Character and Function of Anammox Bacteria under Environmental Stress

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Cover picture: Schematic description of the anammox reaction in an electron microscopy image of an anammox cell

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With all my love to Anna, Nils and John

Abstract

During the last few decades observations of novel processes involved in nitrogen transformations have fundamentally challenged the view of pathways and controlling mechanisms during local and global nitrogen cycling. Anaerobic ammonium oxidation (anammox) constitutes one of these new pathways where autotrophic bacteria oxidize ammonium by nitrite to dinitrogen gas under anaerobic conditions. Anammox provides a shunt during nitrogen transformations as it bypasses the classical pathway of aerobic nitrification coupled to anaerobic denitrification, a reaction scheme previously thought to be the sole source of dinitrogen gas in natural environments. Anammox is now acknowledged as a widespread and a globally important sink for nitrogen in water column and sediment systems.

The first part of this thesis emphasises factors that regulate anammox bacteria in natural environments. Particular focus relates to coastal marine sediments and the importance of anammox for nitrogen removal under environmental stress associated with the temporal availability of oxygen and nutrients. Measurements of anammox and denitrification were made by ^{15}N amendments including both shallow-water illuminated autotrophic (net oxygen producing) sediments and deeper heterotrophic (net oxygen consuming) sediments. While rates of anammox were insignificant in illuminated sediments with primary production by benthic microalgae, anammox was found almost as important as denitrification for total N_2 production in the dark heterotrophic sediments. Long term laboratory incubations under different oxygen conditions confirmed the importance of oxygen availability for the removal of bioavailable nitrogen by N_2 production in surface sediments.

In the second part of the thesis investigations focus on detailed mechanisms involved during anammox. Cutting edge analytical tools of membrane proteomics were utilized to identify and sub-cellularly localize key proteins involved in the anammox reaction. Two proteins, the hydrazine synthase (previously hydrazine hydrolase) and an F-ATPase, were identified by proteomics and LC-MS/MS analysis and subsequently targeted for antibody production. Through immunogold electron microscopy the hydrazine synthase was assigned to the interior of the anammoxosome, the unique “organelle” of anammox bacteria. The F-ATPase was associated with the anammoxosome membrane. These observations not only strengthen the important role of the anammoxosome during anammox metabolism, but also provide experimental support to the idea of the anammoxosome as an energized membrane.

Keywords: Anammox | N-cycle | environmental stress | redox oscillations | anammoxosome | key proteins

Populärvetenskaplig Sammanfattning

Intresset för miljö- och klimatförändringar och deras effekter på en hållbar samhällsutveckling har ökat under de senaste åren. Exempel på förändringar som relaterar till mänsklig aktivitet är global uppvärmning till följd av ett ökat utsläpp av koldioxid (CO_2) och syrebrist i kustnära områden orsakat av övergödning, dvs. tillförsel av näringsämnen rika på kväve (N) och fosfor (P). Kol (C) och kväve är två grundämnen som är livsviktiga för allt levande. Koldioxid tas upp av organismer för uppbyggnad av cellmaterial genom exempelvis fotosyntes och frisläpps genom organismers respiration. I havet bidrar dessa biologiska processer till att reglera koldioxid i atmosfären och tillgången på närsalter och syre i vattnet. Kvävets kretslopp står i direkt relation till kretsloppet av kol då kväve och kol används i stökiometriska proportioner för att bygga upp exempelvis protein och DNA i celler. Biologiskt tillgängligt kväve förekommer vanligtvis i låga koncentrationer i havet vilket innebär att kväve ofta begränsar uppkomst och utbredning av biologisk produktion. Flera nya processer relaterade till kvävets kretslopp har upptäckts under senare tid vilket har lett till ett ökat behov att undersöka vilka faktorer som reglerar kvävets kretslopp under samtidig miljöpåverkan. En av dessa relativt nyupptäckta reaktioner är anaerob ammoniumoxidation (anammox) som tillsammans med denitrifikation är de kvantitativt mest betydelsefulla sätt att bilda kvävgas (N_2). Anammox katalyseras av specialiserade bakterier som bildar kvävgas genom att ammonium (NH_4^-) reagerar med nitrit (NO_2^-) under en reaktionsmiljö som saknar tillgång på syre.

Den här avhandlingen syftar till att undersöka betydelsen av anammox för kvävgasbildning i havet samt hur processen påverkas av förändrade miljöfaktorer såsom tillgång på syre och viktiga näringsämnen. Bland annat studerades hur anammox och denitrifikation påverkas av fotosyntetiserande mikroalger i grunda solbelysta sediment. Under den ljusberoende fotosyntesen varierar tillgängligheten av exempelvis syre och näringsämnen naturligt. Processhastigheterna bestämdes längs en djupgradient inkluderande såväl grunda och solbelysta som djupa och mörka sedimentsystem. Anammox visade sig vara obetydligt för total N_2-produktion i jämförelse med denitrifikation i solbelysta system med aktiv fotosyntes. På djupare vatten dit solljuset inte penetrerar var anammox däremot en viktig process för bortförsel av kväve i sedimentet. Andra undersökningar under kontrollerade förhållanden i laboratoriemiljö har specifikt fokuserat på hur varierande syreförhållanden påverkar betydelsen av anammox och denitrifikation för bortförsel av kväve genom kvävgasbildning. Båda reaktionerna antas bli inhiberade genom närvaro av syrgas. Medan en lång period (140 dagar) av helt syrefria

förhållanden inhiberade anammox fullständigt, bidrog reaktionen signifikant till uppmätt kvävgasbildning, trots full syresättning under hela perioden. Denna observation öppnar upp för fördjupande studier av kontrollerande mekanismer för anammox och denitrifikation på molekylär nivå.

För att i detalj förstå vad som kontrollerar anammox i naturliga system är det betydelsefullt att identifiera molekylära mekanismer, dvs vilka enzym som är inblandade i reaktionen under olika förhållanden. Genom kartläggningen av anammoxbakteriernas genom har ett flertal enzym identifierats som centrala för anammoxreaktionen. Ett tillvägagångssätt att öka förståelsen för hur enzymen samverkar och därmed vilka delreaktioner som är betydelsefulla under olika förutsättningar är att lokalisera var i cellerna de verkar. Anammoxbakterier har en unik celluppbyggnad, med en inre organell-lik membranstruktur, kallad anammoxosom. För att påvisa anammoxosomens betydelse för reaktionsförloppet lokaliserades nyckelprotein med hjälp av avancerade analytiska tekniker, kopplade till elektronmikroskopi för sub-cellulär visualisering. Specifikt utvalda proteinsekvenser identifierades och användes för att skapa guldmärkta antikroppar vilka kunde bindas till enzymen i intakta bakterieceller. Två centrala enzym identifierades genom denna metod. Ett hydrazinsyntas som lokaliserades till innandömet av anammoxosomen samt ett F-ATPas som var specifikt associerat med anammoxosomens membran. Dessa resultat befäste ytterligare att metabolismen för anammox sker i anammoxosomen och att anammoxosommembranet är ett ”energiproducerande” membran. Ett energi-producerande membran är normalt typiskt för mitokondrier i eukaryotiska celler vilket tillsammans med den unika cellstrukturen i anammoxbakterier öppnar för intressanta kopplingar mellan anammoxbakterier och den evolutionära utvecklingen av eukaryotiska celler.

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PART B: List of Publications

This thesis is based on investigations presented in the following papers, hereafter referred to by their roman numerals. The papers are appended at the end of the thesis.

I. Hulth, S., **Bäckman, O.**, Dalsgaard, T., Larson, F. and Sundbäck, K. Nitrogen removal by anammox and denitrification along a depth transect in the Gullmarsfjord, north eastern North Sea. *Geochimica et Cosmochimica Acta.* Accepted pending revisions

II. Bäckman, O., Larson, F and Hulth, S. The importance of oxygen availability and redox conditions for anammox and denitrification in marine sediments. Manuscript for *Limnology and Oceanography.*

III. Karlsson, R., Karlsson, A., **Bäckman, O.,** Johansson, B.R. and Hulth, S. (2009) Identification of key proteins involved in the anammox reaction. *FEMS Microbiology Letters.* **297:** (87-94).

IV. Karlsson, R., Karlsson, A., **Bäckman, O.,** Johansson, B.R. and Hulth, S. Subcellular localization of an ATPase in anammox bacteria using proteomics and immunogold electron microscopy. *FEMS Microbiology Letters.* Submitted

1. Introduction and Objectives

In recent years there has been an increasing focus on climate and environmental change, often considered direct effects of anthropogenic emissions of carbon dioxide (CO_2) to the atmosphere from fossil fuel burning (e.g. IPCC, 2007). Eutrophication by anthropogenic nutrient release to the coastal zone is also of environmental concern due to effects associated with oxygen deficiency and loss of important "ecosystem services" (Diaz and Rosenberg, 2008) Carbon, the most central element for all living organisms, is actively cycled within the biosphere. The biogeochemical loop, i.e. the cycling of carbon and associated elements (e.g. N, P, Si, Fe, Mn, and S) is fundamental in controlling concentrations of CO_2 in the atmosphere. Historical fluctuations of CO_2 have often been linked to changes in this biologically driven loop (e.g. Sarmiento and Toggweiler, 1984; Siegenthaler and Wenk, 1984; Martin, 1990; Brzezinski et al., 2002). As the ocean is constantly equilibrating with atmospheric CO_2 there is a tight coupling between cycling of elements in the marine and atmospheric systems (Takahashi et al., 1997; Fasham et al., 2001).

Nitrogen (N) is an element frequently considered limiting for primary production in large parts of the world's oceans (Ryther and Dunstan, 1971; Howarth, 1988; Gruber and Galloway, 2008). This implies that alterations in the pool of fixed (i.e. biologically available) nitrogen in the sea could have a substantial effect on the spatial and temporal capacity of the oceans to sequester atmospheric CO_2. This feedback suggests that nitrogen cycling is important also for the temporal and spatial evolution and effects from climate change (Capone, 2000). Also, nitrous oxide (N_2O) is a gaseous intermediate mainly produced during nitrification and denitrification (Capone, 2000 and references therein). As N_2O is a highly potent greenhouse gas (Wang et al., 1976) the link between the nitrogen cycle and climate change is further amplified. Additionally, as oxygen availability controls the importance of redox processes the cycling of oxygen is tightly coupled to the speciation of nitrogen and carbon (Berman-Frank et al., 2008).

Biogeochemical feedbacks between the oxygen, nitrogen and carbon cycles, with direct consequences on the evolution of earth's climate and essential ecosystem services, provide important incentives to improve the understanding of processes and mechanisms that control rates and pathways during the cycling of these elements in the biosphere. During the last decades, observations of novel processes (e.g archaeal nitrification, anaerobic ammonium oxidation, eukaryotic denitrification, Thamdrup, 2012) and microorganisms that catalyze these reactions have fundamentally improved the understanding of pathways and controlling mechanisms during transformations of nitrogen in natural environments.

Main objectives of this thesis are to investigate factors that control the importance of anaerobic ammonium oxidation (anammox) for the removal of fixed nitrogen by N_2 production in marine environments. Particular focus relates to effects from environmental stress associated with changes in redox conditions (e.g. availability of oxygen) and availability of nutrients in shallow-water illuminated autotrophic (net oxygen producing) sediments compared to dark heterotrophic (net oxygen consuming) sediments. Net availability of oxygen and nutrients during primary production and mineralization processes are factors that may control activities by anammox bacteria in natural environments (Paper I). Overall the growth of anammox bacteria is suggested to be extremely slow and, as a consequence, they are considered to favor stable environmental conditions. In Paper I, rates of anammox and denitrification were measured along a depth gradient including both photic sediments with primary production by benthic microalgae and aphotic heterotrophic sediments dominated by organic matter mineralization. Anammox was an important process under heterotrophic conditions but was insignificant in photic environments with high photosynthetic activity and where availability of oxygen and nutrients vary on a diurnal time scale. To investigate the long-term importance of redox conditions and availability of oxygen and nutrients for absolute and relative rates of anammox and denitrification (both considered strictly anaerobic) N_2-production rates were in Paper II measured in sediments following a longer period of time (140 days) under different conditions of oxygen in the overlying water. Anammox was not detected in sediments exposed to permanently anoxic conditions. In contrast, anammox could be quantified in samples subjected to fully oxygenated conditions during the experimental period. Anammox bacteria were thus kept viable during long term oxygen exposure which is somewhat contradictory to their supposedly strictly anaerobic metabolism.

Additional focus of the thesis was to visualize the internal structure of the anammox cell and to utilize cutting edge proteomic tools to identify key metabolic enzymes of the anammox reaction as well as to determine their subcellular location by immunogold electron microcopy (Paper III and IV). A lipid based immobilization of membrane associated proteins (the LPI-technology) was used together with immunocytochemistry to experimentally demonstrate that the hydrazine hydrolase (Paper III) and an F-ATPase (Paper IV) were associated with the anammoxosome.

2. The Marine Nitrogen Cycle

Nitrogen is an element essential for all living organisms. Due to the configuration of electrons it has a complex redox cycle relative to most other elements required for life. In natural environments, nitrogen occurs in a multitude of different forms with redox states ranging from –III to +V (Table 1).

Table 1. Examples of N-containing compounds in natural environments. The oxidation state of nitrogen ranges between –III and +V.

Compound	Formula	Oxidation state
Ammonium	NH_4^+	-III
Amino acids	R-NH_2	-III
Urea	NH_2CONH_2	-III
Hydrazine	N_2H_4	-II
Hydroxylamine	NH_2OH	-I
Dinitrogen	N_2	0
Nitrous oxide	N_2O	+I
Nitric oxide	NO	+II
Nitrite	NO_2^-	+III
Nitrogen dioxide	NO_2	+IV
Nitrate	NO_3^-	+V

The largest reservoir of N on Earth is in igneous rocks ($\sim 1.4 \cdot 10^{22}$ g N), primarily as ammonium substituted within potassium-rich minerals, followed by nitrogen in sediments and sedimentary rocks ($\sim 4.0 \cdot 10^{21}$ g N), mostly as ammonium (NH_4^+) in secondary silicate minerals (Canfield et al., 2005 and references therein). Of comparable size is the reservoir of atmospheric dinitrogen gas (N_2; $\sim 3.8 \cdot 10^{21}$ g N). Biologically available inorganic nitrogen mainly occurs as ammonium and nitrate (NO_3^-) where NO_3^- is the major pool and main constituent in oxygenated environments and NH_4^+ is the end-product from mineralization and main component of nitrogen in anoxic systems (Capone, 2000). Trace amounts of the gases nitrous oxide (N_2O) and nitric oxide (NO) as well as different organic (e.g. urea, amines, peptides, aminoacids and proteins) and inorganic (e.g. nitrite, NO_2^-) forms can also be found. While high concentrations are often associated with conservative and non-reactive elements (e.g. Cl^-, Na^+), low concentrations may imply active biogeochemical cycling and a delicate balance between microbial production and consumption processes.

The marine cycling of nitrogen is mainly biologically mediated where transformations are controlled by specific and specialized microorganisms (Capone, 2000). Although a large amount of nitrogen is present in the largest three reservoirs, most of the nitrogen pool is not biologically available. A fraction of the ammonium from the igneous and sedimentary pools may become available by rock weathering. This contribution is, however, normally marginal and occurs locally (Holloway et al., 2001). Similarly, although actively cycled and used as an N-source by a few specialized microorganisms, the N_2 pool is conservative with a slow turnover (Canfield et al., 2005). Assimilation, mineralization and nitrification link the small but actively cycled pools of available dissolved inorganic nitrogen (DIN) with dissolved (DON) and particular (PON) organic nitrogen (Figure 1).

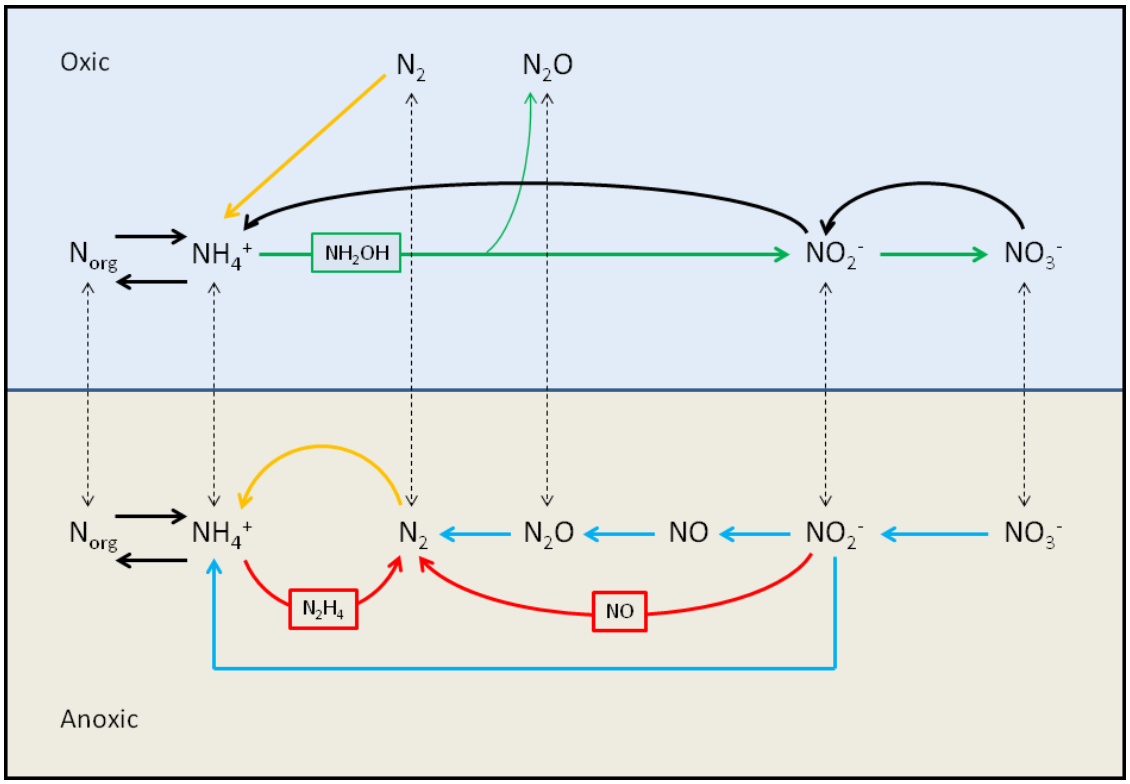

Figure 1. Schematic representation of the major pathways in the marine nitrogen cycle. Yellow arrows represent nitrogen fixation. Black arrows represent assimilation and nitrogen mineralization. Green arrows represent nitrifying processes. Blue arrows represent dissimilatory nitrate and nitrite (denitrification and DNRA) reduction and red arrows represent anammox. Transport of different species between oxic and anoxic environments is represented by black dashed arrows.

2.1. Nitrogen fixation

The large pool of N_2 in the oceans is normally not accessible for most organisms and was for a long period of time considered a minor source in the global nitrogen cycle (Capone, 2000). However, observations have revealed that biological nitrogen fixation (conversion of N_2 to NH_4^+) is a widespread and important source for biologically available nitrogen (Capone, 2001). Nitrogen fixation is carried out by specialized prokaryotes (diazotrophs) that contain the enzyme nitrogenase (Sprent and Sprent, 1990). The feature to utilize N_2 as a source of nitrogen is, however, not restricted to a related group of organisms but is a potential feature for organisms that belong to both the domains *Bacteria* and *Archaea* using a wide range of different metabolic pathways (Canfield et al., 2005). Although these organisms can be either aerobic or anaerobic, the nitrogenase is irreversibly inhibited by molecular oxygen and must therefore be contained in a strictly anoxic environment (Fay, 1992 and references therein). Many filamentous cyanobacteria have solved this problem by heterocysts, specialized cells with thick cell walls that physically limit oxygen diffusion and keep the levels low in nitrogenase containing environments. The heterocysts also differ from the vegetative cells of the filament in that they do not contain photosystem II and thus do not produce oxygen (Fay, 1992 and references therein). Other principles to avoid exposure to oxygen (like in the cyanobacterium *Trichodesmium*) include for example a separation of photosynthesis and nitrogen fixation in time (Berman-Frank et al., 2001a). Furthermore, fixation of N_2 is exergonic (i.e. energy yielding, $\Delta G° < 0$; see section 3.2) at conditions of standard state. To break the triple bond of N_2 ($N \equiv N$) however, requires a significant input of energy.

$$3H_2 + N_2 \rightarrow 2NH_3, \qquad \Delta G° = -33 \text{ kJ mol}^{-1}\, N_2 \qquad (1)$$

Nitrogen fixation is achieved industrially by the "Haber-Bosch" process using high temperature, elevated pressure and the addition of catalysts. In natural environments, nitrogen fixation is mediated enzymatically by the nitrogenase complex with the expense of ATP (Canfield et al., 2005).

$$N_2 + 9H^+ + 8e^- + 16ATP \rightarrow 2NH_4^+ + H_2 + 16ADP \qquad (2)$$

Globally, N_2 fixation in marine environments occurs predominantly in the open ocean with rates on the order of $140 \cdot 10^{12}$ g N y^{-1} (Galloway et al., 2008). *Trichodesmium* and the heterocystic endosymbiont *Richelia* have for long been thought to be the main diazotrophs in marine systems (Zehr et al., 2008). Recently, however, small N_2-fixing unicellular cyanobacteria have been shown to be abundant and potentially important for the global marine N-budget. One group of these global-

ly distributed small cyanobacteria ("UCYN-A") apparently lack photosystem II. They further appear to be photoheterotrophic, generating ATP through photosystem I and seem to lack genes for C-fixation (Zehr et al., 2008) .

Overall, reported rates of nitrogen fixation are comparably low in relation to rates observed for most other pathways during the internal cycling of nitrogen in marine systems (Kirchman, 2012). One factor that may explain that rates of nitrogen fixation and abundance/diversity of diazotrophs are comparably low in relation to the theoretical advantage in nitrogen limited systems is the high energy cost to break the triple-bond of N_2. The high energy cost also explain why high concentrations of ammonium and nitrate often lead to a switch from nitrogen fixation by diazotrophs to the use of dissolved inorganic nitrogen (e.g. NH_4^+ and NO_3^-) as sources of nitrogen. Limitation of N fixation in marine systems is widely associated with requirements of iron (Fe) (Martin, 1990; Berman-Frank et al., 2001). Due to presumed elevated growth requirements of Fe, combined with low atmospheric dust deposition in large parts of the oceans, a major part of diazotrophs in these environments are Fe-limited (Falkowski, 1997; Berman-Frank et al., 2001). Additionally phosphorous (P) seems to be limiting in some areas where iron supply is higher and where diazotrophs also have been shown to be co-limited by P and Fe (Wu et al., 2000; Sañudo-Wilhelmy et al., 2001; Mills et al., 2004).

2.2. Ammonification and ammonium assimilation

Nitrogen is part of organic matter mainly in the reduced amino form (proteins and nucleotides). When organic matter is hydrolyzed and catabolized by heterotrophic organisms, nitrogen is predominately released as NH_4^+. This process is called ammonification or nitrogen mineralization (Herbert, 1999). The released NH_4^+ can either be oxidized or assimilated and incorporated into organic molecules by a variety of aerobic and anaerobic organisms (Herbert, 1999). The rate-limiting step in the mineralization process is the extra-cellular hydrolysis of organic macromolecules. Oligopeptides, amino acids, oligonucleotides and nucleotides resulting from hyrdrolyzation are deaminated by intracellular fermentative and respiratory processes resulting in the release of NH_4^+ (Canfield et al., 2005). Ammonification occurs in both oxic and anoxic aquatic environments and is always coupled to heterotrophic carbon mineralization (Herbert, 1999). The relative importance of aerobic and anaerobic nitrogen mineralization largely depends on the depth of the water column. In shallow coastal areas up to 50% of organic matter mineralization may proceed anaerobically (Canfield et al., 2005). In contrast, in deep sea environments more than 99% of organic matter mineralization occurs in the water column (Suess, 1980). Nitrogen-containing polymers are generally degraded more easily than car-

bon-containing structural cell components like e.g. cellulose and lignin. This preferential nitrogen mineralization results in a gradual increase in C:N ratio of the organic matter remaining to be further mineralized. (Blackburn and Henriksen, 1983).

Nitrogen mineralization is closely coupled to assimilation of ammonium and the net release of NH_4^+ is defined as the difference between gross mineralization and assimilation (Blackburn and Henriksen, 1983). Based on $^{15}NH_4^+$ experiments, assimilation is for example estimated to consume ~30% of the mineralized NH_4^+ in coastal sediments (Blackburn and Henriksen, 1983). Furthermore, reversible adsorption equilibrium of ammonium between pore water and sediment particles is often considered to remove ~50% of ammonium released during benthic mineralization (Mackin and Aller, 1984).

2.3. Nitrification

Ultimately, nitrification describes the sum of processes that lead to the oxidation of NH_4^+ to NO_3^-. Complete nitrification is in practice comprised by two separate reactions, driven by two different functional groups of microorganisms producing a set of nitrogen intermediates (e.g. N_2O and hydroxylamine, NH_2OH; Ward, 2008). The oxidation of NH_4^+ to NO_2^- is governed by the ammonium oxidizing bacteria (AOB) or archaea (AOA), and the subsequent oxidation of NO_2^- to NO_3^- is catalyzed by the nitrite oxidizing bacteria (NOB; Ward, 2008). Nitrification is important for nitrogen dynamics in aquatic environments since the process links the most reduced (NH_4^+) to the most oxidized (NO_3^-) forms of the nitrogen cycle. It thereby provides the key reactant for denitrification and promotes loss of nitrogen from the system (Herbert, 1999). Ammonium and nitrite oxidation during conventional nitrification are aerobic catabolic processes, i.e. N species are oxidized, providing electrons for the energy yielding respiratory chain where molecular oxygen (O_2) serves as electron acceptor.

2.3.1. Ammonium oxidation

Until recently, NH_4^+ oxidation has been considered mainly mediated by aerobic chemolithotrophic (deriving energy from inorganic material) bacteria of the betaproteobacterial genera *Nitrosomonas* (Winogradsky, 1892) and *Nitrosospira* (Winogradsky and Winogradsky, 1933) as well as the gammaproteobacterial *Nitrosococcus* (Winogradsky, 1892). The overall oxidation is written as (Canfield et al., 2005):

$$NH_4^+ + 1/2O_2 \rightarrow NO_2^- + H_2O + 2H^+, \qquad \Delta G^\circ = -272 \text{ kJ mol}^{-1}\ NH_4^+ \qquad (3)$$

This oxidation proceeds in at least two steps with hydroxylamine (NH_2OH) as an intermediate. The first step, involving two electrons, is the oxidation of NH_4^+ to NH_2OH (Canfield et al., 2005):

$$NH_4^+ + 1/2O_2 \rightarrow NH_2OH + H^+, \qquad \Delta G° = +17 \text{ kJ mol}^{-1}\ NH_4^+ \qquad (4)$$

The initial reaction of ammonium oxidation is catalyzed by the membrane-associated enzyme ammonium monooxygenase (AMO; Hollocher et al., 1981; Hyman and Wood, 1985). NH_2OH is normally reactive in aqueous solution and the subsequent oxidation, including four electrons, is catalyzed by the periplasmic enzyme hydroxylamine oxidoreductase (Canfield et al., 2005):

$$NH_2OH + O_2 \rightarrow NO_2^- + H_2O + H^+, \qquad \Delta G° = -289 \text{ kJ mol}^{-1}\ NH_2OH \quad (5)$$

Aerobic ammonia oxidizers may also be important in the production of N_2O, a highly potent greenhouse gas (Anderson and Levine, 1986). The aerobic production of N_2O may be the result of two different reactions: oxidation of NH_2OH, or reduction of NO_2^- to N_2O during "aerobic denitrification" by aerobic ammonia oxidizers (section 2.4.; Poth and Focht, 1985; Stein and Yung, 2003; Schmidt et al., 2004).

Ammonium oxidation by supposed members of marine *Thaumarchaeota*, a recently defined kingdom within the prokaryotic domain *Archaea*, has recently been brought to attention as potentially important for the marine nitrogen cycle (Francis et al., 2005; Nicol and Schleper, 2006). Originally considered constrained to extreme environments including halophiles, thermofiles and methanogens (Woese, 1987), the discovery of archaea in marine environments dramatically challenged the conceptual ideas of controlling mechanisms during key processes involved in element cycling (DeLong, 1992; Fuhrman, 1992; DeLong et al., 1994; Stein and Simon, 1996; Karner et al., 2001). Although nonextremophilic archaea (kingdoms Chrenarcheota and Euryarchaeota) are widespread, there is limited knowledge about their physiology and biogeochemical function (Francis et al., 2005). However, Venter et al. (2004) discovered a unique archaeal ammonia monooxygenase gene when investigating genomic diversity of microorganisms in the Sargasso Sea. This observation implied that archaeal organisms are able to perform ammonium oxidation. Ammonium oxidizing archaea (AOA) has since then been verified and found to be ubiquitous and even dominant during nitrification in several marine environments (Francis et al., 2005; Mincer et al., 2007; Agogue et al., 2008; Beman et al., 2008; Kalanetra et al., 2009). Several species have been identified by genomic studies but only one species of AOA has yet been cultivated under laboratory conditions. This organism is a marine, apparently autotrophic, member of the Thaumarcheota, *Nitrosopumilus maritimus* (Könneke et al., 2005).

In addition to ammonium oxidation by autotrophic organisms there are heterotrophic bacteria capable of oxidizing ammonium. Heterotrophic nitrification is usually coupled to aerobic denitrification (section 2.4) and consumes energy rather than conserving it. The significance of heterotrophic nitrification in natural systems is not well known but this pathway during nitrogen mineralization is thought to be of minor importance in aquatic systems. (see e.g. Robertson and Kuenen, 1990; Ward, 2008)

2.3.2. Anoxic nitrification

Thermodynamic calculations and laboratory experiments have provided indications of chemolithotrophic oxidation of NH_4^+ by manganese (IV) oxide (MnO_2) under anoxic conditons (Luther et al., 1997; Hulth et al., 1999; Bartlett et al., 2008; Javanaud et al., 2011). Two main pathways have been suggested, forming either N_2 or NO_2^- /NO_3^- as end-products (Luther et al., 1997).

$$2NH_4^+ + 3MnO_2 + 4H^+ \rightarrow 3Mn^{2+} + N_2 + 6H_2O, \qquad \Delta G° = -295 \text{ kJ mol}^{-1}\ NH_4^+ \qquad (6)$$

$$NH_4^+ + 4MnO_2 + 6H^+ \rightarrow 4Mn^{2+} + NO_3^- + 5H_2O, \qquad \Delta G° = -317 \text{ kJ mol}^{-1}\ NH_4^+ \qquad (7)$$

Luther et al. (1997) also suggested that iron-oxides (e.g. FeOOH (s)) could serve as a possible oxidant of ammonium in ecosystems with low pH. Although mechanistically described from laboratory manipulations and that isolated bacteria mediating NH_4^+ oxidation by MnO_2 have been identified (Javanaud et al., 2011), the overall significance of these processes for N and Fe/Mn cycling is yet to be verified. Field and laboratory ^{15}N experiments in sediments of a wide range in organic matter reactivity and content of manganese oxides have revealed inconclusive results on the importance of this process (Thamdrup and Dalsgaard, 2000; Engström et al., 2005; Paper II; Hulth pers. comm.)

2.3.3. Nitrite oxidation

NO_2^- oxidation to NO_3^- is primarily accomplished by bacteria that belong to the unrelated genera *Nitrobacter, Nitrococcus, Nitrospina and Nitrospira* (Winogradsky, 1891; Watson and Waterbury, 1971; Watson et al., 1986). These organisms are all capable of chemolithoautothrophic growth, but the alphaproteobacterial genus *Nitrobacter* also implements a heterotrophic metabolism (i.e. organic carbon is the source of C; Delwiche and Finstein, 1965; Smith and Hoare, 1968; Bock, 1976). *Nitrococcus* and *Nitrospina* belong to the Gamma- and Deltaproteobacteria respectively while *Nitrospira* makes up its own phylum (Teske et al., 1994; Ehrich et al., 1995). The NO_2^- oxidation can be described as (Canfield et al., 2005):

$NO_2^- + 1/2O_2 \rightarrow NO_3^-$, $\Delta G° = -76$ kJ mol^{-1} NO_2^- (8)

This reaction is catalyzed by the membrane-bound enzyme nitrite oxidase (Canfield et al., 2005). Although most information on nitrite oxidation stems from studies focused on *Nitrobacter*, *Nitrospira*-species seem the most abundant in waste water and natural systems such as soil and freshwater sediments (Wagner et al., 1996; Bartosch et al., 2002; Altmann et al., 2003). If this is true also for marine environments still remains to be investigated.

Recently, anaerobic oxidation of nitrite to nitrate by phototrophic sulfur bacteria and purple nonsulfur bacteria was demonstrated. In these reactions, nitrite served as electron donor for anoxygenic photosynthesis (Griffin et al., 2007; Schott et al., 2010). The qualitative and quantitative importance of this pathway in natural environments is, however, not well known.

2.4. Denitrification

Denitrification usually refers to the biological process where NO_3^- is reduced to gaseous products (e.g. N_2O or N_2) by heterotrophic bacteria. In contrast to nitrification, denitrification is mainly considered a strictly anaerobic process confined to sediments below the oxic/anoxic interface or in O_2 depleted zones of the water column (Canfield et al., 2005). Reported exceptions include for example the mixotrophic nitrate reducing bacterium *Paracoccus pantotrophus* capable of simultaneously using NO_3^- and O_2 as electron acceptors in up to 90% of air saturation (Robertson and Kuenen, 1984; Robertson et al., 1995) These bacteria also seem capable of heterotrophic nitrification (section 2.3; Robertson et al., 1995). The biogeochemical significance of "aerobic denitrification" is however presently under debate (Ward, 2008; Chen and Strous, 2013). Denitrification is considered a globally important sink for nitrogen since the reaction converts fixed nitrogen to N_2, thus removing it from the system. The process includes a number of respiratory reduction steps (Figure 1) that basically require four enzymes: nitrate reductase (nar), nitrite reductase (nir), nitric oxide reductase (nor) and nitrous oxide reductase (nos). The heterotrophic catabolism of organic material (CH_2O) can be schematically described as:

$5/4CH_2O + NO_3^- + H^+ \rightarrow 5/4CO_2 + 1/2N_2\ 7/4H_2O$, $\Delta G° = -635$ kJ mol^{-1} NO_3^- (9)

Denitrifiers are a diverse group of organisms that belong to several unrelated prokaryotic phyla. Most are facultative anaerobic heterotrophs, i.e. energy is produced by aerobic respiration if oxygen is present. However, under low-oxygen and anoxic conditions they can switch to an anaerobic lifestyle reducing NO_3^- instead of O_2. The

first step of denitrification (dissimilatory reduction of nitrate to nitrite) may, depending on environmental conditions, proceed by further reduction (often by the same organism) to NO, N_2O, and N_2, (or directly to NH_4^+; see section 2.5.). Dissimilatory reduction of nitrate may also stop at nitrite which is then excreted (Zumft, 1997).

Recently it was discovered that eukaryotic organisms within a group of unicellular protists called *Rhizaria* are also capable of denitrification (Risgaard-Petersen et al., 2006). Several genera of the Foraminifera and the genus *Gromia* have been found to be able to assimilate and store NO_3^- which they use during denitrification (Risgaard-Petersen et al., 2006; Høgslund et al., 2008; Piña-Ochoa et al., 2010). These organisms are regarded facultative anaerobes (Piña-Ochoa et al., 2010). This unique feature among eukaryotes is present in many different species inhabiting a wide range of marine habitats. Since they are a widespread and abundant group this discovery may have significant implications for the marine nitrogen cycle on local and global scales (Piña-Ochoa et al., 2010). Measurements of denitrification rates together with observations of e.g. foraminiferal abundance suggests that eukaryotic denitrification locally may contribute up to 70% of total N_2 production (Piña-Ochoa et al., 2010).

In addition to heterotrophic denitrification there are a number of other processes that produce N_2 from NO_3^-. Some are, depending on concentrations of reactants at *in situ* conditions, not thought to be quantitatively important in marine environments. Examples include, for example, the abiotic reduction of NO_2^- at pH ≤5 (e.g. Van Cleemput et al., 1976). Chemolithotrophic denitrifying processes of unknown quantitative importance include for example the oxidation of hydrogen (Smith et al., 1994), hydrogen sulfide (Aminuddin and Nicholas, 1973), thiosulfate (Ishaque and Aleem, 1973), ferrous iron (Benz et al., 1998), and methane (Islas-Lima et al., 2004).

2.5. Dissimilatory nitrate/nitrite reduction to ammonium (DNRA)

Dissimilatory nitrate/nitrite reduction to ammonium (DNRA or nitrite ammonification) may occur under the same environmental conditions as denitrification but does, however, not lead to a loss of nitrogen since N_2 is not produced (Canfield et al., 2005). Nitrite reduction to ammonium are used by organisms for detoxification of NO_2^- (Page et al., 1990) but can also be used as an electron sink during fermentation (Cole and Brown, 1980). Some NO_2^- ammonifiers are also true respirers, reducing NO_2^- to NH_4^+ for conservation of energy (Hasan and Hall, 1975; Sørensen, 1978; Simon, 2002). The first step of both DNRA and denitrification is dissimilatory reduction of nitrate and a biochemical separation of the two processes is first made after

the formation of NO_2^-. Dissimilatory reduction of NO_2^- to NH_4^+ utilizes a few different electron donors (e.g. formate, hydrogen and sulfide) and species capable of this have been found in gamma-, delta- and epsilon-proteobacteria (Simon, 2002). DNRA activity is usually most prominent in highly reduced environments, particularly in presence of free sulfide which seems to be a primary electron donor for dissimilatory nitrite reduction to ammonium and inhibits heterotrophic denitrification (Brunet and Garcia-Gil, 1996; Otte et al., 1999; An and Gardner, 2002; Burgin and Hamilton, 2007). However, recent studies imply that DNRA can be significant in oxygen minimum zones where it is suggested as a pathway of NH_4^+ mobilization for anammox (Hamersley et al., 2007; Lam et al., 2009). In the Peruvian OMZ, where no denitrification could be detected, 67% of the required NO_2^- by anammox came from NO_3^- reduction. DNRA was also detected and supplied significant amounts of NH_4^- for the anammox bacteria (Lam et al., 2009).

2.6. Anammox

Comparatively recently the process of anaerobic oxidation of ammonium (anammox) was identified and acknowledged as a highly important sink for fixed nitrogen in marine environments (Mulder et al., 1995; Thamdrup and Dalsgaard, 2002; Kuypers et al., 2003) Anammox is catalyzed by obligate anaerobic chemolithoautotrophic bacteria that belong to a monophyletic group of the phylum *Planctomycetes* (Strous et al., 1999aa). The reaction can be classified as a denitrifying process since it reduces nitrite and removes fixed nitrogen from the system (Ward, 2008). Detailed reaction mechanisms as well as character and function of anammox bacteria are further discussed in chapters 3 and 4 as well as in paper I-IV.

3. Factors Controlling the Marine Nitrogen Cycle

Biogeochemical processes of qualitative and quantitative importance during nitrogen cycling are tightly coupled to the redox state of the environment and are therefore often classified as oxidation-reduction (redox) reactions. Which processes that control nitrogen transformations under the wide suite of environmental conditions that exist in nature and what kind of microorganisms that mediate these reactions mainly rely on the availability and balance between oxidants and reductants. Concentrations of reactants provide the fundamental base for the thermodynamic state of the environment and thus the energy balance of reactions. As microorganisms utilize energy from reactions for growth and maintenance and as there are direct feedbacks between microbial communities and concentrations of reactants, it is important to acknowledge rates and pathways as well as structure and function of the microbial communities to fully understand factors that control N cycling in marine environments.

3.1. The importance of microorganisms

Microorganisms refer to all single-celled organisms which include representatives from all the three domains of life, *Bacteria, Archaea and Eukarya* (Sherr and Sherr, 2000). *Bacteria* and *Archaea* combined are defined as "prokaryotes", originating from that they normally lack internal membrane bound compartments and a nuclear envelope (Madigan et al., 2000). The prokaryotic organisms dominate microbial abundance and activity in marine systems (Fenchel et al., 1998). In marine water columns abundance of microorganisms typically vary between 10^4 – 10^6 cm^{-3}, and in surface sediments the abundance is usually within the range of 10^8 – 10^{10} cm^{-3} (Canfield et al., 2005). Deeper down in the sediment, there is a progressively decreasing abundance of microorganisms due to the progressively decreasing reactivity of organic matter and, therefore, decreasing net yield of energy from reactions. There may also be consequences from the downward decreasing surface area to volume ratio of particles. However, studies reveal that hundreds of meters down, the abundance can still be in the order of 10^6 – 10^7 cm^{-3} (Parkes et al., 2000). Mineralization of organic matter in marine environments is mainly catalyzed by microorganisms (Fenchel et al., 1998) and a large fraction of organic carbon produced is eventually reoxidized by heterotrophic organisms. Mineralization rates in sediments are usually higher than in the water column as a direct consequence of the higher microbial abundance, primarily due to higher concentrations and availability of organic matter and nutrients (Fenchel et al., 1998).

There is a wide diversity of lifestyles among microorganisms in natural environments. A basic division of metabolic pathways includes *autotrophy* (i.e. C-requirements for growth are obtained from CO_2 and reaction energy is provided by chemical reactions or light) and *heterotrophy* (C-requirements for growth are derived from C-containing organic compounds). Organisms using light as energy source for the production of ATP are referred to as *phototrophic,* and those that gain energy from the oxidation of chemical compounds are denoted *chemotrophic* organisms (Sherr and Sherr, 2000). Further distinction between organisms can be made focusing on the source of elements and electrons for biosynthesis. *Lithotrophic* organisms use inorganic sources while *organotrophic* organisms require organic compounds. Microorganisms able to combine different lifestyles are called *mixotrophic* (Sherr and Sherr, 2000). The diverse set of redox reactions of importance for nitrogen cycling is associated with organisms that can be assigned to a wide combination of lifestyles.

3.2. Redox conditions and availability of oxidants and reductants

Rates and pathways of biogeochemical processes mediated by benthic microorganisms are to a large extent controlled by particulate organic matter (POM) reaching the sea floor (Fenchel et al., 1998). The quantity and quality of this organic matter deposited on the sediment surface are in turn related to properties related to the depositional environment, e.g. physical regime, water depth, temperature and availability of reactants. In shallow coastal ecosystems, a substantial fraction of organic material produced in the photic zone reaches the sediment surface (Berelson et al., 1996) while in the deep parts of the oceans up to 99% of organic material exported from the euphotic zone is degraded in the water column (Suess, 1980). The large amount of organic matter reaching the sediment in coastal environments is often associated with high rates of oxygen consumption during benthic mineralization (as O_2 is the preferred oxidant for mineralization; see below). Passive diffusion of dissolved oxygen from the overlying bottom water results in a penetration depth of just a few millimeters into the surface sediment layer (Revsbech et al., 1980). However, sediment-living macrofauna oxygenate the surface sediment through their feeding, bioirrigatiing, burrowing and tube constructing activities (Aller, 1982). As a consequence macrofauna activity in sediments is generally believed to influence rates and extent of the organic matter mineralization (Aller, 1982).

Respiratory processes during organic matter mineralization are mainly controlled by the net energy yield from reactions oxidizing organic carbon (Claypool and Kaplan, 1974; Froelich et al., 1979). Considering standard state (25˚C, 1M con-

centration for aqueous species and 1 atm pressure for gases) the change in Gibbs free energy ($\Delta G°$) can be calculated as:

$$\Delta G° = \Delta H° - T\Delta S°$$

Where ΔH^o is the change in enthalpy, T is temperature (K) and $\Delta S°$ is the change in entropy at standard state. $\Delta S°$ is >0 and $\Delta G°$ is <0 for a spontaneous reaction. A reaction with a negative $\Delta G°$ is known as an exergonic reaction from which energy is released. In contrast, if $\Delta G°$ is positive the reaction requires energy to proceed in the direction it is written (endergonic). As a direct consequence, however, it is spontaneous in the opposite direction.

Conditions of standard state are normally not observed in natural environments. For example, concentrations of chemical species are generally orders of magnitude less than the ideal unit molar concentration. For any component of a system that deviates from conditions at standard state the free energy of that component can be calculated as:

$$\Delta G = \Delta G°_f + R \times T \times \ln a_i$$

where $\Delta G°_f$ is the change in Gibbs free energy of formation, R is the gas constant (=8,314 J mol^{-1} K^{-1}), T is the temperature (K) and a_i is the activity of species i. a_i is related to concentration (c) through the activity coefficient (γ)

$$a_i = \gamma_i c_i$$

The activity coefficient takes into account the deviations from ideal behavior. The same principle is valid for gases. When a moles of the species A react with b moles of the species B to form c moles of species C and d moles of species D the general reaction evolution can be expressed as:

$$a\mathrm{A} + b\mathrm{B} \rightarrow c\mathrm{C} + d\mathrm{D}$$

The free energy of the reaction (r) is calculated from:

$$\Delta G_r = \Delta G°_f + R \times T \times \ln \frac{a_C^c a_D^d}{a_A^a a_B^b}$$

The probability for certain reactions to take place (ΔG_r) controls the dynamic evolution of reactants in natural environments. In sediments for example, the theoretical

energy yield of respiratory processes results in a well-defined vertical zonation of solutes where the most preferential oxidant (i.e. yielding the most profitable free energy from reaction) is primarily utilized (Burdige, 2006; Table 2). However, since this classical model is described by the net change of free energy at steady state ($\Delta G°$) it is a simplification of in situ conditions and the multi-dimensional distribution of biogeochemical processes in natural environments (Burdige, 2006). Concentrations and accessibility of reactants thus control the thermodynamic succession of reactions in sediments. The comparably high concentrations of oxygen (~250 µM) in seawater, in addition to its high energy yield and wide distribution in the ocean, explain why oxygen is a favourable and preferred oxidant for a major part of organic matter mineralization in marine environments. Nitrate concentrations and rates of supply are generally significantly lower than for oxygen. Although nitrate reduction is an important sink for fixed nitrogen, it is a less important pathway for the oxidation of organic material compared to mineralization using oxygen as electron acceptor (Kirchman, 2012). Similarly, the high concentrations of sulfate ($[SO_4^{2-}]$ = 28 mM) in sea water explain why sulfate reduction is a comparably important pathway for organic matter oxidation in marine environments. Solid phase or colloidal iron oxyhydroxides (FeOOH) and manganese oxides (MnO_2) are also often abundant and therefore important for organic matter mineralization. However, oxidant reactivity can be hampered by their chemical form (Kirchman, 2012). For example, particulate oxides cannot be transported across cell membranes into the bacterial cell. Iron reducing bacteria therefore need to employ other strategies to transport electrons from organic material to the oxidizing agent (e.g. Weber et al., 2006; Roden et al., 2010)

Table 2. The diagenetic sequence of reactions that oxidize organic matter (CH_2O) in marine environments. Reactions are listed in order of decreasing yield of free energy ($\Delta G°$) during standard state. $\Delta G°$ values are in kj mol^{-1} CH_2O. After Berner (1980).

Pathway	**Reaction**	**ΔG°**
Oxygen respiration	$CH_2O + O_2 \rightarrow CO_2 + H_2O$	-475
Denitrification	$CH_2O + \frac{4}{5}NO_3^- \rightarrow \frac{4}{5}HCO_3^- + \frac{1}{5}CO_2 + \frac{2}{5}N_2 + \frac{3}{5}H_2O$	-448
Manganese reduction	$CH_2O + 3CO_2 + H_2O + 2MnO_2 \rightarrow 2Mn^{2+} + 4HCO_3^-$	-349
Iron reduction	$CH_2O + 7CO_2 + 4Fe(OH)_3 \rightarrow 4Fe^{2+} + 8HCO_3^- + 3H_2O$	-114
Sulfate reduction	$CH_2O + \frac{1}{2}SO_4^{2-} \rightarrow \frac{1}{2}H_2S + HCO_3^-$	-77
Methanogenesis	$CH_2O \rightarrow \frac{1}{2}CH_4$	-58

In addition to aerobic mineralization of organic material there is a suite of chemolithoautotrophic and abiotic oxidative processes that include reactants that originate from anaerobic organic matter remineralization (Burdige, 2006). In this sense, O_2 can indirectly be the oxidant for organic matter that is remineralized during anaerobic metabolisms (Burdige, 2006). In sediments this spatial coupling is facilitated by diffusive processes, although macrofaunal activities (i.e. bioturbation and bioirrigation; e.g. Hulth et al., 1999) and high-energetic physical reworking processes can enhance this. Observations of novel alternative metabolic pathways (e.g. anammox and anaerobic nitrification by Mn-oxides) challenge the classical view of nitrogen mineralization and further enlighten the simplification of well-defined sequential patterns of mineralization.

3.3. N-cycling and anammox – local and global perspectives

Following the original discovery in a waste-water reactor (Mulder et al., 1995), the anammox process in natural systems was first observed in sediments of the deepest part of the Skagerrak (Thamdrup and Dalsgaard, 2002). Anammox has since then been detected in a multitude of natural environments including marine sediments, marine water columns and sea ice as well as soil and fresh water systems (Kuypers et al., 2003; Trimmer et al., 2003; Rysgaard and Glud, 2004; Engström et al., 2005; Schubert et al., 2006; Hamersley et al., 2007; Humbert et al., 2010). Until the discovery of anammox, NH_4^+ was considered non-reactive under anoxic conditions and denitrification was thought to be the only sink of fixed nitrogen in marine systems. As of today, extensive research has shown that the anammox process is an important sink for fixed nitrogen not only locally, but also on a global scale. For example, it is estimated that 30-50% of total marine oceanic N_2 production occur in the oxygen minimum zones (Gruber and Sarmiento, 1997; Codispoti et al., 2001). Studies in the Eastern Tropical South Pacific and Eastern Tropical South Atlantic oxygen minimum zones have revealed that anammox can be the dominant or even the only N_2-producing process in large parts of these biogeochemically important environments (e.g. Kuypers et al., 2005; Thamdrup et al., 2006; Hamersley et al., 2007; Lam et al., 2009). Even though denitrification has been shown to dominate total N_2 production in the Arabian Sea (the largest OMZ; Ward et al., 2009), there is still uncertainties regarding the relative importance of anammox and denitrification for total N_2 production in the Arabian Sea as well as in the Estern Tropical North Pacific. Temporal and spatial differences may be important and more investigations of N cycling in these biogeochemically important areas are needed (Lam and Kuypers, 2010). Anammox is also widespread in different estuarine, coastal and offshore sediments (e.g. Thamdrup and Dalsgaard, 2002; Trimmer et al., 2003; Risgaard-

Petersen et al., 2004; Engström et al., 2005; Tal et al., 2005; Paper I) and estimations suggest that its relative contribution to N_2 production is between 25-50%. Locally, however, the relative importance can be up to 80% (Dalsgaard et al., 2005; Engström et al., 2005).

Anammox bacteria derive their energy from the oxidation of NH_4^+ by NO_2^- (van de Graaf et al., 1995). Consequently, the process relies on the availability of these species. NH_4^+ is released during mineralization and while limiting in oxygenated open ocean systems (McCarthy and Carpenter, 1983), it is usually not limiting in benthic environments.. However, in some deep sea sediments NH_4^+ can be depleted in the nitrate reduction zone and here anammox may become limited by ammonium (Trimmer and Engstrom, 2011). NO_2^- rarely accumulates in marine environments and anammox bacteria thus depend on the reduction of NO_3^- by other processes in their proximity (Dalsgaard et al., 2005) or on NO_2^- produced by aerobic ammonia oxidation (Schmidt et al., 2002). In suboxic systems NO_3^- is readily reduced and sediment incubations have demonstrated that NO_3^- reduction is faster than or equal to NO_2^- consumption (Dalsgaard and Thamdrup, 2002; Trimmer et al., 2003; Rysgaard et al., 2004). Thus, NO_2^- is normally not limiting for anammox as long as NO_3^- is available at sufficient concentrations. In anoxic environments, nitrate reducers, often coupled to denitrification and DNRA, are likely candidates for the reduction of NO_3^- to NO_2^- (Dalsgaard et al., 2005). Although DNRA is usually thought to be significant only in highly reduced sediments (e.g. below fishfarms), recent observations in oxygen minimum zones suggest that DNRA can be important in the supply of ammonium to anammox (Kartal et al., 2007b; Lam et al., 2009). Aerobic ammonium oxidizers producing NO_2^- have been shown to exist in close proximity to anammox bacteria in waste water reactors (Schmidt et al., 2002). In these environments nitrite oxidizing bacteria seem to be uncoupled and replaced by anammox bacteria. (Schmidt et al., 2002). The relation between aerobic and anaerobic ammonium oxidation in marine environments is not well constrained. Although these organisms probably compete for ammonia in NH_4^+ limited systems they may well be "natural partners" in ecosystems with limited oxygen supply (Schmidt et al., 2002). Lam et al. (2009) concluded that aerobic ammonia oxidation supplied about 33% of the nitrite needed by anammox bacteria in the Peruvian OMZ suggesting presence of "microaerobic" conditions. Interestly, though both ammonia oxidizing bacteria and archaea were present, a tight association of archaea with anammox activity was observed (Lam et al., 2009). In a laboratory study, mimicking ammonia oxidation in oxygen minimum zones, Yan et al. (2012) also observed cooperation between AOA (as well as AOB) and anammox under oxygen limited conditions.

The relative importance of anammox for total N_2 production in sediments has been shown to be correlated with water column depth (Dalsgaard et al., 2005). This correlation is probably directly connected to the quantity and quality of organic

material reaching the ocean floor. Investigations of anammox in sediments with different rates of mineralization have indicated that there is a negative correlation of relative anammox activity with increasing organic matter reactivity (Thamdrup and Dalsgaard, 2002; Engström et al., 2005; Paper I) This relation might be a result of competition for nutrients between anammox bacteria and the heterotrophic NO_3^- and NO_2^- reducing communities where the latter is supposedly favored in sediments where access to reduced compounds is elevated (Engström et al., 2005). Observations have been made in some estuaries where anammox instead was positively correlated with increasing organic carbon availability but was simultaneously correlated with increasing concentrations of NO_3^- (Trimmer et al., 2003; Nicholls and Trimmer, 2009). Additionally, in shallow-water sediment-systems anammox activity seems suppressed by benthic microalgae (BMA), probably due to a diurnal competition for nutrients and oscillating availability of oxygen and nutrients (Risgaard-Petersen et al., 2004; Risgaard-Petersen et al., 2005; Paper I). NO_3^-/NO_2^- limitation thus seems to be highly unfavorable for anammox in competition with other organisms and the relative significance of anammox for total N2 production increases with water depth. Anammox bacteria are extremely slow growing (Strous et al., 1999bb) which could explain why they seem to favor stable environmental conditions and are suppressed in reactive sediments. Although relative rates of anammox occasionally can be significant in shallow sediments anammox always seems relatively important in deep water environments. If this trend holds true in the major part of the oceans, anammox may be responsible for 2/3 of N_2 production in deep ocean sediments (Dalsgaard et al., 2005).

In Paper I anammox and denitrification activities in the surface sediment were measured using ^{15}N amendments along a depth gradient in the Gullmarsfjord on the Swedish west coast. Observations confirmed earlier observations suggesting that anammox bacteria are absent or of low importance for N removal in environments with primary production and pronounced redox dynamics (Figure 2).

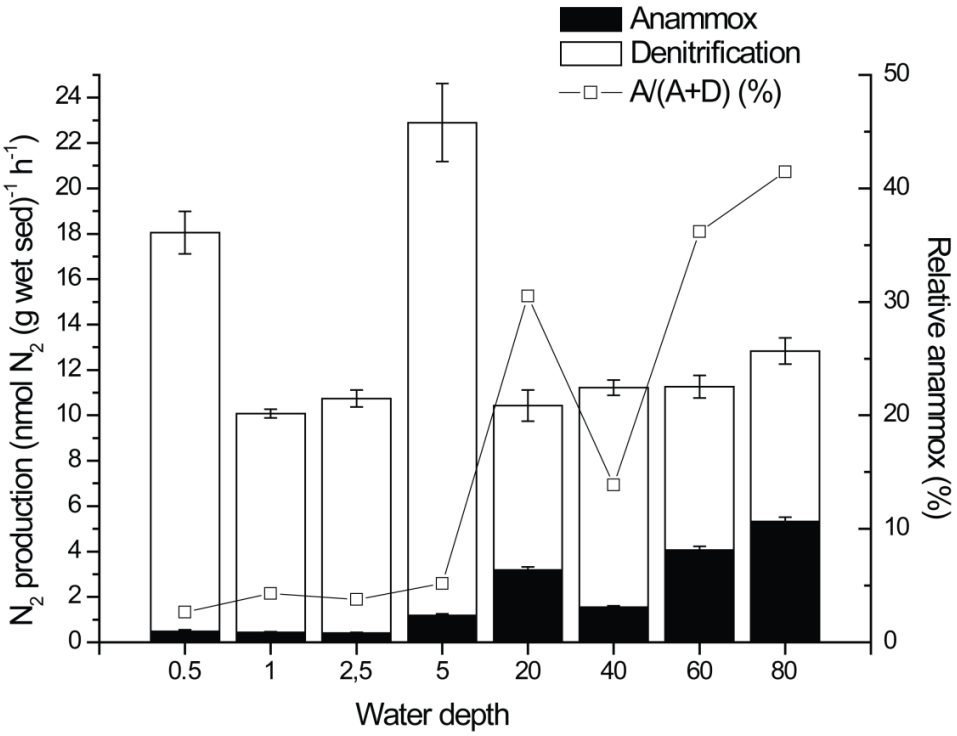

Figure 2. N_2 production rates by anammox and denitrification in sediments along the Sandviken depth transect. The relative importance of anammox seemed strongly and inversely correlated to water depth, mainly reflecting the biogeochemical control by benthic microalgae in autotrophic sediments.

Sediments where anammox is quantitatively important for total N_2 production are most often associated with high concentrations of manganese oxides (Thamdrup and Dalsgaard, 2002; Engström et al., 2005). The overall effects of manganese and iron oxides on the relative importance of anammox and denitrification are however not well known. High concentrations of these metal oxides may extend the inhabitable zone for anammox and heterotrophic denitrification by reducing sulfides. In sediments with high concentrations of Mn(IV) a large amount of organic matter can be oxidized by manganese reduction (Canfield et al., 1993) potentially creating a competitive situation for denitrification and thus favor anammox activity (Thamdrup and Dalsgaard, 2002; Engström et al., 2005). Additionally, reduction of Mn- and Fe-oxides by the anammox bacterium *K. stuttgartiensis* have been observed in laboratory studies (Strous et al., 2006). If the use of metal oxides by marine anammox bacteria is relevant and exactly how the presence of manganese affects the absolute and relative importance of anammox in marine environments still remains to be investigated.

Both anammox and heterotrophic denitrification are inhibited by O_2 and sulfide (S^{2-}) and are therefore usually restricted to a thin zone between oxic and anoxic environments (Trimmer and Engstrom, 2011). Many environments can, however, be

subjected to oscillating conditions with regard to the presence of oxygen e.g. from activities by benthic macrofauna or by phototrophic microalgae (Aller, 1982; McGlathery et al., 2001). Although anammox in waste water systems have been shown to be strictly inhibited by low concentrations of oxygen (1 µM; Strous et al., 1997), marine anammox bacteria seem to be microaerotolerant and have been found to be active at oxygen concentrations up to 10 µM (Kuypers et al., 2005; Jensen et al., 2008).

Hannig et al. (2007) investigated the source of N loss in suboxic waters of the Gotland basin in the Baltic Sea. A massive inflow of oxygenated water caused a shift in nitrogen mineralization from chemolithotrophic denitrification to anammox. A major increase in manganese oxide content due to the inflow of oxygen and subsequent oxidation of hydrogen sulfide by Mn-oxide was suggested to cause this shift and a more favorable environment for anammox. This feedback may have important implications for the controls of anammox and denitrification in manganese rich compared to sulfidic sediments.

In Paper II the availability of oxygen and overall redox status as controlling mechanisms for anammox and denitrification during benthic N mineralization was investigated in surface sediments of the Gullmarsfjord (Alsbäck). Sediment was incubated for 140 days together with overlying water in diffusively open containers (plugs) under continuously oxic, continuously anoxic or oscillating oxic/anoxic conditions in the overlying water . Anammox could not be quantified in sediments continuously exposed to anoxic overlying water. In contrast, and quite surprisingly, anammox was detected in sediments that were completely oxic and continuously exposed to oxygen during the incubation period. Anammox rates were similar under the continuosly oxic compared to the oscillating treatments. Rates of denitrification were similar between the continuous (oxic and anoxic) and the oscillating redox treatments, although a tendency of slightly higher rates in sediments exposed to oscillations between oxic and anoxic conditions in the overlying water. Apparently the anammox bacteria stayed viable during the course of the experiment also under oxic conditions.

4. Structure and Metabolism of Anammox Bacteria

A process where ammonium is removed anaerobically in marine environments has been argued for since the mid sixties. For example (Richards, 1965) observed an unexplainable loss of ammonium under anoxic and suboxic conditions which led to the speculation that NH_4^+ was anaerobically oxidized by NO_3^-. A few years later Broda (1977) predicted that oxidation of NH_4^+ by NO_2^- was plausible to occur in natural environments since it is energetically favorable (Eq 10). In 1995 (Mulder et al.) observed simultaneous depletion of NH_4^+ and NO_3^- with a concomitant increase of N_2 in an anoxic waste water reactor . The same year this phenomenon was found to be a biologically mediated process (van de Graaf et al., 1995). These authors confirmed predictions (Broda, 1977) that NH_4^+ was oxidized by NO_2^- rather than NO_3^-:

$$NH_4^+ + NO_2^- \rightarrow N_2 + 2H_2O, \qquad \Delta G° = -358 \text{ kJ mol}^{-1}\ NH_4^+ \qquad (10)$$

Anammox was attributed to a prokaryotic organism identified as a new order ,*Brocadiales*, branching off deep in the bacterial monophyletic phylum *Planctomycetes* (Strous et al., 1999aa). Since then five different genera of this new order have been identified. *Candidatus* "Brocadia" (e.g. Strous et al., 2002; Kartal et al., 2004), *Candidatus* "Kuenenia" (Schmid et al., 2000), *Candidatus* "Scalindua" (e.g. Kuypers et al., 2003; Schmid et al., 2003), *Candidatus* "Anammoxoglobus" (Kartal et al., 2007a; Liu et al., 2008) and *Candidatus* "Jettenia" (Quan et al., 2008) Candidatus originates from that the bacteria have not been purified by classical standards. So far, marine anammox bacteria observed belong almost exclusively to *Candidatus* "Scalindua" (van de Vossenberg et al., 2013).

Anammox bacteria exhibit several physiological features that are unique or highly unusual relative to other prokaryotes. Although other processes have been identified where ammonium is oxidized under anaerobic conditions (Hulth et al., 1999; Bartlett et al., 2008; Javanaud et al., 2011) anammox is the only process that up to now has been demonstrated to be quantitatively important in marine environments. The stoichiometric reaction of anammox was described by (Strous et al., 1998).

$$1\ NH_4^+ + 1.32\ NO_2^- + 0.066\ HCO_3^- + 0.13\ H^+ \rightarrow 1.02\ N_2 + 0.26\ NO_3^- + 0.066\ CH_2O_{0,5}\ N_{0,15} + 2.03\ H_2O \qquad (11)$$

The main routes of anammox (Figure 3) include the reduction of NO_2^- to NO by a nitrite reductase, the conversion of NO and NH_4^+ to hydrazine (N_2H_4) by a hydrazine synthase (hydrazine hydrolase), the oxidation of hydrazine to N_2 by a hydrazine

oxidizing enzyme (HZO) and the subsequent synthesis of ATP by an ATP synthase using the proton-motive force generated during the anammox cycle (Strous et al., 2006).

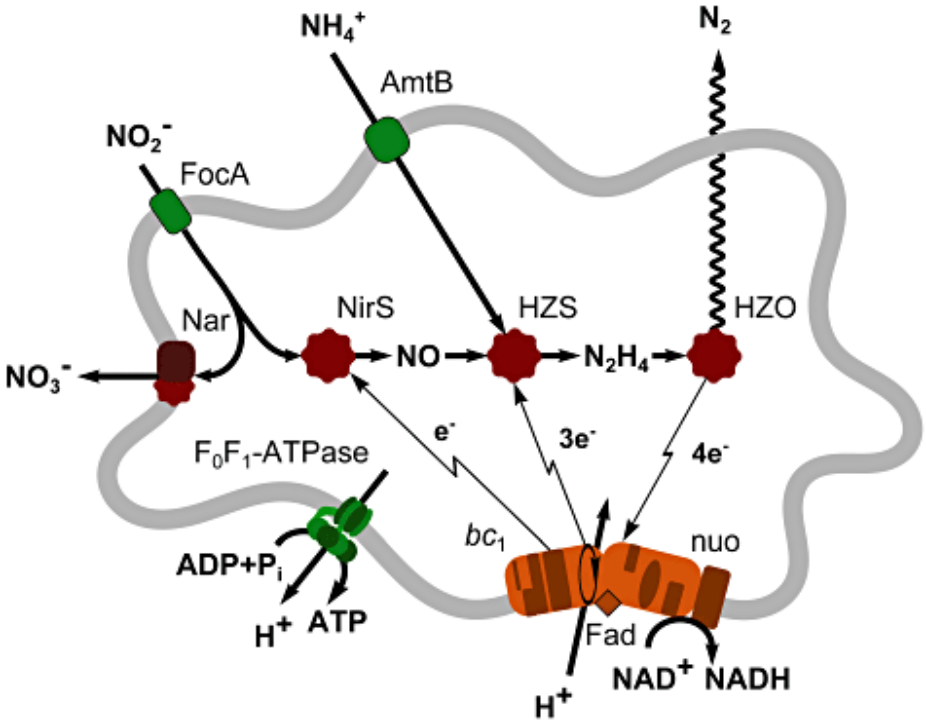

Figure 3. Overview of anammox metabolism in '*Candidatus* Scalindua profunda'. Nar/nxr, nitrite::nitrate oxidoreductase; NirS, nitrite reductase; HZS, hydrazine synthase; HZO, hydrazine oxidoreductase; FocA, nitrite transport protein; amtB, ammonium transport protein; nuo, NADH ubiquinone oxidoreductase (complex I). (Republished with permission of Society for Applied Microbiology and Blackwell Publishing Ltd, from van de Vossenberg et al. (2013); permission conveyed through Copyright Clearance Center, Inc).

The anammox bacteria are exceptionally slow growing, dividing only once in 11-20 days under laboratory conditions (Strous et al., 1999bb). Generation times may be even longer in natural environments under sub-optimal conditions (Jetten et al., 2009).

4.1. Cell plan of planctomycetes

Two basic structural types of living organisms have been recognized, prokaryotes and eukaryotes. Eukaryotic organisms include algae, fungi and protozoa as well as

larger multi-cellular organisms such as animals and plants. Prokaryotes are divided in two major groups of unicellular organisms, *Bacteria* and *Archaea*. In prokaryotic cells the internal organization is relatively simple. There is for example no interior cytoskeleton, which in eukaryotic cells is used for cell support and transport of internal components. An additional major feature of eukaryotic cells is the presence of internal membrane-enclosed structures called *organelles* including a membrane-bound nucleus enclosing the genetic material. Prokaryotes normally lack internal membranous structures with a few exceptions (e.g. Planctomycetes and Cyanobacteria; Fuerst, 2005; Liberton et al., 2006). A typical prokaryotic cell structure includes a cell wall, a cytoplasmic lipid membrane, ribosomes where protein synthesis takes place, occasional inclusions for storage and the nucleoid that contains genetic material (DNA).

Bacteria can be divided in two major groups based on the structure of their cell walls, gram-positive or gram-negative bacteria. Their names originate from a specific staining procedure including a "gram-stain". Gram-positive bacteria are stained because they have a thick cell wall mainly made from the rigid polymer peptidoglycan. The cell wall of gram-negative bacteria is a more complex structure containing only a thin layer of peptidoglycan. Outside this layer, gram-negative bacteria have an outer bilayer membrane of lipids on the inside and lipopolysaccharides on the outside (Madigan et al., 2000). Planctomycete bacteria including all anammox species belong to the gram-negative group (Strous et al., 1999aa). However, the planctomycetes differ from other gram-negative bacteria (and gram-positive bacteria) in that they lack the cell wall polymer peptidoglycan. Instead, their cell wall consists mainly of proteins (König et al., 1984; Liesack et al., 1986; Stackebrandt et al., 1986; Fuerst, 1995). Also, planctomycetes have a cytoplasmic membrane on the inside of the cell wall but, in contrast to other gram-negative bacteria, they do not have an outer lipid/lipopolysaccharide membrane. Typical characteristics for the planctomycetes rather include a cell cytoplasm divided into compartments by an internal membrane, the intracytoplasmic membrane (ICM, Lindsay et al., 2001). Main components in the cell plan of planctomycetes (Figure 4) thus include the proteinaceous cell wall and the cytoplasmic membrane (CM). The space between the CM and the ICM is called the "paryphoplasm" which is devoid of ribosomes and DNA, but has been shown to contain some RNA (Lindsay et al., 1997; Lindsay et al., 2001). The compartment inside the ICM is referred to as the "riboplasm", in some planctomycete genera also called the "pirellusome" (Lindsay et al., 1997). In the genus *Gemmata* and all known anammox genera yet another compartment is present inside the riboplasm. In *Gemmata* this compartment is represented by a nuclear envelope enclosing the nucleoid. The envelope consists of two tightly apposed bilayer membranes thus closely resembling the nucleus of eukaryotic cells (Fuerst and Webb, 1991). In anammox bacteria the intracytoplasmic membranous compartment

has been named the “anammoxosome” originating from that the anammox reaction was suggested to be associated to it (Lindsay et al., 2001). This unique “organelle” takes up the most of the riboplasm and the inside is supposedly devoid of genetic material and ribosomes which is concentrated in the surrounding riboplasm compartment (Lindsay et al., 2001).

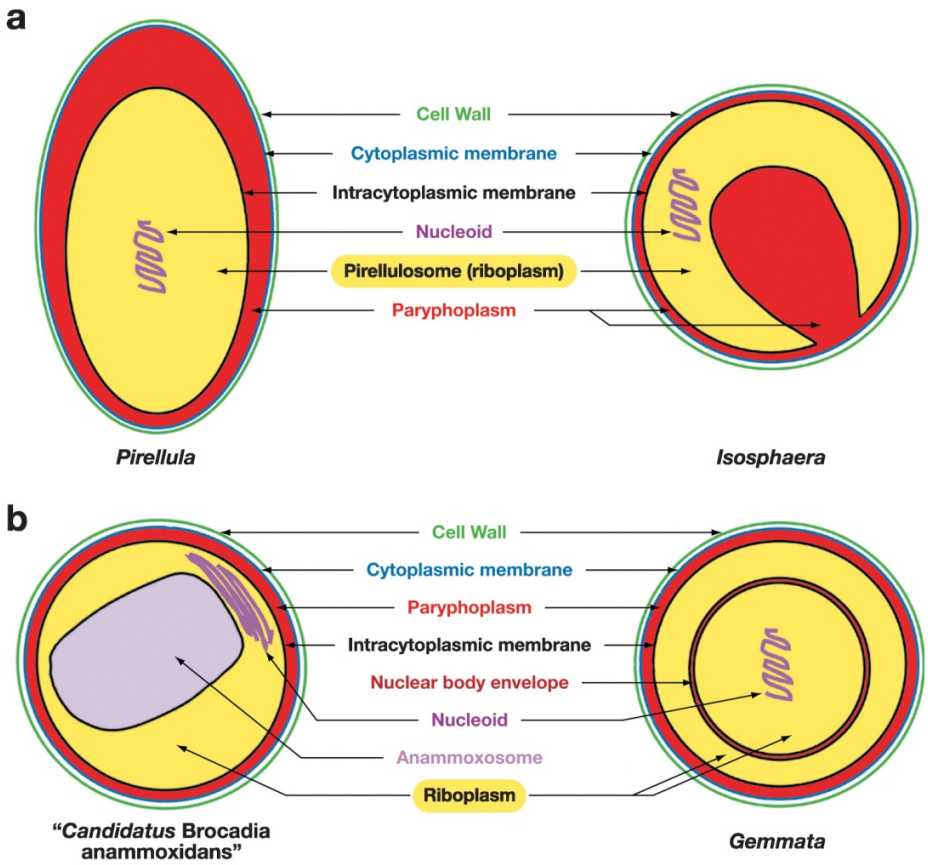

Figure 4. Diagrams of cell organization and compartmentalization in (*a*) *Pirellula* (e.g., *Pirellula staleyi*) and *Isosphaera* (e.g., *Isosphaera pallida*; plan also applies to *Planctomyces maris*) and (*b*) “*Candidatus* Brocadia anammoxidans,” and *Gemmata* (e.g., *G. obscuriglobus*). The varieties of cell compartmentalization found in different planctomycetes, as well as the underlying similarities in topology of their internal organization (i.e., possession of paryphoplasm compartment and intracytoplasmic membrane), are shown. (Republished with permission of Annual reviews, from Fuerst (2005); permission conveyed through Copyright Clearance Center, Inc).

4.2. The anammoxosome – a compartment made from unique lipids

The anammoxosome is one of the most intriguing features of anammox bacteria. The anammoxosome membrane contains unique lipids with sequential structures of four-membered aliphatic cyclobutane rings arranged like a 'staircase' at the end of the hydrocarbon chains (i.e. "ladderane" lipids; Sinninghe Damste et al., 2002; Figure 5). These lipid structures are unprecedented in nature. Another highly interesting feature of these lipids is the presence of ether bonds between the lipids and the glycerol backbone. This is only found in Archea and in a few bacterial species, e.g. deep-branching thermofiles (Sinninghe Damste et al., 2002 and references therein).

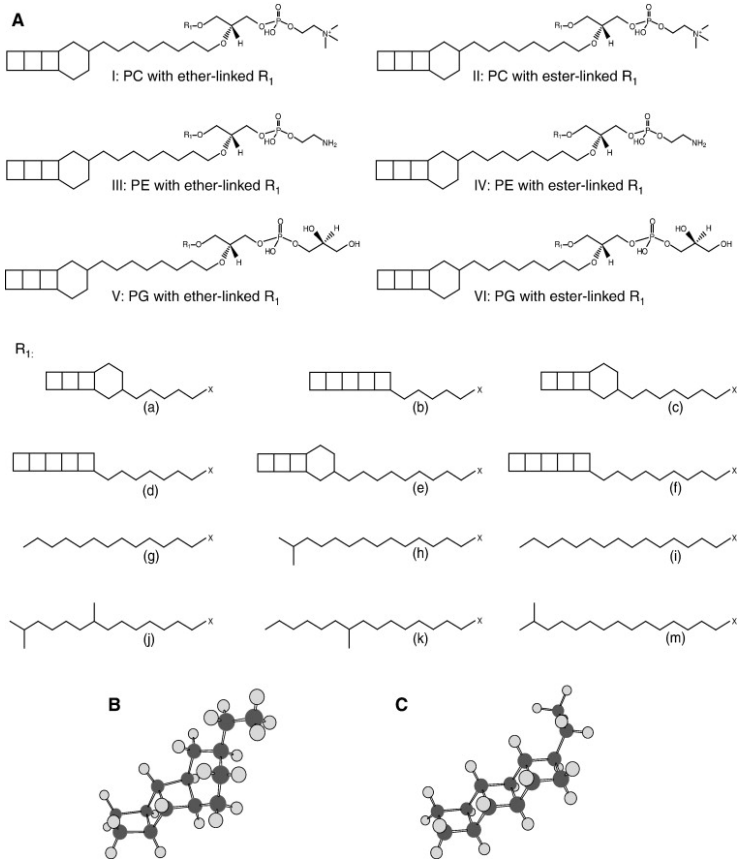

Figure 5. Major phospholipids present in anammox bacteria. (A) The general structures of the PC, PE and PG diether lipids are shown by I, III and V, respectively. Structures II, IV and VI depict the ether–ester lipids of PC, PE and PG, respectively. The R_1 hydrocarbon chain (a–m) are: (a) C_{18}-[3]-ladderane, (b) C18-[5]-ladderane, (c) C20-[3]-ladderane, (d) C_{20}-[5]-ladderane, (e) C_{22}-[3]-ladderane, (f) C_{22}-[5]-ladderane, (g) pentadecane, (h) 14-methylpentadecane, (i) hexadecane, (j) 9,14-dimethylpentadecane, (k) 10-methylhexadecane, (m) 15-methylhexadecane, with X=COOH or CH_3OH. (B) and (C) show the three-dimensional illustration of the [3]- and [5]-ladderane structures, respectively. (Republished with permission of Elsevier, from Boumann (2006); permission conveyed through Copyright Clearance Center, Inc).

The ladderane lipids form an exceptionally dense membrane that likely provides a tight barrier against diffusion (Strous et al., 1999bb). During anammox catabolism, taking place in association with the anammoxosome (paper III and IV), the compound hydrazine (N_2H_4) is produced as an intermediate (van de Graaf et al., 1997). Hydrazine is mutagenic and highly toxic whereby vital parts of the cell, e.g. the genetic material, has to be protected from exposure. Normally, hydrazine diffuses readily across bilayer membranes (Olsen, 1999), but due to the structural properties of ladderane lipids and the resulting lower permeability of the anammoxosome membrane hydrazine is likely retained inside the anammoxosome (Sinninghe Damste et al., 2002). The potential establishment of a proton gradient for energy production, despite the slow anammox catabolism, is another important feature of the anammoxosome that directly relates to the low permeability of the membrane (Sinninghe Damste et al., 2002). Since the ladderane lipids of anammox bacteria are unique in nature they have been targeted as natural candidates for the use as biomarkers in environmental detection of anammox bacteria. In most previous studies however, core lipids (i.e. ladderane alkyl chains lacking the polar headgroup) have been used (Kuypers et al., 2003; Kuypers et al., 2005; Hamersley et al., 2007; Jaeschke et al., 2007). Intact ladderane lipids have been shown to comprise either phosphocoline (PC), phosphoethanolamine (PE) or phosphoglycerol (PG) as the major polar headgroup attached to the glycerol backbone (Boumann et al., 2006; Rattray et al., 2008; Boumann et al., 2009). Intact phospholipids are rapidly decomposed after cell death (White et al., 1979; Harvey et al., 1986) and are therefore thought to be more suitable biomarkers for living and viable microorganisms (Sturt et al., 2004).

4.3. Key proteins of the anammox reaction

As previously described, an important difference between eukaryotic and prokaryotic cells is the presence of intracellular membranous compartments, organelles, in eukaryotes. These are present in all eukaryotic cells and include, for example, the photosynthetic chloroplasts of plants and algae, and the mitochondria where respiration (production of ATP by the oxidation of organic or inorganic material) takes place. In prokaryotic cells the respiratory chain, which consists of a multitude of membrane bound or membrane associated proteins, is normally situated in the cytoplasmic membrane. Generally prokaryotes lack functional organelles, but the anammoxosome of anammox bacteria provides one of the unique exceptions. The presence of a potential energy producing organelle makes anammox bacteria highly unusual and interesting also in an evolutional perspective.

Combined efforts to simultaneously investigate rates and mechanisms of microbial activity, microbial potential and putative functionality provide a unique opportunity to improve the fundamental understanding of controls during N cycling. The discovery that N_2H_4 was an intermediate during anammox catabolism and that it was oxidized to N_2 by a hydroxylamine oxidoreductase-like protein (HAO) (van de Graaf et al., 1997; Schalk et al., 2000), originally led to a postulated pathway involving N_2H_4 and hydroxylamine (NH_2OH). Recently, the metagenome of the anammox bacterium *Candidatus* "Kuenenia stuttgartiensis" was obtained from an enrichment culture (Strous et al., 2006). This enabled the identification of several candidate genes suggesting a number of proteins possibly involved in the catabolic process of anammox (Strous et al., 2006). Key proteins catalyzing the different steps in the anammox process have been suggested leading to a revised biochemical model as described above (Strous et al., 2006). Additionally a nitrate::nitrite oxidoreductase (NarGH) was identified indicating that anammox bacteria also have the potential to oxidize nitrite to nitrate. Experimental evidence has also supported the ability for oxidation of nitrite to nitrate, as well as reduction of nitrate to nitrite (van de Graaf et al., 1997; Schalk et al., 2000; Güven et al., 2005; Kartal et al., 2007b). The oxidation of nitrite is suggested as a source of electrons for CO_2 fixation via the acetyl-coenzyme A (CoA) pathway which was completely detected in the genome (Strous et al., 2006). Also, gene candidates coding for a functional homologue of a dissimilatory nitrite reductase (NrfA) was discovered in the genome. The reduction of NO_2^- to NH_4^+ has been shown to be performed by anammox bacteria under stress (Strous and Jetten, 2004) and explains the experimentally verified capability to produce nitrogen gas in the absence of NH_4^+ with organic acids as the only source of electrons (Güven et al., 2005; Kartal et al., 2007b). Even though it is not known if it is of importance in natural environments, the apparent ability to reduce nitrate to nitrite and ammonium could have impact on the ecological role of anammox bacteria since they would not be solely dependent on the production of nitrite and ammonium from other organisms (Güven et al., 2005; Kartal et al., 2007b).

Efforts have been made in elucidating the role of the anammoxosome and the identification of the responsible proteins in the anammox catabolism. A hydroxylamine oxidoreductase-like protein similar to the HAO of aerobic ammonium oxidizers has earlier been purified in *B. anammoxidans* (Schalk et al., 2000) Similarly, a hydrazine oxidizing enzyme (HZO) was purified from the anammox strain KSU-1 found to have oxidizing activity towards N_2H_4 but not to NH_2OH (Shimamura et al., 2007). The same bacteria was also shown to have a HAO which indicates that both of these proteins are present in anammox bacteria (Shimamura et al., 2008). Lindsay et al. (2001) performed immunogold labeling on the HAO where all positively labeled targets were found inside the anammoxosome. This was the first experi-

mental indication that the anammoxosome membrane is involved in the anammox catabolism.

In this thesis, experiments were made to further pursue the importance of the anammoxosome and to identify and localize key enzymes of anammox catabolism (Paper III and IV) Biomaterial from a waste water treatment plant containing at least 40% anammox bacteria (of which ~80% was identified as *B. anammoxidans*) was analyzed, by proteomics and LC-MS, for peptide sequences associated with proteins suggested as essential for the anammox reaction. Peptides from e.g. the hydrazine synthase (hydrazine hydrolase), the HZO and subunits from an F-ATPase were found. Imunocytochemistry using linear epitopes unambiguously demonstrated labeling of hydrazine hydrolase to the interior of the anammoxosome (Figure 6). This observation further supported the hypothesis that the reaction is associated with the anammoxosome, and that hydrazine is released inside the anammoxosome (Paper III). Furthermore, in paper IV immunocytochemistry and labeling of an F-ATPase using antibodies designed for the α- and β subunits verified association of the ATPase with the anammoxosome membrane (Figure 6). This observation strongly suggests that the anammoxosome is an energized membrane similarly to e.g. the mitochondrion of eukaryotic cells.

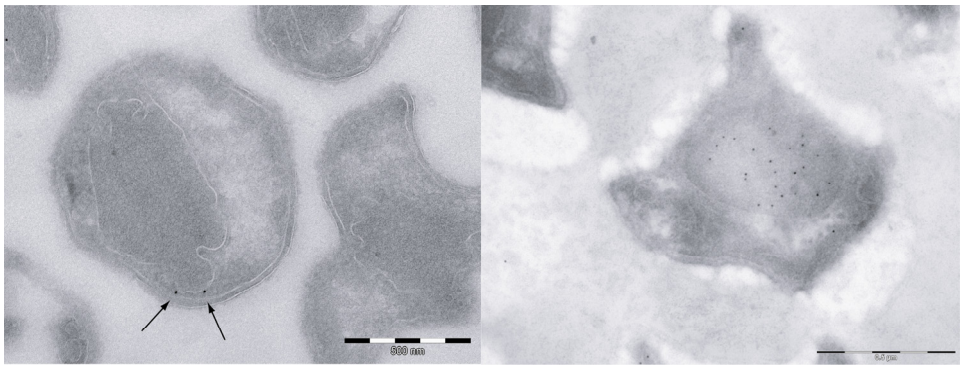

Figure 6. Immunogold labeling of the F-ATPase (paper IV, left) and the hydrazine synthase (hydrazine hydrolase, paper III, right).

5. Techniques for Detection of Anammox Activity and Abundance

5.1. Isotope pairing of ^{15}N

Pairing of the stable isotope ^{15}N following separate ^{15}N amendments has for many years been a standard tool when investigating rates and pathways during N cycling in water and sediment environments (Nielsen, 1992; Thamdrup and Dalsgaard, 2000; Thamdrup and Dalsgaard, 2002).

^{15}N amendments are usually applied on homogenized sediment (slurry), or water samples that are preincubated to consume any in situ O_2 or NO_3^-/NO_2^-. According to the basic principles, three different amendments are made. $^{15}NH_4^+$ (A), $^{15}NH_4^+ + {}^{14}NO_3^-$ (B) and $^{15}NO_3^-$ (C). After time series incubation with these amendments, N_2 gas is extracted and analyzed for specific labeling of ^{15}N ($^{15}N^{14}N$ or $^{15}N^{15}N$) by mass spectrometry. Any ^{15}N labeled gas in A would imply oxidation of NH_4^+ by oxidants other than O_2 or NO_3^-/NO_2^-. Eventual ^{15}N labeled N_2 as ($^{15}N^{14}N$) in amendment B occurs due to a combination of $^{15}NH_4^+$ and $^{14}NO_3^-$ and thus indicates anammox activity. The incubation with only $^{15}NO_3^-$ additions is used to determine rates of potential anammox and denitrification by calculating the production of $^{29}N_2$ (anammox) and $^{30}N_2$ (denitrification) with time of incubation. This approach was used to quantify rates of anammox and denitrification activities in Papers I and II.
Although anaerobic slurry incubations constitutes a frequently used method to quantify pathways of NO_2^-/NO_3^- reduction and the relative importance of anammox and denitrification for total N_2 production, the invasive sampling disrupts natural gradients of reactants and the ambient redox environment. Sampling and handling thus modifie the chemical microenvironment of the microorganisms (Trimmer et al., 2006). Obtained rates should therefore be considered as potential rates of N_2 production.

The isotope pairing technique (IPT) was originally developed by Nielsen (1992) and presents an opportunity to quantify N_2 production in natural environments, including intact sediment cores. According to the original protocol, $^{15}NO_3^-$ (or $^{15}NO_2^-$) is added to the overlying water of the sediment-water system and is allowed to pair with $^{14}NO_3^-$ from the natural pool. Thus $^{28}N_2$, $^{29}N_2$ and $^{30}N_2$ are formed from a combination of the two forms of NO_3^- (Nielsen, 1992). Potential rates can be calculated from the production of $^{29}N_2$ and $^{30}N_2$ and the genuine N_2-production (p_{14}) is then calculated as (Nielsen, 1992):

$$p_{14} = \frac{p^{29}N_2}{2 \cdot p^{30}N_2} \cdot (2 \cdot p^{30}N_2 + p^{29}N_2)$$

The calculation is based on the following assumptions:

1. Addition of $^{15}NO_3^-$ does not affect the production of ^{14}N-N_2
2. The three isotopic N_2 species produced ($^{28}N_2$, $^{29}N_2$ and $^{30}N_2$) are binomially distributed.
3. Requirements for assumption 2 are met if the ratio between $^{15}NO_3^-$ and $^{14}NO_3^-$ is constant throughout the NO_3^- reduction zone.
4. The added $^{15}NO_3^-$ mixes homogenously with the $^{14}NO_3^-$ pool in the sediment.

The original approach was developed before the discovery of anammox in marine environments. In sediments and water samples where denitrification and anammox coexist, the first two assumptions are violated. This implies a potential risk to overestimate N_2-production (Risgaard-Petersen et al., 2003). Based on the additional contribution of anammox to N_2 production, a revised IPT was developed (Risgaard-Petersen et al., 2003). The revised theory uses the parameter r_{14}, i.e. the ratio of $^{14}NO_3^-$ to $^{15}NO_3^-$ in the NO_3^- reduction zone to correct the IPT where anammox is present. However, r_{14} is calculated indirectly either by combining ^{15}N-N_2 production in intact cores with the contribution of anammox to total N_2 production (*ra*; slurry incubations) or by quantifying ^{15}N-N_2 production as a function of increasing $^{15}NO_3^-$ concentration (Risgaard-Petersen et al., 2003).

Furthermore, the revised IPT is associated with potential artifacts under conditions where concentration gradients do not obey one dimensional diffusion patterns, e.g. sediments bioturbated by macrofauna. Trimmer et al. (2006) therefore developed a technique where r_{14} can be measured directly in intact sediment cores. This method is based on the assumption that denitrification, but not anammox, produces ^{15}N-N_2O during $^{15}NO_3^-$ incubations and that the isotopic composition of the produced ^{15}N-N_2O will be binomially distributed and thus directly reflects the ratio of $^{14}NO_3^-$ to $^{15}NO_3^-$ (r_{14}) (Trimmer et al., 2006). A combination of the ^{15}N-N_2O-technique (Trimmer et al., 2006) and the revised IPT (Risgaard-Petersen et al., 2003) likely provides a good measure of N_2 production in intact sediment cores where anammox and denitrification coexists.

5.2. Fluorescence In Situ Hybridization (FISH)

Originally developed by DeLong et al. (1989), Fluorescence In Situ Hybridization (FISH) has grown to be a frequently utilized analytical tool to identify and quantify single bacterial cells without the need for cultivation (Amann et al., 2001). The method is based on the use of oligonucleotide probes where short (15-30 nucleotides) rRNA-targeted strains are covalently attached to fluorescent dye molecules

preferentially on the 5´-end. Since ribosomes are abundant in a living cell, successful binding of the probes usually results in brightly fluorescent cells when viewed in a fluorescence microscope. FISH-probes can be designed for any known rRNA-sequence and can therefore bind to preserved sequences targeting a large group of organisms, or bind to more specific sequences down to species/strain level (Amann et al., 2001). 16S or small subunit (SSU) rRNA sequences have been determined for virtually all described species today (Amann and Fuchs, 2008). Therefore, probes are most commonly designed for specific 16S rRNA sequences.

In principle, FISH includes the following procedural steps. 1) Fixation of cells, to make the membranes permeable to probes, using e.g. paraformaldehyde. 2) Preparation of the sample including eventual pretreatments (e.g. application on glass slides or filters and dehydration of cells). 3) Hybridization with probes targeting the specific nucleotide sequences of the organism of interest. 4) Washing to remove excess unbound probe. 5) Quantification using epifluorescence microscopy or flow cytometry and evaluation of results. A standard FISH procedure was used for identification of anammox species in biomaterial from a waste-water treatment plant in Papers III and IV.

In environmental samples, such as sediments or soils, bacterial cells are usually small (i.e. ~ 0.5 μm) and therefore contain less ribosomes than larger bacteria. This deficit normally leads to a weaker fluorescence signal after hybridization. Sensitivity can be improved by the use of bright carbocyanide dyes like Cy3 (Glockner et al., 1996). Other approaches to enhance the sensitivity have included the use of several oligonucleotide probes binding to different sites (Morris et al., 2002) and multiple labeled polynucleotide probes. Normally, however, the polynucleotide probes lack the specificity of oligonucleotide probes and are hard to use on a genus or species level (Trebesius et al., 1994; Pernthaler et al., 2002a). A preferred approach is the use of horseradish peroxidase-probes in combination with catalyzed reporter deposition (CARD) of fluorescently labeled tyramides (Schonhuber et al., 1997; Pernthaler et al., 2002b). CARD-FISH results in significantly brighter signals but demands larger efforts during membrane permeabilization due to the bulky horse radish peroxide label (Amann and Fuchs, 2008)

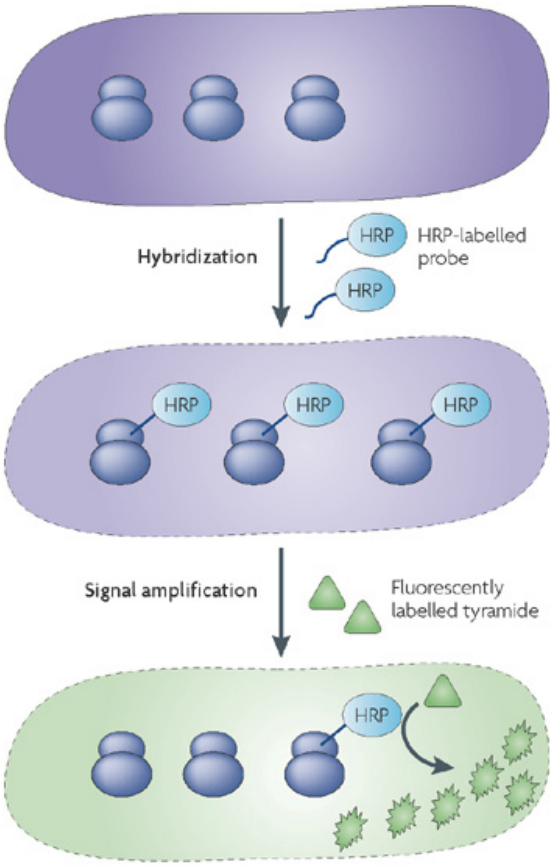

Figure 7. The principle of CARD–FISH (catalysed reported deposition–fluorescence in situ hybridization). CARD–FISH combines CARD of fluorescently labeled tyramides with single-cell identification by FISH. The hybridization involves a single oligonucleotide that is covalently crosslinked to a horseradish peroxidase (HRP) label. Amplification of the signal relative to that achieved with probes that are labelled with a single fluorochrome is based on the radicalization of multiple tyramide molecules by a single horseradish peroxidase. Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Microbiology (Amann and Fuchs, 2008), copyright (2008)

Other approaches include for example a recently developed method that uses single polynucleotide probes of a few hundred base pairs that targets individual genes (Zwirglmaier et al., 2004). The probe carries multiple labels and the method results in a network of probe molecules and peripheral halo-shaped fluorescent signal. This phenomenon has lead to the method being named RING-FISH (Zwirglmaier et al., 2004)

Although there are a wide suite of techniques to address drawback associated with weak fluorescence signals and an unfavorable signal to noise ratio, there are additional potential drawbacks in complex matrices such as sediments. For example, in sediments a positive signal from the fluorescent probe may be hampered by

autofluorescense of organic material or by sediment particles masking the fluorescence from labeled bacteria. Due to the difficulties to detect and accurately quantify bacteria with high sensitivity in sediment samples, a protocol for extracting bacteria from particle surfaces prior to FISH was developed within the framework of this thesis (Klang, 2012). In brief, incubation of surface sediment in pyrophosphate was followed by repeated ultra-sonication and centrifugation. Results were examined by comparing extracted samples with the separated remainder of sediment using the DNA stain 4',6-diamidino-2-phenylindole (DAPI) along with FISH-counts using the eubacterial probe EUB338, targeting almost all known bacterial species. The efficiency of the extraction protocol was amplified about four times in the extracted bacterial suspension (1.4 × 109 cells cm^{-3} sediment) compared to sediment not exposed to pyrophosphate and ultrasonic treatment (3,5 × 108 cells cm-3 sediment). As a consequence, the precision of bacterial quantification was significantly higher after the sample preparation (Table 3).

Table 3. Extraction efficiency (%) of bacteria as a function of number of washing cycles.

Washes	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Mean	SD
1	75,9	78,8	79,9	82	79,1	2,5
2	90,8	92,8	94,2	93,6	92,9	1,5
3	92,3	92,8	96,1	96,5	94,4	2,2
4	96,1	96,8	96,8	97,4	96,8	0,5
5	95,3	-	97,9	98,1	97,1	1,6
6	97,9	-	98,9	98,7	98,5	0,5

5.3. Quantitative real-time polymerase chain reaction (q-PCR)

Identification of specific nucleotide sequences was significantly improved with the introduction of the polymerase chain reaction (PCR, Saiki et al., 1988). PCR is applied on extracted DNA and by the use of specific “primers” and a thermostable DNA polymerase the sequence of interest can be amplified in multiple copies (Saiki et al., 1988). PCR using general 16S rRNA gene-targeted primers is widely used for detection and establishing relationship between organisms and several new techniques for assessments of amplified PCR products have been developed lately (Jenkins and Zehr, 2008). For detection of specific anammox bacteria, PCR using known FISH-primers have been used successfully (Schmid et al., 2005). With the development of quantitative PCR (q-PCR) it became possible to quantify abundance and expression of a specific gene and related sequences or abundances of taxonomic groups (e.g.

Wawrik et al., 2002; Short et al., 2004). Penton et al. (2006) designed a new primer set that was 100% specific against rRNA sequences of marine (*Scalindua*) anammox bacteria. These authors successfully detected anammox bacteria in a wide range of sediments using this primer set. PCR approaches targeting specific genes with regulatory or enzymatic function, "functional genes", have been developed for investigations of biochemical potential and gene expression during N-cycling in natural environments (e.g.; Ward, 1996; Zehr and Capone, 1996; Wawrik et al., 2002). The use of functional gene analysis by q-PCR of specific proteins in anammox and denitrification in sediments from Gullmarsfjorden was initialized but was not finalized within the framework of this thesis.

5.4. Ladderanes as lipid biomarkers

As described (section 4.3.) ladderane lipids are suitable targets to utilize as biomarkers for anammox bacteria due to their unique chemical configuration. Core lipids (extracted fatty acids from ladderane monoethers analyzed by gas chromatography-mass spectrometry, GC-MS) have been used in the Black Sea (Kuypers et al., 2003), the oxygen minimum zones off Namibia (Kuypers et al., 2005) and Peru (Hamersley et al., 2007) as well as the Arabian Sea (Jaeschke et al., 2007). Although they are likely good indicators of anammox bacteria the core lipids do not discriminate between dead and living cells and may thus reflect decomposed debris rather than viable anammox bacteria (Jaeschke et al., 2009). Jaeschke et al. (2009) compared FISH and ^{15}N measurements with analyzes of core lipids and a C_{20}-[3]-ladderane monoether-PC lipid in sediments from the Irish Sea and the Celtic Sea. Although there are analytical concerns with their approach (e.g. only PC-lipids were measured) there was good agreement between microbial activity (^{15}N-measurements) and the content of intact ladderane lipids. This suggests that C_{20}-[3]-ladderane monoether-PC lipids are suitable as biomarkers for viable anammox bacteria.

A new method for the detection of intact ladderane lipids using liquid-liquid extraction (LLE) and solid phase extraction (SPE) followed by liquid chromatography–electrospray ionization-mass spectrometry (LC-ESI-MS) was recently designed (Lanekoff and Karlsson, 2010). This method was, in a study aimed to be included in this thesis, applied on sediment samples from the Gullmarsfjord, Due to methodological drawbacks associated with sensitivity and limit of detection in these specific samples, additional experiments are required.

6. Future work and perspectives

Although investigations of anammox bacteria have increased the general knowledge of N cycling and biogeochemical controls there are still uncertainties related to their regulation in natural environments. One of the most important concerns is their response to variations in oxygen availability following environmental stress. Observations from the studies made in this thesis raise new aspects that require further investigations. For example, obviously anammox organisms can withstand extended periods of completely oxic conditions (paper II). It would be interesting to further explore the potentially diverse metabolic capacity of these seemingly versatile bacteria while exposed to an oxygenated environment. A study was initiated that aimed to investigate effects of naturally oscillating oxygen conditions on the qualitative and quantitative significance of anammox for total N_2-production in the Gullmarsfjord, western Sweden. The bottom water of the fjord seasonally experiences low oxygen conditions. This natural oscillation of oxygen in the bottom water may significantly alter rates and pathways of organic matter mineralization including N_2 production in the sediment. Rates of anammox and denitrification were measured by $^{15}N_2O$ production in intact sediment cores according to Trimmer et al. (2006). It would be highly interesting to pursue the study of potential effects due to this seasonality in oxygen availability using non-invasive sampling protocols.

Additionally, the effect of metal oxides (e.g. MnO_2 and FeOOH) on the absolute and relative importance of anammox is far from understood. The sediment utilized during the studies of this thesis is primarily focused on environments extremely rich in metal oxides. Anammox bacteria are abundant and of high relative importance for N_2 production in such environments. Mechanisms responsible for this relation require further investigation.

A further extension of this research area would be to also include studies of the competition and/or cooperation of aerobic and anaerobic ammonia oxidizers with heterotrophic and autotrophic nitrate reducers. The discovery of ammonium oxidation by archaea and their potential connection with anammox bacteria is intriguing and tempting to explore further. One approach would be the use of Q-PCR for functional genes. Coupling between microbial activities and abundance of microorganisms along with gene expression is a powerful combination. Further, the straight-forward use of the LPI-technique for identification of protein sequences makes it highly interesting to extend also to key proteins of other organisms in natural environments such as marine sediments.

It would also be interesting to explore and apply other sophisticated analytical techniques (for example CARD-FISH) to identify and quantify members of the natural microbial community.

In conclusion, as more information about anammox in natural systems is revealed, more intriguing questions are raised. Not only regarding the specific function and character of anammox bacteria but also of their biogeochemical relation with other processes involved in the nitrogen cycle and elemental cycling overall. Even though we have unraveled a lot there is obviously still so much we don't understand and can learn.

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