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Supplementary material for this article is available [online](#)

Abstract

The Bay of Bengal receives nitrogen inputs from multiple sources and the potential role of nitrogen-metabolizing microbial communities in the surface water is not well understood. The nitrogen budget estimate shows a deficit of $4.7 \pm 2.4 \text{ Tg N yr}^{-1}$, suggesting a significant role of dissolved organic nitrogen remineralization in fuelling ecosystem processes. Unravelling the process of remineralization leading to increasing concentrations of dissolved inorganic nitrogen (DIN) in coastal ecosystems such as in mangroves require a better understanding of the composition of functional resident bacterioplankton communities. Bacterioplankton communities were elucidated from eight stations along different estuaries spanning west to east of northeast coastal Bay of Bengal to understand the influence of DIN on shaping these communities. The eight stations were differentiated into 'low' and 'high' DIN stations based on DIN concentration, with five stations with High DIN concentration ($>45 \mu\text{M}$) and three stations with Low DIN concentration ($<40 \mu\text{M}$). The V3–V4 region of 16S rRNA was amplified and sequenced to elucidate resident bacterioplankton community structure from environmental DNA. Proteobacteria, Bacteroidetes, and Firmicutes were the dominant bacterioplankton phyla across all stations. Nitrogen-fixing groups such as Nitrospirae, Lentisphaerae, Chloroflexi, and Planctomycetes make up about 1% of the bacterioplankton communities. Abundances of Spirochaetes and Tenericutes showed a positive correlation with DIN. Pseudomonadales, Alteromonadales, and Desulfovibrionales were found to distinctly vary in abundance between Low and High DIN stations. Predicted metagenomic profiles from taxonomically derived community structures indicated bacterial nitrate-nitrite reductase to be negatively correlated with prevalent DIN concentration in High DIN stations but positively correlated in Low DIN stations. This trend was also consistent for genes encoding for nitrate/nitrite response regulators and transporter proteins. This indicates the need to delineate functional bacterioplankton community structures to better understand their role in influencing rates and fluxes of nitrogen within mangroves.

Introduction

Nitrogen is essential for all forms of life and is usually present in bioavailable forms including ammonium, nitrite, and nitrate in the sediment and aquatic environments. Dissolved inorganic nitrogen (DIN) is usually the

most abundant bioavailable form of nitrogen in coastal oceans (Veuger *et al* 2004). Limitation in DIN pool influences primary productivity in estuaries and coastal marine ecosystems across temperate and tropical zones (Corredor *et al* 1999, Howarth and Marino, 2006). As nitrogen limits photosynthetically driven primary production in many coastal estuaries, enhanced supply can cause major water quality issues including eutrophication, formation of hypoxic zones, in addition to harmful algal blooms (Burke *et al* 2000, Rabalais, 2002).

Changes in agricultural practices and rapid urbanization have significantly increased the export of nitrogen from rivers into coastal ecosystems (Nixon *et al* 1996, Petrone 2010, Kaushal *et al* 2014). As a result, river-estuarine hydrology can play a crucial role towards increasing the nitrogen load in coastal estuaries (Boynton *et al* 2008). Riverine inputs of DIN pool are usually depleted by numerous processes including denitrification and burial in sediment (Nixon *et al* 1996). Specialized coastal ecosystems such as estuarine mangroves further regulate the export of river-borne nutrients through additional processes including rapid uptake and transformation by resident microbial communities (Wang *et al* 2021). Concentrations of dissolved inorganic nutrients including nitrogen in mangrove environments are controlled by local hydrodynamics, freshwater input, tidal amplitude, and biological activities (Guerrero *et al* 1988, Ovalle *et al* 1990, Alongi *et al* 1992, Bava and Seralathan, 1999). High retention and recycling of nutrients within the mangrove ecosystems results in limited nutrient export, and the adjacent waters are thereby characterized by low inorganic nutrient concentrations (Kristensen *et al* 1995). Mangrove litterfall also acts as a source of nitrogen to coastal waters (Ghosh and Bhadury 2022). Mangrove forests are hence considered either a source or a sink of different forms of nitrogen (inorganic or organic and particulate or dissolved) over a seasonal cycle (Dittmar and Lara, 2001, Valiela *et al* 2018).

Inorganic nutrient dynamics in coastal water are controlled by a set of local factors including differences in size and width of estuaries, creeks, and shape of islands within mangrove ecosystems. Such factors can ultimately influence the fluxes of nutrients and rates of transformation (Alongi *et al* 1992, Dham *et al* 2002). Litterfall from mangrove vegetation, release from sediment, river runoff, groundwater seepage, and pore water exchange can lead to release of forms of nitrogen to estuarine waters (Dittmar 1999, Inoue *et al* 2011, Mandal *et al* 2013, Sadat-Noori *et al* 2017, Ghosh and Bhadury 2022). Biological community dynamics including phytoplankton and bacterioplankton productivity in estuarine mangroves are strongly influenced by concentration of dissolved inorganic nutrients and thereby highlight the importance of mapping spatial and temporal dynamics of dissolved nutrients in this ecosystem (Harrison *et al* 1997, Prasad *et al* 2006). Phytoplankton convert DIN into biomass but bacterioplankton are also thought to assimilate DIN owing to their low C: N ratio (Middelburg and Nieuwenhuize 2000). Heterotrophic bacteria can utilize a variety of nitrogenous compounds including ammonium for growth (Keil and Kirchman 1991); approximately 50% of ammonium in seawater is taken up by bacteria (Fuhrman *et al* 1988 Kirchman *et al* 1989). Several studies have collectively shown that bacterial communities acquire significant fractions of their nitrogen requirements from inorganic available forms of the element (Cotner and Wetzel 1992, Hoch and Kirchman 1995, Jansson *et al* 1996, Thingstad *et al* 1998). In addition to responding directly to inorganic nitrogen enrichment, remineralization and transformation of dissolved organic nitrogen is also a significant driver of bacterioplankton growth and influencing the structure of communities (Goldberg *et al* 2017). Hence, there is a need for functional profiling of genes involved in nitrogen transformation in aquatic ecosystems including in coastal oceans.

Such spatial dynamics of dissolved nitrogen concentrations and the influence on bacterioplankton communities can be ideally studied along the northeast coast of the Bay of Bengal which encompasses the Sundarbans mangroves. Nitrogen budget estimates show an overall N loss of $7.9 \pm 0.6 \text{ Tg N yr}^{-1}$ and N input of $3.15 \pm 2.25 \text{ Tg N yr}^{-1}$ from sources other than N_2 fixation indicating a deficit of $4.7 \pm 2.4 \text{ Tg N yr}^{-1}$ (Löscher *et al* 2019). Previous studies have estimated nitrogen input from N_2 fixation at 1 Tg N yr^{-1} (Naqvi *et al* 2010) hinting towards the potential role of diatoms and diazotrophic communities making this region a potential site of active N_2 fixation. But the large deficit seen in the N budget indicates the possible involvement of other members of the biological communities including bacterioplankton in mediating nitrogen cycling.

The Ganga-Brahmaputra-Meghna Rivers flow into the coastal Bay of Bengal forming a long coastline that houses the Sundarbans (figure 1). Sundarbans is a UNESCO World Heritage Site and a Ramsar Site; home to rich biodiversity including habitat for numerous charismatic fauna including the mangrove horseshoe crab. A large freshwater flow from the complex riverine systems along with saline water inflow from the coastal Bay of Bengal results in typical estuarine conditions within this mangrove. The Indian part of Sundarbans has several large estuaries distributed across 266 kms from the mouth of the River Hooghly to the western border of the River Meghna. Except for the Hooghly and Mooriganga in the west, estuaries formed by north-south flowing rivers are the Saptamukhi, Thakuran, Matla, Bidyadhari, Gomdi, Gosaba, Gona, Harinbanga, and Raimangal in Indian part of the Sundarbans. These estuaries have almost lost their upstream riverine connections due to heavy siltation. These rivers are primarily influenced by diurnal tides entering from the coastal Bay of Bengal but also receive heavy freshwater flow during southwest monsoon (July to September). Freshwater flow thereby strongly



Figure 1. The site map showing the sampling stations located in the northeast coastal Bay of Bengal. Junput ($21^{\circ} 42' 3.2''\text{N}$, $87^{\circ} 47' 57.1''\text{E}$) is located along the Pichaboni estuary. Stn1 ($21^{\circ} 44' 44.4''\text{N}$, $88^{\circ} 08' 49.5''\text{E}$) is located on Chemaguri creek and Stn3 ($21^{\circ} 40' 40.6''\text{N}$, $88^{\circ} 09' 19.2''\text{E}$) is located on Mooriganga estuary and lie in the outer Sundarbans. SBR_S1 ($21^{\circ} 53' 21.99''\text{N}$, $88^{\circ} 34' 38.76''\text{E}$) and SBR_S6 ($21^{\circ} 35' 17.23''\text{N}$, $88^{\circ} 32' 17.60''\text{E}$) are located on Thakuran estuary. SBR_S2 ($22^{\circ} 05' 15.70''\text{N}$, $88^{\circ} 45' 55.60''\text{E}$) is located on the Matla estuary. SBR_S3 ($21^{\circ} 55' 34.85''\text{N}$, $89^{\circ} 01' 54.08''\text{E}$) and SBR_S4 ($22^{\circ} 07' 28.59''\text{N}$, $88^{\circ} 59' 48.39''\text{E}$) are located on Harinbhanga estuary. The SBR stations lie within the Sundarbans Biosphere Reserve. The High DIN stations are marked with blue pins and Low DIN stations are marked with pink pins.

controls inorganic nutrients run-off from the surrounding land and upstream regions. Moving eastward from the Hooghly estuary, the land is considered pristine owing to largely limited human activities. Such rapid changes in hydrological parameters across this region are mirrored in dynamic changes in resident estuarine microbial communities (Sun *et al* 2012). Typically, diverse microbial communities in estuaries are strongly affected by hydrological conditions, and show substantial spatio-temporal heterogeneity (Crump *et al* 2007, Fortunato *et al* 2012). In addition to natural variations, anthropogenic influences such as increased nutrient flow can also result in rapid changes in microbial communities and their functions which in turn impact the health of the estuaries. Changes in the relative abundances of specific taxa, or abundance of functional genes, can hence be indicative of dissolved nutrient dynamics within these estuaries.

The northeast coastal Bay of Bengal displays multiple habitats within a short geographical distance and such ecosystem-level variations primarily result from high freshwater flow, dense human presence in the west in contrast to pristine mangrove forests in the east, among others. We hypothesize that the DIN pool would strongly co-vary within the ecosystem and thereby control the structure of resident bacterioplankton communities and functions. Against this backdrop, the objectives of this study were: (1) to elucidate variations in bacterioplankton community composition in estuaries with contrasting low and high dissolved inorganic nitrogen (DIN) concentrations during monsoon season (July to September), and (2) to explore variations in predicted functional genes that may impact DIN concentrations in northeast coastal Bay of Bengal.

Materials and methods

Study site

The study was conducted across eight stations located along a ~140 km stretches facing the northeast coastal Bay of Bengal (figure 1). All the studied stations have intermediate average salinities ranging between 10–15. The stations are shallow (1–3 m depth in the low tide; the tidal amplitude of 3–4 m) and have unusually high suspended particulate matter (SPM) load ($200\text{--}700\text{ mg l}^{-1}$) due to continuous resuspension of underlying sediment. The westernmost station is located in the Junput town which lies along the bank of Pichaboni estuary. Junput is a sandy clay beach lined by planted *Casuarina* trees. There are some nearby villages with substantial agricultural and aquaculture activities close to Junput. The site is actively used for fishing involving small boats and trawlers and is also an important breeding ground for two species of horseshoe crabs (*Tachypleus gigas* and *Carcinoscorpius rotundicauda*). The Pichaboni River along with anthropogenic activities in the nearby villages including fishing represent active sources of anthropogenic nutrients in the Junput sampling station. Junput marks the western border of the River Hooghly. On the eastern border of River Hooghly lies the largest island of

the Indian Sundarbans, Sagar Island. Two predefined stations, Stn1 and Stn3, lie in Chemaguri creek and Mooriganga estuary respectively, and are part of the Sundarbans Biological Observatory Time Series (SBOTS) (Bhattacharjee *et al* 2013, Samanta and Bhadury 2015, Choudhury *et al* 2015). Chemaguri creek originates in the River Hooghly, transverses the island, and opens into the Mooriganga River. It is a shallow macrotidal creek that increases in depth from ~1 m to ~4 m during diurnal high tides. Stn1 is surrounded by planted mangroves, and nutrients originating from both agriculture and aquaculture farms heavily affect this station (Choudhury *et al* 2015). Stn3 is located on the Mooriganga estuary and has an average depth of ~4 m which increases to ~7 m during diurnal high tide. Located ~1 km away from the coastal Bay of Bengal, this station receives huge amount of freshwater from the Mooriganga estuary, which then mixes with marine water entering from the Bay of Bengal. Large volumes of nutrients, sediments, urban sewage including organic waste, and industrial run-off from the Mooriganga River represent sources of nutrients into this station. Owing to its location, Stn3 receives more saline water from the coastal Bay of Bengal compared to Stn1. Both stations of SBOTS have been monitored every month since 2010, and all studied environmental parameters have been compared annually. Five stations were sampled within the Sundarbans Biosphere Reserve (SBR) which are all surrounded by dense mangrove forests. Two stations, SBR_S1 and SBR_S6 are located on the Thakuran estuary, and SBR_S2 is located in the adjacent Matla estuary. Two stations, SBR_S3 and SBR_S4 are located on the Harinbhanga estuary. The rivers Thakuran, Matla, and Harinbhanga have lost their upward freshwater connection with the River Ganga due to heavy siltation over the years. These rivers only receive freshwater from seasonal precipitation, with saline water coming from the coastal Bay of Bengal. The SBR stations lie within the heavily protected as part of Sundarbans Biosphere Reserve and are not accessible to sampling throughout the year.

Sampling

Sampling was conducted during the monsoon season across all the studied stations: at Stn1 and Stn3 in July 2014, in the five SBR stations in August 2015, and Junput in August 2016. The collection was done approximately 2 h after the highest high tide. Bacterioplankton communities were elucidated from 1 L of surface water samples collected from each of the studied stations following published protocols (Choudhury *et al* 2015). Environmental parameters including air temperature (digital thermometer, Eurolab, Belgium), surface water temperature (SWT; digital thermometer, Eurolab, Belgium), salinity (Salt 6 + salinity probe, Eutech Instruments Pte Ltd., Singapore), pH (Eco testr pH2, Eutech Instruments Pte Ltd., Singapore) and dissolved oxygen (DO; DO meter, Eutech Instruments Pte Ltd., Singapore) were measured in triplicates at each station during the time of sampling. The probes used in this study were ATC enabled and calibrated in the laboratory and also in the field with standards as per manufacturer's instructions before undertaking sampling each time during the study period.

Nutrient analyses

Surface water samples were collected from each station for estimation of dissolved nutrients following published protocols (Choudhury *et al* 2015). Dissolved nutrients including nitrate (Finch *et al* 1998) and ammonium (Liddicoat *et al* 1975) concentrations were measured in triplicates in a UV-vis spectrophotometer (U2900, Hitachi Corporation, Japan). Concentrations of dissolved nitrate and ammonium were collectively considered as DIN. Dissolved nitrite was not considered as it is typically present in very low concentrations compared to dissolved nitrate and ammonium and thus was assumed to be negligible.

Biogeochemical context of the sampling locations

The Junput and SBOTS (Stn1 and Stn3) stations were monitored through pre-monsoon, monsoon, and post-monsoon seasons. Junput station was sampled in March 2016 (pre-monsoon), August 2016 (monsoon), and October 2016 (post-monsoon). Hourly data was recorded encompassing low-high-low tide durations. The SBOTS stations were similarly monitored through pre-monsoon, monsoon, and post-monsoon seasons from 2013 to 2016. Sampling was performed once daily, two hours after the highest high tide. The five stations of SBR lie within the heavily protected regions of the SBR and sampling in these areas required special permission and the presence of armed forest guards. Hence, regular monthly samplings at these stations were not possible, and additionally, these stations were completely inaccessible during days of heavy rainfall. To ensure the reliability of measured dissolved nutrients, samples were collected from additional stations within the vicinity of the main sampling stations based on accessibility.

Environmental DNA extraction

Bacterioplankton communities were elucidated from 1 L surface water sample by filtration through 0.22 μm 47 mm nitrocellulose filter paper (Pall, USA) using standard methodology (Ghosh and Bhadury 2018). Filters were immediately stored at -20°C for downstream processing. The environmental DNA pool was extracted using

the following protocol: a sucrose salt lysis buffer (400 mM NaCl, 50 mM Tris-HCl, 20 mM EDTA, 750 mM Sucrose, 10% SDS; Merck, India) was added to the filter paper, followed by 5 μ l Proteinase K (Amresco, USA) and incubated for 4 h at 55 °C. Then, 10 μ l Lysozyme (ThermoFisher Scientific, Germany) was added and the samples were incubated for 2 h at 37 °C. Phenol: Chloroform (Merck, India) was then added to the lysis buffer in the ratio of 1:1:2 and centrifuged at 16000 rcf (radius 10 cm) for 12 min to separate the aqueous fraction. Samples were then incubated overnight with 3M sodium acetate (Merck, India) and absolute ethanol (Merck, Germany). The solution was pelleted at 16000 rcf (radius 10 cm) for 12 min and the pellet was then air-dried and dissolved in 30 μ l 10 mM Tris-HCl (Merck, India) (Boström *et al* 2004). The extracted environmental DNA was visualized on a 1% agarose gel.

Amplification and sequencing of bacterial 16S rRNA

The V3-V4 regions of the bacterial 16S rRNA were amplified using barcoded primers Pro340F (5'-CCTACGGGNBGCASCAG-3') and Pro805R (5'-GACTACNVGGGTATCTAATCC-3') (Takahashi *et al* 2014) from extracted environmental DNA. Amplicon libraries were prepared using NEBNext Ultra DNA Library Preparation kit (NEB, USA) and purified by 1X AmpureXP beads. Amplicon library parameters were quality checked on an Agilent High Sensitivity (HS) chip on Bioanalyzer 2100 and quantified in a fluorometer by Qubit dsDNA HS Array Kit (ThermoFisher Scientific, USA). Amplicon libraries were loaded onto an Illumina MiSeq platform at concentrations of 10–20 pM. The generated sequences are available from the National Centre for Biotechnology Information (NCBI) Short Read Archive data under accession number SRP092508.

Sequence quality control and operational taxonomic unit (OTU) generation

The pair-end reads were quality filtered and adaptor, barcode, and primer sequences were trimmed. The pair-end reads were merged by using Fast Length Adjustment of SHort reads (FLASH) (Magoč and Salzberg 2011) and chimera sequences were removed using UCHIME in QIIME (Caporaso *et al* 2010, Edgar *et al* 2011). Operational taxonomic units (OTUs) were generated at 97% sequence identity using UCLUST (Edgar 2010) for each dataset. Taxonomic classification obtained was cross-checked using the SILVAngs analysis pipeline (Quast *et al* 2013) to determine accuracy in the determination of bacterioplankton communities.

Functional profiles of the bacterioplankton communities from each station were predicted using the Tax4Fun package (Aβhauer *et al* 2015) to understand the possible influence of variations in DIN concentration on metabolic pathways. Obtaining such information could pave the way for further experimentation to understand nitrogen fluxes in the northeast coastal Bay of Bengal. The taxonomic data obtained from SILVA was run against UProC (Meinicke 2015) and PAUDA (Huson and Xie 2014) to predict functional profiles of the resident bacterioplankton communities. Functional genes indicating involvement in nitrate and ammonium uptakes and utilization by bacterioplankton communities were enlisted separately to observe their changes in terms of abundance.

Statistical analyses

All 16S rRNA read counts were converted to relative abundances by dividing by sample read count totals; these relative abundances were used in subsequent taxonomic and functional profile analyses. Similarity percentage (SIMPER) was performed in R-3.5.3 using *simper* function in *vegan* (Oksanen *et al* 2016) to identify bacterioplankton families that contributed most to the dissimilarity between bacterioplankton communities from different stations. Pearson's correlation analysis was performed in R-3.5.3 using *corrplot* (Wei and Simko, 2021) function to quantify the linear relationship between DIN concentration and bacterioplankton abundance in studied stations. Regression analysis was performed using scatterplots between DIN concentration and relative abundance of bacterioplankton orders to determine the changes in abundance of particular taxa with recorded variation in DIN concentration. The abundance of bacterioplankton phyla across the studied stations was normalized and square-root transformed, and a non-metric multidimensional scaling (nMDS) ordination plot was generated using Bray-Curtis dissimilarity in *vegan* version 2.5–5 (Oksanen *et al* 2019) in R-3.5.3. The abundance of proteins or sub-units of enzymes involved in nitrogen metabolism was correlated with total DIN concentrations using the *ggpairs* function (Schloerke *et al* 2021) using R-3.5.3.

Results

Total dissolved inorganic nitrogen (DIN) concentration profiles

At Junput, in pre-monsoon (March 2016), DIN concentration ranged from 7.8 μ M to 84 μ M (figure S2 (available online at stacks.iop.org/ERC/4/035006/mmedia)), and high concentrations of DIN coincided with both high and low tide timings. The concentration of DIN was higher in the morning hours (between 0600–0900 h) than at any other time of the day. The average concentration of DIN in the morning was \sim 76 μ M

compared to 32 μM during the other times of the sampling program (figure S2). In monsoon (August 2016), the average concentration of DIN was $\sim 73 \mu\text{M}$ with high concentrations (average $\sim 83 \mu\text{M}$) measured between 0630 to 0830 h. Rainfall increased the discharge from the Pichaboni estuary into the Junput station which coincided with an increase in DIN concentration. The average DIN concentration during post-monsoon (October 2016) was about $\sim 55 \mu\text{M}$ (figure S2), and no significant variation in DIN concentration was observed spanning over the time of sampling. Samples used for elucidation of structure of bacterioplankton communities had DIN concentrations of $\sim 83 \mu\text{M}$ and $85 \mu\text{M}$ in August 2016.

The two stations of SBOTS, Stn1, and Stn3, were monitored every month throughout the year from 2013 to 2016. The concentration of DIN did not exhibit significant variation either between the studied seasons or years (figure S2). In the pre-monsoon and monsoon seasons of 2013–2016, DIN concentration remained in the range of ~ 39 to $53 \mu\text{M}$, and in post-monsoon, the average ranged from ~ 43 – $52 \mu\text{M}$. Except April 2013 and March 2014, DIN concentrations between Stn1 and Stn3 were found to be largely comparable (figure S2). Surface water samples collected from Stn1 and Stn3 in July 2014 were used for elucidation of bacterioplankton community structure. The concentration of DIN was 31.6 and $28.8 \mu\text{M}$ at Stn1 and Stn3 respectively for July 2014.

The concentration of DIN determined from 152 samples collected around the monitoring stations ranged from 30 to $90 \mu\text{M}$ (figure S2). For the eighty-five stations along the Thakuran estuary, DIN concentration ranged from 32– $66 \mu\text{M}$, whereas thirteen stations along the Matla estuary remained in the range of 31– $64 \mu\text{M}$. The sixteen stations along the Harinbhanga estuary showed DIN concentration in the range of 38– $60 \mu\text{M}$, with no significant variation found between the upstream stations of SBR_S2 and SBR_S4 and the downstream station of SBR_S3 along the Matla and Harinbhanga estuaries. Two stations along the Thakuran estuary, SBR_S1, and SBR_S6 with an average DIN concentration of $82 \mu\text{M}$ and $90 \mu\text{M}$ respectively were used for elucidation of bacterioplankton community structure. The station on the Matla estuary, SBR_S2, recorded High DIN concentrations (average $\sim 46 \mu\text{M}$) irrespective of the time of sampling. The stations SBR_S3 and SBR_S4 exhibiting 21 μM and 20.5 μM DIN concentrations respectively were subjected to elucidation of bacterioplankton community structure.

DIN concentration-based station differentiation

Annual observations have shown the strong variation of DIN concentration with freshwater inflow in the studied stations. Other measured environmental variations do not appear to have a significant influence on the concentration of DIN in studied stations. Variations in other studied environmental parameters including pH are provided in table S1 and also shown in figure S2.

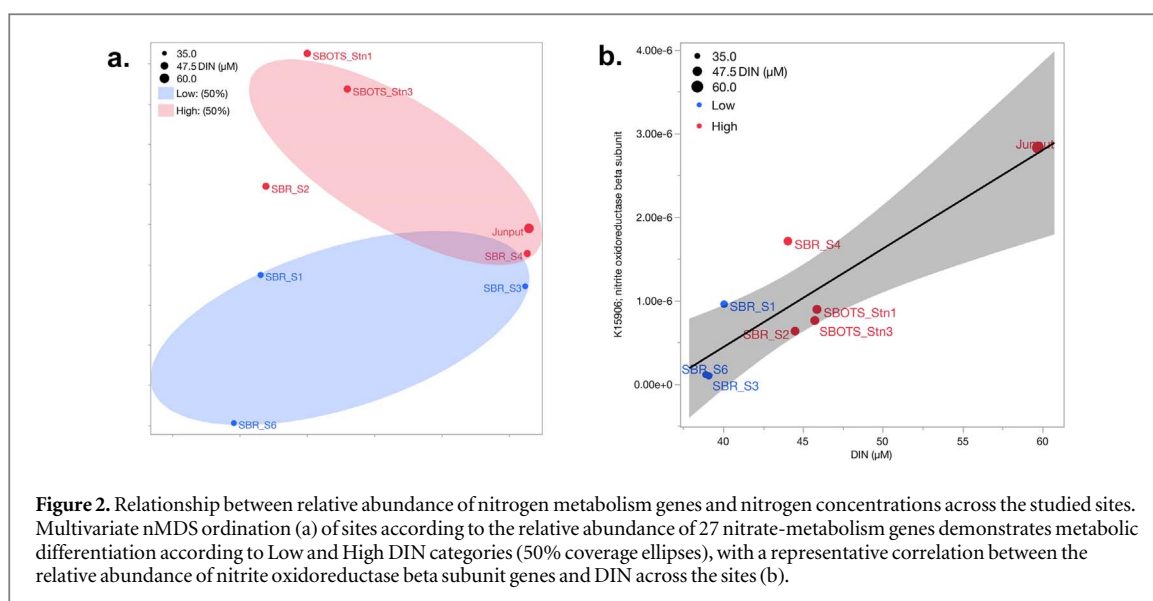
The annual average of DIN concentration in the studied stations was used to differentiate them into High and Low DIN stations. Stations SBR_S1, SBR_S3, and SBR_S6 of SBR were considered as Low DIN ($< 40 \mu\text{M}$) stations. High DIN concentration ($> 45 \mu\text{M}$) stations were Junput, Stn1, and Stn3 of SBOTS and SBR_S2 and SBR_S4 of the SBR (figure 1). The stations with High DIN concentration are referred to as High DIN stations and stations with low DIN concentration are referred to as Low DIN stations throughout rest of the manuscript.

Bacterioplankton community structure in studied stations

In total 32777527 pair-end reads were considered in this study. As evident from OTU numbers, Proteobacteria appears to be the overwhelmingly abundant bacterioplankton phylum across all the studied stations. Apart from Proteobacteria, only Bacteroidetes and Firmicutes showed abundances of $\geq 1\%$ in all the studied stations. All other phyla identified had abundances of $< 1\%$ of the total bacterioplankton communities in eight stations. Bacterioplankton phyla including Acidobacteria, Actinobacteria, Dependuntiae, Gemmatimonadetes, Latescibacteria, Planctomycetes, Spirochaetes, Tenericutes, and Verrucomicrobia were found in all studied stations. The distribution of bacterioplankton classes included a high abundance of Gammaproteobacteria and Deltaproteobacteria (figure S1). Abundant orders ($> 1\%$) identified include Bacteroidales, Cytophagales, Flavobacteriales, Shingobacteriales, Rhizobiales, Desulfovibrionales, Desulfuromonadales, Alteromonadales, Methylococcales, Oceanospirillales and Vibrionales. None of these groups showed comparable abundances across the studied stations, and each station was overwhelmingly dominated by only one bacterioplankton family (e.g. Alteromonadales at Station SBR_S6).

Variation of bacterioplankton communities with DIN concentrations

SIMPER analysis indicated the abundances of Proteobacteria and Bacteroidetes contribute to nearly 80% of the observed difference in bacterioplankton community structure between the high and low DIN concentrations prevalent in studied stations. Further taxonomic classification also indicated similar trends. The following taxa



contributed at least 90% of the differences between DIN groups and were interpreted as being differentially abundant between High and Low DIN stations. At the order level, several orders including Pseudomonadales (Contribution %–25.3), Alteromonadales (17.9%), Desulfovibrionales (17%), Oceanospirillales (10.2%), Vibrionales (7.2%), Cytophagales (2.8%), Cellovibrionales (2.8%), Flavobacteriales (2.7%), Desulfuromonadales (2.3%) and Methylococcales (2%) contributed to the difference in bacterioplankton communities between High DIN and Low DIN stations. Pearson's correlation coefficient showed positive correlation of DIN concentration with only Spirochaetes ($r = 0.49$, $p < 0.05$) and Tenericutes ($r = 0.62$, $p < 0.05$) (figure 5). All other identified phyla from the studied stations showed a weak negative correlation with DIN concentration (figure 5). At the order level, abundances of Flavobacteriales, Sphingobacteriales, Desulfovibrionales, Desulfuromonadales and Alteromonadales showed positive correlation with DIN concentration. Bacteroidales, Cytophagales, Flavobacteriales, Methylococcales, Desulfovibrionales, Vibrionales, and Rhizobiales showed high abundance in stations with High DIN as compared to Betaproteobacteriales and Alteromadales which showed high abundance in Low DIN stations leading to a grouping of stations according to DIN concentrations. The influence of DIN concentrations on shaping bacterioplankton orders was further reinstated by regression analysis (figure S3). The nMDS ordination plot (figure 2(a)) showed two distinct clusters based on DIN concentration where one cluster contained the Low DIN stations (SBR_S1, SBR_S3, and SBR_S6) and the other cluster contained High DIN Stations (Junput, SBR_S4, and Stn3). DIN concentration showed a negative correlation with the bacterial nitrate/nitrite response regulator and transport system proteins across the studied stations. The concentration of DIN showed positive correlation with bacterial nitrite oxidoreductase (figure 2(b)) but was negatively correlated with nitrate and nitrite reductases (figure 4).

Functional level variations with DIN concentrations

The distribution of predicted functional profiles that might be associated with DIN metabolism is shown in figure 3. Predicted functional profiles indicated the presence of bacterial genes coding for periplasmic nitrate reductase and alpha, beta, gamma, and delta subunits of cytosolic nitrate reductase as part of this study. Genes coding ATP-binding nitrate/nitrite transport systems were also found in bacterioplankton communities from all studied stations. Low abundance of bacterial genes coding nitrate oxidoreductase enzyme (alpha and beta subunits) were also identified across the studied stations. The abundance of ammonia monooxygenase gene appears to be low representing the bacterioplankton communities. Of the nitrogen cycle-associated genes, the most abundant one coded for bacterial nitrate reductase enzyme and showed highest distribution in High DIN concentration stations. Other genes including glutamine synthetase and urea transporter proteins did not show variation across the studied stations. Nitrate reductase showed a negative relation with DIN concentration in High DIN stations ($r = -0.7$) but such a negative relation was not observed in Low DIN stations. This trend was also observed for nitrite reductase, nitrate/nitrite response regulators, nitrate/nitrite transport system; all of these genes showed a negative correlation with DIN concentration in High DIN stations but showed a positive correlation in Low DIN stations (figure 4).

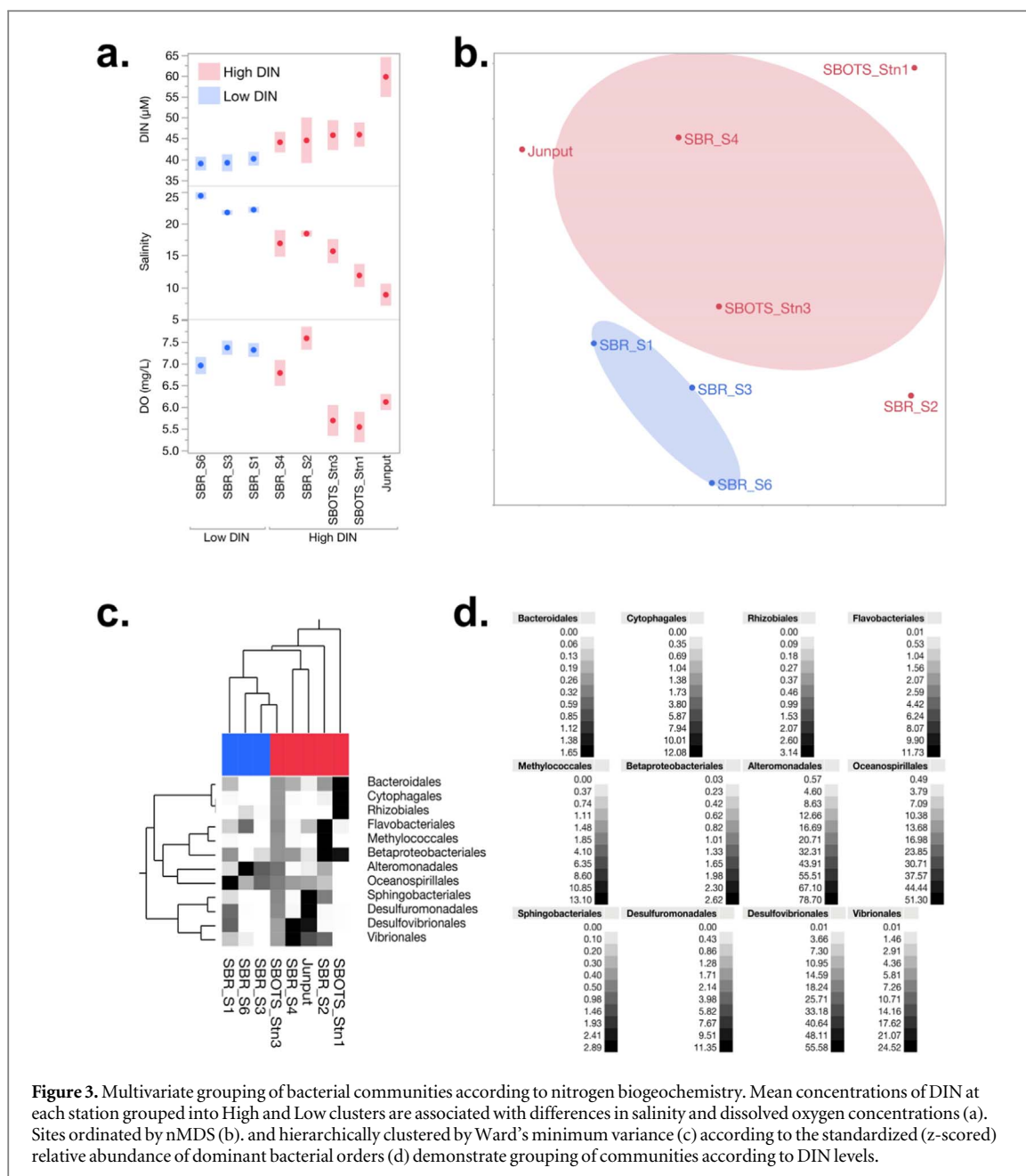
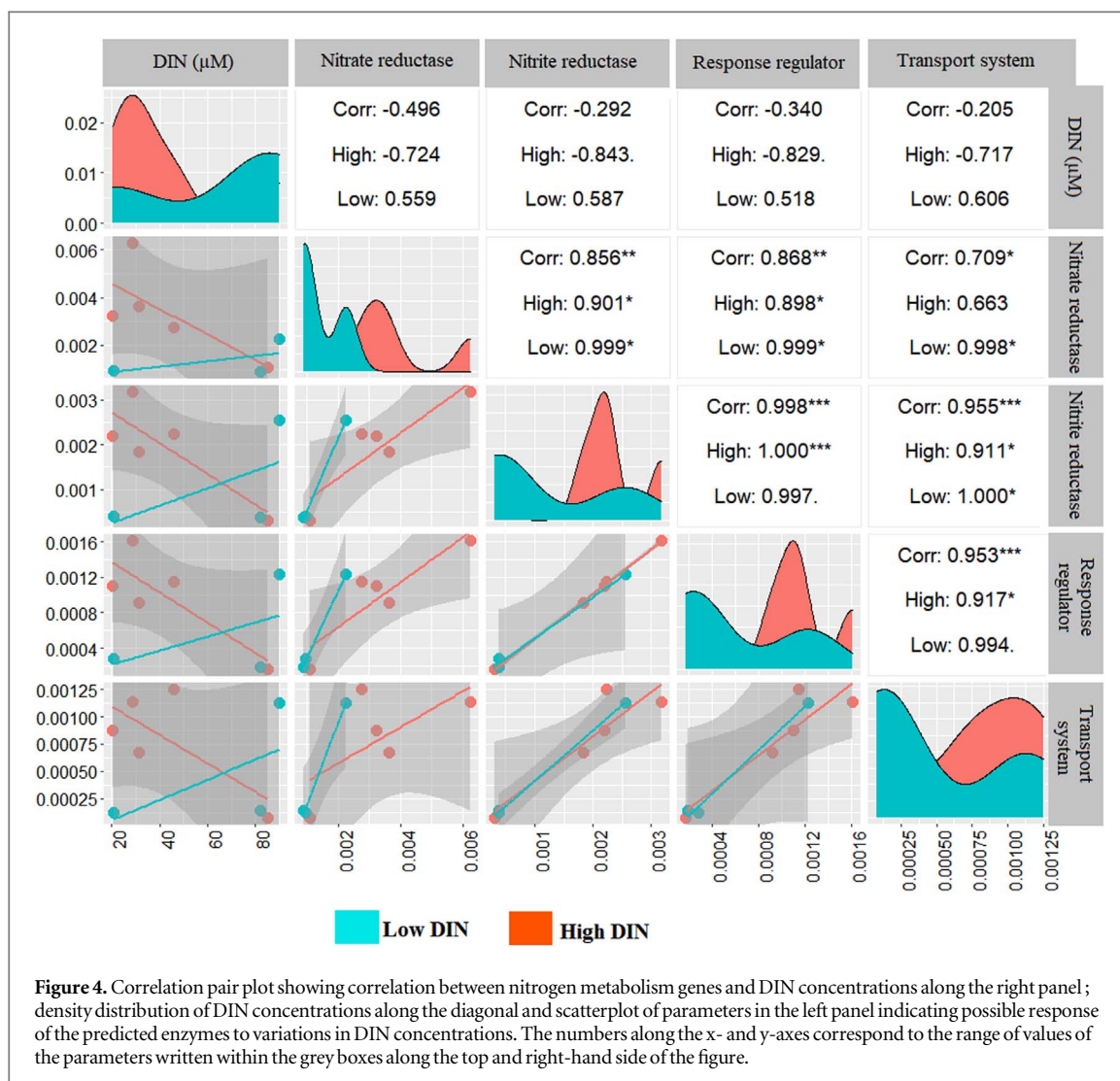


Figure 3. Multivariate grouping of bacterial communities according to nitrogen biogeochemistry. Mean concentrations of DIN at each station grouped into High and Low clusters are associated with differences in salinity and dissolved oxygen concentrations (a). Sites ordinated by nMDS (b), and hierarchically clustered by Ward's minimum variance (c) according to the standardized (z-scored) relative abundance of dominant bacterial orders (d) demonstrate grouping of communities according to DIN levels.

Discussion

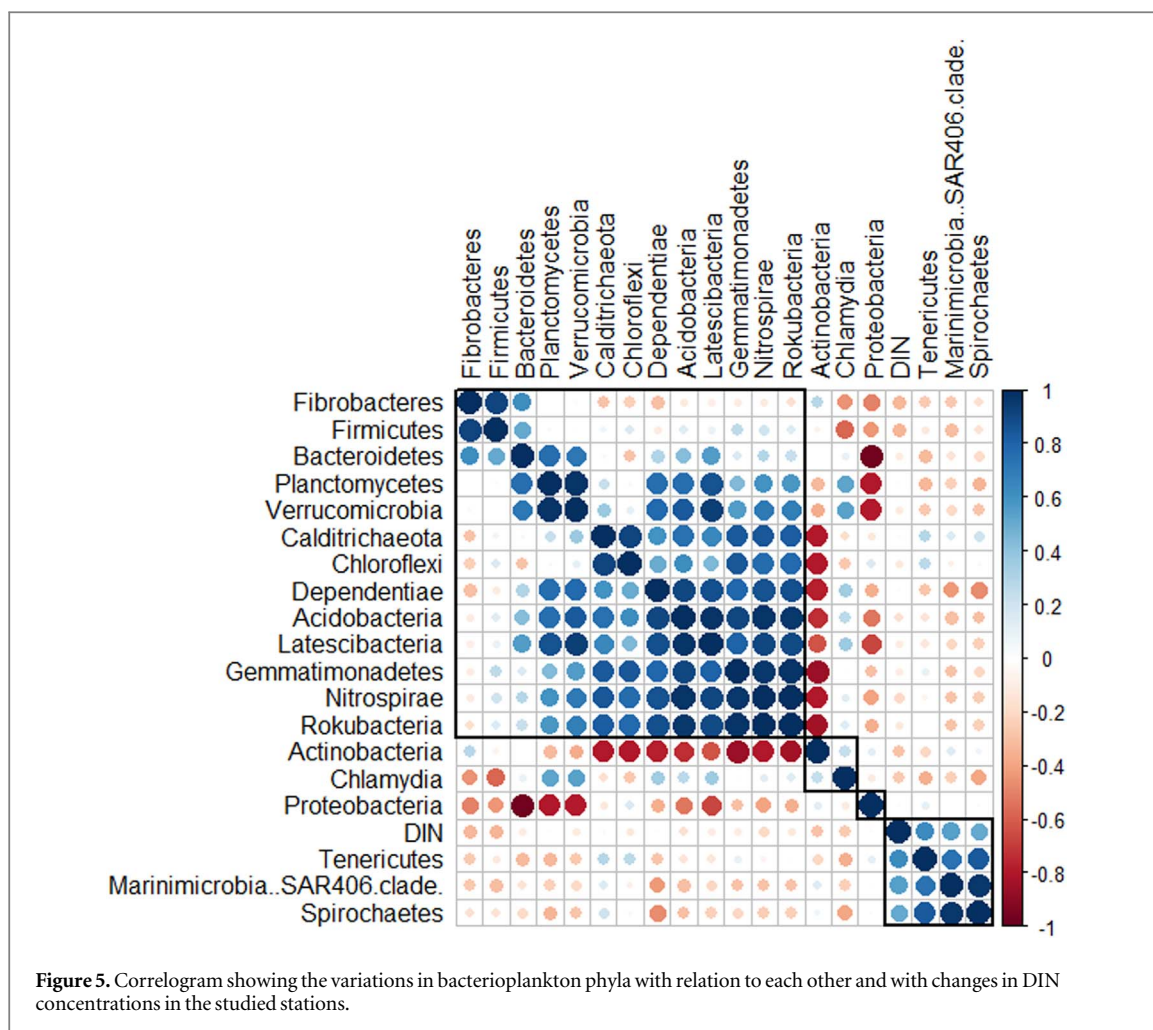
Inorganic nutrient dynamics are influenced by fluvial runoff, groundwater inputs, and estuarine residence time

Owing to the location of the northeast coastal Bay of Bengal, this deltaic region is heavily influenced by southwest monsoon. Huge freshwater flow at local and regional scales during monsoon seasons ($120,000 \text{ m}^3 \text{ sec}^{-1}$) results in rapid changes in environmental conditions. The stations of Junput and SBOTS (Stn1 and Stn3) which lie along the Hooghly and Mooriganga estuaries respectively show clear seasonal trends in surface water salinity variations. These three stations are influenced by diurnal tides from the coastal Bay of Bengal which results in high salinity corresponding to periods of high tides especially in pre-monsoon and post-monsoon seasons. During monsoon, salinity depends strongly on local freshwater input, and further eastward, decrease in freshwater flow from the rivers results in higher salinity. Such trends in surface water salinity in the Sundarbans have been observed over several decades (Banerjee 2013). Observed variations in salinity have been also reflected in terms of changing vegetation cover within the Indian Sundarbans (Joshi and Ghose 2003, Banerjee 2013). Surface water salinity during monsoon in the SBR stations is strongly controlled by local precipitation, and the shallow depth of these stations allows for rapid mixing resulting in typical estuarine conditions, as observed during the monsoon.



The proximity of studied stations to the coastal Bay of Bengal does not appear to be reflected in recorded surface water salinity: SBR_S6 which lies closer to the coastal Bay of Bengal showed lower salinity compared to SBR_S1 located further upstream of the Thakuran River; similarly, SBR_S4 located upstream of SBR_S3 on Harinbhanga River, showed higher salinity values. These patterns may be a result of local freshwater inputs from groundwater discharge across these particular stations. The role of groundwater discharge on influencing salinity has been reported from other parts of Sundarbans (Das and Mukherjee 2019). Intrinsically linked groundwater and surface water systems in coastal ecosystems represent interaction and transformation zones and these are widely reported (Glover 1959, Simpson *et al* 2003). Groundwater discharge from aquifers as well as infiltration of saline water into sediment is induced by tidal activity in other estuarine ecosystems such as in the Chesapeake Bay and the Swan-Canning estuary (Robinson *et al* 1998, Smith and Turner 2001, Acworth and Dasey 2003). Groundwater discharge is also recognized as a significant source of nutrients such as for nitrogen and phosphorus into coastal systems (Simmons, 1992, Krest *et al* 2000, Moore *et al* 2002, Hwang *et al* 2005). Moreover, nitrogen flux from submarine groundwater discharge to local rivers has been reported in the literature (e.g. Georgio shelf by Simmons 1992). Groundwater may also be a significant source of nutrients, including nitrate to surface waters (Valiela *et al* 1990, LaRoche *et al* 1997). Studies in South Carolina and Port Royal Sound have shown that groundwater input can supply as much nitrogen as river discharge (Krest *et al* 2000, Crotwell and Moore 2003). Additionally, high flow events, for example, heavy rainfall, corresponds to high nitrogen loads in estuaries including in Yarra estuary (Roberts *et al* 2016). A strong positive relationship of high nitrogen load with rainfall could explain the observed increase in DIN concentrations across the studied stations facing the northeast coastal Bay of Bengal.

Varying residence time could also influence DIN concentrations among different estuaries located along the northeast coastal Bay of Bengal. Roberts and colleagues attributed high nitrogen concentration to longer residence time under high flow conditions which inhibits uptake of forms of nitrogen (Roberts *et al* 2016). Previous records show that the tidal range can vary from place to place within the estuaries of Sundarbans



(Manna *et al* 2012). Owing to the funnelling effect of estuaries such as those in Sundarbans, tidal fluctuation is smaller in a range near the mouth but increases as the tide pushes inwards (Manna *et al* 2012). The topographical structure of Sundarbans estuaries thereby control tidal fluctuation and in turn, may influence the concentration of DIN. This could explain the variation of DIN concentration recorded between stations located near the mouth of the estuaries with those located more inland, as observed in the Mooriganga estuary. Manna *et al* (2012) also reported tidal patterns to be symmetric near the mouth of the Saptamukhi River but asymmetric more inland of the estuary. Symmetric tides result in no additional residence time of tidal water within the estuary, but asymmetric nature of the tide within the estuary increases tidal water residence time as high tide reaches its maximum height much faster than draining of tidal water during ebb tide (Manna *et al* 2012). Hence, nutrient-rich tidal water has a longer residence time within the estuary than at the mouth of estuary, but draining of tidal water is strongly dependent on current speed and direction, which in turn is controlled by the geomorphology of estuaries (Cooper 2001, BollaPittaluga *et al* 2015).

Previous studies show DIN concentration can range between 2.11 μM to 23.66 μM in the continental shelf of the Hooghly River; however the authors did not find any significant variation in DIN concentration between high and low tide in surface water samples of two stations located in the Hooghly estuary (Das *et al* 2017). In contrast, surface water DIN concentrations were reported to be slightly higher in low tide compared to high tide (Das *et al* 2017). The variation in DIN concentration between stations located parallel to each other in different estuaries could be strongly impacted by the local geomorphology of those estuaries. Variation in freshwater flow, residence time, and local geomorphology could explain the observed variation in DIN concentration recorded in stations namely, SBR_S2, SBR_S3, and SBR_S4.

Influence of biogeochemical dynamics on bacterioplankton community structure and function

The dynamic variations in environmental parameters, including DIN concentration, appear to strongly influence resident abundant bacterioplankton phyla. As observed previously, these phyla have a widespread distribution in the estuaries along the northeast coastal Bay of Bengal (Ghosh and Bhadury 2019). In contrast, previous work from coastal environments has hinted at the likely increase in heterotrophic microbial

assimilation in ecosystems with high DIN concentration (Zieman *et al* 1984). Such heterotrophic bacterioplankton is especially important in ecosystems with large terrestrial inputs (Caraco *et al* 1998). Experiments conducted in the Hudson River conclusively show nitrogen enrichment of organic matter to be mediated by heterotrophic bacterioplankton communities (Caraco *et al* 1998). Bacterial community analysis in the Mondego estuary has also shown an increase in abundance of heterotrophic bacteria with increasing temperature and nitrate concentration (Bacelar-Nicolau *et al* 2003). Previous work also showed strong influence of inorganic nitrogen, particularly nitrate, in shaping bacterioplankton community structure across different estuaries of Sundarbans (Ghosh and Bhadury 2018, 2019). Bacterioplankton phyla including Proteobacteria, Bacteroidetes, and Firmicutes show widespread distribution whereas other phyla showed site-specific distribution as part of the above study. This is in line with the trends of data obtained in the present study. The nMDS ordination plot further reinstated the role of DIN concentrations in shaping bacterioplankton community structure in the northeast coastal Bay of Bengal. Two separate clusters indicated the difference in bacterioplankton communities between High and Low DIN stations. As early as 1994, it was reported that bacterial production in some aquatic ecosystems was strongly influenced by the availability of nitrogen and phosphorus (Caron 1994). This finding was further supported by several other studies (e.g. Kirchman 1994, Rivkin and Anderson 1997, Elser *et al* 2007, Mills *et al* 2008). Bacteria usually uptake substrates with low C: N:P stoichiometry due to low C: N and C:P requirements. Alternately, bacteria supplement their N and P requirements from alternative sources (Mills *et al* 2008). Supplements are required in regions where organic matter released by phytoplankton is not rich enough in N and P to sustain resident bacterial populations (Mills *et al* 2008). Coastal ecosystems usually receive enhanced nutrient loads from river flow, resulting in low C: N ratio DOM release by phytoplankton that can then sustain bacterial production (LaRoche *et al* 1997). However, the dependence of bacterioplankton communities on allochthonous forms of carbon might not be significant in the estuaries of Sundarbans. Previous data has conclusively shown low depth and high SPM load in the estuaries of Sundarbans including at the studied stations resulting in poor light penetration and low phytoplankton cell abundance and diversity (Bhattacharjee *et al* 2013, Choudhury *et al* 2015, Singh and Bhadury 2020). Thereby, organic forms of nitrogen may not play a strong role in shaping bacterioplankton communities in Sundarbans except seasonally (Bhadury and Singh 2020). This would decouple the dependence of bacterioplankton on ambient dissolved organic nitrogen and thus cause an active uptake from the DIN pool.

Phylogenetic studies from different ocean basins including the eastern tropical North and South Pacific Ocean, California Bay, and the Arabian Sea, have shown nitrogen fixers belonging to different clades of Proteobacteria, Clostridia, Spirochaetes, and Chlorobia (Fernandez *et al* 2011, Dekaezemacker *et al* 2013, Gier *et al* 2017, Gaby *et al* 2018, Christiansen and Loescher 2019). These bacterioplankton phyla show widespread presence in the studied estuaries of Sundarbans. Further downstream taxonomic affiliation shows the presence of bacterioplankton genera including *Pseudomonas*, *Azotobacter*, *Alcaligenes*, *Thiobacillus*, *Chromatium*, *Chlorobium*, *Desulfovibrio*, and *Clostridium* which indicates possible ongoing nitrogen fixation in the estuaries of northeast coastal Bay of Bengal. However, it is challenging to ascertain functional attributes from studies based on a single structural marker. Hence, it would be important to use a functional gene linked to bacterial nitrogen metabolism in consort with structural markers such as 16S rRNA to further understand the intricacies of cycling of nitrogen in the northeast coastal Bay of Bengal.

Functional level information indicated a positive correlation of DIN concentration with nitrite oxidoreductase and a negative correlation with both nitrite and nitrate reductases. In regions of continuous severe nitrogen limitation, resident microbial populations are abundant in genera with low demands for nitrogen. These genera survive by allocating low nitrogen to their proteins by enriching amino acids with a relatively low concentration of nitrogen (Grzymalski and Dussaq 2012, Dittberner *et al* 2018). These trends are usually not characteristic of coastal oceans which receive much higher nitrogen inputs from terrestrial and riverine sources as well as from upwelling (Capone and Hutchins 2013). Bacterioplankton in coastal oceans comprises genera that contain membrane proteins such as the nitrite/nitrate transport system. Due to structural constraints introduced by protein folding, membrane transport proteins have low nitrogen content (Berg *et al* 2002). Marine metagenomic datasets have been shown to contain about 12% membrane proteins, and hence are particularly relevant in studying nitrogen allocation (Patel *et al* 2010, Dittberner *et al* 2018). Moreover, these datasets can also aid in understanding nitrogen uptake mechanisms in certain types of coastal ecosystems. Ambient nitrogen concentrations hence differentially influence membrane and non-membrane proteins, which further leads to striking differences in the effects of ambient nitrogen concentration on transmembrane, periplasmic and intracellular domains of proteins involved in nitrogen cycling (Dittberner *et al* 2018).

Glutamine synthetase, an enzyme central to bacterial nitrogen metabolism (Huerger *et al* 2013), controls intracellular nitrogen flow and is directly involved in regulating the uptake of nitrogen from environment (Tanigawa *et al* 2002). Involvement of glutamine synthetase along with transport proteins coded by *urt* ABCDE (urea transporters) and *amt* (ammonia transporters) gene families are activated when nitrogen concentration is low; widely different from the gene network activated in conditions when concentration of nitrogen is high

(Dittberner *et al* 2018). However, both glutamine synthetase and urea transporters showed very low abundance but uniform distribution across all the studied stations. This could indicate the role of unknown proteins in the uptake of forms of nitrogen in estuaries facing the northeast coastal Bay of Bengal.

Genes coding for key enzymes including nitrate, nitrite, nitric oxide, and nitrous oxide reductases could also serve as key molecular markers to study nitrogen cycling. Interestingly, variation in abundance of membrane-bound nitrate reductase (encoded by *narG*) is strongly influenced by concentration of DIN in Low DIN stations ($r = 0.5$, $p > 0.5$) but showed a negative correlation in High DIN stations ($r = -0.7$). This indicates that the expression of genes involved in nitrogen cycling is strictly controlled by ambient nitrogen concentration. Periplasmic nitrate reductase (encoded by *napA*) is widely reported in Gammaproteobacteria (Smith *et al* 2007), and the high abundance of this gene in the estuaries of northeast coastal Bay of Bengal could indicate preferential use for metabolic purposes. Other genes involved in pathways such as ammonification are identified in Epsilonbacteraeota (Kieft *et al* 2018) which could indicate low concentration of dissolved ammonium detected in all the studied stations. Indeed, in previous studies along the coastal Bay of Bengal, it has been reported that dissolved ammonium availability is episodic and usually increases after the end of monsoon season (Ghosh and Bhadury 2017). High nitrate concentrations recorded in Junput and SBR stations could also increase phytoplankton abundance which in turn can increase ammonia concentration by excretion or during degradation of biomass (Valiela 1995). An increase in ammonia further leads to eutrophication followed by depletion in dissolved oxygen, and under such conditions, heterotrophic bacteria are inhibited and they show low abundance as well as diversity. Eutrophication caused by an increase in ammonia in SBR could have adverse consequences for rich coastal fisheries (close to 4 million tons of fish yield in Sundarbans every year) and the linked blue economy of the region and beyond).

Conclusion

Results from our study support the notion that dissolved nitrogen is a key factor controlling the community dynamics in the Bay of Bengal, as seen in previous bacterioplankton studies (Ghosh and Bhadury 2017, 2018, 2019). This present study which focused on the influence of DIN pool on bacterioplankton community structure across a larger geographic area of northeast coastal Bay of Bengal during monsoon showed a strong correlation between DIN and resident bacterioplankton communities. Moreover, this study clearly showed the role of DIN concentrations in shaping bacterioplankton community structures including distinct differences between High and Low DIN stations. Studies involving functional markers can provide deep insights into ecosystem-level functioning of bacterioplankton communities. The use of phylogenetic markers in consort with functional markers would provide a wider understanding of nitrogen recycling in estuaries of northeast coastal Bay of Bengal. Finer details provided by information obtained from functional markers could also help to determine the source of nitrogen and interplay with other environmental parameters such as salinity and pH in highly dynamic estuaries including those of the Sundarbans mangrove ecosystem. Such information would be then valuable to calculate the nitrogen budget of coastal ecosystems and also towards broadening the understanding of health coastal oceans from the view of anthropogenic nitrogen inputs.

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Data availability statement

All data that support the findings of this study are included within the article (and any supplementary files).

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