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ORIGINAL ARTICLE

A comparison of bacterial communities from OMZ sediments in the Arabian Sea and the Bay of Bengal reveals major differences in nitrogen turnover and carbon recycling potential

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ABSTRACT

The Northern Indian Ocean hosts two Oxygen Minimum Zones (OMZ), one in the Arabian Sea and the other in the Bay of Bengal. 16S rRNA gene amplicon sequencing was used to understand the total bacterial diversity in, the surface sediment off Goa within the OMZ of the Arabian Sea, and off Paradeep within the OMZ of the Bay of Bengal. Functional profiling was carried out to pinpoint the occurrence of specific bacterial operational taxonomic units (OTUs) which have been previously described to harbour certain genes/enzymes relevant to biogeochemical cycling of carbon, nitrogen, and sulfur compounds. The dominant phyla identified included Firmicutes (33.08%) and Proteobacteria (32.59%) from the Arabian Sea, and Proteobacteria (52.65%) and Planctomycetes (9.36%) from the Bay of Bengal. Only 30% of OTUs were shared between the sites which make up three-fourth of the Bay of Bengal OMZ bacterial community, but only one-fourth of the Arabian Sea OMZ sediment bacterial community. Statistical analysis indicated the bacterial diversity from sediments of the Bay of Bengal OMZ is ~48% higher than the Arabian Sea OMZ. The community analysis combined with a predictive functional profiling of 16S rRNA amplicons revealed some major differences regarding sediment nitrogen fixation and carbon recycling, and identified a distinct bacterial community structure within the two shallow OMZ sites lying in the east coast and west coast of the peninsular India.

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Introduction

The Northern Indian Ocean consists of two major ocean basins: the Arabian Sea (AS) in the west and the Bay of Bengal (BoB) in the east. Even though both these basins are placed in the same latitude, they differ in many aspects. This includes differences in average salinity, primary productivity, nitrogen loss, the intensity of mesoscale eddies, contrasting transport of dissolved oxygen, and organic matter (McCreary Jr et al. 2013). Both these basins experience intense oxygen depletion below the mixed layer of the water column, where dissolved oxygen (DO) is usually below the detection limit of conventional methods. The AS-OMZ between the water depths of ~100/150–1000/1200 m is the thickest OMZ, and is identified as a primary site of fixed nitrogen loss (Naqvi et al. 2006). In contrast, the BoB-OMZ occurs at shallow depths, more seasonal in nature (Sarma et al. 2013) and has been reported to be less intense than the AS-OMZ (Paulmier 2009) with DO concentrations still present in the nanomolar range (Bristow et al. 2017). Nitrogen loss has been described as rather insignificant and limited by substrate availability resulting from low organic matter supply by primary production (Bristow et al. 2017; Löscher et al. 2020). The sequence of electron acceptor utilization in such an environment, generally follow the thermodynamic energy yield (Froelich et al. 1979). However, recent studies support the possibility of co-occurrence of metabolisms using different electron acceptors in OMZs, one example would be the existence of a cryptic sulfur cycle, which occurs along with nitrogen cycle processes (Canfield et al. 2010; Callbeck et al. 2018).

Surface sediment underlying OMZs entraps all recent microbial signatures of the water column above (Gerdes et al. 2000) in addition to the sediment microbiome; hence it is interesting to explore and compare such benthic OMZ ecosystems, especially those located in shallow zones. OMZs act as niches for microorganisms that can use alternative pathways

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of respiration (Diaz and Rosenberg 2008; Pitcher et al. 2011). In the BoB-OMZ, aerobic communities have been identified to coexist with anaerobic communities (Bristow et al. 2017). In the AS, such coexistence was explained by separate micro-niches in the same environment (Pitcher et al. 2011). Similar studies carried out in the eastern AS-OMZ sediments have identified Proteobacteria (52%) and Planctomycetes (12.7%) as the dominant phyla (Divya et al. 2011). Other integral phyla of soil/sediment habitat are Bacteroidetes, Acidobacteria, Actinobacteria, and Firmicutes (Lv et al. 2014).

It is vital to understand the dominant microbial taxa and also their functional ecology to throw light on the biogeochemistry of these oxygen-depleted zones (Rajpathak et al. 2018). With the advent of molecular techniques over the last decade, a large volume of data has been generated which helped to elucidate the bacterial community structure (Hodkinson and Grice 2015). Phylogenetic profiling, using next-generation sequencing (NGS) techniques, offer high-resolution data from complex environments (Claesson et al. 2010). By using algorithms leveraging functional databases, it is also possible to predict putative functional ecology from 16S rRNA amplicon data. The available data on the bacterial community structure of the northern Indian Ocean OMZ using such high throughput sequencing techniques has been limited to the pelagic realm (Rajpathak et al. 2018; Fernandes et al. 2019), or restricted to some functionally significant groups rather than total bacterial community (Fernandes et al. 2018). Descriptions of OMZ sediment bacterial communities are largely underrepresented and need special attention.

The objective of our work was to compare the surface sediment bacterial taxonomic and functional diversity within two major OMZs in the northern Indian Ocean, the Arabian Sea (AS) and the Bay of Bengal (BoB), using NGS on the v1-v3 hypervariable region of the 16S rRNA gene. Based on this high throughput sequencing dataset, we predicted the metabolic potential present at both sites, the AS and the BoB with a key focus on genes relevant for nitrogen and sulfur turnover, and many fermentative pathways to understand the bacterial role in carbonate precipitation, a possibility least explored.

Materials and methods

Sample collection and site characteristics

Sediment samples were collected in February 2013 off Goa in the AS-OMZ (SSK-046, *RV Sindhu Sankalp*), at the GS1A site located at 15°13′N, 72°56′E, and in August 2014 off Paradeep in the BoB (SSD-002, *RV Sindhu Sadhana*)., at the PS1B site located at 19°57′N, 86° 46′E [Figure 1(A): Plotted in Ocean Data View v.5.2.1.(Schlitzer 2015)]. A profile of nutrient and DO distribution is presented from the AS and the BoB, respectively (Figure 1(B,C)). These profiles represent typical conditions for the two sampling locations but were obtained from other cruises [B: 15°1′N, 72°26′E, collected in February 2018; and C: 19°41′N, 86°45′E, collected in August 2010] and show the distribution of DO, nitrate (NO_3^-), and nitrite (NO_2^-) from the surface to 1000 m water depth in µM. Sampling at both stations covered surface sediments below a ~200 m deep water column, underlying OMZ waters. Though both areas experience intense oxygen depletion with the core of the OMZ located between 150 and 500 m, the maximum NO_x values are twice as high in the AS as compared to the BoB, with a prominent secondary nitrite maxima (SNM). A box corer was used to retrieve the sediment samples. The sediment cores were carefully sub-sampled using acrylic core liners (25 mm ID, ~30 cm length), sub-samples were taken from the centre of the core to avoid mixing of sediment layers. The 0–5 cm subsections of samples were transferred into sterile screw-cap containers. Samples were handled sterile and preserved at −20°C until further analysis. The Temperature/Salinity profiling of the water column above the sediment was carried out using a Sea-Bird Electronics (SBE, USA) conductivity–temperature–depth (CTD) sensor (SBE9), equipped with a Niskin bottle rosette sampling system. The dissolved oxygen (DO) sensor used here (SBE43) yields an accuracy: ±2% of saturation, precision: 1 µM, and detection limit: ≤1µM.

Sediment characterization

The sediment samples were freeze-dried, homogenized, and ground in an agate mortar prior analysis. Total carbon (TC) and total nitrogen (TN) comprising both inorganic and organic forms were analysed in an elemental carbon/ nitrogen (CN) analyzer (Fisons NA 1500) using the method described in Bhushan et al. (2001). The calibration of the CN analyzer was done using a reference standard (NC-soil), and the obtained recovery rate was 96% for TC and 99% for TN. The precision was monitored by carrying out replicates for both samples and was ±1%. The detection limits were two times the blank value. Total organic carbon (TOC) contents were determined with a colorimetric based wet oxidation method (Azam and Sajjad 2005), which is reported to be highly reproducible.

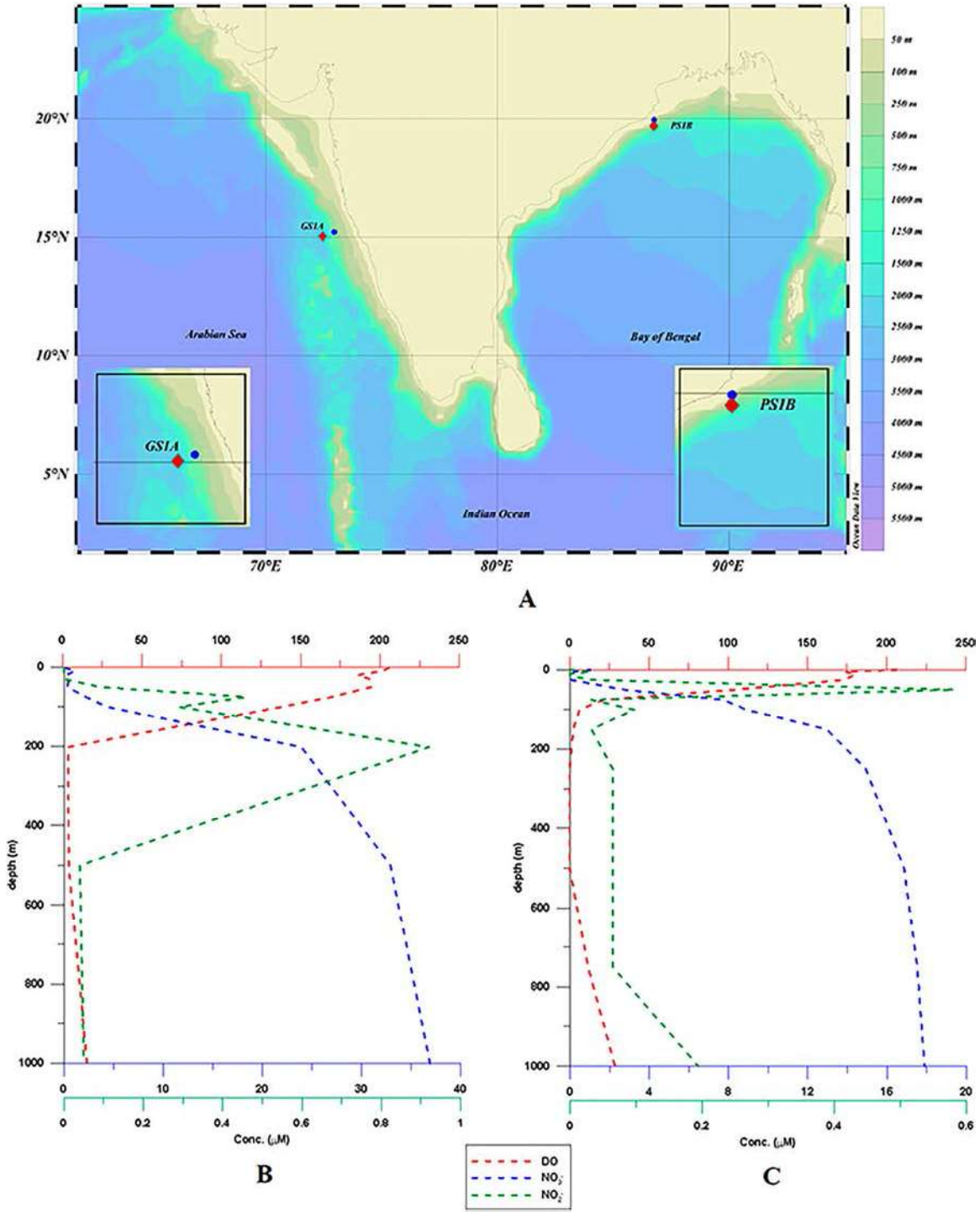

Figure 1. (A) Station map, blue dots indicate sampling stations, red dots are the regions where OMZ water-column characteristics were obtained from. (B) Representative water column profiles of biogeochemical parameters from the AS-OMZ (Unpublished data, personal communication from Dr. G.V.M. Gupa) and (C) the BoB- OMZ (Sarma et al. 2013).

Total inorganic carbon (TIC) was determined as the difference between TC and TOC (Bernard et al. 1995). Organic matter (OM) was calculated by multiplying TOC with the van Bemmelen factor 1.724 (Heaton et al. 2016), based on the assumption that humidified organic matter of soil contains 58% carbon, however, variations of 40-60% have been observed (Nelson and Sommers 1982). For determining $CaCO_3$ abundances, TIC was multiplied with a factor of 8.33 to get the per cent calcium carbonate as described previously (Bernard et al. 1995). The TOC/TN value was converted into molar ratio by multiplying with a

factor 1.167 derived from atomic weights of Carbon and Nitrogen (i.e. 14/12) (Meyers 1994).

Genomic DNA extraction and 454 pyrosequencing

Total genomic DNA was extracted from 400 to 500 mg of the sediment samples in triplicates, using the Fast DNA™ SPIN Kit for Soil (MP Biomedicals, Santa Ana, CA). The purified DNA was quantified using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, USA). DNA was quality checked on an agarose gel (0.8%). The extracted DNA was pooled and amplified using barcoded fusion primers targeting the v1–v3 region of the 16S rRNA gene using the universal primer 27F (GAGTTTGATCMTGGCTCAG) and 518R (ATTACCGCGGCTGCTGG) (Okubo et al. 2009). Mixed amplicons were subjected to emulsion PCR and then deposited on picotiter plates (Agilent, USA). Amplification conditions consisted of an initial denaturation step at 95°C for 5 min, followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and elongation at 72°C for 30 s, with a final elongation at 72°C for 5 min. The detailed procedure of pyrosequencing is described elsewhere (Suh et al. 2014). Sequencing was performed by Chunlab Inc. (Seoul, Korea) using a 454 GS FLX Titanium Sequencing system (Roche Branford, CT, USA) per the manufacturer's instructions.

Sequence data processing

Amplicon pyrosequencing data were processed using the QIIME software package, ver. 1.7 (Caporaso et al. 2010). Chimaeras and primer mismatch sequences were removed from the amplicon dataset using the Amplicon Noise software, version 1.27 (Quince et al. 2011) available from the FLX Titanium sequence data platform, and implemented in QIIME, a using the program CD-HIT (Edgar 2010). The average read length of PCR amplicons was 378 ± 45 bp. The resulting reads were taxonomically classified based on similarity scores in both the basic local search tool (BLASTN) searches (E-value $>10^{-5}$) on the EzTaxon-e 16S rDNA database (2014.07.01) and on the SILVA SSU database, release 132, based on the RDP classifier method (version14) (Im et al. 2012). Relative abundances of taxonomic groups were estimated using the following cut-off values: species ($x \geq 97\%$), genus ($97\% > x \geq 94\%$), family ($94\% > x \geq 90\%$), order ($90\% > x \geq 85\%$), class ($85\% > x \geq 80\%$) and phylum ($80 > x \geq 75\%$). If the similarity was lower than the specific cut-off value, the sequence was characterized unclassified (un) (Chun et al. 2007); sequences that did not have any cultivable representatives were shortened as 'ucl'. The diversity indices and rarefaction curves were calculated at 97% sequence similarity using the Mothur platform v.1.43.0 (Schloss et al. 2009). The CLCommunity™ software version 3.46 was used for data visualization. Venn diagrams were used to compare sediment bacterial taxonomic composition between sampling sites (Figure 2).

Out of 17,784 reads, 43% were filtered out during the quality processing. Altogether, 5944 reads for the AS sediment, and 4125 reads for the BoB sediment sample were available for further analysis with a mean length of approx. 470–480 bp. In marine sediments, pyrosequencing read numbers varied between 5000 and 20,000 per sample in previous studies (Zhu et al. 2013; Choi et al. 2016), the output of our sequencing approach is in the same order of magnitude. The taxonomic assignment done using the SILVA platform (Quast et al. 2012) resulted in classifying the bacterial sequences into several specific clades, the EzTaxon-e data analysis (Chun et al. 2007) was used for species-level taxonomic assignment.

Functional prediction of 16S rRNA amplicons

For the functional prediction of 16S rRNA pyrosequencing amplicons, the OTUs were clustered at 97% sequence similarity. The OTU table and representative sequence fasta files were submitted to the Piphillin pipeline (https://piphillin.secondgenome.com/) (Iwai et al. 2016). The Piphillin algorithm has the advantage to not rely on phylogenetic trees to predict metagenomic contents. It further uses more recent releases of the functional database Kyoto Encyclopedia of Genes and Genomes (KEGG, updated October 2018) and BioCyc as compared to alternative pipelines such as PICRUSt or Tax4Fun (Narayan et al. 2020). It utilizes nearest-neighbor matching 16S rRNA amplicons (or genomes) to predict the representative genomes. The normalized 16S rRNA copy number of each genome is inferred using gene content collected in functional databases (Langille et al. 2013; Iwai et al. 2016; Narayan et al. 2020). The KEGG reference database was used at a 90% cutoff level to predict metabolic functions present in the sequenced microbial community. The final output of this workflow was quantified in terms of predicted gene abundances per number of OTUs per sample. The information extracted was based on a small fraction of the population available from the KEGG database. At 90% similarity cut off, around 338 KEGG pathways were identified from 156 OTU representatives from the AS

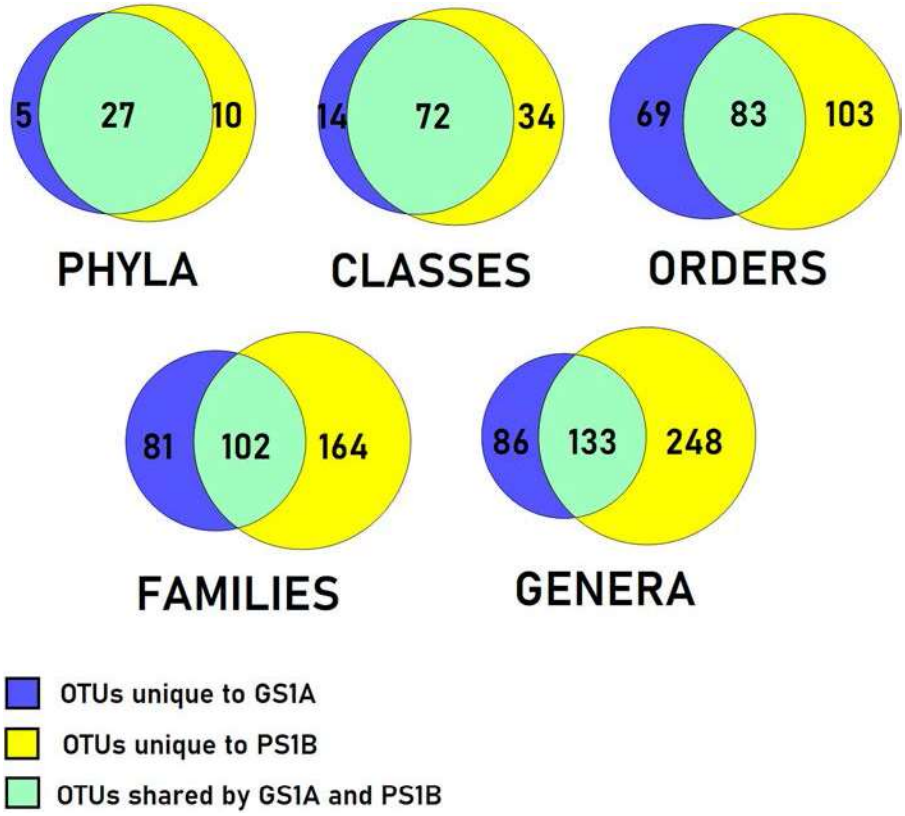

Figure 2. Venn diagram showing OTU number wise comparison of the phylotypes at different taxonomic level assigned through the SILVA database.

and 354 KEGG pathways for 469 OTUs from the BoB. We focused on the KEGG database pathways for nitrogen (ko00910), sulfur (ko00920), and methane (ko00680) turnover, as well as on carbon metabolism (ko01200) with specific focus on fermentation and bioenergetics pathway related to carbon fixation, as they are reported to be prevalent in the OMZ sediment.

Results and discussion

Sediment biogeochemistry

In the present study, both sampling sites showed intense oxygen depletion with dissolved oxygen (DO) concentrations of 2 ± 0.4 µM, at bottom waters which was ~3 m above the sediment sampled. In the shallow zones of the BoB-OMZ and in the AS-OMZ, the DO concentration sometimes falls below the detection limit of conventional methods, especially during the summer monsoon, due to the increased riverine nutrient loading, coastal high primary production and increased respiration (Sarma et al. 2013). Between both sampling sites, bottom water salinity was comparable, but the temperature was 3°C high at the AS sampling site, which may be a seasonal or permanent feature. The sample characteristics of the collected sediment and near-bottom waters are presented in Table I. In brief, total organic carbon was slightly higher in the AS with 3.47%, and 2.24% in the BoB, TN values were 0.28% and 0.16% in the AS and BoB, respectively. The TOC/TN ratio was 8.37 in the AS, and 9.66 in the BoB, thus conditions in the organic matter pool were rather comparable. Here the values are in the typical range of OMZ sediments and higher than non-OMZ surface sediments where the TOC and TN concentration as low as 0.2 and 0.02 wt. % reported (Pattan et al. 2013). OMZs enhance the preservation of organic matter, explaining the reported values of TOC ranging from ~1–2 to 6–7% (Cowie et al. 2014) and TOC/TN ratios within 7.3–12.3 (van der Weijden et al. 1999). Our data is in line with those OMZ ranges, with somewhat lower concentrations for both, TOC and TN, in sediments of the BoB. This may result from generally assumed lower productivity of the BoB waters, compared to the AS rapid nitrogen burial as described for OMZ sediments (Robinson et al. 2012), or different activities in remineralization processes (Bohlen et al. 2011). TIC, which was substantially higher in the AS with 8.11%, compared to

Table I. Sediment and bottom water characteristics for the samples collected from the northern Indian Ocean OMZ.

Sampling details			Sediment characteristics						Near-bottom water profile (CTD)		
Station code	Date	Sampling depth (m)	TOC	TIC	TN %	CaCO3	OM	TOC/TN	DO mM	Temp °C	Salinity PSU
GS1A	Feb-2013	197.3	2.01	8.11	0.28	67.56	3.47	8.37	2.31	15.58	35.35
PS1B	Aug-2014	234.4	1.30	0.29	0.16	2.41	2.24	9.66	1.67	12.33	35.019

the BoB with only 0.29%, while the values falls within the range of OMZ sediments, strikingly higher difference in TIC between the sites could be attributed to the difference in $CaCO_3$ content caused by increased carbon sequestration (Sarma et al. 2007). Additionally, meiobenthic fauna with shells may contribute to the difference in TIC, as this is found to be abundant in sediments of the AS while not abundant in the BoB (Ramaswamy and Gaye 2006). Besides, different microbial communities could explain patterns of carbonate precipitation, a possibility which we will explore in the following.

Benthic bacterial community structure

Between the study sites, the BoB sediments harbour a more diverse bacterial community than the sediments of the AS, which is illustrated not only by the general diversity of taxa and in line with the few available other studies (Dang et al. 2008; Zhu et al. 2013) but also corroborated by various diversity measures as presented in Table II. Given that our rarefaction analysis (A1) showed that our sequencing approach was able to recover ~70% of bacterial phylotypes from the BoB and 90% from the AS sediments, the diversity in the BoB is however still rather underestimated and may be even higher due to the recovery of many rare OTUs.

The dominant communities and their relative percentage remained the same for BLASTN searches using the EzTaxon-e 16S database, and pairwise alignment using the SILVA 132 database. This led to a successful in classification of 44 to 48 phyla, 27 of which were common to both sites. Generally, the dominant bacterial phyla consisted of Firmicutes (33.08%), Proteobacteria (32.59%), Bacteroidetes (17.48%), and Chloroflexi (5.52%) in AS sediments and Proteobacteria (52.65%), Planctomycetes (9.36%), Actinobacteria (7.25%), Firmicutes (5.5%), Acidobacteria (6.74%) and Chloroflexi (4.49%) in BoB sediments. Those abundant taxa contributed >85% to the total bacterial community.

The dominance of Proteobacteria is well documented in marine ecosystems (Wang et al. 2012). In the eastern AS-OMZ surface sediment, nearly 14 phyla were identified in a previous study using the Sanger sequencing technique, the majority of which were Proteobacteria (52%), followed by Planctomycetes (12.7%) and Chloroflexi (8.8%) (Divya et al. 2011). Similarly, in another study carried out utilizing high throughput sequencing confirms Proteobacteria to be the dominant phylum making up 70–75% in all six sites within benthic OMZ of AS followed by Bacteroidetes. Representative sequences affiliated to phyla Chloroflexi and Firmicutes were also recovered in a considerable number (Fernandes et al. 2018). From sediments collected from off Paradeep port, which is roughly 27 nautical miles from our BoB sampling site PS1B, close to 40 bacterial phyla were reported using 454 pyrosequencing approach. The relative contribution of the phylum Proteobacteria was only 17%, which was lesser than Bacteroidetes (23%) and Firmicutes (19%) (Pramanik et al. 2016) indicating a certain patchiness in relative abundance but an overall comparability of the bacterial community composition in the BoB possibly resulting from factors including DO (Stewart et al. 2012), the availability of nutrients or organic carbon (Fierer and Jackson 2006). The BoB receives surplus river water run-off (McCreary Jr et al. 2013), which might be a reason for the observed differences in diversity, as two stations had very different nutrient chemistry, else comparable depth and oxygen depletion levels.

Table II. Summary of pyrosequencing results and statistical analysis of bacterial sequences retrieved from the northern Indian Ocean OMZ surface sediment samples.

		OTU richness[a]				OTU diversity[a]		
Sample name	Optimized reads	Observed	Chao1	ACE	Jackknife	Shannon	Simpson	Good's coverage[b]
GS1A	5944	955	2506	4305	3450	4.37	0.934	89.3%
PS1B	4125	1889	4447	7616	6242	6.97	0.998	69.5%

[a]OTUs (operational taxonomic unit) were calculated using Mothur (3% distance).
[b]Good's coverage is proportional to non-singleton phylotypes.

The candidate phyla GN02, OD1, TM6, TM7, and WS3, were prevalent in ESP (eastern south pacific) pelagic OMZ microbiome as well, implying that they have an essential role in OMZ nutrient cycling (Ulloa et al. 2013; Ganesh et al. 2014). Candidate phyla GN02, OP3, OP8, were unique to both sampled OMZs sediments of the northern Indian Ocean. A total of 13 candidate phyla were obtained in our study. The prevalence of such 'bacterial dark matter' highlights the need to decipher their coding potential, as they cannot be subjected to functional predictions due to a lack of cultivable representatives.

A complete list of taxa is presented in the Supplementary information A2 obtained through SILVA portal. A3 and A4 represents Krona chart of the AS and the BoB sediment bacterial community structure obtained through EzTaxon-e portal. Interestingly, only 28.48% of the identified OTUs were shared between the AS and the BoB, on the genus level (similarity were between 64.29% on the phylum level), leaving 53.10% of unique OTUs in the BoB and 18.42% in the AS (Figure 2). This suggests that the two sediments, while biogeochemically similar, harbour a largely different bacterial community.

The analysis of 58 bacterial classes recovered from our data set showed that there >50% similarities between the phylotypes at the two sites which makes up ~97% of bacteria. The dominant classes in the AS sediment include Bacilli (32.96%), Gammaproteobacteria (18.34%) and Bacteroidia (17.19%). In the BoB sediment, Gamma-, Alpha-, and Delta-Proteobacteria (23.68%, 19.01%, 9.26% respectively) were most abundant, followed by Planctomycetacia (6.72%). Those clades together contribute between 60-70% of the total in the BoB sediment. Dominant bacterial orders recovered exclusively from the AS-OMZ include Bacillales (32.94%), majorly Planococcaceae (26.06%) followed by Flavobacteriales (17.14%), and Oceanospirillales (12.85%). In BoB sediments, Steroidobacterales (7.05%) and Rhizobiales (11.03%) form the most dominant groups. Exploring the taxonomy in more detail, relative abundances for fermenting organisms such as Planococcaceae, Flavobacteriaceae, Bacillaceae, Oceanospirillaceae, Rhodobacteraceae, and Vibrionaceae are strikingly higher in the AS sediment compared to the BoB amongst the abundant clusters (abundant >1%; Figure 3). Those clades are mainly described as heterotroph degraders, mostly able to ferment (Glöckner et al. 1999; Yakimov et al. 2003). The presence of Alcanivoraceae in the AS sediment, and their absence in the sediment of the BoB, could be an important factor in the precipitation of $CaCO_3$, because of their metabolic capability to use ammonification and carbonic anhydrase activity to induce rapid calcium carbonate precipitation (Krause et al. 2018). In the BoB, abundant clades consist mostly of Pseudomonadaceae first described in a deep sea sediment from a Japanese trench [clone AB013829, (Yanagibayashi et al. 1999)] and Desulfobacteraceae, both of which are described as denitrifier groups. Desulfobacteraceae often use acetate (Dyksma et al. 2018) but are also know to degrade other organic compounds (Kümmel et al. 2015). Besides those clades, different proteobacterial clades were found, as well as the purple non-sulfur bacteria Rhodobacteraceae and Rhodospirilliaceae, the latter of which are able to fix molecular nitrogen (Madigan et al. 1984). The double pie-chart provides an overview of both sequenced bacterial communities at the class and family level (Figure 4).

In AS sediments, the most abundant bacterial genus was *Paenisporosarcina* sp. (24.06%), followed by *Salegentibacter* sp. (17%), and as per EzTaxon-e database

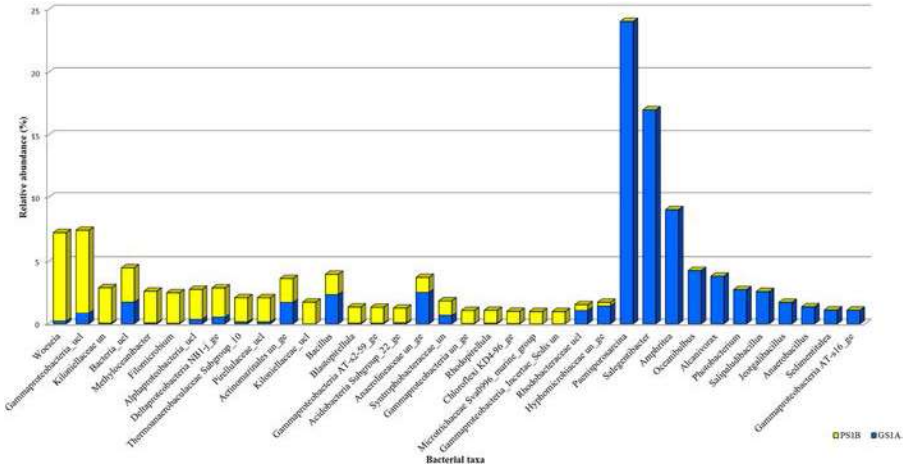

Figure 3. Dominant bacterial taxa retrieved at 1% cut-off based on pairwise alignment in the SILVA SSU database release 132.

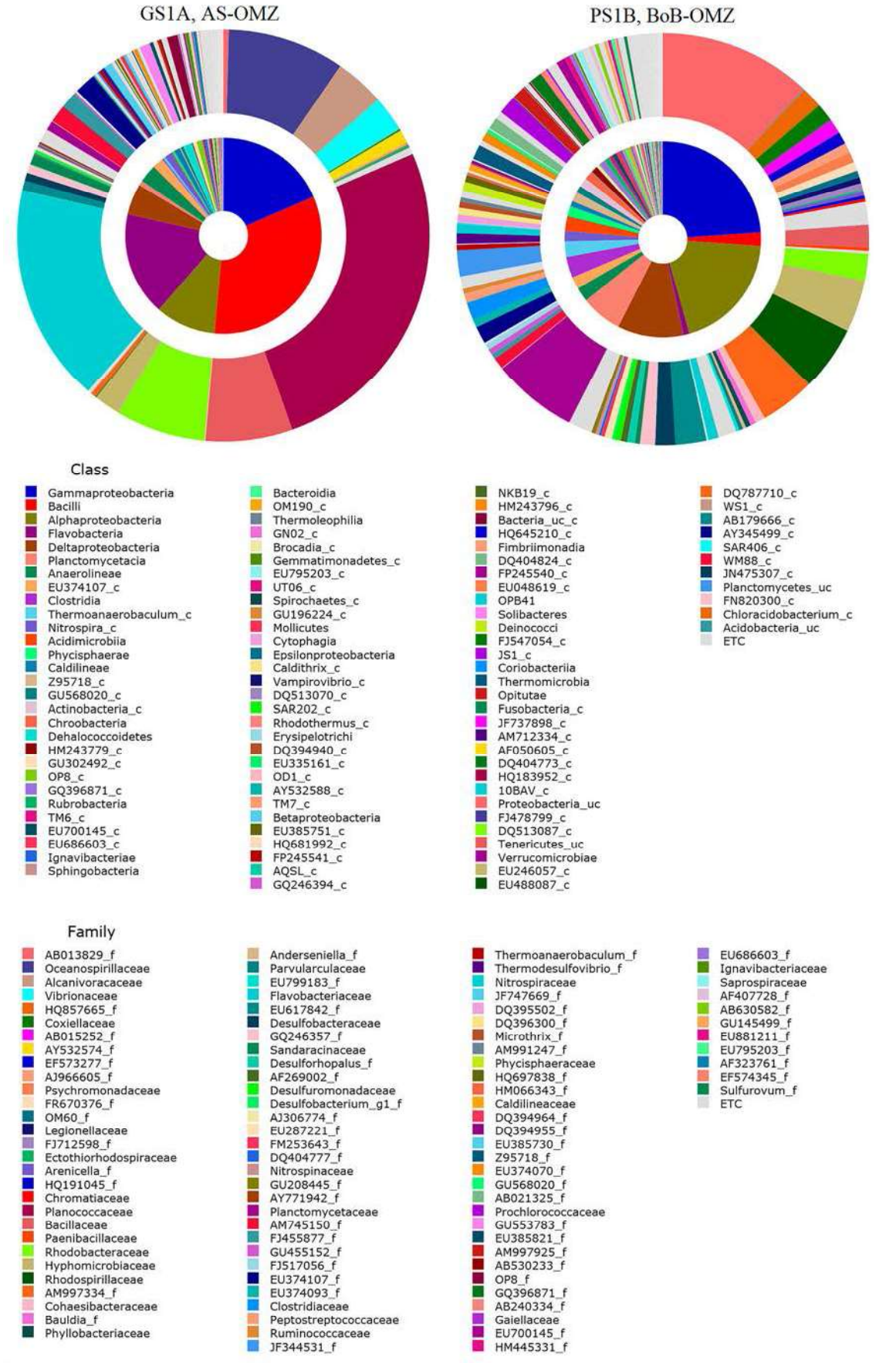

Figure 4. Double Pie chart showing bacterial community composition at the class and family level from the sampling locations based on the EzTaxon-e database.

these were further identified as *Paenisporosarcina quisquiliarum* and *Salegentibacter mishustinae*. Those groups were followed by *Amphritea* (9.02%), *Oceanibulbus* (4.27%), Alcanivorax (3.82%), *Photobacterium* (2.76%) and *Salipaludibacillus* (2.61%). All of these clades are unique to the AS sediment and not present in the BoB sediments. In the BoB sediments, the most abundant taxa are Woeseia (6.98%) and Gammaproteobacteria_ucl (6.5%), with the remaining groups being represented with less than 3%.

The clade Woeseiaceae/JTB255 is recognized as the most abundant clade in marine sediment, having a cosmopolitan distribution. Moreover, analysed metagenomes of JTB255 are known to encode the truncated denitrification pathway to nitrous oxide (Mußmann et al. 2017). Since denitrification mediated nitrogen loss is reported to be dominant in Arabian Sea OMZ, we expected to get more hits in the analysed amplicon dataset (Ward et al. 2009). Though their occurrence was not detected, few representative sequences of JTB31 and JTB38 were identified that might play a similar role.

Predicted functional ecology

For a large proportion of the amplicons, functions could not be assigned clearly, which leads to a rather conservative, and qualitative instead of quantitative estimate of the metabolic potential present at the two sites. The predictive functional profiling of 16S rRNA sequences has identified a high proportion of genes involved in methane cycling, as generally typical for sediments underlying OMZ waters (Fulweiler et al. 2007; Bertics et al. 2013; Gier et al. 2016), followed by genes involved in sulfur and nitrogen cycling (Figure 5). Methane turnover rates are rather high in anoxic shelf sediments and were reported to be correlated with the availability of labile organic matter, and concurrent with sulfate reduction (Maltby et al. 2016), explaining the predicted abundance of genes involved in methane turnover in our samples. Despite the difference in diversity between the two sampling sites, almost all predicted gene functions were identical suggesting an overall similar metabolic potential in the two different sediments.

Nitrogen cycle: In northern Indian Ocean OMZs, nitrogen cycling is reported to be very active (Naqvi et al. 2006). At both our sampling sites, genes coding for major nitrogen cycle pathways including nitrogen fixation, dissimilatory nitrate reduction to ammonia (DNRA), nitrification, and denitrification were predicted. Interestingly, anammox genes were not predicted for either site despite the presence of planctomycetes in our dataset (Figure 6). This may be either due to the low number of species-level identifiable OTUs or to a true absence of anammox-capable planctomycetes as consistent with OMZ sediments from the seasonally anoxic Eckernförde Bay in the Baltic Sea and sediments underlying the Peruvian OMZ (Dale et al. 2011; Bertics et al. 2013). While generally planctomycetes were in the sequence pool, hits corresponding to anammox planctomycetes were indeed exceptionally low at our sampling sites, accounting for 0.03 and 0.3% in the AS and BoB, respectively. A sequencing related bias could, however, have led to an underestimation of *Scalindua*-anammox bacteria as a systematic underrepresentation by sequencing of 16S rDNA v1-v3 regions has been reported (Penton et al. 2006). Specifically,

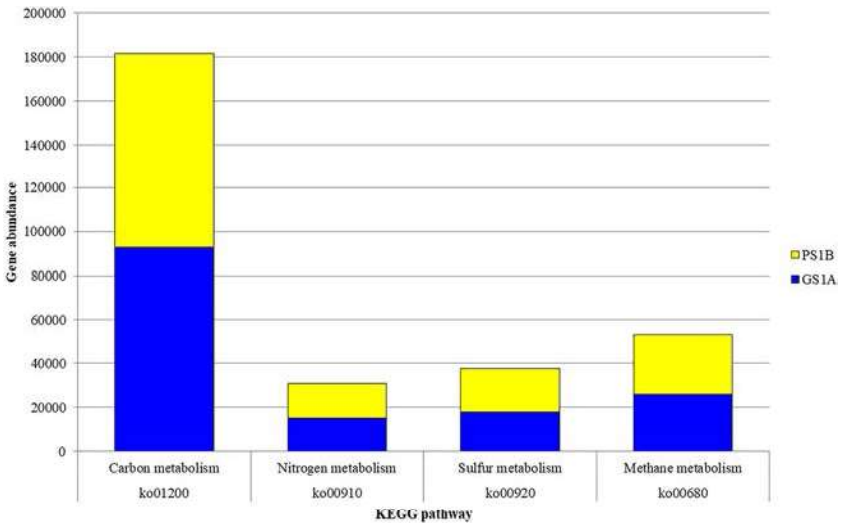

Figure 5. Relative distribution of redox metabolic KEGG pathways identified from our 16S rRNA amplicon pyrosequencing dataset utilizing the Piphillin algorithm.

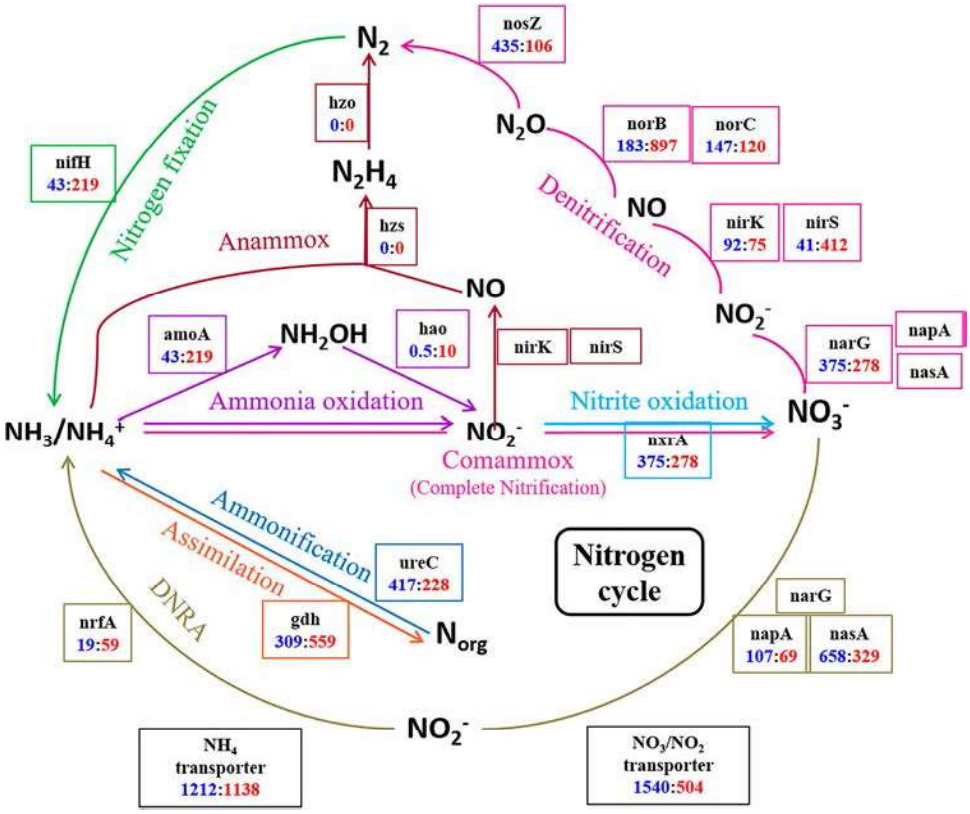

Figure 6. Proposed pathway for OMZ Nitrogen cycling in sediments of northern Indian Ocean OMZ, and the abundance of enzymes with coding genes are indicated in box, where 'blue' and 'red' denotes AS and BoB gene count. Expansion of abbreviation are as follows: **amoA:** Ammonia monooxygenase subunit A; **gdh:** Glutamate dehydrogenase; **hao:** Hydroxylamine oxidoreductase; **hzo:** Hydrazine oxidoreductase; **hzs:** Hydrazine synthase; **napA:** Nitrate reductase (cytochrome); **nasA:** Nitrate reductase; **narG:** Nitrate reductase, alpha subunit; **nifH:** Nitrogenase iron protein; **nirK/nirS:** Nitrite reductase subunit K/S; **norB/norC:** Nitric oxide reductase subunit B/C; **nrfA:** nitrite reductase (cytochrome c-552); **nosZ:** Nitrous oxide reductase; **nxrA:** nitrite oxidoreductase, alpha subunit; **ureC:** Urease subunit alpha. (Color online)

the functional marker gene coding for the hydrazine oxidoreductase was not predicted from our 16S rDNA data. This suggests that the contribution of anammox to the nitrogen cycle in the Indian Ocean sediments, at least at our sampling sites, is rather low similar to the pelagic OMZ of the AS where denitrification is reported to be dominant over anammox (Ward et al. 2009), and the BoB where anammox as well as denitrification could not be detected (Bristow et al. 2017). As Planctomycetales are known to encode a large number of sulfatase genes, which makes them as a specialist for the initial breakdown of sulfated heteropolysaccharides (Wegner et al. 2013), their role in the Indian Ocean sediments could rather be carbon capture in the sediments (Arango et al. 2007; Shao et al. 2010; Dale et al. 2011; Jensen et al. 2011). Here, the predicted gene abundance was 848 and 2901 for AS and BoB microbiome, the predominant form being arylsulfatase, respectively, contributing 65–85% of the sulfatase pool.

The global annual denitrification rate in sediment would be approximately 200 Tg N, and the majority contributed from sediments underlying OMZ, where its reported two to four times higher (Devol 2015). Therefore, nitrogen loss processes would be expected to take place in both, sediments of the AS and the BoB. Denitrification and sulfite reductase genes were prevalent in our prediction possibly favouring sulfur driven autotrophic denitrification (Shao et al. 2010), and as previous studies suggested heterotrophic denitrification (Arango et al. 2007). Other denitrifiers recovered from our sequence dataset are Oceanospirillales, Chromatiales, Nitrospirales, Syntrophobacteriales, and NB1-j which are known to encode denitrification genes including *nirS, norB* and *nosZ* (de Voogd et al. 2015), and contributed 14.05% in the AS sediment, and 4.46% in the BoB sediment, respectively. Similarly, Flavobacteriales are known denitrifiers (Horn et al. 2005), and are abundant in the AS with 17.14% of all 16S rDNA sequences.

Recent studies have also linked methane oxidation to nitrite-based denitrification in the Candidatus phylum NC10 (Padilla et al. 2016). This was supported by studies carried out in a freshwater reservoir, where methane stimulated massive nitrogen loss (Naqvi et al. 2018). The Steroidobacter clades which make up 7% of the BoB-OMZ bacterial community are also known to perform denitrification coupled with methane oxidation (Liu et al. 2014). Though, our predictive functional profiling confirms denitrification is dominated over anammox in the northern Indian Ocean OMZ as reported (Ward et al. 2009; Sarkar et al. 2020), a clear link with methane oxidation cannot be established, since the BoB-OMZ is not an active denitrification site. Further, isotopic studies carried out by Shirodkar et al. (2018) in the seasonally anoxic Shelf waters of the Arabian Sea did not show any effect of additional CH_4 amendments on N_2 production through denitrification.

DNRA was predicted as a potential remineralization pathway in both basins of our study. In seasonally hypoxic Baltic Sea sediments, DNRA accounted for almost 75% of benthic nitrogen flux (Dale et al. 2011). In contrast to the other nitrogen cycle genes, the *nifHDK* operon coding for the functional unit of the key gene for nitrogen fixation, the nitrogenase, was predicted in higher abundance in the BoB compared to the AS, with BoB-nif being five times as many as AS-nif. This is consistent with the higher proportions of known sedimentary nitrogen fixers, such as Desulfobacteraceae and Rhodospirilliaceae. The presence of nitrogen fixers in sediments underlying OMZs has been documented for several regions, including the upwelling system off Mauretania, the Baltic Sea and the eastern tropical South Pacific shelfs, and nitrogen fixing microbes have been shown to be active although at low rates (Bertics et al. 2013; Gier et al. 2016, 2017).

Sulfur cycle: For the sulfur cycle in both, the AS and the BoB, genes for the assimilatory pathway of sulfate reduction were predicted, as well as sulfur oxidation genes of the *sox* operon (Figure 7) in line with a previous study which identified diverse sulfur reducing bacterial and archaeal OTUs in the AS (Fernandes

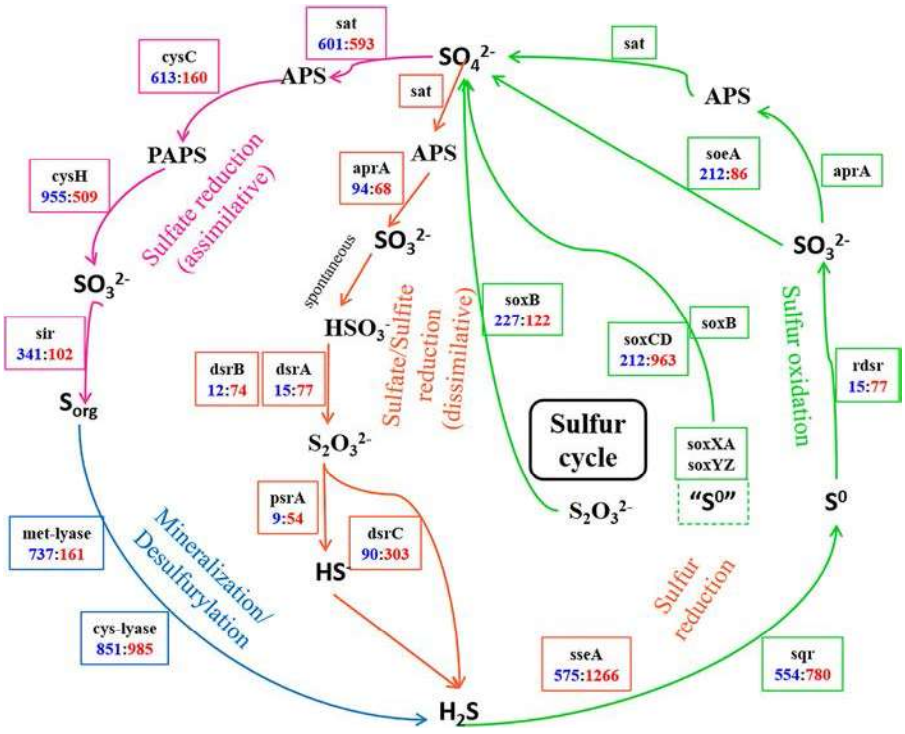

Figure 7. Proposed pathway for OMZ Sulfur cycling in sediments of northern Indian Ocean OMZ, and the abundance of enzymes with coding genes are indicated in box, where 'blue' and 'red' denotes AS and BoB gene count. Expansion of abbreviation are as follows: **aprA:** Adenylylsulfate reductase, subunit A; **dsrA/dsrB:** Dissimilatory sulfite reductase subunit-alpha/beta; **dsrC:** Dissimilatory sulfite reductase related protein; **cysC:** Adenylylsulfate kinase; **cysH:** Phosphoadenosine phosphosulfate reductase; **psrA:** Thiosulfite reductase; **rDsr:** Reverse dissimilatory sulfite reductase; **sat:** Sulfate adenylyltransferase; **soeA:** Sulfite:quinone oxidoreductase; **sir:** Sulfite reductase (ferredoxin); **soxB:** S-sulfosulfanyl-L-cysteine sulfohydrolase; **soxC:** Sulfane dehydrogenase; **soxD:** S-disulfanyl-L-cysteine oxidoreductase; **soxX/A:** L-cysteine S-thiosulfotransferase; **soxY/Z:** Sulfur-oxidizing protein; **sseA:** Thiosulfate/3-mercaptopyruvate sulfurtransferase; **sqr:** Sulfide:quinone oxidoreductase. (Color online)

et al. 2018). In our AS dataset, a potential player in the sulfur cycle could be *Sulfitobacter dubius*, which was represented with 4.32% of all OTUs. All known species of the genus *Sulfitobacter* were isolated from marine habitats and are known to perform sulfite oxidation (Sorokin 1995; Long et al. 2011). *Thermodesulfovibrio* (phylum Nitrospira) accounted for ~1% of sequences at both sites are known sulfate reducers and have been identified from the eastern tropical South Pacific OMZ, before (Schunck et al. 2013). Sequences corresponding to sulfur reducers like Desulfobacterales (AS: 0.87%, BoB: 2.57%) and Syntrophobacterales (AS: 0.67%, BoB: 1.21%) were also recovered from our dataset and were shown to be abundant in sediments of the Black Sea sulfate-methane transition zone as well as in the Arabian Sea OMZ in both pelagic and benthic realms (Fuchs et al. 2005; Leloup et al. 2007; Fernandes et al. 2018).

Carbon fixation: In the BoB sediment, around 1.75% of gene families were predicted to perform photosynthesis, and major contributors would possibly be Chromatiales (0.07%), Rhodospirillales (0.03%), and members of phylum Cyanobacteria (1.65%). Chromatiales, a group of purple sulfur bacteria, can perform anoxygenic photosynthesis (Manske et al. 2005). Similarly, Rhodospirillalesis primarily chemoorganotroph and photoheterotroph (Luo and Moran 2015), can also perform anoxygenic photosynthesis (Manske et al. 2005). It is interesting to note that around 68 Cyanobacterial sequences belonging to order Chroococcales of genus *Prochlorococcus* were retrieved from BoB sediment, where water column depth was ~245 m, but only one representative from the AS sediment, which was located at ~200 m depth. The observed Chroococcales, are assumed to be a low-light adapted group (West et al. 2001) justifying their occurrence in deeper sites, where light penetration is negligible. Since these groups are also known to fix nitrogen (Van Goethem et al. 2017), will justify the observed difference in *nifH* gene abundance. It may also be that this is sunk out debris, with some preserved DNA. The key enzymes responsible for energy metabolism are presented in Table III. In particular, the higher predicted abundance of dehydrogenase enzymes responsible for oxidation of organic matter in the AS points towards a difference in carbon metabolism in the two regions. This is most likely due to the increased availability of carbon in the AS, however, as proposed earlier (Orsi et al. 2017), more efficient organic carbon recycling in the AS may over geological timescales contribute to developing a stronger and more persistent functional anoxia.

Carbon remineralization: As indicated by the high TIC concentration in the AS sediment, carbon remineralization was very active due to the increased availability of organic carbon (Yu et al. 2018). About 20% of the identified bacteria were common soil/sediment inhabitants with a prime role in remineralization of diverse organic carbon compounds (Schimel and

Table III. Distribution of key enzymes relevant in energy metabolism in the northern Indian Ocean surface sediments predicted from 16S rRNA genes.

Energy metabolism	KEGG	Enzymes	GS1A	PS1B
Kreb cycle, NADH + H+	K00161	pyruvate dehydrogenase E1 component alpha subunit [EC:1.2.4.1]	836	454
Kreb cycle, NADH + H+	K00627	pyruvate dehydrogenase E2 component (dihydrolipoamide acetyltransferase) [EC:2.3.1.12]	1279	1108
Kreb cycle, NADH + H+	K00382	dihydrolipoamide dehydrogenase [EC:1.8.1.4]	2476	1889
Kreb cycle, NADH + H+	K00031	isocitrate dehydrogenase [EC:1.1.1.42]	993	1345
Kreb cycle, NADH + H+	K00164	2-oxoglutarate dehydrogenase E1 component [EC:1.2.4.2]	895	850
Kreb cycle, GTP	K01902	succinyl-CoA synthetase alpha subunit [EC:6.2.1.5]	1064	952
Kreb cycle, FADH2	K00239	succinate dehydrogenase / fumarate reductase, flavoprotein subunit [EC:1.3.5.1 1.3.5.4]	902	906
Kreb cycle, NADH + H+	K00024	malate dehydrogenase [EC:1.1.1.37]	1051	881
Glycolysis, ATP	K00927	phosphoglycerate kinase [EC:2.7.2.3]	898	953
Glycolysis, ATP	K00873	pyruvate kinase [EC:2.7.1.40]	955	1012
Glycolysis, NADH + H+	K00134	glyceraldehyde 3-phosphate dehydrogenase [EC:1.2.1.12]	2125	1176
Glyoxylate cycle, NADH + H+	K00024	malate dehydrogenase [EC:1.1.1.37]	1051	881
Glyoxylate cycle, FADH2	K00240	succinate dehydrogenase / fumarate reductase, iron-sulfur subunit [EC:1.3.5.1 1.3.5.4]	916	926
Pentose phosphate pathway, NADPH	K00036	glucose-6-phosphate 1-dehydrogenase [EC:1.1.1.49 1.1.1.363]	678	856
Pentose phosphate pathway, NADPH	K00033	6-phosphogluconate dehydrogenase [EC:1.1.1.44 1.1.1.343]	823	807
Ethylmalonyl pathway, NADP+	K14446	crotonyl-CoA carboxylase/reductase [EC:1.3.1.85]	230	276
Ethylmalonyl pathway, GTPase activity	K01847	methylmalonyl-CoA mutase [EC:5.4.99.2]	1386	669
Ethylmalonyl pathway, NADP+	K00023	acetoacetyl-CoA reductase [EC:1.1.1.36]	309	345
Ethylmalonyl pathway, ADP + Pi	K01965	propionyl-CoA carboxylase alpha chain [EC:6.4.1.3]	575	329
Ethylmalonyl pathway, ADP + Pi	K01966	propionyl-CoA carboxylase beta chain [EC:6.4.1.3 2.1.3.15]	746	351
Malonate semialdehyde pathway, ADP + Pi	K01961	acetyl-CoA carboxylase, biotin carboxylase subunit [EC:6.4.1.2 6.3.4.14]	1212	938
Propanoyl-CoA metabolism, ADP + Pi	K01965	propionyl-CoA carboxylase alpha chain [EC:6.4.1.3]	575	329

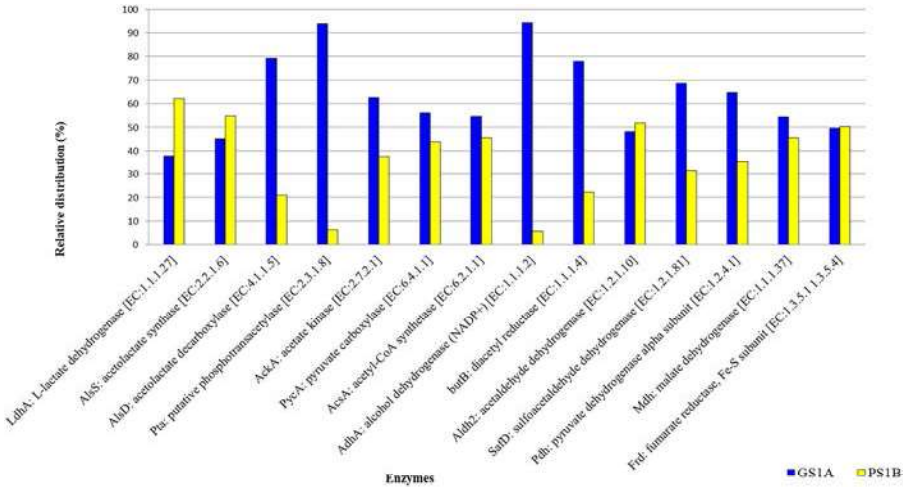

Figure 8. Percentage distribution of key enzymes with coding genes identified in bacterial fermentation (Ramos et al. 2000; Eschbach et al. 2004).

Schaeffer 2015). These include Acidobacteria, Actinobacteria, Bacteroidetes, and Gemmatimonadetes (Janssen 2006). Similarly, Anaerolinaeles (phylum Chloroflexi), which contributed 2–3% of the total hits in our dataset, have also been identified with a similar role and were specific to areas that show incredibly low or zero oxygen. In addition, genes responsible for N-glycan degradation (ko00511) were predicted to occur almost 12 times more often in the AS than in the BoB sample. These genes play a role in cell adhesion and sequestration (Varki and Gagneux 2017). The relative distribution of key enzymes and genes specific to gram-positive and gram-negative bacterial fermenters were compared based on previous reports (Ramos et al. 2000; Eschbach et al. 2004) (Figure 8). Their predicted abundance was higher in AS sediment than in BoB sediments. The connected more complete carbon remineralization which could add an explanation to why the AS-OMZ is more anoxic than the BoB-OMZ as previously suggested (Orsi et al. 2017).

Conclusion

We compared bacterial communities from two sites in the northern Indian Ocean OMZ, in the BoB off Paradeep and a site off Goa in the AS. Less than one-third of the phylotypes were shared between the two sites, leaving a large individual proportion of the bacteria for each site. A higher diversity has been identified from the BoB, compared to the AS, however, our functional prediction identified high abundances of typical heterotrophic degraders in the AS, that were only represented in low proportions or absent in the BoB. We further identified denitrifiers, DNRA bacteria and sulfur cycle bacteria at both sites and predicted the presence of their functional genes. The higher functional diversity for organic matter degradation with fermentation in addition to denitrification and sulfur-compound dependent remineralization may explain, why the AS OMZ is generally more anoxic. Here, the variability in carbon respiration pathways may allow for a more efficient or complete respiration along the electron tower, thus consuming more oxidized compounds. The abundance of *Alcanivorax*-like bacteria in AS sediments may provide an explanation for high $CaCO_3$ precipitation, as this organism has been described to perform this process rapidly when organic nitrogen is available as it is at our sampling site in the AS. A notable finding was the absence of anammox bacteria at both sites. Notably, we predicted nitrogen fixation genes from BoB sediments but not from AS sediments, possibly resulting from higher nitrogen inputs from the water column in the AS.

Despite the limitation of this study with regard to our sample number, we could contribute a first assessment of bacterial diversity and functionality in coastal sediments of the two Indian Ocean basins, as such, we hope to contribute to the general understanding of how these basins function and why they are so different in their biogeochemistry.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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Data availability statement

All pyrosequencing reads were submitted to the NCBI GenBank database under accession number KU821783 – KU831324 and MG860544 – MG860851.

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16S rRNA and hydrazine gene-based profiling of the Candidatus *Scalindua* community from the Arabian Sea hypoxic sediments

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Anammox bacterial diversity and abundance were studied from the organic-rich hypoxic sediments of the Arabian Sea utilizing the partial 16S rRNA, and hydrazine synthase, *hzs*A and hydrazine oxidoreductase, *hzo* genes. Among all the clades obtained, phylotypic diversity was high within the Candidatus genus *Scalindua* with an abundance of $\leq 7 \times 10^4$ copies/g dry wt. As such, *Scalindua* is known to play a significant role in fixed nitrogen removal through anaerobic ammonium oxidation (anammox) pathway. From these analyses, it is inferred that searching for *hzo* gene yields robust evidence for detecting anammox community than the widely used 16S rRNA gene marker.

Keywords: Anammox, community gene-based profiling, hydrazine, hypoxic sediments, *Scalindua*.

THE oxygen-depleted zones (ODZs) are important sites of fixed nitrogen loss, as the scarcity of oxygen, a primary electron acceptor, leads to increased utilization of the next preferred electron sources[1]. Anaerobic ammonium oxidation (anammox) and denitrification are the two major microbial pathways operating in such an environment which are responsible for nitrogen removal[2]. One of the questions being examined in the perennial ODZ localized within the Arabian Sea and the eastern tropical north and the South Pacific Ocean is whether anammox is dominant over denitrification[2,3], and is chiefly inquired utilizing chemical signatures. The anammox reaction, in general, requires ammonia (NH_4^+) and nitrite (NO_2^-) as substrates in a stoichiometric ratio of approximately 1 : 1 and proceeds via two intermediates, nitric oxide (NO) and hydrazine (N_2H_4), and releases di-nitrogen gas (N_2)[4]. Understanding molecular pathways of anammox reactions led to gene-targeted studies, which further made anammox bacterial identification and quantification possible even at species level[5,6]. Yet such information is not utilized properly, and those who attempted molecular characterization have limited themselves to one or two primer sets alone, that too without considering the primer bias.

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The nitrogen loss contribution of anammox to the total denitrification in marine sediments can range from near 0% to 80%, especially in deposits underlying ODZ[7]. In the southeastern Arabian Sea, benthic nitrogen metabolism is driven by sinking organic matter[8] which is exceptionally high during the southwest monsoon period[9]. The prevailing conditions are assumed to favour heterotrophic denitrifying communities that rely on organic sources[10]. Hence the possible occurrence of a chemolithoautotroph like anammox microbes is not studied thoroughly. Besides ammonium ion available *in situ*[11], dissimilatory nitrate reduction to ammonia (DNRA) is reported[1]. In the Oman coast of the Arabian Sea, a coupling of DNRA to anammox resulted in intense nitrogen loss[12], suggesting that anammox occurrence is controlled by the availability of substrates, and the dominance of denitrifiers, in particular, cannot limit anammox bacterial abundance in ODZ.

In comparison to the denitrifying microbial community, little is known about the anammox community. All identified anammox clades have monophyletic origin and are classified under bacterial phylum Planctomycetes and order 'Brocadiales'[13]. To date, seven anammox genera are reported[13–19]: Ca. *Brocadia*, Ca. *Kuenenia*, Ca. *Scalindua*, Ca. *Anammoxoglobus*, Ca. *Jettenia*, Ca. *Brasilis* and Ca. *Anammoximicrobium* (placed under separate order Pireullulaceae). Genus *Scalindua* is the most diverse as well as the dominant anammox community identified in the marine environment. It is characterized as a separate sub-group, as its distribution is primarily governed by high salinity[20]. The phylogenetic 16S rRNA marker gene and functional markers targeting hydrazine genes, a biomarker unique to anammox reaction, have been successfully applied and tested in various habitats to understand the anammox community structure[5,21,22].

In the present study, we utilized five well-established, highly specific anammox-specific primer sets to target partial 16S rRNA, hydrazine synthase gene subunit A (*hzs*A) and hydrazine oxidoreductase gene (*hzo*). The functional gene primers are known to target both *Scalindua* and non-*Scalindua* anammox communities, whereas the 16S rRNA primer specifically targets the *Scalindua*-like anammox community. We screened for multiple

Table 1. Details of PCR primers used in the present study

Gene target	Use	Primer	Sequence	Reference	Base pair	Primary melting temperature
Scalindua 16S rRNA	Clone library	Brod541F Brod1260R	GAGCACGTAGGTGGGT TTGT GGATTCGCTTCACCTCT CGG	29	719	59
Anammox *hzo*	Clone library	HZOF1 HZOR1	TGTGCATGGTCAATTG AAAG CAACCTCTTCWGCAGG TGCATG	30	1000	53
Anammox 16S rRNA	qPCR	Brod541F Amx820R	GAGCACGTAGGTGGGT TTGT AAAACCCCTCTACTTAGTGCCC	33	279	60
Anammox *hzs*A	qPCR	hzA1597F hzA1857R	WTYGGKTATCARTATG TAG AAABGGYGAATCATAR TGGC	5	261	60
*hzo*_cluster2 for non-*Scalindua*	qPCR	hzocl2aF hzocl2aR1	GGTTGYCACACAAGGC TYWACCTGGAACATAC CC	32	289	60
Cloned gene fragment	Sequencing PCR	M13F M13R	GTTTTCCCAGTCACGA C CAGGAAACAGCTATG AC	59	Variable	50

genes to assess phylotypic diversity of anammox community, including the genus Ca. *Scalindua* using multiple sets of primers that targeted to amplify functional (anammox-specific) and ribosomal (taxonomy-specific) fragments and are assumed to occur in high diversity and abundance in oxygen-depleted, organic-rich surface sediments of the southeastern Arabian Sea. In parallel, we also tested the possible occurrence of non-*Scalindua* anammox community. To our knowledge, there are no previous reports from the benthic ODZ of the Arabian Sea exclusively targeting *Scalindua*, a dominant anammox bacterial genus.

Materials and methods

Sampling details

The sediment sample was collected during the SSD-014 cruise of *R V Sindhu Sadhana* from the Arabian Sea at around 600 m water-column depth (9°57′N, 75°32′E). Sampling was carried out in September 2015, which marks the end of the southwest monsoon period. The Van Veen Grab sampler was used for sediment sample collection. The samples were handled aseptically and preserved at –20°C until further analysis. Temperature and salinity profiling of the sampling location was carried out using a Sea-Bird Electronics CTD (conductivity–temperature–depth; model SBE9), fitted with Niskin/Go-Flo bottles. The dissolved oxygen (DO) profile of the location was also obtained using a calibrated sensor (RINKO from ALEC, Japan) attached to the CTD unit.

Chemical characterization of sediment

The sediments were freeze-dried, homogenized and ground to a fine powder in an agate mortar before analysis. Total carbon (TC) and total nitrogen (TN) were analysed using a CN analyser (FISONS NA1500)[23]. Total organic carbon (TOC) was determined using a colorimetry-based wet oxidation method with high reproducibility[24]. Total inorganic carbon (TIC) was estimated by subtracting OC from TC[25]. To estimate organic matter (OM), TOC was multiplied by Van Bemmelen's factor 1.724, based on the assumption that humidified OM of the soil contains 58% carbon, but it could vary from 40% to 60% (ref. 26). For $CaCO_3$ calculation, TIC was multiplied by 8.33 to obtain the relative percentage[25]. The OC/TN ratio was converted into molar ratio by multiplying with a factor 1.167, derived from the atomic weights of nitrogen and carbon[27].

Metagenomic analysis

Total genomic DNA was extracted from 500 mg of the freeze-dried sediment samples using the Fast DNA™ SPIN Kit for Soil (MP Biomedicals, Santa Ana, CA, USA), and for cell lysis the Fastprep 24 cell disruptor was used based on the manufacturer's instructions. Fastprep is one of the most successful and efficient sediment DNA extraction methods that yields reasonably good DNA quality and quantity[28]. The purified DNA was quantified using a nanodrop 2000 spectrophotometer (ThermoScientific, USA) and visualized on an agarose gel (0.8%) to determine the quality of the extracted DNA. The gel was viewed using the AlphaImager Gel documentation system after staining with ethidium bromide (EtBr). PCR was carried out using *Scalindua*-specific 16S rRNA primer set, Brod541F/Brod1260R (ref. 29) and anammox primer set HZOF1/HZOR1 for hydrazine oxidoreductase gene[30]. Table 1 lists the primers used in the present study. The PCR conditions maintained were as follows: initial denaturation at 95°C for 5 min, 35 cycles of denaturation: 94°C for 60 sec, annealing temperature was 59°C for 16S rRNA and 53°C for *hzo* genes for 60 sec, extension: 72°C for 90 sec, followed by a final extension at 72°C for 10 min. The PCR reaction was carried out in a 0.2 ml reaction tube in a final volume of

50 µl. The reaction mix contained 1× PCR buffer, 1.5 mM $MgCl_2$, 10 mM dNTPs, 10 pM each of forward and reverse primers, and 0.32 U of *Taq* polymerase enzyme. About 1 ng of template DNA that was diluted to 0.1–0.2 ng/µl was used. All PCR components were purchased from Genei, Bangalore.

PCR products were purified prior to TA (thymine–adenine) cloning using the GenElute™ PCR clean-up kit (Sigma-Aldrich, USA) and quantified. Cloning was performed using the pGEM®-T Easy Vector System (Promega, USA) based on the manufacturer's instructions. Positive recombinant was screened using the X-gal-IPTG LB indicator plate amended with 100 µg/ml ampicillin. The transformation efficiency for anammox *hzo* and 16S *Scalindua* was 9×10^7 and 1.9×10^8 cfu/µg DNA respectively. Plasmid extraction was performed using GenElute™ Plasmid Miniprep kit (Sigma-Aldrich, USA). Insert size was checked using M13F/M13R primer as well as targeted primers. The positive clones were sequenced using ABI 3130XL genetic analyzer (Applied Biosystems, USA).

Sequence analysis and processing

Sequence quality was checked using SeqScanner software v.1.0 (Applied Biosystems, USA), 2005. Good-quality sequences were further screened for vector contamination through the NCBI VecScreen portal (https://www.ncbi.nlm.nih.gov/tools/vecscreen/) and edited using BioEdit software v.7.2.6.1. Sequence similarity search was carried out using the NCBI BLAST algorithm. Misaligned sequences were corrected using sequence massager online (http://biomodel.uah.es/en/lab/cybertory/analysis/massager.htm). Chimera check was performed during the sequence submission step at GenBank. Most similar hits, as well as standard reference sequences, were included for phylogenetic tree construction. Sequences with a length of ≥500 bp were used for diversity and phylogenetic analysis. The sequences were aligned using CLUSTAL-W multiple sequence alignment tool in BioEdit software v.7.2.6.1. The phylogenetic tree was constructed using MEGA X software neighbour-joining method with 1000 bootstrap replicates. For functional genes, the nucleotide sequences were first translated to amino acids through the ExPASY online portal (https://web.expasy.org/translate/) prior to detailed analysis.

Statistical analysis

Clones were clustered into operational taxonomic units (OTUs) using Mothur v.1.35.1 after generating a distance matrix in BioEdit v.7.2.6.1. A 97% similarity cut-off was used for genus-level clustering. For *Scalindua* 16S rRNA, 0.5% distance was chosen and for *hzo* (gene-translated protein sequence) 1% distance was used[6]. Coverage of clone library was calculated as $C = [1 - n_i/N] \times 100$, where n_i is the total number of organisms of each species and N is the total number of organisms of all species. For diversity calculation, Shannon (H') and Simpson (D') diversity indices were used. Pielou's index (J') was used to understand species evenness. For species richness, abundance-based coverage estimator (S_{ACE}) and evenness bias-corrected (S_{Chao1}) were used. For diversity estimation, statistical software Primer v.6.1.10 and Estimate S v.9.1.0 were used.

Quantification of anammox genes

For qPCR-based gene quantification, three anammox-specific genes were targeted: 16S rRNA, *hzs*A and *hzo*_cluster[2]. Primer pair Brod541F/Amx820R used for 16S rRNA gene is known to target all environmental anammox clusters[30]. However, previous studies reveal that this primer set amplifies only genus *Scalindua*[31]. The functional genes specifically targeted *hzs*A common to all known anammox communities[5] and non-*Scalindua*-specific *hzo*_cluster2 gene[32]. The gene target size varied between 260 and 290 bp for qPCR estimation, while primers used for diversity studies targeted a 720 and 1000 bp fragment, making it not suitable for qPCR-based gene quantification as it generates non-specific fluorescence. The specificity of plasmid clones prepared for the standard curve was checked using BLAST search, and clones with maximum similarity were only used (MG687445 (100%), MG687463 (96%), and MG687465 (83%)). These plasmid standards were generated from the ODZ surface sediments underlying 200 m water-column depths of the Arabian Sea, off Goa site. Standard curves were determined by analysing ten-fold serial dilutions of linear plasmid containing an insert of choice, with linear regression of C_T values plotted against an initial copy number on a log scale from 10^{-1} to 10^{-8}. Amplification factor and PCR efficiency were calculated from the slope using qPCR efficiency calculator available on-line (ThermoFisher, USA). The qPCR was performed in triplicate in 20 µl final reaction volume containing 1–1.25 ng of sediment-extracted DNA diluted to 0.2 ng/µl, 10 µl of 2X Sybr fast master mix (Kapa Biosystems, USA), 1X ROX reference dye (low), 10 pmol each of forward and reverse primers on a 7500 Fast-Real-Time PCR system (Applied Biosystems, CA, USA). The PCR programme was initiated with a denaturation step spanning 15 m at 95°C, followed by 40 cycles of 95°C for 15 sec, 60°C for 60 sec and 72°C for 30 sec. Fluorescence was detected at PCR extension at 72°C. Melt curve analysis was performed at the end of 40 cycles to check the specificity of amplification. Gel electrophoresis confirmed that only the right size fragment was amplified. The gene copy number was calculated from the C_T value applied to the regression formula generated from the standard curve (log

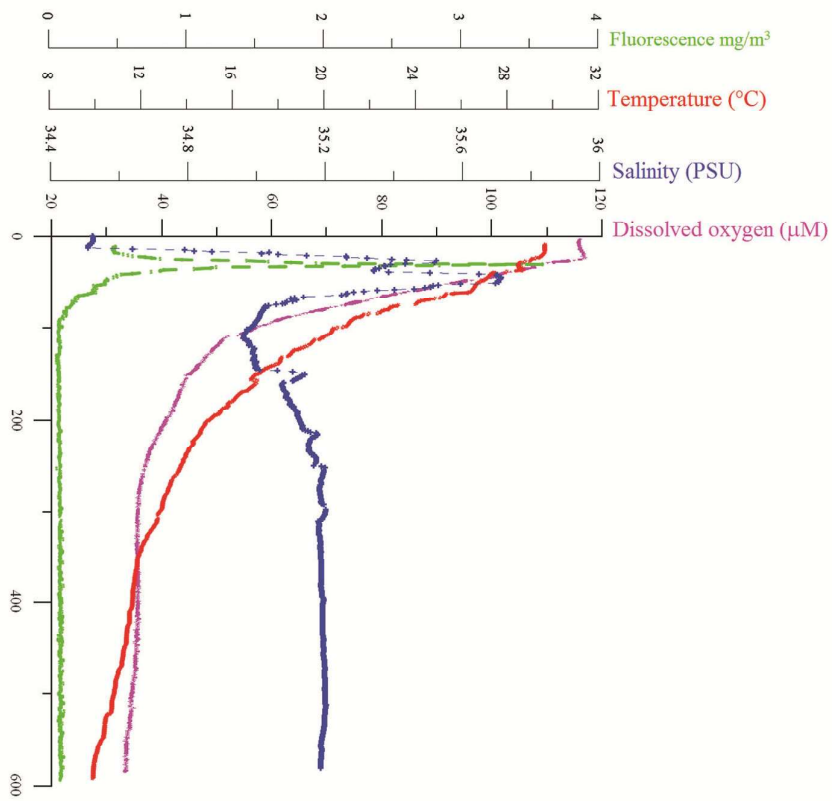

Figure 1. CTD profile of K-600 station showing water column physico-chemical characteristics of the sampled site.

Table 2. Sample characteristics of K-600 station

		Sediment characteristics (%)					Near bottom water profile			
Sampling date	Station	TOC	TIC	TN	OM	TOC/TN	DO (µM)	Temperature (°C)	Salinity (PSU)	Depth (m)
15 September 2015	K-600	4.662	7.131	0.906	8.037	6.005	33.05	9.945	35.188	580

scale). Copy number calculations were made per nanogram of DNA, and results were expressed per gram weight of sediment.

GenBank accession number

Nucleotide accession numbers for anammox-specific clone library obtained are as follows: *Scalindua*16S rRNA library (MG586106-MG586157) and *Scalindua* HZO library (MG687469-MG687487).

Results

Site characteristics

The CTD profile indicates that the near-bottom water (584 m) DO was ~33 µM, which implies stronger hypoxia in the underlying sediments (Figure 1). The bottom-water temperature was 9.95°C and salinity (35.19 PSU) coincided with the average value for the Arabian Sea. The sediment samples had high OC (4.662%) and exceptionally high TN (0.906%). The TIC was also found to be very high (7.13%), along with high OM (8.04%). The observed TOC/TN ratio of 5.14 is common to off shore sediment samples (Table 2).

Scalindua 16S rRNA gene diversity and phylogeny

The 16S rRNA primer set used (Brod541F/Brod1260R) is highly specific to *Scalindua*, and hence all clones obtained using this primer in the study shared maximum similarity with known *Scalindua* sp. only (Figure 2). These sequences were 95–99% identical to each other and 98–100% identical to the top hits in GenBank sequences. Almost 90% of the sequences obtained shared similarities with a wide range of marine sediment habitats. This covers oceanic regions that include Chuckchi Sea (Arctic Ocean), Antarctic Sea (Southern Ocean),

Figure 2. 16S rRNA gene-based phylogenetic tree of *Scalindua* anammox community from surface sediments of the eastern Arabian Sea underlying 600 m deep water column. Primer pair Broad541F/Brod1260R was used for clone library construction, which is known to target only *Scalindu* sp. Neighbor-joining tree was reconstructed taking 1000 bootstrap replicates. Clones are in green colour, anammox reference sequence in blue colour and outgroups in red colour. The most similar hits are in black colour. The evolutionary distances were computed using the maximum composite likelihood method and are in the units of the number of base substitutions per site. This analysis involved 87 nucleotide sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There was total of 2275 positions in the final dataset. Evolutionary analyses were conducted in MEGA X. Bootstrap support is shown in the phylogenetic tree, which corresponds to node diameter and branch width.

Table 3. Diversity analysis of phylogenetic and functional gene clone library

Target gene	*N*	Similarity (%)	OTU	Coverage	Alpha diversity	*J′* (evenness)	*H′* (Shannon)	*D′* (Simpson)	S_{ACE}	S_{Chao1}
Scalindua 16S	39	99.5	26	33.33	6.824	0.9479	3.088	0.031	71.95	59.32
rRNA		99	15	61.54	3.821	0.9089	2.461	0.085	19.86	16.62
		97	02	94.87	0.273	0.2918	0.2023	0.90013	2	2
Anammox *hzo*	17	99	17	0	5.647	1	2.833	0	145	145
		97	14	17.65	4.588	0.9692	2.558	0.0294	63.92	43.05

Jiaozhou Bay of the Yellow Sea located in South China and Mie, Ago Bay in Japan (Indian Ocean), West of Juan de Fuca Ridge and Peruvian oxygen minimum zone (OMZ) (Pacific Ocean) and Gulf of Mexico (Atlantic Ocean). Around 10% of sequences shared similarity with those retrieved from hypersaline groundwater, marshy areas, turning basin, and water conservation areas. Our result displays high diversity in the benthic *Scalindua* community in the sampled site of the Arabian Sea (Table 3). After removal of short-length sequence (≤500 bp), only 39 out of 52 sequences were subjected to further analysis. A total of 26 out of 39 *Scalindua*-specific 16S rRNA OTUs were obtained at a 0.05% sequence dissimilarity distance cut-off; the corresponding *H′* index was 3.09 and predicted OTUs were 60.

Phylotypic diversity and phylogeny of hzo gene fragment

The HZOF1/HZOR1 primer pair-based clone library had 19 positive clones out of 27 total sequences. Their similarity in the positive clones ranged from 94% to 99% with the *hzo* sequences in GenBank. In 17 of these 19 positive *hzo* clones, similarity ranged between 84% and 98%. Two sequences were eliminated due to their shorter than 500bp length. These 17 clones formed 14 distinct OTUs at 97% sequence similarity cut-off (Figure 3). At 99% cut-off, all fell out as separate OTUs. As much as 65% of the sequences clustered into clades bearing similarities with *hzo* sequences retrieved from Soledad basin OMZ sediments and South China Sea sediments. *H′* was 2.83 with a higher estimated richness of ~145.

Anammox gene copy number estimation

Anammox abundance on an average was 10^4 copies per gram dry weight of sediment irrespective of the genes targeted. The PCR efficiency for various anammox-specific genes used here ranged from 102% to 110%. The necessary correction was done to get an amplification factor of 2 before estimating copy number, which was expressed per gram of sediment and per nanogram of total DNA. The *Scalindua* 16S rRNA, anammox *hzs*A and non- *Scalindua hzo*_cluster2 copy numbers were $6.89 \pm 0.14 \times 10^4$, $4.99 \pm 0.35 \times 10^4$ and $3.53 \pm 0.33 \times 10^4$ copies per gram of sediment and 9.4, 6.8 and 4.8 copies/ng of DNA respectively.

Discussion

Majority of the studies related to anammox bacterial abundance and diversity from the Indian Ocean are from pelagic ODZ[33,34] and decidedly less information is available from sediment ODZ[35,36], which is limited to 16S rRNA gene alone. The present multi-primer approach was useful to make realistic estimates of anammox abundance and ascertained the need for using multiple gene markers for reliable quantification of functional and phylotypic members of the anammox process[37]. All the primers used in the present study were able to amplify in single PCR consisting of 35–40 cycles, suggesting quite a high abundance of anammox microbes off Kochi hypoxic zones. In the following discussion, to avoid primer bias and for habitat specificity, the studies emanating from marine environment were carried out utilizing the same primer set only considered.

The Arabian Sea hypoxic zone characteristics

The Arabian Sea is one of the most productive regions of the world's oceans and sediments underlying it are reported to have high OM content[38]. Sampling was carried out during the southwest monsoon period when productivity is the highest[39]. High productivity in surface water and subsequent settling of OM lead to the consumption of DO, and eventually results in the build-up of hypoxia and subsequent alteration of microbial communities[40]. The average percentage TOC and TN values reported from deep-sea sediments of the Gulf of Mexico were 0.9 ± 0.3 and 0.12 ± 0.03 respectively[41] and the maximum reported TOC is 14.5% and TN is 1.6% in OMZ surface sediments[42]. Here the sediment TOC and TN were 4.67% and 0.9% respectively, owing to high productivity-induced hypoxia leading to rapid burial of TOC and TN[43]. After oxygen, as nitrogen is the next preferred electron acceptor, facultative anaerobes relying on processes like anammox and denitrification dominate in the ODZ[1]. In pelagic ecosystem DO and salinity are the major factors controlling the distribution of *Scalindua*[20], whereas in benthic ecosystem their abundance is affected

Figure 3. Hydrazine oxidoreductase, *hzo* gene-based phylogenetic tree of anammox community from surface sediments of the eastern Arabian Sea underlying 600 m deep water column. Primer pair HZOF1/HZOR1 was used for clone library construction, which is known to target all known anammox groups. Neighbour-joining tree was reconstructed, taking 1000 bootstrap replicates. Clones are represented in green colour, anammox reference sequence in blue colour and outgroups in red colour. The most similar hits are in black colour. The evolutionary distances were computed using the Poisson correction method and are in the units of the number of amino acid substitutions per site. This analysis involved 36 amino acid sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There was a total of 673 amino acid positions in the final dataset. Evolutionary analyses were conducted in MEGA X. Bootstrap support is shown in the phylogenetic tree, which corresponds to node diameter and branch width.

by sediment TOC and TN content[44], particularly the availability of nitrogenous substrate nitrite and ammonia[12]. Here the near-bottom DO was higher than amiable; however, there is a possibility that the sediment anammox community must have restricted itself to anoxic microniches as seen in pelagic ODZ[33].

Anammox gene phylogeny and diversity

Scalindua is reported to be a low diverse community in the Arabian Sea pelagic ODZ[45]. In this study, we observed higher diversity and the obtained clones shared ~99% similarity with all seven marine *Scalindua* species previously reported[15,46–49]. These are *S. sorokinii*, *S. brodae*, *S. arabica*, *S. wagneri*, *S. zhenghei* and *S. rubra*. A similar study carried out using the sediment samples from Bohai Sea, had recovered a maximum of only 15 *Scalindua*-specific OTUs, but 24 *hzo*-specific OTUs at 0.5% and 1% cut-off[6]. In the present studies, we obtained 26 *Scalindua*-specific 16S rRNA OTUs and 17 *hzo*-specific OTUs at the respective cut-off values. However, predicted abundance for *hzo* is higher in this study, suggesting that the anammox community in the Arabian Sea sediments might be much more diverse.

Shannon diversity index (H') was 3.078 and 2.994 for the two targeted genes respectively, while in Bohai Sea sediments, the values ranged between 1.46 and 2.95; 2.18

and 3.79 for 16S rRNA and *hzo* genes respectively. In Bohai Sea sediment, another interesting observation was that in samples where *hzo* diversity was high, the *Scalindua* 16S rRNA diversity was low, i.e. 1.46/3.79 and when *Scalindua* 16S rRNA diversity was high, there was not much difference in the diversity of *hzo* gene (2.95/2.85)[6]. Similarly, in this study, diversity index for the two genes did not vary much, suggesting that the *hzo* sequences recovered in this study most likely belongs to *Scalindua*-like anammox community. This is reflected in the phylogenetic analysis as well, as *hzo* sequences clustered only with *Scalindua* sp. Similar results were also reported from the highly productive Peru margin ODZ sediments[50].

This study highlights the need to use primer sets to amplify both taxonomic and functional gene fragments. In spite of the fact that abundance in terms of copy numbers was low, the use of *hzo* primers confirmed the presence of anammox community mostly comprising *Scalindua* OTUs. Using Planctomycetes-specific forward and universal bacterial reverse primers[35], six *Scalindua* OTUs were reported from surface sediments off Kochi[51]. Apparently, looking for specific functional gene/s involved in the anammox process would prove useful in future studies for detecting the community involved as well as for confirming the occurrence of anammox simultaneously with other denitrification reactions.

Hydrazine gene markers are highly unique to the anammox community, and many primers have been successfully tested in diverse habitats targeting hydrazine subunits, namely synthase (*hzs*) and oxidoreductase (*hzo*) genes[5,32]. Recent studies identify the occurrence of hydroxylamine oxidoreductase (*hao*) and hydroxylamine dehydrogenase (*hdh*) as well as hydrazine hydrolase (*hh*) genes in selected species, and primers have been developed using the sequence information[52]. The *hzo* primer set used for this analysis was able to amplify a ~1000 bp gene fragment, thus making it useful for phylogenetic analysis[30]. Similar to16S rRNA gene, majority of the *hzo* gene sequences also shared similarities with those obtained from surface/subsurfaces sediments of the South China Sea and Soledad Basin sediments.

Anammox gene abundance

From North Sea sediments, maximum gene abundance reported for *hzs*A ranged between 10^5 and 10^6, and for the 16S rRNA gene a ten-fold increase was reported at shallow depth[53]. Phylogeny studies confirmed that all clones were similar to *Scalindua* only. While another study from the same location carried out in sandy and muddy shelf sediments reported anammox copy number as low as 10^3 for 16S rRNA, whereas *hzs*A was below detection limit[54]. In the present study, all three genes targeted were present in 10^4 copies/g dry wt of sediment, suggesting that multiple factors control anammox gene distribution within sediment ODZ. For *hzo* gene targeting non-*Scalindua*, no data in particular from marine sediments are reported. An available study from the eastern Indian Ocean ODZ sediments which focused mainly on *Scalindua*-specific 16S rRNA genes detected unusually high abundance, i.e. $3.20 \times 10^4 \pm 1.18 \times 10^3$ ng^{-1} DNA[36], while in the present study we retrieved only 9.6 copies ng^{-1} DNA. However in the methodology, these authors[36] have not mentioned whether they used concentrated DNA or not.

For correlating gene abundance with bacterial abundance, it is essential to understand the copy number variation between the genes. The 16S rRNA gene copy number varies significantly from 1 to 15, an average being 3.6 copies per bacterial cell, making it a less suitable proxy for bacterial abundance estimation[55]. It is suggested that in anammox bacteria, 16S rRNA could be present in a single copy[56]. For *hzs*A genes, the whole-genome study confirmed their occurrence in a single copy[5,57], whereas *hzs*B and *hzs*C subunits occurred in multiple copies[37]. Hence *hzs*A gene proves to be a better proxy for estimating anammox bacterial abundance. Accordingly, we assume that the actual anammox cell abundance in the studied site could be $\geq 4.99 \pm 0.35 \times 10^4$ copies/g dry wt of sediment, based on *hzs*A gene quantification. Few selected *hzs*A clones screened (unpublished data) showed that they must have been derived from an yet to be characterized anammox community. In the case of *hzo* also multiple copies are reported, but since there is not much information on the studied primer set *hzo*_cluster2, we cannot predict anything regarding the non-*Scalindu* cell abundance[4,21]. It is possible that previous studies targeting the 16S rRNA and *hzo* genes must have overestimated the anammox bacterial abundance, and we recommend obtaining data utilizing *hzs*A gene.

Although a 2.4-fold higher value is reported[58], the ratio of *Scalindua* 16S rRNA to *hzs*A was 1.38-fold higher from the present study site. From this observation, it is possible to highlight that only ≤60% of *hzs*A might be from the *Scalindua* anammox community. The ratio of *hzo*_cluster2 to *hzs*A was ~0.71-fold, suggesting that the non-*Scalindua* contribution could be ≥30%. Unravelling the functional capability of these versatile communities is important and, accordingly, new primers must to be designed and tested to get a clear picture of the extent of diversity within the anammox community.

Conclusion

The present multi-gene, multi-primer-based study identifies vast diversity within the Candidatus *Scalindua* community and also provides realistic estimates of anammox abundance in the organic-rich sediments underlying the Arabian Sea hypoxic zones. It further highlights the applicability of functional genes and the advantages of using taxa-specific primers in diversity studies. For

understanding the factual diversity, similar profiling using novel and unique gene markers, and subsequent phylogenetic analysis is required to strengthen the database. Molecular signatures, if used correctly, could refine and contribute to many age-old concepts pertaining to anammox occurrence and dominance in the natural ecosystem.

Conflicts of interest: The authors declare no conflict of interest.

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