

Aberrations in the microbiome of Cyanobacteria from a tropical estuary polluted by heavy metals

Jasmin C,¹ Abdulaziz Anas,^{1*} Dharmesh Singh,² Hemant J. Purohit,² Gireeshkumar T.R.¹ Shanta Nair¹

1 CSIR-National Institute of Oceanography, Regional Centre Kochi, Kochi 682 018, India

2 Environmental Biotechnology and Genomics Division, CSIR-National Environmental Engineering Research Institute, Nehru Marg, Nagpur, India

*Corresponding author: Email: anas@nio.org; phone: +91 4842390814

Abstract

The effect of heavy metal pollution on the microbiome of cyanobacteria in Cochin estuary (CE) on the southwest coast of India is reported in the study. Statistically significant difference in heavy metal concentration were observed between water, suspended particulate matter (SPM) and sediment. The Zn, Cd, Cu, Ni and Cr were 2- 6 times higher in the SPM compared with the sediment, while Pb was 10 to 25 times higher. Although nearly 60 % of the species diversity of microbiome was common between cyanobacteria enriched from the upstream (S1S) and downstream (S11B), there was a difference in the major groups of heterotrophic bacterial associates. Proteobacteria was the dominant phylum (>80 %) in S1S, while it was second only (27.5 %) after Planctomycetes (37.4 %) in S11B. The results of the current study indicate that the pollution can influence an ecosystem at the micro-niche level.

Keywords: Cochin estuary; Cyanobacteria; Pollution; Heavy metal; microbiome

1. Introduction

The effect of heavy metal pollution on the diversity and activities of cyanobacteria in the estuarine and marine environment is a growing ecological concern as it will disturb the productivity and food web dynamics (Pinto et al., 2003). Estuaries are the transition zones connecting fresh- and marine waters and are greatly influenced by terrestrial discharges that introduce a diverse range of materials derived from human activities, leading to disruptions in their normal ecological functions (McLusky, 1989). Cyanobacteria, the primary producer, has been extensively recognized as an evolutionary link between unicellular heterotrophic prokaryotes and photosynthetic multicellular algae (Falcon et al., 2010) and are well known for their role in changing our atmosphere from reducing type to an oxidizing one, about 1.5 million years ago (Hess, 2004; Price et al., 2008). Cyanobacteria contribute >70 % of the productivity of open ocean and are responsible for fixing most of the inorganic carbon that enters the marine biosphere (Muhling et al., 2005). In diverse aquatic environments, cyanobacteria accommodate a plethora of heterotrophic bacteria within their polysaccharide and peptide containing envelopes (Berg et al., 2009; Cole, 1982; Hube et al., 2009; Limei et al., 2009). It is reported in recent literature that many of the ecological roles and biotechnological potentials of cyanobacteria are supported by their association with heterotrophic bacteria. A classic example is *Anabaena* in which the microaerophilic environment required for the functioning of nitrogenase enzyme are provided by heterotrophic bacteria specialized to live inside the heterocyst (Paerl, 1976). Other studies have also shown that the heterotrophic bacteria might be able to affect cyanobacteria either positively by providing beneficial metabolites or negatively by excreting algicidal substances (Hube et al., 2009; Salomon et al., 2003). The chemotactic detection of cyanobacterial hosts by heterotrophic bacteria and the host-specific interactions are discussed in earlier reports (Buchan et al., 2005; Cole, 1982; Delucca and McCracken, 1977). However, the influence of heavy metal pollution on the diversity and activities of heterotrophic bacteria associated with cyanobacteria is rarely discussed.

Microorganisms are known to form a symbiotic association with higher organisms in the marine environment and any alterations in the composition of this association will have repercussions on the health and ecophysiology of host organisms. Classical examples include bleaching of corals (Casey et al., 2015) and disease incidence in culture ponds of shrimp (Cornejo-Granados et al., 2017) due to shifts in the structure of microbiome. Although there are reports on the response of gut microbiome of terrestrial animals to toxic chemicals (Breton et al., 2016), our understanding of similar microbial responses in marine organisms hitherto remains incomplete. Reports on interaction of toxic chemicals with marine organisms are largely restricted to toxicity and bioaccumulation in individual

organisms and bioremediation (Bao et al., 2016; Pereira et al., 2015; Zyadah and Abdel-Baky, 2000). Previously, we found that the unicellular cyanobacterium *Synechocystis* sp. accommodates a plethora of microorganisms with varied enzyme expressions and metal resistance properties (Anas et al., 2016). In the present study, we investigated the impact of heavy metal pollution on the microbiome of a unicellular cyanobacterium isolated from Cochin estuary (CE), the largest wetland ecosystem in the southwest coast of India.

CE receives a significant fraction of ~500 million litres of effluent and sewage through various point and nonpoint sources (Sheeba et al., 2017). The major pollutants reported from the CE are heavy metals, polycyclic aromatic hydrocarbons, pesticides and organic nutrients (Sheeba et al., 2017). In the present study, we considered the influence of heavy metals as they are one of the major pollutants in the CE that impact the diversity and activities of sediment-associated bacteria (Jose et al., 2011), community structure of particle-associated archaea and bacteria (Sheeba et al., 2017) and distribution of cyanobacteria (Anas et al., 2015). Although many of the heavy metals, such as Zn, Mn and Cu, are essential micronutrients for the growth and activities of cyanobacteria, they can be toxic at higher concentrations. Heavy metal pollution can affect the diversity and activities of heterotrophic bacteria associated with cyanobacteria, which may indirectly influence the community structure and activities of cyanobacteria. Therefore, it is important to understand the microbiome of these primary producers found in different environmental conditions to assess the significance of their interactions. In the present study, we investigated the microbiome of a cyanobacterium (*Synechocystis* sp.) isolated from the heavy metal polluted and non-polluted regions of Cochin Estuary, Southwest coast of India.

2. Materials And Methods

2.1 Analysis of heavy metal pollution in CE

Water samples; from the middle of the water column (~6 m for stations near bar mouth and ~2.5 m for other stations) and sediment samples were collected from five locations each in the upstream and downstream parts of the CE during premonsoon season (Figure 1). These stations and heavy metals were selected based on previous reports (Jose et al., 2011; Sheeba et al., 2017). Water samples were filtered through cellulose acetate filter papers (0.45 µm pore size) to collect suspended particulate matter (SPM). Further details of the study area, sample collection and preservation are described elsewhere (Jose et al., 2011; Sheeba et al., 2017). For metal analysis, sediment and SPM collected from each station were digested separately using HF-HClO₄- HNO₃ mixture in a hot plate, filtered and dissolved in 50 ml Milli-Q water. The metals, Zn, Cd, Pb, Cu, Ni, Mn, and Cr were analyzed using an Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES, Thermo

Scientific i-CAP 7000 Series) following the standard protocol (Loring and Rantala, 1992). The dissolved metals were extracted using Ammonium Pyrrolidine Dithio Carbamate (APDC) and Methyl Isobutyl Ketone (MIBK) at pH 4.6 and brought back to aqueous layer by back-extraction with concentrated nitric acid and made up to 20 ml with Milli-Q water (Smith and Windom, 1972). The extracts were analyzed in the ICP OES (ICP-OES, Thermo Scientific i-CAP 7000 Series) for dissolved trace metals. The accuracy of the heavy metal analyses was checked using standard reference material BCSS-1. The results of Cr and Cd were found to be accurate at 96 % compared with the standard, while it was 87, 89, 82, 97 and 98 % respectively for Cu, Mn, Ni, Pb, and Zn. Most of the results were within the uncertainty values certified by National Research Council of Canada.

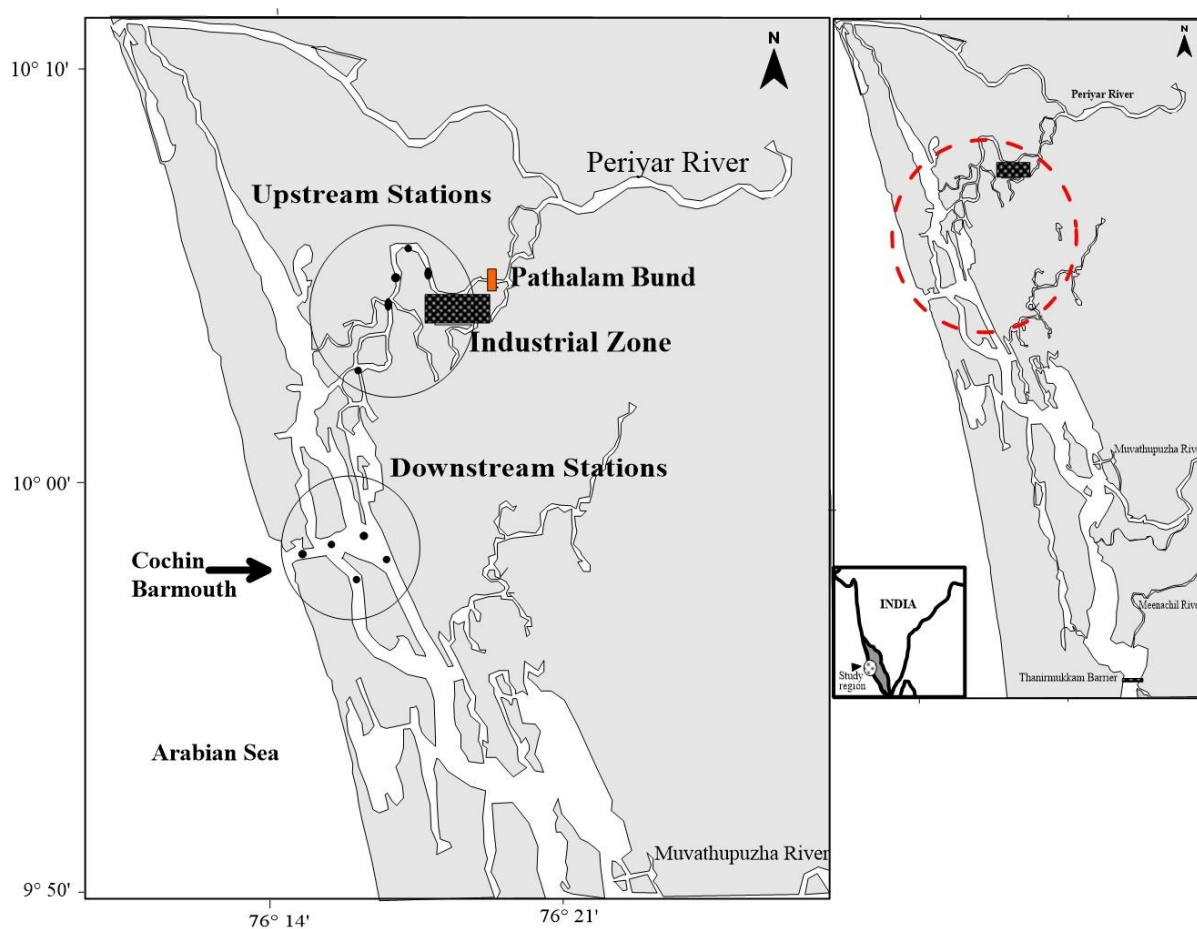


Figure 1: Study area showing five sampling locations each in the upstream and downstream regions of the Cochin Estuary. Comprehensive view of the entire estuary showing the sampling area is given in inset.

2.2 Cyanobacterial isolates

Cyanobacteria were enriched from the water samples collected from upstream (S1S) and downstream (S11B) of the Cochin Estuary. Briefly, each water sample (2L) was filtered sequentially

through 200 µm nylon mesh, 70 and 30 µm cell strainers (Genetix Biotech Asia, India) and 0.45 µm nitrocellulose filter. The filter paper was inoculated into ASN III medium, incubated at 28 ± 2 °C and with a light: dark cycle of 14: 10 hrs. The growth of cyanobacteria was regularly monitored under the 40x objective of an inverted microscope and purified by serial dilution. Since the aim of the study was to isolate the associated microorganisms, antibiotics were not used in the medium. pH of the medium was monitored once a week and maintained at 7.8 ± 2 using 1N HCl or NaOH. Cyanobacteria were maintained by sub-culturing once in every two weeks. Morphological and molecular characterization of this cyanobacteria are reported earlier (Anas et al., 2016).

2.3 DNA extraction

Total genomic DNA was extracted from the cyanobacteria (S1S and S11B) grown in ASN III medium using Nucleospin soil DNA extraction kit (MACHEREY-NAGEL GmbH & Co. KG, Germany). Here the cells were separated from the culture medium by centrifugation at 8000 rpm for 10 min and washed copiously with sterile saline to remove the loosely attached bacteria. The cells were subjected to three cycles of freeze-thawing to disrupt the cell wall before proceeding to DNA extraction using Nucleospin soil DNA extraction kit according to the manufacturer's protocol. The DNA extracted from triplicate samples were pooled, and quantified using a NanoDrop (Thermo Scientific, USA) and maintained at -20 °C until further processing.

2.4 Bacterial diversity study using amplicon Sequencing

Total Genomic DNA was used as the template for 16S rRNA gene PCR for analyzing the microbial diversity using primers, 341F-(5' CCTACGGGAGGCAGCAG 3') and 518R-(5' ATTACCGCGGCTGCTGG 3'), targeting the hypervariable region, V3 (Bartram et al., 2011). The PCR reaction mixture containing 10 ng of sample DNA, 0.4 U Phusion HF DNA polymerase, 10 µM of each primer and 10mM of each dNTPs were subjected to PCR reaction initiated with a denaturation of 94 °C for 2 min followed by 30 cycles of reaction (94 °C for 30 s, 54 °C for 30 sec and 72 °C for 45 sec) and a final extension step at 72 °C for 5 min. Amplicons of the correct size were purified using PureLink Quick Gel Extraction and PCR Purification Combo Kit (Invitrogen) following manufacturers' instructions. Purified products were quantified using a Qubit (Invitrogen). The next-generation sequencing was performed in the Illumina HiSeq platform.

2.5 Data processing

To test the significance of variation in metal concentration between ecological niches (water column, suspended particulate matter, sediment) and location (upstream versus downstream) as well as the interaction of ecological niche and sites, A permutational multivariate analysis of variance

(PERMANOVA) was performed using Primer 6 software package (Plymouth Marine Laboratory, UK). The analysis was conducted with the log transformed and normalized data. Illumina HiSeq data were pair-assembled using PANDAseq (Masella et al., 2012) with an assembly quality score of 0.9, which is the most stringent option to reduce errors. We then followed the Mothur Illumina MiSeq SOP (Kozich et al., 2013) to align, filter, trim, remove chimeras, and classify and assign taxonomy. Sequences were aligned against the Greengenes reference sequences (McDonald et al., 2012), and then the chimeric sequences were removed using UCHIME (Edgar et al., 2011) but only after the pre-clustering command was run to remove erroneous sequences generated due to sequencing errors. Each operational taxonomic unit (OTU), defined at a $\geq 97\%$ cut off of sequence similarity, was classified against the Ribosomal Database Project or RDP database (Cole et al., 2014). Singletons were further removed to eliminate any potentially artefactual reads. All sequences obtained were deposited under the following GenBank SRA Accession numbers SRR3373200 and SRR3401136. Further, similarity percentage (SIMPER) analysis was performed using Primer 6 software package to identify bacterial taxa that explain most of the variation between S1S and S11B.

3. Results

Results indicated that the difference in heavy metal concentrations between upstream and downstream were not significant ($p < 0.05$) while the difference between water, SPM and sediment were highly significant (pseudo $F = 23.8$, $p = 0.001$) (Figure 2). The average concentrations of heavy metals in the SPM and sediment were significantly higher compared to that of water column in the estuary. The concentrations of SPM associated Zn ($808 \mu\text{g g}^{-1}$) and Cd ($2.7 \mu\text{g g}^{-1}$) were higher in the upstream, while Pb ($165 \mu\text{g gm}^{-1}$), Cu ($36 \mu\text{g gm}^{-1}$), Cr ($180 \mu\text{g gm}^{-1}$) and Ni ($48 \mu\text{g gm}^{-1}$) were higher in the downstream of the CE. The levels of sediment associated Zn ($209 \mu\text{g gm}^{-1}$), Cd ($1.3 \mu\text{g gm}^{-1}$), Pb ($7 \mu\text{g gm}^{-1}$) Cu ($12 \mu\text{g gm}^{-1}$), Ni ($43 \mu\text{g gm}^{-1}$), Mn ($169 \mu\text{g gm}^{-1}$) and Cr ($110 \mu\text{g gm}^{-1}$) were higher in the downstream of the estuary. On the contrary, the levels of dissolved Zn ($7 \mu\text{g L}^{-1}$), Cd ($0.2 \mu\text{g L}^{-1}$), Pb ($0.6 \mu\text{g L}^{-1}$), Cu ($1.2 \mu\text{g L}^{-1}$), Ni ($0.4 \mu\text{g L}^{-1}$), Mn ($0.4 \mu\text{g L}^{-1}$) and Cr ($0.3 \mu\text{g L}^{-1}$) were higher in the upstream of CE.

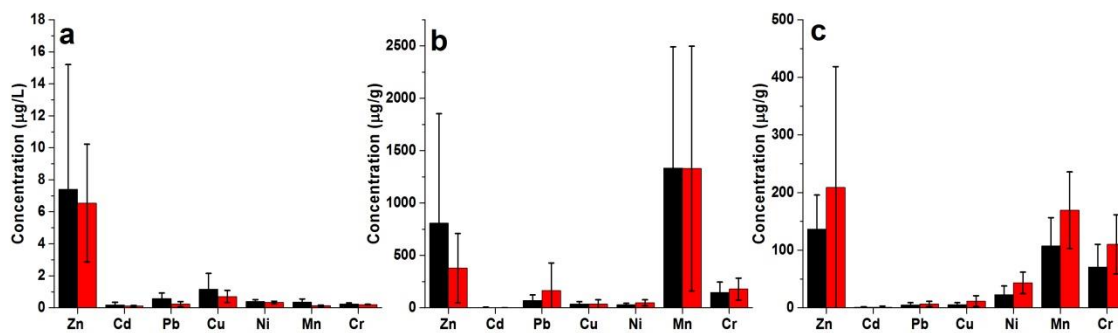


Figure 2: Heavy metal concentrations in water (a), suspended particulate matter (b) and sediment (c) collected from the upstream (black) and downstream (red) of Cochin estuary

The Illumina HiSeq sequencing efforts yielded a total of 90,357 and 492,037 quality paired-end reads, respectively from S1S and S11B. Out of 229,700 sequences that remained after the trimming, aligning, screening, around 90.9 % (208,878) sequences could be classified under the bacterial domain. Singleton removal resulted in a decrease of 20.7 % (36728) and 9.8 % (3067) in total quality reads from S11B and SIS, respectively. After standardizing each sample at 28,176 reads, some 5448 operational taxonomic units (OTUs) were estimated with 4651 and 864 OTUs for S11B and S1S, respectively (Table 1). The table also include estimated richness and diversity indices for both the samples. Rarefaction curve (Fig. 3) indicates a relatively

<i>Sample</i>	<i>OTUs</i> (<i>Richness</i>)	<i>Shannon</i> <i>Index</i> (<i>Diversity</i>)	<i>Chao1</i> (<i>Diversity</i>)
<i>S1S</i>	864	2.8	869.7
<i>S11B</i>	4651	5.7	8723.5

Table 1: Numbers of OTUs and diversity estimates of *Synechocystis* sp. isolated from polluted (S1S) and nonpolluted (S11B) regions of Cochin Estuary

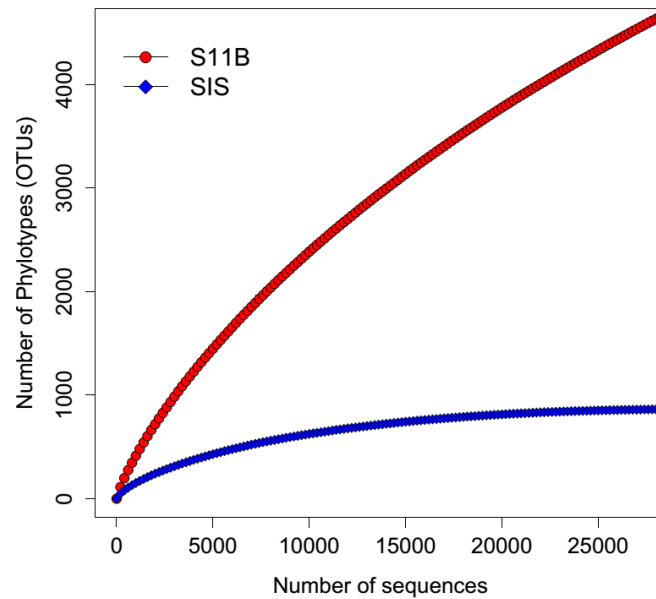


Figure 3: Rarefaction analysis of the bacteria associated with *Synechocystis* sp enriched from upstream (S1S) and downstream (S11B) of CE. The rarefaction curve was calculated for the 0.3 OTU definition (defined at $\geq 97\%$ sequence similarity level) based on pairwise distance.

higher diversity of heterotrophic bacteria associated with S11B, as indicated by the asymptotic nature of the curve compared with S1S. Both richness and diversity were also considerably higher in S11B (Shannon: 5.7) than S1S (Shannon: 2.8) (Table 1). The results revealed immense diversity of heterotrophic bacteria associated with cyanobacterium. Taken together, Proteobacteria emerged as the most abundant bacterial phylum with 74,114 sequences (35.5%), mostly due to acquisition of $>80\%$ sequences in S1S. However, Proteobacteria were only a distant second after Planctomycetes (37.4 %, 66,436 sequences) with only 48,812 sequences (27.5 %) in S11B. Interestingly, bacterial sequences which could not be placed under any phylum came out to be the third most abundant group in S11B (Fig. 4). The most abundant single OTU across S11B belonged to the order Phycisphaerales (Planctomycetes), represented by a total of 39,821 sequences accounting for approximately 22.4 % of total classifiable sequences whereas in S1S it was represented by an unclassified γ -proteobacterial OTU with 17,396 sequences (55.7%). We observed differences in proteobacterial classes associated to S1S and S11B (Fig. 5). S1S was dominated by γ -proteobacteria (73 %) followed by α -proteobacteria (21 %). In contrast, α -proteobacteria (77 %) was dominant in S11B followed by δ - proteobacteria (10 %).

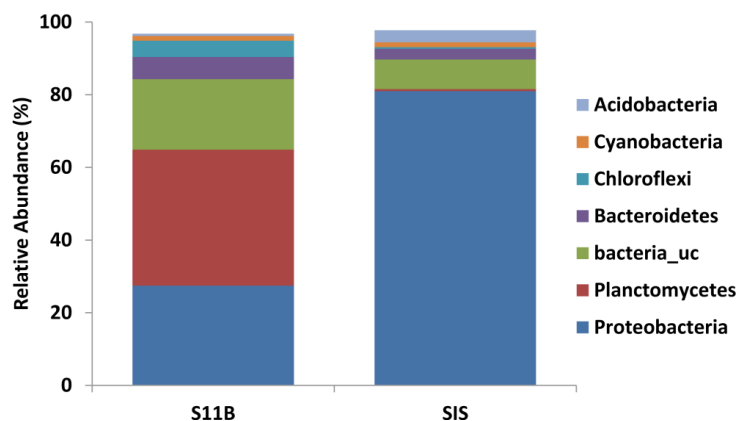


Figure 4: Bar chart showing percentage relative abundance of heterotrophic bacteria (Phylum level) associated with *Synechocystis* sp. enriched from upstream (S1S) and downstream (S11B) of CE.

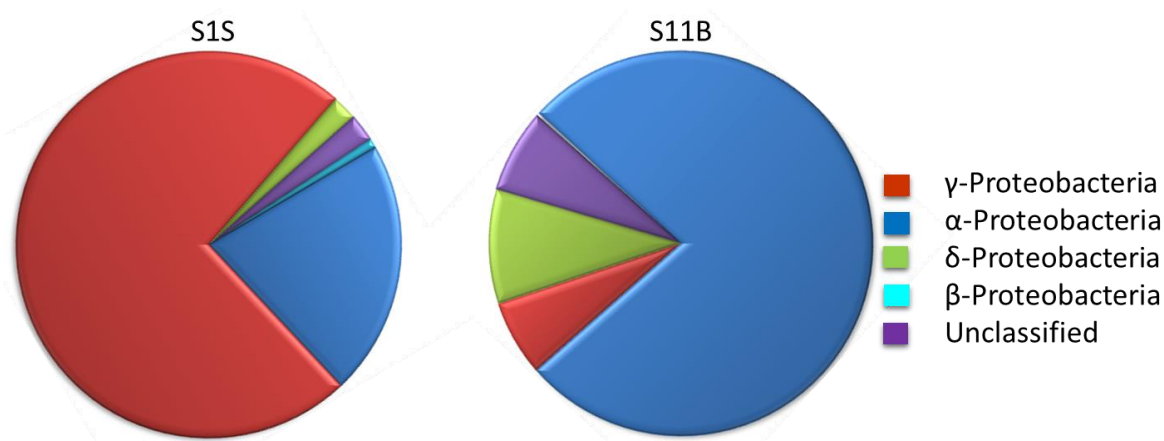


Figure 5: Pie diagrams illustrating the difference in the composition of Proteobacteria associated with *Synechocystis* sp enriched from upstream (S1S) and downstream (S11B) of CE

More than 90 % of the sequences could not be delineated at family and genus level (Suppl. Fig. 1; Suppl. Table 1). Majority of the sequences in S1S were grouped to an unclassified family under class γ -proteobacteria (55.7 %), while unclassified families under order Phycisphaerales (29.7%) and class α -proteobacteria (19.3 %) were dominant in S11B. Rhodospirillaceae (7.3%) and Chitinophagaceae (4.9 %) were the well defined families in the S11B and Rhodobacteriaceae (6.5 %) were the one in S1S. Several sequences which belonged to different families with individual contributions of less than 5%, together (labelled as others) contributed a significant share in S11B (28.9 %) and S1S (21.8 %).

We found 67 OTUs of heterotrophic bacteria common to S1S and S11B, which contributed to 7.8 % and 1.4 % of the total OTUs found in S1S and S11B respectively (Fig. 6). The shared OTUs found among S1S and S11B were; unclassified γ and α -proteobacteria,

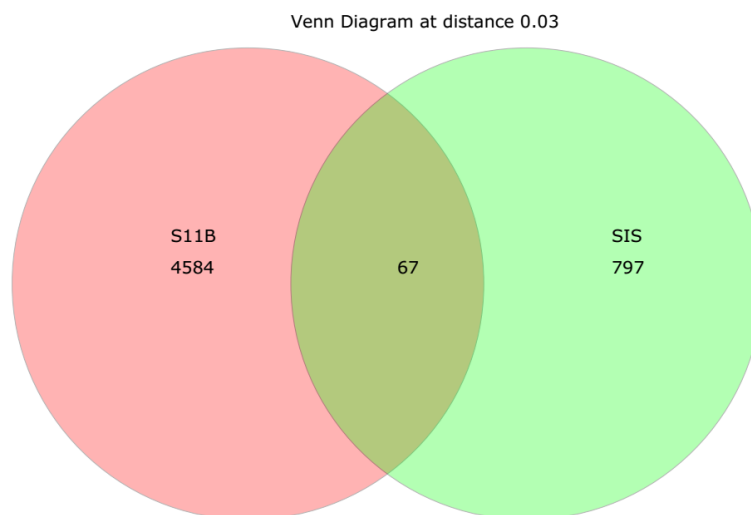


Figure 6: Venn diagrams of shared and unique operational taxonomic units (OTUs) of bacteria associated with *Synechocystis* sp enriched from upstream (S1S) and downstream (S11B) of CE.

Neisseria sp., *Prevotella* sp., *Streptococcus* sp., *Porphyromonas* sp, *Meiothermus* sp. and members of family Synergistaceae. On the other hand, unclassified members of γ and α -proteobacteria, *Thalassobaculum* sp., *Thermoanaerobaculum* sp. and *Marinobacter* sp. were exclusive to S1S. Major share of OTUs found exclusive to S11B were members of order Phycisphaerales of phylum Planctomycetes, family Chitinophagaceae, Rhodospirillaceae and Class Anaerolineae of Chloroflexi. The SIMPER analysis also confirmed γ -proteobacteria and Phycisphaerales of phylum Planctomycetes as the major contributors of the difference between S1S and S11B.

4. Discussion

Here we compared the microbiome of a photosynthetic unicellular cyanobacterium enriched from the upstream and downstream of a tropical estuary impacted with different levels of heavy metal pollution (Jose et al., 2011; Sheeba et al., 2017; Sheeba et al., 2020). Heavy metals are accumulated in the SPM and sediment in the CE as indicated by their higher concentration compared to water. The observed concentrations of heavy metals in sediments were one to two times higher in the downstream compared with the upstream. Interestingly, the concentrations of Zn, Cd and Mn were higher in the SPM collected from the upstream whereas Pb, Cu and Ni were higher in the downstream. The observed concentrations of Zn, Cd, Cu, Ni and Cr were 2-6 times higher in the SPM compared with the sediment, while Pb showed 10 to 25 times increase. Based on the distribution pattern of heavy metals in water,

SPM and sediment, it could be possible to infer the transportation pathway of heavy metals in the CE. The upstream of the estuary receives approximately 1000 m³s freshwater from river Periyar during the dry season, increasing to ~ 12000 m³s⁻¹ during monsoon (wet season). This huge volume of freshwater also carries a significant fraction of ~260 million liters of industrial effluents, rich in heavy metals, polycyclic aromatic hydrocarbons, and nutrients, into the upstream of the CE (Balachandran et al., 2005; Ramzi et al., 2017; Sheeba et al., 2017). Hence, we observed high concentration of dissolved heavy metals in the upstream compared with the downstream of the CE. The heavy metals that are accumulated in the SPM get transported towards the Barmouth of the estuary by water currents. Although the estuary flushes out ~43 times a year, the flushing is relatively slow in the dry season (~once a week)(Vinitha et al., 2015) allowing more time for settling of SPM and accumulation of heavy metals in the downstream of the CE. The accumulation of heavy metals in the sediment might have been taking place over a long period of time, for example a seventy-fold increase in the concentration of Zn has been recorded from sediments in the estuary over three decades (Jose et al., 2011). The accumulation of heavy metals in particulate matter has been reported from world estuaries (Ma et al., 2015; Sheeba et al., 2017). The particulate organic matter has a higher capacity to chelate heavy metals compared to sediments and it is found to be dependent on the biochemical composition of the POC (Quenea et al., 2009; Shi et al., 2018). Protein and carbohydrate components of particulate matter originating from phytoplankton are also found to accumulate Zn, Pb, Ni, Cr and Cu in CE (Sheeba et al., 2017). Phytoplankton forms a significant fraction of SPM in estuaries (Canuel and Hardison, 2016), and *Synechocystis* sp has been recorded as a predominant one in CE (Joseph and Saramma, 2016). The chemically-complicated extracellular polysaccharides (EPS) secreted by cyanobacterium form a mucilaginous micro-niche around the cell and support a plethora of other microorganisms (Pereira et al., 2009). The EPS functions as a cargo vehicle for the transport of metals from external to the internal environment of a micro-niche through charge-based sequestration. This sequestration supports cellular processes of the microbiome within the micro-niche of cyanobacterium under pristine conditions. However, it can become toxic in polluted environments due to the accumulation of toxic metals inside the micro-niche. Under such conditions, the microorganisms may shut down their contributions to ecosystems functioning, such as remineralization of organic matter, and spend most of the energy for maintaining their cellular processes. Although it is widely known that metal-resistant bacteria colonize polluted environments, the impact of heavy metal pollution at the micro-niche level has not been discussed until now. A contrasting diversity of microbiomes associated with cyanobacteria, S1S and S11B isolated from the upstream and downstream of the CE was observed in the present study. There are various host or environment driven factors that control the diversity of heterotrophic bacteria associated with cyanobacteria. Previous studies have focused on such

associations between heterotrophic bacteria and filamentous cyanobacteria such as *Anabaena*, *Nostoc* etc., and have reported the host-driven factors, such as oxygen minimum condition within the heterocyst, selecting the diversity of their heterotrophic bacterial symbionts (Kumar et al., 2009; Paerl, 1977). The difference in community structure of heterotrophic bacteria with the ageing of algal blooms has also been reported (Cai et al., 2014). However, the influence of anthropogenic inputs such as heavy metal pollution on the diversity and activities of the microbiome of cyanobacteria are rarely reported. It is estimated that the heavy metal pollution in the upstream of the CE has increased several fold in the last few decades and influenced the diversity and activities of both micro- and macro-organisms (Jose et al., 2011; Sheeba et al., 2017).

Although nearly 60 % of the species diversity of microbiome was common between S1S and S11B, there was a difference in the major groups of heterotrophic bacterial associates. Proteobacteria were not the dominant phylum in S11B, second only after Planctomycetes. Interestingly, unclassified bacterial sequences were the third most abundant group in S11B. There was also a difference in the composition of Proteobacteria between S1S and S11B with S1S being dominated by γ -proteobacteria while α -proteobacteria dominated the bacterial community in S11B. Generally, Proteobacteria are the ubiquitous group of bacteria found in marine environment and their strong association with cyanobacteria is reported. The evolutionary link between Proteobacteria and cyanobacteria is evident in the literature and many of the genes found in cyanobacteria are from Proteobacteria (Timmis et al., 2004). For example, recent reports of comparative genome analysis showed that 5 % of the genes found in *Synechocystis* sp. are of proteobacterial origin (Encinas et al., 2014). However, the difference in the dominant classes of Proteobacteria between S1S and S11B is interesting. Several species of Proteobacteria were also considered as dominant in sediments polluted with heavy metals and the gut of animals growing there (Chiellini et al., 2013; Gillan and Pernet, 2007). We consider this as a case of selection of the composition of microbiome by the host as an adaptation to protect itself from heavy metal toxicity. This argument is supported by our previous study that heterotrophic Proteobacteria isolated from *Synechocystis* sp. was highly resistant to heavy metals (Anas et al., 2016). Studies during the last few decades have classified the upstream of Cochin estuary as a polluted region in which Zn concentration reached up to $130 \mu\text{gL}^{-1}$ in water (Sheeba et al., 2017), 8.5 mgg^{-1} in the particulate matter (Sheeba et al., 2017) and 2.8 gKg^{-1} in sediment (Jose et al., 2011). It is possible that the metal resistant bacteria belonging to Proteobacteria must have colonized on the S1S isolated from the upstream of CE. In a previous study (Hattenrath-Lehmann and Gobler, 2017), a clear alteration in the assemblage of heterotrophic bacteria on the addition of cyanobacterial filtrate was observed, which strongly indicated the role of cyanobacteria in tuning their microbiome in response to environmental conditions.

The metal resistant *Marinobacter* sp., a γ -proteobacteria, isolated from *Synechocystis* sp. enriched from polluted regions of CE were found to produce higher levels of enzymes compared to those isolated from less polluted regions (Anas et al., 2016). The γ -proteobacteria might be protecting the *Synechocystis* sp. from toxic effects of heavy metals while maintaining their role in food web dynamics. This indicates the possible adaptation of *Synechocystis* sp. to the polluted condition of CE by alteration of their microbiome. The varying hydrography of the environment, in this case, heavy metal pollution, can influence the chemical characteristics of the phycosphere and cyanobacteria living inside it. This variation strongly influences the diversity of heterotrophic bacteria associated with the phycosphere (Cai et al., 2014; Shen et al., 2011).

The *Synechocystis* sp S11B enriched from the downstream also harbored a significant number of other groups of bacteria such as Planctomycetes, Bacterioidetes and uncultured bacteria. It is estimated that the Planctomycete contributed to more than 37 % of the abundance in S11B, while it was reduced to less than 1 % in S1S. Planctomycetes has been recorded as a major heterotrophic bacterial associate of macroalgae in nearly 30 % of all the studies reported (Lage and Bondoso, 2014), and also of cyanobacteria (Cai et al., 2013; Cai et al., 2014). It was reported in previous studies that Planctomycetes have metabolic capabilities to utilize the extracellular polysaccharides secreted by algae such as, sulfated polysaccharides, Laminarin, mannitol, pectin and N-acetyl glucosamine as a carbon source and hence colonize algae (Lage and Bondoso, 2014). Also, there is an evolutionary linkage between Planctomycetes and unicellular cyanobacteria, that in both organisms the ribosomes could be found bound to an internal membrane (Fuerst and Sagulenko, 2012). Further studies focusing on isolation and metal resistance properties of Planctomycetes are required to elucidate the reason for their reduced abundance in S1S.

The present study shows the accumulation of heavy metals in the particulate matter and sediments of a tropical estuary and discusses the possible role of host-microbe interaction in enabling the host to survive in contrasting environmental conditions. In this case, the composition of the microbiome of cyanobacteria enriched from the upstream and downstream of the estuary changes with different levels of heavy metal concentrations. The study also shows that heavy metal pollution can influence an estuarine ecosystem at the micro-niche level, as indicated by the disruption of the microbiome of photosynthetic unicellular cyanobacteria.

References

- Anas, A., Jasmin, C., Sheeba, V., Gireeshkumar, T., Nair, S., 2015. Heavy metals pollution influence the community structure of Cyanobacteria in nutrient rich tropical estuary. *Oceanography open access* 3, 137.
- Anas, A., Sageer, S., Jasmin, C., Vijayan, V., Pavanan, P., Athiyathil, S., Nair, S., 2016. Unicellular cyanobacteria *Synechocystis* accommodate heterotrophic bacteria with varied enzymatic and metal resistance properties. *Journal of Basic Microbiology* 56, 845 - 856.
- Balachandran, K.K., Lalu Raj, C.M., Nair, M., Joseph, T., Sheeba, P., Venugopal, P., 2005. Heavy metal accumulation in a flow restricted, tropical estuary. *Estuarine, Coastal and Shelf Science* 65, 361 - 370.
- Bao, Y., Liu, X., Zhang, W., Cao, J., Li, W., Li, C., Lin, Z., 2016. Identification of a regulation network in response to cadmium toxicity using blood clam *Tegillarca granosa* as model. *Scientific Reports* 6, 35704.
- Bartram, A.K., Lynch, M.D., Stearns, J.C., Moreno-Hagelsieb, G., Neufeld, J.D., 2011. Generation of multimillion-sequence 16S rRNA gene libraries from complex microbial communities by assembling paired-end illumina reads. *Applied and Environmental Microbiology* 77, 3846-3852.
- Berg, K.A., Lyra, C., Sivonen, K., Paulin, L., Suomalainen, S., Tuomi, P., Rapala, J., 2009. High diversity of cultivable heterotrophic bacteria in association with cyanobacterial water blooms. *The ISME Journal* 3, 314 - 325.
- Breton, J., Daniel, C., Vignal, C., Body-Malapel, M., Garat, A., Plé, C., Folligné, B., 2016. Does oral exposure to cadmium and lead mediate susceptibility to colitis? The dark-and-bright sides of heavy metals in gut ecology. *Scientific Reports* 6, 19200.
- Buchan, A., Gonzalez, J.M., Moran, M.A., 2005. Overview of the marine *Roseobacter* lineage. *Applied and Environmental Microbiology* 71, 5665 - 5677.
- Cai, H.-Y., Yan, Z.-s., Wang, A.-J., Krumholz, L.R., Jiang, H.-L., 2013. Analysis of the attached microbial community on mucilaginous cyanobacterial aggregates in the eutrophic lake Taihu reveals the importance of *Planctomycetes*. *Microbial Ecology* 66.
- Cai, H., Jiang, H., Krumholz, L.R., Yang, Z., 2014. Bacterial Community Composition of Size-Fractioned Aggregates within the Phycosphere of Cyanobacterial Blooms in a Eutrophic Freshwater Lake. *PLOS ONE* 9, e102879.
- Canuel, E.A., Hardison, A.K., 2016. Sources, Ages, and Alteration of Organic Matter in Estuaries. *Annual Review of Marine Science* 8, 409-434.
- Casey, J.M., Connolly, S.R., Ainsworth, T.D., 2015. Coral transplantation triggers shift in microbiome and promotion of coral disease associated potential pathogens. *Scientific Reports* 5, 11903.
- Chiellini, C., Iannelli, R., Verni, F., Petroni, G., 2013. Bacterial Communities in Polluted Seabed Sediments: A Molecular Biology Assay in Leghorn Harbor. *The Scientific World Journal* 2013, 165706.
- Cole, J.J., 1982. Interactions between bacteria and algae in aquatic ecosystems. *Annual Review of Ecological Systems* 13, 291- 314.

- Cole, J.R., Wang, Q., Fish, J.A., Chai, B.L., McGarrell, D.M., Sun, Y.N., Brown, C.T., Porras-Alfaro, A., Kuske, C.R., Tiedje, J.M., 2014. Ribosomal Database Project: data and tools for high throughput rRNA analysis. *Nucleic Acids Research* 42, D633-D642.
- Cornejo-Granados, F., Lopez-Zavala, A.A., Gallardo-Becerra, L., Mendoza-Vargas, A., Sánchez, F., Vichido, R., Brieba, L.G., Viana, M.T., Sotelo-Mundo, R.R., Ochoa-Leyva, A., 2017. Microbiome of Pacific Whiteleg shrimp reveals differential bacterial community composition between Wild, Aquacultured and AHPND/EMS outbreak conditions. *Scientific Reports* 7, 11783.
- Delucca, R., McCracken, M.D., 1977. Observations on interactions between naturally collected bacteria and several species of algae. *Hydrobiologia* 55, 71 - 75.
- Edgar, R.C., Haas, B.J., Clemente, J.C., Quince, C., Knight, R., 2011. UCHIME improves sensitivity and speed of chimera detection. *Bioinformatics* 27, 2194-2200.
- Encinas, D., Garcillán-Barcia, M.P., Santos-Merino, M., Delaye, L., Moya, A., de la Cruz, F., 2014. Plasmid Conjugation from Proteobacteria as Evidence for the Origin of Xenologous Genes in Cyanobacteria. *Journal of Bacteriology* 196, 1551-1559.
- Falcon, L.I., Magallon, S., Castillo, A., 2010. Dating the cyanobacterial ancestor of the chloroplast. *The ISME Journal* 4, 777 - 783.
- Fuerst, J.A., Sagulenko, E., 2012. Keys to eukaryality: Planctomycetes and ancestral evolution of cellular complexity. *Frontiers in Microbiology* 3, 1 -12.
- Gillan, D.C., Pernet, P., 2007. Adherent bacteria in heavy metal contaminated marine sediments. *Biofouling* 23, 1-13.
- Hattenrath-Lehmann, T.K., Gobler, C.J., 2017. Identification of unique microbiomes associated with harmful algal blooms caused by *Alexandrium fundyense* and *Dinophysis acuminata*. *Harmful Algae* 68, 17-30.
- Hess, W.R., 2004. Genome analysis of marine photosynthetic microbes and their global role. *Current Opinion in Biotechnology* 15, 191 - 198.
- Hube, A.E., Heyduck-Söller, B., Fischer, U., 2009. Phylogenetic classification of heterotrophic bacteria associated with filamentous marine cyanobacteria in culture. *Systematic and Applied Microbiology* 32, 256-265.
- Jose, J., Giridhar, r., Anas, A., Loka Bharathi, P.A., Nair, S., 2011. Heavy metal pollution exerts reduction/ adaptation in the diversity and enzyme expression profile of heterotrophic bacteria in Cochin estuary, India. *Environmental Pollution* 159, 2775 - 2780.
- Joseph, S., Saramma, A.V., 2016. Species diversity of Cyanobacteria in Cochin estuary. *Journal of Marine Biological Association of India* 58, 55 - 63.
- Kozich, J.J., Westcott, S.L., Baxter, N.T., Highlander, S.K., Schloss, P.D., 2013. Development of a Dual-Index Sequencing Strategy and Curation Pipeline for Analyzing Amplicon Sequence Data on the MiSeq Illumina Sequencing Platform. *Applied and Environmental Microbiology* 79, 5112-5120.
- Kumar, K., Mella-Herrera, R.A., Golden, J.W., 2009. Cyanobacterial Heterocysts, in: Shapiro, L., Losick, R. (Eds.), *Additional perspectives on cell biology of bacteria*. Cold Spring Harbor, p. a000315.

- Lage, O.M., Bondoso, J., 2014. Planctomycetes and macroalgae, a striking association. *Frontiers in Microbiology* 5, 267.
- Limei, S., Yuanfeng, C., Hualin, Y., Peng, X., Pengfu, L., Lingdong, K., Fanxiang, K., 2009. Phylogenetic diversity and specificity of bacteria associated with *Microcystis aeruginosa* and other cyanobacteria. *Journal of Environmental Science* 21, 1581 - 1590.
- Loring, D.H., Rantala, R.T.T., 1992. Manual for the geochemical analyses of marine sediments and suspended particulate matter. *Earth- Science Reviews* 32, 235-283.
- Ma, Y., Qin, Y., Zheng, B., Zhang, L., Zhao, Y., 2015. Seasonal variation of enrichment, accumulation and sources of heavy metals in suspended particulate matter and surface sediments in the Daliao river and Daliao river estuary, Northeast China. *Environmental Earth Sciences* 73, 5107-5117.
- Masella, A.P., Bartram, A.K., Truszkowski, J.M., Brown, D.G., Neufeld, J.D., 2012. PANDAseq: PAired-eND Assembler for Illumina sequences. *Bmc Bioinformatics* 13.
- McDonald, D., Price, M.N., Goodrich, J., Nawrocki, E.P., DeSantis, T.Z., Probst, A., Andersen, G.L., Knight, R., Hugenholtz, P., 2012. An improved Greengenes taxonomy with explicit ranks for ecological and evolutionary analyses of bacteria and archaea. *ISME Journal* 6, 610-618.
- McLusky, D.S., 1989. Estuarine pollution, The estuarine ecosystem. Tertiary level biology. Springer, Dordrecht.
- Muhling, M., Fuller, N.J., Millard, A., Paul, J.S., Marie, D., Wilson, W.H., Scanlan, D.J., Post, A.F., Joint, I., Mann, N.H., 2005. Genetic diversity of marine *Synechococcus* and co-occurring cyanobophage communities: evidence for viral control of phytoplankton. *Environmental Microbiology* 7, 499 - 508.
- Paerl, H.W., 1976. Specific associations of the blue green algae *Anabaena* and *Aphanizomenon* with bacteria in freshwater blooms. *Journal of Phycology* 12, 431 - 435.
- Paerl, H.W., 1977. Role of heterotrophic bacteria in promoting N₂ fixation by *Anabaena* in aquatic habitats. *Microbial Ecology* 4, 215-231.
- Pereira, S., Zille, A., Micheletti, E., Moradas-Ferreira, P., De Philippis, R., Tamagnini, P., 2009. Complexity of cyanobacterial exopolysaccharides: composition, structures, inducing factors and putative genes involved in their biosynthesis and assembly. *FEMS Microbiology Reviews* 33, 917-941.
- Pereira, S.B., Mota, R., Vieira, C.P., Vieira, J., Tamagnini, P., 2015. Phylum-wide analysis of genes/proteins related to the last steps of assembly and export of extracellular polymeric substances (EPS) in cyanobacteria. *Scientific Reports* 5, 14835.
- Pinto, E., Sigaud-kutner, T.C.S., Leitão, M.A.S., Okamoto, O.K., Morse, D., Colepicolo, P., 2003. Heavy metal-induced oxidative stress in algae. *Journal of Phycology* 39, 1008-1018.
- Price, G.D., Badger, M.R., Woodger, F.J., Long, B.M., 2008. Advances in understanding the cyanobacterial CO₂-concentrating-mechanism (CCM): functional components, Ci transporters, diversity, genetic regulation and prospects for engineering into plants, pp. 1441-1461.
- Quenea, K., Lamy, I., Winterton, P., Bermond, A., Dumat, C., 2009. Interactions between metals and soil organic matter in various particle size fractions of soil contaminated with waste water. *Geoderma* 1, 217 - 223.

- Ramzi, A., Habeeb Rahman, K., Gireeshkumar, T.R., Balachandran, K.K., Chacko, J., Chandramohankumar, N., 2017. Dynamics of polycyclic hydrocarbons (PAHs) in surface sediments of Cochin estuary, India. *Marine Pollution Bulletin* 114, 1081 - 1087.
- Salomon, P.S., Janson, S., Graneli, E., 2003. Molecular identification of bacteria associated with filaments of *Nodularia spumigena* and their effect on the cyanobacterial growth. *Harmful Algae* 2, 261 - 272.
- Sheeba, V.A., Anas, A., Gireeshkumar, T.R., Ram, A., Rakesh, P.S., Jasmin, C., Parameswaran, P.S., 2017. Role of heavy metals in structuring the microbial community associated with particulate matter in a tropical estuary. *Environmental Pollution* 231, 589 - 600.
- Sheeba, V.A., Anas, A., Jasmin, C., Vincent, M., Parameswaran, P.S., 2020. Response of particle-associated bacteria to long-term heavy metal contamination in a tropical estuary. *World Journal of Microbiology and Biotechnology* 36, 65.
- Shen, H., Niu, Y., Xie, P., Tao, M., Yang, X., 2011. Morphological and physiological changes in *Microcystis aeruginosa* as a result of interactions with heterotrophic bacteria. *Freshwater Biology* 56, 1065 - 1080.
- Shi, J., Wu, Q., Zheng, C., Yang, J., 2018. The interaction between particulate organic matter and copper, zinc in paddy soil. *Environmental Pollution* 243, 1394-1402.
- Smith, G.R., Windom, H.L., 1972. Analytical handbook for the determination of Ar,Cd,Co,Cu,Fe,Pb,Mn,Hg,Ni,Ag and Zn in the marine and estuarine Environments
- Timmis, J.N., Ayliffe, M.A., Huang, C.Y., Martin, W., 2004. Endosymbiotic gene transfer: Organelle genomes forge eukaryotic chromosomes. *Nature Reviews* 5, 123 - 135.
- Vinitha, J., Shivaprasad, A., Revichandran, C., Manoj, N.T., Muraleedharan, K.R., Binzy, J., 2015. Salinity response to seasonal runoff in a complex estuarine system (Cochin estuary, west coast of India). *Journal of Coastal Research* 31, 869 - 878.
- Zyadah, M.A., Abdel-Baky, T.E., 2000. Toxicity and Bioaccumulation of Copper, Zinc, and Cadmium in Some Aquatic Organisms. *Bulletin of Environmental Contamination and Toxicology* 64, 740-747.

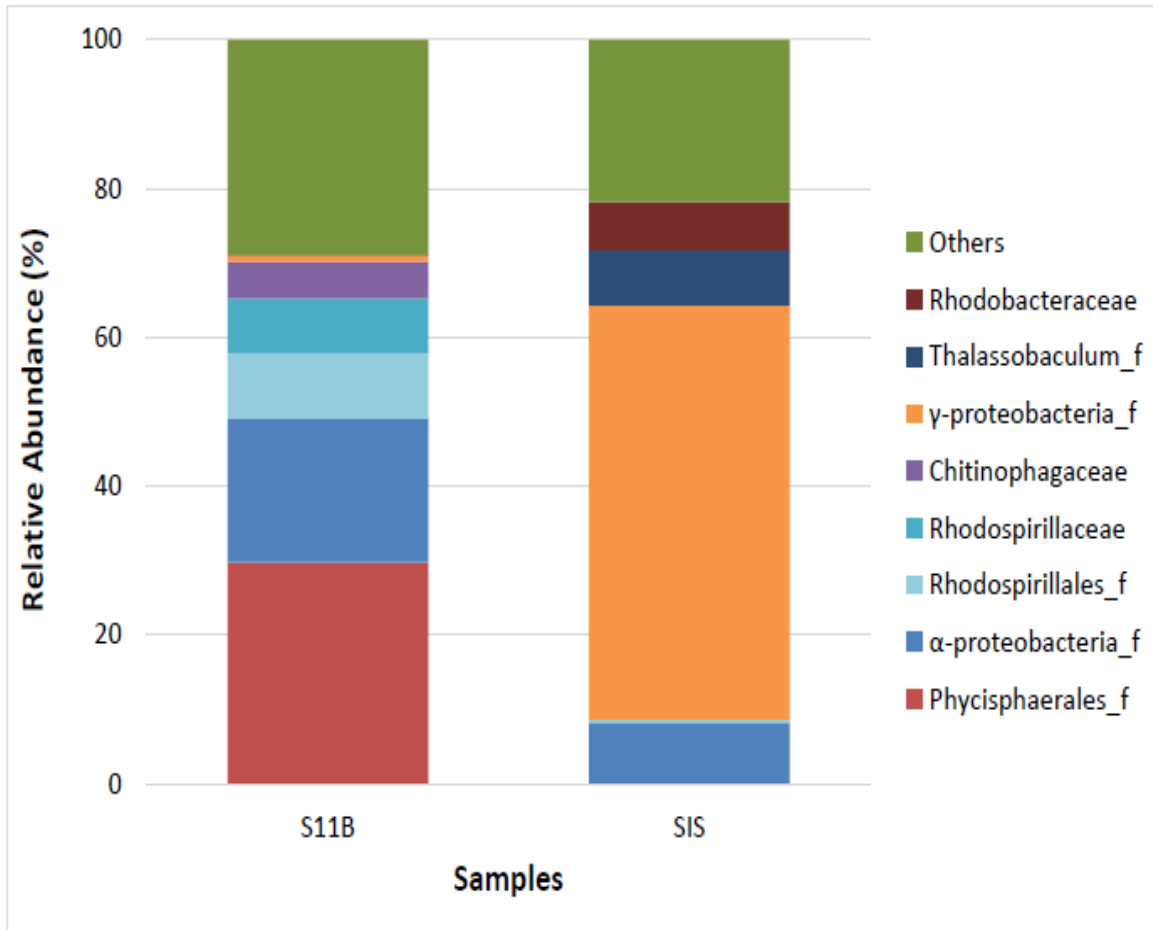
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Conflicts Of Interest

The authors declare no conflict of interest.

Supplementary figure 1: Bar chart showing percentage relative abundance of heterotrophic bacteria (family level) associated with *Synechocystis* sp. enriched from upstream (S1S) and downstream (S11B) of CE



Supplementary table 1: Percentage relative abundance of heterotrophic bacteria (Genus level) associated with *Synechocystis* sp. enriched from upstream (S1S) and downstream (S11B) of CE.

Genus	Relative abundance (%)	
	S11B	SIS
Nitrospira	0.76	0.00
Planctomyces	0.00	0.19
Rhodopirellula	0.00	0.01
Aquisphaera	0.00	0.00
Bartonella	0.00	0.00
Bauldia	0.00	0.00
Devosia	0.00	0.00
Hyphomicrobium	0.00	0.04
Methyloceanibacter	0.00	0.01
Methyloligella	0.00	0.03
Methylobacterium	0.00	0.00
Parvibaculum	0.00	0.60
Pseudaminobacter	0.00	0.00
Labrenzia	0.00	0.01
Henriciella	0.00	0.02
Roseovarius	0.00	0.01
Tistlia	0.00	0.00
Pelagibius	0.24	0.00
Thalassobaculum	0.01	7.12
Novosphingobium	0.00	0.00
Sphingomonas	0.00	0.00
Brevundimonas	0.00	0.00
Phenylobacterium	0.00	0.00
Neisseria	0.02	0.46
Burkholderia	0.00	0.00
Geobacter	0.00	0.00
Haliangium	0.01	0.00
Campylobacter	0.00	0.02
Helicobacter	0.00	0.00
Escherichia	0.00	0.00
Succinatimonas	0.00	0.00
Alcanivorax	0.00	0.32
Halomonas	0.00	0.00
Pasteurella	0.00	0.00
Moraxella	0.00	0.04
Psychrobacter	0.00	0.00
Acinetobacter	0.00	0.02
Pseudomonas	0.00	0.01
Oceanococcus	0.00	0.04

Algiphilus	0.64	0.01
Fontimonas	0.00	0.01
Spongiibacter	0.01	0.02
Marinobacter	0.00	2.51
Pseudoalteromonas	0.00	0.00
Alishewanella	0.00	0.00
Povalibacter	0.00	0.00
Steroidobacter	0.00	0.00
Thiomicrospira	0.00	0.03
Vibrio	0.00	0.00
Pseudoxanthomonas	0.00	0.02
Rhodanobacter	0.03	0.10
Stenotrophomonas	0.00	0.00
Vulcaniibacterium	0.00	0.00
Cardiobacterium	0.00	0.00
Leptonema	0.01	0.00
Treponema	0.00	0.02
Treponema_g1	0.00	0.02
Fretibacterium	0.00	0.02
Saccharimonas	0.00	0.00
Mycoplasma_g4	0.00	0.00
Fervidobacterium	0.00	0.00
Pedosphaera	0.00	0.01
Akkermansia	0.00	0.00
Paludibaculum	0.00	0.00
Solibacter	0.00	0.00
Koribacter	0.00	0.01
Blastocatella	0.00	0.01
Ilumatobacter	0.00	0.03
Solirubrobacter	0.00	0.00
Actinomyces	0.00	0.02
Brachybacterium	0.00	0.00
Microbacterium	0.00	0.00
Rothia	0.00	0.00
Arthrobacter	0.00	0.00
Kocuria	0.00	0.00
Brevibacterium	0.00	0.00
Couchioplanes	0.00	0.00
Nocardioides	0.00	0.00
Propionibacterium	0.00	0.02
Bifidobacterium	0.00	0.00
Corynebacterium	0.01	0.03
Mycobacterium	0.02	0.00
Geodermatophilus	0.00	0.00
Euzebya	0.00	0.01
Flavisolibacter	0.00	0.00

Lewinella	0.03	0.01
Pedobacter_g1	0.00	0.00
Sphingobacterium	0.00	0.00
Phocaeicola	0.00	0.00
Barnesiella	0.00	0.00
Parabacteroides	0.00	0.00
Porphyromonas	0.01	0.15
Tannerella	0.00	0.01
Prevotella	0.04	0.66
Alloprevotella	0.01	0.14
Alistipes	0.00	0.00
Bacteroides	0.08	0.02
Gracilimonas	0.00	0.15
Flexithrix	0.14	0.00
Ekhidna	0.11	0.00
Owenweeksia	0.00	0.01
Muricauda	0.08	0.03
Winogradskyella	0.00	0.00
Capnocytophaga	0.00	0.06
Aphanothece	1.18	0.00
Merismopedia	0.00	0.00
Prochlorococcus	0.00	0.01
Mucispirillum	0.00	0.00
Meiothermus	0.07	0.21
Clostridium_g6	0.00	0.00
Brevibacillus	0.00	0.00
Staphylococcus	0.00	0.00
Planifilum	0.00	0.00
Bacillus	0.14	0.01
Gemella	0.00	0.07
Dolosigranulum	0.01	0.01
Lactobacillus	0.00	0.00
Streptococcus	0.01	0.25
Clostridium	0.00	0.00
Filifactor	0.00	0.00
Helcococcus	0.00	0.00
Peptoniphilus	0.00	0.00
Anaerococcus	0.00	0.00
Hungatella	0.00	0.00
Johnsonella	0.00	0.00
Lachnoanaerobaculum	0.00	0.01
Moryella	0.00	0.01
Oribacterium	0.00	0.00
Shuttleworthia	0.00	0.00
Catonella	0.00	0.01
Peptostreptococcus	0.00	0.01

Oscillibacter	0.00	0.00
Pseudoflavonifractor	0.00	0.00
Subdoligranulum	0.00	0.00
Clostridium_g25	0.00	0.00
Tepidimicrobium	0.00	0.00
Dialister	0.00	0.01
Megamonas	0.00	0.01
Megasphaera	0.00	0.01
Phascolarctobacterium	0.00	0.00
Selenomonas_g1	0.00	0.04
Veillonella	0.00	0.29
Halocella	0.00	0.00
Fusobacterium	0.00	0.14
Leptotrichia	0.00	0.10
unclassified	96.26	85.70