Application of indigenous microbial biofilms for bioremediation of polycyclic aromatic hydrocarbon contaminants of the Indian Sundarbans

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By

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ABSTRACT

In this study, the polycyclic aromatic hydrocarbons (PAHs) distribution of surface water and sediments collected from various geographical locations of the Indian Sundarbans were ascertained. The total concentration of 16 priority PAH pollutants of water and sediments fluctuated from non-detectable to 125 ng/ml and 4880 to 2×10^4 ng/g dry weight, respectively. The concentrations of individual PAHs in Sundarban water were much higher than the limit proposed by WHO. The total PAH concentration of sediments surpassed the Effects Range Low (ER-L) and the recommended Effects Range-Median (ER-M) values, indicating that the PAHs might adversely affect the Sundarbans' biota. The source identification studies using PAH ratios indicated the pyrogenic and petrogenic contaminations in the Sundarban sediments. In addition, a weak positive correlation of PAHs with organic carbon and organic matter indicates the continuous inflow of PAHs.

This study also focused on intertidal biofilms of the Sundarbans and how these biofilms can be exploited to remove polyaromatic hydrocarbon (PAH) contaminants. Six heterotrophic, as well as phototrophic, stably-growing, robust, and well-formed biofilms, were selected, each representing the different geographical locations: (Pg) Purba Gurguria, (Mt) Maipit, (Pp1 and Pp2) Patharpratima, (Nk) Namkhana and (Kk) Kakdwip. These indigenous biofilms were cultivated in two sets of the patented biofilm-promoting culture vessel possessing a hydrophilic glass surface and hydrophobic polymethyl methacrylate (PMMA) surface, containing liquid media spiked with 16 priority PAHs. Heterotrophic biofilm-mediated 97-100%, whereas phototrophic biofilm showed 32-100% removal efficiency of individual PAHs was attained in all media. Significant differences were observed between mean residual PAHs obtained from the liquid media of PMMA and glass flasks cultured with phototrophic biofilms. Residual amounts of acenaphthene (Ace), anthracene (Ant), benzo(a)pyrene (B(a)P) and benzo(ghi)perylene (B(ghi)P) showed notable differences in their sequestration when

cultivated with both the biofilms in flasks having hydrophobic and hydrophilic surfaces. The residual amounts of PAHs obtained from biofilms of both heterotrophic and phototrophic microorganisms cultured in PMMA and glass flasks also displayed a significant difference. The hydrophilic culture of phototrophic biofilm Pp1 and hydrophobic culture of heterotrophic biofilm Pp1, and phototrophic biofilm Kk showed higher PAH sequestration. Morphological analyses of these PAH degrading biofilms showed the presence of purple sulphur bacteria, diatoms, cyanobacteria (*Gleocapsa* sp., *Chloroidium* sp., *Microcystis* sp. and *Phormidium* sp.) as well as *Closterium* sp. like green algae. Composition, structure and abundances of indigenous heterotrophic microbial communities in PAH-sequestering biofilms by amplicon-based metagenomics revealed bacterial phyla including *Proteobacteria*, *Bacteroidetes*, *Firmicutes*, *Actinobacteria*, *Chloroflexi* and *Planctomycetes* as well as members of *Ascomycota* phylum of fungi. The dominance of *Candida tropicalis*, *Clostridium butyricum*, *Sphingobacterium multivorum* and *Paecilomyces fulvus* were established. The biofilm phototrophic community composition did not alter when challenged with PAH; production of biofilm biomass and photosynthetic pigments synthesis and extracellular polymeric substances (EPS) were enhanced

Aim and Objectives

The Sundarbans are the world's largest single-tract intertidal mangrove forest with rich biodiversity. Environmental pollution in the Indian Sundarbans has been the greatest concern in recent years due to natural and anthropogenic activities such as rapid human settlement, deforestation, tourist activities, and increased agricultural and aquaculture practices. A pronounced ecological change in the estuarine environment is due to the discharge of untreated domestic and industrial effluents from the tributary rivers. The delta has become susceptible to chemical pollutants such as heavy metals, polychlorinated biphenyls, and polyaromatic

hydrocarbons (PAH), which may have affected the estuarine geochemistry and the quality of the local coastal environment. PAHs are ubiquitous pollutants, and uptake and accumulation are a threat to the flora & fauna as well as human beings. Bioremediation using microorganisms is the most promising technique compared to other remediation processes. The definite objectives behind this work were as follows

(i) Quantitative determination of PAH levels in water and sediments

Collection of water and sediment samples from various geographical regions of Indian Sundarbans and quantitative estimation of 16 priority PAH pollutants present in both water and sediment samples.

(ii) Isolation of indigenous microbial biofilm mats collected from different regions of Sundarbans.

Collection of biofilm samples from the Sundarban by scrapping the biofilms from the sediment surface, further isolation and culturing of heterotrophic and phototrophic indigenous microbial biofilm mats in different modified culture media at laboratory conditions.

(iii) In vitro estimation of PAH bioremediation by heterotrophic biofilms cultured in the patented biofilm-promoting enhanced surface area conico-cylindrical flasks (ES-CCF)

Culturing the isolated heterotrophic biofilms in two sets of patented ES-CCF possessing hydrophilic glass surface and hydrophobic polymethyl methacrylate (PMMA) surface with enrichment media. Quantification of mean residual PAH to determine the PAH removal efficiency after 30 days of incubation with different concentrations of PAHs spiked into the media.

(iv) Study of the community composition of selected heterotrophic biofilms. Metagenomic sequencing to study the bacterial and fungal community of the selected biofilm, which possesses high PAH degradation potential.

(v) Evaluation of PAH degradation efficiency of phototrophic biofilm and study of the effect of PAHs on characteristic features of selected biofilms.

Culturing the isolated phototrophic biofilms in hydrophilic glass and hydrophobic polymethyl methacrylate (PMMA) ES CCF with modified media and estimating mean residual PAH to determine the PAH degradation potential of the biofilms after 21days of incubation with different concentrations of PAHs spiked into the media. Effect of PAHs on growth, photosynthetic pigments, EPS production, their effect on the biochemical composition of the EPS, and finally, the microscopic study of the biofilm.

Introduction

The world has come across the most critical environmental concern over the past few decades: contaminants with toxic and recalcitrant properties due to various anthropogenic activities. The presence of xenobiotic compounds caused by solid waste deposits, inorganic contaminants like heavy metals, persistent organic pollutants (POPs), namely polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (*PCBs*), pesticides, and unprocessed industrial effluents causes critical environmental as well as health problems (Singh and Haritash, 2019). Direct and indirect release of these compounds into the environment leads to water, soil, and air pollution and further enters the food chain. Among the diverse amounts of pollutants available, PAHs are one of the toxic, noxious wastes affecting aquatic and terrestrial ecosystems adversely.

PAHs are a ubiquitous group of environmentally persistent chemicals with numerous structures with two or more aromatic rings. PAHs cause toxic, mutagenic, and carcinogenic

effects on living beings. PAHs are widely dispersed and repositioned in the environment due to incomplete combustion of organic matter, coal combustion, and automobile exhausts. Above 100 unique PAHs are widely distributed in the surroundings; the U.S. Environmental Protection Agency (US EPA) has recorded 16 PAHs as priority pollutants based on their toxicity (Shukla et al. 2014). With the rising realization of the harmful effects of hazardous pollutants on the environment and human health, internationally, more attentiveness has been received for the remediation of such chemicals.

The biological method of remediation (bioremediation) put forward several advantages over physical and chemical methods, including avoidance of harsh chemicals, minimum or no disruption of land or wildlife in and around the treated site, and also they are economically efficient (Mitra and Mukhopadhyay, 2016). Several microorganisms, such as bacteria, fungi, yeast, and microalgae, have the potential to use and mineralize different types of PAHs. They can convert PAHs into less toxic compounds/water and carbon dioxide (Alexander, 1994). Indigenous microorganisms isolated from polluted sites illustrate more degradation adeptness since they have survived contaminants' toxicity and adapted to the polluted environment (Singh and Haritash, 2019).

From time to time, studies show that specific microorganism lacks the genes required for the enzyme synthesis involved in the complete mineralization of PAHs. These findings led the researchers to focus on developing microbial consortia to degrade pollutants completely. The different microbial species in the consortia of biofilms possess different metabolic degradation pathways and can collectively or individually remove PAHs (Ghosal et al., 2016). Biofilm-mediated degradation of PAHs is more efficient than single microbes since they have a high tolerance toward hazardous pollutants. As PAHs in the environment are found in mixtures, wide varieties of microbial species in the indigenous population

exhibiting differential gene expression help in the formation of nutrients and oxygen and can divide the labor leading to a complex phenomenon known as co-metabolism.

Materials and methods

2.1 Sample collection

Water and sediment samples from Indian Sundarbans estuaries were collected during the month of November 2018. (Figure 1). 1L of surface water and sediment samples were taken at a depth of 5 cm of sediment surface using trowels were collected in amber coloured glass bottles. Simultaneously the biofilm present on the sediment surfaces were scrapped out from the same sites. All the samples were transferred to the laboratory and stored in cold conditions for further experiments.

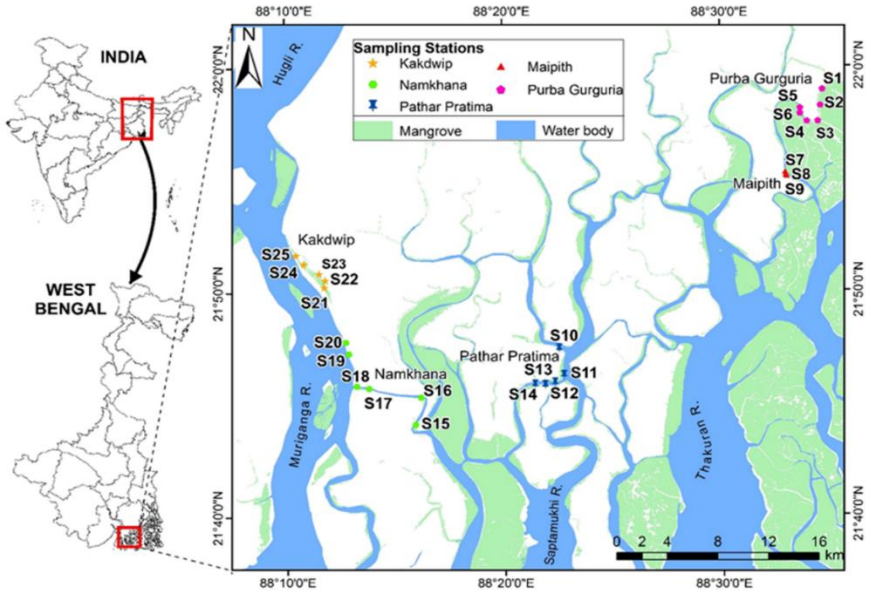


Figure 1: Geographic locations of Indian Sundarbans showing the sampling points.

2.2 In Vitro biofilm culturing

Biofilms collected from Sundarbans regions were stored at -800C and were sub cultured in two different ways: Biofilm samples in the plastic bags were scrapped out using sterile blade and transferred to a mixed nutrient media consisting of GYM without CaCO3 for all

the heterotrophic bacteria, bold basal media enriched with vitamins for eukaryotic microalgae, Czepak dox broth for fungi and ASN III and BG 11 for phototrophic microorganisms. Media were autoclaved and mixed in the ratio of 1:1:1:1:1 (Zammit G.et.al, 2011). Purely phototrophic media consisting of ASN III and BG 11 in the ratio of 1:1. Culturing was done in sterile 250ml conical flask maintained at 27±20C with a light intensity of 36.45µmol photons m-2 s-1 in a light/dark (14/10 hrs.) cycle.

2.3 Physiochemical characteristics of sediments and water

Conductivity of water samples as an indicator of water salinity was determined with Conductivity Meter 36 (Systronics India Ltd.,.India). The OC content of sediments was determined following Walkley and Black (1934). OM content was calculated from the OC (Radojevic and Bashkin, 1999).

2.4 Determination of PAHs in water and sediments

PAHs present in water samples was extracted following the USEPA method 3510C liquid-liquid extraction (1996c) in a separating funnel using dichloromethane (DCM). Sediment samples were air-dried, powdered sieved and the PAHs were extracted following the USEPA method 3540C (1996a) using a Soxhlet apparatus. Extracts were concentrated to 2 ml and the solvent was exchanged to cyclohexane. Silica gel clean-up was carried out following the USEPA method 3630C (1996b) to preclude interference of sulfur compounds. Two microliters of the concentrate was injected to a gas chromatograph equipped with a flame ionization detector (Agilent 7820 A, Agilent Technologies, USA) using nitrogen as the carrier gas following the EPA method 8000 B (1996d).

2.4 Cultivation of biofilms in the patented enhanced surface area conico cylindrical flask (ES-CCF)

Six primarily heterotrophic, stably-growing, robust and well-formed biofilms (heterotrophic and phototrophic) were selected each representing the different geographical

locations: Pg (Purba Gurguria), Mt (Maipit), Pp1 and Pp2 (Patharpratima), Nk (Namkhana) and Kk (Kakdwip). These biofilms were utilized for the removal of the PAHs and were cultured in two sets of the patented ES-CCF possessing hydrophilic glass surface (Set 1) and hydrophobic polymethyl methacrylate (PMMA) surface (Set 2). Approximately 200 mg of biofilm was inoculated in to the sterile mixed medium .After 15 days of incubation, PAHs were spiked in to the glass-ES-CCFs and PMMAES-CCFs containing stable biofilms: 5 ppm for Pg and Mt, 15 ppm forPp1, 10 ppm for Pp2 and Nk, and 25 ppm for Kk biofilms. Another set of biofilms were cultured in the ES-CCFs without the spiked PAHs. Sterile media without PAHs and control flasks containing sterile media spiked with 5, 10, 15 and 25 ppm of PAHs were also incubated. The extent of PAH removal in the liquid medium was determined after 30 days of incubation in duplicate (n = 2) and represented as percent residual PAH. The PAH removal efficiency (%) was: R = (Ci - Cf)/Ci x 100%, where R represents the removal efficiency (%), Ci is the initial and Cf is the final PAH concentration.

2.5 Estimation of PAH contents in biofilms

Biofilms were collected after 30 d and dried by lyophilization (Eyela FDU-1200, Tokyo Rikakikai Co. Ltd, Japan). PAHs were extracted from dried biofilm samples following Froehner et al. (2012). Extraction by ultrasound sonication was carried out using DCM: methanol (2:1) and twice the volume of DCM. Extracts were combined, concentrated to 2 ml and then subjected to silica gel clean up and GC analysis.

2.6 Estimation of photosynthetic pigments

We compared the overall concentration of phototrophic pigments Chl *a*, Chl *b*, Chl *c*, Chl *d*, total Chl and carotenoids from the biofilms cultured in PAH-spiked media with the control biofilms after 21 days incubation. Photosynthetic pigments were extracted in triplicate using reagent-grade methanol. Biofilm samples were weighed, homogenized and

the homogenate extract was centrifuged at 1077 × g at 4 ˚C for 15 min. Pellets were re-suspended in the solvent until they turned colorless. All supernatants were pooled and quantified spectrophotometrically Ritchie (2008).

2.7 Light and scanning electron microscopy of biofilms

Freshly grown biofilms as well as those exposed to PAHs were spread on a clean glass slide, covered with a coverslip and observed at 1000 X magnification using a light microscope (Leica ICC50 HD, Leica Microsystems, Wetzlar, Germany). Biofilm communities growing in control media and media spiked with PAHs were examined by scanning electron microscopy (SEM) using Hitachi FlexSEM 1000 II microscope (Hitachi, Ltd Tokyo, Japan). Biofilms were placed over carbon tape on the SEM sample stub and gold-sputtered (3–5 nm). SEM imaging was done using SEM coupled with detectors for secondary electrons and back-scattered electrons as well as an ultra-variable-pressure detector for imaging non-conductive samples at low vacuum.

2.8 Metagenomic analysis of heterotrophic biofilms showing effective removal of PAHs

Metagenomic study of heterotrophic biofilms were carried out using Illumina sequencing. Sequences are available at the NCBI Sequence Read Archive having accession number PRJNA596375.

2.9 Extraction and characterization of EPS

EPS from control as well as PAH-spiked vessels were measured and characterized. Two fractions of EPS i.e. the released (RPS) as well as the capsular/bound polymeric substances (CPS) from the biofilm samples were extracted. CPS are precipitated in 96% cold ethanol (Di Pippo et al., 2013). RPS from the cell-free culture was quantified following the method prescribed by Gacheva et al. (2013).

Total carbohydrate contents of RPS and CPS fractions were determined spectrophotometrically by the phenol-sulphuric acid method (Dubois et al., 1956). The standard curve was prepared using glucose. Total protein constituents in EPS fractions were spectrophotometrically measured using Lowry's method (Lowry et al., 1951). Bovine serum albumin was used for preparing the standard curve. Uronic acid contents were determined using carbazole in 80% sulfuric acid with sodium borate. Galacturonic acid served as the standard (Taylor and Buchanan-Smith, 1992).

2.10 Estimation of EPS and biofilm hydrophobicity

A biphasic system was applied where 5 ml of control as well as test EPS were suspended in 1% w/v phosphate saline buffer in clean test tubes. Hexadecane (300 µl) was added to the test tubes and OD550 (A0) was obtained. All tubes were vortexed for about 1 min and the phases allowed to separate by keeping them still for 15 min. The lower aqueous phase was carefully removed using a sterile Pasteur pipette and OD550 (A1) was quantified. The hydrophobicity of the EPS was calculated using the formula (Bhatnagar et al., 2014):

$$DH = [(A0 – A1) / A0] \times 100$$

Biofilms at the early stationary phase (between 10 and 15 days of growth) before exposure to PAHs were used for testing the hydrophobicity of the biofilms (Zhang et al., 2011; Veerabadhran et al., 2018). Cultures were thoroughly washed twice using the culture medium. Three milliliters of biofilm suspension was taken in a clean sterile test tube and 3 ml of n-hexadecane was added. The contents were manually agitated for about 1 min and after a five-minute pause, the contents were vortexed for 10s. The tubes were kept stationary for about 10–15 min for the total separation of the two phases. The biofilm cell surface property was assessed by observing adherence to the biphasic (aqueous-hydrocarbon) system as described by Fattom and Shilo (1984).

2.11 Statistical analyses

Analysis of variance (ANOVA), a two-tailed Student t-test, the Pearson correlation and coefficient of variation were performed using SPSS software (version 16.0). The statistical significance was verified at the significance level of $P \leq 0.05$.

Results and discussions

3.1 Physico-chemical analysis of water and sediments

Mean conductivity varied between 24.3 and 45.5 mS/cm and the corresponding mean salinity ranged between 11.9 and 27.5 ppt. Higher values of conductivity implied the presence of inorganic ions Mg^{2+}, Ca^{2+}, CO_3^{2-}, HCO^{3-}, PO_4^{3-} and NO^{3-}. The mean salinity of the sampling sites was similar to previous studies (14-25 ppt) conducted in the Sundarbans by Chaudhuri et al. (2012). The salinity in Kakdwip was comparatively lower due to the continuous influx of freshwater from the Hugli River (Figure.1). OC contents are presented in figure 2. A significant difference in the mean OC and OM contents in the sediments collected at various locations was noted.

3.2 PAHs in water and sediments

Most of the high molecular weight PAHs (HMW PAHs) could not be detected in the water samples but some low molecular weight PAHs (LMW PAHs) were detected. Ace was the most abundant PAH. The mean concentration of LPAHs was 34.7 ng/ml while that of HPAHs was 15.9 ng/ml. Two-three ringed PAHs encompassed 64% while 5-6 ring PAHs comprised 36% of total PAHs in water respectively. Contrary to the PAH levels evidenced in water samples, HPAHs were more dominant than LPAHs in the sediments

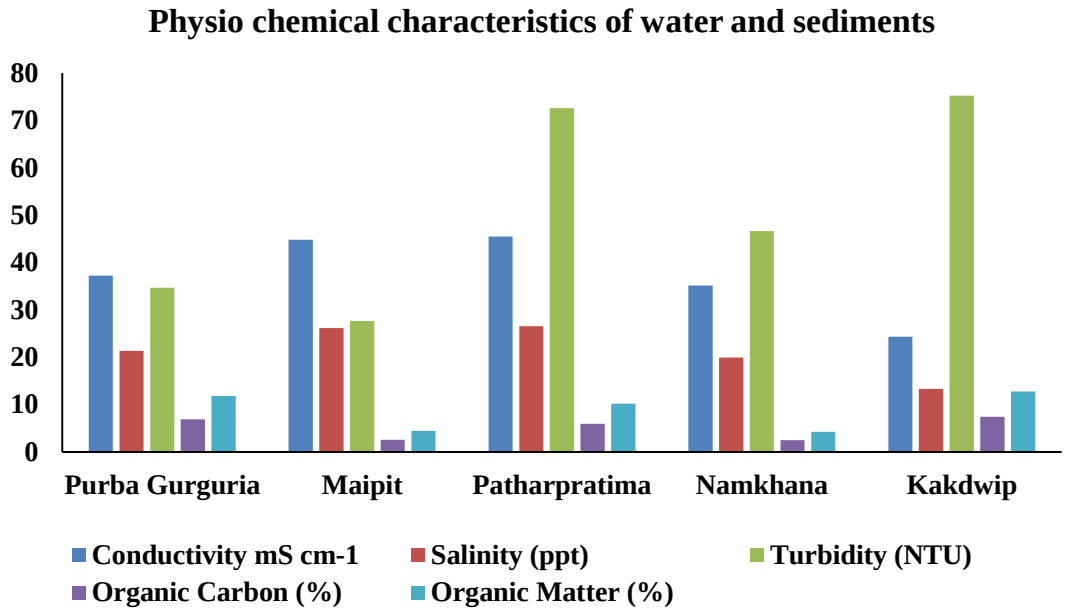


Figure 2: Graphical representation of physiochemical characteristics of water and sediments collected from various geographical locations.

The mean total concentrations were 1.04×10^4 and 2150 ng/g dry weight for HPAHs and LPAHs respectively, which were significantly different ($P \leq 0.05$). Two-three ringed aromatic PAHs comprised 17% whereas 4 ringed PAH covered 23% while 5-6 ringed PAH constituted 60% of the total PAHs (figure 3).

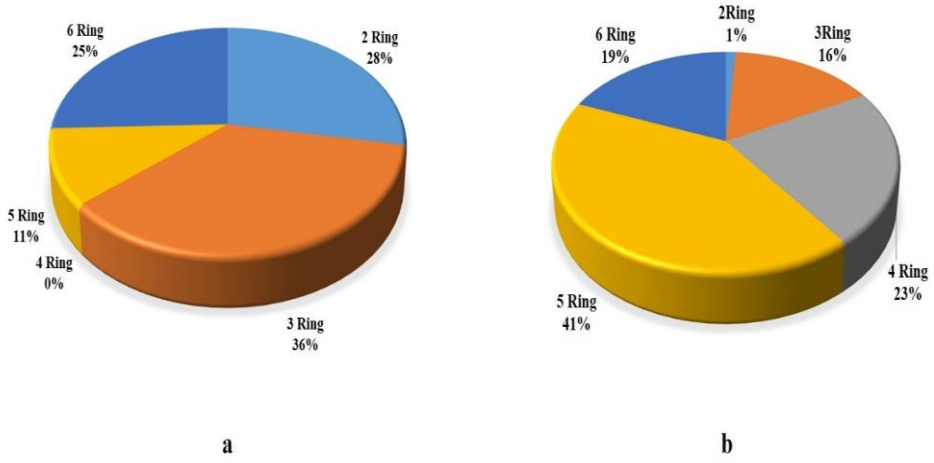


Figure 3: Distribution of the mean concentration of 2-ring, 3-ring, 4-ring, 5-ring, and 6-ring PAHs in **(a)** water and **(b)** sediment samples collected from the Sundarbans

This observation was in concurrence with Raza et al. (2013) who observed higher PAH concentration correlated with increasing salinity of seawater in the Rembaue Linggi estuary, Malaysia. The rate of microbial biodegradation of PAHs decreases with increasing salinity because high salinity can lower microbial utilization rates. Salinity reduces microbial activity, microbial biomass and changes microbial community structure (Yan et al., 2015). Additionally, inverse relationship between ambient salt concentrations and PAH degradation ability of bacterial isolates from the mangrove swamps in Hong Kong was reported by Tam et al. (2002).

Dominguez et al. (2010) demonstrated a similar prevalence of 4e6 ringed PAHs in sediments of the Sundarbans. The hydrodynamic regime of the Saptamukhi river represents a tidal inlet where the mixing of ebb and flood flows because of tidal asymmetry re-suspends the sediments (Dominguez et al., 2010; Sarkar, 2016). This local resuspension process influenced the PAH concentration recorded in Patharpratima.

3.3 Removal of PAHs from PAH-spiked media and heterotrophic biofilms

Biofilms were cultured in hydrophobic PMMA vessels (PMMAES- CCF) and hydrophilic glass vessels (Glass-ES-CCF) (US patent 8,945,917 B2, Sarkar et al., 2015). Concentrations of spiked PAH were in consistency with the total PAHs recorded in their respective geographical locations. Treatment of PAH-spiked media with microbial biofilms collected from different geographical locations did not show any significant differences in residual PAHs (figure 4a). There was no significant difference between the mean residual PAHs from the liquid media between PMMA and glass ES-CCFs ($P \leq 0.05$). However, residual amounts of certain PAHs such as Ace, Ant, B(b)F, B(a)P and B(g,h,i)P showed differences in their sequestration when cultivated in flasks with hydrophobic and hydrophilic surfaces.

The residual amounts of PAHs from biofilms varied between 0 and 106% which showed a greater significant difference at $P \leq 0.05$ in contrast to the residual amounts in the liquid medium (figure 4b). The mean residual amounts of total PAHs in biofilms were in the order Pp1<Kk < Nk < Pp2<Mt < Pg.

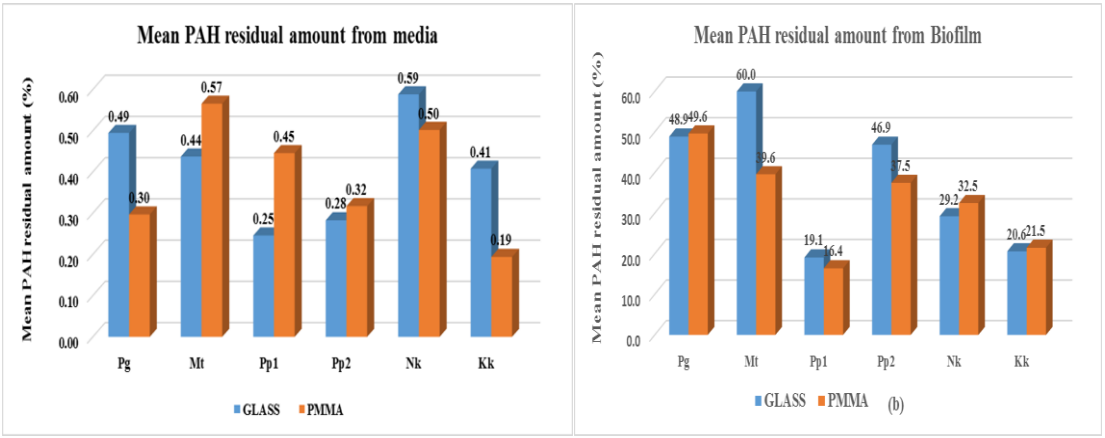


Figure 4: Mean residual amounts (percentage) of 16 priority spiked PAHs from liquid media (a) heterotrophic biofilm biomass (b) developed in glass and PMMA ES-CCF after incubation.

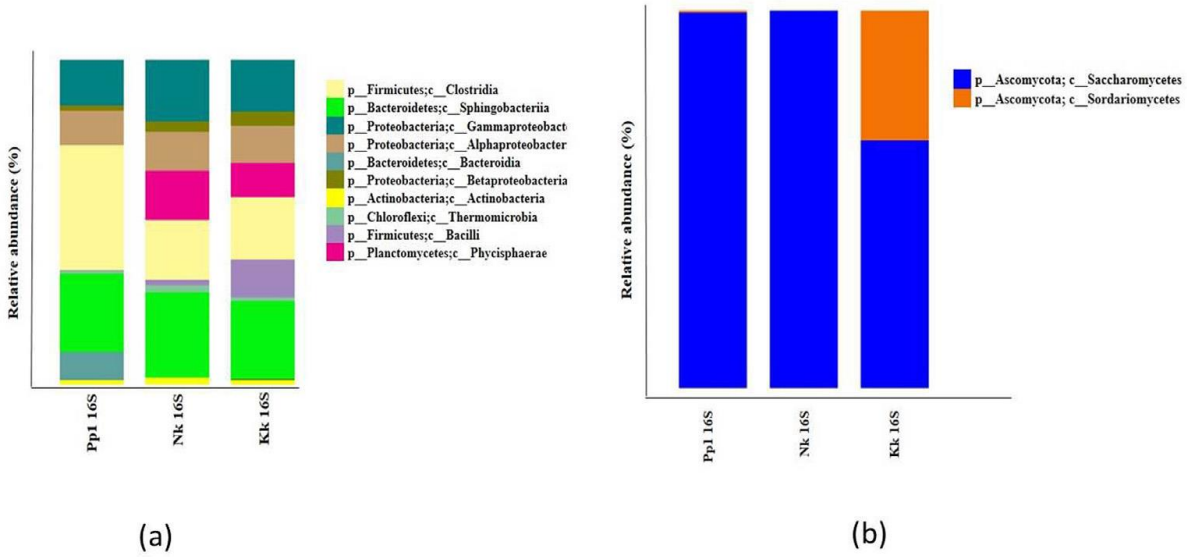
The determination of the water/biomass OC distribution coefficients and diffusion coefficients of the individual PAHs (Wicke et al., 2007) is essential. The composition of the biofilm and the role of external effects on the partitioning of the individual PAHs inside the biofilm and the transformation of the individual PAHs such as enzymatically catalysed inclusion of hydroxyl or carboxyl moieties by different microbial members of the biofilms (Wicke et al., 2008) are necessary.

3.4 Abundance, diversity and composition of PAH-sequestering microbial communities

Relative abundances of different microbial communities (Figure. 5 a and b) revealed the presence of bacterial phyla *Proteobacteria* (26.3-34.1%), *Bacteroidetes* (24.3-32.9%),

Firmicutes (19.9-38.6%), *Actinobacteria* (1.3-2.1%), *Chloroflexi* (0.8-2.3%) and *Planctomycetes* (0-15.3%) as well as members of *Ascomycota* (100%) phylum of fungi.

Biodegradation capacity of strains obtained from hydrocarbon contaminated environments was higher than those isolated from uncontaminated sediments because certain bacteria had acclimatized and adapted themselves to the contaminated environment (Tam et al., 2002). In the present study, biofilms were grown with PAHs as the sole source of carbon. Therefore, only specialized bacteria and fungi which were able to sequester the PAH compounds proliferated. Recently, Lee et al. (2018) reported only three dominant phyla (Proteobacteria, Firmicutes and Bacteroidetes) in the PAH-contaminated Sinduri beach in Korea. We found six phyla in the examined biofilms, higher than that obtained by Lee et al. (2018). Most of the members of the phylum Ascomycota frequently grew in anthropogenically polluted sites (Aranda et al., 2016) and enhanced the bioavailability and degradability of PAHs (Zafra et al., 2014). Hagler et al. (1979) reported Candida as the most frequently isolated genera from a polluted Brazilian estuary.



(a) (b)

Figure. 5:. Class level relative abundance of (a) PAH sequestering bacteria and (b) PAH sequestering fungi in three biofilms (Pp1, Nk and Kk). Amplification of the V3-V4 region

of 16S rRNA bacterial gene and intergenic transcribed spacer (ITS) of fungal gene was carried out.

3.5 Removal of PAHs from PAH-spiked media and phototrophic biofilms

Phototrophic biofilms in this study were able to sequester 98–100% of all the individual spiked PAHs in all liquid media except from the medium cultured with the Pg_p biofilm where the extent of sequestration of the different PAHs ranged from a low 1% to maximum 100%. Significant differences at $p \leq 0.05$ in the mean residual PAHs amounts between the biofilm-cultured vessels with the spiked PAHs and the ones without any spiking were observed.

The mean residual amounts of PAHs inside the phototrophic biofilms after exposure to the PAHs varied between 0% and 100%. Significant differences at $p \leq 0.05$ in the mean residual PAHs amounts between the biofilm-cultured vessels with the spiked PAHs and the ones without any spiking were noticed (figure 6). The ability to remove the 16 priority PAH contaminants by the phototrophic biofilms after 21 days incubation (as evident from the mean residual amount of PAHs) was in the order $Pp1_p$ >Kk_p>Nk_p>Mt_p>$Pp2_p$ >Pg_p. The mean residual PAH was higher in the Pg_p biofilm cultured in the hydrophobic PMMA ES-CCF compared to other biofilms cultured in hydrophobic and hydrophilic vessels, indicating low PAH sequestration potential of the Pg_p biofilm. Hydrophilic culture of the $Pp1_p$ biofilm and hydrophobic culture of the Kk biofilm showed the lowest mean residual amount of PAHs.

Phototrophic biofilms isolated from Patharpratima followed by Kakdwip and Namkhana showed the lowest mean PAH recoveries, which interestingly, were the same geographical locations of the Indian Sundarbans from where the isolated heterotrophic biofilms also demonstrated maximum PAH sequestration activity. Similar to this investigation, there was no substantial divergence between the mean residual PAHs

remaining in the liquid media between hydrophobic and hydrophilic vessels. Initial layers of microalgal cells are more likely to adhere to hydrophobic surfaces than to hydrophilic surfaces (Wang et al., 2018).

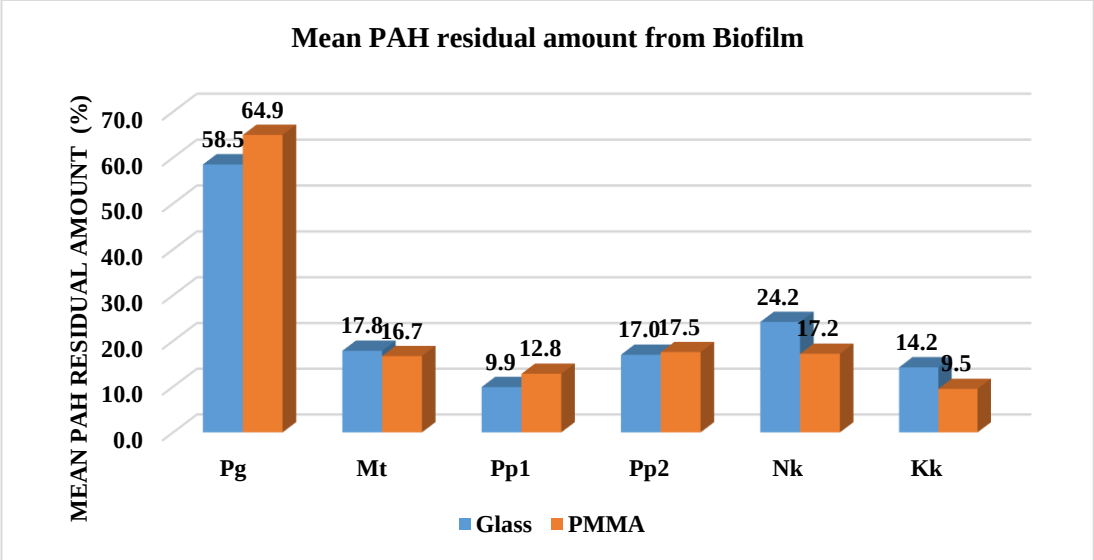


Figure 6: Mean residual amounts (percentage) of 16 priority spiked PAHs from phototrophic biofilm biomass developed in glass and PMMA ES-CCF after incubation.

According to Mantzorou and Ververidis (2019) cell adhesion is favored by hydrophobic materials, although there are arguments disapproving the role of surface hydrophobicity in microalgae adhesion. For example Zhuang et al. (2018) noted that hydrophilic support media.

3.6 Biofilm hydrophobicity and microscopic observations

The hydrophobicity test using n-hexadecane was conducted to establish the surface property (hydrophobic/hydrophilic) of the biofilms. $Pp1_p$, Nk_p and Kk_p biofilms demonstrated hydrophobic characteristics. Biofilms migrated towards the upper hydrocarbon layer in preference to the lower aqueous layer (Figure. 7). Morphological analyses using light microscope revealed the presence of purple sulfur bacteria, cyanobacteria such as *Gloeocapsa* sp., *Chloroidium* sp., pennate diatoms and *Closterium* sp. like green algae in $Pp1_p$ biofilms. Again, cyanobacteria such as *Gloeocapsa* sp.,

Microcystis sp. and purple sulfur bacteria were observed in Nk_p biofilms whereas Kk_p biofilms showed the occurrence of purple sulfur bacteria, cyanobacteria such as *Gleocapsa* sp. *Microcystis* sp. and *Phormidium* sp., *Closterium* sp. like green algae as well as pennate diatoms (Figure. 8). PAH-treated biofilms also showed the presence of similar phototrophic microorganisms. SEM observations (Figure. 9) and comparisons with similar published images (Boelee, 2013; Cole et al., 2014) were suggestive of the biofilm being composed predominantly of filamentous cyanobacteria where the algal cells occurred in clusters embedded in the EPS matrix. Filaments with the associated EPS were observed in the control biofilms. Heterotrophic bacteria formed close association with filamentous phototrophic microorganisms (Fig. 8b). In the PAH-treated biofilms cells were found as clusters and embedded firmly within the produced EPS in response to xenobiotic challenge. Biofilm structures were largely unchanged after 21days of incubation with PAHs. Salt crystals appeared as cube-like structures in SEM images.

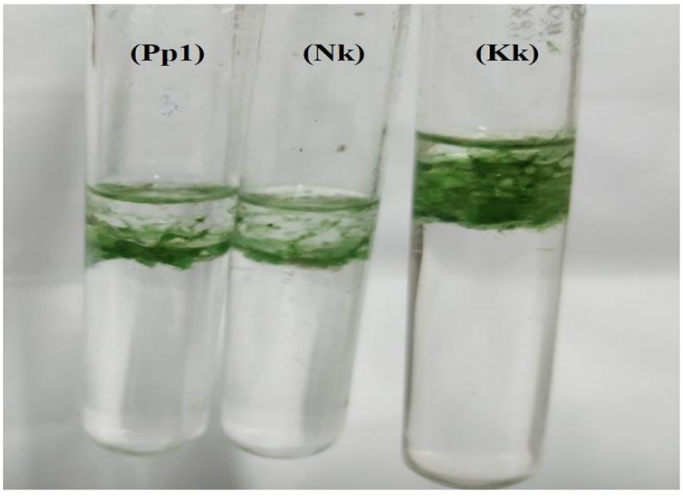


Figure. 7:. Partitioning of phototrophic biofilms in the biphasic system. $Pp1_p$, Nk_p and Kk_p biofilms preferentially adhered to the hydrocarbon layer indicating the hydrophobicity of biofilms.

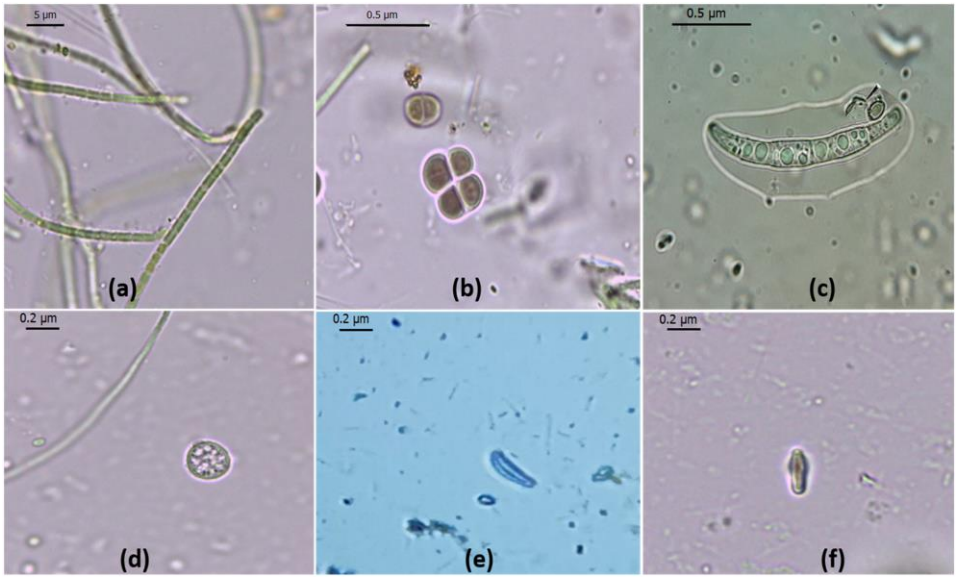


Figure. 8:. Morphological analyses of PAH degrading phototrophic biofilms showed presence of (a) *Phormidium* sp. (b) *Gloeocapsa* sp. (c) *Closterium* sp. (d) *Microcystis* sp. (e) and (f) pennate diatoms (magnification of 100 ×10 X).

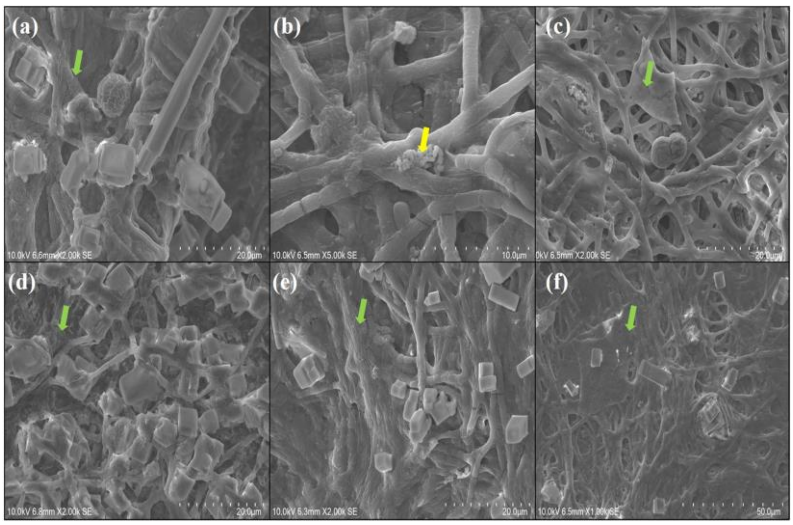


Figure. 9:. Scanning electronic microscope (SEM) micrographs of (a) $Pp1_{\mathbf{p}}$ (b) $Nk_{\mathbf{p}}$ (c) $Kk_{\mathbf{p}}$ phototrophic biofilms without PAHs and (d) $Pp1_{\mathbf{p}}$ (e) $Nk_{\mathbf{p}}$ (f) $Kk_{\mathbf{p}}$ phototrophic biofilms treated with PAHs. Yellow arrow indicates bacteria associated with the biofilm and green arrows denote the EPS matrix.

Phormidium sp., *Gloeocapsa* sp., *Closterium* sp., *Microcystis* sp. and diatoms were identified as photosynthetic organisms in the biofilms. These organisms possess PAH

degradation capability as well as the potential to degrade other pollutants including metals. For example, Ant was degraded by hypersaline *Phormidium tenue* (Zyszka-Haberecht et al., 2019). Abed et al. (2002) also reported presence of *Phormidium* sp. as the predominant cyanobacterium in indigenous microbial communities inhabiting a heavily polluted site in a coastal stream (Wadi Gaza) as well as its PAH degradation potential, especially Phe.

3.7 Effect of PAHs on the biofilm biomass and photosynthetic pigments in hydrophobic and hydrophilic vessels

Addition of PAHs in the phototrophic biofilm growth media showed significant biomass increase in hydrophobic as well as hydrophilic flasks at $p \leq 0.05$ as shown in figure 9. Biomass accrued by the $Pp1_{\mathbf{p}}$ biofilm in the presence of PAHs was higher in the hydrophilic glass flask in comparison to the hydrophobic PMMA flask. The increase in the $Pp1_{\mathbf{p}}$ biomass in the presence of PAHs was 100% in glass ES-CCF and 58% in the PMMA flask. Compared to control biofilms, 19% and 22% increase in $Nk_{\mathbf{p}}$ biofilm biomass were recorded in the glass and PMMA ES-CCFs respectively. For $Kk_{\mathbf{p}}$ biofilms, increase in biofilm biomass was 12% (glass ES-CCF) and 33% (PMMA ES-CCF). The mean residual PAH amounts were lowest in the liquid medium as well as inside the biofilms in the hydrophobic flask where the $Kk_{\mathbf{p}}$ biofilm was cultivated. Interestingly, highest biofilm biomass was recorded in this cultivation.

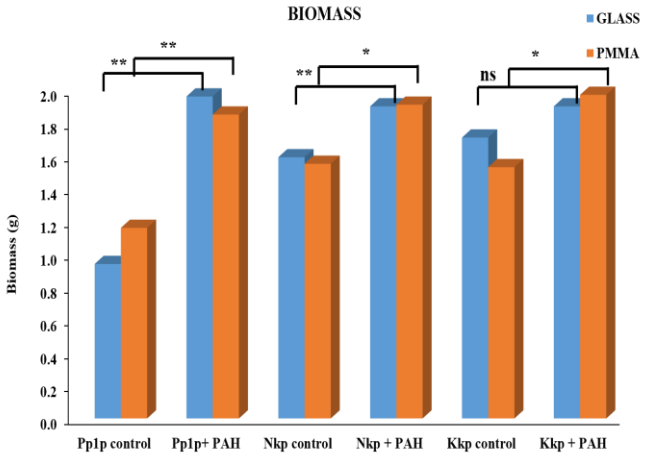


Figure 9: Biomass (g) of selected phototrophic biofilm ($Pp1_{p,}$ $Nk_{p,}$ and Kkp) having high PAH degradation potential cultured with and without spiked 16 priority PAHs in hydrophobic and hydrophilic ES-CCFs.

3.8 Increase in photosynthetic pigments

Total Chl contents in the biofilms cultured with PAHs increased when compared to the controls (figure 10). The highest total Chl content was measured in the hydrophilic culture of $Pp1_{\mathbf{p}}$ biofilm and hydrophobic culture of $Kk_{\mathbf{p}}$ biofilm cultivated in PAH-spiked media. The enhanced total Chl content in these two flasks was well correlated with the lowest mean residual amount of PAHs. Thus, elevated chlorophyll concentrations in PAH-treated biofilms implied an increased photosynthetic efficiency compared to control biofilms that in turn played a role in reducing PAH pollutant levels. Total Chl amounts in PAH-treated Nk biofilms were higher in hydrophobic PMMA flasks compared to hydrophilic glass vessels. Additionally, according to paired *t*-test concentrations of photosynthetic pigments (Chl *a*, *b*, *c*, *d* and carotenoids) in all three biofilms ($Pp1_{\mathbf{p}}$, $Nk_{\mathbf{p}}$ and $Kk_{\mathbf{p}}$) were significantly higher ($p \leq 0.05$) than their respective control treatments

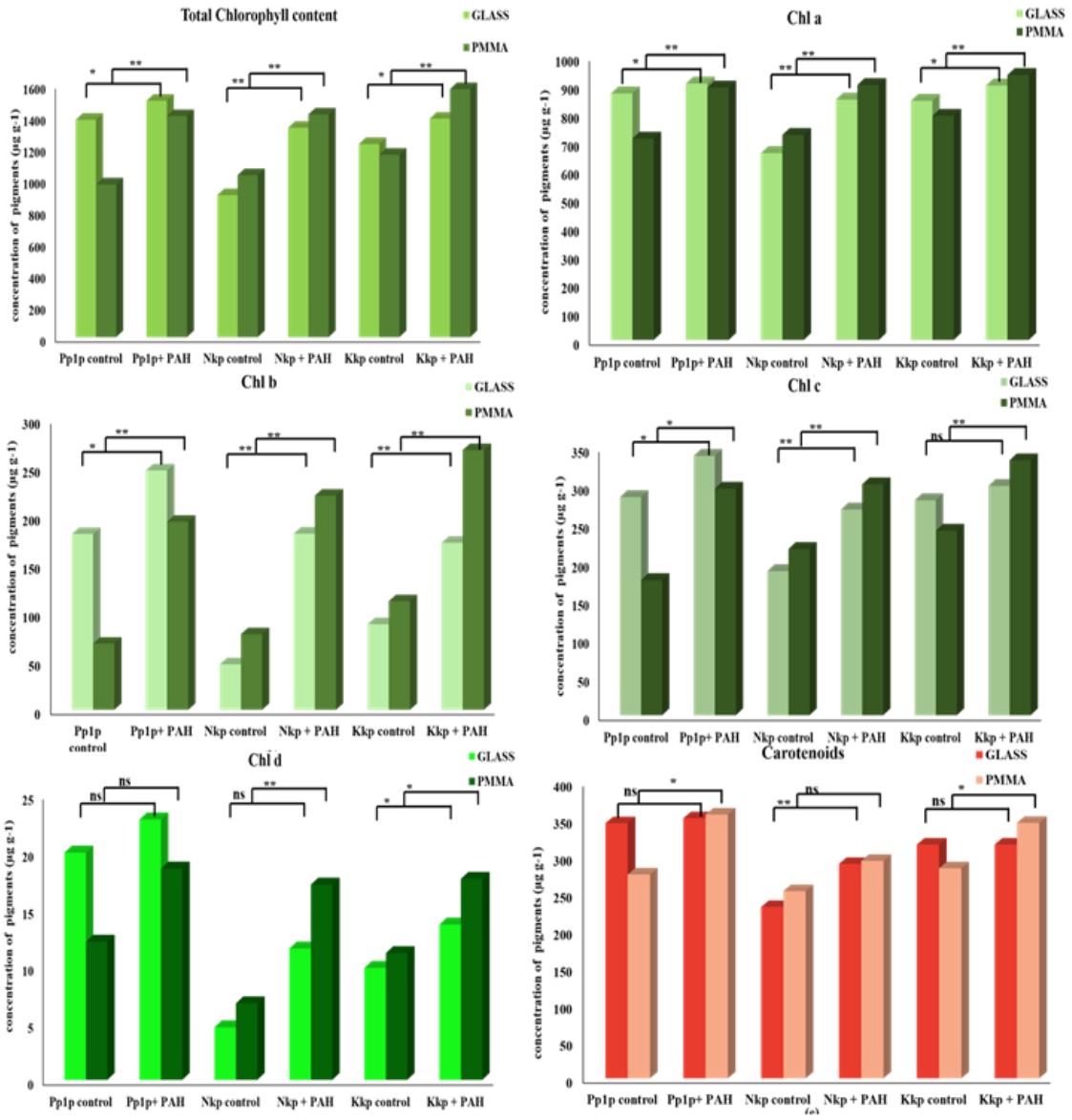


Figure 10: Total chl contents, chl *a*, chl *b*, chl *c*, chl *d*, and carotenoid contents (µg/g) of phototrophic biofilm ($Pp1_{p}$, Nk_{p}, Kk_{p}) cultured with and without spiked 16 priority PAHs in hydrophobic and hydrophilic ES-CCFs.

The present study showed an increase in the photosynthetic pigment concentrations in all the three biofilm samples after growing in media spiked with 16 priority pollutants for 21 days. Increase in Chl *a* content can augment algal photosynthesis, which can accelerate greater energy accretion in the algae and stimulate algal reproduction and growth (Chen et al., 2020). Similar inferences were drawn by several other workers in other phototrophic microorganisms such as the saline cyanobacterium *Phormidium tenue* (Zyszka-Haberecht

et al., 2019). Although these authors reported enhancement of chlorophyll levels in photoautotrophs upon PAH exposure, the reasons for such increase were not discussed.

3.9 Effect of PAHs on EPS and its constituents

RPS and CPS were extracted separately from the PAH-spiked as well as control experiments to evaluate EPS production by the phototrophic biofilms when challenged with the 16 PAHs after 21 days incubation (Table 1). Increased production of the RPS as well as CPS was observed in the biofilms spiked with different concentrations of PAHs. Highest production of RPS was observed in $Nk_{\mathbf{p}}$ biofilms (compared to $Pp1_{\mathbf{p}}$ and $Kk_{\mathbf{p}}$ biofilms) cultured in presence of PAHs in glass ES-CCF and PMMA ES-CCF.

3.9.1 EPS carbohydrates

Carbohydrates, proteins and uronic acid present in the EPS which have characteristic roles in biofilm growth and attachment were determined. The concentration of protein was rather low, less than 15 mg/g of EPS on average, which accounted for only a small portion of the EPS constituents (less than 9% in RPS and 5% in CPS), while uronic acid and carbohydrates were the major biochemical components of the EPS, having proportions of above 91% in RPS and over 95% in CPS. RPS and CPS carbohydrates in biofilms spiked with PAHs increased significantly ($p \leq 0.05$) compared to the controls. Additionally, highest carbohydrate concentration was recorded in the RPS and CPS extracted from $Kk_{\mathbf{p}}$ biofilms cultured in the PMMA flask.

3.9.2 EPS proteins

Measured values of proteins were low compared to the corresponding carbohydrates and uronic acids. Despite their low contents proteins are important because they constitute the enzymes required for metabolism, photosynthesis as well as CO2 fixation. Similar to RPS and CPS carbohydrates the RPS and CPS proteins increased significantly in glass and PMMA ES-CCFs spiked with PAHs ($p \leq 0.05$). Protein concentration in the RPS was

measured in the order Pp1 $_{p}$ >Kk $_{p}$ >Nk $_{p}$ whereas for the CPS protein the series was Kk $_{p}$ >N k $_{p}$ >Pp1 $_{p}$.

3.9.3 EPS uronic acids

Highest concentrations of CPS carbohydrates, uronic acid as well as RPS carbohydrates, proteins and uronic acids in the PMMA flasks cultured with the PAH-treated Kk biofilms was correlated with the lowest mean residual amount of PAHs in the hydrophobic culture of the Kk biofilm. In presence of PAH pollutants, uronic acid contents in RPS as well as CPS in all the biofilms increased significantly ($p \leq 0.05$). The order of RPS as well as CPS uronic acids were Kk $_{p}$ >Nk $_{p}$ >Pp1 $_{p}$. Uronic acid content of EPS (comprising of RPS and CPS) corroborated with the hydrophobicity of the EPS. Hydrophobicity of the EPS increased significantly after spiking with PAHs (Figure. 11).

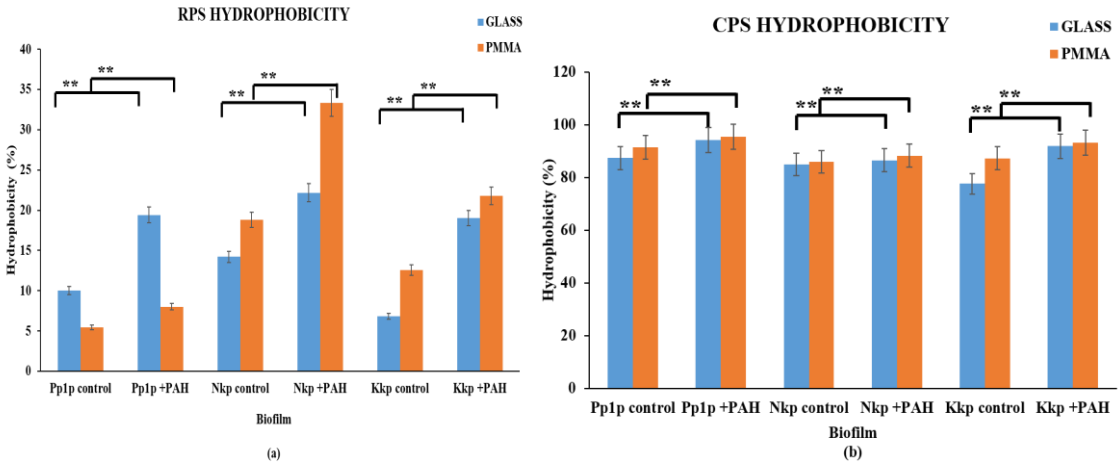


Figure 11: Hydrophobicity of released (RPS) **(a)** as well as capsular (CPS) **(b)** extracellular polymeric substances. While RPS hydrophobicity is altered in response to PAH exposure, CPS hydrophobicity remains constant. Significance was determined through paired t-test at $p < 0.01$.

Table 1: RPS and CPS (mg/g of dry weight of biomass); carbohydrate, protein, and uronic acid contents (mg/g of dry weight of RPS/CPS) and protein/polysaccharide ratio as a percentage (%) in control and experimental biofilms cultured in hydrophilic glass and hydrophobic PMMA ES-CCFs (values are means ± SD; n=3). Protein/ polysaccharide ratios of RPS and CPS were estimated by summing up the carbohydrate and uronic acid contents as total polysaccharides. Bold figures indicate the highest values obtained. ** represents $p<0.01$, * denotes $p<0.05$ and ns stands for non-significant. Differences made on paired *t*-test

Parameters	**$Pp1_p$ control**		**$Pp1_p$ +PAH**		**Nk_p control**		**Nk_p +PAH**		**Kk_p control**		**Kk_p +PAH**	
	GLASS	**PMMA**	**GLASS**	**PMMA**	**GLASS**	**PMMA**	**GLASS**	**PMMA**	**GLASS**	**PMMA**	**GLASS**	**PMMA**
RPS	55.9 ± 0.2	63.1 ± 8.7	78.7± 2.5**	82.3 ± 3.0^{ns}	83.9 ± 6.0	85.7 ± 1.3	**122.0±3.5****	111.4 ±7.3*	59.0 ± 3.7	81.2 ± 3.4	90.6 ± 7.4^{ns}	96.6 ± 8.4*
Carbohydrate	47.6 ± 0.9	50.9 ± 1.8	63.7 ± 2.3*	52.6 ± 2.6^{ns}	77.8 ±2.3	84.7 ± 1.9	92.5±11.4^{ns}	100.3±2.3*	78.6 ± 6.2	88.3 ± 3.1	90.8 ± 2.8^{ns}	**128.2±3.2***
Protein	6.6 ± 0.1	7.4 ± .2	**11.4± 0.3****	9.4 ± 0.6^{ns}	5.7 ± 0.1	5.9 ± 0.1	9.2 ± 0.6**	10 ± 0.6*	5.5 ± 0.0	5.6 ± 0.0	9.8 ± 0.2**	10.4± 0.2**
Uronic acid	43.1 ± 2.8	33.7 ± 3.4	54.8± 2.0**	48.8 ± 0.9*	36.2±1.8	43.1 ± 2.0	64.1 ± 3.7**	68.5 ± 5.9*	31.9 ± 1.6	37.3 ± 0.8	63.7 ± 2.2**	**90.2± 7.4****
Protein/ polysaccharide	7.2	8.7	**9.6****	9.3^{ns}	5.0	4.6	5.9^{ns}	5.8^{ns}	4.8	4.5	6.3*	5.0^{ns}
CPS	51.2 ± 0.9	27.9± 1.4	53.2 ± 3.1^{ns}	42.2 ± 6.2^{ns}	22.5±1.2	31.6 ± 1.2	25.2 ± 2.1^{ns}	31.8 ± 6.2^{ns}	65.1± 2.2	44.2 ± 4.3	**68.3 ± 6.0^{ns}**	53.3±10^{ns}
Carbohydrate	148.3±5.9	169.6±3.2	297.8±4.7**	274.6±4.8**	126.0±4	136.2±1.9	232.6±5.2**	294.6±2.2**	208.0±10.1	178.7±3.0	325.1 ± .5*	**398.4 ± 9****
Protein	5.5 ± 0.8	5 .0 ± 0.1	11.1 ± 0.7**	8.1 ± 0.6*	4.7±0.5	4.9 ± 0.9	8.8 ± 0.9**	10.7 ± 0.3*	5.8 ± 3.3	6.2 ± 0.4	9.1 ± 1.0*	**14.1±3.5****
Uronic acid	30.1 ± 2.8	32.2 ± 0.3	40.9 ± 3.5*	55.0 ± 8.6*	54.1±4.5	56.3 ± 2.6	59.0 ± 0.9**	61.6 ± 5.6^{ns}	34.6 ± 2.4	55.2 ± 4.2	66.1± 4.9**	**90.2 ±8.4****
Protein/ Polysaccharide	3.0	2.5	3.3^{ns}	2.5^{ns}	2.6	2.6	3.0^{ns}	3.0^{ns}	2.4	2.7	2.4^{ns}	2.9^{ns}

A high production of EPS is generally regarded as a protective mechanism against stress factors (Stiefelmaier et al., 2020). Environmental stressors, such as the presence of PAHs in this investigation, affect not only the quantity of produced EPSs, but also the physico-chemical characteristics of the secreted polymer, thus strengthening the adaptation capacity of the biofilm to severe environmental conditions (Rossi and De Philippis, 2015). In this perspective, a mechanism to be considered for the enhancement of the bioavailability of PAHs to the degradative enzymes is the production of biosurfactants and high molecular weight EPS can function as biosurfactants (McGenity et al., 2012). Microbial biosurfactants are also produced in response to heavy metal challenge (Govarthanan et al., 2017). Produced EPS can reduce interfacial tension and interact with hydrophobic PAHs thus enhancing their solubility in water.

Summary and conclusion

This thesis present for the first time **(i)** an assessment of the PAH contamination level in the mangrove ecosystem, which is the center of international conservation efforts. Mangrove forests and estuaries are among the pristine ecosystems existing on the planet. To obtain a comprehensive image of the status of PAH contamination of the Sundarbans, an extensive field sampling including both surface water and sediment was made **(ii)** laboratory application of a patented biofilm-promoting reaction flask to grow the field-collected heterotrophic and phototrophic biofilms in a way to faithfully simulate the field situation **(iii)** determining the biofilms potential to remove spiked PAHs that may indicate their contribution in discovering the PAH contaminants in the sediments. In addition, the role of naturally occurring biofilms in intertidal PAH dynamics was evaluated for the first time in any estuary of the world.

This study revealed the current status of PAH contamination in surface water and sediments of the world's largest tidal mangrove ecosystem, the Indian Sundarbans. The prevalent PAH concentrations in the Sundarbans increased considerably concerning the last recorded value in 2012. Disconcertingly, the PAH concentrations exceed ER-L guidelines' values, suggesting that this mangrove forest's prevailing PAHs are likely to cause adverse biological effects. Therefore, there is an urgent necessity to monitor and take remedial actions to ensure the acceptable quality of the sediments. The current study also reveals the presence of intertidal microbial biofilms (heterotrophic and phototrophic biofilms) with remarkable potential for removing contaminants. Using indigenous microbial biofilms found effective in all the geographical locations tested in the present study may be a helpful bioremediation strategy for removing PAH contamination. Furthermore, the metagenomic study of the heterotrophic biofilms suggested that a diverse microbial population was present in the naturally occurring biofilms of the Sundarbans with PAH degradation potential, which may be utilized for the successful remediation of PAH contamination in the field.

Indigenous phototrophic biofilms of the Sundarbans presented a high potential for PAH sequestration. The lowest mean residual amounts of PAHs in the liquid medium and the biofilms were recorded in the $Kk_{\mathbf{p}}$ biofilm cultivated in the hydrophobic flask. Simultaneously, the highest values of biofilm biomass, total chl, RPS carbohydrates, RPS uronic acids, CPS carbohydrates, CPS proteins, and CPS uronic acids were obtained from the same biofilm cultured in the hydrophobic vessel. A strong association between PAH sequestration and biochemical measurements was not observed in the $Kk_{\mathbf{p}}$ biofilm grown in the hydrophilic vessel. In addition, the cultivation vessel impacted the removal of individual PAHs and the synthesis of photosynthetic pigments and EPS. Analyses of the underlying mechanisms were beyond the scope of this study. However, our observations

emphasise the hydrophobic/hydrophilic property of the culture surface as a major impelling factor in research involving biofilms. Therefore, the surface property of the culture vessel will affect experimental results and inferences drawn from there. It may also be concluded that heterotrophic and phototrophic biofilm communities from the sampling sites with the highest PAH sediment contaminations (Nk and Kk) adapted themselves by developing biochemical responses to the presence of high levels of PAHs. Contaminated sediments pose challenging clean-up and management problems, as conventional environmental dredging techniques are invasive, expensive, and sometimes ineffective or hard to apply to large and diverse sediment sites. Phototrophic biofilms showed huge potential for field applications in sequestering PAH contaminants compared to the heterotrophic biofilms in mangrove forest intertidal sediments. These films should be propagated in preference to heterotrophic ones due to their enhanced extent of PAH removal compared to heterotrophic biofilms. For a better understanding of the PAH degradation process, the composition of the biofilms with PAH degradation potential and the role of external factors which affect the partitioning and transformation of individual PAHs inside the biofilms are essential. The information gained from the present study will significantly advance our knowledge and understanding of environmental biofilms and help us foresee their response(s) to PAH pollution. This study should generate knowledge for future applications of biofilms for the bioremediation of various other pollutants in the estuarine waters and sediments.

Future scope of the study

In mainstream natural environments, microbes are generally associated with surfaces and interfaces in the form of biofilms, multicellular aggregates that bond together with slime secreted by the microbes within the biofilms. Microbes within a biofilm can resist shear

forces, natural deprivation, pH changes, and antibiotics, more evidently due to the mechanical strength of the secreted EPS. The major future scope of this study is translating the laboratory study into the field where PAH confiscation will be assessed through two major bioremediation techniques, such as bio-augmentation and bio-stimulation. The biofilms (Phototrophic and heterotrophic), which showed maximum PAH degradation potential, should be cultivated on a large scale, and the addition of these to the sediments of the intertidal zone of the Indian Sundarbans to attain an acceptable sediment quality—assessing the physico-chemical characteristics and nutrient contents of the sediments and modifying the environment to stimulate the indigenous microbial biofilms present in the contaminated location. Concurrently, microbial diversity and metagenomic study of phototrophic and heterotrophic microbial diversity from the field before and after the bioremediation process helps to characterize the microbes involved in the bioremediation process.

References

Abed, R.M., Safi, N.M., Köster, J., De Beer, D., El-Nahhal, Y., Rullkötter, J. and Garcia-Pichel, F., 2002. Microbial diversity of a heavily polluted microbial mat and its community changes following degradation of petroleum compounds. Appl. Environ. Microbiol. 68(4), pp.1674-1683.

Alexander, M., 1994. Bio-degradation kind bioremediation academic. San Diego, Calif, pp.1-7.

Aranda, E., 2016. Promising approaches towards biotransformation of polycyclic aromatic hydrocarbons with Ascomycota fungi. Curr. Opin. Biotechno, 38, pp.1-8.

Bhatnagar, M., Parwani, L., Sharma, V., Ganguly, J. and Bhatnagar, A., 2014. Exopolymers from *Tolypothrix tenuis* and three *Anabaena* sp. (Cyanobacteriaceae) as novel

blood clotting agents for wound management. Carbohydr. Polym. 99, pp.692-699.

Boelee, N.C., 2013. Microalgal biofilms for wastewater treatment. Wageningen University and Research. ISBN: 978-94-6173-666-6

Chaudhuri, K., Manna, S., Sarma, K.S., Naskar, P., Bhattacharyya, S. and Bhattacharyya, M., 2012. Physicochemical and biological factors controlling water column metabolism in Sundarbans estuary, India. Aquat. Biosyst, 8(1), pp.1-16.

Chen, S., Li, J., Feng, W., Yuan, M., Zhang, W., Xu, H., Zheng, X. and Wang, L., 2020. Biochemical responses of the freshwater microalga *Dictyosphaerium* sp. upon exposure to three sulfonamides. J. Environ. Sci. 97, pp.141-148.

Cole, J.K., Hutchison, J.R., Renslow, R.S., Kim, Y.M., Chrisler, W.B., Engelmann, H.E., Dohnalkova, A.C., Hu, D., Metz, T.O., Fredrickson, J.K. and Lindemann, S.R., 2014. Phototrophic biofilm assembly in microbial-mat-derived unicyanobacterial consortia: model systems for the study of autotroph-heterotroph interactions.Front.microbiol.5, p.109.

Di Pippo, F., Ellwood, N.T., Gismondi, A., Bruno, L., Rossi, F., Magni, P., De Philippis, R., 2013. Characterization of exopolysaccharides produced by seven biofilm-forming cyanobacterial strains for biotechnological applications. J. Appl. Phycol.25 (6), 1697–1708.

Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.T. and Smith, F., 1956. Colorimetric method for determination of sugars and related substances. Anal.Chem. 28(3), pp.350-356

Fattom, A. and Shilo, M., 1984. Hydrophobicity as an adhesion mechanism of benthic cyanobacteria. Appl. Environ. Microbiol. 47, pp 135–143.

Froehner, S., Machado, K.S., Dombroski, L.F., Nunes, A.C., Kishi, R.T., Bleninger, T. and Sanez, J., 2012. Natural biofilms in freshwater ecosystem: indicators of the presence of polycyclic aromatic hydrocarbons. Wat. Air. Soil Poll, 223(7), pp.3965-3973

Gacheva, G., Gigova, L., Ivanova, N., Iliev, I., Toshkova, R., Gardeva, E., Kussovski, V. and Najdenski, H., 2013. Suboptimal growth temperatures enhance the biological activity

of cultured cyanobacterium *Gloeocapsa* sp. J. Appl. Phycol. 25(1), pp.183-194.

Ghosal, D., Ghosh, S., Dutta, T.K. and Ahn, Y., 2016. Current state of knowledge in microbial degradation of polycyclic aromatic hydrocarbons (PAHs): a review. Front. Microbiol., p.1369.

Govarthanan, M., Mythili, R., Selvankumar, T., Kamala-Kannan, S., Choi, D., Chang, Y.C., 2017. Isolation and characterization of a biosurfactant-producing heavy metal resistant *Rahnella* sp. RM isolated from chromium-contaminated soil. Biotechnol. Bioprocess Eng. 22 (2), 186–194.

Grula, E.A., Russell, H.H., Bryant, D., Kenaga, M., Hart, M., Donaldson, E. and Clark, J., 1982. Isolation and screening of clostridia for possible use in microbially enhanced oil recovery. Proceedings of the microbial enhanced oil recovery, Afton, Okla, USA, 1982.

Hagler, A.N., Santos, S.S. and Mendonca-Hagler, L.C., 1979. Yeasts of a polluted Brazilian estuary. Revista de microbiologia.

Lee, D.W., Lee, H., Lee, A.H., Kwon, B.O., Khim, J.S., Yim, U.H., Kim, B.S. and Kim, J.J., 2018. Microbial community composition and PAHs removal potential of indigenous bacteria in oil contaminated sediment of Taean coast, Korea. Environ. Pollu., 234,.503-512.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, pp.265-275.

Mantzorou, A. and Ververidis, F., 2019. Microalgal biofilms: A further step over current microalgal cultivation techniques. Sci. Total Environ. 651, pp.3187-3201.

McGenity, T.J., Folwell, B.D., McKew, B.A. and Sanni, GO, 2012. Marine crude-oil biodegradation: a central role for interspecies interactions. Aquat. Biosyst. 8(1), pp.1-19.

Mitra, A. and Mukhopadhyay, S., 2016. Biofilm mediated decontamination of pollutants from the environment. AIMS Bioeng. 3(1), pp.44-59.

Radojevic, M., Bashkin, V. and Bashkin, V.N., 1999. Practical environmental analysis.

Royal society of chemistry.

Raza, M., Zakaria, M.P., Hashim, N.R., Yim, U.H., Kannan, N. and Ha, SY, 2013. Composition and source identification of polycyclic aromatic hydrocarbons in mangrove sediments of Peninsular Malaysia: indication of anthropogenic input. Environ. Earth Sci., 70(6), pp.2425-2436.

Ritchie, R.J., 2008. Universal chlorophyll equations for estimating chlorophylls a, b, c, and d and total chlorophylls in natural assemblages of photosynthetic organisms using acetone, methanol, or ethanol solvents. Photosynthetica. 46(1), pp.115-126.

Rossi, F. and De Philippis, R., 2015. Role of cyanobacterial exopolysaccharides in phototrophic biofilms and in complex microbial mats. Life. 5(2), pp.1218-1238.

Shukla, S.K., Mangwani, N., Rao, T.S., Das, S., 2014. Biofilm-mediated bioremediation of polycyclic aromatic hydrocarbons. Microbial Biodegradation and Bioremediation. Elsevier Inc., Oxford, United Kingdom, pp. 203-232.

Silva, T.R., Verde, L.C.L., Neto, E.S. and Oliveira, V.M., 2013. Diversity analyses of microbial communities in petroleum samples from Brazilian oil fields. Int. Biodeterior. Biodegrad 81, pp.57-70.

Singh, S.K. and Haritash, A.K., 2019. Polycyclic aromatic hydrocarbons: soil pollution and remediation. Int J Environ Sci Technol, 16(10), pp.6489-6512.

Stiefelmaier, J., Strieth, D., Di Nonno, S., Erdmann, N., Muffler, K. and Ulber, R., 2020. Characterization of terrestrial phototrophic biofilms of cyanobacterial species. Algal Res. 50, p.101996.

Tam, N.F.Y., Guo, C.L., Yau, W.Y. and Wong, Y.S., 2002. Preliminary study on biodegradation of phenanthrene by bacteria isolated from mangrove sediments in Hong Kong. Mar. Pollut. Bull., 45(1-12), pp.316-324.

Taylor, K.A. and Buchanan-Smith, J.G., 1992. A colorimetric method for the quantitation

of uronic acids and a specific assay for galacturonic acid. Anal. Biochem. 201(1), pp.190-196. https://doi.org/10.1016/0003-2697(92)90194-C

United States Environmental Protection Agency 1996a. Method 3540C: Soxhlet extraction, Washington, DC.

United States Environmental Protection Agency, 1996b. EPA-Method 3630C, Silica gel clean-up.

United States Environmental Protection Agency, 1996c. Test Method 3510c: Separatory funnel liquid-liquid extraction.

United States Environmental Protection Agency, 2004. Method 8000B, Determinative chromatographic separations.

Veerabadhran, M., Chakraborty, S., Mitra, S., Karmakar, S. and Mukherjee, J., 2018. Effects of flask configuration on biofilm growth and metabolites of intertidal Cyanobacteria isolated from a mangrove forest. J. Appl. Microbiol. 125(1), pp.190-202.

Walkley, A. and Black, I.A., 1934. An examination of the Degtjareff method for determining soil organic matter, and a proposed modification of the chromic acid titration method. Soil Sci., 37(1), pp.29-38.

Wang, Y., Wang, J., Mu, J., Wang, Z., Cong, Y., Yao, Z., Lin, Z., 2016. Aquatic predicted no effect concentrations of 16 polycyclic aromatic hydrocarbons and their ecological risks in surface seawater of Liaodong Bay, China. Environ. Toxicol. Chem. 35, 1587-1593.

Yan, N., Marschner, P., Cao, W., Zuo, C., Qin, W., 2015. Influence of salinity and water

Zafra, G., Absalón, Á.E., Cuevas, M., Carmen, D. and Cortés-Espinosa, D.V., 2014. Isolation and selection of a highly tolerant microbial consortium with potential for PAH biodegradation from heavy crude oil-contaminated soils. Water, Air, & Soil Pollution, 225(2), pp.1-18.

Zammit, G., Billi, D., Shubert, E., Kasstovskyy, J., Albertano, P., 2011. The biodiversity

of subaerophytic phototrophic biofilms from Maltese hypogea. Fottea 11,187-201.

Zhang, Y., Wang, F., Yang, X., Gu, C., Kengara, F.O., Hong, Q., Lv, Z., Jiang, X., 2011. Extracellular polymeric substances enhanced mass transfer of polycyclic aromatic hydrocarbons in the two-liquid-phase system for biodegradation. Appl. Microb. Biotechnol. 90 (3), 1063–1071.

Zhuang, L.L., Yu, D., Zhang, J., Liu, F.F., Wu, Y.H., Zhang, T.Y., Dao, G.H. and Hu, H.Y., 2018. The characteristics and influencing factors of the attached microalgae cultivation: a review. Renew. Sustain. Energy Rev. 94, pp.1110-1119.

Zyszka-Haberecht, B., Niemczyk, E. and Lipok, J., 2019. Metabolic relation of cyanobacteria to aromatic compounds. Appl. Microbiol. Biotechnol. 103(3), pp.1167-1178.