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Contents

MOLECULAR CHARACTERIZATION OF *SERRATIA MARCESCENS* AND THE DETERMINATION OF THE EFFECT OF PHYSICO CHEMICAL FACTORS ON ITS PIGMENT PRODUCTION

Devapriya K S [1], Jaya Vinny Eapen[2], Nivya Mariam Paul[3*]

[1,3] Department of Microbiology, Mar Athanasius College, Kothamangalam, Kerala-686666
[2] Department of Biotechnology, Mar Athanasius College, Kothamangalam, Kerala-686666
[*]**Corresponding author email id:** nivyamariampaul@gmail.com

Abstract

Microorganisms are known to produce pigments. Red pigmented bacterial strain isolated from flooded soil sample was used for the present study. This motile, gram negative bacteria was identified as *Serratia marcescens* by studying cultural, morphological and performing 16S rRNA gene sequencing. The GenBank accession number for the nucleotide sequence is MW287194. Effect of physico chemical factors on prodigiosin production was carried out. Here the highest yield of prodigiosin pigment production was observed in 9 g NaCl (30^{O}C), pH 9 (30^{O}C). The Rf value of pigment extract was 0.73, it was determined by thin layer chromatography (TLC). Prodigiosin is a promising drug owing to its reported characteristics of having anti-fungal, immunosuppressive, and anti-proliferative activity.

Keywords: *Serratia marcescen*, prodigiosin, physico chemical factors, 16S rRNA

Introduction

Pigments are molecules of great importance for industries such as food, cosmetics, pharmaceuticals, and textiles. At present, there is great interest in the use and consumption of natural compounds, due to benefits associated with

natural pigments (Downham and Collins, 2000; Knecht and Humpf, 2006; Yang *et.al.,* 2006). In nature, there are many sources for pigment production, however, only a fraction of them is available in sufficient quantity to be used at industrial level (Duffosé *et al*., 2005; Mapari *et al*., 2005). Microorganisms are a source of natural pigments. In addition, these organisms can grow into different culture systems (submerged and solid) and can be genetically modified to increase productivity and quality of the pigments produced by them (Campoy *et al.,* 2003; Yan *et al*., 2005).

Microorganisms are known to produce a variety of pigments; therefore, they are a promising source of food colorants (Aberoumand, 2011; Ahmad *et al.,* 2012). Most of the bacteria and fungi are widely studied for their potential as source of food colorants. Natural pigments possess anticancer activity, contain pro-vitamin A and have some desirable properties like stability to light, heat and pH (Joshi *et al.,* 2003). Thus, the food industry has become increasingly interested in the use of microbial technology to produce colors for use in foods. Prodigiosin is a red pigment first isolated from *Serratia marcescens.*

Materials and Methods

Sample collection

Flood affected soil samples were collected in a sterile polythene bag from Kothamangalam, Ernakulam dist., Kerala. The soil sample was then labelled and stored at $4^{O}C$ for further studies.

Primary screening for pigmented bacteria

For primary screening of pigment producing bacteria, 0.1g soil sample was weighed and serially diluted upto 10^{-6} dilutions. 0.1 ml of sample from each dilution was spread plated onto nutrient agar plates (Cecilia *et al*., 2021). The plates were incubated at $37^{O}C$ for 24hrs in an incubator.

Isolation and characterization of microorganism

The morphological characterization of selected pigment producing bacteria was done by colony morphology, gram staining and hanging drop technique. Bacterial colony was characterized by observing the following traits: size, shape, surface, elevation, opacity, pigmentation, consistency gram staining and motility of pigmented bacteria (Cappuccino *et al.,* 2005).

Effect of physico – chemical factors on pigment production

Secondary screening for pigment production was done on nutrient broth under normal growth conditions. To 1000 ml of nutrient broth 3ml of culture was inoculated. This was incubated at 37^{o}C for 15 days and observed for pigment production.

Effect of pH on pigment production

The growth and type of pigment is affected by the pH of the medium in which the microorganisms are grown. 1 ml of glycerol stock culture was inoculated into four 250 ml nutrient broth flasks, which is maintained at pH 2 and 9. pH of the media was set at 2 by adding 0.1N HCl and pH 9 was set by adding 0.1N NaOH. The isolate was incubated at two temperatures, 40^{O}C and room temperature. Pigment production was estimated after 15 days of incubation period.

Effect of NaCl concentration on pigment production

Bacterial growth and production of pigments was affected by salt concentration of the medium. 1 ml of glycerol stock culture was inoculated into four 250 ml nutrient broth flasks. The broth was supplemented with 1 g and 9 g NaCl. The isolate was incubated at 40^{O}C and room temperature. Pigment production was estimated after 15 days of incubation period.

Extraction and purification of bacterial pigment

After 15 days of incubation, the culture was centrifuged at 6,000 rpm for 15 minutes. Supernatant was collected and discarded the pellet. Chloroform and the supernatant were taken in the ratio 1:1, mixed well and kept overnight in a separating funnel for the extraction and purification of the pigment.

Thin layer chromatography

The pigment purification was done by using thin layer chromatography. Thin layer chromatography is a technique used to separate non-volatile mixtures. The prodigiosin pigment was separated using TLC plate coated with silica gel. A drop of sample was spotted on the baseline of the TLC plates at 1 cm interval and then allowed to dry at room temperature. The TLC plates were then placed in a pre-saturated TLC chamber containing mobile phase (methanol/water in the ratio 8:2 v/v). The plate was taken out and dried for few minutes then Rf value was calculated according to the following equation,

Rf=distance travelled by the compound/distance travelled by the solvent

UV-Vis analysis of pigment:

Spectral scan of the pigment was made on a UV-Visible spectrophotometer. Approximately 0.5 ml of pigment extract was taken in a cuvette and was scanned in the wavelength ranging from 200-800 nm. Methanol was used as blank.

Total genomic DNA isolation, PCR amplification and sequencing

Genomic DNA of the isolate was extracted using NucleoSpin® Microbial DNA isolation kit. Agarose gel electrophoresis was used for the separation and visualization of the extracted DNA. 16s rRNA genes were amplified using forward primer A2: 5’ AGAGTTTGATCCTGGCTCAG 3’and reverse primer S8: 5’ TCTACGCATTTCACCGCTAC 3’. The PCR product was detected by

agarose gel electrophoresis and was sent to AgriGenome Labs, Kakkanad, Kochi for sequencing (Dayamrita and Paul, 2021). The 16s rRNA genes were analyzed using NCBI Basic Local Alignment Search Tool (BLAST) and highly similar sequences were found. The sequence was deposited at GenBank and the accession number was obtained.

Results

Isolation of pigmented bacteria from soil sample

The collected soil samples consist of different types of pigment producing bacteria. The bacterial isolate showing bright red pigmentation was selected for further studies (Figure 1). Characteristics of selected bacteria is shown in table 1.

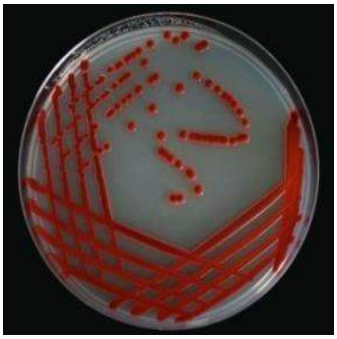


Figure 1. Colony morphology of *Serratia marcescens*

Table 1. Characteristics of *Serratia marcescens*

Colony characteristics	Observation
Size	Small
Shape	Round
Surface	Smooth
Elevation	Raised
Opacity	Opaque
Pigmentation	Bright red
Consistency	Viscous
Gram staining	Gram negative rod
Motility	Motile

Effect of physico – chemical factors on pigment production

The effect of different physico- chemical factors on pigment production was analysed by incubating the culture broth at different pH, salt concentration and temperatures for fifteen days. After 15 days of incubation, the extracted pigment was separated and concentrated using a separating funnel. By keeping pH at normal value, the broth was incubated at 2 different salt concentration (high and low) and at two different temperatures (40^{O}C and 30^{O}C). When incubated at 40^{O}C, culture broth with high salt concentration (9 g NaCl) has little more pigment production than medium with 1 g NaCl. Both medium has a cherry red pigmentation. When incubated at 30^{O}C, culture broth with high salt concentration (9 g NaCl) has more pigment production (deep cherry red) than medium with low salt concentration (pinkish yellow).

Similarly, by keeping salt concentration at normal value, the broth was incubated at 2 different pH (acidic, pH 2 and alkaline, pH 9) and at two different temperatures (40^{O}C and 30^{O}C). At 40^{O}C incubation, culture broth with alkaline pH, has more pigment production (brownish pink with slight growth) than acidic medium (light yellow pigmentation with no growth). At 30^{O}C incubation, medium with pH 9 (alkaline) gives wine red colour with high growth and at pH 2 (acidic), medium turned light yellow with no growth.

When comparing the above results, growth of bacteria and pigment production was favoured in room temperature (30^{O}C) and pigment production was high at alkaline pH and high salt concentration (Figure 2, table 2).

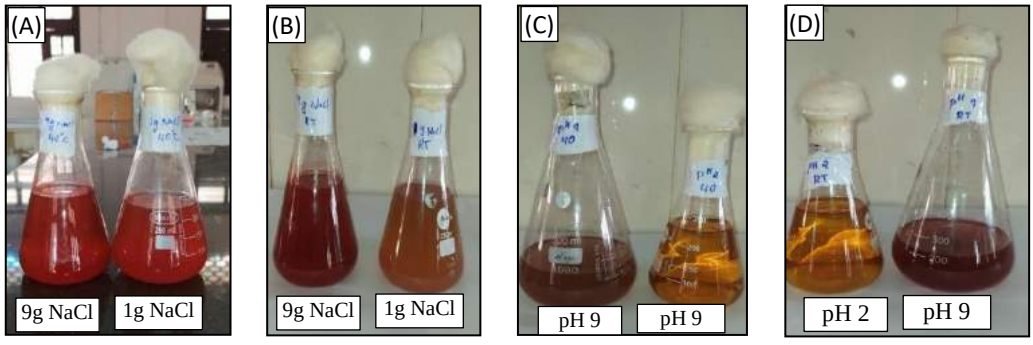


Figure 2. Effect of physico – chemical factors on prodigiosin production. A) pigmentation in 9 g &1 g NaCl conc. (40^{O}C), B) pigmentation in 9g &1g NaCl conc. (30^{O}C), C) pigmentation in pH 9 & pH 2 (40^{O}C), D) pigmentation in pH 2 & pH 9 (30^{O}C).

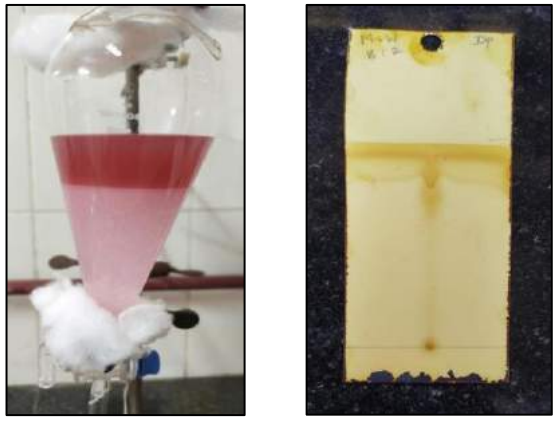
Table 2. Effect of physico chemical factors on pigment production.

Treatment number	pH	NaCl conc.	Incubation temp	Color of pigments
1	normal	9 g	40^{O}C	Cherry red
2	normal	1 g	40^{O}C	Cherry red
3	normal	9 g	30^{O}C	Bright cherry red
4	normal	1 g	30^{O}C	Pinkish yellow
5	9	normal	40^{O}C	Brownish pink
6	2	normal	40^{O}C	Light yellow
7	9	normal	30^{O}C	Wine red
8	2	normal	30^{O}C	Light yellow

Extraction and purification of bacterial pigment

For the extraction and purification of the bacterial pigments, the pigmented broth was mixed with solvent system (Chloroform,1:1) and was kept overnight in the separating funnel. After 24 hours, there formed an aqueous and an alkaline layer, aqueous layer at the bottom and a solvent layer with extracted pigment at the top. The solvent layer was carefully collected for further analysis (Figure 3 A).

The thin layer chromatography using solvent system (methanol: water) separated the pigment and showed a spot-on TLC plate when it is placed in an Iodine chamber. The Rf value of the pigment was calculated as 0.73 (Figure 3 B).



(A) (B)

Figure 3. A) Extraction of prodigiosin pigment B) Purification of prodigiosin pigment

UV-Visible spectral Analysis

The spectral scan of the pigments in broth sample were analysed at 200-800 nm wavelength. The absorbance for the pigment produced in normal growth condition was 321 nm. Maximum absorbance was shown by pigments produced at pH 9, 9g NaCl concentration and 30^{O}C temperature (figure 4).

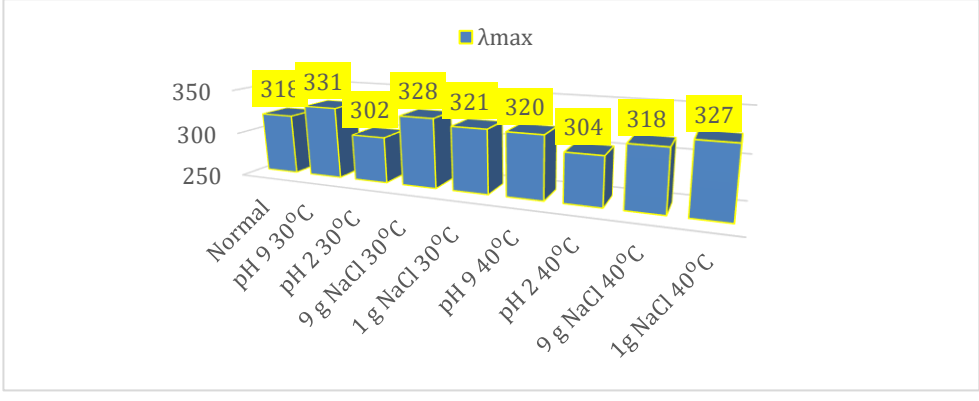


Figure 4. Effect of physico chemical factors on pigment production.

Molecular identification

The isolated genomic DNA from the bacteria was amplified. The 16SrRNA gene was amplified using 16S reverse and 16S forward primer. The 800 bp PCR product was purified for sequencing and clear DNA was visualized under UV transilluminator. NCBI Basic Local Alignment Search Tool (BLAST) was used to search for high scoring sequence alignment between query sequence and 16SrRNA sequence in the database. The nucleotide sequence comparison revealed 100% similarity with *Serratia marcescens* (Figure 5) and the GenBank accession number for the nucleotide sequence is MW287194.

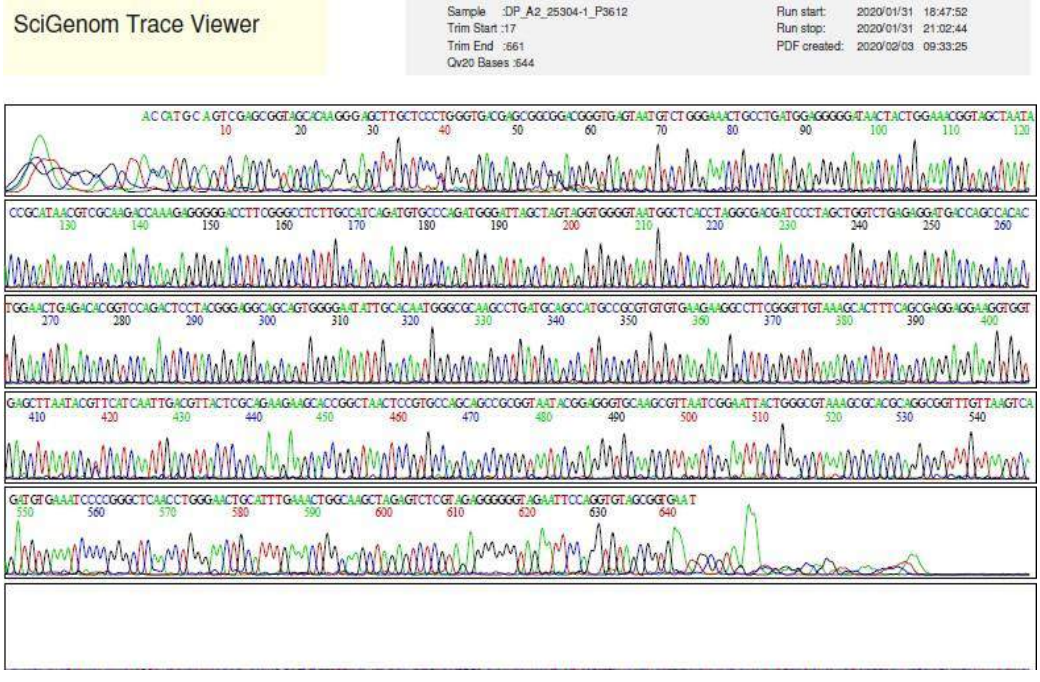


Figure 5. Sequence of *Serratia marcescens*

Discussion

In the present study, the pigment producing bacteria was isolated from flood affected soil. The 16S rRNA amplification of the isolate was sequenced to identify the bacteria. The comparison with 16S rRNA sequence available in the GenBank database revealed that the 16S rRNA sequence had 100 % similarity to that of *Serratia marcescens* and the GenBank accession number for the nucleotide sequence is MW287194.

Serratia marcescens produces a pigment called prodigiosin, it has many applications such as dyeing, antibacterial and anti-fungal activity. Effect of physico chemical factors on prodigiosin production was checked. The highest yield of prodigiosin pigment production was observed medium with 9 g NaCl, pH 9 and 30^{O}C incubation temperature. Extraction of pigment was carried out using Chloroform and TLC was carried out using methanol: water. The Rf value for the pigment was 0.73, which is same Rf value of prodigiosin in other studies (Bharmal *et al.*, 2012*).* Cerdeno *et al.* (2001) and Furstner *et al.* (2003) reported that prodigiosin pigment is a natural compound; it has antifungal, antibacterial, algicidal, antiprotozoal, antimalarial, cytotoxic, anticancer and antiproliferative properties.

Conclusion

In this study, pigment producing bacteria, *Serratia marcescens* was isolated from flood affected soil. Effect of physico chemical factors on prodigiosin production of *Serratia marcescens* was investigated. The highest yield for prodigiosin pigment production was observed in media containing 9 g NaCl, pH 9 and temperature 30^{O}C. Maximum absorbance was obtained for culture grown at pH 9, 30^{O}C (331 nm) and 9g NaCl, 30^{O}C (328 nm).

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ASSESSMENT OF HEAVY METAL CONTENTS IN THE SEDIMENTS OF VEMBANAD LAKE NEAR THANNEERMUKKOM BUND

Anjana Prasad, Chandini P K, Rajathy Sivalingam*, Ratheesh Kumar C S

School of Environmental Studies, Cochin University of Science and Technology, Kalamassery-682022, Kochi, Kerala

***Corresponding author email id: rajkumar@cusat.ac.in**

Abstract

Contamination of lake with accumulation of heavy metals is considered as a major area of specimen studies. One among them is the Vembanad Wetlands which is designated as Ramsar site. It is also classified as "Ecologically Sensitive Zone". Lake sediments of Thanneermukkom bund region of Vembanad lake was chosen as the study area for analysis of heavy metal contents. Analysis of the heavy metal concentration in the study area using Sediment Quality Guidelines (SQG), Geo accumulation Index and Contamination Factor revealed that the study area is not polluted. Aquatic life in the current study habitat indicated low to moderate risk according to Risk Assessment Code (RAC) and CPCB standards. This area is well known for its vast collection of aquatic entities. Bioaccumulation in black clam (*Villorita cyprinoides*), a species endemic to Vembanad lake was also evaluated. Bio-Sediment Accumulation Factor (BSAF) in this species revealed that Cd and As exceed FAO/WHO permissible limit. The study suggests that present chemical characteristics of sediment in the area does not favour remobilisation of metals into water and hence will not be a potential hazard to the biological system. The metal concentration in clam did not mirror the concentration in sediment.

Keywords: Heavy metal, Vembanad Lake, Bioaccumulation, SQG, Geoaccumulation Index.

Introduction

"Heavy metals" is a collective term, applied to a group of metals and metalloids with atomic density greater than 4 g/cm^3, or 5 times or more greater than water. They occur naturally in earth's crust and are found in soils, rocks, sediments, water and microorganisms with natural background concentrations (Tchounwou *et al.,* 2012). Anthropogenic release of heavy metals can give rise to higher concentrations of metals in the environment. Since heavy metals cannot be degraded or destroyed, they persist in environment (Mohammed *et al.,* 2011). Pollution of the biosphere with toxic metals poses major environmental/health problems (Leyval *et al.*,1997) as metals accumulate through food chains forming harmful metabolite compounds that affect biological functions. Terrestrial, aquatic and atmosphere are the entry sources of heavy metals and are carried in dissolved or particulate form. They are distributed in water column and in sediment.

The area in an estuary where fresh and saline waters mix, and the presence of cohesive sediments leads to the accumulation of chemicals. There is immense significance in the occurrence and distribution of metals in estuaries than in open sea. This ecosystem maintains a natural equilibrium due to the interdependence of biotic and abiotic factors among which abiotic factors shows more significance (Turner and Burt, 1985).

Biogeochemical cycling of metals is the substantial factor of estuarine environment. Hence sediments are treated as potential sink and source of organic/inorganic contaminants especially heavy metals depending upon the changes in environment like pH, redox potential, etc. The physicochemical properties of the sediment make it pollution vulnerable. They can hold more than 90% of metals in the aquatic environment thus acting as excellent

metal reservoir. As trace metals are unified and magnified by sediments, flocculation and adsorption processes aid in trapping and accumulation. Metals are removed from water by adsorption onto particulate matter. Settling of particulate matter sequesters sorbed metal into sediment occurs (Hemond and Benoit, 1988).

While assessing the environmental impact of metals, it is necessary to evaluate the whole metal concentration and its different chemical forms. The tendency and rate by which a metal participates in biological or geochemical process depends on the physiochemical form in which it exists. Even though sediments usually act as sink, they can become a source of these metals under certain conditions, especially in heavily contaminated areas or in drastically changing environments. Speciation helps to distinguish metals with a lithogenic origin. The sequential extraction procedure provides information on mobility and bioavailability of metals in the sediment. Heavy metals in both exchangeable (F1) and carbonate (F2) fractions of sediment are relatively mobile and are readily available for biological uptake, a process facilitated by the low pH. Reducible fraction (F3) metals associated with hydrous iron oxides and Mn-oxides, co precipitate or sorption onto pre-existing oxides coating and act as a sink for heavy metals under oxidizing condition. Mobility of metals in this fraction are redox potential dependent, i.e., relatively small changes in redox potential toward reducing conditions would cause reduction of Fe and Mn oxides. This causes dissolution of Fe and Mn oxide, thereby releasing associated metals. Metals in oxidisable (organic matter/sulphide) fraction (F4) are potentially available. They are considered less mobile due to their association with higher molecular weight stable humic substances. Their mobility can be achieved by decomposition processes and degradation of organic matter under oxidizing condition. Lithogenous/residual fraction (F5) is the most stable metal fraction in

soil and sediment since metals in these fractions are occluded in crystal lattice of silicate and well crystallized oxide minerals. They are considered as non-available metals and is natural in origin (Ladigbolu *et al.,* 2014).

Cochin Estuary is facing serious threat due to heavy metal pollution caused by rapid urbanisation/industrialization. Almost every study revealed that it is highly contaminated and the accumulation of heavy metals in the recent past has increased in Vembanad region. Hence this study aims to evaluate the total heavy metal content and degree of pollution in the sediment samples of Vembanad Lake near Thanneermukkom bund, to determine - the distribution pattern of heavy metals in sediments using sequential extraction technique, the bioavailability of heavy metals using RAC, heavy metal concentration in black clam *Villorita cyprinoides*.

Materials and methods

Study area

Thanneermukkom Bund, lying between 9°40'12.59" N 76°23'29.39" E, was constructed in 1974 in the narrower portion of the Vembanad lake to arrest saltwater intrusion into the nearby paddy fields in Alappuzha and is functional since 1976. This divides the lake into two different ecosystems, downstream region (Cochin backwaters) retaining estuarine character and upstream region (Kumarakom backwaters) transforming into freshwater habitat. Tidal flushing of the backwater system does not take place in summer season due to the closure of bund. This may lead to accumulation of toxic contaminants like heavy metals in the sediments of the lake.

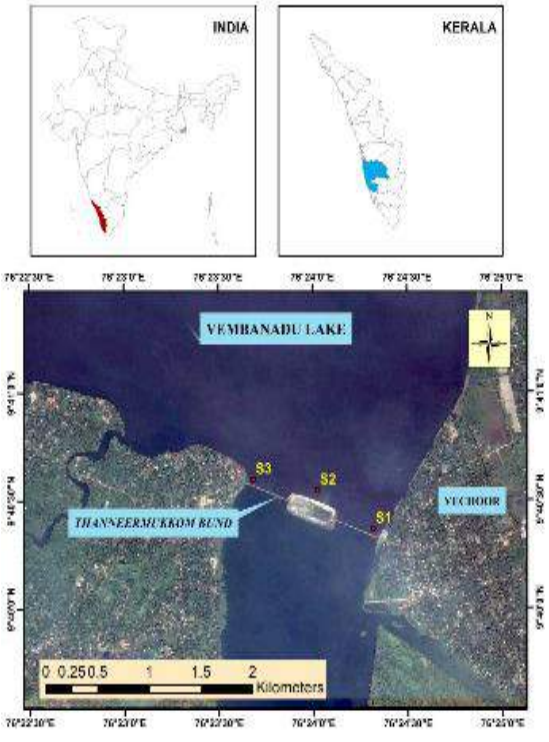

Figure 1. Map showing the study habitat

Sample Collection and Preservation

Surface sediment samples were collected from three locations along the Thanneermukkom bund of Vembanad lake in month of February, 2018 and named them as S_1 (east part of bund - towards Vechoor), S_2 (middle of bund) and S_3 west part of bund – towards Thanneermukkom). The sampling locations are shown in the Figure 1. Shutters of the bund were closed during the sampling period. The closure period is from 15th December to 15th March. Sediment samples were collected using a grab sampler, transferred to plastic bags stored under refrigeration until the analysis was performed. Black clams were collected from the study area. They were sealed in polyurethane bags and stored under refrigeration.

Sample Analysis

Chemical characteristics

Sediment samples were air dried for 4 days and then ground to fine powder using mortar and pestle. It is then sieved through ASTM standard stainless sieve 80 (0.180mm). The powdered samples were analysed for pH and organic carbon (Maiti, 2003). Clams were oven dried at 60˚C for 48 h. The flesh was scraped out of clams and was powdered using mortar and pestle.

Digestion of Metals

Digestion was carried out using aqua regia (1 HNO_3: 3 HCl). 1 g of sediment was taken in a conical flask. 2 drops of distilled water were added followed by the addition of 10 ml aquaregia. To the boiled solution, 5 ml of aquaregia was added. This step was repeated for five times. After cooling, 20 ml distilled water was added and filtered through Whatman 42 filter paper. The solution was made upto 100 ml in a standard flask (Sastre *et al*., 2002; Hseu *et al*., 2002). The metals in digested samples were analysed using Inductively Coupled Plasma – Mass Spectrometry (ICP –MS).

Metal fractionation in sediments

The metal speciation was carried out using sequential extraction scheme as shown in Table 1. (Tessier *et al.*,1979; Jain *et al.*, 2007; Mohan *et al*., 2012). 1g representative sample was used for sequential extraction of heavy metals. In each fractionation step, the extractions were carried out by centrifuging at 2,000 rpm for 30 min. The supernatant was filtered through Whatman No. 41 filter paper, and the residue was washed, shaken with 8 ml of deionized water for 30 min, and centrifuged. The extracted metals were detected with Inductively Coupled Plasma – Optical Emission Spectrometry (ICP –OES).

All the glassware and plastic containers were washed with 15% nitric acid solution and rinsed thoroughly with ultrapure water. The samples, chemical solutions and standards were prepared using ultrapure water. Working standards were prepared from 1,000 ppm standard stock solution of metals. The result of heavy metals in sediment is presented as µg/g dry weight.

Table 1. Sequential extraction procedure

Fractions	Extraction Procedure
Exchangeable (F1)	15ml of 1M $MgCl_2$ (pH = 7) was added to 1g sample at 30°C for 2 hrs with continuous agitation.
Bound to carbonates (F2)	15ml of 1M NaOAc was added to residue from step 1 and adjusted to pH 5.0 with 99.83% acetic acid and continuous agitation for 12 hrs at 30°C.
Bound to Fe- Mn oxides (F3)	Residue from step 2 was extracted with 15ml 0.04M $NH_2OH.HCl$ in 25% (v/v) acetic acid at 96 °C with occasional agitation for 12 hrs.
Bound to organic matter (F4)	5ml of 30% H_2O_2 adjusted to pH 2 with 0.02 M HNO_3 was added to residue from step 3 and was heated at 85˚C in a water bath for 3 hrs with occasional agitation. 1M NH_4OAc was added and kept for 24 hrs at room temperature. Then 3ml of 30% H_2O_2 (pH 2 with HNO_3) was added to the mixture and the sample was heated again in water bath at 85˚C for 3 hrs. After cooling, 5ml of 3.2 M NH_4OAc in 20% (v/v) HNO_3 was added. The sample was diluted to 100 ml and shaken for 30 min.
Residual (F5)	Residue from step 4 was digested with HCl-HNO_3 (3:1) mixture at 180˚C for 30 min, filtered and final volume was made upto 25 ml with ultrapure water.

Pollution extent and risk assessment

Sediment quality guidelines (SQG) applied in this study were CCME-ISQG: Canadian Environmental Quality Guidelines – Interim Sediment Quality Guidelines (2007), NOAA - TEL: National Oceanic and Atmospheric Administration –Threshold Effect Level.

Geoaccumulation index (I_{geo})

The I_{geo} enables the assessment of metal contamination in sediments by comparing current concentrations with pre-industrial levels (Qingjie *et al.*, 2008). I_{geo} is calculated using the following formula (Muller, 1969):

$$I_{geo} = \log_2 \frac{C_n}{1.5B_n}$$

where, C_n is the measured concentration of the metal (n) in the sediment and B_n is the geochemical background of the metal (n). The factor 1.5 is used for the possible variations of the background data due to lithological variations. Average shale standard for different metals reported by Turekian and Wedepohl (1961) was taken as background concentration throughout the study. Muller (1981) classified I_{geo} values into seven grades or classes (Table 2).

Table 2. Classification of contamination levels in sediments using the geoaccumulation index I_{geo} developed by Muller (1981).

Class	Geoaccumulation index (I_{geo})	Pollution status
Class 0	$I_{geo} < 0$	Unpolluted
Class 1	$0 < I_{geo} < 1$	Unpolluted to moderately polluted
Class 2	$1 < I_{geo} < 2$	Moderately polluted
Class 3	$2 < I_{geo} < 3$	Moderately to strongly polluted
Class 4	$3 < I_{geo} < 4$	Strongly polluted
Class 5	$4 < I_{geo} < 5$	Strongly to very strongly polluted
Class 6	$5 < I_{geo} < 6$	Very strongly polluted highest grade reflecting a 100-fold enrichment above baseline values

Contamination Factor (CF)

The level of contamination of lake sediment by given toxic substance (metals) suggested by HaKanson (1980), Goher *et al.* (2014), is expressed in terms of CF and is calculated as follows:

$$\text{CF} = \frac{\text{Metal content in the sediment}}{\text{Background level of metal}}$$

CF values of metals less than 1 indicates the good quality of sediment.

Risk Assessment Code (RAC)

The RAC classification is based on the percentage of metal in the carbonate and exchangeable fractions (Table 3).

Table 3. Classification of risk based on RAC

RAC(%)	Risk
<1	No risk
1 - 10	Low risk
11 - 30	Moderate risk
31 - 50	High risk
>50	Very high risk

Metal bioaccumulation in clam species

The bio-sediment accumulation factor (BSAF) is an index of the ability of a biological species to accumulate a particular metal with respect to its concentration in sediment.

$$\text{BSAF} = \frac{C_{\text{clam}}}{C_{\text{sediment}}}$$

where C_{clam} is the metal concentration in clam (mg/kg dw), and $C_{sediment}$ is the metal concentration in sediment (mg/kg dw]) (Abdallah and Abdallah, 2008; Islam *et al.*, 2015).

Results and discussion

Analysis of chemical characteristics of sediments

pH and percentage of OC (%) are given in table 4. From the present study, pH of S_1, S_2 and S_3 ranges from 5.82 -6.14. All the sediment samples were slightly acidic in nature. Highest OC and lowest pH were observed at S_2. Lowest OC and highest pH were observed at S_3 and S_1 respectively. A decrease in pH will increase the competition between metals and hydrogen ions for binding sites and may dissolve metal complexes, releasing free metal ions into the water column (Elith and Garwood, 2001). An increase in pH is generally accompanied by a decrease of the solubility of many toxic heavy metals in water (Avila Perez *et al*.,1999). It is often assumed that pH is the most important variable controlling the bioavailability of metals; however, OC also influence the availability of exchangeable metals (Arenas Lago *et al*., 2014; Chavez *et al*., 2015). Jo and Koh (2004) stated that organic carbon increased the concentration of heavy metals in soil thus preventing them from being complexed with other ions. Furthermore, there was a trend for increased exchangeable metals with increasing pH once pH > 5. At low-pH values (pH = 4), the release rate of metals into solution was lower than at mid-pH values. In aquatic sediments, the bioavailability of metals to benthic organisms depends not only on the chemical form of metals (Besser *et al.,* 2003; Riba *et al.,* 2004; Simpson, 2005), but also sediment geochemical properties (Nobi *et al.,* 2010; Rainbow, 2007) and varies exposure pathways of the organisms (Simpson *et al.,* 2012). Suspended Organic Matter (SOM), often operationally quantified as TOC, is an important amendment phase for metal-binding in oxidized sediments (Besser *et al.,* 2003). The vertical profile of TOC distribution shows an increase in the upper layer of sediment core but a decrease in the lower

portion (Fernandes *et al.*, 2011). SOM is believed to serve as a principal geo-sorbent for hydrophobic organic contaminants in soils and sediments (Hong *et al.*, 2010). In the fine sediments, particulate OC are well considered for their binding of metals, and its concentrations have been demonstrated to reduce the solubility and toxicity of many metals (Besser *et al.*, 2003; Di Toro *et al.*, 2005; Strom *et al.*, 2011).

Table 4. Chemical characteristics of sediments

Parameters	S_1	S_2	S_3
pH	6.14	5.82	5.89
Total organic carbon (%)	2.2	3	2

Analysis of heavy metals

Cu is relatively abundant in the earth's crust and ranges from 24 to 55 µg/g (Alloway,1990). Their background level in uncontaminated freshwater sediments ranges from 0.8 to 50 µg/g (Forstner and Wittmann, 2012). Previous studies showed that concentrations in the polluted sediment were upto 20.0 to 343.0 µg/g. The least concentration, 1.0 to 13.0 µg/g were observed (Santamaria-Fernandez *et al.*, 2006; Spencer and MacLeod, 2002; Sulivan and Taylor, 2003). The present study falls under unpolluted category as the concentration ranges from 2.79 to 7.24 µg/g.

Pb has an average concentration of nearly 13 µg/g and in uncontaminated soils it is nearly 10 µg/g (Craig,1986). The concentration in our study varied between 0.01 and 0.40 µg/g. Highest concentration was observed by Mohan *et al.*, 2012 from Cochin estuary studies and the value varied from 86.24 to 390.72. Also, Pb concentration of about 26 - 30 µg/g were reported in the sediments of polluted areas (Aluoupi and Angelidis, 2001; Leivuori, 1998; Everrarts and Fischer,1992). Leivuori (1998) found low levels ranging from

0.5–60 µg/g in the regions having unpolluted sediments. As the concentration recorded in the current study is less the sediment samples of study area are considered as unpolluted by Pb.

The average concentration of Zn in the earth's crust is 65 to 80 µg/g. A very high concentration of 4000 µg/g was reported in polluted areas. (Everrarts and Fischer, 1992; Spencer and MacLeod, 2002) and in unpolluted sites it ranged from 9 to 162 µg/g (Ridgway *et al.,* 2003). In comparison to these results, the concentrations of Zn in the samples of the present study (15.68 to 34.96 µg/g) are considered as unpolluted by Zn.

The average concentration of Cr in the earth's crust is about 100 µg/g (Nriagu,1988; Reimann and Caritat, 2012). The concentration varied between 0.15 and 1.76 µg/g. Rosales *et al.,* 2017 noticed low concentration in the range 0.16 to 0.93 µg/g. The highest concentration observed was 20.7–185.7 µg/g in Cochin estuary (Selvam *et al.,* 2012). The sediment samples of present study fall under unpolluted by Cr. Cd occurs in the earth's crust at concentrations between 0.1 and 0.5 µg/g. High levels, 20.5 to 130.0 µg/g were detected in the sediments of polluted areas (Svete *et al.,* 2001; Tack and Verloo, 1999) and low levels reported were 1.56 to 8.0 µg/g (Santamaria-Fernandez *et al.,* 2005; Svete *et al.,* 2001). Mohan *et al.,* 2012 noticed a high concentration in Cochin estuary. Thus, the sediment samples investigated in the present study (0.07 – 0.31µg/g) are considered as unpolluted by Cd.

The average concentration of Ni in the earth's crust is about 84 µg/g. Ni concentration varied between 4.22 to 8.88 µg/g. In unpolluted sites, it ranged from 0.07 to 2.36 µg/g (Rosales *et al.,* 2016) whereas in polluted site, it extended upto 91.8 µg/g (Martin *et al.,* 2012). The current study falls under unpolluted by Ni as the value is below the limit of NOAA sediment quality guidelines.

Mn occurs in the earth's crust at concentration of 950 µg/g. The lowest concentration is in the range 77.1–218.6 µg/g (Venugopal *et al.*,1982). The highest concentration is in the range 380–1946 µg/g (Martin *et al.*, 2012). In comparison to these results, the concentrations of Mn in the present study (84.33 –152.87 µg/g) are considered as unpolluted by Mn.

The average concentration of Mg in earth's crust is 23300 µg/g. High levels, 86.35 – 193.01 µg/g are detected in the present study. Low levels of about 0.02 to 2.3 µg/g were detected (Ratheesh *et al.*, 2010; Robin *et al.*, 2012).

Earth's crust is composed of 82300 µg/g of Al. In the present study about 72.24–231.54 µg/g of Al was detected. The value is smaller when it is compared to the value of Binish *et al.*, 2018, which was about 23968 – 14342 µg/g.

The comparison of concentration of heavy metals with SQG is given in Table 5. SQGs are scientific tools that synthesize information regarding the relationships between the sediment concentrations of chemicals and any adverse biological effects resulting from exposure to these chemicals (CCME, 1998). According to the analysis, even though the concentration of metals in sediment varies with samples, the concentration of metals in the present study is found to be low, even lower than CCME –ISQG and SQC NOAA – TEL guidelines. Chemical concentrations of sediment below ISQG values are not expected to be associated with any adverse biological effects. None of the studied metals found to exceed the value recommended concentration in sediment by CCME–ISQG and SQC NOAA–TEL guidelines. This indicates that the study area is considered as unpolluted as per the standard guidelines. Studies conducted at Sungai Kelantan, Malaysia recorded low concentration of metals in sediments and was free from serious metal pollution as per EPA guidelines (Dasar *et al.*,

2009). Meghna river water can be considered as unpolluted with respect to Cd,Cr,Mn and Zn according to SQG-USEPA,1989 (Hassan *et al.,* 2015). Lake Nasser (Egypt) sediment was not contaminated with Fe,Mn,Zn,Cr,Ni,Cu,Pb and Cd (Goher *et al.,* 2014).

Table 5. Comparison of sample values with sediment quality guidelines (SQG).

Toxic metals	**S_1 (µg/g)**	**S_2 (µg/g)**	**S_3 (µg/g)**	**CCME – ISQG (µg/g)**	**SQC NOAA – TEL (µg/g)**
Cu	4.41	7.24	2.79	35.7	35.7
Pb	0.01	0.4	0.22	35	35
Zn	34.28	34.96	15.68	123	123
Cr	1.29	1.76	0.15	37.3	37.3
Cd	0.19	0.31	0.07	0.6	0.6
Ni	8.87	8.88	4.22	NA	18
As	0.78	1.1	0.6	5.9	5.9

The concentration of metals observed in sediments in Vembanad estuary near Thanneermukkom bund was compared to the concentration of metals reported for Vembanad lake and Cochin estuary which is shown in table 6. The concentration of metal observed in the present study was lower than the values given by earlier studies. Sampling sites are located 43 km south of Cochin estuary. The study agrees with the observation made by Binish *et al.,* 2018 which indicated decreasing trend in concentration of selected heavy metals (Hg,Pb,Cu,Cd,Zn,Mn,Fe,Cr,Al and Ni) from north to south of the lake.

Table 6.Comparison of sediment metal concentrations with other studies and shale value

Location	Cu (ppm)	Pb (ppm)	Zn (ppm)	Cr (ppm)	Cd (ppm)	Ni (ppm)	As (ppm)	Mn (ppm)	Mg (ppm)	Al (ppm)	References
Cochin Estuary	17.1 - 28.2	-	134.3 - 347.9	-	-	22.2 – 59.1	-	77.1 – 218.6	-	-	Venugopal *et al.,* 1982
Cochin Estuary	-	30 - 190	35 - 780	20-145	0.5-8.4	-	-	-	-	-	Ouseph *et al* ., 1987
Cochin Estuary	1.5 - 15.5	6.0 - 18.0	20 - 118	1.0-34.0	0.2-1.6	10.0-46.0	-	-	-	-	Nair *et al* ., 1990
Cochin Estuary *	6.72	35.5	54.2	-	4.33	-	-	-	-	-	Balachandran *et al* ., 2006
Vembanad wetland system	24.1 - 49.4	15.7 - 54.4	136.5 - 211.3	1.8-2.4	0.3-0.7	49.4 – 64.0	-	310 – 860.9	-	-	Harikumar *et al* ., 2009
Cochin Estuary	1.2 - 4.18	0.3 - 34.5	82.5 - 741.9	4.9-89.4	nd-11	2.1-58.2	-	21.9 – 249.2	0.02-1.8	-	Retheesh *et al* ., 2010
Cochin Estuary	7.2 - 123.5	9.7 - 99.6	14.9 -2233	7-379.6	0.9-40.7	2.9-91.8	-	380 – 1946	-	-	Martin *et al* ., 2012
Cochin Estuary	4.6 - 54.6	17.3 - 46.7	29.8 – 973.7	20.7 – 185.7	0.07 – 10.5	9-78.5	-	93 - 1,086	-	-	Paneer selvam *et a* l., 2012
Cochin Estuary	-	31 - 54	-	-	1.2-3.4	-	-	-	0.2-2.3	-	Robin *et al* ., 2012
Cochin Estuary	4.4 - 26.4	3.9 - 26.1	3.13 – 333.8	7.5-64.9	0.7-4.3	6.83 – 61.9	-	48.8 – 263.8	-	-	Deepulal *et al* ., 2012
Cochin Estuary	49.92 - 504.67	86.24 - 390.72	571.2 - 2586.44	-	3.79 - 17.64	-	-	-	-	-	Mohan *et al* , 2012
Vembanad wetland system	58.05 - 137.22	34.65 - 78.35	66.61 - 563.67	88.24 - 156.59	0.27 - 9.09	35.53 - 64.38	-	194.83 - 552.29	-	23968 - 14342	Binish et. al., 2017
Thanneermukkom bund	2.79 - 7.24	0.01 - 0.40	15.68 - 34.96	0.15 - 1.76	0.07 - 0.31	4.22 - 8.88	0.6 - 1.1	84.33 - 152.87	86.35 - 193.01	72.24 - 231.54	Present study
Average shale value	45	20	95	90	0.3	68	13	850	15000	80000	Turekian, Wedepohl; 1961

(Source: Anu et al., 2014)

***** denotes the average value

Geoaccumulation index

According to Muller (1969) formula, calculated results of I_{geo} values of the sediments are given in table 7. As per Muller (1981) scale, I_{geo} values indicated that the sediments are unpolluted (I_{geo}< 0) for three sampling sites. Sediment of this site may be represented as reference for pre-industrial background of Cochin estuary.

Table 7. Geo-accumulation Index (I_{geo}) values of heavy metals of the sediment samples.

Toxic metals	I_{geo} (S_1)	I_{geo}(S_2)	I_{geo}(S_3)	Pollution status
Cu	-1.15	-1	-1.4	Unpolluted
Pb	-3.52	-2	-2	Unpolluted
Zn	-0.61	-0.6	-0.95	Unpolluted
Cr	-2	-2	-3	Unpolluted
Cd	-0.38	-0.16	-0.8	Unpolluted
Ni	-1.05	-1.04	-1.4	Unpolluted
As	-1.4	-1.22	-1.52	Unpolluted
Mn	-1.15	-0.92	-1.15	Unpolluted
Mg	-2	-1.15	-2.39	Unpolluted
Al	-3	-3	-3	Unpolluted

The CF values of all studied metals in the sediment samples were less than 1 as given in table 8 which implies sediment samples are of good quality.

Table 8. Contamination factor of studied heavy metals of sediments

Toxic metals	S_1	S_2	S_3
Cu	0.09	0.16	0.06
Pb	0.0005	0.02	0.01
Zn	0.40	0.37	0.16
Cr	0.01	0.01	0.001
Cd	0.63	0.93	0.23
Ni	0.13	0.13	0.06
As	0.06	0.08	0.04

Sequential extraction for selected heavy metals in sediment

The results of sequential extraction of selected heavy metals in sediment samples are given in Table 9.

Table 9. Concentration of metals in various metal fractions of sediments (a) Zinc (b) Copper (c) Chromium (d) Cadmium (e) Nickel (f) Arsenic

Zn	F1 (µg/g)	F2 (µg/g)	F3 (µg/g)	F4 (µg/g)	F5 (µg/g)
S_1	0.52	3.09	3.68	2.69	18.01
S_2	0.09	3.25	3.78	1.84	20.51
S_3	0.72	0.91	1.89	1.58	8.08

(a)

Cu	F1 (µg/g)	F2 (µg/g)	F3 (µg/g)	F4 (µg/g)	F5 (µg/g)
S_1	0.08	0.49	0.36	0.67	1.81
S_2	0.17	0.15	0.42	0.72	4.28
S_3	0.39	0.34	0.06	0.6	0.97

(b)

Cr	F1 (µg/g)	F2 (µg/g)	F3 (µg/g)	F4 (µg/g)	F5 (µg/g)
S_1	0.0075	0.09	0.2	0.097	0.59
S_2	0.06	0.08	0.31	0.15	0.96
S_3	0.0055	0	0.009	0.038	0.078

(c)

Cd	F1 (µg/g)	F2 (µg/g)	F3 (µg/g)	F4 (µg/g)	F5 (µg/g)
S_1	0.0075	0.02	0.023	0.02	0.089
S_2	0.027	0.04	0.0013	0.03	0.18
S_3	0.0024	0.001	0.009	0.01	0.038

(d)

Ni	F1 (µg/g)	F2 (µg/g)	F3 (µg/g)	F4 (µg/g)	F5 (µg/g)
S_1	0.55	0.13	0.96	0.02	4.87
S_2	0.04	0.05	0.71	0.25	4.95
S_3	0.01	0.03	0.34	0.64	2.61

(e)

As	F1 (µg/g)	F2 (µg/g)	F3 (µg/g)	F4 (µg/g)	F5 (µg/g)
S_1	0.02	0.08	0.19	0.04	0.35
S_2	0.009	0.008	0.13	0.05	0.85
S_3	0.02	0.009	0.08	0.08	0.30

(f)

The results revealed that all the selected heavy metals (Cu,Zn,Cr,Cd, Ni,As) showed higher percentage in the residual fraction which indicates natural origin of heavy metals. Since the concentration of all other fractions is low, the sediment is unpolluted with regards to the metals. This finding is supported by the results concluded through comparison with SQG and I_{geo}.

The sum of total fractions of each metal in various sediment samples is given in table 10. The cumulative amount of metal recovered during the five steps sequential extraction procedure was upto 72.16 – 89.47 % of that obtained by total metal analysis. This supports the near to accuracy of extraction procedure.

Table 10. Comparative analysis of total metal concentration and sum of extraction procedure

Toxic metals	Direct procedure (µg/g)	Sum of five extraction (µg/g)	Recovery (%)
Cu	14.44	11.51	79.09
Zn	84.92	70.09	82.54
Cr	3.2	2.54	72.16
Cd	0.57	0.51	89.47
Ni	21.97	16.16	73.55
As	2.48	2.21	89.11

RAC

The risk type based on the percentage of exchangeable and carbonate fraction is given in table 11. There is a medium risk percentage due to Zn and Cd in the sediment samples.

Table 11. The risk type based on the percentage of exchangeable and carbonate fraction

Metals	Samples	F1 +F2 (%)	Risk based on RAC (%)
	S_1 (µg/g)	16.7	Medium risk
Cu	S_2 (µg/g)	5.57	Low risk
	S_3 (µg/g)	30.56	Medium risk
	S_1 (µg/g)	13.15	Medium risk
Zn	S_2 (µg/g)	11.32	Medium risk
	S_3 (µg/g)	12.36	Medium risk
	S_1 (µg/g)	9.94	Low risk
Cr	S_2 (µg/g)	8.92	Low risk
	S_3 (µg/g)	4.23	Low risk
	S_1 (µg/g)	17.18	Medium risk
Cd	S_2 (µg/g)	23.1	Medium risk
	S_3 (µg/g)	5.67	Medium risk
	S_1 (µg/g)	10.41	Medium risk
Ni	S_2 (µg/g)	1.49	Low risk
	S_3 (µg/g)	1.09	Low risk
	S_1 (µg/g)	14.7	Medium risk
As	S_2 (µg/g)	1.52	Low risk
	S_3 (µg/g)	5.9	Low risk

The total bioavailable fraction is sum of exchangeable, carbonate, reducible and organic fraction. Metals bound to sediments can be released into aquatic environment when reduced sediments are transported to oxic environment due to natural processes such as bioturbation, tidal action, storm events, decreasing pH as well as human activities such as shipping and dredging. Also, the microbially mediated redox reactions result in reduction of some of the insoluble Fe (III) and Mn (II) oxides and release them into water. In this process, may also get into overlying water and benthic biota. The comparison of sum of bioavailable fractions with that of CPCB standards for inland surface water, The Environment Protection Rules, 1986 (µg/g) and WHO

standards for aquatic life (µg/g), Sudhira *et al.*, 2000 is given in table 12. Cu is within the limit of CPCB standards but exceeds WHO standards for aquatic life. Similar pattern is observed for Cr,Cd,Ni and As. But in the case of Zn, it exceeds both the CPCB limits and WHO standards and being most potential metal to cause risk to aquatic life. Similar comparative study was done by Singare *et al.,* 2013. It was based on the observation that heavy metals accumulated in lake sediments may enter the water thereby creating threat to aquatic life. It is expected that sediment analysis performed in the investigation will help in evaluating quality of the total ecosystem of the lake. It will also provide environmentally significant information about natural and anthropogenic influence on the water body.

Table 12. Comparison of sum of bioavailable fractions with that of standards

Toxic metals (µg/g)	Bioavailable fractions (F1+ F2+ F3+ F4)			CPCB standards for inland surface water The Environment Protection Rules, 1986 (µg/g)	WHO standards for aquatic life (µg/g), Sudhira *et al*., 2000
	S_1 (µg/g)	S_2 (µg/g)	S_3 (µg/g)		
Cu	1.6	1.46	1.39	3	0.005
Zn	9.98	8.96	5.10	5	0.03
Cr	0.40	0.60	0.05	2	0.1
Cd	0.07	0.10	1.02	2	0.0002
Ni	1.66	1.05	1.02	3	0.025
As	0.33	0.20	0.19	0.2	0.1

Metal bioaccumulation in clam species

Cd sustained highest BSAF for *Villorita cyprinoides* whereas the lowest are Cu and Ni. The observed concentration of Cd and As in clams was higher than the permissible limit recommended by FAO and WHO (2004). So, it can

be concluded that *Villorita cyprinoides* inhabiting in the present study area is unfit for consumption.

Analysis of heavy metal concentrations in the clam and sediment samples revealed no distinct relationship between heavy metal levels in clam tissues and sediments in which they thrive. Metal accumulation in the clams may not be directly or solely derived from sediments. Other sources of heavy metals in bivalve tissues are derived from living or dead suspended particles and from dissolved metals in the water (Huanxin *et al.,* 2000). The mean concentrations (mg/kg dw) of metals in sediments and clam species as well as mean BSAF is shown in table 13.

Analysis of total metal concentration, metals bound to various fractions and environmental indices showed that sediment of Vembanad lake near Thanneermukkom bund is not polluted. This indicates that the present chemical characteristics does not favour the remobilisation of metals into water and hence will not be a potential hazard to the biological system. The metal concentration in the clam did not reflect the concentration in the sediment.

Table 13. The mean concentrations (mg/kg dry mass) of metals in sediments and mussel as well as mean bio-sediment accumulation factor values (BSAF)

Metals	Concentration in clam (µg/g)	Mean Concentration in sediments (µg/g)	BSAF	FAO/ WHO (µg/g)
Zn	16.085	28.30	0.57	150
Cd	0.731	0.19	3.85	0.2
Cu	0.794	4.81	0.17	10
Ni	0.61	7.32	0.08	NA
Cr	1.128	1.07	1.05	NA
As	0.485	0.83	0.58	0.1

Conclusion

In the present study the sediment and black clam, *Villorita cyprinoides* were collected from Thanneermukkom bund from three sites and analysed heavy metals and fractionation of heavy metals. Cu, Cd, Cr, Ni, Zn, As are detected from the sediment samples of study area. Heavy metals in the sediment samples were low compared to the previous reports from the Vembanad Lake because the sampling locations were confined to the southern part, which is not affected by the industrial pollution from the Cochin Estuary region. The results were compared with various indices and pollution free condition was confirmed. Sequential extraction of selected heavy metals in the lake sediments was done to find the distribution of heavy metals in various fractions and bioavailability. Most of the metals were found in residual fraction. But if there is a change in the physico – chemical characteristics of sediments such as pH, oxic/anoxic conditions, organic and inorganic complexing agents, there is a chance for other fractions to become bioavailable. Based on this reason, Vembanad lake near Thanneermukkom bund is moderately contaminated sediment. RAC and comparison of bioavailable fraction with standards revealed that Cu, Cd, Cr, Zn, Ni and As exceeds WHO standards for aquatic life. *Villorita cyprinoides*, a clam species endemic to Vembanad lake is also studied for the heavy metal accumulation. BSAF in this species revealed that Cd and As exceed FAO/WHO permissible limit. They are one of the most commercially important species collected from Thanneermukkom bund and consumed by the large population daily their daily diet. So, the study recommends further monitoring and risk assessment of edible clams in the Thanneermukkom bund and Vembanad Lake.

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List of abbreviations

Fe	:	Iron
Mn	:	Manganese
Cu	:	Copper
Cr	:	Chromium
Mg	:	Magnesium
Ni	:	Nickel
Zn	:	Zinc
Al	:	Aluminium
As	:	Arsenic
OC	:	Organic Carbon
SOM	:	Suspended Organic Matter
SQG	:	Sediment Quality Guidelines
FAO	:	Food and Agriculture Organization
WHO	:	World Health Organisation
UNEP	:	United Nations Environment Programme
ICP MS	:	Inductively Coupled Plasma Mass Spectrometry
ICP –OES	:	Inductively Coupled Plasma – Optical Emission Spectrometry
CF	:	Contamination Factor
I_{geo} :	:	Geoaccumulation index
RAC	:	Risk Assessment Code

CCME-ISQG	:	Canadian Environmental Quality Guidelines – Interim Sediment Quality Guidelines
NOAA - TEL	:	National Oceanic and Atmospheric Administration– Threshold Effect Level.
BSAF	:	Biota Sediment Accumulation Factor
mg	:	Milligram
kg	:	Kilogram
µg	:	Microgram
g	:	Gram
dw	:	Dry weight

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ASTAXANTHIN FROM SHELL WASTE OF SHRIMP (*Fenneropenaeus indicus*) – ITS EXTRACTION AND IDENTIFICATION

Namitha Bhas, **Neethu M C** and **Anjana J.C**[*]

Department of Bioscience, SNGIST Arts and Science College,
Manakkapady, North Paravur,
Cochin-683520, Kerala.
Corresponding author email id: anjanaajc@gmail.com

Abstract

The single largest industrial waste in India, the shrimp waste processing, can cause diverse environmental problems. The aim of this study was to determine the extraction, characterization, and the antibacterial activity of astaxanthin isolates from waste of white shrimp shell (*Fenneropenaeus indicus*). Astaxanthin was extracted by using organic solvent, hexane: acetone (3:1) (v/v) from shrimp waste and analyzed by Ultraviolet-Visible spectroscopy which showed maximum absorbance at 470 nm. The moisture content, ash content and chitin estimation showed values 16.6 ± 0.03, 25.6 ± 0.8 and 26.67 ± 0.3 respectively. The antibacterial activity was studied on several organisms, and it showed excellent antibacterial activity against *Bacillus* sp. compared to other organisms. The astaxanthin thus isolated has the potential to be environment friendly colourant, apart from its other beneficial uses.

Keywords: Astaxanthin, carotenoids, pigment, spectroscopy, shrimp.

Introduction

Colour is a sensation, a visual perception experienced because of activation of certain classes of photoreceptor by a light wavelength from visible spectrum commonly expressed as VIBGYOR. Natural pigments have drawn the attention of industry as a safe alternative. Among the natural sources of

colourants, microorganisms offer great scope and hope. The different organisms - bacteria, yeast, algae, fungi and actinomycetes appear more efficient and act as an attractive source of biocolourants. Carotenoids are exogenously derived isoprenoid compounds which are responsible for pigmentation in crustaceans. Crustaceans and other aquatic animals are not capable of producing astaxanthin and stimulates astaxanthin production in its body by consuming phytoplankton and some microalgae, which is the primary source of astaxanthin. The red carotenoid, astaxanthin, has been identified as the predominant pigment isolated from *Penaeus* shrimp (Katayama *et al.*, 1971, Katayama *et al.*, 1972). *Fenneropenaeus indicus* is found at depths of 2 to 90 m, inhabiting bottom mud or sand. It is most abundant in shallow waters of less than 30 m depth, on sand or mud.

Astaxanthin is a lipophilic pigment and is also known as terpenes which come under the category of xanthophylls. It consists of two terminal rings connected by a polyene chain. Astaxanthin occurs in microalgae such as *Hematococcus pluvialis* and the yeast *Phaffia rhodozyma*. This high value pigment can be extracted by using organic solvent, acetone: hexane [3:1] (v/v), from shrimp waste such as carapace, cephalothorax, chelipeds and pereiopods etc. Synthetic astaxanthin is not in esterified form, which is the major difference between natural and synthetic astaxanthin. Astaxanthin showed potential effects against various diseases such as cancers, hypertension, diabetes, cardiovascular, gastrointestinal, liver, neurodegenerative and skin diseases. Astaxanthin, a therapeutic carotenoid, has more than 100 potential therapeutic effects (Senthamil and Kumaresan, 2015). Astaxanthin proves its bioactive properties as anti-inflammatory, anticancer and antioxidant.

In recent years, there has been a growing trend towards natural ingredients in food, due to increased concerns for consumer safety and regulatory issues over

the introduction of synthetic chemicals into the food chain. This is also true for the nutraceutical and cosmeceutical markets. While only a negligible part of today's market, the demand for such applications is expected to grow significantly in the near term because of numerous medical studies performed during the last five years in astaxanthin applications. The main objective of the present study is to extract natural pigment from shrimp waste and its identification which in future would help us to apply it as natural colourants in the food and textile industry.

Materials and Methods

Sample collection and Identification of Shrimp Waste

The shrimp was collected from the local market of North Paravur, Kochi, Kerala. This was identified as *Fenneropenaeus indicus* by Central Marine Fisheries Research Institute, Kochi, Kerala.

Preparation of Shrimp Waste

The portions utilized from the shrimp for extraction of Astaxanthin were cephalothorax, chelipeds and pereiopods. The waste was washed under running tap water to remove any adhered flesh and dust particles and dried under shade. Dried shell was weighed, and the samples were packed in polyethylene bags and stored at a temperature of 4^{o}C until use.

Extraction of Astaxanthin

Five grams of shrimp waste was ground with hexane: acetone (3:1) solvent in a laboratory mixer, and the filtrate was filtered using Whatmann filter paper. The filtrate was collected in a conical flask, 12 ml of petroleum ether (BP-40-60°C) and 0.73% sodium chloride was added. The mixture was separated using a filtration method. The filtrate was at the top and sodium

chloride at the bottom, then the epiphase was collected by using a separating funnel. Finally washed with water by mixing equal amounts of distilled water into the epiphase; water separated at the bottom and the above phase was collected. The petroleum ether is evaporated by keeping it in a water bath at 50 °C (Senthamil and Kumaresan, 2015).

Determination of Proximate Composition of Shell Waste (Khanfari et al., 2007)

Moisture Content Determination

Two grams of the sample was dried in a hot air oven at 105°C by heating it for 3 hours. The dried samples were put into desiccators, allowed to cool and reweighed. The process was repeated until a constant weight was obtained. The difference in weight was calculated as a percentage of the original sample.

$$\text{Percentage moisture} = \frac{W2 - W1}{W2 - W3} \text{ x } 100$$

where W1 = Initial weight of empty dish; W2 = Weight of dish + undried sample; W3 = Weight of dish + dried sample

Ash Content Determination

Two grams of sample was heated in a moisture extraction oven for 3h at 100°C before being transferred into a hot air oven at 150°C until it turned white and free of carbon. The sample was then removed from the oven, cooled at room temperature, and reweighed immediately. The weight of the residual ash was then calculated as

$$\text{Ash Content Percentage As} = \frac{\text{Weight of ash}}{\text{Weight of original of sample}} \text{ x } 100$$

Estimation of Chitin

0.5 gram of dry sample was ground and digested in 100 ml of 2% sodium hydroxide at 100°C for one hour. The digested residue was transferred to a beaker, treated for 12 hours at room temperature with 100ml of 5% hydrochloric acid and re-filtered on the sintered glass crucible. The residue was washed with hot distilled water until there was a negative test for chloride. The crucible was dried at 110°C for 16 hours and weighed.

$$\text{The percentage chitin} = \frac{(W2 - W1)}{W} \text{ x } 100$$

where, W1 = weight of the crucible; W2 = weight of the crucible and residue
W = weight of the sample in grams; (W2 - W1) g = weight of residue in grams

Estimation of Proteins

The protein content was estimated according to the method of Lowry et al., 1951 and bovine serum albumin was taken as the standard reference.

Carbohydrate Determination

1 ml of DNS reagent was added to all the test tubes (standard glucose and test sample) and plugged the test tubes with cotton and kept the test tube in a boiling water bath for 5 minutes. Test tubes were cooled and read extinction at 540 mm against the blank (Miller 1959).

UV-Vis Spectrophotometer Analysis

The extracted astaxanthin is scanned under Ultraviolet-Visible scanning spectrometer by measuring the absorbance between 300nm-700nm and the maximum absorbance of the extract was determined.

Determination of amount of Astaxanthin

The amount of astaxanthin present in the filtrate can be identified by using below equation (Senthamil and Kumaresan, 2015)

$$\text{Carotenoid as AST (µg/g BP)} = \frac{A \times D \times 10^6}{100 \times Wx\ E1\ cm}$$

where, A is the absorbance at 470 nm, V is the volume of pigment recovered, D is the dilution factor, W is the weight of sample and E 1% 1cm is the extinction coefficient.

Profiling techniques for identification of astaxanthin in shrimp shell waste
Thin Layer Chromatography Analysis

Analysis of pigment in the shrimp shell extract was done using thin layer chromatography (TLC) (Todd 1998). Acetone and hexane are the major organic solvent used in pigment analysis.

Antimicrobial Assay

Test Pathogens- The cultures used were *Bacillus* sp., *Escherichia coli, Klebsiella* sp., *Pseudomonas* sp. and *Staphylococcus* sp. The isolates were subcultured and stored at 4 ^{o}C.

Agar-Well Diffusion Method

The antibacterial activity of the extract was tested against the selected Gram-positive and Gram negative bacterial strains using agar well diffusion method (Pere et al., 1990). The wells of 6 mm size were cut in the agar plates with the help of sterile cork borer and the wells were loaded with various concentrations of extract [100 µl (1 mg/ml)]. The positive (Amoxicillin and amikacin for gram positive and negative respectively) and negative controls were also used. All the plates were

incubated at 37 ^{o}C for 24 - 48 hours. The zone of inhibition of bacterial growth was measured in millimetres and recorded.

Results

The moisture content, ash content and chitin estimation values of the dried sample obtained were 16.6±0.03, 25.6±0.8 and 26.67±0.33 respectively. 1.3 mg of protein and 0.77 mg of sugar was present in 10.5±1.6 mg dry weight sample. The maximum absorbance of the extract was measured at 470 nm in the UV-VIS spectrophotometer (Figure 1). The carotenoid astaxanthin was quantified in the shrimp shell extract of *Fenneropenaeus indicus* at 470 nm in hexane and acetone was 27.482 µg/g. The presence of astaxanthin was confirmed by orange-coloured spots on thin chromatography analysis. The Rf values obtained were 0.76, 0.65 and 0.33 for spot 1, 2 and 3 respectively. Antimicrobial assay using well diffusion method showed excellent antibacterial activity of the extract against *Bacillus* sp. compared to other organisms (table 1).

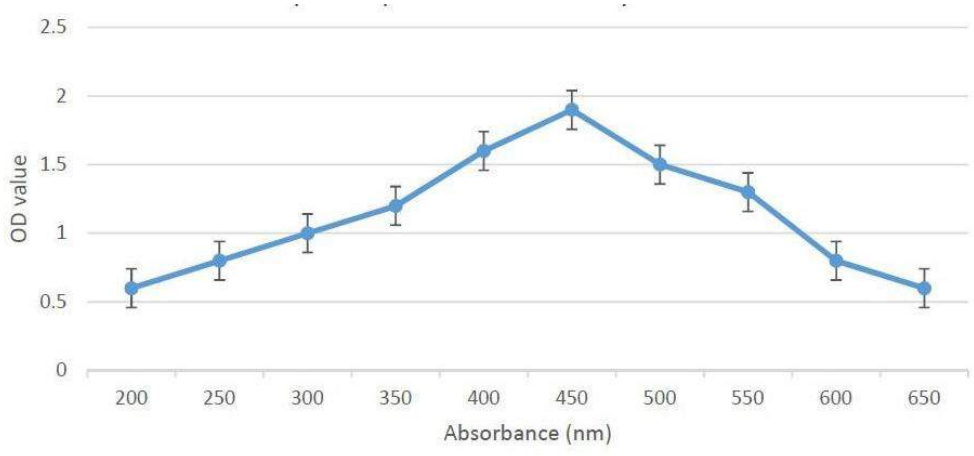

Figure 1. Spectrophotometric analysis of the shrimp extract

Table 1. Table showing Zone of inhibition (mm) of extract

Sl.No.	Bacterial Strains	Zone of Inhibition of extract (mm)	Zone of Inhibition by Antibiotics (mm)
1	*E.coli*	18	20
2	*Klebsiella* sp	19	22
3	*Bacillus* sp	22	20
4	*Pseudomonas* sp	-	18
5	*Staphylococcus* sp	-	19

Discussion

The industrial shrimp shell discards can be utilized for the isolation of important bioactive compounds like natural carotenoids, mainly astaxanthin which has a unique molecular structure that gives it special chemical and biological properties. The proximate composition of dried shrimp shell waste moisture content, ash content and chitin were estimated to be 16.6 ± 0.03, 25.6 ± 0.8 and 26.67 ± 0.3 respectively. Proximate composition of shrimp shell waste varies with species and many other factors. The proximate composition of prawn waste as 75-80 % moisture, 30-35 % ash (dry basis) and 15-20 % chitin (dry basis) was reported (Kobayashi and Sakamoto, 1999). The values observed in the present study also correlated with the above values.

The extracted astaxanthin is scanned under Ultraviolet-Visible scanning spectrometer by measuring the absorbance between 200nm-700nm. In the present study, the maximum absorbance was measured at 470 nm and the solvent used in this study was hexane:acetone (3:1). The carotenoid astaxanthin was quantified in extract at 470 nm and the same was reported by Senthamil and Kumerasan, 2015.

The Thin layer chromatographic separation of carotenoid extracts from *Fenneropenaeus indicus* yielded three distinct bands. The Rf values for the

three bands were 0.33, 0.65, 0.76 which corresponded to astaxanthin, astaxanthin monoester, astaxanthin diester respectively. The Rf values obtained for astaxanthin monoester and astaxanthin diester are in agreement with the results reported by Khanfari et al., 2007, which was 0.60 for astaxanthin monoester and 0.75-0.85 for astaxanthin diester. The Rf value of astaxanthin obtained in the present study was in accordance with the Rf value obtained for the standard astaxanthin reported by Todd 1999.

The antibacterial activity was studied on several organisms like *Bacillus* sp., *Escherichia coli*, *Klebsiella* sp., *Pseudomonas* sp. and *Staphylococcus* sp. The extract showed excellent antibacterial activity against *Bacillus* sp. than the standard Amoxicillin. (Table 1). The reason for the difference in sensitivity between Gram positive and Gram negative bacteria could be attributed to the morphological differences between these microorganisms, Gram negative bacteria having an outer polysaccharide membrane carrying the structural lipopolysaccharide components which makes the cell wall impermeable to lipophilic solutes, unlike the Gram positive bacteria that has only an outer peptidoglycan layer which is not an effective permeability barrier (Scherrer and Gerhardt, 1971).

Thus, the pigment was isolated and characterized for various properties. The biological function and stability of the astaxanthin pigment is more compared to other carotenoids was evaluated for application as a dye in the textile industry and indicated that this pigment could be used as a natural dye for imparting red- orange colour to various grades of textile materials. The current study has put a major effort into extracting Astaxanthin from biological sources instead of synthetic ones and also to increase the productivity of natural Astaxanthin thus making it less expensive. Astaxanthin is considered to have

more than 100 medicinal properties when compared with other carotenoids and nutrients. Moreover the utilization of such large quantities of shrimp processing discards for recovery of bioactive molecules such as carotenoids would not only reduce the disposal problems associated with these wastes, but also enhance the economy of shrimp processing. Especially in recent years, the demand for Astaxanthin has surged in the nutraceutical market due to its potential human health benefits, textile and food industry.

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CHARACTERIZATION OF METABOLICALLY ACTIVE RHIZOSPHERE ASSOCIATED BACTERIA IN THE MANGROVES OF PUTHUVYPEEN, KERALA

Wafha K A, Annie Feby and Baby Divya*

Department of Zoology, St. Xavier's College for Women, Aluva-683101, Kerala
*** Corresponding author email id:** babydivya@stxaviersaluva.ac.in

Abstract

Mangroves play an important role in cycling energy and nutrients and are the crucial exporters of organic matter to estuaries and coasts. These ecosystems provide a unique ecological environment for diverse rhizosphere associated bacterial communities that decompose the mangrove litter and recycle the nutrients making it available for the mangrove trees. In the present study, the metabolic activity and diversity of rhizosphere associated bacteria from the mangrove trees of Puthuvypeen is characterized. Nitrogen fixation was the major activity shown by the isolates with about 64-90% of the isolates from all the rhizospheres showing the activity. The highest activity with 90.4 % was shown in *Avicennia* sp. Nevertheless, the isolates from the rhizosphere of *Rhizophora* sp. showed the highest activity for amylase, cellulase, lipase and potassium solubilisation activity. About 41 different genera were obtained from the four rhizospheres. A few of the major bacterial genera observed were *Paenibacillus* sp, *Bacillus* sp., *Alivibrio* sp, *Burkholderia* sp, *Sphingomonas* sp, *Psuedomonas* sp. *Excoecaria* sp. recorded the highest Shannon Weiner diversity index with 3.09 followed by *Rhizophora* sp. and *Avicennia* sp. Interestingly, *Paenibacillus* was the abundant genus in most of the rhizospsheres followed by *Bacillus* sp. Thus, the present study showed that mangrove rhizosphere is a repertoire of remarkable bacterial metabolic activity and diversity.

Keywords: Bacteria, Rhizosphere, Mangrove, Diversity

Introduction

Mangrove ecosystems cover vast areas of tropical and subtropical coastal zones. Their composition varies and is influenced by the tidal forces as well as high concentrations of dissolved nutrients and decomposing organic matter (Holguin *et al.*, 2001). Numerous microorganisms, especially the autochthonous bacteria, closely associated particularly in the rhizosphere are the key players in the biogeochemical cycling in mangroves (Alongi, 1994, Singh *et al.,* 2005). Mangroves which are in the ecotone of marine and terrestrial ecosystems are prone to wide fluctuations in physico-chemical factors and so are the bacteria closely associated with the roots of mangrove trees which shows high adaptability. These diversified bacteria are important in sequestering nutrients and hence the mangroves are highly productive in the nutrient limited mangrove sediments (Kathiresan and Bingham, 2001). Though the bacterial diversity of the mangrove sediments of Puthuvypeen has been studied (Ammini *et al.,* 2021), the metabolic characterization of bacteria associated with the mangrove rhizosphere has not been conducted. The rhizosphere is a microbial hotspot, and the microbiome interacts with the host plant thereby extending the functional repertoire of the plant (Bakker *et al.,* 2013). Among the various microbial communities surrounding the mangrove rhizosphere, bacteria are considered the largest contributor in the nutrient cycling and occupying large niche (Alongi *et al.,* 1993, Holguin *et al.,* 1999). The bacterial communities are considered as the primary decomposers (Loka Bharathi *et al.,* 1991) in the mangrove sediments, due its fundamental role in nitrogen fixing, methane cycling, ammonia oxidation, phosphate solubilisation, and sulfate reduction (Liu *et al.,* 2018, Bhattacharyya *et al.,* 2015, Ghizelini *et al.,* 2012). The present study attempts to characterize the metabolic activity and diversity of these primary decomposers from rhizospheres of four different mangrove trees in Puthuvypeen.

Materials and Methods

Area of Study

Puthuvypeen (10.01^{o} N, 76.23^{o} S) which is an important mangrove patch with many mangrove trees in western suburb of Kochi City, a part of Vypin Island (24 km long and 2.6 km wide) was selected for the study. It is bordered by Vembanad Lake to the east, Arabian Sea to the west and south and Njarakkal to the north.

Collection of samples and bacterial analysis

The rhizosphere sediments were collected from four mangrove trees such as *Rhizophora sp.*, *Avicennia* sp., *Brugeria* sp. and *Excoecaria* sp. based on their position from seaward side to landward side (Figure 1). The samples were collected during monsoon and non-monsoon seasons.

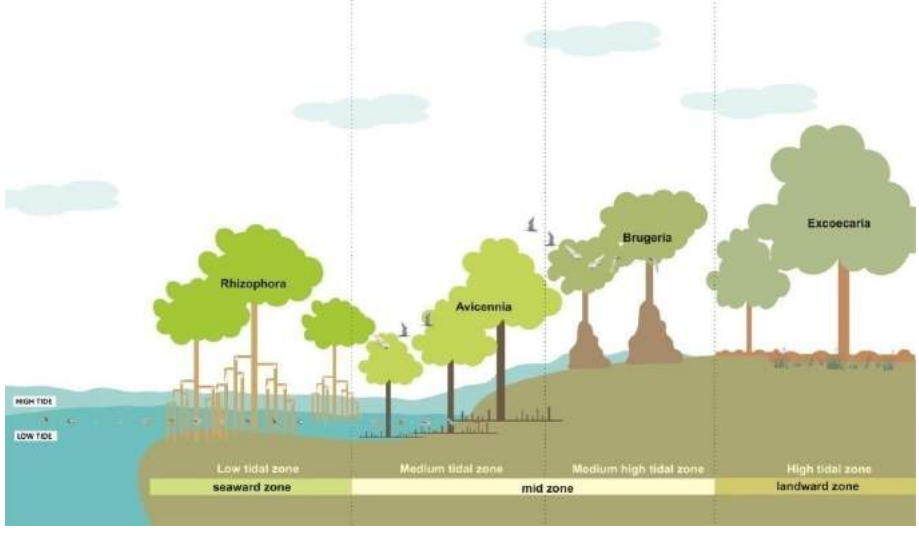

Figure 1. Mangroves (*Rhizophora* sp., *Avicennia* sp., *Bruguiera* sp and *Excoecaria* sp.) selected for the study based on their habitat

The sediment samples were collected aseptically and transported to laboratory under refrigerated conditions. The samples were serially diluted

and were plated on nutrient agar medium. Morphologically similar bacteria were isolated and purified for the study. The isolates were also tested for enzymatic activity for amylase, protease, lipase, cellulase, phosphatase, potassium solubilisation and nitrogen fixation using standard methods (Gerhardt, 1994). The isolates were further characterized by biochemical tests and later identified using ABIS online software (Stoica, 2021) to characterize genus of the organism. Significant variations in metabolic and generic diversity were estimated using statistical tools like ANOVA-one way and diversity was calculated based on Shannon Weiner diversity index using Primer 7 software.

Results

Enzymatic activity was observed in majority of the isolates in all the mangrove trees. The nitrogen fixation was the major activity shown by the isolates. About 64-90% of the isolates from all the rhizosphere showed nitrogen fixation activity (Figure 2). The highest with 90.4 % was shown by *Avicennia* sp. In addition, isolates from the rhizosphere of *Rhizophora* sp. showed the highest for amylase, cellulase, lipase and potassium solubilisation activity. However, the highest phosphatase and protease activity was shown by bacteria associated with Excoecaria (8.1%) and *Avicennia* (19.8 %). Statistical analysis showed significant variations in the metabolic activity by the rhizosphere associated bacteria in the four mangrove trees ($P < 0.05$).

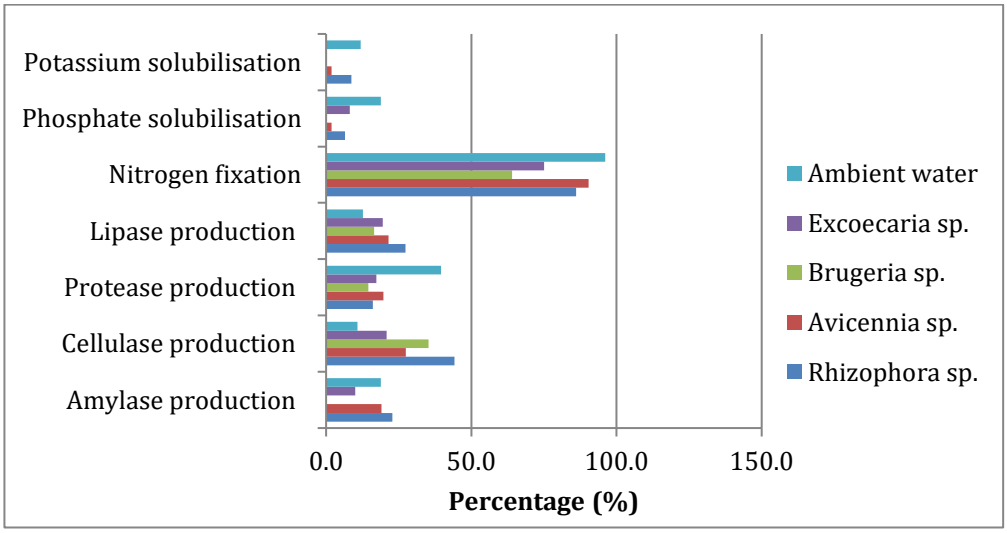

Figure 2. Enzymatic activity of rhizosphere associated bacteria

About 41 different genera were obtained from the four rhizospheres. A few of the major bacterial genera observed were *Paenibacillus* sp., *Bacillus* sp., *Alivibrio* sp., *Burkholderia* sp., *Sphingomonas* sp., *Psuedomonas* sp. *Excoecaria* showed the highest number with 21 genera and lowest by *Brugeria* sp. with 10 genera. Less than 25% of the isolates in *Rhizophora* sp., *Avicennia* sp. and *Brugeria* sp. were in the unidentified group while it was less than 30 % in *Excoecaria* sp.. Interestingly, *Paenibacillus* sp. was the abundant genus in *Rhizophora* with 13.7 %, *Avicennia* sp. 23.4 % and *Excoecaria* sp.16.8 %. This was followed by *Bacillus* sp. with 11.7 % in *Rhizophora* sp., 12.2 % in *Avicennia* sp. and 7.4 % in *Excoecaria* sp.. However, in *Brugeria* sp. the most abundant genus was *Bacillus* sp. with 12.9 % followed by *Ralstonia* sp. with 9.6 %. In the ambient water the most abundant was *Bacillus* 23.8% followed by *Alivibrio* sp. with 13.5%. The average frequency of bacterial distribution analysed and the dominant genera in each mangrove species are depicted in the Figure 2. In addition, as for metabolic diversity, statistical analysis by means of ANOVA showed significant variation even in the generic diversity of the rhizosphere associated bacteria in the four mangrove trees ($P < 0.05$).

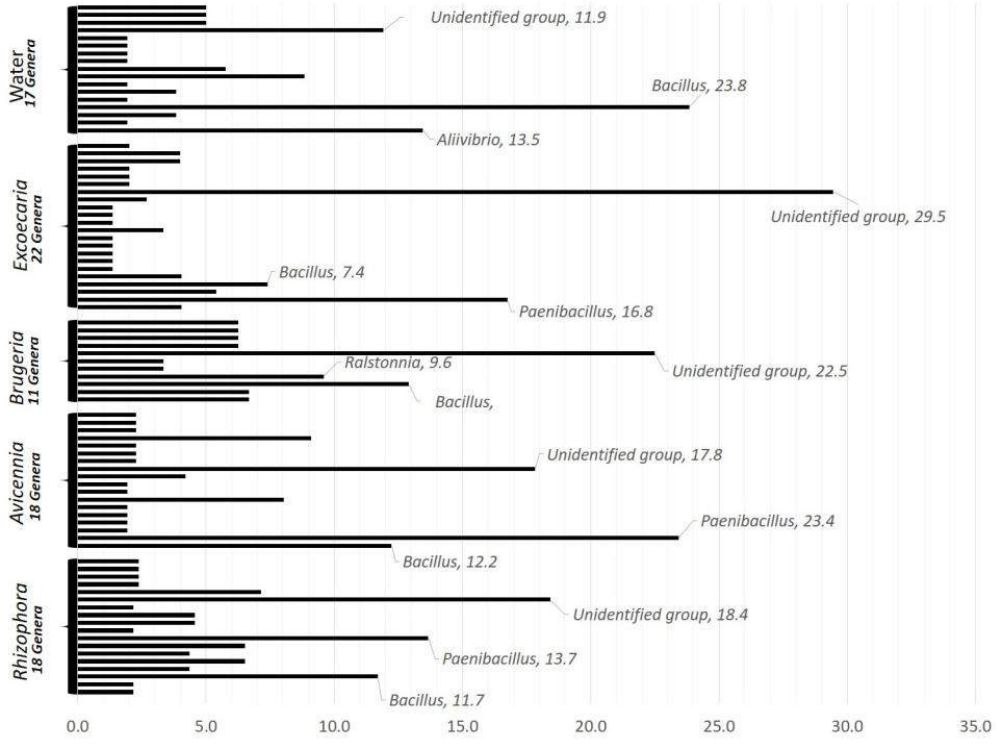

Figure 2. Distribution of rhizosphere associated bacteria in percentage and the dominant genera in each mangrove species

The highest Shannon Weiner diversity index was observed in *Excoecaria* sp. (3.09) followed by *Rhizophora* sp. and *Avicennia* sp. (2.89). The species evenness of the rhizosphere associated bacteria for the four mangroves was the maximum with a value of 1. The various diversity indices observed in the study are given in Table 1.

Table 1. Diversity indices of rhizosphere associated bacteria of the four mangrove species

Indices	***Rhizophora* sp**	***Avicennia* sp.**	***Brugeria* sp.**	***Excoecaria* sp.**
Total Genera (S)	18	18	11	22
Margalef Richness (d)	5.88	5.88	4.17	6.7
Pielou's Evenness (J)	1	1	1	1
Shannon Weiner index (H')	2.89	2.89	2.39	3.09

Discussion

Mangrove forests are regularly flooded with sea water and have to cope with high temperature, low relative air humidity, high and changing salt concentrations, hypoxia and even nutrient availability due to regular inundation (MacFarlane *et al.,* 2007; Agoramoorthy *et al.,* 2008, Robert *et al.,* 2012). These environmental parameters not only influence mangrove vegetation but also alter bacterial population and diversity. The association between mangrove flora and the microbial community plays fundamental role in the efficient functioning of mangrove ecosystem (McLusky, 2013). One of the significant roles of microorganism in the mangrove sediments is the nutritional supply of nitrogen, the most limiting nutrient in mangroves vegetation (Lovelock *et al.,* 2006). Nitrogen (N_2) fixation is common in mangroves mainly carried out by microbes regulated by the litter decomposition and accompanied by the release of nutrients (Wieder and Lang, 1982). In the present study, nitrogen fixation was the major activity shown by majority of isolates. About 64-90% of the isolates from all the rhizosphere showed nitrogen fixation activity. Remarkable bacterial nitrogen fixation in *Avicennia* sp. has been reported from mangrove of Pichavaram and Beachwood Mangrove Nature Reserve (Mann and Steinke, 1992; Baskar and Prabakaran, 2015). In this study the highest with 90.4 % was shown by *Avicennia* sp.

Phosphorous is one of the limiting elements in the mangrove (Reef *et al.,* 2010) as it exists as ionic forms of orthophosphoric acid and contributing to only 0.1% of total soil Phosphorous is available to mangrove. Current study shows highest phosphatase activity by the isolates of *Excoecaria* sp. (8.1%). Usually in mangrove rhizosphere, average 10–15% of bacteria are found to participate in phosphate solubilizing activity (Pupin and Nahas, 2014). These microbes transform the phosphate into usable form, which enhance availability of phosphorous to mangroves (Kucey *et al.,* 1989, Ghosh *et al.,* 2012).

Similarly, potassium is another limiting nutrient in mangroves and the availability of potassium is variable in the sediments (Ukpong, 1997). The high salinity in mangrove sediments can make sodium ions interfere with potassium ion uptake thus making potassium uptake reduced (Maser *et al.,* 2002). Here, the highest potassium solubilisation activity was shown by isolates from *Rhizophora* sp. However, its presence increases the solubility and availability of potassium ions to mangrove plants (Meena *et al.,* 2016).

A wide variety of microbes residing in sediment have significant role in mediating nutrient cycling by means of enzymes (Turan *et al.,* 2017). In mangrove sediments, these microbial enzymes can solubilise nutrients to make available to them and provide a base for food web (Spalding *et al.,* 2010). Bacterial isolates from the rhizosphere could perform many enzymatic activities of which *Rhizophora* sp. showed the highest for amylase, cellulase, lipase production. However, the highest protease activity was shown by *Avicennia* sp. with 19.8 %. Interestingly, these microbes have the potential to synthesize various enzymes in sediments even in high salinity, organic matter and low aeration observed in mangroves (Dias *et al.,* 2009). The mangrove provides large quantities of organic matter in the form of detritus (Bano *et al.,* 1997) and only 30–50% of this organic matter is leachable, the remaining being in unusable form later is degraded by microbial action (Cundell *et al.,* 1979). Bacterial enzymes such as amylases, proteases, esterases and lipases contribute significantly in nutrient cycling and organic matter degradation (Dias *et al.,* 2009). Therefore, in mangrove environment, the microbes themselves play an important role in the decomposition of organic matter and mineralization of organic compounds for plant nutrition (McGuire *et al.,* 2012).

The most predominant bacterial genera observed in this study are *Paenibacillus* sp., *Bacillus* sp., *Alivibrio* sp., *Burkholderia* sp., *Sphingomonas* sp.,

Pseudomonas sp., 34 other genera were observed in the selected rhizospheres. The genera obtained have been similar from those obtained in other studies conducted in the various mangrove system (Vasquez *et al.,* 2000, Sengupta and Chaudhari 1990, 1991, Holguin *et al.*, 1992, 2001, Das *et al.,* 2006, Gray and Herwig 1996, Mishra 2009). Vasquez *et al.,* (2000) isolated various strains of *Avicennia* rhizosphere-associated bacteria such as *Bacillus amyloliquefaciens, B. atrophaeus, Paenibacillus macerans, Xanthobacter agilis, Vibrio proteolyticus, Enterobacter aerogenes, E. taylorae, E.asburiae*, and *Kluyvera cryocrescens* from Mexican mangroves. *Paenibacillus* sp. was the abundant genus in *Rhizophora* sp., *Avicennia* sp. and *Excoecaria* sp. followed by *Bacillus* sp. in this study. *Paenibacillus* sp. and *Bacillus* sp. are known to be highly halo tolerant and associated with rhizosphere of various mangroves, contributing immensely to nutrient cycling and plant nutrition (Dias *et al.,* 2009, Vasquez *et al.,* 2000, Baskar and Prabakaran, 2011, Liu *et al.,* 2017). Almost a quarter of the isolates in all rhizospheres were grouped as unidentified bacteria and further molecular characterization is required to reveal the identity of these microorganisms.

Conclusion

Mangrove rhizosphere provides a unique ecological environment for diverse bacterial communities. Heterotrophic bacteria are very important in mangrove rhizosphere as the bacteria decompose the mangrove litter, recycle the nutrients and produce detritus food for many higher organisms. The present study shed light on the remarkable metabolic and taxonomic diversity of bacteria associated with rhizosphere. These metabolic capacities of the heterotrophic bacteria from mangrove rhizosphere has profound implications on the ecological role they play and their bioprospecting potential in future.

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MARINE MICROPLASTIC ASSOCIATED BIOFILMS: A CRITICAL REVIEW ON THEIR ORIGIN, IMPACT, COLONIZATION AND DEGRADATION

Dayamrita K K*and **Nivya Mariam Paul**

Department of Microbiology, Mar Athanasius College, Kothamangalam-686666, Kerala
* Corresponding author email id: kkdayamrita@gmail.com

Abstract

The oceans are suffering from an unprecedented accumulation of plastic pollutants. Microplastics have become a cause of major concern due to its ubiquitous nature and size. This review focuses on the effect of microplastics on the marine environment from a microbiological point of view mainly consisting of biofilm formation. Microplastics act as a raft for the transfer of pathogens and chemicals and as a reservoir for antimicrobial and metal resistance genes. Owing to its small size these pollutants undergo trophic transfer leading to bioaccumulation and biomagnification, ultimately harming all forms of life. Since microplastics are recalcitrant substances which are insufficient to support prokaryotic metabolism, biodegradation of microplastics will take a very long time. Hence, research on degradable plastics as well as plastic alternatives should be carried out and strategies to combat plastic pollution should be formulated.

Keywords: Microplastics, biofilm, trophic transfer, bioaccumulation, biodegradation

Introduction

Plastic has become essential in varied areas such as packaging, electronics, aeronautics, construction and medicine. A definite portion of this plastic ends up in the global oceans. Almost 80% of the marine plastic debris originates from

land. It may reach the oceans directly via beach- related tourism, by rivers and municipal drainage systems or sewage effluents. Fishing fleet, marine aquaculture and lost cargo containers also act as major sources (Oberbeckmann and Labrenz, 2020).

Large plastic debris undergoes fragmentation due to weathering forces and generate microplastics (MP) which are less than 5mm in size (Rummel *et al.,* 2017). According to an estimate of global plastic pollution, at least 5.25 trillion plastic pieces has been identified and most of its composition is microplastics (Costa *et al.*, 2018). The sources of microplastics can be broadly classified into land-based and sea-based sources. The land-based source is the leading input of microplastics into the oceans.

Primary microplastics

Primary microplastics are barely visible to the naked eye. They are most likely to flow directly from the bathroom drain into drainage systems and escape capture by wastewater treatment plants. They ultimately wind up in the aquatic environment (Vesilind, 2003). They originate from micro-sized plastic beads and industrial production pellets (5 mm diameter) or powders (<0.5 mm). They are extensively used in cosmetics, synthetic textile, and clothing manufacture (Wang *et al.,* 2018). Another important source of microplastics is tyre abrasion (Kole *et al.,* 2017). In drug delivery systems of anticancer agents, polymeric microspheres have been applied. Accidental spillage or leakage of plastic resin pellets during transportation can also act as a source (Patel *et al.,* 2009).

Secondary microplastics

Secondary microplastics form due to fragmentation and degradation of large plastic debris into minute fragments. This occurs because of high solar

UV- radiation and mechanical abrasion as a consequence of physical, chemical and biological processes. Yellowing of plastics can occur over time due to weathering and embrittlement when large plastic debris are exposed to sunlight (Andrady, 2011; Barnes *et al.*, 2009). The rate of biodegradation process will decrease as the depth increases due to low temperature, oxygen level and haline condition in the low energy benthic zone. Hence, many marine biodegradable plastics such as biopolymers and bio-derived/ bio-based plastics cannot be degraded in the ocean which will cause prolonged adverse influences on the ocean (Wang *et al.*, 2018).

Fishing, shipping and offshore industry sectors form the sea-based sources (Moffitt and Cajas-Cano, 2014). Lost, abandoned or worn-out fishing and aquaculture gears and other equipments can introduce microplastics into the ocean. These kill marine life, damage habitats and effect fish stocks and quality (Thomas and Hridayanathan, 2006). Cargo and large shipping vessels act as a significant source of resin pellets besides accidents or operational spills (Cai *et al.*, 2017).

Composition of microplastics

The composition of microplastics reflects the use and disposal of macroplastics like polyolefins (polypropylene and polyethylene), polyurethane, polyethylene terephthalate, polyvinyl chloride, polycarbonate, and polystyrene (Geyer *et al.*, 2017). Initially, most plastic polymers will be biochemically inert due to their high molecular weight and will have low toxicity due to lack of water solubility (Galloway, 2015). But many polymers such as styrene or vinyl chloride, contain low concentrations of unpolymerized monomers which can be toxic and carcinogenic (Teuten *et al.*, 2009). PVC, PU, PS and PC can contain toxic additives or monomers too. These additives can include coloring agents, antimicrobials, plasticizers, fillers and flame retardants (Bolgar *et al.*, 2015).

Biofilms and characteristics

A film of organic and inorganic substances or a conditioning layer is formed on the surface of microplastics via adsorption, within seconds of first contact with water. This initial conditioning film has the capacity to define the colonizing community by amending the surface properties of the material (Loeb and Neihof, 1975). Microorganisms secrete extra polysaccharides including proteins, glycolipids and glycoproteins. These form a matrix around the microorganisms and aid their attachment to various biotic and abiotic surfaces (Tu *et al.,* 2020). Bacterial cells adhere to surfaces and modify its surface physicochemical properties which influences the adhesion of successive colonizers such as cyanobacteria, algae, and protists (de Carvalho, 2018; Dang and Lovell, 2016). Macromolecules adsorb onto a surface within seconds after immersion, followed by bacterial colonization which begins after an hour. Bacteria has been found to be the most significant microbe on marine surfaces and since they are early colonizers, they may determine the structure and purpose of a mature biofilm (Dang *et al.,* 2008, Dang and Lovell, 2016).

Genetic elements control diverse functions including initial attachment, biofilm maturation and return to the planktonic mode of growth. Biofilms have been found to be made of a single microbial species or multiple microbial species (Flemming and Wuertz, 2019). Mixed species biofilms are common in environments. A biofilm can attain large film structures with proper nutrient and carbon substrates. These may be sensitive to physical forces for instance, agitation (Glaser, 2020).

Bacteria in a biofilm communicate with each other using a process termed quorum sensing. It involves the production of and response to secreted or diffusible chemical signal molecules. It varies across different species of

bacteria and is significant to the first stage of encounter between a solid surface and a bacterium (Waters and Bassler, 2005). Quorum sensing enables bacteria to monitor the environment such that they can adjust community behavior in response to community fluctuations in the number and species present. These molecules can synchronize activities for a large group of cells; hence bacteria can coordinate population density-dependent changes in behavior (Whiteley *et al.,* 2017; Abisado *et al.,* 2018).

A new ecological niche

Plastisphere is regarded as a new ecological niche formed due to plastic pollution in the marine environment (Zettler *et al.,* 2013). The development and composition of biofilms on microplastics are influenced by various factors such as location, particle size, surface properties, substrate type and environmental conditions. The plastisphere can include anaerobes, aerobes, non-motile, motile organisms and even extremophiles. The epiplastic community contains species of *Prochlorococcus*, *Synechococcus*, *Comamonadaceae*, *Saprospiraceae*, and *Chitinophagaceae* along with potential pathogenic species like *Mycobacterium* and oil degraders such as *Colwellia* sp. (Rogers *et al.,* 2020; McCormick *et al.,* 2014). Such diverse microbial composition of marine biofilms can lead to interspecies interactions like horizontal gene transfer, competition, symbiosis, influence the stability, behavior and structure of biofilms and plastic colonization and degradation (Oberbeckmann and Labrenz, 2020).

Plastisphere communities

Knowledge about plastisphere community structure and the driving forces behind each succession stage of biofilm formation will help study the impact of plastic pollution on aquatic microbial diversity and load. In situ incubation studies and environmental sampling on biofilms associated with microplastics

in water and sediment have revealed diversity and similarities among diverse plastisphere communities (Rogers *et al.*, 2020). Studies using high-throughput sequencing revealed that bacteria colonizing microplastics are taxonomically different and less diverse than those in the sediment, suspended organic matter or water column (Rummel *et al.*, 2017).

Sub-surface associated plastisphere communities are dominated by photoautotrophic bacteria such as cyanobacteria with the genera *Rivularia* and *Phormidium* (Jacquin *et al.*, 2019). Seafloor and sub-surface plastisphere contain some common taxa such as Bacteroidetes (*Flavobacteriaceae*) and Proteobacteria (*Alcanivoraceae* and *Rhodobacteraceae*). Marine bacteria were dominated by alpha-Proteobacteria (mainly *Pelagibacter* sp.), while plastisphere bacteria were dominated by alpha and gamma-Proteobacteria (Dussud *et al.*, 2018; Caruso, 2020).

Transport of pathogens and anti-microbial resistance AMR genes

Microplastics act as a vector for the transport of pathogens in the marine environment. Due to the high durability of plastics, associated microorganisms can travel longer distances vertically as well as horizontally in the oceans. Pathogens like *Vibrio* sp. hitchhike on microplastics to formerly unaffected ecosystems. Other potentially pathogenic plastic colonizers are *Escherichia* spp., *Pseudomonas* spp., *Arcobacter* spp. and *Colwellia* spp. (Oberbeckmann and Labrenz, 2020).

Plastisphere is a niche for the development, retention and spread of antimicrobial resistance (AMR). The abundance of AMR genes (ARGs) and metal resistance genes (MRGs) in the plastisphere is significantly greater than that of seawater (Yang *et al.*, 2019). The bacteria present in biofilms are efficient at spreading and exchanging ARGs through many HGT mechanisms such as conjugation (Donlan, 2000).

Transport of chemicals through biofilm coated microplastics

Organic and inorganic contaminants harmful to both humans and ecosystem life can be sorbed and accumulated by biofilm microorganisms. Sorptive processes can lead to quick uptake and release of chemicals in microplastics compared to macroplastics due to greater surface to volume ratios. The formation of biofilms is facilitated due to enlarged and weathered surfaces available for colonization. It can influence the persistence and kinetics of hydrophobic organic contaminants (HOCs). Biofilms are therefore able to transport as well as metabolize HOCs (Rummel e*t al.,* 2017, Alimi *et al.,* 2018). EPS produced by biofilm microorganisms contribute to the sorptive capacity of biofilm coated microplastics (Wang *et al.,* 2016). Biofilms have high relevance in the accumulation or removal of plastic associated chemicals via metabolization since a wide range of bacteria, fungi and algae are capable of degrading HOCs. The leaching of antimicrobial agents added to polymers intended to hamper microbial settlement is a major concern as it promotes the spread of resistance in microbial communities (Rummel e*t al.,* 2017).

Transport of microplastics in the ocean

In the ocean, microplastics lighter than the surrounding water float and are transported through water. A mixture of biological and physiochemical processes drives the transport of microplastics from the surface to the sediment (Kaiser *et al.,* 2017; Porter *et al.,* 2018). Microplastics in the coastal regions are prone to wind, waves and tides. Such high energy mechanisms induce surface drifting or vertical mixing causing the deposition of microplastics in beach sediments. Properties of microplastics such as density, shape, dimension and biofilm growth govern the velocity and particle settling (Zhang, 2017). Fouling organisms lead to decrease in buoyancy and increase in density of the particles

(Lagarde *et al.,* 2016). Smaller particles reach its critical sinking density faster. This leads to sedimentation of the particles (Chubarenko *et al.,* 2016; Fazey *et al.,* 2016). Biofilm formation renders microplastics sticky due to the EPS matrix. It promotes the formation of heteroaggregates consisting of microplastic, microorganisms and detritus. Such aggregates alter the sedimentation rates of algal blooms and related microbes. For example, it was observed that when low density microbeads were incorporated into heavy and fast sinking diatom aggregates, their sinking rate decreased, whereas, aggregates increased the sinking rates of light cryptophyte cells (Long *et al.*, 2015). Benthic sediments are not the ultimate sink for microplastics. The biofouling of microplastics can decrease due to its removal or digestion by benthic organisms. This leads to increase in buoyancy which results in submerging and resurfacing cycles (Ye and Andrady, 1991).

Trophic transfer of microplastics

Various organisms such as zooplankton, fish, marine mammals, reptiles, corals and sea birds ingest microplastics which results in its trophic transfer through marine routes. It may ultimately end up being consumed by humans (Bisht *et al.,* 2020; Carbery *et al.,* 2018).

Microplastics coated with nutrient rich biofilms may be ingested by primary consumers in the ocean. Selective feeders such as copepods and shrimps and also passive feeders such as cladocerans ingest these particles (Rummel *et al.,* 2017). Patches of marine snow are explored by zooplankton. They may consume larger quantities of microplastics. For example, the zooplankton *Daphnia magna* which consumes green algae as a vital part of its diet accidently consumes microplastic which end up lining its gut. This can prevent or inhibit the consumption of food which leads to starvation and other

adverse effects (Ogonowski *et al.,* 2016). Microplastics rapidly accumulate on coral reefs which could lead to adverse health effects. Corals are known to mistake microplastics for their natural prey, plankton and end up consuming up to 50 μg of plastic (Hall *et al.,* 2015).

Microplastic biodegradation

Biodegradation of plastic can be defined as a mechanism that results in the partial or total conversion of organic carbon into biogas and biomass associated with a specific community of microbes such as bacteria, fungi or actinomycetes capable of utilizing plastic as a carbon source. Several microorganisms have been reported to have the ability to deteriorate or degrade plastics such as *Corynebacterium, Micrococcus, Pseudomonas, Arthrobacter, Streptomyces* and *Rhodococcus* (Jacquin *et al.,* 2019).

Biodegradation occurs after or simultaneous with physical and chemical or abiotic degradation. The formation of biofilms on the surface of microplastics protect it from photodegradation in the surface of the ocean. It also increases its density leading to microplastic sedimentation. Hence, this shifts the factors that determine the degradation of plastics from physiochemical forces to microbial activity (Weinstein *et al*., 2016; Jahnke *et al.,* 2017).

According to Dussud and Ghiglione (2014) biodegradation can be summarized in four important steps, Bio-deterioration, Bio-fragmentation, Assimilation and Mineralization. Plastics are biodegraded by aerobic metabolism in the pelagic ecosystem, while anaerobic biodegradation pathway is frequent in the sediments. Anaerobic mechanism is slower than aerobic mechanism (Ishigaki *et al.,* 2004). yang.

Conclusion

The burden of microplastic pollution has been increasing for some decades now. Microplastics are now an established colonization surface in the ocean for many microorganisms. This has established the plastisphere as a new ecological niche where several species thrive, exchange genes and increase in biomass. Among these, pathogens use microplastic to form pathogenicity islands and as a long-lasting vector. It also enables the transfer of antimicrobial and metal resistance genes through horizontal gene transfer mechanisms. Microplastics harm the ecosystem by leaching toxic substances, transferring chemicals via sorption. They undergo trophic transfer and damage organisms in all trophic levels, ultimately reaching human life. Furthermore, marine microorganisms are not efficient degraders of plastic and due to the low bioavailability of microplastics in the ocean, microbes may not evolve to significantly degrade plastics any time soon.

To address the challenge posed by microplastics, further research is needed to understand the interactions between plastic materials undergoing weathering and colonization by microorganisms. In order to develop in situ biodegradable materials, further information about microbial pathways linked to plastic degradation is necessary and an efficient cost- effective recycling system that can be applied globally should be devised.

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A STUDY ON THE BUTTERFLY DIVERSITY IN WEST KOCHI, KERALA

Sree Lakshmi S, Meenu Raju and **Meera Menon***

Department of Zoology, Cochin College, Kochi, Kerala-682002
***Corresponding author email id:** meenusn.brm@gmail.com

Abstract

Butterflies are good biological indicators. Hence their conservation is important for maintaining the stability of ecosystem. This is a four-month study conducted from February to May 2020 in West Kochi of Ernakulam district, Kerala. Thirty species of butterflies belonging to five families *viz.*, Nymphalidae, Papilionidae, Lycaenidae, Hesperiidae and Pieridae were recorded. Diversity indices showed higher values suggesting the suitability of the habitat for butterflies. The presence of various host and nectar plants may be the reason for rich butterfly diversity of the study habitat.

Keywords: Nymphalidae, Papilionidae, Lycaenidae, Hesperiidae and Pieridae

Introduction

"Biological diversity" means the variability among living organisms from all sources including, inter alia, terrestrial, marine, and other aquatic ecosystems and the ecological complexes of which they are part; this includes diversity within species, between species and of ecosystems (Weiss, 1992). Concept of diversity is important in many areas of biology. The term biodiversity comes to us from conservation biology (Maclurin and Sterelny, 2008).

Butterflies and moths are insects classified under order Lepidoptera (Class – Insecta). Moths are stout and fuzzy, whereas butterflies are slender and smooth. Generally, butterflies are diurnal, and moth are nocturnal. Some butterflies are

crepuscular, and moths are diurnal. Life cycle of butterfly includes four stages Egg, Larva/Caterpillar, Pupa/Chrysalis, and adult Butterfly (Kasambe, 2018). Butterflies belongs to six family; Papilionidae, Pieridae, Nymphalidae, Riodinidae, Lycaenidae & Hesperiidae. Objectives of this study is to evaluate butterfly diversity of study habitat and to evaluate importance of host and nectar plants in butterfly diversity.

Materials and methods

Study habitat

The study was conducted in the Kerala Water Authority Quarters surroundings situated at Fort Kochi area at Ernakulam district from February to May 2020 (Figure 1). This is a three-acre area with a wide variety of host and nectar plants such as *Borassus flabellifer*, *Arceca catechu*, *Mimosops elanji*, *Delonix regia*, *Aegle marmelos*, *Citrus lemon*, *Ixora coccinea*, *Gomphrena globose*, *Adenanthera pavonina*, *Millingtonia hortensis*, *Turnera ulmifolia*, *Bauhinia acuminate*, *Clerodendrum paniculatum*, *Cassia fistula*, *Millettia pinnata* etc. for butterflies.

Figure 1. Map of the study habitat

Sampling and identification

Random sampling is used as the method of sampling. The butterflies were photographed using Nokia 7 plus mobile camera and these organisms were identified with the help of identification guides (Kasambe, 2018).

Data analysis

The monthly distribution of butterflies in the study area is surveyed and represented graphically. Species richness (Margelef's richness index d), evenness (Pielou's evenness index (J')) dominance (1-Simpson index) and diversity (Shannon Weiner index (H´)) were calculated using Primer software version 7. Species richness (Margelef's richness index (d)) measures the number of species in the habitat, higher the value indicates a greater number of species. Pielou's index is a species evenness index ranges from zero to one, the more value indicates more even distribution of taxa.

Species dominance index (1-Simpson index) ranges from 0 (all taxa are equally present) to 1 (one taxon dominates the community completely). Shannon Weiner index (H´) - diversity index considers the number of individuals as well as number of taxa. It varies from 0 for communities with only a single taxon to high values for communities with many taxa, each with few individuals.

Results and discussion

Thirty species of butterflies were recorded during the study period (table 1). Among them, three butterflies were protected under Schedule I Part IV of Wildlife Protection Act 1972. They are *Papilio clytia* (Common Mime), *Pacliopta hector* (Crimson Rose) and *Hypolimnas misippus* (Danaid eggfly). *Troides minos* (Southern Birdwing/Sahyadri Birdwing), *Papilio demoleus*

(Lime butterfly) and *Papilio polymnester* (Blue Mormon) observed during the study were endemic to Western Ghats (plate 1).

Nymphalidae with nine species was the most dominant family followed by Papilionidae with eight species (Figure 2)

Table 1. Butterflies identified from the study habitat

Family - Papilionidae		**Family - Lycaenidae**	
SCIENTIFIC NAME	**COMMON NAME**	**SCIENTIFIC NAME**	**COMMON NAME**
Troides minos	Southern Birdwing	*Jamides celeno*	Common Cerulean
Graphium agamemnon	Tailed Jay	*Zizula hylax*	Tiny Grass Blue
Papilio polytes	Common mormon	*Jamides alecto*	Metallic Cerulean
Pacliopta hector	Crimson Rose	*Nacaduba pactolus*	Large Four-line Blue
Euploea sylvester	Double-branded Crow	*Rathinda amor*	Monkey puzzle
Papilio polymnester	Blue Mormon	*Chilades pandava*	Cycad Blue
Papilio demoleus	Lime Butterfly	**Family - Pieridae**	
Papilio clytia	Common Mime	**SCIENTIFIC NAME**	**COMMON NAME**
Family - Nymphalidae		*Leptosia nina*	Psyche
SCIENTIFIC NAME	**COMMON NAME**	*Catopsilia pomona*	Common Emigrant
Hypolimnas bolina	Great Eggfly	*Eurema hecabe*	Common Grass Yellow
Hypolimnas misippus	Danaid Eggfly	**Family - Hesperiidae**	
Melanitis leda	Common Evening Brown	**SCIENTIFIC NAME**	**COMMON NAME**
Neptis jumbah	Chestnut-streaked sailer	*Udapes folus*	Grass Demon
Elymnias caudata	Tailed Palmfly	*Barbo cinnara*	Rice Swift
Acraea violae	Tawny Coster	*Lambrix salsala*	Chestnut Bob
Junonia atlites	Grey Pancy	*Matapa aria*	Common Redeye
Melanitis zitenius	Great Evening Brown		
Polyura athamas	Common Nawab		

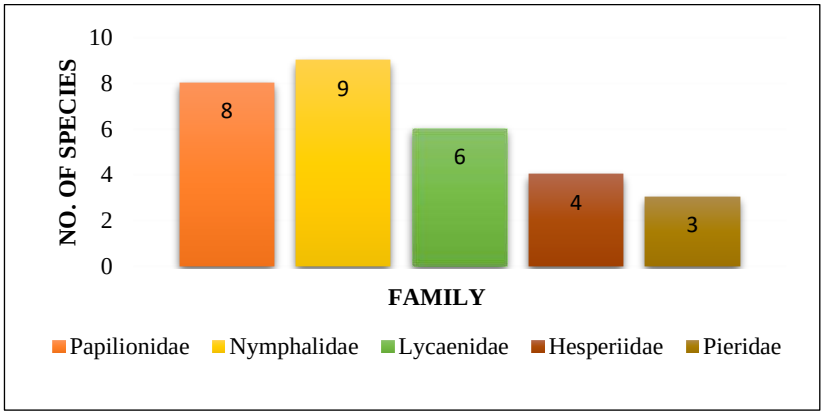

Figure 1. Family wise distribution of butterflies in the study habitat

The abundance of butterfly families showed an increase from February to May during the study period (Figure 2). Nymphalidae and Papilionidae were the most abundant families (table 2). This may be due to the presence of their host plants.

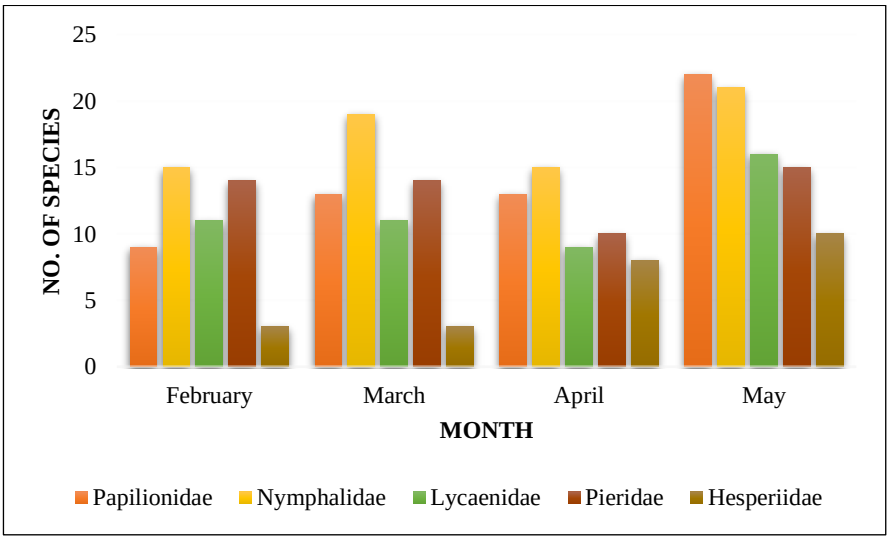

Figure 2. Butterfly families recorded during different months of the study period

Table 2. Monthly distribution of butterflies belonging to five families

Sl. no.	SPECIES	FEBRUARY	MARCH	APRIL	MAY
PAPILIONIDAE					
1	*Troides minos*	1	0	0	0
2	*Graphium Agamemnon*	1	2	3	5
3	*Papilio polytes*	3	6	5	4
4	*Papilio hector*	2	2	1	3
5	*Euploea Sylvester*	1	2	2	4
6	*Papilio polymnester*	0	0	0	2
7	*Papilio demoleus*	0	0	0	1
8	*Papilio clytia*	1	1	2	3
NYMPHALIDAE					
1	*Hypolimnas bolina*	2	3	2	3
2	*Hypolimnas misippus*	0	2	0	1
3	*Melanitis leda*	3	5	4	6
4	*Neptis jumbah*	3	2	3	1
5	*Elymnias caudata*	2	2	2	3
6	*Acraea violae*	3	2	1	2
7	*Junonia atlites*	0	0	0	1
8	*Melanitis zitenius*	1	1	0	2
9	*Polyura athamas*	1	2	3	2
LYCAENIDAE					
1	*Jamides celeno*	6	4	4	5
2	*Zizula hylax*	2	3	1	3
3	*Jamides Alecto*	0	1	0	1
4	*Nacaduba Pactolus*	2	3	2	2
5	*Rathinda amor*	0	0	0	2
6	*Chilades pandava*	1	0	2	3
PIERIDAE					
1	*Leptosia nina*	5	5	3	6
2	*Catopsilia Pomona*	5	6	5	5
3	*Eurema hecabe*	4	3	2	4
HESPERIIDAE					
1	*Udapes folus*	1	1	2	2
2	*Barbo cinnara*	2	2	2	3
3	*Lambrix salsala*	0	0	3	2
4	*Matapa aria*	0	0	1	3

Diversity index values of the study area was calculated and represented in the table given below (Table 3). All the indices showed their peak values during the month of May.

Table 3. Diversity index values of the study habitat

Month	Total species [S]	Total number of individuals [N]	Margalef's Index [d]	Pielou's evenness Index [J']	Shannon's Index [H']	Simpson's Index [1-D]
February	22	52	5.3148	0.94301	2.9149	0.95551
March	22	60	5.129	0.95362	2.9477	0.9565
April	22	55	5.2404	0.96621	2.9866	0.96229
May	29	84	6.3194	0.96323	3.2435	0.96845

The absence of Riodinidae family in this study period may be due the absence of their host plant or may be due to seasonal variation in the study area. *Troides minos* was seen in the month of February and *Papilio polymnester* was seen just before rainy season. Diversity values were high during the month of May. This may be due to the blooming of nectar flowers at this time.

Revathy *et al*., (2014) recorded a Shannon Index of 3.294 after the introduction of nectar plants such as *Lantana camera* and host plants such as Citrus sp., *Murraya koenigii*, Ixora, *Passiflora edulis* etc. Our study recorded 3.243 in the month of May as the area is surrounded by nectar and host plants.

Arun and Azeez (2003) reported that minimising the anthropogenic disturbances can greatly help in improving the status of habitat specific butterflies. In the present study even though the study site is a government office premise, the anthropogenic disturbance towards the natural flora is minimum. In this study 257 butterflies belonging to 17 species of Nymphalidae family were reported. In our study too, the number of species belonging to Nymphalidae are higher.

According to Aneesh *et al.,* (2013), the natural habitat had a greater number of species than man made plantations. They got a greater number of butterflies belonging to Nymphalidae family. According to Mathew *et al.,* (2005) Peechi-Vazhani wildlife sanctuary contains *Cinnamomum spp., Lantana camara, Ixora sp.* etc. and it supports good butterfly diversity. Above mentioned plants are also present in this study area. So, the presence host and nectar plants has an important role in butterfly diversity.

According to Sharp *et al.,* (1974), there was no significance between the host and nectar plants on micro distribution of butterflies but in this study, butterflies in an area seems to be influenced by the presence of their host plants and nectar plants. Yahner (2001), Oliver *et al.* (2006) and Revathy *et al.* (2014) reported that the diversity of butterflies is mainly due to the presence of nectar and host plants, the habitat characteristics affect butterfly diversity and butterfly garden help in caring butterfly population and to maintain natural ecosystem.

Conclusion

Species richness and abundance of butterflies in the study habitat is high when compared to similar studies. It may be because of less anthropogenic activity in the study area. The presence of various host plants and nectar plants are the reason for rich butterfly diversity of the study area. Planting of host and nectar plants and preparing a butterfly garden in homes is essential for the conservation of butterflies. The study period was relatively short for the analysis of trends which may affect their conservation status. Hence a long-term monitoring is needed to identify significant changes in the biodiversity and provide light to its conservation.

Plate 1. Butterflies identified from the study habitat

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