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Benthic nitrogen fixation in oxygen minimum zones

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Hiermit erkläre ich, dass ich die vorliegende Doktorarbeit selbständig und ohne unerlaubte Hilfe erstellt habe. Weder diese noch eine ähnliche Arbeit wurde an einer anderen Abteilung oder Hochschule im Rahmen eines Prüfungsverfahrens vorgelegt, veröffentlicht oder zur Veröffentlichung vorgelegt. Ferner versichere ich, dass die Arbeit unter Einhaltung der Regeln guter wissenschaftlicher Praxis der Deutschen Forschungsgemeinschaft entstanden ist.

Kiel, den..... ……

Jessica Gier

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Diese Arbeit widme ich Vicky Bertics,

danke für alles!

Abstract

Dinitrogen (N_2) fixation is the dominant source for bioavailable nitrogen (N) to the ocean. Only certain prokaryotes (diazotrophs) have the capability to break the triple bonds in N_2 gas and convert it to ammonium. The metabolic processes between N_2 fixation and N removal, (e.g. denitrification), in combination with the other N pathways shape one of the most complex biogeochemical cycles in the marine environment, the N cycle. These pathways between the N sources and sinks define the oceanic N budgets, which seem to be unbalanced, as estimated sources do not equal estimated sinks. Most of the previous research on N_2 fixation focused on the pelagic environment, and knowledge on benthic diazotrophs is limited. The few previous studies on benthic N_2 fixation suggested that this process is more widespread than anticipated, and that it is often coupled to sulfate-reducing bacteria. This hypothesis was further supported by molecular studies. Combining the high abundance of sulfate reducers in the marine environment and their ability to fix N_2, sulfate-reducing bacteria potentially play a key role for introducing new N back into marine sediments. Classically, high sulfate reduction rates are exhibited in oxygen minimum zones (OMZ, < 22 μmol O_2 l^{-1}), making these regions preferential sites to investigate N_2 fixation and a possible coupling to sulfate reduction**.** Previous studies suggested that OMZs are expanding, which could have serious implications for the marine ecosystem. Thus, we need to investigate and understand the effects of expanding OMZs.

In this study, the distribution and magnitude of benthic N_2 fixation in OMZs, and its relevance for the community inhabiting these sediments were investigated. A possible coupling to sulfate reducers was determined by rate measurements of N_2 fixation and sulfate reduction, as well as by molecular analysis in the respective environment. To examine the marine benthic diazotrophic diversity in in several environments, samples from OMZs, methane seeps and brackish water sediments were used for a molecular approach. Investigations showed the following:

1. In sediments of the Peruvian OMZ, N_2 fixation and sulfate reduction occurred throughout the sediment and depth profiles largely overlapped, suggesting a coupling of both processes. This coupling was further supported by the molecular analysis. Detected sequences clustered with known sulfate-reducing diazotrophs; however, a potential coupling to other metabolic processes cannot be ruled out. N_2 fixation was additionally controlled by the organic matter content and sulfide was found to potentially inhibit

diazotrophs. N_2 fixations rates were in the same range as found previously in other organic-rich environments, highlighting the connection between diazotrophs and organic matter to heterotrophic activity.

2. The sediments of the Mauritanian OMZ are characterized by moderate oxygen concentrations, which make them important sites to explore potential effects of anoxia to an ecosystem. N_2 fixation activity often overlapped with sulfate reduction, as well as with ferrous iron concentrations. In addition, the molecular analysis confirmed the presence of sulfate- and iron-reducing bacteria in sediments, what further supports the observation that part of the N_2 fixation could be attributed to these bacteria. Burrowing organisms were found, which potentially create a biogeochemical zonation pattern in sediments that enhanced N_2 fixation in deeper sediment layers. If the Mauritanian OMZ turns anoxic, sediments potentially switch from being a net sink to being a net source of bioavailable N.
3. Further investigations on the diazotrophic diversity in sediments were done in various benthic environments: in the Baltic Sea at Eckernförde Bay and the Gotland basin, in the Atlantic OMZ off Mauritania, in the Arctic off Svalbard, in the Mediterranean Sea at the North Alex Mud Volcano and in the Pacific OMZ off Peru and Chile. Benthic diazotrophs were analyzed by high-throughput sequencing. Results showed a rather small diversity of diazotrophs among the sampling sites. Clusters consisted of potential heterotrophic organisms with a dominance of sulfate-reducing bacteria in all environments. The redundancy analysis model, which was applied to test the correlation between N_2 fixation and environmental parameters, showed a positive correlation between diazotrophs and sulfate reduction, further supporting a link between these processes.

To conclude, the detection of benthic N_2 fixation in OMZs, as well as the diversity study of benthic diazotrophs from different environments, shows that it is a ubiquitous process in the benthic environment. The global distribution of benthic diazotrophs, as revealed by the diversity study, highlights its previously underestimated role in the benthic N cycle, as well as in the marine N budgets. The controlling factors of diazotrophs in marine sediments are: abundance of sulfate reducers; organic matter content in the sediments; sulfide concentration in the porewater, as a potential inhibitor.

Zusammenfassung

Die Fixierung molekularen Stickstoffs (N_2) ist die Hauptquelle für bioverfügbare Stickstoffverbindungen (N) im Ozean. Nur bestimmte Prokaryoten (Diazotrophe) besitzen die Fähigkeit die Dreifachbindung innerhalb des Stickstoffmoleküls zu spalten und dieses in Ammonium umzuwandeln. Stoffwechselprozesse wie N_2-Fixierung und N-Abbau (z.B. durch Denitrifizierung), sowie andere Umwandlungswege von Stickstoffverbindungen bilden einen der komplexesten biogeochemischen Kreisläufe im Meer, den Stickstoffkreislauf. Stickstoffquellen und -senken bestimmen die N-Bilanz im Ozean. Schätzungen zufolge ist diese Bilanz unausgewogen, da ein Missverhältnis zugunsten der N-Senken besteht.

Da sich die bisherige Forschung hauptsächlich auf Stickstofffixierung im Pelagial konzentrierte, ist das Wissen über benthische Diazotrophe begrenzt. Die wenigen bestehenden Studien lassen vermuten, dass benthische Stickstofffixierung häufiger vorkommt als bisher angenommen und nicht selten in Verbindung mit sulfatreduzierenden Bakterien auftritt. Diese Hypothese wurde durch weiterführende molekularbiologische Studien gestützt. Aufgrund der hohen Abundanz von Sulfatreduzierern im marinen Milieu und ihrer Fähigkeit N_2 zu fixieren, spielen diese Bakterien potentiell eine Schlüsselrolle bei der Einführung neuen Stickstoffs in das marine Sediment. Hohe Sulfatreduktionsraten finden sich vor allem in Sauerstoffminimumzonen (SMZ, < 22 µmol O_2 l^{-1}), wodurch diese Regionen besonders geeignet für die Untersuchung von Stickstofffixierung und einer möglicherweise damit in Verbindung stehenden Sulfatreduktion sind. Vorangegangene Studien lassen auf eine Vergrößerung der SMZs schließen, welche erhebliche Veränderungen des marinen Ökosystems nach sich ziehen könnte. Daher müssen die Folgen dieser Ausdehnung entsprechend untersucht und verstanden werden.

In dieser Studie wurde die Verteilung und Ausdehnung benthischer Stickstofffixierung in SMZs und deren Relevanz für die sedimentbewohnenden Lebensgemeinschaften untersucht. Durch die gleichzeitige Bestimmung von Stickstofffixierungs- und Sulfatreduzierungsraten sowie durch molekulare Analysen wurde eine mögliche Verbindung zu Sulfatreduzierern geprüft. Um die Diversität der benthischen Diazotrophen in unterschiedlichen Ökosystemen zu untersuchen, wurden Proben aus SMZs, Methanquellen und aus Brackwasser-Sedimenten für molekulare Analysen verwendet.

Diese Untersuchungen brachten folgende Erkenntnisse:

1. In der peruanischen SMZ traten N_2-Fixierung und Sulfatreduktion im gesamten Sediment auf, wobei sich die Tiefenprofile beider Prozesse überlappten. Es ist demnach zu vermuten, dass eine Verbindung zwischen diesen Vorgängen besteht. Diese Beziehung wurde durch molekulare Analysen bestätigt, in der schon bekannte sulfatreduzierende Stickstofffixierer gehäuft auftraten. Ein Zusammenhang mit weiteren metabolischen Prozessen kann jedoch nicht ausgeschlossen werden. N_2-Fixierung wurde außerdem durch die Menge an organischem Material im Sediment beeinflusst. Die Abundanz der Diazotrophen korrelierte hingegen negativ mit der Sulfidkonzentration. Die hier gemessenen Stickstofffixierungsraten stimmten mit Messungen aus Milieus mit ähnlich hohem Anteil an organischem Material überein. Dies unterstreicht den Zusammenhang zwischen Diazotrophen, organischem Material, sowie heterotropher Aktivität.
2. Die Sedimente der Mauretanischen SMZ zeichnen sich durch moderate Sauerstoffkonzentrationen aus. Dies prädestiniert sie für Untersuchungen bezüglich der potentiellen Auswirkungen von Anoxie auf Ökosysteme. Die Aktivität der Stickstofffixierer überschneidet sich hier häufig mit Sulfatreduktion und der Eisenkonzentration im Porenwasser. Entsprechend wurden Sulfat- und Eisenreduktion den benthischen Diazotrophen zugeschrieben. Molekulare Analysen bestätigten das Vorkommen von sulfat- und eisenreduzierenden Bakterien im Sediment. Des Weiteren konnte gezeigt werden, dass grabende Organismen eventuell eine biogeochemische Zonierung des Sediments erzeugen, welche die N_2-Fixierung in tieferen Sedimentschichten begünstigt. Sollte die Mauretanische SMZ anoxisch werden, würden diese Sedimente möglicherweise von einer N-Abbau Region zu einer Region für N-Quellen werden, welche bioverfügbares N zur Verfügung stellen.
3. Weitere Untersuchungen zur diazotrophischen Diversität wurden in folgenden benthischen Systemen durchgeführt: in der Ostsee (Eckernförder Bucht und Gotlandbecken), in der atlantischen SMZ vor Mauretanien, in der arktischen See vor Spitzbergen, im Mittelmeer um den North Alex Schlammvulkan und in der pazifischen SMZ vor Peru und Chile. Benthische Diazotrophe wurden mittels Hochdurchsatz-Sequenzierung analysiert. Es zeigte sich nur eine geringe Diversität zwischen den Diazotrophen der untersuchten Regionen. Die resultierenden Cluster

bestanden aus möglicherweise heterotrophen Organismen, wobei sulfatreduzierende Bakterien in allen Ökosystem dominierten. Zur Prüfung der Korrelation von N_2-Fixierung und Umweltparametern wurde ein Redundanz-Analyse-Modell genutzt. Dieses konnte eine positive Korrelation von Diazotrophen mit der Reduktion von Sulfat zeigen. Auch dies unterstützt die Hypothese bezüglich einer Kopplung beider Prozesse.

Abschließend kann gesagt werden, dass sowohl die Detektion benthischer N_2-Fixierung in SMZs, als auch die Diversität benthischer Diazotropher in verschiedenen Ökosystemen zeigt, dass N_2-Fixierung ein ubiquitärer Prozess in benthischen Systemen ist. Die in der Diversitätstudie gezeigte globale Verteilung benthischer Diazotrophen unterstreicht ihre bisher unterschätzte Rolle im benthischen Stickstoffkreislauf und in der marinen Stickstoffbilanz. Die Faktoren, die Diazotrophe im marinen Sedimente beeinflussen, sind: die Menge an Sulfatreduzierern, der Anteil an organischem Material im Sediment und die Sulfidkonzentration im Porenwasser als potentiell inhibierender Faktor.

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Chapter 1

General Introduction

1. Introduction

1.1 Fundamentals of the marine nitrogen cycle

The nitrogen (N) cycle possesses a major role in marine biogeochemistry. As one of the most essential elements in the marine environment, N is often limiting the marine productivity and connects to the other biogeochemical cycles, like the carbon, phosphorous, and iron cycles (Howarth et al. , 1988; Gruber, 2008).

Only 6 % of N in the seawater is available for organisms, whereas most of the remaining N exists as dinitrogen gas (N_2) (Gruber, 2008), which is only accessible to certain organisms. These organisms are called diazotrophs and they are able to perform N_2 fixation, which is the reduction of N_2 into ammonium (Capone & Carpenter, 1982; Simpson & Burris 1984; Kim & Rees 1994). In general, the entire sum of bioavailable N forms is called fixed N, which corresponds to only ~0.1% of the total biosphere´s N pool (Vitousek & Howarth, 1991). The main processes that remove fixed N again from the ocean are the anaerobic ammonium oxidation (anammox), which converts ammonium (NH_4^+) with nitrite (NO_2^-) into N_2; and denitrification, which reduces nitrate (NO_3^-) back to N_2 (Gruber & Sarmiento, 1997). The balance between N_2 fixation, denitrification and anammox, in combination with the other N pathways (Figure. 1), defines the oceanic N budget (Gruber & Sarmiento, 1997). Whether these budgets are balanced (Gruber & Galloway, 2008) or unbalanced (Codispoti, 2007) is still under debate (see section 1.3).

Phytoplankton photosynthesis in the upper ocean is the main driver for biogeochemical cycles (Arrigo, 2005). It fixes carbon to organic matter and assimilates further nutrients, such as iron, nitrogen and phosphorous. The produced organic matter is remineralized in the surface ocean; but, some of this organic matter is exported to the deeper ocean, where it is remineralized in the absence of light to inorganic forms, such as NO_3^- and phosphate (Longhurst & Glen Harrison, 1989; Henson et al., 2011). Ocean circulation transports a certain proportion of these inorganic forms back to the upper ocean, where they become available for organisms and are recycled again. This whole process is called the 'biological pump' and describes the coupling between biological and physical transport of organic matter and recycled forms in the water column (Azam, 1998; Longhurst & Glen Harrison, 1989; Henson et al., 2011). The organic matter from the sea surface can also sink through the water column and finally reach the sediment (Knauer & Martin, 1981). The organic

matter flux depends highly on the intensity of primary production in the surface ocean, as well as on the water depth and sinking speed of the particle. While along the continental shelf (0 – 200 m water depth) about 10 – 50% of organic matter is deposited to the sediments, only ~ 1% organic matter reaches the pelagic sediments (~ 6000 m water depth) (Jørgensen, 1983). The sinking of organic matter, in contrast to the convective flux by ocean circulation, is much faster. This is demonstrated in sediments underlying the upwelling region off Peru, where the high primary productivity in the surface result in high organic matter export to the sediments (Pennington et al., 2006; Dale et al., 2015) and consequently an organic matter content between 2 – 7 % dry weight (Degens & Mopper, 1976), while sediments west off the productive current have only 1% dry weight organic carbon content (Degens & Mopper, 1976). Thus, the carbon flux is the major source for N components to the seafloor and these components are either buried deep into the sediment or remineralized (Fenchel & Jørgensen, 1977; Froelich et al., 1979; Jørgensen, 2006). The remineralization of organic components is dependent on the available electron acceptors and in the order of decreasing Gibbs free energy yields (Jørgensen, 1983; Jørgensen & Kasten, 2006) (see section 1.2). The order of electron acceptors in marine sediments can change in oxygen-deficient environments (Canfield, 1989) (see section 1.5). Finally, the degraded compounds are released to the sediment, where they are either utilized by other biogeochemical reactions or transported back to the water column, this interplay is termed ‘benthic-pelagic coupling’ (Graf, 1989; Marcus & Boero, 1998).

Over the recent years, a complex cycle emerged that links the N compounds to the other pathways. In addition, the rapid development of new molecular tools improved our understanding of the microbial community and their connection to the N cycle.

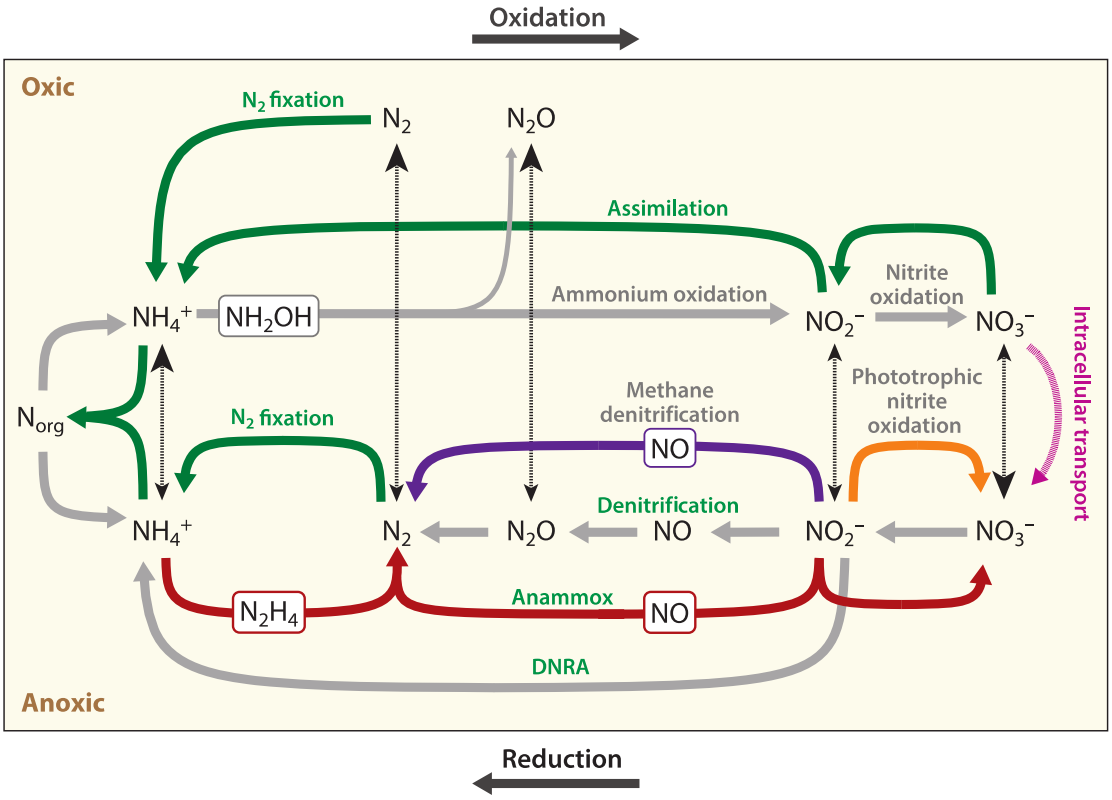


Figure 1: Schematic representation of the N cycle, including major chemical forms and pathways. Assimilation processes are indicated in *green*, while dissimilation processes are *grey* and recently discovered pathways are shown *colored*. Metabolic transformations are represented by thick arrows. The upper part of the figure shows aerobic pathways, while the lower part shows anaerobic pathways. The dashed arrows denote processes that occur between oxic and anoxic environments. The relative size of the arrow heads specifies the dominant direction of transport. Modified from (Thamdrup, 2012).

1.2 Major sources and sinks of nitrogen in the ocean

In the marine environment, N exists in five stable oxidation states: NO_3^- (+V), nitrite (NO_2^-, +III), nitrous oxide (N_2O, +I), N_2 (0), and ammonium (NH_4^+, -III). The complex global N cycle (Figure 1), links all these compounds throughout their different oxidation states (Brandes et al., 2007). Reactions are performed by organisms during the assimilation or dissimilation of N compounds (Gruber, 2008).

In the following, the major biologically mediated reactions are summarized and an overview of the reactions and the corresponding stoichiometry is given (see Table 1).

Table 1: Major biologically mediated processes transforming N in the ocean, stoichiometry, and $C_{106}H_{175}O_{42}N_{16}P$ that indicates the average composition of organic matter in phytoplankton. Stoichiometric ratios of Anderson (1995) are used, modified from Gruber (2008), N_2 fixation equation according to Simpson & Burris (1984).

Process	Stoichiometry
Ammonium assimilation	$106\ CO_2 + 16\ NH_4^+ + HPO_4^{2-} + 48\ H_2O + 14\ OH^- \rightarrow C_{106}H_{175}O_{42}N_{16}P + 118\ O_2$
Nitrate assimilation	$106\ CO_2 + 16\ NO_3^- + HPO_4^{2-} + 78\ H_2O + 18\ H^+ \rightarrow C_{106}H_{175}O_{42}N_{16}P + 150\ O_2$
Ammonification	$C_{106}H_{175}O_{42}N_{16}P + 118\ O_2 \rightarrow 106\ CO_2 + 16\ NH_4^+ + HPO_4^{2-} + 48\ H_2O + 14\ OH^-$
Ammonium oxidation	$2\ NH_4^+ + 3\ O_2 \rightarrow 2\ NO_2^- + 4\ H^+ + 2\ H_2O$
Nitrite oxidation	$2\ NO_2^- + O_2 \rightarrow 2\ NO_3^-$
Denitrification	$C_{106}H_{175}O_{42}N_{16}P + 104\ NO_3^- \rightarrow 106\ CO_2 + 60\ N_2 + H_3PO_4 + 138\ H_2O$
Anammox	$NO_2^- + NH_4^+ \rightarrow 2\ N_2 + 2\ H_2O$
DNRA (benthic)	$NO_3^- + HS^- + H^+ + H_2O \rightarrow SO_4^{2-} + NH_4^+$
DNRA (water)	$NO_3^- + C_2H_3O_2^- + H^+ + H_2O \rightarrow NH_4^+ + 2\ HCO_3^-$
Nitrogen fixation	$N_2 + 8H^+ + 8e^- + 16MgATP \rightarrow 2NH_3 + H_2 + 16MgADP + 16P_i$

The **assimilation of NO_3^- and NH_4^+** is carried out by phytoplankton and describes the conversion of these compounds into organic N biomass (Eppley et al., 1969) (Tab. 1). Whereas NH_4^+ can be incorporated directly into the biomass, NO_3^- has to be converted into NH_4^+ first. The assimilation of NO_3^- requires the reduction of the oxidation state +V to –III and is therefore an energy consuming process; thus, NH_4^+ is the preferred compound for phytoplankton (Zehr & Ward, 2002). Since NO_2^- is an intermediate product during NO_3^- assimilation, phytoplankton that is able to assimilate NO_3^- can also assimilate NO_2^- during NO_3^- assimilation (Wang et al., 2000). In this reaction, the assimilation of NO_3^- and NH_4^+ are strongly linked to carbon fixation because N and carbon compounds are required for the build-up of organic biomass (see average composition of organic matter in Table 1) (Anderson, 1995).

The process of **ammonification** describes the reverse NH_4^+ assimilation, where heterotrophic bacteria release NH_4^+ during the organic matter degradation from particulate organic N (Capone, 1991; Ostrom et al., 2000). In oxygen-rich environments, this reaction can be extended to a two-step process, summarized as **nitrification**. In the first step, NH_4^+ is oxidized by NH_4^+ oxidizers to NO_2^- (ammonium oxidation) and then further to NO_3^- (nitrite oxidation) by chemolithoautotrophic NO_2^- oxidizers (Herbert, 1999). Just recently, the discovery that ammonia-oxidizing archaea contribute to nitrification to a larger extent than

bacteria (Wuchter et al., 2006; Martens-Habbena & Stahl, 2011; Löscher et al., 2012), changed the former view on this process (Jørgensen, 2006).

Denitrification plays a key role in the N cycle, as it removes the fixed N from the environment (Gruber & Sarmiento, 1997). In the ocean, this N pathway is restricted to oxygen-deficient environments, such as the benthic environment and oxygen minimum zone waters (Burdige, 2006) (see section 1.5). Denitrification defines the sequential reduction from NO_3^- to N_2 ($NO_3^- \rightarrow NO_2^- \rightarrow N_2O \rightarrow N_2$) and mostly coupled oxidation of organic matter by bacteria and archaea (Philippot, 2002; Codispoti et al, 2005; Zhang et al., 2009). All steps are performed by different enzymes (Codispoti et al., 2001; Carpenter & Capone, 2008).

New N pathways were discovered in the recent decades, such as the anaerobic NH_4^+ oxidation (**anammox**) in 1999 (Strous et al. 1999). Because this pathway includes the conversion of NH_4^+ and NO_2^- to N_2, this process was, besides denitrification, revealed to be a second sink for bioavailable N (Thamdrup & Dalsgaard, 2002; Kuypers et al., 2003; Brandes et al. 2007). On the contrary to denitrification, anammox is performed by chemoautotrophic bacteria and is mainly occurring in oxygen-deficient environments, where denitrification and nitrification may represent a potential source of NO_2^- for anammox (Kuypers et al., 2003; Kartal et al., 2007; Kalvelage et al., 2011, 2013)**.**

In the global marine N cycle, denitrification and anammox lead to a loss of fixed N from the environment. The recently discovered process of dissimilatory NO_3^- reduction to NH_4^+ (**DNRA**) retains the fixed N in the system by reducing NO_3^- back to NH_4^+ (Kartal et al., 2007). DNRA was found in sediments with strongly reduced conditions and coupled to sulfide (Thamdrup & Dalsgaard, 2008). In the water column, where DNRA was coupled to the oxidation of organc matter, instead of sulfide (Lam et al., 2009; Lam & Kuypers, 2011).

N_2 fixation is the dominant source of bioavailable N in the marine environments (Falkowski et al. 1998; Strous et al. 1999; Brandes & Devol 2002). Biological N_2 fixation is defined as the reduction of N_2 gas to NH_4^+ (Simpson & Burris 1984; Kim & Rees 1994)**.** Diazotrophs have an extremely oxygen sensitive molybdenum-iron-, iron- (Kim & Rees, 1994) or vanadium- (Robsen et al., 1986) enzyme complex, the nitrogenase. The nitrogenase breaks the triple bonds in N_2 gas to form bioavailable N, which involves high metabolic costs of ~ 15 adenosine triphosphate per reduced N_2 (Postgate, 1982; Capone, 1988; Brandes et al.,

2007). The *nifH* gene, which encode for the nitrogenase, is often used for phylogenetic studies among the diazotrophs (Zehr & Paerl, 2008; Sohm et al., 2011). Amazingly, nitrogenase enzymes from various organisms show a remarkable homology, despite large phylogenetic variations of the respective microbes (Ruvkun & Ausubel 1980; Zehr & Paerl 2008). Due to the high oxygen sensitivity of nitrogenases, diazotrophs have developed several strategies to protect the active site of the nitrogenase, such as heterocysts (Jørgensen, 1977; Krekeler et al., 1998; Cypionka, 2000; Wolk et al., 2004).

Generally, diazotrophs are categorized either as free-living, which include e.g. *Desulfovibrio* sp. and *Trichodesmium* sp. (Capone et al. 1997)**,** or as symbiotic diazotrophs (Wagner, 1997). One of the best studied marine diazotrophs is the cyanobacterium *Trichodesmium* sp., (Capone et al., 1997). Recently, N_2 fixation was also found in marine heterotrophic proteobacteria (Fernandez et al., 2011; Farnelid et al., 2013; Löscher et al., 2015), indicating that N_2 fixation is more widespread than previously thought.

1.3 Marine nitrogen budgets

Generally, the global marine N budgets seemed unbalanced, which means that N sources (N_2 fixation) did not equal N sinks (e.g. denitrification) and several N budget estimates exist in the literature (Table 2). For example, the global marine N source side was calculated to be 294 Tg N y^{-1}, while the corresponding global N sink side was at 482 Tg N y^{-1}, which would results in a budget deficit of -188 Tg N yr^{-1} (Codispoti 2001). Gruber (2004) estimated sources to be 265 ± 50 T g N y^{-1} and sinks to be 275 ± 50 T g N y^{-1}, which would result in a roughly balanced global N budget. A recently developed model, which implemented marine geochemical and physical data, revealed an almost balanced N budget with 3 TgN yr^{-1} (Eugster & Gruber, 2012), which clearly argues against the large imbalance that Codispoti et al. (2001) calculated. While Codispoti et al. (2001) estimated the current global N budget; the latest budget calculation refers to the pre-industrial period.

Most studies estimated the oceanic N loss budget higher that the estimate for N input, what suggests that N_2 fixation rates have been underestimated previously (Montoya et al., 1996; Codispoti, 2007). This underestimation is largely owed to a methodological underestimation of N_2 fixation rates in the water column (Großkopf et al., 2012; Löscher et al., 2015), as well as to a restricted knowledge on diazotrophic diversity that has recently been enhanced (Farnelid et al., 2011).

Benthic N_2 fixation has a constant term in the presented N budgets in Table 2. This is due to the fact that all authors considered the same references, i.e. Capone (1988), for the benthic N_2 fixation budget, which primarily considered nearshore environments. This fact highlights that research on benthic N_2 fixation is actually needed, in order to contribute an appropriate term to the global marine N sources side.

Table 2: Global marine N budgets and resulting N deficits of present day (1990) N budgets according to Codispoti et al. (2001), Galloway et al. (2004) Gruber (2004); modified after Gruber (2008).

Process	*Codispoti et al. (2001)*	*Galloway et al. (2004)*	*Gruber (2004)*
		Sources (TG N yr^{-1})	
Pelagic N_2 fixation	117	106	120
Benthic N_2 fixation	15	15	15
River input (DON)	34	18	35
River input (PON)	42	30	45
Atmospheric deposition	86	33	50
Total sources	294	202	265
		Sinks (TG N yr^{-1})	
Organic N export	1	-	1
Benthic denitrification	300	206	180
Water column denitrification	150	116	65
Sediment burial	25	16	25
N_2O loss to atmosphere	6	4	4
Total sinks	482	342	275
Discrepancy	**-188**	**-140**	**-10**

Abbreviations: DON, dissolved organic N; PON, particulate organic N.

1.4 Microbial processes in marine sediments

In marine sediments, the microbial degradation of organic matter is stratified vertically (Figure 2). This stratification is based on the decreasing Gibbs free energy yield per mol organic carbon of the respective oxidant and is called 'redox cascade' (Jørgensen 1983; Iversen & Jørgensen 1985). The redox cascade implies that the major electron acceptor in an oxic environment is oxygen, which yields the most energy in aerobic respiration. After all oxygen is depleted, NO_3^- is the next electron acceptor in the redox cascade and is reduced by denitrification (Burdige, 2006). Denitrification is regarded as a major benthic source for the powerful greenhouse gas N_2O, as it is one of the intermediate reduction compounds and

might be released during denitrification if not further reduced to N_2 (Bange et al., 2010; Naqvi et al., 2010). In the next energy yielding pathways, trace metals (Mn^{2+}, Fe^{2+}) are reduced during manganese and iron reduction, followed by sulfate (SO_4^{2-}) reduction (Froelich et al., 1979; Kasten & Jørgensen, 2000). In most cases, sulfate concentrations in porewater samples result in a concave-down profile. Sulfate reduction is one of the most important anaerobic microbial degradation processes in marine sediments and is performed for the most part by sulfate-reducing bacteria, which take up sulfate and reduce it to sulfide (Figure 2) (Jørgensen 1977; Muyzer & Stams 2008). Once the sulfate pool is depleted, methane (CH_4) accumulates as the end product of anaerobic diagenesis via methanogenesis (Martens & Berner 1974; Jørgensen 1983; Martens et al. 1999). These processes result in a vertical zonation of the sediment, leading to aerobic and anaerobic layers, which may overlap (Jørgensen, 1983).

Porewater chemistry

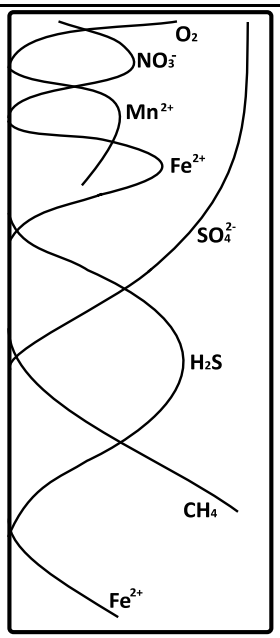
Pathways	Concentration	Stoichiometry	ΔG^0 (kJ mol^{-1})
Oxic/aerobic respiration		$[CH_2O] + O_2 \rightarrow CO_2 + H_2O$	-479
Denitrification		$5[CH_2O] + 4NO_3^- \rightarrow 2N_2 + 4HCO_3^- + CO_2 + 3H_2O$	-453
Manganese reduction		$[CH_2O] + 3CO_2 + H_2O + 2MnO_2 \rightarrow 2Mn^{2+} + 4HCO_3$	-349
Iron reduction		$[CH_2O] + 7CO_2 + 4Fe(OH)^3 \rightarrow 4Fe^{2+} + 8HCO_3 + 3H_2O$	-114
Sulfate reduction		$2[CH_2O] + SO_42^- \rightarrow H_2S + 2HCO_3^-$ $4H^+ + SO_4^{2-} + H^+ \rightarrow 4H_2O$ $CH_3COO^- + SO_4^{2-} + 2H^+ \rightarrow 2CO_2 + HS^- + 2H_2O$	-77 -152 -41
Methanogenesis		$4H_2 + HCO_3^- + H^+ \rightarrow CH_4 + 3H_2O$ $CH_3COO^- + H^+ \rightarrow CH_4 + CO_2$	-136 -28

Figure 2: Biogeochemical zonation of organic matter degradation in marine sediments, modified from Jørgensen & Kasten (2006). Left: Mineralization pathways and porewater concentrations of the related dissolved species, according to Fenchel & Jørgensen, 1977 and Jørgensen, 1983). Right: Stoichiometry and Gibbs free energy (ΔG^0) for each pathway, after Jørgensen & Kasten (2006).

1.5 Nitrogen cycling in marine sediments

The distinction between anaerobic and aerobic processes in the benthic N cycle is not straightforward, as several pathways overlap and the end product of one N pathway is needed to initiate another (Figure 1), e.g. denitrification as NO_2^- source for anammox (Kartal et al., 2007; Lam et al., 2009). Furthermore, NO_2^- is used in five different anaerobic pathways and in one aerobic pathway (Figure 1), thus highlighting the fact that the N cycle is rather a complex network of transformations than a cycle (Thamdrup, 2012).

Especially in the benthic environment, several new discoveries have advanced our understanding of the benthic N cycle in the recent years. For example, foraminifera have been found to perform denitrification (Risgaard-Petersen et al., 2006; Glud et al., 2009). Glock et al., (2013) recently demonstrated the importance of foraminifera in the center of the Peruvian oxygen minimum zone (see section 1.6.4), which contribute up to 50% to benthic denitrification.

In marine sediments, anammox contributed substantially to the N_2 production, with about 24% and 67% to the benthic N pool at two classical continental shelf sites at Skagerrak and Aarhus Bay (Denmark) (Thamdrup & Dalsgaard, 2002). Recently, it was shown that the contribution of anammox to the benthic N pool varies among the environment from <1% to ~80% (Trimmer & Engström, 2011). This finding indicates that the N cycle is strongly dependent on environmental conditions and a general prediction which N process dominates in which environment is difficult to realize.

The discovery of the giant sulfur-oxidizing bacteria of the genera *Thioploca* sp. and *Thiomargarita* sp. (Schulz, 1999) is not so recent anymore; however, they are one of the most fascinating organisms performing DNRA (see section 1.2). These bacteria performed DNRA together with the oxidation of reduced sulfur compounds, thus providing a potential link between the N and the sulfur cycle in marine sediments (Otte et al., 1999). *Thioploca* sp. and *Thiomargarita* sp. occurred in sediments underlying oxygen-deficient environments, for example in sediments of the Peruvian oxygen minimum zone (Schulz, 1999; Levin, 2003). In the Peruvian sediments, microbial mats of the genus *Marithioploca* sp. (Salman et al., 2011) were abundant at water depth between 80 - 400 m (Jørgensen & Gallardo, 1999; Mosch et al., 2012). These bacteria can store up to 500 µM NO_3^- in specialized vacuoles and their filaments can glide several centimeters into the sediment, thereby transporting NO_3^- into the subsurface (Fossing et al., 1995; Schulz & Jørgensen, 2001). These processes could have large

impacts on the benthic N cycle in oxygen minimum zones (Jørgensen & Gallardo, 1999) (see section 1.6.4).

1.6 Benthic N_2 fixation

1.6.1 Exploring N_2 fixation

Methods to measure N_2 fixation in marine sediments are limited and direct measurements are technically not feasible, due to the problem that small changes of N_2 in the large pool cannot be detected (Capone, 1988; Fulweiler et al., 2015). One of the main methods to measure N_2 fixation in sediments is the acetylene reduction assay, which is an *in situ* method to detect nitrogenase activity in a sample, based on the reduction of acetylene to ethylene by the N_2 fixing complex (Capone, 1988). Finally, the nitrogenase activity can be converted to N_2 fixation (Capone, 1983; Capone et al., 2005).

Another N_2 fixation measurement technique is the dissolution method, where $^{15}N_2$ tracer is injected as in water dissolved gas into the sample (Mohr et al., 2010; Großkopf et al., 2012). This method was rather designed for the determination of water column N_2 fixation than for sediments, and it's validity for benthic N_2 fixation remains to be verified.

The incubation of sediment samples with N_2/Argon is another possibility to quantify N_2 fixation, which finally measures the dissolved N_2 and Argon concentration on a quadrupole membrane inlet mass spectrometer that assesses the N_2 change over time (Kana et al., 1994; Eyre et al., 2002). The calculated rates represent a measure of N_2 flux, which means gross denitrification – gross N_2 fixation (Fulweiler & Nixon, 2009; 2012).

Considering the molecular determination of diazotrophs, tools such as the *nifH* gene analysis have been established (Sisler & ZoBell, 1951; Riederer-Henderson & Wilson, 1970; Zehr & Turner, 2001). This has been successfully applied in some benthic environments and the results suggest that sulfate-reducing bacteria, such as *Desulfovibrio*, are responsible for N_2 fixation in these areas (Bertics et al., 2013; Fulweiler et al., 2013). Overall, a critical review of the used methods will be given in chapter 5, section 2.

1.6.2 The potential for sulfate-reducing diazotrophs

While most of the previous research has focused on pelagic N_2 fixation, benthic N_2 fixation gained little attention and knowledge is limited (Bertics et al., 2010; Fulweiler & Nixon, 2011). The occurrence of benthic N_2 fixation has been investigated in recent years, but mostly concentrating on microbial mats and plant rhizospheres (Herbert 1975; Steppe &

Paerl 2002). For example, in productive coastal sea-grass rhizosphere sediments, high rates of N_2 fixation were measured in combination with the sulfate-reducing bacteria *Desulfovibrio sp.* (Herbert, 1975). This discovery suggested that sulfate-reducing bacteria may play a key role in benthic N_2 fixation in marine rhizosphere-systems (Herbert, 1975; Capone 1988).

More recent investigations verified this suggestion, but in other benthic environments. For example, the burrowing ghost shrimp *Neotrypaea californiensis* created a 3-dimensional chemical zonation pattern in coastal sediments, which changed the redox cascade by providing additional electron acceptors into the sediment. Together with the potential removal of NH_4^+ by nitrification, the ghost shrimp may have provided favorable microniches for N_2 fixation (Wenzhöfer & Glud, 2004; Zorn et al., 2006; Bertics et al., 2010). Furthermore, it was shown that bioturbation (Meysman et al., 2006; Kristensen et al., 2012;) activity by the shrimp could lead to enhanced organic matter availability in deeper sediment layers, resulting in high rates of N_2 fixation and sulfate reduction in respective layers (Bertics et al., 2010). Further coupling between N_2 fixation and sulfate reduction 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).

In addition, molecular studies showed that many sulfate-reducing bacteria carry the *nifH* gene (Zehr & Turner, 2001; Muyzer & Stams, 2008; Fulweiler et al., 2013) and actively fix N_2 in culture (Riederer-Henderson & Wilson, 1970). In accordance, activity peaks of benthic N_2 fixation were often found to overlap with peaks of sulfate reduction (Nielsen et al., 2001; Steppe & Paerl, 2002; Bertics & Ziebis, 2010; Bertics et al., 2013), what suggested that sulfate reducers are potentially important supplier of fixed N for the benthic community (Bertics et al., 2010; Sohm et al., 2011; Fulweiler et al., 2013).

Because of the low oxygen concentrations in the water column, upwelling regions (e.g. off Peru) classically exhibit high sulfate reduction rates in the sediment (Canfield, 1989; Fossing, 1990; Brüchert et al., 2003) (section 1.6.4), making these regions an interesting site to investigate N_2 fixation and potentially coupled sulfate reduction.

1.6.3 The role of ammonium as an inhibitor for diazotrophs

Diazotrophs are regarded as being inhibited by high concentrations of bioavailable N compounds, i.e. NH_4^+; (Knapp, 2012 and references therein). Thus, why diazotrophs fix N_2 at high organic N concentrations despite it is a cost intensive process (Capone, 1988), remains unknown. Additionally, if there is an NH_4^+ threshold that finally inactivates benthic N_2 fixation, represents a lack of knowledge (Knapp, 2012). Generally, N_2 fixation is considered to be inhibited or at least suppressed by high fixed N compounds, i.e. NH_4^+ (Capone, 1988; Knapp, 2012; Postgate, 1982). Accordingly, in *Zostera* coastal lagoon sediments, N_2 fixation was reversely correlated to NH_4^+ concentrations (190 - 290 µM) (Welsh et al., 1996). In contrast, 1000 µM NH_4^+ in the porewater of the hypoxic Eckernförde Bay did not fully inhibit N_2 fixation (Bertics et al., 2013) and N_2 fixation was still abundant at 2800 µM ammonium in sediments from an estuary area (Capone, 1988)., suggesting a regulation of benthic diazotrophs by the organic N compound. These findings illustrate the diversity of diazotroph inhibition by NH_4^+. Ultimate sensitivity studies of benthic diazotrophs and regulations by NH_4^+ are still missing.

1.6.4 Diazotrophs in oxygen minimum zones

In general, oxygen minimum zones (OMZ = areas with < 22 µmol O_2 l^{-1} (Wyrtki 1962; Kamykowski & Zentara 1990)) are formed by a combination of processes: upwelling of nutrients; high organic matter availability in the water column; high O_2 demand in the water column; and low water column mixing (Wyrtki, 1962; Kamykowski & Zentara, 1990; Paulmier & Ruiz-Pino, 2009). OMZs are located in the Arabian Sea, at the west coast of South Africa and at the north and south eastern Pacific, with the latter as the largest OMZ (Brandes & Devol, 2002; Levin, 2003; Capone & Knapp, 2007; Karstensen et al., 2008) (Figure 3).

In the todays ocean, the volume of OMZs accounts for 10.3 x 10^6 km^3, what makes up 0.7% of the total ocean volume (Paulmier & Ruiz-Pino, 2009). Previous studies suggested that OMZs are increasing (Diaz & Rosenberg, 1995; Diaz, 2001; Stramma et al., 2008), which can have fatal effects on the ecosystem, as many organisms cannot survive at low oxygen conditions (Rogers, 2000; Weeks et al., 2002; Levin, 2003; Ulloa & Pantoja, 2009). This is why OMZs become an area of great interest and why we need to understand the implications of expanding OMZs on the marine environment.

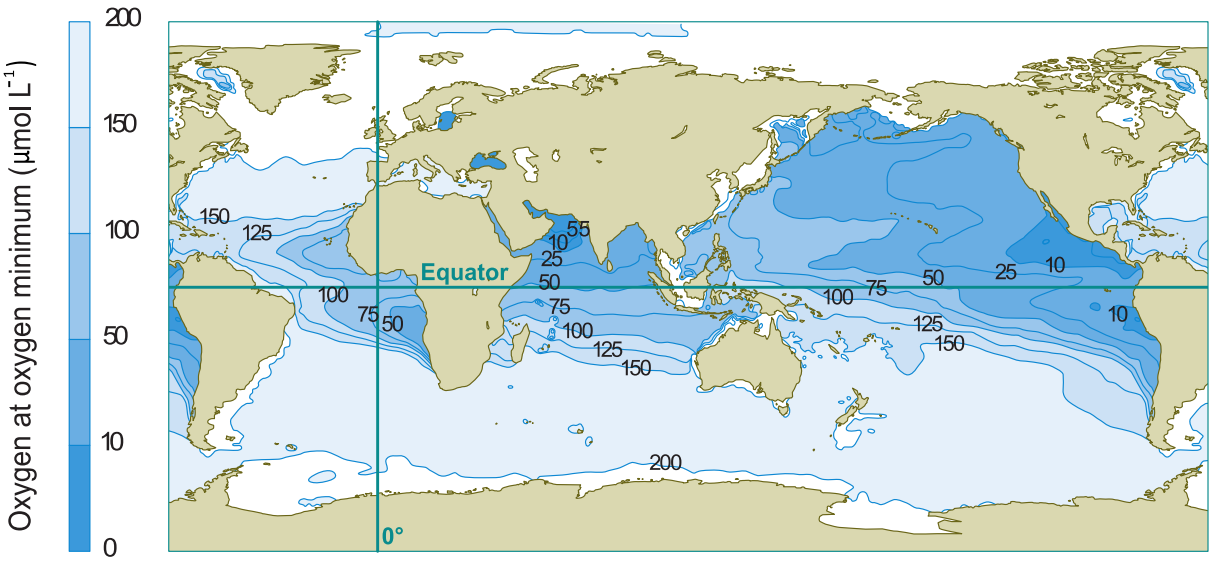


Figure 3: Colors indicate oxygen concentrations at the depth of the minimum oxygen. Modified from Keeling et al. (2010), based on Data from World Ocean Database, 2001 (Conkright et al., 2002).

More than 1 million km² of the benthic area are affected by anoxic conditions (Helly & Levin, 2004). In these sediments, sulfate-reducing bacteria release high amounts of sulfide to the sediments, which are used by sulfide-oxidizing bacteria (*Thioploca* and *Thiomargarita*) (Fossing et al., 1995; Schulz, 1999; Mosch et al., 2012).

In general, OMZs are associated with N loss processes, such as denitrification (Codispoti et al., 2001; Ulloa & Pantoja, 2009; Ward et al., 2009) and anammox (Thamdrup et al., 2006; Lam et al., 2009). However, the Peruvian OMZ was categorized as recycling site for dissolved inorganic N via DNRA at the shelf and as a N sink down slope (Bohlen et al., 2011; Devol, 2015). While several N pathways have been explored already in OMZ sediments, nothing is known about benthic N_2 fixation; still, some studies investigated N_2 fixation in sediments of seasonal hypoxic areas (Bertics et al., 2013).

Because OMZ sediments are colonized by the *Thioploca spp*. (Schulz, 1999; Schulz & Jørgensen, 2001), e.g. off Peru, sediments express high NH_4^+ concentrations, which potentially inhibit benthic diazotrophs (Knapp, 2012). The inhibition of benthic diazotrophs by NH_4^+ is still a topic of much debate and benthic diazotrophs were previously observed to fix N_2 although NH_4^+ was high (Capone, 1988; Bertics et al., 2013). Additionally, the anoxic conditions should favor benthic diazotrophs, because of the oxygen sensitivity of the nitrogenase (Jørgensen, 1977; Krekeler et al., 1998; Cypionka, 2000).

To conclude, combining the anoxic environment and the high benthic sulfate reduction rates in OMZs, with the ability of sulfate reducers to fix N_2, as well as their high abundance in marine sediments, N_2 fixation by sulfate-reducing bacteria could be an important process, introducing new N back into marine sediments underlying OMZs.

2. Objectives

Addressing the previously introduced gaps in knowledge on benthic N_2 fixation, this study had two major objectives.

In the first part of the study, the identification and quantification of benthic N_2 fixation together with potentially coupled heterotrophic processes are investigated. Here, the focus was emphasized on oxygen-deficient sediments, i.e. sediments underlying oxygen minimum zones.
The second part of the study is a molecular approach, which examines the global diversity of the benthic diazotrophic community and possible correlated environmental factors.

For the first objective, two sampling sites were investigated: the oxygen minimum zones off Mauritania and Peru, which experienced moderate oxygen concentrations and fully anoxic conditions, respectively. Laboratory experiments were done, in order to answer the following research questions:

- What are the rates of benthic N_2 fixation within and outside the OMZs?
- Is N_2 fixation linked to other metabolic processes, e.g. sulfate reduction?
- What is the role of benthic N_2 Fixation in the marine N budget
- Are there differences or similarities between the Mauritanian and the Peruvian OMZ regarding N_2 fixation?
- Which implications does an expansion of an OMZ have on benthic diazotrophs?

For the second objective, sediments from seven different locations were used: the Atlantic OMZ, two sites in the Pacific OMZ, the Arctic Ocean, two sites in the Baltic Sea, and a mud volcano in the Mediterranean Sea. Molecular analyses were conducted to answer the following research questions:

- Which diazotrophs are responsible for N_2 fixation in these sediments?

- Do the benthic diazotrophs have a large community diversity?
- Does the benthic community diversity show spatial variation?
- What are the environmental controls of benthic diazotrophic diversity?
- What is the global potential of benthic diazotrophs to contribute new N to the sediments?

3. Thesis outline

In the following 3 chapters the results of my work during my PhD thesis "Benthic nitrogen fixation in oxygen minimum zones" is presented. Each of the chapters is written as a scientific manuscript. While the manuscript in chapter 2 is submitted to a peer-reviewed scientific journal, the manuscripts in chapter 5 and 6 are in preparation for submission. My contribution to each manuscript is described in the following:

Chapter 2: Nitrogen fixation in sediments along a depth transect through the Peruvian oxygen minimum zone

Jessica Gier, Stefan Sommer, Carolin R. Löscher, Andrew W. Dale, Ruth A. Schmitz, and Tina Treude

Published in *Biogeosciences Discussions*

This study was initiated by Tina Treude. Jessica Gier designed the experiments with input from Tina Treude. Jessica Gier carried out sediment sampling and performed the nitrogen fixation experiments. Tina Treude measured sulfate reduction rates. Jessica Gier and Carolin Löscher performed molecular analysis. Porewater measurements were coordinated by Andrew Dale and Stefan Sommer. Jessica Gier wrote the manuscript with contributions from all co-authors.

Chapter 3: Benthic nitrogen fixation through the oxygen minimum zone off Mauritania

Jessica Gier, Andrew W. Dale, Carolin Löscher, Stefan Sommer, and Tina Treude

In preparation for *Frontier in Marine Sciences*

This study was initiated by Tina Treude. Jessica Gier designed the experiments with input from Tina Treude. Jessica Gier carried out sediment sampling and performed the nitrogen fixation experiments. Tina Treude measured sulfate reduction rates. Jessica Gier and Carolin Löscher performed molecular analysis. Porewater measurements were coordinated by Andrew Dale and Stefan Sommer. Andrew Dale performed bioirrigation experiments, modelling and corresponding data analysis. Jessica Gier wrote the manuscript with contributions from all co-authors.

Chapter 4: Novel insights into benthic diazotrophy: Nitrogenase Gene Amplicons from marine sediments reveal a global dominance of sulfate reducers

Jessica Gier, Carolin R. Löscher, Tina Treude

In preparation for *International Society for Microbial Ecology*

This study was initiated by Jessica Gier and Carolin Löscher. Jessica Gier and Tina Treude collected sediment samples. Jessica Gier performed the nitrogen fixation experiments. Tina Treude measured sulfate reduction rates. DNA Extraction was done by Jessica Gier. Carolin Löscher prepared gene libraries, analyzed the sequence data and performed statistical evaluations. Jessica Gier and Carolin Löscher wrote the manuscript with input from Tina Treude.

Beyond the work presented in this thesis I was a co-author of the following manuscripts:

Bertics, V. J., Löscher, C. R., Salonen, I., Dale, A. W., **Gier, J.,** Schmitz, R. A., and Treude, T.: Occurrence of benthic microbial nitrogen fixation coupled to sulfate reduction in the seasonally hypoxic Eckernförde Bay, Baltic Sea, *Biogeosciences*, 10, 1243–1258, doi:10.5194/bg- 10-1243-2013, 2013

Dale, A.W., Sommer, S., Lomnitz, U., Montes, I., Treude, T., Liebetrau, V., **Gier, J.,** Hensen, C., Dengler, M., Stolpovsky, K., Bryant, L. D., and Wallmann, K.: Organic carbon production, mineralisation and preservation on the Peruvian margin, *Biogeosciences*, 12, 1537–1559, doi:10.5194/bg-12-1537-2015, 2015.

Sommer, S., **Gier, J.**, Treude, T., Lomnitz, U., Dengler, M., Cardich, J., and Dale, A.W. (submitted to *Deep-Sea Research Part I*) Depletion of oxygen, nitrate and nitrite in the Peruvian oxygen minimum zone cause an imbalance of benthic nitrogen fluxes.

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Chapter 2

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 of both 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} (L A Codispoti et al., 2001). 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). 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}

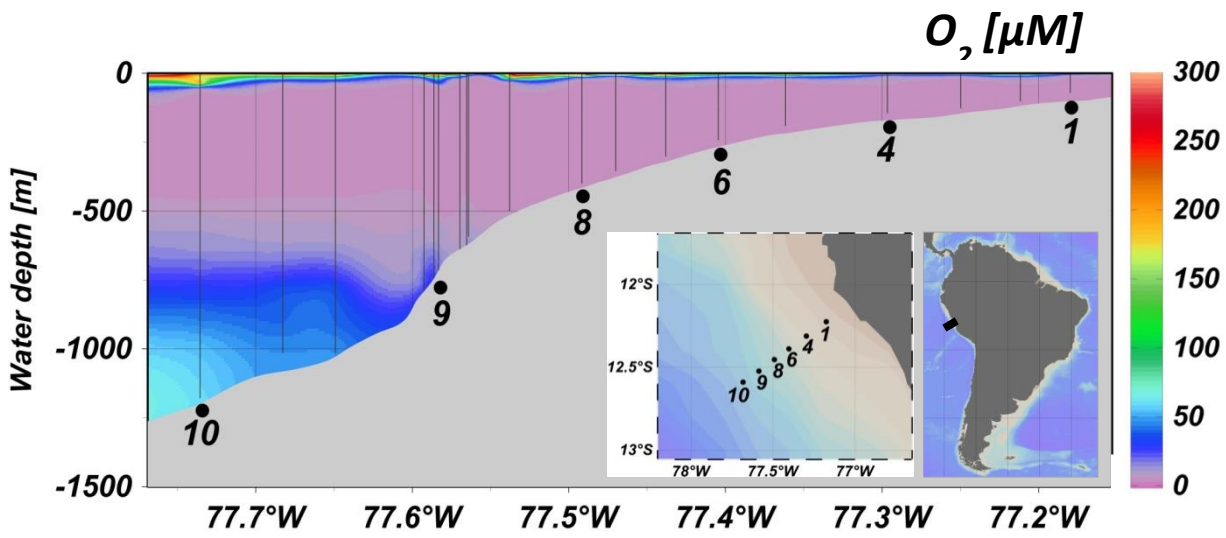

Figure 1: Cross-section of dissolved O_2 concentrations (µM) along the continental margin of the Peruvian OMZ at 12°S. The vertical lines represent CTD cast for O_2 measurement during the cruise M92. Stations 1 to 10 for multicorer (MUC) sampling are indicated by station numbers according to Dale et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

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.

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 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

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.

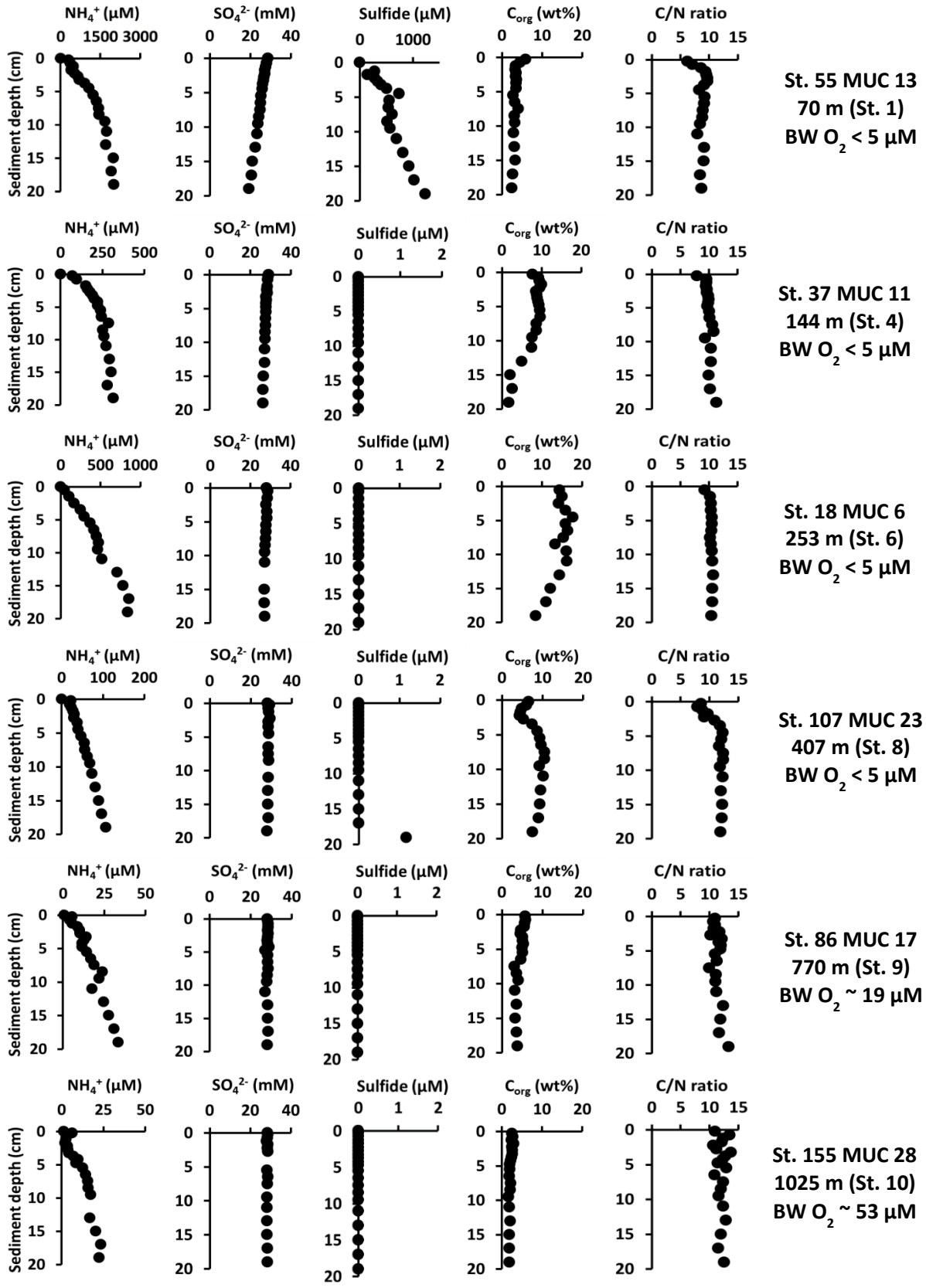

Figure 2: Biogeochemical porewater profiles in MUC cores from sampling stations along the 12°S depth transect. Graphs show NH_4^+ (µM), SO_4^{2-} (mM), sulfide (µM), organic carbon content (C_{org}, wt%) and the C/N ratio (molar). Information about bottom water O_2 concentrations (BW O_2, µM) is provided at the right margin.

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).

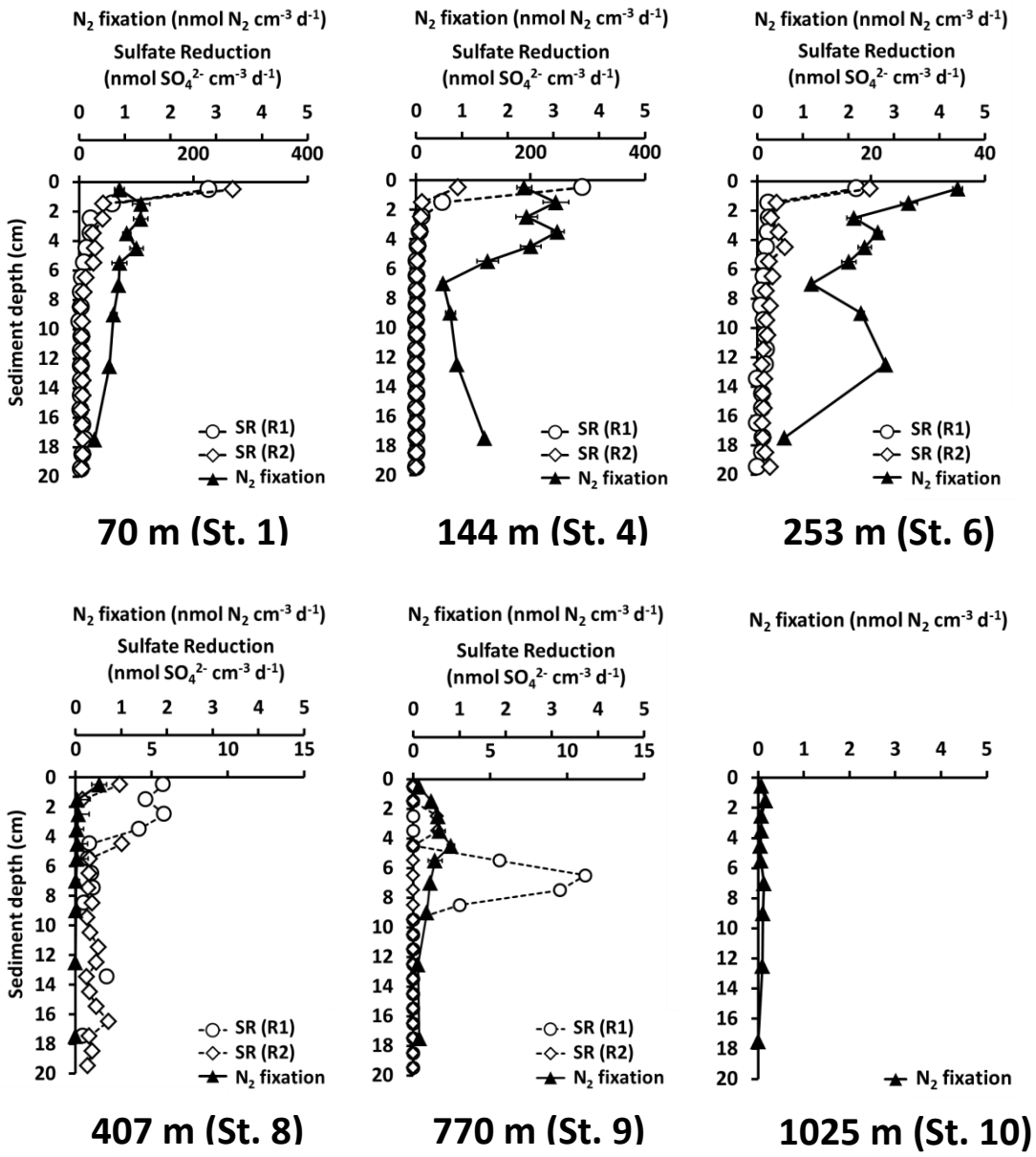

Figure 3: Sediment profiles of N_2 fixation (nmol N_2 cm^{-3} d^{-1}, average of three replicates) and sulfate reduction rates (SR, nmol SO_4^{2-} cm^{-3} d^{-1}, two replicates (R1 and R2)) from 0 - 20 cm at the six stations. The upper x-axis represents the N_2 fixation, while the lower x-axis represents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

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).

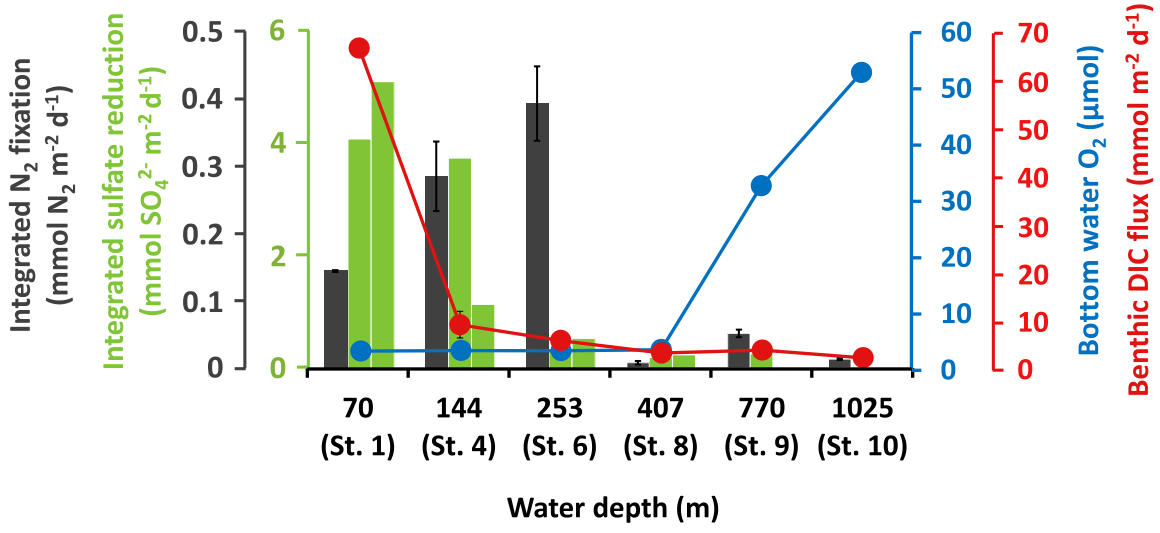

Figure 4: Integrated nitrogen fixation (mmol N m^{-2} d^{-1}, grey bars, average of three replicates) and integrated sulfate reduction (mmol SO_4^{2-} m^{-2} d^{-1}, green bars, two replicates) from 0 - 20 cm, including dissolved inorganic carbon (DIC, mmol m^{-2} d^{-1}, red curve from Dale et al.,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

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.

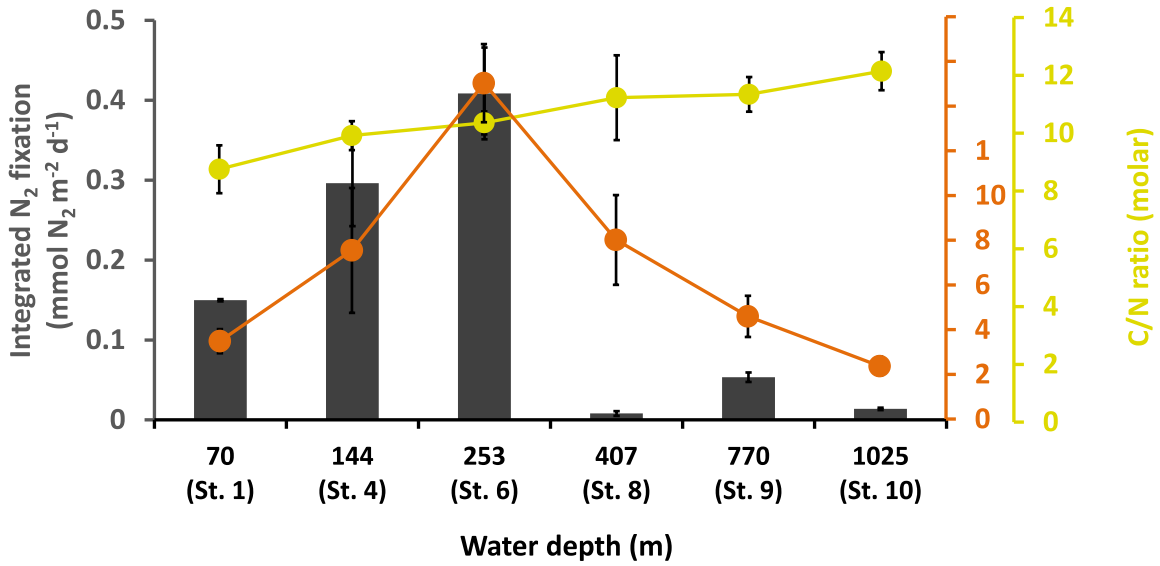

Figure 5: Integrated nitrogen fixation (mmol N_2 m^{-2} d^{-1}, grey bars, average of three replicates), average organic carbon content (C_{org}, wt%, orange curve) and the average C/N ratio (molar, yellow curve) from 0-20 cm along the depth transect (m). Error bars indicate standard deviation.

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 (J P Zehr & Turner, 2001) from the Pacific Ocean.

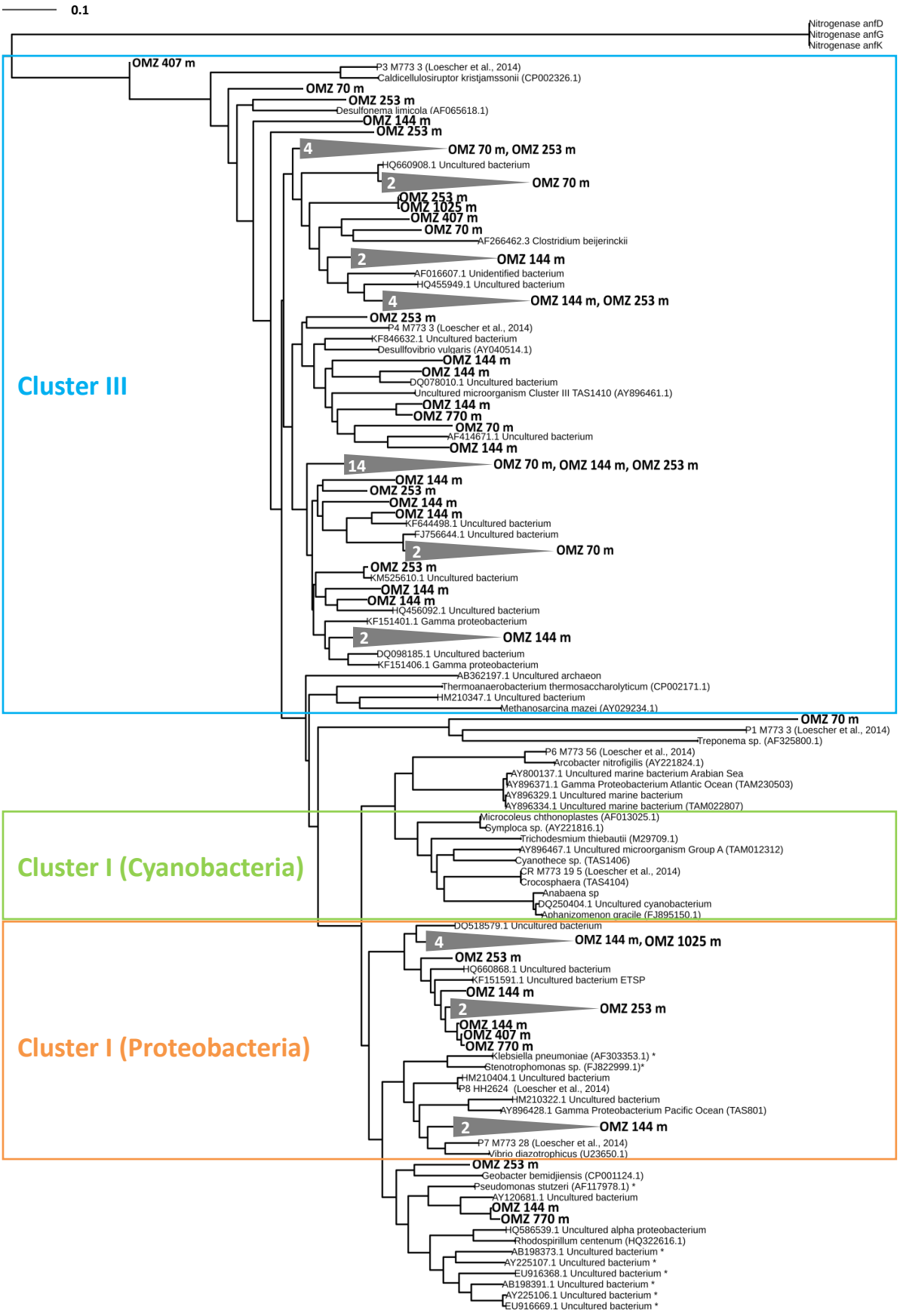

Figure 6: Phylogenetic tree of *nifH* genes based on the analysis of 120 sequences from the six sampling stations between 70 and 1025 m water depth. Novel detected clusters consisting of several

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.

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. 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

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

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 & 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 m 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, this station revealed some of the lowest N_2 fixation activity. We are, however, unable to decipher whether O_2, low organic matter content, and/or the low C/N ratio was responsible for this low activity.

4.3 Comparison of benthic N_2 fixation in different environments

We compiled a list of N_2 fixation rates from different marine environments to gain an overview of the magnitude of N_2 fixation rates measured in the Peruvian OMZ sediments (Tab. 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 (Capone, 1988). The highest integrated N_2 fixation rate determined in our study (0.4 mmol N m^{-2} d^{-1}, St. 6) closely resembles highest rates found in salt marsh surface sediments (0.38 mmol N m^{-2} d^{-1}) and Zostera estuarine sediments (0.39 mmol N 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 m^{-2} d^{-1} , Bertics et al., 2013). Different 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. 6).

Table 2: Integrated rates of nitrogen fixation (mmol 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-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

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.

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Chapter 3

Benthic nitrogen fixation through the oxygen minimum zone off Mauritania

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Abstract

Benthic nitrogen (N_2) fixation and microbial processes were investigated in the Mauritanian oxygen minimum zone (OMZ) at 18°N. Bottom water oxygen concentrations were always above 30 µM at the time of sampling. Sediment samples were retrieved by a multiple corer at six stations along a depth transect between 47 and 1108 m water depth. Benthic N_2 fixation, measured by the acetylene reduction assay, was detected at all sites with highest rates (0.15 ± 0.004 mmol m^{-2} d^{-1}) at the shelf stations (47 and 90 m) and lowest rates (0.08 ± 0.002 mmol m^{-2} d^{-1}) below 412 m water depth. The geochemical data suggest that part of the N_2 fixation could be attributed to sulfate- and iron-reducing bacteria. This observation was further verified by molecular analysis of the gene *nifH* that encodes for the nitrogenase enzyme required for N_2 fixation. Detected *nifH* sequences clustered with sulfate-reducing bacteria, and benthic N_2 fixation activity generally overlapped with sulfate reduction activity. Integrated rates of both processes were highest at 90 m and lowest at 412 m. Bioirrigation activity, measured using an inert tracer, showed high rates at the shelf stations and low rates in deeper waters. The burrowing macrofauna potentially altered the biogeochemical zonation in sediments and introduced organic matter, as well as oxygen deeper into the sediment, thereby providing favorable microniches for N_2 fixation in these layers. N_2 fixation rates in OMZ sediments were low to rates measured in the Peruvian OMZ. Overall, this study may potentially give a hint on how benthic N_2 fixation is likely to change when marine environments are entering anoxic conditions.

1. Introduction

Dinitrogen (N_2) fixation is the dominant source of bioavailable nitrogen (N) to the marine environment (Falkowski et al., 1998; Strous et al., 1999; Brandes & Devol, 2002). Only N_2 fixing prokaryotes (diazotrophs) have the enzymatic capability to convert N_2 to bioavailable N, i.e. ammonium, build it into their biomass, and make it available for non-diazotrophic organisms (Ward & Bronk, 2001; Gruber, 2008). Diazotrophs can be detected using molecular tools such as the *nifH* gene; the key functional marker encoding a subunit of the nitrogenase reductase enzyme, which has been done successfully in the marine environment (Sisler & ZoBell, 1951; Riederer-Henderson & Wilson, 1970; Zehr & Turner, 2001).

So far, most studies on marine N_2 fixation have focused on pelagic environments (e.g., Zehr & Ward, 2002; Galloway et al., 2004; Riemann et al., 2010; Löscher et al., 2014 and

references therein). Relatively little is known about benthic N_2 fixation (Fulweiler et al., 2007; Bertics et al., 2010; Bertics et al. 2013; Gier et al., 2015) and the controlling environmental factors are unclear. One of the major drivers for benthic microbial processes is the availability of organic matter (Jørgensen, 1983; Howarth et al., 1988; Fulweiler et al., 2007; Bertics et al., 2013). Accordingly, benthic N_2 fixation and organic matter were found to correlate in different habitats, such as sediments within the high-productive Peruvian upwelling region (Gier et al., 2015) and coastal sediments inhabited by the bioturbating ghost shrimp *Neotrypaea californiensis* (V. J. Bertics et al., 2010).

Other studies have shown that the physical movement of animals through surface sediments can enhance N_2 fixation. Bioturbation and bioirrigation in sediments increase the rate of organic matter supply to subsurface sediment layers, leading to elevated microbial metabolic rates there (Aller & Aller, 1986; Bertics et al., 2010, 2012). While bioturbation describes the sediment mixing by benthic organisms, bioirrigation encompasses the exchange of seawater with sediment porewater due to the pumping action of burrow-dwelling organisms (Meysman et al., 2006; Kristensen et al., 2012). These processes were associated with increased rates of microbial sulfate reduction (Victoria J. Bertics & Ziebis, 2010) and N_2 fixation (Victoria J Bertics, Sohm, Magnabosco, & Ziebis, 2012). In fact, N_2 fixation was often coupled to sulfate reduction in organic-rich sediments (V. J. Bertics et al., 2013; Victoria J. Bertics & Ziebis, 2010; Gier et al., 2015)

Several studies confirmed that many sulfate reducers carry the *nifH* gene (Zehr & Turner, 2001; Muyzer & Stams, 2008; Fulweiler et al., 2013, Gier et al., 2015) and actively fix N_2 in culture (Riederer-Henderson & Wilson, 1970). In accordance, profile peaks of N_2 fixation are often found to overlap with peaks of sulfate reduction (Nielsen et al., 2001; Steppe & Paerl, 2002; Bertics & Ziebis, 2010; Bertics et al., 2013; Gier et al., 2015). In summary these observations make sulfate reducers a potential important supplier of bioavailable N for the benthic community (Bertics et al., 2010; Sohm et al., 2011; Fulweiler et al., 2013).

In the present study we focused on the highly productive upwelling region off Mauritania, in order to determine the effect of organic matter availability and bioirrigation on benthic N_2 fixation. The region is characterized by a weak oxygen minimum zone (OMZ) with dissolved O_2 concentrations above 30 µM (Karstensen et al., 2008; Dale et al., 2014). The OMZ is

predicted to lose more O_2 in the future at the rate of ca. 0.5 µM y^{-1} (Keeling, Körtzinger, & Gruber, 2010; Lothar Stramma, Johnson, Sprintall, & Mohrholz, 2008). Worldwide, OMZs are expected to increase (Diaz & Rosenberg, 1995; Diaz, 2001; Stramma et al., 2008), which will influence about 1 million km² of seafloor (Helly & Levin, 2004).

As for most continental margins, Mauritanian OMZ sediments are a net sink for dissolved inorganic N due to denitrification (Dale et al., 2014). N loss decreases with increasing water depth, in line with particulate organic carbon flux to the seafloor. The relevance of benthic N_2 fixation for N cycling in the Mauritanian OMZ sediments remains, however, unknown. A variety of bottom dwelling macrofauna was observed in the region, indicating extensive bioirrigation and bioturbation activity (Dale et al., 2014). It is reasonable to suggest, based on the studies listed above, that subsurface microbial activities could be stimulated here, including sulfate reduction and thus N_2 fixation (V. J. Bertics et al., 2013; R W Fulweiler et al., 2007; Gier et al., 2015; B B Jørgensen, 1983). Due to the potential expansion of the OMZ, together with high input of labile organic matter to the seafloor, the Mauritanian OMZ is a key region to understand how benthic N_2 fixation will change under anoxic conditions.

We postulate that a coupling exists between N_2 fixation and sulfate reduction in Mauritanian OMZ sediments, which is stimulated by enhanced benthic organic matter availability due to high carbon export and benthic bioirrigation. The overall goal of the present study was to (1) correlate benthic N_2 fixation and sulfate reduction rates (and potential other heterotrophic processes such as nitrate and iron reduction) along the Mauritanian margin, (2) identify benthic diazotrophs, and (3) investigate the effect of bioirrigation on N_2 fixation and sulfate reduction. Finally, we will compare benthic N_2 fixation in the weak OMZ off Mauritania with benthic N_2 fixation in the anoxic Peruvian OMZ to better understand how marine N cycling may change as O_2 levels diminish.

2. Materials and Methods

2.1 Study area

The region off Mauritania belongs to the extensive eastern tropical North Atlantic upwelling system, which represents a moderate OMZ with lowest O_2 concentrations of ~40 µM (Chavez & Messié, 2009; Karstensen et al., 2008). The upwelling system ranges between 43°N at the Iberian peninsula and 10°N south off Dakar (Schafstall, Dengler, Brandt, & Bange,

2010). While upwelling is present between 20°N and 25°N year-round, upwelling north and south of this region is seasonal, induced by variations in wind forcing related to the migration of the Intertropical Convergence Zone (Barton et al., 1998). Along the continental slope highly nonlinear internal waves, that export fine-grained sediment particles down slope, were observed (Schafstall et al., 2010). The upwelling intensity at 18°N (this study) is strongest between December and April (boreal winter). The 18°N area (50 – 1100 m water depth) features a perennial high primary production (80 – 200 mmol C m^{-2} d^{-1}) (Huntsman & Barber, 1977), probably enhanced by the iron-rich dust input from the Sahara (Baker, Jickells, Witt, & Linge, 2006), making the eastern tropical North Atlantic to one of the most productive marine environments (Carr, 2001).

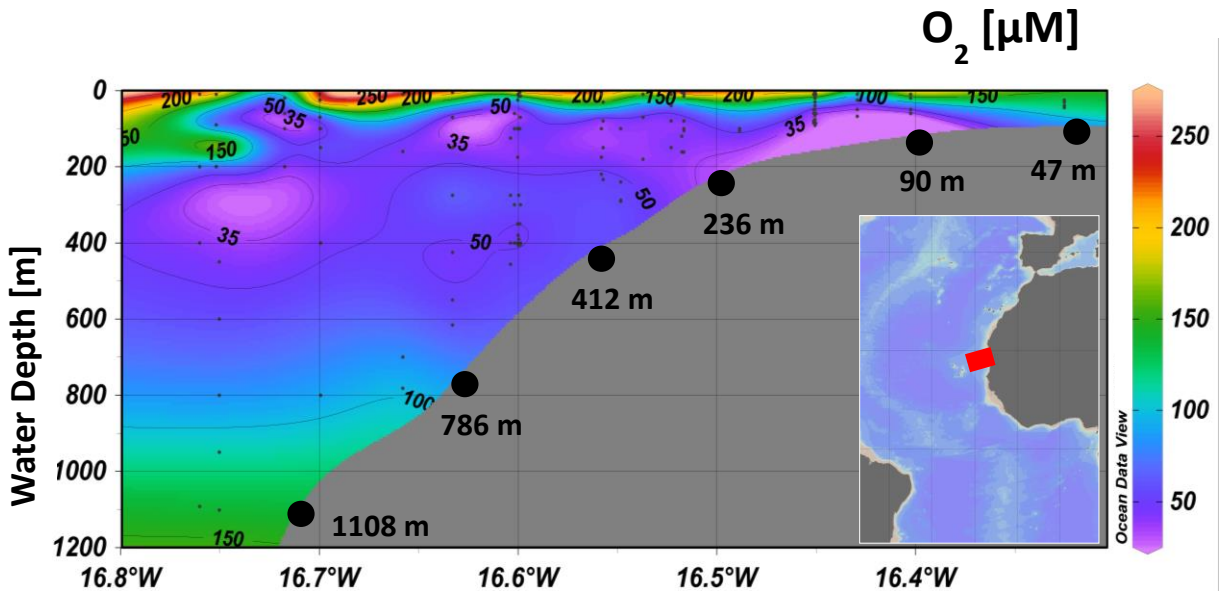

Fig. 1. Contour plot of dissolved O_2 (µM) along the continental margin of the Mauritanian OMZ at 18°N. The black dots represent stations for MUC sampling with corresponding water depth in meters. The inset shows the sampling area in a regional context (red box).

Sediments at 18°N are characterized by an increase of surface particulate organic carbon (0.6 wt% on the shelf and 2.7 wt% at 800 m) with water depth and a decrease of particulate organic carbon with sediment depth (Dale et al., 2014). While the shelf of the 18°N upwelling region is characterized by minor sediment accumulation rates, sedimentation rates between 0.1 and 0.35 cm yr^{-1} were found at the deeper stations. Dale et al. (2014) described the 18°N sediment as muddy sand down to 400 m water depth and as slightly sandy mud from 786 m. Surface porosity was low (0.56–0.62) at the shallow sites (< 100 m) and high (0.83–0.85) at

deeper sites (> 786 m), with grain size calculations (Sokoll, 2013) that indicated permeable sediments down to 400 m water depth (Dale et al., 2014). Permeable sandy sediments were originally considered as biogeochemically inert due to their low organic carbon content (Shum & Sundby, 1996; Boudreau et al., 2001). The discovery that pressure driven advective solute transport and bottom currents interacting with sediment topography (Markus Huettel & Rusch, 2000; Janssen, Huettel, & Witte, 2005; Rusch & Huettel, 2000) supply fresh organic material and biogeochemical important substances to the sediment, has changed this view (Thibodeaux & Boyle, 1987; Huettel & Gust, 1992; Huettel et al., 1996; Huettel & Webster, 2001 and references therein). Sands are now often regarded as potential sites for high metabolic activity (B.P. Boudreau & Westrich, 1984; M Huettel, Roy, Precht, & Ehrenhauss, 2003).

Tab. 1: Sampling stations along the depth transect at 18°N off Mauritania, including station and core ID, sampling date, coordinates and water depth as recorded by the ship´s winch. Bottom water temperature and dissolved O_2 concentrations were measured by the CTD membrane O_2 sensor.

Station ID	Core ID	Date (2015)	Latitude (N)	Longitude (W)	Depth (m)	Temp. (°C)	O_2 (µM)
658	MUC 13	June 23	18°17.299'	16°18.994'	47	19	123
628	MUC 10	June 21	18°15.197'	16°27.002	90	15	30
612	MUC 8	June 20	18°12.945'	16°33.153'	236	14	50
554	MUC 5	June 12	18°12.504'	16°35.583'	412	11	48
534	MUC 3	June 10	18°11.288'	16°39.328'	786	7	98
524	MUC 1	June 09	18°09.991'	16°45.023'	1108	6	138

2.2 Water column and sediment sampling

Sampling was conducted in June 2014 at six stations (47, 90, 236, 412, 786, and 1108 m) along the continental margin off Mauritania at 18°N (Fig. 1) during an expedition on RV Meteor (M107). Dissolved O_2 concentrations in the water column were obtained using a SeaBird CTD rosette system equipped with a Seabird SBE43 membrane O_2 sensor. The sensors were calibrated by Winkler titration with a detection limit of 2 µmol L^{-1}.

Sediment samples for biogeochemical investigations were taken by a TV-guided multiple corer (MUC) equipped with seven core liners. Each core liner had a length of 60 cm and an

inner diameter of 10 cm. The core ID, sampling date and location, water depth, temperature and O_2 concentration for the six stations are listed in Table 1. All sediment cores were immediately transferred to a cold room (12°C) for further processing.

2.3 Geochemical analyses and bioirrigation determination

Measurements for porewater properties and geochemical data analysis are described in detail by Dale et al. (2011; 2015). In short, one replicate core from each MUC sampling was subsampled at anoxic conditions using an argon-filled glove bag to preserve redox sensitive constituents. Concentrations of ammonium, nitrate, ferrous iron, and sulfide were investigated on a Hitachi U2800 UV/VIS spectrophotometer using standard photometric procedures (Grasshoff, Kremlingl, et al., 1999). Sediment porosity, particulate organic carbon and nitrogen were determined on a second replicate core as described by Dale et al. (2014).

A third core was used for bioirrigation experiments. Cores were taken at six sampling sites (47, 90, 169, 412, 786, and 1108 m water depth). Note that the 169 m site does not match with the 236 m N_2 fixation site. Bioirrigation experiments were performed following former procedures (Dale et al., 2013), involving the use of bromide (Br^-) as a dissolved conservative tracer. Cores were incubated for several days, after which the Br^- depth distribution was determined in extracted porewater samples by ion chromatography (Metrohm 761). These data were used to calculate bioirrigation rates using a numerical model that considered Br- transport due to diffusion and advection. The flux due to irrigation was calculated as, where the bromide concentration is in mol l^{-1}:

$$\frac{\partial \varphi \mathrm{Br}^-}{\partial t} = \alpha_{bi} \varphi (\mathrm{Br}_{\mathrm{olw}} - \mathrm{Br}^-) \qquad (1)$$

In this equation, α_{bi} (d^{-1}) is the depth–dependent bioirrigation coefficient describing solute pumping through animal burrows and Br_{olw} (M) is the time–dependent Br^- concentration in the well mixed overlying water. The sediment porosity, φ, was defined using a depth–dependent function (Dale et al., 2013).

The depth–dependence of α_{bi} was described using:

$$\alpha_{bi} = \alpha_{bi1} \frac{\exp(\alpha_{bi2} - z)}{1 + \exp(\alpha_{bi2} - z)} \quad (2)$$

where α_{bi1} (d^{-1}) is approximately equal to the bioirrigation coefficient at the sediment surface and α_{bi2} (cm) is a parameter that controls the irrigation depth. Details of the model are described fully by Dale et al. (2013). The depth integrated irrigation flux, J_{bio} (mmol cm^{-2} d^{-1}) over the upper 30 cm was used for making inter-site comparison, equal to:

$$J_{bio} = \alpha_{bi} \varphi \mathrm{Br}_{\mathrm{olw}} \quad (3)$$

where , Br_{olw} is the concentration at the start of the incubation. J_{bio} were normalized to the fluxes at the deepest site.

Several burrowing species perform bioirrigation and bioturbation simultaneously and may also transport organic matter particle from the water column into the sediment by the ventilation of their burrows (Christensen et al., 2000; Griffen et al., 2004; Quintana et al., 2007; Kristensen et al., 2012). In this study, bioirrigation was used as an indication for bioturbation (Meysman et al., 2006; Kristensen et al., 2012).

2.4 Benthic nitrogenase activity

The sampling procedure and core slicing details for N_2 fixation have previously been described by Gier et al. (2015). In short, at each of the six stations one MUC core was sliced in the cool room (12 °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. In order to quantify the nitrogenase activity, the acetylene reduction assay was applied (D G. Capone, 1993; W. D. P. Stewart, Fitzgerald, & Burris, 1967). Serum vials (60 mL) were flushed with N_2 filled with 10 cm^3 sediment (in triplicate) from each depth horizon, flushed again with N_2 and crimp sealed with a butyl stopper. Samples were injected with 5 mL C_2H_2 and stored in the dark at an average *in situ* temperature (12 °C, see Tab. 1). Two sets of triplicate controls were prepared for every station. One set of controls was not injected with C_2H_2, to test for natural C_2H_4 production. The second set of controls was killed with 1 mL formalin (37.5%) to test for abiotic C_2H_4 production.

The increase of C_2H_4 in each sample was assayed on board over one week (5 time points) by a gas chromatograph. To finally convert from nitrogenase activity to N_2 fixation, the

conversion factor of $3C_2H_4$: $1N_2$ (Patriquin & Knowles, 1972; Orcutt et al., 2001; Capone et al., 2005; Bertics et al., 2013) was applied. Acetylene reduction is termed N_2 fixation in the following. Standard deviations were calculated from three replicates per sediment depth. For integrated N_2 fixation rates, standard deviations were calculated from the three integrated rates per station.

2.5 Sulfate reduction rates

To determine sulfate reduction rates at each of the six stations (Table 1), one push core (length 30 cm, inner diameter 2.6 cm) was taken from one of the MUC cores. Six µl of the carrier-free $^{35}SO_4^{2-}$ radio tracer (dissolved in water, 150 kBq, specific activity 37 TBq $mmol^{-1}$) were injected in 1-cm intervals according to the whole-core injection method (Jørgensen, 1978). Push cores varied in length between 21 and 25 cm. Each push core was incubated in the dark at 12 °C for ~12h. The incubation was stopped by slicing each push core in 1-cm intervals and transferring the sediment into 50 mL plastic centrifuge tubes filled with 20 mL zinc acetate (20% w/w). The controls (in triplicate) were fixed with zinc acetate (20% w/w) before adding the radiotracer. Samples were stored frozen at -20°C (Røy et al., 2014) until further processing in the home laboratory. Sulfate reduction rates were determined using the cold chromium distillation procedure according to Kallmeyer et al. (2004).

2.6 *nifH* gene analysis

Samples for *nifH* gene analysis were collected from the N_2 fixation MUC cores. Sediment (~5 mL) from each sampling depth (except 0 – 1 cm for 47 m and 10 - 15 cm for 786 m) was transferred to plastic whirl-paks® (*Nasco,* Fort Atkinson, USA), frozen at -20 °C and transported back to the home laboratory. To extract DNA, the FastDNA® SPIN Kit for Soil (MP Biomedicals, Carlsbad, CA, USA) was used according to the manufactures instructions, except that the sample homogenization that was done in a Mini-Beadbeater™ (Biospec Products, Bartlesville, USA) for 15 seconds.

Overall, 60 samples were used for *nifH* amplicon sequencing. Nested polymerase chain reactions (PCRs) for *nifH* were performed following established protocols (J P Zehr & Turner, 2001). Modifications of the protocol adjusted for Illumina sequencing preparation have previously been described by Bentzon-Tilia et al. (2015). Illumina indices were added to amplicons in the second PCR round. In addition to the *nifH*1 and *nifH*2 primer sequences, the

primer contained a linker sequence, an 8-base barcode and the Illumina specific region P5 (forward primer) or P7 (reverse primer). Negative controls consisted of the reaction mixture of the addition of DNA. PCRs were performed in triplicate for each sample. Triplicates were then pooled, and purified using the MinElute Gel Extraction Kit (Qiagen, Hildesheim, Germany) and quantified on a spectrophotometer (Nanodrop 1000, Thermo Fisher Scientific, Waltham, MA, USA). Samples were pooled in equimolar ratios and sequencing took place on an Illumina MiSeq Instrument using the MiSeq reagent Kit with V3 chemistry (Illumina, San Diego, CA, USA). Sequences were submitted to a NCBI Sequence Read Archive (Sequence submission in progress).

Sequences were assembled using MOTHUR software version 1.32.1 (Kozich, Westcott, Baxter, Highlander, & Schloss, 2013). Contigs containing ambiguous bases or homopolymers longer than eight bases were removed from the dataset. Redundant sequences were clustered using the command *unique.seqs* and aligned against the functional gene pipeline and repository database (http://fungene.cme.msu.edu/). Sequences not aligning with the seed *nifH* sequence pool were removed. Chimeric sequences were removed with the MOTHUR implemented software UCHIME (Edgar, Haas, Clemente, Quince, & Knight, 2011). Remaining sequences were clustered at 97% nucleotide similarity and reference sequences for the ten most abundant clusters were obtained using BLAST search on the NCBI database. Amplicons and reference *nifH* sequences were consecutively ClustalW aligned using MEGA version 6.0 (Tamura, Stecher, Peterson, Filipski, & Kumar, 2013), and a maximum likelihood tree was constructed and visualized using iTOL (Letunic & Bork, 2011).

3. Results

Data are reported for the 0-20 cm depth section, for which N_2 fixation rates were determined. To determine the total flux due to bioirrigation, the bioirrigation was depth-integrated over 0-30 cm.

3.1 Water column and sediment characteristics and geochemistry

At the 18°N transect, dissolved O_2 was present in the bottom water across the entire transect (Fig. 1). Bottom water O_2 concentration on the shelf station at 47 m was 123 µM and decreased to 30 µM at 90 m, representing the lowest measured concentration along the transect. At 236 m and 412 m, the O_2 concentration was 48 µM and 50 µM, respectively, and increased from 98 µM at 786 m to 138 µM at 1108 m.

The following sediment descriptions are based on recovered MUC cores. Sediment at 47 m was olive-green sand with white foraminiferal ooze throughout the core. Polychaete tubes protruded from the sediment and living shrimps, probably *Mysid* spp. were present in the bottom water. The sediment at 90 m was olive-green sand with clamshell debris. A large red shrimp (ca. 7 cm) was found alive at the surface of the sediment in the recovered MUC and black spots were visible in sediments at 6 – 11 cm depth. Sediment at 236 m were olive-green clay and sand, with an uneven sediment surface in the core. Large polychaete tubes, burrows and white foraminiferal ooze were found throughout the core. At 412 m the sediment consisted of olive-green clay with patches of fine sand throughout the core and black spots at 7.5 cm. The sediment surface in the MUC cores was uneven, indicating bioturbation. Polychaete tubes were visible and white phosphorite nodules with a diameter of 0.5 cm were found. At 786 m the sediment was olive-green soft clay. Black spots were visible at 12–19 cm. Small polychaete tubes, as well as white foraminiferal ooze was found at 0–3 cm sediment depth. The sediment at the deepest 1108 m site was olive-green soft clay with small polychaete tubes at the surface and a polychaete within a burrow at 8–10 cm sediment depth.

Fig. 2 shows the geochemical porewater profiles of ammonium, nitrate, sulfide, organic carbon content (C_{org}), and the C/N ratio in the upper 20 cm at each station. In general, the profiles are very similar to those measured along the same transect in spring 2011 (Dale et al., 2011). Ammonium concentrations increased with sediment depth, with highest concentrations (111 µM) at 1108 m. The lowest ammonium concentration (30 µM) at 20 cm sediment depth of all cores was measured at 236 m. Concentrations of nitrate were highest at the sediment surface (0-1 cm) in all cores, with the highest nitrate concentration (34 µ) at 786 m, and rapidly decreased to zero at around 2 cm. At the 236 m site, the nitrate concentration did not exceed 1 µM throughout the core. At site 1108 m, nitrate was depleted by 2 cm sediment depth. The accumulation of sulfide was detected only at the two shelf stations (47 and 90 m) with peaks of 88 µM at 14 cm and 45 µM at 13 cm, respectively. Sulfide began to accumulate below ca. 10 cm at these sites, with near-zero concentrations closer to the sediment surface. Organic carbon content was at ~1 wt% throughout the cores from 47 m to 412 m and highest surface organic carbon (~3 wt%) at 786 m and 1108 m. The lowest surface value (~0.5 wt%) was measured at 90 m. Benthic C/N ratio scattered between

13 at the surface and 8 at the bottom of the core at the shallowest station (47 m) and remained relatively constant (~9 - 10) throughout the cores at the stations 90 m to 1108 m.

Sediment porosity (data not shown) at the surface was low (0.52) at the shelf stations (47 and 90 m), increased with water depth, and was highest (0.86) at the deepest 1108 m station. Porosity gradually decreased with sediment depth, reaching a porosity of 0.45 at 20 cm sediment depth at 47 m and 0.74 at the deep 1108 m site.

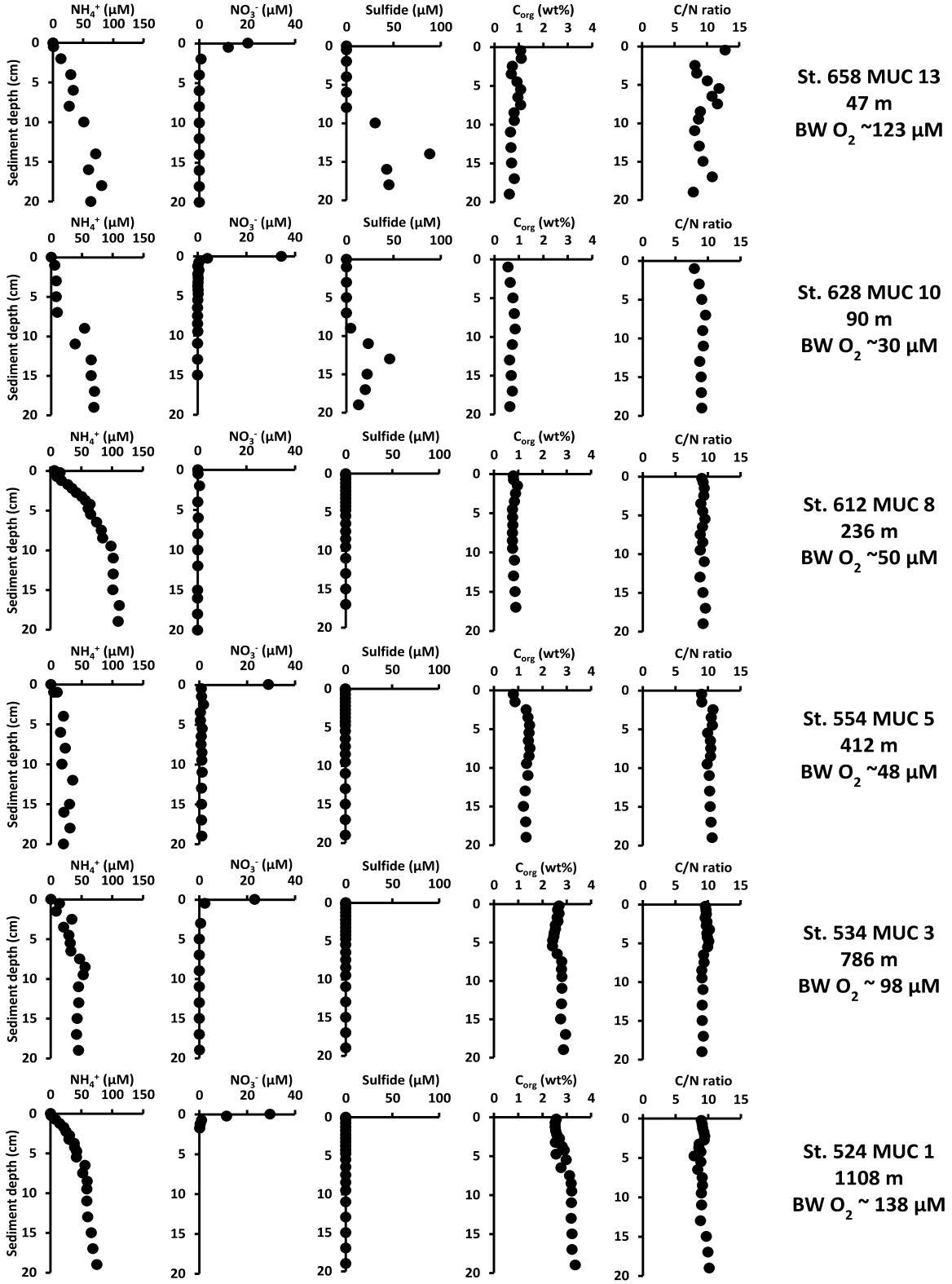

Fig. 2: Biogeochemical porewater profiles in sediments cores from sampling stations along the 18°N transect. Graphs show ammonium (NH_4^+, µM), nitrate (NO_3^-, µM), sulfide (µM), organic carbon content (C_{org}, wt%) and the C/N ratio (molar). Bottom water O_2 concentrations (BW O_2, µM) is provided at the right margin.

Bioirrigation was detected at all sites, with higher fluxes at the shelf stations (47, 90, and 169 m) versus lower ones at the deep sites (412, 786, and 1108 m) (Fig. 3a). At 47 m the highest bioirrigation coefficient (α_{bi1} = >0.5 d^{-1}) was measured along with a high bioirrigation depth parameter (α_{bi2} = 11.4 cm). The lowest bioirrigation coefficient (0.16 d^{-1}), as well as the lowest depth parameter (0.2 cm), were measured at 1108 m. Normalized irrigation fluxes (Fig. 3b) show that the flux at 47 m is almost 40 times greater than that at the deepest site, coincident with high bottom water O_2 (123 µM) (Fig. 1 and 5) and low integrated organic carbon content (0.8 wt%, Fig. 2 and 5).

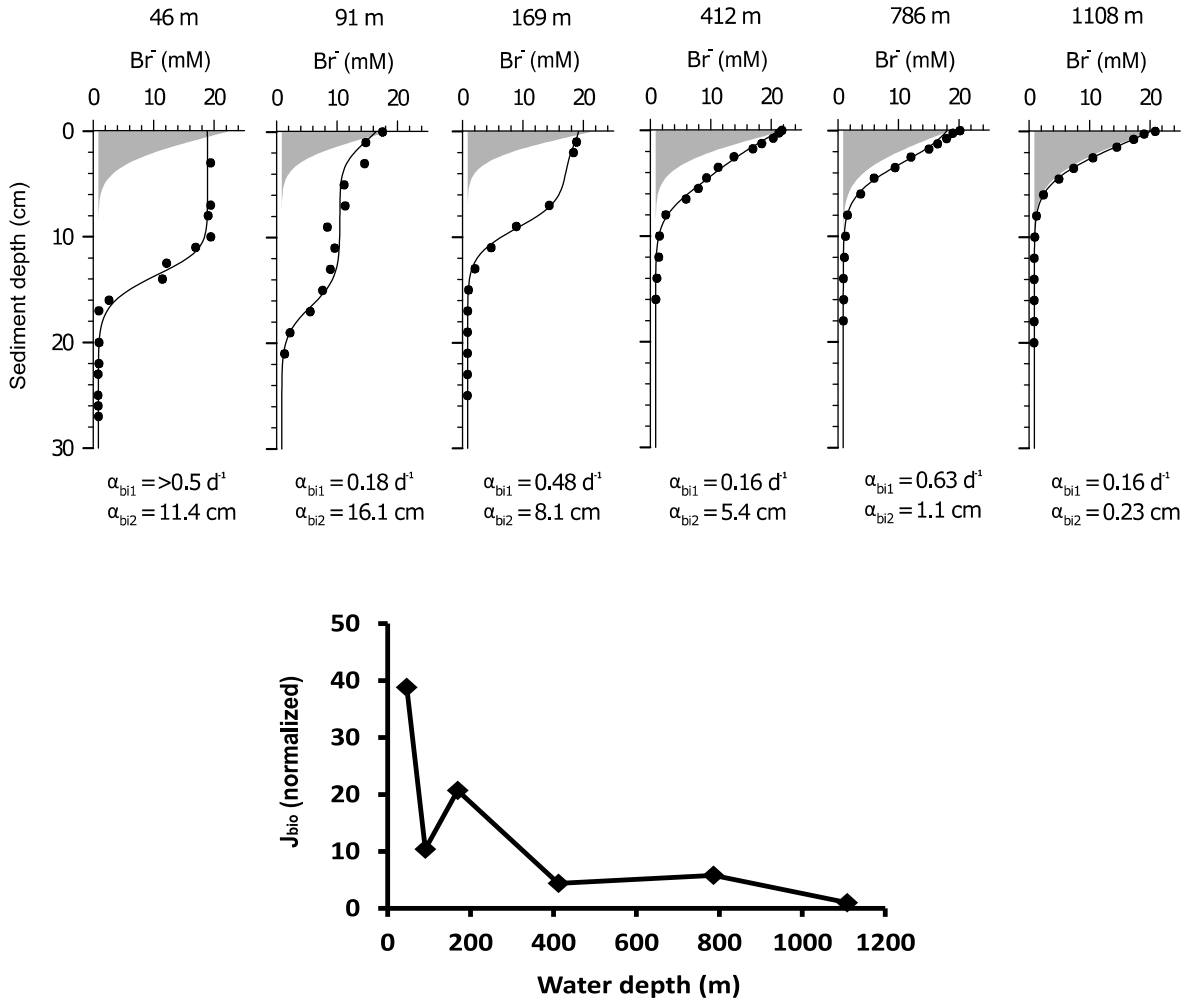

Fig. 3: (a) Sediment depth profiles of measured (symbols) and modeled (curves) bromide (Br^-) concentrations at the end of the bioirrigation experiments along the depth transect (47, 90, 169, 412, 786, and 1108 m). The grey area indicates the transport of Br^- as expected by molecular diffusion only. The coefficients α_{bi1} (d^{-1}) and α_{bi2} (cm) represent the bioirrigation coefficient at the sediment surface and the parameter that controls the bioirrigation depth, respectively. (b) Bioirrigation flux along the depth transect (m) normalized to the deepest site.

3.2 Benthic N_2 fixation and potentially related metabolic processes

Benthic N_2 fixation was measured at all sampling sites and in all sediment depth (Fig. 4). The N_2 fixation rates will be summarized briefly, before detail on N_2 fixation and potentially coupled other heterotrophic processes will follow. In general, N_2 fixation had low activities at the sediment surface, increased in deeper layers and decreased to the bottom of the core. The highest surface N_2 fixation was measured at the three shallow sites (47-236 m, between 0.52 ± 0.09 - 0.57 ± 0.03 nmol N_2 cm^{-3} d^{-1}) , while the lowest surface activity was measured at the three deep sites (412 - 1108 m, between 0.1 ± 0.04 - 0.25 ± 0.02 nmol N_2 cm^{-3} d^{-1}). Peaks of N_2 fixation were at 4-5 cm in 47 m and at 3-4 cm at 90 m, 236 m, and 412 m. Station 786 m had a peak between 1-2 cm, while station 1108 m peaked between 5-6 cm sediment depth. The highest N_2 fixation rate of all stations was measured at 90 m (1.11 ± 0.03 nmol N_2 cm^{-3} d^{-1}), while the overall lowest rate (0.1 ± 0.04 nmol N_2 cm^{-3} d^{-1}) was measured at 1108 m at the surface.

We hypothesized that benthic N_2 Fixation in the Mauritanian sediments is coupled to organoclastic sulfate reduction. But since organic matter is respired using other electron acceptors (O_2, nitrate, ferric iron, manganese), we will investigate a potential coupling to other heterotrophic processes. In the following, the vertical distribution of N_2 fixation will be compared with profiles of nitrate, ferrous iron, and sulfate reduction.

Nitrate, the electron acceptor for denitrification, was depleted within the top 2 cm in most cores, except at 236 m where nitrate was already depleted in the first cm (Fig. 2). The highest nitrate concentration (35 µM) was measured at 786 m. N_2 fixation had low activities in the sediments surface, while nitrate showed a peak in the similar depth (Fig. 4). At 786 m, nitrate concentration (34 µ, 0–1 cm) and N_2 fixation showed opposing depth profiles in the sediment core. At this station the organic carbon content was highest throughout the core (2.7-2.9 wt% at 0 and 20 cm).

The ferrous iron porewater profiles, indicative for iron reduction, showed peaks between 0 and 10 cm at all stations, except at 412 m where ferrous iron increased with sediment depth (Fig. 4). At 47 m and 90 m ferrous iron profiles had concentration peaks at 4 and 3 cm (22 and 17 µM), which were slightly below the N_2 fixation peaks (5-6 and 3-4 cm). At 236 m ferrous iron followed the N_2 fixation depth profile with a peak (31 µM) at 4 – 8 cm, which

overlaps with high N_2 fixation rates (3-4 cm, 0.85 ± 0.05 nmol N_2 cm^{-3} d^{-1}). At 412 m N2 fixation did not overlap with ferrous iron, as activities peaked at 3-4 cm and 19 cm sediment depth, respectively. This site had the highest ferrous iron concentration (49 µM) of all stations. At the 786 m sites, ferrous iron concentrations peaked (30 µM) at 1-6 cm, which overlapped with a peak in N_2 fixation (0.57 ± 0.02 nmol N_2 cm^{-3} d^{-1}) at 2-8 cm. This site had high carbon contents throughout the core (2.7 - 2.9 wt%). Additionally, an overlap of both activities was detected at the 1108 m site. Ferrous iron peaked (27 µM) at 3-9 cm, which overlapped with an activity peak of N_2 fixation (0.66 ± 0.05 nmol N_2 cm^{-3} d^{-1}) at 4 – 5 cm.

Sulfate reduction rates and N_2 fixation were high at the shallow sites (46, 90, and 236 m), and low at the deep sites (412, 768, and 1108 m). At most stations, N_2 fixation and sulfate reduction rates were low at the top and at the bottom of the cores, with N_2 fixation peaks between 3 and 8 cm and sulfate reduction peaks between 9 and 14 cm (Fig. 4).

At 47 m, N_2 fixation and sulfate reduction showed non-conforming profiles in the sediment surface, but aligned towards the bottom of the core. A matching peak (0.71 ± 0.11 nmol N_2 cm^{-3} d^{-1}, 19.3 nmol SO_4^{2-} cm^{-3} d^{-1}) at 12-14 cm was observed. This site had the highest (88 µM, 15cm) sulfide concentration. At 90 m, N_2 fixation did not overlap with sulfate reduction activity. N_2 fixation peaked (1.1 ± 0.03 nmol N_2 cm^{-3} d^{-1}) at 3-4 cm, while sulfate reduction peaked (37 - 43 nmol SO_4^{2-} cm^{-3} d^{-1}) at 7-12 cm. This site had a sulfide peak (46 µM) at 13 cm sediment depth. At the 236 m site, N_2 fixation and sulfate reduction rates showed a corresponding peak (0.85 ± 0.05 nmol N_2 cm^{-3} d^{-1}, 12 nmol SO_4^{2-} cm^{-3} d^{-1}) at 3-4 cm. At 412 m, N_2 fixation and sulfate reduction were low in the surface and had activity peaks at 3 – 4 cm (0.68 ± 0.07 nmol N_2 cm^{-3} d^{-1}, 17.5 nmol SO_4^{2-} cm^{-3} d^{-1}a) at 10-11 cm, respectively. Thus no overlap in activities was observed at this site. Station 786 m had an unusually high sulfate reduction rate (295 nmol SO_4^{2-} cm^{-3} d^{-1}, 13 - 14 cm), which was not detected in N_2 fixation. At this site, the organic carbon content was high throughout the core (2.7 - 2.9 wt% at 0 and 20 cm). At 1108 m, N_2 fixation and sulfate reduction had corresponding depth profiles from the surface down to 8 cm. While sulfate reduction had a second, higher activity peak at 9 – 10 cm (70 nmol SO_4^{2-} cm^{-3} d^{-1}), N_2 fixation decreased below the peak at 6 cm continually.

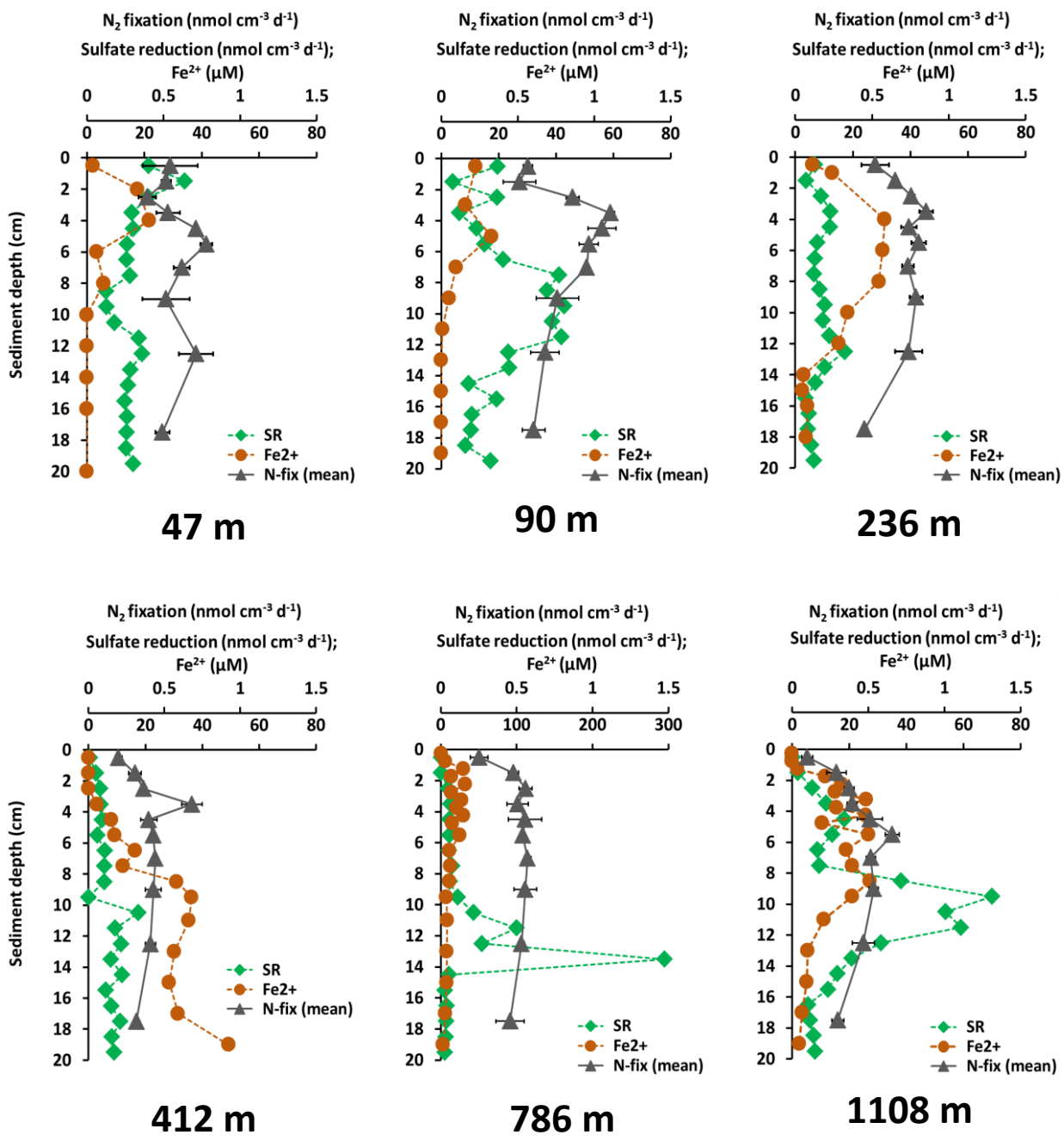

Fig. 4: Depth profiles of N_2 fixation (nmol N_2 cm^{-3} d^{-1}, grey triangles, average of three replicates plus standard deviation), sulfate reduction rates (SR, nmol SO_4^{2-} cm^{-3} d^{-1}, green rhombus, single measurements) and ferrous iron porewater concentrations (Fe^{2+}, µM, brown dots) between 0 and 20 cm at the six stations in Table 1. N_2 fixation is represented by the upper x-axis, while sulfate reduction and ferrous iron concentration are represented by the lower x-axis.

Integrated (0 – 20 cm) N_2 fixation roughly followed integrated (0–20 cm) sulfate reduction rates from 46 m to 1108 m (Fig. 5). Both rates were highest (0.15 ± 0.004 mmol N_2 m^{-2} d^{-1} and 4.2 mmol SO_4^{2-} m^{-2} d^{-1}) at 90 m and lowest at 412 m (0.08 ± 0.002 mmol N_2 m^{-2} d^{-1} and 1.4 mmol SO_4^{2-} m^{-2} d^{-1}, respectively). Averaged (0-20 cm, n = 10-20) organic carbon did not follow the N_2 fixation and sulfate reduction activities along the depth transect. Organic carbon increased from the shelf (46 m, 0.8 wt%) along the continental margin with the highest value (2.9 wt%) at 1108 m. In controls for N_2 fixation and sulfate reduction no activity was detected.

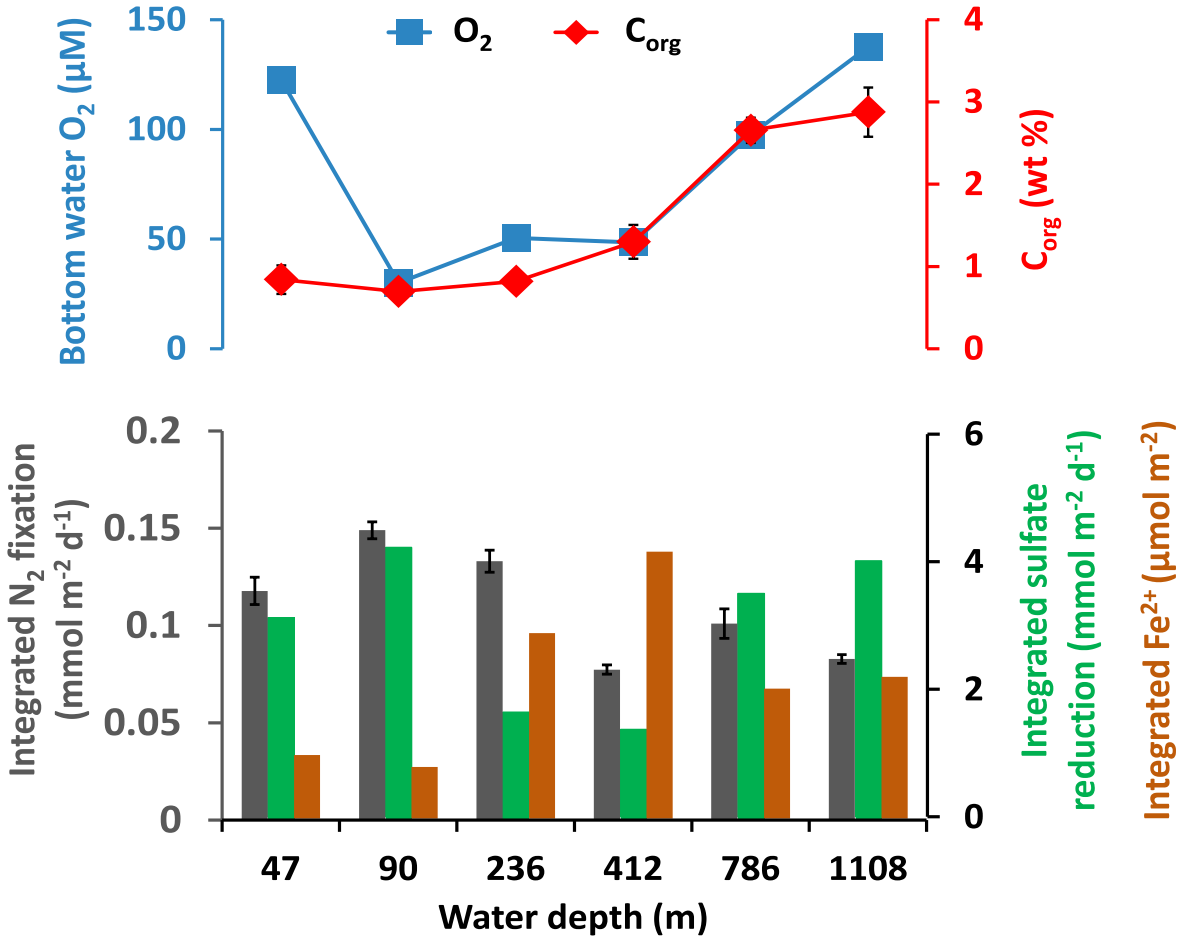

Fig. 5: Top: Bottom water O_2 (µM) and average (0–20 cm, n=10-20) particulate organic carbon content (C_{org}, wt%) along the depth transect. Bottom: Integrated nitrogen fixation (mmol N_2 m^{-2} d^{-1}, grey bars, average of three replicates plus standard deviation), sulfate reduction rate (mmol SO_4^{2-} m^{-2} d^{-1}, green bars, single measurements) and ferrous iron concentration (Fe^{2+}, µmol m^{-2}, brown bars) between 0 and 20 cm along the depth transect (m).

Integrated N_2 fixation did not follow integrated (0 – 20 cm) ferrous iron concentrations along the depth transect (Fig. 5). While N_2 fixation was highest (0.15 ± 0.004 mmol N_2 m^{-2} d^{-1}) at 90 m, integrated ferrous iron was lowest (0.8 µmol m^{-2}) at this station. Further, at 412 m integrated N_2 fixation was lowest (0.08 ± 0.002 mmol N_2 m^{-2} d^{-1}) and integrated ferrous iron was highest (4.2 µmol m^{-2}). The averaged (0–20 cm) organic carbon content did not follow integrated ferrous iron concentrations along the depth transect. While the integrated ferrous iron concentration reached its maximum at 412 m, the organic carbon content was at 1.3 wt%. At 1108 m the averaged carbon was highest (2.9 wt%) and integrated ferrous iron had a medium value (2.0 µmol m^{-2}).

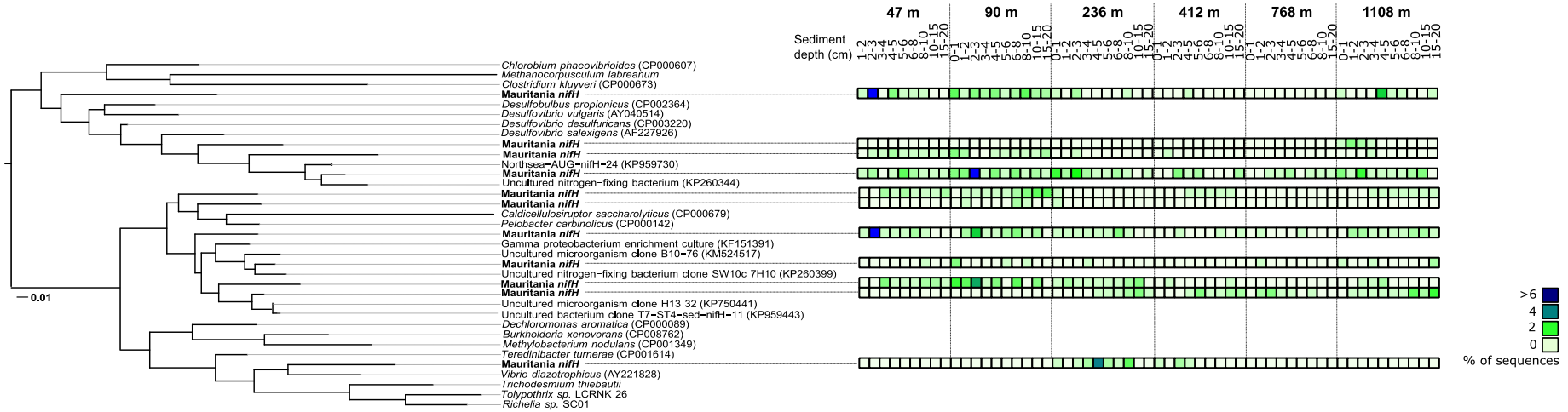

Fig. 6: Phylogenetic tree of expressed *nifH* genes based on the analysis of ~ 8000 sequences and respective abundance from the total data set (see legend of the color code on the right). The six sampling stations are shown with the corresponding sediment depth (cm). The scale bar represents 10% estimated sequence divergence.

3.3 Molecular analysis of the *nifH* gene

In total ~8000 *nifH* gene sequences were obtained that grouped into 10 clusters (Fig. 6). *NifH* sequences were detected at all sampling sites and clustered with Cluster I proteobacterial sequences and Cluster III sequences as defined by Zehr & Turner (2001). No Cluster I cyanobacterial *nifH* sequences were identified.

Detected sequences clustered with several sulfate-reducing bacteria of the genus Desulfovibrio*,* such as *Desulfovibrio desulfuricans* (Steenkamp & Peck, 1981; Lobo et al., 2007)*, Desulfovibrio vulgaris* (Riederer-Henderson & Wilson, 1970; Muyzer & Stams, 2008) and *Desulfovibrio salexigens* (Postgate & Campbell, 1966; van Niel et al., 1996). Detected *nifH* sequences from all stations clustered with these sulfate-reducing bacteria, except for the 768 m site. In particular a high sequence abundance (>6%) of *nifH* amplicons was present at the 46 m site at 2-3 cm sediment depth.

Other organisms clustering with detected nifH sequences (in low (2%) sequence abundance at 46, 90, 412, and 1108 m) belonged to the genera of bacteria using iron and sulfur as electron acceptors, namely *Pelobacter carbinolicus* (Lovley, Phillips, Lonergan, & Widman PK, 1995) and to the species *Caldicellulosiruptor saccharolyticus,* which hydrolyses a variety of polymeric carbohydrates (Rainey et al., 1994). One cluster was related to *Vibrio diazotrophicus*, which was found at 236 m (up to 4% between 4 and 5 cm) and in low sequence abundances (1%) at 412 m.

Additionally, several sequences were phylogenetically related to uncultured microorganisms and were found at all sites, e.g. a *γ*-proteobacterial clone (R. Langlois, Großkopf, Mills, Takeda, & LaRoche, 2015), which had its highest abundance in the sequence pool (>6%) at 46 m between 2 and 3 cm and an uncultured diazotroph (Ribes, Dziallas, Coma, & Riemann, 2015), that was found in highest sequence abundance (>6% of all sequences) at 90 m between 2 and 3 cm sediment depth.

4. Discussion

4.1 Benthic N_2 fixation along the sediment redox cascade

The Mauritanian upwelling has moderately low O_2 concentrations in the water column, as well as a high primary productivity (Baker et al., 2006; Carr, 2001), and extensive bioirrigation/bioturbation (Dale et al., 2014), which are all factors that could increase organic matter availability in the sediments and enhance microbial activities. Previous studies suggests that N_2 fixation and sulfate reduction activity are coupled in numerous benthic environments (Bertics et al., 2013; Fulweiler et al., 2013; Gier et al., 2015). Based on these findings, we hypothesized a coupling of benthic N_2 fixation to sulfate reduction in the Mauritanian OMZ.

N_2 fixation was continuously low in the sediment surface (0 – 2 cm) and increased only in deeper layers (Fig. 4). This observation overlapped with an oxidized water column (O_2 > 30 µM), as well as bioturbation and a low organic carbon content (discussion bioturbation see 4.2.3). These factors together could lead to a relatively deep penetration of O_2 into the sediment (Revsbech et al., 1980; Ziebis et al., 1996; Kristensen, 2000; Bertics & Ziebis, 2009). O_2 is a known inhibitor of the nitrogenase enzyme (Dixon & Kahn, 2004; Postgate, 1998) and an oxic layer at the sediment surface would potentially suppress N_2 fixation activity.

Following the redox cascade, nitrate reduction succeeds the aerobic respiration zone (Jørgensen, 1983; Jørgensen & Kasten, 2006). At all stations N_2 fixation was low in the surface sediment (Fig. 4) and nitrate disappeared between 0 and 2 cm sediment depth (Fig. 2), suggesting nitrate reduction activity. In the molecular analysis denitrifying bacteria did not cluster with *nifH* gene sequences (Fig. 6), ruling this process most likely out for an alliance with N_2 fixation.

Another indication for potential nitrification and an oxic surface layer may provide total oxygen uptake (TOU) rates, which express the benthic respiration. These rates were measured by Dale et al. (2014) at 18°N off Mauritania at similar sampling depth, when compared to our survey. Highest TOU rates were found at the shelf station (-10.3 mmol m^{-2} d^{-1}) in 53 m water depth and were decreasing in a quasi-exponential way to the lowest rate of -3.2 mmol m^{-2} d^{-}1 at the deepest site (1113 m). The highest nitrate concentration (35 µM) of all cores was measured at 786 m, which is not in line with the TOU data; nevertheless, the TOU data shows that benthic respiration existed and this is what we also saw in our data.

Iron reduction is the next major organic matter degradation process after manganese reduction and prior to sulfate reduction (Jørgensen, 1983; Jørgensen & Kasten, 2006). Additionally, we also found manganese peaks in the porewater profiles (data not shown); however, no manganese reducers were detected by the molecular nifH gene analysis (Fig. 6). Therefore, we do not discuss potential manganese reducers.

At all stations, except at site 412 m, the ferrous iron porewater profiles and N_2 fixation activity showed overlapping peaks in the first 10 cm. Additionally, at many sites, the ferrous iron depth profiles showed concentration peaks above sulfate reduction (Fig. 4), suggesting the presence of an iron reduction zone. In accordance with this finding, *nifH* gene sequences clustered in low abundances (2 %) with *Pelobacter carbinolicus* (Fig. 6,), which uses iron and sulfur as electron acceptors (Lovley et al., 1995) and which was previously shown to be involved in N_2 fixation in subtidal sediments of Narragansett Bay (Rhode Island) (Fulweiler et al., 2013), as well as in bioturbated muddy sand sediments at Catalina Island (California) (Bertics et al., 2010). At the 412 m and 1108 m site the sequence abundances of *Pelobacter carbinolicus* sequences had corresponding ferrous iron peaks (Fig. 4 and 6). At the 90 m site, sequence abundance and the ferrous iron depth profile did not overlap. In summary, these observations suggest a potential for iron-reducing bacteria being involved in N_2 fixation.

N_2 fixation activity generally overlapped with sulfate reduction (Fig. 4). Furthermore, integrated rates of N_2 fixation and sulfate reduction revealed similar trends along the depth transect (Fig. 5), with high rates at 90 m and lowest rates at 412 m. Activity peaks in the depth profiles often did not match, which indicated that either N_2 fixation and sulfate reduction are influenced by different environmental factors or that other metabolic processes than sulfate reduction are coupled to N_2 fixation. Phylogenetic analysis of the *nifH* gene indicated the genetic ability of sulfate reducers in the Mauritanian sediments to conduct N_2 fixation (Fig. 6). Sequences clustered with several sulfate reducers of the genus *Desulfovibrio spp.,* which were found previously to be involved in benthic N_2 fixation (V. J. Bertics et al., 2013; R. Fulweiler et al., 2013; Gier et al., 2015). In sediments of the temperate Narrangaset Bay estuary, *nifH* genes related to the sulfate reducer *Desulfovibrio spp*. were identified, suggesting that sulfate-reducing bacteria are responsible for the N_2 fixation in this area (R. Fulweiler et al., 2013). Previously, the sulfate-reducing bacterium *Desulfovibrio vulgaris* clustered with sequences taken in the hypoxic Eckernförde Bay in the Baltic Sea (V.

J. Bertics et al., 2013). In our study, sequence abundances of the genus *Desulfovibrio spp.* and peaks of sulfate reduction activity often overlapped, for example at the 47 m station at 2 – 3 cm, at the 90 m site at 2 – 3 and 4 - 15 cm (Fig. 4 and 6). At the 412 m station maximum sequence abundances of *Desulfovibrio spp.* at 15 – 20 cm overlapped with sulfate reduction activity. Not every sulfate reduction peak overlapped with a high sequence abundance of sulfate-reducing bacteria. For example the 236 m station had two sulfate reduction peaks (3 – 5 cm and 10 – 15 cm), which were not confirmed by high sequence abundance of a sulfate-reducing bacteria in these depth. Overall, the phylogenetic analysis of diazotrophs conducted in our study indicated the potential of sulfate-reducing bacteria to perform N_2 fixation in the Mauritanian sediments.

Further, it should be highlighted that N_2 fixation, sulfate reduction and porewater data were determined from three different replicate cores with a sampling distance of up to 50 cm, which means that N_2 fixation profiles cannot be directly related to sulfate reduction and porewater profiles. Lateral heterogeneity could easily obscure the actual correlations. We are also conscious of potential microbial community shifts induced by the addition of C_2H_2 (Fulweiler et al., 2015). Nevertheless, a community shift would rather lead to an underestimation of absolute N_2 fixation rates.

Finally*, nifH* sequences clustered with *Vibrio diazotrophicus*, which was shown previously to be capable of N_2 fixation (Guerinot et al., 1982) and which was found likewise in the Peruvian OMZ sediment (Gier et al., 2015) and water column (Löscher et al., 2014). We recognized several new *nifH* clusters in the 18°N depth transect, that have not been identified, yet (Fig. 6). These clusters could be related to metabolic processes such as sulfate or iron reduction. Interestingly, also in the Peruvian OMZ several new *nifH* gene sequences were detected that have not been identified (Gier et al., 2015), highlighting the diversity of diazotrophs in marine sediments.

4.2 The role of benthic N_2 fixation

In order to determine the relevance of benthic N_2 fixation in the Mauritanian OMZ, we compared our N_2 fixation rates to the denitrification rates measured by Dale et al. (2014). The Mauritanian sediments are regarded as an N sink for dissolved inorganic N, with denitrification being the main N removal process (Dale et al., 2014).

Compared to our sampling survey, TOU and denitrification were investigated along a similar depth transect (18°N) and in similar water depth (53 – 1113 m). Thus, the results from Dale et al. (2014) are appropriate indicators for the relevance of N_2 fixation in that region. Benthic integrated N_2 fixation was highest (0.15 ± 0.004 mmol m^{-2} d^{-1}) at the shelf (90 m) and lowest (0.08 ± 0.002 mmol m^{-2} d^{-1}) at the deepest site (1108 m). Denitrification rates at the corresponding shelf site (98 m) were 1.8 mmol m^{-2} d^{-1}, while rates at the deepest site (1113 m) were 0.2 mmol m^{-2} d^{-1} (Dale et al., 2014).

Calculating the above mentioned sources and sinks of N, the benthic N_2 fixation could counteract between 8% and 40% of the N loss between the shelf and the deepest site, respectively. In conclusion, benthic diazotrophs in the Mauritanian OMZ are capable to respond to the fixed N loss to a certain extent at particular sites.

4.3. Effects of burrowing organisms on N_2 fixation and related processes

Mauritanian sediments <400 m are classified as permeable sands inhabited by extensively burrowing macrofauna (Dale et al., 2014). Burrowing organisms increase microbial activity in sediments by facilitating burial of organic matter (Aller & Aller, 1986; Christensen et al., 2000; Bertics & Ziebis, 2010; Kristensen et al., 2012). Availability of organic matter was found to be one of the most essential environmental factor that control benthic N_2 fixation (Hartwig & Stanley, 1978; Jørgensen, 1983; Howarth et al., 1988; Fulweiler et al., 2007; Gier et al., 2015). Burrow systems change the redox cascade by providing additional electron acceptors, and the potential removal of ammonium by nitrification may provide favorable microniches for N_2 fixation (V. J. Bertics et al., 2010; Wenzhöfer & Glud, 2004; Zorn, Lalonde, Gingras, Pemberton, & Konhauser, 2006). We therefore hypothesize elevated benthic N_2 fixation in deeper sediment layers, favored by burrowed organic matter and low ammonium concentrations. Benthic N_2 fixation along the Mauritanian depth transect indicate a potential association to bioirrigation and connected processes like organic matter, O_2, and ammonium availability, which will be discussed in the following.

4.3.1 Organic matter

Integrated rates of N_2 fixation were high at the shallow sites (47 – 236 m) (Fig. 5), coinciding with the highest integrated bioirrigation rates (Fig. 3b). No evidence was found for microniches of elevated N_2 fixation in deeper sediment layers. Only the sulfate reduction peak observed at the 90 m site (8 – 12 cm, Fig. 4) coincided with high bioirrigation at this

station and could be a result of organic matter introduction by burrowing activity. N_2 fixation rates do not vary much throughout the sediment cores, which might be the consequence of a consistent input of organics. Though, an increase of organic matter does not necessarily lead to an increase of N_2 fixation, as Fulweiler et al. (2007) found a switch from denitrification to N_2 fixation in the sediments of Narrangaset Bay, caused by a lower organic matter deposition to the sediments. In contrast, organic matter and integrated N_2 fixation correlated well in Peruvian OMZ sediments (Gier et al., 2015). However, Peruvian sediments and Mauritanian sediments are two different types of environments, as the Peruvian sediments are considered as organic rich sandy mud (Dale et al., 2015) and an organic matter content up to 15 wt% (Gier et al., 2015).

4.3.2 Oxygen

Bottom water O_2 along the Mauritanian transect was above 30 µM and reached the highest values of 123 µM and 138 µM at 47 m and 1108 m water depth, respectively (Fig. 1 and 2). As a result of the existing O_2, a diverse burrowing benthic macrofauna community was found at all sampling sites, with the highest integrated bioirrigation rate at 47 m (Fig. 3b) and the highest bioirrigation depth at 90 m (Fig. 3a).

Bioirrigation transports O_2 deeper into the sediment, than it is done by molecular diffusion (Orsi et al., 1996; Ziebis et al., 1996; Dale et al., 2011). Because of the inhibitory effect of O_2 on the nitrogenase enzyme (Postgate, 1998; Dixon & Kahn, 2004) bioirrigation and bioturbation in combination with benthic N_2 fixation have barely been studied (Bertics et al., 2010). Alternatively, several marine diazotrophs have developed strategies to protect the nitrogenase from O_2 (Jørgensen, 1977; Krekeler et al., 1998; Cypionka, 2000). Also some sulfate-reducing bacteria were found to be suppressed by O_2, while others showed an O_2 depended growth (reviewed by Muyzer & Stams, 2008).

Burrowing activities by macrofauna was found to create a 3-dimensional chemical zonation pattern in the sediment, which sequentially changed the redox cascade by providing additional electron acceptors into the sediment. The O_2 only exists inside the burrow holes, where it is consumed quickly or is transported across the burrow walls by molecular diffusion, which limits the O_2 penetration to a few millimeters (Gundersen & Jørgensen, 1990; Revsbech et al., 1980; Ziebis et al., 1996). Additionally, NH_4^+ could be removed by nitrification, providing a favorable microniches for N_2 fixation (Wenzhöfer & Glud, 2004;

Zorn et al., 2006; Bertics et al., 2010). Off Mauritania, burrowing macrofauna was present throughout the depth transect, and potentially creating a 3-dimensional chemical zonation pattern in the sediment, which might have favored N_2 fixation in deeper sediment layers.

4.3.3 Ammonium

In burrowed sediments, the biogeochemical zonation may be altered and other electron acceptors, such as nitrate and ferric iron could be transported deeper into the sediment, as well as processes like nitrification could occur that remove ammonium from the benthic system (Gilbert et al., 1998; Jørgensen & Kasten, 2006). Porewater ammonium concentrations off Mauritania were low and did not exceed 111 µM (Fig. 2, 1108 m), which might be a result of ammonium removal by nitrification in burrow wholes. Overall, the low ammonium concentrations did potentially not inhibit the benthic N_2 fixation, as it is the classical view (Douglas G Capone, 1988; Knapp, 2012; Postgate, 1982).

4.3 Benthic N_2 fixation in the upwelling regions off Mauritania and Peru

As OMZs are predicted to increase globally (Diaz, 2001; Keeling et al., 2010; L. Stramma et al., 2008), it is crucial to understand their biogeochemical concepts to make predictions how relatively oxygenated areas, such as the Mauritanian OMZ (O_2 > 40 µM), will be affected when turning into a strong OMZ (O_2 < 20 µM). We composed a list of N_2 fixation rates and accompanied geochemical parameters from the eastern tropical north Atlantic OMZ off Mauritania and the eastern tropical south Pacific OMZ off Peru (Gier et al., 2015) (Table 2). This comparison should aid in our understanding of the magnitude of N_2 fixation rates in O_2 deficient environments and assess associated environmental factors. The Mauritanian OMZ is considered a weak OMZ with permeable sandy sediments and low organic carbon, while the Peruvian OMZ is fully anoxic with sandy mud, low permeability and high organic carbon.

Bottom water O_2 concentrations were above 30 µM off Mauritania, while concentrations were below the detection limit down to 407 m water depth off Peru. Overall, we found that integrated (0 - 20 cm) N_2 fixation rates from the Mauritanian upwelling were lower (0.08 – 0.15 mmol m^{-2} d^{-1}) than those reported for the Peruvian OMZ (0.01 – 0.41 mmol m^{-2} d^{-1}). The organic matter content was higher in Peruvian sediments (2.4 – 15 wt%) than in Mauritanian sediments (0.7 – 2.9 wt%). Mauritanian sediments are considered to be permeable sands (Dale et al., 2014). Integrated (0 - 20 cm) sulfate reduction rates were higher off Mauritania (1.4 – 6.4 mmol m^{-2} d^{-1}) than off Peru (0.2 – 4.6 mmol m^{-2} d^{-1}). The yielded sulfate reduction

rates off Peru have to be treated with caution due to unfrozen storage, which has recently been shown to result in the re-oxidation of ^{35}S-sulfides and hence an underestimation of sulfate reduction rates (Røy et al., 2014). The lower O_2 concentrations could positively affect N_2 fixation off Peru due to the O_2 sensitivity of the nitrogenase enzyme (Postgate, 1998; Dixon & Kahn, 2004). Off Mauritania, the moderate O_2 concentrations facilitate burrowing macrofauna (Dale et al., 2014), that could finally provide favorable microniches for N_2 fixation. Sulfide in the Mauritanian sediments was highest at site 47 m and still present at the 90 m site. N_2 fixation was not inhibited at these two stations and site 90 m had the highest N_2 fixation rate, but we cannot exclude a partial suppression of N_2 fixation by sulfide. Sulfide in the Peruvian OMZ was detected only at the shelf station, reaching 1229 µM. High concentrations of sulfide in the porewater were reported to potentially inhibit diazotrophy in Peruvian OMZ sediments (Gier et al., 2015). Currently, there are barely enough studies that investigated N_2 fixation and potential inhibition by sulfide (Tam et al., 1982), to make a clear statement about potential relations.

While ammonium concentrations were high in the Peruvian OMZ (up to 1106 µM), ammonium was low in the Mauritanian upwelling (up to 88 µM). However, highest N_2 fixation off Peru was measured in sediments with relatively high ammonium.

Tab. 2: Integrated (0-20 cm) rates of N_2 fixation and sulfate reduction from 0 - 20 cm from this study compared to the Peruvian OMZ (from Gier et al., 2015) as well as environmental parameters. Organic carbon content (C_{org}) and C/N ratio represent mean values including standard deviations. Sulfide and ammonium (NH_4^+) concentrations are maximal concentrations; na = not available.

	Water depth (m)	Integrated N_2 fixation (mmol m^{-2} d^{-1})	Integrated SR (mmol m^{-2} d^{-1})	Bottom water O_2 (µM)	C_{org} (wt%)	C/N ratio (molar)	Sulfide max. (µM)	NH_4^+ max. (µM)
Peru	70	0.15 ± 0.001	4.6	bdl	3.5 ± 0.8	9 ± 0.9	1229	2022
	90	0.30 ± 0.054	2.5	bdl	7.7 ± 2.6	10 ± 0.6	0	316
	253	0.41 ± 0.057	0.5	bdl	14.5 ± 2.4	10 ± 0.3	0	786
	407	0.01 ± 0.003	0.3	bdl	8.0 ± 2.1	11 ± 1.5	1	107
	770	0.05 ± 0.006	0.2	33	4.6 ± 0.9	11 ± 0.7	0	34
	1025	0.01 ± 0.001	na	53	2.3 ± 0.4	12 ± 0.9	0	24

Mauri-tania	47	0.12 ± 0.007	3.1	123	0.8 ± 0.2	10 ± 2.3	88	80
	90	0.15 ± 0.004	4.2	30	0.7 ± 0.1	9 ± 0.5	46	70
	236	0.13 ± 0.006	1.6	50	0.8 ± 0.1	9 ± 0.3	0	31
	412	0.08 ± 0.002	1.4	48	1.3 ± 0.2	10 ± 0.5	0	45
	789	0.10 ± 0.008	6.4	98	2.7 ± 0.2	10 ± 0.3	0	75
	1108	0.08 ± 0.002	4.0	138	2.9 ± 0.3	9 ± 0.4	0	112

The Mauritanian OMZ will potentially encounter expansion and a further decrease of O_2 (Diaz & Rosenberg, 1995; Diaz, 2001; Stramma et al., 2008). Biogeochemical processes in the sediments would change under anoxic conditions. These changes would include the lack of burrowing macrofauna and in turn the lack of organic matter input to deeper sediment layers. The 3-dimensional zonation pattern of burrowed sediments would disappear and as a consequence this would have a negative effect on benthic N_2 fixation activity in the deeper sediments. Alternatively, the biogeochemical zonation of the sediments (Jørgensen, 1983; Jørgensen & Kasten, 2006) would change to purely anoxic microbial degradation processes, which could have in turn a positive effect on benthic N_2 fixation. Fully anoxic sediments would potentially encounter higher sulfide concentrations, what was shown to suppress benthic diazotrophs (Gier et al., 2015; Tam et al., 1982).

Sediments of the Mauritanian OMZ are considered a net sink for dissolved inorganic N by denitrification (Dale et al., 2014). When comparing the Mauritanian sediments to the Peruvian OMZ sediments and taking the above mentioned features into account, the Mauritanian sediments would, under anoxic conditions potentially switch from being a net sink to being a net source of bioavailable N by N_2 fixation.

5. Summary

Our findings add to the growing knowledge of benthic N cycling in upwelling regions and will aid in our understanding of potential environmental factors that control benthic diazotrophs. N_2 fixation occurred throughout the sediment. Iron reducing bacteria were identified as potentially important for benthic diazotrophy, as identified by molecular analysis. N_2 fixation

activity often coincided with sulfate reduction activity. This result was supported by molecular analysis of the *nifH* gene, which confirmed the presence of several sulfate-reducing bacteria of the genus *Desulfovibrio spp*. We further found that burrowing macrofauna created a biogeochemical zonation pattern in the sediments, which potentially enhanced benthic N_2 fixation in deeper sediment layers. However, if the Mauritanian upwelling turns anoxic, burrowing organisms would be absent, further the sediments might switch from a net sink to a net source of bioavailable N.

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Chapter 4

Novel insights into benthic diazotrophy: Nitrogenase Gene Amplicons from marine sediments reveal a global dominance of sulfate reducers.

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Abstract

While there is a growing knowledge of diazotrophic diversity in the oceanic water column, information on diazotrophy in marine sediments has been relatively sparse and can be considered one major gap in knowledge regarding the marine nitrogen (N) cycle. Existing data on benthic diazotrophic diversity were restricted geographically and by limited sequencing depths of the classical Sanger method. However, a potential for benthic dinitrogen (N_2) fixation has previously been demonstrated, e.g., in coastal bioturbated and subtidal heterotrophic sediments, as well as in Peruvian sediments.

Thus, we aimed at characterizing the diversity of diazotrophs in marine sediments of various ocean regions (Arctic, Atlantic, Baltic Sea, Mediterranean Sea, and Pacific), including OMZ and methane seep sites. The diazotrophic diversity was analyzed by applying a high-throughput sequencing approach of the key functional marker gene for N_2 fixation, the *nifH* gene. We generated a dataset of ~80000 reads, and found several clades of diazotrophs in all sediments; however, variations between the different environments were rather small. The investigated diazotrophic community was composed of 30 main clusters, mainly consisting of potential heterotrophic organisms affiliated to clusters II and III, most of them of proteobacterial origin. Among those, sulfate reducers take a key role in all investigated environments, thus indicating an important link between N_2 fixation and sulfate reduction. Along with the detection of active N_2 fixation in the investigated environments, the applied redundancy analysis model indicated a positive correlation between diazotrophs and sulfate reduction. Thus, benthic sulfate-reducing diazotrophs could provide an important source of fixed N in the marine environment. This study provides the first global survey of the *nifH* gene pool in marine sediments, therefore pointing to a potential importance of this process for the world oceanic N budget.

Introduction

Nitrogen (N) is a limiting component for life in the ocean (Howarth et al., 1988) and the large atmospheric pool of dinitrogen gas (N_2) is only accessible to a heterogenic group of prokaryotes, which are called diazotrophs (Capone & Carpenter, 1982). Previously, N budgets in the marine N cycle seemed unbalanced, which means that N sources did not equal N sinks. N budget estimates ranging between an almost balanced budget (Gruber, 2004) to budget deficits up to 200 Tg N yr^{-1} (L. A. Codispoti, 2007). These budget discrepancies were largely owed to a methodological underestimation of N_2 fixation rates (Großkopf et al., 2012; Löscher et al., 2015) and to a limited knowledge on diazotrophic diversity that has grown lately (Farnelid et al., 2011). N_2 fixation, the reduction of N_2 into bioavailable ammonium, has usually been assumed to take place in N depleted surface oceans (Sohm et al., 2011). Knowledge of benthic N_2 fixation is limited (Bertics et al., 2013; Fulweiler, 2013) and detailed molecular studies on benthic diazotrophic diversity from different ocean areas are currently not available. However, the few available recent studies pointed out that benthic diazotrophy is present in sediments; e.g. in the organic rich sediments of the seasonally hypoxic Eckernförde Bay in the Baltic Sea (Bertics et al., 2013) and in subtidal sediments in Narragansett Bay (Rhode Island, USA) (Fulweiler et al, 2007; Fulweiler & Nixon, 2012). Although the above mentioned studies were conducted in two different environments, the detected diazotrophs in both areas were phylogenetically closely related to sulfate reducers (Bertics et al., 2010, 2013; Fulweiler et al., 2013). Further, coupling of N_2 fixation to sulfate-reducing bacteria was observed in bioturbated coastal sediments off Catalina Island (California, USA) (Bertics et al., 2010), as well as in sediments of the Peruvian oxygen minimum zone (OMZ, dissolved oxygen < 20 µmol kg^{-1}, (Fuenzalida et al., 2009)) (Gier et al., 2015). Hence, these sulfate-reducing diazotrophs connect to two major nutrient cycles, namely the N and sulfur cycle.

The discovery from an active deep sea methane seep in the Eel River Basin (California, USA) extended our understanding of a further coupling between the N and the sulfur cycle, to the carbon cycle (Dekas et al., 2009). Diazotrophic deep-sea anaerobic methane-oxidizing archaea (ANME-2 group) were found, that shared the products with their sulfate-reducing symbionts (*Desulfosarcina*/*Desulfococcus*) (Dekas et al., 2009).

Thus, the abundant occurrence of sulfate-reducing bacteria in marine sediments (Jørgensen, 1982), together with the fact that several sulfate reducers have the genetic capability to fix N_2 (Zehr et al., 1995; Steppe & Paerl, 2002), may indicate a global potential of these bacteria to contribute new N to the ocean. In order to provide further insights into benthic N2 fixation and the benthic *nifH* gene pool, we conducted a sampling survey at selected benthic environments.

These sampling locations were chosen according to their special characteristics and with the aim to have a broad variety of environments. OMZ sediments off Peru and off Chile were investigated because both areas are anoxic and could, due to their oxygen-sensitivity (Postgate, 1998; Dixon & Kahn, 2004), provide a favorable habitat for diazotrophs. The Peruvian and Chilean sampling sites are characterized as organic-rich OMZ sediment and as methane seep sediment, respectively (Paulmier et al., 2006; Steeb, 2014; Dale et al., 2015). The Chilean OMZ features anaerobic oxidation of methane in the sediments, which could point to diazotrophs that are anaerobic methane-oxidizing archaea with sulfate-reducing bacterial symbionts (Dekas et al., 2009).

In order to compare the diazotrophic diversity to a weaker OMZ with moderate oxygen concentrations (Karstensen et al., 2008; Chavez & Messié, 2009; Dale et al., 2014), the OMZ off Mauritania was chosen for sediment sampling. Those three OMZ sites share the characteristic of high organic matter content in the sediment, as well as the fact that all of them are continental margin areas.

To further investigate the diazotrophic diversity of methane seep sites, we chose two additional seep locations. These sites were a gas hydrate off West Svalbard in the polar region (Bünz et al., 2012) and a mud volcano in the temperate Mediterranean Sea (Dupré et al., 2007; Feseker et al., 2010). While those sites combined their methane venting and anaerobic oxidation of methane, the sampling locations varied among temperature and water depth.

The sampling sites in the Baltic Sea were chosen to investigate brackish water sediments, with one being seasonally hypoxic (Eckernförde Bay) (Hansen, 1999; Bange et al., 2011), and one being seasonally anoxic (Gotland Basin) (Orsi et al., 1996). The Baltic Sea sites have the potentially low oxygen concentrations in common with the OMZ sites; however, Eckernförde

Bay and the Gotland basin do not represent continental margins but shallow sites and both do not represent seep sites.

As explained above, the available data on benthic diazotrophs indicated that sulfate-reducing bacteria are involved in N_2 fixation. Thus, these bacteria are expected to be correspondingly responsible for N_2 fixation in other environments; but to which extend the distinct environmental parameters of different sites influence sulfate reducers and the benthic diazotrophic diversity remained unknown. Therefore, we investigated the phylogenetic diversity of benthic diazotrophs and the role of the ubiquitous sulfate reducers for diazotrophy, as well as geochemical parameters at distinct locations.

Material and Methods

Study sites

Sediment samples were collected from seven benthic locations (Fig. 1); from the Arctic Ocean off Svalbard; from the Atlantic off Mauritania; two sampling sites in the Baltic Sea in the Eckernförde Bay and the Gotland Basin; in the Eastern Mediterranean Sea at the North Alex Mud Volcano; and in the Pacific off Chile and Peru. For an overview of cruise data, location, sampling date, water depths, N_2 fixation and sulfate reduction rates, as well as environmental parameters of each sampling location see the Tables S2 and S3. In the following, we provide an overview of each sampling site, starting with the locations (Baltic Sea, off Peru, and off Mauritania), where N_2 fixation and sulfate reduction rates have previously been measured (Table S2 and S3), and which allowed the investigation of both processes at these locations.

Eckernförde Bay, located in the South-Western part of the Baltic Sea is a semi-enclosed bay with organic-rich sediments and seasonal hypoxia (Hansen, 1999; Bange et al., 2011) and sulfide-oxidizing bacteria *Beggiatoa* sp. inhabiting the sediments (Preisler et al., 2007). At this site, N_2 fixation was coupled to sulfate reduction, which was demonstrated by the presence of *nifH* sequences, that clustered with known sulfate-reducing bacteria (Bertics et al., 2013).

The Gotland Basin sampling site, located in the central Baltic Sea represents the deepest basin of the Baltic Sea. It is a strongly stratified brackish water body with fine-grained organic rich mud sediments and seasonal anoxic conditions (oxygen below the analytical detection limit: ~ 2

µM) (Orsi et al., 1996) and *Beggiatoa* sp. at the sediment surface (Emeis et al., 2000). Sediments are characterized by laminated horizons of a highly variable depth range (17–300 mm) and a high spatial variability of sedimentation rates with bulk accumulation rates between 10.5 and 527 g m^{-2} yr^{-1} (Hille, Leipe, & Seifert, 2006).

The eastern tropical South Pacific off Peru is characterized by one of the most extensive OMZs worldwide, with oxygen concentrations below the detection limit of conventional methods (Stramma et al., 2008). At the 12°S sampling site, the Peruvian OMZ extents from 50 - 550 m water depth (Dale et al., 2015). Giant sulfur-oxidizing bacteria of the genus *Thioploca sp*. (Schulz, 1999; Schulz & Jørgensen, 2001) colonize the sediment surface and produce high concentrations of ammonium (Gutiérrez et al., 2008; Mosch et al., 2012; Dale et al., 2015). Peruvian sediments have generally a high organic carbon content, as well as high sulfide concentrations (Gier et al., 2015). N_2 fixation and sulfate reduction activity generally overlapped, and *nifH* sequences clustered with sulfate reducers (Gier et al., 2015).

Mauritanian sediments are exposed to a moderate OMZ with oxygen concentrations >40 µM (Karstensen et al., 2008) and high organic carbon (Dale et al., 2014). Sediments are described as permeable sands and represent as a sink for dissolved inorganic N (Dale et al., 2014); however, N_2 fixation was abundant throughout the sediment and was highest at the shelf (J. Gier, unpubl. data). Benthic N_2 fixation overlapped with sulfate and iron reduction activity along vertical depth profiles and bacteria performing these degradation pathways were identified as diazotrophs by *nifH* gene analysis (J. Gier, unpubl. data).

For the Arctic, the Chilean, and the Mediterranean Sea sampling sites, no benthic N_2 fixation measurements were performed; however, sulfate reduction rates are available (Tables S2 and S3).

Arctic sediments were collected in the area off West Svalbard on the Vestnesa Ridge, which characterized by gas hydrates and methane venting (Bünz et al., 2012). This sampling site is located between 1200 – 1300 m water depth and represents one of the most northern gas hydrate environments along the Arctic continental margin (Hustoft et al., 2009). The surface sediment of Vestnesa Ridge was colonized by mats of *Beggiatoa* sp. (Pimenov et al., 2000).

Sediments from the Concepción Methane Seep Area at the continental margin off Chile are exposed to a seasonal OMZ (Paulmier et al., 2006). These site was characterized as soft with bacterial mats on the sediment surface (Steeb, 2014). Sulfate reduction rates were high between 0 - 5 cm sediment depth, with an activity peak at 2.5 cm (660 ± 130 nmol cm^{-3} d^{-1}; n = 3) (Steeb, 2014). Anaerobic oxidation of methane was close to zero at the sediment surface and reached its maximum (712 ± 116 nmol cm^{-3} d^{-1}; n = 3) at 3 cm sediment depth (Steeb, 2014).

The North Alex Mud Volcano sampling site (Eastern Mediterranean Sea) is located at the West Nile Delta in ~ 500 m water depth (Feseker et al., 2010). The mud volcano is about 2000 m in diameter and its highest point is about 50 m above the seafloor (Feseker et al., 2010). Furthermore, the released fluids were dominated by methane and heavier hydrocarbons (Dupré et al., 2007). Surface sediments in the center are colonized by *Beggiatoa* sp. and a steep temperature gradient of 7.09 °C m^{-1} was measured between the sediment surface and 6 m below the seafloor (Makarow, 2010).

Sampling

Except for Chile, sediment samples for *nifH* gene analysis (Fig. 1) were retrieved by a multiple corer equipped with several core liners. Each core liner had a length of 60 cm and an inner diameter of 10 cm. Samples off Chile were retrieved from pushcores (length 30 cm, inner diameter 6 cm) deployed by a remotely operated vehicle (ROV, Kiel 6000, GEOMAR, Shilling Robotics). The water depth, sampling time and sampling depth, as well as the number of extracted DNA samples for every site are listed in Table 1. Sediment cores were sliced in the cool container (temperature adjusted to the respective *in situ* condition). For details on sampling depth intervals for each site see Table S2. For the sampling sites Eckernförde Bay, Gotland Basin, Mauritanian and Peru, approximately 5 mL sediment from each depth horizon was transferred to plastic whirl-paks® (*Nasco,* Fort Atkinson, USA), while sediment samples from Chile, the North Alex Mud Volcano, and Svalbard were transferred to 4 mL cryovials ®. All samples were frozen to -20 °C and transported back to the home laboratory. DNA extraction was done using the FastDNA® SPIN Kit for Soil (MP Biomedicals, CA, USA) following the manufacturer's instructions; however, sample homogenization was achieved using a Mini-Beadbeater™ (Biospec Products, Bartlesville, USA) for 15 seconds.

Geochemical parameters, N_2 fixation and sulfate reduction measurements

Porewater analysis for the Gotland Basin, Mauritania, and Peru) has been described previously by Dale et al. (2015). Briefly, a replicate sediment core was sampled in a cold room (5 - 12 °C) under anoxic conditions, using an argon-filled glove bag. Ammonium and sulfide concentrations were analyzed on a Hitachi U2800 143 UV/VIS spectrophotometer using standard photometric procedures (Grasshoff et al., 1999).

For Eckernförde Bay, porewater analysis has been described by Maltby (2015). In short, porewater was sampled using anaerobic Rhizons and sulfide concentrations were immediately analyzed using standardized photometric methods (Grasshoff, Ehrhardt, & Kremmling, 1999). Sediment porosity was determined samples were then used for analysis of particulate organic carbon content with a Carlo-Erba element analyzer (NA 1500).

Porewater analysis for Chile was described by Steeb (2014). Shortly, sulfide was determined photometrically by the Cline (1969) method according to (Grasshoff, Kremlingl, et al., 1999) Grasshoff et al. (1983).

For all sampling sites, except the North Alex Mud Volcano, the total organic carbon was determined by the weight difference of sediment before and after freeze drying. The dried sediment was then analyzed using a Carbo Erba Element Analyzer (NA1500). The method has been previously described by Dale et al. (2015).

Rate measurements for N_2 fixation (Gotland Basin, Mauritania, and Peru) have been described previously by Gier et al. (2015). In short, a replicate sediment core was sliced (sampling scheme see Table S2) in a cold room (5 - 12 °C). The acetylene reduction assay was applied over one week using gas chromatography (Hewlett Packard 6890 Series II) (Stewart et al., 1967; Capone, 1993). The nitrogenase activity was converted by the factor $3C_2H_4$:$1N_2$ to N_2 fixation (Orcutt et al., 2001; Bertics et al., 2013). We are aware of possible microbial community shifts by C_2H_2 (Fulweiler et al., 2015). However, this shift would rather cause an underestimation of absolute N_2 fixation rates.

Sulfate reduction rates were determined in mini-cores (length 25 cm, diameter 2.6 cm) taken from either a replicate ROV push core (Chile) or a MUC core (all other sites). Six µl of the carrier-free $^{35}SO_4^{2-}$ radiotracer (dissolved in water, ca. 150 kBq, specific activity 37 TBq $mmol^{-1}$) were injected in 1-cm intervals according to the whole-core injection method (Jørgensen, 1978). Mini cores were incubated at in-situ (or average in-situ; Peru and Mauritania) bottom water temperature, sliced, and transferred to 20 mL zinc acetate to stop the incubation. Except for Mauritania, samples were not frozen until further handling, wherefore underestimation of sulfate reduction rates can be expected (Røy et al., 2014), while we still trust the relative distribution of rates. Finally, samples were analyzed in the home laboratory applying the cold chromium distillation procedure (Kallmeyer et al., 2004).

nifH amplicon sequencing

In total, 200 sediment samples were used for *nifH* amplicon sequencing (Tab. 1). Nested PCRs for *nifH* were performed following established protocols (Zehr & Turner, 2001). Modifications of the protocol adjusted for Illumina sequencing preparation have previously been described by Bentzon-Tilia et al., (2015). Illumina indices were added to amplicons in the second PCR round. In addition to the *nifH*1 and *nifH*2 primer sequences, the primer contained a linker sequence, an 8-base barcode and the Illumina specific region P5 (forward primer) or P7 (reverse primer: Table S1 shows the primer sequences). Negative controls consisted of the reaction mixture the addition of DNA. PCRs were performed in triplicates for each sample. Triplicates were then pooled, and purified using the MinElute Gel Extraction Kit (Qiagen, Hildesheim, Germany) and quantified on a spectrophotometer (Nanodrop 1000, Thermo Fisher Scientific, Waltham, MA, USA). Samples were pooled in equimolar ratios and sequencing took place on an Illumina MiSeq Instrument using the MiSeq reagent Kit V3 chemistry (Illumina, San Diego, CA, USA). Sequences are currently in preparation to be submitted to a NCBI Sequence Read Archive.

Sequences were assembled using MOTHUR software version 1.32.1 (Kozich et al., 2013), contigs containing ambiguous bases or homopolymers longer than 8 bases were removed from the dataset. Redundant sequences were clustered using the command *unique.seqs* and aligned against the functional gene pipeline and repository database (http://fungene.cme.msu.edu/). Sequences not aligning with the seed *nifH* sequence pool were removed. Chimeric sequences

were removed with the MOTHUR implemented software UCHIME (Edgaret al., 2011). Remaining sequences were clustered at 97% nucleotide similarity and reference sequences for the most abundant clusters were obtained using BLAST search on the NCBI database. Analysis took place on a 200 base pair fragment. Amplicons and reference *nifH* sequences were ClustalW aligned in MEGA version 6.0 (Tamura et al., 2013), maximum likelihood trees were constructed and visualized using iTOL (Letunic & Bork, 2011).

Statistical analysis

A redundancy analysis model (RDA) for the sampling locations Gotland Basin, Mauritania, and Peru (the other sampling locations were not tested due to reasons mentioned above) has been applied to our metadata set in order to determine most likely explanatory variables for active N_2 fixation at the different sampling sites according to the approach described in Löscher et al, 2014. We tested the parameters sediment depth, sampling location (i.e. geographical position), sulfate reduction, organic carbon, ammonium, and sulfide concentrations. The input table for the applied RDA model is given in the supplemental material (Table S2). A Hellinger transformation was applied to the dataset before RDA was performed.

Finally a biplot was produced, which allowed to display a potential correlation between N_2 fixation and the metadata graphically.

Results and Discussion

Marine diazotrophic diversity

In this study, we investigated a variety of marine sediments (Fig. 1, Tab. 1) across different depth horizons for *nifH* diversity and compared it to existing environmental metadata from those regions (Supplement Tab. 2).

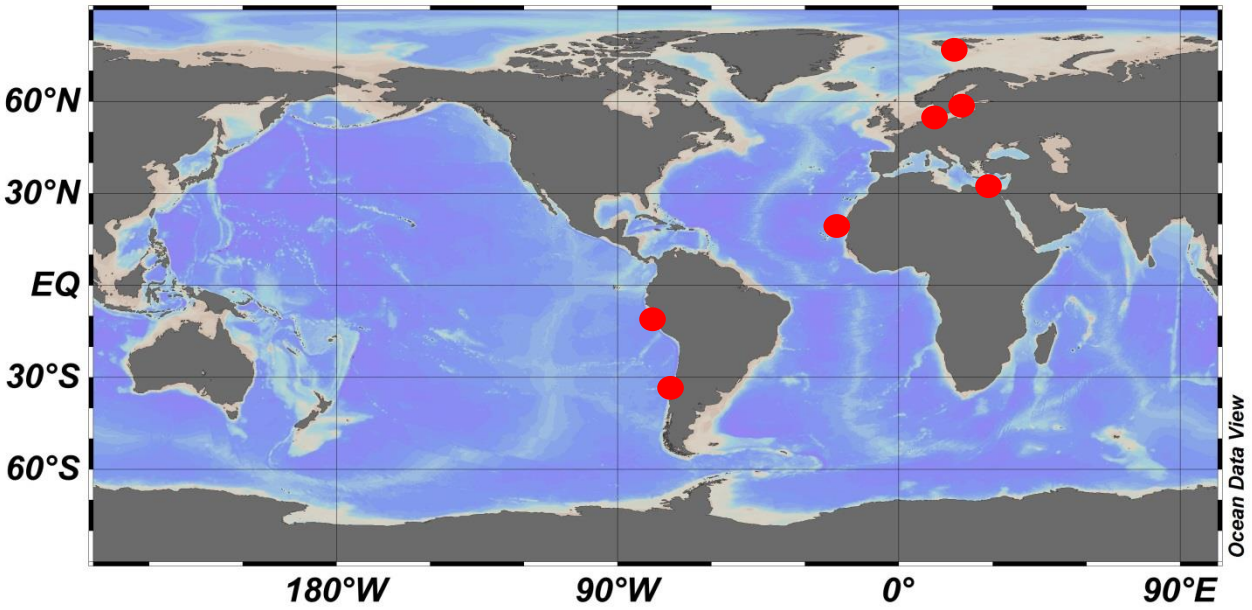


Figure 1: World map of the sampling locations, highlighted by red dots.

We observed a relatively low number of different *nifH* clusters, compared to water column diversity (Farnelid et al., 2011), when analyzing our full sequence dataset, i.e. all sampling sites (Fig. 2). Altogether we identified 30 major clusters when collapsing branches at 97% nucleotide identity. Detected *nifH* sequences from the full dataset clustered with species related to different Cluster III clades as defined by Zehr and Turner (2001), as well as with *Chlorobium, Pelobacter sp., Vibrio sp.*, methanogens, and sulfate-reducing bacteria, such as *Desulfovibrio* sp. and *Desulfobulbus* sp.

Table 1: Sampling location details. For further details see Supplementary Tables S2 and S3.

Sampling location		Water depth (m)	Sediment depth (cm)	# of DNA samples	Available parameters
Atlantic	**Mauretania**	46 – 1108	0 – 20	60	Nfix, SR, Corg, NH_4^+, S
Arctic	**NW Svalbard**	1238	0 – 8	11	SR, Corg, NH_4^+, S
Baltic Sea	**Eckernförde Bay**	28	1 – 5	29	Corg, NH_4^+, S
	Gotland Basin	96 – 173	0 – 20	30	Nfix, SR, Corg, NH_4^+, S
Eastern Mediterr. Sea	**West Nile Delta**	492	1 – 10	5	SR, Corg, NH_4^+,
Pacific	**Chile**	707	0 – 5	5	SR, Corg, S
	Peru	70 – 1025	0 – 20	60	Nfix, SR, Corg, NH_4^+, S

Abbreviations: Mediterr, Mediterranean; Nfix, N_2 fixation; SR, sulfate reduction; Corg, organic carbon content; NH_4^+, ammonium; S, sulfide.

Compared to the diazotrophic diversity reported from ocean waters using classical sequencing methods (Zehr et al., 2003) or novel high-throughput approaches (Farnelid et al., 2011), the observed diversity was with 30 major clusters rather low, although we applied the novel high-throughput technique. This discrepancy may result from the fact that cyanobacterial clades largely contribute to the pelagic diazotrophic diversity (Langlois et al., 2008), while they are missing in the benthic realm.

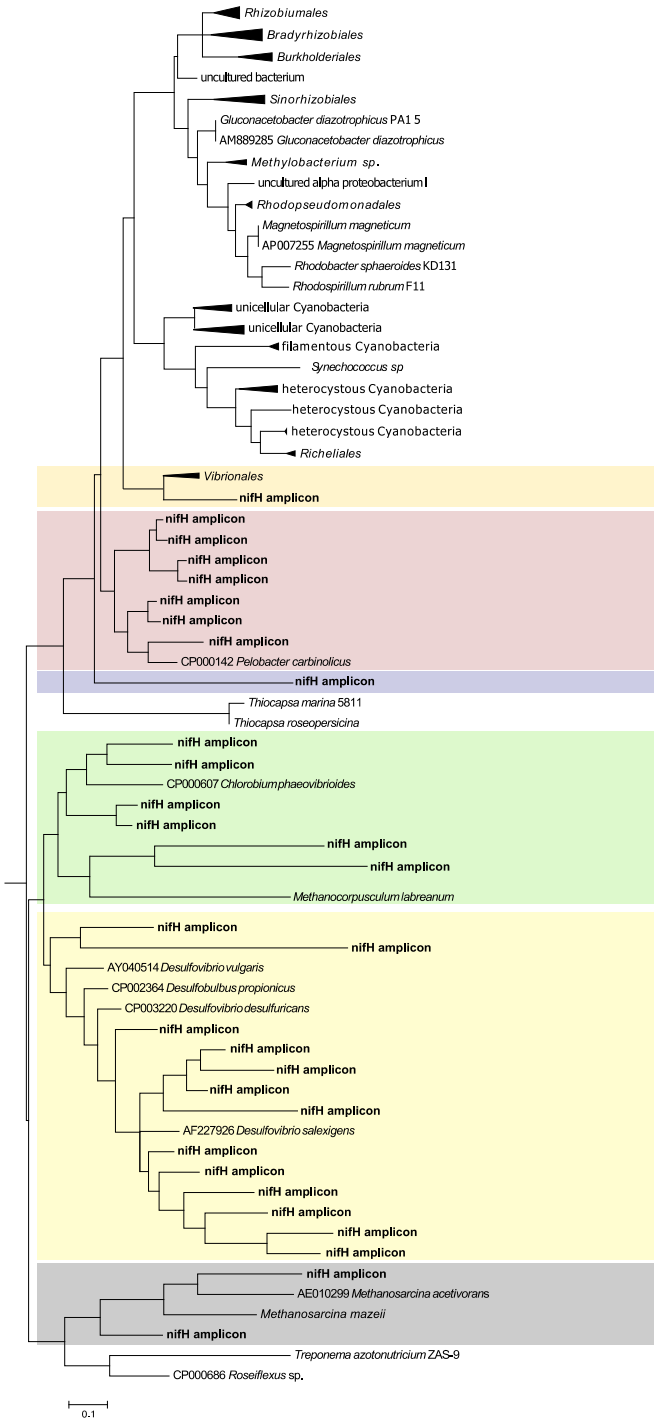


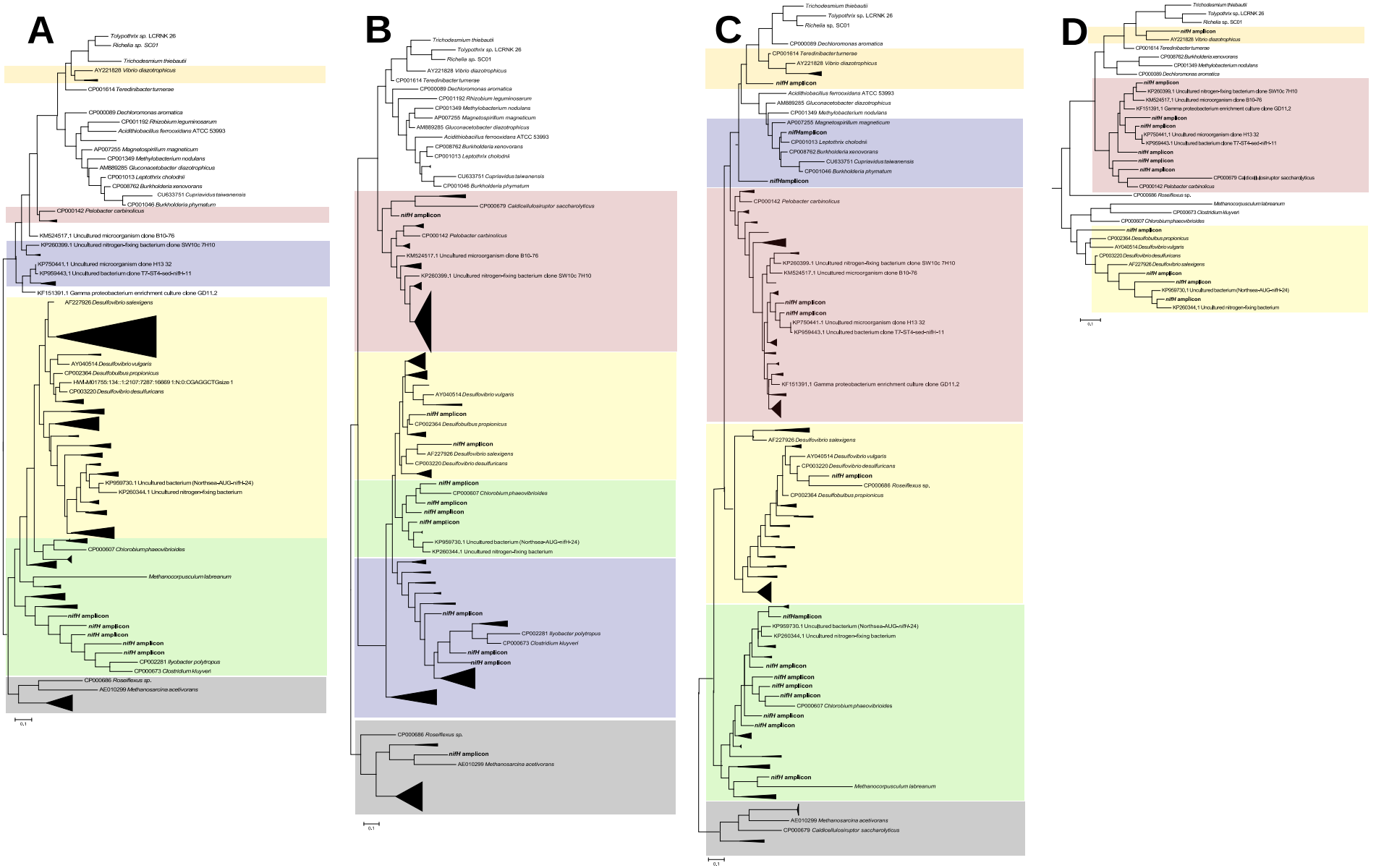
Figure 2: Neighbor- joining tree of *nifH* based on a Muscle alignment of 9445 individual sequences. Clusters were collapsed at a minimum identity level of 97%, and 30 key clusters remained. The sequences amplicon' are given in bold. The colored boxes indicate potential functional groups: Orange for sequences related to *Vibrio sp*., purple denote sequences related to *Pelobacter sp*., blue are other proteobacteria, green denote *Chlorobium* and other Cluster III species, yellow indicates sulfate reducing microbes and grey shows methanogens.

Despite a large variation between the different sites, regarding environmental parameters, such as sulfide, ammonium, and organic matter (Table S2 andS3), the diazotrophic community was relatively similar (Fig. 3A-G). Highest diversity of diazotrophs was present at Eckernförde Bay (Fig. 3A, 331 individual sequences) and in the Gotland Basin (Fig. 3B, 325 individual sequences) and in sediments off Peru (Fig. 3C, 501 individual sequences), while Mauritanian samples showed the lowest grade of diversity with only 11 individual sequences (Fig. 3, D). This overall difference may in parts result from a different sampling strategy, e.g. in Eckernförde Bay, which was sampled over the annual cycle. Thus, phylogenetic groups may occur only under certain conditions once per year, and would therefore be easily overlooked using single cruise observations, are represented in that dataset. However, it may also speak for the development of different diazotrophic communities due to varying environmental factors over time.

Sediments from Svalbard, Chile and the North Alex Mud volcano showed an intermedia grade of diversity with individual sequences of 169, 121 and 102, respectively. The overall difference between these three sites and the sites Eckernförde Bay, Gotland Basin, Peru and Mauritania, may result from the fact that the latter are no methane seep sites. Thus, the phylogenetic nifH diversity at methane sites could be potentially lower than that at the other sampling sites.

Community structure of benthic diazotrophs

In the following, our sequence data is structured according to dominated diversity, thus sulfate reducers, which dominated the nifH gene analysis, are described first.


A
Tolypothrix sp. LCRNK 26
Richelia sp. SC01
Trichodesmium thiebautii
AY221828 Vibrio diazotrophicus
CP001614 Teredinibacter turnerae
CP000089 Dechloromonas aromatica
CP001192 Rhizobium leguminosarum
Acidithiobacillus ferrooxidans ATCC 53993
AP007255 Magnetospirillum magneticum
CP001349 Methylobacterium nodulans
AM889285 Gluconacetobacter diazotrophicus
CP001013 Leptothrix cholodnii
CP008762 Burkholderia xenovorans
CU633751 Cupriavidus taiwanensis
CP001046 Burkholderia phymatum
CP000142 Pelobacter carbinolicus
KM524517.1 Uncultured microorganism clone B10-76
KP260399.1 Uncultured nitrogen-fixing bacterium clone SW10c 7H10
KP750441.1 Uncultured microorganism clone H13 32
KP959443.1 Uncultured bacterium clone T7-ST4-sed-nifH-11
KF151391.1 Gamma proteobacterium enrichment culture clone GD11.2
AF227926 Desulfovibrio salexigens
AY040514 Desulfovibrio vulgaris
CP002364 Desulfobulbus propionicus
HWI-M01755:134::1:2107:7287:16669 1:N:0:CGAGGCTGsize 1
CP003220 Desulfovibrio desulfuricans
KP959730.1 Uncultured bacterium (Northsea-AUG-nifH-24)
KP260344.1 Uncultured nitrogen-fixing bacterium
CP000607 Chlorobium phaeovibrioides
Methanocorpusculum labreanum
nifH amplicon
CP002281 Ilyobacter polytropus
CP000673 Clostridium kluyveri
CP000686 Roseiflexus sp.
AE010299 Methanosarcina acetivorans
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KP260344.1 Uncultured nitrogen-fixing bacterium
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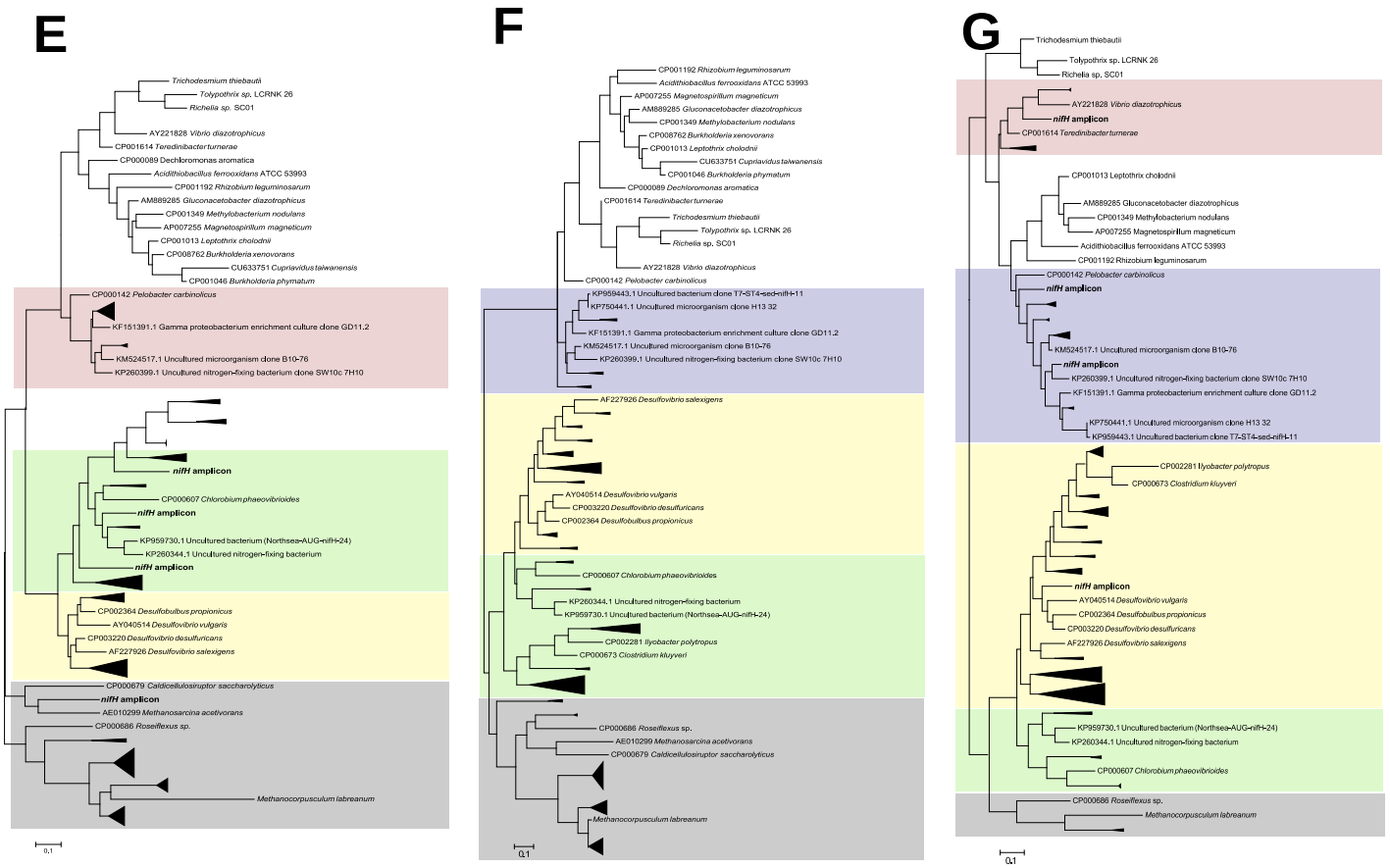

Figure 3: Phylogenetic neighbor- joining tree of *nifH* based on a Muscle alignment for (A) Eckernförde Bay, (B) Gotland basin, (C) Peru, (D) Mauritania, (E) Arctic, (F) Chile and (G) North Alex Mud Volcano. Clusters were collapsed at a minimum identity level of 97%, and 30 key clusters remained. The sequences amplicon' are given in bold. Colors are used as described above.

Several sulfate reducers were shown to fix N_2, in the laboratory, as well as in the environment (Sisler & ZoBell, 1951; Riederer-Henderson & Wilson, 1970; Bertics & Ziebis, 2010). In the Peruvian OMZ, benthic N_2 fixation and sulfate reduction activity depth profiles showed similar qualitative trends, suggesting a coupling of both processes (Gier et al., 2015). Additionally, a coupling of benthic N_2 fixation and sulfate reduction was found in Eckernförde Bay, where integrated rates of both processes correlated, and activity depth profiles overlapped (Bertics et al., 2013). Thus, in the OMZ off Peru and in Eckernförde Bay, N_2 fixation and SR were suggested to be coupled to a large extent.

This suggestion is in line with the dominance of sulfate reducer *nifH* sequences in our dataset (Fig. 3A-G). Sulfate-reducing bacteria of the genus *Desulfobulbus* and *Desulfovibrio* were found our approach at all sampling sites. These genera were highly abundant in previous *nifH* gene analysis, e.g., in the Narragansett Bay (Rhode Island) (Fulweiler et al., 2013), in sediments at Catalina Island (California) (Bertics et al., 2010), and in estuarine sediments in northeast Scotland (Herbert, 1975). Together with our *nifH* sequence analysis, these data strongly indicate a coupling of N_2 fixation to sulfate reduction on a global scale.

The detected *Chlorobium phaeovibrioides* was found in all environments, except Mauritania (Fig. 3D). This is most likely due to the low individual sequence diversity at the respective site. *Chlorobium phaeovibrioides* is a green sulfur bacterium and its photoautotrophic growth occurs with sulfide and sulfur as photosynthetic electron donors (Imhoff, 2003). However, the wide-spread presence of Chlorobium clusters may result rather from dying cells sinking out of the water column, as no light is expected to be present at the sediment surface of the investigated environments. Previously, Bacteria of this type were detected e.g. in the eastern tropical South Pacific (Löscher et al. in prep).

At all sampling sites, except Mauritania and Chile, *nifH* sequences clustered with *Pelobacter carbinolicus* (Fig. 3A-C, E, G). This anaerobic bacterium is an iron and sulfur reducer and has previously been found in the subtidal sediments in Narragansett Bay (Rhode Island) (Fulweiler et al., 2013), as well as in bioturbated muddy sand sediments at Catalina Island (California) (Bertics et al., 2010). Due to its high abundance in *nifH* sequence datasets, which was also shown in our approach, we suggest a possible widespread distribution of *Pelobacter carbinolicus* and it may be one of the main players driving benthic N_2 fixation.

Sequences most likely related to *Vibrio diazotrophicus* clustered only at the sampling sites in Eckernförde Bay, Peru, and Mauritania (Fig. 3A, C, D). Uniquely, *Vibrio diazotrophicus* is capable of N_2 fixation, it reduces nitrate to nitrite (Guerinot et al., 1982) and was found previously in the Peruvian sediments (D'Hondt et al., 2004) and in the Peruvian water column (Löscher et al., 2014). *Vibrio diazotrophicus* is distributed throughout the marine and estuarine environments, and occurs in seawater, sediments, and the gastrointestinal tracts of marine animals (Guerinot et al., 1982), which underlines its abundant occurrence in our sequence approach. However, why this bacterium is absent off Svalbard, the Gotland Basin, Chile and the North Alex Muc Volcano, although it is an ubiquitous organisms, remains unknown.

While N_2 fixation activity has been identified for sulfate reducers in marine sediments before (Fulweiler & Nixon, 2009; Bertics et al., 2013; Gier et al., 2015), the role of N_2 fixation activity by methanogens is largely unclear (Reeve, 2012). We identified a pool of methanoarchaea, mainly consisting of the family *Methanosarcinacea (Methanosarcina acetivorans)* for the environments Eckernförde Bay, Gotland Basin, and Peru (Fig. 3A-C). Why this abundant species was not found in Mauritanian sediments could be due to the low sequence diversity at this particular station. Thus we speculate that *Methanosarcinacea* is also found in Mauritanian sediments but we missed it. *Methanosarcina acetivorans* is a methanogen that was found in diverse environments and is known for its capability to metabolize carbon monoxide to form acetate and formate (Galagan et al, 2002). Additionally a methanogen closely related to *Methanocorpusculum labreanum* was only detected at the three seep sites (Svalbard, Chile, and North Alex Mud Volcano) but not in the other environments. *Methanocorpusculum labreanum* grows while it produces methane from carbonic acid or formate, but not from methanol, methylamines, or acetate(Zhao et al., 1989). *Methanosarcinales*-related anaerobic methanotrophs have previously been found at the Eckernförde Bay sampling site (Treude et al., 2005), which is in line with our sequencing approach.

Statistical analysis and correlation results

The high abundance of sulfate reducers in phylogenetic *nifH* approaches is in line with our multivariate statistical survey on rates of N_2 fixation and environmental descriptors (sulfate reduction, sediment depth, sampling location, ammonium, organic carbon, sulfide, 120

cases, Table S2). Strongest positive correlation was found between N_2 fixation and sulfate reduction (Fig. 4). This is in line with our *nifH* gene analysis, which showed that sulfate reducers were the dominant organisms in all environments. Sulfate reducers have an ubiquitous distribution in the marine sediments (Jørgensen, 1982; Zehr et al., 1995; Steppe & Paerl, 2002) and they were shown to actively fix N_2 in culture experiments (Riederer-Henderson & Wilson, 1970). In conclusion to the above mentioned factors, we suggest a widespread distribution of sulfate-reducing diazotrophs in the benthic environment.

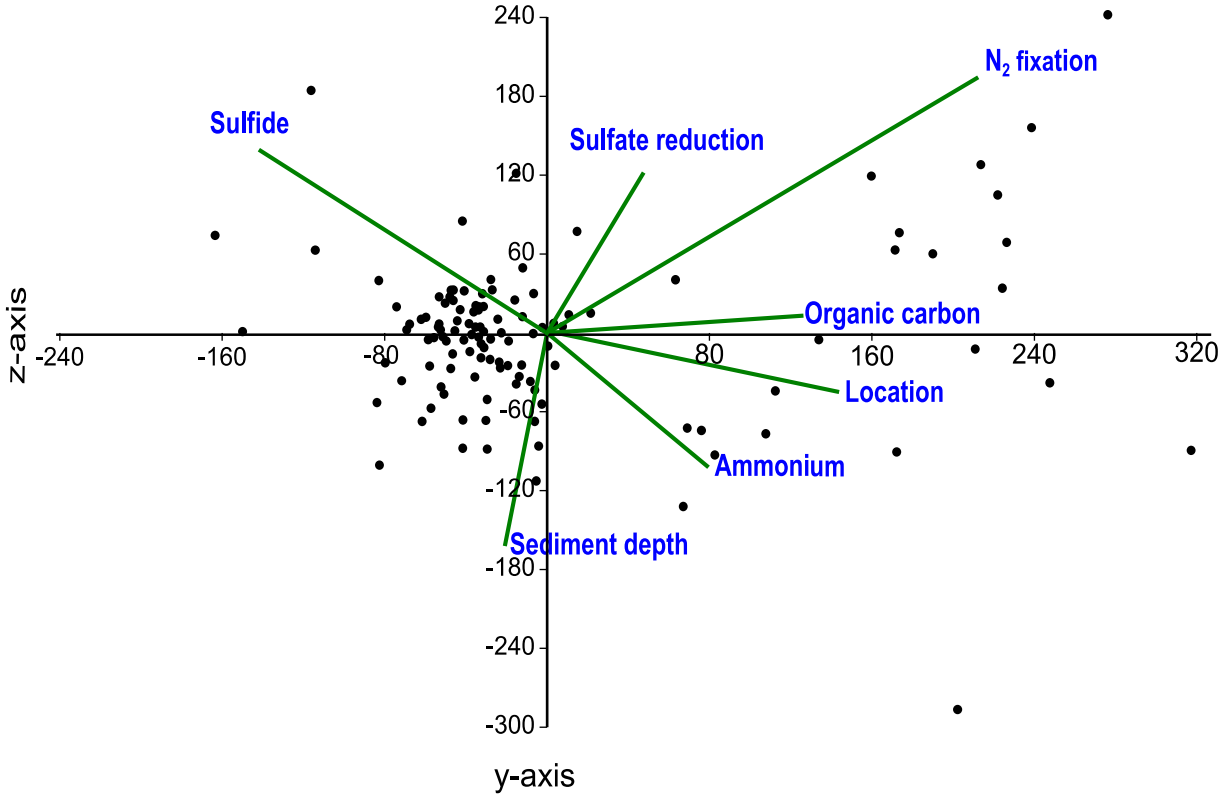

Figure 4: Correlation biplot of N_2 fixation and environmental descriptors. Samples are displayed as dots while variables are displayed as lines. The length of the line provides information about the strength of that correlation. The strongest positive correlation was identified between N_2 fixation and sulfate reduction, as well as organic carbon availability; which are all in the same quadrant. The quadrant opposite of N_2 fixation defines negative correlation, i.e. N_2 fixation was negatively correlated to increasing sediment depths.

Furthermore, the statistical analysis showed a positive correlation between N2 fixation and the organic carbon content (Fig. 4). A strong positive correlation between N_2 fixation and organic carbon was suggested in a previous study from sediments off Peru (Gier et al., 2015).

This result may be also related to the fact that, in our approach, a majority of the detected *nifH* sequences clustered with heterotrophic bacteria strains. In general, microbial processes such as N_2 fixation and sulfate reduction, by potentially heterotrophic organism are dependent on organic carbon (Jørgensen, 1983; Howarth et al., 1988; Fulweiler et al., 2007).

The statistical approach found a negative correlation of N_2 fixation with sediment depth (Fig, 4). This is in line with our observations, as N2 fixation activity profiles decrease with increasing sediment depth (Bertics et al., 2013; Gier et al., 2015), which is in turn dependent on organic matter content (Fulweiler et al., 2007; Bertics et al., 2010;Gier et al., 2015).

Additionally, no correlation between N_2 fixation and the factors location, sulfide, and ammonium were found. This result indicates that diazotrophs are not dependent on location; what we see also in our molecular analysis. However, what is surprising is the fact that diazotrophs do not seem to be negatively impacted by ammonium and sulfide, both classical inhibitors of diazotrophs (Knapp, 2012). However, diazotrophs in combination with ammonium is still a topic of much debate and the remaining question is why they fix N_2 when reduced N species are available. While several environmental parameters, such as, oxygen and organic matter were shown to influence N_2 fixation in a positive or negative way (Fig. 4), uncertainty exist how bioavailable N influences diazotrophs (Knapp et al., 2012 and references therein). For example, N_2 fixation was still active at 2022 µM ammonium in sediments of the Peruvian OMZ (Gier et al., 2015), and in Eckernförde Bay N_2 fixation was found at ammonium levels of up to 1000 µM (Bertics et al., 2013). These findings suggest that N_2 fixation still proceed at considerable high ammonium concentrations, which was also shown by the statistical approach.

The other potential inhibitor of benthic diazotrophs is sulfide, which had no impact on N2 fixation in the RDA model (Fig. 4). Sulfide is an ihibitor for many biological processes (Reis, et al., 1992; Joye & Hollibaugh, 1995) and could potentially affect N_2 fixation (Tam et al., 1982). In the Peruvian OMZ sediments, sulfide was identified as a possible inhibitor of diazotrophs (Gier et al. 2015).

Thus, controlling factors of benthic diazotrophs seem to be very environment dependent and also the type of diazotroph, i.e. which metabolic degradation pathway is performed, seemed to be an important factor.

Does benthic N_2 fixation really matter?

This study provides evidence for a widespread distribution of benthic diazotrophs. Therefore, we suggest a considerable contribution of benthic N_2 fixation to the global marine N budget. To investigate this potential impact of benthic diazotrophs on the marine N budgets, we aimed to calculate the impact of the environments where N sink and N source data are available. These environments were the study sites off Mauritania, Peru, and Eckernförde Bay:

The Mauritanian sediments are regarded as an N sink for dissolved inorganic N (Dale et al. 2014). Integrated benthic N_2 fixation rates in this area had a maximum at the shelf (90 m water depth) with 0.15 ± 0.004 mmol m^{-2} d^{-1} and lowest rates of 0.08 ± 0.002 mmol m^{-2} d^{-1} at the deepest site (1108 m) (J. Gier unpubl. data). Denitrification rates at the shelf (98 m) were 1.8 mmol m^{-2} d^{-1} and 0.2 mmol m^{-2} d^{-1} at the deep site (1113 m) (Dale et al., 2014). According to the above ins and outs of N, N_2 fixation could counteract between 8% and 40% of the N loss between the shelf and the deepest site, respectively. Hence, diazotrophs in the Mauritanian OMZ sediments have, at certain sites, a considerable attenuating effect on the loss of fixed N from the benthic environment.

The Peruvian OMZ sediments were previously regarded as N sinks for fixed N; however, recent results show that shelf sediments off Peru are densely populated by of Marithioploca spp. mats, which were releasing large amounts of dissolved inorganic N, making these sediments rather recycling sites for N (Sommer et al., in prep). Benthic N_2 fixation off Peru (Gier et al., 2015) accounted for <1% of the ammonium flux, while the dissimilatory nitrate reduction to ammonium was estimated to contribute 63% and account for an average of 17 % of the ammonium flux at 142 m (Sommer et al., in prep).

The sampling site in the Eckernförde Bay was previously investigated in terms of N budget calculations; thus, benthic N_2 fixation was measured and compared with modeled N loss rates (Bertics et al., 2013). Fixed N loss rates were calculated to be 0.08 mmol N m^{-2} d^{-1} (Dale et al., 2011), while 0.08 mmol N m^{-2} d^{-1} was measured for benthic N_2 fixation in the same season (Bertics et al., 2013). Thus, N loss equals N gain in this area; however, no direct N budget turnover rates are available for Eckernförde Bay and sediments probably undergo seasonal and temporal variations (Joye & Paerl, 1994).

We conclude that the contribution of diazotrophs to the N sources is environment dependent, and different environmental factors, such as oxygen, sulfide and ammonium, are playing a key role in controlling N_2 fixation (Fulweiler et al., 2007; Gier et al., 2015).

Conclusion

Our study provides the first high-throughput sequencing approach of benthic diazotrophs on a global scale. The detected *nifH* sequences were dominated by sulfate reducers but showed low variation regarding sequence diversity between the investigated sampling sites, what suggests that in most environments, although they are different in terms of environmental parameters, that same diazotrophs perform N_2 fixation. The statistical approach revealed a positive correlation between N_2 fixation and sulfate reduction; thus, further supporting a substantial link between these processes. We conclude that benthic sulfate-reducing diazotrophs contribute, to a so far unknown extent, to the fixed N pool in the ocean. Budget calculations reveal that at certain environmental conditions, benthic N_2 fixation can contribute to the fixed N pool. In order to provide a consistent spatial diazotrophic diversity pattern, future research should aim to include the contribution of benthic N_2 fix into biogeochemical models and N budget calculations to come to more realistic estimates.

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Chapter 5

Final discussion and conclusions

Preface

The presented thesis gives new insights into the distribution, magnitude and controlling processes of benthic diazotrophs, as well as their community diversity. Following environments were investigated:

OMZ environments for the determination of benthic N_2 fixation were:

- sediments off Peru in the eastern tropical South Pacific along the continental margin, with anoxic bottom water conditions
- sediments off Mauritania in the eastern tropical North Atlantic, which had moderate oxygen bottom water concentrations

Diazotrophic diversity studies were carried out with sediment from the:

- seasonally hypoxic Eckernförde Bay in the Baltic Sea
- seasonally anoxic Gotland Basin, located in the central Baltic Sea
- OMZ off Peru, located in the eastern tropical South Pacific
- OMZ off Mauritania, located in the eastern tropical North Atlantic
- Arctic off West Svalbard on the gas hydrates and methane venting Vestnesa Ridge
- Methane seep area at the continental margin off Chile
- North Alex Mud Volcano in the eastern Mediterranean Sea, at the West Nile Delta

In the following sections, I present a summary of the major findings and a final conclusion. Section 2 is a critical review of the applied methods in this thesis, and in the last section an outlook for required research on benthic diazotrophs is given.

1. Benthic N_2 fixation in the marine environment

1.1 Benthic N_2 fixation in OMZ sediments

The results presented in chapter 2, show that benthic N_2 fixation occurred throughout the sediments along a depth transect through the Peruvian OMZ. Those N_2 fixation activities were often overlapped by sulfate reduction depth profiles, suggesting a potential coupling of both processes. Molecular analysis of the *nifH* gene revealed the presence of sulfate-reducing bacteria that have been found to be involved in benthic N_2 fixation in other environments. Controlling factors for benthic diazotrophs were the organic matter content in sediments, as well as sulfide, which potentially inhibited diazotrophs at the respective site.

The data presented in chapter 2, was determined from a depth transect through the upwelling region off Mauritanian. This region does not exhibit anoxic conditions, yet, but is predicted to increase in the future. N_2 fixation rates were abundant throughout the sediments and rates often overlapped with sulfate reduction. However, also iron reduction was identified to potentially being involved in N_2 fixation. The molecular analysis of the *nifH* gene revealed the presence of sulfate- and iron reducers in the sediment. Off Mauritania, burrowing macrofauna was found to potentially create biogeochemical zonation patterns in the sediment, as well as remove bioavailable N compounds, which could enhance N_2 fixation in deeper layers. Additionally, a comparison between Peruvian and Mauritanian microbial rates and environmental parameters was conducted

Overall, this study aimed to answer the research questions stated in the introduction. In the following, these questions will be discussed and reviewed.

1.1.1 Benthic N_2 fixation and coupled microbial processes in the OMZs off Mauritania and Peru

So far, benthic N_2 fixation has been investigated in a limited number of environments (Fulweiler et al., 2007; Bertics et al., 2010, 2012) and has not been measured in the Peruvian and Mauritanian OMZ sediments before. Thus, the controlling environmental factors for diazotrophs in these environments were largely unknown.

In the present study, we detected N_2 fixation at every sampling site and in every sampling depth, in both OMZs (Fig. 1). Both environmental systems, off Peru and off the Mauritanian, revealed N_2 fixation rates of a similar range, which is explained by the fact that both systems

have a high productivity in the water column, resulting in a high sedimentation of organic matter to the seafloor. In both system benthic N_2 fixation was in the range as detected for other organic-rich environments, such as in Eckernförde Bay (0.08 – 0.22 mmol N m^{-2} d^{-1}) (Bertics et al., 2013), see also chapter 1, Table 2, whereas, coral reef sediments exhibit much higher N_2 fixation rates (6.09 ± 5.62 mmol N m^{-2} d^{-1}) (Capone, 1983).

The following data refers to integrated rates over 0 – 20 cm sediment depth. In the Peruvian OMZ, N_2 fixation was high at the shelf stations and low at the three deeper sites, which was also observed for the Mauritanian OMZ (Fig. 1). The lowest N_2 fixation rate was determined at 407 m, while the highest N_2 fixation rate in the Peruvian OMZ was determined at the 236 m site (0.01 – 0.41 mmol m^{-2} d^{-1}). The Mauritanian benthic N_2 fixation was highest (0.08 – 0.15 mmol m^{-2} d^{-1}) at the 90 m site. Overall, N_2 fixation rates were higher in the Peruvian OMZ than in the Mauritanian OMZ. Sulfate reduction rates were higher off Mauritania (1.4 – 6.4 mmol m^{-2} d^{-1}) than off Peru (0.2 – 4.6 mmol m^{-2} d^{-1}) (Fig. 1), what might be a result of unfrozen storage during storage, which was shown to result in an underestimation of rates (Røy et al., 2014).

At both OMZ sites, sulfate-reducing bacteria were identified to be mainly involved in benthic N_2 fixation. Iron reducers were potentially involved in N_2 fixation in the Mauritanian OMZ but they were not found in the Peruvian OMZ.

The reason that iron reducers are possibly involved in benthic N_2 fixation is the different chemical zonation of the sediments off Mauritania, compared to Peru. O_2 defines the redox cascade in marine sediments (Jørgensen, 1983; Jørgensen & Kasten, 2006). While bottom water O_2 concentrations in the Peruvian OMZ were below the detection limit, the Mauritanian OMZ exhibit moderate O_2 concentrations of ~40 µM (Chavez & Messié, 2009; Karstensen et al., 2008). Due to the moderate O_2 concentrations, sediments off Mauritania revealed another chemical zonation pattern (Jørgensen, 1983; Jørgensen & Kasten, 2006) than sediment off Peru. Sediments in the Mauritanian OMZ followed the redox cascade, which means that it is first nitrate reduction that succeeds the aerobic respiration zone, followed by ferric iron-, manganese-, and sulfate reduction. The Peruvian OMZ is anoxic with O_2 concentrations below the detection limit in mid-waters (Stramma et al., 2008). Thus, the redox cascade off Peru follows anaerobic processes, i.e. sulfate reduction.

1.1.2 Relevance of benthic N_2 fixation in the marine N budget

For the Mauritanian OMZ benthic N_2 fixation can account between 8% and 40% of the N loss (i.e. denitrification (Dale et al., 2014)) between the shelf and the deepest site, respectively. Overall, this highlights the underestimated potential for benthic diazotrophs to contribute to the marine N budget.

The Peruvian sediments were regarded as sink sites for N. Recently, the sulfide-oxidizing *Marithioploca* sp. was found off Peru (Salman et al., 2011), which releases large amounts of dissolved inorganic N into the sediments, making them recycling sites for N. In these sediments, benthic N_2 fixation was accounted for <1% of the ammonium flux, while the dissimilatory nitrate reduction to ammonium was estimated to contribute 63% and accounts for ~ 17 % of the ammonium flux at 142 m (Sommer et al., in prep).

Recently, benthic N_2 fixation (0.08 mmol N m^{-2} d^{-1}) was investigated in the seasonal hypoxic Eckernförde Bay and was compared to modeled N loss data (0.08 mmol N m^{-2} d^{-1}) (Dale et al., 2011), which resulted in equal budgets rates (Bertics et al., 2013).

Hence, diazotrophs in the Mauritanian and Peruvian OMZ sediments, as well as in other organic rich sediments, can have a considerable effect on the loss of fixed N from the benthic environment.

1.1.3 Factors controlling benthic diazotrophs in different OMZs and future predictions

The major environmental factors controlling benthic diazotrophs were organic matter availability, sulfide, as well as O_2. O_2 defines the redox cascade, what was discussed in section 1.1.1 (Jørgensen, 1983; Jørgensen & Kasten, 2006). Organic matter is high in both regions, as both are upwelling areas. However, the detected organic matter content was higher off Peru than off Mauritania, which is a potential result of fast turnover rates in the sandy sediment off Mauritania.

Benthic N_2 fixation was potentially inhibited by sulfide in the Peruvian sediment, which has not been seen in Mauritanian sediments. However, there are hardly enough studies that investigated N_2 fixation and inhibition by sulfide (Tam et al., 1982).

Ammonium is regarded as another inhibitor for N_2 fixation (Knapp, 2012). Ammonium concentrations were high in the Peruvian OMZ (up to 1106 µM) and low in the Mauritanian OMZ (up to 88 µM). Highest N_2 fixation off Peru was measured in sediments with relatively

high ammonium. Overall, ammonium concentrations did not seem to inhibit benthic N_2 fixation in sediments of the Peruvian and Mauritanian OMZ.

Another potential controlling factor for benthic diazotrophs is burrowing macrofauna, which was found in the OMZ off Mauritania. The burrowing organisms can increase the organic matter content in deeper sediment layers and enhanced microbial activities in these depths. However, in this study we measured bioirrigation, which is the mixing of overlaying seawater (Meysman et al., 2006; Kristensen et al., 2012). By this process, organic matter could be transferred into the sediment as well and several burrowing species perform bioturbation and bioirrigation simultaneously. Bioirrigation was high at the shelf and low in deeper sites. Additionally, bioirrigating macrofauna can create a three-dimensional zonation pattern in the sediment. Thus O_2 is introduced deeper into the sediment and alters the chemical zonation. This could have enhanced N_2 fixation in the sediments off Mauritania, by providing potential niches by removed N compounds. A coupling of N_2 fixation to sulfate-reducing bacteria was previously observed in bioturbated coastal sediments off Catalina Island (California, USA) (Bertics et al., 2010), further indicating that N_2 fixation is not absent from bioirrigated and bioturbated sediments, as it was the classical view.

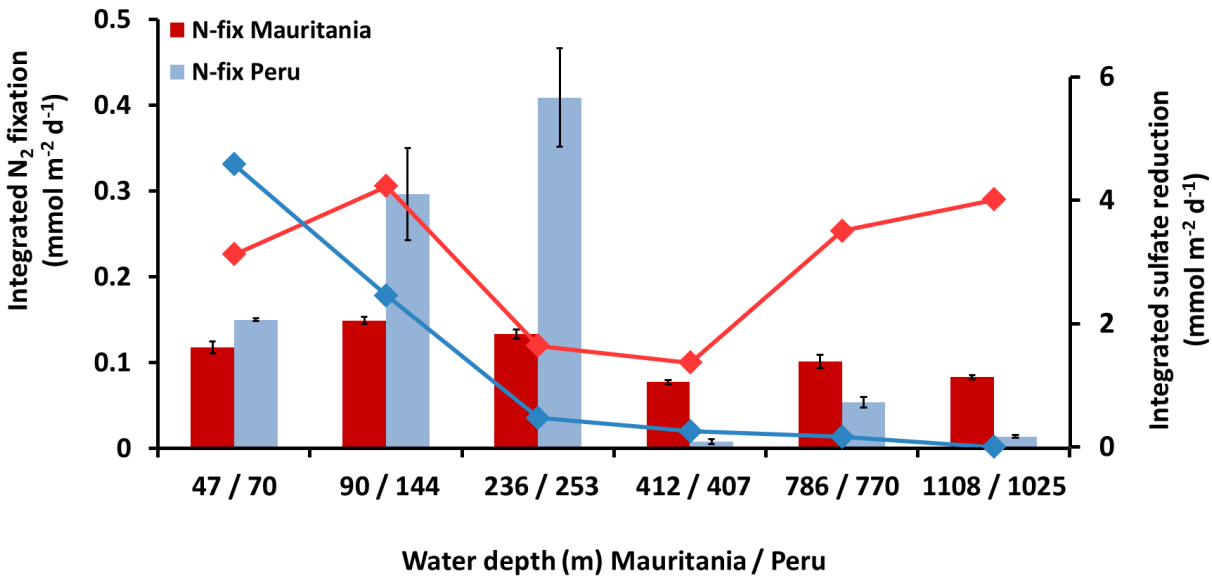

Fig. 1: Benthic integrated (0-20 cm) N_2 fixation and sulfate reduction rates from the Peruvian and the Mauritanian OMZ.

1.2. Benthic diazotrophic diversity in marine environments

Chapter 3 reports the first high-throughput analysis of benthic diazotrophs from various ocean regions. Sediments were taken at 7 different sampling locations and included material from: the Pacific OMZ including Peru and Chile, the Atlantic OMZ off Mauritania, the Arctic seep sediments off Svalbard, a mud volcano in the Mediterranean Sea, and two sampling sites in the Baltic Sea, including the seasonally hypoxic Eckernförde Bay and the seasonally anoxic Gotland Basin. A high-throughput sequencing analysis was determined, which revealed a low diversity between the different sampling locations. The applied redundancy analysis detected a positive correlation of N_2 fixation with sulfate reduction.

1.2.1 Community diversity and structure of benthic diazotrophs

Detected *nifH* genes were dominated by sulfate-reducing bacteria. This was observed for all environments and thus, suggests a great potential for sulfate-reducing bacteria being the main diazotrophs in the benthic environment. Previous studies reported that sulfate reducers were often dominating or abundant in *NifH* gene analysis (Bertics et al., 2010, 2012; Fulweiler & Nixon, 2012). However, due to the various environments in our approach, for example also methane seep areas, a larger diazotrophic diversity was expected. The diazotrophic diversity was rather small and was composed of 30 main clusters, which is low compared to the water column (Farnelid et al., 2011; Löscher et al., 2014). This finding may result from the fact that in water column studies usually several hundred samples are being analyzed, while we had in our survey 200. Water column samples are better to access and to extract than sediment samples, furthermore there is limited sequence data in the gene bank.

It is interesting to note that detected diazotrophs were also related to the iron-reducing bacterium *Pelobacter carbinolicus*, which was found in several benthic environments before. This is in line with the observation from the Mauritanian OMZ. Further detected diazotrophs were related to the family *Methanosarcinacea* and *Chlorobium, as well as to Vibrio diazotrophicus.*

Overall, the accomplished diversity results demonstrate that in most environments, although they are different, N_2 fixation could be performed by the same diazotrophs, i.e. sulfate reducers and potentially iron reducers.

1.2.2 What are the environmental control of the diazotrophic diversity

As the redundancy analysis revealed, one of the environmental factors that controlled the benthic diazotrophs was sulfate reduction, which is in line with our observations of the molecular analysis. Accordingly, sulfate reducers were the main diazotrophs in the *nifH* analysis of all environments. Furthermore, the analysis revealed a positive correlation between N_2 fixation and the organic carbon content. This finding is also in line with our Peru results, where organic matter was also identified as one of the main controlling factors for benthic diazotrophs.

The fact that the availability of the organic material is a major driver for microbial processes such as N_2 fixation and sulfate reduction, has been previously suggested (Jørgensen, 1983; Howarth et al., 1988; Fulweiler et al., 2007).

The observations of the link between benthic diazotrophs and sulfate-reducing bacteria highlight a crucial importance for future research on these bacteria and its potential to provide an important link to the marine N budget.

2. Critical review on used methodology

2.1 Acetylene reduction assay

The acetylene reduction assay (Stewart et al., 1967; Hardy et al., 1968; Capone, 1988) is a widely used method to determine N_2 fixation in marine sediments; however, in recent years it is subject to critical discussion (Fulweiler et al., 2015). The acetylene reduction assay is an *in situ* method to detect activity of the nitrogenase enzyme in a sample, based on the reduction of acetylene to ethylene by the nitrogenase. Thus, N_2 fixation is measured indirectly, by the ability of the nitrogenase to break the triple bond of acetylene. To convert from ethylene to N_2, a ratio of 3:1 is appropriate for benthic samples, whereas a 4:1 ratio gave good agreements with $\delta^{15}N_2$ results for planktonic samples (Capone, 1993). The ratio is an factor that is not consistent throughout the scientific community. While most of the benthic studies use a ratio of 3:1 (Bertics et al., 2012; Cole & McGlathery, 2012; Seitzinger & Garber, 1987), a ratio of 4:1 is used in other benthic studies (Charpy et al., 2007). Thus, a direct comparison between N_2 fixation rates should not be done directly and the applied ratio should be verified.

Other critical points concerning this method deal with the acetylene's capacity to potentially inhibit related microbial processes like denitrification, methanogenesis, sulfate reduction and N_2 fixation itself, as well as the sensitivity of the flame ionization detector to ethylene (Capone, 1988 and references therein). It is recommended to have only a short-term exposure of the sample to ethylene; but with a short term exposure no incubation over time can be accomplished and therefore, no appropriate N_2 fixation rates over time can be determined (Capone, 1988 and references therein).

Recently, the addition of acetylene was revealed to induce a community shift of the benthic diazotrophs (Fulweiler et al., 2015). High-throughput sequencing was applied to detect differences in sediments with and without acetylene. Already after 7 hours, significant differences were found for the microbial activity of all microbes identified in the sediment. Nevertheless, a community shift would rather lead to an underestimation of absolute N_2 fixation rates.

The $\delta^{15}N_2$ method (Montoya et al., 1996) is another approach to determine N_2 fixation rates; however, this method was also revealed to underestimate N_2 fixation rates when $\delta^{15}N_2$ was injected as gas bubble (Mohr et al. 2010). Measuring N_2 fixation with the gas bubble-addition method was always critical for sediments, since the gas is equilibrating too slowly and not very consistent (Capone & Budin, 1982; Dekas et al., 2009).

The newly invented dissolution method was developed to measure N_2 fixation in the ocean (Mohr et al., 2010). In the dissolution method the $\delta^{15}N_2$ tracer is injected not as a gas bubble, but as a dissolved gas in seawater. A comparison between the bubble-addition and the dissolution method, revealed the largest underestimation to be 570% in the Atlantic (Großkopf et al., 2012). The problems in the bubble-addition method with not well dissolved samples are not given any more, since the $\delta^{15}N_2$ is added as a gas that is already dissolved in seawater. This ensures uniformly mixing right after $\delta^{15}N_2$ injection. Therefore, the dissolution method seems to be a step in the right direction to balance the marine N_2 budget. However, if the new method is suitable for marine sediments still needs to be tested.

Methods to measure N_2 fixation in marine sediments are limited and direct measurements are not possible because of the problem to measure the produced N_2 in the large N pool

(Capone, 1988; Fulweiler et al., 2015). Thus, N_2 fixation measurements are still a topic of much debate and due to the fact that the global N budget are not balanced, yet (Gruber, 2008); it will be also in the near future.

Overall, the application of the acetylene reduction assay is cost effective, widely used and a simple method to determine the nitrogenase activity, i.e. N_2 fixation (Capone, 1993), which is why it was used in this study. However, methods using $\delta^{15}N_2$ (Mohr et al., 2010) followed by mass spectrometric measurements of substrates and products and by Nanoscale secondary ion mass spectrometry (nanoSIMS) are more sensitive and are potentially applicable for benthic environments in the future (Capone et al., 2008; Polerecky et al., 2012; Hamersley et al., 2015).

2.2 Radiotracer experiments

For the determination of sulfate reduction rates the whole-core injection method (Jørgensen, 1978; 2006), followed by the cold chromium distillation procedure according to Kallmeyer et al. (2004) was used. Replicate push cores are subsampled from MUC cores, which ensures that the sediment is exposed to only small disturbances while sampling. Additionally, the radioactive tracer is injected in small quantities through silicon filled whole, which further reduces the disturbance of the sample. That samples are not disturbed outweighs the whole-core injection method (Jørgensen, 1978; 2006) versus slurry incubations. Additionally, the whole-core injection method reduces the exposure to oxygen, when compared to e.g. slurry incubations.

Recently, unfrozen storage of sulfate reduction samples was revealed to underestimate sulfate reduction rates (Røy et al., 2014). Storage of samples of up to 3 half-life times of the radio tracer ^{35}S caused the ^{35}S-sulfides to re-oxidatate. In this reaction, FeS is converted to ZnS and the released Fe^{2+} reacts with oygen and forms reactive ferric iron. The ferric iron oxidizes ZnS and FeS, which are the major components of the total reduced inorganic sulfur species, result in the generation of sulfate and hence in an underestimation of sulfate reduction rates.

However, because all sulfate reduction samples in the Peruvian study were treated the same way, we did trust the relative distribution of sulfate-reducing activity along the depth

profiles and recognized potential underestimation of absolute rates. The Mauritanian samples were frozen and thus no underestimation of rates had to be considered.

3. Future research questions

In case of spreading OMZs, future research should measure benthic N_2 fixation and environmental parameters on a regular basis in the OMZs off Peru and Mauritania. These measurements will give information on how these systems may change under anoxic conditions in the future. To verify the distribution, magnitude and relevance of N_2 fixation in these benthic environments, it is important to understand:

- How does the magnitude and distribution of benthic N_2 fixation change when water bodies turn anoxic?
- What are further potential controls for benthic N_2 fixation in oxygen-deficient areas?

This study showed that the ubiquitous sulfate-reducing bacteria are often involved in N_2. In order to get a better estimate for global benthic N_2 fixation rates, future research should aim to investigate additional environments, with different characters. Additionally, the *nifH* gene sequences in this study often clustered with uncultured or unknown diazotrophs. To determine the affiliation and metabolic process of those microorganisms and to get an overall picture of benthic diazotrophs, those organisms should be cultured and sequenced. Regarding these information the following aspects should be addressed:

- Does the diazotrophic diversity change/increase when additional environments are investigated?
- Are sulfate-reducers the main diazotrophs in marine sediments world-wide?
- Which metabolic processes perform the uncultured diazotrophs?

In terms of benthic diazotrophs and ammonium concentrations, future studies should seek into identifying a potential threshold of N_2 fixation inhibition by ammonium, as well as how N_2 fixation is regulated in terms of ammonium:

- Why do diazotrophs fix N_2 when bioavailable N compounds are abundant?
- Is there a certain ammonium concentration that exhibits an activity threshold for benthic diazotrophs?

Up to now, benthic N_2 fixation is not included in any biogeochemical model approach and the only estimate for a benthic N_2 fixation budget that is included in the budget calculations, is from 1988 (Capone, 1988). Thus, future research should include the contribution of benthic N_2 fixation into models, as well as N budget calculations should include more recent estimates. To validate marine biogeochemical models and the marine N budgets, the following research questions should be addressed:

- How can benthic N_2 fixation be implemented into biogeochemical models?
- How do the benthic N_2 fixation rates contribute to the marine N budget?

By answering these research questions, the global importance of benthic N_2 fixation and controlling factors could be validated and uncertainties in benthic N_2 fixation estimates in N budget calculations should be minimized.

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Poster and oral presentations during my PhD study

Oral presentations

- **Gier**, J., Sommer, S., Löscher, C.R., Dale, A.W., Schmitz, R.A. & Treude, T.: Nitrogen fixation in sediments along a depth transect through the Peruvian oxygen minimum zone, Ocean Sciences Meeting, Honolulu, Hawai'i, USA, February 2014
- **Gier**, J., Sommer, S., Löscher, C.R., Dale, A.W., & Treude, T.: Microbial benthic nitrogen fixation inside and below the Peruvian oxygen minimum zone, International Symposium on Environmental Biogeochemistry, Wuhan, P. R. of China, October 2013

Poster presentations

- **Gier**, J., Sommer, S., Löscher, C.R., Dale, A.W., & Treude, T.: Nitrogen fixation in sediments along a depth transect through the eastern boundary upwelling systems, American Geophysical Union, San Francisco, USA, December 2015
- **Gier**, J., Sommer, S., Löscher, C.R., Dale, A.W., Schmitz, R.A. & Treude, T.: Nitrogen fixation in sediments along a depth transect through the Peruvian oxygen minimum zone, Summer School on Oxygen Minimum Zones, Research station Kristineberg, Sweden

Participation on expeditions during my PhD study

- Atlantic: RV "Meteor" 107, 05.-07.2014, Fortaleza (Brazil) – Las Palmas (Canary Islands), project: SFB754
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Supplementary material to chapter 4

Table 1: Primer sequences for MiSeq sequencing containing a tag sequence, the *nifH* specific region and the Illumina specific linker sequence.

primer ID	sequence 5'-3'
Minif-F-46	AATGATACGGCGACCACCGAGATCTACAC AAGGCCTT TATGGTAATT GT TGYGAYCCNAARGCNGA
Minif-F-301	AATGATACGGCGACCACCGAGATCTACAC AGTCTGAC TATGGTAATT GT TGYGAYCCNAARGCNGA
Minif-F-502	AATGATACGGCGACCACCGAGATCTACAC CATGAGGA TATGGTAATT GT TGYGAYCCNAARGCNGA
Minif-F-711	AATGATACGGCGACCACCGAGATCTACAC CTGAAGAG TATGGTAATT GT TGYGAYCCNAARGCNGA
Minif-F-1016	AATGATACGGCGACCACCGAGATCTACAC GGTATAGG TATGGTAATT GT TGYGAYCCNAARGCNGA
Minif-F-87	AATGATACGGCGACCACCGAGATCTACAC ACAGGACA TATGGTAATT GT TGYGAYCCNAARGCNGA
Minif-F-346	AATGATACGGCGACCACCGAGATCTACAC ATCGCCTT TATGGTAATT GT TGYGAYCCNAARGCNGA
Minif-F-521	AATGATACGGCGACCACCGAGATCTACAC CCAAGGAA TATGGTAATT GT TGYGAYCCNAARGCNGA
Minif-F-778	AATGATACGGCGACCACCGAGATCTACAC GAACCATC TATGGTAATT GT TGYGAYCCNAARGCNGA
Minif-F-1064	AATGATACGGCGACCACCGAGATCTACAC GTCAACTG TATGGTAATT GT TGYGAYCCNAARGCNGA
Minif-R-174	CAAGCAGAAGACGGCATACGAGAT ACTCTGTC AGTCAGTCAG CC ADNGCCATCATYTCNCC
Minif-R-362	CAAGCAGAAGACGGCATACGAGAT ATGCGCTA AGTCAGTCAG CC ADNGCCATCATYTCNCC
Minif-R-619	CAAGCAGAAGACGGCATACGAGAT CGTAATCG AGTCAGTCAG CC ADNGCCATCATYTCNCC
Minif-R-830	CAAGCAGAAGACGGCATACGAGAT GACTGAGA AGTCAGTCAG CC ADNGCCATCATYTCNCC
Minif-R-1086	CAAGCAGAAGACGGCATACGAGAT GTCTCTGT AGTCAGTCAG CC ADNGCCATCATYTCNCC
Minif-R-195	CAAGCAGAAGACGGCATACGAGAT AGACCACT AGTCAGTCAG CC ADNGCCATCATYTCNCC
Minif-R-386	CAAGCAGAAGACGGCATACGAGAT ATTAGGCC AGTCAGTCAG CC ADNGCCATCATYTCNCC
Minif-R-642	CAAGCAGAAGACGGCATACGAGAT CGTTGCTT AGTCAGTCAG CC ADNGCCATCATYTCNCC
Minif-R-967	CAAGCAGAAGACGGCATACGAGAT GCTTGGAT AGTCAGTCAG CC ADNGCCATCATYTCNCC
Minif-R-1520	CAAGCAGAAGACGGCATACGAGAT TTGCAAGC AGTCAGTCAG CC ADNGCCATCATYTCNCC

Table S2: Sampling site information and biogeochemical parameters of the collected sediment cores. N_2 fixation and sulfate reduction rate were integrated over 0-20 cm sediment depth. The input data for redundancy analysis is shaded in grey (Gotland Basin, Mauritania, and Peru).

Abbreviation: SR, sulfate reduction

Location	Cruise	Station ID	Core ID	Date	Latitude	Longitude	Water depth (m)	Sediment depth (cm)	N_2 fixation (mmol m^{-2} d^{-1})	SR (mmol m^{-2} d^{-1})	Organic carbon (wt%)	Ammonium (µM)	Sulfide (µM)
Chile	SO210	42	ROV 5	07.10.2010	36° 28.26'	73° 40.74'	707	0.5	-	4.22	1.76	-	0.4
Chile	SO210	42	ROV 5	07.10.2010	36° 28.26'	73° 40.74'	707	1.5	-	4.75	-	-	4
Chile	SO210	42	ROV 5	07.10.2010	36° 28.26'	73° 40.74'	707	2.5	-	6.56	1.82	-	12
Chile	SO210	42	ROV 5	07.10.2010	36° 28.26'	73° 40.74'	707	3.5	-	4.80	-	-	15
Chile	SO210	42	ROV 5	07.10.2010	36° 28.26'	73° 40.74'	707	4.5	-	2.58	1.78	-	18
Gotland	AL 355	361	MUC 9	16.06.2010	57°29.98'	20°56.02'	50	0.5	0.0041	0.77	0.13	25	-
Gotland	AL 355	361	MUC 9	16.06.2010	57°29.98'	20°56.02'	50	2	0.0031	0.59	0.13	34	-
Gotland	AL 355	361	MUC 9	16.06.2010	57°29.98'	20°56.02'	50	4	0.0021	1.03	0.08	-	-
Gotland	AL 355	361	MUC 9	16.06.2010	57°29.98'	20°56.02'	50	7.5	0.0005	0.94	0.07	63	-
Gotland	AL 355	361	MUC 9	16.06.2010	57°29.98'	20°56.02'	50	12.5	0.0007	0.04	0.18	85	-
Gotland	AL 355	365	MUC 10	17.06.2010	57°26.49'	20°43.51'	65	0.5	0.0050	0.47	1.47	20	0.23
Gotland	AL 355	365	MUC 10	17.06.2010	57°26.49'	20°43.51'	65	2	0.0078	0.09	1.09	89	0.20
Gotland	AL 355	365	MUC 10	17.06.2010	57°26.49'	20°43.51'	65	4	0.0026	0.01	1.22	124	0.20
Gotland	AL 355	365	MUC 10	17.06.2010	57°26.49'	20°43.51'	65	7.5	0.0016	0.00	0.51	155	0.20
Gotland	AL 355	365	MUC 10	17.06.2010	57°26.49'	20°43.51'	65	12.5	0.0000	0.00	0.98	214	0.23
Gotland	AL 355	366	MUC 11	17.06.2010	57°20.52'	20°34.22'	111	0.5	0.0152	0.86	7.61	192	106
Gotland	AL 355	366	MUC 11	17.06.2010	57°20.52'	20°34.22'	111	2	0.0042	0.31	4.99	200	143
Gotland	AL 355	366	MUC 11	17.06.2010	57°20.52'	20°34.22'	111	4	0.0052	0.23	5.01	na	171
Gotland	AL 355	366	MUC 11	17.06.2010	57°20.52'	20°34.22'	111	7.5	0.0044	0.16	4.90	663	420
Gotland	AL 355	366	MUC 11	17.06.2010	57°20.52'	20°34.22'	111	12.5	0.0017	0.18	4.74	777	752
Gotland	AL 355	370	MUC 12	18.06.2010	57°20.74'	20°35.35'	94	0.5	0.0105	1.52	-	-	-
Gotland	AL 355	370	MUC 12	18.06.2010	57°20.74'	20°35.35'	94	2	0.0090	0.69	-	-	-
Gotland	AL 355	370	MUC 12	18.06.2010	57°20.74'	20°35.35'	94	4	0.0116	0.40	-	-	-
Gotland	AL 355	370	MUC 12	18.06.2010	57°20.74'	20°35.35'	94	7.5	0.0050	0.21	-	-	-
Gotland	AL 355	370	MUC 12	18.06.2010	57°20.74'	20°35.35'	94	12.5	0.0029	0.07	-	-	-
Gotland	AL 355	371	MUC 13	18.06.2010	57°18.70'	20°33.00'	123	0.5	0.0092	1.87	-	-	-
Gotland	AL 355	371	MUC 13	18.06.2010	57°18.70'	20°33.00'	123	2	0.0137	0.45	-	-	-
Gotland	AL 355	371	MUC 13	18.06.2010	57°18.70'	20°33.00'	123	4	0.0084	0.18	-	-	-
Gotland	AL 355	371	MUC 13	18.06.2010	57°18.70'	20°33.00'	123	7.5	0.0056	0.13	-	-	-
Gotland	AL 355	371	MUC 13	18.06.2010	57°18.70'	20°33.00'	123	12.5	0.0035	0.02	-	-	-
Gotland	AL 355	372	MUC 14	18.06.2010	57°20.80'	20°28.39'	160	0.5	0.0095	0.15	-	-	-
Gotland	AL 355	372	MUC 14	18.06.2010	57°20.80'	20°28.39'	160	2	0.0055	0.03	-	-	-
Gotland	AL 355	372	MUC 14	18.06.2010	57°20.80'	20°28.39'	160	4	0.0030	0.03	-	-	-
Gotland	AL 355	372	MUC 14	18.06.2010	57°20.80'	20°28.39'	160	7.5	0.0041	0.04	-	-	-
Gotland	AL 355	372	MUC 14	18.06.2010	57°20.80'	20°28.39'	160	12.5	0.0000	0.00	-	-	-
Mauritania	M 107	658	MUC 13	23.06.2014	18°17.299'	16°18.994'	47	0.5	0.0054	0.215	1.07	1	0
Mauritania	M 107	658	MUC 13	23.06.2014	18°17.299'	16°18.994'	47	1.5	0.0052	0.341	1.10	13	0
Mauritania	M 107	658	MUC 13	23.06.2014	18°17.299'	16°18.994'	47	2.5	0.0039	0.213	0.73	29	0
Mauritania	M 107	658	MUC 13	23.06.2014	18°17.299'	16°18.994'	47	3.5	0.0053	0.157	0.69	33	0
Mauritania	M 107	658	MUC 13	23.06.2014	18°17.299'	16°18.994'	47	4.5	0.0071	0.162	0.92	27	0
Mauritania	M 107	658	MUC 13	23.06.2014	18°17.299'	16°18.994'	47	5.5	0.0078	0.140	1.07	51	0
Mauritania	M 107	658	MUC 13	23.06.2014	18°17.299'	16°18.994'	47	7	0.0062	0.149	1.06	71	0
Mauritania	M 107	658	MUC 13	23.06.2014	18°17.299'	16°18.994'	47	9	0.0052	0.068	0.81	59	31
Mauritania	M 107	658	MUC 13	23.06.2014	18°17.299'	16°18.994'	47	12.5	0.0071	0.193	0.67	80	88
Mauritania	M 107	658	MUC 13	23.06.2014	18°17.299'	16°18.994'	47	17.5	0.0049	0.138	0.80	63	45
Mauritania	M 107	628	MUC 10	21.06.2014	18°15.197'	16°27.002'	90	0.5	0.0057	0.196	0.55	6	0
Mauritania	M 107	628	MUC 10	21.06.2014	18°15.197'	16°27.002'	90	1.5	0.0051	0.040	0.64	8	0
Mauritania	M 107	628	MUC 10	21.06.2014	18°15.197'	16°27.002'	90	2.5	0.0086	0.194	0.75	8	0
Mauritania	M 107	628	MUC 10	21.06.2014	18°15.197'	16°27.002'	90	3.5	0.0111	0.062	0.80	10	0
Mauritania	M 107	628	MUC 10	21.06.2014	18°15.197'	16°27.002'	90	4.5	0.0105	0.124	0.84	54	0
Mauritania	M 107	628	MUC 10	21.06.2014	18°15.197'	16°27.002'	90	5.5	0.0097	0.150	0.72	39	0
Mauritania	M 107	628	MUC 10	21.06.2014	18°15.197'	16°27.002'	90	7	0.0095	0.411	0.62	65	0
Mauritania	M 107	628	MUC 10	21.06.2014	18°15.197'	16°27.002'	90	9	0.0076	0.429	0.68	65	5
Mauritania	M 107	628	MUC 10	21.06.2014	18°15.197'	16°27.002'	90	12.5	0.0068	0.234	0.73	70	46
Mauritania	M 107	628	MUC 10	21.06.2014	18°15.197'	16°27.002'	90	17.5	0.0060	0.104	0.62	69	20
Mauritania	M 107	612	MUC 8	20.06.2014	18°12.945'	16°33.153'	236	0.5	0.0052	0.068	0.81	10	0
Mauritania	M 107	612	MUC 8	20.06.2014	18°12.945'	16°33.153'	236	1.5	0.0065	0.038	0.96	5	0
Mauritania	M 107	612	MUC 8	20.06.2014	18°12.945'	16°33.153'	236	2.5	0.0075	0.090	0.89	20	0
Mauritania	M 107	612	MUC 8	20.06.2014	18°12.945'	16°33.153'	236	3.5	0.0085	0.122	0.84	16	0
Mauritania	M 107	612	MUC 8	20.06.2014	18°12.945'	16°33.153'	236	4.5	0.0074	0.120	0.76	23	0
Mauritania	M 107	612	MUC 8	20.06.2014	18°12.945'	16°33.153'	236	5.5	0.0080	0.077	0.76	18	0
Mauritania	M 107	612	MUC 8	20.06.2014	18°12.945'	16°33.153'	236	7	0.0073	0.065	0.76	35	0
Mauritania	M 107	612	MUC 8	20.06.2014	18°12.945'	16°33.153'	236	9	0.0079	0.102	0.76	30	0
Mauritania	M 107	612	MUC 8	20.06.2014	18°12.945'	16°33.153'	236	12.5	0.0074	0.172	0.80	21	0
Mauritania	M 107	612	MUC 8	20.06.2014	18°12.945'	16°33.153'	236	17.5	0.0045	0.044	0.90	31	0
Mauritania	M 107	554	MUC 5	12.06.2014	18°12.504'	16°35.583'	412	0.5	0.0020	0.005	0.79	14	0
Mauritania	M 107	554	MUC 5	12.06.2014	18°12.504'	16°35.583'	412	1.5	0.0031	0.026	0.87	9	0
Mauritania	M 107	554	MUC 5	12.06.2014	18°12.504'	16°35.583'	412	2.5	0.0036	0.041	1.32	34	0
Mauritania	M 107	554	MUC 5	12.06.2014	18°12.504'	16°35.583'	412	3.5	0.0068	0.042	1.40	21	0

Mauritania	M 107	554	MUC 5	12.06.2014	18°12.504'	16°35.583'	412	4.5	0.0040	0.048	1.47	30	0
Mauritania	M 107	554	MUC 5	12.06.2014	18°12.504'	16°35.583'	412	5.5	0.0043	0.031	1.45	32	0
Mauritania	M 107	554	MUC 5	12.06.2014	18°12.504'	16°35.583'	412	7	0.0044	0.055	1.48	47	0
Mauritania	M 107	554	MUC 5	12.06.2014	18°12.504'	16°35.583'	412	9	0.0043	0.055	1.34	53	0
Mauritania	M 107	554	MUC 5	12.06.2014	18°12.504'	16°35.583'	412	12.5	0.0041	0.114	1.29	46	0
Mauritania	M 107	554	MUC 5	12.06.2014	18°12.504'	16°35.583'	412	17.5	0.0032	0.111	1.31	42	0
Mauritania	M 107	534	MUC 3	10.06.2014	18°11.288'	16°39.328'	789	0.5	0.0025	0.008	2.62	8	0
Mauritania	M 107	534	MUC 3	10.06.2014	18°11.288'	16°39.328'	789	1.5	0.0048	0.008	2.60	21	0
Mauritania	M 107	534	MUC 3	10.06.2014	18°11.288'	16°39.328'	789	2.5	0.0056	0.099	2.53	31	0
Mauritania	M 107	534	MUC 3	10.06.2014	18°11.288'	16°39.328'	789	3.5	0.0051	0.130	2.49	38	0
Mauritania	M 107	534	MUC 3	10.06.2014	18°11.288'	16°39.328'	789	4.5	0.0056	0.114	2.41	42	0
Mauritania	M 107	534	MUC 3	10.06.2014	18°11.288'	16°39.328'	789	5.5	0.0054	0.113	2.41	42	0
Mauritania	M 107	534	MUC 3	10.06.2014	18°11.288'	16°39.328'	789	7	0.0057	0.155	2.79	52	0
Mauritania	M 107	534	MUC 3	10.06.2014	18°11.288'	16°39.328'	789	9	0.0056	0.224	2.80	59	0
Mauritania	M 107	534	MUC 3	10.06.2014	18°11.288'	16°39.328'	789	12.5	0.0053	0.546	2.77	60	0
Mauritania	M 107	534	MUC 3	10.06.2014	18°11.288'	16°39.328'	789	17.5	0.0046	0.072	2.94	69	0
Mauritania	M 107	524	MUC 1	09.06.2014	18°09.991'	16°45.023'	1108	0.5	0.0010	0.003	2.52	10	0
Mauritania	M 107	524	MUC 1	09.06.2014	18°09.991'	16°45.023'	1108	1.5	0.0029	0.020	2.54	28	0
Mauritania	M 107	524	MUC 1	09.06.2014	18°09.991'	16°45.023'	1108	2.5	0.0037	0.071	2.70	42	0
Mauritania	M 107	524	MUC 1	09.06.2014	18°09.991'	16°45.023'	1108	3.5	0.0039	0.119	2.81	57	0
Mauritania	M 107	524	MUC 1	09.06.2014	18°09.991'	16°45.023'	1108	4.5	0.0051	0.182	2.55	61	0
Mauritania	M 107	524	MUC 1	09.06.2014	18°09.991'	16°45.023'	1108	5.5	0.0066	0.141	2.97	65	0
Mauritania	M 107	524	MUC 1	09.06.2014	18°09.991'	16°45.023'	1108	7	0.0052	0.096	3.11	83	0
Mauritania	M 107	524	MUC 1	09.06.2014	18°09.991'	16°45.023'	1108	9	0.0054	0.700	3.19	98	0
Mauritania	M 107	524	MUC 1	09.06.2014	18°09.991'	16°45.023'	1108	12.5	0.0047	0.312	3.18	102	0
Mauritania	M 107	524	MUC 1	09.06.2014	18°09.991'	16°45.023'	1108	17.5	0.0030	0.063	3.21	112	0
NAMV	PE298	-	MUC 4	11.11.2008	31°58.148'	30°08.173'	492	1.5	-	0.94	1.09	11	-
NAMV	PE298	-	MUC 4	11.11.2008	31°58.148'	30°08.173'	492	2.5	-	0.62	1.05	20	-
NAMV	PE298	-	MUC 4	11.11.2008	31°58.148'	30°08.173'	492	3.5	-	0.33	1.05	17	-
NAMV	PE298	-	MUC 4	11.11.2008	31°58.148'	30°08.173'	492	6	-	0.23	1.08	29	-
Peru	M 92	1	MUC 13	11.01.2013	12°13.492'	77°10.511'	70	0.5	0.0088	2.482	4.56	383	0
Peru	M 92	1	MUC 13	11.01.2013	12°13.492'	77°10.511'	70	1.5	0.0136	0.502	3.39	406	143
Peru	M 92	1	MUC 13	11.01.2013	12°13.492'	77°10.511'	70	2.5	0.0135	0.308	3.46	659	335
Peru	M 92	1	MUC 13	11.01.2013	12°13.492'	77°10.511'	70	3.5	0.0104	0.226	3.63	917	509
Peru	M 92	1	MUC 13	11.01.2013	12°13.492'	77°10.511'	70	4.5	0.0125	0.203	3.61	1084	742
Peru	M 92	1	MUC 13	11.01.2013	12°13.492'	77°10.511'	70	5.5	0.0088	0.173	2.86	1245	550
Peru	M 92	1	MUC 13	11.01.2013	12°13.492'	77°10.511'	70	7	0.0087	0.056	4.10	1439	613
Peru	M 92	1	MUC 13	11.01.2013	12°13.492'	77°10.511'	70	9	0.0076	0.032	3.31	1687	574
Peru	M 92	1	MUC 13	11.01.2013	12°13.492'	77°10.511'	70	12.5	0.0066	0.034	3.12	1723	808
Peru	M 92	1	MUC 13	11.01.2013	12°13.492'	77°10.511'	70	17.5	0.0033	0.077	2.77	1929	1019
Peru	M 92	4	MUC 11	09.01.2013	12°18.704'	77°17.790'	144	0.5	0.0236	1.820	9.31	97	0
Peru	M 92	4	MUC 11	09.01.2013	12°18.704'	77°17.790'	144	1.5	0.0305	0.286	10.04	151	0
Peru	M 92	4	MUC 11	09.01.2013	12°18.704'	77°17.790'	144	2.5	0.0242	0.093	8.63	178	0
Peru	M 92	4	MUC 11	09.01.2013	12°18.704'	77°17.790'	144	3.5	0.0309	0.069	8.91	203	0
Peru	M 92	4	MUC 11	09.01.2013	12°18.704'	77°17.790'	144	4.5	0.0251	0.031	9.33	223	0
Peru	M 92	4	MUC 11	09.01.2013	12°18.704'	77°17.790'	144	5.5	0.0157	0.021	9.57	240	0
Peru	M 92	4	MUC 11	09.01.2013	12°18.704'	77°17.790'	144	7	0.0059	0.012	8.77	246	0
Peru	M 92	4	MUC 11	09.01.2013	12°18.704'	77°17.790'	144	9	0.0075	0.008	7.62	254	0
Peru	M 92	4	MUC 11	09.01.2013	12°18.704'	77°17.790'	144	12.5	0.0089	0.006	5.00	293	0
Peru	M 92	4	MUC 11	09.01.2013	12°18.704'	77°17.790'	144	17.5	0.0149	0.011	2.61	282	0
Peru	M 92	6	MUC 6	07.01.2013	12°23.322'	77°24.181'	253	0.5	0.0440	0.186	14.37	49	0
Peru	M 92	6	MUC 6	08.01.2013	12°23.322'	77°24.181'	253	1.5	0.0333	0.027	15.10	108	0
Peru	M 92	6	MUC 6	09.01.2013	12°23.322'	77°24.181'	253	2.5	0.0213	0.022	14.22	168	0
Peru	M 92	6	MUC 6	10.01.2013	12°23.322'	77°24.181'	253	3.5	0.0265	0.028	15.89	246	0
Peru	M 92	6	MUC 6	11.01.2013	12°23.322'	77°24.181'	253	4.5	0.0236	0.032	17.73	296	0
Peru	M 92	6	MUC 6	12.01.2013	12°23.322'	77°24.181'	253	5.5	0.0201	0.016	15.91	369	0
Peru	M 92	6	MUC 6	13.01.2013	12°23.322'	77°24.181'	253	7	0.0118	0.011	15.35	448	0
Peru	M 92	6	MUC 6	14.01.2013	12°23.322'	77°24.181'	253	9	0.0228	0.014	16.15	465	0
Peru	M 92	6	MUC 6	15.01.2013	12°23.322'	77°24.181'	253	12.5	0.0282	0.011	14.46	710	0
Peru	M 92	6	MUC 6	16.01.2013	12°23.322'	77°24.181'	253	17.5	0.0059	0.010	11.05	857	0
Peru	M 92	8	MUC 23	15.01.2013	12°27.198'	77°29.497'	407	0.5	0.0052	0.043	6.08	20	0
Peru	M 92	8	MUC 23	15.01.2013	12°27.198'	77°29.497'	407	1.5	0.0004	0.026	4.65	28	0
Peru	M 92	8	MUC 23	15.01.2013	12°27.198'	77°29.497'	407	2.5	0.0007	0.058	5.37	30	0
Peru	M 92	8	MUC 23	15.01.2013	12°27.198'	77°29.497'	407	3.5	0.0003	0.042	7.47	38	0
Peru	M 92	8	MUC 23	15.01.2013	12°27.198'	77°29.497'	407	4.5	0.0005	0.020	8.71	41	0
Peru	M 92	8	MUC 23	15.01.2013	12°27.198'	77°29.497'	407	5.5	0.0004	0.009	9.35	47	0
Peru	M 92	8	MUC 23	15.01.2013	12°27.198'	77°29.497'	407	7	0.0001	0.010	10.57	57	0
Peru	M 92	8	MUC 23	15.01.2013	12°27.198'	77°29.497'	407	9	0.0000	0.007	9.28	69	0
Peru	M 92	8	MUC 23	15.01.2013	12°27.198'	77°29.497'	407	12.5	0.0000	0.014	9.61	82	0
Peru	M 92	8	MUC 23	15.01.2013	12°27.198'	77°29.497'	407	17.5	0.0000	0.007	9.06	98	0
Peru	M 92	9	MUC 17	13.01.2013	12°31.374'	77°35.183'	770	0.5	0.0012	0.000	5.90	4	0
Peru	M 92	9	MUC 17	13.01.2013	12°31.374'	77°35.183'	770	1.5	0.0039	0.000	5.57	9	0
Peru	M 92	9	MUC 17	13.01.2013	12°31.374'	77°35.183'	770	2.5	0.0053	0.008	4.61	11	0
Peru	M 92	9	MUC 17	13.01.2013	12°31.374'	77°35.183'	770	3.5	0.0057	0.008	5.19	13	0
Peru	M 92	9	MUC 17	13.01.2013	12°31.374'	77°35.183'	770	4.5	0.0081	0.000	4.98	12	0
Peru	M 92	9	MUC 17	13.01.2013	12°31.374'	77°35.183'	770	5.5	0.0047	0.028	5.19	14	0
Peru	M 92	9	MUC 17	13.01.2013	12°31.374'	77°35.183'	770	7	0.0037	0.048	3.15	19	0
Peru	M 92	9	MUC 17	13.01.2013	12°31.374'	77°35.183'	770	9	0.0029	0.000	4.05	22	0
Peru	M 92	9	MUC 17	13.01.2013	12°31.374'	77°35.183'	770	12.5	0.0010	0.000	3.61	25	0
Peru	M 92	9	MUC 17	13.01.2013	12°31.374'	77°35.183'	770	17.5	0.0013	0.000	3.68	31	0
Peru	M 92	10	MUC 28	13.01.2013	12°35.377'	77°40.975'	1025	0.5	0.0008	-	2.88	3	0
Peru	M 92	10	MUC 28	13.01.2013	12°35.377'	77°40.975'	1025	1.5	0.0016	-	3.07	2	0
Peru	M 92	10	MUC 28	13.01.2013	12°35.377'	77°40.975'	1025	2.5	0.0007	-	2.77	4	0

Peru	M 92	10	MUC 28	13.01.2013	12°35.377'	77°40.975'	1025	3.5	0.0007	-	2.47	7	0
Peru	M 92	10	MUC 28	13.01.2013	12°35.377'	77°40.975'	1025	4.5	0.0004	-	2.06	9	0
Peru	M 92	10	MUC 28	13.01.2013	12°35.377'	77°40.975'	1025	5.5	0.0006	-	2.13	13	0
Peru	M 92	10	MUC 28	13.01.2013	12°35.377'	77°40.975'	1025	7	0.0012	-	2.25	16	0
Peru	M 92	10	MUC 28	13.01.2013	12°35.377'	77°40.975'	1025	9	0.0010	-	1.76	17	0
Peru	M 92	10	MUC 28	13.01.2013	12°35.377'	77°40.975'	1025	12.5	0.0009	-	2.22	17	0
Peru	M 92	10	MUC 28	13.01.2013	12°35.377'	77°40.975'	1025	17.5	0.0000	-	2.03	24	0
Svalbard	PO419	675	MUC 10	28.08.2011	79°00.466'	06°54.279'	1241	1.5	-	0.10	1.40	53	80
Svalbard	PO419	675	MUC 10	28.08.2011	79°00.466'	06°54.279'	1241	2.5	-	0.17	1.33	29	59
Svalbard	PO419	675	MUC 10	28.08.2011	79°00.466'	06°54.279'	1241	3.5	-	0.14	1.07	28	2957
Svalbard	PO419	675	MUC 10	28.08.2011	79°00.466'	06°54.279'	1241	4.5	-	0.11	1.03	37	117
Svalbard	PO419	675	MUC 10	28.08.2011	79°00.466'	06°54.279'	1241	5.5	-	0.07	1.28	29	343
Svalbard	PO419	675	MUC 10	28.08.2011	79°00.466'	06°54.279'	1241	7	-	0.06	1.18	28	3379
Svalbard	PO419	678	MUC 12	29.08.2011	79°00.417'	06°54.131'	1235	0.5	-	2.42	1.73	na	na
Svalbard	PO419	678	MUC 12	29.08.2011	79°00.417'	06°54.131'	1235	1.5	-	2.41	1.66	5	3133
Svalbard	PO419	678	MUC 12	29.08.2011	79°00.417'	06°54.131'	1235	2.5	-	1.52	1.43	5	1132
Svalbard	PO419	678	MUC 12	29.08.2011	79°00.417'	06°54.131'	1235	3.5	-	1.41	1.33	6	764
Svalbard	PO419	678	MUC 12	29.08.2011	79°00.417'	06°54.131'	1235	4.5	-	1.02	1.13	6	667

Table S3: Sampling location, station and core ID, sampling date, latitude and longitude, water depth and integrated N_2 fixation and sulfate reduction rates over 0 - 20 cm sediment depth, as well as the average (0-20 cm) organic carbon content, maximum ammonium and sulfide concentrations at each sampling site.

Location	Station ID	Core ID	Integrated (0-20 cm) N_2 fixation (mmol m^{-2} d^{-1})	Integrated (0-20 cm) SR (mmol m^{-2} d^{-1})	Average carbon (wt%)	Max. Ammonium (µM)	Max. Sulfide (µM)
Chile	42	ROV 5	-	24.2	1.55	-	18
Gotland	361	MUC 9	0.024	12.6	0.12	85	-
Gotland	365	MUC 10	0.034	0.7	1.05	214	0.2
Gotland	366	MUC 11	0.106	4.5	5.45	777	752
Gotland	370	MUC 12	0.074	6.1	-	-	-
Gotland	371	MUC 13	0.116	4.6	-	-	-
Gotland	372	MUC 14	0.047	0.5	-	-	-
Mauritania	658	MUC 13	0.12 ± 0.007	3.1	0.8 ± 0.2	80	88
Mauritania	628	MUC 10	0.15 ± 0.004	4.2	0.7 ± 0.1	70	46
Mauritania	612	MUC 8	0.13 ± 0.006	1.6	0.8 ± 0.1	31	0
Mauritania	554	MUC 5	0.08 ± 0.002	1.4	1.3 ± 0.2	45	0
Mauritania	534	MUC 3	0.10 ± 0.008	6.4	2.7 ± 0.2	75	0
Mauritania	524	MUC 1	0.08 ± 0.002	4.0	2.9 ± 0.3	112	0
NAMV	-	MUC 4	-	8.4	1.05	29	-
Peru	1	MUC 13	0.15 ± 0.001	4.6	3.5 ± 0.8	2022	1229
Peru	4	MUC 11	0.30 ± 0.054	2.5	7.7 ± 2.6	316	0
Peru	6	MUC 6	0.41 ± 0.057	0.5	14.5 ± 2.4	786	0
Peru	8	MUC 23	0.01 ± 0.003	0.3	8.0 ± 2.1	107	1
Peru	9	MUC 17	0.05 ± 0.006	0.2	4.6 ± 0.9	34	0
Peru	10	MUC 28	0.01 ± 0.001	na	2.3 ± 0.4	24	0
Svalbard	675	MUC 10	-	0.8	1.22	53	3379
Svalbard	678	MUC 12	-	15.8	1.46	6	3133