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High Diazotrophic Diversity but Low N_2 Fixation Activity in the Northern Benguela Upwelling System Confirming the Enigma of Nitrogen Fixation in Oxygen Minimum Zone Waters

Christian Furbo Reeder[1], Damian L. Arévalo-Martínez[2,3], Joan A. Carreres-Calabuig[4], Tina Sanders[5], Nicole R. Posth[4] and Carolin Regina Löscher[1,6]*

[1] Nordcee, Department of Biology, University of Southern Denmark, Odense, Denmark, [2] Chemical Oceanography department, GEOMAR Helmholtz Centre for Ocean Research Kiel, Kiel, Germany, [3] Institute for Geosciences, Kiel University, Kiel, Germany, [4] Department of Geosciences and Natural Resource Management (IGN), Geology, University of Copenhagen, Copenhagen, Denmark, [5] Institute for Carbon Cycles, Helmholtz Center Hereon, Geesthacht, Germany, [6] Danish Institute for Advanced Science (DIAS), University of Southern Denmark, Odense, Denmark

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***Correspondence:**
Christian Furbo Reeder
crfurbo@biology.sdu.dk

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Oxygen minimum zones (OMZs) have been suggested as a suitable niche for the oxygen-sensitive process of biological fixation of dinitrogen (N_2) gas. However, most N_2 fixation rates reported from such waters are low. This low N_2 fixation activity has been proposed to result from the unusual community of N_2 fixers, in which cyanobacteria were typically underrepresented. The Northern Benguela Upwelling System (North BUS) is part of one of the most productive marine ecosystems and hosts a well-developed OMZ. Although previous observations indicated low to absent N_2 fixation rates, the community composition of diazotrophs needed to understand the North BUS has not been described. Here, we present a first detailed analysis of the diazotrophic diversity in the North BUS OMZ and the Angola tropical zone (ATZ), based on genetic data and isotope speciation. Consistent with a previous study, we detected a slight N deficit in the OMZ, but isotope data did not indicate any active or past N_2 fixation. The diazotroph community in the North BUS was dominated by non-cyanobacterial microbes clustering with members of gamma-proteobacteria, as is typical for other OMZ regions. However, we found a strikingly high diversity of Cluster III diazotrophs not yet described in other OMZs. In contrast to previous observations, we could also identify cyanobacteria of the clades *Trichodesmium* sp., UCYN-A and *Cyanothece* sp., in surface waters connected to or above the OMZ, which were potentially active as shown by the presence of genes and transcripts of the key functional marker gene for N_2 fixation, *nifH*. While the detection of diazotrophs and the absence of active N_2 fixation (based on isotopic speciation) are consistent with other OMZ observations, the detected regional variation in the diversity and presence of cyanobacteria indicate that we still are far from understanding the role of diazotrophs in OMZs, which, however, is relevant for understanding the N cycle in OMZ

waters, as well for predicting the future development of OMZ biogeochemistry in a changing ocean.

Keywords: nitrogen fixation, diazotrophs diversity, oxygen minimum zone (OMZ), Benguela coastal upwelling system, Angola Basin

1 INTRODUCTION

Nitrogen (N) is an essential nutrient, and limiting for primary production and carbon (C) uptake from the atmosphere in large parts of the ocean (Redfield, 1958; Capone and Carpenter, 1982; Gruber, 2005). Specialized microbes, so-called diazotrophs, can circumvent this shortage due to their ability to fix dinitrogen gas (N_2). Classically, cyanobacteria were considered the most important diazotrophs in the ocean, i.e., the filamentous genus *Trichodesmium* and unicellular genera, including *Crocosphaera* sp. and *Candidatus* Atelocyanobacteriym thalassa (UCYN-A) (Moisander et al., 2010). However, due to their need for light, their habitat is largely restricted to sunlit surface waters. In recent years, a large diversity of non-cyanobacterial diazotrophs has been discovered, many of them from oxygen minimum zone (OMZ) waters (Fernandez et al., 2011; Hamersley et al., 2011; Jayakumar et al., 2012; Löscher et al., 2014; Turk-Kubo et al., 2014; Cheung et al., 2016; Jayakumar et al., 2017; Jayakumar and Ward, 2020; Löscher et al., 2020). These waters are characterized by massive N loss, creating a theoretical need for N_2 fixation by providing excess phosphorous (Ingall and Jahnke, 1997; Deutsch et al., 2007; Kalvelage et al., 2013). Yet studies have confirmed that N_2 fixation is typically minor in such regions (Wang et al., 2019). In addition, anoxic conditions have been shown to reduce the energy constraints of N_2 fixation as compared to nitrate (NO_3^-) uptake, which suggests that N_2 fixation might be an attractive way of N acquisition in oxygen (O_2)-depleted waters (Großkopf and LaRoche, 2012). Indeed, based on the analysis of the key functional marker gene for N_2 fixation, *nifH*, a broad diversity of mostly non-cyanobacterial diazotrophs was found in OMZ waters of the Eastern Tropical North Pacific (ETNP), the Eastern Tropical South Pacific (ETSP), the Arabian Sea (AS), and the Bay of Bengal (BoB) (Fernandez et al., 2011; Halm et al., 2011; Hamersley et al., 2011; Jayakumar et al., 2012; Bonnet et al., 2013; Farnelid et al., 2013; Löscher et al., 2014; Cheung et al., 2016; Löscher et al., 2016a; Jayakumar et al., 2017; Moisander et al., 2017; Christiansen and Löscher, 2019; Löscher et al., 2020; Li et al., 2021).

A recent report of diazotrophs in OMZ waters revealed a biogeographically distinct and diverse community of N_2 fixers throughout the OMZs of the ETNP, ETSP, and AS (Jayakumar and Ward, 2020), but dominated by only a few operational taxonomic units (OTUs) (Jayakumar and Ward, 2020). Interestingly, despite a certain degree of geographical variation in the overall community structure of N_2 fixers, the identified heterotrophic diazotrophs represented similar metabolic types in all OMZ waters (Jayakumar and Ward, 2020). For instance, proteobacteria-like sequences related to *nifH* Cluster I (Zehr et al., 2003) include alpha-proteobacterial methanotrophs and gamma-proteobacterial N_2 fixers such as *Vibrio diazotrophicus* and *Pseudomonas stutzeri*, which have been reported in the OMZ waters of the ETNP, ETSP, AS and BoB (Fernandez et al., 2011; Löscher et al., 2014; Turk-Kubo et al., 2014; Cheung et al., 2016; Jayakumar et al., 2017). Epsilon-proteobacteria related to *Arcobacter nitrofigilis* (Löscher et al., 2014) and sequences related to *Azotobacter vinelandii* and *Teridinibacter tunerae* have been reported (Turk-Kubo et al., 2014) from the ETSP and BoB (Löscher et al., 2020). Besides Cluster I sequences, delta-proteobacterial *nifH* and other more rare *nifH* types of Clusters II, III, and IV (Zehr et al., 2003) have been identified in OMZs, including anaerobic sulphate-reducers in the ETNP (Jayakumar et al., 2017), the ETSP (Löscher et al., 2014; Turk-Kubo et al., 2014; Christiansen and Löscher, 2019), the AS (Jayakumar et al., 2012) and the BoB (Löscher et al., 2020). Cluster II is rarely found and has only been reported in ETSP (Fernandez et al., 2011). Cluster IV, commonly associated with non-functional *nif*-types or paralogue genes, has been reported from the ETNP (Cheung et al., 2016; Jayakumar et al., 2017), the ETSP (Löscher et al., 2014), the AS (Jayakumar et al., 2012) and BoB OMZ waters (Löscher et al., 2020).

Interestingly, while diazotrophs are usually present and diverse in OMZ waters, they only seem to fix N_2 at minimal rates (Bonnet et al., 2013; Dekaezemacker et al., 2013; Löscher et al., 2016b; Jayakumar et al., 2017; Chang et al., 2019; Löscher et al., 2020). One explanation might be the absence of cyanobacterial N_2 fixers from those waters. Only a few sequences related to cyanobacteria have been reported. This includes *Trichodesmium* (<5 sequences) in the ETNP, ETSP, AS and BoB (Jayakumar et al., 2012; Turk-Kubo et al., 2014; Jayakumar et al., 2017; Löscher et al., 2020), UCYN-B, related to *Crocosphaera* in the ETSP (Löscher et al., 2014) and UCYN-A in the ETSP (Turk-Kubo et al., 2014; Li et al., 2021). In addition, UCYN-B related sequences were detected in the AS based on 16S rRNA analysis (Mazard et al., 2004).

To date, the diazotrophic composition of the OMZ waters in the Northern Benguela upwelling system (North BUS) has not been extensively studied. The Benguela current system spans from 14°S to 35°S is one of four major eastern boundary upwelling regions, and is recognized as the most productive upwelling zone in the global ocean (Bakun, 1996; Carr, 2001). The Benguela current system consists of northern (14–26°S) and southern (26–35°S) sectors with active upwelling which are separated by the Lüderitz upwelling cell (25–27°S). North of 14°S, the coastal areas are part of the Angola tropical zone (ATZ). Upwelling zones in this area are not affected by winds as coastal upwelling zones typically are. North BUS is characterized by equatorward winds, low water temperatures, high phytoplankton biomass, and seasonally O_2 depleted waters with locally intense hydrogen sulfide (H_2S) events and massive N loss (Tyrrell and Lucas, 2002; Kuypers et al., 2005; Monteiro and van der Plas, 2006;

Bartholomae and van der Plas, 2007; Hutchings et al., 2009; Nagel et al., 2013; Vizy et al., 2018). At 17°S, the warm tropical waters coming from the north are mixed with cold waters from the south. This is also known as the Angola-Benguela Frontal Zone (Hutchings et al., 2009). This study focused on the ATZ and North BUS. Waters off North BUS are characterized by N loss, together with increased fluxes of phosphate (PO_4^{3-}) from sediments underlying an OMZ to the water column (Ingall and Jahnke, 1997). Creating a low N:P ratio similar to other OMZ regions, this would classically be considered favorable for N_2 fixation (Wasmund et al., 2005; Flohr et al., 2014). Previous studies have identified N_2 fixation from below the detection limit to up to 8 nmol N L^{-1} d^{-1} in surface waters (>70 m) (Staal et al., 2007; Sohm et al., 2011; Wasmund et al., 2015). While previous studies (Staal et al., 2007; Sohm et al., 2011) might have even overestimated N_2 fixation rates dues to the possible use of contaminated $^{15}N_2$ gas (Dabundo et al., 2014), all rates presented in those studies were comparable to other reports of N_2 fixation in OMZ waters with rates of 0.4 nmol L^{-1} d^{-1} to 3.5 nmol L^{-1} d^{-1} found in the ETNP and ETSP (Fernandez et al., 2011; Bonnet et al., 2013; Löscher et al., 2014; Jayakumar et al., 2017). N_2 fixation was also low or undetectable in the OMZ waters of BoB, (Löscher et al., 2020; Saxena et al., 2020) and has not been measured in the OMZ of the AS, but diazotrophs have been found active (Jayakumar et al., 2012). A similar environment has measured 0.07 nmol L^{-1} d^{-1} in the Southern California Bight (Hamersley et al., 2011) and 0.44 nmol L^{-1} d^{-1} in the Baltic Sea (Farnelid et al., 2013).

The last decade has revealed a distinct diversity of heterotrophic diazotrophs in OMZ waters (Bonnet et al., 2013; Dekaezemacker et al., 2013; Löscher et al., 2016b; Jayakumar et al., 2017; Chang et al., 2019; Löscher et al., 2020). Their role and importance, however, in these OMZ waters are not clear. Understanding the community composition of diazotrophs in these waters is the first step to shed light on their potential contribution to N_2 fixation. Here, we present a first report of the diazotrophic community composition and distribution present in the OMZ of the Northern BUS and the ATZ using a combination of isotope analysis, and sequencing of the biomarker gene, *nifH*. Results were compared and compiled into a statistical analysis with similar sequencing techniques from OMZs in the ETNP, ETSP and AS (Jayakumar and Ward, 2020).

2 MATERIALS AND METHODS

2.1 Seawater Sampling

Samples were collected during the cruise M148/1 on the German research vessel *RV Meteor* from 24th of May to 29th of June 2018. The survey crossed a large area with contrasting environmental properties (e.g., temperature) from the ATZ (South ATZ (10-16°S) and North ATZ (north from 5°S) to North BUS (17-23°S). Details regarding the individual stations can be seen in **Table 1** and the **Supplementary Material, Table S1**. Sampling was carried out by means of a CTD-Rosette system equipped with double sensors for salinity and

TABLE 1 | Overview and description of the *nifH* clone library.

Location	Area	Station	Lon (E°)	Lat (N°)	Depth (m)	O_2 feature	O_2 (µM)	Temp (°C)	Sal	NO_3^- (µM)	NO_2^- (µM)	NH_4^+ (µM)	PO_4^{3-} (µM)	$\delta^{15}N$-NO_3^- [‰]	No. of DNA Seq	No. of cDNA seq
North Angola Tropical Zone	Waters off Angola	42	7	-11.7	97	Oxycline	42.2	24.5	35.6	NA	NA	NA	NA	NA	17	0
		46	11.6	-11.7	9.5	Oxic	222.5	24.3	35.7	NA	NA	NA	NA	NA	90	0
		50	12.5	-11.1	97.2	Oxycline	50.7	15.1	35.6	NA	NA	NA	NA	NA	21	0
		80	13.1	-10.7	143.8	Oxycline	44.3	14.5	35.5	NA	NA	NA	NA	NA	11	0
		80	13.1	-10.7	47.7	Oxycline	69.1	20.7	35.9	10.28	0.266	0.11	0.862	NA	16	0
		80	13.1	-10.7	18.3	Oxic	234.9	22.9	35.5	NA	NA	NA	NA	NA	0	91
	Waters off South Angola	87	11.4	-15	9	Oxic	219.8	24.8	35.8	0	0.001	NA	0.164	NA	74	0
		92	11.8	-15.3	17.3	Oxic	154.9	19.1	35.8	12.6	0.27	0.17	0.924	NA	0	0
South Angola Tropical Zone	Waters off Congo	76	11.2	-6.4	336	Oxycline	27.7	21.6	35.9	1.31	0.079	NA	0.296	NA	56	0
		76	11.2	-6.4	29.7	Oxic	143.4	9.5	34.9	40.98	0	NA	2.506	NA	14	23
		76	11.2	-6.4	9.2	Oxic	211.5	20.5	36	10.1	0.644	NA	0.782	NA	6	34
Northern Benguela Upwelling System	Waters off Namibia	98	11.6	-17.5	37.3	Oxic	101.2	15.1	35.5	29.99	0.646	0.2	1.918	5.17	33	0
		98	11.6	-17.5	7.6	Oxic	103.2	15.1	35.5	NA	NA	0.2	NA	5.24	64	0
		99	12.3	-19	100	Oxycline	23	13.7	35.3	30.3	0.278	0.17	2.38	6.43	85	0
		99	12.3	-19	57.9	Oxycline	35.6	13.7	35.3	28.98	0.103	0.17	2.177	6.33	26	0
		99	12.3	-19	37.6	Oxycline	72.2	13.7	35.3	30.98	0.224	0.17	2.346	6.5	30	0
		102	13.8	-22	95.8	OMZ	3.3	12.8	35.2	7.97	0.508	0.05	1.496	15.87	77	0
		102	13.8	-22	71	Suboxic	10.4	12.8	35.2	22.18	0.514	0.25	2.523	9.8	65	0
		101	13.3	-20.9	17.3	Oxic	123.8	14	35.2	26.8	0.1	0.17	2.1	6.44	0	39
		101	13.3	-20.9	92.5	Suboxic	6.7	12.2	35.1	10.28	1.8	0.64	0.99	6.51	0	4
		103	14.2	-22.7	87.1	OMZ	2.7	12.2	35.1	10.28	1.801	1.9	0.99	11.49	82	0

*From each location, CTD corresponds to sampling station in **Figure 1**. O_2 feature refers to oxic waters (>100 µM), the oxycline (20-100 µM), suboxic waters (5-20 µM) and OMZ waters (<5 µM). The total number of sequences along with the corresponding biogeochemical data from each station is presented.*
NA standard for not available.

temperature (Sea-Bird SBE 43), as well as sensors for turbidity and fluorescence (ECO FLNTURTD, Wetlabs) and O_2 (Sea-Bird Electronics, Bellevue, WA). The O_2 sensor was calibrated by manual Winkler titration. Seawater samples for nutrient analyses were obtained from 10 Niskin bottles attached to the CTD-Rosette. Nitrate (NO_3^-), nitrite (NO_2^-), and PO_4^{3-} concentrations (detection limits: 0.01 µM (NO_3^-, NO_2^-, and PO_4^{3-})) were determined on board with a QuAAtro auto-analyzer (SEAL Analytical GmbH, Germany) immediately after sampling following Grasshoff (1999). Ammonium (NH_4^+) concentrations were measured photometrically following Grasshoff (1999) (detection limit: 0.05 µM (NH_4^+)).

2.2 Nucleic Acid Extraction

Seawater samples for nucleic acid extraction were collected from Niskin bottles in a volume of 0.5 -2 L (exact volumes were recorded) and filtered on Millipore Durapore membrane filters (Millipore, Billaterica, USA) with a pore diameter of 0.2 µm using a gentle vacuum. After a maximum of 20 min of filtration time, filters were stored at -80°C until further analysis. Filters were flash-frozen in liquid nitrogen and nucleic acids (DNA and RNA) were extracted using the Qiagen DNA/RNA AllPrep Kit (Qiagen, Hildesheim, DE) as described in Löscher et al. (2012). The nucleic acid concentration and quality were checked spectrophotometrically on a MySpec spectrofluorometer (VWR, Darmstadt, Germany). Genomic DNA was eliminated from RNA by treating it with DNase I (Invitrogen, Thermo Fisher Scientific United Kingdom). RNA was checked for purity by a *nifH* nested PCR. As no DNA contamination could be seen, RNA was transcribed to cDNA using Qiagen QuantiTect Reverse Transcription Kit (Qiagen, Hildesheim, DE).

2.3 Molecular Methods

Environmental DNA and cDNA samples (48 samples in total) were subjected to *nifH* PCR-amplification using a nested PCR with primers and conditions described by Zani et al. (2000). Both nested PCR runs were carried out with Taq DNA polymerase (Thermo Scientific, Waltham USA). For the first PCR round, Bovine Serum Albumin (BSA, 1.6 µg in 25 µl) (Thermo Scientific, Waltham, USA) were added to the PCR mix. *nifH* amplicons were purified from an agarose gel using the E.Z.N.A. Gel Extraction Kit (Omega Bio-Tek, Norcross, USA). In total, 21 *nifH* products were recovered covering waters from ATZ (northern part off Congo (5°S), southern part off Angola (10-16°S)), North BUS (off Namibia (17-23°S)) (**Figure 1**) and different O_2 ranges (oxic waters >100 µM O_2, oxycline 20-100 µM O_2, suboxic 5-20 µM O_2, and OMZ <5 µM O_2. *nifH* amplicons were Topo TA cloned (Thermo Fisher Scientific,

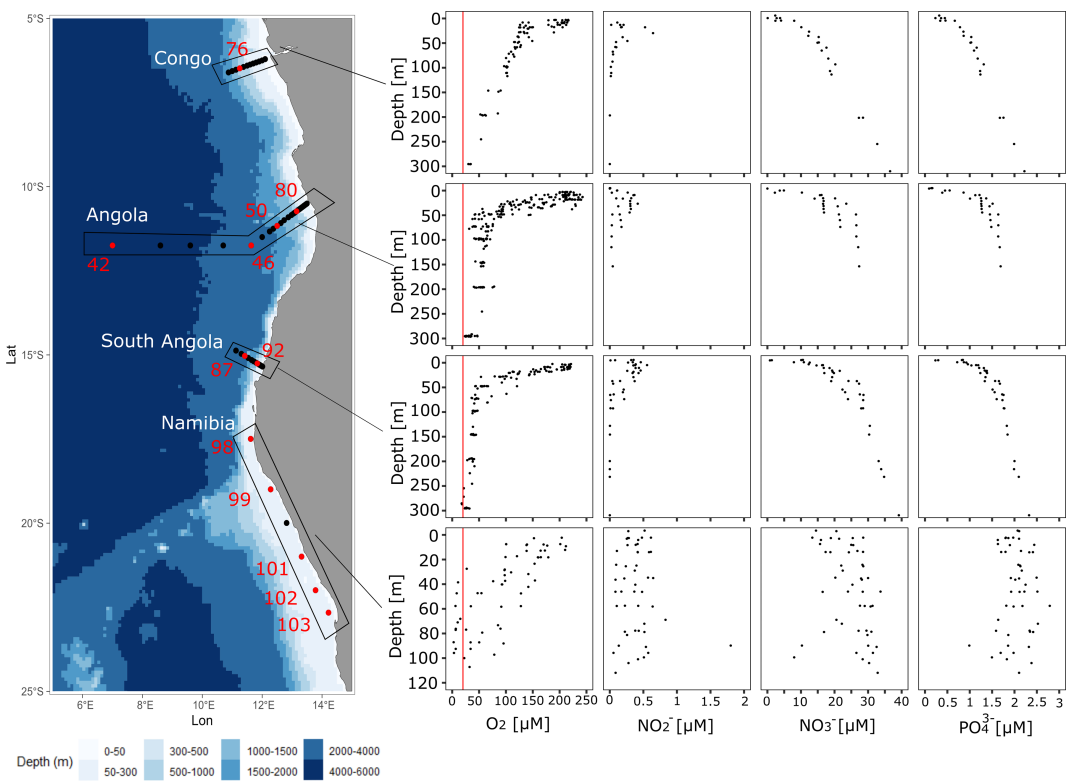

FIGURE 1 | Left panel: Topographical map of the North ATZ (north of 10°S) and South ATZ (between 10 and 15°S) and the North BUS (15-25°S). The four coastal sections of the cruise track are indicated with black dots. Red dots indicate stations from which *nifH* amplicons were generated. Right panels: Oxygen and nutrient depth profiles from each transect as indicated with black boxes on the map. The red line indicates an O_2 concentration of 20 µM.

Waltham, US). Sequencing was carried out as a service of the Institute of Clinical Molecular Biology in Kiel, Germany. In total, 958 *nifH* sequences (191 transcripts and 767 gene sequences) were obtained. Sequences were quality-checked and trimmed to a length of 321 bp in MEGAX (Kumar et al., 2018). The sequences were submitted to NCBI with accession ID OK082431-OK083221, OL412005-OL412134 and OM453718 - OM453821.

2.4 Phylogenetic Analysis and OTU Generation

nifH amplicons were translated into amino acids and aligned in MEGAX (Kumar et al., 2018). Based on the nucleotide alignment OTUs were defined using Mothur (Schloss et al., 2009). For comparability, OTUs were generated following Jayakumar and Ward (2020). Briefly, a distance matrix was created using *pairwise.seqs* followed by assigning sequences to OTUs using *cluster.classic* at the default setting (furthest neighbor clustering algorithm). *nifH* richness was calculated using the command *rarefaction.single*, from each location and O_2 feature. OTUs were defined at a threshold of 0.03% which is similar to the level for species definition used for 16S rDNA (Gaby et al., 2018). Different alignment constraints (delay divergent cut-off (%) at 30, 60, 80, 95) and gap penalties (10 and 20) were tested. There were no observed changes in OTU outcome. In total, 82 OTUs were generated, which were aligned with published *nifH* sequences using *Methanosarcina lacustris* (AAL02156) as the outer group. A maximum-likelihood phylogenetic tree was constructed using the Poisson model with 1000 bootstraps replicate. Finally, a phylogenetic tree was visualized in iTOL (Letunic and Bork, 2019).

2.5 Natural Abundance of $\delta^{15}N$ in the Particulate Organic Material (POM) and NO_3^-

For each sample, 2 L of seawater was filtered onto pre-combusted (450°C, 4-6 hours) 45 mm 0.7 GF/F filters (GE Healthcare Life Sciences, Whatman, USA) at 200 mbar vacuum. Prior to analysis, filters were stored in a -20°C freezer. Natural abundances of $\delta^{15}N$ in POM were determined as follows. Filters were acidified over fuming HCl overnight, oven-dried, and duplicates were analyzed on an Elemental Analyzer Flash EA 1112 series (Thermo Fisher, USA) coupled to an isotope ratio mass spectrometer (Finnigan Delta Plus XP, Thermo Fisher, USA) against a caffeine standard.

Isotope ratios of NO_3^- (plus NO_2^-) were determined by the denitrifier method (Casciotti et al., 2002; Sigman et al., 2001). This method is based on a mass spectrometric measurement of isotopic ratios of N_2O produced by the bacterium *Pseudomonas aureofaciens*. Briefly, 20 nmol of NO_3^- were injected in a 20 mL vial containing *P. aureofaciens*. Two international standards were used (IAEA NO_3^- δ15N= +4.7 ‰, USGS34 $\delta^{15}N$ = −1.8 ‰) for a regression-based correction of isotope values. For further quality assurance, an internal standard was measured with each batch of samples. The standard deviation for $\delta^{15}N$ was better than 0.2 ‰. Samples were measured on a Delta Plus XP mass spectrometer coupled to a Gasbench II (Thermo Fisher, USA).

2.6 Statistical Analysis

The most abundant OTUs from North BUS along with environmental factors (NO_2^-, NO_3^-, PO_4^{3-}, O2, NH_4^+, isotope ($\delta^{15}N - NO_3^-$), depth, temperature, turbulence, salinity and fluorescence) were used for a redundancy analysis (RDA) using the vegan package in R (Oksanen et al., 2020). Briefly, environmental factors from OTUs mixed from different stations were included by taking the average between the stations contributing above 5% to the OTU. Environmental factors were Hellinger-transformed using *decostand*. Then, the most parsimonious RDA model was determined using vegan's function *ordistep*. The RDA model for OTUs derived from this study included depth, $\delta^{15}N - NO_3^-$, salinity, O_2, NH_4^+ and NO_3^- as explanatory variables. Turbulence, fluorescence, NO_2^- and PO_4^{3-} was removed as those parameters turned out to be repetitive. For cross-comparison between OMZ regions, OTUs from the ETNP, ETSP and AS taken from Jayakumar and Ward, 2020 were included in an additional RDA. Compiling OTUs from all OMZ waters, temperature, depth, O_2, NO_2^- and NO_3^- were identified as explanatory variables. Raw data can be accessed in **Table S5**.

3 RESULTS AND DISCUSSION

3.1 Hydrochemistry in the ATZ and North BUS

We investigated the community diversity and distribution of N_2 fixers in the ATZ and the OMZ off the North BUS and compared our results to previous studies on OMZ N_2 fixers. During the time of the cruise, we observed a meridional gradient in O_2 with decreasing concentrations from 5˚S to 16˚S. O_2 concentrations in the ATZ reached a minimum of around 20 µM O_2 at 300 m water depth with an oxycline (20-100 µM O_2) starting at around 50-100 m (**Figure 1**). Only in the Northern BUS, a strong OMZ with O_2 concentrations below 5 µM O_2 could be detected (**Figure 1**).

Along with decreased O_2 concentrations in South ATZ (10-16 °S), a small secondary NO_2^- maximum was detected at a water depth of 75 m (**Figure 1**), possibly indicative for N loss processes, such as denitrification or anammox (Kalvelage et al., 2013). Comparable to the ATZ (5-16 °S), NO_2^- concentrations in the Northern BUS were generally elevated with concentrations of up to 0.8 µM and a single datapoint of 1.8 µM at a depth of 87 m (**Figure 1**; **Table 1**). NO_3^- and PO_4^{3-} showed a vertical distribution with concentrations increasing below the oxycline reaching concentrations of up to 39 µM and 2.5 µM, respectively, in our deepest samples from 300 m water depth in the ATZ. In the Northern Benguela upwelling system OMZ waters, where O_2 concentrations were below 20 µM, some samples showed a decrease of NO_3^- over depth along with increased NO_2^- concentrations, again suggesting beginning denitrification within the water column or the sediment below (**Table 1**; **Figure 1**). Overall, the northern BUS seemed to be N limited compared to the ATZ, as indicated by the negative intercept of the trendline for the N:P ratio

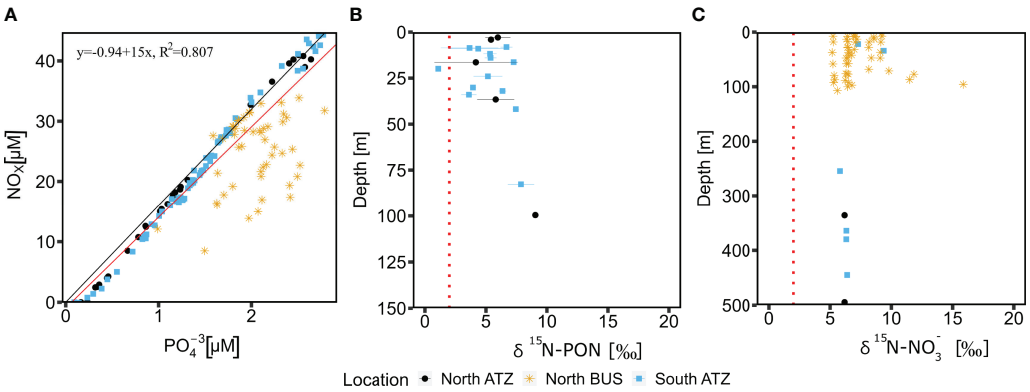

FIGURE 2 | NO_x over PO_4^{3-} concentrations from all sampled stations **(A)**. The N:P ratio is around the Redfield ratio of N:P = 16:1 (black line). The linear regression equation (red line) has a negative intercept suggesting a slight N limitation. Samples from the North BUS OMZ are an exception, as those show an undersaturation of N suggesting active N loss processes. δ^{15}N-PON **(B)** and $\delta^{15}N - NO_3^-$ **(C)** shows no indication of active N_2 fixation but rater N-loss (denitrification, anammox).

(**Figure 2A**). The presence of N loss processes is corroborated by the detected surplus of PO_4^{3-} compared to fixed N assuming Redfield stoichiometry (Redfield et al., 1963) (**Figure 2A**). Alternatively, the surplus might result from enhanced P flux at anoxia (Ingall and Jahnke, 1997).

Classically, a surplus of PO_4^{3-} over fixed nitrogen in combination with low O_2 concentrations would be considered favorable for O_2-sensitive diazotrophs (Wasmund et al., 2005; Deutsch et al., 2007; Flohr et al., 2014). However, our δ^{15}N-PON (3‰ to 7‰) and $\delta^{15}N - NO_3^-$ (5‰ to 17‰) signatures indicates an absence of N_2 fixation (**Figures 2B, C**).Our data rather points towards active denitrification in the north BUS OMZ water, as active N_2 fixation would be expected to result in a lighter δ^{15}N signature of -2‰ – 2‰ (Delwiche and Steyn, 1970; Montoyal et al., 2002; Dähnke and Thamdrup, 2013). Cautiously, one must note that δ^{15}N signatures are a mixed signal of many processes and might be impacted by other factors and processes, including denitrification, annamox or N import *via* dust and rivers. Our measurements are, however, in line with a previous study from the North BUS reporting an isotope enrichment from 1 - 10‰ in the OMZ waters of this region (Nagel et al., 2013) and from other OMZ regions with δ^{15}N-PON signatures from 2‰ - 8‰ (Kumar et al., 2004) or 5-8‰ (Löscher et al., 2020) in the BoB and 4‰ -11.6‰ in the ETNP (Voss et al., 2001).

3.2 Diazotrophic Diversity in the Northern Benguela Upwelling System

While we did not find any clear evidence for active N_2 fixation in either the North/South ATZ or the Northern BUS during our cruise, we found the genetic potential and presence of transcripts for N_2 fixation using the key functional marker gene *nifH*. Altogether, we obtained 958 *nifH* amplicon sequences, which include 191 transcripts from an area covering the North ATZ (waters off Congo 6°S) (133 sequences), the South ATZ (waters off Angola [11°S-15°S)] (320 sequences), the North BUS (waters off Namibia [17-22°S)] (505 sequences) and oxic features, oxic (>100 µM O_2) (435 sequences), oxycline (20-100 µM O_2), suboxic (5-20 µM O_2) and OMZ (<5 µM O_2). See **Tables S1, S2** for details.

Compared to a previous study, which used a similar approach and a higher number of input sequences for *nifH* sequence analysis from OMZs of the ETNP, ETSP, and AS (505 from the North BUS OMZ compared to 787 sequences combined from the ETSP, ETSP and AS, (Jayakumar and Ward, 2020)), we found a higher diversity based on the number of OTUs (in total 82 OTUs combining both ATZ and North BUS compared to 59 OTUs in the previous study) (**Figures S1**, **S2**). This diversity might be somewhat biased as our samples were derived from a wider range of biogeochemical conditions, including the ATZ and the higher obtained total number of sequences. If taking only sequences from the North BUS suboxic (5-20 µM O_2) and OMZ (<5 µM O_2) conditions into account, a similar diversity (41 OTUs in this study and 38 OTUs in Jayakumar and Ward, 2020) can be observed in both studies (**Table S2**; **Figure S1**). Interestingly, we found the highest N_2 fixer diversity in waters of the Northern BUS, which also showed strong OMZ conditions with O_2 concentration below 5 µM O_2 (**Figure 1**; **Table S1**). However, we also recovered more sequences from those waters as compared to our other study sites and compared to the individual OMZs explored by Jayakumar and Ward (2020). The 15 most abundant OTUs and their phylogeny are shown in **Table 2**. Nine of the OTUs were associated with *nifH* Cluster I, which consists of cyanobacteria and proteobacteria mainly (**Table 2**; **Figure 4**). The remaining six OTUs were associated with Cluster III (**Table 2**; **Figure 5**).

3.2.1 Cyanobacterial Diazotrophs in the Northern Benguela Upwelling System

With above 99% coverage and identity, the most abundant OTU (OTU-1), consisting of 284 sequences, was associated with the filamentous cyanobacterium *Trichodesmium IMS101* on DNA and protein level (**Table 2**; **Figure 4**). OTU-1 was mainly found in samples from the South and North ATZ (65% and 22%, respectively) and to a lesser extent in samples from the OMZ in

TABLE 2 | OTU identities of the 15 (out of 83) most abundant OTUs (>10 Seq).

				DNA			
	No. of Seq	***nifH* cluster**	**Phylogenetic affiliation**	**Closets NCBI hit**	**Accession no.**	**Identity (%)**	**Coverage (%)**
OTU1	**284**	1	Cyanobacterium	*Trichodesmium sp IMS101*	AF167538.1	99.69	100
OTU2	**90**	1	Alpha	*Magnetococcus marinus MC-1*	CP000471.1	80.06	99
OTU3	**59**	234	Delta	*Desulfobacter curvatus*	AF065619.1	76.97	93
OTU4	**52**	1	Gamma	*Shewanella dokdonensis*	CP074572.1	81.88	99
OTU5	**35**	1	Gamma	*Marine proteobacterium*	AF046833.1	83.18	100
OTU6	**34**	234	Delta	*Sulfurospirillum sp.*	AP014723.1	74.43	59
OTU7	**33**	1	Alpha	*Bradyrhizobium japonicum*	GQ289567.1	80.75	99
OTU8	**33**	234	Delta	*Solidesulfovibrio carbinolicus*	CP026538.1	83.89	92
OTU9	**30**	1	Cyanobacterium	*Candidatus Atelocyanobacterium Thalassa (UCYN-A)*	KF806612.1	99.38	100
OTU10	**26**	234	Delta	*Pelobacter carbinolicus*	CP000142.2	84.74	100
OTU11	**25**	NA	NA	*Unidentified bacterium*	AF016613.2	98.75	100
OTU12	**18**	234	Delta	*Desulfotomaculum nigrificans*	AY221823.1	84.85	30
OTU13	**18**	234	Delta	*Pseudodesulfovibrio sp. SF6*	AP024485.1	87.54	100
OTU14	**13**	1	Cyanobacterium	*Cyanothece sp. WH8904*	AY620241.1	100	100
OTU15	**10**	1	Actinobacteria	*Frankia sp. D11*	HM026366.1	76.77	61
				Protein			
OTU1	**284**	1	Cyanobacterium	*Trichodesmium erythraeum IMS101*	O34106.1	100	100
OTU2	**90**	1	Gamma	*Azotobacter chroococcum*	AYE20514.1	100	96.26
OTU3	**59**	234	Delta	*Desulfobacteraceae*	RJP85619.1	91.59	100
OTU4	**52**	1	Gamma	*Vibrio sp. HA2012*	WP_100798453.1	100	100
OTU5	**35**	1	Gamma	*Pseudomonas stutzeri*	CBW30543.1	93.46	100
OTU6	**34**	234	Delta	*Solidesulfovibrio carbinolicus*	WP_129352166.1	88.79	100
OTU7	**33**	234	Kiritimatiellaeota	*Pontiellaceae desulfantans*	WP_136081448.1	93.33	100
OTU8	**33**	234	Bacteroidetes	*Lutibacter agarilyticus*	WP_089382364.1	88.79	100
OTU9	**30**	1	Cyanobacterium	*Candidatus Atelocyanobacterium Thalassa (UCYN-A)*	AHH30822.1	98.13	100
OTU10	**26**	234	Delta	*Desulfuromonas sp.*	PLX91276.1	95.33	100
OTU11	**25**	1	Gamma	*Pseudomonas stutzeri*	CBW30543.1	93.69	100
OTU12	**18**	234	Delta	*Desulfobacula sp.*	NJM03842.1	91.59	100
OTU13	**18**	234	Delta	*Maridesulfovibrio frigidus*	WP_031482840.1	95.33	100
OTU14	**13**	1	Cyanobacterium	*Cyanothece sp. UBA12306*	HAC63251.1	97.2	100
OTU15	**10**	1	Planctomycetes	*Planctomyceataceae*	MBV8488868.1	92.52	100

The closets cultivated species and accession number from the NCBI database based on DNA and protein is presented along with phylogenetic affiliation and identity/coverage score. NA standard for not available.

the North BUS (13%, **Figure 3**; **Table S4**). Of those 284 sequences. 23.5% were derived from cDNA and were found in surface waters of the North ATZ (13% cDNA) and the North BUS (10% cDNA) (**Tables S3, S4**). Only below 0.1% were derived from cDNA extracted in the South ATZ (**Tables S3, S4**). Earlier studies found no evidence of *Trichodesmium* sp. and blooms were not detectable by satellite imaging (Shannon and Pillar, 1986; Pitcher et al., 1991; Tyrrell et al., 2003; Westberry and Siegel, 2006; Sohm et al., 2011). More recent studies, however, detected *Trichodesmium* only in low biomass or in a concentration of 1–10 colonies per m^3 (Nagel et al., 2013; Wasmund et al., 2015). Low numbers did not appear to be a result of iron (Fe), vitamin or other trace metal limitations in those earlier studies, but rather of low water temperatures (20-25°C), which limited the growth and activity of *Trichodesmium* (Staal et al., 2007; Wasmund et al., 2015; Tang and Cassar, 2019) (**Figure S3**).

The small unicellular, cosmopolitan cyanobacterium UCYN-A (OTU-9) was detected in the oxic waters of the North ATZ (70%) and North BUS (30%) (**Table 2**; **Figure 3**) with >99% coverage and identity. UCYN-A were not detected in the South ATZ. OTU-9 consisted of 90% transcripts and was found in surface waters down to 30 m water depths (58% cDNA from the North ATZ and 30% from the North BUS), suggesting UCYN-A contributing to N_2 fixation (**Tables S3, S4**). Thus, highlighting the importance of this clade for N_2 fixation. UCYN-A has been found all over the globe, from polar to tropical regions, often playing a major role in N_2 fixation (Short and Zehr, 2007; Martínez-Pérez et al., 2016; Harding et al., 2018; Tang and Cassar, 2019; Gradoville et al., 2020; Mills et al., 2020). Previously, there was one report of UCYN-A from the Benguela upwelling system where it has been found at water depths of 40-110 m off Angola (14.75°S) (Sohm et al., 2011). Otherwise, UCYN-A has not been found in OMZs except for the ETSP and the BoB (Turk-Kubo et al., 2014; Li et al., 2021).

Cyanothece sp. *WH8904* (OTU-14) was detected only in the North BUS and 15% of the sequences were derived from cDNA, suggesting that *Cyanothece* sp. may be active in the North BUS. Additionally, we detected four cyanobacterial OTUs, one clustering closely with *Dolichospermum* spp. (previously known as *Anabaena* sp. [Wacklin et al., 2009)] (OTU-18) and three singletons (OTU-61, OTU-66, OTU-76) related to *Trichodesmium thiebautii* (**Figure 4**). In this study, we found a regional difference in cyanobacterial diazotroph presence and *nifH* gene expression within the Benguela current system (ATZ

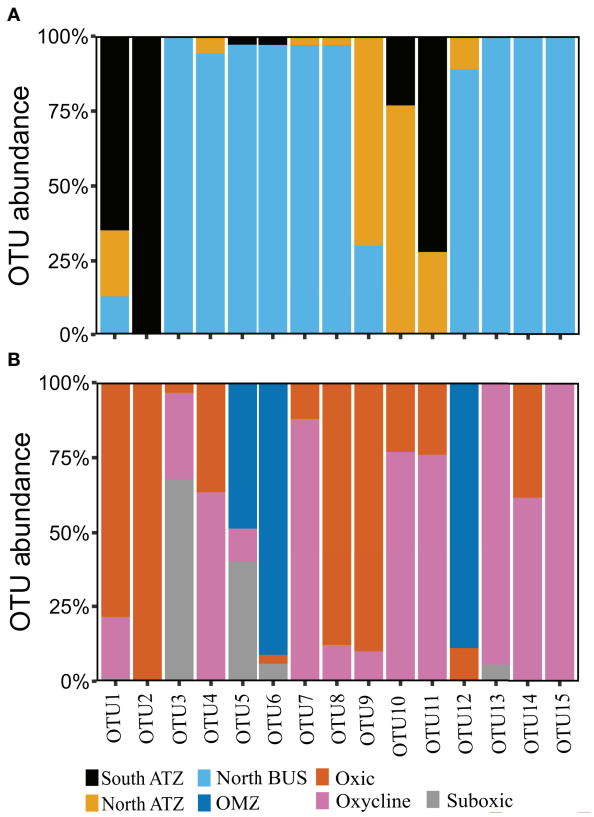

FIGURE 3 | Histogram of the 15 most abundant OTUs (above 10 sequences) divided by location **(A)** and O_2 feature **(B)**. OTUs were defined at a threshold of 3% nucleotide differences and are presented in descending abundance with OTU1 being the most abundant. ATZ stands for Angola Tropical zone and BUS for Benguela Upwelling System.

and North BUS). Moreover, we report for the first time not only the abundance of *nifH* genes but also the detection of *nifH* transcripts related to *Trichodesmium*, *Cyanothece* and UCYN-A in surface waters adjacent to an OMZ.

3.2.2 Heterotrophic Diazotrophs in the Northern Benguela Upwelling System

Besides cyanobacterial N_2 fixers, we found several OTUs related to common OMZ heterotrophic diazotrophs (**Table 2**; **Figure 4**). Based on protein level, several gamma-proteobacteria were detected (OTU-2, 4, 5 and 11) and were related to *Azotobacter* sp., *Vibrio* sp. and *Pseudomonas stutzeri* (*P. stutzeri*) (above 90% identity and coverage) and *nifH* sequences from the BoB OMZ and gamma-proteobacterial sequences from the South Pacific (Moisander et al., 2014). (**Table 2**; **Figure 4**). Related sequences have been reported within both ETNP, ETSP, AS and BOB (e.g., Jayakumar and Ward, 2020). Based on DNA comparison (identity score of 80%), OTU-2 and 7 were related to alpha-proteobacteria, *Magnetococcus marinus* and *Bradyrhizobium* respectively. Based on protein comparison OTU-2 were related to *Azotobacter* sp. with 100% identity. Phylogenetic analysis further establishes OTU-2 being related to gamma-proteobacteria. OTU-7 did not cluster to alpha-proteobacteria or *nifH* cluster I, but rather with *nifH* cluster III sequences related to *Pontiellace*ae.

Like in other OMZ waters, we detected sequences related to *Vibrio* sp. (OTU-4) and *P. stutzeri* (OTU-5) in the North BUS (94% and 97%, respectively). Alpha-proteobacterial sequences have been reported the OMZs of the ETNP, ETSP, AS and BoB (e.g., *M. palustris*, *S. melioti* and *S. azotifegens*), but were not confidently detected in this study, nor were beta-proteobacterial sequences (**Figure 4**) (Halm et al., 2011; Hamersley et al., 2011; Jayakumar et al., 2012; Farnelid et al., 2013; Turk-Kubo et al., 2014; Cheung et al., 2016; Jayakumar et al., 2017). 23 out of 82 OTUs belonged to *nifH* Cluster I. 13 of those OTUs were found in the North BUS (**Figure 4**). These proteobacteria include diazotrophs commonly reported in OMZs (Jayakumar et al., 2012; Löscher et al., 2014; Cheung et al., 2016; Löscher et al., 2020), low O_2 waters of the Southern Californian Bight (Hamersley et al., 2011) and anoxic waters in the Baltic Sea (Farnelid et al., 2013). Gamma-proteobacteria of the Atlantic clade (OTU-11 and -70) (Langlois et al., 2008) (**Figure 4**) were only detected and expressed their *nifH* gene in the ATZ. As previously suggested, they are not commonly active in OMZ or low-O_2 waters (Langlois et al., 2015; Moisander et al., 2017).

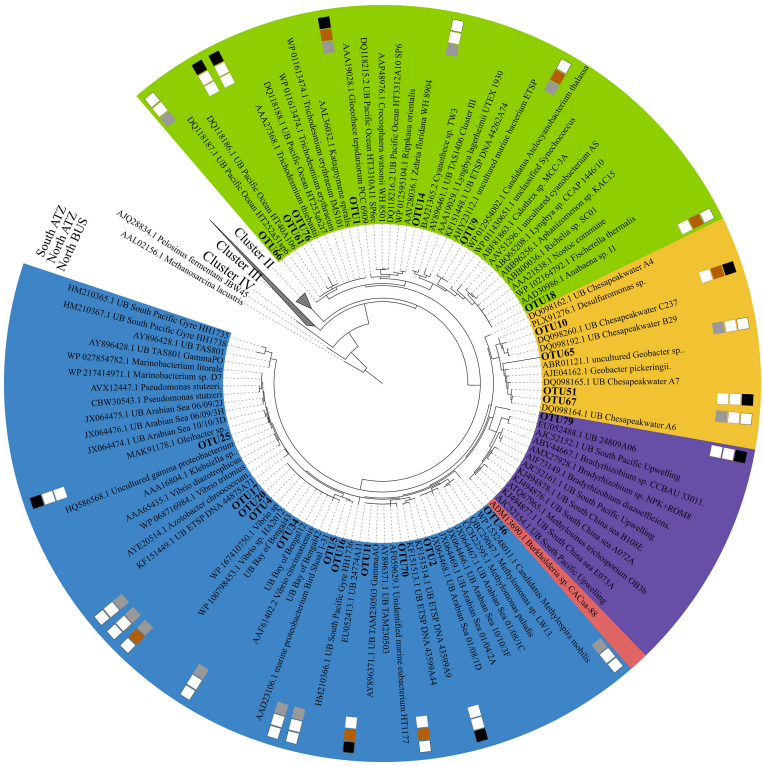

FIGURE 4 | Maximum likelihood tree of Cluster I *nifH* amino acid sequences. The phylogenetic tree is based on a Poisson model with 1000 bootstraps. OTUs are shown in bold nearest to their closest relative. Locations from where sequences were derived from are shown in the outer heatmap, fark brown represents the Northern Angola Tropical Zone, black indicates the Southern Angola Tropical Zone and grey indicates the Northern Benguela Upwelling Zone. Cyanobacteria are shown in green, gamma-proteobacteria are shown in blue, epsilon-proteobacteria are shown in yellow, alpha-proteobacteria are shown in red and beta-proteobacteria are shown in purple.

While gamma-proteobacteria *M. paludis*, a methanotroph (Danilova et al., 2013) have been reported in both ETNP, ETSP and AS (Fernandez et al., 2011; Cheung et al., 2016; Jayakumar and Ward, 2020). We did not find any evidence of methanotrophs in the North BUS. Unlike in the AS and ETNP, we found four OTUs clustering among epsilon-proteobacteria which has also been reported before in ETSP and BoB. However, only two singletons were identified in the North BUS (OTU-65 and 67) (**Table S3**). The more abundant OTU-10 clustered with Chesapeakwater and shares high similarity to *Desulfuromonas* sp. (above 95% identity) and was only found in the ATZ (north 77%, south 23%).

Overall, compared to other OMZ regions (Jayakumar and Ward, 2020), we found a similarly small diversity of heterotrophic diazotrophs in the North BUS OMZ waters. In contrast to most other OMZ waters, we found potentially active UCYN-A and *Trichodesmium* in OMZ-adjacent surface waters. While the role of heterotrophic microbes is unclear, it does not seem that heterotrophic Cluster I diazotrophs play a major role in N_2 fixation in the Northern Benguela upwelling system. Rather, cyanobacterial diazotrophs might play a bigger role due to their presence and activity in the OMZ surface waters.

3.2.3 Delta-Proteobacterial Diazotrophs in the Northern Benguela Upwelling System

In addition to Cluster I diazotrophs, we found a vast diversity of Cluster III sequences (59 OTUs out of 82 OTUs) related to Delta-proteobacteria, Bacteroides, Chlorobi, Spirochaetes, Verrucomicrobiae/Kiritimatiellales and Thiospirochaetes (**Figure 5**). Notably, we did not detect any Cluster II and Cluster IV sequences, which represent e.g., archaeal sequences or non-functional N_2 fixers. Only four out of 59 OTUs consisted of sequences from stations in the ATZ (**Figure 5**). Among the top 15 OTUs associated with Cluster III, only OTU-10 was found in the ATZ (OTU-10: south 23%, north 77%).

OTU-3, 6, 7, 8, 10, 12 and 13 scored above 90% of identity on protein level and closely related to *Desulfobacteraceae* sp., *Solidesulfovibrio* sp., *Pontiellaceae* sp., *Lutibacter* sp., *Desulfuromonas* sp., *Maridesulfovibrio* sp. and *Desulfobacula* sp., respectively (**Table 2**). OTU-3 was mainly found in suboxic waters (68%), while OTU-10 and 13 were found in the oxycline (77% and 94%, respectively), and OTU-6 and 12 were found in the OMZ (91% and 88%) (**Table 2**, **S4** and **Figure 3**, **5**). Despite a wide range of Cluster III sequences, only OTU-13, in the top 15 OTUs, contained transcript sequences (5.5%). OUT-13 were only found in North BUS in oxycline and suboxic

conditions (94.5% and 5.5%, respectively). The low number of *nifH* transcripts is somewhat expected in OMZs as N_2 fixation rates are typically low, too. Although there were few high identity scores (based on DNA) among the top 15 OTUs to known species, four out of six were either on DNA or protein level affiliated with sulphate-reducing microbes (**Table 2**). This speaks for the importance of sulphate reduction associated with N_2 fixation in North BUS. Such co-occurrence resulting in a coupling between sulphate-reduction and N_2 fixation has been previously described in OMZ waters and underlying sediments (Bertics et al., 2013; Gier et al., 2016), mostly represented by sequences clustering closely with *Desulfovibrio* [**Figure 5**, (Fernandez et al., 2011; Löscher et al., 2014; Turk-Kubo et al., 2014)]. Indeed, this coupling seems to be consistence between all OMZ waters stressing the importance of this co-occurrence (Jayakumar and Ward, 2020).

Besides sulphate-reducing clades, we also found sulfide-oxidizing clades, such as *Thiospirochaeta* and *Spirochaetes* (**Figure 5**). OTU-6 consisted mainly of OMZ derived sequences (91%) is related to *Sulfurospirillum* sp., or *Solidesulfovibrio* sp. (**Table 2**), while the phylogenetic analysis determined it as being closely related to the sulfide-oxidizing group Spirochaetes (**Figure 5**). OTU-23, the most abundant OTU related to *Thiospirochaeta*, was only found in the North BUS in the OMZ region. Chlorobi and Spirochaetes identified in our study, have previously been described in the ETNP, ETSP and anoxic waters of the Baltic Sea (Farnelid et al., 2013; Christiansen and Löscher, 2019; Jayakumar and Ward, 2020) (**Figure 5**). Our identified Bacteroidetes sequences were related to sequences from the BoB (**Figure 5**) and have previously been reported in e.g., the sulfidic OMZ off the coast off Peru (Schunck et al., 2013). Also, a recent 16S rRNA-based study from the Southern Benguela upwelling system revealed a similar community with Bacteroides and sulfate-reducing clades present in low-O_2 waters (Rocke et al., 2020). Overall, the diversity of Cluster III microbes could result from locally occurring H_2S events favoring anaerobic sulphate-reducing and sulfide-oxidizing microbes. Like for Cluster I diazotrophs, there is a distinct difference between the North BUS OMZ (waters off Namibia) and the ATZ (waters off Congo and Angola).

3.3 Biogeochemical Controls on Diazotroph Distribution and Diversity

The waters off North BUS were the only ones during this cruise with an OMZ with O_2 concentrations below 5 μM in its core waters. As one main goal of this study was to explore OMZ diazotrophs in North BUS, an RDA based on OTUs (from the top 15), and environmental variables was carried out from those waters specifically. A model containing depth, salinity, the isotope signature of $\delta^{15}N-NO_3^-$, NO_2^-, O_2, NH_4^+ and NO_3^-

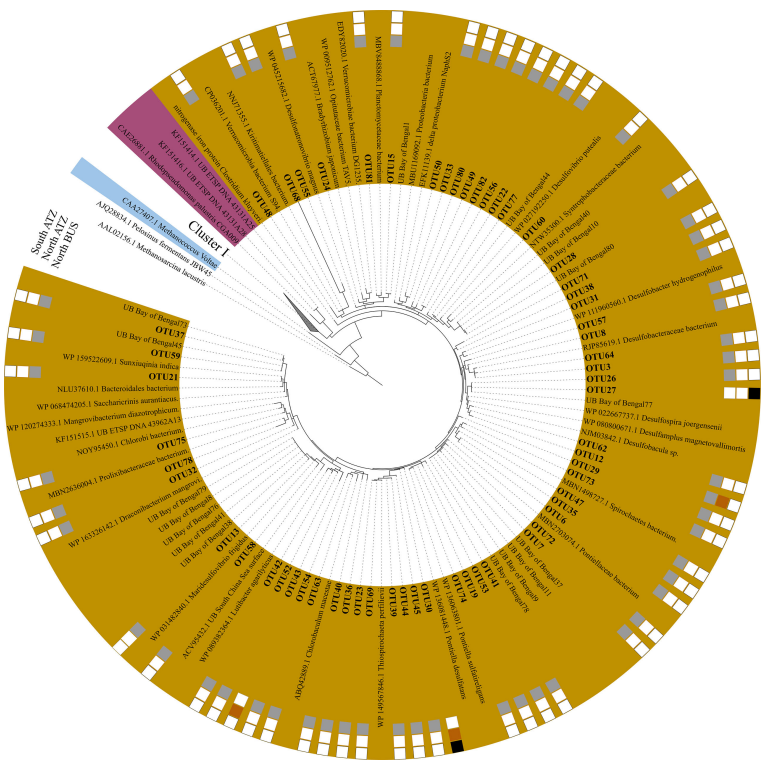

FIGURE 5 | Maximum likelihood tree of Cluster II, III, and IV *nifH*-amino acid sequences. The phylogenetic tree is based on a Poisson model with 1000 bootstraps. OTUs are shown in bold nearest to their next cultivated relatives. Cluster II is shown in purple, Cluster III in brown and Cluster IV in blue.

could explain up to 99% of the total variability (**Figures 6A, B**). While a clear difference in OTUs between the ATZ and the North BUS (**Figure 3**) could be observed, the geographical factors within North BUS seems also evident in the redundancy analysis.

The diazotroph community was divided into three clusters which positively correlated with either O_2 (OTU-4 and 14), NO_3^- (OTU-7, -13 and -15) and $\delta^{15}N - NO_3^-$ along with NH_4^+ (OTU-5, -6 and -12). OTU-4 (*Vibrio* sp.) and 14 (*Cyanothece* sp.) were derived mainly from waters with >20 µM O_2 and positively correlated with O_2. OTU-7 (*Pontiellaceae* sp.), -13 (*Maridesulfovibrio* sp.) and -15 (*Plantomyceataceae* sp.), derived from oxycline features, were positively correlated to depth and NO_3^- and negatively to $\delta^{15}N - NO_3^-$ and NH_4^+. While including the third component (RDA3), OTU-7, -13 and -15 showed the same tendency and were positively correlated to NO_3^- but negatively correlated to O_2, (**Figures 6A, B**). OTU-5 (*P. stutzeri*), -6 (*Solidesulfovibrio* sp.) and -12 (*Desulfobacula* sp.) were identified as suboxic and OMZ-derived OTUs. They correlated positively with $\delta^{15}N - NO_3^-$ along with NH_4^+ and negatively to NO_3^- and O_2. The remaining OTUs (8, 9 and 3) could not be sufficiently explained by our statistical approach. Thus, the diverse explanatory factors for OTU-8 (*Lutibacter* sp.), -9 (UCYN-A), and -3 (*Desulfobacteraceae* sp.) suggest a lack of factors (e.g., organic matter or Fe) that could help to further explain the dataset. Interestingly, our statistical approach suggests that NO_3^- plays a role in separating OTUs derived from oxycline waters (~20-100 µM O_2) from OTUs derived from suboxic/OMZ waters (<5 and 5-20 µM O_2) (**Figures 6A, B**). OTU-5, -6 and -12 correlating to $\delta^{15}N - NO_3^-$ and NH_4^+ indicate that they are present in denitrifying waters enriched $\delta^{15}N - NO_3^-$ and in potentially sulfidic waters typically enriched with NH_4^+. While this correlation does not indicate coupling of denitrification and N_2 fixation in OMZs as previously proposed (Deutsch et al., 2007),

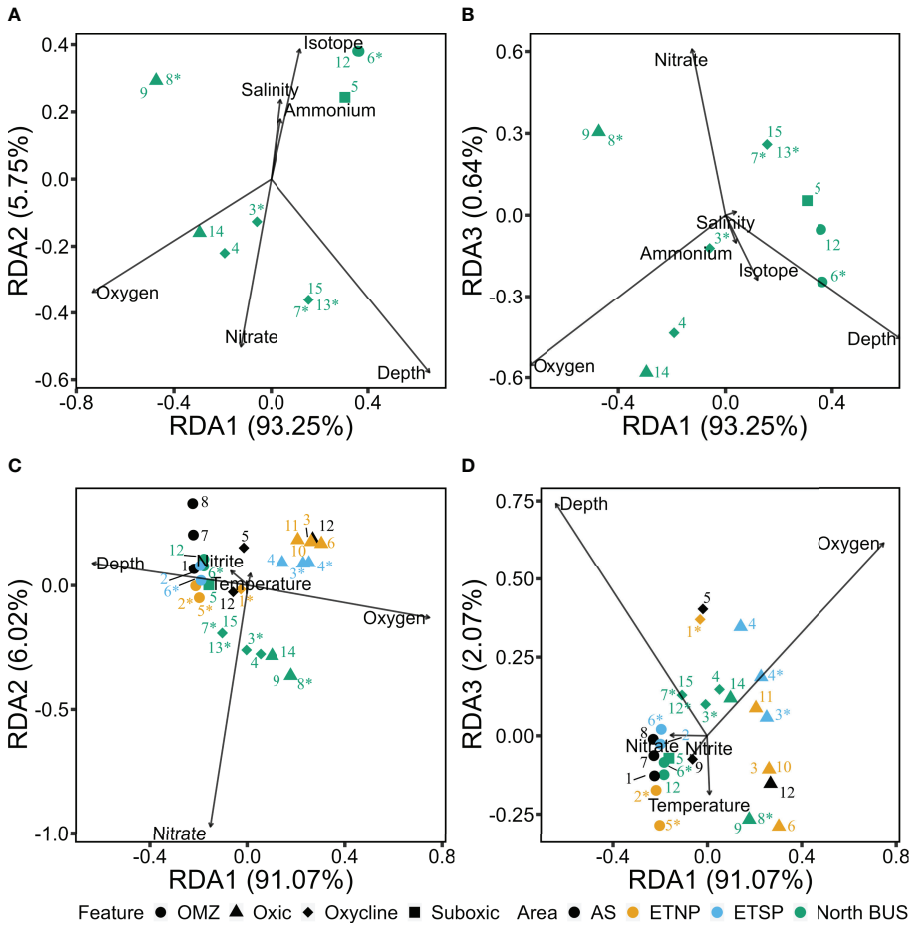

FIGURE 6 | Redundancy analysis (RDA) plot illustrating the relationship between biogeochemical variables and OTUs within the coastal waters the Northern Benguela Upwelling System (North BUS, waters off Namibia) **(A, B)** and between different OMZ areas **(C, D)**. Numbers indicate OTU identifiers, different shapes denote the O_2 feature. Numbers with * are OTUs belonging to Cluster III, other OTUs belong to Cluster I. The Arabian Sea is denoted in black, the ETNP in yellow, the ETSP in blue and waters off Namibia in green. **(A, B)** show only the top 15 abundant OTUs derived majorly from Namibia (>5%). **(C, D)** show OTUs generated from the ETNP, ETSP and AS (Jayakumar and Ward, 2020) along with OTUs from the Northern Benguela Upwelling system.

it suggests a spatial coupling between the habitat of diazotrophs within OMZs and denitrification, and a possible link to sulfate-reduction (Jayakumar and Ward, 2020). Lastly, when comparing across OMZ waters, OTUs derived from OMZ core correlate positively to NO_2^- and negatively to O_2 and NO_3^- (**Figures 6C, D**). OTUs derived from the North BUS correlated positively to NO_3^- (**Figure 6C**). We see between OMZ regions (ETNP, ETSP, AS) a similar pattern where OMZ OTUs correlated positively to NO_2^- and oxic derived OTUs with O_2 or positively with temperature, as it showed collinearity with depth (**Figures 6C, D**).

Compared to other OMZ areas, the most striking difference is the positive correlation between high-NO_3^- and low-O_2 derived OTUs from the North BUS (**Figures 6A, B**). Assimilation of NO_3^- has been suggested to be energetically not of advantage as compared to N_2 fixation in low O_2 environments (Großkopf and LaRoche, 2012). However, it is unclear whether this explains the observed difference between suboxic and OMZ OTUs. The correlation of diazotroph presence to NO_3^- might also be indicative of a correlation with any other compound in the same water mass. As the NO_2^- signal could be sedimentary, it could be similar to a signal of dissolved Fe concentrations from coastal upwelling, or of organic matter possibly stimulating diazotrophic growth (Löscher et al., 2014; Benavides et al., 2015; Benavides et al., 2020). As typical for OMZs, the diazotroph community collected during this cruise consisted mainly of heterotrophic microbes. While we see a segregation of the community in response to macronutrients, it is important to consider that this might rather be an indicator for a general niche within those waters than for NO_3^- directly. It has previously been suggested that heterotrophic N_2 fixation is independent of the Redfield-based N deficiency as long as PO_4^{3-} and iron (Fe) is available (Löscher et al., 2014; Bombar et al., 2016).

Fe can always be considered an important factor for N_2 fixation because the Fe requirement of diazotrophs is 60 times that of other microbes (Berman-Frank et al., 2001). In the Northern BUS, the sedimentary release of Fe is rather low (less than 1 wt.% Fe compared to other OMZ regions between 2-3 wt.% Fe) (Scholz et al., 2016; Böning et al., 2020). This potential Fe limitation might explain why *Trichodesmium* was mainly detected in the ATZ and to a lesser extent the North BUS. It might also explain low N_2 fixation rates (Staal et al., 2007; Sohm et al., 2011; Wasmund et al., 2015) and δ^{15}N-PON, along with $\delta^{15}N - NO_3^-$, being outside of the range indicative for N_2 fixation (**Figure 2**) (Nagel et al., 2013). Sohm et al., 2011 measured up to 2.4 nM Fe in the North BUS and 0.08-0.34 nM in surface waters of the ATZ (north of 14°S). Compared to ETSP OMZ waters with Fe concentrations of 10-60 nmol kg^{-1} and upwelled surface waters with Fe concentrations of up to 267 nmol kg^{-1}, the concentrations measured in the Benguela region indeed appear to be very low (Löscher et al., 2014). On the other hand, nutrient addition experiments did not increase N_2 fixation in Benguela waters (Staal et al., 2007; Wasmund et al., 2015). Thus, other factors that could limit N_2 fixation should be taken into account, such as micronutrients (e.g. molybdenum, zinc, vitamins), macronutrients (e.g., organic matter) or physical factors such as light, temperature, or turbulence (Arrigo, 2005; Saito et al., 2008). Though based on our RDA model, turbulence did not explain the dataset significantly, while temperature only explained a minor part.

4 CONCLUSION

In this study, we explored the diazotrophic community and distribution along the ATZ and the North BUS spanning from the waters off Congo (5°S) to Namibia (23°S). Typically, OMZ regions across the world contain a considerable *nifH* diversity in both surface and OMZ waters, thus it was of little surprise that we could also recover a broad diversity of Cluster I and III diazotrophs. However, in contrast to other OMZs, we did not find any representatives of other *nifH* clusters. Interestingly, *Trichodesmium, Cyanothece* and UCYN-A were both present and potentially active in the ATZ (Coast off Congo and Angola). Moreover, we report a distinct pattern of cyanobacterial in the OMZ waters of the North BUS. While *Trichodesmium* was mainly found in the ATZ; it was, although to a lesser extent, also found potentially active in OMZ surface waters of the North BUS. UCYN-A was detected in the North BUS and the ATZ in both the gene and the transcript pool, even in surface waters of the OMZ waters. Lastly, *Cyanothece* were only found in the North BUS surface waters, potentially active. Cluster I heterotrophic diazotrophs were related to gamma-proteobacteria such as *P. stutzeri, Vibrio* sp., and *Azotobacter* sp. and were mainly found in the North BUS OMZ waters. Unlike in other OMZs, we did not detect any alpha – or beta-proteobacterial diazotrophs. The most striking difference to other OMZs was the vast diversity of Cluster III diazotrophs, consisting of delta-proteobacteria, Bacteroides, Chlorobium, Spirochaetes and Thiospirochaeta. All of them were found in the OMZ environment.

Combining our data with reports from other OMZ regions, we observe a similar pattern with OMZ derived OTUs responding positively to the secondary NO_2^- peak. However, looking at the North BUS OMZ waters, NO_2^- did not play a significant role. Contrary, we did observe a positive correlation with NO_3^- to OTUs derived from low-O_2 waters (~20 µM O_2). While assimilation of NO_3^- is energetically unfavorable in low-O_2 waters, the correlation might indicate a response to either upwelled Fe or organic matter, stimulating diazotrophic growth. OMZ derived OTUs positively correlated with NH_4^+ and $\delta^{15}N - NO_3^-$ which might support the idea of spatially coupled denitrification to N_2 fixation and a link between sulfate-reduction and N_2 fixation.

While we did not measure N_2 fixation rates, earlier reports confirm low rates in waters above 70 m in line with our δ^{15}N isotope speciation data. Like in other OMZs, low or absent rates are related to a specific OMZ diazotroph community with cyanobacteria being underrepresented and heterotrophic diazotrophs fixing N_2 only at low rates. Therefore, the diversity of diazotrophs especially of Cluster III in the North BUS suggests that they find a niche in OMZ waters not because of their ability

to fix N_2, but because of other metabolic capabilities, e.g., sulphate reduction or oxidation. Thus, it is still unclear why those microbes keep their genetic ability to fix N_2, as they rarely seem to use it.

DATA AVAILABILITY STATEMENT

The sequences were submitted to NCBI with accession ID OK082431-OK083221, OL412005-OL412134 and OM453718 - OM453821.

AUTHOR CONTRIBUTIONS

CL, CR, and DA-M designed the study. JC-C, TS, CR, and DA-M carried out field and laboratory experiments. CR performed statistical and molecular analysis. JC-C and TS carried out isotopic measurement. CR wrote the manuscript with contributions from all co-authors. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2022.868261/full#supplementary-material

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