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

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Abstract

Benthic nitrogen (N_2) fixation and sulfate reduction (SR) were investigated in the Peruvian oxygen minimum zone (OMZ). Sediment samples, were retrieved by a multiple corer at six stations (70 - 1025 m water depth) along a depth transect at 12°S, covering anoxic and hypoxic bottom water conditions. Benthic N_2 fixation was detected at all sites using the acetylene reduction assay, with high rates between 70 m and 253 m and lower rates at greater depth. SR rates decreased with increasing water depth.. Benthic N_2 fixation and SR depth profiles in sediments showed similar qualitative trends, suggesting a coupling ofboth processes. Potential N_2 fixation by sulfate-reducing bacteria was verified by the molecular analysis of *nifH* genes. Detected *nifH* sequences, i.e., the key functional gene for N_2 fixation, encoding for the nitrogenase enzyme, clustered with sulfate-reducing bacteria that have been demonstrated to fix N_2 in other benthic environments. Depth-integrated rates of benthic N_2 fixation and SR showed no direct correlation along the transect, suggesting that the benthic diazotrophs in the Peruvian OMZ is controlled by additional environmental factors such as organic matter and free sulfide. It was further found that N_2 fixation in OMZ sediments was not inhibited by high ammonium concentrations. N_2 fixation rates in OMZ sediments were similar to rates measured in other organic-rich sediments. Overall, this study improves our knowledge on fixed N sources and N cycling in oxygen deficient environments.

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 & 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 & 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 ~265 Tg N yr^{-1} for N sources and ~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 oxygen 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; Bertics et al. 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 & 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 & ZoBell, 1951; Riederer-Henderson & Wilson, 1970; Zehr & Turner, 2001) and were shown to actively fix N_2 in culture experiments (Riederer-Henderson & Wilson, 1970). However, information on sulfate-reducing bacteria and their contribution to N2 fixation in the environment is rather sparse and makes this one of the remaining questions to be solved.

So far, the distribution of benthic N_2 fixation and its relevance for N cycling in the Peruvian (OMZ), defined by dissolved oxygen < 20 µmol 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 in the benthic N cycle (Bohlen et al., 2011). This process is mediated by the filamentous sulfide-oxidizing *Thioploca* bacteria (Schulz, 1999; Schulz & Jørgensen, 2001). Along with dissimilatory NO_3^- reduction to NH_4^+, benthic denitrification by foraminifera between 80 and 250 m water depth occurs in the Peruvian OMZ (Glock et al., 2013). These authors calculated a potential NO_3^- flux rate of 0.01 to 1.3 mmol m^{-2} d^{-1} via this pathway and suggested that foraminifera could be responsible for most of the benthic denitrification.

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 & Wilson, 1970; Muyzer & 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 potentially coupled SR. Additionally, the identification of bacteria facilitating these processes will help to understand the diazotrophic community structure of these sediments. The overall knowledge gained is useful 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 & Zentara, 1990). The Peruvian OMZ ranges between 50 m 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 µM O_2 (Gutiérrez et al., 2008). O_2 concentrations (Fig. 1, Tab. 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 time-scales (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 m 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 lower boundary of the OMZ (Dale et al. 2015). Between 70 m 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 order to access hydrogen sulfide (Fossing et al., 1995; Jørgensen & Gallardo, 1999; Schulz, 1999). Sediments at the lower boundary (770 m 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 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 subsamples 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 – 6 cm, in 2-cm intervals from 6 – 10 cm, and in 5-cm intervals from 10 – 20 cm. The acetylene reduction assay (Capone, 1993; Bertics et al. 2013) was applied, to quantify nitrogenase activity. To convert from nitrogenase activity to N_2 fixation, a conversion factor of 3 C_2H_4:1 N_2 was applied (Patriquin & 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 Tab. 1). Two sets of triplicate controls (10 cm^3) were processed for every station. Sediment was collected from each core liner from 0 – 5 cm, 5 – 10 cm, and from 10 – 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 one week (in total 5 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 for depth profiles was calculated from three replicates per sediment depth and error bars for standard deviation of integrated N_2 fixation were calculated from three integrated rates per station.

2.5 Sulfate reduction rates

One MUC core per station was used for determination of SR activity. 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 - 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 ~12h 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 seconds. 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 m, 144 m, 253 m, 407 m, 770 m, 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 & Bork, 2007, 2011). Reference sequences were obtained using BlastX on the NCBI database. Sequences were submitted to Genbank (Accession numbers: KU302519 - KU302594).

3. Results

3.1 Sediment properties

Although sediment description and porewater sampling was done down to the bottom of the core, the focus here is on sediments from 0 – 20 cm where benthic N_2 fixation was investigated.

Sediments at the shelf station (St.) 1 (70 m) were black between 0 – 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 reached down to a sediment depth of 36 cm in the sediment cores. The sediment on the outer shelf St. 4 (144 m) was dark olive green from 0 – 13 cm and dark grey until 20 cm. At St. 6 (253 m), which was within the OMZ core, sediment appeared dark olive green between 0 – 17 cm and olive green with white patches between 17 – 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 cm-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 *Thioploca* mats were found at St. 8. St. 9 (770 m) was below the OMZ. Sediments were brown to dark olive green with white particles between 0 – 12 cm and appeared 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 sediment cores. The deepest St. (10; 1025 m) had dark olive green sediment from 0 – 20 cm and black patches from 17 – 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 – 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 µM 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 µM and 22 µM at 20 m sediment depth, respectively.

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

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 (~15 wt%) was found at St. 6, whereas the lowest (~2.6 wt%) was detected at the deep St. 10. The average (0 - 20 cm) organic carbon value (Fig. 5) increased from St. 1 to St. 6 (15 ± 1.7 wt%) and decreased from St. 6 to the lowest value at St. 10 (2.4 ± 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 (SR)

For a straightforward comparison of SR rates with benthic N_2 fixation only the sediment depths between 0 – 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 the shallow St. 1, 4, and 6 (70 m, 144 m, 253 m) and lowest at the deep St. 8 – 10 (407 m, 770 m, 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 sites, reaching 248 nmol SO_4^{2-} cm^{-3} d^{-1} at 0 – 1 cm, N_2 fixation was not highest at this station. Only St. 1 had considerable porewater sulfide concentrations and a decrease of SO_4^{2-} concentration with increasing sediment depth, as well as the highest NH_4^+ concentrations throughout the core. At St. 4 (144 m), both N_2 fixation and SR revealed peaks close to the surface. N_2 fixation decreased between 0 – 8 cm and increased below 8 cm. This increase was not observed in SR rates, which were highest in 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 ± 0.5 nmol N_2 cm^{-3} d^{-1} in the surface cm m. Although N_2 fixation and SR had corresponding depth 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 ± 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}). 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 cm 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 ± 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 ± 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 ± 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 ± 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 ± 0.005 N_2 m^{-2} d^{-1} and 0.01 ± 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 Molecular analysis of the *nifH* gene

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 & Turner (2001) (Fig. 6). In Cluster I and Cluster III, three and seven novel clades were detected, respectively. In general, most of the previously unidentified clades belong 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). In this study, detected sequences clustered with sulfate-reducing bacteria, such as *Desulfovibrio vulgaris* (Riederer-Henderson & Wilson, 1970; Muyzer & Stams, 2008) and *Desulfonema limicola* (Fukui et al., 1999). One cluster (OMZ 144 m) was closely related to *Vibrio diazotrophicus* (Guerinot et al., 1982), which has the unique property for a known *Vibrio* species to perform N_2 fixation and which was found previously in the water column of the OMZ off Peru (Löscher et al., 2014). The other organisms with which OMZ sequences clustered belonged to the genera of bacteria using fermentation, 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 & Turner, 2001) from the Pacific Ocean.

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 it's coupling to sulfate reduction (SR). We

confirmed the presence of N_2 fixation in sediments at all sampled stations along the depth transect. This activity was generally 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. This discrepancy indicates that N_2 fixation might be partly coupled to processes other than SR (see *nifH* discussion below). But it should be kept in mind that the N_2 fixation and SR were determined in replicate MUC cores, which had a sampling distance of up to 50 cm, depending on where the core liners were situated in the multiple corer. Nonetheless, it appears that the observed N_2 fixation is not directly fueled by SR activity. We are also aware of potential microbial community shifts driven by the addition of C_2H_2 (Fulweiler et al., 2015). However, a community shift would be expected to cause rather an underestimation of absolute N_2 fixation rates. Further, incubation with acetylene can lead to a potential lack of fixed N; however, to the best of our knowledge this is the standard method used for the determination of N_2 fixation in sediments (Bertics et al., 2013).The more surprising finding 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 4.2).

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 & 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; Steppe & 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 *nifH* gene sequences obtained in our study strongly indicated the genetic capability of sulfate reducers in the Peruvian sediments to conduct N_2 fixation. They clustered with the sulfate-reducing bacteria *Desulfovibrio vulgaris,* which is a confirmed diazotroph (Sisler & ZoBell 1951; Riederer-Henderson & Wilson 1970)**,** as well as *Vibrio diazotrophicus*, which recently clustered with sequences from the Peruvian OMZ water column (Fernandez et al.,

2011; Löscher et al., 2014). Sequences taken from the seasonally hypoxic Eckernförde Bay in the Baltic Sea also clustered with *Desulfovibrio vulgaris* (Bertics et al., 2013), suggesting a major involvement of sulfate-reducing bacteria in N_2 fixation in organic-rich sediments underlying OMZs. Interestingly, we detected several new *nifH* gene clusters in the Peruvian OMZ that have not been identified yet (Fig. 6).

The molecular analysis further indicates that not all of the benthic diazotrophs are known sulfate-reducing organisms. Therefore, a coupling of N_2 fixation also to processes other than SR is possible, which might explain some of the discrepancies between N_2 fixation and SR activity (see above). Other relevant processes may include the usage of reduced carbon compounds as previously suggested for diazotrophic organisms in the water column of the Peruvian OMZ (Dekaezemacker et al., 2013; Löscher et al., 2014).

4.2 Environmental factors potentially 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 quantity and quality

A major driver for microbial processes such as SR and N_2 fixation by potentially heterotrophic organism 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). Thus, organic matter availability appears to be a major factor controlling N_2 fixation at this study site. Low N_2 fixation rates were previously shown to be related to low organic matter content in slope sediments in the Atlantic Ocean (Hartwig & Stanley, 1978). This pattern is supported 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 from N sink to N source was caused by a decrease of organic matter deposition

to the sediments, which was in turn triggered by low primary production in the surface waters.

Besides quantity also the quality of organic matter in sediments is a major factor influencing microbial degradation processes (Westrich & 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). However, an increase of the C/N ratio with depth would suggest highest integrated N_2 fixation rate at the shallowest St. 1 (70 m), which however is not in line with our observation that shows an increase in rate from St. 1 (70) to St. 6 (253 m) (Fig. 5). Similarly, DIC fluxes measured using benthic chambers at the same stations can be used as an indicator for organic matter degradation rates (Dale et al., 2015). The DIC flux did not correlate with integrated N_2 fixation rates, but instead roughly followed the pattern of SR rates along water depth (Fig. 4). 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 mainly represent the long duration of unfrozen storage of the 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 & 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). This co-occurrence of *Marithioploca* and N_2 fixation was puzzling since high concentrations of NH_4^+, could 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. However, we cannot exclude that a partial suppression occurred. Inhibition experiments of N_2 fixation with NH_4^+ have been conducted in several environments with different results. For example, benthic N_2 fixation was measured in the Carmens River estuary (New York) with ambient NH_4^+ concentrations of 2800 µM (Capone, 1988). In general, these studies suggested that the impact of NH_4^+ on N_2 fixation is more complex than previously thought and poorly understood.

One explanation for why diazotrophs still fix N under high NH_4^+ concentrations could be that bacteria try to preserve the intracellular redox state by N_2 fixation functioning as an excess for electrons, particularly with a deficient Calvin–Benson–Bassham pathway, as it was shown for photoheterotrophic non-sulfur purple bacteria (Tichi & Tabita, 2000). Previous studies on benthic environments propose that the organic carbon availability can reduce an inhibition of N_2 fixation by abundant NH_4^+ (Yoch & Whiting, 1986; McGlathery et al., 1998). In the study of Yoch & Whiting (1986), enrichment cultures of *Spartina alterniflora* salt marsh sediment showed different N_2 fixation inhibition stages for different organic matter species. Another explanation could be that microniches, depleted in NH_4^+ exist between the sediment grains, which we were unable to track with the applied porewater extraction techniques (Bertics et al., 2013). Such microniches are found in the form of localized organic matter hot spots (Brandes & Devol, 2002; Bertics & Ziebis, 2010), and could also supply NH_4^+.

4.2.3 Sulfide

Sulfide is a known inhibitor for many biological processes (Reis, et al., 1992; Joye & 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 might explain why N_2 fixation was lower at St. 1 compared to St. 6, despite the higher quality, i.e. lower C/N ratio, of organic matter at this station. Because SR rates were highest at St. 1 (Fig. 4), we exclude direct inhibition on SR, although the effect has generally been

457 reported (Postgate, 1979; McCartney & Oleszkiewicz, 1991). Interactions of sulfide with
458 benthic N_2 fixation have so far not been investigated, and hence we cannot rule out a
459 partial inhibition of N_2 fixation by sulfide.

460 **4.2.4 Oxygen**

461 Dissolved O_2 can have a considerable influence on N_2 fixation due to the O_2 sensitivity of
462 the key enzyme nitrogenase (Postgate, 1998; Dixon & Kahn, 2004). Bioturbating and
463 bioirrigating organisms can transport O_2 much deeper into sediments than molecular
464 diffusion (Orsi et al., 1996; Dale et al., 2011). In coastal waters, the bioturbation and
465 bioirrigation activity of ghost shrimps was found to reduce N_2 fixation when sediments
466 were highly colonized by these animals (Bertics et al., 2010). While bottom water O_2
467 concentrations in the Peruvian OMZ were below the detection limit at St. 1 to 8 (70 m to
468 407 m), thereby mainly excluding benthic macrofauna, O_2 concentrations increased to
469 above 40 µM at St. 10 (1025 m) where a diverse bioturbating and bioirrigating benthic
470 macrofauna community was observed (Mosch et al. 2012). Accordingly, this station
471 revealed some of the lowest N_2 fixation activity. We are, however, unable to decipher
472 whether O_2, low organic matter content, and/or the low C/N ratio was responsible for this
473 low activity.

474 **4.3 Comparison of benthic N_2 fixation in different environments**

475 We compiled a list of N_2 fixation rates from different marine environments to gain an
476 overview of the magnitude of N_2 fixation rates measured in the Peruvian OMZ sediments
477 (Tab. 2). We found that N_2 fixation rates from the Peruvian sediments exceed those
478 reported for open ocean sediments (2800 m) (Howarth et al., 1988), bioturbated coastal
479 lagoon sediment (Bertics et al., 2010) and sediments >200 m water depth (Capone, 1988).
480 The highest integrated N_2 fixation rate determined in our study (0.4 mmol N m^{-2} d^{-1}, St. 6)
481 closely resembles highest rates found in salt marsh surface sediments (0.38 mmol N m^{-2} d^{-1})
482 and Zostera estuarine sediments (0.39 mmol N m^{-2} d^{-1}) (Capone, 1988). Further, our rates
483 were characterized by a similar range of N_2 fixation rates that were previously measured in
484 an organic-rich hypoxic basin in the Baltic Sea (0.08 - 0.22 mmol N m^{-2} d^{-1}, Bertics et al.,
485 2013). Different to the above examples, our N_2 fixation rates were 8.5 times lower
486 compared to shallow (< 1 m) soft-bottom sediment off the Swedish coast (Andersson et al.,
487 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. 6).

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 elevated activity often overlapped with peaks of SR. The molecular analysis of the *nifH* gene confirmed the presence of heterotrophic diazotrophs at all sampling sites. Sequences clustered with sulfate-reducing bacteria, such as *Desulfovibrio vulgaris*, which is a known diazotroph in sediments. In combination, our results suggest that N_2 fixation and SR were coupled to a large extend, but additional coupling to other metabolic pathways cannot be ruled out completely. 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^+ concentrations, 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 contribution

J. G. and T. T. collected samples and designed experiments. J. G. performed nitrogen fixation experiments and T. T. conducted sulfate reduction experiments. S. S. and A. W. D. measured porosity, DIC, organic carbon content and C/N. J. G., T. T., C. R. L. and S. S. analyzed the data. J. G. and C. R. L. performed molecular analysis. J. G. prepared the manuscript with contributions from all co-authors and T. T. supervised the work.

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Figure captions

Fig. 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).

Fig. 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). Information about bottom water O_2 concentrations (BW O_2, µM) is provided at the right margin.

Fig. 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 - 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.

Fig. 4: Integrated nitrogen fixation (mmol N 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 - 20 cm, including dissolved inorganic carbon (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.

Fig. 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 ratio (molar, yellow curve) from 0-20 cm along the depth transect (m). Error bars indicate standard deviation.

Fig. 6: Phylogenetic tree of *nifH* genes based on the analysis of 120 sequences 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.

Tables

Tab. 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 by 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 09	12°18.704’	77°17.790’	144	13.4	bdl
6	MUC 6	January 07	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

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

Benthic Environment	**N-fixation (mmol N m^{-2} d^{-1})**	**Depth of integration (cm)**	**Method**	**Reference**
PERU OMZ	0.08 – 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 sediment	0.03 – 3.4	0 – 1	ARA	Andersson et al., 2014
Coral reef sediment	6.09 (± 5.62)	-	-	Capone 1983
Eelgrass meadow sediment	0.15 – 0.39	0 – 5	ARA	Cole and McGlathery, 2012
Eutrophic estuary	0 – 18	0 – 20	MIMS	Rao and Charette, 2012
Mangrove sediment	0 – 1.21	0 – 1	ARA	Lee and Joye, 2006
Salt marsh surface sediment	0.38 (± 0.41)	-	-	Capone 1983
Subtidal sediment	0.6 – 15.6	0 - 30	MIMS	Fulweiler et al., 2007
Zostera estuarine sediment	0.39	-	-	Capone 1983
OPEN OCEAN				
Atlantic ocean (2800 m)	0.00008	-	-	Howarth et al., 1988
< 200 m sediments	0.02 (± 0.01)	-	-	Capone 1983
Mauritania OMZ	0.05 – 0.24	0 – 20	ARA	Bertics and Treude, unpubl

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Figures

Fig. 1

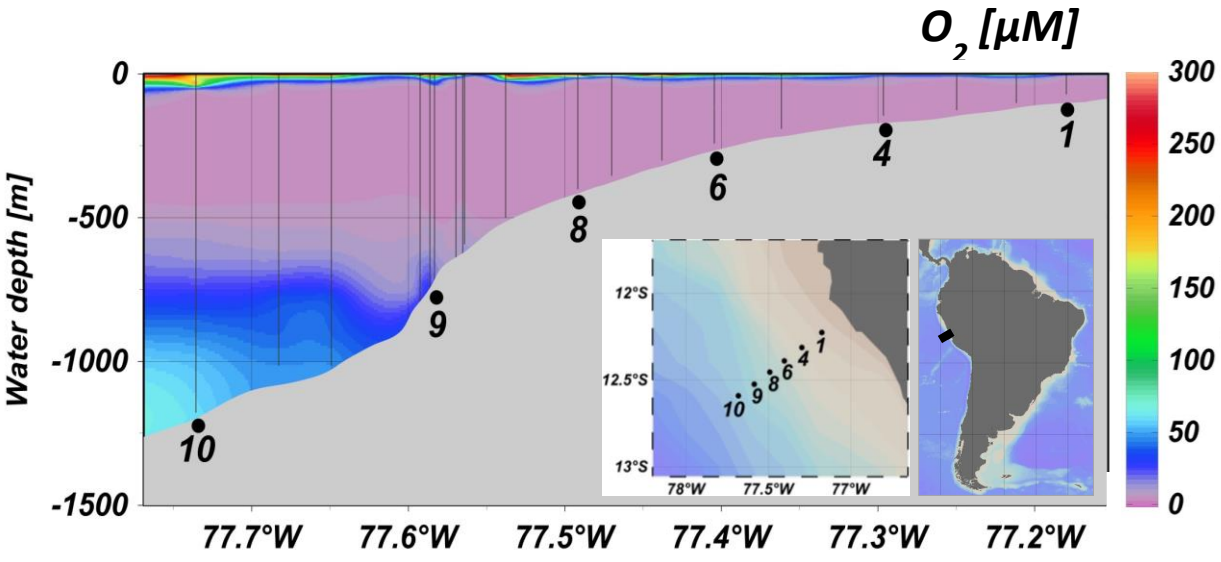


Fig. 2

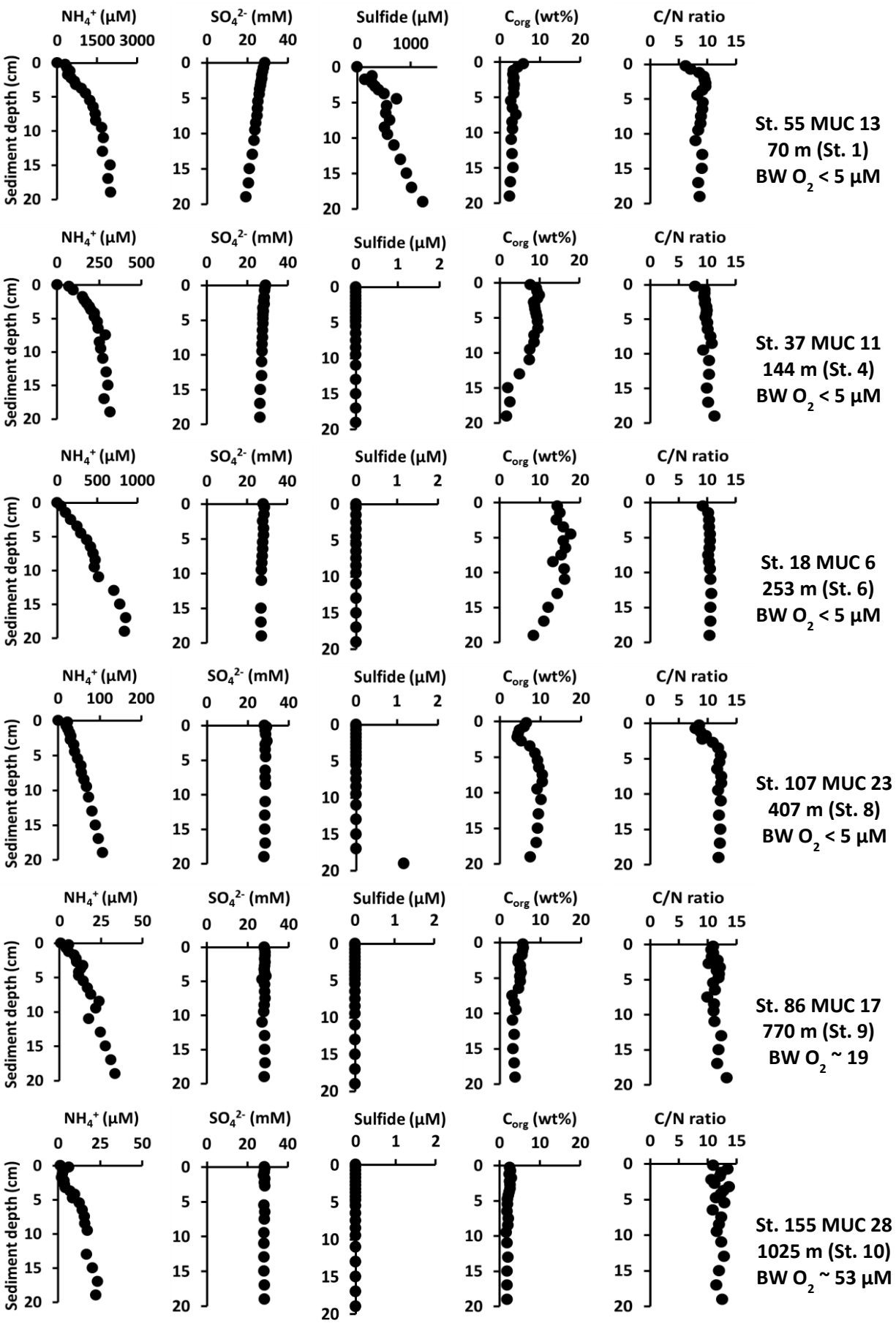

NH4+ (µM)
SO4 2- (mM)
Sulfide (µM)
Corg (wt%)
C/N ratio
Sediment depth (cm)
St. 55 MUC 13
70 m (St. 1)
BW O2 < 5 µM
St. 37 MUC 11
144 m (St. 4)
BW O2 < 5 µM
St. 18 MUC 6
253 m (St. 6)
BW O2 < 5 µM
St. 107 MUC 23
407 m (St. 8)
BW O2 < 5 µM
St. 86 MUC 17
770 m (St. 9)
BW O2 ~ 19
St. 155 MUC 28
1025 m (St. 10)
BW O2 ~ 53 µM

Fig. 3

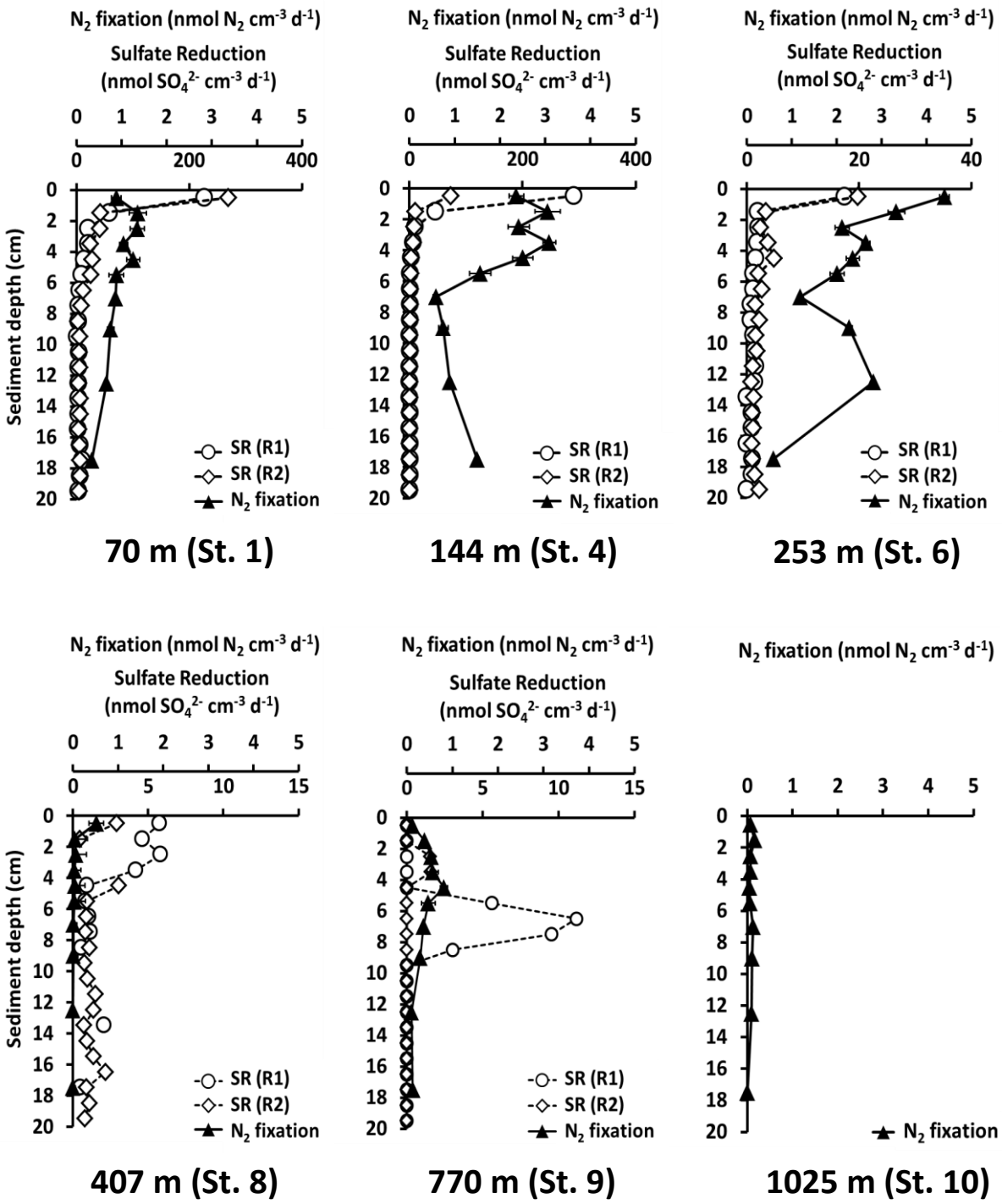

N2 fixation (nmol N2 cm-3 d-1)
Sulfate Reduction (nmol SO4 2- cm-3 d-1)
Sediment depth (cm)
SR (R1)
SR (R2)
N2 fixation
70 m (St. 1)
144 m (St. 4)
253 m (St. 6)
407 m (St. 8)
770 m (St. 9)
1025 m (St. 10)

Fig. 4

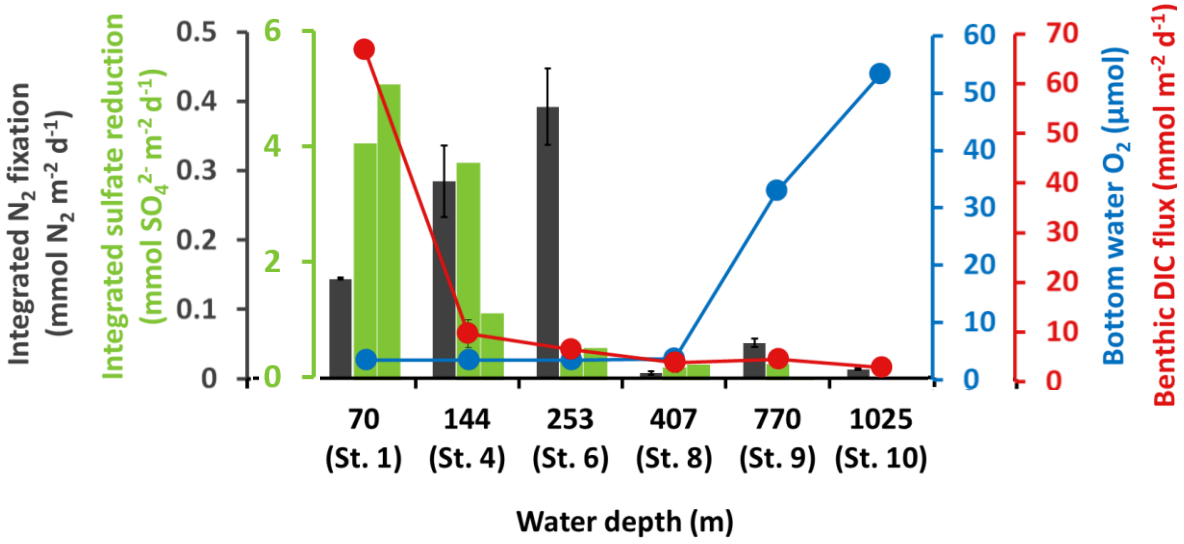
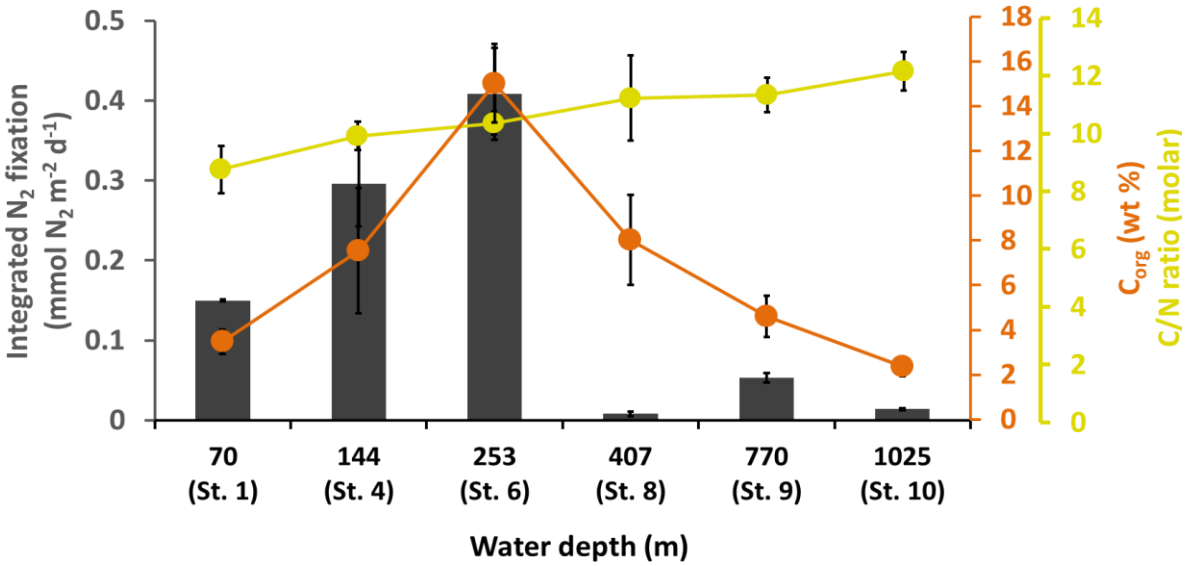

Integrated N_2 fixation (mmol N_2 m^{-2} d^{-1})
Integrated sulfate reduction (mmol SO_4^{2-} m^{-2} d^{-1})
Bottom water O_2 (µmol)
Benthic DIC flux (mmol m^{-2} d^{-1})
0
0.1
0.2
0.3
0.4
0.5
0
2
4
6
0
10
20
30
40
50
60
0
10
20
30
40
50
60
70
70 (St. 1)
144 (St. 4)
253 (St. 6)
407 (St. 8)
770 (St. 9)
1025 (St. 10)
Water depth (m)

Fig. 5


Integrated N_2 fixation (mmol N_2 m^{-2} d^{-1})
0.5
0.4
0.3
0.2
0.1
0
18
16
14
12
10
8
6
4
2
0
14
12
10
8
6
4
2
0
C_{org} (wt %)
C/N ratio (molar)
70 (St. 1)
144 (St. 4)
253 (St. 6)
407 (St. 8)
770 (St. 9)
1025 (St. 10)
Water depth (m)

1020 **Fig. 6**

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