No nitrogen fixation in the Bay of Bengal?

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The Bay of Bengal (BoB) has long stood as a biogeochemical enigma with subsurface waters containing extremely low, but persistent, concentrations of oxygen in the nanomolar range which -for some, yet unconstrained reason- are prevented from becoming anoxic. One reason for this may be the low productivity of the BoB waters due to nutrient limitation, and the resulting lack of respiration of organic material at intermediate waters. Thus, the parameters determining primary production are key to understanding what prevents the BoB from developing anoxia. Primary productivity in the sunlit surface layers of tropical oceans is mostly limited by the supply of reactive nitrogen through upwelling, riverine flux, atmospheric deposition, and biological dinitrogen (N_2) fixation. In the BoB, a stable stratification limits nutrient supply via upwelling in the open waters, and riverine or atmospheric fluxes have been shown to support only less than one quarter of the nitrogen for primary production. This leaves a large uncertainty for most of the BoB's nitrogen input, suggesting a potential role of N_2 fixation in those waters.

Here, we present a survey of N_2 fixation and carbon fixation in the BoB during the winter monsoon season. We detected a community of N_2 fixers comparable to other OMZ regions, with only a few cyanobacterial clades and a broad diversity of non-phototrophic N_2 fixers present throughout the water column (samples collected between 10 m and 560 m water depth). While similar communities of N_2 fixers were shown to actively fix N_2 in other OMZs, N_2 fixation rates were below the detection limit in our samples covering the water column between the deep chlorophyll maximum and the OMZ. Consistent with this, no N_2 fixation signal was visible in $\delta^{15}N$ signatures. We suggest that the absence of N_2 fixation may be a consequence of a micronutrient limitation or of an O_2 sensitivity of the OMZ diazotrophs in the BoB. To explore how the onset of N_2 fixation by cyanobacteria compared to non-phototrophic N_2 fixers would impact on OMZ O_2 concentrations, a simple model exercise was carried out. We observed that both, photic zone-based and OMZ-based N_2 fixation are very sensitive to even minimal changes in water column stratification, with

stronger mixing increasing organic matter production and export, which would exhaust remaining O_2 traces in the BoB.

Introduction

Primary production in large areas of the surface ocean is limited by the availability of fixed nitrogen (Moore et al., 2013). This deficiency in nitrogen creates a niche for dinitrogen (N_2) fixation, an energy-costly process carried out only by certain prokaryotes, also referred to as diazotrophs, which are phylogenetically highly diverse. N_2 fixation in the ocean has been described quantitatively as most important in the oligotrophic surface waters of the subtropical gyres (Sohm et al., 2011;Luo et al., 2012;Wang et al., 2019) where cyanobacterial N_2 fixers dominate. Over the last decade, the development of novel molecular tools revealed that non-cyanobacterial N_2 fixers are widely distributed throughout ocean waters (Farnelid et al., 2011;Farnelid et al., 2013;Fernandez et al., 2011;Luo et al., 2012;Riemann et al., 2010;Zehr et al., 1998) and sediments (Fulweiler et al., 2007;Andersson et al., 2014;Bertics et al., 2013;Gier et al., 2017;Gier et al., 2016). Their quantitative importance for global N_2 fixation, however, is not yet clear. In oxygen minimum zones (OMZs) of the eastern tropical North and South Pacific Ocean, hypoxic basins in the Californian Bay and the Arabian Sea, those N_2 fixers form a unique community consisting of different clades of proteobacteria, clostridia, spirochaetes, chlorobia, and methanogenic archaea (Christiansen and Loescher, 2019;Dekaezemacker et al., 2013;Fernandez et al., 2011;Gaby et al., 2018;Gier et al., 2017;Goebel et al., 2010;Halm et al., 2012;Hamersley et al., 2011;Jayakumar et al., 2012;Jayakumar et al., 2017;Löscher et al., 2014). In contrast, cyanobacterial N_2 fixers and diatom-diazotroph-associations (DDAs), which are commonly considered the most important N_2 fixers in the surface ocean, were either absent or were detected only in low abundances in OMZs (Turk-Kubo et al., 2014;White et al., 2013;Jayakumar et al., 2012). Both, the presence of diazotrophs clustering with proteobacteria, clostridia, spirochaetes, chlorobia, and methanogenic archaea, and the underrepresentation of cyanobacterial and DDA N_2 fixers could thus be considered characteristic for OMZ environments.

Nutrient stoichiometry and model predictions (Deutsch et al., 2007) suggest that oxygen-deficient waters are a potentially important niche for N_2 fixation. Based on this suggestion, several studies have focused on N_2 fixation in the large and persistent OMZ of the eastern tropical South Pacific. In this region N_2 fixation rates vary, however, with maximum rates of 840 µmol N $m^{-2}d^{-1}$ detected in nitrogenous sulfidic waters off the coast of Peru (Löscher et al., 2014), and 117 µmol N $m^{-2}d^{-1}$ in the oxygen-depleted zone (Bonnet et al., 2013). Low N_2 fixation rates close to the detection limit were reported from the same area (Chang et al., 2019), another set of N_2 fixation rates estimated from sediment trap analyses were in the range of 0-23 µmol N $m^{-2}d^{-1}$ (Knapp et al., 2016). Taken together, these rates suggest either a strong temporal variation or spatial patchiness. A similar variation in N_2 fixation rates was described for the eastern tropical North Pacific ranging from close to the detection limit in the OMZ (Jayakumar et al., 2017) up to 795 µmol N $m^{-2}d^{-1}$ in episodic diazotroph blooms (White et al., 2013). This apparent temporal or spatial variation in N_2 fixation rates may originate from unresolved environmental controls on N_2 fixation and makes it difficult to quantify N_2 fixation in OMZ waters.

The Bay of Bengal (BoB) is a region with a strong seasonality driven by the Asian monsoon system. Massive rainfall and river discharge with maximum freshwater inputs in September (e.g. Mahadevan (2016)) cause a considerable lowering of surface water salinity during and after the monsoons throughout the whole basin (Subramanian, 1993). This, together with increased surface water temperatures, leads to intensive and persistent stratification of the water column (Kumar et al., 2004), restricting nutrient fluxes to the surface from below and promoting a strong OMZ (McCreary et al., 2013;Paulmier and Ruiz-Pinto, 2009;Sarma, 2002) with minimum oxygen (O_2) concentrations in the lower nanomolar range (Bristow et al., 2017).

The potential importance of N_2 fixation in the BoB can be derived from a simple N budget estimate with an overall N loss of 7.9 ± 0.6 Tg N yr^{-1} and N sources other than N_2 fixation of 3.15 ± 2.25 Tg N yr^{-1} (Tab. 1, data from (Naqvi, 2008;Naqvi et al., 2010;Bristow et al., 2017;Singh et al., 2012;Krishna et al., 2016;Srinivas and Sarin, 2013;Suntharalingam et al., 2019)). This implies a deficit of 4.7 ± 2.4 Tg N yr^{-1} within the given range of uncertainty indicating the potential importance of N_2 fixation assuming a coupling of nitrogen loss and N_2 fixation as proposed by (Deutsch et al., 2007). Naqvi et al. (2010) proposed N_2 fixation to contribute 1 Tg N yr^{-1} in the BoB, while Srinivas and Sarin (2013) interpolated a contribution of 0.6- 4 Tg N yr^{-1} from phosphate availability. Measurements of N_2 fixation rates from the BoB are not available, isotope analysis of sediment trap samples indeed suggests that the BoB is a site of active N_2 fixation. Indeed, the composition of the organic material produced in BoB surface waters is characterized by a high portion of biogenic opal (20%) and a low $\delta^{15}N$ nitrate signal (3.2 - 5‰, Gaye-Haake et al. (2005)). This points towards a production of a considerable part of organic matter produced by diatoms in symbiotic association with or in close proximity to diazotrophs (Subramaniam et al., 2008). Only few studies report the presence of diazotrophs including *Trichodesmium* in the BoB (Wu et al., 2019;Shetye et al., 2013;Sahu et al., 2017;Jyothibabu et al., 2006;Mulholland and Capone, 2009), with only one of them using a functional gene approach.

To investigate the diazotrophic community and to quantify N_2 and carbon fixation in the BoB OMZ, we used a combination of gene sequencing and quantification, rate measurements, isotope tracing and box modeling.

Methods

Geochemical sampling

Samples were collected from the top 500 m of the water column during the SK-308 cruise with the ORV *Sagar Kanya* to the BoB during the winter monsoon between 24 January and 3 February 2014. Seawater samples were collected using 5 L and 30 L-Niskin bottles on a CTD- rosette equipped with a Seabird SBE 43 oxygen sensor and a WET Labs ECO-AFL/FL chlorophyll sensor as previously described in Bristow et al. (2017). To resolve oxygen dynamics below the Seabird sensor's detection limit a STOX (Switchable Trace amount OXygen) amperometric oxygen sensor was used (Revsbech et al., 2009), which had a detection limit of 7 – 12 nmol L^{-1} during this sampling campaign (Bristow et al., 2017). Nutrients, including nitrate, nitrite and phosphate were determined according to Grasshoff (1999).

N_2/C-fixation rate measurements

Seawater was collected from the Niskin bottles and filled into 2.4-L glass bottles or 2.8-L polycarbonate bottles for (near-) anoxic and all other (oxic) waters, respectively. Bottles were capped with black rubber stoppers (anoxic waters) or Teflon-coated butyl rubber septa (oxic waters). Incubations were performed with the method developed by Mohr et al. (2010) as described in (Grosskopf et al., 2012). Batches of $^{15}N_2$ gas (Cambridge Isotopes, USA) –enriched water was prepared with degassed water from two – three of the six sampling depths. Each incubation bottle was supplemented with 50 mL of the $^{15}N_2$-enriched seawater. Discrete samples for the measurement of the $^{15}N_2$ concentration were taken from each incubation bottle and were measured by membrane-inlet mass spectrometry (MIMS). Final $^{15}N_2$ enrichments were on average 1.65 atom % ^{15}N. For carbon fixation measurements, $NaH^{13}CO_3$ was dissolved in sterile MilliQ water (1g/117mL), and 5 mL were added to each incubation (~8 atom% final, based on total DIC of 2.2 mM). Bottles with water from the upper two depths were kept in surface seawater-cooled on-deck incubators. Bottles from the lower depths were incubated at 13-15°C in the dark. Incubations were stopped after approximately 24 h (samples with less than 20h incubation time were excluded from our analysis). Volumes between 2.1 and 2.7 L of seawater were filtered onto pre-combusted (450°C, 4-6 hours) 25 mm diameter GF/F filters (Whatman, GE Healthcare, Chalfont St Gile, UK) under gentle vacuum (200 mbar). Filters were either frozen at -20°C and oven-dried prior to processing or oven-dried (50°C) directly for 24 h and stored dry until analysis. Untreated seawater was filtered and prepared as described above to obtain background natural abundance values. For elemental and isotopic analysis, GF/F filters were acidified over fuming HCl overnight in a desiccator to remove inorganic C. Filters were then oven-dried for 2 hours at 50°C and pelletized in tin cups. Samples for particulate organic carbon and nitrogen (POC and PON) and C and N isotopic composition were analyzed on an Elemental Analyzer Flash EA 1112 series (Thermo Fisher) coupled to a continuous-flow isotope ratio mass spectrometer (Finnigan Delta Plus XP, Thermo Fisher). A table of N_2 and C fixation rate measurements is given in the supplementary material. Data sets were deposited on PANGAEA.

Molecular methods

Nucleic acid samples were collected at stations 1, 4 and 5 (Fig. 1). Between 5 and 27 L of seawater were filtered in two size fractions (3 µm and 0.22 µm pore size, Supor PES membrane disc filters; Pall, Portsmouth, UK), exact filtration volumes were recorded. Filters were stored in 2.7 mL sucrose lysis buffer at −20 °C.

DNA was extracted using an established protocol based on a phenol/chloroform extraction (Giovannoni et al., 1996). The quality and concentration of the purified DNA was checked spectrophotometrically and using the Quant-iT PicoGreen dsDNA kit (Invitrogen, Carlsbad, USA).

A metagenome from the deep chlorophyll maximum (DCM, 84m water depth) at station 4 was Illumina HiSeq-sequenced using a 2x125bp read length on a NexteraXT library at the Institute for Clinical Microbiology (IKMB) at Kiel University, Germany. Sequencing resulted in 321Mbp. Sequences were analyzed using the MetPathways pipeline (Konwar et al., 2013), a modular annotation and analysis pipeline for predicting diversity and metabolic interaction from environmental sequences consisting of a quality control, an open reading frame prediction and annotation, diversity analysis, and environmental pathway reconstruction. Phylogenetic identification of OTUs was derived via a comparison with the RefSeq and Greengenes databases (DeSantis et al., 2006). After quality check, 6,454 sequences of ribosomal RNA were identified, 622,286 sequences (27.56%) of proteins with known functions, and 1,628,841 sequences (72.15%) were predicted proteins with unknown function.

nifH gene amplification was performed using a nested PCR protocol (Zehr et al., 1998). PCRs were performed using the GoTaq kit (Promega, Fitchburg, USA) adding one additional µL BSA (20 mg mL^{-1} (Fermentas, Waltham, USA). The TopoTA Cloning® Kit (Invitrogen, Carlsbad, USA) was used for cloning of PCR amplicons, according to the manufacturer’s protocol. Sanger sequencing (340 *nifH* sequences) was performed by the Institute of Clinical Molecular Biology, Kiel, Germany. Negative controls were performed using the PCR mixture as described without template DNA; no amplification was detected. Samples from the particulate fraction >3 µm were consistently negative for *nifH* gene copies and were thus not further investigated. Sequences were ClustalW aligned in MEGA 7 (Kumar et al., 2016) and a maximum likelihood tree was constructed on a 321 base pair fragment. Reference sequences were obtained using BlastX on the NCBI database. Sequences were submitted to Genbank, submission ID 2245434. The metagenome has been submitted to the NCBI's sequence read archive, accession number SRR9696254.

Quantitative real time PCRs for *nifH* were performed using cluster specific TaqMan-probe qPCRS as described in Löscher et al (2014), with primers, probes, environmental standards and PCR conditions as presented in table S1. Samples were run in duplicates on a Biorad qPCR machine (Biorad, Hercules, USA).

Box model exercise

We used a simplistic five-box representation of an upwelling system with a deep and intermediate water iron source, with primary and export production as well as respiration derived from the original models (Canfield, 2006;Boyle et al., 2013). The model was used to distinguish a N_2 fixation state of the BoB and a non-N_2 fixation state with primary production driven by recycled dissolved nitrogen compounds. In contrast to the previous model versions, we applied a non- Redfield-based N_2 fixation scenario. Ammonia concentrations were set to zero in all boxes, in accordance with our direct measurements. Fe concentrations were set to 0.1 µmol L^{-1} in the deep and intermediate water boxes and 0.00044 µmol L^{-1} in the productive zone (Grand et al., 2015a;Grand et al., 2015b). Oxygen concentrations were adjusted to our measurements with 220, 0.02 and 50 µmol L^{-1} in the surface, OMZ and deep water, respectively (Bristow et al., 2017). Phosphate and nitrate concentrations were taken from our measurements with phosphate concentrations of 0, 2.7 and 2.5 µmol L^{-1} in the surface, OMZ and deep boxes, respectively; and oxidized nitrogen compounds (nitrate+ nitrite) at a concentration of 0, 38 and 35 µmol L^{-1} in the surface, intermediate and deep boxes, respectively. Further information on the model stoichiometry is given in the supplementary material.

Results and discussion

We explored the diversity, distribution and activity of N_2 fixing microbes and carbon fixers in the OMZ of the northern BoB during the Northeast monsoon in January 2014. During the time of the cruise, low sea surface temperatures (SST) and low surface water salinity reaching from the coasts of India, Bangladesh and Myanmar southwards to approximately 16°N were present (Figure 1A, B). At the coast, this low salinity/low SST plume co-occurred with increased chlorophyll concentrations (Fig 1C), thus suggesting a stimulation of primary production by waters possibly of riverine origin (Fig 1C). This is in line with earlier suggestions of riverine nutrient runoff promoting primary production close to the shelf, where nutrients are consumed rapidly thus preventing their offshore transport (Kumar et al., 2004;Singh et al., 2012;Singh and Ramesh, 2011;Krishna et al., 2016). Chlorophyll concentrations in the BoB during the time of the cruise detected via satellite monitoring ranged between 0.08 mg m^{-3} in open waters and 15 mg m^{-3} at the northern coast and were consistent with previous in-situ measurements during low productivity periods in the BoB (Kumar et al., 2010).

The sampling stations were located offshore in the central BoB (Fig. 1), where waters were strongly stratified with low sea surface salinity, but warmer SST compared to the coast, and a steep oxycline reaching O_2 concentrations close to anoxia at around 100 m water depth. No in-situ chlorophyll measurements are available from the cruise, but a fluorescence sensor attached to the CTD showed a maximum of up to 0.8 mg m^{-3} between 32-90 m water depth (Fig. 2). Satellite derived chlorophyll concentrations in the coastal BoB were in the range from 0.08 to 0.35 mg m^{-3}, slightly higher than in a previous study of this region (0.06 mg m^{-3}, Kumar et al. (2002)). Carbon fixation rates ranged between 286-1855 nmol C L^{-1} d^{-1} at the depth of the DCM (Fig. 2), however, our rate measurements did not cover the water column above 60 m water depth where rates may have been higher. Consistent with previous descriptions of primary producers at our study site (Loisel et al., 2013) and with satellite imaging (Fig. S1), we identified cyanobacteria related t*o Synechococcus* and *Prochlorococcus* as the most abundant primary producers in the in our metagenome from the BoB DCM, accounting for 3.3% of OTUs while eukaryotic phytoplankton accounted for only 0.3% of OTUs (Table S2).

Similar to chlorophyll, particulate organic carbon (POC, Tab. S3; see also Fig. S2 for a distribution of POC in the BoB) concentrations were low, ranging between 4.96 and 7.84 µmol C L^{-1} in surface waters, and resulting in an average POC:chlorophyll ratio of 68:1 to 115:1 at the depth of the DCM (Fig. 1). This ratio, is comparable to POC:chlorophyll ratios reported from cyanobacteria-dominated communities (74:1–126:1; e.g., (Lorenzoni et al., 2015;Sathyendranath et al., 2009)), but it is higher compared to other OMZ regions (e.g. 50:1 in the eastern tropical South Pacific (Chavez and Messié, 2009;Chavez et al., 1996)). Similarly, carbon fixation rates were 1-2 orders of magnitude lower compared to the Arabian Sea, the tropical South Pacific and tropical Atlantic (e.g. Longhurst et al. (1995)). While our POC concentrations from DCM are one order of magnitude higher than the satellite-derived POC estimates (Fig. S2) from surface waters indicating that POC and primary production in surface waters was not higher than in the DCM, it must be noted that our measurements did not cover the entire mixed layer and are thus likely a rather conservative minimum estimate.

N_2 fixation in the upper water column and the oxycline

Based on the dissolved inorganic nitrogen (NO_3^- + NO_2^-) to phosphate (PO_4^{3-}) ratio which has a negative intercept with the y-axis (Fig. 3; Benitez-Nelson (2000)), primary production in BoB waters appeared nitrogen limited during the cruise assuming Redfield stoichiometry. This nitrogen limitation would be expected to create a niche for N_2 fixation, but except for two samples for which in both cases only one out of three technical replicates showed an isotope enrichment, N_2 fixation rates were below the detection limit (Tab. S1). Consistent with this, $\delta^{15}N$ signatures of both the nitrate and the particulate organic nitrogen (PON) pool were only slightly decreased in the top 100 m of the water column to 5-8‰ (Fig. S3), thus not clearly supporting active N_2 fixation which would be expected to create light $\delta^{15}N$ signatures of -2- 2‰ (e.g. Dähnke and Thamdrup (2013)). Several clusters of N_2 fixing microbes were, however, identified by screening for the key functional marker gene *nifH* (Fig. 4). Only a few *nifH* sequences were associated with cyanobacteria commonly abundant in ocean surface waters. This pattern seems to be typical for OMZ areas (Fernandez et al., 2011;Jayakumar et al., 2012;Löscher et al., 2014) and for the eastern Indian Ocean (Wu et al., 2019), where cyanobacterial *nifH* sequences are also rare. Similar to earlier studies, which identified *Trichodesmium* in BoB surface waters (Bhaskar et al., 2007;Hegde, 2010;Wu et al., 2019), we detected *nifH* copies related to *Trichodesmium* in our samples, both by sequencing and by qPCR (Fig. 4, Tab. S4). These sequences clustered closely to *Trichodesmium-nifH* previously recovered from the Arabian Sea (Jayakumar et al., 2012;Mazard et al., 2004), where those N_2 fixers were found in low abundances, but possibly actively fixing N_2 as indicated by *nifH* presence in a cDNA library. No sequences related to the different groups of unicellular cyanobacterial diazotrophs (UCYN-A, -B, or –C; Zehr et al. (2001)) were present in our *nifH* dataset. UCYN-A and UCYN-B have previously been found in the Arabian Sea, but only at oligotrophic stations with warm water temperatures >30°C (Mazard et al., 2004). While UCYN-A may occur at temperatures below 25°C, *Trichodesmium* and UCYN-B may be limited by the water temperatures at our sampling stations, which were possibly too low with around 25°C. *Trichodesmium* is usually abundant in high-iron input regions such as the tropical Atlantic Ocean (Martínez-Pérez et al., 2016). The absence of *Trichodesmium* and other cyanobacterial N_2 fixers may thus also result from an insufficient iron source (Moore et al., 2013). Additionally, light limitation due to severe atmospheric pollution (known as the 'South Asian Brown Cloud') which lasts over the BoB from November to May (e.g. Ramanathan et al. (2007)) may influence the distribution of cyanobacteria in the BoB (Kumar et al., 2010). While earlier studies also detected *Chaetoceros* (Bhaskar et al., 2007;Hegde, 2010;Wu et al., 2019), a diatom known to live in association with diazotrophs, no diatom-associated N_2 fixers could be identified from our sequences. Thus our data does not directly support previous suggestions of those specific diazotrophs producing low $\delta^{15}N$ nitrate signatures along with high opal concentrations previously detected in sediment trap samples (Gaye-Haake et al., 2005).

N_2 fixation in the OMZ

In the OMZ, we detected again the genetic potential for N_2 fixation, but N_2 fixation rates were below the detection limit and $\delta^{15}N$ signatures of nitrate and PON indicated nitrogen loss instead of N_2 fixation (Fig. S3). The community of N_2 fixers in the BoB consisted mostly of the non-phototrophic, proteobacterial representatives of *nifH*- clusters I and III (Fig. 4), most of them related to previously identified OMZ diazotrophs (Fernandez et al., 2011;Jayakumar et al., 2012;Löscher et al., 2014).

A statistical comparison of BoB *nifH* sequences with OMZ diazotroph communities from the Arabian Sea, the ETSP, ETNP and hypoxic basins in California Bay revealed a strong similarity suggesting that certain diazotrophs are characteristic for OMZs (Fig. 5). Those typical OMZ-clusters include uncultured ɣ-, ɗ- and ε-proteobacteria and clostridia. Only one cluster was uniquely represented in the BoB and absent from the other OMZ datasets, with only three individual sequences related to *Azotobacter chroococum*. Another difference between the BoB and in the other OMZ diazotroph communities was the composition of Cluster IV *nifH* sequences, which are present but cluster in different groups as compared to for instance the Arabian Sea Cluster IV community. It is, however, unlikely that Cluster IV diazotrophs are important for N_2 fixation in the BoB or other OMZs because they were never shown to be transcribed (Fernandez et al., 2011;Jayakumar et al., 2012;Löscher et al., 2014) and Cluster IV-*nif* is generally considered to encode non-functional *nif* or paralogous sequences (Gaby and Buckley, 2014;Angel et al., 2018). In addition, the presence of Cluster IV *nifH* sequences has previously been ascribed to PCR-contamination (Zehr et al., 2003). Thus, the importance of this cluster for N_2 fixation in OMZs is generally debatable and the different composition of the Cluster IV diazotroph community does likely not explain the absence of N_2 fixation in the BoB.

While diazotroph communities highly similar to the identified BoB diazotrophs promote active N_2 fixation in other OMZ waters, we have no consistent indication for N_2 fixation in the BoB (Table S1). One explanation for the absence of N_2 fixation could be the sensitivity of the BoB OMZ diazotrophs to O_2 as opposed to the relative O_2 tolerance of cyanobacterial N_2 fixers. We identified BoB diazotrophs closely related to cultivated N_2 fixers, including *Vibrio diazotrophicus* and *Desulfonema limnicola*, which fix N_2 only under strictly anaerobic conditions (Urdaci et al., 1988;Bertics et al., 2013;Gier et al., 2016). Further, communities of diazotrophs from other OMZs highly similar to the BoB diazotroph community were described to transcribe their *nifH* gene and to actively fix N_2 only under strictly anoxic or anoxic-sulfidic conditions (Löscher et al., 2016;Löscher et al., 2014;Jayakumar et al., 2012;Jayakumar et al., 2017), and are unable to fix N_2 in the presence of even minimal concentrations of O_2 (reviewed in Bombar et al. (2016)). N_2 fixation in our samples (Tab. S1) may therefore be directly inhibited by the detected traces of O_2. Thus, our data suggest that even only nanomolar O_2 concentrations such as present in the BoB may prevent non-phototrophic N_2 fixers from actively fixing N_2, which could ultimately limit the supply of new nitrogen to the BoB.

Role of Fe and mesoscale activities (eddies)

The high iron (Fe) requirement of N_2 fixing microbes (60 times higher compared to other marine organisms, Gruber and Galloway (2008)) limits N_2 fixation in large parts of the ocean (Moore et al., 2013). However, aeolian Fe fluxes to surface waters of the southern BoB were estimated to be comparable to those detected underneath Saharan dust plumes in the Atlantic (290 ± 70 µmol m^{-2} yr^{-1}; Grand et al. (2015a)). Indeed, dissolved Fe (dFe) accumulates in the BoB OMZ reaching comparably high concentrations of up to 1.5 nM (Grand et al., 2015b;Chinni et al., 2019). In surface waters, dFe concentrations were described to range from 0.4 nM in the area of the cruise to up to 0.5 nM towards the north of the BoB, with increasing concentrations coinciding with decreasing salinity north of 15°N (Grand et al., 2015a;Grand et al., 2015b;Chinni et al., 2019). While the reported Fe concentrations do not indicate Fe limitation of N_2 fixation in the OMZ, surface primary production and N_2 fixation may be limited by any other micro-nutrient. Indication for such a limitation can be derived from eddy-induced Ekman pumping, mesoscale dynamics and the summer monsoon current have been shown to trigger plankton blooms with high productivity (Jyothibabu et al., 2015;Vinayachandran and Mathew, 2003;Chen et al., 2013;Fernandes et al., 2009) possibly induced by upwelling of certain nutrients to surface waters. Besides locally increasing surface water chlorophyll concentrations, erosion of the strong stratification and subsequent nutrient input to surface waters result in a change of phytoplankton size class (Prasanna Kumar et al., 2004). While usually smaller phytoplankton dominate the primary producer pool (60 – 95 % of the total chlorophyll), the contribution of larger phytoplankton has been observed to double in the regions influenced by the summer monsoon current and in mesoscale eddies, which impacts the vertical organic carbon flux in the BoB temporally and locally (Jyothibabu et al., 2015;Prasanna Kumar et al., 2004;Huete-Ortega et al., 2010;Gomes et al., 2016). The resulting increase of organic matter production, the modified composition of organic matter (i.e. production fresh and labile POM), a faster export and subsequent respiration could promote anoxic OMZ conditions in the BoB. This may subsequently allow for O_2-sensitive processes to take place, which may include N_2 fixation and nitrogen loss processes (Johnson et al., 2019), locally or regionally. Rapid changes in dissolved O_2 induced by increased surface productivity and organic matter export were reported in the context of mesoscale water mass dynamics in the BoB (Johnson et al., 2019), and also in other eddy systems in the Atlantic, which showed rapid O_2 exhaustion in otherwise oxic waters (Fiedler et al., 2016;Löscher et al., 2015). Episodes of increased biological productivity have also been reported from the BoB during both the pre-southwest monsoon and northeast monsoon (Kumar et al., 2004). Under those scenarios, large parts of the BoB's surface waters exhibited a strong $p\text{CO}_2$ undersaturation compared to the atmosphere (~350 µatm), resulting in an air-sea $p\text{CO}_2$ gradient sometimes exceeding 100 µatm. This gradient is explainable only by an increase in biological primary production fueled by temporal external nutrient input (Kumar et al., 2004). As Singh et al. (2012) pointed out, these high productivity episodes cannot be explained by riverine or atmospheric deposition of nutrients alone, but that upwelling or N_2 fixation would be required to sustain the nitrogen demand.

Feedbacks between N_2 fixation and OMZ intensity

We used a simple model to test the conditions allowing for N_2 fixation in the surface waters and in the OMZ of the BoB, and the interplay of N_2 fixation with primary production in response to changes in stratification (i.e. upwelling). We further explored in how far N_2 fixation controls O_2 concentrations in the BoB OMZ. We simulated a nitrate-driven primary production, and a N_2 fixation-dependent primary production, which is representative of N_2 fixation in the photic zone and governed by excess phosphorus and Fe availability as previously used in Canfield (2006) and Boyle et al. (2013). In addition, we simulated primary production that is dependent on OMZ-associated N_2 fixation, which in contrast to the classical N_2 fixation scenario is independent of a Redfield-based nitrogen deficit with N_2 fixation being active as long as phosphorus and Fe are available in concentration > 0 (Bombar et al., 2016;Löscher et al., 2014). One weakness of this model simulation is that it only includes Fe as potentially limiting nutrient for N_2 fixation, which is according to the available datasets (Grand et al., 2015b;Chinni et al., 2019) not necessarily correct but may be valid as an indicator for any other unrecognized micro-nutrient limitation. Consistent with the previous deep-time models of Canfield (2006) and Boyle et al. (2013), our model exercise revealed that additional nitrogen supply by N_2 fixation or other external nitrogen sources would generally exhaust the remaining traces of O_2 with increasing upwelling (Fig. 6). According to our model, this would lead to denitrification, which is in line with O_2-manipulated experiments as presented in Bristow et al. (2017) and consistent with the available isotope records from the OMZ (Fig. S3). A weaker stratification (in the model depicted as increased upwelling fluxes) would have the strongest effect on oxygen exhaustion and the onset of denitrification if primary production is dependent on N_2 fixation in the photic zone, followed by OMZ-located N_2 fixation, and last by nitrogen recycling. Given that OMZ regions are sites of massive nitrogen loss characterized by a nitrogen deficit in the water column (Deutsch et al., 2007), the similar diazotroph community in the OMZ paired with an absence of N_2 fixation in the euphotic zone suggest that OMZ-associated N_2 fixation is the most likely scenario. Thus, nitrogen limited primary production in the BoB and in OMZs in general would be susceptible to changes in stratification, with increased upwelling of nutrient-rich waters causing O_2 exhaustion. Considering the potential O_2 sensitivity of OMZ diazotrophs based on the comparison with other OMZs, the interplay between

O_2 concentrations, stratification and N_2 fixation may act as a stabilizing feedback on the BoB OMZ, preventing full O_2 depletion.

One factor possibly disturbing a possible stabilizing feedback is the external anthropogenic supply of nitrogen to the northern Indian Ocean. This additional nitrogen source is projected to increase over the next decades (Duce et al., 2008) potentially accelerating primary production in the future ocean including the BoB. An atmospheric input in the range of 1.1 (model-based) to 1.6 Tg N yr^{-1} (observation based) has been reported, which will likely increase in the future (Suntharalingam et al., 2019). This additional nitrogen fertilization would cause the same effect as N_2 fixation in our model, thus exhausting the present traces of O_2 in the OMZ rapidly. Until an increased supply of atmospheric or riverine nitrogen would become significant, changes in water column stratification, however, likely impose the strongest control on N_2 fixation and primary production, and thus on respiration, nitrogen loss processes and ultimately on the O_2 status of the OMZ in the BoB.

Conclusion

We detected a diazotrophic community similar to those from other OMZ regions, however, we could not obtain consistent evidence for active N_2 fixation in the BoB. Coming back to our original question 'No N_2 fixation in the BoB?' our data suggest 'No.'. In other OMZs, N_2 fixation has been observed to largely vary temporally and spatially but never reaching rates comparable to oligotrophic open ocean systems such as the Pacific gyres. Episodes of N_2 fixation, however, could be induced by changes in water mass dynamics, riverine or atmospheric nutrient input. Resulting increased N_2 fixation and primary production would possibly lead to O_2 exhaustion in the BoB, which otherwise doesn't become fully anoxic.

Previous observations describing the absence of nitrogen loss processes in the BoB were explained by the remaining traces of O_2 (Bristow et al., 2017) and possibly by a nitrogen deficiency relative to carbon in the organic matter pool. While we acknowledge that our dataset represents only a snapshot of the BoB's biogeochemical setting, our observations may help to predict the future development of N_2 fixation in the BoB and of the BoB OMZ with regard to increasing atmospheric dust deposition and ocean fertilization (Duce et al., 2008), altered ocean circulation patterns (Yeh et al., 2009), and deoxygenation of the ocean as a consequence of global warming (Schmidtko et al., 2017;Stramma et al., 2008).

Code/Data availability: Sequence data is available from Genbank, submission ID 2245434 and from NCBI's sequence read archive, accession number SRR9696254. The model code and other biogeochemical data are available from the Pangaea database (https://doi.pangaea.de/10.1594/PANGAEA.905498, https://doi.pangaea.de/10.1594/PANGAEA.905496).

Author contribution: CRL carried analyzed the data together with WM, CRL ran the model simulations and wrote the manuscript with substantial contributions from all co-authors.

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Tables:

Table 1: Nitrogen fluxes in the BoB (Tg N yr^{-1}); N loss fluxes are given in black, N sources are given in in gray; DIN = dissolved inorganic nitrogen, PON = particulate organic nitrogen, IO = Indian Ocean. N_2 loss by denitrification was excluded by Bristow et al. (2017). Naqvi et al. (2010) reported possible N loss to the atmosphere in the form of N_2O.

	Flux [Tg N yr^{-1}]	reference
Net exchange with the IO	3.3	Naqvi , 2010
Sedimentary denitrification	3 - 4.1	Naqvi , 2008; Naqvi 2010
PON burial	1	Naqvi , 2010
Water column N loss to the atmosphere	0 - 0.07	Naqvi , 2010; Bristow, et al. 2017
Atmospheric deposition	0.5 - 1.6	Naqvi , 2010; Singh et al., 2012; Suntharalingam et al., 2019
Riverine/ land input	0.4 - 4	Naqvi , 2010; Singh et al., 2012; Krishna et al., 2016
N_2 fixation	0.6-11.3	Naqvi , 2010; Srinivas & Sarin, 2013

Figures:

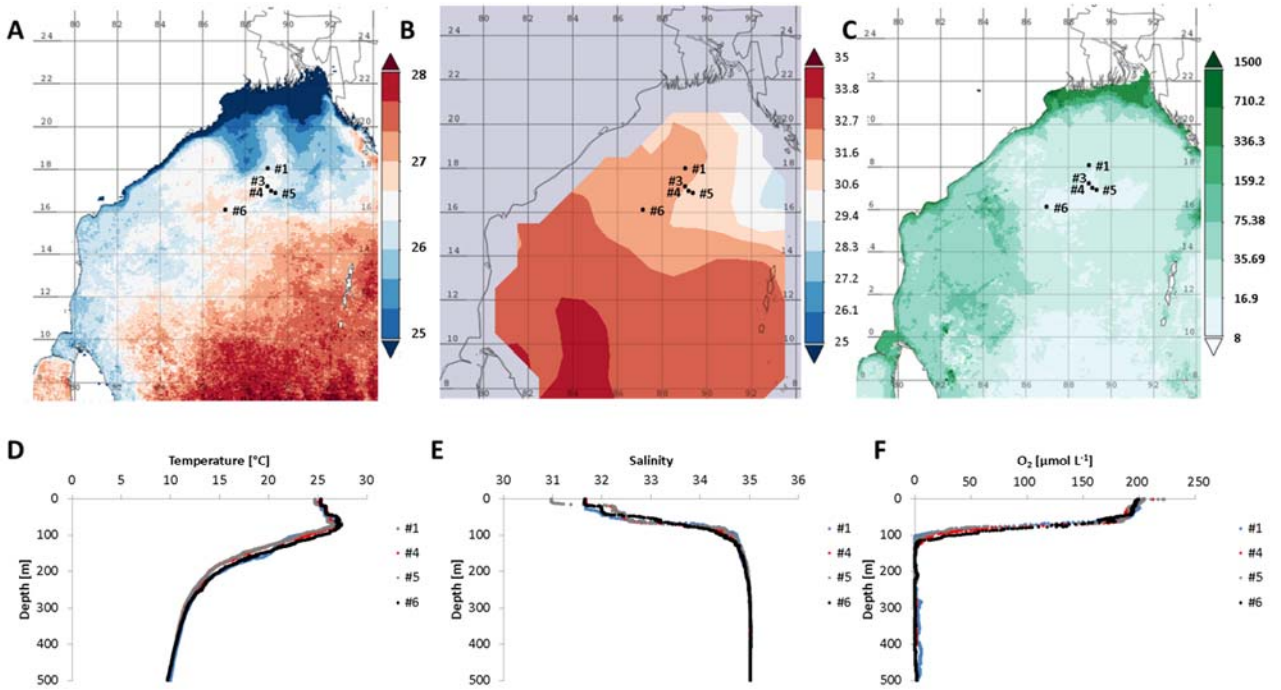

Fig. 1: Time-averaged maps from 15. Jan 2014 – 15. Feb 2014 of (A) sea surface temperature (SST in °C (night only, 8-daily, 4 km resolution obtained from MODIS-Aqua, https://giovanni.gsfc.nasa.gov), (B) sea surface salinity, (C) chlorophyll a concentration in 10^{-2} mg m^{-3}, note the log scale (8-daily, 4 km resolution obtained

from MODIS-Aqua, https://giovanni.gsfc.nasa.gov). (D) CTD data-based water temperature in °C, (E) salinity at the cruise stations. (F) O_2 (in µmol L^{-1}) over the top 500 m of the water column, data from Bristow et al. (2017)

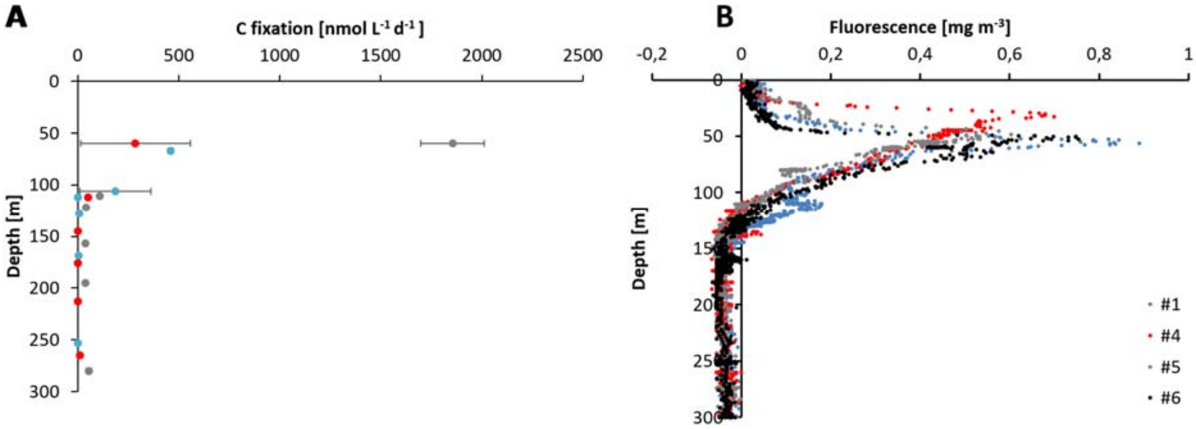

Fig. 2: (A) Carbon fixation rates at stations 1, 4 and 5, and (B) sensor-based fluorescence measurements from station 1, 4, 5 and 6.

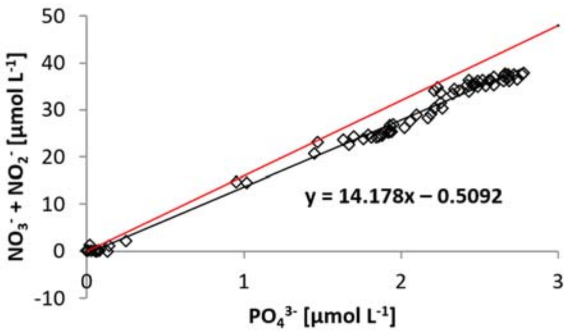

Fig. 3: N:P ratio at station 1, 4, 5 and 6, with the Redfield ratio of N:P = 16:1 indicated with a red line, the negative intercept of the trendline indicates a deficit in dissolved inorganic nitrogen.

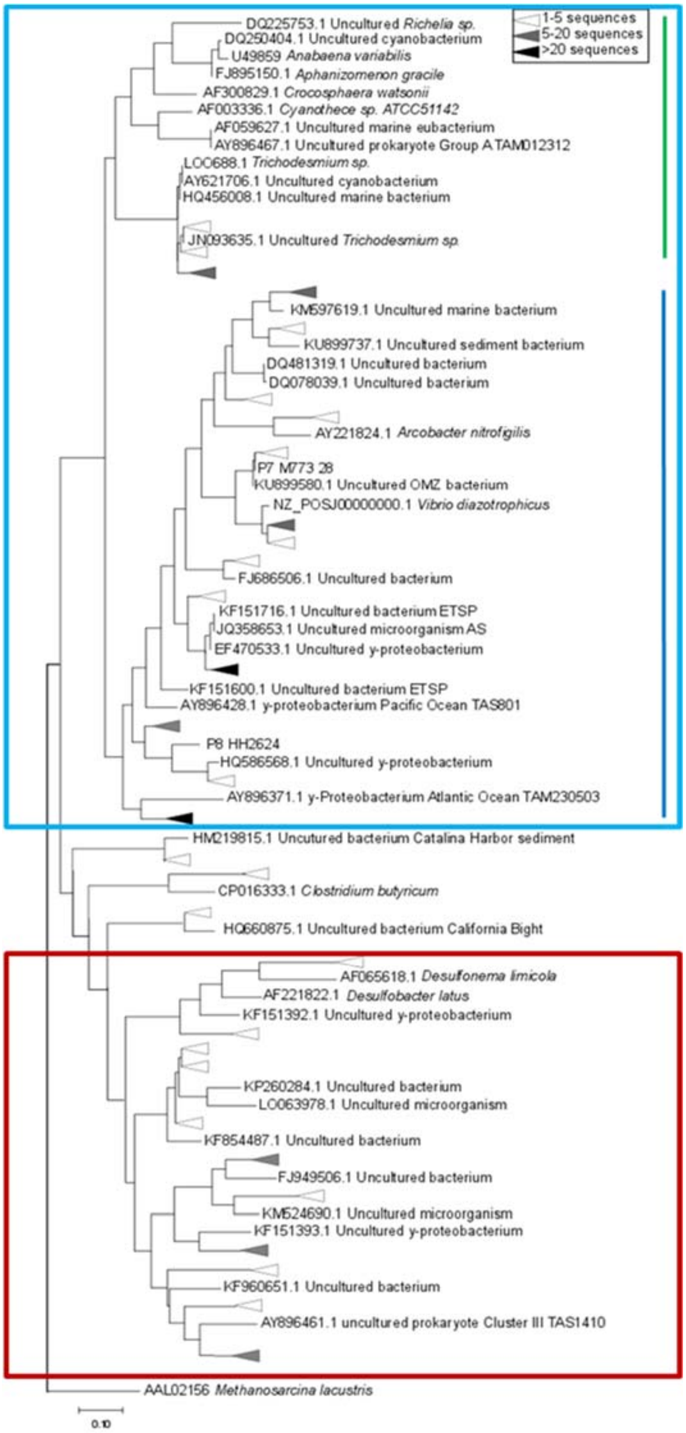

Fig. 4: Maximum likelihood tree of a 321 bp fragment of the *nifH* gene. Clusters identified by Sanger sequencing are indicated with triangles with colors denoting the sequence abundances in our dataset. The light blue box indicates Cluster I sequences including cyanobacteria (green line) and proteobacteria (blue line). Cluster III sequences are indicated with a red box.

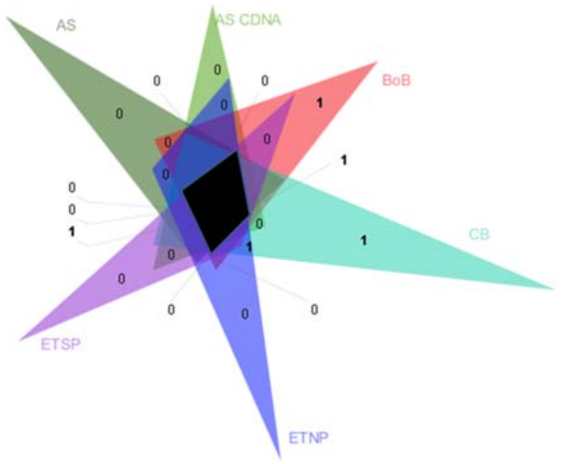

Fig. 5: Venn diagram of *nifH* clusters present in Arabian Sea DNA libraries (AS), and in cDNA libraries (AS cDNA), clusters identified in the BoB, in O_2-depleted basins of the Californian Bay (CB), the eastern tropical North Pacific (ETNP) and the eastern tropical South Pacific (ETSP). Clusters as depicted by triangles in Fig. S 6) were collapsed based on a 98% identity. The black area shows the clusters present in all OMZs. Numbers indicate the individual clusters in fields which would otherwise appear unproportionally large.

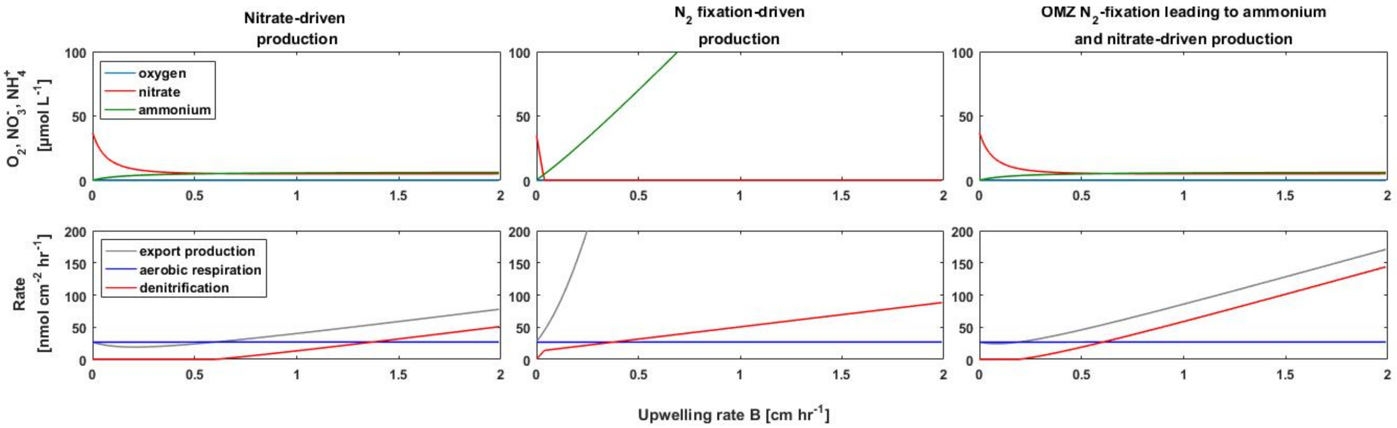

Fig. 6: Model of the response of the BoB OMZ to a weaker stratification corresponding to increased upwelling in this model, under a non-N_2 fixation scenario with nitrate driven production, a photic zone N_2 fixation-dependent primary production, and a scenario of N_2 fixation in the OMZ, which would result in built-up of a nitrogen stock and export to the productive surface if stratification becomes weaker.