Niche differentiation between ammonia-oxidizing bacteria in aquatic environments

(with a summary in English)

Nichedifferentiatie van ammoniumoxiderende bacteriën
in aquatische ecosystemen

Differenziazione di nicchia fra batteri ammonio-ossidanti
in ambienti acquatici

(con una sintesi in Italiano)

Proefschrift

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Preface

Niche differentiation between ammonia-oxidizing bacteria of the beta–subclass of the Proteobacteria in aquatic environments, from freshwater to marine, has been the object of several studies in the past. However, only few of them focussed on stratified deep lakes and estuarine habitats and even less on shallow freshwater lakes. In addition, the role of the epiphyton of submerged macrophytes, colonizing shallow freshwater lakes, as a niche for ammonia-oxidizing bacteria in freshwater habitats is still unrecognized. Furthermore, studies on selection, adaptation or niche differentiation of ammonia-oxidizing communities in freshwater environments subjected to environmental perturbations, whether natural or anthropogenic, are still scarce.

The present thesis aimed to contribute in the first place to the identification of the ammonia-oxidizing communities in the epiphyton of submerged macrophytes and to their recognition as essential components of the nitrification process in shallow freshwater lakes. In the second place, it aimed to increase the knowledge on the effect of salinity as driving factor for the niche differentiation and ecological succession of ammonia-oxidizing communities from freshwater to saline habitats. Evidence of the response of freshwater ammonia-oxidizing communities to anthropogenic perturbations was also found and therefore discussed.

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Chapter I

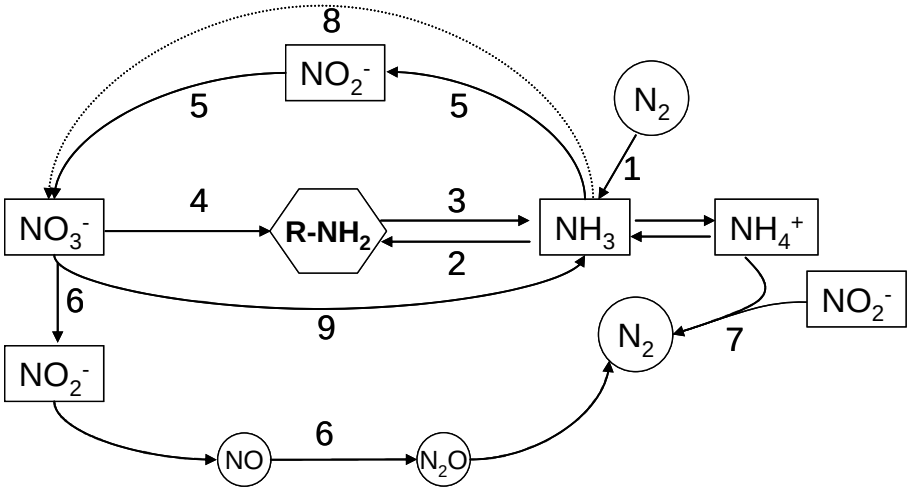
General introduction

The nitrogen cycle

Nitrogen is an essential component of all living organisms. A typical bacterial cell for example contains about 12-15% nitrogen by dry weight [1], as components of proteins, amino-sugars, nucleic acids and several other constituents of the cell. In nature, the nitrogen occurs mainly in the lithosphere and as inert gas in the atmosphere. Only a small part is found in the biosphere either in reduced forms as ammonium-ammonia and amine groups or in many oxidized forms from nitric oxide to nitrates, covering a wide range of oxidation states. Reduction-oxidation reactions between the different stages of oxidation offer the potential of energy-generation by microorganisms. A schematic representation of the biogeochemical cycle of nitrogen is given in **Figure 1**. The main redox reactions involving microorganisms are indicated.

The gaseous nitrogen is fixed from the atmosphere into reduced nitrogen compounds by free-living and symbiotic microorganisms, and then assimilated into organic forms. In oxic environments, ammonia is converted into nitrite and nitrate during the nitrification process, for which mainly two different groups of microorganisms are responsible, the ammonia-oxidizing and the nitrite-oxidizing bacteria. The nitrate is further assimilated into organic material or reduced to nitrogen oxides by denitrifying bacteria or completely into ammonia by the process of nitrate ammonification, mainly operated by fermentative microorganisms. In anoxic environments, ammonia and nitrite are converted into nitrogen gas by anaerobic ammonia oxidation (Anammox), of which microorganisms of the order *Planctomycetales* are responsible [2].

Figure 1. Schematic representation of the nitrogen cycle and the main processes in which microorganisms are involved. 1. Nitrogen fixation; 2. Ammonia assimilation; 3. Ammonification/mineralization; 4. Assimilatory nitrate reduction; 5. Nitrification; 6. Denitrification; 7. Anaerobic ammonia oxidation (Anammox); 8. Hypothetical complete ammonia oxidation (Comammox); 9. Nitrate ammonification.



Our understanding of the nitrogen cycle is however far from complete, for example with respect to the microorganisms that are involved; new processes and players in the cycle evolve and are just beginning to be investigated and understood [3]. Moreover, over the past hundred years, human activity has dramatically altered the global nitrogen cycle in several ways [4], for instance by increasing inputs of inorganic and organic nitrogen through severe fertilization, by releasing nitrogen oxides in the atmosphere by industrial combustion of fossil fuels, by acidifying soils, streams and lakes, etc…[5]. Consequentially, human activities are altering the tendency of the processes of the nitrogen cycle to balance each other in natural ecosystems [6]. This suggests that important knowledge on the nitrogen cycle is still missing and, even more, that classical knowledge may need a reassessment, finally leading to a better understanding of the overall nitrogen cycle. In this perspective, studies on the

microorganisms involved in the nitrogen cycle appeared to be noteworthy.

The nitrification process

Nitrification is defined as the conversion of the most reduced form of nitrogen (*i.e.* NH_3) to its most oxidized form (*i.e.* NO_3^-). Soon after the identification of nitrification as a biological process [7], Sergei Winogradsky succeeded in 1891 in isolating nitrifying bacteria [8]. He also confirmed the pioneer observation of Warington [9] that two different groups of bacteria cooperated in the process of nitrification, namely the ammonia- and the nitrite-oxidizing bacteria. Their isolation revealed also their aerobic nature and obligate chemolitho-autotrophic metabolism, except for the nitrite oxidizer genus *Nitrobacter* which showed also chemo-organotrophic growth [10]. The ammonia-oxidizing bacteria convert ammonia into nitrite (**equation 1**), which is subsequently oxidized into nitrate by the nitrite-oxidizing bacteria (**equation 2**):

$$NH_3 + 1.5\ O_2 \rightarrow HNO_2 + H_2O \quad (\Delta G_0' = -235\ KJ\ mol^{-1}) \qquad (\mathbf{1})$$

$$NO_2^- + 0.5\ O_2 \rightarrow NO_3^- \quad (\Delta G_0' = -54\ KJ\ mol^{-1}) \qquad (\mathbf{2})$$

The first reaction is often considered the rate-limiting step of the nitrification process, since nitrite rarely accumulates in oxic environments.

At present, bacteria able to oxidize ammonia directly into nitrate have not been described, although Costa et el. [11] have recently postulated the existence of bacteria able to carry out this hypothetical process, *i.e.* complete oxidation of ammonia (Comammox).

Nitrification is also known to be performed by several heterotrophic bacteria and fungi [12], able to convert either organic or inorganic reduced nitrogen forms into more oxidized states. However, in heterotrophic microorganisms the process is not likely to be generating energy. The impact of heterotrophic nitrification seems restricted to acid forest and heathland soils [13, 14].

In addition to aerobic nitrification, ammonium can also be oxidized in anoxic environments by anaerobic ammonia

oxidation (Anammox). The anammox process was discovered in a fluidized bed reactor [15] and initially received considerable attention in engineered systems. Bacteria responsible of anammox have not been obtained in pure culture yet and are maintained in anoxic enrichments thanks to which the unique features of these bacteria have been revealed. By applying molecular techniques, anammox bacteria have been classified as autotrophic members of the order of the *Planctomycetales* [16]. In the last few years, anammox has been documented in a wide range of aquatic environments, such as marine, coastal and estuarine sediments, anoxic basins, sea-ice and freshwater lakes [17-24]. When the experimental work described in this thesis started, anammox was assumed to be significant only under strictly anoxic or low-oxygen conditions. Aerobic ammonia-oxidizing bacteria were therefore considered to play a crucial role in the nitrification process in oxic freshwater and estuarine environments and are therefore the main subject of the microbiological analyses described in this thesis.

The nitrification process has long been thought to be restricted to the kingdom of the Bacteria. However, this paradigm has been recently overturned by the observation of archaeal nitrification genes [25] and by the isolation of the first chemolitho-autotrophic ammonia-oxidizing crenoarchaeote, *Nitrosopumilus maritimus* [26]. By cultivation-independent techniques, sequences related to non-thermophilic Creanoarcheota have been retrieved in temperate terrestrial, marine and freshwater systems. However, not all Creanoarchaea are capable of oxidizing ammonia [27]. To date, while archaeal nitrification is suggested to play a major role in especially marine environments, there is no such evidence for freshwater habitats.

Nitrification, as defined in the beginning of the paragraph, has significant relevance in terrestrial and aquatic, natural and engineered environments. For example, nitrification contributes to the loss of nitrogen from the ecosystems via nitrate runoff and by the coupled nitrification-denitrification process; it contributes to the emission of the greenhouse gas nitrous oxide and to eutrophication. On the other hand nitrification is essential in the removal of nitrogen compounds in waste water treatment plants and in wetland ecosystems. Again, considering the

nowadays concerns for detrimental environmental changes affecting also the nitrogen cycle, studying nitrification and the responsible microorganisms appeared noteworthy.

Aerobic ammonia oxidation

Ammonia-oxidizing bacteria are chemolitho-autotrophic bacteria that use the oxidation of ammonia to nitrite as energy source and carbon dioxide as the major carbon source, fixed via the Calvin-Benson cycle [28]. The reactions involved in the oxidation of ammonia are described in the following equations:

$NH_3 + O_2 + 2H^+ + 2e^- \rightarrow NH_2OH + H_2O$ (**3**)
($\Delta G_0' = -120$ KJ mol^{-1})
$NH_2OH + H_2O \rightarrow NO_2^- + 5H^+ + 4e^-$ (**4**)
($\Delta G_0' = +23$ KJ mol^{-1})
$0.5O_2 + 2H^+ + 2e^- \rightarrow H_2O$ (**5**)
($\Delta G_0' = -114$ KJ mol^{-1})

Basically, ammonia is oxidized firstly to hydroxylamine by the membrane-bound ammonia-monooxygenase (AMO) enzyme. Hydroxylamine is further oxidized to nitrite, by the periplasmatic enzyme hydroxylamine oxidoreductase (HAO). The last is the real electron-generating reaction and yields in total 4 electrons. Two electrons are required for the activity of the AMO enzyme; the other two enter the electron transport chain with concomitant generation of a proton-motive force. The low energy gained by the oxidation of ammonia as well as the reversed electron flow for the generation of reducing power for the carbon fixation explain the low specific growth rate of these bacteria. A doubling time of 8 hours has been reported for the well studied ammonia-oxidizing bacterium, *Nitrosomonas europaea*, in batch culture [29]. Cultivation-based methods for the detection of these bacteria in the environment are therefore time-consuming and difficult when applied to these slowly growing-bacteria.
To date, the oxidation of ammonia is regarded as the only energy source for aerobic ammonia-oxidizing bacteria. They are however able to oxidize several apolar compounds such as methane, carbon monoxide and aliphatic and aromatic

hydrocarbons, due to the lack of a high substrate specificity of the enzyme ammonia-monooxygenase. However, there is no evidence of the growth of ammonia oxidizers on these alternative electron donors other than ammonia.
More interestingly, ammonia oxidizers showed relatively high denitrification activity when cultivated under oxygen-limited conditions [30]. Their simultaneous nitrification and denitrification activity resulted in the significant formation of gaseous nitrogen compounds. In addition to the production by this so called nitrifiers denitrification, these gaseous oxidized nitrogen compounds seem to be formed from hydroxylamine by non-specific reactions. The production of gaseous nitrogen oxides has been shown to be proportional to the increasing ammonium concentrations in the environment [31-33].

Phylogeny of ammonia-oxidizing bacteria

Ammonia-oxidizing bacteria are traditionally placed into the Gram-negative family of Nitrobacteraceae, Buchanan 1917 [34]. Within this family, 5 genera and 16 species have been recognized, based on the morphology of the cells and the arrangement of intracytoplasmatic membranes. The genera are: *Nitrosomonas*, *Nitrosolobus*, *Nitrosococcus*, *Nitrosospira* and *Nitrosovibrio*. However, phenotypic characteristics are not effective for differentiating these microorganisms.
Phylogenetic analyses based on 16S rRNA gene sequences distinguished two monophyletic groups of ammonia oxidizers [35]. One group is affiliated with the gamma-subclass of the Proteobacteria and includes two species *Nitrosococcus oceani* [36-38] and *Nitrosococcus halophilus* [39]. The other group is affiliated with the beta-subclass of the Proteobacteria and contains the other four genera: *Nitrosomonas*, *Nitrosospira*, *Nitrosovibrio* and *Nitrosolobus,* the latter three being closely related to each other [35]. To date, members of the gamma-subclass of Proteobacteria are considered to be restricted to marine ecosystems or saline terrestrial habitats. In contrast, members of the beta-subclass of Proteobacteria are present in a wide range of habitats, *i.e.* in natural terrestrial and aquatic habitats as well as in artificial systems. Aerobic ammonia-oxidizing bacteria of the beta-subclass of Proteobacteria are the main subject of this thesis.

The *amoA* gene, coding for the alpha subunit of the ammonia-monooxygenase, represents a marker molecule alternative to the 16S rRNA gene for the detection of ammonia oxidizers in the environment and also for the establishment of their phylogeny. Results of phylogenetic analyses based on the *amoA* gene fragment are highly congruent with the 16S rRNA based phylogeny [40, 41].

Ecology and niche differentiation among ammonia-oxidizing bacteria

The concept of niche to which this thesis refers has been defined by G. E. Hutchinson [42], for whom a niche is a region ("n-dimensional hypervolume") in a multi-dimensional space of environmental factors or resources that affect the welfare of a species. In order words, the niche is the full range of environmental conditions (biological and physical) suitable for the existence of that particular species (fundamental niche). However, as a result of naturally occurring intra- and inter-specific competition and interaction, species are forced to use a narrower space of this hypothetical "hypervolume", the so called realized niche. By natural selection, then, species are forced to differentiate their niches, thereby allowing the coexistence of species with similar functions but different physiological requirements.

This concept of niche is not always easily applicable to microorganisms [43]; however ammonia-oxidizing bacteria may represent an interesting exception in this respect.

Although having a generally uniform basic metabolism and an essential requirement for ammonium as energy source, ammonia oxidizers seemed to occupy different niches in the environment according to the physiological properties of the species or group of species [40, 44, 45]. This hypothesis emerged and increased in strength because, in contrast to other microbial groups, all non-cultivated environmental ammonia-oxidizing strains are affiliated to different groups of ammonia oxidizers for which cultivated species with known physiological properties are generally available [46-56].

Several studies have investigated the link between environmental characteristics and the distribution of ammonia oxidizers, often

describing changes in the microbial community composition under environmental perturbations [57-66]. By mimicking environmental perturbation under controlled laboratory conditions, the response of the microbial ammonia-oxidizing community to a single factor has also been studied [67].

Ammonia-oxidizing bacteria are essential components of the nitrogen cycle, therefore changes in their community composition may affect the nitrifying activity, given the differences in activity observed between AOB strains [29, 68]. Several studies have addressed this aspect [67, 69], which is of particular interest considering the increasing concern of the effect of anthropogenic activity on the nitrogen cycle. Lately, it has been shown that the diversity of ammonia oxidizers is reduced in fertilized soils [65, 70] and that the changes in the community composition are associated with changes in potential rates of nitrification [71]. Furthermore, ammonia oxidizers were shown to respond to global change, by decreasing in number under elevated CO_2 and precipitation or by increasing the relative abundance of strains that metabolize ammonium more efficiently under increasing nitrogen concentrations [72].

The overall emerging picture is that diversity and eco-physiology of ammonia oxidizers can be linked to single factors as well as to complex environmental perturbations, making this bacterial group a potential model in microbial ecology studies [41]. Moreover, such a diversity of ammonia oxidizers in the environment suggests that both adaptation and selection are possible within the ammonia oxidizers' communities [66]. In this perspective, the study of ammonia-oxidizing bacteria is an appealing subject to study niche differentiation and driving factors involved.

Driving factors for niche differentiation

Among the factors than can drive the niche differentiation between ammonia oxidizers, the availability of ammonium is certainly a major one, since ammonia is used for their basic metabolism. Differential ammonia concentrations in activated sludge, lake water and sediment are suggested to drive the evolution of different subpopulations of ammonia oxidizers [73-75]. The prolonged exposure to either low or high ammonium

concentrations, as well as the competition for ammonium between ammonia oxidizers, heterotrophic nitrifiers and plants and algae, may also select for specific groups of ammonia oxidizers in terrestrial and aquatic environments [6, 76, 77].
In addition to the availability of ammonium, several other environmental parameters appeared to drive the niche differentiation between ammonia-oxidizing bacteria, namely oxygen, pH, temperature, salinity, concentration of organic carbon, presence of toxic compounds, tidal dynamics, soil heterogeneity caused by plants, the effect of substratum [61, 62, 78-81]. Likely, the more complex the environment is, the higher the number of factors contributing to the changes in the ammonia-oxidizing community.

Niche differentiation among ammonia-oxidizing bacteria in aquatic habitats

Difference in dominancy of ammonia-oxidizing bacteria occurred also between marine and freshwater environments, due to different salt requirement, tolerance or sensitivity of distinct species [40].
In addition to ammonia oxidizers of the gamma–subclass of the Proteobacteria, marine environments are inhabited by members of the *Nitrosomonas marina* lineage, which comprises obligate or salt-tolerant species. Species of the *N. europaea* lineage were occasionally enriched from marine samples, but were notably present in an alkaline, hypersaline closed-basin lake [51, 82]. Within the range of ammonia-oxidizing bacteria of the beta-subclass inhabiting marine environments, differences have been found between particulate material-associated and planktonic species [83].
In freshwater environments, results of repeated isolations as well as of molecular surveys showed that members of the *Nitrosomonas oligotropha* lineage are generally the dominant ammonia oxidizers. This might be explained by the high affinity for ammonium demonstrated by members of this lineage in laboratory experiments [84-87]. As for the marine environments, differences in diversity, numbers and activity between ammonia oxidizers of the beta-subclass of Proteobacteria have been found

within freshwater bodies, for example between the sediment and the water column [74, 88, 89].
The AOB niche differentiation has been intensively studied also along the freshwater-marine gradient of estuarine environments, where gradients of salinity, ammonia concentration and dissolved oxygen level may lead to the coexistence of different strains of ammonia oxidizers [58, 66, 73, 90, 91]. Among the estuarine systems investigated with respect to the ammonia-oxidizing bacteria communities, the estuaries of the Scheldt [66] and the Seine river [90] were of particular interest. Both estuaries were inhabited by different subpopulations of ammonia oxidizers along the oxygen and saline gradient. Moreover, both estuarine systems drain large, highly populated areas and receive waste water inputs directly from the city of Brussels and Paris, respectively, or from smaller influents. Waste water inputs affected significantly the ammonia-oxidizing communities' dynamics and activity. In the Scheldt estuary, moreover, nitrification activity was shown to be the main source of the greenhouse gas N_2O [92].

Shallow freshwater lakes colonized by submerged macrophytes

Among the freshwater habitats, shallow lakes have been rarely subject of studies with respect to the niche differentiation of ammonia-oxidizing bacteria [47, 84]. Unlike stratified lakes, shallow lakes are defined as freshwater bodies with an average depth of less than 3 meter, which do no stratify in summer for long periods, whose nutrient dynamics are influenced by the resuspension of sediment particles, and in which the presence of submerged macrophytes is crucial for the whole ecosystem functioning. Easily subjected to eutrophication as consequence of high nutrient inputs, shallow freshwater lakes appeared to have two alternative stable states: an algae-driven turbid state and a macrophytes-driven clear state [93]. The equilibrium between the two states involved many ecological mechanisms. All mechanisms are centered on the interaction between submerged vegetation and turbidity, caused by phytoplankton and by resuspension of sediment particles. Simplistically,

submerged macrophytes enhance water clarity, while turbidity hampered their growth by light limitation.
The macrophytes driven-clear state is likely the pristine state of shallow freshwater lakes as well as the ideal target for restoration measures. The latter include hydrological management, reduction of phosphate and biomanipulation and have been particularly intense in the Netherlands. In the Netherlands shallow lakes are important for the hydrology, the water balance and the agriculture in the surrounding reclaimed areas, as well as for the recreational function of the lakes themselves [94, 95].
The Border Lakes, in Dutch *Randmeren* (Figure 1, **Chapter II**), represent a complex system of seven man-made interconnected freshwater lakes, obtained by the reclamation of 1419 km^2 land in the centre of the Netherlands. Lakes differed between each other in the nutrient content of both the sediment and the water and in the distribution and coverage of submerged macrophytes species, which are all somehow connected to restoration measures that were applied with different degrees to the lakes. In the past, severe restoration measurements were applied to 3 of the 7 lakes, namely Lake Nuldernauw, Wolderwijd and Veluwemeer, resulting in the establishment of a clear water state driven by Charophytes meadows. The other lakes, namely Gooimeer, Eemmeer, Drontermeer and Vossemeer, present a more natural situation, with higher diversity of macrophytes species and higher nutrient contents, but differently controlled water regimes.

Epiphyton of submerged macrophytes: unexplored niche for ammonia-oxidizing bacteria

To the best of our knowledge, few studies focused on the niche differentiation of ammonia-oxidizing bacteria in shallow freshwater lakes. However, differences in diversity and activity between sediment-associated and pelagic AOB have been already described in relatively shallow freshwater bodies [73].
Nevertheless, the epiphyton of submerged macrophytes as a niche for ammonia oxidizers has long been overlooked. In the past Körner [96] demonstrated that the epiphyton of submerged macrophytes can harbor considerable numbers of nitrifying bacteria. Lately, Eriksson et al. [97-99] measured nitrification

activity in the epiphyton of submerged macrophytes and demonstrated the importance of the epiphyton in the coupled nitrification-denitrification process in wetland systems. This has already found several applications in the removal of nitrogen compounds by using plants, *i.e.* phytoremediation, in natural and constructed wetlands [100-108]. The epiphyton of submerged macrophytes therefore might offer a niche to ammonia oxidizers in shallow freshwater lakes, in addition to the sediment and the water column. Dynamics between the three compartments with respect to the niche differentiation of AOB are still unexplored.

Outlines of the thesis

The aim of this thesis was to investigate the niche differentiation of ammonia-oxidizing bacteria in aquatic environments and to give insight in the reaction of the ammonia-oxidizing communities on environmental perturbations.

The thesis can be divided in two main parts. The first part, which includes **Chapter II**, **III** and **IV**, focused on the niche differentiation of ammonia-oxidizing bacteria in shallow freshwater lakes. Most emphasis has been given to the effect of the establishment of submerged macrophytes due to anthropogenic measures.

Ideally, freshwaters lakes can be divided in three different compartments, *i.e.* the benthic, the pelagic and the epiphytic compartments, corresponding to the sediment, the water column and the epiphyton of submerged macrophytes, respectively. **Chapter II** provides a detailed comparison of the diversity of the ammonia-oxidizing bacterial communities in the three compartments of 7 different shallow freshwater lakes. The AOB community composition was analysed by means of denaturing gradient gel electrophoresis (DGGE) of 16S rRNA gene fragments obtained by polymerase chain reaction PCR using sets of primers specific for ammonia oxidizers. Multidimensional scaling analysis was applied to define the dominant AOB members in the different compartments. The epiphyton of submerged macrophytes was presented as a new niche for ammonia oxidizers.

In **Chapter III**, three lakes were chosen as most representative of the freshwater system previously investigated. The lakes have been differently subjected to biomanipulation measures and hydrodynamic controls. The ammonia-oxidizing communities were described using the 16S rDNA based PCR-DGGE approach that appeared most efficient in the analyses presented in **Chapter II**, as well as by means of the Fluorescent In Situ Hybridization (FISH) technique and by analyses of *amoA* clone libraries. Numbers of ammonia oxidizers in the different compartments were assessed by Real-Time PCR based on the 16S rRNA gene and related to potential ammonia-oxidizing activities. The effect of restoration measures on the diversity,

potential activity and numbers of ammonia-oxidizing communities was evident.

Chapter IV focused on the dynamics of colonization of the epiphyton of a model submerged macrophyte by benthic and pelagic AOB. A microcosm experiment was designed to follow the dynamics. Attempts to measure nitrification rates in the benthic, pelagic and epiphytic compartments in the microcosms were also done.

The second part of the thesis focused on the niche differentiation of AOB along a freshwater-saline gradient. In particular, the succession of AOB subpopulations along the estuary of the Seine river after the discharge of the effluent of a waste water treatment plant was investigated in **Chapter V**. The spatial and temporal dynamics of the response of a benthic freshwater AOB community to increasing salinity were tested under controlled laboratory conditions in a microcosm experiment presented in **Chapter VI**.

Chapter II

Epiphyton as a niche for ammonia-oxidizing bacteria: detailed comparison with the benthic and pelagic compartments in freshwater shallow lakes

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Abstract

Next to the benthic and pelagic compartments, epiphyton of submerged macrophytes may offer an additional niche for ammonia-oxidizing bacteria in shallow freshwater lakes. In our study we explored the potential activity and community composition of ammonia-oxidizing bacteria of the epiphytic, benthic and pelagic compartments of seven shallow freshwater lakes, which differed in trophic status, submerged macrophytes distribution and restoration history. PCR-DGGE analyses demonstrated that the epiphytic compartment was inhabited by members of cluster 3 of the *Nitrosospira* lineage and of the *Nitrosomonas oligotropha* lineage, which also inhabited the pelagic and the benthic compartment. Potential activity differed between compartments. Moreover, both ammonia-oxidizing bacterial community composition and potential activity were influenced by the restoration status of the different lakes investigated.

Introduction

By converting ammonia into nitrite, ammonia-oxidizing bacteria (AOB) are responsible for the first step of the nitrification process [109, 110]. Comparative 16S rRNA gene sequence analyses revealed the assemblage of AOB in two monophyletic groups [35, 111]. The main group, containing the genera *Nitrosomonas*, *Nitrosospira*, *Nitrosovibrio* and *Nitrosolobus*, is affiliated with the beta-subclass of the Proteobacteria. The second group is affiliated with the gamma-subclass and includes two species of the genus *Nitrosococcus*, retrieved and isolated exclusively from marine environments.

AOB of the beta-subclass have been detected in many different environments from aquatic habitats [50, 51, 75, 84, 88, 112-115], to soils [41, 47, 49, 116] and building stones [117]. Distribution patterns of distinct species of AOB depend on physiological differences between distinctive representatives [44] and on environmental parameters like ammonia concentration [73, 75, 85], pH and temperature [62, 68], oxygen availability [29, 78, 118] and salinity [73, 119]. Hortz et al. [72] demonstrated that ammonia-oxidizing bacteria from a grassland soil are also able to respond to multifactorial global changes. AOB are therefore considered a model group in microbial ecology studies [41]. However, it remains difficult to couple results obtained from physiological experiments conducted in the laboratory with isolated strains to nitrification activities observed in the environment from which the strains were isolated [41].

AOB are known to grow either free-living or attached. For example, compared with the water column of aquatic environments, solid-phase habitats such as sediment, particulate material and aquatic macrophytes seemed to be differently [50, 83, 113] and more densely populated with nitrifying bacteria [120-122]. This surface attached growth has been proposed to offer resilience against environmental constrains [123]. Moreover the formation of a biofilm on surfaces provides significant benefit for AOB of the *Nitrosomonas* lineages able to produce extracellular polymeric materials.

Hence, aquatic macrophytes may provide a habitat for attached growth of microorganisms [124]. In order to create clear lakes without noxious algae, water managers aim for macrophytes to

dominate the community of nutrient-consuming primary producers in shallow freshwater lakes. Evidence of nitrification activity on submerged macrophytes leaves was given by Eriksson et al. [97, 99, 101] in microcosm experiments and by Körner and Matulewitch [96, 120] who detected nitrifying bacteria on submerged macrophytes by enumeration methods. However, to our knowledge, there is no information available on the composition of the ammonia-oxidizing bacterial community in the epiphyton of aquatic macrophytes.

This study aims to describe in detail the ammonia-oxidizing bacterial communities of the epiphyton of submerged macrophytes, and to compare it with the benthic and pelagic compartments in a complex hydrological system composed of interconnected shallow freshwater lakes.

Based on previous studies conducted in the same sampling area [84, 125, 126] and on the known distribution pattern of AOB [40, 44], we hypothesized that the respective communities would differ between them. Moreover, we hypothesized that the colonization of the epiphyton might occur both by pelagic AOB species, which could benefit from the surface-attached conditions and by benthic AOB species resuspended from the sediment by the wind in these shallow freshwater lakes. Attempts have been done to link community structures as well as potential activities to several environmental variables characterizing the benthic, pelagic and epiphytic compartments in a complex hydrological system that has been subjected to biomanipulation processes in the past.

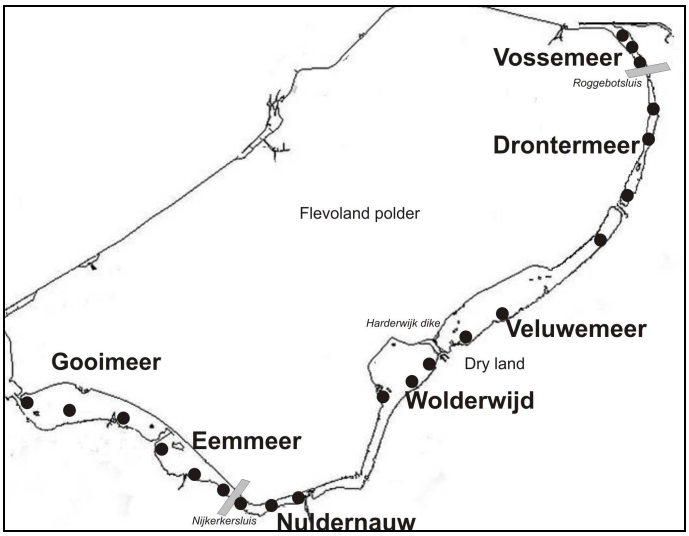
Material and methods

Description of study area

The Border Lakes are man-made, shallow freshwater lakes, resulting from the reclamation of 1419 km^2 land (*i.e.* the Flevoland polder, 52°31' N; 5°29' E) in a former brackish lagoon, in the centre of the Netherlands. Two sluices divide the series of 8 lakes into three hydrological units (from south to north): the southern lakes (Gooimeer and Eemmeer); the central lakes (Nuldernauw, Wolderwijd, Veluwemeer and Drontermeer)

and the northern lakes (Vossemeer and Ketelmeer). **Figure 1** schematically represents the lake area. Lake Ketelmeer has been omitted from the analyses as it is directly influenced by the River IJssel.

Figure 1. Map of the area of the Dutch Border Lakes. Black dots indicate the sampling stations, labeled in the text as A, B and C from south to north. Grey rectangles indicate the sluices that determine the three hydrological units. Lake names were abbreviated in the codes assigned to the samples by the first character (*i.e.* G, E, N, W, D,) or by the first two characters (*i.e.* Ve and Vo).



Water retention times were 3, 14, 30, 45, 60, 90, 150 days for Lakes Vossemeer, Eemmeer, Drontermeer, Nuldernauw, Veluwemeer, Wolderwijd and Gooimeer, respectively. Interestingly, Lakes Nuldernauw, Wolderwijd and Veluwemeer, have been subjected to restoration measures in the past (e.g. dredging of phosphate-rich sediment, flushing with phosphate-pour water, and removal of the stock of benthivorous fishes) [95] that resulted in the establishment of a clear water state with *Chara* sp. as macrophyte-dominating species.

Sample collection

In June 2004, samples were collected from the sediment, the water column and the leaves of submerged macrophytes, hereby

indicated as benthic, pelagic and epiphytic samples, in the 7 different shallow freshwater lakes. In every lake, three stations (A, B, C) were chosen based on macrophytes distribution maps [127]. At every station, 5 replicate sampling plots were determined randomly by throwing a 1 m^2 polyvinyl square on the water surface. Sampling and Secchi depths, pH and temperature of lake water were registered on board.

Physical and chemical characterization of sediment samples

Two sediment cores were collected in every of the 5 replicate 1m^2 plots at every station and the upper 5 cm portions were homogenized and stored in sealed glass jars to reduce oxygen diffusion. All analyses described below were done within 2 days after sampling.

pH was measured in 1:5 (wt/v) sediment/water slurries shaken for 1 hour at 150 rpm at room temperature. Specific gravity was calculated from the volume increase due to the addition of 20 g dried and sieved sediment to 50 ml of water in a graduated cylinder. Gravimetric water content (%) and dry-weight of the sediment were determined by oven drying of the samples at 60°C for 48 hours. Weight % of organic carbon and nitrogen and their molar ratio were measured from freeze-dried and grinded samples combusted at 1010°C, with an Elemental Analyzer NA-1500 equipped with a Hyasep-Q column (Carlo Erba Instruments, Milan, Italy). Phosphate content (mmol/kg) was measured with an ICP-Spectrometer (ICP-Optima 3300DV, Perkin Elmer, USA), after acid and microwave destruction of sediment samples. For molecular analyses of microbial communities, sediment samples were freeze-dried and stored in screw-cups at -20°C until analyses.

Physical and chemical characterization of water samples

Water samples were collected in 5 liter plastic containers, previous to sediment sampling to reduce resuspension of sediment particles. No replicate sampling plots per station were defined for the pelagic compartment. Data on lake water were provided by Mrs. K. Oostinga of the Public Service for the IJssel

Lagoon of the Department of Waterways and Public Works. Water was filtered over 0.45 μm membrane filters for the potential nitrification activity assay and over 0.2 μm membrane filters for the molecular analyses of microbial communities.

Macrophytes species composition, abundance and sampling

Submerged macrophytes composition and coverage percentages were visually assessed within the 1 m^2 plots. Basically, two species dominated the Border Lakes: *Potamogeton pectinatus* and *Chara* sp.. Additional species retrieved and sampled were *Potamogeton perfoliatus* and *Zannichellia palustris*. The coverage percentages of the macrophyte species per station are summarized in **Table 1**. Per macrophyte species, samples of flooding leave were collected within the 5 replicate plots and stored in airtight plastic bags filled with lake water and used fresh for the activity assay and freeze-dried for the molecular analyses.

Table 1. Coverage percentages of macrophytes species at different station of the Border Lakes sampled in June 2004. Values represent averages of 5 replicate plots.

Lake	Station	Macrophyte coverage (%)			
		P. pectinatus	*Chara* spp.	*Z. palustris*	*P. perfoliatus*
Gooimeer	A	30			5
	B	30			
	C	30			10
Eemmeer	A			50	
	B			50	
	C	30			
Nuldernauw	A	20	70		10
	B		100		
	C	30	70		
Wolderwijd	A	No plant			
	B	40	60		
	C	40	60		
Veluwemeer	A		100		
	B		100		
	C		100		
Drontermeer	A	70			
	B	50	10		
	C	50	10		
Vossemeer	A	50			
	B	50			
	C	50			

Determination of potential ammonia-oxidizing activities

Potential ammonia-oxidizing activity (PAA) in the benthic compartment was measured in slurries of 5 g wet sediment in sterile 250 ml flasks containing 50 ml mineral medium and incubated on a rotary shaker at 25°C and 150 rpm. Replicate samples corresponded to the 5 sampling plots defined per station. The medium contained 1 mM $(NH_4)_2SO_4$, 0.80 mM K_2HPO_4, 0.20 mM H_2PO_4, adjusted to pH 7.5 with 1 N NaOH [128, 129]. Subsamples were taken every 3 hours within 24 hours and every 6 hours until 160 hours incubation. NH_4^+ and NO_2^- plus NO_3^- concentrations were measured colorimetrically with a Technicon Auto-Analyzer (TRAACS 800, Bran Luebbe, Germany). Potential activity and lag phase have been determined by using the coefficient of a linear regression calculated from nitrite plus nitrate produced per hour. Results were normalized for volume loss during sampling and expressed as nmol of NO_2^- plus NO_3^- $cm^{-3}\,h^{-1}$.

Potential activity in the pelagic compartment was measured in triplicate from 0.45 μm filter incubations as described above; activity has been expressed in μmol NO_2^- plus NO_3^- $l^{-1}\,h^{-1}$.

Triplicate samples of 1 g fresh macrophyte leaves, gently dried on paper, were used to measure activity in the epiphytic compartment. 4 mM HEPES (4-2-hydroxyethyl-1-piperazineethanesulfonic acid) and 0.04 % bromothymol blue solution were added to the previously described medium as pH buffer and indicator, respectively. Changes of pH in the medium were restored by adding 5% $NaHCO_3$ solution. Subsamples for determination of inorganic nitrogen concentrations were taken after every acidification event for a maximum of 15 days. Presented values correspond to the total NO_2^- plus NO_3^- production that occurred. No potential activity has been calculated but net NO_2^- plus NO_3^- production per dry weight macrophyte leaves and related to the percentage of macrophytes coverage.

Statistical analysis on potential activities

Statistical analyses on potential activities in the benthic and pelagic compartments and on net NO_2^- plus NO_3^- production in the epiphytic compartment were conducted with the data analysis software system STATISTICA, version 7.1 (StatSoft, Inc., OK, USA). After checking for normality and homogeneity of variances, followed by transformation if necessary, analyses of variance on the activities were conducted to test for the effect of the factors lake, station and plot as well as for the effects of the environmental parameters associated with the different compartments.

DNA extraction

Environmental DNA was extracted from 0.5 g freeze-dried sediment or from 0.3 g of freeze-dried macrophytes leaves. 250 ml of water samples were filtered over a 0.2 µm membrane filter and DNA was extracted from half of the filter. The protocol described by Henckel et al. [130] was modified as follows. Briefly, cells were dissociated from surfaces and lysed by bead-beating with approximately 1 g of sterilized zirconia-silica beads in a sodium phosphate and SDS buffer. After 5 minutes of centrifugation at 10.000 g, 600 µl supernatant were collected in clean screw-cups. The procedure was repeated twice per sample, with no addition of SDS during the second extraction. To precipitate proteins and debris, 0.4 volumes ammonium acetate were added per sample and the solution was maintained on ice for 5 minutes. After 5 minutes of centrifugation at 10.000 g at 4°C, 1 ml supernatant was collected into new screw-cups containing 0.7 volumes of pure isopropanol and centrifuged for 45 minutes at 13.000 g at 4°C to precipitate DNA. Isopropanol was decanted and 1 ml ethanol (-20°C) was added for the washing step. After 10 minutes of centrifugation at 13.000 g at 4°C and decantation, the DNA pellet was dried under vacuum centrifugation (DNA 110 SpeedVac® Concentrator, Sovant Instruments Inc, Farmigdale, NY, USA*)*, resuspended in 100 µl pre-heated (65°C) sterile purified water and concentrated in 50 µl final volume with the AMPure® PCR Purification System according to manufacture's instruction (Agencourt Bioscience

Corporation, Beverly, MA, USA). Quantification was done spectrophotometrically using 2 µl DNA samples (Nanodrop® ND-1000, Nanodrop Technology, Wilmington, DE, USA).

Direct and nested Polymerase Chain Reactions (PCR)

The diversity of the ammonia-oxidizing community in the three compartments was analyzed by means of PCR-DGGE assays. **Table 2** summarizes the four different approaches used, here named as I, II, III and IV. 16S rDNA- (I, II and III) and *amoA* gene-based (IV) approaches designed for ammonia-oxidizing Proteobacteria of the beta-subclass were applied. Specificity and sensitivity of the primer sets have been described before [40, 49, 113, 131].

Table 2. Summary of used PCR approaches with the different primer sets applied. Codes have been assigned to the different approaches of which two involved a nested amplification and two a direct one. The asterisk (*) indicates the presence of a 40-bp GC-clamp at the 5' end of the oligonucleotides. Abbreviations S, W and M indicate the benthic, pelagic and epiphytic compartment, respectively.

Code	PCR type	I Primer set	II Primer set	Target	Compartment
I	nested	βAMOf, βAMOr	*CTO189f, CTO 654r	16S rRNA	S, W, M
II	nested	27f, 907r	*CTO189f, CTO 654r	16S rRNA	S, W, M
III	direct	_	*CTO189f, CTO 654r	16S rRNA	S
IV	direct	_	*AmoA-1F, AmoA-2R-TC	*AmoA* gene	S

100 ng of purified DNA were used as template for 25 µl PCR reaction containing 1 X Mg-free Buffer, 0.5 µM of each primer (1.0 µM in case of βAMOF to compensate for ambiguities), 200 µM of each deoxynucleotide triphosphate, 1.75 mM $MgCl_2$, 400 ng/µl BSA (Bovine Serum Albumine), 2 U Taq-polymerase. Thermocycling programs consisted of 5 minutes denaturation at 94°C, followed by 35 or 25 cycles (for approach III, IV and I, II, respectively) of 30 seconds of denaturation at 94°C, 45 seconds specific annealing (at 57, 59 and 60°C for primer sets CTO - βAMO and amoA, respectively, and touchdown 65-55°C for primer set 27f-907r), and 30 seconds elongation at 72°C; 10 minutes final elongation were performed in all reactions. Nested

amplifications (approach I and II) of 20 cycles were performed from 1:100 dilutions of PCR products as described above. DNA samples of *Nitrosomonas europaea* and *Nitrosomonas ureae* were used as positive controls. All reactions were verified by UV illumination of 1.2 % agarose gels stained in ethidium bromide solution.

Denaturing Gradient Gel Electrophoresis (DGGE)

PCR amplicons obtained from direct and nested amplifications were separated on denaturing gradient gels. A denaturing gradient of 30-55 % was used for approaches I, II and III, while a 20-60% denaturing gradient was used for approach IV [49, 132, 133]. For all approaches, electrophoresis was conducted for 17 hours at 60°C in 0.5X TAE buffer. Gels were stained for 1 hour in 0.05 μg/ml ethidium bromide solution and visualized with UV. Bands of interest were cut in their middle portions and after elution from polyacrylamide overnight at 4°C, reamplified with maximum 20 cycles PCR (CTO primer set for approach I, II and III and *amoA* primer set for approach IV) and reloaded on denaturing gradient gel till obtaining single and pure bands suitable for sequencing reactions.

Analyses of sequences of ammonia-oxidizing bacteria related bands

Sequences were aligned with the fast-aligner tool of the software ARB for phylogenetic analyses [134]. 16S rRNA phylogenetic trees were constructed based on previous published neighbor-joining trees of the two major subgroups of the beta-subclass of the AOB (*i.e. Nitrosomonas* spp. and *Nitrosospira* spp.). Band sequences were added to ammonia oxidizers' sequences subsets using the parsimony criterion and *ad hoc* created filters. Sequences that appeared to be duplets were not considered in the phylogenetic analyses neither submitted to the EMBL-bank.

Nucleotide sequences accession numbers

Sequences obtained from bands related to ammonia oxidizers were submitted to the EMBL–bank under the following accession numbers: **AM18429-AM418440**; **AM418441-**

AM418445 and **AM418446-AM418447** for bands obtained from the benthic, pelagic and epiphytic compartment, respectively.

Analysis of ammonia-oxidizing bacterial communities within and between compartments

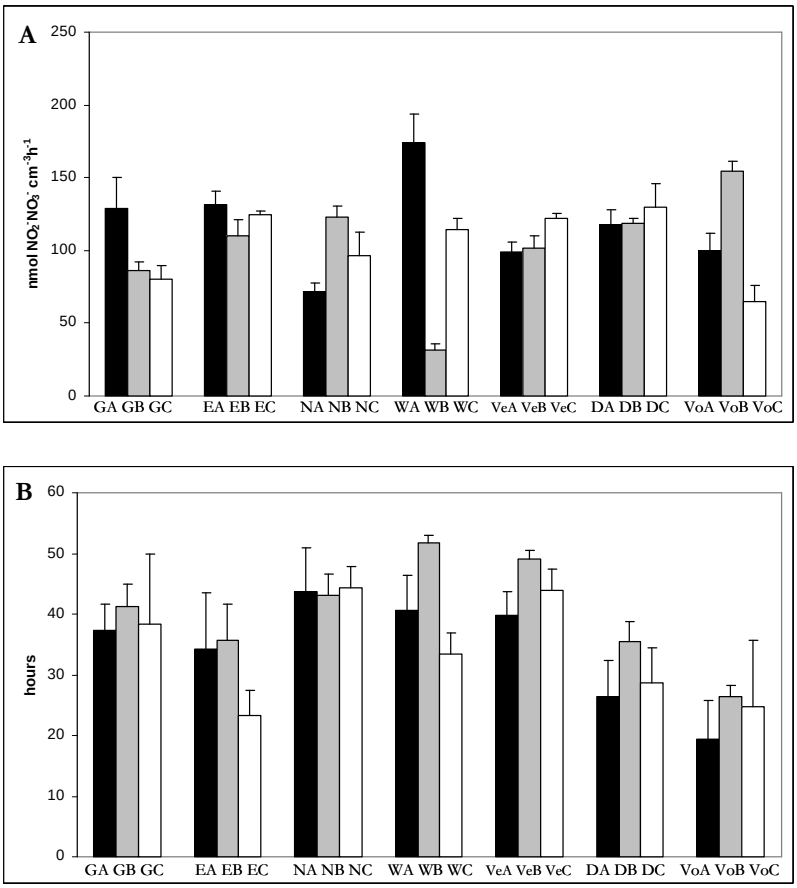
Ammonia-oxidizing bacterial communities structure obtained by means of DGGE were analyzed with PRIMER software, version 5 (Plymouth Routines In Multivariate Ecological Research) [135]. Bands related to AOB were used to build similarities matrices (Bray-Curtis coefficient) based on presence-absence of single bands as well as on abundance of clusters and lineages' members. Matrices were used as inputs for analyses of similarities, hierarchical clustering and non-metric multi dimensional scaling to test for the effect of different factors on community composition within compartments and to compare communities between different compartments. Possible effects of environmental variables on the AOB community were tested by combining similarities matrices with Euclidean distance-based matrices of log-transformed environmental variables (BIO-ENV procedure).

Results

Potential activity in the benthic compartment

Results on potential ammonia-oxidizing activity (PAA) assay of sediment samples showed a highly negative correlation between NH_3 consumption and NO_2^- plus NO_3^- production rates (Pearson Correlation coefficient range= -0.91, -0.99). PAA values varied from a minimum of 31.52 to a maximum of 173.86 nmol NO_2^- plus NO_3^- cm^{-3} h^{-1} (**Fig. 2A**). Lag phase values ranged from a minimum of 19 hours to a maximum of 51 hours. Samples from lakes subjected to restoration in the past (*i.e.* Lakes Nuldernauw, Wolderwijd and Veluwemeer, **Fig. 2B**), all had lag phase times of more than 35 hours. PAA values and lag phase showed a significant negative correlation ($p < 0.05$). Moreover, PAA values were negatively correlated with specific gravity of the sediment and positively with % tot-N and % org-C.

Figure 2. Potential ammonia-oxidizing activities (**A**) and lag phases (**B**) determined for the benthic compartments. Values are averages of 5 replicate samples.



Univariate analyses were performed to test for the effect of different variables on PAA and lag phase. The factor lake had a significant effect on both activity ($p= 0.048$; **Table 3**) and lag phase ($p= 0.000$), the latter also being highly significantly influenced by the factor station ($p=0.000$). The factor plot did not significantly affect PAA or lag phase. Effects of the different environmental variables on PAA have been assessed with Main effect ANOVA: % tot-N, % org-C, C/N, PO_4^{2-} concentration, % clay content, water retention time and wind exposure had all a significant positive effect on the PAA (**Table 3**).

Table 3. Summary of the effects of lake, station, plot, macrophyte species and environmental factors on potential ammonia-oxidizing activities (PAA) in the benthic and pelagic compartments and on net NO_2^- plus NO_3^- production in the epiphytic compartment. Activity per compartment was treated as dependent variable. The effects of the factors lake, station, plot and macrophytes were treated as an independent variable at the significant level of $p<0.05$. Abbreviations stand for n.s. = not significant; n.t. = not tested.

Dependent variable and compartment	P values of the variables tested				Significant environmental variables	Statistical test
	Lake	Station	Plot	Macrophyte species		
Benthic PAA	0.048	n.s.	n.s.	n.t.	% N, % org C, C/N, $[PO_4^{-2}]$, % clay, retention time, wind exposure	Univariate ANOVA
Pelagic PAA	0.000	n.s.	n.s.	n.t	$[O_2]$, $[PO_4^{-2}]$, Kj-N, retention time	Kruskal-Wallis ANOVA
Epiphytic net NO_2^- plus NO_3^- production	0.000	0.029	n.s.	0.000	Retention time	Kruskal-Wallis ANOVA

Potential activity in the pelagic compartment

In the incubation of water samples from Lakes Nuldernauw, Wolderwijd and Veluwemeer, again the lakes subjected to restoration in the past, no NO_2^- plus NO_3^- production was measured, indicating no or below-detection limit activity in the water column of these lakes. NH_3 consumption rates and NO_2^- plus NO_3^- production rates were negatively correlated in water samples of Lakes Gooimeer, Eemmeer, Drontermeer and Vossemeer (Pearson Correlation coefficient range= -0.99, -0.86). Activity values in these lakes ranged from 0 to 1 μm NO_2^--NO_3^- l^{-1} h^{-1} (**Fig. 3**) Responses to the addition of ammonium, expressed as lag phase, were above 65 hours. The Kruskal-Wallis test identified a significant effect ($p=0.000$) of the factor lake on pelagic PAA (**Table 3**). The factors station and plot were not of significant influence on PAA. Among the environmental variables, PO_4^{2-}, Kjeldahl-N and O_2 concentrations and water retention time had a positive significant effect (**Table 3**).

Net NO_2^- plus NO_3^- production in the epiphytic compartment

Incubations of macrophyte leaves from lakes Veluwemeer, Wolderwijd, Drontermeer and Vossemeer did not show any detectable nitrification activity. Therefore, we might assume that if present, the epiphytic ammonia-oxidizing bacterial communities of these lakes were not sufficiently active. Net

production in incubations from lakes Gooimeer, Eemmeer and Nuldernauw varied from 0 to a maximum of 175 mmol NO_2^--NO_3^- dry-wt^{-1} related to percentage of macrophyte coverage (**Fig. 4**).

Kruskal-Wallis ANOVA demonstrated (**Table 3**) that the factors lake and macrophyte species were highly significant (p=0.000), the factor station was significant (p= 0.029), while the factor plot showed no significant effect. Of the environmental variables related to water quality tested, only water retention time had a positive significant effect (**Table 3**).

Figure 3. Potential ammonia-oxidizing activities determined for the pelagic compartments. Values are averages of 3 replicate samples.

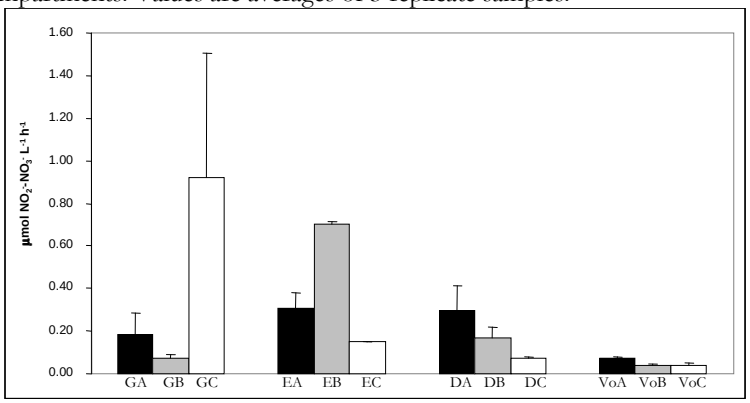
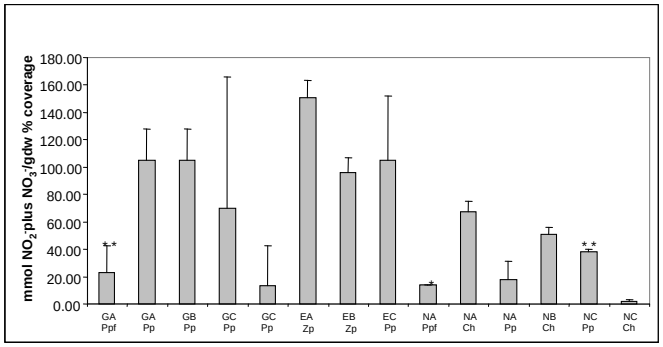


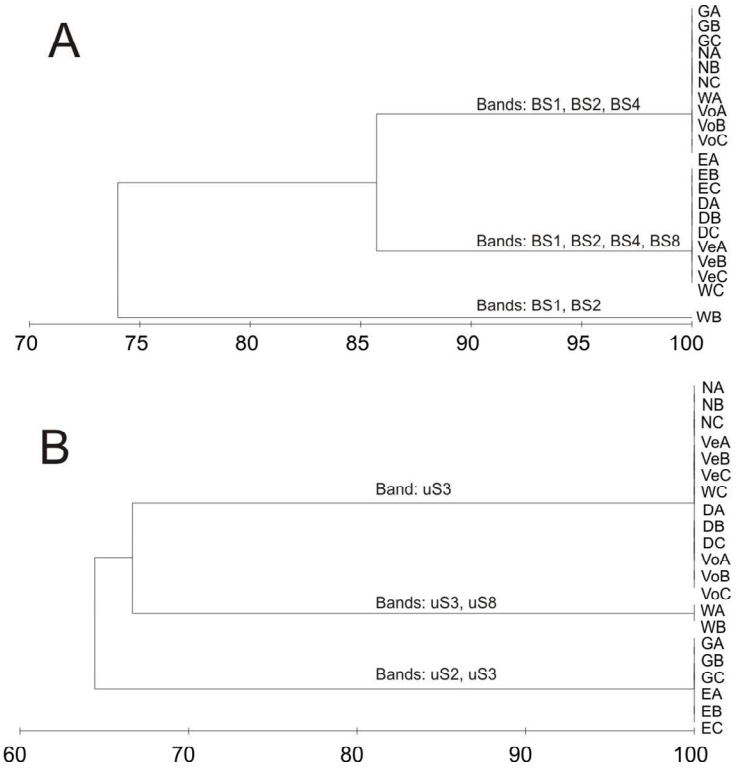
Figure 4. Net NO_2^- plus NO_3^- production related to macrophyte percentage coverage per lake and station. Abbreviations: Ch: *Chara* sp; Pp: *Potamogeton pectinatus*; Ppf: *Potemogeton perfoliatus*; Zp: *Zannichellia palustris*. Values represent averages of 5 replicate samples, except for samples GA Ppf and NC Pp (**) where only 3 replicates were available and for sample NC Ppf (*) where only 1 sample was available.



Ammonia-oxidizing community composition and hierarchical cluster analyses

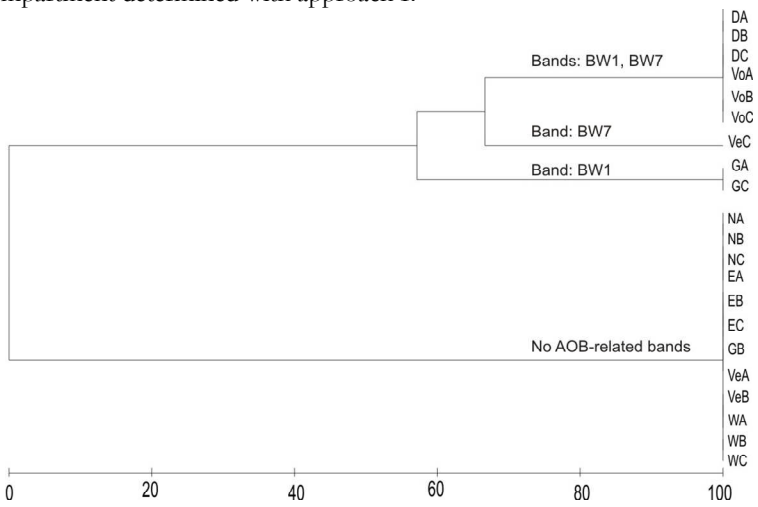
The composition of the ammonia-oxidizing bacterial community was analysed by means of denaturing gradient gel electrophoresis using 4 different PCR approaches for each compartment. The direct amplification assays (**Table 2**: approaches III and IV) were applied for all compartments, but gave products only for the benthic compartment. This might be due to the fact that numbers of AOB are highest in the sediment samples (unpublished quantitative PCR data, M. Coci). Hereby, we present only the results obtained with approaches I and II. In the benthic compartment, a total of 4 AOB-related bands were detected with approach I (**Fig. 5A**), of which band BS1 belonged to the *Nitrosomonas oligotropha* lineage, band BS2 to cluster 3 of the *Nitrosospira* lineage and bands BS4 and BS8 to cluster 0 of the *Nitrosospira* lineage [40]. A total of 3 bands were detected with approach II (**Fig. 5B**); band uS3 belonged to the *N. oligotropha* lineage, band uS2 belonged to the *Nitrosomonas* sp. Nm143 lineage and band uS8 to cluster 3 of the *Nitrosospira* lineage. On the basis of approach I, AOB communities showed high similarity percentages between samples (87%, **Fig. 5A**), with the exception of sample WB of station B from Lake Wolderwijd (75 %). On the basis of approach II, similarity percentages were lower (< 70 %, **Fig. 5B**). In order to test whether the description of the benthic AOB community composition has been affected by the choice of the PCR-DGGE approaches, analyses of similarity were performed on matrices based on abundance of members of AOB lineages (*i.e.* number of bands per lineage or cluster). This turned out to be significant.

Figure 5. Dendrograms of cluster analyses of the samples from the benthic compartment determined from DGGE band patterns with approaches I and II (panel A and B, respectively). Values at the x axis represent percentages of Bray-Curtis similarities based on presence-absence of bands that are indicated on the respective branches. In the dendrograms, the first part of the band code, *i.e.* R04, used in the phylogenetic trees and in the sequences submitted to the EMBL-bank (**Fig. 8** and **9**), has been omitted.



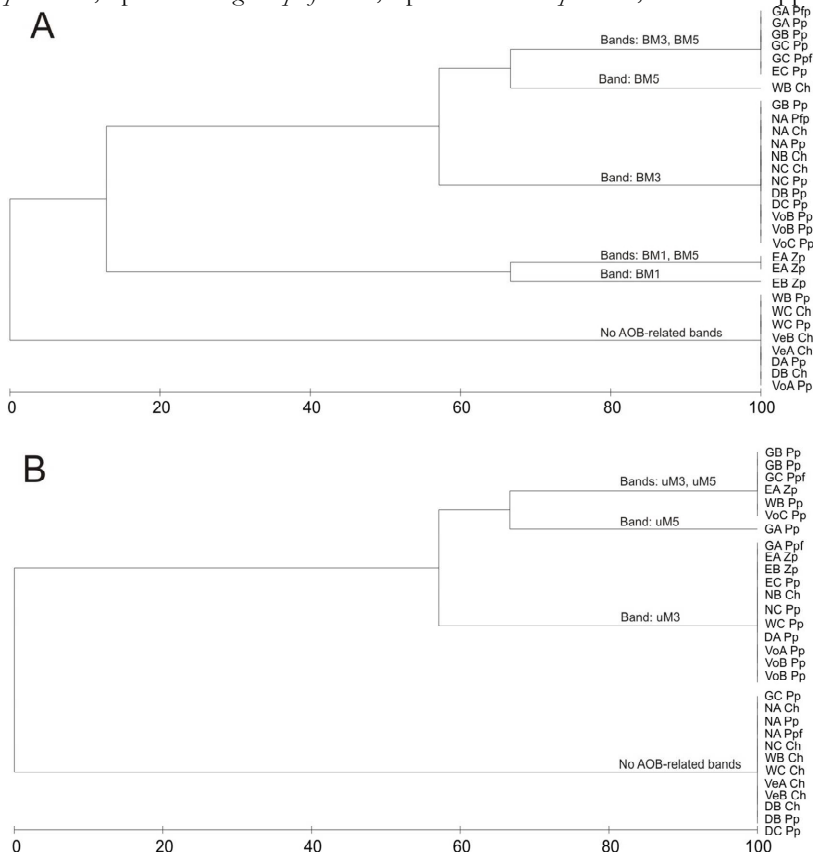
In the pelagic compartment, approach II failed to detected AOB-related bands. On the basis of approach I (**Fig. 6**), which was successful in detecting AOB-related bands in 58% of the samples, a total of 2 bands (BW1 and BW7) were detected both belonging to the *N. oligotropha* lineage that apparently dominated the pelagic ammonia-oxidizing bacterial communities.

Figure 6. Dendrogram of the cluster analyses of the samples from the pelagic compartment determined with approach I.



In the epiphytic compartment (**Fig. 7**), a total of 3 bands were detected with approach I. One band (*i.e.* BM3) belonged to cluster 3 of the *Nitrosospira* lineage and was the only band detected in 50% of the samples. Two other bands (BM1 and BM5) that belonged to the *N. oligotropha* lineage were retrieved in epiphytic samples of *Z. palustris*, present at stations A and B of Lake Eemmeer, of *P. pectinatus* at station A, B and C of Lake Gooimeer, and of *Chara* sp. at station B of Lake Wolderwijd. On the basis of approach II, 2 bands were detected: band uM3, that belonged to cluster 3 of the *Nitrosospira* lineage, was again the only band detected in 50% of the samples. Band uM5, which belonged to the *N. oligotropha* lineage, was detected in epiphytic samples of *Zannichellia palustris* at station A of Lake Eemmeer, and of *Potamogeton pectinatus* at stations A and B of Lake Gooimeer, at station B of Lake Wolderwijd and at station C of Lake Vossemeer. No AOB-related bands were detected with approach I and II in 26 and 36% of the samples, respectively. Analyses of similarities showed no effect of the approach used on the description of epiphytic community composition. However, the detection of bands related to the *N. oligotropha* lineage was affected by the choice of PCR approaches (**Fig 7**).

Figure 7. Dendrograms of the cluster analyses of samples from the epiphytic compartment determined with approaches I and II, panel A and B, respectively. Abbreviations of macrophyte species stand for: Pp = *Potamogeton pectinatus*; Ppf= *Potamogeton perfoliatus*; Zp= *Zannichellia palustris*; Ch= *Chara* spp.



Despite the differences within the compartments, analyses of affiliation of bands revealed similarity percentages above 96% between bands obtained with different PCR approaches that grouped together within the *Nitrosomonas* or the *Nitrosospira* lineages, as evident in the respective phylogenetic trees of the two major subdivisions of the beta-subclass of the ammonia-oxidizing Proteobacteria (**Fig. 8** and **9**). Moreover, bands retrieved in different compartments showed also high similarity values (> 93%) when clustered together.

Figure 8. Phylogenetic tree of ammonia-oxidizing bacteria of the *Nitrosomonas* lineages. Bands marked in bold were retrieved in this study from benthic (S), pelagic (W) or epiphytic (M) compartments. First part of the band code (R04) correspond to the Border Lakes sampling campaign of the year 2004, followed by an abbreviation for the approach, *i.e. B* for the nested approach I based on the βAMO primer set, *u* for the nested approach II based on the 27f-907r primer set.

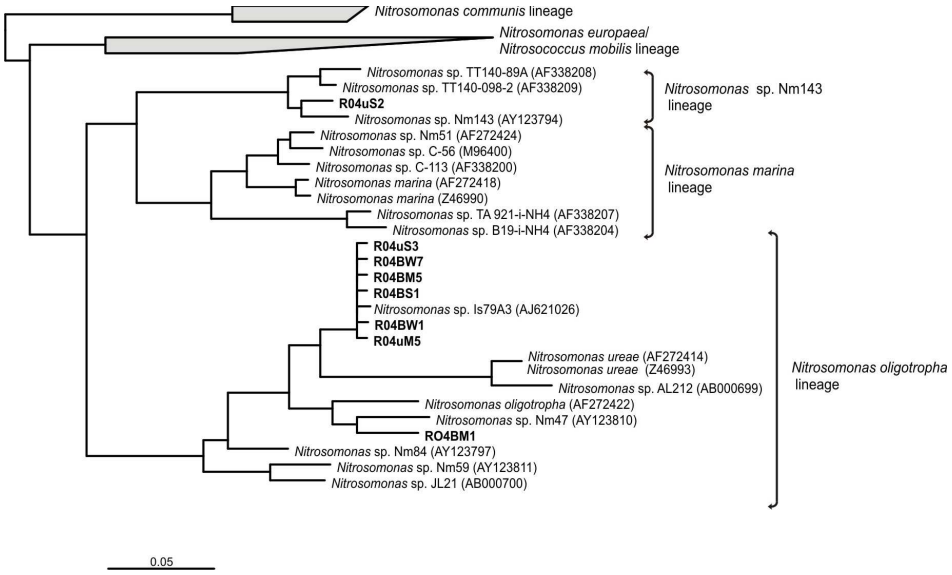
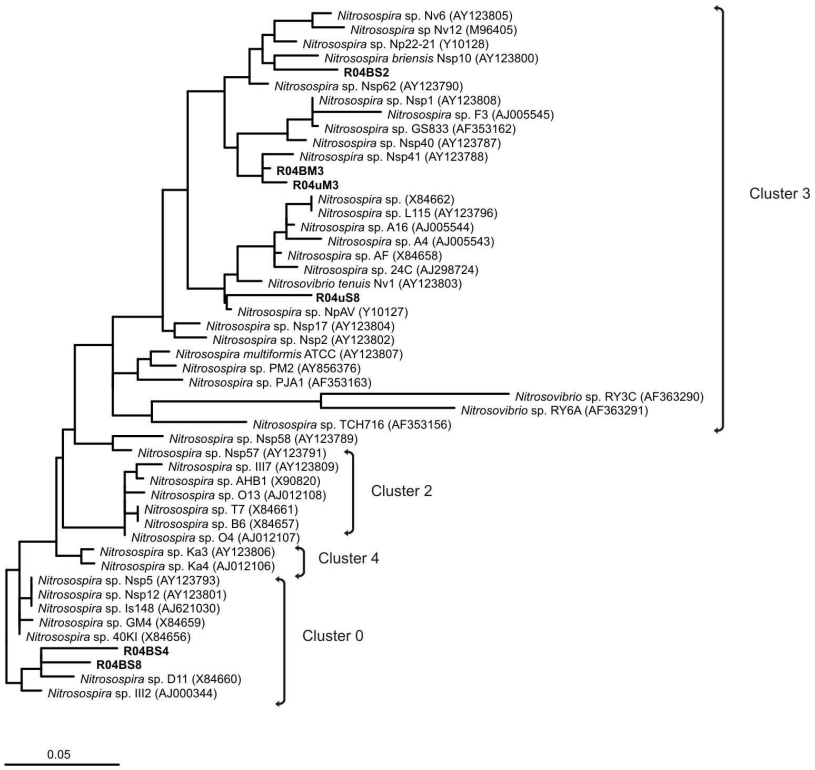


Table 4. Summary of the analyses of variance on similarities matrices, constructed from PCR-DGGE approaches I and II, to test for the effects of the factors lake, station and macrophyte species on the ammonia-oxidizing communities of the benthic, pelagic and epiphytic compartments. Significance was assessed at 5% level (*). Abbreviation n.t. stands for not tested.

Compartments and approaches	Factors and % significance		
	Lake	Station	Macrophyte
Benthic			
Approach I	0.5*	62	n.t.
Approach II	0.1*	100	n.t.
Pelagic			
Approach I	0.1*	90.1	n.t.
Epiphytic			
Approach I	0.1*	94	4.7
Approach II	5.6	94	1.9

Figure 9: Phylogenetic tree of the ammonia-oxidizing bacteria of the clusters of the *Nitrosospira* lineage.

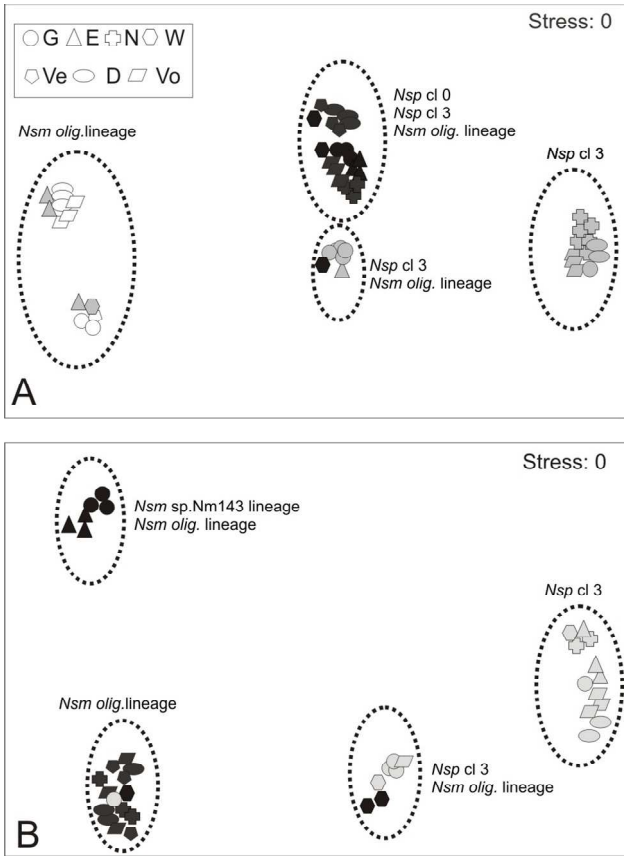


Analyses of similarities to test the effect of the factors lake, station and, in case of the epiphytic compartment, macrophytes species on the composition of the AOB communities revealed a significant effect of the factor lake in the benthic compartment when analysed with approaches I and II, and in the pelagic and epiphytic compartments when analyzed with approach I (**Table 4**).

Comparison of ammonia-oxidizing bacterial communities between compartments and the effects of environmental variables

Similarities matrices based on presence-absence of bands detected with the nested PCR approaches I and II were converted into abundance matrices of AOB species of the above

Figure 10. Multidimensional scaling plot of ammonia-oxidizing bacterial (AOB) communities of the benthic, pelagic and epiphytic compartments based on PCR approach I (A) and II (B). Calculations were done from similarities matrices based on abundance of AOB-related bands per AOB lineages or clusters. Different shapes were used to indicate samples from different lakes, as explained in the figure. Colors, black, white and grey correspond to the benthic, pelagic and epiphytic compartments, respectively.



mentioned lineages and clusters to allow comparison between compartments. Figure 10 showed the results obtained by non-metric multidimensional scaling analyses. Only the results of approach I allowed the comparison between the 3 compartments (**Fig 10A**), while approach II allowed comparison between the benthic and epiphytic compartments (**Fig. 10B**).

Based on approach I, it was shown that members of the *N. oligotropha* lineage and the *Nitrosospira* clusters 3 and 0 inhabited

the benthic compartment, while members of the *N. oligotropha* lineage dominated the pelagic zone. The epiphytic compartment was populated both by members of the *Nitrosospira* cluster 3 and the *N. oligotropha* lineage. In particular, the majority of epiphytic samples were colonized by members of the *Nitrosospira* cluster 3. The epiphytic AOB communities of all samples from Lake Eemmeer were colonized only by members of the *N. oligotropha* lineage, while five out of six epiphytic samples from Lake Gooimeer where colonized by members of both mentioned groups.

Based on approach II, the benthic compartment resulted inhabited by members if the *N. oligotropha* lineage as well as on the *Nitrosomonas* sp. Nm 143 lineage. Members of cluster 3 of the *Nitrosospira* lineages appeared to be a minor component of the benthic AOB community, in comparison with the results obtained with approach I. Again, members of the *Nitrosospira* cluster 3 populated the epiphytic compartments. Four out of six epiphytic samples from Lake Gooimeer were colonized also by members of the *N oligotropha* lineage.

Matrices based on abundance of species in the AOB clusters were combined with Euclidean-distance based dissimilarity matrices of environmental variables with BIO-ENV procedure of PRIMER. Best matches were obtained with the combination of the concentrations of nitrogen and phosphate ($\rho = 0.65$ and $\rho = 0.57$ for approach I and II based matrices, respectively).

Discussion

This study focused on the activity and composition of the communities of ammonia-oxidizing bacteria of the beta-subclass of the Proteobacteria in the benthic, pelagic and epiphytic compartments of seven shallow freshwater lakes characterized by submerged macrophytes. The ability of the benthic ammonia-oxidizing bacterial communities to nitrify appeared quite poor, as demonstrated by the high lag phase values, which were all above 20 hours. In particular, benthic samples from Lakes Nuldernauw, Wolderwijd and Veluwemeer showed lower potential activities and higher lag phase values. The significant effect of the factor lake in addition to the effects of nutrient concentrations (N, C and P), clay content, water retention time

and wind exposure, suggest that the potential activities of the ammonia-oxidizing bacterial communities depend on the environmental conditions that characterize the lakes. In the pelagic compartment, potential activities were lower in comparison with the benthic compartment and absent in samples from Lakes Nuldernauw, Wolderwijd and Veluwemeer. Again, the factor lake turned out to be highly significant as well as nutrient (N and P) and oxygen concentrations and water retention time in the lakes themselves. The size of the nitrite plus nitrate production in the incubation of epiphytic samples was comparable to the potential nitrification activity measured by Eriksson and Andersson [101] from litter of macrophytes. Again, the factor lake appeared to be highly significant in the analyses of variances between samples. Here, retention time was the only environmental variable with a significance effect on the activity.

The potential activity assay chosen does not allow direct comparison of actual activities between compartments. For this the use of 15-N stable isotope techniques are preferable over all other techniques [136]. The potential nitrification assesses the number of potentially active cells present in the compartments and reflects therefore the environmental history of the cells more than the immediate *in situ* activity. It might be of interest to notice that lakes with a history of restoration (*i.e.* manipulation of nutrients), namely Lakes Nuldernauw, Wolderwijd and Veluwemeer, appeared to have lower activities in all compartments. Pauer and Auer [137] found little evidence of nitrification in the water column of hypereutrophic lakes and strongly suggested that nitrification is a sediment-based processes. However, they excluded epiphytic AOB communities from their study. Eriksson et al. [97, 100] suggested that macrophytes litter strongly supported or restrained epiphytic nitrification depending on macrophyte species studied. Our results confirmed their observations.

Our investigation of the composition of ammonia-oxidizing bacteria of the beta-subclass of the Proteobacteria had limitations, which are related to the use of molecular assays based on the PCR-DGGE procedure. When using this procedure with AOB, heteroduplexes and other artifacts are

easily formed [126] and therefore caution has to be taken in drawing conclusions on AOB community compositions based on minor variations in nucleotide sequences. To reduce possible distortions, different approaches have been used in our study and overestimation of richness have been overcome by grouping single AOB-related bands into AOB clusters and subjected these to analyses of variance. It was shown that the approach chosen had an effect on the final assessment of the bacterial community compositions. Approach II, based on a combination for universal primer and AOB-specific primer set, failed to detect members of cluster 3 of the *Nitrosospira* lineage in the benthic compartment and was not suitable for the pelagic compartment. [46]By applying two primer sets both specific for AOB, *i.e.* βAMO and CTO in approach I, the diversity of the ammonia-oxidizing bacterial community was better described, as also demonstrated by Mahmood et al.[138]. However, the approaches I and II gave comparable results on the epiphytic community composition.

To our knowledge, this is the first time AOB species were retrieved from epiphytic communities by means of molecular techniques. Presence of AOB in the epiphytic compartment of submerged macrophytes has been reported by Körner [96] on the basis of MPN counts and has been assessed by Eriksson et al [97, 100] using nitrification activity assays. As hypothesized, the benthic, pelagic and epiphytic communities differed among each other and the epiphytic compartment appeared to be inhabited both by benthic and pelagic ammonia oxidisers.

We intentionally did not relate the diversity of AOB communities with activity measurements because of the limitation implied in the use of molecular assays that are not based on functional gene expression [139, 140]. As a matter of fact the mere retrieval of a 16S rDNA sequence of AOB proves neither that this organism is abundant nor that is physiologically active [40], but it definitely indicates the presence of AOB in a particular environment.

Belonging to one or the other lake was apparently an important factor both for the potential activity and for the community composition of the ammonia-oxidizing bacteria. Several environmental variables were tested, and nitrogen and phosphate

concentration as well as water retention time turned out to play a major role in the explaining differences between lakes. All these factors are subject to changes according to the restoration history of the lakes. Hence, the AOB community characteristics as assessed in our study are linked to the trophic status of the lake, thereby offering the potential of being used in monitoring programs. Our sampling area, constituted of 7 shallow freshwater lakes, is representative for a typical wetland-like environment, where the presence of stable stands of submerged macrophytes is desirable for the maintenance of a clear water state against algal dominance.

Our study gave evidence of the importance of the epiphytic compartment as a niche for ammonia-oxidizing bacterial community in addition to the niches offered by the benthic and pelagic compartments. Since the establishment of dense stands of macrophytes is a target for the water management to repress the growth of noxious algae and cyanobacteria, the role of the epiphytic compartment in the nitrogen turnover of shallow freshwater lakes must be carefully considered in the biomanipulation processes.

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Chapter III

Qualitative and quantitative assessment of ammonia-oxidizing communities in the sediment, the water column and the epiphyton of submerged macrophytes in shallow lakes

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Abstract

The diversity, numbers and activity of epiphytic ammonia-oxidizing bacteria in epiphyton of submerged macrophytes has long been overlooked in shallow freshwater lakes. In the present study we demonstrated the presence of ammonia-oxidizing bacterial cells in the epiphyton of the submerged macrophyte *Potamogeton pectinatus* by FISH microscopy images. Apparently, the composition of the epiphytic ammonia-oxidizing communities depended on the colonization by both benthic and pelagic cells. By Real-Time PCR of 16S rRNA genes, we also showed that total numbers of ammonia oxidizers in the epiphytic compartment were comparable to those in the pelagic compartment. Numbers of both epiphytic and pelagic ammonia-oxidizing cells were significantly lower than the benthic ones. However, the environmental variables associated with the shallow lakes and the different degrees of anthropogenic influence affected significantly the ammonia-oxidizing bacterial communities from a qualitative and a quantitative perspective.

Introduction

Ammonia-oxidizing bacteria (AOB) are responsible for the rate-limiting step of the nitrification process, therefore playing an important role in the nitrogen turnover in a wide range of habitats, from natural and agricultural soils, to natural and artificial fresh and marine aquatic environments [41]. Their monophyletic nature allowed the rapid development and the successful application of molecular techniques based on the genes of 16S rRNA and of ammonia monooxygenase A subunit (*amoA*). Hence, they have been considered an ideal model group in molecular microbial ecology [41]. In the last decade the number of studies focusing on diversity [50, 70, 141-144], niche differentiation and related driving factors [47, 57, 60, 62, 66, 119, 145], as well as on the abilities of ammonia-oxidizing bacteria to cope with contaminants [61, 146, 147], environmental stresses [148-150] and global change [72, 151] has significantly increased.
Moreover, the discovery of the anaerobic ammonia oxidization process [15] together with the most recent isolation of marine Crenarchaeota able to oxidize ammonia [26] is currently changing and deepening the knowledge and understanding of microorganisms involved in the nitrogen cycle, indicating that a lot is still undiscovered.
With respect to the "classical" aerobic ammonia-oxidizing bacteria, molecular study in freshwater ecosystems suggested the dominance of AOB of the beta-subclass of Proteobacteria [152]. However, the knowledge is far from complete, like for freshwater systems colonized by submerged macrophytes.
Our study focused only on aerobic ammonia-oxidizing bacteria of the beta-subclass of Proteobacteria present in the three different compartments in which a shallow freshwater lake might ideally be divided: *i.e.* the benthic, the pelagic and the epiphytic compartment. With these terms we referred to the sediment, the water column and the surface of submerged macrophytes, respectively.
Quantification of AOB in freshwater environments has been done using most-probable number techniques [96, 122, 137, 153] and competitive PCR [154]. However, quantitative studies of AOB in freshwater habitats are limited in relation to comparable

studies in soils and activated sludge, where several other molecular techniques have been successfully applied, for example quantitative PCR [155-157], Fluorescent In Situ Hybridization (FISH) [114, 158, 159] and in situ hybridization of mRNA sequences [160]. Furthermore, the AOB inhabiting the epiphytic compartment have been the subject of a very limited number of studies. Eriksson and Andersson [97, 99, 101] measured nitrification rates on leaves and litter of submerged macrophytes, while Körner [96] reported considerable numbers of AOB colonizing the leaves of different species of submerged macrophytes by means of MPN counts. In a recent detailed study on AOB in shallow freshwater lakes (**Chapter II** of this thesis), we described the communities colonizing the epiphyton of submerged macrophytes as composed by members of the *Nitrosomonas oligotropha* lineage and of cluster 3 of the *Nitrosospira* lineage [50] that dominated the pelagic and benthic compartments, respectively.

Regarding the activity of nitrifiers, it is generally assumed that nitrification in aquatic systems is associated with the sediment rather than with the overlying water [45]. However, when integrating total numbers of bacteria over the water column, Vincent and Downes [161] obtained better estimates of the impact of the pelagic community in the total nitrification process of lakes. Nevertheless, those measurements were not based on absolute numbers of ammonia-oxidizing cells and where performed in deep lakes where stratification occurs. In shallow freshwater lakes populated by large stands of macrophytes, the role of epiphytic nitrification must be taken into account since submerged macrophytes can provide a large accessible surface area for attached microorganisms [162].

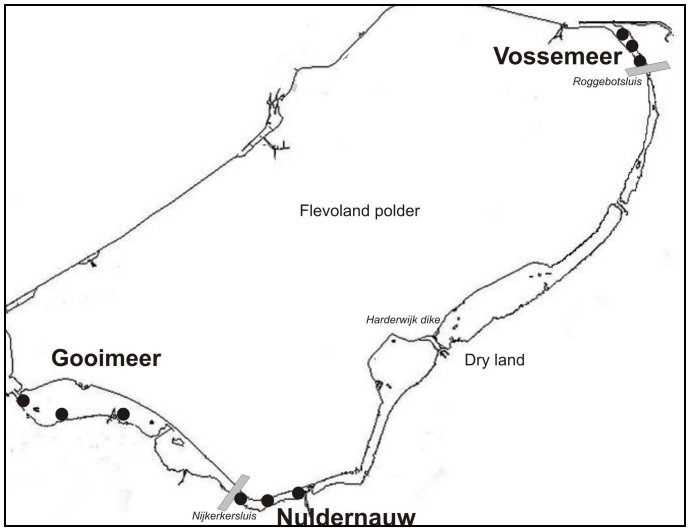
This study aims to provide qualitative and quantitative information about ammonia- oxidizing bacteria in the benthic, pelagic and epiphytic compartment of shallow freshwater lakes and to assess the presence of AOB in the epiphytic compartments by direct microscopy images. Evidence for the impact of restoration and managing procedures of shallow freshwater lakes on the AOB communities will also be presented.

Material and Methods

Site and samples collection

In July 2005, samples of sediment, water and submerged macrophyte leaves were collected from 3 shallow freshwater lakes (*i.e.* Lakes Gooimeer, Nuldernauw and Vossemeer) in the centre of the Netherlands. A map of the sampling area is provided in **Figure 1**. The lakes belong to a more complex hydrological system composed of 7 interconnected artificial shallow freshwater lakes, created from the reclamation of 1490 km^2 of brackish area.

Figure 1. Schematic map of the sampling area, with the name of the lakes sampled in July 2005, namely Lake Gooimeer, Nuldernauw and Vossemeer. In every lake, three stations were chosen, indicated by black circles. To every station a code was assigned, including the first letters of the name of the lake (G, N and Vo) and the character A, B and C, from the southern to the northern location in every lake**.**



The three lakes were chosen as the most representative of the whole area (**Table 1**). Lake Gooimeer is a relatively large freshwater body of approximately 2757 ha of which 34% is less than 1.5 m deep, with mainly silty sediment and a water retention time of approximately 150 days. The dominant submerged macrophyte species is *Potamogeton pectinatus*. Lake Nuldernauw is a relatively smaller freshwater body (600 ha) of which 56% is less than 1.5 m deep. It has mainly sandy sediment and a water retention time of 45 days. Lake Nuldernauw has been subjected to severe restoration procedures, which lead to the establishment of dense Charophytes meadows and subsequent higher Secchi depths and lower resuspension rates. Lake Vossemeer is the smallest (400 ha) and most shallow (61% is less than 0.5 m deep) of the lakes sampled, with a mixed sandy and silty sediment and a water retention time of only 3 days.
In every lake, 3 stations (A, B, C) were selected on the basis of the distribution and coverage percentages of two submerged macrophyte species, which were *P. pectinatus* and *Chara* sp. [163]. The stations were differently exposed to the dominant wind, *i.e.* South-West, in relation to their geographic coordinates and their vicinity to the shore. Classes of exposure to wind were assigned and are summarized in Table 1. Per station, water samples were collected from the boat by means of plastic bottles; 250 ml water was filtered on board over three 0.2 μm membrane filters for DNA isolation. 500 ml of water from Lake Vossemeer and 1 liter of water from Lakes Nuldernauw and Gooimeer were filtered over 0.45 μm membrane filters and over glass fiber filters for activity measurement and FISH analyses, respectively. At every station, 5 replicate sampling areas were defined by means of a 1 m^2 polyvinyl square sampling device. Per sampling area, the upper 5 cm of 2 sediment cores were collected, homogenized and stored in glass jars, and leaves of submerged macrophytes were collected in airtight plastic bags, for activity measurements and molecular analyses. Analytical analyses of sediment and water samples were performed as described in the Materials and Methods section of **Chapter II** of this thesis.

Table 1. Summary of environmental parameters characterizing the lakes studied. Characters G, N and Vo stand for Lakes Gooimeer, Nuldernauw and Vossemeer, respectively. Characters w or s in brackets indicate the water column and the sediment, respectively. [a] Water retention time (days); [b] Classes of exposure to the South-West dominant wind direction, the numbers indicate the angle degree of the stations in relation to the SW wind direction; S indicate the proximity of the station to the South shores of the lakes; [c] sampling depth; [d] Kjeldahl nitrogen, i.e., organic N and N-NH_3; [e] coverage percentage of *Potamogeton pectinatus* (Pp) and *Chara* sp. (Ch); [f] values are averages of 5 replicate samples; [g] pH in 1 mM KCl sediment extraction.

	RT[a]	Win dexp [b]	Depth (cm) [c]	Secchi depth (cm)	T °C (w)	pH (w)	O_2 mg/l (w)	KjN mg/l [d] (w)	P mg/l (w)	% Pp[e]	% Ch [e]	% N [f] (s)	%orgC [f] (s)	C/N[f] (s)	P mg/l[f] (s)	% silt[f] (s)	pH[g] (s)
GA	150	45S	120	120	20.4	8.2	8	2.8	0.3	100	0	0.015	0.116	9.47	0.054	10.00	7.3
GB	150	45S	100	80	19.4	8.2	8	2.8	0.3	80	0	0.010	0.099	12.24	0.038	12.25	7.6
GC	150	45S	100	80	19.5	8.6	8	2.8	0.3	80	0	0.405	5.895	17.05	0.362	14.9	7.2
NA	45	20S	80	100	17.6	8.3	9.3	0.9	0.12	0	100	0.052	0.821	18.41	0.029	5.16	7.7
NB	45	20	120	100	18.9	8.6	9.3	0.9	0.12	0	100	0.011	0.135	13.87	0.043	0.83	7.8
NC	45	45S	100	100	18	8.8	9.3	0.9	0.12	0	100	0.025	0.216	10.44	0.056	1.89	7.7
VoA	3	45	40	50	18.8	8.2	10.4	1.4	0.02	60	0	0.097	1.691	20.17	0.305	4.46	7.7
VoB	3	45	90	20	18.7	8.2	10.4	1.4	0.02	60	0	0.243	3.247	15.59	0.836	7.93	7.4
VoC	3	45	50	25	19.8	8.4	10.4	1.4	0.02	20	0	0.021	0.228	12.28	0.193	1.47	7.2

Determination of potential ammonia-oxidizing activity

To estimate the numbers of actively ammonia-oxidizing cells, potential ammonia-oxidizing activities (PAA) have been determined. PAA in the benthic compartment was measured by incubating 2.5 g of 5 replicate sediment samples in 50 ml of medium containing 1 mM NH_4^+, according to the protocol of Belser and Mays [128], modified by Verhagen and Laanbroek [129]. PAA in the pelagic and epiphytic compartments was measured in triplicate from 0.45 μm membrane filters and 1 g dried macrophyte leaves, respectively. Ammonia and nitrite plus nitrate concentrations were measured colorimetrically in sub-samples collected every 6 hours during 96 hours of incubation. Potential activity was determined using slope of linear regression curves.

DNA extraction and quantification

Environmental DNA was extracted from 0.5 g freeze-dried sediment, 125 ml lake water filtered over a 0.2 μm membrane filter or 0.1 g freeze-dried macrophyte leaves, in triplicate samples per station. Samples were homogenized by vortexing with 1 ml CTAB buffer [164] and 0.5 g sterilized zirconia-silica beads (0.1 mm diameter) and subsequently subjected to disruption by bead-beating at 5.0 m/s rotation for 60 seconds. After the addition of 5 μl proteinase K (20 mg/ml), samples were homogenized and incubated for 30 minutes at 37 °C, with quick vortexing after 15 minutes. After the addition of 150 μl of 20% SDS solution, samples were incubated for 1 hour at 65 °C in a Thermoblock apparatus, with quick vortexing every 15-20 minutes. After centrifugation at 10.000 g for 10 minutes, 600 μl of supernatant was collected into 2 ml clean screw-cap tubes. The rest of the sample was re-extracted with 450 μl CTAB buffer and 50 μl of 20% SDS solution, vortexed for 10 seconds, incubated for 10 minutes at 65 °C and centrifuged at 6000 g for 10 minutes. Again 600 μl were collected, added to the previous extracted supernatant, mixed with 1 ml phenol: chloroform: isoamyl alcohol solution (25:24:1 v/v) and centrifuged at 6000 g for 10 minutes. 1 ml supernatant was collected into new screw

cup tubes containing 700 µl isopropanol and incubated for 1 hour at 24 °C. After 20 minutes of centrifugation at 15.000 g, isopropanol was decanted and the pellet was resuspended and washed with 1 ml 20% cold ethanol. This was followed by 5 minutes of centrifugation at 15000 g, decantation of the ethanol and drying of the pellet under vacuum centrifugation and final resuspension in 100 µl water (Sigma®). All sample were purified and concentrated in a final volume of 50 µl with the AMPure® PCR purification system according to the manufacture's instruction (Agencourt Bioscience Corporation, Beverly, MA, USA). Quantification was done on 2 µl DNA samples using the Nanodrop® ND-1000 (Nanodrop Technology, Wilmington, DE, USA).

Polymerase Chain Reaction (PCR) and Denaturing Gradient Gel Electrophoresis (DGGE)

The composition of the ammonia-oxidizing bacterial communities in the benthic, pelagic and epiphytic compartments was assessed by a nested PCR-DGGE approach, involving two primer sets specific for AOB of the beta-subclass of Proteobacteria. Purified environmental DNA samples (100 ng) were used as template for a first amplification round with the primer set βAMOf-βAMOr [113]. PCR products obtained were diluted to a final concentration of 50 ng and amplified with the primer set CTO189f(GC clamp)-CTO654r [49]. Thermocycling programs consisted of 5 minutes denaturation at 94 °C, followed by 30 or 25 cycles (for βAMOf-βAMOr and CTO189f(GC)-CTO654r primer sets, respectively) of 30 seconds of denaturation at 94 °C, 45 seconds specific annealing at 57 °C, and 30 seconds elongation at 72 °C; final elongation lasted 10 minutes. PCR products were loaded on 1.2 % agarose gels, stained in 0.05 µg/ml ethidium bromide solution and verified by UV illumination. DGGE of CTO amplicons were performed on 8% polyacrylamide gels along a 30-55% denaturing gradient [49]. Electrophoresis in 0.5X TAE buffer lasted 17 hours at 60 °C. Gels were stained for 1 hour in ethidium bromide solution and visualized with UV. Images were taken with an exposure time of 1.200 seconds (IMAGO, B&L Systems, Netherlands). Bands of

interest were cut in their middle portions and after elution from polyacrylamide overnight at 4 °C, reamplified with maximum 20 cycles PCR with the primer set, CTO189f-CTO654r, reloaded on gels till the appearance of single and pure bands, suitable for sequencing reactions.

Band-pattern analyses and statistics

DGGE gel images were analyzed with the software Phoretix™ 1D advanced version 5.1 (Non Lynear Dynamic Ltd, Newcastle upon Tyne, UK) as described by Massieux [165]. Finally, 3 band-percentage matrices were calculated, *i.e.* for the benthic, pelagic and epiphytic compartment, each including triplicate samples per station. Band percentage matrices were subjected to hierarchical cluster and statistical analyses using the software PRIMER version 5 [166]. The effect of the factors compartment, lake, station and exposure to SW wind on the composition of the AOB communities was tested by analysis of similarities (ANOSIM procedure [167]). The statistic test R (where $R = \frac{\bar{r}_B - \bar{r}_W}{n(n-1)/4}$) calculated the differences between the average of all the rank dissimilarities between band-percentages profiles between compartments, lakes or stations ($\bar{r}_B$) and the average of all the rank dissimilarities between band-percentage profiles within compartment, lake or station ($\bar{r}_W$). R was scaled within the -1 and +1 range. $R > 0$ indicated higher significant differences between groups than within groups. The reverse was indicated by $R < 0$. For $R = 0$, no significant differences occurred. First, the R test was applied to the whole dataset (Global R). If significant, pair-wise procedures were applied to test the main between-group differences.

Non-metric multidimensional scaling analysis was performed for different clusters and lineages of ammonia-oxidizing bacteria, including only the samples were AOB-related bands were detected. To relate the AOB community compositions of different compartments to their respective environmental variables, we used the BIO-ENV procedure. Briefly, the procedure calculated Spearman's rank correlation coefficient (ϱ) between the distances of the response matrix (in our case, the

Bray-Curtis similarity matrices of AOB communities of the three compartments) with normalized Euclidean distance matrices of appropriately transformed environmental parameters associated to the compartments [168]. We used the BIO-ENV procedure to calculate the ϱ values of every possible combination of predictor variables and the "best fit", *i.e.* the single variable or the combination of variables that, simplistically, best explained the composition of the ammonia-oxidizing bacterial communities. We presented only the highest ϱ values and their associated combinations of environmental variables.

Construction and analyses of clone libraries based on the a*moA*-gene

Three *amoA* clone libraries were constructed by cloning PCR fragments obtained from the benthic, the pelagic and the epiphytic samples of station A of Lake Gooimeer. Environmental DNA samples were firstly amplified with the Whole Genome Amplification Kit (WGA) Genomic PhiV2 DNA (GE Helthcare, UK). Amplifications with the primer set *amoA*-1F and *amoA*-2R [131] were performed in a final volume of 50 µl, containing 1X Mg-free Buffer (Invitrogen Inc., CA, USA), 0.5 µM of each primer, 200 µM of each deoxynucleotide triphosphate, 1.75 mM $MgCl_2$, 400 ng/µl BSA (New England, Biolabs, Inc, MA, USA), 2.5 U *Taq* DNA polymerase (Invitrogen Inc., CA, USA). The thermocycling program consisted of 5 minutes of denaturation at 94 °C, 35 cycles of: 30 seconds of denaturation at 94 °C, 45 seconds of annealing at 57 °C and 30 seconds of elongation at 72 °C. Final elongation at 72 °C step lasted 30 minutes. The expected 491-bp DNA fragments were excised and eluted from 1% agarose gels using QIAquick spin columns (Quiagen, Hilden, Germany). Ligation in pCR® 2.1-TOPO® plasmid and transformation by heat shock in *Escherichia coli* TOP10 cells were performed with TOPO TA Cloning® kit (Invitrogen Inc., CA, USA) according to the manufacturer's instruction. Cells were plated onto agar supplemented with 20 mg/l X-Gal, 5 mg/l IPG, 20 mg/l ampicillin and 80 mg/l methicillin. Plasmids were isolated after overnight culturing of transformed colonies in liquid LB medium, using the PureLink™ Quick Plasmid Miniprep Kit (Invitrogen, CA, USA).

From each library, 50 clones were randomly selected and used as template for PCR-colonies with primer set *amoA*-1F and *amoA*-2R [131] and then digested with the restriction enzyme *MspI* (New England BioLabs, MA, USA). 10 µl of the digested products were analyzed on 2% agarose gel; 20-25 randomly selected clones per compartment were chosen for sequence analyses.

Phylogenetic analyses and nucleotide sequences accession numbers

Sequences obtained from DGGE bands and from *amoA* clones were aligned with published 16S rDNA and *amoA* sequences of cultured ammonia-oxidizing bacteria, respectively, by means of the fast aligner tool of the ARB software and added to their respective neighbour-joining trees by using the parsimony criterion and *ad hoc* created filters [134]. Partial 16S rRNA gene sequences obtained from bands were submitted to the EMBL-Bank under the following accession numbers: **AM503957**-**AM503962**. The longest sequences obtained from *amoA* clones were submitted under the following accession numbers: **AM700587** (R05S_amoA1), **AM700588** (R05S_amo2), **AM700589** (R05S_amo15), **AM700590** (R05W_amo11), **AM700591** (R05M_amo6).

Fluorescence in situ hybridization, epifluorescent and confocal laser scanning microscopy

The fluorescent in situ hybridization technique (FISH) was used to detect AOB on filters of water samples and on leaves of submerged macrophytes. No FISH technique was applied for the detection of AOB in sediment samples. Samples were fixed in 4% paraformaraldehyde solution. Small pieces of macrophytes leaves were placed on Teflon-coated slides and embedded into approximately 10 µl of 0.1% agarose solution, while portions of filters were hybridized in 1.5 ml centrifuge tubes and placed on the slides afterward. Hybridization steps were performed according to the specific requirements of the oligonucleotidic probes [169]. The following oligonucleotidic probes were used in combination: probe NSV443, specific for the *Nitrosovibrio*-

Nitrosolobus-Nitrosospira group [169], probes NSO190 and NSO1225 covering all sequences of ammonia oxidizers of the beta-subclass [169], and probe BET42a specific for all Beta-Proteobacteria [170]. Oligonucleotides were synthesized and fluorescently labeled at the 5'-end with a hydrophilic sulfoindocyanine dye, CY3 or CY5, or with 5(6)-carboxyfluorescein-*N*-hydroxysuccinimide ester (FLUOS) by Thermohybaid Inc. (Germany). Slides were washed briefly with ddH_2O, air dried and embedded with mounting medium Vectashield® containing DAPI stain. For images screening and acquisition, the epifluorescence microscope ZEISS Axioplan 2 (Zeiss, Jena, Germany) and the confocal laser scanning microscope LEICA DMIRE2 (Leica, Mycrosystem, Germany) were used. All image analyses were performed with the standard microscope software packages.

Real-Time PCR assay

Quantification of ammonia-oxidizing bacteria from benthic, pelagic and epiphytic samples was performed by Real-Time PCR with the Taqman assay as described by Hermansson and Lindgren [156]. Modification in the concentration of the mixture components in final volume of 25 µl were as follow: half volume IQ™ Super mix (Bio-Rad Laboratories Inc., CA, USA), 1 µM each primer (2:1 CTO189fA/B:CTO 189fC and RT1), 0.5 µM probe TMP1, 200 ng/µl BSA, and 10 ng DNA template. Reactions were performed and monitored with the thermocycler DNA Engine (Opticon 2, MJ Research, MA, USA). Standard curves were calculated in every run based on 10 known concentrations of DNA from *Nitrosomonas europaea*, used also in spiking experiments to optimize the DNA concentrations of sediment samples.

Statistical analyses

Statistical analyses of the potential ammonia-oxidizing activity and of the quantitative data of the ammonia-oxidizing communities were performed using the software package STATISTICA 7 (Statsoft., Inc., OK, USA). Data were checked for normality by means of the Wilk-Shapiro test and either

logarithm or square root transformed to obtain homogeneity of variance. Correlations between measured variables and potential activity measurements or numbers of AOB in sediment samples were determined by means of Spearman-Rank correlation tests. ANOVA analyses were used to test for the effects of water quality parameters on the potential activity measured in macrophytes incubations and on numbers of AOB in the pelagic and epiphytic compartments. ANOVA analyses were also used to test for the effects of the water retention time and the exposure to SW wind, as well as to test for the effects of different factors (lake, station and plot). Tukey's HSD tests were performed to calculate significant differences between mean values.

Results

Potential ammonia-oxidizing activity

Determination of ammonia-oxidizing activities was possible from the incubations of sediment and macrophyte samples (**Table 2**); no NO_2^- plus NO_3^- production has been observed in the incubation of filters. Depending on the sampling station, benthic potential ammonia-oxidizing activities (PAA) ranged from 7.33 (± 1.41) to 22.0.5 (± 1.95) mmol NO_2^- plus NO_3^- m^{-2} h^{-1}. The factor lake was highly significant for the benthic PAA (main effect ANOVA, df = 2, F = 57.71, $p < 0.001$), while the factor station was almost significant (df = 2, F = 2.9, p = 0.06) and the factor plot was not significant (df = 4, F = 0.5, p = 0.67).

As summarized in **Table 3**, the potential ammonia-oxidizing activities in the benthic compartment were positively correlated with the percentages of silt, nitrogen and organic carbon and with the concentration of phosphate measured in the sediment. Physical parameters, *i.e.* Secchi disc and sampling depth, water retention time and exposure to SW wind, had no significant effect on benthic PAA. The potential activities in stations A, B and C of Lake Nuldernauw, with the lowest silt percentages, were significantly lower than in Lake Gooimeer and Vossemeer (**Table 2**). The highest activity values were measured in station B of Lake Vossemeer that had the highest nutrient content.

Table 2. Potential ammonia-oxidazing activities in the benthic and epiphytic compartment of Lakes Gooimeer, Nuldernauw and Vossemeer. Abbreviation n.d. stands for not detectable. Values are average of 5 and 3 replicate samples for the incubations of sediment and macrophyte, respectively. Different characters in brackets indicate significant differences between mean values of activities within compartment measured in different stations and lakes (Tukey's HSD tests, $p < 0.05$)

Station	Benthic (n=5) mmol (NO_2-NO_3) m^{-2} h^{-1}	Epiphytic (n=3) mmol (NO_2-NO_3) m^{-2} h^{-1}
GA	15.79 ± 0.83 (a)	8.67 ± 1.55 (a)
GB	16.66 ± 0.99 (a)	7.21 ± 0.59 (a)
GC	12.58 ± 1.47 (a)	7.63 ± 0.42 (a)
NA	8.06 ± 0.96 (b)	n.d
NB	7.55 ± 0.59 (b)	n.d
NC	7.33 ± 1.41 (b)	n.d
VoA	16.18 ± 1.84 (a)	5.78 ± 0.79 (ab)
VoB	22.05 ± 1.95 (c)	5.48 ± 0.57 (b)
VoC	12.40 ± 0.59 (a)	3.41 ± 0.75 (c)

Table 3. Spearman-rank correlation test between the potential ammonia-oxidizing activity measured in the incubations of sediment samples and the variables measured in the sediment. Correlations marked in bold were significant at the 0.05 level.

Variables	PAA Sediment mmol m^{-2} h^{-1}
% silt	**0.307**
% Nitrogen	**0.424**
% org Carbon	**0.429**
C/N	0.289
Phosphate	**0.522**
pH	-0.267

The potential ammonia-oxidizing activity (PAA) in the incubations of macrophyte leaves of *P. pectinatus* collected from Lakes Gooimeer and Vossemeer (Table 2) ranged from 3.41 (± 0.75) to 8.67 (± 1.55) mmol NO_2^- plus NO_3^- m^{-2} h^{-1}. PAA was not detectable in the incubations of leaves of *Chara* sp. collected from Lake Nuldernauw. The factors lake and station were significant for the epiphytic PAA (main effect ANOVA; factor lake: df = 1, F = 70.17, $p < 0.001$; factor station: df = 2, F = 7.84, $p < 0.001$). Factors nutrient and oxygen concentration, water retention time, Secchi and sampling depth and exposure to SW wind had a significant effect on the epiphytic PAA (**Table 4**). In particular, the activities were significantly higher in the incubations of macrophytes collected from Lake Gooimeer, which had highest phosphate and nitrogen concentrations in the water.

Table 4. Results of main effect ANOVA analyses (Degree of Freedom, F and p values) on the effect of water quality and physical parameters on the potential ammonia-oxidizing activity measured on the incubation of macrophytes colleted from Lake Gooimeer and Vossemeer. Asterisks indicate significant differences between mean values (Tukey's HSD test, $p < 0.05$).

Parameters	**PAA macrophytes mmol m^{-2} h^{-1}**		
	DF	**F**	**P**
Phosphate	1	45.36	**0.000***
Kjeldahl Nitrogen	1	45.36	**0.000***
O_2	1	45.36	**0.000***
Retention time	1	45.36	**0.000***
Secchi depth	4	29.75	**0.000**
Sampling depth	4	29.75	**0.000**
Exposure to SW wind	1	45.36	**0.000***

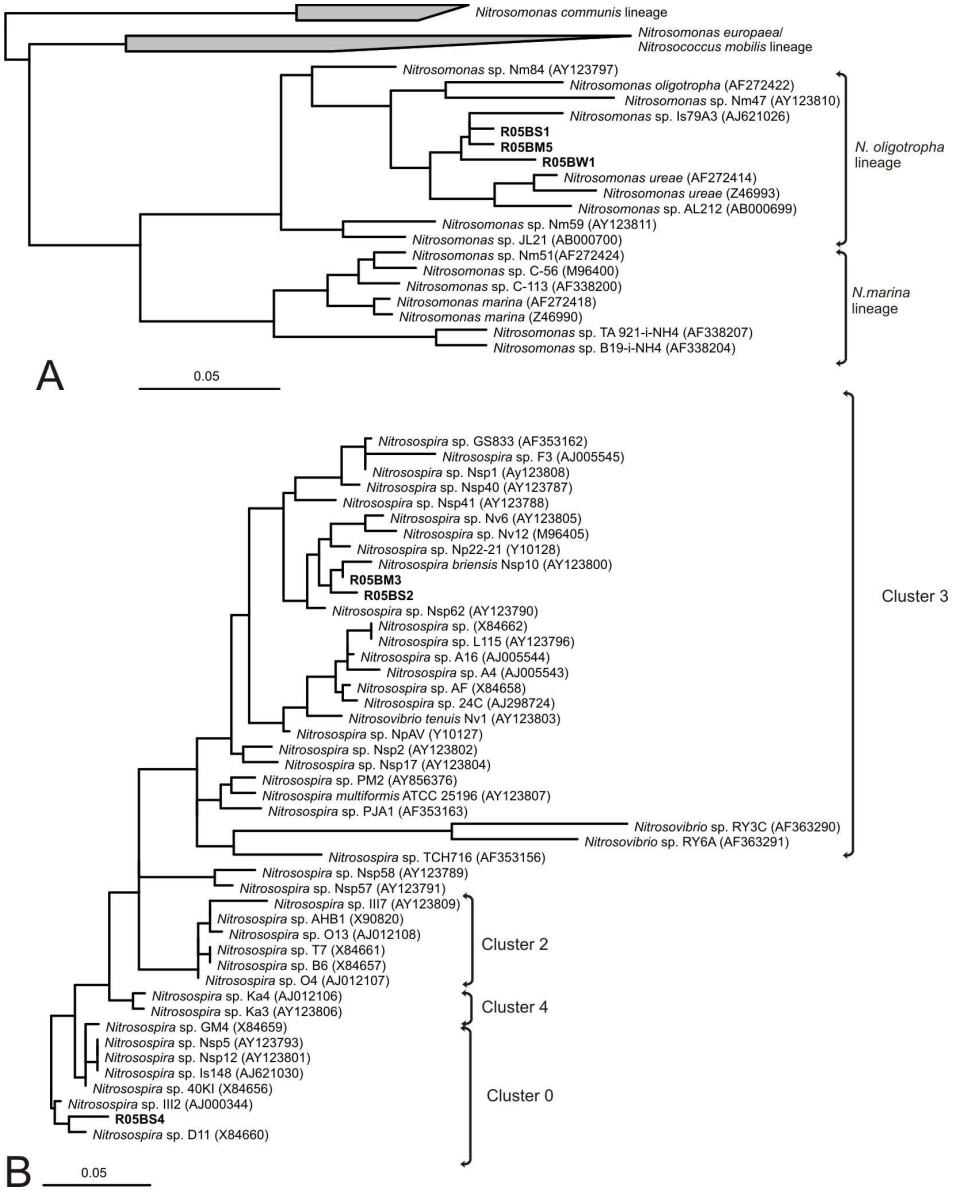
Composition and analyses of ammonia-oxidizing bacterial communities based on 16S rDNA PCR-DGGE

Replicate samples per station showed consistent band patterns in the three compartments, with coefficients of variance calculated for percentages of bands varying from 0 to a maximum of 10% (data not shown). A total of 3, 2 and 1 bands were detected in the benthic, epiphytic and pelagic compartment, respectively. Results of the phylogenetic analyses (Fig. **2A** and **B**, respectively) showed that sequences affiliated with members of the *N. oligotropha* lineages were recovered in the three compartments; sequences affiliated to the cluster 3 of the *Nitrosospira* lineage were recovered in the benthic and epiphytic compartments, while sequences affiliated to the cluster 0 of the *Nitrosospira* lineage were recovered only in the benthic compartment. Names of the bands retrieved in the different compartments and their affiliation to lineage or cluster of ammonia oxidizers [40] are summarized in **Table 5**.

Table 5. Summary of bands retrieved in this study on denaturing gradient gels in the three different compartments, grouped by lineages and clusters of ammonia-oxidizing bacteria of the beta-subclass of the Proteobacteria. [1]The first part of the band code (R05) indicate the sampling campaign of the year 2005, followed by a character B indicating the nested-PCR approach (based on the primer sets βAMOf-βAMOr and CTO189f-CTO654r), and by the character S, W or M indicating the benthic, pelagic and epiphytic compartment, respectively. Accession numbers obtained by the EMBL-Bank are given in parenthesis.

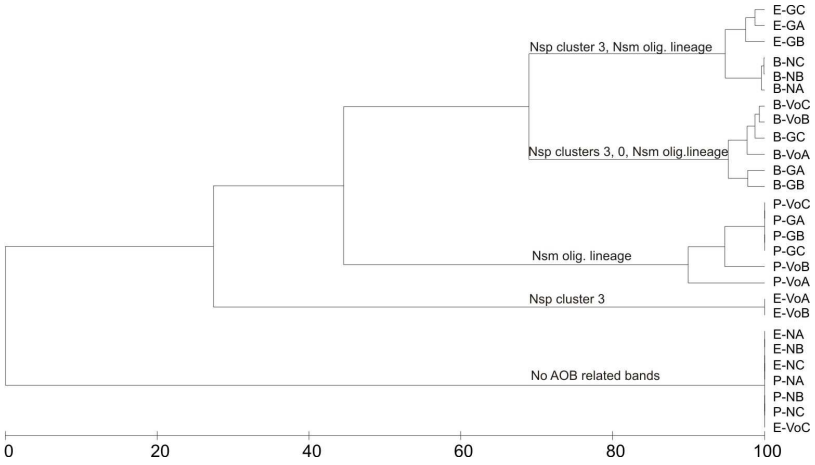
Compartment / AOB-lineages or clusters	Benthic	Pelagic	Epiphytic
N.oligotropha lineage	R05BS1[1] (**AM503957**)	R05BW1 (**AM503960**)	R05BM5 (**AM503961**)
Cluster 3 *Nitrosospira* lineage	R05BS2 (**AM503958**)	---	R05BM3 (**AM503962**)
Cluster 0 *Nitrosospira* lineage	R05BS4 (**AM503959**)	---	---

Figure 2. Phylogenetic trees based on 16S rDNA sequences of selected *Nitrosomonas* spp. (A) and *Nitrosospira* spp. (B). Bands were added to neighbor joining trees containing sequences of ammonia-oxidizing cultured strains (> 1400 bp), with the fast aligner tool of the ARB software by the maximum parsimony criterion. Bands retrieved in this study are depicted in bold. Scale bars represent 5% estimated sequence divergence.



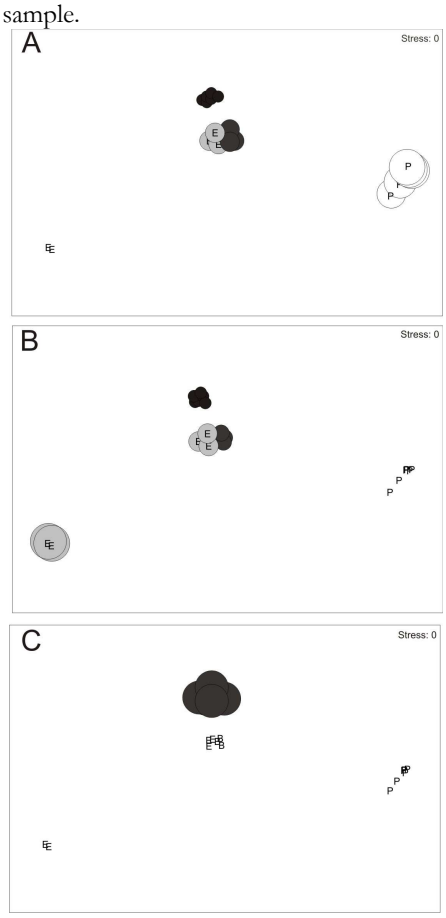
Results of the cluster analyses (**Fig. 3**) demonstrated that samples from the benthic compartments (indicated by the suffix B in Fig. 3) were more than 70% similar in their composition of AOB communities, while samples from the pelagic compartments (suffix P, **Fig. 3**) of Lakes Gooimeer and Vossemeer were more than 90% similar. The composition of the ammonia-oxidizing community of epiphytic samples (suffix E, **Fig. 3**) differed more strongly between lakes. Epiphytic samples of Lake Gooimeer were 95% similar to the benthic samples of the same lake, while epiphytic samples of Lake Vossemeer grouped together, remotely from the other samples. In the pelagic and epiphytic samples of Lake Nuldernauw, as well as in one epiphytic sample (E-VoC) of Lake Vossemeer, no ammonia oxidizers were detected.

Figure 3. Dendrogram of the cluster analyses of percentages of band present in triplicate samples from the benthic, pelagic and epiphytic compartments indicated with the suffixes B, P and E, respectively. Names of clusters and lineages of ammonia oxidizers to which the retrieved bands belonged are indicated on the branches. Abbreviations: Nsm olig stands for *Nitrosomonas oligotropha*; Nsp stands for *Nitrosospira*.



Non-metric multidimensional scaling analyses (**Fig. 4**) revealed the more frequent occurrence of members of the *Nitrosomonas oligotropha* lineage in the pelagic in comparison to the benthic and the epiphytic compartments. Members of the *Nitrosospira* cluster 3 inhabited both the benthic and epiphytic compartment and occurred more frequently in the samples of the epiphytic compartment of Lake Vossemeer.

Figure 4. Non-metric multidimensional scaling plots showing relative similarities of ammonia-oxidizing bacterial communities in the benthic (black symbols), epiphytic (grey symbols) and pelagic compartment (white symbols), belonging to different clusters and lineages of ammonia oxidizers, namely the *Nitrosomonas oligotropha* lineage (A), cluster 3 of the *Nitrosospira* lineage (B) and cluster 0 of the *Nitrosospira* lineage (C). The sizes of the circles indicate the average percentages of presence of bands in triplicate DGGE-band profiles of that particular sample.



Analyses of similarity (**Table 6**) revealed an overall significant difference of ammonia-oxidizing communities between compartments. The R_{pw} (R values for pairwise test) indicated a higher significant difference between the benthic and the pelagic compartments than between, again, the benthic and the epiphytic compartment. The epiphytic compartment was not significantly different from the pelagic one. However, considering the p value at the edge of significance (p = 0.06) obtained by the latter pair wise comparison, we repeated the analyses by excluding the pelagic and epiphytic samples where no AOB-related bands were detected. In that case pelagic and epiphytic AOB communities differed significantly from each other (R_{pw} = 0.62, p = 0.002, data not shown in **Table 6**).

Table 6. R test for dissimilarities between composition of ammonia-oxidizing bacterial communities between and within different groups, namely Compartment, Lake and Station. Three different R tests were run for each grouping procedure. The global R values (^{a}) indicate the overall significant difference of each test. In these tests samples in which no ammonia-oxidizing bacteria were retrieved were also taken into account. The $_{pw}$ characters indicate pair-wise comparison. Values in bold indicate significant differences. A detailed explanation of the R test is given in the Materials and Methods. Section.

Factor	**Pair wise comparison**	**R_{pw}**	**P_{pw}**
Compartment Global R^a = 0.348 P= **0.001**	Benthic Vs Pelagic	0.59	**0.002**
	Benthic Vs Epiphytic	0.39	**0.001**
	Pelagic Vs Epiphytic	0.10	0.063
Lake Global R^a = 0.228 P= **0.004**	Gooimeer Vs Nuldernauw	0.46	**0.007**
	Gooimeer Vs Vossemeer	-0.04	0.69
	Nuldernauw Vs Vossemeer	0.20	**0.036**
Station Global R^a = - 0.087 P= 0.1	A Vs B	-0.10	0.98
	A Vs C	-0.07	0.98
	B Vs C	-0.08	0.98

An overall significant difference between ammonia-oxidizing bacterial communities of different lakes was also obtained (**Table 6**). In particular, the communities of Lake Nuldernauw differed more from the communities of Lake Gooimeer (R_{pw} = 0.46) than from the communities of Lake Vossemeer (R_{pw} = 0.26). The latter two communities turned out not to be significantly different. Ammonia-oxidizing bacterial communities were not significantly different between stations.

Effect of environmental variables on the ammonia-oxidizing community composition

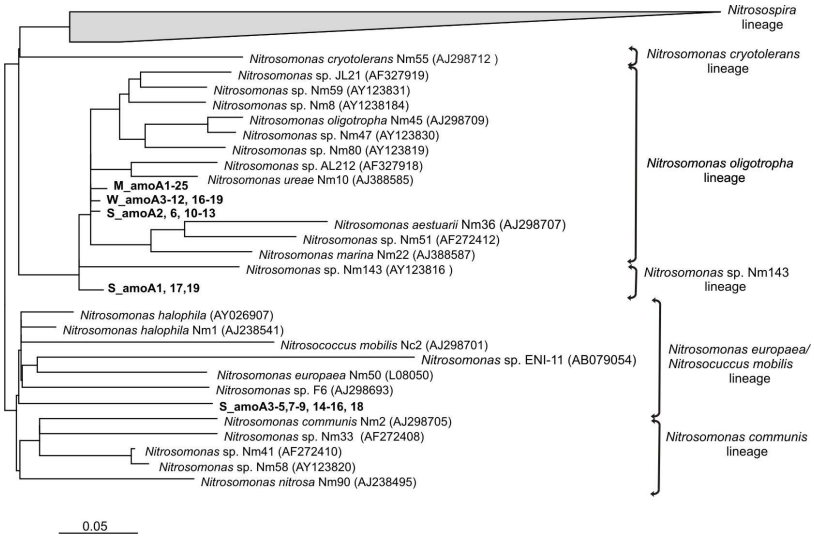
By applying the BIO-ENV procedure of the software PRIMER, we found that the composition of the benthic ammonia-oxidizing bacterial community was positively correlated with silt percentage (ϱ = 0.31) among the physical variables and with the phosphate concentration (ϱ = 0.51) among the chemical variables measured in the sediment. The pelagic and epiphytic AOB community compositions were both positively correlated with concentration of nitrogen (ϱ_p = 0.39; ϱ_e = 0.59). The exposure to South West wind had no effect on the AOB community compositions in any of the compartments.

Composition and analyses of ammonia-oxidizing bacterial communities based on a*moA*-gene clone libraries

Analyses of *amoA* clones from the three compartments of station A of Lake Gooimeer gave different results in comparison to PCR-DGGE analyses based on 16S rRNA gene. A total of 25 clones have been analyzed per compartment. Analysis of restriction enzyme fragments gave 3 different patterns for the benthic clones and only 1 for the pelagic and the epiphytic samples. In the benthic compartment, 6 out of 25 sequenced clones gave uncommon sequences not related to ammonia-oxidizers, 6 were affiliated with the *Nitrosomonas oligotropha* lineage, 3 with the *Nitrosomonas* sp. Nm 143 lineage and 10 with the *Nitrosomonas europaea* lineage. Surprisingly, no sequences affiliated with the *Nitrosospira* lineage were retrieved. In the pelagic and epiphytic compartments, 14 and 25 clones,

respectively, were affiliated with the *Nitrosomonas oligotropha* lineage. The remaining 11 clones from the pelagic compartment gave sequences not related to AOB. The results of phylogenetic analyses of *amoA* sequences are shown in **Fig. 5**.

Figure 5. Neighbor-joining phylogenetic tree based on *amoA* gene fragments of selected sequences from cultured *Nitrosomonas* and *Nitrosospira* strains. Three different libraries were constructed for the benthic, pelagic and epiphytic compartments of station A of Lake Goimmeer. Sequences obtained from the clones were added to the tree with the fast aligner tool of the ARB software by the maximum parsimony criterion. Sequences of clones obtained in this study are depicted in bold. Characters S, W and M indicate the benthic, pelagic and epiphytic compartment, respectively. Clones were numbered from 1 to 25 in each library.



Localization of ammonia-oxidizing bacteria determined with FISH

Detection of ammonia-oxidizing bacterial cells on the leaves of *P. pectinatus* was difficult by means of epifluorescence microscopy due to the autofluorescence of the macrophyte surfaces. By means of confocal laser microscopy, cells belonging to the *Nitrosospira* genus were observed on the leaves of *P. pectinatus* at stations A, B and C of Lakes Gooimeer and Vossemeer (**Fig. 6A** and **6B**). No ammonia-oxidizing bacteria-like cells were

observed on *Chara* sp. that dominated the stations of Lake Nuldernauw.

Figure 6. Images of fluorescent in situ hybridizations of ammonia-oxidizing bacterial cells in the epiphytic compartments (See Appendix for full color images). **Panel A**: Confocal laser scanning microscopy field of *Nitrosospira*-like cells (inside the marked area) on leaves of *Potamogeton pectinatus* from station C of Lake Gooimeer, double hybridized with the Cy3-labeled probe Nsv443 and the Cy5-labeled probe BET42a, stained with DAPI and viewed by phase–contrast (right image).

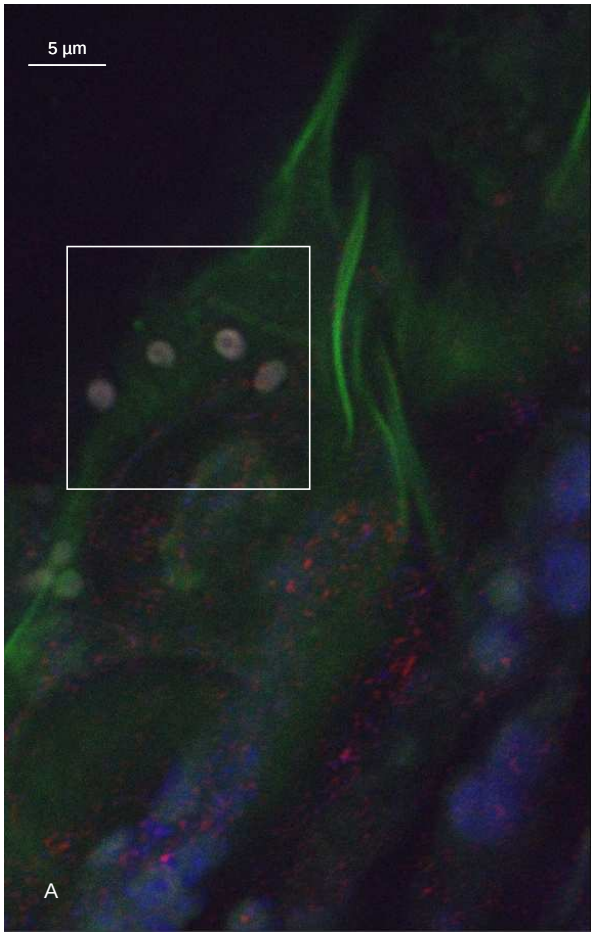
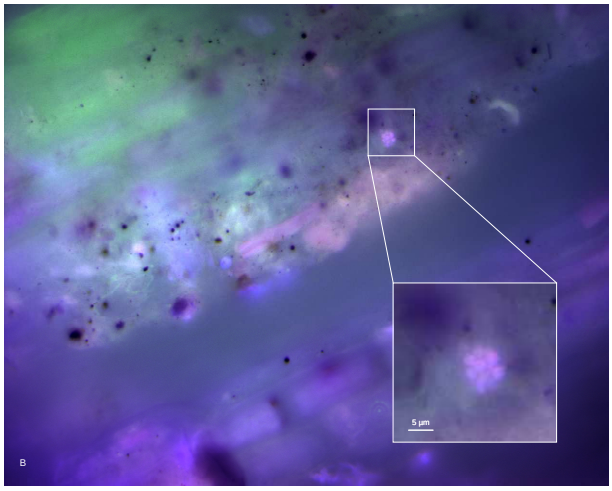
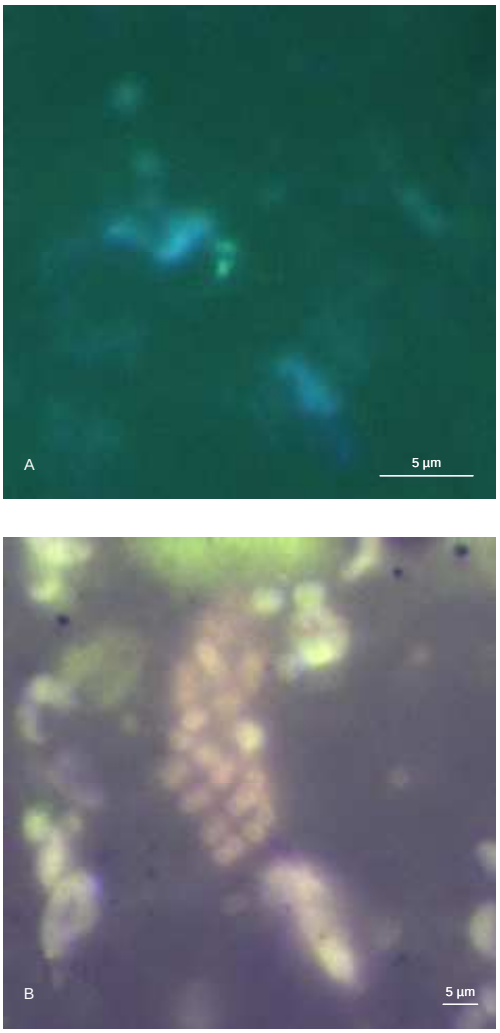


Figure 6. Images of fluorescent in situ hybridizations of ammonia-oxidizing bacterial cells in the epiphytic compartments (See Appendix for full color images). **Panel B**: Epifluorescent microscope image of a *Nitrosospira*-like cell cluster (colored in red by image analysis) and its enlarged detail, on a *Potamogeton pectinatus* leave from station A of Lake Vossemeer; image of cells double hybridized with the Cy3-label probe Nsv443 and the Cy5-labeled probe BET42a, stained with DAPI and viewed by phase–contrast.



Filters over which water samples were collected were analysed by epifluorescent microscopy. *Nitrosomonas*-like cells were detected on filters from stations A, B and C of Lake Gooimeer (**Fig 7A**), while *Nitrosospira*-like cells were observed at station C of Lake Vossemeer (**Fig 7B**). No observation of AOB cell was feasible on filters from water samples of stations A and B of Lake Vossemeer due to the excessive autofluorescence of algae. No ammonia-oxidizing bacterial cells were observed in the water samples collected from Lake Nuldernauw.

Figure 7. Images of fluorescent in situ hybridizations of ammonia-oxidizing bacterial cells in the pelagic compartment (See Appensix for full color images). **Panel A**: Epifluorescent microscope image of *Nitrosomonas*-like cells (colored in turquoise by image analysis) on filter samples from station A of Lake Gooimeer, double hybridized by Fluos-labeled probes NSO190/NSO1225 and Cy5-labeled probe BET42a and stained with DAPI. **Panel B**: Epifluorescent microscopic image of *Nitrosospira*-like cells (colored in red by image analysis) on filter samples of Lake Vossemeer, double hybridized by the Cy3-labeled probe Nsv443 and the Cy5-labeled probe BET42a and stained with DAPI.



Quantification of ammonia-oxidizing bacteria by real-time PCR

Numbers of ammonia-oxidizing bacterial cells obtained by quantitative PCR of 16S rRNA gene fragments measured from samples of sediment, macrophytes and water were converted into numbers of AOB per ml (**Fig. 8**), assuming one 16S rDNA copy per cell [171] and considering the first 5 cm of sediment and the biomass values of the macrophytes at every station. Numbers of ammonia-oxidizing bacterial cells were highly significantly different between compartments (one-way ANOVA, df= 2, F = 71, $p < 0.001$).

Results of Tukey's tests (**Table 7**) showed that numbers of AOB in the benthic compartment were significantly higher than numbers of pelagic and epiphytic AOB, being the latter two not significantly different from each other. The factor lake had also an overall significant effect (df= 2, F = 20, $p < 0.001$) on numbers of AOB, while the factor station showed no significant effect.

Figure 8. Numbers of ammonia-oxidizing bacterial cells measured by quantitative PCR of 16S rRNA gene fragments from every station and compartment of the three different lakes. Values are averages of triplicate samples. The benthic (black bars), the pelagic (white bars) and the epiphytic (grey bars) compartment are indicated.

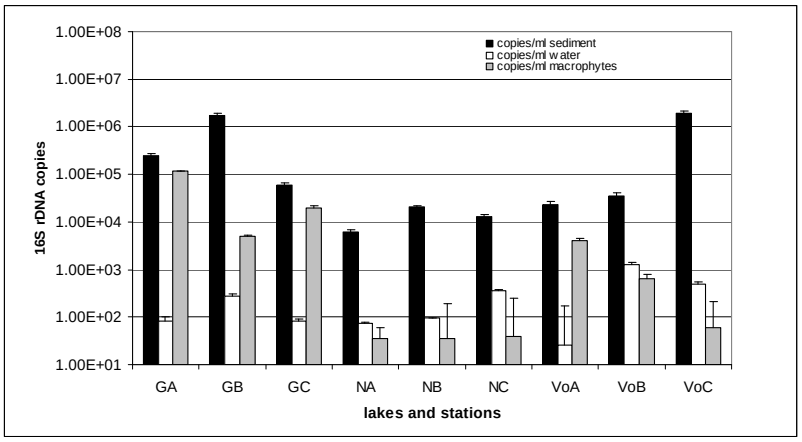


Table 7. Results of Tukey's test for honestly significant differences between mean values of numbers of ammonia-oxidizing bacterial cells per sample grouped by compartments. Values marked in bold were significant at the 0.05 level.

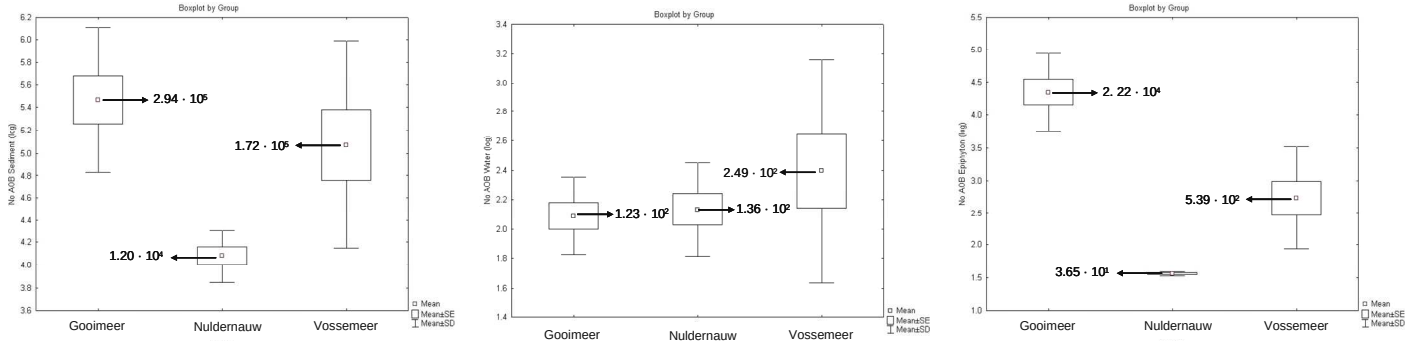
Compartment	Benthic	Pelagic
Pelagic	**0.000**	**---**
Epiphytic	**0.000**	0.079

Table 8. Results of Kruskal-Wallis test for significant differences between mean values of numbers of ammonia-oxidizing bacterial cells in the benthic, pelagic and epiphytic compartments, grouped per lake. Values marked in bold indicate significant differences in pair wise comparison test for significant differences.

Compartment	Lake	Gooimeer	Nuldernauw
Benthic	Nuldernauw	**0.000**	**---**
	Vossemeer	0.931	**0.008**
Pelagic	Nuldernauw	1.000	---
	Vossemeer	0.572	0.813
Epiphytic	Nuldernauw	**0.000**	---
	Vossemeer	**0.048**	**0.048**

The effect of the factor lake was further tested within compartments (**Fig. 9** and **Table 8**). In the benthic compartments, highest numbers of ammonia-oxidizing bacterial cells were recorded at both Lake Gooimeer and Lake Vossemeer, whose values did not significantly differ between each other (**Fig. 9**, first plot on the left and **Table 8**). However, the mean values for benthic AOB were higher in Lake Gooimeer in comparison with Lake Vossemeer. The latter showed higher variability as indicated by the size of standard errors boxes and standard deviation lines in the box plot graph. The numbers of benthic AOB in Lake Nuldernauw were significantly lower in comparison with the other two lakes (**Table 8**). Finally, mean values of numbers of benthic ammonia-oxidizing bacterial cells were $2.94 \cdot 10^5$, $1.20 \cdot 10^4$ and $1.72 \cdot 10^5$ cells ml^{-1} in Lakes Gooimeer, Nuldernauw and Vossemeer, respectively.

Figure 9. Numbers of ammonia-oxidizing bacteria cells in the benthic, pelagic and epiphytic compartment (from left to right) grouped per lake, indicated on the horizontal axis. Vertical axis is on a logarithmic scale. Boxes indicate the standard error and error bars indicate the standard deviations. In each box the average values are indicated by small boxes, connected by an arrow to mean values in the scientific notation.



In the pelagic compartments, no significant differences were recorded between lakes (**Fig. 9**, second plot on the left and **Table 8**). Mean values of numbers of pelagic ammonia-oxidizing bacterial cells were slightly higher in Lake Vossemeer that again showed higher variability. Finally, mean values of pelagic ammonia oxidizers were 1.23 10^2, 1.36 $\cdot 10^2$ and 2.49 $\cdot 10^2$ cells ml^{-1}, in Lakes Gooimeer, Nuldernauw and Vossemeer, respectively.
Apparently, numbers of ammonia-oxidizing bacterial cells measured in the epiphytic compartments showed a pattern similar to the benthic ammonia oxidizers. This was confirmed by the significant positive correlations between numbers of benthic and epiphytic AOB cells ($r = 0.93$). In particular (**Fig. 9**, third plot on the left and **Table 8**), the highest numbers of AO bacterial cells were recorded on the leaves of *Potamogeton pectinatus* collected from Lake Gooimeer. These values differed of about 2 and 3 orders of magnitude from the numbers of epiphytic AOB in Lake Vossemeer and Lake Nuldernauw, respectively. The latter were also significantly different between each other. Finally, mean values of epiphytic ammonia oxidizers were 2.22 10^4, 3.65 $\cdot 10^1$ and 5.39 $\cdot 10^2$ cells ml^{-1}, in Lakes Gooimeer, Nuldernauw and Vossemeer, respectively.

Effect of environmental variables on the numbers of ammonia-oxidizing bacteria

Spearman-rank correlation analyses (**Table 9**) showed that the numbers of benthic AOB were significantly positively correlated with the percentage of silt and negatively correlated with the C/N measured in the sediment. Numbers of benthic AOB were not influenced by the physical parameters of the lakes, *i.e.* water retention time, sampling and Secchi depth or by the exposure to the SW wind.
The numbers of pelagic ammonia-oxidizing bacteria were significantly affected by Secchi depth (one-way ANOVA, $df = 5$; $F = 19.73$; $p < 0.001$) and by the degree of exposure to SW wind (one-way ANOVA, $df = 3$; $F = 3.94$; $p < 0.05$), but were not influenced by water quality parameters or the water retention time. The numbers of pelagic AOB were significantly higher in the water column of stations B and C of Lake Vossemeer, with lowest Secchi depth and higher exposure to SW wind.

The numbers of epiphytic AOB were affected by nitrogen and phosphate concentration in the water (one-way ANOVA, nitrogen, df = 2, F = 53.93, $p < 0.001$; phosphate, df = 2, F = 53.93, $p < 0.001$), by the water retention time (one-way ANOVA, df =2, F= 53.93, $p < 0.000$) and by the degree of the exposure to SW wind (one way ANOVA, df =3; F = 6.48, $p < 0.05$).

Table 9. Results of Spearman rank correlations between number of benthic ammonia-oxidizing bacteria and chemical and physical variables measured in the sediment. Correlations marker in bold were significant at the 0.05 level.

Variables	Numbers AOB in ml sediment
% Nitrogen	-0.303
% Org Carbon	-0.252
C/N	**-0.407**
Phosphate	-0.208
% silt	**0.388**
pH	0.133

Discussion

The aim of this study was to investigate, both quantitatively and qualitatively, the ammonia-oxidizing bacterial communities in the three compartments in which shallow lakes dominated by submerged macrophytes might be ideally divided, namely the benthic, the pelagic and epiphytic compartments. The epiphyton of submerged macrophytes, in particular, represents a recently discovered niche in these ecosystems (**Chapter II** of this thesis). Furthermore and to the best of our knowledge, this study is the first report of a systematic assessment of ecological niches offered to ammonia oxidizers in freshwater lakes subjected to different degrees of restoration that appeared to influence the ammonia-oxidizing bacteria in the three compartments.

Qualitative assessment of ammonia-oxidizing bacteria

In term of diversity, the benthic ammonia-oxidizing bacterial communities were richer than those in the epiphytic compartments and above all in the pelagic compartments.

Members of the *Nitrosomonas oligotropha* lineage were present in the three compartments, and appeared to dominate the pelagic compartment, while members of cluster 3 of the *Nitrosospira* lineage were present in the benthic and the epiphytic compartments. Finally, members of cluster 0 of the *Nitrosospira* lineage were exclusively detected in the benthic compartments of both Lakes Gooimeer and Vossemeer. The dominance of members of the *N. oligotropha* lineage in the pelagic compartment is in line with various isolations of members of this lineage from freshwater habitats [73, 172], as well as with other studies conducted in the same sampling area [84], based both on 16S rRNA and *amoA* genes retrieval. Shallow freshwater habitats appeared to be different from marine environments where particles associated sequences were predominantly related to the *N. europaea* lineage, while planktonic sequences were related to cluster 1 of the *Nitrosospira* lineage, for which there is no cultured representative yet [83]. Ammonia-oxidizers of the recently defined cluster 0 of the *Nitrosospira* lineage [141] are mainly present in undisturbed and unfertilized soils [57, 70], but they have been also retrieved from the same sampling area used in this study by Speksnijder et al. [84]. The retrieval of 16S rRNA sequences of members of the cluster 0 of the *Nitrosospira* lineage proves neither that the organism is abundant nor that is physiologically active [40], but it shows its presence in that particular environment. Considering that the area we investigated is receiving groundwater from the nearby land, we might suggest that the species has a terrestrial origin and is therefore not autochthonous for freshwater environment.

More interestingly, we characterized the epiphytic ammonia-oxidizing bacterial communities as composed both by members of the *N. oligotropha* lineage and the cluster 3 of the *Nitrosospira* lineage. That latter was mostly present in the epiphyton of *P. pectinatus* leaves collected at Lake Vossemeer, which was the shallowest of the lakes investigated and it was heavily exposed to

the action of the SW wind. It seems likely that resuspension of sediment particles by wind action enhanced the colonization of the macrophytes surfaces by AOB originating from the sediment in Lake Vossemeer. Another interesting aspect is that members of the *N. oligotropha,* possibly originating from the water column, were detected only in epiphytic samples of Lake Gooimeer characterized by the highest retention time value. Some members of the *N. oligotropha* are known to produce exopolymeric materials [173] that contribute to the establishment of biofilms at different surfaces [159, 174]. Hence, submerged macrophytes may provide a surface for the attachment of members of this AO bacterial lineage. However, as for the waste water, long enough retention times are necessary to avoid washing away of the pelagic bacterial cells [175]. In contrast with *Potamogeton pectinatus*, leaves of *Chara* sp. resulted negative to the detection of AOB cells, at least with 16S rDNA based PCR-DGGE assay.

Regarding the presence of ammonia-oxidizing bacteria in the epiphyton of *P. pectinatus*, this study provided also the first report of microscopy images of ammonia-oxidizing bacteria hybridized with a combination of oligonucleotidic probes with nested specificity and good sensitivity, as suggested by Purkhold et al [141] after the successful study of Mobarry et al. [169]. Generally, the results of microscopy analyses of FISH reactions supported the description of AO bacterial communities based on 16S rDNA-DGGE analyses. *Nitrosospira* cells were detected on the leaves of *P. pectinatus* in samples from both Lake Gooimeer and Lake Vossemeer, while members of the *N. oligotropha* lineage were detected on membrane filters containing materials from water samples of Lake Gooimeer. In contrast, membrane filters with materials from water samples of Lake Vossemeer contained high numbers of cells of the *Nitrosospira* lineage that were never detected in the pelagic compartment by 16S rRNA PCR-DGGE analyses. However, methodological factors may have influenced our results. In a diversity study between particle-associated and planktonic AOB, Phillips et al. [83] suggested that differences in size between *Nitrosomonas* and *Nitrosospira* cells could affect differential filtration, finally leading

to a greater relative abundance of *Nitrosospira* sequences associated with particles.

Unfortunately, our data on the composition of AOB community assessed by 16S rDNA-based DGGE and FISH analyses were not supported by the results of the *amoA* clone libraries. To overcome the difficulties encountered in the direct amplification of *amoA* fragments from DNA samples, we applied a pre-PCR, whole-genome amplification (WGA) [176], based on multiple displacement amplification (MDA) of the Φ29 Taq Polymerase. For the 16S rDNA we used instead a nested approach involving two sets of primers specific for the AOB. Recently, it has been shown that the WGA performance is significantly affected by the initial DNA concentrations [177] and that an amount of DNA template less than 10 ng produces artefacts during the MDA amplification [178]. Even more, in a simulated environmental sample of eight different isolates, Abulencia et al. [179] showed that *N. europaea* was preferentially amplified. Those results might explain the discrepancy between the AOB communities composition described in our study by *amoA* clones and by 16S rDNA analyses. In addition, the DGGE analyses of the 16S rRNA genes produced almost identical patterns for triplicate samples in the benthic, pelagic and epiphytic compartments, which strengthen the results.

The ongoing hypothesis of the distribution of different strains of ammonia-oxidizing bacteria in relation to different habitats [44] appeared to be again confirmed by our study. In this perspective the epiphyton of submerged macrophytes might represent a favourable niche for ammonia-oxidizers in shallow freshwater environments dominated by certain species of submerged macrophytes. Furthermore, our results demonstrated the importance of the overall condition of the lakes on the diversity of the AO bacterial communities. The communities of the most restored lake, *i.e.* Lake Nuldernauw, were significantly poorer in term of diversity from the communities of Lakes Gooimeer and Vossemeer. The factor station was not significant for the different communities investigated in our study, while the factor lake had a highly significant effect. This implies that the overall conditions of the lake influenced the diversity of the ammonia-oxidizing community more strongly than the conditions at each

single station. The composition of the benthic community was correlated with the percentage of silt and the concentration of phosphate in the sediment. Both factors seemed to have a positive effect on the diversity of the benthic ammonia-oxidizing bacterial communities that in fact was lower in the sediment of Lake Nuldernauw, which has more sandy sediment and the lowest phosphate concentrations, as a result of heavy restoration measures. To the best of our knowledge, there are no studies on the effect of both sediment texture and phosphate on the diversity of AOB. However, the adsorption of ammonia by clay minerals may provide localized surface-associate ammonia oxidation [29], thereby enhancing the nitrification process. This has already been applied in waste water plants and constructed wetlands for the removal of nitrogen [180, 181]. In our case, the lower concentration of phosphate must be considered as an indicator of a higher degree of anthropogenic impact, which might have caused a lower diversity in the AOB communities. Similarly, Webster et al. [65] demonstrated lower AOB diversity in grassland soil subjected to fertilization measure. Finally, ammonia-oxidizing bacteria seemed to respond to the restoration measures, indicating their potential to be used in monitoring programs.

Quantitative assessment of ammonia-oxidizing bacteria

As reported in other studies [73, 89, 96], the numbers of ammonia-oxidizing bacterial cells were higher in the benthic compartments, in comparison with the pelagic and epiphytic compartment, which resulted not significantly different from each other. This study provides the first report of numbers of AOB in freshwater habitats assessed by quantitative PCR rather then Most Probable Numbers. In addition, correlations were found between numbers of AOB and the environmental variables characterizing the lakes. The latter affected the communities of AOB substantially both quantitatively and qualitatively.

In the pelagic compartment, the overall effect of lake did not affect the numbers of ammonia-oxidizing bacterial cells substantially. Nevertheless, the numbers of pelagic ammonia

oxidizers showed higher variability in the samples of Lake Vossemeer, where the shallowness and the exposure to wind might have been responsible for the resuspension of sediment particles, and consequentially of attached bacteria in the water column, as confirmed by the significant effect of the Secchi depth and the exposure to wind in our analyses.

As already said, numbers of ammonia-oxidizing bacterial cells measured in the epiphytic compartments showed a pattern similar to benthic ammonia oxidizers. Of particular interest is the difference between the numbers of AOB cells present on the two different submerged plants investigated, namely *Chara* sp., dominating Lake Nuldernauw, and *P. pectinatus,* that colonized Lakes Gooimeer and Vossemeer, however reaching different biomass values in relation to the shallowness of the lakes. *P. pectinatus* is a vascular plant with high shoot surface area, therefore being easily accessible by microorganisms for the attachment, in comparison to individuals of *Chara* sp. that are anatomically highly developed green algae with lower surface area: volume ratio. Moreover *Chara* spp. contain high concentration of sulphur compounds [182] that could inhibit the nitrification process [183]. Furthermore, no AOB were retrieved on the leaves of *Chara* sp. by 16S rDNA analyses, suggesting a presence at the edge of the detection limit of the assay. At present, there are only few studies on AOB associated with different plant species [184-186]. The fact that there are none on freshwater environments nor on the actual numbers of bacteria makes a comparison with other studies difficult. However, the consequences of a specific association of bacteria to particular macrophytes might be suggested by our potential activity measurements that again showed the non-supporting role of *Charophytes* for AOB, hence for nitrification. In a study on potential nitrification on litter of macrophytes, Eriksson [101] found that potential activity of indigenous attached nitrifying bacteria differed between macrophytes species and advocated that this was caused by the differences in physical and chemical attributes of the macrophytes themselves, as well as their effect of environmental variables within macrophyte beds. Both reasons may be supported by our results, since the presence of particular species of macrophytes is both a cause and a

consequence of the trophic status as well as the hydro-dynamics of the lakes. As a matter of fact, Charophytes were introduced in Lake Nuldernauw during the restoration process, for their strong positive effect on water transparency. Charophytes decrease the sediment resuspension by creating dense and thick rhizoid layer as well by taking up of nutrients, increasing zooplankton grazing and releasing allelopathic substances [95, 187-189]. Studies on the nitrogen uptake by Charophytes [190] indicated their preferential uptake of NH_3 rather than NO_3^- by both from the below- and above-ground parts of the macroalgae, evoking a possible competition for NH_4 with epiphytic AOB. Moreover, a significant depletion of NH_3 from the sediment caused by *Chara* sp. [190] suggested a competitive role to the N demand also with benthic ammonia oxidizers. This might contribute to explaining the significantly lower PAA rate measured in the benthic samples of Lake Nuldernauw, in addition to the effect of the environmental variables.

Simultaneous estimates of numbers of ammonia-oxidizing bacterial cells and their potential activities in different compartments might allow correlations and inference on the impact of nitrification in shallow freshwater habitats. However, we found no significant correlations between the two measurements. In this study, quantitative data were obtained on 16S rRNA gene analyses, while studies on the expression of the *amoA* might provide accurate data on the active portion of ammonia-oxidizing bacterial cells within complex communities. More recently, Freitag et al. [58] used stable isotope probing on nucleic acids of AOB, providing a new tool for the correct assessment of active and inactive ammonia-oxidizing bacterial populations.

Conclusions

The communities of ammonia-oxidizing bacteria in the benthic, pelagic and epiphytic compartments differed between each other both qualitatively and quantitatively. The epiphyton of certain species of macrophytes offers an ideal niche for the attachment of both benthic and pelagic ammonia-oxidizing bacteria.

Ammonia-oxidizing bacteria seemed to respond to restoration procedures by decreasing in diversity, numbers, and potential

activities. The importance of the epiphytic ammonia-oxidizing bacterial communities must be recognized as essential for the internal nitrogen turnover, as well as possible source of nitrifying cells to cope with increasing nitrogen inputs in shallow freshwater lakes.

Chapter IV

The origin of epiphytic ammonia-oxidizing bacteria: in freshwater shallow lakes

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Abstract

The presence of ammonia-oxidizing bacteria in the epiphyton of the submerged macrophyte *P. pectinatus,* in natural shallow freshwater lakes has been recently assessed, but the origin of the epiphytic ammonia-oxidizing community remained unexplained. In a microcosm experiment, we demonstrated that both benthic and pelagic ammonia-oxidizing bacteria (AOB) were able to colonize the epiphyton of submerged macrophytes. In particular benthic AOB of the *Nitrosospira* lineage colonized the plants during their sprouting as well as during the resuspension of sediment particles. Interestingly, the epiphyton offered a niche to pelagic ammonia-oxidizing bacteria of the *Nitrosomonas oligotropha* lineage, otherwise not detectable in the water. Members of this lineage became attached to the macrophytes. The impact of the epiphytic ammonia-oxidizing communities on the nitrification process was not assessable, due to the uptake of nitrogen by the submerged macrophytes in the system. However, evidences of the colonization of submerged macrophyte by AOB were provided as bases for future studies on the role of the epiphytic AOB communities in shallow freshwater lakes.

Introduction

Ammonia-oxidizing bacteria (AOB) are Gram-negative, obligate aerobic chemolithotrophic microorganisms, which generate their energy by oxidizing ammonium to nitrite. At present, two phylogenetically distinct groups of AOB are defined [35, 40]. One group is affiliated with the gamma-subclass of the Proteobacteria and includes two species *Nitrosococcus oceani* [36-38] and *Nitrosococcus halophilus* [39]. Both species seem to be restricted to marine environments [82]. The other group is affiliated with the beta-subclass of the Proteobacteria and contains four genera: *Nitrosomonas*, *Nitrosospira*, *Nitrosovibrio* and *Nitrosolobus,* the latter three being closely related with each other [35]. The development of molecular techniques had overcome many of the difficulties inherent to cultivation-based methods applied to these slow-growing chemolithotrophic bacteria. Additionally, the combination of different molecular techniques has led to the detection of ammonia-oxidizing bacteria in a great variety of environments [48, 68, 73, 113, 115, 191, 192]. This knowledge has further contributed to the hypothesis that different ammonia-oxidizing bacteria are associated with specific ecological niches [44, 45].

In freshwater environments in particular, members of the *Nitrosomonas oligotropha* lineage, addressed also as cluster 6a [50], are considered to be dominant, based on repeated isolation [73, 172] and on 16S rRNA and *amoA* sequence retrieval [84, 89, 90]. However, members of the *Nitrosospira* lineage are also found in freshwater environments [47, 89]. In addition, when defining an ammonia-oxidizing bacterial population as dominant in an environment, it should always be carefully considered whether the retrieved species are autochthonous or allochthonous members of the analyzed ecosystem [40].

Recently, we described in detail the diversity of the ammonia-oxidizing bacterial communities inhabiting the sediment and the water column of man-made shallow freshwater lakes (Chapters II and III of this thesis). The benthic ammonia-oxidizing bacterial community was composed of members of clusters 0 and 3 of the *Nitrosospira* lineage, as well as of members of the *N. oligotropha* lineage. The latter were the only members of the pelagic community. In addition, we described the epiphyton of

submerged macrophytes as a novel niche for ammonia-oxidizing bacteria containing members of both the benthic and pelagic communities. However, at that time, the exact origin of the epiphytic ammonia-oxidizing communities and the process of colonization were not established.

Submerged macrophytes are often a major component in shallow freshwater lakes and several studies recognized their importance in the nitrogen turnover of these environments, firstly by the direct uptake and assimilation of nitrogen [107, 193, 194] and secondly by offering a large accessible area for the attachment and proliferation of epiphytic communities, which directly contribute to the nitrogen turnover [162, 195]. Epiphytic communities can sustain high nitrification-denitrification rates [97, 99, 103, 196, 197]. However, nitrification and consequentially the coupled process of nitrification-denitrification, is sensitive to environmental stresses and often markedly suppressed by human activities that finally lead to eutrophication [145].

Here we present a study in which a microcosm experiment was designed to i) assess the origin of epiphytic ammonia-oxidizing bacteria, ii) measure nitrification rates in the epiphytic compartment, iii) test the effect of different concentrations of ammonium on the colonization of submerged macrophytes by ammonia-oxidizing bacteria.

Material and Methods

Submerged macrophyte

Potamogeton pectinatus (Linnaeus 1753, common name Fennel pondweed) is a submerged aquatic macrophyte, mostly present in oligotrophic, eutrophic and even polluted, standing or moderately turbulent waters. In Lake Vossemeer, which is a shallow freshwater lake in the centre of the Netherlands, *P. pectinatus* is basically the only submerged macrophyte present and it grows in stands in confined areas near the shore with coverage percentages ranging from 20 up to 80 %. Tubers of *P. pectinatus* were collected at the end of the growing season. Tubers were surface sterilized as described by Bodelier et al. [198]. Absence of fungi on the tubers was checked on Tryptic Soy Agar

(Difco™) plates incubated for 3 days at 28 °C in the dark. Selected seeds were stored for 2 days in sterile water at 4 °C till seeding.

Experimental set-up: initial phase and general conditions

To investigate the ability of pelagic and benthic ammonia-oxidizing bacteria to colonize the surface of *P. pectinatus* individuals, we used fresh sediment and water collected from Lake Vossemeer. For the experiment 64 sterile glass bottles were used, with a total volume of approximately 950 ml, a bottom diameter of 8 cm and a height of 19 cm. Two types of microcosm were prepared: the suspended sediment type (SS) and the bottom sediment type (BS). The 32 bottles, prepared as SS type microcosms, were filled with approximately 400 g dry sterile sand. 3 g of fresh sediment were added simultaneously with the concentrated media (see below). The sand itself was covered with textile discs with a central hole to allow the seeding and sprouting of one tuber of *P. pectinatus*. Sterile quartz stones (about 1.5 cm diameter) were placed on the discs to prevent resuspension of sand during the mixing events with a disposable plastic stirrer, necessary for the resuspension of the sediment. In this type of microcosm the resuspended sediment mimicked the sediment particles in the water column of the lake suspended by the action of the wind.

The other 32 bottles, prepared as the BS type microcosms, were filled with 300 g of fresh, moist sediment collected from Lake Vossemeer. In this type of microcosm the benthic AOB were associated with the sediment located in the bottom and could only colonize the macrophytes during the sprouting of the tubers. Sand and fresh sediment in the SS and BS types of microcosms, respectively, had an approximate volume of 250 ml and served as substratum for the growth of *P. pectinatus*.

The SS and BS type of microcosms were further divided into 4 treatments named PL, L, P and C, depending on the presence of *P. pectinatus* plants (P) and lake water (L). Treatment C represented a control, without *P. pectinatus* and lake water. A summary of the experimental design is given in **Table 1**. Each treatment was performed in 4 replicate bottles; three with ^{14}N-

ammonium added and one as control. During the initial phase, 300 ml sterile water was added to treatments PL and L and 600 ml to P and C, to allow the sprouting of the tubers. The experiment was conducted in a closed chamber at 20-23 °C, with a light intensity of 224-226 μmol s^{-1} $m^{-}2$ (LI-189, light meter, LI-COR, Lincoln, Neb., USA), with a light-dark cycle of 12 h. The experiment lasted for 35 days, plus 7 initial days needed for the sprouting of the tubers.

Table 1. Summary of the experimental setup. Two different types of microcosms were prepared, namely the suspended and the bottom sediment types. Four treatments (PL, L, P, C) were applied differing with respect to the presence of *Potamogeton pectinatus* and natural water from the lake. Four replicate microcosms were used for each type of treatment of which 3 contained ^{15}N labeled media, and one contained unlabeled media. The experiment has been done with two concentrations of ammonium, *i.e.* 0.2 and 2.0 mM. [a] Numbers of replicate indicated in the table referred to only one concentration of ammonium.

Microcosm type	Treatment			# replicate[a] ^{15}N-NH_4^+	# replicate[a] control
	code	*P. pectinatus*	*Lake water*		
Suspended Sediment (SS)	PL	+	+	3	1
	L	-	+	3	1
	P	+	-	3	1
	C	-	-	3	1
Bottom Sediment (BS)	PL	+	+	3	1
	L	-	+	3	1
	P	+	-	3	1
	C	-	-	3	1

Experimental set-up: addition of pelagic ammonia-oxidizing bacteria and media

The basic medium contained 10 mM NaCl, 0.2 mM $MgSO_4 \cdot 7H_2O$, 1 mM $CaCl_2 \cdot 2H_2O$, 1 mM KCl, 0.4 mM KH_2PO_4 (sterilized separately) and 1 ml l^{-1} of the trace element solution described by Verhagen and Laanbroek [129]. Two different concentrations of ammonium were used, namely 0.2 and 2 mM, added as $(NH_4)_2SO_4$. 48 microcosms were prepared with ^{15}N-labeled media containing 2.5 atom % ^{15}N-NH_4Cl (98 atom %) as part of the 0.2 or 2.0 mM $(NH_4)_2SO_4$ solutions. The 16 microcosms without ^{15}N-labeled media were used as control for

the stable isotope analysis. 100 ml of 7X concentrated media were added to the microcosms after the sprouting of the tubers.
The experiment started when 300 ml of lake water and 3 g of fresh sediment were added to the appropriate microcosms as inoculum of pelagic and benthic ammonia-oxidizing bacteria, respectively. Based on quantitative PCR data, 3 gram of fresh sediment from Lake Vossemeer contained approximately $5 \cdot 10^4$ ammonia-oxidizing bacterial cells (**Chapter III** of this thesis).
In order to maintain the concentrations of ammonium at the values of 0.2 and 2 mM in the appropriate microcosms, ammonium concentrations in the microcosms were measured with the colorimetric assay described by Kandeler and Gerber [199]. Measurements were performed at the initial phase (T_{in}), immediately after the addition of the media (T_0) and then weekly. Ammonium concentrations were restored to the initial values by adding the appropriate volumes of 7X concentrated media.

Monitoring of environmental conditions

The pH and the concentration of oxygen in the water were monitored during the experiment. Neutral pH and oxic conditions were necessary for the growth and activity of the ammonia-oxidizing bacteria. The concentration of oxygen and the pH were measured with Oxi330i and pH330i portable meters (WTW, Weilheim, Germany), respectively. Measurements were done once during the period of the germination of the tubers, 24 hours after the addition of media, suspended sediment and lake water, and further weekly till the end of the experiment, alternatively in the light and in the dark phases.
During the experiment, we observed the growth of algae in the microcosms. Therefore, on Day 20 of the experiment (T_{20}) samples were collected from the water phase of all microcosms and fixed with 0.01% paraformaldehyde and 0.1 % glutaraldehyde (final volume), stored at 4 °C in the dark for no longer then 2 days and analysed using the flow cytometer model MoFlo (Dako, Ft. Collins, Co., USA). Cell clusters of cyanobacteria, eukaryotic algae and prochlorophytes were distinguished by their differential clustering in the field characterized by orange and red fluorescence, while bacterial cells and detritus particles were counted in the same fraction. All

groups were normalized for the total flow cytometer counting events and expressed in percentages. To better estimate the differences between the microcosms containing 0.2 and 2.0 mM NH_4^+, the percentages of the different algal groups were normalized for the total of counting events of algae, excluding the counting events of bacterial cells and detritus particles.

Harvesting and processing of plants, water and sediment samples

To avoid any possible bias during harvesting, which took 3 days, replicate microcosms were equally distributed in 3 sets. Where present, plants of *P. pectinatus* were removed using sterile tweezers and quickly dried on blotting paper. After cutting off the roots, shoots and roots were weighted for biomass. The shoots were then freeze-dried in plastic bags for DNA isolation. Stones and textile discs from the SS type microcosms were collected with sterile tweezers, placed in plastic beakers containing 100 ml sterile water and shaken at 180 rpm water for 48 hours. The water phase was centrifuged at 6000 g for 2 hours and the pellet was stored at –20 ºC for DNA isolation. This material represented the suspended sediment. Aliquots of sediment and sand were also freeze-dried for DNA isolation.

The water phase of each microcosm was processed for different analyses. 100 ml of water were filtered over glass fiber filter (diameter 47 mm) to measure the biomass of seston. Another 100 ml of water were first sieved over a 125 μm mesh and then filtered over 0.2 μm membrane filters. Both filter types were used also for DNA isolation from the seston and the water, respectively. The remaining volume of the water was used to measure the final NH_4^+ and NO_2^- plus NO_3^- concentrations with a Technicon Auto-Analyzer (Traacs 800) and for the stable isotope analyses, as described in detail below.

DNA isolation and PCR-DGGE analyses of the ammonia-oxidizing bacterial communities

DNA was isolated from fresh sediment and water immediately after collection from Lake Vossemeer (AOB community at T_0). DNA was isolated from samples of sediment, suspended sediment, freeze-dried macrophytes and seston, and from the 0.2

μm membrane filters collected at the end of the experiment. The DNA isolation protocol combined mechanical disruption, chemical lyses of cells and extraction with phenol-chloroform-isopropanol solution. The protocol has been described in detail in the Materials and Methods section of Chapter III of this thesis. The composition of the ammonia-oxidizing bacterial communities was analyzed by PCR-DGGE. DNA amplifications were done by using two sets of primers specific for the 16S rDNA V3 region of ammonia-oxidizing bacteria of the beta-subclass of Proteobacteria in a nested way, namely i) the primer sets βAMOf- βAMOr [49, 113] and ii) CTO189f (with GC clamp)-CTO654r [49]. Thermocycling conditions were applied as described by the authors. The CTO fragments obtained were separated on gels with a denaturing gradient of 30-55% for 16 h at 60 °C at 75 V in 0.5 X TAE solutions. Agarose gels, used to verify PCR products, and polyacrilamide gels, used to separate CTO-PCR products, were stained in a ethidium bromide solution and visualized with UV. Bands separated on denaturing gradient gels were cut in their middle portions, eluted overnight from acrylamide, reamplified for 20 cycles with the CTO primer set and reloaded on denaturing gradient gels to obtain single and pure bands suitable for sequencing.

Nucleotide accession numbers

Sequences obtained from bands separated on denaturing gradient gels were aligned with selected cultures strains of ammonia-oxidizing bacteria by using the fast aligner tool of the ARB software [134] and added to phylogenetic neighbour-joining trees using the maximum parsimony method and *ad hoc* created filters [134]. Sequences were submitted to the EMBL-Bank under the following accession number: **AM503570** (band dggecoe1); **AM503571** (band dggecoe8); **AM503572** (band dggecoe9); **AM503573** (band dggecoe11).

^{15}N stable isotope measurements

Nitrogen isotopic ratios were expressed in the delta notation ($\delta^{15}N$ ‰). $\delta^{15}N$ values were measured directly from freeze-dried samples of sand, sediment and macrophytes, weighted into tin cups pre-washed in a 50/50 (v/v) methanol/chloroform solution. $\delta^{15}N$ values in the water columns were measured directly from the seston collected on glass fiber filters. $\delta^{15}N$ of NH_4^+ and NO_3^- pools were measured from the water using the modification of the diffusion method described by Holmes et al. [200]. Briefly, water was filtered over glass fiber filters. 200 and 100 ml of filtered water were used for the 0.2 and 2.0 mM NH_4^+ types, respectively. Volumes were transferred to 500-ml bottles (Fisherbrand, Fisher Scientific, Netherlands). The diffusion method allowed trapping of NH_3 on an acidified 10 mm GF/D filter, hermetically closed between two Teflon filters, in a basic environment, created by adding MgO to the filtered water. The filters sandwiches remained floating on the water surface in closed bottles for approximately 10 days after which they were removed and dried for 2 days. $\delta^{15}N$ values measured from these filters corresponded to the ^{15}N-NH_4^+ pool. New acidified filters were placed in the bottles and Devarda's Alloy was added to convert NO_2^- plus NO_3^- into NH_3, which was again trapped on the filters. $\delta^{15}N$ values measured from these filters corresponded to the ^{15}N-NO_2^- plus ^{15}N-NO_3^- pool. All samples were measured using a Carlo Erba 1006 Elemental Analyzer coupled on line with a Finnigan Delta-S Isotope Ratio Mass Spectrometer (IRMS). Stable isotopes ratios expressed in δ-notation were corrected with the international standard value for nitrogen ($^{15}N/^{14}N$ ratio= 0.003676 Air). The ^{15}N fractions were calculated from the $^{15}N/^{14}N$ ratios of the samples subtracted by $^{15}N/^{14}N$ ratios of the relative controls. The absolute amounts of ^{15}N label incorporated (^{15}N) were calculated as follow: $^{15}N = (F_s^{15} - F_c^{15}) \times N$, where F^{15} indicate the ^{15}N fraction of the labeled samples and the controls (F_s^{15} and F_c^{15}) and N indicate the total N concentration of the samples [201].

The nitrogen content was assumed to be 15% of the dry mass of cells of the seston. Since no nitrate was measured at the end of

the experiment with the automatic analyzer, we assumed a nitrate concentration below the detection limit of the instrument, *i.e.* 10 μM. Percentages of ^{15}N recovered in the different compartments were calculated considering the total amount of ^{15}N label added during the 5 weeks of the experiment.

Statistical analyses

Statistical analyses were performed using the software STATISTICA 7 (Statsoft Inc., Tulsa, Oklahoma). Data were checked for normality and transformed to obtain homogeneity of variance and allow analyses of variance. Effects of different factors were tested with ANOVA analyses. Tukey's HSD tests was used to calculated significant differences between mean values. Correlation analyses between measured variables were performed with Spearman–rank correlation tests.

Results

Monitoring of the environmental conditions

During the 35 days of the actual experiment, pH values and oxygen concentration were maintained in the ranges optimal for the growth and the activity of ammonia-oxidizing bacteria. pH values oscillated from 6.0 ± 0.2 to 9.2 ± 0.2 units, in the dark and light phases, respectively. No statistically significant differences were observed between the two different types of microcosms and between the two concentrations of ammonium applied, or between the treatments. Oxygen concentrations oscillated during the day-light cycle from 1.12 mg l^{-1} in the dark to 13 mg l^{-1} in the light. pH and oxygen values were positively correlated with each other.

Flow cytometer analyses of the seston component showed that the percentages of cyanobacteria, eukaryotic algae, prochlorophytes and bacterial cells plus detritus particles differed significantly depending on the microcosm type, treatment and concentration of ammonium, as shown in **Table 2**.

Table 2. Results of 3-way ANOVA analyses (degrees of freedom, F and p values) showing the effects of the factors microcosm type, treatment and ammonium concentration and their combined effects on the percentages of cyanobacteria, eukaryotic algae, prochlorophytes and small particles including bacterial cells plus detritus. Values marked in bold were significant.

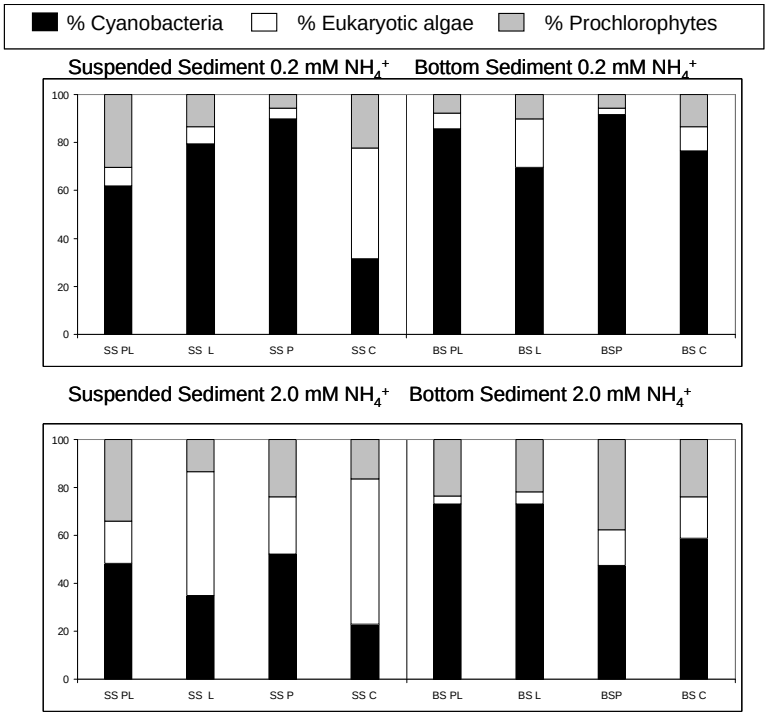
Factor	df	F	P
microcosm type	4	9.543	**0.000**
treatment	12	10.051	**0.000**
ammonia	4	7.143	**0.000**
type*treatment	12	3.657	**0.000**
type*ammonia	4	5.052	**0.002**
treatment*ammonia	12	1.309	0.222
type*treatment*ammonia	12	1.130	0.343

However, the bacterial cells and detritus particles represented approximately 70-80% of the seston community, except for the SS and BS microcosms subjected to treatment P, where they reached only 50% of the total, independently upon the concentration of ammonium (data not shown). The algal component of the seston (**Fig. 1**) included cyanobacteria to a total of approximately 65-95% in the microcosms containing 0.2 mM NH_4^+, except for the SS-C microcosms where cyanobacteria represented only 25% of the total algal component. The relative percentages of eukaryotic algae and prochlorophytes increased from a minimum of 10 to a maximum of 60% in the microcosms containing 2.0 mM NH_4^+, in comparison with the microcosms containing 0.2 mM NH_4^+.

During the experiment, the concentrations of NH_4^+ were restored four times (T_{10}, T_{17}, T_{24} and T_{31}) in all microcosms, as shown in **Figure 2**. In the SS type of the microcosms containing 0.2 mM NH_4^+ in the water (**Fig. 2**, upper left panel), the profiles of ammonium in the treatments PL, L and P were similar and ammonium was totally periodically consumed. In contrast, in the microcosms, without *P. pectinatus* and lake water (*i.e.* C), the concentrations of ammonium never dropped to zero, but reached a minimum concentration of 0.04 mM. In the BS type of microcosms containing also 0.2 mM NH_4^+ (**Fig. 2**, lower left

panel), the concentrations of ammonium periodically dropped to zero in treatments PL, L and P.

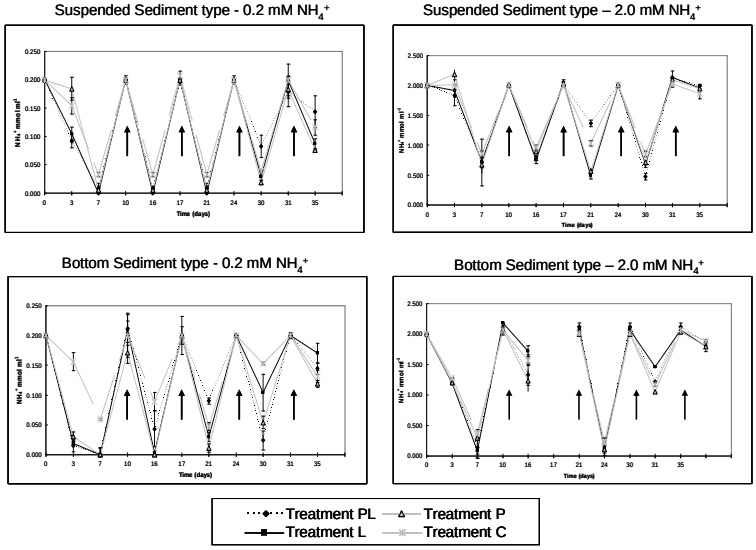
Figure 1. Percentages of cyanobacteria, eukaryotic algae and prochlorophytes measured by flow cytometry in the seston of different types and treatments of microcosms containing 0.2 or 2.0 mM NH_4^+. Percentages indicated were normalized for the total percentage of the algal component of the seston, without considering the bacterial cells and detritus particles. Characters at the bottom of the bars indicate the type of microcosms and the treatments applied (see **Table 1**). Values are averages of four replicate microcosms.



Again, microcosms of treatment C showed a lower consumption rate of ammonium. The SS type of microcosms subjected to 2.0 mM NH_4^+ (**Fig. 2**, upper right panel) showed a regular consumption of ammonium down to 0.5 mM. The changes in the concentration of ammonium in the BS type of microcosms **(Fig. 2**, lower right panel) are assumed to have the same profile as in the SS type of microcosms containing also 2.0 mM NH_4^+, despite the lack of data at T_{16}. In general, the whole system never experienced a limitation of ammonium and microcosms without

P. pectinatus and lake water, *i.e.* treatment C, showed always a lower consumption of ammonium, independently upon the type of microcosms or the concentration of ammonium.

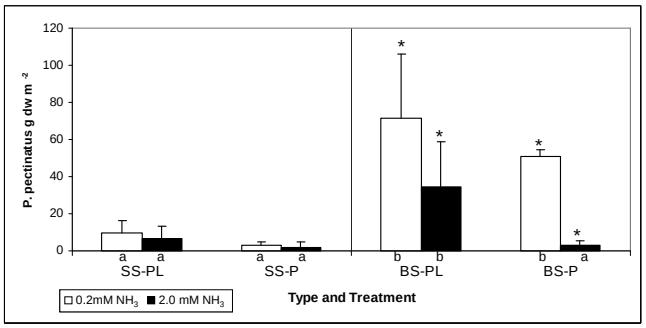
Figure 2. Changes in NH_4^+ concentrations in different types of microcosms and treatments. T_0 corresponds to the initial addition of media to the microcosms. Thick solid arrows indicate the points at which medium was added to restore the concentrations of ammonium to 0.2 and 2 mM in the respective microcosms. Values of ammonium concentrations at T_{16} in the Bottom Sediment type of microcosm containing 2 mM NH_4^+ are missing. Values are averages of 4 replicate microcosms.



Biomass of *Potamogeton pectinatus* and of seston

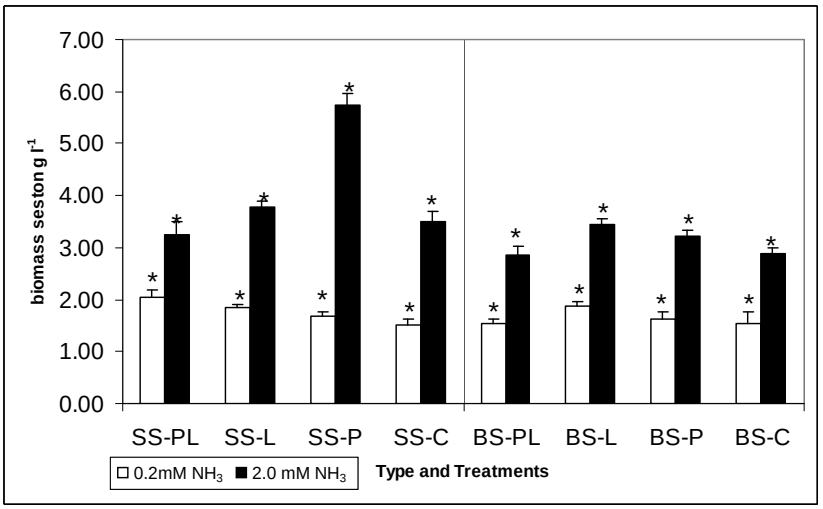
100% of the sterile tubers sprouted during the initial phase, using the energy source contained in the tubers themselves. Biomass values of *P. pectinatus* reached at the end of the experiment are shown in **Figure 3**. Biomass values were significantly higher in the BS type of microcosms in comparison with the SS type, as confirmed by univariate ANOVA (factor type, DF = 1; F= 21; $p < 0.05$; Tukey's HSD test, $p < 0.05$). Biomass values were significantly higher in the BS microcosms containing 0.2 mM NH_4^+, as shown by univariate ANOVA (factor ammonium, DF = 1; F= 5.59; $p < 0.05$; Tukey's HSD test, $p < 0.05$). No significance differences were observed between biomass values in relation to the treatment applied.

Figure 3. Biomass of *Potamogeton pectinatus* measured at the end of the experiment in the Suspended Sediment (SS) and Bottom Sediment (BS) type of microcosms of treatments PL and P with the two different ammonium concentrations of 0.2 and 2.0 mM. Values are averages of four replicate samples. Different letters indicate significant differences between means of biomass values in the Suspended and Bottom sediment type of microcosms. Asterisks indicate significant differences between the 0.2 and 2.0 mM NH_4^+ concentrations.



In general, biomass values of the seston appeared similar in the SS and BS types of microcosms (**Fig. 4**). However, biomass values of the seston were significantly different depending on the concentration of ammonium in the media, being 1.70 (± 0.1) and 3.59 (± 0.68) g l^{-1} on average at 0.2 mM and 2 mM NH_4^+, respectively. One-way ANOVA revealed an effect of the factor microcosm type on the biomass of seston at the edge of significance (DF = 1; F= 3.83; p = 0.055), in contrast with a highly significant effect of the factor ammonium (DF = 1; F= 135; $p < 0.001$; Tukey's HSD test $p < 0.001$). The factor treatment turned out not to be significant for the biomass of the seston. Interestingly, a significant negative correlation was found between the biomass of the seston and of *P. pectinatus* in the microcosms of treatments PL and P (Spearman-rank correlation, $r = -0.58$, $p < 0.05$).

Figure 4. Biomass of the seston components measured at the end of the experiment in the Suspended Sediment (SS) and Bottom Sediment (SS) type of microcosms of all treatments at the two different ammonium concentrations of 0.2 and 2.0 mM. Values are averages of four replicate samples. Asterisks indicate significant differences between the 0.2 and 2.0 mM NH_4^+ concentrations.



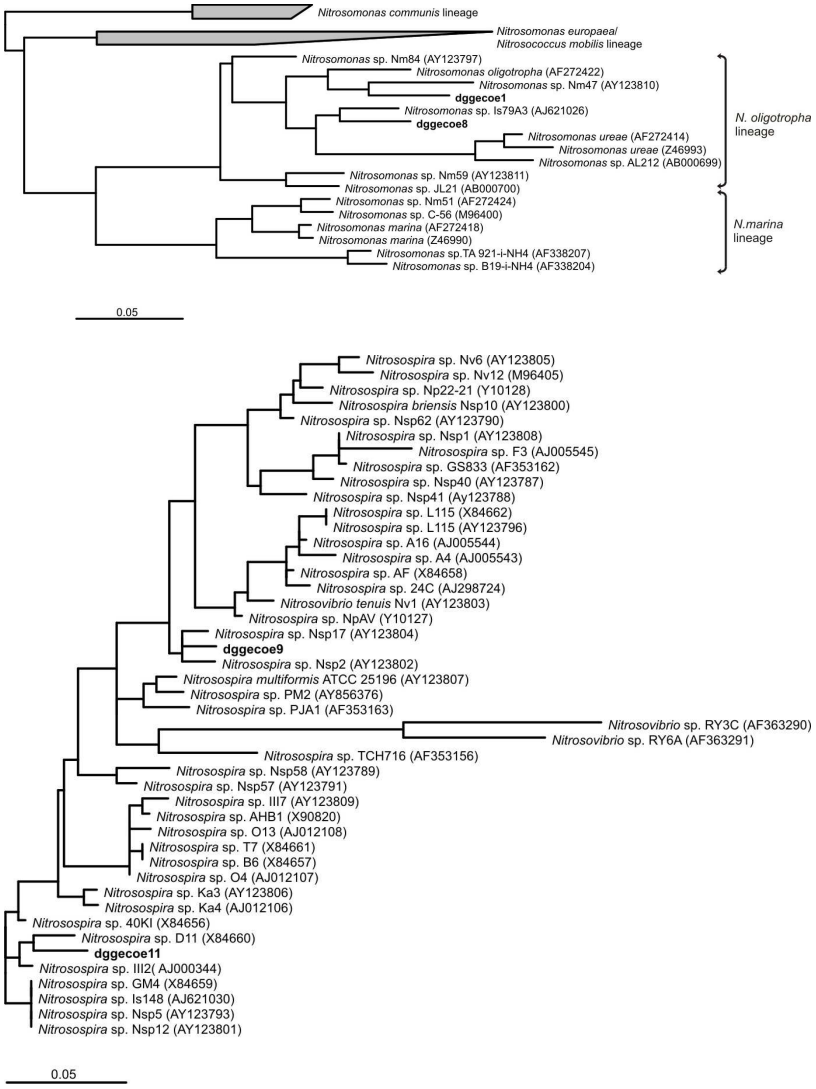
Ability of pelagic and benthic ammonia-oxidizing bacteria to colonize *P. pectinatus* leaves

A summary of the affiliation of the AOB-related sequences retrieved from DGGE bands in this study is given in **Table 3**. Results of phylogenetic analyses are presented separately for the "Nitrosomonas" and the "Nitrosospira" groups (**Fig. 5**).

Table 3. List of bands related to ammonia-oxidizing bacteria detected on denaturing gradient gels with their respective symbols used to label the bands on the gels (**Fig. 4-8**). The percentages of similarity together with the closest matching sequences of isolates of ammonia oxidisers deposited in GenBank are indicated.

Band name (accession no.)	Symbol	No of bases similar/total (percentage of similarity)	Closest AOB isolate in GenBank (Accession no.)
dggecoe1 (**AM503570**)	✠	415/427 (97%)	*Nitrosomonas oligotropha*, isolate AS1 (**EF016119**)
dggecoe8 (**AM503571**)	□	404/409 (98%)	*Nitrosomonas* sp., isolate 79A3 (**AJ621026**) (*N. oligotropha* lineage)
dggecoe9 (**AM503572**)	◇	396/404 (98%)	*Nitrosospira* sp., isolate Nsp17 (**AY123804**) (cluster 3)
dggecoe11 (**AM503573**)	△	377/398 (94%)	*Nitrosospira* sp., isolate III2 (**AJ000344**) (cluster 0)

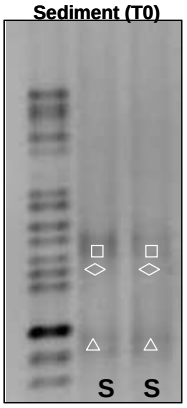
Figure 5. 16S rRNA-based neighbour-joining phylogenetic trees of the ammonia-oxidizing species of the *Nitrosomonas* and *Nitrosospira* lineages of the beta-subclass of the Proteobacteria. Bands retrieved in this study are marked in bold. Scale bars represent 5% estimated sequence divergence.



The ammonia-oxidizing bacterial community inhabiting the sediment of Lake Vossemeer at T_0 (**Fig. 6**) was composed of strains belonging to three different groups of ammonia-oxidizing

bacteria, namely the *Nitrosomonas oligotropha* lineage (band dggecoe8) and the clusters 0 and 3 of the *Nitrosospira* lineage (band dggecoe9 and dggecoe11, respectively). Ammonia-oxidizing bacteria in the water were probably below the detection limit of our assay.

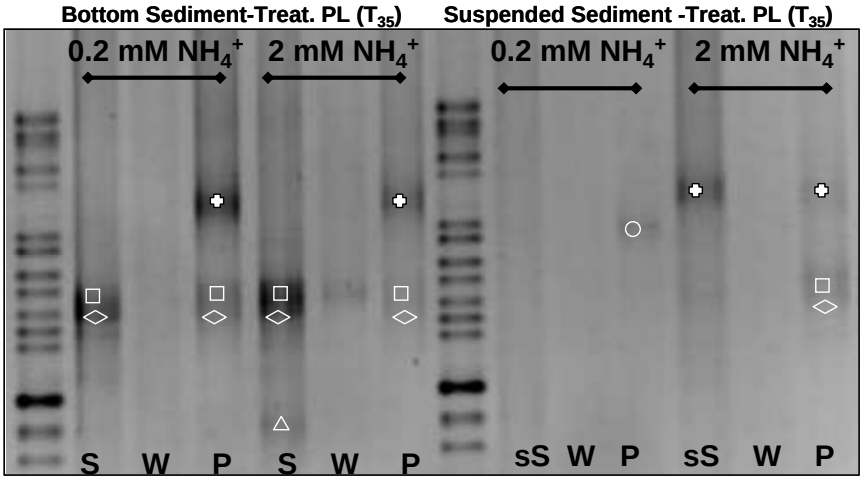
Figure 6. Detail of a denaturing gradient gel showing the ammonia-oxidizing bacterial communities of two replicate sediment samples at T_0. S stands for Sediment. Symbols assigned to the bands are explained in **Table 4.**



At the end of the experiment (T_{35}), members of the *N. oligotropha* lineage and of cluster 3 of the *Nitrosospira* lineage were still present in the sediment, but were detected also on the leaves of *P. pectinatus* at both concentrations of ammonium in the BS-PL microcosms (**Fig. 7**, left). In addition, ammonia-oxidizing bacteria belonging to cluster 0 of the *Nitrosospira* lineage were still present in the sediment of the microcosm subjected to 2 mM NH_4^+. Ammonia-oxidizing bacteria in the water samples were probably again below the detection limit of our assay (**Fig. 7**). The same occurred for the seston samples of the four microcosms (BS and SS at 0.2 and 2 mM NH_4^+) that were run on a separate gel (not shown). Interestingly, an additional band (dggecoe1, indicated by a cross in **Fig. 7** was detected on the leaves of *P. pectinatus*. This band belonged to the *N. oligotropha* lineage and was 95% similar to band dggecoe8 (indicated by a square in **Fig. 6**) detected in the sediment and that also belonged to the *N. oligotropha* lineage. In the SS-PL microcosms (**Fig. 7**, right), no ammonia-oxidizing bacteria were detected either in the

suspended sediment or in the water or on the leaves of *P. pectinatus* at 0.2 mM NH_4^+. In contrast, at 2 mM NH_4^+, ammonia-oxidizing bacteria present in the sediment at T_0, were also detected on the leaves of *P. pectinatus*. Moreover, in this microcosm type, the additional ammonia oxidizer, member of the *N. oligotropha* lineage, (indicated by a cross in **Fig. 7**), was detected on both the suspended sediment and the leave of *P. pectinatus*.

Figure 7. Denaturing gradient gel showing the ammonia-oxidizing bacteria present in the different compartments of four different microcosms containing *Potamogeton pectinatus* and lakes water (PL) of the Bottom Sediment (left panel) and the Suspended Sediment (right panel) type of microcosm at the end of the experiment (T_{35}). For every type of microcosm, replicates subjected to the two different concentrations of ammonium are shown and indicated by horizontal arrows on the top of the gel. Abbreviations stand for: sediment (S), suspended sediment (sS), water (W), and leave of *P. pectinatus* (P). The lane with no indication is a marker lane. Symbols used to label the bands are explained in Table 4. The circle indicates a band not affiliated to ammonia-oxidizing bacteria.



Finally, at T_{35} in the BS-P microcosms (**Fig. 8**), ammonia-oxidizing bacteria of the *N. oligotropha* lineage and of cluster 3 of the *Nitrosospira* lineage were still present, but only in the sediment of the microcosm containing 2 mM NH_4^+. In contrast, in the SS-P microcosms (**Fig. 9**), ammonia oxidizers present at T_0 in the sediment were detected on the suspended sediment and

on the leaves of *P. pectinatus* at both concentrations of ammonium. In both the BS and the SS type of microcosms subjected to treatment P (**Fig. 8** and **9**), no ammonia oxidizers were detected in the water or in the seston.

Figure 8. Denaturing gradient gel showing ammonia-oxidizing bacteria present in the different compartments of two microcosms of the Bottom Sediment type subjected to treatment P and two different concentrations of ammonium at the end of the experiment (T_{35}). The sample of the *Potamogeton pectinatus* leave in the microcosm containing 2mM NH_3 is missing. Abbreviations stand for: sediment (S), water (W), seston (St) and leave of *P. pectinatus* (P). Symbols used to label the bands are explained in **Table 4**.

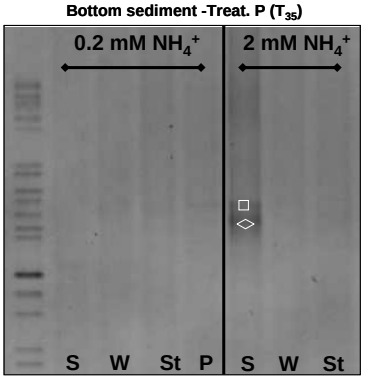
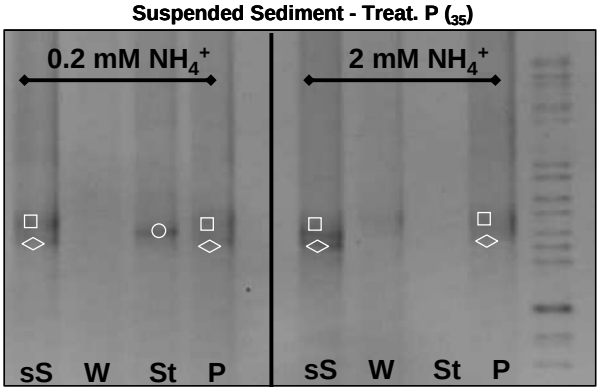


Figure 9. Denaturing gradient gel showing the ammonia-oxidizing bacteria present in the different compartments of two microcosms of the Suspended Sediment type subjected to treatment P and two concentrations of ammonia at the end of the experiment. (T_{35}). Abbreviations stand for: suspended sediment (sS), water (W), seston (St) and leave of *Potamogeton pectinatus* (P). Symbols used to label the bands are explained in Table 4. The circle indicates a band not affiliated to ammonia-oxidizing bacteria**.**



Stable isotope measurements and statistical analyses

One of the objectives of this study was to asses the nitrification rates in the benthic, the pelagic and the epiphytic compartment in which a freshwater environment dominated by submerged macrophytes can be divided. In our microcosms, the so called benthic and epiphytic compartments corresponded to the sediment and to the *P. pectinatus* plants, respectively. The pelagic compartment included the seston, collected on glass fiber filters, as well as the NH_4^+ and NO_3^- pools measured from prefiltered water subjected to the ammonia diffusion method.

In our experiment, only the BS microcosms containing 0.2 mM NH_4^+ sustained plant biomasses of *P. pectinatus* comparable to natural values, *i.e.* 73.2 g dry weight [202] (**Fig. 3**). Therefore, we present data on the stable isotope measurements performed in this type of microcosms, since they best mimicked a natural situation.

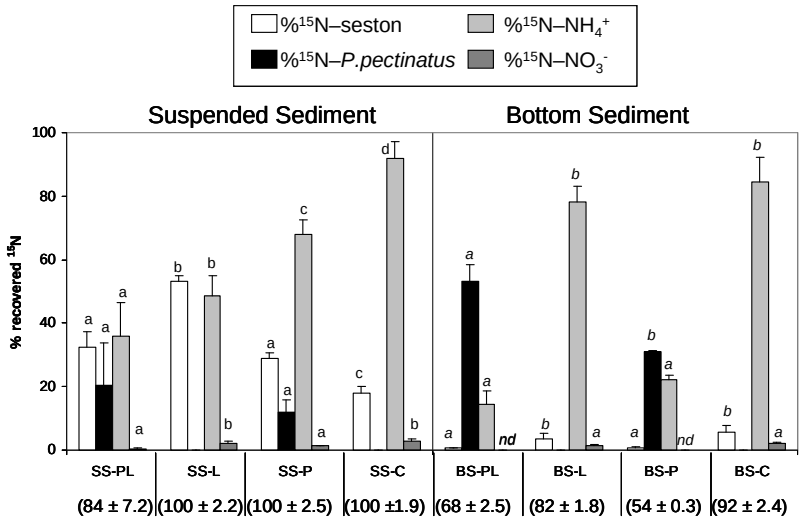
Unfortunately, ^{15}N label was not measurable directly from sediment samples and were therefore not taken into account in the calculation for the total percentage of recovered ^{15}N label. At the end of the experiment, NO_3^- in the water was not detectable. The percentages of ^{15}N recovered in the NO_3^- pool were therefore calculated assuming a final concentration of NO_3^- below 10 μM, and were not considered in the statistical analyses.

The loss of nitrogen observed in our microcosms was likely to be due to the coupled nitrification-denitrification process occurring in the sediment. In order to account for this loss, the percentages of ^{15}N recovered in the BS type of microcosms were compared with those in the SS type of microcosms that had not an active benthic compartment.

Nearly all the ^{15}N added to the system was recovered in the SS type of microcosms at the end of the experiment. The percentages of recovery ranged from 84 to 100 (**Fig. 10**). In contrast, the percentages of ^{15}N recovered in the BS type of microcosms ranged from 54 to 94. The factor microcosm type had an overall significant effect on the total percentage of ^{15}N recovered (ANOVA, DF = 1, F = 24, $p < 0.001$). In particular, by comparing mean values of ^{15}N recovered in the different compartments of the SS and BS type of microcosms, it appeared

that the percentages of ^{15}N recovered in the seston and in the NH_4^+ pool were significantly higher in the SS than in the BS microcosms (HSD Tukey' test, $p < 0.005$). In contrast, the percentages of ^{15}N recovered in the *P. pectinatus* plants were significantly higher in the BS than in the SS microcosms (HSD Tukey' test, $p < 0.005$).

Figure 10. Percentages of ^{15}N recovered in different compartments of the microcosms subjected to the four different treatments (PL, P, L and C) in the presence of 0.2 mM ammonium of both the suspended sediment (SS, left panel) and the bottom sediment type (BS, right panel). Values are averages of 3 replicates microcosms. Different characters on the top of the bars indicate significant differences (HSD Tukey's test, $p < 0.05$) between means of percentages of ^{15}N recovered in the different compartments within the same type of microcosms. Characters in regular and italic fonts referred to the suspended sediment and bottom sediment microcosm type, respectively. Abbreviation *n.d.* stands for not detected. Values in parenthesis at the bottom indicate the average total percentages (±SE) of ^{15}N recovered in the microcosms of that particular type and treatment.



The presence of plant and lake water had a significant effect both in the SS and BS types of microcosms (**Table 4**). However, the presence of lake water was more significant in the SS than in BS microcosms. Within the SS microcosms, the percentages of ^{15}N recovered in the seston were significantly higher in the L treatments, containing lake water and were positively correlated

with the biomass of the seston itself (**Fig. 10**, left panel and **Table 5**).

Table 4. Results of factorial ANOVA analyses (F and P values) showing the effect of the presence of *Potemogeton pectinatus* (the factor plant) and of natural water from the lake (the factor lake) on the percentage of ^{15}N recovered in the Suspended Sediment and Bottom Sediment types of microcosms. Values marked in bold were significant at the 0.05 and 0.001 level. Tukey's HSD tests were performed to test significant differences between mean values for the factors lake and plant. Asterisks indicate significant differences between mean values at the 0.05 level.

Factor	**Microcosm type**			
	SS		**BS**	
	F	P	F	P
Plant	7.3	**0.000***	297	**0.000***
Lake	141	**0.000***	9	**0.016**
Plant*lake	69	**0.019***	10	**0.011***

Table 5. Spearman-Rank correlations of the percentages of recovered ^{15}N label in different compartments and the biomass of *Potemogeton pectinatus* (*P. pect*) and of the seston measured in the microcosms of the suspended sediment type (SS), containing 0.2 mM NH_4^+. Correlations marked in bold were significant at the 0.05 level.

Variable	**%^{15}N GF filter**	**%^{15}N *P. pect***	**%^{15}N NH_4^+**	**Biomass *P.pect* (g)**
% ^{15}N *P. pectinatus*	-0.011			
% ^{15}N NH_4^+	**-0.671**	-0.485		
Biomass *P. pectinatus* (g)	-0.018	**0.992**	-0.447	
Biomass seston	**0.706**	0.470	**-0.930**	0.410

The percentage of ^{15}N recovered from *P. pectinatus* plants in the PL and P treatments were similar between each other and were positively correlated with the biomass of the plant. The percentages of ^{15}N recovered in the NH_4^+ pool were higher in

the control microcosms without plant and lake water and negatively correlated with the percentages of ^{15}N recovered in the seston as well as with its biomass.
Within the BS microcosms (**Fig. 10**, right panel and **Table 6**), the percentages of ^{15}N recovered in the seston were higher in treatment L and C, both without *P. pectinatus*. They were positively correlated with percentages of ^{15}N recovered in the NH_4^+pool and negatively correlated with the percentages of ^{15}N recovered in *P. pectinatus*, as well as with its biomass. The percentages of ^{15}N recovered in the NH_4^+ pool were significantly lower in treatments PL and P, both containing *P. pectinatus*. They were positively correlated with ^{15}N recovered in the NO_3^- pool and negatively correlated with the percentage of ^{15}N recovered in *P. pectinatus* and with its biomass.

Table 6. Spearman-Rank correlations of the percentages of recovered ^{15}N label in different compartments and the biomass of *Potemogeton pectinatus* (*P. pect*) and of the seston measured in the microcosms of the bottom sediment type (BS), containing 0.2 mM NH_4^+. Correlations marked in bold were significant at the 0.05 level.

Variable	**%^{15}N GF filter**	**%^{15}N *P. pect***	**%^{15}N NH_4^+**	**Biomass *P.pect* (g)**
% ^{15}N *P. pectinatus*	**-0.877**			
% ^{15}N NH_4^+	**0.790**	**-0.921**		
Biomass *P. pectinatus* (g)	**-0.862**	**0.968**	**-0.869**	
Biomass seston	-0.069	-0.231	0.321	-0.209

Discussion

This microcosm experiment was in the first place designed to clearly assess the origin of the epiphytic ammonia-oxidizing bacteria that colonize the leaves of a cosmopolitan submerged macrophyte such as *Potamogeton pectinatus*, in the second place to measure nitrification rates in the different compartments, and in the third place to test the effect of the ammonium concentration, major energy source for ammonia-oxidizing bacteria, on the colonization behaviour.

The results of this study clearly show that the ammonia-oxidizing bacteria inhabiting the epiphyton of *P. pectinatus* originated from the sediment and colonized the plant both during the sprouting of the tubers (as revealed by the BS type of microcosms) as well as during the resuspension of sediment (as demonstrated by the SS type of microcosms). In our microcosms the resuspension of sediment naturally caused by the wind action, was simulated by artificial mixing. The association of nitrifying bacteria of the water column and of the sediment with particulate matter has been already reported [203]. Even more interestingly, the epiphyton of *P. pectinatus* offered a niche to another ammonia-oxidizing bacterium, probably present in the water, but below the detection limit. This ammonia oxidizer belonged to the *Nitrosomonas oligotropha* lineage and was detected both on the leaves of *P. pectinatus* as well as on the suspended sediment, but only in the microcosms that contained water from the lake, namely the SS and BS types of microcosm subjected to treatment PL. Its absence on the leaves of *P. pectinatus* and on sediment particles in the microcosm that did not contain natural lake water (treatment P), demonstrated that this strain of AOB of the *N. oligotropha* lineage must originate from the lake water itself. Apparently, the presence of a surface offered this strain the possibility to attach and to produce a number of cells detectable by the molecular technique applied. Supportive of this idea, is the known ability of some members of the *N. oligotropha* lineage to produce remarkable amounts of exopolymeric substances [173]. The production of exopolymers might contribute to the attachment of bacteria and to the subsequent establishment of a microenvironment around the cells more favorable for the maintenance of the community itself [204]. In our microcosm, the optimal conditions for the growth of ammonia oxidizers as well as the longer water retention time might have stimulated the growth of the exopolymeric-producing ammonia-oxidizing bacteria.

The second objective of this study, *i.e.* the establishment of nitrification rates in the different compartments, was unfortunately not completely fulfilled. As plants, eukaryotic algae and cyanobacteria may consume nitrate that had been produced by the ammonia-oxidizing bacteria, any activity of these latter

microorganism might have been masked, especially under nitrogen-limited conditions as occurred in the treatments with 0.2 mM NH_4^+. In addition, a coupled nitrification-denitrification process in the sediment [205] might have obscured the production of nitrate by the ammonia-oxidizing bacteria. Nevertheless, our data might provide additional and complementary information to other studies dealing with the same subject [96, 97, 99, 101, 102, 206].

In our study, biomasses of *P. pectinatus* comparable to the natural situation [202], were reached only in the microcosms containing fresh sediment in the bottom and 0.2 mM NH_4^+ in the water. In a study on *P. pectinatus*, Barko and Smart [207] showed that dissolved organic carbon in the sediment is essential for the growth of plants. In our experiment, a carbon limitation might have occurred to *P. pectinatus* plants of the suspended treatment microcosms, where plain sand could apparently not support the development of a reasonable amount of plant biomass. Furthermore, *P. pectinatus* plants were outcompeted by cyanobacteria, eukaryotic algae and prochlorophytes in the microcosms containing 2 mM NH_4^+ due to light limitation, as naturally also occurs in shallow freshwater lakes with a turbid state [208].

The higher percentages of recovery of ^{15}N label in the SS microcosms containing sand, instead of fresh sediment, suggested the occurrence of the coupled nitrification-denitrification process in the sediment of the BS microcosms. Denitrification is known to occur in the sediments and biofilms where low oxygen concentration can be maintained by simultaneous oxygen consumption combined with low solubility of oxygen in overlaying water [209-211]. Denitrification is also known to be higher in stagnant water [97] where the oxic-anoxic interfaces represent the ideal habitat for denitrification [212]. Even more, the coupled nitrification-denitrification is enhanced by submerged macrophytes and their epiphytic biofilms [99, 197]. This is a well-established concept in the functioning of constructed wetland for the removal of nitrogen [100, 102, 103, 105, 213-215].

As a matter of fact, the mayor loss of nitrogen occurred in the microcosms that contained sediment and higher biomasses of *P.*

pectinatus (BS-PL and BS-L). Moreover, the biomass of *P. pectinatus* constituted the major discriminatory factor for the distribution of ^{15}N label in the different compartments of the BS microcosms. The biomass of *P. pectinatus* affected negatively the biomass of the seston components and consequently the percentage of ^{15}N recovered in the seston itself, as well as the percentage of ^{15}N recovered in the NH_4^+ pool. *P. pectinatus* plants are physiologically adapted to growth with their roots in the sediment and their shoots in the water column [216]. Both the roots and the shoots are involved in the uptake of nitrate and ammonium by submerged macrophytes, but the only significant transport is from the roots to the shoots [217, 218]. Nevertheless, Barko and Smart [207] suggested that macrophytes are opportunistic and will consume nitrogen from the water phase in significant amounts when available. However, high removal efficiency of nitrogen though assimilative uptake by plants can only be achieved by frequent harvesting [219].

Possibly, in our system as already demonstrated in field studies [220], *P. pectinatus* plants took up nitrogen either as ammonium or nitrate from the sediment and the water; plants stimulated nitrification in the sediment-water interface by oxygenating the sediment and enhanced nitrification in the water column by providing the surface for the attachment of ammonia-oxidizing bacteria. Additionally, they stimulated the coupled nitrification-denitrification process in the sediment, finally leading to the loss of nitrogen in the system.

In addition to *P. pectinatus*, algae also take up nitrogen compounds. This was mainly evident from the SS microcosms where highest percentages of ^{15}N were recovered in the seston component. Furthermore, the biomass of seston affected the recovery of ^{15}N label in the NH_4^+. Cyanobacteria dominated of the algal component of seston at 0.2 mM NH_4^+. Cyanobacteria can use both ammonium and nitrate as nitrogen source, being however ammonium the preferred source. [221, 222]. This might explain the fact that the percentages of ^{15}N recovered in the NH_4^+ pools were higher in the microcosms subjected to treatment P and C, that did not contain water from the lake, hence less seston.

To make it even more complicated, ammonia-oxidizing bacteria of both the "*Nitrosomonas*" and the "*Nitrosospira*" groups are able to produce N_2O, proportionally to the available ammonium concentrations [30-32]. This might also have contributed to the incomplete nitrogen balance in our system.

The dynamic of nitrogen in our system was obviously quite complex. We were not able to assess nitrification rates, as a simple conversion of $^{15}N\text{-}NH_4^+$ into $^{15}N\text{-}NO_3^-$, neither could we assess whether the percentage of ^{15}N recovered in the plants came directly from plant uptake or assimilation or indirectly from the activity of ammonia-oxidizing bacteria present on their leaves. However, in a similar experiment, Eriksson and Andersson [101] showed that shoots of *P. pectinatus* significantly enhanced nitrification and suggested that shoots provided the surface for the attachment of nitrifying microorganisms. Hence, our study could contribute to this observation by providing a molecular-based assessment of the actual presence of ammonia-oxidizing bacterial cells on the surface of submerged macrophytes and even more by establishing the origin of the inocula of the epiphytic ammonia-oxidizing bacterial cells.

Regarding the effect of ammonium on the colonization behavior of ammonia-oxidizing bacteria, we must say that at the end of the experiment the higher concentration of ammonium, i.e. 2 mM, resulted as a stress factor for the overall functioning of our microcosms, due to the growth of cyanobacteria, eukaryotic algae and prochlorophytes that outcompeted *P. pectinatus*. This phenomenon observed in our microcosms resembled in a small scale a well known natural process, *i.e,* eutrophication. In particular, the shift between the macrophytes-driven clear state and the algal-driven turbid state has actually been a real and negative phenomenon in the past in the area we choose to study. As a matter of fact, the reestablishment of submerged macrophytes is a target of water managing policy and it is often regulated by a strict control of the water retention time in the lake. In our system the excess of ammonium plus the absence of water flow has caused its collapse. Ammonia-oxidizing bacteria were however retrieved in the benthic and epiphytic compartment of the both the SS and BS type of microcosms that contained 2 mM NH_4^+ in the water; but this provided no

information on the actual functioning of the ammonia-oxidizing community.

Conclusion

To summarize, ammonia-oxidizing bacteria that colonize the epiphyton of submerged macrophytes have both a benthic and pelagic origin. In particular, benthic ammonia oxidizers colonize *P. pectinatus* both during the sprouting of the tubers and during resuspension of the sediment itself. In addition, the epiphyton offers an additional niche to pelagic ammonia-oxidizing bacteria that might otherwise not grow to larger numbers in the pelagic compartment. As consequence the presence of submerged macrophytes might enhance the nitrification process by directly providing a substratum for the attachment of bacterial cells. However, the actual contribution of the epiphytic ammonia-oxidizing bacteria to the nitrification process was not perceptible, due to the unrivalled impact of submerged macrophytes in the removal of nitrogen from the system by their direct assimilation of nitrogen and above all by enhancing the coupled nitrification-denitrification process in the sediment.

Chapter V

Denaturing Gradient Gel Electrophoretic Analysis of Ammonia-Oxidizing Bacterial Community Structure in the Lower Seine River: Impact of Paris Wastewater Effluents

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Abstract

The Seine River is strongly affected by the effluents from the Achères wastewater treatment plant (WWTP) downstream of the city of Paris. We have shown that the effluents introduce large amounts of ammonia and inoculate the receiving medium with nitrifying bacteria. The aim of the present study was to investigate the diversity of the ammonia-oxidizing bacterial population by identifying autochthonous bacteria from upstream and/or allochthonous ammonia-oxidizing bacteria from the WWTP effluents. Measurements of potential nitrifying activity, competitive PCR, and denaturing gradient gel electrophoresis (DGGE) of 16S ribosomal DNA fragments specific to ammonia-oxidizing bacteria (AOB) were used to explore the succession and shifts of the ammonia-oxidizing community in the lower Seine River and to analyze the temporal and spatial functioning of the system at several different sampling dates. A major revelation was the stability of the patterns. The CTO primers used in this study (G. A. Kowalchuk, J. R. Stephen, W. D. Boer, J. I. Prosser, T. M. Embley, and J. W. Woldendorp, Appl. Environ. Microbiol. 63:1489-1497, 1997) were shown not to be completely specific to AOB of the ß subclass of *Proteobacteria*. We further demonstrated that when DGGE patterns are interpreted, all the different bands must be sequenced, as one major DGGE band proved to be affiliated with a group of non-AOB in the ß subclass of *Proteobacteria*. The majority of AOB (75 to 90%) present in the lower Seine river downstream of the effluent output belong to lineage 6a, represented by *Nitrosomonas oligotropha*- and *Nitrosomonas ureae*-like bacteria. This dominant lineage was represented by three bands on the DGGE gel. The major lineage-6a AOB species, introduced by the WWTP effluents, survived and might have grown in the receiving medium far downstream, in the estuary; it represented about 40% of the whole AOB population. The other two species belonging to lineage 6a seem to be autochthonous bacteria. One of them developed a few kilometers downstream of the WWTP effluent input in an ammonia-enriched environment, and the other appeared in the freshwater part of

the estuary and was apparently more adapted to estuarine conditions, i.e., an increase in the amount of suspended matter, a low ammonia concentration, and high turnover of organic matter. The rest of the AOB population was represented in equal proportions by *Nitrosospira*- and *Nitrosococcus mobilis*-like species.

Introduction

Effluents discharged from wastewater treatment plants (WWTPs) into rivers may have detrimental environmental effects, because they are a source of high levels of nutrients, organic matter, and bacteria (10, 20, 51). The lower Seine River, downstream of the city of Paris, is greatly affected by the discharge of the effluents from the Achères WWTP, which treats the wastewater from 6.5 million inhabitant equivalents. These effluents contain large amounts of ammonium (NH_4^+), which is completely oxidized into nitrite (NO_2^-) and subsequently converted to nitrate (NO_3^-) by the nitrification process along the 300 km of the Seine River continuing to the estuary. Autotrophic nitrification is accomplished in two stages by two distinct groups of bacteria: ammonia oxidizers and nitrite oxidizers. Ammonia oxidation due to chemolithotrophic ammonia-oxidizing bacteria (AOB) is the first and often the rate-limiting step of nitrification; it is essential for the removal of nitrogen from the environment (44). Aerobic autotrophic ammonia-oxidizing bacteria are found within two phylogenetic groups based on comparative analyses of 16S rRNA sequences (45, 62). One group comprises strains of *Nitrosomonas* and *Nitrosospira* spp. within the class of ß-*Proteobacteria*, and the other contains *Nitrosococcus oceani* and *Nitrosococcus halophilus* within the class of gamma-*Proteobacteria*. All ß-proteobacterial ammonia oxidizers belong to a phylogenetically coherent group, within which all the organisms have the same basic physiology as far as we know. A continually expanding database of AOB 16S ribosomal DNA (rDNA) gene sequences has produced descriptions of distinct lineages and clusters within the genera *Nitrosomonas* and *Nitrosospira* of the ß-*Proteobacteria* (31, 45). Pure culture representatives have been isolated for all groups, except for *Nitrosospira* cluster 1 and *Nitrosomonas* lineage 5, where only clone sequences are available. A number of studies suggest that there are physiological and ecological differences between the different AOB genera and lineages and that environmental factors such as salinity, pH, and concentrations of ammonia and suspended particulate matter (SPM) select for certain species of AOB (16, 30, 34, 43, 60). These external factors, which include

the impact of WWTP effluents, may therefore influence the range of AOB diversity and consequently the structure and function of the ammonia-oxidizing community. Most research has focused on AOB community composition in WWTP activated sludge (15, 17, 28, 29, 48, 52). Only a few studies on AOB diversity in freshwater environments or estuaries are available (7, 16, 26, 27, 56, 57). The diversity of AOB has been analyzed previously at two sites in the Seine River: one station just downstream of the WWTP input and another in the upper freshwater estuary (12). *amoA* gene library analysis showed that a majority of AOB at the two sampling stations were affiliated with lineage 6a, represented by *Nitrosomonas oligotropha*- and *Nitrosomonas ureae*-like bacteria, while the other AOB lineages are present in smaller proportions. However, the differences in AOB composition between the two stations did not allow any conclusion to be drawn either on the variety of AOB input by the Achères WWTP effluents or on their fate in the receiving medium. AOB, generally found in small numbers in natural environments, have low growth rates and low biomass yields and are therefore difficult to isolate in a pure culture (44). Molecular techniques allow a more complete understanding of the diversity and distribution of AOB in natural environments than that offered by cultivation-based methods alone (2, 28, 64). Therefore, we combined PCR of 16S rRNA (rDNA) gene fragments using AOB-selective primers with denaturing gradient gel electrophoresis (DGGE) to allow comparative analysis of the dominant AOB populations in the lower Seine River. Moreover, we complement the use of competitive PCR (cPCR) and the determination of the nitrification rates. The aims of this study were (i) to investigate the proportion of the AOB from upstream that is autochthonous and/or the proportion from the WWTP effluents that is allochthonous, (ii) to better understand the successions of these bacteria in the lower Seine River, highlighting possible shifts in the AOB populations, and (iii) to analyze the temporal and spatial functioning of the system with respect to ammonium oxidation by using observations made on several sampling dates.

Materials and methods

Description of the research area.

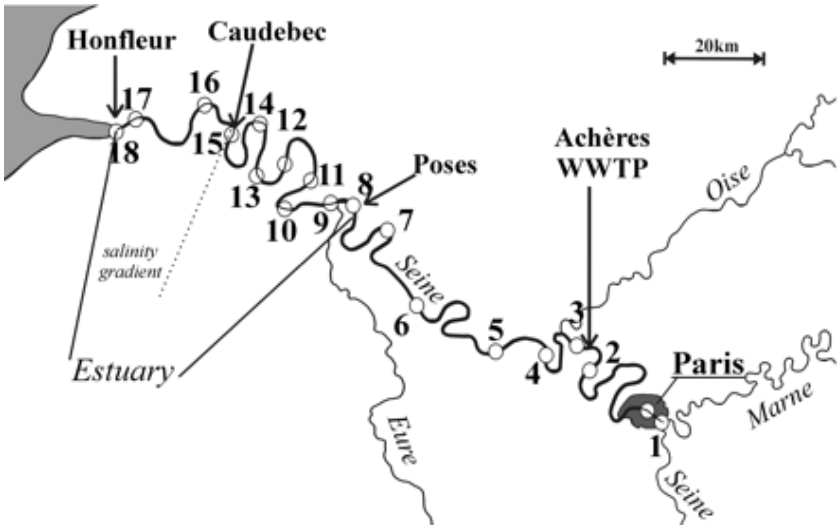
A 356-km Seine River transect was studied from upstream of the city of Paris (St. Maurice at the outlet of the Marne River, set at km 0) to the mouth of the estuary (Honfleur, at km 356); the weir at Poses (km 202) represents the physical limit between the riverine and estuarine sections (**Fig. 1**). There is a salinity gradient between Caudebec (km 310) and Honfleur (km 356), which is influenced by tides and river flow. The lower Seine River and estuary receive the pollution from the whole drainage basin, greatly impacted by industrial activity, intensive agriculture, and the population density concentrated in the Paris region (**Fig. 1**). The domestic effluents from 6.5 million inhabitants of Paris and its suburbs undergo a secondary treatment including activated sludge in the wastewater treatment plant of Achères. The treated, but ammonium-rich, Achères effluents are discharged 70 km downstream from Paris. The dissolved oxygen profiles reflect the microbial activities taking place in the lower Seine River (12, 23) .

Sample collection

Water samples were collected at 18 stations in July 2002 and at 16 stations in September 2002 and September 2003 along the Seine River from St. Maurice (km 0, immediately upstream of Paris) to Honfleur (km 356, in the estuary) (**Fig. 1**); the sampling stations are referred to by number, from 1 to 18. Two wastewater samples (raw and treated) were also collected. During the period from July to September, when water flow rates are low (185 to 250 $m^3\ s^{-1}$) and temperatures are high (18 to 21°C), nitrifying activity is always at its maximum. Five to 10 liters of water were collected for chemical, biochemical, and molecular analyses and were brought to the laboratory within 2 to 3 h. The water was filtered through glass fiber membrane filters (GF/F; Whatman) and frozen until it could be analyzed for inorganic nitrogen compounds. Ammonium, nitrite, and nitrate levels were

determined spectrophotometrically; ammonium and nitrite concentrations were determined according to the procedure of Slawyck and MacIsaac (54), and nitrate levels were determined after Cd reduction to nitrite (46). Suspended matter was weighed on GF/F filters dried at 450°C. Potential nitrifying activities were determined by two methods directly after the sampling (see below). For molecular analysis, 150 to 250 ml of water (depending on the amount of SPM) was filtered through 0.22-μm-pore-size nitrocellulose filters (diameter, 45 mm; Durapore) in triplicate, and the filters were frozen (–20°C) until DNA extraction.

Figure 1. Description of the study site: the Seine River from Paris (km 0) to the mouth of the estuary (km 356). Numbers 1 to 18 represent the sampling stations, as follows: 1, Saint Maurice; 2, Maison Laffites; 3, Conflans; 4, Triel; 5, Porcheville; 6, Vernon; 7, Les Andelys; 8, Poses; 9, Pont Arche; 10, Elbeuf; 11, Oissel; 12, Bassin des Docks; 13, La Bouille; 14, Duclair; 15, Heurtauville; 16, Caudebec; 17, Tancarville; 18, Honfleur. Caudebec represents the limit of saline intrusion.



Measurement of potential nitrifying activity and potential ammonia oxidation rate

Potential nitrifying activities (i.e., the sum of ammonia and nitrite oxidation rates) were measured according to the methods described by Cébron et al. (12). Potential nitrifying activities were determined by the difference in $H^{14}CO_3^-$ incorporation between samples with and without specific nitrification inhibitors, all of which were incubated under optimal conditions, i.e., 7.5 mg of $O_2 \cdot liter^{-1}$, 20°C, and 2 mM NH_4Cl (9). The inhibitors were allylthiourea (10 mg $liter^{-1}$) and sodium chlorate (10 mM), which inhibit the oxidation of ammonia and nitrite, respectively. Determination of ammonia oxidation rates was based on measurements of the changes in nitrite concentration in samples containing chlorate as an inhibitor that were incubated under optimal conditions (see above).

DNA extraction

DNA was extracted from the filters by a bead-beating method with the FastDNA spin kit for soil (Bio 101, La Jolla, Calif.) according to the manufacturer's instructions. DNA extracts were then stored at –20°C until purification on a Sephadex G-200 column and ethanol precipitation. Nucleic acids were quantified by comparison between 1 μl of an undiluted sample and a range of known DNA concentrations (various dilutions of SuperLadder; Eurogentec) on an agarose gel stained with ethidium bromide. To obtain suitable PCR amplicons, 10- to 100-fold dilutions of crude DNA were used as templates for subsequent PCRs. DNA was visualized by UV transillumination (Gel Doc 200; Bio-Rad Laboratories; Hercules, Calif.). Digital images of the gels were obtained with a charge-coupled device camera controlled by Quantity One software (Bio-Rad).

cPCR for AOB quantification

Ammonia oxidizers were estimated as *amoA* gene copy numbers by cPCR as described by Stephen et al. (58) and Cébron et al.

(12). PCR amplification of a 491-bp fragment of the *amoA* gene and a 428-bp fragment of a DNA competitor was carried out by using primer set *amoA*-1F (5'-GGG GTT TCT ACT GGT GGT-3')-*amoA*-2R (5'-CCC CTC KGS AAA GCC TTC TTC-3'), which is specific to ammonia oxidizers belonging to the ß subclass of *Proteobacteria* (47). PCR amplifications were carried out in a total volume of 50 μl in 0.2-ml tubes by using a DNA thermocycler (GeneAmp 2400 PCR system; Perkin-Elmer Cetus), according to the thermal profiles described in **Table 1**. Reaction mixtures were prepared in a 1x buffer [75 mM Tris (pH 9.0), 20 mM $(NH_4)_2SO_4$, 0.01% Tween 20] with 1.5 mM $MgCl_2$, 10 ng of DNA, and 0.5 U of *Taq* DNA polymerase (RedGoldtar; Eurogentec). Aliquots of amplification products were analyzed by gel electrophoresis on 2% (wt/vol) agarose gels (Eurogentec). DNA band intensities were estimated with imaging and analysis software (Quantity One; Bio-Rad). The cPCR for *amoA* was performed in triplicate for 10 samples to establish the experimental variability of the method and to determine standard deviations for the results.

Table 1. Summary of primer characteristics and PCR conditions.

Primers	Target (specificity)	PCR conditions	References
amoA-1F-*amoA*-2R	*amoA* gene (AOB Beta-*Proteobacteria)*	5 min at 94°C, followed by 42 cycles of 1 min at 94°C, 1 min 30 s at 60°C, and 1 min 30 s at 72°C, followed by a 10-min final extension at 72°C	46
CTO189F-GC or CTO189F-CTO654r	16S rRNA gene (AOB Beta-Proteobacteria)	3 min at 94°C, followed by 38 cycles of 30 s at 92°C, 30 s at 57°C, and 45 s at 72°C, followed by a 5-min final extension at 72°C	35
357F-GC or 357F-518R	16S rRNA gene (universal)	5 min at 94°C, followed by 25 cycles of 1 min at 94°C, 1 min at 65°C (–1°C each 2 cycles and 5 cycles at 55°C), and 1 min at 72°C, followed by a 5-min final extension at 72°C	38

PCR amplification for DGGE

All PCRs for DGGE were conducted according to the work of Kowalchuk et al. (32) with an equimolar mixture of three forward primers, each with a GC clamp, to increase the PCR sensitivity (CTO189fA-GC, CTO189fB-GC, and CTO189fC-GC [5'-CCG CCG CGC GGC GGG CGG GGC GGG GGC

ACG GGG GGA GRA AAG YAG GGG ATC G-3'] [underlining indicates GC clamp; Y is C or T, and R is A or C]) and with the reverse primer CTO654r (5'-CTA GCY TTG TAG TTT CAA ACG C-3'), containing a single ambiguous base. These primers were designed to amplify partial 16S rDNA sequences (465 bp) from beta-subclass AOB. The PCR was run under the conditions described in **Table 1**. A nested PCR was performed on the PCR products obtained from CTO primers with a second primer pair allowing the amplification of an internal fragment of 196 bp. These universal primers (357f-GC [5'-C GCC CGC CGC GCG CGG CGG GCG GGG CGG GGG CAC GGG GGG CCT ACG GGA GGC AGC AG-3'] and 518r [5'-ATT ACC GCG GCT GCT GG-3']) were used for the PCR under the conditions described by Muyzer et al. (38) (**Table 1**). PCR products were examined by standard agarose gel electrophoresis (1.2% agarose, 0.5x Tris-borate-EDTA) with ethidium bromide staining to confirm the product size and estimate the DNA concentration. PCR products were loaded for DGGE analysis according to the protocol described by Muyzer et al. (38) and modified by Kowalchuk et al. (32). Polyacrylamide gels (8% polyacrylamide, 1.5 mm thick, 16 by 16 cm) were run in a 1x TAE buffer (40 mM Tris-acetate, 1 mM EDTA [pH 8.3]). The denaturant gradient ranged from 30 to 55% denaturant (100% denaturant was 7 M urea and 40% formamide in the 1x TAE buffer). Gels were run at 60°C on a PROTEAN II xi system (Bio-Rad Laboratories) for 16 h at 75 V. The DNA was stained with ethidium bromide and visualized by UV transillumination. DGGE patterns were visually analyzed, and the intensity of the bands was estimated by imaging and software analysis (Quantity One; Bio-Rad). Double-band formation due to heteroduplex formation had been checked and confirmed by reamplification of the two bands with CTO primers.

DGGE Band excision and sequence analyses.

For subsequent DNA reamplification, the central sections of selected DGGE bands were excised. Each gel fragment was placed overnight, at 4°C, in a 2-ml screw-cap tube containing 50 μl of MilliQ H_2O. Tenfold dilutions of these DNA band samples

were used as templates for subsequent PCR, using the same PCR primer sets. From each PCR, 5 μl was subjected to DGGE as described above to confirm the recovery of the desired band, thus obtaining a pure sample for the sequencing step. The PCR products submitted to sequencing resulted from amplification with CTO189F-CTO654R or CTO357F-CTO518R without a GC clamp according to the conditions described in **Table 1**. The name of each sequence consists of C or N (for the result of PCR with CTO primers or nested PCR); J2, S2, or S3 for the sampling date (July 2002, September 2002, or September 2003); b1 to b8 or A to L for the corresponding DGGE band letter; and finally, the name of the sampling station from which the band has been extracted.

Phylogenetic analysis and nucleotide sequence accession numbers.

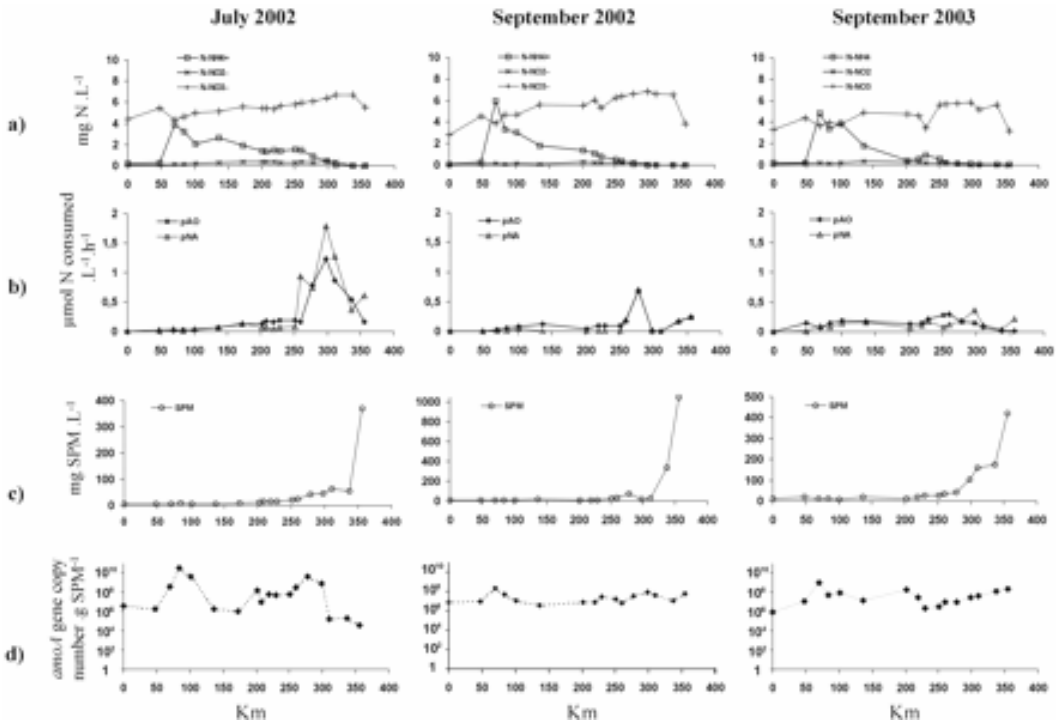
The partial 16S rRNA gene sequences from the Seine River samples were aligned with published 16S rRNA gene sequences from ß-proteobacterial AOB and related non-AOB sequences by use of Clustal X, version 1.8. Phylogenetic algorithms and tree design (DNA-DIST, NEIGHBOR, and SEQBOOT) were operated using the PHYLIP 3.5 package, written by J. Felsenstein (19). Bootstrap analysis was based on 1,000 replicates. Phylogenetic distance trees were generated with non-AOB sequences as an outgroup. The sequences determined in this study were deposited in the GenBank database under accession numbers **AY583655** to **AY583690** and **AY584247**.

Results

Variations in inorganic nitrogen, potential nitrifying activities, and *amoA* gene copy numbers along the lower Seine River. Immediately downstream from the Achères WWTP effluent input, there was a significant increase in ammonium levels, from less than 0.25 to 4 to 6 mg of $N\text{-}NH_4 \cdot liter^{-1}$ (**Fig. 2a**). Depending on residence times and temperatures, these amounts were totally consumed by nitrification at distances of 240, 190, and 130 km downstream of the effluent input in July 2002,

September 2002, and September 2003, respectively. The net nitrate production represented approximately 2.5 mg of $N\text{-}NO_3^-$ produced · $liter^{-1}$ between Conflans (station 3) and Tancarville (station 17), which is less than the total ammonium consumed. This was probably due to (i) dilution by two major tributaries of the Seine River (i.e., the Oise and the Eure) and (ii) denitrification at the water-sediment interface (49). At the last station (station 18; Honfleur), the nitrate concentration decreased rapidly, mainly due to dilution by seawater rather than to denitrification activity (49).

Figure 2. Variations along the Seine river from Paris (km 0) to the mouth of the estuary (km 356) in (a) ammonium (NH_4^+), nitrite (NO_2^-), and nitrate (NO_3^-) levels, (b) potential ammonia oxidation rates (pAO) and potential nitrification activities (pNA), (c) SPM concentrations, and (d) *amoA* gene copy numbers determined by cPCR for three sampling dates (July 2002, September 2002, and September 2003).



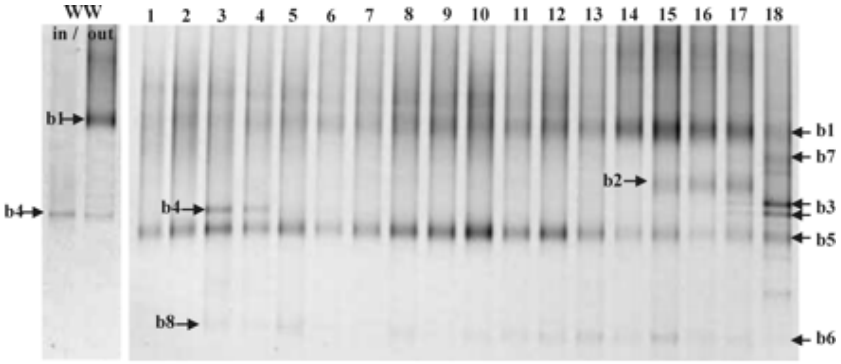
The longitudinal profiles resulting from measurements of potential nitrification activities (^{14}C technique) and of ammonium oxidation rates showed patterns similar to each other for each sampling date (**Fig. 2b**). These patterns, however, differed among the three sampling dates (**Fig. 2b**). In July 2002, potential nitrifying activities averaged 0.1 μmol of N · liter^{-1} · h^{-1} in the lower Seine when impacted by WWTP effluents and reached a maximum of 1.8 μmol of N · liter^{-1} · h^{-1} in the upper freshwater estuary 300 km downstream of Paris, whereas in September 2002, the maximum potential nitrifying activity and ammonium oxidation rate of 0.7 μmol of N · liter^{-1} · h^{-1} occurred at 278 km. In September 2003, the values were quite constant along the whole transect, between 0.1 and 0.3 μmol of N · liter^{-1} · h^{-1}, from the effluent output to the estuary. Invariably, the potential nitrifying activities increased slightly in the saline estuarine sector, where turbidity was maximal. Maximum potential nitrifying activities within the freshwater estuary were associated with a significant increase in suspended particulate matter concentrations, which then increased drastically at the turbidity maximum of the saline estuary (**Fig. 2c**). An increase in the *amoA* gene copy number as determined by cPCR was observed just downstream of the WWTP input for the three sampling dates (**Fig. 2d**). Although the *amoA* gene copy number increased at the maximum of the potential activities, no significant correlation could be found. The standard deviation calculated for *amoA* gene copy number results led to variation coefficients of 1.75 to 5.1% at stations with high *amoA* gene copy numbers and 11.1 to 14.5% at stations with low *amoA* gene copy numbers, so that the observed spatial changes are significant. However, further knowledge of the diversity, physiology, and gene regulation of ammonium-oxidizing bacteria is needed in order to interpret these temporal and spatial patterns in detail.

Analysis of CTO and nested PCR products by DGGE

Because analysis of the CTO PCR products by DGGE revealed that the profiles for the three sampling dates were quite similar, results are shown only for the July 2002 samples (**Fig. 3**). We

identified eight different bands (b1 to b8 [**Fig. 3**]). It seems that bands b5 and b6 (**Fig. 3**) were present in all the samples; b5 was very intense, and b6 was very weak. Band b1 was not well defined upstream of stations 4 and 5 but was very intense in the treated wastewater sample (WWout) and even more so toward the downstream sector. Band b4, which was clearly present in both wastewater samples, was also observed at station 3 (Conflans; km 72), apparently due to seeding of bacteria from WWTP effluents. Band b8 is probably also provided by WWTP effluents; these bands disappeared again 30 km downstream. In the estuary, a new band, b2, appeared from station 15 to station 17; this bacterial group is probably adapted to particular estuarine conditions. At the last station (station 18; Honfleur), which is strongly influenced by seawater, two additional bands appeared (b3 and b7), leading to a DGGE profile quite different from those of the upstream sites and probably characterizing a new community of halotolerant and halophilic ammonia-oxidizing bacteria.

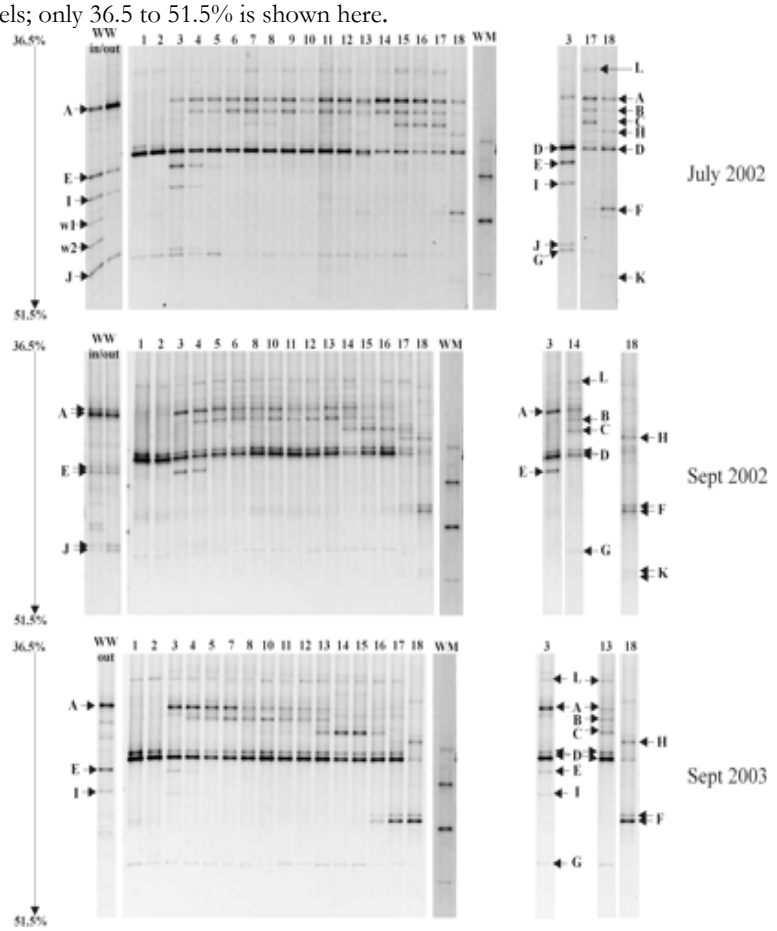
Figure. 3. DGGE profiles of ammonia-oxidizing bacteria of the ß subclass of the *Proteobacteria*, obtained by CTO PCR on July 2002 samples. Lanes 1 to 18 correspond to the 18 different sampling stations; lanes "WW in" and "WW out" are samples from domestic wastewater before and after treatment, respectively. The principal bands are labeled b1 to b8 (bands that were cut and sequenced). The denaturing gradient is from 39 to 52%.



The DGGE profiles produced after nested PCR on CTO PCR products showed that banding patterns obtained from the three sampling dates were rather similar, with the dominant bands

(from A to L) showing the same overall distribution from Paris to the estuary (**Fig. 4**).

Figure 4. DGGE profiles of ammonia-oxidizing bacteria of the ß subclass of the *Proteobacteria*, obtained by nested PCR on July 2002, September 2002, and September 2003 samples. Lanes 1 to 18 correspond to the 18 different sampling stations; lanes "WW in" and "WW out" represent samples from domestic wastewater treatment plants before and after treatment. Lanes WM, weight markers. The principal bands observed are labeled with capital letters (A to L). All these bands have been cut and sequenced to determine the phylogeny of each ammonia-oxidizing bacterium and to compare the three sampling dates. The denaturing gradient is the same for the three DGGE gels; only 36.5 to 51.5% is shown here.



These results show that the diversity of the bacteria varied little over time. Band D (double band due to heteroduplex formation) was present in all the samples from station 1, upstream of Paris, to station 18 in the Seine estuary. It dominated everywhere, except in some estuarine samples. Bands G and L were also omnipresent, but at lower intensities. The impact of the WWTP effluents was visible on all three sampling dates. Immediately downstream from the WWTP input at station 3, four new bands (bands A, E, I, and J) appeared, which were also present in the wastewater from the Achères treatment plant. Bands E, I, and J quickly disappeared again in the river, indicating that these bacteria are well adapted to the conditions in the WWTP but do not survive in the receiving medium. In contrast, band A persisted much longer in the river and was present as far as stations 18, 16, and 13 in July 2002, September 2002, and September 2003, respectively. Band B, which was not present in the effluents but appeared at the Triel station, 15 km downstream from the effluent output, might represent an autochthonous bacterium that thrived on the massive input of ammonia. Finally, band C was present only in the upper freshwater estuary, at stations 13, 14, and 15 in September 2003, September 2002 and July 2002, respectively. This ammonia-oxidizing bacterium seems well adapted to low ammonia concentrations and large amounts of suspended particulate matter. At the last marine station in the mouth of the estuary, new bands, H, F, and K, appeared on the three sampling dates, confirming the presence of halotolerant or halophilic AOB.

Phylogenetic analyses

After confirmation of the purity of the bands (see Materials and Methods), one to three copies of each band were sequenced. Sequence analyses of 404-bp 16S rDNA fragments for CTO PCR products and 152-bp 16S rDNA fragments for nested-PCR products allowed us to build two phylogenetic trees (**Fig. 5** and **6**). The sequences of most of the bands retrieved from the two different DGGE analyses correspond to each other as follows: band b1 corresponds to band A, b2 to C, b3 to F, b4 to E, b5 to D, b6 to G, b7 to H, and b8 to J. The sequence analysis shows

that several bands were not affiliated with known AOB from the beta subclass of the *Proteobacteria*. Bands b4, b5, and b8, which belong to the beta-*Proteobacteria*, are closely related to *Aquaspirillum serpens* (98% homology with b4), *Methylophilus leisingeri* (94% homology with b5), and a *Dechloromonas* sp. (97% homology with b8). Similarly, bands D, E, I, J, w1, and w2 are not affiliated with AOB from the beta subclass of the *Proteobacteria*. The majority of these bacteria were found in the wastewater samples, except for band D, which was present only in all river samples. All these bands will be considered artifacts, due to the fact that the CTO primers apparently are not completely specific to AOB (45).

Figure 5. Phylogenetic neighbor-joining tree based on a comparison of 404 bp of 16S rRNA gene sequences from AOB of the beta subclass of the *Proteobacteria* and some other beta-proteobacteria. The tree is rooted with two sequences of AOB belonging to the γ subclass of the *Proteobacteria*. Our DGGE sequences are boldfaced. Their designations are composed of C, for CTO PCR products; J2, S2, or S3, for a sampling date of July 2002, September 2002, or September 2003; b1 to b8, for the band as labeled in **Fig. 3** and the name of the sampling station from which the sequence originated.

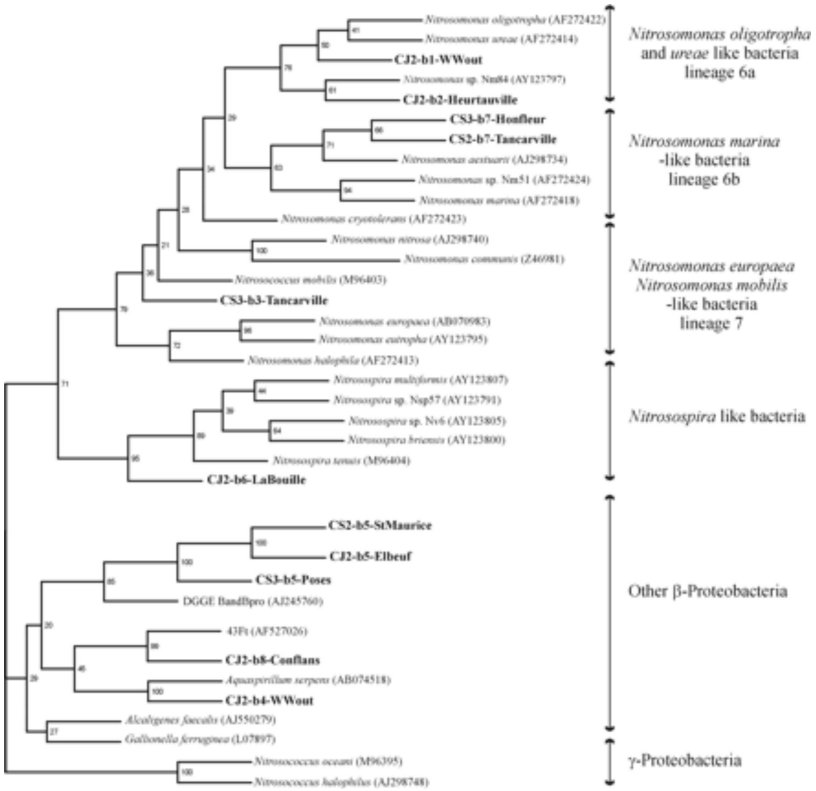
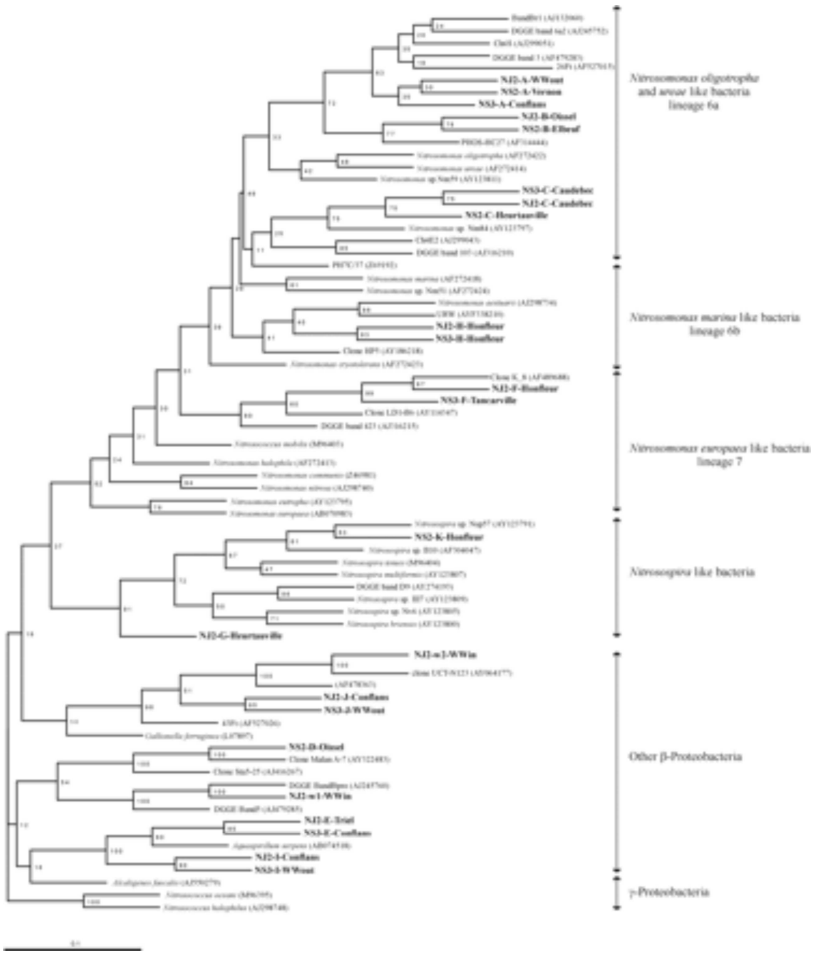


Figure 6. Phylogenetic neighbor-joining tree based on a comparison of 152 bp of 16S rRNA gene sequences from AOB of the beta subclass of the *Proteobacteria* and some other beta-proteobacteria. The tree is rooted with two sequences of AOB belonging to the γ subclass of *Proteobacteria*. Our DGGE sequences are boldfaced. Their designations are composed of N, for nested PCR products; J2, S2, or S3, for a sampling date of July 2002, September 2002, or September 2003; A to L, w1, or w2, for the band as labeled in **Fig. 4**; and, at the end, either the name of the sampling station from which the sequence originated or WWin or WWout (explained in the legend to **Fig. 4**).



All the other bands are affiliated with ammonia-oxidizing lineages belonging to the beta-subclass of the *Proteobacteria*. Band b1 (band A) is affiliated with lineage 6a, represented by *N.*

oligotropha- and *N. ureae*- like bacteria. We recovered one band b1 (CJ2-b1-WWout), and three different bands A (NJ2-A-WWout, NS2-A-Vernon, and NS3-A-Conflans) from the three sampling dates and different stations that showed 100% homology in the 152-bp 16S rDNA sequences. Two of these sequences were from treated-effluent samples (WWout), and two others were from the river; this means that the WWTP effluents have inoculated the lower Seine River with the ammonia-oxidizing species represented by band A. We analyzed two different band B sequences (NJ2-B-Oissel, NS2-B-Elbeuf) that belong to lineage 6a and have a mutual homology of 100% in the 152-bp 16S rDNA sequence but a three-base difference from the band A sequence. In low abundance, this ammonia oxidizer may originate from the upstream part of the Seine but would not be observed as a DGGE band B in the first three samples of the longitudinal profile. Band B could also originate from the Oise River, which joins the Seine between stations 3 and 4. CTO band b2 (CJ2-b2-Heurtauville), which appeared only in the freshwater part of the estuary, has 100% homology with the three bands C (NJ2-C-Caudebec, NS2-C-Heurtauville, NS3-C-Caudebec) in the 152-bp sequence. This ammonia oxidizer belongs to lineage 6a and is closely related to *Nitrosomonas* sp. strain Nm84 (98% homology) and other uncultured beta-proteobacterial AOB sequences (6, 7). Its source was not identified, but as in the case of band B, it might be an autochthonous bacterium present in very low proportions in the upper part of the Seine, or it might originate from lateral tributaries such as the Eure, from other wastewater inputs, or from freshwater estuarine mudflat exchanges (24). Bands b7 and H (CS3-b7-Honfleur, CS2-b7-Tancarville, NJ2-H-Honfleur, and NS3-H-Honfleur) were also completely homologous in the 152-bp sequence. The corresponding bacteria, present only in samples with high salinity at the mouth of the estuary, were related to lineage 6b, represented by *Nitrosomonas marina*- and *Nitrosomonas aestuarii*-like bacteria, known to be halophilic. These bacteria, isolated from the marine environment, are definitely not specific to the Seine but are introduced by the seawater. The homologous bands b3 and F correspond to an AOB closely related to *Nitrosococcus mobilis*; phylogenetically, the sequences are situated between the

typical halophilic lineages 6b and 7, the latter represented by *Nitrosomonas europaea*-like bacteria. *N. mobilis*, probably introduced by tidal cycles, was found in samples taken within the salinity gradient (1 to $8^0/_{00}$) and was characteristic of the downstream estuary at low tide. The closest relatives reported were some uncultured AOB such as clone K_8 (99% homology), collected in surface sediments from Denmark Fjord, where the salinity value was $14^0/_{00}$ (40); clone LD1-B6 (98% homology), found in anoxic marine sediments (21); and DGGE band 423 (98% homology), obtained from the Schelde estuary water with a low ammonia level and high salinity (7). Band L did not correspond to any CTO DGGE band. The NJ2-L-Poses and NS2-L-Elbeuf sequences (100% homology) were close to *N. mobilis* (97% homology). This band, found in all river samples, may belong to an autochthonous AOB that has become adapted to environmental changes along the Seine River continuum. Band G, similar to CTO band b6 (100% homology between CS2-b6-LaBouille and NJ2-G-Heurtauville), belonged to *Nitrosospira*-like bacteria and displayed environmental behavior identical to that of band L. These two species probably represent the upstream bacterial community, because no other AOB band was visible by DGGE analysis for stations 1 and 2. The fact that these bacteria are invariably present in the same low proportion at all sampling stations and dates argues that these species are only weakly dependent on environmental variations.

Discussion

Nonspecificity of CTO primers

The CTO primer set described by Kowalchuk et al. (35) was designed from a large database of sequences from cultured representatives and cloned sequences from various environments. Purkhold et al. (45), have clearly demonstrated that there is no perfect primer set for studying AOB, since none of the published primers that are intended to target all beta-subclass AOB shows both 100% sensitivity (targeting all beta-subclass AOB) and 100% specificity (excluding all non-beta-subclass AOB). Consequently, some of the bands in the DGGE

patterns were not related to AOB, as revealed by sequencing aimed at confirming the phylogenetic affiliations of the DGGE bands. Many bands, initially thought to represent AOB, were in fact identified as uncultured members of other branches of beta-*Proteobacteria*; these bands were identical to many sequences obtained in previous studies using the same primers (for example, clone ME-15 [**AJ003772**] (56) is similar to band b5, and DGGE band 5 [**AF479285**] (8) is similar to band w1). Since the AOB sequence that is closest to these bands is the sequence of *Nitrosospira* sp. strain III7, with less than 84% homology, we considered that none of these bands represented ammonia-oxidizing bacteria.

Limits of the PCR-DGGE technique

The limitations inherent in any electrophoresis technique also apply to DGGE. The number of bands generated by DGGE may not accurately reflect the number of different sequences present in a given mixture. Indeed, DNA of bacteria that are present in relatively low numbers in the environment might escape amplification by PCR. Furthermore, one DGGE band might represent several species, or several bands can be generated by the same bacterium due to degenerate primers (35), heteroduplex formation, or multiple heterogeneous rRNA operons (13). Consequently, sequencing must be used to confirm the identity of the bands. We sequenced one to three bands that had the same position on the DGGE gel for the three sampling dates and showed that all the bands at the same position had the same nucleotide composition. The strategy adopted here can reasonably be considered to lead to the same bacterium for any sample and sampling date. Determination of the intensities of DGGE bands by image analyses enables us to calculate the relative proportion and the percentage of each band and AOB lineage within the whole AOB community. This result is only indicative: the double amplification (nested PCR) can increase the bias, and the presence of band D (non-AOB), representing nearly half of the products of PCR in all the samples, can bias the band intensities. Although Kowalchuk et al. (35) demonstrated that the ratio of the products from the CTO

primers was in good agreement with the concentrations in the starting template, Suzuki and Giovannoni (61) argued that this equivalence would not hold for all primer-template combinations in more-complex mixtures. As a result, the intensity of the bands in a DGGE pattern would provide only limited insights into the microbial community diversity (18). Typical features of nitrification and ammonium-oxidizing bacteria in the lower Seine River. The temporal and spatial differences observed in potential nitrifying activity profiles and *amoA* gene copy numbers cannot be explained by changes in the AOB populations. At three summer time points, the same AOB species were identified in the lower Seine, and the same bacterial shifts and successions were observed along the river transect with banding patterns very close to each other. This result shows a temporal stability in the genetic diversity of the functional bacterial population, which indicates that similar hydrological (residence time), meteorological (temperature), nutritional (NH_4^+ concentrations), and bacterial WWTP input conditions have led to the same selection and adaptation of AOB species in the environment. The major difference between the situations investigated was spatial shifts in the presence or absence of AOB species, which can be explained by slight differences in the controlling factors mentioned above.

Analyzing the diversity of AOB in the lower Seine River

To understand the relationship between environmental variables and ecosystem functioning, it is essential to investigate microbial diversity (63). The relative abundances of sequences obtained by PCR amplification from environmental samples, illustrated by DGGE patterns, suggest that the distribution of different bands might be linked to environmental factors. For example, Suwa et al. (60) showed that various *Nitrosomonas* strains isolated from activated sludge could be either sensitive or insensitive to high ammonium concentrations. Temperature may also affect the community structure of ammonia oxidizers (3) either directly or indirectly. From upstream to downstream in the lower Seine River, the first change in DGGE patterns appeared at station 3,

due to AOB seeding (mainly band A) by Achères WWTP effluents. The corresponding bacterial strain represented a large proportion (about 40%) of the AOB community in the river and persisted down to the freshwater estuary, showing that it could survive or adapt to a variety of ammonium concentrations, suspended-matter levels, and other environmental conditions. This feature distinguishes it from most heterotrophic bacteria, which, when released from domestic wastewater, rapidly decreased in the Seine River (22). Band B appeared at station 4 and probably represents an autochthonous river species, originating from upstream in the Seine or from the Oise tributary. Unfortunately, no DGGE patterns from the Oise were studied to ascertain whether the band B species, initially in very low proportions in the Seine, was favored by increased substrate concentrations or was already present in high numbers in the Oise. Furthermore, in the freshwater estuary, a new AOB (band C) was selected in the sector of maximum nitrifying activity where oxygen and ammonia concentrations decrease and suspended-matter concentrations increase. Whereas many studies have shown that oxygen has no effect on the composition of the AOB community (4, 7, 32), the "band C" population may thrive on freshly produced ammonia, organic compounds, or new mineral ions (14) specific to the estuary, where the organic-matter turnover is generally high (23, 50). This niche differentiation may be due to physiological differences such as the ability to produce extracellular polymeric substances (57), allowing an attached versus free-living AOB selection (42). Finally, the shift from freshwater to halophilic bacteria, related to *N. marina* and *N. aestuarii* (bands F and H), at the mouth of this macrotidal estuary (stations 17 and 18) can easily be explained by a permanent mixing with seawater, where such species are selected (21, 27, 37). An additional and/or alternative source for these halophilic bacteria could be the lateral saline mudflats, which are permanently dynamically interacting with the turbidity maximum within the channel. All three major nested DGGE bands (A, B, and C), i.e., 75 to 90% of the AOB community from the WWTP output to the estuary, belong to lineage 6a, represented by *N. oligotropha*- and *N. ureae*-like bacteria. The fact that identical PCR conditions are able to detect lineage 6a

sequences in soil, rhizosphere, wastewater, and brackish water samples (32, 36, 40, 48, 59) suggests that the frequent detection of *Nitrosomonas* lineage 6a sequences in the Seine River and estuary was not simply due to a bias in the PCR. This population might have a selective advantage over the other populations in the Seine. Speksnijder et al. (56) found that *Nitrosomonas* lineage 6a was the only sequence lineage of beta-subclass ammonia-oxidizing bacteria to be found at all water and sediment sites. *Nitrosomonas* lineage 6a is well adapted to low ammonia concentrations because of its low K_m for ammonia (5, 57, 60). These bacteria outcompeted other AOB at low ammonia concentrations in the Schelde river (5, 6); note that these authors also found that *N. oligotropha*- and *N. ureae*-like bacteria were dominant in the freshwater part of the estuary and that *N. marina* and *N. aestuarii* were present in the brackish zone. Lineage 6a is known to be inhibited at ammonia concentrations exceeding 10 mM, which is 20 to 30 times higher than those found in the Seine River downstream of the WWTP effluent discharge (6, 31, 57, 60). *Nitrosospira*-like sequences are typical AOB populations present in soil fertilized with amended water (41), while *Nitrosomonas*-like sequences are typically found in effluents of treated urban sewage. This supports our results, i.e., the presence of *Nitrosospira* in low proportions in all the samples. The *Nitrosospira* sequence (band G) might come from upstream in the Seine River and is probably introduced by soil leaching. *Nitrosospira* is generally regarded as the most ubiquitous genus in forest soils (25), and it dominates in grasslands and agricultural ecosystems (11, 36, 59). In the transect of the Seine River that was studied, an *N. mobilis*-like sequence (band L) belonging to lineage 7 was also present in all the samples and had the same behavior as *Nitrosospira*. Species belonging to lineage 7 are often found in rich media with high ammonia concentrations such as activated sludge (28, 48) and would have been introduced into the Seine by upstream WWTPs. However, these bacteria have also been found in both oligotrophic and eutrophic lakes (26, 55). It is worth mentioning that many other AOB species in very low numbers might exist in the river without being detected, since approximately 1% of the target community is below the PCR-DGGE detection limit (39). Because no major temporal

change in AOB community composition occurred during our study (July and September 2002, September 2003), these results were compared with those obtained in September 1998 (12), where the *amoA* gene diversity was obtained from cloning and sequencing of two gene libraries from station 4 (Triel), immediately downstream of the WWTP input, and from station 14 (Duclair), in the freshwater part of the estuary. The comparison is relevant because the phylogeny of the *amoA* gene, which encodes the α-subunit of the ammonia monooxygenase enzyme, was found to correspond largely to the phylogeny of the 16S rRNA gene in ammonia oxidizers (1, 34, 45). The two studies in the Seine River showed the same major lineage, i.e., 60 to 80% and 75 to 90% of AOB, respectively, belonging to lineage 6a, but a lower diversity was found in the samples taken in 2002 to 2003 and analyzed by DGGE (12). Whereas three different sequence types (bands A, B, and C) were found here within lineage 6a, four different sequences were previously found at the Triel station (compared to the two different sequences here) and three were found at Duclair in both studies. To increase the knowledge of the functional community of AOB in the Seine River, its active bacteria could be investigated by RNA DGGE. In reality, analysis of 16S rDNA does not distinguish between metabolically active and quiescent cells. Boon et al. (8) reported that some very active populations visible in the RNA DGGE patterns can hardly be detected in the DNA DGGE fingerprints, and they concluded that whereas an RNA DGGE analysis is appropriate for a rapid examination of changing microbial communities, DNA DGGE analysis is suited to slowly evolving, stable communities, such as those in the Seine river system. Furthermore, analysis of environmental 16S rRNA by using reverse transcription-PCR would provide greater sensitivity because of the higher target copy number and might indicate which members of the community are more metabolically active, i.e., have a high ribosome content (21, 33, 65). An alternative approach could be the use of a translated functional gene as a molecular marker (53). The primer pair (*amoA*-1F-*amoA*-2R) developed by Rotthauwe et al. (47) amplifies a fragment of the *amoA* gene α-subunit. Recently Oved et al. (41) and Nicolaisen and Ramsing (40) have applied *amoA* amplicons to DGGE

analysis using newly designed PCR primers. Although these authors succeeded in the optimization of the assay, application of their *amoA* PCR-DGGE approach to our samples led to patterns with too many bands, not separable as single bands; furthermore, any changes in the technical approach failed to give valid results (M. Coci, unpublished data). Moreover, the fact that Oved et al. (41) found a band (w2406) that was not an *amoA* sequence tends to support the statement that this technique must be used with caution. As a whole, besides demonstrating the surprisingly stable AOB community patterns in the lower Seine River which are strongly impacted by the Achères WWTP effluents, this study identified the ammonia-oxidizing bacteria introduced by the effluents. In addition, in contrast to many typical allochthonous heterotrophic bacteria from WWTPs, such as coliforms, that are not adapted to surface water, the AOB introduced by the effluents seem to be able to maintain their biomass in the receiving medium and even to grow, probably favored by the ammonia pollution in the river or by the estuarine conditions.

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Chapter VI

Effect of salinity on temporal and spatial dynamics of ammonia-oxidizing bacteria from intertidal freshwater sediment

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Abstract

Temporal and spatial dynamics within an ammonia-oxidizing community from intertidal, freshwater sediments were studied in microcosms simulating flooding twice a day with fresh, brackish and marine waters. The microcosms had been filled with the upper 5 cm of intertidal freshwater sediment from the river Scheldt. Changes in community composition were examined by denaturing gradient gel electrophoresis of amplified DNA from the community. In the first week of incubation the initially present members of the *Nitrosomonas oligotropha* lineage were replaced by other members of the same lineage in the top layer of the sediment subjected to flooding with freshwater. Prolonged incubation extended niche differentiation to a depth of 5 cm. In the microcosms flooded with saline media, the initially present members of the *N. oligotropha* lineage were replaced by strains belonging to the *Nitrosomonas marina* lineage, but only in the top 1 cm. Shift in community composition occurred earlier in the marine microcosms than in the brackish microcosms and was slower than the change in the freshwater microcosms. Irrespective of the nature of the flooding medium, shifts in community composition were always consistent among replicate microcosms. We conclude that salinity is an important steering factor in niche differentiation among ammonia-oxidizing bacteria and also that changes within the community of this functional group of bacteria may occur at different rates.

Introduction

Ammonia-oxidizing bacteria (AOB) are chemolithoautotrophic microorganisms that generate their energy by oxidizing ammonia to nitrite. Ammonia oxidation is the first and usually rate-limiting step in the nitrification and fulfils a critical role in global nitrogen cycle. Due to the vital role of AOB in elemental cycling and in environmental issues like acidification, eutrophication and greenhouse gas emission, a wealth of studies have been performed to elucidate ecological and physiological aspects of these bacteria in various environments [1–4]. Especially the use of cultivation-independent techniques has enhanced the knowledge on the biogeography of this very specialised group of bacteria [2,5,6]. AOB are among the rare cases in microbial ecology where pure cultures of nearly all recognised lineages are available. The latter makes it possible to connect ecophysiology with diversity and distribution of these bacteria [1,2]. Based on structural (16S rRNA) as well as functional (amoA) gene relationships, seven major lineages of AOB are recognised [6–8]: *Nitrosospira*, *Nitrosomonas europaea/Nitrosococcus mobilis*, *Nitrosomonas communis*, *Nitrosomonas marina*, *Nitrosomonas oligotropha*, *Nitrosomonas cryotolerans* and *Nitrosomonas* sp. Nm143. Despite the fact that all these lineages carry out the same basic energy-generating biochemical reaction, AOB seem to occupy clearly different niches and can therefore be classified into ecophysiological groups [1]. The former cluster 6, as defined by Stephen et al. [5] and comprising the *N. oligotropha* (6a) and the *N. marina* (6b) lineages, is predominantly found in aquatic environments. The *N. marina* lineage comprises obligate halophilic and salt-tolerant species, whereas members of the *N. oligotropha* lineage are found under oligotrophic freshwater conditions. The latter lineage seems to contain species growing relatively well at low ammonia concentrations [9,10] but also contains strains found in wastewater treatment plants [11]. Speksnijder and co-workers [12] detected a high micro-variation within the former cluster 6a, suggesting different eco-types and probably niche differentiation within this group. Niche differentiation within AOB communities has also been observed with respect to salinity [13–16]. In a survey of the diversity within the ammonia-oxidizing *Betaproteobacteria* along a salinity gradient in the Scheldt estuary, a

change in community composition has been observed at the transition zone between the freshwater and the brackish water parts of the estuary [13]. As indicated by enrichment experiments under different conditions of salinity, the freshwater part contained salt-sensitive as well as salt-tolerant ammonia-oxidizing bacteria, whereas the brackish water samples contained only salt-tolerant ammonia-oxidizing bacteria [9,14]. Due to the prevailing conditions in the freshwater part of the river, salt-sensitive bacteria were apparently dominant. The salt-sensitive and the salt-tolerant ammonia-oxidizing bacteria belonged to the *N. oligotropha* and the *N. marina* lineages, respectively [1,5]. In a study of ammonia-oxidizing bacteria in intertidal freshwater sediments more upstream of the river, sequences related to *N. oligotropha* lineage were the most dominant among the sequences belonging to the betaproteobacterial ammonia-oxidisers ([12] and Speksnijder, personal communication). Studies on selection, adaptation or niche differentiation of AOB communities while environmentally perturbed are very scarce. Community changes or replacement of species may lead to changes in diversity, which may yield a community with different ecophysiological properties, certainly in the case of AOB, with possible consequences for the biogeochemical process of nitrification. However, it is not known how quick such changes occur, especially under natural conditions. The aim of this study was to investigate how AOB communities change upon environmental perturbations. We focussed on the response of AOB communities from intertidal freshwater sediment to tidal floods with media of different salinities and on the possible niche differentiation among and within the lineages. Changes in community composition were followed in time and with depth in the sediment using PCR and denaturing gradient gel electrophoresis (DGGE) assays for ammonia oxidisers.

Material and methods

Description of the sampling site

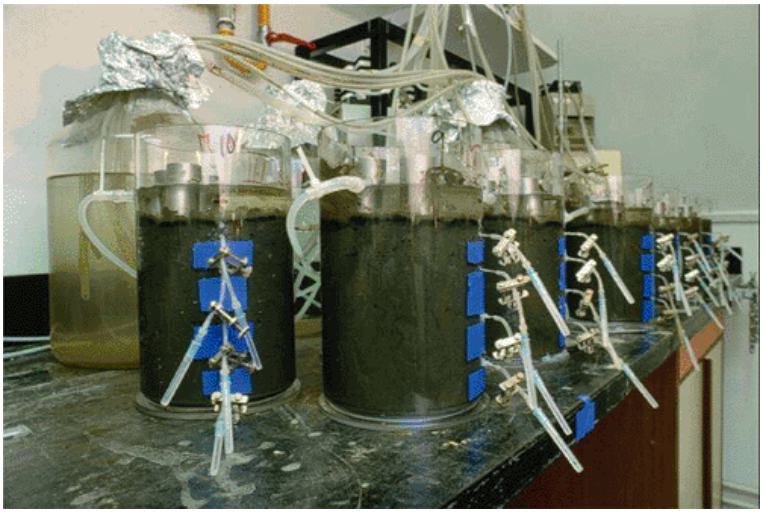
The sampling site was a freshwater intertidal marshland near the village of Appels (51°01'N, 4°03'E, Belgium) that is located more

than 100 km upstream of the Scheldt mouth. In June 2002, sediment from the upper 5 cm of the non-vegetated zone was collected during low tide and then stored in buckets at 4 °C until the filling of the microcosms. The mean tidal difference at Appels is 6 m and the average ammonia concentrations amount to 0.2 mM in the water and to 0.2–1.5 mM in the upper 10 cm of the sediment (Christelle Hyacinthe, personal communication). Salinity at Appels is 0.5 ppt and it does not change with the tides.

Description of the microcosms and sampling

Microcosms consisted of polymethylmethacrylate (PMMA) cylinders of 15 cm diameter and 25 cm height with a total volume of more than 4 l. The microcosms were filled with mixed sediment up to a height of 15 cm. In each microcosm four custom made rhizon soil solution samplers (Eijkelkamp, Giesbeek, The Netherlands), as described by Bodelier et al. [17], were mounted to enable sampling of the pore water at 1, 4, 8 and 12 cm depth, considering 0 cm at the interface with the flooding water. Each microcosm had also an inflow device (0.5 cm diameter) at 4 cm above the sediment surface, through which the flooding medium entered the system. The outflow device was located on the opposite side at the surface sediment level. The outflow device was protected by a metallic sieve to prevent sediment from leaving the microcosm system. Fig. 1 gives an overview of the microcosm system with the marine replicate microcosms in the foreground. On every sampling day, a stainless steel cylinder (3 cm diameter and 20.5 cm height) was pushed into the sediment of the microcosms where it remained till the end of the experiment. Inside this stainless cylinder a second cylinder of PMMA (2.5 cm diameter and 23.5 cm height) was pushed and used to take a sediment core. In this way the sediment profile in the microcosm itself was not disturbed. Sediment cores were cut with a sharp tool in slices of 1 cm each; slices from different depths were used for analysis.

Figure 1. Overview of the microcosms: 12 microcosms in total with four replicates per treatment. The marine microcosms are in the foreground. (See Appendix for full color image).



Microcosm experiment

The microcosms were subjected to a tidal regime using flooding media that differed mutually in salinity. The basic medium was a mineral salt medium containing 1 mM $(NH_4)_2SO_4$, 1 mM KCl, 0.2 mM $MgSO_4 \cdot 7H_2O$, 1 mM $CaCl_2 \cdot 2H_2O$, 0.1 mM KH_2PO_4[18] and 1 ml SL8 trace element solution according to Verhagen and Laanbroek [19]. The basic medium was supplemented with 10, 260 or 520 mM NaCl. The microcosms exposed to these media will be referred to as freshwater microcosms (FM), brackish microcosms (BM) and marine microcosms (MM) (four replicates per treatment). The media were adjusted to pH 7.5–7.8 with 1 N NaOH before autoclaving for 25 min at 121 °C. Sterile KH_2PO_4 (0.4 mM) was added after separate autoclaving. Microcosms were incubated at 25 °C in the dark for 35 days. The whole system was designed as a recycling system of 10 l flooding media that were replaced by fresh media once every week, after sampling of the microcosms. The flooding media covered the sediment surfaces for about 2 cm with a volume of approximately 350 ml. Tides were simulated

according to the standard regime of 6 h high and 6 h low tide. NH_4^+ and NO_2^- plus NO_3^- concentrations were daily measured in the three different 10 l reservoirs during one week and in the overlying water during one tidal cycle. NH_4^+ concentration in the media and overlying water was 1 mM in the whole experiment and this excludes ammonia to be a niche-differentiating factor.

Analysis of sediment water content and pore water analysis

Water content in percentage of weight was measured gravimetrically after drying 5 g sediment for 48 h at 60 °C. Measurements were done in sediment slices from 1, 2, 3, 4, 5 and 10 cm depth at day 7, day 24 and day 35 from four replicate microcosms per treatment. Pore water samples were collected at day 0, 7, 14, 24, 35 from three replicate microcosms of each treatment type. Pore water samples were taken at 1, 4, 8, 12 cm depth in 3 ml Silicone Vacuum Venoject vials (Terumo) and immediately frozen at −20 °C. NH_3 and NO_2^- plus NO_3^- concentrations were measured with an Auto-Analyzer Traacs 800 (Technicon Instrument, Canada).

Potential nitrification activity

Potential nitrification activity was measured at day 0 and day 35 at different depths from two replicates per treatment. Measurements were done in slurries containing 2 g wet sediment in 20 ml mineral medium and incubated on a rotary shaker at 25 °C at 150 rpm. The sterilised medium contained 2.5 mM $(NH_4)_2SO_4$, 0.80 mM, K_2HPO_4, 0.20 mM KH_2PO_4, adjusted to pH 7.5 with 1 N NaOH [20]. Sub-samples from the slurries were taken after 1, 2, 4, 6, 12 and 24 h incubation. Nitrite and nitrate concentrations were measured colorimetrically with a Technicon Auto-Analyzer (Traacs 800). Potential nitrification activity in the intertidal freshwater sediment itself was measured as described above in slurries of sediment collected at the sampling site.

DNA extraction and purification

DNA was extracted from 0.5 g sediment with the mechanical disruption protocol described by Henckel [21]. Subsequently the extracted DNA was repurified with a commercial purification kit (Wizard DNA Clean-up system, Promega Corporation, USA) according to the manufacturer' s recommendations. Purified DNA was resuspended in deionised water. Extraction and purification were verified by electrophoresis of 5 µl of the DNA solution in 1.2% agarose gel and 0.5X TBE buffer (5X Tris-Borate: 54 g Tris base, 27.5 boric acid, 20 ml 0.5 M EDTA) and then visualised by ethidium bromide fluorescence.

Direct PCR amplification of ammonia-oxidizing bacteria

Direct amplification of 16S rRNA gene fragments was performed for different replicates of each treatment type. The AOB-specific primer set, CTO 189f-GC (an equimolar mixture of three forward primers) and CTO 654r, was used as described by Kowalchuk et al. [22]. The primer set amplified an approximately 465 bp fragment of the 16S rRNA gene of the betaproteobacteria. In this manuscript we refer to this fragment as the CTO fragment. The primers are degenerated with specificity and sensitivity clearly defined [2,22,28]. PCR amplification was performed in a total volume of 50 µl containing 1X PCR Mg-free buffer, 200 µM of each deoxynucleotide, 1.75 mM $MgCl_2$, two units of *Taq* DNA Polymerase (Invitrogen, Tech-Line USA), 400 ng μl^{-1} BSA (purified bovine serum albumin, New England BioLabs, Beverly, MA, USA), 0.5 µM of each primer, and 80–100 ng of purified DNA as template. Reactions were performed in a Multiblock Thermocycler System (Hybaid, USA) according to the following program: 3 min denaturation at 94 °C; 35 cycles of: 30 s denaturation at 92 °C, 30 s annealing at 57 °C and 45 s elongation at 72 °C; the last step was 5 min elongation at 72 °C. All the amplification reactions were verified loading 5 µl amplification products in 1.2% agarose gel, separated by

electrophoresis in 0.5X TBE, stained in ethidium bromide solution and visualised with UV.

Nested PCR approach

As described by Bollmann and Laanbroek [14], 1:100 diluted CTO-PCR fragments were used as template for a nested PCR, using the eubacterial primers 357F with GC clamp and 518R [23]. CTO-PCR products (2.5 µl of 1:100 diluted) were used for 25 µl of master mix, whose components and concentrations were described above. The thermocycling program has been described by Muyzer [23] as touchdown approach. In brief, the annealing temperature was set 10 °C above the expected annealing temperature and then lowered with 1 °C every second cycle until 55 °C.

DGGE analyses of ammonia-oxidizing communities

Approximately 200–300 ng of PCR products were separated by DGGE with a Protean II system (Bio-Rad, USA) according to the protocol of Muyzer [23] as adapted by Kowalchuk et al. [22] for the study of ammonia-oxidizing bacteria. The denaturant gradient to separate CTO fragments as well as PCR products resulting from the nested PCR approach were 30–55%, with a solution of 8% acrylamide, 7 M urea and 40% formamide defined as 100% denaturing. Gels were run for 16–17 h at 60 °C in 0.5X TAE buffer (50X Tris-acetate: 242 g Tris, 57.1 ml glacial acetic acid, 100 ml 0.5 M EDTA per liter).

Sequencing of DNA fragments from DGGE and phylogenetic analysis

From the denaturing gradient gels, selected bands were cut out from the middle parts. The acrylamide pieces were stored in water overnight at 4 °C for the elution of PCR fragments [14]. Bands were used 1:10 diluted as template for a further 25 cycles

PCR. Obtained PCR products were separated again with DGGE to confirm recovery of the desired single bands [24]. Single bands were sequenced with internal Cy 5 labelled primers, 357F and 518R, with a Thermo Sequenase Primer Cycle Sequencing Kit and ALF Express II Instrument (Amersham Biosciences, UK). Bands at the same height on the gel had the same nucleotide sequences and they were therefore submitted only once to the EMBL database. Recovered sequences were aligned with the ARB program package (http://www.arb-home.de[25]). Alignments were refined by visual inspection and the Bioedit (Biological Sequence Alignment Editor) program. Phylogenetic trees were constructed in the Treecon program (version 1.3b, Van de Peer and de Watcher 1994) using the neighbour-joining method with the Jukes and Cantor correction. Gaps were not taken into account in the analysis. Bootstrap analyses were based upon 1000 replicates. For the phylogenetic analysis, we considered only the CTO fragments and not the nested amplicons of CTO fragments.

Nucleotide sequence accession numbers

Partial 16S rRNA gene sequences determined in this study have been deposited into the EMBL sequence database with the following accession numbers: **AJ629220** (band N1), **AJ628354** (band FM1), **AJ628355** (band FM2), **AJ628356** (band FM3), **AJ628357** (band BM4), **AJ628358** (band MM5), **AJ628359** (band N3), **AJ628360** (band N4), **AJ628361** (band N5).

Statistical analysis

The effect of salinity and sediment depth on ammonium concentrations in pore water was tested per sampling day using a two-way ANOVA (n = 3). Salinity and depth were independent variables and ammonium concentration in pore water was the dependent variable. Potential nitrification activities of two replicates per treatment were compared between day 0 and day 35 with a *T*-test for dependent samples. One-way ANOVA analysis was used to test the effect of salinity on potential

nitrification activities at the two different sampling days. All data were analysed for normality (plots of SD vs. means) and were checked for homogeneity of variance (Levene' s test). All the statistical analyses were made with the STATISTICA software package, version 6.0 for Windows (Tulsa, USA).

Results

Physical observations of the sediment

The sediment used to fill the microcosms contained many indigenous worms belonging to the class Oligochaetes, which constitute more than 95% of the total density of the benthic fauna of the river Scheldt [26]. Worms were active in the freshwater microcosms, bioturbating and thereby creating a oxidised sediment down to approximately 5 cm depth. In **Fig. 2A**, the burrows of the worms are clearly visible on a picture taken from a freshwater replicate microcosm. One cm sediment slides were washed with water on 210 μm diameter sieve and worms were counted by eyes; counting gave a density of 2–4 individuals per cm^3. In brackish and marine microcosms worms did not survive, probably due to osmotic stress. The absence of worm activity led to very different redox profiles in the brackish and marine treatments. In **Fig. 2B**, it is clearly visible that 1 cm below the sediment surface a layer of black deposit is formed. This putative iron sulphide layer marks the oxic–anoxic transition zone. **Fig. 1** shows the blackish layer in the replicate marine microcosms. Another indication of the physical effect of worm activity might be the water content of the sediment. At day 7, the average water content in percentage of dry weight in the freshwater microcosms was 60% at 1 cm depth and about 40% from 2 down to 10 cm depth. At day 24 and at day 35, we observed an increase in the water content within the first 5 cm of the sediment. In the brackish and marine microcosms, the average water content was maintained within a range of 38–45% along the whole depth profile without any significant change during the incubation period. Irrespective of sampling day, the water content decreased slightly with depth in the latter microcosms.

Pore water characteristics

To reveal the effect of treatment on ammonium availability, pore water samples were retrieved from different depth in the sediment. At day 0, the average ammonium concentrations in the pore water of freshwater, brackish and marine microcosms were around 1 mM along the whole depth profile (Fig. 3). In the freshwater microcosms (**Fig. 3A**), ammonium concentrations increased during the incubation period to 2–2.5 mM at 1 and 4 cm depth and to 3–4 mM at 8 and 12 cm depth. However, in the brackish and marine microcosms (**Fig. 3B** and **C**) the steep increase in ammonium concentrations occurred already at 4 cm depth after 7 days and reached 5–6 mM at the end of the incubation. Nitrite and nitrate were not detected in the pore water samples. Statistical analysis showed that ammonium concentrations in the pore water depended significantly on salinity and depth. *P* values for salinity and depth were always below 0.05.

Potential nitrification activity

At day zero, potential nitrification activity was within the range of 2.0–3.0 μmol g^{-1} h^{-1} in the three treatments. At day 35, the potential nitrification activities decreased to a range of 0.5–1.5 μmol g^{-1} h^{-1}. This decrease was significant for all treatments (*T*-test for dependent samples, $P < 0.05$). However, salinity as such had no significant effect on potential nitrification activity (One way ANOVA). Measured PNA values of potential nitrification activity at the end of the incubation were in the range of 0.2–1.5 μmol g^{-1} h^{-1}, as were analysed at the sampling site.

Figure 2. Detail of sediment profiles. **A**: In the freshwater microcosm, the burrows of worms are visible down to approximately 5 cm. **B**: In the marine microcosm a black layer is visible at approximately 1 cm from the sediment surface. (See Appendix for full color image).

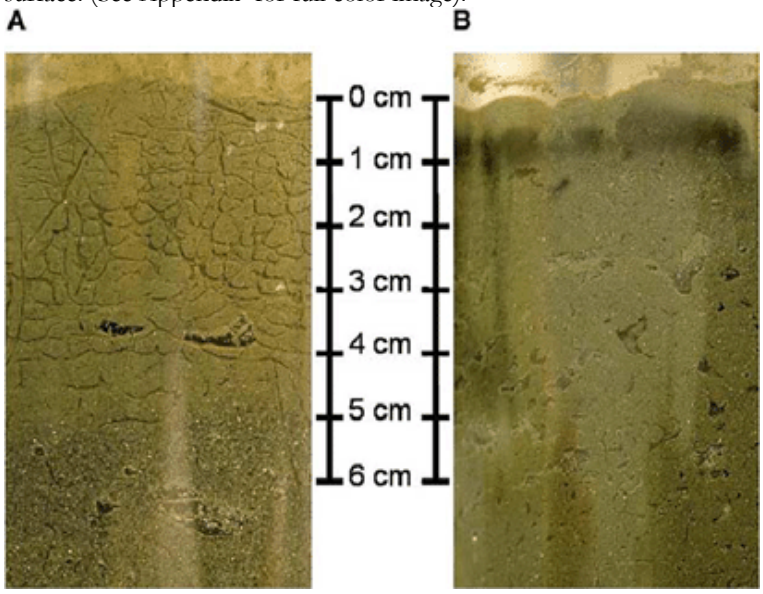
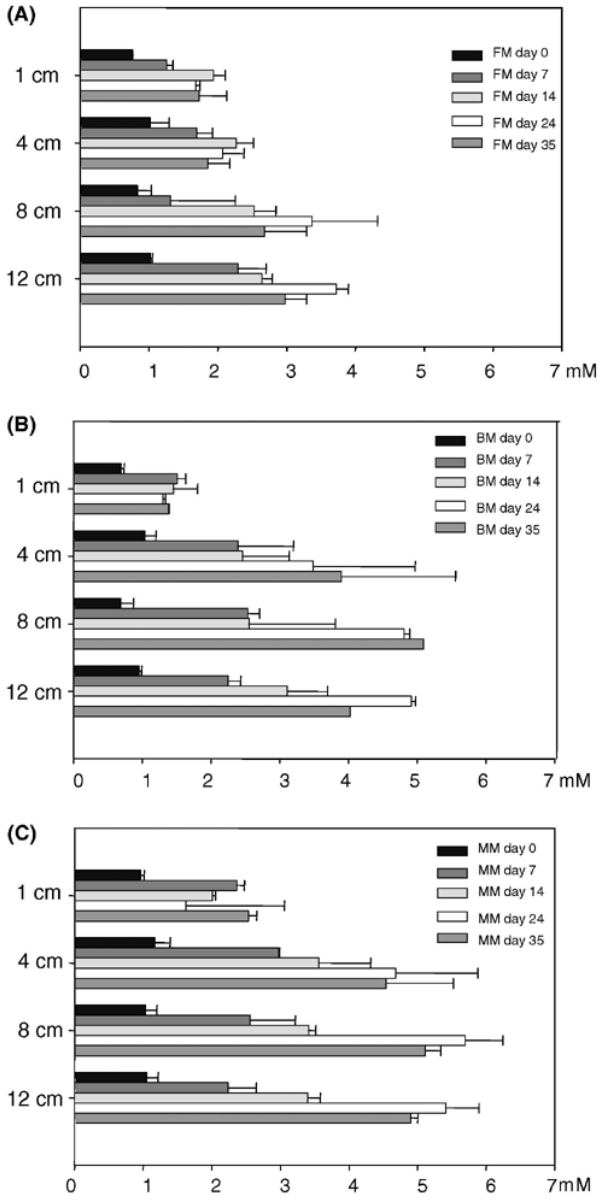


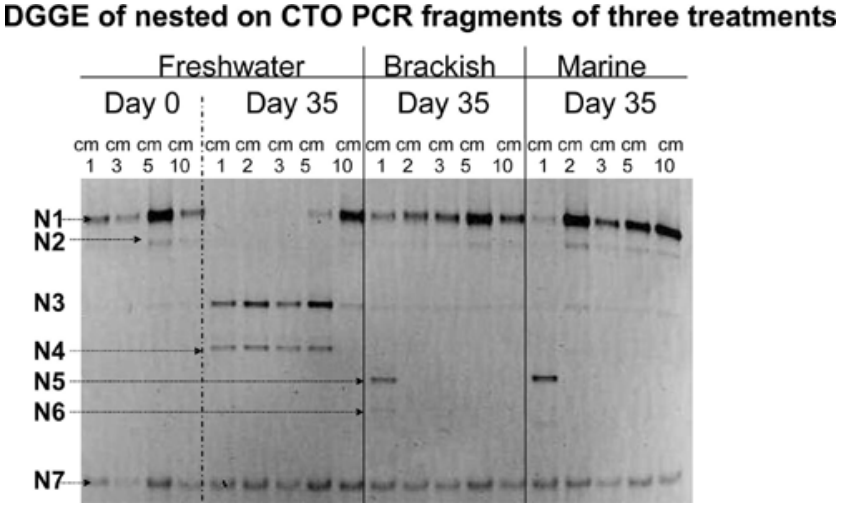
Figure 3. Effect of treatment on the ammonium concentrations (mM) in the pore water at different sediment depths. Values are averages of three replicates per treatment. A: FM, freshwater microcosms; B: BM, brackish microcosms; C: MM, marine microcosms.



Dynamics of ammonia-oxidizing community revealed by nested PCR-DGGE

Temporal and spatial dynamics within the ammonia-oxidizing community were investigated using denaturing gradient gel profiles of direct and nested PCR products. DGGE profiles of nested PCR amplicons (see Section 2.8; **Figs. 4** and **5**) showed that changes in the ammonia-oxidizing community occurred both in the freshwater microcosms and in the microcosms flooded with saline media. Moreover, the changes in the freshwater microcosms clearly differed from those in the brackish and marine microcosms. The initial ammonia-oxidizing community was represented by band N1 at all depths (**Fig. 4**).

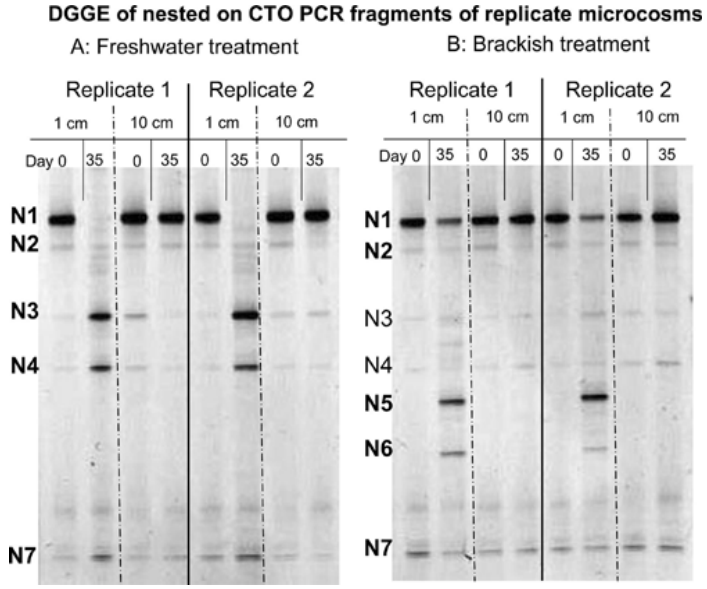
Figure 4. DGGE of nested PCR of CTO fragments. One replicate per treatment (freshwater, brackish and marine) is shown. The spatial profile at day zero (1, 3, 5 and 10 cm) is only shown for the freshwater replicate. The spatial profile at day 35 is shown for the three treatments.



In the freshwater microcosms the prominent band N1 had completely disappeared after 35 days from 1 to 3 cm depth and two new bands, N3 and N4, had appeared; band N1 persisted at 5 and 10 cm depth. After the same period of time, the intensity of band N1 decreased slightly in the brackish and in the marine

microcosms at 1 cm depth and two new bands appeared at positions N5 and N6. In the brackish and marine microcosms no changes occurred at other depths. The changes occurring in replicate freshwater microcosms (**Fig. 5A**) were identical. Similarly, no differences were observed between replicates of the brackish treatment; in both treatments no changes occurred at 10 cm depth (**Fig. 5B**).

Figure 5. DGGE of nested PCR of CTO fragments. A: Replicate microcosms 1 and 2 of the freshwater treatment. B: Replicate microcosms 1 and 2 of the brackish treatment. Per replicate, two different depths (1 and 10 cm) at two sampling days (day 0 and day 35) are displayed. Bands are numbered from N1 to N7.



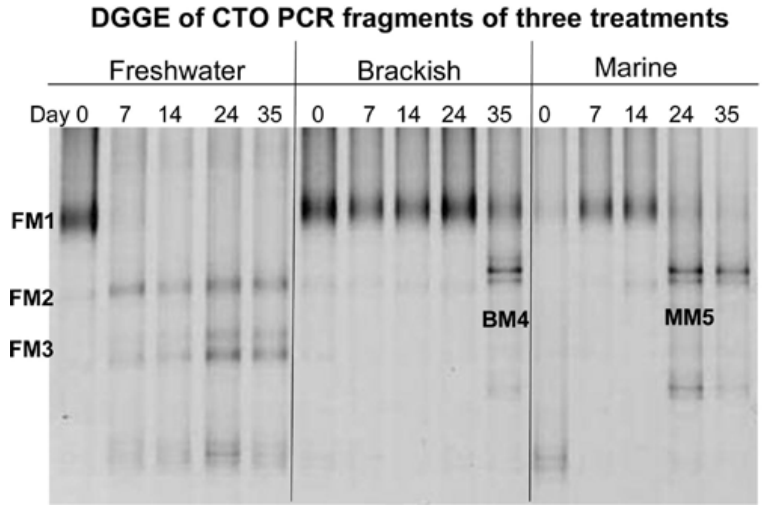
On the basis of BLAST analysis, band N1 belonged to the *N. oligotropha* lineage and showed 99% identity with clone 26Ft recovered from a wastewater treatment plant [27] and 97% identity with band FM1 recovered in our study with the direct PCR-DGGE approach (see below). Band N3 also belonged to the *N. oligotropha* lineage and showed 100% identity with *Nitrosomonas* strain Is 79 A3, which had been enriched with 0.25 mM from freshwater lake sediment (A. Bollmann, personal communication). Band N5, recovered in the marine microcosm

after 35 days belonged to the *N. marina* lineage. It had 99% identity with clone 11 Ft recovered from a wastewater treatment plant [26] and 99% identity with bands BM4 and MM5 recovered in this study with the direct PCR-DGGE approach (see below). Band N4 was not related to ammonia oxidisers' sequences and showed less then 95% identity with an environmental clone of the *Methylophilus* spp. lineage. Recovery of non-ammonia oxidisers-related sequences has been reported in other studies and explained by degenerated positions in the CTO primer nucleotides [22,28]. Reamplification of bands N2, N6 and N7 after excision failed. The limited length of sequences obtained from bands N1, N3 and N5 prohibited direct inclusion into the phylogenetic tree (**Fig. 8**).

Ammonia-oxidizing community using DGGE profiles of CTO amplicons

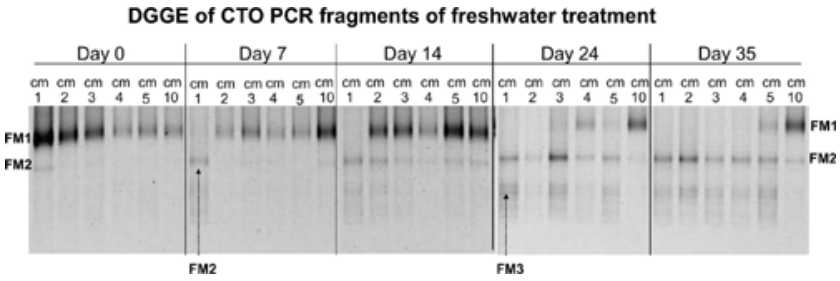
To obtain sufficient sequence information from DGGE bands for phylogenetic analysis, we also analysed the DGGE profiles of the samples through direct amplification from community DNA with the CTO primers (see Section 2.7**; Figs. 6** and **7**). The disadvantage of this direct procedure is that somewhat fuzzy bands are usually obtained. DGGE profile of CTO amplicons (**Fig. 6**) showed the changes in the initial freshwater ammonia-oxidizing community over time at 1 cm depth. At day zero, band FM1 was retrieved in all microcosms. In the freshwater microcosm, band FM1 has disappeared after 7 days and two new bands, FM2 and FM3, appeared and persisted until day 35. A new band, BM4, appeared in the brackish microcosm after 35 days in addition to band FM1, while in the marine microcosm band MM5 had appeared already at day 24. The double-band pattern (BM4 and MM5 in **Fig. 6**) in the saline treatments is the result of the ambiguities in CTO oligonucleotides [22,28].

Figure 6. Denaturing gradient gel of PCR amplified CTO fragments. Per treatment (freshwater, brackish and marine) samples at 1 cm depth are shown at five sampling days (day 0, 7, 14, 24 and 35).



Because DGGE profile of nested PCR on CTO fragments (**Fig. 4**) showed no changes in the ammonia-oxidizing community in the deeper layers of the brackish and marine microcosms, the complete spatial and temporal DGGE profile of CTO amplicons was analysed only for the freshwater microcosms (**Fig. 7**). Again, band FM1 was dominant at all depth at day zero. Band FM2 had appeared at 1 cm depth after 7 days, at 2 cm after 14 days and at 5 cm depth after 24 days. The unclear band FM3 appeared at day 24 at all depths. Band FM1 belonged to the *N. oligotropha* lineage with 97% and 95% identity to *N. oligotropha* and *Nitrosomonas ureae*, respectively. Band FM2 also belonged to the *N. oligotropha* lineage with 96% and 95% identity to *N. oligotropha* and *N. ureae*, respectively. Bands FM1 and FM2 both showed more than 97% identity to *Nitrosomonas* strain Is. 79 A3. Band FM3 was mostly related to a *Decholoromonas* species, which is not an ammonia oxidiser, and is thus not presented in the phylogenetic tree (**Fig. 8**). Band BM4 and MM5 were identical (100%) and belonged to the *N. marina* lineage; they showed 99% identity with *Nitrosomonas* strain Is. 343, which has been isolated from the brackish part of the Scheldt estuary (A. Bollmann, personal communication).

Figure 7. Denaturing gradient gel of CTO-PCR fragments (direct amplification). A complete profile in a freshwater replicate microcosm over time (day 0, 7, 14, 24 and 35) at six different depths (1, 2, 3, 4, 5 and 10 cm) is shown.

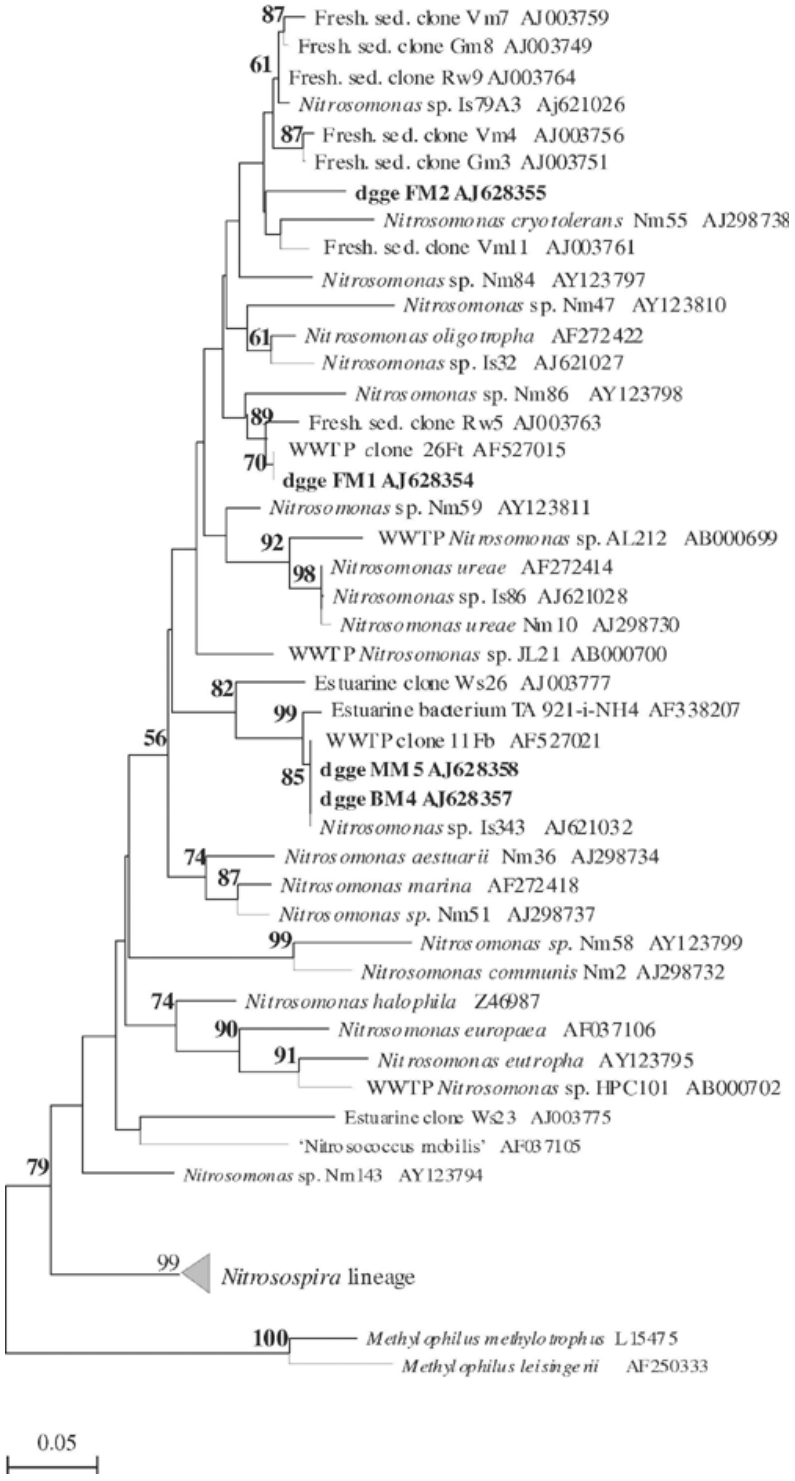


Discussion

The aim of this study was to assess spatial and temporal dynamics of AOB communities under environmental perturbations. The microcosms system designed for the experiment facilitated the assessment of the response of freshwater AOB communities to tidal flooding with saline water under nearly natural, but controlled conditions. Bacteria from the *N. oligotropha* lineage of the betaproteobacteria appeared to dominate the initial ammonia-oxidizing community of the intertidal freshwater sediment. It is indeed known that in natural freshwater environments, members of the *N. oligotropha* lineage are generally the dominant representatives of AOB [2,12]. Our results match studies on other estuarine systems, such as the Seine [16] and the Elbe [15] estuaries, as well as with previous studies on the Scheldt estuary [13,14]. The initial salt-sensitive population was clearly replaced by salt-tolerant or halophilic ammonia oxidisers, when the sediments were flooded with saline water; their proliferation occurred after 14–24 days. Changes occurred earlier in the marine than in the brackish microcosms; apparently, higher salinity of the marine microcosms led to a faster shift from salt-sensitive members of the *N. oligotropha* lineage to salt-tolerant or even halophilic AOB members of the *N. marina* lineage. This difference indicates that AOB that are appearing are more likely halophilic than salt-tolerant. Enrichment of AOB from the brackish part of the Scheldt

estuary yielded, on the contrary, salt-tolerant bacteria [14]. The *N. oligotropha* and the *N. marina* lineages of ammonia oxidisers are considered phylogenetically different [7,8] and have pronounced differences in distribution patterns and ecophysiological characteristics [1]. Obviously the conditions in the freshwater sediment of Appels are not such that species belonging to the *N. marina* lineage can become dominant members of the AOB community; these AOB remain in these sediments and wait for conditions that match their physiology such as higher salt concentration before emerging. Unexpectedly, flooding with freshwater also induced changes in the ammonia-oxidizing community. In the freshwater microcosms a shift occurred within the *N. oligotropha* lineage. This change occurred already in the first week of incubation at 1 cm depth.

Figure 8. Neighbour-joining tree of selected 16S rRNA genes of ammonia oxidisers from *Betaproteobacteria*. Sequences determined in this study are printed in bold and codes correspond to Figs. 6 and 7. The phylogenetic tree was based on 417 informative positions. The scale bar represents 0.05 fixed position mutations per nucleotide sequence position. Values at the node indicate bootstrap percentage over 1000 replicate trees. Trees were rooted using *E. coli* (U00006) and *Agrobacterium tumefaciens* (AJ389886) as outgroup.


87 Fresh. sed. clone Vm7 AJ003759
Fresh. sed. clone Gm8 AJ003749
61 Fresh. sed. clone Rw9 AJ003764
Nitrosomonas sp. Is79A3 Aj621026
87 Fresh. sed. clone Vm4 AJ003756
Fresh. sed. clone Gm3 AJ003751
dgge FM2 AJ628355
Nitrosomonas cryotolerans Nm55 AJ298738
Fresh. sed. clone Vm11 AJ003761
Nitrosomonas sp. Nm84 AY123797
Nitrosomonas sp. Nm47 AY123810
61 Nitrosomonas oligotropha AF272422
Nitrosomonas sp. Is32 AJ621027
Nitrosomonas sp. Nm86 AY123798
89 Fresh. sed. clone Rw5 AJ003763
WWTP clone 26Ft AF527015
70 dgge FM1 AJ628354
Nitrosomonas sp. Nm59 AY123811
92 WWTP Nitrosomonas sp. AL212 AB000699
98 Nitrosomonas ureae AF272414
Nitrosomonas sp. Is86 AJ621028
Nitrosomonas ureae Nm10 AJ298730
WWTP Nitrosomonas sp. JL21 AB000700
82 Estuarine clone Ws26 AJ003777
99 Estuarine bacterium TA 921-i-NH4 AF338207
56 WWTP clone 11Fb AF527021
85 dgge MM5 AJ628358
dgge BM4 AJ628357
Nitrosomonas sp. Is343 AJ621032
74 Nitrosomonas aestuarii Nm36 AJ298734
87 Nitrosomonas marina AF272418
Nitrosomonas sp. Nm51 AJ298737
99 Nitrosomonas sp. Nm58 AY123799
Nitrosomonas communis Nm2 AJ298732
74 Nitrosomonas halophila Z46987
90 Nitrosomonas europaea AF037106
91 Nitrosomonas eutropha AY123795
WWTP Nitrosomonas sp. HPC101 AB000702
Estuarine clone Ws23 AJ003775
'Nitrosococcus mobilis' AF037105
79 Nitrosomonas sp. Nm143 AY123794
99 Nitrosospira lineage
100 Methylophilus methylotrophus L15475
Methylophilus leisingerii AF250333
0.05

In a study of ammonia-oxidizing bacterial population of the Scheldt estuary, Bollmann and Laanbroek [14] showed that ammonia oxidisers grew better in their natural water supplemented with ammonia compared to mineral medium. They suggested that the presence of homoserine lactones or small organic compounds or ions, not provided in the mineral medium but present in the natural environment, could enhance the growth of strains different from those dominant under natural conditions. Another explanation for the observed community shift in the freshwater microcosms may be temperature. In addition to a partial effect of soil ammonia concentration, Avrahami and Conrad [29] demonstrated a direct effect of temperature on the composition of the ammonia-oxidizing community in soils. Temperature at the sampling site was about 8–10 °C, while the experiment was conducted at 25 °C. The higher temperature could have selectively facilitated the growth of ammonia oxidisers that were negligible under the natural conditions. Although the data on the potential nitrification activities have to be considered with caution, because they were measured only at the beginning and at the end of the experiment in only two out of the four replicated treatments, we observed a clear decrease of activity with incubation time. Such a decrease in activity might be expected in the brackish and marine treatments due to osmotic stress and induced anoxicity, but it was quite surprising in the freshwater treatment. It might indicate that the original freshwater community had suffered from the mineral medium used for incubations and possibly also from higher temperature. However, at the end of the experiment, the potential nitrification activity was still within the range of values measured in sediment slurries collected at the sampling site. We clearly observed that changes in the AOB community in the freshwater microcosms occurred down to a depth of 5 cm, while changes in the brackish and marine microcosms were detected only at 1 cm depth. This is interestingly linked with the presence of a blackish layer, hence of reduced and anoxic conditions, at the 1-cm depth in both brackish and marine treatments after 7 days of incubation. Since changes in the AOB community in the freshwater microcosms were observed down to 5 cm depth, it can be assumed that

oxygen was not a limiting factor in this zone. It is significant to point out that in this part of the sediment and down to 6–8 cm, the activity of worms took place. Worms persisted actively in the freshwater microcosms but not in the brackish and marine microcosms. The direct effect of the Oligochaetes on the microbial communities in our microcosms is not known. However, detailed studies demonstrate the strong impact that tubificids have on grain size distribution, erosion, water content, diffusion, permeability and oxygen demand of the sediments [26]. Data on the water content might be an indication of the impact of worms; in brackish and marine microcosms, water content was maintained constant during the time and along the depth. In contrast, the water content in the freshwater microcosms increased manifestly down to 6–8 cm during the incubation. This zone correlates strongly with the oxygenated layer we observed. We are aware that our observations can not explain the shift that occurred in the freshwater microcosms because worms will also play a role in the natural environment, but the results open new questions about the discrete or combined role of various factors on niche differentiation of ammonia oxidisers even among closely related strains. In conclusion, our results confirmed that the composition of ammonia-oxidizing community changes under environmental perturbations; further we showed that community changes or niche differentiation under natural conditions can take place within days and that salinity is a steering factor in selecting *Nitrosomonas* species in the sediment.

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Chapter VII

General discussion

Studies on niche differentiation of ammonia-oxidizing bacteria are relatively numerous in comparison with other functional groups. Unlike most functional groups, ammonia-oxidizing bacteria (AOB) are restricted to two monophyletic groups within the beta- and gamma-subclasses of the Proteobacteria, respectively [35]. This allowed the successful application of molecular techniques, mainly based on 16S rRNA and *amoA* genes amplification, for investigating the diversity of AOB in the environment and for studying the phylogenetic relationships between different species.

Furthermore, the phylogenetically defined lineages or clusters of ammonia-oxidizing bacteria generally contain cultured species, for which physiological properties are known [40, 44]. Sequences retrieved in the environment can be related to specific ammonia oxidizers allowing tentative identification of the microorganisms they can be assigned to. This has led to formulate the hypothesis that different strains of ammonia-oxidizing bacteria, *i.e.* ecotypes, are associated with particular environments according to their physiological requirements [40, 44, 45].

Molecular techniques however have intrinsic biases that are unavoidable. The diversity described, for example, by PCR and cloning approaches may not represent the actual diversity of the microbial community investigated. Micro-variations introduced by PCR and cloning approaches especially occur when targeting a monophyletic group like the ammonia-oxidizing bacteria of beta-subclass of the Proteobateria [126]. Furthermore, the sensitivity and specificity of primers and probes used, for example, for PCR or Fluorescent In Situ Hybridization (FISH) bias the outcome of molecular surveys of ammonia oxidizers in the environment [40, 41]. However, the increasing amount of

information obtained by molecular surveys brings continuous feedback on the molecular techniques themselves, finally leading to the improvement of the already exploited ones and the setting up of new ones [223, 224].

In the case of slowly growing bacteria such as ammonia oxidizers, molecular techniques had significantly contributed to overcome the difficulties of cultivation-based techniques and produce knowledge about this functional group, revealing an unexpected and intriguing diversity. On the other hand, to unequivocally prove the presence and viability of AOB species molecularly detected in the environment, the isolation of species is still necessary. Researchers increased their effort in enriching AOB strains from the environment [114], but, again, enrichment techniques have also limitations and can results in the isolation of species that are actually not dominating the environment examined. Over the time, the combination of both cultivation and molecular approaches strengthened the basic hypothesis of the existence of ecotypes among AOB that occupy different niches in the environment [40, 44]. From an ecological point of view, ammonia oxidizers represent therefore a model group for ecological studies [41].

Despite the common basic metabolism, different species of ammonia-oxidizing bacteria required distinct environmental conditions for their existence; in other words, they occupy different niches in the environment (**Chapter** I). For instance, members of the *Nitrosomonas oligotropha* lineage appear to dominate freshwater environments [40], due to their adaptation to low ammonium concentration and their sensitivity to salt. However, members of the *Nitrosospira* lineage were also detected in freshwater environments (for detailed review [40, 41]. Moreover, within freshwater environments there are several habitats and possible niches for ammonia-oxidizing bacteria. Next to the sediment and water column [73, 84], the epiphyton of submerged macrophytes should also be considered as a possible niche for ammonia oxidizers. With respect to this, particularly interesting is the study of Körner [96], who counted significant numbers of ammonia oxidizers on the leaves of submerged macrophytes, as well as the study of Eriksson et al. [99], who showed that submerged macrophytes stimulated

nitrification rates. Eriksson et al. suggested that submersed macrophytes might offer accessible surface areas for the attachment of nitrifying bacterial cells. The proof of this was however missing. Hence, the following questions arose:

1. Do submerged macrophytes offer a niche to ammonia-oxidizing bacteria in addition to the benthic and pelagic compartments?
2. In addition, how diverse are the epiphytic communities of different submerged macrophytes from the benthic and pelagic communities?

Indeed, submerged macrophytes colonizing shallow freshwater lakes offered a niche to ammonia-oxidizing bacteria in addition to the benthic and the pelagic compartment (**Chapters II**, **III** and **IV**). The epiphyton of submerged macrophytes was inhabited by members of the *N. oligotropha* lineage and of cluster 3 of the *Nitrosospira* lineage. The benthic compartment contained both members of clusters 0 and 3 of the *Nitrosospira* lineage as well as members of the *N. oligotropha* lineage. The pelagic compartment instead contained only members of the *N. oligotropha* lineage. The communities were different between compartments, but were also significantly influenced by the overall conditions of the lakes sampled. This latter observation will be discussed in more detail later on in this Chapter.

The sampling effort done in the study presented in **Chapter II** included 7 different shallow freshwater lakes and 4 macrophytes species and gave a first insight into the diversity of the epiphytic communities over a large collection of sample. The study also served to evaluate the most suitable technique to address the questions. The basic choice fell on the denaturing gradient gel electrophoresis technique, since this technique allowed the rapid and simultaneous comparison of multiple samples [41]. DGGE was used to separate amplification products obtained with four different PCR approaches specific for ammonia oxidizers of the beta-subclass of the Proteobacteria. Finally, a choice was also made with respect to the PCR-approach to be used. Only the nested-PCR approach that used the primer sets βAMOf-βAMOr and CTO189f-CTO654r allowed amplification of AOB from all compartments (**Chapter II**). Nested PCR-based approaches are indeed a common practice on low-copy number

targets in environmental samples [225]. Results obtained with the nested approach were confirmed in a study conducted one year later (**Chapter III**), on 3 out of the 7 lakes, with triplicate DGGE profiles per sample. Even though the sampling effort was reduced to 3 out of 7 lakes, the lakes chosen were the most representative of the whole system. Moreover, the 16S rDNA-DGGE results were supported by microscopy analyses of FISH showing the presence of members of the *N. oligotropha* and of the *Nitrosospira* lineages on the leaves of the submerged macrophyte *Potamogeton pectinatus*. In contrast, the other dominating submerged plant, *Chara* sp., showed to hardly support an AOB community.

These observations of ammonia-oxidizing bacteria in the epiphyton of submerged macrophytes gave rise to the following questions:

3. Given that certain submerged macrophytes offered a niche to ammonia oxidizers, what is the size of the epiphytic ammonia-oxidizing community in comparison with the benthic and the pelagic communities?
4. What is the relationship between pelagic and benthic ammonia-oxidizing bacteria with respect to the colonization behavior, in other words which is the origin of epiphytic AOB?

By applying Real-Time PCR on the 16S rRNA gene (**Chapter III**, protocol by Hermansson [156]), numbers of AOB in different compartments were assessed. Epiphytic AOB were comparable in number to pelagic AOB but lower than benthic AOB. Other studies showed that the sediment [73, 89] contained higher numbers of ammonia oxidizer cells. Sediments are richer in nutrient content and allow more complex microbial interaction at the oxic-anoxic interface. Similarly to the community composition, numbers of ammonia- oxidizing bacteria were significantly affected by the status of the lakes investigated.

The origin and colonization mechanism of epiphytic ammonia-oxidizing bacteria were assessed in a microcosm experiment using *P. pectinatus* as a model plant (**Chapter IV**). Epiphytic AOB originated from the benthic compartment and colonized the plant during its sprouting but also during the sediment

resuspension. Interestingly, pelagic AOB were also colonizing the plant. As a matter of fact, the pelagic AOB colonizing the plant were below the detection limit in their original environment, and become attached to the plant likely by exopolymer-production for biofilm formation. The main difference between the microcosms and the natural situation was indeed the water retention time, of 35 and 3 days, respectively. As already known from the establishment of waste water treatment plant, sufficient long retention times are necessary for the correct establishment of nitrifying biofilm.

Assessing the diversity and numbers of ammonia-oxidizing bacteria and defining their niches are basic to study the interaction between the AOB microbial community and the characteristics of their host environments. The dynamics of this interaction may lead to the formulation of hypotheses on the effect of the environment on the process of nitrification itself. These types of studies are definitely the main target of true molecular ecology of microbial systems. The final aim is to assess the impact of the microbial communities on the ecosystems and possibly predict future scenarios of responses of the microbial communities to continuously changing environments. With respect to this knowledge is far from complete. However, AOB again appeared as a model group for molecular microbial ecology studies and a suitable subject to answer new questions arose:

7. Is the environment, viewed as a complex set of interacting variables, a driving force of niche differentiation?
8. Are the niches distributed along natural environmental gradients?
9. Do ammonia oxidizers respond to environmental perturbations?
10. If so, what is the response to anthropogenic influences?
11. What is, for example, the effect of the trophic status of a lake on the diversity and numbers of ammonia-oxidizing bacteria? Is there a relationship between the submerged macrophyte species, whose distribution is the result of past restoration measures, and the AOB community?

The known hypothesis of G. M. Baas Becking: *"Alles is overall, maar het milieu selecteert"* ("Everything is everywhere but the environment selects") is still more a question than a statement. Are all ammonia-oxidizing species present everywhere? Does the diversity revealed by molecular surveys truly resemble the natural diversity of AOB communities? Is then the environment to enhance the relative dominance of certain species among the others? What happened to AOB and subsequently to the process of nitrification if the environment changes?

By molecular surveys it resulted that not all ammonia-oxidizing bacteria are everywhere but that different species are associated with defined environments. For example, in Chapter V the species-environment association was described along a freshwater-saline gradient, where the succession of subpopulations of ammonia–oxidizing bacteria occurred along with the salinity and oxygen gradient. In particular, the AOB freshwater community composed by members of the *N. oligotropha* lineage was replaced along the estuaries by brackish and marine communities, constituted by salt-tolerant or even halophilic species. Thus, the environment selects species.

Among the environmental variables, salinity represents a steering force in the niche differentiation occurring in natural environments. Therefore, the direct effect of salinity was tested on a benthic freshwater AOB community (**Chapter VI**). What happens to the benthic freshwater AOB community under increasing salt concentrations? Much to my surprise, salt-tolerant AOB species were enriched from a freshwater sediment and substituted the original freshwater community as fast as the increase of salinity was steep. In addition to this shift, the original freshwater community responded also to the mineral medium treatment. What has been said before, *i.e.* that not all ammonia-oxidizing bacteria are everywhere, finds already its first objection. Freshwater sediment appeared to contain salt tolerant species. Why? One simple hypothesis could be that the diversity of AOB guarantees ecosystem stability, given that nitrification is a key process in the nitrogen cycle. Theory indeed predicts functional redundancy, intended as a measure of the number of different species within functional groups, for a correct ecosystem functioning [226-229].

Even though I can just speculate on the reasons that drive community changes, it remains clear that AOB are adapted to environmental gradients and respond to natural perturbations (**Chapter V** and **VI**) [57, 60-62, 78, 79, 119, 230-234]. However, what is the response of AOB to environmental changes caused by human activities?

AOB are shown to respond to global change [72] and to changes in land uses, for example by decreasing in diversity [65, 67]. The results of the research presented in this thesis are in the same line. Already in the extended sampling survey presented in **Chapter II** there was evidence of lower diversity of AOB communities in the freshwater bodies subjected to past restoration measures. The results presented in **Chapter III** confirmed those evidences: not only diversity, but also numbers and potential activities of ammonia-oxidizing bacteria were lower in the benthic, pelagic and epiphytic compartments of the lakes restored. In the microcosm experiment presented in **Chapter IV**, a longer retention time allowed the colonization of the epiphyton by pelagic AOB, otherwise not detected in their original compartment. In Chapter V the waste water treatment plant effluent inoculated the estuarine system with AOB, otherwise not detected in the autochthous populations. Finally, in **Chapter VI** the use of mineral media caused a shift in the original freshwater AOB community.

Given that AOB communities respond to the environmental changes and are affected by human activities, a final question arises:

12. What happens to the process for which AOB are responsible, *i.e.* nitrification?

Unfortunately this thesis has no answer to this question. Attempts to measure nitrification in the different compartments of shallow lakes were done in the model system described in **Chapter VI**. However, nitrification, as the conversion of ammonium into nitrate was not measurable, due to the loss of nitrogen caused by the submerged macrophyte *P. pectinatus*. Aquatic macrophytes, in general, affect the nitrogen cycle in many ways: for examples by direct uptake and assimilation of nitrogen, by lowering the water velocity thereby enhancing sedimentation of nitrogen containing particulate matter, and also

by stimulating the coupled nitrification-denitrification process, which finally leads to the permanent removal of nitrogen from the system [6, 98, 99, 103, 106, 108, 151, 194, 196, 197, 220, 235-238]. Even though nitrification was not established, the molecular assessment of the presence of AOB on the leaves of submerged macrophytes was provided. The presence of AOB was highly influenced by the type of submerged macrophytes, which were *Chara* sp. and *P. pectinatus*. From an applied perspective, it might be important to know that not all macrophytes species harbor ammonia oxidizers and also that the submerged macrophytes ideal for restoration and aesthetic reasons, namely Charophytes, might be not equally beneficial for the ecosystem functioning.

Moreover, nitrification is responsible for the production of greenhouse gases, proportionally to the increase of ammonia in the environment. In this respect, future scenarios of global change are not encouraging. It might therefore be not unrealistic to hypothesize an increase of greenhouse gases by nitrification. In addition, nitrification is considered detrimental in agriculture because it causes nitrogen depletion in soils where its final product, *i.e.* nitrate, is used by denitrifying bacteria or leached to groundwater where it may cause pollution of drinking water. As anthropogenic inputs of fixed nitrogen continue to expand to meet the demands of a growing global population, intimate knowledge of the nitrification process and the microorganisms that control this process will be necessary to address environmental nitrogen imbalances [24]. These future scenarios are generally negative. Encouraging is the fact that the scientific community is continuously elucidating the metabolism of AOB and of several other microorganisms involved in the nitrogen cycle, with the always increasing awareness of the impact on a global scale [3, 24]. Integrating different disciplines with microbial ecology and environmetal microbiology is among the greatest research challenges [226]. At present, and in a much smaller scale, this thesis may contribute to the knowledge on the ecology of ammonia-oxidizing bacteria and their niche differentiation, hopefully adding a new tile to the puzzle of science.

Future perspective

The application of molecular techniques enabled the exploration of diversity of ammonia-oxidizing bacteria in a great variety of environments. In contrast culture-depend microbiological studies are time-consuming and effort-intensive when applied to slowly growing microorganisms like AOB. However, well defined cultures are basic to assess the mechanisms of niche differentiation and effort should be done to obtain relevant species from their host environments. For example, the physiological properties of the likely exopolymer-producing AOB detected in the epiphyton need a more detailed investigation. Laboratory experiments, mimicking the natural or perturbed environmental conditions, might help assessing the mechanisms of niche differentiation among relevant species observed in the environment. Particular attention should be paid in defining the real active *in situ* AOB population within the community present in the environment. Microsensor measurements, ^{15}N stable isotope techniques have been successfully applied in this respect [136, 196, 206, 239]. Equally, stable isotope probing combined with molecular techniques can distinguish between active and non-active populations [58, 240, 241]. Analyses of mRNA expression of the *amoA* gene can be also used to assess in situ activity [140]. To date, whole genome sequences of 3 ammonia-oxidizing species are available [242, 243] and several others are on the way. Genomic data might provide information on the genes and their expression and translation into proteins which may be at the very basis of mechanisms of niche differentiation, for example in the formation of biofilm on different surfaces and in response to changes in the environment. Finally, all information obtained in laboratory experiments should be directed towards the assessment of the impact of the nitrifying microbial communities and theirs interaction with other microbial groups involved in the nitrogen cycle, at the proper scale. Niche differentiation in micro-niches can only be understood comprehensively when studying at the micro or sub-micron scale. The discovered niche, *i.e.* the epiphyton, is another step into the direction of achieving better insights in the functioning AOB, being a crucial link in the global nitrogen cycle.

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SUMMARY IN ENGLISH

Niche differentiation between ammonia-oxidizing bacteria in aquatic environments

The aim of the studies presented in this thesis was the search for niche differentiation between the ammonia-oxidizing bacteria in aquatic environments. Ammonia-oxidizing bacteria are chemolitho-autotrophic microorganisms responsible for the first, mostly rate-limiting step of the nitrification process, *i.e.* the conversion of ammonia into nitrite. The recent development of molecular techniques had overcome the difficulties inherent to the classic cultivation-based methods applied to these slow-growing bacteria. Finally, a large diversity of ammonia-oxidizing bacteria has been uncovered in several aquatic environments. However, only a few studies focused on niche differentiation between these bacteria within the same estuary or freshwater environment. The studies reported in this thesis demonstrated the existence of an intriguing niche differentiation between ammonia-oxidizing bacteria driven by different factors, such as ammonia and oxygen availability, salinity, tidal regime, physical-chemical characteristics of the substrate for the biofilm formation and anthropogenic influences.

In particular, in shallow freshwater lakes dominated by submerged macrophytes ammonia-oxidizing bacteria appeared to utilize the epiphyton as a niche in addition to the benthic and pelagic compartments (**Chapters II** and **III**). The molecular detection of ammonia-oxidizing bacteria on the leaves of submerged macrophytes, like *Potamogeton pectinatus*, is the first time ever to be reported. Diversity, numbers and activity of benthic, pelagic and epiphytic ammonia-oxidizing bacteria was influenced by the characteristics of both the compartments and the lakes in which the samples were collected (**Chapter III**).

Ammonia-oxidizing bacteria showed higher diversity, numbers and activity in the benthic compartment in comparison to the pelagic compartment (**Chapter III**). Members of the *Nitrosomonas oligotropha* lineage dominated the pelagic compartment and were present in the benthic section together with members of the clusters 0 and 3 of the *Nitrosospira* lineage. The epiphyton was colonized by both pelagic and benthic ammonia-oxidizing bacteria. The dynamics of colonization of

the epiphyton by benthic and pelagic ammonia-oxidizing bacteria were investigated in a microcosm experiment (**Chapter IV**). Results showed that benthic ammonia oxidizers colonized the surface of the macrophytes both during the sprouting of the tubers and the resuspension of sediment particles. Being attached to the macrophytes surface appeared to be favorable for pelagic species that were only present at low numbers in their original compartment.

Restoration procedures applied to the lakes in the past at different intensities, appeared to influence the overall fitness of ammonia-oxidizing bacteria negatively (**Chapter III**). Briefly, the best restored lake, i.e. Lake Nuldernauw, nowadays colonized by a Charophytes meadow showed the lowest diversity, numbers and potential activity of ammonia-oxidizing cells.

The influence of anthropogenic inputs on the niche differentiation between ammonia-oxidizing bacteria was also shown in a study along a estuarine gradient in addition to the effects of salinity as well as ammonia and oxygen availability (**Chapter V**). Salinity showed to be a steering factor for the succession from salt-sensitive to salt-tolerant ammonia-oxidizing species in freshwater sediment during a microcosm experiment with controlled environmental perturbations (**Chapter VI**).

Finally, the studies presented in this thesis contributed to the knowledge on the niche differentiation between ammonia-oxidizing bacteria in shallow lakes and estuarine ecosystems by describing the epiphyton of submerged macrophytes as a novel niche for ammonia oxidizers and by describing the response of these bacteria to natural and anthropogenic environmental perturbations.

SINTESI IN ITALIANO

Differenziazione di nicchia dei batteri ammonio-ossidanti in ambienti acquatici

Oggetto della ricerca riportata in questa tesi è stata la differenziazione di nicchia dei batteri ammonio-ossidanti in ambienti acquatici. I batteri ammonio-ossidanti sono microorganismi Gram negativi, chemolito-autrotrofi, aerobi obbligati, responsabili della conversione dell'ammoniaca in nitriti durante il primo step del processo di nitrificazione. I nitriti vengono successivamente trasformati in nitrati dai batteri nitrito-ossidanti. L'enzima chiave per il processo di ossidazione dell'ammoniaca è l'ammonio-monossigenasi. Il basso rendimento energetico della reazione di ossidazione, in aggiunta alla necessità di creare potere riducente tramite trasporto inverso di elettroni a livello di membrana sono alla base del lungo tempo di generazione di questi microrganismi (circa 8 ore per *Nitrosomonas europaea)*. Per questa ragione, lo studio dei batteri ammonio-ossidanti mediante tecniche microbiologiche "classiche", quali l'arricchimento e l' isolamento, è stato per lungo tempo difficile e poco efficiente. Negli ultimi decenni, lo sviluppo e l'applicazione delle tecniche di biologia molecolare basate sui geni per la subunità ribosomiale 16S e sui geni codificanti la subunità a dell'enzima ammonio-monoossigenasi hanno reso possibile lo studio dei batteri ammonio-ossidanti in ambiente naturale. I batteri ammonio-ossidanti hanno rivelato una sorprendente differenziazione di specie in tutti gli ambienti acquatici, naturali ed artificiali, e di acqua dolce, salmastra e salata, nonché in ambiente terrestre. I batteri ammonio-ossidanti sono rapidamente diventati un gruppo microbico modello per lo studio dei processi di differenziazione di nicchia. Le numerose specie che vi appartengono, infatti, pur possedendo un identico metabolismo di base, hanno dimostrato una chiara distribuizione nei diversi ambienti che generalmente riflette le diverse caratteristiche fisiologiche delle specie stesse. Nel caso dei batteri ammonio-ossidanti sembra quindi possibile parlare di differenziazione di nicchia o di eco-distribuzione. Tuttavia e al meglio delle nostre conoscenze, i processi di differenziazione di

nicchia fra batteri ammonio-ossidanti nell'ambito di uno stesso ambiente finora non erano stati oggetto di studio.
I risultati riportati in questa tesi dimostrano l'esistenza di una differenziazione di nicchia tra i batteri ammonio-ossidanti all'interno di uno stesso ambiente acquatico. Lo studio ha individuato numerosi fattori significativamente responsabili del processo di differenziazione di nicchia. In particolare, la salinità e la disponibilità di ossigeno erano alla base della successione tra microoganismi ammonio-ossidanti di non-alofili, alotolleranti ed alofili lungo l'estuario del fiume Senna (**Capitolo V**). L'immissione di acque di scolo da un impianto situato a Nord della città di Parigi nell'estuario della Senna inoltre alterava la diversità della comunità ammonio-ossidante inoculando l'estuario di specie alloctone all'estuario stesso. In condizioni controllate di laboratorio la salinità è stata confermata come fattore determinante per la differenziazione di nicchia (**Capitolo VI**), congiuntamente all'effetto delle maree e possibilmente alla omogenizzazione ed ossigenazione dei sedimenti ad opera di macroinvertebrati.
L'ipotesi della differenziazione di nicchia fra batteri ammonio-ossidanti è stata investigata in dettaglio nei laghi di acqua dolce, poco profondi, quindi mai stratificati e fortemente influenzati dalla risospensione dei sedimenti per azione del vento (**Capitoli II, III** e **IV**). In questi laghi, la vegetazione acquatica sommersa gioca un ruolo di primaria importanza nel mantenere le acque limpide ed evitare la proliferazione di alghe spesso tossiche. Laghi di questo tipo sono presenti in gran numero nei Paesi Bassi, dove l'acqua è vincolata in sistemi di canali artificiali, necessari per l'approvvigionamento idrico dell'intero paese, nonché per la navigazione e per scopi ricreativi.
Il sistema acquatico scelto per lo studio consisteva di 7 laghi seminaturali, interconnessi tra loro a mezzo di dighe, soggetti nel passato in misura differente a processi di biomanipolazione (per esempio, la rimozione dei sedimenti ricchi in fosfato o di pesci bentivori responsabili della risospensione dei sedimenti) per debellare la proliferazione di cianobatteri tossici e promuovere il ripristino della vegetazione acquatica sommersa. Idealmente, nei laghi sono stati individuate tre differenti nicchie spaziali, cioè il sedimento, la colonna d'acqua e la superficie delle piante

acquatiche sommerse, rispettivamente corrispondenti al dominio bentonico, pelagico e all'epifito.
Mediante tecniche molecolari di PCR-DGGE e FISH, specie differenti di batteri ammonio-ossidanti sono state ritrovate nelle tre zone. Precedenti studi avevano già descritto la diversità dei batteri ammonio-ossidanti nei sedimenti e nella colonna d'acqua, ma questo studio riporta il primo ritrovamento mediante tecniche molecolari di batteri ammonio-ossidanti nell'epifito di piante sommerse della specie *Potamogeton pectinatus.*, dimostrando inequivocabilmente che l'epifito rappresenta una ulteriore nicchia per i batteri ammonio-ossidanti in aggiunta ai dominii bentonico e pelagico nei laghi poco profondi (**Capitoli II, III** e **IV**). In condizioni controllate di laboratorio è stato anche dimostrato che batteri ammonio-ossidanti di origine sia bentonica che pelagica colonizzavano l'epifito di *P. pectinatus*. La colonizzazione dell'epifito ad opera di batteri ammonio-ossidanti di origine bentonica avveniva nella fase di germinazione e anche tramite risospensione del sedimento (**Capitolo IV**). Le macroalghe del genere *Chara* spp. invece sembravano non favorire la colonizzazione di batteri ammonio-ossidanti.
La diversità in specie, il numero di cellule e l' attività potenziale dei batteri ammonio-ossidanti venivano influenzate dalle caratteristiche dei diversi habitats (bentonico, pelagico ed epifitico). Il dominio bentonico è risultato più ricco in termini di diversità e numero di cellule e attività potenziale, soprattutto in contrapposizione al dominio pelagico.
Anche lo stato trofico dei laghi, al tempo stesso causa ed effetto della vegetazione acquatica sommersa, risultava influenzare in maniera negativa diversità, numero e attività dei batteri ammonio-ossidanti. Essi infatti risultavano significativamente ridotti nei laghi sottoposti in passato a processi di biomanipolazione. Quest' ultimo aspetto desta sicuramente interesse e rende i batteri ammonio-ossidanti un possibile modello non solo per studiare la differenziazione di nicchia ma anche per descrivere la risposta della comunità microbica agli, spesso negativi, effetti degli interventi antropici nell'ambiente.

SAMENVATTING IN HET NEDERLANDS

Ammoniumoxiderende bacteriën spelen een belangrijke rol in de stikstofkringloop. Zij zijn namelijk verantwoordelijk voor de eerste stap in de oxidatie van ammonium tot nitraat en dragen daardoor bij aan de eutrofiëring van terrestrische en aquatische ecosystemen. Onder de laatsten zijn ondiepe zoetwatermeren en estuaria wijdverbreid en belangrijk. Ze bieden niches aan verschillende ammoniumoxiderende bacteriën door de gradienten die er van nature voorkomen.

Het onderzoek beschreven in dit proefschrift heeft aangetoond dat ammoniumoxiderende bacteriën hun niches kiezen op grond van verschillen in fysiologische eigenschappen en heersende milieufactoren. In de Seine werd de nichedifferentiatie vooral bepaald door het zoutgehalte en de beschikbare zuurstof. Het zoutgehalte bleek ook een sturende factor in een experiment met sedimentmonsters uit de Schelde.

In de Nederlandse randmeren bleken ammoniumoxiderende bacteriën voor te komen in het sediment en in het water, maar ook aangehecht op ondergedoken waterplanten. Dit laatste is nieuw en kan van belang zijn voor het functioneren van de randmeren. Activiteit, aantallen en soortensamenstelling bleken verschillend voor de verschillende compartimenten van de randmeren. Innovatief in het onderzoek was de vraag naar de herkomst van de ammoniumoxiderende bacteriën op de ondergedoken waterplanten. Interessant was de waarneming dat de maatregelen in de randmeren voor herstel van de waterkwaliteit een duidelijk effect hadden op de verspreiding van de soorten ammoniumoxiderende bacteriën.

De resultaten van het onderzoek dragen bij aan de kennis van de ecologie van ammoniumoxiderende bacteriën in aquatische milieus, maar zij vormen ook de basis voor nieuwe onderzoeksvragen.

Acknowledgments/ Ringraziamenti/ Agradecimientos

Finally...I did it, better We did it! Indeed, I am conscious that I would not been able to arrive so far without the help of many of you.

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A Sergei Winogradsky...
Alla Sicilia, l'Itaca dei miei pensieri...

Acicastello, Gennaio 2007

Curriculum Vitae

Manuela Coci was born on April 7, 1978, in the city of Catania on the Ionian coast of Sicily. After obtaining her gymnasium diploma at the Liceo Classico Mario Cutelli, she started her study in Biology at the University of Catania. During her forth academic year, she went to the Universidad de Ciencias del Mar, in Càdiz, Spain with an Erasmus Student grant. There she started her specialization in marine biological sciences. Once back to Catania, she prepared the master thesis at the laboratory of Prof. S. Stefani in Marine Microbiology studying the nitrifying bacteria of commercial start-up solution for biological filters used in the aquaria. She then continued for another 6 months at the same laboratory working with different molecular techniques. In the meanwhile, she passed the national examination for Biologist and she obtained a student grant to go abroad. With that grant, she started working at the Department of Microbial Ecology at the Centre for Limnology, in the Netherlands. She performed the molecular analyses on the ammonia-oxidising bacterial communities subject to increasing salinity with a tidal regime simulated in a microcosm experiment. In the meantime, Prof. H.J. Laanbroek and Dr. P. Bodelier offered her a position as junior scientist. She therefore started her own project on the niche differentiation of ammonia-oxidising bacteria in the benthic, pelagic and epiphytic compartments of shallow freshwater lakes dominated by submerged macrophytes. During her PhD training, she attended several post-graduated courses, she collaborated with the University Pierre and Marie Curie in Paris and with the Universities of Torino and Nijmegen. She also went to the laboratory of Prof. J. I. Prosser at the University of Aberdeen to perform quantitative PCR on ammonia oxidizers. For that visit she obtained the Schure-Beijerinck-Popping Fonds. She also presented her PhD studies at several international congresses. Since October 2006, Manuela is giving classes in Environmental Microbiology at the University of Catania and trying to develop her own research line in the laboratory of Prof. S.Stefani.

List of publications

Denaturing gradient gel electrophoresis analysis of ammonia-oxidizing bacterial community structure in the lower Seine river: impact of Paris waster water effluent. Cebron, A.., Coci, M., Garnier, J., Laanbroek, H.J.. (2004). Applied and Environmental Microbiology, 70: 6726-6737.

Effect of salinity on temporal and spatial dynamics of ammonia-oxidizing bacteria from intertidal freshwater sediment. Coci, M., Riechmann, D., Bodelier, P.L.E., Stefani, S., Zwart, G., Laanbroek, H.J.. (2005). FEMS Microbiology. Ecology, 53: 359-368.

Effect of substratum on the diversity and stability of ammonia-oxidising communities in a constructed wetland used for wastewater treatment. (2007). Gorra, R., Coci, M., Ambrosoli, R., Laanbroek, H.J. Journal of Applied Microbiology. (Published article, online: 25-Apr-2007)

Epiphyton as a niche for ammonia-oxidizing bacteria: detailed comparison with benthic and pelagic compartments in shallow freshwater lakes. Coci, M., Bodelier, P.L.E, Laanbroek, H.J.. (submitted to Applied and Environmental Microbiology, 2007)

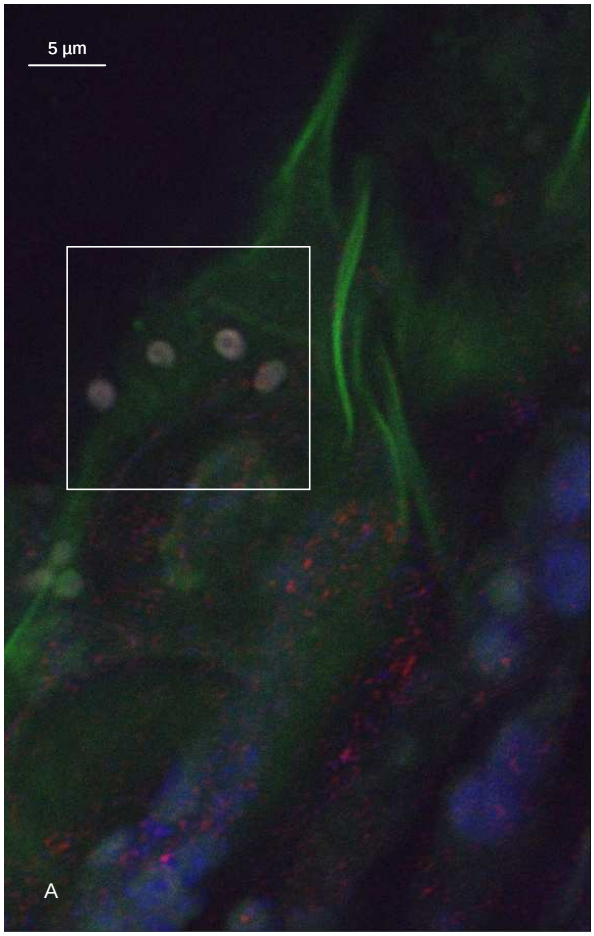
List of partecipation to International Congresses

September 2002. SAME-8, Symposium of Aquatic Microbial Ecology. Taormina, Italy. Oral presentation: "Biological filter: a model for studying microorganisms able to metabolize ammonia, nitrite and nitrate in aquatic environment".
June 2003. FEMS-1, Congress of European Microbiologists. Ljubljana, Slovenia. Poster presentation: "Niche differentiation of Ammonia-oxidising bacterial communities along a salinity gradient in microcosm experiment", supported by a FEMS-Congress Attendance Grant.
August 2004. ISME-10, International Symposium on Microbial Ecology. Cancun, Mexico. Poster presentation: "Epiphytic nitrifying communities on submerged macrophytes in shallow freshwater lakes".
August 2005. SAME-9, Symposium of Aquatic Microbial Ecology. Helsinki, Finland. Oral presentation: "Niche differentiation of nitrifying bacteria in shallow freshwater lakes", supported by a Congress Attendance Grant.
August 2006. ISME-11, International Symposium on Microbial Ecology. Vienna, Austria. Abstract title: "Ammonia-oxidising bacteria in the benthic, pelagic and epiphytic compartments of shallow freshwater lakes".
July 2007. SEFS5, Symposium of European Freshwater Science. Palermo, Italy. Oral presentation: "Ecology of ammonia-oxidising bacteria in shallow freshwater lakes". Award as best PhD student.
September 2007. SAME-10, Symposium of Aquatic Microbial Ecology , Faro, Portugal. Oral presentation: "Ammonia-oxidizing bacteria in shallow freshwater lakes", supported by a FEMS Congress Attendance Grant.

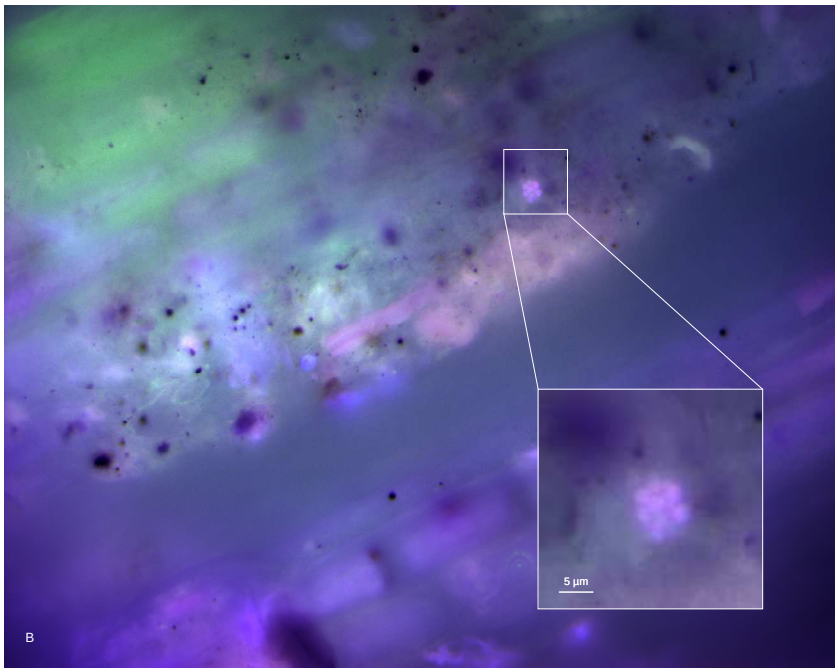
Appendix

Full color images

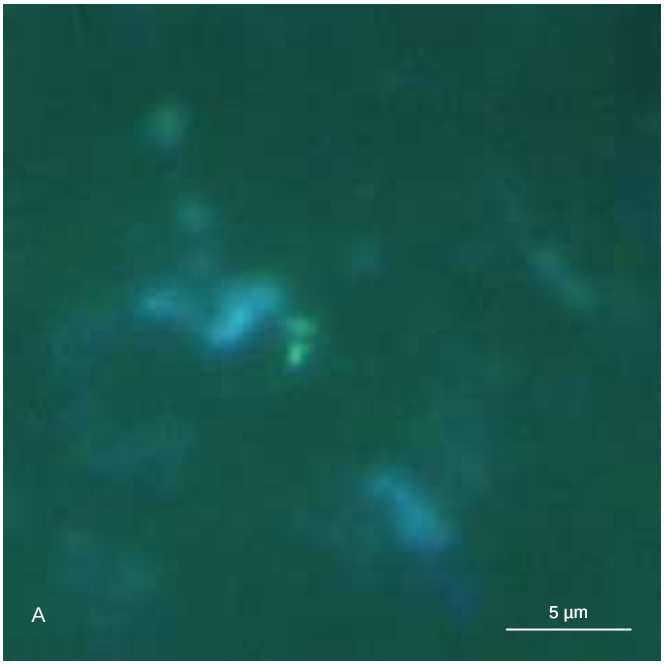
Chapter III. Figure 6. Images of fluorescent in situ hybridizations of ammonia-oxidizing bacterial cells in the epiphytic compartments.
Panel A: Confocal laser scanning microscopy field of *Nitrosospira*-like cells (inside the marked area) on leaves of *Potamogeton pectinatus* from station C of Lake Gooimeer, double hybridized with the Cy3-labeled probe Nsv443 and the Cy5-labeled probe BET42a, stained with DAPI and viewed by phase–contrast.



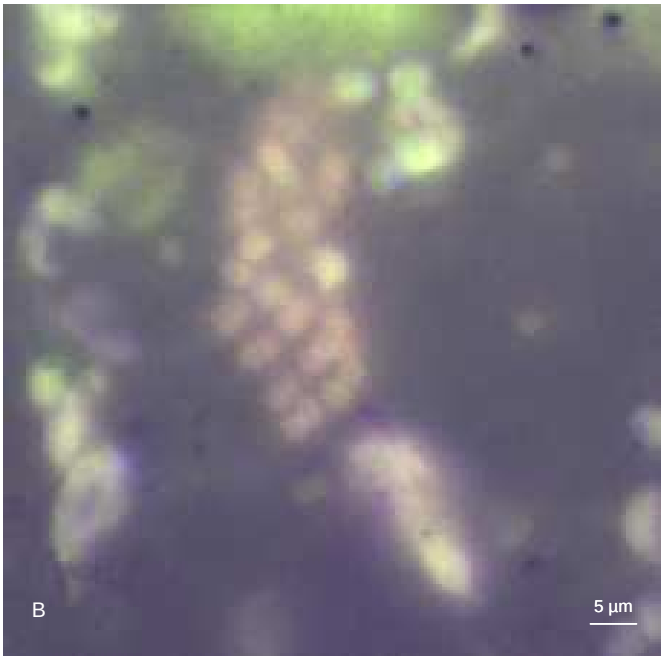
Chapter III Figure 6. Images of fluorescent in situ hybridizations of ammonia-oxidizing bacterial cells in the epiphytic compartments (See appendix I for full color images). **Panel B**: Epifluorescent microscope image of a *Nitrosospira*-like cell cluster (colored in red by image analysis) and its enlarged detail, on a *Potamogeton pectinatus* leave from station A of Lake Vossemeer; image of cells double hybridized with the Cy3-label probe Nsv443 and the Cy5-labeled probe BET42a, stained with DAPI and viewed by phase–contrast.



Chapter III. Figure 7. Images of fluorescent in situ hybridizations of ammonia-oxidizing bacterial cells in the pelagic compartment.
Panel A: Epifluorescent microscope image of *Nitrosomonas*-like cells (colored in turquoise by image analysis) on filter samples from station A of Lake Gooimeer, double hybridized by Fluos-labeled probes NSO190/NSO1225 and Cy5-labeled probe BET42a and stained with DAPI.



Chapter III. Figure 7. Images of fluorescent in situ hybridizations of ammonia-oxidizing bacterial cells in the pelagic compartment.
Panel B: Epifluorescent microscope image of *Nitrosospira*-like cells (colored in red by image analysis) on filter samples of Lake Vossemeer, double hybridized by the Cy3-labeled probe Nsv443 and the Cy5-labeled probe BET42a and stained with DAPI.



Chapter VI. Figure 1. Overview of the microcosms: 12 microcosms in total with four replicates per treatment. The marine microcosms are in the foreground.

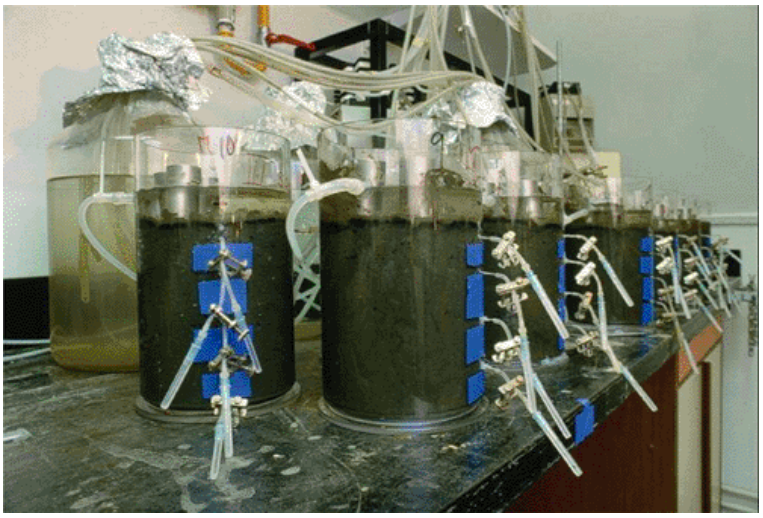


Figure 2. Detail of sediment profiles. **A**: In the freshwater microcosm, the burrows of worms are visible down to approximately 5 cm. **B**: In the marine microcosm a black layer is visible at approximately 1 cm from the sediment surface.