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Spatial variation of microfouling community on artificially immersed cement panels at three different habitats of Chennai, Tamil Nadu, India

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Abstract
In this study, the composition of microfouling bacteria and the chemical components of conditioning biofilm was examined on artificially immersed cement panels exposed to three different habitats *viz*. Korattur lake (freshwater), Muttukadu backwaters (brackish) and Chennai port (marine) Tamil Nadu, India. Test panels were deployed in the surface waters at the depth of 1m in three study sites. Microfouling material which formed in these panels were analysed for microfouling bacteria using various morphological and biochemical parameters. Additionally, hydrographical parameters at three study sites were assessed. Culture-based method was used in the quantification of bacterial species formed on the surface of the cement panel. A total of 7 biofilm forming bacteria was recorded on the cemented panels immersed in Korattur lake (Freshwater), 9 biofilm forming bacterial species in Chennai Port (Marine) and 12 biofilm forming bacteria and 3 non-biofilm bacteria were observed in Muttukadu backwaters (Brackish). The result highlights that bacterial communities were more heterogeneous in Muttukadu backwaters and conversely, community composition in Korattur lake and Chennai port was less heterogeneous. This paper will give an insight on how microorganisms like bacteria spatially distributed in contrasting environments with reference to fouling.

Keywords: Microfouling, korattur lake, muttukadu backwaters, chennai port, biofilm

Introduction

The undesirable attachment and growth of organisms on natural and artificial structures present in the water is known as biofouling. Several biofouling studies has been done using different substrates such as plastics (Muthu Krishnan *et al*., 2019), wood (Pati *et al*., 2015) [2], metallic substrates (Veda Prakash *et al*., 2013; D'Souza and Bhosle, 2003) [4], artificial biological reef structures (Mohamed *et al*., 2023) [5], ship hull (Kavitha and Vimala, 2018) [6] and in boat surfaces (Nandini and Revathi, 2016). The process of biofouling occurs due to divergent group of organisms from bacteria to invertebrates which follows a pattern of growth and development.

The various stages of biofouling community occur in the following sequence: 1. formation of conditioning film, 2. primary film, 3. microfouling and 4. macrofouling (Nandakumar and Yano, 2003) [8]. This succession is largely influenced by the geographical location, material used, depth of the water and season of the year. All these factors direct the biofouling growth, dynamics, species composition and accumulation rate (McDougall,1943; Kawahara,1969; Keough,1989; Abelson and Denny,1997 & Nair,1999) [9, 10, 11, 12, 13]. Among the physical factors, water temperature, sunlight, turbidity are the most crucial factors that regulate the growth of organisms in the biofouling community (Smedes,1984) [14].

The fouling community in an aquatic environment has several ecological functions. Costlow and Tipper (1984) [15] explained about the role of fouling communities both firstly, as deteriorators of man-made structures in the water and secondly, as biological indicators in the polluted waters. Microfouling starts within few hours of deployment of substrates in the water. The community of microfouling comprises of bacteria, green algae, cyanobacteria, diatoms and protozoans (Sandrock et. al., 1991) [16]. The biofilm formation is regarded as the significant step in the biofouling process. The quantification of biochemical constituents of the biofilm formed during the fouling process is one of the common methods to measure the biomass present in it.

Bacteria constitute as one of the important parts of the microfouling assemblage. The biofilm is developed by bacteria which adhere to any immersed surfaces or structures present in an aqueous environment. These bacteria produce slime as extracellular polymeric substances (EPS) which helps in adhesion. The production of EPS is influenced by various factors such as environmental conditions encountered by the bacteria (Marshall, 1976; Watnick and Kolter, 2000) [17, 18]. Hence, bacteria are the initial colonisers of surfaces exposed to the water and provides the suitable habitat to upcoming foulants. Biofilms developed on submerged surfaces have significant environmental and economic consequences (Jeliani et. al., 2022) [19, 36]. The composition of the microfouling products plays a crucial role in subsequent colonization and development of macrofouling organisms.

Spatial and temporal differences in fouling compositions are due to seasonal changes in the water quality (Pati *et al*., 2011) [20]. The hydrographical parameters such as temperature, pH, salinity, dissolved oxygen and nutrients such as nitrate, nitrite and phosphate of a particular habitat determines its faunal distribution and habitat productivity. The salinity act as a prime factor among the most important environmental parameters in the distribution of living organisms (Chandran, 1984) [21]. Therefore, it is significant to explore the correlations between fouling bacteria and various environmental parameters which would provide an understanding into the ecosystem response to future changes in the environment.

The main objective of this study is to observe the microfouling community in three different habitats *viz*. freshwater, brackish and marine waters of Chennai, Tamil Nadu, India. The hydrological parameters and biochemical constituents of the biofilm at different habitats were also analysed in relation to microfouling. This is the first study which was conducted in three different habitats of Chennai to analyse the microfouling activity. This paper will give an insight on spatial distribution of microorganisms like bacteria in contrasting environments with reference to fouling and further investigation of this study will help in developing the antifouling strategies to prevent the fouling process in initial stages.

Materials and Methods

Study area

Three sites located around the city of Chennai, Tamil Nadu (Figure 1), exhibiting different hydrographical parameters were selected in order to study microfouling with reference to bacteria.

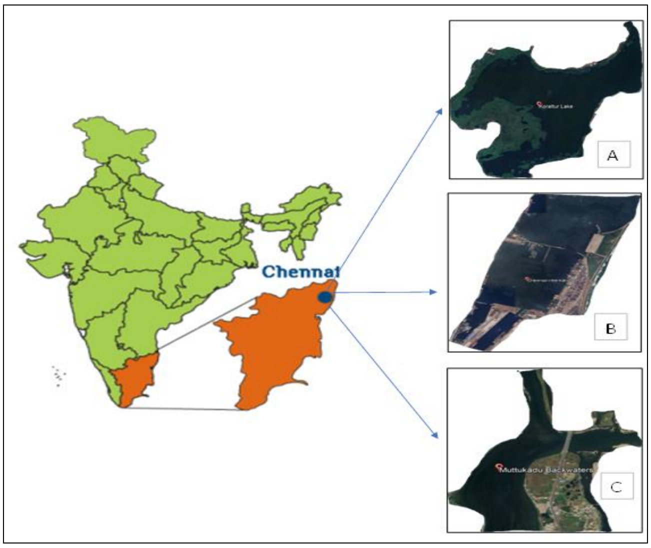


Fig 1: Location of three study sites. (A) Korattur Lake (B) Chennai port (C) Muttukadu backwaters

a **Korattur Lake:** Korattur Lake (13.12330°N and 80.18047°E) is one of the largest lakes in the western part of the city(Figure 1A). The freshwater lake is situated to the north of the railway line of Chennai–Arakkonam. This lake is one of a chain of three water bodies, including the Ambattur Lake and the Madhavaram Lake.

b **Chennai port:** Chennai Port is located in the southeastern part of India at 13.0815° N and 80.2921° E. The Port has three docks – Bharathi, Jawahar and Dr. Ambedkar. This study was carried out at Dr. Ambedkar Port (13°5"24.82764 N and 80°17"37.65192 E) (Figure 1B).

c **Muttukadu backwaters:** The Muttukadu backwaters is an important intertidal area, which is located approximately 36 km south of Chennai (Figure 1C). It lies within latitude 12.8006ºN and longitude 80.2363º E. This region is divided into different microhabitats like tidal mudflats, sand bars, intertidal zones, mangroves and sand beach.

Submersion and retrieval of cement panels

Test cement panels (Figure 2) with dimensions 21cm x 21cm were submerged at a depth of 1m in water at three study sites. Before immersion, the cement panels were cleaned thoroughly using distilled water, dried and rinsed with 70% alcohol (Satheesh and Wesley, 2010) [22]. The cement panels were then retrieved after seven days to analyse the microfouling assemblages and biofilm formed on the cement panels at three study sites. After retrieval of the panels from water, they were taken to the laboratory using a sterile container filled with water collected from the respective study sites. The samples were collected in a conical flask using sterile nylon brush (Wesley and Satheesh, 2009) [23] and the scrapped microfouling assemblage was divided into two 100ml samples: first 100ml sample was used for the analysis of bacteria and the second was used for the analysis of biofilm nutrients.

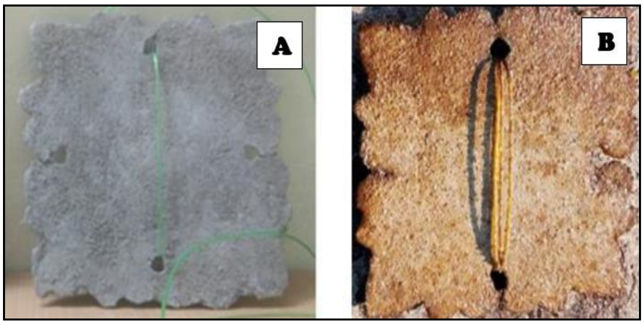


Fig 2: Cement panel used for the microfouling study. A- before immersion B- after retrieval

Analysis of hydrographical parameters

For this study, the hydrographical parameters such as temperature, salinity, pH, dissolved oxygen and nutrients like nitrite, nitrate, and phosphate were assessed. The surface water samples were collected in a sterilized bottle which were taken to the laboratory and utilized for the estimation of above-mentioned parameters. Temperature, salinity and dissolved oxygen were assessed at the study site itself. Salinity was measured using salinity refractometer (ERMA, Japan). pH of the sample was measured using a portable pH meter (HANNA HI98107). The determination of dissolved oxygen was carried out by performing standard Winkler's method. The other physicochemical parameters such as nitrite, nitrate and phosphate were studied using a kit developed by TWAD (Tamil Nadu Water supply and Drainage Board) which follows the procedure of APHA (APHA, 2012). The mean values of hydrographical parameters for three study sites were calculated and given as box plots.

Analysis of biochemical constituents of biofilm

In the present study, spatial variation in the concentration of biochemical components like total carbohydrate, protein, nitrate, nitrite, phosphate, calcium and magnesium were analysed in the study sites. In order to analyse the biochemical constituents of the microfouling sample, the second sample collected from the study sites was filtered using GF/C membrane filter (0.45, Whatman). The total carbohydrate content was estimated using Phenol - Sulfuric Acid method (Dubois *et al*., 1956) [25]. The protein content was estimated using Folin phenol reagent (Lowry *et al*.,1951) [26]. The methods described by Venugopalan and Paulpandian (1989) [27] have been applied for the quantification of nitrate, nitrite, phosphate, calcium and magnesium.

Isolation and identification of bacteria

A-Nutrient Agar **B- Mac Conkey Agar** **C- Blood Agar**

D- Chocolate Agar **E- Congo red Agar** **F- Zobell Marine Agar**

Fig 3: Culture agar plates used for the isolation of microfouling bacteria.

Cultivable bacteria formed on the test panels were cultured using Nutrient agar, Mac Conkey agar, blood agar, chocolate agar and Congo Red agar for isolation. The Figure 3 shows the culture agar plates used for the isolation of microfouling bacteria. The samples collected from Chennai port and Muttukadu backwaters were cultured using ZoBell Marine agar in addition to above mentioned media. The biofilm sample of 1ml was serially diluted using water collected from respective habitats. These agar plates were incubated at $28^{0}C \pm 2$ for 24-48h.After the incubation period of 48h, the total viable colonies were counted.The bacterial colonies which showed biofouling activity was characterized using various morphological and biochemical parameters (Dhanasekaran *et al*., 2008). Gram staining was performed for preliminary identification of the isolate (Allegrucci and Sauer, 2007) [29]. Morphological characters and biochemical tests were carried out (Dalton *et al*., 1994) [30] and confirmed using Bergey's Manual (Krieg *et al* 2010) [31].

Data Analysis
In order to evaluate the significance of the various results obtained, statistical analysis was done by using Microsoft Excel 2019.

Results
Culturable bacteria isolated from cement panels from three study sites during this analysis are given in Table 1(A)-1(C). The current study findings revealed that a total of 7 biofilm forming bacteria belonging to 4 families were recorded on the cemented panels immersed in Korattur lake (Freshwater) (Table 1A), 9 biofilm forming bacterial species belonging to 2 families in Chennai Port (Marine) (Table 1B), 15 bacteria belonging to 9 families in Muttukadu backwaters (Brackish) (Table 1C). Among them 12 are biofilm producing bacteria. The diversity of microfouling bacteria was maximum in Muttukadu backwaters and least in Korattur lake. The diversity of microorganisms varied depending on the nutritive status of submerged water. The results of biochemical tests for identification of the bacteria isolates collected from three habitats are presented in Appendix 1.

Table 1A: List of bacteria isolated from cement panels deployed in Korattur lake

Sample no.	Class	Order	Family	Species
1	Actinomycetes	Micrococcales	Micrococcaceae	*Micrococcus luteus*
2	Bacilli	Bacillales	Bacillaceae	*Bacillus cereus*
3	Bacilli	Bacillales	Bacillaceae	*Bacillus licheniformis*
4	Bacilli	Bacillales	Staphylococcaceae	*Staphylococcus xylosus*
5	Bacilli	Bacillales	Staphylococcaceae	*Staphylococcus hominis*
6	Gammaproteobacteria	Enterobacterales	Enterobacteriaceae	*Escherichia coli*
7	Gammaproteobacteria	Enterobacterales	Enterobacteriaceae	*Klebsiella pneumoniae*

Table 1B: List of bacteria isolated from cement panels deployed in Chennai Port

Sample no.	Class	Order	Family	Species
1	Bacilli	Bacillales	Bacillaceae	*Bacillus altitudinis*
2	Bacilli	Bacillales	Bacillaceae	*Bacillus cereus*
3	Bacilli	Bacillales	Bacillaceae	*Bacillus licheniformis*
4	Bacilli	Bacillales	Bacillaceae	*Bacillus pumilus*
5	Bacilli	Bacillales	Bacillaceae	*Bacillus subtilis*
6	Bacilli	Bacillales	Bacillaceae	*Bacillus thuringiensis*
7	Bacilli	Bacillales	Staphylococcaceae	*Staphylococcus epidermidis*
8	Bacilli	Bacillales	Staphylococcaceae	*Staphylococcus hominis*
9	Bacilli	Bacillales	Staphylococcaceae	*Staphylococcus warneri*

Table 1C: List of bacteria isolated from cement panels deployed in Muttukadu backwaters

Sample no.	Class	Order	Family	Species
1	Gammaproteobacteria	Aeromonadales	Aeromonadaceae	*Aeromonas hydrophila*
2	Bacilli	Bacillales	Paenibacillaceae	*Bacillus brevis*
3	Bacilli	Bacillales	Bacillaceae	*Bacillus circulans*
4	Bacilli	Bacillales	Bacillaceae	*Bacillus megaterium*
5	Betaproteobacteria	Burkholderiales	Burkholderiaceae	*Burkholderia cepacia*
6	Gammaproteobacteria	Enterobacterales	Enterobacteriaceae	*Citrobacter freundii*
7	Gammaproteobacteria	Enterobacterales	Enterobacteriaceae	*Citrobacter koseri*
8	Gammaproteobacteria	Enterobacterales	Enterobacteriaceae	*Klebsiella pneumoniae*
9	Gammaproteobacteria	Enterobacterales	Enterobacteriaceae	*Cedecea davisae*
10	Gammaproteobacteria	Enterobacterales	Enterobacteriaceae	*Escherichia albertii*
11	Gammaproteobacteria	Enterobacterales	Enterobacteriaceae	*Shigella sonnei*
12	Gammaproteobacteria	Enterobacterales	Erwiniaceae	*Pantoea agglomerans*
13	Gammaproteobacteria	Enterobacterales	Hafniaceae	*Hafnia alvei*
14	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	*Acinetobacter baumannii*
15	Gammaproteobacteria	Enterobacterales	Morganellaceae	*Providencia alcalifaciens*

The Figure 4 represents the Venn diagram of species composition at three study sites and the distribution of common species in all the habitats. Three bacterial species such as *Bacillus cereus, Bacillus licheniformis* and *Staphylococcus hominis* were common in both Korattur lake and Chennai port. *Klebsiella pneumoniae* was observed in Korattur lake and Muttukadu backwaters. No common species was observed in all three habitats.

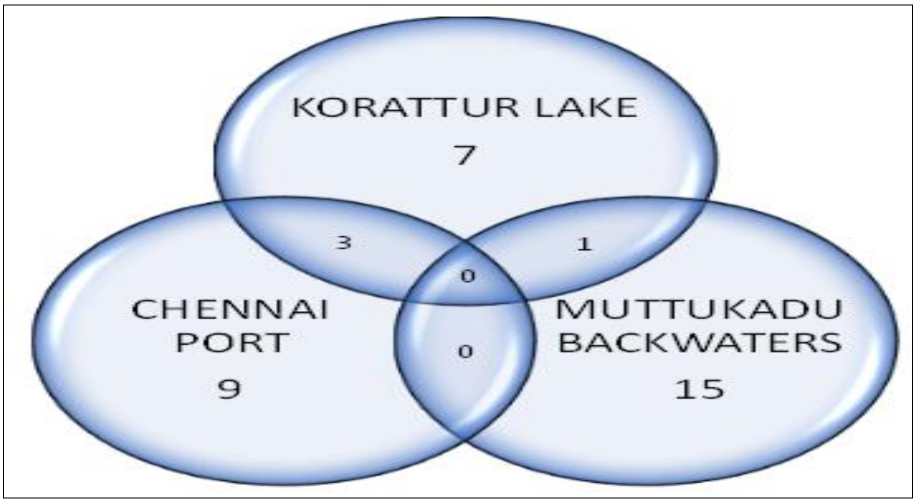


Fig 4: Venn diagram of species composition at three study sites and the distribution of common species in all the habitats.

The Figure 5 shows the proportion of bacterial families identified in microfouling community at three study sites. In Korattur lake, the families Bacillaceae, Staphylococcaceae and Enterobacteriaceae represented with 2 species by each family. The Chennai Port has highest number of species in family Bacillaceae. The Enterobacteriaceae family with 6 species constitutes the highest among all families observed in Muttukadu backwaters. The variations in the hydrographical parameters at three study sites during the sampling period are shown in Figure 6. Temperature was high in Korattur lake and pH was high in Muttukadu backwaters. The salinity, dissolved oxygen, nitrate, nitrite and phosphate was high in Chennai port.

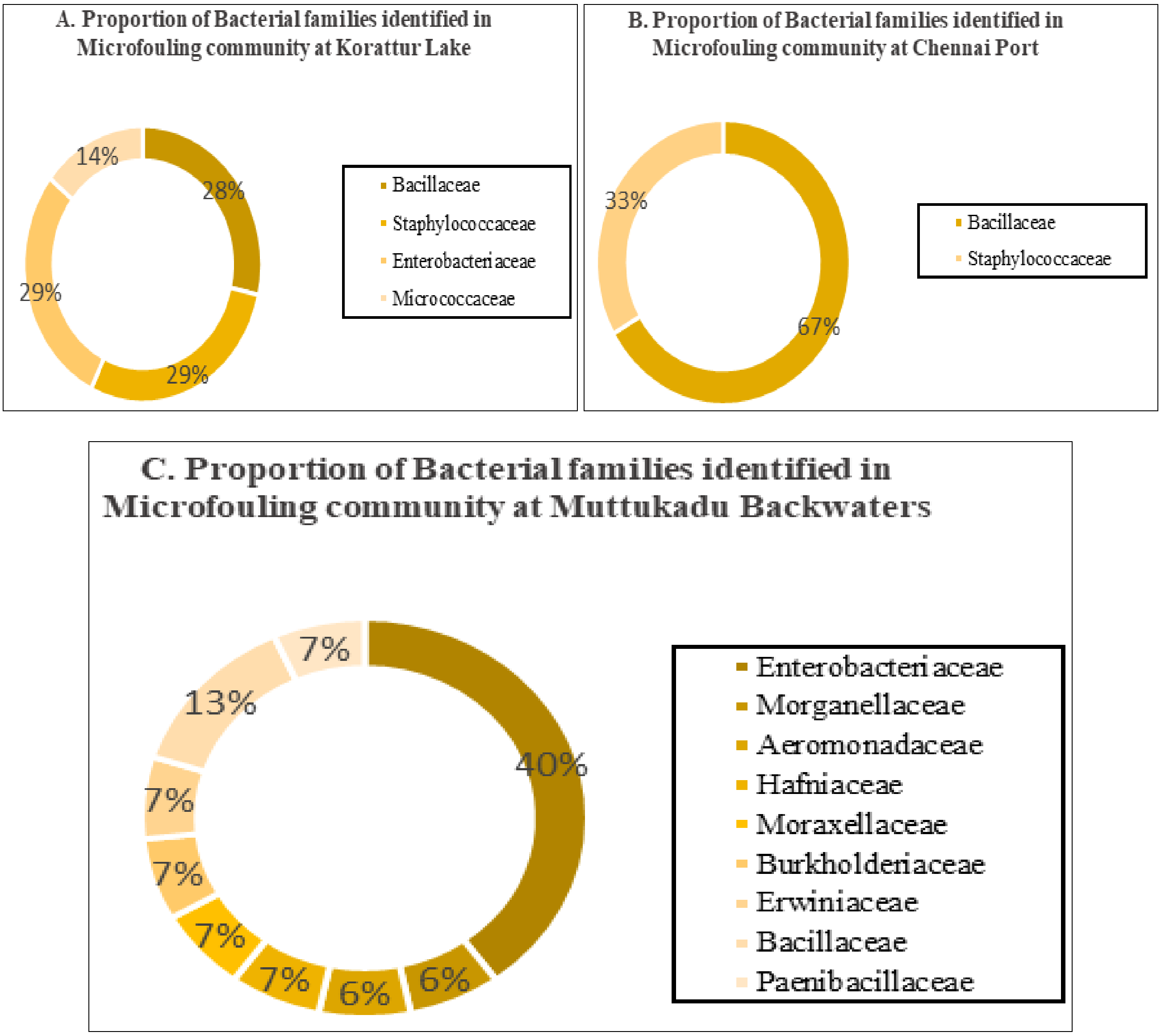
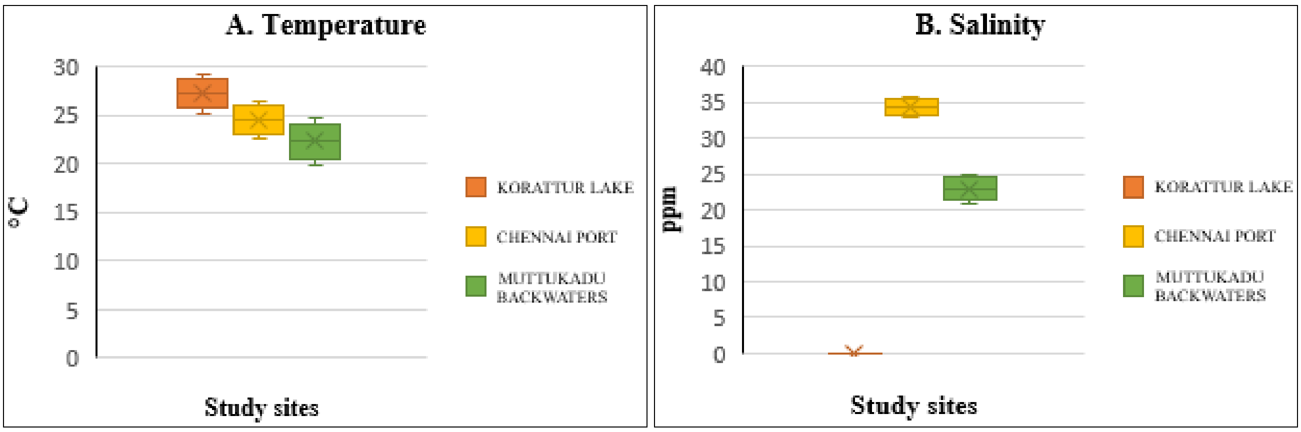


Figure 5: Proportion of bacterial families identified in microfouling community at three study sites. (A- Korattur Lake, B- Chennai Port, C- Muttukadu Backwaters)



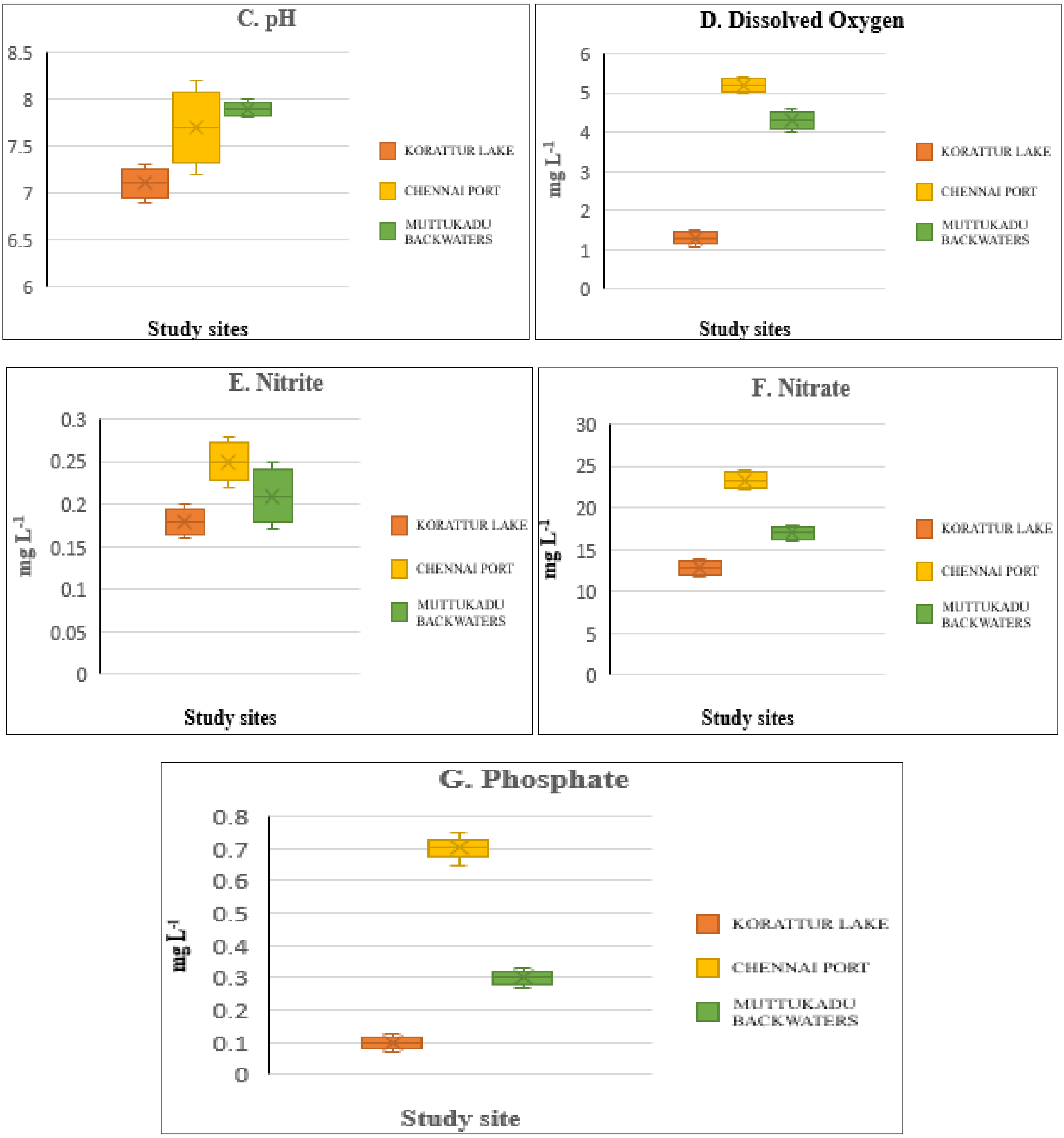


Fig 6: Box plot showing the analysis of hydrographical parameters observed during the sampling period at three study sites (A-G).

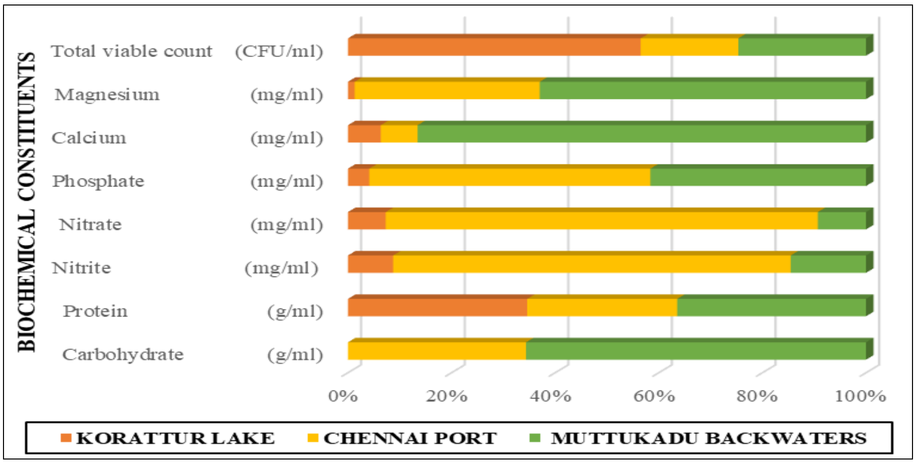


Fig 7: Biochemical constituents of biofilm isolated from the concrete panels of three study sites.

The biochemical constituents of biofilm developed on the deployed test cement panels were analysed and the results are presented in the Figure 7. The carbohydrate content, protein, calcium and Magnesium was high in Muttu Kadu backwaters. The Nitrite, Nitrate and Phosphate was high in Chennai port.

Discussion

This paper highlights about the spatial distribution of microfouling bacteria on cement panels suspended in three different habitats of Chennai and specifically embarks to determine the biofilm forming bacteria and the influence of environmental parameters on the prevailing species was also explored. Microfouling is influenced by several factors such as the time of exposure of substratum, the climatic conditions of the environment, the physico-chemical parameters of the water surface available for settlement and biology of fouling organisms. Among all biofouling species, fouling bacteria forms the basis of biofouling in all aquatic environments, since they are ubiquitous in nature, which overlap all exposed surfaces and then become platform for the attachment of larval fouling organisms (Chen *et al*., 2022) [32].

Microfouling was observed highly on the cement panel immersed at Muttu Kadu backwaters (brackish water) compared to Chennai port (marine) and Korattur lake (freshwater). The results revealed a higher diversity of bacteria in saltwater (Table 1A &B), highlighting that marine water have been shown to harbour higher bacterial diversity than freshwater (Table 1C). Serving as buffer zones, backwaters are exposed to a host of physiochemical disturbances and they exhibit natural environmental gradients, therefore making them ideal habitat for exploring the impact of various environmental parameters on fouling communities (Liu *et al*., 2014) [33]. The three study locations significantly affected the diversity and abundance of bacteria on the panels. In this study, the different morphological characters and biochemical tests were performed for characterization and identification of bacterial isolate. Among all, the order Bacillales and Enterobacter ales were the most abundant in all three habitats. This observation showed that these species were the stable colonisers in the panels deployed in the three study sites.

Environmental parameters play a crucial role in shaping the structural and functional characteristics of biofilm. Unlike planktonic communities, the impact of seasonal variation of hydrographical parameters on microfouling communities is minimum since they develop biofilm which protects all the bacterial cells (Guo *et al*., 2020) [34]. So, they should be considered in order to elucidate biofilm community on artificial surfaces immersed in an aquatic environment. Although they are harboured in surrounding areas of Chennai, the three study sites chosen exhibited dissimilar characteristics. As expected, salinity was significantly higher in Chennai port and Muttu Kadu backwaters compared to Korattur lake. Muttu Kadu backwaters showed variable salinity patterns due to freshwater inputs. This freshwater entry influenced the nature of the hydrographical parameters at this site. Chennai port waters exhibited coastal characteristics with high temperature and salinity. The water characteristics of Korattur lake showed stable freshwater values.

Several studies have described the effect of concentrations of nutrients on the slime films. Previous studies have focused on the biochemical constituents of biofilm developed on various surfaces deployed in different waters. (D'Souza and Bhosla, 2003; Wesley and Satheesh, 2009 and Balqadi *et al*., 2018) [4, 23, 35]. The concentration of phosphate, nitrite, nitrate, calcium and magnesium in the biofilm showed spatial variations. These variations can be due to the concentrations of these nutrients in the respective waters present in the study sites. As the nutrient contents of the Chennai port and Muttu Kadu backwaters was high, the accumulation of the nutrients in the biofilm developed on the submerged test cement panel also showed higher values in these study site. According to (Wesley and Satheesh, 2009) [23], the nutrient load of the submersion medium determines the concentration of the biofilm nutrient.

Biofilms developed on submerged surfaces have significant environmental and economic consequences (Jeliani *et al*., 2022) [19, 36]. During the initial period, immersed substrate influences the composition of microfouling organisms (Characklis and Cooksey,1983) [37]. The substrate adsorbs organic and inorganic matters present in the submerged medium which recruits the initial fouling organisms (Taylor *et al*., 1997) [38]. The results showed that calcium and magnesium was the highest organic constituent among the nutrients in the biofilm developed on the test cement panels submerged in all the three study sites. It supported the fact of divalent cations such as $Ca2^{+}$ and $Mg2^{+}$ are important for the formation of bacterial biofilms, contributing to bacterial adhesion to substrates and increasing biofilm stability. (Koerstgens *et al*., 2001) [39]. The total carbohydrate content was high in Muttu Kadu backwaters than Chennai port and absent in Korattur lake. The nutrients like nitrate, nitrite and phosphate showed slight spatial variation compared to other parameters. Considerable variation in the nutrient concentration of biofilm was observed in relation to exposure of cement panels between different sampling sites. Except carbohydrate, all other compounds showed indicates strong spatial variability in the organic and inorganic composition of biofilms. The observed results showed that the spatial variability may be partly influenced by the concentration of these nutrients in the surrounding medium.

The microfouling bacteria have already been reported from different habitats. The establishment and growth of fouling communities were studied by (Tempesti *et al*., 2022) [40] in three Northern Tyrrhenian port systems (Mediterranean Sea) which focussed on the relationship between the fouling structure components and the associated fouling community. Previous study by Jeliani *et al*. (2022) [19, 36] investigated the diversity of biofilm-forming bacteria in a mangrove ecosystem along the Persian Gulf and this study revealed the comparison of fouling bacteria developed on fishing nets and on the trunks of plants in the mangrove forest. A study by Chen *et al*. (2023) depicted the diversity and distribution of 37 species culturable fouling bacteria belonging to 25 genera were isolated from shellfish, fish and non-mariculture zones in Daya bay of South China. Previous studies (Liu *et al*. 2014) [33] have reported that studies of composition of fouling bacterial community in freshwater have received less attention compared to those in marine. Hence an attempt has been made to study the diversity of fouling bacteria in different habitats *viz*. freshwater lake, brackish and marine waters around Chennai.

Conclusions

Microfouling studies with the emphasis on bacteria were carried out on test cement panels in comparison with three different habitats of Chennai, Tamil Nadu, India. The present study revealed a significant spatial variation in the accumulation of biochemical constituents of biofilm formed on the test cement panels submerged in three study sites. The results of the study cleared showed the spatial effects on the composition of fouling bacterial communities with lower estimates at Korattur lake and higher estimates in Muttukadu backwaters and Chennai port. Therefore, bacterial communities were more heterogeneous in Muttukadu backwaters and conversely, community composition in Korattur lake and Chennai port was less heterogeneous.

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Credit authorship contribution statement

Ramya Shree N: Conceptualization, Methodology, Software, Formal analysis, Investigation, Writing – original draft, Project administration.

Vani Ganapathy R: Conceptualization, Methodology, Investigation, Writing – original draft, Project administration.

Malathi E: Conceptualization, Writing – review & editing, Supervision, Project administration.

Disclosure statement

No potential conflict of interest was reported by the authors.

Data availability statement

Data will be made available on request.

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