Diversity and distribution of Nitrogen Fixation Genes in the Oxygen Minimum Zones of the World Oceans

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Abstract

Diversity and community composition of nitrogen fixing microbes in the three main oxygen minimum zones (OMZs) of the world ocean were investigated using operational taxonomic unit (OTU) analysis of *nifH* clone libraries. Representatives of the all four main clusters of *nifH* genes were detected. Cluster I sequences were most diverse in the surface waters and the most abundant OTUs were affiliated with Alpha- and Gammaproteobacteria. Cluster II, III, IV assemblages were most diverse at oxygen depleted depths and none of the sequences were closely related to sequences from cultivated organisms. The OTUs were biogeographically distinct for the most part – there was little overlap among regions, between depths or between cDNA and DNA. Only a few cyanobacterial sequences were detected. The prevalence and diversity of microbes that harbour *nifH* genes in the OMZ regions, where low rates of N fixation are reported, remains an enigma.

Introduction

Nitrogen fixation is the biological process that introduces new biologically available nitrogen into the ocean, and thus constrains the overall productivity of large regions of the ocean where N is limiting to primary production. The most abundant and most important diazotrophs in the ocean are cyanobacteria, members of the filamentous genus *Trichodesmium* and several

unicellular genera, including *Chrocosphaera sp*. and the symbiotic genus *Candidatus* Atelocyanobacterium thalassa (UCYN-A). Although these cyanobacterial species are wide spread and have different biogeographical distributions (Moisander et al. 2010), they are restricted to surface waters, mainly in tropical or subtropical regions.

Because diazotrophs have an ecological advantage in N depleted waters, and because those conditions occur in the vicinity of oxygen minimum zones, due to the loss of fixed N by denitrification, it has been proposed that N fixation should be favoured in regions of the ocean influenced by OMZs (Deutsch et al. 2007). It has also been suggested that the energetic constraints on N fixation might be partially alleviated under reducing, i.e., anoxic, conditions (Großkopf and LaRoche 2012). In response to these ideas, the search for organisms with the capacity to fix nitrogen has been focused recently in regions of the ocean that contain OMZs. That search usually takes the form of characterizing and quantifying one of the genes involved in the fixation reaction, *nifH*, which encodes the dinitrogenase reductase enzyme. Diverse *nifH* assemblages have been reported from the oxygen minimum zone of the Eastern Tropical South Pacific (Turk-Kubo et al. 2014, Loescher et al. 2016, Fernandez et al. 2011) and the Costa Rica Dome, at the edge of the OMZ in the Eastern Tropical North Pacific (Cheung et al 2016). The search for non cyanobacterial diazotrophs has resulted in discovery of diverse *nifH* genes, but they have not been associated with significant rates of N fixation (Moisander et al. 2017). Here we report on the distribution and diversity of *nifH* genes in all three of the world ocean's major OMZs, including samples from both surface and anoxic depths, and both DNA and cDNA (i.e., both presence and expression of the *nifH* genes).

Materials and Methods:

Samples analysed for this study were collected from the three major OMZ regions of the world oceans (32 total samples, Table1 and 2) from surface, oxycline and oxygen depleted zone (ODZ) depths. Particulate material from water samples (5 – 10 L), collected using Niskin samplers, mounted on a CTD (Conductivity-Temperature-Depth) rosette system (Sea-Bird Electronics), was filtered onto Sterivex capsules (0.2 μm filter, Millipore, Inc., Bedford, MA) immediately after collection using peristaltic pumps. The filters were flash frozen in liquid nitrogen and stored at -80°C until DNA and RNA could be extracted. For samples from the Arabian Sea, DNA extraction was carried out using the PUREGENE™ Genomic DNA Isolation Kit (Qiagen, Germantown, MD) and the RNA was extracted using the ALLPrep DNA/RNA Mini Kit (Qiagen, Germantown, MD). For samples collected from ETNP and ETSP DNA and RNA were simultaneously extracted using the ALLPrep DNA/RNA Mini Kit (Qiagen, Germantown, MD). SuperScript III First Strand Synthesis System (Invitrogen, Carlsbad, CA, USA) was used to synthesise cDNA immediately after extraction following purification of RNA using the procedure described by the manufacturer, including RT controls. DNA was quantified using PicoGreen fluorescence (Molecular Probes, Eugene, OR) calibrated with several dilutions of phage lambda standards.

PCR amplification of *nifH* genes from environmental sample DNA and cDNA was done on an MJ100 Thermal Cycler (MJ Research) using Promega PCR kit following the nested reaction (Zehr et al. 1998), with slight modification as in Jayakumar et al. (2017). Briefly, 25μl PCR reactions containing 50 pmoles each of outer primer and 20-25ng of template DNA, were amplified for 30 cycles (1 min at 98°C, 1 min at 57°C, 1 min at 72°C), followed by amplification with the inner PCR primers 50 pmoles each (Zehr and McReynolds 1989). Water for negative controls and PCR was freshly autoclaved and UV-irradiated every day. Negative controls were run with every PCR experiment, to minimize the possibility of amplifying contaminants (Zehr et al. 2003). The

72 PCR preparation station was also UV irradiated for 1 hour before use each day and the number of
73 amplification cycles was limited to 30 for each reaction. Each reagent was tested separately for
74 amplification in negative controls. *nifH* bands were excised from PCR products after electrophoresis
75 on 1.2% agarose gel, and were cleaned using a QIAquick Nucleotide Removal Kit (Qiagen). Clean
76 *nifH* products were inserted into a pCR®2.1-TOPO® vector using One Shot® TOP10 Chemically
77 Competent *E. coli*, TOPO TA Cloning® Kit (Invitrogen) according to manufacturer's specifications.
78 Inserted fragments were amplified with M13 Forward (-20) and M13 Reverse primers from
79 randomly picked clones. PCR products were sequenced at Macrogen DNA Analysis Facility using
80 Big Dye™ terminator chemistry (Applied Biosystems, Carlsbad, CA, USA). Sequences were edited
81 using FinchTV ver. 1.4.0 (Geospiza Inc.), and checked for identity using BLAST. Consensus *nifH*
82 sequences (359 bp) were translated to amino acid (aa) sequences (108 aa after trimming the primer
83 region) and aligned using ClustalX (Thompson et al. 1997) along with published *nifH* sequences
84 from the NCBI database. Neighbor-joining trees were produced from the alignment using distance
85 matrix methods (PAUP 4.0, Sinauer Associates). Bootstrap analysis was used to estimate the
86 reliability of phylogenetic reconstruction (1000 iterations). The *nifH* sequence from *Methanosarcina*
87 *lacustris* (AAL02156) was used as an outgroup. The accession numbers from GenBank for the *nifH*
88 sequences in this study are Arabian Sea DNA sequences JF429940‑JF429973 and cDNA sequences
89 accession numbers JQ358610‑JQ358707, ETNP DNA sequences KY967751-KY967929 and cDNA
90 sequence KY967930-KY968089, and ETSP DNA sequences MK408165‑MK408307 and cDNA
91 sequences MK408308‑MK408422.
92
93 The *nifH* nucleotide alignment (of 787 sequences) was used to define operational
94 taxonomic units (OTUs) on the basis of DNA sequence identity. Distance matrices based on this

nucleotide alignment were generated in MOTHUR (Schloss and Handlesman 2009). The relative *nifH* richness within each clone library was evaluated using rarefaction analysis. OTUs were defined as sequences which differed by ≤3% using the furthest neighbor method in the MOTHUR program (Schloss and Handlesman 2009). The 3% OTU definition is similar to the level at which species are conventionally defined using 16S rDNA sequences, so it may overestimate the meaningful diversity of the functional gene. Redundancy analysis was performed in R using the vegan package. Environmental variables were transformed using decostand.

Results and Discussion:

DNA and cDNA sequences (787 in total) derived from the OMZ regions of the Arabian Sea (AS), Eastern Tropical North Pacific (ETNP) and Eastern Tropical South Pacific (ETSP) were subjected to OTU and phylogenetic analyses to compare the diversity and community composition, biogeography and gene expression, of *nifH* possessing microbes among the three OMZ regions. Phylogenetic analysis of the sequences from the AS, ETNP and ETSP were reported previously (Jayakumar et al. 2012, Jayakumar et al. 2017, Chang et al. 2019), but the sequences have been combined for additional analyses here. We compared the threshold OTU definitions at 3 and 10% and found that the number of OTUs decreased, as expected, as the resolution decreased. Even at the 3% threshold, however, OTUs tended to separate by depth and location, indicating a functionally useful distinction at this level. Thresholds of 3 – 5% as the OTU definition correspond to within and between species level distinctions for *nifH* (Gaby et al. 2018). The sequences from the OMZ regions represented all four sequence clusters (I, II, III, IV) described by Zehr et al. (1998).

Cluster I *nifH* OTU distributions: Diversity analysis of the *nifH* cluster 1 sequences for the three OMZs based on OTUs using MOTHUR identified 41 OTUs at a distance threshold of 3% (Supplemental Table 1A and B). The number of sequences and the number of OTUs varied widely among depths and stations, so the results are grouped by region (AS, ETNP, ETSP) or depth horizon (surface or OMZ, including upper oxycline depths) or cDNA vs DNA (Table 2). The OTUs are numbered in order of decreasing abundance in the clone library, i.e., OTU-1 was the most common OTU.

For all regions and depths combined, the number of OTUs detected (41) was less than the sum of OTUs detected when each region was analyzed separately (45), indicating that there was some overlap of OTUs among regions. The overlap was not large, however. Only three of the 12 most abundant OTUs contained sequences from more than one region and none contained sequences from all three regions (Figure 1A). When sequences for all three regions were combined, only four of the 12 most abundant OTUs contained sequences from both depth horizons (Figure 1B). Most OTUs represented a single depth, and many a single sample. Interestingly, Cheung et al. (2016) used 454-pyrosequencing to obtain a similar number of OTUs (37 total) from the Costa Rica Dome, and all of the 15 samples investigated by Cheung et al. (2016) were dominated (>50%) by one of five major OTUs.

The Arabian Sea was strikingly less diverse than other regions and sample subsets (Figure 2). For example, when all DNA and cDNA sequences for all depths are grouped together, the Arabian Sea (OTUs = 14, Chao = 21) contains less species richness than the combined surface samples from all three regions (OTUs = 25, Chao = 52), despite having a similar number of total sequences (178 for the Arabian Sea, 198 for all surface samples

141 combined). This lack of diversity in the AS data may be partly due to the preponderance of
142 cDNA sequences, which generally contained less diversity than a similar number of DNA
143 sequences (see below).

144 Although similar numbers of sequences were obtained for cDNA (255) vs DNA (257),
145 the OTU "density", i.e., number of OTUs per number of sequences analyzed, was higher for
146 DNA (0.136 for DNA, 0.094 for cDNA). The Chao statistic verified this observation for the
147 combined data from each region in predicting higher total numbers of OTUs for DNA (Chao =
148 42) than for cDNA (Chao = 24). This difference could indicate that some of the *nifH* genes
149 present were not expressed at the time of sampling, but the cDNA sequences were not simply a
150 subset of the DNA community. Half of the 12 most abundant OTUs contained either cDNA or
151 DNA (Figure 1C), meaning that some genes were never expressed and some expressed genes
152 could not be detected in the DNA.

153 For all regions combined, similar numbers of OTUs were detected in surface waters
154 (OTUs = 25) and in OMZ samples (OTUs = 23), although a larger number of sequences was
155 analyzed for the OMZ environment (198 vs. 314 sequences for surface and OMZ depths,
156 respectively). It might be expected that the presence of phototrophic diazotrophs in the surface
157 water would lead to greater diversity there, but only one OTU representing a known
158 cyanobacterial phototroph (OTU-12 = *Katagmynene spiralis* or *Trichodesmium*) was identified,
159 so most of the additional diversity must be present in heterotrophic or unknown sequences.

160 Rarefaction curves (Figure 2) indicate that sampling did not approach saturation either for
161 region or depth. The Chao statistic also indicated that much diversity remains to be explored,
162 despite the great uncertainty in these estimates. The total number of OTUs detected, the shape of
163 the rarefaction curve and the diversity indicators (Figure 2, Table 2) all indicate that the greatest

164 *nifH* diversity occurred in surface waters, and much of that diversity was in singletons, i.e., not
165 represented in the 12 most abundant OTUs, which represented 441 (86 %) of the total 512 *nifH*
166 Cluster 1 sequences analyzed. Most of that diversity was contained in the ETNP, not solely a
167 function of number of sequences analyzed (Figure 2).

168 **Cluster I *nifH* Phylogeny:** Phylogenetic affiliations at both DNA and protein level are
169 shown for the 12 most abundant OTUs in Table 3. The most abundant OTU (129 sequences),
170 OTU-1, contained Gammaproteobacterial DNA and cDNA sequences from both surface and
171 OMZ depths of the ETNP and cDNA sequences from oxycline and OMZ depths in the Arabian
172 Sea (Figure 3). Although very similar to each other, none of these sequences had higher than
173 91% identity at the DNA level (96% at AA level) with cultivated strains and were most closely
174 related to *Pseudomonas stutzeri*. *P. stutzeri* is a commonly isolated marine denitrifier, but it is
175 also known to possess the capacity for N fixation (Krotzky and Werner 1987). OTU-4, OTU-6
176 and OTU-8 also contained Gammaproteobacterial sequences. All had high identity with
177 cultivated strains at the protein level but none were >91% identical to cultivated strains at the
178 DNA level.

179 Gammaproteobacterial sequences with very close identities to *Azotobacter vinelandii* have
180 been reported from the Arabian Sea ODZ and also from the ETSP (Turk-Kubo et al. 2014). This
181 group of *nifH* sequences with close identities to *A. vinelandii* was also retrieved from the English
182 Channel, Himalayan soil, South Pacific gyre, Gulf of Mexico, mangrove soil and many other
183 environments (Figure 3). *Azotobacter*- like sequences were included in OTU-6 but were not closest
184 identity at the DNA level. Although a large number of clones were analyzed here, no sequence that
185 was closely associated with *A. vinelandii* was retrieved from the three regions. None of the g-

244774A11 sequences, Gammaproteobacterial relatives that were abundant in the South Pacific (Moisander et al. 2014), were detected in this study.

OTUs-2, 3, 5, 10, and 11 all represented Alphaproteobacterial sequences, with closest identities to various *Bradyrhizobium, Sphingomonas* and *Methylosinus* species. Thus, Alphaproteobacterial sequences (206 sequences) were the most abundant in the clone library. OTU-2 contained almost exclusively ETSP ODZ DNA and cDNA sequences (plus one AS ODZ DNA sequence). OTU-3 contained DNA sequences from ETNP surface waters. OTU-5 contained exclusively Arabian Sea DNA sequences from Station 3, while OTU-10 contained only surface samples from the ETNP. An OTU threshold of 11% grouped all (179 sequences in five OTUs) of these Alphaproteobacterial sequences together, but the 3% threshold is consistent with the phylogenetic tree, which shows small scale biogeographical separation of sequence groups.

OTUs-7 and -9 were identified as Betaproteobacteria with closest identities to *Rubrivivax gelatinosum* and *Burkholderia*, 91 and 90% respectively at the DNA level. However, at the AA level, these sequences were 99 and 100% identical to *Novosphingobium malaysiense* and *S. azotifigens,* both Alphaproteobacteria, and again were biogeographically distinct. OTU-7 contained 25 DNA sequences from the ODZ depths in the Arabian Sea, and OTU-9 contained 17 *Burkholderia*-like sequences from the oxycline at Station 1 in the Arabian Sea. No Betaproteobacterial *nifH* sequences were detected in the ETNP or ETSP, but sequences similar to *Burkholderia phymatum*, *Cupriavidus sp.* and *Sinorhizobium meliloti* were reported from ETSP previously (Fernandez et al. 2015). Consistent with our previous report, however, there is no clear separation between the alfa and the beta groups in *nifH* phylogeny (Jayakumar et al 2017).

Most of the Cluster I ETSP sequences from this study were contained in two OTUs (2 and 4). OTU-2 contained 89 Alphaproteobacterial sequences with >98% identity to *nifH* sequences from

209 *Bradyrhizobium* sp. Uncultured bacterial sequences retrieved from the South China Sea, English
210 Channel, mangrove sediment, wastewater treatment and grassland soil were related to these ETSP
211 sequences. OTU-4 contained 29 Gammaproteobacterial sequences retrieved from both surface and
212 ODZ depths. Four of the remaining ETSP Cluster I sequences were grouped together as OTU-17
213 (Alphaproteobacteria, 89 and 96% identities with *Methyloceanibacter sp.* and *Bradyrhizobium sp.* at
214 the DNA and AA level respectively), three were in OTU-23 (*Bradyrhizobium* 100% identity) and
215 two were singletons. One of the singletons was most closely related to uncultured soil and sediment
216 sequences and to *Azorhizobium sp*. (86%) and one had 97% identity with *Bradyrhizobium*
217 *denitrificans* and many sequences from marine sediments.
218 OTU-22 represents the Deltaproteobacterial group. This novel group was reported
219 previously from the ETNP (Jayakumar et al. 2017) and has three sequences from Arabian sea (OTU-
220 22) and two singletons from ETNP surface waters. *nifH* possessing Deltaproteobacteria have been
221 reported not only from all the three ODZs but also in several other marine environments including
222 Chesapeake Bay water column, microbial mats from intertidal sandy beach in a Dutch barrier island,
223 Jiaozhou Bay sediment, Rongcheng Bay sediment, Bohai Sea, Mediterranean Sea, Narragansett Bay,
224 and the south Pacific gyre.
225 Proteobacteria-like sequences are the most frequently reported *nifH* sequences from the
226 OMZs studied here and similar environments. Thirty one of 37 OTUs detected by Cheung et al
227 (2016) in the Costa Rica Dome OMZ were Proteobacteria, the two most common OTUs being
228 closely related to Alphaproteobacterium *Methylocella palustris* and the Gammaproteobacterium
229 *Vibrio diazotrophicus*. Loescher et al. (2014, 2016) also found *V. diazotrophicus*-like sequences, as
230 well as several other Gammaproteobacteria in the ETSP. *V. diazotrophicus* was reported previously
231 in the Arabian Sea (Jayakumar et al. 2012) but was not prominent in the present study.

232 *Bradyrhizobium spp*., one of the most common genera reported here and in surface waters of the
233 Arabian Sea (Bird and Wyman 2013) and by Fernandez et al. (2011) in the ETSP, were also detected
234 in the Costa Rica Dome OMZ and were the dominant OTU at 1000 m at one station (Cheung et al.
235 2016). In addition to *Bradyrhizobium*-like and *Teredinibacter*-like *nifH* sequences, Turk-Kubo et al.
236 (2014) found four other abundant Gammaproteobactera-like *nifH* sequences, which were entirely
237 novel. The "Gamma A", which are commonly reported non-cyanobacteria diazotroph *nifH*
238 sequences from non-OMZ environments (Langlois et al. 2015, Moisander et al. 2017), were
239 represented by a singleton from the ETNP in the present study.
240 *nifH* sequences related to various Alphaproteobacterial methylotrophs are commonly found
241 in OMZs: *Methylosinus trichosporium*-like sequences, which are reported here in OTU-5 from the
242 Arabian Sea at both surface and ODZ depths, were also reported by Fernandez et al. (2011) in the
243 ETSP. *Methylocella palustris*-like *nifH* genes comprised the most common OTU in the ODZ core
244 depths in the Costa Rica Dome (Cheung et al. 2016). *M trichosporium* and *M. palustris* represent
245 obligate and facultative methanotrophs, respectively, both also obligately aerobic. Detection of *nifH*
246 genes closely related to those of methanotrophs does not prove that methanotrophy is present or
247 important in the anoxic environment of the ODZ but the consistency of this finding across sites
248 motivates further investigation on the potential for methane production and consumption in ODZs.
249 The pattern of high diversity of *nifH*-bearing mostly heterotrophic microbes, but dominance
250 in each sample by one or a small number of *nifH* OTUs, suggests a bloom and bust pattern of
251 organic matter-supported growth. That is, we suggest that organic matter, which is supplied
252 episodically in the upwelling regimes, stimulates the growth of copiotrophic microbes that respond
253 rapidly in bloom like fashion. This bloom scenario has been described for denitrifying bacteria
254 based on the OTU patterns observed in the *nirS* and *nirK* genes as a function of the stage of

denitrification in both natural assemblages and incubated samples from OMZs (Jayakumar et al. 2009). The role of *nifH* in these heterotrophic microbes is unclear, especially because rates of nitrogen fixation in these locations in the absence of cyanobacteria is often very low (Turk-Kubo et al. 2014, Loescher et al. 2016, Chang et al. 2019).

Although *Trichodesmium*-like clones have been retrieved from the surface waters of the Arabian Sea and the ETNP OMZs, only ten clones (OTU-12) in the combined clone library analyzed here were related to *Trichodesmium* (98% identity), including both cDNA and DNA from the Arabian Sea and cDNA from the ETNP. These sequences were actually 100% identical to *Katagnymene spiralis*, a close relative of *Trichodesmium* isolated from the South Pacific Ocean. Turk-Kubo et al. (2014) also retrieved only a few cyanobacterial sequences from the ETSP. No other cyanobacterial *nifH* sequences were identified.

Clusters II, III, IV *nifH* OTU distributions: The other three *nifH* clusters were combined for OTU analysis due to the limited number of sequences and OTUs obtained. A total of 18 OTUs were identified in the combined set of 275 sequences with a 3% distance threshold (Table 3). Most of the Cluster II, III, IV sequences were from the ETNP and ETSP. As with the Cluster I sequences, there was very little geographic and depth overlap among these OTUs (Figure 4A, 4B). Only OTU-1 contained sequences from more than one site, the ETNP and the ETSP. OTU-2 contained only cDNA sequences representing ODZ depths at both ETNP stations. OTU-3 contained exclusively ETSP DNA sequences from surface and cDNA sequences from ODZ depths. Only 10 of the Cluster II, III, IV sequences were from the Arabian Sea, and they formed three separate OTUs, a greater "OTU density" than was present at either of the Pacific sites. As observed for Cluster I, most of the OTUs that were detected in the DNA were not being expressed, and those that were expressed were not detected in the DNA (Figure 4C).

278 Rarefaction curves (Figure 5) indicate that sampling for Cluster II, III, IV did not
279 approach saturation. The Chao statistic also indicated that much diversity remains to be
280 explored, despite the great uncertainty in these estimates. Unlike the Cluster I analysis, there
281 were relatively few singletons in the Cluster II, III, IV data and the assemblages were dominated
282 by a few types.

283 **Clusters II, III, IV *nifH* phylogeny:** Three large OTUs (OTU-1, -4 and -6) in Clusters II,
284 III, IV belonged to *nifH* Cluster IV and Alphaproteobacteria/Spirochaeta and Deltaproteobacteria
285 were the dominant phylogenies (Table 3, Figure 6). The largest OTU, OTU-1, contained 88 DNA
286 sequences from the ETNP ODZ depths from both stations and from both depths in the ETSP. This
287 OTU had no similarity to any cultured microbe. OTU-4 contained 30 sequences from the ETSP, all
288 cDNA from one surface station, in *nifH* Cluster IV.

289 OTU-2 (75 sequences) in Cluster II contained only cDNA sequences, all from ODZ
290 samples in the ETNP (both stations), and had no close relatives among cultivated species. Turk-
291 Kubo et al. (2014) also retrieved a few clones identified as belonging to Cluster II from the
292 euphotic zone of the ETSP. OTU-3 contained 35 sequences in Cluster III and was dominated by
293 DNA sequences from surface depths of the ETSP. OTU-5 represented Deltaproteobacteria in
294 *nifH* Cluster III and contained 18 identical DNA sequences from 90 m at Station BB1 in the
295 ETNP. Thus, of the five most common OTUs (89% of the total Cluster II, III, IV sequences
296 analyzed), only one could be identified to a closely related genus (i.e., OTU-4 with 90% identity
297 with *R. palustris*) and there was no overlap between DNA and cDNA OTUs from the same
298 depths.

299 The other 13 OTUs in the Cluster II, III, IV sequences represented either Cluster III or IV.
300 None of these were very closely related to any cultivated sequences. OTU-6 contained both DNA

301 and cDNA from the OMZ at one ETSP station. OTU-7 contained four sequences from ETNP
302 surface waters with close identities with a sequence retrieved from Bohai sea. OTU-11, had one
303 DNA and one cDNA sequences from the ETSP. All of the other sequences were less than 84%
304 identical to any sequence in the database and could only be loosely identified as Firmicutes or
305 Proteobacteria.

306 Although there were few high identities with known species, many of the Cluster II, III, IV
307 sequences (OTUs -2, -5, -7, -9, -10) were most closely affiliated with sulfate reducing clades at
308 either the DNA or protein level. Four OTUs with highest identity to known sulfate reducers were
309 reported by Cheung et al. (2016) and one of them comprised nearly 40% of the sequences in one
310 anoxic sample. *nifH* sequences that cluster with *Desulfovibrio spp.* are often reported from ODZ
311 samples (Turk-Kubo et al. 2014, Loescher et al. 2014, Fernandez et al. 2011). Consistent reports of
312 *nifH* genes associated with obligate anaerobes involved in sulfate reduction suggests a role for this
313 metabolism in the ODZ, again motivating further research on the significance of both sulfate
314 reduction and associated N fixation in ODZ waters.

315 **Biogeography and Environmental Correlations:** The dominant factor determining OTU
316 composition and distribution is clearly biogeography (Figure 4). That geographical factor is also
317 evident in the redundancy analysis (Figure 7). (Only sites that contained sequences from one of the
318 top OTUs are represented in the plots, so the number of site symbols is less than 32 for both plots.)
319 For example, Cluster I OTU-5 containing only Arabian Sea surface sequences was positively
320 correlated with both T and S and all of the Arabian Sea samples clustered in the quadrant associated
321 with high T and S (Figure 7A). Surface samples from the ETSP were also in that quadrant, but
322 surface ETNP samples were negatively correlated with S. The surface ETNP samples correlated
323 with OTUs-3. -6, -10 and -11, all of which contained exclusively surface samples. The two largest

Cluster I OTUs were associated with the deep samples from the ETNP and ETSP and correlated positively with nitrite concentration and negatively with oxygen – a signature of the ODZ. Nitrate concentration and depth did not increase the power of the analysis and were omitted from the Cluster I RDA. Most of the sites and five of the most common Cluster I OTUs were not well differentiated by any of the usual environmental parameters.

The Arabian Sea contained very few sequences in Clusters II, III, IV and none of them were in the top six OTUs, so only ETNP and ETSP samples are represented in the RDA for these clusters (Figure 7B). The two largest OTUs in Clusters II, III, IV were negatively correlated with T and S but separated along the second RDA axis, demonstrating opposite relationships with oxygen, nitrite, and nitrate concentrations. OTU-1 included ETSP surface sequences, as well as ODZ sequences from both ETNP and ETSP, while OTU-2 contained only ODZ sequences but both OTUs were phylogenetically related to anaerobic clades (Table 2). Inclusion of all six environmental variables was necessary to obtain maximum separation of the sites and OTUs for Clusters II, III, IV.

Conclusions

The OMZ regions of the world ocean contain substantial *nifH* diversity, both in surface waters and oxygen depleted intermediate depths. Surface waters contained greater diversity for Cluster I, but the ODZ held the highest diversity for Clusters II, III, IV. Cyanobacterial sequences were rare in the combined dataset and were not detected in the ETSP. The ETSP contained the least diversity of Cluster I sequences, while Cluster II, III, IV were least abundant and least diverse in the Arabian Sea. Most of the sequences in all four Clusters of the conventional *nifH* phylogeny were not closely related to any sequences from cultivated Bacteria or Archaea. The most abundant OTUs in Cluster I and in Clusters II, III, and IV could be assigned to the Alphaproteobacteria, followed by the

347 Gammaproteobacteria for Cluster I and Deltaproteobacteria accounted for Clusters II, III, IV
348 sequences. Most of the OTUs were not shared among regions, depths or DNA vs cDNA and
349 sometimes were restricted to individual samples. Some Cluster I sequences had high identity to
350 known species (e.g., *Bradyrhizobium*, *Trichodesmium*) but most of the Cluster II, III, IV sequences
351 were only distantly related to any cultured species.

352 The assemblage composition of *nifH*-bearing microbes is mainly explained by region, but
353 OTU composition was also consistent with the influence of key environmental parameters such as
354 oxygen and temperature, and reflects association with the secondary nitrite maximum for deep
355 samples. Most of the sites/depths, both in this study and in others from OMZ regions, are
356 dominated by one or a few OTUs, which suggests bloom-type dynamics within a diverse
357 background assemblage. Microbes occupying very similar niches and present at low population
358 levels might respond differentially to episodic inputs of organic matter, resulting in spatially and
359 temporally varying dominance by a few clades. Thus we find similar metabolic types represented
360 across all the OMZs, although the specific species and genus level affiliations differ. The consistent
361 detection of *nifH* sequences related to those found in known sulfate reducers and methanotrophs
362 suggests the need for further investigation of these pathways in ODZs.

363 While measurements of N_2 fixation rates are not reported here, the abundance of cDNA
364 sequences suggests that the cells harboring these genes are active. Low, but analytically significant,
365 rates have been detected in ODZ depths in the ETNP (Jayakumar et al. 2017) and ETSP (Chang et
366 al. 2019), which suggests that non-cyanobacterial N fixation could make a minor contribution to the
367 nitrogen budget of the ocean. It is therefore important in future work to determine how the diversity
368 described here actually contributes to biogeochemically significant reactions and what

environmental and biotic factors might influence or control the activity of diazotrophs in the dark ocean.

373 **Figure Legends**

374 Figure 1. Histogram of the 12 most common OTUs from Cluster I *nifH* clone libraries from the
375 three OMZ regions. OTUs were considered common if the total number of sequences in an
376 OTU was ≥2% of the total number of *nifH* clones analyzed (The common OTUs contained 441
377 of the 512 Cluster I sequences). OTUs were defined according to 3% nucleotide sequence
378 difference using the furthest neighbor method. OTU designation is from most common (OTU-1)
379 to least. A) OTU distribution among regions. B) OTU distribution between OMZ (including
380 core of the ODZ and the upper oxycline depths) and surface depths (oxygenated water). C)
381 OTU distribution of cDNA vs DNA clones.

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383

384 Figure 2. Rarefaction curve displaying observed OTU richness versus the number of clones
385 sequenced for Cluster I *nifH* sequences (cDNA and DNA). OTUs were defined and designated as
386 in Figure 1. Chao estimators (individual symbols) are shown for each of the same subsets
387 represented in the rarefaction curves.

388

389 Figure 3. Phylogenetic tree of Cluster 1 based on amino acid sequences. Positions of the OTUs
390 are shown relative to their nearest neighbors from the database. Individual sequence identities
391 comprising each OTU are listed in Supplemental Table 2.

392

393 Figure 4. Histogram of the 6 most common OTUs from Cluster II, III, IV *nifH* clone libraries
394 from the three OMZ regions. OTUs were considered common if the total number of sequences
395 in an OTU was ≥2% of the total number of *nifH* clones analyzed (the common OTUs contained

252 of the 275 Cluster II, III, IV sequences). OTUs were defined according to 3% nucleotide sequence difference using the furthest neighbor method. OTU designation is from most common (OTU-1) to least. A) OTU distribution among regions. B) OTU distribution between OMZ (including core of the ODZ and the upper oxycline depths) and surface depths (oxygenated water). C) OTU distribution of cDNA vs DNA clones.

Figure 5. Rarefaction curve displaying observed OTU richness versus the number of clones sequenced for Cluster II, III, IV *nifH* sequences (cDNA and DNA). OTUs were defined and designated as in Figure 4. Chao estimators (individual symbols) are shown for each of the same subsets represented in the rarefaction curves.

Figure 6. Phylogenetic tree of Clusters II, III, IV based on amino acid sequences. Positions of the OTUs are shown relative to their nearest neighbors from the database. Individual sequence identities comprising each OTU are listed in Supplemental Table 2.

Figure 7. RDA plots for (A) Cluster I and (B) Clusters II, III, IV illustrating the relationships among OTUs (green circles) and sites. DNA = squares; cDNA = circles. Arabian Sea = cyan (surface) and blue (OMZ); ETNP = pink (surface) and red (deep); ETSP = yellow (surface) and orange (deep). (A) Twelve most abundant OTUs for Cluster I and the four most independent environmental variables. T = temperature, S = salinity, NO2 = nitrite concentration, O2 = oxygen concentration. (B) Six most abundant OTUs for Clusters II, III, IV and all six environmental variables. NO3 = nitrate concentration, Z = depth.

Tables

Table 1. Sampling regions and depths and sequences derived from each depth

Table 2. OTU summary for both clusters

Richness and diversity statistics for *nifH* clone libraries from three OMZ regions. ACE and Chao are non-parametric estimators that predict the total number of OTUs in the original sample.

Table 3. OTU identities for both clusters

Cultivated species with closest nucleotide identity to the OTUs identified in the *nifH* clone libraries from three OMZ regions. Only the 12 most common OTUs (out of 41 total) are listed for Cluster 1 sequences, and the six most common (out of 18 total) for the Clusters II, III, IV libraries.

Supplemental

S Table 1A and B. List of sequences in each OTU for both clusters

S Table 2

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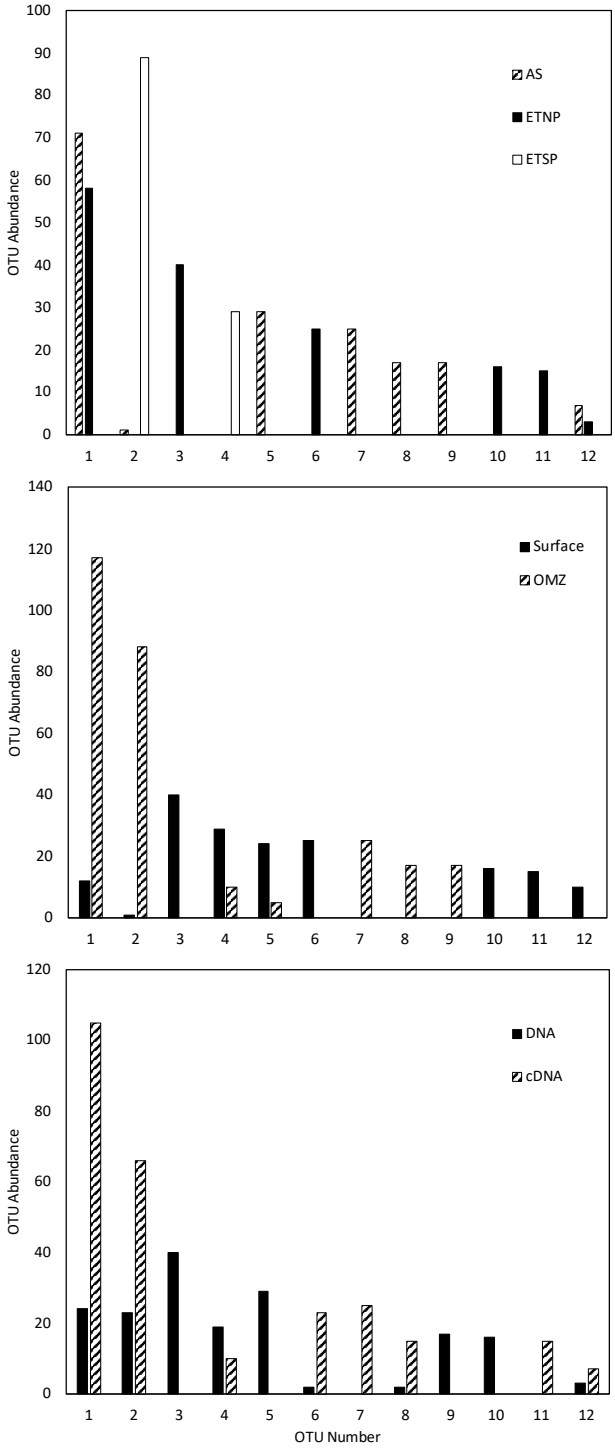
519 Figure.1



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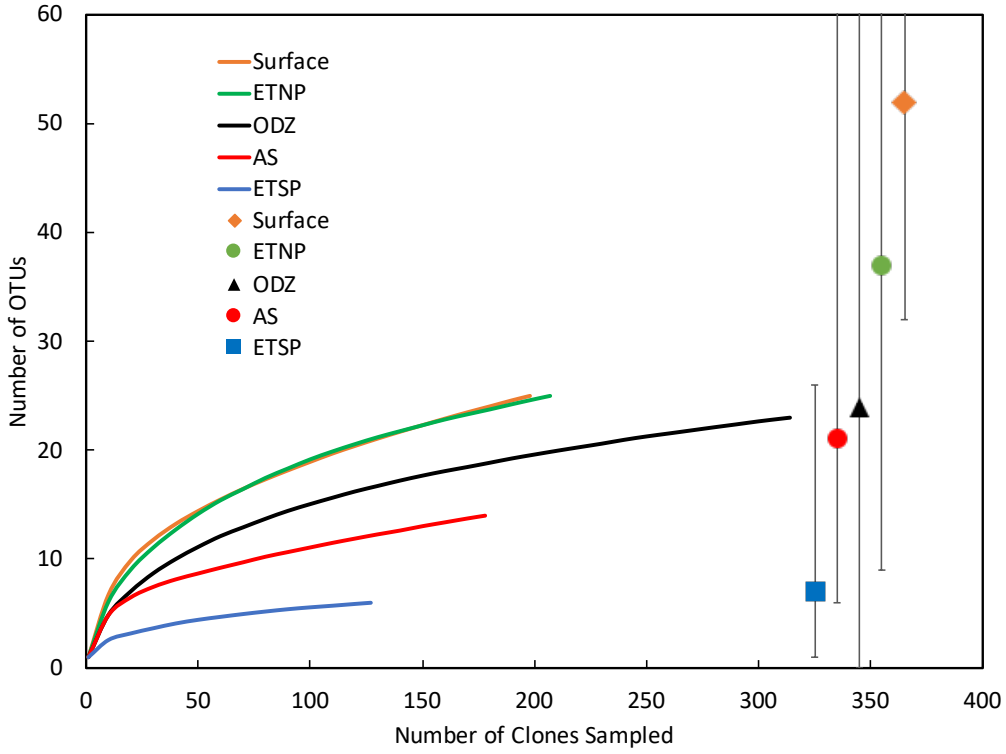

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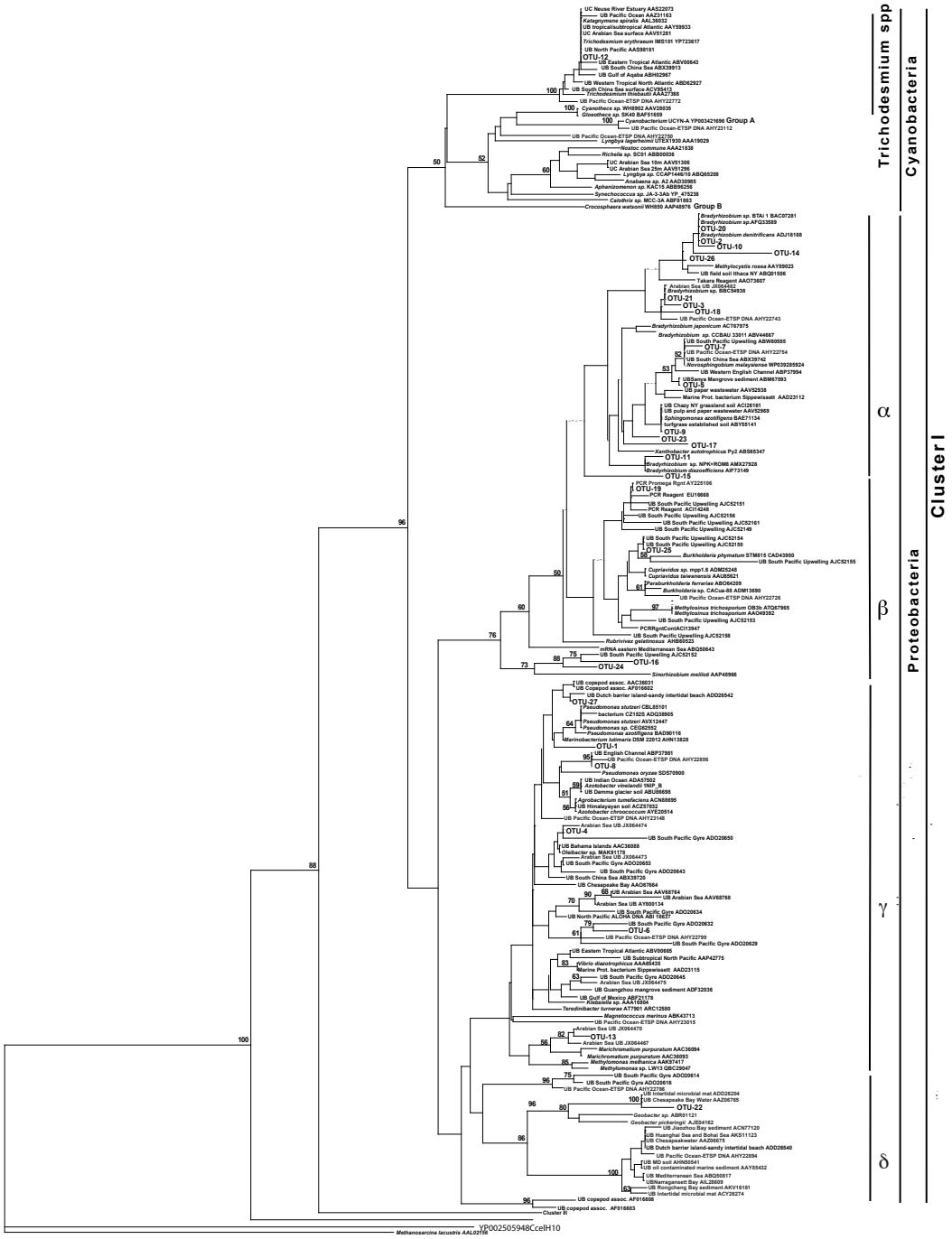

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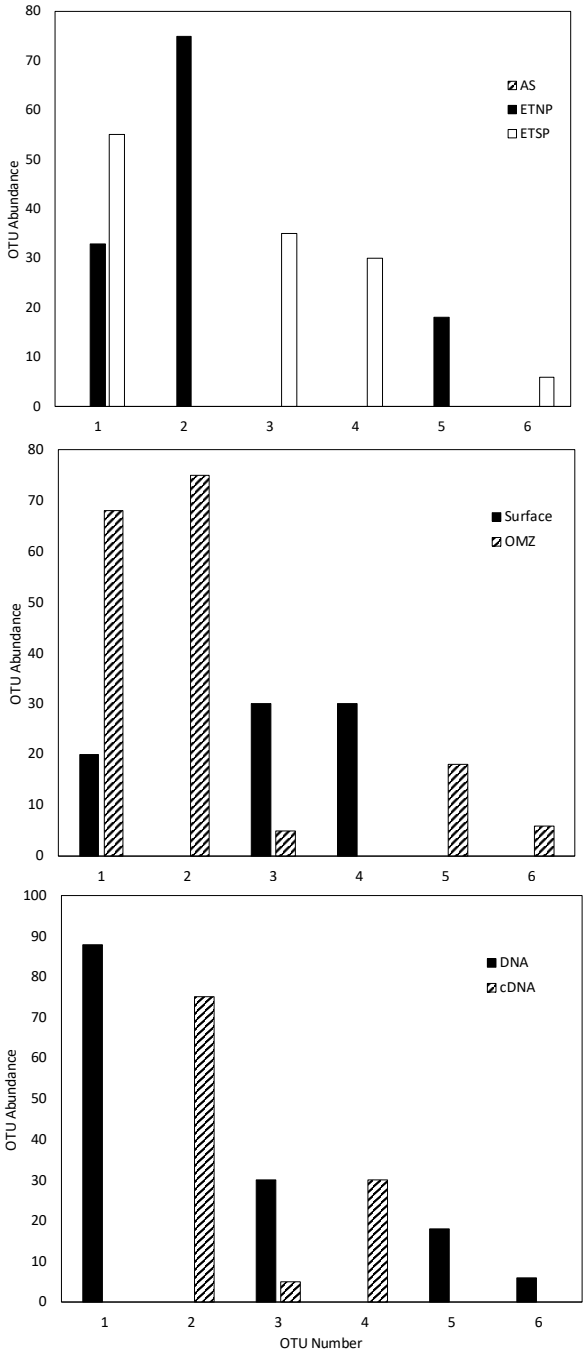

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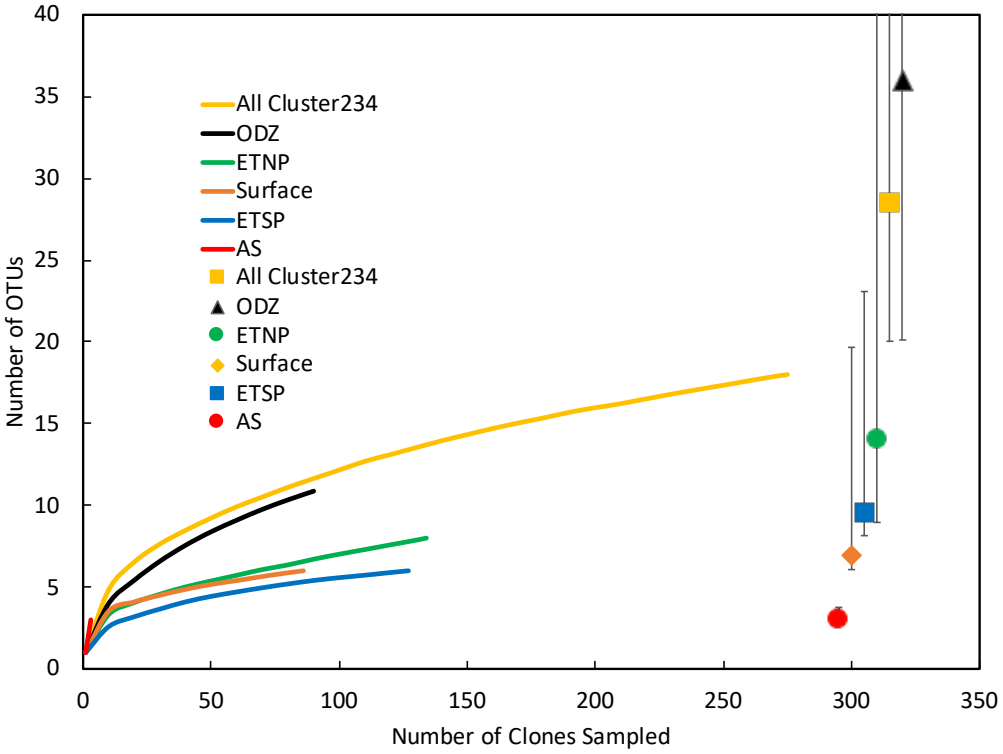

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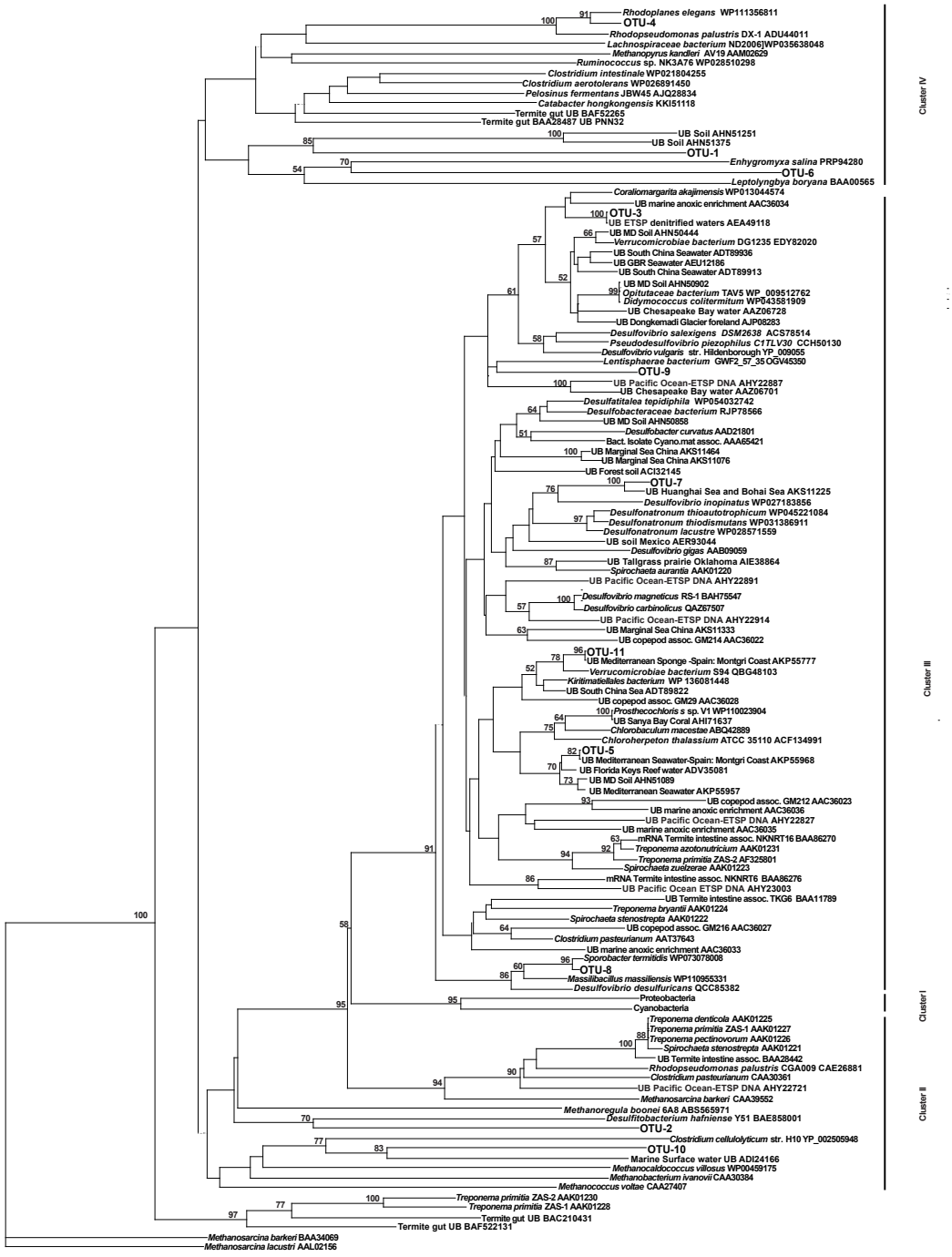

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Figure 7A

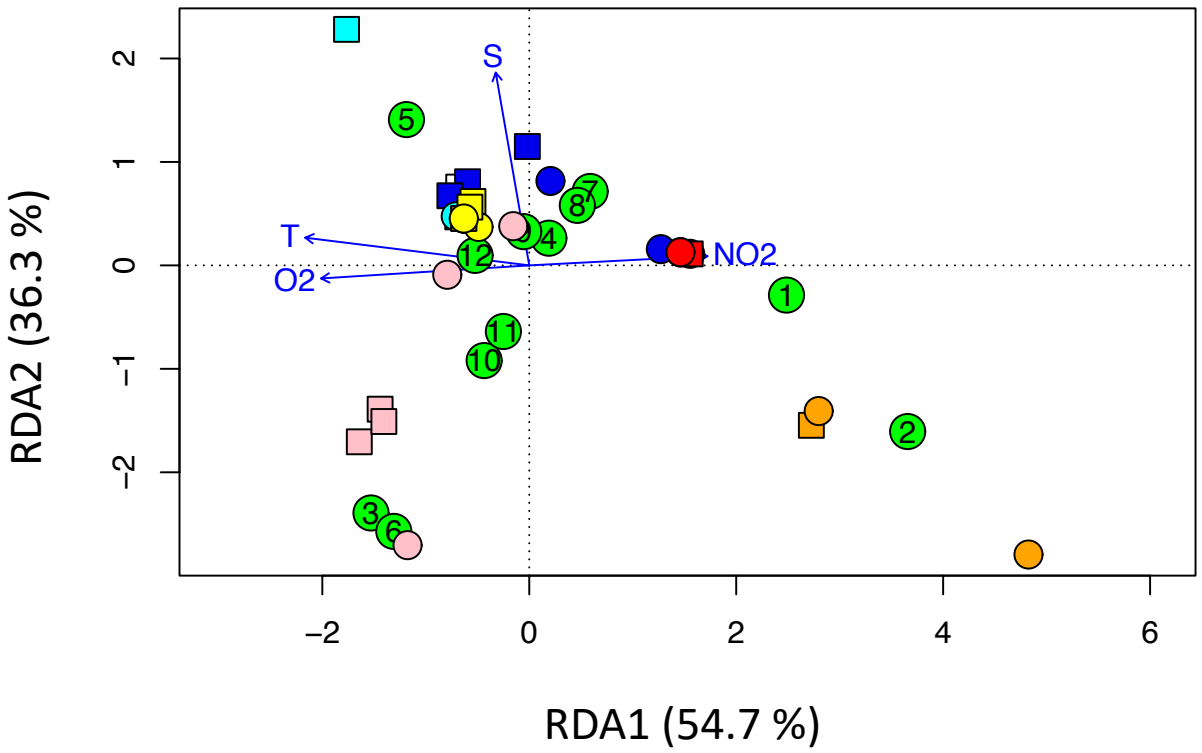

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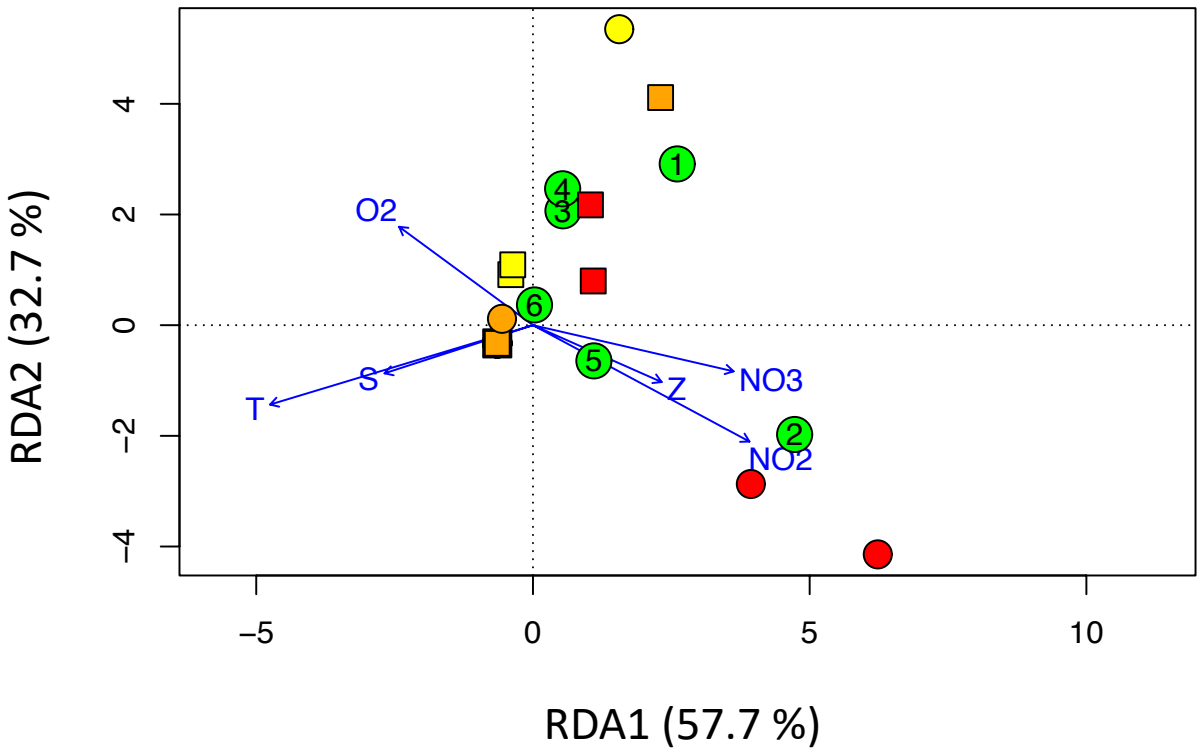
Figure 7B



538 Table 1 Sampling position details

OMZ Region	Station	Latitude	Longitude	Depth (m)	DNA Seqs	cDNA Seqs
Arabian Sea	S1	19°N	67°E	10	3	0
Arabian Sea	S1	19°N	67°E	60	20	0
Arabian Sea	S1	19°N	67°E	150	23	25
Arabian Sea	S1	19°N	67°E	175	10	22
Arabian Sea	S2	15°N	64°E	150	4	25
Arabian Sea	S3	12°N	64°E	10	25	4
Arabian Sea	S3	12°N	64°E	110	4	23
ETNP	BB1	20 9.6°N	106°W	0	26	5
ETNP	BB1	20 9.6°N	106°W	18	24	17
ETNP	BB1	20 9.6°N	106°W	90	42	38
ETNP	BB2	16 31°N	107 6.8°W	0	40	35
ETNP	BB2	16 31°N	107 6.8°W	150	47	67
ETSP	BB1	13 59.9°S	81 12.0°W	2	29	1
ETSP	BB1	13 59.9°S	81 12.0°W	130	46	44
ETSP	BB2	20. 46.1°S	70 39. 5°W	20	45	30
ETSP	BB2	20. 46.1°S	70 39. 5°W	115	23	40

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Table 2 OTU Summary

Sample subset	Depths, regions included	No. of Sequences	No. of Unique Sequences	No. of OTUs (cutoff ~3)	OTU /seq	Shannon	Simpson	Chao	Ace
Cluster I									
AS	Arabian Sea, all depths	178	36	14	0.079	1.8	0.22	21	45
ETNP	ETNP, all depths	207	80	25	0.121	2.37	0.14	37	34
ETSP	ETSP, OMZ depths	127	51	6	0.047	0.87	0.53	7	8
All ClusterI	Three regions, all depths	512	165	41	0.080	2.7	0.11	59	67
All ClusterI DNA	Three regions, all depths	257	97	35	0.136	2.8	0.08	42	45
All ClusterI cDNA	Three regions, all depths	255	75	24	0.094	1.7	0.25	24	27
All ClusterI Surface	Three regions, surface depths	198	73	25	0.126	2.5	0.10	52	75
All ClusterI OMZ	Three regions, all depths	314	98	23	0.073	0.9	0.23	30	37
Clusters II, III, IV									
AS	Arabian Sea, all depths	10	6	3	0.300	1.09	0.27	3	3
ETNP	ETNP, all depths	134	49	8	0.060	1.19	0.39	14	38
ETSP	ETSP, all depths	131	64	8	0.061	1.37	0.30	9	19
All Clusters II,III,IV	Three regions, all depths	275	117	18	0.065	1.88	0.21	28	26
All Clusters II,III,IV DNA	Three regions, all depths	155	65	12	0.077	1.20	0.37	22	17
All Clusters II,III,IV cDNA	Three regions, all depths	120	56	9	0.075	1.11	0.45	12	15
All Clusters II,III,IV Surface	Three regions, surface depths	86	46	6	0.070	1.32	0.29	7	13
All Clusters II,III IV OMZ	Three regions, OMZ depths	189	76	15	0.079	1.57	0.29	46	24

545 Table 3
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	No. of Sequences	Phylogenetic Affiliation	Closest cultured relative (DNA)	Identity DNA %	Coverage %	Closest cultured relative (Protein)	Identity AA %	Coverage %
Cluster I								
OTU-1	129	Gamma	*Psuedomonas stutzeri*	91	98	*Pseudomonas stutzeri strain SGAir0442*	95.8	99
OTU-2	89	Alpha	*Bradyrhizobium sp*	99	100	*Bradyrhizobium denitrificans strain LMG 8443*	99	99
OTU-3	40	Alpha	*Bradyrhizobium sp. TM124*	94	98	*Bradyrhizobium sp. MAFF 210318*	99	98
OTU-4	29	Gamma	*Marinobacterium lutimaris*	87	100	*Oleibacter sp*	100	99
OTU-5	29	Alpha	*Methylosinus trichosporium*	92	99	*Sphingomonas azotifigens*	99	100
OTU-6	25	Gamma	*Azotobacter chroococcum* strain B3	81	99	*Psuedomonas stutzeri*	94	99
OTU-7	25	Beta/Alpha	*Rubrivivax gelatinosus*	91	99	*Novosphingobium malasiense*	99	100
OTU-8	17	Gamma	*Psuedomonas stutzeri*	91	98	*Azotobacter chroococcum* strain B3	97	100
OTU-9	17	Beta/Alfa	*Burkholderia*	90	100	*Sphingomonas azotifigens*	100	100
OTU-10	16	Alpha	*Bradyrhizobium*	97	98	*Bradyrhizobium sp. ORS 285*	99	99
OTU-11	15	Alpha	*Bradyrhizobium*	97	98	*Bradyrhizobium diazoefficiens*	98	99
OTU-12	10	Cyanobacterium	*Katagnymene spiralis*	100	99	*Trichodesmium erythraeum*	100	99
Clusters II, III IV								
OTU-1	88	Alpha/Spirochaetaceae	*Rhizobium sp*	74	59	*Treponema primitia ZAS-1]*	55	98
OTU-2	75	Delta/Firmicutes	*Geobacter*	73	43	*Desulfitobacterium hafniense*	98	61
OTU-3	35	Verrumicrobia	*Opitutaceae bacterium*	82	99	*Coraliomargarita akajimensis*	95	99
OTU-4	30	Alpha	*Rhodopseudomonas palustris*	90	98	*Rhodoplanes elegans*	96	99
OTU-5	18	Delta/Chlorobi	*Desulfovibrio piezophilus*	79	99	*Prosthecochloris sp. V1, Chloroherpeton thalassium, Chloroherpeton thalassium*	92	99
OTU-6	6	Beta/Delta	*Azoarcus communis*	70	88	*Enhygromyxa salina*	70	74
OTU-7	4	Delta	*Desulfovibrio carbinolicus strain DSM 3852*	81	99	*Desulfovibrio inopinatus*	90	99
OTU-8	4	Delta/Firmicutes	*Desulfovibrio desulfuricans strain IC1*	77	100	*Sporobacter termitidis*	99	99

OTU-9	3	Delta/Lentisphaerae	*Desulfovibrio magneticus RS-1 DNA*	84	100	*Lentisphaerae bacterium GWF2_57_35, Desulfatitalea tepidiphila, Desulfobacteraceae bacterium*	84	100
OTU-10	3	Delta/Methanococci	*Desulfovibrio desulfuricans strain IC1*	77	100	*Methanocaldococcus villosus*	65	99
OTU-11	2	Verrucomicrobia	Verrucomicrobia bacterium S94	87	100	Verrucomicrobia bacterium S94	97	99

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