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.

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

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

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^{TM} 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^{TM} 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^{TM} 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*_cluster2. 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
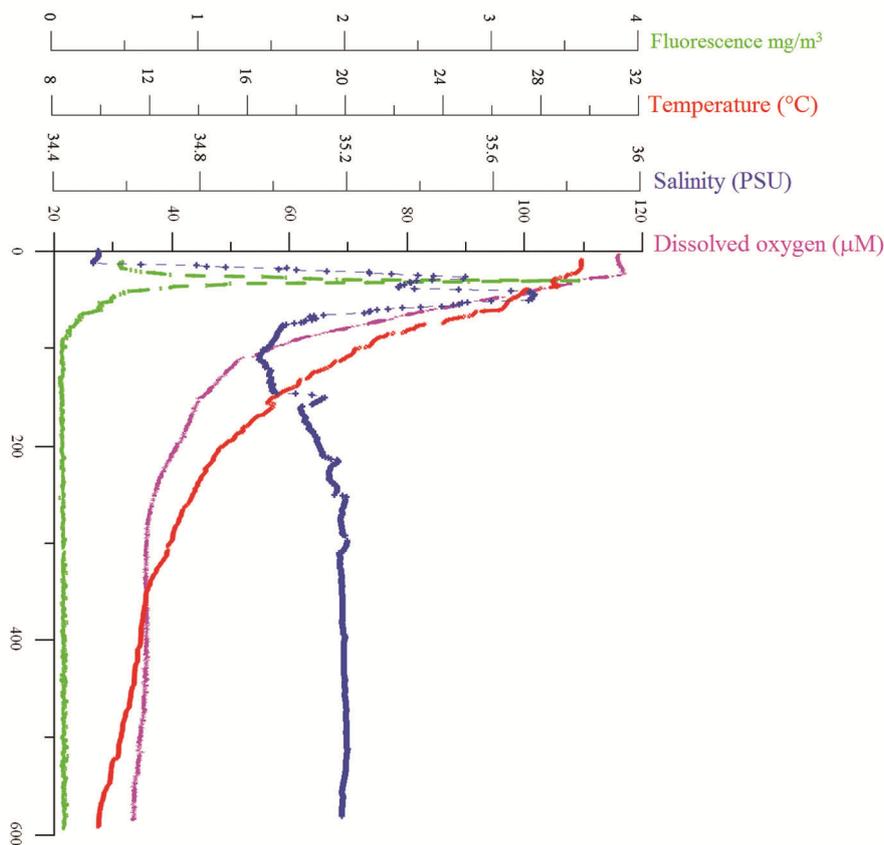
Figure 1. CTD profile of K-600 station showing water column physico-chemical characteristics of the sampled site.

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 to 16S 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 $\leq 60\%$ 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 $\geq 30\%$. 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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