Impacts and Importance of Ammonia- and Nitrite Oxidation in the Marine Nitrogen Cycle

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Jessika Füssel

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1. Gutachter: **Prof. Dr. Marcel M.M. Kuypers**

2. Gutachter: **Priv. Doz. Dr. Holger Daims**

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Summary

Nitrification, the stepwise oxidation of ammonia to nitrite and nitrate, is a key process in the oceanic nitrogen cycle. Nitrification produces the most abundant form of bioavailable nitrogen in the ocean, which is also a major electron acceptor in the oxidation of organic matter. The latter role of nitrate as an electron acceptor becomes crucial in the absence of oxygen. Such conditions prevail in eastern boundary upwelling systems, where nutrient rich, deep water fuels high rates of primary production in the surface waters. Subsequently, subsurface aerobic remineralization of organic matter results in the formation of oxygen minimum zones (OMZs) at midwater depths (80-900 m). Here, nitrogen cycling organisms determine the fate of nitrate, whether by reducing it to dinitrogen gas, whereupon it is lost from the ocean, or by reducing it to nitrite or ammonia followed by the re-oxidation to nitrate, whereupon it is retained as dissolved inorganic nitrogen.

One major aim of this thesis is to investigate the importance of nitrite oxidation in OMZ nitrogen cycling. Nitrite oxidation was detected throughout the Namibian OMZ and appears unaffected by low to non-detectable oxygen concentrations. Instead, high rates of nitrite oxidation are fuelled by ammonia oxidation and, most importantly, nitrate reduction to nitrite. Nitrite oxidation could recycle up to 100% of reduced nitrate, thereby retaining nitrogen in the system and potentially reducing N-loss.

Nitrite oxidizing bacteria belonging to the genera *Nitrospina* and *Nitrococcus* constituted up to 9% of the microbial community. Their abundance however did not correlate to rates of nitrite oxidation. *Nitrococcus* for example was especially abundant in zones where no nitrite oxidation was detected. The high abundance of *Nitrococcus* in the Namibian OMZ can likely be explained by its versatile metabolic capabilities. While *Nitrococcus* grows chemolithoautotrophically via nitrite oxidation in the presence of oxygen, it gains energy via organoheterotrophic nitrate reduction to nitrite in the absence of oxygen. Various sulfur metabolizing enzyme complexes encoded in the genome of Nitrococcus might enable its participation in sulfur cycling and potentially sulfide detoxification in OMZ waters.

Intriguingly, *Nitrococcus* is also able of nitrite reduction to nitrous oxide and, as a result of its high abundance in OMZ waters, might substantially contribute to oceanic nitrous oxide production.

In the oxygenated ocean, on the other hand, ammonia and nitrite oxidation were until now believed to be the only dissimilatory nitrogen cycling processes. They constitute the major chemolithoautotrophic process and significantly contribute to oceanic carbon fixation. Nevertheless little is known on their regulation and interaction in productive coastal ecosystems. In the Mauritanian upwelling rates of both processes were related to particle abundance in the water column, indicating that ammonia and nitrite oxidizers might occur in association with marine aggregates. While rates of ammonia oxidation were often insufficient to sustain rates of nitrite consumption by nitrite oxidizing bacteria, an alternative source of nitrite was provided by nitrate reduction to nitrite. The anaerobic reduction of nitrate to nitrite is likely facilitated by oxygen reduced microniches within marine aggregates.

Taken together, our findings provide detailed insights into the importance of ammonia and nitrite oxidation in the global nitrogen cycle and reveal the direct and indirect influence of nitrite oxidizing bacteria on nitrogen availability in the ocean.

Zusammenfassung

Nitrifizierung, die schrittweise Oxidation von Ammoniak zu Nitrit und Nitrat, bildet einen Schlüsselprozess im Marinen Stickstoffzyklus. Nitrifizierung bringt die häufigste Form von biologisch verfügbarem Stickstoff hervor, die außerdem einen wesentlichen Elektronenakzeptor zur Oxidation von organischem Material darstellt. Nitrat wird in der Abwesenheit von Sauerstoff als Elektronenakzeptor genutzt. Entsprechende Bedingungen herrschen in den östlichen tropischen Ozeanen, wo nährstoffreiches Tiefenwasser auftreibt und zu einer hohen Primärproduktion im Oberflächenwasser führt. Die nachfolgende, aerobe Remineralisierung des sinkenden organischen Materials führt zu intensiver Sauerstoffzehrung und der Entstehung von Sauerstoff Minimum Zonen (SMZ) in mittleren Wassertiefen. Hier bestimmen mikrobiell vermittelte Stickstoffumwandlungsprozesse ob Nitrat zu Stickstoffgas reduziert, und so aus dem Ozean entfernt wird, oder ob Nitrat zu Nitrit und Ammonium reduziert und zu Nitrat re-oxidiert wird, und so im Ozean verbleibt. Ein wesentliches Ziel dieser Arbeit ist es die Bedeutung von Nitritoxidation im Stickstoffkreislauf von SMZ zu untersuchen. Nitritoxidation wurde in der gesamten Namibischen SMZ festgestellt, unabhängig von den niedrigen oder nicht detektierbaren Sauerstoffkonzentrationen. Stattdessen wurden hohe Nitritoxidationsraten durch Ammoniakoxidation und im Besonderen durch Nitratreduktion zu Nitrit befördert. Nitritoxidation konnte bis zu 100% des reduzierten Nitrats re-oxidieren und so Stickstoff im System erhalten und möglicherweise Stickstoffverlust reduzieren. Nitrit oxidierende Bakterien der Gattungen *Nitrospina* und *Nitrococcus* machten bis zu 9% der mikrobiellen Gemeinschaft aus. Ihre Abundanz korrelierte allerdings nicht mit Nitritoxidationsraten. *Nitrococcus* war zum Beispiel besonders in solchen Zonen häufig, in denen keine Nitritoxidation gemessen wurde.

Das zahlreiche Vorkommen von *Nitrococcus* lässt sich vermutlich durch seine vielseitigen metabolischen Kapazitäten erklären. Während *Nitrococcus* in der Anwesenheit von Sauerstoff durch chemolithoautotrophe Oxidation von Nitrit lebt, gewinnt er unter anaeroben Bedingungen Energie über die organoheterotrophe Reduktion von Nitrat zu Nitrit. Verschiedene Enzyme des Schwefelmetabolismus, die im Genom von *Nitrococcus* codiert sind, könnten außerdem eine

Teilnahme am Schwefelkreislauf und insbesondere der Entgiftung von Sulfid in SMZ Gewässern ermöglichen. Spannenderweise hat *Nitrococcus* die Fähigkeit Nitrit zu Stickstoffmonoxid zu reduzieren und könnte durch sein häufiges Vorkommen entscheidend zur Marinen Stickstoffmonoxid-Produktion beitragen.

Im Gegensatz zu SMZ, wurden Nitrit- und Ammoniakoxidation im sauerstoffreichen Ozean bisher als einzige aktive dissimilatorische Stickstoffzyklusprozesse angenommen. Nitrifizierung stellt den wichtigsten chemolitoautotrophen Prozess im Ozean dar, der signifikant zur Marinen Kohlenstofffixierung beiträgt. Trotzdem ist wenig über Regulation und Interaktion dieser Prozesse in produktiven Küstengewässern bekannt. Im Mauretanischen Auftriebsgebiet konnte ein Zusammenhang zwischen Ammoniak und Nitritoxidation mit der Abundanz von Partikeln in der Wassersäule beobachtet werden, was auf eine Partikelassoziation beider Prozesse hindeutet. Während Ammoniakoxidationsraten häufig nicht ausreichten um den Nitrit Verbrauch von Nitrit oxidierenden Bakterien zu decken, bot die anaerobe Nitratreduktion zu Nitrit eine alternative Nitrit Quelle. Dieser anaerobe Prozess wurde vermutlich durch Sauerstoffreduzierte Mikronischen innerhalb Mariner Aggregate ermöglicht.

Zusammengenommen bieten unsere Ergebnisse detaillierte Einblicke in die Bedeutung von Ammoniak und Nitritoxidation im globalen Stickstoffzyklus und demonstrieren den direkten und indirekten Einfluss von Nitrit oxidierenden Bakterien auf die Stickstoff Verfügbarkeit im Ozean.

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Chapter 1

Introduction

1. Nitrification in the global ocean

Diazotrophic nitrogen fixation adds ~ 135 Tg new nitrogen to the ocean every year (Gruber and Sarmiento 1997; Gruber 2004; Codispoti *et al*., 2001, 2007). Once incorporated into biomass the fixed nitrogen is metabolized through the food web and eventually released as ammonia. Yet, ammonia is rarely detected in the pelagic ocean. Instead, nitrate constitutes > 99% of the dissolved inorganic nitrogen (DIN) species in the ocean (Gruber 2008). In the oxygenated ocean, Nitrate is solely produced by nitrification, the biologically mediated oxidation of ammonium to nitrite and nitrite to nitrate.

In the deep sea, nitrate accumulates to concentrations of up to 50 µM, indicating past and/or present occurrence of ammonia and nitrite oxidation. Direct rate measurements of nitrification below 250 m are however scarce (Ward *et al*., 1987). Since only ~20% of organic matter reaches the deep ocean (Denman *et al*., 2007; Fowler and Knauer, 1986) nitrification rates are likely low compared to the productive surface ocean. Instead, nitrate accumulates as a consequence of long residence times of water masses and the absence of major nitrogen sinks. This situation is advantageous to many organisms as comparably high levels of ammonium and nitrite would harm higher animals as well as microorganisms (e.g. Handy and Poxton, 1993; Jensen 2003; Stein and Arp, 1998). Nitrate thus serves as a stable, non-toxic form of bioavailable nitrogen.

In the surface ocean, DIN is largely depleted and its availability plays a key role in shaping global oceanic phytoplankton production (Tyrell *et al*., 1999; Falkowski 1997) (Figure 1). The two major DIN compounds in the surface ocean are ammonia and nitrate. Nitrification in surface waters was however long thought to be inhibited by light (Horrigan *et*

al., 1981; Olson, 1981; Venzella *et al*., 1989; Guerrero *et al*., 1996) and thus restricted to depth below the euphotic zone. Consequently, nitrate was assumed to reach surface waters primarily by vertical transport from depth, while ammonia is released from on-site remineralization of organic matter. Based on this vertical separation, Dugdale and Goering (1967) introduced the f-ratio ($P_{new}/(P_{new} + P_{regen})$ that describes the productivity of an oceanic system and thus estimates its export production. The fixation of nitrogen gas and the incorporation of nitrate by phytoplankton were termed "new production" (P_{new}), while incorporation of ammonia would lead to "regenerated production" (P_{regen}). In steady state, the export of organic nitrogen can not exceed the supply rate of "fresh" nitrogen and the incorporation of nitrate relative to ammonium was therefore considered as an indicator for export production.

The concept of the f-ratio was however negated by the more recent detection of ammonia and nitrite oxidation within the sunlit surface ocean (e.g. Ward *et al*., 1982, 1989, Ward 2005, Dore and Karl, 1996; Bianchi *et al*., 1994, 1997; Clark *et al*., 2007, 2008, Wankel *et al*., 2007). These studies contradict the earlier, mostly culture based studies and show that nitrifying organisms are not completely inhibited by light and instead successfully compete with phytoplankton for ammonium. As a consequence, phototrophic organisms often depend on nitrate as their major nitrogen source. Nevertheless, nitrifying activity within the surface ocean generally increases at the base of the euphotic zone, where competition for ammonium and light inhibition become minimal.

The occurrence of nitrification within the euphotic zone affects the f-ratio especially in oligotrophic environments like the subtropical gyres, where recycled nitrogen constitutes by far the dominant nitrogen source (Yool *et al*., 2007). Even high rates of nitrification in the euphotic zone however hardly influences the f-ratio in upwelling regions, where nitrate rich, deep water flows to the surface ocean and supports high primary production (Figure 1). These upwelling areas consequentially constitute hotspots of carbon export from the surface ocean (Sarnthein, *et al*., 1988; Wefer and Fischer, 1992) and the immense stock of deep sea nitrate indirectly facilitates sequestration of atmospheric carbon dioxide to the ocean via the biological pump.

Over the last decades, more than 30 studies measured rates of nitrification in various environments (e.g. Ward *et al*., 1982, 1987, 1989a, 1989b, Bianchi *et al*., 1993, 1997, 1999; Dore and Karl, 1996; Reimbault *et al*., 1999; Lam *et al*., 2004; 2009; Ward 2005; Clark *et al*.,

2007, 2008; Fuessel *et al*., 2012; Beman *et al*., 2012; 2013; Dahm *et al*., 2002) and its ubiquitous occurrence renders nitrification the dominant chemolithoautotrophic process in the pelagic ocean. Thus, nitrifying organisms are important players in the oceanic carbon cycle. Raven (1996) estimated the inorganic carbon fixation of ammonia and nitrite oxidizing bacteria to ~ 0.19 Pg y^{-1} , while ammonia oxidizing archaea have been suggested to fix ~ 0.4 Pg y^{-1} (Wuchter *et al*., 2006). Although rates of phototrophic inorganic carbon assimilation (~ 50 Pg y^{-1}, Field *et al*., 1998) exceed those of nitrification several fold, nitrification significantly contributes to oceanic inorganic carbon assimilation. Furthermore, Nitrifying organisms were found to add new particulate organic carbon (POC) to sinking aggregated material in the mesopelagic zone (~ 500 m), thereby directly influencing carbon transport to the deep sea (Karl *et al*., 1984).

While several factors influencing ammonia and nitrite oxidation have been described, including light (see above), salinity (Pakulski *et al*., 1995; 2000; Somville, 1984) and temperature (Dahm *et al*., 2002), their effects tend to vary in time and space and no consistent relation has been observed yet. Nitrifying activity also shows no apparent direct correlation with either ammonia concentrations or primary productivity (Ward 1985; Bianchi *et al.,* 1999; Ward *et al*., 2005; Clark *et al*., 2008). For example high rates of ammonia and nitrite oxidation have been reported from the oligotrophic South Pacific (~ 140 nM d^{-1}), (Dore and Karl, 1996), but in the highly productive waters of the Canary Upwelling nitrifying microorganisms oxidized < 20 nM d^{-1} ammonia and nitrite. Therefore, other factors must influence the distribution and activity of nitrifying organisms.

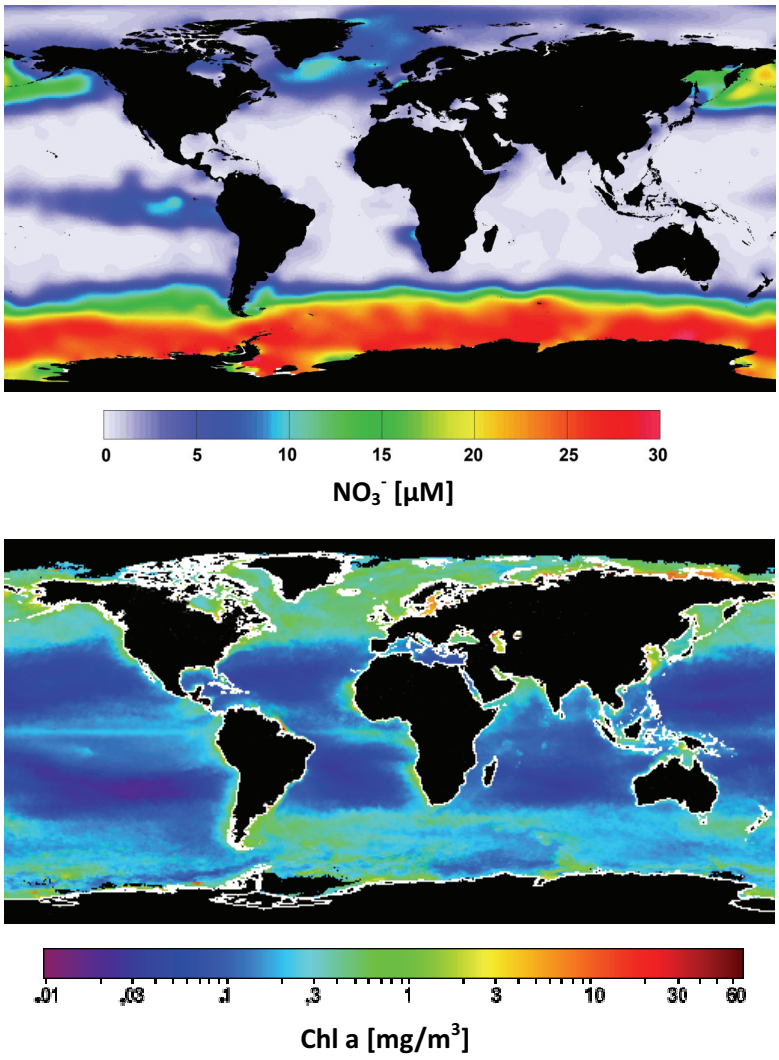

Figure 1: Annual surface Nitrate concentrations in the global ocean derived from World Ocean Atlas (2009) in comparison to anual *chlorophyll a* concentrations recorded by Modis in 2009. High Nitrate concentrations indicate upwelling of deep, nitrate rich water resulting in highly productive ecosystems.

2. Identity and ecology of nitrifying microorganisms

Insights into the microbial diversity and physiology of nitrifying organisms are an important prerequisite to decipher the role of ammonia and nitrite oxidation in the marine nitrogen and carbon cycles. Physiological characteristics in combination with the environmental distribution of different ammonia and nitrite oxidizer species can provide information on the importance of nitrification in certain habitats and can be indicative of environmental conditions.

2.1 Ammonia oxidizing microorganisms

Until recently, ammonia oxidation has been solely attributed to bacteria belonging to a few groups within the β- and γ-Proteobacteria: *Nitrosomonas, Nitrosospira* and *Nitrosococcus.* Despite the significant contribution of ammonia oxidation to biogeochemical cycling of pelagic environments, ammonia oxidizing bacteria (AOB) often constitute less than 0.1% of the microbial community (Ward, 2000). Recently, the important role of extremely abundant and diverse Archaea in ammonia oxidation became evident (Venter *et al*., 2004; Schleper *et al*., 2005; Könneke *et al.*, 2005; Francis *et al.*, 2005). The greater abundance of archaeal amoA genes in open ocean settings (Wuchter *et al.*, 2006; Mincer *et al*., 2007; Prosser *et al*, 2008), as well as productive coastal environments such as oxygen minimum zones (Francis *et al*., 2005; Lam *et al*., 2007; 2009; Beman *et al*., 2008), and their correlation with ammonia oxidation rates (Wuchter *et al*., 2006; Beman *et al.*, 2012) strongly indicate a major contribution of archaea to ammonia oxidation in the ocean. Although the relative importance of ammonia oxidizing archaea and bacteria in different marine environments has not been totally resolved, more and more recent studies point to niche seperation between them (Mosier and Francis 2008; Martens-Habbena *et al.,* 2009; Cao *et al*., 2011, 2012; Beman *et al*., 2012; Wang *et al*., 2013). Extraordinarily low K_m values and substrate threshold for ammonium uptake (0.13 µM nM and 0.01 µM, respectively) of some ammonia oxidizing archaea predestine these microorganisms to thrive under oligotrophic conditions, outcompeting not only ammonia oxidizing bacteria (K_m values between 8-54 µM (Ward, 1987b; Suzuki *et al*., 1974; Keener and Arp, 1983) but also heterotrophic Bacteria in the uptake of ammonia (Martens-Habbena *et al*., 2009). In contrast, particles sinking through the water column such as marine snow appear to be enriched in ammonia oxidizing Bacteria (Karl *et al*., 1984; Phillips *et al*., 1998), while ammonia oxidizing archaea tend to be free living (Woebken *et al*., 2008). Particles or aggregates could thus provide ammonia enriched microniches (Allredge and Cohen, 1987; Allredge and Gotschalk, 1990) for ammonia oxidizing Bacteria. Moreover, ammonia oxidizing bacteria were found responsible for nitrite production in the oxygen deficient zone of the Black Sea, while marine *Thaumarchaeota* dominate in the oxic part of the water column (Lam *et al.*, 2007). Hence, the combination of ammonia availability and oxygen concentrations may dictate niche separation for ammonia

oxidizing Bacteria and Archaea (Martens-Habbena *et al*., 2009; Schleper 2010).

2.2 Nitrite oxidizing bacteria

In contrast to ammonia oxidizing microorganisms, marine nitrite oxidizing bacteria (NOB) only recently started receiving broad attention and so far, only few species belonging to the genera *Nitrobacter, Nitrospira, Nitrococcus* and *Nitrospina* have been identified (e.g. Watson *et al*., 1986; Watson and Waterbury, 1971; Bock *et al*., 1983; Ehrich *et al*., 1995; Alawi *et al.*, 2007; Sorokin *et al*., 2012), while no marine representatives of the genera *Nitrotoga* (Alawi *et al*., 2007) and *Nitrolancetus* (Sorokin *et al*., 2012) have been detected yet.

The genus *Nitrobacter* covers the most well investigated group of nitrite oxidizers, and includes four described species (Spieck and Bock, 2005), yet, the only marine isolate of this genus has not been described (Vanparys *et al.*, 2007). Although *Nitrobacter* has been detected in the Eastern Tropical South and North Pacific via immunofluorescence (Ward and Carlucci, 1985, Ward *et al*., 1989), no further reports on their abundance in the ocean exists, and their contribution to marine nitrogen cycling remains obscure.

The first cultured representative of the genus *Nitrospira* has been isolated from a marine sample (Watson *et al*., 1986), but most marine representatives of *Nitrospira* appear to be associated with marine sponges (Off *et al*., 2010; Karlinska-Batres and Wörheide, 2013), except for a new species that has recently been enriched from a coastal North-Sea water samples (Haaijer *et al*., 2013). It is likely that there are more free-living *Nitrospira* which have escaped our previous detection.

Based on our current knowledge, the two genera *Nitrococcus* and *Nitrospina* comprise the most abundant NOB in the ocean. Both genera were first isolated from seawater and have solely been detected in marine environments to date (Watson and Waterbury, 1971). *Nitrococcus mobilis* was isolated from a “foamy surface slick” and the *Nitrospina gracilis* culture originated from surface waters (13 m depth) (Watson and Waterbury, 1971). *Nitrococcus* has been detected in the highly productive oxygen minimum zone off Peru and in the California Bight alongside with *Nitrobacter* (Ward and Carlucci, 1985; Ward *et al.,* 1987; Ward *et al*., 1989), and *Nitrococcus* occurs simultaneously with *Nitrospina* in the Namibian oxygen minimum zone (OMZ) (Füssel *et al*., 2012). *Nitrospina* is widely distributed in the ocean and exists in oligotrophic waters from the epipelagic to the

bathypelagic zone (DeLong *et al.,* 2006; Mincer *et al*., 2007, Santoro *et al.,* 2006), in mesotrophic surface waters (Fuch *et al*., 2005) and in highly productive OMZs (Labrenz *et al.,* 2007; Fuchsmann *et al.,* 2011, Füssel *et al.*, 2012).

Although *Nitrospina* appears dominant in marine settings, many studies specifically screen for *Nitrospina* and thus may have overlooked other NOB (Mincer *et al.*, 2007; Santoro *et al.*, 2006; Beman *et al*., 2013). Furthermore, few representatives of each genus have so far been isolated and described and new genera of NOB still await to be discovered (Alawi *et al.*, 2007; Sorokin *et al*., 2012).

2.2.1 Physiology of Nitrospina and Nitrococcus

Nitrococcus and *Nitrospina* are the only NOB that have been regularly detected in oceanic samples and their physiologies might be indicative of their adaptations to environmental settings. Each of both genera include a well described, cultured species from the marine environment: *Nitrococcus mobilis* and *Nitrospina gracilis* (Watson and Waterbury, 1971). Although culture conditions as applied by Watson and Waterbury (1971) are hardly comparable to marine environments, basic physiological characteristics observed in cultured microorganisms have provided invaluable insights into their optimal growth conditions.

Nitrospina appears to be a specialist for low substrate environments, whereas *Nitrococcus* is a fast growing opportunist. While nitrite concentrations of 20 mM inhibited growth of *Nitrospina gracilis, Nitrococcus mobilis* tolerated more than 60 mM nitrite. The motile cocci of *Nitrococcus* are characterized by extensive cytomembrane systems, densely packed with nitriteoxidoreductase (NXR) complexes that facilitate the rapid turnover of nitrite (Spieck and Bock, 2005) (Figure 2). Under ideal culture conditions, *Nitrococcus* can thus double more than twice as rapidly as *Nitrospina.* In contrast, the slender, long rods of *Nitrospina* lack a cytomembrane system (Figure 2) (Watson and Waterbury, 1971). Their adaptation to low substrate concentrations appears to be facilitated by an NXR that is anchored in the cytoplasmic membrane and, in contrast to *Nitrococcus*, faces the periplasm. Thus, two protons are released into the periplasm when nitrite is oxidized, and they are added to the proton motive force required for energy conservation by the ATPase (Spieck *et al.*, 1998; Spieck and Bock 2005; Lücker *et al.*, 2010, 2013).

Annotation of the complete genome of *Nitrospina gracilis* has provided further insight into their physiology (Lücker *et al*., 2013). No genomic potential for mixotrophic or heterotrophic growth was found, which confirms the obligate chemolithoautotrophic lifestyle of *Nitrospina* observed in culture. Their genome exhibited various adaptations to microaerophilic conditions, such as the expression of a highly oxygen affine terminal oxidase and an oxygen sensitive carbon fixation pathway. No such information is available for *Nitrococcus mobilis*, whose genome has been fully sequenced within the frame of the Gordon and Betty Moore Foundation Marine Microbiology Initiative in 2006, but awaits annotation and analyses.

Like Ammonia oxidizing bacteria and archaea, the apparently opposing metabolic strategies employed by *Nitrococcus* and *Nitrospina* might indicate niche separation between these NOB. While *Nitrococcus* appears to thrive under high substrate levels, *Nitrospina* is adapted to low nutrient environments. However, such hypothesis remain speculative and only little is known on the distribution and physiology of especially *Nitrococcus*.

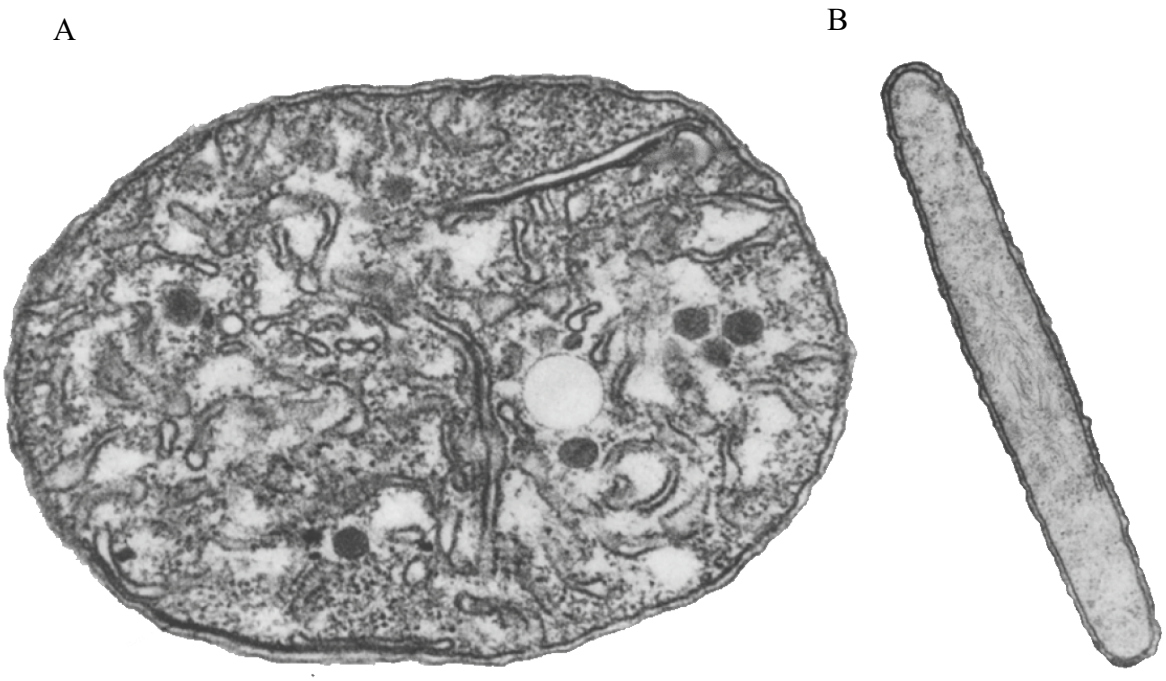

Figure 2: Electron micrograph of fixed and stained sections of Nitrococcus mobilis (A) and Nitrospina gracilis (B). A: *Nitrococcus mobilis* possesses a dense cytoplasmic tubular membrane system housing the NXR system of *Nitrococcus*. B *Nitrospina gracilis* in contrast lacks any cytomembrane system and the NXR systems are anchored in the periplasmic membrane facing the periplasmic space. Adapted from Watson and Waterbury (1971).

3. Nitrogen cycling in oxygen minimum zones

In > 99% of the pelagic ocean, oxygen concentrations > 4.5 µM do supposedly not allow for the occurrence of anaerobic nitrogen cycling processes. In < 1% of the ocean, however, oxygen deficient conditions render nitrate and nitrite the thermodynamically most favorable electron acceptor in the oxidation of organic matter. These oxygen deficent waters, known as oxygen minimum zones (OMZs), are often associated with eastern boundary upwelling systems that persist in the Eastern Tropical North and South Pacific (ETNP and ETSP) and the Eastern Tropical South Atlantic off the Namibian coast. Wind blowing parallel to the coastline results in offshore Ekman transport of surface waters and consequently upwelling of deep, nutrient rich waters that fuel high rates of primary production. Subsequent subsurface oxygen consumption during the respiration of organic matter in combination with sluggish ventilation result in the gradual decrease of oxygen and the formation of OMZs at midwater depths (80-900 m)

In OMZs, the microbially mediated nitrogen cycle comprises the major energy conserving pathways. Here, nitrate is ultimately converted to N_2 via heterotrophic denitrification and anammox. Despite the minor spatial extend of OMZs, they account for 30-50% of oceanic N-loss and thus have critical influence on nitrogen availability and primary productivity in the surface ocean (Codispoti, 2007; Gruber, 2008).

3.1 The role of nitrite in organic matter remineralization and N-loss

Ammonia and nitrite oxidation represent key processes in this complex marine nitrogen cycle as they transform fixed nitrogen from its lowest (-III) to its highest (+III, +V) oxidation states (Figure 3). In OMZs, dissimilatory ***nitrate reduction to nitrite*** is the dominant process in the oxidation of sinking organic matter (Figure 3) (Lipschultz *et a*l., 1990; Lam *et al*., 2009; Füssel *et al.*, 2012; Kalvelage *et al*., 2013). Nitrate reduction to nitrite is a widespread capability in microbes and many aerobic heterotrophic microorganisms are able to switch to dissimilatory nitrate reduction under oxygen limitation (Gonzales *et al*., 2006; Zumft *et al*., 1997). Though generally considered as an anaerobic process, nitrate reduction to nitrite has been found active at oxygen concentrations of at least 27 µM (Kalvelage *et al*., 2011) and thus co-occurs

with oxic remineralization over a broad range of oxygen concentrations.

Nitrite produced during dissimilatory nitrate reduction to nitrite can be further reduced in the oxidation of organic matter via dissimilatory nitrite reduction to ammonia ***(DNRA)*** or sequential ***heterotrophic denitrification*** to nitrogen gas (Figure 3). ***DNRA*** releases a similar amount of energy per mol organic carbon oxidized as nitrate reduction to nitrite (Table 1). The importance of DNRA in OMZs, however, remains controversial. While Lam *et al.* (2009) and Kartal *et al*. (2007) measured rates of DNRA in the ETSP and close to the seafloor in the Namibian OMZ, Füssel *et al.* (2012) only sporadically measured very low rates of DNRA in the Namibian OMZ and Kalvelage *et al.* (2013) could not detect DNRA in the ETSP.

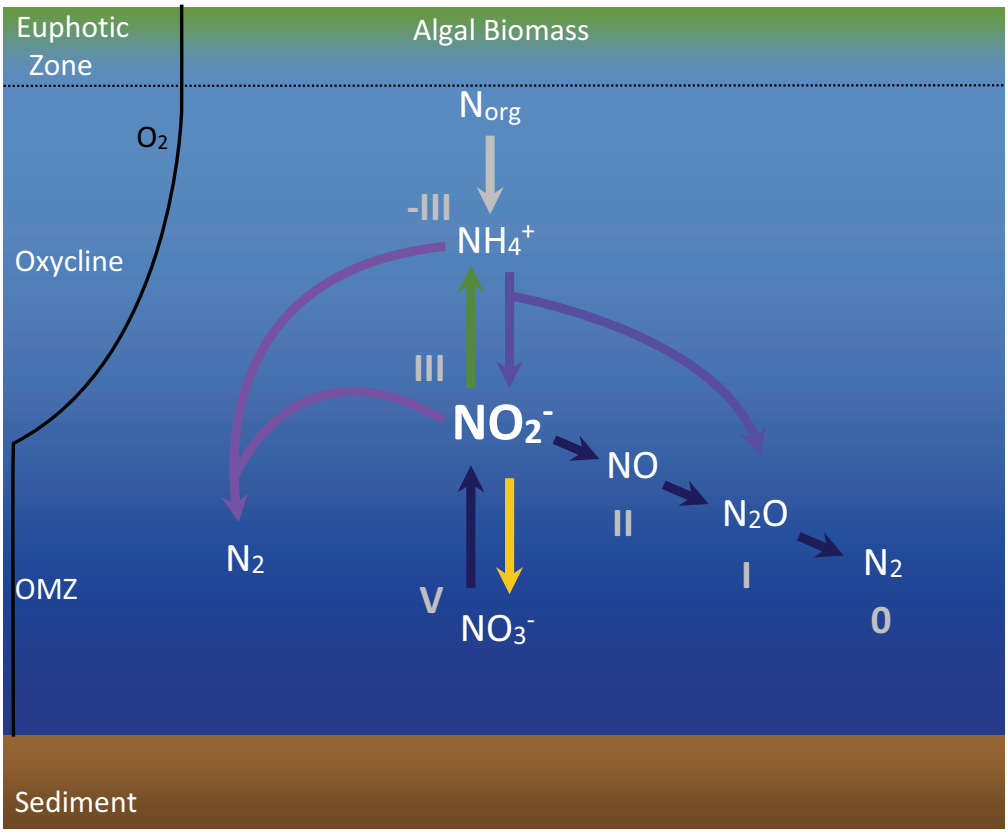

Figure 3: Oxygen concentrations in oxygen minimum zones allow for the co-occurrence of aerobic and anaerobic processes (Table 1). Nitrite represents the crossroad between N-loss and N-retention in OMZs. Dissolved inorganic nitrogen can be recycled between different oxidation states with nitrite as the key intermediate (indicated by grey numbers). These processes include **ammonia and nitrite oxidation** (violet and yellow arrows, respectively) as well as **nitrate reduction** and **DNRA** (blue and green arrows, respectively). Alternatively, nitrite is reduced via **anammox** (light blue arrows) or **denitrification** (black arrows), resulting in N_2 production and N-loss.

Heterotrophic denitrification, the sequential reduction of nitrite to nitric oxide (NO), nitrous oxide (N_2O) and nitrogen gas (N_2), releases more energy per mol carbon oxidized than nitrate reduction and DNRA (Figure 3; Table 1). DNRA however uses nitrate more efficiently and transfers eight electrons per nitrate reduced in contrast to five electrons that are transferred during heterotrophic denitrification. Thus, DNRA could be more favorable under high labile carbon availability but low nitrate concentrations (Tiedje 1988; Bonin, 1996; Nijburg *et al*., 1997). The relative importance of both pathways likely varies over time according to the prevailing conditions.

Exceptionally high rates of heterotrophic denitrification have been measured sporadically (Ward *et al*., 2009; Thamdrup *et al*., 2013), but substantial denitrification has rarely been observed in OMZs (Kuypers *et al.*, 2005; Thamdrup *et al*., 2006; Hamersley *et al*., 2007; Galan *et al*., 2009, Ward *et al*., 2009). All known denitrifying microorganisms are facultative and able to utilize oxygen as well as nitrate in the oxidation of organic matter. Complete denitrification however requires four enzyme complexes in addition to the core modules of the respiratory chain (Betlach and Tiedje, 1981), which might be kinetically disadvantageous (Chen and Strous, 2013). Furthermore, the cytoplasmic nitrate reductase is the only enzyme involved in denitrification that contributes to the proton motive force (PMF) and thus conserves energy (Bertero *et al*., 2003). This probably explains the much lower ATP synthesis observed from denitrification than would be expected from free energy changes (Strohm *et a.l,* 2007). Hence, these facultative aerobes likely mainly reduce nitrate to nitrite under oxygen deficient conditions and only when an excess of electron donor persists and the water column is anoxic, complete denitrification becomes locally important (Ward *et al*., 2008, Dalsgaard *et al.*, 2012). Overall, complete denitrification appears to be of minor relevance in organic matter remineralization in OMZs.

Instead, the major fraction of nitrogen gas production in OMZs is the result of anaerobic ammonia oxidation (***anammox)*** with nitrite to dinitrogen gas (Figure 3), an ubiquitously active process throughout suboxic waters OMZs (Kuypers *et al*., 2003, 2005; Dalsgaard *et al*., 2003; Thamdrup *et al.*, 2006; Hamersley *et al*., 2007; Dalsgaard *et al*., 2012; Kalvelage *et al.*, 2013). In contrast to heterotrophic denitrifiers, anammox bacteria are chemolithoautotrophs and only indirectly depend on the supply of organic matter (Strous *et al*., 1999; Kalvelage *et al.*, 2013). Both N-loss processes are restricted to the core of OMZs

(Kalvelage *et al*., 2011; Codispoti *et al*., 2001)

3.1.1 Nitrification in OMZs

OMZs are not entirely dominated by anaerobic nitrogen cycling processes. Instead, aerobic ammonia and nitrite oxidation appear largely unperturbed by the low oxygen concentrations and both processes co-occur with anaerobic ammonia oxidation and nitrate reduction (Lipschultz *et al*., 1990; Füssel *et al*., 2012; Kalvelage *et al.*, 2013).

Ammonia oxidation has been measured in the OMZ of the ETSP and ETNP as well as in the Namibian OMZ, where both ammonia oxidizing archaea and bacteria are abundant (Ward *et al*., 1989; Lipschultz *et al*., 1990; Lam *et al*., 2007, 2009; Woebken *et al*., 2008; Beman *et al*., 2012, Stewart *et al.*, 2012, Füssel *et al*., 2012). Hence, ammonia produced during the respiration of organic matter is directly re-oxidized within the OMZ. Maximal rates are generally observed in the upper OMZ, after which the rates decline towards the OMZ core (Lipschultz *et al.,* 1990, Beman *et al*., 2008; Lam *et al*., 2009, Füssel *et al*., 2012). Ammonia oxidation and nitrate reduction provide nitrite for DNRA, denitrification, and anammox, directly linking the remineralization of organic matter to N-loss processes.

Not all nitrite is reduced to nitrogen gas or ammonium. A substantial fraction of the produced nitrite is oxidized to nitrate by nitrite oxidizing bacteria (Lipschultz *et al*., 1990, Füssel *et al*., 2012; Kalvelage *et al.*, 2013; Beman *et al*., 2013, Casciotti *et al*., 2013). ***Nitrite oxidation*** appears to be even less sensitive to low oxygen concentrations than ammonia oxidation. Nitrite oxidizing bacteria even remain active in the core of OMZs, where oxygen concentrations typically drop below the detection limit of conventional oxygen sensors (~ 2 µM) (Lipschultz *et al*., 1990; Füssel *et al.*, 2012; Kalvelage *et al*., 2013; Beman *et al.*, 2013) and that have been described as functionally anoxic (< 10 nM O_2) previously (Thamdrup *et al*., 2012). In contrast to ammonia oxidizing microorganisms, the NXR of nitrite oxidizing bacteria does not require oxygen as an enzymatic substrate. Oxygen is only utilized by the terminal oxidase as an electron acceptor to support oxidation of nitrite to nitrate. Thus, if nitrite oxidizing bacteria could utilize alternative electron acceptors, nitrite oxidation could proceed in the absence of oxygen. Although suitable electron acceptors still have to be determined, species such as Iodate as well as iron (III) and manganese (IV) oxides would be thermodynamically feasible.

Rates of nitrite oxidation generally significantly exceed rates of ammonia oxidation in OMZs (Lipschultz *et al*., 1990; Füssel *et al*., 2012; Kalvelage *et al.,* 2013; Beman *et al*., 2013) and a major fraction of nitrite is provided by nitrate reduction (22-325% in the Namibian OMZ). Thus, nitrite oxidizing bacteria can bypass ammonia oxidation where there are additional sources of nitrite. Intriguingly, nitrite oxidation in OMZs directly recycles up to 100% of previously reduced nitrate (Füssel *et al*., 2012), the major electron acceptor in the remineralization of organic matter. Hence, nitrite oxidizing bacteria retain oxidized nitrogen species in the OMZ and may thereby reduce direct N-loss from the system (Füssel *et al*., 2012; Casciotti *et al.*, 2013).

Table 1: Nitrogen cycling microorganisms either conserve energy by the oxidation of organic substrates (Organotrophy) (Nitrate reduction, DNRA, denitrification) or by the oxidation of reduced nitrogen species (chemolitotrophy) (Anammox, ammonia and nitrite oxidation). While ammonia and nitrite oxidation presumably require oxygen (aerobic), all other processes of the nitrogen cycle are anaerobic and restricted to oxygen deficient environments.

	Reaction	Equation (Stoichiometry based on Redfield)	ΔG° (kj/mol reaction)
Anaerobic	Nitrate reduction	$(CH_2O)_{106}(NH_3)_{16}H_3PO_4 + 212\ NO_3^- + 16\ H^+ \rightarrow 106\ CO_2 + 16\ NH_4^+ + 212\ NO_2^- + 106\ H_2O + H_3PO_4$	-244
	DNRA	$(CH_2O)_{106}(NH_3)_{16}H_3PO_4 + 53\ NO_3^- + 122\ H^+ \rightarrow 106\ CO_2 + 69\ NH_4^+ + 53\ H_2O + H_3PO_4$	-257
	Denitrification	$(CH_2O)_{106}(NH_3)_{16}H_3PO_4 + 106\ NO_3^- + 16\ H^+ \rightarrow 106\ CO_2 + 16\ NH_4^+ + 106\ N_2 + 212\ H_2O + H_3PO_4$	-398
	Anammox	$NH_4^+ + NO_2^- \rightarrow N_2 + 2H_2O$	-358
Aerobic	Ammonia oxidation	$NH_3^+ + 1/2\ O_2 \rightarrow N_2 + 2HNO_2 + H_2O$	-278
	Nitrite oxidation	$NO_2^- + 1/2\ O_2 \rightarrow NO_3^-$	-82

4. Marine nitrous oxide production by nitrifying microorganism

While most of the nitrogen that is lost from OMZs is lost as dinitrogen gas (N_2), a small

proportion reaches the atmosphere as nitrous oxide (N_2O). Nitrous oxide is a potent greenhouse gas and a major ozone-depleting substance reducing the stratospheric ozone layer (Lashof and Ahuja, 1990; Ravishankara *et al*., 2009). Globally, the ocean is an important source of atmospheric nitrous oxide, contributing about 30% to the "natural" N_2O production, of which 20-50% is attributed to OMZs (Nevison *et al*., 2003; Suntharalingan *et al*., 2000).

Heterotrophic denitrifiers release nitrous oxide when the nitrous oxide reductase is inactivated in the presence of oxygen (Ferguson *et al*., 1994; Pomowski *et al*., 2011). Furthermore, ammonia oxidizing microorganisms produce nitrous oxide in the course of nitrifier denitrification (Poth and Focht, 1985; Ostrom *et al*., 2000; Codispoti *et al*., 2005). In nitrifier denitrification, autotrophic ammonia oxidizing microorganisms oxidize ammonia to nitrite and subsequently reduce nitrite to nitrous oxide (Poth and Focht, 1985) and further to dinitrogen gas (Poth, 1986; Muller *et al*., 1995). Both pathways of nitrous oxide production are restricted to oxygen deficient environments. On the other hand, ammonia oxidizing microorganisms also persistently release nitrous oxide in the course of ammonia oxidation, though the relative fraction of N_2O to NO_2^- increases with decreasing oxygen concentrations (Cohen and Gordon, 1978, Goureau 1980; Löscher e*t al*., 2012). Based on the frequently observed correlation between apparent oxygen utilization (AOU) and ΔN_2O (N_2O excess) (Oudot *et al*., 1990; Nevison *et al.*, 2003; Ryabenko *et al*., 2011), oceanic N_2O production has largely been attributed to nitrification by ammonia oxidizing microorganisms and, according to some recent studies, specifically to archaea (Santoro *et al*., 2011; Löscher *et al*., 2012).

The ability to produce nitrous oxide has rarely been explored in nitrite oxidizing bacteria, but one species of the genus *Nitrobacter* has been found to heterotrophically reduce nitrate to nitrous oxide under anaerobic conditions (Freitag *et al*., 1987). Nonetheless, Nitrobacter is not abundant in marine environments. The closely related genus *Nitrococcus* (Lücker *et al*., 2013), on the other hand, is present in great numbers at least in OMZs (Füssel *et al*., 2012) and it is physiologically similar to Nitrobacter in many regards (Spieck and Bock, 2005). Hence, potential nitrous oxide production by *Nitrococcus* might play an important yet thus far overlooked role in nitrous oxide production in the marine environment. The substantial role of marine nitrous oxide in climate change and ozone depletion are compelling reasons to study the role of nitrite oxidizing bacteria in its production.

Aims and Objectives

Marine nitrite oxidation has so far gathered little attention over the last decades, mainly because ammonia oxidation, the first step of nitrification, has long been considered as the rate limiting step in nitrification. Even though nitrate reduction has the potential to provide an alternative nitrite source for nitrite oxidizing bacteria under oxygen deficient conditions, it was widely thought that aerobic and anaerobic processes were spatially segregated in OMZs according to their respective oxygen demands (Codispoti and Packard, 1980; Anderson *et al*., 1992). Intensive research on nitrogen cycling in OMZs has, however, shown the co-occurrence of aerobic and anaerobic nitrogen cycling processes over a broad range of oxygen concentrations (~0-20 µM) (e.g. Lam *et al*., 2007, 2009; Kalvelage *et al.*, 2011). In OMZs, nitrite represents the link between nitrogen loss and dissolved nitrogen retention. It is a substrate, product or intermediate of most dissimilatory N-cycling processes, and the reduction of nitrite mostly results in nitrogen loss (DNRA is the only nitrite reducing pathway that retains dissolved inorganic nitrogen as ammonium) (Figure 3). Thus, nitrite oxidation might play an important role in retaining dissolved inorganic nitrogen in OMZs. One major object of this thesis research is therefore to investigate the role of nitrite oxidation within the complex nitrogen cycle of OMZs.

We chose the ***Benguela upwelling system*** in the tropical South Atlantic Ocean that generates an OMZ over the Namibian shelf as a model system. In the Namibian OMZ, oxygen is often depleted below water depths of 70-80 m (Figure 4 A) (Mohrholz *et al*., 2007). The OMZ is restricted to the continental shelf where the water column is shallow, such that the OMZ directly interacts with the underlying sediment. Although anoxia usually persists during upwelling events over the Namibian shelf, the upwelling system is highly dynamic, resulting in oxygen and nutrient concentration variations over time scales of days to months (Chapmann and Shanon, 1987; Bailey and Chapman, 1991). Overall, the Namibian OMZ

provides an ideal setting to investigate interactions, regulation and adaptation of nitrogen cycling microorganisms.

In **chapter two** we quantitatively evaluate the importance of nitrite oxidation by measuring rates of all nitrogen cycling processes in parallel ^{15}N incubation experiments in water samples from the Namibian OMZ. Moreover, we investigate which nitrite oxidizing bacteria are present and determine their abundance and distribution in catalyzed reported deposition-fluorescence *in situ* hybridization (CARD-FISH). Nitrite oxidation was found highly active in bottom waters of the Namibian OMZ, where ammonia oxidation rates were low or not detectable and furthermore oxygen concentrations often drop below the detection limits of conventional oxygen sensors (~ 2 µM). Of the two genera of nitrite oxidizing bacteria present in the OMZ, *Nitospina* and *Nitrococcus*, especially *Nitrococcus* is unexpectedly and highly abundant in bottom water samples.

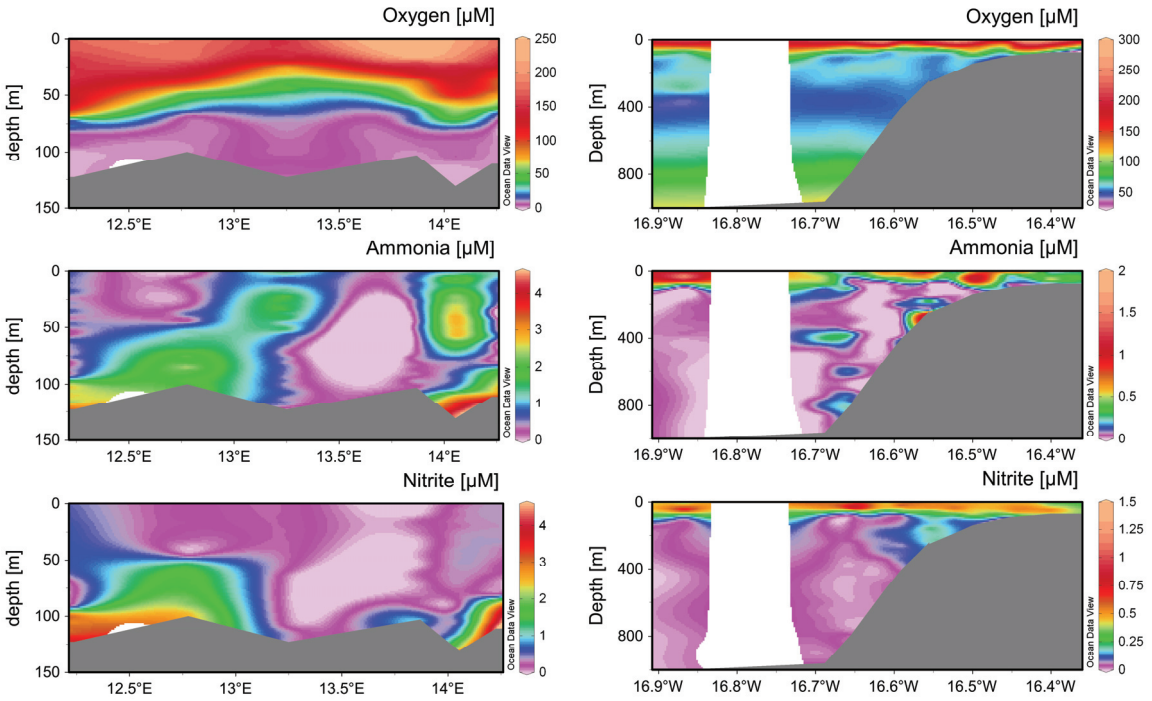

Figure 4: Vertical distributions of oxygen (upper panels) ammonium (middle panels), and nitrite (lower panels) in the Benguela (A) and the Mauritanian (B) upwelling system. The Benguela upwelling results in an intense OMZ over the Namibian shelf characterized by low to non detectable oxygen concentrations below ~ 80 m. The Namibian OMZ directly interacts with the underlying sediments nitrite and ammonia in accumulate bottom waters (< 4.6 µM). Oxygen concentrations in the Mauritanian upwelling do not decrease below 40 µM and ammonia and nitrite concentrations in the Mauritanian upwelling are maximal in surface waters and decrease along the water column. Only shelf waters exhibit elevated ammonia and nitrite concentrations throughout the water column that range < 1µM. (Data plotted with Ocean data view, Schlitzer, 2011)

In chapter three we further explore how *Nitrococcus* thrives in the oxygen depleted, organic rich bottom waters of the Namibian OMZ. We analyze the relation of *Nitrococcus* abundance with oxygen concentrations and nitrite oxidation rates, but no correlation becomes evident. Instead, *Nitrococcus* abundance shows a correlation with rates of nitrate reduction to nitrite. To elucidate the success of *Nitrococcus*, we therefore explore its metabolic potential *in situ* in pure cultures of *Nitrococcus mobilis* 231 and *in silico* by the annotation of those genes relevant in the nitrogen and carbon metabolism of *Nitrococcus*. We investigate whether *Nitrococcus* is able to anaerobically oxidize nitrite or whether these bacteria employ other, unanticipated pathways to conserve energy. The relevance of these culture and genome based findings is explored by Nano SIMS analyses of single *Nitrococcus* cells from the Namibian OMZ.

In chapter four we change scenery and investigate nitrogen cycling in the highly productive, but oxygenated ***Mauritanian upwelling system.*** In contrast to the Namibian OMZ, a better overall ventilation prevents the formation of and oxygen deficient zone and oxygen concentrations do not drop below 40 µM within the water coloumn (Fig 4) (Karstensen *et al*., 2008), such that ammonia and nitrite oxidation are supposedly the only redox active N-cycling processes.

In the oxygenated ocean, ammonia oxidizing microorganisms rely on the regeneration of ammonia by aerobic respiration of organic matter, while nitrite oxidizing bacteria are limited by rates of ammonia oxidation to nitrite. This linear relation is only interrupted in the euphotic zone, where phototrophic organisms also release nitrite in the course of assimilatory nitrate reduction (Lomas and Lipschultz, 2006). In this study we aim to investigate the relationships between aerobic respiration, ammonia oxidation and nitrite oxidation in the dark ocean. We explore the influence of concentrations of oxygen, ammonia, nitrite and particulate organic carbon on their activity. Concentrations of particulate organic carbon appear to largely influence not only rates of aerobic respiration, but also of ammonia and nitrite oxidation. Our data further indicate that nitrite oxidizing bacteria do not rely on ammonia oxidation alone, but also explore a thus far overlooked source of nitrite.

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Chapter 2

Nitrite oxidation in the Namibian oxygen minimum zone

Jessika Füssel[1], Phyllis Lam[1,2], Gaute Lavik[1], Marlene M. Jensen[1,3], Moritz Holtappels[1], Marcel Günter[1] and Marcel M.M. Kuypers[1]

[1] Max Planck Institute for Marine Microbiology, Bremen, Germany

[2] Current address: National Oceanography Centre Southampton, University of Southampton, England

[3] Current address: Nordic Center of Earth Evolution and Institute of Biology, University of Southern Denmark, Odense, Denmark

J.F, P.L, G.L and M.M.M.K designed research and project outline, P.L, G.L and M.M.J and M.H collected samples and performed incubation experiments, J.F. and M.G performed rate measurements, J.F performed CARD-FISH; J.F., P.L and M.M.M.K. conceived, wrote and edited the manuscript

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Abstract

Nitrite oxidation is the second step of nitrification. It is the primary source of oceanic nitrate, the predominant form of bioavailable nitrogen in the Ocean. Despite its obvious importance, nitrite oxidation has rarely been investigated in marine settings. We determined nitrite oxidation rates directly in ^{15}N-incubation experiments and compared the rates with those of nitrate reduction to nitrite, ammonia oxidation, anammox, denitrification, as well as dissimilatory nitrate/nitrite reduction to ammonium in the Namibian oxygen minimum zone (OMZ). Nitrite oxidation (≤ 372 nM NO_2^- d^{-1}) was detected throughout the OMZ even when *in situ* oxygen concentrations were low to non-detectable. Nitrite oxidation rates often exceeded ammonia oxidation rates, while nitrate reduction served as an alternative and significant source of nitrite. Nitrite oxidation and anammox co-occurred in these oxygen-deficient waters, suggesting that nitrite-oxidizing bacteria (NOB) likely compete with anammox bacteria for nitrite when substrate availability became low. Amongst all of the known NOB genera targeted via catalyzed reporter deposition fluorescence *in situ* hybridization, only *Nitrospina* and *Nitrococcus* were detectable in the Namibian OMZ samples investigated. These NOB were abundant throughout the OMZ and contributed up to ~9% of total microbial community. Our combined results reveal that a considerable fraction of the recently recycled nitrogen or reduced NO_3^- was re-oxidized back to NO_3^- via nitrite oxidation, instead of being lost from the system through the anammox or denitrification pathways.

Introduction

Nitrogen is the limiting element for primary production in many parts of the Ocean, and its availability is therefore closely coupled to the biological sequestration of atmospheric carbon dioxide and the oceanic carbon cycle (Gruber *et al*, 2004). Nitrate (NO_3^-) is the most abundant form of bioavailable inorganic nitrogen (NH_4^+, NO_2^-, NO_3^-) in the Ocean. It is formed via nitrification, a biologically mediated two-step process comprising the oxidation of ammonia (NH_3) to nitrite (NO_2^-), and that of NO_2^- to NO_3^-, each of which is catalyzed by distinct groups of microorganisms. The majority of oceanic NO_3^- is locked up in the deep sea, where it is no longer assimilated by phytoplankton and thus accumulates to high concentrations of 20-50 µM. In the surface ocean, NO_3^- is usually depleted. Only in regions with strong diapycnal mixing or upwelling, deep-sea NO_3^- re-enters the euphotic zone and stimulates surface primary production. This in turn results in enhanced sinking of organic matter and subsequent remineralization that releases ammonium (NH_4^+), especially around the base of the euphotic zone. Consequently, nitrification is stimulated therein and NH_4^+ is recycled back to NO_3^-.

In certain highly productive regions, respiration of organic matter below the euphotic zone can be so intense that oxygen minimum zones (OMZs) develop. Upon oxygen-depletion in the OMZs, NO_3^- becomes thermodynamically the most favourable electron acceptor for the respiration of organic matter that may ultimately lead to the loss of oceanic nitrogen via denitrification or anammox (Codispoti *et al*., 2005; Lam and Kuypers, 2011). Upwelling was generally considered to be the only important source of NO_3^- in these waters, whereas nitrification was considered to be of negligible significance within the oxygen-deficient OMZs. However, recent studies reported active ammonia oxidation within the eastern tropical south Pacific (ETSP) OMZ (Lam *et al.* 2009; Molina and Farias, 2009). Hence, a considerable portion of NO_3^- in the OMZs may have come from recycled nitrogen. Nevertheless, the actual significance of nitrification as a NO_3^- source in the OMZs cannot be fully assessed without evaluating the activities of nitrite oxidation, the second and final step of nitrification.

Direct rate measurements for nitrite oxidation in an OMZ have been reported only once (Lipschultz *et al*., 1990). In that study in the ETSP OMZ, nitrite oxidation rates were detected even at very low (≤ 2.5 µM) oxygen levels. Since then, little research has been done

on marine nitrite oxidation in general. Although the importance of nitrite oxidation has recently been suggested for the Arabian Sea OMZ based on a reaction diffusion model, no direct rate measurements have been made for that region (Lam *et al*., 2011).

Ammonia oxidation is generally presumed to be the rate-limiting step in nitrification, thereby its rates are usually taken as the overall nitrification rates (e.g. Ward 2005; Wuchter *et al*. 2006). In the suboxic OMZs, nonetheless, NO_2^- can also be produced via nitrate reduction ($NO_3^- \rightarrow NO_2^-$). Nitrate reduction may in theory support nitrite oxidation beyond or even in the absence of ammonia oxidation. In other words, the two steps of nitrification are not necessarily coupled in the OMZs. Meanwhile, the suboxic conditions in the OMZs also allow the occurrence of other reductive NO_2^--consuming processes like anammox, denitrification and dissimilatory nitrite reduction to ammonium (DNRA) (Lam *et al*., 2009, Lam and Kuypers, 2011). Whether nitrite oxidation competes with these concurrent nitrogen transformations or how the overall nitrite balance may be maintained in the OMZs, remains largely unexplored.

The diversity and distribution of nitrite-oxidizing bacteria (NOB) in oceanic settings are also poorly known. Five genera of NOB have been identified so far: *Nitrospira*, *Nitrospina*, *Nitrococcus*, *Nitrobacter* and the newly discovered single-species genus *Nitrotoga* (Alawi *et al.*, 2007). All genera except for *Nitrotoga* have been detected in marine environments, though *Nitrobacter* is generally not abundant in oceanic settings (Koops, 2001). *Nitrospira* shows a relatively widespread distribution across various habitats but few were actually marine surveys (Hoffmann *et al*., 2009; Off *et al*., 2010). *Nitrococcus* and *Nitrospina* have been found exclusively in marine habitats to date. *Nitrospina* appeared to be quite abundant in some open-ocean settings in the North Pacific, yet the abundance of other NOB genera was not investigated in parallel (Mincer *et al.*, 2007; Beman *et al*., 2010; Santoro *et al*., 2010). There has been only one study to date that examined the distribution of NOB in the OMZs (Ward *et al.* 1989), in which only two species, *Nitrobacter sp*. and *Nitrococcus mobilis*, were targeted based on immunofluorescence. Both species were found to be present where nitrite oxidation was detected in the ETSP OMZ (Ward *et al.* 1989, Lipschultz *et al*., 1990).

In the current study, we investigated nitrite oxidation in the OMZ of the Benguela upwelling system off the Namibian coast. We optimized a method that allowed us to determine nitrite oxidation rates in the same series of ^{15}N-incubation-experiments, as used

for the rate measurements of other nitrogen cycling processes occurring in the OMZs. Parallel incubation experiments were conducted to evaluate for the first time the contribution of nitrite oxidation to the overall NO_2^- turnover in OMZ waters. In particular, we compared the rates of nitrite oxidation with rates of nitrate reduction to nitrite, 'aerobic' ammonia oxidation, anammox, as well as dissimilatory nitrate/nitrite reduction to ammonium (DNRA). Moreover, we determined the abundance and distribution of all five known NOB genera by using catalyzed reporter deposition fluorescence in situ hybridization (CARD-FISH) with specific 16S rRNA targeted probes.

Material and Methods

Water sampling was conducted onboard the *R/V Meteor* in May/June 2008 (M76/2) over the Namibian shelf between 22°59.87´S/14°3.12´E and 19°1.0´S/12°13.74´E (Fig. 1). Salinity, temperature, dissolved oxygen and chlorophyll *a* fluorescence were measured with a conductivity-temperature-depth (CTD) system, equipped with an oxygen sensor and a fluorometer (Sea Bird Electronics). Oxygen data were calibrated against Winkler titration. Water samples for high-resolution vertical nutrient profiling and ^{15}N-incubation-experiments were collected with a pump-CTD system (Kuypers *et al.*, 2003). Additionally, the benthic boundary layer (BBL) was sampled at 6 depths from 30 cm to 2 m above seafloor using a bottom water sampler (Sauter *et al*., 2005, Holtappels *et al.*, 2011 a). NO_2^- and NH_4^+ were measured on board spectrophotometrically (Grasshoff *et al*., 1999, detection limit 0.01 µM) and fluorometrically, respectively (Holmes *et al*. 1999, detection limit 0.01 µM). Water samples were frozen for later analyses of NO_3^- and PO_4^{3-} with an autoanalyzer in a shore-based laboratory (0.1 µM detection limit) (TRAACS 800, Bran & Lubber).

^{15}N-incubation experiments

Incubation experiments were conducted for 5-6 depths at 5 stations (Table 1). For each incubation experiment, a 250 ml seawater sample was collected from specific depths with the pump-CTD system or with the bottom water sampler for samples from the BBL. Different combinations of ^{15}N- and ^{14}N-substrates were added for the rate determinations of various

nitrogen cycling processes (Table 1). Production of ^{15}N-labeled N_2 via anammox or denitrification was determined in all incubation experiments prior to other measurements (for further details, see Holtappels *et. al.*, 2011b). Nitrite oxidation rates were determined as the net production of $^{15}NO_3^-$ from $^{15}NO_2^-$-amended incubations. DNRA was assessed as $^{15}NH_4^+$ production from $^{15}NO_2^-$+$^{14}NH_4^+$. Ammonia oxidation and nitrate reduction were measured as the production of $^{15}NO_2^-$ from incubations with $^{15}NH_4^+$+$^{14}NO_2^-$ and $^{15}NO_3^-$ +$^{14}NO_2^-$ respectively. Except for the latter two, all samples were purged with helium for 15 minutes prior to incubations, to reduce O_2 down to ≤ ~0.5 µM (Dalsgaard *et al.*, 2003; Jensen *et al.*, 2008, 2011). To examine the effect of oxygen on NO_2^- oxidation, additional experiments were conducted for two samples (St. 206-100m and St. 252-105m) with O_2 adjusted to four different controlled levels (~1-11 µM). In these cases, a known amount of O_2-saturated water was added to the He-purged samples, and the achieved O_2 concentrations were checked with a microsensor or the highly sensitive STOX (Switchable Trace amount OXygen) sensor (Revsbech *et al.*, 2009). O_2 concentrations were also monitored using the same sensors at discrete time intervals in a parallel set of incubation vials. As described in detail in Holtappels *et al.*, 2011 b, each ^{15}N(/^{14}N)-amended sample was immediately transferred into five 12-ml exetainer vials (Labco, High Wycombe, Buckinghamshire, U.K.), by introducing an overpressure of helium into the serum bottle such that the amended water sample was displaced via a 1-ml syringe into the bottom of each exetainer vial, which was then filled carefully from the bottom without bubbles. These samples were incubated for up to 48 h in the dark and at *in situ* temperatures. At each time interval (approximately 0, 6, 12, 24 and 48 h), incubation was terminated in one exetainer by removing 2 ml of sample while replacing it with helium and by adding 100µl of saturated mercuric chloride solution to stop biological activities. Samples were stored upside down in the dark at room temperature until further processing in a shore-based laboratory.

Nitrite oxidation rate measurements - method optimization

Due to the absence of a sensitive, direct method to determine nitrite oxidation rates in small-volume samples, only a limited number of field studies on this process have been performed in marine environments to date. The modified ^{15}N stable isotope pairing technique (Nielsen *et al.*, 1996, Thamdrup and Dalsgaard, 2002) has successfully been

applied to distinguish multiple concurrent N-transformations in the OMZs (Lam *et al*. 2009). Therefore, we optimized and combined several techniques to quantify $^{15}NO_3^-$ production in the same series of ^{15}N-incubation experiments to determine nitrite oxidation rates from incubations with $^{15}NO_2^-$ (Table 1).

Table 1: Summary of stations, sampling depths and ^{15}N incubation experiments conducted. Prior to the analyses for the targeted products listed, $^{15}N^{15}N$:$^{14}N^{14}N$ and $^{14}N^{15}N$:$^{14}N^{14}N$ ratios of the produced N_2 were determined in all treatments in order to measure denitrification and anammox rates. Asterisks (*) indicate helium-purging of the samples; 'a': same depths as listed for the treatment above, 'b': additional oxygen manipulation experiments were conducted for this treatment.

Station	*bottom depth [m]*	*sampling depths [m]*	*Substrate additions [µM]*
206	131	90, 100, 110, 129, 130, 131	$^{15}NH_4^+$ (5)*,
		a	$^{15}NO_2^-$ (5)* + O_2
		a	$^{15}NO_2^-$ +$^{14}NH_4^+$ (5)*
225	119	50, 70, 90, 117, 118, 119	$^{15}NH_4^+$ (5)*,
		a	$^{15}NO_2^-$ (5)*
		a	$^{15}NO_2^-$ +$^{14}NH_4^+$ (5)*
230	100	50, 70, 90, 98, 99	$^{15}NH_4^+$ (5)*,
		a	$^{15}NO_2^-$ (5)*
		a	$^{15}NO_2^-$ +$^{14}NH_4^+$ (5)*
		a	$^{15}NH_4^+$ + $^{14}NO_2^-$ (5)
243	103	80, 90, 97, 101, 102, 103	$^{15}NH_4^+$ (5)*,
		a	$^{15}NO_2^-$ (5)*
		a	$^{15}NO_2^-$ +$^{14}NH_4^+$ (5)*
		a	$^{15}NH_4^+$ + $^{14}NO_2^-$ (5)
		a	$^{15}NO_3^-$ (40) + $^{14}NO_2^-$ (5)
252	111	76, 95, 105, 109, 110, 111	$^{15}NH_4^+$ (5)*[b],
		a	$^{15}NO_2^-$ (5)* + O_2
		a	$^{15}NO_2^-$ +$^{14}NH_4^+$ (5)*
		a	$^{15}NH_4^+$ + $^{14}NO_2^-$ (5)
		a	$^{15}NO_3^-$ (40) + $^{14}NO_2^-$ (5)

To assess the N-isotopic ratio of NO_3^-, NO_3^- first was chemically converted to NO_2^- and subsequently to N_2 or N_2O, which are the forms measurable on the highly sensitive gas chromatography-isotopic ratio mass spectrometer (GC-IRMS). In order to distinguish the produced $^{15}NO_3^-$ from any unused $^{15}NO_2^-$-amendments, the latter needs to be removed completely prior to the reduction of $^{15}NO_3^-$ to $^{15}NO_2^-$ and the eventual conversion to N_2 or N_2O. We tested the efficiency of sodium azide and sulfamic acid as reducing agents for NO_2^-. Sodium azide efficiently reduces NO_2^- to N_2O (McIlvin and Altabet 2005), but forms highly toxic gases under the acidic reaction conditions; while the high solubility of N_2O in water hinders its complete removal. Sulfamic acid reduces NO_2^- to N_2, and has successfully been applied in analyses for the natural stable isotopic composition of NO_3^- in seawater samples and in earlier nitrite oxidation measurements (Granger *et al*. 2009, Lipschultz *et al.,* 1990). Sulfamic acid is not toxic and N_2 is less soluble in water than N_2O, thereby ensuring its efficient removal and minimizing interference with subsequent isotopic analyses for NO_3^-. The incubation of samples with 16.5 mM sulfamic acid (final concentration) for ~12 h achieved better NO_2^- removal efficiency than the reduction with azide, and so became our method of choice for subsequent nitrite oxidation rate measurements (Fig. S1). Following NO_2^- removal, sample pH was adjusted to 8-9 and spongy cadmium was added to 6 ml of experimental subsamples in order to reduce NO_3^- to NO_2^- (Margerson *et al.,* 1980, McIllvin and Atlabet, 2005). They were then incubated for at least 12 h on a horizontal shaker at room temperature (Gal *et al*., 2004). Subsequently, samples were transferred to fresh 6 ml exetainers with 2 ml headspace and flushed with helium for 10 min to remove any N_2 produced from $^{15}N\ NO_2^-$. Finally, the NO_2^- converted from NO_3^- via cadmium was further reduced to N_2 by the addition of sulfamic acid as previously described, and were neutralized with NaOH afterwards. The resultant N_2 was then ready for isotopic analyses.

Stable Isotopic Analyses of N_2 and N_2O

Nitrogen stable isotopic ratios of N_2 and N_2O were determined by GC-IRMS (VG Optima, Manchester, UK). Prior to any chemical conversions to determine the N-isotopic compositions of nitrite, nitrate or ammonium, the $^{15}N^{15}N$:$^{14}N^{14}N$ and $^{15}N^{14}N$:$^{14}N^{14}N$ ratios of N_2 produced via denitrification and/or anammox were measured in all treatments. Ammonia oxidation rates and nitrate reduction rates were determined as the $^{15}NO_2^-$ production over

time from $^{15}NH_4^+$ ($+^{14}NO_2^-$) and $^{15}NO_3^-$($+^{14}NO_2^-$), respectively, with the reduction of NO_2^- by sodium azide to N_2O for GC-IRMS analyses (McIllvin and Altabet, 2005). To determine DNRA rates, the N-isotopic composition of NH_4^+ was determined in 5 ml subsamples (+ 5 µM added $^{14}NH_4^+$) by converting NH_4^+ to N_2 with hypobromite (Warembourg 1993, Lam *et al*, 2009). Rates of all processes were calculated from the slopes of linear regression with $^{15}N^-$ production as a function of time, and only when the production was instantaneous (Fig. S3). All rates presented were calculated from 0-48 h of incubations ($p<0.05$), except for a few samples in which there was no more substantial nitrate production after 12 h due to likely nitrite depletion then. All rates reported here were calculated as net rates, and have been corrected for the ^{15}N-labeling percentages of initial substrate pools. However, in incubation experiments for the rate determination of DNRA, ammonia oxidation and nitrate reduction, the addition of unlabeled (^{14}N) 'products' NH_4^+, NO_2^- and NO_2^-, respectively, alongside ^{15}N-labeled substrates would likely have minimized immediate consumption of the ^{15}N-labeled products during the incubation period. Hence, these measured rates are expected to be closer to gross rates.

Catalyzed reporter deposition in situ fluorescence hybridization (CARD-FISH)

Water samples for CARD-FISH were fixed in 2% (final concentration) paraformaldehyde in phosphate buffered saline solution for 8-12 h at 4°C, prior to filtration onto polycarbonate membrane filters (GTTP, 0.22 µM pore size, 47 mm diameter, Millipore, Eschborn, Germany). CARD-FISH was performed following the protocol by Pernthaler *et al*. (2002). Briefly, cells were immobilized on the GTTP filters by embedding in 0.2% agarose. Then, cells were permeabilized by 60 min incubation at 37°C in 10mg ml^{-1} lysozyme in 50 mM EDTA and 100 mM Tris-HCL. Hybridization with horseradish peroxidise labelled oligonucleotide probes (Biomers, Ulm, Germany) was conducted for 2 h at 46°C at varying formamide concentrations, according to the oligonucleotide applied (Table 2). This was followed by tyramide signal amplification for 15 min at 46°C and subsequent staining of cells with DAPI (4′, 6′-diamidino-2-phenylindole). Positive hybridization signals and DAPI-stained cells were enumerated with epifluorescence microscopy (Axioplan 2, Zeiss, Jena, Germany).

Table 2: NOB specific 16S rRNA targeted oligonucleotide probes and the respective formamide concentrations in the hybridization buffer applied in this study.

Probe	Sequence (5' → 3')	Formamide [%]	Species	Source
Ntcoc-84	TCG CCA GCC ACC TTT CCG	20	*Nitrococcus mobilis*	Juretschko, 2000
Ntspn-693	TTC CCA ATA TCA ACG CAT TT	20	*Nitrospina gracilis*	Juretschko, 2000
Ntspa-662	GGA ATT CCG CGC TCC TCT	35	*Nitrospira spp.*	Daims *et al*., 2001
NIT3	CCT GTG CTC CAT GCT CCG	40	*Nitrobacter spp.*	Wagner *et al*.,1996
Ntoga122	TCC GGG TAC GTT CCG ATA T	40	*Nitrotoga sp.*	Lücker, 2009
FGall178	TCC CCC TYA GGG CAT ATG	30	*Gallionellaceae*	Lücker, 2009

Results and Discussion

Hydrochemical Setting of the Namibian OMZ

All five stations investigated in this study were located over the Namibian shelf from 19° S to 23° S, with bottom depths ranging between 103 and 130 m (Fig. 1). They were generally characterized by high primary production in the euphotic zone as indicated by the high surface chlorophyll *a* concentrations (Fig. 1F). Dissolved oxygen concentrations in surface waters were in the range of 170-245 µM, and declined to ≤ 4 µM by 80-90 m water depth (Fig. 1B). At most stations investigated, prominent NO_2^- and NH_4^+ maxima of up to 4.4 and 4.6 µM, respectively, were present in the lower OMZ; while NO_3^- concentrations showed a reverse trend with local minima as low as 12 µM within the OMZ (Fig. 1C,D,E).

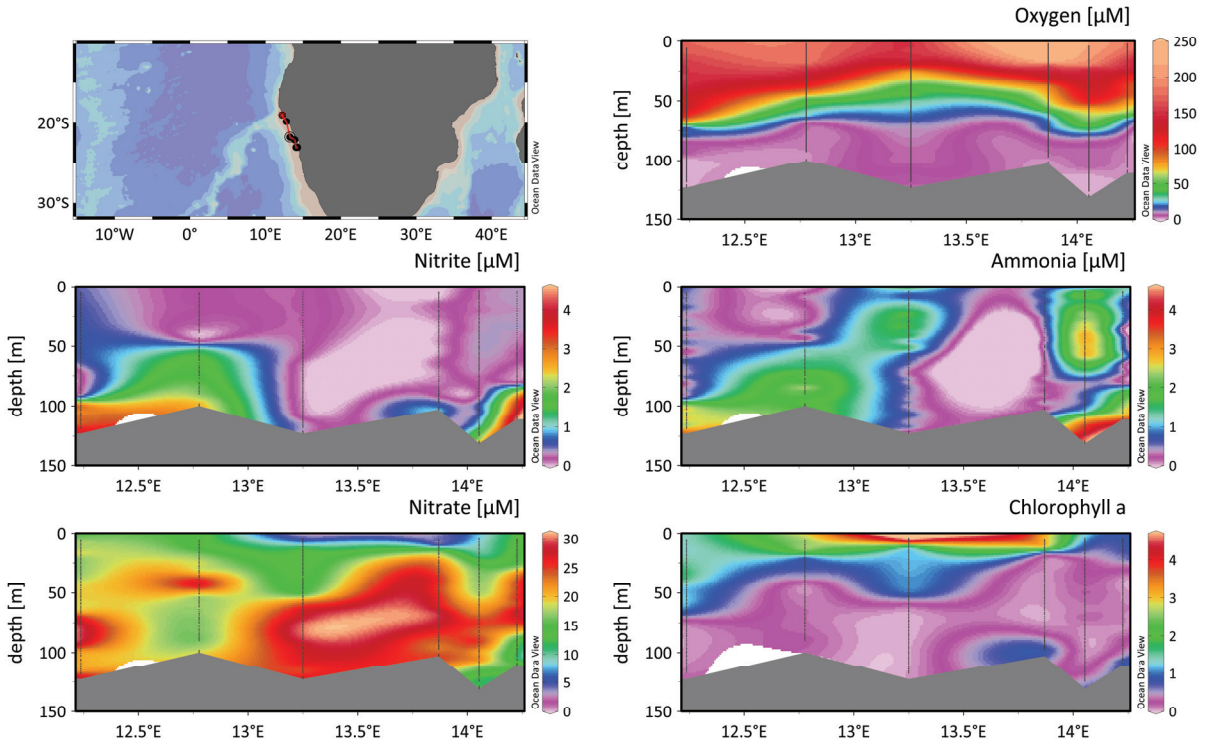

Figure 1: (A) Location of sampling stations (solid circles with station numbers) over the Namibian shelf in relation to the bathymetry of the region. The full water depths at these stations ranged between 103 m and 131 m. Vertical distribution of oxygen (B), nitrite (C), nitrate (D), ammonium (E) and chlorophyll a (measured as fluorescence in arbitrary units, without cross calibration with absolute quantities of chlorophyll) (F), along a NW-SE transect over the Namibian shelf. Sampling sites are indicated by black dots along the water column.

Nitrite Oxidation Rates

Nitrite oxidation rates were detected at all five stations investigated, at depths spanning from the oxycline to the benthic boundary layer (BBL) (Fig. 2 and S2). The measured rates ranged from 14 to 372 nM d^{-1} (Fig. 2). These values were within the range of previous observations from the Eastern Tropical South Pacific (ETSP) OMZ, where maximum rates reached ~170-600 nM d^{-1} at OMZ depths at 4 different stations (Lipschultz *et al*., 1990). No systematic trends could be discerned with respect to depth or oxygen availability in our dataset, with some stations exhibiting maximum nitrite oxidation rates in the lower OMZ (e.g. St. 252) and some in the BBL (e.g. St. 225) (Fig. 2). At other stations, nitrite oxidation decreased with water depth (Station 243) or was not detectable in the BBL (St. 206) (Fig. S2).

Interestingly, the highest rates observed during this study (372 ± 45 nM d^{-1} at St. 252 and 343 ± 40 nM d^{-1} at St. 225) seemed to occur at very low O_2 levels (< 1µM) in the lower OMZ and the BBL, respectively (Fig. 2). Since these rates were in fact determined in helium-purged water samples, in which oxygen had been reduced down to ≤~0.5 µM as verified in the current and previous studies with microsensors (Dalsgaard *et al.*, 2003; Jensen *et al.*, 2008, 2011), our results showed that nitrite oxidation occurred at microaerobic or apparently anoxic conditions.

The effect of O_2 on nitrite oxidation was further examined in ^{15}N-labeling experiments with varying O_2 concentrations (0-11 µM) in the Namibian OMZ waters. Although nitrite oxidation rates were 1-2 fold higher under elevated O_2 levels (≤ 11.3 µM; Fig.3), considerable nitrite oxidation rates were measured in incubations where O_2 levels were close to detection limit or at levels equivalent to ambient conditions. Similar observations of high nitrite oxidation rates associated with low O_2 (≤2.5 µM) have also been reported for the ETSP OMZ (Lipschultz *et al.*, 1990). Therefore, nitrite-oxidizing communities in the OMZs appear to be well adapted to oxygen-deficient conditions.

Nitrite availability or competition for nitrite with other processes might affect nitrite oxidation rates in the OMZ. Although the highest nitrite oxidation rates in this study were obtained from the NO_2^- maxima at stations 225 and 252 (Figs. 1 and 2), there appeared to be no correlation between NO_2^- availability and nitrite oxidation rates when considering the entire dataset (Fig. S4). However, we cannot fully exclude the possibility of activity stimulation by the amendment of 5 µM of $^{15}NO_2^-$ in samples containing low (i.e.nanomolar) NO_2^- concentrations. To identify the exact mode of rate regulation on nitrite oxidation, further sampling and experimentation in higher spatial and temporal resolution are necessary, including manipulative experiments with variable substrate levels to examine the kinetics of nitrite oxidation. Regardless, the current dataset shows that nitrite oxidation occurred at substantial rates throughout the Namibian OMZ.

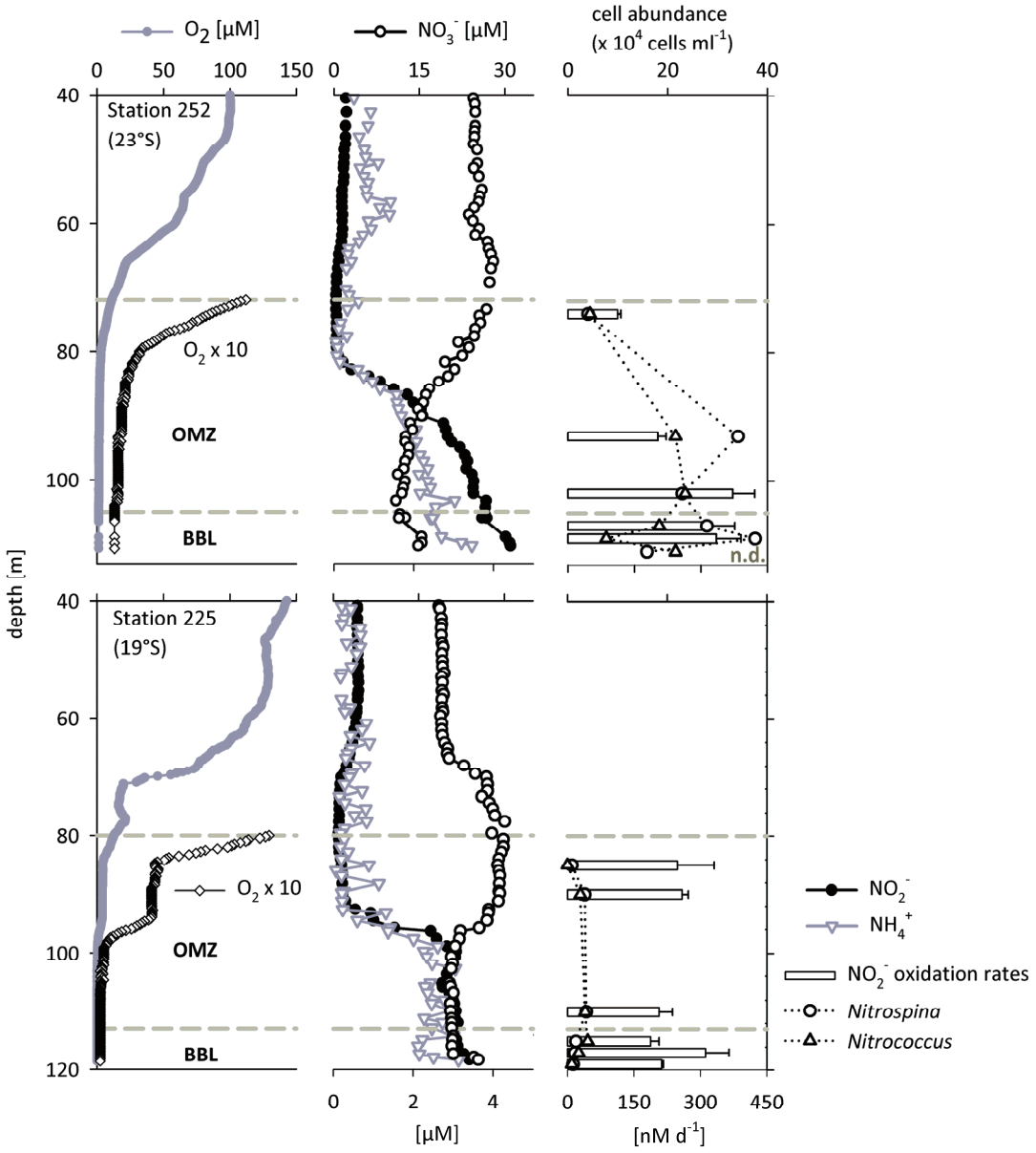

Figure 2: Stations 225 and 252: High-resolution vertical profiles of oxygen and 10x its concentration in the OMZ and BBL (O_2 grey circles, O_2 x 10 open diamonds) (left panels) and of nutrients (NO_2^-, black solid circles; NH_4^+, grey triangles; NO_3^-, open circles) (central panels). NO_2^- oxidation rates measured as $^{15}NO_3^-$ production are indicated by horizontal bars (right panels). At each depth, cellular abundance of *Nitrococcus* (triangle) and *Nitrospina* (circle) are shown. Most nitrite oxidation rates presented are derived from significant slopes ($p<0.05$) in corresponding linear regression. On occasions, $^{15}NO_3^-$ was produced so rapidly that a maximum was reached within as little as ~12 h, so rates were calculated only from these first 12h. Despite their substantial and instantaneous $^{15}NO_3^-$ production and the apparently high correlation between $^{15}NO_3^-$ and time ($r^2>0.90$), the low degrees of freedom resulted in slightly higher p-values (0.07-0.20) from one-way ANOVA in a few experiments – St. 225: 85m and 118 m; Station 252: 105 m, 109 m and 110.7 m). 'n.d.' denotes non-detectable reaction rates.

Nitrite-Oxidizing Bacteria in the Namibian OMZ

The abundance of potential nitrite-oxidizing bacteria (NOB) in the Namibian OMZ waters was assessed via 16S rRNA-based CARD-FISH, targeting all NOB genera known to date (*Nitrobacter, Nitrococcus, Nitrospira, Nitrotoga* and *Nitrospina*). Amongst these genera, only *Nitrospina* and *Nitrococcus* were detectable in the samples investigated. These NOB were abundant throughout the Namibian OMZ, indicating that these genera might substantially contribute to the measured nitrite oxidation rates in these waters (Fig. 2, S2). *Nitrospina* and *Nitrococcus* constituted up to 5.4% and 4.9% of total microbial abundance, respectively. Together, these two NOB genera represented 0.3–9% of total microbial community, equivalent to 0.09-5.5 x 10^5 cells ml^{-1}. In general, the NOB abundance in the present study was several orders of magnitude higher than that reported in an ETSP OMZ study, in which a maximum of 1.1x10^3 cells ml^{-1} were detected with immunofluorescence (Ward *et al.*, 1989). The applied antisera in the latter study had been produced against few cultured strains, which might thus be too specific to cover all NOB species present in the environment and resulted in underestimation. Our results are within the same range as those found in the central Californian current, where up to 1x10^4 16S rRNA gene copies ml^{-1} of the genus *Nitrospina* were detected by quantitative PCR, yet other NOB genera were not targeted in that study (Santoro *et al*., 2010).

Although the highest nitrite oxidation rates measured during this study corresponded well with the highest NOB abundance (4.37 x 10^5 and 5.32 x 10^5 cells ml^{-1} at 105 m and 1.12 m above seafloor respectively at St.252), NOB abundance did not show a significant correlation with nitrite oxidation rates (Spearman rank correlation, $p>0.05$) (Fig. 3B). The oligonucleotide probes used in our CARD-FISH analyses should in theory cover all NOB genera available in public databases, but diversity surveys for marine nitrite oxidizers have been few so that these probes may not cover all NOB species in nature. Additionally, dense populations of both *Nitrococcus* and *Nitrospina* cells were sometimes observed in large aggregates (≤250 µm wide) with other microorganisms (Fig. 4A and B). Consequently, NOB were difficult to enumerate in such cases and their abundance was likely underestimated at those depths. These factors might partly explain the lack of clear correlation in samples where nitrite oxidizer abundance was relatively low while nitrite oxidation rates were high. At the same time, NOB abundance was sometimes found to be high though nitrite oxidation

activities were barely or not detectable (Fig.3 B). These NOB might be utilizing an alternative pathway to gain energy. Regardless, the combination of molecular and biogeochemical results obtained in the current study provides strong evidence for the importance of NOB for N-cycling in the Namibian OMZ.

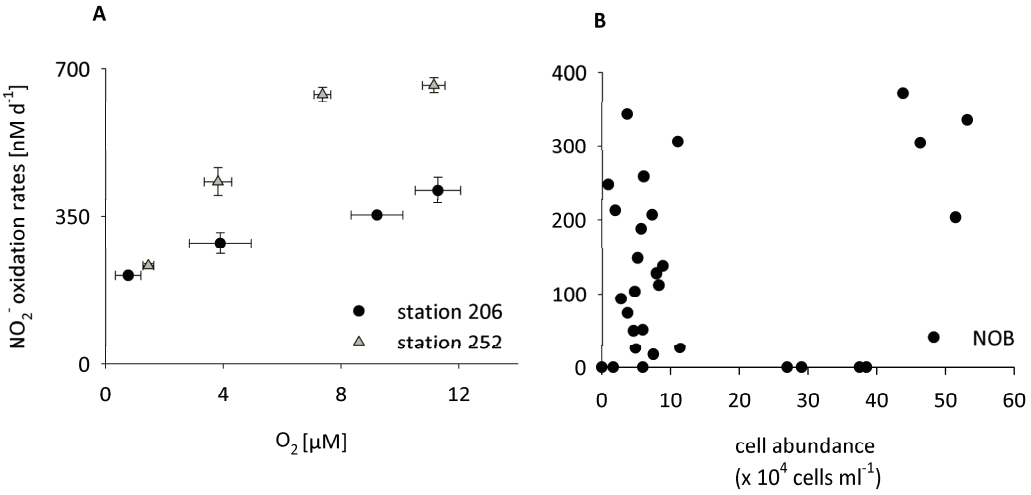

Figure 3: (A) Influence of O_2 concentration on nitrite oxidation: $^{15}NO_3^-$ production rates were determined in incubations with various controlled O_2 levels in two water samples – station 206 at 100 m and station 252 at 105 m. (B) Nitrite oxidizing bacteria (NOB) abundance as detected with 16S rRNA based CARD-FISH in relation to the measured nitrite oxidation rates.

Decoupling of nitrite oxidation from ammonia oxidation

Ammonia oxidation and nitrite oxidation are regarded as successive steps in nitrification, with ammonia oxidation being the first and presumably rate-limiting step. Therefore, ammonia oxidation rates should in principle be as high as nitrite oxidation rates. Ammonia oxidation rates were compared with nitrite oxidation rates at three stations for which measurements for both processes were available at the same depths (Fig. 5, S2). Nitrite oxidation often exceeded ammonia oxidation by as much as two to three folds, except for some depths at station 243, where ammonia oxidation rates were higher instead (Fig. 5, S2). Although ammonia oxidation experiments were conducted in unpurged samples whereas nitrite oxidation experimental subsamples were He-purged, parallel oxygen sensitivity experiments indicate that ammonia oxidation rates remained unchanged from purged to unpurged samples and up to 12 µM O_2 (Kalvelage *et al.*, 2011). Similarly, nitrite oxidation

rates were found to be several-fold greater than ammonia oxidation rates in the ETSP OMZ, based on ^{15}N-tracer method (Lipschultz *et al.* 1990). Results from both OMZs show that both nitrification processes were actively recycling significant proportions of nitrogen back to the NO_3^- pool in the OMZs. However, the two reactions were not necessarily coupled, as the measured ammonia oxidation could not provide sufficient NO_2^- to support the detected nitrite oxidation rates. Therefore, another source of NO_2^- was required for nitrite oxidation.

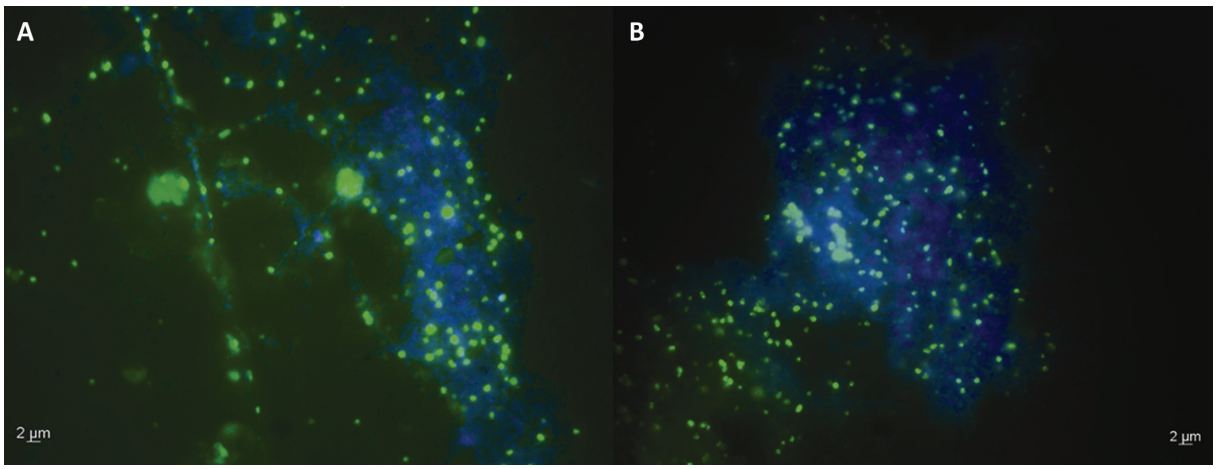

Figure 4: Epifluorescence micrographs showing dense populations of *Nitrospina* (green) (A) and *Nitrococcus* (green) (B) cells in large aggregates with other microorganisms (blue) at station 206 in 110 m water depth, as detected with 16S rRNA based CARD-FISH.

Nitrate reduction to NO_2^-, though often regarded as the first step in denitrification, also occurs as an independent reaction. It has been found as an important process in the ETSP and Arabian Sea OMZ (Lipschultz 1990, Lam *et al*., 2009, 2011). Nitrate reduction rates were measured at two stations in our study (St. 243 and 252). Active nitrate reduction was detected throughout the OMZ, at rates of 17-469 nM d^{-1} (Fig. 5). Nitrate reduction rates exceeded ammonia oxidation rates by 1-3 fold at St. 252. At this station, nitrate reduction could provide up to 325% of the NO_2^- consumed in nitrite oxidation and up to 100% of total NO_2^- production. Ammonia oxidation alone produced less than ~60% of the NO_2^- required by nitrite oxidation at station 252. Meanwhile, ammonia oxidation at St. 243 was responsible for 35-74% of total NO_2^- production, while the contribution from nitrate reduction was generally lower (26-66%) (Fig. 5). In other words, both ammonia oxidation and nitrate reduction were important NO_2^- sources for nitrite oxidation in the Namibian OMZ. Based on

these results, nitrification should not be regarded as one single process, but rather as two independent reactions, at least in suboxic settings like the OMZs.

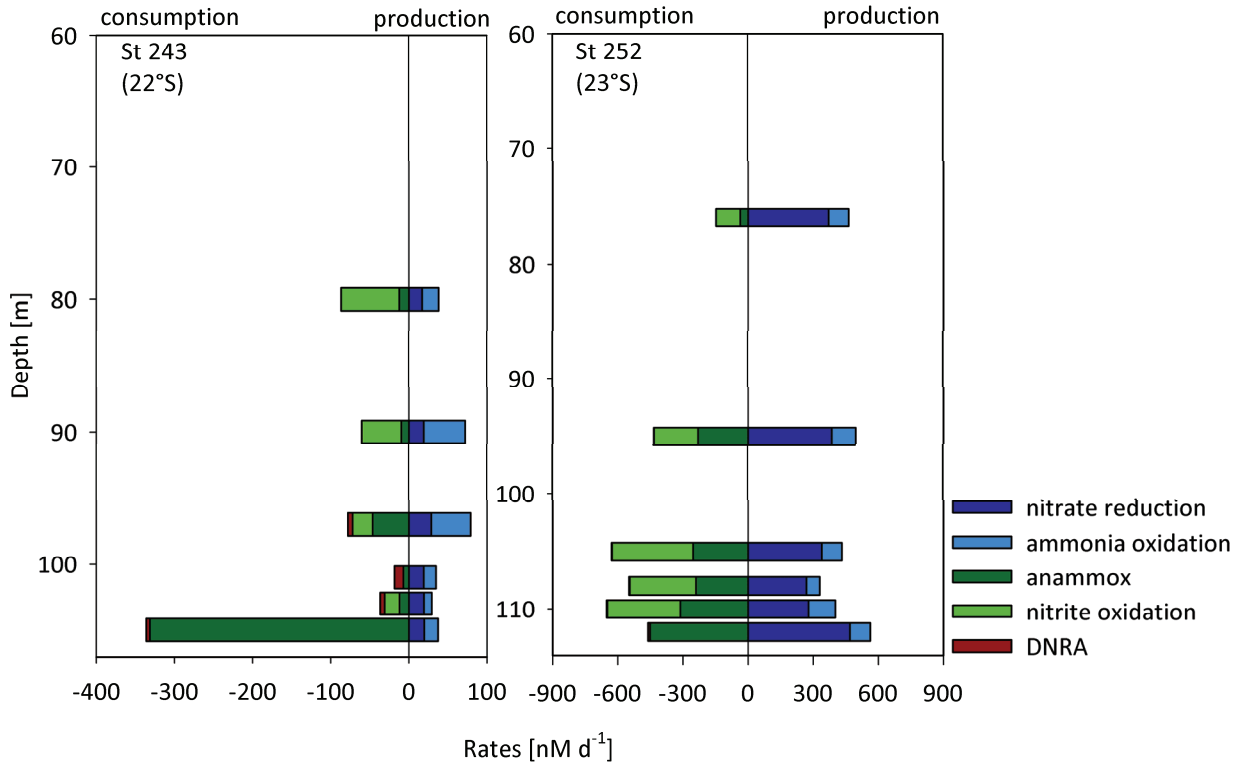

Figure 5: Comparison of nitrite sources and sinks at stations 243 and 252. Rates of NO_2^- consuming processes include anammox (dark green), nitrite oxidation (light green) and DNRA (red). NO_2^- producing processes include ammonia oxidation (light blue) and nitrate reduction (dark blue). The rates were determined from parallel incubation experiments that have been conducted with the same set of samples. . Most nitrite oxidation rates presented are derived from significant slopes ($p<0.05$) in corresponding linear regression. On occasions, $^{15}NO_3^-$ was produced so rapidly that a maximum was reached within as little as ~12 h, so rates were calculated only from these first 12h. Despite their substantial and instantaneous $^{15}NO_3^-$ production and the apparently high correlation between $^{15}NO_3^-$ and time ($r^2>0.90$), the low degrees of freedom resulted in slightly higher p-values (0.07-0.20) from one-way ANOVA in a few experiments – Nitrite oxidation: St. 243: 97m; Station 252: 105 m, 109 m and 110.7 m; Nitrate reduction: St 252 76 m and 109 m. 'n.d.' denotes non-detectable reaction rates.

Competition with other nitrite consuming processes

Nitrite can be consumed by nitrite oxidation, anammox, denitrification and DNRA. The occurrence of DNRA has been reported from the Namibian OMZ, but actual rates were not determined (Kartal *et al*., 2007). In this study, DNRA rates were determined at five stations, but significant rates were measurable only at few depths (Fig. 5). They never exceeded 10 nM d^{-1} and were usually 1-2 orders of magnitude lower than anammox and nitrite oxidation. Therefore, DNRA did not appear to be an important nitrite sink in the Namibian OMZ, at least at the time and location of our sampling. However, DNRA might become more important during more prolonged anoxia such that the true significance of DNRA in the OMZ remains to be further explored.

Previous investigations with ^{15}N-labelling experiments revealed high rates of anammox in the Namibian OMZ, while denitrification was only detected in the presence of sulphide (Kuypers *et al*. 2005; Lavik *et al*., 2009). In the current study, only low denitrification rates were detected at two bottommost BBL samples (~30 cm above the sediment) at stations 206 and 231,but sulphide was not measurable (data not shown). Overall, anammox was the main N_2-producing process at all investigated stations at the time of our sampling (Figs. 2, S2). Anammox occurred at almost all depths where nitrite oxidation rates were determined, while nitrite oxidation rates often exceeded anammox rates.

When all NO_2^- sources and sinks are compared, there was roughly a nitrite balance within the mid-depths of the OMZ, where nitrite concentration was at its lowest (Fig. 5). Net NO_2^- consumption was calculated usually for depths closest to the seafloor where NO_2^- levels were highest. However, the presented crude nitrite budget (Fig. 5) has not taken into account any external inputs and outputs. Since BBLs are characterized by intense turbulent mixing and sharp gradient of nutrients (e.g. Holtappels *et al*., 2011 a), additional NO_2^- could have been provided by the underlying sediments at these depths. Nitrite might also be transported laterally by both cross-shelf and along-shelf currents (Lass and Mohrholz, 2005). In order to fully assess the true nitrite and nitrogen budget in the Namibian OMZ, both water circulation and sediment-water fluxes need to be taken into consideration.

Conclusions

The optimized method to determine nitrite oxidation rates in seawater presented in this study enables us to directly measure low nitrite oxidation rates from small-volume samples used in ^{15}N-incubation experiments. This method has allowed us for the first time to quantitatively assess the role of nitrite oxidation in the nitrogen cycle of the Namibian OMZ. The ^{15}N-incubation experiments revealed the occurrence of nitrite oxidation throughout the Namibian OMZ. Even in the apparent absence of oxygen, nitrite oxidation proceeded at significant rates, indicating either microaerobic activities of nitrite-oxidizers or the occurrence of anaerobic nitrite oxidation. The exact use of electron acceptors in such conditions remains to be determined.

Nitrospina and *Nitrococcus* were abundant throughout the Namibian OMZ, indicating that these genera might have substantially contributed to the measured nitrite oxidation rates in these waters. However, the low abundance of these NOB at depths where high rates of nitrite oxidation were measured, suggested that more organisms were involved in nitrite oxidation in the Namibian OMZ. On the other hand, where the cell densities were too high to be explained by the determined rates, these NOB might have gained energy from processes other than nitrite oxidation.

Nitrite oxidation rates often exceeded ammonia oxidation rates, such that the two steps of nitrification are not necessarily coupled within the OMZ. Nitrate reduction, on the other hand, served as an alternative and significant NO_2^- source under these suboxic conditions. Nitrite oxidation and anammox co-occurred in these oxygen-deficient waters, with NOB potentially competing with anammox bacteria for NO_2^- when this substrate becomes limiting.

Our combined results indicate that a considerable fraction of the recently recycled nitrogen or reduced NO_3^- was re-oxidized back to NO_3^- by nitrite oxidation. Hence, NOB in oxygen deficient waters may play a critical role in the global N-cycle, potentially diminishing the amount of N-loss from OMZs.

Acknowledgements

We sincerely thank cruise leader Kay Emeis (University of Hamburg) and the crew of the *R/V Meteor* (cruise M 76/2) for their support at sea. We are grateful for the conscientious technical and analytical assistance of Gabriele Klockgether, Daniela Franzke, Tim Kalvelage, Jan Fischer, Andreas Ellrott and Volker Meyer (Max Planck Institute for Marine Microbiolgoy). Funding came from the Max Planck Gesellschaft and Deutsche Forschungsgemeinschaft (for Meteor cruise M76/2).

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Supplementary information

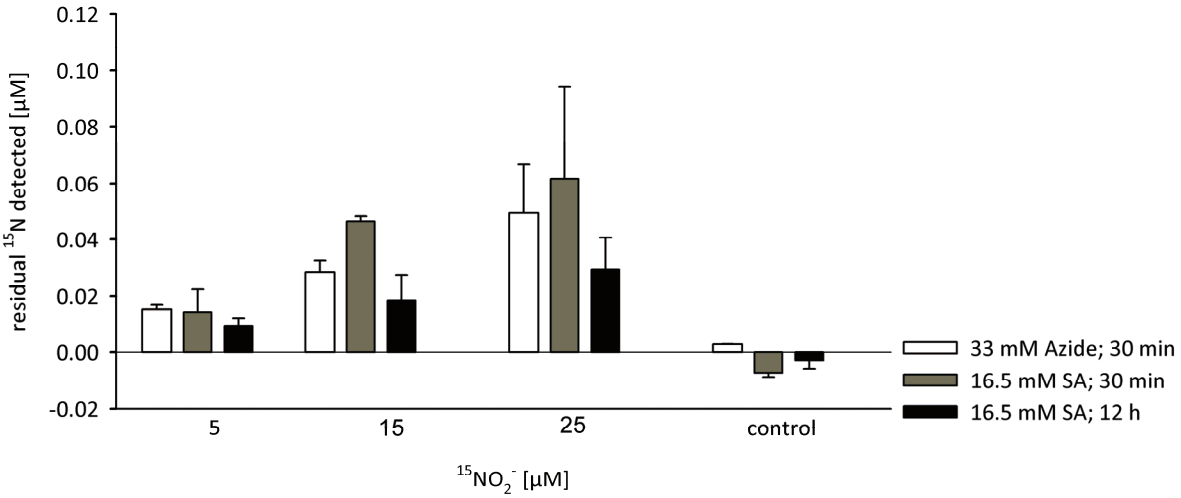

Figure S1: Effectiveness of four different approaches on nitrite removal. Initially, 5 to 25 µM of $^{15}NO_2^-$ was added and the mixtures were subjected to removal by one of the two methods: Incubation with (1) 33 mM sodium azide for 30 min (white bars), (2) 16.5 mM SA for 30 min (grey bars), and (3) 16.5 mM SA for ~ 12 h (black bars). All samples initially contained 500 µM $^{14}NO_3^-$ that were converted to $^{14}NO_2^-$ via cadmium reduction prior to $^{15}NO_2^-$ removal. No $^{15}NO_2^-$ was added in the controls

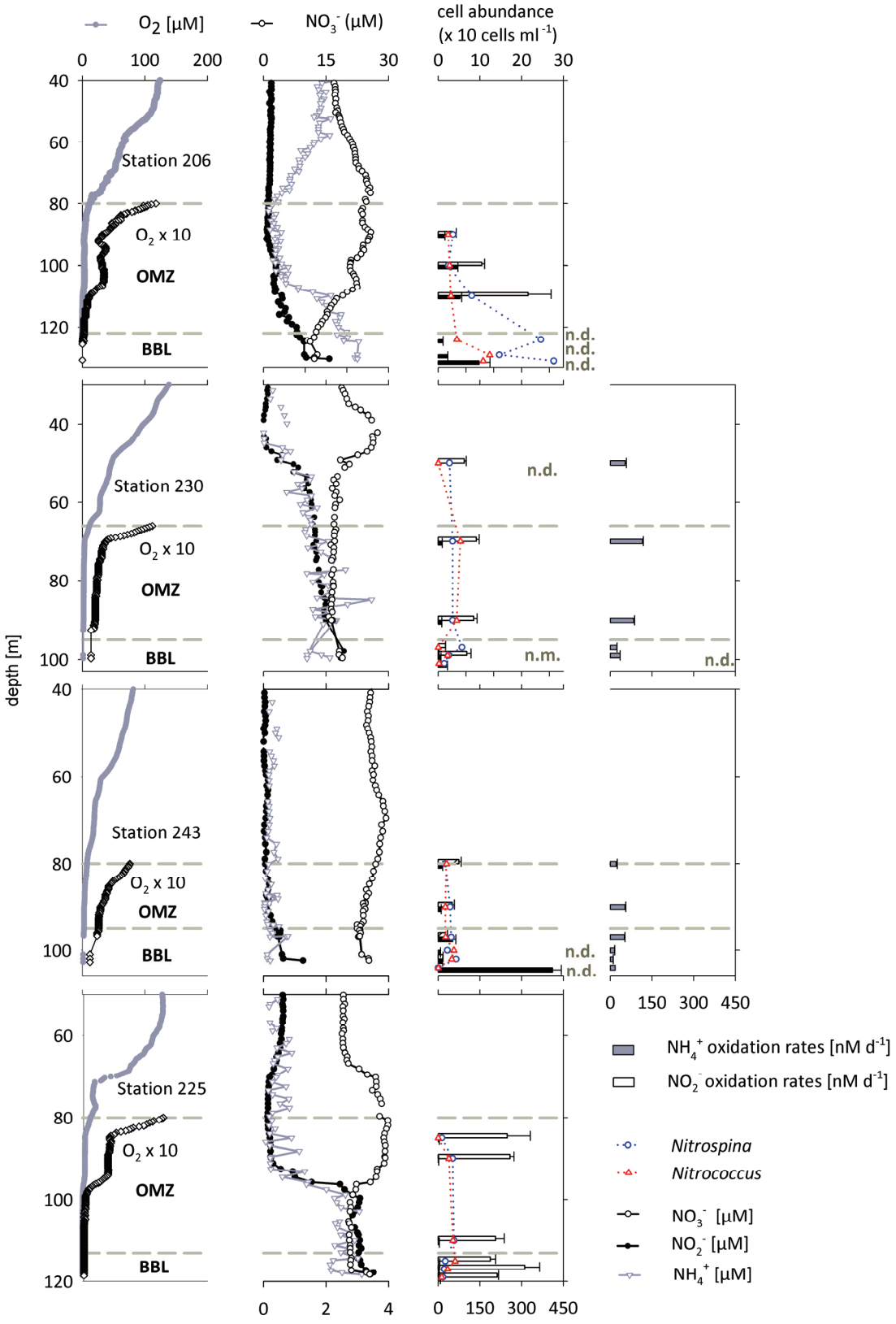
O2 [µM]
NO3- (µM)
cell abundance
(x 10 cells ml-1)
Station 206
Station 230
Station 243
Station 225
O2 x 10
OMZ
BBL
depth [m]
n.d.
n.m.
NH4+ oxidation rates [nM d-1]
NO2- oxidation rates [nM d-1]
Nitrospina
Nitrococcus
NO3- [µM]
NO2- [µM]
NH4+ [µM]

Figure S2: High-resolution vertical profiles of oxygen and 10x its concentration (O_2 grey circles, O_2 x 10 open diamonds) (first panels) and of nutrients (NO_2^-, black solid circles; NH_4^+, grey triangles; NO_3^-, open circles) (second panels) from midwater depths to the BBL at stations 206, 230, 243 and 225. NO_2^- oxidation rates measured as $^{15}NO_3^-$ production at six depths are indicated by white horizontal bars, anammox rates are indicated by black bars (third panels). Ammonia oxidation rates measured at stations 230 and 243 are indicated by grey horizontal bars (fourth panel). Also shown in each experimental depth are the cellular abundance of *Nitrococcus* (triangle) and *Nitrospina* (circle) (third panel).

Most nitrite oxidation rates presented are derived from significant slopes ($p<0.05$) in corresponding linear regression. On occasions, $^{15}NO_3^-$ was produced so rapidly that a maximum was reached within as little as ~12 h, so rates were calculated only from these first 12h. Despite their substantial and instantaneous $^{15}NO_3^-$ production and the apparently high correlation between $^{15}NO_3^-$ and time ($r2>0.90$), the low degrees of freedom resulted in slightly higher p-values (0.07-0.20) from one-way ANOVA in a few experiments – Nitrite oxidation St. 206: 110 m; Station 225: 85 m and 118 m St. 243: 97 m; Anammox: St 225: 110 m)

'n.d.' denotes non-detectable reaction rates, while 'n.m.' denotes not measured.

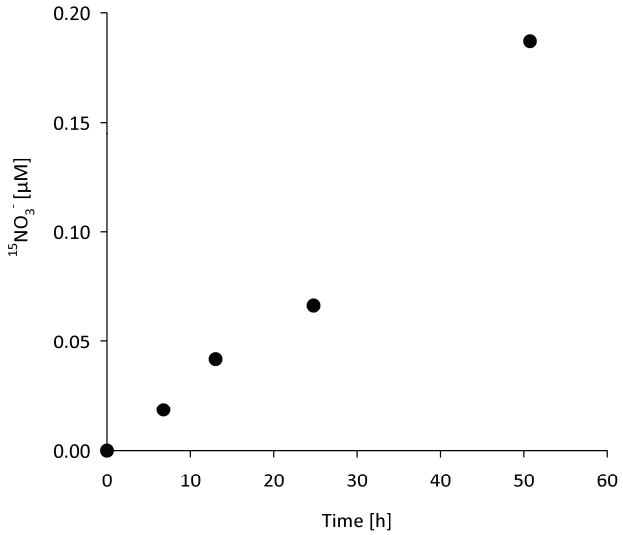

Figure S3: An example of linear increase of $^{15}NO_3^-$ production with time, as observed for 50 m water depth at station 230 (black circles). The corresponding dashed line indicates the linear regression slope (R_2: 0.981) that has been used for nitrite oxidation rate calculation.

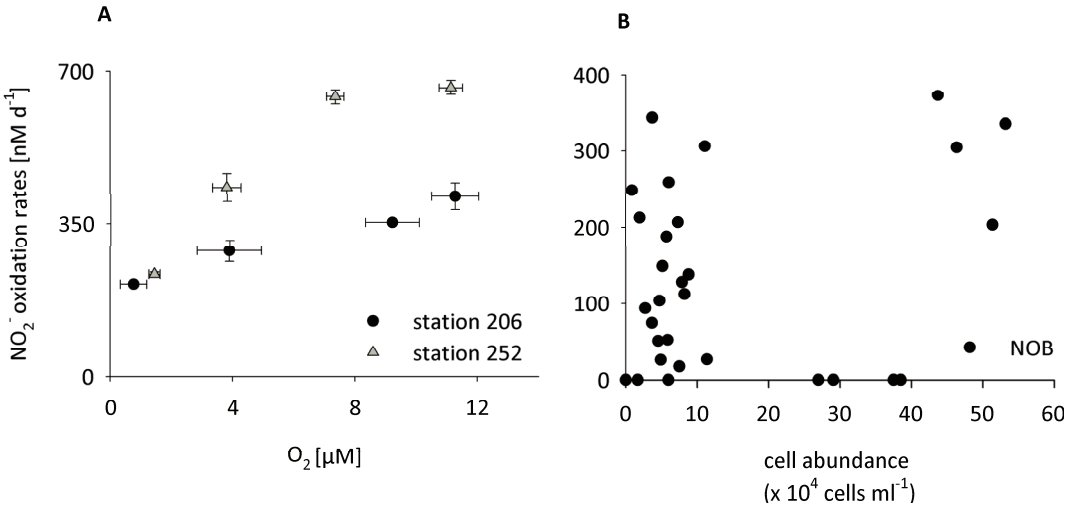

Figure S4: Nitrite oxidation rates in relation to ambient nitrite concentrations (A) and ambient oxygen concentrations (B). Samples from the benthic boundary layer (BBL) are indicated by open triangles, while those from the water column are shown as solid black circles.

Chapter 3

Metabolic versatility of a globally distributed nitrite oxidizer, *Nitrococcus sp.*

Jessika Füssel[1] and Boris Nowka[2], Sebastian Lücker[3], Phyllis Lam[1,4], Eva Spieck[2], Philipp Hach[1], Sten Litman[1] and Marcel M M Kuypers[1]

[1] Max Planck Institute for Marine Microbiology, Bremen, Germany

[2] Boris Nowka, Department of Microbiology and Biotechnology, Biocenter Klein Flottbek, University of Hamburg, Germany

[3] Sebastian Lücker, Division of Microbial Ecology, University of Vienna, Austria

[4] Current address: National Oceanography Centre Southampton, University of Southampton, England

Contribution to the manuscript:

J.F, B.N, P.L., E.S and M.M.M Kuypers designed research and project outline, J.F and P.H. performed ^{15}N incubation experiments with samples from the Namibian OMZ, J.F. and B.N performed pure culture incubation experiments, S.Li. performed NanoSIMS analyses, S.L. performed genome annotation and analyses, J.F, S.L., P.L and M.M.M.K conceived, wrote and edited the manuscript.

In preparation for *Proceedings of the National Academy of Sciences*

Abstract

Aerobic nitrite oxidation is a key process in the nitrogen cycle of oxygen minimum zones (OMZs), where it recycles a substantial portion of reduced nitrate, thereby retaining nitrate in the system and potentially reducing N-loss. *Nitrococcus* has been identified as a major nitrite oxidizer in OMZs. Nevertheless, little is known about its physiological adaptation to the oxygen deficient conditions prevailing in OMZs. We combined environmental and pure culture incubation experiments, single cell analyses and genome annotation to investigate their metabolic adaptation mechanisms. The chemolithoautotrophic bacterium *Nitrococcus* was found to adapt to anoxic, organic rich conditions by adopting an organoheterotrophic lifestyle. The presence of various sulfur metabolizing enzyme complexes within the genome further indicates that *Nitrococcus* participates in sulfur cycling and sulfide detoxification in OMZ waters. Intriguingly, incubation experiments showed that *Nitrococcus* can reduce nitrite to nitrous oxide, a potent greenhouse gas and major ozone depleting substance.

Taken together, our observations suggest the participation of *Nitrococcus* in oxidative as well as reductive branches of the nitrogen cycle in OMZs and may indicate a substantial contribution of these highly abundant nitrite oxidizing bacteria to oceanic nitrous oxide production.

Introduction

Nitrification plays a key role in the marine nitrogen cycle, returning remineralized nitrogen back to nitrate, the most abundant form of bioavailable inorganic nitrogen in the ocean. It is a two-step process comprising the oxidation of ammonia to nitrite and that of nitrite to nitrate. The aerobic oxidation of ammonia is carried out by distinct groups of Proteobacteria as well as members of the phylum Thaumarchaeaota (Brochier-Armanet *et al*., 2008; Spang *et al*., 2011). Nitrite oxidation as a standalone process has however rarely been investigated in oceanic settings and our knowledge about the abundance and diversity of marine nitrite oxidizing bacteria (NOB) is limited. Molecular approaches indicate that the genus *Nitrospina* is widespread in various marine settings, including the open ocean (Delong *et al*., 2006; Mincer *et al*., 2007; Santoro *et al*., 2010; Beman *et al*, 2010), sediments (Hunter *et al*., 2006; Davis *et al*., 2009; Joergenensen *et al*., 2013) as well as suboxic water columns (Fuchs *et al*., 2005; Labrenz *et al*., 2007; Fuchsman *et al*., 2011; Fuessel *et al*., 2012, Beman *et al*., 2013). The few environmental studies investigating the abundance of other NOB revealed the presence of members of the genus *Nitrococcus* in oxygen minimum zone (OMZ) waters (Ward *et al*., 1989), where it can be more abundant (up to 4.9 % of DAPI) than *Nitrospina* (Füssel *et al*., 2012).

The oxygen regimes in OMZs allow for the co-occurrence of nitrite oxidation with other aerobic processes such as aerobic ammonia oxidation as well as anaerobic processes such as nitrate reduction to nitrite and anammox (Lipschultz *et al*., 1990; Lam and Kuypers, 2012; Füssel *et al*., 2012, Kalvelage *et al*. 2013, Beman *et al*., 2013). Here, ammonia and nitrite oxidation are largely decoupled as NO_2^- is predominately produced by nitrate reduction to nitrite. Nitrite oxidation directly recycles a substantial proportion of reduced NO_3^- and thus potentially reduces the amount of N-loss from the system (Füssel *et al*., 2012; Casciotti *et al*.,2013).

Nitrite oxidation appears to be largely unaffected by the low to non detectable oxygen concentrations prevailing in OMZs and high rates were observed even in apparently anoxic OMZ cores (Ward *et al*., 1989; Lipschultz *et al*. 1990, Lam *et al*. 2009, Newell *et al*., 2011; Füssel *et al*. 2012, Kalvelage *et al*. 2013; Beman *et al*., 2013). Intriguingly, nitrite oxidation rates did also not correlate with NOB abundance and sometimes substantial NOB

populations (4.9 % of DAPI) were observed even without the detection of nitrite oxidation activity (Füssel *et al*., 2012). Such lack of correlation suggests the potential of some NOB to use electron acceptors other than oxygen or to employ alternative metabolisms under oxygen limitation. Flexible adaptation strategies may enable these organisms to survive and even thrive in dynamically changing environments like OMZs, where oxygen and carbon availability can fluctuate dramatically within days (Bailey and Chapman, 1991; Chavez *et al*., 1997; Morales *et al*, 1999, McChain *et al*., 2002; Paulmier *et al*, 2006, Gutiérrez *et al*, 2008).

Particularly *Nitrococcus* appears to thrive in OMZs but it is so far unclear why. Interestingly, the nitrite oxidoreductase (NXR) of *Nitrococcus* is closely related to that of *Nitrobacter,* a NOB genus found in waste waters, fresh water and soils. *Nitrobacter* can grow chemolithoautotrophically by nitrite oxidation as well as organoheterotrophically by nitrate reduction to nitrite (Steinmüller and Bock, 1976) or to nitrous oxide (Freitag *et al*., 1987). The resemblance between the NXRs of *Nitrococcus* and *Nitrobacter* might suggest similar capabilities of *Nitrococcus*, but such alternative respiration and carbon acquisition pathways remain to be verified.

The objective of this study was to examine the metabolic adaptation of *Nitrococcus* to the suboxic and organic-rich conditions encountered in OMZ waters, exemplified here by the Namibian OMZ. The potential for anaerobic nitrite oxidation as well as the ability to switch between organohetero- and lithoautotrophic growth was investigated through a combination of incubation experiments and single-cell analysis via halogen *in situ* hybridization - secondary ion mass spectrometry (HISH SIMS) (Musat *et al.,* 2008) in the isolate *Nitrococcus mobilis* strain 231 and in Namibian OMZ seawater samples. Furthermore, the genome of *Nitrococcus mobilis* sequenced within the frame of the Gordon and Betty Moore Foundation Marine Microbiology Initiative in 2006, was annotated and investigated for its organoheterotrophic potential.

Results and Discussion

Nitrite oxidation under apparent anoxia

While recent molecular studies mostly focus on the distribution of *Nitrospina* in marine settings (e.g. Mincer *et al*., 2007; Santoro *et al*., 2010; Beman *et al*., 2013), the global ocean

sampling (GOS) data and the SILVA 16S library indicate a wide distribution of *Nitrococcus* in the ocean and its important role in productive coastal ecosystem nitrogen cycling as well as in OMZs (Figure 1).

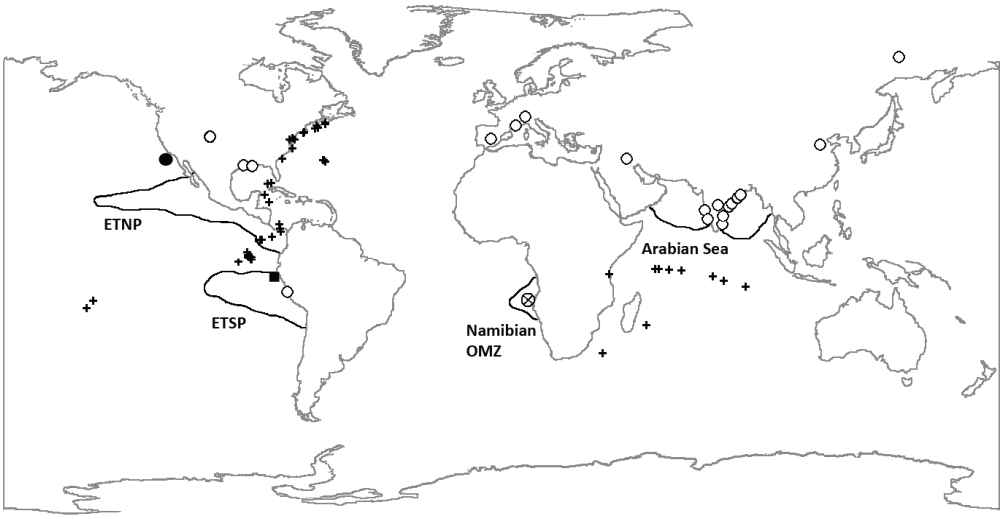

Figure 1: World map depicting the occurrence of *Nitrococcus* in the ocean. *Nitrococcus* 16S rRNA gene sequences were detected in metagenomes of the global ocean sampling (GOS) campaign (black crosses) and within the 16S SILVA database (white circles) (Pruesse *et al*., 2007). Moreover, *Nirococcus* has been detected via immunofluorescence in the Southern California Bight (black circle) (Ward and Carlucci, 1985; Ward 1987) and the Eastern Tropical South Pacific OMZ (black square) (Ward *et al*., 1989) and via CARD FISH in the Namibian OMZ (crossed circle) (Füssel *et al*., 2012). Major OMZs, including the Eastern Tropical North and South Pacific (ETNP and ETSP, respectively) the Arabian Sea and the Namibian OMZ are outlined by black lines. The geo-blast function with blastn algorithm provided by negx.net was used to search and plot the GOS and SILVA datasets (Kottman *et al*., 2010).

Nitrococcus appears to significantly differ from *Nitrospina* in its metabolic strategy. *Nitrospina gracilis* can sustain growth under low nitrite availability by exporting their NXR enzyme complexes to the periplasmic space, such that the two protons liberated during nitrite oxidation directly contribute to the proton motive force (PMF) (Spieck *et al.*, 1998; Spieck and Bock 2005, Lücker *et al.,* 2010; Lücker *et al*., 2013) (For genomic information on nitrogen uptake in *Nitrcoccus* see SI 4.1). In contrast, neither NxrA nor NxrB of *Nitrococcus mobilis* contains predicted signal peptides for protein secretion, indicating a cytoplasmic orientation of these subunits as was also described for *Nitrobacter* and *Nitrolancetus*

hollandicus (Starkenburg *et al*., 2008; Sorokin *et al*., 2012) (Further genomic information on the NXR complex of *Nitrococcus* can be found in SI 4.2). This has severe implications on energy yield, as the protons liberated have to be actively pumped across the membrane for the formation of a PMF and a large part of the derived energy needs to be invested in PMF generation. Furthermore, the high O_2 affinity of the cbb3 type terminal oxidase expressed by *Nitrospina* likely facilitates microaerophilic growth (Lücker *et al*., 2013). In comparison, the aa_3 type cytochrome c oxidase expressed by *Nitrococcus* has a relatively low O_2 affinity (Massari *et al*., 1996) (Figure 2).

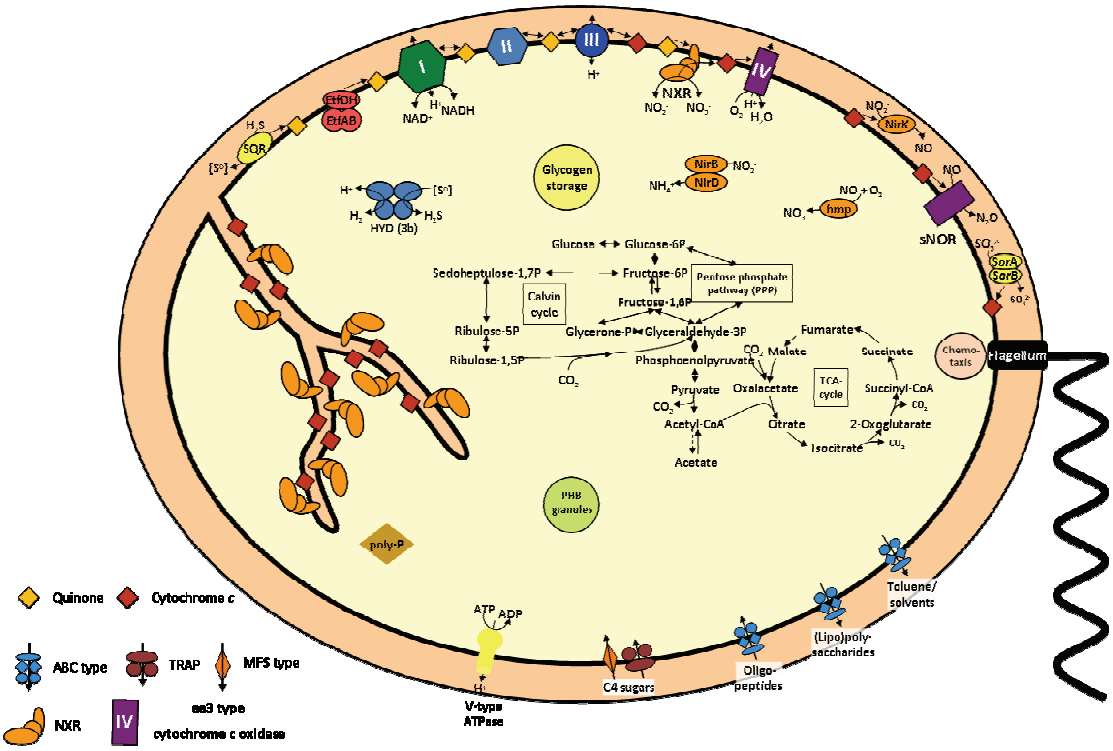

Figure 2: Cell metabolic cartoon based on the annotation of the *Nitrococcus mobilis* 231 genome. **Etf**: electron-transport flavoprotein, **hmp**: NO dioxygenase, **HYD**: hydrogenase, **NirBD**: assimilatory nitrite reductase, **NirK**: copper-containing nitrite reductase (NO-forming), **NXR**: nitrite oxidoreductase complex, **sNOR**: NO reductase, **PHB**: polyhydroxybuterate, **poly-P**: polyphosphates, **SOR**: sulfite dehydrogenase, **SQR**: sulfide:quinone oxidoreductase. Enzyme complexes of the electron transport chain are labeled by Roman numerals. Red and orange diamonds represent cytochrome *c* proteins and quinones, respectively. The legend indicates transport protein classifications.

For these reasons, *Nitrospina* thrives as K-strategists adapted to oligotrophic settings and can likely sustain growth by nitrite oxidation under microaerophilic conditions. *Nitrococcus*, in contrast resemble *r*-strategists that grow quickly whenever they encounter high substrate concentrations (Schramm *et al.*, 1999).

In the Namibian OMZ waters, Nitrococcus and *Nitrospina* were the only NOB detected (0.1 to 1.3 % DAPI and 0.7-1.3 % DAPI, respectively). While *Nitrococcus* abundance increased in the lower OMZ. *Nitrospina* was more abundant in the oxycline and the upper OMZ (SI Figure 1). A similar trend was found in the Namibian OMZ in 2009, where *Nitrococcus* constituted 4.9% of the microbial community in the lower OMZ (Füssel *et al.,* 2012). In both studies, *Nitrococcus* abundance was maximal were oxygen concentrations were below detection limits of conventional sensors and *Nitrococcus* was not detected at oxygen levels > 20 µM (Figure 3A). These results could indicate that *Nitrococcus* has the ability to utilize electron acceptors other than O_2 in the oxidation of NO_2^-.

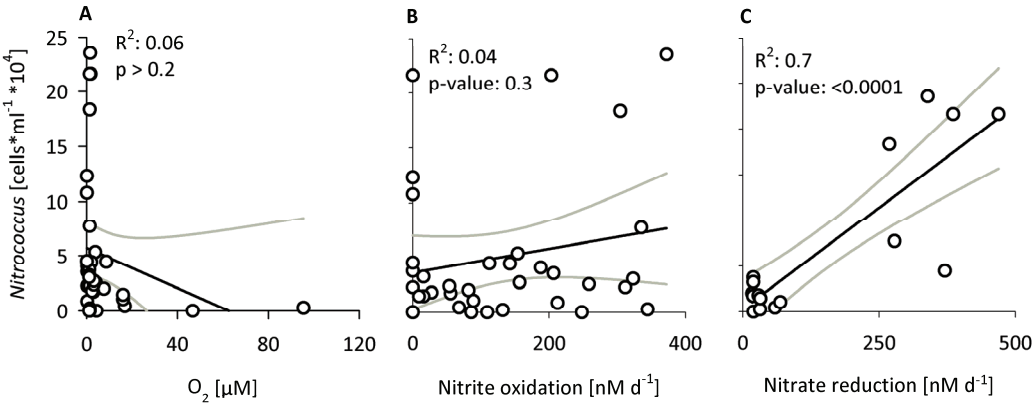

Figure 3: Abundance of *Nitrococcus* affiliated cells in the Namibian OMZ based on CARD FISH counts related to ambient O_2 concentrations (A), to nitrite oxidation rates (B) and to nitrate reduction rates (C). Data points are shown as white circles with the corresponding linear regression (black lines) and the 95% confidence interval (grey lines)

While anaerobic oxidation of nitrite by Fe(III) and Mn(IV) is thermodynamically feasible, their low abundance in the water column (Thamdrup *et al*., 1994) render them unlikely candidates as electron acceptors in pelagic environments. Chlorate (ClO_3^-) is known to serve as alternative electron acceptor in nitrite oxidation, the reaction product chlorite, however, efficiently inhibits NOB (Lees and Simpson, 1956) and ClO_3^- rarely occurs naturally. Iodate (IO_3^-) is a structural analog of chlorate and the anaerobic oxidation of NO_2^- with IO_3^-

would be thermodynamically feasible ($3NO_2^- + IO_3^- \rightarrow 3NO_3^- + I^-$; $\Delta G0' = -48.6$ kJ mol^{-1}). Concentrations of IO_3^- in seawater range between 0.1-0.6 µM and in anoxic basins IO_3^- appears to be biologically reduced to I^- resulting in subsurface I^- maxima (Farrenkopf *et al*., 1997 a; Truesdale and Bailey, 2003; Farrenkopf *et al*., 1997 b).

To investigate the potential for nitrite oxidation with IO_3^-, degassed environmental samples from the Namibian OMZ were amended with IO_3^-. In samples from the lower OMZ (O_2 < 20 µM) NO_2^- oxidation rates increased significantly ($p<0.05$; Wilcoxon matched pairs test; Figure 4), potentially indicating the utilization of IO_3^- by NOB. A similar pattern was however also observed in the absence of IO_3^- when samples were solely purged with helium prior to incubation ($p<0.05$; Wilcoxon matched pairs test; Figure 4). Thus, when the ambient O_2 concentrations were low (<20 µM), the reduction of oxygen concentrations in the incubation vessels enhanced nitrite oxidation. Oxygen contaminations inevitably occur during sampling with a CTD rosette and appear to inhibit NOB from the lower OMZ. Hence, these results imply the activity of highly adapted microaerophilic NOB in the lower OMZ. Since both, IO_3^- addition and oxygen reduction had the same enhancing effect on nitrite oxidation, the utilization of IO_3^- as electron acceptor can not be confirmed by our observations.

Anaerobic oxidation of nitrite could alternatively be facilitated via nitrite disproportionation to N_2 and NO_3^- ($5\ NO_2^- + 2H^+ \rightarrow N_2 + 3\ NO_3^- + H_2O$; $\Delta G' = -68.6$ kJ/mol). The different reaction stoichiometries of nitrite disproportionation and canonical denitrification ($2\ NO_3^- + 10\ e^- + 12\ H^+ \rightarrow N_2 + 6\ H_2O$) would allow for the differentiation of both processes in our ^{15}N labeling experiments. In our incubation experiments 11 to 600 times more $^{15}NO_3^-$ than $^{29}N_2$ was produced, with the exception of one bottom water sample that exhibited a 1:1 ration of both products. Production of $^{30}N_2$ could not be detected. Thus, neither nitrite disproportionation nor canonical denitrification appear to contribute to N_2 production in the Namibian OMZ. The parallel occurrence of aerobic or anaerobic nitrite oxidation and nitrite disproportionation however would increase the ratio of $^{15}NO_3^-$ over $^{29}N_2$ and based on the current data we can not exclude the occurrence of nitrite disproportionation.

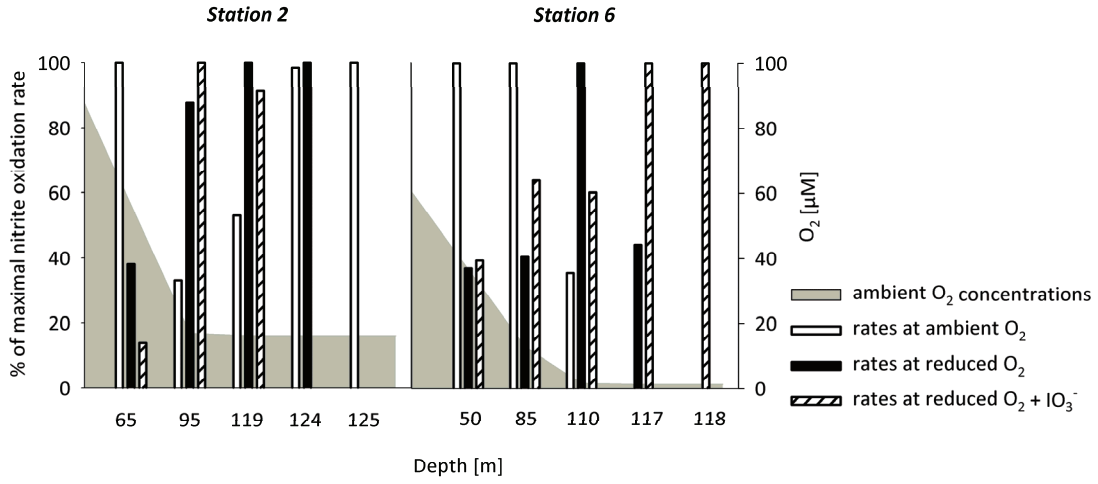

Figure 4: Rates of Nitrite oxidation measured as the production of $^{15}NO_3^-$ from $^{15}NO_2^-$ over time at two stations in the Namibian OMZ including 5 depths each are indicated by vertical bars. Samples incubated under ambient O_2 (white bars), reduced O_2 (black bars) and reduced O_2 concentrations plus IO_3^- (striped bars). Maximal nitrite oxidation rates measured at each depth corresponds to 100%. Rates measured under different incubation conditions are depicted as proportions of the maximal rate observed.

Experiments with pure cultures of *Nitrococcus mobilis* 231 did not show any detectable NO_3^- production in the absence of O_2 (Figures 5A and SI Figure 2A). Thus, *Nitrococcus mobilis* 231 is neither able of nitrite disproportionation nor anaerobic nitrite oxidation with IO_3^-. Nevertheless, other strains or species of *Nitrococcus* might be able of anaerobic nitrite oxidation. From 16 clones isolated from the Namibian OMZ, two were affiliated to *Nitrococcus*. *Nitrococcus* clone I could be ascribed to *Nitrococcus mobilis* (98.9% 16S rRNA gene sequence identity) while *Nitrococcus* clone II (96.6-97.7 % 16S rRNA gene sequence identity) represents a distinct *Nitrococcus* species (SI Figure 3). Since metabolic capabilities of different species belonging to one genus can greatly differ, it can not be excluded that some *Nitrococcus* species in the Namibian OMZ might posses the capability to oxidize nitrite anaerobically.

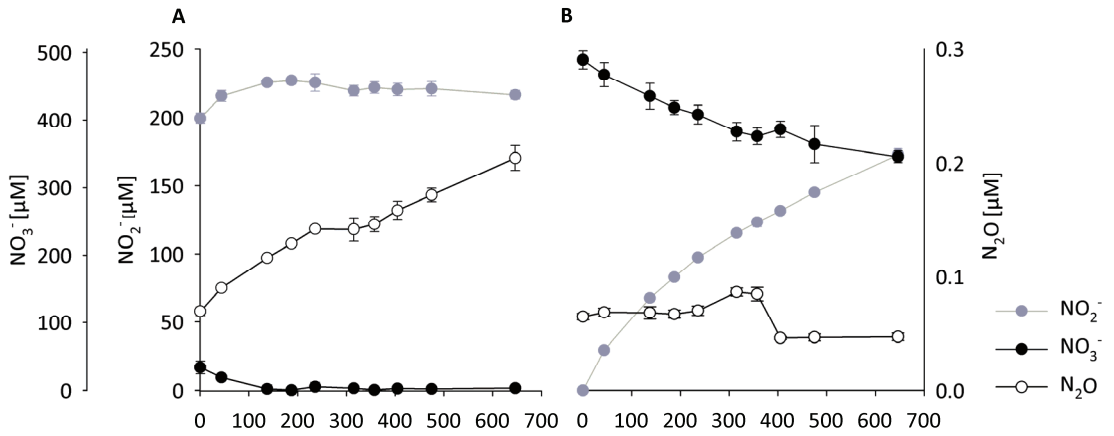

Figure 3: Incubation experiments with *Nitrococcus mobilis* strain 231: NO_2^- (grey circles), NO_3^- (black circles) N_2O (open circles), were measured in over a time course of 700h in incubation experiments with *Nitrococcus mobilis* under lithoautotrophic conditions (A): 200 µM $^{15}NO_2^-$, anaerobic and organoheterotrophic conditions (B): 500 µM $^{15}NO_3^-$, 250 µM acetate, 250 µM formate, anaerobic.

Organotrophic nitrate reduction

Bottom waters of the Namibian OMZ are characterized by the accumulation of NO_2^- (~2-7 µM) that could facilitate high nitrite oxidation rates and thus sufficient energy conservation to sustain growth of *Nitrococcus*. The terminal oxidase of *Nitrococcus* however likely prevents efficient nitrite utilization under the low to non detectable oxygen levels. Moreover, *Nitrococcus* abundance did not correlate with nitrite oxidation rates in the Namibian OMZ (Wilcoxon matched pairs test; $p>0.6$) (Figure 3B). Nevertheless, *Nitrococcus* outnumbered *Nitrospina* under these conditions. These observations indicate metabolic capacities of *Nitrococcus* other than nitrite oxidation. Interestingly, *Nitrococcus* abundance and nitrate reduction rates in the Namibian OMZ were significantly correlated (Figure 3C) (Wilcoxon matched pairs test; $p< 0.0002$), indicating the contribution of *Nitrococcus* to nitrate reduction.

The potential for nitrate reduction in *Nitrococcus* could be confirmed in pure cultures of *Nitrococcus mobilis* strain 231, that instantaneously switched to NO_3^- reduction under anoxic conditions when organic substrates were amended. When formate and acetate were provided as electron donors, reduction of NO_3^- was enhanced during the first ~230 h of

incubation (~17 µM d^{-1}) and subsequently decreased to <1 µM d^{-1}. After ~230 h, ~150 µM NO_3^- had been reduced to NO_2^-. Based on the 2:1 ratio of nitrate reduction to organic carbon oxidation, neither formate nor acetate could have (125 µM) become limiting. Instead, the accumulation of nitrite to ~150 µM presumably inhibited *Nitrococcus mobilis* (Figure 5 B).

In incubations with complex DOM obtained from algal biomass, nitrate reduction decreased from ~4.5 µM d^{-1} to ~ 1 µM d^{-1} after ~ 180 h of incubation, when ~40 µM NO_3^- had been reduced. Inhibition of *Nitrococcus* by the comparably low NO_2^-concentrations (see above) can be excluded, instead *Nitrococcus* could likely only utilize a small fraction of the substrate mixture (SI Figure 2B).

These observations indicate that the NXR of *Nitrococcus mobilis* 231, that is most similar to the enzyme systems found in *Nitrobacter* and *Nitrolancetus*, as well as to the nitrate reductase system of *Candidatus* Methylomirabilis oxyfera (Spieck and Bock, 2005; Lücker *et al*., 2010; Sorokin *et al*., 2012; Lücker *et al*., 2013) is also capable of nitrate reduction. The genome of *Nitrococcus mobilis* further encodes a second copy of the NXR alpha subunit (NxrA2), which has an amino acid identity of 89% to NxrA1 and is not clustered with other genes involved in nitrite oxidation. This duplication of the catalytic subunit also was observed in all *Nitrobacter* genomes and in *Nitrolancetus hollandicus* (Starkenburg *et al*., 2008; Sorokin *et al*., 2012), but its function so far has not been elucidated. These NXR homologues might display different preferred reaction directions, such that they can reduce nitrate under anoxic organotrophic conditions by reversal of NXR.

The genome annotation further revealed the potential of *Nitrococcus* to assimilate acetate and possibly ethanol (or other primary and secondary alcohols), indicating the ability of *Nitrococcus* to utilize these organic compounds as electron donor. Furthermore, the presence of diverse C_4 carboxylate transporters might allow transport and utilization of mono- and dicarboxylic acids (Figure 2) (For genomic information on CO_2 fixation in Nitrococcus, se SI 4.3). These low molecular weight organic acids are important intermediates in many biological reactions, such as the tricarboxylic acid cycle and fermentation. Their concentrations in seawater are comparable to other labile organic compounds (Steinberg and Bader, 1984) and they constitute a substantial fraction of dissolved organic carbon (DOC) in marine sediments (Barcelona, 1980).

The environmental relevance of our culture and genome based results was further investigated via single cell analyses of *Nitrococcus* in samples from the Namibian OMZ. Upon

incubation with ^{13}C DOM or ^{13}C HCO_3^- , single *Nitrococcus* cells were targeted and analyzed via Halogen *in situ* hybridization-secondary ion mass spectrometry (HISH SIMS). The ^{13}C enrichment of on average 1.6% ± 0.3 AT% in *Nitrococcus* cells incubated with ^{13}C DOM confirmed their heterotrophic lifestyle under oxygen deficient conditions. Parallel incubation experiments with ^{13}C HCO_3^- did not result in a significant ^{13}C enrichment of *Nitrococcus*. Thus, remineralization of ^{13}C DOM and subsequent uptake of ^{13}C HCO_3^- within the incubations can be excluded (Figure 6). Heterotrophic growth in *Nitrococcus* is facilitated by the complete oxidative TCA cycle and all genes involved in glycolysis, glyconeogenesis and the oxidative branch of the pentose phosphate pathway are present (Figure 2). These pathways provide building blocks for anabolic biosynthesis reactions, but also are involved in the formation and degradation of storage compounds. Indeed, genes for production and utilization of glycogen and polyhydroxybutyrate (PHB) could be identified and both storage compounds were already noticed by Watson and Waterbury (1971). The genome annotation further revealed genes for respiratory complexes I – III (Figure 4), which are not required for nitrite oxidation but will function in energy conservation during oxidation of organic substrates and in the reverse electron flow from nitrite to NAD+ during chemolithoautotrophic growth. Furthermore, an electron transfer flavoprotein (ETF) complex and an ETF:quinone oxidoreductase probably couple beta-oxidation of fatty acids to the respiratory chain.

Our results clearly indicate the ability of *Nitrococcus* to preserve energy via the reduction of nitrate to nitrite under oxygen limiting conditions. Given their previously observed ability to utilize acetate as carbon source, (Watson and Waterbury, 1971), these organisms are able to grow lithoautotrophically via nitrite oxidation as well as organoheterotrophically via nitrate reduction.

Potential involvement in sulfur cycling

Intense benthic sulfate reduction sporadically results in the accumulation of sulfide in bottom waters of the Namibian OMZ (Brüchert *et al*., 2006; Lavik *et al*., 2008) and occasionally sulfide even accumulates in the water column with disastrous effects for marine life (Diaz and Rosenberg, 1995; Malakoff 1998). Sulfide also inhibits various microbial

processes, including anammox, ammonia and nitrite oxidation (Joye and Hollibaugh 1995; Lam and Kuypers, 2011).

Interestingly, *Nitrococcus* appears to be adapted to elevated sulfide concentrations, as its genome encodes several sulfur-metabolizing enzyme systems that could facilitate its participation in sulfur cycling (Figure 5). First, a sulfide:quinone oxidoreductase might oxidize hydrogen sulfide in the periplasmic space to elemental sulfur or polysulfide S^0, with the concomitant reduction of quinone. Second, a periplasmic sulfite dehydrogenase could oxidize sulfite to sulfate and transfer the electrons onto cytochrome c. Last, a cytoplasmic type 3b bidirectional hydrogenase (Vignais and Billoud, 2007) is also present. These so called sulfhydrogenases can catalyze the reversible oxidation of H_2 with $NAD(P)^+$ (Silva *et al*., 2000), but also the reduction of S^0 to H_2S (Ma *et al*., 2000). Through the action of this hydrogenase, S^0 might serve as electron sink during anaerobic growth on low-potential electron donors like organic carbons and in the absence of a more favorable terminal electron acceptor. These observations indicate that *Nitrococcus* might participate in the previously described detoxification of sulfide within the oxygen depleted bottom waters (Lavik *et al*., 2008). Overall, the presence of these sulfur-metabolizing enzyme systems in *Nitrococcus* unveils an unexpected link of the marine carbon, nitrogen, and sulfur cycle in one single organism.

Partial denitrification

OMZs are major sources of oceanic nitrous oxide (N_2O), a potent greenhouse gas and ozone depleting substance (Lashof and Ahuja, 1990; Ravishankara *et al*., 2009; Nevison *et al*., 2003; Suntharalingan *et al*., 2000). In the Namibian OMZ, ~17 nM d^{-1} N_2O where produced in bottom waters where NO_2^- accumulated, oxygen concentration was minimal and *Nitrococcus* abundance maximal (SI Figure 1). Production of N_2O is usually ascribed to denitrifying and ammonia oxidizing microorganisms, whereas the latter organisms release N_2O either as a byproduct of ammonia oxidation or in the course of nitrifier denitrification. Here, no canonical denitrification was detected, indicating a minor relevance of denitrification in N_2O production. Ammonia oxidation amounted to 35 nM d^{-1} and could not account for the observed N_2O production, indicating the presence of an additional N_2O source. Pure cultures of *Nitrococcus mobilis* 231 continuously produced small but significant amounts of N_2O during anoxic incubations with NO_2^- or NO_2^- and IO_3^- (Figure 5 A, SI Figure 2A). No organic

substrates were amended in these incubations and nitrite reduction was likely facilitated by the ambient DOC in the sterilized seawater (SI Table 2). Thus, potential N_2O production rates of *Nitrococcus* might be underestimated from our experiments. Rates of denitrification to N_2O from *Nitrococcus mobilis* were similar to those from *Nitrobacter vulgaris* (Figure 3A, SI Figure 2+4, A and A+B, respectively) (< 8 nM d^{-1} during the first 140 h) and amounted to ~ 0.2 % of nitrate reduction rates. When the NO_3^- level was significantly higher than that of NO_2^- (>0.4 mM and 0-140 µM, respectively), no N_2O production was observed, despite the amendment of organic compounds (Figure 5B, SI Figure 2B). Thus, nitrate is preferentially reduced and nitrite reduction to N_2O might be initiated by the accumulation of NO_2^- or the depletion of NO_3^-. Such conditions often prevail in the core of OMZs, where NO_2^- accumulates to concentrations of up to ~12 µM and NO_3^- is depleted. Given the high abundance of *Nitrococcus* in the Namibian OMZ, these organisms might substantially contribute to N_2O production in oxygen deficient environments.

Nitrite reduction in *Nitrococcus mobilis* is facilitated by a copper-containing NO-producing nitrite reductase (NirK), but no canonical cytochrome *c* or quinone-dependent nitric oxide reductase (NOR) was identified. Instead, annotation revealed the presence of a two-subunit oxidase that belongs to a distinct phylogenetic cluster within the haem-copper oxidase family which also contains proteins from *Nitrobacter*, *Nitrolancetus*, and most genome-sequenced ammonia-oxidizing bacteria (Figure 2). This family was designated as sNOR family and a function as NO reductase to detoxify NOx intermediates produced in the periplasm during the metabolism of AOB was suggested (Stein *et al*., 2007). To our knowledge, however, this is the first study actually showing anoxic organotrophic denitrification by an organism containing this type of NOR only, thus giving evidence that this enzyme family indeed functions as NOR and can further be used for energy conservation. In agreement with the lack of N_2 formation by *Nitrococcus*, a nitrous oxide reductase is not present in the genome.

Conclusions

The combination of molecular, biogeochemical and *in silico* methods enabled us to gain insights into the metabolic potential of *Nitrococcus* as well as into its environmental relevance. These NOB appear to be metabolically highly flexible as they are able to switch

between lithoautotrophic NO_2^- oxidation and organoheterotrophic NO_3^- as well as NO_2^- reduction, depending on the prevalent biogeochemical conditions, explaining the apparent discrepancy between NOB abundances and nitrite oxidation rates found in OMZ environments. Intriguingly, *Nitrococcus* can recycle the 'waste' product of its main energy generating reaction once oxygen levels drop below a critical threshold. Furthermore, the presence of various sulfur metabolizing enzyme complexes in the genome of *Nitrococcus* might facilitate growth even under sulfidic conditions that sporadically occur in OMZs (Lavik *et al*., 2008; Schunck et al., 2013). Thus, these bacteria are ideally adapted to dynamic systems with varying organic matter and O_2 levels such as coastal OMZs, which are characterized by episodic upwelling events on a timescale of hours or days (Bailey and Chapman, 1991; Chavez *et al*., 1997; Service *et al*., 1998; Pennington and Chavez *et al*., 2000, Mc Clain *et al*, 2002).

Due to the presence of a cytoplasmic NXR system that requires comparably high nitrite concentrations for sufficient energy generation, this metabolic versatility was likely necessary in order to be competitive with other NOB like *Nitrospina*, which are better adapted to the mostly oligotrophic conditions found in the open ocean. Thus, while *Nitrospina* appears to be the dominant NOB in such settings, *Nitrococcus* might play an important role in niche ecosystems such as surface slicks (Watson and Waterbury, 1971) or marine snow aggregates and might be overlooked in large scale metagenomic studies, possibly do to the size fractionation method usually employed in these study setups.

Nitrococcus are widely distributed in OMZs, were 25-50% of oceanic N_2O production occurs (Santharalingan *et al*., 2000; Nevison *et al.*, 2003) that is mostly attributed to ammonia-oxidizing microorganisms (e.g.Nevison *et al*., 2003; Farias *et al*., 2007, Santoro *et al*., 2011) and denitrifiers (e.g. Naqvi *et al*., 2000; Nicholls *et al*., 2007; Farias *et al*., 2009;). In this study we now demonstrate that marine *Nitrococcus* affiliated NOB also are able to reduce NO_2^- to N_2O but lack the nitrous oxide reductase necessary for complete denitrification to N_2. N_2O production by these organisms might occur over a wide range of O_2 concentrations and would in prior studies have wrongly been attributed to ammonia oxidizers. The wide distribution and high abundance of *Nitrococcus* in OMZs as well as the often several fold higher potential NO_2^- than NH_3 oxidation rates imply a significant contribution of NOB to oceanic N_2O production.

Material and Methods

Water sampling was conducted onboard the R/V Maria S. Merian in October 2011 (MSM19/1c) over the Namibian shelf between 21°59.9 S/13°40.92 E and 28°30 S/15°45.01 E. Salinity, temperature, dissolved oxygen and chlorophyll a fluorescence were measured with a conductivity-temperature- depth (CTD) system, equipped with an oxygen sensor and a fluorometer (Sea Bird Electronics, Bellevue, WA, USA). Water samples were obtained with 12L Niskin bottles attached to the CTD system. Oxygen data were calibrated against Winkler titration. The benthic boundary layer (BBL) was sampled at 3 depths from 30cm to 2m above seafloor using a bottom water sampler (Sauter *et al*., 2005; Holtappels *et al*., 2011a). NO_2^- and NH_4^+ were measured on board spectrophotometrically (Grasshoff *et al*., 1999) and fluorometrically, respectively (Holmes *et al*., 1999). Water samples were frozen for later analyses of NO_3^- and PO_4^{3-} with an autoanalyzer in a shore-based laboratory (TRAACS 800, Bran & Lubbe, Hamburg, Germany). Detection limits for NH_4^+, NO_2^-, NO_3^- and PO_4^{3-} were 0.01, 0.01, 0.1 and 0.1 µM, respectively.

Incubation experiments

Incubation experiments were conducted with water collected from two stations in the Namibian OMZ, located at 22° S, 13°4 E and 22° 6 S, 16° 6 E. Water samples (250 ml) were collected from five to six depths with the CTD system or the bottom water sampler for samples from the BBL. Different combinations of ^{15}N- and ^{14}N-substrates were added for the rate determinations of ammonia oxidation, nitrite oxidation and nitrate reduction (SI Table 1). Samples were purged with helium for 15 minutes to eliminate any O_2 contamination from the CTD system (Dalsgaard *et al*., 2003). For NH_3 and NO_2^- oxidation a second set of incubation experiments without helium purging were conducted. Each ^{15}N(/^{14}N)-amended sample was immediately transferred into five 12-ml exetainer vials (Labco, High Wycombe, Buckinghamshire, UK). These samples were incubated for up to 38 h in the dark at *in situ* temperatures. At each time interval (approximately 0, 6, 12, 24 and 38 h), incubation in one exetainer was terminated by adding 100 ml of saturated mercuric chloride solution to stop biological activities. Samples were stored upside down in the dark at room temperature until

further processing in a shore-based laboratory (For a more detailed description, see Holtappels *et al*., 2011 b).

Stable isotope analyses of N_2

Nitrogen stable isotopic ratios of N_2 were determined by GC-IRMS (VG Optima, Manchester, UK). Ammonia oxidation rates and nitrate reduction rates were determined as the $^{15}NO_2^-$ production over time from incubations amended with $^{15}NH_4^+/^{14}NO_2^-$ or $^{15}NO_3^-/^{14}NO_2^-$ via the conversion of NO_2^- by sulfamic acid (Granger and Sigmann, 2009). Nitrite oxidation rates were determined in incubations amended with $^{15}NO_2^-/^{14}NO_3$. After the removal of residual NO_2^-, NO_3^- was reduced to NO_2^- with cadmium and subsequently to N_2 via sulfamic acid as previously described (Füssel *et al*., 2012). Process rates were calculated from the slopes of linear regressions with $^{15}N_2$-production as a function of time, and only when the production was instantaneous. All rates were calculated as net rates from five samples obtained over the course of incubation (0–38 h) ($P<0.05$) and have been corrected for the ^{15}N-labeling percentages of initial substrate pools. In the incubation experiments for rate determinations of ammonia oxidation and nitrate reduction, the addition of $^{14}NO_2^-$ alongside the ^{15}N-labeled substrates will minimize immediate consumption of the ^{15}N-labeled products during the incubation period and the measured rates thus are expected to be closer to gross rates.

Catalyzed reporter deposition in situ fluorescence hybridization (CARD-FISH)

Water samples were fixed with 2% (final concentration) paraformaldehyde in phosphate buffered saline solution for 8-12 h at 4°C followed by filtration onto polycarbonate membrane filters (GTTP, 0.22 µM pore size, 47 mm diameter, Millipore, Eschborn, Germany). CARD FISH was performed according to the protocol by Pernthaler *et al*., (2002). First, cells were immobilized on the GTTP filters by embedding in 0.2% agarose followed by permeabilization in 10 mg ml^{-1} lysozyme in 50 mM EDTA and 100 mM Tris-HCL. Labelled oligonuctleotide probes (Biomers, Ulm, Germany) were added (SI Table 4) and allowed to hybridize for 2 h at 46° at varying formamide concentrations, according to the oligonucleotide applied (SI Table 4). This was followed by tyramide signal amplification for 15 min at 46°C and subsequent staining of cells with DAPI (4′, 6′-diamidino-2-phenylindole).

DAPI-stained cells and positive hybridization signals were enumerated with epifluorescence microscopy (Axioplan 2, Zeiss, Jena, Germany).

Pure culture experiments

Pure cultures of *Nitrococcus mobilis* strain 231 and *Nitrobacter vulgaris* strain Ab1 were grown aerobically in a batch culture for 32 days in 3.5 L of seawater and freshwater medium, respectively (SI Table 2). NO_2^- was repeatedly resupplied to a final concentration of 3 mM when the substrate had been consumed. The cultures had reached stationary phase when the experiments were conducted. For anoxic incubation experiments, cultures were pelleted by centrifugation and transferred into 2 L of anaerobic medium (SI Table 2). Subsequently, each experimental step was conducted in an anaerobic chamber (90%N_2/10%CO_2 atmosphere) to avoid oxygen contamination. 150 ml of culture were transferred into 250 ml serum bottles and various substrates were added (SI Table 3). All experiments were conducted in triplicate. To test for abiotic reactions, dead controls with autoclaved cultures were included (SI Figures 2 C). The bottles were sealed with butyl stoppers and incubated at 28°C in the dark. For the duration of 28 days, a total of 10 samples composed of 3 ml culture and 2 ml headspace were obtained every 2-3 days. The sampling volume was replaced with 90% N_2/10% CO_2 from the anaerobic chamber to avoid negative pressure in the incubation flasks.

Concentrations of NO_x and NO_2^- were measured in culture samples with a NOx analyzer (CLD 63, ecophysics, Munich, Germany) and ^{15}N nitrite and nitrate were determined as described above via isotopic ratio mass spectrometry.

N_2O and N_2 measurements

The gas samples were injected into sterile, water filled 3 ml exetainers and the water was displaced via a compensation needle. The concentration of N_2O in the headspace was measured by a gas chromatograph with a 63Ni electron capture detector (Shimadzu GC-8A). The linear increase over time was used to calculate the N_2O production rates by *Nitrococcus* and *Nitrobacter.* Nitrogen stable isotopic ratios of N_2 ($^{15}N^{15}N$:$^{14}N^{14}N$ and $^{15}N^{14}N$:$^{14}N^{14}N$) was

determined by GC-IRMS (VG Optima, Manchester,UK). Rates were calculated from the slopes of linear regressions with $^{29}N_2/^{30}N_2$ and N_2O production as a function of time.

NanoSIMS analyses

During the cruise to the Namibian OMZ, 250 ml water samples were purged with He for 15 min and amended with 5 µM $^{15}NO_2^-$ and either ^{13}C-labeled dissolved organic matter (^{13}C-DOM) or 200 µM ^{13}C-HCO_3^- , alongside incubations for rate measurements. After ~29 h of incubation, samples were fixed with 1 % paraformaldehyde (PFA, final concentration) over night at 4°C. Subsequently, 11 ml of sample were filtered onto gold-palladium pre-coated polycarbonate filters (type GTTP, 0.2 µm pore size, 25 mm-diameter, Millipore, Eschborn, Germany) and stored at -80°C until further analyses in a shore-based laboratory.

In pure culture experiments, *Nitrococcus mobilis* strain 231 was incubated with either complex ^{13}C-DOM from algal biomass or 250 µM ^{13}C-acetate/^{12}C-formate. Subsamples were obtained after ~13 days of incubation, followed by PFA-fixation and filtration as described above.

Nitrococcus cells were hybridized on filter with *Nitrococcus*-specific CARD FISH probes (for detailed description, see Füssel *et al*. 2012) and analyzed with a NanoSIMS 50L (Cameca, Gennevilliers, France). To obtain a stable ion emission rate and to clean the sample from any contamination, the area of interest was pre-sputtered with a Cs^+ primary ion beam of 100 pA. The cells were analysed by rastering a primary Cs+ ion beam with a beam current of 0.8-1 pA and a beam diameter of 50-100 nm. Secondary ion images of $^{12}C^-$, $^{13}C^-$, $^{19}F^-$, $^{12}C^{14}N^-$ and $^{32}S^-$ were recorded in parallel. The analysed areas ranged from 10 x 10 µm to 20 x 20 µm with an image size of 256 x 256 pixels and a dwell time of 1 ms per pixel. Tuning of the instrument for high mass resolution (~7000 MRP) reduced the interference for ^{13}C. Data analyses were performed with the freeware "Look@nanosims" (Polerecky *et al*., 2012).

Genome Annotation of Nitrococcus mobilis

The genome of *Nitrococcus mobilis* 231 was sequenced by the J. Craig Venter Institute in the framework of the Gordon and Betty Moore Foundation Marine Microbiology Initiative. The sequence was deposited in the GenBank database in 2006 and consists of 43 assembled

contigs. The draft genome has a size of 3,617,638 bases and contains 3552 open reading frames (ORFs) encoding for 3503 protein coding genes (coding sequence, CDS), one complete rRNA operon, and 45 tRNA genes for all 20 amino acids, between 1 and 5 for each tRNA type. The average GC content is 59. 97%.

The draft genome of *Nitrococcus mobilis* 231 (GenBank project PRJNA13475) was integrated into the MicroScope annotation platform (Vallenet et al., 2013). After automated prediction and annotation of coding sequences (CDS), the annotation of all CDS in key pathways, including those for nitrite oxidation, respiration, and carbon fixation, was manually refined by using the respective tools of MaGe (Vallenet *et al*., 2006) as described in detail elsewhere (Lücker *et al*., 2010).

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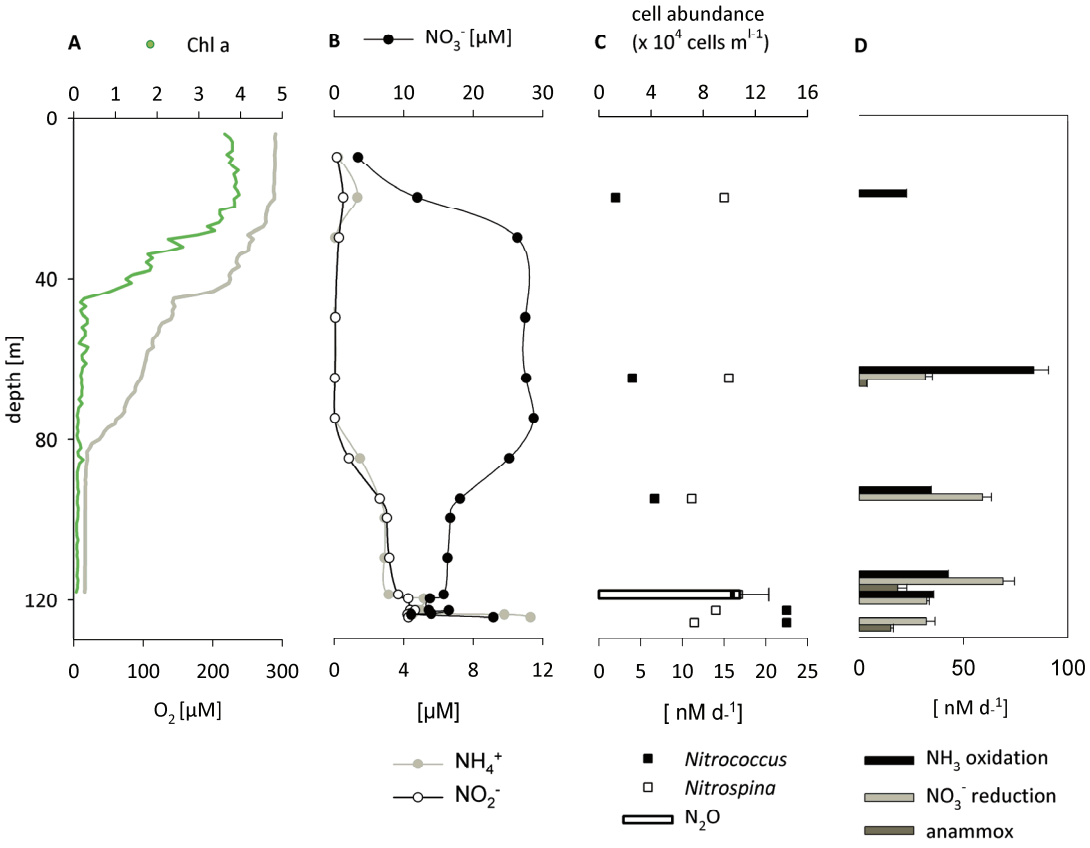
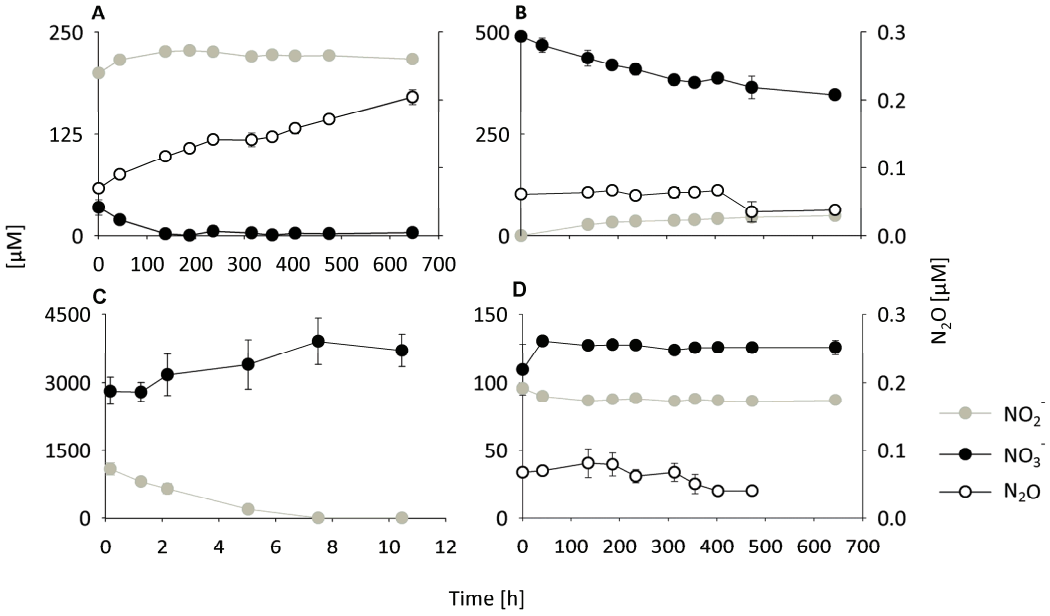
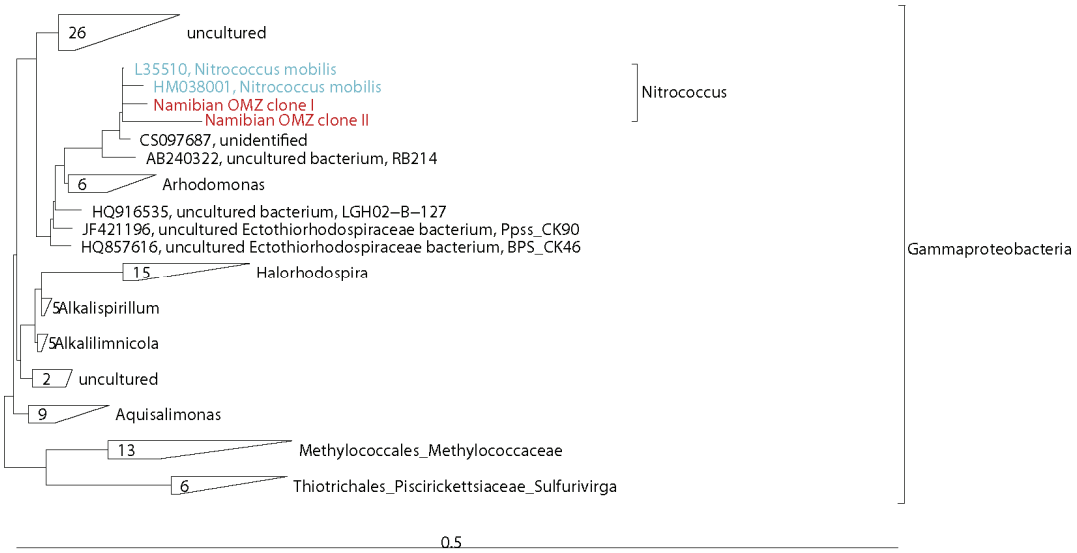
Supplementary Information



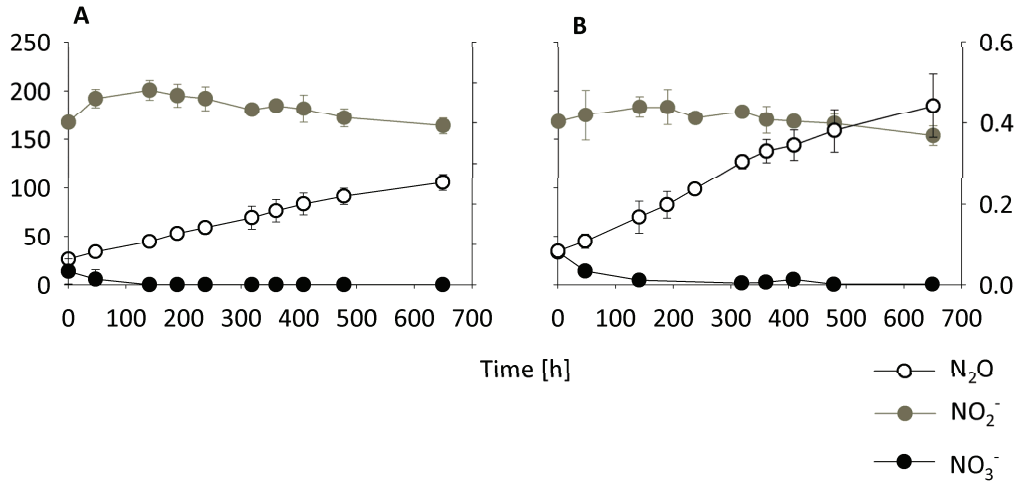
SI Figure 1: Station 2: Vertical profiles of oxygen (grey line) and chlorophyll (green line) (left panel) and of nutrients: NO_2^-, (open circles), NH_4^+ (grey circles) and NO_3^- (black circles) (second panel). Production rates of N_2O measured as $^{45}N_2O$ and $^{46}N_2O$ over time from incubations with $^{15}NO_2^-$ are indicated as white bars (third panel). Abundance of *Nitrococcus* and *Nitrospina* affiliated cells are depicted as black and white squares, respectively (third panel). Ammonia oxidation rates (black bars), nitrate reduction rates (grey bars) and anammox rates where determined in parallel experiments from incubations with $^{15}NH_4^+/^{14}NO_2^-$, $^{15}NO_3^-$ and $^{15}NH_4^+/^{14}NO_2^-$ and measured as the production of $^{15}NO_2^-$, $^{15}NO_2^-$ and $^{29}N_2$, respectively (right panel). All rates presented here are derived from significant slopes ($p<0.05$) in corresponding linear regression. All rates were derived from significant slopes ($p<0.05$) in corresponding linear regressions.



SI Figure 2: Incubation experiments with *Nitrococcus mobilis* strain 231: NO_2^- (grey circles), NO_3^- (black circles) N_2O (open circles), were measured over a time course of 700h in incubation experiments with A: 200 µM $^{15}NO_2^-$ and 80 µM IO_3^-, anaerobic; B: 500 µM $^{15}NO_3^-$, ~500 µM DOM, anaerobic; C: 1 mM $^{15}NO_2^-$ (12 h incubation time), aerobic; D: 200 µM $^{15}NO_2^-$, 80 µM IO_3^-, autoclaved culture, anaerobic.



SI Figure 3: Small subunit ribosomal RNA based phylogenetic tree visualizing the relation of two *Nitrococcus* clones detected in sweater samples from the Namibian OMZ, Namibian clone I and II. Aligned sequences were added to the SSU_RefNR_115 guide tree using the ARB parsimony tool, with a 50% positional variability filter for Bacteria (Ludwig *et al*., 2004; Pruesse *et al*., 2007)



SI Figure 4: Incubation experiments with *Nitrobacter vulgaris* Ab1: NO_2^- (grey circles), NO_3^- (black circles) N_2O (open circles), $^{15}NO_3^-$ (crossed circles) and $^{15}NO_2^-$ (half circles) were measured in over a time course of 700h in incubation experiments with different substrate combinations: Plot A: 200 µM $^{15}NO_2^-$, anaerobic; Plot B: 200 µM $^{15}NO_2^-$, 80 µM IO_3^-, anaerobic.

SI Table 1: Summary of stations, sampling depths and ^{15}N incubation experiments conducted. Before the analyses of the listed products, $^{15}N^{15}N$:$^{14}N^{14}N$ and $^{14}N^{15}N$:$^{14}N^{14}N$ ratios of the produced N_2 were determined in all he-purged treatments in order to measure denitrification and

Station	*bottom depth [m]*	*sampling depths [m]*	*Substrate additions [µM]*
2	125	65,95,119,123.3, 124.7	$^{15}NH_4^+ + {}^{14}NO_2^-$ *,
2	125	a	$^{15}NO_2^- + {}^{14}NO_3^-$ *
2	125	a	$^{15}NO_2^- + IO_3^- + {}^{14}NO_3^-$
2	125	a	$^{15}NO_3^- + {}^{14}NO_2^-$
6	118	50, 85, 110, 116.8, 117.7	$^{15}NH_4^+ + {}^{14}NO_2^-$ *,
6	118	a	$^{15}NO_2^- + {}^{14}NO_3^-$ *
6	118	a	$^{15}NO_2^- + IO_3^- + {}^{14}NO_3^-$
6	118	a	$^{15}NO_3^- + {}^{14}NO_2^-$

* indicate incubations that have been conducted with helium purged as well as with non helium purged water

SI Table 2: Composition of freshwater and seawater media used to grow *Nitrobacter vulgaris* and *Nitrococcus mobilis*, respectively. Seawater Medium was supplemented with 70% sterile filtered seawater.

Component	*Freshwater medium [g l^{-1}]*	*Seawater medium [g l^{-1}]*
NaCl	0.50	
$CaCl_2$ x 2 H_2O		$5 *10^{-3}$
KH_2PO_4	0.15	$17 *10^{-3}$
$MgSO_4$ x 7 H_2O	0.05	0.10
CaCO3	$7 *10^{-3}$	
$FeSO_4$ x 7 H_2O	$0.97 *10^{-3}$	$1 *10^{-3}$
H_3BO_3	$49.4 *10^{-6}$	
$ZnSO_4$ x 7 H_2O	$43,1 *10^{-6}$	
$MnSO_4$ x 1 H_2O	$38.8*10^{-6}$	
$(NH_4)_6$ Mo_7O_{24}	$37,1 *10^{-6}$	
$CuSO_4$ x 5 H_2O	$25 *10^{-6}$	$6*10^{-6}$
Na_2MoO_4 x 2 H_2O		$25*10^{-6}$
$MnCl_2$ x 4 H_2O		$5*10^{-6}$
$CoCl_2$ x 6 H_2O		$0.5*10^{-6}$
$ZnSO_4$ x 7H_2O		$25*10^{-6}$

SI Table 4: Table 2: NOB specific 16S rRNA targeted oligonucleotide probes and the respective formamide concentrations in the hybridization buffer applied in this study.

Probe	Sequence (5' → 3')	Formamide [%]	Species	Source
Ntcoc-84	TCG CCA GCC ACC TTT CCG	20	*Nitrococcus mobilis*	Juretschko, 2000
Ntspn-693	TTC CCA ATA TCA ACG CAT TT	20	*Nitrospina gracilis*	Juretschko, 2000
Ntspa-662	GGA ATT CCG CGC TCC TCT	35	*Nitrospira spp.*	Daims et al., 2001
NIT3	CCT GTG CTC CAT GCT CCG	40	*Nitrobacter spp.*	Wagner et al.,1996
Ntoga122	TCC GGG TAC GTT CCG ATA T	40	*Nitrotoga sp.*	Lücker, 2009
FGall178	TCC CCC TYA GGG CAT ATG	30	*Gallionellaceae*	Lücker, 2009

SI 4 Material Genomic analysis

4.1 Nitrogen uptake

For transport of nitrite into the cytoplasm, *Nitrococcus mobilis* encodes three transporters, one NirC-type nitrite importer, and two NarK-type nitrate/nitrite transporters that function as antiporters and simultaneously export nitrate.
Nitrogen assimilation is facilitated by a NirBD-type nitrite reductase and by direct uptake of ammonium by an Amt-type ammonium transporter (Figure 4). Further, a cyanase might also supply the cell with ammonium, most likely as side effect of cyanate detoxification (Sung *et al*., 1987). No assimilatory nitrate reductase could be identified in the *Nitrococcus mobilis* genome, but this function will be performed by NXR in the absence of nitrite

4.2. Nitritroxidoreductase of Nitrococcus

In NOB, the central enzyme complex for energy conservation during aerobic chemolithoautotrophic growth with nitrite and carbon dioxide as sole energy and carbon sources, respectively, is the nitrite oxidoreductase system (NXR). NXR belongs to the type II group in the dimethyl sulfoxide reductase family of molybdopterin-cofactor-binding enzymes (Meincke *et al*., 1992), also referred to as complex iron-sulfur molybdoenzyme (CISM) family (Rothery *et al*., 2008) or more recently as the Mo/W-*bis*PGD enzymes family (Grimaldi *et al.,* 2013). These oxidoreductase systems typically consist of three subunits, a catalytic alpha subunit containing the molybdenum *bis* molybdopterin guanine dinucleotide (Mo-*bis*-MGD) cofactor and one [4Fe–4S] iron-sulfur (Fe-S) cluster, an electron-transporting beta subunit with one [3Fe-4S] and three [4Fe-4S] clusters which shuttles the electrons between the alpha and the usually membrane-integral gamma subunit. The gamma subunit functions as membrane anchor for the holoenzyme and transfers the electrons to and from the electron chain via one or two haems (Rothery *et al*., 2008), but a lot of variations to this theme exist (Grimaldi *et al*., 2013). The genome of *Nitrococcus mobilis* 231 contains one genomic region encoding all three structural NXR genes as well as the delta subunit, which functions as chaperone in inserting the molybdenum cofactor into the alpha subunit (Blasco *et al.,* 1998). The NXR of *Nitrococcus mobilis* 231 is most similar to the enzyme systems found in

Nitrobacter and *Nitrolancetus*, as well as to the nitrate reductase system of *Candidatus* Methylomirabilis oxyfera (Lücker *et al*., 2010; Sorokin *et al*., 2012; Lücker *et al*., 2013). The gene order in all these organisms is highly conserved and includes besides the NXR alpha, beta, delta, and gamma (*nxrABDC*) subunits also a gene for a cytochrome *c*-like protein with binding patterns for two haems *c* upstream of *nxrA* (Sorokin *et al.,* 2012). As electrons derived from nitrite oxidation enter the respiratory chain at the level of cytochrome *c*, this dihaem protein likely serves as primary electron acceptor for NXR.

4.3 Carbon dioxide fixation

Carbon dioxide fixation in *Nitrococcus mobilis* 231 is carried out by the Calvin-Benson-Bassham (CBB) cycle. The large and small subunits of the key enzyme ribulose bisphosphate carboxylase/oxygenase (RubisCO) are encoded by the genome, as are the phosphoribulokinase and all other enzymes involved in the CBB cycle. The RubisCO is of the green form I and most similar to its homologs in *Cyanobacteria*, but also to one of the RubisCO paralogs in *Nitrobacter* and *Nitrosomonas* (Sorokin *et al*., 2012). The genome encodes a second RubisCo-like protein, but this copy is of the type IV which has been shown to be involved in the methionine salvage pathway (Tabita *et al*., 2007). Genome annotation further revealed all genes necessary for carboxysome formation, which is a common mechanism for concentrating CO_2 in organisms using the CBB cycle (Yeates *et al*., 2008). Genes for glycolate salvage and the glyoxylate shunt of the tricarboxylic acid (TCA) cycle are also present and allow metabolizing the phosphoglycolate byproduct that is formed when RubisCO reacts with O_2 at low CO_2 concentrations (Bowes *et al*., 1971). Furthermore, a phosphoenolpyruvate carboxylase replenishes oxaloacetate in the TCA cycle.

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Chapter 4

Nitrification associated with O_2 respiration and particle abundance in the Mauritanian upwelling

Jessika Füssel[1], Moritz Holtappels[1], Gaute Lavik[1], Tim Kalvelage[1,2], Marcel M. M. Kuypers[1]

[1] Max Planck institute for Marine Microbiology, Bremen, Germany

[2] Hallifax Marine research Institute, Dalhousie University, Halifax, Canada

Contributions to the manuscript

J.F., M.H., G.L and M.M.M.K. designed research and project outline, M.H., G.L., and T.K. collected samples and performed ^{15}N incubation experiments, J.F measured rates of N-cycling processes, J.F and M.H performed data analyses, J.F., M.H. and M.M.M.K conceived, wrote and edited the manuscript

This chapter is in preparation

Abstract

The redox-activ nitrogen cycle in the oxygenated ocean is controlled by nitrification, a two step process comprising the oxidation of ammonia to nitrite and nitrite to nitrate. Nitrification is ubiquitously active in the pelagic ocean and its product, nitrate, constitutes the major bio-available nitrogen source. Nevertheless, the knowledge about environmental factors and how they influence the distribution and activity of nitrification remains fragmentary. Here, we investigate nitrogen cycling in the productive Mauritanian upwelling by measuring rates of ammonia regeneration, ammonia and nitrite oxidation as well as nitrate reduction. We analyze relations between these N-cycling processes and explore the influence of environmental variables, including substrate levels and POC concentrations. Our data indicate a close association of ammonia and nitrite oxidation activity with the particulate organic matter in the water column. Although nitrite oxidation significantly exceeded ammonia oxidation, both processes were tightly linked to the aggregate associated respiration of organic matter. As an alternative nitrite source, anaerobic reduction of nitrate to nitrite was detected despite the high ambient (~50-140 µM) oxygen concentrations and was likely facilitated by oxygen depleted microniches in marine aggregates. These observations suggest that our current perception of the nitrogen cycle in the oxygenated ocean might be too narrow and that nutrient rich and oxygen depleted microniches largely influence activity and distribution of nitrogen cycling processes.

Introduction

Nitrification, the microbially mediated oxidation of ammonia to nitrite and of nitrite to nitrate, represents a key process in the oceanic nitrogen cycle. Its product, nitrate, constitutes > 99% of the dissolved inorganic nitrogen (DIC) pool (Gruber 2008) and nitrate is a major terminal electron acceptor in the oxidation of organic matter under oxygen deficient conditions. Nitrification appears ubiquitous in the pelagic zone and could be detected in productive coastal regions and estuaries (e.g. Bianchi *et al.*, 1994a, de Bie *et al*., 2002; Ward 2005) oxygen minimum zones (OMZ) (Ward *et al*., 1989; Lipschultz *et al*., 1990; Molina *et al*., 2005, Lam *et al*., 2009; Füssel *et al*., 2012; Kalvelage *et al*., 2013) as well as in oligotrophic oceanic gyres (Yoshida *et al*., 1989; Dore and Karl, 1996; Bianchi *et al*.,1999; Clark *et al*., 2008).

Several factors have been found to influence the distribution and activity of nitrification, such as light (Guerrero and Jones, 1996; Horrigan and Springer, 1990), salinity (Pakulski *et al*., 1995; 2000; Somville, 1984) and substrate concentrations (Ward 1985). The influence of these environmental variables on nitrification appears however to be limited to certain ecosystems and no "global" regulatory factor could be identified yet (Ward 2008). During a transatlantic expedition, Clark *et al*. (2008) for example detected more pronounced nitrifying activity in the oligotrophic subtropical gyres than in the productive Canary upwelling system and also the comparison of nitrification rates from different environments could not reveal major factors influencing its activity (Yool *et al.,* 2007).

A more coherent pattern is usually observed for the vertical distribution of nitrification in the upper water column (250 m). Rates are generally low to non-detectable in surface waters and peak at the base of the euphotic zone. Here, pronounced ammonia oxidation activity often coincides with the accumulation of NO_2^- in the so called primary nitrite maximum (PNM) (Beman *et al*. 2013, Ward *et al*., 1984, 1986, Bianchi *et al*., 1994b). The typically low rates of nitrification in the sunlit surface ocean are usually attributed to either light inhibition of nitrifying bacteria, substrate limitation due to competition with phytoplankton, or a combination of both (Olson *et al.*, 1981a; Ward *et al*., 1986).

Although the two steps of nitrification, ammonia and nitrite oxidation, are catalyzed by distinctly different types of bacteria, they are usually considered to be tightly linked. Thus, many studies solely rely on determining ammonia oxidation as a measure for

nitrification (e.g. Bianchi *et al*., 1994a; Ward 2005; Beman *et al*., 2010). In contrast, those studies measuring both processes separately often find significant discrepancies between ammonia and nitrite oxidation rates. While ammonia oxidation appears more active within the euphotic zone, nitrite oxidation often exceeds ammonia oxidation below this zone (e.g.Clark *et al*., 2008; Beman *et al*., 2013; Ward *et al*., 1987). In the oxygenated ocean, nitrite is produced by ammonia oxidizers and by phototrophic organisms in the course of assimilatory nitrate reduction. Because the latter process is restricted to the euphotic zone, nitrite oxidation below the euphotic zone should not exceed ammonia oxidation. The only alternative nitrite producing pathway in the dark ocean, nitrate reduction to nitrite, has been shown to be active at O_2 concentrations at least as high as 25 µM (Kalvelage *et al*. 2011) but has so far not been measured in permanently oxygenated water masses.

During this study we aimed to investigate regulatory factors of ammonia and nitrite oxidation, focusing on processes producing NH_4^+ and NO_2^- and on the role of particulate organic carbon (POC). We measured concentrations of nutrients, oxygen, Chlorophyll a, POC and particulate organic nitrogen (PON) as well as rates of ammonia and nitrite oxidation, oxic respiration and nitrate reduction.

To cover gradients of primary productivity, nutrient concentrations and particulate organic matter (POM) concentration, we collected samples throughout the water column of stations along transects from the Senegalese to the Mauritanian coast (12°50 to 18°13 N) and from the Mauritanian coast towards the open ocean (16°45 to 18°32 W). Here, the Canary current forms one of the most productive eastern boundary upwelling systems (Carr *et al*., 2003), that is characterized by high aggregate densities within the water column (Karakas *et al*., 2009; Iversen *et al*., 2010). In contrast to other major eastern boundary upwelling systems, such as the Eastern and Northern Tropical South Pacific, subsurface O_2 levels usually do not drop below ~ 40 µM due to a better overall ventilation (Karstensen *et al*., 2008), offering ideal conditions for nitrifying microorganisms.

Material and Methods

Water sampling was conducted onboard the R/V Maria S. Merian off the Senegalese and Mauritanian Coast between 12°29 N to 18°13 N and 16°27 W to 18° 32 W, with bottom depths ranging between 54 and 3010 m. (Figure 1 A). Salinity, Temperature, dissolved oxygen and chlorophyll a fluorescence were measured with a conductivity-temperature-depth system (CTD), equipped with an oxygen sensor and a fluorometer (Sea Bird Electronics, Bellevue, WA, USA). Oxygen data were calibrated against O_2 concentrations determined by Winkler titration. Chlorophyll a fluorescence was calibrated against discrete samples following acetone extraction. Water samples for nutrient, POC and PON measurements and for ^{15}N incubation experiments as well as for oxic respiration rate determinations were collected with 12 l Niskin bottles attached to the CTD system. Additionally, 3 depths from 30 cm to 2 m above seafloor were sampled with a bottom water sampler (Sauter *et al*., 2005; Holtappels *et al*., 2011a).

NO_2^- and NH_4^+ were measured on board spectrophotometrically and fluorometrically, respectively, with a detection limit of 0.01 µM (Grasshoff *et al.*, 1999; Holmes *et al*., 1999). Water samples were frozen for later analyzes of NO_3^- and PO_4^{3-} with an autoanalyzer in a shore-based laboratory (TRAACS 800, Bran & Luebbe, Hamburg, Germany, detection limit 0.1 µM).

^{15}N Incubation experiments

Incubation experiments were conducted for 4-6 depths at 8 stations (SI Table 1). For each incubation experiment a 250 ml sample was collected from specific depth with a Niskin bottle attached to the CTD rosette or with the bottom water sampler. To determine rates of ammonia oxidation 4.8 µM $^{15}NH_4^+$ and 4.8 µM $^{14}NO_2^-$, of nitrate reduction 20 µM $^{15}NO_{3-}$ and 4.8 µM $^{14}NO_2^-$ and of nitrite oxidation 4.8 µM $^{15}NO_2^-$ were amended. Additionally, $^{15}NO_2^-$ production was determined in samples incubated with 0.4 µM ^{15}N labelled dissolved organic matter (DOM) and from few experiments incubated with ^{15}N DOM and $^{14}NH_4^+$ to investigate the direct utilization of small organic compounds in ammonia oxidation.

Immediately after substrate addition, the sample was homogenized and transferred into five 12 ml exetainers (High Wycombe, Buckinghamshire, UK) without headspace. Samples were

incubated for up to 50 h in the dark at *in situ* temperatures. At each time interval (~ 0, 6, 12, 24 and 50 h) one exetainer vial was sacrificed and biological activity was stopped by the addition of 100 µl saturated mercuric chloride solution. The experimental procedure is described in detail in Holtappels *et al.,* 2011b. Samples were stored in the dark at room temperature till further analyzes in a shore based laboratory.

Stable isotopic analyzes

Nitrogen stable isotope ratios of N_2 were determined by GC-IRMS (VG Optima, Manchester, UK). Rates of ammonia oxidation and nitrate reduction were measured as the production of $^{15}NO_2^-$ over time and rates of nitrite oxidation as the production of $^{15}NO_3^-$ over time. Nitrite and ammonia oxidation rates were determined as follows: 4 ml of sub-sample were transferred into 6 ml exetainer vials and purged with helium for 10 min to remove dissolved N_2 and O_2. Subsequently, 50 µl of 0.4% (w/v) sulfamic acid in 10% HCl were injected through the septum of each vial to reduce NO_2^- to N_2. Samples were incubated upside down over night and the $^{15}N^{14}N$:$^{14}N^{14}N$ ratio was determined the following day. To determine $^{15}NO_3^-$ produced by nitrite oxidation, the remaining $^{15}NO_2^-$ had to be removed prior to the reduction of NO_3^-. 4.5 ml sample were transferred into 15 ml vials (Sarstedt, Nuembrecht, Germany) and 50 µl sulfamic acid (see above) were added. After ~ 12 h of incubation, sample pH was adjusted to 8-9 by the addition of ~ 40 µl 6M NaOH subsequently 0.3-0.4 g spongy cadmium, that had previously been activated with 6M HCl for 1 min, were added to each sample to reduce NO_3^- to NO_2^-. To guarantee complete reduction of NO_3^- to NO_2^-, samples were incubated for > 16 h (Gal *et al*., 2004). Finally, 4 ml of the sample were transferred into 6 ml exetainers and samples were handled as described above to convert NO_2^- to N_2 (Fuessel *at al*., 2012).

Rates of all processes were calculated from the increase of ^{15}N concentration over time using linear regression analysis. All rates presented here were instantaneous and linear over time, except for few samples, in which ^{15}N production decreased after 12 or 24 h, due to unknown reasons. Only the linear increase was considered in rate calculations. All rates reported are derived from significant ($p< 0.05$) slopes in corresponding linear regression. Rates have been corrected for the ^{15}N labeling percentages of the initial substrate pools. In incubation experiments for the rate determination of ammonia oxidation and nitrate

reduction unlabeled $^{14}NO_2^-$ was added alongside with ^{15}N substrates to minimize the immediate consumption of ^{15}N labeled nitrite during the incubation period. For ammonia oxidation rates determined from incubations with ^{15}N DOM, the ambient ^{14}N DOM was not taken into account and thus rates are likely underestimated.

Aerobic Respiration

To determine rates of aerobic respiration, samples were incubated in 100 ml Winkler flasks equipped with non-invasive optode-sensor spots to measure oxygen concentrations ranging between 0-1400 µM (full range spots, PST6). Oxygen consumption was measured with the corresponding fiber optics and electronics from PreSens (Precision Sensing GmbH, Regensburg, Germany). While the spots where fixed on the inside of the bottle with silicon glue, the fluorescent signal was read from outside. To minimize interferences from temperature changes on optode readings, bottles were incubated in a water bath in the dark at constant (± 0.1°C) insitu temperatures. Slow stirring of a glass coated magnet at 30-60 rounds per minute ensured mixing of the water during the incubation. Oxygen concentrations were measure every 5sec and rates were calculated from the linear decrease of oxygen over a time within the first 24 hour of the incubation. A detailed method description can be found in Hotappels *et al.* (submitted to PLOS ONE).

Few (3) measurements (> 1.6 µM d^{-1}) were excluded from analyses because degrading copepods were found in these samples.

Ammonia regeneration

Assuming that the major fraction of organic matter respiration was associated to particulate organic matter the release of ammonia during aerobic respiration was calculated from measured O_2 respiration rates and the carbon and nitrogen content of the particulate organic matter (POM) sampled in the same depths using the simplified stoichiometry:

$$(CH_2O)x(NH_3)y + (x+2y)O_2 + yH+ \rightarrow xCO_2 + yNO_3^- + (x+y)H_2O + 2yH^+$$

where x and y is defined by the C:N ratio of POM (x/y = C/N). Assuming that ammonia is fully oxidized to nitrate in the respiration rate measurement, the ammonia release rate is calculated by multiplying the O_2 respiration rate with the factor y/(x+2y).

Results and Discussion

Hydrochemical setting

Five coastal-offshore transects between 18°N to 12°N and 16°27W to 18°32W were sampled within the Mauritanian upwelling (Figure 1A). While the northernmost stations exhibited exceptionally high Chl a concentrations of 40-60 mg/m^2 (depth integrated over the water column), the concentrations at stations south of 18°N ranged between 20-40 mg/m^2 (Figure 1B). High NO_3^- levels in surface waters indicated upwelling at all stations (> 8 µM) but NO_3^- levels gradually decreased from coastal to open ocean stations (Figure 1C). Ammonia and nitrite concentrations ranged from non detectable to 1.3 and 0.8 µM, respectively. Both, NH_4^+ and NO_2^- levels were enhanced in bottom waters of shallow shelf stations, likely due to benthic fluxes (Figure 2, example for a coastal-offshore transect off the Mauritanian coast).

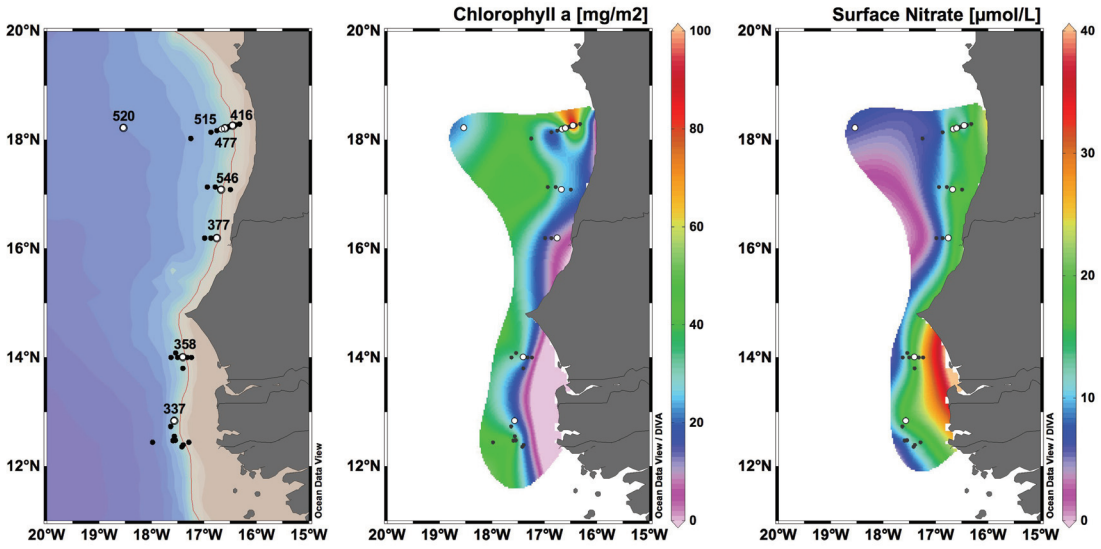

Figure 1: *panel A*: Location of sampling stations along the Mauritanian and Senegalese coast. Stations where nutrients, Chlorophyll a fluorescence, POC, PON and oxygen were measured at 9-24 depths are indicated by black and white dots. Rates of ammonia and nitrite oxidation, nitrate reduction and oxic respiration where determined at 1-7 depths at stations indicated by white dots.
Panel B: Depth integrated chlorophyll a concentrations as a proxy for primary productivity
Panel C: Surface nitrate concentrations determined at depths ranging between 1 and 6 m. Figure was produced with ocean data view (ODV) (Schlitzer, 2011).

Oxygen concentrations decreased from 122-330 µM in surface water to 37-82 µM at midwater depths, exhibiting a prominent oxygen minimum zone of 27m thickness over the shallow shelf (Figure 2) and up to 650 m thick at station 520 (3010 m). High POC and PON concentrations of up to 33 µM and 4.6 µM, respectively, were observed in surface waters. Concentrations decreased with increasing depths and were minimal in midwater depths at station 520 (2.7 µM POC, 0.3 µM PON). A distinct increase could be observed in bottom water samples with maximal concentrations of 140 µM POC and 6.6 µM PON (Figure 2). Atomic ratios of C over N were nearly constant in the water column (~7 on average) and increased significantly in bottom waters of shallow shelf stations and below 2000m water depth at station 520 (up to 28).

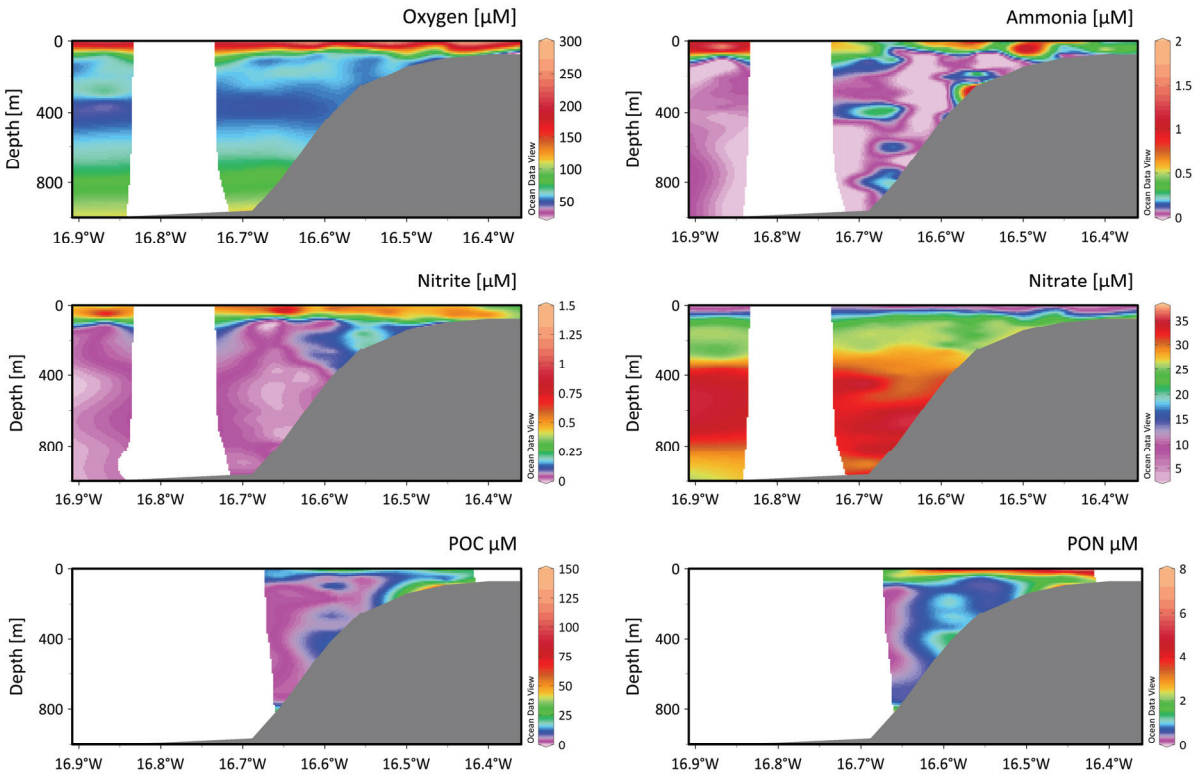

Figure 2: Vertical distribution of oxygen, ammonia, nitrite, nitrate, POC and PON along a transect perpendicular to the Mauritanian coast at 18° N. Figure was produced with ocean data view (ODV) (Schlitzer, 2011)

Remineralization of organic nitrogen

Aerobic respiration, the major remineralization pathway in the oxygenated ocean, consumed 0.3-1.8 µM O_2 d^{-1} and released 16-170 nM ammonia d^{-1}. In combination with observations from previous studies our results strongly indicate that a major fraction of aerobic respiration and thus ammonia regeneration occurred in association with marine aggregates. Firstly, their vertical distribution corresponds to the particle abundance along the water column of the Mauritanian upwelling (Iversen *et al.,* 2010), with maximal rates in the shallowest sampling depth at each station that decreased with increasing depth and peaked again just above the seafloor (30 cm). Secondly, rates of aerobic respiration and ammonia regeneration clearly correlated with POC concentrations in the water column (Figure 3 A + B) (p-value: < 0.0001; R^2: 0.6; p-value: <0.0001, R^2: 0.6, respectively) as well as in bottom water samples (BWS) (Figure 3 C+D) (p-value: < 0.0001; R^2: 0.9; p-value: 0.0002, R^2: 0.8). Finally, the average carbon specific respiration rates calculated from our bulk water incubations (0.13 ± 0.07 d^{-}) and POC concentrations are identical to previously determined carbon specific respiration rates of aggregates (0.13 d^{-1} ± 0.09) sampled in the same region (Iversen *et al*., 2010). For the following analyses we thus assume that aerobic respiration in our incubation experiments was mainly associated with marine aggregates or particles.

Regulation of ammonia and nitrite oxidation

Average Ammonia and nitrite oxidation activity was more pronounced in the most productive region investigated (north of 18° N) (34 nMd^{-1} and 130 nM d^{-1}, respectively) than in the slightly less productive area south of 18°N (11 nM d^{-1} and 40 nMd^{-1}, respectively) (SI Table 2). A more detailed analysis however reveals a patchy distribution of both processes with few exceptionally high rates over the Mauritanian shelf (> 100 nM d^{-1} and > 400 nM d^{-1}, respectively). Despite the accumulation of ammonia and nitrite in the water column, ammonia oxidation shows no relation with NH_4^+ concentrations (p-value: 0.9; R^2: 0.0009; Figure 4A) while ambient NO_2^- concentrations were correlated with nitrite oxidation rates, however explaining only 50% of the variability of the rates (p-value: < 0.0001; R^2: 0.5; Figure 4B). This suggests no influence of the ambient ammonia pool and some influence of the nitrite pool on the activity of ammonia and nitrite oxidizing microorganisms. In contrast,

rates of ammonia and nitrite oxidation were clearly correlated (p-value: < 0.0001; R^2: 0.8; Figure 4C), indicating a close coupling of the two steps of nitrification.

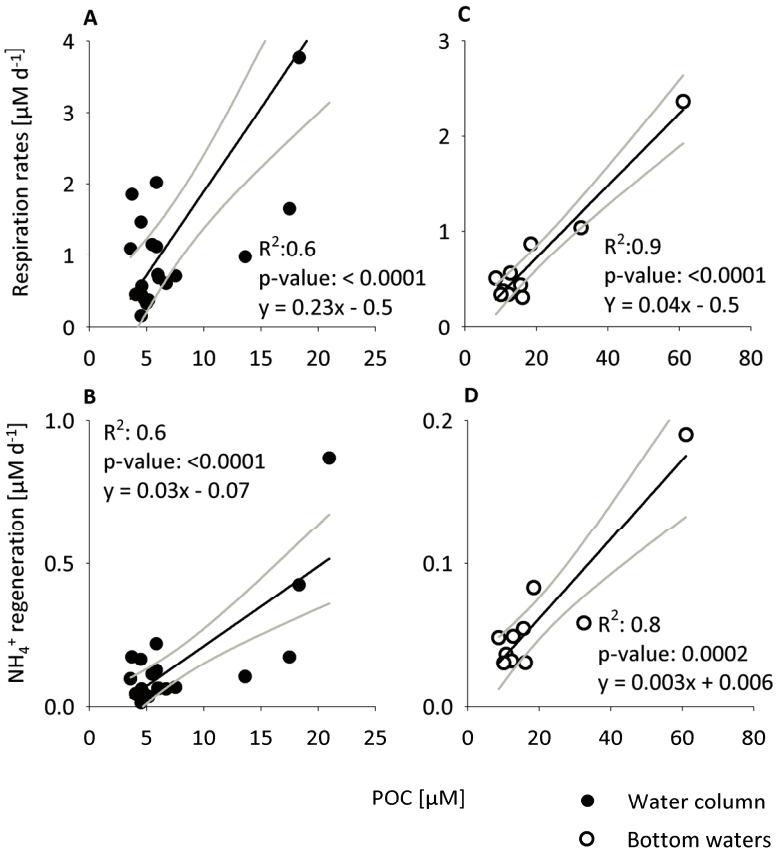

Figure 3: Linear regression analyses of aerobic respiration (A+C) and ammonia regeneration rates (B+D) as a function of POC concentrations. Samples from the water column are depicted by black circles, those from bottom waters by white circles. Slopes of linear regressions are shown as black lines and the grey lines indicate the corresponding 95% confidence interval. Rates of aerobic respiration and ammonia regeneration were significantly correlated with POC concentrations within the water column (A + B) and within bottom water samples (C + D) of the Mauritanian upwelling. For correlation analyses of bottom water samples and POC (two outliers were excluded from analyses).

Ammonia oxidizing microorganisms depend on the remineralization of organic matter and the regeneration of ammonia by heterotrophic organisms while nitrite oxidizing bacteria (NOB) rely on nitrite supply by ammonia oxidation. Consequently, rates of ammonia and nitrite oxidation were distinctly correlated with rates of aerobic respiration (p-value: 0.0004; R^2: 0.7; p-value 0.0001, R^2: 0.8) and with rates of ammonia regeneration (p-value 0.0004; R^2:

0.7; p-value: 0.0004; R^2: 0.7). This apparently direct coupling of aggregate associated respiration and nitrification and the lack of correlation with ambient NH_4^+ concentrations indicate an association of ammonia and nitrite oxidation with particulate organic matter.

These indications are further supported by previous studies that reported the preferential attachment of ammonia oxidizing Bacteria to particles (Stehr *et al.,* 1995; Owens 1986; Abril *et al.,* 2000; Karl *et al*., 1984). Moreover, genes associated with motility and chemotaxis, prerequisites to exploit such nutrient-rich microniches, have been detected in marine ammonia oxidizing Bacteria and Archaea (Blainey *et al*., 2011, Spang *et al.*, 2012).

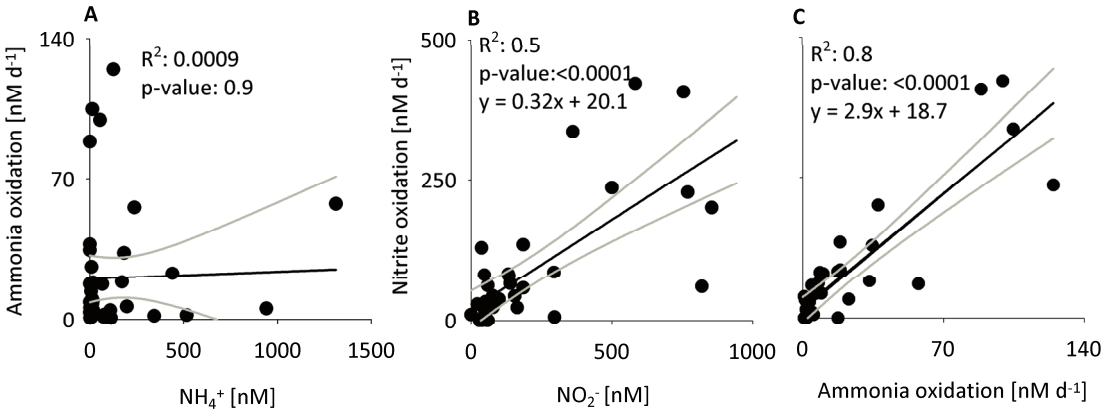

Figure 4: Linear regression analyses of ammonia oxidation rates with ambient NH_4^+ concentrations (A), of nitrite oxidation rates with ambient NO_2^- concentrations (B) and rates of ammonia and nitrite oxidation (C). Slope of linear regressions are shown as black lines with the corresponding 95% confidence interval (grey lines). Ammonia oxidation rates showed no correlation with the ambient NH_4^+ concentrations (A) while nitrite oxidation rates showed a relatively weak correlation with ambient concentrations of NO_2^- (B). The coupling of ammonia and nitrite oxidation is indicated by the clear correlation of ammonia and nitrite oxidation rates (C).

While ammonia oxidizing Archaea (AOA) appear as the major ammonia oxidizers in the ocean and have been detected in great numbers in productive coastal regions (Francis *et al*., 2005; Santoro *et al*., 2010; Lam *et al*., 2007; Beman *et al*., 2008), ammonia oxidizing Bacteria (AOB) often contribute less than 0.1% of the microbial community (Ward, 2000). AOA are adapted to low ammonia concentrations (<10 nM substrate threshold, K_M value: 134 nM), while AOB require higher concentrations of ammonia than are usually observed in the pelagic zone (i.e. K_M values of 8-48 µM; Ward *et al*., 1987; Suzuki *et al*., 1974). However,

high abundance does not necessarily indicate high turnover rates and AOB attached to NH_4^+ enriched aggregates might be highly active and thus substantially contribute to ammonia oxidation despite their low abundance.

The nitrite budget

As discussed above, NOB appear to depend on ammonia oxidation as their source of NO_2^-. Nevertheless, NOB consumed ~2.9 times more nitrite than was provided by ammonia oxidation (Figure 4C). Although the selective stimulation of nitrite oxidation over ammonia oxidation by the addition of ^{15}N NO_2^- can not be entirely excluded, the highly significant correlation of ammonia and nitrite oxidation should have been masked in such case. Likewise, neither Olson *et al.*, (1981b) nor Ward *et al.* (1987) could detect stimulation of nitrite oxidation by the addition of NO_2^- in incubation experiments. Furthermore, Clark *et al*. (2008) also observed nitrite oxidation rates often exceeding those of ammonia oxidation several fold when measuring dilution of $^{15}NO_2^-$ and $^{15}NO_3^-$ pools through ammonia and nitrite oxidation, respectively.

Instead, our analyses may indicate the underestimation of ammonia oxidation. Despite the direct coupling of ammonia oxidation rates to NH_4^+ regeneration rates, ammonia oxidizers only utilized ~64% of the provided substrate (Figure 5B, indicated by the 1:1 slope). A systematic underestimation of ammonia oxidation could provide an explanation for the missing NO_2^- required to sustain the observed nitrite oxidation rates. Such underestimation might be related to methodological constraints. The intracellular hydrolization of organic N compounds and subsequent oxidation to NO_2^- (Hallam *et al*., 2006; Alonso-Saez *et al*., 2012) for example would be overlooked in incubations with $^{15}NH_4^+$. To explore the direct oxidation of naturally occurring small organic nitrogen compounds, we incubated seawater with ^{15}N labeled (~95%) dissolved organic matter (DOM) extracted from algae grown on $^{15}NO_3^-$. $^{15}NO_2^-$ production was detected in 10 out of 18 samples, ranging between 0.45-17 nM d^{-1} (Mean: 6 nM d^{-1}).

Although the production of $^{15}NO_2^-$ in these incubations was instantaneous, incubation in the first sub sample was only stopped after 30 min and the remineralization of ^{15}N-DOM to $^{15}NH_4^+$ could have supported ammonia oxidation. To minimize potential oxidation of $^{15}NH_4^+$ produced from ^{15}DOM, $^{14}NH_4^+$ was additionally provided in parallel experiments. The

$^{14}NH_4^+$ should dilute the $^{15}NH_4^+$ produced in the course of ^{15}DOM respiration several folds, such that $^{15}NO_2^-$ would only be produced if DOM had been utilized directly in ammonia oxidation. Here, $^{15}NO_2^-$ production was detected in 4 out of 8 samples, ranging between 1.2-2 nM d^{-1} (mean: 1.7 nM d^{-1}). Overall, the addition of $^{14}NH_4^+$ resulted in a substantial decrease of $^{15}NO_2^-$ production compared to incubations solely amended with ^{15}N-DOM (Figure 6). These low ammonia oxidation rates from ^{15}N DON were not sufficient to close the gap in the NO_2^- budget, indicating a minor relevance of this process in the Mauritanian upwelling. Uptake and hydrolyzation of urea requires energy and presumably becomes relevant only when the availability of ammonium is low (Sáez *et al.*, 2012).

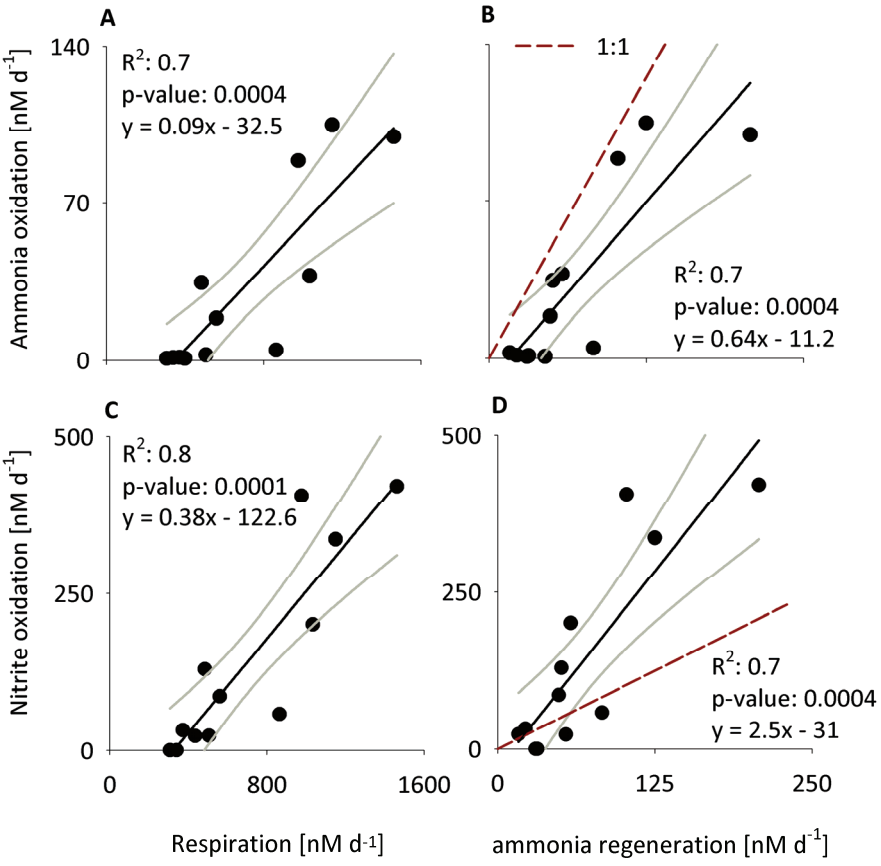

Figure 5: Linear regression analyses of ammonia and nitrite oxidation rates as a function of aerobic respiration (A+C) and ammonia regeneration (B+D).. Ammonia and nitrite oxidation rates were distinctly correlated with rates of aerobic respiration (A+C) and ammonia regeneration (B+D). Data points are shown as black circles with the corresponding linear regression (black lines) and the 95% confidence interval (grey lines). The red, dashed line shows the 1:1 ratio of ammonia remineralization over ammonia oxidation, indicating the maximum ammonia oxidation that can be supported by rates of ammonia remineralization.

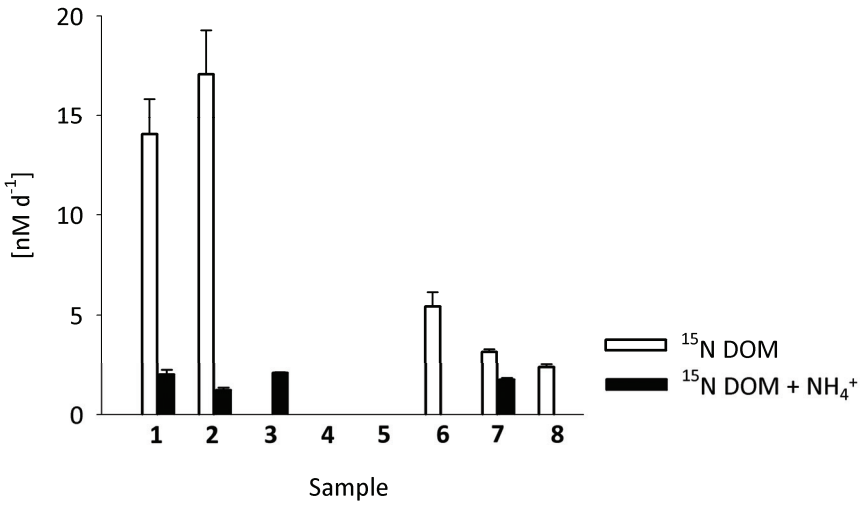

Figure 6: Rates of $^{15}NO_2^-$ production from samples incubated with ^{15}N DOM (white bars) and ^{15}N DOM + $^{14}NH_4^+$ (black bars). Parallel incubation experiments were performed for eight samples.

Instead, the particle or aggregate association of ammonia oxidizing microorganisms could explain the systematic underestimation of ammonia oxidation rates. The intense local respiration and ammonia regeneration associated with marine aggregates dilutes the amended $^{15}NH_4^+$ within the aggregate and enhances the relative fraction of $^{14}NH_4^+$ oxidation over $^{15}NH_4^+$ oxidation. Moreover, the close proximity of ammonia release and uptake might favor the direct shuttling from microorganism to microorganism and thereby bypass the $^{15}NH_4^+$ present in the surrounding waters. The resulting production of $^{14}NO_2^-$ would not be captured by our measurements of ammonia oxidation and could lead to substantial underestimation of rates.

Assuming steady state conditions, the maximal ammonia oxidation activity can however not exceed rates of ammonia release. Thus, ammonia oxidation rates can maximally be underestimated by ~ 36% (see slope in Fig.5b) and even the complete oxidation of remineralized ammonia could not sustain rates of nitrite oxidation in our incubation experiments (see slope in Fig.5d).

Nitrate reduction to nitrite

Below the euphotic zone, the only NO_2^- producing pathway other than ammonia oxidation is the anaerobic reduction of nitrate. Nitrate reduction has been detected at oxygen

concentrations of up to 25 µM in the Eastern Tropical South Pacific OMZ (Kalvelage *et al*., 2011), but has never been observed in the permanently oxygenated ocean. In our incubations nitrate reduction was detected in half of the investigated samples at rates ranging between 2.6-18 nM d^{-1} at ambient O_2 concentrations of 54-140 µM, indicating the occurrence of anaerobic nitrogen cycling processes at elevated oxygen concentrations (Figure 7). Maximal reduction rates were observed in the shallowest sampling depths and coincided with maximal rates of aerobic respiration. Rates were rarely (5 times) measured in samples form bottom waters (Figure 7). Maximal activity (18 nM d^{-1}) was detected at 30 m at station 377 (99m) and at 90 m at station 515 (578 m) at O_2 levels of 142 and 57 µM, respectively (Figure 7).

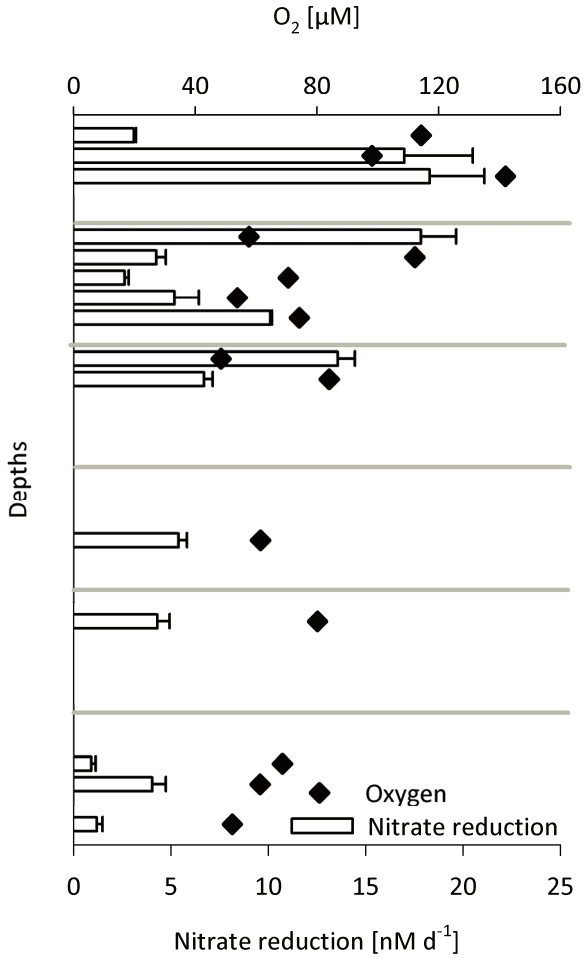

Figure 7: Nitrate reduction to nitrite (white bars) was detected in half of the investigated samples at oxygen concentrations ranging between 142-57 µM (black diamonds). Maximal rates were observed in the shallowest sampling depths (1+2) investigated and were often below detection at midwater depths (3) as well as in bottom water samples (Depths 4, 5+6). Depths 3 and 4 were only investigated at three stations. Due to the high variability of water depths, depths were clustered according to relative sampling depths along the water column.

Nitrate reduction could be facilitated in suboxic or anoxic microniches within marine aggregates. These micro niches can develop depending on the O_2 consumption inside the aggregate and the diffusive O_2 flux into the aggregate, the latter is a function of the aggregate size, the thickness of the diffusive boundary layer around the aggregate and the effective diffusivity inside the aggregate (Ploug *et al*. 1997). Below a certain O_2-concentration threshold outside the aggregate, anoxic microniches will start to appear.

By applying and rearranging the analytical solution for solute transport and reaction in a sphere as presented by Ploug *et al.* (1997), the O_2 concentration threshold can be estimated from:

$$C_{thres} = R_0 r^2 / (6 D) \quad (1)$$

where r denotes the radius of the aggregate, D is the diffusivity within the aggregate and R_0 is the non-limited O_2 consumption rate of the aggregate. For simplicity we neglected the DBL around the sphere and we assumed zero order O_2 consumption. For a range of carbon specific respiration rates (0.05 d^{-1} to 0.25 d^{-1}) we estimated the threshold O_2 concentration using data from a recent study on aggregate degradation in the upwelling region off Mauritania (Iversen *et al*. 2010). The threshold concentration estimated for an aggregate of 2 mm in diameter ranged from 20 to 100 µM (Fig. 8) suggesting that niches allowing for the occurrence of nitrate reduction can persist even at elevated O_2 concentrations. In *situ* measurements of particle sizes from the same study (Iversen *et al.* 2010) revealed that 30-60% were larger than 1 mm in diameter, indicating that nitrate reduction in oxygenated waters could be of relevance, especially in regions with high export production.

Moreover, aerobic denitrification has been observed in pure cultures as well as in environmental settings and seems to be promoted under dynamic or oxygen limiting conditions. Chen and Strous (2013) propose the parallel occurrence of NOx and O_2 reduction under excess substrate supply, when the production of NADH is more rapid than the supply of oxygen. Such conditions may prevail in marine aggregates that contain fresh organic matter and thus nitrate reduction might also occur in sinking aggregates under high (~ 140 µM) O_2 levels.

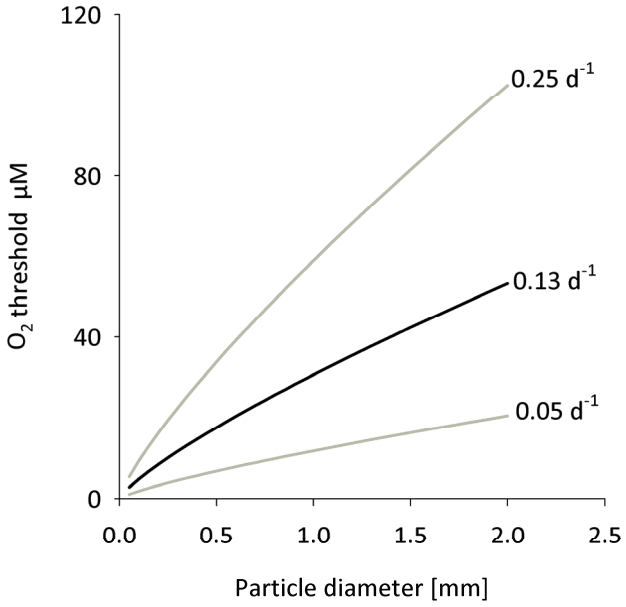

Figure 8: Theoretical threshold concentrations of O_2 in the ambient water below which anoxic micro niches are expected within aggregate. Threshold concentrations are plotted as a function of particle diameter and for a range of observed carbon specific respiration rates. The average specific respiration rate measured in this study as well as in single aggregate studies (Iversen et al. 2010) was 0.13 d^{-1}.

Since aggregates are easily destroyed during sampling and subsequent sample processing aggregate-associated nitrate reduction might be substantially underestimated in our measurements. On the other hand, particles in our incubation experiments quickly settle and become stagnant, which results in a reduced mass transfer compared to sinking aggregates (Ploug and Iversen, 1999), such that the formation of anaerobic microniches is facilitated. Although the relative contribution of nitrate reduction to NO_2^- production cannot be reliably assessed from the present dataset, nitrate reduction might play an important role in organic matter remineralization in oxygen depleted (<90 µM) pelagic environments. These oxygen minimum zones currently constitute ~10% of the ocean volume. In light of the ongoing expansion of OMZs (Stramma *et al*., 2008), the contribution of nitrate reduction in organic matter remineralization might increase even further.

Conclusions

Our data depict a more complex picture of the nitrogen cycle in productive coastal oceans than previously assumed. A strong coupling between sources and sinks of ammonia is reflected by the significant correlation between ammonia release from O_2 respiration and the independently measured oxidation of ammonia to nitrite. This suggests that ammonia oxidizing microorganisms attached to particles or aggregates are responsible for a major fraction of ammonia oxidation rates. Here, the direct and continuous supply of ammonia likely allows for maximal activity. In contrast, ammonia oxidation does not correlated with ambient ammonia concentrations which may suggest that free living ammonia oxidizing microorganisms are of minor relevance. Although we can only speculate on the contribution of AOA and AOB, AOA of the phylum Thaumarchaeota tend to be free living (Woebken *et al*., 2008) while AOB, especially of the genus *Nitrosomonas*, are often enriched on particles or aggregates (e.g. Philipps *et al*., 1999; Lam *et al*., 2004). Thus, AOB might play an important role in marine systems characterized by high particle densities.

Along the same lines, the correlation of ammonia release and nitrite oxidation suggests that nitrite oxidation is also associated with particles abundance. However, NOB activity was also related to the ambient nitrite concentrations in the water column, indicating that particle attached as well as free living NOB are of equal relevance. Although little is known on the diversity and abundance of NOB, bacteria belonging to the genus *Nitrococcus* are, like *Nitrosomas*, r-strategists with high substrate demands and might prevail on aggregates (Fuessel *et al*., in prep), while *Nitrospina*, typical k-strategists, likely dominate nitrite oxidation in the water column (Spieck *et al*., 1998; Spieck and Bock, 2005; Lücker *et al*., 2013).

Moreover, nitrate reduction to nitrite constituted an additional source of nitrite and was likely facilitated by oxygen depleted microniches in marine aggregates (Aldredge and Cohen, 1989). Our data indicate the contribution of nitrate reduction to the oxidation of organic matter especially in shallow depths, were fresh organic matter forms aggregates and aerobic respiration is most intense. Rates of nitrate reduction are however likely underestimated in our incubation experiments and its role in organic matter remineralization remains to be elucidated.

Marine aggregates are hot spots of carbon and nitrogen cycling that allow for various direct interactions of microorganisms and might largely influence activity and occurrence of N-linked processes. Detailed studies of N-cycling on marine aggregates are necessary to evaluate the relevance of these small scale processes for the global ocean.

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Supplementary information

Table S 1: Geographical position of the 8 investigated stations during this study alongside with the depth of each station and the Incubation depths at which rates of nitrite and ammonia oxidation, nitrate reduction and oxic respiration were determined. Sampling depths are subdivided into three vertical zones relative to bottom depth of each station: Surface ocean, midwater depths and bottom waters.

				Incubation depths [m]		
Station	***Bottom depth [m]***	***Latitude***	***Longitude***	***surface***	***midwater***	***bottom water***
337	54	12°49,997	17°33,848	39.00	45, 51	52, 53, 53.7
358	81	14°00,124	17°24,076	53	70, 78	79, 80. 80.7
377	91	16°11,445	16°45,027	30,	50, 85	89, 90, 90.7
416	99	18°15,319	16°27,023	40.7	74	97, 98.7
477	409	18°12,401	16°35,500	88.00	260	407, 408.7
515	787	18°11,302	16°39,300	93.00	380	785, 786.7
546	110	17°05,014	16°40,080	30.00	60	110, 107.7
520	3010	18°13,002'	18°32,917'	50,	375, 425, 500	2600, 2900*, 3010

* Indicates samples obtained from water depths below 2500 m at station 520 that are listed under “bottom water samples” but are referred to during this study as “deep sea” samples

Table 2: Rates of ammonia and nitrite oxidation measured in the shallowest sampling depth, often coinciding with the PNM, midwater depths and bottom water samples.

	Station	**337**	**358**	**377**	**416**	**477**	**515**	**546**	**520**
surface water	ammonia oxidation [nM d^{-1}]	5.4	10.3	57.5	124.6	17.5	34.6	0.0	55.8
	nitrite oxidation [nM d^{-1}]	5.8	79.5	60.4	236.1	0.0	129.7	228.2	n.m.
midwater depths	ammonia oxidation [nM d^{-1}]	12.5°	8.7°	25.4 °	104.7	8.6	3.4	99.5	10.5°
	ammonia oxidation [nM d^{-1}]	16.6°	52.7°	74.3°	336.6	81.7	28.9	421.6	n.m.
bottom waters	ammonia oxidation [nM d^{-1}]	0.6°	1.5°	8.4°	11.6°	0.9°	2.3°	63.1°	6.6*°
	ammonia oxidation [nM d^{-1}]	13.3°	31.1°	50.7°	96.6°	0°	5.1°	303.7*	n.m.

° indicates rate measurements averaged over 2-3 depths from midwater depths or bottom waters; * Indicates samples obtained from water depths below 2500 m at station 520 that are listed under “bottom water samples” but are referred to during this study as “deep sea” samples

Outlook

The work presented in this thesis provides new insights into the role of ammonia and nitrite oxidation in OMZs and productive coastal oceans and furthers our understanding of the interactions between nitrogen cycling microorganisms. Our results raise yet unresolved questions and provide directions for future research.

1. Role of Nitrite oxidation in OMZs

1.1 How does nitrite oxidation take place in functionally anoxic OMZ cores?

Our data indicate that nitrite oxidation is active even within the core of OMZs, that have been reported as functionally anoxic (<10 nM O_2) previously (Thamdrup *et al*., 2012). Here, nitrite oxidation appears to prevent a substantial amount of NO_2^- from being reduced to N_2 and lost from the system. This is consistent with observations by Casciotti *et al*., (2013), who even showed that without active nitrite oxidation in the OMZ core, NO_3^- would be nearly entirely consumed over time. Yet, the question remains how does nitrite oxidation occur in functionally anoxic cores of OMZs? While our incubation experiments with pure cultures of *Nitrococcus mobilis* 231 yielded no indication for anaerobic nitrite oxidation, this strain, which has been in culture under stable oxic, autotrophic conditions since >40 years, may have lost this capacity (Wiser *et al*., 2013) and might not be representative of all NOB species/genera present in OMZs. Further environmental experiments under tightly controlled anoxic conditions and amendment with potential electron acceptors such as IO_3^-, Mn^{IV} or Fe^{III} could resolve this mystery. Alternatively, the enrichment of NOB from an OMZ and subsequent physiological experiments would offer most reliable and direct evidence.

1.2 Does nitrite oxidation indirectly influence benthic N-loss in OMZs?

Our data indicate that NOB attach to larger particles or aggregates (Figure 1) in the Namibian OMZ where they might directly interact with nitrate reducing microorganisms. As marine aggregates are hotspots of respiration, nitrate, the favorable electron acceptor in the oxidation of organic carbon under oxygen deficiency, might be rapidly depleted within such aggregates despite the high (~ 8-36 µM) concentrations of nitrate in the bulk water column. Here, the efficient recycling of nitrate could enhance rates of nitrate reduction and organic carbon oxidation (Cartoon 1). Thereby, less labile organic carbon would eventually reach the sediment to fuel heterotrophic denitrification.

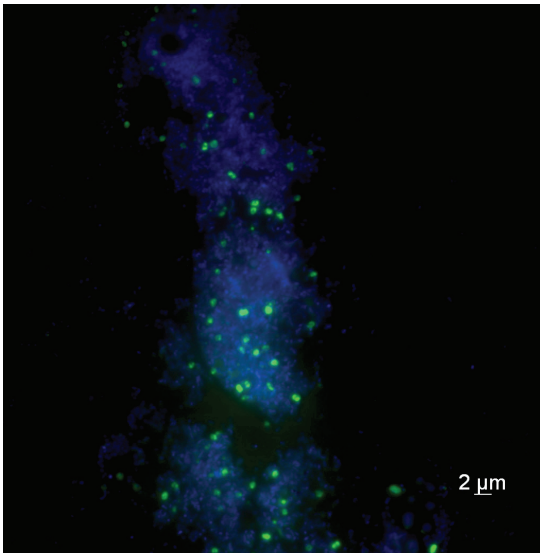

Figure 1: Epifluorescence micrograph of a particle from the Namibian OMZ densely populated by *Nitrococcus* (green) and other microorganisms (blue).

Such small-scale interactions could best be investigated in scuba collected aggregates by means of incubation experiments mimicking sinking velocities of aggregates as well as realistic nitrate and oxygen concentrations. Subsequently, a time dependent model of OMZ N-cycling including the aggregate specific recycling mechanism could explore the importance of aggregate associated nitrite oxidation and coupled nitrate reduction.

1.3. What is the role of nitrite oxidation in future scenarios of OMZ N-cycling?

Continuously increasing anthropogenic carbon dioxide emissions since the industrial revolution have increased the atmospheric CO_2 levels from ~280 ppm to ~400 ppm. As a consequence the greenhouse effect intensifies and global mean temperature have already risen by 1°C since 1850 (Solomon *et al*., 2007). Reduced O_2 solubility with increasing temperature and enhanced thermal stratification cause an expansion and intensification of oxygen minimum zones (Stramma *et al*., 2008; Keeling *et al.*, 2010). Even moderate decreases of O_2 concentrations in the upper ocean could already multiply the suboxic ocean volume (Deutsch *et al*., 2011) and result in substantially increased N-loss from OMZs (Codispoti, 2010). Although nitrification does not directly influence the oceanic nitrogen budget it might indirectly affect the magnitude of N-loss from the system. The vertical expansion of oxygen minimum zones for instance would also result in an earlier onset of anaerobic respiration of sinking organic matter and consequently an enhanced consumption of nitrate**.** It would be essential to investigate how the recycling of nitrite to nitrate under such conditions would influence N-loss. Such future scenarios can only be assessed based on the mechanistic comprehension of nitrite oxidation in OMZs, as described in the previous sections.

2. Role of particles and aggregates in pelagic nitrogen cycling

2.1 Is our view of the nitrogen cycle in the oxygenated ocean too narrow?

Our observations suggest that a substantial fraction of nitrogen cycling occurs in association with marine aggregates in productive coastal oceans. As a result, unanticipated processes of the nitrogen cycle, such as anaerobic nitrate reduction to nitrite, occur in the oxygenated pelagic zone. Destruction of particles during sampling procedures might negatively affect rates, especially those processes restricted to oxygen reduced microniches such as e.g. nitrate reduction to nitrite. ^{15}N labeling experiments with e.g. Scuba collected marine aggregates would offer further insights into small-scale nitrogen cycling on and in these aggregates. Such experiments may reveal the occurrence of a considerably more complex nitrogen cycle in oxygenated environments than previously assumed.

2.2 Do nitrifying microorganisms influence the efficiency of the biological pump?

Marine aggregates are major transport vehicles for organic carbon to the deep sea, where carbon may be sequestered for centuries to millennia (e.g. Stuiver *et al*., 1983; Falkowski *et al*., 1998). Activity of autotrophic bacteria, such as ammonia and nitrite oxidizers, has been suggested to add particulate organic carbon (POC) to sinking marine aggregates in the mesopelagic zone of the North Pacific Ocean (Karl *et al*., 1984).

However, no such observations are available for coastal oceans, although they disproportionately influence carbon export to the ocean interior (Wlash 1991; Muller-Karger *et al*., 2005). While our results indicate a substantial contribution to nitrification by aggregate associated microorganisms in these areas, their influence on carbon export remains to be elucidated. Identification and quantification of microorganisms populating sinking aggregates in combination with aggregate specific carbon fixation and nitrification rate measurements could provide first insights.

3. Role of Nitrococcus *and* Nitrospina *in pelagic N-cycling*

3.1 Where are they? How many are there? How active are they?

Our results indicate the adaptation of *Nitrococcus* to nitrite and organic rich environments such as oxygen minimum zones while *Nitrospina* exhibits adaptations to substrate limited environments. As the ocean is largely oligotrophic and *Nitrospina* has often been detected by molecular methods such as qPCR and environmental metagenome analyses (e.g. DeLong *et al.,* 2006; Mincer *et al*., 2007; Fuch *et al*., 2005; Santoro *et al.,* 2010), *Nitrospina* is considered to be the dominant nitrifying microorganisms in the ocean (e.g. Lücker *et al*., 2013; Beman *et al*., 2013). Nevertheless, nutrient rich microniches such as sinking particles and aggregates also exist in oligotrophic regions (Pilskaln *et al*., 2005; Close *et al*., 2013) and might largely influence the activity and distribution of microorganisms (Azam and Long, 2001). Microorganisms attached to aggregates might previously have escaped detection in bulk seawater analyses due to the size fractionation often employed. Moreover, microorganisms that exploit nutrient rich microniches might be overall less abundant but would nevertheless significantly influence turnover rates. While the presence of *Nitrospina* as well as *Nitrocoocus* in samples from the oligotrophic open ocean and from highly productive

coastal ecosystems indicates their simultaneous activity, only comparative analyses on the abundance and distribution of both genera, including microorganisms attached to aggregates or particles, could reveal their respective importance in nitrogen cycling.

3.2 Does Nitrococcus significantly contribute to oceanic N_2O production?

We could show that *Nitrococcus*, an abundant NOB especially in OMZs, produces N_2O when subjected to anoxia. However, we have not yet identified the oxygen threshold that triggers the onset of nitrate or nitrite reduction in *Nitrococcus*. Concentrations of nitrate, nitrite and organic substrates also likely influence the metabolism of *Nitrococcus*, and these regulatory factors should be investigated under conditions comparable to those found in the environment. To asses the role of *Nitrococcus* in oceanic N_2O production, extensive environmental analyses on the distribution and abundance of *Nitrococcus* in OMZs are also needed.

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