binding was used to purify the sequenced sample. For priming and loading, an Oxford Nanopore SpotON flow cell was utilised.

2.5. Sequencing annotation and library coverage calculation

The EPI2ME 16 s analysis workflow empowers users to attain genus-level identification from individual reads, providing access to base-called files for thorough examinations at both the species and sub-species levels. After obtaining blast results, a phylogenetic analysis was conducted, comparing the query sequence with closely related sequences, followed by multiple sequence alignment. The process's goal is to match base call sequences with the NCBI 16 s bacterial database, which include 16 s sequences belonging to various species. Based on

Table 1
The nutrient composition of the estuarine regions of Chaliyar and Anjarakkandiriver, 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**

identification and percentage coverage, each read is categorised. The 16S process is useful for identifying pathogens in a mixture sample or learning more about the makeup of a microbial community.

2.5.1. Sequence analysis

The raw sequence of Fastq format was uploaded to Kaiju webserver. Kaiju is a software designed for precise taxonomic classification of high-throughput sequencing reads originating from metagenomic whole-genome sequencing or metatranscriptomics experiments. It accomplishes this by matching each sequencing read to a taxonomic category within the NCBI taxonomy through comparisons with a reference database that includes microbial and viral protein sequences. Krona chart was prepared for further analysis.

2.6. Statistical analysis

The data were presented as the mean ± standard deviation from three distinct sample locations in each estuary zones. When comparing the nutritional quality of water samples from both locations, the statistical analysis employed a paired *t*-test, where a difference of $p < 0.05$ is deemed statistically significant.

3. Results

3.1. Nutrient composition of the estuaries

Table 1 lists the changes in the different physical and chemical parameters as well as the biological elements of the water quality from the Chaliyar River and Anjarakkandi estuary zones. The data show that the temperature of the water in both estuary zones varied minimally, while the dissolved oxygen content of the Anjarakkandi river was significantly lower. Nitrate, ammonium, phosphate, and sulphate levels were found to be elevated together. Furthermore, these samples had considerably greater BOD and COD values than expected (Table 1).

3.2. Bacterioplankton diversity of the estuaries

Tables 2 and 3 list the diversity of free-living and particle-associated bacterioplanktons found in the samples. The estuarine regions of Anjarakkandi river exhibited a notably greater diversity of bacteria in comparison to Chaliyar River's estuarine parts. Additionally, the diversity of firmicutes (17.24 %) and proteobacteria (67.07 %) in the Anjarakkandi River estuaries was revealed by the phylum level investigation. In addition, the Chaliyar river has less abundant proteobacteria and more firmicutes than Anjarakkandi river, with 46.35 % and 31.51 %, respectively. Bacillus the subtilis, a faecal bacterium, was identified at the species level in estuaries of the Anjarakkandi and Chaliyar rivers at significantly higher percentages 16.19 % and 10.07 %, respectively (Table 3).

3.3. Bacterial diversity and composition of Chaliyar river

Particle associated bacteria:11015 out of 36701 reads were classified. Shown are taxa that comprise at least 0.1 % of classified reads. Bubble plot shows some major phyla of bacteria. Actinobacteria dominates over other phyla of bacteria in the sample, second dominating phyla is proteobacteria. Plot shows the presence of Cyanobacteria in the particle associated bacteria community (Fig. 1b). Krona chart shows the presence some distinctive class like Alphaproteobacteria, Gammaproteobacteria in major share of 30 % of classified reads (Fig. 1a).

Free living bacteria:2367 out of 10658 reads were classified. Shown are taxa that comprise at least 0.1 % of classified reads.Bubble plot shows some major phyla of bacteria. Proteobacteria dominates over other phyla of bacteria in the sample. Second dominating phyla is Actinobacteria. Plot shows the presence of large amount of Cyanobacteria in the Particle associated bacteria community than the other samples (Fig. 2b). Krona chart shows 78 % of unclassified reads and within the 22 % classified reads, predominant strains are Phenylobacteriumzucineum, Rhizobiales,Actinomycetia and Bacteroidetes (Fig. 2a).

3.4. Bacterial diversity and composition of Dharmadom

Particle associated bacteria:35090 out of 89214 reads were classified. Shown are taxa that comprise at least 0.1 % of classified reads.

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

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.

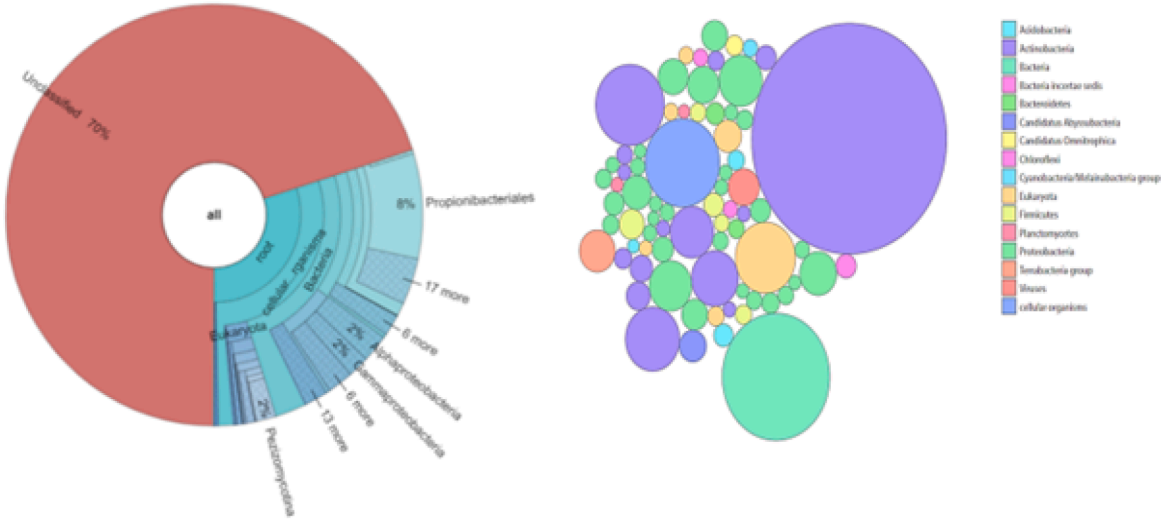

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Fig. 1. (a) Krona pie chart showing particle- associated Bacteria present in estuary of Chaliyar. (b) Bubble plot showing particle-associated Bacteria present in estuary of Chaliyar.

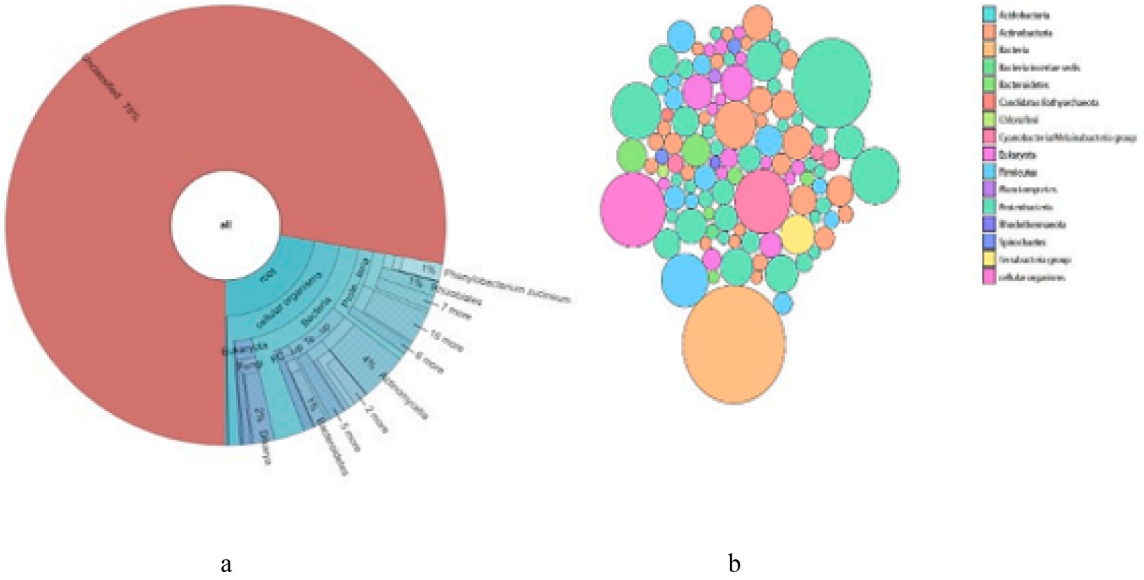

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Fig. 2. (a) Krona pie chart showing free-living Bacteria present in estuary of Chaliyar. (b) Bubble plot showing free-living Bacteria present in estuary of Chaliyar.

The bubble plot shows some major phyla of bacteria. Actinobacteria dominates over other phyla of bacteria in the sample, second dominating phyla is Proteobacteria. Plot shows least abundance of Cyanobacteria in the particle associated bacterial community (Fig. 3b). Krona chart shows 61 % of unclassified reads,and within 39 % of classified reads, Propionibacteriales, Micrococcales, Alphaproteobacteia, Gammaproteobacteia and Pezizomycotinawere found to be prominent (Fig. 3a).

Free-living bacteria: 12348 out of 40998 reads were classified. Shown are taxa that comprise at least 0.1 % of classified reads. Bubble plot shows the dominance of Actinobacteria, Proteobacteria, Acidobacteria and Firmicutes (Fig. 4b). Krona chart shows prominent presence of Propionibacteriale sand Gammaproteobacteria (Fig. 4a).

4. Discussion

Bacterioplanktons are important in an aquatic ecosystem due to their roles in mineral cycling, improving overall productivity of the system as well as in pollution control (Pommier, 2011; Grossart, 2010). The current study discovered a notably increased quantity of Proteobacteria, Actinobacteria, and Cyanobacteria in the estuary waters of the Anjarakkandi River. Elevated concentrations of water nutrients, including nitrates, nitrites, and phosphates, correlate with a higher proportion of bacterioplankton taxa, predominantly consisting of Actinobacteria, Cyanobacteria, and Proteobacteria. (Kiersztyn et al., 2019; Mou et al.,2013a). Firmicutes identified in the samples indicates the faecal contamination in the water (Paruch et al., 2019a; Paruch et al., 2019b). We also discovered that a range of nitrogen-breaking bacteria, including Sulfitobacter, Nitratiruptor, and Roseobacter, were present. One common bacterioplankton that is well-known for breaking down different

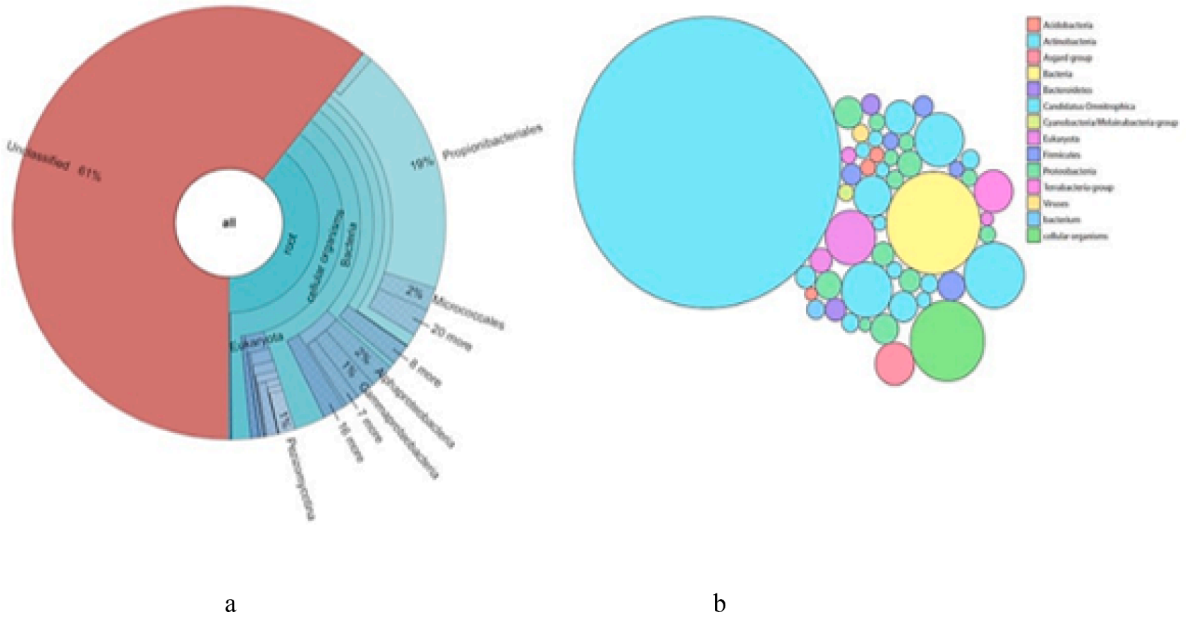

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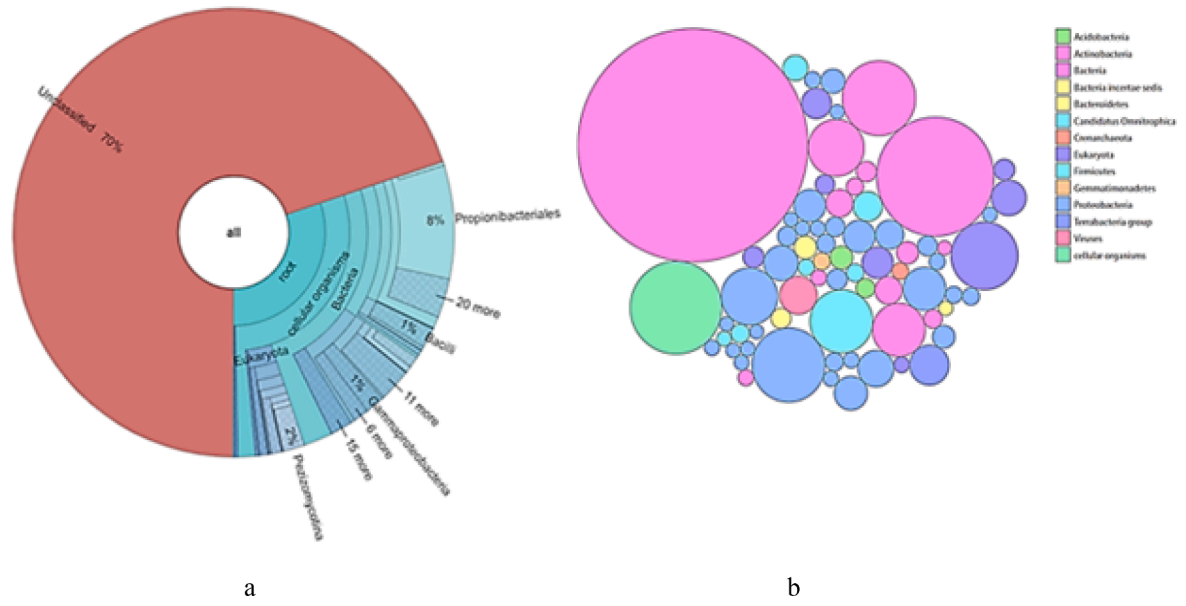
Fig. 3. (a) Krona pie chart showing particle- associated Bacteria present in Darmadam estuary. (b) Bubble plot showing particle- – associated Bacteria present in Darmadam estuary.



a b

Fig. 4. (a) Krona pie chart showing free-living Bacteria present in Darmadam estuary. (b) Bubble plot showing free-living Bacteria present in Darmadam estuary.

types of organic materials is the Roseobacter.(Pohlner et al., 2019). Likewise, Nitratiruptor is a nitrite reducing bacteria, which is capable of metabolising the nitrate in the water (Nakagawa et al., 2005). Sulfitobacter and Pseudoruegeria are other two strains observed in both the Chaliyam and Dharmadom estuarine regions. It is reported that the Sulfitobacter strains are predominantly distributed in the areas of oil polluted marine environments;it has been associated with hydrocarbon removal from seawater (Neethu et al., 2019). Pseudoruegeria is associated with the metabolic pathways for vitamin Bcomplexes (such as B1, B7, B12)(Cho et al., 2020).Further isolation and culturing of these bacteria may yield a source for vitamin B complex biosynthesis. Identifying characteristic strains from particle -associated and free-living communities is vital for their specific applications. Most of the diverse bacterioplankton reported here are capable of utilizing the dissolved organic matter for their growth and functioning. Isolation of such bacterial communities is having great significance in the aspects of wastewater treatment and pollution abatement(Dinasquet et al., 2018; Watanabe et al., 2009). Abundance of members like Actinobacteria, Cynobacteria and Proteobacteria(Table 3) act as biological elements of water quality assessment (Pinto et al., 2021). There are reports of how water contaminations has changed the community compositions of bacteria. However, certain organisms are adapting to the pollutants. Bacterioplanktons are actively participating in pollution control. A few species of Betaproteobacteria are involved in degradation of of microcystin, which is a cyanobacteria induced pollutant (Mou et al. 2013). Some Species belongs to Gammaproteobacteria are involved in hydrocarbon degradation and Rodococcus like species are capable of degrading organic pollutants and they form a biofilm which reduce the mobility of pollutants (Gontikaki et al., 2018; Martínková et al., 2009).

Estuaries exhibit variations not only in bacterial abundance but also

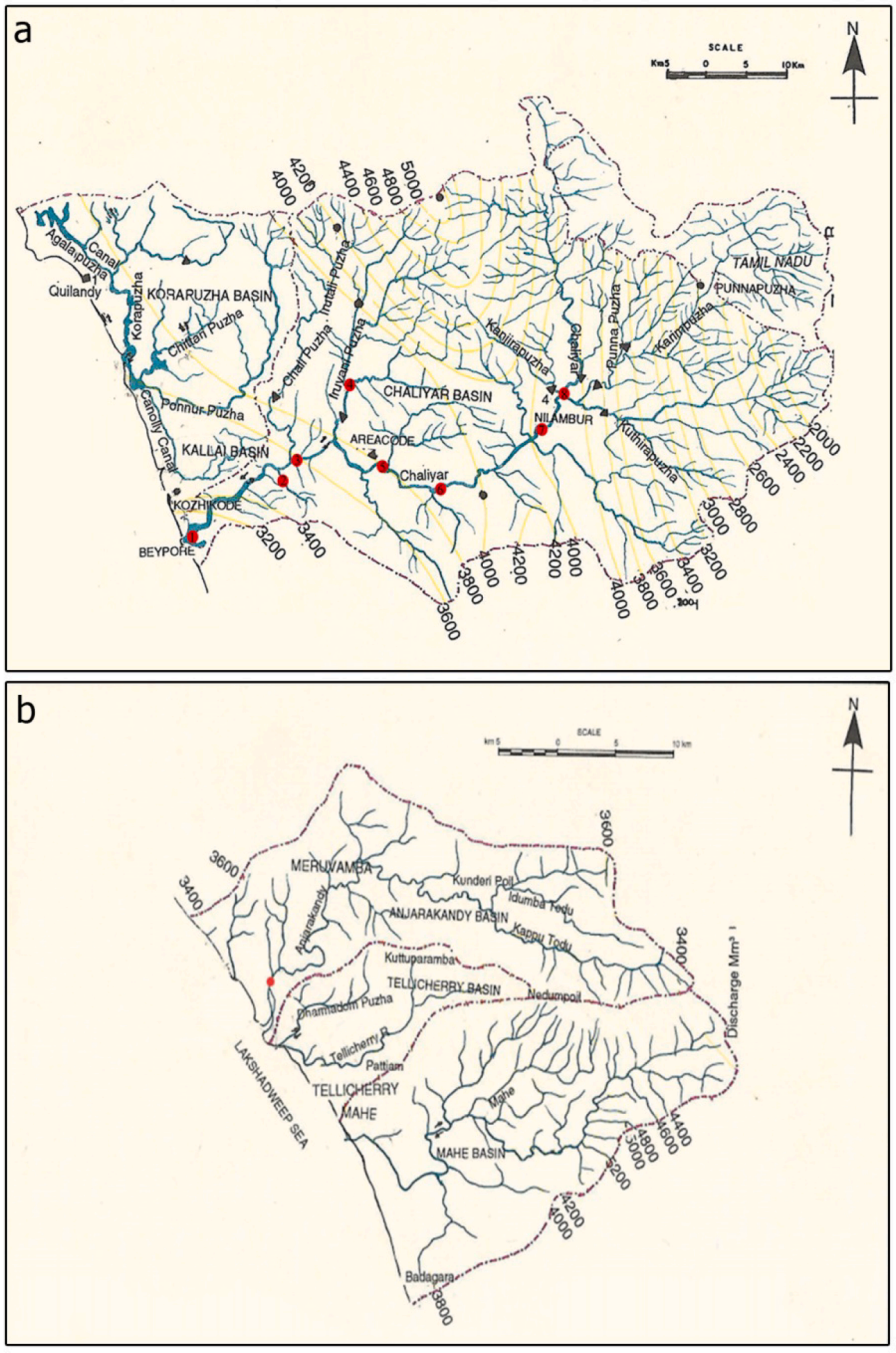


Fig. 5. The site map of estuarine regions of Chaliyar river (a) and Anjarakkandi river (b).

in nutrient parameters, with the Anjarakandy River estuary displaying higher levels of physiochemical parameters compared to the Chaliyar River estuary. Specifically, nutrients such as calcium, magnesium, chloride, iron, nitrate, ammonium, sulfate, and phosphate are elevated in the Anjarakandy estuary, along with a higher percentage of organic carbon content.

Various scientific studies have investigated how nutrient parameters impact the bacterial composition of water bodies, rivers, and similar aquatic ecosystems. These studies aim to understand how factors such as nutrient levels, including nitrates, phosphates, and organic carbon, influence the types and abundance of bacteria present in these environments. (Yang et al., 2020; Mou et al., 2013b). Certain investigations have focused on seasonal variations, revealing shifts in bacterioplankton composition and richness influenced by changing environmental conditions. Additionally, these studies explore the role of bacterioplankton in mediating the bioavailability of organic matter, crucial for nutrient cycling within aquatic ecosystems (Xiao et al., 2021,Stephens et al., 2020) We did not observe any major difference in diversity of particle associated bacterioplanktons in the two estuaries investigated. However, there was a significant variation observed in the diversity of free living bactrioplankton attributed to changes in physio-chemical parameters.

5. Conclusions

In conclusion, the study conducted in the Anjarakkandi River estuary in Dharmadom emphasises the critical functions that bacterioplankton play in aquatic environments. These waters' high Proteobacteria,

Actinobacteria, and Cyanobacteria abundances indicate that the surrounding environment has an abundance of nutrients. The detection of Firmicutes hints at the possibility of fecal contamination, prompting concerns regarding the water's quality. When comparing bacterioplankton abundance and nutrient parameters between the Chaliyar and Anjarakandy river estuaries, we observed a higher abundance of bacteria and elevated nutrient levels in the Anjarakandy river estuary. These findings suggest a potential link between nutrient availability and bacterioplankton abundance. The diversity of bacterioplankton in natural environments is impacted by various physio-chemical factors, underscoring the importance of ongoing research into these microbial communities for their unique environmental and industrial potential. Overall, the comparison has been easy to draw because metagenomics has yielded a comprehensive understanding of the composition of the communities in both estuaries.

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CRediT authorship contribution statement

D. Nikhitha: . **Arunaksharan Narayanankutty:** Writing – review & editing. **Manoj Mathews:** Writing – review & editing, Methodology, Funding acquisition, Conceptualization. **Deepa Sathee:** Writing – review & editing, Formal analysis. **Ahmed Alfarhan:** Software. **Rajakrishnan Rajagopal:** Software. **Jisha Jacob:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

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.

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Availability of data and material

Upon proper request, the data will be made available.

Ethics approval

There are neither human nor animal subjects in this study.

Consent for publication

The research does not incorporate any external materials or third-party content.

NCBI Accession Number

The data have been deposited with links to BioProject accession number PRJNA750037 in the NCBI BioProject database (https://www.ncbi.nlm.nih.gov/bioproject/).

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