Biofilm Biochemistry Induced by Heavy Metal Sequestration: A Field Study and Their Role in Sedimentary Structure Formation and Bio-Stabilization

DIVYANGANA LAHIRI

Roll Number: 002130904011

Exam Roll Number: M4EBT23003

MASTER OF TECHNOLOGY

IN

ENVIRONMENTAL BIOTECHNOLOGY

SCHOOL OF ENVIRONMENTAL STUDIES

FACULTY OF INTERDISCIPLINARY STUDIES, LAW AND MANAGEMENT

(F.I.S.L.M)

JADAVPUR UNIVERSITY

JADAVPUR, KOLKATA

Biofilm Biochemistry Induced by Heavy Metal Sequestration: A Field Study and Their Role in Sedimentary Structure Formation and Bio-Stabilization

A thesis

Submitted in partial fulfilment of the requirements for the award of degree of

M. Tech in Environmental Biotechnology

Jadavpur University

by

DIVYANGANA LAHIRI

Environmental Biotechnology

Roll Number: 002130904011

Exam Roll Number: M4EBT23003

Under the supervision of

Prof. Joydeep Mukherjee

Professor

&

Dr. Reshmi Das

UGC Assistant Professor

School Of Environmental Studies

Faculty Of Interdisciplinary Studies, Law and Management

(F.I.S.L.M)

Jadavpur University

Jadavpur, Kolkata

Declaration

I hereby declared that the work presented in this thesis report title **"Biofilm Biochemistry Induced By Heavy Metal Sequestration: A Field Study And Their Role In Sedimentary Structure Formation And Bio-Stabilization**" submitted to Jadavpur University, Kolkata in partial fulfilment of the requirements for the award of the degree of M.Tech is a bonafide record of the research work carried out under the supervision of Prof. Joydeep Mukherjee and co-supervision of Dr. Reshmi Das. The contents of Thesis report in parts, have not been submitted to and will not be submitted by me to any other Institute or University in India or abroad for the award of any degree or diploma.

Signature

Divyangana Lahiri 15/06/23

Divyangana Lahiri
Roll no.: 002130904011
Exam Roll no: M4EBT23003

Environmental Biotechnology

School of Environmental Studies
Jadavpur University, Kolkata

Certificate

This is to certify that the Thesis Report titled "**Biofilm Biochemistry Induced By Heavy Metal Sequestration: A Field Study And Their Role In Sedimentary Structure Formation And Bio-Stabilization**", submitted by Divyangana Lahiri (002130904011) to Jadavpur University, Kolkata for the award of degree of M.Tech, is a bonafide record of the research work done by her under my supervision. To the best of my knowledge, the constituents of this report, in full or in parts, have not been submitted to any other Institute or University for the award of any degree or diploma.

Signature

Thesis Supervisor

Prof. Joydeep Mukherjee

Co-Supervisor

Dr. Reshmi Das

TO WHOM IT MAY CONCERN

It is hereby notified that this thesis titled "**Biofilm Biochemistry Induced By Heavy Metal Sequestration: A Field Study And Their Role In Sedimentary Structure Formation And Stabilization**", is prepared and submitted for the partial fulfilment of the continuous assessment of Master of Technology in Environmental Biotechnology course of Jadavpur University by Divyangana Lahiri (002130904011), a student of the said course for session 2021-2023. It is also declared that no part of this thesis has been presented or published elsewhere.

(Signature with Seal)

Director

Prof. Tushar Jash

School of Environmental Studies

Jadavpur University, Kolkata

(Signature with Seal)

Thesis Supervisor

Prof.Joydeep Mukherjee

School of Environmental Studies

Jadavpur University, Kolkata

(Signature with Seal)

Dean, Faculty of Interdisciplinary Studies Law and Management

Prof. Tushar Jash

Jadavpur University, Kolkata

CERTIFICATE OF APPROVAL

This foregoing thesis is hereby approved as a credible study of an engineering subject carried out and presented in a manner satisfactorily to warranty its acceptance as a prerequisite to degree for which it has been submitted. It is understood that by this approval the undersigned do not endorse or approve any statement made or opinion expressed or conclusion drawn therein but approve the thesis only for purpose for which it has been submitted.

_____

Final examination for evaluation of thesis

_____

Signature of the Examiners

AKNOWLEDGEMENT

I want to express my sincere gratitude to my supervisor, Dr. Joydeep Mukherjee, Professor, School of Environmental Studies, Jadavpur University, and Co-supervisor, Dr. Reshmi Das, UGC Assistant Professor, School of Environmental Studies, Jadavpur University, for their unwavering guidance, inspiration, and support throughout my M.tech study and research. My study and thesis writing have benefited from their vast expertise and patience.

Along with my advisors, I'd also want to thank Prof. Tarit Roy Chowdhury and Prof. Subarna Bhattacharya for their guidance, inspiration, and insights.

I also like to thank all the research scholars, especially Tanaya Bhowmick and Shantanu Bhunia, for their assistance, encouragement, and support throughout my M. Tech project work.

I am thankful to my fellow batchmates of my department for assisting me whenever I needed them.

Finally, I want to express my eternal gratitude to my parents for their inspiration and unwavering support during this period.

DIVYANGANA LAHIRI

Table of Contents

LIST OF FIGURES:

LIST OF TABLES:

ABSTRACT

Intertidal region is a sensitive indicator of climate change along with extensive anthropogenic activities and their effects on the surrounding ecosystem. Heavy metals which are accumulated in the sediments, when leached out in the water become bio-available and can bioaccumulate causing subsequent biomagnification. Intertidal biofilms, a highly structured and dynamic microbial community has shown its resilience towards different pollutants and toxic heavy metals. Different in-situ removal mechanisms of biofilms and their biochemical response in variable metal concentration have been identified from laboratory-based cultures. Our focus of research is to find out the response of biofilms characteristics to heavy metals in natural environment.

1. INTRODUCTION

The atmospheric carbon dioxide (CO_2) level has increased nearly 40% over the past 250 years, mainly due to the rapid growth of industrialization, fossil fuel combustion, and deforestation(Doney *et al.*, 2009). Such anthropogenic activities have led to the present atmospheric CO_2 concentration of 420 ppmv (parts per million volume), which is surging at a rate of approximately 0.5% per year (Fabry *et al.*, 2008). Other than the rapid rise in temperature around the globe, ocean acidification is also a parallel consequence of this escalating atmospheric CO_2 level. Human influence has severely affected the estuarine and coastal ecosystems, which are integral parts of human development in terms of their massive contribution to general ecological and economic services(Ivanina and Sokolova, 2015). The net air-to-sea flux of excess CO_2 can cause a significant shift in the biogeochemical cycles of various elements and the general chemistry of the sea water by increasing the partial pressure of CO_2 (pCO_2) and decreasing the carbonate concentration and pH(Zeng, Chen and Zhuang, 2015).

Ocean acidification, associated with global climate change, is an emerging concern in the coastal and estuarine ecosystems since industrialization has severely impacted the oceanic pH, which has dropped to approximately 8.1, the lowest of all time in the history of the earth. If this situation persists, it is predicted that the pH level can decline to 7.4 to 7.5 by the year 2300 (Gattuso and Hansson, 2011; Ivanina and Sokolova, 2015). Non-CO2anthropogenic inputs also serve as a potential threat to this ocean acidification phenomenon. Chemical fertilisers, pesticides, and different waste products from industrial activities get accumulated in the coastal ecoregion, which contributes to the influx of various heavy metals, eutrophication nutrient elements, oil pollutants, hydrocarbons, and many more in the marine water (Doney *et al.*, 2009; Zeng, Chen and Zhuang, 2015).Metals that enter the coastal environment through various routes are very common contaminants that originate from both natural and anthropogenic sources. Some heavy metals, such as cadmium (Cd), copper (Cu), lead (Pb), zinc (Zn), and nickel (Ni), have greater anthropogenic inputs relative to their natural sources (Ivanina and Sokolova, 2015). These trace metals belong to the non-biodegradable category and have the potential to accumulate in any environment. They impart a severe toxic effect on the intertidal eco-region. Reduction of oceanic pH can affect the solubility and distribution of metal ions, leading to the desorption of metal ions from the

sediments and elevated levels of metals in the water column (De Orte *et al.*, 2014). Speciation of the desorbed metals increases their bioavailability, which has direct implications for bioaccumulation and biomagnification in marine organisms(Riba *et al.*, 2003). The accumulation of contaminants in this ecosystem is disturbing the overall biodiversity, and the persistence of such contaminants is exerting a shift in community composition at different trophic levels by altering the enzymatic functionality and disrupting the metabolic pathways (Quero *et al.*, 2015). Microbial consortiums of marine ecosystems manage to maintain balance in the ecosystem in spite of its severe confrontation with various adverse situations with their sustainable and rapid adaptable features. Adaptability is obtained by microorganisms by changing their growth rate with a change in metabolic and enzymatic activities. The formation of biofilm in the marine and coastal environment is one example of adaptability facilitated by marine microorganisms (Dash *et al.*, 2013).

Biofilm is a community of various microorganisms having a microcolony structure, comprising both prokaryotic and eukaryotic cells embedded in an extracellular matrix (Egan, Thomas and Kjelleberg, 2008). These biofilms are considered pivotal elements of the coastal ecosystem because they are the base of the ecological pyramid, protect the intertidal beds from erosional stress, and have bio-stabilising capabilities along with remarkable possibilities in the bioremediation of organic pollutants and heavy metals (Gerbersdorf and Wieprecht, 2015). Biofilms and their mucilaginous matrix is essential in heavy metal in-situ bioremediation. This metal sequestering ability is being studied for its potential to protect intertidal and coastal ecosystems from heavy metal toxicity and lower the likelihood of heavy metals entering the food chain.

Other than heavy metal bioremediation they establish interaction with the physical sedimentary dynamics and other various environmental constraints which result into different distinctive sedimentary structures and biosignatures. Microbially induced sedimentary structures (MISS) and stromatolites are examples of such structures that provide us with knowledge of the early earth (Noffke and Awramik, 2013). MISS are the physical depositional structures formed by microorganisms, whereas stromatolites are chemical depositional structures formed by the precipitation and mineralization of calcium carbonate with the help of microbial EPS. This stromatolite structure, mainly composed of microbial biofilms, also confers biostabilizing properties (Decho, 2000).

Sunderban, the largest delta situated in India and Bangladesh, is a UNESCO world heritage site and falls under the RAMSAR convention. It is one of the most important coastal ecosystems in the world and is highly vulnerable to climate change, pollution, and exploitation of natural resources. It is thus under great international attention for its protection and preservation(Ramanathan *et al.*, 2009). Previously, various studies had been conducted on the role of intertidal biofilms in the sequestration of polycyclic aromatic hydrocarbons and their biochemical response to the increasing concentration of heavy metals such as Cd and Co by Balu 2020 and 2022 and Dutta et al. 2022.

For this study, we focused on the intertidal biofilms collected from the few of the places in Sunderban that are highly polluted either for being a ferry terminal or a fishing harbour or an industrial area where the constant movement of motorized launch, trawlers are witnessed on an everyday basis. We tried to observe different biochemical parameters of the biofilms collected from the field and compared it with the metal content obtained from the bioavailable fraction of the sampled biofilms. An empirical study was performed along with the field study to find out a way to obtain an artificial culture on the sediment surface.

2. LITERATURE REVIEW

2.1 Environmental biofilm: Composition and Characteristics

Estuarine and coastal environments, also known as intertidal mudflats, are considered the coastal defence system because of their great resilience to changing ecosystems. These mudflats are primarily made of clay and silt, which have charged surfaces and are therefore covered in organic layers made of single and/or multicomponent microbial aggregation, also known as biofilms, which are thought to be one of the most widely distributed life forms on the planet (Yin et al., 2019). Prokaryotic and eukaryotic bacteria aggregate into biofilms when they are combined with soil particles and a self-produced mucilaginous matrix. Numerous microorganisms, including bacteria, diatoms, algae, fungus, protozoa, etc., contribute to these biofilms. Depending on the microorganism that makes them up, they can be either phototrophic or heterotrophic. These mucilaginous extrapolymeric substances secreted from the biofilm mediate a cohesive interaction with the sedimentary particles that confers the binding of the biofilms with the surface (Decho, 2000). Intertidal mudflats are continuously subjected to shifting hydrological, chemical, and morphological factors, which results in variation in the compositional aspects of the environment, including the physical and chemical characteristics of biofilms (Sahan *et al.*, 2007). The content and release of extrapolymeric compounds, in particular, might change due to seasonal variations, which can also affect the characteristics of biofilm. According to the convenience of the researchers, the ambiguity of the phrase is taken into account universally despite the possibility that the terminology's interpretation varies among fields. The microbial assemblage and matrix that cover hard or rocky surfaces are primarily defined by the term "biofilm," according to some research studies. On the other side, "microphytobenthos" refers to the presence of microbial communities on soft sediments like tidal planes. Biomats is more of a macroscopic phrase than biofilm, which is typically thought of as a microscopic term. Intertidal microbiota is referred to more broadly as a "microbial community" (Thompson, Norton, and Hawkins, 2004; Beninger and Paterson, 2018; Mandal et al., 2021). A diatom-rich microbial community known as an epipelic biofilm migrates to the sediment during the photic period (when it is daylight) and clings to it. Muddy sediment profiles in mudflats make it easy to study the epibenthic microbial community. Due to the quantity of cyanobacteria and diatoms that make up microphytobenthos, they are also known as phototrophic biofilms (Beninger and Paterson, 2018).

2.1.1 Biofilm Development and Associated Microbial Interaction:

The formation of biofilm is a dynamic and intricate activity. Encapsulation of numerous microbial communities in their own exopolymeric substances usually comprises five major stages. As a result of reversible adsorption mechanisms and weak interactional forces like the Vander Waal force, bacteria bind to surfaces of substrata to begin the creation of biofilms. With the aid of its flagella and pili, polysaccharides, and several adhesive proteins, the bacterium develops a strong and irreversible attachment with the substrate to aid in colonisation. After microorganisms have colonised an area, the biofilm begins to develop, and the growth is sparked by the release of EPS. Maturation of the biofilms is highly dependent on inter- and intra-species interaction among the constituting microorganisms. This cellular interaction mechanism is also known as "quorum sensing," where cells interact by producing, detecting, and responding to autoinducers (small diffusible signalling molecules). For the survival and proliferation of complex consortia, bacteria have developed great interactivity with their neighbouring species, ranging from competition for nutrients to collaborative cross-feeding to protective shielding (Decho, 2000; Dufour, Leung, and Lévesque, 2010). Various intrinsic and extrinsic factors promote the dispersal of the components of mature biofilms along with the enzymatic degradation and reconfiguration of biofilms, leading to surface recolonization (Flemming *et al.*, 2016).

2.1.2 Exopolymeric Substances and Their Significance:

The building blocks of these biofilms are their exopolymeric substances and regarded as the essential elements that govern the physicochemical and biological characteristics of biofilms since they are primarily responsible for maintaining the structural and functional integrity of biofilms by creating a cohesive matrix with the sedimentary particles and assist to foster a strong interface with the microorganisms and sedimentary constituents(Flemming and Wingender, 2001).90% of the components of biofilm are EPS, while the remaining 10% are bacterial cells. By changing density, porosity, water content, charge, sorption characteristics, hydrophobicity, and mechanical stability, EPS affects the immediate conditions of life of biofilm cells residing in this biological setting (Flemming et al. 2007).

The biomolecules that make up EPS include mono and polysaccharides, proteins, lipids, glycoproteins etc. A surprising volume of extracellular DNA is sometimes constrained by these mucilaginous substances along with phospholipids, nucleic acids, and additional

biomolecules. Despite the fact that some EPS are neutral macromolecules, the majority are polyanionic due to the inclusion of either uronic acids (glucuronic acid, mannuronic and galacturonic) or ketal-linked pyruvate. inorganic remnants, phosphate or, less frequently, Environmental biofilms are primarily composed of polysaccharides, which is why they are sometimes referred to as extracellular polysaccharides. Extracellular polysaccharides are important to counter environmental stress, including oxidative stress and ion chelation. The exopolymeric compounds also contain several functional groups, such as phosphoric, carboxylic, hydroxyl, and amino groups (Flemming and Wingender, 2001; Sutherland, 2001)

EPS can be classified into two major categories, those bound to the cell surface (CPS) alongside those released into the environment (released polysaccharides, or RPS). According to the thickness, consistency, and appearance of the EPS glued to the cell surface, these can be designated as sheaths, capsules, or slimes. The RPS are soluble portions of polysaccharide material that have been released into the medium, either from the outer layer(s) or as a consequence of a biosynthetic process independent of the production of EPS.The sugar compositions of CPSs can differ significantly from those of RPS showing various biosynthetic pathways that are, in turn, depending on environmental variables(Pereira *et al.*, 2009; Di Pippo *et al.*, 2013a).

The EPS matrix performs a variety of tasks, including adhesion to surfaces, bacterial cell aggregation into flocs and biofilms, stabilisation of the biofilm's structural integrity along with the stabilisation of the adhering substratum of the same, formation of a protective barrier that offers resistance to biocides and other harmful effects, retention of water, sorption of exogenous organic compounds for the absorption of nutrients from the environment, and accumulation of enzymatic activities, such as the digestion of exogenous macromolecules for nutrient (Laspidou and Rittmann, 2002; Neu and Lawrence, 2010)

2.2 Heavy metal sequestration by biofilm:

Heavy metal contamination in developing countries' biodiversity-rich coastal ecosystems is increasing at an alarming rate (Qian et al., 2015), with remediation techniques primarily relying on degradation and ex-situ treatment procedures (Mulligan et al., 2001). Electrolysis, ion exchange, flotation, reverse osmosis, membrane process precipitation, and ultrafiltration are examples of heavy metal removal procedures. These are expensive methods and can cause damage to the contaminated sites(Camacho-Chab *et al.*, 2018)

The problems with ex-situ approaches can be solved by in-situ bioremediation of heavy metals using immobilised algae and other microbial communities found in biofilms (Camacho-Chab *et al.*, 2018). Due to the presence of natural diffusion barriers, multispecies biofilms and microbial mats are relevant in many applications because they provide greater resistance than individual planktonic cells to tolerate potentially toxic pollutants like heavy metals, chlorine, and antibiotics. The interaction between a microbe and a metal ion can take place through a variety of methods that can be divided based on metabolism, such as active and passive uptake of metal ions depending on the interaction(Alluri *et al.*, 2007). These interactions biologically transform them either into less toxic or less available (less utilizable) form, or immobilize them to prevent their breach into bio-systems.

2..2.1 Biosorption and bioaccumulation mechanisms of biofilms

Under heavy metal stressed conditions microbial mat consisting of diversified microorganisms have developed multiple resistance mechanisms. The bacterial cells and other microorganisms in the biofilm can acquire heavy metals in both particulate and soluble forms.Usually, the biosorption mechanism sequesters metal pollutants found in the intertidal water and sediments in the microbial cells. A specific structural and chemical makeup of EPS, which is created by the bacteria producing the biofilm as a self-defence mechanism, favours the sequestration of metal ions and prevents the metal ions from reaching the cell surface(Zamboulis *et al.*, 2011; Gupta and Diwan, 2017). Other mechanisms implicated in the bioaccumulation of metal ions by microbial cells include cell surface adsorption and intracellular absorption, in addition to adsorption on EPS. Humic acid, citric acid, and EDTA are a few examples of chelating agents that are prevalent in nature and in biofilms that can enhance the bioaccumulation of heavy metals. The ionizable functional groups, such as amino, hydroxyl, phosphate, carboxyl, and sulphates, are usually responsible for biosorption through the negatively charged EPS. By interacting with sulfate-reducing bacteria (SRB)

found in the biofilm and phosphate groups found in the lipopolysaccharides found in EPS, respectively, metals can precipitate as their sulphide and phosphate forms. Different enzymatic processes can degrade metals and trap them as metal oxides(Mandal *et al.*, 2021).

2.2.2 Metallothionein protein in heavy metal biodegradation:

Some heavy metals are essential for the enzymatic regulations to certain extent and some are poisonous in limited amount for any biological entity. Heavy metals can trigger several deadly processes within the biological system such as the production of reactive oxygen species, destruction of enzymatic function and many more once it penetrates the cell wall as enters the cytoplasm. To maintain cellular homeostasis microbial cells have developed plethora of defence mechanisms, which includes the intercellular sequestration of metals with the help of 'Molecular Soldiers' that are cytosolic, cysteine rich largest metal binding protein that links with the toxic metal and prevent it from reaching essential cellular molecular and foster in maintenance of healthy physiology(Cui *et al.*, 2021). With the influx of heavy metal metallothionein expression in the bacteria increases. Thus they can used as a biochemical tool capable of bioremediation. Very few bacterial genes that code for MT have been studied for their biological function. The *Synechococcus elongates*SmtA gene is crucial for detoxifying zinc. The potential of the marine bacterium *Pseudomonas aeruginosa* N6P6, which possesses the bmtA gene and exhibits resistance to a number of heavy metals, including Pb, Cd, Hg, Cr, and Zn, has been investigated. According to a study, bmtA gene expression increased in a 48-hour metal-spiked biofilm culture. (Chatterjee *et al.*, 2020)

2.3Previous investigations:

There aren't many studies looking at how metal poisoning affects the biochemical expression of biofilms. In a lab experiment, grown biofilms spiked with increasing amounts of Cd and Co revealed a discernible increase in EPS content as the metal concentration increased. This observation can be explained by the consortium being protected from metal toxicity by the microorganisms present in the biofilm community. However, the biofilm's ability to survive is directly proportional to how much chlorophyll it contains. The culture's chlorophyll concentration was seen to increase somewhat with increasing concentration, although it quickly drops off at high concentrations.As a result, the tolerance is set at a specific concentration level. Beyond that point, the viability of the biofilm rapidly decreases.

2.4 Biofilms in Biostabilization and Sedimentary Structure Formation:

Biofilms are one of the important features of the intertidal and coastal and significant mediator of the erosive response. Biofilms cover the large area of the sediment and protect it from erosion. This stabilization of sediment by the biofilms has been termed as 'biostabilization'. This phenomenon is not only restricted to the surface layer but also can be seen in the subsurface area during an event of flood or reservoir flushing (Gerbersdorf and Wieprecht, 2015).Biofilms intricately bind to the particles of sediments and during the incoming tide and high tide period they prevent the sediments from washing away(Redzuan and Milow, 2019). Their growth and metabolic activity interacts with the substrate, promotes precipitation and cementation process thus rendering the increase of substrate stability(Stal, 2010, p. 20). Biofilm matrix formation have substantial impact on the erosion-transport-deposition-consolidation(ETDC) cycle of the sediment and can consequently have further implications such as nutrient storage and pollutant uptake.(Paterson *et al.*, 2018).

The significance of microorganism induced mechanical properties of the fine sediments were first introduced by Meadow and Anderson(Meadows and Anderson, 1968) following which investigations were conducted on the erosion threshold of the water-sediment interface. Microbes cannot participate in this stabilization mechanism individually. Colonized structure formation such as biofilm formation by microbes and algae and development of cell-cell or cell-substratum adhesion by extrapolymeric substance matrix filling up the void pore space can help in decreasing erosional threshold(Paterson *et al.*, 2018).Growth and metabolic activity of the biofilms allow interaction with the substrate particles and subsequent precipitation and cementation of the minerals render and/or increases the substrate stability(Stal, 2010).

2.4.1 Role of Cyanobacteria:

Filamentous cyanobacteria trap the sediment particles by entangling them in the filamentous networks and produce a strong and coherent structure. Sediment bed level is also increased as sediment particles deposit on the biomats, trapped by the filamentous cyanobacteria and migrates towards the top of the mat by phototaxis movement (Stal, 2010).Other than the release of the extrapolymeric substances, the filamentous cyanobacteria that have rope like bundles promote greater affinity towards(Figure 1a) the increase of the erosion threshold of the intertidal sediments. This rope like bundle is a multifilament structure where the filaments

are tightly woven together. These rope builders are pioneers in the colonization of unstable sedimentary surface. Intertidal cyanobacterial biomats are often dominated by *Microcoleuschthonoplastes,* cosmopolitan cyanobacteria that forms a thick bundle by binding the polysaccharides with trichomes. M. *chthonoplastes*, *Schizothrix*spp. were observed in the intertidal and subtidal marine carbonate sediments respectively can take part in the stabilization action of unconsolidated sediments(Stal, 2003; Garcia-Pichel and Wojciechowski, 2009).

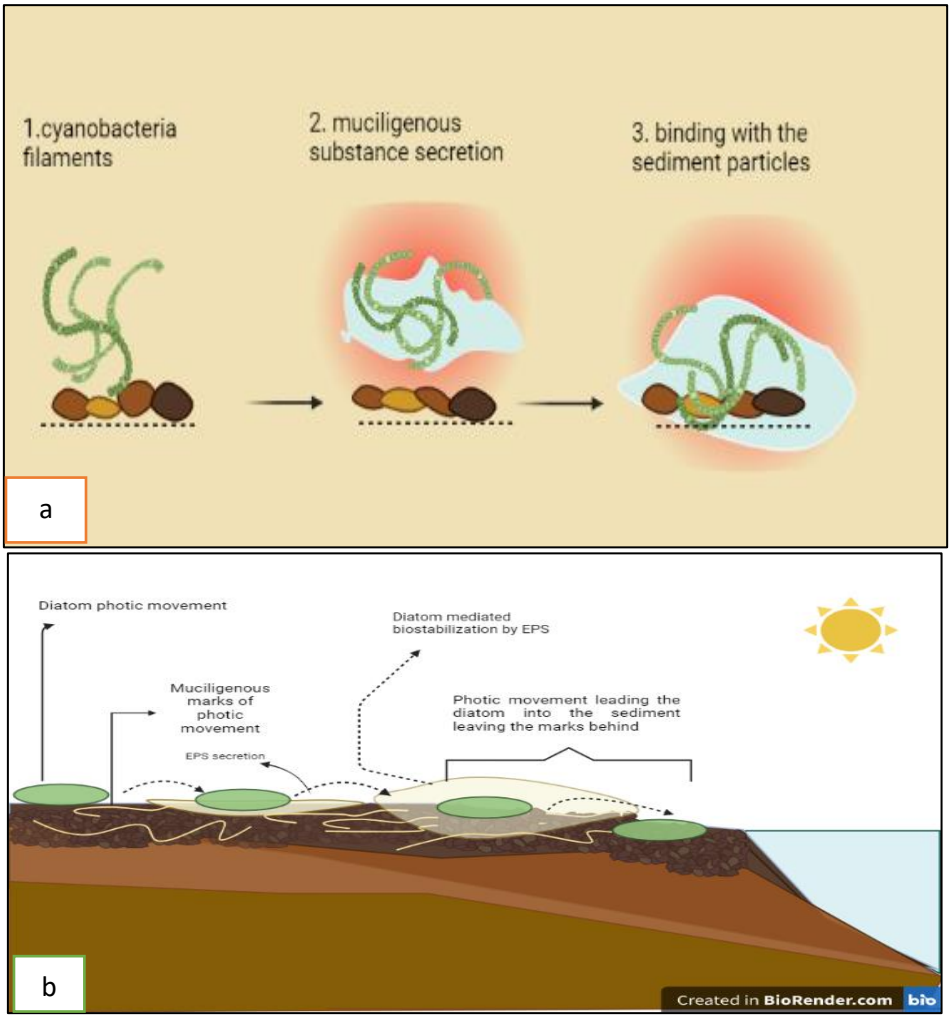

Figure 1 a. cyanobacteria mediated sediment stabilization; b. diatom photic movement leading to sediment stabilization

2.4.2 Role of Diatom:

The diatom mediated sediment stabilization occurs due to the secretion of mucopolysaccharide during its active movement stage. The migratory rhythm is synchronized

with the diurnal tidal activity. During emersion or low tide period diatoms move upwards to the photic zone and accumulate on the sediment surface (Figure 1b). Before the high tide they move downward into the sediment. This upward and downward movement is one of the major mechanisms involved in the diatom mediated sediment stabilization. This type of sediment stabilization by the migrating diatoms is mainly observed in the intertidal mudflats as these tide-synchronized movements leave a network of mucilage strands on the surface of the sediments(Madsen, Nilsson and Sundbäck, 1993).

2.4.3 Biofilm in formation of sedimentary structures:

Microbial biofilms also have significant role in providing the impression of evolution on the surface of earth. In the right environmental condition these biofilms can form solid, reef like structures known as stromatolites. Other than stromatolites, different microbially induced sedimentary structures (MISS)are also evidenced as sedimentary features developed with the help of microorganisms. They also participate in the sediment stabilization process. MISS and stromatolites are considered as the first traces of life since the Archean time(Noffke, 2008).

2.4.3.1 Stromatolites:

This three-dimensional sedimentary structure dates back to Archean time and originated in an hypersaline environment with the ability to stabilize sediments developed due to the chemical precipitation by the sediment microbial interaction. Various process is responsible for the development of this laminated structure where the basic process includes entrapment of sediment particles with the help of organic layer of microbial exopolymers and subsequent mineralization by inorganic precipitation of carbonate. Microbes that are associated with stromatolite formation includes cyanobacteria six functional groups of microbes: (i) oxygenic phototrophs (cyanobacteria) are the primary producers, anoxygenic phototrophs (purple and green bacteria), aerobic heterotrophic bacteria, fermenters, anaerobic heterotrophs, predominantly SRB, sulfide oxidizing bacteria (Dupraz and Visscher, 2005). Photosynthesis by different phototrophic microorganisms and respiration by other microorganism can cause carbon precipitation and dissolution by interfering with the carbon equilibrium of the interstitial water. Metagenomic analysis and 16S rRNA sequencing of the various stromatolites all over the world shows that Cyanobacteria, Proteobacteria, Bacteroidetes,

planctomycetes are the dominating phyla in majority of this sedimentary structure(Foster and Green, 2011).

2.4.3.2 Microbially Induced Sedimentary structures (MISS):

It is also an ancient structure recording the evolving microbial activity in earth's history. These two-dimensional structures are mainly the result of physical interaction of microbial mats with the sedimentary particles thus can be seen in any terrestrial and marine dominated by siliciclastic facies. Different studies on fossil traces of MISS formed almost 3 billion years ago provides us with the fact that the mobile and phototrophic bacteria constituted this structure and these are the evidence of locating ancient photic zones. These structures are also the clue for reconstruction of paleoenvironmental settings. Most of these phototrophic bacterial colonies found in the MISS in ancient shallow water are composed of cyanobacteria.

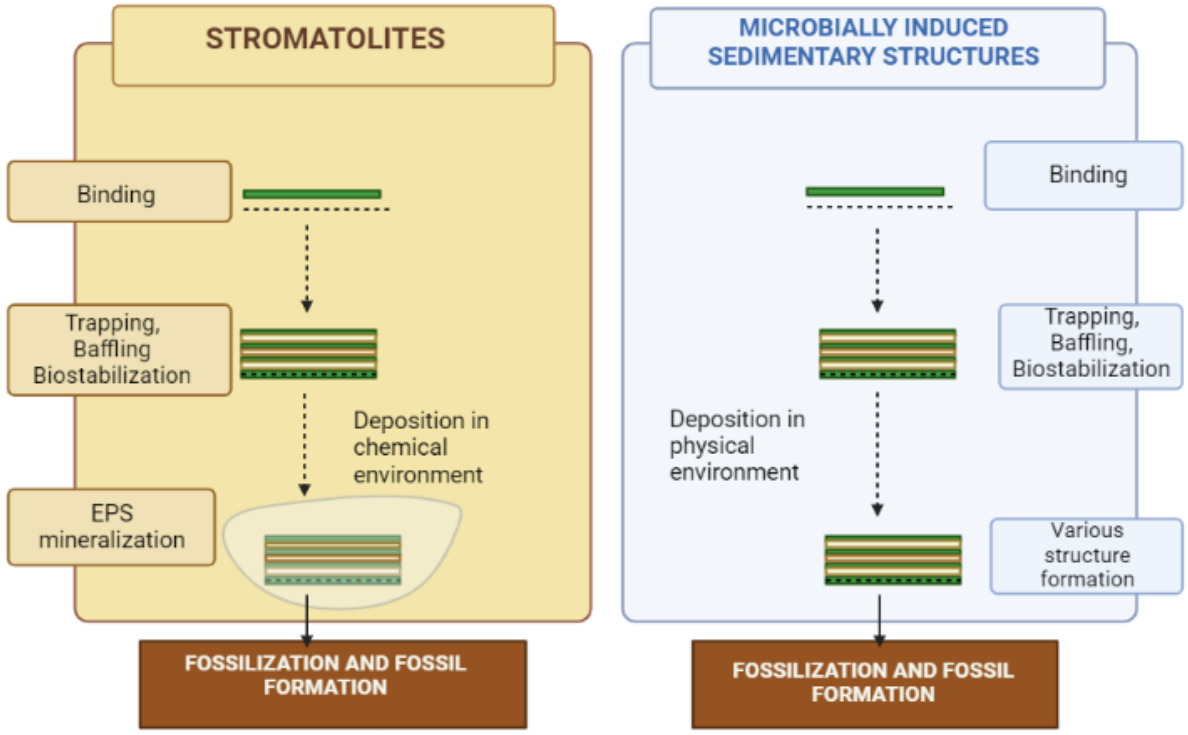

Figure 2 Graphical representation of different stages of stromatolites and MISS formation [adapted from (Noffke and Awramik, 2013)]

2.4.3.4 Lithification in stromatolites influenced by physicochemical parameters:

In depth investigation of these stromatolites suggests that they can be of both lithifying and non-lithifying in nature. Lithification occurs mainly where the precipitation of minerals mainly carbonate minerals suppresses the dissolution of such(Dupraz and Visscher, 2005; Khodadad and Foster, 2012). Metabolic activity leading to biochemical changes of microbial community participating in stromatolite formation render the precipitation of minerals in its natural environment. The biochemical changes as well the abiotic factors of the natural environment such as the pH, Temperature and salinity impart their significant contribution in the mineralization phenomenon. For instance, with the increase in pH calcium carbonate precipitation increases. Negatively charged bacterial cells and the acidic monomers present in the secreted EPS get attached with the free calcium ions and in the event of increase in pH calcium carbonate precipitation take place (Janssen *et al.*, 2022).Cyanobacteria and some photosynthetic microorganisms have an integral role in lithification process. Increase of such microorganisms increases the lithification and as a result lithified mat in stromatolites are formed(Zhu and Dittrich, 2016). In cases where this lithification process is inhibited because of some alteration of biochemical process or absence of certain microorganisms responsible for lithification, nonlithified structure starts to develop. In the modern stromatolites along with the abundance of lithified ones non-lithified stromatolites are also been observed majorly in the upper intertidal zone including two main type such as smooth and pustular(Babiloni*et al.*, 2018)

3. MATERIALS AND METHOD

2.1 Site of Study:

Three different riverine intertidal sites of Indian Sunderbans, situated in the district of South 24 Parganas were targeted for the collection of the water, sediment and Biofilms. In a two-day sampling period total 10 number of samples were collected. Three different sampling regions includes Patharpratima (3 samples), Namkhana (2 samples) and Kakdwip (3 samples). This on field sampling was conducted during the month of January and all the details are mentioned in the table below.

3.2 Sampling procedure:

Biofilms from the extensive microbial mats on the surface of the sediments exposed during low tide were collected covering the 3 different sampling locations along with the water and sediment. The intertidal areas of the Sunderbans were sampled at several locations, and the samples were gathered in plastic containers. Plastic spades were used to collect the biofilm samples in order to prevent metal contamination and data contradictions. Immediately following the collection of the sample, the salinity, conductivity, and temperature of the water were each measured using a refractometer, a conductivity metre, and a thermometer. Later, a pH metre was used to determine the pH of the water samples in the lab. Based on the location, the field data are described below, and the table below includes the latitude and longitude as well.

Table 1- Details of sampling location and water characteristics

Serial No	Sample Site	Sample Details			Water			
		Sample Name	Latitude	Longitude	Temperature (°C)	Salinity (‰)	pH	Conductivity (mS/cm)
1	Patharpratima	P1	21.76611º	88.378442º	20	24	7.94	16.51
2		P2	21.76609º	88.378428º	20	24	7.78	18.25
3		P3	21.72001º	88.342856º	20	22	7.82	18.18
4	Namkhana	N4	21.74286°	88.270523°	22	13	8.06	10.37
6	Kakdwip	K6	21.85958º	88.175666º	17	14	7.45	11.82
7		K7	21.85822º	88.17703º	17	15	7.44	13.12
8		K8	21.85387º	88.189952º	17	6.5	7.63	7.2

Due of the extremely low biofilm growth in January, only one sample was once again obtained from the Kakdwip sampling station (K6), and most estimations for this research were made using that sample

3.3 In-vitro Biofilm Culture

Biofilm samples collected from the sampling sites were transferred into 250 ml mixed media containing ASN III and BG11 (1:1) [as mentioned in the table below]. These biofilm samples were cultured in a Conico Cylindrical flask (CCF) made up of polymethyl methacrylate (PMMA) in which around 150mg of sample was inoculated in the sterile mixed media. These cultures were kept under fluorescent irradiance (50 µmol photons m^{-2} s^{-1}) with 14:10 h light: dark photoperiod at 25 ± 1 °C.

Figure 3. Biofilm Cultured in CCF flask

3.4 Pilot Experiments Conducted for Biofilm Growth on Sediment Surface on a Static Condition in a Laboratory Environment

3.4.1 First Method: (Growth on A Plastic Sheet)

For the first case study, the primary aim was to grow biofilm mat on a plastic sheet sprayed with mixed media and after the plastic sheet is fully covered with the biofilm mat, it would have been transferred to a sediment surface in a plastic box.

The main reason of trying out this method is the prior evidence of affinity of biofilm growth to the plastic surface.

For this experiment a container was lined with a plastic sheet and a generous amount of ASNIII and BG 11 mixed media was sprayed onto it. Biofilms were taken in a small amount (better adhesion) with a tweezer from the field samples and were placed on the sheet. Then the container was tightly covered to ensure moisture retention. The process of media spraying was repeated as per requirement. This system was kept under fluorescent irradiance (50 μmol photons m^{-2} s^{-1}) with 14:10 h light: dark photoperiod at 25 ± 1 °C in an undisturbed condition.

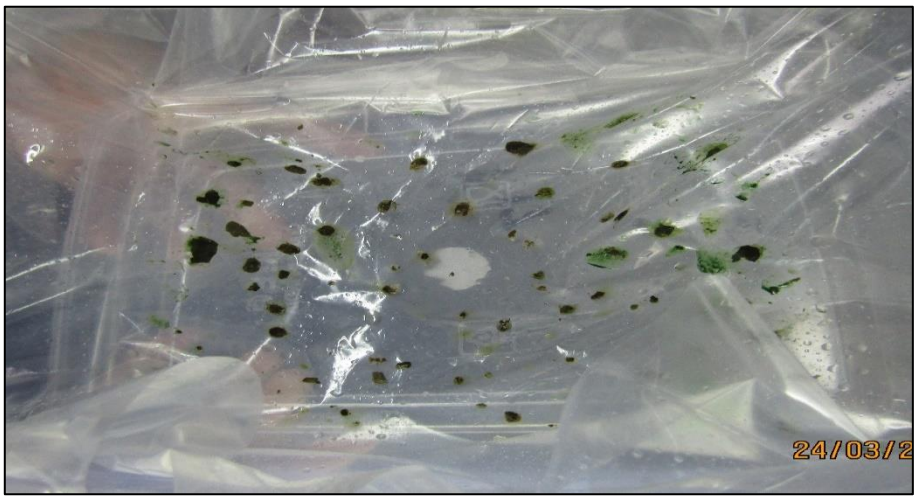

Figure 4: Biofilm Growth on a plastic sheet

3.4.2 Second Method:

In this case whole biofilm samples along with the sediment collected during the sampling was used. The mixed media was poured carefully to ensure that the sample gets partially submerged without losing its integrity. Then the whole container was tightly covered to minimize moisture loss and was kept in an undisturbed condition.

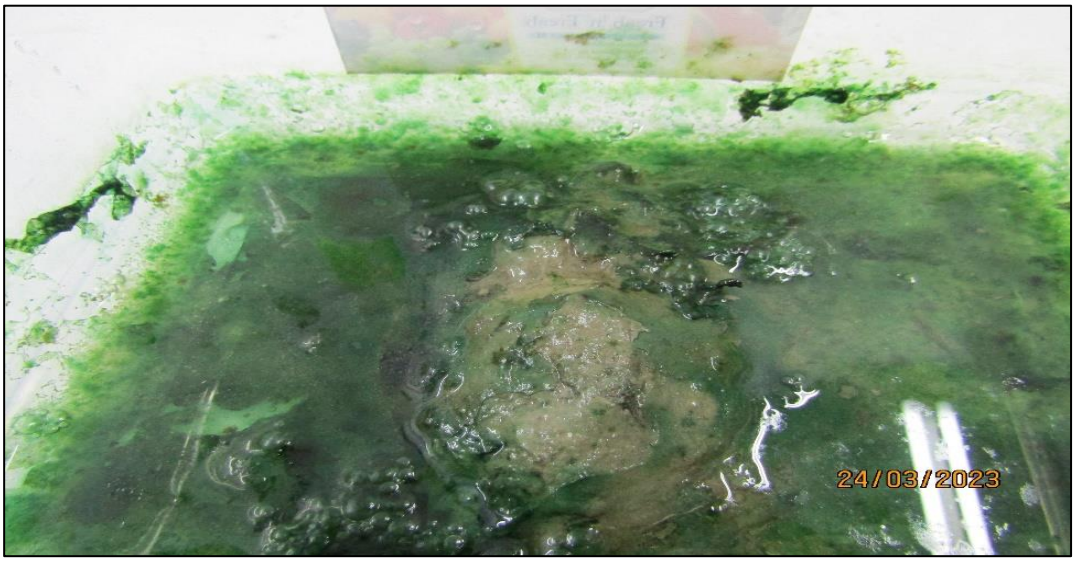

Figure 5 Biofilm cultivation on a sediment with the help of artificial sea media

3.4.3 Third method:

In this case sediment collected during the sampling period was used and it was spread as a slant inside of a container. ASN III and BG 11 mixed media (1:1) was used to mimic the estuarine water and the sediment slant was partially submerged in the media. This design of artificial biofilm growth was particularly done to mimic the intertidal system. Then around 100mg of biofilm was inoculated in the media. This system was kept in an airtight condition to ensure moisture retention under fluorescent irradiance (50 µmol photons m^{-2} s^{-1}) with 14:10 h light: dark photoperiod at 25 ± 1 °C in an undisturbed condition.

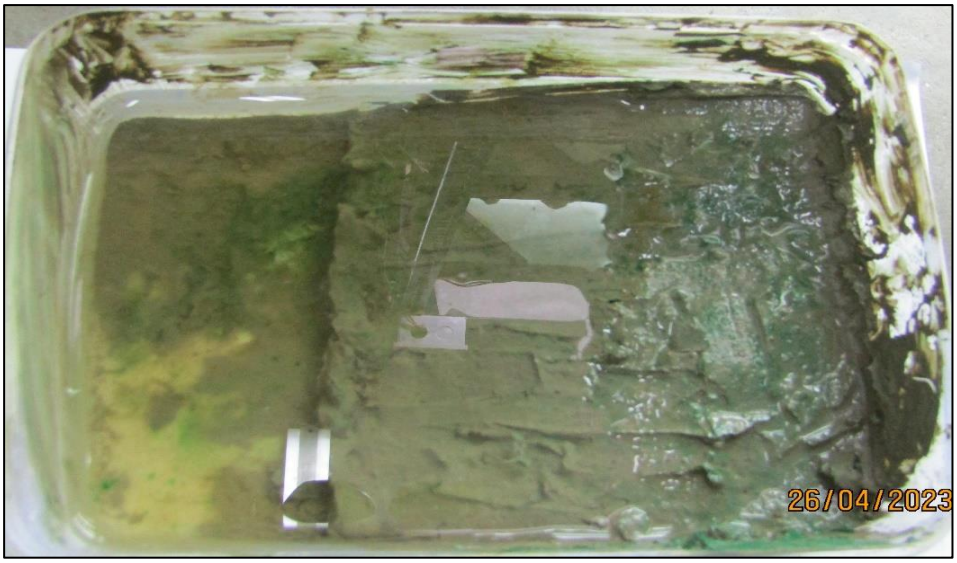

Figure 6 Growth of biofilm on a slant of sediment with the help of artificial sea media

3.5 Estimation of Chlorophyll: From Both Field Samples And In Vitro Cultured Biofilms

Biofilms were scraped out of the sediment along with some sediment particles because of their tightly woven nature. Total 60 mg of the mass was scraped out and then grounded with 3ml of the methanol. The soup was then taken in falcon and centrifuged for 5 minutes at 10000 RPM. After centrifugation supernatant was taken and then absorbance was spectrophotometrically quantified at five different wavelengths(632nm, 652nm, 665nm, 696nm, 750nm) and different types of chlorophylls and the amount of total chlorophyll was measured using the procedure described in (Ritchie, 2008) which is given below.

Same procedure was applied for the extraction of chlorophyll from the in-vitro biofilm cultures and absorbance was spectrophotometrically measured using the same set of wavelengths and the calculation procedure.

Chl a ($\mu g\ ml^{-1}$) = − 2·0780×(A632-A750) − 6·5079×(A652-A750) +16·2127× (A665-A750) − 2·1372×(A696-A750) (±0·0070)

Chl b ($\mu g\ ml^{-1}$) = − 2·9450 × (A632-A750) +32·1228×(A652-A750) − 13·8255× (A665-A750) − 3·0097× (A696 -A750) (±0·0212)

Chl c (µg ml^{-1}) = 34·0115 × (A632-A750) − 12·7873×(A652-A75) +1·4489× (A665-A750) − 2·5812×(A696-A750) (±0·0120)

Chl d (µg ml^{-1}) = − 0·3411 × (A632-A750) +0·1129×(A652-A750) − 0·2538× (A665-A750) +12·9508×(A696-A750) (±0·0031)

Total Chl (µg ml^{-1}) = Chl a+ Chl b+ Chl c+ Chl d

3.6 Estimation Of Extracellular Polymeric Substances

The Biomass obtained from the cultured biofilms both in the CCF and the with sediment culture were utilized for the extraction and estimation of CPS and RPS following the protocol (Di Pippo *et al.*, 2013b). Biomass harvested from both the flask and the sediment samples were centrifuged at 5000 rpm for 10 minutes. RPS was separated in the supernatant and the CPS and other cells were separated as the pellet.

3.6.1 CPS Extraction:

Pellet containing the cell bound portion of the extra polymeric substances was incubated with 0.1M sulfuric acid at 95^0 C for 1hour. Samples were centrifuged at 3500 rpm for 5 minutes after incubation period. The resultant supernatant containing the cell bound exopolysaccharides was separated from the pellet (cells) and precipitated in 96% ethanol.

3.6.2 RPS Extraction:

For supernatant containing RPS, 99% chilled ethanol in a ratio 1:3(v/v) was used for the precipitation of EPS fraction.

Both for CPS and RPS, these were centrifuged at 10000 X g for 10 minutes and after that the pellets were washed with 65% ethanol to remove any contamination. After washing pellets were freeze dried and the stored at -20℃.

3.7 Estimation of Metallothionein Concentration from Cultured Samples

Metallothionein concentration in the biofilm samples were estimated using acidic ethanol/chloroform fractionation of biofilm tissue homogenate. This process is designed in such a way to ensure complete metallothionein precipitation and to avoid oxidation of sulfhydryl group. Mainly three steps are followed for maximization of metallothionein precipitation and consecutive estimation.

Step 1- Tissue Homogenization – Biofilm biomass weighing around 100mg is homogenized by a manual tissue homogenizer using 3 volumes of homogenization buffer. This homogenization buffer is composed of 0.5M Sucrose, 20mM Tris-HCl buffer of pH 8.6, 0.5mM phenylmethylsulfonyl fluoride (PMSF), 0.006mM leupeptin solution and 0.01% β-mercaptoethanol. Homogenate is distributed in aliquots.

Step 2 – Concentration of metallothionein solution- Homogenates are centrifuged at 30000xg for 20 minutes (or 11500xg for 54 minutes). The supernatant containing metallothionein is collected after centrifugation. 1.05 ml of chilled (-20 °C) absolute ethanol and 80µl chloroform per ml of resultant supernatant was added to the previously collected supernatant and again centrifuged in at 6000xg for 10 minutes at 0-4°C. Three volumes of cold ethanol and 40µl HCl were added to resulting supernatant and stored at -20 °C for 1-24 hours for the denaturation of the proteins.

Step 3- Purification and Estimation of Metallothionein- after 1-24 hours the stored supernatant was centrifuged at 6000xg for 10 minutes(0-4°C). The resulting pellet was washed with Ethanol (cold -20°C): Chloroform: homogenization buffer (without β mercaptoethanol) (87:1:12 v/v) and again centrifuged at 6000xg for 10 minutes at 0-4°C. Supernatant was removed and the pellet obtained was dried with liquid nitrogen.

Step 4- Resuspension of metallothionein enriched fraction- pellet was resuspended with 150µl 0.25M NaCl and 150µl 4mM of EDTA containing1N HCl. This resuspended fraction of metallothionein was added to a 4.2ml solution of 0.43mM 5,5-dithiobisnitrobenzoic acid (DTNB) in dissolved in 0.2 phosphate buffer of pH 8 containing 2M NaCl. As DTNB is photosensitive chemical compound the solution in kept in dark for some time and then absorbance was measured using a spectrophotometer at a wavelength of 412nm.

3.8 Principal Component Analysis on Microbial Diversity of Stromatolites by Various Physicochemical Parameters

To demonstrate the possible corelation between the microbial abundance and associated physicochemical parameters a Principal Component Analysis (PCA) was performed in R package. PCA is a multivariate statistical approach that basically represents the original dataset in a new orthogonal system. Here variables were considered as influential which represented corelation coefficient above 0.6. To perform PCA data were collected from different publications as mentioned in Table 2. The analysis was conducted separately for lithified and nonlithified stromatolite structures.

We created a graphic representation (Figure 7) based on the literature that demonstrates the variety of chemical processes involved in the photosynthesis process of cyanobacteria and how they lead to the following carbonate precipitation through calcium mineralization (Kamennaya *et al.*, 2012). To examine the degree of lithification (precipitation of carbonate in the cyanobacterial inorganic conversion of carbon dioxide) affected by the microbial consortia under different climatic fluctuation (mainly affected by the changes in pH and CO_2 concentration before and after the industrialization) CellNetAnalyzer (CNA) was used, a MATLAB toolbox that offers a graphical user interface and various (partially unique) computational methods and algorithms for exploring structural and functional properties of metabolic, signalling, and regulatory networks. We used interactive network maps (GUI) for computation of the rate of calcium precipitation with respect to varying pH and CO_2.

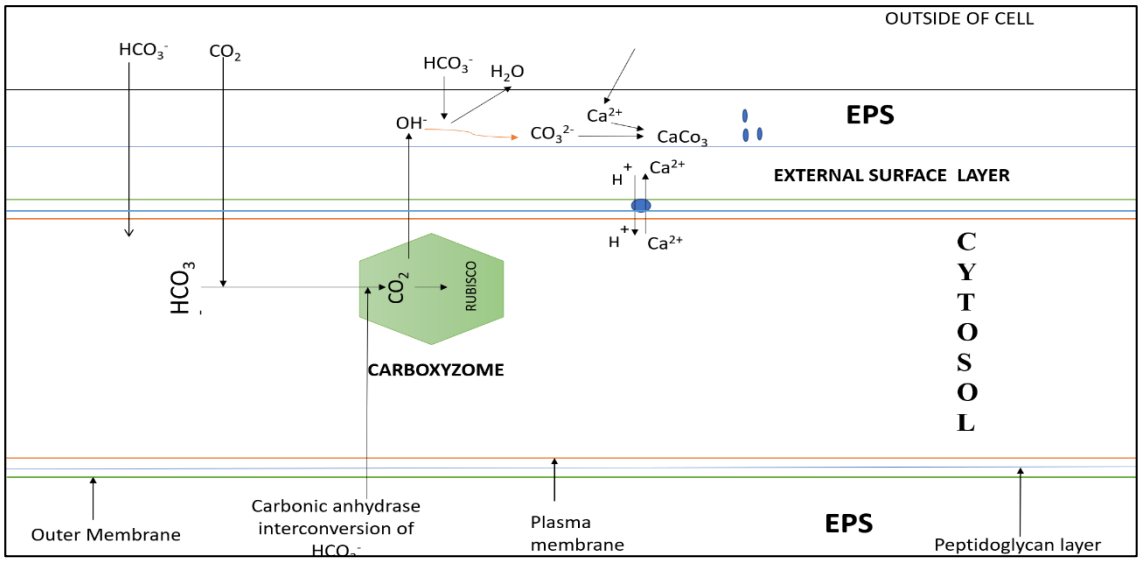

Figure 7 Graphical model of chemical reactions of cyanobacteria during photosynthesis

Table 2- Microbial abundance and associated abiotic factors under lithified and nonlithified mat types

Type Of Mat	Study location	pH	Temperature	Salinity	Cyanobacteria	Proteobacteria	Bacteroidetes	References
Non Lithified	Shark Bay, Australia	8.1	26	55	29	69	7	Babilonia et al. 2018
	Highborne Cay, Bahamas	8.9	24	38	27	19	8.6	Khodad and Foster 2012
	Shark Bay, Australia	8.1	27	55	11	62	8	Goh 2009
	Shark Bay, Australia	8.1	29.7	55	12	30	24	Ruvindy 2015
	Shark Bay, Australia	8.1	29.7	55	14	36	15	Ruvindy 2015
Lithified	Highborne Cay, Bahamas	9.2	24	38	27	18	8.5	Khodad and Foster 2012
	Laguna Interna, Salar de Atacama (Chile)	8	28	38	14.21	54	17.69	Osman 2021
	Little Darby Island, Bahamas	8.9	26	90	31.6	62.2	3.4	Casaburi et al. 2016
	Rottnest Island, Australia	8.6	26	200	9.9	52.9	20.8	Mendes 2019
	Lagoa Vermelha, Brazil	8.2	30	38	8	77	35	Popall 2020

4. RESULT AND DISCUSSION

4.1 Results of Pilot Experiments Conducted for Biofilm Growth on Sediment Surface on a Static Condition in a Laboratory Environment:

4.1.1 Method 1: (Growth on a plastic surface)

After a week of continuous media spraying, initial attachment was seen, however it was found that the consortium may take a very long time to entirely adhere and spread on the plastic's surface forming a mat like structure. This method is not very practical because it is a time-consuming process.

4.1.2 Method 2: (Cultivation of biofilm on partially submerged sediment)

In this case we observed that the growth initially started to spread on and around the plastic surface which was completely submerged in the media and then it started to adhere sediment surface. Micro colony formation was observed on that part which was partially submerged in the media. A thick mat like structure was developed.

4.1.3 Method 3: (Biofilm growth on a inclined sediment surface that mimics intertidal zone)

For this system also, the preliminary growth occurred on completely submerged plastic surfaces and then proceeded to from the bottom to the upper layer of the sediment slant.

4.2 Chlorophyll Estimation:

4.2.1 For Field Samples:

Estimation of chlorophyll performed from the samples collected during the Field investigation shows that the greater amount of chlorophyll was present in the samples collected from the region of Kakdwip rather than the samples of Patharpratima and Namkhana. Amongst the samples collected from the Kakdwip region the location K6 had the highest amount of total chlorophyll**.**

Table 3 Chlorophyll Concentration of Field samples

Sample	Total Average Chlorophyll (µg/g)
P1	31.05474813
P2	31.9525806
P3	142.4841614
N4	47.16166079
K6	1090.511884
K7	505.9340336
K8	241.2205503

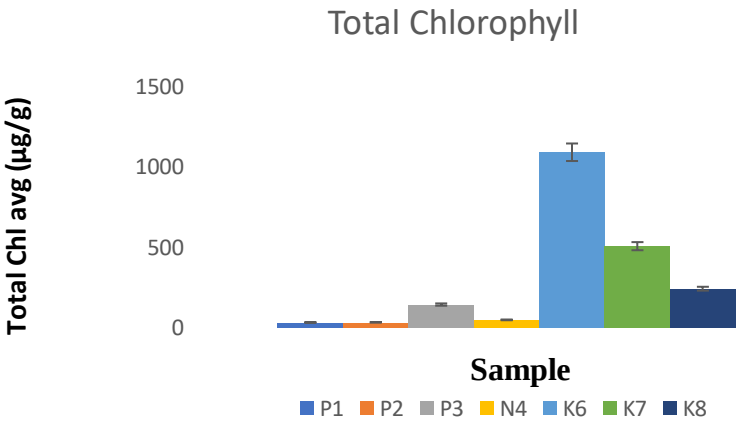

Figure 8 Bar graphs representing various chlorophyll concentration of field samples

4.2.2 For In-Vitro Biofilm Culture (CCF and Biofilm growth on sediment matrix):

Referring to an assessment of the chlorophyll of the biofilm samples that were cultured in the lab with the use of mixed media, the cultivated biofilm in the CCF flask exhibits a greater chlorophyll value than the one cultivated on the sediment surface for a similar amount of analyte (Table 4 – and Figure 8).

Table 4 Chlorophyll concentration from cultured samples

SAMPLE	Total Average Chlorophyll (µg/g)
Biofilm Cultivated On sediment surface	7728
Biofilm Cultivated in CCF	11568

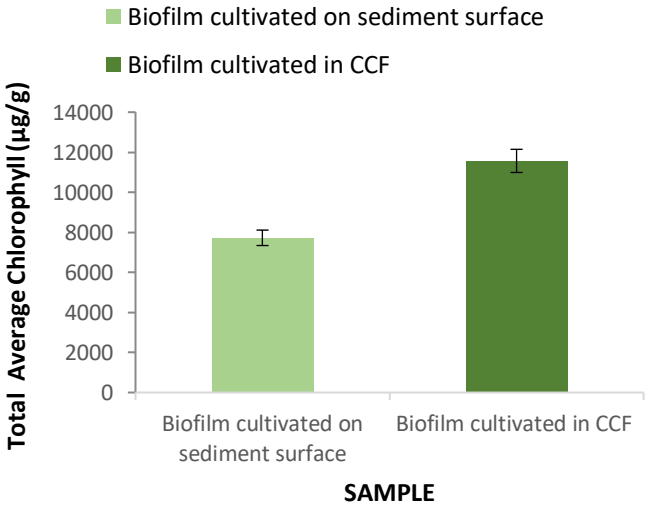

Figure 9 Bar graphs representing various chlorophyll concentration of lab grown biofilm cultures

4.3 EPS Estimation from the cultured samples:

The amount of exopolysaccharide (EPS) recovered from the sample was evaluated, and it was observed that for an equivalent amount of sample analyzed, the sample grown on the sediment surface had a higher exopolysaccharide content than the biofilm cultivated in the CCF flask.

Table 5 EPS content of biofilm samples:

SAMPLE	Capsular EPS CONTENT (mg/g)
Biofilm Cultivated On sediment surface	20.75
Biofilm Cultivated in CCF flask	15.37

4.4 Metallothionein Estimation from In-Vitro cultures:

Comparing the biofilm sample grown on the sediment surface to the sample grown in the CCF flask, the metallothionein assay reveals that the CCF flask sample has a higher concentration of this metal binding protein (Table 6 and Figure 9).

Table 6 Metallothionein concentration of samples

Sample	Metallothionine Concentration (µg/G)
Biofilm Cultivated On sediment surface	75.60
Biofilm Cultivated in CCF flask	91.8

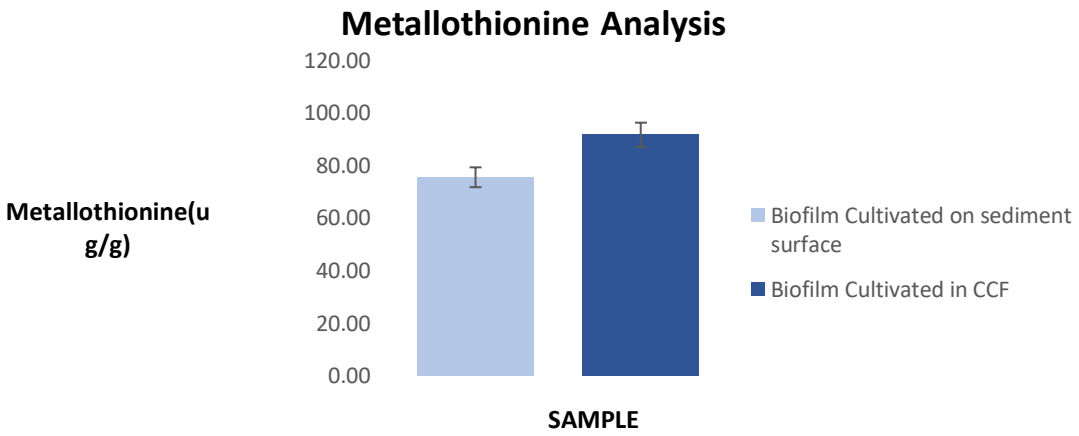

Figure 10 Graphical representation of metallothionein concentration in two samples

4.5 Principal Component Analysis on Microbial Diversity of Stromatolites and Various Physicochemical Parameters

As shown in Table 2 the population of cyanobacteria was comparatively higher in lithified beds, where the members of phylum Proteobacteria and Bacteroidetes dominated nonlithified stromatolite structures. The outcome of PCA established that cyanobacteria represented a strong positive correlation with the increasing pH in lithified structures (PCA=0.759), while cyanobacterial abundance is loosely impacted by the pH in nonlithified beds. On the other hand, the growth of cyanobacterial members is negatively regulated with the increasing salinity (PCA= -0.213) and temperature (PCA= -0.686) in lithified beds, although a strong positive correlation between salinity and growth of the members of Proteobacteria (PCA= 0.632) and Bacteroidetes (PCA=0.306) is evidenced in nonlithified structures.

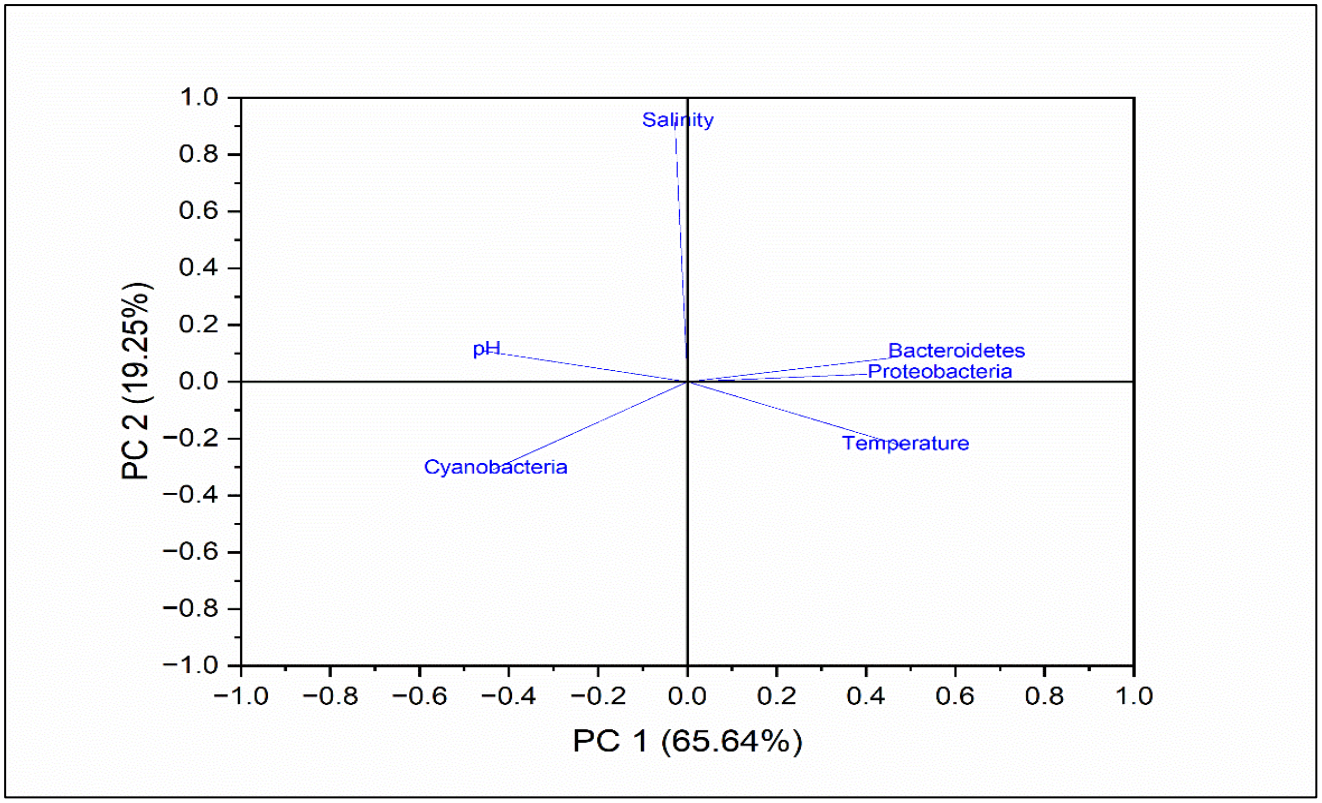

Figure 11 PCA analysis for Lithified Mat type

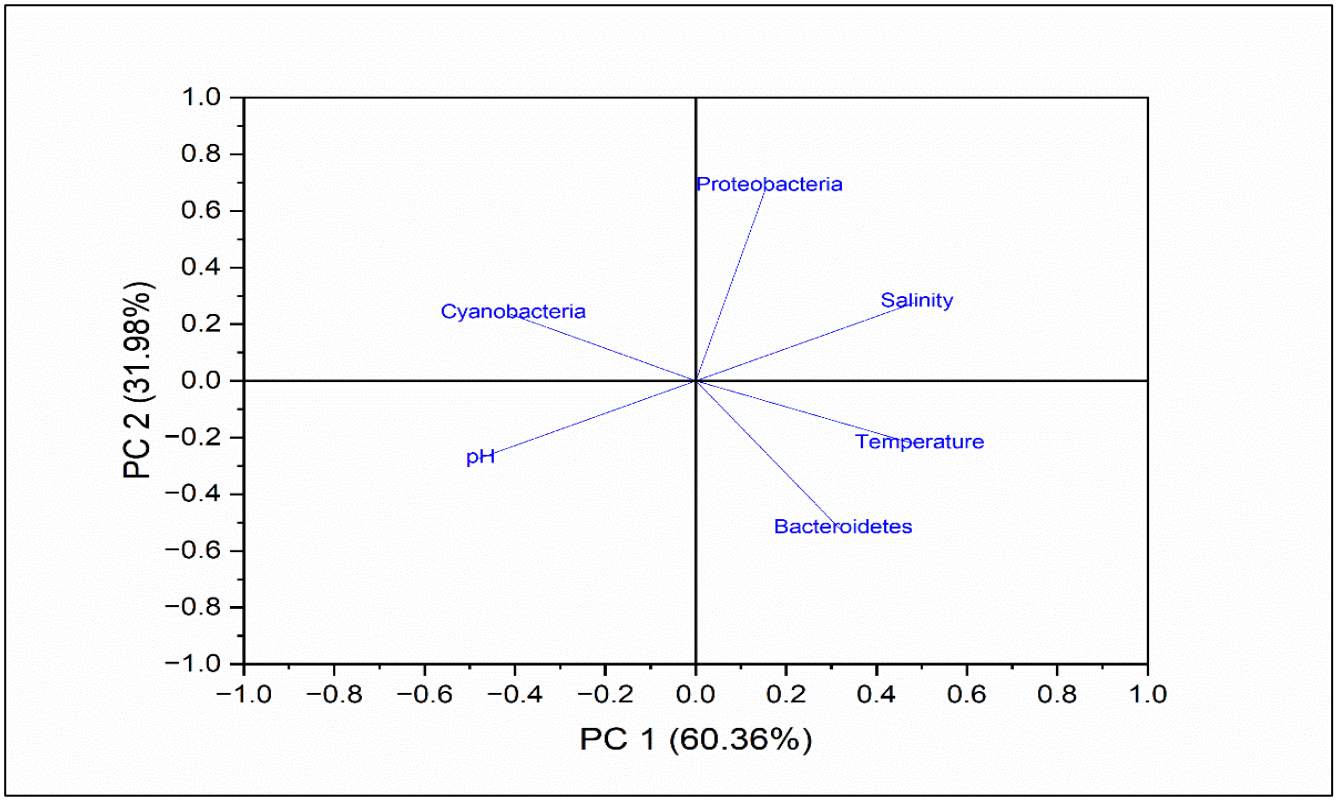

Figure 12 PCA analysis for Non-lithified Mat type

4.6 Model of calcification in cyanobacterial cell during photosynthesis:

Cyanobacteria can thrive in the majority of ecosystem habitats on Earth; they successfully populate freshwater and marine environments, hot springs, and cold dry valleys, coping with extremes in salinity, light quality and availability, UV radiation, pH, dryness, desiccation, temperature.

From literature survey it was observed that cyanobacteria are one of the major potential contributors in the carbonate precipitation via EPS mineralization and holds vast biogeochemical and ecological significance. Even our PCA evaluation also supports this fact that lithification is dominated by cyanobacterial population. Many cyanobacteria can mineralize CO_2 to recalcitrant carbonates along with the conversion of CO_2 to organic compounds during photosynthesis.

Solubility of Carbon dioxide in water is very high and when dissolved in water it reacts and forms carbonic acid. This carbonic acid dissociates to form bicarbonate while releasing H+ in the solution and a fraction of HCO_3^- again dissociated to form carbonate (CO_3^{2-})

$$CO_2 + H_2O = HCO_3 \quad (1)$$

$$H2CO_3^- = HCO_3^- + H^+ \quad (2)$$

$$HCO_3^{-} = CO_3^{2-} + H^+ \quad (3)$$

If the solution is in natural water for example sea water carbonate anions can interact with the cations present in the solution to form insoluble carbonate, for instance, if Ca2+ is present then CaCO3 will form.

$$Ca_2^+ + 2HCO_3^- = CaCO_3 + H_2O \quad (4)$$

$$Ca_2^+ + CO_3^- = CaCo_3 \quad (5)$$

CO_2 enters the cell of cyanobacteria through HCO_3^-/Na+ symports and diffusion of Co2 subsequently converting into HCO_3^-. Cytosolic HCO_3- gets imported into the carboxysome and is converted into CO_2 by carboxysomal Carbonic anhydrase (CA).

Cyanobacteria may catalyse the carbonate mineralization reaction(s) by increasing the saturation state of the environment intimately associated with the cell, with respect to the mineral. This can be done by one or both of two means metabolic activity altering a pH of the environment and passive and/or active ion concentration. The photosynthetic electron

transport in the thylakoids and the CA activity in the carboxysome both consume cytosolic H^+, resulting in a net increase of OH− in the cytosol. Neutralization of this imbalance for example, by the activity of a Ca^{2+}/H^+ antiport, generates an alkaline microenvironment on the outer cell surface. The alkaline pH shifts the equilibria of the bicarbonate buffer system to the right and generates localized regions of increased CO3 2− concentrations at the cell exterior. Recruitment of Ca^{2+} to the cell surface occurs from the surrounding medium and also via the export of Ca^{2+} through the Ca2+/H+ translocator. A second mechanism by which cyanobacteria can catalyse carbonate precipitation is by the presence of ordered Ca^{2+}/Mg^{2+}- binding groups on the cell surface for instance glutamate and aspartate residues, or carboxylates and sulfonates. Those groups serve as nucleation sites for initiation of the $CaCO_3$ precipitation.

Through a variety of processes, EPS can significantly affect carbonate precipitation. For instance, negatively charged EPS groups can draw in cations like Ca^{2+}, enabling the production of carbonate. On the other hand, the EPS can prevent carbonate forms by strongly binding bivalent cations. Additionally, the formation of heterogeneous microdomains that support various microbial metabolisms and the provision of energy and carbon to heterotrophic bacteria can also help carbonate precipitation. The mineralogy of the precipitated $CaCO^3$ crystals is also influenced by the characteristics of the EPS. Thus, it is usually discovered that EPS is connected to the mineralization processes because it has both the highly hydrated character that facilitates ion accumulation and concentration and the negatively charged residues. It was also demonstrated that the cyanobacterial surface layer (S-layer), which offers organised ion-binding groups and is typically the outermost defining layer of the cell, goes through mineralization. Additionally, it has been noted that cyanobacteria remove the encrusted regions of S-layer, perhaps in an effort to avoid total encasing in mineral and eventual cell death.

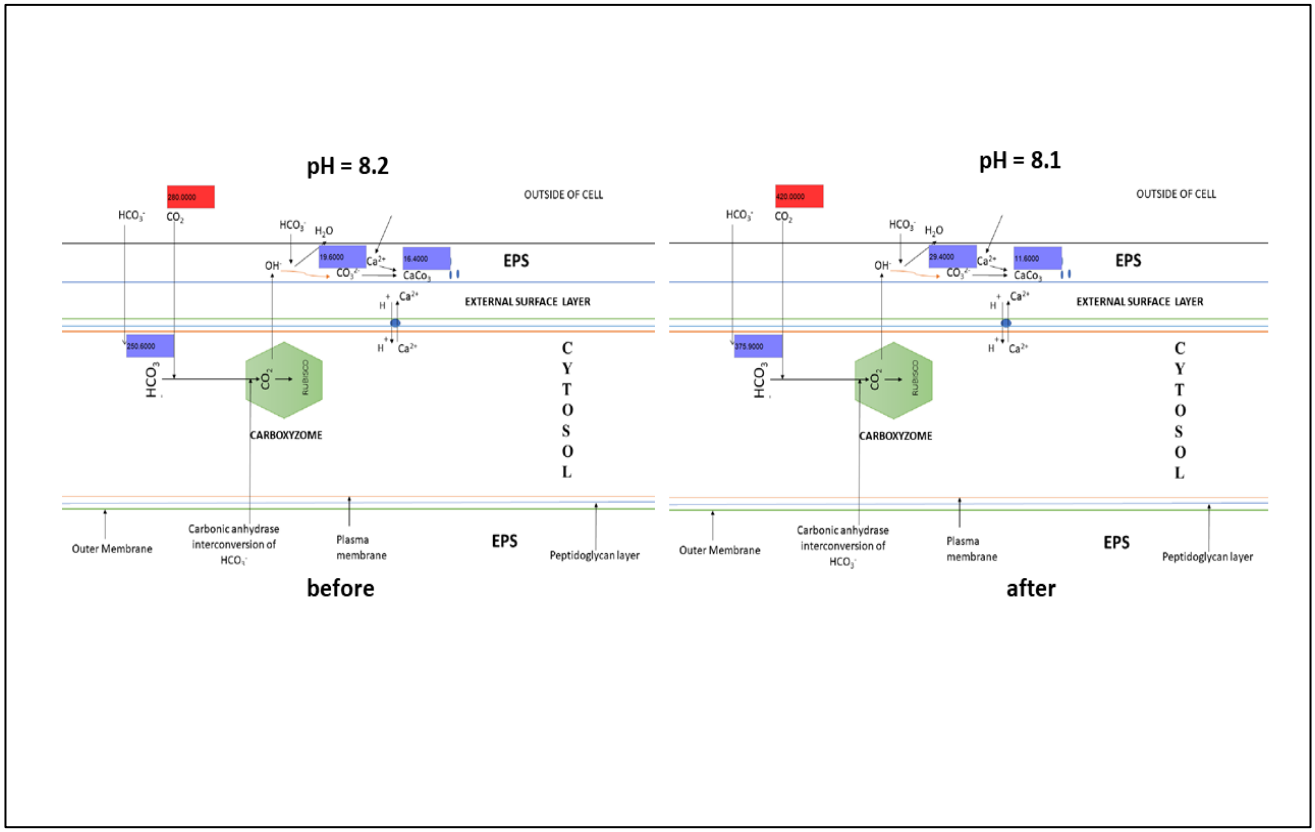

Figure 13 Model of calcification in cyanobacterial cell during photosynthesis

Rapid industrialization caused a drastic rise in the atmospheric CO_2 concentrations which subsequently affected the oceanic water chemistry. Atmospheric CO_2 that gets dissolved in the water dissociates into HCO_3^- and H^+. when the oceanic pH is around 8.2, the H^+ concentration is relatively low. Addition of more excess CO_2 in the water followed by rising atmospheric temperature lowers the pH by increasing the H^+ ions and lowering the availability of HCO_3^- ion and thus the carbonate precipitation decreases(Obasanjo *et al.*, 2023). The model that we demonstrated in the Figure 13 developed by CellNetAnalyzer using MATLAB shows that cyanobacteria are capable of precipitating calcium carbonate through their photosynthesis process at a higher pH and lowers precipitation rate decreases with the decrease of pH and supports the fact of the effect of ocean acidification on the rate of carbonate precipitation (Kamennaya *et al.*, 2012). Also, it corroborates to our PCA data which depicts that the rate of lithification by the cyanobacterial members contributing in the formation of stromatolites increases with the increasing pH.

4.5 Discussion:

4.5.1 Field Observation and sampling

Biofilm samples were collected from the Sunderbans, areas that are in peril of severe environmental pollution due to the constant influx of pollutants brought on by the presence of ferry terminals, fish harbours, and other large-scale water transportation systems, which result in massive amounts of waste disposal and oil spillage. According to the sampling session's observations, Kakdwip region was significantly more contaminated than the other sites, and comparably thicker mats were collected from this location. Chlorophyll extraction from the field samples also demonstrates that the samples of the Kakdwip had higher biomass due to their higher chlorophyll concentration. Heavy metal present in the bio-available fraction of the sampled sediments were analyzed and their individual Enrichment factors were calculated by my co-worker. The information is shown in the table below (Table 6). From the analysis, we can see that all the sampling locations of Kakdwip have extremely high enrichment factor for Cadmium (EF > 40 is extremely high enriched) whereas other metals are present in the range of moderate to very high enrichment (2-5 moderately enriched; 5-20 very high; 20-40-significantly high).

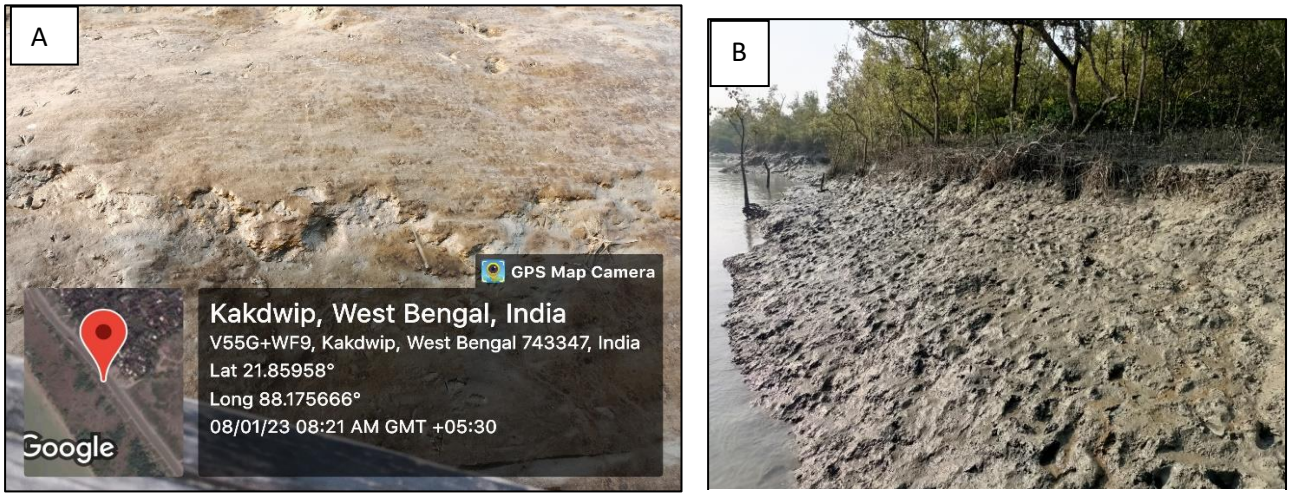

Figure 14 Sampling Site in Sunderban A. Kakdwip site showing abundance of biofilm. B. on the way to Namkhana ferry terminal from Patharpratima.

Because of such high enrichment factor and the availability of higher biomass, K6 sampling location was chosen for resampling and later this sample was utilized for developing various

methods for biofilm cultivation on the sediment surface in laboratory environment and then multiple biochemical assays were conducted on them.

Table 7 Enrichment factor of different analyzed heavy metals from the bio-available fraction in the sampling sites

SAMPLE	ENRICMENT FACTORS OF DIFFERENT HEVY METAL IN BIOAVAILABLE FRACTION													
	Cd	Co	Cr	Cu	Fe	Ga	In	Li	Mg	Ni	Pb	Sr	Tl	Zn
P1	109.7	1.1	2.1	14.7	1.5	3.7	4568.7	40.0	38.9	2.3	0.9	63.0	126.0	3.9
P2	74.7	1.0	1.6	5.7	1.4	1.6	3492.1	21.1	20.7	2.1	2.1	31.8	63.0	3.0
P3	15.1	0.7	1.7	13.8	1.6	5.4	3791.6	43.2	48.9	2.6	8.6	73.8	187.4	3.6
N4	101.1	0.8	1.6	14.8	1.5	4.2	5300.6	38.9	51.2	2.4	9.8	55.6	128.3	6.4
K6	391.9	2.2	1.8	15.7	1.5	4.5	4615.5	29.3	47.8	3.3	1.0	64.8	149.6	4.8
K7	168.0	1.3	1.9	14.6	1.5	2.1	9297.0	28.2	44.1	1.8	10.5	61.7	142.6	4.1
K8	302.8	6.6	3.9	19.2	2.2	37.1	42657.6	33.7	130.9	3.3	50.1	494.6	1296.1	10.8

4.5.1 Pilot Experiments Conducted for Biofilm Growth on Sediment Surface on a Static Condition in a Laboratory Environment

A successful experimental finding was observed in case of method 2 and 3 where the biofilms were successfully cultivated on the sediment surface in a partially submerged condition after 28 days of inoculation. It was also noted that initial attachment of the biofilm started from the plastic surface that was completely submerged with the artificial sea water media. Previous investigation(De-la-Pinta *et al.*, 2019; Balu*et al.*, 2020) showed that, biofilm growth had a greater opportunity to attach to hydrophobic surfaces such as plastic than the hydrophilic surfaces such as sediment and glass surfaces. In this pilot experiment we observed that the biofilms primarily started their attachment with the hydrophobic surface and they proceeded towards the hydrophilic one.

4.5.2 For the Chlorophyll Analysis:

Since we worked with phototrophic biofilm, the primary indicator of growth of such biofilm is their photosynthetic activity. Several research papers stated that most of the toxicants target photosynthetic activity of the bacterial and algal consortium present in the biofilms to inhibit their growth. This condition is influenced by the induction of shade type chloroplast. In case of field chlorophyll estimation, we observed that the sample collected from most polluted sampling station K6 showed the highest value (Bhowmick*et al.*, 2021). Enrichment factor of the bioavailable fraction of K6 shows moderate to high enrichment of various heavy metals, especially for Cd. It can be interpreted that stress condition induce shade type chlorophyll a synthesis, to increase their photosynthetic activity as a survival mechanism.

The biofilm growth might be impacted by the substratum's quality. We found that the biofilm sample grown in the PMMA-CCF flask had more total chlorophyll content than the biofilm grown on a sediment surface. Although the hydrophobic properties of both plastic and PMMA surfaces encourage the formation of biofilms(Balu *et al.*, 2020), the CCF flask's larger hydrophobic surface area fosters more biofilm growth. In addition, the CCF flask has greater micronutrient availability because of uniform distribution of the same when compared to the sediment surface cultivation.

4.5.3 For EPS analysis:

Extrapolymeric compounds stimulate the development of biofilms via the production of a mucilaginous matrix. Additionally, it acts as a key defence mechanism to shield microorganisms from various stressful situations and promote better survival. Comparing the sediment cultivated sample to the CCF culture it was seen that there was a slight increase in CPS per gram of the biomass of the former, may be due to the presence of some amount of sediment during extraction of CPS.

4.5.4 Metallothionein Assay Analysis:

Metallothionein protein expression is one other method of protection against metal stress in the organisms that bind with the metal and maintains the cell homeostasis (mainly Zn and Cu) and also partake in the detoxification action of certain heavy metals such as Cd. Cyanobacterial metallothionein gene SmtA identified in the species *Synechococcus* is the widely studied metallothionein gene of prokaryotes(Cui *et al.*, 2021). Different researches on this largest family of metal binding protein shows that the level of this protein can change with the availability of trace amount of zinc and Copper. Experimental analysis revealed that biofilm cultured in the CCF displayed slightly higher concentration of metallothionein compared to those cultivated on a sediment surface (Table5 and Figure 9). This observation suggests that consistent availability of both Zn and Cu used as a micronutrient in the culture media along with some other essential heavy metals in the CCF potentially contributes to the elevated metallothionein level. In contrast, biofilm grown on sediment surface encountered an inconsistency and lesser quantity of media components. As biofilms can bioaccumulate soluble heavy metal from the water and liquid media and induce metallothionein expression for metal binding, the consistent distribution of media components in CCF flask may have caused increased metallothionein concentration.

Table 8 List of micronutrients present in the ASNIII+ BG11 media (1:1)

Micronutrients	**Metal present**	**Metal concentration(gm/l)**	**Metal concentration in CCF (mg/0.25ml)**
$MnCl_2.4H_2O$	Mn	1.81	0.45
$ZnSO_4.7H_2O$	Zn	0.22	0.055
$CuSO_4.5H_2O$	Cu	0.079	0.019
$Co(NO_3)_2.6H_2O$	Co	0.049	0.0122

4.5.5 Principal Component Analysis on Microbial Diversity of Stromatolites and Various Physicochemical Parameters

According to the PCA analysis, the rise in pH is directly correlated with cyanobacterial abundance. Since cyanobacterial quantity was high in lithified beds, it may be deduced that the lithification rate rises as cyanobacterial abundance does. The functioning of each community present in the mat and their interactions with physicochemical parameters can also be interpreted as an influence to the pace of lithification.

The findings of Dupraz and Visscher, 2005 and Kamennaya *et al.*, 2012 also corroborate with our interpretation.

5. CONCLUSION:

From the findings of both field and laboratory it can be concluded that, abundance of inorganic pollutants and heavy metal (essential and non-essential both) has directly impacted on the growth and proliferation of the field biofilms leading to their uptake of heavy metal to certain concentration. From our field observation it was seen that the Kakdwip was the most polluted sampling location in comparison to the others but in spite of being highly polluted this place was observed with thicker biomats and even their chlorophyll content was greater than any other location (especially K6). Laboratory experiments on their biochemical characteristics also shows that they increase their chlorophyll content by the induction of shade type chloroplast activity and metallothionein concentration by up taking heavy metals from the surroundings and shows high resilience.

Biofilms are capable of developing various sedimentary structure which includes stromatolites and MISS. Stromatolites, early evidence of life on earth is also developed by biofilm community. Different physicochemical parameters can affect the expression of microbial community of this system and contribute in lithification. From the analysis we can see that in high pH condition cyanobacterial community plays an integral role and induces lithification rate.

Future perspective:

Microbially induced sedimentary structures being a biomarker along with the stromatolites different microbial cells and filaments can also help in the prediction of the existence of life in the neighbouring planets of the earth such as Mars. Their presence can confirm biogenicity. This kind of study of MISS is still in a nascent stage(Noffke, 2015).

6. REFERENCES:

Alluri, H.K. *et al.* (2007) 'Biosorption: An eco-friendly alternative for heavy metal removal', *African Journal of Biotechnology*, 6(25). Available at: https://doi.org/10.4314/ajb.v6i25.58244.

Babilonia, J. *et al.* (2018) 'Comparative Metagenomics Provides Insight Into the Ecosystem Functioning of the Shark Bay Stromatolites, Western Australia', *Frontiers in Microbiology*, 9. Available at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.01359 (Accessed: 9 May 2023).

Balu, S. *et al.* (2020) 'Assessment of polycyclic aromatic hydrocarbon contamination in the Sundarbans, the world's largest tidal mangrove forest and indigenous microbial mixed biofilm-based removal of the contaminants', *Environmental Pollution*, 266, p. 115270. Available at: https://doi.org/10.1016/j.envpol.2020.115270.

Bhowmick, T. *et al.* (2021) 'Assessing the effect of herbicide diuron on river biofilm: A statistical model', *Chemosphere*, 282, p. 131104. Available at: https://doi.org/10.1016/j.chemosphere.2021.131104.

Camacho-Chab, J.C. *et al.* (2018) 'Biosorption of Cadmium by Non-Toxic Extracellular Polymeric Substances (EPS) Synthesized by Bacteria from Marine Intertidal Biofilms', *International Journal of Environmental Research and Public Health*, 15(2), p. 314. Available at: https://doi.org/10.3390/ijerph15020314.

Chatterjee, S. *et al.* (2020) 'Diversity, structure and regulation of microbial metallothionein: metal resistance and possible applications in sequestration of toxic metals', *Metallomics*, 12(11), pp. 1637–1655. Available at: https://doi.org/10.1039/d0mt00140f.

Cui, J. *et al.* (2021) 'Deciphering and engineering photosynthetic cyanobacteria for heavy metal bioremediation', *Science of The Total Environment*, 761, p. 144111. Available at: https://doi.org/10.1016/j.scitotenv.2020.144111.

Dash, H.R. *et al.* (2013) 'Marine bacteria: potential candidates for enhanced bioremediation', *Applied Microbiology and Biotechnology*, 97(2), pp. 561–571. Available at: https://doi.org/10.1007/s00253-012-4584-0.

De Orte, M.R. *et al.* (2014) 'Metal mobility and toxicity to microalgae associated with acidification of sediments: CO2 and acid comparison', *Marine Environmental Research*, 96, pp. 136–144. Available at: https://doi.org/10.1016/j.marenvres.2013.10.003.

Decho, A. (2000) 'Decho AW.. Microbial biofilms in intertidal systems: an overview. Cont Shelf Res 20: 1257-1273', *Continental Shelf Research*, 20, pp. 1257–1273. Available at: https://doi.org/10.1016/S0278-4343(00)00022-4.

De-la-Pinta, I. *et al.* (2019) 'Effect of biomaterials hydrophobicity and roughness on biofilm development', *Journal of Materials Science. Materials in Medicine*, 30(7), p. 77. Available at: https://doi.org/10.1007/s10856-019-6281-3.

Di Pippo, F. *et al.* (2013a) 'Characterization of exopolysaccharides produced by seven biofilm-forming cyanobacterial strains for biotechnological applications', *Journal of Applied Phycology*, 25(6), pp. 1697–1708. Available at: https://doi.org/10.1007/s10811-013-0028-1.

Di Pippo, F. *et al.* (2013b) 'Characterization of exopolysaccharides produced by seven biofilm-forming cyanobacterial strains for biotechnological applications', *Journal of Applied Phycology*, 25(6), pp. 1697–1708. Available at: https://doi.org/10.1007/s10811-013-0028-1.

Doney, S.C. *et al.* (2009) 'Ocean Acidification: The Other CO $_2$ Problem', *Annual Review of Marine Science*, 1(1), pp. 169–192. Available at: https://doi.org/10.1146/annurev.marine.010908.163834.

Dupraz, C. and Visscher, P.T. (2005) 'Microbial lithification in marine stromatolites and hypersaline mats', *Trends in Microbiology*, 13(9), pp. 429–438. Available at: https://doi.org/10.1016/j.tim.2005.07.008.

Egan, S., Thomas, T. and Kjelleberg, S. (2008) 'Unlocking the diversity and biotechnological potential of marine surface associated microbial communities', *Current Opinion in Microbiology*, 11(3), pp. 219–225. Available at: https://doi.org/10.1016/j.mib.2008.04.001.

Fabry, V.J. *et al.* (2008) 'Impacts of ocean acidification on marine fauna and ecosystem processes', *ICES Journal of Marine Science*, 65(3), pp. 414–432. Available at: https://doi.org/10.1093/icesjms/fsn048.

Flemming, H.-C. and Wingender, J. (2001) 'Relevance of microbial extracellular polymeric substances (EPSs) - Part I: Structural and ecological aspects', *Water Science and Technology*, 43(6), pp. 1–8. Available at: https://doi.org/10.2166/wst.2001.0326.

Foster, J.S. and Green, S.J. (2011) 'Microbial Diversity in Modern Stromatolites', in V. Tewari and J. Seckbach (eds) *STROMATOLITES: Interaction of Microbes with Sediments*. Dordrecht: Springer Netherlands (Cellular Origin, Life in Extreme Habitats and Astrobiology), pp. 383–405. Available at: https://doi.org/10.1007/978-94-007-0397-1_17.

Garcia-Pichel, F. and Wojciechowski, M.F. (2009) 'The Evolution of a Capacity to Build Supra-Cellular Ropes Enabled Filamentous Cyanobacteria to Colonize Highly Erodible Substrates', *PLOS ONE*, 4(11), p. e7801. Available at: https://doi.org/10.1371/journal.pone.0007801.

Gattuso, J.-P. and Hansson, L. (2011) *Ocean Acidification*. OUP Oxford.

Gerbersdorf, S.U. and Wieprecht, S. (2015) 'Biostabilization of cohesive sediments: revisiting the role of abiotic conditions, physiology and diversity of microbes, polymeric secretion, and biofilm architecture', *Geobiology*, 13(1), pp. 68–97. Available at: https://doi.org/10.1111/gbi.12115.

Gupta, P. and Diwan, B. (2017) 'Bacterial Exopolysaccharide mediated heavy metal removal: A Review on biosynthesis, mechanism and remediation strategies', *Biotechnology Reports*, 13, pp. 58–71. Available at: https://doi.org/10.1016/j.btre.2016.12.006.

Ivanina, A.V. and Sokolova, I.M. (2015) 'Interactive effects of metal pollution and ocean acidification on physiology of marine organisms', *Current Zoology*, 61(4), pp. 653–668. Available at: https://doi.org/10.1093/czoolo/61.4.653.

Janssen, K. *et al.* (2022) 'The complex role of microbial metabolic activity in fossilization', *Biological Reviews*, 97(2), pp. 449–465. Available at: https://doi.org/10.1111/brv.12806.

Kamennaya, N.A. *et al.* (2012) 'Cyanobacteria as Biocatalysts for Carbonate Mineralization', *Minerals*, 2(4), pp. 338–364. Available at: https://doi.org/10.3390/min2040338.

Khodadad, C.L.M. and Foster, J.S. (2012) 'Metagenomic and Metabolic Profiling of Nonlithifying and Lithifying Stromatolitic Mats of Highborne Cay, The Bahamas', *PLOS ONE*, 7(5), p. e38229. Available at: https://doi.org/10.1371/journal.pone.0038229.

Laspidou, C.S. and Rittmann, B.E. (2002) 'A unified theory for extracellular polymeric substances, soluble microbial products, and active and inert biomass', *Water Research*, 36(11), pp. 2711–2720. Available at: https://doi.org/10.1016/S0043-1354(01)00413-4.

Madsen, K.N., Nilsson, P. and Sundbäck, K. (1993) 'The influence of benthic microalgae on the stability of a subtidal sediment', *Journal of Experimental Marine Biology and Ecology*, 170(2), pp. 159–177. Available at: https://doi.org/10.1016/0022-0981(93)90150-M.

Mandal, A. *et al.* (2021) 'Role of intertidal microbial communities in carbon dioxide sequestration and pollutant removal: A review', *Marine Pollution Bulletin*, 170, p. 112626. Available at: https://doi.org/10.1016/j.marpolbul.2021.112626.

Meadows, P.S. and Anderson, J.G. (1968) 'Micro-organisms attached to marine sand grains', *Journal of the Marine Biological Association of the United Kingdom*, 48(1), pp. 161–175. Available at: https://doi.org/10.1017/S0025315400032501.

Neu, T.R. and Lawrence, J.R. (2010) 'Extracellular polymeric substances in microbial biofilms', in *Microbial Glycobiology*. Elsevier, pp. 733–758. Available at: https://doi.org/10.1016/B978-0-12-374546-0.00037-7.

Noffke, N. (2008) 'Turbulent Lifestyle: Microbial Mats on Earth's Sandy Beaches—Today and 3 Billion Years Ago', *GSA Today*, 18(10). Available at: https://doi.org/10.1130/GSATG7A.1.

Noffke, N. (2015) 'Ancient Sedimentary Structures in the <3.7 Ga Gillespie Lake Member, Mars, That Resemble Macroscopic Morphology, Spatial Associations, and Temporal Succession in Terrestrial Microbialites', *Astrobiology*, 15(2), pp. 169–192. Available at: https://doi.org/10.1089/ast.2014.1218.

Noffke, N. and Awramik, S.M. (2013) 'Stromatolites and MISS—Differences between relatives', *GSA Today*, 23(9), pp. 4–9. Available at: https://doi.org/10.1130/GSATG187A.1.

Obasanjo, C.A. *et al.* (2023) 'High-rate and selective conversion of CO2 from aqueous solutions to hydrocarbons', *Nature Communications*, 14(1), p. 3176. Available at: https://doi.org/10.1038/s41467-023-38963-y.

Paterson, D.M. *et al.* (2018) 'Form, function and physics: the ecology of biogenic stabilisation', *Journal of Soils and Sediments*, 18(10), pp. 3044–3054. Available at: https://doi.org/10.1007/s11368-018-2005-4.

Pereira, S. *et al.* (2009) 'Complexity of cyanobacterial exopolysaccharides: composition, structures, inducing factors and putative genes involved in their biosynthesis and assembly',

FEMS Microbiology Reviews, 33(5), pp. 917–941. Available at: https://doi.org/10.1111/j.1574-6976.2009.00183.x.

Quero, G.M. *et al.* (2015) 'Patterns of benthic bacterial diversity in coastal areas contaminated by heavy metals, polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs)', *Frontiers in Microbiology*, 6. Available at: https://www.frontiersin.org/articles/10.3389/fmicb.2015.01053 (Accessed: 29 March 2023).

Ramanathan, A. *et al.* (2009) 'Textural characteristics of the surface sediments of a tropical mangrove Sundarban ecosystem India', *IJMS*, 38, pp. 397–403.

Redzuan, N.S. and Milow, P. (2019) 'The potential of microphytobenthos in sediment biostabilisation of aquatic ecosystems: an overview', 12(3).

Riba, I. *et al.* (2003) 'Bioavailability of heavy metals bound to estuarine sediments as a function of pH and salinity values', *Chemical Speciation & Bioavailability*, 15(4), pp. 101–114. Available at: https://doi.org/10.3184/095422903782775163.

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. Available at: https://doi.org/10.1007/s11099-008-0019-7.

Sahan, E. *et al.* (2007) 'Community structure and seasonal dynamics of diatom biofilms and associated grazers in intertidal mudflats', *Aquatic Microbial Ecology*, 47, pp. 253–266. Available at: https://doi.org/10.3354/ame047253.

Stal, L.J. (2003) 'Microphytobenthos, their Extracellular Polymeric Substances, and the Morphogenesis of Intertidal Sediments', *Geomicrobiology Journal*, 20(5), pp. 463–478. Available at: https://doi.org/10.1080/713851126.

Stal, L.J. (2010) 'Microphytobenthos as a biogeomorphological force in intertidal sediment stabilization', *Ecological Engineering*, 36(2), pp. 236–245. Available at: https://doi.org/10.1016/j.ecoleng.2008.12.032.

Sutherland, I. (2001) 'Biofilm exopolysaccharides: a strong and sticky framework', *Microbiology (Reading, England)*, 147(Pt 1), pp. 3–9. Available at: https://doi.org/10.1099/00221287-147-1-3.

Zamboulis, D. *et al.* (2011) 'Metal ion separation and recovery from environmental sources using various flotation and sorption techniques', *Journal of Chemical Technology & Biotechnology*, 86(3), pp. 335–344. Available at: https://doi.org/10.1002/jctb.2552.

Zeng, X., Chen, X. and Zhuang, J. (2015) 'The positive relationship between ocean acidification and pollution', *Marine Pollution Bulletin*, 91(1), pp. 14–21. Available at: https://doi.org/10.1016/j.marpolbul.2014.12.001.

Zhu, T. and Dittrich, M. (2016) 'Carbonate Precipitation through Microbial Activities in Natural Environment, and Their Potential in Biotechnology: A Review', *Frontiers in Bioengineering and Biotechnology*, 4. Available at: https://doi.org/10.3389/fbioe.2016.00004.