

**Diversity and abundance of denitrifying and anammox bacteria from the Arabian Sea oxygen minimum zone**

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**Abstract**

Bacterial community involved in nitrogen transformations in the oxygen minimum zones (OMZ) with dissolved oxygen (DO) levels below 0.5ml L<sup>-1</sup> is ascribed to be responsible for the reported 40% losses of fixed nitrogen. The Arabian Sea (AS) OMZ is among the largest suboxic regions accounting for a substantial loss of fixed nitrogen. Sampling was carried out at the Arabian Sea Time Series (ASTS) location (17°0.126' N, 67°59.772'E) during three different seasons to document the diversity and abundance of both denitrifying and anammox bacteria, based on their molecular markers. Quantitative PCR (qPCR) was done to assess the abundance and distribution of genes encoding nitrite reductase (*nirS*) in denitrifying bacteria and hydrazine oxidoreductase (*hzo*) in anammox bacteria. Copy numbers of these genes had their maxima in the core OMZ depths of 250 and 500m. The preponderance of *nirS* to the tune of 0.35 x10<sup>6</sup> copy numbers L<sup>-1</sup> and *hzo*, of 1.5 x10<sup>6</sup> L<sup>-1</sup> is indicative of simultaneous denitrification and anammox processes. The abundance of *hzo* was higher than *nirS* during all three seasons implying probable dominance of anammox process at this sampling location. Phylogenetic analysis revealed that *nirS* gene was present in bacteria belonging to *Gammaproteobacteria*, *Alphaproteobacteria* and *Deltaproteobacteria*. All the *hzo* sequences obtained in this study apparently imply that *Candidatus Scalindua* is the dominant anammox bacteria at the ASTS location.

**Keywords:** Denitrification, Anammox, oxygen minimum zone, Arabian Sea, quantitative PCR

## 1. Introduction

Oceanic zones with dissolved oxygen (DO) concentrations below detection limits harbor anaerobic heterotrophic nitrate ( $\text{NO}_3^-$ ) reducers and chemolithotrophic ammonia oxidizing (anammox) bacteria which reduce nitrate to nitrogen gas. Denitrification is a process [reduction of  $\text{NO}_3^-$  to dinitrogen ( $\text{N}_2$ )] performed by a diverse group of heterotrophic microbes (Zumft 1997). Whereas anammox, [anaerobic oxidation of ammonium ( $\text{NH}_4^+$ ) and nitrite ( $\text{NO}_2^-$ ) through a hydrazine intermediate] leading to the  $\text{N}_2$  formation, is carried out by a limited number of bacterial taxa. Thus, microbe mediated marine denitrification and anammox are the major biogeochemical pathways accounting for ~35% of the fixed nitrogen loss from the world oceans (Codispoti 2007). Though studies on N loss via anammox are few, off late, it is considered to be of high significance (Strous et al. 1998, Thamdrup and Daalgaard, 2002) compared with denitrification process.

Two functionally and physiologically vital respiratory nitrite reductases, the copper-containing *nirK* and the cytochrome *cd1*-containing *nirS* are critical enzymes in denitrification (Zumft 1997). The *nirK* gene is reported from diverse heterotrophs, but *nirS*, mostly from *Proteobacteria*, and *Planctomycetes* (Strous et al. 2006). Though believed to appear to have spread by horizontal gene transfer, the phylogeny of nitrite reductase is incompatible with that obtained by 16S rRNA gene sequencing (Song and Ward, 2003). On the contrary, the anammox bacteria are deeply branching groups of *Planctomycete* (Strous et al. 1999) with five *Candidatus* genera so far reported (Strouss et al. 1998, Schmid et al. 2000, Schmid et al. 2003 and Quan et al. 2008). Harhangi et al. (2012) suggested that 16S rRNA gene primers do not capture the total diversity of the anammox bacteria although they reliably differentiate this unique functional group (Schmid et al. 2005). Therefore, molecular methods to detect functional gene markers based on hydrazine oxidoreductase (*hzor*), a crucial enzyme catalyzing the oxidation of the intermediate hydrazine ( $\text{N}_2\text{H}_4$ ) to  $\text{N}_2$ , are useful.

The Arabian Sea (AS) is a region of intense upwelling and high productivity (Wyrтки 1973, Madhupartap et al. 1996, Naqvi and Jayakumar, 2000) with low/no exchange of intermediate waters. Anaerobic metabolism including denitrification and anammox dominate the biogeochemistry of this intermediate oxygen-depleted water column (150-1000m). Many researchers have reported higher abundance and diverse assemblages of denitrifying and anammox bacteria in these suboxic layers (Castro-Gonzalez et al. 2005, Jayakumar et al. 2004, 2013, Schmid et al. 2007, Woebken et al. 2008, Spinrad et al. 1989 and Ward et al. 1998). Some of these studies are carried out using cultured bacterial isolates. Given that most marine bacteria are non-culturable; these studies only capture a

partial picture. In this regard, efforts to look for metagenomic DNA-based phylogeny and quantification of heterotrophic denitrifiers and anammox bacteria would prove worthy.

Analyses of diversity, distribution, and abundance of two marker genes *nirS* and *hzo* were carried out for this study to appraise if they are involved in the removal of fixed nitrogen from the AS-OMZ. Through qPCR, the predominance of *hzo* bearing anammox bacteria and the exclusive presence of *nirS* bearing denitrifying bacteria in the AS-OMZ is evidenced by this study. Quantification and phylogenetic analyses of *nirS* and *hzo* genes prove valuable to recognize microbial community extant in the core depths of the AS-OMZ, vital for both denitrification and anammox processes.

## **2. Materials and methods**

### **2.1 Sampling and Physico-chemical parameters measurements**

Systematic sampling was carried out during May 2012 (representing Spring Intermonsoon [SIM]), September 2012 (Fall Intermonsoon [FIM]), and February 2013 (Northeast monsoon [NEM]), from the Arabian Sea Time Series (ASTS) station (17°0.126' N, 67°59.772'E). Water samples were collected from five depths [surface (5m), DCM (45m), core omz (250m, 500m) and lower denitrification zone (1000m)]. Details of the collection of water samples and their measurements of nutrients and DO are published earlier in Jain et al. (2014).

### **2.2 DNA extraction and PCR amplification**

Water samples were passed through sterivex (Millipore Corp, USA) filters and stored at -80°C until nucleic acid extraction was performed in the laboratory usually within a month of sampling. Extraction of DNA was carried out by Ferrari and Hollibaugh (1999) method. All DNA extracts were subjected to PCR amplification using the primer set and annealing temperatures listed in **Table 1**. PCR reaction was performed in 50 µl volume using a Veriti (Applied Biosystem, USA) thermal cycler containing 1µl of extracted DNA (5 to 50 ngµl<sup>-1</sup>), 1 µl of each primer (Forward and Reverse) at a concentration of 10 pmolµl<sup>-1</sup> each, 25 µl of Ready Mix *Taq* PCR mix (1.5 U *Taq* DNA polymerase; 10mM Tris-HCl, 50mM KCl, 1.5mM MgCl<sub>2</sub>, 0.001% gelatin, 0.2mM deoxynucleoside triphosphate [dNTP], stabilizers, Sigma Aldrich) and 22µl of Milli-Q water. Details of PCR conditions for *nirS* and *hzo* gene are mentioned in Braker et al. (1998) and Li et al. (2010) respectively. Nuclease-free water used as negative controls. Electrophoresis confirmed positive amplicons from each DNA sample on a 1% agarose gel.

### 2.3 Cloning and sequencing

Amplicons were purified using UltraClean PCR Clean-up kit (GENAXY, Biosciences), cloned into pCR<sup>®</sup>4-TOPO vector (Invitrogen) and transformed into *E. coli* TOP-10 competent cells as per the protocol mentioned in TOPO-TA cloning kit (Invitrogen, USA). At least 40 positive clones from each sample were randomly picked out and subjected to the colony-PCR with primers sets pucM13F/pucM13R using temperature conditions as per manufacturer's instructions. Colony-PCR products were purified using UltraClean PCR Clean-up kit (GENAXY, Biosciences) and sequenced in an ABI 3130XL genetic analyzer (Applied Biosystems, USA).

### 2.4 Sequence analysis of amplified *nirS* and *hzo* genes

As a standard protocol, all the sequences were assembled into contigs using DNA Baser Sequence assembly software version 2. Non-chimeric consensus sequences without vector and primer residues and with a quality score of 20 (which translates into more than 99.5% correct bases, 1), were used for further analyses. The classification of sequences was done using 1000 pseudo-bootstrap replications with a bootstrap value of 80%, which results in a standard error of only 1.3%. Assembled sequences compared with NCBI database. Alignments were automatically trimmed using Gblocks (Castresana 2000) to remove poorly aligned positions and divergent regions. These sequences were then assigned to a phylum if their identity was >80% with database sequences. Sequences obtained is submitted to the GenBank database under the accession number KP153530 - KP153534, KP317841 - KP317905 and KX078645 - KX078768 for *nirS* gene, and KT211367 - KT211397 and KU572781 - KU573035 for the *hzo* gene.

### 2.5 Determination of OTUs and construction of phylogenetic tree

Obtained *hzo* and *nirS* gene sequences were translated into amino acid sequences using Bioedit. The sequences were assigned to operational taxonomic units (OTUs) applying the average neighbour rule (Schloss and Westcott, 2011) using MOTHUR. NirS sequences obtained from different samples collected during three different seasons from 250 and 500m were combined as *CnirS* for obtaining the possible OTUs at 95% sequence similarity. Similarly, *hzo* clone libraries from 250 and 500m combined as *Chzo* sequences. A representative sequence from each OTU of *CnirS* and *Chzo* and the top-hit amino acid sequences from cultivated known strains from the GenBank identified via BLASTp search were used to build the phylogenetic tree. Neighbor-joining tree of both *CnirS* and *Chzo* was constructed using MEGA 6.0 with 500 replicate bootstrap analyses.

## 2.6 Quantitative PCR (qPCR) assay

Quantification of *nirS* gene was carried out using primer pair nirS1F/nirS6R. This is because primer pair, nirS1F/nirS3R failed to show positive amplification. Primer pair hzoF1/hzoR1 was used in quantification of anammox bacteria. Triplicates of each DNA sample was subjected to qPCR using the ABI 7500 Real-Time PCR system (Applied Biosystems, USA). Each gene-reaction performed in a 20- $\mu$ l volume containing four  $\mu$ l (10 ng  $\mu$ l) of DNA template, 0.5  $\mu$ l of each primer (20  $\mu$ M), and 12.5  $\mu$ l of 5 $\times$  qARTA Green qPCR Mix (AXYGEN, USA). PCR was carried out using modified method of Levy-Booth and Winder, (2010), cycle started with 15 min at 95°C, followed by 40 cycles of 15s at 95°C, and 1 min at 60°C. Plasmids carrying target gene fragment cloned into pCR4-TOPO vector was quantified using a Nanodrop (Thermo Scientific, USA). The copy number of the target gene was calculated directly from the concentration of the extracted plasmid DNA. Ten-fold serial dilutions of a known copy numbers of the plasmid DNA were subjected to qPCR assay in triplicates to generate an external standard curve and qPCR efficiency for both *nirS* and *hzo* genes was calculated.

## 2.7 Diversity/Richness analysis and rarefaction curves

Spearman's correlation coefficient test was used to assess the significance of association between environmental parameters and copy numbers during each season. To determine the significance of temporal variation in diversity, one-way analysis of variance (ANOVA) was done. The diversity indices (Simpson's and Shannon's indices), richness estimators (Chao 1 and ACE), and rarefaction curve were derived using MOTHUR (Schloss et al. 2009). The three nonparametric richness estimators were used to extrapolate the total richness of the clone libraries from the observed number of OTUs. Diversity and richness estimators were calculated for individual clone libraries of *nirS* and *hzo* gene as well as combined samples (*CnirS* and *Chzo*).

## 3. Results

### 3.1 Physico-chemical characteristics of the sampling site

There were distinctly different concentrations of DO and nutrients above and below the OMZ and in the core of OMZ. Sub-surface peaks of  $\text{NH}_4^+$  and  $\text{NO}_2^-$  concentrations were observed in the euphotic zone during all three seasons (**Fig. 1**). The highest concentrations of  $\text{NO}_2^-$  (2.57  $\mu\text{mol L}^{-1}$ ) and  $\text{NH}_4^+$  (0.27  $\mu\text{mol L}^{-1}$ ) were observed within the core of OMZ during NEM and FIM respectively. Surface and DCM depths (43-50m) were well oxygenated followed by a sharp oxycline at 250m. The average DO concentration in the oxygenated surface, and DCM depths in the core of OMZ was  $185.24 \pm 31.1 \mu\text{mol L}^{-1}$  and  $5.56 \pm 5.5 \mu\text{mol L}^{-1}$  respectively.

## 3.2 Phylogenetic analyses

Phylogenetic analyses were used to discern the evolutionary differences between OTUs and the representative sequences. All 250m and 500m samples showed PCR amplification for both the genes. From a total of 479 non-chimeric sequences, 194 of them were of *nirS* and, 285 of the *hzo* gene.

### 3.2.1 Phylogeny of denitrifying bacteria

The amino acid sequences of *nirS* gene obtained in this study show 44–99% identity with previously published sequences from the AS. They were >75% identical to each other and 75–85% similar to the closest match in GeneBank sequences. Most of the present sequences matched the closest with those reported from the deep sea sediments. Sequence similarity of the clones from the present study with those in NCBI database showed that all the OTUs belonged to the phylum *Proteobacteria*. The OTUs from 250m and 500m together were similar to the *nirS* sequence of the cultivated denitrifiers of *Halomonas*, *Xanthomonas*, *Thiohalomonas*, *Marinobacter*, *Rhodospirillales*, and *Deltaproteobacteria*. The closest matches were those of clone sequences obtained from the estuary sediment and cold oxic sub-seafloor aquifer.

Of the 194 clones sequenced from both 250m and 500m samples, a total of 13 OTUs were generated (**Fig. 2**). Of these nine OTUs (69%, 134 sequences) were affiliated to the genera *Halomonas*, *Xanthomonas*, *Thiohalomonas* and *Marinobacter* of the class *Gammaproteobacteria*, 3 OTUs (23%, 45 sequences) were affiliated to *Rhodospirillales* of the class *Alphaproteobacteria* and 1 OTU (8%, 15 sequences) was affiliated to class *Deltaproteobacteria*.

### 3.2.2 Phylogeny of anammox bacteria

Phylogenetic analysis reveals that at 95% sequence similarity all 285 non-chimeric *hzo* sequences match with hydrazine oxidoreductase sequence of the anaerobic ammonia oxidizing bacteria. The *hzo* sequences from the ASTS location were 80–99% identical to each other and 76–90% similar to the closest-match sequences in the GenBank. Correspondingly, the deduced *hzo* amino acid sequences were 84–100% identical to each other and 74–93% identical to the sequences retrieved from engineered and natural habitats.

Of the 285 sequences from 250m and 500m samples 10 OTUs were formed. The phylogenetic tree constructed (**Fig. 3**) show that all the OTUs were affiliated to *Candidatus Scalindua*. These matched with the sequences reported from the sediments of the South China Sea, Okhotsk Sea and Jiaozhou Bay.

### 3.3 Quantification (qPCR) of *nirS* and *hzo* gene copy numbers

Independent standard curves were constructed and qPCR efficiency for *nirS* (80.23%) and *hzo* (81.01%) gene was calculated respectively. The non-template control had Ct values at least ~9 cycles higher than the most diluted plasmid containing the target gene. The post-amplification melting curve analysis showed that there was no target gene contamination in the reagents. **Fig. 4** shows the seasonal distribution of *nirS* and *hzo* genes.

#### 3.3.1 Copy numbers of *nirS*

In the surface samples, the distribution pattern of *nirS* copy numbers was identical during all three seasons, with their number ranging from 0.01 to 0.02  $\times 10^6 \text{L}^{-1}$ . Quantifiable copy numbers from DCM samples were scarce/absent during all three seasons. Copy numbers in the core of OMZ depths ranged from 0.16 to 0.35  $\times 10^6 \text{L}^{-1}$ . Copy numbers were the highest during NEM (0.35  $\times 10^6 \text{L}^{-1}$ ) followed by FIM (0.16  $\times 10^6 \text{L}^{-1}$ ) and SIM (0.2  $\times 10^6 \text{L}^{-1}$ ). Copy number at 1000m was in the range of 0.02 to 0.04  $\times 10^6 \text{L}^{-1}$ .

#### 3.3.2 Copy numbers of *hzo*

The *hzo* gene copy numbers ranged from 0.04 to 0.05  $\times 10^6 \text{L}^{-1}$  in the surface samples during all seasons. No significant values of copy numbers were observed in the DCM either. The *hzo* copy numbers were the highest in the core OMZ depth ranging from 0.1  $\times 10^6 \text{L}^{-1}$  to 1.5  $\times 10^6 \text{L}^{-1}$ . During SIM, the highest copy numbers were 1.5  $\times 10^6 \text{L}^{-1}$  followed by FIM (0.5  $\times 10^6 \text{L}^{-1}$ ) at 500m whereas during NEM (0.8  $\times 10^6 \text{L}^{-1}$ ) the highest was recorded at 250m. Copy numbers at 1000m were quite small during all three seasons.

### 3.4 Statistical Analysis

During SIM, DO ( $r = -0.872$ ;  $p < 0.05$ ) and nitrite ( $r = -0.821$ ;  $p < 0.05$ ) correlated negatively with *hzo* gene numbers. Copy numbers of *nirS* correlated negatively with TOC ( $r = -0.900$ ;  $p < 0.01$ ) during FIM season. DO negatively influenced the distribution of both *nirS* ( $r = -0.872$ ;  $p < 0.05$ ) and *hzo* ( $r = -0.894$ ;  $p < 0.01$ ) gene copy numbers during NEM (**Table 2**).

For recognizing the relationship between sampling effort and diversity, rarefaction analysis was performed on 194 *nirS* and 285 *hzo* gene clones and their OTUs (**Table 3**). Shannon and Simpsons indices suggest that diversity of *CnirS* was higher than that of *Chzo* in the core of OMZ. Similarly, Chao 1 and ACE richness estimators infer that the estimated OTUs in *CnirS* are more than those of *Chzo* (**Table 3**).

The numbers of *nirS* OTUs were more during SIM followed by FIM and NEM. The highest number of *nirS* OTUs was detected during SIM at 250m. Shannon and Simpson's indices, Chao 1 and ACE richness estimators suggest higher diversity of *nirS* gene at 250m during all seasons. (**Table 3**). Saturation of rarefaction curve (**Fig. 5(A)**) at the recovered OTU level with an evolutionary distance of 5% implies that our sampling efforts adequately covered the diversity.

The numbers of *hzo* OTUs were highest during SIM. Shannon and Simpson's indices indicate that the diversity was the maximum during SIM followed by NEM and FIM. Contrary to *nirS* the *hzo*, gene based OTU diversity was the highest at 500m during SIM and FIM except for NEM. The nonparametric, Chao 1 and ACE estimators revealed that the estimated OTUs are higher at 500m during SIM and FIM (**Table 3**). Saturation of rarefaction curves of the *hzo* OTUs (**Fig. 5(B)**) is useful to infer that our sampling was adequate. ANOVA showed a significant ( $P < 0.05$ ) seasonal variation in the diversity of *nirS* and *hzo* genes only during FIM ( $p < 0.05$ ) and NEM ( $p < 0.05$ ).

#### **4. Discussion**

Denitrification is recognized as an important process in the OMZs of the AS and Eastern Tropical South Pacific (ETSP, Ward et al. 2009, Dalsgaard et al. 2012). However, the discovery of anammox (Mulder et al. 1995) revealed additional pathway for fixed nitrogen loss from the marine ecosystem. Since the unusual circulation pattern and nutrient-rich waters promote very high primary production in the AS (Madhupratap et al. 1996), decomposition of the organic matter from these highly productive surface waters culminates in total consumption of DO at 150-1200m leading to denitrification and anammox (Naqvi et al. 1990) in these depths.

##### **4.1 Denitrifying and Anammox bacteria community structure in the AS-OMZ**

There was no amplification of both *nirS* and *hzo* genes in the surface (5m), DCM (50m), and 1000m during all three seasons even after repeated attempts of PCR. The failure to detect *nirS* in the surface waters is an important new observation and is implicit of the fact that denitrifying bacteria are facultative aerobes that fail to compete with the strict aerobes that dominate surface waters as also documented by Jayakumar et al. (2013). These authors reported no amplification of *nirS* from surface water samples collected both from AS and ETSP. Similar observation for anammox (*hzo*) gene was reported in the Chilean open ocean OMZ (Chang et al. 2010) where the surface waters revealed fewer transcripts of anammox bacteria compared to the prominent anammox gene transcripts in the core OMZ.



#### 4.1.1 The *nirS* Phylogeny

Stepwise and subsequent reduction of nitrate as an electron acceptor is carried out by denitrifying bacteria. Due to its high redox state, the OMZ is selective for the growth and multiplication of denitrifying bacteria. All *nirS* gene fragments from AS-OMZ are affiliated with Proteobacteria as also reported by Divya et al. (2017) and Wang et al. (2017). Distribution of ubiquitous Proteobacteria is well known and their dominance was reported in AS OMZ depths (Jain et al. 2014, Bandekar et al. 2017). Similar to results reported by Zheng et al. (2014), all ASTS OTUs aligned into three classes: *Gammaproteobacteria*, *Alphaproteobacteria* and *Deltaproteobacteria*. All sequences from the ASTS matched with uncultured sequences of the *nirS* gene. A few of them are identical to those reported from Baltic Sea (Hannig et al. 2006), Black Sea (Oakley et al. 2007), the OMZ of the ETSP (Castro-Gonzalez et al. 2005), and from sediment samples of the North West Pacific (Braker et al. 2000).

The *nirS* gene sequences in the AS-OMZ bear striking similarity to those of *Halomonas* are reported by Yan et al. (2003) from uranium- and nitrate- contaminated groundwater environment. *Marinobacter* and *Xanthomonas* bearing *nirS* gene sequences similar to those in AS-OMZ are reported from the Eastern South Pacific-OMZ and pyrite-fed denitrifying reactor by Castro et al. (2005) and Torrentó et al. (2011) respectively. Further, *nirS* sequences from ASTS are 75-85% similar to the cultivated denitrifiers of *Thiohalomonas*, *Rhodospirillales*, and *Deltaproteobacteria*. *Chromatiales* as the active sulfide-utilizing denitrifiers (Beller et al. 2006; Park et al. 2011) are widely distributed in OMZs (Beman and Carolan, 2013), and their abundance is tightly coupled to DO concentrations. Dimitry et al. (2007) reported that *Thiohalomonas denitrificans* of the order *Chromatiales* reduced nitrate completely to N<sub>2</sub> with intermediate production of nitrite and N<sub>2</sub>O. Detection of *Rhodospirillales* (*Alphaproteobacteria*) in this study is in agreement with the observation of Luke et al. (2016) wherein *Rhodospirillales* comprised 4.1% of the community in the OMZ core. An environmental clone, exhibiting close identity to *nirS* from cultured denitrifier's species, was also observed in a recent study of the River Colne estuary sediments, in which identical *nirS* sequences were obtained from a *Flavobacterium* isolate (Nogales et al. 2002) and two RT-PCR mRNA clones.

#### 4.1.2 The *hzo* Phylogeny

The use of *hzo* gene as a marker is a recent approach to delineate anammox bacterial diversity. It is a highly reliable marker for detecting anammox bacteria from environmental samples (Wang and Gu. 2013). Being a functional gene unique to anammox bacteria (Schmid et al. 2008;

Quan et al. 2008; Li et al. 2010) thus an appropriate biomarker for their phylogenetic analyses. From the very high similarity percentages, it is suggested that the AS-OMZ harbors anammox bacteria exclusively similar to uncultured *Planctomycete* sequences obtained previously from the AS and Chilean OMZs (Woebken et al. 2008, Galán et al. 2009). Further, Jayakumar et al. (2009) and Bao-lan et al. (2011) also reported *Planctomycete* as the dominant anammox bacteria in the AS-OMZ.

Beman and Carolan (2013), Pedros-Alio (2012) reported that anammox bacteria contribute to less than 4% of the bacterial community in the OMZs. Consequently, anammox gene surveys in OMZ have recognized very less diversity comprising mostly of “*Candidatus Scalindua*” (Chang et al. 2010). The *hzo* sequences obtained in this study are 74–93% similar to sequences reported from marine sediments. Most OTUs from 250m and 500m showed affiliation to the genus *Candidatus Scalindua*. Such abundances are useful to suggest that these bacteria contribute to the anammox process in the AS-OMZ. The phylogenetic analysis of *hzo* protein sequences implies that *Candidatus Scalindua* is the dominant anammox bacteria in the water samples from the ASTS location which is in agreement with the previous finding that most of the 16S rRNA gene sequences obtained from AS-OMZ is closely related to *Candidatus Scalindua* (Luke et al. 2016). It is likely that depending on the hydro chemical features distinct to each OMZ, specific groups of anammox or any other functional group may be the dominant ones.

#### **4.2 Distribution of denitrifying and anammox bacteria in the core OMZ depths of AS**

In spite of many repeated attempts of PCR, the surface, DCM and 1000m did not show the presence of both *nirS* and *hzo* genes. Unambiguously, the bacteria possessing *nirS* and *hzo* genes are relatively rare in the well-oxygenated waters. Even increased template DNA concentrations did not yield positive PCR for both genes. Ct values of these samples (5m, DCM, and 1000m) were very low ( $0.04 \times 10^6 \text{L}^{-1} \text{ nirS}$  and  $0.08 \times 10^6 \text{L}^{-1} \text{ hzo}$ ) than those recorded from the core OMZ. Thus, we assume that there are fewer *nirS* and *hzo* gene bearing bacteria at these depths and hard to detect via conventional PCR. Although qPCR results show the signatures of the presence of both these groups of bacteria, their abundances are quite small. However, we acknowledge detection of these genes in water samples from 5m; DCM and 1000m may be due to non-specific amplicons even though the melt curves, the melting temperature, and all other protocol were verified to confirm that amplification in our qPCR was not an artefact. Also, as reported in several studies (Staggemeier et al. 2015; Nagdev et al. 2015; Zemtsova et al. 2015; Paiva-Cavalcanti et al. 2010) we cannot rule out that the sensitivity of qPCR is higher than the conventional PCR.

Distribution of both *nirS* and *hzo* genes was consistent with the environmental settings that define the OMZs. The highest abundance of *nirS* gene copy numbers was in the core OMZ depths (250m-500m) of the AS. Although different species of denitrifiers are present in a few numbers, the population is dominated by organisms that do not possess *nirS* genes. At the ASTS, the oxygen concentration is at threshold (1–4  $\mu\text{M}$ ) level, ideal enough to favor denitrifiers and denitrification to happen as suggested by Codispoti et al. (2005) and, Devol et al. (1978). At this location, the abundance of *nirS* gene is significantly lower than that reported by Jayakumar et al. (2013) from other sites in the AS-OMZ swath. This abundance suggests the existence of perennially active nitrite reducing populations.

Copy numbers of *hzo* genes reported by Ward et al. (2009) and Pitcher et al. (2011) from the AS-OMZ are much higher than that of *hzo* observed in this study. It is to be noted however that reports on the distribution of anammox gene by Ward et al. (2009) are limited to the secondary nitrite maxima (SNM) depths whereas samples analyzed for this study were from the core OMZ depths. The highest copy number of the *hzo* gene during SIM may be because the organic carbon is a significant substrate supporting the anammox bacterial communities in the core OMZ depths (Dang et al. 2010). As suggested by Ramaiah et al. (2000) it is apparent that bacterial communities are sustained by the slow-to-degrade dissolved organic carbon (DOC) during SIM which is because; SIM is a transitional period where primary production is low (Madhupratap et al. 1996) mainly due to stratification and prevalence of oligotrophic conditions.

#### **4.3 Implications of higher abundance of anammox genes over denitrifying genes**

One of the most striking features at the ASTS location is the greater abundance of *hzo* over *nirS* gene copy numbers. Earlier reports on denitrification and anammox in the AS-OMZ and ETSP have suggested denitrification as a dominant pathway in the removal of fixed nitrogen (Ward et al. 2009, Dalsgaard et al. 2012). One of the possible reasons for detection of low concentrations of *nirS* in this study may be due to the length of the amplified fragment used for quantification of *nirS*. Earlier reports on *nirS* copy numbers from the AS-OMZ use primers which produce a much shorter fragment (Ward et al. 2009, Jayakumar et al. 2009) in comparison to the longer fragment used in this study which could probably lead to an underestimate of the actual copy number. However, results from this study may be taken to denote that anammox might be playing an equal role at the ASTS leading to such high percentage of fixed nitrogen loss. Incubation experiments by Kuypers et al. (2005), in the oxygen-deficient waters off Namibian Shelf suggest higher rates of anammox with no detection of denitrification.

Richards (1965) and Richards et al. (1965) state that ammonium should accumulate in waters if nitrogen loss is solely due to heterotrophic denitrification. No accumulation of  $\text{NH}_4$  was evident at the ASTS. Low oxygen concentrations, increased nitrite concentration and reduced ammonia concentration (Dalsgaard et al. 2003) appear to provide an ideal condition in the ASTS for the presence of anammox bacteria.

#### **4.4 Richness and diversity analyses**

The two different diversity indices and richness estimators ACE and Chao 1 indicates higher degree of diversity of both denitrifying and anammox bacteria in the core OMZ depths. The overall diversity of denitrifying bacteria at the ASTS location was greater than that of the anammox bacteria. Although the sampling size in this study is not very large, it is evident from the saturation of rarefaction curves that the diversity of denitrifying and anammox bacterial communities is adequately captured, and the dominant phylotypes sufficiently represented. The *nirS* and *hzo* gene sequences from the AS-OMZ, when compared with other marine environments, imply the presence of denitrifying and anammox bacteria, the latter albeit not so very diverse.

#### **Conclusion**

Quantitative analyses and distributions patterns of *nirS* and *hzo* genes helps to signify their role in critical biogeochemical processes leading to reported N losses from the AS-OMZ. Copy numbers of *hzo* were found to be far higher than the *nirS* gene copy numbers. Thus, anammox could be playing a major role in the  $\text{N}_2$  loss at the ASTS location. The diversity of anammox bacteria was lesser than that of denitrifying bacteria. Results from this study also indicate that the Proteobacteria are identified as the predominant denitrifiers in the AS-OMZ. The anammox and denitrifying bacterial communities are relatively in low diversity and their distribution limited to the core of the OMZ (Kong et al. 2013). Further, as reported by Bryant et al. (2012), low concentrations of organic matter in marine OMZs reduce the number of niches available for microbes. Results of this study provide much-needed information to demonstrate the anammox process in oxygen deficient intermediate waters of the AS-OMZ.

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## LEGENDS TO FIGURES

**Figure 1.** Vertical distribution of ammonia ( $\text{NH}_4$ ) and nitrite ( $\text{NO}_2$ ) concentrations during spring intermonsoon (SIM), fall intermonsoon (FIM), and northeast monsoon (NEM) at the Arabian Sea Time Series (ASTS) location.

**Figure 2.** Neighbor-joining phylogenetic tree constructed using *CnirS*-amino acid OTU sequences obtained from 250m and 500m at the Arabian Sea Time Series (ASTS) location. during spring intermonsoon (SIM), fall intermonsoon (FIM), and northeast monsoon (NEM). The sequences in bold are from the present study.

**Figure 3.** Neighbor-joining phylogenetic tree constructed using *Chzo*-amino acid OTU sequences at 250m and 500m at the Arabian Sea Time Series (ASTS) location during spring intermonsoon (SIM), fall intermonsoon (FIM), and northeast monsoon (NEM). The sequences in bold are from the present study.

**Figure 4.** Seasonal and depth wise distribution of copy numbers of (A) *nirS* and (B) *hzo* gene. See Fig. 1 for other abbreviations.

**Figure 5.** Rarefaction analysis of denitrifying and anammox bacterial communities based on *nirS* (A) and *hzo* (B) gene sequences. See Fig. 1 for other abbreviations.

**Table 1.** Primers used in this study for PCR amplification and qPCR.

<b>Primer</b>	<b>Primer sequence (5'–3')</b>	<b>Annealing temperature</b>	<b>Product size (bp)</b>	<b>Reference</b>
nirS1F	CCTA(C/T)TGGCCGCC(A/G)CA(A/G)T	63°C	890	Braker et al.(1998)
nirS6R	CGTTGAACTT(A/G)CCGGT			
hzoF1	TGTGCATGGTCAATTGAAAG	60°C	740	Li et al. (2010)
hzoR1	CAACCTCTTCWGCAGGTGCATG			

**Table 2.** Correlation coefficient (r values) between physico-chemical parameters and gene copy numbers at the Arabian Sea Time Series Station

<b>Location</b>	<b>Season</b>	<b>Genes</b>	<b>Variable</b>	<b>r</b>	<b>p value</b>	
<b>ASTS</b>	<b>SIM</b>	<b>NirS</b>	Depth	0.354	-	
			Dissolved oxygen	-0.707	-	
			Ammonia	0.707	-	
			Nitrite	-0.707	-	
			TOC	-0.354	-	
			<b>Hzo</b>	Depth	0.667	-
	Dissolved oxygen	<b>-0.872</b>	<b>0.05</b>			
	Ammonia	0.462	-			
	Nitrite	<b>-0.821</b>	<b>0.05</b>			
	TOC	-0.667	-			
	<b>FIM</b>	<b>NirS</b>	<b>NirS</b>	Depth	<b>0.900</b>	<b>0.01</b>
	Dissolved oxygen			-1.000	-	
Ammonia	-0.051			-		
Nitrite	-0.354			-		
TOC	<b>-0.900</b>			<b>0.01</b>		
<b>Hzo</b>	Depth			-0.447	-	
Dissolved oxygen	0.224		-			
Ammonia	0.229		-			
Nitrite	0.000		-			
TOC	0.447		-			
<b>NEM</b>	<b>NirS</b>		<b>NirS</b>	Depth	0.667	-
				Dissolved oxygen	<b>-0.872</b>	<b>0.05</b>
		Ammonia		-0.544	-	
		Nitrite		0.564	-	
		TOC		-0.667	-	
		<b>Hzo</b>		Depth	0.224	-
	Dissolved oxygen	<b>-0.894</b>	<b>0.01</b>			
	Ammonia	-0.395	-			
	Nitrite	0.447	-			
	TOC	-0.224	-			

P < 0.05; P < 0.01; P < 0.001—levels of significance

**Table 3.** Comparison of *nirS* and *hzo* diversity in clone libraries constructed from the OMZ during spring intermonsoon (SIM), fall intermonsoon (FIM), and northeast monsoon (NEM) at the Arabian Sea Time Series (ASTS) location

	No. of sequences	No. of OTUs	Richness estimation			
			Chao	ACE	Shannon	Simpsons
<b><i>nirS</i> gene</b>						
<i>CnirS</i>	194	13	13.14	14.92	1.24	0.35
SIM-250	32	9	10.20	13.00	1.42	0.39
SIM-500	33	7	7.50	10.56	0.98	0.54
FIM-250	31	5	5.50	6.79	0.82	0.60
FIM-500	34	4	4.00	4.69	0.71	0.64
NEM-250	34	4	4.00	4.69	0.80	0.56
NEM-500	30	4	4.00	4.49	0.71	0.64
<b><i>hzo</i> gene</b>						
<i>Chzo</i>	285	10	8.00	8.39	1.12	0.55
SIM-250	47	2	2.00	2.00	0.49	0.68
SIM-500	49	7	7.33	8.53	1.30	0.36
FIM-250	47	2	2.00	0.00	0.10	0.96
FIM-500	47	3	3.00	4.11	0.34	0.84
NEM-250	48	3	3.00	4.76	0.48	0.74
NEM-500	47	1	1.00	0.00	0.00	1.00

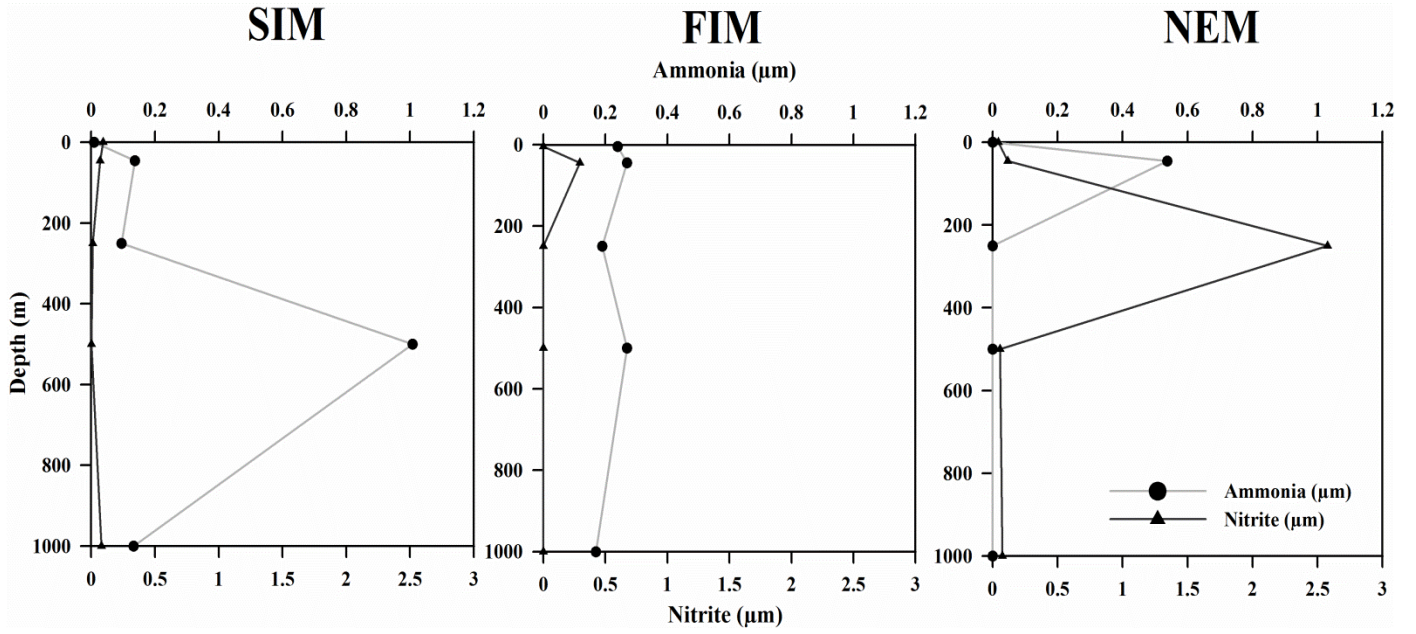
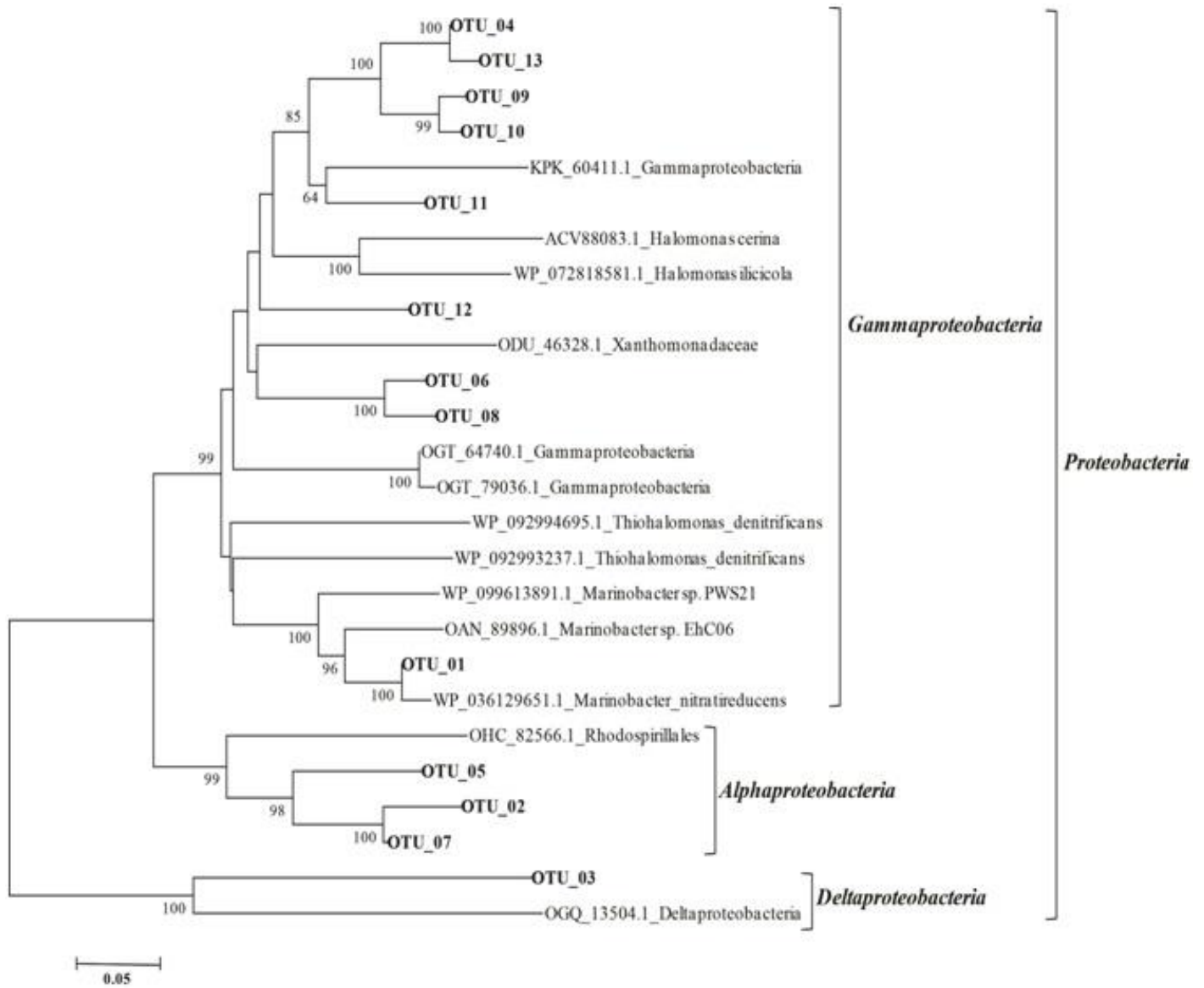
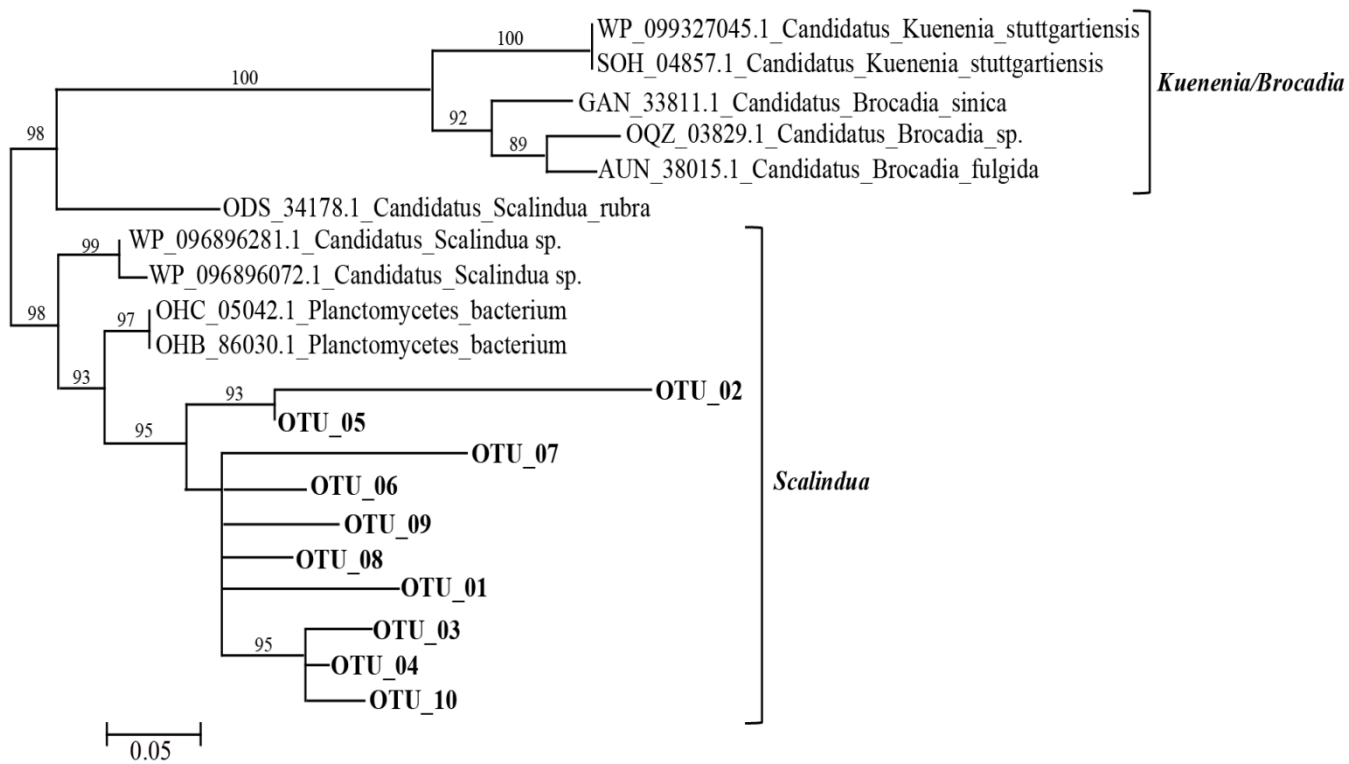


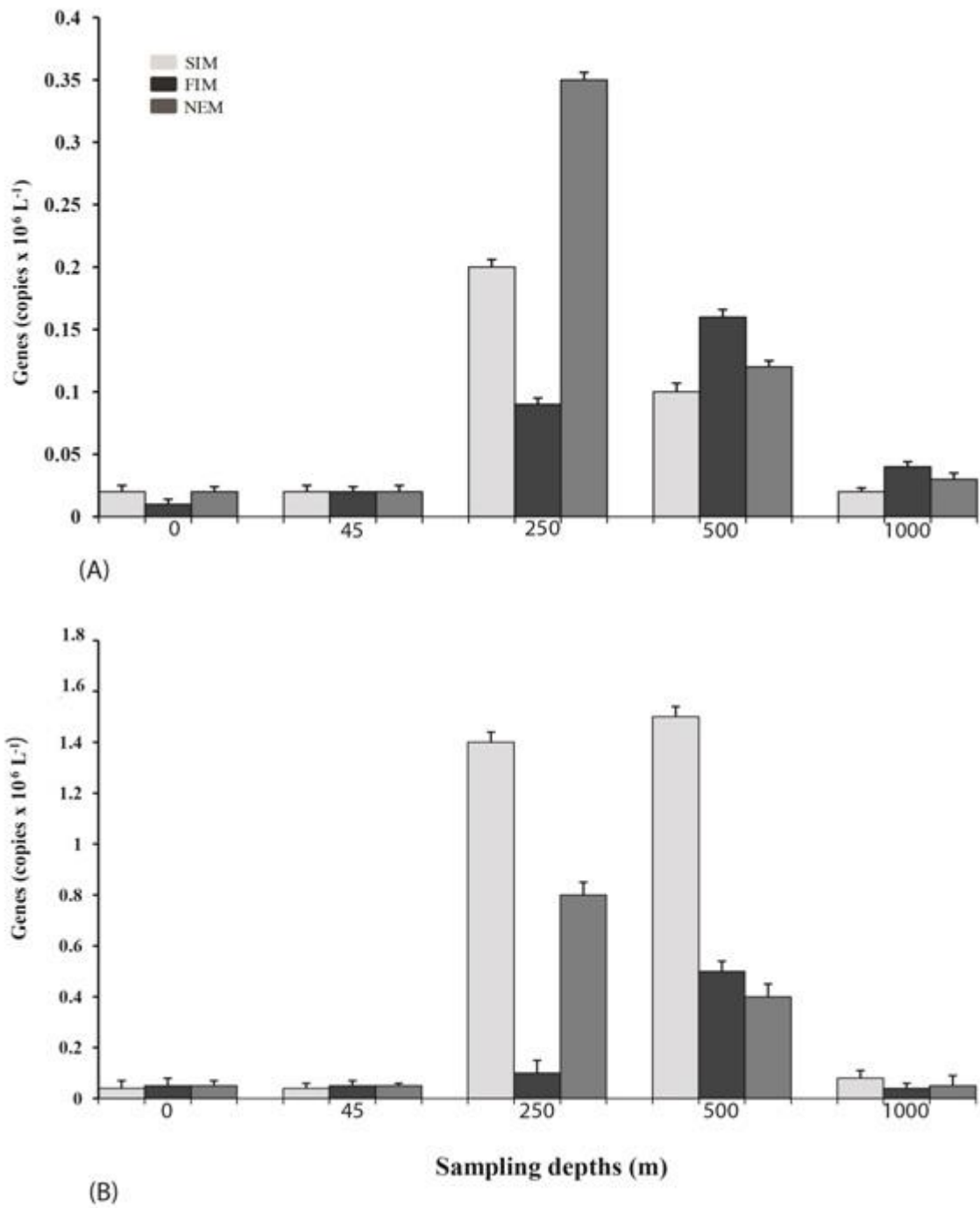
Figure 1



**Figure 2**

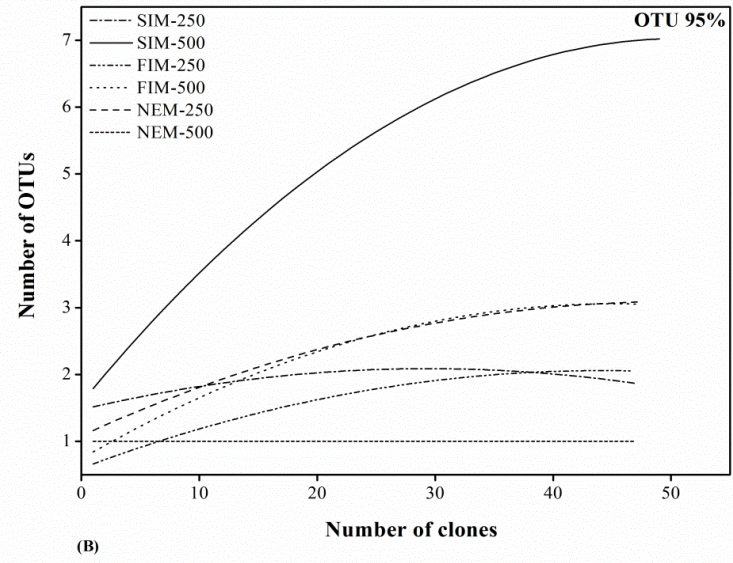
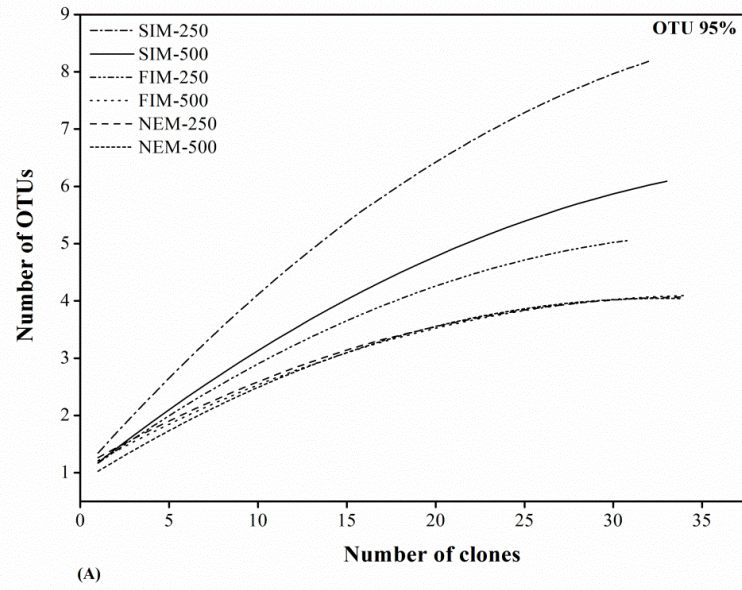


**Figure 3**



**Figure 4**





**Figure 5**