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Discovery of anammox bacteria in terrestrial ecosystems

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Discovery of anammox bacteria in terrestrial ecosystems

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Summary/Résumé

Keywords: anammox, nitrogen cycle, oxic-anoxic interfaces, terrestrial ecosystems, activity, abundance, ^{15}N-isotope incubations, real-time PCR

Until this study, the anammox (anaerobic ammonium oxidation) process has been only studied in waste water treatment plants and aquatic environments, including sediments. However, nothing is known so far about the distribution, diversity, abundance and activity of anammox bacteria in terrestrial ecosystems. In this study, we provided molecular evidence for the presence of anammox bacteria in wetlands, sediments of marshes, a Reductisol profile, lake shores, a permafrost soil and a porous aquifer. Phylogenetic analysis of the 16S rRNA gene sequences showed that anammox bacteria from terrestrial ecosystems are affiliated to *Candidatus* 'Brocadia', 'Kuenenia', 'Scalindua', 'Jettenia' and 'Anammoxoglobus', as well as two unidentified clusters. They were widely distributed in the different terrestrial environments indicating a higher diversity than in marine water column environments. Anammox bacteria were not present in every sampled environments and soil fractions demonstrating their heterogeneous distribution and their specific ecological requirements as the presence of long term oxic/anoxic interfaces and inorganic nitrogen compounds. We quantified Anammox bacteria using a new developed qPCR approach applied to the different soil environments and their abundance ranged from 10^4 to 10^6 copies/g of soil. Finally, the Reductisol has been selected for a detailed analysis of their activity along the soil profile by ^{15}N-isotope incubation experiments. For each sampling date, production of $^{29}N_2$ was observed at all depths in the soil profile demonstrating the presence of active anammox bacteria. The amount of N_2 produced by anammox is less than 14% of the total N_2 production. This study provides the first evidence that anammox bacteria are present, diverse and active in terrestrial ecosystems.

Mots-clés: anammox, cycle de l'azote, interfaces oxique-anoxique, écosystèmes terrestres, activité, abondance, ^{15}N-isotope incubations, PCR en temps réel

Avant cette étude, le processus anammox (oxydation anaérobie de l'ammonium) était uniquement étudié dans les usines de traitement des eaux usées et dans les milieux aquatiques, sédiments inclus. Cependant, rien n'était connu encore sur la distribution, la diversité, l'abondance et l'activité des bactéries anammox dans les écosystèmes terrestres. Dans cette étude, nous apportons l'évidence, par approche moléculaire, de la présence de bactéries anammox dans les sols de zones humides, les sédiments des marais, le profil de sol d'un Reductisol, des sols de rives de lacs, un sol sur Permafrost et un aquifère poreux. L'analyse phylogénétique des séquences du gène ARNr 16S a démontrée que les bactéries anammox présentes dans les écosystèmes terrestres sont affiliées à *Candidatus* 'Brocadia', 'Kuenenia', 'Scalindua', 'Jettenia' and 'Anammoxoglobus' ainsi qu'à deux groupes non identifiés. Ces candidats anammox étaient largement distribués dans les différents environnements terrestres indiquant une plus grande diversité que dans les colonnes d'eau des milieux marins. Les bactéries anammox n'étaient pas présentes dans tous les milieux et fractions de sol échantillonnés, l'analyse démontrant leur distribution hétérogène et leurs besoins écologiques spécifiques comme la présence d'interfaces oxique / anoxique à long terme et de composés azotés inorganiques. Nous avons quantifié les bactéries anammox dans ces différents environnements en développant une nouvelle approche qPCR spécifique anammox, et leur abondance variait de 10^4 à 10^6 copies / g de sol. Finalement, le Réductisol a été sélectionné pour réaliser une analyse détaillée de l'activité anammox le long du profil de sol par des expériences d'incubation à l'isotope ^{15}N. Pour chaque date d'échantillonnage, une production de $^{29}N_2$ était observée à toutes les profondeurs du Réductisol, démontrant la présence de bactéries anammox actives. La contribution d'anammox à la production totale de N_2 était inférieure à 14%. Cette étude fournit la première preuve que les bactéries anammox sont présentes, diverses et actives dans les écosystèmes terrestres.

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Chapter I

General Introduction

Notice to the reader

In introduction, I wanted to let the reader discover successively the scenery, the actors, their interactions, the main theme, and finally the principal actor of this anammox story. This chapter was constructed in order to allow you to obtain a global view of this fascinating process and finally to better understand its importance within the terrestrial nitrogen cycle.

First, let's begin with the discovery of the scenery which refers to terrestrial ecosystems particularly with its soil compartment and its microbial actors and their inherent interactions.

Terrestrial ecosystems

Definition

An ecosystem is defined as an area in which organisms (plant, animals, and microorganisms) interact with each other and with the physical (abiotic) factors of the environment. The English botanist Arthur Tansley, a pioneer of ecology, describes it as the whole system, including not only the organism-complex, but also the whole complex of physical factors forming what we call the environment (Tansley, 1935). The ecosystems are represented by various kinds and sizes of natural (forest, land, ocean) or anthropogenic (cultivated fields, waste water treatment plants), temporary (pond) or permanent (grassland), terrestrial (tropical forest) or aquatic (ocean), small (tree) or large (ocean) systems. Moreover, they correspond to a functional unit in which biotic and abiotic factors are linked together through nutrient cycle and energy flow in their local environment (Odum, 1971). Other parameters could define ecosystems at their spatial scale (e.g. biomes, ecotopes) and temporal extent (e.g. period of stability). All the actors of an ecosystem develop a network of interdependences allowing the preservation and the development of life (Tansley, 1939).

A terrestrial ecosystem consist thus of a biological community (biocenose) and its physical environment (biotope) present on the land of continents and islands (Fig. I. 1).

Figure I. 1. A *Fraxinion* forest in the vicinity of the Lake Le Loclat (CH) representing a terrestrial ecosystem that was prospected for the presence of anammox bacteria.

Terrestrial ecosystems occupy 28.2% of Earth's surface representing a smaller portion than marine ecosystems. As they differ from aquatic ecosystems by the lower quantity and availability of water, thus water is considered as a limiting factor in such ecosystems (Hordon, 1977). Terrestrial ecosystems have been a major site of adaptations for plants and animals as for example plant have developed strategies for water retention, body support in the atmosphere, resistance to extreme temperature, wind, humidity, as well as pollen transportation. Terrestrial environments are composed by land surface and subsurface which provide water and nutrients in interaction with the atmosphere from which gases could be obtained. In such environments, terrestrial organisms interact with each other trough food chains allowing energy and chemical flows transfer (Chapin, 2002).

The following under-chapters were mainly inspirited by The Living Soil (Gobat *et al*., 2004) and Le Sol Vivant (Gobat *et al*., 2010) and develop the importance of the soil and its microbial actors.

Importance of soil

In terrestrial ecosystems, the soil is an important compartment representing the interface between the abiotic (rock, minerals) and the biotic (vegetation). It is also the site in which the vegetation is firmly rooted. The soil represents an exchange interface of material and energy between the below- and aboveground (Chesworth, 2008)(Fig. I. 2).

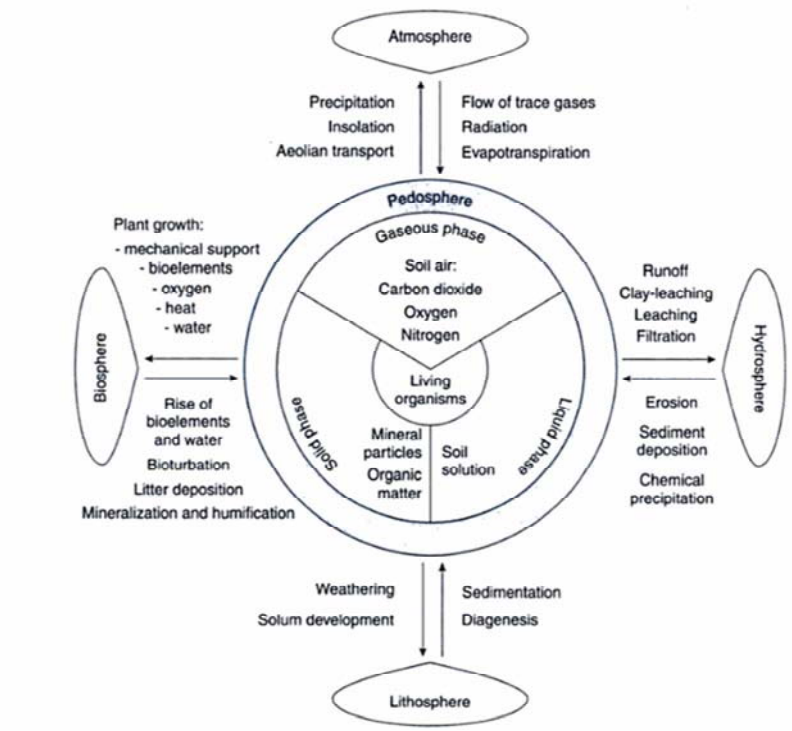


Figure I. 2.
The soil in the center of interactions between the lithosphere, hydrosphere, atmosphere, and the biosphere. From Gobat *et al*., 2004.

The soil is constituted by three phases: the solid phase with mineral and organic components; the liquid phase composed by the soil solution; the gaseous phase represented by the soil atmosphere. The solid skeleton of the soil is composed by mineral constituents originating primary from the bedrock layer or secondary from its alteration and by organic constituents coming from fresh litter and humification. The mineral components derive from alteration rocks as sand and silt, which are the products of physical alteration and clay which is the product of chemical alteration. The texture is a fundamental soil property constituted by the mineral fraction, which refers to sand, silt and clay composition (Fig. I. 3) and by an organic fraction corresponding to the fiber proportion.

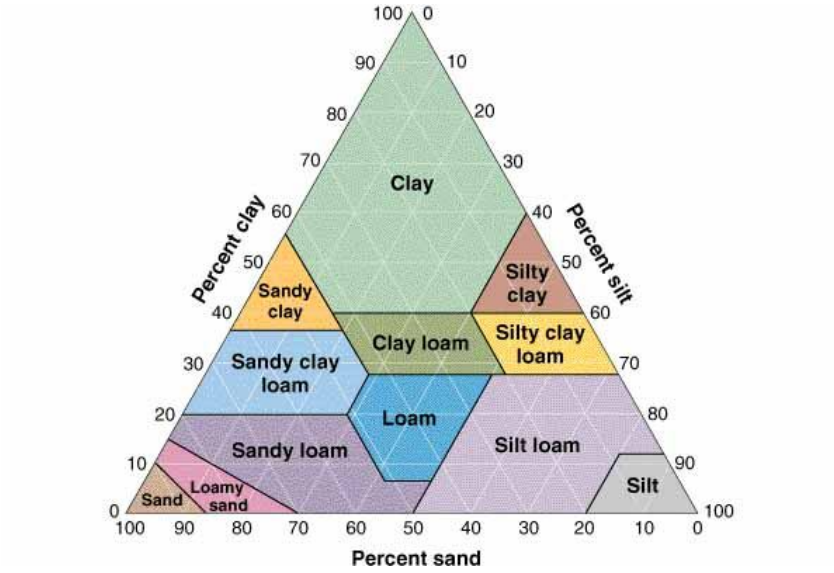


Figure I. 3.
The soil texture triangle represents the proportion of minerals present in the soil determining the soil type (USDA, 1951).

Furthermore, the soil texture plays an important role in the structure and porosity of the soil. The arrangement of soil particles into aggregates (micro- or macro-aggregates) and the remaining soil pore spaces defined the soil structure (Marshall and Holmes, 1979). The structure of the soil is essential for its aeration, water flow, nutrients availability, plant growth and microorganism's activity. Between all the soil particles, clay particles play a major role in soil aggregation (Leeper and Uren, 1993). Its proportion influences the formation of the clay-humus complex which is the base for the soil micro-aggregates cohesion. This organo-mineral association plays an important role in the soil structure, water and nutrient retentions favorable for the soil fertility. The water with its dissolved substances represents the soil solution which plays an important role in the surrounding soil for nutrient transport and supply for plants, lixiviation as well as

solubilization. Free or dissolved gases compose the soil atmosphere where they significantly contribute to the exchange with the external atmosphere (Fig. I. 4).

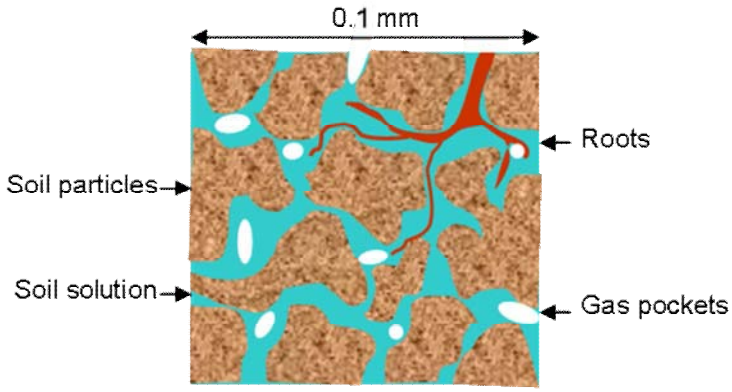


Figure I. 4. Soil structure filled with pore spaces containing the soil solution and atmosphere providing to the plant its nutrients. Modified from Bellingham, 2009.

In addition to all these constituents, a major part of the soil consists of living organisms from microflora (microorganisms) to megafauna (moles, rabbits). Soil biota initiates soil bioturbation especially ecosystems engineers (earthworms, ants, termites, plants) creating burrows and pores which allow the transfer of moisture and gases to deeper layers. Conversely, nutrients are transported from deeper layers to the upper part of the soil through the open channels created by plant roots or earthworm galleries. In addition, roots exudation or decomposition offer organic nutrients to the surrounding microorganisms and act also as cement helping the soil to aggregate (Walker *et al.*, 2003). Soil living organisms obtain nutrients and energy by feeding primary producers and each other (Fig. I. 5). Thus, soil living organisms promote the soil structure, porosity, and stability, which contribute to improve the soil health. As a result, soil is a heterogeneous compartment offering various habitats, nutrients, pool of organic and mineral matter, as well as water availability for living organisms with their related functions.

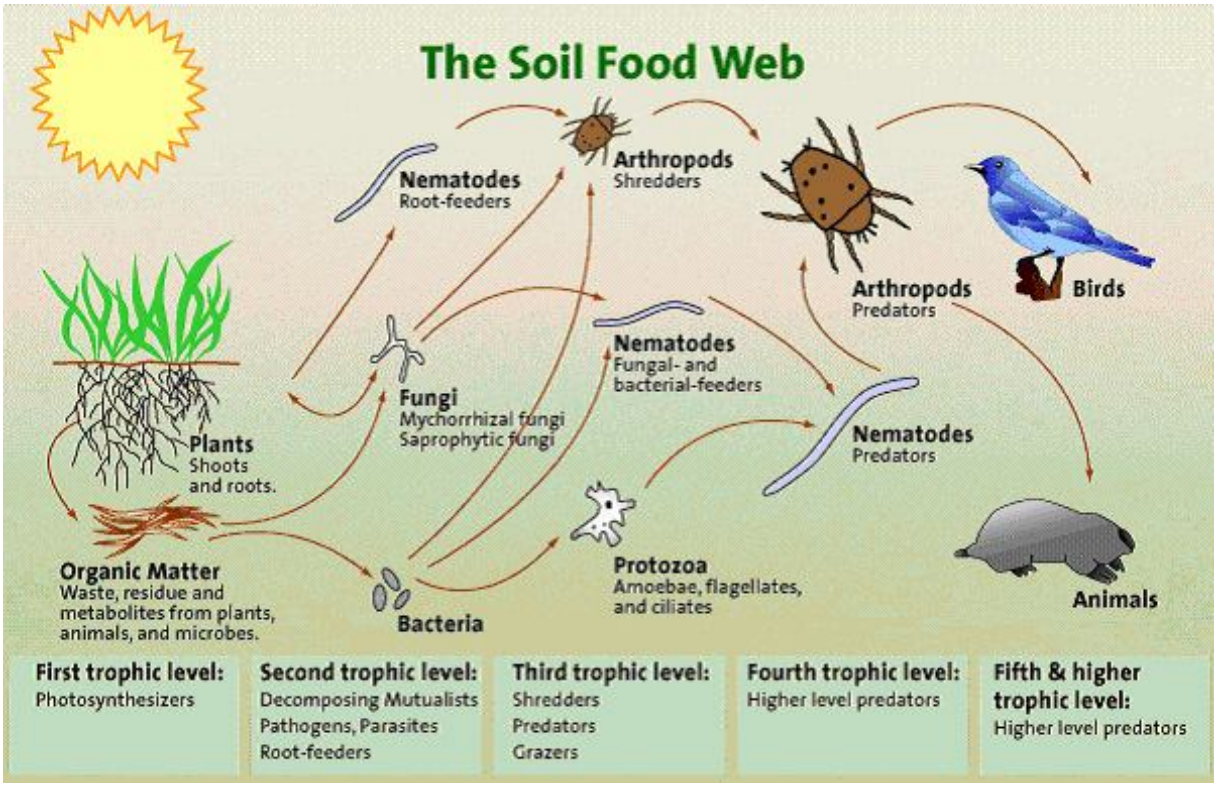


Figure I. 5. Soil food web diagram representing the trophic level of interactions (USDA, 2004).

Plants and microbial interactions within the soil

Soil is a living compartment where many interactions occur between plants and microorganisms (bacteria, fungi, archaea and protists) (e.g. Fig. I. 5). Plants interact above- and belowground with the soil through their leaves and roots, respectively. Plants produce fresh organic matter whose degradation and further mineralization delivers organic and mineral components back to the soil. In the deeper part of the soil, roots exchange permanently water and secreted substances in the surrounding soil (Fig. I. 6). Plant-soil interactions are so important that the part of the soil which is under the direct influence of the root was named the rhizosphere. In the rhizosphere, the growth and activity of the root release into the soil organic compounds also called 'rhizodeposition', which confer to the surrounding soil carbon and energy flows (Nguyen, 2003).

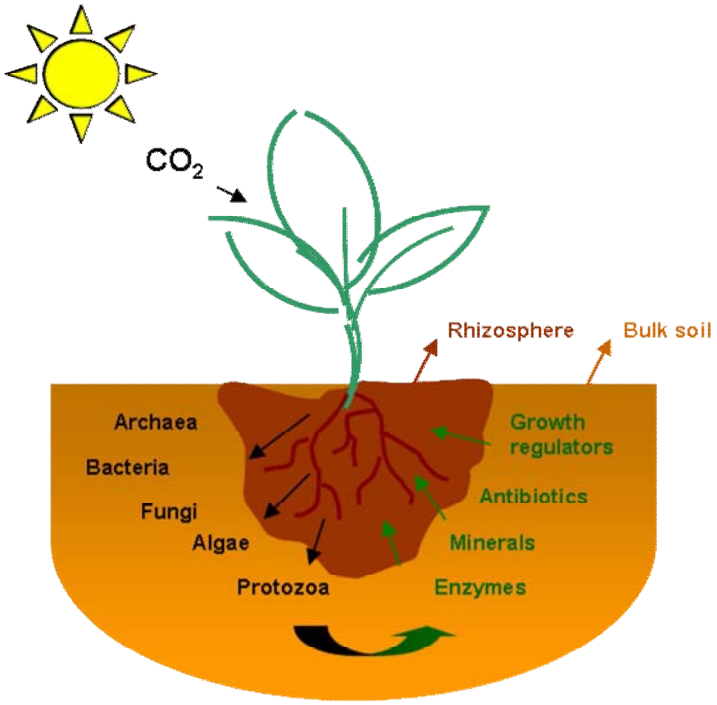


Figure I. 6.

In the rhizosphere, rhizodepostion can be assimilated by mainly heterotrophic soil organisms which in turn may secrete useful signal compounds which are absorbed through the roots.

Soil microorganisms (bacteria, fungi, archaea, and protists) are involved in a lot of important interactions within the biogeochemical cycles such as the decomposition of vegetal or animal organic matter, the oxidation or reduction of mineral substances, nitrogen-fixation, and in the alteration of the bedrock. For example, the first process involved in the nitrogen cycle is the nitrogen fixation, which is driven only by bacteria. This microbiological process is very important as molecular nitrogen has first to be fixed and transformed to become available for plants or animals.
In conclusion, plants and microorganisms are essential key players acting together and render the soil its life and properties.

Now that I have placed the scenery and the actors with their interactions, I want to present you the main theme in which the anammox story occur: 'the nitrogen cycle', which was mainly inspired by the book: Biology of the Nitrogen Cycle (2007).

Biology of the nitrogen cycle

Overview

Nitrogen is a chemical element essential for life constituting amino and nucleic acids, proteins, organic nitrogen compounds (e.g. amines and urea). Nitrogen is found in different forms as molecular nitrogen, ammonium, nitrogen oxides, azides and hydrazines. These nitrogen compounds are used in many different ways, for example, nitrate is applied as a fertilizer, or used in explosives, hydrazine is used as rocket fuel, nitrous oxide as a partial anesthetic, and finally, nitrogen is an important constituent of drug molecules. Biologically, nitrogen represents an essential nutrient for all living organisms. While present in molecular form in the atmosphere it cannot be directly used by them. Molecular nitrogen has first to be fixed biologically by certain bacteria and archaea in order to become available for plant or animals. This process represents the first step of the nitrogen cycle (Fig. I. 7).

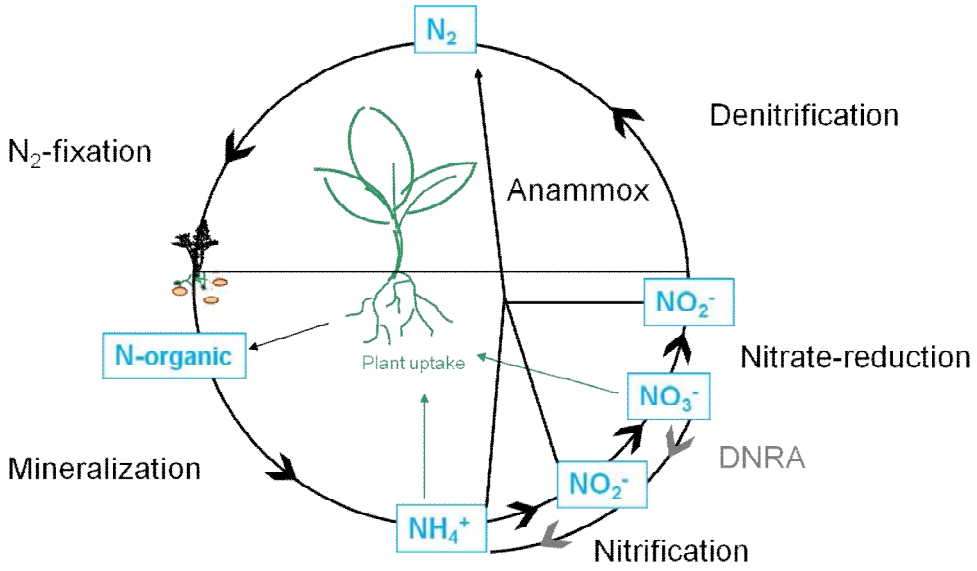
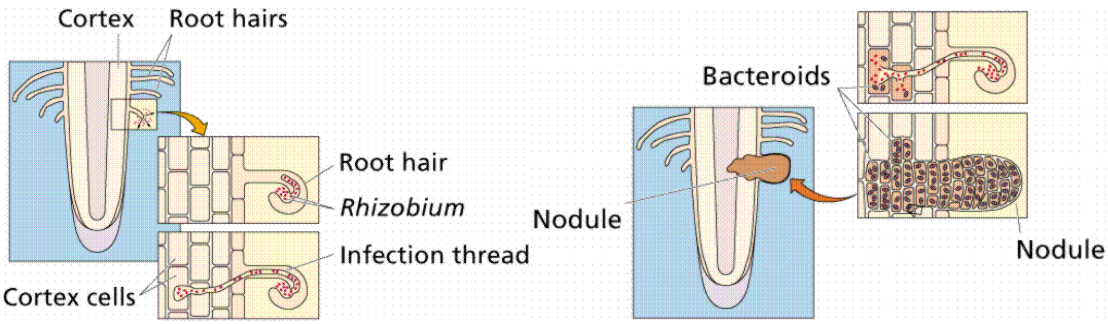


Figure I. 7. The biological nitrogen cycle in soil, with the processes of N_2-fixation, mineralization, nitrification, dissimilatory nitrate reduction to ammonium (DNRA), nitrate reduction, denitrification and anammox. All these processes provide nitrogen supply for plant growth and for other process leading to competition for substrate availability between them.

The nitrogen cycle represents one of the most important nutrient cycles in natural ecosystems (Fig. I. 7). Most of its transformations are driven by microorganisms

(bacteria, archaea and fungi) and atmospheric N_2 represents its largest reservoir. The nitrogen fixation is driven by certain free-living bacteria in soils (i.e. Azotobacter) or in symbiosis with plants (i.e. *rhizobia* with leguminous plants or *Frankia* with non-leguminous plants) (Fig. I. 8).

A



B

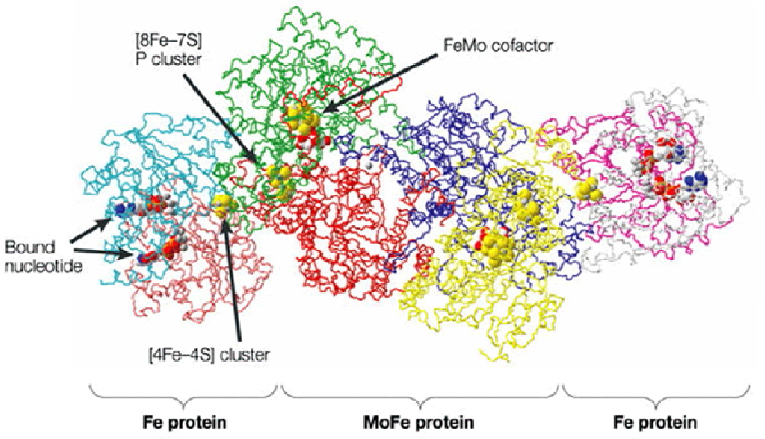


Image from Dixon and Kahn, 2004. Nature Microbiology.

Figure I. 8. A) Root nodule formation in which the biological nitrogen fixation is carried out by bacteria using a specific enzymatic complex called 'nitrogenase' (B), which converts the molecular nitrogen into ammonia. Image A from Purves *et al*., 1992.

The nitrogen fixation is catalyzed by a specific enzyme named nitrogenase which performs the following reaction (Equation 1; Burgess and Lowe, 1996):

$$N_2 + 8\,H^+ + 8\,e^- + 16\,ATP \rightarrow 2\,NH_3 + H_2 + 16\,ADP + 16\,P_i \qquad (1)$$

Plants incorporate fixed nitrogen into their biomass mostly in the form of proteins. They represent, thus, the first source of available nitrogen for superior organisms. When plants are degraded under oxic or anoxic conditions, the organic N is released into the soil and is further transformed by microorganisms to NH_4^+ (mineralization)(Berg and Matzner, 1997). Then, under oxic conditions, ammonium is oxidized in two steps, first to nitrite (nitritation, *Nitrosomonas*) and further to nitrate (nitratation, *Nitrobacter*) during the process of nitrification (Purkhold *et al*., 2000). This process plays an important role within the nitrogen cycle as it links organic matter decomposition to the production of

assimilable nitrate. The nitrate can consequently be assimilated by plants and microorganisms. Following the cycle, denitrification is defined as the process by which mostly heterotrophic bacteria reduce nitrate to molecular nitrogen under anoxic conditions (Zumpft, 1997). The produced N_2 returns to the atmosphere and closes the nitrogen cycle. Nitrate can also be converted to ammonium. This process is named dissimilatory nitrate reduction to ammonium (DNRA) and is driven, as denitrification, by heterotrophic bacteria (An and Gardner, 2002).

A novel process which converts ammonium anaerobically to dinitrogen with nitrite as electron acceptor to dinitrogen was discovered in wastewater treatments plants (Mulder *et al*., 1995) and aquatic ecosystems (Thamdrup and Dalsgaard, 2002). This process named 'anammox' is driven by bacteria in oxic/anoxic transition zones. As anammox represents also a dinitrogen forming process it may contribute to the loss of N from ecosystems (Kuypers *et al*., 2005).

All the nitrogen compounds involved in the nitrogen cycle have an impact on life in different ways. As the availability of nitrogen depends on the biological nitrogen fixation, thus it is considered as a limiting factor for living organisms in soil (Aldén *et al*., 2001). On the other hand, nitrite, nitrate, and ammonium may be present in excess in soils and then be leached down to the groundwater generating eutrophisation of aquatic systems or affecting human health. Moreover, nitrate losses from soil by leaching or microbial activity can lead to the loss of cations important for plant nutrition and to soil acidification (Aber *et al*., 1992; 1998). During nitrification and most commonly denitrification, different gases are produced, which affect our atmosphere. For example, nitrous oxide (N_2O) and nitric oxide (NO) may destroy the ozone layer contributing to global warming also defined as the greenhouse effect.

Finally, a better understanding of the terrestrial nitrogen cycle is important due to the wide implication of its processes on ecology, agriculture, wastewater treatment, health, and the Earth atmosphere.

In the following two under-chapters, I would like to focus more precisely on two important processes involved in the nitrogen cycle which interact with the principal actor of the anammox story.

Nitrification

Nitrification represents the aerobic oxidation of ammonia (NH_3) to nitrite (NO_2^-) and further to nitrate (NO_3^-) (Equation 2).

$$\text{Nitrous bacteria: } NH_3 + O_2 \rightarrow NO_2^- + 3H^+ + 2e^-$$

$$\text{Nitric bacteria: } NO_2^- + H_2O \rightarrow NO_3^- + 2H^+ + 2e^- \qquad (2)$$

This process is principally driven by chemo-litho-autotrophic bacteria. This process provides oxidized nitrogen compounds for anammox bacteria, denitrification and plants. Autotrophic ammonium oxidizing bacteria belong to 2 major subclasses of *Proteobacteria*, the *Gammaproteobacteria* as e.g. *Nitrosococcus sp.* and the *Betaproteobacteria* as e.g. *Nitrosomonas sp.*. These bacteria are present in natural habitats especially in soils, freshwater, seawater, and in man-made habitats like wastewater treatment plants. Nitrite is rarely found at high concentrations in nature because it is often directly used and doesn't accumulate so much in environments. Nitrite oxidizing bacteria are more difficult to study, nevertheless, four genera were described within the *Proteobacteria*: *Nitrobacter*, *Nitrococcus*, *Nitrospina*, and *Nitrospira*. However, recently, new groups of microorganisms performing this reaction were discovered: Archaea, heterotrophic bacteria, fungi and anammox bacteria (Könneke *et al*., 2005). Ammonium oxidizing Archaea (AOA), which are representatives of the *Crenarchaeota*, are also common in terrestrial and aquatic environments (Nicol and Schleper, 2006). These archaeal ammonium oxidizers were found to be more abundant than bacterial ammonium oxidizers in some ecosystems (Leininger *et al.,* 2006; Martens-Habbena *et al*., 2009).

Despite all the studies on autotrophic nitrification, heterotrophic nitrifiers also exist. Many heterotrophic bacteria and fungi perform the oxidation of organic N compounds (Daum *et al*., 1998; Hayatsu, 2008).

In conclusion, nitrification plays an important role in the nitrogen cycle by linking organic matter degradation and ammonium production to nitrate formation which represents the principal electron acceptor for denitrification. It plays thus also a central role in the elimination of nitrogen during wastewater treatment.

Denitrification

Denitrification completes the nitrogen cycle by returning N_2 to the atmosphere (Equation 3). This reaction of nitrate reduction is driven by heterotrophic bacteria.

$$4NO_3^- + 5(CH_2O) + 4H^+ \rightarrow 2N_2 + 5CO_2 + 7H_2O \quad (3)$$

This process reducing nitrate creates competitions for this substrate availability especially between plants and nitrate reducers. The reduction of nitrate to nitrite is the first important step for nitrate assimilation and dissimilatory nitrate reduction. The former is a main process performed by plants, fungi, algae, and many bacteria. The second is divided in two distinct processes: denitrification, reduction of nitrate to nitrite and further to gaseous nitrogen (NO, N_2O or N_2) and DNRA, the reduction of nitrate to ammonium. Both denitrification and DNRA occur under oxygen-limited or strictly anoxic conditions. Denitrification is commonly performed by heterotrophic bacteria but also a few autotrophic bacteria (e.g. *Thiobacillus* denitrificans) (Baalsrud and Baalsrud, 1954). Denitrifiers are found in main phylogenetic groups and several of them belong to *Pseudomonas* spp. (Zumpft, 1997). They have been frequently isolated from soil and marine environments (Gamble *et al*., 1977). Denitrification is not only performed by bacteria but also by Archaea (Crenarcheota, Euryarcheota), fungi (*Fusarium oxysporum*, Shoun and Tanimoto, 1991) and, as recently discovered, by foraminifers (Risgaard-Petersen *et al*., 2006).

Denitrification consists of four steps in which nitrate is sequentially reduced to dinitrogen gas (Equation 4) by specific enzymes which are only produced under anoxic conditions.

$$\underset{\text{nitrate}}{NO_3^-} \rightarrow \underset{\text{nitrite}}{NO_2^-} \rightarrow \underset{\text{nitric-oxide}}{NO} \rightarrow \underset{\text{nitrous-oxide}}{N_2O} \rightarrow \underset{\text{reductases}}{N_2\,(g)} \quad (4)$$

Under oxic conditions, O_2 is preferably used as electron acceptor. Denitrification is believed to be the largest sink for combined nitrogen and responsible for important nitrogen losses from agricultural systems (Carter *et al*., 1995). Moreover, nitric- and nitrous- oxides are intermediate products of denitrification and may be released to the atmosphere. Both gases play an important role in atmosphere chemistry. Nitrous oxide (N_2O) is an important greenhouse gas and nitric oxides (NO) cause ozone (O_3) production in the lower and ozone destruction in the upper atmosphere (Skiba *et al*., 1993). However, combined with nitrification, denitrification represents the second important process involved in the nitrogen removal from wastewater. Denitrification could be a useful process for the decontamination of ecosystems where nitrate is

present in excess through anthropogenic input, for example, in contaminated aquifers, wetlands, riparian zones, and agricultural fields.

Denitrification was believed to be the only mechanism of N_2 production in the global N-cycle. Recently, however, a novel microbial process of dinitrogen formation was discovered in wastewater treatment systems and called “anammox” process for anaerobic ammonium oxidation (Mulder *et al*., 1995).

I am very proud to introduce you the principal actor of this whole story: ‘The anammox process ’.

The Anaerobic ammonium oxidation (Anammox) process

Overview

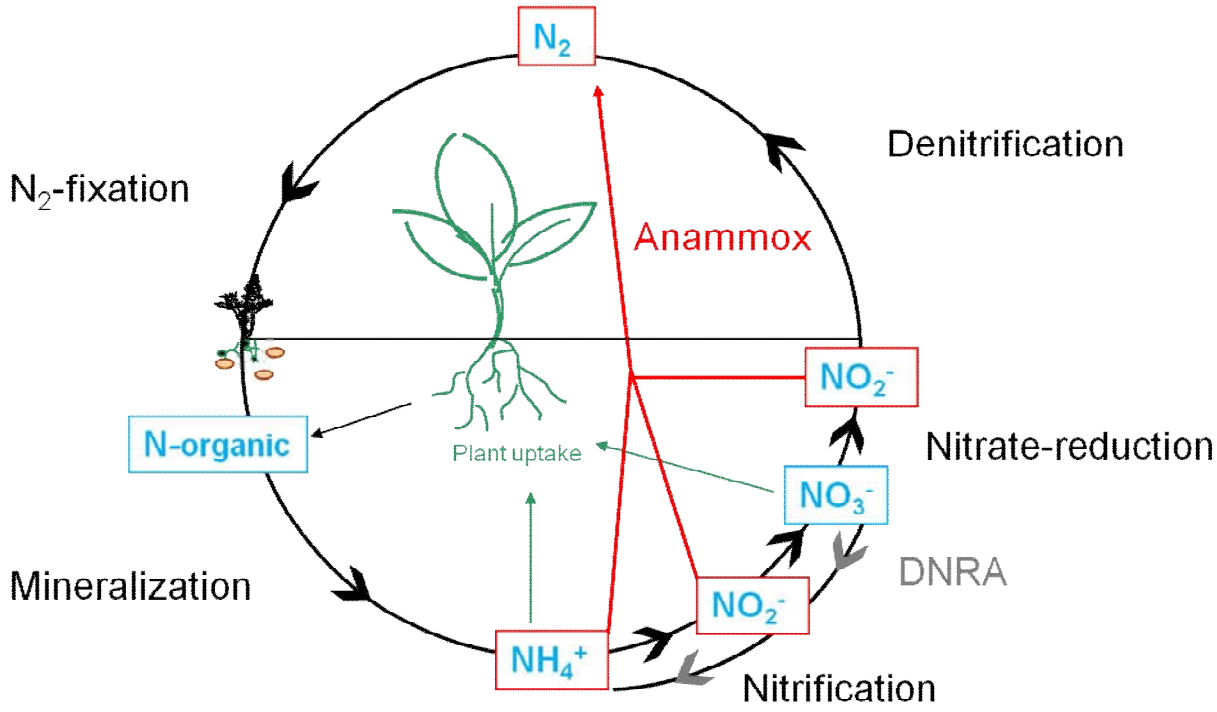


Figure I. 11. The anammox process within the nitrogen cycle interacting with others processes and plants. Anammox process closes also the nitrogen cycle by returning N_2 to the atmosphere.

Anammox represents the anaerobic conversion of ammonium to dinitrogen with nitrite as electron acceptor (Kuenen, 2008)(Fig. I. 11; Equation 5; van de Graaf *et al*., 1997).

$$NH_4^+ + NO_2^- \rightarrow N_2 + 2\,H_2O \qquad (\Delta G_0 = -357\ KJ/mol) \qquad (5)$$

Anammox bacteria are members of the order of *Planctomycetales*, which are found in aquatic environments but are also important members of soil microbial communities (Neef *et al*., 1998; Strous *et al*., 1999). Typical properties of this bacterial group, are the absence of peptidoglycan in their cell walls, and may include cell surfaces with "crateriform structures", budding reproduction, and internal cell compartmentalization (Fuerst, 1995; Lindsay *et al*., 2001; Strous *et al*., 1999). Until now, only five candidate genera, *Candidatus* 'Brocadia', 'Kuenenia', 'Jettenia', 'Anammoxoglobus' and 'Scalindua', have been identified (Schmid *et al*., 2003; Kartal *et al*., 2007). Anammox bacteria are active at redox transition zones in various aquatic ecosystems where they contribute significantly to N_2 production (Thamdrup and Dalsgaard, 2002; Kuypers *et al*., 2005; Schmid *et al*., 2007). Although, this process is of great importance in ammonium removal in wastewater systems and in dinitrogen formation in the world's oceans, the diversity, abundance, distribution and activity of anammox bacteria in terrestrial ecosystems needs still to be explored more in detail.

Cell structure and physiology

Anammox bacteria are anaerobic, slow-growing organisms with doubling times of around 11 days (Jetten *et al*., 1998). So far, only enrichment cultures exist, but no pure culture was isolated. They have been described initially as strictly autotrophic organisms that fix CO_2 with nitrite as reductor and produce thereby anaerobically nitrate (Equation 6; Strous *et al*., 1998).

$$NH_4^+ + 1.32\ NO_2^- + 0.066\ HCO_3^- + 0.13\ H^+ \rightarrow 0.26\ NO_3^- + 1.02\ N_2 + 0.066\ CH_2O_{0.5}N_{0.15} + 2.03\ H_2O \qquad (6)$$

However, it has been shown that some anammox bacteria (*Anammoxaglobus, Brocadia, Kuenenia*) possess a more mixotrophic metabolism than presumed. They may as well oxidize organic acids by dissimilatory nitrate reduction to ammonium (Guven *et al*., 2005; Kartal *et al*., 2007a-b; Kartal *et al.,* 2008*)* and perform the anaerobic respiration of manganese and iron oxides (Strous *et al*., 2006). Anammox bacteria are active between 6 up to 43°C with an optimum at 37°C but it was recently observed that this process occurs also at higher temperatures until 52°C in hot springs (Jaeschke *et al*., 2009) and even at 85°C in hydrothermal vents (Byrne *et al*., 2008). The anammox process was

found to occur within a pH range of 6.7 to 8.3, with an optimum at pH 8 (Jetten *et al*., 1999). The substrate affinity for ammonium and nitrite is high with affinity constants of less than 10 μM (Kuenen and Jetten, 2001; Strous *et al*., 1999). Anammox activity is reversibly inhibited at oxygen concentrations > 0.5% air saturation (Strous *et al*., 1997) and irreversibly inhibited at nitrite concentration higher than 20 mM (Jetten *et al*., 1999).

Anammox bacteria possess an atypical cell structure. The anammox cytoplasm is separated in three distinct compartments bound by a specific membrane (Fig.I. 12).

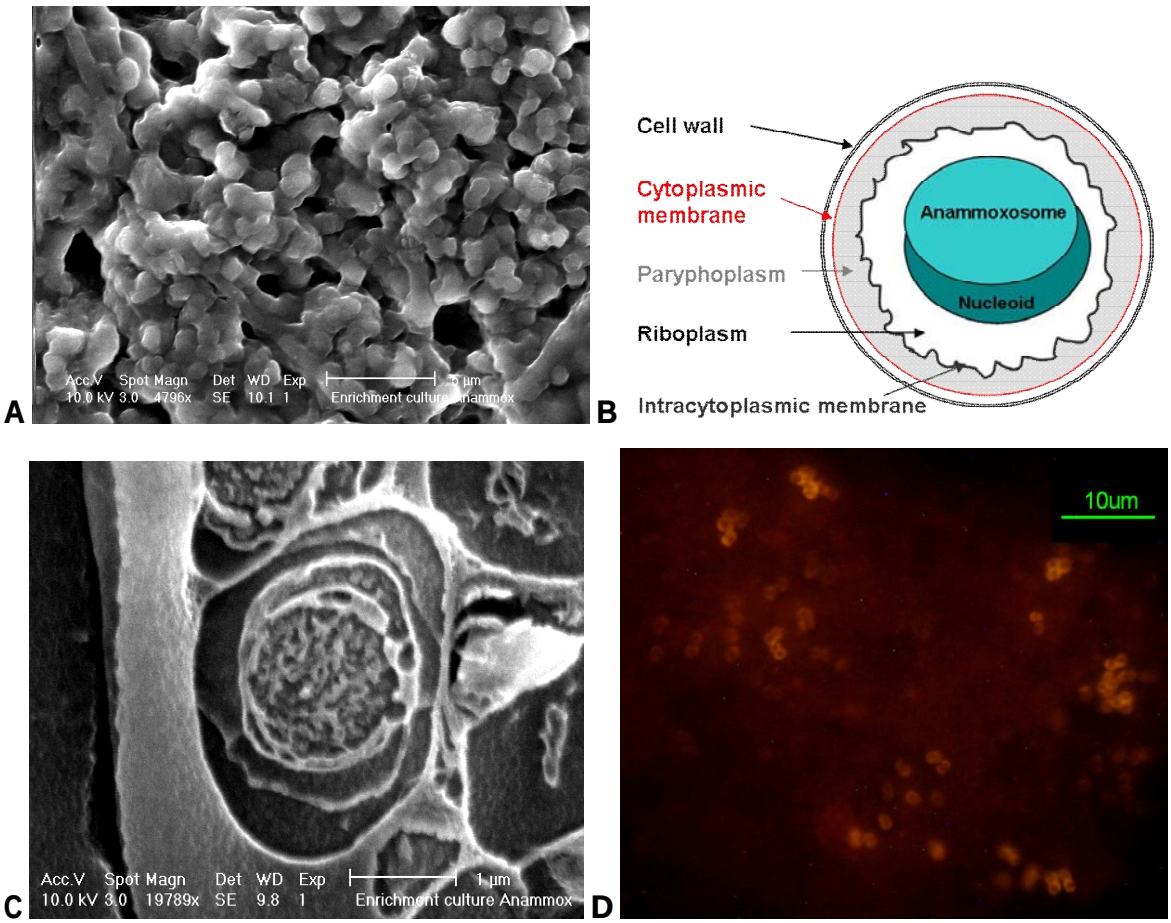


Figure I. 12. A) Cryo-SEM picture of the anammox enriched culture from activated sludge set up in the laboratory. Individual anammox cells are hold together by exopolymeric substances (EPS), B) Schema of anammox cell compartmentalization, modified from Jetten *et al*., 2001, C) Cryo-picture showing the internal anammox cell compartmentalization, D) FISH applied on the anammox enrichment culture using A820 probes showing the typical 'doughnut' shape of anammox cells. (SEM pictures C.G and S.H)

Most of the cell volume is occupied by the anammoxosome, which is bounded by the anammox membrane. This compartment is surrounded by a riboplasm-cytoplasm containing ribosome particles and a nucleoid. The last cytoplasmic compartment is the paryphoplasm which is bounded by the cytoplasmic membrane. After this last membrane, a cell wall without peptidoglycane is present (van Nifrtik *et al*., 2008a). Another typical property of anammox bacteria is the presence of ladderane lipids in the anammoxosome membrane (Fig I. 13)(Damsté *et al*., 2002).

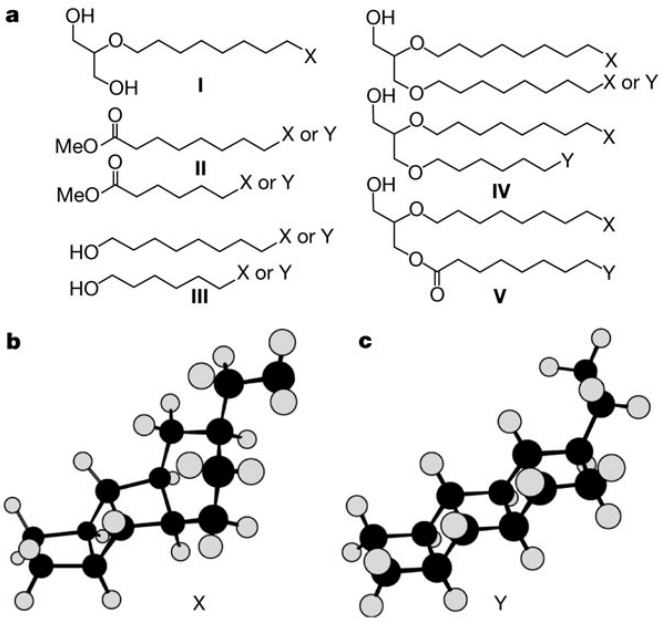


Figure I. 13. Structures of ladderane lipids composing the anammoxosome membrane. **a**, General chemical structures of unique membrane lipids of anammox bacteria. **b**, **c**, Three-dimensional representation of ring structures X and Y. Carbon and hydrogen atoms are represented by black and grey balls, respectively. In **b** and **c** the first two carbon atoms of the side chains of these ring structures are indicated for reference (Damsté *et al*., 2002).

This membrane constitution may protect the anammox cell from the toxic intermediates produced during their metabolisms such as hydrazine and hydroxylamine (van de Graaf *et al*., 1997) or may enlarge the area where their metabolic pathways occur to enhance their activity (van Nifrtik *et al.,* 2008a).

The anammox reaction occurs in the anammoxosome and represents a biological process through which a proton motive force over the anammoxosome membrane is generated (Fuerst *et al*., 1995; Lindsay *et al*., 2001, van Niftrik *et al*., 2008a). Initially, the anammox catabolic pathway was proposed to involved hydrazine and hydroxylamine as intermediates (van de Graaf *et al*., 1997) (Fig. I. 14A). Recently, however, an alternative model has been postulated to involved several iron-containing heme enzymes which are principally located within the anammoxosome membrane (Lindsay *et al*., 2001, van

Niftrik *et al*., 2008b; Strous *et al*., 2006) (Fig. I. 14B). In this model, hydroxylamine is no more involved.

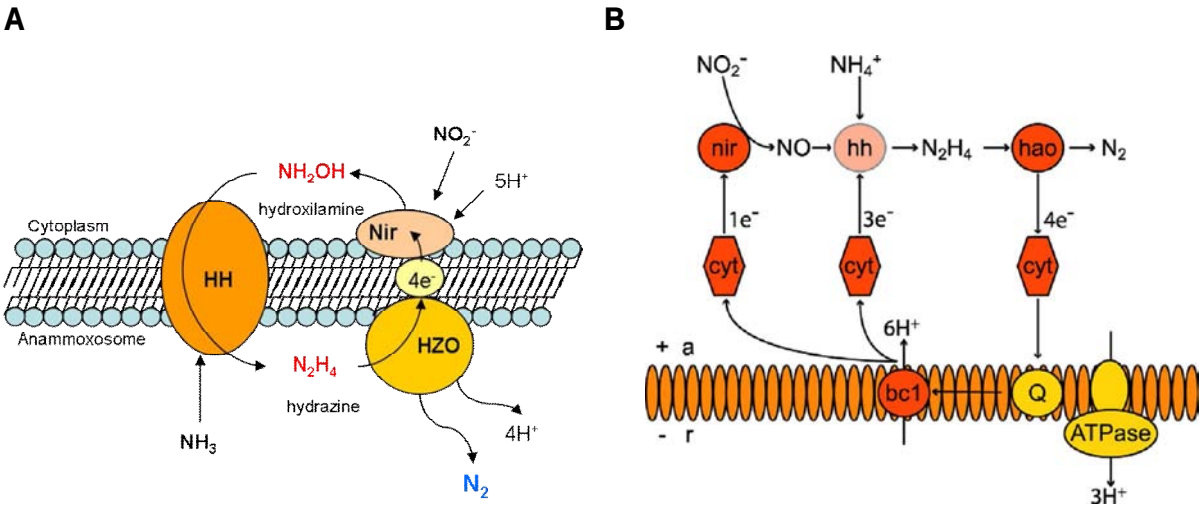


Figure I. 14. A) Postulation of catabolic pathway involves also hydroxylamine besides hydrazine as intermediates. Image modified from Jetten *et al*., 2001. B) Postulated model of anammox reactions where nitrite is reduced to nitric oxide, which is combined with ammonium to hydrazine and further oxidized to N_2 (Strous *et al*., 2006, van Nifrtik 2008b). The reaction generates four reducing equivalents, which are used for ATP synthesis. nir, nitrite reductase; hh, hydrazine hydrolase; hao, hydroxylamine oxidoreductase; hzo, hydrazine oxidoreductase; cyt, cytochrome; bc1, cytochrome bc1 complex; Q, coenzyme Q; a, anammoxosome compartment; r, riboplasm compartment.

Habitats and diversity

Anammox activity was first detected in wastewater treatment plants (Mulder *et al*., 1995) where the bacteria performing this process were identified (Strous *et al*., 1999). Thereafter, the first evidence of anammox activity and bacteria was confirmed in the water column of the Black Sea (Kuypers *et al*., 2003). So far, anammox bacteria have been identified in a wide range of natural aquatic ecosystems such as anoxic marine water columns (Kuypers 2005; Hammerslay 2007; Lam *et al*., 2007; Woebken *et al*., 2008) and marine (Thamdrup and Dalsgaard, 2002; Risgaard-Petersen *et al*., 2004; Tal *et al*., 2005; Amano *et al*., 2007; Schmid *et al*., 2007; Rich *et al*., 2008) and freshwater sediments (Penton, 2006; Zhang *et al*., 2007) or water column (Schubert *et al*., 2006). Phylogenetic analysis have identified so far five genera which perform the anammox reaction: *Candidatus* "Brocardia", "Scalindua" , "Kuenenia", "Jettenia", and "Anammoxoglobus" (Schmid *et al*., 2003; Kartal *et al*., 2007). All of them have been detected in wastewater treatment systems and only 'Scalindua' was typically identified

in natural aquatic ecosystems (Penton *et al*., 2006; Schmid *et al*., 2007; Woebken *et al*., 2008). However, recent findings of *Ca. Brocadia* and *Kuenenia* like sequences in a river estuary in Japan (Amano *et al*., 2007), in USA (Dale *et al*., 2009) as well as in freshwater sediment in China (Zang *et al*., 2007) showed that there is a wider diversity of anammox bacteria in nature than previously presumed.

Ecology and importance

Since its discovery, the anammox process has been applied for nitrogen removal in wastewater treatment plants (WWTPs). Precisely, the application of the anammox process combined with nitrification contributes to the ammonium removal in wastewater systems. This approach mainly called SHARON-Anammox (van Dongen *et al*., 2001) involves partial nitrification, where half of the ammonium is oxidized to nitrite, followed by anammox, where the remaining ammonium is converted to N_2 with the produced nitrite (Fig. I. 15).

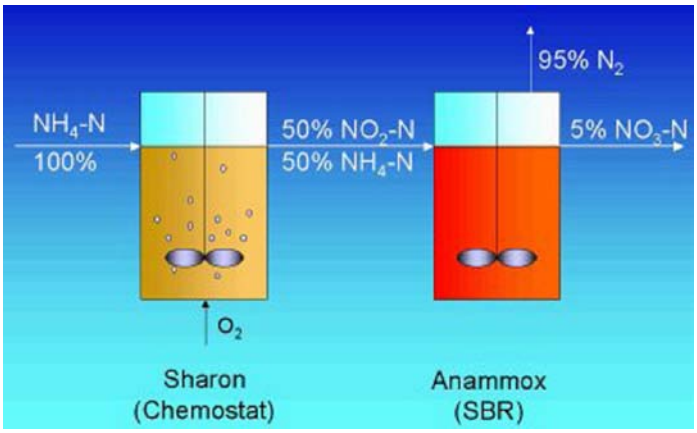


Figure I. 15. Principle of the combined Sharon-anammox process as it is applied in a WWTP of Rotterdam (van Dongen *et al*., 2001).

Another application of this combination of process is called CANON for 'Completely Autotrophic Nitrogen removal Over Nitrite' (Sliekers *et al*., 2003; Third *et al*., 2005) where aerobic ammonium oxidizing bacteria (nitrifiers) with anammox bacteria perform simultaneously a two step reaction under oxygen-limited conditions. However, this process is limited to the laboratory and pilot-plant scale due to the complex controlling conditions and the slow growth of anammox bacteria (van Loosdrecht, 2004). Since the addition of organic carbon substrates and energy for aeration are not required for both nitrogen removal processes, they are more cost effective and environment friendly since less greenhouse gases are produced than the conventional N-elimination by sequential nitrification/denitrification (Volcke, 2006).

Anammox bacteria were first considered to be minor players in the global nitrogen cycle but their relative importance in N_2 production in marine ecosystems has changed this point of view. Using ^{15}N-isotope incubation experiments, anammox activity in nature was first determined in marine sediments where it accounts for up to 67% of the produced N_2 (Thamdrup and Dalsgaard, 2002). The highest amounts of N_2 production by anammox ranged between 20% to 70% and were found in suboxic water columns (Dalsgaard *et al*., 2003; Kuypers *et al*., 2003; 2005), in oceanic oxygen minimum zones (Thamdrup *et al*., 2006; Hammersley *et al*., 2007) and in shelf sediments (Thamdrup and Dalsgaard, 2002; Trimmer and Nicholls, 2009). Conversely, anammox contributes to less than 20% of total N_2 production in coastal, and estuarine sediments (Engström *et al*., 2005; Hietanen and Kuparinen, 2008; Rich *et al*., 2008; Dale *et al*., 2009), in sea ice (Rysgaard *et al*., 2008) and in meromictic lakes (Schubert *et al*., 2006). Anammox accounted significantly in shelf sites whereas compared to denitrification its relative importance is minor in organic rich coastal sediments. Thus, the importance of anammox activity relative to denitrification depends on the availability of NOx-, the presence of anammox cells, and the quantity and quality of organic matter (Dalsgaard *et al*., 2005; Engström *et al*., 2005; Meyer *et al*., 2005; Trimmer *et al*., 2005). In contrast to aquatic systems, the detection and measurement of anammox activity is still lacking in terrestrial ecosystems.

The project

Hypothesis

As anammox bacteria need the concomitant presence of oxidized and reduced nitrogen compounds in their environment close interactions with other N cycling bacteria is suspected. In soils, ammonium is provided by the organic matter degradation *via* mineralization. Moreover, ammonium could be produced by anaerobic- or anammox-bacteria performing the dissimilatory nitrate reduction to ammonium (DNRA) under anoxic conditions (Kartal *et al*., 2007; Fig. I. 7). Nitrite can be produced by aerobic ammonium oxidizers at oxic/anoxic interface or by nitrate reducing bacteria under anoxic conditions (Fig. I. 7). Moreover, the significant occurrence of anammox activity in the wide range of aquatic environments suggests that this process occurs in any N-

containing ecosystems with oxic/anoxic zone. Terrestrial ecosystems present favorable environments with also oxic/anoxic interfaces where anammox bacteria could be found. A wide range of terrestrial ecosystems were selected based on the following hypothesis: terrestrial oxic/anoxic interfaces would provide appropriate habitats for anammox bacteria (Fig. I. 16). Precisely, oxic/anoxic interfaces may include, in 'oxic' soils (Fig. I. 16A): the rhizosphere where the oxygen concentration is reduced compared to distant soil due to respiration of plant roots and microorganisms; the bulk soil where anoxic pockets exist within soil macro-aggregates; and the soil-groundwater-table interface including its fluctuation zone. In water saturated soils (Fig. I. 16B), such conditions are met in the rhizosphere of marsh plants, where oxygen is transferred through the aerenchyme into the otherwise anoxic submersed soil (Brune *et al.*, 2000).

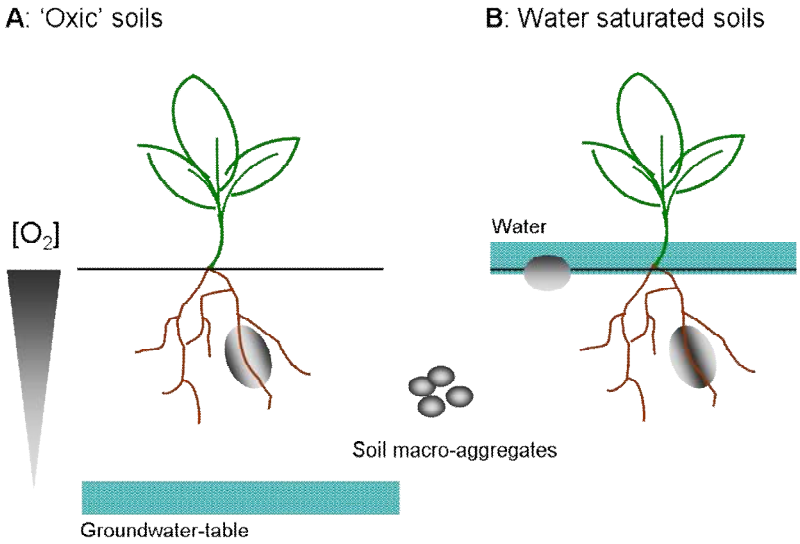


Figure I. 16. Potential oxic/anoxic interfaces present in A) oxic soils and B) water saturated soils favorable for the growth of anammox bacteria.

Aim of the project

This thesis was focused on exploring the anammox part of the global terrestrial nitrogen cycle. To better understand the distribution, diversity, abundance and activity of anammox bacteria in terrestrial ecosystems a combination of molecular tools, biogeochemistry methods, and ^{15}N-incubations experiments have been established. The study was conducted following different questions in order to obtain a better view and understanding of this process in the terrestrial realm:

- Are anammox bacteria present in terrestrial ecosystems?
- Who are they? What is their diversity? How are they distributed?

- How many anammox bacteria are present in terrestrial environment?
- Are they active? What is their contribution to total N_2 formation?

Chapter II

Are anammox bacteria present in terrestrial ecosystems?

Molecular detection of anammox bacteria in terrestrial ecosystems

Chapter based on the published article:
Humbert S, Tarnawski S, Fromin N, Mallet MP, Aragno M, and Zopfi J. (2010). Molecular detection of anammox bacteria in terrestrial ecosystems: distribution and diversity. ISME J. 4: 450-454

Abstract

The role of anaerobic ammonium oxidation (anammox) in nitrogen cycling has been studied widely in marine and freshwater redox transition zones, including chemoclines of stratified water columns and surface sediments. Yet nothing is known to date about distribution, diversity, and activity of anammox bacteria in the terrestrial realm. Based on the hypothesis that the abundant redox transition zones in soils provide also suitable habitats, a wide range of soil types and plant-soil systems were sampled and tested for anammox bacteria using a nested-PCR approach. From several soils 16S rDNA sequences were retrieved that were phylogenetically closely related to known anammox candidate genera. Anammox bacteria were not ubiquitously present but were only detected in certain soil types and at particular depths, thus reflecting their ecological requirements. So far, sequences of *Candidatus* 'Brocadia', 'Kuenenia', 'Scalindua', and 'Jettenia' were detected in marshes, lakeshores, a contaminated porous aquifer, permafrost soil, agricultural soil, and in samples associated with nitrophilic or nitrogen fixing plants. This suggests a higher diversity of anammox bacteria in terrestrial than in marine ecosystems and could be a consequence of the larger variety of suitable niches in soils. As opposed to water columns habitats where *Candidatus* 'Scalindua' dominates anammox guilds, 'Kuenenia' and 'Brocadia' appear to be the most common representatives in terrestrial environments.

Introduction

Anammox bacteria form a deep-branching, monophyletic group within the Planctomycetes and oxidize anaerobically ammonium to dinitrogen gas with nitrite as electron acceptor (Kuenen, 2008). This conversion involves reactive intermediates such as hydrazine and hydroxylamine and takes place in a separate cell compartment called anammoxosome (van de Graaf *et al*., 1997; Lindsay *et al*., 2001). Shortly after its discovery (Mulder *et al*., 1995), anammox was applied to inorganic nitrogen removal in wastewater treatment (e.g. Strous *et al*., 1997; Paredes *et al*., 2007) but its importance in natural ecosystems was recognized only recently (Thamdrup and Dalsgaard, 2002; Francis *et al*., 2007). Anammox bacteria are active at redox transition zones in various aquatic environments, particularly in oceanic oxygen minimum zones (e.g. Dalsgaard *et al*. 2003; Kuypers *et al*. 2003; Stevens and Ulloa, 2008) and in marine surface sediments (e.g. Hietanen and Kuparinen, 2008; Rich *et al*., 2008), but also in sea ice (Rysgaard *et al*.,

2008), and meromictic lakes (Schubert *et al*., 2006). The contribution of anammox to N_2 production can range from a few to more than 60% (Thamdrup and Dalsgaard, 2002; Kuypers *et al*., 2005; Schmid *et al*., 2007) whereby its relative importance depends on the availability of NO_x-, the presence of anammox cells, and the quantity and quality of organic matter (Dalsgaard *et al*., 2005; Engström *et al*., 2005; Meyer *et al*., 2005; Trimmer *et al*., 2005).

Since anammox depends on the concomitant presence of both oxidized and reduced inorganic nitrogen compounds under anoxic conditions, we hypothesize that oxic/anoxic interfaces in terrestrial ecosystems provide appropriate habitats for anammox bacteria (Fig. II. 1). In 'oxic' soils, this may include: the rhizosphere where the oxygen concentration is reduced compared to distant soil due to respiration of plant roots and microorganisms; the bulk soil where anoxic pockets exist within soil macro-aggregates, and the soil-groundwater-table interface including its fluctuation zone. In water saturated soils, such conditions are met in the rhizosphere of marsh plants, where oxygen is transferred through the aerenchyme into the otherwise anoxic submersed soil (Brune *et al*., 2000).

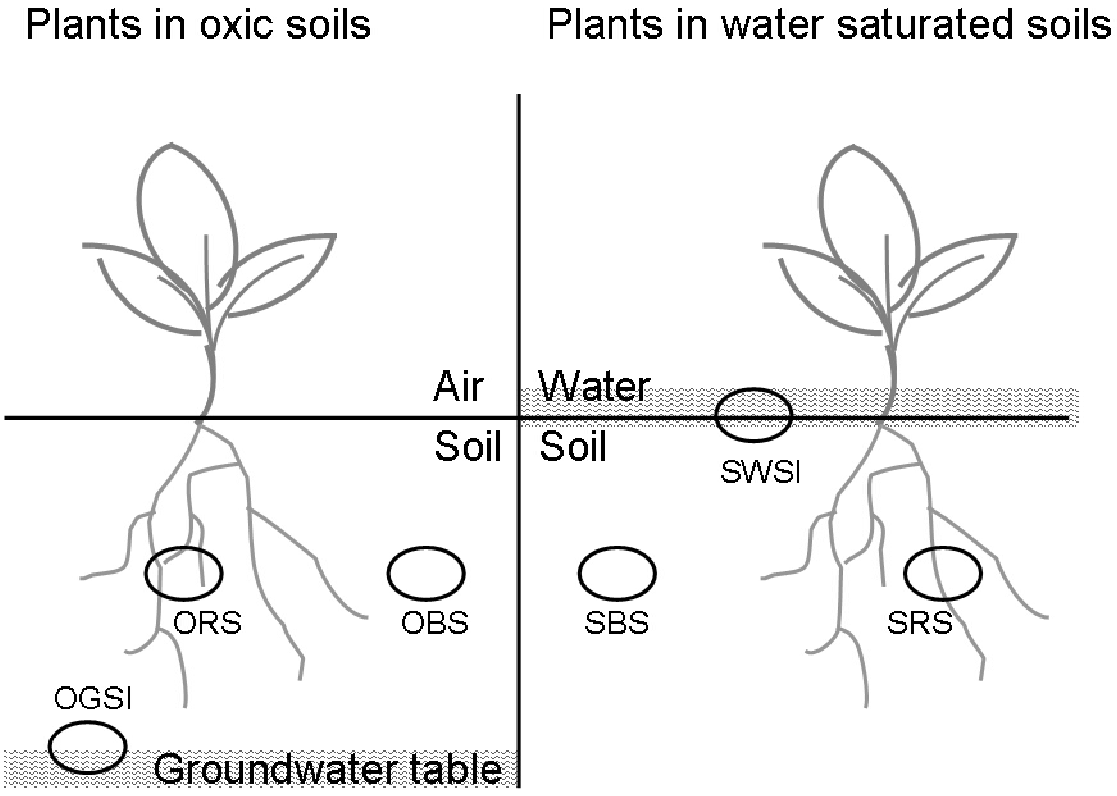


Figure II. 1. Terrestrial microenvironments potentially favourable for anammox bacteria: In oxic soils: ORS) rhizosphere soil, OBS) bulk soil, OGWSI) groundwater table; In water saturated soils: SBS) anoxic bulk soil, SRS) flooded rhizosphere soil, SWSI) water-soil interface. Abbreviations correspond to the soil fractions in Table II. 2.

The goals of this study were to test whether anammox bacteria occur in soils, to assess their environmental distribution, and to determine their diversity at selected sites. A two step molecular screening approach was established, consisting of an initial PCR amplification of Planctomycetales 16S rRNA followed by a second PCR targeting the 16S rRNA gene of anammox bacteria. Subsequent sequence analysis of cloned PCR products was performed to determine their phylogenetic affiliation. We show that soils are potential habitats for anammox bacteria and harbour a greater genus level diversity than in marine water column environments. These results represent a first step towards a global understanding of the anammox process and the biogeography of anammox bacteria.

Material and methods

Choice of sites and samples

According to the hypothesis that anammox bacteria may thrive at oxic/anoxic interfaces in soils, different locations encompassing a wide variety of suitable ecosystems were selected for sampling:

(i) Wetlands in the Camargue (southern France), in the « Grande Cariçaie » (Swiss plateau), and in the « Alpe di Cadagno » (Swiss Alps, Canton of Ticino, CH).

(ii) Lake shores where water table fluctuations create temporally and spatially dynamic oxic/anoxic interfaces (Lake Neuchâtel and Lake Loclat, Swiss plateau).

(iii) Anthropogenic N-enriched sites, such as an ammonium-contaminated porous aquifer in the Rhone valley (Canton of Valais, CH) as well as an intensively N-fertilized agricultural field in Boudry (Canton of Neuchâtel, CH).

(iv) Soils subjected to freeze/thawing cycles resulting in oxic/anoxic transients: including a permafrost soil (Creux-du-Van, Jura Mountains, Canton of Neuchâtel, CH) and a glacier forefield (Morteratsch glacier, Engadin, Canton of Graubünden, CH).

These sites allowed sampling of different types of microenvironments, plant-related or not, providing conditions *a priori* favorable to anammox bacteria at oxic/anoxic

interfaces (Table II. 1). In oxic soils: rhizosphere fraction (ORS) of *Urtica dioica*, *Alnus viridis, Rumex alpinus, Filipendula ulmaria* and *Carex davalliana*; bulk soils (OBS) in the neighbourhood of *Alnus incana, Fraxinus excelsior* and *Zea mays*, as well as in the groundwater table-soil interface (OGSI). In water saturated soils: rhizosphere fraction (SRS) of *Phragmites australis* and *Cladium mariscus*, as well as anoxic bulk soils (SBS) and water-soil interfaces (SWSI) in marsh sediment or Salisodisol.

Sampling

The geographical coordinates of the sampling sites including information about vegetation and sampled soil fractions are presented in Table 2. II together with the anammox screening results. Two soil fractions were sampled whenever possible: rhizosphere soil (RS) was the fraction of soil remaining attached to the roots after gentle shaking, whereas bulk soil (BS) was sampled at distance from the roots. Soil and sediment samples were frozen on-site in liquid nitrogen, transported to the laboratory and stored at -80°C until DNA was extracted.

DNA extraction and PCR amplification

Total genomic DNA was extracted from about 0.5 g (ww) of soil with the FastDNA SPIN kit for soil (BIO 101, Qbiogene Inc., Carlsbad, CA, USA) according to the protocol of the manufacturer (Annex A-1). The amplifiability of the extracted DNA was subsequently tested by PCR with universal primers targeting the 16S rDNA of *Eubacteria* (Muyzer *et al.*, 1993; Annex A-2). Different combinations of the primers (Table II. 1.) were tested for the amplification of *Planctomycetales* and anammox 16S rDNA, whereby a sequential PCR approach was finally chosen based on the amplification yield and the absence of unspecific PCR products with positive controls. In the first PCR round *Planctomycetales* 16S rDNA was amplified with Pla46f as forward and Univ1390r as reverse primer (Annex A-3). The second, anammox specific PCR was performed by using Amx368f and either Amx820r or BS820r (Schmid *et al.*, 2005; Annex A-4). The first PCR reaction mixture was prepared in a final volume of 20 µl, containing 2mM $MgCl_2$, 1X PCR buffer, 0.2mM of each dNTP, 0.3 µM of each primer, 0.5 U GoTaq polymerase (Promega AG, Dübendorf, Switzerland) and 2 µl of 1-5ng/µl DNA extract. The nested PCR mixture was similar to the first one except that 0.25 mM of each dNTP, 0.25 µM of each primer and 2 µl of tenfold diluted PCR product were used. PCR was done on a PTC-200 Bioconcept (MJ

Research Inc., Watertown, MA, USA) thermocycler using the following parameters for both PCR amplifications: initial denaturation step of 2 min at 95°C, followed by 30 cycles of denaturation at 95°C for 45 s, annealing at 62°C for 50 s and elongation at 72°C for 1 min 22 s. The final elongation step was set at 72°C for 5 min. Negative controls (*Escherichia coli* and *Pseudomonas fluorescens* DNA) and positive controls (DNA of anammox enrichment cultures from M. Schmid (Nijmegen, NL) and from waste water treatment plants of Neuchâtel and Visp (CH)) were included routinely. The expected amplicon length was 1350 bp for the *Planctomycetales* PCR and 480 bp for the anammox PCR.

Table II. 1: PCR primers combination used for 16S rRNA gene sequence amplification of *Planctomycetales* and anammox bacteria.

Primer combination	Target group	Primer sequence (5'-3')	Annealing temperature	Reference
Pla46f	*Planctomycetales*	GGATTAGGCATGCAAGTC	62 C°	Neef *et al*., 1998
Univ1390r	*Bacteria*	GACGGGCGGTGTGTACAA		Zheng *et al*., 1996
Amx368f	Anammox organisms	TTCGCAATGCCCGAAAGG	62 C°	Schmid *et al*., 2003
Amx820r	'*Ca. Brocadia*' and '*Ca. Kuenenia*'	AAAACCCCTCTACTTAGTGCCC		Schmid *et al*., 2000
Amx368f	Anammox organisms	TTCGCAATGCCCGAAAGG	62 C°	Schmid *et al*., 2003
BS820r	'*Ca. Scalindua wagneri*'' and '*Ca.Scalindua sorokinii*'	TAATTCCCTCTACTTAGTGCCC		Kuypers *et al*., 2003

Evaluation of PCR protocol

A PCR approach has been established to provide a method of screening environmental samples for the presence of anammox bacteria. Based on published PCR primers for anammox or *Planctomycetales* bacteria, different combinations of primer sets (Table II. 1) have been tested. Since direct amplification of anammox 16S rRNA genes with Amx368f/Amx820r was not successful with most soil samples (data not shown), an additional PCR step was usually required to increase the template number of *planctomycetes* (Schmid *et al*., 2005). The suitability of two reverse primers (Amx820r

and BS820r, Table II. 1) was further tested using DNA extracts from an ammonium-contaminated porous aquifer. Two clone libraries were constructed and the clone sequences retrieved using both primer sets were affiliated to anammox bacterial candidate genera and were highly similar (99% sequence similarity). Similar results were reported by Amano *et al*. (2007) who observed no significant differences between clone libraries constructed with either of these two reverse primers.

Finally, a sequential PCR approach was selected for the amplification of 16S rRNA genes of anammox organisms using primers sets Pla46f/Univ1390r and Amx368f/Amx820r at an annealing temperature of 62°C. PCR products of expected size were obtained following this protocol with DNA from enrichment cultures as well as from soil and sediment samples. In samples where anammox 16S rRNA gene were low abundant or absent, unspecific amplifications were sometimes observed (Fig. II. 2-3). Since the amplifiability of the extracted DNA had been verified beforehand, failure to produce any PCR product of the correct size was taken as below the detection limit.

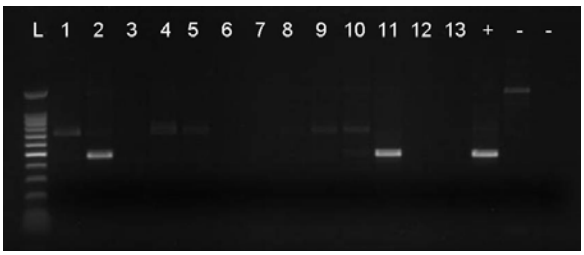


Figure II. 2. Anammox nested PCR results of Lake Loclat shore samples.

L : Ladder 100 bp
1. *Phragmites australis,* roots
2. *Phragmites australis,* rhizosphere
3. *Fraxinus excelsior,* rhizosphere 0-20cm
4. *Fraxinus excelsior,* rhizosphere 20-40cm
5. *Fraxinus excelsior,* rhizosphere 40-60cm
6. *Fraxinus excelsior,* rhizosphere 60-80cm
7. *Fraxinus excelsior,* rhizosphere 80-100cm
8. Reductisol, 0-5cm
9. Reductisol, 5-10cm
10. Reductisol, 10-20cm
11. Reductisol, 20-35cm
12. *Urtica dioica,* rhizosphere
13. *Filipendula ulmaria,* rhizosphere
\+ Positive control, enrichment culture
\- Negative control, *E.coli*
\- Negative control, water

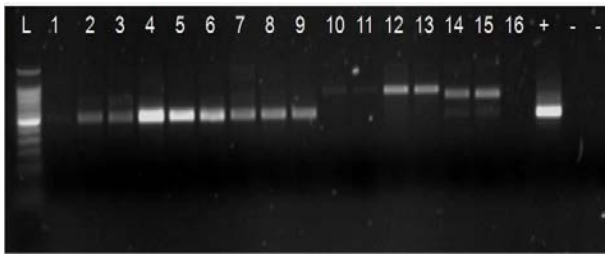


Figure II. 3. Anammox nested PCR results from Camargue samples.

L : Ladder 100 bp
1. *Phragmites australis,* bulk soil
2. *Phragmites australis,* roots
3. *Phragmites australis,* rhizosphere
4. Marsh sediment, water-soil interface
5. Marsh sediment, bulk soil
6. Marsh sediment, bulk soil
7. *Phragmites australis,* bulk soil
8. *Phragmites australis,* roots
9. *Phragmites australis,* rhizosphere
10. Fallow field, water saturated
11. Fallow field, water saturated
12. Grassland, water saturated
13. Grassland, water saturated
14. *Phragmites australis* roots
15. *Phragmites australis* rhizosphere
16. Salisodisol
\+ Positive control, enrichment culture
\- Negative control, *E.coli*
\- Negative control, water

Clone library production, analysis and sequencing

PCR products were purified with the Wizard SV Gel and PCR Clean-up System (Promega), ligated into pGEM-T vector (Promega) and used for transformation of electrocompetent *E. coli* XL1 cells (Annexes A-5 and A-6). After blue/white screening 6 to 50 transformants per sample were randomly selected to constitute the clone library. The inserted DNA was amplified using primers T7 / SP6, and submitted to RFLP analysis using *Msp*I (Promega; Annexes A-7 and A-8). Clone inserts displaying identical restriction patterns were grouped into Operational Taxonomic Units (OTUs). Plasmids were extracted and purified using the Wizard®Plus SV Miniprep System (Promega; Annex A-9). Two clones per OTU and environment were sent to sequencing at MWG-Biotech (Ebersberg, Germany), whereby only OTUs consisting of more than two clones were considered. The phylogenetic affiliation of corresponding organisms was determined using BLAST (Altschul *et al*., 1997) and Ribosomal Database Project-II (Cole *et al*., 2005). A total of 440 nucleotide positions per sequence were aligned using the ClustalW implementation of MEGA4 (Tamura *et al*., 2007), which was also used to calculate NJ and ML trees. Nucleotide sequences have been deposited in the EMBL sequence database under accession numbers FM174251 to FM174320.

Results and Discussion

Environmental distribution of anammox

A wide range of environments were tested for the presence of Planctomycetales and anammox bacteria: Among the 112 samples collected at 9 different geographical locations in Switzerland and France, 82 yielded PCR products for Planctomycetales and 60 for anammox bacteria (Table II. 2). Anammox PCR products were detected in different wetlands, lake shores, a contaminated porous aquifer, permafrost soil, agricultural soil, and in samples associated with nitrophilic or nitrogen fixing plants (Table II. 3). This implies that anammox bacteria are also present in terrestrial environments mostly associated with water and/or high nitrogen contents.

Table II. 2: PCR detection of anammox bacteria in terrestrial ecosystems.

Location	Sampled Environment	Soil fraction	Sample name	Total of analyzed samples	Positive nested-PCR products	Anammox confirmed
Camargue (F) (43°29'37''N, 4°38'57''E)	*Phragmition* (*Phragmites* [a] *australis*)	SBS[b]	CaPh4	6	6	-[c]
		SRS	CaPh5	2	2	-
	Marsh sediment	SBS	CaMs4	2	2	+
		SWSI	CaMs6	1	1	+
	Water saturated fallow field	SWSI	CaFf	2	1	-
	Water saturated grassland	SWSI	CaG	2	0	
	Salisodisol	SBS	CaS4	2	2	n.d.
		SWSI	CaS6	1	1	-
	Rice field	SRS	CaR	4	4	-
Grande Cariçaie (CH) (46°58'32''N, 7°02'36''E)	*Phragmition* (*Phragmites australis*)	SBS	GcPh4	3	3	-
		SRS	GcPh5	3	2	-
	Cladietum (*Cladium mariscus*)	SBS	GcC4	1	1	n.d.
		SRS	GcC5	3	1	+
	Fraxinion (Alnus incana)	ORS	GcA	2	0	
Cadagno (CH) (46°32'53''N, 8°42'04''E)	*Rumicion alpini* (*Rumex alpinus)*	ORS	CadR	3	1	-
	Caricion fuscae /Rhododendro-Vaccinion (Sphagnum sp.)	SRS	CadS	3	3	-
	Alnenion viridis (Alnus virids)	ORS	CadA	1	1	+
	Caricion davallianae (Carex davalliana)	ORS	CadC1	1	0	
		OBS	CadC2	2	0	
Wallis (CH) (46°17'52''N, 7°55'12''E)	Porous aquifer (2m60-16m50)	OGSI	WaA	22	9	+
Shore Lake Neuchâtel (CH) (46°55'60''N, 6°50'21''E)	*Fraxinion (Alnus incana)*	OBS	LnA	6	6	+
	Convolvulion (*Urtica dioica*)	ORS	LnU	1	1	-
Boudry (CH) (46°57'48''N, 6°50'04''E)	Agricultural field (*Zea mays*)	ORS	BoZ1	2	1	n.d
		OBS	BoZ2	1	1	-
	Planted grassland	OBS	BoPg	2	0	
Creux-du-Van (CH) (46°56'15''N, 6°43'28''E)	Permafrost	OBS	CdvP	7	6	+
Shore Lake Loclat (CH) (47°01'07''N, 6°59'57''E)	*Fraxinion (Fraxinus excelsior)*	OBS	LlF	5	1	+
	Reductisol (0-35cm)	OBS	LlR	4	1	+
	Phragmition (*Phragmites australis*)	SRS	LlPh	2	2	-
	Convolvulion (Urtica dioica)	ORS	LlU	1	0	
	Filipendulion (Filipendula ulmaria)	ORS	LlFu	1	0	
Morteratsch glacier forefield (CH) (46°26'19''N, 9°56'07''E)	Glacier forefield *(Epilobium fleischeri)*	ORS	MoE1	10	0	
		OBS	MoE2	6	0	

[a] The sampled plant species in the respective plant community (alliance, sous-alliance, association) is given in parenthesis; [b] Sampled soil fraction as explained in Fig. II.1. [c] +) one sample confirmed, i.e. retrieved 16S rDNA sequences fall into anammox cluster; -: not confirmed; n.d.: not determined

Table II. 3. Soil sample characteristics from where anammox bacterial 16S rRNA gene sequences were retrieved.

Location	Sampled envrionment [a]	Porosity (%)	pH [H_2O]	Corg.tot [%]	N tot. [%]
Grande Cariçaie (CH)	*Cladietum* (*Cladium mariscus*)	83	7.1	34	1.3
Wallis (CH)	Porous aquifer (2m60-16m50)	17	7.7	1.5	1.7
Shore Lake Neuchâtel (CH)	*Fraxinion (Alnus incana)*	31	7.4	6.8	0.2
Creux-du-Van (CH)	Permafrost	10	5.4	44.6	1.3
Shore Lake Loclat (CH)	*Fraxinion (Fraxinus excelsior)*	59	7.3	26.3	1.7
	Reductisol (0-35cm)	49	7.5	12.7	0.2

[a] The sampled plant species in the respective plant alliance or association is given in parenthesis

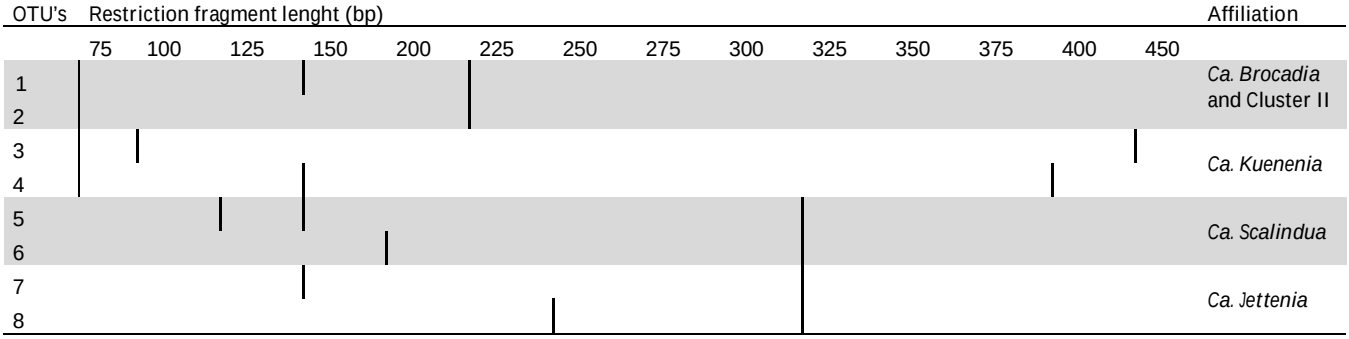
While anammox bacteria may be widespread, 8 out of 9 locations were anammox positive, they are not ubiquitously detected. Usually, not all samples from a given location or environment yielded anammox bacterial PCR products. As in stratified water columns or in sediments where anammox activity is restricted to particular layers (Dalsgaard *et al*., 2003; 2005), anammox sequences were detected at particular depths along a soil profile (Fig. II. 2). Moreover, rhizosphere samples of *Urtica dioica* and *Alnus incana* collected at different locations resulted in positive as well as negative anammox bacterial PCR results. This may suggest that the global environmental conditions (e.g. soil water regime, nitrogen content), rather than the microscale-environmental conditions promote the enrichment of anammox bacteria to a detectable level. Environments where no anammox bacterial PCR products were observed included water-saturated grassland in the Camargue where the conditions were probably too reducing and NOx- thus lacking, planted grassland in Boudry, and rhizosphere soil of *Epilobium fleischeri* in the Morteratsch glacier forefield. Here, oxic conditions and particularly low nitrogen contents may have impeded growth of anammox bacteria.

Phylogenetic identification of 16S rRNA gene sequences

In order to determine the phylogenetic affiliation of the detected anammox bacteria, 43 nested-PCR products of the correct size were used for phylogenetic analysis. These samples represented 17 different environments from 8 Locations. Among the 581 clones

obtained, 107 OTUs were distinguished after RFLP analysis using *Msp*I (Table II. 4). Finally, 200 clones were selected for sequencing according to their OTU affiliation and environmental distribution.

Table II. 4. Principal restriction profiles of anammox bacterial 16S rRNA gene sequences affiliated with known anammox bacteria using MspI.



Phylogenetic analysis revealed that 29% of the clone sequences were closely related to the known anammox genera *Candidatus* 'Brocadia', 'Kuenenia', 'Scalindua', and 'Jettenia'. The remaining environmental clone sequences were related to *Planctomycetes* 16S rRNA gene sequences branching outside the "anammox bacterial cluster" (Fig. II. 4). This cluster was defined on the basis of a limited number of available sequences from described anammox enrichment cultures, which were obtained from a narrow range of environments (e.g. Schmid *et al*. 2003; Kartal *et al*., 2007b; 2008). Furthermore, the "external" sequences have no close representatives among cultivated organisms (Fig. II. 4). It is thus impossible to exclude that at least part of them belong to so far uncultivated anammox bacteria, which could well exist in soils with their inherent heterogeneity and diversity of niches. If they are not, it means that the primer sets used in this study, which were primarily developed as FISH probes (Schmid *et al*., 2005) are not narrowly specific for anammox bacteria. Increasing the number of verified anammox 16S rDNA sequences from enrichment cultures from a variety of soils or metagenomic studies could ultimately lead to a wider definition of the "anammox cluster", and aid developing better adapted primers. In this study, we considered only clones branching within the present "anammox bacterial cluster" as representative of anammox bacteria.

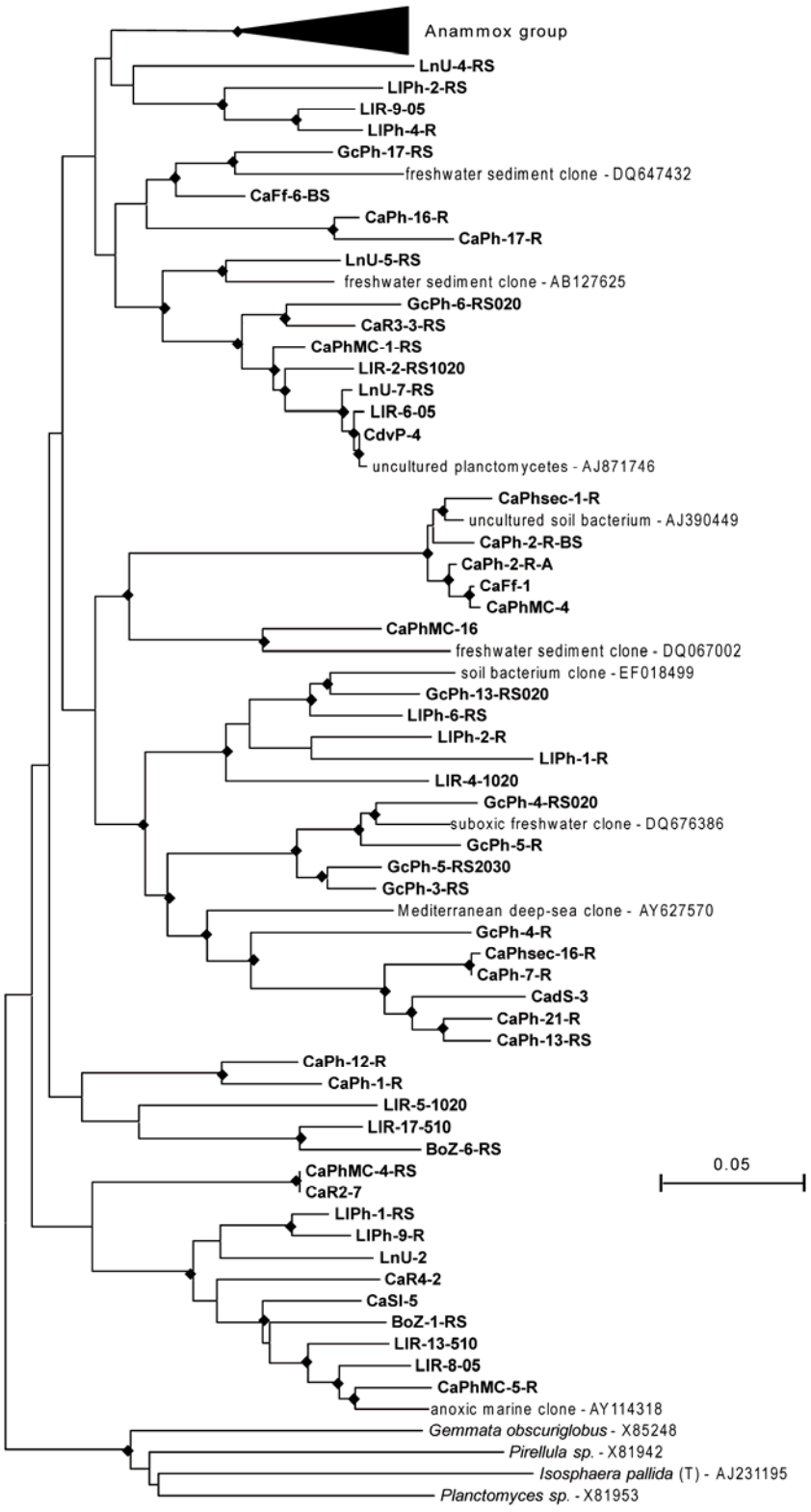


Figure II. 4. Neighbour-joining tree showing the phylogenetic relationship between the 16S rDNA sequences retrieved from different terrestrial environments, known anammox bacteria, and representatives of cultivated *Planctomycetes*. Clones are labelled as follows: sample name (as in Table II. 2)-clone number-soil fraction. The scale bar represents 5% sequence divergence and filled diamonds at nodes indicate bootstrap values above 50% (1000 replicates).

A neighbour joining phylogenetic tree was constructed with environmental and 16S rRNA gene sequences of the described anammox bacterial genera (Fig. II. 5).

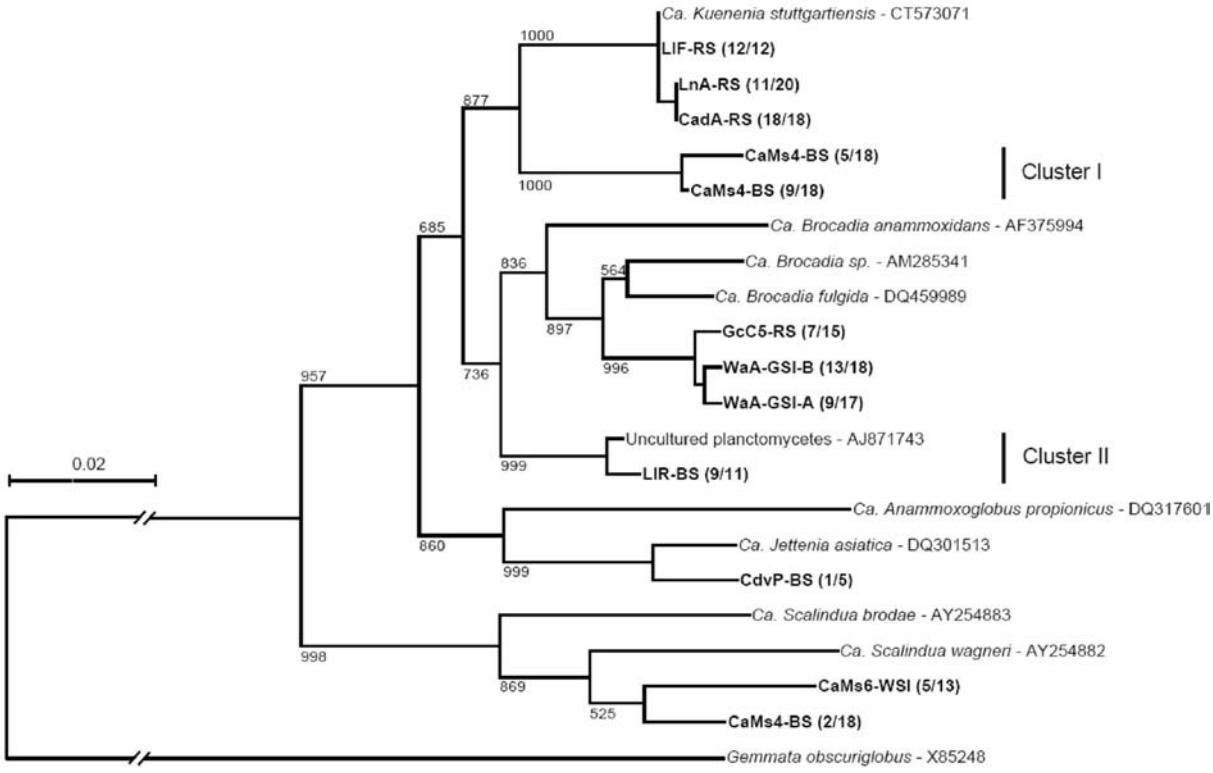


Figure II. 5. Neighbour-joining tree showing the relationship between known anammox bacteria and closely related 16S rRNA gene sequences retrieved from the different terrestrial environments. Clone names are composed as follows: Sample name, followed by soil fraction according to Table II.1. The number of times a sequence was detected among all tested clones of a sample is indicated in parentheses. For the WaA samples clone names are complemented with abbreviations for the reverse primers used for the sequential PCR (A: Amx820r, B: BS820r). Bootstrap values (1000 replicates) higher than 50% are shown and the scale bar represents 2% of sequence divergence.

Four of the five candidate genera were represented in our samples; (1) clone sequences from rhizosphere soil from *Fraxinus excelsior* (shore of Lake Loclat), *Alnus viridis* (Cadagno) and *Alnus incana* (shore of Lake Neuchâtel) were related to *Ca. Kuenenia* with more than 99% of similarity; (2) sequences from the porous aquifer and *Cladium mariscus* rhizosphere from « La Grande Cariçaie » clustered with *Ca. Brocadia* with more than 96% of similarity; (3) sequences from marsh sediment from the Camargue were associated to *Ca. Scalindua* with 94% similarity; and finally (4) sequences from permafrost from the Creux-du-Van were affiliated to *Ca. Jettenia* with 97% similarity. Two groups of clones could not be affiliated unambiguously to any described anammox genera yet and formed distinct clusters within the anammox group (Fig. II. 5). Cluster I

consisted uniquely of sequences obtained from a salt marsh in southern France (Fig. II. 5, CaMs4-SBS) while Cluster II contained sequences from a reductisol (Fig. II. 5, LIR-OBS) and a ammonium-contaminated aquifer (AJ871743; Smits *et al*., 2009). Sequences from both clusters shared equal similarities of 95% with both *Ca. Kuenenia* and *Ca. Brocadia* and could represent so far undescribed anammox bacterial genera. *Brocadia* and *Kuenenia* species are traditionally found in wastewater treatment plants and bioreactors (Schmid *et al*., 2005). It has thus been suggested that the presence of these two genera in estuarine sediments might be because of urbanization (Dale *et al*., 2009). We show here that particularly *Ca. Brocadia* and *Kuenenia* are frequently detected in soils unaffected by any human activity. In most cases, only one anammox taxon was detected in a sample; but as only a limited number of clones have been tested per environment this result must be interpreted cautiously. The only exceptions among the sampled locations were the Camargue marsh sediments, which harboured both *Ca. Scalindua*-related representatives and members of Cluster I. The finding of *Ca. Scalindua* in this saline ecotone that links the marine and the terrestrial realm is consistent with the previous observation that *Ca. Scalindua* predominates the marine anammox guild (for example, Penton *et al*., 2006; Schmid *et al*., 2007; Woebken *et al*., 2008). Similarly, studies on the biogeography of anammox bacteria in river estuaries revealed that *Ca. Scalindua* was the most abundant anammox genus at higher salt contents whereas *Ca. Brocadia* and *Kuenenia* were negatively correlated with salinity (Amano *et al*., 2007; Zhang *et al*., 2007; Dale *et al*., 2009). These findings might be interpreted as a continental signal of anammox bacteria in an environment typically dominated by *Ca. Scalindua*.

Conclusion

In this study, we provide evidence for the presence of anammox bacteria in a wide range of soil environments. The higher number of detected anammox bacterial genera in terrestrial as compared with the relatively homogenous marine water column environments may reflect the larger variety of offered anammox niches in soils. Anammox bacteria were not detected everywhere, showing that they require ecological minimum conditions such as oxic/anoxic interfaces and inorganic nitrogen compounds. Yet, the environmental conditions that control anammox activity in soil and determine which anammox phylotypes thrive in an ecosystem are unknown. Whether soil

anammox bacteria indeed perform the classical anammox process remains also to be shown. Recent physiological studies suggest that their metabolism is more versatile than presumed. They may grow heterotrophically and perform dissimilatory nitrate reduction to ammonium (Guven *et al*., 2005; Kartal *et al*., 2007a, 2008). Genome analysis of *Kuenenia stuttgartiensis* further suggested the possibility for anaerobic respiration of iron and manganese oxides (Strous *et al*., 2006). Future studies will therefore focus on the abundance and activity of anammox bacteria in soils to better understand their quantitative contribution to the terrestrial nitrogen cycle.

Chapter III

How many anammox bacteria are present?

Quantification of anammox bacteria in different terrestrial ecosystems by a new qPCR method

Chapter based on the publication in preparation for submission to Applied and Environmental Microbiology:
Humbert S, Zopfi J., and Tarnawski S. (2010). Quantification of anammox bacteria in different terrestrial ecosystems by a new qPCR method. Appl. Env. Microbiol.

Abstract

The diversity and activity of anammox bacteria has been studied in various environments yet knowledge about their abundance and population dynamics is scarce. Data are particularly lacking for soils, where previously described quantitative detection methods failed to deliver reliable results due to limited specificity. A SYBR Green qPCR protocol was developed for the quantification of anammox bacterial 16S rRNA gene copies using newly designed primers (A438f and A668r). This primer set allows specific detection of all currently known anammox candidate genera. The amplification is linear over at least 8 orders of magnitudes with a detection limit of about 10 target copies per reaction tube. Several soil types were investigated and anammox bacterial 16S rRNA gene copy numbers of 4.01×10^4 to 6.74×10^6 per gram of soil were found. Anammox bacteria were also quantified along a Reductisol profile at three different sampling dates. The copy numbers varied significantly with depth but only weakly between the different seasons, indicating that anammox bacteria represent a minor but stable proportion of microbial communities in this soil. The results demonstrate that the newly developed qPCR method is sufficiently sensitive and specific to quantify anammox bacteria in environmental samples in order to better understand their spatial distribution and dynamic.

Introduction

Anaerobic ammonium oxidation (anammox) is a microbial metabolism that combines ammonium and nitrite to molecular nitrogen. As denitrification, the anammox process closes the biogeochemical nitrogen cycle and may be an important process for the removal of nitrogen from ecosystems (Francis *et al*., 2007; Kuenen, 2008). The potential of anammox for nitrogen elimination in technical systems was rapidly recognized (Jetten *et al*., 1998; Strous *et al*., 1997; Third *et al*., 2005; van Loosdrecht and Salem, 2006), however, its role in the natural environment remained still enigmatic. The use of ^{15}N stable isotopes finally permitted quantification of anammox process rates in sediments and water columns (Kuypers *et al.,* 2005; Thamdrup and Dalsgaard, 2002). It was found that anammox is indeed a significant process in nature and may account for up to 67% of total N_2 formation in the oxygen minimum zones of upwelling areas and shelf slope sediments, respectively (Jetten, 2005; Kuypers *et al*., 2005; Trimmer and Nicholls, 2009). Recently, it was also shown that anammox bacteria are widespread in soils and more

diverse than in most other environments but no quantitative data on anammox soil abundance and process rates are available to date (Humbert *et al*., 2010).

Anammox bacteria have resisted isolation in pure culture so far but the study of highly enriched mixed-cultures permitted the physiological characterization of some representatives of anammox bacteria and led to the description of five candidate genera (Kartal *et al*., 2007b; Quan *et al*., 2008; Schmid *et al*., 2000; Schmid *et al*., 2003; Strous *et al*., 1999): *Ca. Kuenenia*, *Brocadia*, *Scalindua*, *Anammoxoglobus*, and *Jettenia*. The diversity and abundance of these bacteria have been investigated mostly by PCR amplification of anammox bacterial 16S rRNA genes, cloning/sequencing (Dale *et al*., 2009; Li *et al*., 2010) and fluorescence *in situ* hybridization (FISH, Schmid *et al*., 2005). In recent years, quantitative real-time PCR (qPCR) methods based on ribosomal (Dale *et al*., 2009, Hamerslay *et al*., 2007; Lam *et al*., 2007; Tsushima *et al*., 2007) or functional genes as *hzo* (hydrazine oxidoreductases) have been developed (Li *et al*., 2010, Quan *et al*., 2008, Schmid *et al*., 2008; Schimamura *et al*., 2007). They represent suitable alternatives to FISH, particularly when the target organisms are in low abundance or when high background fluorescence of environmental samples impairs enumeration. Several primer sets were used for detecting anammox bacterial 16S rRNA genes in different environments (Penton *et al*., 2006; Schmid *et al*., 2005). Because only a few sequences were initially available for the definition of anammox 16S rRNA gene consensus oligonucleotides, they suffered from limited specificity and targeted also other uncultured *Planctomycetes* (Amano *et al*., 2007, Dale *et al*., 2009, Humbert *et al*., 2010, Rich *et al*., 2008).

In this study we developed a specific and sensitive real-time PCR (qPCR) method that allows detection of the 16S rRNA genes of all currently known anammox candidate genera. We quantified successfully anammox bacteria in different terrestrial ecosystems. To the best of our knowledge these are the first quantitative data on anammox bacteria in soils. The presented approach will be useful to screen for and monitor anammox bacteria in different ecosystems and to assess the quantitative importance of anammox populations.

Material and methods

Soil samples and DNA extraction

Soil samples were collected from different terrestrial ecosystems and include: i) Wetlands in the Camargue (F, 43°29'37''N, 4°38'57''E), the «Grande Cariçaie» (CH, 46°58'32''N, 7°02'36''E), the «Alpe di Cadagno» (CH, 46°32'53''N, 8°42'04''E), and in Bellefontaine, French Jura (F, 46°34'8.76''N, 6°04'53.28''E); ii) Forest soil from the shore of Lake Neuchâtel (CH, 46°55'60''N, 6°50'21''E); iii) A porous aquifer in the Rhone valley (CH, 46°17'52''N, 7°55'12''E); iv) Reductisol from the shore of the eutrophic Lake Le Loclat (CH, 47°01'07''N, 6°59'57''E). Genomic DNA was extracted from about 1 g of fresh soil using the FastDNA SPIN kit for soil (BIO 101, Qbiogene Inc., Carlsbad, CA, USA) according to the manufacturer's instructions (Annex A-1). The amplifiability of the extracted DNA was subsequently tested by PCR with universal primers targeting the 16S rRNA gene of *Eubacteria* (Muyzer *et al*., 1993; Annex A-2). Further details on the sampling sites, DNA extraction, and molecular methods can be found elsewhere (Humbert *et al*., 2010; Annexes A-1-3-4).

Design and evaluation of Anammox-specific primers

Nine 16S rRNA gene sequences of the 5 known anammox genera *Ca. Brocadia* (DQ459989, AM285341, AF375994), *Ca. Kuenenia* (CT573071), *Ca. Jettenia* (DQ301513), *Ca. Anammoxoglobus propionicus* (DQ317601), *Ca. Scalindua* (AY254882, AY257181, AY254883) were retrieved from GenBank (http://www.ncbi.nlm.nih.gov/genbank/). Additionally, 17 different sequences of anammox related environmental clones (similarity with corresponding candidate genus ≥ 94%; FM174251 to FM174267) and 10 non-anammox clones (similarity with anammox cluster ≤ 84%; FM174268, 70, 75, 78, 80, 84, 96 and FM174306, 11, 13) were taken from a previous study (Humbert *et al*., 2010). A 482 bp fragment of all 16S rRNA gene sequences was aligned in MEGA4 (Tamura *et al*., 2007) and conserved regions were identified for designing forward and reverse PCR primers. The theoretical specificity of the primers was assessed with the 'Probe Match' tool of the Ribosomal Database Project-II (Cole *et al*., 2005) and the nucleotide BLAST in the GenBank database (Altschul *et al*., 1997).

Quantitative real-time PCR (qPCR) protocol

Adjustments were done to improve specificity and efficiency of the qPCR protocol. Optimal amplification conditions were determined by testing, in final concentrations, $MgCl_2$ from 1.5 to 3 mM in 0.5 mM increments and primers at 150, 300, 600, 1200, 1500 nM. The optimal annealing temperature was defined by temperature gradient PCR from 51°C to 60°C. Amplification was done on a Rotor-gene™ 3000 (Corbett Research, Sydney, Australia) using 10 µl reaction volumes. The optimized reaction mixture consisted of: 0.5X "SensiMix*Plus* SYBR®" qPCR master mix (Quantace Ltd, London, UK), 1200 nM of forward primer A438f, 300 nM of reverse primer A668r, and 1 µl of template DNA, which was either serially diluted plasmid DNA or purified environmental DNA (Annex A-10). Environmental DNA extracts were diluted 0, 4, 8, and 16 times in sterile nuclease-free water (Qiagen, Hilden, Germany) in order to detect potential inhibitory effects of co-extracted soil compounds on the amplification efficiency. The quantity of DNA extract added to the qPCR mix was 1-15 ng. All dilutions of DNA samples were run in triplicate. The following amplification parameters were used: initial activation of the hot-start polymerase at 95°C for 15 min, followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 55.5°C for 15 s and elongation at 72°C for 30 s. Control reactions without template were included in every qPCR run. A melting curve analysis was performed after the last amplification step of each run to detect primer dimers and unspecific amplifications (Rotor-Gene software 6, Corbett Research).

Calibration and concentration calculations

Standard curves (Fig. III. 1A) were established with serial dilutions of plasmid DNA harboring the partial 16S rDNA of a *Ca. Brocadia* related environmental clone (FN691945). The number of gene copies in a known amount of plasmid DNA was determined according to Ritalahti (Ritalahti *et al.*, 2006). Briefly, the calculation was based on the quantification of plasmid DNA at 260 nm using a NanoDrop™ spectrophotometer (Thermo scientific, Wilmington, USA), the total plasmid size of 3482 bp, which corresponds to the anammox 16S rRNA gene cloned into pGEM-T vector, and the average molecular weight per bp (660g/mol). A target gene concentration of $4.6x10^{10}$ copies/µl of plasmid DNA was determined using this approach. The standard curves in the range of $4.6x10^{1}$ to $4.6x10^{8}$ copies/µl DNA were run. Calibration curves, cycle thresholds (C_T), and amplification efficiencies (E) were determined using Rotor-

Gene software 6 (Corbett Research, Sydney, Australia) (Fig. III. 1). The number of *rrn*-operons in anammox bacteria genomes is not known to date and may differ between species. The determined 16S rRNA gene copy numbers are, therefore, not necessarily equal to cell numbers. The target gene copy number/µl of template DNA was determined based on the standard curve. The concentration per gram of fresh soil (copies/g) was calculated as follows: number of gene copies/µl multiplied by the dilution factor of the template DNA and the volume (µl) of extracted DNA divided by the soil sample fresh weight (g). The number of gene copies/µl multiplied by the dilution factor and divided by the concentration of the DNA extract (ng/µl) yielded the number of molecules per ng of soil DNA (copies/ng DNA).

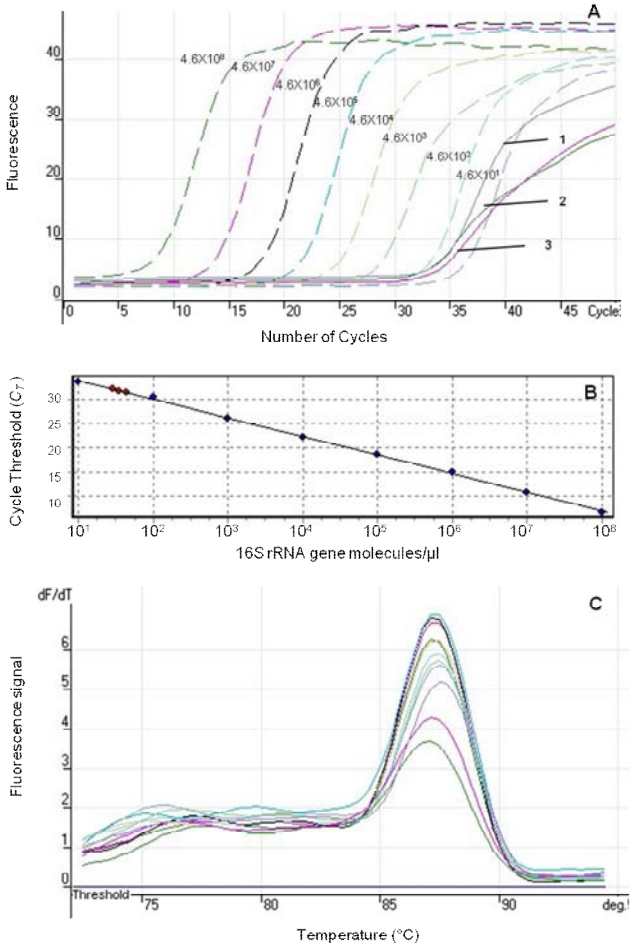


Figure III. 1. Example of anammox bacterial 16S rRNA gene quantification from environmental samples using the qPCR method developed in this study. A) Serial dilution standard curve ($4.6x10^8$-$4.6x10^1$ molecules/µl) and DNA from different ecosystems samples: 1) Wetlands (*Cladietum*) in Grande Cariçaie (CH), 2) Marsh sediment in Camargue (F), 3) Forest soil (*Fraxinion*) in Shore Lake Neuchâtel (CH). B) Linear regression line between the C_T and the amount of 16S rRNA gene molecules. The correlation coefficient of the regression line was 0.999 and the efficiency was 0.83. C) The peak obtained with the melting-curve analysis. The peak represents each standard dilutions and DNA samples melting at 87.2 to 87.5°C.

Cloning, sequencing, and phylogenetic analysis of qPCR products

Positive qPCR products (248 bp length) from two different soil DNA extracts a minerotrophic fen and a Reductisol were ligated into pCR®2.1-TOPO® vector and transformed into *E. coli* TOP10F' using TOPO TA cloning® kit following the manufacturer's instructions (Invitrogen, Carlsbad, CA). After blue/white screening 50 transformants per sample were randomly picked to constitute a clone library. The inserted DNA was amplified using primers T7 and Sp6. PCR products of 31 clones per sample were purified using MSB Spin PCRapace system (Invitek, Berlin, Germany) and sent for sequencing (Eurofins MWG Operon, Ebersberg, Germany). The most closely related sequences were identified using BLAST (Altschul *et al*., 1997). Phylogenetic analyses were done in MEGA4 (Tamura *et al*., 2007) as described previously (Humbert *et al*., 2010). Nucleotide sequences were deposited in the EMBL sequence database under accession numbers FN908027 to FN908044**.**

Results and discussion

Previous studies have demonstrated that current primers used for detecting anammox bacteria in environmental samples are not as specific as expected (Amano *et al*., 2007, Dale *et al*., 2009, Humbert *et al*., 2010, Penton *et al*., 2006, Rich *et al*., 2008).This was particularly true for soils where up to 71% of the retrieved sequences fell outside the anammox bacterial cluster (Humbert *et al*., 2010) and cloning/sequencing was necessary to confirm unambiguously the presence of anammox bacteria in a sample. The first goal of this study was, therefore, to develop new primers that allow a more specific amplification of the anammox bacterial 16S rRNA genes and, at the same time, take into account the increased diversity of anammox bacteria in soils (Humbert *et al*., 2010).

Primers design and *in silico* tests

The design of the new anammox-specific primers was based on the alignment of partial 16S rRNA gene sequences retrieved from the GenBank database and the above-cited foregoing study (Humbert *et al*., 2010). Conserved target regions specific for anammox bacteria were identified between positions 438-456 for the forward primer A438f and between positions 650-668 for the reverse primer A668r (Table III. 1).

Table III. 1: PCR primers used in this study for 16S rRNA gene sequence amplification of *Planctomycetales* order and anammox bacteria

Primer	Target group	Sequence (5'-3')	Reference
Pla46f	*Planctomycetales*	GGATTAGGCATGCAAGTC	Neef *et al*., 1998
Univ1390r	*Bacteria*	GACGGGCGGTGTGTACAA	Zheng *et al*., 1996
Amx368f	Anammox organisms	TTCGCAATGCCCGAAAGG	Schmid *et al*., 2003
Amx820r	*Ca. Brocadia* , *Ca. Kuenenia*	AAAACCCCTCTACTTAGTGCCC	Schmid *et al*., 2005
A438f	Anammox organisms	GTCRGGAGTTADGAAATG	This study
A668r	Anammox organisms	ACCAGAAGTTCCACTCTC	This study

The specificity of the primers was assessed *in silico* using BLAST and the online primer testing tools "probeCheck" (Loy *et al*., 2008) and "Probe match" of RDPII. Analysis revealed that no sequences outside of the anammox bacteria cluster are targeted without mismatch to either one of the two primers. There are no mismatches between primer A438f and the template sequences from *Ca. Kuenenia*, *Ca. Brocadia fulgida*, *Ca. Brocadia sp.* and one mismatch to the target sequences of *Ca. Brocadia anammoxidans, Ca. Scalindua sorokini*, *Ca. Scalindua brodae, Ca. Anammoxoglobus*, *Ca. Jettenia* and three mismatches with *Ca. Scalindua wagneri*. Primer A668r shows perfect match to *Ca. Kuenenia*, *Ca. Brocadia fulgida*, *Ca. Brocadia sp.*, *Ca. Brocadia anammoxidans, Ca. Anammoxoglobus*, *Ca. Jettenia, Ca. Scalindua wagneri* and one mismatch with *Ca. Scalindua sorokini*, *Ca. Scalindua brodae* target sequences (Table III. 2).

Furthermore, both primers have no or at least one mismatches with environmental anammox related 16S rRNA gene sequences and at least 3 mismatches with non-anammox 16S rRNA sequences (Table III. 2), which were amplified in a previous study using the "anammox-specific" primer set Amx368f/Amx820r (Table III. 1). The *in silico* analysis, thus, suggested that the primers are a priori specific and suitable for the detection of all anammox candidate genera.

The designed primer set has compatible melting temperatures of 52 and 54 °C, respectively, and was used to optimize a qPCR protocol for the amplification of a 230 bp fragment of the anammox 16S rRNA gene. Standard curves were established with serial dilutions of a plasmid carrying a 482 bp fragment of *Ca. Brocadia* related 16S rDNA. Concentrations from $4.6x10^8$ to $4.6x10^1$ copies/µl DNA were used for the calibration curve (Fig. III. 1A). The amplification was linear over at least 8 orders of magnitudes and the limit of detection method was below 10 copies/µl of reaction mixture. This result showed that the qPCR protocol is highly sensitive and permits detection of small

amounts of target gene copies in a sample. The stability of the method was tested by 3 different operators: The amplification efficiencies ranged between 0.78 and 0.92 whereby a perfect amplification corresponds to a value of 1.0 (Bach *et al*., 2002). The correlation between the threshold cycle numbers (C_T values) and the log numbers of anammox bacterial 16S rRNA gene molecules was R^2=0.996 on the average (Fig. III.1B). Furthermore, melting curve analysis showed only one peak at 87.6 ± 0.4°C, confirming the absence of non-specific PCR products or primer-dimers (Fig. III. 1C).

Table III. 2: Mismatches in environmental anammox and non-anammox related clone sequences with respect to primers used for the qPCR

16S rRNA sequence name	Related genera	Similarity (%)[b]	5'-3' Forward primer GTCRGGAGTTADGAAATG	5'-3' Reverse primer GAGAGTGGAACTTCTGGT
Ca. Brocadia fulgida (DQ459989)			.....	.....
Ca. Brocadia sp. (AM285341)			.....	.....
Ca. Brocadia anammoxidans (AF375994)			.....C..	.....
Ca. Kuenenia stuttgartiensis (CT573071)			.....	.....
Ca. Jettenia asiatica (DQ301513)			.....G..	.....
Ca. Anammoxoglobus propionicus (DQ317601)			.....G.	.....
Ca. Scalindua wagneri (AY254882)			....A.G.....A	.....
Ca. Scalindua sorokini (AY257181)			.....A	....A.....
Ca. Scalindua brodae (AY254883)			......G.....	....A.....
Wa-A-1-A[a] (FM174253)	*Ca. Brocadia*	96	.....	.....
CadA-1-RS[a] (FM174255)	*Ca. Kuenenia*	99	.....	.....
CaMs-4[a] (FM174267)	*Ca. Scalindua*	94	......G.....	.....
EC-1[a] (FM174251)	*Ca. Jettenia*	97	.....G..	.....
Summer-a-9[a, c](FN691945)	*Ca. Brocadia*	97	.....	.....
CdvP-4[d] (FM174275)	-	-	.....CT.GG.....TG.	.....T.C.A...
LnU-4-RS[d] (FM174268)	-	-	....C.TTC.....CC.	...T.GAACT.CAGGT.G
RSL-7[d]	-	-	......G.GG.....GC	...CTGT....T.C....
CamRros-2[d]	-	-	.....CT.AG.....TCT	.....GAA.G.GGAATTC

a: clones previously retrieved in an environmental study (Humbert *et al*., 2010). Accession number is given in parentheses; b: Similarity of the clone with the respective anammox bacterial genera; c: clone used in this study as standard for calibration curve; d: clone sequence retrieved from environmental samples using primers Amx368f/820r (Humbert *et al*., 2010) clustering outside the anammox group with less than 84% of similarity

In vitro testing of primers and qPCR protocol

Five environmental clones with 16S rRNA inserts related to each of the known anammox genera, 4 environmental clones with inserts of non-anammox *Planctomycetes* (<84% of extracts from where these 9 clones have been retrieved, were tested using the A438f/A668r primer set and the optimized qPCR protocol. All positive control clones

and their environmental source DNA were amplified and yielded a single PCR product as indicated by the melting curve analysis and the unique peak at 87.6 ± 0.4°C (e.g. Fig. III. 1C). Two of the negative control sequences (LIU-4 and CamRros-2, Table III. 2) did not lead to any amplification, however, in two cases (CdvP-4 and RSL-7, Table III. 2) an unexpected amplification signal was observed. The 16S rRNA sequence of these two clones show more than 3 mismatches with both primer sequences (Table III. 2) and their C_T values were at least 1.5 times higher than the C_T values obtained for the same amount of clone DNA with perfectly matching primers. Moreover, qPCR tests with the environmental DNA extracts from where these 2 clones had been retrieved yielded no amplification signal, which suggests that the risk of a false positive signal is small due to the weak amplification efficiency.

In order to illustrate the improved specificity of the new qPCR protocol we compared it with the widely used nested PCR approach (Amano *et al*., 2007, Dale *et al*., 2009, Humbert *et al*., 2010,), where an initial *Planctomycetes* specific PCR with primer set Pla46f/Univ1390r (Table III. 1), is followed by specific anammox bacterial 16S rDNA amplification with primers Amx368f and Amx820r (Table III. 1). Using DNA extracts from different depths from a Reductisol profile as template, the sequential PCR protocol yielded amplification products for all samples (Fig. III. 2A); yet sequence analysis revealed that only amplicons in wells 5 to 12 were affiliated with anammox bacteria. The products in wells 1 to 4 were *Planctomycetes* but did not fall into the anammox cluster (Humbert *et al*., 2010). With the new primers a single PCR product of the correct size was only obtained with the DNA extracts where the presence of anammox bacteria had been confirmed by cloning/sequencing (Fig. III. 2B, samples 5 to 12).

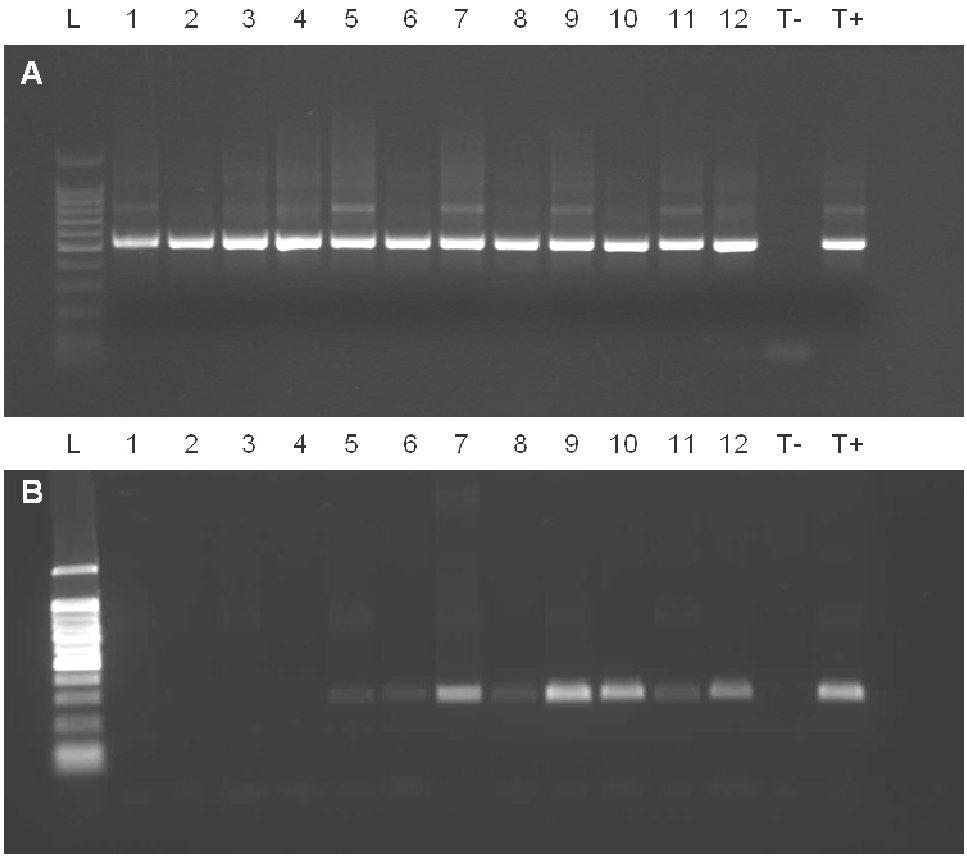


Figure III. 2. Anammox nested PCR results from the Reductisol profile in autumn 2008. A) PCR products obtained with the previous nested-PCR protocol (Humbert *et al*., 2010). B) PCR products obtained with the nested-PCR protocol using the new primer set from this study. L: Ladder (Benchtop 100 bp). Each DNA extract was diluted 10X and 100X and successively loaded on the gel: 1, 2) 0-10 cm; 3, 4) 10-20 cm; 5, 6) 20-30 cm; 7, 8) 30-40 cm; 9, 10) 40-50 cm; 11, 12) 50-60 cm; T-) negative control, sterile desionized water; T+) positive control, anammox bacterial DNA from enrichment culture.

Post PCR controls for the new qPCR protocol

In order to confirm the specificity of the primer set and to exclude false positive amplifications, the qPCR products of two different soil DNA extracts were sequenced (Fig. III. 3). Results show that 100% (n=31) of the clones retrieved from a minerotrophic fen, and 97% (n=31) of the clones retrieved from a Reductisol fell into the anammox bacterial cluster. Sequences were most closely related to *Ca. Brocardia* and a currently not well defined new anammox sequence cluster, which may represent a new genus (Humbert *et al*., 2010, Quan *et al*., 2008; Tal *et al*., 2005). This result is considerably better than with the previous nested PCR approach where only 28% (Reductisol) and 58% (fen) of the retrieved clones were related to anammox bacteria (data not shown). Control experiments show thus consistently that the newly developed primers and qPCR method are suitable to detect and quantify anammox bacterial16S rRNA gene copies in environmental samples.

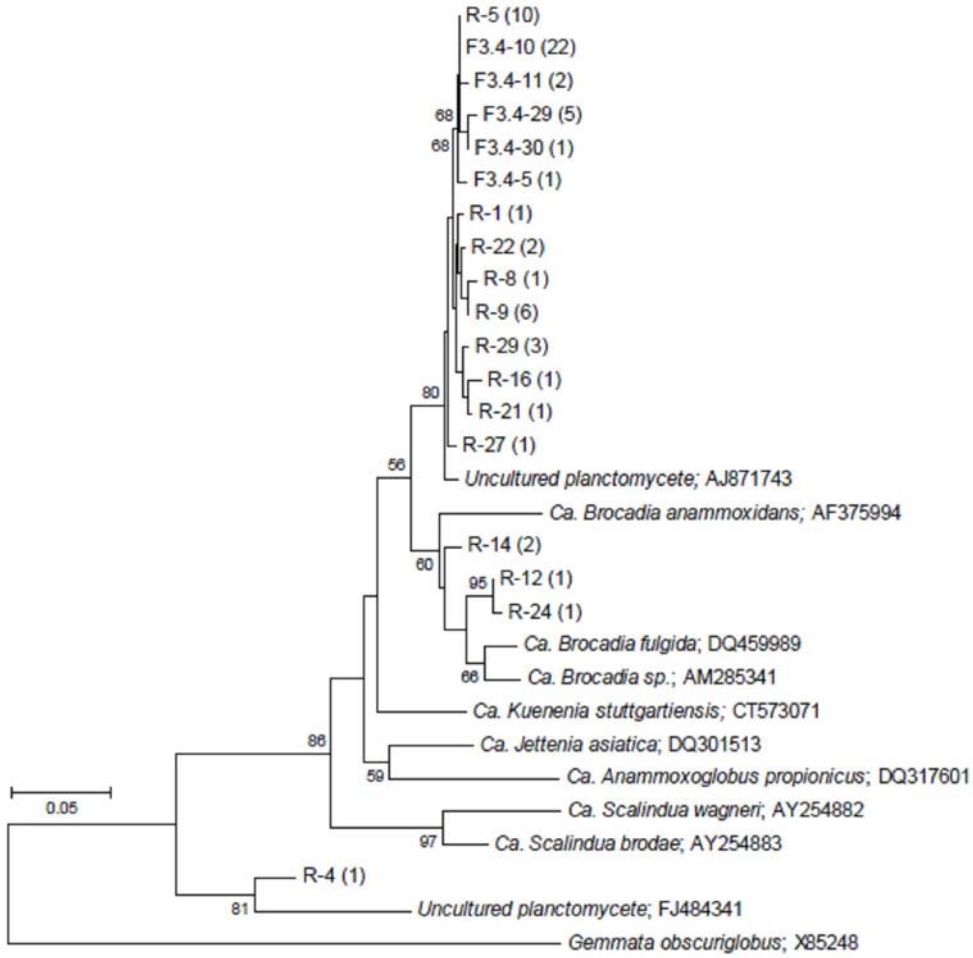


Figure III.3. Neighbour-joining tree showing the relationship between known anammox bacteria and closely related 16S rRNA gene sequences retrieved from the minerotrophic fen (F3.4) and a Reductisol (R). The number of clones with identical sequences is given in parentheses. Bootstrap values (1000 replicates) higher than 50% are shown and the scale bar represents 5% of sequence divergence.

Abundance of anammox bacteria in soils

Anammox 16S rRNA copy numbers were successfully quantified in all examined soil samples, including wetland and forest soils and sediment from a porous aquifer (Table III.3). It should be noted that absolute quantification (copy numbers/g soil) is always based on different assumptions and that the interpretation should be done with care. Copy numbers expressed per nanogram of extracted DNA are e.g. less affected by variations in DNA extraction efficiencies and soil water content. They represent more correct values but for a better comparison with previous studies we discuss mainly absolute values. The determined anammox densities in the different soils varied in a relatively narrow range from 4.01×10^4 to 6.74×10^6 copies/g (or 3 to 230 copies/ng DNA) (Tables III. 3 and 4) and variations between several samples were significant. Different copy numbers are not surprising because these soils cover a wide range of

physicochemical conditions and are not directly comparable with the exception of a shallow and fluctuating groundwater table that induces transient oxic/anoxic conditions. A previous study suggested that such conditions might be favorable for the development of anammox bacteria in wetland soils (Humbert *et al*., 2010). Only limited quantitative data for anammox bacteria in natural ecosystems are available and none exist for soils to date. Much larger variations than in soils were observed in sediments, where reported values are mostly based on direct cell counts using anammox-specific FISH probes (Schmid *et al*., 2007; Tal *et al*., 2005). The reported abundances range from $3x10^2$ cells/ml in Baltimore harbor sediments where only low anammox rates were measured (Tal *et al*., 2005) to $8x10^7$ cells/ml in Barents Sea surface sediment where anammox bacteria account for 8.8% of total cells (Schmid *et al*., 2007). Assuming that anammox bacteria possess only one 16S rRNA gene copy per genome as observed in the genome of *K. stuttgartiensis* (Strous *et al*., 2006) one may conclude that the maximum abundances in soils are at least 35 times lower than in the Barents Sea sediments. The anammox densities determined in this study, however, are in the same range as found in estuarine sediments (1.3×10^5 to 8.4×10^6 cells / g of sediments) using Taqman qPCR (Dale *et al*., 2009). It is not clear to date whether the lower values obtained by qPCR methods represent real ecological differences or are due to a reduced DNA extraction efficiency in soils and sediments or other biases of the different quantification methods (Dale *et al*., 2009).

Table III.3: Quantification of anammox bacterial 16S rRNA gene copies in different soil envrionments.

Location	Sampled environment	Copies/ g of soil ww	Copies/ ng DNA	Anammox genus
Cadagno (CH)	Alnenion viridis (*Alnus viridis*)	$2.02 \times 10^6 \pm 8.50 \times 10^5$ a	85 ± 36 c	*Ca. Kuenenia*
Camargue (F)	Marsh sediment	$4.01 \times 10^5 \pm 1.35 \times 10^5$ bd	3 ± 1 a	*Ca. Scalindua*
Shore Lake Neuchâtel (CH)	Fraxinion (*Alnus incana*)	$6.48 \times 10^4 \pm 3.42 \times 10^4$ c	1 ± 1 d	*Ca. Kuenenia*
Wallis (CH)	Porous aquifer (3.6 m depth)	$1.83 \times 10^5 \pm 8.23 \times 10^4$ d	44 ± 20 c	*Ca. Brocadia*
Grande Cariçaie (CH)	Cladietum (*Cladium mariscus*)	$2.14 \times 10^5 \pm 1.19 \times 10^5$ bcd	2 ± 1 ad	*Ca. Brocadia*
Bellefontaine (F)	Caricion davallianae (25 cm depth)	$1.82 \times 10^6 \pm 1.21 \times 10^6$ ab	15 ± 10 a	*Ca. Jettenia* + *Brocadia*
	Caricion davallianae (44 cm depth)	$2.29 \times 10^6 \pm 4.89 \times 10^5$ a	36 ± 8 b	*Ca. Brocadia* + *Anammoxogl.*

a, b, c, d: Mean values were compared using *t* test. Significant differences of gene copy numbers ($P<0.05$) between environments are indicated by different letters

Table III. 4: Quantification of anammox bacterial 16S rRNA gene copies in a Reductisol soil profile at three differents dates. Samples were collected from the shore of the Lake Loclat (CH) in 2008.

	Spring (May 6)		Summer (July 16)		Autumn (October 10)	
Depth (cm)	Copies/g of soil ww	Copies/ng of DNA	Copies/g of soil ww	Copies/ng of DNA	Copies/g of soil ww	Copies/ng of DNA
0-10	ND	ND	$1.78 \times 10^5 \pm 1.66 \times 10^5$ a	6 ± 5 a*	ND	ND
10-20	-	-	-	-	ND	ND
20-30	$4.01 \times 10^4 \pm 9.85 \times 10^3$ aA	3 ± 1 aA	$2.16 \times 10^5 \pm 2.06 \times 10^4$ aB	11 ± 1 a*B	$6.05 \times 10^5 \pm 4.33 \times 10^5$ a*AB	21 ± 12 a*AB
30-40	-		-	-	$6.74 \times 10^6 \pm 2.70 \times 10^6$ b	230 ± 92 b*
40-50	-		-	-	$4.67 \times 10^6 \pm 6.12 \times 10^5$ b	169 ± 22 b
50-60	$1.82 \times 10^6 \pm 4.19 \times 10^5$ bA	183 ± 42 bA*	$4.98 \times 10^5 \pm 2.67 \times 10^5$ aB	1049 ± 562 b*B*	$4.36 \times 10^5 \pm 7.06 \times 10^4$ aB	197 ± 32 bA*

ND: No fluorescence signal detected in qPCR; "-": not analyzed

a, b: Mean values were compared using *t* test. Significant differences of gene copy numbers ($P<0.01$; * for $P<0.05$) between depths are indicated by different letters

A, B: Mean values were compared using *t* test. Significant differences of gene copy numbers ($P<0.01$; * for $P<0.05$) between sampling dates are indicated by different letters

Repeated quantification in a Reductisol revealed a consistent picture of the distribution of anammox bacteria along a soil profile (Table III. 4). Anammox bacteria were absent or in low abundance at the soil surface in spring and autumn at 0-10cm and increased significantly with depth, which was particularly evident when copy numbers were expressed per ng of extracted DNA. The data were sometimes blurred when absolute numbers were considered. For example in summer, when the anammox 16S rDNA copies increased from 6 to 1049 copies/ng DNA no significant differences were observed in the copy numbers/g of fresh soil.

A complete soil profile from 0 to 60 cm was analyzed in fall 2008 whereby anammox 16S rRNA genes were detected from 20 to 60 cm depth (Table III.4). Maximum average copy numbers of 5.7 x 10^6 copies/g were determined between 30 and 50 cm, which corresponded to the groundwater level at the time of sampling. This result supports the hypothesis that oscillating oxic/anoxic conditions in soils may favor the growth of anammox bacteria (Humbert *et al*., 2010). Conversely, the upper soil sections do not appear to be preferred habitats for anammox bacteria. They may be outcompeted for inorganic nitrogen compounds by growing plants and heterotrophic nitrate and nitrite reducers. The predominance of anammox bacteria in deeper soil sections may be the main reason why this process was overlooked in soils so far.

The seasonal variations in 16S rRNA gene abundances were less pronounced and only significant for the spring anammox abundances, which were lower than during the other seasons (Table III.4). At 20-30 cm depth, anammox bacterial 16S rRNA gene abundances increased by about one order of magnitude from spring to fall. The results clearly show that anammox bacteria represent permanent and, in terms of abundances, stable members of the soil microbial community in this Reductisol.

Conclusion

Two new primers for the specific detection of anammox bacterial 16S rRNA genes were designed and a sensitive qPCR protocol was developed. The method was successfully applied on different environmental samples and delivered the first quantitative data of anammox bacteria in soils. The determined anammox bacterial 16S rDNA copy numbers were in the same range as observed in estuarine sediments. Copy numbers were lowest in soil layers influenced by plant activity but increased typically with depth. Measurements at different sampling dates suggest that soils can harbor persistent

populations of anammox bacteria at specific depths. In future studies, a reverse transcriptase-qPCR protocol may be applied to quantify active anammox bacteria in a wide range of terrestrial ecosystems to better understand their spatial distribution, activity, and populations dynamic.

Chapter IV

Are terrestrial anammox bacteria active?

Seasonal variation of anammox and denitrification activities along a Reductisol profile

Chapter based on the publication submitted to FEMS Microbiology Ecology:
Humbert S, Tarnawski S., Seth B., and Zopfi J. (2010). Seasonal variation of anammox and denitrification activities along a Reductisol profile. FEMS Microbiol. Ecol.

Abstract

Although anaerobic oxidation of ammonium (anammox) represents an important source of N_2 in some marine environments, nothing is known to date about its quantitative importance in terrestrial ecosystems. In this study, we present anammox and denitrification rates in a Reductisol using anoxic ^{15}N-isotope incubations with water-saturated soil samples taken at different seasons. In parallel the community structure of anammox bacteria was assessed by PCR targeting anammox bacterial 16S rRNA genes and cloning/sequencing. Anammox activity was detected at different depths and seasons but denitrification was always the dominant N_2 forming process. Potential anammox rates increased significantly with soil depth but did not exhibit clear seasonal variations. The relative contribution of anammox to total N_2 formation was greatest in the deepest soil section and varied between 6.5% in spring and 10.8% in autumn. Conversely, negligible anammox activity was usually observed in the surface soil at 0-10 cm. In agreement with limited seasonal variation of anammox activity, the composition of the anammox guild did not change significantly between sampling dates and consisted of 2 taxa: one being phylogenetically related to *Candidatus* 'Brocadia' and the other one forming a separate cluster within the known anammox bacteria. This is the first study providing evidence for a stable and active anammox bacterial community in a Reductisol. Results suggest that anammox bacteria may be active also in other natural wetland soils, particularly in deeper soils layers where a shortage of fresh organic matter may limit the denitrifying community.

Introduction

Anaerobic ammonium oxidation (anammox) represents the conversion of ammonium with nitrite as electron acceptor to dinitrogen gas under anoxic conditions. Shortly after its discovery (Mulder *et al*., 1995), anammox was applied to inorganic nitrogen removal in wastewater treatment systems (Strous *et al*., 1997; Paredes *et al*., 2007) but its importance in natural ecosystems was recognized only recently (Thamdrup and Dalsgaard, 2002; van de Vossenberg *et al*., 2008; Kuenen, 2008).

Anammox bacteria are active at redox transition zones in various aquatic ecosystems where the contribution of anammox to total N_2 production may range from a few to 67% (Thamdrup and Dalsgaard, 2002; Kuypers *et al*., 2005; Schmid *et al*., 2007). The largest contributions by anammox were found in suboxic water columns (Dalsgaard *et al*., 2003;

Kuypers *et al*., 2003; 2005; Thamdrup *et al*., 2006; Hammersley *et al*., 2007) and in shelf slope sediments (Thamdrup and Dalsgaard, 2002; Trimmer and Nicholls, 2009). Conversely, anammox was responsible for less than 20% of total N_2 production in shallow coastal and estuarine sediments (Engström *et al*., 2005; Hietanen and Kuparinen, 2008; Rich *et al*., 2008; Dale *et al*., 2009), sea ice (Rysgaard *et al*., 2008), and meromictic lakes (Schubert *et al*., 2006). The relative importance of anammox activity depends on the availability of NO_x^-, the presence of anammox cells, and the quantity and quality of organic matter (Dalsgaard *et al*., 2005; Engström *et al*., 2005; Meyer *et al*., 2005; Trimmer *et al*., 2005).

Recently, anammox bacteria were also detected in a wide range of terrestrial environments including marshes, lakeshores, a porous aquifer, permafrost soil, and in soil samples associated with nitrophilic or nitrogen fixing plants (Humbert *et al*., 2010). The observed diversity of terrestrial anammox guilds was higher than usually observed in aquatic habitats. However, their activity and quantitative contribution to N_2 production was not investigated. Consequently, knowledge about the role of anammox in terrestrial N-cycling is still very limited (Humbert *et al*., 2010). Only a few studies quantified anammox in natural terrestrial environments, including wetlands (Matheson *et al*., 2003), mangroves (Meyer *et al*., 2005) and tidal marsh sediments (Koop-Jakobsen and Giblin, 2009), and found that anammox was of minor importance compared to denitrification. Conversely, significant anammox activity was detected in constructed wetlands (Paredes *et al*., 2007; Erler *et al*., 2008).

The objectives of this study were to quantify the activity of anammox bacteria and determine their contribution to N_2 formation in a soil profile. A Reductisol, characterized by intermittent oxidized and reduced conditions due to fluctuations in groundwater level, was investigated using PCR targeting anammox bacterial 16S rRNA genes and ^{15}N-isotope incubations to quantify anammox and denitrification rates. Measurements were done in spring, summer, and autumn 2008 in order to identify seasonal variations in the potential activities of anammox and denitrification.

Material and methods

Study site and Sampling

This study was performed in a Reductisol situated about 2 m from the shore of the eutrophic Lake Loclat (Switzerland, Fig. IV. 1). Three soil cores of 60 cm length were

collected. Sections corresponding to the depth intervals 0-10 cm, 20-30 cm, and 50-60 cm were retained for analysis.

Figure IV.1. The shore of the Loclat Lake (CH) where the Reductisol profiles were sampled at three different sampling dates. The picture was taken in summer 2008.

The soil profile has no distinct horizons (Fig. IV. 2). Therefore, we selected depths where anammox activity was detected in pre experiments (data not shown). Selected sections were separately homogenized and stored in sterile plastic bags (ca. 300 g) at 4°C. For the incubation experiments with isotopically labeled substrates we used 180 g; 120 g were used for the characterization of soil physico-chemical parameters. On site, about 0.3 g of each soil section was immediately fixed in 10 ml of 1 M HCl for iron determination. Ten grams were directly placed into 30 ml of 2 M KCl for quantification of inorganic nitrogen compounds. On site sampling for determining iron and inorganic nitrogen compounds was done in triplicate. Sampling was repeated in spring (May 06, 2008), summer (July 16, 2008) and autumn (October 10, 2008).

Figure IV. 2. Soil profile sampling at the Loclat Lake (CH): A) Material preparation before the soil core sampling, B) Soil core collecting in autumn 2008, C) Soil core profile from 0 to 60 cm of length showing no distinct horizons, D) Soil core separated every 10 cm by Sonia Tarnawski, E) Soil fractions homogenized and collected into plastic bags with Jakob Zopfi.

Physicochemical analyses of the Reductisol

For each soil section, total iron, ammonium, nitrite and nitrate, organic carbon, and total nitrogen contents were determined (Annexes A-14 to A-21). Soil extracts were obtained by shaking (1h at 200 rpm) 50 ml Falcon tubes containing the soil samples and a) 10 ml of 1 M HCl for reactive iron extraction, and b) 30 ml of 2 M KCl for determining nitrogen compounds. The tubes were centrifuged at 4000 rpm for 10 min and supernatant was filtrated through glass fiber filters (GF/F, Whatman). The extracts were conserved at 4°C until analysis within 24 h or frozen at -20°C for longer conservation. Different forms of reactive iron (Fe^{II}/Fe^{III}) were determined by the colorimetric ferrozine method (Viollier *et al*., 2000; Annex A-18). Ammonium and nitrite in the KCl extracts were determined by the hypochlorite-phenol method (Chaney and Marbach, 1962; Annex A-15) and the sulfanilamide assay (Hansen and Koroleff, 1999; Annex A-16). Nitrate was quantified using a modified cadmium reduction protocol (Annex A-17). To 1 ml of KCl soil extract 100 µl of boric acid buffer (pH=10.5) and about 1/3 of a Nitraver 6 sachet (Hach Lange, Switzerland) were added. The sample was agitated for 1h at 175 rpm, whereby nitrate was reduced to nitrite and subsequently quantified by the sulfanilamide assay. Nitrate

concentrations were calculated by subtracting the nitrite concentrations initially measured in the sample.

Soil water content was determined by weighing soil samples before and after drying for 2 days at 105°C (Annex A-20). Soil $pH_{[H2O]}$ was determined on air dried and sieved (2 mm) soil samples, that had been agitated for 1 hour in deionized water (Annex A-19). Total carbon (C_{tot}) and total nitrogen (N_{tot}) contents were measured with an element analyzer (EA 1108 CHNS-O Element Analyzer; Annex A-21). Total organic carbon ($C_{org.tot.}$) was determined by Rock-Eval (Disnar *et al*., 2003; Sebag *et al*., 2006) on dried and agate-crushed soil samples. The measurement of soil temperature was done on site with a Pt-100 temperature probe inserted into the intact soil core. All chemical analyses were done on triplicate samples.

DNA extraction and PCR amplification

Total genomic DNA extraction and PCR amplification of anammox bacterial 16S rDNA were done as described previously (Humbert *et al*., 2010; Annex A-1 to A-4). Briefly, about 0.5 g (wet weight) was extracted with the FastDNA SPIN kit for soil (BIO 101, Qbiogene Inc., Carlsbad, CA, USA). Anammox bacteria were detected by a nested PCR approach. In the first PCR round *Planctomycetales* 16S rRNA gene was amplified with Pla46f (Neef *et al*., 1998) as forward and Univ1390r (Zheng *et al*., 1996) as reverse primer. The second, anammox specific PCR was performed by using Amx368f and Amx820r (Schmid *et al*., 2005).

Clone library production, analysis and sequencing

Anammox 16S rRNA gene clone libraries were produced and analyzed as described in detail in Humbert *et al*. (2010; Annex A-5 to A-9). Briefly, anammox 16S rRNA gene PCR products were cloned and 50 transformants were selected randomly per analyzed soil depth and sampling date to create the clone library. The inserted DNA was amplified using T7 / SP6 primer set and 18/50 insert-confirmed PCR products were submitted to Restriction Fragment Length Polymorphism (RFLP) analysis using MspI endonuclease (Promega). Clone inserts displaying identical restriction pattern were grouped into the same Operational Taxonomic Unit (OTU). Two clones per OTU were sent for sequencing to MWG-Biotech (Ebersberg, Germany) and only OTU's with more than two clones were considered. Phylogenetic analyses were conducted using BLAST (Altschul *et al*., 1997)

and MEGA v.4 software (Tamura *et al*., 2007). Nucleotide sequences have been deposited in the EMBL sequence database under accession numbers FN691936 through FN691958.

^{15}N-isotope incubations-anammox and denitrification activities measurements

Twenty gram of homogenized soil from each section was placed in 100 ml glass bottle (Schott) containing 40 ml of anoxic (purged with N_2), sterile water (Fig. IV. 4A; Annex A-22). Nine bottles per section were prepared. Three consecutive evacuation/purging cycles with N_2 were performed to replace the gas atmosphere and remove any traces of O_2 trapped in the soil matrix (Fig. IV. 4B). The bottles with the anoxic, water-saturated soil were pre-incubated for 24h in the dark at 16°C. They were moved from time to time in order to ensure complete consumption of O_2 and unlabelled substrates (NO_3^-, NO_2^-) before the start of the incubation experiment.

A **B**

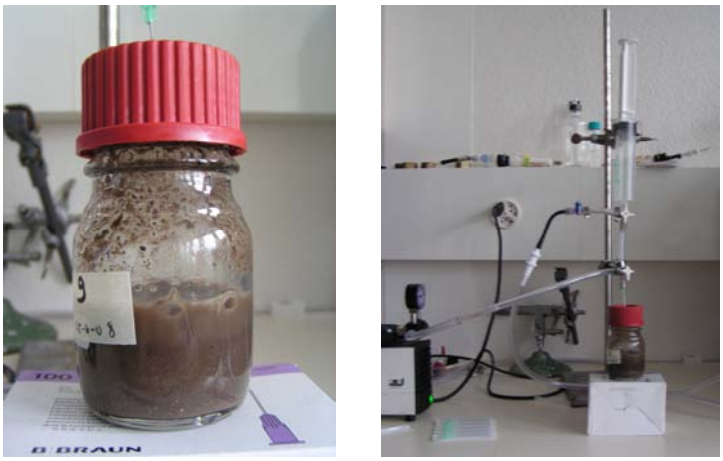


Figure IV. 4. Bottles preparation for ^{15}N-isotopes incubations containing A) homogenized soil faction and anoxic sterile water; B) Gas removal by vacuum and nitrogen gas input (gas flow connected with the top of the bottle by a syringe).

A ^{15}N-isotopic incubation experiment consisted of 3 treatments realized in triplicate: 1) control treatment with addition of $^{15}NH_4^+$, 2) anammox treatment with addition of $^{15}NH_4^+$ and $^{14}NO_3^-$, 3) denitrification treatment with addition of $^{15}NO_3^-$ (Annex A-23). The substrates were added to a final concentration of about 100 µM from anoxic, N_2-flushed stock solutions. Bottles were incubated during 12 h in the dark at 16 °C. Gas and liquid phases were sampled every 3 hours and analyzed for $^{29}N_2$, $^{30}N_2$, ammonium, nitrite and nitrate concentrations (Fig. IV. 5; Annex A-24). Prior to gas sampling, bottles were agitated briefly to equilibrate the gas and water phases. Four milliliters of headspace gas were withdrawn with a syringe (previously flushed with N_2) (Fig. IV. 5A) and injected

into 10 ml gas-tight Exetainer vials (Gifco) that had been completely filled with Ar saturated water (Fig. IV. 5B). Through a second needle in the septum, the entering gas displaced water. The vials were afterwards stored upside-down at room temperature until analysis by GC-IRMS.

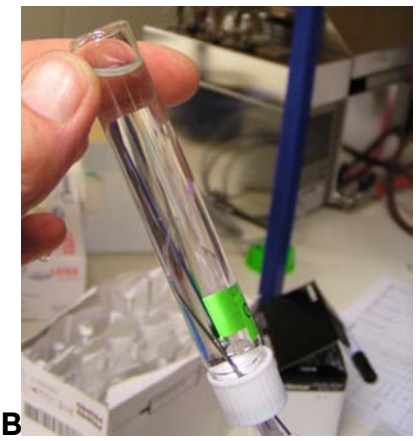
A   B

Figure IV. 5. A) Gas headspace sampled in the incubation bottles by Nicole Jeanneret, B) sampled-gas injected (bubbles) into the vial containing anoxic water which was displaced by the gas through a second needle.

Aqueous phase samples were also taken with a N_2-flushed syringe to avoid any contamination of the bottle content with atmospheric oxygen (Fig. IV. 6; Annex A-24). An initial sample was collected before the addition of the labeled substrates. Liquid samples (2 x 2 ml) were transferred to centrifuge tubes (Eppendorf) and immediately frozen at -20°C until dissolved nitrogen compounds were analyzed. Concentration of ammonium, nitrite, and nitrate were later measured in the thawed and centrifuged samples as described above.

Figure IV. 6. Red arrow in the left picture shown the liquid phase which is going to be sampled as in picture right.

N_2 analyses and calculations

Nitrogen isotopes ($^{29}N_2$ and $^{30}N_2$) were obtained using a Thermo Finnigan DELTA^{plus} XP continuous flow mass spectrometer equipped with GasBench II and CONFLO III at the Institute of Environmental Geosciences, University of Basel (Fig. IV. 7A). Headspace gas from the Exetainer vials was withdrawn and replaced by pure Helium using a double-hole flushing needle injecting the Exetainer rubber septum. This gas was captured in a 2 µL-loop, transferred through the GasBench GC column (PoraPLOT Q, 30 m x 0.32 mm) and injected into the mass spectrometer using the CONFLO III open split system.

For our purpose it is not crucial to measure absolute but relative (change over time) isotope ratios and concentrations and, therefore, we did not measure reference material with known respective values. Instrumental bias during each measuring session (12h) and analytical sensitivity for mass 30 ($^{15}N^{15}N$) were monitored by analysis of a working standard, which we produced (in duplicate A and B) from labeled (99%) $^{15}NH_4Cl$ using NaOBr following the procedure after Rittenberg (1946) (Fig. IV. 7B). These mixtures were diluted with pure N_2 down to 600 ppm (for $^{30}N_2$). Measurements of the working standards A and B each bracketed batches of ten samples and gave mean values for the $^{29}N_2/^{30}N_2$ ratio during a 12-hours-measuring session of 0.66 ± 0.01 and 0.74 ± 0.01 (SD, n=7), respectively.

The mean peak area of mass 30 (SD ~1%) and the concentration of the working standard A (600 ppm) was used to calculate the increase of $^{30}N_2$ over time. The concentration of produced $^{29}N_2$ was calculated as excess above the natural abundance. Rates of anammox and denitrification were calculated from $^{29}N_2$ and $^{30}N_2$ accumulated during the incubation time in samples with addition of $^{15}NO_3^-$. Anammox and denitrification rates were obtained from linear regressions fitted to the respective amounts of N_2 formed by each process over time. The contribution of anammox to total N_2 production was calculated as the rate of anammox divided by the sum of anammox and denitrification rates and multiplied by 100.

A   B

Figure IV. 7. Analyses of the gas headspace sampled during the ^{15}N-isotopic incubation by A) GC-IRMS (Chemical Elemental Analyzer (TCEA), Thermo-Finningan Delta Plus XP) under the direction of B) Prof. Franz Conen and Dr. Barbara Seth preparing standard N_2 gases.

Results

Environmental parameters of the Reductisol

Measurements of the biogeochemical parameters (temperature, water content, $pH_{[H2O]}$, nitrogen compounds, $C_{org.tot.}$, $C_{tot.}$, $N_{tot.}$, reactive Fe^{II}/Fe^{III}) were done to characterize the soil environment of anammox bacteria (Table IV. 1).

Table IV.1: Environmental parameters measured at three different depths along the Reductisol profile at three seasons sampling dates.

	Spring			Summer			Autumn		
Parameters	0-10 cm	20-30 cm	50-60 cm	0-10 cm	20-30 cm	50-60 cm	0-10 cm	20-30 cm	50-60 cm
Temperature (°C)	11.8±0.6	9.0±0.4	8.2±0.5	18.0±0.6	17.3±0.4	16.7±0.2	13.8±0.6	13.4±0.4	13±0.3
Water content (%)	54	49	68	53	51	64	62	47	64
pH [H_2O]	5.9	6.6	7.5	7.3	7.5	7.1	6.3	6.9	7
NH_4^+ (µmol/gsoil)	0.03 ± 0.0021	0	0.06 ± 0.003	0.11± 0.03	0.06±0	0.04±0.019	0.03±0.013	0.01±0.004	0.02±0.006
NO_3^- (µmol/gsoil)	0.13± 0.0187	0.02±0.0018	0.01±0.0024	0.03±0.01	0.02±0.008	0.02±0.03	0.01±0.002	0	0.01±0.009
NO_2^- (µmol/gsoil)	0.01±0.0008	0	0	0.03±0.012	0.01±0.006	0.01±0.001	0.01±0.001	0	0
C org.tot. (%)	6.77	2.24	8.13	6.14	3.35	4.62	6.19	1.45	4.15
C tot. (%)	16.5	12.6	16.4	15.8	13.6	14.2	16.1	12.8	15.4
N tot. (%)	0.6	0.2	0.5	0.5	0.3	0.3	0.6	0.1	0.4
Fe^{2+} (µmol/soil)	5.8±1.4	3.9±1.5	10.1±1.0	3.5±1.4	7.9±2.4	16.9±8.0	2.9±0.0	8.05±6.6	8.84±0.8
Fe^{3+} (µmol/soil)	8.3±1.8	1.7±2.7	0	7.0±1.5	1.1±0.3	0	12.95±3.7	2.75±1.5	0.69±0.3

In spring and summer the temperature decreased gradually about 3°C with depth while in autumn the temperature was constant along the entire sampling depth. The water content by weight was on average 50%, whereas the upper and lower parts of the soil profile were wetter. The $pH_{[H2O]}$ ranged from 5.9 to 7.5 with the lowest value obtained in spring at 0-10 cm depth. Amounts of mineral nitrogen (ammonium, nitrate, and nitrite) ranged generally from 0 to 0.13 µmol g^{-1} soil. Concentration of ammonium varied significantly between depths for each season and tended to decrease along the soil profile in summer and autumn. The amount of nitrate and nitrite decreased with depth in spring while in summer and autumn no significant variations between depths were observed. Percentage of $C_{org.tot.}$ ranged from 1.45 to 8.13 with the lowest values obtained at 20-30 cm. In general, the C_{tot} and N_{tot} decreased from the upper part to the middle of

the soil profile and then increased. For all seasons, the Fe(II) increased with depth by more than 50% along the soil profile whereas Fe(III) decreased with depth.

Detection and identification of anammox 16S rRNA gene sequences

The nested PCR for anammox bacterial 16S rRNA genes yielded products of the expected size with all soil DNA extracts except for the spring sample at 0-10 cm depth.

The phylogenetic affiliation of the PCR products was determined by cloning/sequencing. Inserts of 144 clones were restricted with *Msp*I: 28 different OTUs (n≥2) were identified and 46 clones were selected for insert sequencing. BLAST analysis revealed that 15 sequences were related to *Candidatus* 'Brocadia' with more than 96% of similarity and 13 sequences were affiliated with an equal similarity of 95% with both *Ca. Kuenenia* and *Ca. Brocadia*. These clone sequences were related to an uncultured *Planctomycete* (AJ871743; Smits *et al*., 2009) with 99% similarity. The remaining sequences were affiliated with less than 84% similarity to the anammox group and were mostly related to uncultured *Planctomycetes* 16S rRNA genes sequences. A Neighbor-Joining phylogenetic tree was constructed with the sequences retrieved from the Reductisol and sequences of known anammox genera (Figure.1). Cluster I consisted of sequences retrieved in spring from soil samples at 20-30 cm and 50-60 cm depths, and in summer at 0-10 cm, 20-30 cm and 50-60 cm depths. This cluster was affiliated to *Ca. Brocadia* (Bootstrap value > 50%) with a similarity of 97%. Sequences of Cluster I shared among each other more than 98% similarity. Sequences retrieved in spring and in summer from 20-30 cm and 50-60 cm depth, and in autumn from 50-60 cm depth formed a second cluster (Cluster II). The similarity among the sequences forming Cluster II exceeded 98%. The closest anammox candidates of this cluster were *Ca. Anammoxoglobus* and *Ca. Jettenia* but the bootstrap support of this branching was not significant (Bootstrap value < 50%). Finally, clone sequences retrieved from a specific depth and season were usually affiliated with both *Ca. Brocadia* and Cluster II, except clone sequences from summer at 0-10 cm, which were only related to *Ca. Brocadia,* and clone sequences from autumn at 50-60 cm, which belong to Cluster II. Sequences (n=18) branching outside the anammox group were not included in the final tree.

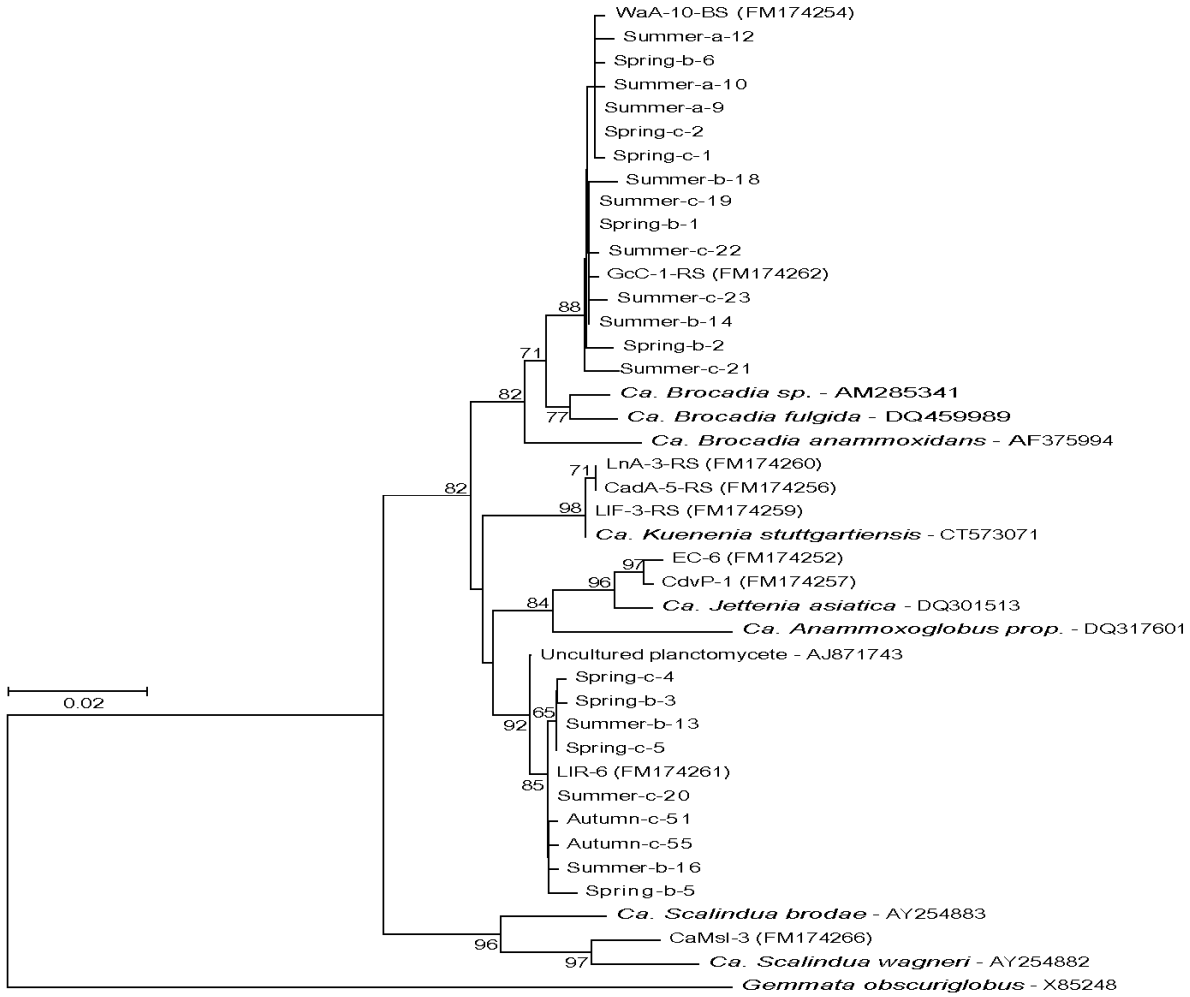


Figure IV. 8. Neighbour-joining tree showing the relationship between known anammox bacteria, environmental 16S rRNA gene sequences retrieved from a previous study (Humbert *et al*., 2010) and closely related 16S rRNA gene sequences retrieved from the Reductisol profile at 3 different depths for three seasons. Clone names are composed as follows: Sampling season – soil depth (a = 0-10cm, b = 20-30 cm and c = 50-60cm of depth) - clone number. Bootstrap values (1000 replicates) higher than 50% are shown and the scale bar represents 1% of sequence divergence.

Potential anammox and denitrification activities along the Reductisol profile

Measurements of nitrogen compounds, anammox and denitrification rates were done in incubations representing all three treatments, depths and seasons (Annexes B-1 to B-9). The first treatment involved the addition of $^{15}NH_4^+$ and served as a control. The second involved the addition of $^{15}NH_4^+ + {}^{14}NO_3^-$ and was used to confirm anammox activity. The third treatment with addition of $^{15}NO_3^-$ was done to compare anammox and denitrification rates under the same conditions. The presented data of the mineral nitrogen compounds in the incubations (Fig. IV. 9, right panel) are from the $^{15}NO_3^-$ treatments and provide information on the soil conditions before the start and during the course of the experiment. For all seasons, ammonium was always present but

concentrations were lower in the 20-30 cm and 50-60 cm incubations. Conversely, nitrite and nitrate pools were depleted before the isotope addition. After injection of $^{15}NO_3^-$, ammonium concentrations remained stable or, as sometimes observed in surface soil incubations, continued to increase due to the mineralization of fresh organic matter. Conversely, nitrate decreased rapidly in the 0-10 cm samples and gradually slower in the deeper samples. For all depths and seasons, nitrite concentrations increased transiently during the consumption of nitrate.

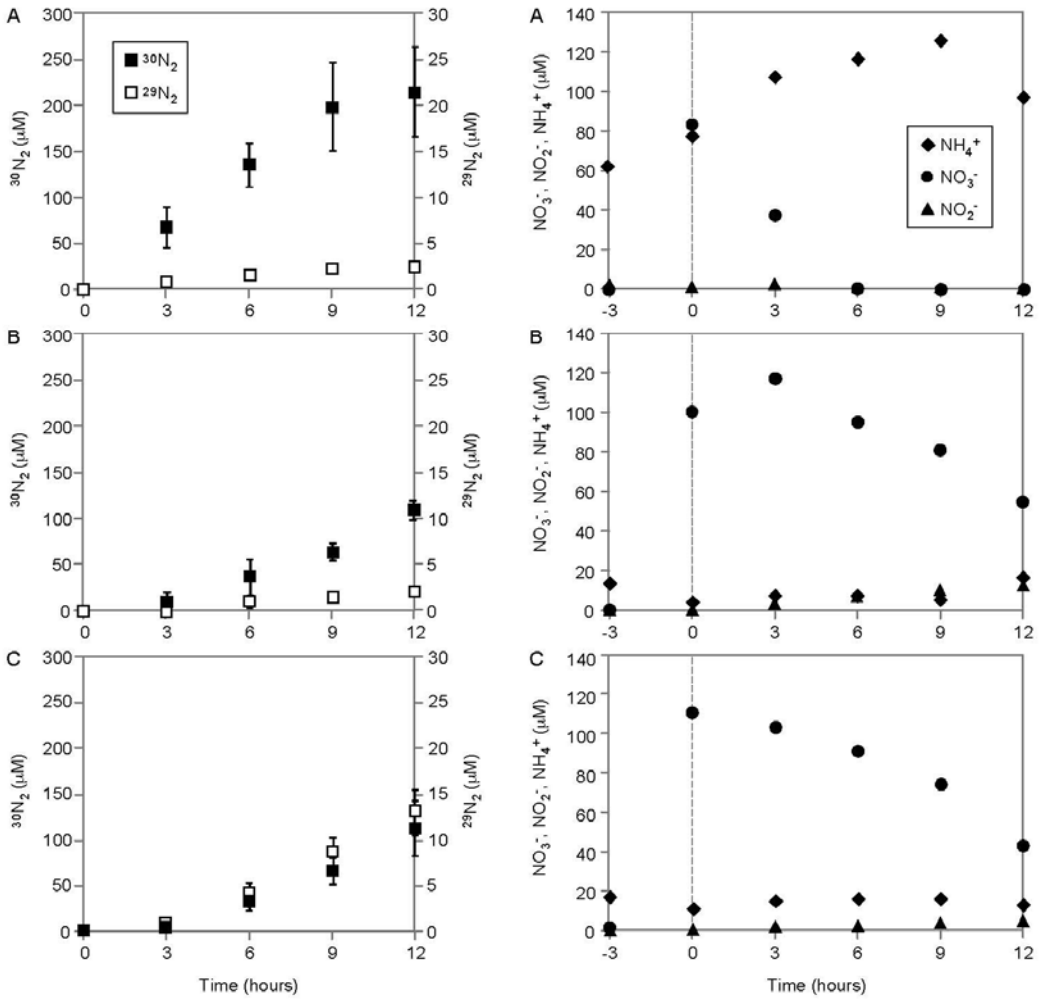


Figure IV. 9. Potential anammox and denitrification activities in soil samples from a Reductisol profile with addition of $^{15}NO_3^-$ treatment. Left panels: production of $^{29}N_2$ (empty squares) and $^{30}N_2$ (bold squares) at A) 0-10 cm, B) 20-30 cm and C) 50-60 cm of depth; right panels, amount of ammonium (lozenges), nitrate (circles), and nitrite (triangles) at A) 0-10cm, B) 20-30cm and C) 50-60cm of depth. The data are from autumn sampling date.

Potential anammox and denitrification activities were estimated based on the isotopic composition of the produced N_2 gas (Annexes B-1 to B-9). No production of $^{29}N_2$ was observed in the control treatments of all depth and seasons. In the microcosm with the

$^{15}NH_4^+ + {}^{14}NO_3^-$ treament small quantities of $^{29}N_2$ were detected suggesting the presence of anammox in the soil profile for all sampling dates. Detection of both $^{29}N_2$ and $^{30}N_2$ in the $^{15}NO_3^-$ treatment demonstrated the simultaneous activity of anammox and denitrification (e.g. in autumn, Fig. IV. 9, panel left), whereby the quantity of $^{29}N_2$ produced was 3 to 100 times larger than with $^{15}NH_4^+ + {}^{14}NO_3^-$ (Annexes B-2; B-5; B-8).

The potential anammox and denitrification rates were calculated from the respective amounts of $^{29}N_2$ and $^{30}N_2$ produced per unit time in the $^{15}NO_3^-$ incubations (Table IV. 2). For all seasons, denitrification rates decreased by 20 to 70% with depth but they were always 8 to 250 times higher than the anammox rates. The highest rate of denitrification was measured at 0-10 cm in spring while the lowest was found at 50-60 cm depth in autumn. Moreover, in spring at 0-10 cm of depth, the denitrification rate was significantly higher (p-value<0.01) than in summer or autumn potentially showing a seasonal dynamic of potential denitrification activity. In contrast, anammox rates increased significantly (p<0.01) by more than 82% with depth at every sampling date. The highest anammox rate was observed in summer at 50-60 cm and the lowest at 0-10 cm of depth in spring and 20-30 cm in autumn. Although significant differences (p<0.05) of anammox rates were sometimes observed between sampling dates the seasonal variations remained weak. The contribution of anammox to total N_2 production increased significantly (p<0.01) with depth by a factor of 7 in summer and 17 in spring (Table IV. 2).

Table IV. 2: Denitrification and anammox rates in a Reductisol at three different dates in 2008. Rates and % contribution of anammox to total N_2 formation were calculated from the $^{29}N_2$ and $^{30}N_2$ formation rates, respectively, in anoxic soil microcosms spiked with $^{15}NO_3^-$.

Depth	Spring (May 6)			Summer (July 16)			Autumn (October 10)		
	Rates (nmol N_2 g^{-1} ww soil h^{-1})		Contribution	Rates (nmol N_2 g^{-1} ww soil h^{-1})		Contribution	Rates (nmol N_2 g^{-1} ww soil h^{-1})		Contribution
	Anammox	Denitrification	% Anammox	Anammox	Denitrification	% Anammox	Anammox	Denitrification	% Anammox
0-10 cm	0.64±0.05 aA	164.91±20.07 aA	0.39±0.02 aA	0.89±0.14 aB	77.81±2.63 aB	1.13±0.16 aB	0.73±0.04 a*B	68.70±12.14 aB	1.08±0.26 aB
20-30 cm	1.10±0.1 bA	51.23±4.35 bA	2.11±0.09 bA	1.39±0.11 bB	67.29±7.59 bB	2.04±0.26 bAB	0.64±0.05 bC	35.85±0.63 bC	1.75±0.11 bB
50-60 cm	4.61±0.31 cA	66.12±4.85 cA	6.52±0.3 cA	5.25±0.28 cB	61.71±3.63 bA	7.85±0.46 cB	4.10±0.73 cA	35.57±9.10 bB	10.76±3.54 cAB

a, b, c: Mean values were compared using *t* test. Significant differences of rates or contribution (P<0.01; * for P<0.05) between depths are indicated by different letters

A, B, C: Mean values were compared using *t* test. Significant differences of rates or contribution (P<0.05) between sampling dates are indicated by different letters

Discussion

Measurements of environmental parameters demonstrated the presence of inorganic nitrogen compounds essential for anammox and denitrification activities along the Reductisol profile. The presence of anammox bacteria was confirmed for all three sampling dates, except for 0-10 cm in spring and autumn and also at 20-30 cm in autumn. Anammox activity was observed at these depths, although only low rates, which suggests that anammox template DNA concentration was too low to be detected by the applied PCR protocol (Humbert *et al*., 2010). Phylogenetic analysis of the sequenced clones revealed the presence of the anammox *Candidatus* 'Brocadia'. Originally, this genus was found only in wastewater treatment systems (Third *et al*., 2005; Van Loosdrecht and Salem, 2006). However subsequent detection in coastal (Amano *et al*., 2007), estuarine (Dale *et al*., 2009), and river sediments (Zang *et al*., 2007) as well as in soils (Humbert *et al*., 2010) suggested that they are widespread and occupy a broader ecological niche than presumed so far. Twenty eight percent of the sequences formed a separate cluster (Cluster II) within the anammox group and may belong to a new genus. These clone sequences were mostly retrieved from the samples with the highest anammox activity.

Anammox and denitrification activities were detected at all sampling dates along the soil profile demonstrating the co-existence of the two processes. A competition between denitrifying and anammox bacteria is possible since both depend on the availability of NO_x^-. Anammox bacteria are slow-growing organisms (Jetten *et al*., 1998) and thus likely less competitive for nitrate than denitrifiers in organically rich environments like soils. Alternatively, coexistence also raises the possibility that anammox bacteria benefit from the intermediate formation of nitrite during denitrification as it was observed in the incubation experiments.

For all sampling dates the lowest anammox rates were detected at 0-10 cm depth, where denitrification rates were highest, which is explained by rhizodeposition of labile organic matter fuelling denitrifying heterotrophs (Landi *et al*., 2006; Haichar *et al*., 2008; Berg and Smalla, 2009). This observation was confirmed by the rapid consumption of nitrate and nitrite upon $^{15}NO_3^-$ addition in the 0-10 cm soil incubations (Fig. 2). The contribution of anammox to total N_2 production was 1% or less in the surface soil incubations. Nevertheless, the lowest anammox rates in the Reductisol (Table 2) were close to the highest rates measured in Cape Fear Estuary sediment (0.66 nmol N_2 g^{-1} of

sediment h^{-1}; Dale *et al*., 2009) and within the range of values observed in Thames Estuary sediment (0.2 to 10 nmol N_2 ml^{-1} of wet sediment h^{-1}; Trimmer *et al*., 2003). Also denitrification rates were within the range reported for estuarine sediments (40 to 170 nmol N_2 ml^{-1} of wet sediment h^{-1}, Trimmer *et al*., 2003; 31 to 137 nmol N cm^{-3} h^{-1}, Risgaard-Petersen *et al*., 2004).

For all three sampling dates highest anammox rates were observed at 50-60 cm where denitrification rates were lowest. Similar ranges of anammox rates were observed in constructed wetlands (3.1 ± 1.33 nmol N_2 cm^{-3} h^{-1}, Erler *et al*., 2008), in marine (4.125 nmol N_2 cm^{-3} h^{-1}, Thamdrup and Dalsgaard 2002), in estuarine (3.8 to 5.9 nmol N cm^{-3} h^{-1}, Risgaard-Petersen *et al*., 2004), in mangrove (4 to 8 nmol N cm^{-3} h^{-1}, Meyer *et al*., 2005), and in river sediments (2.5 to 5.8 nmol N cm^{-3} h^{-1}, Rich *et al*., 2008). The lowest rates of denitrification were close to the rates detected in Chesapeake Bay and Choptank river sediments (27 to 40 nmol N cm^{-3} h^{-1}, Rich *et al*., 2008).

The anammox contribution to N_2 formation significantly increased with depth as denitrification rates diminished (Table 2). The influence of the sampling date on the rates was also significant but less important than sampling depth. Both results suggested that autotrophic anammox bacteria become more competitive in deeper soil layers where organic matter is more refractile and less accessible for heterotrophic denitrifiers. This is consistent with earlier findings from marine sediments, where the relative importance of the two N_2 forming processes was regulated by the availability of their respective substrates (Thamdrup and Dalsgaard, 2002, Risgaard-Petersen *et al*., 2004, Meyer *et al*., 2005, Hietanen and Kuparienen, 2008, Trimmer and Nicholls, 2009). However, unlike sediments that are typically anoxic below a few millimeters of depth (Zopfi *et al*., 2001), soils may fall dry during parts of the year and experience highly oxic conditions. Hence, one may speculate that in addition to the substrate requirements mentioned above, the more stable, anoxic conditions in deeper soil layers are favorable for the development of slowly growing anammox bacteria.

The highest contribution of anammox to N_2 production in the Reductisol reached 14% and was observed in autumn at 50-60 cm depth. This value is within the range reported for comparable environments like constructed wetlands (3 to 9%, Erler *et al*., 2008), estuarine (5 to 24%, Risgaard-Petersen *et al*., 2004; 3.8 to 16.5%, Dale *et al*., 2008), mangrove (0 to 9%, Meyer *et al*., 2005), as well as river sediments (0 to 22%, Rich *et al*., 2008).

Conclusion

This study provides the first quantitative data on anammox and denitrification activities in a natural wetland soil. Results show that anammox bacteria form a minor but stable portion of the soil microbial community, both in terms of activity and genetic diversity. The presence of *Ca. Brocadia*, which was originally related to wastewater treatment systems, highlights the capacity of this candidate to exploit different ecological niches. Moreover, the detection of new anammox related sequences (Cluster II) in a soil with shown anammox activity suggests that the anammox guild is more diverse than previously thought. However, further studies in other terrestrial ecosystems are needed to better understand the factors controlling the distribution and activity of anammox bacteria in soils.

Chapter V

Detailed study of anammox along a *Catena* in the Jura (F)

Chapter based on the manuscript in preparation for submission to FEMS Microbiology Ecology:
Bagnoud A, Humbert S., Hai B., Schloter M., Conen F., Seth B., and Zopfi J. (2010). Anammox in soil: distribution, diversity and activity along a soil transect in the Jura Mountains (Bellefontaine, F). FEMS Microbiol. Ecol.

Abstract

Anaerobic ammonium oxidation (anammox) has been recognized as important process in aquatic ecosystems, yet virtually nothing is known about its role in terrestrial environments. In this study we investigated the ecology of anammox bacteria along a soil transect with opposing ecological gradients of nitrogen and water contents. Soil profiles of an amended pasture, a nitrophilic wet meadow, a minerotrophic fen, and an ombrothrophic bog were investigated. Physico-chemical properties of the soil and interstitial water were followed during a complete annual cycle with the aid of multilevel piezometers. Abundances of aerobic and anaerobic ammonium oxidizing microorganisms (Anammox bacteria, AOB, AOA) were determined by quantitative PCR and cloning-sequencing to assess the diversity of anammox bacteria. Anammox bacteria were only present in soil sections, which did not fall dry during the year and seemed to depend on long-term anoxic conditions and the presence of nitrate. 16S rRNA gene sequences of the three known anammox genera *Ca. Kuenenia*, *Ca. Brocadia* and *Ca. Jettenia* were detected plus sequences of an as yet unidentified new cluster. Unlike anammox bacteria, aerobic ammonium oxidizing *Bacteria* and *Archaea* were detected in all soils and soil sections and were generally more abundant. Incubation experiments using ^{15}N labeled substrates revealed that denitrification was the dominant N_2 forming process in the tested soil sections. Anammox did not contribute by more than 2% to total N_2 formation. Results suggest that ammonium oxidation in these organic-rich soils is essentially an aerobic process and that anammox plays also only a minor role for N-elimination.

Introduction

Until recently, the oxidation of ammonium was known as strictly aerobic process, performed by only a few genera of autotrophic *Eubacteria* (AOB, ammonium oxidizing Bacteria). This perception is changing at a rapid pace and at different levels: Firstly, besides *Bacteria*, aerobic ammonium oxidizing *Archaea* were isolated (Könneke *et al*., 2005) and detected by molecular methods in the environment (Nicol and Schleper, 2006; Leininger *et al.,* 2006). Ammonium oxidizing Archaea (AOA) have been reported to be quantitatively as or even more abundant than AOB (Leininger *et al.,* 2006) and it was proposed that AOA may outcompete AOB under oligotrophic conditions (Martens-Habbena *et al*., 2009; Erguder *et al*., 2009). Secondly, anaerobic ammonium oxidation

(anammox), a bacterial process initially proposed based on thermodynamic considerations (Broda, 1977), was recently shown to be important in nature (e.g. Thamdrup and Dalsgaard, 2002). Anammox bacteria convert ammonium to molecular nitrogen with nitrite or nitrate as oxidant. They were detected in marine sediments (e.g. Thamdrup and Dalsgaard, 2002), oceanic oxygen minimum zones (Kuypers *et al*., 2003) and chemoclines of permanently stratified freshwater lakes (Schubert *et al*., 2006). The low contents of organic matter in some aquatic ecosystems limit the activity of denitrifying bacteria (Thamdrup and Dalsgaard, 2002) but may favor anammox. It is estimated that anammox bacteria may account for 30 to 50% of the total oceanic N_2 production (Devol, 2003). A priori, there is no reason why anammox bacteria should not be present in soils. The substrates of anammox bacteria, ammonium, nitrite and nitrate occur naturally or are added in large quantities in form of fertilizer. Moreover, the heterogeneity of soils includes a variety of oxic/anoxic interfaces, which represent potential habitats for anammox bacteria since they offer concomitant access to reduced and oxidized nitrogen species under anoxic conditions. However, as opposed to aquatic environments, only few studies investigated the role of anammox bacteria in soils (Humbert e*t al*., 2010). In order to learn more about the ecology of terrestrial anammox bacteria, we investigated their distribution, diversity and abundance along a soil transect that encompassed two major ecological gradients: soil nitrogen content and soil water content. The studied soil types included an amended pasture, a nitrophilic wet meadow, a minetrotrophic fen and a peat bog. Anammox bacteria were quantified by specific qPCR targeting the anammox bacterial 16S rRNA gene and the results were related to data on the abundance of aerobic ammonium oxidizing *Bacteria* and *Archaea* from *amoA* qPCR. Anoxic soil incubations using ^{15}N labeled substrates were used to quantify anammox and denitrification rates. Furthermore, the study was complemented by a seasonal assessment of the physico-chemical conditions in the different soils using multilevel piezometers.

Material and methods

Study site

The studied transect is located close to Bellefontaine in the French part of the Jura Mountains (Figure V. 1). Four stations were defined with the help of the vegetation record of Gallandat (1982): an amended pasture (Soil 1; N 46°34'12.30'', E

006°04'50.94''), a nitrophilic wet meadow (Soil 2; N 46°34'11.40, E 006°04'51.00''), a minerotrophic fen (Soil 3; N 46°34'8.76'', E 006°04'53.28''), and a ombrotrophic peat bog (Soil 4; N 46°34'6.36'', E 006°04'55.20'') (Figure V. 1).

Figure V. 1. The *Catena* represented by four stations distributed along a slope that have been studied in Bellefontaine (F). 1) Amended pasture, 2) Nitrophilic wet meadow, 3) Minerotrophic fen, 4) Ombrotrophic peat bog.

Soil 1, a Brunisol Eutric according to the French classification system (Baize and Girard, 2009), was a carbonate-rich soil with a clayey texture due to the parental material issued from Oxfordian marls (Guillaume *et al*., 1963). Soil 2 was similar but was classified as a Reductisol Typique since reduction/oxidation processes caused by groundwater table fluctuations led to iron redistribution. Soil 3, a Histosol Saprique, was seasonally water saturated up to the surface, which leads to an accumulation of doughy organic matter. Soil 4, described as Histosol Fibrique was also permanently water saturated, acidic and oligotrophic. This soil consists essentially of dead *Sphagnum* moss giving a fibrous texture to the organic matter. Table V. 1 summarizes some general bulk soils characteristics. Soils 2, 3, and 4 were water saturated at least part of the year, which allowed the use of piezometers to characterize physically and chemically the interstitial water of the soils.

Physico-chemical analysis of the free-water

In situ measurements of temperature, electrical conductivity, pH, and pO_2 of the free-water were done every 10 cm by lowering a multi-meter (HQ40d, Hach Lange GmbH,

Germany) in a piezometer. To determine concentrations of dissolved compounds (e.g. NH_4^+, NO_2^-, NO_3^-) soil water was sampled every 10 cm using multi-level piezometers. From each level, water was withdrawn with the help of a peristaltic pump and airtight tubings (PharMed®). The outlet of the tube was connected directly to a syringe, allowing sample collection without any contact with atmospheric oxygen. The water samples were directly filtered through 0.45 µm nylon membranes and stored at 4 °C in the dark. For nitrite, a sample of 6.6 ml was fixed with 3.3 ml of sulphanilamide on site and analyzed within 24h in the laboratory using the colorimetrical method of Griess (1879) (Annex A-16). Nitrate was quantified by a modified cadmium reduction protocol (Annex A-17): 100µl sodium-borate buffer (pH 11) and about one third of a NitraVer®6 Nitrate Reagent bag (Hach Lange GmbH, Germany) were added to 1 ml of sample. Then the sample was shaken for 1 hour and the produced nitrite was measured again with the sulphanilamide assay (Annex A-16). Ammonium was measured by the hypochlorite-phenol reaction (Chaney and Marbach, 1962; Annex A-15). Concentrations of major cations and anions were determined by ion chromatography (Dionex DX-120, USA).

Table V. 1. Summary of general soil characteristics: Median (plus minima and maxima) of total organic carbon (% TOC), organic carbon to nitrogen-ratio (C_{org}/N_{tot}-ratio), percentage of humic substances in organic matter (% Humic), pH_{H2O}, and ammonium, nitrite, and nitrate contents.

	% TOC	C_{org}/N_{tot}	% Humic substances	pH H_2O	NH_4^+ [µmol·g^{-1} dry soil]	NO_2^- [µmol·g^{-1} dry soil]	NO_3^- [µmol·g^{-1} dry soil]
Soil 1 Amended pasture	3.7 (1.8 -9.1)	10.8 (9.8-13.1)	74.8 (60.2-75.4)	6.6 (6.3 - 7.0)	0.10 (0.06-0.16)	0.00 (0.00-0.00)	0.01 (0.01 - 0.03)
Soil 2 Wet meadow	4.7 (1.8-14.3)	12.8 (12.1-14.6)	73.7 (52.2-93.8)	7.0 (6.6 - 7.4)	0.12 (0.09-0.27)	0.00 (0.00-0.00)	0.02 (0.01 - 0.06)
Soil 3 Fen	29.0 (24.8-34.6)	13.2 (11.8-17.4)	52.4 (49.3-52.4)	6.9 (6.9 - 7.0)	0.48 (0.40-0.53)	0.01 (0.00-0.01)	0.02 (0.01 - 0.03)
Soil 4 Peat bog	40.7 (37.5-43.0)	64.4 (58.9-67.2)	32.6 (28.3-34.5)	5.2 (5.0 - 5.4)	1.12 (0.96-1.44)*	0.00 (0.00-0.00)	0.29 (0.24 - 0.43) *

*High concentrations supposed to be due to the KCl extraction method liberating stored NH_4^+ and NO_3^- from the *Sphagnum* cells.

Pedological and geochemical characterization of the soils

Soil samples were collected in 10 cm intervals using a core sampler. For pH and organic matter analysis, samples were dried at 40 °C and sieved at 2 mm. The soil pH (H_2O) was measured with pH-electrodes in an equilibrated (1 h on a rotary shaker) suspension of 50 g of dried soil in 30 mL deionized water. Organic carbon to total nitrogen ratios (C_{org}/N_{tot}), total organic carbon (TOC), and the content of humic substances were determined on a Carlo Erba CHN analyzer and by Rock-Eval (Disnar *et al*., 2003 ; Sebag *et al*., 2006) on dried and agate-crushed soil samples (Annex A-21). For quantification of adsorbed inorganic nitrogen compounds (NH_4^+, NO_2^-, NO_3^-) 15 g soil were directly placed in 30 ml of 2 M KCl and shaken for 1 h and kept on ice until analysis within 24 h. The inorganic nitrogen compounds were determined as described above whereby no negative effect of high KCl concentrations on the photometric methods was observed. For Soil 4, concentrations of NH_4^+ and NO_3^- were unusually high, supposedly because the KCl extraction method released these compounds from the *Sphagnum* cells.

Molecular analyses

Soil samples for molecular analyses were frozen on site in liquid-nitrogen. DNA was extracted using the FastDNA SPIN kit for soil (BIO 101, Qbiogen Inc., Carlsbad, CA, USA) following the manufacturers protocol (Annex A-1). A previously described nested-PCR approach was used to determine the presence and diversity of anammox bacteria in soil samples (Humbert et *al*., 2010). *Planctomycetes* 16S rDNA was amplified by PCR using Pla46f and Univ1390r primers (Annex A-3). Anammox bacterial 16S rDNA was amplified by Amx368f and Amx820r primers (Annex A-4). To assess the diversity of anammox bacteria, samples were cloned and sequenced. PCR products were ligated into pGEM-T vector (Promega) and used for transformation of electrocompetent *E. coli* XL1 cells (Humbert et *al*., 2010; Annex A-5 to A-6). Purified plasmids were sent for sequencing at MWG-Biotech (Ebersberg, Germany). The phylogenetic affiliation of the retrieved sequences was determined using BLAST (Altschul *et al*., 1997) and Silva (Preusse, 2007). The checked and edited sequences were aligned in the ClustalW implementation of MEGA4 (Tamura *et al*., 2007). Neighbor joining-trees were created using 1'000 bootstrap repetitions (Hall, 2008).

For the qPCR runs, all DNA extracts were purified with Wizard® SV Gel and PCR Clean-Up System (Promega). For each sample, genes coding for the sub-unit A of the

ammonium monooxygenase (amoA) of AOB and AOA were quantified using the Power SYBR® Green PCR Master Mix (Applied Biosystems®). AOB amoA were amplified by *amoA*-1f and *amoA*-2r primers, and AOA amoA were amplified by 19f and CrenamoA616r48x (Hai *et al*., 2009; Leininger *et al*., 2006). Anammox bacteria were quantified in all samples, which showed positive results after anammox specific nested-PCR screening (Humbert *et al*., 2010). Copy numbers of anammox bacterial 16S rRNA genes were determined using primers A438f and A668r, the SensiMixPlus SYBR® qPCR master mix (Quantace, Biolabo), and the amplification conditions as described elsewhere in detail (Humbert *et al*., submitted; Annex A-10).

^{15}N incubations experiments

Anammox and denitrification rates were determined in triplicates with 20 g of fresh soil under anoxic conditions in 100 ml glass bottles closed with thick butyl-rubber stoppers. The soil material was mixed with anoxic water and trapped O_2 in soil micropores was removed by 3 consecutive evacuation/N_2-flushing cycles. Then, bottles were pre-incubated for 3 days at 16 °C in the dark to deplete the intrinsic NO_3^- pool (Annex A-22). The experiment was started by adding different substrates from anoxic stock solutions: 1) addition of $^{15}NH_4^+$, 2) addition of $^{15}NH_4^+$ and $^{14}NO_3^-$, 3) addition of $^{15}NO_3^-$ (Annex A-23). Labelled and unlabelled compounds were added to a final concentration of about 100 µM and bottles were incubated for 12 hours in the dark at 16 °C. Every 3 hours gas samples were collected from the headspace just after shaking briefly the bottles (Annex A-24). Gas was withdrawn with previously N_2-purged syringes and directly injected into Vacutainers (Gifco), which were completely filled with argon-flushed water. Concentrations of $^{29}N_2$ and $^{30}N_2$ were measured by irGC-MS analysis. The excess of $^{29}N_2$ and $^{30}N_2$ above their natural abundances represent the produced N_2 (Thamdrup and Dalsgaard, 2002). Rates of anammox and denitrification were calculated based on the linear regression of the $^{29}N_2$ and $^{30}N_2$ accumulated in the $^{15}NO_3^-$ incubation.

Results

Interstitial water chemistry

Free interstitial water could only be sampled in Soils 2, 3, and 4 whereby data from October 2008 are presented in Figure V. 2 to illustrate the general characteristics of the different soils. Soil 2 contained more inorganic nitrogen than the others (Figure V. 2).

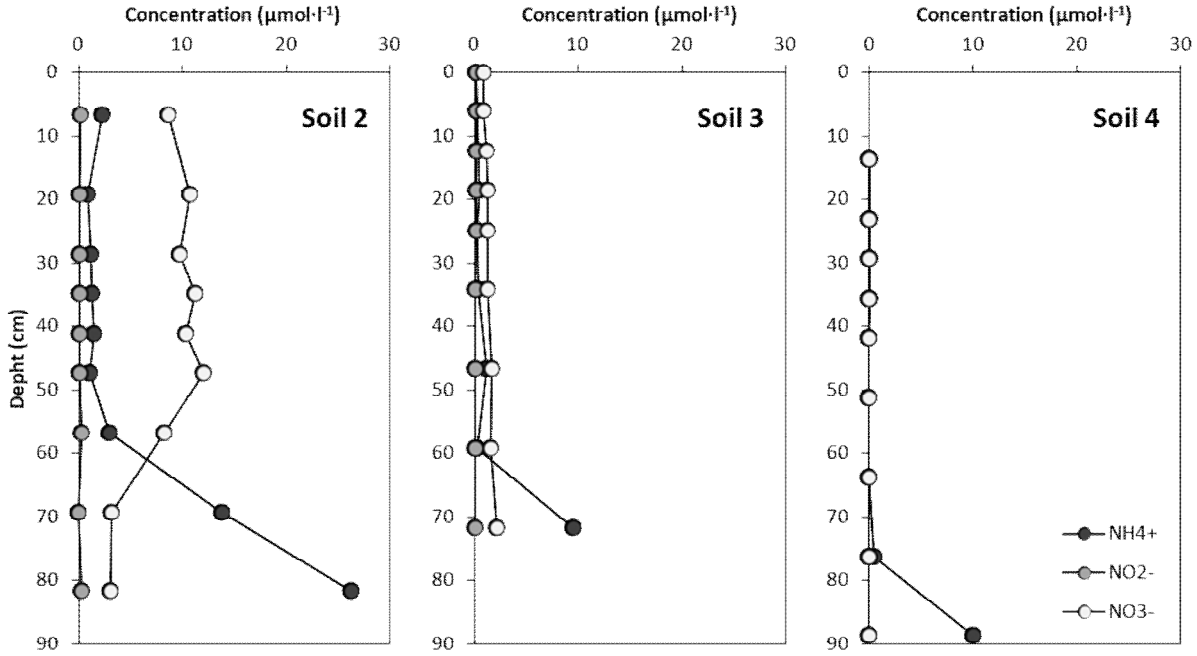


Figure V. 2. Depth profiles (October 2008) of dissolved ammonium, nitrite, and nitrate in the interstitial water of soil 2 (nitrophilic wet meadow), 3 (minerotrophic fen), and 4 (peat bog).

Concentrations of ammonium were around 1 µmol·l^{-1} in the upper part but increased to 26 µmol·l^{-1} at 82 cm. Nitrate concentrations were around 10 µmol·l^{-1} but decreased below 50 cm, to reach 3 µmol·l^{-1} at 28 cm. The lowest concentrations were measured for nitrite, which was below 0.4 µmol·l^{-1} for the whole profile. In the interstitial water of Soil 3 ammonium concentrations were around 0.3 µmol·l^{-1} in the upper part of the core but increased below 35 cm to 10 µmol·l^{-1} at 70 cm. Nitrate increased gently from 0.9 µmol·l^{-1} at the surface to 2.2 µmol·l^{-1} at 70 cm. Nitrite concentrations were similar as in Soil 2 and close to the detection limit. No inorganic nitrogen was usually detected in the interstitial water of Soil 4 with the notable exception of ammonium, which became detectable below 75 cm and reached 10 µmol·l^{-1} at 90 cm depth.

Dissolved Fe^{2+} and Fe^{3+} as well as sulfate and sulfide (H_2S) provide additional information on the redox conditions in the soils (data not shown). Sulfate concentrations in oxic interstitial water decreased from Soil 2 (43 µM) to Soil 3 (20 µM). In Soil 4, sulfate concentrations were only 1.7 µM in uppermost 13 cm and were below the detection limit underneath. Decreasing SO_4^{2-} concentrations below 55 cm in Soil 2 indicates net sulfate reduction. However, the reduction of sulfate was not associated with a detectable accumulation of dissolved sulfide in the interstitial water. In fact, free

sulfide was never detected in any of the cores. Nevertheless, at the same depth where sulfate concentrations decreased, Fe^{2+} concentrations increased from 0 µM to 57 µM in Soil 2 at 81 cm and to 24.4 µM in Soil 3 at 71 cm, respectively. The excess of iron could therefore precipitate with sulfide thus explaining its absence in the interstitial water. Ferrous iron concentrations along the profile of Soil 4 varied between 0 and 4 µM. Significant concentrations of Fe^{3+} in the interstitial water, either in colloidal form or complex with organic matter, were only detected in Soil 3. Concentrations varied between 0 and 5.9 µM, whereby the highest concentrations were typically observed at 20 cm during the vegetation period.

Annual nitrogen cycling

Periodic studies of the physical and chemical conditions in the free-water of soil 2 and 3 revealed the presence of two contrasting situations (Figure V. 3, data only shown for Soil 3). In autumn and winter the water level, pH, pO_2, and nitrate concentrations were high while the temperature and ammonium concentrations are low. The situation changed rapidly in spring when plant growth started and the microbial activity in the soil gained momentum: dissolved O_2 concentrations decreased rapidly and nitrate, which reached 10 µM (Soil 3) and 55 µM (Soil 2) in February (data not shown), dropped to <1 µM. The conditions returned again to their winter-values at the end of summer. Strong seasonal groundwater fluctuations were only observed for Soil 2, were the water table dropped to 74 cm bl.s. in August and even to 84 cm bl.s. in late October. Essentially no seasonal variations were observed in the interstitial water chemistry and the water level of the peat bog (Soil 4).

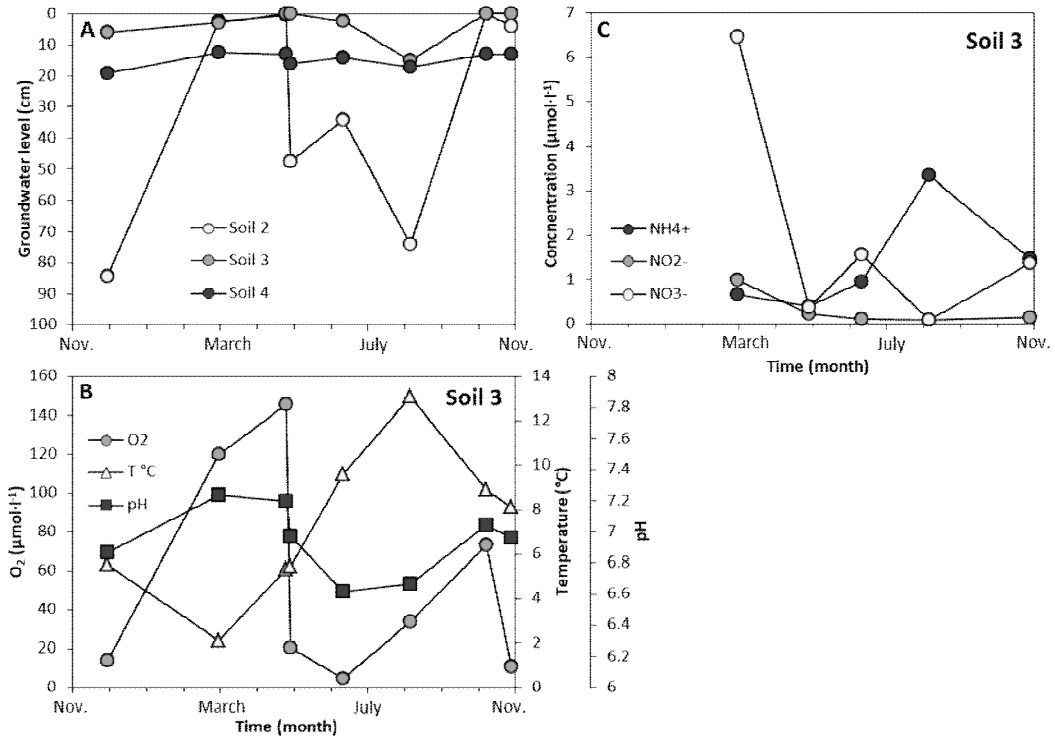


Figure V. 3. (A) Seasonal variations of the groundwater level in soil 2 (nitrophilic wet meadow), 3 (minerotrophic fen), and 4 (peat bog). Seasonal changes of the mean values of the physical and chemical parameters of the interstitial water in the soil 3: (B) temperature, dissolved O_2 and pH; (C) ammonium, nitrite, and nitrate.

Distribution and abundance of aerobic and anaerobic ammonium oxidizers

The anammox nested-PCR yielded only positive amplification of a 477 pb fragment in Soil 3 below 20 cm and in Soil 2 below 55 cm. Everywhere else, the anammox nested-PCR was considered negative (Figure V. 4): in Soil 1 and at the top of Soil 2, because the PCR products were very weak and showed multiple bands. No PCR amplification products were obtained with samples from 30 and 55 cm in Soil 2 and with the samples from Soil 4.

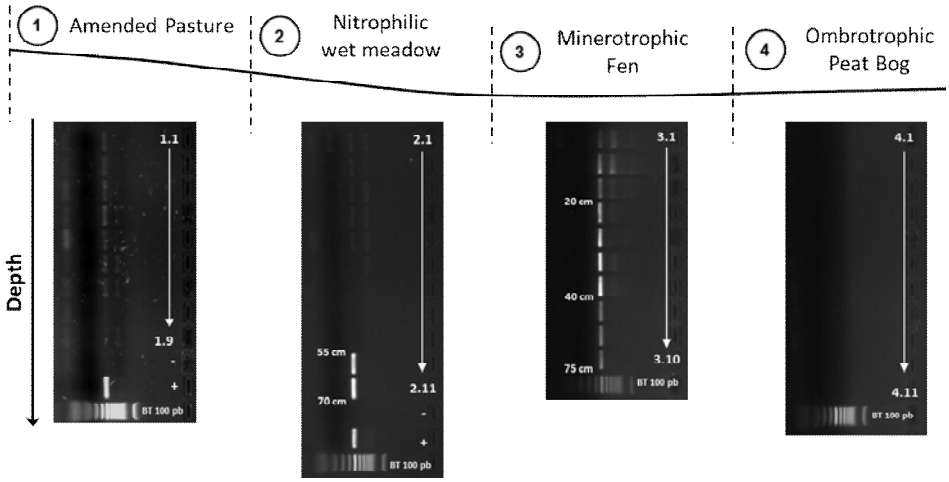


Figure V. 4. Results of the anammox PCR along the soil profile at different depth. The ladder used is "Benchtop 100 pb" and "+" stands for the positive and "-" for the negative PCR controls.

Quantitative PCR results also showed that anammox bacteria are present below 55 cm in Soil 2, and below 20 cm in Soil 3 (Figure V. 5). In Soil 2, the number of 16S rDNA of anammox bacteria reached 7×10^5 copies per gram of dry soil, and in soil 3 2.7×10^7 copies per gram dry weight. For AOB and AOA *amoA* qPCR results indicated that these organisms were presents in all four soils and at every depth. The copy numbers of *amoA* genes were between 4.3×10^6 and 3.1×10^9 copies per gram of dry soil (Figure V. 5). Except for Soil 4, copy numbers of AOB *amoA* decreased with depth. Conversely, AOA *amoA* copy numbers increased with depth or, if this was not the case, decreased less than AOB. For Soil 4, the quantities of both organisms increase with depth. In Soil 1, AOA were 4 times more abundant than AOB and in Soil 3 about 30 times. In Soil 2, AOB were 2 times more abundant than AOA, whereas they were equally abundant in Soil 4.

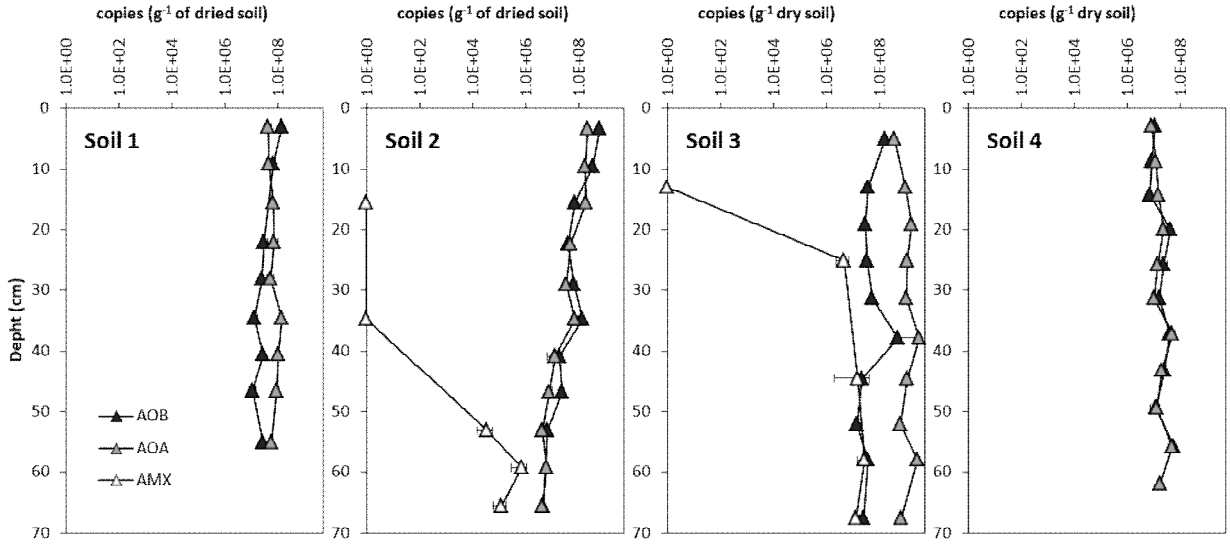


Figure V. 5. Number of amoA gene copies of ammonium oxidizing *Bacteria* (AOB) and *Archea* (AOA), and number of 16S rRNA gene copies of anammox bacteria (AMX), per gram of dry soil. Errors bars indicate the standard deviation of triplicates

Diversity of anammox bacteria

Phylogenetic analysis of retrieved anammox PCR sequences from three samples of the Soil 3 (22-28 cm, 41-48 cm, 60-75 cm) and one sample of Soil 2 (56-62 cm) formed four distinct clusters that fell clearly into the anammox group (Figure V. 6). A fifth group was composed of sequences, which were not related to anammox bacteria. The phylogenetic tree revealed that the Cluster II was not very close to a known anammox candidate genus, but to a sequence belonging to the clone FN691953, (Humbert *et al*., 2010), and to a sequence belonging to the clone AJ871743, which is an uncultured *Planctomycete* from an ammonium-contaminated aquifer (Smits *et al*., 2009). Cluster I was similar to

Candidatus 'Brocadia fulgida' and *Candidatus* 'Brocadia sp.'. Cluster III was close to *Candidatus* 'Jettenia asiatica' and Cluster IV close to *Candidatus* 'Anammoxoglobus propionicus'.

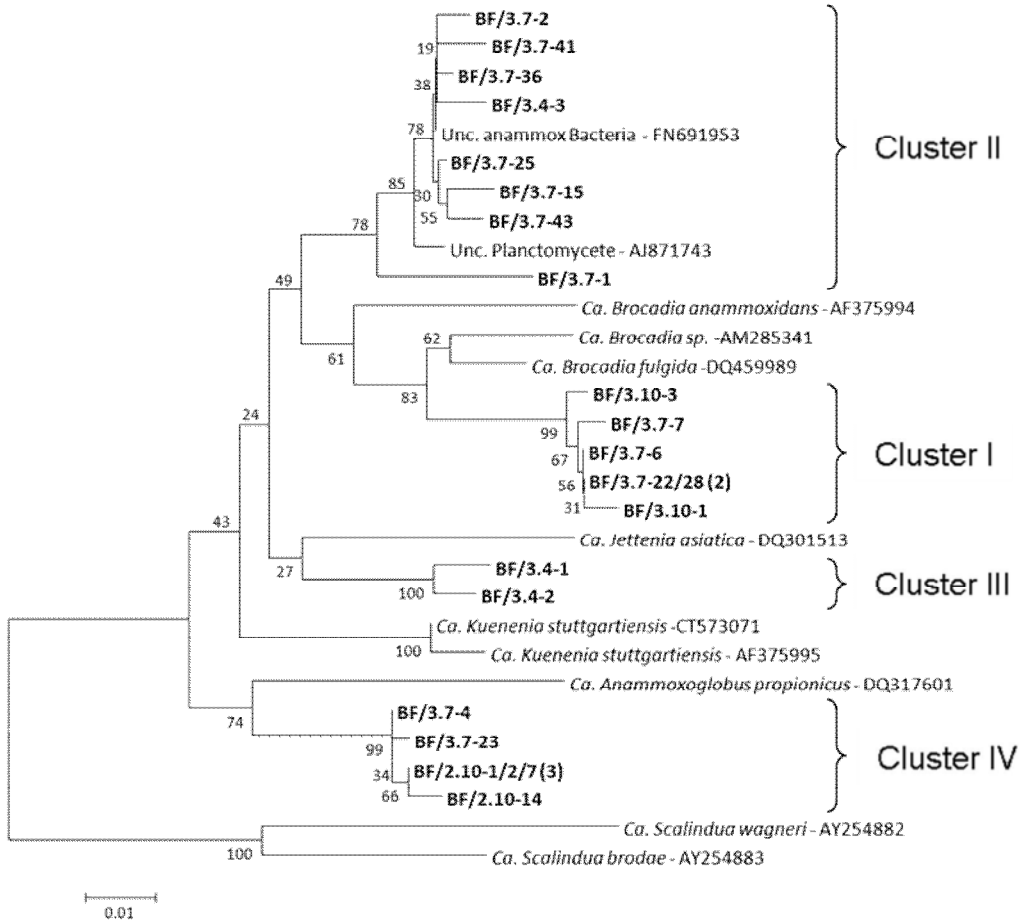


Figure V. 6. Neighbour-joining tree based on the Silva alignment of the products of the Amx nested-PCR, showing the phylogenetic relationship between known anammox bacteria and closely related 16S rRNA gene sequences retrieved from the nitrophilic wet meadow (soil 2) and the minerotrophic fen (soil 3). Clone names are composed as follows: Soil number (2 or 3) – sampling depth (a = 22-28 cm, b = 41-48 cm, c = 56-62 cm, and d = 60-75 cm) - clone number. Bootstrap values (1'000 replicates) are indicated for each node and the scale bar represents 1% of sequence divergence.

The proportion of each of the cluster in the sequenced samples is presented in Table V. 2. In Soil 3 about 42% of the retrieved clones from the uppermost sample (22-28 cm) were not affiliated with anammox bacteria. Below that depth almost all retrieved sequences were related to anammox bacteria. Furthermore, the sample from 41-48 cm was the most diverse as it contained sequences belonging to three different anammox clusters. With increasing depth diversity anammox bacteria got reduced again. In Soil 3 only sequences from Cluster I, related to *Ca. Brocadia sp.,* were found. This seemed also

to be the case in Soil 2 where at 56-62 cm only sequences related to *Ca. Anamoxoglobus* were detected (Cluster IV).

Table V. 2. Phylogenetic repartition of the clones retrieved from different depths of soil 2 and 3

Cluster	Soil 3			Soil 2
	22-28 cm	41-48 cm	60-75 cm	56-62 cm
I	0.00 %	61.54 %	95.24 %	0.00 %
II	47.37 %	30.77 %	0.00 %	0.00 %
III	10.53 %	0.00 %	0.00 %	0.00 %
IV	0.00 %	7.69 %	0.00 %	100.00 %
Non-anammox	42.11 %	0.00 %	4.76 %	0.00 %
Number of clones	26	19	21	16

Activity of anammox

The ^{15}N incubations were done in triplicates with a sample of Soil 2 (50-60 cm) and of Soil 3 (40-50 cm), respectively, where the sequencing results indicated the presence of anammox bacteria. After 12 hours of incubation, the production rates of $^{30}N_2$ in the $^{15}NO_3^-$ incubations were 45 and 60 times higher than the $^{29}N_2$ production rates in Soil 2 and 3, respectively (Figure V. 7). For the other incubations ($^{15}NH_4^+$ and $^{15}NH_4^+ + {}^{14}NO_3^-$) and for both soils, the production of $^{30}N_2$ was 30 times more important than the $^{29}N_2$ production, whereby the production of $^{30}N_2$ in the $^{15}NH_4^+$ incubation of Soil 3 stopped after 6h.

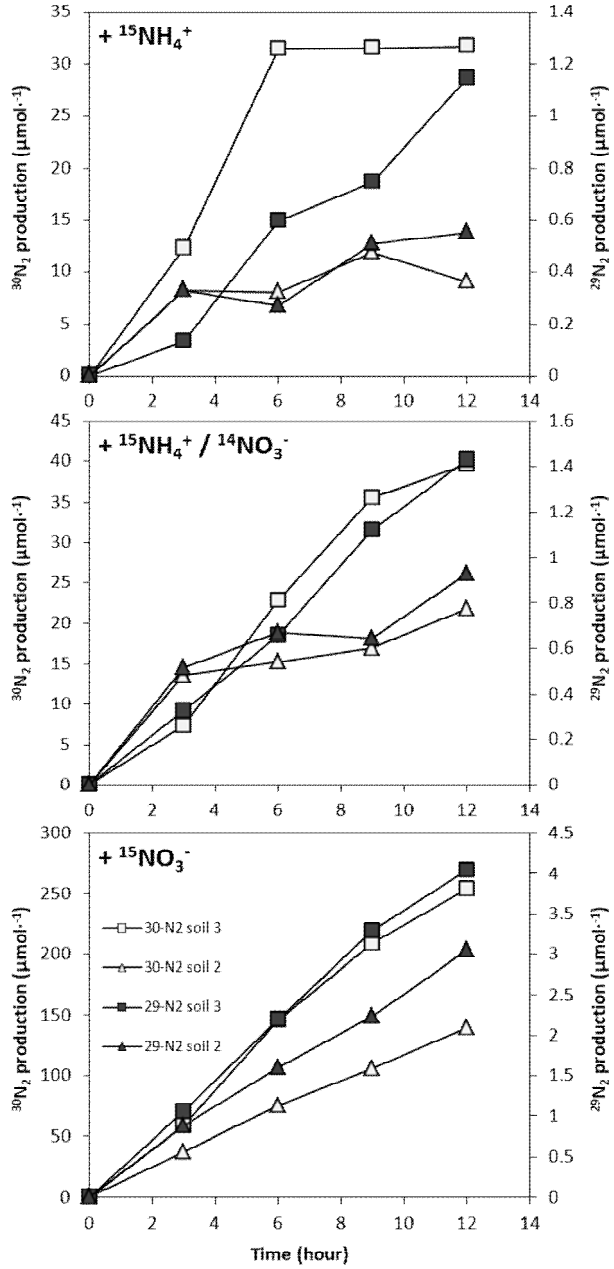


Figure V. 6. Production of $^{29}N_2$ (black) and $^{30}N_2$ (white) in anoxic incubations with $^{15}NH_4^+$, $^{15}NH_4^+$ + $^{14}NO_3^-$ and $^{15}NO_3^-$ additions, respectively, to soil 2 (triangles, nitrophilic wet meadow) and soil 3 (squares, minerotrophic fen).

Discussion

Chemical conditions in the soils

Interstitial water concentrations of inorganic nitrogen compounds of all four soils were highest in the nitrophilic wet meadow (Soil 2), which is located next to an amended pasture at the foot of a gentle hill slope. Part of the nitrogen brought out as manure reaches Soil 2 by lixiviation. The nitrophilic wet meadow serves thus as a buffer zone between the amended pasture and the fen (Soil 3) and the oligotrophic peat bog (Soil 4). Inorganic nitrogen contents in the interstitial water decreased from Soil 2 to 4. In the peat bog, concentrations of the dissolved nitrogen compounds never exceeded 1 µM,

which is due to its ombrotrophic nature and the presence of *Sphagnum* sp., sequestering nutrients in their cells (Gobat *et al.*, 2010). Measurements of inorganic nitrogen compound using a KCl extraction procedure, which releases adsorbed and intracellular compounds, revealed that nitrate and particularly ammonium are present in the peat bog despite the negligible concentrations in the interstitial water.

Ammonium and nitrate concentrations along a depth profile are closely related to the degree of oxygenation and, therefore, depend essentially on the biological activity in the soil and the groundwater level. Water saturated conditions reduce the diffusive transport of oxygen to deeper soil sections and facilitate the development of anoxic conditions and the establishment of anaerobic metabolisms like fermentation, nitrate-reduction, sulfate-reduction and methanogenesis. During wintertime, the groundwater level was highest due to increased precipitation and the absence of evapotranspiration. At the same time, the biological activity (i.e. heterotrophic respiration) in the soil was low, which explains the oxic conditions and high nitrate concentrations in Soil 2 and Soil 3. Ammonium liberated during the degradation of organic matter in autumn and winter is oxidized to nitrate under these conditions.

With the beginning of the vegetation period in spring, conditions changed rapidly and in a concerted manner. The respiration of plant roots and of microorganisms, stimulated by rising temperatures and rhizodeposition, reduced pO_2. Nitrate was assimilated by the growing plants or used by denitrifying microorganisms. Furthermore, in Soil 2 evapotranspiration lowered the groundwater table rapidly to about 40-50 cm bl.s. (April, June) and even to 80 cm bl.s. in August. Above the water table the soil is considered oxic, whereas it is anoxic underneath, as indicated by the presence of ammonium and the nearly absence of nitrate in the interstitial water. Hence, Soil 2 and 3 are characterized by periods of oxic and anoxic conditions that alternate predictably on a seasonal scale, but may also change on a short-term scale according to the meteorological conditions.

Distribution and abundance of aerobic and anaerobic ammonium oxidizers

The comparison of anammox PCR (nested and qPCR) results with the measurements of nitrogen compounds shows that the presence of nitrite, which is the primary substrate of anammox bacteria, may not explain their distribution. Nitrite was never detected in the bulk soil and the interstitial water of Soils 2 and 3 during the vegetation period.

Nitrate, on the other hand, never drops below 1 $\mu mol \cdot g^{-1}$ of dried soil where anammox bacteria were detected. This implies a reduction of nitrate to nitrite, which can be done by the anammox bacteria themselves or by other nitrate-reducing microorganisms. Also ammonium does not seem to play a decisive role for the presence of anammox bacteria due to its omnipresence in the studied soils.

High contents of organic matter may favor nitrate-reducing bacteria yet they do not hinder growth of anammox bacteria. Anammox bacteria were detected in the Soil 3, which consists of more than 60% of organic matter. The quality, i.e. degradability, of organic matter, rather than the absolute quantity is likely the determining factor for the distribution and activity of anammox and denitrifying bacteria. For example in Soil 3, the presence of anammox bacteria was anti-correlated to soil respiration (determined as BOD_5), which rapidly decreased from 10.90 mg $O_2 \cdot g^{-1}$ of dry soil at 5 cm to 3.50 mg $O_2 \cdot g^{-1}$ of dry soil at 25 cm and to 0 mg $O_2 \cdot g^{-1}$ of dry soil at 65 cm (data not shown).

Except for Soil 2, AOB were less abundant than AOA but observed differences were less pronounced than reported by Leininger *et al*. (2006), where there were up to 3000 times more AOA than AOB. The general increase with depth of the ratio AOA/AOB is consistent with the findings of Leininger *et al*. (2006). However, it is unclear why in Soil 2 AOB are more abundant than AOA since this soil is very similar to Soil 1 in terms of structure, composition and nitrogen content. Copy numbers of AOA and AOB *amoA* were generally 1-2 orders of magnitudes higher that maximum values of anammox bacterial 16S rRNA gene copy numbers. Assuming one *rrn* operon per anammox bacterial genome, it means that aerobic ammonium oxidizing microorganisms are clearly more abundant than the anaerobic ones.

Diversity of anammox bacteria

Despite the difficulties of obtaining a stable phylogenetic tree, the four highlighted clusters remained well defined with high bootstrap supports of 99% for Clusters I, III, IV; and 78% for Cluster II. The different clusters stayed with the different tested treeing methods and with the presence/absence of outgroup sequences (results not shown). These results are consistent with earlier findings of an increased phylogenetic diversity of anammox bacteria in terrestrial (Humbert *et al*., 2010) as opposed to aquatic ecosystems (Schmid *et al*., 2007). Similar observations were made in river estuary sediments, where the highest diversity was found in the least saline part of the estuary

(Dale *et al*., 2009). A reduced ecological pressure and the heterogeneity of the soil (and sediments), as opposed to the more homogenous water column habitat, may explain this higher diversity.

It is difficult to link a certain cluster with a known genus of anammox bacteria because of the relatively short length of the amplified 16S rRNA gene sequence. Nevertheless, Cluster II is the most remote and may represent a new anammox candidate genus. The fact that this cluster is very close to clone sequence FN691953 and to others sequences retrieved by Humbert *et al*. (2010) supports this idea. To confirm the existence of a new genus, however, nearly complete 16S rRNA gene sequences will be required. Cluster I is close to *Candidatus* 'Brocadia sp.', with a similarity higher than 96% but Clusters III and IV could not be linked unambiguously to a known genus. Both are between two known genera: *Candidatus* 'Kuenenia' and *Candidatus* 'Jettenia', which may explain, in concert with the short sequences, the fragility of the phylogenetic tree.

Activity of anammox

The production of $^{30}N_2$ in the $^{15}NH_4^+$ incubation, which is the negative control, shows that the added ammonium is oxidized without oxygen (Figure V. 7). The incubation bottles had been pre-incubated for 3 days to deplete the intrinsic NO_3^- pool and all possible precautions had been taken to avoid any oxygen contamination. Still, a background of intrinsic NO_3^- remained detectable throughout the experiment (about 4 µM). This might have been due to an anaerobic oxidation of the ammonium with Fe(III) as oxidant as postulated previously (Clément *et al*., 2005), but further investigation is needed to solve this question. Despite the anaerobic oxidation of NH_4^+ the $^{15}NO_3^-$ incubation can be used to quantify denitrification and to estimate at least the maximum contribution of anammox to N_2 formation, respectively. This incubation shows that the denitrification is much more efficient than anammox in both soil samples. In the sample of Soil 2 (40-50 cm), denitrifying bacteria produced N_2 at a rate of 11.7 µmol·l^{-1}·h^{-1} of N_2 and the anammox bacteria at 0.25 µmol·l^{-1}·h^{-1}, which corresponds to 0-2.2% of the total N_2 production. This situation is almost the same in the sample of Soil 3 (50-60 cm). The anammox bacteria produce 0.3 µmol·l^{-1}·h^{-1} of N_2 (0-1.6% of total N_2 production) whereby the denitrifying bacteria produce 21.1 µmol·l^{-1}·h^{-1} of N_2. This may be due to the higher organic matter content in soils than in aquatic environments. Similar results have been found in marine sediments where anammox was hardly detectable in eutrophic

bay and shelf sediments, but got progressively more important with distance from the shore (Dalsgaard *et al.*, 2005). In organic poor shelf slope sediments from Skagerrak anammox contributed by more than 70% to total N_2 formation (Dalsgaard *et al.*, 2005).

Conclusion

Anammox bacteria can be found in soils mostly in deeper soils sections were the ecological conditions are favorable for their growth. In the studied soils, anammox bacteria appear to depend on long-term anoxic conditions and on a source of inorganic nitrate. A yet unidentified process might contribute additionally to the anaerobic oxidation of ammonium, possibly by using iron(III) as oxidant. Anammox bacteria are less abundant and active than aerobic ammonium oxidizing microorganisms and denitrifying bacteria. But the interesting fact is the high diversity of those bacteria in the soils. Four distinct clusters were identified and one may represent even a new genus.

Chapter VI

Supplementary experiments

Introduction

In this chapter, supplementary experiments are presented which were done to establish and improve the different methods used in this study.

An anaerobic continuous enrichment culture for anammox bacteria was set up in the laboratory mainly to obtain a positive control for the DNA extraction and to optimize the nested-PCR protocol for the amplification of the 16S rRNA gene of *Planctomycetales* and anammox bacteria (See chapter II). The molecular detection of anammox bacteria in terrestrial ecosystems shown in chapter II, revealed the presence of environmental clone sequences affiliated to other, non-anammox, *Planctomycetales* sequences. To date there exist no closely related cultivated relatives to these sequences. A continuous enrichment culture with different soil samples was, therefore, set up in order to: i) enrich for anammox organisms and to assess their phylogenetic affiliation; ii) link the presence of phylotypes, which do not belong to one of the currently know anammox genera to anammox activity; iii) confirm viability of the anammox bacteria in soils.

Material and methods

Set up of the enrichment cultures

The anaerobic enrichment of activated sludge for anammox bacteria was set up and started in February 2006 until May 2008 in a flow through glass reactor (Fig. VI. 1).

Figure VI. 1. Glass reactor filled with glass wool, sterile sand and the activated sludge. Red arrow indicates the fresh medium in-flow and blue arrow indicates the medium out-flow. A one way continuous flow was applied to the reactor.

The reactor consisted essentially of a glass tube, filled at one end (entry) with a sterile glass wool, covered by sterile sand. Then 50 grams of activated sludge (WWTP of Neuchâtel and Visp (CH)) where anammox bacteria were suspected were added to the reactor and covered again with sterile sand. Finally, sterile sand was put at the top of reactor. The reactor was amended with a continuous one way flow of anoxic mineral medium containing 2 mM ammonium, 2.5 mM nitrite and bicarbonate as carbon source (Annex A-13). The media was flushed with N_2/CO_2 (90/10%) gas to obtain anoxic conditions and was fed into the reactor at a flow rate of 13 ml/h with a peristaltic pump; resulting in a hydraulic retention time of 3 hours. The reactor was kept at 37°C in dark to avoid algal growth. All these conditions represented optimal growth conditions for anammox bacteria (Egli *et al*., 2001; Isaka *et al*., 2006; Third *et al*., 2005).
Anammox activity was detected by comparing ammonium and nitrite concentrations in the in- and out-let of the reactor. For this, 2 ml of medium were sampled with a syringe and either directly measured or frozen at -20°C for further analysis. Ammonium concentrations were determined by the hypochlorite-phenol reaction (Annex A-15) and nitrite concentrations were measured by the sulfanilamide method (Annex A-16). The concentration measurements were done usually every second week during two years.

The anaerobic enrichment of soil samples for anammox bacteria was set up in 10 ml glass syringe (Fig. VI. 2). The medium and conditions were the same as for the continuous enrichment culture of activated sludge.

Figure VI. 2. The eight microcosm enrichment cultures (left) with the pump delivering continuously the anoxic mineral medium (right).

The eight different soil samples were consisting in:

A1) Rhizosphere of *Cladium mariscus* from Grande-Cariçaie (CH)
A2) Sediment from Neuchâtel Lake shore (CH)
A3) Forest soil (*Fraxinus excelsior* L.) from Loclat Lake shore (CH)
A4) Sediment from ammonium-contaminated porous aquifer in Wallis (CH)
NA1) Rhizosphere of *Phragmites sp.* from Grande-Cariçaie (CH)
NA2) Rhizosphere of *Urtica dioica* from Neuchâtel Lake shore (CH)
ND1) Rhizosphere of *Urtica dioica* from Loclat Lake shore (CH)
CII) Sediment from Loclat Lake shore (CH)

The different soil samples were selected based on the results obtained in the chapter II. Microcosms A1, A2, A3, and A4 were set up to observe anammox activity in a soil from where the retrieved 16S rRNA gene sequences were related to know anammox bacteria. Microcosms NA1 and NA2 were selected to link the presence of phylotypes which do not fall into currently know anammox genera with anammox activity. Microcosm ND1 was established to test whether an enrichment for anammox bacteria is possible with a soil from where PCR products were not retrieved, possibly due to the low abundance of the anammox bacteria. Microcosm CII was set up to observe the presence of anammox activity as clone sequences retrieved from this environment formed an independent cluster within the anammox group also called the cluster II (see chapter II and IV).
A detailed description of the soils and foregoing nested-PCR results for anammox bacterial 16S rRNA gene diversity can be found in chapter II.

Nested-PCR protocol evaluation

The reaction mixtures of the nested-PCR are explained more in details in chapter II. The optimal conditions of the nested-PCR were determined using the enrichment culture as positive control and *E. coli* or *Pseudomonas fluorescens* DNA extracts as negative controls. The optimal annealing temperature was determined using a gradient temperature between 50 to 65°C. The $MgCl_2$ concentration was tested from 1.5 to 3 mM in 0.5 increments. The optimal annealing temperature was set at 62°C and optimal $MgCl_2$ concentration at 2 mM. These adjustments improved significantly the specificity

(single band at the corrected size 480 bp) and the efficiency (amplification yield increased) of the nested-PCR protocol.

In order to determine the phylogenetic affiliation of the enriched anammox bacteria, positive nested-PCR products of the correct size were used for phylogenetic analysis. The PCR products were cloned and sequenced following the protocol explained in chapter II. The positive PCR products were retrieved from the enrichment culture after five months and after one year of continuous enrichment. Among the 50 clones obtained for each samples, 18 were submitted to RFLP analysis using *Msp*I. Finally, at least 2 clones per OTU per sample were selected for sequencing. The retrieved sequences were aligned using the ClustalW implementation of MEGA4 (Tamura *et al*., 2007), which was also used to calculate NJ trees.

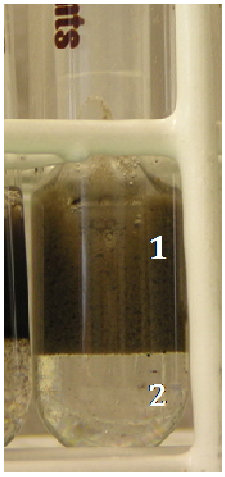
Visualization of anammox cells

Microscopy was used to observe anammox bacterial cells in the enrichment culture. For this observation, about 1 g of enriched sludge was sampled and bacterial cells were extracted in 10ml of 0.2% hexamethaphosphate solution, then fixed in 2% final concentration of formaldehyde solution and filtered using a polycarbonate membrane filter of $0.2\ \mu m$ (Pernthaler *et al*., 2001). DAPI (4-6 diamidinophenylindole) staining was done directly on the filters. In addition, *Fluorescence In Situ Hybridization* (FISH) protocol (Annex A-12) using PLA886 probe with its competitor cPLA886 (Neef *et al*., 1998) were applied to detect precisely the presence of bacteria belonging to the order of *Planctomycetales* and Amx368 probe was used for all anammox bacteria genera detection (Schmid *et al*., 2005). Finally, the hybridized- and DAPI- stained cells were observed using an epi-fluorescence microscope *Leica* (DMR HC) equipped with a *Nikon* DIGITAL CAMERA DXM1200. For the hybridized cells, filter Y3 was used as the probes possess a Cy3 fluorochrome. Filter 2 was selected for the DAPI stained cells. The retrieved pictures were observed using ACT-1 software.

Cells extraction protocol

DAPI staining revealed that there were still a lot of organic residues and cell aggregates that caused high background fluorescence on the filter making observation difficult. Thus, a cells extraction protocol was applied (Caracciolo *et al*., 2005) in order to separate any residual organic matter or inorganic particles from the cells (Annex A-11).

Briefly, sample was collected and put in an extraction solution containing phosphate buffer with pyrophosphate and Tween 0.5% final concentration then homogenized with an Ultra-Turrax (IKA) during 2 min at 17'500 u/min. The soil extract was put in a centrifuge tube in which Histodenz (density=1.3g/ml) was carefully added with a syringe at the bottom of the centrifuge tube in order to obtain two distinct phases (Fig. VI. 3). The detailed cells extraction protocol can be found in Annex A-11.

A 

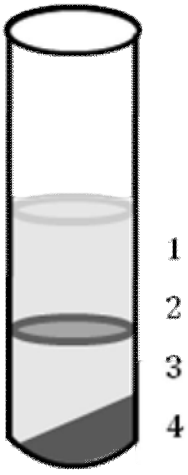
B 

Figure VI. 3.

A) The two distinct phases obtained after addition of Histodenz in the centrifuge tube containing the soil extract. 1) Soil extract, 2) Histodenz. B) Schema of the sample after centrifugation. 1) Supernatant, 2) Cell layer, 3) Histodenz, 4) Pellet.

After one hour and half of centrifugation at 13'000 rpm at 4°C, the remaining cell layer was recovered using a micropipette, fixed with 2% of formaldehyde (final concentration) and then filtered using a polycarbonate membrane filter of 0.2 μm. The filter was stained with DAPI and FISH methods as explained previously (§ visualization of anammox cells).

Results and discussion

Enrichment of activated sludge for anammox bacteria

The enrichment culture started in February 2006 and lasted until May 2008. After three month, the presence of gas bubbles within the sludge suggested gas producing microbial activity in the enrichment culture. This gas production was likely due to the activity of anammox bacteria that produced N_2. Denitrification, which is also a N_2 producing process, should not be present in the reactor as no organic carbon source was added to the medium. In addition, the color of the enrichment, which was black at the beginning of the incubation, became light brownish which is also a good indicator of the presence of anammox bacteria (Third *et al.*, 2005). However, to verify if anammox activity was

established, nitrite and ammonium concentrations were measured in the in- and out- let of the reactor (Table VI. 1).

Table VI.1: Measurements of ammonium and nitrite concentrations in the medium before and after the enrichment culture.

Sampling dates	measured compounds	Inflow [mM]	Outflow [mM]
03.07.2006	Nitrite	0.87	0.001
	Ammonium	2.71	1.68
02.08.2007	Nitrite	2.02	0.0019
	Ammonium	2.27	0.24
13.12.2007	Nitrite	2.54	0.003
	Ammonium	1.84	0.033
10.04.2008	Nitrite	2.53	0.001
	Ammonium	2.02	0.002

The results (Table VI. 1) demonstrate a clear nitrite and ammonium consumption under anoxic conditions, representing clear evidence for the installation of anammox activity after five months. Thereafter, the concentration of nitrite in the medium was progressively increased from 0.87 mM to 2.54 mM until all ammonium was consumed. Similar ratio of substrates utilization was observed in a sequencing batch reactor (SBR) under substrate-limiting conditions (Strous *et al*., 1998). Based on the SBR results, the stoichiometry of the anammox process was established to be: 1 NH_4^+ + 1.32 NO_2^- → 1.02 N_2 + 0.26 NO_3^-.

After two years of enrichment, the color of the sludge had changed to brownish-red (Fig. VI. 4). This red color was due to the high content of haeme containing enzymes in the anammox cells (Jetten *et al*., 1999). This change of color has been described in several studies enriching for anammox bacteria (Strous *et al*., 1999; Egli *et al*., 2001; Third *et al*., 2005).

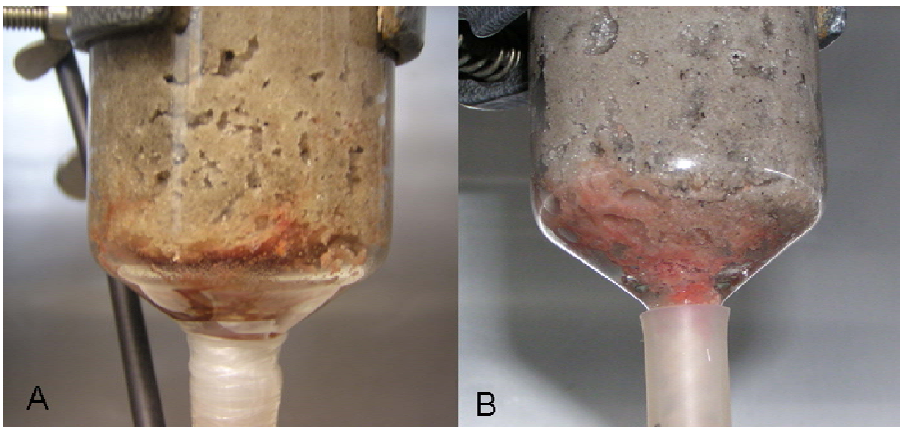


Figure VI. 4. Enrichment cultures of anammox bacteria after A) one year and B) two years of incubations. The red color is due to the cytochrome pigmentation of anammox bacteria.

In addition to this observation, the ammonium and nitrite content measurements demonstrated that still 99.9% of both substrates were consumed. To identify the anammox bacteria in the reactor, DNA was extracted and anammox bacterial 16S rRNA gene was amplified using nested-PCR as described in chapter II. Positive PCR products of the correct size (480bp) were obtained (Fig. VI. 5.).

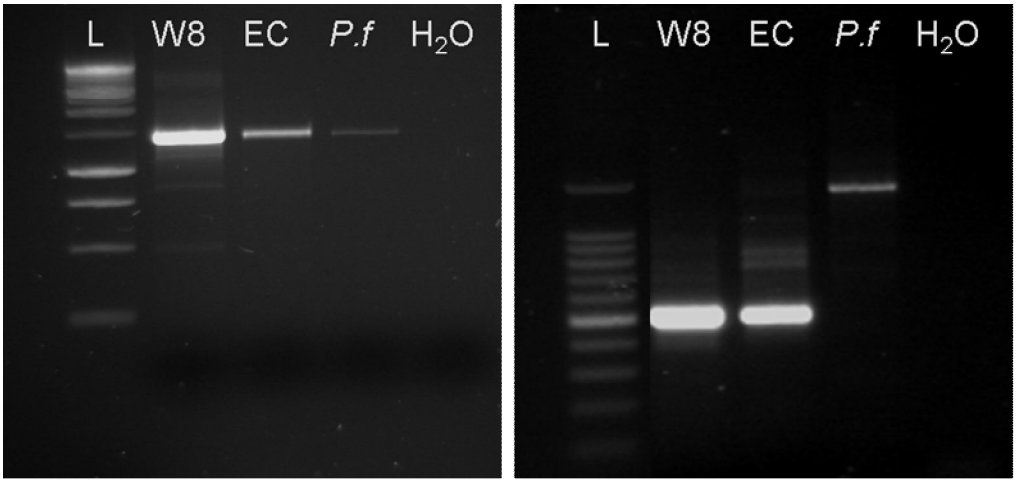


Figure VI. 5. Positive and negative amplification products after specific *Planctomycetales* (picture left) and anammox specific (picture right) 16S rRNA gene amplification. L: ladder (BenchTop 1Kb (left) and BenchTop 100pb (right)), W8: positive control from Dr. M. Schmid (Vienna) enrichment culture, E.C: enrichment culture, *P.f*: negative control (*Pseudomonas fluorescens*), H_2O: no template control (water).

Phylogenetic analysis of the culture after five months of enrichment showed that 85% of the retrieved clones were related to *Candidatus* 'Jettenia asiatica' with 97% of similarity. After two years of continuous culture, 100% of the clones were related to *Candidatus* 'Brocadia anammoxidans' with 96% of similarity. This result shows that the population of anammox bacteria in the enrichment culture changed during the incubation suggesting that the diversity was more important at the beginning of the experiment than presumed (Fig. VI. 6). It also shows that a minor member of the initial anammox bacterial community may become dominant with the current enrichment conditions and highlights the great bias of the enrichment methods. Furthermore, one might speculate that *Ca. Jettenia* is a mixotrophic anammox bacterium depending on organic component present in the inoculums that gets replaced by *Ca. Brocadia* after a prolonged period of autotrophic growth.

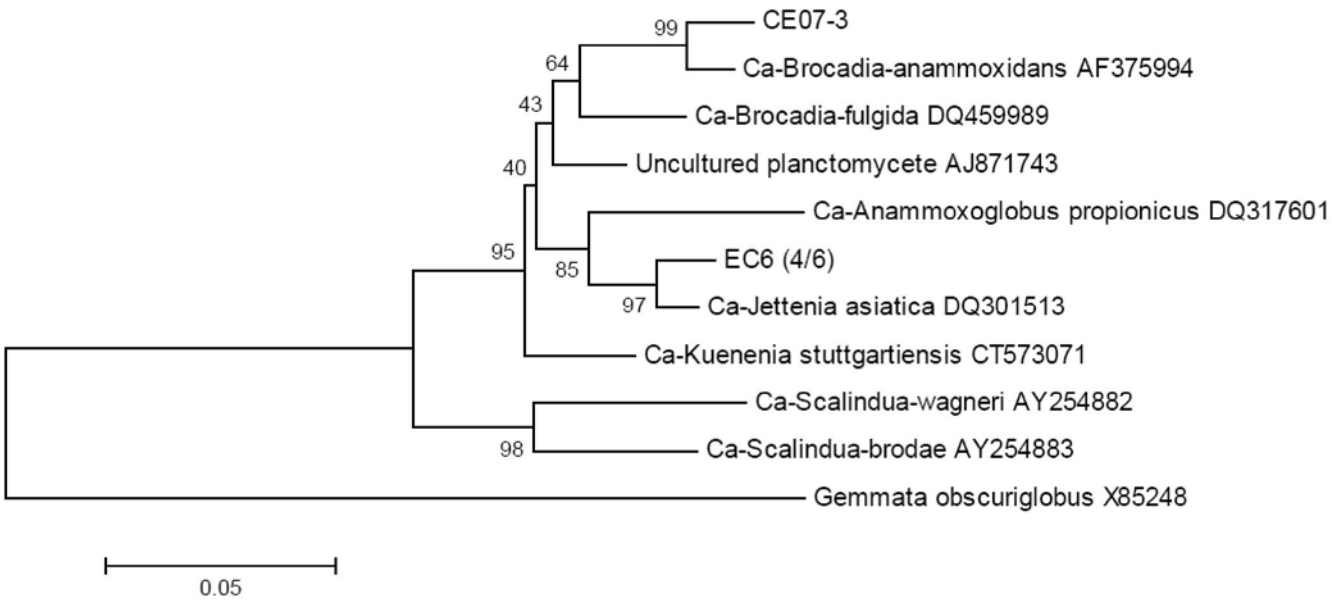


Figure VI. 6. Neighbour-joining tree showing the relationship between known anammox bacteria and closely related 16S rRNA gene sequences retrieved from the enrichment culture after five months (EC6) and one year (CE07-3) of continuous incubation. Bootstrap values (1000 replicates) are shown and the scale bar represents 5% of sequence divergence.

Regarding to ammonium and nitrite consumptions (Table VI. 1), enrichment conditions of the culture may have changed during the two years and probably inducing favorable conditions for the growth of another anammox specific genus. Moreover, during the first year, the production of gas has conduct to an over-pressure in the reactor. Two solutions were possible for limiting this problem, the first was to take a bigger reactor and the second was to diminish the media flow. The second solution has been selected and the flow has been decreased from 27 ml/h to 13 ml/h. Thus the availability of nitrogen nutrient in the reactor has changed which may explain the change of the anammox population. This could indicate that an anammox genus required specific available supplies for it growth.

The cell extraction protocol improved in this study has provided a thin cell layer without any residual organic matter or particles (Fig. VI. 7).

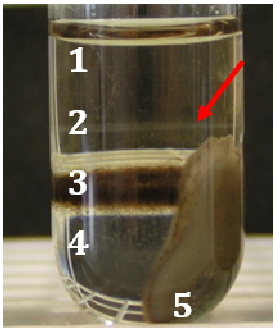


Figure VI. 7. The soil extract-Histodenz sample appearance after the centrifugation step during the bacterial cell layer extraction protocol. The detached bacterial cells are present in the cell layer indicated with the red arrow. 1) Supernatant, 2) Cell layer, 3) Slurry, 4) Histodenz, 5) Pellet.

The cell layer was observed in optical microscopy. The results obtained by this method shows that the cell layer contain bacteria cells only and no residual organic matter or inorganic particles. This procedure gives a powerful technique for the separation of bacteria cells from the soil matrix. The microscope observation has also allowed determining the volume of the cell layer that was further filtered. This cell extraction protocol could be subsequently used for epi-fluorescent microscopy.

The anammox bacterial cells visualization by epi-fluorescent microscopy has allowed obtaining some pictures of cells in the enrichment culture. The DAPI staining method has allowed to observe the typical 'donught shape' of the anammox cells (Fig. VI. 8). This typical characteristic is due to their ribosome's localization around the anammoxosome (Lyndsay *et al*., 2001).

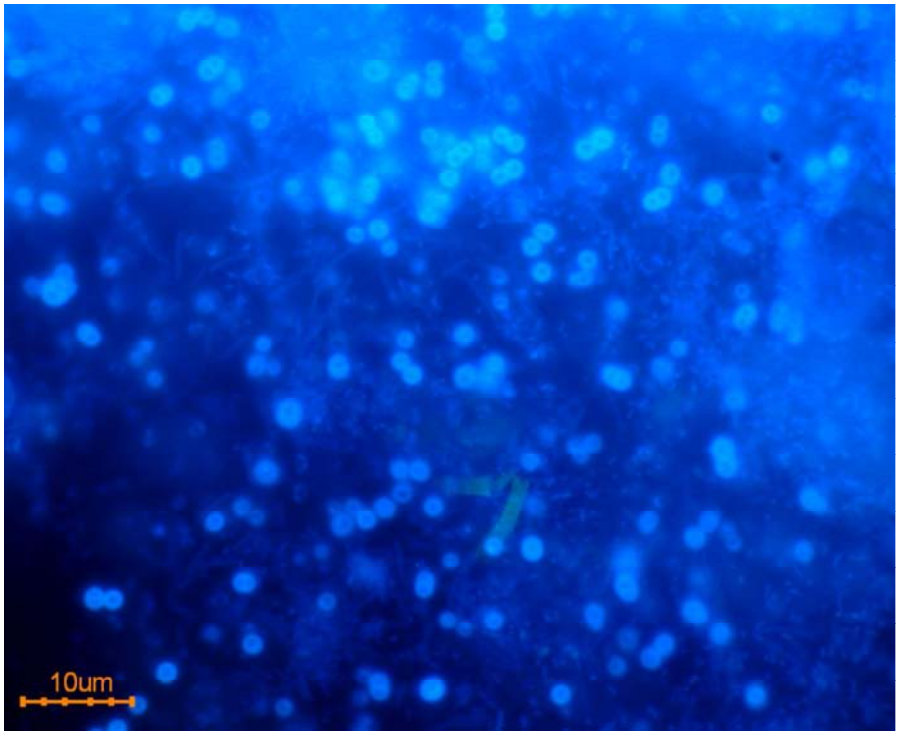


Figure VI. 8. DAPI applied on samples from the enrichment culture showing typical doughnut-shaped anammox cells.

Positive hybridization of *Planctomycetales* and anammox probes with some cells allowed their detection in the enrichment culture and their typical 'donught shape' observation (Fig. VI. 9). However, the results obtained with the FISH probes were very deceiving. Indeed, the fluorescence intensity was very bad and the probes were lacking of specificity. Consequently, the FISH observation did not allow further anammox bacteria quantification. Thus, the molecular technique 'qPCR` have been developed and

improved in this study to succeed in quantifying anammox bacteria in soil samples (see chapter III).

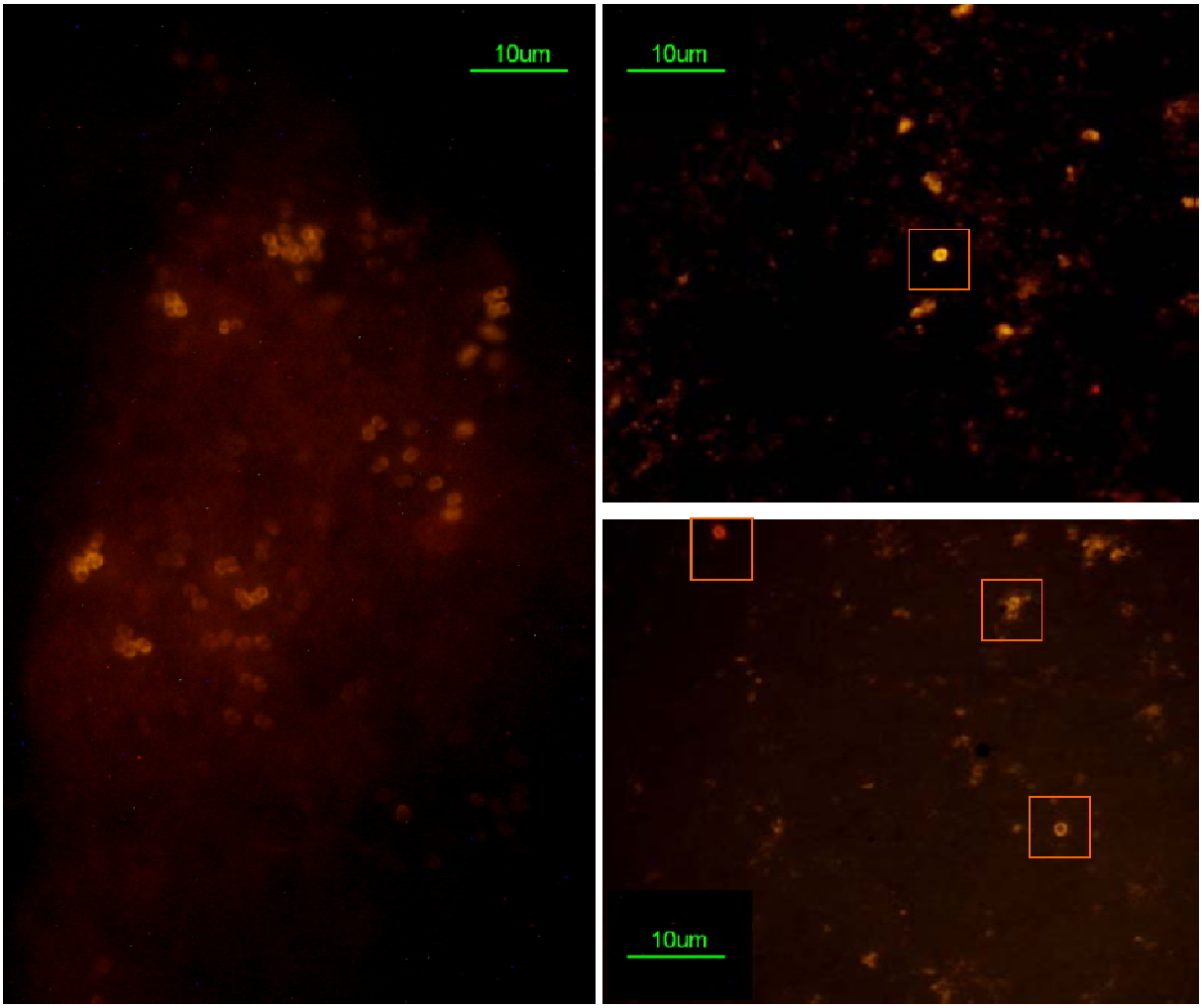


Figure VI. 9. Detection of anammox bacteria cells by FISH in the enrichment culture. Squares indicate typical anammox cells. Picture left, anammox cells in the culture after two years of enrichment. Pictures right, anammox cells after five months of enrichment; upper picture *Planctomycetales* cell detected with PLA886 probe, down picture: anammox cells detected using Amx368 probe.

Enrichment of soil samples for anammox bacteria

The continuous enrichment of anammox bacteria in the different microcosms started in May 2007 and lasted until April 2008. After five months among 30% of the initial ammonium concentration and less than 10% of initial nitrite concentration were consumed in microcosms NA1, NA2, A1, A2, A4, and CII (Table VI. 2). In microcosm ND1 the initial nitrite concentration decreased by 70% and in microcosm A3 by 40% whereas the ammonium decreased by 30% as in the other microcosms. These results

suggest that after five months anammox activity was slowly establishing in all microcosms.

Table VI. 2: Ammonium and nitrite concentrations measurements in the in- and out-flow of the eight microcosms.

Dates	Compounds	Inflow [mM]	Outflow [mM]							
			NA1	NA2	A1	A2	A3	A4	CII	ND1
07.06.2007	Nitrite	1.19	1.3	0.61	1.57	1.02	1.25	1.45	1.34	0.95
	Ammonium	2.51	2.36	1.81	2.03	2.38	2.41	2.45	2.42	2.47
03.10.2007	Nitrite	1.01	1.08	0.91	0.89	0.94	0.57	1.05	1.09	0.28
	Ammonium	2.66	1.91	1.75	1.99	1.91	1.86	1.83	1.99	1.8
10.04.2008	Nitrite	2.61	1.9	2.31	1.8	1.9	2.28	2.44	2.36	0.05
	Ammonium	2.04	1.65	1.64	1.71	1.58	1.57	1.58	1.69	0.01

After one year, in all microcosms the initial ammonium concentration decreased only by about 20% except in microcosm ND1 where ammonium was completely consumed (99.5%). The initial nitrite concentration decreased by less than 40% in microcosms NA1, A1, A2, less than 20% in NA2, CII, A3 and less than 10% in microcosm A4. However, in microcosm ND1, 98% of the initial nitrite was consumed. These results demonstrated that anammox activity was clearly established in microcosm ND1 whereas absent, lost or not clearly detectable in the other microcosms. These results might suggest that the hydraulic retention time of 3h was too short to allow the complete consumption of nitrite and ammonium in all microcosms except ND1. After one year of enrichment, the presence of gas bubbles and red spots (Fig. VI. 10) in microcosm ND1 evidenced anammox activity.

Figure VI. 10. The presence of red aggregates and gas pockets in microcosm NDI after one year of continuous enrichment evidences the presence of anammox bacteria.

Positives PCR products were obtained after enrichment in three out of the eight microcosms ND1, A2, and CII. The samples from microcosms, ND1 and CII, submitted to the nested-PCR before the beginning of the enrichment did not yield any anammox PCR products. This suggested that in some samples anammox bacteria are only detectable by the nested-PCR protocol after enrichment, which points to a low abundance of these organisms in their habitats. In microcosms NA1, NA2, A1, A3, A4, no anammox bacterial PCR products were obtained after enrichment suggesting that anammox bacteria are clearly not present. This result is sustained with the substrates concentrations measurements which have shown that anammox activity was not present after enrichment in these microcosms. However, in microcosms A2, and CII, anammox bacteria were detected whereas their activity was in terms of ammonium and nitrite consumption not detected.

Phylogenetic affiliations of the clones retrieved from microcosms ND1, A2, and CII are presented in figure VI. 11.

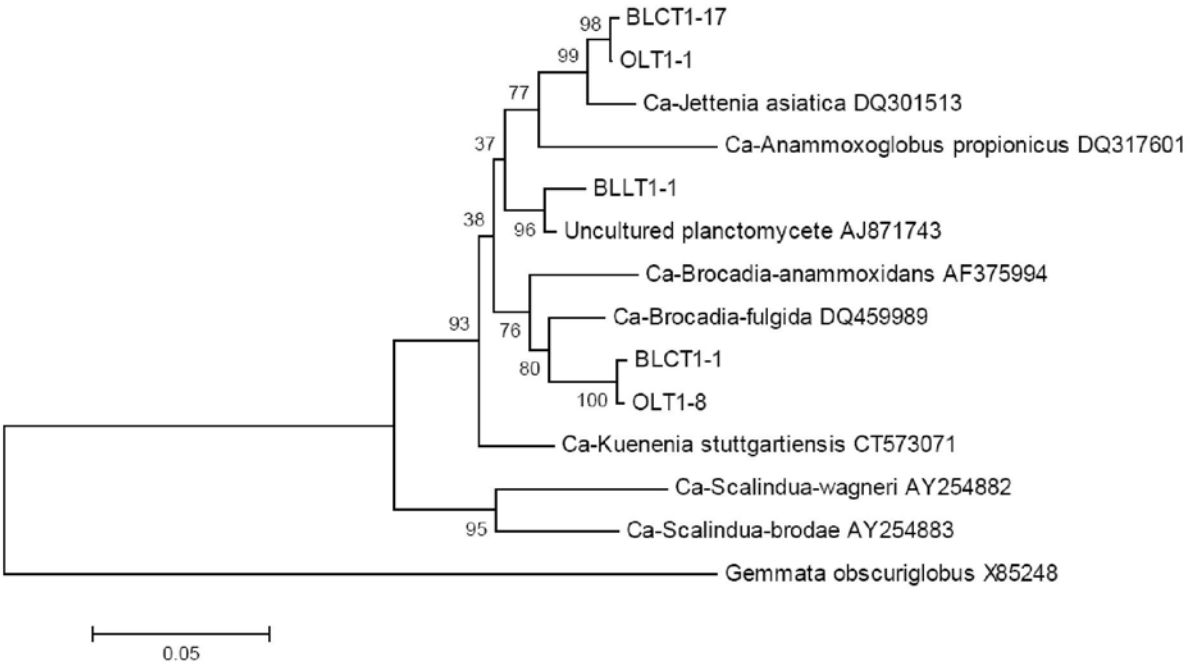


Figure VI. 11. Neighbour-joining tree showing the relationship between known anammox bacteria and closely related 16S rRNA gene sequences retrieved from the three microcosm's enrichment culture; BLCT1: A2, OLT1: ND1, BLLT1: CII Bootstrap values (1000 replicates) are shown and the scale bar represents 5% of sequence divergence.

Environmental clone sequences obtained from the microcosm ND1 were affiliated with *Ca. Brocadia fulgida* at 96% similarity (34% of retrieved clones) and *Ca. Jettenia asiatica* at 97% similarity (66% of clones). 16 % of clones from the microcosm A2 were related to *Ca. Jettenia asiatica* with 97% similarity and 84% of clones to *Ca. Brocadia fulgida*

with 95% similarity. Sequences from microcosm CII shared equal similarities of ~95% with both *Ca. Kuenenia* and *Ca. Brocadia* and could represent so far undescribed anammox genera named cluster II (chapter II and IV). These results confirmed the presence of anammox bacteria and demonstrated that in one environment different anammox genera can be present. Moreover, the identification of *Ca. Jettenia asiatica* for the first time in such environments increased its potential favorable niches. So far, *Ca. Jettenia asiatica* was only found in a granular sludge anammox reactor (Quan *et al.*, 2008).

In addition, clone sequences retrieved from microcosm ND1 (*Urtica dioica* from Loclat Lake shore (CH)) and microcosm A2 (sediments from Neuchâtel Lake shore (CH)) are both related to *Ca. Brocadia fulgida* and *Ca. Jettenia asiatica* whereas the distribution of clones differ between both microcosms. In microcosm ND1 67% of clones (n=18) were affiliated to *Ca. Jettenia asiatica* whereas this candidate was related to only 17% of the clones than microcosm A2. As anammox activity was only detected in microcosm ND1 it could be suspected that this genus is more efficient and contribute more to the anammox process than *Ca. Brocadia fulgida* in such environment and specific conditions. However, this result must be taken with caution as only 18 clones were phylogenetically analyzed.

Conclusion

The combination of the different observations like gas bubble production, red color development, chemical and molecular analyses confirmed the enrichment of anammox bacteria from activated sludge from a WWTP in Neuchatel. The presence of *Ca. Jettenia* and later *Ca. Brocadia* suggested that the initial anammox guild was more diverse than suspected and that *Ca. Brocadia* out-competed *Ca. Jettenia* under the chosen conditions.

Anammox bacteria are slow growing organisms whose activity takes time to get established. Terrestrial anammox bacteria were not detectable in some soils before the enrichment whereas their presence was confirmed afterwards. This shows that anammox bacteria were initially only low abundant yet alive. Furthermore it suggests that these organisms can play a role in nature as soon as favorable conditions for their development exist for an extend period of time. The presence of anammox bacteria was confirmed by specific PCR in some microcosms where no activity was observed suggesting that anammox bacteria may be present but not active or their activity was

minor thus undetectable. The presence of two candidates in one environment demonstrates that this environment provide favorable conditions to sustain a higher diversity. In environments presenting the same diversity, the structure of the population was different between them. This difference of distribution underlined the specific niches provide by the environment in each microcosm. Thus, it underlies the importance of ecological niches for the growth anammox bacteria. Anammox activity differs in environments where different population structures of anammox bacteria have been observed. This supposed that one genus is more efficient in specific environmental conditions than the other demonstrating its specific requirements. This result suggested a niche differentiation of anammox bacteria in specific environments.

Finally, all this experiment of continuous enrichment culture of different soil samples has increased our knowledge and understanding of terrestrial anammox bacteria.

Chapter VII

General discussion

Introduction

In this thesis anammox bacteria were detected in a wide range of terrestrial ecosystems and five anammox bacterial genera could be identified. Despite the increased diversity of anammox bacteria as compared to marine environments, anammox bacteria in the studied soils contributed not more than 14% to the total N_2 formation. The main results of the thesis are summarized in Table VII. 1 in order to provide an overview.

Table VII.1: Summary of all the results obtained during this study.

Sampled Environment	Sample	Anammox genera	Abundance[a]	Activity[b]
Cladium mariscus	RS	*Brocadia*	2.14×10^5	-
Fen, *Caricion davallianae*	BS (25 cm)	*Brocadia*, *Jettenia*, cluster II	1.82×10^6	-
Fen, *Caricion davallianae*	BS (44 cm)	*Brocadia*, *Anammoxoglobus*	2.29×10^6	-
Porous aquifer	BS	*Brocadia*	1.83×10^5	-
Reductisol	Spring 0-10 cm	*Brocadia*, cluster II	ND	0.4
	Spring 20-30 cm	*Brocadia*, cluster II	4.01×10^4	2.2
	Spring 50-60 cm	*Brocadia*, cluster II	1.80×10^6	6.8
	Summer 0-10 cm	*Brocadia*, cluster II	1.78×10^5	1.3
	Summer 20-30 cm	*Brocadia*, cluster II	2.16×10^5	2.3
	Summer 50-60 cm	*Brocadia*, cluster II	5.00×10^5	8.3
	Autumn 0-10 cm	ND	ND	1.3
	Autumn 20-30 cm	*Brocadia*, cluster II	6.05×10^5	1.9
	Autumn 50-60 cm	Cluster II	4.36×10^5	14.3
Reductisol	BS enrichment	Cluster II	-	-
Urtica dioica	RS enrichment	*Brocadia*, *Jettenia*	-	+
Alnus incana	BS enrichment	*Brocadia*, *Jettenia*	-	-
WWTP NE and Wallis	Enrichment	*Brocadia*, *Jettenia*	-	+
Fraxinus excelsior	BS	*Kuenenia*	-	-
Alnus viridis	RS	*Kuenenia*	2.02×10^6	-
Alnus incana	BS	*Kuenenia*	6.48×10^4	-
Marsh sediment	Sediment	*Scalindua*	4.01×10^5	-
	GWTI	*Scalindua*	-	-
Permafrost	BS	*Jettenia*	-	-

a: Abundance of anammox bacterial 16S rRNA copies/g of fresh soil
b: Maximum percentage of anammox contribution to total N_2 formation
RS: Rhizosphere soil, BS: Bulk soil, GWTI: Groundwater table interface
ND: not detected, -: not determined, +: observed by nitrite and ammonium consumption

Presence

The presence of anammox bacteria was confirmed in different environments precisely in the bulk soil of a *Fraxinion* on the shore of Lake Neuchâtel, a *Caricion davallianae* in a fen, and a Reductisol on the shore of Lake Loclat, in sediment of marshes in the Camargue, in a porous aquifer, in a permafrost and, finally, in the rhizosphere of *Cladium mariscus* and *Alnus incana*. The detection of terrestrial anammox bacteria in all fractions of these different environments confirm our basic hypothesis that rhizosphere, bulk soil, and soil-groundwater table interfaces provide oxic/anoxic interfaces and/or represent nitrogen-rich environments, which offer favorable habitats for anammox bacteria. It was shown that in some environments (Reductisol, fen) the presence of anammox bacteria was correlated with the groundwater level. This conclusion is supported by the observation that anammox bacteria were not detected in some cultivated environments like sweet corn crop and grassland, demonstrating that anoxic conditions must prevail a long to maintain a detectable population of anammox bacteria due to their slow growth.

Terrestrial anammox bacteria were not detected in all tested soil fractions from a given ecosystem and were only present at a specific sampling date season (cf. chapter IV e.g. always presents at 50-60 cm depth and sometime at 0-10 cm depth). These results confirm that the local conditions such as too high oxygen concentration, the absence of spatially or temporally well established oxic/anoxic interfaces, low nitrogen concentrations or low water content were not favorable to sustain a detectable population of anammox bacteria.

The different results obtained in this thesis have improved our understanding of the conditions required by anammox bacteria. Ammonium is not a limiting factor for growth of anammox bacteria as it is always present in natural environments due to the degradation of organic matter (c.f. chapter IV, Table IV. 1, [NH4+]). Very little or no nitrite were measured in soil samples from where anammox bacteria were retrieved suggesting that this compound is completely used by soil microorganisms. Nitrification was present in soil samples down to 35 cm depth (data not shown) and may provide nitrite as substrate for anammox bacteria which were usually present in the lower part of the soil profile. Therefore, a synergetic interaction between both processes may exist. Moreover, nitrite which is used by anammox bacteria is not present a long time in soils as principally known to be a metabolic intermediate generally rapidly consumed. Nitrate could be reduced in nitrite under anoxic conditions providing the constant flow of

nitrite. Nitrate was always present in samples where anammox bacteria were retrieved suggesting that this substrate is present under not limiting conditions. However, anammox bacteria were not detected in some samples with high nitrate content, demonstrating that other conditions are necessary for their occurrence. Nitrate is involved in many N-cycling processes as e.g. denitrification, DNRA, anammox. Nitrate serves also in plant nutrition, thus multiple competitions for this compound are possible. Additionally, anammox bacteria are suspected to perform the DNRA in which they reduce nitrate to ammonium (Kartal *et al*., 2007). These suggest that anammox bacteria could thrive in environments with high nitrate content. Finally, the discovery of terrestrial anammox bacteria has increased the knowledge of the number of ecosystems where these bacteria exist.

Diversity

The five known anammox genera *Candidatus* 'Brocadia', 'Kuenenia', 'Jettenia', 'Scalindua', and 'Anamoxoglobus were retrieved in 9 out of 24 environments (chapter II, chapter III, chapter V and chapter VI). This result demonstrates that there is a higher diversity of anammox bacteria in terrestrial than in aquatic ecosystems where only *Ca.* 'Scalindua', 'Brocadia' and 'Kuenenia' were identified. In the ecologically homogeneous water column and marine water columns only *Ca. Scalindua* was detected.

This study revealed for the first time the presence of all five known anammox genera in natural ecosystems. This greatly increased the knowledge of their potential habitats since they were so far only related to bioreactors, wastewater treatment plants (WWTP's), as well as in marine and freshwater ecosystems. More precisely, *Ca. Kuenenia* and *Ca. Brocadia* first discovered in WWTP's, were recently also detected in river and estuary sediments (Amano *et al*., 2007; Dale *et al*., 2009; Zang *et al*., 2007). Both candidates were found in rhizosphere and bulk soil of different environments (Table VII. 1) suggesting that they thrive also in more heterogeneous environments where oxic/anoxic interfaces and nitrogen content are present. This may also suggest that they could prosper in such environments due to their versatile metabolism (Guven *et al*., 2005; Kartal *et al*., 2007b; Kartal *et al*., 2008; see introduction chapter I). The results obtained in this study demonstrate that both genera dominate in terrestrial environments (Table VII. 1) suggesting that *Ca. Brocadia* and *Ca. Kuenenia* may possess a better adaptation capacity in such ecosystems than the 3 other candidates. The evidence

of the presence of *Ca. Scalindua* in terrestrial ecosystems was obtained in marsh sediments from the Camargue. This demonstrates that this candidate also lives in a less saline and more heterogeneous environment as compared to the homogenous marine water column (Penton *et al.*, 2006; Schmid *et al.*, 2007). *Ca. Jettenia* and *Ca. Anammoxoglobus* were also retrieved in terrestrial ecosystems suggesting that such ecosystems are also favorable habitats for both candidates relative to WWTP's where they were discovered.

In some cases (enrichment cultures, Reductisol, and fen), two or more genera were detected in the same sample. It has been also demonstrated that the structure of the anammox guild differs between environments (chapter VI). This result may also reflect a niche differentiation of anammox bacteria in specific environments as found, for example, along a salinity gradient in estuarine sediment (Dale *et al.*, 2009).

Abundance

The qPCR protocol developed in this study allowed the quantification of anammox bacteria in some terrestrial ecosystems (chapter III, V, Table VII. 1). The amount of anammox bacterial 16S rRNA gene copies per gram of fresh soil ranged up to 6.74 x 10^6 copies/g. This represents the first quantification of these bacteria in such ecosystems as only reported previously in marine ecosystems (Dale *et al.*, 2009; Hamerslay *et al.*, 2007; Lam *et al.*, 2007; Li *et al.*, 2010) and enrichment culture (Tsushima *et al.*, 2007; Quan *et al.*, 2008; Schmid *et al.*, 2008).

The qPCR method has allowed the quantification of all five known anammox genera and the potential new anammox candidate (Cluster II). The highest abundances of anammox bacteria were detected in the rhizosphere of *Alnus viridis* (Cadagno), at 30-40 cm depth in the Reductisol (Loclat Lake), and at 25 to 44 cm depths in the *Caricion davallianae* (Bellefontaine). The former environment represents a wet soil where the rhizosphere provides an oxic/anoxic interface due to roots and microorganism respiration. Both last environments possess a fluctuating water table that leads also to oxic/anoxic interfaces. This result confirms that this condition is sufficient to maintain anammox bacteria density about 10^6 copies/g of fresh soil. At 30-40 cm depth in the Reductisol (Loclat Lake) and at 25 to 44 cm depths in the *Caricion davallianae* (Bellefontaine), the diversity of anammox bacteria was higher as two candidates were detected in the same sample.

In environments where long term anoxic condition are less or not present it could result in a lower abundance of anammox bacteria. The optimal temperature and pH for the growth of anammox bacteria in bioreactor were found to be 37°C and pH 8 (Jetten *et al*., 1999; Strous *et al*., 1999). In this study, the temperature of soil samples where anammox bacteria were present was between 8 -18°C and the pH was between 5.9 and 7.5. These results suggest that anammox bacteria could grow under such conditions in natural ecosystems whereas in a low abundance.

This study has provided a suitable tool for the quantification of anammox bacteria in terrestrial ecosystems thus allowing a better understand of their distribution in specific environment.

Activity

The activity of terrestrial anammox bacteria was determined using ^{15}N-isotope incubations. This was the first application of this method in soil samples. The determination of anammox activity in terrestrial ecosystems was done at a Reductisol and three sampling dates (spring, summer, and autumn), and in a fen, where also the diversity and the abundance of anammox bacteria were determined (Table VII. 1).

Anammox activity was detected at all sampling dates and depths in Reductisol and ranged from 0.4% to 14.3% of the total N_2 production in the soil. Anammox did not contribute by more than 2% to total N_2 formation in the soil from the fen. These results show that anammox bacteria are active in soils whereas their contribution to N_2 formation is minor. The anammox activity was less important than in water column and within the ranges observed in constructed wetlands (3 to 9%, Erler *et al*., 2008), estuarine (5 to 24%, Risgaard-Petersen *et al*., 2004; 3.8 to 16.5%, Dale *et al*., 2008), mangrove (0 to 9%, Meyer *et al*., 2005) as well as river sediments (0 to 22%, Rich *et al*., 2008). These environments are also water-saturated environments and more heterogeneous than the marine water column where anammox could account up to 67% of total N_2 formation (Thamdrup and Dalsgaard, 2002).

The highest contribution to total N_2 formation was found in the deeper layers of the Reductisol profile. Indeed, in the upper part of the soil the organic matter is present and could therefore be used by heterotrophic denitrifiers. This result suggests a competition in the upper part of the soil profile by nitrate using microorganisms or plants. In the deeper part of the soil profile, the organic matter is still present but less degradable. The

availability of electron donor is therefore reduced for heterotrophic bacteria. Thus denitrification is weak in the bottom of the soil profile leading to the presence of inorganic nitrogen content which could be used by anammox bacteria. These results demonstrate that the availability and quality of organic matter are more important than its absolute quantity and suggests that autotrophic anammox bacteria are more competitive than heterotrophic denitrifiers in deeper soil layers.

Here, it's important to remember that the sampling for the activity was done at three different sampling dates (spring, summer, and autumn) and that the results should not be viewed as a complete seasonal study. However, it's not false to postulate that the season has an impact on the environmental conditions as reported in many studies (Dale *et al*., 2009; Thamdrup and Dalsgaard, 2002, Risgaard-Petersen *et al*., 2004, Meyer *et al*., 2005, Hietanen and Kuparienen, 2008, and Trimmer *and* Nicholls, 2009) but this needs still to be analyzed more in detail. The season has an impact on the environmental parameters as oxic/anoxic interfaces, inorganic nitrogen compounds and organic matter degradability whereas it did not influence their availability. This suggests that the relative importance of both processes to N_2 production seems to be regulated by substrates availability over the season. Such results have been also reported by Thamdrup and Dalsgaard, 2002, Risgaard-Petersen *et al*., 2004, Meyer *et al*., 2005, Hietanen and Kuparienen, 2008, and Trimmer and Nicholls, 2009. All these results demonstrate that despite the competition for the same substrates, anammox and denitrification occur in the same environment whereas the contribution to dinitrogen production is dominated by denitrification in soil. However, the anammox rates measured in this study were within the range values observed in constructed wetlands (Erler *et al*., 2008), in marine (Thamdrup and Dalsgaard 2002), in estuarine (Trimmer *et al*., 2003; Risgaard-Petersen *et al*., 2004; Dale *et al*., 2009), in mangrove (Meyer *et al*., 2005), and in river sediments (Rich *et al*., 2008).

The contribution of anammox to total N_2 production increased with depth as well as the number of anammox bacterial 16S rRNA gene copies per ng of DNA (Chapter III). This result suggests that there is a positive correlation between abundance and activity of anammox bacteria in the Reductisol as also observed and reported in the oxygen minimum zones (Hamerslay *et al*., 2007; Kuypers *et al*., 2005; Lam *et al*., 2008), marine and estuarine sediments (Schmid *et al*., 2007; Dale *et al*., 2009), as well as hydrothermal vents (Byrne *et al*., 2009).

Chapter VIII

Synthesis and perspectives

The main objective of this thesis was to shed light on a newly discovered process within the biogeochemical nitrogen cycle: the anaerobic oxidation of ammonium, which has received some attention in aquatic but not in terrestrial ecosystems. The results presented in the different chapters provide a better understanding of this process in the terrestrial realm.

It was shown in chapter II, that also oxic/anoxic interfaces in soils can sustain detectable populations of anammox bacteria, independently of the soil type (oxic soils or water saturated soil). However, the molecular tools used for their detection were not sufficiently specific and new PCR primers needed to be developed. In chapter III, these primers were applied in a new nested-PCR approach to detect more specifically anammox bacterial 16S rRNA genes in different ecosystems. This improved detection protocol could now be applied to a large variety of terrestrial environments. Thus, the presence of anammox bacteria could be tied to a wider range of potentially favorable habitats, possibly leading to a better comprehension of the ecological requirements of anammox bacteria. In chapter II and III, it was also demonstrated that the heterogeneity of soil offers a larger variety of favorable niches than the homogeneous marine and freshwater columns, where mostly only *Ca. Scalindua* has been detected. As a consequence, up to five known anammox genera can be present in soils. Moreover, a new potential anammox candidate (Cluster II) was discovered, suggesting that the anammox guild is more diverse than suspected. Whereas the phylogenetic confirmation as a new genus needs still to be done, was its activity shown by the ^{15}N-incubations experiment. Thus, in further studies, complete 16S rRNA gene sequence have to be recovered and submitted to supplementary sequences analysis in order to confirm the discovery of a new genus within the anammox genera.

In chapter VI, the enrichment culture experiments revealed the succession of two different anammox bacterial populations in an environment, where the conditions were modified over time. Additionally, enrichments cultures showed that different structures of anammox populations could be retrieved from different environments. This suggests that the distribution and diversity of anammox bacteria are influenced by specific environmental requirement and a competition between the different genera may be suspected. The further study of the population dynamics between the genera could be important in order to evaluate the inter-genera competition or their potential equilibrium. Thus, the design of specific primers for each genus and their application in

a qPCR approach could lead to a specific quantification of each anammox population in order to better understand their distribution and dynamic. Moreover, the study of their metabolism in enrichment culture under controlled conditions will provide a better characterization of their specific requirements. Correlating a specific anammox population with the environmental conditions may allow a more global understanding of their distribution in terrestrial ecosystems. Until now, the environmental conditions that determine which anammox types thrive in an ecosystem are unknown. Therefore, the measurement of environmental parameters in a wide range of terrestrial ecosystems could be compared allowing a better understanding of the environmental parameters (e.g. nitrogen content, pH, organic compounds) favorable for the presence of anammox bacteria. In chapter II it was suggested that certain anammox bacteria (*Ca. Kuenenia* and *Brocadia*) possess a more versatile metabolism leading to a better adaptation in heterogeneous niches than the other genera. Thus the complete analysis of the genome of each genus using molecular tools could provide new data on each specific metabolism and enhance the comprehension of their distribution in terrestrial ecosystems. In addition, the set up of anoxic enrichment cultures with addition of carbon sources or alternative electron acceptors as propionate, acetate, Fe(III), Mn(IV), and SO_4^{2-}, respectively, could allow the definition of their metabolic versatility.

To better understand the specific ecological requirements of anammox bacteria, a Reductisol and fen profiles were studied in detail (Chapters III, IV, and V). Thus the following synthesis is constructed on the results obtained at these particular sites. Results show that these Reductisol and fen harbor a stable community of anammox bacteria, both in terms of diversity and abundance. Anammox bacteria were only low abundant at the soil surface but increased in numbers by about 1 to 2 orders of magnitude with depth. The abundance of anammox bacteria seems to be controlled by the temporality of the anoxic conditions: Short term anoxic conditions as present in soil macro-aggregate and the rhizosphere sustain only a low anammox density, whereas long term anoxic conditions, provided mainly by the water level, sustain a higher abundance. The comparison between total bacterial and anammox abundances using qPCR or FISH methods, in the future, could allow a better comparison or correlation between different terrestrial environments.

The measurement of potential anammox and denitrification activities have allow underlining the importance of two essential ecological factors, the quality of organic

matter and the availability of inorganic nitrogen compounds. At depth, denitrification rates are lower due to the poorer quality of organic matter, allowing the establishment of higher anammox activity relative to the top of the soil profile. In environments where denitrifiers are less active, the contribution of anammox to the total N_2 production was higher suggesting that competition for substrates may be an important factor controlling the abundance and activity of anammox bacteria in terrestrial environments. In future studies, the potentials activities could be correlated with the amount of anammox DNA or cells present in the soil. Thus, providing a good indicator of anammox bacterial cell efficiency in specific environments.

The study of nitrification, anammox and denitrification in a selected site during an annual cycle will provide knowledge about their activity dynamics. These different processes involved in nitrogen cycling influence the presence and availability of nitrogen contents. Thus the measurement of inorganic nitrogen contents will help to define the specific seasonal conditions in which anammox bacteria thrives favorably. Moreover, as suggested in chapter III, the presence of the *hzo* gene (hydrazine oxidoreductase), which was discovered in the *Ca. Kuenenia* genome, needs still to be confirmed in the other genera and to be related to anammox activity. In this study, clone sequences retrieved from different environmental samples were affiliated with the five known genera. Therefore, the DNA extracts from which the anammox sequences were retrieved could be used to confirm the presence of the *hzo* gene in the other candidates. After confirmation of the involvement of this gene in anammox activity, an RT-qPCR method could be developed in order to link the presence and abundance with activity. Moreover, the activity could be confirmed by ^{15}N-incubations and correlated with abundance of anammox bacteria.

In conclusion, the present study represents the first evidence for the presence, diversity, and activity of anammox bacteria in diverse terrestrial ecosystems, which enhances greatly our understanding of the importance of this process in the global nitrogen cycle. Terrestrial environments offer an important variety of niches where the five known anammox bacteria and the new potential candidate could prosper and be active to a detectable level. Current evidence suggests that anammox bacteria can represent a stable and active proportion of the soil microbiota. However, the classical processes within the nitrogen cycle, nitrification and denitrification, are quantitatively more important in the tested soils. Anammox may, however, play a greater role in deeper soil

sections or aquifers, where the organic matter is poorly degradable or where its contents are extremely low and denitrification activity becomes limited.

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Annexes A: Protocols

1. Soil DNA extraction and purification
2. 16S rRNA gene amplification of V3 region
3. 16S rRNA gene amplification of *Planctomycetales*
4. Nested-PCR for 16S rRNA gene amplification of anammox bacteria
5. PCR product purification and quantification
6. Cloning using E. coli XL1 and p-GEM-T Vector
7. Amplification of clones insert using T7-Sp6 PCR
8. Restriction with *MspI*
9. Plasmids extraction and purification
10. qPCR of the anammox bacterial 16S gene
11. Bacteria extraction and sample preparation from soil samples for FISH analysis
12. FISH
13. Medium for enrichment culture and microcosms
14. Soil sampling and preparation for physico-chemical characterization
15. Ammonium quantification by the indophenol blue assay
16. Nitrite quantification by the sulfanilamide assay
17. Nitrate quantification by a modified cadmium reduction assay
18. Iron quantification by the Ferrozine assay
19. Measurements of pH H_2O and pH KCl
20. Water content and residual humidity
21. Determination of total carbon and total nitrogen
22. Soil sampling and samples preparation for ^{15}N-incubation experiments
23. ^{15}N-incubation experiments
24. Liquid and gas sampling during ^{15}N-incubation experiments

Annex A-1: Soil DNA extraction and purification

Products and solutions:

- Multimix 2 Tissue Matrix Tubes (BIO 101, ref 6560-200)
- Spin Filter™ (BIO 101, ref 6560-210)
- PBS (Sodium Phosphate Buffer, ref 6540-403)
- MT buffer (BIO 101, ref 6511-202)
- PPS (BIO 101, ref 6540-403)
- Binding matrix (BIO 101, ref 6540-408)
- Wash buffer (SEWS-M de Bio 101 : Salt Ethanol Wash)
- TE pH = 8.0, H_2O nanopure

Protocol:

Extraction was realized using FastDNA® SPIN Kit for soil BIO 101according to the manufacturer.

- Put 0.5-0.7g of soil in *Multimix 2 Tissue Matrix Tube*
- Add 978 µl of *PBS* and 122 µl of *MT buffer*. Mix gently by inversion
- Mix the tubes at 5.5 ms^{-1} in the Bead beater machine during 30 seconds
- Centrifuge at 13000 rpm for 5 min
- Transfer the supernatant (1000 µl) in a sterile tube
- Add 250 µl of *PPS* and mix by inversion 10X
- Centrifuge at 13000 rpm for 15 minutes
- Transfer the supernatant (450 µl) in a sterile tube
- Vortex the *Binding Matrix solution*
- Add same volume of *Binding Matrix* (450 µl) and homogenized by inversion (10X)
- Incubate for 5 min at room T°C
- Transfer the mix (900 µl) in a *SpinFilter*
- Centrifuge at 13000 rpm for 1 min and eliminate the elution
- Wash the *Binding matrix* with 500 µl of *Wash buffer*
- Centrifuge at 13000 rpm for 1 min and eliminate the elution, repeat this step 2X
- Centrifuge at 13000 rpm for 10 sec to dry
- Transfer the *SpinFilter* in a new tube and let drying for 5 min at room T°C
- Suspend the extract DNA in 200 µl of nanopure sterile water and let 5 min
- Centrifuge at 13000 rpm for 3 min
- The elution contain the DNA extract
- Conserve the DNA extract at 4°C for direct used or at -20°C for longer conservation

Annex A-2: 16S rRNA gene amplification of V3 region

Primers:

Univ338f: 5' AC TCC TAC GGG AGG CAG CAG 3'

Univ520r: 5' ATT ACC GCG GCT GCT GG 3'

Mix:

mix (20 µl)	1 tube (µl)	Concentration
H_2O	10	
$MgCl_2$ (25 mM)	2.4	3mM
Buffer (5x)	4	1X
dNTPs (10 µM)	0.5	250 nM
Primer f (10 µM)	0.5	250 nM
Primer r (10 µM)	0.5	250 nM
GoTaq Promega(5U/µl)	0.1	0.025U/ µl
Total :	**18**	
Template :	**2**	

Program:

Step 1	94°C for 5 min	Initial denaturation
Step 2	94°C for 1 min	Denaturation
Step 3	55°C for 30 s	Annealing
Step 4	74°C for 1 min	Elongation
Step 5	Go to 2, 14 times	15 cycles
Step 6	74°C for 10 min	Final elongation
Step 7	10°C forever	
Step 8	End	

Annex A-3: 16S rRNA gene amplification of *Planctomycetales*

Primers:

Pla46f: 5' GGA TTA GGC ATG CAA GTC 3'

Univ1390r: 5' GAC GGG CGG TGT GTA CAA 3'

Mix:

mix (20 µl)	1 tube (µl)	Concentration
H_2O	10	
$MgCl_2$ (25 mM)	2.4	3mM
Buffer (5x)	4	1x
dNTPs (10 µM)	0.5	250 nM
Primer f (10 µM)	0.5	250 nM
Primer r (10 µM)	0.5	250 nM
GoTaq Promega (5U/ µl)	0.1	0.025U/ µl
Total :	**18**	
Template :	**2**	

Program:

Step 1	95°C for 2 min	Initial denaturation
Step 2	95°C for 45 s	Denaturation
Step 3	62°C for 50 s	Annealing
Step 4	72°C for 1 min 22	Annealing
Step 5	Go to Step 2, 29 times	30 cycles
Step 6	72°C for 5 min	Final elongation
Step 7	4°C forever	

Annex A-4: Nested-PCR for 16S rRNA gene amplification of anammox bacteria

Primers:

A368f: 5' TTC GCA ATG CCC GAA AGG 3'

A820r: 5' AAA ACC CCT CTA CTT AGT GCC C 3'

Mix:

mix (20 µl)	1 tube (µl)	
H_2O	10.8	
$MgCl_2$ (25 mM)	1.6	2mM
Buffer (5x)	4	1X
dNTPs (10 µM)	0.5	250 nM
Primer f (10 µM)	0.5	250 nM
Primer r (10 µM)	0.5	250 nM
GoTaq Promega (5U/ µl)	0.1	0.025U/ µl
Total :	**18**	
Template :	**2**	

Program:

Step 1	95°C for 2 min	Initial denaturation
Step 2	95°C for 45 s	Denaturation
Step 3	62°C for 50 s	Annealing
Step 4	72°C for 1 min 22	Annealing
Step 5	Go to Step 2, 29 times	30 cycles
Step 6	72°C for 5 min	Final elongation
Step 7	4°C forever	

Annex A-5: PCR product purification and quantification using *Wizard® SV Gel and PCR Clean-Up system*

Products and solutions:

- Membrane binding solution
- SV minicolums
- Membrane wash solution
- Nuclease free water

Protocol:

1) Fixation

- Add same volume of *Membrane binding solution* to the PCR product
- Place a *SV minicolumn* in a sterile tube
- Transfer the Membrane binding solution-PCR product in the mini-column
- Incubate for 1 min
- Centrifuge at 13'000 rpm for 1 min
- Put off the elution

2) Purification

- Add 700 µl of *membrane wash solution* in the column
- Centrifuge at 13'000 rpm for 1 min
- Put off the elution
- Add 500 µl of *membrane wash solution*
- Centrifuge at 13'000 rpm for 5 min
- Put off the elution
- Centrifuge at 13'000 rpm for 1 min
- Incubate for 2 min

3) Elution

- Transfer the column in a new sterile tube
- Add 20 to 50 µl of *nuclease-free water*
- Incubate for 1 min
- Centrifuge at 13'000 rpm for 2 min
- Conserve the purified PCR product at -20°C until further use

4) Quantification

- Quantified the purified PCR product at 260 nm using a NanoDrop™ spectrophotometer (Thermo scientific, Wilmington, USA)

Annex A-6: Cloning using *E. coli* XL1 and p-GEM-T vector

Products and solutions:

- p-GEM-T vector kit Promega (-20°C)
- Sterile tips conserved at -20°C
- Ice
- Oven at 37°C
- 1.5 ml sterile tubes
- Electro competent *E. coli* XL-1 cells conserved at -80°C
- IPTG (-20°C)
- X-Gal conserved in black tubes (-20°C)
- S.O.C solution (4°C):
  - 2g Tryptone
  - 0.5 g Yeast Extract
  - 1ml NaCl 1M
  - 0.25 ml KCl 1M
  - 1ml Mg^{2+} 2M
  - Add to 100 ml water

- Glycerol 15% (w/v) /1 mM MOPS solution:
  - MOPS (3-morpholinopropane-sulfonic acid) 0.1046 g
  - H_2O desionized 500 ml
  - Glycerol 75 g (61.12 ml)
  - Autoclave and store at 4°C

Media:

LB

- Tryptone 10 g
- Yeast Extract 5 g
- NaCl 10 g
- H_2O add to 1000 ml

Adjust pH at 7, autoclave

LB agar with ampicilline

- Tryptone 10 g
- Yeast Extract 5 g
- NaCl 10 g
- Agar 15 g
- H_2O add to 1000 ml

Adjust pH at 7, autoclave

- Let cool to 45°C
- Add Ampicilline (150 mg/ml) 1 µl /ml

Protocol:

1) Competent cells for electroporation

- Incubate overnight a bacterial culture of *E. coli* at 37°C in 10 ml of LB medium
- Add 2 ml of the culture to 200 ml of LB medium
- Incubate under agitation until 0.5 < OD 600 nm > 1
- Centrifuge at 4000 rpm at 4° C for 10 min
- Drain the supernatant
- Suspend cell pellet in 10 ml of glycerol 15% /1 mM MOPS solution, on ice
- Centrifuge at 7000 rpm at 4° C for 10 min
- Repeat the two last steps twice
- Drain the supernatant
- Suspend pellet in 1 ml of glycerol 15% /1 mM MOPS solution, on ice
- Aliquot 50 µl per tube
- Store at -80° C or use directly for electroporation

2) Ligation

- Calculate the volume of PCR product needed for the reaction as follow:
  (50 ng vector x insert size (bp)/ vector size (bp)) x 6 = ng of PCR product to be use in 3 µl final volume
  In the case of anammox PCR products (480 bp) = ((50X480)/ 3)) X 6= 48 ng in 3 µl
- Mix the different following products in 500 µl tubes
  a. Ligase 2x buffer 5 µl
  b. p-GEM-T vector (50 ng) 1 µl
  c. PCR product 3 µl
  d. Add H_2O if necessary
  e. T4 DNA ligase (1U/1 µl) 1 µl
- Incubate for 1 hour at ambient °C and at 4°C overnight

3) Transformation

- Let defrosting on ice a tube of competent cells during 15 min
- Transfer gently 2 µl of ligation product into a tube of competent cells
- Incubate 1 min on ice
- Put the mix in a electroporation tube
- Place the tube in the electroporation machine
- Pulse using the *E. coli* 2 programm (2.5kV)
- Add 900 µl of S.O.C solution and mix very carefully
- Incubate 1 hour at 37 °C
- Add 125 µl d'IPTG and 50 µl of X-Gal on a Petri dish containing LB-agar with ampicilline
- Put the Petri dish for at least 30 min at 37°C
- After the incubation time, centrifuge transformed cells at 6000 rpm for 10 min
- Put off the supernatant and suspend the cells in 200 µl of S.O.C solution
- Put this on the pre-warmed Petri dish
- Incubate over night at 37°C
- Incubate min 4 hours at 4°C to enhance the blue stained colonies
    - → Blue colonies possess the plasmid without the insert
    - → White colonies posses the plasmid with insert

Annex A-7: Amplification of clones insert using T7-Sp6 PCR

Primers:

Sp6 5' CGA TTT AGG TGA CAC TAT AG 3'

T7 5' TAA TAC GAC TCA CTA TAG GG 3'

Mix:

mix (25 µl)	1 tube (µl)	Concentration
H_2O	15	
$MgCl_2$ (25 mM)	3	3mM
Buffer (5x)	5	1X
dNTPs (10 µM)	0.625	250 nM
Primer f (10 µM)	0.625	250 nM
Primer r (10 µM)	0.625	250 nM
GoTaq Promega (5U/ µl)	0.125	0.025U/ µl
Total :	**25**	
Template :	**Picked colony**	

Program:

Step 1	94°C for 4min30	Initial denaturation
Step 2	94°C for 30 s	Denaturation
Step 3	51°C for 30 s	Annealing
Step 4	74°C for 1 min	Annealing
Step 5	go to step 2 , 34 times	35 cycles
Step 6	74°C for 5 min	Final elongation
Step 7	10°C forever	
Step 8	End	

Annex A-8: Restriction with *MspI*

Products and solution:

- Restriction enzyme Kit Promega
- Buffer
- BSA
- PCR product
- Sterile nanopure water

Protocol:

The restriction was done using the restriction Kit from Promega according to the manufacturer instructions.

In a 500 µl tube, add the following products:

- 8 µl of PCR products
- 1.2 µl of Buffer
- 0.3 µl of *Msp*I enzyme
- 0.3 µl of BSA
- 2.2 µl of sterile nanopure water
- Let react for 3h at 37°C
- Prepare a gel of 2% agarose
- Migration of the restricted product: at 110 V for 1h30 (5 V/cm)

Annex A-9: Plasmids extraction and purification

Products and solutions:

- 1.5 and 2 ml Eppendorf tubes
- Spin column
- Catch tubes
- Cells suspension solution
- Cell lysis solution
- Alkaline protease solution
- Neutralization solution
- Wash solution
- LB with ampicilline liquid medium (4ml)

Protocol:

The plasmid extraction and purification was done with the Wizard® Plus SV minipreps DNA purification system of Promega.

1) Extraction

- Prepare a liquid culture of the clones:
  - → Inoculate a fresh clone colony in a tube containing 4 ml of LB ampicilline medium
  - → Incubate overnight under agitation (180 rpm) at 37°C
- Put 2 ml of the liquid culture in a Eppendorf of 2 ml
- Centrifuge the tube for 5 min at 13'000 rpm
- Put off the supernatant and return the tubes on an absorbent paper
- Add 250 µl of *cell suspension solution* and vortex (from this step do not vortex anymore)
- Add 250 µl of *cell lysis solution* and mix by inversion
- Incubate for maximum 5 min at room temperature
- Add 10 µl of *alkaline protease solution* and mix by inversion
- Incubate for 5 min at ambient temperature
- Add 350 µl of *neutralization solution* and mix by inversion
- Centrifuge for 15 min at 13'000 rpm

2) Purification

- Transfer the maximum of supernatant to a *spin column* previously inserted in a *catch tube*
- Centrifuge for 2 min at 13'000 rpm and discard the elution
- Add 750 µl of *wash column solution*

- Centrifuge for 1 min at 13'000 rpm and discard the elution
- Add 250 µl of *wash column solution*
- Centrifuge for 1 min at 13'000 rpm and discard the elution
- Centrifuge for 2 min at 13'000 rpm
- Transfer the spin column in a 1.5 ml Eppendorf
- Add 100 µl of sterile nanopure water
- Incubate 2 min at room temperature
- Centrifuge 2 min at 13'000 rpm and put off the spin column
- The elution contain the plasmid DNA
- Conserved at – 20°C until further use

3) Control and Quantification

- Plasmid DNA extract was checked on an agarose gel of 0.8%
- Quantification was done with the Lambda *Hind* III ladder

Annex A-10: qPCR of the anammox bacterial 16S gene

Primers:

A438f: 5' GTC(AG)GG AGT TA (AGT) GAA ATG 3'

A668r: 5' ACC AGA AGT TCC ACT CTC 3'

Mix:

Mix (10 µl)	1 tube (µl)	Concentration
H_2O Rnase free	5	
Primer f (10 µM)	1.2	1200nM
Primer r (10µM)	0.3	300nM
Master mix (2X)	2.5	0.5X
Total :	**9**	
Template (µl)	**1**	

Program:

Step 1	95°C for 15 min	Hot start enzyme activation
Step 2	95°C for 30 s	Denaturation
Step 3	55.5°C for 15 s	Annealing
Step 4	72°C for 30 s	Elongation
Step 5	go to step 2 , 39 times	40 cycles

Annex A-11: Bacteria extraction and sample preparation from soil samples for FISH analysis

Extraction solution:

The solution contains:

- 7.598 g of NaCl, final concentration 130 mM
- 1.26 g of Na_2HPO_4 x $12H_2O$, final concentration 7 mM
- 0.414 g of NaH_2PO_4 x $2H_2O$, final concentration 3 mM
- 0.5% of Tween 20
- 1 g of sodium pyrophosphate, final concentration 1g/l
- add 1000 ml H_2O deionized
- pH of 7.4

Sample preparation and cell extraction

- 1 g of soil sample placed in a sterile 50ml tube
- add 10 ml of extraction solution
- Use the Ultra-Turrax for 2 min at 17'500 u/min
- Transfer 1 ml of supernatant in a 2 ml centrifuge tube
- Add 1 ml of Histodenz (density=1.3g/ml)
- Centrifuge for 1h30 at 13'000 rpm at 4°C
- Put off the supernatant
- Take the cell layer

Cell fixation

- Add x ml of formaldehyde 36% solution to obtained a final concentration of 2%
- Incubate for 1 hour
- Centrifuge for 5 min at 10'000 rpm
- Put off the supernatant
- Suspend in 1 ml of PBS 1X
- Centrifuge for 5 min at 10'000 rpm
- Repeat last two steps
- Put off the supernatant
- Add 1.5 ml of PBS:EtOH 1:1
- Conserved at -20°C until analysis

Filtration

- Vortex vigorously the cell extraction solution before filtartion
- Place support filter (0.45 μm pore size, cellulose nitrate, 47mm diameter) and a membrane filter (0.2 μm pore size, polycarbonate, 47mm diameter) into a filtration tower
- Rinse the filtration tower with sterile H_2O
- Add 100 μl of sample to 2 ml of sterile H_2O
- Filter the sample by applying a gentle vacuum
- After complete sample filtration wash with 10 ml of sterile H_2O
- Put the membrane filter in a Petri dish, cover and allow air-drying
- Store at -20°C until further analysis

Annex A-12: FISH

With a razor blade cut different pieces of the filter and proceed to the hybridization step.

Hybridization:

- For each Buffer concentration prepare 2 ml of each hybridization buffer:
  - 360 µl of NaCl 5M
  - 40 µl of TRIS/HCl 1M
  - Formamide: 300 µl 600 µl 700 µl 800 µl
  - H_2O milliQ: 1300 µl 1000 µl 900 µl 800 µl
  - Conc. Formamide: **15** % **30** % **35** % **40** %
  - 2 µl of SDS 10 %
- Put the filter piece on a parafilm piece previously put on a glass strip
- In the dark, mix 20 ul of hybridization buffer and 2 ul of FISH probes
- Put the mix delicately on the piece of filter
- -Place the glass strip in a 50 ml falcon tube with a Kleenex wetted with the rest of the hybridization buffer
- Incubate for 2h30 at 46°C

Washing step:

- The NaCl concentration in the washing buffer depends of the hybridization buffer concentration.
  - 3180 µl of NaCl for 15 % formamide concentration
  - 1020 µl of NaCl for 30 % formamide concentration
  - 700 µl of NaCl for 35 % formamide concentration
  - 460 µl of NaCl for 40 % formamide concentration
- Prepare 50 ml of washing buffer in a falcon tube
  - x ml of NaCl 5 M
  - 1 ml of TRIS/HCl
  - 500 µl of EDTA 0,5 M
  - Add to 50 ml with MilliQ water
  - 50 µl of SDS 10 %
- With the buffer at 15 % formamide no EDTA is necessary
- Previously put the washing buffer in a water bath at temperature of 48°C
- Put the filter in the washing buffer for 15 min
- Rinse them twice in a deionized water bath

- Let air-dried for 5 min
- Put 20 ul of DAPI on the filter for 5 min
- Rinse in water followed by a EtOH 80% bath
- Let air-drying
- Put Citifluor on the air-dried filter
- Observed with the epi-fluorescent microscope
- The filters could be conserved in the dark at -20° for few days

Annex A-13: Medium for enrichment culture and microcosms

Medium:

Add the following substrates to a 10 liters bottle:

- 9.7 liters of nanopure water
- 1.32 g of $(NH_4)_2SO_4$
- 2 g of $KH2PO_4$
- 4 g $MgCl_2 \cdot 6H_2O$
- 1.5 g $CaCl_2 \cdot 2H_2O$
- 5 g KCl
- 10 g NaCl
- Autoclave at 121°C for 1h
- Let cool for a moment
- Flush 45 min with gas N_2/CO_2
- Add 7.5 ml of anoxic $NaNO_2$ (2 M) solution (previously flushed with N_2)
- Add 300 ml of $NaHCO_3$ (1 M) and 1 ml of element trace solution
- Set the peristaltic pump to obtain a flow of 13 ml/h
- Open the in-flow medium exit and start the pump

Annex A-14: Soil sampling and preparation for physico-chemical characterization

1) Soil sampling for nitrogen (ammonium, nitrite, and nitrate) compounds analysis

- Put 30 ml of KCl 2M in 50 ml 'Falcon' tubes
- Weighted them exactly
- On site, place 10-15 g of fresh soil in the tube
- Put the tube directly into the dark on ice
- Agitate for 1 hour on a rotary-shaker
- Weight the tube containing the soil sample and KCl to obtain exactly the fresh soil weight
- Centrifuge for 10 min at 4'000 rpm
- Filter the supernatant with a glass fiber filter (GF/F or GF/A)
- Conserve the soil extract at -20°C until further analysis

2) Soil sampling for iron (FeII, FeIII) determination

- Put 10 ml of HCl 5M in 50 ml 'Falcon' tubes
- Weight them exactly
- Put about 1 g of fresh soil in the tube
- Put the tube immediately into the dark
- Agitate
- Weight the tube containing the soil sample and HCl to obtain exactly the fresh soil weight
- Centrifuge for 10 min at 4'000 rpm
- Filter the supernatant with a glass fiber filter (GF/F or GF/A)
- Conserve the soil extract at -20°C until further analysis

Annex A-15: Ammonium quantification by the indophenol blue assay

Based on the reaction of ammonium, hypochlorite and phenol to form indophenol blue.
(Nach Chaney, A.L.;Marbach, E.P.; 1962. Modified reagents for the determination of urea and ammonia.Clin.chem.8:130-132)

Reagents:

Reagent **A**:
- 1.5 g Phenol
- 1.5 mg Sodium-Nitroprussid
- add to 50 ml with nanopure water

Reagent **B**:
- 1 g NaOH
- 40 ml H_2O nanopure
- cool
- add 2 ml Sodium hypochlorite (12%)
- add to 50 ml with nanopure water

- Standard NH_4^+ solution (10 mmol):
  - NH_4Cl is dried 1 h at 100°C
  - 0.535 g NH_4Cl
  - 1 liter H_2O nanopure

Protocol:

- 1 ml sample
- Add 0.1 ml reagent **A**
- Add 0.1 ml reagent **B**
- Incubate 1 hour in dark (color stable for 24h)
- Centrifuge at 13'000 rpm for 5 min
- Take 1 ml of the supernatant
- Measure at 635 nm

Annex A-16: Nitrite quantification by the sulfanilamide assay

Based on reaction of nitrite with sulfanilamide in an acid solution to form a diazo compound. (H.P. Hansen and F. Koroleff: *Determination of nutrients*. In : M. Grasshoff, M. Gerhardt and K. Kremling (eds). Methods of seawater analyses, 3rd Ed. Wiley VCH, Weinheim; pp 159-228.)

Reagents:

- Sulphanilamide:
  - 600 ml H_2O nanopure (volumetric flask)
  - 100 ml HCL concentrated
  - 10 g crystalline sulphanilamide
  - After cooling, add to 1 liter with H_2O nanopure

Store in dark at < 8°C. Stable for 1 month.

- NED:
  - 0.5 g NED (1-(N-naphtyl)ethylenediamine dihydrochloride)
  - 500 ml H_2O nanopure (volumetric flask)

Store in brown bottle at < 8°C. Stable for 1 month.

- Standard (10 mM):
  - 0.6900 g of NaNO2
  - dried at 100°C for 1 hour
  - 1 litre H_2O nanopure

Store in dark at < 8°C. Stable for 3 months.

Protocol:

- 0.5 ml of sample (with or without filtration) in a tube
- Add 0.25 ml of Sulphanilamide, mix 1 min
- Add 0.25 ml of NED, mix and wait for the reaction 15 min
- Measure the absorbance at 540 nm

Annex A-17: Nitrate quantification by a modified cadmium reduction assay

Reagents:

- Boric Buffer pH=10.5:
  - 7.456 g of KCl
  - 6.184 g of H_3BO_3
  - 4 g of NaOH
  - Add to 1000 ml with nanopure water
  - Adjust the pH at 10.5 with HCl 5M
  - Conserve the buffer in the fridge

- Sulphanilamide:
  - Add among 600 ml of nanopure water
  - Add 100 ml of concentrated HCl
  - 10 g de sulphanilamide
  - Cool and complete to 1000 ml with nanopure water

Store in brown bottle at < 8°C. Stable for 1 month.

- NED:
  - 0.5 g NED (1-(N-naphtyl)ethylenediamine dihydrochloride)
  - 500 ml H2O nanopure (volumetric flask)

Store in brown bottle at < 8°C. Stable for 1 month.

- Standard (10 mM):
  - 1.0110 g of KNO_3, dried for 1 hour at 100°C
  - Add to a final volum of 1000 ml with nanopure water

Store in brown bottle at < 8°C. Stable for 3 months.

Protocol:

- Analysis should be done in triplicates
- Put 1 ml of sample in a 2 ml Eppendorf tube
- Add 100 µl of boric buffer
- Add 1/3 of Nitraver 6 (« for 5 ml sample and for low nitrate concentration »; n° article 1412099 Hach Lange, CH)
- Incubate under agitation for 1 hour at 175 rpm
- Take 0.5 ml of the sample
- Add 0.25 ml of Sulphanilamide, mix 1 min
- Add 0.25 ml of NED, mix and wait for the reaction 15 min

- Measure the absorbance at 540 nm

Calculations:

Determine the nitrate concentration by subtracting the nitrite concentration previously measured in the same extract sample.

Annex A-18: Iron quantification by the Ferrozine assay

Reagents:

Ferrozine solution 100 mM of HEPES:	- Wash a volumetric flask of 1 liter with HCl 6M
	- Rinse with nanopure water
	- Add 500 ml of nanopure water
	- Add 23.83 g of HEPES (100 mM)
	- Add 1 g of Ferrozine
	- Adjust the pH at 7 with NaOH
HCl solution 0.5 mM:	- Wash a volumetric flask of 1 liter with HCl 6M
	- Rinse with nanopure water
	- Add 500 ml of nanopure water
	- Add 41.55 ml of HCl 37%
	- Adjust to 1000 ml with nanopure water
Hydroxylamine solution 1.5 M:	- Wash a volumetric flask of 250 ml with HCl 6M
	- Rinse with nanopure water
	- Add a part of HCl 0.25 M
	- Add 26.1 g of hydroxylamine
	- Adjust to 250 ml of HCl 0.25 M
Fe^{2+} stock solution of 1 mM:	- Wash a volumetric flask of 1 liter with HCl 6M
	- Rinse with nanopure water
	- Add 500 ml of nanopure water
	- Add 41.55 ml of HCl 37%
	- Add 392.14 mg of Iron sulfate and ammonium ($FeH_8N_2S_2O_8.6H_2O$)
	- Dissolved and complete to 1 liter
Fe^{3+} stock solution of 1 mM:	- Wash a volumetric flask of 1 litre with HCl 6M
	- Rinse with nanopure water
	- Add 500 ml of nanopure water
	- Add 41.55 ml of HCl 37%
	- Add 526.80 mg of Iron sulfate and ammonium ($FeH_4N_4S_2O_8.12H_2O$)
	- Dissolved and complete to 1 liter

Protocol:

1)Iron (II) determination:

- Take 100 µl of sample
- Add 900 µl of Ferrozine solution
- Immediately measure the absorbance at 562 nm

2)Iron (III) determination:

- Take 100 µl of sample
- Add 150 µl of hydroxylamine 1.5 M
- Add 900 µl of Ferrozine solution
- Measure the absorbance at 562 nm

Annex A-19: Measurements of pH H_2O and pH KCl

Material and reagents

- Glass recipient of 100 ml
- Glass stick
- pH measurer with electrodes
- Deionized water
- KCl 1M: dissolve 74.55 g of KCl in 1 liter of deionized water
- Buffer solutions of pH=4 and pH=7

Protocol:

- Dry soil sample and sieve at 2 mm
- Put 20 g of dried and sieved soil in the glass recipient
- Add 50 ml of deionized water (proportion of 1:2.5 between sample and solution)
- Homogenize the soil-water solution with the glass stick
- Incubate for 1h
- Calibrate the pH-meter
- Measure the pH of samples
- Between each measurement rinse the electrode

- For the measurement of pH[KCl], do the same but with KCl 1M solution

Annex A-20: Water content and residual humidity

Material

- Glass or recipient of glass for weighting
- Analytical balance (0.0001 g)
- Oven set up at 105 °C
- Dessicator

Protocol:

1) Water content:

- Weight about 20 g of fresh soil which correspond to **fw** for fresh weight
- Put the recipient for 24 hours at 105°C
- Remove the recipient from the oven and put it for cooling in the dessicator
- Weight the dried soil sample which correspond to w105
- Calculate the water content as followed:

$$\text{Water content (\%)} = \frac{\text{fw - w105}}{\text{fw - tare}} \text{x100}$$

- Conserve the dried soil sample for further RockEval analysis

2) Residual Humidity:

- Repeat the same protocol as for water content but use air-dried soil sample in place of fresh soil

Annex A-21: Determination of total carbon and total nitrogen

Material:

- Recipient
- Sieve 2mm
- Agate crusher
- Small plastic bags
- Analytical balance
- Small aluminum recipient

Sample:

- Among 10 g of each soil sample

Protocol:

- Soil samples are air dried for few days
- Sieve the air dried soil samples with a 2 mm sieve
- Crush the sample with an agate crusher
- Weight 2 mg of each soil sample
- Put in the small aluminum recipient

Analysis:

- The samples are combusted on an element analyzer (EA 1108 CHNS-O Element Analyzer)
- N tot and C tot are measured after combustion on the element analyzer

Annex A-22: Soil sampling and samples preparation for ^{15}N-incubation experiments

Soil sampling:

- 3 soil profiles were collected
- For each profile, 3 different soil samples were selected based on the cm of depth: i) 0-10 cm, ii) 20-30cm, iii) 50-60 cm
- 100 g per depth were collected from each soil profile
- 3x100 g soil samples (representing the same depth from each profile) were put in plastic bags
- Soil samples were conserved at 4°C before experiment

Samples preparation:

- Homogenized 300 g of fresh soil sample
- For one soil sample prepare 9 bottles as follows:
- Put 20 g of soil sample in 100 ml glass bottle
- Add 40 ml of anoxic sterile water previously flushed with N_2
- Remove the gas in the bottle by applying a vacuum until water boils
- Replace the atmospheric gas with N_2
- Repeat the last two step 3 times
- Preincubate the bottle for 24h in the dark at 16°C
- Shake bottle occasionally

Annex A-23: ^{15}N-incubation assays for anammox and denitrification activities determination

For one soil sample, the ^{15}N-isotopic incubations experiment consisted of 3 treatments realized in triplicate: 1) control treatment with addition of $^{15}NH_4^+$, 2) anammox treatment with addition of $^{15}NH_4^+$ and $^{14}NO_3^-$, 3) denitrification treatment with addition of $^{15}NO_3^-$.

Stock solutions:

- Calculate the concentration of the stock solution in order to obtain a final concentration in the microcosm of 100 µM of each treatment.

4mM of $^{15}NH_4Cl$: dissolve 2.12 mg of $^{15}NH_4Cl$ in 10 ml of deionized water

4mM of $K^{14}NO_3^-$: dissolve 3.94 mg of $K^{14}NO_3^-$ in 10 ml of deionized water

4mM of $K^{15}NO3^-$: dissolve 3.98 mg of $K^{15}NO_3^-$ in 10 ml of deionized water

Liquid sampling before the isotope experiment:

- Sample 2 ml of liquid from each bottle as explained in the annex A-24.

Protocol:

- Each solution was flushed with N_2
- Add 1 ml of stock solution in the respective bottles (3 treatments x 3 replicates)
- Shake vigorously each bottle
- Incubate the bottle in the dark at 16°C for 12 hours
- Every 3 hours, liquid and gas phases were sampled as explained in the annex A-24

Annex A-24: Liquid and gas sampling during ^{15}N-incubation experiments

Liquid sampling:

Samples of the liquid phase were collected using a syringe previously flushed with N_2 and always collected before the gas sampling.

- Sample 4 ml of liquid within the bottle with a syringe
- Transfer the liquid in 2 x 2ml centrifuge tubes
- Freeze immediately at -20°C until further analysis
- Concentrations of nitrite, nitrate, and ammonium were done on thawed and centrifuged samples as explained in annexes A-15-16-17.

Gas sampling:

Before gas sampling, bottle were shaken to equilibrate the gas between the headspace and water phase. Gas samples were collected using a syringe previously flushed with N_2.

- For each sample, prepare a 5 ml vial containing argon flushed water
- Collect 4 ml of the gas phase
- Put a needle in the septum of the vial cap
- Inject the gas in the vial
- The water in the vial is displaced by the entering gas through the second needle
- Store the vials upside down at room temperature until further GC-ir-MS analysis

Annexes B: Data

1. $^{29}N_2$, $^{30}N_2$, ammonium, nitrate, and nitrite measurements in $^{15}NH_4^+$ treatment from spring Reductisol samples
2. $^{29}N_2$, $^{30}N_2$, ammonium, nitrate, and nitrite measurements in $^{15}NH_4^+$+$^{14}NO_3^-$ treatment from spring Reductisol samples
3. $^{29}N_2$, $^{30}N_2$, ammonium, nitrate, and nitrite measurements in $^{15}NO_3^-$ treatment from spring Reductisol samples
4. $^{29}N_2$, $^{30}N_2$, ammonium, nitrate, and nitrite measurements in $^{15}NH_4^+$ treatment from summer Reductisol samples
5. $^{29}N_2$, $^{30}N_2$, ammonium, nitrate, and nitrite measurements in $^{15}NH_4^+$+$^{14}NO_3^-$ treatment from summer Reductisol samples
6. $^{29}N_2$, $^{30}N_2$, ammonium, nitrate, and nitrite measurements in $^{15}NO_3^-$ treatment from summer Reductisol samples
7. $^{29}N_2$, $^{30}N_2$, ammonium, nitrate, and nitrite measurements in $^{15}NH_4^+$ treatment from autumn Reductisol samples
8. $^{29}N_2$, $^{30}N_2$, ammonium, nitrate, and nitrite measurements in $^{15}NH_4^+$+$^{14}NO_3^-$ treatment from autumn Reductisol samples
9. $^{29}N_2$, $^{30}N_2$, ammonium, nitrate, and nitrite measurements in $^{15}NO_3^-$ treatment from autumn Reductisol samples

Annex B-1: $^{29}N_2$, $^{30}N_2$, ammonium, nitrate, and nitrite measurements in $^{15}NH_4^+$ treatment from spring Reductisol samples.

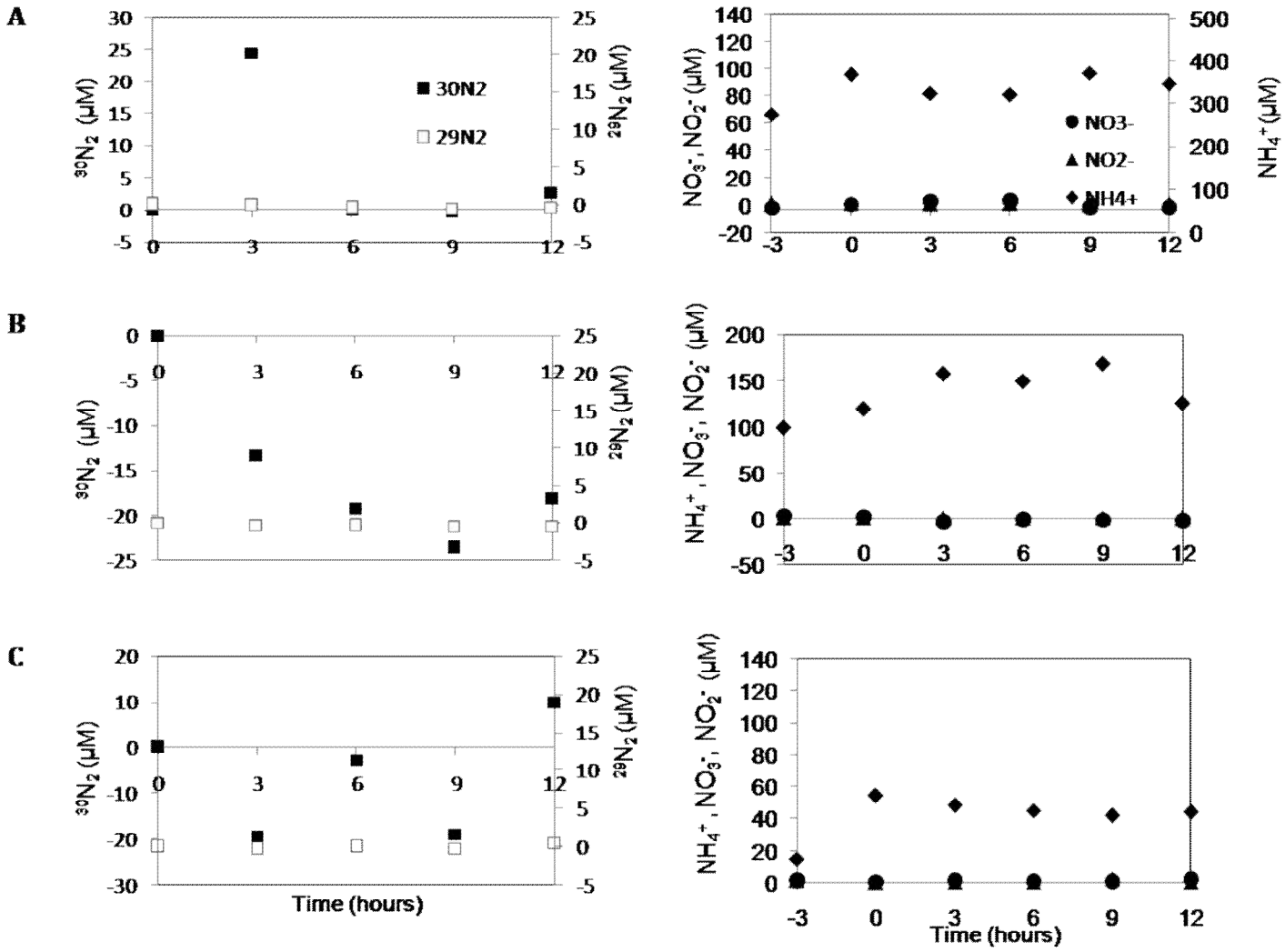

Figure 1: Anammox and denitrification activities in soil samples from a Reductisol profile with addition of $^{15}NH_4^+$ treatment. Panels left: production of $^{29}N_2$ (empty squares) and $^{30}N_2$ (bold squares) at A) 0-10cm, B) 20-30cm and C) 50-60cm of depth; right, amount of ammonium (lozenges), nitrate (circles), and nitrite (triangles) at A) 0-10cm, B) 20-30cm and C) 50-60cm of depth.

Annex B-2: $^{29}N_2$, $^{30}N_2$, ammonium, nitrate, and nitrite measurements in $^{15}NH_4^+ + ^{14}NO_3^-$ treatment from spring Reductisol samples.

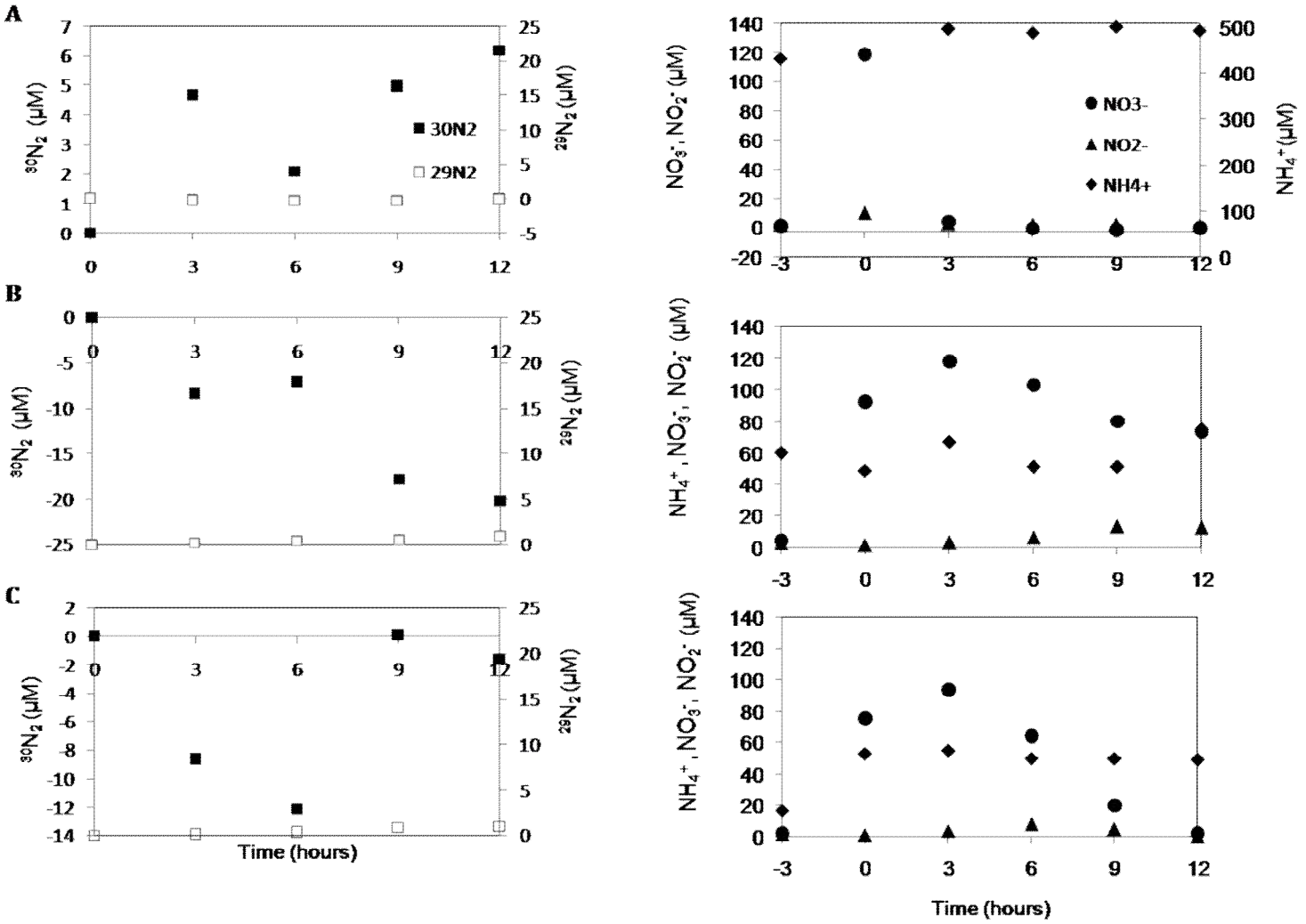

Figure 2: Anammox and denitrification activities in soil samples from a Reductisol profile with addition of $^{15}NH_4^+ + ^{14}NO_3^-$ treatment. Panels left: production of $^{29}N_2$ (empty squares) and $^{30}N_2$ (bold squares) at A) 0-10cm, B) 20-30cm and C) 50-60cm of depth; right, amount of ammonium (lozenges), nitrate (circles), and nitrite (triangles) at A) 0-10cm, B) 20-30cm and C) 50-60cm of depth.

Annex B-3: $^{29}N_2$, $^{30}N_2$, ammonium, nitrate, and nitrite measurements in $^{15}NO_3^-$ treatment from spring Reductisol samples.

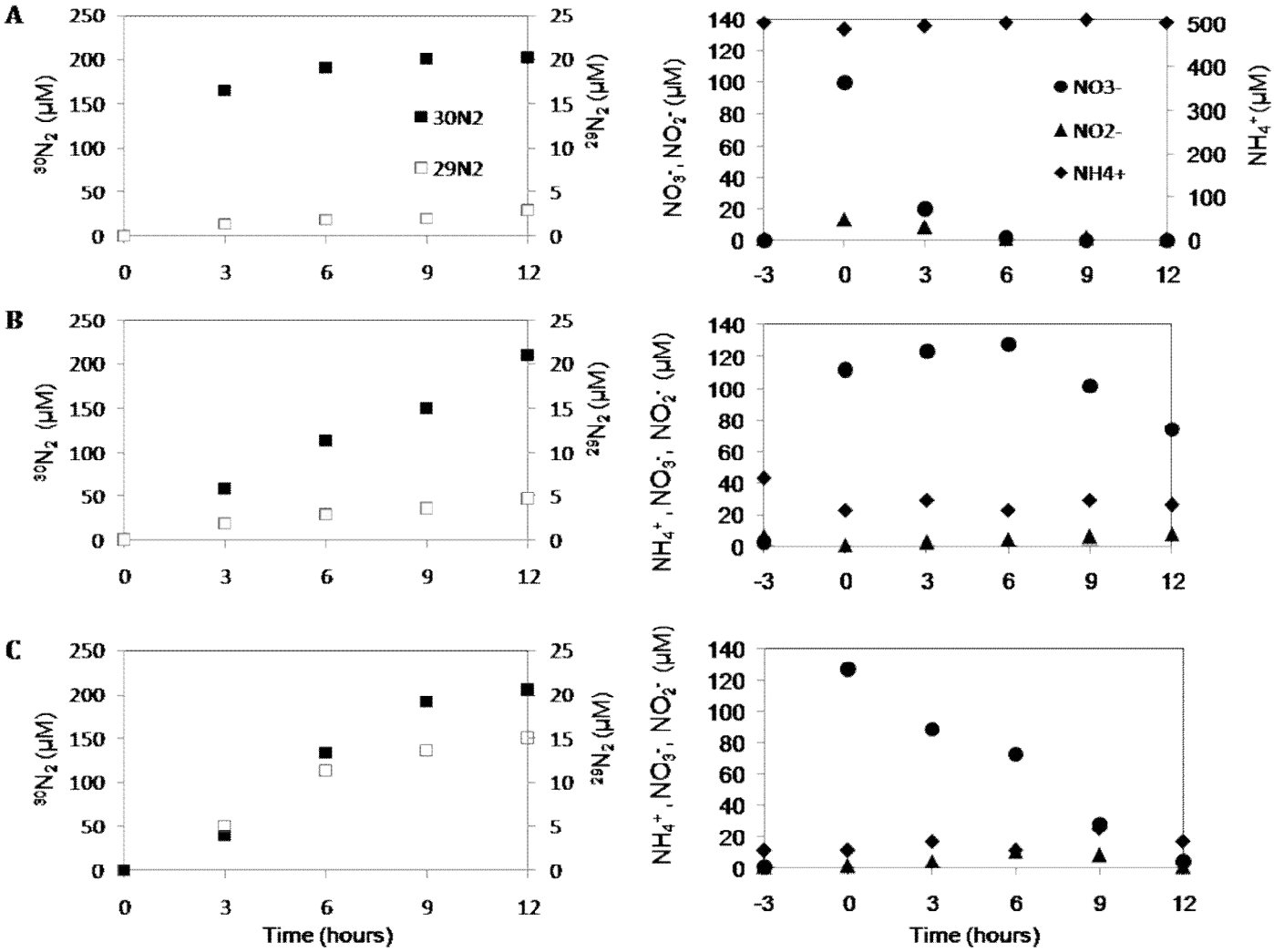

Figure 3: Anammox and denitrification activities in soil samples from a Reductisol profile with addition of $^{15}NO_3^-$ treatment. Panels left: production of $^{29}N_2$ (empty squares) and $^{30}N_2$ (bold squares) at A) 0-10cm, B) 20-30cm and C) 50-60cm of depth; right, amount of ammonium (lozenges), nitrate (circles), and nitrite (triangles) at A) 0-10cm, B) 20-30cm and C) 50-60cm of depth.

Annex B-4: $^{29}N_2$, $^{30}N_2$, ammonium, nitrate, and nitrite measurements in $^{15}NH_4^+$ treatment from summer Reductisol samples.

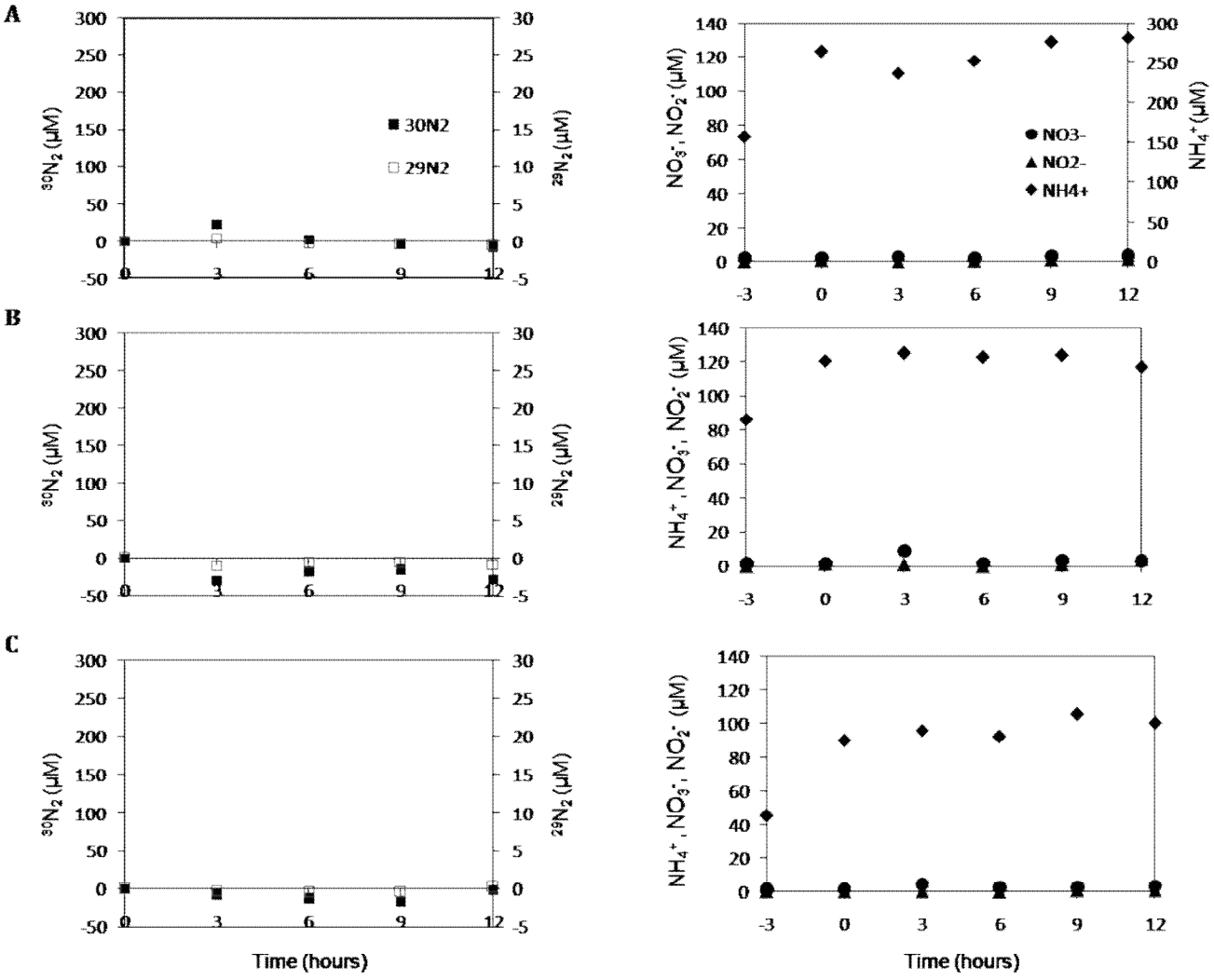

Figure 4: Anammox and denitrification activities in soil samples from a Reductisol profile with addition of $^{15}NH_4^+$ treatment. Panels left: production of $^{29}N_2$ (empty squares) and $^{30}N_2$ (bold squares) at A) 0-10cm, B) 20-30cm and C) 50-60cm of depth; right, amount of ammonium (lozenges), nitrate (circles), and nitrite (triangles) at A) 0-10cm, B) 20-30cm and C) 50-60cm of depth.

Annex B-5: $^{29}N_2$, $^{30}N_2$, ammonium, nitrate, and nitrite measurements in $^{15}NH_4^+ + ^{14}NO_3^-$ treatment from summer Reductisol samples.

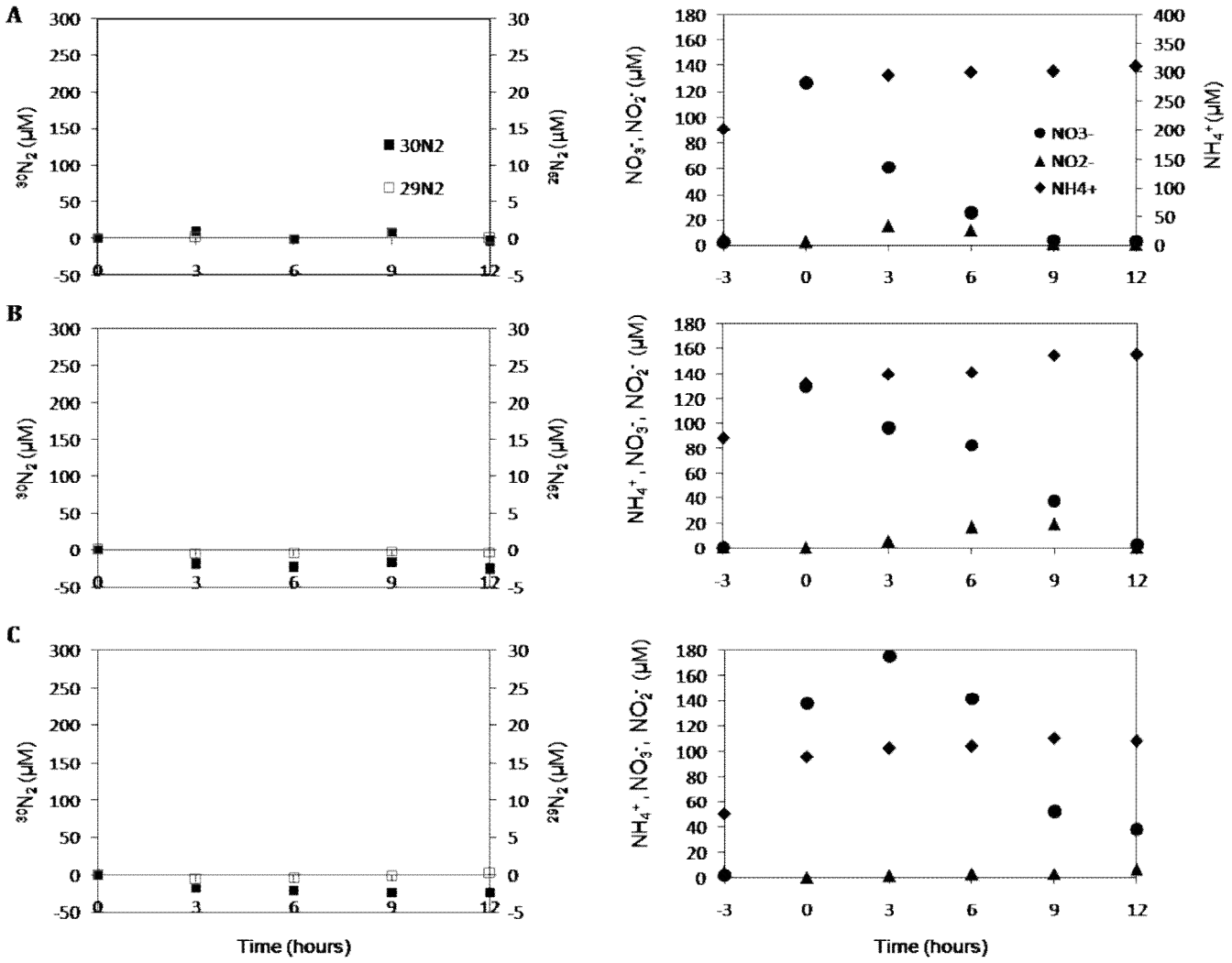

Figure 5: Anammox and denitrification activities in soil samples from a Reductisol profile with addition of $^{15}NH_4^+ + ^{14}NO_3^-$ treatment. Panels left: production of $^{29}N_2$ (empty squares) and $^{30}N_2$ (bold squares) at A) 0-10cm, B) 20-30cm and C) 50-60cm of depth; right, amount of ammonium (lozenges), nitrate (circles), and nitrite (triangles) at A) 0-10cm, B) 20-30cm and C) 50-60cm of depth.

Annex B-6: $^{29}N_2$, $^{30}N_2$, ammonium, nitrate, and nitrite measurements in $^{15}NO_3^-$ treatment from summer Reductisol samples.

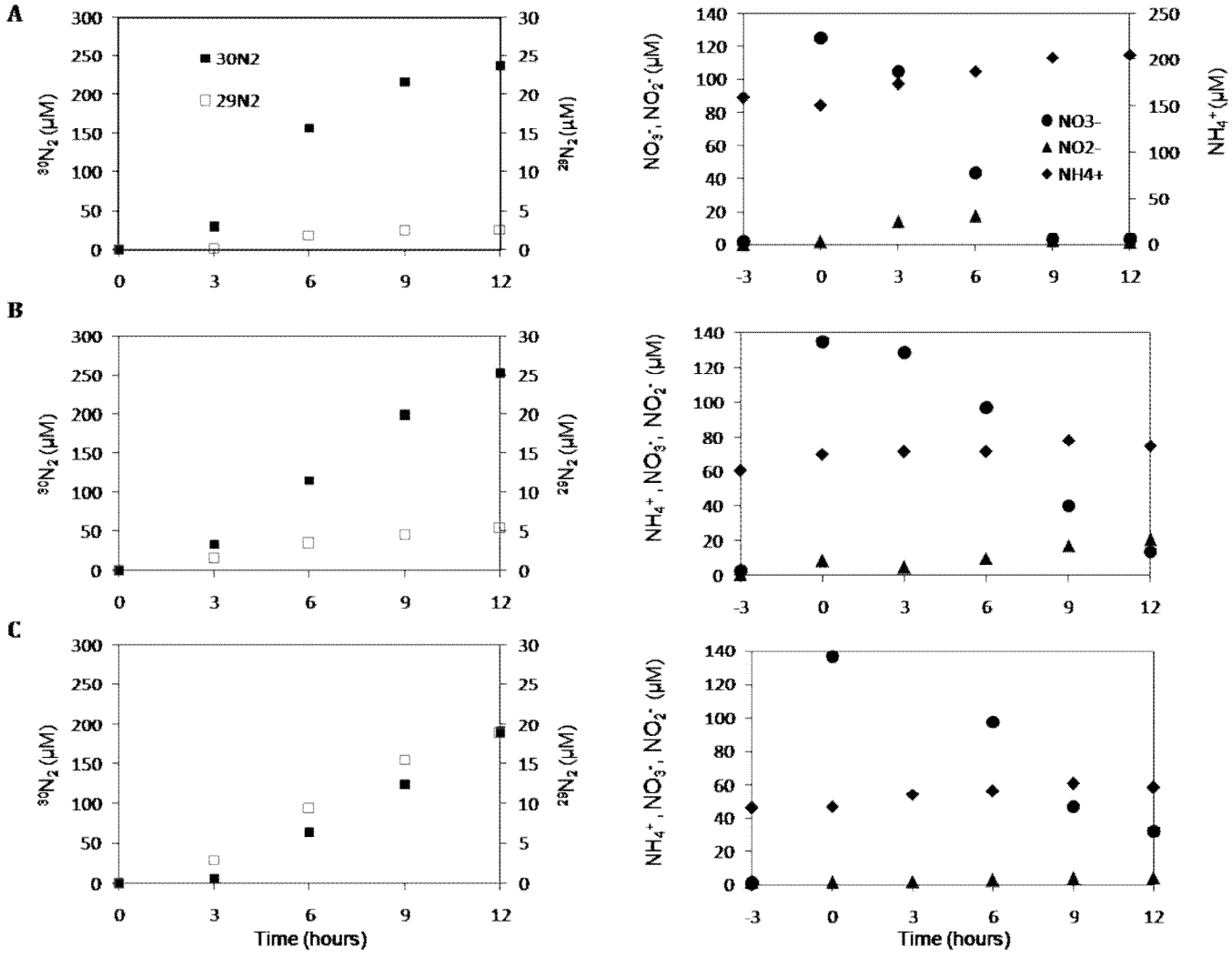

Figure 6: Anammox and denitrification activities in soil samples from a Reductisol profile with addition of $^{15}NO_3^-$ treatment. Panels left: production of $^{29}N_2$ (empty squares) and $^{30}N_2$ (bold squares) at A) 0-10cm, B) 20-30cm and C) 50-60cm of depth; right, amount of ammonium (lozenges), nitrate (circles), and nitrite (triangles) at A) 0-10cm, B) 20-30cm and C) 50-60cm of depth.

Annex B-7: $^{29}N_2$, $^{30}N_2$, ammonium, nitrate, and nitrite measurements in $^{15}NH_4^+$ treatment from autumn Reductisol samples.

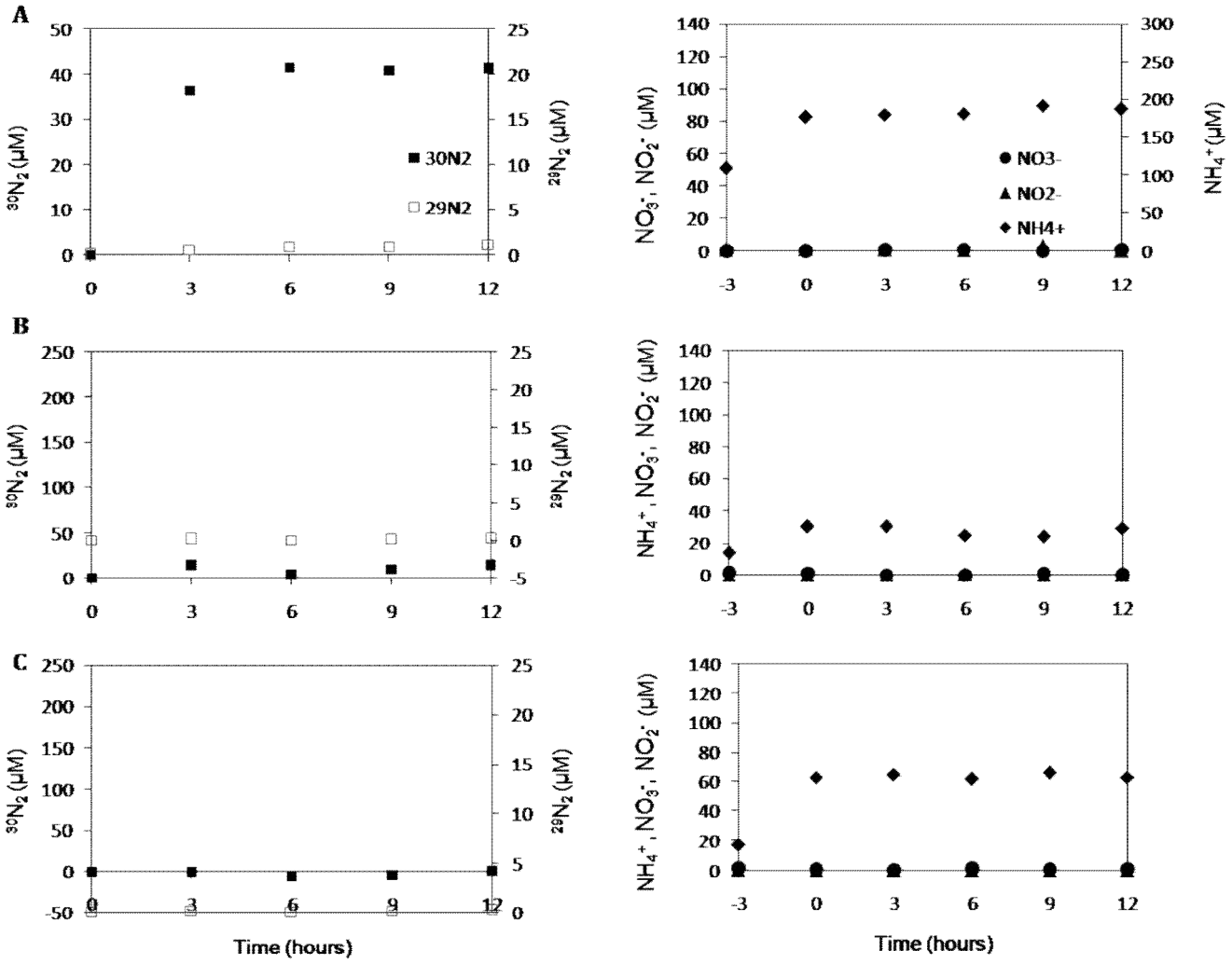

Figure 7: Anammox and denitrification activities in soil samples from a Reductisol profile with addition of $^{15}NH_4^+$ treatment. Panels left: production of $^{29}N_2$ (empty squares) and $^{30}N_2$ (bold squares) at A) 0-10cm, B) 20-30cm and C) 50-60cm of depth; right, amount of ammonium (lozenges), nitrate (circles), and nitrite (triangles) at A) 0-10cm, B) 20-30cm and C) 50-60cm of depth.

Annex B-8: $^{29}N_2$, $^{30}N_2$, ammonium, nitrate, and nitrite measurements in $^{15}NH_4^+ + ^{14}NO_3^-$ treatment from autumn Reductisol samples.

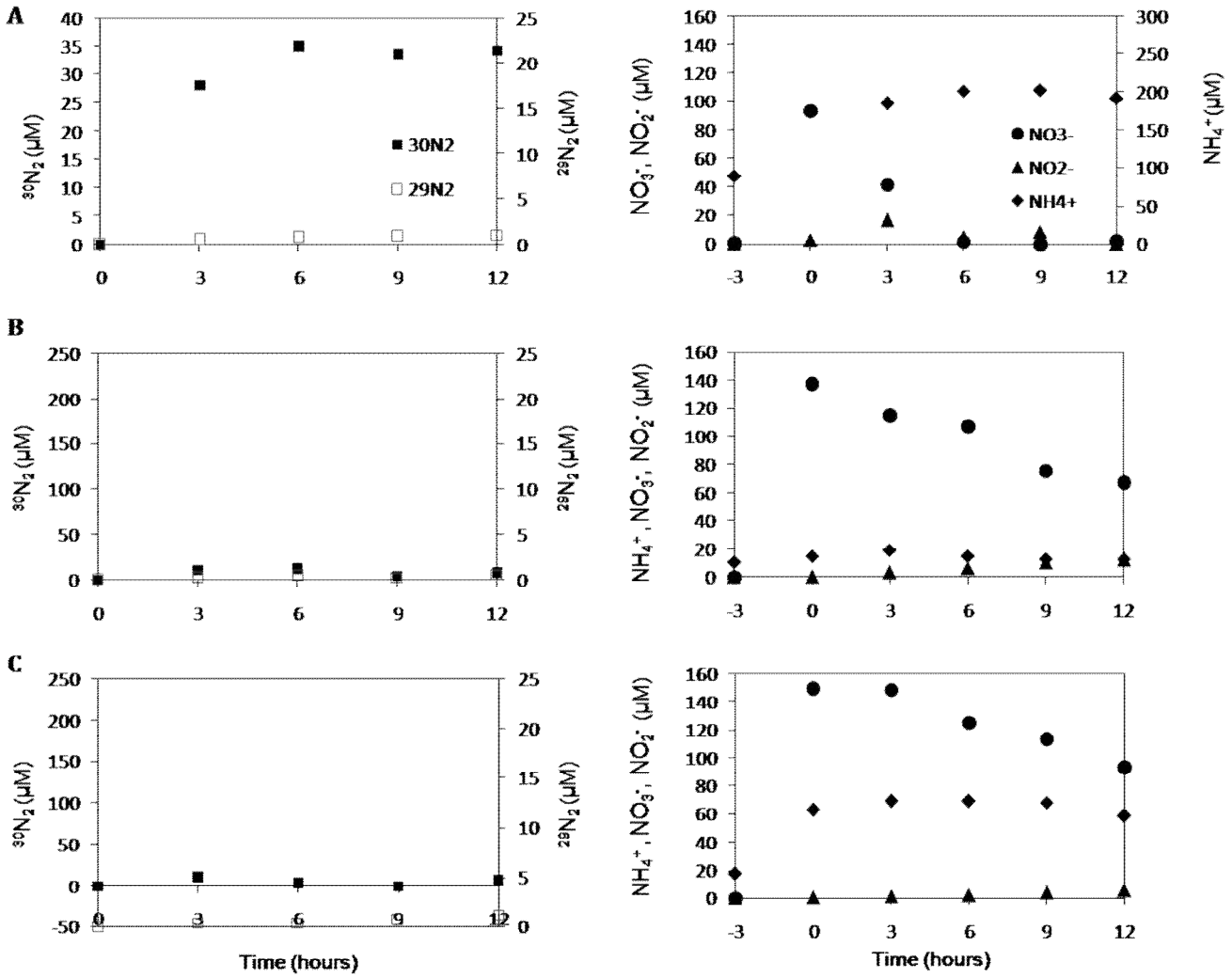

Figure 8: Anammox and denitrification activities in soil samples from a Reductisol profile with addition of $^{15}NH_4^+ + ^{14}NO_3^-$ treatment. Panels left: production of $^{29}N_2$ (empty squares) and $^{30}N_2$ (bold squares) at A) 0-10cm, B) 20-30cm and C) 50-60cm of depth; right, amount of ammonium (lozenges), nitrate (circles), and nitrite (triangles) at A) 0-10cm, B) 20-30cm and C) 50-60cm of depth.

Annex B-9: $^{29}N_2$, $^{30}N_2$, ammonium, nitrate, and nitrite measurements in $^{15}NO_3^-$ treatment from autumn Reductisol samples.

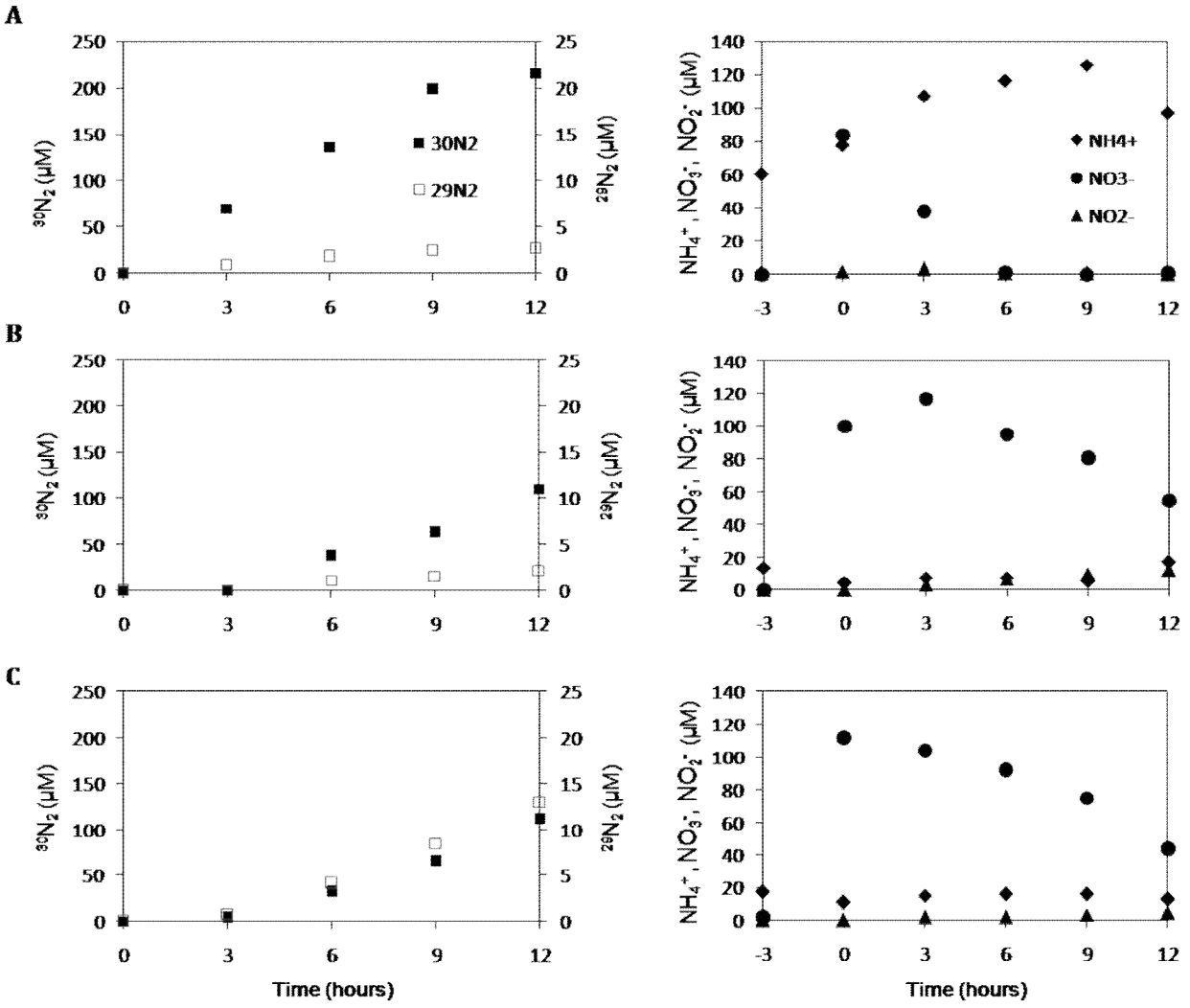

Figure 9: Anammox and denitrification activities in soil samples from a Reductisol profile with addition of $^{15}NO_3^-$ treatment. Panels left: production of $^{29}N_2$ (empty squares) and $^{30}N_2$ (bold squares) at A) 0-10cm, B) 20-30cm and C) 50-60cm of depth; right, amount of ammonium (lozenges), nitrate (circles), and nitrite (triangles) at A) 0-10cm, B) 20-30cm and C) 50-60cm of depth.