## Title

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# Nitrogen fixation in sediments along a depth transect through the Peruvian oxygen minimum zone

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**Abstract.** The potential coupling of nitrogen ($N_2$) fixation and sulfate reduction (SR) was explored in sediments of the Peruvian oxygen minimum zone (OMZ). Sediment samples were retrieved by a multiple corer at six stations along a depth transect (70–1025 m water depth) at 12° S, covering anoxic and hypoxic bottom water conditions. Benthic $N_2$ fixation, determined by the acetylene reduction assay, was detected at all sites, with highest rates between 70 and 253 m and lower rates at greater depth. SR rates decreased with increasing water depth. $N_2$ fixation and SR overlapped in sediments, suggesting a potential coupling of both processes. However, a weak positive correlation of their activity distribution was detected by principle component analysis. A potential link between $N_2$ fixation and sulfate-reducing bacteria was indicated by the molecular analysis of *nifH* genes. Detected *nifH* sequences clustered with the sulfate-reducing bacteria *Desulfonema limicola* at the 253 m station. However, *nifH* sequences of other stations clustered with uncultured organisms, Gammaproteobacteria, and Firmicutes (Clostridia) rather than with known sulfate reducers. The principle component analysis revealed that benthic $N_2$ fixation in the Peruvian OMZ is controlled by organic matter (positive) and free sulfide (negative). No correlation was found between $N_2$ fixation and ammonium concentrations (even at levels $>2022\,\mu M$). $N_2$ fixation rates in the Peruvian OMZ sediments were in the same range as those measured in other organic-rich sediments.

## 1 Introduction

Only 6 % of nitrogen (N) in seawater is bioavailable (Gruber, 2008). This bioavailable N is mainly present in the form of nitrate ($NO_3^-$), whereas the large pool of atmospheric dinitrogen gas ($N_2$) is only available for $N_2$ fixing microorganisms (diazotrophs). N often limits marine productivity (Ward and Bronk, 2001; Gruber, 2008) and the largest source of bioavailable N (i.e., ammonium; $NH_4^+$) in the marine environment is $N_2$ fixation (Falkowski et al., 1998; Strous et al., 1999; Brandes and Devol, 2002).

To date, the quantitative contribution of diazotrophs in the marine N cycle remains unclear and numerous estimates of global sources and sinks of global N have led to an unbalanced budget with deficits of around $200\,Tg\,N\,yr^{-1}$ (Codispoti, 2007). This suggests that either previous $N_2$ fixation rate determinations have been underestimated (Großkopf et al., 2012) or that N loss processes are overestimated (Codispoti, 2007). However, also balanced budgets such as $\sim 265\,Tg\,N\,yr^{-1}$ for N sources and $\sim 275\,Tg\,N\,yr^{-1}$ for N sinks exist (Gruber, 2004). These budget discrepancies illustrate that the current knowledge on diazotrophy and the marine N cycle is still limited.

Recent investigations argue that $N_2$ fixation in the water column cannot be totally attributed to phototrophic cyanobacteria, but that also heterotrophic prokaryotes contribute substantially (Riemann et al., 2010; Farnelid et al., 2011; Dekaezemacker et al., 2013; Löscher et al., 2014; Fernandez et al., 2015). This was shown for the Peruvian oxy-

gen minimum zone (OMZ), where proteobacterial clades dominated with heterotrophic diazotrophs, indicating that cyanobacterial diazotrophs are of minor importance in this area (Löscher et al., 2014).

Pelagic $N_2$ fixation has been studied mostly in the oligotrophic surface oceans, but it was not until the past decade that benthic habitats began to receive more attention (Fulweiler et al., 2007; Bertics et al., 2010, 2013). Most studies on benthic $N_2$ fixation focused on coastal environments (Capone et al., 2008 and references therein). For example, subtidal sediments in Narragansett Bay (Rhode Island) were found to switch from being a net sink in the form of denitrification to being a net source of bioavailable N by $N_2$ fixation, caused by a decrease of organic matter deposition to the sediments (Fulweiler et al., 2007). Shallow brackish-water sediments off the Swedish coast revealed benthic $N_2$ fixation along with a diverse diazotrophic community (Andersson et al., 2014). $N_2$ fixation was positively influenced by a variety of environmental factors, such as salinity and dissolved inorganic Nitrogen, while wave exposure had a negative influence. Recent work revealed that benthic $N_2$ fixation is often linked to sulfate-reducing bacteria. For instance, bioturbated coastal sediments showed enhanced $N_2$ fixation activity mediated by sulfate-reducing bacteria, adding new dissolved inorganic N to the system (Bertics et al., 2010; Bertics and Ziebis, 2010). Further coupling of $N_2$ fixation to SR was observed in organic-rich sediments of the seasonal hypoxic Eckernförde Bay (Baltic Sea, Bertics et al., 2013), as well as in the sub-tidal, heterotrophic sediments of Narragansett Bay (Rhode Island, USA; Fulweiler et al., 2013). Several sulfate-reducing bacteria carry the functional gene marker for $N_2$ fixation, the *nifH* gene (Sisler and Zo-Bell, 1951; Riederer-Henderson and Wilson, 1970; Zehr and Turner, 2001) and were shown to actively fix $N_2$ in culture experiments (Riederer-Henderson and Wilson, 1970). However, information on sulfate-reducing bacteria and their contribution to $N_2$ fixation in the environment is still sparse and restricted to a small selection of environments.

So far, the distribution of benthic $N_2$ fixation and its relevance for N cycling in the Peruvian oxygen minimum zone (OMZ), defined by dissolved oxygen $< 20\,\mu mol\,kg^{-1}$ (Fuenzalida et al., 2009), are unknown. The shelf and the upper slope in the Peruvian OMZ represent recycling sites of dissolved inorganic N with dissimilatory $NO_3^-$ reduction to $NH_4^+$ being the dominant process ($\sim 15\,mmol\,N\,m^{-2}\,d^{-1}$) in the benthic N cycle (Dale et al., 2016). This process is mediated by the filamentous sulfide-oxidizing *Thioploca* bacteria (Schulz, 1999; Schulz and Jørgensen, 2001). Benthic denitrification, which is mediated by foraminifera at water depth between 80 and 250 m of the Peruvian OMZ, represent a sink for bioavailable N in sediments, accounting for a potential $NO_3^-$ flux, i.e., N loss, of 0.01 to $1.5\,mmol\,N\,m^{-2}\,d^{-1}$ (Glock et al., 2013; Dale et al., 2016).

The high input of labile organic carbon to Peruvian OMZ sediments (Dale et al., 2015) and subsequent SR should favor benthic $N_2$ fixation. Sulfate-reducing bacteria could considerably contribute to $N_2$ fixation in these organic-rich OMZ sediments, given that several sulfate-reducing bacteria (e.g., *Desulfovibrio* spp.; Riederer-Henderson and Wilson, 1970; Muyzer and Stams, 2008) carry the genetic ability to fix $N_2$, and provide an important bioavailable N source for non-diazotrophic organisms (Bertics et al., 2010; Sohm et al., 2011; Fulweiler et al., 2013). We therefore hypothesize a possible coupling of $N_2$ fixation and SR in sediments off Peru. The aim of the present study was to identify and quantify benthic $N_2$ fixation along a depth transect through the Peruvian OMZ, together with SR, and compare its distribution with environmental factors, such as organic matter, to study its control mechanisms. The identification of bacteria carrying the genetic ability to perform $N_2$ fixation should further deliver information about benthic diazotrophic community structures at the different stations. The overall knowledge gained is needed to better constrain benthic N cycling in OMZs and to improve our knowledge on sources and sinks of fixed N.

## 2 Materials and methods

### 2.1 Study area

The most extensive OMZ worldwide is found in the eastern tropical south Pacific Ocean at the central Peruvian coast (Kamykowski and Zentara, 1990). The Peruvian OMZ ranges between 50 and 700 m water depth with oxygen ($O_2$) concentrations below the detection limit in the mid-waters (Stramma et al., 2008). The mean water depth of the upper OMZ boundary deepens during intense El Niño Southern Oscillation years and can reach a depth of 200 m (Levin et al., 2002) with oxygenation episodes reaching concentrations of up to $100\,\mu M\,O_2$ (Gutiérrez et al., 2008). $O_2$ concentrations (Fig. 1, Table 1) off Peru are modulated by coastal trapped waves (Gutiérrez et al., 2008), trade winds (Deutsch et al., 2014) and subtropical–tropical cells (Duteil et al., 2014), and can vary on monthly to interannual timescales (Gutiérrez et al., 2008).

At 12° S, the OMZ extends from water depths between 50 and 550 m (Dale et al., 2015; Fig. 1). During our field work, bottom water $O_2$ concentrations varied greatly with water depth and were below the detection limit (5 µM) at stations from 70 to 407 m water depth. Bottom water $O_2$ increased to 19 µM at 770 m water depth and 53 µM at 1025 m water depth, indicating the increase of dissolved $O_2$ below the lower boundary of the OMZ (Dale et al., 2015). Between 70 and 300 m water depth, the sediment surface was colonized by dense filamentous mats of sulfur-oxidizing bacteria, presumably of the genera *Marithioploca* spp. These bacteria are able to glide up to $1\,cm\,h^{-1}$ through the sediment in

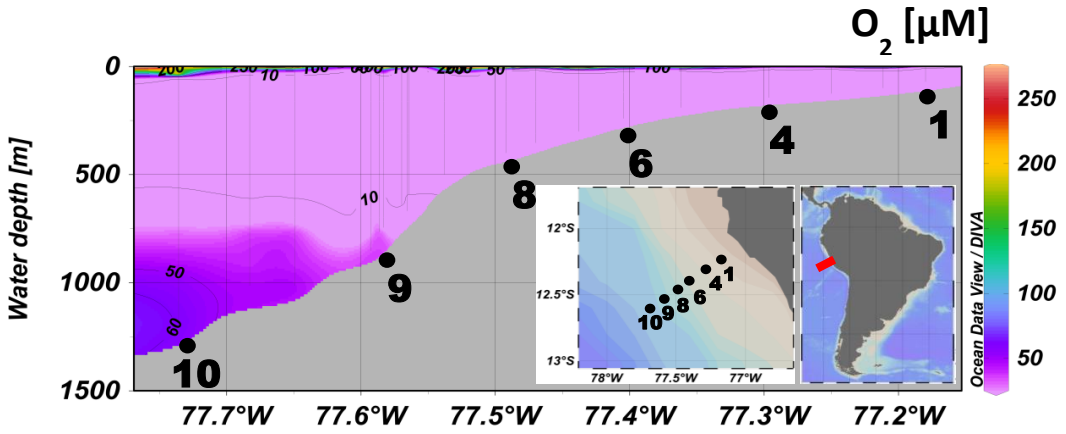


**Figure 1.** Cross-section of dissolved $O_2$ concentrations (µM) along the continental margin of the Peruvian OMZ at 12° S. The vertical lines represent CTD cast for $O_2$ measurement during the cruise M92. Stations 1 to 10 for multicorer (MUC) sampling are indicated by station numbers according to Dale et al. (2015).

**Table 1.** Sampling deployments, including station number according to Dale et al. (2015), core ID, sampling date and coordinates. Water depth (m) recorded by the ship's winch and bottom water temperature (°C) and bottom water $O_2$ concentration (µM; bdl = below detection limit: 5 µM) measured on the CTD.

| Station | Core ID | Date (2013) | Latitude (S) | Longitude (W) | Depth (m) | Temp. (°C) | $O_2$ (µM) |
|---|---|---|---|---|---|---|---|
| 1 | MUC 13 | January 11 | 12°13.492′ | 77°10.511′ | 70 | 14 | bdl |
| 4 | MUC 11 | January 9 | 12°18.704′ | 77°17.790′ | 144 | 13.4 | bdl |
| 6 | MUC 6 | January 7 | 12°23.322′ | 77°24.181′ | 253 | 12 | bdl |
| 8 | MUC 23 | January 15 | 12°27.198′ | 77°29.497′ | 407 | 10.6 | bdl |
| 9 | MUC 17 | January 13 | 12°31.374′ | 77°35.183′ | 770 | 5.5 | 19 |
| 10 | MUC 28 | January 19 | 12°35.377′ | 77°40.975′ | 1025 | 4.4 | 53 |

order to access hydrogen sulfide (Fossing et al., 1995; Jørgensen and Gallardo, 1999; Schulz, 1999). Sediments at the lower boundary (770 and 1025 m) of the OMZ host a variety of macrofaunal organisms, e.g., ophiuroids, gastropods, and crustaceans (Mosch et al., 2012).

The 12° S region is in the center of an extensive upwelling zone and features high primary productivity (Pennington et al., 2006). Sediments at 12° S have higher rates of particulate organic carbon accumulation (2–5 times) compared to other continental margins and a high carbon burial efficiency, indicating preferential preservation of organic matter in the Peruvian OMZ (Dale et al., 2015). The shelf (74 m) of the Peruvian OMZ is characterized by high sedimentation rates of 0.45 cm $yr^{-1}$, while mid-waters and below the OMZ show rates between 0.07 and 0.011 cm $yr^{-1}$.

## 2.2 Sampling

Sediment samples were taken in January 2013 at six stations (70, 144, 253, 407, 770, and 1025 m) along a depth transect at 12° S in the OMZ off Peru (Fig. 1) during an expedition on RV *Meteor* (M92). January represents austral summer, i.e., the low upwelling, high productivity season in this area (Kessler, 2006). Samples were retrieved using a TV-guided multiple corer (MUC) equipped with seven core liners. The core liners had a length of 60 cm and an inner diameter of 10 cm. Location, water depth, temperature, and $O_2$ concentration (from Dale et al., 2015) at the six sampling stations are listed in Table 1. Retrieved cores for microbial rate measurements were immediately transferred to cold rooms (4–9 °C) for further processing.

## 2.3 Geochemical analyses

Porewater analysis and the determination of sediment properties and geochemical data have been previously described in detail by Dale et al. (2015). In short, the first core was subsampled under anoxic conditions using an argon-filled glove bag, to preserve redox sensitive constituents. $NH_4^+$ and sulfide concentrations were analyzed on a Hitachi U2800 UV/VIS spectrophotometer using standard photometric procedures (Grasshoff et al., 1999), while sulfate ($SO_4^{2-}$) concentrations were determined by ion chromatography (Methrom 761).

The second replicate core was sampled to determine porosity by the weight difference of the fresh sediment sub-

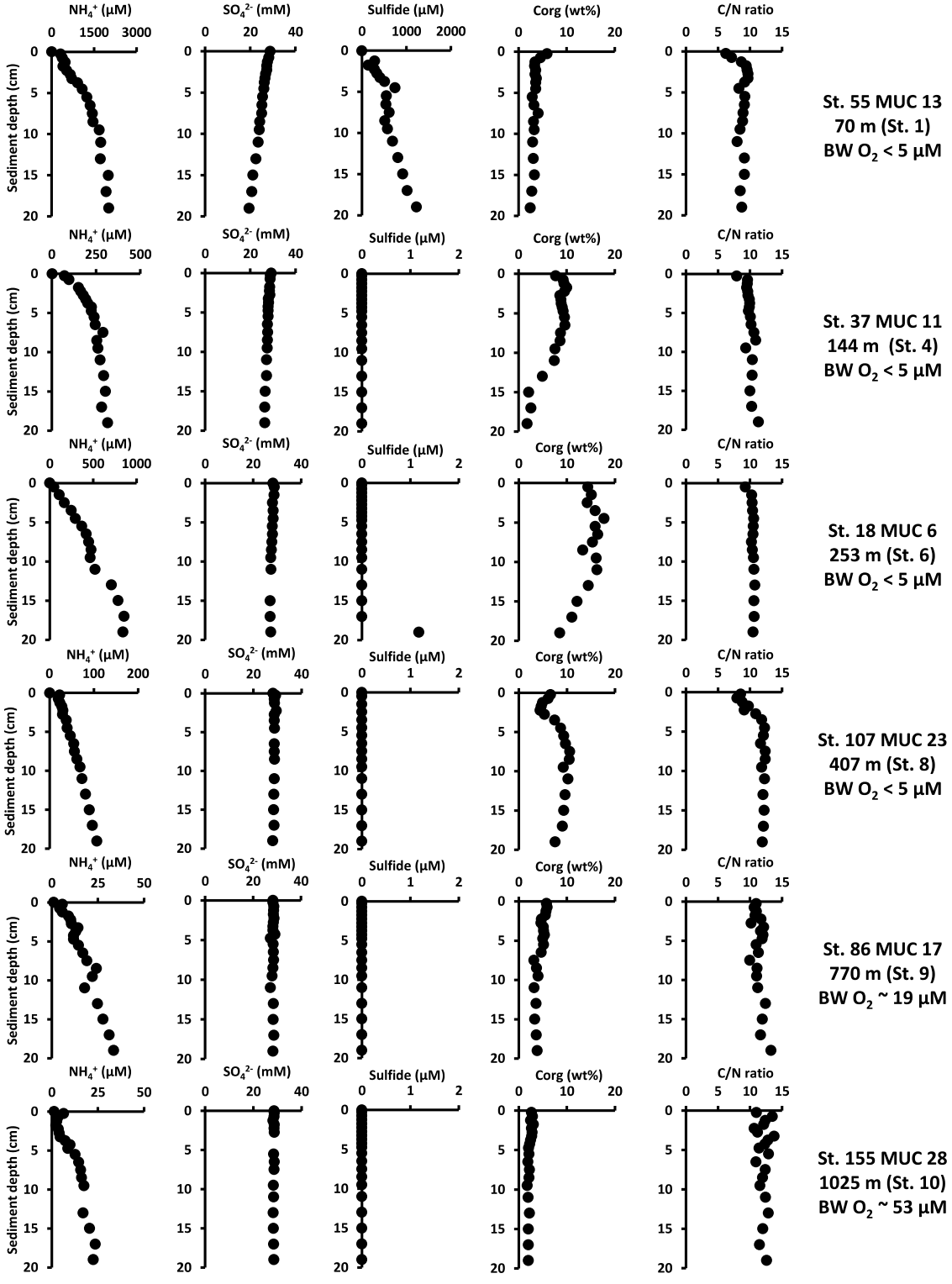


**Figure 2.** Biogeochemical porewater profiles in MUC cores from sampling stations along the 12° S depth transect. Graphs show $NH_4^+$ (µM), $SO_4^{2-}$ (mM), sulfide (µM), organic carbon content ($C_{org}$, wt %) and the C / N ratio (molar). Water depths and bottom water $O_2$ concentrations (BW $O_2$, µM) are detailed on the right.

samples before and after freeze-drying. Particulate organic carbon and particulate organic nitrogen contents were analyzed using a Carlo-Erba element analyzer (NA 1500).

### 2.4 Benthic nitrogen fixation

At each of the six stations, one MUC core was sliced in a refrigerated container (9 °C) in 1 cm intervals from 0 to 6 cm, in 2 cm intervals from 6 to 10 cm, and in 5 cm intervals from 10 to 20 cm. The acetylene reduction assay (Capone, 1993; Bertics et al., 2013) was applied to quantify nitrogenase activity. This application is based on the reduction of acetylene ($C_2H_2$) to ethylene ($C_2H_4$) by the nitrogenase enzyme (Dilworth, 1966; Stewart et al., 1967; Capone, 1993). To convert from nitrogenase activity to $N_2$ fixation, a conversion factor of 3 $C_2H_4 : 1$ $N_2$ was applied (Patriquin and Knowles, 1972; Donohue et al., 1991; Orcutt et al., 2001; Capone et al., 2005), which was previously used to measure $N_2$ fixation in sediments (Welsh et al., 1996; Bertics et al., 2013).

Serum vials (60 mL) were flushed with $N_2$ and filled with 10 $cm^3$ sediment from each sampling depth (triplicates). The samples were flushed again with $N_2$, crimp sealed with butyl stoppers and injected with 5 mL of $C_2H_2$ to saturate the nitrogenase enzyme. Serum vials were stored in the dark at 9 °C, which reflected the average in situ temperature along the transect (compare with Table 1). Two sets of triplicate controls (10 $cm^3$) were processed for every station. Sediment was collected from each core liner from 0 to 5, 5 to 10, and from 10 to 20 cm and placed in 60 mL serum vials. One set of controls was used to identify natural $C_2H_4$ production without the injection of acetylene, and the second control set was fixed with 1 mL 37.5 % formaldehyde solution.

The increase of $C_2H_4$ in each sediment slice was measured onboard over 1 week (in total five time points, including time zero) using gas chromatography (Hewlett Packard 6890 Series II). From each serum vial, a 100 µL headspace sample was injected into the gas chromatograph and the results were analyzed with the HP ChemStation gas chromatograph software. The gas chromatograph was equipped with a packed column (Haye SepT, 6 ft, 3.1 mm ID, Resteck) and a flame ionization detector. The carrier gas was helium and the combustion gases were synthetic air (20 % $O_2$ in $N_2$) and hydrogen. The column had a temperature of 75 °C and the detector temperature was 160 °C.

Standard deviation of individual $N_2$ fixation rates was calculated from three replicates determined per sediment depth in one multicorer. Standard deviation of depth-integrated $N_2$ fixation was calculated from the three replicate integrated rates.

It should be mentioned that the incubation with $C_2H_2$ can potentially lead to a lack of fixed N caused by the saturation of the nitrogenase enzyme, which leads to a reduction of cell viability and consequently $N_2$ fixation (Seitzinger and Garber, 1987). These effects are expected to cause an underestimation of $N_2$ fixation rates. However, the acetylene reduction method is to the best of our knowledge still the standard method for the determination of benthic $N_2$ fixation (Bertics et al., 2013). The $\delta^{15}N$ rate determinations are not feasible in sediments, as they would require incubation times of several weeks to months to achieve signals that are statistically above the natural $\delta^{15}N$ abundance of sediments.

We are further aware that our samples might have experienced a potential microbial community shift during the $N_2$ fixation determination, which was shown to be driven by the addition of $C_2H_2$ (Fulweiler et al., 2015). Again, a community shift would be expected to cause rather an underestimation of absolute $N_2$ fixation rates.

### 2.5 Sulfate reduction rates

One MUC core per station was used for determination of SR activity (same MUC cast as for $N_2$ fixation, but different core). First, two replicate push cores (length 30 cm, inner diameter 2.6 cm) were subsampled from one MUC core. The actual push core length varied from 21 to 25 cm total length. Then, 6 µL of the carrier-free $^{35}SO_4^{2-}$ radio tracer (dissolved in water, 150 kBq, specific activity 37 TBq $mmol^{-1}$) was injected into the replicate push cores in 1 cm depth intervals according to the whole-core injection method (Jørgensen, 1978). The push cores were incubated for ~ 12 h at 9 °C. After incubation, bacterial activity was stopped by slicing the push core into 1 cm intervals and transferring each sediment layer into 50 mL plastic centrifuge tubes filled with 20 mL zinc acetate (20 % $w/w$). Controls were done in triplicates from different depths and first fixed with zinc acetate before adding the tracer. Rates for SR were determined using the cold chromium distillation procedure according to Kallmeyer et al. (2004).

It should be mentioned that the yielded SR rates have to be treated with caution due to long (up to 3 half-life times of $^{35}S$) and unfrozen storage. Storage of SR samples without freezing has recently been shown to result in the re-oxidation of $^{35}S$-sulfides (Røy et al., 2014). In this reaction, FeS is converted to ZnS. The released $Fe^{2+}$ reacts with $O_2$ and forms reactive Fe(III). The Fe(III) oxidizes ZnS and FeS, which are the major components of the total reduced inorganic sulfur species, resulting in the generation of $SO_4^{2-}$ and hence an underestimation of SR rates. However, because all SR samples in the present study were treated the same way, we trust the relative distribution of activity along sediment depth profiles and recognize potential underestimation of absolute rates.

### 2.6 *nifH* gene analysis

Core samples for DNA analysis were retrieved from the six stations and were sliced in the same sampling scheme as described for benthic $N_2$ fixation. Approximately 5 mL sediment from each depth horizon was transferred to plastic whirl-paks® (Nasco, Fort Atkinson, USA), frozen at −20 °C and transported back to the home laboratory. To

check for the presence of the *nifH* gene, DNA was extracted using the FastDNA® SPIN Kit for Soil (MP Biomedicals, CA, USA) following the manufacturer's instructions with a small modification. Sample homogenization was done in a Mini-Beadbeater™ (Biospec Products, Bartlesville, USA) for 15 s. PCR amplification, including primers and PCR conditions, was done as described by Zehr et al. (1998), using the GoTaq kit (Promega, Fitchburg, USA) and additionally 1 µL bovine serum albumin (20 mg $mL^{-1}$; Fermentas). The TopoTA Cloning® Kit (Invitrogen, Carlsbad, USA) was used for cloning of PCR amplicons, according to the manufacturer's protocol. Sanger sequencing (122 *nifH* sequences) was performed by the Institute of Clinical Molecular Biology, Kiel, Germany. For the sampling sites 70, 144, 253, 407, 770, and 1025 m water depth the number of obtained sequences was 22, 24, 24, 13, 18, and 21, respectively. Negative controls were performed using the PCR mixture as described without template DNA; no amplification was detected. Sequences were ClustalW aligned in MEGA 6.0 (Tamura et al., 2007), and a maximum likelihood tree was constructed on a 321 base pair fragment and visualized in iTOL (Letunic and Bork, 2007, 2011). Reference sequences were obtained using BlastX on the NCBI database. Sequences were submitted to Genbank (Accession numbers: KU302519 – KU302594).

### 2.7 Statistical analysis

A principle component analysis (PCA) was applied to microbial rates and environmental parameters to determine most likely explanatory variables for active $N_2$ fixation at the sampling St. 1 to 9. The deepest St. 10 was excluded from the analysis because at this site SR rates were below the detection limit and the PCA only allows complete datasets, which otherwise would have resulted in the exclusion of all SR rates. Prior to PCA, the dataset was Hellinger transformed in order to make it compatible with PCA. The PCA was performed in R v3.0.2 by using the R package "Vegan" (Oksanen et al., 2013) according to the approach described in Löscher et al. (2014).

For the depth profiles of $N_2$ fixation rates (mmol $m^{-2}$ $d^{-1}$) the variables water depth (m), sediment depth (cm), sulfate reduction (mmol $m^{-2}$ $d^{-1}$), organic carbon content (wt %), C / N ratio (molar), ammonium (µM), and sulfide (µM) were tested. A PCA of integrated (0–20 cm) $N_2$ fixation rates (mmol $m^{-2}$ $d^{-1}$) and environmental parameters could not be done due to the lack of sufficient data points.

Finally, two biplots for the depth profiles were produced, which allowed having two different views from two different angles, i.e., one biplot for principle component 1 and 2, and one biplot for principle component 2 and 3. These biplots graphically reveal a potential negative, positive or zero correlation between $N_2$ fixation and the tested variables.

## 3 Results

### 3.1 Sediment properties

Although sediments were sampled down to the bottom of the core, the focus here is on the 0–20 cm depth interval where benthic $N_2$ fixation was investigated.

Sediments at the shelf station (St.) 1 (70 m) were black between 0 and 1 cm and then olive green until 20 cm. Only a few metazoans (polychaetes) were observed in the surface sediment. The sediment surface was colonized by dense filamentous mats of sulfur-oxidizing *Marithioploca* spp. These bacteria extended down to a sediment depth of 36 cm. The sediment on the outer shelf St. 4 (144 m) was dark olive green from 0 to 13 cm and dark grey until 20 cm. At St. 6 (253 m), which was located within the core of the OMZ, the sediment appeared dark olive green between 0 and 17 cm and olive green with white patches between 17 and 20 cm. At this station, *Marithioploca* spp. was abundant. Uniquely, surface sediments (0–3 cm) at St. 8 (407 m), consisted of a fluffy, dark olive-green layer mixed with white foraminiferal ooze. This layer also contained centimeter-sized phosphorite nodules with several perforations (ca. 1–3 mm in diameter). Below 2 cm, the sediment consisted of a dark olive green, sticky clay layer. No *Marithioploca* mats were found here. St. 9 (770 m) was below the OMZ, and sediments were brown to dark olive green with white particles between 0 and 12 cm, and brown to olive green without white particles below this depth. Organisms such as anemones, copepods, shrimps and various mussels were visible with the TV-guided MUC and in the sediment cores. The deepest St. (10; 1025 m) had dark olive green sediment from 0 to 20 cm and black patches from 17 to 20 cm. The sediment was slightly sandy and was colonized with polychaete tubes at the surface and organisms that were also present at St. 9. For further sediment core descriptions see also Dale et al. (2015).

Geochemical porewater profiles of $NH_4^+$, $SO_4^{2-}$, sulfide, organic carbon content, and organic C / N ratio between 0 and 20 cm at the six stations are shown in Fig. 2. In all cores, $NH_4^+$ concentrations increased with sediment depth. The highest $NH_4^+$ concentration was reached at St. 1 (70 m), increasing from 316 µM in the upper cm to 2022 µM at 20 cm. St. 4 and 6 showed intermediate $NH_4^+$ concentrations between 300 and 800 µM at 20 cm, respectively. At St. 8 (407 m) the $NH_4^+$ concentration increased from 0.7 µM at the surface to 107 µM at 20 cm. The two deep stations (St. 9 and 10) had the lowest $NH_4^+$ concentrations with 33 and 22 µM at 20 m sediment depth, respectively.

The $SO_4^{2-}$ concentrations remained relatively constant in the surface sediments along the transect. A decrease was only observed at St. 1; from 28.7 µM in the surface layer to 19.4 µM at 20 cm. In parallel with the decrease in $SO_4^{2-}$, only St. 1 revealed considerable porewater sulfide accumulation, whereby sulfide increased from 280 µM at the surface sediment to 1229 µM at 20 cm.

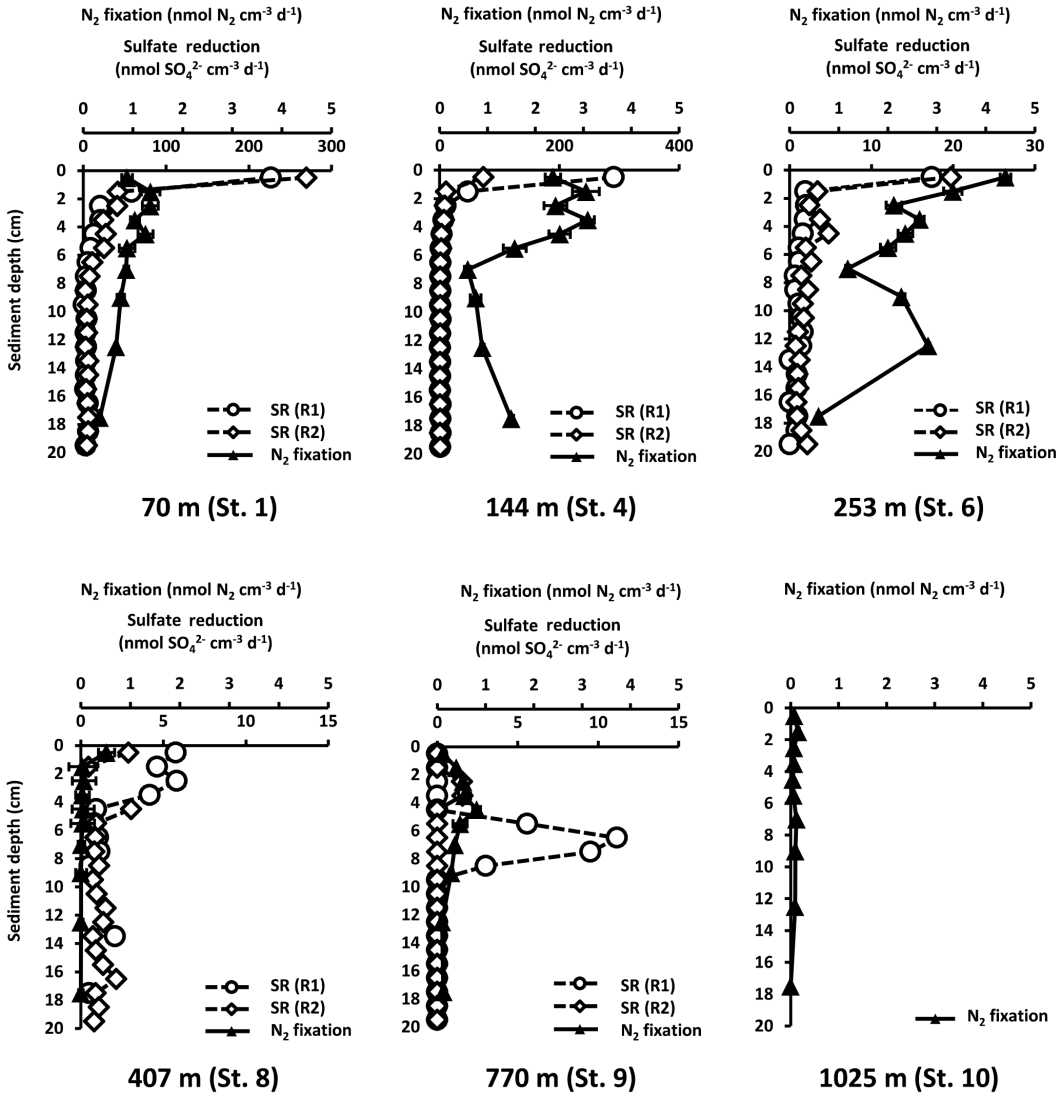


**Figure 3.** Sediment profiles of $N_2$ fixation (nmol $N_2$ $cm^{-3}$ $d^{-1}$, average of three replicates) and sulfate reduction rates (SR, nmol $SO_4^{2-}$ $cm^{-3}$ $d^{-1}$, two replicates; R1 and R2) from 0 to 20 cm at the six stations. The upper $x$ axis represents the $N_2$ fixation, while the lower $x$ axis represents the SR. Error bars indicate standard deviation of $N_2$ fixation.

Organic carbon content decreased with increasing sediment depth at St. 1 (70 m), 9 (770 m), and 10 (1025 m). The highest surface organic carbon content ($\sim 15$ wt %) was found at St. 6, whereas the lowest ($\sim 2.6$ wt %) was detected at the deep St. 10. The average (0–20 cm) organic carbon content (Fig. 5) increased from St. 1 to St. 6 ($15 \pm 1.7$ wt %) and decreased from St. 6 to the lowest value at St. 10 ($2.4 \pm 0.4$ wt %).

C / N ratios, as a proxy for the freshness of the organic matter, increased with increasing sediment depth (Fig. 5). The lowest surface C / N ratio (6.2) was measured at the shallow St. 1, while the highest surface C / N ratio (11) was found at St. 10.

### 3.2 Benthic nitrogen fixation and sulfate reduction

For a straightforward comparison of SR rates with benthic $N_2$ fixation only the sediment depths between 0 and 20 cm are considered. Sediment depth profiles are expressed as $N_2$ fixation, that is, with the conversion factor of $3\,C_2H_4 : 1\,N_2$.

Highest $N_2$ fixation and SR rates were detected in the surface sediments (0–5 cm) and both rates tended to decrease with increasing sediment depth (Fig. 3). $N_2$ fixation and SR rates were high at St. 1, 4, and 6 (70, 144, 253 m) and lowest at the deeper St. 8–10 (407, 770, 1025m).

At St. 1, $N_2$ fixation and SR rates showed different trends in the top layer of the cores, but depth profiles were more aligned below. Although St. 1 had the highest SR rates of all

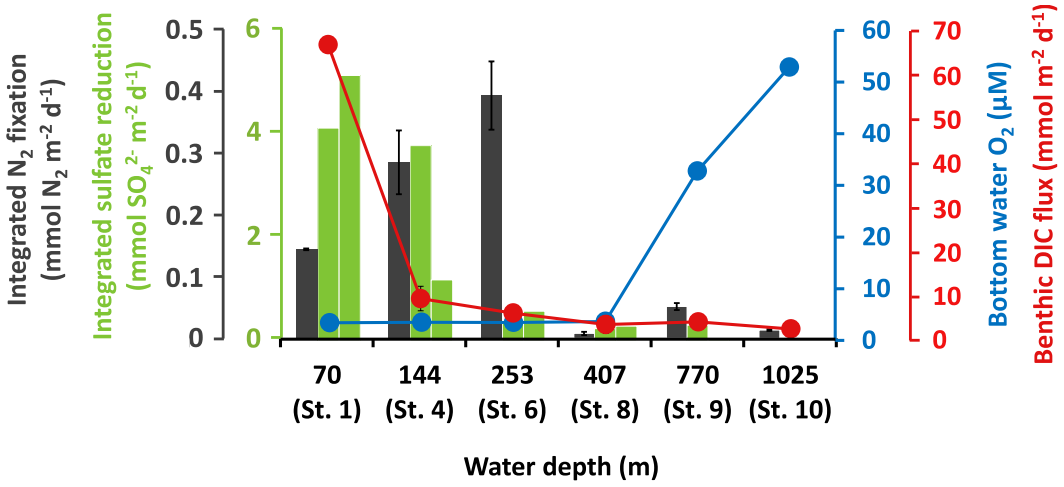


**Figure 4.** Integrated nitrogen fixation (mmol $N_2$ $m^{-2}$ $d^{-1}$, grey bars, average of three replicates) and integrated sulfate reduction (mmol $SO_4^{2-}$ $m^{-2}$ $d^{-1}$, green bars, two replicates) from 0 to 20 cm, including dissolved inorganic carbon flux (DIC, mmol $m^{-2}$ $d^{-1}$, red curve from Dale et al., 2015) and bottom water $O_2$ (µM, blue curve) along the depth transect (m). Error bars indicate standard deviation of $N_2$ fixation.

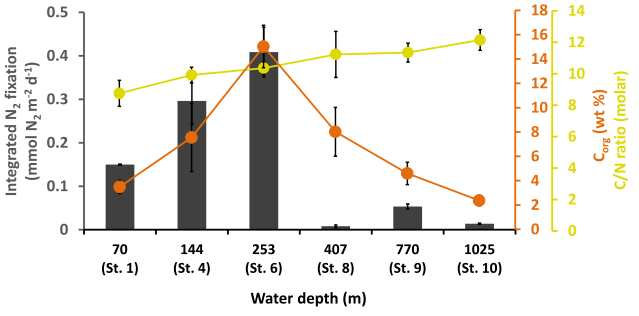


**Figure 5.** Integrated nitrogen fixation (mmol $N_2$ $m^{-2}$ $d^{-1}$, grey bars, average of three replicates), average organic carbon content ($C_{org}$, wt %, orange curve) and the average C / N molar ratio (yellow curve) from 0 to 20 cm along the depth transect (m). Error bars indicate standard deviation.

sites, reaching 248 nmol $SO_4^{2-}$ $cm^{-3}$ $d^{-1}$ at 0–1 cm, $N_2$ fixation was not highest at this station. At St. 4 (144 m), both $N_2$ fixation and SR revealed peaks close to the surface. $N_2$ fixation decreased between 0 and 8 cm and increased below 8 cm. This increase was not observed in SR rates, which were highest at the surface (181 nmol $SO_4^{2-}$ $cm^{-3}$ $d^{-1}$) and decreased towards the bottom of the core. St. 6 (253 m) had the highest $N_2$ fixation of all stations, with rates of $4.0 \pm 0.5$ nmol $N_2$ $cm^{-3}$ $d^{-1}$ in the surface centimeter. Yet, although $N_2$ fixation and SR had overlapping activity profiles, the highest SR rate of all stations was not detected at St. 6. Very low $N_2$ fixation rates were measured at St. 8 (407 m; $0.5 \pm 0.25$ nmol $N_2$ $cm^{-3}$ $d^{-1}$ in the surface), as well as very low SR rates (0–4.3 nmol $SO_4^{2-}$ $cm^{-3}$ $d^{-1}$). As mentioned, this station was unique due to the presence of foraminiferal ooze, phosphorite nodules and a sticky clay layer below 2 cm. $N_2$ fixation and SR rates showed a peak at 5 and at 7 cm, respectively. At St. 9 (770 m) $N_2$ fixation was low in the surface and at 20 cm sediment depth, with a peak in activity at 4–5 cm ($0.8 \pm 0.08$ nmol $N_2$ $cm^{-3}$ $d^{-1}$). At St. 10 (1025 m), $N_2$ fixation rates were low throughout the sediment core, not exceeding $0.16 \pm 0.02$ nmol $N_2$ $cm^{-3}$ $d^{-1}$. This site had the lowest organic carbon content throughout the core (between 2.6 wt % at the surface and 1.9 wt % at 20 cm), as well as low $NH_4^+$ concentrations. At St. 9 (below 9 cm depth) and St. 10 (entire core) SR rates were below detection, which could point either to the absence of SR or to the complete loss of total reduced inorganic sulfur due to the long, unfrozen storage (see methods).

Integrated $N_2$ fixation (0–20 cm) increased from St. 1 to St. 6, with the highest rate ($0.4 \pm 0.06$ $N_2$ $m^{-2}$ $d^{-1}$) at St. 6 (253 m), and decreased from St. 6 (407 m) to St. 10 (1025 m; Fig. 4). Integrated SR rates (0 to 20 cm) ranged from ~ 4.6 mmol $SO_4^{2-}$ $m^{-2}$ $d^{-1}$ at St. 1 to below detection at St. 9 (Fig. 4). Overall, integrated SR rates decreased with increasing water depth. Integrated $N_2$ fixation rates and SR were in general inversely correlated between St. 1 and St. 6, and followed the organic carbon content from St. 1 to St. 6 (70–253 m; Fig. 5). Both parameters had the highest value at St. 6. This pattern did not hold for the relatively low integrated SR rate at St. 6. The C / N ratio, averaged over 20 cm, increased with increasing water depth (Fig. 5). Regarding the three deep stations, the lowest integrated $N_2$ fixation rate ($0.008 \pm 0.002$ $N_2$ $m^{-2}$ $d^{-1}$) was detected at St. 8 (407 m). Also the integrated SR rate was low at this site (~ 0.46 mmol $SO_4^{2-}$ $m^{-2}$ $d^{-1}$). At St. 9 and 10 (770 and 1025 m), integrated $N_2$ fixation was low at $0.05 \pm 0.005$ and $0.01 \pm 0.001$ $N_2$ $m^{-2}$ $d^{-1}$, respectively, and integrated SR rates were also lowest at St. 9 (770 m). From St. 8 to 10 a decrease of integrated $N_2$ fixation and SR together with the average organic carbon content was detected.

No activity was detected in controls for $N_2$ fixation and SR.

### 3.3 Statistical analysis

The PCA of $N_2$ fixation depth profiles (Fig. 6a and b) showed a weak positive correlation with sulfate reduction rates (Fig. 6a) and a strong positive correlation between $N_2$ fixation and the organic matter content in sediments (Fig. 6b). A negative correlation between $N_2$ fixation and sediment depth (Fig. 6a), as well as between $N_2$ fixation and sulfide concentration for St. 1 (Fig. 6b) was found. Furthermore, a weak negative correlation was detected between $N_2$ fixation and the C / N ratio (Fig. 6a). No correlation was found between $N_2$ fixation and ammonium concentration and water depth (Fig. 6a and b).

### 3.4 Molecular analysis of the *nifH* gene

Sequences for the *nifH* gene analysis were pooled for each of the six stations, making about 20 sequences per sample and 120 in total. *NifH* gene sequences were detected at all six sampling sites and clustered with Cluster I proteobacterial sequences and Cluster III sequences as defined by Zehr and Turner (2001) (Fig. 7). In Cluster I and Cluster III, three and seven novel clades were detected, respectively. In general, most of the previously unidentified clades belonged to uncultured bacteria. One distinct novel clade was found for St. 1–6. No Cluster I cyanobacterial *nifH* sequences were detected and no potential PCR contaminants were present (Turk et al., 2011). Sequences clustered with only one identified sulfate-reducing bacterium, *Desulfonema limicola* (Fukui et al., 1999, OMZ 253). Other sequences from several stations (OMZ 70, 144, 253, 770) were distantly related to *Desulfovibrio vulgaris* (Riederer-Henderson and Wilson, 1970; Muyzer and Stams, 2008). One cluster (OMZ 144 m) was closely related to the anaerobic marine bacterium *Vibrio diazotrophicus* (Guerinot et al., 1982). Other organisms with which OMZ sequences clustered belonged to the genera of fermenting bacteria, namely *Clostridium beijerincki* (Chen, 2005), and to the genera of iron-reducing bacteria, namely *Geobacter bemidjiensis* (Nevin et al., 2005). In addition, several sequences were phylogenetically related to a gamma proteobacterium (Zehr and Turner, 2001) from the Pacific Ocean.

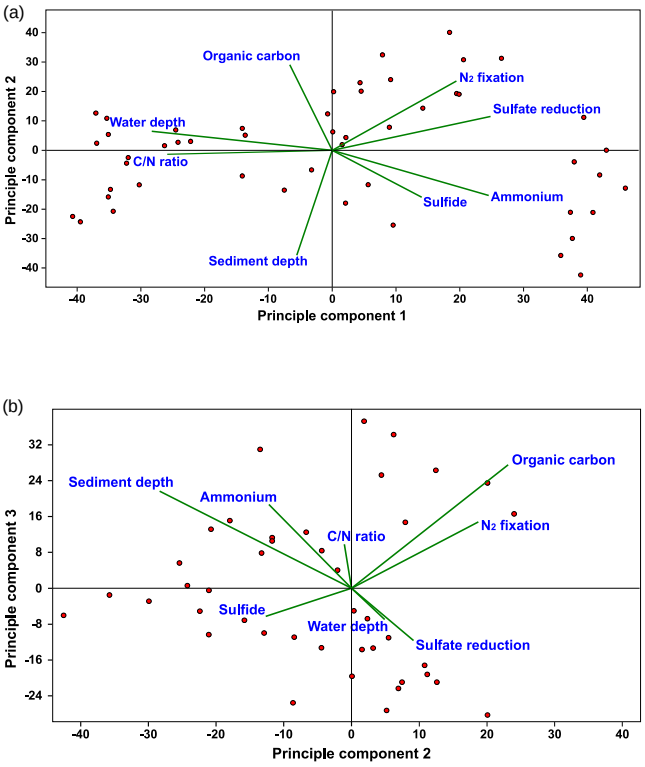


**Figure 6.** Principle component analysis (PCA) from two different angles of Hellinger transformed data of $N_2$ fixation and environmental parameters along vertical profiles. Correlation biplots **(a)** of principle components 1 and 2 and of **(b)** principle components 2 and 3 in a multidimensional space are shown. Samples are displayed as dots while variables are displayed as lines. Parameters pointing into the same direction are positively related; parameters pointing in the opposite direction are negatively related.

## 4 Discussion

### 4.1 Coupling of benthic nitrogen fixation and sulfate reduction

Based on the high organic matter input to Peruvian sediments underneath the OMZ we hypothesized a presence of $N_2$ fixation and its coupling to sulfate reduction (SR). We confirmed the presence of $N_2$ fixation in sediments at all sampled stations along the depth transect. $N_2$ fixation activity was often enhanced where SR peaked and sometimes both activity depth profiles revealed similar trends. However, while peaks in SR were very pronounced, maximum $N_2$ fixation showed a much broader distribution over depth. These findings are in line with the PCA of depth profiles, which revealed a weak positive correlation between activities of $N_2$ fixation and sulfate reduction. But it should be kept in mind that the $N_2$ fixation and SR were determined in replicate MUC cores, which were taken up to 50 cm apart, depending on where the core liners were situated in the multicorer. Nonetheless, it appears that the observed $N_2$ fixation is not exclusively fueled by SR activity.

The coupling between $N_2$ fixation and SR has been previously suggested for coastal sediments off California, where $N_2$ fixation significantly decreased when SR was inhibited (Bertics and Ziebis, 2010). Different studies confirmed that sulfate-reducing bacteria, such as *Desulfovibrio vulgaris* can supply organic-rich marine sediments with bioavailable N through $N_2$ fixation (Welsh et al., 1996; Nielsen et al., 2001;

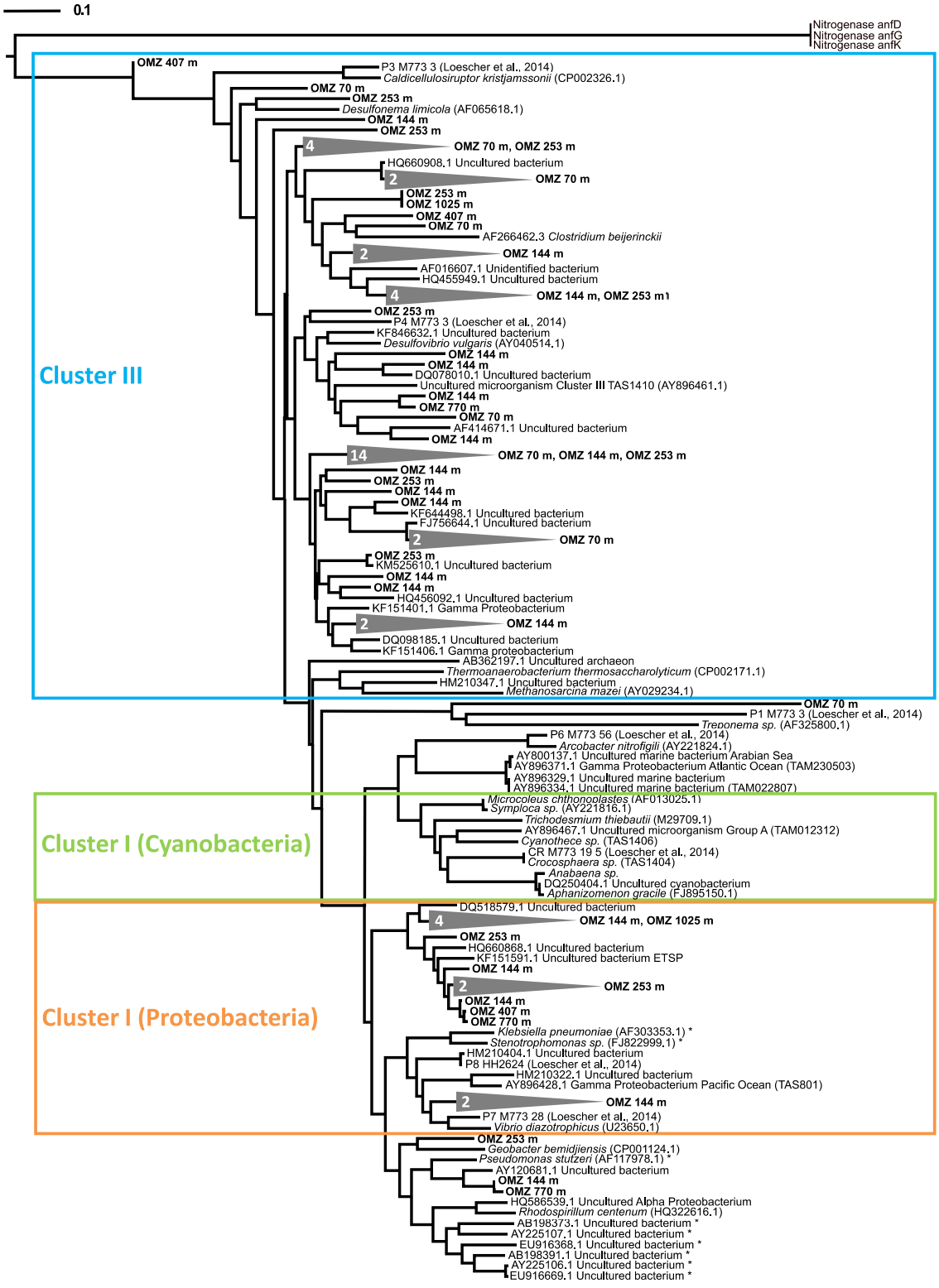


**Figure 7.** Phylogenetic tree of *nifH* genes based on the analysis of 122 sequences (~ 20 sequences per sample) from the six sampling stations between 70 and 1025 m water depth. Novel detected clusters consisting of several sequences from the same sampling depth are indicated by grey triangles. Reference sequences consist of the alternative nitrogenase $anfD$, $anfG$, $anfK$. Cluster III sequences as defined by Zehr and Turner (2001) are highlighted in blue; Cluster I cyanobacterial sequences are highlighted in green and Cluster I proteobacterial sequences are highlighted in orange. The scale bar indicates the 10 % sequences divergence. Sequences marked with an asterisk represent potential PCR contaminated products, with novel clusters distant from those clusters. Sequences determined in this study are termed OMZ plus the corresponding water depth.

Steppe and Paerl, 2002; Fulweiler et al., 2007; Bertics et al., 2013; Fulweiler et al., 2013). Fulweiler et al. (2013) conducted a study in sediments of the Narrangaset Bay and found several *nifH* genes related to sulfate-reducing bacteria, such as *Desulfovibrio* spp., *Desulfobacter* spp. and *Desulfonema* spp., suggesting that sulfate-reducing bacteria were the dominant diazotrophs.

The more surprising finding in this study is that integrated rates of $N_2$ fixation and SR showed opposite trends at the three shallowest stations, pointing to potential environmental control mechanisms (see Sect. 5.2). Overall, these findings indicate that $N_2$ fixation might be partly coupled to processes other than SR or that the two processes are controlled by different parameters. The *nifH* gene sequence analyses indicated only a weak potential of sulfate reducers to conduct $N_2$ fixation in the Peruvian sediments. Sequences clustered only with the sulfate-reducing bacteria *Desulfonema limicola* (Fukui et al., 1999) exclusively at the 253 m Station. *D. limicola* is known from other benthic environments through *nifH* gene analyses (Mussmann et al., 2005; Bertics et al., 2010, 2013). A distant relation to the confirmed diazotrophic sulfate reducer *Desulfovibrio vulgaris* (Sisler and ZoBell, 1951; Riederer-Henderson and Wilson, 1970) was detected at several stations. *D. limicola* and *D. vulgaris* clustered with sequences taken from the seasonally hypoxic Eckernförde Bay in the Baltic Sea (Bertics et al., 2013), suggesting a major involvement of these sulfate-reducing bacteria in $N_2$ fixation in organic-rich sediments. Further, sequences related to *Vibrio diazotrophicus* were detected, which has the unique ability for a known *Vibrio* species to perform $N_2$ fixation and which was found previously in the water column of the OMZ off Peru (Fernandez et al., 2011; Löscher et al., 2014). Interestingly, we detected several new *nifH* gene clusters in the Peruvian OMZ that have not been identified yet and which have, consequently, yet unknown metabolic processes (Fig. 7). Thus, a coupling of $N_2$ fixation to processes other than SR is also possible, which might also explain some of the discrepancies between $N_2$ fixation and SR activity (see above). However, the coupling to heterotrophic metabolic processes such as denitrification or methanogenesis was not supported by our molecular data.

### 4.2 Environmental factors controlling benthic $N_2$ fixation

The observed differences between integrated $N_2$ fixation and SR along the depth transect indicate potential environmental factors that control the extent of benthic $N_2$ fixation, which will be discussed in the following section.

#### 4.2.1 Organic matter

A major driver for microbial processes such as SR and $N_2$ fixation by potentially heterotrophic organisms is the availability of the organic material (Jørgensen, 1983; Howarth et al., 1988; Fulweiler et al., 2007). Integrated $N_2$ fixation and average organic carbon content showed similar trends along the Peruvian OMZ depth transect (Fig. 5), and a strong positive correlation was detected by PCA in the sediment depth profiles (Fig. 6). Thus, organic matter availability appears to be a major factor controlling $N_2$ fixation at this study site. Low organic matter content was previously shown to result in low $N_2$ fixation rates in slope sediments in the Atlantic Ocean (Hartwig and Stanley, 1978). Correlation to organic matter was further confirmed by the study of Bertics et al. (2010), which showed that burrow systems of the bioturbating ghost shrimp *Neotrypaea californiensis* can lead to enhanced organic matter availability in deeper sediment layers, resulting in high rates of $N_2$ fixation. However, high organic matter availability does not always result in enhanced $N_2$ fixation rates. Subtidal sediments in the Narragansett Bay were found to switch from being a net sink via denitrification to being a net source of bioavailable N via $N_2$ fixation (Fulweiler et al., 2007). This switch was caused by a decrease of organic matter deposition to the sediments, which was in turn triggered by low primary productivity in the surface waters.

Besides quantity also the quality of organic matter in sediments is a major factor influencing microbial degradation processes (Westrich and Berner, 1984). In the Peruvian OMZ sediments, the average C / N ratio increased with water depth indicating that the shallow stations received a higher input of fresh, labile organic material compared to the deeper stations. Similar trends were reported for a different depth transect off Peru (Levin et al., 2002). The C / N ratios did not follow the pattern of integrated $N_2$ fixation (Fig. 5), which is in line with the PCA of depth profiles, which showed a weak negative correlation between $N_2$ fixation and the C / N ratio. These results indicate that the C / N ratio is not a major factor controlling $N_2$ fixation in Peruvian OMZ sediments.

DIC fluxes, which were determined in benthic chamber lander incubations at the same stations and during the same expedition as our study (Dale et al., 2015), can be used as an indicator for organic matter degradation rates, e.g., by SR. The DIC flux did not follow the pattern of the integrated $N_2$ fixation rates (Fig. 4) and thus does not indicate that $N_2$ fixation and SR are coupled. Instead, the benthic DIC flux roughly followed the pattern of SR rates along the depth transect. The highest integrated SR rate and DIC flux were found at St. 1 (70 m), whereas the lowest occurred at St. 10 (1025 m). Assuming that SR is largely responsible for organic matter remineralization in the sediments below the OMZ (Bohlen et al., 2011; Dale et al., 2015), the difference between integrated SR and DIC flux is expected to be mainly caused by the loss of $^{35}$S-sulfides during the long duration of unfrozen storage of the SR samples (see methods).

#### 4.2.2 Ammonium

Interestingly, the highest $N_2$ fixation was measured in sediments colonized by the sulfur-oxidizing and nitrate-

reducing filamentous bacteria *Marithioploca* spp. (Schulz, 1999; Schulz and Jørgensen, 2001; Gutiérrez et al., 2008; Salman et al., 2011; Mosch et al., 2012). *Marithioploca* facilitates dissimilatory $NO_3^-$ reduction to $NH_4^+$, which preserves fixed N in the form of $NH_4^+$ in the environment (Kartal et al., 2007). OMZ sediments off Peru are generally rich in $NH_4^+$ (Bohlen et al., 2011; Dale et al., 2016). This co-occurrence of *Marithioploca* and $N_2$ fixation was puzzling since high concentrations of $NH_4^+$ were expected to inhibit $N_2$ fixation (Postgate, 1982; Capone, 1988; Knapp, 2012). It remains questionable why microorganisms should fix $N_2$ in marine sediments, when reduced N species are abundant. Some doubt remains as to the critical $NH_4^+$ concentration that inhibits $N_2$ fixation and whether the inhibitory effect is the same for all environments (Knapp, 2012). For example, $NH_4^+$ concentrations up to 1000 µM did not fully suppress benthic $N_2$ fixation in a hypoxic basin in the Baltic Sea (Bertics et al., 2013), indicating that additional environmental factors must control the distribution and performance of benthic diazotrophs (Knapp, 2012). We observed high porewater $NH_4^+$ concentrations at the shallow St. 1 with 316 µM at the sediment surface (0–1 cm) increasing to 2022 µM at 20 cm (Fig. 2), while no inhibition of $N_2$ fixation was found. This observation is verified by the PCA, which showed no correlation with ammonium for the $N_2$ fixation depth profiles. Hence, ammonium did not seem to have a significant influence on benthic $N_2$ fixation rates in the Peruvian OMZ.

One debated explanation for why diazotrophs still fix N under high $NH_4^+$ concentrations is that bacteria fix $N_2$ to remove excess electrons and to preserve their intracellular redox state, particularly with a deficient Calvin–Benson–Bassham pathway, as shown for photoheterotrophic nonsulfur purple bacteria (Tichi and Tabita, 2000). Another explanation could be that microniches, depleted in $NH_4^+$, exist between sediment grains, which we were unable to track with the applied porewater extraction techniques (Bertics et al., 2013).

#### 4.2.3 Sulfide

Sulfide is a known inhibitor for many biological processes (Reis, et al., 1992; Joye and Hollibaugh, 1995) and could potentially affect $N_2$ fixation (Tam et al., 1982). The shallow St. 1 was the only station with sulfide in the porewater, reaching 280 µM in surface sediments and 1229 µM in 20 cm (Fig. 2). The presence of relatively high concentrations of sulfide at St. 1 might explain why $N_2$ fixation was lower at this site when compared to St. 6, which had the highest $N_2$ fixation rates. Statistically, depth profiles of $N_2$ fixation and sulfide showed a negative correlation (Fig. 6b). Generally, interactions of sulfide with benthic $N_2$ fixation have so far not been investigated, and the PCA did not provide a complete pattern, as sulfide was not widespread in the sediments along the transect and thus does not allow robust interpretation.

#### 4.2.4 Oxygen

Dissolved $O_2$ can have a considerable influence on $N_2$ fixation due to the $O_2$ sensitivity of the key enzyme nitrogenase (Postgate, 1998; Dixon and Kahn, 2004). Bioturbating and bioirrigating organisms can transport $O_2$ much deeper into sediments than molecular diffusion (Orsi et al., 1996; Dale et al., 2011). In coastal waters, the bioturbation and bioirrigation activity of ghost shrimps was found to reduce $N_2$ fixation when sediments were highly colonized by these animals (Bertics et al., 2010). While bottom water $O_2$ concentrations in the Peruvian OMZ were below the detection limit at St. 1 to 8 (70 to 407 m), thereby mainly excluding benthic macrofauna, $O_2$ concentrations increased to above 40 µM at St. 10 (1025 m) where a diverse bioturbating and bioirrigating benthic macrofauna community was observed (Mosch et al., 2012). Accordingly, St. 10 revealed some of the lowest $N_2$ fixation activity. We speculate that the low organic matter content at this St. was mainly responsible for the low $N_2$ fixation rates and not the high bottom water $O_2$ concentrations, as the statistics showed a positive correlation between integrated $N_2$ fixation and organic carbon content.

### 4.3 Comparison of benthic $N_2$ fixation in different environments

We compiled a list of $N_2$ fixation rates from different marine sedimentary environments to gain an overview of the magnitude of $N_2$ fixation rates measured in the Peruvian OMZ sediments (Table 2). We found that $N_2$ fixation rates from the Peruvian sediments exceed those reported for open ocean sediments (2800 m; Howarth et al., 1988), bioturbated coastal lagoon sediment (Bertics et al., 2010) and sediments > 200 m water depth from various sites worldwide (Capone, 1988). The highest integrated $N_2$ fixation rate determined in our study (0.4 mmol $N_2$ $m^{-2}$ $d^{-1}$, St. 6) closely resembles highest rates found in salt marshes (0.38 mmol $N_2$ $m^{-2}$ $d^{-1}$) and Zostera estuarine sediments (0.39 mmol $N_2$ $m^{-2}$ $d^{-1}$) (Capone, 1988). Further, our rates were characterized by a similar range of $N_2$ fixation rates that were previously measured in an organic-rich hypoxic basin in the Baltic Sea (0.08–0.22 mmol $N_2$ $m^{-2}$ $d^{-1}$, Bertics et al., 2013). In contrast to the above examples, our $N_2$ fixation rates were 8.5 times lower compared to shallow (< 1 m) soft-bottom sediment off the Swedish coast (Andersson et al., 2014) and 17 times lower than coral reef sediments (Capone, 1988). However, in these environments, phototrophic cyanobacterial mats contributed to benthic $N_2$ fixation. Given the dark incubation, $N_2$ fixation of the present study seems to be attributed to heterotrophic diazotrophs, which is additionally confirmed by the *nifH* gene analysis, where none of the sequences clustered with cyanobacteria (Fig. 7).

**Table 2.** Integrated rates of benthic $N_2$ fixation (mmol $N_2$ $m^{-2}$ $d^1$) in the Peruvian OMZ sediments from this study compared to other marine benthic environments. Only the highest and lowest integrated rates are shown, as well as the integrated sediment depth (cm) and the method used (ARA = acetylene reduction assay, MIMS = membrane inlet mass spectrometry).

| Benthic environment | $N_2$ fixation (mmol $N_2$ $m^{-2}$ $d^{-1}$) | Depth of integration (cm) | Method | Reference |
|---|---|---|---|---|
| Peru OMZ | 0.01–0.4 | 0–20 | ARA | This study |
| Coastal region | | | | |
| Baltic Sea, hypoxic basin | 0.08–0.22 | 0–18 | ARA | Bertics et al. (2013) |
| Bioturbated coastal lagoon | 0.8–8.5 | 0–10 | ARA | Bertics et al. (2010) |
| Brackish-water | 0.03–3.4 | 0–1 | ARA | Andersson et al. (2014) |
| Coral reef | 6.09 (±5.62) | – | – | Capone (1983) |
| Eelgrass meadow | 0.15–0.39 | 0–5 | ARA | Cole and McGlathery (2012) |
| Eutrophic estuary | 0–18 | 0–20 | MIMS | Rao and Charette (2012) |
| Mangrove | 0–1.21 | 0–1 | ARA | Lee and Joye (2006) |
| Salt marsh | 0.38 (±0.41) | – | – | Capone (1983) |
| Subtidal | 0.6–15.6 | 0–30 | MIMS | Fulweiler et al. (2007) |
| Zostera estuary | 0.39 | – | – | Capone (1983) |
| Open ocean | | | | |
| Atlantic Ocean (2800 m) | 0.00008 | – | ARA | Howarth et al. (1988) |
| < 200 m, various sites | 0.02 (±0.01) | – | – | Capone (1983) |
| Mauritania OMZ | 0.05–0.24 | 0–20 | ARA | Bertics and Treude, unpubl. |

## 5 Summary

To the best of our knowledge, this is the first study combining $N_2$ fixation and SR rate measurements together with molecular analysis in OMZ sediments. We have shown that $N_2$ fixation occurred throughout the sediment and that activity often overlapped with SR. The PCA showed a weak positive correlation between activity depth profiles of $N_2$ fixation and SR. The molecular analysis of the *nifH* gene confirmed the presence of heterotrophic diazotrophs at all sampling sites, but only a few of the sequences were related to known sulfate reducers. Instead, many sequences clustered with uncultured organisms. In combination, our results indicate that $N_2$ fixation and SR were coupled to some extent, but additional coupling to other metabolic pathways is very likely. The major environmental factor controlling benthic diazotrophs in the OMZ appears to be the organic matter content. Sulfide was identified as a potential inhibitor for $N_2$ fixation. We further found no inhibition of $N_2$ fixation by high $NH_4^+$ concentration, highlighting gaps in our understanding of the relationship between $NH_4^+$ availability and the stimulation of $N_2$ fixation. $N_2$ fixation rates determined in the Peruvian OMZ sediments were in the same range of other organic-rich benthic environments, underlining the relation between organic matter, heterotrophic activity, and $N_2$ fixation.

*Author contributions.* Jessica Gier and Tina Treude collected samples and designed experiments. Jessica Gier performed nitrogen fixation experiments and Tina Treude conducted sulfate reduction experiments. Stefan Sommer and Andrew W. Dale measured porosity, DIC, organic carbon content and C / N. Jessica Gier, Tina Treude, Carolin R. Löscher and Stefan Sommer analyzed the data. Jessica Gier and Carolin R. Löscher performed molecular analysis and statistical analysis. Jessica Gier prepared the manuscript with contributions from all co-authors and Tina Treude supervised the work.

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