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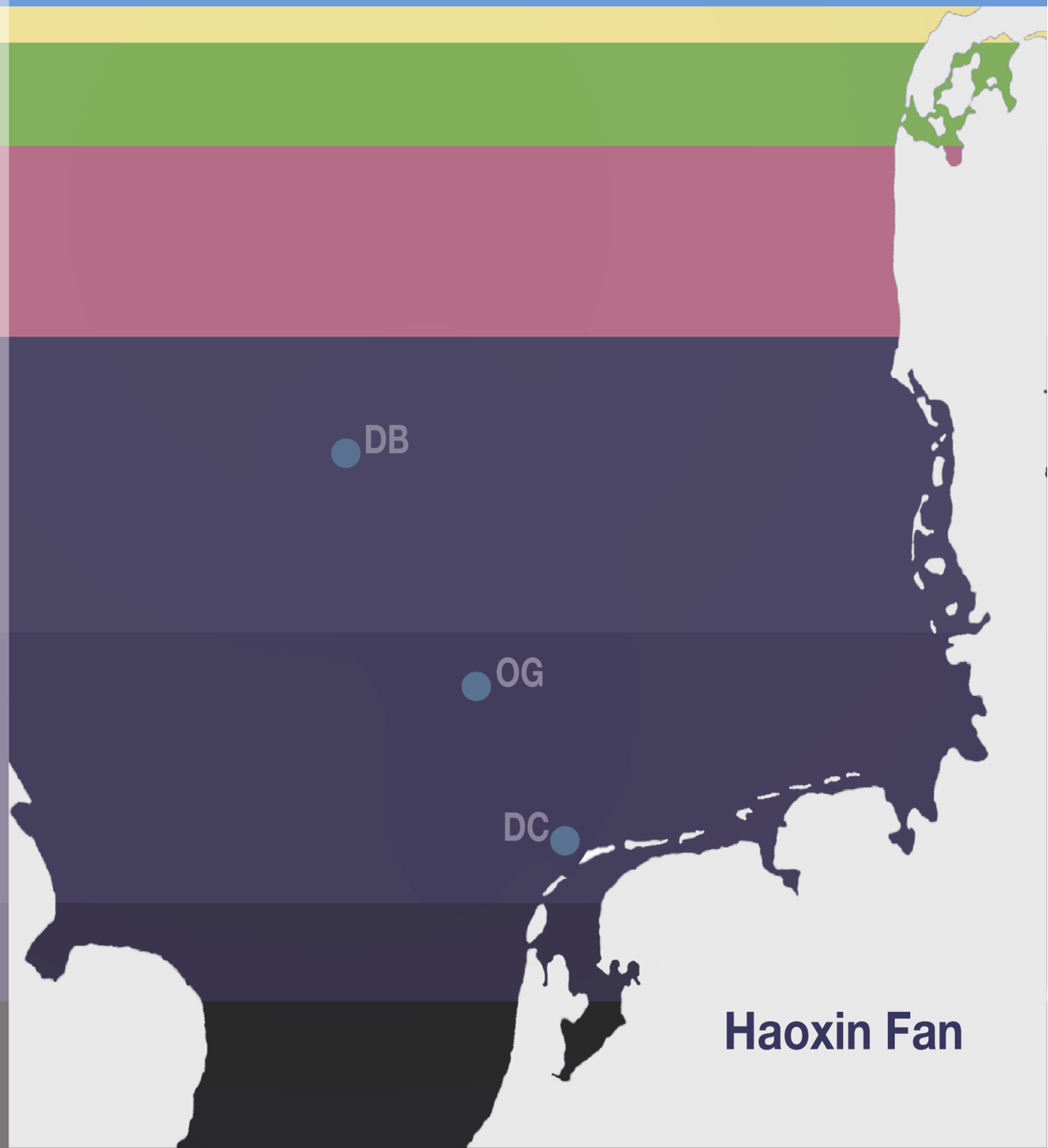

Haoxin Fan

Investigations on the nitrogen cycle in the coastal North Sea

ACADEMISCH PROEFSCHRIFT

ter verkrijging van de graad van doctor

aan de Universiteit van Amsterdam

op gezag van de Rector Magnificus

prof. dr. D.C. van den Boom

ten overstaan van een door het College voor Promoties ingestelde commissie,

in het openbaar te verdedigen in de Agnietenkapel

op woensdag 30 maart 2016, te 12:00 uur

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Faculteit der Natuurwetenschappen, Wiskunde en Informatica

Investigations on the nitrogen cycle in the coastal North Sea

ISBN: 978-94-91407-32-1

Cover by: Yu Liu sephinx@gmail.com

The research reported in this thesis was carried out at the Department of Marine Microbiology of the Royal Netherlands Institute for Sea Research in Yerseke. This research was part of the project NICYCLE, which was financially supported by the Dutch Organization for Scientific Research, Earth and Life Science (NWO-ALW) grant 839.08.332 in the framework of the program Sea and Coastal Research (ZKO).

Contents

Chapter 1

General Introduction

Nitrogen cycle processes

Nitrogen is essential for organisms and follows carbon as the most abundant element in living biomass. Proteins represent the bulk of the cellular nitrogen, which is present in the amino groups in amino acids that form with their peptide bonds the backbone of these polypeptides. Nucleic acids, pigments such as chlorophyll, and the peptidoglycan in the bacterial cell wall are other components that contain a considerable amount of the cellular nitrogen.

Nitrogen exists in different redox states and between the most oxidized and most reductive forms there is a difference of eight electrons. Therefore, nitrogen is subject to oxidation and reduction reactions that are predominantly mediated by bacteria and archaea. Oxidized forms of nitrogen such as nitrate or nitrite may serve as electron acceptors and may be reduced during anaerobic respiration, while reduced forms such as ammonium may serve as source of energy when oxidized by chemolithotrophic microorganisms. The major processes of the nitrogen cycle are summarized in Fig.1. This study focuses on four main processes that are relevant in coastal environments: nitrogen fixation, nitrification, denitrification and anaerobic ammonium oxidation (anammox) (Fig. 1). Below, each of these processes is introduced in detail.

Nitrogen fixation

Nitrogen (N_2) fixation is the reduction of atmospheric dinitrogen gas to biologically available ammonium by either high-temperature abiotic (chemical) reactions (lighting or the industrial Haber-Bosch process) or biologically via specialized N_2-fixing microbes. Biological N_2 fixation is an important contributor to the pool of biological available nitrogen in many ecosystems and is the only biological process in the nitrogen cycle that compensates for the losses of nitrogen through denitrification and anammox (see below). Only bacteria and archaea that possess the capacity to synthesize nitrogenase are capable of N_2 fixation. Nitrogenase is an enzyme complex consisting of dinitrogenase (also called the MoFe enzyme) encoded by *nifDK* and dinitrogenase reductase (also called the Fe-enzyme) encoded by *nifH* (Zehr *et al*., 2003). The biomarker nitrogenase gene, *nifH*, is widely used to investigate the diazotrophic community composition and activity.

N_2 fixation occurs in the pelagic as well as in various benthic habitats including photosynthetic microbial mats (Severin and Stal, 2008), sea grass sediments (Herbert, 1999; McGlathery *et al*., 1998) and estuarine and shallow marine sediments (Bertics *et al*., 2013; Fulweiler *et al*., 2007). N_2 fixation is an important process in nitrogen depleted freshwater and brackish water bodies and in the (sub)tropical ocean. In freshwater and brackish water bodies, N_2 fixation is mainly driven by heterocystous cyanobacteria while in the warmer (sub)tropical ocean non-heterocystous (filamentous and unicellular) cyanobacteria are the predominant

nitrogen fixers. Previously, N_2 fixation was thought to be negligible in coastal waters because of ample availability of combined nitrogen (Galloway *et al*., 2004), but this viewpoint is changing.

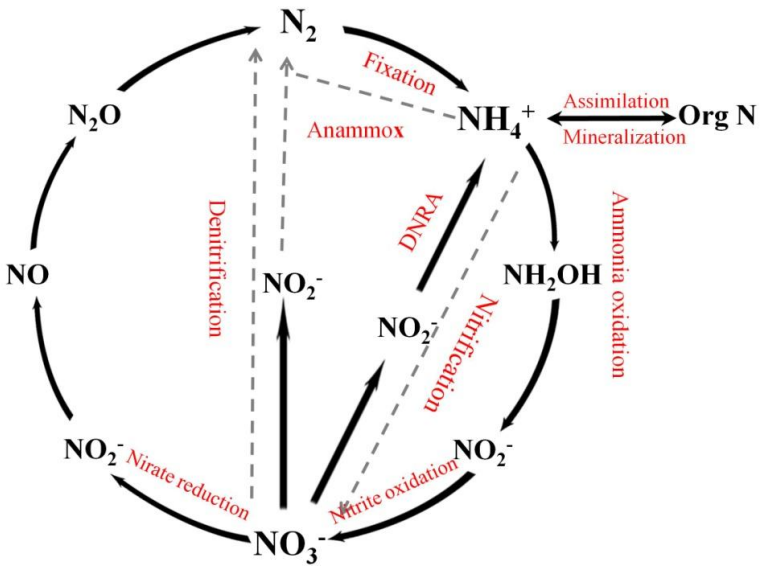

Figure 1. The nitrogen cycle. The arrows mark the transformation processes among all N forms. N_2 fixation is the biological conversion of N_2 into ammonia, which is incorporated into organic N forms. Biological processes transform organic N and release ammonium as the result of mineralization. Ammonium, the most reduced form of N, is either biologically assimilated or oxidized to nitrate via nitrite through nitrification. Nitrate, the most oxidized form of N, is either biologically assimilated (assimilatory nitrate reduction) or is dissimilatory reduced through a series of intermediate gaseous nitrogen oxide products (i.e. NO, N_2O) and is ultimately converted into N_2 gas via denitrification. In addition, nitrate/nitrite can also be reduced to ammonium via dissimilatory nitrate reduction to ammonium (DNRA). Ammonium can be oxidized and converted to N_2 via anaerobic ammonium oxidation with nitrite (anammox).

In the past five years, N_2 fixation has been observed in the Mekong River plume (Grosse *et al*., 2010), in the South China Sea (Dang *et al*., 2013), in the surface water of the English Channel (Rees *et al*., 2009), and in the North American coastal waters between Cape Hatteras and Georges Bank (Mulholland *et al*., 2012). In addition, there is an increasing awareness that diazotrophic microorganisms other than cyanobacteria may be involved in many of these aquatic environments. It has been suggested that gammaproteobacteria might be among the most important heterotrophic diazotrophs (Halm *et al*., 2012; Moisander *et al*., 2014). The progress

that has been made with regard to N_2 fixation in the marine environment was mostly attributed to processes and organisms in the (sub)tropical ocean. For instance, the North Sea has not been investigated for N_2 fixation, except for the microbial mats that develop on the intertidal sediments and beaches along its coast (Severin and Stal, 2008; Severin and Stal, 2010a). A study of the English Channel showed high rates of N_2 fixation and a diverse community of diazotrophic microorganisms (Rees *et al*., 2009). This indicates that a more extensive survey of nitrogen fixation in the North Sea and the microorganisms involved is justified and necessary in order to make accurate estimations of the N budget in this region.

Most studies of benthic N_2 fixation focused on environments exposed to light, such as photosynthetic microbial mats (Joye and Paerl, 1993; Severin and Stal, 2008; Severin *et al*., 2010). High rates of N_2 fixation have been reported from several microbial mats. Nitrogen fixation is essential for the development and productivity of the microbial mats because this environment is usually depleted of nitrogen. However, the contribution of microbial mats to the N budget of coastal marine ecosystems is minor because of their restricted areal distribution (Herbert, 1999). Cyanobacteria and proteobacteria are the main contributors to the *nifH* pool in photosynthetic microbial mats (Severin and Stal, 2010b; Zehr *et al*., 1995). The diazotrophic communities and their nitrogen-fixing activities vary in time and in space. For example, Severin and Stal analyzed *nifH* expression of different groups of diazotrophs in three types of microbial mats on the beach of the North Sea barrier island Schiermonnikoog and found distinct patterns during a 24 h day in each of them (Severin and Stal, 2010a). These authors found that at a station near the dunes, cell specific *nifH* expression of unicellular Cyanobacteria was highest among five groups of phototrophs that exhibited higher expression during the day. At the intertidal station, characterized by mature microbial mats, the *nifH* expression associated with filamentous *Oscillatoria*-type and unicellular cyanobacteria were both high. The *nifH* expression levels related to *Oscillatoria* reached maxima at sunset and shortly before sunrise. The expression levels by unicellular cyanobacteria were higher at night. The diazotrophic community in a North Carolina (USA) cyanobacterial mat shifted from diazotrophic cyanobacteria in the summer to heterotrophic diazotrophic organisms in the fall and winter (Zehr *et al*., 1995).

Despite the notable exception of intertidal microbial mats, marine sediments (e.g. North Sea bottom sediments) serve as a nitrogen sink (Seitzinger, 1988). A few publications report heterotrophic N_2 fixation in coastal waters (Bertics *et al*., 2010; Fulweiler *et al*., 2007; Gardner *et al*., 2006). This suggests that marine sediments may be a net source of N at least during part of the year (Fulweiler *et al*., 2007; Lee and Joye, 2006). Therefore, even when marine sediments do not overwhelmingly become a source of fixed nitrogen, they may be less of a sink as hitherto thought (Knapp, 2012). Several studies suggested that sulfate-reducing bacteria are the main

organisms responsible for benthic marine N_2 fixation in anaerobic bottom sediments. In Chesapeake Bay (USA) sediments, many *nifH* DNA sequences phylogenetically grouped with *nifH* sequence from sulfate reducers, such as *Desulfovibrio salexigens* and *Desulfobacter curvatus* and nitrogenase activity decreased substantially when sulfate reduction was inhibited (Burns *et al*., 2002). Fulweiler *et al*. (2013) found that *nifH* mRNA transcripts were limited to two phylogenetic groups related to *Pelobacter carbinolicus* and *Desulfovibrio vulgaris*, anaerobes that reduce sulfur and sulfate, respectively, in the estuarine sediments of Narragansett Bay (USA). These reports suggest that benthic N_2 fixation in marine sediments other than intertidal microbial mats has been overlooked.

Denitrification and anaerobic ammonium oxidation

Benthic N_2 production is the main sink of bound nitrogen in the marine nitrogen cycle, accounting for up to 70% of combined nitrogen escaping as gas to the atmosphere (Codispoti *et al*., 2001; Galloway *et al*., 2004). According to Codispoti (2007), this loss of bound nitrogen from shelf sediments accounts for more than 60% of the total oceanic dinitrogen production. Therefore, the shelf plays an important role in the marine nitrogen cycle. Denitrification and anaerobic ammonium oxidation (anammox) are the two processes that are responsible for the conversion of bound nitrogen into gaseous dinitrogen.

Denitrification is an anaerobic process that stepwise reduces NO_3^-/ NO_2^- via NO to N_2O and eventually to N_2. Denitrification is considered as the major re-mineralization process in suboxic environmental settings, i.e. organic matter is respired using nitrate as electron accepter after O_2 is depleted. Denitrification coupled to the oxidation of a reduced inorganic compound such as sulfide may support autotrophic CO_2 fixation. Denitrifying microorganisms belong to a variety of different subclasses of the Bacteria (Philippot and Hallin, 2005). There are many genes coding for proteins involved in denitrification, but *nirS* and *nirK*, which code for nitrite reductase, are commonly used to characterize denitrifying communities (Braker *et al*., 2000; Hallin *et al*., 2006; Mosier and Francis, 2010; Philippot and Hallin, 2005; Prieme *et al*., 2002; Santoro *et al*., 2006). The *nirS* and *nirK* genes are coding for two functional equivalent but structurally distinct nitrite reductases in denitrifying bacteria (Zumft, 1997). These are Cytochrome cd1 (Cd-Nir), encoded by *nirS* and a copper nitrite reductase (Cu-Nir), encoded by *nirK*. There are no organisms known that carry both genes and therefore these two enzymes are thought to be mutually exclusive (Zumft, 1997). In coastal sediments, denitrifier communities and their activities vary frequently along nitrate-, salinity-, and tidal gradients (Francis *et al*., 2013; Santoro *et al*., 2006). Environmental factors that determine the distribution and activity of denitrifiers include salinity, organic matter, sulfide and dissolved inorganic nitrogen (Dong *et al*., 2009; Francis *et al*., 2013; Jones and Hallin, 2010; Liu *et al*., 2003).

Anammox is carried out by a group of chemolithoautotrophic bacteria belonging to the phylum planctomycetes (Jetten *et al*., 2005). These bacteria oxidize ammonium anaerobically using nitrite as electron acceptor and converting both nitrogen compounds to dinitrogen (Mulder *et al*., 1995; Strous *et al*., 1999; Vandegraaf *et al*., 1995). Anammox in the marine environment was first reported in the sediment of the Skagerrak using a modified isotope paring technique that distinguishes N_2 production by anammox and denitrification (Thamdrup and Dalsgaard, 2002). It is now clear that anammox is a quantitatively important sink of nitrogen in the marine environment (Dalsgaard *et al*., 2003; Kuypers *et al*., 2005). In marine sediments, anammox is responsible for <1% to 80% of the total N_2 production, but it is considered of little importance in shallow estuarine and coastal sediments due to the high carbon re-mineralization rates (Engström *et al*., 2005; Thamdrup and Dalsgaard, 2008). Several studies showed that the distribution and abundance of anammox bacteria is dependent on temperature, content and type of organic matter, concentration of nitrite, and type of sediment (Dalsgaard and Thamdrup, 2002; Dang *et al*., 2010a; Jaeschke *et al*., 2009; Neubacher *et al*., 2011).

Nitrification

Nitrification is a two-step process, in which ammonium is oxidized via nitrite to nitrate by two distinct groups of primarily chemolithoautotrophic microorganisms. The energy generated from the oxidation of ammonia and nitrite is partly used for the fixation of CO_2 to synthesize organic matter. The product of nitrification, NO_3^-, may serve as a source of nitrogen and assimilated by organisms, or used as a terminal electron acceptor for denitrification or anammox, or reduced via dissimilatory nitrate reduction to ammonia (DNRA) (Thamdrup and Dalsgaard, 2008). Nitrification links the mineralization of organic nitrogen to the conversion to dinitrogen through denitrification and anammox (Kuypers *et al*., 2005; Lam *et al*., 2007). Nitrification is the connection of oxidative and reducing pathways in the N cycle (Joye and Hollibaugh, 1995; Zehr and Ward, 2002).

Nitrification is carried out by different types of microorganisms. The ammonia oxidizing bacteria (AOB; Rotthauwe *et al*., 1997) and ammonia oxidizing archaea (AOA; Francis *et al*., 2005) perform the first reaction and oxidize ammonium to nitrite. The second reaction is performed by the nitrite oxidizing bacteria. The oxidation of ammonia is the rate-limiting step in nitrification (Prosser and Nicol, 2008). Diversity of ammonium oxidizers is usually assessed from the *amoA* gene, which encodes the α-subunit of ammonia monooxygenase. This gene has been evaluated from a wide range of ecosystems (Caffrey *et al*., 2007; Christman *et al*., 2011; Mincer *et al*., 2007; Wuchter *et al*., 2006). The ecological importance of AOA and AOB differs among environments. Several studies suggested that AOB are more important than AOA in nitrification in the marine environment. This conclusion was based on the higher abundance of AOB gene copies (Christman *et al*., 2011; Mosier

and Francis, 2008). However, other studies came to the opposite conclusion (Beman *et al*., 2010; Caffrey *et al*., 2007; Santoro *et al*., 2008).

The nitrogen cycle in microbial mats

Microbial mats are found in various environments (Severin and Stal, 2010c). Coastal microbial mats are compact, highly structured, small-scale ecosystems (Stal, 2001) and typically formed by different functional groups of microorganisms that occur in vertically stratified layers according to their preference along the physicochemical gradient that prevail in these mats. The depth orientation of different phototrophic organisms that possess different pigmentation results in a visible lamination of the mat (Figure 2). When present, diatoms are usually on top followed by cyanobacteria that form a distinct blue-green layer. Due to their photosynthetic and autotrophic mode of life, cyanobacteria are the primary producers and form the basis of the microbial food web in this small-scale ecosystem (Severin and Stal, 2010a). Purple (and sometimes also green) sulfur bacteria are underneath the cyanobacterial layer. The principle electron donor for green and purple sulfur bacteria is H_2S. Hence, these organisms are usually found in the anoxic zone of microbial mats. Due to a higher tolerance towards H_2S and more efficient light harvesting, green sulfur bacteria in microbial mats are usually positioned below purple sulfur bacteria. Photosynthesis is restricted to the top few millimeters due to the attenuation of light. It drives primary production that provides the ecosystem with a source of organic carbon. Below the purple layer there is a permanently anoxic black horizon of iron sulfide. Chemotrophic bacteria are involved in a variety of biogeochemical processes and accumulate at the optimal conditions along the physicochemical gradients. The integrated and combined activity of microbial mat organisms results in a highly productive and self-sustained ecosystem (Stal, 2001).

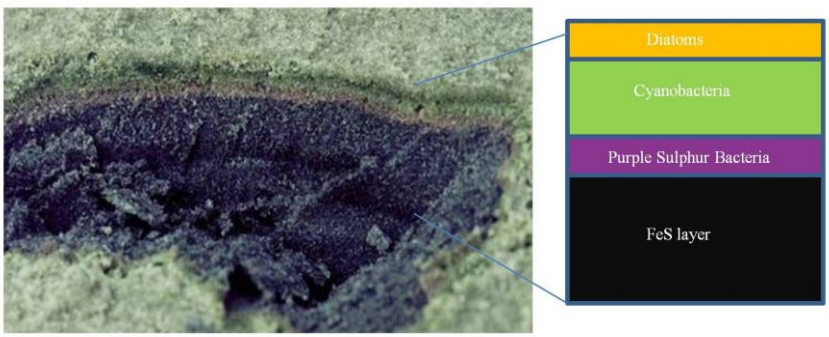

Figure 2. Microbial mat cross section.

In this thesis, I studied microbial mats on the North Sea beaches of the Dutch barrier island Schiermonnikoog. These microbial mats play an important role in coastal morphodynamics and protection. Previous studies, using massive parallel 16S rRNA tag sequencing, showed that these mats are among the most diverse marine microbial ecosystems (Bolhuis and Stal, 2011). These authors distinguished three types of microbial mats developing along the tidal gradient, perpendicular to the coast. The three mat types differed in morphology and community composition (Bolhuis *et al*., 2013). Temperate coastal regions are generally low in nitrogen (Paerl *et al*., 1996) and this limits the primary production and development of microbial mats (Paerl *et al*., 2000). Therefore, nitrogen fixation provides a source of new nitrogen that supports the growth and persistence of the mat (Stal *et al*., 1985). Cyanobacteria and Proteobacteria have been shown to contribute to nitrogen fixation in microbial mats (Moisander *et al*., 2007; Severin *et al*., 2010; Zehr *et al*., 1995). Previous studies also showed the strong spatial and temporal variability of the diazotrophic community and nitrogenase activity (Severin and Stal, 2008; Severin and Stal, 2010b; Zehr *et al*., 1995). In comparison with the wealth of knowledge obtained from nitrogen-fixing bacteria in microbial mats, knowledge about other processes is limited to a few studies that focused mainly on denitrification. Joye and Paerl (1994) found that denitrification accounted for 15% nitrogen loss on an annual basis in the microbial mats of Tomales Bay (California, USA). While in a hypersaline microbial mat (Camargue, France) denitrification was lower than N_2 fixation in summer but exceeded N_2 fixation in winter when it turned the mat into a sink for nitrogen (Bonin and Michotey, 2006). These observations lead to the formulation of the hypothesis that the nitrogen cycle in microbial mats may be out of balance and that this imbalance may be the result of the separation of different processes of the nitrogen cycle in different ecosystem compartments (spatially) or in different seasons (temporally).

Nitrogen cycle in the southern North Sea

The North Sea is a shallow sea of the European continental shelf, situated between the United Kingdom in the west and Belgium, The Netherlands, Germany, Denmark and Norway in the south and east. The southern part of the North Sea is a shallow and continuously mixed region that receives most of the riverine nutrient loads (Voss *et al*., 2011). The increased nutrient load has resulted in a severe eutrophication and related negative consequences for the quality of the water such as increased frequency of phytoplankton blooms (Radach and Pätsch, 1997), foam accumulation (Lancelot, 1995) and hypoxic conditions in bottom water (Greenwood *et al*., 2010).

Denitrification causes a loss of combined nitrogen from the ecosystem and thus may help to decrease the effect of eutrophication (Seitzinger *et al*., 2006). Most of the studies on denitrification in the North Sea focused on rate measurements (Law and Owens, 1990; Lohse *et al*., 1993; Lohse *et al*., 1996) and geochemical modeling

(Hydes *et al*., 1999; Middelburg *et al*., 1996; van Raaphorst *et al*., 1990; Seitzinger and Giblin, 1996; van Raaphorst *et al*., 1992). Denitrification varied from 0.9 to 255 mmol N m^{-2} $year^{-1}$ (Brion *et al*., 2004) and is the dominant sink for nitrogen under low oxygen conditions in the North Sea (Middelburg *et al*., 1996). Moreover, the coupling between denitrification and nitrification is incomplete and varied between location and season (Lohse *et al*., 1993). In addition to denitrification, anammox accounted for 10-20% of the total N_2 production in the southern North Sea (Neubacher *et al*., 2011). It was thought that biological N_2 fixation is a minor source of nitrogen in the North Sea (Brion *et al*., 2004). Rees *et al*. (2009) reported N_2 fixation rates ranging from 18.9 to 20.0 nmol N L^{-1} d^{-1} in surface water in the western English Channel. Hitherto no report has been published that showed that nitrogen fixation occurs in the North Sea, neither in the water column nor in the bottom sediments, with exception of the microbial mats that grow on the intertidal sandy beaches.

Aims and outline

This dissertation reports about studies on the nitrogen cycle in intertidal microbial mats and bottom sediments of the southern North Sea. The main hypothesis of this study was that the major processes of the nitrogen cycle occur on different spatial and temporal scales and is rarely complete and balanced at one location at one point in time in the southern North Sea. In order to test this hypothesis, the four main processes of the nitrogen cycle were studied in three contrasting ecosystems, including intertidal microbial mats, North Sea water column, and bottom sediments during four seasons. These contrasting environments allow for the comparison of the nitrogen cycle in different but interconnected marine ecosystems and provide a comprehensive picture of the nitrogen cycle in North Sea coastal area. This study includes rate measurements using ^{15}N stable isotope tracers and assesses the diversity and activity of the microorganisms by analyzing functional genes and their transcripts.

Chapter 2 reports the study of denitrification in microbial mats and compares this to the composition of the denitrifying community in microbial mats. In order to understand whether different mat types contain different types of denitrifying bacteria and whether a relationship exists between the denitrifier community and the potential rate of denitrification, I measured the potential rates of denitrification and identified the denitrifying communities (both *nirS* and *nirK*) in three different mat types during three different seasons. In addition, I related environmental parameters to *nirS* and *nirK* composition and explored the potential ecological roles of *nirS* and *nirK* possessing bacteria.

Chapter 3 reports on the study of nitrification in three mat types. The potential rate of nitrification was measured and the diversity and abundance of *amoA* gene of both

AOB and AOA was assessed in these mat types during four different seasons. Moreover, the key environmental variables were monitored and linked to changes in the community composition and abundance of ammonia oxidizers (*amoA* gene). The results elucidate the main players in nitrification and how they are driven by environmental factors.

Chapter 4 provides a detailed account of the functionalities of the microbial mat community and uncovers the core functional gene potential and complexity of intertidal microbial mats by using a functional gene array (GeoChip). The functional genetic potential of microbial communities involved in nutrient cycling, including carbon-, nitrogen- and sulfur- cycling as well as the stress tolerance strategies for mat organisms to thrive in intertidal microbial mats, were analyzed. This chapter complements our understanding of the ecology of the microbial mat community.

Chapter 5 and Chapter 6 deal with N_2 fixation, denitrification and anammox in the surface (only N_2 fixation) and the bottom sediments of the southern North Sea at different sites and during different seasons. The potential rates of N_2 fixation, denitrification and anammox were measured. Alongside, the community composition of the diazotrophic, denitrifying and anammox bacteria communities were determined and the abundance and activity of the organisms were measured and linked to a number of environmental variables. The results elucidate the presence of these processes of the nitrogen cycle and the microorganisms involved. In addition, it reveals the spatial and temporal trends of N_2 fixation, denitrification and anammox in the bottom sediments of the North Sea.

Chapter 7 provides a summary of the overall conclusions of this thesis and puts these in a wider context. This chapter also provides an outlook and perspective of future research questions on the nitrogen cycle that still await answers.

Chapter 2

Denitrification and denitrifiers in coastal microbial mats

Haoxin Fan[1], Henk Bolhuis[1], and Lucas J. Stal[1,2]

[1]Department of Marine Microbiology, Royal Netherlands Institute for Sea Research, Yerseke, The Netherlands
[2]Department of Aquatic Microbiology, Institute of Biodiversity and Ecosystem Dynamics, University of Amsterdam, The Netherlands

Published as: Fan, H., Bolhuis, H., and Stal, L.J. (2015). Denitrification and the denitrifier community in a coastal microbial mat. *FEMS Microbiol. Ecol.* 91 (3): fiu033.

Abstract
Denitrification was measured in three structurally different coastal microbial mats by using the stable isotope technique. The composition of the denitrifying community was determined by analyzing the nitrite reductase (*nirS* and *nirK*) genes using clone libraries and the GeoChip. The highest potential rate of denitrification (7.0 ± 1.0 mmol N m^{-2} d^{-1}) was observed during summer at station 1 (supra-littoral). The rates of denitrification were much lower in the stations 2 (marine) and 3 (intermediate) (respectively 0.1 ± 0.05 and 0.7 ± 0.2 mmol N m^{-2} d^{-1}) and showed less seasonality when compared to station 1. The denitrifying community at station 1 was also more diverse than that at station 2 and 3, which were more similar to each other than either of these stations to station 1. In all three stations, the diversity of both *nirS* and *nirK* denitrifiers was higher in summer when compared to winter. The location along the tidal gradient seems to determine the composition, diversity and activity of the denitrifier community, which may be driven by salinity, nitrate/nitrite and organic carbon. Both *nirS* and *nirK* denitrifiers are equally present and therefore they are likely to play a role in the denitrification of the microbial mats studied.

Keywords: denitrification, microbial mat, *nirK*, *nirS*

Introduction

Denitrification is a bacterial process during which nitrate or nitrite is stepwise reduced through a few intermediate gaseous nitrogen compounds to dinitrogen (Zumft, 1997). Nitrite reductase is present in all denitrifying bacteria and mediates the reduction of nitrite to nitric oxide and is considered as the key enzyme of denitrification. There are two functional equivalent but structurally distinct nitrite reductases known in denitrifying bacteria (Zumft, 1997). These are Cytochrome cd1 (Cd-Nir), en- coded by *nirS* and a copper nitrite reductase (Cu-Nir), encoded by *nirK*. There are no organisms known that carry both genes and therefore these two enzymes are thought to be mutually exclusive (Zumft, 1997). Nitrite reductase genes have been used as functional molecular markers for denitrification in natural environments and have revealed the diversity of denitrifying bacteria in a variety of habitats such as soil (Prieme *et al*., 2002; Throback *et al*., 2007), estuarine sediments (Santoro *et al*., 2006), marine sediments (Braker *et al*., 2000; Hannig *et al*., 2006) and seawater (Jayakumar *et al*., 2004; Castro-Gonzalez *et al*., 2005; Oakley *et al*., 2007). Environmental factors were identified that shaped the denitrifier community composition (Braker *et al*., 2000; Hallin *et al*., 2009). Moreover, the type of habitat determined the presence or dominance of *nirS*- and *nirK-type* denitrifiers (Hannig *et al*., 2006; Oakley *et al*., 2007).

Coastal microbial mats are compact, highly structured, small-scale ecosystems (Stal, 2001). These mats are built by cyanobacteria, oxygenic phototrophic bacteria, which through primary production enrich the sediment with organic matter. This organic matter forms the basis of a complex, multi-layered microbial ecosystem. Previous studies of nitrogen cycling in microbial mats have focused mainly on the nitrogen fixation and only a few studies documented denitrification in microbial mats. Joye and Paerl (1994) studied denitrification in microbial mats of Tomales Bay (California) and found that it removed only 15% of the N_2 that was fixed on an annual basis in these mats. In a hypersaline microbial mat, denitrification was lower than N_2 fixation in summer, but exceeded N_2 fixation in winter when it turned the mat into a sink for nitrogen (Bonin and Michotey, 2006). Desnues *et al*. (2007) reported spatio–temporal distribution of denitrifying bacteria in a hypersaline microbial mat. These studies focused on the rates of denitrification and did not give information on the diversity and the temporal and spatial distribution of the denitrifying bacteria and their activities and therefore provided only an incomplete view on this process in microbial mats.

We investigated microbial mats that proliferate at the North Sea coast of the Dutch barrier island Schiermonnikoog. Based on morphological, microscopic and molecular genetic differences, we distinguish three major types of microbial mats that develop along the tidal gradient. The bacterial, archaeal and eukaryal community composition and microbial diversity were intrinsic of the mat type and depended on the location

along the tidal salinity gradient (Bolhuis and Stal, 2011; Bolhuis *et al*., 2013). Previously, it was shown that N_2 fixation and the diazotrophic community varied along the same lines at these three stations (Severin and Stal, 2010). The variation of N_2 fixation may be attributed to different environmental conditions in microbial mats, which changes spatially (location along the tidal gradient) and temporally (tide, day–night and seasonal). We expect that the same factors exert also a selective force on the denitrifying community and its activity. At the different positions along the tidal gradient, the mats would allow the development of different community compositions, which would also alter the potential rate of denitrification that can be achieved (Philippot and Hallin, 2005). In this study, we measured the potential rates of denitrification in the three different mat types during different seasons. Alongside, we identified the denitrifying communities and measured relevant environmental variables in order to elucidate (1) whether mats along the tidal gradient contain different types of denitrifying bacteria; (2) whether a relationship exists between the denitrifier community and the potential rate of denitrification; (3) which environmental factors affect denitrification and the composition of the denitrifier communities.

Material and methods

Study area and sampling

Table 1. The geographical coordinates and description of the mats investigated in this study.

Station	Geographical coordinates	Description
Station 1 (ST1)	53°29.445′N, 6°8.718′E	Mainly freshwater influenced site, close to the dunes. Irregularly inundated.
Station 2 (ST2)	53°29.460′N, 6°8.309′E	Seawater influenced site, developing microbial mat. At the low water mark.
Station 3 (ST3)	53°29.445′N, 6°8.342′E	Seawater and freshwater influenced site, located between ST1 and ST2, at the edge of the salt marsh

The study site was located on the North Sea coast of the Dutch barrier island Schiermonnikoog. The geographical locations and descriptions of the three types of microbial mats (stations) that were sampled for this study are given in Table 1. The sample stations were situated along a transect perpendicular to the beach covering the tidal gradient. Sampling was done at four different seasons during 2010 and 2011. Samples were taken from the top 2.5–3 cm of the mat using custom-made transparent Lexan cylinder cores of 50 mm inner diameter and 60 mm height. The cores were transported back to the laboratory within 4 h of sampling and kept at ambient temperature and light. The incubations started within 24 h after sampling. Additional samples were taken for nucleic acid extraction. These samples were taken from the

top 1 cm of the mat by using a 10 ml truncated syringe as a corer. These mat samples were divided into four equal parts, put into cryovials and immediately frozen in the field in liquid nitrogen.

Chemical analyses

For nutrient analyses, 5 g mat sample (top 1 cm) was extracted with 40 ml 2 M KCl. The extracts were filtered through What- man GF/F filters and the filtrates were kept at −20◦C until analysis (within a month). Nutrient concentrations were measured on an automated segmented flow analyzer using standard analytical procedures. Other mat samples were freeze-dried for the determination of total nitrogen (TN), total organic carbon (TOC) and C/N ratio by EA-IRMS (DELTA V Advantage; Thermo Fisher Scientific, Bremen, Germany).

Measurement of potential denitrification

Subsamples of 1.2 cm^2 (10 mm thickness) of the cores of the mats were placed into 12.5 ml Exetainers (Labco Limit, Buckinghamshire, England) by using a 5-ml syringe as a corer. The measurements were carried out according to Thamdrup and Dalsgaard (2002) with some modifications. Briefly, the Exetainers were completely filled with artificial seawater (NaCl 20.5 g, Na_2SO_4 3.4 g, KCl 0.58 g, KBr 0.084 g and H_3BO_3 0.022 g, $MgCl_2.6H_2O$ 0.05 mol, $CaCl_2.2H_2O$ 0.01 mol in 1000 ml Milli-Q water). Addition of 100 µM $Na^{15}NO_3$ (98.5%, ^{15}N atom%; Sigma-Aldrich, 100 µM $^{15}NH_4Cl$ (99.2%, ^{15}N atom%; Sigma-Aldrich) and 100 µM $^{15}NH_4Cl$ +100µM $Na^{14}NO_3$ were applied to the Exetainers, respectively. Before the addition of the ^{15}N tracer, the Exetainers were placed in dark for 2 h to allow the depletion of NO_x^- and any residual oxygen. All Exetainers, including one set without any addition, were incubated for 24 h in the dark at ambient temperature (Table 2). At 4-h intervals during 24 h two replicate Exetainers from each treatment were fixed by injecting 200 µl 50% (w/v) $ZnCl_2$. The Exetainers were stored at 4°C in the dark upside down until analysis (within a week). The isotopic composition of the dinitrogen of He-equilibrated headspace (2 ml in the 12.5-ml Exetainer vials) was determined by an EA-IRMS (DELTA V Advantage; Thermo Fisher Scientific, Bremen, Germany) equipped with a Haysep Q column. The potential rate of denitrification was calculated from the linear production of excess $^{29}N_2$ and $^{30}N_2$ according to Risgaard-Petersen *et al*. (2002).

DNA extraction, PCR, cloning and sequencing

DNA was extracted using the MoBio UltraCLEAN soil DNA kit (MoBio Laboratories, Inc., Carlsbad, CA, USA) according to the manufacturer's instructions. Fragments of the genes *nirS* and *nirK* were amplified using the primer pairs cd3aF-R3cd for *nirS* and F1aCu-R3Cu for *nirK* (Throbäck *et al*., 2004). PCR conditions for the two sets of primer pairs were 2 min at 95°C, 35 cycles of 50 sec 95°C, 50 sec 53°C, and 50 sec at 72°C, followed by a final extension of 10 min at

72°C. PCR products were checked on a 1% agarose gel. PCR products were cloned using the TOPO-TA cloning kit with the pcR2.1 vector and TOP10 competent cells (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. Transformants were randomly picked from each clone library and screened by PCR using T3 and T7 vector primers following the recommended PCR conditions (Invitrogen, Carlsbad, CA, USA). Forty-eight clones were randomly selected for sequencing with the T7 vector primer using ABI PRISM 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). The total number of sequences obtained from each clone library varied (28-70 sequences) due to the variable quality of the sequencing reads. Sequences have been submitted to NCBI (accession numbers KJ738332 - KJ739305).

Sequence analysis

Sequences were edited, aligned and translated using MEGA 5 (Molecular Evolutionary Genetics Analysis, http://www.megasoftware.net/mega5/mega.html) and manually checked. Neighbor-joining trees were produced and the reliability of the phylogenetic reconstructions was evaluated by bootstrapping (1000 replicates). The program Mothur (http://www.mothur.org/wiki/Main Page) was used to calculate the non-parametric richness estimators and the Shannon diversity index and to determine the differences in nucleic acid sequences. Operational taxonomic units (OTUs) were defined by a 5% difference in nucleic acid sequence for the purpose of community analysis. Based on the OTUs from each library, sequence data were transformed into binary data (presence/absence) for community composition analysis.

GeoChip analysis

The GeoChip is a high-throughput functional gene array covering 289 functional gene families involved in the biogeochemical cycling of carbon, nitrogen, phosphorus and sulfur (He *et al*., 2010). For the analysis using the GeoChip we used DNA extracted in triplicate from the three types of microbial mats sampled in July and in January. The DNA was purified using UltraClean 15 DNA purification Kit (MoBio Laboratories, Inc., Carlsbad, CA, USA) in order to achieve the quality necessary for hybridization on the chip. The DNA quantity was measured by an ND-1000 spectrophotometer (Nanodrop Inc., Wilmington, DE). The procedures for DNA labeling and microarray hybridization followed the previously established protocols (Wu *et al*., 2006). Briefly, 800 ng of environmental DNA was labeled with fluorescent dye Cy-5 by random priming. The labeled DNA was re-suspended in 50 µl hybridization solution [40% formamide, 5 x SSC, 5 µg of unlabeled herring sperm DNA (Promega, Madison, WI), and 0.1% SDS] and 2 µl universal standard DNA (0.2 pmol μl^{-1}) labeled with the fluorescent dye Cy-3 (Liang *et al*., 2010), denatured at 95°C for 5 min and maintained at 50°C until loaded onto the microarray slides. Arrays were hybridized on a MAUI Hybridization Station (Roche, South San Francisco, CA) for 12 h at 42^{o}C. Hybridized microarrays were scanned by a ScanArray Express Microarray scanner (Perkin-Elmer,

Wellesley, MA) at 95% laser power and 85% photomultiplier tube gain. The resulting images were analyzed by ImaGene with signals processed as SN>2.0 (signal to noise ratio).

Statistical analysis

In order to summarize the gene overlap at station and season level, the detected genes from the GeoChip in the three replicates of each station from July and January were deployed as one pool (mean value from the three replicates). The analyses of overlapping genes, unique genes and diversity indices were performed using an online pipeline (http://ieg.ou.edu/). The proportion of overlapping genes was calculated as the number of overlapping genes divided by the total number of genes detected in both stations. The proportion of unique genes at each station was calculated as the number of unique genes at each station divided by the total number of genes detected at that station.

Statistical analyses of the multi-response permutation procedure (MRPP) and canonical correspondence analysis (CCA) (see below) were performed based on community data from the clone libraries as well as from the GeoChip data. MRPP using Bray–Curtis distance was used to test for significant differences in community composition. The MRPP A-statistics describes the within and between group relatedness relative to what is expected by chance. A *P-value* <0.05 and an A-statistics >0.1 is considered as significant difference between groups (McCune *et al*., 2002). To test the relationship between the denitrifier community and the environmental variables, CCA was carried out. The significance of the whole canonical model was tested by 999 permutations. All statistical analyses were carried out in the open source-software R (R Development Core Team, 2011), using the vegan package (Oksanen, 2013). Stepwise regression was carried out to test the influence of the environmental factors and denitrifier community on potential denitrification rates using SigmaPlot (SigmaPlot, Version 12). Denitrifier communities were converted into univariate variables based on the sample scores for the first two CCA axes.

Results

Physicochemical characteristics

Table 2 lists the seasonal and annual mean values of the physicochemical parameters of the sample sites and the potential denitrification rates in the three mats. The physicochemical parameters fluctuated seasonally and some parameters showed differences between the three mat types. Ammonium concentration was lowest at Station 1 (ST1) and highest at ST3, except in April. Nitrate/nitrite concentrations were in the same range at all stations, albeit with slightly higher concentrations in July and April and slightly lower concentrations in September and January. Phosphate

concentration was highest in April and lowest in September at all stations. TOC and TN were similar at ST2 and ST3 but always lowest at ST1.

Potential denitrification rates

$^{29}N_2$ and $^{29}N_2$ were produced at the expected ratio for denitrification given the addition of 99.2 atom%-enriched $^{15}NO_3^-$. Denitrification rates (N_2 production) showed remarkable differences between the three stations and varied also seasonally. For ST1 (supratidal, close to the dunes), the potential denitrification rates ranged from 0.1 ± 0.05 to 7.0 ± 1.0 mmol N m^{-2} d^{-1} (Table 2). The denitrification rate was highest in July (7.0 ± 1.0 mmol N m^{-2} d^{-1}) and much lower (1.6 ± 0.3 mmol N m^{-2} d^{-1}) in September. Denitrification was lowest (0.1 ± 0.05 mmol N m^{-2} d^{-1}) in April. The seasonal trend of denitrification at the littoral site (ST3) was slightly different from that at ST1. At ST3, the highest denitrification rate (0.7 ± 0.2 mmol N m^{-2} d^{-1}) was in July and was lowest in September (0.1 ± 0.05 mmol N m^{-2} d^{-1}). A higher rate (0.5 ± 0.2 mmol N m^{-2} d^{-1}) was again observed during January. Unlike the other two sites, the highest rate of denitrification at ST2 (low water mark) (1.6 ± 0.4 mmol N m^{-2} d^{-1}) was observed in January and the lowest rate (0.1± 0.05 mmol N m^{-2} d^{-1}) occurred in July and September. The annual average denitrification rate was highest at the supralittoral (near the dunes, ST1) (2.8 mmol N m^{-2} d^{-1}) and significantly higher ($P < 0.05$) than at the other two stations, which were low and not significantly different from each other (0.5 mmol N m^{-2} d^{-1} at ST2 and 0.4 mmol N m^{-2} d^{-1} at ST3).

nirS and *nirK* diversity and composition

nirS and *nirK* sequences from the three stations were analyzed. Combining the clone libraries, 76 unique *nirS* OTUs and 74 unique *nirK* OTUs (at a 5% distance cut-off) were retrieved. The richness and diversity estimators showed different *nirS* and *nirK* gene richness at the three stations. *nirS*-denitrifier community was richest at ST3, while the other two stations showed a similar richness. *nirK*-denitrifier community was richest at ST1 and poorest at ST3 (Table 3).

Table 2. Physicochemical parameters and potential denitrification rates in the microbial mats during the 2010 -2011 sampling period.

	July (2010)	September (2010)	January (2011)	April (2011)
Station 1				
Temperature (°C, sediment)	17	10	0	8
NH_4^+ (µmol/l)	128.9±3.0	83.7±17.3	191.3±23.4	233.1±23.7
NO_X^- (µmol/l)	23.3±4.6	8.4±1.1	9.6±4.6	25.9±3.4
PO_4^{3-} (µmol/l)	20.1±6.1	3.1±0.5	n.d.	25±3.8
TOC (%)	0.04	0.06	0.04	0.04
TN (%)	0.006	0.01	0.007	0.007
C/N	6.7	6.0	5.7	5.7
Salinity (psu)	18	19	15	17
Denitrification (mmol N $m^{-2}d^{-1}$)	7.0±1.0	1.6±0.3	2.4±0.3	0.1±0.05
Station 2				
Temperature (°C, sediment)	17	10	0	8
NH_4^+ (µmol/l)	587.9±41.2	216.2±69.8	736.8±199.6	486.1±61.3
NO_X^- (µmol/l)	22.4±14.7	8.7±1.4	6.2±1.1	20.6±4.5
PO_4^{3-} (µmol/l)	19.7±2.0	3.2±0.9	n.d.	58.6±26.3
TOC (%)	0.19±0.01	0.20±0.03	0.20±0.03	0.17±0.02
TN (%)	0.03	0.04	0.03	0.03
C/N	6.3	5.0	6.7	5.7
Salinity (psu)	28	28	30	28
Denitrification (mmol N $m^{-2}d^{-1}$)	0.1±0.05	0.1±0.05	1.6±0.4	0.2±0.1
Station 3				
Temperature (°C, sediment)	17	10	0	8
NH_4^+ (µmol/l)	217.3±102.3	255.9±68.4	510.2±62.2	475.8±3.5
NO_X^- (µmol/l)	16.0±3.7	7.6±1.9	2.8±0.6	19.6±4.0
PO_4^{3-} (µmol/l)	28.5±15.2	3.9±1.2	n.d.	48.5±10.6
TOC (%)	0.11±0.03	0.15±0.02	0.15±0.02	0.13±0.02
TN (%)	0.02	0.03	0.03	0.02
C/N	5.5	5.0	5.0	6.5
Salinity (psu)	25	25	22	23
Denitrification (mmol N $m^{-2}d^{-1}$)	0.7±0.2	0.1±0.05	0.5±0.2	0.1±0.05

Abbreviations: n.d., no data; TOC, total organic carbon; TN, Total nitrogen.

Table 3. Richness and diversity statistics of *nirS* and *nirK* clone libraries based on 95% cutoffs.

	No. of clones	No. of OTUs	ACE	Chao1	Shannon	Simpson
nirS						
ST1	55	20	56	42	2.1	0.25
ST2	48	19	131	49	2.2	0.18
ST3	66	32	48	47	3.2	0.03
nirK						
ST1	70	35	481	194	3.3	0.05
ST2	69	27	122	84	2.6	0.18
ST3	52	17	41	30	2.1	0.13

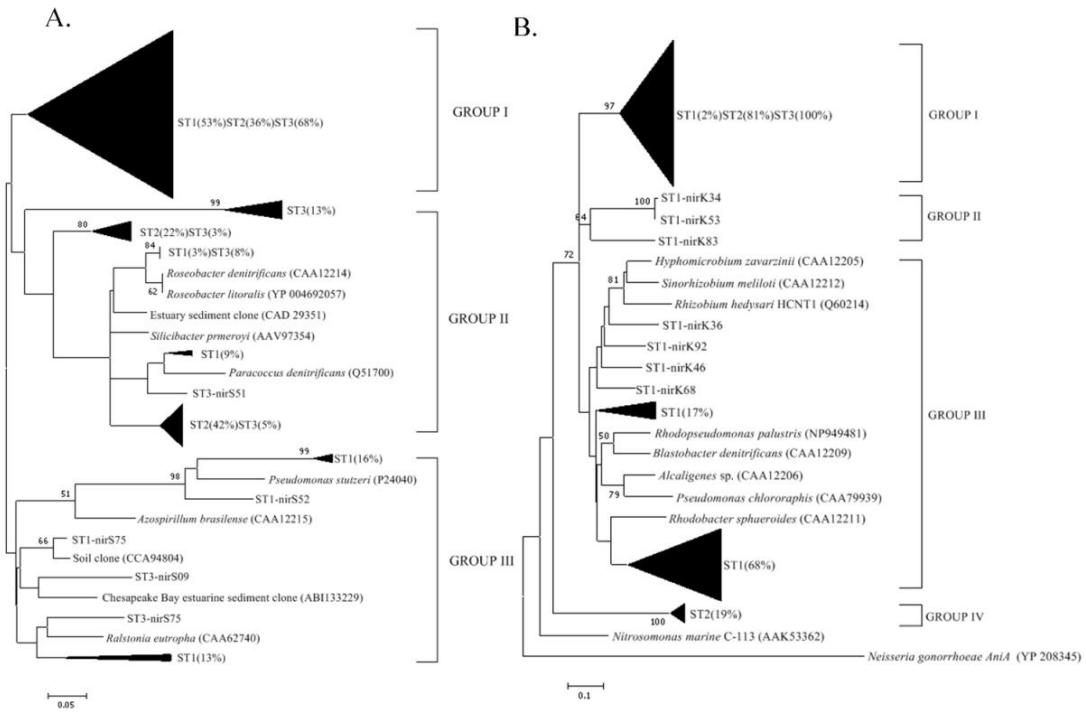

Figure 1. Phylogenetic trees for *nirS* (A) and *nirK* (B) genes, based on the translated amino acid sequence, constructing by neighbor-joining method in MEGA 5. Sequences from this study were shown as the percentage of environmental clones from each station. Significant bootstrap values (>50) are shown at branch nodes.

Phylogenetic analyses of deduced amino acid sequences for *nirS* and *nirK* gene fragments are shown in Fig. 1. The *nirS* sequences clustered into three distinct groups (Fig. 1A). Group I contained 53, 36 and 68% of the total sequences from ST1, ST2

and ST3, respectively. The sequences in this group were closely related to the cultivated denitrifier *Marinobacter* sp. U31 (CAF25138) as well as to environmental clones from a hyper- saline microbial mat (e.g. CAL69009, CAL69007), an estuarine sediment (e.g. AEK77712, ABY52470) and from the Baltic Sea (e.g. CAJ87449). Group II contained 12, 64 and 30% sequences from ST1, ST2 and ST3, respectively. Sequences in Group II clustered closely with those of a variety of cultivated denitrifiers including *Roseobacter denitrificans*, *Paracoccus denitrificans* and *Silicibacter pomeroyi*. The third group contained 35 and 2% of the sequences of ST1 and ST3. These sequences were closely related to *Pseudomonas stutzeri*, *Azospirillum brasilense* and *Ralstonia eutropha*.

nirK sequences clustered into four groups (Fig. 1B). Group I contained 2, 81 and 100% of the total *nirK* sequences from ST1, ST2 and ST3, respectively. The sequences belonging to group I were most closely related to environmental clones from San Francisco Bay estuarine sediment (ADM93883) and from the Arabian Sea oxygen minimum zone (ACT98741). Three OTUs from ST1 fell into Group II, showing the best hit of 87% nucleotide sequence similarity with a sequence retrieved from a Chinese agricultural soil (HM628810). Group III contained sequences from ST1 (7, 17, 68%, Fig. 1B) and were related to a variety of cultivated denitrifiers, such as *Rhodobacter sphaeroides* (CCA12211) and *Rhodopseudomonas palustris* (NP949481). Group IV comprised only sequences (19%) from ST2. These sequences are closely related to environmental clones from San Francisco Bay estuarine sediment (ADM93844, ADM93870) and remotely related to the cultivated *Alcaligenes* sp. (75% similarity and 48% sequence coverage).

Diversity of *nirS* and *nirK* genes based on GeoChip analysis is summarized in Table 4. A total of 264 *nirS* sequences showed a hybridization signal in at least one of mat. The average number of *nirS* sequences (richness) at ST1 (July), ST2 (July), ST3 (July), ST1 (January), ST2 (January) and ST3 (January) was 253, 206, 146, 215, 161 and 125, respectively (Table 4). Thirty-three sequences were unique and detected only at one of the stations and during one season. In July, ST1 harbored 26 unique sequences, which was 10.3% (26/253) of the total number detected. ST2 and ST3 harbored 2.4% (5/206) and 0.7% (1/146) unique sequences, respectively. In January, the number of unique sequences in ST1 dropped to 3, which was only 1.4% (3/215) of the total number detected. In January, no unique sequences were observed at ST2 and ST3. Pairwise comparison of *nirS* sequences showed a high number of overlapping *nirS* sequences between summer and winter as well as between the stations: 81% (ST1 July and January), 72% (ST2 July and January), 70% (ST3 July and January), 74–77% (ST1 and ST2), 57% (ST1 and ST3) and 68–70% (ST2 and ST3).

Table 4. Summary of *nirS* and *nirK* genes detected by GeoChip, including the number and percentage of overlapping (italic) and unique (bold) sequences, the diversity indices and abundance for each station.

	ST1_July	ST2_July	ST3_July	ST1_Jan.	ST2_Jan.	ST3_Jan.
nirS						
ST1_July	**26(10.3%)**	*200(77.2%)*	*144(56.5%)*	*210(81.4%)*	*159(62.4%)*	*124(48.8%)*
ST2_July		**3(1.5%)**	*145(70.1%)*	*185(78.4%)*	*154(72.3%)*	*123(59.1%)*
ST3_July			**1(0.7%)**	*139(62.6%)*	*123(66.9%)*	*112(70.4%)*
ST1_Jan.				**3(1.4%)**	*161(74.9%)*	*124(57.4%)*
ST2_Jan.					**0(0.00%)**	*116(68.2%)*
ST3_Jan.						**0(0.00%)**
Richness*	253	206	146	215	161	125
Shannon-Weaver (H)	5.5	5.3	5	5.4	5.1	4.8
Abundance (%)**	8.2	7.7	7.4	7.9	7.8	7.4
nirK						
ST1_July	**26(10.2%)**	*200(76.9%)*	*139(53.9%)*	*212(82.2%)*	*147(56.8%)*	*124(47.9%)*
ST2_July		**1(0.5%)**	*139(67.5%)*	*184(78.6%)*	*144(68.6%)*	*126(61.5%)*
ST3_July			**1(0.7%)**	*134(60.6%)*	*117(67.2%)*	*116(76.3%)*
ST1_Jan.				**1(0.5%)**	*145(66.2%)*	*122(55.7%)*
ST2_Jan.					**1(0.7%)**	*116(72.1%)*
ST3_Jan.						**0(0.00%)**
Richness *	256	204	141	214	150	127
Shannon-Weaver (H)	5.5	5.3	4.9	5.4	5	4.8
Abundance (%)**	8.3	7.6	7.3	7.9	7.3	7.6

* richness was determined as probe numbers detected.

**abundance was determined by dividing the hybridization intensity of *nirS* or *nirK* on the GeoChip by the total signal of all nitrogen cycling genes detected on the array.

Similar results were obtained for *nirK* (Table 4). We detected 264 *nirK* sequences in the mats. Most *nirK* sequences were detected in summer at ST1 (256). We detected 204, 141, 214, 150 and 127 *nirK* sequences at ST2 (July), ST3 (July) ST1 (January), ST2 (January) and ST3 (January), respectively (Table 4). In July, ST1 harbored 26 unique sequences, which was 10.2% (26/256) of the total detected sequences. ST2 and ST3 harbored 1.5% (3/204) and 0.7% (1/141) unique sequences of the total detected number, respectively. In January, only one unique sequence was detected both at ST1 and ST2. No unique sequence was detected at ST3. 77% of the total detected *nirK* sequences were shared by ST1 and ST2, 54% was shared by ST1 and ST3 and 67% was shared by ST2 and ST3.

The diversity indices for *nirS* and *nirK* were assessed by richness and Shannon–Weaver index (Table 4). At all stations, the values for both diversity estimators were higher in summer. The highest richness was observed in summer at

ST1 and the lowest value was found in summer and winter at ST3 ($P < 0.01$). The highest abundance for both *nirS* and *nirK* were observed in summer at ST1. The lowest abundance for *nirS* was found at ST3 and for *nirK* was found in summer at ST2 and winter at ST3.

MRPP statistics was carried out to test the differences of the composition of the microbial community between the stations based on *nirS* and *nirK* OTUs obtained from the clone libraries and the GeoChip. MRPP testing of these two data sets gave consistent results. Distinctly different denitrifier communities were found in ST1 when compared to the other two stations ($P < 0.05$) from the two data sets. ST2 and ST3 did not contain significantly different communities ($P > 0.05$) when the analysis was based on data from the GeoChip but were significantly different when using the data from the clone libraries ($P > 0.05$). This was the case for both *nirS* and *nirK* (Table 5). There were no seasonal differences in the denitrifier communities in any of the stations (data not shown). These results were confirmed by CCA analyses (Fig. 2).

Table 5. MRPP A-vales of the denitrifier community composition.

Difference between group		
Spatial differences	A-value (clone library)	A-value (GeoChip)
nirS		
ST1 vs ST2	0.753 (p=0.024)*	0.190 (p=0.002)*
ST1 vs ST3	0.749 (p=0.018)*	0.400 (p=0.001)*
ST2 vs ST3	0.667 (p=0.027)*	0.059 (p=0.108)
nirK		
ST1 vs ST2	0.604 (p=0.029)*	0.242 (p=0.004)*
ST1 vs ST3	0.721 (p=0.045)*	0.454 (p=0.002)*
ST2 vs ST3	0.501 (p=0.020)*	0.66 (p=0.096)

* means $p<0.05$ (statistical difference between whole *nirS* and *nirK* profiles assessed using multi-response permutation procedure).

Environmental control of denitrifying mat community and activity

No relationship between potential denitrification rates, environmental factors and the denitrifier community were revealed based on stepwise regression analysis. CCA was applied in order to discover patterns in the composition of the denitrifying community. Using CCA, we analyzed the *nirS* and *nirK* sequence data obtained from the clone libraries and from the GeoChip with the related environmental factors (Table 2).

Fig. 2A shows the results of the CCA from the *nirS* sequences obtained from clone libraries. In the diagram, denitrifiers were distinctly grouped according to sample station. There was no effect of the season. The community composition was significantly correlated with all selected variables in the adapted CCA model ($P = 0.02$) (based on 999 permutations test). In the CCA diagram (Fig. 2A), the first two

axes explained 71% of relation- ship between the total *nirS-containing* community and the environmental factors. The first canonical axis explained 42.5% of total variations (P = 0.008) and was dominated by the environmental variables TOC (P < 0.05), TN and ammonium concentration (P < 0.05). The second canonical axis explained an additional 28.5% of the constrained variations and was dominated by phosphate (P < 0.05) and the nitrate + nitrite concentration. The *nirS*-containing community in ST1 was distinctly different from those in ST2 and ST3 along the first canonical axis (axis 1), while the *nirS*-containing communities in ST2 and ST3 separated along the second canonical axis (axis 2). ST2 and ST3 were influenced by both the first and second canonical axes and positively correlated with TOC (P < 0.05), TN, phosphate (P < 0.05) and ammonium concentrations (P < 0.05), but negatively correlated with nitrate + nitrite concentration (P < 0.05). ST1 was primarily influenced by the first canonical axis, reflecting the role of TOC (P < 0.05), TN and ammonium concentrations (P < 0.05). For each individual variable, a significant correlation was found between community and TOC (r^2 = 0.89, *P* = 0.003) and TN (r^2 = 0.87, *P* = 0.003), which is indicated by the length of the arrows in the CCA diagram.

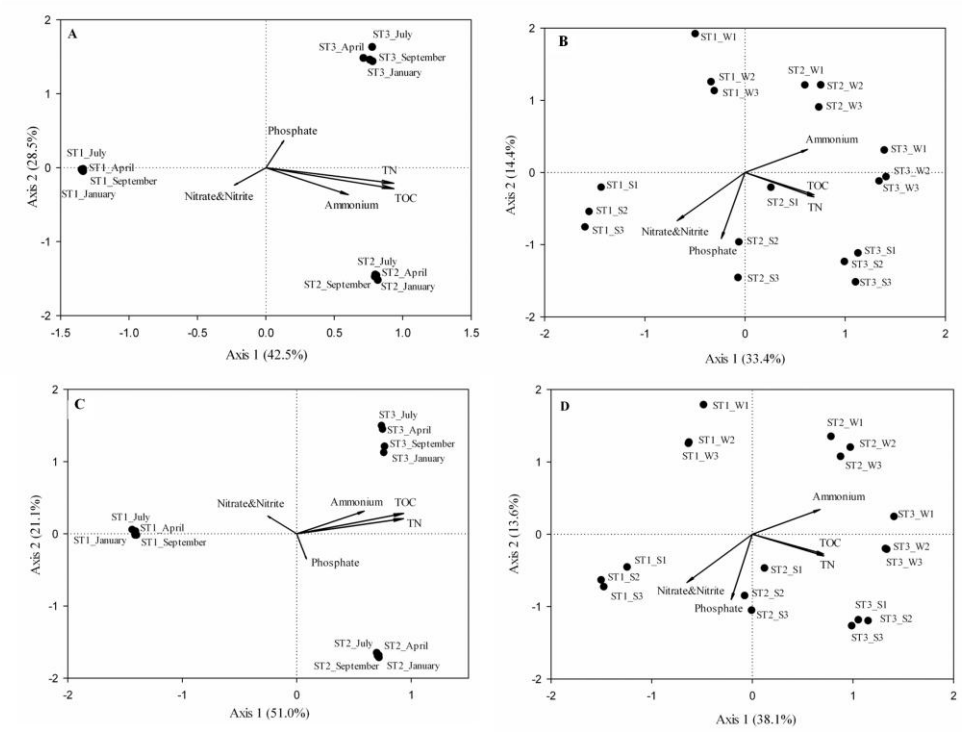

Figure 2. Canonical correspondence analysis of the denitrifier community composition of mat samples. (A) and (C): analysis based on *nirS* and *nirK* clone data and points represent the denitrifier community from seasonal samples at indicated station. (B) and (D): analysis based on *nirS* and *nirK* GeoChip data and points represent replicated denitrifier community from summer and winter samples at indicated station (S: summer; W: winter). Arrows represent the relationship between environmental parameters with the denitrifier communities.

Fig. 2B shows the CCA profiles based on the seasonal *nirS* community data as obtained from the GeoChip. GeoChip analyses were only performed on the summer and winter samples. Spatially, *nirS* communities from different stations were separated along the first axis of the CCA diagram. Temporally, *nirS* communities of the summer samples were separated from those of the winter samples along the second axis. In general, the community composition was significantly correlated with all selected variables in the adapted CCA model ($P = 0.001$) (based on 999 permutations test). Due to multicollinearity, the C:N ratio, salinity and the potential rate of denitrification were removed. Therefore, five environmental factors were selected and are depicted in the diagram. The first two axes explained 47.7% of the relationship between the total *nirS* community composition and the environment. The first canonical axis explained 33.4% of the total variation ($P = 0.001$). The first axis was dominated by the environmental variables TOC ($P < 0.001$), TN ($P < 0.001$) and ammonium concentration ($P < 0.001$). The second axis explained the rest 14.4% of the total variation ($P = 0.001$) and was dominated by phosphate ($P < 0.001$) and the nitrate + nitrite concentration ($P < 0.001$). For ST1, the *nirS* community was influenced by all factors taken into account and showed negative correlation with TOC, TN and ammonium concentration. The *nirS*-containing community in the summer samples correlated positively with nitrate + nitrite and phosphate concentrations, while the community in the winter samples was negatively correlated with these factors. For ST2, *nirS* in the summer samples was only influenced by the factors reflected by second axis. The *nirS* community in the winter samples was influenced by all the selected factors and was positively correlated with TOC, TN and ammonium concentrations but negatively correlated with phosphate ($P < 0.001$) and nitrate + nitrite concentrations. For ST3, the *nirS* community was positively correlated with TOC, TN and ammonium concentrations. The *nirS* in the summer samples also showed a positive correlation with phosphate ($P < 0.001$) and nitrate + nitrite concentrations (although this was not significant), while *nirS* in the winter samples was not strongly influenced by any of the environmental variables on the second axis.

Fig. 2C and D depict the results of the CCA from the *nirK*-containing communities. For both the cloning and GeoChip data, the first two axes explained the community composition better than the observations of the *nirS*-containing community. The axes 1 and 2 explained 72.1 and 51.7% of the total variation of the *nirK*-containing community, respectively. The *nirS- and nirK*-containing communities responded similarly to spatial and temporal variation of the environmental factors.

Discussion

The few existing studies on denitrification in microbial mats used the acetylene inhibition (AI) technique. The published rates of denitrification in microbial mats ranged from 0 to 3.14 mmol N m^{-2} d^{-1} (Joye and Paerl, 1994; Bonin and Michotey, 2006; Desnues *et al*., 2007). In the present study, we measured potential

denitrification rates by the isotope pairing (IP) technique using small cores of the mat from 0.06 to 7.00 mmol N m^{-2} d^{-1} and, hence were in the same range. We do realize that such comparisons fall short because of differences that are inherent of the technique as well as different incubations (i.e. intact cores versus slurries). For example, Lohse *et al*. (1996) concluded that the AI technique underestimated the rate of denitrification by a factor of 2 when compared to the IP technique. Also, Bonin and Michotey (2006) measured denitrification in a microbial mat in the Camargue using both the AI and IP techniques and found that the latter gave 10-fold higher rates. However, the AI technique is not adequate when denitrification depends on nitrification in the sediment, because acetylene blocks the latter. Our measurements did not depend on nitrification since we added ample nitrate.

A previous study on the same mats revealed that in summer nitrogen fixation was 2.0, 0.5 and 2.1 mmol N m^{-2} d^{-1} for the stations 1, 2 and 3, respectively (Severin and Stal, 2008). Similar mats on the German Wadden Sea barrier island Mellum fixed 3.2, 0.2 and 1.0 mmol N m^{-2} d^{-1} for the stations that were representative for those studied here (station 1, 2 and 3, respectively) (Stal *et al*., 1984). Hence, these values indicate that denitrification and N_2 fixation are in the same range in the coastal mats studied here. Joye and Paerl (1994) measured denitrification by the AI technique and showed that denitrification was only 15% of N_2 fixation on an annual basis in the mats of Tomales Bay. However, given that denitrification is underestimated by the AI technique, denitrification may have been responsible for a much higher proportion of the loss of the fixed N_2. Also, Bonin and Michotey (2006) found that denitrification exceeded N_2 fixation in winter in the hypersaline mat in Camargue. Hence, we conclude that denitrification can be an important sink for the fixed nitrogen in microbial mats.

Spatial and temporal heterogeneity of potential denitrification rates were observed in the present study and have also been documented for other microbial mats (Joye and Paerl, 1994; Bonin and Michotey, 2006). ST1 and ST3 showed the highest potential denitrification rates in summer (July), which was consistent with what has been reported for the hypersaline mats in the Camargue (Bonin and Michotey, 2006) and for the mudflat mats in Tomales Bay (Joye and Paerl, 1994). These results suggest that the nitrogen cycle in different phototrophic microbial mats behaves in a similar way. This might be due to the fact that microbial processes in phototrophic microbial mats are fundamentally the same and driven by the physicochemical gradients typically existing in these ecosystems (Stal, 2012).

The potential rate of denitrification in each of the mats can most likely be attributed to the dissimilar denitrifier communities. Denitrifiers are phylogenetically diverse and therefore it is expected that the physiology and enzyme affinities may vary considerably (Philippot and Hallin 2005). Consequently, shifts in community composition would lead to changes in the potential rate of denitrification and this has

actually been shown in several cases (Cavigelli and Robertson 2000; Rich *et al*., 2003; Jayakumar *et al*., 2004).

We used GeoChip and clone libraries to investigate the denitrifier community in microbial mats. Clone libraries offer the possibility to discover novel species of denitrifiers (assessed by evaluating and analyzing the *nirS* and *nirK* genes) in the microbial mats. However, the limited numbers of clones that were sequenced and the bias of the PCR approach targeting mainly dominant groups could have underestimated the rare types. Therefore, we used in addition the GeoChip. This chip provides a high coverage of the *nirS* and *nirK* genes that are not sufficiently abundant to be retrieved by clone libraries, provided that their probes were included on the chip. The combination of these two approaches allowed us to obtain a comprehensive diversity of the *nirS* and *nirK* denitrifiers in the microbial mats. The results from both analyses were in agreement with each other.

The phylogenetic analysis of the denitrifier community using *nirS* and *nirK* revealed that denitrifiers inhabited all three types of microbial mats. Most of the *nirS* and *nirK* genes retrieved in this study were unrelated to known denitrifying bacteria but shared considerable phylogenetic similarity with sequences from diverse environments including estuarine (Santoro *et al*., 2006), marine habitats (Castro-Gonzalez *et al*., 2005) as well as soil (Throbäck *et al*., 2007) and sludge (Osaka *et al*., 2006). This suggests that a large number of the denitrifiers in these microbial mats have not yet been cultivated. The high diversity of the denitrifier community may be due to a variety of potential environmental niches present in the microbial mats, which would allow diverse denitrifiers to proliferate. The deduced amino acid sequences of *nirS* and *nirK* fragments retrieved from clone libraries made from each of the sample stations were more similar to each other than to those of the other stations and hardly overlapped with the sequences from other stations. Although the *nirS* phylogenetic tree did not show a clear division of the clones according to the stations from which they originated, as was the case for the *nirK* tree, CCA analyses confirmed that both *nirS*- and *nirK*-denitrifier communities partitioned according to the different mat types. This shows that the conditions that prevail in a certain mat type selects for the type of denitrifier. This is in agreement with a clone library based study of a denitrifier community along a salinity and nitrate gradient in a coastal aquifer. Santoro and coworkers found that both NirS and NirK were distinct for certain communities, exhibiting little overlap between stations (Santoro *et al*., 2006). This habitat specificity of *nirS*- and *nirK*-denitrifier communities was observed in various other environments such as the Baltic Sea and freshwater lakes (Kim *et al*., 2011) or soils (Prieme *et al*., 2002).

Diversity estimates (Shannon–Weaver) based on the clone library GeoChip indicated that ST1 harbored a more diverse *nirK*- type denitrifying community than the other

two stations. With respect to *nirS* diversity, estimates made by clone libraries and the GeoChip were not consistent and we are therefore unable to draw a conclusion for this gene. The stringency of hybridization was optimized (Wu *et al*., 2006). The group-specific probes matched perfectly with their targets and the false positive signal was negligible. Most unique *nirS* and *nirK* were detected at ST1 suggesting that this station is different from the other two. Moreover, the phylogenetic trees of translated *nirS* and *nirK* from ST2 and ST3 show that a large number of sequences fell into the same clusters, suggesting that the denitrifying communities of these two stations were similar. The *nirS* and *nirK* sequences from ST1 formed distinct clusters. MRPP analysis based on clone libraries and GeoChip of *nirS* and *nirK* confirmed that the similarity was higher between ST2 and ST3 than either of these stations to ST1. Hence, in this respect the dissimilarity of the denitrifying communities in the three stations did not follow the pattern of the whole microbial community (Bolhuis and Stal, 2011). These authors found that the microbial community of ST2 was more dissimilar from that of ST1 and ST3. The *nirS* and *nirK* denitrifiers showed higher diversity during summer and a lower diversity during winter. This is in agreement with the development of a mat, which grows to maturity during summer and growth stops during winter when the mat is degraded (Stal *et al*., 1985).

The spatial organization of the denitrifying community in the microbial mats was likely the result of the different environmental conditions. Salinity has been proposed as the major driver of the microbial community composition for these microbial mats (Bolhuis *et al*., 2013). This might also apply to the denitrifier community. Jones and Hallin (2010) concluded that the global distribution pattern of *nirS* and *nirK* genes corresponded to salinity. The lower salinity at ST1 may explain the higher diversity of the denitrifier community. This has also been observed in a benthic denitrifier community along the estuarine gradient in Chesapeake Bay (Bulow *et al*., 2008). These authors found the highest *nirS* diversity at a freshwater station and the lowest diversity at a station with high salinity. Nitrite reductase genes in a wastewater treatment system showed that salinity decreased the diversity of both *nirS*- and *nirK-containing* denitrifying bacteria (Yoshie *et al*., 2004). Similar observations have been made for other functional genes of the N-cycle. Severin *et al*. (2012) investigated the same mats as in this study and found that the proportion of cyanobacterial *nifH* transcripts de- creased with increasing salinity. Likewise, Bernhard *et al*. (2010) found that the loss of diversity of ammonia-oxidizing bacteria correlated with increasing salinity in the Plum Island Sound estuary. Possible factors for the denitrifier compositional changes were partly associated with but not exclusively driven by salinity.

CCA of the *nirS* and *nirK* genes indicated that organic substrates and nitrate/nitrite are also important environmental factors influencing the denitrifier community composition but in opposite ways. Nitrite is the electron acceptor in denitrification.

The nitrate/nitrite concentration at ST1 was slightly higher than at the other two stations and this might be the underlying reason for the different denitrifier community in this station (Table 2). This is in line with the observation of Liu *et al*. (2003), who found that denitrifying communities were similar when the nitrate concentrations were at the same level. Organic carbon is the primary electron donor for heterotrophic denitrifiers (Zumft, 1997). We showed that the highest diversity of the denitrifier community was at the station with the lowest concentration of organic matter (Table 2). Most of this organic matter is recalcitrant polymeric material (Stal, 2003). We conceive that this would increase the diversity of denitrifiers. When organic matter is available, diversity will be low because of strong competition and out-competing of the less adapted species.

The two dimensions of CCA explained only part of the total variance of the denitrifier community (Fig. 2). This implies that there are also other factors that contribute to the composition of microbial community. For example, interaction and competition for resources with other microorganisms could be additional factors. In the microbial mats, denitrifiers compete for nitrate + nitrite with the primary producers such as cyanobacteria and diatoms that represent dominant groups in these mats (Severin *et al*., 2010; Bolhuis and Stal, 2011). The diversity of the *nifH* gene in these mats varied in a similar way (Severin and Stal, 2010). These authors found that the diazotrophic communities of ST2 and 3 were more similar to each other than to ST1. These results suggest that regardless different functional genes (*nifH*, *nirS* and *nirK),* the structure of the mats and its position in the littoral gradient overwhelmingly drive the diversity of the community, rather than single geochemical factors.

NirS and NirK nitrite reductases are functionally equivalent, but there is a debate going on as to whether the two types of denitrifiers are ecologically distinct (Jones and Hallin, 2010). Smith and Ogram (2008) found that *nirS* and *nirK* denitrifiers responded differently to environmental gradients. In this study, we found that *nirS* and *nirK* denitrifiers were similarly affected by environmental variables (Fig. 2). However, although this does not exclude the possibility that the two types of denitrifiers inhabit different niches, we have also no evidence for the opposite that they occupy the same niche. Desnues *et al*. (2007) investigated the vertical zonation of *nirS* and *nirK* denitrifiers in a hypersaline mat. These authors found that *nirS* was mainly localized in the permanent anoxic layer whereas *nirK* occurred throughout the whole mat and seemed to be better adapted to environmental fluctuations. Shannon index based on *nirS* and *nirK* sequences indicated a higher diversity of *nirS* clones com- pared to *nirK* clones at station 3. This finding agrees with observations from another ecosystem (Mosier and Francis 2010). However, the opposite was found for the stations 1 and 2. The seasonal changes of abundance of *nirS* and *nirK* were not consistent and varied between stations. This would imply that *nirS* and

nirK denitrifiers adapt differently to the environment. As illustrated in a previous study (Santoro *et al*., 2006), caution is needed because comparisons by using richness estimates may vary ac- cording to sample size. The GeoChip results showed a similar diversity and richness for *nirS* and *nirK*. It has been shown that in some ecosystems *nirS* denitrifiers were more abundant than *nirK* denitrifiers (Mosier and Francis, 2010). In our study, *nirS* and *nirK* denitrifiers were equally abundant in the GeoChip analysis, suggesting that both types of denitrifiers play important roles in denitrification in the microbial mats.

Chapter 3

Nitrification and nitrifiers in coastal microbial mats -Bacteria are the main players of ammonia oxidation in a coastal microbial mat

Haoxin Fan[1], Henk Bolhuis[1], and Lucas J. Stal[1,2]

[1]Department of Marine Microbiology, Royal Netherlands Institute of Sea Research, Yerseke, The Netherlands
[2]Department of Aquatic Microbiology, Institute of Biodiversity and Ecosystem Dynamics, University of Amsterdam, The Netherlands

Submitted to Frontiers in Microbiology

Abstract
The first step of nitrification, the oxidation of ammonia to nitrite, can be performed by ammonia-oxidizing archaea (AOA) or ammonium-oxidizing bacteria (AOB). We investigated the presence of these two groups in three structurally different types of coastal microbial mats that develop along the tidal gradient on the North Sea beach of the Dutch barrier island Schiermonnikoog. The abundance and transcription of *amoA*, a gene encoding for the alpha subunit of ammonia monooxygenase that is present in both AOA and AOB, were assessed and the potential nitrification rates in these mats were measured. The potential nitrification rates in the three mat types were highest in autumn and lowest in summer. AOB and AOA *amoA* genes were present in all three mat types. The composition of the AOA and AOB communities in the mats of the tidal and intertidal stations, based on the diversity of *amoA*, were similar and clustered separately from the supratidal microbial mat. In all three mats AOB *amoA* genes were significantly more abundant than AOA *amoA* genes. The abundance of neither AOB nor AOA *amoA* genes correlated with the potential nitrification rates, but AOB *amoA* transcripts were positively correlated with the potential nitrification rate. The composition and abundance of *amoA* genes seemed to be partly driven by salinity, ammonium, temperature and the nitrate/nitrite concentration. We conclude that AOB are responsible for the bulk of the ammonium oxidation in these coastal microbial mats.

Keywords: ammonia-oxidation, *amoA*, microbial mat, nitrification, salinity

Introduction

Coastal microbial mats are compact, highly structured, small-scale ecosystems (Stal *et al*., 1985). These mats are built by cyanobacteria, oxygenic phototrophic bacteria, which through primary production enrich the sediment with organic matter. This organic matter forms the basis of a complex, multi-layered microbial ecosystem. An important process in these microbial mats is the fixation of dinitrogen (N_2) (Severin and Stal, 2010). N_2 fixation has been intensively studied in microbial mats, but very little is known about the fate of the fixed nitrogen and about the functioning of the nitrogen cycle in microbial mats. In this study we investigated the oxidation of ammonium and assessed the seasonal variations in microbial mats located along a tidal salinity gradient.

Nitrification is the oxidation of ammonium to nitrate, which occurs in two steps, each carried out by specialist aerobic bacteria (Kowalchuk and Stephen, 2001). The first step, the oxidation of ammonia to nitrite (nitritification), is carried out by two distinct groups of microorganisms: ammonia-oxidizing bacteria (AOB) (two specific groups in Beta- and Gammaproteobacteria) and ammonia-oxidizing archaea (AOA). The second step is the oxidation of nitrite to nitrate (nitratification) and is carried out by a specialist group of bacteria. No archaea are known to carry out this second reaction. The oxidation of ammonia to nitrite is the rate-limiting step in nitrification. Nitritification is also important because it provides the oxidant for anaerobic ammonium oxidation (anammox) (Jetten *et al*., 1998). Moreover, nitrite can also be reduced by denitrification (Davidson and Seitzinger, 2006). Both processes eventually lead to the formation of dinitrogen and thus represent a loss of bound nitrogen from the microbial mat ecosystem.

Metagenomic studies (Venter *et al*., 2004) and the isolation and cultivation of *Nitrosopumilus maritimus* (Könneke *et al*., 2005), a marine AOA, (now placed within the *Thaumarchaeota* (Brochier-Armanet *et al*., 2008)) suggested an important role for this group of organisms in ammonia oxidation in the marine environment. This finding challenged the view that bacteria are the main players of microbial ammonia oxidation and has led to a large volume of research for the presence of AOA and AOB in a wide range of ecosystems. The presence of ammonia oxidizers is usually determined through the detection of *amoA*, the gene encoding the alpha subunit of ammonia monooxygenase, an enzyme that performs the first step in ammonia oxidation in both AOA and AOB. The ecological importance of AOA and AOB in nitrification has been determined in several studies. On the one hand, some studies reported that the archaeal *amoA* genes outnumbered those of bacteria by orders of magnitudes (as was the case for example in the North Atlantic Ocean and in the North Sea (Wuchter *et al*., 2006), in Monterey Bay and near Hawaii (Mincer *et al*., 2007), and in several estuaries (Caffrey *et al*., 2007). On the other hand, some studies reported that bacterial *amoA* genes were more abundant than the archaeal

amoA (Mosier and Francis, 2008; Christman *et al*., 2011). Since also gene or cell abundance do not necessarily reflect activity, the relative contribution of AOA and AOB to ammonia oxidation in coastal sediments remains uncertain. There is however good evidence for different niches for AOA and AOB. The former possesses a high affinity (low K_m) for ammonia and therefore seems to particularly dominate environments that are very low in it, while the latter seems to prefer environments with high ammonia concentrations (Martens-Habbena *et al*., 2009). Compared to many terrestrial and marine environments, the ecology of ammonia oxidizer communities and their role in nitrification in coastal microbial mats has been poorly studied. AOA and AOB may be subject to different selection pressures that result from biotic and abiotic conditions and the different physiology that characterizes these organisms. A suite of environmental parameters may control nitrification in coastal sediments. These include besides ammonia, oxygen- and sulfide concentrations, the rate of carbon metabolism, and the presence or absence of vegetation or macro-fauna (Herbert, 1999). Coastal microbial mats harbor a multitude of potential environmental niches as the result of the large daily fluctuations of the key geochemical parameters such as: oxygen, pH, and sulfide (Revsbech *et al*., 1983).

The aims of this study were to identify the ammonia oxidizing communities in the three types of microbial mats and to elucidate the factors that determine the abundance and activity of ammonia oxidizers. We measured therefore the potential rate of nitrification and investigated the diversity and abundance of *amoA* for AOB and AOA in three different mat types during four different seasons. We monitored the key environmental variables and linked them to changes in ammonia oxidizer communities and their activities (*amoA* gene transcripts).

Material and methods

Sampling

The study site was located on the North Sea beach of the Dutch barrier island Schiermonnikoog. The geographical locations and descriptions of the three types of microbial mats (stations) that were sampled during this study as well as the vegetation and primary cyanobacterial species at these stations are presented in Table 1. The stations were located along a transect perpendicular to the beach covering the tidal gradient. Sampling was done five times during 2010 and 2011 to cover the four seasons. Samples were taken from the top 2.5-3 cm of the mat using custom-made transparent Lexan cylinder corers of 50 mm inner diameter and 60 mm height. The cores were transported back to the laboratory within 4 h of sampling and subsequently kept at ambient temperature and light. Incubation experiments for measuring the potential nitrification rate started within 24 h after sampling. Additional samples were taken from the natural mats for nucleic acid extraction. These samples were taken from the top 1 cm of the mat by using as a corer a 10-ml

syringe from which the needle connector was removed. These mat samples were divided into four equal parts using a scalpel, put into cryo-vials, and immediately frozen in the field in liquid nitrogen.

Chemical analyses

For nutrient analyses 5 g mat sample (top 1 cm) was extracted with 40 ml 2 *M* KCl. The extracts were filtered through Whatman GF/F filters and the filtrates were kept at -20 °C until analysis (within a month). Nutrient (DIN and phosphate) concentrations were measured by a standard colorimetric method using an automated Segmented Flow Analyzer. Other mat samples were freeze-dried for the determination of total nitrogen (TN), total organic carbon (TOC) and C/N ratio by EA-IRMS (DELTA V Advantage; Thermo Fisher Scientific, Bremen, Germany).

Table 1. The geographical coordinates and description of the mats investigated in this study.

Station	Geographical coordinates	Description	Vegetation	Dominant cyanobacterial species
Station I	53°29.445′N, 6°8.718′E	Mainly freshwater influenced site, close to the dunes. Irregularly inundated.	*Elymus arctus* *Juncus gerardi* *Glaux maritima* *Ammophila arenaria* *Scirpus maritimus*	*Nostoc* *Calothrix* *Anabaena* *Spirulina* *Nodularia* *Synechocystis* *Merismopedia* *Gloeocapsa*
Station II	53°29.460′N, 6°8.309′E	Seawater influenced site, developing microbial mat. At the low water mark.	No vegetation	*Lyngbya* *Leptolyngbya*
Station III	53°29.445′N, 6°8.342′E	Seawater and freshwater influenced site, located between St1 and St2, at the edge of the salt marsh.	*Salicornia* sp. *Puccinellia distans*	*Microcoleus* *Lyngbya*

Potential nitrification rate

The potential rate of nitrification was determined by using the ^{15}N isotope dilution method by the addition of ^{15}N-labelled nitrate (Kirkham and Bartholomew, 1954). The measurements were performed in triplicate in the intact sediment cores. The ^{15}N

nitrate solution was injected into the sediment core. The needle was inserted fully into the sediment core and the syringe plunger was depressed while the needle was withdrawn out of the sediment as to distribute the label equally in the sediment. Four injections were made in each sediment core. Three cores from each station were frozen (-20°C) immediately after the injection. The other cores were incubated at *in situ* temperature (Table 2) under a 12-12 h light-dark cycle for 24 h. Subsequently, the inorganic nitrogen was extracted from the cores by 2 *M* KCl. The nitrogen isotopic composition of NO_x^- was determined using the ammonia diffusion procedure according to Gribsholt *et al*. (2005). The rate of nitrification was calculated according to the equation of Norton and Stark (2011).

Nucleic acid extraction and GeoChip analysis.

DNA and RNA were extracted using the MoBio UltraCLEAN soil DNA kit and the RNA PowerSoil® Total Isolation Kit, respectively (MoBio Laboratories, Inc., Carlsbad, CA, USA) according to the manufacturer's instructions. The quantity and quality were determined and checked by Nanodrop (Nanodrop ND1000, Thermo Scientifica, Wilmington, DE, USA) and agarose gel electrophoresis, respectively. The RNA extracts were immediately treated with RNase free DNase I (Deoxyribonuclease I, Amplification Grade, Invitrogen Corporation, Carlsbad, CA, USA). Remaining DNA contamination of the RNA extracts was checked by PCR using the RNA extract as a template. RNA concentration and quality were checked again as described above. The DNA-free RNA was reverse transcribed to copy DNA using Superscript II Reverse Transcriptase and random primers (Invitrogen Corporation, Carlsbad, CA, USA) following the manufacturer's manual. Two controls were performed that either lacked reverse transcriptase or RNA. PCR reactions were performed to check the transcription to cDNA and controls were included as described above. The synthesized cDNA was kept at -20 °C until further use.

In addition, we used a functional gene microarray system, the GeoChip (Tu *et al*., 2014), containing probes for genes involved in the majority of important biogeochemical nutrient cycles. For this study, we extracted data from the GeoChip for *amoA* genes. We analyzed DNA samples from July 2010 and January 2011 by the GeoChip 4.2. DNA was extracted from triplicate samples from each of the three types of microbial mats. The DNA was purified using UltraClean 15 DNA purification Kit (MoBio Laboratories, Inc., Carlsbad, CA, USA) in order to achieve the quality necessary for hybridization on the chip. The DNA quantity was measured using the Nanodrop ND-1000 system. The procedures for DNA labeling and microarray hybridization followed previously established protocols (Wu *et al*., 2006). Briefly, 800ng DNA was labeled with fluorescent Cy-5 dye by random priming and re-suspended in 50 µl hybridization solution (40% formamide, 5× SSC, 5 µg of unlabeled herring sperm DNA (Promega, Madison, WI), and 0.1% SDS) and 2 µl

Table 2. Physicochemical parameters in the microbial mats during the 2010-2011 sampling period.

	July (2010)	September (2010)	November (2010)	January (2011)	April (2011)
ST1					
Temperature					
(°C,sediment)	17	10	9	0	8
NH_4^+(μmol/l)	128.9±3.0	83.7±17.3	252.4±25.6	191.3.5±23.4	233.1±23.7
NO_X^-(μmol/l)	23.3±4.6	8.4±1.1	11.4±2.1	9.6±4.6	25.9±3.4
TOC(%)	0.04	0.06	0.04	0.04	0.04
TN(%)	0.006	0.01	0.006	0.007	0.007
C/N	6.7	6.0	6.7	5.7	5.7
Salinity(psu)	18	19	15	15	17
ST2					
Temperature					
(°C,sediment)	17	10	9	0	8
$NH4^+$(μmol/l)	587.9±41.2	216.2±69.8	1094±91.3	736.8±199.6	486.1±61.3
NO_X-(μmol/l)	22.4±14.7	8.7±1.4	9.8±3.2	6.2±1.1	20.6±4.5
TOC(%)	0.19±0.01	0.20±0.03	0.13±0.02	0.20±0.03	0.17±0.02
TN(%)	0.03	0.04	0.02	0.03	0.03
C/N	6.3	5.0	6.5	6.7	5.7
Salinity(psu)	28	28	29	30	28
ST3					
Temperature					
(°C,sediment)	17	10	9	0	8
NH_4+(μmol/l)	217.3±102.3	255.9±68.4	782.6±158.5	510.2±62.2	475.8±3.5
NO_X-(μmol/l)	16.0±3.7	7.6±1.9	6.6±1.9	2.8±0.6	19.6±4.0
TOC(%)	0.11±0.03	0.15±0.02	0.18±0.02	0.15±0.02	0.13±0.02
TN(%)	0.02	0.03	0.03	0.03	0.02
C/N	5.5	5.0	6.0	5.0	6.5
Salinity(psu)	25	25	23	22	23

Abbreviations: TOC, total organic carbon; TN, Total nitrogen.

universal standard DNA (0.2 pmol μl^{-1}) labeled with the fluorescent Cy-3 dye (Liang *et al*., 2010), denatured for 5 min at 95°C and maintained at 50°C until loaded onto the microarray slides. Arrays were hybridized on a MAUI Hybridization Station (Roche, South San Francisco, CA) for 12 h at 42°C. The hybridized microarrays were scanned by a ScanArray Express (Perkin- Elmer, Wellesley, MA) at 95% laser power and 85% photomultiplier tube gain. The resulting images were analyzed by ImaGene with signals processed as SN>2.0 (signal to noise ratio).

Quantitative PCR (qPCR) analysis

qPCR analyses were run on a Corbett Rotor-Gene 6000TM (Corbett Life Science, Sydney, Australia). The copy numbers of AOB and AOA were determined by primers amoA-1F and amoA-2R (Tm=53°C) (Rotthauwe et al., 1997) and by CrenAmoAQ-F (Mincer *et al*., 2007) and Arch-AmoA-R (Tm=51°C) (Francis *et al*., 2005), respectively. We determined the gene copy number in the mat samples in triplicate. Standard curves were made by dilution series of linearized plasmids (quantified by Nanodrop before using as standard for quantification) containing the target genes and were run parallel with each analysis as well as non-template controls. The reaction mixture (15 µl) contained 7.5 µl of Absolute ™ QPCR SYBR® Mix (Thermo Fisher Scientific, Rockford, IL, USA), 0.2 pmol/µl primers, 1 µl template and extra sterilized MQ water. Cycling conditions were as follows: 95°C 15 min, 45 cycles of 15 sec 95°C, 20 sec Tm, and 20 sec at 72°C, followed by melting curve analysis (50-95°C). The standard curves spanned a range from 15 to 1.7×10^6 copies per µl for the β-AOB and 2.2 to 1.2×10^6 copies per µl for the AOA. PCR efficiencies (E) and correlation coefficients for β-AOB were 78-88% and R^2=0.99 and for AOA were 85-98% and R^2=0.99.

Sequences and statistical analysis

Ammonia monooxygenase alpha subunit amino acid sequences obtained from GeoChip hybridization and some reference sequences retrieved from GenBank were used to produce neighbor-joining trees and the reliability of the phylogenetic reconstructions was evaluated by bootstrapping (1000 replicates) using MEGA 6 (Molecular Evolutionary Genetics Analysis, http://www.megasoftware.net/mega6/mega.html) (Tamura *et al*., 2013).

In order to summarize the gene overlap at station and season level, the genes detected in the three replicates of each station from July 2010 and January 2011 by the GeoChip were deployed as one pool (mean value from the three replicates). The determination of the overlapping genes, unique genes and diversity indices was done using an online pipeline (http://ieg.ou.edu/). The proportion of overlapping genes of two stations was calculated as the number of overlapping genes divided by the total number of genes detected in these stations. The proportion of unique genes at each station was calculated as the number of unique genes at each station divided by the total number of genes detected at that station.

Cluster analysis, multi-response permutation procedure (MRPP) and canonical correspondence analysis (CCA) (see below) were made based on the community data from the GeoChip. Cluster analysis of the community composition was done using PAST (http://folk.uio.no/ohammer/past/). MRPP using Bray-Curtis distance was used to test for significant differences in community composition. The MRPP was carried out in the open source software R (Team, 2011), using vegan package

(Oksanen, 2011). The MRPP A-statistics describes the within and between group relatedness relative to what is expected by chance. A p-value <0.05 and an A-statistics >0.1 is considered to be a significant difference between groups (McCune *et al*., 2002). In order to test the relationship between the community composition and the environmental variables, CCA was carried out using Canoco 4.5 for Windows (ter Braak, 1989). The significance of the whole canonical model was tested by 999 permutations. One-way ANOVA, correlation test (Spearman and Pearson), and stepwise regression were carried out in SigmaPlot (Version12). Stepwise regression was carried out to test the influence of the environmental factors and abundance of *amoA* gene and transcript on potential nitrification rates.

Results

Phylogeny of *amoA*

In all microbial mats, AOB *amoA* genes were detected belonging to Beta- and Gammaproteobacteria (Fig. 1A). The majority (>98% signal intensities) AOB *amoA* sequences retrieved from the GeoChip hybridizations belonged to Betaproteobacteria (β-AOB). Among the Betaproteobacteria, the sequences clustered with *Nitrosospira* and *Nitrosomonas*. The sequences belonging to *Nitrosospira* can be subdivided into three major clusters. *Nitrosospira* cluster A contained 9.8% of the sequences obtained from the GeoChip and all of them were environmental clones. Cluster A is related to *Nitrosospira* sp. Nsp12 (AY123823). *Nitrosospira* cluster B contained 34.8% of the sequences obtained from the GeoChip and most of them were environmental clones. Several cultivated species, such as *Nitrosospira multiformis* (AAC25057), *Nitrosospira briensis* and *Nitrosospira* sp. REGAU (AAV34189) were detected. *Nitrosospira* cluster C contained 8.0% of the sequences obtained from the GeoChip. *Nitrosovibrio* sp. FJ182 (ABB69924) and some uncultured clones were detected that were grouped in cluster C. The sequences belonging to *Nitrosomonas* can be sub-divided into four major clusters. The nomenclature suggested by Purkhold *et al*. (2003) and Francis *et al*. (2003) was used. *Nitrosomonas marina* lineage, *Nitrosomonas* like, *Nitrosomonas communis* lineage and *Nitrosomonas europaea* accounted for 4.5, 10.7, 6.3 and 11.6% of the sequences obtained from the GeoChip, respectively. In all samples, *Nitrosospira* clusters A, B, C and *Nitrosomonas*-like, *Nitrosomonas marina* lineage, *Nitrosomonas communis* lineage and *Nitrosomonas europaea* lineage accounted for 9.0-10.9%, 34.5-42.6%, 7.5-9.8%, 8.3-10.3%, 1.2-4.1%, 4.6-6.6% and 10.5-12.5% of the total AOB *amoA* signal intensities, respectively, whereas the remaining 10.5-14.6% of AOB *amoA* signal intensities were from other species (Fig. 1A). The shifts between different groups are minor at all stations. Phylogenetic analysis revealed a broad distribution of archaeal *amoA* genes (Fig. 1B). The nomenclature suggested by Pester *et al*. (2012) was used. 41.1, 14.7, 37.9 and 6.3% of the total number of sequences obtained from the GeoChip fell into *Nitrosopumilus*, *Nitrosotalea*, *Nitrososphaera*, and others, respectively. Based on GeoChip signal intensities, the *Nitrosopumilus*

and *Nitrososphaera* were predominant and accounted for respectively 39.6-45.5% and 28.7-34.3% of total AOA *amoA* gene signal intensities. *Nitrosotalea* and other groups accounted for the remaining 16.5-20.3% and 6.9-9.3% of total AOA *amoA* gene signal intensities. In none of the stations shifts between different groups were observed.

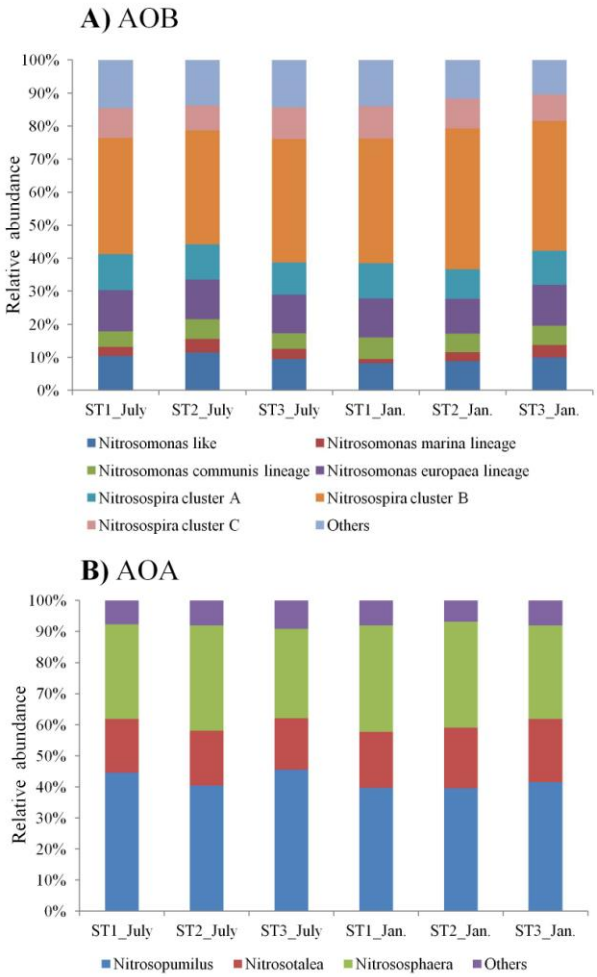

Figure 1. Relative abundance of A) probes affiliated with the major bacterial *amoA* clusters suggested by Purkhold *et al*. (2003) and Francis *et al*. (2003) in the analysed microbial mats; B) probes affiliated with the major archaeal *amoA* clusters suggested by Pester *et al*. (2012) in the analysed microbial mats.

Table 3. Summary of AOB and AOA *amoA* detected by GeoChip, including the number and percentage of overlapping (italic) and unique (bold) sequences, and the diversity indices for each station.

	ST1_July	ST2_July	ST3_July	ST1_Jan.	ST2_Jan.	ST3_Jan.
AOB						
ST1_July	**9(8.74%)**	*88(82.95%)*	*59(57.48%)*	*83(79.05%)*	*65(61.32%)*	*47(44.76%)*
ST2_July		**3(3.19%)**	*60(62.50%)*	*77(75.49%)*	*66(68.75%)*	*49(52.13%)*
ST3_July			**1(1.61%)**	*54(58.06%)*	*51(64.56%)*	*46(70.77%)*
ST1_Jan.				**1(1.18%)**	*64(77.91%)*	*46(50.56%)*
ST2_Jan.					**0(0.00%)**	*46(64.79%)*
ST3_Jan.						**0(0.00%)**
Richness*	103	94	62	85	68	49
Shannon-Weaver	4.8	4.7	4.3	4.7	4.5	4.2
AOA						
ST1_July	**5(6.3%)**	*69(78.4%)*	*52(61.2%)*	*68(77.3%)*	*53(61.6%)*	*46(54.1%)*
ST2_July		**3(3.9%)**	*56(71.8%)*	*65(73.9%)*	*57(72.2%)*	*50(64.1%)*
ST3_July			**1(1.8%)**	*51(62.2%)*	*47(68.1%)*	*44(68.8%)*
ST1_Jan.				**4(5.3%)**	*57(73.1%)*	*49(62.8%)*
ST2_Jan.					**0(0.00%)**	*47(74.6%)*
ST3_Jan.						**1(2.0%)**
Richness*	80	77	57	76	59	51
Shannon-Weaver	2.9	2.9	2.7	2.9	2.7	2.7

* richness is determined as probe numbers detected.

AOB and AOA diversity and community composition

Diversity of AOA and AOB *amoA* genes based on analysis with the GeoChip is summarized in Table 3. A total of 112 AOB *amoA* sequences showed hybridization. The average number of AOB *amoA* sequences (richness) at Station 1 (July), Station 2 (July), Station 3 (July), Station 1 (January), Station 2 (January) and Station 3 (January) was 103, 94, 62, 85, 68, and 49, respectively (Table 3). Fourteen sequences were unique, meaning that they were detected only at one station and in one season. In July, Station 1 harbored 9 unique sequences, which were 8.7% (9/103) of the total number detected. Station 2 and Station 3 harbored 3.2% (3/94) and 1.6% (1/62) unique sequences, respectively. In January, the number of unique sequences in Station 1 dropped to 1, which was only 1.2% (1/85) of the total number detected. No unique sequences were observed in January at Station 2 and Station 3. Pairwise comparison of AOB *amoA* sequences showed a high number of overlapping AOB *amoA* sequences between July and January as well as between the stations: 79.1% (Station 1 July and January), 68.8% (Station 2 July and January), 70.8% (Station 3 July and January), 61.3%-83.0% (Station 1 and Station 2), 44.8- 57.5% (Station 1 and Station 3) and 52.1%-62.5% (Station 2 and Station 3).

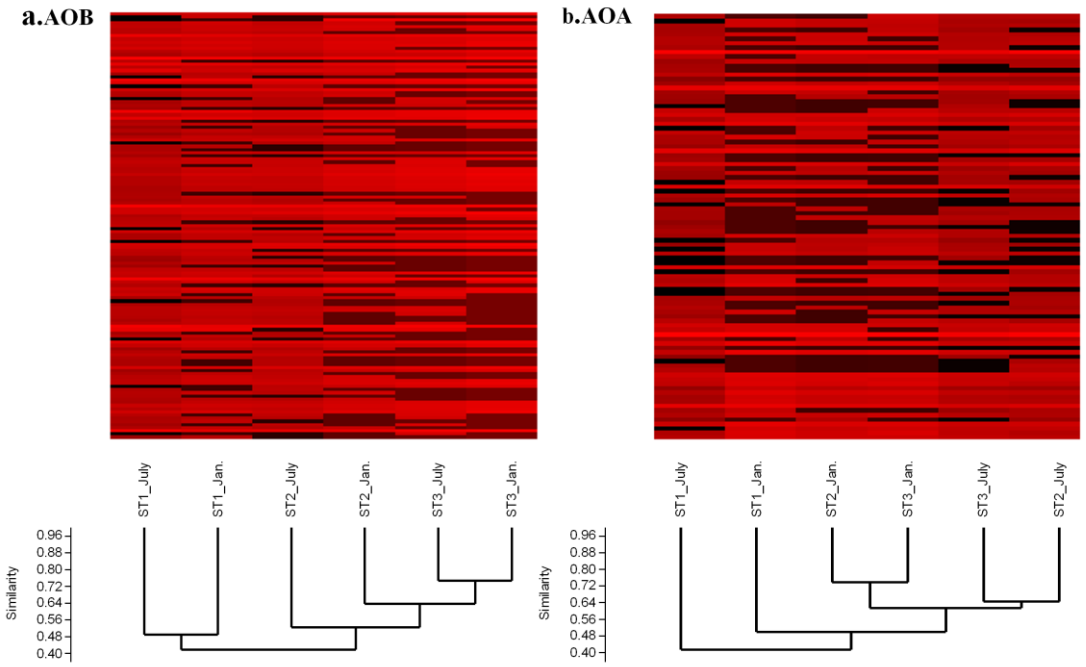

Figure 2. Cluster analysis of *amoA* community composition at the different sampling stations. (a) Cluster analysis of betaproteobacterial *amoA* gene composition (b) Cluster analysis of archaeal *amoA* gene composition. Sample codes consist of the sampling station and the month of sampling.

We detected 95 AOA *amoA* sequences in the mats. The highest richness of AOA *amoA* sequences was detected in July at Station 1 (80). We detected 77, 57, 76, 59 and 51 AOA *amoA* sequences at Station 2 (July), Station 3 (July), Station 1 (January), Station 2 (January) and Station 3 (January), respectively (Table 3). A considerable number of AOA *amoA* sequences were also shared between stations and seasons (Table 3). The highest Shannon index was observed at Station 1 and the lowest was at Station 3, irrespective the time of sampling.

Cluster analysis revealed that the AOB community composition (based on the *amoA* gene diversity) was largely the same in summer and winter in each of the Stations 1 and 3. Station 2 and Station 3 clustered and were dissimilar from Station 1 (Fig. 2A). A similar distribution was also found for the AOA community: Station 2 and Station 3 were more similar and separated from the community of Station 1. In addition, the July AOA community composition was separated from that of January in the Stations 2 and 3 (Fig. 2B).

Table 4. Multi-response permutation procedure A-values of the ammonia oxidizing community composition.

Difference between group	
Spatial differences	A-value
AOA	
ST1 vs ST2	0.135 (p=0.032)*
ST1 vs ST3	0.357 (p=0.004)*
ST2 vs ST3	0.065 (p=0.095)
AOB	
ST1 vs ST2	0.135 (p=0.03)*
ST1 vs ST3	0.365 (p=0.005)*
ST2 vs ST3	0.04 (p=0.165)

MRPP statistics was carried out to test the differences of the AOB and AOA *amoA* composition between the stations and seasons (July and January) based on the GeoChip data. When the data from July or January were analyzed individually, no significant differences were observed between stations. When the data from the two seasons were combined, distinct communities of AOB and AOA were found in Station 1 when compared to the other two stations ($p<0.05$) (Table 4). The Stations 2 and 3 did not show significant different ammonia-oxidizer communities ($p>0.05$) (Table 4). There were no seasonal differences in the ammonia-oxidizer communities in any of the stations (data not shown).

Potential nitrification rates (PNR)

The potential nitrification rates were measured using the isotope (^{15}N nitrate) dilution method (Kirkham and Bartholomew, 1954) and covered the four seasons during 2010 and 2011. The potential rate of nitrification varied between stations as well as between seasons and ranged from 34 to 537 μmol N $m^{-2}d^{-1}$ (Fig. 3) (Table 5). At all three stations the potential rate of nitrification showed a similar seasonal pattern with higher rates occurring in November and January and lowest rates occurring in July and September (Fig. 3) (Table 5). The highest rate was measured at Station 3 (537 μmol N $m^{-2}d^{-1}$) while the lowest was detected at Station 2. The potential rate of nitrification was always the lowest at Station 2, irrespective of the season.

AOB and AOA abundance and activity

Abundance of archaeal and bacterial *amoA* genes was quantified in three stations from different seasons. Because of a negligible signal for nitrifiers belonging to the Gammaproteobacteria, we focus on β-AOB. β-AOB *amoA* genes were detected in all three stations and in all seasons except at Station 1 in September. The numbers ranged from below detection level to 1.7×10^7 copies g^{-1} (sediment) (Fig. 3) (Table

5). The highest number of β-AOB *amoA* genes was always observed in January at all stations. AOB *amoA* gene was undetectable in Sep. and April at Station 1. The lowest β-AOB *amoA* gene abundance at Stations 2 and 3 were observed in November and April, respectively.

β-AOB *amoA* gene abundance increased from the station at the dunes to the low water mark in three of the four seasons tested, the exception being that in November the highest value was found in the intertidal Station 3. AOA *amoA* copies ranged from below detection level to 1.2×10^4 copies g^{-1} sediment. AOA *amoA* was undetectable at Station 1, irrespective of the season, and in April at Station 3. The highest number of AOA *amoA* copies was detected in January at Station 2. The β-AOB *amoA* copy number was significantly ($p<0.05$) higher in all samples except in September at Station 1 when neither β-AOB nor AOA *amoA* genes were detected. β-AOB *amoA* abundance was significantly correlated (Spearman, $r=0.70$, $p<0.01$) with AOA *amoA* abundance. The expression of β-AOB *amoA* was detected in four of the five months at Station1 (undetectable in November) and Station 3 (undetectable in July) with highest expression in April and November, respectively. At Station 2, the β-AOB *amoA* expression was only detected in September and November. Gene expression of AOA *amoA* was below the limit of detection in all samples.

Relationship of ammonia oxidizer community with environmental variables

Canonical correspondence analysis showed that ammonia oxidizer (AOA and AOB) communities were significantly correlated with salinity, temperature, ammonium and nitrate content (based on 999 Monte Carlo permutations, $p=0.001$) (Fig. 4). The variables in the first and second axes explained 49.6% and 47.2 % of the total variation of AOB and AOA composition, respectively.

Environmental variables influenced also the abundance of ammonia oxidizers. Across all samples, both β-AOB (Spearman, $r=0.59$, $p<0.01$, $n=15$) and AOA (Spearman, $r=0.73$, $p<0.01$, $n=15$) *amoA* abundance was positively correlated with salinity and organic matters (AOB: Spearman, $r=0.87$, $p<0.01$, $n=15$; AOA: Spearman, $r=0.60$, $p<0.05$, $n=15$). Abundance of β-AOB *amoA* gene also correlated with ammonium content (Spearman, $r=0.78$, $p<0.05$, $n=15$). No significant correlations were found between abundance of ammonia oxidizers and other environmental variables (measured in this study).

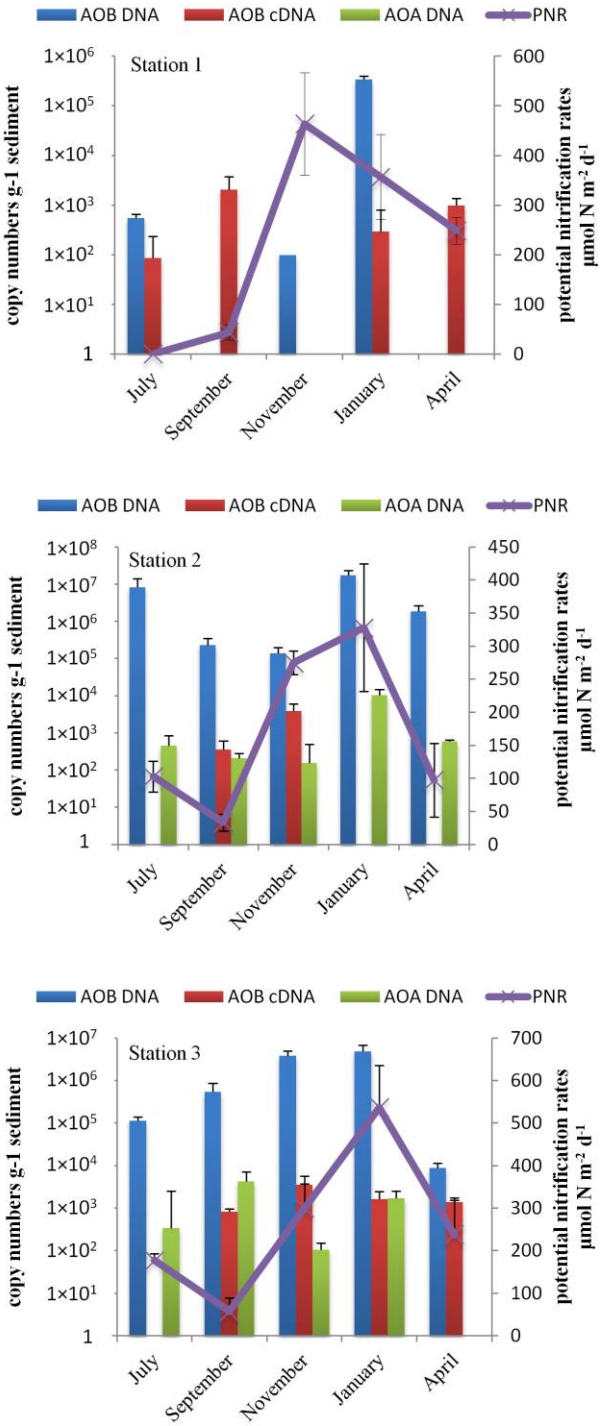

Figure 3. Mean (±standard error, n=3) potential nitrification rate (PNR) and abundance of *amoA* (of β-AOB and AOA) and their transcripts at Station 1, Station 2 and Station 3 during the 2010-2011 sampling period (July, September, November, January and April).

Table 5. Potential nitrification rate (PNR) (μmol N m^{-2} d^{-1}) and abundance of *amoA* (of β-AOB and AOA) and their transcripts in the microbial mats during the 2010-2011 sampling period (copies g^{-1} sediment).

	July		September		November		January		April	
		SD		SD		SD		SD		SD
Station 1										
PNR	n.d.		43.5	15.6	463.7	103.7	356.9	86.3	248.7	27.0
AOB DNA	550	100	n.d.		99		3.4×10^5	5.9×10^4	n.d.	
AOB cDNA	86	150	2.0×10^3	1.7×10^3	n.d.		290	510	970	390
AOA DNA	n.d.		n.d.		n.d.		n.d.		n.d.	
AOA cDNA	n.d.		n.d.		n.d.		n.d.		n.d.	
Station 2										
PNR	102.8	23.6	33.6	12.8	274.9	17.8	327.8	96.4	97.5	55.4
AOB DNA	8.2×10^6	5.8×10^6	2.3×10^5	1.2×10^5	1.4×10^5	5.6×10^4	1.7×10^7	6.2×10^6	1.8×10^6	8.5×10^5
AOB cDNA	n.d.		360	250	3.9×10^3	2.2×10^3	n.d.		n.d.	
AOA DNA	460	390	210	66	150	340	1.0×10^4	4.6×10^3	5.9×10^3	55
AOA cDNA	n.d.		n.d.		n.d.		n.d.		n.d.	
Station 3										
PNR	177.4	15.2	57.5	31.7	300.3	56.8	537.0	99.2	237.7	86.4
AOB DNA	1.1×10^5	2.6×10^4	5.4×10^5	3.3×10^5	3.8×10^6	1.2×10^6	4.9×10^6	1.9×10^6	8.9×10^3	2.5×10^3
AOB cDNA	n.d.		830	140	3.5×10^3	2.1×10^3	1.6×10^3	830	1.4×10^3	180
AOA DNA	330	2.2×10^3	4.3×10^3	2.8×10^3	110	47	1.7×10^3	800	n.d.	
AOA cDNA	n.d.		n.d.		n.d.		n.d.		n.d.	

Abbreviations: SD: standard deviation. n.d., below detection

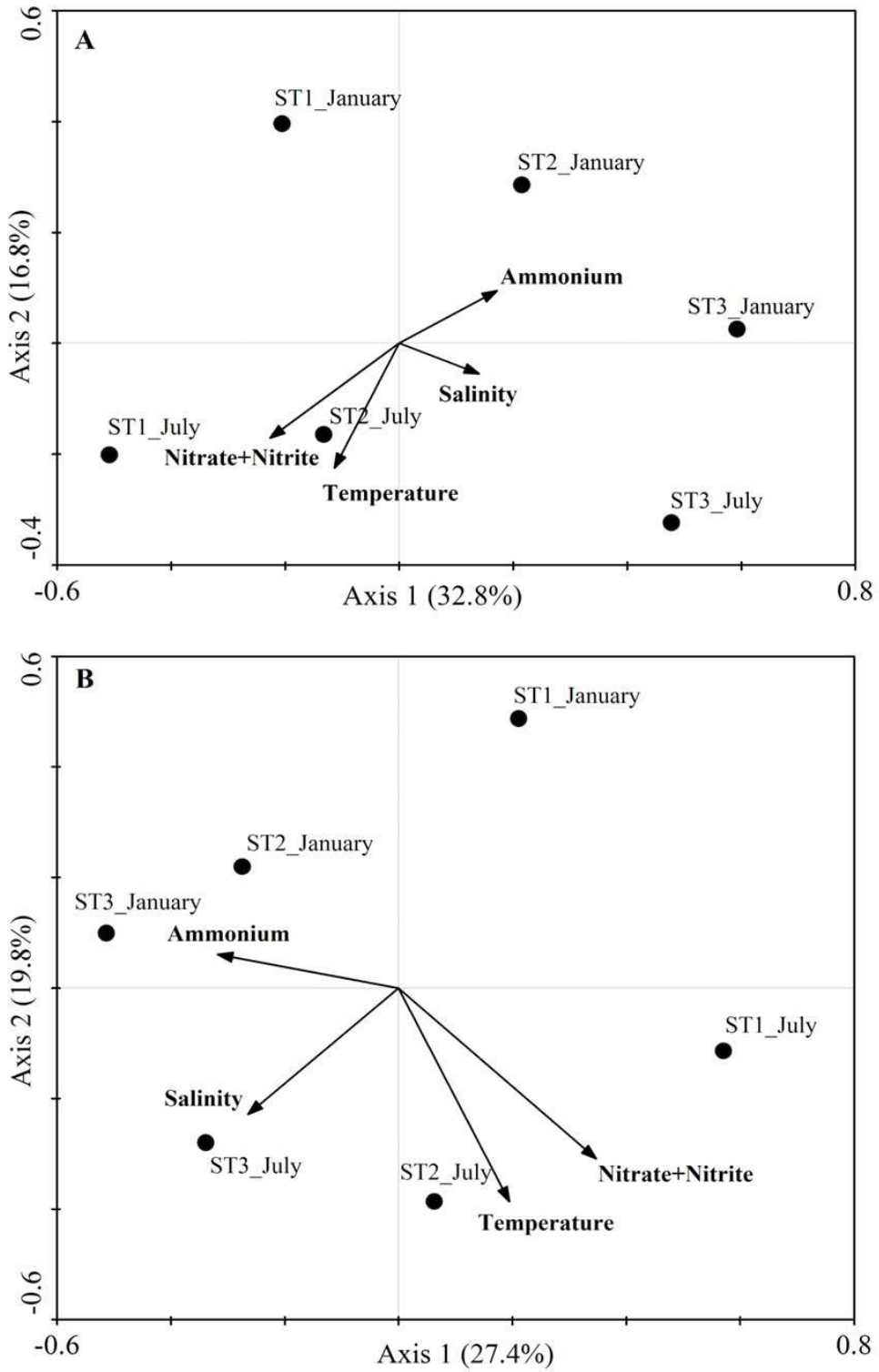

Figure 4. Canonical correspondence analysis of *amoA* community composition of mat samples. (a) β-AOB *amoA* gene, (b) AOA *amoA* gene. Points represent the *amoA* gene community from seasonal samples at the indicated station. Arrows represent the relationship between environmental parameters with the *amoA* communities.

Discussion

The GeoChip analyses revealed that ammonia-oxidizing bacteria and archaea are common in the microbial mats of the North Sea barrier island Schiermonnikoog. AOB and AOA *amoA* genes that were detected by GeoChip were distributed throughout the phylogenetic trees of AOB and AOA *amoA*. The AOB *amoA* sequences in the microbial mat were dominated by clusters B and C, which both relate to *Nitrosospira* cluster 3, first described by (Purkhold *et al*., 2003). This is consistent with what other studies reported. *Nitrosospira* cluster 3 is found predominantly in brackish and marine environments (Francis *et al*., 2003; Bernhard *et al*., 2005; Freitag *et al*., 2006; Wankel *et al*., 2011). A considerable number of sequences were found belonging to *Nitrosomonas*. The AOB in *Nitrosomonas* spanned a wide range of physiological types and inhabit oligohaline to polyhaline environments (Koops and Pommerening-Roser, 2001; Francis *et al*., 2003). The AOA *amoA* sequences detected in the microbial mats investigated in this study have been found in a variety of other habitats, such as corals (Brochier-Armanet *et al*., 2008), hot springs (Zhang *et al*., 2008), estuaries (Santoro *et al*., 2008), marine sediments (Francis *et al*., 2005), and soil (Onodera *et al*., 2009). In a previous 16S rRNA gene based amplicon sequencing study of the same microbial mats (Bolhuis and Stal, 2011) a small number of AOB sequences (less than 0.01% of the total community) related to the Nitrosomonadales were found. In two metagenomic datasets of these microbial mats the AOB genus *Nitrosococcus* and *Nitrosomonas* were found at ~0.5% and ~0.2% of the total identified genus, respectively (unpublished). In none of these studies AOA sequences were found. However, due to the different nature of the experiments in these and the present study (DNA hybridization vs PCR bases gene amplification vs whole genome sequencing) any direct comparisons are difficult. The high diversity of ammonia oxidizers that we observed in the coastal microbial mats can be explained by the large variety of potential microhabitats in the microbial mats that are generated by the daily fluctuations of geochemical parameters and by the activity of the mat microbes.

The richness and diversity estimators (Shannon-Weaver) indicated that Station 1 harbored a more diverse ammonia-oxidizing community than the other two stations. Cluster analysis showed that the bacterial and archaeal *amoA* composition both grouped Station 2 and Station 3 apart from Station 1 (Fig. 2). MRPP analysis of the ammonia oxidizer community (AOB and AOA) confirmed that Station 2 was more similar to Station 3 than either of these stations to Station 1. This agrees with what was found for the community of denitrifiers (*nirS* and *nirK*) in these mats (Fan *et al*., 2015) (Chapter 2) and for the community of nitrogen fixers (*nifH*) (Severin *et al*., 2012). This suggests that the community composition of the different functional groups of microorganisms involved in the nitrogen cycle is determined by the same physical and geochemical factors that prevail in the three types of microbial mats. Previous studies of the eukaryal, bacterial and archaeal diversity (based on ribosomal RNA genes) of these mats revealed that community composition and microbial

diversity were intrinsic of the mat type (Bolhuis and Stal, 2011; Bolhuis *et al*., 2013). Here we show that this also applies for a functional gene for ammonia oxidation.

Despite the lack of statistically significant differences in *amoA* (β-AOB and AOA) community composition, the richness and diversity of bacterial *amoA* were more vulnerable to seasonal changes, while those of archaeal *amoA* remained unchanged except in Station 2. The fluctuation of richness and diversity for archaeal *amoA* at Station 2 may be due to the tidal inundation by seawater. We expected that the richness and diversity might be higher in winter because other studies showed that AOA were more abundant in winter in the North Sea (Wuchter *et al*., 2006). However, our results showed that this scenario did not occur in the coastal microbial mats. Cluster analysis showed that the AOB composition at Station 2 was more affected by seasonal changes. The possible explanation is that the mat is disappearing at Station 2 during the year, while the other mats are perennial.

AOB *amoA* copy numbers were similar to those reported from estuaries (Caffrey *et al*., 2007; Santoro *et al*., 2008), salt marshes (Dollhopf *et al*., 2005) and coastal aquifer sediments (Mosier and Francis, 2008), whereas AOA *amoA* copy numbers in our study were comparable with those in the cold seep surface sediment (10^2-10^4 copies g^{-1} sediment) (Dang *et al*., 2010b) but were two to three orders of magnitude lower than reported for other estuarine and coastal sediments (10^4-10^7 copies g^{-1} sediment) (Mosier and Francis, 2008; Santoro *et al*., 2008). The *amoA* copy number of neither AOB nor AOA correlated with the potential rate of nitrification. This has also been found in other studies (Caffrey *et al*., 2007; Santoro *et al*., 2010; Wankel *et al*., 2011). For instance, Caffrey *et al*. (2007) did not find a correlation between the number of AOA in the sediment and the potential nitrification rate at four out of six sites in an estuary. Likewise, Santoro *et al*. (2010) did not find a correlation of either AOB or AOA *amoA* abundance with nitrification rate in the central California Current. The lack of a correlation may have various reasons. Firstly, it is possible that different types of ammonia-oxidizers have different potential rates of ammonia oxidation per cell (Santoro *et al*., 2010). It is not precisely known whether certain phylotypes, defined by their 16S rRNA gene sequence, share the same physiological characteristics (Prosser and Nicol, 2012). Secondly, it is possible that only part of the nitrifying populations was active and responsible for the nitrification in microbial mats. The lack of positive correlation between AOB *amoA* gene abundance and its transcripts in this study supports the explanation that only part of the nitrifying community was active. Moreover, there is evidence that AOB and AOA are capable of mixotrophic or heterotrophic growth (Jia and Conrad, 2009), thus the presence of AOB or AOA does not necessary mean that they oxidize ammonia. In addition, the function of archaeal ammonia monooxygenase is not clear (Prosser and Nicol, 2012). Therefore, AOB or AOA abundance and diversity should not be considered as a proxy for nitrification. Ammonia oxidizers that belong to the Gammaproteobacteria might

also contribute to nitrification (Lam *et al*., 2007). However, the GeoChip showed a negligible signal for nitrifiers of the Gammaproteobacteria when compared to β-AOB and AOA. Therefore, it was concluded that it is unlikely that Gammaproteobacteria play a role of importance in ammonia oxidation in the studied microbial mats.

Some studies reported that AOA are more abundant than AOB in marine (Mincer *et al*., 2007) and terrestrial ecosystems (Leininger *et al*., 2006; Shen *et al*., 2008). Meanwhile, mounting evidence from various estuarine and coastal studies suggested that in certain regions AOB *amoA* gene abundance is higher than that of AOA *amoA* (Caffrey *et al*., 2007; Mosier and Francis, 2008; Santoro *et al*., 2008; Wankel *et al*., 2011). We show that in coastal microbial mats AOB were two to four orders of magnitude more abundant than AOA. On the one hand, AOA *amoA* was not expressed at detectable levels in the microbial mats despite the high diversity of the gene. On the other hand, the AOB *amoA* transcripts positively correlated with potential nitrification rates (Pearson, $r=0.638$, $p<0.05$, $n=13$). Moreover, multiple stepwise linear regressions showed that β-AOB *amoA* transcription was the only valid predictor (out of the variables measured in this study) of potential nitrification rates ($r^2= 0.516$, $p<0.05$). AOB *amoA* transcripts variation explained 51% of the potential nitrification rate. This evidence suggests that AOB are the predominantly responsible for nitrification in the microbial mats investigated in this study.

A study on the archaeal diversity in the same mats as in this study revealed that sequence reads assigned to *Thaumarchaeota* were present in low numbers, hence, confirmed the low abundance of this group in the mats (Bolhuis and Stal, 2011). There are several explanations for the minor importance of AOA in nitrification in the mat. Firstly, salinity appears to play a role in the relative distribution of AOA and β-AOB. Mosier and Francis (2008) found that in coastal aquifer sediments with high salinity (22-31 psu) and low (2-15 μM) ammonia concentration AOB were more abundant than AOA but that at low salinity (0.2- 9 psu) the latter prevailed. A similar study across a groundwater seawater beach interface also revealed that AOB *amoA* abundance exceeded AOA *amoA* abundance with proximity to the ocean and higher salinity (Santoro *et al*., 2008). The salinities of the microbial mats in this study were generally polyhaline. Secondly, the pore water of the mat contains relatively high ammonium concentrations. The majority of AOA found in this study belonged to *Nitrosopumilus*. This lineage is represented by *Nitrosopumilus maritimus*, which appears to be adapted to growth at low ammonia concentrations (Martens-Habbena *et al*., 2009). This may also be the case for AOA in our mats, although some *Nitrososphaera* strains tolerate higher ammonium concentrations (Verhamme *et al*., 2011). It is possible that AOA are not obligate ammonia-oxidizers and would explain the positive correlation between abundance of AOA *amoA* gene and organic matter (Mußmann *et al*., 2011). Alves *et al*. (2013) showed that AOA belonging to *Nitrososphaera* are functional heterogeneous and that some would not exclusively

grow at the expense of ammonia oxidation.

Constrained correspondence analysis showed that the community composition of both AOA and AOB was influenced by the same factors: salinity, temperature and DIN. The lower salinity at Station 1 may explain the high diversity and distinct ammonia oxidizer community compared to the other two stations. Bernhard *et al*. (2010) observed that the loss of diversity of ammonia-oxidizing bacteria correlated with increasing salinity in the Plum Island Sound estuary. Salinity influenced not only the composition of the ammonia-oxidizing community but also the abundance of ammonia oxidizers. In this study, AOA and β-AOB *amoA* abundance were both significantly and positively correlated with salinity. The literature does not reveal consistent conclusions with respect to the effect of salinity of ammonium oxidizers. In San Francisco Bay, AOA *amoA* abundance was negatively correlated with salinity and β-AOB *amoA* abundance was positively correlated with salinity (Mosier and Francis, 2008). However, Caffrey *et al*. (2007) reported that AOA *amoA* abundance was positively correlated with salinity, while no correlation was observed between AOB *amoA* abundance and salinity. These discrepancies indicate that the factors that control the ammonia oxidizer community and *amoA* abundance are complex and not well understood. Salinity alone does certainly not explain the observations sufficiently.

Ammonium concentration is a crucial factor that may determine the community composition of ammonia oxidizers because they differ largely in affinity and tolerance towards ammonia (Prosser and Nicol, 2012). For instance, ammonium concentration influenced AOB community composition and AOB abundance more than in the case of AOA. Particularly, the relative contribution of *Nitrosospira* cluster B (is related to *Nitrosospira* cluster 3 as described by Purkhold *et al*. (2003)) is positively correlated with ammonium concentration (Pearson, r=0.95, p=0.01, n=5). This is in line with the observation that *Nitrosospira* cluster 3 responded best to high ammonia concentration (Verhamme *et al*., 2011). The two dimensions of CCA explained only part of the total variance of the ammonia oxidizer community. This implied that other factors must be involved. Many physical and geochemical factors have been proposed including oxygen availability, sulfide concentration (Joye and Hollibaugh, 1995), light (Horrigan and Springer, 1990), and trace metal availability (Mosier and Francis, 2008). All those factors are important in microbial mats but were not taken into account in this study.

The seasonal patterns of potential nitrification observed in the three stations were similar. The low potential nitrification rates in July may be due to competition for ammonium between ammonia oxidizers and cyanobacteria that use it as nitrogen source (in Station 2 also diatoms may compete for the ammonia). Cyanobacteria are the main structural component of these coastal microbial mats. The microbial mat

reaches maturity in summer and becomes less productive and the standing stock biomass decreases afterwards (Stal *et al*., 1985). Nitrification is an aerobic process and therefore in summer can only happen in the light when the Cyanobacteria evolve oxygen as the result of photosynthesis. However, the Cyanobacteria then also fix CO_2 and assimilate ammonium for growth (Stal, 2003). Hence, the competition pressure in summer may lead to the lower potential nitrification rate compared to other seasons, when the mats are less active and presumably do not become anaerobic. Also in the coastal Arctic Ocean potential nitrification rates were higher in winter than in summer (Christman *et al*., 2011). These authors hypothesized that the lack of competition for ammonium with phytoplankton and other microorganisms would stimulate nitrification in winter. This is also in line with Risgaard-Petersen *et al*. (2004b) who concluded that benthic algae are superior to AOB when it comes to competition for ammonium. The seasonal pattern of potential nitrification rates may also be attributed to the dynamics of the community composition of ammonia oxidizers. CCA indicated a seasonal trend for both bacterial and archaeal ammonia oxidizers that correlated particularly to the concentrations of ammonium and nitrate/nitrite. We observed signal intensity shifts in the GeoChip for different types of bacterial ammonia oxidizers between July and January. Because different ammonia oxidizers will have different physiological characteristics, the shift in the community composition may eventually result in the seasonality of nitrification.

Chapter 4

Ecosystem function of intertidal microbial mats

Haoxin Fan[1], Henk Bolhuis[1], and Lucas J. Stal[1,2]

[1]Department of Marine Microbiology, Royal Netherlands Institute of Sea Research, Yerseke, The Netherlands
[2]Department of Aquatic Microbiology, Institute of Biodiversity and Ecosystem Dynamics, University of Amsterdam, The Netherlands

To be submitted

Abstract
Microbial mats are small-scale and largely closed self-sustaining benthic ecosystems that comprise the major element cycles. Here, we identified the functional genetic potential of microbial mat communities involved in carbon-, nitrogen-, and sulfur-cycling as well as the stress tolerance strategies that allow microorganisms to thrive in intertidal microbial mats. We applied a metagenomic approach using a GeoChip-based functional gene array to establish the metabolic capabilities of an intertidal microbial mat. The overall diversity was similar among the three sampled stations but the functional community composition depended on mat type and season. The number of functional genes detected at fresh water station (Station 1) was always highest. In all three stations, the number and abundance of genes was higher in July when compared to January. However, the overall functionality was similar regardless mat type or season. The results suggested a global make up of marine microbial mats with a conserved functional composition. A suite of stress response pathways, oxygen, osmotic, and nutrient limitation responses were identified and related to environmental stressors, offering clues to the mechanisms behind the enduring success of microorganisms in this microbial mat.

Keywords: ecosystem function, GeoChip, microbial mat, nutrient cycling, stress response

Introduction

Coastal microbial mats are compact, highly structured, largely closed small-scale ecosystems characterized by steep physicochemical gradients (Stal, 2001). These gradients are generated by the combination of physical factors (light and molecular diffusion) and the metabolic activities of microorganisms that form the microbial mat. Microbial mat organisms position themselves within these physicochemical gradients and occupy a niche that is suitable for their physiological requirements. As a result, microbial mats typically produce a clearly visible vertical lamination.

Cyanobacteria are oxygenic phototrophic microorganisms that are often seen as the key organisms that build microbial mats in illuminated environments. Due to their photosynthetic and autotrophic mode of life, cyanobacteria are the primary producers and form the basis for the microbial food web in this small-scale ecosystem (Severin and Stal, 2010). Cyanobacteria are usually found at the mat surface or sometimes just underneath a layer of diatoms, when these are present, and form a distinct green layer. Cyanobacteria are therefore conspicuously present. As photoautotrophs they fix carbon dioxide, while many species also fix dinitrogen. Underneath the cyanobacteria, anaerobic anoxygenic phototrophic purple sulfur bacteria may form a purple layer. Photosynthesis is restricted to the top few millimeters due to the attenuation of light and fuels the primary production that provides the system with a source of organic carbon. Below the purple layer there is a permanently anoxic black horizon of iron sulfide. Chemotrophic bacteria are involved in a variety of biogeochemical processes and accumulate at the optimal conditions along the physicochemical gradients. The integrated and combined activity of microbial mat organisms results in a highly productive and self-sustained ecosystem. For example, sulfate-reducing bacteria use the organic matter produced by the primary producers and respire it anaerobically using sulfate as electron acceptor resulting in the formation of sulfide. Sulfide is used as electron donor for anoxygenic photosynthesis or in case oxygen is present it can be oxidized by colorless sulfur bacteria (Stal, 2001).

Coastal microbial mats can be found on the North Sea beaches of the Wadden Sea barrier islands. Microbial mats on the island of Schiermonnikoog have been particularly well investigated (Bolhuis and Stal, 2011; Bolhuis *et al*., 2014). These mats play an important role in coastal morphodynamics and protection. Previous studies using massive parallel 16S rRNA gene tag sequencing have shown that these mats are among the most diverse marine microbial ecosystems (Bolhuis and Stal, 2011). These authors distinguished three types of mats along the tidal gradient, which differ in morphology and community composition. These differences were also reflected in the composition and diversity of the nitrogen-fixing (Severin *et al*., 2010) and denitrifying communities and their activities (Fan *et al*., 2015). These three mat types also showed distinctly different daily patterns of nitrogenase activity and the highest rates of nitrogen fixation were found in the low-salinity supra-littoral mats

(Severin and Stal, 2008). Despite several in depth studies of community composition and nitrogen cycling, still little is known about the genetic potential of metabolism in microbial mats, in particular with respect to carbon, nitrogen and sulfur cycling. Due to the cycles of inundation and exposure causing desiccation and strong variations in salinity and temperature, intertidal areas are considered as extreme environments that are also low in nutrients (Stal, 2001). In microbial mats, physicochemical gradients change dramatically over a daily cycle. Hence, it is an interesting question on how microorganisms have adapted to these harsh environmental conditions and to explore the stress tolerance mechanisms in them.

The GeoChip is a functional gene array containing (GeoChip 4.0) 82 000 probes from 410 functional gene families related to microbial element cycling (including carbon, nitrogen, sulfur and phosphorus), energy metabolism, antibiotic resistance, metal resistance, organic remediation, stress responses, bacteriophage and virulence (Tu *et al*., 2014). The GeoChip is particularly useful for providing direct linkages of microbial populations to ecosystem processes and functions. The GeoChip has been successfully applied to analyze samples from a variety of environments, including marine sediments, bioreactors, soils, hydrothermal vents, and groundwater. The results indicate that the GeoChip is a powerful high throughput metagenomic tool for monitoring microbial community dynamics in environmental samples and assess metabolic functions of microbial communities.

The main aim of this study was to explore the functional genetic potential of microbial communities involved in nutrient cycling including carbon-, nitrogen-, and sulfur- cycling as well as the stress tolerance strategies that allow microorganisms to thrive in intertidal microbial mats. We provide a detailed account of functionalities of the microbial mat community and uncover the core functional gene potential and complexity of intertidal microbial mats. This study complements our understanding of the ecology of the microbial mat community.

Table 1. The geographical coordinates and description of the mats investigated in this study.

Station	Geographical coordinates	Description
Station1 (ST1)	53°29.445′N, 6°8.718′E	Mainly freshwater influenced site, close to the dunes. Irregularly inundated.
Station2 (ST2)	53°29.460′N, 6°8.309′E	Seawater influenced site, developing microbial mat. At the low water mark.
Station3 (ST3)	53°29.445′N, 6°8.342′E	Seawater and freshwater influenced site, located between St1 and St2, at the edge of the salt marsh

Materials and methods

Study area and sampling

The study site was located on the North Sea beach of the Dutch barrier island Schiermonnikoog. The geographical locations of the three types of microbial mats (stations that were sampled for this study) are shown in Table 1. The sample stations were situated along a transect perpendicular to the beach along the tidal gradient. Sampling was done in July 2010 and January 2011. Samples for nucleic acid extraction were taken from the top 1 cm of the mat by using a 10 ml syringe, from which the needle connector was removed, as a corer. These mat samples were divided into four equal parts, put into cryo-vials, and immediately frozen in liquid nitrogen in the field. Nucleic acid extracts from three randomly taken samples from each mat were used for hybridization on separate chips.

Nucleic acid extraction and GeoChip analyses.

DNA was extracted from three samples from each of the three types of microbial mats using the MoBio UltraCLEAN soil DNA kit (MoBio Laboratories, Inc., Carlsbad, CA, USA) according to the manufacturer's instructions and further purified using the UltraClean 15 DNA purification Kit (MoBio Laboratories, Inc., Carlsbad, CA, USA) in order to achieve the quality necessary for hybridization on the chip. The quantity and quality were determined and checked by Nanodrop (Nanodrop ND1000, Thermo Scientifica, Wilmington, DE, USA) and agarose gel electrophoresis, respectively. DNA samples from July 2010 and January 2011 were selected for GeoChip (version 4.2) analysis (n=18). GeoChip 4.2 is a moderate expansion of GeoChip 4.0 (Tu *et al*., 2014) and contains ~103,685 50-mer oligo probes in relation to microbial carbon-, nitrogen-, sulfur-, and phosphorus cycling, energy metabolism, antibiotic resistance, metal resistance, organic remediation, stress responses, bacteriophage, fungal function, soil benefit, soil borne pathogen, bioleaching and virulence. The procedures for DNA labelling and microarray hybridization followed previously established protocols (Wu *et al*., 2006). Briefly, 800 ng DNA was labelled with fluorescent dye Cy-5 by random priming and re-suspended in 50 µl hybridization solution (40% formamide, 5× SSC, 5 µg of unlabelled herring sperm DNA (Promega, Madison, WI), and 0.1% SDS) and 2 µl universal standard DNA (0.2 pmol μl^{-1}) labelled with the fluorescent dye Cy-3 (Liang *et al*., 2010), denatured for 5 min at 95°C and maintained at 50°C until loaded onto the microarray slides. Arrays were hybridized on a MAUI Hybridization Station (Roche, South San Francisco, CA) for 12 h at 42^{o}C. The hybridized microarrays were scanned by a ScanArray Express (Perkin- Elmer, Wellesley, MA) at 95% laser power and 85% photomultiplier tube gain. The signal intensities of spots on the GeoChip were normalized across samples by the mean of Cy-5 labelled universal standard signal intensities and the sum of Cy-3 labelled signal intensities of hybridized spots in samples. The resulting images were analysed by ImaGene with signals processed as SN>2.0 (signal to noise ratio).

Statistical analyses

The generated data sets from the microarray image analyses were considered as gene variant abundance presented as signal intensity of each gene. Relative abundance was calculated by dividing signal intensities of each individual gene (or gene category) by the sum of signal intensities for a given set. In order to summarize the gene overlap at station and season level, the mean value of genes detected from the GeoChip in the three replicates of each station from July 2010 and January 2011 were used. The analyses of overlapping genes, unique genes, diversity indices and phylogenetic distributions were performed using an online pipeline (http://ieg.ou.edu/). The proportion of overlapping genes was calculated as the number of overlapping genes divided by the total number of genes detected in both stations. The proportion of unique genes at each station was calculated as the number of unique genes at each station divided by the total number of genes detected at that station. The significance of compositional differences between mat types was analyzed by analysis of similarity. The contribution of each functional gene to the total dissimilarity between mat types was determined using Similarity Percentage (SIMPER) (Clarke, 1993). Detrended correspondence analysis (DCA), ANOSIM, SIMPER and one-way ANOVA (analysis of variance) were performed in a statistical software PAST (Hammer *et al*., 2001).

Results and discussion

Overview of total functional diversity

A high-throughput microarray approach was employed to assess the diversity of microbial functional genes in microbial mats. A total of 40,963 functional genes were detected. For individual samples, the number of detected genes was between 19,632 and 36,621 (Table 2). In general, the diversity (Shannon-Weaver index) and evenness were similar across these samples. But the number of genes detected was significantly decreased (one-way ANOVA, $p<0.01$) in samples from ST1 to ST3 and from July to January. The higher number of functional genes in mat samples taken in July compared to those of January is in agreement with the succession of the development of the mat community, which reaches maturity during summer and become less productive and low in biomass during winter (Stal *et al*., 1985). A similar trend was observed in the phylogenetic diversity of these mats (Bolhuis and Stal, 2011). The overlapping genes were largely shared among the samples and the percentage of overlapping genes ranged from 48.5% to 92.2% (Table 2). Furthermore, the overall relative abundance of genes detected in different functional categories is similar among samples (Fig. 1). Similarity Percentage (SIMPER; Clarke *et al*., 1993) was used to distinguish which functional categories (or genes) contributed to the compositional dissimilarity among stations. Total functional profiles exhibited only a mean dissimilarity of 13.2% among three mat types. These results suggest that the overall functionality as deduced from the expression of genes belonging to different functional groups is similar and independent of the mat type and the season.

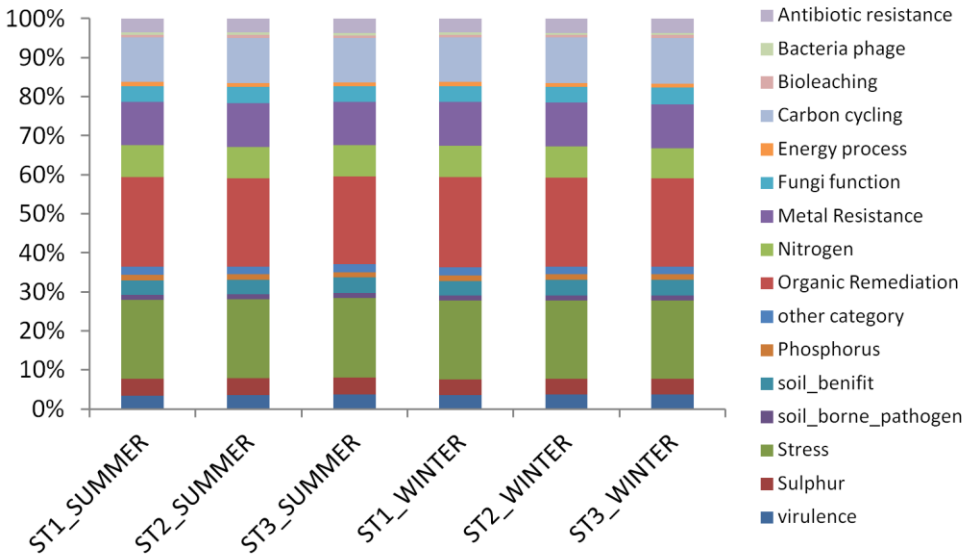

Figure 1. Relative abundance of genes belonging to different functional categories that detected on GeoChip. Signal intensity was normalized by the number of the probes for each gene.

Table 2. Percentage of overlapping genes and diversity indices of total functional genes in microbial mat samples.

Sample	ST10601	ST10602	ST10603	ST20601	ST20602	ST20603	ST30601	ST30602	ST30603	ST10101	ST10102	ST10103	ST20101	ST20102	ST20103	ST30101	ST30102	ST30103
ST10601																		
ST10602	89.4%																	
ST10603	92.3%	87.8%																
ST20601	73.7%	73.0%	74.6%															
ST20602	77.6%	77.2%	78.3%	89.0%														
ST20603	73.1%	75.4%	73.7%	82.9%	88.0%													
ST30601	60.1%	61.3%	61.2%	72.5%	70.0%	71.2%												
ST30602	53.1%	54.9%	54.0%	65.5%	62.2%	65.7%	84.8%											
ST30603	55.7%	56.9%	56.9%	67.6%	65.2%	66.2%	89.5%	78.2%										
ST10101	79.0%	80.1%	78.9%	74.5%	78.1%	76.5%	65.0%	58.7%	60.6%									
ST10102	74.0%	76.7%	73.2%	68.7%	72.8%	73.6%	61.9%	57.4%	58.0%	85.8%								
ST10103	78.8%	80.0%	78.3%	74.4%	78.2%	76.5%	64.9%	58.5%	60.4%	90.8%	86.5%							
ST20101	58.2%	60.8%	58.7%	63.7%	65.6%	70.1%	66.5%	65.4%	63.6%	66.8%	70.2%	67.0%						
ST20102	60.9%	63.4%	61.5%	68.0%	69.1%	73.4%	69.4%	67.3%	66.0%	69.7%	72.5%	69.9%	86.3%					
ST20103	61.5%	64.1%	61.9%	66.9%	69.0%	73.5%	68.0%	65.7%	64.7%	70.0%	73.7%	70.4%	88.3%	91.7%				
ST30101	51.4%	53.3%	52.1%	60.4%	59.5%	63.1%	70.9%	71.2%	70.2%	58.7%	58.6%	58.8%	71.3%	72.5%	70.7%			
ST30102	50.0%	52.2%	50.7%	57.9%	57.7%	61.8%	67.6%	68.3%	66.4%	57.4%	59.5%	57.5%	75.2%	74.5%	73.7%	88.0%		
ST30103	48.5%	50.3%	49.2%	57.1%	56.1%	59.6%	68.5%	69.9%	67.9%	55.2%	55.3%	55.3%	68.0%	68.5%	67.3%	85.6%	82.6%	
Richness	36621	34940	36029	30507	32202	30163	24379	21587	22744	31935	30487	32181	23556	24421	24937	20816	20154	19632
Shannon-Weiner index	10.5	10.5	10.5	10.3	10.4	10.3	10.1	10.0	10.0	10.4	10.3	10.4	10.1	10.1	10.1	9.9	9.9	9.9
Evenness	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8

The DCA of the GeoChip data showed that the samples studied in this study were well separated by mat type and by season (Fig. 2), which was confirmed by dissimilarity test using ANSOSIM. ANOSIM analyses of overall functional gene profiles indicated significant variation of composition in microbial communities from different mat types (R=0.73, P = 0.0001, n=18, permutation=9999) as well as from different seasons (July and January) (R=0.31, P = 0.01, n=18, permutation=9999). Hence, the results suggest that, although there was only a slight difference between the three mat types regarding the relative contribution of different gene categories to total abundance detected by GeoChip, the intrinsic functional community composition was affected by mat type and season. For example, ST1 harbors a higher number of different gene sequences for the same functions, e.g. *nirS* and *nirK* (Fan *et al*., 2015) (Chapter 2), suggesting a higher functional redundancy. The stability of a function has been shown to depend on trait redundancy across members of the microbial community (Griffiths and Philippot, 2013).

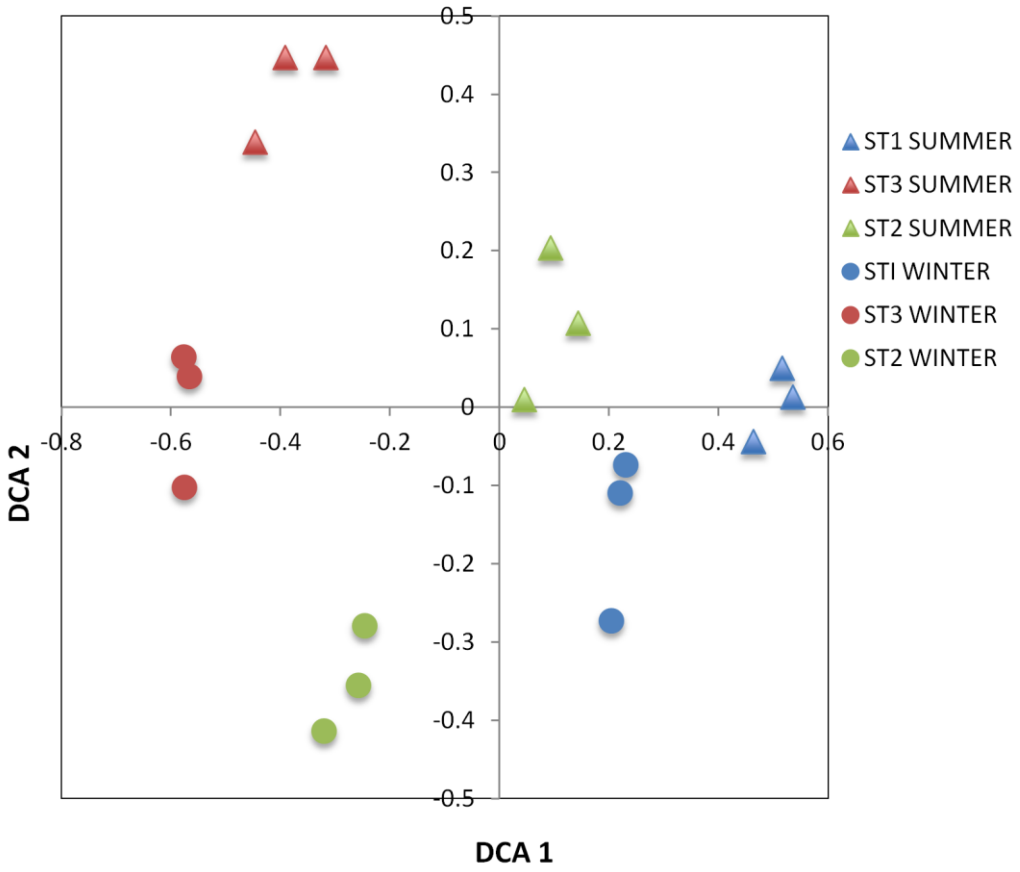

Figure 2: Detrended correspondence analysis of overall functional genes

Our results support the theory of assembly of the bacterial community by its function rather than by species (Burke *et al*., 2011). Bonilla-Rosso *et al*. (2012) compared several microbial mats possessing a similar structure but which developed under

different environmental conditions. These authors found few shared organisms but a higher similarity at higher taxonomic level. This is also true for microbial mats in extreme environments that have significant lower diversity (e.g. hot spring and hypersaline mats) (Klatt *et al*., 2007; Bolhuis *et al*., 2014). These results suggest a global make up of marine microbial mats with a conserved functional composition. Most likely any (marine) microbial mat will show a similar functional composition. The major steady components of marine microbial mats are their dependence on light, a sandy and course grained sediment, the irregular inundation by seawater and therefore the steady input of sulfate that fuels the sulfur cycle, the steep physicochemical gradients and the low concentrations of the nutrients N and P. These environmental conditions apparently select for the same functional composition that we see worldwide. There might be variation in the species composition of primary producers and the chemotrophic community, but they are functionally similar.

This similarity in function can also be explained in terms of the "insurance hypothesis" which states that increasing biodiversity insures ecosystems against declines in their functioning caused by environmental fluctuations (Yachi and Loreau, 1999). The observed large species richness in these mats (Bolhuis and Stal, 2011) should lead to a decreased variability in ecosystem processes. This becomes apparent in this study where the samples from January compared to those from July reveal overall lower signal intensity, in agreement with a lower biomass but maintain their functional composition. The implication of this hypothesis is that for any given sandy sediment in a tidal system we should always find a similar functional composition unless perhaps environmental extremes may decrease the species richness and diversity that remove/eradicate/diminish some less abundant or non-essential functions.

Among the genes detected, those involved in organic remediation were most abundant (22.4-23.2%), followed by genes involved in stress strategies (19.9-20.4%), carbon cycling (11.3-11.7%), nitrogen cycling (7.6-8.1%) and sulfur cycling (3.9-4.4%). In SIMPER analysis, genes involved in organic remediation, stress strategies, carbon cycling, metal resistance, nitrogen cycling contributed to 24.3%, 19.7%, 11.0%, 11.0% and 8.5% of total functional dissimilarity (13.2%), respectively. The relative high contribution from organic remediation could be attributed to the high number of probes associated with these functions present on the GeoChip microarray slide. Nevertheless, these gene groups might be important reservoirs for novel biotechnology products with potential applications in bioremediation. Microbial groups related to carbon, nitrogen, sulfur and stress strategy are of major interest, given their importance for microbial mat maintenance.

Microbial community composition

The gene encoding DNA gyrase subunit-B (*gyrB*) was used to determine the phylogenetic diversity. In this study, 97.9–98.4% of total signal intensity of *gyrB* genes was derived from bacteria and the rest from archaea. The proteobacteria was the predominant phylum of bacteria, contributing to 51.2–56.2% of total signal intensity of *gyrB*. Actinobacteria, firmicutes, cyanobacteria and bacteroidetes were the subdominant bacterial groups and contributed to 12.7–15.4%, 10.6–14.6%, 3.3–5.0% and 2.6-3.1% of total signal intensity of *gyrB*, respectively (Fig. 3A).

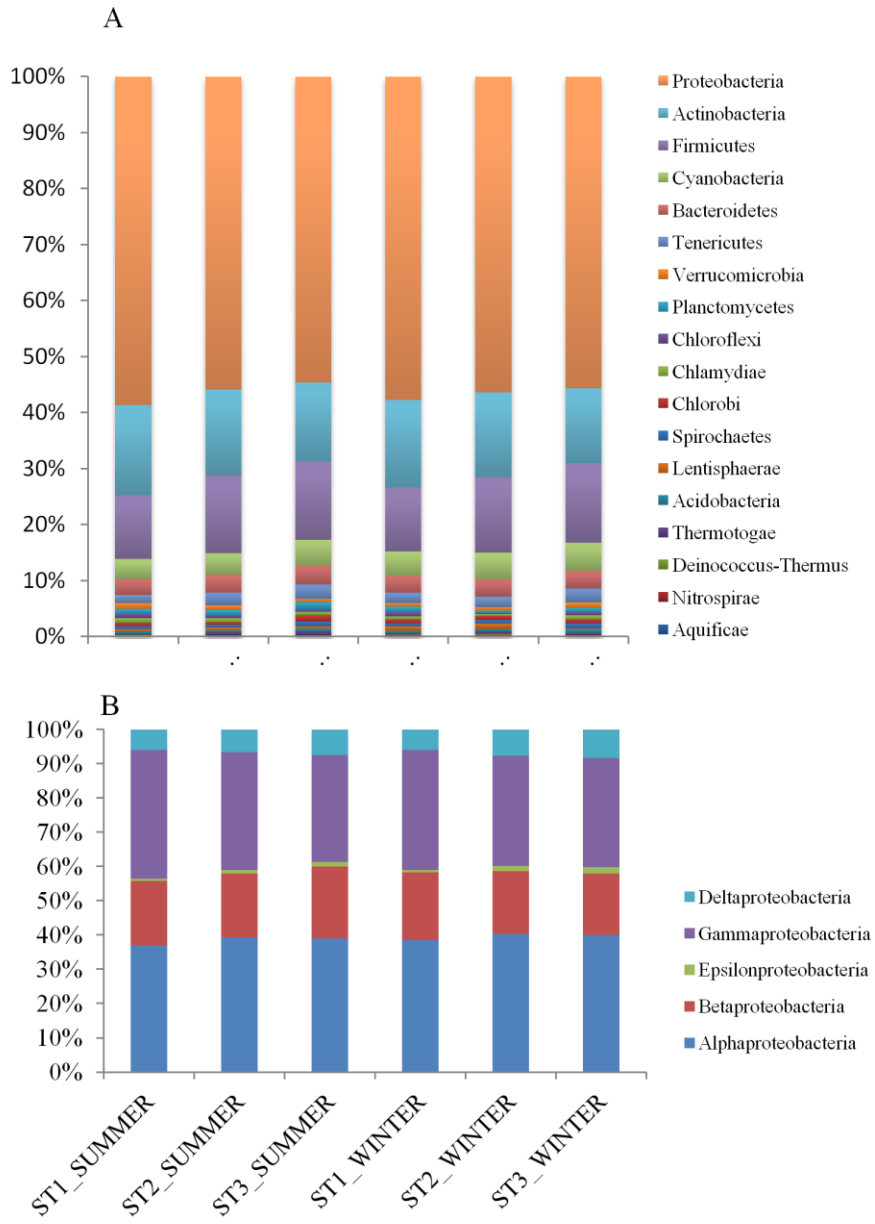

Figure 3: Relative distribution of the dominant A) bacterial phyla B) proteobacterial class in different stations based on *gyrB* gene. Signal intensity was normalized by the number of the probes for each gene.

Alphaproteobacteria, betaproteobacteria and gammaproteobacteria contributed to 36.6-41.4%, 17.9-20.9% and 31.2-37.9% of the total intensity of proteobacterial *gyrB* genes, respectively (Fig. 3B). This largely agrees with the conclusions drawn in previous studies, which documented the taxonomic diversity in the same mats by 16S rRNA gene clone libraries and massive parallel tag sequencing (Severin *et al*., 2010; Bolhuis and Stal, 2011). These phyla are abundant in most other microbial mats (Bolhuis *et al*., 2014 and references therein). However, the contribution of Cyanobacteria is surprisingly low compared to that reported by Bolhuis and Stal (2011). Besides, these authors showed that cyanobacterial sequences were abundantly present at ST1 and ST3 while they were largely absent at ST2. In this study, we did not find a distinct spatial variation of cyanobacteria. We can not exclude the possibility that this discrepancy is due to insufficient or inefficient probes present on GeoChip microarray slide targeting cyanobacteria.

Carbon cycling

A total of 4,739 genes involved in carbon cycling were identified. We categorized these genes as autotrophy (carbon fixation), carbon degradation, methanogenesis, methane oxidation and acetogenesis. Among the genes detected, those involved in carbon degradation were most abundant (77.0-79.1%) followed by genes involved in autotrophy (17.3-18.6%) (Fig. 4A).

In the category of carbon fixation, genes coding for ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) in the Calvin – Benson – Bassham (CBB) cycle, the propionyl-CoA/acetyl-CoA carboxylase (ppc) in the 3-hydroxypropionate/malyl-CoA (3-HP) cycle, the ATP citrate lyase (aclB) and the carbon-monoxide dehydrogenase (CODH) in the reductive acetyl-CoA (rTCA) pathway were detected in all mat samples, representing the key enzymes in autotrophic carbon fixation pathways. The majority of the genes in this category belong to the Bacteria. The relative contribution of genes involved in autotrophy from different phyla were similar among samples and were mainly attributed to proteobacteria (54.0-57.8%), actinobacteria (21.4-24.4%), firmicutes (4.1-5.3%), bacteroidetes (4.0-5.2%) and cyanobacteria (1.3-2.4%). The surprisingly low contribution from cyanobacteria may due to insufficient number of probes of this group on the GeoChip and this may be one of shortcomings of applying the GeoChip to microbial mats. The most abundant gene in carbon fixation in all samples was *pcc*, followed by genes coding for RuBisCO, and CODH (Fig. 4B). We would expect that the CBB is the dominant CO_2 fixation pathway but surprisingly, the *pcc* gene was the most abundant one among genes involved in the different carbon fixation pathways. The 3-hydroxypropionate/malyl-CoA (3-HP) pathway has also been found in microbial mats of Shark Bay in Western Australia and in hot spring microbial mats (Klatt *et al*., 2007). However, given the fact that the protein for which this gene is coding is also involved in fatty acid metabolism (Strauss and Fuchs, 1993), *pcc* alone

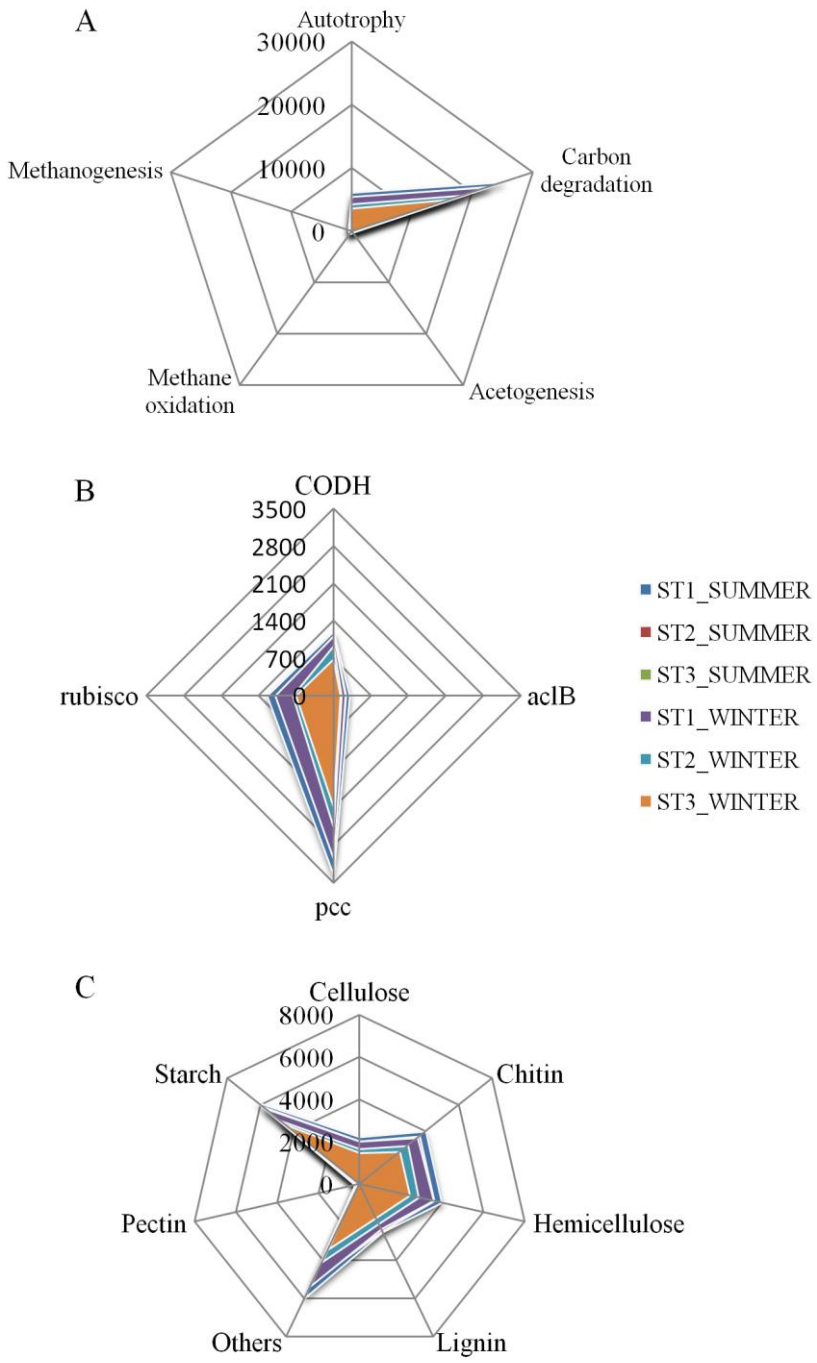

Figure 4: Total signal intensity of various carbon cycling genes A) grouped by the major functional processes; B) involved in carbon fixation; C) involved in carbon degradation but grouped by different metabolic pathways. Signal intensity was normalized by the number of the probes for each gene. A list of targeted genes for each category is provided in Tu *et al*. (2014) (See Table S1).

is not a reliable diagnostic gene for the 3-HP pathway (Klatt *et al*., 2007). Therefore, the presence of 3-HP in these mats is speculative. The importance of the 3-HP with respect to other carbon fixation pathways remains unclear.

The Calvin Cycle is the pathway for inorganic carbon fixation used by many chemoautotrophic and photoautotrophic bacteria including proteobacteria, cyanobacteria, chloroflexi, verrucomicrobia, firmicutes, thermus, actinobacteria, and all photosynthetic eukaryotes. Abundance of both type I and type II of RubisCO genes are similar in the microbial mats studied by us, reflecting that autotrophic organisms in these mats are highly adapted to the steep O_2 and CO_2 gradients of this microbial ecosystem. Type II RubisCO is considered to be most effective in microaerobic or anaerobic environments while type I RubisCO behaves the opposite (Chen and Spreitzer, 1992). Cyanobacterial photosynthesis results in oxygen supersaturation during the day, while at night, anoxic conditions prevail due to continued activity of heterotrophic microorganisms (Van Gemerden, 1993). Many organisms possessing type II RubisCO also have type I RubisCO encoded in their genomes. The former is expressed under anoxic conditions while the latter is expressed under oxic conditions. For example, purple non-sulfur alphaproteobacteria fix inorganic carbon via anoxygenic photosynthesis and often contain both types I and II RubisCO in their genomes (Lu *et al*., 2010). These organisms primarily utilize the faster type II RubisCO under high CO_2 conditions, and revert to using the type I enzyme when CO_2 is limiting. In addition, the rTCA cycle represents another pathway of carbon fixation. *aclB* was much less abundant than RubisCO genes in our mat samples, suggesting that the reverse TCA cycle might not be as widely used as the CBB cycle in microbial mats.

Approximately half of the bacteria in these microbial mats are chemoorganotrophs that depend on organic substrates provided by the primary producers (Bolhuis and Stal, 2011). This would explain the high abundance of genes involved in carbon degradation. Genes involved in degradation of various carbohydrates including starch, cellulose, hemicellulose, chitin, lignin, pectin and aromatics were detected in all mat samples and the relative abundance of genes involved in degradation of different substrates was similar among samples (Fig. 4C), suggesting similarity of functional genes in these mats and versatile ability of heterotrophs to utilize the carbon sources. Chitin, derived from cell walls of fungi and the exoskeletons of crustaceans, insects, and spiders, is second most abundance organic substance in nature and the most abundant polymer in the marine ecosystem (LeCleir *et al*., 2004). This can explain high abundance of genes involved in chitin degradation in microbial mats. This can be related to large proposition of actinobacteria in the mats, which are known for producing a variety of extracellular hydrolytic enzymes especially in degradation of chitin (Bolhuis and Stal, 2011). Notwithstanding the fact that chitin is the most abundant polymer in marine systems expression of starch genes appeared to be more

prominent (Fig 4C). This could be due to the fact that the GeoChip contains more genes related to starch degradation than to chitin degradation. Nevertheless, starch (and glycogen) is an important polymer in microbial mats that is subject to degradation. Genes for the transformation of lignin and other complex organic aromatic substrates were identified, notably involved in the super pathway of the aromatic compound cleavage. This might indicate that these microbial mats possess the potential of degradation of even the most recalcitrant compounds.

Finally, genes involved in methane oxidation (*mcrA*) and methanogenesis (*mmoX* and *pmoA*) were also detected in all samples. Phylogeny (Bolhuis and Stal, 2011) and other reports on metabolic activities in microbial mats confirmed the presence of methanogens. However, methanogenesis and methane oxidation possibly play a minor role in the carbon cycling in the mats when comparing to other pathways. This may be the result of the presence of sulfate-reducing bacteria (SRB). SRB are superior in their affinity for the substrates such as acetate, ethanol and hydrogen, and sulfate reduction yields not only more energy but sulfate is also abundantly available (28 mM in seawater) (Stal, 2001). Therefore, methanogens are outcompeted by SRB for these substrates. However, there are also non-competitive methanogenic substrates not used by SRB, such as methylamines and dimethylsufide (Oremland *et al*., 1982; Visscher *et al*., 1991; Jonkers *et al*., 1998). Nevertheless, the superiority of SRB in these microbial mats is illustrated by the detection of large proportion of *drsA* and *drsB* genes that are associated with sulfate-reducing pathways. Moreover, genes involved in methanogenesis such as, *dmsA* were present in negligible amounts.

Nitrogen cycling

As expected, the nitrogen cycle was prominently present in the intertidal microbial mats as witnessed by the presence of a variety of functional genes involved in this cycle. A total of 3,325 genes involved major pathways of in nitrogen cycling, including nitrogen fixation, denitrification, anaerobic ammonium oxidation (anammox), nitrification, dissimilatory nitrogen reduction and ammonification were detected (Fig. 5A). The genes involved in denitrification were most abundant, following those involved in nitrogen fixation, nitrification, and ammonification. Among genes detected in nitrogen cycling, *narG* was most abundant gene detected followed *nifH*, *amoA*, *ureC*, *nirS* and *nirK* (Fig. 5B). The abundance of *amoA*, *nirS*, *nosZ* and *ureC* was significantly different between stations (one-way ANOVA, $p<0.01$). The relative abundance of *nirB* was significantly lower at ST3 (one-way ANOVA, $p<0.01$). The relative abundance of *nifH* was significantly higher at ST2 (one-way ANOVA, $p<0.01$). It was not surprising that *nifH* gene was abundant. These mats are known for their nitrogen-fixing activity and diversity of diazotrophs (Severin and Stal, 2008; Severin *et al*., 2010). Several other published reports showed the high diversity of diazotrophs in microbial mats (Omoregie *et al*., 2004; Steppe and Paerl, 2005). Consistent with the measurements of process rates, denitrification outcompeted

anammox in microbial mats (Fan *et al*., 2015) (Chapter 5). Anammox bacteria compete for substrates with other mat organisms. For example, anammox bacteria compete for nitrite with denitrifiers and for ammonium with nitrifiers. These groups are abundant present according to the GeoChip results. In contrast, the number of anammox bacteria seems to be negligible. Risgaard-Petersen *et al*. (2005) studied the effect of microphytobenthos on denitrification and anammox and demonstrated that anammox plays a minor role in sediments colonized by microphytobenthos.

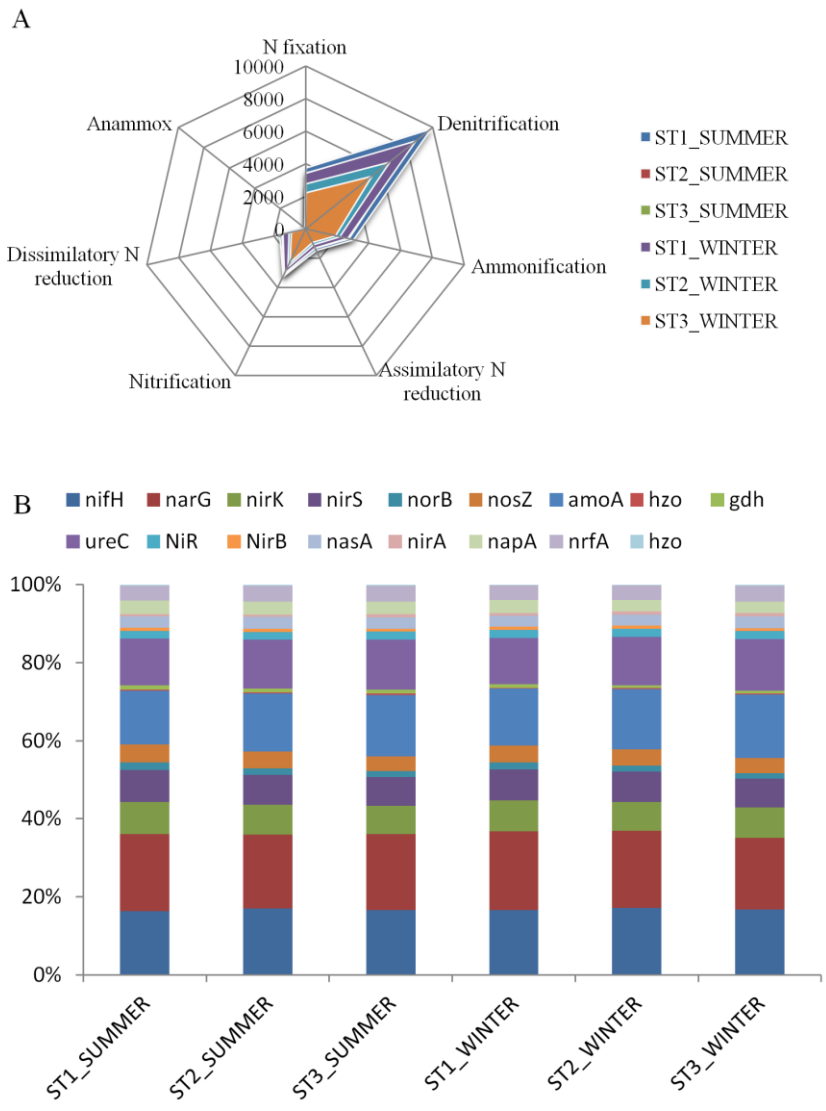

Figure 5. Summary of genes involved in nitrogen cycling detected on GeoChip. A) total signal intensity of various nitrogen genes grouped by the major transformation pathways. B) relative abundance of each gene involved in nitrogen cycling. Signal intensity was normalized by the number of the probes for each gene. A list of targeted genes for each category is provided in Tu *et al*. (2014) (See Table S1).

Sulfur cycling

The significance of sulfur metabolism in microbial mats has been well documented (Bolhuis *et al*., 2014). The GeoChip revealed a range of diverse metabolic pathways involved in sulfur cycling and confirmed their importance. A total of 1,794 genes coding for sulfite reductase, adenylyl-sulfate reductase, sulfur oxidation, and sulfide oxidation were detected. Among the genes that were detected, *drsA* was the most abundant followed by *drsB* (Fig. 6). The *drsA* and *drsB* genes that were detected showed a dominance of deltaproteobacteria, especially of *Desulfobacteraceae*. These sulfate-reducing bacteria have also been found in some hypersaline mats of Guerrero Negro (Mexico) and appear to be the dominant sink for fermentation products under dark conditions in GN-S mats (Lee *et al*., 2014). In fact, studies have shown that in marine stromatolites the carbon products of photosynthesis are rapidly utilized by chemotrophic bacteria, including sulfate-reducing bacteria (Reid *et al*., 2000; Gallagher *et al*., 2012). Genes involved in sulfur oxidation were also present in our mats. Previous studies have shown that anoxygenic photosynthesis accounted for 10–40% of the carbon fixation in GN-S mats (Finke *et al*., 2013) and were assumed to be fueled through photosynthetic sulfide oxidation. These results suggest that sulfur and carbon cycling are closely coupled in microbial mats.

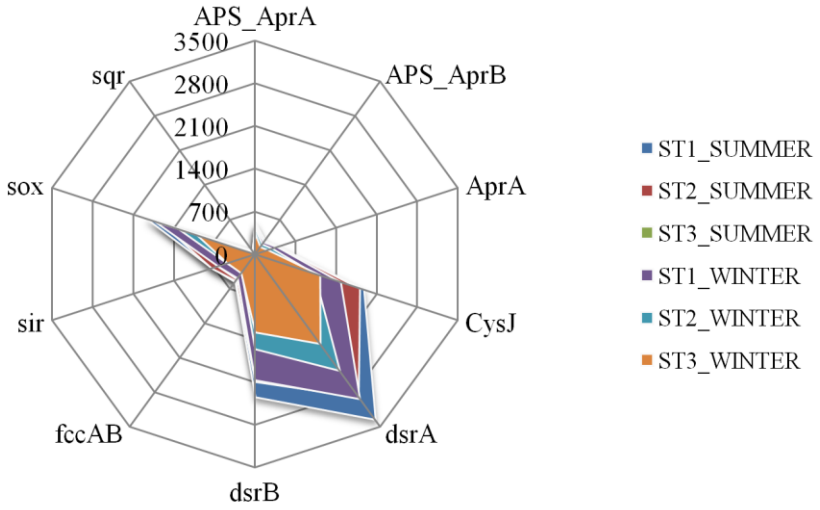

Figure 6. Total intensity of genes involved in sulfur cycling in different stations. Signal intensity was normalized by the number of the probes for each gene.

Stress response

The microbial mats studied here experience an array of harsh environmental conditions such as strong fluctuations of temperature, salinity, desiccation and UV-irradiation. Consequently, the microorganisms inhabiting these mats need to respond to these stresses in order to be able to tolerate them. A total of 8,339 genes

were detected that are involved in stress strategies including cold shock, heat shock, nutrient limitations, osmotic shock and radiation. In most samples, genes involved in oxygen stress were most abundant (23.2-24.6%) (except samples from January at ST3) followed by genes involved in the response to phosphate limitation (22.2-24.8%), sigma factors (20.9-21.6%) and nitrogen limitation (7.7-8.0%) (Fig. 7). In the category of phosphate limitation, oxygen stress, nitrogen limitation and radiation stress, genes were widespread and particularly abundant among proteobacteria, actinobacteria, firmicutes and cyanobacteria. All mat types contained broadly similar functional genes involved in coping with environmental stress, particularly related to oxidative stress, phosphate limitation, and sigma factors. Photosynthesis by cyanobacteria causes oxygenation of the top layer. The high oxygen concentration in the mat surface layer exerts oxidative stress, since molecular oxygen is a potent inhibitor of key microbial processes, such as photosynthesis, N_2 fixation, and sulfate reduction (Paerl and Pinckney, 1996). Sigma factors are general regulons that induce several other genes in response to different stresses, including acid, salt and starvation (Volker *et al*., 1999). Sigma B genes were observed in cyanobacterial mats in the Antarctic, which was attributed to the osmotic stress during freeze-up of Antarctic ponds (Varin *et al*., 2012). The high abundance of sigma factors possibly indicated severe salt stress on these mats. One of the mechanisms that microorganism cope with high salinity is that the osmotic pressure is balanced by the production of compatible solutes (Pikuta *et al*., 2007). On the GeoChip, *opuE* (osmo-protectant-uptake, a single component sodium/solute symporter with high affinity for proline) and the ProU system genes *proX*, *proV*, and *proW* (with a broad substrate specificity for osmoprotectants but a clear preference for glycine betaine and proline betaine) were selected for monitoring microbial responses to osmotic stress (Zhou *et al*. 2013). These genes were detected in all samples. It is not clear why the brackish ST1 exhibited the highest abundance of genes involved in osmotic stress. Moreover, salt stress does not only consist of ionic and osmotic stress. Oxygen and water availability decreases with increased salinity. Hence, salinity adaptation of microorganisms in microbial mats may be a combination of several strategies.

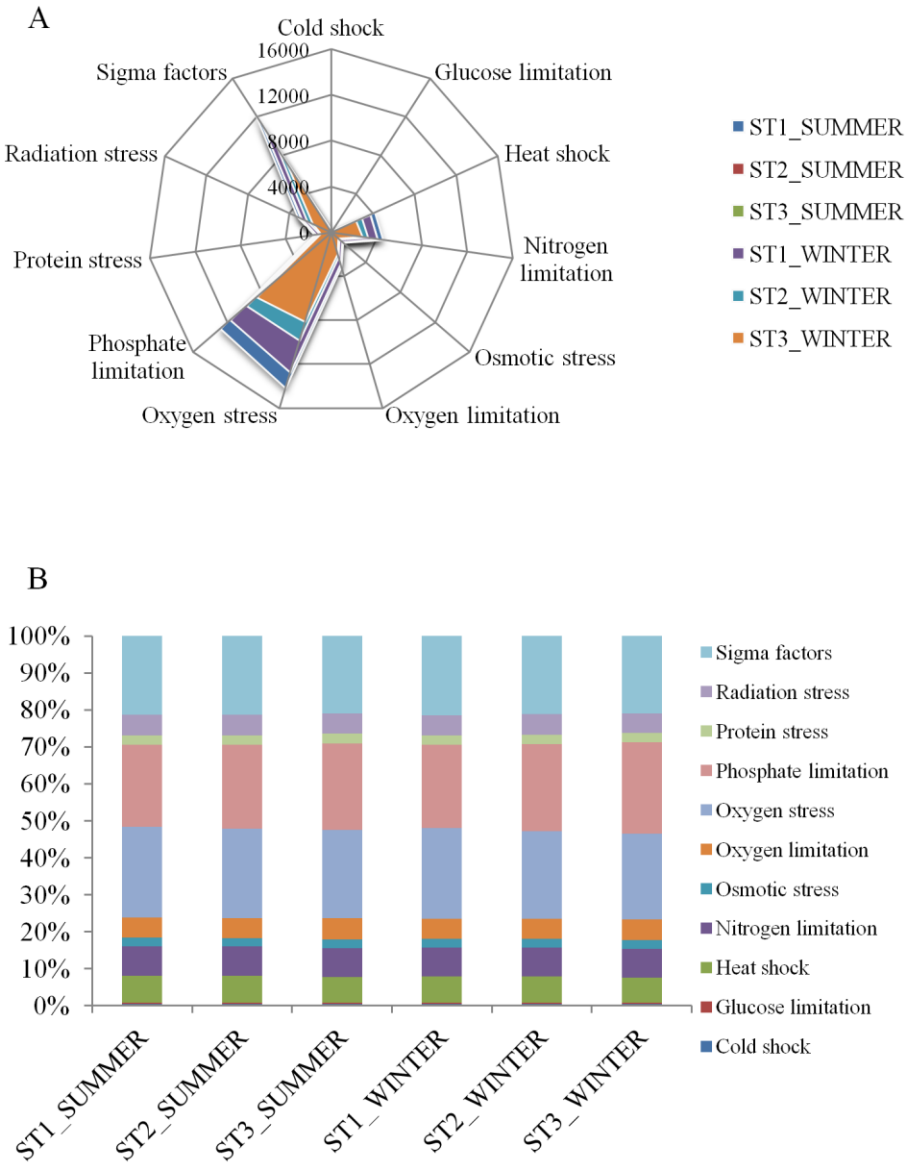

Figure 7 Total intensity (A) and relative abundance of stress response gene (B). Signal intensity was normalized by the number of the probes for each gene. A list of targeted genes for each category is provided in Tu *et al*. (2014) (See Table S1)

Table S1. Summary of probe and covered coding sequence information of GeoChip 4 based on gene categories (Tu *et al*., 2014)

Gene category	No. genes or enzymes	No. probes	No. sequence-specific probes	No. group- specific probes	No. covered CDS
Carbon cycling	41	11 034	3204	7830	18 071
Acetogenesis	1	122	35	87	250
Methane cycling	3	498	244	254	1577
Carbon fixation	4	1620	288	1332	3148
Carbon degradation	33	8794	2637	6157	12 996
Cellulose	4	702	268	434	1246
Chitin	3	1473	519	954	2295
Hemicellulose	5	1380	363	1017	2026
Lignin	4	934	569	365	1028
Pectin	1	72	50	22	63
Starch	8	2432	613	1819	3439
Others	8	1801	255	1546	2899
Nitrogen cycling	17	7386	3090	4296	10 744
Ammonification	2	969	210	759	1554
Anammox	1	47	2	45	282
Assimilatory N reduction	4	469	106	363	692
Dissimilatory N reduction	2	646	193	453	1341
Nitrification	2	1419	497	922	118
Denitrification	5	2612	1318	1294	4534
Nitrogen fixation	1	1224	764	460	2223
Phosphorus utilization	3	1341	351	990	2261
Sulfur	6	3113	1784	1329	4049
Adenylsulfate reductase	3	563	301	262	549
Sulfite reductase	2	2084	1336	748	2831
Sulfur oxidation	1	466	147	319	669
Energy process	4	853	436	417	1131
Cytochrome	1	619	390	229	728
Hydrogenase	2	197	40	157	348
P450	1	37	6	31	55
Metal resistance	44	9272	1295	7977	17 198
Aluminium	1	162	30	132	270
Arsenic	5	905	208	697	1551
Cadmium	2	876	99	777	1284
Cadmium, cobalt, zinc	3	1333	145	1188	2637
Chromium	1	1067	129	938	1949
Cobalt	1	62	8	54	118
Cobalt, nickel	3	16	5	11	29
Copper	5	1729	199	1530	3063
Lead	3	69	13	56	119
Mercury	7	733	169	564	1237
Nickel	1	36	4	32	71
Selenium	1	4	2	2	6
Silver	4	402	45	357	934
Tellurium	4	1290	136	1154	2542
Zinc	2	555	99	456	1321
Miscellaneous	1	33	4	29	67
Organic remediation	184	17 056	4692	12 364	28 716
Aromatics	132	12 831	3732	9099	21 056
Aromatic carboxylic acid	38	6068	1900	4168	9637
BTEX and related aromatics	21	1131	225	906	2044
Chlorinated aromatics	11	717	205	512	1227
Heterocyclic aromatics	9	154	68	86	234
Nitro aromatics	11	981	187	794	1782
Polycyclic aromatics	19	1138	375	763	1547
Other aromatics	23	2642	772	1870	4585

Table S1 (continued)

Gene Category	No. genes or enzymes	No. probes	No. sequence-specific probes	No. group-specific probes	No. covered CDS
Chlorinated solvents	6	600	161	439	1060
Herbicides related compounds	13	1408	283	1125	2373
Pesticides related compounds	5	492	96	396	928
Other hydrocarbons	14	746	241	505	1368
Others	14	979	179	800	1931
Antibiotic resistance	11	3,334	589	2,745	5533
Transporters	5	2316	294	2022	3979
b-lactamases	4	665	220	445	964
Others	2	353	75	278	590
Stress	45	21 541	1313	20 228	40 635
Cold shock	4	70	0	70	134
Heat shock	5	1655	215	1440	2616
Glucose limitation	2	87	3	84	134
Nitrogen limitation	3	1461	11	1450	3939
Osmotic stress	4	457	41	416	997
Oxygen limitation	7	994	121	873	2020
Oxygen stress	7	4765	255	4510	10 375
Phosphate limitation	6	5484	185	5299	9083
Protein stress	2	587	14	573	1316
Radiation stress	1	1264	54	1210	2695
Sigma factors	4	4717	414	4303	7326
Bacteria phage	40	1071	195	876	1987
Replication	25	666	113	553	1188
Lysis	7	133	22	111	289
Structural	6	230	42	188	452
Host recognition/structural	2	42	18	24	58
Virulence	13	3726	315	3411	7444
Other (gyrB, bchY)	2	2347	834	1513	4226
Total	410	82 074	18 098	63 976	141 995

Chapter 5

Drivers of the dynamics of diazotrophs and denitrifiers in North Sea bottom waters and sediments

Haoxin Fan[1], Henk Bolhuis[1], and Lucas J. Stal[1,2]

[1]Department of Marine Microbiology, Royal Netherlands Institute of Sea Research, Yerseke, The Netherlands
[2]Department of Aquatic Microbiology, Institute of Biodiversity and Ecosystem Dynamics, University of Amsterdam, The Netherlands

Published as: Fan H, Bolhuis H and Stal LJ (2015) Drivers of the dynamics of diazotrophs and denitrifiers in North Sea bottom waters and sediments. *Front. Microbiol*. 6:738.

Abstract
The fixation of dinitrogen (N_2) and denitrification are two opposite processes in the nitrogen cycle. The former transfers atmospheric dinitrogen gas into bound nitrogen in the biosphere, while the latter returns this bound nitrogen back to atmospheric dinitrogen. It is unclear whether or not these processes are intimately connected in any microbial ecosystem or that they are spatially and/or temporally separated. Here, we measured seafloor nitrogen fixation and denitrification as well as pelagic nitrogen fixation by using the stable isotope technique. Alongside, we measured the diversity, abundance, and activity of nitrogen-fixing and denitrifying microorganisms at three stations in the southern North Sea. Nitrogen fixation ranged from undetectable to 2.4 nmol N $L^{-1}d^{-1}$ and from undetectable to 8.2 nmol N $g^{-1}d^{-1}$in the water column and seafloor, respectively. The highest rates were measured in August at Doggersbank, both for the water column and for the seafloor. Denitrification ranged from 1.7 to 208.8 µmol m^{-2} d^{-1} and the highest rates were measured in May at the Oyster Grounds. DNA sequence analysis showed sequences of *nifH*, a structural gene for nitrogenase, related to sequences from anaerobic sulfur/iron reducers and sulfate reducers. Sequences of the structural gene for nitrite reductase, *nirS*, were related to environmental clones from marine sediments. Quantitative polymerase chain reaction (qPCR) data revealed the highest abundance of *nifH* and *nirS* genes at the Oyster Grounds. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) data revealed the highest *nifH* expression at Doggersbank and the highest *nirS* expression at the Oyster Grounds. The distribution of the diazotrophic and denitrifying communities seems to be subject to different selecting factors, leading to spatial and temporal separation of nitrogen fixation and denitrification. These selecting factors include temperature, organic matter availability, and oxygen concentration.

Keywords: N_2 fixation, denitrification, *nifH* gene, *nirS* gene, North Sea

Introduction

The microbial biogeochemical cycle of nitrogen transfers atmospheric dinitrogen gas (N_2) to bound nitrogen in the biosphere and back to N_2 (Revsbech *et al*., 2006). Dinitrogen fixation is the reduction of N_2 to ammonia, which is subsequently assimilated into amino acids and proteins to synthesize biomass. There are two processes that return bound nitrogen back to atmospheric N_2. Denitrification reduces nitrate or nitrite stepwise to dinitrogen (Zumft, 1997), while anaerobic ammonium oxidation (anammox) also produces N_2 gas, using nitrite as oxidant (Jetten *et al*., 1998). Nitrification is the aerobic oxidation of ammonia to nitrite and nitrate, substrates for both denitrification and anammox. Denitrification and anammox are anaerobic processes. The former seems to be quantitatively more important than the latter in most habitats, although in certain environments anammox has been shown to out rate denitrification (Kuypers *et al*., 2003; 2005). It is unknown whether or not in any microbial ecosystem the nitrogen cycle is functional at the same spatial and temporal scales or that they are (partly) occurring separated. Here, we investigated denitrification and dinitrogen fixation in the North Sea in order to answer this question.

Denitrification mainly takes place in the sediments of the seafloor of the coastal shelf (Seitzinger and Giblin, 1996; Codispoti *et al*., 2001). Coastal shelf seas are therefore major sinks for bound nitrogen and have been estimated to account for up to 67% of the global denitrification (Codispoti *et al*., 2001). The North Sea is such a coastal sea located on the European continental shelf, bordered by the United Kingdom in the west and Belgium, The Netherlands, Germany, Denmark and Norway in the east. Denitrification in the North Sea bottom sediments varies from 0.9 to 255 mmol m^{-2} $year^{-1}$ (Brion *et al*., 2004) and is the most important sink of nitrogen under hypoxic conditions (Middelburg *et al*., 1996).

Denitrification is carried out by a variety of different bacteria. The key intermediate step during denitrification is the reduction of nitrite to nitric oxide, which is catalyzed by either NirS (Cytochrome cd1) encoded by *nirS* or NirK (copper nitrite reductase) encoded by *nirK* (dissimilatory nitrite reductase). Nitrite reductase genes have been used as molecular markers for denitrification in natural environments. Phylogenetic analyses revealed the diversity of denitrifying bacteria in a variety of habitats such as soil (Prieme *et al*., 2002; Throbäck *et al*., 2007), estuarine sediments (Santoro *et al*., 2006), marine sediments (Braker *et al*., 2000; Hannig *et al*., 2006), and seawater (Jayakumar *et al*., 2004; Castro-Gonzalez *et al*., 2005; Oakley et al., 2007). Studies on denitrification in the North Sea were limited to rate measurements (Law and Owens, 1990; Lohse *et al*., 1993; 1996) or geochemical modeling (Van Raaphorst *et al*., 1990, 1992; Middelburg *et al*., 1996; Seitzinger and Giblin, 1996; Hydes *et al*., 1999). Not much is known about the diversity of denitrifier communities in the North Sea. In

order to assess the denitrifier community composition we targeted the *nirS* gene at three sites in the North Sea that differed in depth and seafloor sediment composition.

N_2 fixation occurs in the pelagic as well as in various benthic habitats including photosynthetic microbial mats (Severin and Stal, 2008), sea grass sediments (McGlathery *et al*., 1998; Herbert, 1999), and estuarine and shallow marine sediments (Fulweiler *et al*., 2007; Bertics *et al*., 2013). N_2 fixation is an important process for nitrogen depleted freshwater and brackish water bodies and in the warmer (sub)tropical ocean where it is driven by heterocystous (freshwater and brackish) and non-heterocystous (filamentous and unicellular; tropical ocean) cyanobacteria. Surprisingly, N_2 fixation is largely absent from temperate marine waters. N_2 fixation seems to be negligible in the North Sea, including the seafloor, although cyanobacterial microbial mats in intertidal sediments are a notable exception (Severin and Stal, 2008). Apparently, the nitrogen demand of the North Sea waters and sediments is covered from run-off and wet- and dry deposition (Brion *et al*., 2004), although this does not seem to cover the demand and therefore does not fully explain the absence of diazotrophs. There have been some reports of heterotrophic N_2 fixation in coastal waters (Gardner *et al*., 2006; Fulweiler *et al*., 2007; Bertics *et al*., 2010). Thus, diazotrophic microorganisms other than cyanobacteria may have been overlooked and might play a more important role than previously thought (Dang *et al*., 2013).

The reduction of N_2 to ammonia is catalyzed by nitrogenase, an enzyme complex composed of dinitrogenase and dinitrogenase reductase and that is similar among all diazotrophs (Sohm *et al*., 2011). The gene encoding nitrogenase reductase, *nifH*, is commonly used as a marker of diazotrophs in ecological studies (Zehr and Capone, 1996). In shallow marine sediments, N_2 fixation is mainly attributed to sulfate-reducing bacteria (Bertics *et al*., 2010; 2013; Brown and Jenkins, 2014). Hitherto, N_2 fixation has not been measured in the southern North Sea. In general, little information is available on the importance of biological N_2 fixation in temperate coastal waters (Brion *et al*., 2004).

The aim of this study was to measure N_2 fixation and denitrification in the bottom sediments of three different stations in the southern North Sea during three seasons. The diazotrophic and denitrifying communities and their activities were determined at the same time together with metadata of a range of environmental variables. With this research we elucidated: (1) N_2 fixation does occur in the southern North Sea bottom sediments and in the water column; (2) the identity of the microorganisms involved in N_2 fixation and denitrification; (3) the spatial and temporal trends of N_2 fixation and denitrification in the bottom.

Materials and methods

Study area and sampling

Four cruises were completed aboard the R/V Pelagia between November 2010 and August 2011. The study sites were located along the "Terschelling Transect" in the North Sea and the geographical coordinates are given in Bale *et al*. (2014) (Chapter 6) (Fig. 1). The Dutch Coast station (DC) is located in the well-mixed water of the coastal area where the sediments are typically sandy and have a low organic content. The Oyster Grounds station (OG) is a large circular depression in the central southern North Sea. The bottom sediments at OG are muddy sands and the organic carbon content is an order of magnitude higher compared to the DC and DB (follows) sites. The water column at the Dogger Bank station (DB) is 30 m deep. The bottom sediment at DB is sandy and contains a low amount of organic matter. The properties of the sediments at the three sampling stations are described in Bale *et al*. (2014) (Chapter 6). Sampling of the bottom sediment and the overlying water was performed using a box corer. Intact sediment cores were collected from the box corer using custom-made cores (20 cm long × 5 cm i.d.) and were used to measure denitrification and N_2 fixation. Physicochemical parameters were measured in the water column and in the sediment as described by Bale *et al*. (2014) (Chapter 6).

Figure 1. Map of the North Sea with the three sampling stations marked.

Denitrification and N_2 fixation

The rates of denitrification and N_2 fixation were measured by the ^{15}N stable isotope technique. The procedures for measurement and calculation of the rate of denitrification were described previously (Bale *et al*., 2014). The dissolution of $^{15}N_2$ in medium for the measurement of N_2 fixation was performed according to Mohr *et al*. (2010) with some modifications. Briefly, 500 ml ASW (artificial seawater) (NaCl 20.5 g, Na_2SO_4 3.4 g, KCl 0.58 g, KBr 0.084 g and H_3BO_3 0.022 g, $MgCl_2.6H_2O$ 10.2 g, $CaCl_2.2H_2O$ 1.1 g in 1000 ml Milli-Q water) was degassed by vacuuming for 45

min (KNF Neuberger, type N726.3 FT.18) in an ultrasonic bath. The degassed ASW was transferred to 300 ml Schott bottles until overflow and sealed after which 3 ml $^{15}N_2$ (98%) were injected. The bottle was shaken overnight before it was used for enriching samples with $^{15}N_2$. Fifty milliliter of the $^{15}N_2$-enriched ASW was added to 450 ml ASW, which was subsequently used for the slurry incubations. For the measurement of N_2 fixation, sediment from the top 5 cm was homogenized to slurry (equal volumes of sediment and ASW). Ten milliliters of slurry were put into 50-ml serum bottles, which were subsequently filled with the $^{15}N_2$-enriched ASW. The bottles were sealed with butyl stoppers while avoiding air bubbles. The bottles were incubated for 24 h in the dark at *in situ* temperature. Incubations were terminated by removing the overlaying water and freeze the sediment at −20°C. *In situ* denitrification rates were measured as described in Bale *et al*. (2014). Nucleic acids were extracted from the top 5 cm of the sediment collected by box cores from the three stations. The sediment samples were taken from the box cores using 15-ml plastic screw cap tube and stored immediately at -80°C until analysis.

Nucleic acid extraction, PCR, cloning, and sequencing

DNA and RNA from the water column were extracted according to the procedures described by Bale *et al*. (2013). DNA and RNA were extracted from the sediments using the MoBio UltraCLEAN soil DNA and RNA kit (MoBio Laboratories, Inc., Carlsbad, CA, USA), according to the manufacturer's instructions.

The quantity and quality of RNA were determined and checked by Nanodrop spectrophotometer (Nanodrop ND1000, Thermo Scientifica, Wilmington, DE, USA) and agarose gel electrophoresis, respectively. The RNA extracts were immediately treated with RNase free DNase I (Deoxyribonuclease I, Amplification Grade, Invitrogen Corporation, Carlsbad, CA, USA). DNA contamination was checked by PCR using the DNase-treated RNA extract as template. After the DNase treatment and the confirmation of the absence of DNA the RNA concentration and quality were checked again. The DNA-free RNA was reverse transcribed to cDNA using Superscript II Reverse Transcriptase and random primers (Invitrogen Corporation, Carlsbad, CA, USA) following the manufacturer's manual. Controls were run that either lacked reverse transcriptase or the RNA extract and should not give a product. The synthesized cDNA was kept at −20°C until further use.

For amplification of *nifH* and its transcripts we used a nested PCR with inner primer pair nifH 1 (5′ TGY GAY CCN AAR GCN GA 3′) and nifH 2 (5′ ADN GCC ATC ATY TCN C 3′) (Zehr and McReynolds, 1989) and outer primers nifH 3 (5′ ATR TTR TTN GCN GCR TA 3′) nifH 4 (5′ TTY TAY GGN AAR GGN GG) (Zani *et al*., 2000). The PCR conditions have been described in Severin *et al*. (2010).

Both *nirS* and *nirK* were initially tested, however, we subsequently only targeted *nirS* as this gene is preferentially found in marine sediment, while *nirK* is more common in soil (Braker *et al*., 2000). Fragments of *nirS* were amplified using the primer pairs cd3aF and R3cd (Throbäck *et al*., 2004). PCR conditions for this primer pair were 2 min at 95°C, 35 cycles of 50 s 95°C, 50 s 53°C, and 50 s at 72°C, followed by a final extension of 10 min at 72°C. PCR products were checked on a 1% agarose gel. PCR products were cloned using the TOPO-TA cloning kit with the pCR2.1 vector and TOP10 competent cells (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. Transformants (99 for *nifH* and 50 for *nirS*) were randomly picked from each clone library and screened by PCR using T3 and T7 vector primers following the recommended PCR conditions (Invitrogen, Carlsbad, CA, USA). PCR products were purified and checked as described by Severin *et al*. (2010) and sequenced with the T7 vector primer using ABI PRISM 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

Quantitative real-time PCR

Quantitative real-time PCR (qPCR) analyses were run on a Corbett Rotor-Gene 6000TM (Corbett Life Science, Sydney, Australia). The copy numbers of *nifH* and *nirS* were determined by primers nifH_q1 (5′CgYggYgTTATCACYgCYATCAACTT 3′) and nifH_q2 (5′ CgAAACCRCCRCARACAACgTC 3′) (Tm = 53°C) and by primer pair cd3aF and R3cd (Throbäck *et al*., 2004) (Tm = 53°C), respectively. For the quantification of the *nifH* gene, primers nifH_q1 and nifH_q2 were designed by aligning 200 sequences (main groups as revealed by phylogenetic analysis) obtained from cloning the PCR products amplified by using the Zehr and Zani primers. In order to confirm the specificity, 48 amplification products from sediment samples were cloned and sequenced. All of these amplicons encode a *nifH* gene and the majority was identical and clustered amongst those listed in Table S1. DNA (dilution 1:10) and cDNA samples were run in triplicate. Standard curves were made by dilution series of linearized plasmids (quantified by Nanodrop before using as standard for quantification) containing the target genes and were run parallel to each analysis. Non-template controls were also included in each run. The reaction mixture (15 µl) contained 7.5 µl of Absolute™ QPCR SYBR® Mix (Thermo Fisher Scientific, Rockford, IL, USA), 0.2 pmol/µl primers, 1 µl template and sterilized MQ water. Cycling conditions were as follows: 95°C 15 min, 45 cycles of 15 s 95°C, 20 s Tm, and 20 s at 72°C, followed by melting curve analysis (50–95°C). The standard curves spanned a range from 22 to 2.2×10^6 copies per µl for the *nifH* and 12 to 1.2×10^6 copies per µl for the *nirS*. PCR efficiencies (E) and correlation coefficients for *nifH* were 85% and $R^2 = 0.99$ and for *nirS* were 81% and $R^2 = 0.99$.

Sequence and statistical analysis

Sequences were manually checked, aligned, and translated using MEGA 6 (Tamura *et al*., 2013). Neighbor-joining trees were produced and the reliability of the phylogenetic reconstructions was evaluated by bootstrapping (1000 replicates). The Prodist program within Phylip v.3.6 (Felsenstein, 2005) generated the distance matrix files of amino acid sequences. These files were used to calculate the non-parametric richness and diversity estimators and to determine the differences in nucleic acid sequences applying the program Mothur (Schloss *et al*., 2009). Operational Taxonomic Units (OTUs) were defined as a 5% difference in amino acid sequences for the purpose of community analysis. The principal coordinate analyses were generated using Mothur. The coverage of the clone library was calculated as $C = [1-(n/N)] \times 100$, where n denotes the number of unique OTUs (95% amino acid cutoff was used) and N denotes the total number of sequences examined (Good, 1953). The Pearson correlation test was performed using software the SigmaPlot™ v12.0.

Nucleotide sequence accession numbers

Sequences were submitted to NCBI (accession numbers KP959349–KP959733).

Results

Physicochemical parameters

Physicochemical parameters in the water column were described in Bale *et al*. (2013). Ammonium concentrations in the pore water of the bottom sediment (5 cm) ranged between 4.1 and 23.4 μM and were highest in August at all stations (Table 1). Nitrate concentrations were between 4.9 and 46.2 μM and were always highest in February. Pore water nitrite concentration ranged between 0.2 and 1.3 μM and was highest in August at DC. The concentration of phosphate in the pore water ranged between 1.0 and 3.0 μM and the highest value was detected in August at DB.

Table 1. Sediment pore water nutrients and bottom water temperatures (top 5 cm).

	February			May			August		
	DB	OG	DC	DB	OG	DC	DB	OG	DC
Ammonium (μM)	7.5	7.6	4.8	4.1	13.7	4.7	9.7	23.5	22.4
Nitrate (μM)	18.5	19.7	46.2	5.7	15.0	9.4	6.6	4.9	6.7
Nitrite (μM)	0.2	0.3	0.5	0.3	0.6	0.8	0.2	0.6	1.3
Phosphate (μM)	1.3	1.8	1.1	2.4	1.9	1.0	3.0	1.6	1.0
Temperature (°C)	5.2	5	4.9	11.4	8.6	14	15.4	15.4	18.3

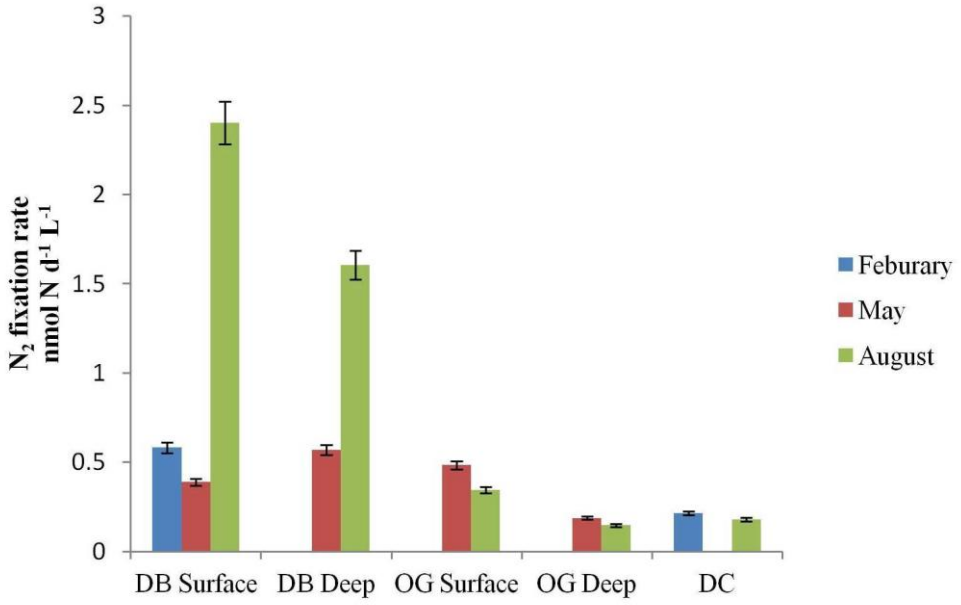

Figure 2. N_2 fixation (nmol N L^{-1} d^{-1}) in surface and deep water at the stations DB, OG and DC in February, May and August.

N_2 fixation in the water column

N_2 fixation was detected at all stations in the water column as well as in the bottom sediments. In the water column, the rates ranged from 0 to 2.4 nmol N $L^{-1}d^{-1}$ (Fig. 2). The highest rate of N_2 fixation was measured in August at the water surface at station DB. Among the three stations, DB always recorded the highest rate of N_2 fixation with a peak in August. In February we did not detect N_2 fixation at station OG and in May not in DC. In August N_2 fixation was detected in all stations but was highest in station DB.

Abundance and activity of diazotrophs in the sediment

In the top 5 cm of the bottom sediments, N_2 fixation was in the range of 0–8.1 nmol N d^{-1} g^{-1} wet sediment (Fig. 3A). The highest rate of N_2 fixation was recorded in August at station DB. N_2 fixation was undetectable in February at station OG and at all three stations in May. The abundance of diazotrophs and denitrifiers was evaluated through the quantification of the *nifH* and *nirS* genes, respectively. Gene copy numbers of *nifH* were more or less constant throughout the seasons. The abundance of *nifH* was highest at station OG (on average 10^6 copies g^{-1} wet sediment). The stations DB and DC contained on average 4.8×10^5 and 1.9×10^5 *nifH* gene copies g^{-1} wet sediment, respectively (Fig. 3B). At all stations expression of the *nifH* gene was detected in the bottom sediment. The values ranged from 5.3×10^2 to 1.8×10^4 and the highest number was recorded in February at station DB (Fig. 3C). The number of *nifH* transcripts was 13 times higher at station DB (on average 1.2×10^4 transcripts g^{-1} wet

sediment) than that at station OG (on average 8.7×10^2 transcripts g^{-1} wet sediment). We have only data for August at station DC (8.3×10^3 transcripts g^{-1} wet sediment).

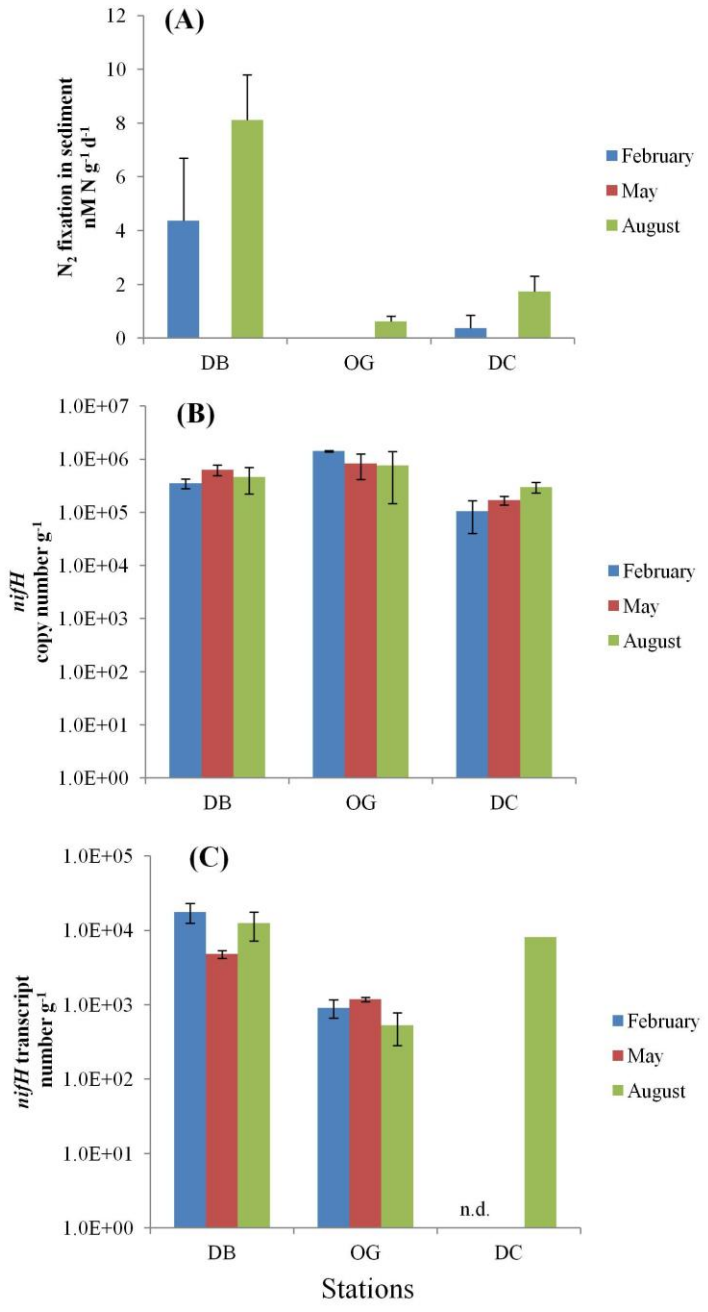

Figure 3. N_2 fixation in the bottom sediments at the stations DB, OG and DC in February, May and August. (A) potential nitrogen fixation rates (nmol N day^{-1} g^{-1} wet sediment). (B) *nifH* copy number (g^{-1} wet sediment). (C) *nifH* transcript number (g^{-1} wet sediment). n.d. no data.

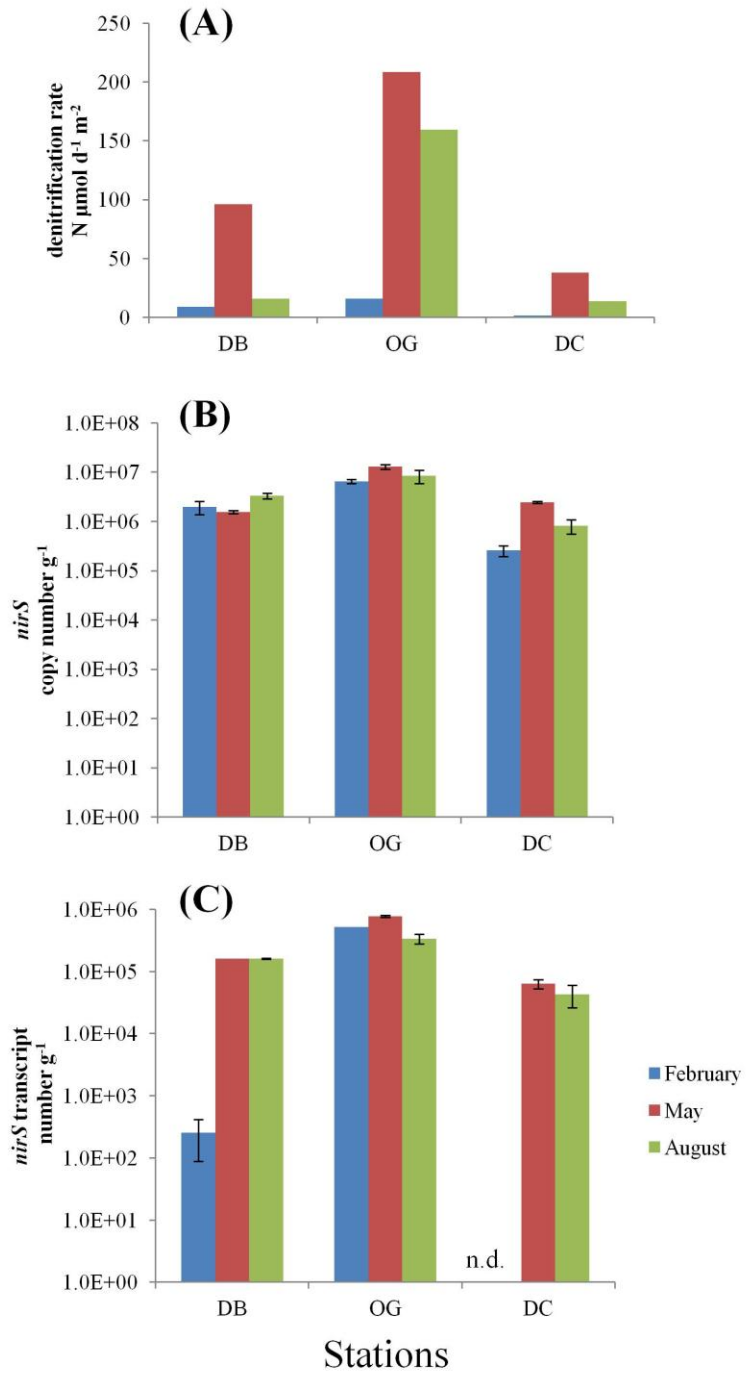

Figure 4. Denitrification in the bottom sediments at the stations DB, OG and DC in February, May and August. (A) *in situ* denitrification (µmol m^{-2} day^{-1}). (B) *nirS* copy number (g^{-1} wet sediment). (C) *nirS* transcript number (g^{-1} wet sediment). n.d. no data.

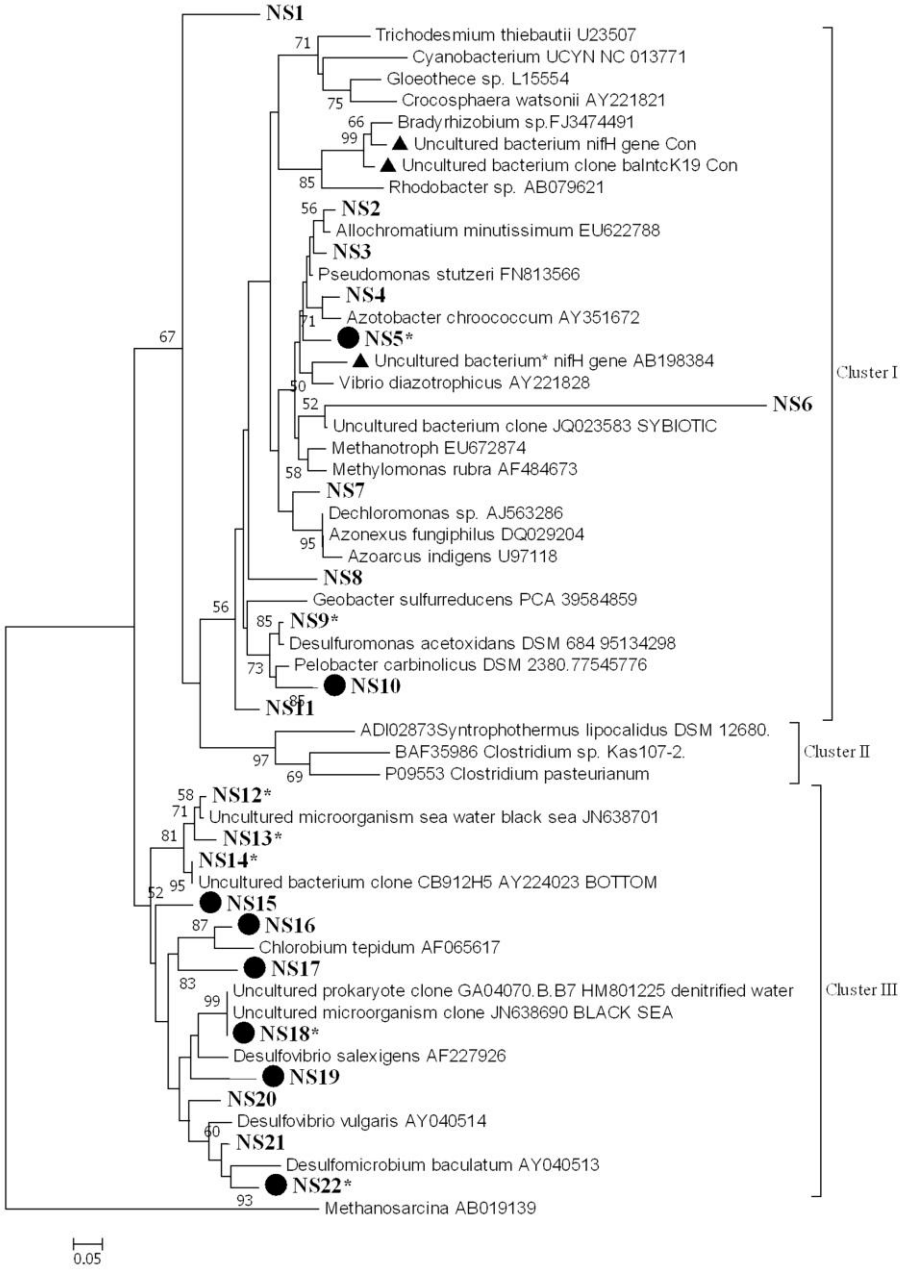

Figure 5. Phylogenetic tree of *nifH* based on the translated amino acid sequence, constructed by the neighbor-joining method in MEGA 6. The scale bar indicates the number of sequence substitution per sites. Sequences retrieved in this study fell into 22 groups (NS1-NS22) and are shown in bold. Table S1 gives all sequences in groups NS1–NS22. Groups that contain sequences from both genomic DNA and cDNA are marked by a solid cycle. Asterisks indicate groups that contain sequences from the water column. *nifH* sequences that have been reported previously as potential contaminants in RT-PCR reagents are marked by a solid triangle.

Abundance and activity of denitrifiers in the sediment

Denitrification in the top 10 cm of the bottom sediment was in the range of 1.7–208.8 μmol N m^{-2} d^{-1}. The highest rate was recorded in May at station OG and the lowest rate in February at station DC (Fig. 4A). Denitrification was always highest at station OG regardless in which of the three seasons studied.

The *nirS* gene abundance followed the same spatial trend as *nifH* and was also highest at station OG, irrespective the season (Fig. 4B). At the stations DB and OG the *nirS* gene abundance was more or less constant during the seasons (on average 2.3×10^6 and 9.3×10^6 gene copies g^{-1} wet sediment, respectively), whereas at station DC the numbers increased 9.4-fold between February and May (on average 1.2×10^6 copies g^{-1} wet sediment).

The expression of *nirS* was detected in all samples and ranged $2.5 \times 10^2 – 7.7 \times 10^5$ transcripts g^{-1} wet sediment (Fig. 4C). The number of transcripts was highest at station OG (on average 5.4×10^5 transcripts g^{-1} wet sediment) and lowest in DC. We did not observe seasonality at the stations OG and DC whereas the number of transcripts at station DB was three orders of magnitude lower in February than in May and August.

Diversity, phylogeny, and community composition of diazotrophs and denitrifiers (*nifH* and *nirS* sequences)

nifH diversity and expression were examined in samples collected from the water column (surface and deep) at all stations in August. We examined 15–20 sequences from each library. Amplified *nifH* gene sequences (from DNA and cDNA) fell within the clusters I and III, according to the phylogenetic classification proposed by Zehr *et al*. (2003) (Fig. 5). The cDNA sequences fell mainly into group NS22 (Fig. 5). These sequences did not cluster according to stations or to depths. A large proportion of the sequences (60%) belong to cluster III and almost all expressed *nifH* belongs to this cluster.

The diversity of *nifH* and *nirS* was evaluated in sediment samples collected in August. In total 246 sequences were obtained from three *nifH* clone libraries and resulted in 55 OTUs at the 95% identical amino acid level. The coverage of these libraries ranged from 66 to 78%. Based on diversity indices (H, 1/D), the station OG had the lowest diversity and the coastal station DC had highest diversity. The richness estimators S_{ACE} and Chao1 are consistent with these results (Table 2).

Table 2. Biodiversity and predicted richness of the sediment NifH and NirS amino acid sequences from the sampling stations of the Southern North Sea based on 95% cutoffs.

	No. of clones	No. of OTUs	Coverage (%)	ACE	Chao1	Shannon	Simpson
NifH							
DB	85	23	73	43	43	2.3	0.21
OG	73	16	78	30	25	1.4	0.50
DC	88	30	66	76	43	2.9	0.09
NirS							
DB	34	14	59	47	21	2.3	0.12
OG	41	32	22	719	307	3.2	0.03
DC	46	14	70	25	19	2.0	0.12

Phylogenetic analysis of the deduced amino acid sequences of the *nifH* amplicons revealed that they fell within clusters I and III. Of the 246 *nifH* sequences from the sediment, 63% belonged to cluster I (64, 86, and 42% of the *nifH* sequences from station DB, OG, and DC, respectively). Of these, another 63% could be affiliated to *Pelobacter carbinolicus* (accession number WP_011341851) (48, 86, and 23% of the *nifH* sequences from station DB, OG, and DC, respectively). The rest of the *nifH* sequences belong to cluster III. At all stations *nifH* was expressed and *nifH* sequences from cDNA libraries are subsets of the gene copy libraries.

A total of 118 *nirS* DNA sequences was obtained consisting of 60 OTUs at 95% identity at the amino acid level. The coverage ranged from 22 to 70% with the poorest one observed at station OG. In contrast to *nifH*, the highest diversity and richness were found at station OG while the lowest values were found at station DC (Table 2). Phylogenetic analysis of the deduced amino acid sequences for *nirS* gene fragments showed that the majority of sequences clustered and are related to environmental clones from a variety of environments (e.g., ABI33733 from Chesapeake Bay, CAL69007 from a hypersaline microbial mat and CAJ87449 from the Baltic Sea) (Fig.6). The sequences were closest related to those belonging to *Thiothrix lacustris* (AGO45492) (similarity 83%) *Azoarcus tolulyticus* (AAL86941) (similarity 72%).

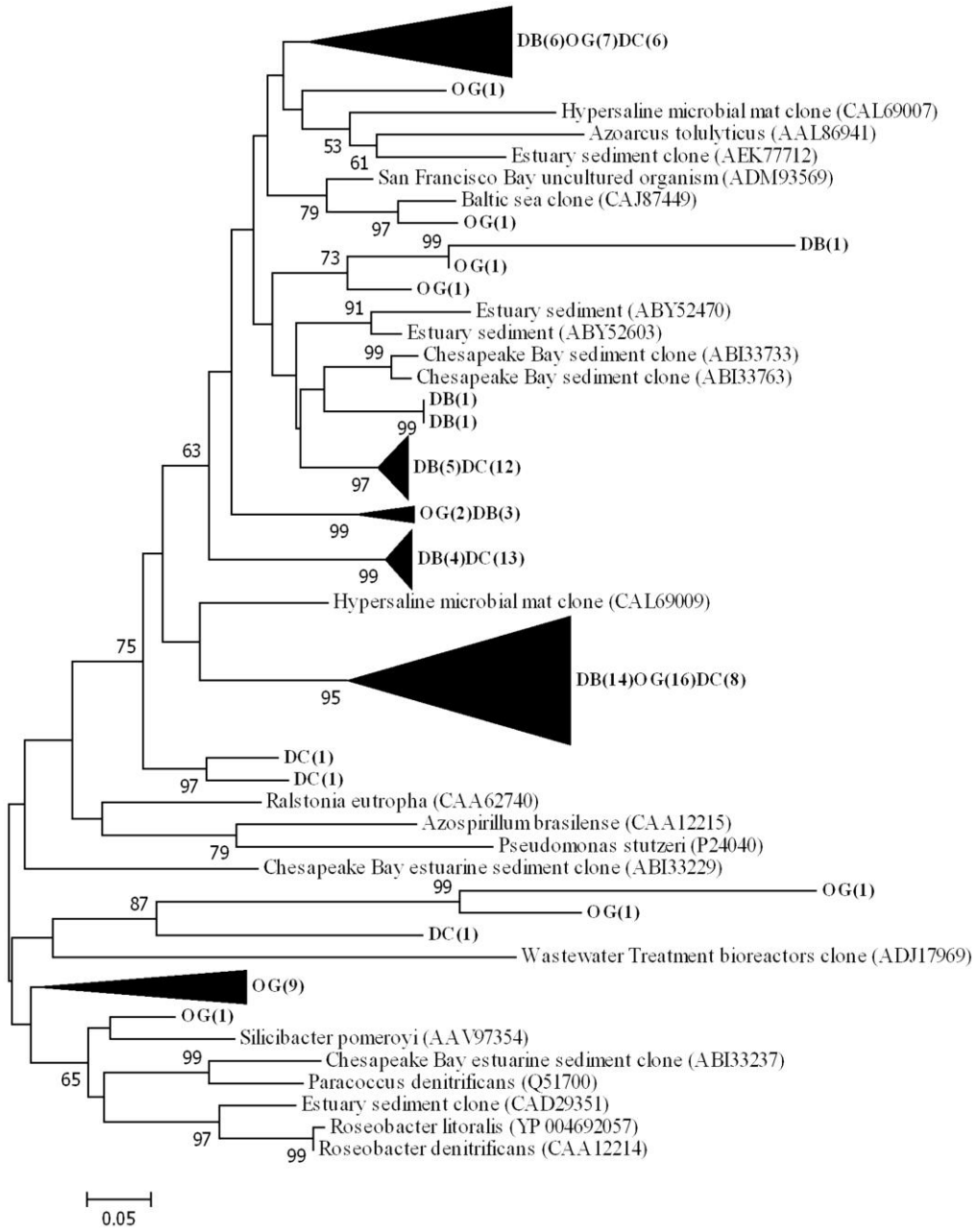

Figure 6. Phylogenetic tree of *nirS* based on the translated amino acid sequence, constructed by the neighbor-joining method in MEGA 6. The scale bar indicates the number of sequence substitution per sites. Sequences retrieved in this study are shown in bold. The numbers in the parenthesis following the station name indicate the number of sequences within this cluster at that station.

Principal coordinate analysis was performed to show the differences in composition of diazotrophic and denitrifying communities between stations. As shown in Fig. 7, the diazotrophic community composition at station DC was different from the other two stations. The denitrifying community compositions at station DB and station DC were more similar to each other than to station OG.

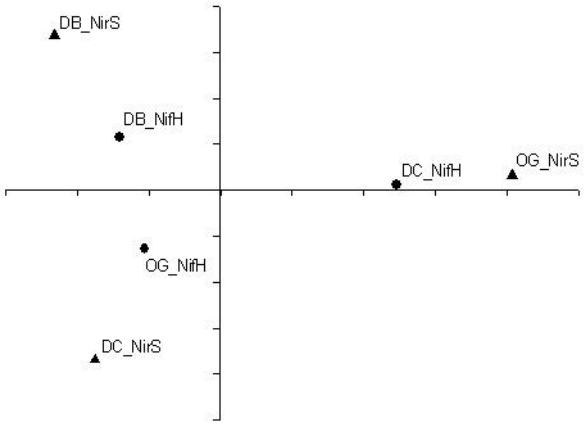

Figure 7. Principal coordinate analyses of *nifH* (solid cycle) and *nirS* (solid triangle) translated amino acid sequences at the 95% cutoff

Discussion

Recent studies expanded biological N_2 fixation to include temperate coastal waters (Rees *et al*., 2009; Mulholland *et al*., 2012). The nitrogen fixation rates (0–2.4 nmol N L^{-1} d^{-1}) in the water column of the southern North Sea that we report here were in the same range as those that have been reported for the tropical Atlantic Ocean (0.6–1.1 nmol N L^{-1} d^{-1}) (Falcón *et al*., 2004), the subtropical and tropical eastern Atlantic Ocean (0–1.4 nmol N L^{-1} d^{-1}) (Staal *et al*., 2007) and from some stations of the western Atlantic coastal waters (0.2–76.8 nmol N L^{-1} d^{-1}) (Mulholland *et al*., 2012) but substantially lower than those reported for the western English Channel (18.9–20.0 nmol N L^{-1} d^{-1}) (Rees *et al*., 2009). Depth integrated rates of nitrogen fixation ranged from 1.25 to 62.5 μmol N m^{-2} d^{-1} at the stations of the southern North Sea. These rates of N_2 fixation were detected throughout the year but may appear low relative to other coastal ecosystems such as microbial mats, coral reefs, sea grass meadows, and intertidal marshes. N_2 fixation also occurred in the bottom sediments of the stations. This is the first time that N_2 fixation is reported from the water column and bottom sediments in the cold waters of the North Sea. The rates of denitrification that we observed in the North Sea (1.7–208.8 μmol N m^{-2} d^{-1}) compared well with other reports [240–320 μmol N m^{-2} d^{-1}, (Lohse *et al*., 1996) and 700 μmol N m^{-2} d^{-1}, Hydes *et al*., 1999]. When comparing the rates of N_2 fixation and denitrification we conclude that these processes seem to be in balance.

Sequence analysis revealed that *nifH* expressed in the water column belong to clusters I and III. Cluster I sequences that are closely related to deltaproteobacteria have been found in the English Channel (EF470531) (Rees *et al*., 2009). Cluster III sequences affiliated to *Desulfovibrio salexigens* have been found in the western mid-Atlantic coastal waters (FJ756655) (Mulholland *et al*., 2012). Hence, these diazotrophs seem to be common in Atlantic coastal waters. We did not found *nifH* sequences belonging to cyanobacteria or γ-proteobacteria although these groups are regarded as the dominant diazotrophs in the marine environment (Capone *et al*., 1997; Montoya *et al*., 2004; Halm *et al*., 2012; Moisander *et al*., 2014) and were also detected in English Channel (Rees *et al*., 2009). The absence of these groups would explain the low rates of N_2 fixation rates measured in our study compared to those recorded in the western English Channel. The fact that the *nifH* sequences retrieved in oxygenated surface water hints to the presence of anaerobic groups requires an explanation. Given that some of the retrieved *nifH* phylotypes have also been reported from coastal microbial mats (Severin *et al*., 2010), we speculate that benthic microorganisms might have been brought into suspension. But we cannot exclude the possibility that anaerobic diazotrophs thrive in anoxic microniches such as in aggregates (Ploug, 2001), or that the sequences belong to other aerobic organisms.

A large proportion of *nifH* homologs obtained from the sediment belongs to *P*. *carbinolicus*, similar to what has been found in other studies (Fulweiler *et al*., 2013; Brown and Jenkins, 2014). *P*. *carbinolicus* couples the oxidation of organic matter or metals to the dissimilatory reduction of Fe(III) or S (elemental sulfur) (Lovley *et al*., 1995; Holmes *et al*., 2004b). *P*. *carbinolicus* is a strictly anaerobic bacterium belonging to the deltaproteobacterial family of *Peleobacteraceae*. Organisms of this family are known for their bioremediation potential. It is currently unknown whether this organism fixes N_2 but genome sequencing revealed the presence of genes encoding proteins that are involved in nitrogen fixation including a cluster containing *nifHD* homologs, genes involved in molybdenum biosynthesis and several other nif-genes (Aklujkar *et al*., 2012). There are also no reports of studies on N_2 fixation by *P*. *carbinolicus* although the phylogenetically related *Geobacter metallireducens* has been shown to fix N_2 (Bazylinski *et al*., 2000). Holmes *et al*. (2004a) found that N_2 fixation is a highly conserved trait in the *Geobacteraceae* and proposed that it gives the members of this family the advantage to compete in environments that are being bio-remediated. But it remains to be seen whether this is also the case in *P*. *carbinolicus*.

The sequencing results suggest that sulfate reducers such as *D*. *salexigens* and *Desulfovibrio vulgaris* may be the dominant diazotrophs in the bottom sediments of the southern North Sea. Many sulfate-reducing bacteria possess the genetic potential to fix dinitrogen (Zehr *et al*., 1995). These diazotrophic organisms were also found in the sediments of the Baltic Sea (Bertics *et al*., 2013), Narragansett Bay (Fulweiler *et*

al., 2013), and coastal California (Bertics *et al*., 2010). Previous studies found that nitrogenase activity decreased substantially when sulfate reduction was inhibited (Burns *et al*., 2002; Bertics *et al*., 2013). In addition, Bertics *et al*. (2013) and Brown and Jenkins (2014) complemented these findings with genetic data showing that the *nifH* sequences retrieved were closely related to sulfur and sulfate reducers *Desulfovibrio* and *Desulfobacter* spp. This supports the idea that sulfate-reducing bacteria are key players in sedimentary N_2 fixation.

It has been shown that denitrification and nitrogen fixation are both controlled by the same common factors such as temperature, oxygen, and substrate availability (e.g., organic matter and nitrite for denitrification) (Joye and Paerl, 1994; Nowicki *et al*., 1997; Kana *et al*., 1998; Staal *et al*., 2003; Fulweiler *et al*., 2007). In this study we observed different seasonality and spatiality of sedimentary nitrogen fixation and denitrification, suggesting that the individual response is different.

Spatial variation of denitrification is primarily attributed to the distribution of organic carbon in the bottom surface sediments. Trimmer and Nicholls (2009) have shown that sedimentary denitrification correlated positively with the concentration of organic carbon in the surface sediments along a transect in the North Atlantic. Station OG is a recognized deposition area with muddy sands and contains an order of magnitude more organic carbon compared to the other two stations (Bale *et al*., 2013). This would explain the higher rate of denitrification at station OG. Temporally, denitrification was highest in spring and this seasonality coincided well with the abundance of the *nirS* gene transcripts. This result is also consistent with a previous study in the southern North Sea. Van Raaphorst *et al*. (1992) estimated the denitrification at two stations in the southern North Sea and showed that denitrification was highest in spring and early summer and lowest in winter. Different seasonality of denitrification has also been observed in other studies. For instance, Tuominen *et al*. (1998) reported highest denitrification in Baltic Sea bottom sediments in late summer and early autumn while Hietanen and Kuparinen (2008) observed the highest rates in autumn and early winter in the sediment in the Gulf of Finland. High rates of denitrification in late spring could be attributed to an elevated temperature and an increased supply of fresh organic carbon deposited from spring blooms (Joint and Pomroy, 1993). Moreover, during late spring and summer, the increased availability of organic matter would stimulate the consumption of O_2, which would enhance denitrification. The bottom water at station OG can become hypoxic in summer (Weston *et al*., 2008; Greenwood *et al*., 2010), and consequently this would decrease the oxygen penetration depth in the sediment. The low oxygen concentration in the sediment at station OG may be responsible for the observed high rate of denitrification. In addition, a previous study has shown that nitrification is also higher in summer than in winter in the North Sea sediment (Lohse *et al*., 1993). When the source of nitrate/nitrite in the water column is limited in summer (nitrate/nitrite

concentrations in bottom water have been reported in Bale *et al*., 2013), nitrification becomes the primary source of nitrate/nitrite for denitrification.

In contrast to denitrification, station DB exhibited the highest pelagic and sedimentary rates of nitrogen fixation as well as the highest number of *nifH* transcripts when compared to the other two stations. Station DB is located at the shallow Dogger Bank. Previous studies indicated that the Dogger Bank is a special ecological area with distinct biological characteristics compared to the surrounding regions in the North Sea (Kröncke and Knust, 1995). Throughout the year the Dogger Bank exhibits high rates of primary production (Howarth *et al*., 1993). This introduces fresh organic matter to the water column and the bottom sediment, fuelling heterotrophic N_2 fixation. This would explain why N_2 fixation occurred even in February in the water column and bottom sediment at the sandy station DB. O'Neil and Capone (1989) found that N_2 fixation in coarse-grained marine sediments is higher in eutrophic than in oligotrophic environments and is generally stimulated by the addition of organic matter.

In this study we observed that N_2 fixation is generally higher in summer than in winter and this also the case for denitrification for the same reasons as discussed above. The exception was that benthic N_2 fixation was undetectable in spring (May) when denitrification was highest. This difference suggests that N_2 fixation and denitrification respond differently to the post bloom deposition. Fulweiler *et al*. (2007) showed that denitrification responded rapidly and positively to the deposition of organic matter. Van Luijn *et al*. (1999) investigated nitrogen fluxes and processes in the bottom sediments of a shallow eutrophic lake and found that denitrification increased with increasing contents of fresh organic matter but then deceased when a certain concentration of organic matter was exceeded. These observations coincided well with our seasonal denitrification trend. It is not clear why N_2 fixation did not respond to the post bloom deposition in spring. Fulweiler *et al*. (2013) proposed that the quality of organic matter plays a role in controlling N_2 fixation. It is possible that a different timing of the phytoplankton blooms alters both the quantity and quality of the deposited organic matter (Nixon *et al*., 2009) and we speculate that benthic diazotrophs depend on a restricted range of organic matter and/or its concentration. The positive correlation (Pearson, $\rho = 0.95$, $p < 0.05$, $n = 9$) between pelagic and benthic N_2 fixation suggests that both respond in the same way to environmental factors.

The spatial and temporal separation of denitrification and nitrogen fixation is also projected on the composition of chemotrophic diazotrophic and denitrifying communities. We hypothesize that the characteristics of the sediment may be a factor that determines the diazotrophic and denitrifying community compositions. Both the abundances of *nifH* (Pearson, $\rho = 0.82$ $p < 0.05$, $n = 9$) and *nirS* (Pearson, $\rho = 0.90$, $p <$

0.05, *n*= 9) genes are positively correlated with total organic matter (Bale *et al*., 2013). However, the diazotrophic community composition at the three stations appears to correlate with geographic location (coastal and offshore) rather than with sediment type (sandy and muddy). In contrast, sediment type determined denitrifying community composition (based on *nirS* gene). The community composition of denitrifiers at station DB and station DC are more similar to each other than to station OG. Low organic carbon content and a large grain size are characteristic for the former two stations (Bale *et al*., 2014) (Chapter 6).

High abundance of *nifH* copies at station OG indicated the genetic potential of N_2 fixation at this station. Nevertheless, the number of *nifH* transcripts was low and we did not find N_2 fixation at station OG while the opposite was true for station DB. There are several explanations for this discrepancy. First, heterotrophic N_2 fixers may have to compete for carbon with other heterotrophic microorganisms such as denitrifiers, which were highly active at station OG. Second, the quality and quantity of organic matter may determine the activity of diazotrophs. It has been shown that the water mass at the three stations are not the same (Bale *et al*., 2013). This could lead to a deposition of organic matter that differs in quality and quantity. Third, although organic matter would promote oxygen consumption in sediment, the degradation of organic matter also leads to the accumulation of nitrate and ammonium, which could lead to inhibition of N_2 fixation. Whether or not this latter possibility is realistic is unclear. In this study as well as in other published reports, N_2 fixation took place in the presence of considerable levels of dissolved inorganic nitrogen (DIN). The thresholds of DIN at which N_2 fixation under natural conditions is inhibited have not been precisely determined. N_2 fixation activity has been detected in diverse pelagic and benthic environments while the DIN concentrations were higher than those in our study. Mulholland *et al*. (2012) found N_2 fixation in the presence of DIN and low phosphate concentrations in a temperate marine system. Haines *et al*. (1981) found N_2 fixation in a coastal sediment in Alaska in the presence of an ammonium concentration of 177 μM. Bertics *et al*. (2010) detected nitrogenase activity in the subsurface of bioturbated sediments when ambient ammonium concentrations were >50 μM and Bertics *et al*. (2013) even detected N_2 fixation by sulfate reducing bacteria in the presence of 1 mM ammonium. However, contrary to these observations, Joye and Paerl (1994) found that N_2 fixation decreased when ambient DIN concentrations increased. Similar observations of N_2 fixation in sea-grass-bearing sediments coincided with an annual minimum ammonium concentration of 190 μM (Welsh *et al*., 1996). We found no evidence of inhibition of N_2 fixation by DIN and this is consistent with other studies (Haines *et al*., 1981; Bertics *et al*., 2010; 2013). Nitrogen fixation may also benefit from high phosphate concentration. It has been shown that DIN may not be inhibiting N_2 fixation in the euphotic zone of marine waters, especially when phosphate and trace metals are abundant (Knapp, 2012, and references therein). The positive correlation (Pearson, $\rho = 0.84$, $p < 0.05$, $n= 9$)

between benthic N_2 fixation and phosphate concentration in our study supports the view that phosphate plays a crucial role. It is not clear why the energetically expensive N_2 fixation occurs while sufficient DIN is available. This would be understandable when N_2 fixation would serve another function such as an electron sink as suggested by Tichi and Tabita (2000). Moreover, even at high bulk ammonium concentrations N_2 fixation may be favored in microzones that are depleted of ammonia for instance because ammonia oxidizers decrease the concentration of ammonium locally and there may be also other microorganisms that compete for this source of nitrogen.

In conclusion, N_2 fixation and denitrification were temporally and spatially separated. The former was highest in August in the DB station, a sandy area with low organic content, while the latter was high in May in the OG station, a muddy depression in the North Sea with high organic content. Nevertheless, both processes were more or less in balance. The rates of both processes coincided with the expression of the functional genes *nifH* and *nirS*, but not with the number of gene copies present. A high number of gene copies indicated the potential for N_2 fixation and denitrification but was not a good indicator of the actual process. N_2 fixation was mainly attributed to the anaerobic sulfate reducing bacteria. The functional gene representing denitrification, *nirS*, could not be assigned to a specific group of microorganisms.

Supplementary table

Table S1. List of *nifH* gene sequences from this study.

Group Name	Sequence sources	Gen Bank accession numbers
NS1	DC	KP959586
NS2	DC	KP959524, KP959534
NS3	DB	KP959426
NS4	DC	KP959560
NS5	DB,DC,WATER	KP959367*,KP959375,KP959387,KP959390,KP959549*,KP959597, KP959731
NS6	DC	KP959593
NS7	DC	KP959518, KP959560, KP959567, KP959569
NS8	DB	KP959422
NS9	WATER	KP959729
NS10	DB, OG,DC, WATER	KP959349-KP959356*, KP959359-KP959363, KP959369, KP959374-KP959375,KP959378, KP959381-KP959382, KP959384-KP959386, KP959389,KP959392-KP959394, KP959396-KP959397, KP959399, KP959402,KP959404-KP959406, KP959410-KP959411, KP959413-KP959416, KP959430-KP959431,KP959434-KP959436, KP959438-KP959440, KP959442-KP959445, KP959447, KP959449 -KP959467, KP959470-KP959473, KP959475-KP959478,KP959480, KP959483-KP959494, KP959497-KP959504, KP959506-KP959508, KP959510-KP959511, KP959513，KP959515, KP959522, KP959527, KP959536-KP95953, KP959539, KP959542, KP959545, KP959552-KP959553, KP959556, KP959564-KP959566, KP959568, KP959571, KP959577,KP959582, KP959584, KP959588, KP959590, KP959721-KP959722, KP959728-KP959729, KP959733, KP959349-KP959356, KP959359-KP959363, KP959369, KP959374-KP959375, KP959378, KP959381-KP959382, KP959384-KP959386, KP959389, KP959392-KP959394, KP959396-KP959397, KP959399, KP959402,KP959404-KP959406, KP959410-KP959411, KP959413-KP959416*, KP959430-KP959431,KP959434-KP959436,KP959438-KP959440, KP959442-KP959445, KP959447, KP959449-KP959467, KP959470-KP959473, KP959475-KP959478, KP959480*, KP959483-KP959494*, KP959497-KP959504, KP959506-KP959508, KP959510-KP959511, KP959513, KP959515, KP959522, KP959527, KP959536-KP959537, KP959539*, KP959542, KP959545, KP959552-KP959553, KP959556,KP959564-KP959566*, KP959568, KP959571, KP959577,KP959582, KP959584, KP959588, KP959590, KP959721-KP959722,KP959728-KP959729,KP959733*

NS11	DB	KP959378, KP959381-KP959382, KP959384-KP959386, KP959389
NS12	WATER	KP959723
NS13	WATER	KP959730
NS14	WATER	KP959724
NS15	OG,DC	KP959474*, KP959482, KP959495-KP959496, KP959509*, KP959516, KP959519,KP959523, KP959525, KP959528-KP959529, KP959533, KP959535, KP959543, KP959546-KP959548,KP959554-KP959555, KP959557-KP959559, KP959572*, KP959574- KP959576*, KP959578-KP959579, KP959581, KP959583*, KP959587*, KP959589*, KP959591-KP959592, KP959594-KP959595
NS16	DB	KP959365*, KP959368, KP959391, KP959398, KP959409, KP959419
NS17	DB, OG, DC	KP959366*, KP959377, KP959379, KP959417-KP959418*, KP959420-KP959421, KP959428, KP959433, KP959468, KP959526, KP959541, KP959596*
NS18	WATER	KP959732
NS19	DB, DC	KP959358*, KP959371-KP959372, KP959380*, KP959383, KP959387*, KP959395*, KP959407, KP959427, KP959520-KP959521
NS20	DC	KP959531
NS21	DB,DC	KP959423, KP959540, KP959563, KP959580
NS22	DB,OG, DC, WATER	KP959364*, KP959388, KP959400, KP959469, KP959505, KP959514, KP959530, KP959544, KP959550, KP959570, KP959573, KP959585*, KP959599- KP959600*, KP959719- KP959720, KP959725- KP959727*

* sequences detected in both DNA and cDNA. WATER indicates sequences from the water column

Chapter 6

Occurrence and activity of anammox bacteria in surface sediments of the southern North Sea

Nicole J. Bale,[1] Laura Villanueva,[1] Haoxin Fan,[2] Lucas J. Stal,[2,3] Ellen C. Hopmans,[a] Stefan Schouten,[1] Jaap S. Sinninghe Damsté[1]

[1]Department of Marine Organic Biogeochemistry, Royal Netherlands Institute for Sea Research, Den Helder the Netherlands
[2]Department of Marine Microbiology, Royal Netherlands Institute of Sea Research, Yerseke, The Netherlands
[3]Department of Aquatic Microbiology, Institute of Biodiversity and Ecosystem Dynamics, University of Amsterdam, The Netherlands

Published as: Bale NJ, Villanueva L, Fan H, Stal LJ, Hopmans EC, Schouten S *et al*. (2014). Occurrence and activity of anammox bacteria in surface sediments of the southern North Sea. *FEMS Microbiol Ecol* **89:** 99-110.

Abstract

We investigated the occurrence and activity of anaerobic ammonia oxidation (anammox) bacteria in sandy and muddy sand sediments of the southern North Sea. The presence of anammox bacteria was established through the detection of the specific phosphocholine monoether ladderane lipids, 16S rRNA and hydrazine synthase (*hzs*A) genes. Anammox activity was measured in intact sediment cores (*in situ* rate) and in sediment slurries (potential rate) as the rate of N_2 evolution from ^{15}N-labeled substrates and compared to the transcriptional activity of genes of anammox bacteria. The contribution of anammox to N_2 production ranged between 0 and 29%. The potential rate of anammox agreed well with the abundance of anammox bacteria 16S rRNA and *hzs*A gene copies and the transcriptional activity of the anammox bacteria 16S rRNA gene. We found a higher abundance and activity of anammox bacteria in sediments with higher organic carbon content and also activity was higher in summer than in winter. The abundance of anammox bacteria and their potential anammox rates were similar to those reported for other marine coastal sediments, suggesting that potentially they are important contributors to the nitrogen cycle in sandy sediments of shallow continental shelf areas.

Keywords: Lipid biomarkers, ladderane lipid, hydrazine synthase, nitrogen cycle, sandy sediments.

Introduction

Our understanding of the marine nitrogen cycle has been redefined in recent years by the discovery of anaerobic ammonia oxidation (anammox), carried out by bacteria belonging to the phylum *Planctomycetes* (Jetten *et al*., 2005). This unique process involves the oxidation of ammonium with nitrite to form dinitrogen gas (N_2), thus causing a loss of fixed nitrogen from the environment (Kuypers *et al*., 2003; 2005; Francis *et al*., 2007). Anammox bacteria are present and active in a wide range of marine environments including sediments from the Baltic Sea (Hannig *et al*., 2007), continental shelf and slope sediments from northwest Africa (Jaeschke *et al*., 2010), water column and sediments of oxygen minimum zones (OMZ) (Kuypers *et al*., 2003; Jaeschke *et al*., 2007; Lam *et al*., 2007; Jensen *et al*., 2008; Galan *et al*., 2009; Ward *et al*., 2009), fjord sediments (Risgaard-Petersen *et al*., 2004a; Gihring *et al*., 2010; Brandsma *et al*., 2011), estuaries (Tal *et al*., 2005; Trimmer *et al*., 2005; Li *et al*., 2011; Rooks *et al*., 2012;), eutrophic bays (Thamdrup and Dalsgaard, 2002; Dang *et al*., 2010a), deep ocean surface sediment (Hong *et al*., 2011), Arctic sediment (Rysgaard *et al*., 2004), Arctic sea ice (Rysgaard and Glud, 2004), and deep sea hydrothermal vents (Byrne *et al*., 2009).

Coastal shelf seas are major sinks for nitrogen and are estimated to account for up to 67% of global denitrification (Codispoti *et al*., 2001). Several studies have investigated the importance of the anammox process in areas such as the Skagerrak straight between the Baltic Sea and the North Sea (Dalsgaard and Thamdrup, 2002; Thamdrup and Dalsgaard, 2002; Engstrom *et al*., 2005), Long Island Sound, USA (Engstrom *et al*., 2005), in the Irish and Celtic Seas (Jaeschke *et al*., 2009; Trimmer and Nicholls, 2009), in the East China Sea (Song *et al*., 2013) and in the southern North Sea (Neubacher *et al*., 2011; 2013). Several environmental factors, such as temperature, total organic carbon (TOC) content, nitrite concentration, organic matter mineralization rate, water depth, oxygen saturation and sedimentology play a role in the distribution, abundance and activity of anammox bacteria in sediments (Dalsgaard and Thamdrup, 2002; Thamdrup and Dalsgaard, 2002; Engstrom *et al*., 2005; Dale *et al*., 2009; Jaeschke *et al*., 2009; Nicholls and Trimmer, 2009; Dang *et al*., 2010a; Jaeschke *et al*., 2010; Li *et al*., 2011; Neubacher *et al*., 2011). However, most of these studies have focused on fine grained sediments while the potential role of anammox bacteria in shallow sandy sediments, representing 70% of the continental shelf areas (Vance-Harris and Ingall, 2005), has received less attention. Furthermore, anammox bacteria were previously thought to only exist in stable, non-bioturbated, anoxic sediments, but recent studies have indicated their presence at high oxygen levels (up to 25 μM), and suggested that they could be active possibly by thriving in anaerobic microniches (Kuypers *et al*., 2005; Woebken *et al*., 2007). It has been suggested that bioturbation could even promote anammox bacteria by extending the area of nitrate reduction, thereby increasing the availability of nitrite (Meyer *et al*., 2005).

In this study, we investigated the seasonality of the abundance and activity of anammox bacteria in two sandy sediments and two muddy sand sediments of the southern North Sea, in order to evaluate the potential importance of anammox in these sediments. Our study site, the southern North Sea (Fig. 1), is a shallow (generally maximum 50 m depth) sea bounded in the north by the Dogger Bank (DB; characterized by sandy sediment) and in the east by the Dutch coast (DC; also sandy and shallow). Between these two regions there is a depression in the seabed known as the Oyster Ground (OG), which is a temporary deposition center for sediment (van Raaphorst *et al*., 1998) and plays an important role in the carbon and nitrogen cycles in the region (Weston *et al*., 2008). At the southeastern edge of the OG is the Frisian Front, a transition zone between the shallow DC and the deeper OG. Here, particulate matter from the sandy Southern Bight, which is transported by the anticlockwise currents caused by incoming Atlantic water through the English Channel (van Engeland *et al*., 2010), deposits, resulting in muddy sand with high amounts of organic matter that supports abundant benthic fauna (Creutzberg, 1986; van Raaphorst *et al*., 1992). A previous study by Neubacher *et al*. (2011) measured the anammox process at three sites in the southern North Sea using ^{15}N labeling of intact cores. They concluded that the anammox process accounted for 10–20% of the total N_2 production and that it was highest during late summer and early autumn. Here, we determined the abundance and activity of anammox bacteria in the surface sediment of four locations in the southern North Sea and during four seasons. We applied a more extensive approach than Neubacher *et al*. (2011) since we used a combination of intact polar lipid (IPL) analysis, nucleic acids (DNA/RNA), and ^{15}N isotope labeling. The abundance of anammox bacteria was measured through the quantification of specific phosphocholine (PC)-monoether ladderane lipid (Boumann *et al*., 2006; Jaeschke *et al*., 2009), as well as copy numbers of the anammox bacteria 16S rRNA and the hydrazine synthase, *hzs*A, genes (Wang *et al*., 2012). The activity of anammox bacteria was assessed by quantifying the gene transcripts of anammox bacteria 16S rRNA and *hzs*A genes, as well as the rate of anammox measured as the production of N_2 in sediment slurries as well as in intact cores. Our results provide solid evidence for the presence and activity of anammox bacteria in sandy and muddy sand sediments of the southern North Sea.

Material and methods

Cruises and study site.

Sampling was carried out during four cruises on board the *R/V* Pelagia; in November 2010, February 2011, May 2011 and August 2011. The cruises followed the 'Terschelling transect' (Peeters and Peperzak, 1990), which runs from the North Sea barrier island Terschelling to 235 km offshore and sampling was performed at four stations: the Dogger Bank (DB), the Oyster ground (OG), the Frisian Front (FF) and at the Dutch coast (DC) (Fig. 1). The stations were also sampled for sediment characteristics: grain size, total organic carbon (TOC) as well as pore water nutrients

(except station FF).

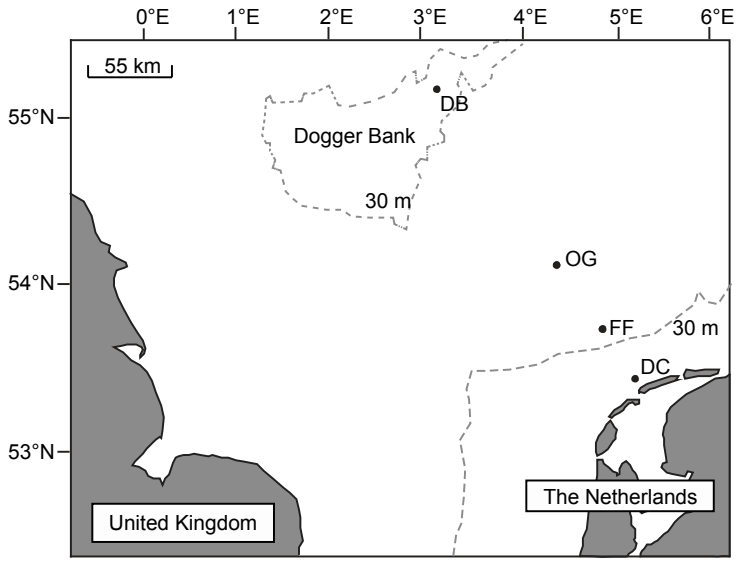

Figure 1. Map of the study site the southern North Sea. Stations labels: the Dogger Bank (DB), the Oyster ground (OG), the Frisian Front (FF) and the Dutch coast (DC). Approximate bathymetry at 30 m illustrated with gray dashed line.

Physicochemical conditions.

The temperature and salinity of the bottom water were determined using a conductivity–temperature–depth system attached to a rosette sampler containing 24 × 12 L Niskin bottles. Pore water collection was done in a thermally controlled mobile laboratory (container) maintained at *in situ* temperature. Sediment cores were collected using a multicorer and sliced using an automatic hydraulically operated slicer. Pore water was extracted from 2.5-mm sediment slices from 0 to 1 cm depth by centrifugation (Centrikon T-42K, Germany) for *c*. 4000 g, for 5 min through a 0.45 µm 25 mm Acrodisc HT Tuffryn Membrane syringe filter and subsequently stored in the dark at 4°C in polyethylene vials. Nutrient concentrations were analyzed on board within 18 h on a QuAAtro autoanalyzer. Samples not analyzed onboard were frozen at -18°C except those for silicate analysis, which were stored at 4°C. The standard deviation of samples measured between different runs was: PO_4^{3-} 0.007 µM; NH_4^+ 0.013 µM; $NO_3^- + NO_2^-$ 0.041 µM and NO_2^- 0.005 µM. For total organic carbon (TOC) analysis, sediment was freeze-dried and subsequently decalcified with a 2 N HCl solution for *ca*. 18 h. The sediment was then washed three times with double-distilled water before the sediment was freeze-dried again. Analysis was carried out using a Flash 2000 series Elemental Analyzer (Thermo Scientific) equipped with a TCD detector.

IPL analysis.

Sediment for IPL analysis was collected at each station using 10-cm-diameter, 60-cm-length multicores or push cores into box cores. The cores were sliced into 1 cm slices using a hydraulic or manually operated slicer. Each slice was stored separately in a geochemical bag and immediately frozen at –80°C. For this study, we focused on the 0–1 cm (surface sediment) layer. Upon arrival in the laboratory, sediment samples were stored at –40ºC until they were freeze-dried and extracted. After freeze-drying, the sediment samples were homogenized. The extractions were carried out using a modified Bligh Dyer extraction (Pitcher *et al*., 2011). Briefly, sediment was extracted in methanol (MeOH): dichloromethane (DCM): phosphate buffer (2:1:0.8, v:v:v) using an ultrasonic bath for 3 × 10 min. Between sonication the extract and residue were separated by centrifugation (Froilabo SW12, France) at 1,000 *g* for 2 min. DCM and phosphate buffer were added to induce phase separation and the extracts were centrifuged again at 1,000 × *g* for 2 min. The DCM phase was collected and the MeOH/phosphate buffer phase was washed twice with DCM. The combined DCM phases were concentrated under rotary vacuum and evaporated to dryness under a stream of N_2. Detection and quantification of the C_{20}-[3]-monoether ladderane lipid with a phosphocholine (PC) head group (hereafter referred to PC-monoether lipid) was carried out as described previously (Jaeschke *et al*., 2009) on an Agilent (Palo-Alto, CA, US) 1100 series LC equipped with a thermostated autoinjector, coupled to a Thermo TSQ Quantum EM triple quadrupole mass spectrometer equipped with an Ion Max source with ESI probe. Separation was achieved on a LiChrospher diol column (250 × 2.1 mm, 5 µm particles; Alltech) maintained at 30°C. For details of gradient, source parameter optimization and ESI setting see Jaeschke *et al*. (2009).

Nucleic acid extraction

The top layer (0-1 cm) of the sediment core was sampled at the same time and under the same conditions as those used for lipid analysis and stored at –80°C. DNA and RNA from the top layer of the sediment core were extracted by using DNA or RNA PowerSoil® Total Isolation Kit, respectively (Mo Bio Laboratories, Inc., Carlsbad, CA). Nucleic acid concentrations were quantified spectrophotometrically (Nanodrop, Thermo Scientific, Wilmington, DE, USA) and checked by agarose gel electrophoresis for quality. Extracts were kept frozen at –80°C. The RNA extracts were treated with RNase free DNase (DNA-*free*™, Ambion Inc., Austin, TX). The quality and concentration of RNA were estimated with the Experion RNA StdSens Analysis Kit (Bio-Rad Laboratories). DNA contamination was checked by PCR using RNA as a template.

Reverse transcription PCR

Reverse transcription (RT) was performed with an Enhanced Avian First Strand synthesis kit (Sigma-Aldrich Co., St Louis MO) using random nonamers as described

previously (Holmes *et al*., 2004). Two negative controls lacking either reverse transcriptase or RNA were included. PCRs were performed as previously described by Pitcher *et al*. (2011) to confirm the transcription to complementary DNA (cDNA) and the negative controls using the product of the RT reaction as a template.

Quantitative PCR (qPCR) analysis

qPCR analyses were performed on a Biorad CFX96^{TM} Real-Time System/C1000 Thermal cycler equipped with CFX Manager^{TM} Software. The copy numbers of anammox 16S rRNA and hydrazine synthase (*hzs*A) genes were estimated by using primers Brod541F/Amx820R (Li *et al*., 2010) and *hzs*A_1597F/*hzs*A1857R (Harhangi *et al*., 2012) respectively. PCR products were purified, cloned and sequenced as described in Bale *et al*. (2013). The specificity of the anammox bacteria 16S rRNA and the hydrazine synthase (*hzs*A) gene primer pairs were tested by sequencing the amplification product obtained from the surface sediment of station OG in August, which gave 16S rRNA gene sequences closely related to *Candidatus* Scalindua sp., and *hzs*A protein sequences 99% identical to *hzs*A protein sequences of uncultured anammox bacteria recently detected in cold hydrocarbon-rich seeps and hydrothermal vent sediments (Russ *et al*., 2013). All qPCRs were carried out in triplicate using standard curves from 10^0 to 10^7 molecules per microliter. Standard curves were generated as described previously (Pitcher *et al*., 2011) by using either anammox 16S rRNA or *hzs*A gene fragments amplified from genomic DNA extracted from the samples used in this study, cloned and confirmed by sequencing. Gene copies were determined in triplicate on diluted DNA extracts or cDNA. The reaction mixture (25 µl) contained 1 U of Pico Maxx high fidelity DNA polymerase (Stratagene, Agilent Technologies, Santa Clara, CA, USA) 2.5 µl of 10x Pico Maxx PCR buffer, 2.5 µl 2.5 mM of each dNTP, 0.5 µl BSA (20 mg ml^{-1}), 0.02 pmol µl^{-1} of primers, 10,000 times diluted SYBR Green® (Invitrogen) (optimized concentration), 0.5 µl 50 mM of $MgCl_2$ and ultra-pure sterile water. All reactions were performed in iCycler iQ^{TM} 96-well plates (Bio-Rad, Hercules CA, US) with optical tape (Bio-Rad). One µl of diluted DNA or cDNA was added to 24 µl of mix in each well. Specificity of the reaction was tested by a gradient melting temperature assay. The cycling conditions for the qPCR reaction were as follows: 95°C, 4 min; 40–45× [95°C, 30 s; Tm, 40 s; 72°C, 30 s]; final extension 80°C, 25 s. Specificity for qPCR reaction was tested on agarose gel electrophoresis and with a melting curve analysis (50°C–95°C; with a read every 0.5°C held for 1 s between each read) in order to identify unspecific PCR products such as primer dimers or fragments with unexpected fragment lengths. Melting temperatures (Tm), PCR efficiencies (*E*) and correlation coefficients for standard curves are listed in Table S1.

Anammox rate measurements

Experiments were performed at stations DB, OG and DC, incubating the top 10 cm of the sediments. We used sediment slurry incubations according to (Thamdrup and

Dalsgaard, 2002) for measuring potential anammox rates. For *in situ* rates of anammox we incubated intact cores (Risgaard-Petersen *et al*., 2003). Sediment from the top 10 cm was homogenized to slurry using equal amounts of wet sediment and artificial seawater (NaCl 20.5 g, Na_2SO_4 3.4 g, KCl 0.58 g, KBr 0.084 g and H_3BO_3 0.022 g in 1000 ml Milli-Q water) of which 1 ml aliquots were added to 12.6 ml Exetainers (Labco, Ceredigion, UK). The slurries were pre-incubated for 12 h in the dark in order to eliminate the background nitrate/nitrite (Dalsgaard and Thamdrup, 2002; Risgaard-Petersen *et al*., 2004a). Then four treatments were carried out with these slurries: 1) addition of 100 µM $Na^{15}NO_3$ (98.5%, ^{15}N atom%; Sigma-Aldrich); 2) addition of 100 µM $^{15}NH_4Cl$ (99.2%, ^{15}N atom%; Sigma-Aldrich); 3) addition of 100 µM $^{15}NH_4Cl$ and 100µM $Na^{14}NO_3$; 4) no addition. All ^{15}N solutions were pre-flushed with N_2 and filled into Exetainers taking care that no headspace was left. The slurries were handled in a glove bag under an N_2 atmosphere and at bottom water temperature (the laboratory container was acclimatized according to the season). Slurries were incubated in the dark at bottom water temperature for 24 h. Exetainers were sacrificed at 4 h intervals over a 24 h period. For each time point and treatment the incubation was stopped in two Exetainers by injecting 200 µl 50% w/v $ZnCl_2$. The anammox rate and the potential contribution of anammox to the production of N_2 was derived from treatment (1) (Dalsgaard and Thamdrup, 2002; Risgaard-Petersen *et al*., 2004a), while the treatments (2) and (3) were used as controls to confirm the occurrence of anammox. The ^{15}N atom % of NO_3^- in the sediment incubation was determined by concentration difference, with and without NO_3^- addition, and corrected for the ^{15}N atom % of stock solutions.

For intact core incubations, the top 10 cm of the sediment was sampled from box-cores using custom-made cores (core tube: i.d. 50 mm, height 200 mm). We carried out two sets of triplicate incubations. Ten centimeters of the bottom water was added on top of the sediment. In one set of incubations the bottom was enriched water with 100 µM ^{15}N-nitrate. After the addition of the tracer, the overlying water was pre-incubated for 30 min while stirring to allow equilibration with the sediment pore water. Subsequently, the cores were sealed with rubber stoppers making sure that no headspace was left. The cores were incubated in the dark for 24 h at temperatures similar to those of the bottom waters from where the sediments were recovered. Data on oxygen consumption for August (not shown) indicated that after 24 h the maximum dissolved oxygen depletion was 35% of air equilibration and thus the cores were unlikely to have become anoxic.

After incubation, the cores were inversed 5 times in order to mix the water phase above the core and allowed to stand for 15 min before the overlying water was collected and the activity stopped in a 12.6 ml helium-flushed Exetainer containing 100 µl of 50% (w/v) $ZnCl_2$. The completely filled Exetainers were capped leaving no headspace and the isotopic composition of the N_2 analyzed back in the laboratory (see

below). The core set with no additions was used for determining the natural ^{15}N background.

The isotopic composition of dissolved dinitrogen (N_2) was determined by introducing a helium headspace in the sample vials (2 ml He headspace in the 12.6 ml vials). Subsequently, the headspace was sampled and analyzed by an EA-IRMS (DELTA V Advantage; Thermo Fisher Scientific, Bremen, Germany) equipped with a Haysep Q column. The potential rate of the anammox process in the slurry incubations was calculated from the production of $^{29}N_2$ and $^{30}N_2$ in treatment with $^{15}NO_3$- by using the equations of (Thamdrup and Dalsgaard, 2002). For the intact core incubation, the total *in situ* N_2 production was measured according to (Risgaard-Petersen *et al*., 2003) and the contribution of anammox was estimated from the slurry incubations.

Results

Physicochemical conditions

Bottom water temperature varied between 4.9 and 18.3°C and bottom water salinity ranged between 30.1 and 34.9 practical salinity units (psu). The sediment from DB and DC was composed mainly of fine and medium sandy particles (M. Le Guitton, pers. comm.) and were low in total organic carbon (TOC; 0.03%) (Table 1). The sediment at OG and FF, while still sandy, contained a higher proportion of clay and silt particles and TOC than DB and DC (0.30 and 0.46% TOC, respectively, Table 1). The TOC values at each station were similar throughout the year varying by, on average, 0.03% in total (data not shown). Ammonium concentrations in the pore water of the top 1 cm (average of 4 × 2.5 mm resolution measurements) ranged between 2.3–14 μM and were highest in February at DB and in August at OG and FF (Table 2). Pore water nitrate was between 5–44 μM and was also highest in February at DB (22 μM), while it peaked in May at OG (20 μM) and February at DC (44 μM). Pore water nitrite was highest in August in all three stations and ranged between 0.2–2 μM. The concentration of phosphate in the pore water was fairly constant between stations and seasons falling in the narrow range of 0.9–1.9 μM with the exception of DB in August when 3.9 μM was measured. Silicate concentrations in the pore water was more variable and ranged from 0.3–59.9 μM. In all stations the highest concentrations of silicate were measured in August. Oxygen penetration measurements could not be completed due to significant probe damage from shells in the sediment. The single measurement was from OG in November, which indicated an oxygen penetration depth of 4–7 mm (F. Meysman, pers. comm.).

Table 1. Properties of sediment, location and water depth of stations. DB = Dogger Bank, OG = Oyster Ground, FF = Frisian Front, DC = Dutch Coast. Including data previously reported in Bale *et al*. (2013).

	DB	OG	FF	DC
% Clay and silt (< 63 μm)	0	30	35	0
% Very fine sand (63-125 μm)	6	48	32	1
% Fine sand (125-250 μm)	63	23	28	58
% Medium sand (250-500 μm)	31	0	6	41
TOC %	0.03	0.30	0.46	0.03
Median grain size (μm)	210	87	93	235
Water depth (m)	31	49	38	9
Latitude (decimal deg)	55.170	54.130	53.767	53.401
Longitude (decimal deg)	3.150	4.330	4.767	5.150

Anammox bacteria abundance

The PC-monoether lipid was detected at all four stations and in all seasons, ranging between 0.05–2.4 ng g^{-1} sediment (Fig. 2d; Table S2). Anammox 16S rRNA gene abundance ranged from below detection level (*c*.100 copies g^{-1}) to 6.2×10^7 copies g^{-1} at stations DB and DC (Fig. 2a). The anammox bacteria functional gene, hydrazine synthase (*hzs*A), was only detected in sediments at stations OG and FF (detection limit *c*.100 copies g^{-1}), in which it was approximately an order of magnitude lower in abundance (4.2×10^5–1.4×10^6 copies g^{-1}) than the anammox bacteria 16S rRNA gene copy number (Fig. 2c).

Anammox bacteria transcriptional activity and anammox rate measurements

Anammox 16S rRNA gene transcripts were only detected in FF in November, February and August, in OG in February and August and in DB in August. The 16S rRNA gene transcript abundance ranged from 3.0×10^3 to 1.5×10^7 g^{-1} (Fig. 2). *hzs*A gene transcripts were not detected in any station or during any season. N_2 generation by anammox was undetected when measured using intact sediment cores *in situ* at DB, OG and DC, in November or in May at DB (Fig. 2). In all other cases, the *in situ* anammox rates were between 0.3 and 26 μmol N m^{-2} day^{-1} (Fig. 2e). The potential N_2 production attributed to anammox bacteria, as measured by slurry incubations, varied from 0.5 to 3.0 nmol N cm^{-3} h^{-1} (Fig. 2f) with the highest rates measured at OG in any season, while it was undetectable at BD in November and May. The relative contribution of anammox to total N_2 production (from anammox and denitrification) ranged from 0 to 29% (Table 3) and was on average highest at station 4 and on average highest in February.

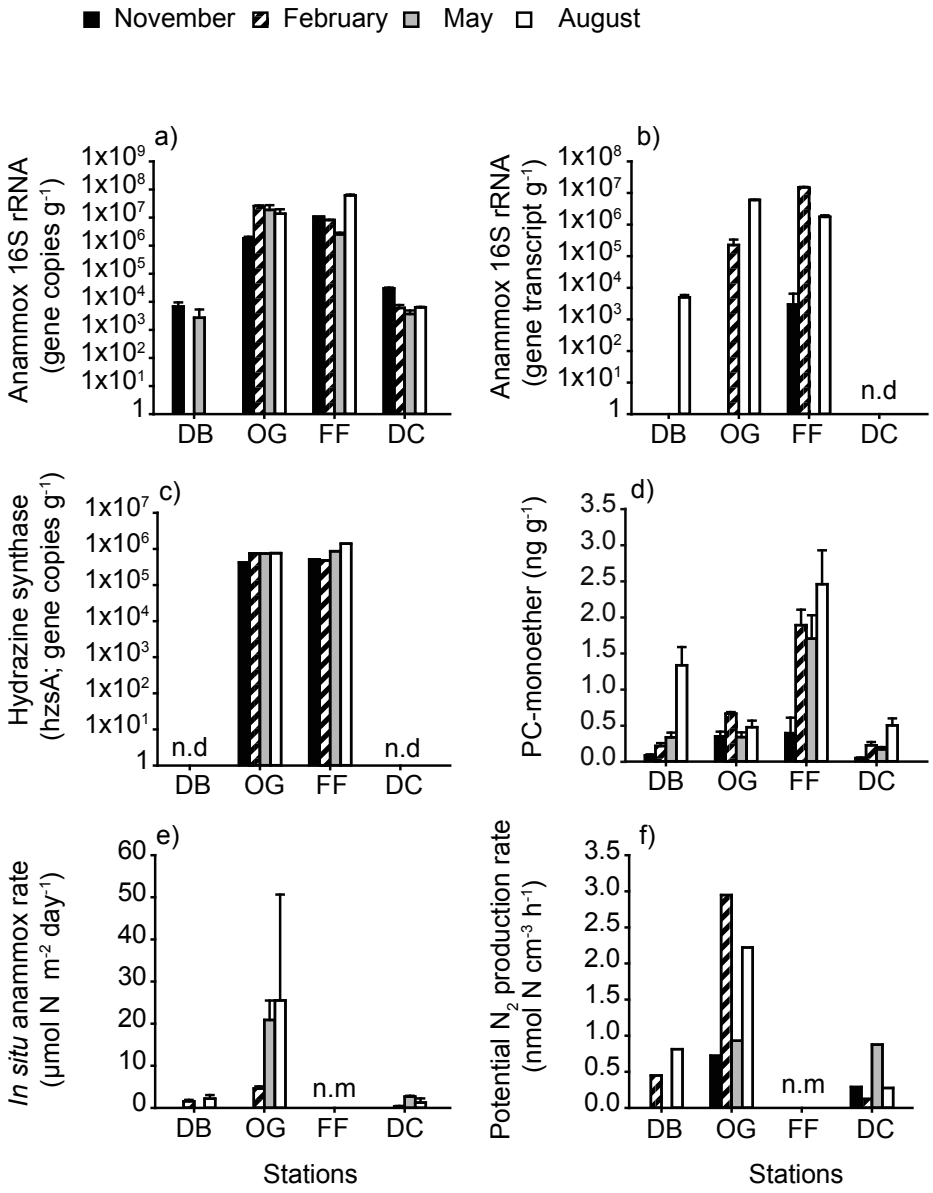

Figure 2. Abundance of anammox bacteria markers at the Dogger Bank (DB), the Oyster ground (OG), the Frisian Front (FF) and the Dutch coast (DC) stations in different seasonal campaigns. a) abundance of anammox bacteria 16S rRNA gene copies per gram of sediment; b) abundance of anammox bacteria 16S rRNA gene transcripts per gram of sediment, c) abundance of hydrazine synthase (*hzs*A) gene copies per gram of sediment, d) concentration of PC-monoether lipid in ng per gram of sediment, e) *in situ* anammox rate in µmol N m^{-2} day^{-1} and f) the potential N_2 production rate from anammox in nmol N cm^{-3} h^{-1}. Note the logarithmic scale in a), b) and c). Error bars give standard deviation of replicate analysis

Table 2. Properties of pore water at stations. DB = Dogger Bank, OG = Oyster Ground, FF = Frisian Front, DC = Dutch Coast. n.m. = not measured. Including data previously reported in Bale *et al.* (2013).

	November				February				May				August			
	DB	OG	FF	DC	DB	OG	FF	DC	DB	OG	FF	DC	DB	OG	FF	DC
Ammonium (µM)	3.0	6.0	n.m	8.2	7.4	3.1	n.m	6.2	2.3	9.4	n.m	10.2	4.6	11.4	n.m	14.5
Nitrate (µM)	5.3	18	n.m	21.9	22.3	16.9	n.m	44.2	8.7	20.3	n.m	11.8	14.7	8.4	n.m	6.8
Nitrite (µM)	0.3	0.5	n.m	0.4	0.3	0.2	n.m	0.6	0.4	0.4	n.m	1.5	0.5	0.9	n.m	1.7
Phosphate (µM)	1.0	1.4	n.m	1.3	1.5	1.5	n.m	1.0	1.9	1.7	n.m	1.5	3.9	1.4	n.m	0.9
Silicate (µM)	0.3	43.2	n.m	0.6	0.5	26.6	n.m	24.9	8.0	42.3	n.m	8.3	11.1	56.0	n.m	59.9
Temperature (°C)	8.9	10.3	10.1	7.9	5.2	5.0	5.0	4.9	11.4	8.6	12.1	14.0	15.4	15.4	16.9	18.3
Salinity (psu)	34.6	34.5	34.6	31.7	34.8	34.4	34.5	30.1	34.9	34.3	34.6	32.2	34.7	34.7	34.4	31.7

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Table 3. Seasonal relative contribution (in %) of anammox to the total production of N_2. DB = Dogger Bank, OG = Oyster Ground, DC = Dutch Coast. n.d. = not detected.

Station	November	February	May	August	Station average
DB	n.d	18	0	14	11±9
OG	18	29	10	16	18±8
DC	10	20	7	9	12±6
Seasonal average	14±6	22±6	6±5	13±4	

Sandy sediments are potentially permeable to advection, and this could affect the determination of anammox rates. The sediments were analyzed for signs of advection. Although the high median grain size at DC (235 μm) suggests that advection may occur in the sediment at this station, the sediment composition was nonhomogenous, which would impede advection (the relative content of fine sand was 58%, while 41% was medium sand; Rocha *et al.* (2005); Table 1). The pore water concentration profile of silicate also argued against advection. The pore water concentration profile of silicate is governed by the ratio of chemical dissolution to transport. Therefore, the silicate concentration profile near the sediment surface can serve as an indicator of enhanced transport in the surface sediment. The depth profile of the silicate concentration in the pore water at DC (not shown) was convex rather than sigmoidal, indicating that no significant advection occurred. In this context, the quote of Rocha *et al.* (2005) is notable. These authors examined the permeability of sandy sediment from the southern North Sea at a station close to DC and wrote 'very low permeability of the topmost 2 mm sediment effectively clogs the inter- face, making this particular stretch of bottom sediment impervious to advection'. Hence, we conclude that this sediment was not permeable to advection and that the incubation conditions used to determine the rate of anammox as used here were appropriate.

Spearman rank correlations

Spearman rank correlation coefficients were calculated for the relationships between anammox markers and with environmental parameters (Table 4). Of the pore water nutrient concentrations only silicate exhibited a correlation with the anammox markers greater than 0.6 (Table 4). Sedimentary characteristics, such as grain size and TOC content, and the water depth also exhibited some correlation with two anammox markers.

Table 4. Spearman rank correlation coefficients (ρ) for the anammox markers with each other and with environmental parameters carried out over the whole data set (n=16 except where n=12, indicated with an *) showing statistical correlations ($P \leq 0.01$ indicated in bold and $P \leq 0.05$ indicated in italics). Only coefficients higher than 0.6 are reported.

Anammox markers	*hszA*	Lipid	*In situ* anammox rate	Potential N_2 production rate	Season	Pore water silicate	Grain size	% clay and silt	% very fine sand	% fine sand	% medium sand	TOC	Water depth
16S rRNA gene abundance	**0.88**						**-0.73**	**0.81**	**0.73**	**-0.85**	**-0.73**	**0.81**	**0.73**
16S rRNA transcriptional activity		**0.69**	*0.63**	*0.66**									
hszA gene abundance		**0.67**	*0.60**	**0.76***		*0.61**	**-0.80**	**0.90**	**0.80**	**-0.80**	**-0.80**	**0.90**	**0.80**
Lipid concentration						**0.71***		**0.70**				**0.70**	
In situ anammox rate				**0.85***	*0.62**								
Potential N_2 production rate								**0.72***		*-0.69**		**0.72***	

TOC (total organic carbon).

Discussion

Comparison of different anammox markers

Some differences were detected between anammox bacterial abundance and activity markers, which became apparent when they were correlated with each other using Spearman rank correlation (Table 4). The abundance of anammox bacteria 16S rRNA gene transcripts and the specific anammox bacteria metabolic gene hydrazine synthase correlated positively with the *in situ* rate of anammox and with the potential N_2 production from slurry incubations. However, despite the good correlations between the *in situ* rate of anammox and the potential N_2 production by the anammox process with the 16S rRNA and *hsz*A gene abundance, it should be noted that, for technical reasons, the anammox rate measurements were done using the top 10 cm of the sediment and not the 0–1 cm sample depth as for the other analyses. Furthermore, comparison of the two types of anammox rate measurements that we applied should be made with caution since the potential N_2 production rates were obtained from mixed slurry, while the *in situ* anammox rates were obtained from intact cores.

The PC-monoether lipid correlated to anammox bacterial transcriptional activity, both of the abundance of anammox bacteria 16S rRNA gene (ρ = 0.69, P ≤ 0.01, n=16) and of the *hsz*A gene (ρ = 0.67, P ≤ 0.01, n=16). However in some cases, such as the sandy sediments of the DB and DC (Fig. 2), the PC-monoether lipid and anammox bacteria gene markers remained stable over the seasons while the transcriptional activity decreased, suggesting that the anammox bacteria may have been dormant or otherwise inactive. Alternatively, the lipid and DNA biomarkers may have partially originated from fossil remains in the sediment. Brandsma *et al*. (2011) already considered this possibility when observing an increase in the amount of PC-monoether ladderance lipid per anammox cell with sediment depth in Gullmar Fjord marine sediments. Hence, the authors cautioned that due to the lyso-nature of this intact polar lipid (IPL) the PC-monoether ladderane lipid may represent a degradation product formed from a non-lyso-IPL during cell lysis or by diagenesis.

Spatial and seasonal variability of anammox bacteria

The presence of the PC-monoether lipid, the anammox bacterial 16S rRNA and *hzs*A genes unambiguously shows that anammox bacteria were present in all four sediments of the southern North Sea. We note that abundances based on anammox bacteria 16S rRNA and *hzs*A copy numbers differ by one order of magnitude. This discordance between the anammox bacteria gene markers has been previously observed in environmental samples (Harhangi *et al*., 2012) and could be due to primer biases. Intriguingly, *hzs*A gene transcriptional activity was under the limit of detection of our analysis in all sediment tested. This suggests that the regulation of the *hzs*A gene is not directly coupled to an active anammox process and we could speculate that the expression of *hzs*A gene is low and constitutive with independence of changes in the anammox reaction. However, further studies would need to clarify this point.

Table 5. Overview of reported *in situ* anammox rates, potential N_2 production from anammox rates and the relative contribution of anammox to N_2 production in marine sediments.

Study	*In situ* anammox rate (μmol N m^{-2} day^{-1})	Potential N_2 production rate (nmol N cm^{-3} h^{-1})	PC-monoether lipid (ng g^{-1})	Relative contribution of anammox to N_2 production
Arctic fjord sediments from Svalbard, Norway (Gihring *et al*., 2010)	10–26	0.08–0.42	n.r.	5 - 23
Coastal station, Gulf of Finland (Hietanen and Kuparinen, 2008)	10–30	n.r.	n.r.	0 - 15
Randers Fjord and Norsminde Fjord, Denmark (Risgaard-Petersen *et al*., 2004a)	0–504†	3.8–11	n.r.	5 - 24
Continental shelf and slope sediments, North Atlantic (Trimmer & Nicholls, 2009; Jaeschke *et al*., 2009)	2.6–60	n.r.	0–0.04	15 - 65
Marine sediments of the Gullmar Fjord, Sweden (Brandsma *et al*., 2011)	n.r.	< 0.5–7.6*	2.0–3.0**	23 - 47
Sediment from a subtropical tidal river system (Meyer *et al*., 2005)	n.r.	0.5–8	n.r.	0 - 9
Arctic marine sediments (Rysgaard *et al*., 2004)	1–92	0.15–15.1	n.r.	1 - 35
Sandy sediments, southern North Sea (Neubacher *et al*., 2011)	4.8–137	n.r	n.r	10 - 20
Sandy sediments, southern North Sea (This study)	0–26	0–3	0.1–2.4	0 - 29

† Calculated with the revised isotope pairing technique (Risgaard-Petersen *et al*., 2004a). n.r. = not reported. *Reported based on assumption that 1 g sediment = 1 cm^3. **Value range for the upper 2 cm only.

The *in situ* rates of anammox (0–26 μmol m^{-2} day^{-1}) were similar to those measured in other coastal marine sediments (Table 5; See Trimmer and Engstrom (2011) for reported anammox rates in a wider range of environments). Even in the low TOC (0.03%), sandy sediments from stations DB and DC the anammox rates were as high as 2.6 μmol m^{-2} day^{-1}. The potential N_2 production by anammox bacteria as measured in slurry incubations in this study (0–3 nmol N cm^{-3} h^{-1}) and the relative contribution of anammox to the total N_2 production (which includes denitrification) (Table 3) were also in a similar range as those reported for other marine sediments (Table 5). The concentration of the PC-monoether lipid compared well with a previous study of marine sediments of the Gullmar Fjord, Sweden (Brandsma *et al*., 2011) but were much higher than those reported for continental shelf and slope sediments from the North Atlantic (Jaeschke *et al*., 2009) (Table 5). These results suggest that anammox bacteria are as abundant and active as in other marine sediments and may be an important component of the sedimentary nitrogen cycle in the southern North Sea.

Regarding the spatial variability, three of the anammox bacteria markers were on average higher at the two muddy sand stations, FF and OG, than at the two sand stations DB and DC: the concentration of the anammox bacterial 16S rRNA gene abundance (t-test, p= <0.001), the *hsz*A gene abundance (t-test, p= <0.001), and the PC-monoether lipid concentration (t-test, p= 0.015). Although not carried out at FF, the potential N_2 production by anammox in slurry was on average higher at OG relative to DB and DC (t-test, p= 0.016).

Seasonality in the abundance and activity of anammox was not distinct and disagreed between the different markers. However, when all markers for activity (the *in situ* anammox rate, the potential N_2 production rate and the 16S rRNA transcriptional activity) were examined together (n=10) August and November were statistically different (paired t-test, p=0.008), with the majority of activity markers being higher in August than in November. This suggests that the anammox bacterial community in the surface of the bottom sediments of the shallow coastal North Sea is influenced by seasonality, with the highest activity in summer. This result corroborates that of Neubacher *et al*. (2011) who found that production of N_2 gas by anammox process was maximum at OG in summer and early autumn.

Factors controlling the abundance and activity of sedimentary anammox bacteria

The presence and activity of anammox in sediment is dependent on a range of environmental variables, a number of which, e.g. competition or synergism with other microorganisms (Ding *et al*., 2013), were not measured in this study. Here, we examined the impact of environmental variables by correlating the different anammox bacteria markers with environmental variables (Table 4). Almost no significant Spearman rank correlations were observed between the six markers for anammox bacteria and the pore water nutrient concentrations for the upper 1 cm of sediment,

suggesting that the concentration of nutrients in the pore water did not affect the abundance or activity of the anammox bacteria (Table 4). Only the *hzs*A gene abundance and the lipid abundance correlated with the concentration of silicate (ρ = 0.61, P ≤ 0.05 and ρ = 0.71, P ≤ 0.01 respectively, both n=12) for reasons that are not clear. Temperature is another important factor controlling anammox activity (Dalsgaard and Thamdrup, 2002). However, we did not find a correlation of the anammox bacteria markers with temperature, neither when all data were examined as a set nor when the four stations were examined separately (Table 4). In contrast, the anammox bacteria 16S rRNA and *hsz*A gene abundances correlated well with sedimentary characteristics, for example the *hsz*A gene abundance and the percentage of clay and silt in the samples (ρ = 0.90, P ≤ 0.01 n=16). The PC-monoether lipid and the potential N_2 production from the anammox process as measured in slurry correlated with the percentage of clay and silt and the TOC. Water depth correlated with abundance of both the anammox bacteria 16S rRNA and the *hzs*A genes (ρ = 0.73 and ρ = 0.80 respectively, both P ≤ 0.01, n=16), but neither bottom water temperature nor salinity correlated with the anammox bacteria markers. This suggests that sediment characteristics rather than pore water or bottom water conditions play a role in determining the abundance and control the activity of anammox bacteria in the surface sediments of the southern North Sea.

A study of anammox along the Thames Estuary also showed a correlation between the potential N_2 production from anammox as measured in slurry and the TOC contents of the sediment (Trimmer *et al*., 2003). The authors of that study proposed that this correlation could be attributed to the increased organic carbon deposition, which would lead to an increased sedimentary reactivity causing increased nitrite production. Indeed, the enhanced deposition of organic matter at OG and FF would also stimulate processes such as denitrification and dissimilatory nitrate reduction to ammonium in deeper anoxic sediments producing nitrite in the pore waters. Several other studies concluded that the nitrite supply controls the abundance of anammox bacteria in marine sediments (Risgaard-Petersen *et al*., 2004a; Rysgaard *et al*., 2004; Meyer *et al*., 2005; Dang *et al*., 2010a) . While we did not find a correlation between nitrite concentration and the *in situ* rate of anammox, we expect that increased microbial activity would play a role in determining anammox activity.

Conclusion

Our results reveal the presence and activity of anammox bacteria in different sediments of the southern North Sea. The spatial and seasonal variability in the abundance and activity of anammox bacteria are most likely linked to the sediment characteristics, in particular to the TOC content. The anammox rates and contribution to total N_2 production are similar to those measured in other studies focusing on different sediment types, suggesting that anammox may be an important component of the sedimentary nitrogen cycle in the North Sea bottom sediments. Because bottom

sediments of shallow coastal shelf seas are mainly composed of sand, we conclude that anammox is an important process in the sedimentary nitrogen cycle of this type of environment

Supplementary tables

Table S1. Melting temperatures (Tm), PCR efficiencies (*E*) and correlation coefficients of quantitative PCR standard curves.

qPCR assay	Tm	*E*	R^2
Anammox 16S rRNA Brod541F/Amx820R	59	97.7	0.995
Hydrazine synthase *hzs*A_1597F/*hzs*A1857R	51	80	0.997

Table S2. Anammox markers. DB = Dogger Bank, OG = Oyster Grounds, FF = Frisian Front, DC = Dutch Coast. n.m. = not measured. n.d. = not detected.

	PC-monoether lipid (ng g^{-1})	**Anammox 16S rRNA gene (10^4 copies g^{-1})**	**Anammox 16S rRNA gene transcripts (RNA) (10^4 copies g^{-1})**	**Hydrazine synthase *hzs*A gene (10^3 copies g^{-1})**	***In situ* anammox rate (µmol N m^{-2} day^{-1})**	**Potential N_2 production rate (nmol N cm^{-3} h^{-1})**
November						
DB	0.1 ± 0.0	0.7 ± 0.3	n.d.	n.d.	n.d.	n.d.
OG	0.3 ± 0.1	180 ± 25	n.d.	420 ± 1	n.d.	0.7
FF	0.4 ± 0.1	1070 ± 0.2	0.3 ± 0.4	510 ± 1	n.m	n.m
DC	0.1 ± 0.0	3.0 ± 0.2	n.d.	n.d.	n.d.	0.3
February						
DB	0.2 ± 0.0	n.d.	n.d.	n.d.	1.6 ± 0.3	0.5
OG	0.6 ± 0.1	2600 ± 150	23 ± 11	760 ± 1	4.6 ± 0.5	3.0
FF	1.9 ± 0.4	820 ± 16	1500 ± 50	480 ± 1	n.m	n.m
DC	0.2 ± 0.0	0.6 ± 0.2	n.d.	n.d.	0.3 ± 0.1	0.1
May						
DB	0.3 ± 0.1	0.3 ± 0.3	n.d.	n.d.	n.d.	n.d.
OG	0.3 ± 0.1	1900 ± 870	n.d.	740 ± 1	21 ± 4.6	0.9
FF	1.7 ± 0.3	250 ± 37	n.d.	870 ± 0	n.m	n.m
DC	0.2 ± 0.0	0.4 ± 0.1	n.d.	n.d.	2.7 ± 0.2	0.9
August						
DB	1.3 ± 0.3	n.d.	0.5 ± 0.1	n.d.	2.2 ± 0.9	0.8
OG	0.5 ± 0.1	1400 ± 608	600 ± 17	766 ± 0	26 ± 25	2.2
FF	2.4 ± 0.5	6200 ± 380	180 ± 21	1400 ± 2	n.m	n.m
DC	0.5 ± 0.1	0.6 ± 0.0	n.d.	n.d.	0.6 ± 0.5	0.3

Chapter 7

Synthesis

Microbial mats host metabolically diverse groups of microorganisms that contribute to critical steps in the biogeochemical cycles of these small-scale ecosystems. Despite the differences in diversity and abundance of each mat (Bolhuis and Stal, 2011), the overall functionality as revealed by the DNA array GeoChip is similar and seemed to be independent on the mat type or the season. The results support the theory of assembly of bacterial communities by functional level rather than by species (Burke *et al*., 2011). Bonilla-Rosso *et al*. (2012) compared several microbial mats with similar microbial ecosystems but developed under different environmental conditions. These authors found a few shared organisms but observed a greater similarity at a higher taxonomic level. Most likely, any (marine) microbial mat might turn out to have a similar functional composition. The major components of the microbial mat will occupy the available ecological niches along the biogeochemical gradients such as using photosynthesis, respiration, denitrification, sulfate reduction and fermentation (Chapter 4).

The low availability of nitrogen in coastal marine ecosystems limits primary production (Howarth and Marino, 2006). Therefore, the functioning and control of the nitrogen cycle is critical for the ecosystem and for the food web. The major steps in the nitrogen cycle are carried out by bacteria and archaea and representatives of those that are involved in the nitrogen cycle have been found in the Wadden Sea and the neighboring North Sea. For example, it has been shown that Thaumarchaeota play an important role in the Wadden Sea and North Sea (Herfort *et al*., 2007). Studies of microbial mats that develop on the North Sea beaches of the Wadden Sea barrier islands revealed high rates of nitrogen fixation and a diverse diazotrophic community (Severin and Stal, 2008; Severin *et al*., 2010). Yet, these studies were confined to a single process in an ecosystem and focused on a specific group of microorganisms. Especially in the North Sea, the ecology of some newly discovered processes and groups of microorganisms involved in the nitrogen cycle such as anaerobic ammonium oxidation (anammox) and nitrogen fixation are poorly understood. I have investigated major processes of the nitrogen cycle in contrasting coastal ecosystems (water column, bottom sediment and intertidal sandy sediment colonized by microbial mats) and during different seasons. I studied N_2 fixation, denitrification, anammox and nitrification. I concluded that these processes and the responsible microorganisms vary both spatially (different ecosystem compartments) and temporally (different seasons).

N_2 fixation

N_2 fixation is the only biological process that compensates for the loss of bio-available nitrogen from an ecosystem by denitrification or anammox. Its importance varies depending on whether an ecosystem receives sufficient combined nitrogen, such as is the case when a system suffers from eutrophication.

Microbial mats develop on nitrogen-depleted intertidal sandy beaches and therefore N_2 fixation plays a pivotal role in these ecosystems. Many studies have documented that microbial mats exhibit high N_2 fixation rates and this is also true for the mats from Schiermonnikoog (Severin and Stal, 2008). A new discovery was that N_2 fixation also occurs in the water column and seafloor of the southern North Sea, although the rates of N_2 fixation were low compared to microbial mats and other coastal ecosystems such as rocky shores (Chapter 5). This may be attributed to the high input of combined nitrogen in the water column and in the sediment from riverine discharge.

The different rates of nitrogen fixation in each of the studied ecosystems are most likely attributed to the intrinsic diazotrophic community. Several studies have shown a high diversity of diazotrophs in microbial mats (Omoregie *et al*., 2004; Steppe and Paerl, 2005; Severin and Stal, 2010;). In agreement with previous reports, Cyanobacteria and Proteobacteria are the main contributors to the *nifH* pool of the microbial mats of Schiermonnikoog, although the relative contributions to *nifH* varied in the different mat types (Severin *et al*., 2010). This was confirmed by the high diversity and abundance of the *nifH* gene detected by the GeoChip (Chapter 4). As reported in Chapter 5, an analysis of the diazotrophic community of the water column of the southern North Sea revealed that the dominant diazotrophs were deltaproteobacteria. I did not find *nifH* sequences belonging to cyanobacteria or to gammaproteobacteria, even though these groups are regarded as the dominant diazotrophs in the marine environment (Capone *et al*., 1997; Montoya *et al*., 2004; Halm *et al*., 2012; Moisander *et al*., 2014). The absence of these groups perhaps explains the low rates of N_2 fixation that were observed in these samples compared to those recorded for other pelagic habitats such as the western English Channel (Rees *et al*., 2009). The fact that the *nifH* sequences from anaerobic groups were retrieved from oxygenated surface water also requires an explanation. Given the fact that some of the retrieved *nifH* phylotypes have also been reported from coastal microbial mats (Severin *et al*., 2010), we speculate that benthic microorganisms might have been brought into suspension. But we cannot exclude the possibility that anaerobic diazotrophs thrive in anoxic micro-niches such as in aggregates of phytoplankton (Ploug, 2001), or that the sequences belong to other, hitherto unknown, aerobic diazotrophic microorganisms, that could have obtained the gene by horizontal gene transfer (Bolhuis *et al*., 2010). The sequencing results suggest that sulfate reducers such as *Desulfovibrio salexigens* and *Desulfovibrio vulgaris* may be the dominant

diazotrophs in the bottom sediments of the southern North Sea. Many sulfate-reducing bacteria possess the genetic potential to fix dinitrogen (Zehr *et al*., 1995). These diazotrophic organisms were also found in the sediments of the Baltic Sea (Bertics *et al*., 2013), Narragansett Bay (Fulweiler *et al*., 2013) and coastal California (Bertics *et al*., 2010). Previous studies found that nitrogenase activity decreased substantially when sulfate reduction was inhibited (Burns *et al*., 2002; Bertics *et al*., 2013). In addition, Bertics *et al*. (2013) complemented these findings with genetic data showing that the *nifH* sequences retrieved were closely related to *nifH* sequences from sulfur and sulfate reducers *Desulfovibrio* and *Desulfobacter* spp. These data suggest that sulfate-reducing bacteria are key players in sedimentary N_2 fixation.

Besides the composition and nature of the diazotrophic community, several physicochemical factors may affect N_2 fixation (Staal *et al*., 2003; Fulweiler *et al*., 2007). Chapter 5 recorded spatial and temporal variations of the rates of N_2 fixation in North Sea sediments and these variations might be partly attributed to organic matter. Herbert (1999) reviewed the rates of N_2 fixation in non-vegetated marine sediments and found that the highest rates were reported from sediments rich in organic carbon indicating that its availability is the key factor limiting the nitrogen-fixation potential of non-vegetated sediments. In terms of spatial distribution, station DB exhibited the highest pelagic and sedimentary rates of nitrogen fixation as well as the highest number of *nifH* transcripts when compared to the other two stations in the North Sea. Station DB is located at the shallow Dogger Bank. Previous studies indicated that the Dogger Bank is a special ecological area with distinct biological characteristics compared to the surrounding regions in the North Sea (Kröncke and Knust, 1995). Throughout the year, the Dogger Bank exhibits high rates of primary production (Howarth *et al*., 1993). This introduces fresh organic matter to the water column and to the bottom sediment, fuelling heterotrophic N_2 fixation. This would explain why N_2 fixation occurred even in February in the water column and bottom sediment at the sandy station DB. Temporally, N_2 fixation rates are generally higher in summer than in winter in the bottom sediments of southern North Sea. The higher temperature might have been the reason for that, but, alternatively, the increased supply of organic carbon deposited from the spring and summer blooms could have contributed, or the combination of both (Joint *et al*., 1993). However, N_2 fixation was not detected in May. It is not clear why N_2 fixation did not respond to the post bloom deposition in spring (in May). Fulweiler *et al*. (2013) proposed that the quality of organic matter plays a role in controlling N_2 fixation. It is possible that a different timing of the phytoplankton blooms alters both the quantity and quality of the deposited organic matter (Nixon *et al*., 2009) and we speculate that heterotrophic diazotrophs depend on a restricted range of organic carbon and/or its concentration. Station OG is a recognized deposition area with muddy sands and contains an order of magnitude more organic carbon compared to the other two stations (Bale *et al*., 2013). However, there is no N_2 fixation detected, notwithstanding the fact that the *nifH* gene is

abundantly present. There are several possible explanations. First, heterotrophic N_2 fixers may have to compete for carbon with other heterotrophic microorganisms such as denitrifiers, which were highly active at station OG. Second, although organic matter would promote oxygen consumption in sediment, the degradation of organic matter also leads to the accumulation of nitrate and ammonium that could lead to a repression of N_2 fixation. Whether or not this latter possibility is realistic is unclear. A high abundance of *nifH* gene copies at station OG indicates that, whilst there is a genetic potential for nitrogen fixation, the lack of suitable carbon substrates prevents it from being used. As a consequence, the contribution of these benthic diazotrophs to the nitrogen budget in the bottom sediments may be small (Herbert, 1999).

Denitrification and anammox

Denitrification and anammox are two processes in the nitrogen cycle that lead to a loss of combined nitrogen from an ecosystem. The contribution of denitrification and anammox to the evolution of dinitrogen gas varied among ecosystems. In the microbial mats of the barrier island Schiermonnikoog, measurements showed that the bulk of N_2 production was attributed to denitrification (Chapter 2). This observation was further supported by the results obtained from analyses using the GeoChip. This DNA microarray, which contains probes for most of the known genes involved in biogeochemical pathways, showed that genes involved in denitrification were abundant while genes involved in anammox were virtually absent (Chapter 4). An explanation may be that anammox bacteria are outcompeted in microbial mats. For example, anammox bacteria compete for nitrite with denitrifiers and for ammonium with nitrifiers. These groups are abundantly present according to the GeoChip results. This is in agreement with another study (Risgaard-Petersen *et al*., 2005). These authors demonstrated that anammox is not ubiquitously present in sediments colonized by microphytobenthos, not only because of their photosynthetic O_2 evolution but also because of their high demand for nitrogen. In the bottom sediments of the southern North Sea, anammox is responsible for up to 29% of the total N_2 production (Chapter 6). These data support the idea that anammox is of less importance than denitrification in shallow estuarine and coastal sediments (Engstrom *et al*., 2005; Thamdrup and Dalsgaard, 2008).

Denitrification is the dominant sink for combined nitrogen in the microbial mats of Schiermonnikoog and it is in the same range as the fixation of N_2. Previous studies have repeatedly shown that denitrification and anammox are insignificant when compared to N_2 fixation in microbial mats. For example, Joye and Paerl (1994) showed that denitrification was only 15% of N_2 fixation on an annual basis in the mats of Tomales Bay (USA), which are comparable with those of Schiermonnikoog. However, one should keep in mind that different methodologies were used to measure the denitrification. Joye and Paerl (1994) and others used the acetylene inhibition technique (AIT) to measure denitrification while I used the isotope pairing (IPM)

method (Nielsen, 1992). The AIT may underestimate denitrification while IPM is considered to be more accurate (Lohse *et al*., 1993). Therefore, denitrification may have been responsible for a much higher proportion of the loss of the fixed N_2 in microbial mats that were investigated by using the AIT (Chapter 2).

Several physicochemical and biological factors are important for controlling denitrification in coastal marine sediments. These include temperature, salinity, oxygen, availability of substrates (organic matter and nitrate), and the community of denitrifiers. Spatial and temporal heterogeneity of potential denitrification rates were observed in the microbial mats of Schiermonnikoog (Chapter 2), which has also been documented for other microbial mats (Joye and Paerl, 1994; Bonin and Michotey, 2006). The stations 2 (marine station) and 3 (intermediate brackish station) revealed much lower rates of denitrification, which showed also less seasonality when compared to station 1 (fresh water). The denitrifying community (both *nirS* and *nirK* containing) at station 1 was more diverse than those at the stations 2 and 3. The denitrifying communities of the stations 2 and 3 were more similar to each other than either of these stations to station 1. These results indicate that the different patterns of potential denitrification rates in each of the mat types are most likely attributed to the dissimilar denitrifier communities. Several studies have also suggested that differences in composition of the denitrifier community that might have influenced the rate of denitrification (Philippot and Hallin, 2005 and references therein). The spatial organization of the denitrifying community in the microbial mats is likely the result of changing environmental conditions. Salinity has been proposed as the major driver of the microbial community composition for these microbial mats (Bolhuis *et al*., 2013). This also applies to the denitrifier community. Jones and Hallin (2010) concluded that the global distribution pattern of *nirS* and *nirK* genes corresponded to salinity. The lower salinity at station 1 may explain the higher diversity of the denitrifier community. This has also been observed in a benthic denitrifier community along the estuarine gradient in Chesapeake Bay (Bulow *et al*., 2008). These authors found the highest *nirS* diversity at a freshwater station and the lowest diversity at a station with high salinity. According to canonical correspondence analysis, salinity is only responsible for part of the total variance of the denitrifier community. This implies that other factors also count. For example, availability of substrates and interaction and competition for resources with other microorganisms could be additional factors. In the microbial mats, denitrifiers compete for nitrate+nitrite with the primary producers such as cyanobacteria and diatoms that represent dominant groups in these mats (Severin *et al*., 2010; Bolhuis and Stal, 2011).

In the bottom sediments of the southern North Sea (Chapter 4), spatial variation of denitrification is primarily attributed to the distribution of organic carbon. Trimmer and Nicholls (2009) have shown that sedimentary denitrification correlated positively with the concentration of organic carbon in the surface sediments along in the North

Atlantic. Hence, both denitrification and N_2 fixation in the bottom sediments of the North Sea benefit from the availability of organic matter and may therefore be coupled. Station OG is a recognized deposition area with muddy sands and contains an order of magnitude more organic carbon compared to the other two stations (Bale *et al*., 2013). This would explain the higher rate of denitrification at station OG. Temporally, denitrification was highest in spring and this seasonality coincided well with the abundance of the *nirS* gene transcripts. This result is also consistent with a previous study in the southern North Sea. Van Raaphorst *et al*. (1992) estimated denitrification at two stations in the southern North Sea and showed that it was highest in spring and early summer and lowest in winter. This seasonality of denitrification could be attributed to an elevated temperature and an increased supply of fresh organic carbon deposited from spring blooms (Joint *et al*., 1993). Moreover, during late spring and summer, the increased availability of organic matter would stimulate the consumption of O_2, which would favor the facultative anaerobic denitrifying community. The bottom water at station OG can become hypoxic in summer (Weston *et al*., 2008; Greenwood *et al*., 2010) and, consequently, this would decrease the oxygen penetration depth in the sediment. The low oxygen concentration in the sediment at station OG may be responsible for the observed high rate of denitrification. In addition, a previous study has shown that nitrification is also higher in summer than in winter in the North Sea sediment (Lohse *et al*., 1993). When the source of nitrate/nitrite in the water column is limited in summer (low nitrate/nitrite concentration in bottom water has been reported in Bale *et al*. (2013)), nitrification becomes the primary source of nitrate/nitrite for denitrification. In addition, the community composition of denitrifiers at station DB and station DC are more similar to each other than either of them to station OG. Low organic carbon content and a larger grain size are characteristic for the former two stations (Bale *et al*., 2014) (Chapter 6). These results indicate that sediment type is one of the driving factors that determined the denitrifying community composition.

Nitrification (Chapter 3)

The seasonal patterns of potential nitrification observed in the three mat types were similar. The low potential nitrification rates in summer may be due to competition for ammonium between ammonia oxidizers and cyanobacteria or diatoms that use it as nitrogen source. The microbial mat reaches maturity in summer and becomes less productive and the standing stock biomass decreases afterwards (Stal *et al*., 1985). Nitrification is an aerobic process and therefore can only happen in the light when the cyanobacteria and/or diatoms evolve oxygen as the result of photosynthesis, which is mostly confined to the summer. However, the cyanobacteria also fix CO_2 and assimilate ammonium for growth (Stal, 2003). Hence, the competition pressure in summer may lead to the lower potential nitrification rate compared to other seasons, when the mats are less active and presumably do not become anaerobic in the top few millimeters. Also, in the coastal Arctic Ocean potential nitrification rates were higher

in winter than in summer (Christman *et al*., 2011). These authors hypothesized that the lack of competition for ammonium with phytoplankton and other microorganisms would stimulate nitrification in winter. Also in line with this is the conclusion of Risgaard-Petersen *et al*. (2004b) that benthic algae are superior to AOB when it comes to competition for ammonium. The seasonal pattern of potential nitrification may also be attributed to the dynamics of the community composition of ammonia oxidizers. Canonical correspondence analysis indicated a seasonal trend for both bacterial and archaeal ammonia oxidizers that correlated particularly to the concentrations of ammonium and nitrate/nitrite. I observed signal intensity shifts in the GeoChip for different types of bacterial ammonia oxidizers between summer and winter. Because different ammonia oxidizers will have different physiological characteristics, the shift in the community composition may eventually result in the seasonality of nitrification.

Lipsewers *et al*. (2014) reported that AOA play a more dominant role in aerobic ammonia oxidation in bottom sediments of southern North Sea. In contrast, in coastal microbial mats AOB were two to four orders of magnitude more abundant than AOA. On the one hand, AOA *amoA* was not expressed at detectable levels in the microbial mats despite the high diversity of the gene. On the other hand, the AOB *amoA* transcripts positively correlated with potential nitrification rates. Moreover, multiple stepwise linear regressions showed that β-AOB *amoA* transcription was the only valid predictor of potential nitrification. AOB *amoA* transcripts variation explained 51% of the potential nitrification. All evidence suggests that AOB are the predominantly responsible for nitrification in the microbial mats investigated in this study. There are several explanations for the minor importance of AOA in nitrification in the mat. Salinity appears to play a role in the relative distribution of AOA and β-AOB. Mosier and Francis (2008) found that in coastal aquifer sediments with high salinity (22-31 psu, practical salinity units) and low (2-15 μM) ammonia concentration AOB were more abundant than AOA but that at low salinity (0.2-9 psu) the latter prevailed. A similar study across a groundwater - seawater beach interface also revealed that AOB *amoA* abundance exceeded AOA *amoA* abundance with proximity to the ocean and higher salinity (Santoro *et al*., 2008). The microbial mats in this study were generally polyhaline (salinity: 18-30 psu). Furthermore, the pore water of the mat contains high ammonium concentrations. The majority of AOA found in this study belonged to *Nitrosopumilus*. This lineage is represented by *Nitrosopumilus maritimus*, which appears to be adapted to growth at low ammonia concentrations (Martens-Habbena *et al*., 2009). This may also be the case for AOA in our mats, although some *Nitrososphaera* strains grow also well at higher ammonium concentrations (Verhamme *et al*., 2011). It is possible that AOA are not obligate ammonia-oxidizers. Alves *et al*. (2013) showed that AOA belonging to *Nitrososphaera* are functional heterogeneous and that some would not exclusively grow at the expense of ammonia oxidation.

Conclusions and outlook

The nitrogen cycle was prominently present in the microbial mats of Schiermonnikoog as indicated by the presence of a variety of functional genes involved in this cycle. Denitrification was highest in the supra-littoral mat (Station 1) and was in the same order of magnitude as nitrogen fixation. Therefore denitrification is an important sink of combined nitrogen in these mats. Nitrification was highest in autumn and lowest in summer and it seems therefore that it occurred temporally separated from nitrogen fixation and denitrification. Investigation of the *amoA* gene revealed that ammonium-oxidizing bacteria outcompete archaea and play a dominant role in aerobic ammonia oxidation. The diversity of the denitrifiers (*nirK* and *nirS*) and ammonium-oxidizers (*amoA*) in these mats varied in a similar way. Based on their genes, microbial communities of Station 2 and 3 were more similar to each other than each of them to Station 1, which also applied to the diazotrophic community (*nifH*). Thus regardless different functional genes (*nifH*, *nirS*, *nirK* and *amoA*), microbial mat type overwhelmingly drives the diversity of the community, while mat type along the intertidal transect may be driven by salinity (Bolhuis *et al*., 2013).

In the southern North Sea, N_2 fixation and denitrification were temporally and spatially separated. N_2 fixation was highest in August in the offshore DB station, a sandy area with low organic content, while denitrification was high in May in the OG station, a muddy depression in the North Sea with high organic content. The rates of both processes coincided with the expression of the functional genes *nifH* and *nirS*. N_2 fixation was mainly attributed to the anaerobic sulfate reducing bacteria. The functional gene representing denitrification, *nirS,* could not be assigned to a specific group of microorganisms. The spatial and temporal separation of denitrification and nitrogen fixation is also projected on the composition of chemotropic diazotrophic and denitrifying communities. The diazotrophic community composition at the three stations appears to correlate with geographic location (coastal and offshore) while sediment type determined denitrifying community composition.

This thesis shows that a number of factors may drive observed changes in main processes in nitrogen cycle. However, a precise identification of the dominant drivers would require quantitative experimental studies of each of the processes in the nitrogen cycle as well as the microbial communities behind them in different environmental settings in mesocosms. It is for the first time that N_2 fixation was investigated in the temperate waters of the North Sea and its occurrence was an unexpected and novel observation. However, in this study only a few contrasting sampling stations were selected. In order to fully understand the importance of N_2 fixation in temperate waters such as the southern North Sea it would require more extensive measurements in different systems, over a larger area, and at a higher temporal resolution. Moreover, the effect of environmental conditions need to be tested experimentally and the key microorganisms need to be isolated and their

properties studied in the laboratory.

Summary

This thesis focuses on the nitrogen cycle in Dutch coastal waters and sediments. The main hypothesis of this study was that the different steps of the nitrogen cycle occur spatially and temporally separated from each other rather than that the cycle is closed in the same place and time. To verify this hypothesis, multiple experimental approaches were applied to investigate N_2 fixation, denitrification, anammox and nitrification in the some selected ecosystems of the Dutch coast.

Chapters 2, 3 and 4 investigate N_2 fixation, denitrification, anammox and nitrification in an intertidal microbial mat. Microbial mats are benthic communities of vertically stratified functional groups of microorganisms. The oxygenic phototrophic cyanobacteria are conspicuously present, and by fixing carbon dioxide and dinitrogen, they form the basis of the microbial food web in this small-scale ecosystem. Microbial mats develop on nitrogen-depleted intertidal sandy beaches and therefore nitrogen fixation plays an important role in supplying the mats with bound nitrogen. This aspect has received considerable attention but not much is known about the fate of the fixed nitrogen, the responsible microorganisms, and the factors that control the nitrogen cycle in microbial mats.

The GeoChip is a DNA microchip that contains probes for virtually all known genes involved in the major biogeochemical processes. The study of the microbial mats of the Dutch barrier island Schiermonnikoog using this GeoChip revealed that the overall functionality of these mats is similar and seemed to be independent on the mat type or the season. The nitrogen cycle was prominently present as indicated by the presence of a variety of functional genes involved in this cycle. Especially, the genes involved in denitrification were abundant, following those involved in nitrogen fixation and nitrification (Chapter 4).

N_2 production was measured in microbial mats that develop along a tidal and salinity gradient from the supra littoral to the low water mark (Chapter 2). Denitrification is the main sink for nitrogen in these mats. The seasonality of denitrification is not consistent in different types of mats. Station 2 (marine station) and Station 3 (intermediate station) revealed much lower rates of denitrification, which showed also less seasonality when compared to Station 1. The denitrifying community at Station 1 was more diverse than that at Station 2 and Station 3. The denitrifying communities of these stations were more similar than either of these stations with Station 1. Therefore, the seasonality of denitrification may be mainly attributed to the denitrifying community composition. The spatial organization of the denitrifying community in the microbial mats was likely the result of changing environmental conditions. Further investigation on the potential rate of nitrification and the diversity and abundance of

amoA gene of AOB and AOA in these mats showed that AOB are responsible for most of the aerobic ammonium oxidation in the mats (Chapter 3). Salinity has been proposed as the major driver of the microbial community composition for these microbial mats. This also applies to the denitrifier and aerobic ammonia-oxidizer community. In conclusion, nitrogen fixation and denitrification occur predominantly in summer in these microbial mats. Denitrification may benefit from the supply of organic matter that is produced by the photoautotrophs. Nitrification was highest in autumn and lowest in summer and therefore it occurred temporally separated from nitrogen fixation and denitrification.

Chapter 5 and Chapter 6 report on studies on N_2 fixation, denitrification, and anammox in the surface of the water column (only N_2 fixation), and the bottom sediments of the southern North Sea. In contrast to microbial mats, N_2 fixation was thought to be uncommon in the southern North Sea waters due to the high nitrogen availability in these regions. It was shown that N_2 fixation occurred in the water column and in the surface sediment and was mainly attributed to the anaerobic sulfate reducing bacteria. Compared to anammox, denitrification was responsible for the main nitrogen loss from the system. The contribution of denitrification to N_2 production varied among stations and between seasons. The functional gene representing denitrification, *nirS*, could not be assigned to a specific group of microorganisms. In the sediment, N_2 fixation and denitrification were temporally and spatial separated. The former was highest in August in the offshore DB station, a sandy area with low organic content, while the latter was high in May in the OG station, a muddy depression in the North Sea with high organic content. The rates of both processes coincided with the expression of the functional genes *nifH* and *nirS*. The spatial and temporal separation of denitrification and nitrogen fixation is also projected on the composition of chemotrophic diazotrophic and denitrifying communities. The diazotrophic community composition at the three stations appeared to correlate with geographic location (coastal and offshore) while the denitrifying community composition was determined by sediment type. Chapter 6 studied anammox and anammox bacteria during four seasons. It was shown that higher abundance and activity of the anammox bacteria were associated with higher organic carbon and elevated temperature.

In conclusion, N_2 fixation, denitrification anammox and nitrification and the responsible microorganisms occur on different spatial and temporal scales in ecosystems of the Dutch coast. A number of factors may drive the observed changes in the main processes in the nitrogen cycle. These may include composition and nature of the diazotrophic community and physicochemical factors including temperature, salinity, oxygen, and availability of substrates. Future studies are recommended in order to provide knowledge on the effects of environmental

conditions on the nitrogen cycle in coastal environments, using quantitative experiments in mesocosms and laboratory experiments with the key microorganisms.

Samenvatting

Dit proefschrift gaat over de stikstofcyclus in het Nederlandse kustgebied, waarbij zowel de waterkolom als het sediment (getijdensediment en zeebodem) werd onderzocht. De belangrijkste hypothese van deze studie was dat de verschillende stappen in de stikstofcyclus zowel ruimtelijk als in de tijd gescheiden verlopen en niet, zoals vaak gedacht wordt, dat de cyclus gesloten is op een bepaalde plaats en tijd (dat wil zeggen dat alle stappen van de stikstofcyclus in een gegeven microbieel ecosysteem tegelijkertijd plaatshebben). Om deze hypothese te testen werden verschillende benaderingen gekozen om de binding van luchtstikstof (via ammonium naar microbiële biomassa), de denitrificatie (de omzetting van nitraat naar luchtstikstof), de anaerobe ammonium oxidatie (anammox), en de nitrificatie (de aerobe oxidatie van ammonium) te onderzoeken en te meten in een aantal geselecteerde microbiële ecosystemen van de Nederlandse kust.

De hoofdstukken 2, 3 en 4 gaan over de stikstoffixatie, denitrificatie, anammox en nitrificatie in een microbiële mat in het intergetijdengebied. Microbiële matten zijn benthische (op of in het sediment levende) gemeenschappen van verticaal gestratificeerde (gelaagde) functionele groepen van micro-organismen. De oxygene (zuurstof-producerende) fototrofe (fotosynthese bedrijvende) cyanobacteriën (ook bekend als ‘blauwwieren’) zijn opvallende organismen in deze matten. Doordat cyanobacteriën koolzuur en stikstof fixeren produceren ze organische stof en vormen zij zodoende de basis van de microbiële voedsel web van dit kleinschalige ecosysteem. Microbiële matten ontstaan in stikstofarme zandige getijdenstranden en daarom is de eigenschap dat micro-organismen in de mat luchtstikstof kunnen binden en deze oneindige stikstofbron gebruiken voor de vorming van biomassa en groei van groot belang en zonder dit proces zou een microbiële mat zich niet ontwikkelen. Dit aspect heeft daarom ook veel aandacht genoten in de wetenschappelijke literatuur. Echter, heel weinig is bekend over het lot van de gefixeerde stikstof, de organismen die betrokken zijn bij de stikstof cyclus en de factoren die bepalend zijn voor deze cyclus in microbiële matten.

De GeoChip is een DNA microchip dat oligonucleotide sondes (‘probes’) heeft voor vrijwel alle bekende genen die betrokken zijn bij de belangrijkste biogeochemische processen. Door het gebruik van deze GeoChip voor het onderzoek aan microbiële matten van het Waddenzee eiland Schiermonnikoog kon worden vastgesteld dat de allesomvattende functionaliteit van verschillende typen microbiële matten in feite hetzelfde is en dat er ook geen verschillen tussen zomer en winter bestaan. Het detecteren van een groot aantal verschillende genen die betrokken zijn bij de stikstofcyclus liet zien dat deze cyclus prominent aanwezig is in de onderzochte microbiële matten. Vooral genen die betrokken zijn bij de denitrificatie waren volop

aanwezig, gevolgd door die, die betrokken zijn bij de stikstoffixatie en nitrificatie (Hoofdstuk 4).

De microbiële matten die gevormd worden langs de getijde- en zoutgradiënt van het supra litoraal (bij de duinen) tot de laagwaterlijn, produceerden alle distikstofgas (N_2) (Hoofdstuk 2). Er kon geconcludeerd worden dat in deze matten denitrificatie de belangrijkste put voor stikstof is. Denitrificatie vertoont echter geen consistent gedrag. Station 2 (het marine station bij de laagwaterlijn) en Station 3 (station tussen 1 en 2 en een echt tussengetijde gebied) vertoonden relatief lage (vergeleken bij Station 1) denitrificatie waarden die bovendien niet seizoensgebonden bleken te zijn. Ook de denitrificerende micro-organismen in beide stations leken meer op elkaar dan elk van deze stations leek op Station 1. Daarom zou het kunnen zijn dat een seizoenafhankelijke denitrificatie een gegeven is die vooral samenhangt met de samenstelling van de gemeenschap van micro-organismen die dit proces uitvoeren. Evenzo heeft de ruimtelijke verdeling van de denitrificerende micro-organismen wellicht te maken met de verschillende milieucondities.

Verder onderzoek naar de potentiële nitrificatie snelheid en de diversiteit en abundantie van het *amoA* gen van ammonia-oxiderende bacteriën (AOB) en ammonia-oxiderende archaea (AOA) resulteerde in de conclusie dat AOB de belangrijkste organismen zijn die zorg dragen voor de oxidatie van ammonia in deze microbiële matten (Hoofdstuk 3). Het zoutgehalte is waarschijnlijk een belangrijke factor voor de samenstelling van zowel de denitrificerende als ook de aerobe ammonium oxiderende bacteriën. De conclusie is gerechtvaardigd dat stikstoffixatie en denitrificatie vooral gedurende de zomer plaatsvinden en dat de laatste profiteert van het organische materiaal dat gedurende de zomer wordt geproduceerd door de fotosynthetische organismen. Nitrificatie is het meest actief gedurende de herfst en het minst actief gedurende de zomer en treedt dus in de tijd gescheiden op van stikstoffixatie en denitrificatie.

Hoofdstuk 5 en 6 gaan over stikstoffixatie, denitrificatie en anammox in het oppervlaktewater (alleen stikstoffixatie) en de zeebodem van de zuidelijke Noordzee. In tegenstelling tot microbiële matten, is steeds aangenomen dat stikstoffixatie in gematigde zeeën zoals de zuidelijke Noordzee geen factor van belang is in verband met de hoge beschikbaarheid van stikstof in deze wateren. Maar niettemin kon ik aantonen dat stikstoffixatie zowel in de waterkolom als ook in de zeebodem belangrijk was en dat het voornamelijk de anaerobe sulfaat-reducerende bacteriën waren, die daarvoor verantwoordelijk waren. Anammox bleek van ondergeschikt belang en denitrificatie werd aangewezen als het voornaamste proces dat verantwoordelijk is voor het verlies van stikstof uit het ecosysteem. De bijdrage van denitrificatie aan de productie van distikstof (N_2) verschilde tussen de verschillende onderzochte stations maar ook binnen een station. Het gen dat de functie denitrificatie

representeert, *nirS*, kon niet worden gekoppeld aan een specifieke groep micro-organismen. Stikstoffixatie en denitrificatie in het sediment verliepen zowel in de tijd als in de ruimte van elkaar gescheiden. De stikstoffixatie was het hoogst in augustus in het offshore station Doggers Bank, dat een zandige bodem heeft waar weinig organisch materiaal in zit. Denitrificatie was hoog in mei in het station Oestergronden, een modderige diepte in de Noordzee met een hoog gehalte aan organische stof. De snelheid van beide processen kwam overeen met de expressie van de genen *nifH* en *nirS*, die coderen voor eiwitten die achtereenvolgens bij de stikstoffixatie en denitrificatie betrokken zijn. De ruimtelijke en tijdelijke scheiding van denitrificatie en stikstoffixatie komt ook tot uiting in de samenstelling van de chemotrofe stikstoffixerende en denitrificerende microbiële gemeenschappen. De samenstelling van de stikstoffixerende gemeenschappen in de drie stations leek te correleren aan de geografische positie (kust en offshore), terwijl de samenstelling van de denitrificerende gemeenschappen meer leek te zijn bepaald door het type sediment. In Hoofdstuk 6 is aandacht besteed aan de seizoenvariaties van het anammox proces en de anammox bacteriën. Het bleek dat de abundantie en activiteit van de anammox bacteriën overeenkwamen met een situatie waarbij veel organisch materiaal aanwezig is en bij hogere temperatuur.

Concluderend kan ik vaststellen dat de stikstoffixatie, denitrificatie, anammox en nitrificatie, alsmede de micro-organismen die voor deze processen verantwoordelijk zijn, in de Nederlandse kustecosystemen op verschillende ruimtelijke en tijdelijke schalen plaatshebben. De waargenomen veranderingen in de stikstofcyclus worden gedreven door verschillende factoren. Daaronder zijn de identiteit van de betrokken micro-organismen en de samenstelling van de microbiële gemeenschappen en fysicochemische factoren zoals temperatuur, zoutgehalte, zuurstof, en de beschikbaarheid van substraat van belang. Ik beveel verdere studies aan om de kennis te vergroten die nodig is om de effecten van milieucondities op de stikstofcyclus in kustecosystemen te kunnen inschatten. Daarvoor zijn kwantitatieve experimenten in gesloten, gecontroleerde natuurlijke systemen (‘mesocosm’) nodig als ook laboratorium experimenten met de sleutelorganismen.

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Acknowledgements

I have to say, though my name appears on the cover of this dissertation, a great many people have contributed to its production. I would like to thank all those people who have made this thesis possible, and because of whom my experience has been one that I will cherish forever.

First of all, I would like to express my greatest gratitude to my promoter and supervisor, Prof. Lucas J.Stal, who has vision, passion and as well as a sense of humor. He gave me an opportunity to work in the Marine microbiology department at our institute with such a nice atmosphere of easy collaboration. He opened the door for me to the field of N-cycling and microbial mats and shed the light for me through the darkness. Do learn from him how to dig the importance out of the numerous irrelevances. I am always amazed by his industriousness, acumen and to-the-point comments. His critical commentary and seemingly inexhaustible patience in reviewing my manuscripts are deeply appreciated. His support and patience helped me overcome the crisis and finish this thesis.

I would like to thank Dr. Henk Bolhuis as co-supervisor who greatly supported me in numerous ways. I'm specially thankful to Henk for teamwork and fun time during Schiermonnikoog expeditions, his supervision on data analyses, carefully reading and commenting on countless revisions of the manuscripts.

This thesis would have gone nowhere without the support from MM members (past and present). I'm grateful to Dr. Akiko Tomitani for teamwork in Schiermonnikoog expeditions and great times during and after work. I'm grateful to Dr. Eric Boschker for sharing the experience of technical details of stable isotope, supporting for the isotope measurements. I'm grateful to Juliette. She has been a great office mate and friend and helped me in every aspect of my life. Many thanks go to Veronique, Julia and Michele and for their assistances in expeditions, collaboration and sharing their experiences. Many thanks go to Ina, Tetsuro, Diana, Christine, Clara, Anita, Tanja, Sven and Jacco for their collegiality and sharing knowledge. I also would like to thank Alessia, Lara, Marie, Dorina from Ecosystem study for the collaborations of sharing their experience, technical supports and joyful time in the "NICYCLE cruises. I want to thank all technicians in analytical department for their helps and technical assistances in the nutrient and stable isotope analyses. In particular, many thanks go to Peter for his expertise, encouragement and help. His assistance is invaluable. All dear colleagues in the NIOZ-Yerseke are thanked for their warmth and open minds, which make me feel like home.

I am also grateful to the partners in the NICYCLE project, especially, Dr. Nicole J. Bale for arranging the logistics during the four cruises. I would like to thank the captains and crew of the R/V Pelagia for their support during four cruises as well as Marine Technology Electronics and Marine Technology Mechanics departments (NIOZ) for their technical support on board.

I am grateful to Prof. Dr. Jizhong (Joe) Zhou for allowing me to carry out the GeoChip analyses at the University of Oklahoma. I want to thank Maggie, Jason, Ye, Qichao and Yuan from Joe's group for their technical support and helps with logistics during my stay in Norman

I would like to thank all my friends, especially Juliette, Alessia, Qiuyin, Hui, Quanxing, Zhengchang, Zhigang, Akiko and all friends from NIOZ-Yerseke, for their helps and supports in these years, bringing the sunshine into my life and accompanying with me through difficulties. I am always grateful to my families for their love, encouragements and solid supports.