Studies on aerobic ammonia oxidation and denitrification in stratified habitats

Dissertation zur Erlangung des Doktorgrades der Naturwissenschaften

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Zusammenfassung

Das Hauptziel dieser Arbeit war es die Denitrifikation und aerobe Ammoniakoxidation in stratifizierten Habitaten (mikrobielle Aggregate, mikrobielle Matten und Sedimente) im Hinblick auf den Einfluss von Salinität und/ oder O_2 zu studieren.

Proben mikrobieller Aggregate, wie sie in Wasseraufbereitungsanlagen genutzt werden, wurden entweder salin oder nicht salin in denitrifizierenden, anoxisch gehaltenen Aufstrom-Schlammbettreaktoren angezogen. Salinitätseffekte konnten somit anhand eines kleinmaßstäbigen stratifizierten Systems (1-3 mm Durchmesser pro Aggregat) studiert werden, während andere die Denitrifikation beeinflussende Faktoren, wie O_2-Schwankungen und Substratlimitierung, ausgeschlossen werden konnten. Der Einfluss von Salinität auf die Denitrifikation in mikrobiellen Aggregaten hang stark von den vorhergehenden Wachstumsbedingungen ab. Unter Erhöhung der Salzkonzentration wurde in nicht salin angezogenen Aggregaten, eine starke Abnahme der Denitrifikationsraten und -effizient beobachtet, was andere Studien bestätigte, die eine negative Korrelation von Salinität und Denitrifikation gezeigt haben. Dieses ist von Nachteil, wenn die Denitrifikation für die Aufbereitung von Brackwasser oder Sole eingesetzt wird. Salin gezogene Aggregate zeigten eine leichte Überanpassung an Salinität. Ihre Aktivität nahm zu bei Salzkonzentrationen, die größer waren als die ursprüngliche Wachstumskonzentration. Dieses legt nahe, dass der Salzgradient, der sich über den Zellmembranen der Zellen aufbaut, für Na^+/H^+-Antiporter –basierten Substrattransport genutzt wird. Von einem angewandten Standpunkt betonen die aufgeführten Ergebnisse die Notwendigkeit salinitätsangepasster, denitrifizierender mikrobieller Gemeinschaften zur Aufbereitung von Brackwasser und Sole.

Die Aggregate bestehen v.a. aus mikrobiellen Zellen und extrazellulären polymeren Substanzen, was auch für mikrobielle Matten zutrifft. Letztere wurden thematisiert in der hier

aufgeführten Studie zur aeroben Ammoniakoxidation unter hypersalinen Bedingungen. Die mikrobiellen Matten waren in ihren natürlichen Habitaten tageszeitlichen Unterschieden und Tiden ausgesetzt, welche Veränderungen verschiedener physikalisch-chemischer Gradienten herbeiführen. Hier sind vor allem Salinität, Schwefelwasserstoff und O_2 von Bedeutung. Letzterer wird für die Oxidation von Ammoniak benötigt. Schwefelwasserstoff inhibiert die Ammoniakoxidation und Salinität scheint sich nachteilig darauf auszuwirken. Dementsprechend haben viele Studien ein Fehlen von aerober Ammoniakoxidation unter hypersalinen Bedingungen, wie sie z.B. in mikrobiellen Matten gegeben sind, beobachtet. Es ist uns gelungen die Präsenz und Aktivität von Ammoniakoxidierern in hypersalinen Matten zu zeigen, die von geographisch verschiedenen Orten stammen. Die gemessenen Ammoniak- Oxidationsraten und die mikrobielle Diversität waren eher klein und gekennzeichnet durch die Dominanz von Betaproteobakterien über Archaeen, was sich deckt mit Beobachtungen, die in Ästuaren gemacht wurden. Ästuarine mikrobielle Gemeinschaften, sowie Gemeinschaften mikrobieller Matten, sind beide Salinitätsschwankungen ausgesetzt, wenn auch in unterschiedlichem Maße. Dieses könnte darauf hinweisen, dass Betaproteobakterien bei gegebenen Salinitätsschwankungen überwiegen.

O_2, welcher für aerobe Ammoniakoxidation benötigt wird, kann die Denitrifikation inhibieren, weil O_2 zum einen ein kompetetiver Elektronenakzeptor der Nitratrespiration sein kann und zum anderen werden Schlüsselenzyme der Denitrifikation bereits von kleinen O_2-Konzentrationen inhibiert. Nichtsdestotrotz wurde die Präsenz aerober Denitrifikation in einigen Laborstudien gezeigt, aber die Bedeutung der aeroben Denitrifikation in der Umwelt blieb unklar. In der letzten hier präsentierten Studie werden signifikante Raten aerober Denitrifikation in permeablen sandigen Sedimenten gezeigt. Dieses legt nahe, dass gezeiteninduzierte O_2-Oszillation im Sediment eine O_2-Anpassung der Denitrifikation fördern. Co-Respiration von

NO_x^- und O_2, ist ein vorgeschlagener Mechanismus zur Erklärung der aeroben Denitrifikation in den studierten sandigen Sedimenten.

Summary

The main aim of this thesis was to study the effect of salinity and /or O_2 on denitirification and aerobic ammonia oxidation in stratified habitats (microbial aggregates, microbial mats and sediments).

Samples of microbial aggregates, as used in waste water treatment, were grown either under saline or non-saline conditions, in denitrifying anoxic upflow-sludge blanket reactors. Salinity effects could therefore be studied in a small-scale stratified system (typical aggregate diameter 1-3 mm), whilst excluding other denitrification influencing factors, such as O_2 fluctuations or substrate limitation, as they occur in the environment. The influence of salinity on denitrification in microbial aggregates was strongly dependent on prior growth conditions. As expected, strong decreases in denitrification rates and denitrification efficiency were seen for non-saline grown microbial communities upon increasing salinity. This observation corroborates other studies, showing a negative correlation of salinity and denitrification in microbial aggregates, which is disadvantageous when applied within waste water treatment of brackish water or brine. Saline grown aggregates were observed to have a slight over-adaptation to salinity. Their activity increased upon increases of salinity higher than their initial growth salinity. This may indicate that the increased salinity gradient along their cell membranes is used for Na^+/H^+- antiporter substrate transport into the cell. From an applied point of view, the presented results highlight the importance of applying salinity-adapted denitrifying microbial communities in wastewater treatment of brackish water and brine.

The aggregates consist mainly of microbial cells and extracellular polymeric substances, which is also the case for microbial mats. The latter were subject to the here presented study on aerobic ammonia oxidation under hypersaline conditions. Microbial mats were in their natural habitats exposed to diel and tidal changes, which induce changes in various physico-chemical

gradients, especially salinity, sulfide and O_2. The latter is required for aerobic ammonia oxidation. Sulfide inhibits aerobic ammonia oxidation, and salinity has been observed to have an adverse effect on it. Accordingly, many studies observed an absence of aerobic ammonia oxidation under hypersaline conditions as e.g. given in microbial mats. We could show the presence and activity of ammonium oxidizers in hypersaline microbial mats originating from different geographical locations. Measured ammonia oxidation rates and microbial diversity were rather low, showing a predominance of betaproteobacteria over archaea, which coincides with observations made in estuaries. Both, microbial communities from estuaries and from microbial mats are exposed to salinity fluctuations, albeit within different ranges. This might indicate that ammonia oxidizing betaproteobacteria prevail, when salinity fluctuates.

O_2, which is required for aerobic ammonia oxidation, can inhibit denitrification, because O_2 can act as a competing electron acceptor for NO_3^- respiration and key enzymes of the denitrification pathways are inhibited by relatively small amounts of O_2. Nevertheless, the presence of aerobic denitrification was shown in several laboratory studies, but its significance in the environment remained unclear. The last study presented in this thesis shows the occurrence of significant rates of aerobic denitrification in permeable sandy sediments. This suggested that tidally induced oscillations of O_2 in the sediment, facilitate an adaptation of denitrification to O_2. Co-respiration of NO_x^- and O_2 is a proposed mechanism for aerobic denitrification in the studied sandy sediments.

Table of Contents

Chapter 1

Introduction

Introduction

1.1 Stratified systems, redox gradients and microbial metabolic zones

Limited physical vertical mixing results in stratification in many natural and man-made environments. The extents of such stratification can range from hundreds and thousands of meters to micrometers, including oceans, lakes, sediments, soils, microbial mats, biofilms and microbial aggregates. Sharp geochemical gradients often ensue across the strata in these systems. In particular, if oxygen is prevalent only towards either the upper or lower boundaries, exponential decrease in oxygen concentrations is commonly observed, followed by the sequential depletion of nitrite and nitrate, manganese oxides, iron oxides, sulfate and carbon dioxide. This sequence is directly related to the effective electrode potentials of these above-mentioned electron acceptors, and thus their preferential use in redox reactions. While the initial presence of many of these chemical species in the environments is geochemical and abiotic in nature, their subsequent consumptions in redox reactions are, however, often mediated by microorganisms. Hence, the tendency of microbial communities to stratify in nature can be explained by a competition for substrate, but the ability of microorganisms to compete with each other is determined within the boundaries of their ability to gain energy (Canfield et al., 2005). From a thermodynamic point of view, the gain of energy from a redox reaction (Gibbs free energy, ΔG) is positively correlated with the difference in electrode potentials (E'_0) between the electron donor and the electron acceptor. Under most environmental conditions, oxygen is energetically the most favorable of all electron acceptors for the respiration of organic matter (electron donor). The use of alternative electron acceptors with less negative E_o' than oxygen would thus yield lower energy. Because the atmosphere and most water bodies in modern Earth systems are well oxygenated, organisms dependent on oxic respiration usually populate the layers towards the surfaces exposed to oxygen. When oxygen supplies run low, those organisms

capable of utilizing the next favorable and available electron acceptors, would then flourish (Figure 1). In other words, a sequence of respiration processes, or metabolic zones, would develop within stratified systems due to potential energy yields roughly following the sequence: oxic respiration > nitrate/nitrite reduction > manganese reduction > iron reduction > sulfate reduction > methanogenesis.

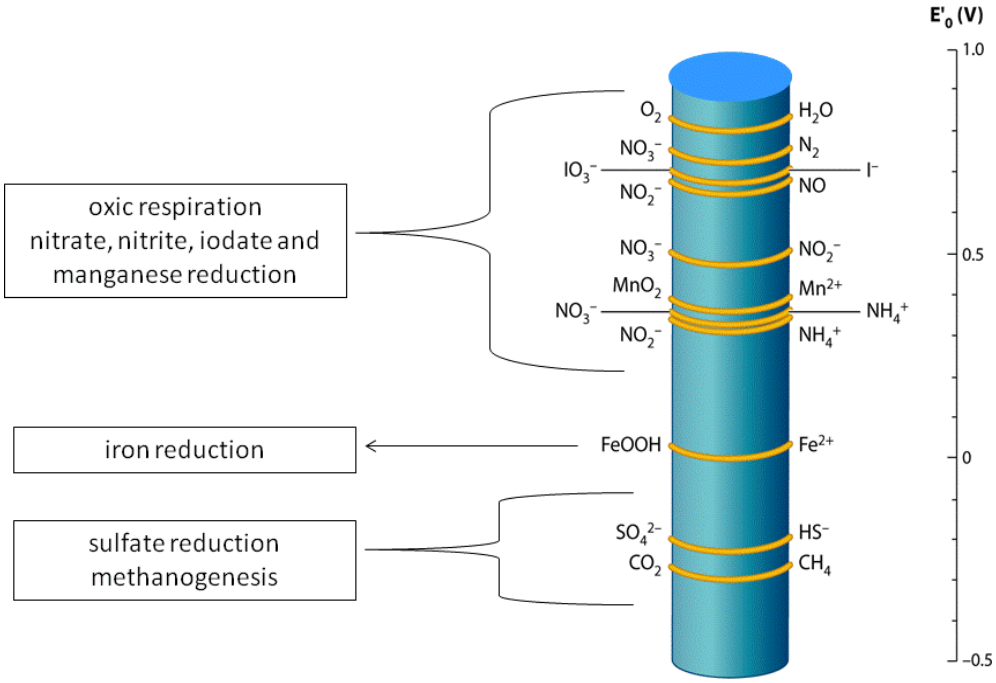

Figure 1. The electron tower illustrating the electrode potentials (E'_0) of various redox couples. The values are calculated for pH 7 and otherwise standard conditions. Modified after Lam and Kuypers (2011).

Nevertheless, the demarcation of these metabolic processes or zonation is not always clearly cut. Depending on the particular systems, other environmental factors may become important, e.g. availability of electron donors and acceptors, pH, light, temperature, abiotic geochemical reactions. Moreover, specific adaptive strategies employed by different microbial communities or functional groups, may also significantly alter such vertical metabolic and chemical zonation (Canfield et al., 2005).

The stratified systems, which were studied within this thesis, are microbial aggregates, microbial mats and sandy sediments, ranging in size from millimeters (microbial aggregates)

over centimeters (vertical depth of microbial mats) up to kilometers (vertical depth of sediments).

In the studied mm-sized microbial aggregates, which were grown under anoxic conditions, the focus was on salinity induced differences on denitrification, showing a vertical stratification of nitrogen cycling along a micrometer-scale. Within the studied hypersaline microbial mats is a potential vertical depth range of centimeters given, but it was not clear if the studied ammonia oxidizers with their typically low energy yield would at all find a niche within the salinity adaptation requiring hypersaline systems (which are additionally dominated by strong changes in various physico-chemical gradients). In the study on Wadden Sea sediments, the effect of pore water advection in combination with tidally induced oxygen oscillations in coarse grained sandy sediments caused an overlap of zones of NO_x^- and O_2 penetration up to several centimeters depth, which is proposed to facilitate the measured rates of aerobic denitrification.

Taken together, the studied systems might range overall from millimeters to kilometers, but the main focus here is on aspects of stratification within a micrometer-scale, even within the sediments, as the grain size of the sediments (and the resulting permeability) facilitates the described tidally induced redox oscillations, and the overlab of NO_x^- and O_2 penetration zones.

All described stratified systems are additionally characterized by temporal changes in salinity and/or oxygen, and were examined with a focus on nitrogen cycling processes, which are introduced in the following sections.

1.2 Nitrogen cycling processes

Nitrogen is a constituent of many biomolecules, and therefore pivotal to all living organisms, but most of the nitrogen on earth is not biologically available (Canfield et al., 2005). The most common biologically available inorganic forms in aquatic and terrestrial environments are dissolved NH_4^+, NO_2^- and NO_3^-. However, quantitatively they only account for about 0.006% compared to the atmospheric N_2 gas, and only for about 25% of the nitrogen, which is buried as

dead organic detritus (Vitousek et al., 1997; Canfield et al., 2005). Because nitrogen can exist in different oxidation states ranging from –III to +V, its various forms can be involved in many redox reactions mediated by disparate groups of microorganisms (Thamdrup and Dalsgaard, 2008). Figure 2 gives an overview on the nitrogen cycle, as it can occur in sediments (Thamdrup and Dalsgaard, 2008), the water column and microbial mats.

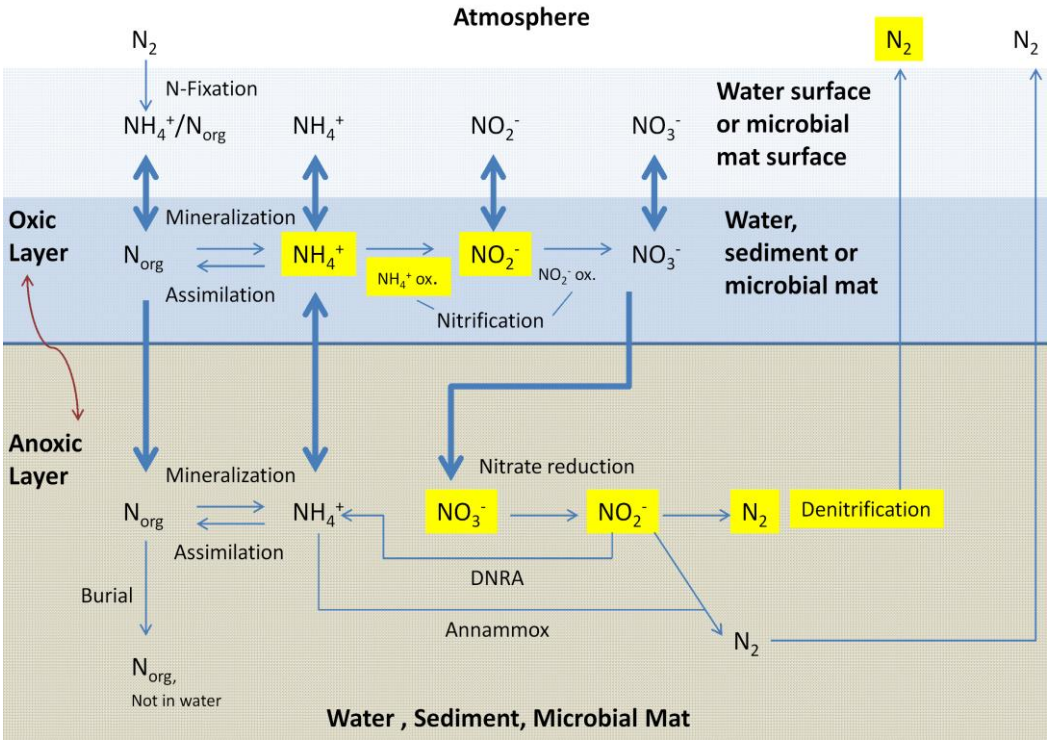

Figure 2: The nitrogen cycle, modified and extended after Thamdrup and Dalsgaard, 2008, excluding heterotrophic nitrification. Blue thick arrows indicate transport processes and blue thin arrows indicate microbial transformations. Red arrow indicates shifts of the oxic-anoxic interface, which might also exist as a more gradual "suboxic" zone. Aerobic ammonium oxidation and denitrification are highlighted yellow.

1.2.1 Biological nitrogen fixation

Nitrogen fixation is the conversion of atmospheric N_2 into bioavailable NH_3/NH_4^+. Three types of nitrogen fixation are known: (1) atmospheric nitrogen fixation by lightning, (2) the industrial Haber-Bosch process, which requires temperatures between 300 and 400 °C and pressures between 35 and 100MPa, and (3) biologically mediated nitrogen fixation which is performed by diazotrophs. It is the third that acts as the major source of fixed nitrogen in the

biosphere (Canfield et al., 2005; Newton, 2007). All diazotrophs contain the oxygen-sensitive enzyme nitrogenase, which enables them to reduce the triple-bonded N_2 gas into NH_3 /NH_4^+ (Sprent and Sprent, 1990) at normal environmental temperatures and pressures. Diazotrophs are exclusively prokaryotes. They would release their fixed N as NH_4^+ directly, or as organic N that is eventually microbially degraded to NH_4^+ (Ward, 2008). The produced NH_4^+ can subsequently be either assimilated or nitrified by other organisms.

In microbial mats are cyanobacteria believed to be the main N_2-fixers (Bebout et al., 1993; Paerl et al., 1991), performing N_2-fixation in the absence of light, due to photosynthetically produced O_2, which is suppressing nitrogenase activity (Bothe, 1982; Bebout et al., 1993). Often are N_2-fixing filamentous non-heterocystous cyanobacteria (e.g. *Microcoleus sp.*, *Oscillatoria sp.*, *Lyngbiya sp.*) found at the surface of microbial mats, but there are also coccoid species, such as *Gloeocapsa sp.*, *Cyanotheke sp.* and *Synechococcus sp*. (D´Amelio et al. 1989; Paerl et al., 1991; Bebout et al., 1993; Steunou et al., 2006; Woebken et al., 2012). Heterotrophic bacteria are also known microbial mat N_2-fixers, but their overall significance within these systems is not yet clarified (Bebout et al., 1993; Zehr et al., 1995; Steunou et al., 2006), and the common observation of indirectly light-inhibited nitrogen fixation (e.g. Bautista et al., 1985; Bebout et al., 1987) corroborates with the idea of cyanobacteria being in most microbial mat systems the main nitrogen fixers.

In the open ocean, *Trichodesmium* and to some extent symbionts of diatoms have been believed to be the predominant N_2 fixers (Villareal, 1991; Zehr and Ward, 2002), but the estimates of N_2-fixation rates and *Trichodesmium* abundance do not always coincide as expected (Lipschultz and Owens, 1996; Michaels et al., 1996; Gruber and Sarmiento, 1997; Zehr, 2001). Recent studies revealed in fact high N_2 fixation rates by unicellular diazotrophs in the oceans (e.g. Montoya et al., 2004).

Meanwhile, N_2-fixing communities in global oceans are now known to be much more diverse (Zehr et al., 1998, 2000; Capone, 2001, Langlois et al., 2005), especially with the unicellular photoheterotrophic and heterotrophic bacteria likely playing much greater roles than once thought (Zehr et al., 2008, Moisander et al., 2010, Halm et al., 2011). Despite the conventional thinking that diazotrophic activities are restricted to mainly oligotrophic regions, Deutsch and colleagues (2007) deduced from nutrient inventories and global circulation models that oceanic N_2 fixation might instead be spatially coupled to nitrogen loss activities. Overall, with recent improvements in N_2-fixation rate measurements (Mohr et al., 2010; Gandhi et al. 2011) and diversity surveys for diazotrophic communities, global marine N-gain via N_2-fixation might in reality be a lot closer to a balance with total marine N-loss than previously considered (Codispoti et al., 2001).

In addition, was the abundance of *Trichodesmium spp.* probably in some cases underestimated, as shown by Davis and McGillicuddy Jr. (2006) using an underwater digital microscope, which was towed across the North Atlantic, revealing especially a higher subsurface abundance of *Trichodesmium spp.*, than previously thought. The number of studies changing the view on so far conservative estimates of N-input in the ocean is rising.

1.2.2 Ammonium assimilation and ammonification/minearlization

Ammonium is the preferred form of nitrogen for assimilation by most organisms. The initial product of ammonium assimilation is usually glutamate. Glutamate production might either take place with the enzyme glutamate dehydrogenase (GDH), or it might be produced within an ATP-consuming two- step pathway involving the enzymes glutamine synthetase (GS) and glutamate synthase (GOGAT). The latter GS-GOGAT pathway is on one hand more costly for the cell, but on the other hand it is showing a higher affinity to NH_4^+ than the GDH-pathway. For diazotrophs, nitrogenase activity can be suppressed by high levels of intracellular ammonium, and thus rapid consumption of NH_4^+ within these organisms is essential (Gottschalk, 1986). The

assimilated nitrogen is used within the prokaryotic cell predominantly for proteins and polynucleotides (Madigan et al., 2003).

Upon cell deaths or exudations, degradation of organic nitrogen either as respiration of organic matter or fermentation, will lead to liberation of inorganic nitrogen in the form of ammonium. This process is also referred to as (re)mineralization or ammonification, which can occur under oxic and anoxic conditions (Herbert, 1999). Therefore, ammonium plays a key role in connecting the organic and inorganic pools of nitrogen. The produced NH_4^+ from ammonification might then either be assimilated again by other organisms, or oxidized to nitrite/nitrate by nitrifying bacteria or archaea, or oxidized to dinitrogen gas by nitrite via the anammox process.

1.2.3 Nitrification

Nitrification describes the complete oxidation of NH_3/NH_4^+ (most reduced) to NO_3^- (most oxidized) in two consecutive steps: the oxidation of NH_3/NH_4^+ to NO_2^-, followed by the oxidation of NO_2^- to NO_3^-. Both nitrification steps occur primarily under aerobic conditions, with ammonia oxidation usually regarded as the rate-limiting step (Kaplan, 1983). Nitrification is the major oxidative branch of the nitrogen cycle. It plays a critical role in connecting the ammonium released from the organic pool, to the most oxidized form that could be assimilated or used as electron acceptors for dissimilatory processes and ultimately lost from the systems as gaseous products. However, most nitrifiers are chemolithoautotrophs, depending on CO_2 as their major carbon source. CO_2 fixation in nitrifying bacteria accounts for approximately 80% of their energy budget (Forrest and Walker, 1971; Kelly, 1978), but the oxidation of NH_4^+ and NO_2^- gives only low energy yields (Lam and Kuypers, 2011), thus resulting in relatively long generation times. For example, the ammonia-oxidizing *Nitrosomonas* and nitrite-oxidizing *Nitrobacter* require at least 7-13 h (Bock et al., 1989), while the ammonia-oxidizing archaeon, *Nitrosopumilus maritimus*, requires at least 21h (Könneke et al., 2005).

1.2.3.1 Aerobic ammonia oxidation

The understanding of ammonia oxidation, the 1st step of nitrification, has changed tremendously in the past few years. For more than a century, since the first isolation of ammonia-oxidizing bacteria by Winogradsky (Meiklejohn, 1949), the capability of ammonia oxidation has long been thought only possible in some chemolithoautotrophic bacteria. It is now known, however, that some heterotrophic bacteria as well as more recently some archaea also carry this capacity (Richardson et al., 1998; Könneke et al., 2005, Ferguson et al., 2007; Prosser, 2007). These three groups are distinctly different from each other, and perform ammonia oxidation to NO_2^- in different ways. The heterotrophic ammonium oxidation by bacteria and fungi is beyond the scope of this thesis, and will not be further discussed.

Ammonia-oxidizing bacteria (AOB) fall into two monophyletic groups based on 16S rRNA: (1) the beta-proteocbacterial *Nitrosomonas* and *Nitrosospira*, and (2) the gamma-proteobacterial *Nitrosococcus* (Head et al., 1993; Teske et al., 1994). Consistent phylogenetic relationships among AOB is further provided by sequence analysis of *amoA*, which encodes the active subunit of the ammonia-monooxygenase (Purkhold et al., 2000). A few years ago, a copy of the *amoA* was found in an archaeal metagenome in the Sargasso Sea (Venter et al., 2004), thus first suggesting the potential of archaeal ammonia oxidation in the oceans. This potential was confirmed unequivocally in a culture study upon the first successful isolation of a chemolithoautotrophic ammonia-oxidizing archaeon, which showed a similar stoichiometry of NH_3 oxidation to nitrite as in AOB (Könneke et al., 2005). Because archaeal *amoA* appeared to be rather ubiquitous in marine environments (Francis et al., 2005), and marine archaea are generally more abundant than AOB in pelagic ocean (Karner et al., 2000), ammonia-oxidizing archaea (AOA) have since been postulated to be the major player in marine ammonia oxidation instead of AOB (Francis et al., 2007).

The bacterial ammonia oxidation to NO_2^- involves hydroxylamine as an intermediate. The first step is catalyzed by the membrane-bound enzyme ammonia monooxygenase (AMO), which contains copper and probably also iron in its active form (Zahn et al., 1996). It requires molecular oxygen as well as 2 e^- for oxidation ($NH_3 + O_2 + 2H^+ + 2e- => NH_2OH + H_2O$), and the process is usually carried out at a pH of 7-8. The subsequent conversion of hydroxylamine to NO_2^- ($NH_2OH + H_2O => NO_2^- + 5H^+ + 4e-$) is catalyzed by the periplasmic enzyme hydroxylamine oxidoreductase, and this is the energy- and e^-- gaining step of aerobic ammonia oxidation (Kaplan, 1983).

For ammonia-oxidizing archaea, however, the genes encoding the enzyme hydroxylamine oxidoreductase have never been detected. From whole-genome analyses of *N. maritimus*, Walker and colleagues (2010) proposed instead nitroxyl as an intermediate, and that it is oxidized by a putative nitroxyl-oxidoreductase, which is potentially one of the several soluble periplasmic multi-copperoxidase proteins annotated in the genome. AOA show in general much higher affinity for ammonia than AOA (Martens-Habbena et al., 2009). In both, the oxidation of hydroxylamine (AOB) and nitroxyl (AOA), oxygen is consumed by a terminal oxidase, which facilitates electron transport for ATP generation (Ward, 2008; Walker et al., 2010).

1.2.3.2 Nitrite oxidation

The second nitrification step is the oxidation of nitrite to nitrate (the most oxidized form) mediated by nitrite-oxidizing bacteria belonging to the genera *Nitrobacter, Nitrospira, Nitrococcus, Nitrospina* and *Nitrotoga* (Herbert, 1999; Alawi et al., 2007).

The energy yield of nitrite oxidation is even lower than the energy yield of ammonium oxidation, requiring even more NO_2^- in order to fix only small amounts of carbon. However, the biochemistry of the NO_2^- oxidation to NO_3^- ($NO_2^- + H_2O => NO_3^- + 2H^+$), which is catalyzed by nitrite oxidoreductase is much simpler than the previously described biochemistry of NH_3 oxidation to NO_2^-, as it requires only a transport of two electrons, and no intermediates are

produced. As in NH_3 oxidation, oxygen is consumed by a terminal oxidase to facilitate electron transport for ATP generation ($2H^+ + 0.5\ O_2 => H_2O$) (Ward, 2008).

1.2.3.3 Environemental regulating factors on nitrification

The activity and distribution of aerobic nitrifiers in stratified systems is determined by various environmental factors, including oxygen, sulfide, ammonium, temperature, salinity, pH and light (Canfield et al., 2005, Prosser, 2007). In general, nitrification can be maintained within a wide range of changes of environmental factors, but these usually go hand in hand with community shifts, as different species of nitrifiers have different optima and different degrees of adaptability to various environmental factors. The established community within a habitat thus determines the actual ranges in which nitrification can occur, as long as these species are not outcompeted by others.

1.2.3.3.1 Oxygen, sulfide, light and ammonium

Chemolithotrophic nitrifiers require molecular oxygen for respiration and reactions in the nitrification pathway. They are considered to be obligate aerobes, with some probably microaerophiles. Adaption to low oxygen could be important in interface environments, such as the sediment water interface, oxygen minimum zones of the ocean (Ward, 2008), oxic-anoxic interfaces of stratified lakes (Voytec and Ward, 1995), biofilms, and microbial mats. For instance, some AOB species in cultures were shown to grow and nitrify under oxygen tension down to 0.5-1% O_2 while producing N_2O (Goreau et al., 1980), though with some interspecies variations. In sediments, nitrifiers were even capable of surviving periods of anoxia, and could recover instantly after O_2 exposure (Henriksen et al., 1981). This is likely attributed to their ability to utilize a non-oxidative, dissimilatory metabolism when oxygen concentrations are very low or zero. For example, growth on organic compounds under anoxic conditions has been observed for the NH_3 oxidizer *Nitrosomonas sp*. producing NO, N_2O and N_2 (Stuven et al.,

1992), and for NO_2^- oxidizers, which grew via dissimilatory nitrate reduction (Freitag et al., 1987).

Nitrification or ammonia oxidation in particular, is probably irreversibly inhibited by sulfide (Kaplan, 1983; Joye and Hollibaugh, 1995). A chelation reaction with copper, which is located at the active site of the enzyme ammonia monooxygenase (AMO), is the likely explanation for the inhibitory effect of H_2S (Bedard and Knowles, 1989). Many other more complex S-containing compounds also have an inhibitory effect on AMO and each works to different degrees (Juliette et al., 1993; Ginestet et al., 1998; McCarty et al., 1999), but they are not considered further here as they are beyond the scope of this thesis. Together with oxygen, chemolithotrophic nitrifiers need to find a position along a vertical gradient within a stratified habitat that has sufficient oxygen available, but low or zero sulfide concentrations.

While light drives primary production, and so stimulates oxygen production in a stratified system, light is also a direct inhibitor for ammonia oxidation, and probably also for nitrite oxidation (Olson, 1981b; Vanzella et al, 1989, Guerrero and Jones, 1996a, b). Hence, in a dynamic stratified system like a photosynthetic microbial mat, where there is strong oxygenation during the day followed by a rise of the sulfide front at night due to heterotrophic activities (Jørgensen et al., 1983; Van Gemerden, 1993; Wieland and Kuehl, 2000; Kohls, 2010), nitrification is only feasible within a very narrow sub-surface layer: away from light and sulfide yet sufficiently oxygenated.

However, ultimately NH_4^+ would have to be available for the nitrifiers, and its availability is often the most important regulator for ammonia oxidation, which is considered as a first-order reaction. Different species of nitrifiers exhibit different substrate affinity, and are also adapted to a certain extent to the particular substrate availability in their immediate habitats (Focht and Verstaete, 1977; Olson, 1981a; Koops and Pommerening-Röser, 2001; Canfield et al., 2005; Martens-Habbena et al., 2009). It is the substrate affinity that determines the nitrifier's ability to

compete with other NH_4^+ consuming organisms - e.g. heterotrophic bacteria (Strauss et al., 2002) or phytoplankton (Risgaard-Petersen et al., 2004). Additionally, the ability to compete might be influenced by the diel cycle or seasonal patterns. An example for potential substrate competition can be seen in a stratified water column: NH_4^+ assimilation by phototrophs dominates mainly in the euphotic zone, whilst ammonia oxidation prevails towards the base of the euphotic zone or in the dark (Ward, 1987). This can be interpreted as phytoplankton outcompeting nitrifiers towards the surface, or it can be a result of light inhibition on nitrification, or of the higher amounts of NH_4^+ remineralized at the base of the euphotic zone, or a combination of various factors (Ward, 2008). However, in shallow sediments it was shown that benthic algae were indeed the superior competitors over nitrifying communities (Risgaard-Petersen et al., 2004).

1.2.3.3.2 pH and temperature

Growth and activity of most chemolithoautotroph nitrifiers are optimal at a pH range of 7.5-8 (Focht and Verstraete, 1977). Because it is the molecular NH_3 and not the charged ion NH_4^+ that is used as a substrate for the enzyme AMO, a low pH would reduce the substrate availability due to the NH_3:NH_4^+ disequilibrium (pK_a = 9.25) and thus ammonia oxidation (Suzuki et al., 1974, Frijlink et al.,1992). Declines in ammonia oxidation rates at low pH have been evident in lakes, soils, activated sludge and the open ocean (e.g. Painter and Loveless, 1983, Rudd et al., 1988, De Boer and Kowalchuk 2001, Beman et al., 2011). However, the presence of too much NH_3 and alkaline salts at high pH are also toxic to nitrifiers, albeit nitrification is seldom inhibited completely by extreme pH values. Nitrification activities along with both ammonia-oxidizers and nitrite-oxidizers have been detected in acidic environments – e.g. *Nitrosospira* and *Nitrosovibrio* from acid soils and sediments showed activity at ~pH 4 (De Boer and Laanbroek, 1989) and more recently an acidophilic ammonia-oxidizing archaeon (Lehtovira-Morley et al., 2011). Several mechanisms have been proposed for nitrifiers to cope with suboptimal pH, including pH-neutral microenvironments, biofilm and aggregate formation, urease activities and close

interactions with ammonifiers (Killham 1986; De Boer et al. 1989, 1991; Allison and Prosser 1993; Burton and Prosser 2001; De Boer and Kowalchuk 2001; Giesecke et al. 2006). Similarly, both ammonia-oxidizers and nitrite-oxidizers have been isolated from sediments of soda lakes with pH ~10 (Sorokin et al., 1998; 2001).

With respect to temperature, the growth optima of nitrifiers vary in pure cultures between 25-35 °C, showing growth between 3-45 °C (Herbert, 1999). Although thermophillic chemolithoautotrophic nitrification has been believed to be rare, thermophilic ammonia-oxidizing bacteria and archaea, as well as nitrite-oxidizers, have recently been detected in various hot springs (Lebedeva et al. 2005, 2011; De la Torre et al. 2008; Hatzenpichler et al. 2008). In nature, nitrifiers adapt to the temperature of their environment with seasonal variations in activity (Canfield et al., 2005). Activity in the summer is usually higher due to the Q10s for nitrification and various metabolic reactions. It might also be partially linked to the higher primary production and thus higher amounts of NH_3 remineralized from organic matter – as long as O_2 concentrations are sufficient for the nitrifying communities (Hansen et al., 1981; Macfarlane and Herbert, 1984; Seitzinger et al, 1984; Canfield et al., 2005). However, higher temperatures could also reduce the solubility of oxygen and thereby also the oxygen penetration into stratified systems like sediments, and thereby having an indirect negative effect on nitrification. Higher respiration rates can increase the demand in oxygen, and the upward shift of the oxygenated zone might induces additional competition with heterotrophic bacteria for NH_4^+, and an increase of sulfide, which is inhibiting nitrification (Canfield et al., 2005). There are also nitrifiers that are adapted to cold temperatures. For example, in a study on Arctic sediments, the temperature optima for nitrification could range from 14 to 40°C, depending on the *in situ* temperature of the environment from which the samples were collected (Thamdrup and Fleischer, 1998). Altogether, the dependence of nitrification on temperature can be observed in many systems, but the temperature adaptability of nitrifiers to their particular habitats means that

nitrification is usually regulated in the environment by factors other than temperature (Ward, 2008).

1.2.3.3.3 Salinity

Salt concentrations in the open ocean are fairly stable, therefore the sensitivity to salinity changes is not an important variable in this habitat. However, it is clearly an important factor in riverine and estuarine environments (Ward, 2008). Community shifts and activity changes along a salinity gradient were observed in many studies (Rysgaard et al., 1999; De Bie et al., 2001; Bollman and Laanbroek, 2002, Francis et al., 2003, Magalhaes et al., 2005). The freshwater portion of the Schelde estuary was dominated by *Nitrosomonas ureae* and *N. oligotropha*. In the brackish area, sequences of a novel group of *Nitrosomonas*-like bacteria and relatives of *N. marina* and *N. aestuarii* were found. In summary, in the presence of salt, relatives of already known halophilic AOB dominated, and in the absence of salt were relatives of already known freshwater AOB dominating, even though relatives of already known halophilic AOB were shown to be rather halotolerant than halophilic (Bollmann and Laanbroek, 2002), what might be an advantageous adaption, when salinities decrease. This is consistent with observations of Caffrey et al. (2007) studying six different estuaries. They showed that highest rates of potential nitrification were negatively correlated with salinity (in combination with other factors). There were also a strong positive correlation between AOA abundance and potential nitrification rates, as well as between archaeal amoA gene abundance and salinity.

1.2.4 Dissimilatory nitrate reduction

Dissimilatory nitrate reduction to nitrite ($NO_3^- + 2H^+ + 2e^- \Rightarrow NO_2^- + 2H_2O$) describes the reduction of NO_3^- to NO_2^- in the absence of oxygen. This reaction can be performed by many organisms, which might not show the ability to denitrify or ammonify the produced nitrite (Gonzalez et al. 2006, Zumft 1997). Therefore, it should be considered as a stand-alone process. It is a major remineralization pathway in e.g. oceanic oxygen minimum zones (Lam and

Kuypers, 2011). The product of this reductive process, NO_2^-, can subsequently be reduced to N_2 via denitrification or anammox, which are important N-loss processes in sediments and the water column (Thamdrup and Daalsgard, 2008; Lam and Kuypers, 2011), and both are applicable in waste water treatment for nitrogen removal. Additionally, NO_2^- can be further reduced to ammonium (Bonin et al.,1998; Thamdrup and Dalsgaard, 2002; Canfield et al., 2005) via DNRA, or it can be recycled back to nitrate via nitrite oxidation under suboxic conditions (Füssel et al., 2011).

1.2.5 Anaerobic ammonium oxidation (Anammox)

Anammox (Anaerobic ammonium oxidation) is carried out by specific groups of chemolithoautotrophic *Planctomycetes* (Strous et al., 1999, Schmid et al., 2000), in which NH_4^+ is oxidized by NO_2^- to form N_2 gas ($NH_4^+ + NO_2^- \Rightarrow N_2 + 2H_2O$) (Van de Graaf et al., 1995), and the energy gained is used to fix CO_2 into organic carbon. Originally found in wastewater treatments (Mulder et al, 1995, van de Graaf et al., 1995), anammox bacteria and their activities have now been detected in a wide variety of suboxic aquatic environments, and it is responsible for significant amount of nitrogen loss from especially suboxic marine water columns (Lam et al., 2009, Jensen et al., 2011) and to a certain extent marine sediments (Trimmer and Engström, 2011). Unlike conventional wastewater treatment for nitrogenous wastes that employs a combination of nitrification and denitrification reactors, application of anammox has the advantage of having NH_4^+ and NO_2^- removed simultaneously in one single treatment (Jetten et al., 2002). Anammox is now a patented technology for industrial-scale wastewater treatment (Paques, the Netherlands).

1.2.6 Dissimilatory nitrate reduction to ammonium (DNRA)

Dissimilatory nitrate reduction to ammonium ($NO_3^- \Rightarrow NO_2^- \Rightarrow NH_4^+$) is widespread among chemoautotrophic and heterotrophic bacteria, and used by facultative or obligate anaerobes to detoxify NO_2^-, or as an electron sink during fermentation in order to oxidize organic substrates

and thereby generate ATP via substrate-level phosphorylation (Thamdrup and Dalsgaard, 2008), or to gain energy via respiration (Welsh et al., 2001; Canfield et al., 2005). Organsims capable of DNRA may be involved in other dissimilatory pathways, e.g. sulfate (Simon, 2002), and thus link the nitrogen cycle to other elemental cycles (Lam and Kuypers, 2011). Besides, DNRA is interlinked with anammox by providing ammonium for the anammox-reaction ($NH_4^+ + NO_2^- => N_2 + 2H_2O$). It was estimated to account for up to 100% of the ammonium requirements for anammox over the shelf regions in the Peruvian and Arabian Sea oxygen minimum zones (Lam et al., 2009, Jensen et al., 2011). Meanwhile, at least some anammox bacteria have also been found to perform DNRA themselves, with the NH_4^+ produced from DNRA directly channeled to the anammox reaction within the same cells, thus mimicking the end results of denitrification (Kartal et al, 2007). DNRA can occur under similar conditions as denitrification, and in theory it has a lower energy yield than the latter. Nevertheless, a recent study indicated that the energy yield of denitrification is not efficiently conserved in the series of four enzymatic reactions, such that higher growth yield is in reality achieved via DNRA instead (Strohm et al. 2007).

1.2.7 Denitrification

Denitrification describes the stepwise reduction of NO_3^- to N_2, which consists of a number of respiratory reduction steps ($NO_3^- => NO_2^- => NO => N_2O => N_2$), carried out by four functional groups of reductases. These reductases catalyze four respiratory processes, which need to be simultaneously active in order to reduce NO_3^- to N_2 without intermediate accumulation (Zumft, 1997). It is important as a favorable alternative pathway of respiration after oxic respiration, with a similar $\Delta G°$ (-402 kJ for oxic respiration vs. -398 kJ for denitrification, calculated for 4 e- transfer at pH 7, 25 °C and acetate as e-donor) (Lam and Kuypers, 2011), which is of course only given if denitrification is carried out completely. Inefficient coordination of enzymatic activities results often in an accumulation and release of intermediates, especially NO_2^- and N_2O. The major requirement for complete denitrification is a full assemblage of all four enzymes, which is

not always given, e.g. N_2O reductase is absent in some *Pseudomonas* strains. Furthermore N_2O reductase is only fully active at pH 7. Trace elements are required for the enzyme biosynthesis, e.g. molybdenum in nitrate reductase (Hille, 1996), or iron in one type of nitric oxide reductase (Shiro et al., 1995). However, various environmental factors can also significantly influence the activities and distribution of denitrifying microbial populations, as discussed below.

While most studies on dentrification deal with heterotrophic denitrification, autotrophic denitrification (chemolithoautotrophic denitrification) can also be carried out by some sulfide/sulfur oxidizers, which utilize NO_3^-/NO_2^- as electron acceptors and release N_2 and/or N_2O. The gained energy is used to fix inorganic carbon. It can also be linked to hydrogenotrophy, methanotrophy, and iron oxidation (Lam and Kuypers, 2011). Autotrophic denitrification is beyond the scope of this thesis and will mainly not be further elaborated.

1.2.7.1 Substrate availability and oxygen

Denitrifying organisms can use a large variety of electron donors including various kinds organic matter (Payne,1981; Paul et al. 1989), in addition to several reduced inorganic compounds, such as hydrogen (Smith et al. 1994), hydrogen sulfide (Aminuddin and Nicholas 1973), thiosulfate (Ishaque and Aleem 1973), ferrous iron (Benz et al. 1998; Straub et al. 1996; Weber et al. 2006), carbon monoxide (King, 2006), and methane (Raghoebarsing et al. 2006). The respiration of NO_3^- to N_2 is thought to require an absence or nearly an absence of oxygen (Canfield et al., 2005). In benthic environments, there are two possible sources of NO_3^-: (1) diffusion of NO_3^- from the overlying water into the sediments, and (2) *in situ* production of NO_3^- within the sediment by nitrification. The denitrifying microbial community is expected to be located in an anoxic layer, and preferably not far under the oxic zone (Nielsen et al., 1990). The size of the oxic zone determines the substrate supply of denitrification, as the longer the diffusional path of nitrate through the oxic zone, the smaller the diffusional supply of NO_3^- to the underlying denitrifier communities (Jenkins and Kemp, 1984). The NO_3^- produced within the

sediment is believed to be minor in comparison, because it is expected to diffuse into the water and into the sediment with varying percentages, respectively. Additonally, increases in O_2 penetration depth were also shown to reduce the NO_3^- gradient within the sediment and thus its flux into the sediments (Seitzinger, 1988; Rysgaard et al., 1994). The oxygen induced increase of coupled nitrification-denitrification was shown to be modest, and it could not outcompete the NO_3^- loss, which was determined by the oxygen penetration depth (Jensen et al., 1994).

However, oxygen and denitrification are not necessarily always negatively correlated. It has been shown that the expression of genes encoding for denitrification enzymes is inhibited by oxygen concentrations >1µM (Zumft, 1997), but denitrification was also observed under well oxygenated conditions. For example, *Paracoccus pantotrophus* can use NO_3^- and O_2 simultaneously as electron acceptors at up to 90% air saturation (Robertson and Kuenen, 1984). Nevertheless, aerobic denitrification was only seldom reported in environmental studies and about its significance in nature is only little known (Canfield et al., 2005).

1.2.7.2 The impact of salinity on denitrification

Several studies have demonstrated different effects of salinity on denitrification but without consensus. On the one hand, studies on activated sludge showed a negative correlation between denitrification and salinity (Glass and Silverstein, 1999; Dinçer and Kargi, 1999). On the other hand, studies in natural environments with varying salinity gradients suggest a high adaptive potential of microbial communities to different salinities (Fear et al., 2005; Magalhães et al., 2005), even though Fear and co-workers showed that the variability in denitrification rate data increased as salinity increased, potentially reflecting the varying impact of activity within different microbial communities. The seemingly discrepant outcomes of these studies are potentially related to differences in microbial adaptation, and will be further elaborated within this thesis.

1.3 Scope and framework

The stratified habitats investigated in this thesis include microbial mats, artificial microbial aggregates and intertidal sand flats. The stratification of these systems is driven by different physico-chemical gradients, which define the basic conditions and zonations for various nitrogen cycling processes.

The first presented study (Chapter 2) is a comparative study on four hypersaline microbial mats with the goal to elucidate the importance of aerobic ammonia oxidation within these systems, as high salinities are considered to be inhibitory to nitrification. Biogeochemical gradients associated with stratification within microbial mats are more complicated than observed within the aggregates examined in Chapter 3. Many redox reactions occur within these mats, driven by various functional groups of microorganisms, which interact with each other according to redox-potentials or metabolic zones as described earlier (section 1.1.). The occurrence of nitrification within these microbial mats could be considered as a trade-off among the accessibility to oxygen towards the surface, the avoidance of light from above, and the avoidance of the inhibiting sulfide from below. Meanwhile, the presence of a strong oxygen gradient permits the concurrence of reductive nitrogen transformations in close proximity within the mats, which might complicate the detection of nitrifying activities. Hence, ^{15}N-labeled substrates were used as tracers to determine nitrification rates within various hypersaline mats, which were further corroborated by the detection of the active microbial players (ammonia-oxidizing bacteria and archaea) involved via their signature functional genes.

The studied microbial aggregates (Chapter 3) are the least complex of the three systems examined. The aggregates were taken from model upflow-sludge blanket reactors fed under anoxic conditions with either saline or non-saline nitrate-rich solutions in order to facilitate growth and activity of denitrifying microbial communities. Stratification was therefore mainly characterized by strong gradients in substrate availability and salinity. The degree of

stratification, however, differed between the saline and non-saline grown aggregates – the former appeared as dense granules with more pronounced stratification, while the latter formed flocks and were thus less clearly stratified. Consequently, the degrees of nitrate and substrate penetration into the two types of microbial aggregates also differed. In general, salinity affects ionic composition, specific gravity, surface tension, specific heat, viscosity, conductivity, pH, gas solubility, gas diffusion coefficients and water activity (Javor, 1989; Garcia-Pichel et al., 1999; Kohls, 2010). Some of these changes may further affect the resident microbial communities by causing e.g. community shifts (Bebout et al. 2002) and changes in microbial diversity (Abed et al., 2007), which in turn may induce changes in activity. The effect of salinity on the denitrifying activity of saline and non-saline grown microbial aggregates is one aspect studied within the frame work of this thesis, in order to find out how salinity influences complete denitrification, and nitrate reduction to nitrite. Both processes are important from an applied point of view with respect to desalination based wastewater treatment. Hence, incubation experiments with ^{15}N-labeled substrates were used in combination with membrane inlet mass spectrometry (MIMS) to determine N-cycling rates in the saline and non-saline grown aggregates. These were accompanied by corresponding nitrate and nitrite concentration measurements, which gave an insight into salinity-dependent nitrate consumption and nitrite accumulation during denitrification. Additional microsensor measurements showed the detailed stratification of N-cycling processes in saline grown aggregates.

The third study (Chapter 4) investigated the impact of O_2 dynamics on N-loss by denitrification in permeable marine sediments of the Wadden Sea. While some environmental studies suggest possible occurrence of denitrification in the presence of O_2 (Carter et al., 1995; Bateman and Baggs, 2005; Rao et al., 2007), denitrification is still largely considered an exclusively anaerobic process in marine sediments controlled by the depth of the vertical oxygen gradient (Brandes et al., 2007). High substrate input rates would facilitate denitrification, but

tidally induced changes in oxygen penetration depth, would be expected to inhibit denitrification under oxic condition. In contrast, laboratory studies showed that denitrification occurs under aerobic conditions in pure cultures of bacteria (Robertson and Kuenen, 1984; Robertson et al., 1995). Hence, the potential occurrence and rates of denitrification under oxic conditions were determined using multiple experimental set-ups, including ^{15}N-isotope pairing experiments in intact core incubations, slurry incubations, a flow-through stirred retention reactor and microsensor profiling.

Chapter 2

Aerobic ammonia oxidation in hypersaline microbial mats

Contributions to this chapter

Concept by O. Svitlica

Experimental specification by Olivera Svitlica with input of R. Amann, P. Lam and G. Lavik.

Practical work by O.Svitlica with help of N.Rödiger (co-work on clone libraries) and support of technical staff and student assistants (Hiwi).

Data analysis by O.Svitlica with support of G. Lavik and P. Lam (phylogenetic tree in co-work with P. Lam).

Writing by O. Svitlica with editorial help of G.Lavik and P. Lam.

Aerobic ammonia oxidation in hypersaline microbial mats

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Abstract

Aerobic ammonia oxidation plays a central role in global nitrogen cycling. It can be found in a wide variety of environments, but excluding in many cases hypersaline habitats such as microbial mats. Aerobic ammonia oxidation was studied in four different hypersaline microbial mats in order to verify the presence or absence of aerobic ammonia oxidation within these systems. Sequence analyses of ammonia monooxygenase gene subunit A (*amoA*) showed the presence of ammonia-oxidizing microbial communities in three out of four microbial mats. They were dominated by *Nitrosospira*-like sequences (*β*-proteobacteria), while ammonia-oxidizing archaea were only detected in one mat, and γ-proteobacterial ammonia-oxidizers were not detectable at all. Incubation experiments with ^{15}N-labeled substrates provides unequivocal evidence for the occurrence of ammonia oxidation within all four microbial mats, and the average measured rates ranged from 0.6 to 1.7 nmol g^{-1} d^{-1}. Corresponding net NOx consumption rates were 2 to 3 orders of magnitudes greater than ammonia oxidation rates, showing that nitrate reduction by far exceeds the NOx production in all mats. This suggests that nitrification provided only a minor part of the NOx consumed within microbial mats and there had to be additional nitrate supply from the overlying water. However, ammonia oxidation rates could also be underestimated due to the concurrent N-cycling processes, which would need to be measured for a full assessment on the actual contribution of various NOx sources within these microbial mats.

1 Introduction

Hypersaline microbial mats can be characterized by a high microbial diversity (Ley et al., 2006), but at the same time microbial mats in general are known to be dominated by only a few functional groups of microbes mainly driving the primary production, and carbon- and sulfur cycling of the mat (Van Gemerden, 1993; Kohls, 2010), resulting in steep vertical gradients of oxygen and sulfide (Van Gemerden, 1993). These gradients undergo diel cycles in oxygen and sulfide, as photosynthetic production increases the oxygen penetration during daytime, while anoxic conditions predominate at night due to continued activity of heterotrophic microorganisms (Revsbech et al 1983; Van Gemerden, 1993). Many biogeochemical studies in microbial mats conducted so far have focused on carbon and sulfur cycling (Jørgensen et al., 1979, 1983; Revsbech et al., 1983; Canfield and DesMarais, 1991, 1993; Jørgensen, 1994; Van Gemerden, 1993; Conrad et al., 1995; Teske et al., 1998; Ludwig, 2004). With respect to nitrogen cycling, many focus almost exclusively on N_2-fixation, the conversion of atmospheric N_2 gas into bioavailable ammonium, by diazotrophic cyanobacteria (Bebout et al., 1987, 1993; Paerl et al., 1993, 1996; Steppe et al., 1996; Currin and Paerl, 1998), even though a broad range of N-transformations, including denitrification and nitrification, can also occur within these mats (Van Gemerden, 1993; Bonin and Michotey, 2006).

Nitrification is a two-step process performed by distinctly different microbes. The first step is the aerobic oxidation of ammonia to nitrite. It can be performed by some members of β-*Proteobacteria* belonging to the *Nitrosomonas* and *Nitrosopira* clade, members of γ-*Proteobacteria* (so far represented by only three *Nitrosococcus spp*.) (Campbell et al., 2011), as well as *Thaumarchaeota* (Könneke et al., 2005, Francis et al., 2005, Brochier-Armanet et al. 2008). Ammonia oxidation is the step that oxidizes nitrogen from its most reduced form to NO_2^- (Herbert, 1999). The second nitrification step is the oxidation of nitrite to nitrate (the most

oxidized form) mediated by nitrite-oxidizing bacteria belonging to the genera *Nitrobacter*, *Nitrospira*, *Nitrococcus*, *Nitrospina* and *Nitrotoga* (Herbert, 1999; Alawi et al., 2007).

Nitrification is often inhibited by light (Vanzella et al., 1989; Guerrero and Jones, 1996a,b; Merbt et al., 2012) and sulfide (Kaplan, 1983; Joye and Hollibaugh, 1995). Consequently, the potential occurrence of nitrifiers within microbial mats is a trade-off between the accessibility to sufficient oxygen concentrations, a minimum exposure to light towards the upper layers, while avoiding the sulfide from below. For hypersaline microbial mats, the ability of nitrifiers to compete with phototrophs for ammonium was described as rather low. De Beer (2001) was assuming a generally higher substrate affinity of phototrophs over nitrifiers, stating that the lowest reported *Km* (Michaelis-Menten saturation constant) for nitrifiers is 40µM and for phototrophs 2µM. This general assumption is contradicted by *Km* values found for nitrifiers from oligotrophic open ocean and lake environments ranging from 0.1-5µM (Olson, 1981; Koops and Pommerening-Roser, 2001), and especially by the more recent study of Martens-Habbena and colleagues (2009) showing a *Km* value of 133nM of total ammonium for the ammonia oxidizing archaeon *Nitrosopumilus maritimus.*

Because of the low energy yields of both nitrification steps ($\Delta G°$ in kJ per reaction = -278 and -82 respectively for ammonia- and nitrite- oxidation, Lam and Kuypers, 2011) relative to the high energy demands for CO_2 fixation, nitrifiers grow rather slowly. Under hypersaline conditions, additional energy is required for osmoregulation strategies, which include either the synthesis of compatible organic solutes or the maintenance of cellular salt concentrations (mainly potassium chloride) in order to provide osmotic balance with the outside medium (Oren, 2001,2002).

In the hypersaline Mono Lake, ammonia oxidation was examined by comparing dark $^{14}CO_2$ fixation rates in the presence or absence of methyl fluoride, an inhibitor of NH_3 oxidation, and the corresponding organisms have been detected as well via 16S rRNA sequencing (Joye et al.,

1999; Ward et al., 2000). Nitrate turnovers have also been reported in solar salterns at a salinity of 37% (Joint et al., 2002). Any NOx resulting from nitrification might be assimilated to organic matter and eventually recycled back to ammonium, or directly reduced by DNRA, or lost as gaseous products N_2 (and N_2O) via anammox or denitrification. Such NOx consumption might partially explain why nitrification is often subject to speculations upon its presence or absence in microbial mats (DeBeer et al., 2001; Hinck et al., 2007; Joye and Paerl, 1994).

The aim of this study was to verify the occurrence of aerobic ammonia oxidation within different hypersaline microbial mats, excluding potential artifact rate measurements based on seawater diffusion into the mat. Furthermore, ammonia oxidation rates were directly measured by assessing the production of ^{15}N-stable isotope labeled nitrite from incubation experiments with ^{15}N-labeled ammonium. In parallel, the responsible archaea and bacteria in ammonia oxidation were detected and identified via analyses of ammonia monooxygenase gene subunit A (*amoA*), a biomarker functional gene of this process, in order to get an insight into the diversity of the ammonia-oxidizing communities within microbial mats.

2 Material and methods

2.1 Origin of mat samples

Microbial mats were collected from Australia, Brazil and the United Arab Emirates. They were all characterized by a cynoabacterial top-layer, and they were all exposed to hypersaline conditions (Lovelock et al., 2009; Kohls et al., AlNajjar et al. 2009, Vasconcelos et al., 2006).

The cynaobacterial mat, further assigned with an "A", was collected in October, 2008 in Exmouth Gulf, Western Australia. It is moist during spring tides (>2.4m), receiving only about 10 days a year tidal water (Lovelock et al., 2009; Personal communication A. Grinham, co-author of Lovelock). The mat is mainly exposed to hypersaline conditions, but salinity varies greatly. On-site seawater salinity is 35‰, and therefore the lowest salinity the mats are exposed

to, excluding very seldom events of rainfall. When they dry out, salinity increases far above 50‰ until the water is fully evaporated, resulting in a surface salt crust. It was kept after collection most of the time in an aquarium at 26° C without water. North Sea water with a salinity of 33‰ was used to mimic tidal regime as given at the sampling site (Lovelock et al., 2009; Personal communication A. Grinham, co-author of Lovelock; AlNajjar et al., 2011).

The cyanobacterial mat, further assigned as "V", was collected in September 2007 from an intertidal flat near Saadiyat Island (United Arab Emirates). It was kept similarly as mat "A", but it was supplied with water ones a week, which evaporated fully from the aquaria, forming a salt crust.

The cyanobacterial mat, further assigned with a "P", was collected in 2008 in Lagoa Vermelha, a small shallow hypersaline lagoon showing semi-aride characteristics, located 100 km eastern of Rio de Janeiro (Vasconcelos et al., 2006). Salinities were ranging up to hypersaline conditions. It was kept under the same conditions as mat V.

The cyanobacterial mat, further assignd with a "K", was collected from an intertidal zone during low tide in December 2006 in Abu Dhabi (Arabian Gulf coast, United Arab Emirates) (Kohls et al. 2009). The ambient salinity varied between 6 and 22%, due to tidal fluctuations. The mat pieces were kept at 26 °C. North Sea water was adjusted to 10% salinity with NaCl. The salinity was ranging from 10% to max. 25% (Kohls et al., 2009).

2010/2011 was the keeping of the mats unified. They all maintained in the 26 °C room. North Sea water was added to them on a weekly basis. It evaporated inducing hypersaline conditions and salt crusts. Afterwards was distilled water added in order to dissolve the salt crusts and mimic tidal regime, as described before. The North Sea water was replaced every 4-6 weeks. Due to remaining bits of salt crusts was the average salinity increased after fresh North Sea Water supply to approximately 4-6%. All mats were therefore exposed to salinities of at least 4%, increasing during the week up to hypersaline conditions, and finally salt crust formation.

The only mat which was kept mainly dry, according to the prior description was mat A, as it turned out to decompose, if exposed to water on a weekly basis throughout the year.

Detailed information about the characteristics of the exact sampling location can be found in previous publications and references within (Vasconcelos et al., 2006; AlNajjar et al., 2009, 2011; Kohls et al. 2009; Lovelock et al., 2009).

2.2 Incubation and sampling

Hypersaline microbial mat pieces of 4 different locations were cut in half. To assess the possible influence from nitrifying microbes from the added seawater on the respective mats, one half was pre-incubated for 45 h in sterile-filtered (0.22 µm pore-size) seawater and the other half in non-filtered seawater. The salinity of the water increased to ~ 6% after addition of the mat pieces, due to the salt on the mats. Subsequently, the mats were cut into mm sized pieces, in order to increase the oxygen penetration into the mat tissue, and incubated in 400 ml sterile filtered seawater (15.8 µM NO_3^- concentration) for about 24 h.

As substrate for nitrification, ^{15}N-labeled ammonium was added to an end concentration of 100-200µM. Concentrations per gram of mat-sample are shown in Table 1. Less ammonium was mistakenly added to two of the duplicate experiments, kfd (approx. 10µM) and vfd (approx. 0.3µM). The mats were incubated in the dark at 26° C for up to 96 hours. 8 to 12 samples were taken to monitor the changes in NOx (nitrate and nitrite) concentrations and the production of $^{15}NO_2^-$ of each incubation (Table 2). At each time interval 2ml water samples were taken from each incubation and frozen at -20 °C until analyzed for nitrite and nitrate. Additional 6-ml samples were taken from each experiment and transferred into gas-tight exetainers (Labco, UK) without headspace with addition of 100µl of saturated mercury chloride solution. These exetainers were stored at 26 °C (ambient RT) until further stable isotopic analyses. In addition, control incubation experiments were performed by incubating and sampling filtered (negative control) and non-filtered seawater (positive control), as described for the mat incubations.

Table 1: Average concentration and standard deviation (SD) of $^{15}NH_4^+$ in microbial mat incubations.

Microbial mats	µM $^{15}NH_4^+$, average	µM $^{15}NH_4^+$, SD
All A mats	19.09	2.61
All P mats	21.58	0.95
Three V mats	11.52	1.89
vfd mat	0.02	
Three K mats	24.9	1.65
kfd mat	1.15	

Table 2: Hourly displayed sampling frequency over 5 days for the specific incubations. **Inc. 1 =** sampling time of mat incubations / [2] sample only taken for P-mat; **Inc. 2 =** sampling time of replicate mat incubations; **Sw. inc.** = sampling time of seawater incubations.

day	Inc. 1 [h]	Inc. 2 [h]	Sw. inc. [h]
1	0	0	0
	1	3	4
	[2]		8
	5		
	9		
2	23	18	22
	34	29	34
3	47	41	47
	58	53	58
4	72	66	72
	83		
5	95	89	96

2.3 ^{15}N-stable isotopic analyses

Aerobic ammonia oxidation was measured as the production of ^{15}N- labeled NO_2^-. The converted nitrite was reduced to ^{15}N labeled N_2 upon addition of sulfamic acid, as follows: 1ml of the 6 ml-samples was discarded in order to set a Helium headspace. 50µl sulfamic acid was added into each gas-tight exetainer with a gas-tight syringe, shaken vigorously and incubated for 12 hours at 20 °C. This was followed by the addition of 40µl of 6M NaOH, in order to neutralize and thus terminate the sulfamic acid reaction. The efficiency of the sulfamic acid reaction was checked with a standard (15µM labeled nitrite was added to filtered sea water in an exetainer).

The formation of $^{29}N\text{-}N_2$ was measured on an Gas Chromatography Isotope Ratio Mass Spectrometer (GC-IRMS) (VG Optima, Manchester UK) after the conversion of nitrite to N_2 by sulfamic acid (Granger et al., 2009) following the modified protocol for labeling experiments (Füssel et al, 2011).

2.3.1 Correction of ^{15}N-nitrite measurements

High net NOx consumption, which occurred in most incubations made a linear correction of the $^{15}NO_2^-$ production rates necessary. The proportion of net NOx consumption relative to 0h was added to the measured $^{15}NO_2^-$ at each time point. Sampling induced volume reduction has also been taken into account. Linear increase in $^{15}NO_2^-$ was observed over 18-64 hours, and has been taken into account for rate calculations including a linear regression analysis (Excel Linest Array function) providing amongst other the standard error and the R^2 of the rate.

2.4 NOx (nitrate + nitrite) and nitrite measurements

NOx was measured following the approach of Braman and Hendrix (1989), which is based on the reduction of NOx to nitric oxide in an acidified Vanadium (III) chloride solution followed by a chemiluminescent reaction with ozone (Cox, 1980). Nitrite was measured following the approach of Dunham et al. (1995), which is based on an acidified iodide conversion of NO_2^- to NO. A CLD 60 NO/NOx analyzer (Eco Physics) was used for all measurements.

2.5 AmoA gene clone libraries

2.5.1.1 DNA extraction, PCR, Cloning and Sequencing

Approximately 0.2-0.4 mg mat sub-samples were taken for DNA extractions from the mat surface down to a depth of maximal ~ 3-4 mm. DNA was extracted with the bio101 FastDNA® SPIN Kit for Soil and the MoBio PowerBiofilm™ DNA Isolation Kit and cleaned prior further applications with the Promega Wizard® DNA Clean-Up System following the provided standard

protocols of the suppliers. PCR products were obtained with the 5Prime Master Taq Kit. The reaction mixture contained per 50µl reaction 0.5 Units Taq DNA Polymerase, 5µl 10x Taq Buffer with Mg^{2+}, 2.5 mM d'NTP´s, 50pM primer reverse and forward and 1-2µl DNA template (undiluted or 1/10 dillutions). Thermal cycling was carried out by an initial denaturation step at 95°C for 5 minute, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 53°C (exception: AmoA gamma proteobacteria were targeted with the *amoA*-3F and *amoB*-4R –primer pair with an annealing temperature of 50°C) and elongation at 72°C for 1min (McTavish et al., 1993; Rotthauwe et al., 1997; Alzerreca et al. 1999; Purkhold et al.,2000; Francis et al., 2005). Cycling was completed by a final elongation step at 72°C for 15 min. Table 3 shows the applied primer pairs.

Table 3: Applied primer pairs (McTavish et al., 1993; Rotthauwe et al., 1997; Alzerreca et al. 1999; Purkhold et al.,2000; Francis et al., 2005)

Primer	Annealing	Target	Sequence
amoA_Arch_F	53°C	Archaeal	STAATGGTCTGGCTTAGACG
amoA_Arch_R	53°C	Archaeal	GCGGCCATCCATCTGTATGT
amoA_Bac_F	53°C	Betaproteobacterial	GGGGTTTCTACTGGTGGT
amoA_Bac_R	53°C	Betaproteobacterial	CCCCTCKGSAAAGCCTTCTTC
amoA-3F	50°C	Gammaproteobacterial	GGTGAG TGGGYTAACMG
amoB-4R	50°C	Gammaproteobacterial	GCTAGCCACTTTCTGG

Triplicats of 50µl reactions were reduced in volume down to approximately 20 µl with a SpeedVac® Concentrator (Savant SpeedVac® Plus, SC110A) and applied to 1% Agarose gel with TAE buffer, cut out after Gel electrophoresis and dissolved in 50µl 70 °C warm PCR water. This mixture was used without further purification in the TOPO TA Cloning® Kit for Sequencing, following the suppliers protocol for performing a cloning reaction with chemically competent E.coli-cells with an amended increase of PCR-product (10µl instead of 2µl) and Salt solution (2-3µl instead of 1µl). One Shot® TOP10 and DH5α™-T1R Competent Cells were transformed following the suppliers protocol. They were spread at least in two quantities (60µl and 120µl) after transformation on 37 °C pre-warmed LB-Agar-plates with 100µg/l Ampicillin

and incubated overnight. Grown colonies were picked with sterile tooth picks and incubated overnight at 37 °C on 96 well plates filled with 100-120µl LB medium per well. 5µl per well were removed on the next day for inoculation of 96 Agar-well plates provided by GATC Biotech AG, Germany. The inoculation was incubated at 37°C overnight and sent out to GATC for sequencing.

2.5.2 Sequence analysis

Distance matrix files of the obtained archaeal and bacterial *amoA* gene sequences were created in ARB and exported to mothur (Open-source software for describing and comparing microbial communities), which was used to determine the number of operational taxonomic units (OTU) at various % sequence identity cut-off values for bacterial and archaeal *amoA* gene sequences (Schloss, et al., 2009). Additionally, it was used to determine representative sequences of each OTU.

Rarefaction analyses were also performed using the '*rarefaction.single*' command to generate intra-sample rarefaction curves using a re-sampling without replacement approach. Rarefaction curves are suitable to compare the richness observed in different samples, and to assess the coverage of sequencing efforts to sufficiently represent each clone library. The number of estimated OTU´s is shown as a function of the number of samples, which are in this case sequences. The last value on the x-axis is corresponding to the number of samples/sequences taken within this study. The coverage of sequencing is considered satisfactory when the rarefaction curve approaches a certain plateau or saturation.

Nucleic-acids based phylogenetic trees for archaeal and bacterial *amoA* gene sequences were constructed by using the ARB software package (Ludwig et al., 2004). The used database was updated prior calculations with all available *amoA* sequences from the public database until January 2012 (altogether ca. 25000 archaeal and ca. 15000 bacterial sequences). Calculated nucleic acid trees were gradually trimmed down to 75 - 80 sequences per tree, containing

representative sequences of the defined OTU´s (99% cut-off; mothur analysis used to define representative sequences) of the studied microbial mat, their closest relatives and representatives of selected major clusters from published studies. Maximum likelihood, maximum parsimony and distance matrix algorithms were used for tree calculations, and the branching patterns were cross-checked.

3 Results

3.1 Incubation experiments

A total of 18 experiments –16 with microbial mats and two controls without microbial mats – were performed to determine the potential for aerobic ammonia oxidation to nitrite in hypersaline microbial mats. They were collected from 4 different locations, namely A (Australia), P (Brazil), V (UAE, Saadiyat Island) and K (UAE, Abu Dhabi), mats were pre-incubated either with non-filtered seawater or sterile-filtered (0.2 µm pore-size) seawater, and subsequently incubated exclusively in filtered seawater.

The results of the microbial mat incubations show the same general patterns. NOx was rapidly consumed and in most experiments substantially depleted towards the end of the experiment (Fig. 1). Although nitrite constituted only a small portion of the NOx pool, a transient net accumulation was sometimes observed in the beginning <30 hours of incubation, after which it was again depleted (Fig. 2).

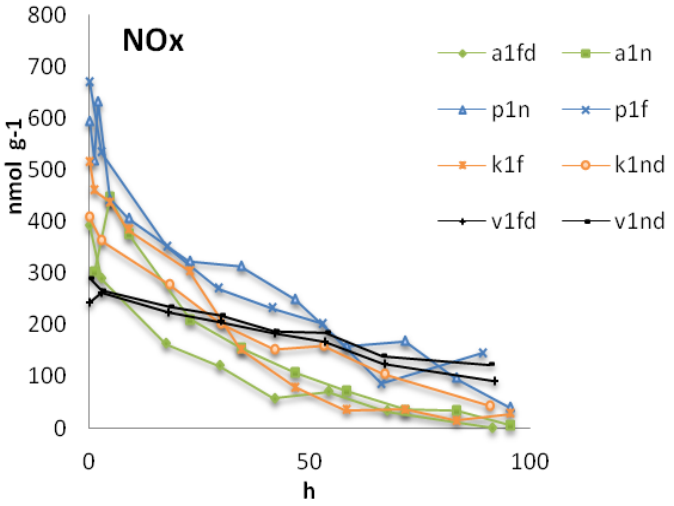

Figure 1: Concentration changes of non-labeled NOx over time in a representative filtered and non-filtered mat incubation of every microbial mat.

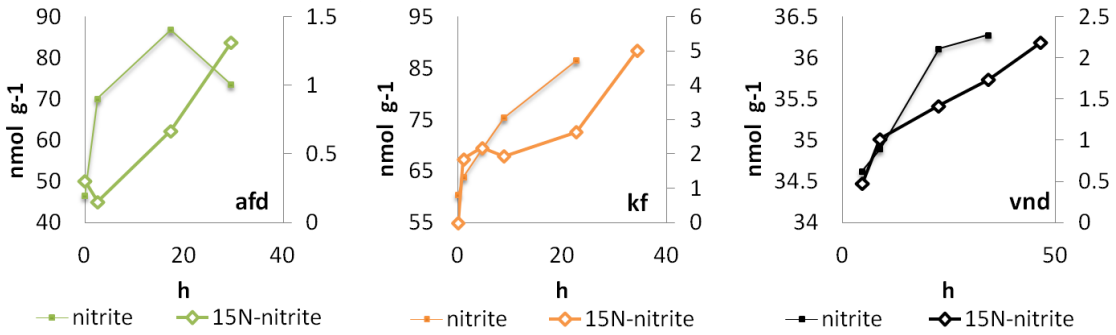

Figure 2: Concentration changes of non-labeled NO_2^- (primary axis) and ^{15}N labeled NO_2^- (secondary axis) over time in mat incubation afd (mat A, filtered), kf (mat K, filtered) and vnd (mat V, non-filtered), showing examples of transient NO_2^- accumulation.

The presence of ammonia oxidation was evidenced by the production of ^{15}N-labeled NO_2^- from incubations with ^{15}N-NH_4^+. All rates presented have been normalized by the weight of the mats and calculated when production remained linear with time (Table 4). The $^{15}NO_2^-$ accumulation rates have been corrected for the overall net NOx consumption in the experiments (Fig. 1). Additionally, a rate was measureable for the non-filtered seawater control incubation.

Overall, no systematic difference in nitrate reduction rates or $^{15}NO_2^-$ production rates could be observed between the filtered and non-filtered treatments (Table 4), hence all observed rates can be attributed to the activities of the respective microbial mats. Consequently, we can consider the four incubations with each type of mat as quadruplicates and combine the results (Table 4).

Table 4: Production or consumption (-) of NO_2^- and $^{15}NO_2^-$ in nmol g^{-1}*(of mat)* day^{-1} with standard error (SE) and R^2 calculated based on a regression analyses (Excel linest, array funtion) for four incubations of four microbial mats, respectively, including a mat specific average with standard deviation (SD). Nomenclature: a, k, v or p designates the respectiv mat; n =non-filtered, f=filtered; d=duplicate; sn= non filtered seawater.

Incubation	NO_x	SE	R^2	$^{15}NO_2$	SE	R^2
af	-275	40	0.92	2.61	0.39	0.92
afd	-174	32	0.91	1.46	0.32	0.87
an	-226	64	0.81	0.60	0.14	0.85
and	-292	79	0.93	1.98	0.15	0.99
average A	-242			1.66		
SD A	53			0.85		
kf	-215	13	0.98	3.86	0.70	0.86
kfd	-228	62	0.87	0.56	0.04	0.98
kn	-157	14	0.98	1.00	0.25	0.98
knd	-211	49	0.86	0.50	0.03	0.99
average K	-203			1.48		
SD K	32			1.60		
vf	-24	6	0.64	0.62	0.12	0.82
vfd	-44	3	0.98	0.11	0.02	0.83
vn	-34	7	0.70	0.99	0.07	0.97
vnd	-43	3	0.96	0.65	0.09	0.89
average V	-36			0.60		
SD V	9			0.36		
pf	-138	22	0.85	0.77	0.10	0.90
pfd	-197	39	0.86	0.63	0.17	0.78
pn	-285	85	0.74	0.38	0.11	0.74
pnd	-138	29	0.85	2.94	0.64	0.84
average P	-190			1.18		
SD P	69			1.18		
sn	-537	167	0.60	16.82	0.86	0.98

3.2 Analysis of amoA gene sequences

Sequences of the ammonia monooxygenase subunit A (amoA), a functional gene biomarker for aerobic ammonia oxidation, were detected in all mats except for mat V. Exclusively beta-proteobacterial gene sequences were found, while no gamma-proteobacterial sequences were found in any of the mat samples. Archaeal *amoA* gene sequences were found only in mat A. The rarefaction curves showed that the obtained sequences had relatively sufficient coverage for

ammonia oxidizing communities, but the diversity is rather limited, which is also reflected in the number of defined OTU´s at different identity cut-off values (Fig. 3, Table 5).

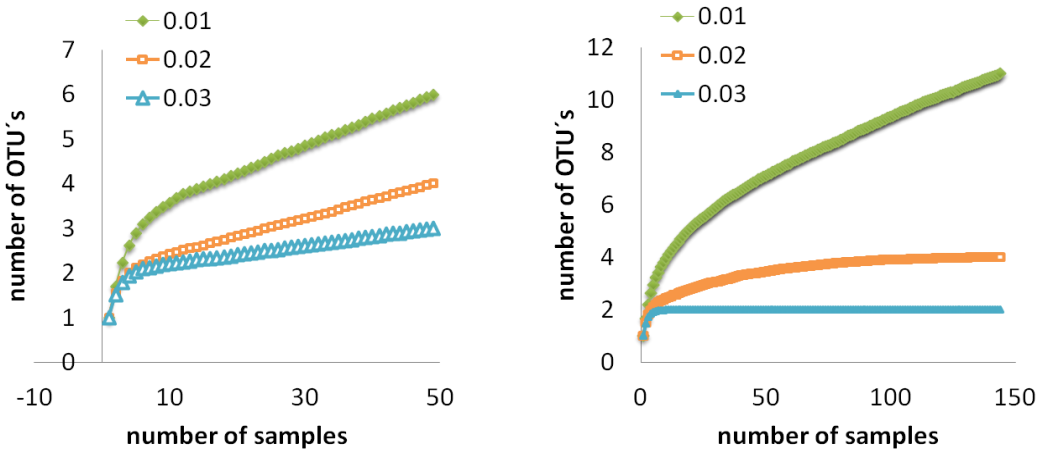

Figure 3: Nucleic acid based rarefaction curves for archaeal (a) and bacterial (b) amoA gene sequences at 99% (0.01), 98% (0.02), and 97% (0.03) cut-off.

Bacterial amoA gene sequences were subdivided into 11 OTU´s at a 99% identity cut-off, decreasing down to only 4 OTU´s at 98%, and only one OTU is left at 96% identity cut-off. For archaeal *amoA* gene sequences, only 6 OTU´s were determined at 99% identity cut-off, 4 OTU´s at 98% identity cut-off, and only one OTU remained at a fairly high 80% cut-off (Table 5).

Table 5: Number of OTU´s at different cut-offs for bacterial and archaeal amoA gene sequences.

Archaeal amoA gene		**Bacterial amoA gene**	
cut-off %	number of OTU´s	cut-off %	number of OTU´s
unique	32	unique	88
99	6	99	11
98	4	98	4
97	3	97	2
92	2	96	1
80	1		

The OTU´s obtained for 99% and 98% identity cut-off within bacterial *amoA* gene sequences were assigned to microbial mats, showing that mat P is characterized by a slightly higher *amoA* gene diverstity, than mat A and K (Table 6).

Table 6: Number of OTU´s at 98% and 99% cut-off for bacterial amoA gene sequences with the corresponding numbers of sequences per OTU (no. of seq/OTU) and the corresponding numbers of unique sequences per OTU (no. of seq/OTU) assigned to respective microbial mats.

All MAT (A+K+P)			**MAT A**		**MAT K**		**MAT P**	
cut-off %	OTU´s	Seq./OTU all	Seq./OTU all	Seq./OTU unique	Seq./OTU all	Seq./OTU unique	Seq./OTU all	Seq./OTU unique
99	1	62	31	6	11	7	20	20
	2	1					1	1
	3	1					1	1
	4	1	1	1				
	5	1					1	1
	6	18	5	3	4	4	9	6
	7	2					2	2
	8	1					1	1
	9	4					4	4
	10	10			9	3	1	1
	11	43	23	7	6	6	14	14
98	1	84	37	10	15	11	32	29
	2	2					2	2
	3	5					5	5
	4	53	23	7	15	9	15	15

The representative sequences of archaeal and bacterial *amoA* genes were displayed with representatives of their closest relatives and main known clusters in a “best out of 100” maximum likelihood phylogenetic tree searches (Figure 4, Figure 5). Archaeal microbial mat *amoA* sequences intermingle first of all exclusively with sequences from seawater, including Central Californian Current, Nambian and Peruvian oxygen minimum zones, East and South China Seas as well as North Pacific Subtropical Gyre (Figure 4) (e.g. Francis et al., 2005; Moraru et al., 2010; Hu et al., 2011, Santoro et al. 2011). Betaproteobacterial microbial mat *amoA* gene sequences cluster predominantly with marine and estuarine *Nitrosospira*-like sequences, which were mainly found in marine, estuarine and salt-marsh sediments, as well as the Arctic and Antarctic water columns (Figure 5) (e.g. Mosier and Francis, 2008; Kalanetra et al., 2009; Moin et al., 2009; Christman et al., 2011).

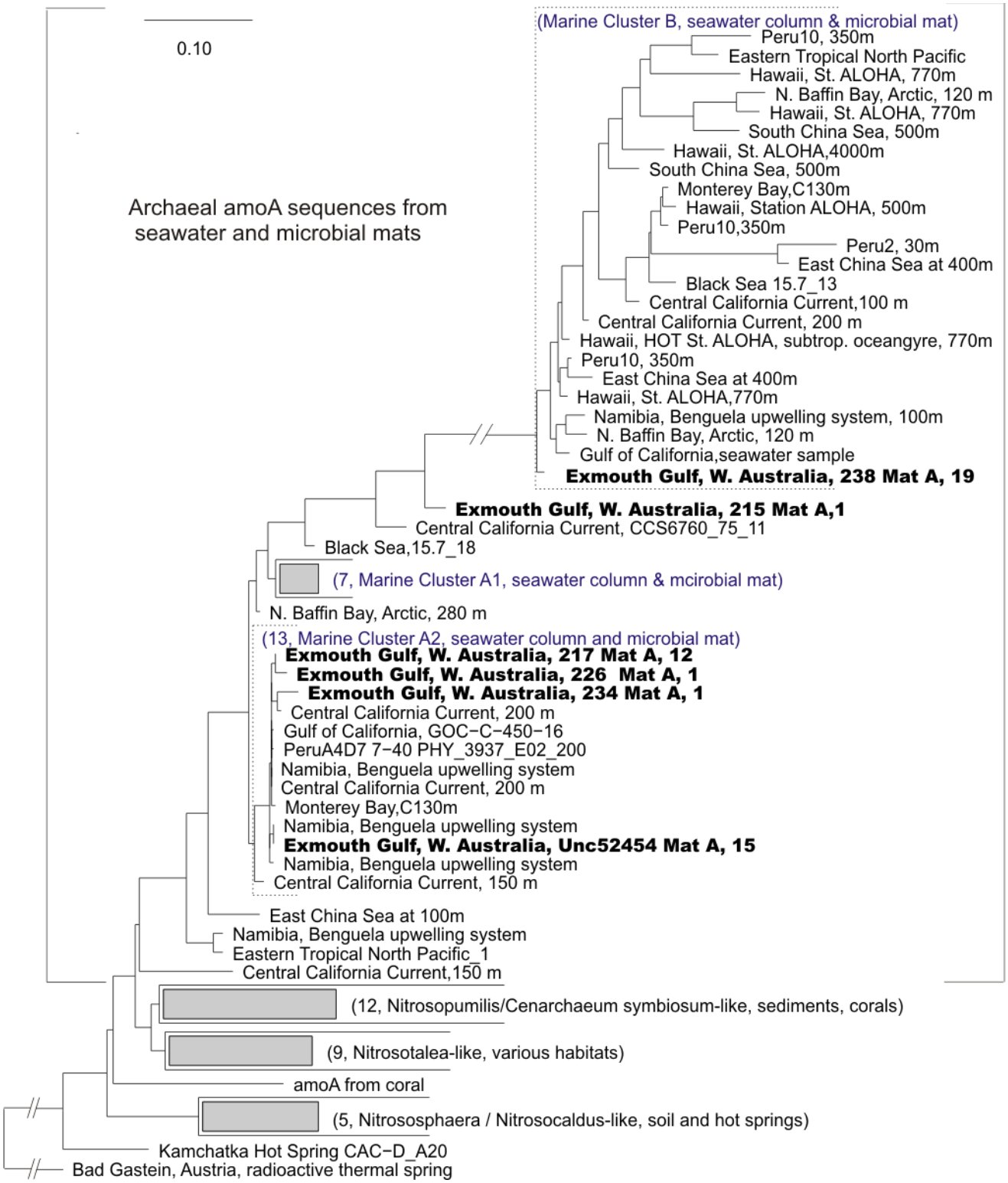

Figure 4: Phylogenetic relationships among archaeal amoA sequences from mat A (representative sequences in bold) and their closest relatives displayed in a maximimum likelihood "best out of 100"-phylogenetic tree. Numbers of sequences used in final tree calcuations are given in front of grouped sequences. Behind the representative microbial mat sequence is the number of corresponding clones per OTU shown (99% identity per OTU). The scale bar shows the substitution/site. Please note the breaks in scale for certain long branches, which accounts for the same length as given by the scale bar that shows the subtituiton/site.

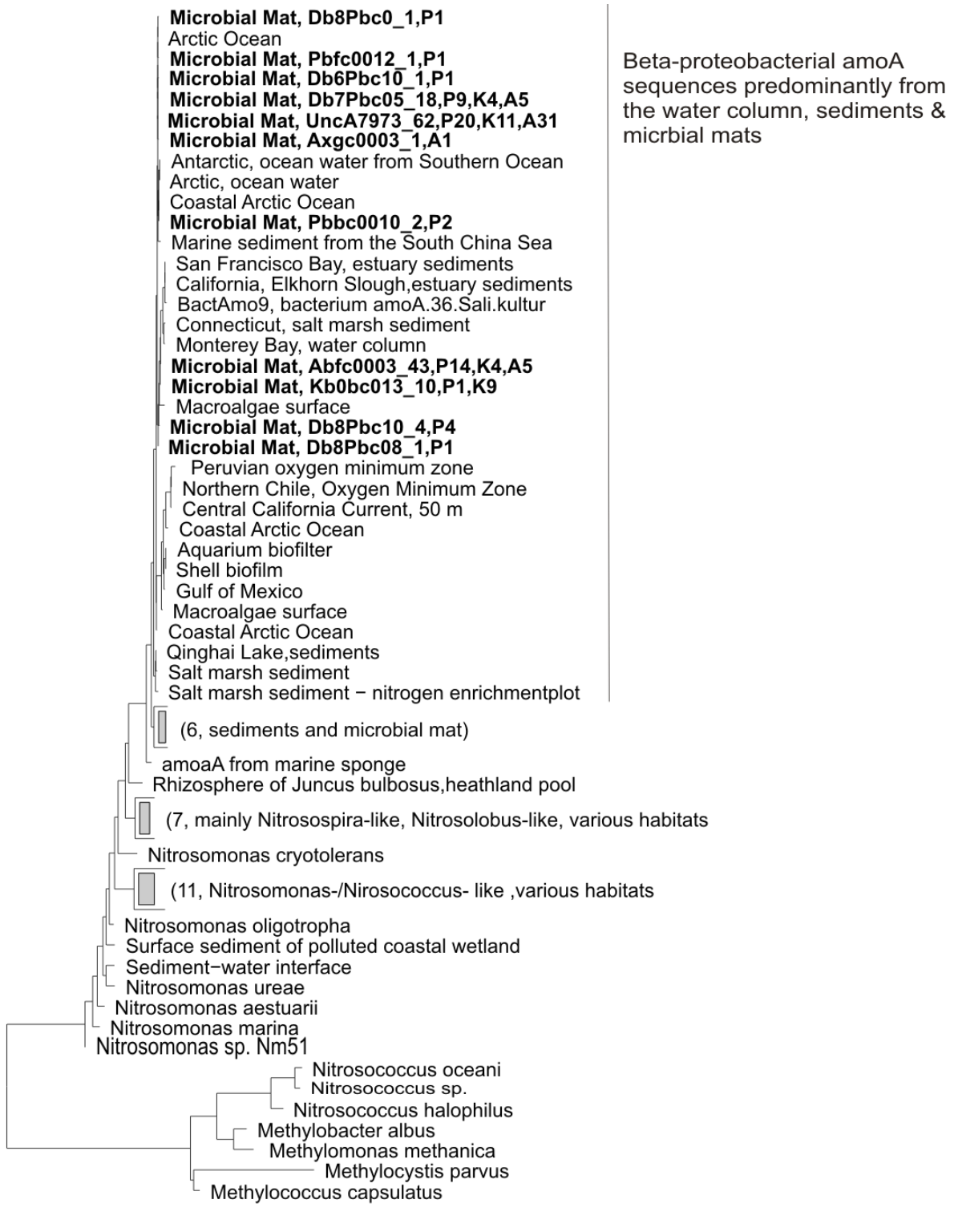

Figure 5 (previous page): Phylogenetic relationships among betaproteobacterial amoA sequences from mat A, K and P (representative sequences in bold) and their closest relatives displayed in a maximimum likelihood "best out of 100"-phylogenetic tree. Numbers of sequences used in final tree calcuations are given in front of grouped sequences. Behind the representative microbial mat sequence is the number of corresponding clones per OTU shown (99% identity per OTU) together with the correspondg microbial mat. The scale bar shows the substitution/site.

4 Discussion

Based on both, ^{15}N-incubation experiments and analyses of the biomarker functional gene *amoA*, results from the current study provide direct evidence for the occurrence of ammonia oxidation, the first step of nitrification, within hypersaline microbial mats. However, the measured rates in the studied mats are fairly low (Table 4), compared to rate measurements from other habitats. In aquatic sediment systems, ammonia oxidation rates range from 0.005-1600 µmol l^{-1} d^{-1} (Mortimer et al., 2004; Ward et al., 2008). Direct ^{15}N tracer based rate measurements in the stratified hypersaline Mono Lake, where salinity usually ranged between 68 and 79 g kg^{-1} throughout the water column, demonstrated that ammonia oxidation occurred with up to 480 nmol L^{-1} d^{-1} near the bottom of the oxycline (Carini and Joye, 2008). Our ammonia oxidation rates were considerably low in comparison (0.06 - 1.7 nmol g^{-1} d^{-1} in average), and fell within the lower range of oligotrophic seawater (1-10 nmol l^{-1} d^{-1}) (Clark et al., 2008). However, it should be noted that we compare here the activity of microorganisms distributed in one gram of microbial mat with the activity of microorganisms distributed within one liter of water.

Overall, net NOx consumption rates were 2 to 3 orders of magnitudes greater than ammonia oxidation rates determined via $^{15}NH_4^+$-incubations amongst all four microbial mats (Figure1, Figure 2, Table 4), implying that a considerable part of the produced nitrite was consumed inside the mat. In other words, our measured ammonia oxidation rates as $^{15}NO_2^-$ production would have likely underestimated the true ammonia oxidation rates *in situ*. In addition, high respiration and thus remineralization would release large amounts of non-labeled ammonium in the mat. Its subsequent oxidation would not be detected in the $^{15}NO_2^-$ pool, and so our measured ammonia oxidation rates would be further underestimated. It is impossible at this point of time to estimate how large the total underestimation of the nitrification rates is, without additional measurements of other internal as well as external fluxes. However, it is clear that the NOx reduction by far exceeds the $^{15}NO_2^-$ production in all mats. This implies that nitrification provided only a minor

part of the NOx consumed within the mat, and there had to be external supplies of NOx from the overlying waters.

Further support for ammonia oxidation activities in these hypersaline microbial mats came from the detection of ammonia-oxidizing bacteria and archaea, showing an ubiquitous distribution of betaproteobacterial *amoA* gene sequences within three out of four mats, but only one mat contained *amoA* gene sequences of archaeal decent. This observation corroborates with previous observations, showing a dominance of AOB over AOA in estuarine and coastal sediments (Caffrey et al., 2007; Mosier and Francis, 2008; Santoro et al., 2008; Magalhaeas et al., 2009), which might indicate that beta-proteobacterial AOB prevail in environments characterized by salinity fluctuations, as found here within the studied microbial mats. However, the salinity ranges within microbial mats up to hypersaline conditions, which is not the case within the above-mentioned estuarine and coastal sediments.

The closest relatives of the archaeal *amoA* sequences from mat A originated exclusively from seawater (Figure 4), and fell therefore into the water column cluster, as defined by Francis et al. (2005). Mat A, was the only mat in the experiments that was exposed to long drought periods to simulate *in situ* conditions (Lovelock, 2009).

The only source of ammonia oxidizing archaea was in the laboratory given by seawater, but archaea were not found in any other microbial mat. The establishment of archaeal ammonia oxidizers within this mat might be attributed to the long drought periods, which hamper fluxes of various substrates throughout the layers of a microbial mat. Hence, the effective ammonium concentrations available for ammonia oxidation within the layers of this particular mat might usually be lower than in the other mats. This lower ammonium availability may then explain, at least in part, the presence of archaeal ammonia-oxidizers, as they appear to be better adapted for low ammonium conditions, with a reported half-saturation constant of K m = 133 nM (Martens-Habbena et al., 2009).

The betaproteobacterial *Nitrosospira*-like amoA gene sequences seem to cluster predominantly with other *amoA* sequences of marine descent (Figure 5). In particular, they are related to sequences retrieved from marine, estuarine and salt-marsh sediments, as well as the Arctic and Antarctic water columns. However, the extremely low sequence diversity of their next neighbours does not allow a clear habitat based differentiation, as given for the archaeal water column cluster.

Nitrosospira-like sequences predominate many clone libraries from oceanic environments (Bano and Hollibaugh, 2000; O'Mullan and Ward, 2005), with many attribute this to a possibily higher potential by *Nitrosospira* to adapt to salinity than *Nitrosomonas*. In the eutrophic Schelde estuary, the AOB present were almost exclusively *Nitrosomonas*-like, but at more seaward sites, *Nitrosospira*-like sequences were also found (De Bie et al., 2001). Similarly, *Nitrosospsira* cluster 1-like sequences predominated marine sites in a Scottish estuary, while the brackish waters were characterized by the concurrence of the halophilic *Nitrosomonas marina*, the halotolerant *Nitrosomonas* sp. Nm143, as well as the *Nitrosomonas oligotropha* of freshwater origin, and the latter predominated freshwater sites (Freitag et al., 2006). In salt marsh sediments from New England, exclusively found were also *Nitrosospira*-like sequences (Moin et al., 2009). Hence, the above-mentioned studies seem to point to more commonly abundant *Nitrosospira*-like strains showing halotolerance or halophilia in marine and brackish habitats.

Nevertherless, *Nitrosomonas* isolates from the Elbe River estuary showed species dependent differences in halotolerance (Stehr et al., 1995). In an extreme case, the cultured isolate of *Nitrosomonas halophila* (formerly *Nirosococcus halophilus*) grows optimally at a salinity of ~40 ‰ and only stops growing when salinity reached values >90 ‰ (Koops et al., 1990). Previous molecular surveys in the hypersaline Mono Lake water column have also found the majority of ammonia-oxidizers to be *Nitromomonas*-like (Ward et al., 2000; Carini and Joye, 2008). Therefore, different *Nitrosomonas* species may be capable of adapting to a wide range of

salinity, while factors other than salinity might also have an impact on the distribution of *Nitrosomonas*-and *Nitrosospira*-like sequences in saline habitats. For example, in sediment samples from the Elkhorn Slough Estuary, *Nitrosospira*-like *amoA* gene sequences predominated when nitrification rates were high, while *Nitrosomonas*-like sequences were predominant when nitrification rates were low (Wankel et al., 2011). These observations suggest rather a substrate-based niche separation for the relative distribution of *Nitrosomonas* and *Nitrosospira* , instead of salinity being the primary or only structuring factor for the AOB community.

While the questions on which osmoregulation strategies ammonia oxidizing archaea and bacteria employ, or how they exactly meet the additional energy demand of osmoregulation with their normally meagre energy yields, remain to be further explored, our combined results clearly indicate that ammonia oxidation takes place within hypersaline microbial mats. Further ^{15}N-labelling experiments in the light, and in the dark, and with or without the redox gradients maintained (undisrupted mat) would be necessary to elucidate the relative importance of various nitrogen fluxes in the different layers of the hypersaline mats. Furthermore, rate measurements at different salinity levels would give more insight into the effects of tidally induced salinity changes on ammonia oxidation rates.

5 Summary and Conclusions

We could measure ammonia oxidation rates in all four hypersaline microbial mats that were further corroborated by the detected *amoA* genes as signatures for the presence of bacterial and archaeal ammonium-oxidizers. The ammonia-oxidizing communities were dominated by *Nitrosospira*-like species, while ammonia-oxidizing archaea were only detected in the mat that normally experienced longer drought periods, and γ-proteobacterial ammonia-oxidizers were not detected at all. The respective distribution of these ammonia oxidizers may reflect their differences in physiological adaptations, but further research is needed to assess their halotolerance under different salinity levels, including qualitative as well as quantitative analyses

of the gene abundance and expression of the individual groups, and experimentation with samples originating from habitats with different *in-situ* salinity ranges.

Overall, we have shown the occurrence of aerobic ammonia oxidation within hypersaline microbial mats carried out by unequivocally established nitrifying microbial mat communities, despite the unfavorable energetic considerations.

6 Acknowledgement

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Chapter 3

Effect of salinity on denitrification in saline and non-saline grown microbial aggregates

Contributions to this chapter

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Basic data analysis shared between O.Svitlica, P.Stief, M.Kuypers; refined overall data analysis by O. Svitlica with advice of G.Lavik on MIMS gas drift corrections.

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Effect of salinity on denitrification in saline and non-saline grown microbial aggregates

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Abstract

Microbial aggregates were grown under saline (salinity of 10‰) and non-saline conditions in upflow-sludge blanket (USB) reactors in order to assess the effect of salinity on denitrification ($NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$). Denitrification rates in saline grown microbial aggregates increased with increasing salinities, followed by a decrease down to ~3/4 of the original denitrification rate at 30‰ salinity. Intriguingly, the highest denitrification rates were measured at 16.5‰, 20.5‰ and 25‰ salinity, which are all higher than the growth salinity. Denitrification rates of non-saline grown aggregates decreased already at a salinity of 3.5‰ down to ~1/3 of the original denitrification rate. Nitrate reduction to nitrite was only minimally affected by salinity for either of the two aggregate-types, resulting in an accumulation of toxic nitrite when activities of subsequent reactions were reduced. Nitrite accumulated in non- saline grown microbial aggregates (73µM - 99 µM) more than in saline grown aggregates (34µM - 59µM). Its consumption decreased with increasing salinities in both aggregate types, but with a smaller rate in saline grown aggregates. The efficiency of denitrification was assessed by correlating the nitrite accumulation with denitrification, showing an antithetic effect of salinity on denitrification efficiency in saline and non-saline grown aggregates, respectively. Additionally, the surface of the microbial aggregates was identified as the source of the excess nitrite.

Consequently, the surface-to-volume ratio of the aggregates might have a regulatory effect on the imbalance of nitrite production and consumption, beside other factors.

Keywords: Denitrification, microbial aggregates, salinity, nitrite, nitrate

Nomenclature

ATU: Allylthiourea

FTSRR: Flow-Through Stirred Retention Reactor

MIMS: Membrane Inlet Mass Spectrometry / Spectrometer

NO_x: Nitrate + nitrite

RO: Reverse osmosis

SD: standard deviation

USB reactor: Upflow-sludge blanket reactor

1 Introduction

Increasing fresh water shortage in many parts of the world and salt water intrusions into freshwater systems, have increased the eco- and socioeconomic importance of desalination for potable water production. Countries that have reached the limits of their renewable water resources include Malta, Israel, Cyprus and Saudi Arabia (Molle and Vallee, 2009). One of the most common desalination methods is reverse osmosis (RO). Salts, viruses and microorganisms cannot pass the membrane, so they are removed from the clean water and enriched in the brine (a saline or hypersaline by-product). Nitrogen compounds, like nitrate and nitrite, also accumulate in the brine. The final concentration of nitrate depends on the water quality of the feed water and the type of membrane used. Cellulose triacetate RO membranes typically reject 50 –70% of nitrate, which is fairly low compared to thin film composite RO membranes, which show typically a rejection of 90-95% nitrate (Applied Membranes, Inc. 2007). Consequently, the use

of RO filters produces large quantities of brines (10-15% of the well water remains as brine) which are also highly enriched in nutrients like nitrate and nitrite. These nutrient-contaminated brines are currently mainly disposed in coastal waters, removed by deep-well injection in inland areas or disposed in evaporation ponds. However, such brine disposal may cause various environmental problems that are either related to eutrophication, to further salt intrusion into fresh water systems, or to the accidental release of toxic compounds, which are enriched in the disposed brine (Ahmed et al., 2001). The risk of eutrophication or the accidental release of toxic compounds into e.g. aquifers could be minimized by prior brine treatment. Future changes to water quality regulations may even impose an extensive treatment prior to disposal (Beliavski et al., 2010).

Nitrate and the more toxic nitrite could be removed from the brine by denitrification, a common way of nitrate and nitrite removal from waste waters ($NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$). However, the efficiency of denitrification may decrease with increasing salinity, and thereby pose problems for its application on the brine treatment. Several studies have demonstrated the effects of salinity on denitrification, but without consensus. Studies on activated sludge showed a negative correlation of denitrification and salinity within a range of 12‰ to 38‰ at pH 9 (Glass and Silverstein, 1999), and within a range of 0‰ to 60‰ salinity at a feed water pH of 7 (Dinçer and Kargi, 1999).

Similarly, a study on the influence of salinity on denitrification of sediments from a Mississippi River freshwater diversion site (Davis Pond, Louisiana) of a Louisiana estuary, also showed a negative correlation of denitrification rate and salinity, with salinities varying between 0‰ and 36‰ (Seo et al., 2008). The highest salinity tested in this study showed a decrease in denitrification rate down to 30.8% of its original activity (salinity 0%). On the contrary, other studies in estuaries reported an absence of a regulatory effect of salinity on denitrification rates (Fear et al., 2005; Magalhães et al., 2005). Denitrification rates in the eutrophic Neuse River

estuary, which had a persistent salinity gradient ranging from 0 to 20‰, showed that the variability in the denitrification rate data increased as salinity increased, but altogether, denitrification rates did not vary significantly across the salinity gradient (Fear et al., 2005). Denitrification rates in intertidal sediments and rocky biofilms of the Douro River estuary, exposed to variations in salinity between 0‰ and 35‰, showed that the "activity of denitrifying bacteria within intertidal sandy sediments and rocky biofilms was not influenced by the presence of sea salts, indicating that halotolerant denitrifying bacteria inhabit both environments" (Magalhães et al., 2005). These opposing observations of salinity-effects on denitrification rates emphasize salinity as a driving force in the selection on halotolerant and halophilic microbial communities.

Following these studies, the effect of salinity on denitrification was investigated in saline and non-saline grown microbial aggregates from upflow-sludge blanket (USB) reactors. In contrast to environmental samples from saline and non-saline habitats is the activity of denitrifying communities from the studied aggregates not influenced by tidal, seasonal or diel variations of oxygen or substrate gradients.

Incubation experiments with ^{15}N-labeled substrates were used in combination with membrane inlet mass spectrometry (MIMS) to assess N-cycling processes, in particular for denitrification rates from different salinities (0-30‰). Changes in nitrate and nitrite concentrations were measured along with denitrification rates, in order to evaluate the salinity effect on two different steps of denitrification, namely nitrate reduction to nitrite, and nitrate reduction to di-nitrogen gas. Additional microsensor measurements were performed to examine the detailed stratification of N-cycling processes within the saline grown microbial aggregates, which were forming granules.

2 Material and Methods

2.1 Origin of microbial aggregates and aggregate description

The experiments were carried out in October and November 2007 using microbial aggregates grown in two model USB reactors that were operated at the Faculty of Civil and Environmental Engineering at the Technion in Haifa, Israel. One reactor was operated under saline conditions, fed with a 1% saline solution consisting of tap water, 128.3 mM NaCl, 3.7mM $MgCl_2$, 5.1mM $CaCl_2$, and 0.1mM KH_2PO_4. The second reactor was operated under non-saline conditions, and it was fed with a solution consisting of tap water and 0.1mM KH_2PO_4. The flow rate in the USB reactors was 30 l d^{-1} with a loading rate of 9.52mMol d^{-1} $NaNO_3^-$ and 8mMol d^{-1} acetic acid as carbon source, which was added in stoichiometric amounts according to Equation1.

$$0.84\ CH_3COOH + NO_3^- \rightarrow 0.08\ C_5H_7O_2N + 1.3\ CO_2 + OH^- + 0.92\ H_2O + 0.46\ N_2 \quad (1)$$

The biomass concentration of the two aggregate types differed. The saline grown aggregates had approximately four-fold higher biomass content than the non-saline grown microbial aggregates (Beliavski et al., 2010). The pH was regulated with NaOH to approximately 7. The reactors were equipped with PVC tubing and intermittent stirring devices. They were operated at 25 °C with continuous flow using a peristaltic pump. The resultant aggregates were 1-3 mm in diameter. The auto-granulating brownish saline grown microbial aggregates were elliptically shaped granules and occupied approximately 60% (1.6-1.8 l) of the total reactor volume (2.8 l). The non-saline grown aggregates were whitish flock forming aggregates with less good settling characteristics and a higher washout rate compared to the granular microbial aggregates. The non-saline grown flocks occupied only 0.4-1.5 l, equivalent to approximately 15 -50% of the total reactor volume.

All experiments were carried out under nearly anoxic conditions.

2.2 Experimental Set-up

2.2.1 Flow-Through Stirred Retention Reactor (FTSRR)

Incubation Experiments were conducted in a custom-designed Flow-Through Stirred Retention Reactor (FTSRR), which allows continuous real-time measurements of denitrification via Membrane Inlet Mass Spectrometry (MIMS). The main polycarbonate chamber has an outlet which connects the reactor with a peristaltic pump and the membrane inlet of the MIMS. The filter which is attached to the punctured rubber stopper, avoids dirt being pumped into the Membrane Inlet. The plunger of the top-cut glass syringe allows volume and pressure compensation, when liquid is removed from the reactor or injected into the reactor. Liquid removal without pressure changes is possible as long as the plunger does not reach the surface of the perforated disc, below which a magnetic stirrer is placed.

Up to two glass syringes can be attached to the system. Microsensors and other devices can be attached by drilling additional holes through the rubber stoppers (Figure 1).

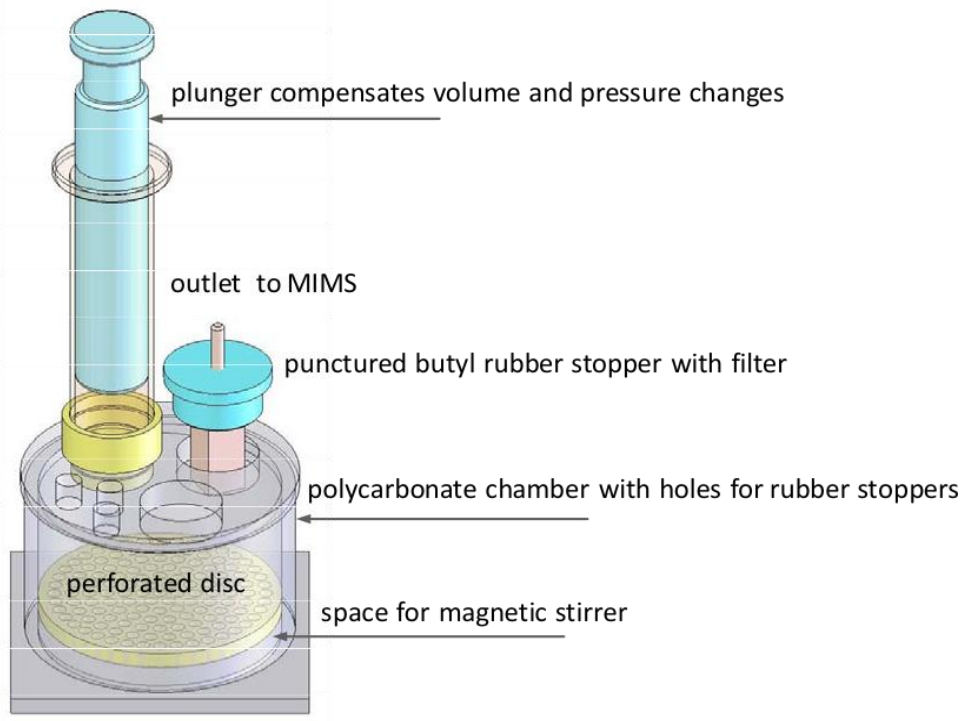

Figure 1: Flow-Through Stirred Retention Reactor (FTSRR).

2.2.2 Denitrification experiments at different salinity levels

Denitrification was determined with the ^{15}N-isotope pairing technique (Nielsen, 1992) by incubating saline or non-saline grown aggregates in an FTSRR, which was directly connected to a membrane inlet mass spectrometer (MIMS; GAM 200, IPI) (Gao et al., 2010, Corrigendum, 2011). 10 ml of settled aggregates and 10 ml of medium (depleted in electron- acceptor and –donor) were taken directly from the USB reactor and transferred into the FTSRR with 355ml fresh prepared medium , as used for the USB reactor (section 2.2.1.). The medium was purged with helium to remove oxygen.

^{15}N labeled $NaNO_3$ (~ 190 µM) and acetic acid (~ 160 µM) were added as electron-acceptor and -donor for denitrification. The labeled nitrate was reduced by the microbial aggregates to labeled N_2 and pumped from the FTSRR (Figure 1) into the membrane inlet mass spectrometer (GAM200, IPI) as described in Gao et al. (2010). ^{15}N-labeled nitrogen gas and other dissolved gas concentrations (of at least helium, oxygen, non-labeled dinitrogen gas, and argon) were determined from intensities of the mass spectrometer signals in the multiple ion detection mode (Kana, 1994) in the quadrupole ion trap of the MIMS. The salinity was raised by injecting saline medium through a rubber stopper into the reactor. The non-saline grown microbial aggregates were exposed to a stepwise increase in salinity up to a maximum value of 17‰ and the saline grown microbial aggregates were exposed to an increase in salinity up to a maximal value of 30‰. The reactor was kept under anoxic condition throughout the experiments. Denitrification rates were calculated based on the linear production of $^{30}N_2$ and $^{29}N_2$ excess (Nielsen, 1992).

2.2.3 Nitrate and nitrite measurements

NOx (nitrate + nitrite) samples were taken simultaneously during denitrification experiments (see section 2.2.2.) every five minutes. NOx concentrations were measured with a 42C Chemiluminescence NO-NO2-NOx Analyzer (Thermo Environmental instruments Inc.) following the approach of Braman and Hendrix (1989). NOx was reduced to nitric oxide in an

acidified Vanadium (III) chloride solution followed by a chemiluminescent reaction with ozone (Cox, 1980). Nitrite was measured using the method described in Dunham et al. (1995), which is based on a acidified iodide conversion of NO_2^- to NO.

2.2.4 The N-ratio – Evaluating the efficiency of denitrification

The efficiency of denitrification was further evaluated by calculating the N-ratio, which is defined as the ratio of two distinct concentrations of N-atoms. The concentration of N atoms converted to N_2 by denitrification at the point of time were the highest nitrite accumulation was measured is described in equation 2 as N_{N2}.The corresponding concentration of N atoms which were accumulated as nitrite is defined as N_{NO2-}.

$$\text{N-ratio} = N_{N2} / N_{NO2-} \qquad (2)$$

The higher the concentration of N-atoms derived from denitrification relative to the concentration of N-atoms derived from nitrite accumulation, the bigger the N-ratio. A ratio of 1 means equal concentrations of N-atoms from denitrification and nitrite accumulation. A ratio < 1 shows the presence of more N-atoms from nitrite accumulation than from denitrification. The smaller the N-ratio the higher the relative imbalance of nitrite production and consumption compared to the denitrification rate. This means that from an applied point of view, denitrification is inefficient and toxic nitrite is strongly accumulating when the N-ratio is small. A high N-ratio indicates efficient denitrification and low or non nitrite accumulation.

2.2.5 Microsensor experiments

Further incubation experiments were conducted with detailed microsensor profiling within saline grown microbial aggregates, in order to decipher the stratification of N-conversion activities.

The saline grown aggregates were embedded in modeling clay which was fixed to the bottom of a flow cell exposed to a laminar stream, which was driven by a submersible pump (Stief and

Eller, 2006). The microsensors were mounted on a motorized micromanipulator (MM 33, Märzhäuser, Wetzlar, Germany), which was automated by the software program µ-profiler (Polerecky et al, 2005). NO_x^-, NO_2^-, N_2O, and O_2 microsensors were used in experiments to determine high-resolution concentration profiles (Revsbech et al., 1989; Larsen et al., 1997; Andersen et al. 2001; Nielsen et al., 2004). The sensors were calibrated each day, before and after the measurements. NO_x biomicrosensors are sensitive to NO_3^-, NO_2^-, and N_2O (Larsen et al. 1997). Because NO_2^- and N_2O rarely exceeded concentrations of ~3 µM in the medium and in the aggregates, NO_x microprofiles are referred to as NO_3^- microprofiles henceforth. Each microsensor profile started at least 500 µm above the surface of a granular aggregate (i.e., in the artificial brine) and continued through at least half of the aggregate in 50µm steps. At least 10 data points were recorded at every depth.

During all measurements, the aggregates were exposed to the same medium as the reactor influent of the USB reactors, with adjustments in salinity (5‰ or 18‰) and addition of acetic acid as electron donor and sodium nitrate (final concentration approximately 40 µM) as electron acceptor for denitrification. The electron donor and acceptor were added approximately 30 minutes before microsensor measurements started.

Local rates were calculated for N_2O and NO_2^- concentration profiles in order to estimate the net production / consumption rate at each depth as described in Stief and De Beer (2002). Diffusion coefficients of 2.36 x 10^{-5} cm² s^{-1} for N2O and 1.91 x 10^{-5} cm² s^{-1} for NO_2^- at 25 ° C were assumed, respectively (Broecker and Peng 1974, Li and Gregory 1974).

Samples of the medium were taken during the measurements and analyzed for NOx (nitrate + nitrite) as described earlier (section 2.2.3.). The non-saline grown microbial aggregates were not suitable for microsensor depth profiles, because of their texture and their bad settling conditions. They were not granules, but soft flocks (easy to squash) with a high tendency to float.

2.2.6 Total sulfide, sulfate, ammonium concentrations and anaerobic ammonium oxidation

Total sulfide concentrations of samples from FTSRR incubation experiments were determined colorimetrically following the approach of Pachmayr (1960), as described by Trüper and Schlegel (1964). For the saline grown aggregates, the sulfate concentration of the in-and out-flow of the USB reactor was additionally determined using a Metrohm 761 ion chromatograph (IC) equipped with a Metrosep A Supp 5 anion separating column and suppressor using a CO_3/HCO_3 eluent accounting for the activity of 1.7 l microbial aggregates. Ammonium concentrations were determined colorimetrically for the outflow according to Willis et al., 1996.

Anaerobic ammonium oxidation (anammox) was evaluated within the same set-up as denitrification (section 2.2.1. and 2.2.2.) by adding 70 µM ^{15}N labeled NH_4^+ and 70 µM non-labeled NO_3^- without acetic acid, as electron-donor and –acceptor, respectively. The formation of labeled $^{29}N_2$ would indicate the presence of anammox.

3 Results

3.1 FTSRR based experiments

FTSRR based incubation experiments showed a pattern of salinity induced changes in aggregate denitrification. Denitrification rates and the salinity effects differed according to the origin of the aggregate-type. The rates of saline grown aggregates were less affected by an increase in salinity than non-saline grown aggregates. The highest rate (218 µM h^{-1} N2) for saline grown aggregates was measured at a salinity of 16.5‰, which is higher than the rate measured at their growth salinity 10‰ (186 µM h^{-1} N2). Increases in salinity up to 20.5‰ and 25‰ induced only slight decreases in denitrification rate, but a clear rate reduction at salinities of 5‰ (146µM h^{-1}) and 30‰ (146µM h^{-1}) (Figure 2).

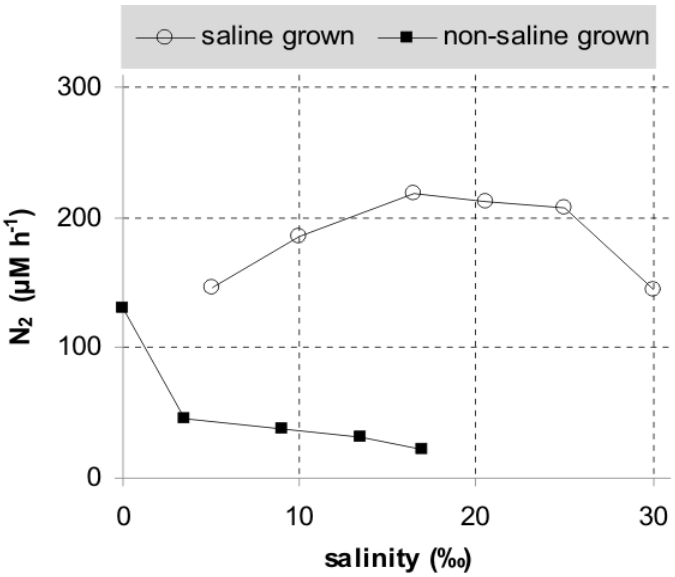

Figure 2: Denitrification rates of saline and non-saline grown aggregates as a function of salinity measured with an FTSRR attached to a MIMS. All rate measurements are based on at least 100 consecutive concentration measurements ($R^2 \geq 0.99$ in all cases, with one exception $R^2 = 0.98$).

The non-saline grown microbial aggregates show a strong decrease in denitrification rate with increasing salinity (Figure2). A mere increase of 3.5‰ salinity has already reduced the denitrification rate by 65%. The denitrification rate dropped from 131µM h^{-1} under non-saline conditions down to 22µM h^{-1} at 17‰ salinity. The lowest rates within saline grown aggregates make up for 79% and 78% of the original denitrification rate, whilst the lowest rate determined in non-saline grown aggregates made up for only 17% of the original denitrification rate within the tested salinity range.

Samples for nutrient analyses were taken during denitrification rate measurements and analyzed for nitrate and nitrite (section 2.2.3.). Both aggregate types consumed on average 555µM h^{-1} ± 33µM h^{-1} (mean ± SD) nitrate with all tested salinities (Figure3, Figure4). In contrast, nitrite measurements of saline and non-saline grown aggregates showed distinct differences upon increasing salinities. Nitrite concentrations in saline grown aggregates increased instantaneously when nitrate concentrations decreased (Figure 3). The highest nitrite concentrations in saline grown microbial aggregates varied between 34µM and 59µM, at

salinities of 16.5‰ and 5‰, respectively (Figure 3, Table S.1). These values were higher for non-saline grown microbial aggregates, varying between 73µM and 99µM (Figure 4, Table S.1).

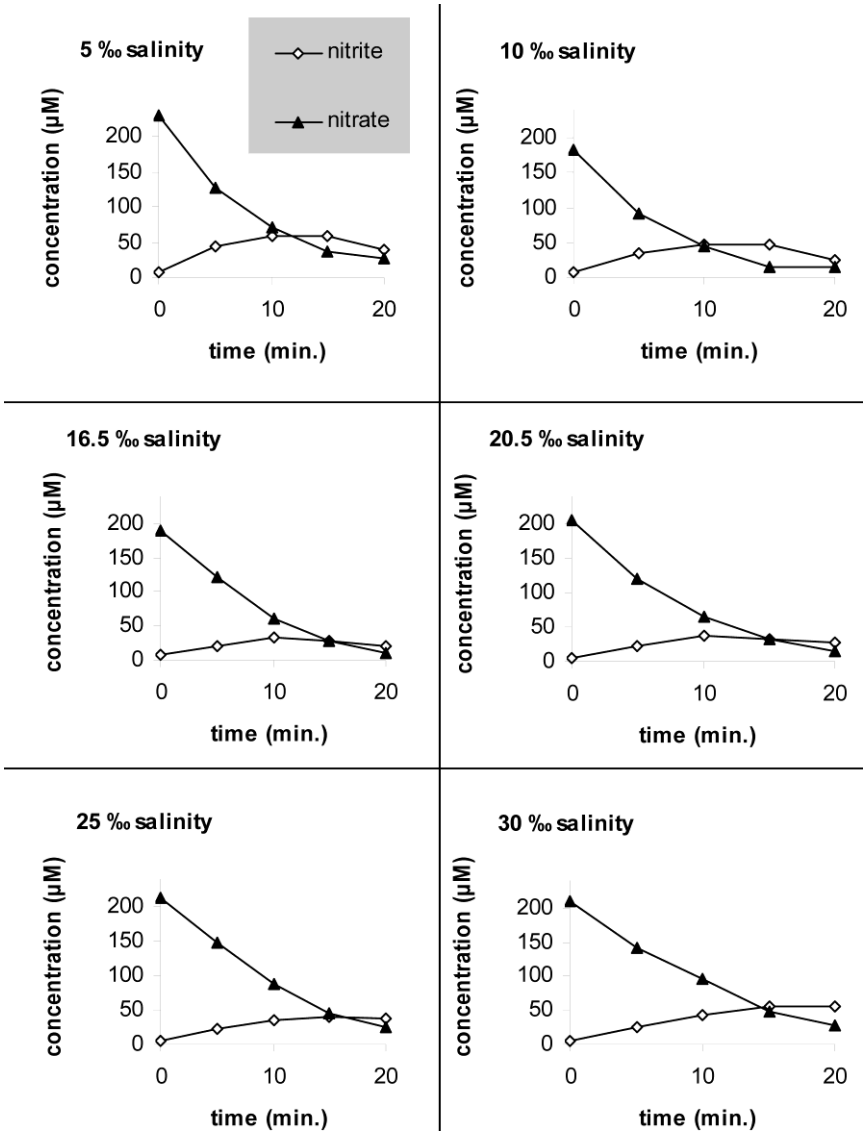

Figure 3 Time-series showing nitrate consumption and nitrite accumulation in saline grown microbial aggregates at different salinities.

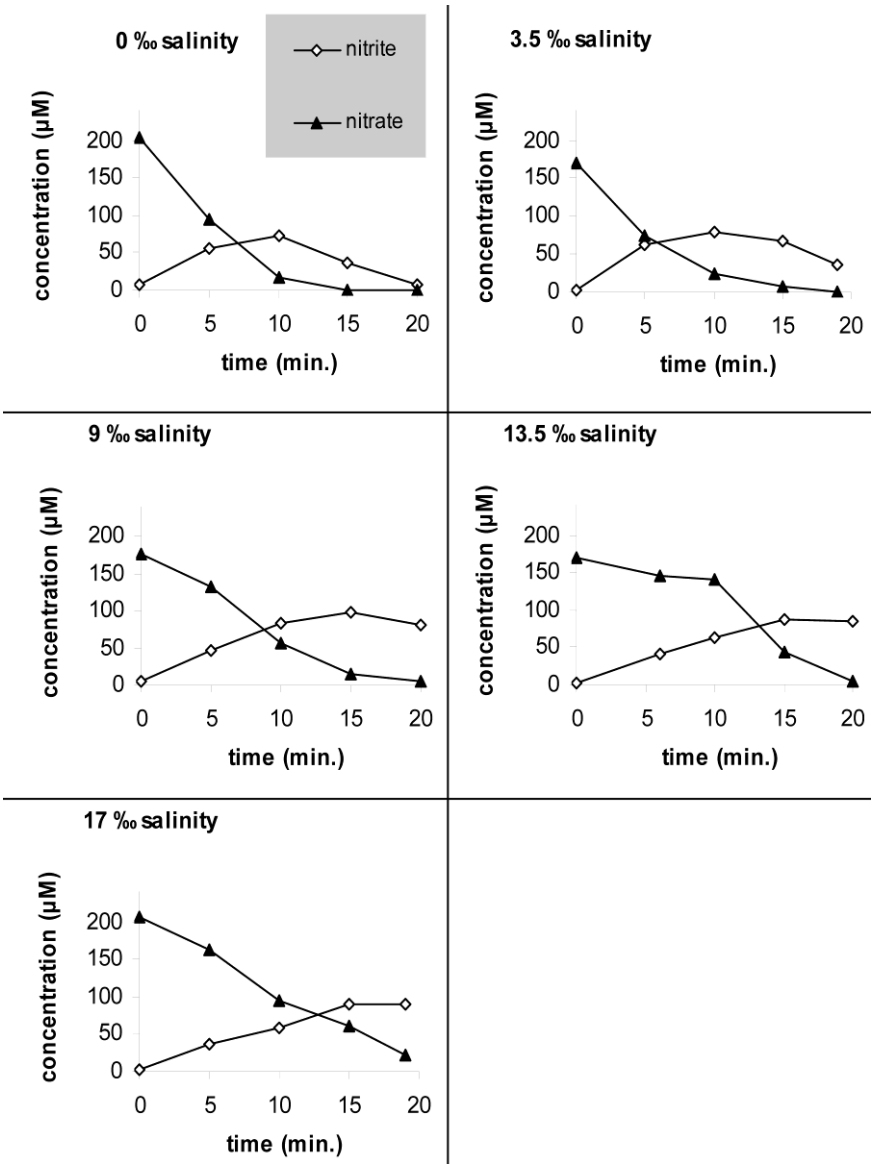

Figure 4: Time-series showing nitrate consumption and nitrite accumulation in non-saline grown microbial aggregates at different salinities.

However, the lower the salinity, the higher was the nitrite removal after nitrite accumulation in saline and non-saline grown microbial aggregates (Table S.1, Figure 5).

All N-ratios of saline grown microbial aggregates are higher than N-ratios of non-saline grown microbial aggregates (Figure 6). The N-ratio of saline grown aggregates is increasing with increasing salinities, and it decreased only at a salinity of 30‰ (Figure 6), which is three times higher than the growth salinity of the aggregates. The non-saline grown aggregates showed a decrease in N-ratio to a third of the original value after a first addition of salt (3.5‰ final concentration). Further increases in salinity caused smaller decreases of the N-ratio (Figure 6).

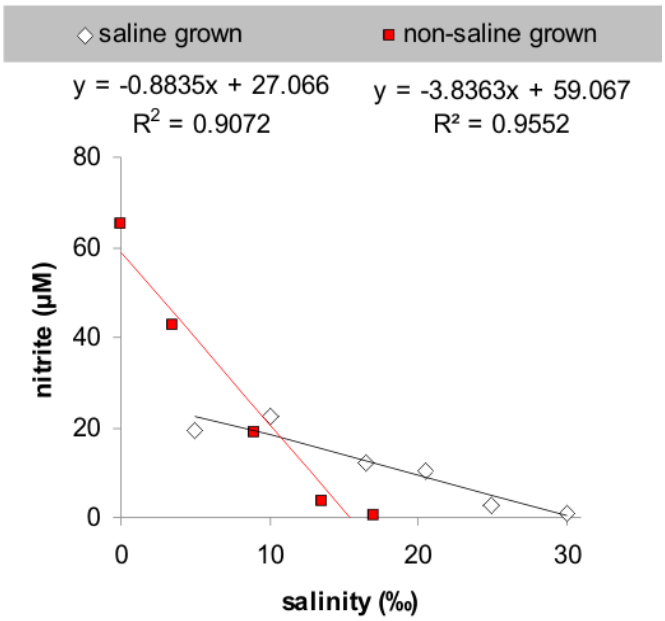

Figure 5: Nitrite consumption after accumulation shown for saline and non-saline grown aggregates as a function of salinity. Reference data displayed in Table S.1 (X-axis = column C, Y-axis = column A-B).

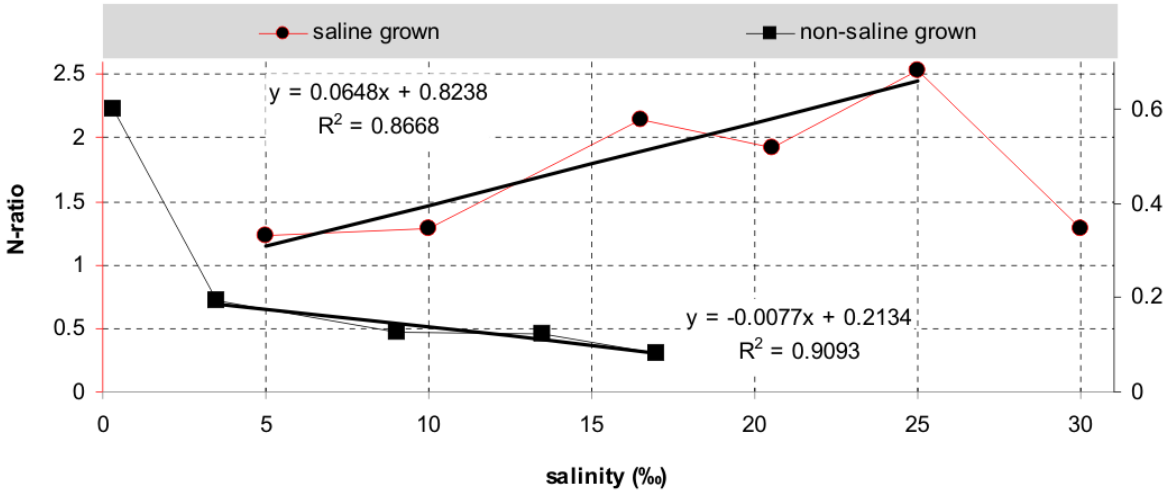

Figure 6: N-ratio displayed as function of salinity for saline and non-saline grown microbial aggregates, showing a constant decrease with increasing salinity after the first addition of salt in non-saline grown aggregates, and showing an increase with increasing salinity (up to a 25‰) in saline grown aggregates.

3.2 Microsensor experiments

Oxygen microprofiles of saline grown microbial aggregates measured at ~5‰ salinity showed a sharp decrease from 16µM ± 8µM in the overlying medium to 5µM ± 4µM (mean ± SD) at

the surface of the aggregate. The microsensor set-up could not be prevented from slight oxygen penetration into the system, although no oxygen was detectable at a depth of 50 µm within the aggregate (Figure 7d).

Nitrate profiles showed a sharp decrease of nitrate concentration at the aggregate surface (Figure 7a). The addition of 10% acetylene, which is an inhibitor of nitrous oxide reductase (enzyme mediating N_2O => N_2), caused an increase in N_2O within the aggregates (Figure 7b), which is indicative for the denitrification pathway. The highest increase in N_2O was not measured at the surface, where most of the nitrate consumption took place, but in a subsurface layer (Figure 7b). Local rate calculations (section 2.2.5) revealed that there was already net N_2O production at 50 µm within the aggregate (Figure 7c,). Nitrite microprofiles showed a peak right at the surface of the aggregate, which did not change upon the addition of ~ 82µM of allylthiourea (ATU), an inhibitor of ammonia oxidation, but it increased drastically when the triple amount of carbon source was added to the medium (Figure 8a, 8b). The latter strongly suggests the occurrence of heterotrophic dissimilatory nitrate reduction to nitrite. The calculated local rates, which were based on mean nitrite microprofiles taken at a salinity of 5‰, show a layer of net-nitrite production at the surface of the aggregate down to a depth of approximately 100µm inside the aggregate (Figure 8 c).

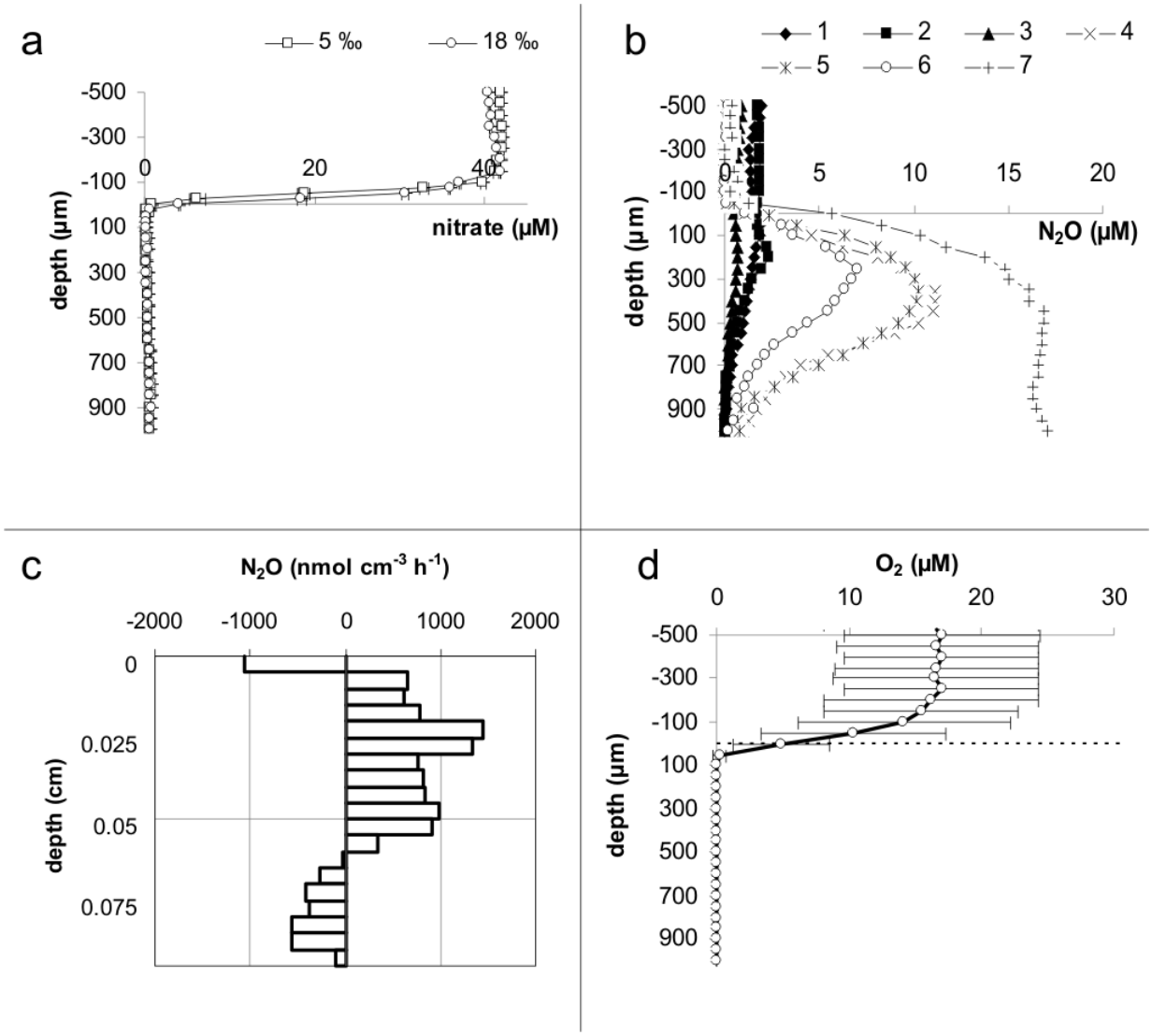

Figure 7: Mean microprofile of **(a)** nitrate in saline grown microbial aggregates exposed to 5‰ and 18‰ salinity (n=3; Error bars show SD); **(b)** N_2O microprofiles measured at a salinity of 18‰ show increase in N_2O after acetylene treatment (4-7) compared to non-treated profiles (1-3); (**c)** local rate calculated from mean N_2O microprofile after acetylene addition (n=4), and **(d)** mean microprofile of oxygen in saline grown microbial aggregates exposed to 5‰ salinity (n=4; Error bars show SD).

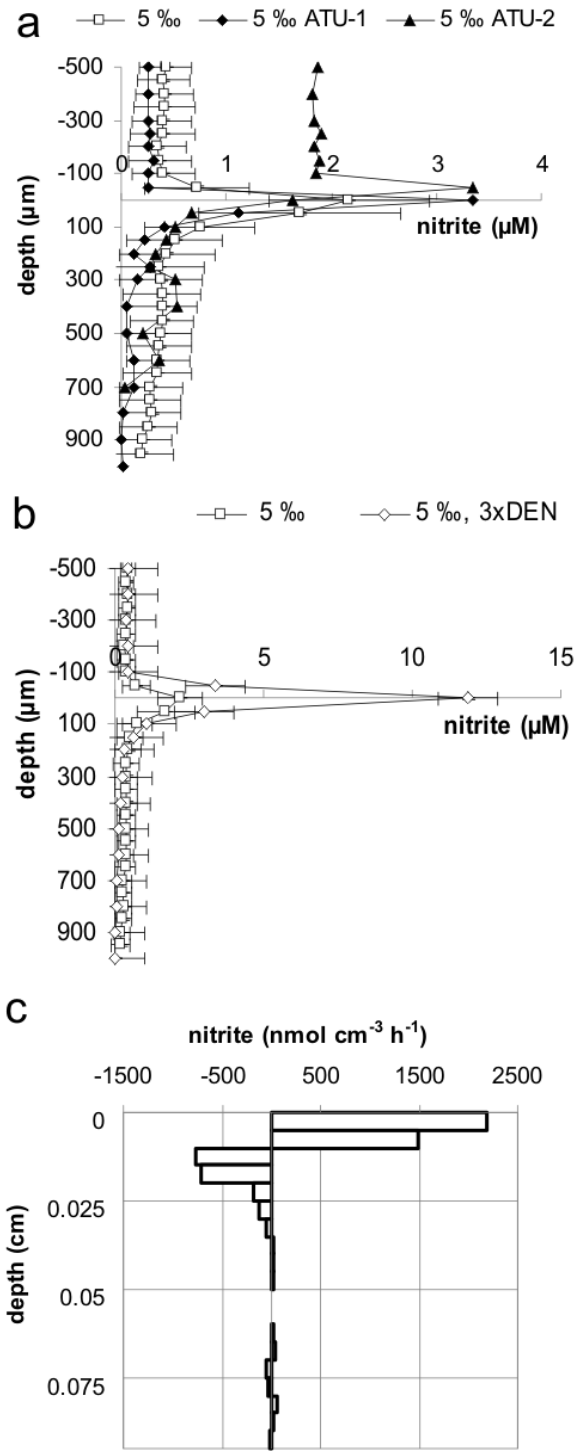

Figure 8: Mean nitrite microprofile **(a)** measured on saline grown microbial aggregates at a salinity of 5‰ under addition of nitrate as electron acceptor and acetic acid as electron donor (n=3; Error bars show SD). The addition of ATU did not cause any significant change in peak size (two ATU profiles). **(b)** The addition of the triple amount of carbon source (3xDEN) caused an increase in peak size (n=3; Error bars show SD). **(c)** Corresponding local rates of nitrite microprofiles taken at a salinity of 5‰.

3.3 Total sulfide, sulfate, ammonium concentrations and anammox

Total sulfide concentrations were colorimetrically not detectable following the Pachmeyr approach. The observations of the in- and out-flow concentrations of sulfate, which account for ~1.7 l of saline grown microbial aggregates showed within up to 28 days only very small and insignificant differences in sulfate concentrations (Figure S1). The average out-flow concentration was 3.07mM ± 0.12mM with an in-flow concentration of 3.16mM ± 0.30mM and an average corresponding ammonium concentration of 64µM ± 26µM (mean ± SD). Anammox turned out to be negligible, as well (Figure S2).

4 Discussion

4.1 The effect of salinity on denitrification

Several studies reported a negative correlation on denitrification and salinity (Dinçer and Kargi, 1999; Glass and Silverstein, 1999; Seo et al., 2008), whilst other studies did not find a negative correlation (Fear et al., 2005; Magalhães et al., 2005). The latter studies, however, were conducted in environments with fluctuating salinity, showing that salinity is a selective force for halotolerant and halophilic denitrifying bacteria.

Denitrification rates in saline grown microbial aggregates were overall higher compared to non-saline grown aggregates, but the saline microbial aggregates had also an approximately four-fold greater biomass. Biomass-normalized denitrification rates were still higher in saline grown aggregates, except for those measured at 0‰ and 3.5‰ salinity (in non-saline grown aggregates). The normalized rates accounted for 184 µM at 3.5‰ and 524 µM at 0‰ salinity (Table S.2). The latter was the overall highest rate measured amongst all experiments in this study, and it was approximately three times higher than any denitrification rate determined with saline grown microbial aggregates. This suggests that salinity may indeed have an overall adverse effect on denitrification. Denitrifying microorganisms may not contribute equally to the

total microbial communities in respective aggregates, but changes in community structure and density, were also induced by salinity, the only variant factor in this study. Different carbon sources as reason for different denitrification rates (Akunna et al., 1993) can be excluded, because all experiments were carried out with acetic acid as carbon source.

When different salinity levels were introduced, the effect of salinity on denitrification rates within saline grown aggregates was fairly low compared to the effect of salinity on non-saline grown aggregates (Figure 2). A first increase in salinity of 3.5‰ in non-saline grown aggregates caused a decrease in denitrification rate down to 35% of the original denitrification rate, followed by further decreases down to only 17% of the original rate with increasing salinity (Figure 2). These findings were consistent with previous studies on activated sludge showing a negative correlation of salinity and denitrification rate (Dinçer and Kargi, 1999; Glass and Silverstein, 1999).

Studies on samples from estuaries that were exposed to salinity fluctuations, showed an absence of a significant salinity induced regulatory effect on denitrification rates (Fear et al., 2005; Magalhães et al., 2005). The saline grown aggregates, in the current study, were grown at a constant brackish salinity of 10‰, but the highest denitrification rates were measured within a range of 16.5 -25‰ salinity (Figure2), which 12 - 18% greater denitrification rates than at their growth salinity.

This implied that saline grown denitrifying communities had a higher optimal salinity than their growth salinity, showing a slight over-adaptation to salinity. In the FTSRR salinity incubations, nitrite concentrations increased as nitrate concentrations decreased (Figure 3, Figure 4). The non-saline grown microbial aggregates showed on average approximately two-times higher maximum nitrite accumulations than saline grown microbial aggregates (46µM ± 10µM vs. 86µM ± 10µM), but they also showed a higher nitrite consumption after prior nitrite accumulation (Figure 3, Figure 4, Table S.1). In both aggregate types, nitrite removal decreased

with increasing salinities, which was especially pronounced in non-saline grown aggregates (Figure 3, Figure 4, Figure 5). This facilitates increases of nitrite up to toxic concentrations, if denitrification rates decrease induced by salinity. Hence, these results indicate that nitrate reduction to nitrite is much less affected by salinity than subsequent steps of denitrification, as also reflected in the overall nitrate reduction rate of 555µM ± 33µM (mean± SD) for saline and non-saline grown aggregates. There are many more organisms known to be capable of nitrate reduction to nitrite other than denitrifiers (Richardson et al., 2001). Consequently, it is likely that there are more halotolerant nitrate reducers than denitrifiers. The higher efficiency of denitrification in saline grown aggregates is further revealed by their N-ratio. The higher N-ratios of saline grown microbial aggregates implied a relatively low nitrite accumulation with fairly high corresponding denitrification rates compared to non-saline grown microbial aggregates (Figure 6). Even more striking is that the N-ratio of saline grown aggregates increased with increasing salinities, and decreased only at a salinity of 30‰. In comparison, denitrification efficiency in non-saline grown aggregates, as reflected by the N-ratio, decreased down to a third of the original value, just after the first increase in salinity. Further increases in salinity reduced the N-ratio (Figure 6) consistent with the overall trend of the measured denitrification rates. Together, these combined results highlight, from an applied point of view, the necessity to adapt denitrifying microbial communities to salinity prior to treatment of saline waste waters or brine.

4.2 The stratification of N-cycling processes within saline grown microbial aggregates

The FTSRR-based experiments gave an overview on the influence of salinity on overall denitrification (NO_3^- => N_2), and specifically on nitrite production and consumption in saline and non-saline grown microbial aggregates, whilst high resolution microsensor profiles were performed to examine the detailed stratification of N-cycling processes within saline grown microbial aggregates.

Microsensor depth profiles of saline grown aggregates did not show any salinity induced difference in nitrate microprofiles at 5‰ and 18‰ salinity (Figure 7), but a full depletion of nitrate already at the surface of the aggregate. This implies either that denitrification takes exclusively place on the surface of the aggregate (assuming that it is the most significant nitrate and nitrite removing process) or it implies a low substrate feed charge. The addition of acetylene caused a strong N_2O accumulation in the sub-surface layer of the aggregate up to a concentration of ~ 17 µM in deeper layers (Figure 7 b,c). This accumulation is indicative for denitrification (Balderston et al., 1976; Kristjansson et al., 1980), and it takes place at a depth of 50-600 µm inside the microbial aggregates. Consequently, the observed net-nitrate uptake at the surface of the aggregate is not indicating the spatial position of the main denitrifying community, but it simply evolved from a low substrate feed charge (Figure 7 a-c).

Nitrite microprofiles showed a peak at the surface of the microbial aggregate, revealing the spatial position of the source of nitrite accumulation within the saline grown microbial aggregates. However, oxygen microprofiles showed that we could not perfectly prevent the microsensor set-up from oxygen penetration into the aggregates (Figure 8), so nitrification, which occurs under oxic conditions, was also a possible source of nitrite production.

The addition of ATU should have caused a decrease in peak size, if this would have been the case. Approximately 82 µM ATU was added, which is a concentration likely to inhibit bacterial nitrification (Hall, 1984), but it did not show any effect. In contrast, the addition of the triple amount of acetate caused an increase in peak size (Figure 8).

This suggests that the surface of the aggregate is indeed the source of the nitrite accumulation, which was already observed under anoxic conditions in the FTSRR. This spatial separation of nitrite accumulation indicates that the relative surface of the granules might have a regulatory effect on nitrite production and consumption, beside other factors, i.e. salinity, as shown in the current study, or ambient nitrate, nitrite and oxygen concentrations (Körner und Zumpft, 1989).

However, a reduction of the surface-to-volume ratio may decrease excess nitrite production. Further research is needed to evaluate the significance of this observation and its potential applicability to waste water treatment.

5 Conclusions

In this study, higher salinities than the growth salinity had a positive effect on denitrification rates and denitrification efficiency for saline grown aggregates, showing a slight over-adaptation to salinity. In contrast, strong decreases in denitrification rates and denitrification efficiency were seen for non-saline grown microbial communities upon increasing salinity. This highlights the importance of using pre-adapted denitrifying communities in the treatment of brackish water or treatment of brine.

However, the highest biomass-normalized denitrification rate was measured in non-saline grown microbial aggregates under non-saline conditions, so salinity might have indeed an overall decreasing effect on denitrification rates, even if microbial communities are adapted to salinity. The surface of the microbial aggregates was identified as the source of the excess nitrite. Consequently, reducing the surface-to-volume ratio of the aggregates might reduce as well the excess nitrite production to some extend, although further research is needed to elucidate this observation.

6 Supplementary Material

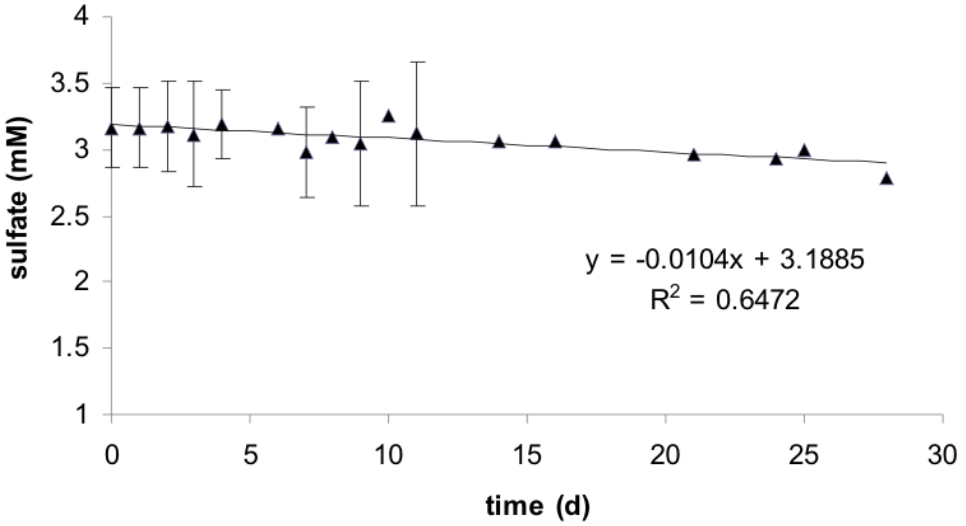

Figure S1: Insignificant decrease in sulfate concentrations (R^2 = 0.65) observed over 28 days accounting for the activity of ~1.7 l of saline grown microbial aggregates.

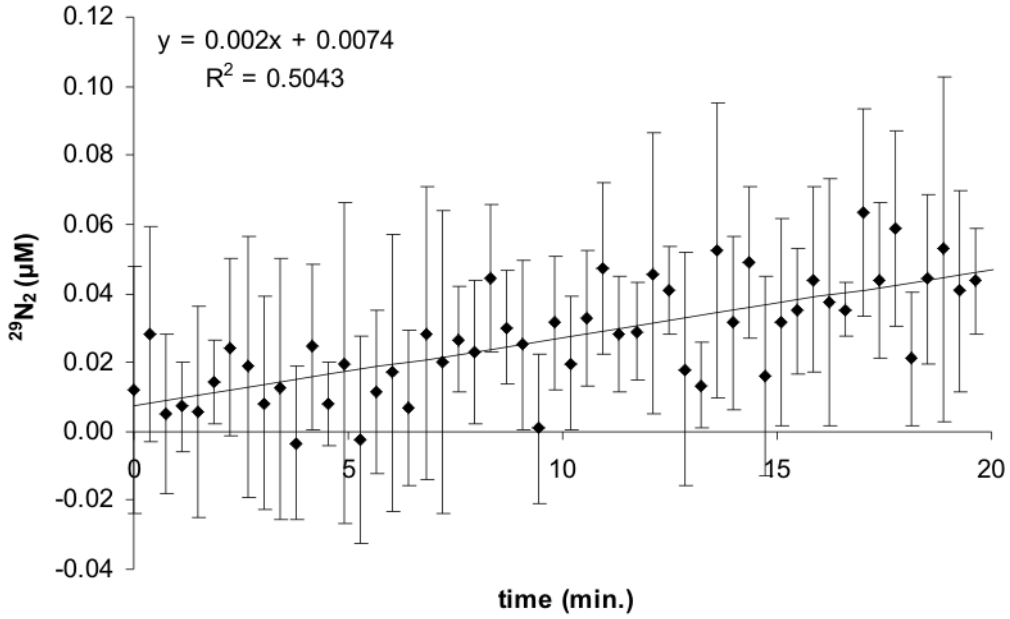

Figure S2: Insignificant increase in $^{29}N_2$ concentration (anammox rate 0.12µM h^{-1}, R^2 = 0.50) within the FTSSR reactor after addition of 70 µM ^{15}N labeled NH_4^+ and 70µM non-labeled NO_3^- without acetic acid. Every data point is a mean of four data points.

Table S.1: The highest nitrite accumulation (column A) subtracted from the nitrite concentration measurement after 20 minutes (column B and column A-B) is inversely correlated to salinity (column C). The corresponding Pearson product-moment correlation coefficients are r = -0.95 for saline grown aggregates and r = -0.98 for non-saline grown aggregates.

Saline grown aggregates			
A	**B**	**A-B**	**C**
highest NO_2^- accumul. (µM)	NO_2^- accumul. after 20 min.(µM)	NO_2^- consumption (µM)	salinity ‰
59	39	19	5
48	26	22	10
34	21	12	16.5
37	27	10	20.5
41	38	3	25
56	55	1	30
Saline grown aggregates			
A	**B**	**A-B**	**C**
highest NO_2^- accumul. (µM)	NO_2^- accumul. after 20 min.(µM)	NO_2^- consumption (µM)	salinity ‰
73	8	65	0
79	36	42	3.5
99	80	19	9
87	84	4	13.5
90	89	1	17

Table S.2: Denitrification rates (DR) shown in µM h^{-1}, expressed in % relative to the respective growth DR, and shown with corresponding salinities. For non-saline grown aggregates are denitrification rates corrected for biomass.

Saline grown aggregates		
salinity ‰	DR as N2, µM h-1	% of DR at growth salinity (gs)
5	146	79
10	185	100
16.5	218	118
20.5	213	115
25	208	112
30	144	78

Non-saline grown aggregates			
salinity ‰	DR as N2, µM h-1	% of DR at gs	Biomass corr. DR
0	131	100	524
3.5	46	35	184
9	37	28	148
13.5	32	24	128
17	22	17	88

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Chapter 4

Aerobic denitrification in permeable Wadden Sea sediments

Contributions to this chapter

Concept and design of the Flow through stirred retention reactor (FTSRR) by O.Svitlica

Experimental specification of the FTSRR incubation experiment with contribution of O.Svitlica.

Practical work relating the FTSRR incubation experiment by H. Gao with help of O.Svitlica

Aerobic denitrification in permeable Wadden Sea sediments

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Abstract

Permeable or sandy sediments cover the majority of the seafloor on continental shelves worldwide, but little is known about their role in the coastal nitrogen cycle. We investigated the rates and controls of nitrogen loss at a sand flat (Janssand) in the central German Wadden Sea using multiple experimental approaches, including the nitrogen isotope pairing technique in intact core incubations, slurry incubations, a flow-through stirred retention reactor, and microsensor measurements. Results indicate that permeable Janssand sediments are characterized by some of the highest potential denitrification rates (≥ 0.19 mmol N m^{-2} h^{-1}) in the marine environment. Moreover, several lines of evidence showed that denitrification occured under oxic conditions. In intact cores, microsensor measurements showed that the zones of nitrate/nitrite and O_2 consumption overlapped. In slurry incubations conducted with $^{15}NO_3^-$ enrichment in gas-impermeable bags, denitrification assays revealed that N_2 production occurred at initial O_2 concentrations of up to ~90 µM. Initial denitrification rates were not substantially affected by O_2 in surficial (0-4 cm) sediments, while rates increased by two fold with O_2

depletion in the at 4-6 cm depth interval. In a well mixed, flow-through stirred retention reactor, $^{29}N_2$ and $^{30}N_2$ were produced and O_2 was consumed simultaneously, as measured on-line using membrane inlet mass spectrometry. We hypothesize that the observed high denitrification rates in the presence of O_2 may result from the adaptation of denitrifying bacteria to recurrent tidally-induced redox oscillations in permeable sediments at Janssand.

Keywords: aerobic dentrification / nitrogen loss / permeable sediments / simultaneously NO_x^- and O_2 respiration

1 Introduction

Nitrogen (N) is primarily removed from coastal ecosystems by microbially-mediated denitrification that occurs on the seafloor (Hulth et al., 2005). Continental shelf sediments are important sites of N-removal, which may account for 50-70% of oceanic N-loss (Codispoti et al., 2001). Although the majority of continental margins is covered by coarsed-grained relict sediments (Emery, 1968; Johnson and Baldwin, 1986), most previous biogeochemical research has focused on muddy or fine-grained sediments. Pore-water advection, driven mainly by pressure gradients from wave action and bottom currents interacting with surface topography, causes rapid solute exchange and allows direct transfer of suspended particles into permeable sediment strata. Recent studies indicate that advective transport leads to an acceleration of organic matter mineralization and a stimulation of biogeochemical cycling proportional to the extent of pore-water exchange (Huettel and Rusch, 2000; de Beer et al., 2005; Werner et al., 2006). Up- and downward flow of pore-water associated with migrating sandy sediment ripples generates vertical oscillations in oxic and anoxic conditions as redox zones move horizontally through the surface layer of the bed. The dynamic redox conditions found in permeable marine sediments resemble those found in wastewater treatment plants (Gray, 1990º). In other words, high transport rates of organic matter and electron acceptors from the water column into the

seafloor and the presence of oscillating oxic / anoxic conditions allow marine sands to act as an efficient nutrient filter that may facilitate N removal. However, few studies have investigated N-loss by denitrification in coastal permeable sediments; of these studies, fewer still have considered the effects of advective pore water flows on the rates of denitrification (Cook et al., 2006; Hunter et al., 2006; Rao et al., 2007; 2008). Further research is needed to determine the rates and controls of N-removal from permeable marine sediments.

The current paradigm is that denitrification is an anaerobic process in marine sediments, and oxygen is believed to act as a major control of the process (Brandes et al., 2007). Denitrification is considered to require completely anoxic conditions due to the fact that O_2 acts as a competing electron acceptor for NO_3^- respiration and key enzymes of the denitrification pathways are inhibited by relatively small amounts of O_2 (Tiedje et al., 1982; Zumft, 1997; Shapleigh, 2006). However, in contrast to the observations made in natural environments, a large number of laboratory studies have reported that denitrification occurs under aerobic conditions in pure cultures of bacteria (Robertson and Kuenen, 1984; Ronner and Sorensson, 1985; Trevors and Starodub, 1987; Robertson et al., 1995). Such findings suggest that denitrification may not always be so effectively inhibited by O_2. Microbiologists have defined aerobic denitrification as the co-respiration or co-metabolism of O_2 and NO_3^-. Physiological studies show that microorganisms are able to use branches of their electron transport chain to direct electron flow simultaneously to denitrifying enzymes as well as to O_2 (Robertson and Kuenen, 1988; Huang and Tseng, 2001; Chen et al., 2003). Although some environmental studies suggest that denitrification can occur in the presence of O_2 (Carter et al., 1995; Bateman and Baggs, 2005; Rao et al., 2007), substantial rates of aerobic denitrification have not yet been verified in the natural marine environment. Through a combination of new techniques employing stable N isotopes for the direct determination of denitrification rates as well as the rapid quantification of aqueous gases over short time scales, the study of aerobic denitrification becomes more feasible.

The main objective of this study was to investigate the impact of O_2 dynamics on N-loss by denitrification in permeable marine sediments of the Wadden Sea. Taking advective transport into account, we investigated denitrification rates in permeable sediments under near *in situ* conditions using a variety of experimental approaches. Surprisingly, multiple lines of evidence indicated that denitrification was not inhibited in the presence of substantial oxygen concentrations but rather the co-respiration of O_2 and NO_x^- occurred. Therefore, we hypothesized that where NO_x^- and O_2 cooccur, O_2 may not act as the primary or exclusive control of N_2 production in permeable sediment environments.

2 Materials and Methods

2.1 Site description

The Janssand sand flat (13 km^2) is in the back barrier area of Spiekeroog Island in the German Wadden Sea. The western edge of the flat faces the 17 m-deep, tidal channel separating the barrier islands Spiekeroog and Langeroog. The entire Janssand flat is inundated with ~2 m of seawater for 6-8 h during each semi-diurnal tidal cycle, becoming exposed to air for 6-8 h during low tide, depending on the tidal range.

The central region of Janssand comprises the main area of the sand flat and is termed the upper flat due to the sloping margin downwards to the low water line. The upper flat is itself almost level and the physical appearance is homogeneous, consisting mainly of well-sorted silica sand with a mean grain size of 176 μm (Billerbeck et al., 2006a). The mean permeability of 7.2 – 9.5 × 10^{-12} m^2 (upper 15 cm of sediment; (Billerbeck et al., 2006a) permits advective pore water flows (Huettel and Gust, 1992). Detailed descriptions of the Janssand flat are available in Billerbeck et al. (2006a; 2006b), Røy et al. (2008) and Jansen et al. (2009).

The sampling site (53°44.11'N, 7°41.95'E) is situated on the northeastern margin of the upper flat, about 80 m upslope from the mean low water line. Flat-bottom ships, the Spes Mea and

Doris von Ochtum, were used to investigate the site in March 2007 and April 2008, respectively. All sediment core and seawater sampling was conducted at the upper Janssand tidal flat in March, 2007, unless otherwise indicated.

2.2 Dissolved inorganic nitrogen (DIN) in sediment pore water

Rhizon samplers (Seeberg-Elverfeldt et al., 2005) were utilized to extract pore water directly from sediment cores on the deck of the ship. Cores were sampled by pushing Plexiglas core liners (I.D., 9.5 cm) with side ports into the sediment, and Rhizons were then inserted horizontally into the ports at 1 cm intervals to 10 cm depth. Site seawater was also collected during low tide and filtered through a 0.2 um syringe filter. All samples were immediately frozen onboard ship at -20 °C for later analysis. Dissolved ammonium (NH_4^+) concentrations were determined using a flow injection analyzer (Hall and Aller, 1992). Nitrate + nitrite (NO_x^-) was determined by chemiluminescence after reduction to NO with acidic vanadium(II) chloride (Braman and Hendrix, 1989).

2.3 Intact core incubations

Denitrification rates were determined by the isotope pairing technique (Nielsen, 1992) in intact core incubations modified according to De Beer et al. (2005) to simulate *in situ* pore water advection in the permeable sediments. A set of 15 sediment cores were collected in parallel to obtain 15 cm of sediment and 15 cm of overlying water each from a 1-m^2 area using Plexiglas push-cores (I.D., 3.5 cm; height, 28 cm). Seawater was also collected in parallel from the site. Water overlying the sediment was removed and replaced with $^{15}NO_3^-$-amended site seawater (final concentration of 50 μM). Rubber bottom stoppers were fitted with valves to allow for pore water perfusion over the upper 5 cm depth, and each core was percolated by 20 ml $^{15}NO_3^-$-amended aerated seawater at the perfusion speed of 12 ml/min. Cores were immediately sealed without any headspace by rubber stoppers after percolation, incubated at *in situ* temperature (8-

10°C) and were destructively sampled in triplicate at regular intervals between 0 and 6 hours. The overlying water of all cores was mixed continuously at approximately 60 rpm during the incubations by externally-driven magnetic stirring bars. Cores were sacrificed in reverse order of percolation. Briefly, 1 ml of zinc chloride (50% w/v) $ZnCl_2$ was added to the sediment surface. The cores were resealed with no headspace prior to mixing by inversion. After allowing the sediment particles to settle, an aliquot of water was removed from each core and transferred to a 12 ml Exetainer ™ (Labco, UK) pre-filled with 200 µl saturated $HgCl_2$ for $^{29}N_2$ and $^{30}N_2$ determinations. The concentrations of excess $^{29}N_2$ and $^{30}N_2$ were calculated from $^{29}N_2$:$^{28}N_2$ and $^{30}N_2$:$^{28}N_2$ ratios of He-equilibrated headspace in Exetainers determined by gas chromatography-isotope ratio mass spectrometry (GC-IRMS; VG Optima). Denitrification rates were calculated based on the linear production of excess $^{29}N_2$ and $^{30}N_2$ according to Nielsen (1992).

Another set of intact core incubations was conducted to provide direct evidence for the co-respiration of O_2 and NO_x^- using multiple microsensors. An O_2 microsensor and a NO_x^- biosensor were simultaneously applied to freshly sampled sediment cores. Advective transport in sediments cores was simulated by percolation as described above.

Oxygen microsensors were constructed as described previously (Revsbech, 1989). A 2-point calibration method for the O_2 sensor was performed using the signal in saturated overlying site seawater and anoxic sediments, and the O_2 solubility was corrected for the ambient water temperature (18 °C) and salinity (32 ‰) using the spreadsheet supplied by Unisense (www.unisense.dk). The NO_x biosensor was constructed, according to Larsen et al. (1997), with a tip diameter of 100 µm and was calibrated in seawater with additions of increasing amounts of NO_3^- to confirm linearity of the response (0-500 µM). For the calculation of pore water concentrations, the slope and offset were corrected for NO_3^- concentrations in the overlying water determined as described above.

For simultaneous measurements of vertical concentration profiles, the O_2 and NO_x sensors were mounted on a 3-axis micromanipulator (MM 33; Märzhäuser, Wetzlar, Germany). The vertical axis was motorized for µ-positioning and controlled by µ-Profiler software described in Polerecky et al. (2005). The microsensor tips were aligned carefully to the same horizontal axis. The even sediment topography allowed alignment of both sensor profiles to the sediment surface with a precision of 1 mm, using the initial decrease of O_2 in the diffuse boundary layer. Microsensor measurements were made at 0.5 cm and 1 cm below the sediment surface during each percolation. Data were recorded over a time series to determine rates of potential O_2 uptake and NO_x^- consumption under oxic and anoxic conditions.

2.4 Slurry incubations in gas-tight bags

The depth-specific response of denitrification to O_2 was initially examined in bag incubations using the ^{15}N tracer isotope pairing technique according to Thamdrup and Dalsgaard (2002). Sediments were sampled using Plexiglas push-cores (I.D., 9.5 cm; height, 60 cm) and sectioned into 2-cm depth intervals to a depth of 6 cm. Afterward, sediment and air-saturated, site seawater were mixed at a ratio of 1:1 in the gas-tight bag while expelling all air bubbles. The slurries were amended with $^{15}NO_3^-$ to a final concentration of 200 µM, and the bags were incubated at *in situ* temperature (same as for intact core incubations). During the incubation, the bags were periodically shaken to ensure that the labeled N species were homogenously distributed. Sub-samples of the interstitial water were withdrawn from the bags at regular intervals and preserved in 6 ml Exetainer ™ vials (Labco, UK) pre-filled with 100 µl saturated $HgCl_2$. An initial subsample was taken immediately after the addition of the tracer and at regular intervals to 16 hours.

The aqueous O_2 concentration of the subsamples was determined using an O_2 microelectrode (MPI, Bremen) as described above. The 6 ml Exetainer vials were opened only briefly during the

measurement, and were afterwards stored with no headspace for further analysis of dissolved N_2 by GC-IRMS as described above.

2.5 Flow-through stirred retention reactor (FTSRR) incubation experiment

To provide further corroboration for the co-respiration of O_2 and NO_3^- in a sediment slurry, aqueous gases (O_2 and N_2) were directly determined in line by membrane inlet mass spectrometry (MIMS; GAM200, IPI) in a FTSRR system. Surface sediments (0-3 cm) and site seawater were collected from the upper flat in April 2008, and stored at 4°C during transport to the laboratory. Sediments and site seawater were mixed at a ratio of 1:3 in the gas-tight FTSRR without headspace. The slurry was vigorously mixed at 200 rpm by a magnetic stir bar, and the incubation was carried out in the dark at room temperature. The FTSRR system consisted of a sealed cylinder chamber (Plexiglas, inner diameter 9 cm, height 6cm) fitted with three ports for the input and output of water. The effluent pumped through a filter by one port from the chamber was injected directly into the membrane inlet using a peristaltic pump with the pumping speed of 0.5 ml/min. Gas-tight syringes connected to the chamber by the other two ports, each filled with 50 ml of air-saturated site sea water, provided replacement water during pumping.

Simultaneous online measurements of mass 28 ($^{14}N^{14}N$), 29 ($^{14}N^{15}N$), 30 ($^{15}N^{15}N$), 40 (Ar), 31 (^{15}NO), 32 (O_2), 44 ($^{14+14}N_2O/CO_2$), 45 ($^{14}N^{15}NO$) and 46 ($^{15}N^{15}NO$) were obtained by MIMS. A standard calibration curve was constructed, based on measurements obtained under both air-saturated and anoxic conditions using a two-point calibration for each. The slurry in the incubator was amended with $^{15}NO_3^-$ to a final concentration of ~150 μM. Mass abundance signals were recorded by MIMS at one second time intervals and the flow-through samples were collected in 2-ml vials and stored at -20°C for DIN analysis as described above **(2.2).**

3 Results

3.1 DIN and O_2 penetration in permeable sediments

Zones of O_2 penetration and NO_x^- depletion largely overlapped in the upper 2 to 3 cm of tidal flat sediments. During the winter-spring, NO_x^- concentrations with a mean value of ~67 µM were observed in the overlying seawater, while NH_4^+ concentrations were comparably 10 times lower (< 7 µM) (Figure 1A). In surficial sediments, pore water NO_x^- decreased rapidly with depth to ~40 µM at 1 cm depth, and a minimum concentration was observed below 3 cm depth. Concomitantly, pore water NH_4^+ increased to ~70 µM from the surface to 3 cm depth and remained consistently high (70-105 µM) below that depth (Figure 1A). *In situ* O_2 measurements in the upper flat from March 2006, showed that, O_2 penetrated to ~3 cm during tidal inundation (Figure 1B) and O_2 penetration depths of up to 5 cm were observed at other locations on Janssand tidal flat (Billerbeck et al., 2006b; Jansen et al., 2009). The decrease in NO_x^- was equivalent to approximately half of the observed increase of NH_4^+ with depth (Figure 1A).

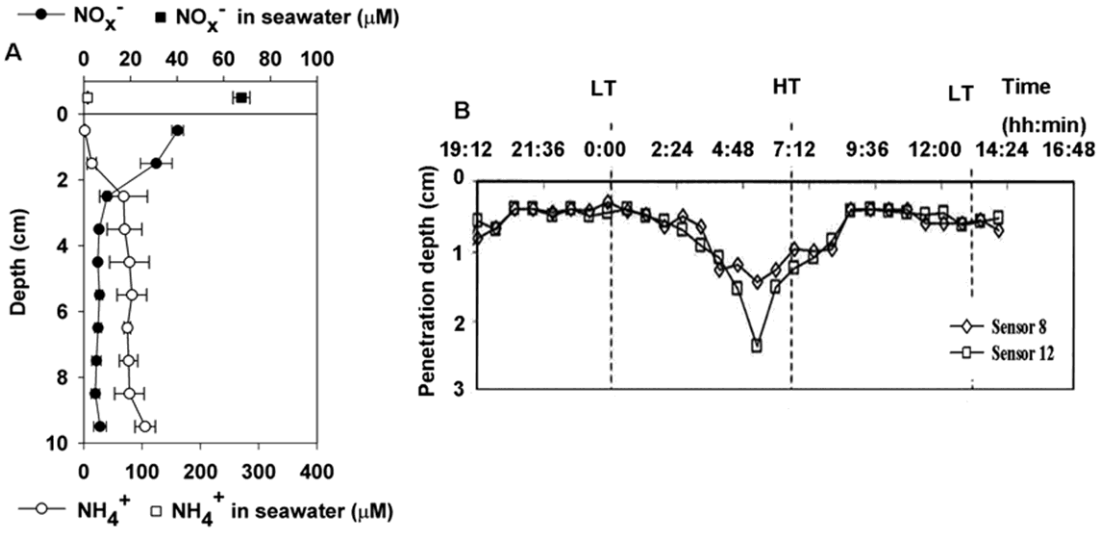

Figure 1: Distribution of dissolved inorganic nitrogen species [NH4+ and NOx- (NO3- + NO2-)] and O_2 penetration in sediments from the upper flat at Janssand. **(A)** NO_x^- (closed circles) and NH_4^+ (open circles) concentration profiles in permeable sediments in March 2007 during exposure. The daily mean values of NO_x^- and NH_4^+ concentrations in overlying seawater are depicted as closed and open squares, respectively. **(B)** O_2 penetration depth during a tidal cycle measured by two oxygen sensors in March 2006 (modified from Jansen *et al*., 2009).

3.2 Denitrification potential in intact cores and gas-tight bag incubations

Following with the overlap in O_2 penetration and NO_x depletion, we observed the immediate and rapid production of ^{15}N-labeled N_2 in both incubations amended with $^{15}NO_3^-$ throughout the first 4 hours of incubation under oxic conditions (Figure 2).

Our study of the intact core incubations was motivated by a previous study of O_2 consumption using the same pore water percolation method that observed substantial O_2 was present during the first 1-2 hours of intact core incubations in March (Polerecky et al., 2005; Billerbeck et al., 2006b). In the present study, $^{29}N_2$ and $^{30}N_2$ were produced linearly (r^2_{29N2} = 0.93 and r^2_{30N2} = 0.91, respectively) without any lag during the first 2 hours of incubation in the presence of O_2 (Figure 2A). In bag incubation experiments conducted in parallel, high potential denitrification rates were observed in sediment slurries from the 0-2 cm, 2-4 cm and 4-6 cm depth intervals in which initial O_2 concentrations of ~95, 30 and 35 µM were observed, respectively. Higher $^{29}N_2$ and $^{30}N_2$ production rates were observed in incubations from the 0 to 4 cm depth intervals where higher NO_x^- concentrations are observed in sediment pore waters (Figure 1A, Figure 2B - D).

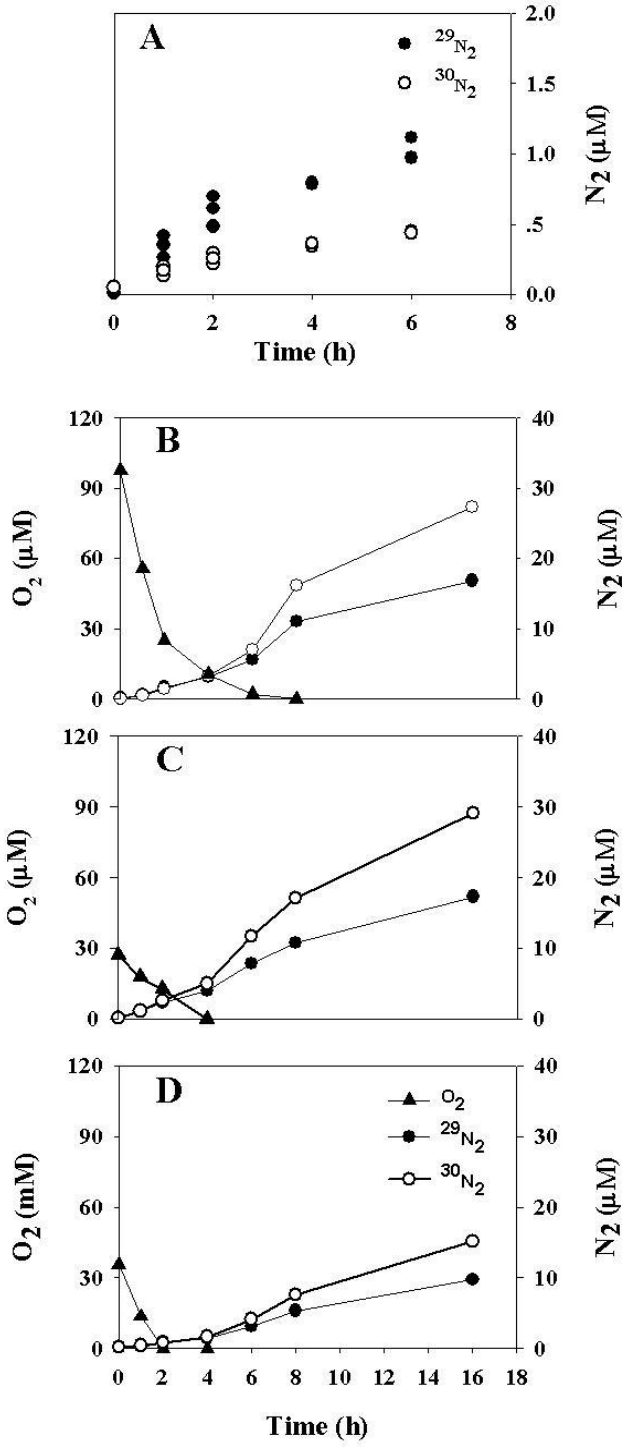

Figure 2: ^{15}N-labeled N_2 production and oxygen consumption in whole-core and bag incubations. **(A)** $^{29}N_2$ (black circles) and $^{30}N_2$ (open circles) production from $^{15}NO_3$ amendments in percolated whole-core incubations. O_2 consumption and ^{15}N-labeled N_2 production versus time in $^{15}NO_3^-$ - amended, oxic, gas-tight bag incubations with sediment from **(B)** 0-2 cm, **(C)** 2-4 cm and **(D)** 4-6 cm depth intervals.

No significant change in the denitrification rates was observed in the incubations under oxic conditions (during the first 4 h) in comparison to anoxic conditions (during the last 12 h) (Table

1). In the incubation from the deepest depth interval (4-6 cm) where NO_x^- was depleted, the lowest denitrification rates were observed while O_2 depleted earliest (~2 h) (Table 1). Moreover at 4 to 6 cm depth, the rate under anoxic conditions was 2.3 times higher as that under oxic conditions (Figure 2D and Table 1). When extrapolated over the 0-5 cm depth interval, potential denitrification rates measured in percolated intact cores and bag incubations were in the same range (Table 1).

3.3 Microelectrode and biosensor measurements

Similar to the observations in the bag incubations, time series measurements by microsensors upon percolation of air-saturated and NO_3^--rich sea water showed that NO_x^- and O_2 were consumed simultaneously at 0.5 to 1 cm depth in intact sediment cores (Figure 3; Table 1).

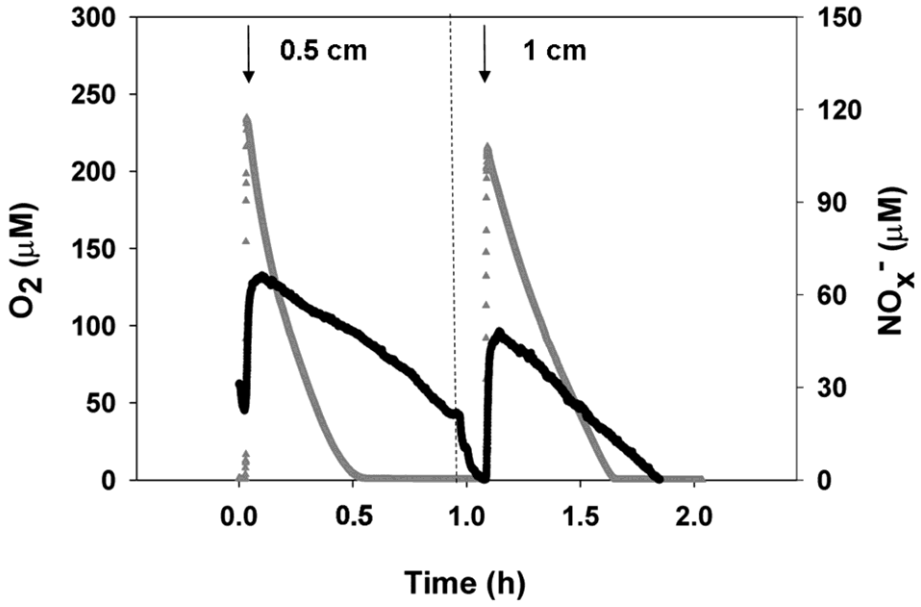

Figure 3: Time series of O_2 (grey) and NO_x^- (black) concentrations in an intact sediment core after percolation (indicated by arrows) with air-saturated overlying seawater containing ~60 µM NO_x^-. O_2 and NO_x^- microsensor tips were adjusted on a horizontal axis and measurements were carried out simultaneously. The first percolation treatment started at 0 h when sensors were positioned at 0.5 cm depth. The sensors were moved from 0.5 cm to 1 cm after 0.9 h and the second percolation began at 1.1 h when sensors were positioned at 1 cm depth.

Table 1: Summary of denitrification rates measured in all incubations

Measurement	Investigated depth (cm)	Denitrification / NOx – consumption (mmol N $m^{-3}_{sediment}$ h^{-1})	
		Oxic	Anoxic
Intact core incubation	5	4.60 ± 0.46	
	Integrated to 5	0.23 ± 0.02*	
Intact core by multi – microsensors	0.5	15.5 ± 0.04	21.9 ± 0.05
	1	22.0 ± 0.04	21.5 ± 0.04
Slurry incubation	0 – 2	6.40 ± 0.37	10.57 ± 3.20
	2 – 4	8.27 ± 0.32	9.63 ± 1.15
	4 – 6	2.72 ± 0.16	6.28 ± 0.84
	Extrapolated to 5	0.32 ± 0.01*	0.47 ± 0.07*
Constant mixing, flow-through retention reactor incubation	0 - 3	6.23 ± 0.07	
	Extrapolated to 3	0.187 ± 0.002*	

The mean porosity of sediments in upper flat is 35% (Billerbeck et al., 2006b); * the unit is mmol N $m^{-2}_{sediment}$ h^{-1}

O_2 and NO_x^- - rich seawater was transported downward into the sediment by percolation, which increased concentrations at 0.5 cm to ~240 µM O_2 and 50 µM NO_x^-, and at 1 cm to ~230 µmol l^{-1} O_2 and 40 µM NO_x^-. Under those high initial O_2 concentrations, a slight accumulation of 3-6 µM NO_x^- was detected after 2-3 min, followed by a substantial linear decrease in NO_x^- over the next 0.5 to 1.0 hour of incubation in the presence of O_2. NO_x^- was consumed at a higher rate at 1 cm than at 0.5 cm under oxic conditions (Table 1). After O_2 was completely consumed, the NO_x^- reduction rate increased slightly at 0.5 cm depth, however, NO_x^- consumption rates showed no significant difference under oxic and anoxic conditions at 1 cm depth (Table 1), which corresponded to the results observed in the bag incubations with sediments from 0-4 cm depth.

3.4 Aerobic denitrification in a FTSRR

To provide further evidence for the simultaneous consumption of NO_x^- and O_2 in permeable sediments, an incubation was conducted in a stirred retention reactor, in which the slurry was vigorously and continuously mixed. Under constant mixing, substantial $^{30}N_2$ production was again observed by real-time MIMS measurements in the presence of 32 µM O_2 (Figure 4).

$^{15}NO_3^-$ was amended to the continuously stirred chamber 20 minutes after the start of the incubation in the presence of 128 µM O_2. Online MIMS analyses indicated that after an initial lag period of 1.1 hours, significant $^{30}N_2$ production occurred in the presence of 40 µM O_2. Concomitantly, O_2 consumption slowed below that concentration. Simultaneously, there was a slight accumulation of NO_x^- (data not shown) during $^{30}N_2$ production. However, during that period, $^{29}N_2$ production was not concurrent with $^{30}N_2$ production and the increase of NO_x^-. In contrast, $^{29}N_2$ began to accumulate only when NO_x^- decreased at 1.5 hours of incubation in parallel with a 7-fold higher rate of $^{30}N_2$ production (Figure 4).

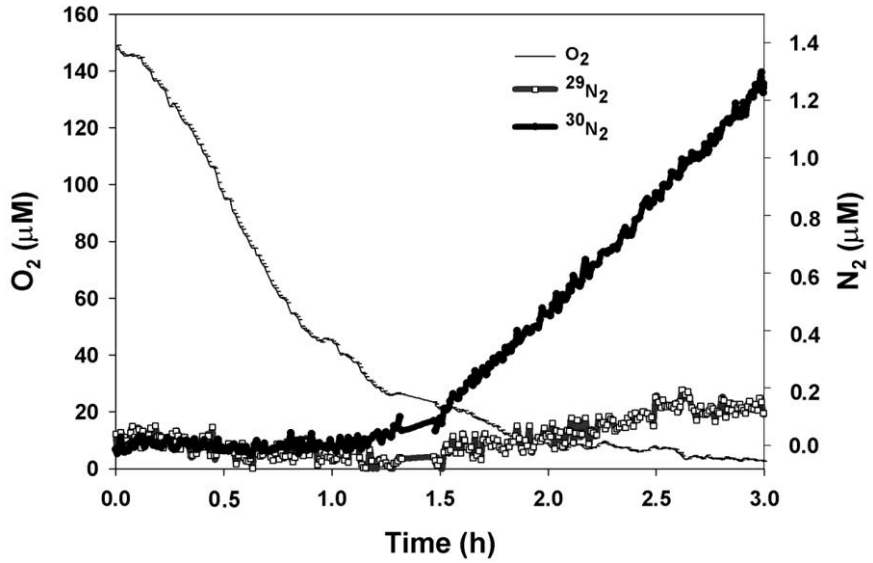

Figure 4: ^{15}N-labeled N_2 production and oxygen concentration versus time during the incubation of permeable sediments in the flow-through stirred retention reactor (FTSRR). Sediments were sampled from the 0-3 cm depth interval of the upper flat during April 2008. $^{29}N_2$, $^{30}N_2$ and O_2 concentrations are depicted as open circles, black squares and open triangles, respectively.

4 Discussion

In permeable marine sediments of the Wadden Sea, zones of NO_x^- and O_2 penetration often overlap to several centimeters depth due to pore water advection (Figure 1) (Werner et al., 2006; Billerbeck et al., 2006a; 2006b; Jansen et al., 2009). Further, previous O_2 percolation experiments that incorporated pore water advection, showed that during the spring season when

NO_x^- is at high concentration in the overlying seawater, O_2 persisted in the bulk pore water over the first 1 to 2 hours of incubation in intact cores of Wadden Sea sediments (Polerecky et al., 2005; Billerbeck et al., 2006b). From these observations, it could be inferred that where NO_x^- and O_2 cooccur, O_2 may not act as the primary or exclusive control of N_2 production in permeable sediment environments. To test this assumption, we investigated N-loss by denitrification in relation to O_2 dynamics. Several lines of independent evidence collected with multiple experimental approaches under near *in situ* conditions showed that denitrification occurs in the presence of oxygen. We observed the immediate and rapid consumption of NO_x^- under air saturated pore water in the intact core, and the directly determined production of ^{15}N-labeled N_2 in the presence of up to 90 µM O_2 in slurry incubations. Further, the rapid production of labeled N_2 was not diminished in a vigorously stirred, flow-through retention reactor. Thus, our results strongly suggest that aerobic denitrification makes a substantial contribution to N-loss in permeable marine sediments.

The rates and mechanisms of N-removal in permeable marine sediments remain in question. Few studies have quantified N_2 production in coastal permeable sediments, and the rate measurements in this small but growing database vary widely, ranging from 0.1 to 3 mmol m^{-2} d^{-1} (Laursen and Seitzinger, 2002; Vance-Harris and Ingall, 2005; Cook et al., 2006; Rao et al., 2007; 2008). However, researchers have now become aware of the fact that in experiments where pore water advection is absent or impeded, a realistic determination of diagenetic processes is not achieved (Jahnke et al., 2000; Cook et al., 2006). At present, at least two mechanisms have been proposed to explain denitrification in the presence of oxygen: 1) co-respiration of NO_x^- and O_2 (Bateman and Baggs, 2005), and 2) closely coupled nitrification-denitrification in microenvironments isolated from bulk sediment pore water (Rao et al., 2007). Bateman and Baggs (2005) provided one of the few observations of the contribution of aerobic denitrifying bacteria to denitrification potential in the environment. Using a combined stable

isotope and acetylene inhibition approach, they were able to distinguish the relative contribution of nitrification and denitrification to N_2O production in arable soil. The results suggested that aerobic denitrification occurred at 20% water-filled pore space.

Although biogeochemical evidence exists for denitrification in the presence of oxygen in the marine environment (Hulth et al., 2005; Hunter et al., 2006; Brandes et al., 2007; Rao et al., 2007), significant rates of aerobic denitrification have not been verified until now. New techniques such as NO_x biosensors and stable N isotope tracers applied in conjunction with MIMS allowed for the further confirmation of aerobic denitrification. Rao et al. (2007; 2008) incorporated the effects of pore water advection, and in corroboration with our results, observed high rates of N_2 production in flow-through columns of oxic permeable sediments. In oligotrophic continental shelf sediments of the South Atlantic Bight, Rao et al. (2007) observed that pore water nitrate was only above detection in the oxic zone. Nitrogen released as N_2 accounted from 80 to 100 % of remineralized N, and the C:N ratio of regeneration supported the interpretation of N_2 produced by denitrification. In the Rao et al. study, the addition of ^{15}N-nitrate caused only a small and gradual rise in $^{29}N_2$ and $^{30}N_2$ production in sediment columns over up to 12 days of incubation. Only columns with anoxic outflow showed substantial $^{29}N_2$ or $^{30}N_2$ production. Thus, Rao et al. (2007) concluded that their evidence for aerobic denitrification was equivocal, and N_2 production more likely occurred from coupled nitrification-denitrification in microenvironments.

In the present study, we observed the rapid and immediate production of ^{15}N-labelled N_2 in the presence of O_2 under a variety of experimental conditions. Oxygen and NO_x^- dynamics were directly determined in real time under well mixed conditions in sediment slurries and intact core incubations. Microsensor measurements showed that NO_x^- and O_2 consumption occurred simultaneously in intact cores (Figure 3). Further direct evidence for the co-respiration of O_2 and NO_x^- was provided using ^{15}N tracer experiments in slurries which were constructed with

sediments from different depths. Successive incubation experiments showed the reliability and uniformity of aerobic denitrification rates, despite the fact that the experimental setup differed (including the amount of sediments, volume of associated water, and starting concentration of labeled nitrate; Supplementary Table 1). Although the concentrations of $^{29}N_2$ and $^{30}N_2$ in the associated water varied, the denitrification rates normalized to sediment volume were in the same range, with the exception of the higher rate measured by the microsensor, which incorporated NO_3^- assimilation as well as denitrification (Table 1). Under the experimental conditions used, $^{29}N_2$ could be attributed to coupled nitrification-denitrification or anammox in the slurry (Thamdrup and Dalsgaard, 2002). In contrast, $^{30}N_2$ would only be produced by complete denitrification. Anammox was shown to comprise only a small percentage of N_2 production in parallel slurry experiments conducted in gas-tight bags (Gao et al., in preparation). Therefore, we conclude that the ^{15}N-labeled N_2 production is mainly contributed by denitrification, and the occurrence in the presence of O_2 provided evidence for aerobic denitrification.

At each depth examined in slurry incubation, the potential denitrification rate under aerobic conditions was similar to that measured under anaerobic conditions. Moreover, the maximum denitrification rate was not observed in the deepest depth interval with the lowest initial O_2 concentration, but rather in the surface 0 to 4 cm depth. This suggests that the overlapping NO_x^- concentration may act together with O_2 to control the denitrification rate. On the Janssand tidal flat during winter/spring, rapid denitrification is likely to be supported by the constant supply of NO_x^- advected from the overlying seawater (Gao et al., in preparation). In short, O_2 dynamics did not strongly affect N-loss by denitrification in the presence of abundant NO_x^-, but rather denitrification co-existed with O_2 respiration in permeable Wadden Sea sediments affected by advection.

In order to further exclude the possibility of anoxic microniches forming in our sediment incubations, we conducted an experiment in a vigorously mixed FTSRR. The initial production of $^{30}N_2$ in the presence of 40 µM O_2 (Figure 4) provided evidence for the process of aerobic denitrification. The concomitantly suppressed O_2 consumption may indicate that nitrate acts as a competitive electron acceptor to facultatively aerobic denitrifying bacteria. Whereas at lower O_2 concentrations later in the incubation (where an increased ratio of unlabeled NO_3^- was observed), the production of $^{29}N_2$ indicated denitrification coupled to nitrification. Due to the variability in the mass abundance signals, we cannot exclude the possibility that some $^{29}N_2$ was also produced in the early stages of the FTSRR incubation. Thus, we observed the mechanism for rapid denitrification under oxic conditions depended on two pathways- aerobic denitrification and dentrification coupled to nitrification.

During the FTSRR incubation, the bulk porewater was vigorously flushed by aerated seawater and the labeled isotope ratio was kept constant. Thus, the possibility that denitrification occurred in anoxic microzones can be completely excluded. In corroboration with our results, previous studies on the formation of anoxic microzones in particles and aggregates showed that the respiration capacity is simply not sufficient to create anoxia under high ambient O_2 concentration, and anoxic microzones more likely form at around 10 % of air saturation (under ~ 25 µM O_2 in the bulk phase; (Schramm et al., 1999; Ploug, 2001). In our study, at O_2 concentrations of ~20 % air saturation and above, the establishment of anoxic microzones would be unlikely. Given the larger grain sizes present in marine sands, O_2 transport is enhanced by advection / interstitial fluid flow, which produces less steep O_2 gradients at the sediment-water interface and within particles / aggregates compared with those that develop under pure diffusion conditions (Ploug, 2001). The abovementioned experiments were conducted in a vertical flow system under nonturbulent uniform flow conditions. Thus, for the coarse-grained sediments in our well-mixed retention reactor experiments where the sediment slurry is exposed to turbulent

aerated flow, anoxic microzones would not form. Therefore, we conclude that substantial N-loss occurs by aerobic denitrification in the permeable Wadden sediments.

Denitrification has long been considered as an anoxic biogeochemical process in marine and aquatic environments, and oxygen has been shown to inhibit denitrifying enzyme activity (Tiedje et al., 1982; Hulth et al., 2005; Brandes et al., 2007). However, a phylogenetically and physiologically diverse group of microorganisms has been shown to denitrify in the presence of oxygen in laboratory studies (Zehr and Ward, 2002; Hayatsu et al., 2008). Bacteria capable of aerobic nitrate respiration were cultured in abundance from freshwater soils and sediments (Carter et al., 1995). Aerobic denitrifiers were further isolated from a variety of managed and natural ecosystems (Patureau et al., 2000). Thus, the influence of oxygen on nitrate respiration activity appears to vary between microorganisms, with some strains able to respire nitrate at or above air saturation (Lloyd et al., 1987; Hayatsu et al., 2008). Microbiological studies have gone so far as to suggest that the capacity for denitrification under aerobic conditions is the rule rather than the exception amongst ecologically important denitrifying microbial communities (Lloyd et al., 1987).

Previous studies indicate that the diversity, as well as the metabolic activity, of bacterial communities is high in permeable sediment environments, likely due to increased transport of growth substrates and the removal of metabolites by advective exchange with the overlying water column (Hunter et al., 2006; Mills et al., 2008; Boer et al., 2009). Denitrification in the marine environment is believed to be mediated by a group of facultative anaerobes that display a wide range in phylogenetic affiliation and metabolic capabilities (Zehr and Ward, 2002). In pristine ecosystems, nitrate concentrations are typically too low to select for large populations of denitrifying organisms, and denitrifiers are thought to rely on aerobic heterotrophy in conjunction with their denitrification capacity (Tiedje, 1988). In permeable marine sediments,

up- and downwelling of pore water associated with sandy sediment ripples generates redox oscillations that may promote the microbially-mediated oxidation and reduction of N species.

Although the consensus is that low or no O_2 is required for the initiation of denitrification, most information on the O_2 level at which denitrification starts comes from pure cultures. Denitrification has been observed in the laboratory at O_2 concentrations approaching air saturation (Zehr and Ward, 2002), but previous environmental studies are equivocal with regard to the impact of O_2 dynamics on denitrification. Large differences are observed in the expression and regulation of denitrification genes between species studied in pure culture (Shapleigh, 2006). The expression of denitification genes was shown to require O_2 in some cases, and the presence of denitrification intermediates may impact the denitrification rate in the presence of O_2. A possible explanation is that the accumulation of intermediates slows O_2 respiration, particularly at low O_2 levels, thereby slowing down the aerobic-anaerobic transition and allowing the expression of O_2-requiring denitrification genes (Bergaust et al., 2008).

We hypothesize that the co-respiration of nitrate and O_2 represents an adaptation of denitrifiers to recurrent tidally-induced redox oscillations in permeable sediments of the Wadden Sea. Some evidence from pure cultures of denitrifying bacteria supports this hypothesis. For example, when the selective pressure of environmental redox changes was removed, the aerobic denitrification ability of *Paracoccus denitrificans* decayed (Dalsgaard et al., 1995; Robertson et al., 1995). Further, Bergaust et al. (2008) proposed that denitrifiers adapt to recurrent oscillations in oxygen concentrations through a protection mechanism, which consists of the coordinated expression and activity of the denitrification enzymes for survival during the rapid transition from oxic to anoxic conditions. A “bottle neck effect” was also proposed, whereby nitrifying and denitrifying bacteria react to oxygen and nitrate in the environment by coordinating their respective activities. Schmidt et al. (2003) observed that the onset of the aerobic denitrification did not depend on oxygen sensitivity of the corresponding enzymes, but rather on regulation of

redox-sensing factors at the transcriptional level. Our biogeochemical evidence corroborates microbiological studies to indicate a clear need to elucidate the significance and the controls of aerobic denitrification in permeable marine sediments.

In contrast to the paradigm that denitrification is an exclusively anaerobic process, our experiments point to aerobic denitrification and indicate that O_2 may not act as a primary or exclusive control of N_2 production in permeable marine sediments. We propose that the availability of NO_x^- as well as O_2 limit the denitrification rate at depths of marine sands that are impacted by pore water advection. We can only speculate on the mechanism of aerobic denitrification at this time. Co-metabolism would imply that both NO_x^- and O_2 are used simultaneously as electron acceptor in a single organism. Alternatively, separated denitrifying and oxygen respiring populations may be active within the community. In the first case one would expect a competition for electrons within the electron transport chain, thus an enhanced denitrification upon oxygen depletion. In the second case, denitrification would be uncoupled entirely from the presence of oxygen, as denitrification is not kinetically inhibited by oxygen, nor can oxygen compete for electrons. In the FTSRR, we observed a pronounced effect of oxygen on denitrification rate whereas in other incubations less of an effect was found, indicating that both mechanisms may be present. Further research is needed to elucidate the true mechanisms of aerobic denitrification in permeable marine sediments.

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Chapter 5

Discussion

Discussion

5 Discussion

Studies on the bacterial colonization of marine sand grains date back to the 1960's (Meadows and Anderson, 1966), exemplifying the preference of microorganisms to aggregate, either exclusively with each other, forming microbial aggregates, or they aggregate with each other attached to a surface (e.g. sediment), often embedded in viscous excretions known as extracellular polymeric substances (EPS) (Vandevivere and Bavaye, 1992; Ludwig, 2004). These viscous excretions are especially obvious components of biofilms, microbial aggregates and microbial mats. The formation of biofilms and aggregates can be interpreted as a first attempt to stratify. From a thermodynamic and ecological point of view, the tendency of microbial communities to stratify in nature can be explained by a competition for substrate, but the ability of microorganisms to compete with each other is determined within the boundaries of their ability to gain energy (Van Gemerden, 1993; Ludwig, 2004; Canfield et al., 2005).

The EPS layer of biofilms, microbial mats and aggregates can provide a competitive advantage for the resident microorganisms in many ways. The formation of a physical, diffusive barrier increases the resistance to environmental stress, such as biocide/antibiotic exposure or salinity stress (Costerton et al 1987, 1999; Prosser et al., 2007; Kohls, 2009), and it conveys the presence of quorum sensing, which can for example accelerate the recovery from starvation, as e.g. shown for *Nitrosomonas europaea* (Batchelor et al., 1997).

The stratified systems which were studied in this thesis are microbial aggregates, microbial mats and intertidal sandy sediments, which were characterized by temporal changes in salinity and/or oxygen, and were examined with a focus on aerobic ammonia oxidation and denitrification.

5.1 Adaptation to hypersaline conditions and the influence on aerobic ammonia oxidation

Salinity adapted microorganisms face high salt concentrations, which induces low water activity (Oren, 2002). Two main strategies are known to maintain turgor pressure, which is at least to some extent pivotal for expansion and growth of cells (Brown, 1978, 1990, Oren, 2002). The osmotic balance of a cell can be maintained by adjustments on cellular salt concentrations (mainly potassium chloride, KCl) to provide osmotic balance with the outside medium. This way of turgor pressure maintainance is only chosen by a minority of halophiles including halophilic archaea (Order *Halobacteriales*), anaerobic halophilic bacteria (Order *Halanaerobiales*) and *Salinobacter ruber* (Oren, 2002).

In most halophilic and halotolerant bacteria and methanogenic archaea, low molecular weight organic compounds (compatible solutes) are used to provide an osmotic balance and turgor (Brown and Simpson, 1972). The maintenance of the turgor pressure does not require salt adapted enzymes in this case (Da Costa et al., 1998; Galinski, 1993). However, in bacteria, the intracellular Na^+ and K^+ concentrations do increase, in response to externally increasing salinity, but taken together, intracellular salt concentrations are usually not sufficient to balance the osmotic pressure of the medium, which is mainly provided by the already mentioned compatible solutes (Oren, 2002).

In general, some metabolic types present in nature are not expected to play a major role in hypersaline habitats according to their energy gains. For example, high energy-yielding processes like denitrification ($\Delta G° = -398$ kJ per reaction), as well as anoxygenic photosynthesis and aerobic respiration ($\Delta G° = -402$ kJ), can occur close to NaCl saturation; whilst low energy-yielding processes such as autotrophic ammonia ($\Delta G° = -278$ kJ) and nitrite oxidation ($\Delta G° = -82$ kJ) do not seem to thrive under similar conditions. In other words, an organism can make a living at high salt concentrations, if the amount of generated energy within its dissimilatory metabolism is high enough to cover the additional costs of osmotic adaptation beside their

normal metabolic activities (Oren 2002; ΔG° values from Lam and Kuypers, 2011, calculated for 25 ºC pH 7, with unit activity for all reactants and products. ΔG° for respiratory pathways are standardized to a 4 e− transfer equivalent to the oxidation of 1 mol of organic carbon).

Apart from the additional energy demand for halotolerance or halophilia, all organisms living within the mat face further challenges on substrate acquisition. The close proximity of various functional groups within the mats, and the very restricted spatial distribution along strong vertical physico-chemical gradients, pose potentially strong competition among major players for substrate availability, in order to maintain their energy demands. Potentially high substrate affinity, and apparently low activity of nitrifiers, might contribute to their survival under oligotrophic hypersaline conditions. Especially, nitrifiers have been known to survive long periods of dormancy despite apparent substrate and energy deprivation (Jones and Morita, 1985; Bollmann et al., 2002). This specific situation might explain why it is possible to show the presence and activity of nitrifiers within mat-systems, even though salinity increases up to the point where the surrounding body of water is fully saturated with salt, and eventually evaporated.

On one hand, nitrification has already been shown in habitats that are described as hypersaline, such as Mono Lake at a salinity of up to 8.8% (Ward et al., 2000). On the other hand, it was not shown at very high salinities above 15% in the Great Salt Lake (Post and Stube, 1988), nor in marsh waters (Rubentschick,1929, cited by Hof, 1935). However, Post and Stube (1988) do not exclude the possibility that nitrification takes place at a very slow rate, which is exactly what is shown at least for ammonia oxidation in the here-presented study on microbial mats (Chapter 2). At the same time, the fairly high NOx consumption, which is 2-3 orders of magnitude higher than the observed ammonium oxidation rates, additionally confirms the observation of Oren (2002) that some metabolic types such as denitrification, can occur close to NaCl saturation, whilst other processes like autotrophic ammonia oxidation do not seem to thrive under similar conditions (Oren, 2002). However, Oren (2002) suggested that nitrifiers cannot

persist at all at salinities above 15%, assuming that the amount of generated energy within its dissimilatory metabolism is not high enough to cover the additional energy costs of osmotic adaptation, what is clearly contradictory to our observations. Nevertheless, it is not clear from our study if the observed ammonia-oxidizing activities only represent potentials for periods when salinities decrease due to mimicked tidal regimes, but it is clear that nitrifiers can persist under hypersaline conditions.

5.2 Oxygen supply in the aerobic ammonium oxidation study

Gas solubility decreases with increasing salinity, so oxygen penetration depth into stratified systems is also expected to be decreased under hypersaline conditions. This might have an additional influence on the distribution of aerobically growing microorganisms, as the here studied ammonia-oxidizers (Chapter 2), but this aspect is only of minor or even negligible importance. Aerobic ammonia oxidation requires oxygen, but oxygen demands are usually fairly low, and aerobic ammonia oxidation is therefore often observed in habitats with low oxygen concentrations (Henriksen and Kemp, 1988; Voytek and Ward, 1995; Kalvelage et al., 2011). Additionally, nitrifiers are expected to be fairly close to the surface layer of the microbial mat, in order to avoid sulfide inhibition from below.

However, the oxygen penetration into the microbial mats was hampered by dark incubations (suppressing the activity of photosynthetic organism), which were chosen in order to avoid potetntial photoinhibiton of ammonia oxidation. This practice is typically chosen for the establishment of enrichment cultures of ammonia oxidizers. However, enrichment cultures are usually not started with big pieces of biomass as used in this study, so the potential of oxygen depletion here might remain comparably high. Microbial mat pieces were further chopped into smaller units prior to our incubation experiments, in order to increase the mat surface area and thereby to enhance the O_2 penetration into the system, as trade-off for the lack of oxygen supply via photosynthesis.

5.3 AmoA gene distribution upon salinity fluctuations

The archaeal and bacterial ammonia-oxidizers in the hypersaline microbial mats, as detected via their signature *amoA* (encoding ammonia monooxygenase subunit A) sequences, were characterized by a rather limited diversity, with only a small number of OTU´s even with a 99% nucleic acid sequence identity cut-off.

The betaproteobacterial *Nitrosospira*-like *amoA* gene sequences seem to cluster predominantly with other *amoA* sequences of marine or estuarine descent, in particular with sequences retrieved from sediments and the Arctic and Antarctic water columns (Moiser and Francis, 2008; Kalanetra et al., 2009; Moin et al., 2009 Christman et al., 2011; Wankel et al., 2011). *Nitrosospira*-like sequences dominate many clone libraries from oceanic environments (Bano and Hollibaugh, 2000; O'Mullan and Ward, 2005), and *Nitrosospira* spp. are postulated to generally have a higher potential to adapt to salinity than *Nitrosomonas*. In the eutrophic Schelde estuary, the identified ammonia-oxidizing bacteria were almost exclusively *Nitrosomonas*-like, but at more seaward sites, *Nitrosospira*-like sequences were also found (De Bie et al., 2001). Similarly, *Nitrosospsira* cluster 1-like sequences predominated marine sites in a Scottish estuary, while the brackish waters were characterized by the concurrence of the halophilic *Nitrosomonas marina*, the halotolerant *Nitrosomonas* sp. Nm143, as well as the *Nitrosomonas oligotropha* of freshwater origin, and the latter predominated freshwater sites (Freitag et al., 2006). Moreover, the *amoA* sequences found in salt marsh sediments from New England were exclusively *Nitrosospira*-like (Moin et al., 2009), which might again point to more commonly abundant *Nitrosospira*-like strains showing halotolerance or halophilia. However, *Nitrosomonas* isolates from the Elbe River estuary were characterized by species-dependent differences in halotolerance (Stehr et al., 1995), and *Nitrosomonas*-like sequences have also been detected in the hypersaline Mono Lake (Ward et al., 2000), suggesting that different *Nitrosomonas* spp. may be adapted to a wide range of salinity. Besides, factors other than

salinity might also have an impact on the distribution of *ammonia-oxidizing bacteria* in saline habitats. For example, the predominance of *Nitrosospira*-like *amoA* gene sequences over *Nitrosomonas*-like sequences in sediments of the Elkhorn Slough Estuary was shown in conjunction with high nitrification rates, which might be a result of substrate based niche separation (Wankel et al., 2011).

Archaeal *amoA* sequences were only found in one of the hypersaline mats investigated. The closest relatives of the archaeal *amoA* sequences originate exclusively from seawater, falling all into the water column cluster (Francis et al., 2005). Mat A was the mat that was in its natural environment mainly exposed to draught periods, and in the laboratory it was also mainly kept dry. The main source of ammonia-oxidizing archaea was in this case probably given by seawater in the laboratory, but the same ammonia-oxidizing archaea were not found in any other microbial mats. The establishment of archaeal ammonia oxidizers within this mat might thus be attributed to the long draught periods, which decrease the substrate availability within the mat, and thereby form a niche for archaeal ammonia oxidizers, with their typically higher substrate affinity than their bacterial counterparts (Martens-Habbena et al., 2009).

Altogether, ammonium-oxidizing activity was detectable in four microbial mats, which was corroborated by the detection of the biomarker functional genes for ammonia oxidizers in at least three mats. Consistent with observations in estuarine and coastal sediments, bacterial *amoA* genes were more readily detectable than archaeal *amoA* genes (Caffrey et al., 2007; Mosier and Francis, 2008; Santoro et al., 2008; Magalhaeas et al., 2009), which might indicate that ammonia-oxidizing bacteria predominate upon environments characterized by salinity fluctuations, as they are given within the examined microbial mats.

5.4 Adaptation of denitrification to salinity

In chapter 3, the effect of salinity on denitrification was studied by comparing saline and non-saline grown microbial communities. Microbial aggregates were grown under controlled conditions in upflow-sludge blanket reactors, in order to exclude all other factors than salinity in posing changes on denitrification. Oxygen was not present in the reactors, substrate limitation was not given, seasonal or diel patterns can be excluded, and marked S-cycling was not observed either, what simplifies the evaluation of salinity effects on denitrification (compared to estuaries).

The salinity adaptation of denitrifying organisms is not quite well understood, even though it has been broadly studied. On the one hand, it seems that salinity barely has an influence on denitrification compared to nitrification. Magalhaes et al. (2005) reported in a study on estuary nitrification and denitrification on intertidal sediments and rocky biofilms, clear salinity induced changes only in nitrification activity. Denitrification seemed to be rather unaffected by salinities, which varied at the study site between 0 and 3.5%. Based on these results, it was concluded that halotolerant denitrifying bacteria prevail in this environment.

Similarly, the denitrification rates measured in the eutrophic Neuse River estuary, which had a persistent salinity gradient ranging from 0 to 2%, showed that despite the increasing variability in denitrification rate data with increasing salinity, altogether the denitrification rates themselves did not vary significantly across the salinity gradient (Fear et al., 2005). Nevertheless, the increase in the variability of denitrification rates might coincide with the activity of different microbial communities, which show different salinity optima.

In comparison, reactor studies showed clear salinity induced decreases in denitrification rates (Dincer and Kargi, 1999; Glass and Silverstein, 1999). Glass and Silverstein operated the reactors for 4 months with stepwise increase in ionic strength, and still observed a decrease in nitrate reduction down to 38% of the initial nitrate reduction rate. Complete denitrification did

occur, even though transient nitrite accumulation was also observed. The negative correlation between denitrification and salinity, and at the same time the observation of complete denitrification, might reflect on-going adaptive changes in denitrifying microbial communities, which have not yet fully adjusted to the imposed changes in salinity. Extension in adaptation time could probably allow further adjustment to higher nitrate reduction rate and reduced nitrite accumulation.

In the here presented study (Chapter 3), we show that salinity exerts a strong effect on non-adapted denitrifying microbial communities, imposing right away a strong decrease of denitrification rate and increases in nitrite accumulation upon increasing salinities. At the same time, it seems that salinity adapted microbial aggregates are thriving better at salinities slightly above their growth salinity (1% growth salinity versus higher denitrification rates at 1.65% , 2,05 % and 2.5% salinity). This apparent 'over-adaptation' might be related to their specific type of salinity adaptation or an increase in activity of parts of the microbial community, which are characterized by a "sub-optimal over-adaption" at ambient salinity conditions. Halophiles, which are having a strong Na^+/H^+ antiporter activity, can maintain lower intracellular sodium concentrations than given in the surrounding medium. This poses a gradient across the membrane, which can be used for substrate transport into the cell. Many studies showed salinity-gradient mediated amino acid transport into microbial cells (Stevenson, 1966; Lanyi et al., 1976; MacDonald et al., 1977; Birkeland and Ratkje, 1985), but acetate (the C-source in our study) was also observed to be transported via salinity mediation within *Natronococcus occultus* (Kevbrina et al., 1989). Altogether, an increase in denitrification rate at higher salinities than the original growth salinity might arise from a more efficient substrate transport into the cells, as a rise in external salinity increases the salinity gradient across the membrane and thereby facilitates more efficient substrate uptake.

However, microsensor measurements showed a nitrite peak at the surface of the aggregates, followed by the main area of denitrification in a sub-surface layer. A reduction of the surface-to-volume ration may decrease the relative size of the niche of exclusively nitrite producing microorganisms, and thereby it might also decrease the amount of produced nitrite, which would be desirable in waste water treatment. In general, more organisms are capable of maintaining an energy metabolism by exclusively reducing nitrate to nitrite (Gonzalez et al. 2006, Zumft 1997), without any further reduction to N_2 ($\Delta G° = -244$), what may explain why nitrite accumulates at all. Moreover, it is accumulating stronger within non-saline grown aggregates upon increasing salinities, than in saline grown aggregates. This observation suggests that nitrate reduction to nitrite is less affected by salinity increases, than consecutive steps of denitrification. The occurrence of DNRA (dissimilatory nitrate/nitrite reduction to ammonium) cannot be excluded either within the aggregates (DNRA with NO_3^- => $\Delta G° = -257$ or DNRA with NO_2^-=> $\Delta G° = -222$). Less energy yield is expected through DNRA than denitrification, but efficient denitrification relies on well coordinated functioning of 4 enzymes at the same time (Zumft, 1997). Results show especially within the non-saline grown aggregates an unbalanced budget between NOx consumption and N_2 production, which might indicate the prevalence of DNRA when denitrification rates decrease. All $\Delta G°$ in this section are from Lam and Kuypers, 2011.

5.5 Adaptation of denitrification to oxygen

Denitrification, which is considered to be an anaerobic process, is shown to occur under oxic conditions (Chapter 4). Rates were measured in the presence of oxygen, suggesting that adaptation of denitrifying bacteria to tidally-induced redox oscillations took place within the permeable sediments at Janssand, which might be either attributed to co-respiration of NO_x^- and O_2 (Bateman and Baggs, 2005) or closely coupled nitrification-denitrification in microenvironments isolated from bulk sediment pore water (Rao et al., 2007). Slurry incubations showed denitrification activities at initial oxygen concentrations as high as ~ 90µM, which

would not exclude the possibility of either co-respiration of NO_x^- and O_2, nor closely coupled nitrification-denitrification in microenvironments. However, it is also possible that anoxic microenvironements form around particles, and thereby bias the provided rate information. The likelihood of an influence of such anoxic microenviroenments is however reduced by performing experiments in a vigorously mixed Flow Through Stirred Retention Reactor (FTSRR). Within the FTSRR, the initial production of $^{30}N_2$ was observed in the presence of ~40 µM O_2, which accounts for ~ 44% of the oxygen concentration determined in prior slurry incubations, suggesting that anoxic microenvironment formation might indeed play a role, but co-respiration of NO_x^- and O_2 seemed to occur as well.

Concomitant suppressed O_2 consumption was observed, which might indicate that nitrate acted as a competitive electron acceptor to facultatively aerobic denitrifying bacteria. At significantly lowered O_2 concentrations, the production of $^{29}N_2$ was observed, suggesting denitrification coupled to nitrification. Taken together, a mechanism for rapid denitrification under oxic conditions is probably depended on two pathways: (1) aerobic denitrification and (2) dentrification coupled to nitrification.

Microbial diversity and metabolic activity are high in permeable sediment environments, which are attributed to increased transport of growth substrates and the removal of metabolites by advective exchange with the overlying water column (Hunter et al., 2006; Mills et al., 2008; Boer et al., 2009). These active and diverse communities likely include denitrifying communities (Zehr and Ward, 2002). Additionally, Schmidt et al. (2003) observed that the onset of aerobic denitrification depended on the regulation of oxygen- and redox-sensing factors at the transcriptional level, and not on the oxygen sensitivity of the corresponding enzymes. In concert, these points suggest that O_2 may not act as a primary or exclusive control of N_2 production in permeable marine sediments, but the availability of NO_x^- might limit the denitrification rate at depths of marine sands that are impacted by pore water advection.

Furthermore, many laboratory studies have shown the occurrence of aerobic denitrification, in contrast with the paradigm that denitrification is an exclusively anaerobic process. A phylogenetically and physiologically diverse group of microorganisms has been shown to denitrify in the presence of oxygen (Zehr and Ward, 2002; Hayatsu et al., 2008), and aerobic denitrifiers were isolated from a variety of managed and natural ecosystems (Patureau et al., 2000). The influence of oxygen on nitrate respiration activity varies among microorganisms, with some strains able to respire nitrate at or above air saturation (Lloyd et al., 1987; Hayatsu et al., 2008). A study in oceanic oxygen minimum zones showed that in fact aerobic and anaerobic N-cycle pathways can co-occur over a large range of O_2 concentrations, with nitrate reduction at one study site remaining fully active at 25 µM O_2 (Kalvelage et al., 2011). Additionally, large differences were observed in the expression and regulation of denitrification genes between species studied in pure culture (Shapleigh, 2006). The expression of denitification genes was even shown to require O_2 in some cases (Bergaust et al., 2008).

Altogether, the adaptive potential to denitrify under oxic condtions is given within denitrifying microbial communities, and substrate availability does not seem to be a limiting factor in the studied habitat. Hence, the only potentially limiting factor would be oxygen, which is likely to select for aerobic denitrification under given environmental conditions.

6 Conclusions

The presented studies provided an insight into aerobic ammonia oxidation and denitrification within stratified systems, which were all characterized by temporal changes in salinity and/or oxygen. The presence and activity of ammonia oxidizers was shown in hypersaline microbial mats (Chapter 2), despite the unfavorable energetic conditions given by meager energy yields, which were further decreased by the metabolic need to maintain some kind of salinity adaptation. The fairly low ammonia oxidation rates, which were measured, explain why ammonia oxidation was often assumed to be absent in hypersaline environments, or why it was often only subject to

speculations. The low diversity of *amoA* gene sequences, suggest a strong salinity induced selection within ammonia oxidizing communities. The general predominance of bacterial *amoA* gene sequences over archaeal gene sequences corroborates with observations made in estuaries, which are also exposed to salinity fluctuations like the studied microbial mats.

Denitrification was studied within two types of stratified habitats. One study focused on the influence of salinity (Chapter 3), and the other on the influence of oxygen (Chapter 4). The influence of salinity on denitrification in microbial aggregates (Chapter 3) was highly dependent on prior growth conditions. Saline grown aggregates were best adapted to salinities slightly above their growth salinity, whilst strong decreases in denitrification rates and denitrification efficiency were seen for non-saline grown microbial communities upon increasing salinity. The increased salinity gradient along the membrane of the saline grown aggregates might be used for substrate transport into the cell, and thereby explain why salinities higher than the original growth salinity increase denitrification rates. From an applied point of view, it highlights the importance of using pre-adapted denitrifying communities in the treatment of brackish water or treatment of brine. Furthermore, the surface of the microbial aggregates was identified as the source of the excess nitrite, suggesting that a potential reduction of the surface-to-volume ratio of the aggregates might reduce as well the excess nitrite production to some extent.

Oxygen was so far considered as having an adverse effect on denitrification, due to the fact that O_2 acts as a competing electron acceptor for NO_3^- respiration and key enzymes of the denitrification pathways are inhibited by relatively small amounts of O_2 (Payne, 1981; Zumft, 1997; Shapleigh, 2006). Nevertheless, in permeable sandy sediments was the presence of aerobic denitrification shown (Chapter 4), suggesting that O_2 may not act as a primary or exclusive control of N_2 production in permeable marine sediments, while the availability of NO_x^- might limit the denitrification rate at depths of marine sands that are impacted by pore water advection.

Co-respiration of NO_x^- and O_2 (Bateman and Baggs, 2005) is proposed as a likely mechanisms of aerobic denitrification in the intertidal permeable sandy sediments.

7 Outlook

The impact of salinity fluctuations on ammonia oxidizers could be further studied, including aspects as salinity adaptations, salinity induced community shifts, diversity changes and activity changes upon saline and non-saline conditions. Ammonia oxidizing microbial communities would potentially show different adaptive strategies to salinity changes, e.g. compatible solutes vs. high intracellular KCl-concentrations. The function of Na^+/H^+ pumps could be compared within ammonia oxidizing archaea and bacteria, and more detailed with focus on potentially different strategies of *Nitrosomonas* spp. and *Nitrosospira* spp., as the observation of Wankel et al. (2011) showing a predominance of *Nitrosospira* associated with higher nitrification rates in a saline habitat might indicate differences in the activity of Na^+/H^+ antiporters, which might be used to increase substrate transport into the cell. This difference in Na^+/H^+ antiporter activity would also be relevant in order to examine the over-adaptation to salinity changes within saline grown microbial aggregates (Chapter 3).

In addition to the remaining questions already mentioned in chapter 3, the influence of salinity on methylamine production should also be further investigated. Saline microbial aggregates, which were kept for a longer period of time outside of a flow-through system, as e.g. an USB reactor, produced large amounts of methylamine when exposed to denitrification inducing substrates. This observation is not yet properly understood and might be related to the degradation of glycine betaine, which is an osmotic stabilizer of many prokaryotic halophiles (Oren, 2002). The physiological trait, which induces strong methylamine production, whilst N_2 production rates are fairly low or not measureable, might be an alternative physiological pathway, applied upon environmental stress. However, it is not clear how the added ^{15}N-labeled substrate is used in this case, as the measured methylamine turned out to be non-labeled. DNRA

would be a simple and straightforward explanation, which could be easily verified, by measuring the production of ^{15}N-labeled ammonium.

Enrichment cultures from the studied Wadden Sea sediments (Chapter 4) might be a good way to get first information on the microbial players involved in aerobic denitrification in permeable marine sediments. The culturing could take place under varying controlled oxygen levels, revealing potential oxygen-induced differences in the physiologies, metabolic pathways and composition of microbial communities. These enrichment cultured studies could be further coupled with molecular ecological analyses, e.g. community gene expression and proteomics, to further illustrate the responses of potential aerobic denitrifiers to changing oxygen regimes. Pure cultures might also be isolated from these enrichment cultures with aerobic and anaerobic media, as well as gradient tubes. The latter are likely to show oxygen tolerant denitrifying cultures closer to the surface of the tube. These cultures could be used to further elucidate the mechanisms of aerobic denitrification.

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Eidesstattliche Erklärung

Gem. § 6(5) Nr. 1-3 PromO

Hiermit erkläre ich, dass ich

1. die Arbeit ohne unerlaubte fremde Hilfe angefertigt habe,
2. keine anderen als die von mir angegebenen Quellen und Hilfsmittel benutzt habe und
3. die den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen als solche kenntlich gemacht habe.

Olivera Svitlica, Bremen, den 19. Februar 2012