Factors controlling nitrification and nitrous oxide production in the Schelde estuary

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C H A P T E R 1

General introduction

Estuaries are located at the interface of seas and rivers. The Schelde estuary is the last true estuary remaining in the delta region of south-west Netherlands. The Schelde estuary is characterised by high organic loads due to waste and industrial water inputs, which cause oxygen depletion in the water column of the upper estuary. The oxygen concentration is of importance to various steps in the nitrogen cycle. This thesis is focussed on nitrification, one of the processes in the nitrogen cycle that is dependent on oxygen. Nitrification, oxidation of NH_4^+ to NO_3^-, is an oxygen-consuming process and is therefore partly responsible for the oxygen depletion in the upper estuary. Nitrification under low oxygen conditions may lead to the formation of nitrous and nitric oxides. Nitric oxide (NO) is a highly reactive gas and contributes to greenhouse effect by formation of ozone. Due to its reactive character, its atmospheric residence time is short; 1.5 days. Nitrous oxide (N_2O) is not reactive and has a residence time of 100-150 years. N_2O is also a greenhouse gas and it is involved in the destruction of the ozone layer.

Nitrification is the only microbial process that can alter nitrogen to a more oxidized state. The availability of oxidized nitrogen in the form of nitrate is of crucial importance for the "self-cleaning" system of the estuary, i.e. denitrification. The severe state of nitrogen pollution in the Schelde estuary demands a better understanding of factors controlling nitrification and denitrification, and of the micro-organisms that are involved in these processes.

The Nitrogen Cycle

Introduction

Nitrogen can occur in numerous oxidation states and has stable valences ranging from -3, as in ammonia (NH_3), to +5, as in nitrate (NO_3^-). One of the major reservoirs of nitrogen in the biosphere ($3.8*10^{15}$ metric tons) is N_2-gas in the atmosphere. Various types of rocks contain even larger amounts of nitrogen in the form of non-exchangeable ammonia. The triple bond between the two nitrogen atoms in N_2 makes it very difficult to access for organisms. To make the nitrogen available as a nutrient, it has to be converted to nitrogen salts by nitrogen fixation. The inorganic nitrogen salts, ammonium, nitrite and nitrate, are highly water-soluble and form actively cycling reservoirs in aquatic ecosystems. Under low oxygen conditions,

chemically bound nitrogen can be removed by denitrification. Consequently, nitrogen is often a limiting factor for primary production in many ecosystems. Nitrogen is an important component of all living organisms, because it makes a substantial part of proteins and amino acids. Hence, living and dead organic matter also provide relatively small but very actively recycled reservoirs.

Micro-organisms play a central role in almost all processes of the global nitrogen cycle. A general scheme of the cycle is shown in Figure 1. Four important processes of the cycle are: nitrogen fixation, ammonification, nitrification, i.e. ammonium plus nitrite oxidation and denitrification, which will be discussed hereafter.

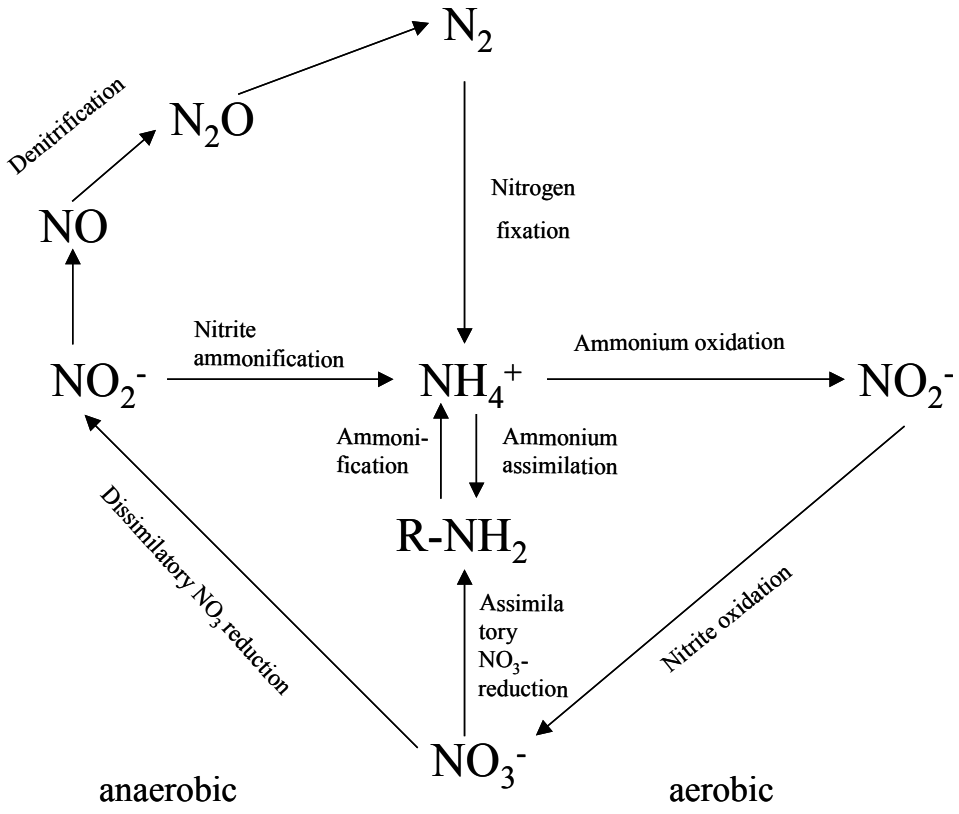


Figure 1. The nitrogen cycle, showing the chemical forms of nitrogen components. Key processes of nitrogen transformations are indicated with arrows. The left part of the figure represents anaerobic, the right side aerobic processes. R-NH_2 represents organically bound nitrogen.

Budget calculations on the global nitrogen cycle are of interest to scientists of a variety of disciplines. As many other nutrient cycles, the nitrogen cycle is drastically changing since industrialisation, which demands a continuous update and recalculation of the global budget. In the eighties, the amount of anthropogenically fixed nitrogen exceeded natural nitrogen fixation (Vitousek 1994). A recent recalculation of the ocean's nitrogen budget

(Codispoti et al. 2001, Table 1) shows a nitrogen deficit of about 200Tg yr^{-1}, which indicates major shortcomings in our present understanding of global nitrogen cycling. Besides increased anthropogenic nitrogen fixation, new insights indicate that also oceanic nitrogen fixation is more extensive than previously believed. Estimates of oceanic nitrogen fixation in 1994 were 5-20 Tg yr^{-1} (Carpenter & Capone 1983). More recent estimates are 6-25 higher (Codispoti et al. 2001). The global increase of biologically available nitrogen consequently influences nitrification and denitrification, which are the most important biological processes controlling nitrous oxide fluxes. The increasing atmospheric nitrous oxide concentration has led to concern because of its effect on the global climate. To better understand the effects of elevated nitrogen cycling on nitrification and nitrous oxide production, the factors regulating these processes have to be surveyed and quantified.

Table 1 Nitrogen budget for the ocean, according to Codispoti et al. (2001).

Process	Tg N yr^{-1}
Sources	
Pelagic N_2-fixation	110
Benthic N_2-fixation	15
River input (DN)	34
River input (PON)	42
Atmospheric deposition (Net)	30
Atmospheric deposition (DON)	56
TOTAL SOURCES	**287**
Sinks	
Organic N export	1
Benthic denitrification	300
Water column denitrification	150
Sedimentation	25
N_2O loss	6
TOTAL SINKS	**482**

Nitrogen fixation

Nitrogen fixation is the only way to utilize the large reservoir of atmospheric nitrogen and is the only process that puts new nitrogen into the biological nitrogen cycle. The energy-costing process of nitrogen fixation is generally initiated under circumstances of nitrogen limitation. Nitrogen fixation is restricted to bacteria which might either be free-living (like the cyanobacteria *Anabaena, Nostoc* and *Trichodesmium* sp) or form symbiotic associations with

plants (e.g. *Rhizobium, Azotobacter* and *Azospirillum* spp) and other organisms like termites and fungi (lichens) (Atlas & Bartha 1987). The symbiotic association of nitrogen fixing bacteria with plants provides the plants with necessary nitrogen in nutrient-poor soils. Some termites maintain a nitrogen-fixing flora in their guts to balance the carbon-nitrogen ratio in their cellulose-based diet.
Besides this biological pathway, small amounts of ammonia are produced from N_2 in the atmosphere by lightning. Moreover, nitrogen is fixed industrially to produce fertilizers.

Ammonification

Nitrogen is mostly present as reduced amino-groups in living and dead organic matter. During the decomposition of organic matter, ammonification is the process in which organically bound nitrogen is converted to ammonia. In water, ammonia will be protonated to ammonium depending on the pH. Many plants and micro-organisms assimilate ammonia, which is directly incorporated into amino-acids and other nitrogen-containing biochemicals.

Ammonia can also be formed as a result of dissimilatory nitrate reduction. Some bacteria can use nitrate as an alternative electron acceptor instead of oxygen to produce ammonia. Finally, ammonia is formed as an intermediate in the assimilation of nitrate by autotrophic plants and micro-organisms. (Kuenen & Robertson 1987)

Nitrification

Nitrification is defined as the oxidation of reduced nitrogen compounds and is an energy-yielding microbial process. There are two groups of bacteria that obtain energy for growth from nitrification; ammonia oxidizers and nitrite oxidizers. The first group oxidizes NH_3 to NO_2^-, while the second oxidizes NO_2^- further to NO_3^-.

Ammonia-oxidation: $NH_3 + 1½\ O_2 \rightarrow NO_2^- + H^+ + H_2O$ (ΔG_0' = -272 kJ)

Nitrite oxidation: $NO_2^- + ½\ O_2 \rightarrow NO_3^-$ (ΔG_0' = -75 kJ)

Nitrifying bacteria are chemolithotrophic and are able to grow with inorganic carbon in the form of CO_2 as their sole carbon source, which is fixed via the Calvin cycle. Assimilation of small organic compounds by ammonia oxidizers has been shown (Frijlink et al. 1992), but does not contribute to energy yield. The oxidation of ammonia is a multistep process and involves the intermediate hydroxylamine (NH_2OH). From studies with $^{18}O_2$ it became evident

that the oxygen incorporated in NH_4^+ is derived from O_2, while the oxygen used to produce NO_2^- is derived from H_2O (Bedard & Knowles 1989). Nitrite oxidation is a single step process and yields only small amounts of energy.

Two enzymes are involved in the process of ammonia oxidation. The first one, ammonia monooxygenase (AMO) converts ammonia (rather than ammonium) into hydroxylamine and the second one, hydroxylamine oxidoreductase converts hydroxylamine to nitrite (Wood 1986). Ammonia monoxygenase is a versatile enzyme capable of oxidizing a variety of substrates, including methane (Bedard & Knowles 1989).

Different microbial populations carry out the two steps of nitrification. However, the two processes are closely coupled and accumulation of NO_2^- does usually not occur. Organisms capable of the first reaction (ammonium-oxidation) are named *Nitroso-*, like: *Nitrosomonas europaea, Nitrosococcus oceanus* and *Nitrosolobus multiformis.* Further oxidation to nitrate is carried out by organisms which are named *Nitro-*: *Nitrobacter winogradskyi, Nitrospira gracilus* and *Nitrococcus mobilis* are examples of this group. Studies of the genotypic diversity have revealed that representatives of the ammonia-oxidizing bacteria are found in both the ß- and γ-subclasses of the Proteobacteria (see Koops & Pommerening-Röser 2001 for a review). The ß-subclass of proteobacteria contains 14 described species in two clusters, while only one genus has been described in the γ-subclass.

Nitrifying bacteria are difficult to study using traditional cultivation techniques because they have long generation times. The development of molecular techniques offers new opportunities to explore the physiology and ecology of nitrifying bacteria and application of these techniques has led to new insights in nitrifyer population dynamics. Traditionally, bacterial populations are studied by enrichment cultures. Organisms to be studied are selected in selective media, often with optimal, i.e. high nutrient concentrations. This procedure tends to enrich organisms that are adapted to these optimal conditions, which are not necessarily the ecologically most important organisms. It was not before molecular techniques were established that the real extent of microbial diversity was revealed (Giovannoni et al. 1990, Ward et al. 1990). A recent study shows that the number of genotypes of ammonia oxidizers found by conventional Most Probable Number (MPN) techniques was 1-3 orders of magnitude lower than that obtained by specific PCR (Polymerase Chain Reaction) amplification of target DNA (Philips et al. 2000). This implies that our knowledge of the physiology of nitrifying bacteria is based on a minor, not necessarily representative part of the natural community. The importance of new isolation and enrichment techniques in

combination with molecular techniques has therefore been stressed (Bollmann & Laanbroek 2001, Koops & Pommerening-Röser 2001).
Bacteria involved in both ammonia and nitrite oxidation generally have lower specific growth rates at low oxygen concentration as compared to heterotrophic bacteria. However, it has become evident that nitrification may occur at very low oxygen concentrations (Kuenen & Robertson 1987, Muller 1994). Ammonia oxidation at low oxygen concentrations may be accompanied by reduction of nitrite to nitric and nitrous oxide (Poth & Focht 1985).

Denitrification

Denitrifying bacteria are capable of reducing nitrate through nitrite, nitric oxide (NO), and nitrous oxide (N_2O) to molecular nitrogen (N_2). Nitrate is used by these organisms as an alternative electron acceptor when oxygen is not or poorly available. The nitrate necessary for denitrification in natural systems is provided by local production by nitrification or advection of nitrate-rich (ground) water. Besides nitrate or nitrite, a source of organic carbon is required for the reduction of these oxidized inorganic compounds. Biological denitrification is mostly carried out under anoxic conditions, although denitrification under reduced oxygen tension is also observed (Kuenen & Robertson 1987).
Nitrification and denitrification are not necessarily separate processes (Kuenen & Robertson 1994). An example of a combined nitrification-denitrification process is anaerobic ammonium oxidation (Anammox) (Mulder et al. 1995), in which nitrate is serving as the electron acceptor under anaerobic conditions. Anammox is taking place according to the following reaction:

$$5NH_4^+ + 3NO_3^- \rightarrow 4N_2 + 9\,H_2O + 2H^+$$

The process is energy yielding and has shown to be biologically mediated (Van de Graaf et al. 1995, Jetten 2001a,b). The process was discovered in a denitrifying (waste water) reactor and the responsible organism has been identified to be a new planctomycete (Strous et al. 1999). Ammonium can also be transformed to N_2 through oxidation by MnO_2 but this does not necessarily involve bacterial mediation (Luther et al. 1997). Manganese (Mn^{2+}) may also contribute to denitrification by the reduction of nitrate.
During denitrification, nitrogen is removed from the aquatic or terrestrial environment and emitted to the atmosphere, which is why it has been of particular interest in studies regarding nitrate-polluted waters. This sink of nitrogen in aquatic systems appears to vary from a few to

more than 40% of the inorganic nitrogen inputs (Seitzinger 1993), depending on environmental factors, in particular the water residence time.

Human impact on the global nitrogen cycle

> *"Our understanding of abundance, distribution, and behavior of nitrogen in estuarine systems began with a very practical concern about the effects of pollution. Some of the earliest systematic measurements of nitrogen in estuarine waters were those obtained by William Joseph Dibdin, chemist to the London Metropolitan Board of Works. Beginning in 1885, he carried out routine monitoring of ammonia levels in the tidal Thames, off the major sewage outfall at Crossness." (Nixon & Pilson 1983)*

As with all cycles, the global nitrogen cycle should be in balance, with about equal amounts of nitrogen entering the biological pool by fixation and of nitrogen removed to the atmospheric reservoir by denitrification. However, the global nitrogen cycle has been influenced in several ways by human activity. Human activities like production of nitrogen fertilizer and cultivation of legume crops alter global biogeochemistry and contribute to eutrophication (Howarth et al. 1996, Vitousek 1994). In addition, nitrogen stored in biomass of fossil fuels is mobilised by burning (Crutzen & Andreae 1990, Vitousek 1994). Export of inorganic nitrogen by world rivers and estuaries was estimated to be 20.8 Tg N yr^{-1} in 1990, of which 75% was estimated to be of anthropogenic origin (Seitzinger & Kroeze 1998). On a global scale, the North Sea region has the most disturbed N-fluxes, with increased fluxes of 6-20 fold as compared to pre-industrial times (Howarth et al. 1996)

The increase of inorganic nitrogen, and especially ammonium, in the environment leads to enhanced nitrification and therefore to various pollution effects like acidification of soil and water, acid rain, corrosion of building materials and statues and increase of nitrate and nitrite in ground- or drinking waters. Ammonium ions are positively charged and adsorb to clay particles in soils, while nitrate is easily washed out to ground waters. Nitrification in agricultural soils is therefore an undesirable process, not only leading to a loss of nitrogen source for crops, but also contamination of ground-, surface- and eventually drinking waters with nitrate and nitrite. In agricultural practices, nitrification inhibitors are used to prevent nitrification to take place. In reaction to the effects of nitrogen alterations, efforts have been made to eliminate as much nitrogen as possible in waste water treatment plants as well as to minimize further increase of human induced nitrogen fixation and mobilisation, for example by use of natural fertilizers and decrease of fossil fuels use. Modern wastewater

treatment plants can remove up to 80-90% of the nitrogen that was present in the influent (http://www.ecology.su.se/dbhfj).

Nitrogen is often the limiting nutrient for primary production in marine waters. Increased nitrogen inputs from estuaries into coastal waters stimulate phytoplankton growth. This results in higher productivity and biomass, but often lower algal species diversity. Moreover, a change in phytoplankton species composition may have drastic effects on the food web structure. The increased primary productivity can lead to massive blooms that may be nuisance or even toxic leading to a risk for exploration of fisheries and coastal tourism. Collapse of phytoplankton blooms lead to anoxic bottom water and sediments. Decreased oxygen concentrations negatively affect the environment in several ways, of which the potential production of nitrous oxide is one (Jørgensen et al. 1984, Poth & Focht 1985).

The Schelde estuary

> *"Many people consider this river as not much more than a river in coma, an environmental patient that doesn't house fish anymore. Others look at the Schelde as a navigation channel alone, which demands expensive dredging operations. For people living on her shores, the Schelde becomes threatening and unpredictable in case of storm floods. Nevertheless, the Schelde deserves an international label of quality for her nature values." (Schelde informatiecentrum)*

The river Schelde originates on the Saint Quentin plateau in northern France. It is 355 km long, drains about 19,500 km^2 in France, Belgium and the Netherlands and has a discharge into the North Sea of 120 $m^3\ s^{-1}$ on average (Belmans 1991). The river discharge is mainly governed by rainfall and can fluctuate from 20 $m^3\ s^{-1}$ during summer to 400 $m^3\ s^{-1}$ during winter. The freshwater runoff from the river Schelde is mixed with the salt water of the North Sea in the estuarine part of the Schelde, which is the topic of interest in this thesis. The Schelde estuary, of which the part that is situated in the Netherlands is called "Westerschelde", is defined to extend over a distance of about 100 km from the North Sea at Vlissingen to the mouth of the river Rupel (see Figure 2). The mean tidal difference is 3.8 meter at Vlissingen and up to 5 meter in Antwerpen. Tidal changes are noticeable as far as to the city of Gent. The estuary is characterised by a strong horizontal salinity gradient, high turbidity and a suboxic water column in the most upstream part during most of the year. Complex flood and ebb channels exist and the water column is well mixed. Estuaries are often characterised by high nutrient and organic loads (Binnerup et al. 1992, Feliatra & Bianchi 1993, Jørgensen & Sørensen 1988). This is particularly true for the Schelde estuary

because the river Schelde and its tributaries are heavily polluted by the discharge of the cities Gent, Antwerpen and Brussel, and active industrial areas along their course. The watershed of the Schelde constitutes the living and working environment for about 10 million people. In addition, intensive stock farming contributes to pollution in the northern part of the watershed (Billen et al. 1985). Another important characteristic of the Schelde estuary is the extensive dredging and dumping of sediment that is performed to maintain the navigation channels and harbours of Antwerpen open for shipping activities. This contributes to the yet high turbidity. A clear turbidity maximum, which is common in most estuaries, is difficult to detect in the Schelde estuary (Paucot & Wollast 1997).

The function of a coastal ecosystem is strongly influenced by estuarine inputs. A recent example is the massive dead of mussels in the Oosterschelde, as a result of anoxic waters after the collapse of a *Phaeocystis* bloom (Collombon & Poelman 2001). Depletion of oxygen has become a major problem in many eutrophic environments including estuaries. The low

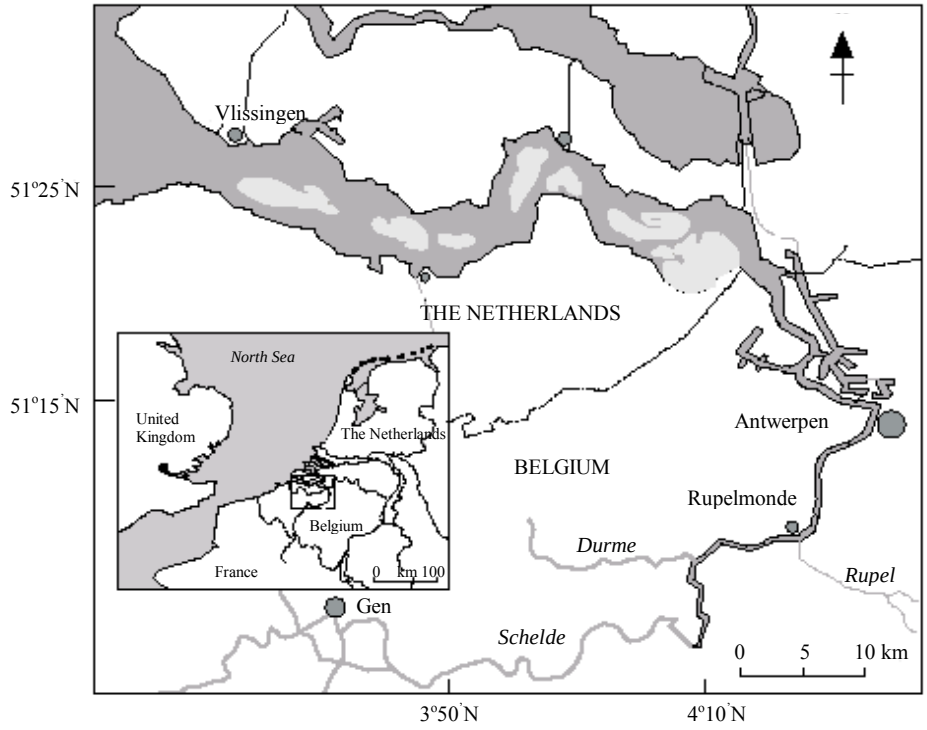


Fig 2. Map of the Schelde estuary.

oxygen tension is mostly caused by heterotrophic microbial activity. If NH_4^+ is available in substantial amounts, however, chemolithoautotrophic nitrifying bacteria may also contribute significantly to oxygen consumption (Seitzinger 1987, Soetaert & Herman 1995). In eutrophic estuaries like the Schelde, heterotrophic bacterial production may exceed phytoplankton production, indicating that allochthonous sources of organic matter are more important than phytoplankton in supporting bacterial growth (Goosen et al. 1995, Hoch & Kirchman 1993).

Estuaries can play an important role in controlling the organic and inorganic nutrient load of open seas, and act as a filter system between rivers and oceans. Only one third of the nitrogen load of the Schelde watershed finally reached the sea (Billen et al. 1985). Sediments play an important role in the nitrogen cycling in the estuary, because of the occurrence of denitrification. Denitrification comprises a natural sink and mitigates the increasing nitrate discharge in coastal environments (Jørgensen & Sørensen 1988, Nixon et al. 1996). In balanced systems, denitrification is depending on nitrate that is produced by nitrification. In estuaries however, where the external NO_3^--input sometimes exceeds the endogenous NO_3^--production from nitrification, the nitrate-level in the water may primarily determine the denitrification activity in sediments. (Jørgensen & Sørensen 1988, Middelburg et al. 1996). Attention is also paid to the influence of vascular plants in estuarine sediments. It is thought that their growth can significantly affect N-cycling in sediments. Nitrification is enhanced in vegetation zones, probably as a result of oxygen release from roots of submersed plants in the anoxic sediment (Bodelier et al. 1996). As a result of the enhanced nitrate input, denitrification can also be stimulated (Caffrey & Kemp 1992, Nijburg & Laanbroek 1997).

In recent years, several projects have been started in order to improve the water quality in the Schelde estuary. One of them is the SIGMA project, which is preliminary meant to buffer increased water levels by means of the construction of controlled flooding areas. In addition, studies are undertaken to determine the capacity of these controlled flooding areas, which are often vegetated with so called halophyte filters consisting of reed, bulrush and willow to increase nitrogen removal from the estuary.

Methods of measuring nitrification

Inhibition

A commonly used method to determine nitrification rates is to add specific inhibitors to samples. Nitrification inhibitors were developed as agrochemicals to prevent ammonium loss from crop cultivation (Prasad & Power 1995), but have been used in laboratory and environmental studies as well. Changes in concentrations of inorganic nitrogen compounds due to the working of the inhibitors give insight in nitrification processes.

One of the inhibitors used is acetylene, which inhibits ammonium oxidation irreversibly at concentrations >10 Pa by reacting with the ammonia-monooxygenase. Besides nitrification, acetylene also inhibits the final step in denitrification. Denitrification is then measured by the accumulation of nitrous oxide. Acetylene is commonly used in studies in soils and sediments (Sloth et al. 1992). Other inhibitors of ammonia oxidation are nitrapyrin (2-chloro-6-trichloromethyl-pyridine) also called N-serve, allylthiourea (ATU) and thiourea. These compounds block the first step in nitrification i.e. oxidation of ammonia to hydroxylamine (Bedard & Knowles 1989, Hall 1984). Disadvantage of the use of these compounds is that not all species and strains of nitrifying bacteria are similarly affected by a given compound. For seven strains belonging to three different genera of ammonia-oxidizers nitrapyrin concentrations required for complete inhibition ranged from 0.9 to 43 μM (Bedard & Knowles 1989). Nitrapyrin can also be lost through volatilisation or degradation, which is probably of little importance in short term incubation. Moreover, it is sparingly soluble in water and has to be dissolved in an organic solvent before adding. This carrier itself may have inhibitory effects (Bedard & Knowles 1989, Hall 1984). Nevertheless, inhibitor studies have yielded estimates of nitrification, which agree within an order of magnitude with measurements made by using other approaches (Bedard & Knowles 1989, Sloth et al. 1992).

Since many of the inhibitors used also inhibit other oxidation processes like the oxidation of ammonium and methane by methanotrophs it is often not possible to conclude that observed nitrification is due to nitrifying bacteria alone (Bedard & Knowles 1989). Recently, supposedly more selective inhibitors have been tested. Methyl fluoride (Bodelier & Frenzel 1999) and dimethyl ether inhibit NO_2^- and N_2O production from NH_4^+ at concentrations of 10% [vol/vol headspace] and 25% [vol/vol headspace] respectively (Miller et al. 1993). The two gases also inhibit methane oxidation but are of no influence in dissimilatory nitrate

reduction to ammonium and oxidation of nitrite and hydroxylamine. Besides these gaseous compounds, the water-soluble allylsulfide showed to inhibit nitrification (Juliette et al. 1993).

Dark ^{14}C-bicarbonate incorporation

The dark ^{14}C-bicarbonate incorporation method to measure nitrification was first described by Somville (Somville 1978). The method is based on the fact that nitrifying bacteria, being chemoautotrophs, incorporate dissolved bicarbonate as a carbon source during growth. By adding radioactively labelled bicarbonate to natural samples, the amount of incorporated bicarbonate in cells can easily be measured. Unfortunately, bicarbonate is not only incorporated by nitrifying bacteria. It is therefore necessary to prevent non-target organism from taking up of bicarbonate during the experiment. By incubating in the dark, the incorporation by photoautotrophs is minimised. However, photoautotrophs are able to incorporate carbon even in the dark via anapleurotic routes, and bicarbonate might also be incorporated by other chemoautotrophic organisms. It is therefore of importance to use an appropriate nitrification inhibitor together with this method.

While measuring nitrification, the rate of ammonium oxidation is generally of more interest than the bicarbonate incorporation rate. A conversion factor is needed to obtain ammonium oxidation rates from results obtained with the dark ^{14}C-bicarbonate incorporation method. Several authors have measured conversion factors for nitrification empirically: 14.31 (river) to 12.34 (sea) (Feliatra & Bianchi 1993), 5.95 obtained with estuarine isolates of ammonium oxidisers (Berounsky & Nixon 1990, Owens 1986) and 8.3, measured in the Schelde estuary (Somville 1978). Because these conversion factors include the whole nitrification process, it is necessary to add an inhibitor for nitrite oxidation, i.e. chlorate (Owens 1986) to samples as well. The amount of ammonium necessary for growth can vary substantially among different growth phases or species of nitrifying bacteria. It has been shown that the composition of the nitrifying population is changing along the estuarine gradient (de Bie et al. 2001), and it is therefore possible that N/C ratios for nitrification may differ along this gradient as well. To measure ammonium oxidation rates directly, stable isotope techniques are necessary.

Stable isotope techniques

The availability of the stable isotope ^{15}N offers opportunities to determine ^{15}N content of inorganic nitrogen compounds accurately by mass spectrometry. By addition of $^{15}NH_4^+$ to the water sample nitrification can be determined by following the development of $^{15}NO_3^-$

concentration. One of the problems with this method in studies in which sediments or high particulate matter concentrations are involved, is the disappearance of $^{15}NH_4^+$ immediately after addition to particles because of adsorption and its subsequent reappearance during incubation (Blackburn 1993, Laima 1993).

Nitrous oxide

The greenhouse effect and the destruction of the ozone layer are two important issues in global change. The greenhouse effect is a naturally occurring mechanism that increases the temperature on earth to levels suitable for life. The greenhouse effect is the absorption of infrared radiation from the earth by the so-called greenhouse gases (of which carbon dioxide, methane and nitrous oxide are the most important ones besides water vapour). The destruction of the ozone layer leads to increased radiation, especially in the ultraviolet part of the spectrum. Changes in ultraviolet radiation affect life on earth by for example an increase of risk for skin cancer, eye damages, increase of breakdown of dissolved and particulate organic matter and increase of photoinhibition of near-surface aquatic micro-organisms.

Nitrous oxide is involved in both processes discussed above (Bange 2000) and atmospheric concentrations are increasing. Much attention has therefore been paid to the global production and consumption of nitrous oxide during the past decade. Nitrous oxide is chemically stable in the troposphere and its atmospheric residence time is estimated to be 100-150 years (Crutzen 1981). The history of atmospheric nitrous oxide concentration is analysed in air bubbles trapped in ancient ice (Khalil & Rasmussen 1988) or snow packs (Battle et al. 1996). Analysis of ice cores showed that nitrous oxide concentration in the atmosphere was relatively stable around 285 nmol mol^{-1} during pre-industrial times, but has since 1860 increased to reach about 315 nmol mol^{-1} at present (AGAGE 1999). The atmospheric concentration of nitrous oxide is relatively low compared to the concentration of other greenhouse gases, but it contributes nevertheless 6% to the global warming, due to its high infrared absorption capacity and relatively long atmospheric residence time (Kroeze 1993). The long atmospheric residence time of nitrous oxide allows part of the nitrous oxide to reach the stratosphere, where it is photochemically converted to reactive NO_x species which are involved in the destruction of ozone. Oxidation and photolysis in the stratosphere are the only sinks of importance of nitrous oxide.

About 45 % of the total N_2O emission is attributed to human activities. Important anthropogenic sources of nitrous oxide are agricultural soils (in particular rice paddies), biomass and fossil fuel burning, industrial sources, wastewater treatment plants and cattle.

Aquatic systems, both salt and fresh water, contribute 25-30 % of the total global N_2O emission (IPCC 1996). Estuaries are calculated to account for approximately 60% of total marine nitrous oxide production/emission (Bange et al. 1996). In natural systems, the microbial processes of nitrification and denitrification are considered to be the major sources of nitrous oxide. The first step of nitrification, the oxidation of ammonia to nitrite, can be a source of nitrous oxide, in particular at low oxygen concentrations (Anderson & Levine 1986, Goreau et al. 1980, Jørgensen et al. 1984, Kester et al. 1997, Stüven et al. 1992). The concentration range of oxygen, at which significant quantities of nitrous oxide are produced, is very narrow. At oxygen concentrations higher than this critical range, oxidation to nitrite and subsequently nitrate is completed, while ammonium oxidation is not occurring at all at oxygen concentrations below the range for nitrous oxide production. During denitrification nitrate is reduced via the intermediates NO_2, NO and N_2O to molecular nitrogen gas (N_2). The enzyme nitrous oxide reductase is, at least in some denitrifying species, more sensitive to oxygen than the other reduction steps (Betlach & Tiedje 1981, Zumft & Kroneck 1990). This can lead to accumulation of nitrous oxide at low oxygen concentrations. At lower oxygen concentrations, denitrification acts as a sink for nitrous oxide by reduction to N_2 (Firestone & Tiedje 1980).

Outline of the thesis

The research presented in this thesis is focussed on nitrification in the water column of the Schelde estuary. The subject is approached by a combination of field measurements and laboratory experiments.

The spatial and temporal variation of nitrification activity in monthly measurements in the field during a period of 15 months is addressed in Chapter 2. The method of measuring nitrification rates is discussed, with emphasis on the use of specific nitrification inhibitors. Factors determining nitrification rates in the Schelde estuary are discussed (Chapter 2) and the community structure and dynamics of the ammonia-oxidising bacteria are analysed by use of Denaturing Gradient Gel Electrophoresis (Chapter 3).

The concentration of nitrous oxide is measured, showing that the estuary provides a continuous source of nitrous oxide to the atmosphere (Chapter 4 and 5). Fluxes of nitrous oxide to the atmosphere are estimated and compared with historical nitrous oxide data of the Schelde (Chapter 4). Factors controlling the production of nitrous oxide are examined by field measurements (Chapter 4), and by laboratory experiments (Chapter 5). The importance of nitrification as a source of nitrous oxide is studied, as well as the role of oxygen. Finally, the

role of nitrification in estuaries is discussed, with emphasis on population dynamics and nitrous oxide production (Chapter 6).

C H A P T E R 2

Nitrification in the Schelde estuary: methodological aspects and factors influencing its activity

Monique J.M. de Bie, Mathieu Starink, Henricus T.S. Boschker, Jan J. Peene & Hendrikus J. Laanbroek.

We present a 15-months dataset on nitrification measurements in the Schelde estuary (Belgium and the Netherlands). Nitrification was estimated using the N-serve sensitive dark ^{14}C-bicarbonate incorporation technique. A peak of nitrification activity was observed in the freshwater part of the estuary. Downstream of this peak, nitrification declined, probably because of ammonium limitation. A range of nitrification inhibitors was tested on both a Nitrosomonas europaea culture and estuarine samples. It was found that methyl fluoride and acetylene stimulated dark ^{14}C-bicarbonate incorporation and those inhibitors were therefore considered inappropriate nitrification inhibitors in combination with this technique. The effect of the inhibitor N-serve was studied on the dark incorporation of ^{13}C-bicarbonate into polar lipid derived fatty acids (PLFA) to further identify the dominant chemoautotrophic processes. Inhibition of PLFA labelling in the presence of N-serve was complete suggesting that nitrifying bacteria dominated the chemoautotrophic community.

Submitted for publication

Introduction

Eutrophication of estuaries is a common phenomenon. The high nutrient concentration in the output of these estuaries, leads to algal blooms in predominantly N-limited coastal waters, which in turn might lead to anoxia. At the same time, estuaries are described to contain a well-developed self-cleaning system. Vast organic and inorganic nitrogen reductions occur due to intense microbial activity. This nitrogen removing function of estuaries is therefore of crucial importance to the water quality of coastal seas. Denitrification, which is the main process removing nitrogen from the estuary permanently, reduces nitrate to gaseous dinitrogen, which disappears to the atmosphere. Nitrate is thus an essential substrate for the removal of nitrogen from the estuary. This makes bacterial nitrification a key process because it is the only biological process that converts ammonium to nitrate. The nitrifying consortium consists of two different groups of chemoautotrophic bacteria. The first one oxidizes ammonia to nitrite, while the second one oxidizes nitrite to nitrate.

Estuarine nitrification activity typically shows a peak at intermediate salinity (Helder & de Vries 1983, Iriarte et al. 1996, Owens 1986, Pakulski et al. 1995, Somville 1984, de Wilde & de Bie 2000). This increase in activity with increasing salinity is reported to be coupled to the oxygen condition in the estuary (Somville 1984), indicating that nitrifying bacteria are oxygen limited upstream of the peak. The cause of the collapse in activity downstream of the peak is less clear, but might be explained by ammonia limitation or intolerance against the increasing salt concentration by the freshwater adapted community (Helder & de Vries 1983, Somville 1984). Another explanation for the appearance of the peak of activity is that it coincides with the maximum turbidity zone and that ammonia-oxidisers prefer to be attached to particles instead of being free-living (Abril et al. 2000, Owens 1986). Nitrifying bacteria tend to form clusters or attach to particulate material and form flocks. In the Elbe estuary, between 50–100% of the nitrifying bacteria were attached to flocks (Stehr et al. 1995). In the Urdaibai estuary nitrification activity was associated with the fraction >3µm (Iriarte et al. 1996). Particulate material has a longer residence time than water (Soetaert & Herman 1995a) and attachment of bacteria to flocks will increase their residence time, providing them a longer stay at one section in the estuary.

There are a number of studies that report nitrification rates in estuarine waters (Berounsky & Nixon 1990, 1993, Bianchi et al. 1994, Billen 1975, Feliatra & Bianchi 1993, Iriarte et al. 1996, Owens 1986, Pakulski et al. 1995, Somville 1984, de Wilde & de Bie 2000). Many of these use a specific inhibitor to measure the nitrification activity. Nitrification inhibitors have

been developed as agrochemicals to prevent loss of nutrient salts through nitrification after fertilisation. Nitrapyrine (N-serve, 2-chloro-6-trichloromethyl-pyridine) (See Oremland & Capone (1988) and references therein) is a commonly used inhibitor, which works by blocking the first step in nitrification i.e. the oxidation of ammonia to hydroxylamine (Bedard & Knowles 1989). Disadvantage of N-serve is that it is sparingly soluble in water so that it has to be dissolved in an organic solvent. A range of other inhibitors has been used, (Joye et al. 1999, Table 1), but comprehensive comparative studies including several inhibitors are scarce.

This study presents an intercomparison of different nitrification inhibitors for a pure culture and a natural community of the Schelde estuary. Nitrification rates were measured once a month during a period of 15 months, using the N-serve-sensitive ^{14}C-bicarbonate incorporation technique. In addition, we studied the effect of N-serve on the incorporation ^{13}C-bicarbonate into polar lipid derived fatty acids (PLFA) to further identify the dominant chemoautotrophic processes and populations (Boschker et al. 1998). Factors governing nitrification will be identified by comparing *in situ* nitrification rates with potential nitrification rates based on experimental manipulation of ammonium.

Method

Research area

The Schelde estuary (see Fig. 1) drains an estimated 21.000 km^2 of Northern France, Belgium and the Netherlands, an area with approximately 10 million inhabitants, and receives a nitrogen load of 56000 ton N Yr^{-1} (Soetaert & Herman 1995b). Salinity ranges from about 0.5 at Temse, the upper limit of saltwater intrusion, to about 30 at Vlissingen near the mouth of the estuary (Fig. 1). The estuary is vertically well mixed, although slight stratification can occur near Antwerpen (Wollast 1988). The average freshwater outflow of 105 m^3 s^{-1} (Billen et al. 1985) is small relative to the tidal exchange of about 45000 m^3 s^{-1} (Middelburg & Nieuwenhuize 1998). Turbidity is high in the entire upper estuary with suspended matter concentrations ranging from 15-350 mg l^{-1}.

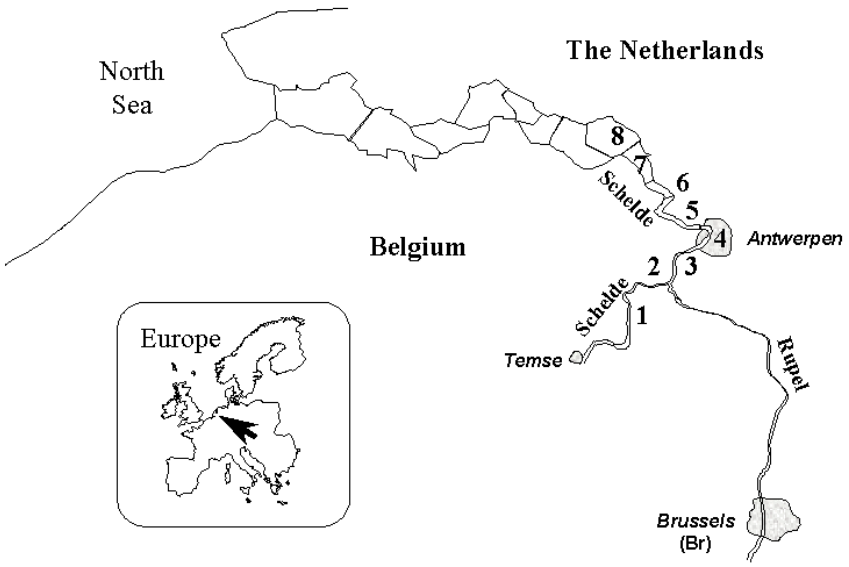


Fig. 1 Map of the Schelde estuary. Numbers indicate sample locations. The distance form the city of Antwerpen to the mouth of the estuary is approximately 100 km.

Sample collection and chemical analysis

Once a month from April 1997 till June 1998, surface water samples in the estuary were collected aboard the RV Luctor in the navigation channel at the indicated sample sites (Fig. 1). Concomitant with the samples for nitrification measurements, samples for nutrient analysis were taken. Ammonium, nitrite and nitrate were analysed with a SKALAR segmented flow autoanalyser system with a precision of approximately 1 %. Salinity, temperature, and oxygen were measured with a CTD system, equipped with a polarographic oxygen sensor (THISHYDRO H2O).

Dark ^{14}C-bicarbonate incorporation measurements

Nitrification activity was measured by the dark ^{14}C-bicarbonate incorporation method (Somville 1978). With this method the carbon fixation by autotrophic ammonia-oxidising bacteria was estimated by measuring the difference of inorganic carbon incorporation during dark incubation with and without a specific inhibitor of nitrification activity. At each station, 50 ml glass screw cap bottles were filled so that no headspace was left. To these bottles, 100

µl of 0.925 MBq ml^{-1} ^{14}C-$NaHCO_3$ was added. One set of bottles was treated with a combination of N-serve (5 mg l^{-1} final concentration) and chlorate (10 mg l^{-1} final concentration). A stock solution of N-serve was prepared in 80% ethanol and added to the empty incubation bottles about 4 hours before sampling, to allow the ethanol to evaporate (Brion & Billen 1997). To prevent nitrite oxidizing bacteria from taking up bicarbonate, chlorate was added to inhibit nitrite oxidation (Belser & Mays 1980). The water-soluble chlorate was directly added to the samples. Samples were incubated in triplicate for 20 hours (Brion & Billen 1997) in a rotating incubator in the dark at *in situ* temperature. Filtration and counting was carried out according to the protocol of Kromkamp & Peene (1995).

During October 1997 to June 1998, each month a triplicate set of bottles was incubated to distinguish between *in situ* and potential nitrification rates. Potential nitrification rates can be considered as a measure for the nitrifying biomass present at the time of sampling (Bodelier et al. 1996). The bottles for *in situ* measurements were incubated with a minimal air headspace to prevent additional oxygen to dissolve during incubation. These bottles were incubated at *in situ* temperature. For determination of potential nitrification activity (PNA) bottles were incubated at room temperature (20-22°C) with 50% (v/v) air headspace and addition of 2 mM NH_4^+ (final concentration). In May 1998, an additional series of bottles was incubated with only 2 mM NH_4^+ (final concentration) added, with a minimal headspace, to study the effect of ammonium on nitrification activity along the estuary.

In order to investigate the relative importance of flock-attached and free-living bacteria, experiments were carried out where flocks in the water sample were allowed to settle for 1 hour. Filtration was not feasible as a method to separate particulate from free activity, due to the high turbidity, which immediately cause clogging of filters. After 1 hour of settling, potential nitrification activity was determined in the upper, clear water layer as described above. Obtained nitrification rates were compared with total sample measurements (with flocks) and particulate matter content was determined in both samples.

Standard errors on nitrification rates were calculated assuming a t-distribution of the results according to:

$$\Delta_{\bar{N}} = \frac{t_{\alpha,n-1}}{\sqrt{n}} \sqrt{\sigma_x^2 + \sigma_y^2}$$

where Δ_N= standard error of the nitrification rate, $t_{\alpha, n-1}$ =critical value of *t* at probability α and n-1 degrees of freedom, where n= number of replicate measurements, σ_x= standard

deviation of control measurements and σ_y= standard deviation of measurement with inhibitor.

Inhibitor comparison experiment

A range of known nitrification inhibitors was tested in the dark ^{14}C-incorporation-method. Concentrations used and references are listed in Table 1. The background effect of the solvent dimethyl sulfoxide (DMSO**)**, which is used to dissolve allylsulfide, dicyandiamide and allylthiourea, was also tested. The acetylene used in the experiment was purified by passing it through a trap containing sulfuric acid followed by a trap containing 5M NaOH according to Hyman and Arp (1987). The inhibition of ammonia-oxidation was determined on a pure culture of *Nitrosomonas europaea* and on an estuarine water sample from Station 7, May 1998. Incubation was performed in triplicate as described above in the dark at room temperature with 2 mM ammonium (final concentration) and an air headspace of 50% of the total bottle volume.

Table 1. List of inhibitors used in the inhibitor experiment

Inhibitor	Concentration used	Reference	Comments
DMSO		Roy & Knowles 1995	Solvent
N-serve	50 µM	Bedard & Knowles 1989, Roy & Knowles 1995	Dissolved in 80% ethanol
Allylsulfide	200 µM	Juliette et al. 1993, Roy & Knowles 1995	Dissolved in DMSO
Dicyandiamide (DCD)	2 mM	Roy & Knowles 1995	Dissolved in DMSO
Acetylene (C_2H_2)	0.01 and 1 %	Bedard & Knowles 1989, Roy & Knowles 1995, Sloth et al. 1992	
Methyl fluoride	10% (vol/vol)	Miller et al. 1993, Oremland & Culbertson 1992	
Allylthiourea (ATU)	50 µM	Roy & Knowles 1995	Dissolved in DMSO

^{13}C-bicarbonate incorporation into PLFA

In April 1997 and June 1998, we studied the ^{13}C-bicarbonate label incorporation into polar lipid derived fatty acids (PLFA) to determine the dominant autotrophic processes and populations at station 3. This station in general showed near maximum nitrification activities. Duplicate 500 ml serum bottles were incubated on deck at *in situ* temperature for 3 to 4 hours and received the following treatments: non-labelled control incubations, ^{13}C-bicarbonate-

labelled in the light, and ^{13}C-labelled dark incubations with and without N-serve and chlorate as specific inhibitors of nitrification. The final ^{13}C-bicarbonate concentration was 0.2 mM (Isotec, >99% ^{13}C), which led to a 3.0% ^{13}C-labelling of the total inorganic carbon pool, and inhibitors were used at concentrations as in the ^{14}C-assay. At the end of the incubations, suspended material was collected on pre-combusted GF/F glass-fibre filters (Whatman), which were directly added to the PLFA extraction solvents to stop all activities.

PLFA on the filters were extracted and analysed as in Boschker et al (Boschker et al. 2001). In short, lipids were extracted in chloroform/methanol/water using a modified Bligh and Dyer method and fractionated on silicic acid into different polarity classes. The most polar fraction containing the PLFA was derivatized to yield fatty acid methyl esters (FAME). Concentrations and isotopic composition of individual FAME were determined with a gas-chromatograph combustion-interface isotope-ratio mass spectrometer (GC-c-IRMS). Stable carbon isotope ratios for individual FAME were corrected for the one carbon atom in the methyl group that was added during derivatisation.

The rate of ^{13}C-label incorporated into a specific PLFA was calculated as: incorporation rate = $((F_{tx} - F_{t0}) \times [PLFA]_{tx})$/incubation time, with the PLFA concentration in pmol C/l and the fraction ^{13}C at the start (F_{t0}) and the end (F_{tx}) of the incubations as: $F = {}^{13}C/({}^{13}C+{}^{12}C) = R/(R+1)$. The carbon isotope ratio (R) was derived from the measured δ^{13}C values as: $R = (\delta^{13}C/1000 + 1) \times R_{VPDB}$, with $R_{VPDB} = 0.0112372$.

Results

Estuary characteristics

Ammonium concentrations (Fig 2, closed triangles) decreased from 200-500 µM in the upper reaches of the estuary to about 10 µM at station 8. At the mouth of the estuary, the ammonia concentration was around 5 µM. Ammonium concentrations were typically higher in autumn months. Oxygen concentrations reached a maximum of about 300 µM (Fig 2 open triangles) and never exceeded 100% air saturation in the entire sampling area. Oxygen concentrations were much lower during summer than during winter.

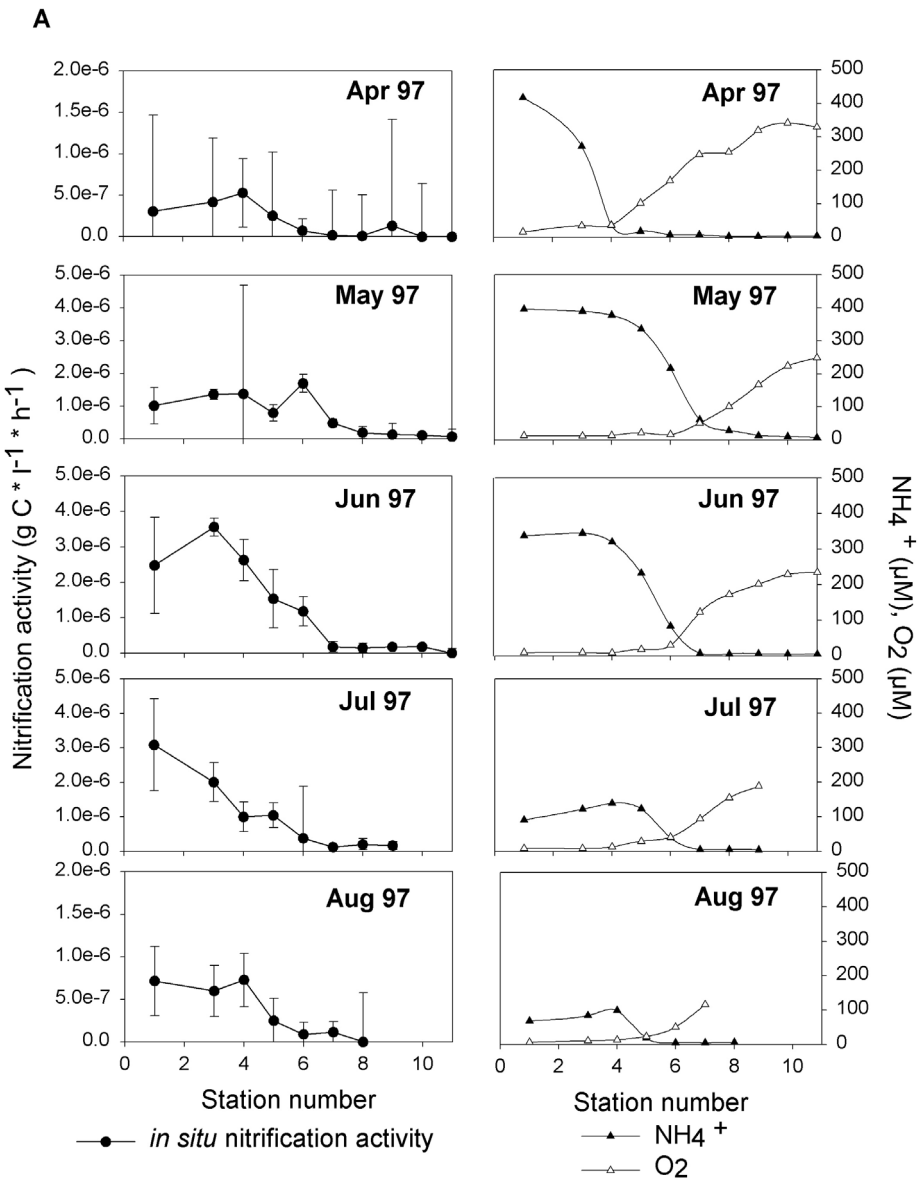
Salinity varied from below 1 at station 1 to about 16 at station 8, our most downstream sample station. At the mouth of the estuary the salinity was 30.

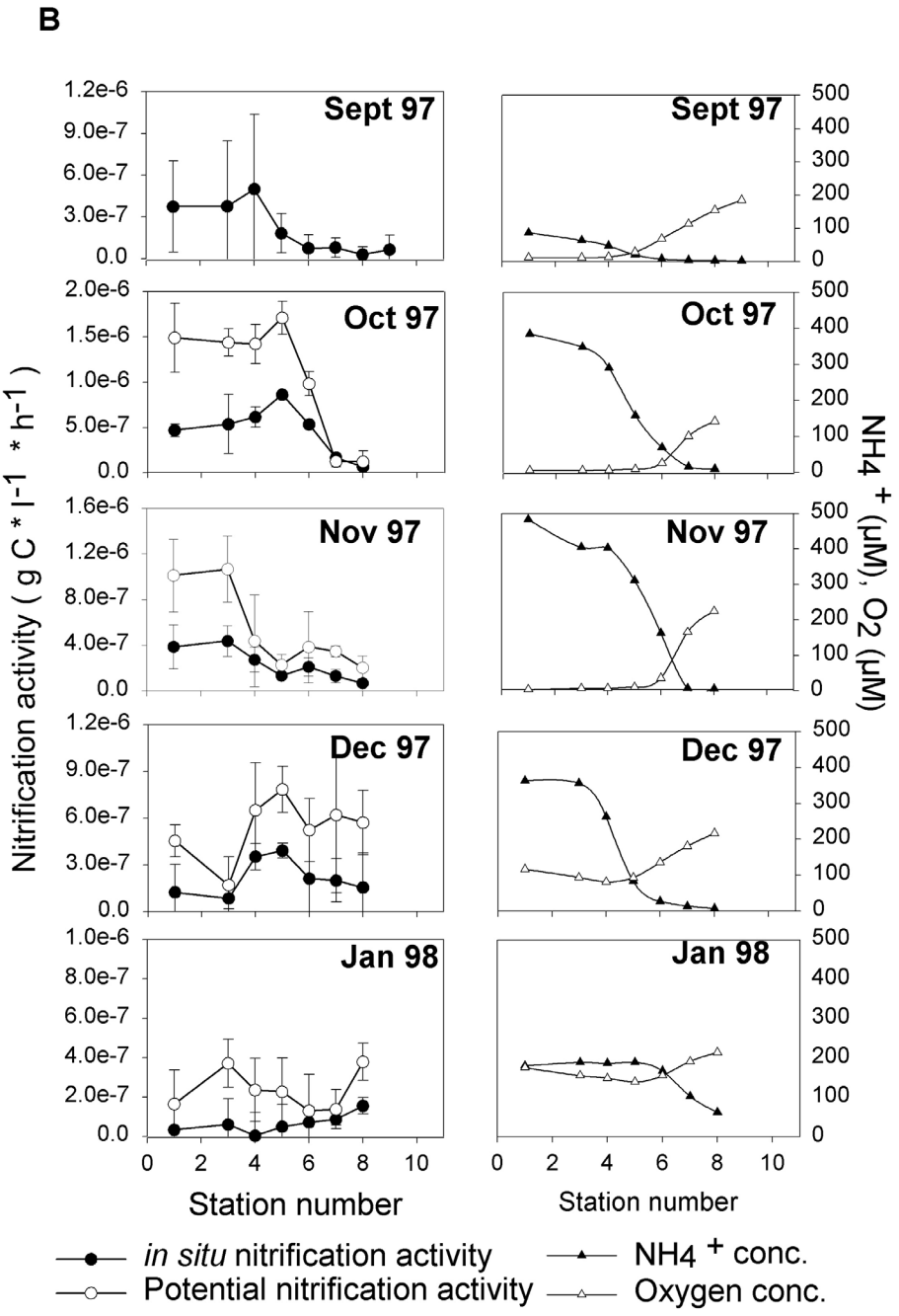
Temperature was 21°C in June 98 but was never lower than 6°C even in winter months when ambient temperatures were close to zero. Temperature differences along the estuarine gradient were usually not more than 2 °C (Data not shown).

Suspended particulate matter varied from more than 300 mg liter^{-1} in the upper estuary to about 15 mg liter^{-1} at lower stations. A clear turbidity maximum could not always be detected but suspended matter concentrations were typically highest between salinity 1 and 5. Suspended particulate matter averaged 82.5 mg l^{-1} with maximal values of 363 mg l^{-1} in October 97 (Station 1), 193.3 mg l^{-1} in February 1998 (Station 5) and 132 in May 1998 (station 6)

Nitrification rates

Nitrification activity generally reached a maximum at one of the most upstream stations (Fig. 2, closed circles). Besides biomass, oxygen and ammonium concentrations, tidal currents further influence the exact location of this peak. Nitrification was low during winter and reached a maximum of 3.7 µg C liter^{-1} hour^{-1} in June (both 1997 and 1998, Fig. 2, closed circles). This maximal value was reached in a part of the estuary where oxygen concentrations were less than 100 µM, i.e. in the upper estuary in summer. This indicates that no severe oxygen limitation in this part of the estuary exists. Potential nitrification rates (Fig. 2, open circles) exceeded the *in situ* activity in the months October 1997 to April 1998, which is probably due to lower *in situ* temperatures. Differences between potential nitrification rates and *in situ* rates decreased gradually from the upper estuary downwards. . In a more detailed study on the effect of ammonium it was shown that in the lower estuary, nitrification activity increased significantly ($p<0.05$) upon addition of ammonium, indicating ammonium limitation at stations 6, 8 and 10 (Fig. 3). Particle associated nitrification activities were 70, 86, 59, 80 and 57 % for the months January, February, March, April and May, respectively.


A
Apr 97
May 97
Jun 97
Jul 97
Aug 97
Nitrification activity (g C * l-1 * h-1)
NH4 + (µM), O2 (µM)
Station number
Station number
in situ nitrification activity
NH4 +
O2


B
Sept 97
Oct 97
Nov 97
Dec 97
Jan 98
Nitrification activity (g C * l-1 * h-1)
NH4 + (µM), O2 (µM)
Station number
Station number
in situ nitrification activity
Potential nitrification activity
NH4 + conc.
Oxygen conc.

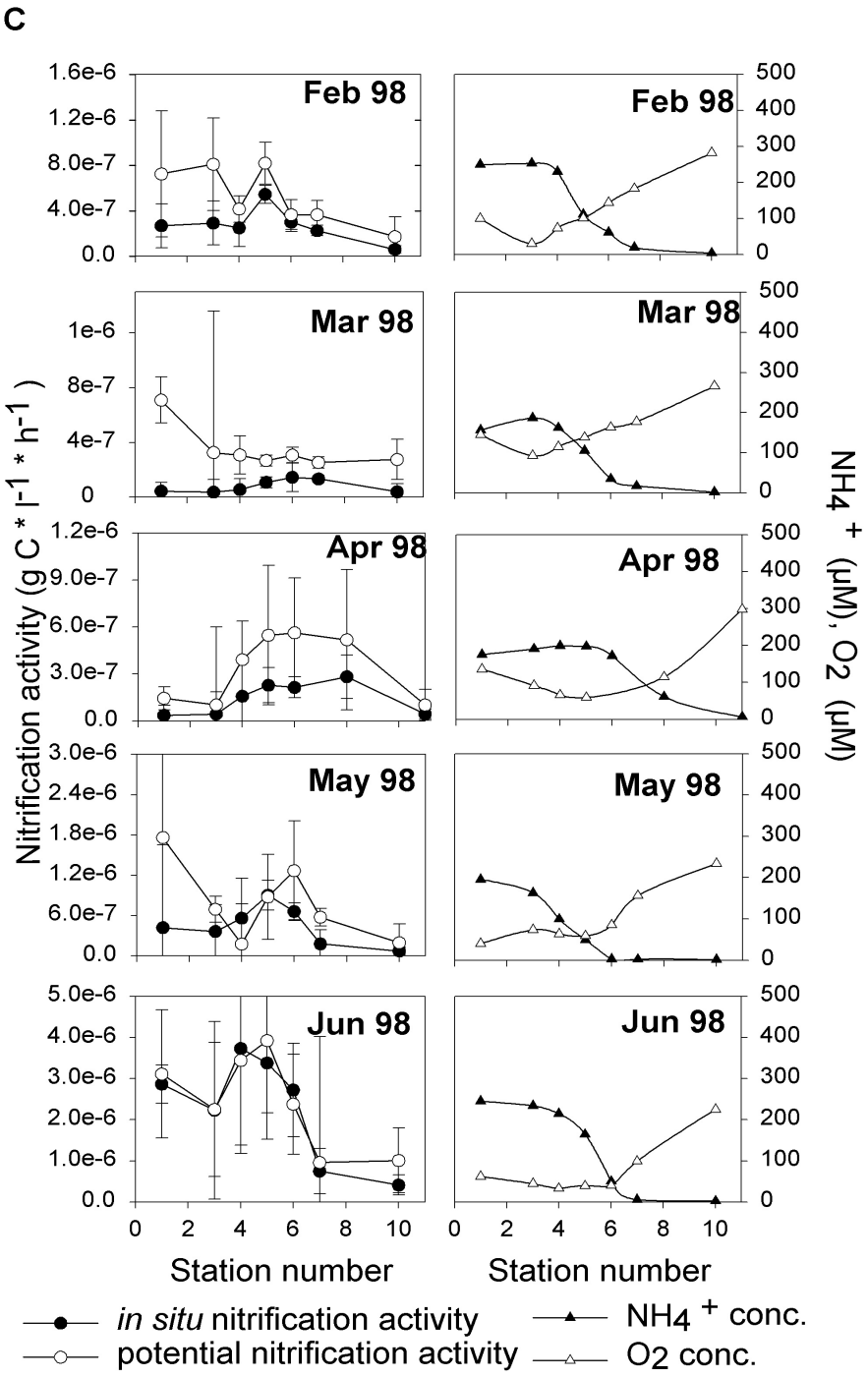


Fig 2 *In situ* nitrification activity (closed circles, n=3) and potential nitrification activity (open circles, n=3). Error bars are calculated for with a 95% confidence limit as explained in the text. Ammonia (closed triangles) and oxygen (open triangles) concentration along the estuary. Mind scale differences.

Table 2. Average bicarbonate incorporation (n=3) as influenced by different inhibitors in a pure culture of *Nitrosomonas europaea* and an estuarine sample. All inhibitor treated samples differ significantly from the control (p<0.05, ANOVA).

Inhibitor	Nitrosomonas europaea Incorporation (dpm)	% inhibited	Natural sample Incorporation (dpm)	% inhibited
Control	206186		4976	
DMSO	110927	46	2969	40
Allylsulfide	13994	93	2564	48
N-serve	19926	90	3097	38
DCD	9252	96	3246	35
ATU	8172	96	3433	31
C_2H_2 (0.01%)	9636	95	7236	-45
C_2H_2 (1 %)	9832	95	7220	-45
Methyl Fluoride	10109	95	7967	-60

Inhibitor comparison

In the *Nitrosomonas europaea* culture, bicarbonate incorporation was inhibited by all inhibitors tested (Table 2), with efficiencies varying between 90 and 96%. Highest inhibition was found with DCD and ATU (96%), but acetylene and methyl fluoride also inhibited 95 % of C-incorporation of the pure culture.

In the natural estuarine sample dark bicarbonate incorporation was inhibited by only 31-48% by the inhibitors allylsulfide, N-serve, DCD and ATU. This lower inhibition rate compared to the pure culture was expected since more C-fixing organisms are present in the natural sample than nitrifiers alone. Acetylene and methyl fluoride caused a remarkable increase of bicarbonate incorporation in natural samples by 45 and 60% respectively

The solvent DMSO inhibited ^{14}C-incorporation by 46 and 40 % in the pure culture and the natural community respectively.

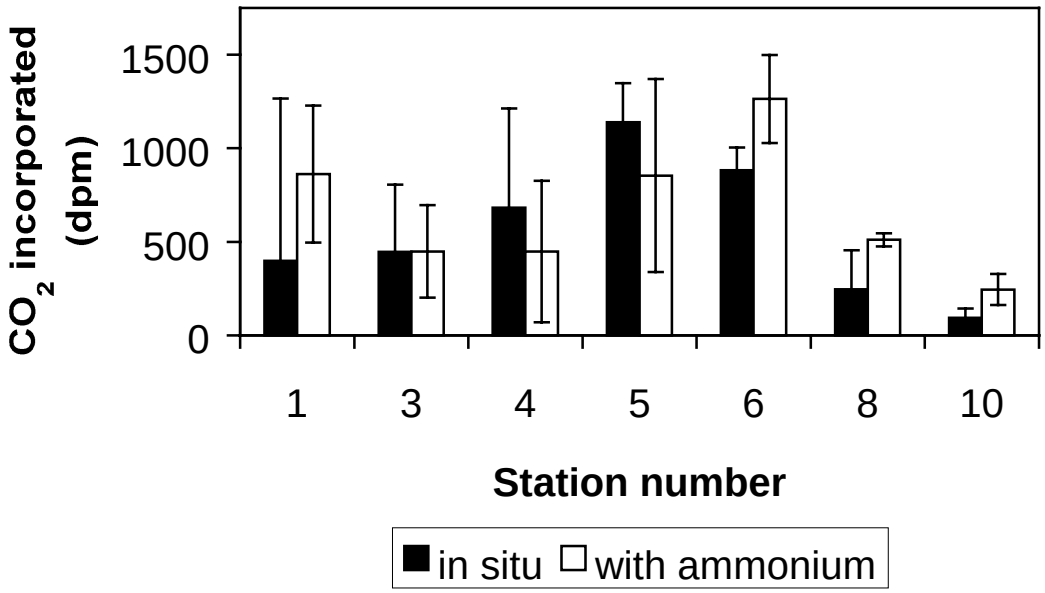


Fig. 3 Effect of ammonium addition on nitrification activity as measured in May 98. Error bars represent 95% confidence limit, n=3.

^{13}C-bicarbonate incorporation into PLFA

Results of the PLFA study were similar in April 1997 and June 1998 and only the latter data are shown here (Fig. 4). Concentrations of PLFA were high and both specific bacterial markers (e.g. i15:0 and a15:0) and poly-unsaturated algal compounds (e.g. 20:5ω3) were detected in substantial amounts (Fig 4A). PLFA labelled in the dark were mainly 16:0 and 16:1ω7c with some label in 16:1ω5c and 18:1ω7c (Fig 4B). The two dominantly labelled PLFA are very general and occur in both bacteria and eukaryotes. However, the simple labelling pattern with two dominant compounds and the absence of label in poly-unsaturated PLFA shows that dark incorporation was by bacteria and not by phytoplankton. N-serve in combination with chlorate effectively inhibited the ^{13}C incorporation into PLFA (92% inhibition, Fig 4B), indicating that most of the dark incorporation was by nitrifying bacteria. Labelling patterns in the light were very different with most of the label in poly-unsaturated PLFA like 16:2, 16:3, 18:2, 18:3, 18:4, 20:5ω3 and 22:6ω3 (data not shown, total incorporation rate into PLFA 7700 ± 330 pmol ^{13}C l^{-1} h^{-1}), which are typical fatty acids in green algae and diatoms that dominate the phytoplankton at the studied salinity (TRIPOS 1999, Volkman et al. 1989). Total dark incorporation rate into PLFA was 96 ± 4 pmol ^{13}C l^{-1} h^{-1}, which translates to a carbon based nitrification rate of about 1 μg C l^{-1} h^{-1} (calculated using with the percentage ^{13}C labelling of bicarbonate pool and a general bacterial PLFA to

biomass conversion factor of 0.04 g PLFA C/g biomass C (Brinch-Iversen & King 1990)). This rate is within the range as found with the ^{14}C-method (Fig 2) given the uncertainties in the PLFA to carbon conversion factor.

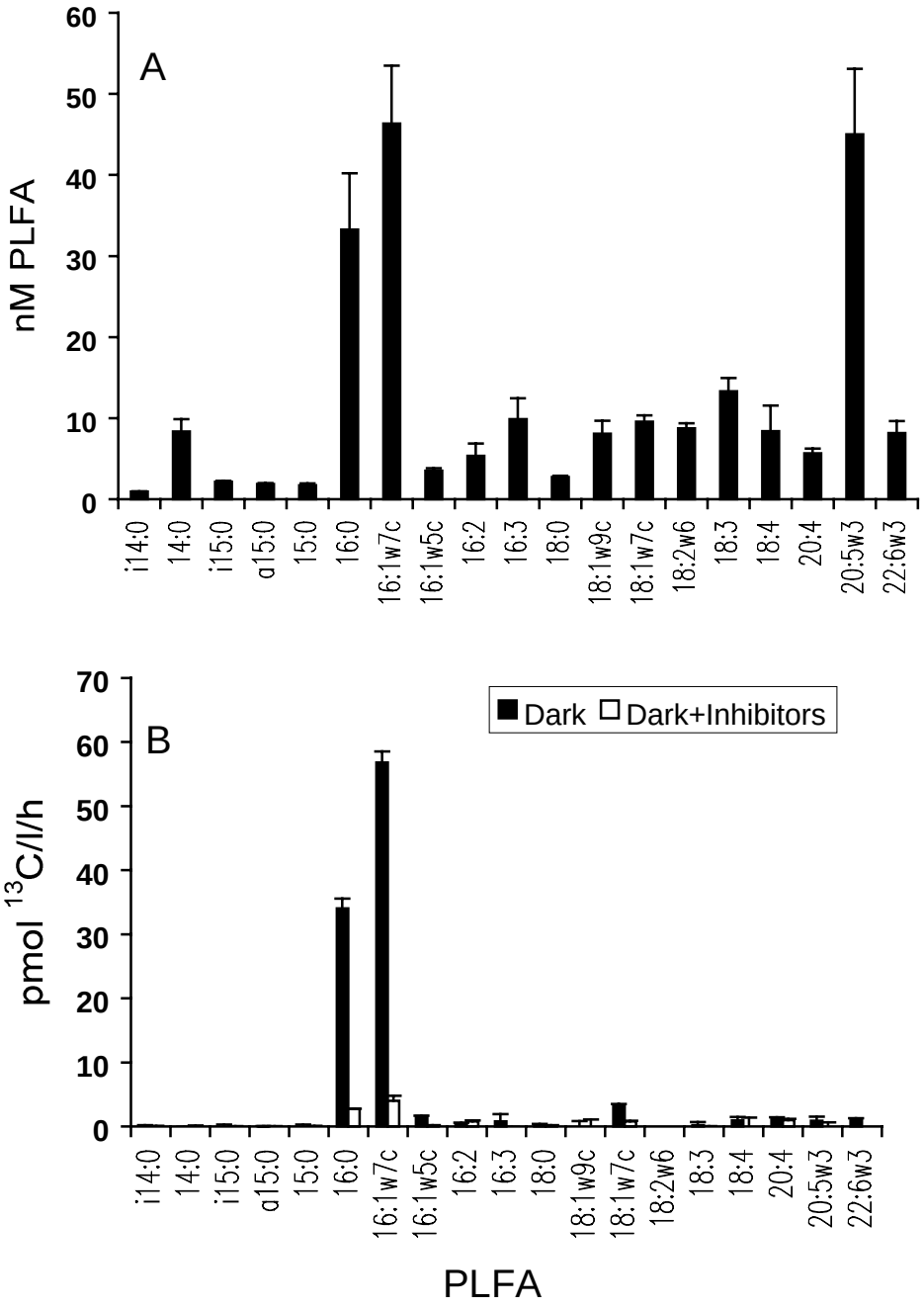


Fig. 4 Results of ^{13}C-bicarbonate incorporation into individual PLFA at station 3 in June 1998. Shown are the PLFA concentrations (4A) and the ^{13}C incorporation rates in the dark with (white bars) and without (black bars) nitrification inhibitors (4B).

Discussion

Nitrification rates

We measured a maximum in-situ nitrification rate of 3.7 µg C l^{-1} h^{-1} in June. Earlier measurements in the Schelde estuary showed maximum nitrification rates of 4.9 µg C l^{-1} h^{-1} (80 µM N l^{-1} day^{-1} in September 1977 (Somville 1984, Table 3). Our results show that nitrification rates are about a factor 10 lower in September as compared to June, which suggest that actual maximum values in 1977 may have been much higher than our present results. The peak activity in 1977 was observed around station 7 Somville 1984), which is more downstream in the estuary than where the present maximal nitrification activity is observed (station 3-5). This observation is explained by the water quality history of the Schelde estuary. Waste loadings in the estuary used to be high, which induced intense microbial degradation leading to very high heterotrophical bacterial production rates (Goosen et al. 1995) and accompanying oxygen depletion (Wollast 1988). In recent years the water quality in the Schelde estuary has improved (van Damme et al. 1995, de Wilde & de Bie 2000), which has caused a lower nitrification activity, with the peak activity located more upstream in the estuary. Secondary wastewater treatment in the catchment area has increased, which has resulted in decreased carbon and nitrogen loadings. Wastewater treatment might also have resulted in a decreased nitrifying biomass in the estuary, since untreated wastewater might be an important source of nitrifying bacteria (Brion & Billen 1997, Somville 1984).

Table 3. Comparison of published nitrification activities (maximal reported values). If data were reported as amount carbon incorporated we converted to nitrogen using a conversion factor of 8.3 mol N/mol C (Somville 1978).

Estuary	Nitrification activity (µmol N $liter^{-1}$ day^{-1})	NH_4 concentration (µM)	Method	Reference
Rhone river plume	1-2	1-10	^{14}C, ATU	Feliatra & Bianchi 1993
Narragansett Bay:	up to 11	8	^{14}C, N-serve	Berounsky & Nixon 1990, 1993
Long island sound	6.3	6	^{13}N	Capone et al. 1990
Urdaibai estuary	up to 4.6		^{14}C, ATU	Iriarte et al. 1996
Tamar estuary	3	5	^{14}C, N-serve	Owens 1986
Chesapeake Bay	32	30	^{15}N	Horrigan et al. 1990
Schelde estuary	80	500	^{14}C, N-serve	Somville 1984
Schelde estuary	45	150	^{14}C,N-serve	This study

Comparison with other estuaries (Table 3) shows that nitrification rates in the Schelde are still among the highest reported, despite the improved water quality of the estuary. In order to be able to compare the different nitrification rates with one another, reported nitrification rates have been recalculated (Table 3) using a conversion factor of 8.3 mol N:mol C (Somville 1984). Several authors have measured conversion factors for nitrification empirically: 14.3 (river) to 12.3 (sea) (Feliatra & Bianchi 1993), 5.95 obtained with estuarine isolates of ammonium oxidisers (Berounsky & Nixon 1990, Owens 1986) and 8.3, measured in the Schelde estuary (Somville 1978). The amount of ammonium necessary for growth can vary substantially among different growth phases or species of nitrifying bacteria. It has been shown that the composition of the nitrifying community is changing along the estuarine gradient (de Bie et al. 2001), and it is therefore plausible that N/C ratios for nitrification differ along this gradient as well. We therefore prefer to present the data in amounts of carbon incorporated.

In the upper estuary, *in situ* nitrification activity and potential activity were almost alike during May and June 1998 (Fig. 2). During this period, the difference in incubation temperature between *in situ* and potential measurements is very low and nitrification therefore appeared not be limited by either ammonium or oxygen concentrations. This also implies that neither oxygen nor ammonium was limiting in the incubation bottles during the *in situ* measurements despite the sometimes low concentrations. The difference between potential and *in situ* activity is largest in autumn months. This is probably due to temperature-limited nitrification in autumn. More downstream, there is an indication for ammonium limitation of nitrification activity (Fig. 3) as bacterial activity increases upon addition of ammonium to the incubation bottles. Although the influence of increasing salinity cannot be excluded (Billen 1975), ammonium limitation seems a more likely explanation of the lower nitrification rates in this part of the estuary. Furthermore, the lower residence times in the lower estuary play a role in decreased activity. The distribution and activity of the nitrifying population is also influenced by their attachment to particulate material (Prosser 1989). We found that 57 to 86% of the nitrifying biomass is attached to particles, which is in accordance with observations in the Elbe estuary (Stehr et al. 1995). The tendency to attach is a known phenomenon of (nitrifying) bacteria. Free-living bacteria are subject to the residual seaward flow in an estuary, which means that they have little time to adapt to the particular circumstances at a given section in the estuary. Attachment to particles increases their residence time (Soetaert & Herman 1995a).

Inhibitor experiments and PLFA labeling

CO_2-fixation in the pure culture *Nitrosomonas europaea* was inhibited by 90-96% by all inhibitors. In estuarine samples, inhibition in the ^{14}C-assay never exceeded 48%, indicating that more than half of the dark bicarbonate incorporation in natural samples is the result of other chemolithotrophic bacteria or dark-fixation by heterotrophic bacteria or phytoplankton (Kromkamp & Peene 1995). However, incorporation of ^{13}C-bicarbonate into PLFA was almost completely inhibited with N-serve and chlorate. The dark PLFA labelling is specific for chemoautotrophic organisms, as dark fixation by anapleurotic routes in heterotrophic bacteria and algae is not channelled to fatty acid synthesis. The complete inhibition of the PLFA labelling with nitrification specific inhibitors therefore strongly suggests that nitrifying bacteria dominated the chemoautotrophic community. The ^{13}C-PLFA labelling pattern was dominated by 16:0 and 16:1ω7c and is similar to the fatty acids found in most ammonium-oxidising bacteria belonging to the β and γ-subclasses of the Proteobacteria (Blumer et al. 1969). More detailed identification of the active populations by ^{13}C-PLFA labelling is unfortunately not possible.

The inhibitors acetylene and methyl fluoride had a stimulating effect on ^{14}C-incorporation in natural samples, while they almost completely inhibited bicarbonate incorporation in the *Nitrosomonas europaea* culture. Both acetylene and methyl fluoride (Caffrey & Miller 1995, Miller et al. 1993) have successfully been tested on natural soil and sediment samples. In combination with ^{14}C-incorporation methyl fluoride has been used in natural samples from an alkaline, saline lake (Joye et al. 1999). It is unclear what the reason could be for the increased incorporation with methyl fluoride and acetylene in our field sample, but it might be that bacteria able of degrading these inhibitors were stimulated and that these bacteria partially us carbon dioxide for growth similar to the Type II methanotrophs containing the serine pathway (Hanson & Hanson 1996). Many of the inhibitors used have the disadvantage of being insoluble in water. The use of a solvent is therefore necessary. During the inhibitor test, we used DMSO. Although DMSO has been described not to have inhibiting effects on *Nitrosomonas europaea* (Powell & Prosser 1985), we found an inhibiting effect on bicarbonate incorporation with this species, as well as with natural assemblages of bacteria. Because the gaseous inhibitors failed to work in our natural samples, we used the inhibitor N-serve with ethanol as a solvent during the *in situ* measurements (Brion & Billen 1997).

Inhibitors were tested at one concentration level only in this study. It has been described earlier that not all species or strains of nitrifying bacteria are similarly affected by a given

inhibitor (Bedard & Knowles 1989). For example N-serve concentrations required for complete inhibition ranged from 0.9 to 43 μM for seven strains belonging to three different genera of ammonia oxidisers. The 50 μM we used was on the higher side of this range. The almost complete inhibition as found with the PLFA method suggests that this inhibitor concentration was indeed effective despite the possible decreased availability of the inhibitor by particle adsorption in this turbid estuary.

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C H A P T E R 3

Shifts in the dominant populations of ammonia-oxidizing ß-subclass Proteobacteria along the eutrophic Schelde estuary.

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The community structure of ammonia-oxidizing bacteria of the ß-subclass Proteobacteria was investigated with respect to environmental gradients along the Schelde, a eutrophic estuary system. A dominance of *Nitrosomonas*-like sequences was detected using molecular techniques targeting the 16S rRNA gene on 3 separate sampling dates, and different *Nitrosomonas*-like populations were most dominant at different locations along the estuary. The most frequently detected ammonia oxidizer-like sequences in the freshwater part of the estuary were associated with a sequence cluster previously designated as *Nitrosomonas* cluster 6a. This group, which is closely affiliated with the cultured species *N. ureae*, has previously been detected as the dominant ammonia oxidizer group in various freshwater systems, and was also the dominant recovered sequence cluster from a contributory, untreated sewage effluent sample. The 16S rDNA recovered from brackish locations further downstream was dominated by a group of novel *Nitrosomonas*-like sequences. *Nitrosospira*-like sequences represented only a small minority of those detected for all samples. The shift in dominant ammonia oxidizer populations occurred in the estuarine region with the sharpest observed gradients in salinity, oxygen, and ammonia. These results provide evidence in support of the differential selection of physiologically distinct *Nitrosomonas*-like groups by the environmental gradients encountered along the estuary.

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Introduction

The discharge of large amounts of nitrogen into the environment has led to the eutrophication of many estuarine systems. In the Schelde estuary, domestic and industrial waste products (both treated and untreated) as well as runoff from fertilized agricultural lands have contributed to nitrogen enrichment. Nitrogen enters the estuary predominantly in its reduced form, ammonia (NH_3, or ammonium [NH_4^+] in its protonated form), which can be oxidized to nitrite (NO_2^-) by chemolithotrophic ammonia-oxidizing bacteria. Ammonia oxidation is the first, and often rate-limiting step, in the removal of nitrogen from environmental systems (Prosser 1989), and nitrification is quantitatively important in the estuary, in terms of both oxygen and ammonia consumption (Soetaert & Herman 1995a). Estuarine environments contain gradients of salinity, ammonia concentration, and dissolved oxygen levels. Thus, as bacteria travel with the residual seaward current, they encounter changing environmental conditions. The residence time of water in the total estuary is about 60 d (Soetaert & Herman 1995b), although this may be extended via attachment to particles or by (temporary) sedimentation (Owens 1986). The mean residence time of particles in one compartment of the estuary (see Fig. 1) is comparable with the generation time of many cultured ammonia-oxidizing bacteria (Helder & Vries 1983). Thus, competition and selection may occur between distinct ammonia oxidizer populations as they travel through the Schelde estuary. Alternatively, ammonia-oxidizing bacteria may possess the ability to adapt to the environmental gradients encountered. Clues into which of these processes most affect ammonia oxidizer populations might therefore be gained by examining their community structure along the estuarine region where these key environmental gradients are observed.

Ecological studies of ammonia-oxidizing bacteria have been hampered by the difficulties and biases associated with the isolation and manipulation of these organisms in pure culture (Koops & Harms 1985, Prosser 1989). The monophyletic nature of the ß-subclass ammonia-oxidizing bacteria has however facilitated the development of nucleic acid-base techniques for their detection and characterization (Kowalchuk et al. 1997, McCaig et al. 1994, Mobarry et al. 1996, Rotthauwe et al. 1997, Schramm et al. 1998, Stephen et al. 1998, Voytek & Ward 1995, Wagner et al. 1996). Phylogenetic analysis of 16S rDNA sequence data defines 2 genera within this clade, *Nitrosospira* and *Nitrosomonas*, each of which can be further subdivided into at least 4 distinct sequence clusters (see present Fig. 3; Maidak et al. 1999, Stephen et al. 1996). Members of the species *Nitrosococcus oceani*, of the γ-subclass Proteobacteria, also possess the property of autotrophic ammonia oxidation. Although these

bacteria have been described in marine habitats (Ward 1982), very few cultured strains have been described using molecular tools, impeding the development of similar nucleic acid-based analyses for their detection and phylogenetic characterization.

The separation of mixed polymerase chain reaction (PCR) products, generated by specific amplification of 16S rRNA genes, by denaturing gradient gel electrophoresis (DGGE) has become a powerful technique for the rapid comparison of multiple bacterial communities over space and time (Muyzer & Smalla 1998, Muyzer et al. 1993). The interpretation of DGGE banding patterns has been facilitated by hybridization using specific oligonucleotide probes for band identification (Teske et al. 1996). Alternatively, excision of DGGE bands, followed by DNA extraction, re-amplification, and sequence analysis, has also aided in the phylogenetic placement of DGGE bands (Ferris et al. 1996). These techniques have recently been applied to the analysis of ß-subclass ammonia oxidizer-like 16S rDNA sequences recovered by PCR specifically targeting the *Nitrosomonas / Nitrosospira* clade (Kowalchuk et al. 1997, Stephen et al. 1998). These studies have correlated the dominance of certain phylogenetic clusters with specific environmental factors, suggesting that physiological differences between clusters affect their distribution across environmental gradients (Kowalchuk et al. 1998, McCaig et al. 1999, Speksnijder et al. 1998, Stephen et al. 1996, 1998, Whitby et al. 1999). This study continues the process of relating the structure of environmental β-subclass Proteobacteria ammonia oxidizer communities to ecological parameters.

The specific aim of this study was to relate the community structure of ammonia-oxidizing bacteria to the dynamic environmental conditions encountered along the Schelde estuary. Eight study locations were chosen to sample across the region of the estuary with the sharpest gradients with respect to salinity, ammonia availability, and dissolved oxygen content (Fig. 1). An untreated sewage sample, typical of that discharged into the estuary, was also included in the investigation. Estuarine samples were taken on 3 separate occasions to investigate seasonal and year-to-year differences, and key environmental factors were monitored for all samples. PCR, specifically targeting ß-subclass ammonia oxidizer-like 16S rDNA, and DGGE were used to analyze changes in ammonia oxidizer community structure. Specific hybridization and sequence analysis of DGGE bands were also employed to determine the phylogenetic cluster composition of the samples examined. Ammonia oxidizer community composition, as judged by PCR-assisted sequence retrieval, is discussed with reference to the observed environmental gradients along the dynamic estuarine system.

Materials and Methods

Description of the research area

The Schelde estuary (also known as the 'Western' Scheldt) drains an estimated 21 000 km^2 of Northern France, Belgium and the Netherlands, an area with approximately 10 million inhabitants and a nitrogen load of 56 000 t N Yr^{-1} (Soetaert & Herman 1995b). The estuary proper is defined to extend from Temse, the uppermost point of salt-water intrusion, to the mouth into the North Sea near Vlissingen (Fig. 1). Sampling stations were selected in the upper part of the estuary, where gradients of oxygen, ammonia and nitrate are steepest (Fig. 2, Table 1). The pH of all samples ranged between 7.5 and 7.9 (results not shown). An additional sample was taken from untreated wastewater of the city of Brussels, at its point of entry into the Rupel, a tributary of the Schelde (Brion, pers. comm.; Fig. 1). The Brussels wastewater is one of the main contributors to pollution in the upper Schelde estuary.

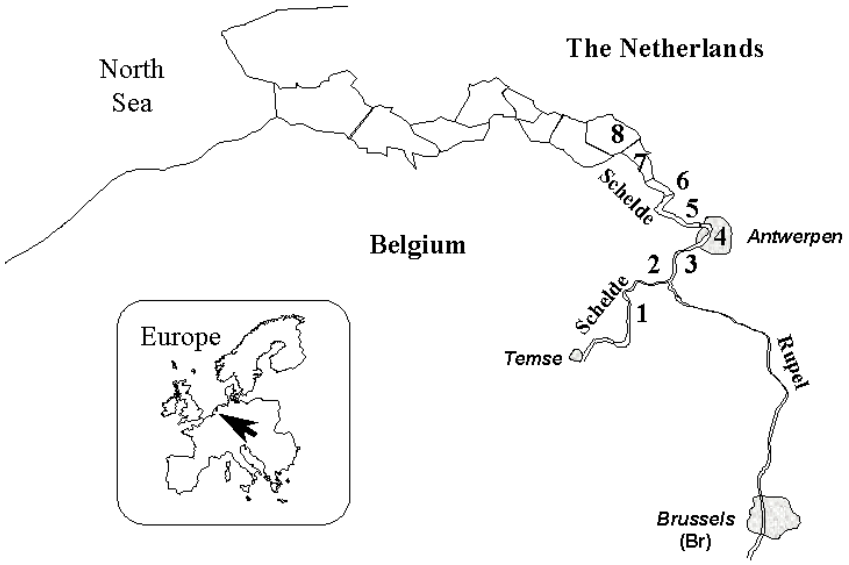

Fig. 1 Map of the river Schelde and the Schelde estuary. Numbers indicate sample locations. Dotted lines across the estuarine indicate compartment borders as used previously in a modeling study of nitrogen dynamics (Soetaert & Herman 1995b). The distance from the city of Antwerp to the mouth of the estuary is approximately 100 km.

Sample collection

Surface water samples were taken aboard the RV Luctor at the indicated sample sites (Fig. 1) in June 1995, July 1996 and October 1996. The amount of vertical stratification in this system is minimal, and surface water samples were therefore regarded as providing a good representation fo the entire water column (Wollast 1988). Samples in different years and seasons were taken to provide a gross estimate of the stability of the β-proteobacterial ammonia-oxidizing community along the estuary. Sampling was performed within in the navigational channel by use of a sterile 1 liter screw-cap bottle. It should be noted that the sample furthermost downstream used for the moleculair analysis of the June 1995 sample was taken at Stn 8 (Fig. 1) instead of Stn 7. Samples were filtered through 0.2 µm pore size nitrocellulose filters (approximately 250 ml water per filter; Schleicher and Schuell BA83, diam.=25 mm). Filters were wrapped in aluminum foil and frozen immediately (-20ºC). Filters were transferred to -80ºC upon arrival at the laboratory. Salinity, temperature and oxygen concentrations were measured with a CTD (conductivity, temperature, depth) system, equipped with a polarographic oxygen sensor (THISHYDRO H2O; Lokeren, Belgium). Nutrient analyses were performed with a segmented flow auto-analyzer system (SKALAR, Breda, the Netherlands). The amount of suspended particulate matter (SPM) in the water samples was estimated by filtering a known volume of sample through preweighed glassfiber (GF/F) filters. Filters with SPM were dried for 24 hours at 40°C, cooled in a desiccator and weighed again. SPM was calculated by the increase of weight of the filters.

DNA isolation

DNA isolation used a mechanical disruption protocol (Stephen et al. 1996). Half of each nitrocellulose sample filter was added to a 2-ml screw-cap tube containing 0.5 ml TE (Tris 10mM/EDTA 1mM pH 7.6) buffer, 0.5 ml TE saturated phenol pH 8.0 (Gibco Laboratories, Detroit, MI, USA) and 0.5 g 0.1 mm-diam. acid-washed zirconium beads (Biospec Products, Bartlesville, OK, USA). The tubes were shaken at 5000 rpm for 3 times 30 s in a Mini-Beadbeater (Biospec Products) and kept on ice between shaking intervals. After centrifugation for 5 min at 5000 × *g*, 0.5 ml of the aqueous layer was removed and extracted twice with 0.5 ml phenol/chloroform/isoamylalcohol 25:24:1 (pH 8.0; Gibco). The remaining aqueous layer (0.4 ml) was recovered and DNA precipitated with 0.1 vol 3 M sodium acetate (pH 5.2), 2 vol 96% ethanol and 2 µl glycogen (Boehringer Mannheim, Germany). Precipitation was for 16 hours at -20°C. DNA was pelleted at 13000 × *g* for 30 min and

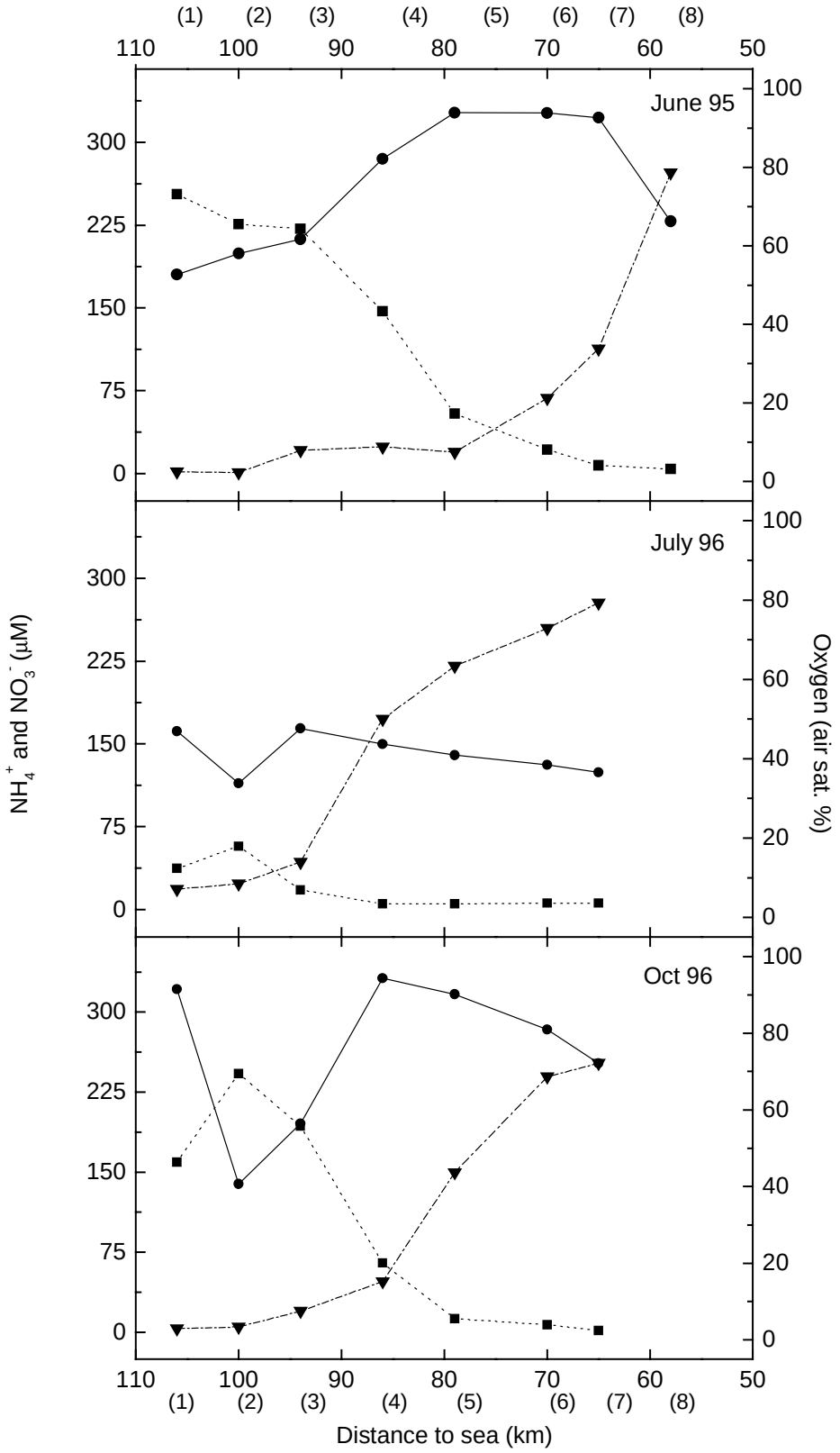

Fig 2. Oxygen (triangles), ammonia (squares) and nitrate (circles) concentrations as a function of distance from mouth of the estuary in June 1995 and July and October 1996. Stations numbered as indicated in Fig. 1 (in parentheses, above top abscissa and below bottom abscissa)

washed once with ice-cold 70% ethanol. After drying (2 min Savant Speedvac DNA 110), the pellet was resuspended in 100 µl TE buffer (pH 8.0). Further purification was performed using the Wizard DNA clean up kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. DNA was eluted from the columns with 100 µl 80°C TE buffer, and stored at -80°C.

PCR, DGGE and hybridization analyses

PCR was conducted using the Expand High-Fidelity polymerase system mix (Boehringer Mannheim, Germany) according to the manufacturer's specifications using the conditions described below. Each 50 µl reaction mixture contained 5 µl template DNA (approximately 30 ng), 5 µl 10 × reaction buffer, 200 µM of each deoxynucleotide, 5 pM each of the CTO189f-GC and CTO654r primers (Kowalchuk et al. 1997), 2 µl 10 mg ml^{-1} non-acetylated bovine serum albumin (New England Biolabs, Beverley, MA, USA), and 2.5 units Expand DNA polymerase. Reaction mixtures were overlaid with an equal volume of molecular biology grade mineral oil (Sigma, St. Louis, MO, USA) and PCR was performed on a Hybaid Omnigene thermocycler (Teddington, UK) in simulated tube mode according to the following thermocycling regime: 1 × (60s 94°C); 35 × (30s 92°C, 60s 57°C, and 45s + 1 s $cycle^{-1}$ 68°C); and 1 × (5 min 68°C). PCR products were examined by 2% agarose gel electrophoresis (2% agarose; 0.5 × TBE; 1 × TBE = 90 mM Tris-Borate, 2 mM EDTA, pH 8.3) with standard ethidium bromide staining to confirm product size and estimate DNA concentration.

Approximately 200 ng PCR product was loaded per sample for DGGE analysis according to the protocol described by Muyzer et al. (1993) as modified by Kowalchuk et al. (1997). DGGE gels used a gradient of 35% to 50% denaturant (100% denaturant = 7 M urea and 40% formamide) and were run at 60°C on a Protean II electrophoresis system (BioRad Laboratories; Hercules, CA, USA) for 16 h at 75 V. DNA fragments of known ammonia oxidizer sequence cluster affinity (present Fig. 3 and Stephen et al. 1996) were run alongside environmental samples to act as controls for subsequent hybridization analysis. DNA was stained using ethidium bromide and rinsed twice for 15 min in 0.5 x TAE buffer (48.22 g Tris Base, 2.05 g anhydrous sodium acetate, 1.86 g $Na_2EDTA.2H_2O$, pH 8 in 1 liter deionized water) prior to UV transillumination. Gel images were captured digitally using The Imager System (Appligene; Illkirch, France). DNA from DGGE gels was transferred to Hybond-N^+ Nucleic Acid Transfer Membranes (Amersham International; Bucks, UK), using a BioRad Semi-Dry Transblotter SD according to Muyzer et al. (1993). Transferred DNA was

subsequently denatured (DNA-side down) and simultaneously cross-linked to the membrane by soaking in 0.4M NaOH, 0.6M NaCl on Whatman 3MM (Whatman; Kent, UK) filter paper. Membranes were similarly neutralized with 1 M NaCl, 0.5 M TRIS/HCl (pH 8,0). Hybridization analyses and quantification of hybridization signals were conducted according to Stephen et al. (1998), using a hierarchical set of oligonucleotide probes designed for the identification of the previously recognized sequence clusters within the ß-subclass ammonia oxidizer clade (Fig. 3). Recent evidence now allows for the specific detection of sequence subgroups within *Nitrosomonas* cluster 6 as proposed by Stephen et al. 1996. This sequence cluster has previously been detected in a variety of environments (McCaig et al. 1994, Stephen et al. 1996), and a subgroup of this cluster, currently termed cluster 6a, has recently been postulated (Speksnijder et al. 1998). This monophyletic group of 16S rDNA sequences has been recovered from soil and freshwater sediment environments, and the oligonucleotide probe, NmoCL6a_205, has been used in conjunction with specific PCR-DGGE for their detection (Speksnijder et al. 1998, Stephen et al. 1996, 1998). The phylogenetic relationship of strains and sequences grouped into *Nitrosomonas* cluster 6b is as yet uncertain, and this group currently represents all *Nitrosomonas* cluster 6 sequences that do not fall within the *Nitrosomonas* cluster 6a clade (Fig. 3). The DGGE pattern obtained for the Brussels wastewater sample was not subjected to hybridization analysis. The relative intensities of DGGE bands for this sample were estimated by quantification of ethidium bromide staining using the ImageMaster Elite software package (Version 3.01; Amersham Pharmacia Biotech; Uppsala, Sweden).

DGGE band excision and sequence analysis

Only the central section of selected DGGE bands was excised for subsequent DNA re-amplification. Each gel fragment (approximately 2 mm^3 acrylamide) was placed in a 2.0 ml screw-cap tube, containing 300 µl TE buffer and 0.3 g 1 mm-diam. zirconium beads. Tubes were shaken for 30 s (5000 rpm) in a Mini-Beadbeater and incubated for 3 h at 4°C. After gel fragments had been collected at the bottom of the tube by 15 s centrifugation, 5 µl of the supernatant was recovered to act as template DNA for subsequent PCR. Each 25 µl PCR mixture contained 1.25 units Taq DNA polymerase (Boehringer), 1.5 mM $MgCl_2$, 10 mM Tris/HCl (pH 8.3), 50 mM KCl, 0.01% w/v gelatin, 200 µM of each deoxynucleotide, and 5 pM each of the CTO189f-GC and CTO654r primers (Kowalchuk et al. 1997). Amplification was performed using a thermocycling regime of 28 × (60 s 95°C, 60 s 57°C, 45 s + 1 s $cycle^{-1}$

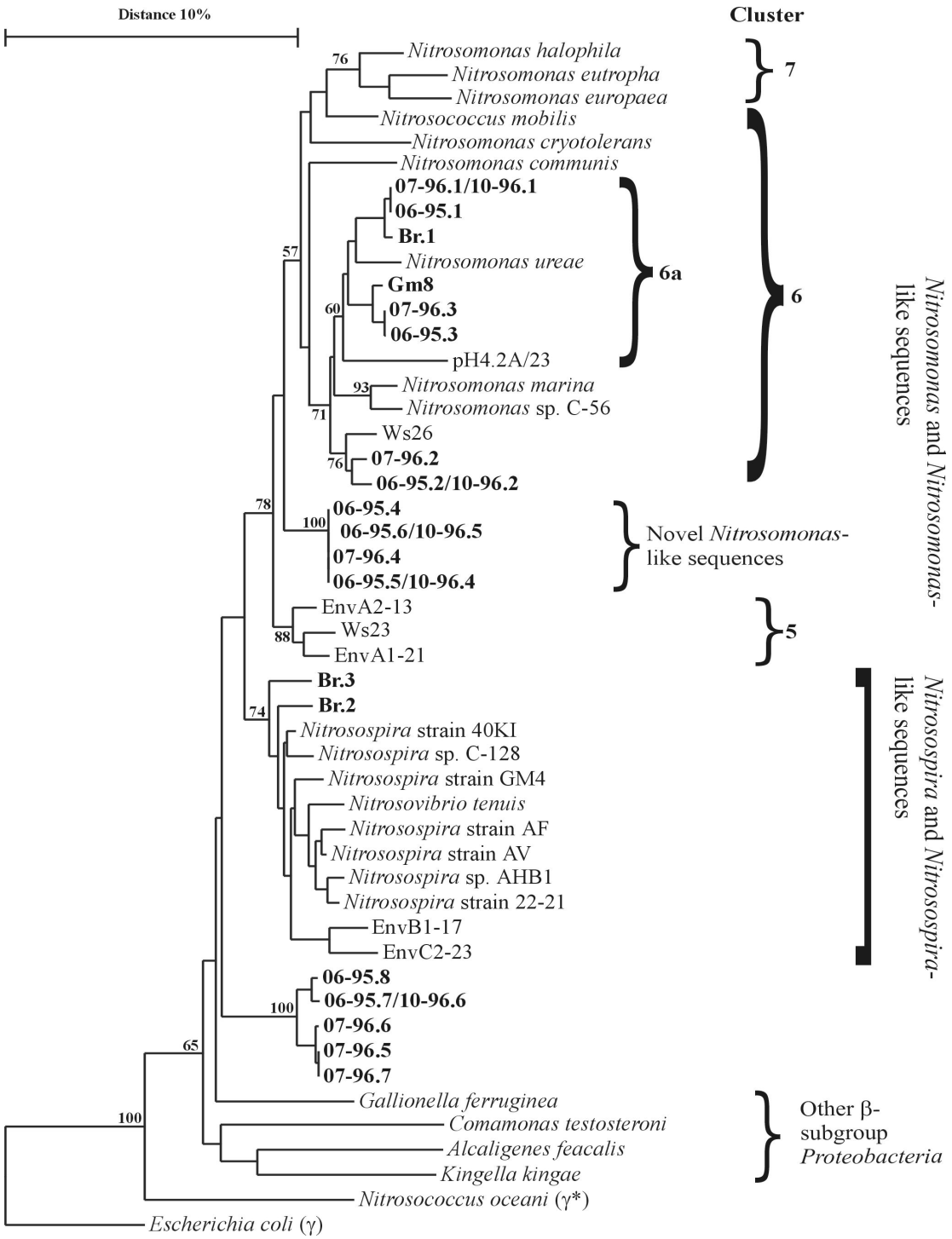

Fig. 3 Neighbor joining tree of selected ß-subclass Proteobacteria 16S rDNA sequences. The tree highlights the ammonia oxidizer-like sequences found and is constructed using partial 16S rDNA sequences as described in the text. Sequences with number designations correspond to the DGGE bands indicated in Fig. 4. Band and sequence designations were as follows: 'month-year.band#'. For example, 07-96.4 would indicate the fourth DGGE band position from the July, 1996 samples. "Br" designations correspond to excised bands from the Brussels wastewater DGGE pattern shown in Fig. 4c. Comparative environmental clone sequences were recovered from marine sediments (prefixed with Env; Stephen et al. 1996), soil with low pH (pH4.2A/23; Stephen et al. 1996), freshwater lake sediment (Gm8; Speksnijder et al. 1998), and estuarine water (Ws26 and Ws23; Speksnijder et al. 1998).

72°C). From each PCR, 5 µl was subjected to DGGE as described above to confirm recovery of the desired band. Sequencing reactions used 5 µl of each reaction template without further purification. Sequencing was performed with the Thermosequenase kit (Amersham) according to the manufacturer's recommendations using the bacterial-specific 16S rDNA primers, 357f and 518r (Edwards et al. 1989), labeled with Texas Red. Sequencing reactions were run and analyzed on a Vistra DNA sequencer 725 (Amersham). Sequence data was edited and assembled in the Sequencer 3.0 software package (Gene Codes Corporation, Ann Arbor, MI, USA), and format conversions were carried out in Seqapp 1.9a169 (Gilbert 1993, available by ftp.bio.indiana.edu.)

Phylogenetic analysis of recovered 16S rDNA sequences was performed as described previously (Speksnijder et al. 1998). Sequence alignments included sequences from the Ribosomal Database Project (RDP; Maidak et al. 1999) and spanned 430 nucleotide positions. Optimization of alignments was performed in the Dedicated Comparative Sequence Editor program (van de Peer et al. 1997, de Rijk & de Wachter 1993) using recognized 16S rRNA secondary structures (van de Peer et al. 1997). Sequence comparisons used 432 informative positions of 16S rDNA sequence that could be unambiguously aligned for all recovered sequences and selected databank reference sequences. Tree construction was performed with the Treecon program (van de Peer & de Wachter 1994) using neighbor-joining analysis and matrix calculation according to the method of (Jukes & Cantor 1969). Gaps were not taken into account, and bootstrap analysis was based upon 100 replicates. Novel partial 16S rDNA sequences determined in this study have been deposited into the EMBL sequence databank under accession numbers AJ132047-AJ132062.

Results

Characterization of environmental conditions along the estuary

The upper reaches of the estuary typically contained low dissolved oxygen levels, low salinity, and a high ammonia to nitrate ratio (Fig. 2, Table 1). The situation was reversed in lower, more saline parts of the estuary. Variation between sampling dates with respect to salinity and other environmental gradients was mostly influenced by tides, as previously found in the Schelde (Soetaert & Herman 1995a, b). The brackish zone (near the city of Antwerp) contained sharp gradients in all of these variables (Fig. 2, Table 1). It is in this portion of the estuary where maximum nitrification has been observed (Somville 1984, de

Wilde & de Bie 2000). The locations of the steepest gradients in nutrients and oxygen concentration were between Stns 4-8, 3-5, and 3-6 for the June 1995, July 1996, and October 1996 sampling dates, respectively.

TABLE 1: Salinity (SAL), nitrite (NO_2) and suspended particulate matter (SPM) values in water samples along the Schelde estuary. Station numbering is according to Fig. 1

	JUN 95			JUL 96			OCT 96		
Stn number	SAL (PPT)	NO2 (µM)	SPM (mg/l)	SAL (PPT)	NO2 (µM)	SPM (mg/l)	SAL (PPT)	NO2 (µM)	SPM (mg/l)
1	0.5	35	12	1.1	12	131	0.8	18	65
2	0.7	29	18	1.7	11	122	1	24	77
3	1.2	39	30	4.9	10	42	1.9	23	43
4	2.9	34	65	10.3	4	20	6	26	27
5	4.1	35	14	12.7	3	21	10.3	12	19
6	5.8	29	77	14.6	3	22	14	4	29
7	7.5	24	11	16	3	19	14.3	3	15
8	14.4	18	43						

Recovery of 16S rDNA and DGGE analysis

All DNA extractions from filtered samples were performed at the same time. Thus, DNA extractions were performed using samples that had been stored at -80ºC for different periods of time. Although it was observed that older filters yielded slightly less DNA than fresher filters, it is not known whether the 1995 samples contained less DNA, or if prolonged storage affected the efficiency of DNA recovery (results not shown). Specific PCR amplification of 16S rDNA using the CTO189f-GC and CTO654r primers yielded positive results in all cases.

DGGE analysis of recovered PCR products revealed 3 to10 bands per sample (Fig. 4). For upstream sample sites, the most dominant bands occurred relatively high in the gel, showing migration within the range of controls for *Nitrosomonas* clusters 6a, 6b, and 7 (*N. europaea* and *N. eutropha* belonging to the last). At more seaward sites, bands lower in the gel, comparable in migration to *Nitrosomonas* cluster 5 and several *Nitrosospira* sequence cluster controls, became most dominant. Although all sampling dates showed this trend, the exact position and extent of this shift varied in a fashion similar as the nutrient profiles described above. The Brussels wastewater sample produced a very strong band high in the gel, suggestive of *Nitrosomonas* clusters 6a, 6b or 7, as well as 3 additional bands lower in the gel, migrating within the range of several *Nitrosospira* controls (Fig. 4c).

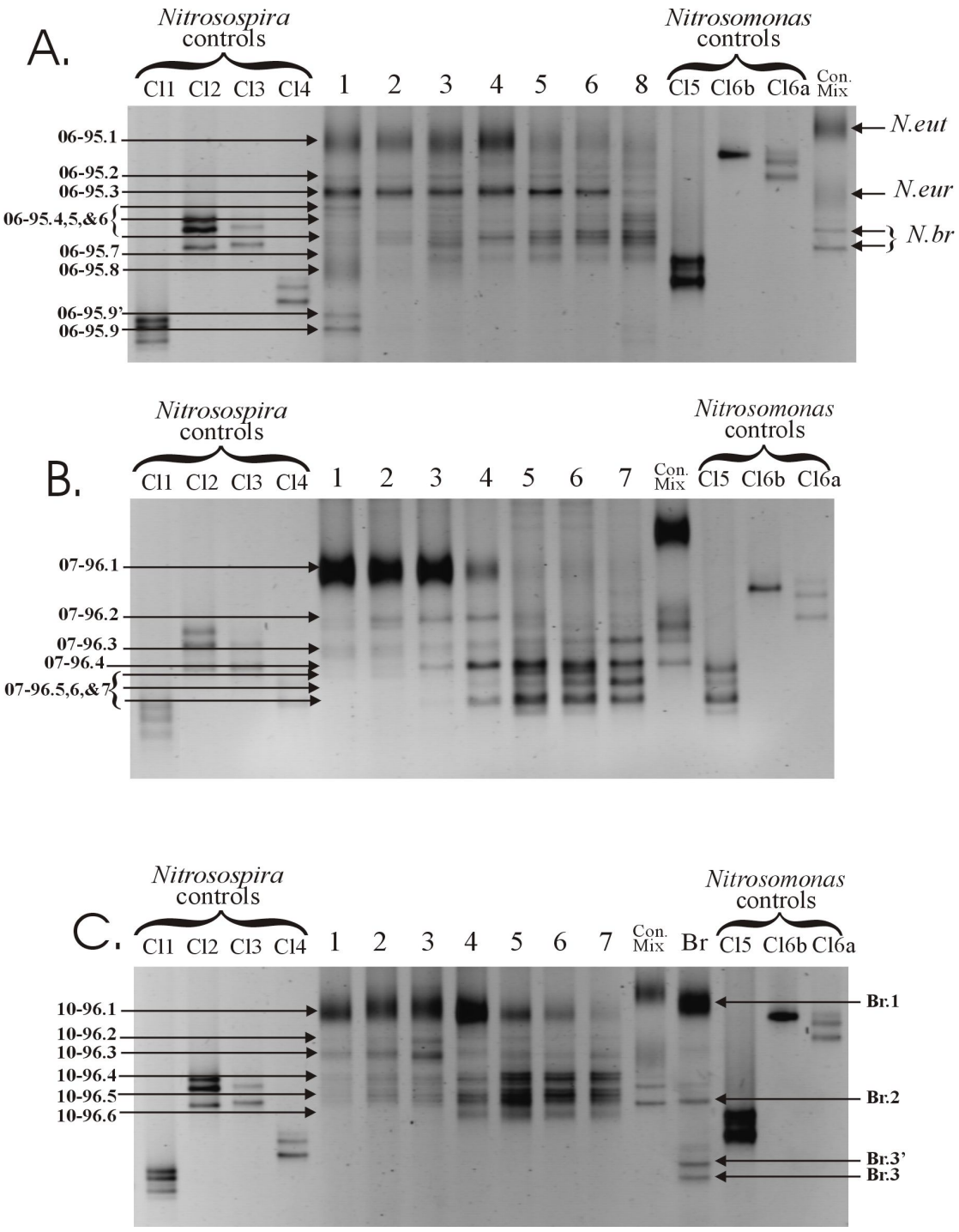

Fig. 4 PCR-DGGE analysis of ammonia oxidizer-like 16S rDNA fragments along the Schelde estuary. DGGE analyses are for June 1995 (A), July 1996 (B) and October 1996 (C), and the numbers above the lanes, 1-8, correspond to the station numbers indicated in Fig. 1. Cloned sequence cluster controls, labeled "Cl", are numbered according the sequence cluster designations of Stephen et al. (1996), as described in Kowalchuk et al. (1997). Bands labeled with a prime (') contain a one base pair difference with their namesake sequences, which was introduced due to an ambiguous position in the CTO654r primer (Kowalchuk et al. 1997). Sequences from such bands were not included in the phylogenetic analysis. Lanes labeled "Con. Mix." were from control PCRs, where the template consisted of a mixture of chromosomal DNA from three pure culture strains, *Nitrosomonas eutropha*, *Nitrosomonas europaea* and *Nitrosospira briensis.* The lane marked "Br." in panel C contains DNA recovered from the Brussels wastewater sample. Arrows indicate the position of the bands that were excised for sequence determination. The phylogenetic placement of DGGE band sequences and a description of band labeling are found in Fig. 3.

Hybridization and sequence analysis of DGGE banding patterns

Previous DGGE studies have shown that migration behavior alone is not a good predictor of phylogenetic affinity with respect to ammonia oxidizer 16S rDNA fragments (Kowalchuk et al. 1997, 1998). We therefore characterized DGGE bands by hybridizing DGGE banding patterns with a battery of 16S rDNA-targeted oligonucleotide probes, specific at different taxonomic levels within the ß-subclass ammonia-oxidizing bacteria (Stephen et al. 1998). However, an all ß-subclass ammonia oxidizer-specific probe, ß-AO233 (Stephen et al. 1998), failed to hybridize with some DGGE bands, suggesting that these might not contain sequences falling within the *Nitrosospira* / *Nitrosomonas* radiation. Excision, re-amplification, sequence determination, and phylogenetic analysis of such bands (06-95.7-9', 07-96.5-7, and 10-96.6; Figs. 3 & 4) placed their sequences outside the *Nitrosospira* / *Nitrosomonas* clade. These bands were excluded from further analyses. The recovery of some non-ammonia oxidizer sequences with these primers has been noted previously (Kowalchuk et al. 1998, 2000), and their presence did not interfere with the further analysis of sequences within the ß-subclass ammonia oxidizer radiation.

The diversity within 16S rDNA sequences belonging to the genus *Nitrosomonas* is greater than that found for the *Nitrosospira* genus (Pommerening-Röser et al. 1996). Given the current available sequence information, it is not yet possible to assess the specificity and accuracy of probes designed for the detection of phylogenetic lineages within the genus *Nitrosomonas* (Stephen et al. 1998, Utaaker & Nes 1998). We therefore excised all the bands from environmental DGGE patterns for sequence analysis to confirm hybridization results (see below) and allow for the phylogenetic placement of recovered *Nitrosomonas*-like sequences. Bands with the same DGGE mobility across sample locations produced identical sequences, and were included only once in the phylogenetic analysis (Fig. 3). Quantification of hybridization signals and sequence analysis of excised bands revealed a shift in the predominant ß-subclass ammonia oxidizer-like sequences detected along the estuary transect (Fig. 5). Upstream regions of the estuary contained a dominance of 16S rDNA sequences classified as *Nitrosomonas* cluster 6a (Fig. 5). In contrast, a novel *Nitrosomonas*-like sequence group was observed in lower reaches of the estuary. This shift was observed for all 3 sampling dates, although not always to the same degree or at the exact same position along the estuary. Moving seaward, the point in the estuary where the novel *Nitrosomonas*-like sequences first became most abundantly detected was between Stns 6 and 8 for the June 1995 sample, at Stn 4 for the July 1996 sample, and at Stn 5 for the October 1996 sample. The

position of the sharpest ammonia oxidizer community shift for all 3 sampling dates corresponded to the point in the estuary where salinity values were approximately 10, oxygen saturation levels were around 40 %, and ammonia concentration dropped below 15 µM (Fig. 2, Table 1).

Nitrosomonas-like sequences, which were most closely related to the culture strain *Nitrosomonas marina* Nm22 (06-95.2, 07-96.2, and 10-96.2), were also detected along the entire estuary transect sampled. Hybridization signals for these bands represented 0 to 10% of the total ß-subclass ammonia oxidizer-specific signal, and no trends with respect to location or sampling date were apparent. No clear *Nitrosospira*-like DGGE bands were apparent in the estuarine samples by ethidium bromide staining, due to the weak nature of the signal and the overlap with other, more dominant DGGE bands. *Nitrosospira*-specific hybridization signals were characterized as *Nitrosospira* cluster 3. These signals constituted less than 2% of the total recovered PCR product in all estuarine samples. Sequence results from excised bands agreed with the phylogenetic predictions based upon hybridization analysis in all cases examined.

DGGE bands from the Brussels sample were also sequenced. The sequence of the uppermost band (Br.1) placed it within *Nitrosomonas* cluster 6a (Fig. 3). The remaining three bands (Br.2, Br.3, and Br.3') produced *Nitrosospira*-like sequences. The Br.2 sequence contained the probe site characteristic of members of *Nitrosospira* cluster 4 (Stephen et al. 1998). However the existence of several ambiguities in the nucleotide sequence did not allow for accurate phylogenetic analysis to the sequence cluster level. The lowermost bands (Br.3 and Br.3') also contained several ambiguous nucleotide positions, which corresponded to highly variable regions of the 16S rRNA gene, here again, sequence cluster level characterization was not possible. These bands may have consisted of multiple *Nitrosospira*-like sequences.

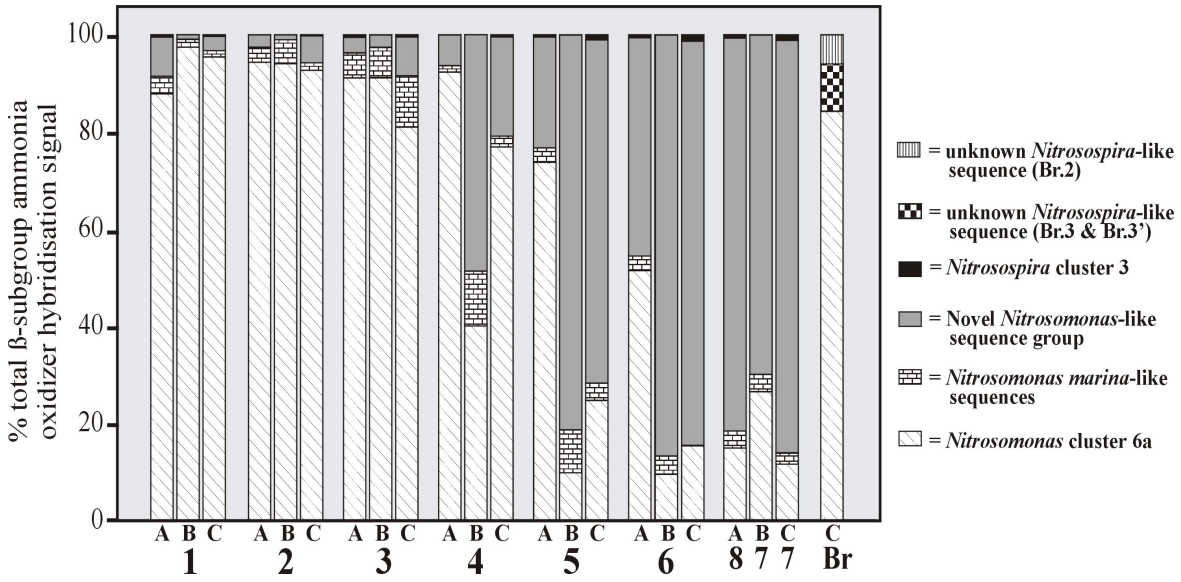

Fig. 5 Distribution of detected ß-subclass ammonia oxidizer-like sequences across the Schelde estuary. The DGGE gels shown in Fig. 4 were hybridized with a hierarchical set of oligonucleotide probes for identification and quantification of DGGE bands (Stephen et al. 1998). Numbers, 1-8, indicate sampling station, and the letters A, B, and C correspond to the sampling dates June 1995, July 1996, and October 1996, respectively. The relative amount of each specific sequence cluster, as detected by specific hybridization, is given with respect to the hybridization signal produced by the all ß-subclass ammonia oxidizer probe, ß-AO233 (Stephen et al. 1998). The cumulative signal from sequence cluster-specific probes could explain 95-103% of the all ß-subclass ammonia oxidizer hybridization signal. Results were standardized to 100% for graphical presentation. Values for the Brussels wastewater sample were determined by the quantification of ethidium bromide fluorescence.

Discussion

A shift was observed in the ß-subclass ammonia-oxidizer populations detected along the estuary within the region where gradients with respect to salinity, dissolved O_2, and ammonia were sharpest, and where ammonia oxidation was highest (de Wilde & de Bie 2000). The physiological significance of detecting different phylogenetic groupings of ammonia oxidizer-like 16S rDNA sequences is not yet known. However, that different ammonia oxidizer groups were detected under the different environmental conditions along the estuary, and that these changes were stable with regard to the steepest environmental gradients over sampling dates, implies that these changing conditions may affect ammonia oxidizer growth, activity and / or survival in the estuary. Bacteria showing affinity with *Nitrosomonas* cluster 6a were the most dominantly detected ß-subclass ammonia oxidizers in the upper reaches of the estuary, where freshwater, low oxygen and high ammonia conditions prevail. Sequences showing affinity with this sequence cluster have previously been detected in soil, freshwater, and freshwater sediment (Stephen et al. 1996, 1998). These authors suggested that the distribution of this specific *Nitrosomonas* lineage may be restricted to non-marine environments, and the decrease in *Nitrosomonas* cluster 6a detection in the lower estuary

samples would support this hypothesis. (Speksnijder et al. 1998) also detected this group of sequences in nearly anoxic sediment layers, suggesting a level of tolerance to low oxygen conditions. Although it has been demonstrated that ammonia-oxidizing bacteria can adapt to low-oxygen environments, this ability may not be equally present in all phylogenetic groups (Bodelier et al. 1996, Smorczewski & Schmidt 1991). Given the methods used, the activity of the detected bacteria could not be assessed, and some may be present in inactive forms, potentially more resistant to adverse environmental conditions. The dominance of *Nitrosomonas* cluster 6a in upstream estuary samples may also be affected by input of ammonia-oxidizing bacteria from untreated wastewater into the estuary. Although it should be stressed that the Brussels waste water samplerepresents but one of many potential sources of nitrifying bacteria into the estuary. Such wastewater samples have previously been shown to contain high ammonia oxidizer biomass (Brion & Billen 1997), and a representative wastewater sample was also dominated by *Nitrosomonas* cluster 6a. It may be that such organisms survive well after being released into the upper estuary, despite the long stretch of the estuary before entering the region studied. Their decline lower in the estuary might be explained by either a lack of tolerance to changing environmental conditions or a decreased ability to compete with other organisms for substrate. Previous studies of activated sludge samples have also detected high numbers of *Nitrosomonas*-like bacteria, although these populations belonged to the *Nitrosomonas* cluster 7 lineage (Mobarry et al. 1996, Wagner et al. 1996).

Nitrosomonas cluster 6a is displaced by a novel *Nitrosomonas*-like group further down the estuary system. This shift coincides with sharp increases in salinity and O_2 concentration, as well as a sharp decrease in ammonia concentration in the estuary. The exact curve of the nutrient and salinity values is the result of several biological and physical processes (Soetaert & Herman 1995b). While nitrogen salts in the upper estuary are mainly controlled by microbiological activity, the location of maximum activity, both with regard to Stn number and salinity, is influenced by the tidal regime, freshwater discharge, and wind. As shown in Table 1, suspended matter values vary along the estuary, and the distribution of the novel *Nitrosomonas*-like group coincides with lower SPM-values in the downstream reaches of the estuary. Thus, the distribution of different ammonia oxidizer populations throughout the estuary may be influenced by differential strategies of either free-living or particle-bound lifestyles. Separate analyses of particulate and pelagic samples would address this issue (Philips et al. 1999). Strains affiliated with the novel *Nitrosomonas* lineage may be well adapted to the environmental conditions present in the lower regions of the estuary. However,

the relative shift between phylogenetic groupings is not strong evidence of growth of specific nitrifier types, as the decrease in the overall number of nitrifiers at the most downstream locations may lead to the dilution of certain populations (Billen 1975). The increase in the relative proportion of these *Nitrosomonas*-like sequences lower in the estuary could also be due to ammonia oxidizer inoculation *via* land run-off or other point or non-point sources along its way to the sea. Although one cannot exclude this possibility, 2 lines of evidence speak against this explanation. Firstly, previous studies on nutrient concentration along the region of the estuary under study have never revealed nutrient point sources that measurably increased local nutrient concentrations (Soetaert & Herman 1995a, b). Also, the city of Antwerp, situated at the point where ammonia-oxidizer population shifts were observed, utilizes advanced waste-treatment systems, which releas only negligible bacterial biomass and nutrients into the estuary. In contrast, wast from the urban area of Brussels is untreated, providing a far greater potential source of both nitrifying organisms and nutrients (Brion & Billen 1997). Secondly, terrestrial environments, similar to the agricultural soil systems adjacent to the Schelde estuary, have previously been shown to contain ammonia-oxidizer communities dominated by *Nitrosospira*-like bacteria (Hastings et al. 1998, Stephen et al. 1996, 1998).

The physiological differences between the 2 main *Nitrosomonas* lineages detected across the estuarine transect studied are not yet known, and no pure cultures are available yet to examine their differential responses to the environmental gradients encountered in the Schelde estuary. However, knowledge of the distribution of these populations should help in the development of the necessary enrichment and isolation strategies.

Minority populations of *Nitrosospira* cluster 3 and a *Nitrosomonas marina*-like bacteria were also identified across the estuarine system. The former group has previously been detected in terrestrial and freshwater environments (Hastings et al. 1998, Hiorns et al. 1995, Stephen et al. 1996, 1998), but only represents a small fraction of the total recovered ß-subclass ammonia oxidizer sequences in this estuary environment. As the name suggests, *Nitrosomonas marina* Nm22, and closely related strains, have previously been detected in marine environments and are obligatorily halotrophic (McCaig et al. 1999, Pommerening-Röser et al. 1996). The detection of the sequences 06-95.2, 07-96.2, and 10-96.2 in the freshwater reaches of the estuary suggests that strains closely related to *Nitrosomonas marina* might not be strictly limited to saline environments.

Despite the importance of nitrification in estuarine habitats (Owens 1986, Soetaert & Herman 1995b), few studies to date have addressed ammonia oxidizer community structure

in this habitat (Murray et al. 1996). A previous study, based on the cloning of ammonia oxidizer-like 16S rDNA sequences after semi-specific PCR, also detected *Nitrosomonas*-like sequences at sampling Stn 4 within the Schelde estuary (Speksnijder et al. 1998). Among the limited number of ammonia oxidizer-like clones examined, only sequences showing affinity with the genus *Nitrosomonas* were recovered, including sequences from the *Nitrosomonas* cluster 6a and *Nitrosomonas marina* lineages detected in the present study (Fig. 3). Nitrifying bacteria have also been studied in the Elbe estuary, where several *Nitrosomonas*-like lineages have been isolated (Stehr et al. 1995) or detected by fluorescent in situ hybridization (Wagner et al. 1996). Although *Nitrosospira*-like bacteria were observed, most detected strains were related either to *Nitrosomonas ureae* or *Nitrosomonas europaea*.

As is the case with most molecular studies of ammonia-oxidizers, the present study has focussed upon bacteria of the ß-subclass of the Proteobacteria. However, ammonia-oxidizing bacteria of the genus *Nitrosococcus*, within the γ-subclass Proteobacteria, may also be present in the estuarine system examined. This possibility is especially relevant to the lower, more saline, reaches of the estuary, given the currently recognized distribution of this genus (Ward & Carlucci 1985). The development of similar methods to those used here for the direct detection of γ-subclass ammonia-oxidizing bacteria may prove essential to improving our understanding of their role in the environment. It should also be stressed that the present study targeted 16S rDNA, and therefore could not discriminate between active and dormant cells. Studies designed to detect active cells, for instance by targeting 16S rRNA (Felske et al. 1996) or ammonia oxidizer-specific mRNAs by reverse transcriptase PCR should be helpful in this respect.

The efficiency of DNA extraction may affect the accuracy of community fingerprinting techniques such as PCR-DGGE. Ammonia-oxidizing bacteria tend to form tight clusters or attach to particulate matter, which can prevent their lysis during DNA isolation procedures (Schramm et al. 1998). For this reason, we used a highly rigorous method for DNA isolation, the addition to which of freeze-thaw steps or additional bead-beating did not lead to the liberation of more DNA or additional DGGE bands (results not shown). In addition to environmental gradients, the distribution of other microbial community members may influence ammonia oxidizer populations. Although a previous study of ammonia oxidizing bacteria in freshwater sediment environments demonstrated that ammonia oxidizer and eubacterial communities can vary independently (Speksnijder et al. 1998), this possible influence certainly cannot be discounted.

Several environmental parameters vary across the estuarine transect studied. Given the bacterial residence time in the estuary, both adaptation and selection are possible within the ammonia oxidizer communities present. Although some ammonia-oxidizing bacteria are known to be able to adapt to low oxygen environments (Bodelier et al. 1996, Kowalchuk et al. 1998, Speksnijder et al. 1998), exposure to low oxygen conditions can also affect ammonia oxidizer diversity and community structure (Smorczewski & Schmidt 1991). Similarly, adaptation to increasing salt concentrations is possible for some ammonia-oxidizers (Helder & Vries 1983, Somville 1984). However, certain strains appear to be particularly well suited to high salt conditions (Pommerening-Röser et al. 1996), and different ammonia oxidizer populations have been detected in the comparison of freshwater and saltwater aquaria (Hovanec & DeLong 1996) . Ammonia-oxidizing bacteria also undergo physiological adaptations in response to low ammonia availability (Laanbroek & Woldendorp 1994). Despite these adaptive capabilities, phylogenetic differences within the genus *Nitrosomonas* are known to be reflected in their ammonia sensitivities and half-saturation constant (K_S) values of ammonia oxidation (Suwa et al. 1997). In addition to the environmental gradients discussed above, other less readily apparent environmental factors may also influence the distribution of ammonia oxidizer populations in this estuarine system. Thus, although the current study suggests that the environmental gradients encountered in the estuarine environment affect ammonia oxidizer community structure, it is not yet possible to determine the influence of individual environmental factors. However, knowledge of the distribution and diversity of ß-subclass ammonia oxidizer populations along the Schelde estuary now allows for the design of experiments to test which environmental factors most influence their distribution, and provides clues into strategies for their enrichment and isolation.

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C H A P T E R 4

Nitrous oxide in the Schelde estuary: production by nitrification and emission to the atmosphere

Hein P.J. de Wilde, Monique J.M. de Bie

Concentrations of nitrous oxide (N_2O), oxygen, nitrate and ammonium, as well as nitrification activity were determined along the salinity gradient of the Schelde Estuary, North West Europe, in October 1993, March 1994, and July 1996. The entire estuary was always supersaturated with N_2O. Concentrations ranged from 338 nmol dm^{-3} (31 times supersaturated) in the O_2-poor upper estuary, down to about 10 nmol dm^{-3} (slight supersaturation) at the mouth of the estuary. Nearly all N_2O was lost to the atmosphere within the estuary rather than being transported to the open sea. The mean annual emission from the Schelde estuary to the atmosphere was estimated to be 2.8 x 10^8 g. Per unit area, this flux is large when compared to data published for other estuaries.

Nitrification in the water column was the main source of N_2O. The oxygen gradient along the estuary controlled the location and intensity of nitrification. In the upper estuary the maximum nitrification activity was 6.40 µmol N dm^{-3} h^{-1}. The N_2O yield, associated with nitrification, varied spatially and seasonally. Between 0.1 and 0.4% of the oxidised NH_4^+ was converted to N_2O. The total amount of N_2O in the estuary appeared to be controlled by the NH_4^+ concentration of the river water which entered the estuary.

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Introduction

Nitrous oxide (N_2O) is an atmospheric trace gas that contributes to global warming and ozone depletion (Wang et al. 1976; Crutzen 1970). The atmospheric N_2O concentration has increased by about 15% since pre-industrial times and stabilisation at the present level of about 313 ppb would require a reduction in the anthropogenic emission of more than 50% (Houghton et al. 1996). Concern about the global N_2O budget has stimulated research to quantify the sources and sinks. Terrestrial sites have received more attention than the aquatic environment. Marine N_2O emissions are insufficiently constrained (Nevison et al. 1995). Even less is known about estuarine N_2O emissions, as measurements are scarce and restricted to a small number of estuaries (Bange et al. 1996). Presently, however, the distribution of several trace gases in nine European estuaries, is being investigated within the framework of the EU-ELOISE project BIOGEST (BIOGas transfer in ESTuaries).

Estuaries often receive high loadings of nutrients and organic matter, while the tidal circulation generally causes a long residence time of the water. As a consequence, turnover of nitrogen and carbon usually is more intense in estuaries than in either rivers or the open ocean. Since N_2O production is positively related to nitrogen and carbon turnover (Firestone & Davidson 1989), estuaries potentially are strong sources of N_2O.

N_2O is produced as a by-product during several microbiological processes including (1) nitrification, the oxidation of ammonium to nitrite, and subsequently to nitrate, (2) denitrification, the reduction of nitrate, via nitrite and N_2O, to N_2, and (3) dissimilatory nitrate reduction to ammonium (DNRA). Nitrification and denitrification appear to be the dominant sources of N_2O in most natural systems (Firestone & Davidson 1989). The precise mechanisms of N_2O production, however, are still unclear (Conrad 1996). Low oxygen concentrations, which are characteristic for polluted estuaries, can increase the N_2O production by both nitrification and denitrification (Anderson & Levine 1986; Codispoti & Christensen, 1985; Goreau et al. 1980; Jørgensen et al. 1984). During nitrification, N_2O may be formed under oxygen limiting conditions by reduction of nitrite (Poth & Focht 1985; Anderson & Levine 1986), or hydroxylamine (Stüven et al. 1992). N_2O production by denitrification is maximal when the oxygen concentration is very low, but not zero (Codispoti & Christensen 1985; Otte et al. 1996).

The relative contribution to N_2O production by nitrification and denitrification is often hard to determine, especially in estuaries where N_2O can be supplied by many internal and external sources. Denitrification is reported to be the main source of N_2O in many estuaries (Bange et al. 1996; Butler et al. 1987; Jørgensen et al. 1984; Kerner 1996; Law et al. 1992; Robinson et al. 1998; Seitzinger & Nixon 1985). There are, however, also reports on the importance of nitrification in estuarine N_2O production (McElroy et al. 1978; Deck 1981; Nixon & Pilson 1983).

We present here a study on the N_2O distribution in the Schelde estuary in relation to nitrification in the water column. Additional chemical and physical data were collected, to determine the factors controlling the N_2O production. The objectives of our study were: (1) to determine the distribution of N_2O along the Schelde estuary in several years; (2) to locate the nitrification activity along the longitudinal axis of the estuary; (3) to assess the contribution of this process to the production of N_2O; (4) to estimate the N_2O emission into the atmosphere; (5) to assess the contribution of estuaries to the marine N_2O budget; and (6) to evaluate the coupling between N_2O production and N-loading to the estuary.

Methods

Description of the research area

The Schelde estuary is one of the most eutrophied estuaries in Europe (Wollast 1988) as a result of urban waste water drainage and runoff from agriculture. Since the residence time of the water is about 50 - 70 days (Soetaert & Herman 1995a), the waste loadings induce intense microbial degradation in the estuary. Heterotrophic bacterial production rates in the Schelde are among the highest reported in literature (Goosen et al. 1995), leading to an oxygen depleted zone which can extend over a length of 30 to 70 km (Wollast 1988). Salinity ranges from about 0.5 at Temse, the upper limit of salt water intrusion, to about 30 at Vlissingen near the mouth of the estuary (Fig. 1). Vertical stratification is usually small (Wollast 1988). The average freshwater outflow of 105 $m^3\ s^{-1}$ (Billen et al. 1985) is small relative to the tidal exchange of about 45000 $m^3\ s^{-1}$ (Middelburg & Nieuwenhuize 1998). Turbidity is high in the entire upper estuary since suspended matter concentrations range from 25-200 mg dm^{-3}. Although a distinct turbidity maximum cannot always be detected (Paucot & Wollast 1997),

turbidity generally is highest between a salinity of 1 and 5 (Wollast 1988). Cruises were made in October 1993, March 1994, and July 1996.

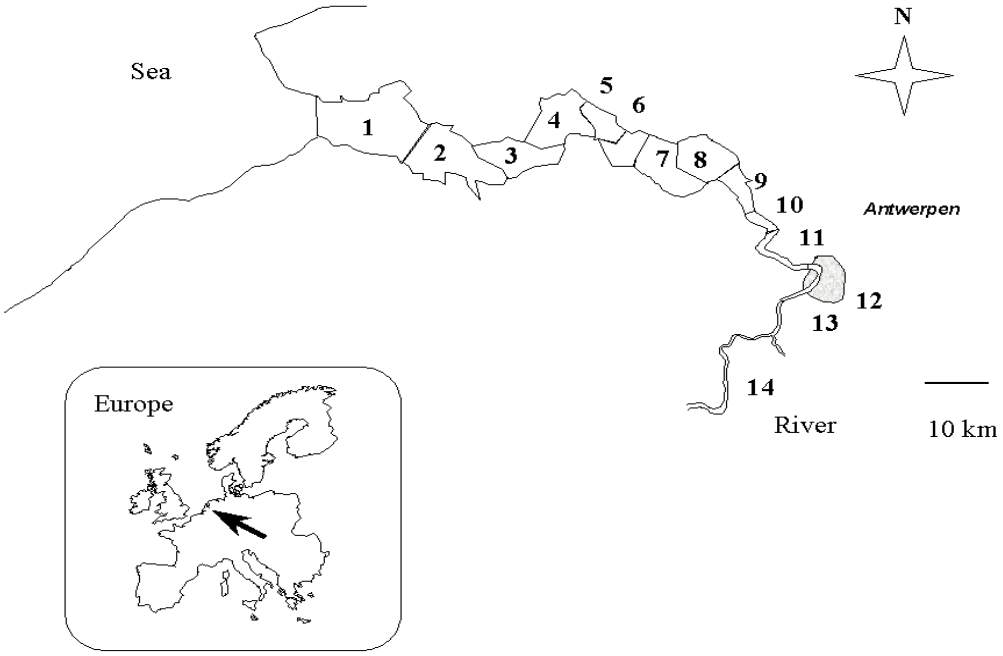

Fig. 1.Map of the Schelde estuary. Compartments are indicated by numbers (see text).

Analysis of N_2O

During cruises with RV 'Luctor', water for N_2O analysis was pumped from a depth of about 1.5 m, while the ship was sailing in the navigation channel. For logistic reasons, N_2O was measured by different methods during the cruises. The results, obtained with the various methods, have been proved to agree within a few percent by simultaneous application of the different methods (De Wilde & Helder 1997; De Wilde & Middelburg, unpublished results). In October 1993 and July 1996, water was continuously sprayed through a headspace of air in an equilibrator (De Wilde & Helder 1997). The dissolved N_2O in the influent of the equilibrator partitions between the water and the headspace according to the N_2O solubility, which is a function of temperature and salinity (Weiss & Price 1980). Analysis of the N_2O concentration in the equilibrator headspace, along with measurements of temperature and salinity, therefore enables calculation of the dissolved gas concentration in the estuary. In October 1993, the air in the equilibrator headspace was sampled and analysed by means of automated gas chromatographic analysis with ECD-detection (Chrompack CP9000), as described by De Wilde & Helder (1997). In July 1996, a photo-acoustic analyser (Bruel and

Kjaer type 1302) was applied to determine the N_2O concentration in the equilibrator headspace (Middelburg et al. 1995; Velthof & Oenema 1995). During the cruise in March 1994, discrete water samples were collected and poisoned with 0.22 volume % saturated $HgCl_2$ solution. All samples were stored under water at about 3 °C. Within 36 hours all samples were measured by headspace equilibration at 3.0 °C and subsequent gas chromatographic analysis, according to the protocol of De Wilde & Helder (1997).

The precision of the various measurements, deduced from repetitive measurement of the same water mass, was about 1.5% for the continuous gas chromatographic analysis, about 2% for the continuous photo-acoustic analysis, and about 2.5 % for the gas chromatographic analysis of discrete samples. The accuracy of the data sets is based on calibration with 1 to 4 standard gases out of a range (0.252, 0.506, 1.475, and 5.0 µmol N_2O per mol synthetic air; Hoekloos and National Institute of Standards), as described in De Wilde & Helder (1997) for the gas chromatographic analysis and in Middelburg et al. (1995) for the photo-acoustic analysis, respectively.

Nitrification activity.

Nitrification activity was measured by two different methods. In October 1994, the ^{14}C-bicarbonate incorporation method was applied (Somville 1978). With this method the growth of autotrophic ammonium oxidising bacteria is estimated by measuring their inorganic carbon incorporation, during incubation with and without a specific inhibitor of nitrification activity. At each station, surface water was collected with a bucket, which was used to fill 4 bottles with 50 ml water. To these bottles, 100 µl of 0.925 MBq ml^{-1} ^{14}C-$NaHCO_3$ was added. Two bottles were treated with 5-10 % (vol/vol) methylfluoride (Air Products), a specific inhibitor of nitrification activity (Miller et al. 1993). Samples were incubated for 2 hours in a rotating incubator in the dark at *in situ* temperature. Filtration and counting of ^{14}C activity were carried out according to the protocol of Kromkamp & Peene (1995). The difference in bicarbonate incorporation between samples treated with methylfluoride and untreated samples was multiplied by a factor 8.3 (moles N/moles C), to achieve nitrification rates expressed in µmol N dm^{-3} h^{-1} (Somville 1984).

In July 1996, at the same time as the N_2O measurements, 500 ml of surface water was incubated in 1 litre flasks on a rotating incubator in the dark at *in situ* temperature. Subsamples, taken at regular intervals for 2 hours, were analysed for ammonium and nitrate.

Nitrification activity was estimated from the initial slope of the ammonium decrease in the incubations.

Ancillary measurements

Salinity, temperature, and oxygen were measured with a CTD system, equipped with a polarographic oxygen sensor (THISHYDRO H2O). In addition, the temperatures of surface water and in the equilibrator system were measured with PT100 sensors with an accuracy better than 0.1 °C, enabling to correct for small solubility changes resulting from warming of the water in the pumping system (De Wilde & Helder 1997). Ammonium and nitrate were analysed with a SKALAR segmented flow autoanalyser system with a precision of approximately 1 %.

Calculation of N_2O emissions

The total N_2O flux from the estuary was calculated by adding up the fluxes from compartments of the estuary. The estuary was divided in 14 compartments with known areas and volumes (Fig. 1), according to the hydrographically based partitioning of Wattel & Schouwenaar (1991). The N_2O exchange between a compartment and the atmosphere was estimated from:

$$F = k \cdot A \cdot (cN_2O - cN_2O_{eq}) \qquad (1)$$

Where F (mol s^{-1}) is the aerial flux between a compartment of the estuary and the atmosphere, k (m s^{-1}) is the gas transfer velocity across the water-air interface, A (m^2) is the area of a compartment at mean tide, cN_2O (mol m^{-3}) the measured N_2O concentration in surface water, and cN_2O_{eq} (mol m^{-3}) the dissolved N_2O concentration, which would have been in equilibrium with the atmosphere. The value of cN_2O_{eq} (nM), was calculated from temperature and salinity according to the relationship of Weiss & Price (1980). The measured local atmospheric N_2O concentration in 1993 and 1996 ranged from 314 to 320 ppb. This variation is insignificant compared to the large supersaturation of the estuarine water. Therefore the atmospheric concentration was assumed to correspond to the global mean atmospheric concentration during the time of each cruise; i.e. about 300 ppb in 1978, and approximately 312-313 ppb in 1993, 1994, and 1996 (Houghton et al. 1996). The transfer

velocity, *k*, was calculated from wind speed, according to the relationship of Clark et al. (1995). The relationship of Clark et al. was applied rather than the often used relationships of Liss & Merlivat (1986) and Wanninkhof (1992), since it contains a non-zero intercept which accounts for gas exchange resulting from tidally induced turbulence in the estuary:

$$k^* = 2.0 + 0.24u^2 \quad (2)$$

Where u (m s^{-1}) is the wind speed (5 days average value prior to each cruise) at a height of 10 m above the water surface. Note that formula (2) gives k* in the dimension (cm hr^{-1}), rather than in (m s^{-1}). Wind speed data from the mouth of the estuary at Vlissingen (Royal Netherlands Meteorological Institute, De Bilt), were used to calculate fluxes in compartments 1 to 8 (Fig. 1). Wind speed data from the upper estuary near Antwerpen (Climatological Survey of Belgium, Bruxelles), were used to calculate fluxes in compartments 9 to 14 (Fig. 1). The value of *k** in formula (2) is valid for CO_2 at 20 °C in fresh water. Values of *k* for N_2O at the local temperature and salinity conditions, are deduced from *k** by applying a correction for the local N_2O diffusivity and water viscosity (Ledwell 1984; Wanninkhof 1992):

$$k = k^* (Sc/600)^{-0.5} \quad (3)$$

Where the Schmidt number, *Sc*, is the ratio of the kinematic viscosity of water over the diffusivity of N_2O. The number 600 refers to the *Sc* value at the reference conditions of CO_2 in fresh water at 20 °C. Local values of *Sc* were calculated from temperature and salinity according to the polynomial fit given by Wanninkhof (1992).

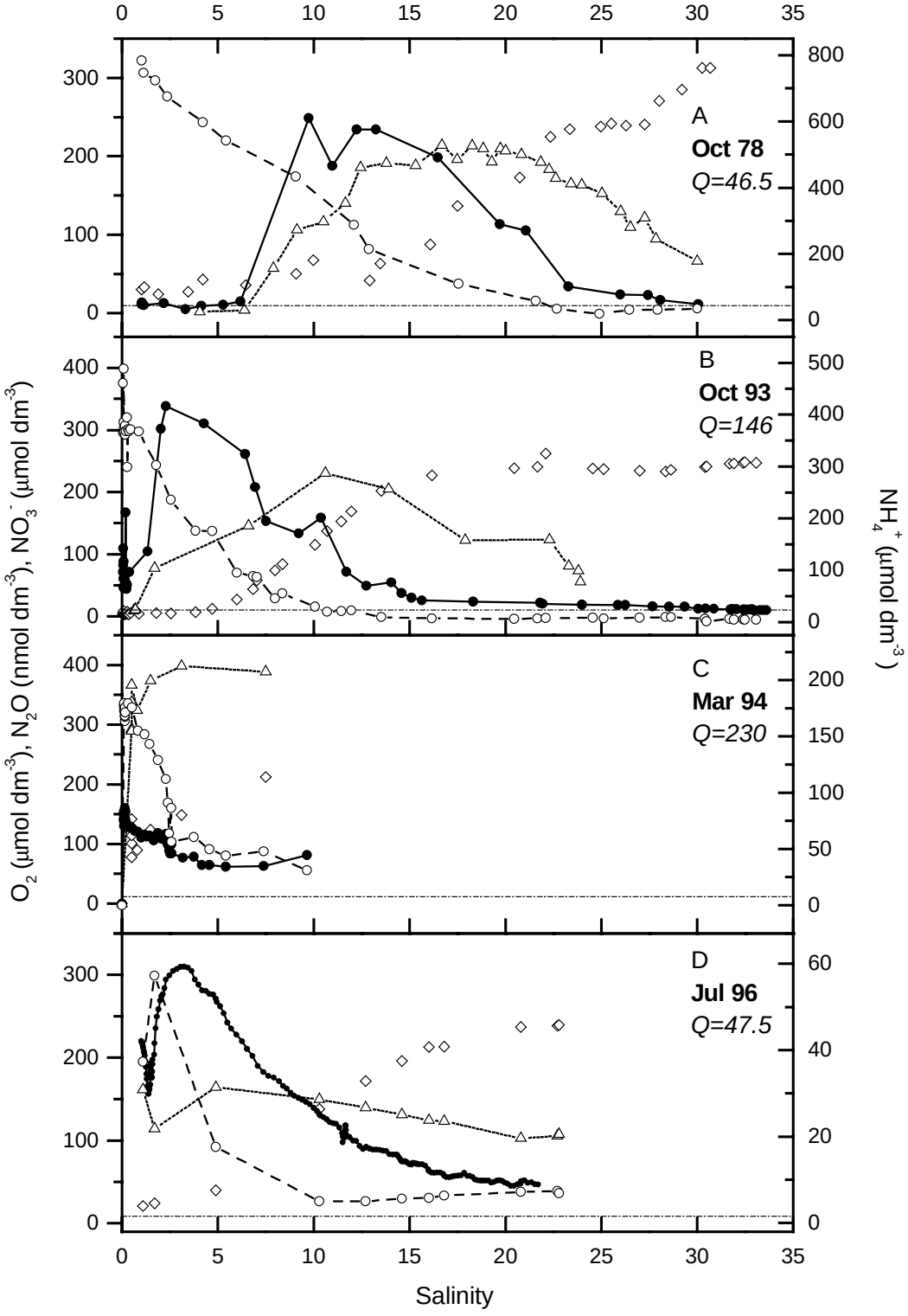

Fig. 2.Distribution of: N_2O (solid circles), O_2 (diamonds), ammonium (open circles), and nitrate (triangles) *versus* salinity; (a) October 1978, (b) October 1993, (c) March 1994, and (d) July 1996. The 1978 data are taken from Deck (1981). Note the different vertical scales for the various years. The dashed-dotted line in each panel indicates the equilibrium saturation of N_2O with respect to the atmospheric concentration. The river discharge, *Q* (m^3s^{-1}), averaged over one week prior to each cruise, is indicated in each panel.

Results

Dissolved N_2O, oxygen, ammonium, and nitrate were measured during October 1993, March 1994, and July 1996. A similar data set was already obtained in October 1978 (Deck 1981). The data from 1978 are presented in the results section of this paper (with permission of the author), in order to allow for optimal comparison with our own data.

Distribution of N_2O along the estuary

The N_2O profiles along the salinity gradient vary from year to year but show the same trends (Fig. 2A-D). All data sets indicate supersaturation with respect to the atmosphere, along the entire salinity gradient of the estuary. In October 1978, a broad N_2O maximum, reaching values up to 250 nmol dm^{-3}, was located between a salinity of 8 and 20 (Fig. 2A). Both downstream and upstream of the maximum, concentrations were close to equilibrium with the atmosphere (about 10 nmol dm^{-3}).

In October 1993, the N_2O peak was very sharp and located far upstream of the position of the 1978 maximum (Fig. 2B). Concentrations increased from about 10 nmol dm^{-3} in coastal waters at salinity 30, to a pronounced maximum of 338 nmol dm^{-3} (31 times supersaturated) around a salinity of 2-3. Upstream of salinity 2, the N_2O concentrations dropped sharply to values around 50 to 100 nmol dm^{-3} (about 4 - 9 times supersaturated).

In March 1994, the N_2O concentration ranged from about 150 nmol dm^{-3} (12 times supersaturated) at the fresh water endmember, down to about 75 nmol dm^{-3} at a salinity of 10 (Fig. 2C). The N_2O peak at salinity 2-3 was very small.

The N_2O distribution in July 1996 (Fig. 2D) compares well to the situation in October 1993 (Fig. 2B). Both data sets show a similarly shaped N_2O peak, located at salinity 2-3. However, in July 1996 the N_2O concentrations upstream and downstream of the maximum, were higher by a factor 2, as compared to the situation in October 1993.

Oxygen

The oxygen concentration along the salinity gradient of the estuary ranged from nearly saturated conditions in the salt water zone at the mouth of the estuary, down to highly undersaturated conditions in the low salinity region (Fig 2A-D). In summer and autumn, oxygen concentrations near the fresh water endmember sometimes reached values below 5

µmol dm^{-3} (Fig. 2B). In early spring, when the river discharge was higher, the oxygen depletion was less severe (Fig. 2C).

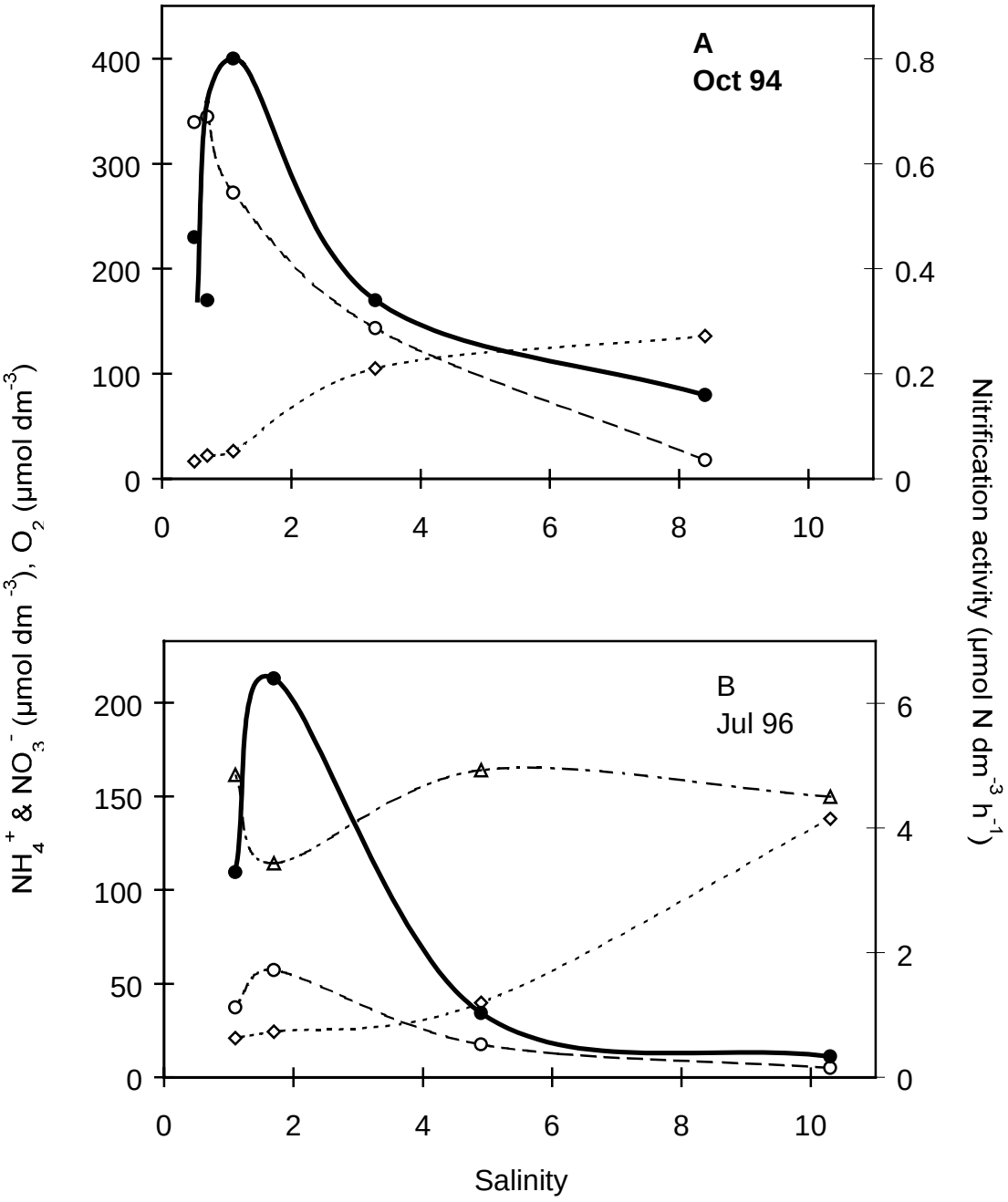

Fig. 3 Distribution of: nitrification activity (solid circles), O_2 (diamonds), ammonium (open circles), and, only for panel b, nitrate (triangles) *versus* salinity; (a) October 1994, (b) July 1996. Lines are added for clarity.

Ammonium and nitrate

Ammonium concentrations were highest at the fresh water endmember (Fig 2A-D). Slightly downstream, ammonium concentrations rapidly dropped, coinciding with an increase of concentrations of N_2O and nitrate. After the largest ammonium drop, oxygen concentrations increased in the seaward direction. Since 1978, the observed riverine ammonium concentration has decreased substantially. The ammonium concentration at the upper limit of the estuary amounted nearly 800 µmol dm^{-3} in October 1978 (Fig. 2A), whereas in October 1993 (Fig. 2B) this value had dropped to about 400 µmol dm^{-3}. During March 1994 and July 1996, the observed ammonium concentration had further decreased to values around 175 µmol dm^{-3} (Fig. 2C) and 55 µmol dm^{-3} (Fig. 2D), respectively.

The nitrate distribution along the Schelde was always characterised by a maximum in the middle or upper estuary (Fig. 2A-D). Maximum nitrate values were about 200 µmol dm^{-3}, except for March 1994 when oxygen depletion was limited and the nitrate concentration reached values up to 400 µmol dm^{-3} in the upper part of the estuary. During all cruises, nitrate concentrations gradually decreased in the lower estuary, due to dilution with seawater with a lower nitrate content.

Nitrification activity

The nitrification activity along the estuary showed a clear peak around a salinity of 1-2 (Fig. 3A-B). The highest nitrification activity in July 1996 (Fig. 3B) coincided with a decreasing ammonium concentration as well as with increasing concentrations of N_2O, nitrate and oxygen (Fig. 2D). In July 1996 nitrification activities were nearly an order of magnitude higher than in October 1994. The nitrification activity reached maximum values of 0.8 µmol N dm^{-3} h^{-1} during October 1994, and 6.4 µmol N dm^{-3} h^{-1} during July 1996 (Fig. 3A-B).

Discussion

Nitrification and N_2O

The profiles of N_2O, nitrification activity, oxygen, ammonium, and nitrate *versus* salinity, suggest that a major part of the N_2O production in the Schelde results from nitrification. In the upper estuary, a decrease of the ammonium concentration in the seaward direction coincided with a sharp increase of the N_2O concentration (Fig. 2). Slightly more downstream,

concentrations of nitrate and oxygen increased too. This pattern suggests that ammonium oxidation by nitrifying bacteria resulted in the subsequent production of N_2O and nitrate. A plot of the pelagic nitrification activity *versus* salinity supports this conclusion (Fig. 3). The location of the maximum nitrification activity at salinity 1-2, virtually coincided with the location of the N_2O maximum in 1996 (Fig 2). It is therefore likely that most of the N_2O production in the estuary resulted from nitrification in the water column. The role of benthic nitrification is probably limited since the N_2O maximum moves back and forth with the tide, rather than being associated with a certain geographical position. In line with this observation, Billen (1975) and Somville (1984) reported that nitrate production in the water column is higher by about one order of magnitude than nitrate production in the underlying sediment. In contrast, N_2O production in other estuaries has generally been attributed to the sediments (Bange et al. 1996; Butler et al. 1987; Law et al. 1992; McElroy et al. 1978; Robinson et al. 1998; Seitzinger 1988).

The nitrification activities observed in July 1996 were an order of magnitude higher than the activities measured at the end of October 1994. High nitrification rates are usually reported for the summer season (Berounsky & Nixon 1993; McElroy et al. 1978). The observed differences in nitrification activity between October 1994 and July 1996 could partly result from the application of different methods during these cruises (see Methods). The bicarbonate incubation method, used in October 1994, depends on the factor that converts incorporated carbon to nitrogen oxidised. The conversion factor, however, may vary with different bacterial growth rates and nutrient conditions. We used a conversion factor of 8.3 (moles N/moles C) on the basis of extensive research in the Schelde by Somville (1984). In order to avoid the uncertainties associated with the conversion factor, ammonium decrease resulting from nitrification was measured directly during the cruise in 1996. Since part of the ammonium consumption could theoretically result from other processes, this latter method possibly gives an upper limit. Potential bias associated with both methods is probably limited, since previous nitrification measurements in the Schelde, using the same technique as we did in October 1994, support our observations and fit within the apparent seasonal trend. Compared to nitrification measurements in the Schelde during September 1977 (Somville 1984), the activity measured at the end of October 1994 was about 5 times lower, whereas the activity in July 1996 was about twice as high.

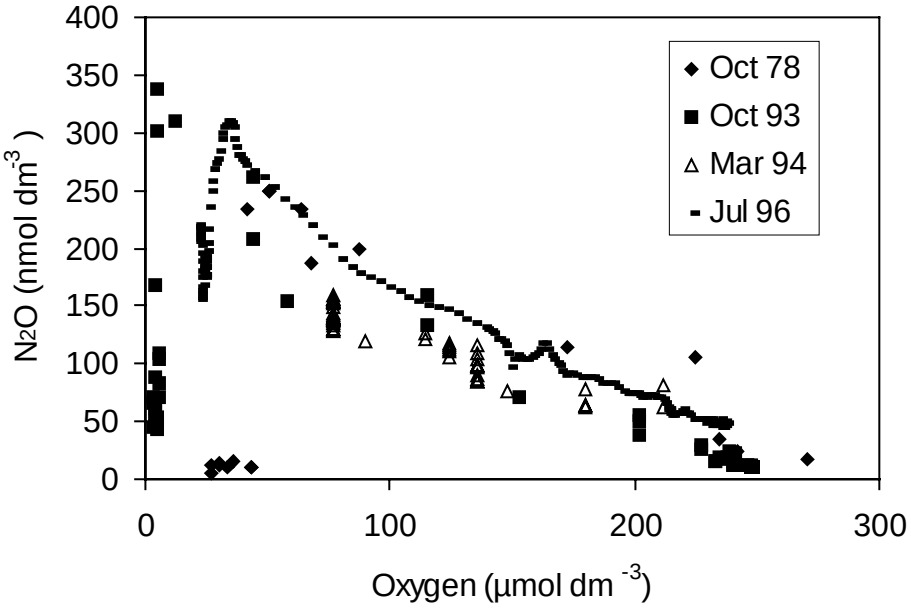

Fig. 4 Relationship between N_2O and O_2 for the data sets of October 1978, October 1993, March 1994, and July 1996. The 1978 data are taken from Deck (1981).

The role of oxygen

The river water entering the estuary is severely depleted in oxygen as a result of strong mineralisation of organic matter (Frankignoulle et al. 1996). In contrast, the coastal waters are oxygen saturated (Fig. 2). The resulting oxygen gradient along the estuary controls the occurrence and intensity of the processes involved in N_2O production. In Figure 4, N_2O is plotted *versus* oxygen for all 4 data sets available. N_2O and oxygen appear to be closely related, despite the fact that their residence times in the water column may differ. Going from high to low oxygen concentrations, Fig. 4 can be divided in three zones with a different relationship between N_2O and oxygen:

1) At oxygen concentrations in excess of about 70 µmol dm^{-3}, N_2O is linearly related to oxygen, suggesting that in this part of the estuary the N_2O concentration can be predicted from the oxygen concentration. The mean slope of the regression lines, corresponding to the four data sets plotted in Fig. 4, is -0.91 nmol N_2O/µmol oxygen ($r^2 = 0.93$).

2) At oxygen concentrations decreasing to values below about 70 µmol dm^{-3}, the ratio between N_2O and oxygen tends to increase (Fig. 4). This observation can be explained by an increased N_2O yield during incomplete nitrification at low oxygen concentrations. A similar increase of the N_2O yield during nitrification at low O_2 concentrations has been reported for the open ocean (Cohen & Gordon 1978; De Wilde & Helder 1997), estuaries (McElroy et al. 1978; Deck 1981), as well as in laboratory studies (Goreau et al. 1980).

3) At oxygen concentrations below an apparent threshold value, ranging from 5 - 40 µmol dm^{-3}, the N_2O/O_2 ratio drops to very low values, indicating that N_2O production in the corresponding water mass is small. It is not completely clear why the minimum oxygen concentration required for N_2O production, ranged from 5 µmol dm^{-3} in 1993 up to 40 µmol dm^{-3} in 1978. Apart from the bulk oxygen concentration, nitrification is probably also influenced by other factors such as salinity, temperature, or the composition and size of suspended particles.

Additional N_2O sources

Apart from nitrification, the N_2O production in the Schelde estuary may concurrently be influenced by several other factors, including denitrification in the water column or sediments or production within anoxic microsites like flocs. Furthermore N_2O may be supplied by production in intertidal flats or external sources such as N_2O-rich tributaries or supply by groundwaters. The mentioned possibilities are discussed hereafter.

a) Denitrification. High denitrification rates in the sediments and the water column of the Schelde during the years 1975-1982 have been reported by Billen et al. (1985). In a model study Soetaert & Herman (1995b) indicated that the largest amount of nitrogen in the Schelde is lost by pelagic rather than by benthic denitrification. However, this model was calibrated with data from the years 1980-1986, when the O_2 concentration in the upper estuary was still low, compared to more recent conditions (Van Damme et al. 1995). At present, denitrification in the estuarine part of the Schelde is rare (Regnier et al. 1997). Denitrification in the riverine part of the Schelde, however, may result in substantial N_2O production. Part of this N_2O escapes from the river to the atmosphere, whereas the remaining N_2O may contribute to the relatively high N_2O concentration at the upper limit of the estuary (Fig. 2B-D).

b) Anoxic microsites. Nitrifying bacteria mostly occur attached to particles (Stehr et al. 1995). Particle aggregates provide microsites in which oxygen concentrations are usually lower than in the surrounding water (Plough et al. 1997). In addition, increasing particle concentrations may influence N_2O production simply by increasing the nitrifying biomass (Owens 1986). Since the concentration of suspended particles is as high as 25 - 200 mg dm^{-3} in the entire upper estuary (Paucot & Wollast 1997; Zwolsman et al. 1997), suspended particles may enhance pelagic N_2O production in the Schelde. It is unlikely, however, that the N_2O maximum is closely related to a maximum in particle density. Particle density generally is highest upstream of salinity 4 (Wollast 1988), whereas the N_2O maximum in 1978 was located far more downstream.

c) *Intertidal mud flats.* Middelburg et al. (1995) quantified the N_2O flux between the intertidal mud flats in the Schelde estuary and the atmosphere. The mean N_2O loss to the atmosphere ranged from about 14 µmol m^{-2} day^{-1} in the fresh water part of the estuary up to almost zero at a salinity of 27. These fluxes are lower by 1 to 2 orders of magnitude as compared to the N_2O emission from the water surface of the Schelde estuary.

d) Contribution of tributaries. The most important tributary, the Rupel, joins the Schelde about 90 km from sea. The N_2O concentrations at the mouth of the Rupel were always comparable to the concentrations in the adjacent waters of the Schelde. In October 1993, similar N_2O concentrations were measured on both sides of the entire lower estuary, showing minor influence of tributaries.

e) Drainage water and ground water. Drainage water from agricultural land can contain high concentrations of N_2O (Dowdell et al. 1979). Since the Schelde traverses an intensively fertilised agricultural region, it is likely that drainage waters are a source of N_2O to the Schelde. Similarly, ground waters and aquifers, are known to contain considerable amounts of N_2O (Ronen et al. 1988), and therefore may be a source of N_2O to the estuary. The contribution by both sources is unknown, however. The largest contribution is expected upstream of the freshwater endmember.

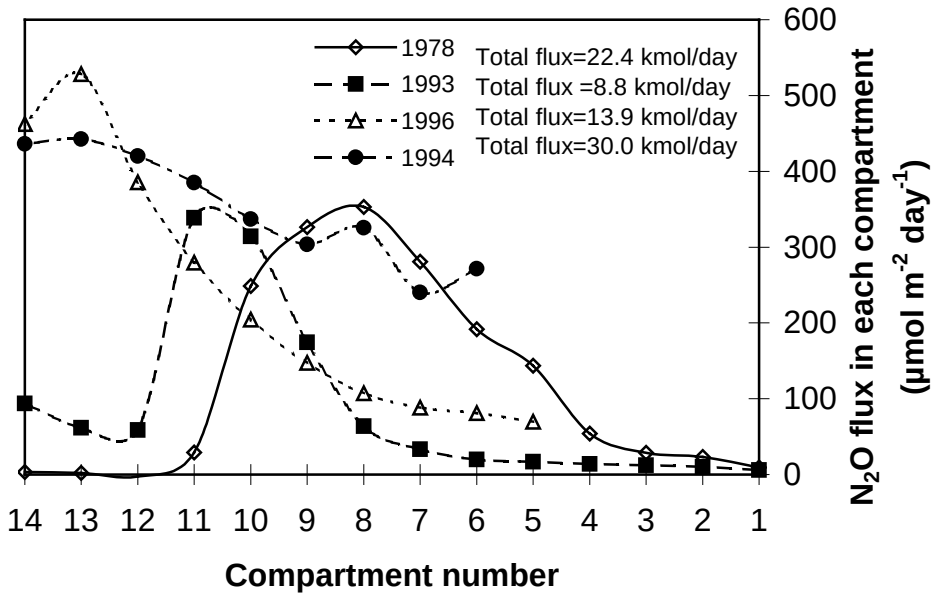

Fig. 5 N_2O flux in each compartment during October 1978, October 1993, March 1994, and July 1996. The total flux from the entire estuary during each cruise is indicated in the legend. The 1978 fluxes were calculated from the data of Deck (1981).

Long term trend

The Schelde is characterised by substantial seasonal variation in discharge, temperature, and wind speed (this study; Zwolsman et al. 1997; Regnier et al. 1997; Somville & De Pauw1982; Billen 1975; Van Damme et al. 1995). In response, the distribution of oxygen, N_2O, nutrients, and other parameters varies too. A compilation of several data sets clearly indicates that the oxygen-depleted zone in the Schelde has shifted in upstream direction during the past two decades (Van Damme et al. 1995). During the same time span the ammonium concentration at the fresh water endmember has decreased by about a factor two, whereas the nitrate concentration has roughly doubled (Somville 1984; Van Damme et al. 1995). Since the N_2O production is largely controlled by the local oxygen concentration, it is expected that the N_2O production has decreased and moved upstream during the last two decades. The present situation (Fig 2B-D) can only be compared with the data collected by Deck (1981) in October 1978 (Fig 2A), since to our knowledge no other data exist for the Schelde. Despite potential bias by short term variability, the expected decadal change in the N_2O distribution is supported by the available data. In 1978 the N_2O peak was located around a salinity of 10 (Fig. 2A), whereas the measurements in 1993, 1994 and 1996 show that the

N_2O maximum was located around a salinity of 2-3 (Fig. 2B-D). This upstream shift along the salinity gradient corresponds to a distance of about 20 km.

N_2O emission to the atmosphere

The profiles of N_2O *versus* salinity (Fig. 2) are characterised by concave curves, downstream of the N_2O peak. The concave profile indicates that N_2O in the estuary decreases faster than would be expected from conservative mixing with the N_2O-poor coastal waters. It is unlikely that N_2O is consumed at oxygen concentrations above approximately 2 µmol dm^{-3} (Codispoti & Christensen 1985). Consequently, the N_2O decrease results mainly from gas emission to the atmosphere. The rate of gas exchange with the atmosphere is largely determined by wind speed (Liss & Merlivat 1986; Wanninkhof 1992). In tidal waters, however, gas exchange at wind speeds below about 3 m s^{-1} is generally controlled by tidally induced turbulence (Clark et al. 1995).

The mean N_2O flux in each compartment, calculated according to formulas 1, 2, and 3, is given in Fig. 5 for all cruises. The total daily N_2O flux from the entire estuary, during each cruise, is listed in the legend of Figure 5. The data sets of 1994 and 1996 did not cover the entire lower estuary. The fluxes from the missing compartments were estimated by linear extrapolation of the N_2O flux towards zero, i.e. atmospheric N_2O saturation, in compartment (1) at the mouth of the estuary.

High N_2O emissions were calculated for October 1978 and March 1994 (22 and 30 kmol day^{-1}, respectively), whereas lower emissions were calculated for October 1993 and July 1996 (9 and 14 kmol day^{-1} respectively). The high N_2O emission in October 1978 was expected, since the zone with a high N_2O concentration (Fig. 2) covers a large area in the broad middle estuary, corresponding to compartments 5 to 10 (Fig. 1). In contrast, the high emission in March 1994 largely resulted from high wind speed. In March 1994 the wind speed at the mouth of the estuary (Vlissingen), averaged over 5 days prior to the cruise, amounted 10.0 ms^{-1}, as compared to wind speeds of 5.3, 4.9, and 5.5 ms^{-1} in October 1978, October 1993, and July 1996, respectively. Because gas exchange is approximately proportional to the square of the wind speed (Formula 2), the relatively low N_2O concentrations in March 1994 (Fig. 2C) corresponded to a high N_2O emission (Fig. 5).

Based on the average of the estimated emissions during the cruises in October 1993, March 1994, and July 1996 (Fig. 5), we estimated the N_2O flux from the Schelde in recent years to be 6.4 x 10^6 mol $year^{-1}$, which equals 2.8 x 10^8 g $year^{-1}$. Given the strong variability observed, our estimate for the annual N_2O emission from the Schelde contains considerable uncertainty. Further constraining the magnitude of the N_2O emission from the Schelde estuary would require frequent measurements with high spatial coverage. In addition it is to be realised that windspeed and the N_2O concentration are strongly coupled. High wind speeds will result in rapid ventilation of the dissolved N_2O to the atmosphere and consequently decreasing N_2O concentrations in the estuary. In contrast, low wind speeds will result in a low ventilation rate and accumulation of the produced N_2O. The use of instantaneous or daily wind speeds for flux calculations can lead to large uncertainties, because the wind speed can change much faster than the N_2O concentration in the estuary. Therefore, the 'history' of the wind speed, during the time prior to the measurements, should be taken into account when calculating fluxes from local wind speeds. As a compromise we used the 5 days mean wind speed prior to each cruise, since this value corresponded to the mean ventilation time of the water column, i.e. the ratio between the water depth and the average transfer velocity. The N_2O ventilation time of the water column is short, relative to the residence time of about 2 months of the water mass associated with the highest N_2O production at a salinity of 2-3 (Soetaert and Herman 1995a). Consequently, nearly all N_2O is lost to the atmosphere within the estuary, rather than being transported to the open sea.

N_2O yield

The yield of N_2O during nitrification can be defined as the ratio of N_2O production relative to ammonium consumption. We estimated the N_2O yield in compartments 11 to 14 during July 1996 by assuming that (1) pelagic nitrification is the main N_2O source, and (2) N_2O emission to the atmosphere in a compartment is larger than gas exchange with the adjacent compartments. Given these assumptions, we calculated the N_2O yield for each compartment from the ratio of N_2O emission (Fig. 5) on the one hand and the product of nitrification activity (Fig. 3B) and compartment volume on the other hand. he N_2O yield in compartments 11, 12, 13, and 14 amounted 0.42, 0.15, 0.04, and 0.07% (mol N_2O per mol NH_4^+), respectively, coinciding with oxygen concentrations of 138, 40, 24, and 21 µmol dm^{-}

^{3}. These N_2O yields correspond to the production of 1 molecule of N_2O during the oxidation of 240 to 2500 moles of NH_4^+. Our crude estimate of the N_2O yield in the Schelde is in good agreement with the limited values published by others. Elkins et al. (1978) reported a mean N_2O yield of 0.14 % (mol N_2O per mol NH_4^+) during nitrification in freshwater and saltwater systems. De Wilde & Helder (1997) reported N_2O yields of 0.15 to 0.18 % (mol N_2O per mol NH_4^+) for the Indian Ocean (Somali Basin). For cultures of nitrifying bacteria, N_2O yields range from about 0.15% (mol N_2O per mol of NH_4^+) at oxygen saturated conditions, up to nearly 5% at an oxygen concentration of 5.6 µmol dm^{-3} (Goreau et al. 1980).

Global extrapolation

Recent measurements in the framework of the BIOGEST project indicated that the N_2O concentration in the Schelde is roughly 2-8 times higher than in other European estuaries, such as the Rhine, Gironde, Eems, Douro, and Sado (De Wilde, unpublished data). A comparison with 10 other estuaries, as summarised by Bange et al. (1996), confirms that the N_2O concentration in the Schelde is relatively high. Therefore, extrapolation of the N_2O emission from the Schelde to a global scale will give an indication of the upper limit of the total estuarine N_2O emission to the atmosphere. We extrapolated the emission from the Schelde on the basis of aerial coverage at mean tide. Based on the area of the Schelde (262 x 10^6 m^2, Wattel & Schouwenaar 1991), relative to the total area covered by estuaries (1.4 x 10^{12} m^2, Whittaker & Likens 1975), the total estuarine flux was estimated to be 1.5 Tg yr^{-1}. Our estimate is low as compared to the total estuarine N_2O emission of 3.7 to 5.7 Tg yr^{-1}, as reported by Bange et al. (1996). Since we consider our estimate to be an upper limit, the value reported by Bange et al. (1996) seems rather high. Our upper limit fits better to the reported total estuarine N_2O emission of 0.11 to 1.1 Tg yr^{-1} by Seitzinger & Kroeze (1998). Regardless of the exact magnitude of the total estuarine emission, it is clear that estuaries are an important N_2O source relative to their aerial coverage. The oceans are estimated to emit 6.3 ± 4.4 Tg N_2O yr^{-1} (Nevison et al. 1995) from an area of 361 x 10^{12} m^2 (Chester 1990). Consequently, per unit area, the estuarine N_2O emission is higher by about two orders of magnitude than the oceanic N_2O emission. In contrast, the few emissions reported for freshwater systems so far (e.g. McMahon & Dennehy 1999; Mengis et al. 1997), generally fall in the range of estuarine emissions.

The quantification of the N_2O emission from estuaries could be improved by using knowledge on the factors that control the N_2O emission in estuarine ecosystems. This approach is not affected by uncertainties in the relationship between wind speed and gas exchange. Firestone & Davidson (1989), pointed out that the turnover rate of nitrogen and carbon in ecosystems is positively related to N_2O production. Matson & Vitousek (1987) found a relationship between soil fertility and N_2O emission from tropical forests. Subsequently, Matson & Vitousek (1990) used soil fertility as a controlling factor to estimate the global N_2O budget of tropical forests. Likewise, the factors that control the N_2O emission in estuarine ecosystems could be used to improve the estuarine N_2O budget. The main factor controlling the N_2O emission from estuarine ecosystems appears to be the nitrogen load (Seitzinger & Nixon 1985; Seitzinger 1988; Middelburg et al. 1995). However, the coupling between N-loading and the resulting N_2O emission is variable and difficult to constrain, since estuaries are open and dynamic ecosystems. Consequently, Seitzinger & Kroeze (1998) had to make several assumptions when estimating the total N_2O emission from rivers and estuaries on the basis of external nitrogen inputs.

In the Schelde estuary, riverine NH_4^+ appears to be an important factor controlling the N_2O production. A comparison between October 1978 and October 1993 indicates that a decrease of 41% of the NH_4^+ concentration of the river water entering the estuary corresponded to a decrease of 39% of the total amount of N_2O in the estuary. Additional field work is required to improve our understanding of the factors controlling N_2O production in the Schelde and other estuaries. In this way the global estuarine N_2O budget can be improved and the effect of future changes in nitrogen loading can be predicted.

Acknowledgements

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C H A P T E R 5

Factors controlling nitrous oxide at the microbial community and estuarine scale

Monique J. M. de Bie, Jack J. Middelburg, Mathieu Starink, and Hendrikus.J. Laanbroek

This paper examines the effect of oxygen on nitrous oxide concentrations in estuarine waters. Nitrous oxide has been measured year-round in the Schelde estuary, a high-nitrogen, low-oxygen macrotidal system. Nitrous oxide concentrations were above atmospheric equilibrium levels indicating that this estuary represents a source to the atmosphere. The distribution of nitrous oxide showed consistent and systematic relationships with distribution patterns of ammonium, oxygen, nitrite and nitrification activities. A controlled laboratory experiment with a natural bacterial community from the Schelde estuary revealed maximum nitrous oxide production to occur at oxygen concentrations of about 5 µM. This production was inhibited by acetylene, a nitrification inhibitor. Maximum nitrous oxide concentration in the field occurred at oxygen concentration below 35 µM. It appears that low oxygen concentrations in estuarine water trigger nitrous oxide production, if ammonium is present in sufficient amount. This conclusion is further illustrated by data from the Thames, Loire and Gironde estuaries.

Submitted for publication

Introduction

Nitrous oxide (N_2O) affects the global climate in two ways (Bange 2000). In the lower atmosphere N_2O acts as a greenhouse gas that is 200-300 times more powerful in warming potential than CO_2. Nitrous oxide is chemically stable in the troposphere and reaches the stratosphere where it forms NO radicals in photochemical reactions that are involved in the destruction of ozone. In this way, N_2O contributes indirectly to the destruction of the ozone layer. Atmospheric mixing ratios of N_2O have been increasing steadily during the past 100 years (Battle et al. 1996). The accumulation of the gas in the atmosphere is the result of unbalanced sources and sinks of N_2O. Aquatic systems, both salt and fresh water, contribute 25-30 % of the total global N_2O emission (IPCC 1996). Estuaries are calculated to account for approximately 60% of total marine nitrous oxide production/emission (Bange et al. 1996). In order to understand the biogeochemistry of N_2O in estuaries, both quantification of N_2O concentrations and fluxes, and understanding of factors controlling the production of N_2O are necessary.

In aquatic systems nitrous oxide can be produced during both nitrification and denitrification. The first step of nitrification, the oxidation of ammonia to nitrite, can be a source of nitrous oxide, in particular under low oxygen concentrations (Anderson & Levine 1986, Goreau et al. 1980, Jørgensen et al. 1984, Kester et al. 1997, Stüven et al. 1992). The concentration range of oxygen, under which significant quantities of nitrous oxide are produced, is very narrow. At oxygen concentrations higher than this critical range, oxidation to nitrite and subsequently nitrate is completed, while the ammonium oxidation reaction is not occurring at all at oxygen concentrations below the range for nitrous oxide production. Besides this oxidative pathway, nitrous oxide may also be formed in the reductive process of denitrification. During denitrification nitrate is reduced to molecular nitrogen gas (N_2) via the intermediates NO_2, NO and N_2O. Nitrous oxide reduction is, at least in some denitrifying species, more sensitive to oxygen than the other reduction steps (Betlach & Tiedje 1981, Zumft & Kroneck 1990). This can lead to accumulation of nitrous oxide at low oxygen concentrations. Oxygen concentration apparently has a strong regulating influence on nitrous oxide production. Additionally, ammonia concentration, nitrite concentration, pH, the physical environment and the physiological characteristics of the microbial community determine the amount and extent of the eventual emission of nitrous oxide to the atmosphere. The overall factor controlling N_2O production and emission seems to be dependent on the specific conditions and scale of the system studied.

At the global scale nitrous oxide production and emission from aquatic systems is correlated to N-loading (Seitzinger & Kroeze 1998), as illustrated by the observation that 90% of the nitrous oxide emission of rivers and estuaries is in the northern hemisphere. Similarly, the main factor controlling N_2O emission from estuarine sediments is nitrogen load (Middelburg et al. 1995, Seitzinger & Nixon 1985).

At the level of isolated populations (Anderson & Levine 1986, Anderson et al. 1993, Goreau et al. 1980) and natural communities (Jørgensen et al. 1984) oxygen is governing nitrous oxide yield during nitrification and denitrification. Consistent with these laboratory studies, Yoh et al. (1983) reported accumulation of nitrous oxide in oxygen deficient layers of freshwater lakes and Naqvi et al. (2000) reported enhanced nitrous oxide production in low oxygen zone of the Indian continental shelf. There appear to be different controlling factors of nitrous oxide production at different scales, with nitrogen loading governing nitrous oxide at the global scale and across ecosystem scales, and oxygen being the key factor in laboratory studies and within individual aquatic ecosystems.

In this study, we present a seasonal study of nitrous oxide concentrations in the Schelde estuary, a high-nitrogen, low-oxygen macrotidal system. Furthermore, a natural community from the Schelde estuary was used in controlled laboratory experiments to identify the controlling factor of N_2O-production. These field and experimental data will be used to argue that oxygen is the controlling factor provided ammonium is abundant. Finally, we present dissolved nitrous oxide data for a number of additional macrotidal European estuaries (Thames, Loire and Gironde) to strengthen our arguments.

Material and Methods

Research areas

Samples were collected in the context of BIOGEST, a EU-supported programme on biogeochemical processes and trace-gas production in a number of European tidal estuaries (Middelburg et al. 2001). The Schelde estuary (also known as the ‘Westerschelde”) drains an estimated 21.000 km^2 of Northern France, Belgium and the Netherlands, an area with approximately 10 million inhabitants and a nitrogen load of $5 * 10^9$ mol N yr^{-1} (Soetaert & Herman 1995). Turbidity is high in the entire upper estuary, which is illustrated by the observed suspended matter concentrations (10-350 mg l^{-1}). The Thames estuary is a turbid, tidal estuary on the east coast of the United Kingdom, entering the North Sea at Southend on

Sea. The drainage area of the river Thames is rather small (14000 km^2) but hosts a population of about 12 million, including London and has a dissolved inorganic nitrogen load of 2.4 * 10^9 mol N yr^{-1} (Trimmer et al. 2000). The river Loire drains a major part of central France and its estuary is well mixed and very turbid with suspended matter concentrations over 1 g l^{-1}. The rivers Garonne and Dordogne that drain a large part of south-western France feed the Gironde estuary. It is a well-mixed, highly turbid estuary with suspended matter concentrations over 1 g l^{-1}. The Thames, Loire and Gironde estuaries have been studied only once each in February 1999, September 1998 and June 1997, respectively. The Schelde estuary has been examined at monthly resolution from April 1997 to April 1998.

N_2O measurements

Dissolved nitrous oxide concentrations were measured in surface waters while sailing or on station using a continuous flow gas equilibrator connected to a photoacoustic gas analyzer (Bruel & Kjaer type 1302). This method has been described elsewhere (Abril et al. 2000, Middelburg et al. 2001, De Wilde & De Bie 2000). Headspace gas concentrations were recalculated to water concentrations using the temperature and salinity dependent partitioning coefficient (Weiss & Price 1980). The precision of dissolved nitrous oxide concentrations was better than 3 % and the accuracy is based on calibration with certified standards traceable to the US National Institute of Standards.

Ancillary measurements

Salinity, temperature, and oxygen were either measured with a CTD system, equipped with a polarographic oxygen sensor (THISHYDRO H2O) or obtained from Middelburg & Nieuwenhuize (2000) and Middelburg et al. (2001). Nutrients have been measured using automated colorimetric techniques (Middelburg & Nieuwenhuize 2000, De Wilde & De Bie 2000). Nitrification activity was measured by the ^{14}C-bicarbonate incorporation method (Somville 1978). Briefly, carbon fixation by autotrophic ammonia-oxidising bacteria is quantified by the difference of inorganic carbon incorporation during incubation with and without N-serve, a specific inhibitor of nitrification activity. These data are discussed in detail by de Bie et al. (2001).

Incubation experiments

Estuarine water with a salinity of 1 from the upper part of the Schelde estuary was collected in April 1998 and subsequently incubated in a 4 liter glass incubation vessel that was kept at *in situ* temperature (10 ^{o}C) by a cooling spiral connected to a water bath. The water was stirred by a magnetic stirrer and continuously bubbled with a gas mixture, of which the composition was accurately controlled by mass flow control valves (Brooks Instruments) connected to N_2, air and acetylene bottles. Oxygen concentrations in the vessel were monitored by an oxygen electrode (ECOLAB, Maarssen, the Netherlands). Nitrous oxide concentrations were monitored in the headspace using the photoacoustic gasanalyzer that was connected in a closed circuit to the vessel so that no gas exchange with air outside the experimental setup occurred during sampling. Water samples could be taken by means of a syringe in the vessel lid, where a gas tight valve prevented gas exchange.

At the start of the experiment, ammonium was added to a final concentration of 2 mM and the vessel was flushed with air until 100% air saturation and a steady state in nitrous oxide was reached. After that, oxygen concentration was stepwise brought down to 70, 40, 20, 10, 5, 1, 0 % air saturation respectively by adjusting the incoming gas mixture until a new steady state in N_2O concentration was established. Samples for ammonium, nitrite and nitrate were taken regularly.

The experiment was repeated as described above, with the addition of 1 % acetylene (C_2H_2) to the incoming gas mixture. Acetylene was purified by passing it through a trap containing sulfuric acid followed by a trap containing 5M NaOH according to Hyman & Arp (1987). Acetylene is a known inhibitor of nitrification at low concentrations. At higher concentrations (10% v/v) it also inhibits the last step of denitrification, resulting in the accumulation of N_2O.

Results

Experiments

Experiments with the natural community from the upper Schelde estuary revealed that nitrous oxide was predominantly formed at low oxygen concentrations. Nitrous oxide concentrations were maximal at an oxygen concentration of about 5 µM (0.5-2 % air saturation) (Fig. 1). At lower oxygen concentrations, nitrous oxide concentration decreased,

although some nitrous oxide was produced even when oxygen was completely absent. In the presence of acetylene, there was no maximum in nitrous oxide production at oxygen levels of about 5 µM oxygen. However, high nitrous oxide concentrations were observed under anoxic conditions. Acetylene is an inhibitor of the firs step of nitrification (ammonium oxidation) and the last step of denitrification (nitrous oxide reduction to dinitrogen). The absence of nitrous oxide production at low, non-zero oxygen in the presence of the nitrification inhibitor acetylene provides evidence that nitrification is the major source of nitrous oxide in this experiment and, since we used a natural bacterial population, also in the Schelde estuary. The low nitrous oxide production in the absence of oxygen without acetylene strengthens this conclusion. The importance of nitrification was further illustrated by the immediate increase in production of nitrous oxide upon addition of ammonium (data not shown).

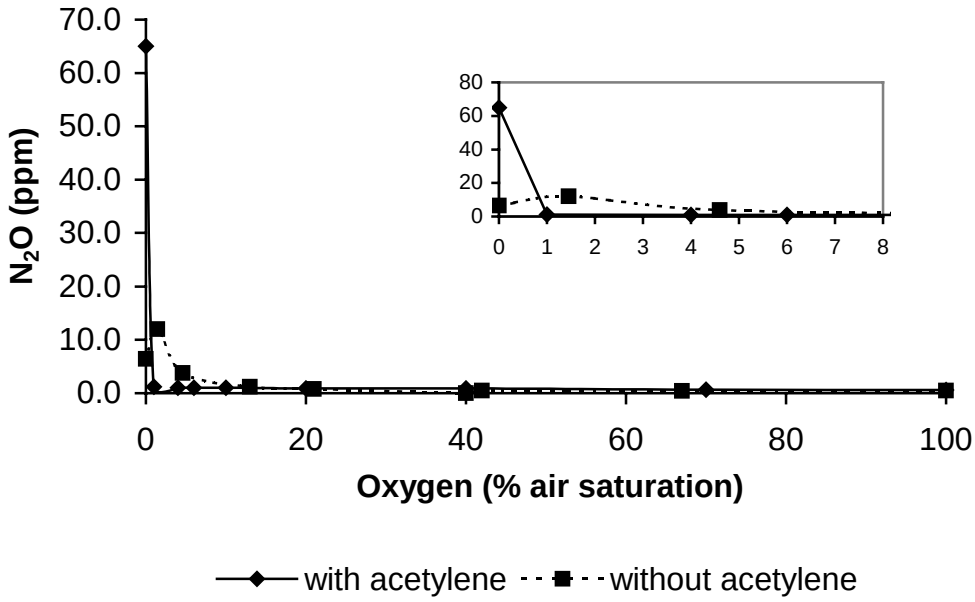

Fig. 1. Steady state nitrous oxide concentrations at different oxygen concentrations in the laboratory experiment with natural community. The inset shows a blow-up of the data in the oxygen range between 0 and 8 % air saturation.

Field observations

The Schelde estuary is a highly heterotrophic system with low oxygen levels and high nitrogen loading. Figure 2 shows a detailed set of logitudinal profiles for April 1997. Ammonium concentrations were highest in the river Schelde (417 µM) and rapidly decreased with increasing salinity (Fig. 2A). This decrease in ammonium concentration was accompanied with a proportional increase in nitrate from 146 µM in the river to a maximum

of 429 μM at salinity 7.6 (Fig. 2B). At higher salinities, nitrate concentrations decreased again due to dilution with relatively nitrate-poor North Sea water. Nitrification activity was highest in the river 0.025 μmol C l^{-1} h^{-1} and increased up to 0.055 μmol C l^{-1} h^{-1} at salinity 7.6. Nitrification activities rapidly dropped further downstream (Fig. 2C). Oxygen saturation levels were below 20 % in the low salinity zone with high nitrification activities, while oxygen was supersaturated in the lower estuary due to a spring bloom. Nitrification of ammonium to nitrate in the low salinity, low-oxygen zone was likely incomplete because there was significant production of nitrite (uniform concentration of 15 μM; Fig. 2B) and nitrous oxide (245 nM at salinity 7.6; Fig. 2A).

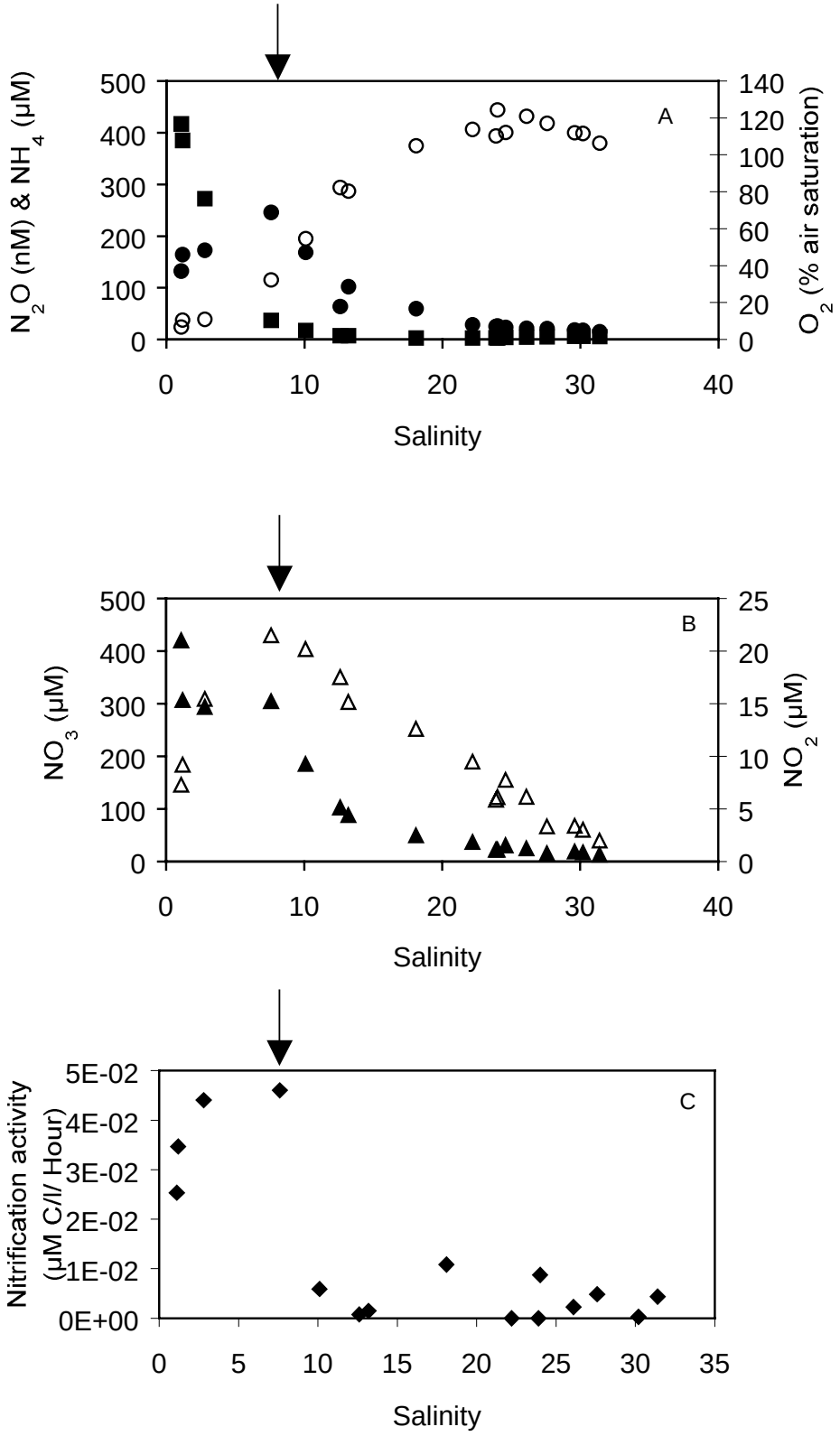

Fig. 2 Property versus salinity plots for the Schelde estuary in April 1997. A: Oxygen- (open circles), ammonium- (squares) and nitrous oxide concentration (closed circles). B: Nitrate- (open triangles) and nitrite concentration (closed triangles). C: Nitrification activity (diamonds). The arrow indicates the location of the nitrous oxide peak.

This basic pattern of oxygen and nitrogen species was consistently observed over the 13-month period of observation (Fig. 3), though concentration levels and profiles shapes varied in response to variability in river discharge, temperature and other environmental factors. Ammonium concentration in the upper estuary varied from 86 µM in September to 483 µM in November. Concentrations of nitrous oxide in the tidal river varied between 8.7 nM in November and 1457 nM in August. Nitrous oxide showed prominent peaks in the period April till September with maximum concentrations during August. For all samples, but the river in November, the estuary was a net source of nitrous oxide to the atmosphere with a median saturation ratio (observed/equilibrium concentration) of 7.1 (atmospheric equilibrium levels varied from 6.9 to 14 nM).

In accordance with the experimental results (Fig. 1), nitrous oxide concentrations were related to oxygen levels in a non-linear way with peak concentrations of nitrous oxide at oxygen saturation levels between about 2 and 15 % and lower nitrous oxide concentration above and below this oxygen level (Fig. 4A). Ammonium concentrations in the Gironde estuary varied from 0.4 to 1.6 µM with no clear trend with salinity (Fig. 5A). Oxygen increased from 65% saturation in the river to close to saturation in the most saline part. Nitrous oxide concentrations are relatively low and varied from 9.8 to 36.7 nM, consistent with observations by Bange et al. (1996). Ammonium concentration in the Loire estuary were also relatively low (2 to 5.5 µM). Oxygen concentration in the Loire estuary were rather low with two oxygen minimum zones, one down to 21 % saturation at salinity 7.5 and one down to 45 % saturation at salinity 16 to 22 (Fig. 5B). Nitrous oxide concentrations were rather low (7.3 to 21 nM), but showed a maximum in the oxygen minimum zones at salinity 18 (Fig. 5B). Ammonium concentrations in the river Thames and upper part of the estuary were high (30 to 43 µM) but decreased rapidly with increasing salinity (Figure 5C). Oxygen concentrations were lowest in the upper estuary but were always above 50 % saturation. Nitrous oxide concentration ranged from 49 nM in the river to a maximum of 93 nM at salinity 2.7 and then steadily decreased down to 11.2 nM in the mouth of the estuary (Fig. 5C).

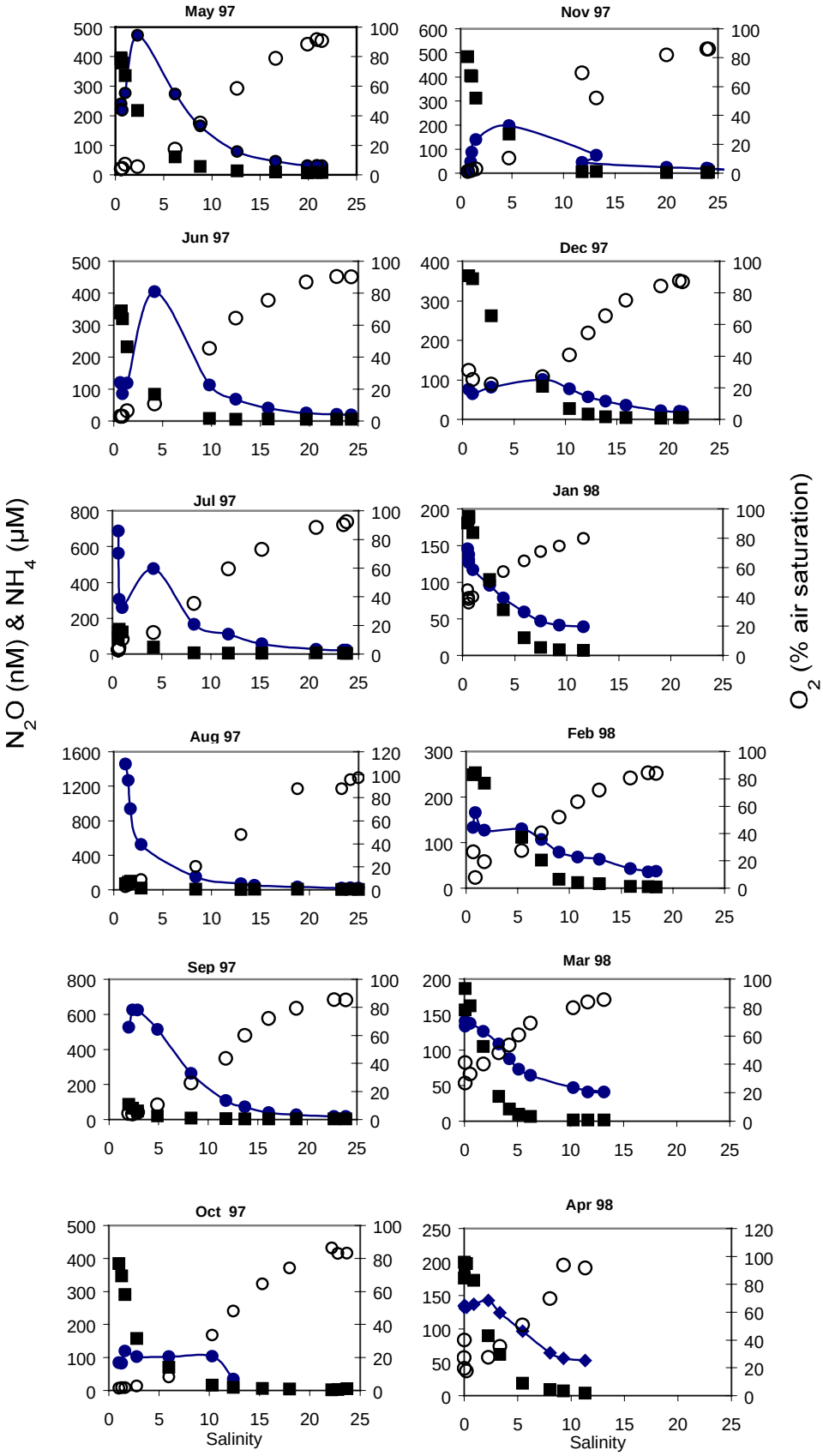

Fig. 3 Ammonium (squaures), oxygen (open circles) and nitrous oxide (closed circles) versus salinity in the Schelde estuary from May 1997 to April 1998. Notice the differences in scale.

Discussion

Concentrations of dissolved nitrous oxide are the result of production as well as consumption in the water bodies, inputs from the sediments, outgassing to the atmosphere and dispersal. Our data clearly revealed distinct nitrous oxide maxima in the Schelde estuary indicating either that nitrous oxide is locally produced or that there are large local inputs from the sediments. Robinson et al. (1998) have reported that benthic denitrification represents the major source of nitrous oxide in the nitrate-rich Colne Estuary. However, it is unlikely that sediment release is the major source of nitrous oxide in the Schelde estuary. Firstly, nitrous oxide maxima move along with the tides indicating a water-column source. Secondly, Middelburg et al. (1995) reported nitrous oxide fluxes from intertidal sediments of the Schelde estuary. These sedimentary fluxes are highly variable but at least about one order of magnitude too low to sustain the flux of nitrous oxide from the water (De Wilde & De Bie 2000). Consequently, in the Schelde estuary most of the nitrous oxide is produced in the water column.

High concentrations of nitrous oxide in the Schelde estuary were consistently observed at a restricted range of salinity, which is also the zone of oxygen depletion (Figures 2 and 3). This might suggest that salinity could be the controlling factor of nitrous oxide production. In a previous study, we discussed this possibility while comparing our data with nitrous oxide measurements from 1978 (De Wilde & De Bie 2000). At that time, the oxygen depleted zone of the estuary was more extensive and the nitrous oxide peak was observed at a higher salinity (i.e. more downstream), but at the same oxygen concentration. This indicates that salinity is more an indicator of the re-aeration status of the estuarine water rather than that the salt concentration does have a direct influence on nitrous oxide production.

An oxygen control of nitrous oxide production is also consistent with most literature observations. Experimental studies with pure cultures (Anderson et al. 1993, Goreau et al. 1980, Kester et al. 1997) and suspended marine sediments (Jørgensen et al. 1984) have clearly shown maximum nitrous oxide production at a narrow range of low, but non-zero oxygen levels. We found similar results in the experiment with the natural estuarine population (Fig 1) as well as in our field observations (Fig 4a). On the basis of these nitrous oxide-oxygen relationships alone it is difficult to conclude whether nitrous oxide production in the natural population is the result of nitrification or denitrification. Addition of acetylene resulted in complete inhibition of nitrous oxide production above 1% oxygen saturation, indicating that the nitrous oxide produced above this oxygen concentration is the product of

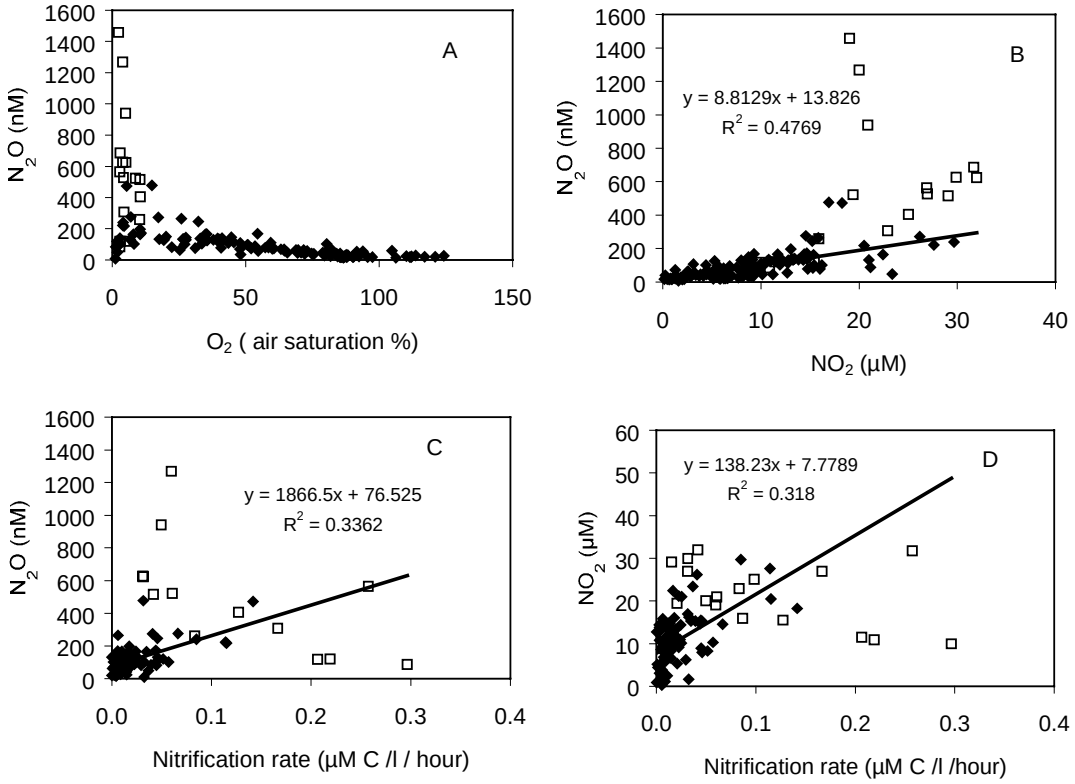

Fig. 4. Relation of nitrous oxide with oxygen (A), nitrite (B) and nitrification (C) and relation of nitrite and nitrification (D) of all data presented in Figures 2 and 3. Squares represent samples possibly affected by denitrification (i.e. with air saturation lower than 10% for the months June, July, August and September), diamonds represent all other samples. Regression lines are based on samples with air saturation higher than 10 % (diamonds).

nitrification. The importance of nitrification as a source of nitrous oxide has also been shown in the Humber estuary through quantification of the transformation of ^{15}N-labelled ammonium into ^{15}N-labelled nitrous oxide (Barnes & Owens 1998).

A consortium of ammonia- and nitrite oxidising bacteria carries out the complete process of nitrification. Nitrite oxidising bacteria usually have a lower affinity for oxygen than ammonia oxidising bacteria (Laanbroek et al. 1994), which may then lead to the transient accumulation of nitrite at low oxygen levels (Fig. 2B; Helder & Vries (1983)). Since nitrite and nitrous oxide can both be formed during nitrification at low oxygen levels, one might expect a correlation. Figure 4B shows that they are indeed correlated (r^2==0.42; N=146, regression line not shown), another indication that nitrous oxide is formed by nitrification. However, nitrite and nitrous oxide are also intermediates in denitrification and we can not exclude some contributions by this process. To explore this possibility, we have excluded samples possibly affected by denitrification (oxygen saturation ≤ 10 % for the months June,

July, August and September; squares in Fig. 4). The resulting correlation between nitrous oxide and nitrite is much tighter (r^2=0.48; N=129; Figure 4B) with about 8.8 nM of nitrous oxide for 1 µM of nitrite being produced by nitrifiers (yield of about 0.09%). A direct comparison of nitrous oxide (Fig. 4C) and nitrite (Fig 4D) with rates of nitrification revealed significant correlations, the low oxygen, summer samples were again excluded from the regression analysis. The low oxygen samples from the summer months are strongly enriched in nitrous oxide (Fig. 4A), have low ammonium concentrations (Fig. 3) and have nitrous oxide concentrations significantly higher than expected on their nitrite concentration (Fig. 4B). This might indicate that nitrous oxide in these samples is partly derived from denitrification. In particular during August when nitrification rates are limited by oxygen and ammonium (De Bie et al. 2001) and nitrous oxide concentration are highest (Fig. 3, 4C).

When comparing oxygen concentrations at which nitrous oxide is produced in laboratory experiments (Fig. 1) and in estuaries (Figures 2, 3 and 5), it is clear that the nitrous oxide peaks in the field are occurring at higher oxygen concentrations than in the laboratory experiment. Maximum nitrous oxide levels in the Schelde estuary were observed at *in situ* oxygen concentrations in the range from 5-35 µM (2-15 % air saturation, Figures 2, 3, 4), while this range was from about 2-7 µM in the laboratory experiment (0.5-2 % air saturation, Fig. 1). Maximum nitrous oxide concentrations in the Thames, Loire and Gironde estuaries occur at even higher oxygen saturation levels (Fig. 5).

In our incubations, we vigorously stirred our water samples to optimise gas exchange between water and the headspace that was analysed. This stirring also prevented the development of suboxic microsites so that all micro-organisms likely experienced the same, imposed oxygen level. Oxygen concentrations measured in estuarine waters are not necessarily representative for the microsites at which nitrous oxide formation might occur: e.g. in flocculate material (Billen et al. 1985) or in fluidised mud layers (Abril et al. 2000). Anoxic microsites in the abundant flocculate material have been proposed as the site for denitrification in the Schelde estuary (Billen et al. 1985, Soetaert & Herman 1995) and suboxic microsites may thus provide niches for nitrous oxide production. The structure of flocculate material is readily destroyed by experimental manipulation and was certainly destroyed in our experiments. The Loire and Gironde estuaries are characterised by the temporal presence of dynamic, biogeochemical active fluidised mud layers (Abril et al. 2000). These fluidised mud layers are partly anoxic and suboxic and have been shown to be a site of nitrous oxide production, nitrification and denitrification (Abril et al. 2000). Erosion of these mud layers and dispersal of the produced nitrous oxide would account for the observed

nitrous oxide distribution. Both, the abundance of flocculate materials and fluidised mud layers are maximal in the maximum turbidity zone of estuaries (Herman & Heip 1999) and could explain the correlation between nitrous oxide and turbidity in the Humber estuary (Barnes & Owens 1998).

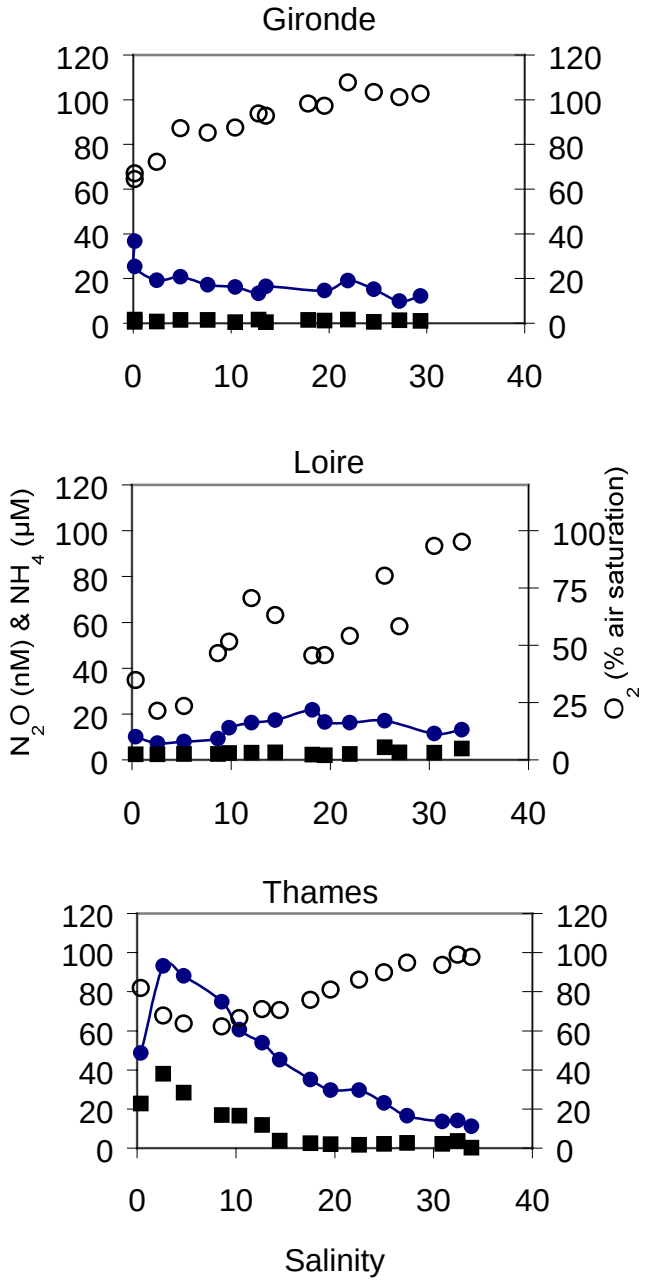

Fig. 5 Ammonium (squares), oxygen (open circles) and nitrous oxide (closed circles) data versus salinity in the Gironde, Loire and Thames estuaries. Samples were taken in June 1997, September 1998 and February 1999, respectively.

On the basis of our results we propose the following mechanism for nitrous oxide production in estuarine waters. Nitrifying bacteria travelling in the estuary may be oxygen-limited in the upstream part, where ammonium concentrations are high (De Bie et al. 2001). As a consequence of estuarine mixing there is aeration and oxygen concentrations increase. Once the oxygen concentration has reached a level at which ammonium oxidation can occur, nitrifying bacteria turn active (Billen et al. 1985) and nitrite accumulates in the water, because of the low affinity for oxygen of nitrite-oxidizing bacteria (Helder & Vries 1983). Since the oxygen concentration is still sub-optimal for ammonium oxidation, part of the oxidation products is released in the form of nitrous oxide. More downstream in the estuary, when oxygen has increased further, the nitrification reaction is carried out completely, as indicated by increasing nitrate concentrations and decreasing ammonium and nitrite concentrations. Nitrous oxide is no longer formed, and the nitrous oxide in the water is either emitted to the atmosphere or consumed by other processes in the water or sediment and diluted with sea water (Barnes & Owens 1998). This mechanism is perfectly illustrated by the Schelde-data and may also apply to the Thames estuary (Fig 5). When ammonium concentrations are too low, like in the Gironde and Loire, nitrous oxide does not reach high levels. When oxygen concentrations are very low, i.e. during summer conditions in the upper Schelde estuary (Fig. 3) or in the fluidised muds of the Gironde estuary (Abril et al. 2000), nitrification is oxygen limited and denitrification of nitrate may cause additional nitrous oxide production (Fig. 4).

Although our study has clearly revealed that oxygen is the major factor controlling nitrous oxide in estuarine systems, it is not in conflict with the nitrogen loading model of Seitzinger & Kroeze (1998). Integrated over large spatial and temporal domains, there are always areas in, or periods during, which oxygen supply is limited and denitrification occurs: i.e. in estuarine and coastal sediments that are rich in labile carbon. In coastal sediments denitrification, and consequently nitrous oxide production, is primarily limited by bottom-water nitrate concentrations (i.e. nitrogen loading; (Middelburg et al. 1996)). Nitrous oxide in estuarine systems with oxygenated water columns then depends on the nitrate concentration in the water that effects nitrous oxide production processes in sediments (e.g. the Colne estuary in the United Kingdom, Robinson et al. (1998)) or in fluidised mud layers (e.g. the Gironde estuary in France, Abril et al. (2000)). In estuarine systems with low oxygen concentrations there is additional nitrous oxide formation resulting from nitrification which is controlled primarily by oxygen and ammonium availability. There appear to be multiple controlling factors: nitrate availability for benthic nitrous oxide production during denitrification and oxygen (and ammonium) for pelagic nitrous oxide production during

nitrification. These processes are not exclusive but rather additive and may alternate in their relative importance depending on the system. This may then explain why Cole & Caraco (2001)found only a weak correlation between average nitrate concentrations and nitrous oxide fluxes across a number of estuarine systems.

In conclusion, this study has clearly identified the non-linear relation between low oxygen concentration and nitrous oxide production by nitrifying bacteria, both in the field and experimentally. The combination of low oxygen conditions with high nitrogen loads is not uncommon in estuaries and coastal zones, which illustrates the importance of estuaries as a source of nitrous oxide.

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C H A P T E R 6

General discussion

Nitrification in estuaries

Estuaries act as a filter system between nutrient-rich, polluted rivers and the relative nutrient-poor sea. Nitrification is the only microbial process that can alter nitrogen to a more oxidized state, and is therefore of crucial importance for the supply of nitrate as substrate for denitrification. Consequently, nitrification can be considered as one of the key processes in the self-cleaning system in estuaries. Under conditions of low oxygen, nitrification may lead to the production of nitrous oxide, a gas that is involved in both the greenhouse effect and the destruction of atmospheric ozone (Chapters 4 and 5). Nitrogen transformation in estuaries can thus have both positive and negative effects on the environment. Yet, little is known about factors controlling nitrification and nitrous oxide production in estuaries. This thesis approaches these controlling factors by a combination of field measurements and laboratory experiments.

BOX 1

Nitrification in estuaries according to Billen (1975)

Nitrifying bacteria from terrigenous origin enter the ammonium-rich but oxygen-poor river water and remain inactive because of the unfavourable conditions during transport to the estuary. As soon as the oxygen concentration has reached a certain threshold, a sudden peak in nitrification activity is observed. However, the growth of the fresh-water organisms is strongly reduced by the increasing salinity, and they are diluted by sea-water faster than they can increase in number by division.

Several mechanisms for nitrification in estuaries have been presented (See box 1 and 2). In Owens´ hypothesis, the nitrification maximum co-occurs with the maximum turbidity zone. Although Owens´ (Box 2) hypothesis that nitrifying bacteria are attached to particles could be confirmed (Chapter 2), the absence of a pronounced maximum turbidity zone makes it difficult to apply this mechanism to the situation in the Schelde. The terrigenous origin of nitrifying bacteria in the Schelde has also been confirmed (Chapter 3), and the mechanism described in Box 1 could therefore be valid as far as the inactivity of bacteria during their course to the estuarine part is concerned. However, the peak of nitrification once oxygen concentration has reached a certain level is not as sharp anymore as in the seventies (Billen 1975, Chapter 2). Furthermore, it has become clear that bacteria are not limited by increased salinity only, but also by a lack of ammonia (Chapter 2). Moreover, although the freshwater

population might not survive in the more saline water, the nitrifying community shows a shift to other, perhaps better adapted genotypes (Chapter 3).

BOX 2

Nitrification in estuaries according to Owens (1986)

Nitrifying bacteria are attached to the particles in the maximum turbidity zone. These particles are sedimented and resuspended in the tidal cycle. The turbidity maximum is considered to act like a fluidized bed reactor, which is periodically injected into the water column where nitrification conditions are optimal.

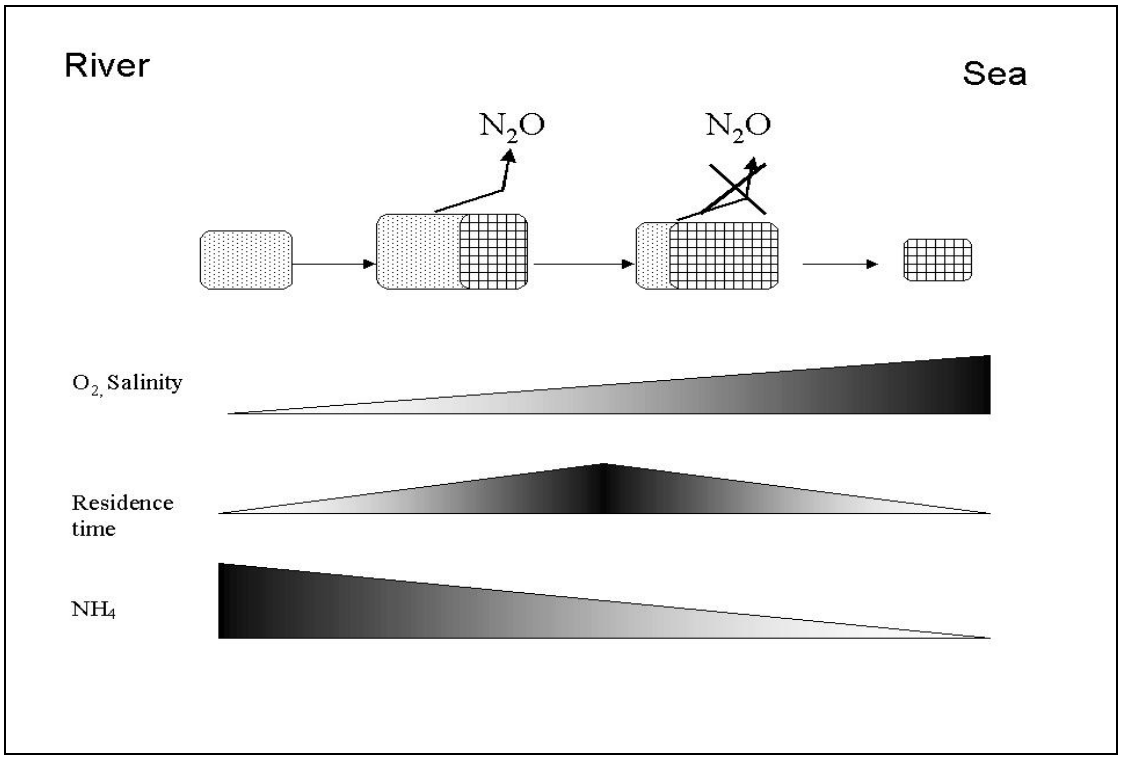
Controlling factors

Besides a strong salinity gradient, the Schelde estuary also contains a distinct oxygen gradient. This thesis provides evidence for the dominant role of oxygen in controlling processes involved in the nitrogen cycle. Although the chemolithotrophic nitrifying bacteria are aerobic and need oxygen for their activity, nitrification activity was observed at low (below 20 µM) oxygen concentrations (Chapter 2). Apart from the biochemical need of oxygen for the nitrification process, oxygen may also act as a controlling factor for the selection of different genotypes within the nitrifying population (Chapter 3). The intensive nitrification at low oxygen concentration further leads to the formation of nitrous oxide in the Schelde estuary (Chapter 4 and 5). Nitrous oxide concentration in the water is nearly always oversaturated and leads to an estimated annual emission to the atmosphere of $2.8*10^8$ g (Chapter 4), which is relatively high compared to 10 other estuaries (Bange et al. 1996). Nitrous oxide production was highest at an oxygen concentration of 5-35 µM in the field and oxygen concentration of 5 µM in laboratory experiments (Chapter 5).

Oxygen alone is not determining the ultimate outcome of the nitrogen cycle in the Schelde estuary and other systems. Ammonium is of course a requirement for nitrification and is the limiting factor in the downstream part of the estuary (Chapter 2). A change in the community structure of ammonia-oxidizing bacteria coincides with the zone of maximum nitrification rates (Chapter 3), but a correlation with ammonium concentration was not found in that study. Low oxygen concentration is the prerequisite for nitrous oxide production. However, if ammonium concentrations are low, the resulting flux of nitrous oxide remains small. Consequently, ammonium concentration determines the amount of nitrous oxide produced, given that the oxygen concentration is in the right range (Chapter 5).

Nitrification and nitrous oxide production occur over the complete salinity range from fresh to oceanic water. Consequently, the salinity gradient only affects the nitrogen cycle by affecting the micro-organisms involved (Chapter 3). The determining role of salinity on the community shift in the Schelde estuary has elegantly been shown in a recent study by Bollmann and Laanbroek (submitted). Several studies have described the possibilities of ammonia-oxidizing bacteria to adapt to increasing salt concentrations in batch cultures (Helder & Vries 1983, Somville 1984). In the Schelde estuary, however, a community structure change was observed instead of physiological adaptation in reaction to higher salinities (Chapter 3). On the other hand, it might be that population changes did occur in the batch culture experiments mentioned above, given the difficulties observing such changes with the techniques available at that time these experiments were carried out.

Nitrifying bacteria tend to attach to particles (Box 2, Chapter 2). The high concentration of particles in the Schelde estuary therefore provides an excellent environment for nitrifying bacteria. The dynamic nature of the Schelde contributes to the steady resuspension of particles, which increases the access to dissolved ammonium and oxygen for the bacteria attached to these particles. Furthermore, attachment to particles increases the residence time of bacteria in a certain section of the estuary as compared to the residence time of free-living bacteria. This offers better opportunity for the ammonium-oxidizing community to develop a substantial biomass and to react to the rapidly changing conditions in the estuary (Chapter 3).



Box 3. Schematic representation of factors controlling nitrification and nitrous oxide production in the Schelde estuary. Scales are non-linear. The nitrifying freshwater population, indicated by the dotted square, is transported along the Schelde river, but is hampered in its activity by the low oxygen concentration and the short residence times in this narrow part of the river. When the oxygen concentration is increasing, nitrification starts. At the same time, the salinity is increasing, which influences the community structure (Bollmann & Laanbroek, submitted), as indicated by the checked square. In this part of the estuary, the residence times are sufficient long to make such a community change possible. Because the oxygen concentration is still very low, part of the nitrification products are released in the form of nitrous oxide. Further downstream the estuary, oxygen concentration increases to such levels that significant nitrous oxide production is no longer occurring. Finally, ammonium concentration is limiting nitrification, the nitrifying community becomes less active and is diluted by seawater.

Present situation in the Schelde estuary

The water quality in the Schelde estuary has improved in recent years (Damme et al. 1995). Billen (1985) suggested that increased wastewater treatment, mainly based on the removal of organic load without tertiary nitrogen removal could result in increasing total dissolved inorganic nitrogen concentrations in the Schelde. A lower input of organic matter would increase the oxygen concentration which would increase nitrification rates in the upper estuary but simultaneously would reduce estuarine denitrification rates. Although denitrification rates have not been measured in the framework of this thesis, dissolved inorganic nitrogen concentrations decreased instead of increased since 1983. Despite the decreased ammonium and nitrate concentrations, nitrous oxide concentrations in the estuary presented in this thesis are in the same order of magnitude as the data of 1978 (Chapter 4),

with that difference that the location of the nitrous oxide peak has moved in a more upstream direction. The fact that the oxygen gradient also moved in this direction, further stresses the role of oxygen concentration as the main controlling factor for nitrous oxide production. Furthermore, the historically similar nitrous oxide concentrations (Chapter 4) show that suboxic sites still exist in the estuary, in spite of the improved oxygen conditions. Taking these observations into account, it is very plausible that denitrification still occurs in the estuary, although rather in the sediments and (vegetated) shores than in the water column.

Conclusions

Oxygen concentration is the factor controlling nitrous oxide production in the Schelde; the ammonium concentration determines nitrification rates and the community structure is controlled by salinity.

Methyl fluoride and acetylene are inappropriate as inhibitors of nitrification in combination with the dark ^{14}C-bicarbonate incorporation technique in natural samples.

The ammonia-oxidizing bacterial community in the Schelde estuary is well adapted to the governing conditions, and reacts to the dynamic estuarine conditions, especially salt concentration, by changing its composition.

The Schelde estuary is a continuous source of nitrous oxide and nitrification is the main source of nitrous oxide in the upper estuary.

Further research

This study reveals multiple factors controlling nitrification and nitrous oxide production in the Schelde estuary. However, much is left to be done. To gain accurate estimates of nitrification rates that can be used in ecological models, stable isotope techniques that directly measures ammonium oxidation should be used. Application of stable isotope techniques will limit the amount of samples that can be handled because of the laborious sample handling. An accurate determination of the conversion factor from incorporated bicarbonate as compared to ammonium oxidized should therefore be prioritised. The use of ^{15}N is also recommended to reveal the source of nitrous oxide, as has been pioneered by (Barnes & Owens 1998).

The oxygen concentration at which nitrous oxide production occurs is much lower in the laboratory compared to the field (Chapter 5). To achieve the exact location of nitrous oxide production, an integrated study should address the role of flocs, sediments and intertidal vegetated sediments.

References

Abril G, Riou SA, Etcheber H, Frankignoulle M, Wit R de, Middelburg JJ (2000) Transient, tidal time-scale, nitrogen transformations in an estuarine turbidity maximum-Fluid mud system (The Gironde, South-west France). Estuar Coast Shelf Sci 50:703-715

AGAGE (1999): Annual arithmetic average based on 1999 monthly, non-pollution values from the Advanced Global Atmospheric gases Experiment, Mace Head, Ireland monitoring site. http:/cdiac.esd.ornl.gov/ndps/alegage.html.

Anderson IC, Levine JS (1986) Relative rates of nitric oxide and nitrous oxide production by nitrifiers, denitrifiers and nitrate respirers. Appl Environ Microbiol 51(5):938-945

Anderson IC, Poth M, Homstead J, Burdige D (1993) A comparison of NO and N_2O production by the autotrophic nitrifier *Nitrosomonas europaea* and the heterotrophic nitrifier *Alcaligenes faecalis*. Appl Environ Microbiol 59:3525-3533

Atlas RM, Bartha R (1987) Microbial ecology. Benjamin/Cummings Publishing Company, Menlo Park, California

Bange HW (2000) It´ not a gas. Nature 408:301-302

Bange HW, Rapsomanakis S, Andreae MO (1996) Nitrous oxide in coastal waters. Global Biochem Cycles 10(1):197-207

Barnes J, Owens NJP (1998) Denitrification and nitrous oxide concentrations in the Humber estuary, UK, and adjacent coastal zones. Marine Pollution Bulletin 37:247-260

Battle M, Bender M, Sowers T, Tans PP, Butler JH, Elkins JW, Ellis JT, Conway T, Zhang N, Lang P, Clarke AD (1996) Atmospheric gas concentrations over the past century measured in air from firn at the South Pole. Nature 383:231-235

Bedard C, Knowles R (1989) Physiology, biochemistry and specific inhibitors of CH^4, NH^4 and CO oxidation by methanotrophs and nitrifiers. Microbiol Rev 53(1):68-84

Belmans H (1991) Debieten van het Scheldebekken. Maand- en jaargemiddelden 1981-1990. Ministerie van de Vlaamse Gemeenschap,, Atwerpse Zeehavendienst (in Dutch)

Belser LW, Mays EL (1980) Specific inhibition of nitrite oxidation by chlorate and its use in assesing nitrification in soils and sediments. Appl Environ Microbiol 39:505-510

Berounsky VM, Nixon SW (1990) Temperature and the annual cycle of nitrification in waters of Narragansett Bay. Limnol Oceanogr 35(7):1610-1617

Berounsky VM, Nixon SW (1993) Rates of nitrification along an estuarine gradient in Narragansett Bay. Estuaries 16:718-730

Betlach MR, Tiedje JM (1981) Kinetic explanation for accumulation of nitrite, nitric oxide, amd nitrous oxide during denitrification. Appl Environ Microbiol 42(6):1074-1084

Bianchi M, Bonin P, Feliatra F (1994) Bacterial nitrification and denitrification rates in the Rhône River plume (northwestern Mediterranean Sea). Mar Ecol Prog Ser 103:197-202

Bie MJM de, Speksnijder AGCL, Kowalchuk GA, Schuurman T, Zwart G, Stephen JR, Diekmann OE, Laanbroek HJ (2001) Shifts in the dominant populations of ammonia-oxidizing β-subclass Proteobacteria along the eutrophic Schelde estuary. Aquat Microb Ecol 23:225-236

Bie MJM de, Starink M, Boschker HTS, Peene J, Laanbroek HJ (2001) Nitrification in the Schelde estuary: methodological aspects and factors influencing its activity. FEMS Microbiol Ecol submitted

Billen G (1975) Nitrification in the Scheldt estuary (Belgium and the Netherlands). Est Coast Mar Sci 3:79-89

Billen G, Somville M, Becker E de, Servais P (1985) A nitrogen budget of the Scheldt hydrographical basin. Neth J Sea Res 19(3-4):223-230

Binnerup SJ, Jensen K, Revsbech NP, Jensen MH, Sorensen J (1992) Denitrification, dissimilatory reduction of nitrate to ammonium, and nitrification in a bioturbated estuarine sediment as measured with ^{15}N and microsensor techniques. Appl Environ Microbiol 58(1):303-313

Blackburn TH (1993) Turnover of $^{15}NH_4^+$ tracer in sediments. In: Kemp PF, Sherr BF, Sherr EB, Cole JJ (eds) Handbook of methods in aquatic microbial ecology. Lewis, USA, p 643-648

Blumer M, Chase T, Watson SW (1969) Fatty acids in the lipids of marine and terrestrial nitrifying bacteria. J Bacteriol 99:366-370

Bodelier PLE, Frenzel P (1999) Contribution of methanotrophic and nitrifying bacteria to CH_4 and NH_4^+ oxidation in the rhizosphere of rice plants as determined by new methods of discrimination. Appl Environ Microbiol 65:1826-1833

Bodelier PLE, Libochant JA, Blom CWPM, Laanbroek HJ (1996) Dynamics of nitrification and denitrification in root-oxygenated sediments and adaptation of ammonia-oxidizing bacteria to low-oxygen or anoxic habitats. Appl Environ Microbiol 62:4100-4107

Bollmann A, Laanbroek HJ (2001) Continuous culture enrichments of ammonia-oxidizing bacteria at low ammonium concentrations. FEMS Microbiol Ecol :1-11

Bollmann A, Laanbroek HJ (Submitted) Influence of oxygen partial pressure and salinity on the community composition of ammonia-oxidizing bacteria in the Schelde estuary. Aquat Microb Ecol submitted:

Boschker HTS, Graaf W de, Köster M, Meyer-Reil L-A, Cappenberg TE (2001) Bacterial populations and processes involved in acetate and propionate consumption in an anaerobic sediment. FEMS Microbiol Ecol 35:97-103

Boschker HTS, Nold SC, Wellsbury P, Bos D, Graaf W de, Pel R, Parkes RJ, Cappenberg TE (1998) Direct linking of microbial populations to specific biogeochemical processes by ^{13}C-labelling of biomarkers. Nature 392:801-805

Brinch-Iversen J, King GM (1990) Effects of substrate concentration, growth state and oxyge availability on relationships among bacterial carbon, nitrogen and phospholipid phosporus content. FEMS Microbiol Ecol 74:345-356

Brion N, Billen G (1997) Wastewater as a source of nitrifying bacteria in river systems: tha case of the river Seine downstream from Paris. In: Brion N (ed) Etude de processus de nitrification á l´échelle de grands réseaux hydrographiques anthropisés. Université Libre de Bruxelles, Brussels, p 26-37

Butler JH, Jones RD, Garber JH, Gordon LI (1987) Seasonal distributions and turnover of reduced trace gases and hydroxylamine in Yaquina Bay Oregon. Geochim Cosmochim Acta 51:697-706

Caffrey JM, Kemp WM (1992) Influence of the submersed plant, *Potamogeton perfoliatus*, on nitrogen cycling in estuarine sediments. Limnol Oceanogr 37(7):1483-1495

Caffrey JM, Miller LG (1995) A comparison of two nitrification inhibitors used to measure nitrification rates in estuarine sediments. FEMS Microbiol Ecol 17:213-220

Capone DG, Horrigan SG, Dunham SE, Fowler J (1990) Direct determination of nitrification in marine waters by using the short-lived radioisotope of nitrogen, ^{13}N. Appl Environ Microbiol 56:1182-1184

Carpenter EJ, Capone DG (1983) Nitrogen fixation by marine oscillatioria (*Trichodesmium*) in the world´s oceans. In: Carpenter EJ, Capone DG (eds) Nitrogen in the marine environment. Academic Press, New York, p 65-103

Chester R (1990) Marine Geochemistry. Unwin Hyman London 698p

Clark JF, Schlosser P, Simpson HJ, Stute M, Wanninkhof R, Ho DT (1995) Relationship between gas transfer velocities and wind speeds in the tidal Hudson River determined by the dual tracer technique. In: Jähne B, Monahan E (eds) Selected papers from the

Third International Symposium on Air-Water gas Transfer. AEON Verlag & Studio Hanau ,Germany, pp 785-800

Codispoti LA, Brandes JA, Christensen JP, Devol AH, Naqvi SWA, Paerl HW, Yoshinari T (2001) The oceanic fixed nitrogen and nitrous oxide budgets: Moving targets as we enter the anthropocene? Sci Mar 65:85-105

Codispoti LA, Christensen JP (1985) Nitrification denitrification and nitrous oxide cycling in the Eastern Tropical South Pacific Ocean. Mar Chem 16:277-300

Cohen Y, Gordon LI (1978) Nitrous oxide in the oxygen minimum of the eastern tropical North Pacific: evidence for the consumption during denitrification and possible mechanisms for its production. Deep Sea Res 25:509-524

Cole JJ, Caraco NF (2001) Emissions of nitrous oxide (N_2O) from a tidal, freshwater river, the Hudson river, New York. Environ Sci Technol 35:991-996

Collombon M, Poelman M (2001): Mass mortality of *Mytilus edilus* in the Oosterschelde, Netherlands, May 2001. Report nr. c045/01, RIVO-CSO, the Netherlands. 13 p.

Conrad R (1996) Soil microorganisms as controllers of atmospheric trace gases (H_2 CO CH_4 OCS N_2O and NO. Microbiol Rev 60:609-640

Crutzen PJ (1970) The infuence of nitrogen oxides on the atmospheric ozone content. Quaterly J of the Royal Meteorol Society 96:320-325

Crutzen PJ (1981) Atmospheric chemical processes of the oxides of nitrogen, including nitrous oxide. In: Delwiche CC (ed) Denitrification, nitrification and atmospheric nitrous oxide. John Wiley & Sons, New York, p 17-44

Crutzen PJ, Andreae MO (1990) Biomass burning int he tropics, impacts on atmospheric chemistry and biogeochemical cycles. Science 250:1669-1678

Damme S van, Meire P, Maeckelberghe H, Verdievel M, Bourgoing L, Taveniers E, Ysebaert T, Wattel G (1995) De waterkwaliteit van de zeeschelde: evolutie in de voorbije dertig jaa.r Water 85 :44-256 (In Dutch with a summary in English)

Deck BL (1981) Nutrient element distribution in the Hudson estuary. PhD Dissertation Columbia University, New York, 396p

Dowdell RJ, Burford JR, Crees R (1979) Losses of nitrous oxide dissolved in drainage water from agricultural land. Nature 278:342-343

Edwards U, Rogall T, Blöcker H, Emde M, Böttger EC (1989) Isolation and direct complete nucleotide determination of entire genes. Characterization of gene coding for 16S ribosomal RNA. Nucl Acids Res 17:7843-7853

Elkins JW, Wofsy SC, McElroy MB, Kolb CE, Kaplan WA (1978) Aquatic sources and sinks for nitrous oxide. Nature 275:602-605

Feliatra F, Bianchi M (1993) Rates of nitrification and carbon uptake in the Rhone river plume (Northwestern Mediterranean Sea). Microb Ecol 26:21-28

Felske A, Engelen B, Nübel U, Backhaus H (1996) Direct ribosome isolation from soil to extract bacterial rRNA for community analysis. Appl Environ Microbiol 62:4162-4167

Ferris MJG, Muyzer G, Ward DM (1996) Denaturing gradient gel electrophoresis profiles of 16S rRNA-defined populations inhabiting a hot spring microbial mat community. Appl Environ Microbiol 62:340-346

Firestone MK, Davidson EA (1989) Microbiological basis of NO and N_2O production and consumption in soil. In: Andreae MO, Schimel DS (eds) Exchange of trace gases between terrestrial ecosystems and the atmosphere. John Wiley & Sons Inc, New York, pp 7-21

Firestone RB, Tiedje JM (1980) Nitrous oxide from soil denitrification: factors controlling its biological production. Science 280:749-751

Frankignoulle M, Bourge I, Wollast R (1996) Atmospheric CO_2 fluxes in a highly polluted estuary (the Scheldt). Limnol Oceanogr 41:365-369

Frijlink MJ, Abee T, Laanbroek HJ, De Boer W, Konings WN (1992) Secundary transport of amino acids in *Nitrosomonas europaea*. Arch Microbiol 157:389-393

Giovannoni SJ, Britschgi TB, Moyer CL, Field KG (1990) Genetic diversity in Sargassso Sea bacterioplankton. Nature 345:60-63

Goosen NK, Van Rijswijk P, Brockmann U (1995) Comparison of heterotrophic bacterial production rates in early spring in the turbid estuaries of the Scheldt and the Elbe. Hydrobiologia 311:31-42

Goreau TJ, Kaplan WA, Wofsy SC, McElroy MB, Valois FW, Watson SW (1980) Production of NO_2^- and N_2O by nitrifying bacteria at reduced concentrations of oxygen. Appl Environ Microbiol 40:526-532

Graaf AA van de, Mulder A, De Bruijn P, Jetten MSM, Robertson LA, Kuenen JG (1995) Anaerobic oxidation of ammonium is a biologically mediated process. Appl Environ Microbiol 61:1246-1251

Hall GH (1984) Measurement of nitrification rates in lake sediments: comparison of the nitrification inhibitors nitrapyrin and allylthiourea. Microb Ecol 10:25-36

Hanson RS, Hanson TE (1996) Methanotrophic bacteria. Microbiol Rev 60:439-471

Hastings RC, Saunders JR, Hall GH, Pickup RW, McCarthy AJ (1998) Application of molecular biological techniques to a seasonal study of ammonia oxidation in a eutrophic freshwater lake. Appl Environ Microbiol 64:3674-3682

Helder W, Vries RTP de (1983) Estuarine nitrite maxima and nitrifying bacteria (Ems-Dollard estuary). Neth J Sea Res 17(1):1-18

Herman PMJ, Heip CHR (1999) Biogeochemistry of the maximum turbidity zone of estuaries (MATURE): some conclusions. J Mar Systems 22:89-104

Hiorns WD, Hastings RC, Head IM, McCarthy AJ, Saunders JR, Pickup RW, Hall GH (1995) Amplification of 16S ribosomal RNA genes of autotrophic ammonia-oxidizing bacteria demonstrates the ubiquity of nitrosospiras in the environment. Microbiology 141:2793-2800

Hoch MP, Kirchman DL (1993) Seasonal and inter-annual variability in bacterial production and biomass in a temporate estuary. Mar Ecol Prog Ser 98:283-295

Horrigan SG, Montoya JP, Nevins JL, McCarthy JJ, Ducklow HW, Goericke R, Malone T (1990) Nitrogenous nutrient transformations in the spring and fall in the Chesapeake Bay. Estuar Coast Shelf Sci 30:369-391

Houghton JT, Meira Filho LG, Callander BA, Harris N, Kattenberg A, Maskell K (eds) (1996) Climate Change 1995. The Science of Climate Change. Published for the Intergovernmental panel on climate change, Cambridge University Press, pp1-572

Hovanec TA, DeLong EF (1996) Comparitive analysis of nitrifying bacteria associated with freshwater and marine aquaria. Appl Environ Microbiol 62:2888-2896

Howarth RW, Billen G, Swaney D, Townsend A, Jaworski N, Lajtha K, Downing JA, Elmgren R, Caraco N, Jordan T, Berendse F, Freney J, Kudeyarov V, Murdoch P, Zhu ZL (1996) Regional nitrogen budgets and riverine n&p fluxes for the drainages to the north atlantic ocean: natural and human influences. Biogeochemistry 35:75-139

Hyman MR, Arp DJ (1987) Quantification and removal of some contaminating gases from acetylene used to study gas-utilizing enzymes and microorganisms. Appl Environ Microbiol 53:298-303

IPCC (1996) Climate Change 1995. The science of climate change. Contribution of Working group I to the second assesment report of the Intergovernmental Panel on Climate Change. Cambridge University Press, Cambridge, UK

Iriarte A, Madariaga I de, Diez-Garagarza.F, Revilla M, Orive E (1996) Primary plankton production, respiration and nitrification in a shallow temperate estuary during summer. J Exp Mar Biol Ecol 208:127-151

Jetten MSM (2001a) New pathways for ammonia conversion in soil and aquatic systems. Plant and Soil 230:9-19

Jetten MSM (2001b) Microbiology and application of the anaerobic ammonium oxidation (`anommox´) process. Current Opinion in Biotechnology 12:283-288

Jøgensen KS, Sørensen J (1988) Two annual maxima of nitrate reduction and denitrification in estuarine sediment (Norsminde Fjord, Denmark). Mar Ecol Prog Ser 48:147-154

Jørgensen KS, Jensen HB, Sørensen J (1984) Nitrous oxide production from nitrification and denitrification in marine sediment at low oxygen concentrations. Can J Microbiol 30:1073-1078

Joye SB, Connell TL, Miller LG, Oremland RS, Jellison RS (1999) Oxidation of ammonia and methane in an alkaline, saline lake. Limnol Oceanogr 44:178-188

Jukes TH, Cantor CR (1969) Evolution of protein molecules. In: Munro HN (ed) Mammalian protein metabolism. Academic Press, New York, p 21-132

Juliette LY, Hyman MR, Arp DJ (1993) Inhibition of ammonia oxidation in *Nitrosomonas europaea* by sulfur compounds - Thioethers are oxidized to sulfoxides by ammonia monooxygenase. Appl Environ Microbiol 59:3718-3727

Juliette LY, Hyman MR, Arp DJ (1993) Mechanism-based inactivation of ammonia monooxygenase in *Nitrosomonas europaea* by allylsulfide. Appl Environ Microbiol 59:3728-3735

Kerner M (1996) Sink and source reactions of N_2O within the sediment-water interface formed by settling seston from the Elbe estuary. Est Coast Shelf Sci 43:419-432

Kester RA, Boer W de, Laanbroek HJ (1997) Production of NO and N_2O by pure cultures of nitrifying and denitrifying bacteria during changes in aeration. Appl Environ Microbiol 63:3872-3877

Khalil MAK, Rasmussen RA (1988) Nitrous oxide: Trends and global mass balance over the 3000 years. Annal Glaciology 10:73-79

Koops H-P, Harms H (1985) Deoxyribonucleic acid homologies among 96 strains of ammonia-oxidising bacteria. Arch Microbiol 141:214-218

Koops HP, Pommerening-Röser A (2001) Distribution and ecophysiology of nitrifying bacteria emphasizing cultured species. FEMS Microbiol Ecol 37:1-9

Kowalchuk GA, Bodelier PLE, Heilig GHJ, Stephen JR, Laanbroek HJ (1998) Community analysis of ammonia-oxidising bacteria, in relation to oxygen-availability in soils ande root-oxygenated sediments, usin PCR, DGGE and oligonucleotide probe hybridisation. FEMS Microbiol Ecol 27:339-350

Kowalchuk GA, Stephen JR, Boer W de, Prosser JI, Embley TM, Woldendorp JW (1997) Analysis of ammonia-oxidizing bacteria of the â-subdivision of the class *Proteobacteria* in coastal sand dunes by Denaturing Gradient Gel Electrophoresis and sequencing of PCR-amplified 16S ribosomal DNA fragments. Appl Environ Microbiol 63:1489-1497

Kowalchuk GA, Stienstra A, Stephen JR, Speksnijder AGCL, Heilig GHJ, Woldendorp JW (2000) Changes in community structure of ammonia-oxidising bacteria during secondary succesion of calcereous grasslands. Environ Microbiol 2:99-110

Kroeze C (1993): Global warming by halocarbons and nitrous oxide. Ph.D. Thesis, University of Amsterdam, Amsterdam, the Netherlands.

Kromkamp JC, Peene J (1995) Possibility of net phytoplankton primary production in the turbid Schelde Estuary (SW Netherlands). Mar Ecol Prog Ser 121:249-259

Kuenen JG, Robertson LA (1987) Ecology of nitrification and denitrification. In: Cole JA, Ferguson S (eds) The nitrogen and sulfur cycles. Cambridge University press, UK, p 162-218

Kuenen JG, Robertson LA (1994) Combined nitrification-denitrification processes. FEMS Microbiology Reviews 15:109-117

Laanbroek HJ, Bodelier PLE, Gerards S (1994) Oxygen consumption kinetics of *Nitrosomonas europaea* and *Nitrobacter hamburgensis* grown in mixed continuous cultures at different oxygen concentrations. Arch Microbiol 161:156-162

Laanbroek HJ, Woldendorp JW (1994) Activity of chemolithotrophic nitrifying bacteria under stress in natural soils. In: Gwynfryn Jones J (ed) Advances in Microbial Ecology. Plenum Press, New York, p 275-304

Laima MJC (1993) Recovery of $^{15}NH_4^+$ in labelling experiments on coastal marine sediments. Mar Chem 44:31-42

Law CS, Rees AP, Owens NJP (1992) Nitrous oxide: estuarine sources and atmospheric flux. Est Coast Shelf Sci 35:301-314

Ledwell JJ (1984) The variation of gas transfer velocity with molecular diffusivity. In: Brutsaert W, Jirka G (eds) Gas transfer at water surfaces. Reidel, Dordrecht, pp 293-302

Liss PS, Merlivat L (1986) Air-sea gas exchange rates: introduction and synthesis. In: Buat-Menard P (ed) The role of air-sea exchange in geochemical cycling. Reidel, Dordrecht, pp 113-127

Luther GW, Sundby B, Lewis BJ, Brendel PJ, Silverberg N (1997) Interactions of manganese with the nitrogen cycle: alternative pathways for dinitrogen formation. Geochim Cosmochim Acta 61:4043-4052

Maidak BL, Cole JR, Parker CTJ, Garrity GM, Larsen N, Lilburn BLiTG, McCaughey MJ, Olsen GJ, Overbeek R, Pramanik S, Schmidt TM, Tiedje JM, Woese CR (1999) A new version of the RDP (Ribosomal Database Project). Nucl Acids Res 27:171-173

Matson PA, Vitousek PM (1987) Cross-system comparisons of soil nitrogen transformations and nitrous oxide flux in tropical forest ecosystems. Glob Biogeochem Cycles 1:163-170

McCaig AE, Embley TM, Prosser JI (1994) Molecular analysis of enrichment cultures of marine ammonia oxidisers. FEMS Microbiol Lett 120:363-368

McCaig AE, Phillips CJ, Stephen JR, Kowalchul GA, Harvey M, Herbert M, Embley TM, Prosser JI (1999) Nitrogen cycling and community structure of â-subgroup ammonia-oxidising bacteria withing polluted, marine fish-farm sediments. Appl Environ Microbiol :

McElroy MB, Elkins JW, Wofsy SC, Kolb CE, Durán AP, Kaplan WA (1978) Production and release of N_2O from the Pontomac estuary. Limnol Oceanogr 23:1168-1182

McMahon PB, Dennehy KF (1999) N_2O Emissions from a nitrogen-enriched river. Environ Sci Technol 33:21-25

Mengis M, Gächter R, Wehrli B (1997) Sources and Sinks of nitrous oxide (N_2O) in deep lakes. Biogeochemistry 38:281-301

Middelburg JJ, Klaver G, Nieuwenhuize J, Markusse RM, Vlug T, Van der Nat FJWA (1995) Nitrous oxide emissions from estuarine intertidal sediments. Hydrobiologia 311:43-55

Middelburg JJ, Nieuwenhuize J (1998) Carbon and nitrogen stable isotopes in suspended matter and sediments from the Schelde Estuary. Mar Chem 60:217-225

Middelburg JJ, Nieuwenhuize J (2000) Nitrogen uptake by heterotrophic bacteria and phytoplankton in the nitrate-rich Thames estuary. Mar Ecol Prog Ser 203:13-21

Middelburg JJ, Nieuwenhuize J, Iversen N, Hoegh N, Wilde HPJ de, Helder W, Seifert R, Christof O (2001) Methane distribution in European tidal estuaries. Biogeochem In press:

Middelburg JJ, Soetaert K, Herman PMJ, Heip CHR (1996) Denitrification in marine sediments: a model study. Global Biochem Cycles 10:661-673

Miller LG, Coutlakis MD, Oremland RS, Ward BB (1993) Selective inhibition of ammonium oxidation and nitrification-linked N_2O formation by methyl fluoride and dimethyl ether. Appl Environ Microbiol 59(8):2457-2464

Mobarry BK, Wagner M, Urbain V, Rittmann BE, Stahl DA (1996) Phylogenetic probes for analyzing abundance and spatial organization of nitrifying bacteria. Appl Environ Microbiol 62(6):2156-2162

Mulder A, Vandegraaf AA, Robertson LA, Kuenen JG (1995) Anaerobic ammonium oxidation discovered in a denitrifying fluidized bed reactor. FEMS Microbiol Ecol 16:177-183

Muller E (1994): Bacterial energetics in aerobic wastewater treatment. Ph.D. Dissertation, Free University, Amsterdam.

Murray AE, Hollibaugh JT, Orrego C (1996) Phylogenetic composition of bacterioplankton from two California estuaries compared by Denaturating Gel Electrophoresis of 16S rDNA fragments. Appl Environ Microbiol 62:2676-2680

Muyzer G, Dewaal EC, Uitterlinden AG (1993) Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16s rRNA. Appl Environ Microbiol 59:695-700

Muyzer G, Smalla K (1998) Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. Anton Leeuwenhoek Int J Gen Microbiol 73:127-141

Naqvi SWA, Jayakumar DA, Narvekar PV, Naik H, Sarma VVSS, D´Souza W, Joseph S, George MD (2000) Increased marine production of N_2O due to intensifying anoxia on the Indian continental shelf. Nature 408:346-349

Nevison CD, Weiss RF, Erickson III DJ (1995) Global oceanic emissions of nitrous oxide. J Geophys Res 100:15809-15820

Nijburg JW, Laanbroek HJ (1997) The fate of ^{15}N-nitrate in healthy and declining *Phragmites australis* stands. Microb Ecol 34:254-262

Nixon SW, Ammerman JW, Atkinson LP, Berounsky VM, Billen G, Boicourt WC, Boynton WR, Church TM, Ditoro DM, Elmgren R, Garber JH, Giblin AE, Jahnke RA, Owens NJP, Pilson MEQ, Seitzinger SP (1996) The fate of nitrogen and phosphorus at the land sea margin of the north atlantic ocean. Biogeochemistry 35:141-180

Nixon SW, Pilson MEQ (1983) Nitrogen in estuarine and coastal marine ecosystems. In: () Nitrogen in the marine environment. Academic Press,, p 565-648

Oremland RS, Capone DG (1988) Use of "specific' inhibitors in biogeochemistry and microbial ecology. Advances in Microbial Ecology 10:285-383

Oremland RS, Culbertson CW (1992) Evaluation of methyl fluoride and dimethyl ether as inhibitors of aerobic methane oxidation. Appl Environ Microbiol 58(9):2983-2992

Otte S, Grobben NG, Robertson LA, Jetten MSM, Kuenen JG (1996) Nitrous oxide production by *Alcaligenes faecalis* under transient and dynamic aerobic and anaerobic conditions. Appl Environ Microbiol 62:2421-2426

Owens NJP (1986) Estuarine nitrification: A naturally occurring fluidized bed reaction? Est Coast Shelf Sci 22:31-44

Pakulski JD, Benner R, Amon R, Eadie B, Whiltledge T (1995) Community metabolism and nutrient cycling in the Mississippi River plume: evidence for intense nitrification at intermediate salinities. Mar Ecol Prog Ser 117:207-218

Paucot H, Wollast R (1997) Transport and transformation of trace metals in the Scheldt estuary. Mar Chem 58:229-244

Peer Y van de, Jansen J, Rijk P de, Wachter R de (1997) Database on the structure of small ribosomal subunit RNA. Nucl Acids Res 24:111-116

Peer Y van de, Wachter R de (1994) TREECON for Windows: a software package for the construction and drawing of evolutionary trees for the Microsoft Windows environment. ComputApplic Biosci 10:569-570

Philips CJ, Paul EA, Prosser JI (2000) Quantitative analysis of ammonia oxidising bacteria using competitve PCR. FEMS Microbiol Ecol 32:167-175

Philips CJ, Smith Z, Embley TM, Prosser JI (1999) Phylogenetic differences between particle-associated and planktonic ammonia-oxidizing bacteria of the â-subdivision of the class proteobacteria in the Northwestern Mediteranean Sea. Appl Environ Microbiol 65:779-786

Plough H, Kühl M, Buchholz-Cleven B, Jørgensen BB (1997) Anoxic aggregates - an ephemeral phenomenon in the pelagic environment ? Aquat Microb Ecol 13:285-294

Pommerening-Röser A, Rath G, Koops H-P (1996) Phylogenetic diversity within the genus *Nitrosomonas*. System Appl Microbiol 19:344-351

Poth M, Focht DD (1985) ^{15}N Kinetic analysis of N_2O production by *Nitrosomonas europaea*: an examination of nitrifier denitrification. Appl Environ Microbiol 49:1134-1141

Powell SJ, Prosser JI (1985) The effect of nitrapyrin and chloropicolinic acid on ammonium oxidation by Nitrosomonas europaea. FEMS Microbiol Lett 28:51-54

Prasad R, Power JF (1995) Nitrification inhibitors for agriculture, health and the environment. Adv in Agron 54:233-281

Prosser JI (1989) Autotrophic nitrification in bacteria. Adv Microbiol Physiol 30:125-181

Regnier P, Wollast R, Steefel CI (1997) Long-term fluxes of reactive species in macrotidal estuaries: Estimates from a fully transient multicomponent reaction-transport model. Mar Chem 58:127-145

Rijk P de, Wachter R de (1993) DCSE v2.54, an interactive tool for sequence alignment and secondary structure research. ComputApplic Biosci 9:735-740

Robinson AD, Nedwell DB, Harrison RM, Ogilvie BG (1998) Hypernutrified estuaries as sources of N2O emission to the atmosphere: the estuary of the river Colne, Essex, UK. Mar Ecol Prog Ser 164:59-71

Ronen D, Magaritz M, Almon E (1988) Contaminated aquifers are a forgotten component of the global N_2O budget. Nature 335:57-59

Rotthauwe J-H, Witzel K-P, Liesack W (1997) The ammonia monooxygenase structural gene *amoA* as a functional marker: molecular fine scale analysis of natural ammonia oxidizing populations. Appl Environ Microbiol 63:4704-4712

Roy R, Knowles R (1995) Differential inhibition by allylsulfide of nitrification and methane oxidation in freshwater sediment. Appl Environ Microbiol 61(12):4278-4283

Schramm A, Beer D de, Wagner M, Amann R (1998) Identification and activities in situ of *Nitrosospira* and *Nitrospira* spp. as dominant populations in a nitrifying fluidized bed reactor. Appl Environ Microbiol 64:3480-3485

Seitzinger SP (1987) Nitrogen biogeochemistry in an unpolluted estuary: the importance of benthic denitrification. Mar Ecol Prog Ser 41:177-186

Seitzinger SP (1988) Denitrification in freshwater and coastal marine ecosystems: Ecological and geochemical significance. Limnol Oceanogr 33:702-724

Seitzinger SP (1993) Denitrification and nitrification rates in aquatic sediments. In: Kemp PF, Sherr BF, Sherr EB, Cole JJ (eds) Handbook of methods in aquatic microbial ecology. Lewis, USA, p 633-641

Seitzinger SP, Kroeze C (1998) Global distribution of nitrous oxide production and N inputs in freshwater and coastal marine ecosystems. Global Biochem Cycles 12:93-113

Seitzinger SP, Nixon SW (1985) Eutrophication and the rate of denitrification and N_2O-production in coastal marine sediments. Limnol Oceanogr 30:1331-1339

Sloth NP, Nielsen LP, Blackburn TH (1992) Nitrification in sediment cores measured with acetylene inhibition. Limnol Oceanogr 37(5):1108-1112

Smorczewski WT, Schmidt EL (1991) Numbers, activities, and diversity of autotrophic ammonia-oxidizing bacteria in a freshwater, euthrophic lake sediment. Can J Microbiol 37:828-833

Soetaert K, Herman PMJ (1995a) Estimating estuarine residence times in the Westerschelde (The Netherlands) using a box model with fixed dispersion coefficients. Hydrobiologia 311:215-224

Soetaert K, Herman PMJ (1995b) Nitrogen dynamics in the Westerschelde estuary (SW Netherlands) estimated by means of the ecosystem model MOSES. Hydrobiologia 311:225-246

Somville M (1978) A method for the measurement of nitrification rates in water. Wat Res 12:843-848

Somville M (1984) Use of nitrifying activity measurements for describing the effect of salinity on nitrification in the Scheldt estuary. Appl Environ Microbiol 47(2):424-426

Somville M, De Pauw N (1982) Influence of water temperature and river discharge on water quality of the Western Scheldt estuary. Water Res 16:1349-1356

Speksnijder AGCL, Kowalchuk GA, Roest K, Laanbroek HJ (1998) Recovery of a *Nitrosomonas*-like 16S rDNA sequence group from freshwater habitats. System Appl Microbiol 21:321-330

Stehr G, Böttcher B, Dittberner P, Rath G, Koops H-P (1995) The ammonia-oxidizing nitrifying population of the River Elbe estuary. FEMS Microbiol Ecol 17:177-186

Stephen JR, Kowalchuk GA, Bruns M-A, McCaig AE, Phillips CJ, Embley TM, Prosser JI (1998) Analysis of â-subgroup proteobacterial ammonia oxidizer populations in soil by Denaturing Gradient Gel Electrophoresis analysis and hierarchical phylogenetic probing. Appl Environ Microbiol 64:2958-2965

Stephen JR, McCaig AE, Smith Z, Prosser JI, Embley TM (1996) Molecular diversity of soil and marine 16s rRNA gene sequences related to beta-subgroup ammonia-oxidizing bacteria. Appl Environ Microbiol 62:4147-4154

Strous M, Fuerst JA, Kramer EHM, Logemann S, Muyzer G, Van de Pas-Schoonen KT, Webb R, Kuenen JG, Jetten MSM (1999) Missing lithotroph identified as a new planctomycete. Nature 400:446-449

Stüven R, Vollmer M, Bock E (1992) The impact of organic matter on nitric oxide formation by *Nitrosomonas europaea*. Arch Microbiol 158:439-443

Suwa Y, Sumino T, Noto K (1997) Phylogenetic relationships of activated sludge isolates of ammonia oxidizers with different sensitivities to ammonium sulfate. J Gen Appl Microbiol 43:373-379

Teske A, Wawer C, Muyzer G, Ramsing NB (1996) Distribution of Sulfate-Reducing bacteria in a stratified fjord (Mariagerfjord, Denmark) as eveluated by most-probable number counts and denaturing gradient gel electrophoresis of PCR-amplified ribosomal DNA fragments. Appl Environ Microbiol 62:1405-1415

Trimmer M, Nedwell DB, Sivyer DB, Malcolm SJ (2000) Seasonal benthic organic matter mineralisation measured by oxygen uptake and denitrification along a transect of the inner and outer river Thames estuary, UK. Mar Ecol Prog Ser 197:103-119

TRIPOS (1999) Biomonitoring can fytoplankton in de Nederlandse zoute en brakke wateren 1998. Rijkswaterstaat, Rijksinstituut voor Kust en Zee (RIKZ)

Utaaker JB, Nes IF (1998) A qualitative evaluation of the published oligonucleotides specific for the 16S rRNA gene sequences of the ammonia-oxidizing bacteria. System Appl Microbiol 21:72-88

Velthof GL, Oenema O (1995) Nitrous oxide fluxes from grassland in the Netherlands: 1 Statistical analysis of flux chamber measurements. Eur J Soil Sci 46:533-540

Vitousek PM (1994) Beyond global warming: ecology and global change. Ecology 75(7):1861-1876
Volkman JK, Jeffrey SW, Nichols PD, Rogers GI, Garland CD (1989) Fatty acid and lipid consumption of 10 species of microalgae used in mariculture. J Exp Mar Biol Ecol 128:219-240
Voytek MA, Ward BB (1995) Detection of ammonia-oxidizing bacteria of the beta-subclass of the class *Proteobacteria* in aquatic samples with the PCR. Appl Environ Microbiol 61:1444-1450
Wagner M, Rath G, Koops HP, Flood J, Amann R (1996) *In situ* analysis of nitrifying bacteria in sewage treatment plants. Water Science and Technology 34:237-244
Wang WC, Yung YL, Lacis AA, Mo T, Hansen JE (1976) Greenhouse effects due to man-made perturbations of trace gases. Science 194:685-690
Wanninkhof R (1992) Relationship between wind speed and gas exchange over the ocean. J Geophys Res 97:7373-7382
Ward BB (1982) Oceanic distribution of ammonia oxidizing bacteria determined by immunofluorescence. J Mar Res 40:1155-1172
Ward BB, Carlucci AF (1985) Marine ammonia- and nitrite-oxidizon bacteria: sereological diversity determined by immunofluorescence in culture and in the environment. Appl Environ Microbiol 50:194-201
Ward DM, Weller R, Bateson MM (1990) 16S rRNA sequences reveal numerous uncultured microorganisms in a natural comunity. Nature 345:63-65
Wattel G, Schouwenaar A (1991) SAWES-report 9106, Rijkswaterstaat Dienst Getijde Wateren, Middelburg, The Netherlands
Weiss RF, Price BA (1980) Nitrous oxide solubility in water and seawater. Mar Chem 8:347-359
Whitby CB, Saunders.JR, Rodriguez J, Pickup RW, McCarthy A (1999) Phylogenetic differentiation of two closely related i Nitrosomonas spp. that inhabit different sediment environments in an oligotrophic freshwater lake. Appl Environ Microbiol 65:4855-4862
Whittaker RH, Likens GE (1975) The biosphere and man. In: Lieth H, Whittaker RH (eds) Primary production of the biosphere. Springer-Verlag, Berlin, pp 305-328
Wilde HPJ de, Bie MJM de (2000) Nitrous oxide in the Schelde estuary: production by nitrification and emission to the atmosphere. Mar Chem 69:203-216
Wilde HPJ de, Helder W (1997) Nitrous oxide in the Somali Basin: The role of upwelling. Deep Sea Res II 44:1319-1340
Wollast R (1988) The Scheldt Estuary. In: Salomons W, Bayne BL, Duursma EK, Förstner U (eds) Pollution of the North Sea an assessment. Springer Verlag, Berlin-Heidelberg, pp183-193
Wood PM (1986) Nitrification as a bacterial energy source. In: Prosser JI (ed) Nitrification. IRL Press, Oxford UK, p 39-64
Yoh M, Terai H, Saijo Y (1983) Accumulation of nitrous oxid in the oxygen deficient layer of freshwater lakes. Nature 301:327-329
Zumft WG, Kroneck PMH (1990) Metabolism of nitrous oxide. In: Revsbech NP, Sørensen J (eds) Denitrification in soil and sediment. Plenum Press, New York, p 37-55
Zwolsman JJG, Van Eck BTM, Van Der Weijden CH (1997) Geochemistry of dissolved trace metals (cadmium copper zinc) in the Scheldt estuary southwestern Netherlands: Impact of seasonal variability. Geochim Cosmochim Acta 8:1635-1652

Summary

Estuaries form the transition zone between river and sea and are often, like in the case of the Schelde estuary eutrophic, dynamic environments. The Schelde estuary is characterised by high nitrogen loads, low oxygen concentration and high turbidity. Nitrification, the oxidation of ammonium to nitrite and nitrate, is a key process in the self-cleaning system of the estuary by denitrification. During denitrification, nitrate is, via several intermediates, reduced to dinitrogen gas. Nitrous oxide can be formed as an intermediate or by-product of both nitrification and denitrification. Nitrous oxide is a greenhouse gas and is involved in the destruction of the ozone layer. The steady increase of nitrous oxide in the atmosphere has therefore led to concern, and the contribution of natural systems to the nitrous oxide budget has been acknowledged. This thesis aims to determine the main factors that are controlling nitrification and nitrous oxide production in the Schelde estuary.

The spatial and temporal variation of nitrification activity is addressed in Chapter 2 in monthly measurements during a period of 15 months. Nitrification was measured by $^{14}CO_2$-incorporation, using a specific inhibitor for nitrification, N-serve to distinguish the incorporation by this process from other C-fixing processes. The functioning of the specific inhibitor is thus crucial. Comparison of a range of inhibitors showed that the inhibitors methyl fluoride and acetylene stimulated ^{14}C-incorporation in estuarine samples, while they indeed inhibited C-incorporation in a pure culture of *Nitrosomonas europaea*. The mechanism for this surprising result remains unclear.

Nitrification activity showed a peak in the upstream part of the estuary (Chapter 2). The peak was located at a point where the combination of ammonium and oxygen is advantageous for growth. In the months May and July, the conditions for nitrification were optimal, because no difference could be detected between potential nitrification rate measurements and *in situ* measurements. Upstream of the maximum activity, nitrification is probably limited by oxygen concentration, while ammonia limitation, dilution by seawater and decreased residence time cause decreased nitrification activity in the downstream part of the estuary.

In Chapter 3 the community structure and dynamics of the ammonia-oxidising bacteria along the salinity gradient were analysed by use of Denaturing Gradient Gel Electrophoresis of PCR products obtained from naturally occurring DNA. The results show that a change in the community structure occurs along the estuarine gradient. In the upstream part of the estuary, bacteria belonging to *Nitrosomonas* cluster 6a were dominant, while a novel group of *Nitrosomonas*-like sequences was more common in the downstream part. *Nitrosomanas*

cluster 6a was also detected in an untreated wastewater sample from Brussels, indicating that wastewater might be one of the sources for nitrifying bacteria in the estuary.

The concentration of nitrous oxide was measured during an extensive sample program, showing that the estuary acts as a continuous source of nitrous oxide to the atmosphere (Chapter 4 and 5). Most of the abundant nitrous oxide was lost to the atmosphere, rather than transported to the sea. The total flux was estimated to be $2.8*10^8$ g per year (Chapter 4). Factors controlling the production of nitrous oxide were examined by field measurements (Chapter 4 and 5), and by laboratory experiments (Chapter 5). The field measurements showed that the peak in nitrous oxide concentration usually coincides with the peak in nitrification. The hypothesis that nitrification is the main source of nitrous oxide could be confirmed in an experiment with a specific nitrification inhibitor. Maximum nitrous oxide production occurred at 5 µM oxygen in the laboratory experiment, while the peak in the field occurred at 35 µM oxygen. Vigorously stirring in the experimental set-up, which disturbs the microstructure of flocks containing micro-organisms, might be the reason for this observed discrepancy. In conclusion the following mechanism is proposed for nitrification in the Schelde estuary (Chapter 6). The nitrifying freshwater population is transported along the Schelde river, but its development is hampered by the low oxygen concentration and the short residence times caused by high current velocities in this narrow part of the river. When the oxygen concentration is increasing, nitrification is stimulated and nitrifying bacteria can start to grow. At the same time, the salinity is increasing, which influences the community structure of nitrifying bacteria. At this point, the residence times are sufficient long to facilitate a community change. Because the oxygen concentration is still very low, parts of the nitrification products are released in the form of nitrous oxide. Further downstream the estuary, oxygen concentration increases to such levels that significant nitrous oxide production is no longer occurring. The low ammonium concentration is then limiting nitrification; the nitrifying community becomes less active and is diluted by seawater.

Although the water quality in the Schelde has improved drastically since the seventies, nitrogen loads, and consequently nitrification rates and nitrous oxide concentration still belong to the highest reported in comparison with other estuaries. Much is left to be done before the ecological value of the Schelde will catch up with the economical value that is has today.

Samenvatting

Een estuarium kan worden gedefinieerd als de plaats waar een rivier en de zee elkaar ontmoeten. In het estuarium worden de twee watermassa´s, de ene zoet, de andere zout met elkaar gemengd door de stroming van de rivier, de getijdenbeweging, de wind en golfslag. Deze mengzone kan vele kilometers lang zijn, en biedt ruimte aan een unieke fauna en flora, die is aangepast aan het brakke water. In zuidwest Nederland is de Westerschelde het enige overgebleven estuarium na de voltooiing van de Deltawerken. De Westerschelde, of het Schelde estuarium, zoals het in dit proefschrift wordt genoemd, wordt gekenmerkt door hoge stikstofconcentraties. De verhoogde stikstofconcentraties zijn het gevolg van het veelal ongezuiverde afvalwater van o.a. de steden Brussel, Antwerpen en Gent dat op de Schelde wordt geloosd, afvalwater van de industriegebieden langs de oevers en overbemesting van de langs de Schelde gelegen landbouwgebieden. Het water in estuaria is vaak erg troebel, doordat als gevolg van de dynamische krachten van het getij en de rivierstroom veel bodemdeeltjes worden opgewerveld. In de Westerschelde dragen de continue baggerwerkzaamheden om de vaargeul naar Antwerpen open te houden bij aan een verhoogde troebelheid van het water.

De eutrofiëring, overbemesting van de Schelde heeft er toe geleid dat in grote delen van het estuarium de zuurstofconcentratie erg laag is. Dit is vooral het geval in het stroomopwaartse, smallere deel van de Westerschelde, in de buurt van Antwerpen. In het bredere, stroomafwaartse deel van de Westerschelde wordt de zuurstofconcentratie geleidelijk aan hersteld door menging met schoner Noordzee water. Het troebele karakter en de slechte waterkwaliteit hebben de Westerschelde lang een “dood” estuarium gemaakt. Recentelijk echter zijn er aanzienlijke verbeteringen in de waterkwaliteit opgetreden en is het zuurstofgehalte toegenomen.

Er zijn vele factoren van invloed op de waterkwaliteit. Dit proefschrift richt zich op de rol van stikstof. Stikstof kan voorkomen in de vormen ammonium (NH_4^+), nitriet (NO_2^-), nitraat (NO_3^-), lachgas (N_2O) of stikstofgas, N_2. Bacteriën spelen een belangrijke rol in de stikstofkringloop, de omzettingen van deze stikstofvormen. De belangrijkste processen zijn nitrificatie en denitrificatie. Nitrificatie is een cruciaal proces in de stikstofkringloop omdat het het enige proces is waarbij ammonium geoxideerd wordt tot nitriet en nitraat. Dit nitriet en nitraat kan dan door denitrificatie worden omgezet in stikstofgas. Het stikstofgas dat in het water wordt gevormd, verdwijnt naar de atmosfeer. Denitrificatie is daarom een belangrijk proces in de natuurlijke waterzuivering van het estuarium. Behalve het onschadelijke

stikstofgas kan er tijdens nitrificatie en denitrificatie ook lachgas (distikstofoxide) worden gevormd. Lachgas is een broeikasgas en is bovendien betrokken bij de afbraak van de ozonlaag. De huidige toename van de concentratie lachgas in de atmosfeer, die vooral wordt veroorzaakt door industriële activiteiten, is daarom zorgwekkend. Natuurlijke systemen dragen echter ook in belangrijke mate bij aan lachgasproductie, en het is daarom van belang om de processen die leiden tot deze productie te bestuderen.

In dit proefschrift worden de factoren die van invloed zijn op nitrificatie en lachgasproductie in de Westerschelde nader bestudeeerd. Daartoe is de nitrificatie activiteit in de Westerschelde onder een langere tijd gemeten (Hoofdstuk 2). Daarbij is tevens aandacht besteed aan de methode om nitrificatie te meten en zijn experimenten uitgevoerd om te onderzoeken waardoor de nitrificatieactiviteit wordt beïnvloed. Nitrificatie werd gemeten met behulp van radioactief kooldioxide $^{14}CO_2$. Nitrificerende bacteriën nemen kooldioxide op als koolstofbron voor groei. Omdat ook andere bacteriën kooldioxide opnemen moet een specifieke remmer voor nitrificatie worden toegevoegd om te weten welk aandeel er door de nitrificeerders is gebruikt. In dit proefschrift is de remmer N-serve gebruikt. Door de CO_2 inbouw in monsters met en zonder N-serve van elkaar af te trekken kon worden uitgerekend hoeveel CO_2 en uiteindelijk ammonium er door de nitrificeerders is gebruikt. Deze meting is dus erg afhankelijk van de specificiteit van de remmer. Daarom zijn ook andere remmers getest. In hoofdstuk 2 wordt aangetoond dat de remmers methylfluoride en acetyleen een stimulerende i.p.v. een remmende werking hebben op de CO_2 inbouw door nitrificeerders in estuarien water, terwijl de beide remmers wel goed werken met reincultures van nitrificeerders. Waarschijnlijk treedt er in het estuariene water een reactie op waardoor deze remmers een verhoogde CO_2-inbouw geven. De resultaten in Hoofdstuk 2 laten verder zien dat de hoogste nitrificatie activiteit vrijwel altijd te zien is in het stroomopwaartse deel van het estuarium, in de buurt van Antwerpen. In dit deel zijn de omstandigheden voor nitrificatie optimaal. Meer stroomopwaarts in het estuarium is er te weinig zuurstof om nitrificatie mogelijk te maken, terwijl er meer naar zee toe niet genoeg ammonium beschikbaar is. Verdunning met zeewater en een kortere verblijfstijd zijn andere oorzaken van de afname van nitrificatie naar de zee toe.

In hoofdstuk 3 wordt aangetoond dat de soortensamenstelling van nitrificerende bacteriën in de loop van het estuarium verandert. Dit werd gedaan met behulp van een moleculaire methode waarbij het DNA van de nitrificerende bacteriën werd geanalyseerd. Nitrificerende bacteriën zijn niet met een microscoop van andere bacteriën en van elkaar te onderscheiden. Moleculaire technieken bieden daarom de enige mogelijkheid om iets over de identiteit van

de bacteriële gemeenschap te zeggen. Het feit dat er een verandering in de soortensamenstelling optreedt geeft aan dat de nitrificeerders een actieve populatie vormen die de dynamische omstandigheden en de veranderingen in zout, zuurstof en ammoniumgehalte in de loop van het estuarium goed kunnen opvangen. De meest voorkomende DNA-sequentie behorende tot een ammoniumoxideerder was die van een *Nitrosomonas*-soort. Deze soort is nauw verwant met de soort *Nitrosomonas urea*, die gecultiveerd is. Deze *Nitrosomonas*-sequentie werd ook aangetroffen in een monster van Brussels rioolwater, wat aantoont dat de nitrificerende gemeenschap in het estuarium mogelijk met dit afvalwater werd geënt. In het brakke en zoute deel van het estuarium werd de sequentie van een andere *Nitrosomonas*-soort aangetroffen die nog niet eerder beschreven was.

In hoofdstuk 4 worden lachgasconcentraties in de Westerschelde beschreven. Berekeningen aan de lachgasconcentratie laten zien dat de Westerschelde bijna altijd oververzadigd is met lachgas. Het in het estuarium gevormde lachgas verdween naar de atmosfeer. De totale lachgasemissie werd geschat op 280 miljoen gram per jaar.

In hoofdstuk 5 worden de oorzaken van lachgasproductie verder onderzocht. Welk proces is eigenlijk verantwoordelijk voor de hoge lachgasconcentraties in de Westerschelde? Uitgebreide veldmetingen toonden aan dat de maximale lachgasconcentraties samenvallen met de plek waar ook de nitrificatiepiek voorkomt. De hypothese dat nitrificatie een belangrijke bron van lachgas is kon worden bevestigd in een experiment met een specifieke remmer voor nitrificatie. In dit experiment werd verder aangetoond dat de lachgas productie het hoogst is bij zeer lage zuurstofconcentraties. Zuurstof is dus een zeer belangrijke factor die bepalend is of er lachgasproductie plaatsvindt. De hoeveelheid ammonium is vervolgens bepalend voor de hoeveelheid lachgas die geproduceerd wordt.

De waterkwaliteit van de Schelde is in de afgelopen 30 jaar erg verbeterd, hetgeen onder andere blijkt uit de hogere zuurstofconcentraties. Vergelijking met andere estuaria in de wereld laat echter zien dat de Westerschelde nog steeds het estuarium is waar de hoogste stikstof-en lachgasconcentraties worden aangetroffen. Hoewel de waterkwaliteit van de Schelde aan de beterende hand is, is er dus nog een lange weg te gaan tot een gezond estuarium.

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Curriculum vitae

Monique de Bie werd geboren op 23 december 1969 in De Moer, gemeente Loon op Zand. Zij volgde het basisonderwijs in De Moer en doorliep daarna van 1981 tot 1988 het VWO aan het Dr. Schaepmancollege in Dongen. In 1988 begon zij haar biologiestudie aan de Rijksuniversiteit Groningen. Van de afstudeervakken werd er een uitgevoerd bij het Nederlands Instituut voor Oecologisch Onderzoek, Centrum voor Estuariene en Mariene Oecologie in Yerseke met als onderwerp: Remineralisation of nutrients as caused by grazing of flagellates. Het tweede afstudeervak werd uitgevoerd bij het NIOO, Centrum voor Limnologie, Nieuwersluis met als onderwerp: Oxygen respiration and production in the littoral zone of Lake Gooimeer, field results and modelling. In 1991 nam zij deel aan de Netherlands Indian Ocean Program, Monsoons and pelagic ecosystems in Kenya. Het doctoraal examen microbiële oecologie werd met goed gevolg afgelegd in 1993.

In december 1993 trad zij in dienst van de Koninklijke Nederlandse Academie van Wetenschappen als AIO bij het Nederlands Instituut voor Oecologisch Onderzoek, Centrum voor Estuariene en Mariene Oecologie, in Yerseke. De resultaten daarvan zijn in dit proefschrift beschreven.

Vanaf september 1998 woont zij in Kalmar, Zweden en sinds februari 2000 is zij werkzaam als EU-liaison officer en student exchange coordinator aan de University of Kalmar.