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Nitrogen cycling in a turbid, tidal estuary

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Nitrogen cycling in a turbid, tidal estuary

De stikstofkringloop in een troebel getijdenestuarium

(met en samenvatting in het Nederlands)

Proefschrift

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1 Introduction

1.1 General introduction

In the beginning of the 20th century two men made an invention that came to affect mankind and nature ever after. Fritz Haber and Carl Bosch developed a process, later called the Haber-Bosch process, to produce ammonia from hydrogen (H_2) and nitrogen (N_2) gases. This made it possible for farmers to manifold their harvests since the ammonia produced in the Haber-Bosch process could be oxidized to make nitrates and nitrites essential for the production of nitrate fertilizer, which then could be produced at large scale. Today, global output of ammonia is about 130 million tonnes a year and four-fifths of this goes into fertilizers (Smil, 1999), sustaining 40% of the Earth's population, as well as having various deleterious environmental consequences (Fryzyk, 2004).

Nitrogen is biologically very important since it is needed by all living organisms, e.g. for DNA and RNA and required to make proteins. Nitrogen is only a minor constituent of living matter, compared to carbon, hydrogen and oxygen. These three however, can easily be taken from their large natural reservoirs in the air and water, while nitrogen can not, despite the fact that 78% of the air we breath is nitrogen in the form of N_2 gas. Humans, other animals and most plants can not use N_2 gas in air as a source of nitrogen since it is relatively inert due to the stable triple bond that keeps the molecule together. Only specialized groups of prokaryotes which have the enzyme nitrogenase and include both autotrophs and heterotrophs that can fix nitrogen from N_2 gas. The Haber-Bosch process however, made it possible for us humans to produce bio-available nitrogen.

The massive introduction of reactive nitrogen into soils and waters has had strong consequences for the environment. The problems caused by nitrogen introduction range from affecting local health e.g. through contamination of groundwater, to global changes e.g. production of the po-

tent climate-active gas nitrous oxide, and extend from deep underground to high in the stratosphere. The excess of fertilizers is transported to lakes, marshes and the ocean where they cause eutrophication. There is no clear definition of what eutrophication is, even though the term is frequently used. The word "eutrophication" originates from Greek where "eu" means "well" and "trope" means "nourishment". One common definition is increased supply of organic matter (Nixon, 1995) or increase in nutrient loading. Primary production is most often limited by the availability of light and nutrients. Eutrophication contributes to increased primary production and subsequently increased respiration or sedimentation of organic matter (Cloern, 2001; Gray et al., 2002). Degradation of organic matter leads to an increased oxygen demand which, under certain circumstances such as stratified water bodies or in the sediments causes oxygen depletion and release of toxic hydrogen sulfide (H_2S). Oxygen depletion or anoxia can change the community structure or lead to death of the benthic fauna. High concentrations of nutrients can increase growth of opportunistic macroalgae species which out-compete e.g. sea grasses and result in a change in macroalgae community structure.

The complexity of the nitrogen cycle has inspired many scientists to devote their careers to the fate of nitrogen. A lot is known about the nitrogen cycle but still today new pathways and processes are found, keeping scientists intrigued. The aim of this thesis is to investigate the nitrogen cycle in estuaries, and the factors governing the transformation rates of nitrogen. Different methods will be compared and as many others have done before, we aim to shed some light over some of the mysteries of the nitrogen cycle.

1.2 The nitrogen cycle

Nitrogen can undergo a series of oxidation/reduction reactions which changes the oxidation state from -3 in ammonia (NH_4^+) to +5 in nitrate (NO_3^-), (Table 1.1).

Table 1.1: Chemical species of nitrogen

Species	Molecular Formula	Oxidation Number of Nitrogen
Nitrate ion	NO_3^-	+ V
Nitrite ion	NO_2^-	+ III
Nitrous oxide gas	N_2O	+ I
Nitric oxide gas	NO	+ II
Nitrogen gas	N_2	0
Ammonia gas	NH_3	- III
Ammonium ion	NH_4^+	- III
Organic amine	RNH_2	- III

1.2.1 Nitrification

Ammonia can be oxidized to nitrite (NO_2^-) and further to nitrate through nitrification (Figure 1.1). This is one of the key processes in the nitrogen cycle, performed under oxic conditions by 2 groups of micro-organisms, one group of Bacteria and Archaea that oxidize ammonium to nitrite and another group of Bacteria that further oxidize nitrite to nitrate (Wuchter et al., 2006). Our understanding of the importance of nitrification has improved considerably over the past decade and it is now recognised that oxidation of ammonium plays an important role in linking organic matter mineralization (ammonification) and nitrogen removal through denitrification. Nitrifying bacteria are chemolithoautotrophic organisms which use energy from the oxidation of NH_4^+ or NO_2^- to fix inorganic carbon into their biomass. Accordingly, nitrification links the flows of nitrogen with those of oxygen and carbon.

Many different methods have been developed to estimate nitrification rates and several are based on the use of 'specific' inhibitors, e.g. nitrapyrine (N-serve), allylthiourea and chlorate (Herbert, 1999, and references therein). The inhibitors N-serve and allylthiourea prevent the first step of the nitrification and nitrification rates are estimated by measuring the difference in ammonium concentration in samples with and without inhibitor after incubation. An alternative to this technique is dark in-

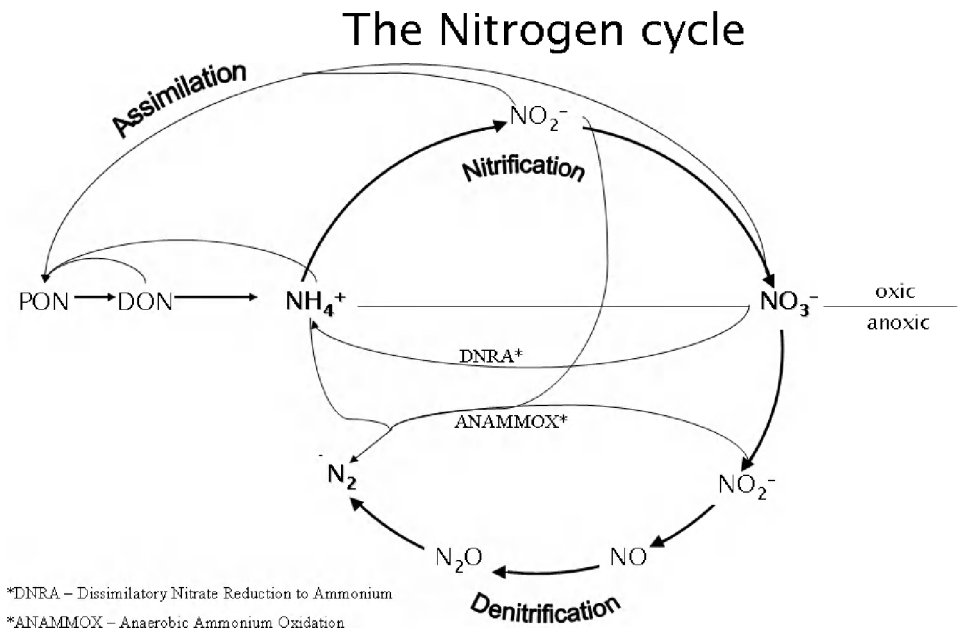

Figure 1.1: The nitrogen cycle.

corporation of labeled carbon, as ^{14}C-bicarbonate, in the presence and absence of an inhibitor. Nitrification rates are calculated using a N:C ratio, to transform carbon incorporation to ammonium oxidation. One drawback with this method is that the N:C ratio can vary between 4 to 40 depending on the system (Herbert, 1999). These problems have initiated the developing of new techniques to measure nitrification, e.g. using stable isotopes of ^{15}N-NH_4^+ and ^{15}N-NO_3^- (Koike and Hattori, 1978).

1.2.2 Denitrification

Anoxic reduction of NO_3^- to N_2 gas, denitrification, is a key biogeochemical process in which bioavailable nitrogen can be removed from a system as the gaseous end-products N_2 and N_2O that diffuse into the atmosphere (Figure 1.1). Denitrification is strongly linked to nitrification via coupled nitrification/denitrification where the NO_3^- originates from nitrification. The main factors governing N_2 production in aquatic systems are bottom water concentrations of NO_3^- and oxygen, temperature and organic matter loading (Seitzinger, 1988; Middelburg et al., 1996b; Cornwell et al., 1999).

Various methods have been used to measure denitrification e.g. mass balance methods (Lomstein et al., 1998; Seitzinger, 1987), acetylene inhibition technique (AIT) (Yoshinari and Knowles, 1976), direct N_2 production measurements (Seitzinger et al., 1984) and lately, ^{15}N-tracer techniques (Nielsen, 1992). All methods used previously have some disadvantages, limiting the possibility to obtain accurate denitrification rates. Mass balance techniques, where rates are calculated as differences between N-inputs and outputs, are cumbersome since differences are very small in comparison to inputs and outputs and prone to large errors since the errors from each estimate accumulate in the denitrification rate estimate. The AIT suffers from incomplete inhibition at low nitrate concentrations and simultaneous inhibition of nitrification resulting in underestimated denitrification rates (Lohse et al., 1996). Direct measurements of N_2 production should in theory be a straightforward and reliable technique. However, the change in N_2 concentration is often too low to be detected above the large background. ^{15}N-tracer techniques have proven to be successful. One example is the isotope pairing technique, developed by Nielsen (1992), where total denitrification rates along with the relative importance of coupled nitrification/denitrification can be estimated at the same time. ^{15}N-labelled

NO_3^- is added to the overlying water of a sediment core and the production of labeled N_2 is measured. The production of $^{29}N_2$ and $^{30}N_2$ provides information about the quantity of nitrate denitrified. However, in sediments with high anammox activities interpretation is cumbersome and rates are inflated, since anammox violates one of the central assumptions on which the IPT is built (Hietanen, 2007). According to Risgaard-Pedersen et al. (2003) the contribution of anammox accounts for < 6 % of N_2 production in estuarine sediments, thus, this is generally of little concern in these sediments. Another recent technique to estimate production of N_2 is determination of the N_2/Ar ratio using a membrane inlet mass spectrometer (MIMS) (Kana et al., 1994). Due to the high background concentration of N_2 in relation to a relatively small change resulting from denitrification during incubation, measurement of N_2 concentration changes alone is not enough. Normalizing N_2 with argon (Ar) however, makes it possible to detect the N_2 produced during the incubation because Ar is not affected by biological processes and therefore at equilibrium with the water (Figure 1.2). Normalizing N_2 with Ar cancels all fluctuations in N_2 concentration that not are due to biological processes.

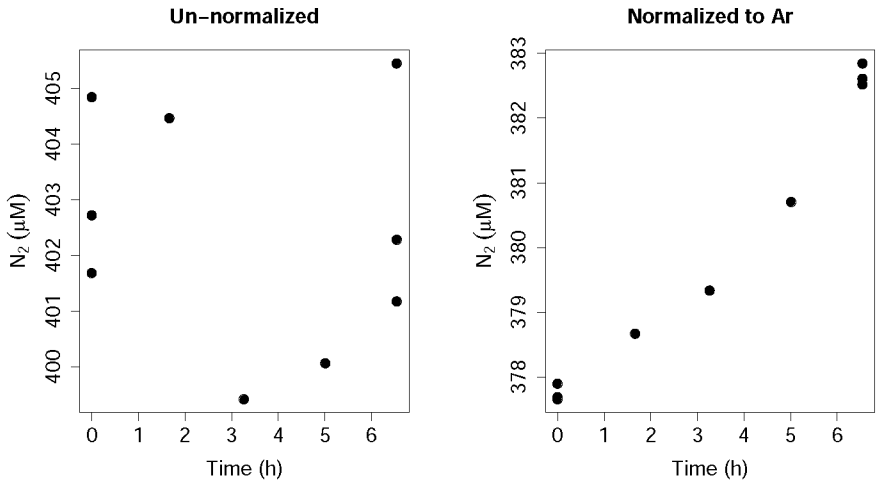

Figure 1.2: The left graph show measured concentrations of N_2 during an 7 hours incubation. The right graph show N_2 concentrations normalized versus argon from the same incubation.

1.2.3 Uptake of nitrogen

Dissolved organic nitrogen (DON) remains one of the unknowns of the nitrogen cycle. Less than 20% of the DON pool has been identified (Benner, 2002; Bronk, 2002). Known components are urea, amino acids (AA), proteins, oligopeptides, purines, nucleic acids and humic substances. Since the exact composition of the DON pool is unknown it is difficult to estimate the importance of this pool as a source of nitrogen. The concentration of DON frequently exceeds that of DIN. Historically, DON has been considered unavailable as source of N nutrition for phytoplankton, bacteria and macrophytes (Berman and Bronk, 2003). Recent findings have revealed that DON plays an active role in supporting N nutrition directly or indirectly. This has shifted the focus to DON in uptake studies. Some forms of DON, e.g. urea and amino acids, have been found to be an important N source for phytoplankton (Palenik and Morel, 1990; Bronk and Glibert, 1993; Berman and Chava, 1999; Fan and Glibert, 2005) as well as for heterotrophic bacteria (Wheeler and Kirchman, 1986; Hoch and Kirchman, 1995; Middelboe et al., 1995; Rosenstock and Simon, 2001).

1.3 Stable Isotopes

Isotopes of an element have nuclei with the same number of protons but different numbers of neutrons. There are several isotopes of nitrogen, ^{13}N, ^{14}N, ^{15}N and ^{16}N. The most abundant one is ^{14}N which constitutes 99.634% of all nitrogen in nature. ^{13}N and ^{16}N are short-lived radioactive isotopes, making them difficult and cumbersome to use in experiments. The stable isotope ^{15}N has a natural abundance of 0.366 % with small variations due to isotopic fractionation. Stable isotopes can be used in two ways, either by studying variations in their natural abundance or in deliberate tracer experiments. The work in this thesis focuses on the use of stable isotopes as deliberate tracers. A substrate highly enriched with the heavy isotope is added to a system and the heavy isotope is traced into the compartments of interest, e.g. addition of ^{15}N labelled NH_4^+ and following the appearance into particulate organic matter or NO_3^-. Isotopes are generally analyzed by gas-source isotope ratio mass spectrometry (IRMS). The stable isotopic composition of most particulate material is relatively simple to measure

using an elemental analyzer to convert particulate N into N_2, but isotope ratio measurements in dissolved inorganic nitrogen pools are more difficult. In order to measure e.g. ^{15}N-NH_4, the NH_4^+ must be removed from solution and concentrated in a form that can be introduced to a mass spectrometer.

The use of stable isotopes has opened a world of possibilities to increase our understanding about the mystery of nitrogen. The fate of one specific compound can be investigated and whole ecosystems can be labelled. Adding e.g. labeled NH_4^+, uptake into phytoplankton and bacteria can be quantified. Other transformations such as nitrification and coupled nitrification/denitrification can be determining by detecting labeled NO_3^- and N_2.

1.4 Estuaries

Our earth is equipped with systems such as estuaries and wetlands, which naturally clean eutrophied waters and counteract the dramatic impacts of increased nitrogen loading. An estuary is semi-enclosed with rivers flowing into it at one end and an open connection to the sea at the other end. There is a strong salinity gradient in an estuary, from fresh water in the inner part, to marine conditions at the mouth. The estuary acts as a filter between land and sea, nutrient rich water flows into the estuary via rivers and land runoff in the inner part of the estuary. A large fraction of the nitrogen that is delivered to estuaries is removed by burial of organic nitrogen in sediments and mostly by denitrification (Nixon et al., 1996). On the way through the estuary towards the sea, natural processes e.g. denitrification, lower the amount of nutrients and bioavailable nitrogen (Brion et al., 2007).

If no chemical or biological process would affect a compound transported through the estuary, concentrations would follow the pattern of conservative mixing (Figure 1.3, left graph). In estuaries however, the pattern of the middle graph in Figure 1.3 is found for compounds that are being consumed during transport to the sea, e.g. NH_4^+. Ammonium is consumed by nitrification and uptake by phytoplankton and bacteria. Conversely, NO_3^- is being produced in the estuary by nitrification (Figure 1.3, right graph). The combined result of all processes removing nutrients from estuaries is often called the estuarine filter (Soetaert et al., 2006). Estuaries

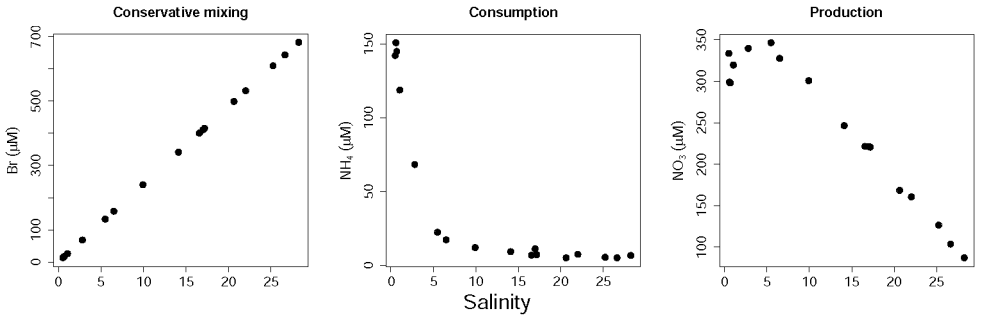

Figure 1.3: Left graph shows the expected pattern of conservative mixing as Cl vs. salinity. Middle graph shows the yearly average of NH_4^+ concentration in the Scheldt estuary as an example of a compound consumed during transport to the sea. The right graph shows the yearly average of NO_3^- as an example of a compound produced during transport to the sea. Data are for the year 2000.

are not the only example of natural systems counteracting eutrophication, marshes are another example. Marshes are a type of wetland and wetland systems are believed to be important sinks for nutrients (Gribsholt et al., 2005, 2006; Verhoeven et al., 2006).

1.4.1 Study sites

The experimental work presented in this thesis has been executed in the Scheldt estuary, the Oosterschelde and Tielrode fresh water marsh (Figure 1.4).

The Scheldt

The Scheldt estuary is located in the southwest of the Netherlands and Belgium and is among the most nutrient rich systems in the world (Soetaert et al., 2006). The estuary is fed by the river Scheldt, which originates in the northern part of France (St. Quentin) and flows into the North Sea near Vlissingen (The Netherlands). The Scheldt estuary is turbid and well mixed, with a water residence time of about 2 months (Heip, 1988). The

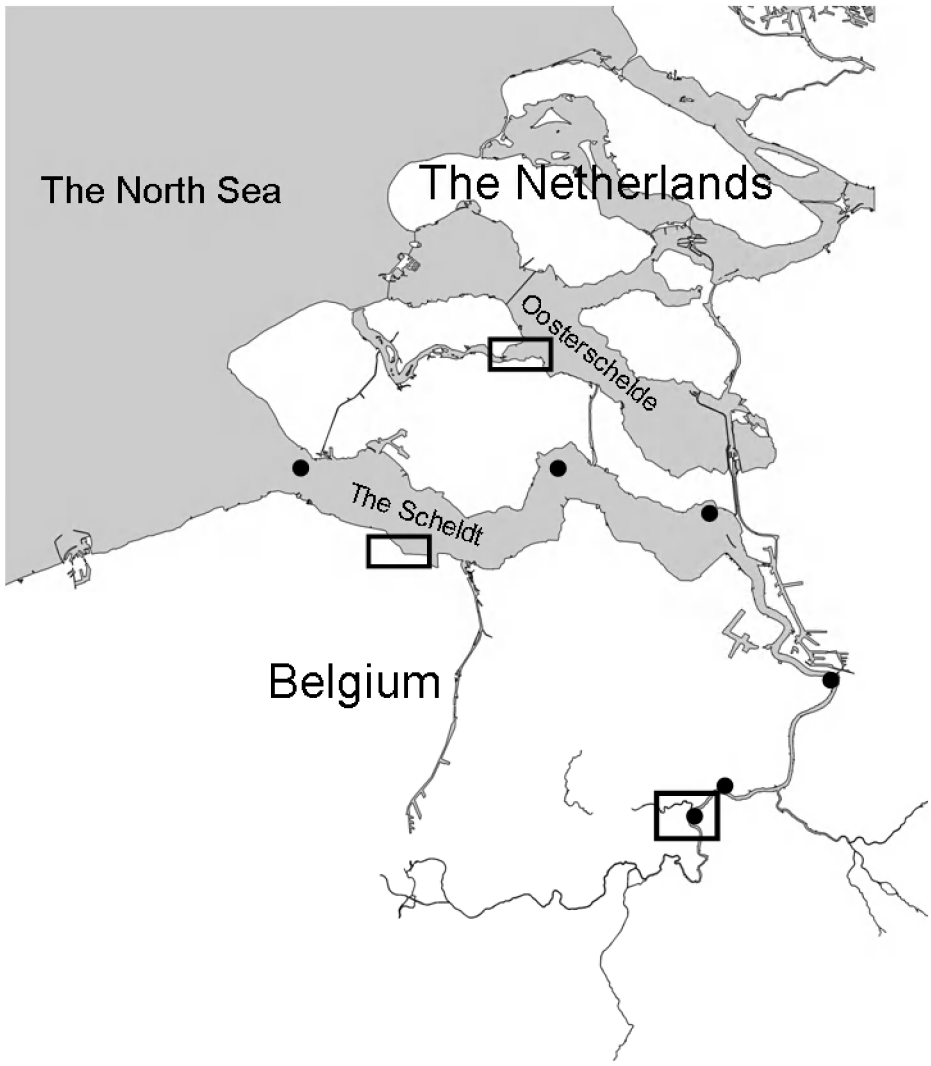

Figure 1.4: Study sites: squares indicate benthic studies and solid dots represent pelagic sampling locations.

tidal amplitude is high, ranging from 3.8 m in the western to 5.2 m in the eastern part, and the estuary is about 160 km long covering an area of $\sim$300 km^2. The total catchment area of the Scheldt river is 22000 km^2, with a population of more than 10 million people, and containing several large industrial areas. The three main tributary rivers joining the Scheldt estuary are the Dender, the Durme and the Rupel. The Rupel brings partially untreated waste water from the densely populated Brussel region (Soetaert et al., 2006). Water quality is poor in the greater part of the river and the eastern part of the estuary (Baeyens et al., 1998). Large efforts for industrial and municipal waste water treatment have been undertaken during the last decade, but still untreated municipal waste water is being discharged into the estuary, with the city of Brussel as the most prominent example (Van Damme et al., 2005). The Scheldt estuary includes many wetlands and is a typical example of an estuary that serves as a natural filter, "cleaning" the water on the way to the sea. The high amounts of nutrients that enter the inner part are reduced to much lower concentrations than what would be expected by conservative mixing. Before the seventies, a large part of the effluents from the industry and domestic waste were discharged into the Scheldt river and estuary, both directly and via the sewage systems. This caused severe oxygen deficiencies and major efforts were undertaken to reduce the nutrient load. The efforts have been successful and the nutrient load has decreased and the oxygen conditions have improved. Before 1995 the estuary behaved as a source of DIN while nowadays it acts as a sink, removing DIN (Soetaert et al., 2006).

The Oosterschelde

The Oosterschelde is a tidal inlet in the southwest of the Netherlands and a part of the former Delta area created by the rivers Rhine, Meuse and Scheldt before entering the North Sea. Before 1987 the Oosterschelde was an estuary but after the construction of a storm-surge barrier at the mouth and two auxiliary compartment dams near the head, the freshwater input was closed off and the Oosterschelde changed from an estuary into a tidal bay (Nienhuis and Smaal, 1994). The building of the storm-surge barrier had a large impact on the Oosterschelde. As a result, the water exchange with the North Sea was lowered with 28 %, which lead to decreased current

velocities and turbidity. The water quality and salinity have increased and nutrient loadings have decreased due to the lowered freshwater input. Gradients in salinity and nutrient concentrations along the estuary have disappeared (Smaal and Nienhuis, 1992). The water residence time in the Oosterschelde varies between 20 to 135 days depending on physical conditions and distance from the North Sea (i.e. shorter residence time closer to the North Sea) (Dame and Prins, 1998). The tidal range is on average 3 m (Smaal and Nienhuis, 1992).

Tielrode fresh water marsh

The tidal freshwater marsh located where the small tributary Durme flows into the Scheldt is approximately 100 km from the mouth of the Scheldt estuary. Because of the funnelshaped morphology of the Scheldt estuary, the tidal range (5.3 m) and turbidity are here at their maxima. The studied area is a triangular section of the marsh (3500 m^2) with dikes on two sides and water entering on the third. The side where water enters the marsh was screened of by wooden screens except for a 4.5 m wide opening. Along this opening a sampling and tracer release platform was constructed. The marsh contained a dense cover of typical freshwater marsh vegetation with reed, willows and ruderal vegetation. The studied area is only flooded at the highest tides.

1.5 Outline of this thesis

The aim of this thesis was to investigate nitrogen cycling in estuaries. The relationship between the processes studied in this thesis is displayed in Figure 1.5 with number indicating relevant chapter. The first two chapters focus on pelagic processes with NH_4^+ playing a central role. Nitrification, oxidation of NH_4^+ to NO_3^-, was studied in Chapter 2. These nitrification rates were related to uptake of NH_4^+ into particulate matter, in Chapter 3, in order to estimate the fate of NH_4^+. This matter was further investigate on a whole ecosystem level in Chapter 6, in combination with processes regarding other nitrogen compounds. Nitrogen transformations occurring in the sediment constituted the centre of interest in Chapters 4 and 5, focusing on N_2 production and factors regulating this production.

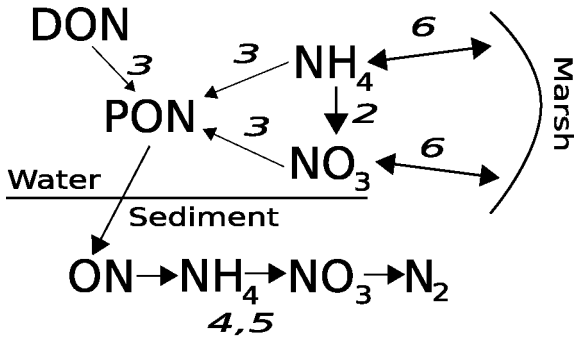

Figure 1.5: Processes of the nitrogen cycle studied in this thesis. Numbers in italics indicate in which chapter the process was studied.

In **Chapter 2** nitrifier growth (measured as carbon incorporation) was compared to nitrifier activity (ammonium oxidation to nitrate). Estimates of nitrification are often based on the ^{14}C labeled bicarbonate incorporation, using N:C conversion ratios. This technique was compared to production of NO_3^- from ^{15}N labeled NH_4^+ in the Scheldt estuary, at five station ranging in salinity from 0 to 28, during four occasions. The aim of this study was to investigate the validity of nitrification rates based on carbon incorporation using a single N:C ratio, in a variable system such as the Scheldt estuary.

Uptake of dissolved inorganic nitrogen, NH_4^+, NO_3^- and NO_2^-, was compared to uptake of dissolved organic nitrogen, in the form of amino acids and urea (**Chapter 3**). The relative use of the organic compounds as sources for nitrogen and carbon was also assessed. The study was conducted in the Scheldt estuary at five stations over a salinity gradient during four sampling campaigns.

Chapter 2 and 3 treat nitrogen processes in the pelagic zone. **Chapter 4** focuses on a key process in the sediment, production of N_2. Temperature and NO_3^- concentration are known as two of the main factors governing

the production of N_2 in sediments. In this chapter the effects of NO_3^- concentration and temperature were studied in sediment cores sampled in the Oosterschelde. To test the effect of *in situ* temperature, experiments were conducted during both winter and summer.

Another important factor affecting N_2 production is assessed in **Chapter 5**: presence of benthic fauna. *In situ* defaunation was achieved through anoxia by covering the experimental plots with thick plastic sheets. N_2 production rates were measured at control plots and where fauna was initially absent and slowly recovering over time. Temporary anoxia can occur as a consequence of eutrophication and we studied the eventual consequence of eutrophication induced hypoxia on N_2 production.

In **Chapter 6** the fate and transport of watershed-derived NH_4^+ in a freshwater marsh was quantified in a whole-ecosystem ^{15}N labeling experiment. ^{15}N-NH_4^+ was added to the floodwater entering the marsh area. NH_4^+ processing and retention were traced in six subsequent tide cycles. Changes in concentration together with isotopic enrichment of NO_3^-, NO_2^-, N_2O, N_2, NH_4^+ and suspended particulate nitrogen were measured.

The results obtained and conclusions reached are summarized in **Chapter 6.4.5**.

2 Comparison of nitrifier activity versus growth in the Scheldt estuary - a turbid, tidal estuary in northern Europe

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Aquatic Microbial Ecology, 42:149-158, 2006

2.1 Introduction

Nitrification, the microbial oxidation of ammonium (NH_4^+), is one of the key nitrogen transformation processes. It is performed under oxic conditions by 2 groups of bacteria, one group that oxidizes ammonium to nitrite (NO_2^-) and a second group that further oxidizes nitrite to nitrate (NO_3^-). Nitrification links organic matter mineralization (ammonification) and nitrogen removal through denitrification (conversion of nitrate to nitrogen gas). Nitrifying bacteria are chemolithoautotrophic organisms which use energy from the oxidation of NH_4^+ or NO_2^- to fix inorganic carbon in their biomass. Accordingly, nitrification links the flows of nitrogen with those of oxygen and carbon. In ammonium-rich systems, nitrification may make significant contributions to, or even dominate, total oxygen consumption and carbon fixation. Nitrifiers were responsible for up to 60 % of the total O_2 consumption in the Seine estuary, France (Garnier et al., 2001). Nitrification is the most important carbon fixation process in the upper part of the Scheldt estuary (Soetaert and Herman, 1995), where it accounts for 12 to 78 % of oxygen consumption (Gazeau et al., 2005). One by-product formed during nitrification is nitrous oxide gas (N_2O), which contributes to global warming and ozone depletion. de Wilde and de Bie

(2000) showed that a major portion of N_2O production in the Scheldt estuary results from nitrification, and that almost all of it is lost to the atmosphere within the estuary and is not transported out to sea. In recognition of the pivotal role of nitrification in estuarine biogeochemistry, research has been initiated to identify the organisms and governing factors of this process so that it may be accurately quantified (Bollmann and Laanbroek, 2002; de Bie et al., 2002b; Caffrey et al., 2003). One of the most common methods to measure nitrification rates is the N-serve sensitive ^{14}C-bicarbonate incorporation technique (Somville, 1978; Brion and Billen, 1998), henceforth referred to as the ^{14}C method. It is based on the incorporation of inorganic carbon by growing nitrifiers. Nitrification rates measured with the ^{14}C method are expressed as the amount of carbon incorporated over time. However, many researchers are interested in nitrogen transformations (rather than nitrifier growth) and use a conversion factor to express these carbon incorporation rates into nitrogen oxidation rates. This approach requires (1) a tight coupling between nitrifier activity (NH_4^+ oxidation to NO_2^- or NO_3^-) and growth (carbon incorporation), and (2) a fixed stoichiometry between the quantity of nitrogen transformed and carbon fixed. Although these 2 requirements are essential for the conversion of C incorporation rates into N oxidation rates, their validity has not been tested thoroughly in natural conditions.

Nitrifying bacteria are relatively slow growing, growth rates between 0.04 and 0.06 h^{-1} (Helder and de Vries, 1983), implying that nitrifying communities need time to adapt to environmental changes. Organisms in estuarine systems are submitted to variable biogeochemical conditions. Seasonal factors like temperature, nutrient availability and oxygen concentration can affect nitrifier activity and growth (Carlucci and McNally, 1969; Goreau et al., 1980; Berounsky and Nixon, 1993; Bodelier et al., 1996; Bollmann et al., 2002). Estuarine nitrifier populations are subject to large environmental variations because of tides and strong salinity gradients across estuaries. Different populations of ammonium oxidizing bacteria occur at estuarine sites with different salinities (de Bie et al., 2001; Bollmann and Laanbroek, 2002). In turbid estuaries, nitrifying bacteria are attached to suspended particulate matter in a manner similar to that in a "fluidized bed reactor" (Owens, 1986; Brion and Billen, 2000). Particle-attached nitrifiers experience a longer residence time in the estuary, which allows them to develop their population (Brion et al., 2000).

However, these particles with associated nitrifiers are subject to repeated cycles of settling and resuspension. Given these multiple causes of environmental variability, one might question whether nitrifier growth (as measured with the ^{14}C method) and activity are coupled. The main aim of the present study was to assess whether nitrifier activity and growth are so tightly coupled that nitrogen oxidation can be estimated from carbon incorporation in combination with conversion factors. In parallel with the ^{14}C method, nitrification rates in the Scheldt estuary were measured with a stable isotope technique where ^{15}N labeled NH_4^+ was added and the appearance of ^{15}N in NO_3^- after incubation was measured.

2.2 Materials and methods

2.2.1 Study Area

The Scheldt estuary is located in the southwest of the Netherlands and Belgium. The estuary is fed by the Scheldt river which originates in northern France (St. Quentin) and flows into the North Sea near Vlissingen (Netherlands). The Scheldt estuary constitutes a dynamic environment: it is turbid and well mixed, with a water residence time of about 2 months (Heip, 1988). The tidal amplitude is high, ranging from 3.8 m in the western to 5.2 m in the eastern part, and the estuary is about 100 km long covering an area of ~300 km^2. The total catchment area of the Scheldt river is 22000 km^2, contains several large industrial areas, and supports a population of approximately 10 million (Soetaert et al., 2006). Compared to other tidal estuaries, the maximum turbidity values in the Scheldt are low and the zone of maximum turbidity less pronounced. The river and its tributaries are a major drain for industrial and domestic waste discharges, which are not all treated in waste water treatment plants. Water quality is poor in the greater part of the river and the eastern part of the estuary (Baeyens et al., 1998). Until the mid 1970s discharges caused an increase in nutrient levels, but after the end of the 1970s nutrient loading decreased and the oxic conditions of the Scheldt estuary improved (Soetaert et al., 2006). In the early 1970s denitrification occurred in the water column, but since 1980 water column denitrification has been reduced and a nitrification front has progressed toward the freshwater section. Water

column denitrification has not occurred since 1990, and since 2000 most nitrification has occurred in the upstream region (Soetaert et al., 2006). Nevertheless, nitrification is the most pervasive process of the nitrogen cycle in the Scheldt estuary.

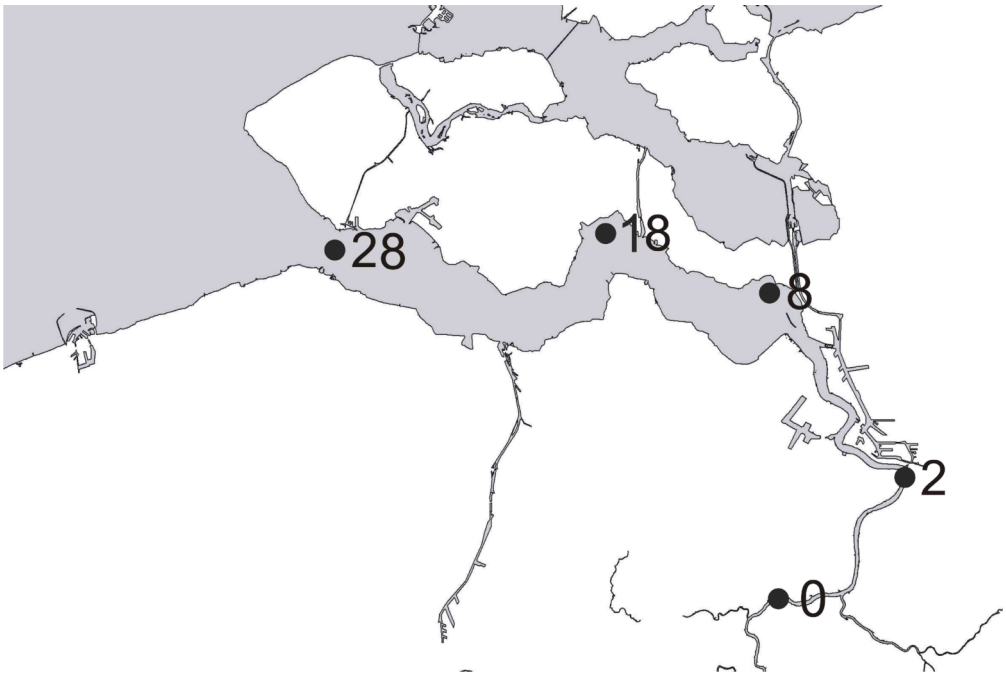

Figure 2.1: Scheldt estuary. Numbers indicate salinity and approximate location of stations

2.2.2 Sampling

During 2003, 4 cruises were conducted with RV 'Luctor' in January, April, July and October, 1 in each of 4 seasons. Five stations were sampled during every cruise. The stations were of fixed salinity but their exact location varied depending on tide and discharge. The salinities of the 5 stations were 0, 2, 8, 18 and 28, and these numbers are used as station names from this point onwards. Station 0 was located close to Dendermonde, Belgium, 122 km from the mouth of the estuary, whereas Station 28 was located at the mouth of the estuary, close to Vlissingen, the Netherlands

(Figure 2.1). Since the stations were situated along a salinity gradient, nutrient and oxygen concentrations and bacterial communities differed among the 5. Water was sampled in 20 L Niskin bottles from approximately 2 m depth and subsampled immediately after retrieval of each bottle.

2.2.3 Concentration measurements

Samples were taken from 17 fixed monitoring stations along a transect through a salinity gradient that ranged from 0 to marine. The water was filtered through preweighed, precombusted Whatman GF/F filters (47 mm), stored frozen, and then analyzed for ammonium, nitrate and nitrite using automated colorimetric techniques. The filters were weighed and analyzed for concentrations of suspended particulate matter (SPM) and particulate organic carbon and nitrogen using a Carlo Erba NA 1500 elemental analyzer (Nieuwenhuize et al., 1994). Salinity, temperature and oxygen were measured at all 17 stations.

2.2.4 Nitrification measurements

^{15}N method

Water samples were spiked with 98 % ^{15}N labeled NH_4^+, at levels that approximated 2.5 % of ambient NH_4^+ concentration. Samples were incubated at *in situ* temperature in dark bottles in a tank with running estuarine water. Incubations were terminated after 3, 6, 9, 12 and 24 hours by filtration through precombusted Whatman GF/F filters (20 mm). MgO (3 g L^{-1}) and (depending on salinity of the sample) NaCl were added to the water immediately after filtration, to a final salinity of 35. Measurements of $\delta^{15}NH_4$ and $\delta^{15}NO_3$ were based on the diffusion method of Sigman et al. (1997) and Holmes et al. (1998), as modified by Middelburg and Nieuwenhuize (2001) in order to extract the NH_4^+ from the water. The NH_4^+ dissolved in the water sample was converted to ammonia gas (NH_3) under alkaline conditions. The NH_3 was then trapped as ammonium sulfate on an acidified precombusted Whatman GF/D 10 mm filter, sandwiched between two 2.5 cm, 10 μm pore size Teflon membranes. After trapping NH_4^+ in the sample, Devarda's alloy was added to convert all

the NO_3^- to NH_4^+, which was then extracted as described above. Since all the original NH_4^+ was removed during the first step, NH_4^+ trapped on the second filter was completely derived from NO_3^-. The ^{15}N content of the GF/D filters was determined using a Fisons NA 1500 elemental analyzer coupled to a Finnigan Delta S masspectrometer via a Conflo II interface. During incubation, the ^{15}N label added as NH_4^+ was oxidized through nitrification and appeared in NO_3^-, see Figure 2.3. Control experiments (to which the nitrification inhibitors N-serve and chlorate [see below] were added) showed no transfer of ^{15}N from the NH_4^+ to NO_3^- pool during 24 h.

When the increase of ^{15}N in NO_3^- was linear (see Figure 2.3A) a linear regression was fitted, but in some cases a curve gave a better fit (See Figure 2.3B, 'Results'):

$$Y = a_0 - a_1 e^{-kt} \tag{2.1}$$

Results from these regressions were used to calculate nitrification rates (R_N) using the following equation (Dugdale and Goering, 1967):

$$R_N = \frac{b}{(\alpha_{\mathrm{NH_4^+}} - \alpha_{\mathrm{n}})}[\mathrm{NO_3^-}] \tag{2.2}$$

where b is the slope obtained from the linear regression or the initial slope obtained with the fitted curve (atom % ^{15}N h^{-1}), $\alpha_{NH_4^+}$ is the initial ^{15}N content in NH_4^+ after addition of ^{15}N, α_n is the natural abundance of ^{15}N in NH_4^+, and $[NO_3^-]$ is the average *in situ* concentration of NO_3^- during incubation, which was measured on the filtered water sample as described above. The estimated errors of R_N were obtained from the variance/covariance matrix of the fitted parameters and error propagation. Eq. 2.2 does not take other processes such as uptake or regeneration into account. Uptake of NH_4^+ should not affect the fraction of ^{15}N in NH_4^+ and consequently not influence the nitrification rate. If a high uptake of NO_3^- occurred, the calculated nitrification rates would be underestimated. Parallel measurements of the uptake of NH_4^+ and NO_3^- demonstrated that NH_4^+ is taken up to a much larger extent than NO_3^-. Regeneration of NH_4^+ would dilute the ^{15}N content of the NH_4^+ and cause nitrification rates to be underestimated at high regeneration rates.

^{14}C method

Carbon incorporation by autotrophic nitrifiers was measured with the inhibitor based ^{14}C-bicarbonate incorporation technique as described by Brion and Billen (1998). Water samples, taken from the same Niskin bottle as the samples used for the ^{15}N method, were spiked with ^{14}C-bicarbonate (50 mCi mmol^{-1}, Amersham) to a final concentration of 5 μmol L^{-1} and incubated in dark bottles in the tank mentioned above. Carbon incorporation was measured at 3 time intervals over a 9 to 24 hours period. Incorporation due to nitrifier growth was determined using the difference between the amount of $H^{14}CO_3^-$ incorporated in samples with and without nitrification specific inhibitors. N-serve (2-chloro-6-trichloromethyl pyridine; 5 mg mL−1) and sodium chlorate ($NaClO_3$; 10 mM) were used to inhibit the ammonium and nitrite oxidation, respectively. Nitrification rates (expressed in nMC h^{-1}) were calculated as the slope difference of linear regression lines of C incorporated versus time between samples with and without the inhibitor.

Pilot study

Prior to initiation of the field experiments, a pilot experiment was conducted. In November 2002, water from a fresh water station in the Scheldt estuary was sampled and nitrification rates were measured using the ^{15}N and ^{14}C method (as described above) in a laboratory where more extensive sampling was possible than during a cruise. Moreover, incubations with ^{15}N method were performed under dark and light (approximately 200 μmol photons m^{-2} s^{-1}) conditions to assess the light dependence of nitrification.

2.3 Results

2.3.1 Concentration measurements

Concentrations of NH_4^+ and NO_3^- + NO_2^- showed a similar pattern in all seasons (Figure 2.2). NH_4^+ concentrations were highest in the fresh water section of the estuary ($\leq$ 150 μM) and decreased with increasing salinity.

The decrease was faster during July and October. NO_3^- + NO_2^- concentrations in the fresh water section were around 300 μM, either increased or were uniform until a salinity of ~10, and from there decreased throughout the estuary. The decrease of NH_4^+ and increase of NO_3^- in the upper estuary reflected intensive nitrification. The O_2 concentration varied among seasons and also along the estuary transect. Oxygen concentrations were generally low in the upper part of the estuary, even hypoxic during July and October. Oxygen concentrations increased toward the mouth of the estuary and reached saturation levels and even supersaturation (415 μM) in April. The SPM varied from 250 mg L^{-1} in freshwater in October to 10 mg L^{-1} in marine water in January. Water temperature varied with season and was recorded at 4, 10, 20, 14 °C in January, April, July and October, respectively.

2.3.2 Nitrification rates

In most samples, the ^{15}N in NO_3^- showed a linear increase with time (Figure 2.3B), but for some (Figure 2.3A) the increase was non-linear. Non linearity could be explained by substrate limitation, as shown in the comparison of NO_3^- production at Stations 0 and 2. At Station 2 the NH_4^+ concentration at the start of the incubation was 14 μM and the nitrification rate was 124 nM h^{-1}, which meant that only 16 % of the initial NH_4^+ was consumed during the 24 h incubation. At Station 0 the initial NH_4^+ concentration was 4 μM and the nitrification rate was 621 nM h^{-1}. At this rate, the initial stock of NH_4^+ was consumed within 6 h of the incubation. Regeneration of NH_4^+ during incubation may also have contributed to the non-linear increase of ^{15}N in NO_3^-: in this case, ^{15}N label in the NH_4^+ pool would have become diluted. Although further transfer of ^{15}N from NH_4^+ to NO_3^- could not be detected after 6 h (Figure 2.3A), nitrifier growth and likely nitrification of regenerated NH_4^+ still continued for at least several hours at the same rate (Figure 2.3C). Highest nitrification rates were recorded with both methods in the fresh water region of the estuary and decreased toward the North Sea, similar to the observed pattern of NH_4^+ concentrations (Figure 2.4 and Figure 2.2). With the ^{15}N method, nitrification rates ranged from 700 nM N h^{-1} in October to 150 nM N h^{-1} in April in fresh water, and from 20 nM N h^{-1} to not detectable in the marine water. With the ^{14}C method, rates at Station 0

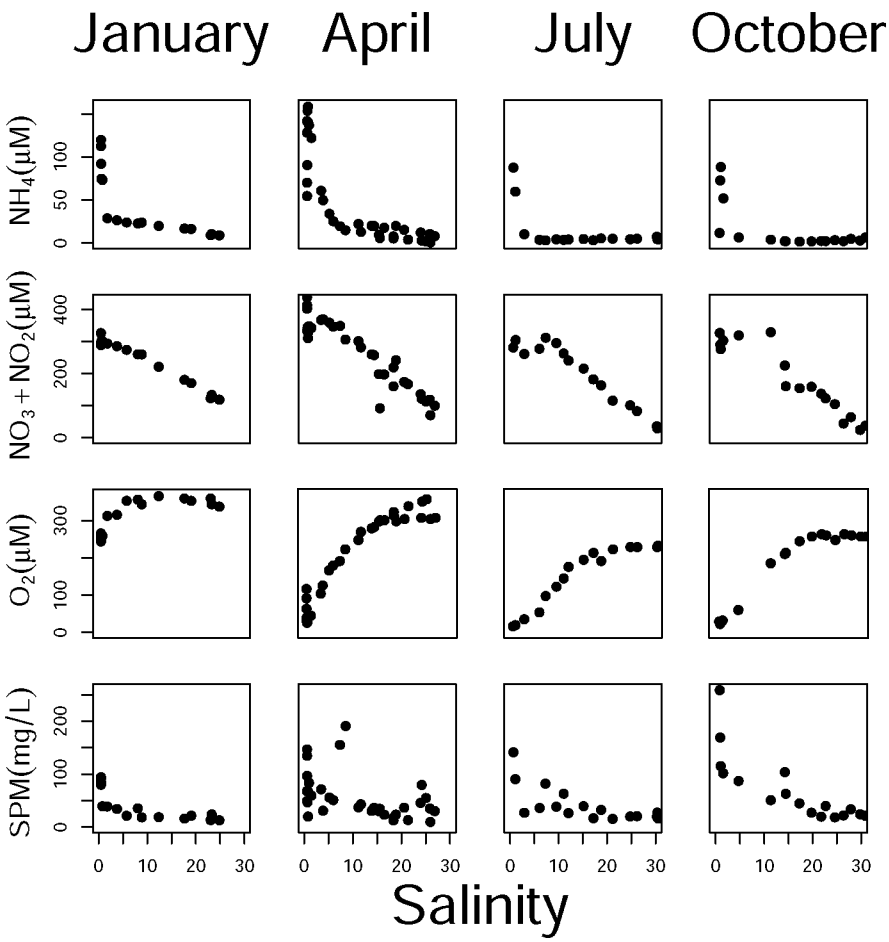

Figure 2.2: Concentrations of NH_4^+, NO_3^- + NO_2^-, O_2 and suspended particulate matter (SPM) in the pelagic zone of the Scheldt estuary during 2003.

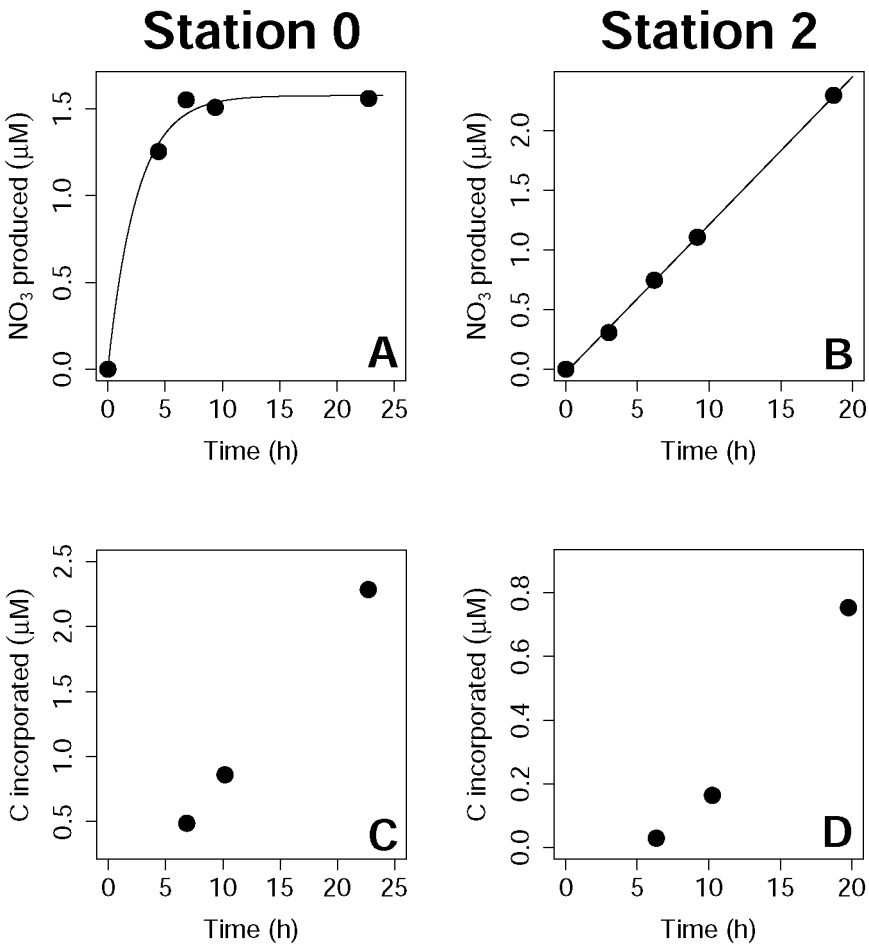

Figure 2.3: (A, B) NO_3 produced and (C, D) carbon incorporated at Stations 0 and 2 in July.

ranged from 150 nM C h^{-1} in October to not detectable in January. Nitrification rates decreased toward Station 28 where values from 1 nM C h^{-1} in January to 10 nM C h^{-1} in October were measured (Table 2.1).

In January, high rates were measured with the ^{15}N method and not detectable or very low rates with the ^{14}C method (Figure 2.4, Table2.1). In April, the ^{15}N method also showed relatively high activity compared to growth when measured using the ^{14}C method at Station 8. This corresponded to an increase in SPM content at this station during this season (Figure 2.2). The error was larger when a fitted curve was used to calculate nitrification rates (Figure 2.4 and Table 2.1). This observation should be taken into account when interpreting the results.

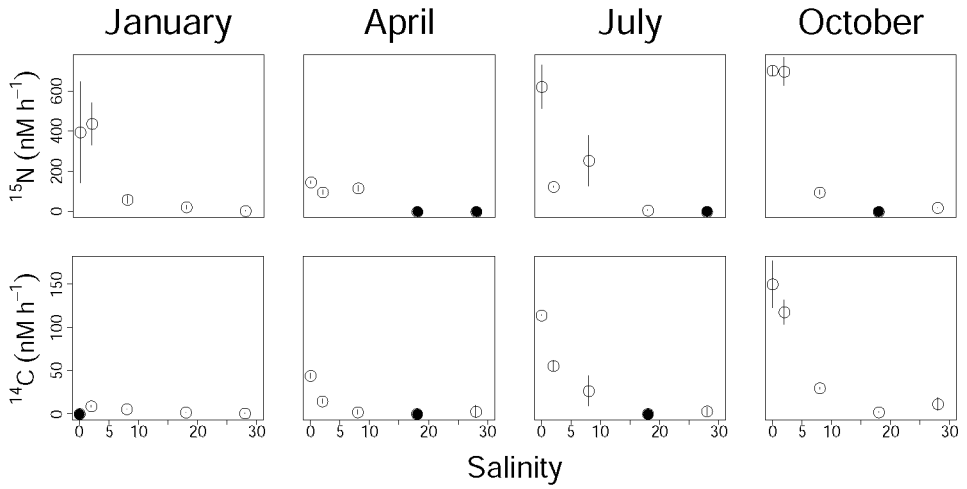

Figure 2.4: Nitrification rates measured with the (top row) ^{15}N method or (bottom row) ^{14}C method during 4 seasons over a salinity gradient. For details of the 2 methods, see 'Material and methods'. (•) Not detectable; errors are SE calculated from fitted parameters

2.3.3 Pilot study

NO_3^- produced during the light and dark incubations is shown in Figure 2.5A. It appears that nitrification was linear up to 24 hours and is a

Table 2.1: Comparison of nitrification rates measured with ^{15}N and ^{14}C methods (see 'Materials and methods') at 5 salinities (Sal.) during 4 seasons. Curve (C): nitrification rate for ^{15}N method calculated using fitted curve, Line (L): nitrification rate for ^{15}N method calculated using fitted line, nd: not detectable, errors are SE calculated from fitted parameters.

Sal.	Month	^{15}N nM N h^{-1}	^{14}C nM C h^{-1}	N/C ratio	Curve/ Line
0	January	395.2 ± 251.0	nd	nd	C
	April	146.6 ± 5.0	44.1 ± 3.0	3.3 ± 0.3	L
	July	620.9 ± 106.8	114.0 ± 0.3	5.4 ± 0.9	C
	October	701.5 ± 22.1	150.0 ± 27.0	4.7 ± 0.9	L
2	January	437.3 ± 105.2	8.9 ± 1.2	49.2 ± 13.5	L
	April	97.2 ± 10.4	14.6 ± 2.5	6.7 ± 1.4	L
	July	124.3 ± 1.9	55.5 ± 6.3	2.2 ± 0.3	L
	October	696.8 ± 68.7	117.8 ± 14.2	5.9 ± 0.9	L
8	January	59.5 ± 18.8	5.5 ± 0.1	10.8 ± 3.4	C
	April	116.7 ± 14.0	1.9 ± 2.5	60.2 ± 77.2	L
	July	254.0 ± 125.0	26.5 ± 17.3	9.6 ± 7.8	C
	October	96.5 ± 10.5	29.8 ± 1.0	3.2 ± 0.4	L
18	January	23.5 ± 9.3	1.7 ± 0.2	13.7 ± 5.8	C
	April	nd	nd	nd	
	July	6.9 ± 1.8	nd	nd	L
	October	nd	2.0 ± 0.6	nd	
28	January	4.0 ± 1.1	0.6 ± 0.2	6.8 ± 2.7	L
	April	nd	2.6 ± 7.2	nd	
	July	nd	2.9 ± 5.0	nd	
	October	19.7 ± 0.7	11.3 ± 7.6	1.7 ± 1.2	L

light sensitive process. NH_4^+ concentration at the start of the incubation was 102 μM and nitrification rates from light and dark incubations were 443 $\pm$ 9 and 792 $\pm$ 17 nM N h^{-1}, respectively. Accordingly, 44 % of the nitrification activity was inhibited by the light. Parallel incubations in the dark with the ^{14}C method revealed a rate of 197 $\pm$ 16 nM C h^{-1} (Figure 2.5B) . When we combined ^{14}C and ^{15}N methods for dark incubations we observed that 4.0 $\pm$ 0.3 moles of nitrogen were transformed for each mole of carbon fixed.

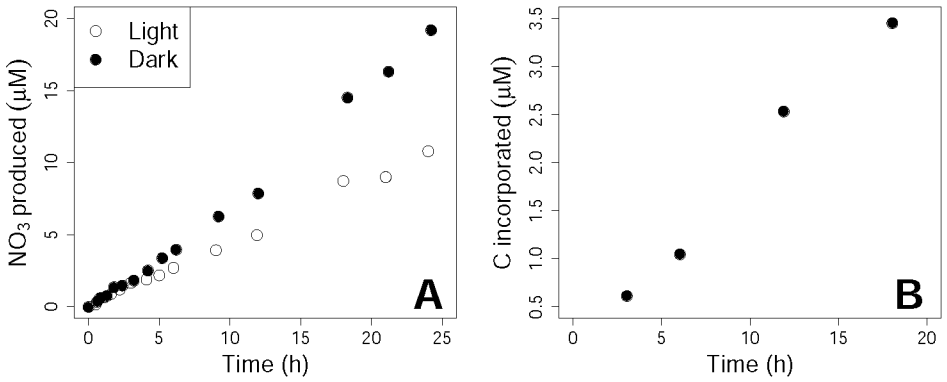

Figure 2.5: (A) NO_3 produced during light and dark incubations, ^{15}N method. (B) Carbon incorporated during dark incubations, ^{14}C method. For details of the 2 methods, see 'Materials and methods'

2.4 Discussion

Given that nitrification plays a pivotal role in estuarine biogeochemistry, it is essential to have a reliable and accurate technique to quantify the actual activity of ammonium and nitrite oxidizing bacteria. This is especially true when the results are to be used to calculate nitrogen budgets. The ^{14}C method (Somville, 1978) is probably the most common way to measure nitrification and is based on quantifying presence and growth of nitrifying bacteria (Brion and Billen, 1998). This approach is an excellent way to determine whether nitrifying bacteria are growing, but it can not be

used directly as a quantitative measure of nitrification. Nitrification rates obtained from the ^{14}C method (in carbon units) need to be converted into nitrogen units to be relevant in a nitrogen context. This conversion requires knowledge of how much carbon is fixed by the nitrifier community in order to oxidize a known amount of ammonium to nitrate, and was the subject of extensive research during the 1970 and 1980s (Table 2.2).

Literature values for conversion ratios have often been determined under optimal laboratory growth conditions on pure strains of oxidizing bacteria, e.g. *Nitrosomonas* or *Nitrospira* strains for ammonium oxidation and *Nitrobacter* strains for nitrite oxidation. These studies (Table 2.2) revealed highly variable results, with N:C ratios ranging from 6.0 (Owens, 1986) to 18.9 (Helder and de Vries, 1983). It is questionalbe whether these ratios are applicable in nature where environmental conditions as well as bacterial population and composition are variable. Factors such as temperature (Bianchi et al., 1997), salinity (Feliatra and Bianchi, 1993), oxygen concentrations (Carlucci and McNally, 1969; Goreau et al., 1980), and substrate availability (Belser, 1984) can affect the N:C ratio. The assumption that slow-growing nitrifiers (Helder and de Vries, 1983) have a conversion factor similar to nitrifiers under optimal growth conditions, in such a heterogeneous and highly variable system as an estuary, is quite possibly erroneous. Considering that even optimal growing populations have variable N:C ratios (Table 2.2), the applicability of a single, constant conversion factor is even more dubious.

Several studies have expressed strong concerns over this issue. For example, one study conducted under optimal growth conditions produced a low N:C ratio, because the bacteria needed little energy for growth under optimal conditions. Therefore, use of this factor provides a minimum estimate of nitrification activity *in situ* (Owens, 1986). The ^{14}C method can be used as a relative index of nitrification, but accurate estimation of the rate of nitrogen oxidation can only be deduced if a constant ratio exists for natural populations of nitrifying bacteria (Billen, 1976). As stated by Hall (1982), "It is unlikely that one ratio could possibly cover a range of environmental conditions and that the absence of reliable data casts doubt on the general applicability of the ^{14}C method as a quantitative measure of nitrification".

Some attempts have been made to quantify the *in situ* N:C ratio through parallel measurements with the ^{14}C method and alternative methods for

Table 2.2: Comparison of N:C ratio values

N:C	Bacterial community	Studied area	Source
8.3	Enrichment cultures	North Sea	Billen (1976)
18.9	Cultures of pure *Nitrosomonas* and *Nitrobacter winogradskyi* strains	Ems-Dollard estuary	Helder and de Vries (1983)
9.1	Cultures of *Nitrosomonas europaea* and *Nitrospira* sp. strains and *Nitrobacter winogradskyi* strains	Cultured bacteria	Belser (1984)
6.0	Enrichment cultures	Tamar estuary	Owens (1986)
8.6-9.8	Natural samples	Rhone estuary	Feliatra and Bianchi (1993)
2-60	Natural samples	Scheldt estuary	Present study

nitrification (Enoksson, 1986; Feliatra and Bianchi, 1993; Dore and Karl, 1996; Bianchi et al., 1997). A ^{15}N labeling approach similar to the one used in this paper was used by Enoksson (1986), but the addition of labeled NH_4^+ increased the concentration far above ambient concentrations. Consequently, potential rates were obtained and this complicated a direct comparison between methods. Nevertheless, the main conclusion was that the ^{15}N method and ^{14}C method should be used in parallel in future studies to determine whether the N:C ratio is constant. When comparing the ^{14}C method with direct measurments of changes in NO_2^- and NO_3^- concentrations in incubated samples, and using an N:C ratio of 8.3 (Billen, 1976), Dore and Karl (1996) found that the 2 independent methods agreed reasonably well in open ocean waters despite exhibiting a high degree of variability. Nitrification has also been assessed with the ^{14}C method and via the change in NO_2^- concentration using specific inhibitors that block the first and the second step of nitrification, respectively (Feliatra and Bianchi, 1993; Bianchi et al., 1997). These studies demonstrated that the N:C ratio varied with salinity, and decreased from the river to the sea (Feliatra and Bianchi, 1993). In addition, a strong negative correlation between the N:C ratio (values ranging between 3 to 9) and temperature was observed in the Indian sector of the Southern Ocan, confirming that the ratio fluctuates with environmental conditions (Bianchi et al., 1997).

Although these studies report variable N:C ratios and concerns about the validity of using 1 fixed ratio in a fluctuating environment, many other studies continue to report nitrification rates in nitrogen units are based on the ^{14}C method and converted with a ratio provided by those studies referred to above: e.g. Indrebo et al. (1979); Joye et al. (1999); Brion et al. (2000), and de Bie et al. (2002b) used the N:C ratio of Billen (1976); Berounsky and Nixon (1990, 1993); Iriarte et al. (1997) and Iriarte et al. (1998) used the N:C ratio of Owens (1986); and Jiang and Bakken (1999b) used the ratio from Belser (1984).

The relationship between nitrifier activity and nitrifier growth observed in our study is shown in Figure 2.6. A constant conversion factor imposes a straight line with a slope corresponding to the quantity of nitrogen transformed per unit carbon fixed. In the Scheldt estuary a conversion factor of 8.3 is often used (Billen, 1976), which is shown as a dashed line in Figure 2.6. We observed large, systematic deviations from the N:C ratio of 8.3 for January and October samples taken in the upper estuary (Fig-

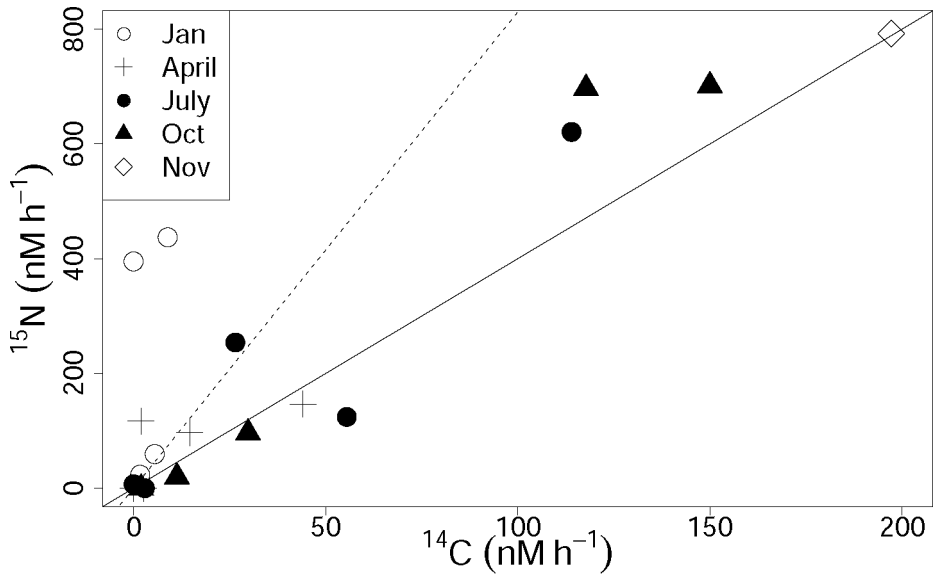

Figure 2.6: ^{15}N method versus ^{14}C method (for details of methods, see 'Materials and methods'). Dashed line: conversion factor 8.3 (Billen, 1976); solid line: conversion factor of 4.0 (our pilot study), November data: result from pilot study.

ure 2.6). Rates measured in January showed high nitrification activity and no or very low growth. In January, the water temperature was 4 °C and a high N:C ratio agreed with the strong negative correlation between N:C ratio and temperature observed by Bianchi et al. (1997). Temperature decreases have been shown to induce bacteria to increase their substrate requirement for optimal growth (Wiebe et al., 1992). Accordingly, it may very well be that N:C ratios of nitrifying communities are seasonally variable in temperate systems. Nitrification can efficiently proceed at low temperatures (even < 0 °C) despite the general impression that nitrifiers are inactive at temperatures below 4 to 5 °C (Collos et al., 1988). High activity without growth might also be due to heterotrophic nitrification, which can constitute a significant fraction of ammonium oxidation under favorable conditions (Zhao et al., 1999). This process would not be detected using the ^{14}C method. A number of common denitrifying bacteria have the ability to carry out heterotrophic nitrification (Castignetti and Hollocher, 1984). Heterotrophic nitrifiers occur among algae, fungi and bacteria and, in comparison to autotrophic nitrifiers, rates of heterotrophic nitrification are low, and occur preferentially under conditions not favorable for autotrophic nitrification (Schmidt et al., 2003). Low water temperatures in January could constitute such a condition. Complete heterotrophic growth has been demonstrated for both *Nitrosomonas* spp. and *Nitrobacter* spp. (Hall, 1982, and references therein).

In contrast, October rates based on the ^{14}C method were higher than would be predicted from an N:C ratio of 8.3. Our pilot experiment with tidal freshwater was conducted in November 2002 and produced an N:C ratio of 4.0 (solid line, Figure 2.6), and October data from 2003 appeared to follow this trend. This result implies that bacteria grow faster during these periods than the energy from nitrification at N:C ratio of 8.3 would allow. There are several possible explanations for this: (1) bacteria may use an additional energy source or stored energy reserves, (2) community composition of ammonium-oxidizing bacteria in the estuary may vary over time and/or space, and these different communities may have variable optimal N:C ratios; metabolic activity and growth rates can differ among cultures (Jiang and Bakken, 1999a) and thus also among different communities, (3) our N:C ratios may have been biased towards low values because of our neglect of ammonium regeneration that caused dilution of ^{15}N, and (4) low N:C ratios coincided with low oxygen concentrations i.e.

< 100 μM. Higher carbon assimilation per unit nitrogen oxidized at low oxygen concentrations has been observed in several studies (Carlucci and McNally, 1969; Goreau et al., 1980). Our results were consistent with these findings, which demonstrated a significant ($p = 0.007$, $r^2 = 0.64$) positive correlation between N:C ratio and oxygen concentration (Figure 2.7).

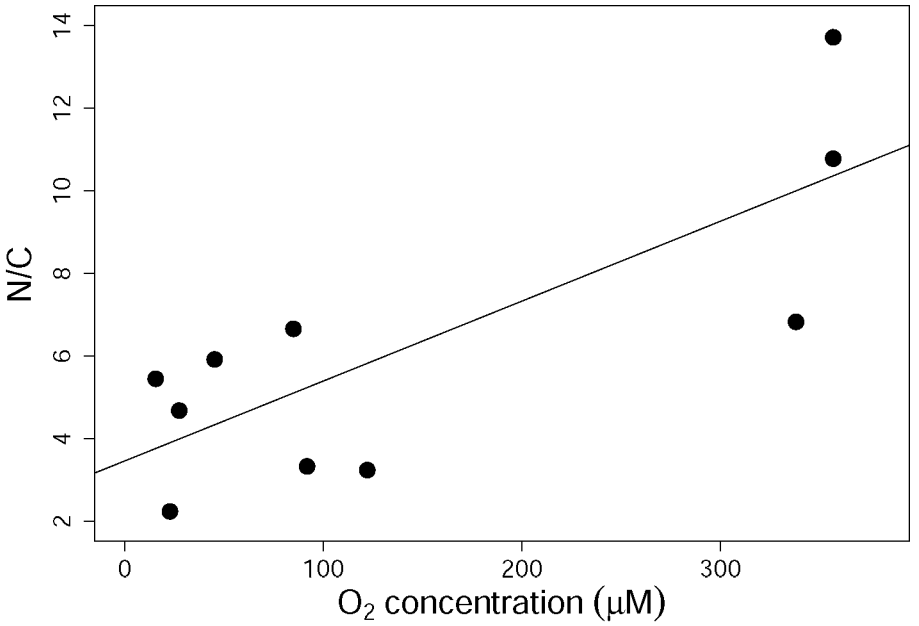

Figure 2.7: N:C ratio < 25 and relative error < 50 % versus O_2 concentrations

Based on our results and previously published studies, we propose that a clear distinction should be made between nitrification activity expressed in units of nitrogen and nitrifier growth expressed in units of carbon. It is only under optimal steady-state conditions, such as in the open ocean or in a lake with long residence time, that a uniform N:C ratio is to be expected. Accordingly, we propose that the ^{15}N method is preferable when nitrifier activity is of interest, while the less costly and less labour intensive ^{14}C method provides a good measure of the growth of chemoautotrophic nitrifying bacteria. In addition to providing a direct measure of

nitrifying activity in nitrogen units, the ^{15}N method has 2 other advantages over the ^{14}C method: (1) the ^{15}N method is independent of specific inhibitors, and (2) it enables light dependent studies of nitrification. Use of specific inhibitors has been questioned because these may not be 100 % selective, i.e. they may inhibit other processes as well, or because they may not completely block all nitrifying activity (Oremland and Capone, 1988). Not all natural communities of nitrifying bacteria are affected to the same extent by a given inhibitor, and responses to inhibitor concentration may vary among communities (de Bie et al., 2002b). Moreover, several inhibitors are not soluble in water and require organic solvents for application, which may affect nitrification rates. One draw back of the ^{15}N method is that in waters with very low ambient NH_4^+ concentrations, the rates measured will be potential rather than actual *in situ* nitrification rates. We observed that light inhibited more than 40 % of nitrifying activity and, depending on the system studied, it might be highly relevant to measure light dependence. Earlier studies demonstrated an important relationship between nitrification rate and light intensity (Ward, 2005). In cultures of both oceanic and estuarine isolates, NH_4^+ oxidizers in oceanic communities were inhibited by light to a much greater extent than estuarine isolates (Horrigan and Springer, 1990). This result was attributed to high NH_4^+ concentrations and high nitrification rates in estuaries. Our light inhibition of 44 % agreed with the findings of Horrigan and Springer (1990) for 1 of the 3 estuarine isolates studied.

2.5 Conclusions

The present study came to the following conclusions:
(1) A comparison between the ^{14}C and ^{15}N method for nitrification revealed that growth and activity can be uncoupled and consequently that N:C ratios can vary (from 2 to 60 in our study); (2) Oxygen concentration and temperature govern N:C ratios; (3) It is preferential to use ^{15}N techniques over ^{14}C method to measure actual nitrification rates; (4) Nitrification is a light sensitive process: 44 % of nitrifying activity was observed to be inhibited by light in this nitrogen-rich, turbid estuary.

3 Uptake of dissolved inorganic nitrogen, urea and amino acids in the Scheldt estuary: comparison of organic carbon and nitrogen uptake

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Aquatic Microbial Ecology, 44:303-315, 2006

3.1 Introduction

All organisms need nitrogen, and both dissolved inorganic and organic nitrogen (DIN and DON) can be used as substrates by micro-organisms. Measurements of DIN uptake are straightforward using ^{15}N labeled ammonium (NH_4^+), nitrate (NO_3^-) and nitrite (NO_2^-) and a large body of data exists (e.g. Dugdale and Goering, 1967; McCarthy et al., 1977; Middelburg and Nieuwenhuize, 2000b; Tobias et al., 2003a). Uptake of DON is difficult to quantify since it contains a large number of compounds, such as proteins, oligopeptides, purines, nucleic acids, and humic substances, and the exact composition of the pool is unknown. As a consequence, application of ^{15}N labeled substrates is cumbersome and few data exist (Bronk, 2002). To increase our understanding of nitrogen (N) cycling in aquatic systems it is important to quantify the importance of DIN and DON uptake at the same time. Nitrification, oxidation of NH_4^+ to NO_3^-, is another key process in estuarine nitrogen cycling but parallel studies of other processes consuming or producing NH_4^+ and NO_3^- are rare.

Many studies have shown DON to be an important N source for phytoplankton (Palenik and Morel, 1990; Bronk and Glibert, 1993; Berman and Chava, 1999; Fan and Glibert, 2005) as well as for heterotrophic bacteria (Wheeler and Kirchman, 1986; Hoch and Kirchman, 1995; Middelboe et al., 1995; Rosenstock and Simon, 2001). The majority of these studies either measured uptake of total DON in laboratory cultures or quantified the uptake of well-defined substrates (e.g. amino acids) in batch cultures or in natural samples. Some micro-organisms can use DON-derived N directly (Berg et al., 1997; Mulholland et al., 2002) while others use DON indirectly, i.e. by using extracellular enzymes to mobilize N from DON (Palenik and Morel, 1990; Berg et al., 2002; Mulholland et al., 2003). NH_4^+ can also be released from DON through inorganic reactions, such as photochemical oxidation (Bushaw et al., 1996). It is not clear whether N-containing dissolved organic compounds are used as substrates exclusively for N, for carbon (C), or for both. Previous studies addressing this question on natural samples are few. Petersen et al. (2004) showed that both heterotrophic and nitrifying bacteria can incorporate a small amount of C from urea. Jørgensen (2006) reported urea uptake by estuarine bacteria and concluded that uptake is variable and rather unpredictable. For phytoplankton, the results are variable and sometimes conflicting (Mulholland et al., 2002, 2003, 2004; Fan and Glibert, 2005). Fan and Glibert (2005) found urea to be a C substrate during a dinoflagellate bloom, while Mulholland et al. (2004) concluded that urea was not used as a substrate for C during a phytoplankton bloom. Mulholland et al. (2003) reported uncoupled uptake of amino acid, i.e. N was taken up preferentially to C along an estuarine gradient. This contradicted their previous findings regarding uptake in the phytoplankton *Aureococcus anophagefferens*, whereby both C and N were incorporated from amino acids (Mulholland et al., 2002). Clearly, more observations are required to identify the differential assimilation of C and N by natural communities.

Another key question is to what extent dissolved organic matter (DOM) contributes to N uptake by bacteria and phytoplankton compared to dissolved inorganic nutrients, since there are few data on this. In a recent review, Berman and Bronk (2003) stated that there is still a need for greater appreciation and understanding of the potential role of DON in aquatic systems. Estuaries receive DIN and DON via riverine input, direct land runoff, atmosphere inputs and from the sea. Moreover, during

transport through an estuary to the sea, these compounds undergo many transformations such as uptake, regeneration, nitrification and denitrification. Most estuaries receive high N loads from anthropogenic activities in the watershed. Depending on the nature of these inputs (agricultural nitrate or organic nitrogen from sewage), this will affect estuarine N cycling in different ways (Heip and Herman, 1995) and it is therefore necessary to identify DIN and DON pathways. Bacteria are considered the major sink for DON in marine environments (Wheeler and Kirchman, 1986). Studies in estuarine and coastal systems, however, have found that N uptake in the form of urea is predominantly by phytoplankton rather than by bacteria (Berman and Bronk (2003) and references therein) but few data exists (Jørgensen, 2006). Most previous studies of DON uptake have used batch cultures, and the utilization of DON under *in situ* conditions is still unclear. Few studies have examined how bacteria and phytoplankton respond to multiple sources of N under natural conditions. Using stable isotopes and labeling one source at a time makes it possible to study the effect of each substrate under *in situ* conditions.

The aim of this study was to simultaneously quantify the uptake of DIN, as NH_4^+, NO_3^- and NO_2^-, and urea and dissolved free amino acids (DFAA) over the whole salinity range of the Scheldt estuary during different seasons. These results could then be compared with concomitant nitrification measurements (Andersson et al., 2006). The importance of DON as a N or a C substrate in natural samples was also investigated in detail using ^{13}C labeled urea and DFAA in parallel with ^{15}N labeled equivalents.

3.2 Materials and Methods

3.2.1 Study area

The Scheldt estuary is located in the SW of the Netherlands and Belgium and is among the most nutrient-rich systems in the world (Soetaert et al., 2006). The estuary is fed by the Scheldt river, which starts in northern France (St. Quentin) and flows into the North Sea near Vlissingen (the Netherlands). The total catchment area of the Scheldt river is 22000 km^2, with a population of more than 10 million people, and containing several

large industrial areas. The river is used as a major drain for industrial and domestic wastes, of which a substantial portion is not treated in a wastewater treatment plant. The Scheldt estuary is shallow (mean depth about 10 m), turbid and well mixed with a residence time of about 2 months (Heip, 1988). The estuary is about 100 km long with an area of 300 km^2. The tidal amplitude is high, ranging from 3.8 m in the western to 5.2 m in the eastern part.

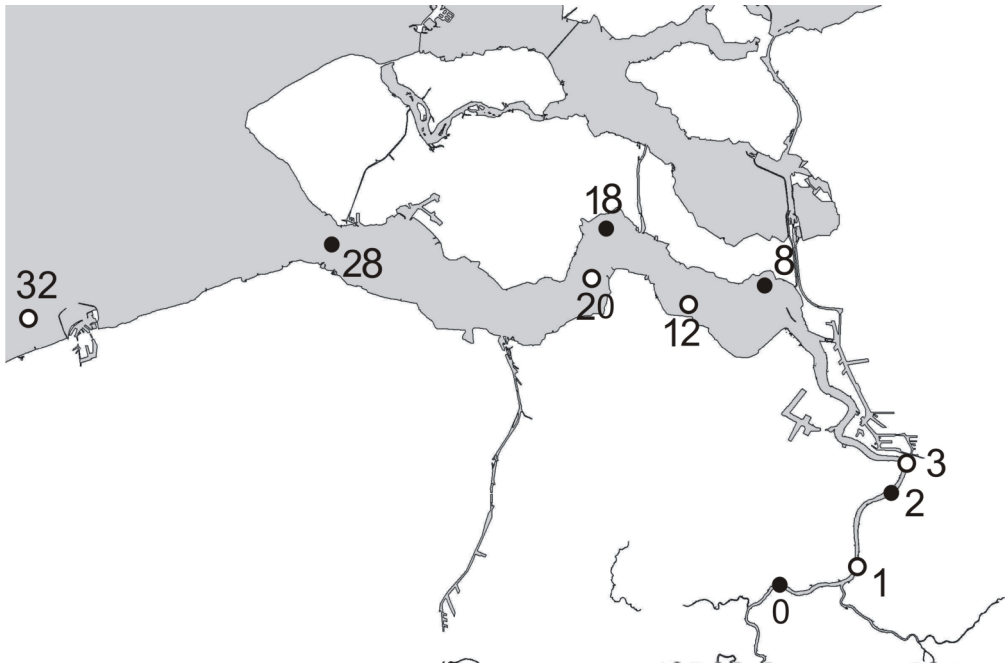

Figure 3.1: Scheldt estuary. Numbers show approximate location of the stations and represent their salinity. (•) Stations visited during VLANEZO campaigns; (○) stations visited during EUROTROPH campaigns

3.2.2 Sampling

Within the VLANEZO (Dutch-Flemish cooperation on sea research) project, 4 cruises were conducted with RV 'Luctor' in January, April (April^b), July and October 2003. Five stations were sampled throughout the salinity

range during every cruise: Stations 0, 2, 8, 18 and 28, with the numbers reflecting their salinity (Figure 3.1). Water samples were taken with a 20 L Niskin bottle from approximately 2 m depth and were subsampled on board immediately after retrieval of the Niskin bottle. Samples were also taken from 17 fixed monitoring stations along a transect covering the whole salinity gradient from freshwater in the inner estuary to marine conditions at the mouth. Additional data for DON uptake originate from 2 cruises conducted within the EUROTROPH project (Gazeau et al., 2005), 1 in November 2002 and 1 in early April 2003 (April[a]), 2 weeks before the RV 'Luctor' cruise. During these cruises 5 or 6 stations were also sampled, covering the whole salinity range (Figure 3.1).

3.2.3 Nutrients

Water was filtered through pre-weighed, pre-combusted Whatman GF/F filters (47 mm), stored frozen, and analyzed for NH_4^+, NO_3^- and NO_2^- using automated colorimetric techniques. The filters were weighed for suspended particulate matter (SPM) analysis and concentrations of particulate organic nitrogen (PON) and carbon (POC) were measured with a Carlo Erba NA 1500 elemental analyzer (Nieuwenhuize et al., 1994). At all stations, salinity, temperature and oxygen were measured. At the VLANEZO stations, DFAA concentrations were measured by HPLC (Fitznar et al., 1999) from the cruises in July and October. Concentrations of urea were measured from the cruises in January, July and October using automated colorimetric techniques (Middelburg and Nieuwenhuize, 2000a). Water was filtered through GF/F filters, which were stored frozen until analysis of chlorophyll *a* (chl *a*) by HPLC (Barranguet et al., 1997).

3.2.4 Nitrification measurement

Nitrification rates were measured using ^{15}N labeled NH_4^+ as described in Andersson et al. (2006). Briefly, water samples were spiked with ^{15}N labeled NH_4^+ and, after incubation, the appearance of ^{15}N in NO_3^- was measured based on the diffusion technique of Sigman et al. (1997) and Holmes et al. (1998). NH_4^+ and subsequently NO_3^- (converted into NH_4^+ using Devarda's alloy) in the sample was trapped on a GF/D filter. The ^{15}N content of the GF/D filters was determined using a Fison NA 1500

elemental analyzer coupled to a Finnigan Delta S masspectrometer via a Conflo II interface.

3.2.5 Uptake measurements

N uptake was measured using ^{15}N labeled NH_4^+, NO_3^- and NO_2^-, urea and a mixture of 17 algal amino acids (Cambridge Isotope Laboratories, NLM-2161). The amino acid mixture consisted of alanine (7%), arginine (7%), aspartic acid (10%), glutamic acid (10%), glycine (6%), histidine (2%), isoleucine (4%), leucine (10%), lysine (14%), methionine (1%), phenylalanine (4%), proline (7%), serine (4%), threonine (5%), tyrosine (4%) and valine (5%) (giving an average C:N ratio of 4 in the amino acid mix). Urea and DFAA uptake was also quantified using ^{13}C labeled urea and DFAA with the same composition as mentioned above. The water samples were spiked with the tracer to a final concentration of 0.1 μM (except for 1 μM of NO_3^-) and incubated for approximately 2 h in polystyrene bottles at *in situ* water temperature in the dark and at ambient light conditions. The incubations were terminated by filtration through precombusted (450 °C for 4 h) Whatman GF/F filters (20 mm). The filters were stored frozen until analysis of isotope content of the SPM using a Fisons NA 1500 elemental analyzer coupled to a Finnigan Delta S isotope ratio mass spectrometer (EA-IRMS). Rates for specific uptake (V, h^{-1}) of NH_4^+, NO_3^- and NO_2^-, urea and DFAA were calculated by dividing the excess ^{15}N or ^{13}C on the Whatman GF/F filter by the excess ^{15}N or ^{13}C in the solution h^{-1} incubation time according to Dugdale and Wilkerson (1986). Absolute uptake rates U, (μmol N L^{-1} h^{-1} or μmol C L^{-1} h^{-1}) were calculated by multiplying V with PON or POC (μmol l^{-1}), depending on which label was added. The rates reported are average values for light and dark incubations since these showed no statistically significant difference, consistent with previous studies in this and other turbid, tidal estuaries (Middelburg and Nieuwenhuize, 2000a,b). The errors reported herein represent the standard deviation of replicate incubations (in both the dark and light [n = 4], except for January [n = 2]). Uptake rates were not corrected for isotope dilution because we lacked measurements, and estimated correction factors (based on the Kanda et al., 1987, approach) were very small, as we have reported earlier (Middelburg and Nieuwenhuize, 2000b,a).

3.3 Results

3.3.1 Concentrations

The concentration versus salinity plots show the dynamic cycling of nitrogen in the Scheldt estuary (Figure 3.2). NH_4^+ concentrations were highest in the inner part of the estuary, with concentrations up to 150 μM, and then rapidly decreased with increasing salinity. The decrease was highest during July and October. NO_3^- concentrations in the inner part were around 300 μM and either increased or were uniform until a salinity of around 10, thereafter decreasing throughout the estuary. The decrease in NH_4^+ and increase in NO_3^- in the inner estuary reflect intensive nitrification (Andersson et al., 2006). NO_2^- levels were low throughout the estuary, with high concentrations (up to 30 μM) in the inner part. Urea and DFAA concentrations varied with season, with a fairly stable concentration over the salinity gradient (around 4 μM N for urea, 2 μM N for DFAA).

O_2 concentration varied between seasons and also along the salinity gradient of the estuary. The general trend was low concentrations (even hypoxic during July and October) in the inner part with increasing concentrations toward the mouth of the estuary, reaching saturation levels and even super saturation (415 μM) in April[b]. SPM varied from 250 mg L^{-1} in the fall in the inner part of the estuary to 10 mg L^{-1} in the marine part in January. POC followed the pattern of SPM and varied from 1200 μM in the fall in the inner stations to 30 μM at the marine stations in January. pH values were around 7.5 at the inner stations and increased throughout the estuary to above 8 at the marine stations. Chl *a* levels were below 10 μg L^{-1} throughout the whole estuary in January and at the more marine stations in July and October. In the inner estuary the concentrations of chl *a* reached values of up to 100 μg L^{-1} in July and October, and 10 to 40 μg L^{-1} were measured throughout the estuary in April[b] . Comparing algal C (converted from chl *a* using a carbon:chl *a* ratio of 30; Wetsteyn and Kromkamp, 1994) with total POC we obtained a first-order estimate of the algal contribution to the POC pool. A bloom of algae was measured in the marine part of the estuary in April[b] (Dijkman and Kromkamp, 2006), with algae contributing about 75 % to POC. During the other months and in the inner part in April[b], algae contributed between 2 and 30 % of the POC, bacteria and detritus accounting for the

rest of the POC pool. Dijkman and Kromkamp (2006) used phospholipid-derived fatty acids (PLFA) to measure biomasses of phytoplankton and bacteria during the VLANEZO cruises. These authors showed that in January bacteria were 3 times more abundant than algae while during the other 3 months bacterial biomass was between one-third and half of algal biomass, with higher biomasses at mid salinities. Accordingly, detritus constituted around 80 % of the POC in January and approximately 50 % during July and October. The water temperature varied with season: 4, 10, 20, 14 °C in January, April^b, July and October, respectively.

3.3.2 Uptake of DIN and DON

There was no significant difference (ANOVA: p-values 0.07 to 0.37) between uptake rates measured in the dark and at ambient light. Therefore these data were combined and considered as replicates. With very few exceptions, highest uptake rates were those of NH_4^+ ranging from 1 nM h^{-1} at the highest salinity in October to 1 μM h^{-1} at Station 2 in April^b (Table 3.1). There were no clear trends for uptake of any of the substrates, although rates were usually lowest at the mouth of the estuary. Uptake of NO_3^- ranged from 270 nM h^{-1} in July to 6 nM h^{-1} in October. Generally, the lowest uptake was for NO_2^-: 110 nM h^{-1} in July down to 1 nM h^{-1} in January. Uptake of urea and DFAA showed rates comparable to and often higher than those of DIN. Urea uptake varied from 700 nM N h^{-1} in July to 2 nM N h^{-1} in January and DFAA uptake from 200 nM N h^{-1} in July to 13 nM N h^{-1} in October. Unfortunately, data for DFAA uptake from January and April^b were not available. The standard deviation of replicate samples and light/dark incubations were rather high, in some cases even higher than the measured uptake rate (Table 3.1).

The relative contribution of the different substrates to their summed uptake varied over the salinity gradient and with season (Figure 3.3A,C). In July NH_4^+ made its highest contribution at Station 8 (51 %), with values decreasing both toward the inner estuary and the mouth of the estuary. A complete opposite pattern was found in October, when the lowest relative contribution was at Station 8, with NO_3^- showing the highest contribution (48 %). Urea showed a contrasting pattern to NH_4^+, being highest in July at Station 0 (43 %), and DFAA displayed its highest relative importance (up to 29 %) at the more marine stations. The relative contribution of

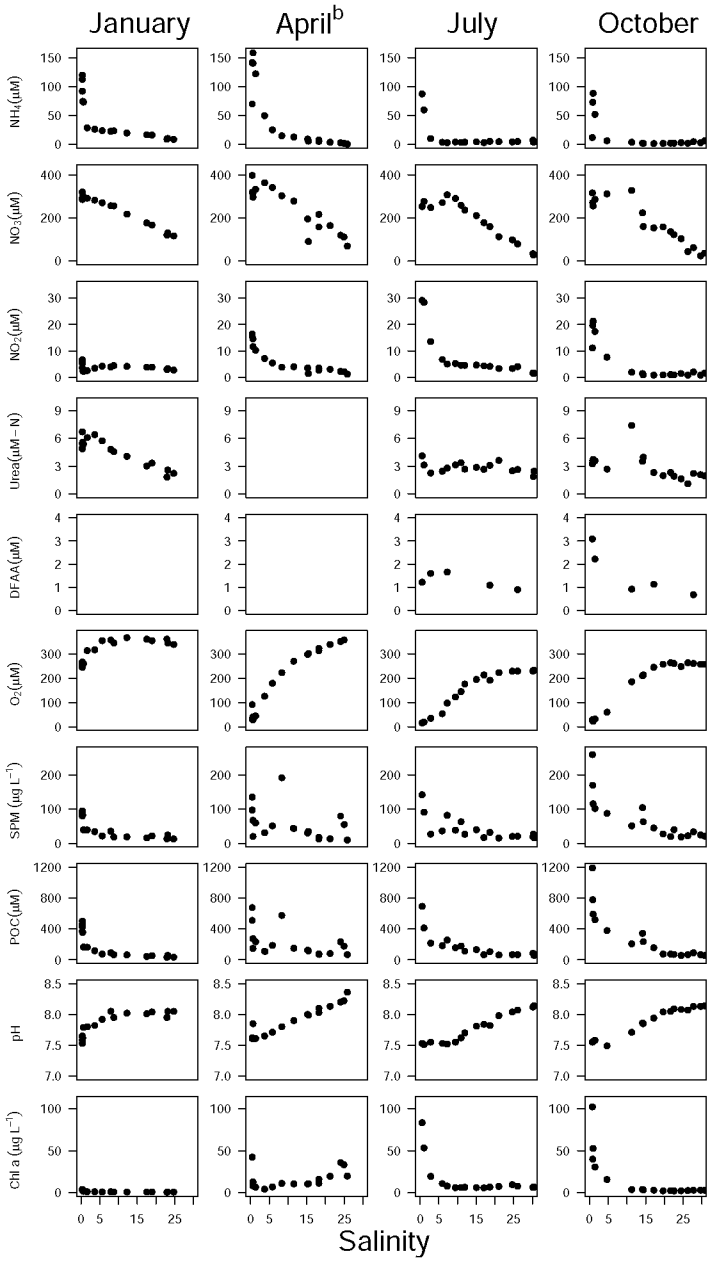

Figure 3.2: Parameters measured in the Scheldt estuary 2003. Urea was not measured in April[b] (RV 'Luctor' cruise) and DFAA were not measured in January and April[b]. Urea is given as μmol N-urea L^{-1}, DFAA as μmol N-DFAA L^{-1}. DFAA: dissolved free amino acids; SPM: suspended particulate matter; POC: particulate organic carbon

Table 3.1: Uptake rates (nM h^{-1}) of NH_4^+, NO_3^-, NO_2^-, urea and DFAA at 5 salinities during 4 seasons. Data are means ± SD of 2 replicate samples each from light and dark incubations, except for January when single samples were incubated in light and dark. DFAA: dissolved free amino acids; nd: no data

Salinity	NH_4^+ nM h^{-1}	NO_3^- nM h^{-1}	NO_2^- nM h^{-1}	Urea-N nM N h^{-1}	Urea-C nM C h^{-1}	DFAA-N nM N h^{-1}	DFAA-C nM C h^{-1}
January							
0	438 ± 254	90 ± 63	32 ± 24	21 ± 12	59 ± 41	nd	nd
2	195 ± 17	95 ± 33	5.7 ± 2.3	20 ± 8.8	15 ± 8.7	nd	nd
8	154 ± 101	46 ± 44	3.7 ± 2.9	29 ± 20	39 ± 55	nd	nd
18	61 ± 25	15 ± 4.8	4.2 ± 3.4	5.7 ± 1.3	0.8 ± 0.5	nd	nd
28	11 ± 0.6	8.0 ± 7.6	1.0 ± 0.3	2.3 ± 0.3	1.1 ± 1.2	nd	nd
April[b]							
0	464 ± 172	66 ± 6.7	7.4 ± 3.9	nd	nd	nd	nd
2	1283 ± 525	35 ± 24	5.9 ± 8.5	nd	nd	nd	nd
8	248 ± 26	188 ± 13	22 ± 2.0	nd	nd	nd	nd
18	94 ± 35	44 ± 4.9	5.7 ± 0.8	nd	nd	nd	nd
28	35 ± 13	30 ± 32	2.4 ± 1.7	nd	nd	nd	nd
July							
0	452 ± 146	268 ± 51	34 ± 2.0	701 ± 86	208 ± 139	195 ± 44	226 ± 96
2	461 ± 139	161 ± 51	113 ± 53	148 ± 175	6.0 ± 5.4	110 ± 18	228 ± 103
8	239 ± 54	87 ± 36	17 ± 5.5	58 ± 43	7.9 ± 11	66 ± 10	115 ± 68
18	112 ± 47	35 ± 22	5.8 ± 2.3	45 ± 22	7.0 ± 9.6	40 ± 5.0	116 ± 30
28	48 ± 21	7.0 ± 1.5	2.3 ± 0.3	30 ± 9.7	3.7 ± 4.2	29 ± 2.3	54 ± 7.5
October							
0	348 ± 122	79 ± 18	53 ± 18	93 ± 37	29 ± 37	110 ± 29	775 ± 355
2	355 ± 39	125 ± 10	55 ± 11	57 ± 13	13 ± 8.9	86 ± 26	213 ± 68
8	43 ± 7	105 ± 15	13 ± 1.0	36 ± 4.8	10 ± 11	20 ± 4.1	55 ± 23
18	26 ± 2.2	36 ± 5.8	2.0 ± 0.8	25 ± 7.8	4.9 ± 6.1	37 ± 18	108 ± 17
28	1.3 ± 1.8	6.0 ± 6.2	1.9 ± 2.0	11 ± 12	8.9 ± 7.1	13 ± 14	45 ± 17

NO_2^- was low throughout the estuary (11 % at highest) with lowest values at the mouth (2 %). The absolute uptake rates were higher at stations with lower salinities (Figure 3.3B,D). Uptake of NH_4^+ and NO_3^- was similar in July and October while uptake of urea was higher in July, resulting in a higher total N uptake in July.

3.3.3 Uptake of dissolved organic matter

Absolute uptake rates of urea and DFAA are only available for the July and October cruises (Table 3.1, Figure 3.3). However, for all cruises results can be presented as excess ^{15}N or ^{13}C after incubation (nM h^{-1}), and these allow a direct comparison of N and C uptake. The regression lines in Figure 3.4 show the expected relationship between excess ^{13}C and ^{15}N were the whole urea molecule (with a C:N ratio of 0.5) to be taken up and both C and N to remain in the cell and be assimilated during the 2 h incubation. Large differences can be seen for the 6 sampling occasions (Figure 3.4). In November and January urea uptake rates were low and urea was mainly used as a C source. In early spring, April^a uptake of urea was lower than later the same month, April^b. In July the highest utilization of urea was measured, with rates decreasing again in October. Throughout April to October urea was used as a substrate for both N and C. Moreover, no differences were found for samples incubated in ambient light and samples incubated in dark (data not shown).

The algal amino acid mixture used in this study had on average a C:N ratio of 4; the regression lines in Figure 3.5 represent the values expected if the whole amino acid molecule were taken up and both C and N assimilated. DFAA utilization of N and C was diametrically to that of urea. In November, January and April the whole molecule seems to have been used. Later in the season, particularly in July and October, there was a shift toward using DFAA as a N substrate. Remarkably, those months with low urea uptake (January, April^a and November) all had a higher uptake of DFAA than the other three months.

3.3.4 Nitrification

Highest nitrification activity was measured in the inner part of the estuary, with rates decreasing toward the mouth. Nitrification was generally higher

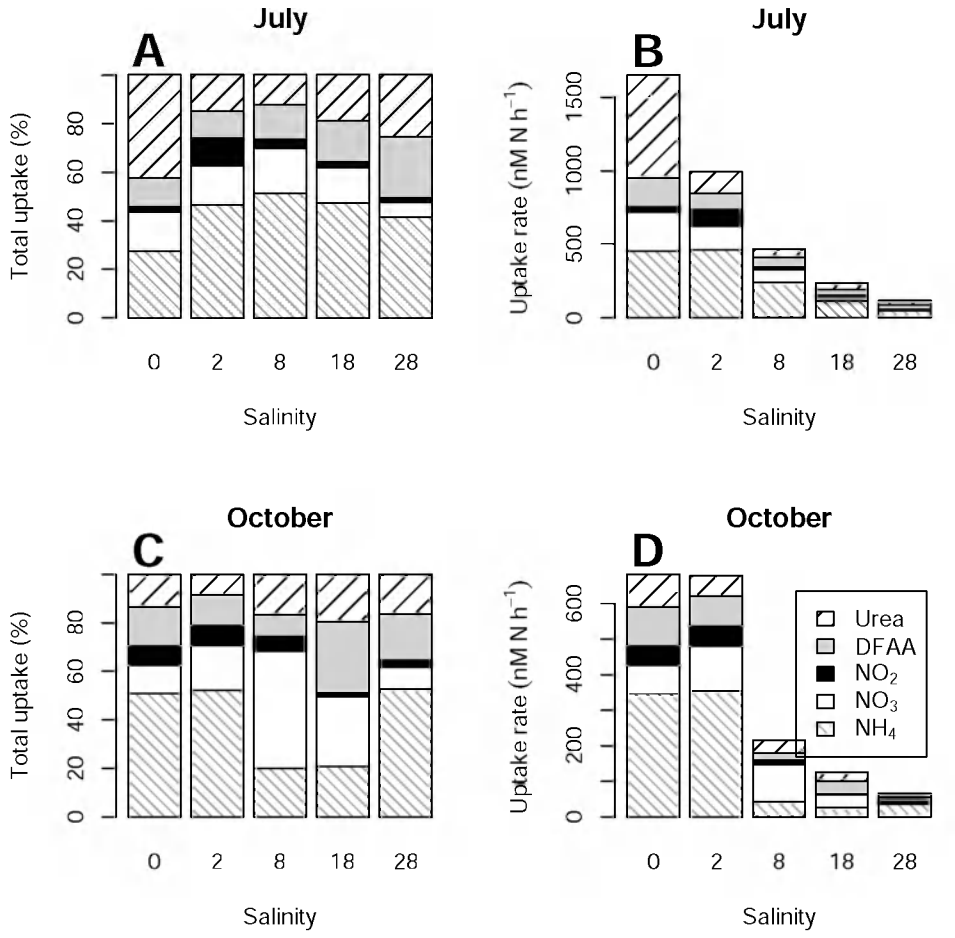

Figure 3.3: Relative contribution to (A,C) total uptake and (B,D) uptake rates of the 5 nitrogen sources along the salinity gradient. Note differences in scale for uptake rates.

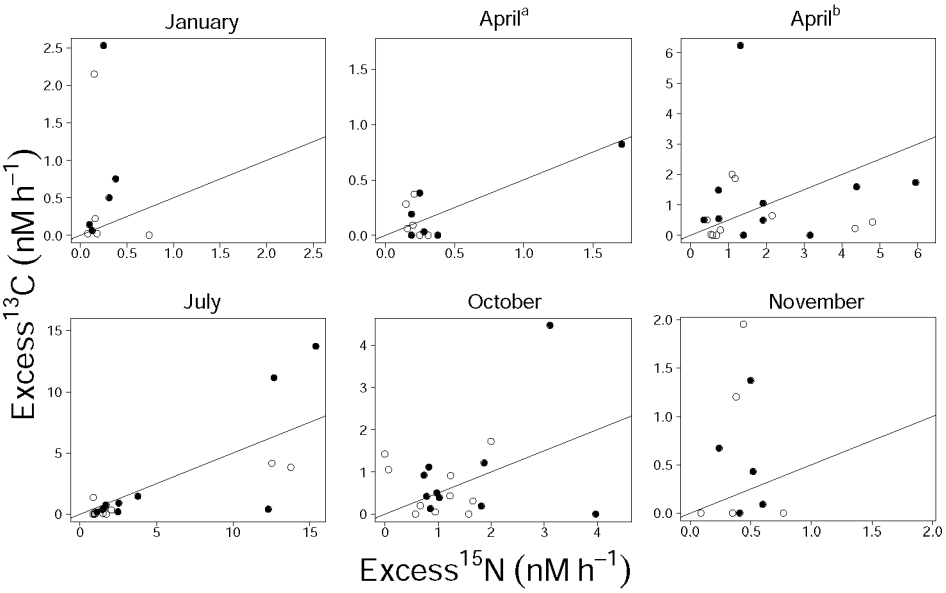

Figure 3.4: Excess ^{13}C vs. excess ^{15}N during incubations with labeled urea. Uptake in ambient light (○) and in the dark (●). Regression line shows value expected if a complete urea molecule, with C:N ratio of 0.5, were taken up. April[a]: EUROTROPH project, 2 weeks before April[b] sampling by RV 'Luctor'

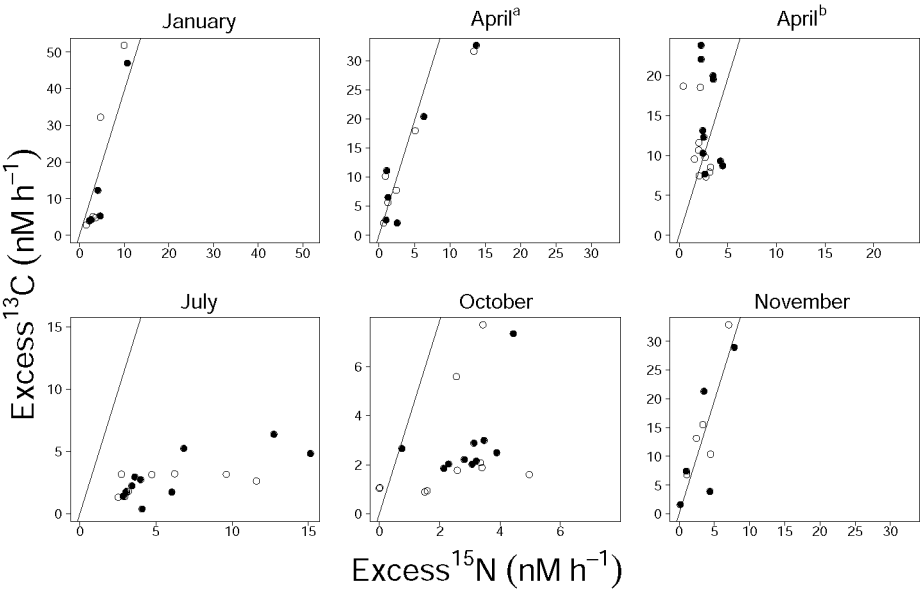

Figure 3.5: Excess ^{13}C vs. ^{15}N during incubations with labeled DFAA. Uptake in ambient light (○) and in the dark (●). Regression line shows value expected if a whole average amino acid molecule, with C:N ratio of 4 were taken up. April^{a}: EUROTROPH project, 2 weeks before April^{b} sampling by RV 'Luctor'

in July and October, corresponding to higher temperature and lower NH_4^+ concentration, which ranged from 700 in October to 150 nM h^{-1} in April^b in the fresh water stations and from 20 nM h^{-1} to undetectable in the marine stations. Nitrification rates were in the same order of magnitude as NH_4^+ uptake rates. The most obvious difference was at Stations 0 and 2 in April^b, when NH_4^+ uptake was much higher than both nitrification and NO_3^- uptake (Figure 3.6).

3.4 Discussion

The Scheldt estuary is a turbid, tidal, heterotrophic system (Goosen et al., 1999; Boschker et al., 2005) with high nutrient concentrations and organic loading (Kromkamp et al., 1995). Algal C constituted a small part of the POC (on average 15 % during our study) and bacteria were 3 times more abundant than algae in January while during the other months bacterial biomass made up between one-third and half of the algal biomass (Dijkman and Kromkamp, 2006). Input of allochthonous organic matter and nutrients in the inner estuary are high (Kromkamp et al., 1995), and during our study most N pools did not show a conservative behaviour due to mixing (Figure 3.2) during transport to the sea, but were clearly affected by processes within the estuary. Two major transformation pathways for NH_4^+ in the estuary are nitrification, whereby NH_4^+ is oxidized to NO_3^- via NO_2^-, and uptake by phytoplankton and bacteria. NO_3^- and NO_2^- can also be taken up by phytoplankton and bacteria or be denitrified to N_2 gas (in the sediment). Urea and DFAA can be taken up by phytoplankton and bacteria as a substrate for N, C or both.

Comparison of our NH_4^+ uptake rates with parallel measurements of nitrification rates during the same cruises (Andersson et al., 2006) show them to be of the same order of magnitude (Figure 3.6). The ratio of NH_4^+ uptake to nitrification ranges from 0.5 to 25 (where 1 represents equal uptake and nitrification rates). In April^b, during the spring bloom, NH_4^+ uptake was much higher than NH_4^+ oxidation by the nitrifiers. In July the community of nitrifiers had time to develop, and roughly the same amount of NH_4^+ was taken up as was nitrified. There are few parallel studies of nitrification and NH_4^+ uptake. Lipschultz et al. (1986) showed nitrification to be the major process affecting NH_4^+ distribution in the Delaware

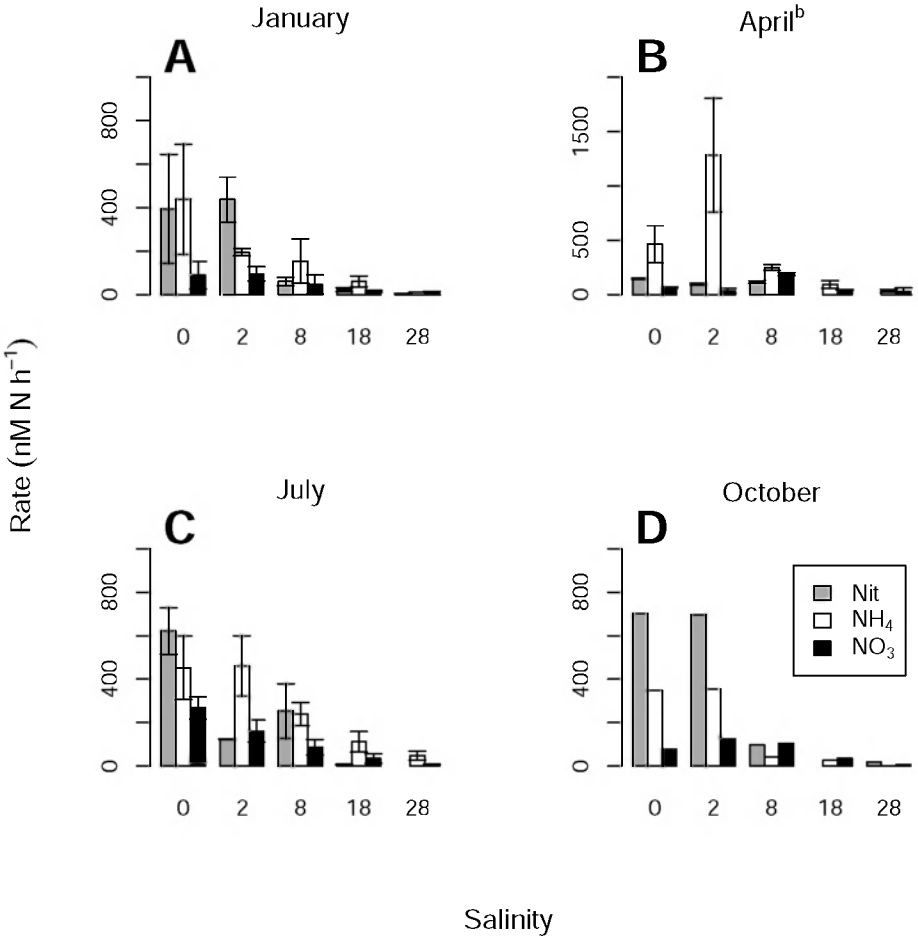

Figure 3.6: Nitrification rate and uptake rates of NH_4^+ and NO_3^- during 4 months in 2003 at 5 different salinities. Note scale for April[b] is twice that for the other 3 months

River. Ward (2005) investigated NH_4^+ oxidation and assimilation in the upper 70 m of Monterey Bay, California, USA, an eastern boundary upwelling system, and found that NH_4^+ assimilation was much larger than NH_4^+ oxidation in the upper 25 m, but that rates were similar at greater depths. Depth-integrated rates of NH_4^+ assimilation (5.1 μmol m^{-2} d^{-1}) were higher than those of NH_4^+ oxidation (2.0 μmol m^{-2} d^{-1}). Comparison the NO_3^- production rate via nitrification with the NO_3^- uptake rate (Figure 3.6) revealed that, with a few exceptions, most NO_3^- produced via nitrification was not taken up, consistent with the NO_3^- vs. salinity plots of net NO_3^- production in Figure 3.2).

Uptake rates in the light were not significantly different from those in the dark for all substrates and light and dark incubation data were therefore combined. This similarity between light and dark uptake rates has been reported before, in particular for turbid systems with relatively low algal biomass and high concentrations of bacteria with respect to total community uptake (Middelburg and Nieuwenhuize, 2000b,a). The Scheldt estuary is a prototype heterotrophic, turbid estuary in which particle-attached bacteria play a dominant role in moderating C and N flows (e.g Boschker et al., 2005). Partitioning of nitrogen uptake between phytoplankton and heterotrophic bacteria in turbid estuaries such as the Scheldt Estuary is difficult since size fractionation by pre- or post-incubation filtration is not feasible because of filter clogging and is not appropriate because of the tight association of the bacteria with particles. Alternatively, prokaryotic inhibitors might be used to distinguish between eukaryotic and prokaryotic activity, although these are never 100 % selective or 100 % effective (Oremland and Capone, 1988). Although we have already used this approach successfully in the turbid Loire and Thames estuaries (Middelburg and Nieuwenhuize, 2000b,a), we obtained highly variable, inconclusive results in the Scheldt estuary. We were therefore unable to partition nitrogen uptake among the bacteria and phytoplankton but, given the lack of light-dark difference, the heterotrophic status of this estuary, and its high bacterial biomass (Boschker et al., 2005), we believe bacteria to be major contributors to the nitrogen uptake.

In regards to the absolute uptake rates of the different substrates, the rates of NH_4^+ uptake were highest, with a few exceptions, partly due to the much higher concentrations of NH_4^+ compared to DFAA and urea. To investigate preference for different substrates, many authors (Phillips and

Hurd, 2003; Fan et al., 2003; Weston et al., 2004; Bode et al., 2005) have used the relative preference index (RPI) (McCarthy et al., 1977), defined as

$$RPI_x = \frac{U_x}{\sum U} / \frac{C_x}{\sum C} \tag{3.1}$$

where RPI_x = relative preference index of Substrate x, U_x = Uptake rate of Substrate x, C_x = concentrations of Substrate x and $\sum U$ and $\sum C$ = the summed uptake and concentration, respectively, for all substrates (i.e. NH_4^+, NO_3^-, NO_2^-, urea and DFAA). The RPI for the different substrates

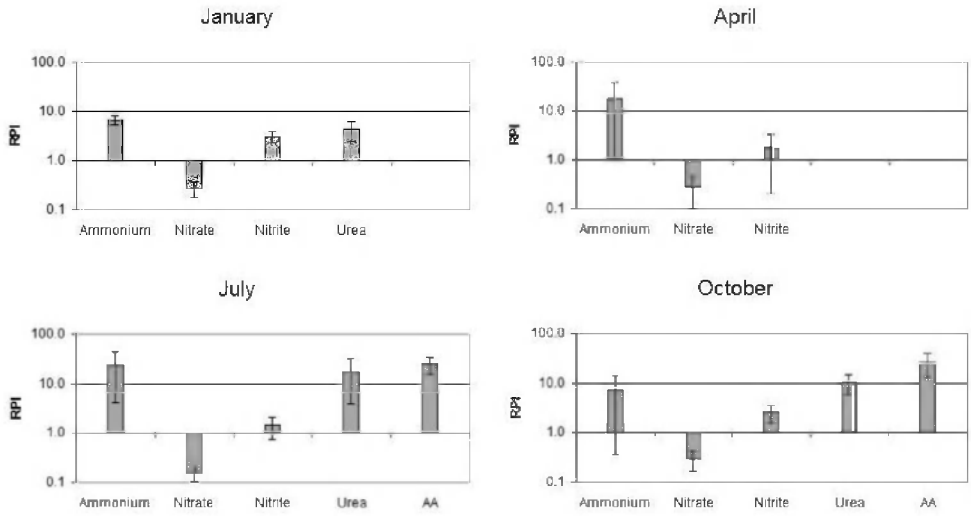

Figure 3.7: Relative preference index (RPI) values, averaged over the salinity gradient, for NH_4^+, NO_3^-, NO_2^-, urea and DFAA in the Scheldt estuary during 2003.

is shown in Figure 3.7. An RPI of 1 reflects utilization equal to availability, >1 preference for substrate, and <1 preference against that substrate. Our results clearly shows that NO_3^- is the least preferred substrate (Figure 3.7) with RPI values <1 in all seasons, implying that it would be used only if other forms of N were less available. Urea and DFAA have RPI values in the same range as, or higher than, those of NH_4^+, which means that these substrates are used to the same extent when equally available.

NO_2^- was less preferred than NH_4^+, urea and DFAA but more preferred than NO_3^-. The utilization of RPI has been questioned as being strongly dependent on substrate concentration and weakly related to physiological preferences (Dortch, 1990; Stolte and Riegman, 1996). This could be the case for our findings of NO_3^- rejection arising from high NO_3^- concentration, but the differences recorded for the other substrates were likely due to physiological preferences, since their concentrations were similar (except for NH_4^+ in the freshwater).

Our uptake rates are similar to and often higher than previous rates recorded in the literature for estuarine systems. Our uptake rates of NH_4^+ and NO_3^- fall within the range found by Middelburg and Nieuwenhuize (2000b) in 6 turbid, tidal estuaries including the Scheldt estuary. Other studies showed lower uptake of NH_4^+ and higher or similar uptake of NO_3^- (Lipschultz et al., 1986; Bronk and Glibert, 1993; Bronk et al., 1998; Middelburg and Nieuwenhuize, 2000a; Veuger et al., 2004), corresponding to lower and higher substrate concentrations, respectively. The uptake rates of urea and DFAA in our study are within the range reported in an extensive comparison of urea and DFAA uptake rates from various systems by Bronk (2002), and compare best to rates found in the Chesapeake bay estuary. The importance of urea as a N substrate varies (depending on the system) from 5 to 64 % of total N uptake (Bronk, 2002) and on average urea contributes more to total N uptake than does DFAA. DFAA contributes approximately 10 % to total N uptake (Bronk, 2002), but can contribute more than this, e.g. in the marine part of the Thames estuary DFAA accounted for about 90 % of total N uptake (Middelburg and Nieuwenhuize, 2000a). In the Scheldt estuary, urea contributed between 8 and 43 % and DFAA between 9 and 29 % to total N uptake in July and October (the only months where absolute uptake rates for urea and DFAA were available).

DON comprises a diverse mixture of compounds such as proteins, oligopeptides, purines, nucleic acids and humic substances. Usually < 20 % of the DON pool can be identified, including urea and DFAA, while up to 70 % of DON is often potentially bioavailable (Seitzinger et al., 2002; Stepanauskas et al., 2002). Since urea and DFAA constitute a small fraction of DON, we have very likely underestimated total DON uptake using our approach. In previous studies, 7 to 30 % of DON uptake arose from urea utilization in the spring and 74 % in the fall (Bronk and Glib-

ert, 1993). Despite our likely underestimation of total DON uptake, it is obvious that calculation of DON uptake is necessary for accurate quantification of total N uptake and that some of the compounds within the DON pool are used preferentially. A few previous studies used ^{15}N labeled DON found that this complex DON was taken up in the same order of magnitude as DIN (Bronk and Glibert, 1993; Veuger et al., 2004). When studying the importance of total ambient DON as a source of N compared to DIN, this may be a more useful approach, where as the use of individual DON compounds such as urea and DFAA can increase our knowledge of the mechanisms of DON uptake.

Having shown urea and DFAA to be similar in importance to DIN for microbial N acquisition, we further investigated whether urea and DFAA constitute a N substrate only, or if they also can be used as an energy source. It has been shown that both heterotrophic bacteria (Middelboe et al., 1995; Rosenstock and Simon, 2001) and microalgae (Palenik and Morel, 1990; Bronk and Glibert, 1993; Berman and Chava, 1999; Fan and Glibert, 2005; Linares, 2006) utilize DON, although the questions as to how, why and when they do so remain unclear. Utilization of urea requires energy, since urease needs to be synthesized to enable hydrolyzation of this substrate. The importance of urea as a N source for phytoplankton and bacteria has been well documented (Berman and Bronk, 2003; Jørgensen, 2006). However, the importance of urea as a C source is unclear, and has been studied rarely and with contradictory results (Petersen et al., 2004; Jørgensen, 2006). In the 1980s, investigators used radioactive ^{14}C labeled urea in parallel with ^{15}N labeled urea (Price and Harrison, 1988, and references therein) to measure urea uptake, and an uncoupling between N and C uptake from urea was often found. More recent literature has also shown variable results, depending on the system or organism studied. During a bloom of a brown tide pelagophyte on the east coast of the USA, urea was used as a source for N but not for C (Mulholland et al., 2004). However, during a bloom of a dinoflagellate in Chesapeake Bay, urea was used mostly as a source of C (with a molar C:N uptake ratio of 2.3; Fan and Glibert, 2005), indicating greater retention of C than N. During phytoplankton blooms, uptake measurements of DFAA have shown that these are used as a substrate for N as well as C to a variable extent (Mulholland et al., 2002).

One urea molecule contains 1 C and 2 N atoms and, when taken up,

is split into 1 CO_2 molecule and 2 NH_4^+ molecules by the enzyme urease (Price and Harrison, 1988; Mobley and Hausinger, 1989; Zehr and Ward, 2002). If urea were utilized as a whole molecule, 2 N atoms per 1 C would be taken up (indicated by regression line in Figure 3.4. Our results for urea uptake were not easily interpreted. In January and November, C was incorporated to a much larger extent than would be expected through uptake of whole molecules, implying that N was released, probably as NH_4^+. In the other months the data generally followed the same trend as for uptake of the whole molecule and we thus cannot conclude that urea was used preferentially as a source of N or C. Additional research will be required to attain a more complete understanding of the utilization of urea as a substrate for N and C.

The importance of DFAA for N uptake has been well studied (Wheeler and Kirchman, 1986; Palenik and Morel, 1990; Hoch and Kirchman, 1995; Nilsson and Sundback, 1996; Middelburg and Nieuwenhuize, 2000a; Mulholland et al., 2002), and a few of studies have addressed the question as to whether DFAA constitute a N or a C source (Jørgensen et al., 1993; Middelboe et al., 1995; Jørgensen et al., 1999; Mulholland et al., 2002). Bacteria are still considered to be most important for DFAA uptake (Hoch and Kirchman, 1995); however, it is now apparent that many phytoplankton also can use DFAA as N source (Berman and Bronk, 2003). DFAA can be incorporated via direct uptake of the whole molecule or can be oxidized extracellularly and the released NH_4^+ and organic carbon can subsequently be utilized (uncoupled uptake). To our knowledge Mulholland et al. (2002) were the first to use dually labeled DFAA to estimate C and N uptake by natural communities of the algae *Aureococcus anophagefferens*. These authors showed both coupled and uncoupled uptake in this pelagophyte,with DFAA constituting 50 % of the total N uptake and about half of the associated C being taken up. In a later study, Mulholland et al. (2003) found uncoupled uptake rates of DFAA C and N, with DFAA N being taken up preferentially to C along an estuarine gradient in the Chesapeake Bay. In Lake Constance, Germany, DFAA and protein supported 58 % and 80 % of the bacterial C and N demand, respectively, making them the most important bacterial C and N sources (Rosenstock and Simon, 2001). Using a combination of ^{15}N and ^{13}C labeled DFAA it is possible to distinguish between uptake of the whole molecule and stoichiometric assimilation on the one hand and partial uptake or selective

assimilation of N or C on the other hand.

Our results support previous findings that DFAA are used as a source for both C and N. Moreover we observed seasonal variation in the coupling or uncoupling of N or C uptake. The regression lines in Figure 3.5 show the ratio expected between C and N uptake if the whole amino acid molecule were utilized (based on an average C:N ratio of 4 in the labeled algal mixture). In January, April and November, the uptake data were scattered around this line, indicating that DFAA were used as both a N source and a C source. In July and October, DFAA were used as a N rather than a C source, likely through extracellular amino acid oxidation. It should be noted, however, that the activities were lower during these months. The most striking difference between July and October and the other months was the availability of NH_4^+ (Figure 3.2). When NH_4^+ availability was low, bacteria, phytoplankton or both utilized the N content of DFAA to a larger extent. High amino acid oxidase activities in phytoplankton have been shown to coincide with low NH_4^+ levels and with high biomass levels indicating an algal bloom (Mulholland et al., 1998). Furthermore, Pantoja and Lee (1994) showed that extracellular amino acid oxidation only occurs at higher temperature, consistent with our findings that in July and October the N portion of the DFAA was preferentially used.

Urea and DFAA may be preferred as substrates under limited growth conditions, such as reduced light intensity or lowered temperature (Berg et al., 1997), since they can supply the cell with both organic C and N. This might explain why in November, January and April DFAA were particularly used as a source of both C and N (Figure 3.5). During these months low water temperature and reduced light were limiting autotrophic growth. During the months when DFAA were used as a source for both N and C, urea was, in general, used primarily as a C source. When uptake rates of DFAA were high (in January, April[a] and November), uptake rates of urea were lower than in the 3 other months. This seasonal variability might be related to seasonal changes in the community of phytoplankton and bacteria, but nutrient concentrations, light availability, oxygen conditions also vary during the year.

Since the C and N of urea and DFAA may or may not be taken up in proportion to their occurrence, and since this may depend on the organisms involved (photoautotrophs vs. heterotrophs) and their physiological status, C-based uptake rates cannot always be converted into N units, or

vice versa. The use of dually labeled substrates is necessary to increase our insight into the role of DON cycling in pelagic ecosystems and to further our understanding of DON acquisition strategies by microbes. To take this relatively unexplored field of research one step further and study in detail which organisms are involved in the uptake of inorganic versus organic substrates, biomarkers such as PLFA (Boschker and Middelburg, 2002; Petersen et al., 2004) and D-alanine (Veuger et al., 2005) can be valuable tools in combination with dually labeled substrates. Heterotrophs (bacteria) and photoautotrophs differ with respect to their uptake of C and N. Photoautotrophs can assimilate DIC during photosynthesis in the light and incorporate N throughout the entire day, resulting in a temporarily uncoupled uptake of C and N. Heterotrophs grow on organic substrates and usually take up C and N at the same time, but their relative use of C and N may differ. It is likely that during the summer months uptake by photoautotrophs was more important in our study than during the other months, since uptake rates of C and N in DFAA were uncoupled. During the other months heterotrophic uptake may have been of greater importance. This is consistent with the findings of Dijkman and Kromkamp (2006), especially for January, when bacteria were more abundant than in the other months. Organisms that are most suited to utilize DON are mixotrophs, which can use inorganic nutrients and light energy (as photoautotrophs) for growth during the day, and organic substrates for both nutrients and energy (as heterotrophs) during the day and night (Mulholland et al., 2004). The Scheldt estuary constitutes a perfect environment for such organisms, with limited light availability below the surface and both high nutrient and DOM availability.

3.5 Conclusions

DON uptake contributes significantly to total N uptake by natural estuarine communities and should therefore be included in N uptake studies. Like urea and DFAA, it can constitute a source for both N and C, and thus the use of single labeled substrates might result in a biased uptake estimate and incomplete understanding of DON cycling.

The uptake of C and N from DON varies seasonally: in January, April and November, C is taken up to a larger extent than during July and Octo-

ber, periods when NH_4^+ availability is relatively low. Rates of nitrification and uptake of NH_4^+ are of similar of magnitude.

4 Response of sediment nitrogen cycling to changing temperature and nitrate concentration

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4.1 Introduction

Production of N_2 is a key biogeochemical process since this is the dominant way bioavailable nitrogen can be removed from a system. It is therefore a natural process counteracting eutrophication. In aquatic systems, the main factors governing N_2 production by denitrification and Anammox are bottom water concentrations of nitrate (NO_3^-), nitrite (NO_2^-) and oxygen, temperature and organic matter loading (Seitzinger, 1988; Middelburg et al., 1996b; Cornwell et al., 1999). Denitrification (the microbial reduction of NO_3^- to N_2 via NO_2^-, Figure 4.1 c) and dissimilatory nitrate reduction to ammonium (DNRA, Figure 4.1 d), are competing for the NO_3^- available. The two NO_3^- consuming processes have different ecosystem consequences. The NH_4^+ produced by DNRA can be used by plants, algae and bacteria and thus sustain eutrophication while the N_2 produced by denitrification is not available for assimilation and thus this process counteracts eutrophication. Sørensen (1978) already suggested that denitrification and DNRA might be of similar importance in coastal marine sediments. Several studies have since shown DNRA to be of similar importance (Bonin et al., 1998; Kelly-Gerreyn et al., 2001) or more important (Gilbert et al., 1997; An and Gardner, 2002; Karlson et al., 2005) than

denitrification regarding NO_3^- removal. The relative importance of N_2 formation by denitrification and Anammox (Figure 4.1e) is unknown subject of intensive study (Hietanen, 2007).

In tidal systems the sediment can experience large tidal driven differences with respect to salinity, temperature, oxygen and nutrient concentrations in the overlying water. Many studies have shown sediment denitrification rates to be strongly depending on the concentration of NO_3^- in the overlying water (e.g. Christensen et al., 1990; Meyer et al., 2001) and that increasing concentrations of NO_3^- have stimulating effect on denitrification (Rysgaard et al., 1994; Kana et al., 1998) even if NO_3^- produced within the sediment is the major substrate for denitrification (Seitzinger, 1988; Middelburg et al., 1996b). The aim of this study was to experimentally investigate the response of sediment nitrogen cycling to sudden increases in the NO_3^- concentrations in the overlying water alone and in combination with elevated temperatures. The response was investigated at two different seasons with whole core sediment incubations using membrane inlet mass spectrometer (MIMS) measurements to measure net N_2 concentrations changes. This study focuses on the relative contribution of N_2 versus NH_4^+ production and release and will therefore lump denitrification and Anammox below.

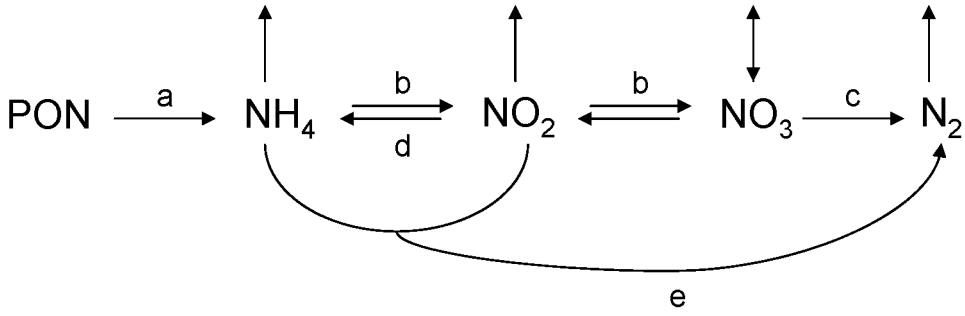

Figure 4.1: Processes which can occur in the sediment and in the water where nitrogen is transformed include a) ammonification b) nitrification c) denitrification d) dissimilatory nitrate reduction to ammonium (DNRA) and e) Anammox, anaerobic oxidation of ammonium.

4.2 Materials and Methods

4.2.1 Study area and sampling

Sediment cores were collected in the Oosterschelde, a tidal inlet situated in the southwest of the Netherlands and a part of the Delta area, created by the rivers Rhine, Meuse and Scheldt entering the North Sea. Before 1987 the Oosterschelde was an estuary but after the construction of a storm-surge barrier at the mouth and two auxiliary compartments dams in the rear ends, the Oosterschelde changed from an estuary into a tidal bay (Nienhuis and Smaal, 1994). The water residence time in the Oosterschelde varies between 20-135 days depending on physical conditions and distance from the North Sea (Dame and Prins, 1998). The tidal range is on average 3 meters (Smaal and Nienhuis, 1992). The samples for this study were collected close to Zandkreekdam (Figure 4.2).

Undisturbed sediment cores were taken by hand at low tide in winter (November/December) 2004 and summer (July) 2005. During the summer dense algae mats were present at the sediment surface. Here cores were taken in open patches with little algae cover to be comparable with the cores taken during the winter when no algae were present. 12 Plexiglas cores (winter: 7.5 cm i.d. and summer: 10 cm i.d.) were gently inserted into the sediment and withdrawn without disturbing the sediment surface. Within one hour the cores were transferred to a climate controlled room held at constant temperature. The *in situ* water temperature was 10 and 15 °C during winter and summer respectively, and the salinity around 20 at all sampling occasions. Oosterschelde water (water height 2/3 and sediment height 1/3 of the core) was carefully added to the cores, avoiding resuspension of the surface sediment. Cores were placed uncapped, submerged in an open tank containing aerated Oosterschelde water, to pre-incubate for 48 hour in darkness. Teflon coated magnets, rotated by a central magnet, were placed approximately 5 cm above the sediment surface in each core to ensure movement of the water and to prevent anoxia at the sediment-water interface. All equipment used for the incubations (lids, Teflon tubings, Luer stopcocks) were pre-incubated in Oosterschelde water to avoid introduction of new surfaces for O_2, N_2 and argon (Ar) adsorption and desorption.

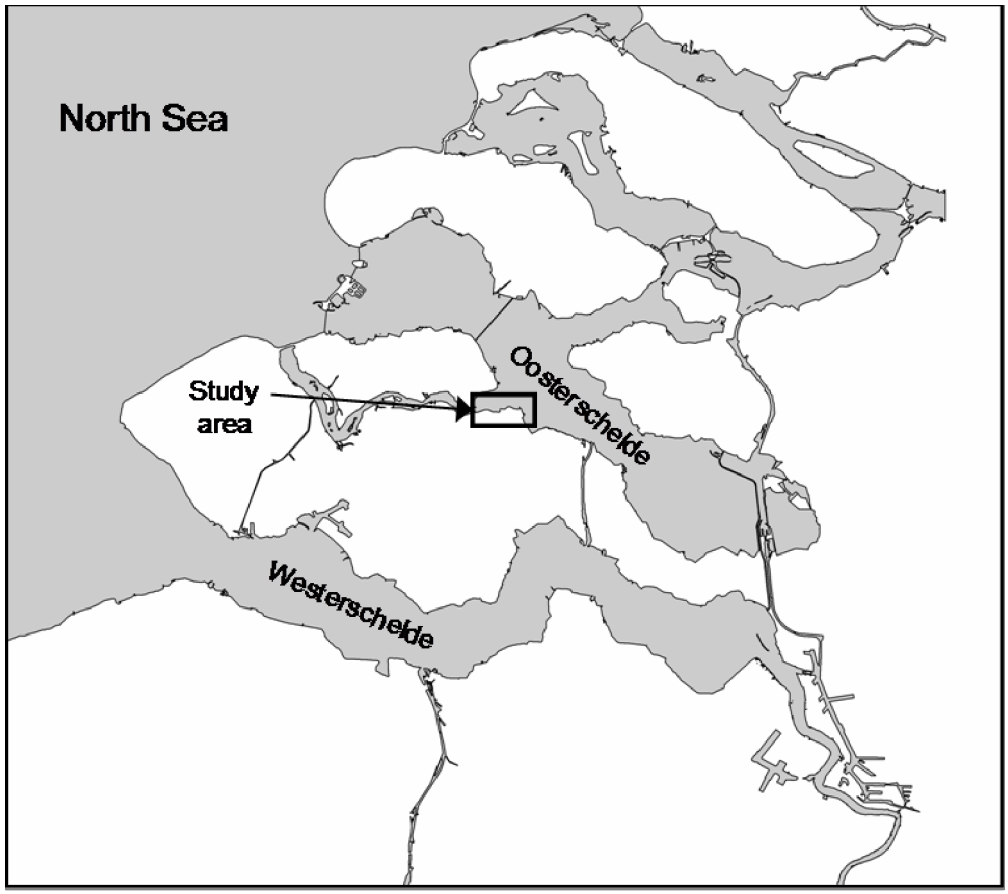

Figure 4.2: Map showing the Oosterschelde bay in the southwest of the Netherlands. The study area Zandkreek is indicated with a square.

4.2.2 Flux measurements

After 48 hours of pre-incubation each core was closed with a gas tight lid. Triplicate cores were amended with 25, 50 or 75 μM NO_3^- (0.5 M $NaNO_3$ solution), respectively at the start of the incubations. The remaining three cores were unamended. The cores were incubated under dark conditions and the incubations lasted between 6-10 hours. The incubation time depended on the decrease of oxygen in the overlying water, average oxygen decrease during incubation was 25 % (maximum decrease was 45 %). Incubations were made at three different temperatures per season; ambient, ambient + 5 °C and ambient + 10 °C, corresponding to 10, 15 and 20 °C in winter and 15, 20 and 25 °C in summer.

Before the incubations were started, but after NO_3^- addition, samples were taken for the start values. During the incubation samples for N_2, O_2, dissolved inorganic carbon (DIC, summer only) and nutrients were withdrawn from the overlying water via Luer stopcocks sample ports with a gas tight glass syringe. Refill water was allowed to flow in via Luer stopcocks from the reservoir tank water surrounding the cores. For N_2 and Ar triplicate samples were collected at the start and end of the incubation while duplicate samples were collected at intermediate sampling occasions. The samples were transfered to 10 mL Pyrex glass test tubes with glass stoppers avoiding introduction of air bubbles, preserved with 20 μL $HgCl_2$ (saturated solution) and stored submerged in Oosterschelde water at the incubation temperature. After the incubations some of the cores were sieved (1 mm sieve) to give us an idea of the macro fauna present.

The samples for N_2 gas were analyzed within one week using membrane inlet mass spectrometry (MIMS). N_2 and Ar were detected using an Omnistar QMS 200 quadropole mass spectrometer (Baltzers Instruments, Liechtenstein). The inlet tube consists of a 60 cm stainless steel tube (1/16 inch O.D., 1 mm I.D.) with 14 holes of 0.7 mm close to the silver soldered closed end, and was kept at 150 °C. Medical silicon tubing (Raumedic, 1 mm O.D., 0.5 mm I.D.) covering the 14 holes forms the gas permeable inlet. Due to the high background concentration of N_2 in relation to a relatively small change, N_2 was normalized versus Ar to detect the N_2 produced during the incubation. Ar concentrations were assumed to be at equilibrium with the water at the measured temperature and salinity because Ar is not affected by biological processes (Kana

et al., 1994). N_2 fluxes across the sediment-water interface were calculated by linear regression of the concentration data, corrected for the refill water (Figure 4.3). The rates are presented as average values ± standard deviations of replicate cores.

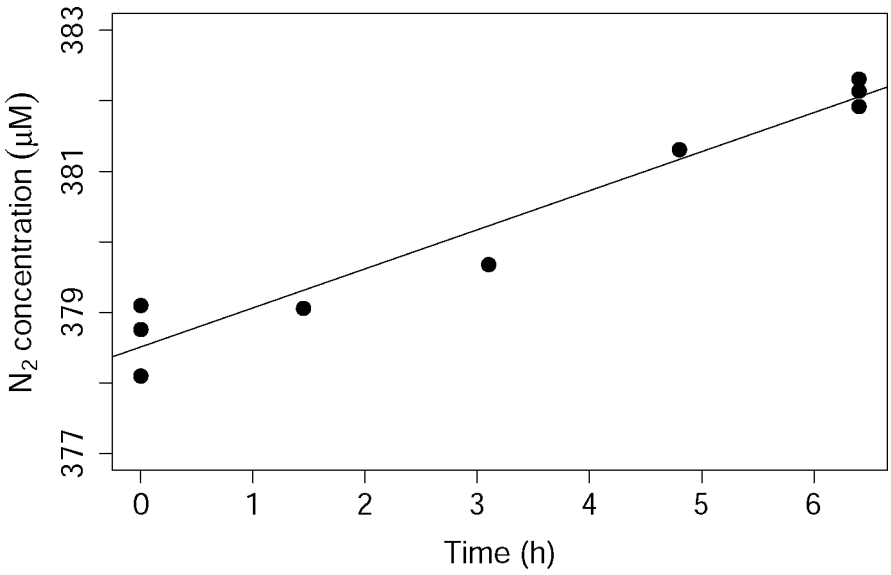

Figure 4.3: Representative example of change in N_2 concentration, after normalizing versus Ar, during an incubation. Line is drawn according to least square linear regression of all sampling points.

Water was filtered through pre-weighed, pre-combusted Whatman GF/F filters (47 mm), stored frozen and analyzed for NH_4^+, NO_3^- and NO_2^- using automated colorimetric techniques. Fluxes of nutrients across the sediment-water interface were calculated as mentioned above. Oxygen concentrations in all cores at the start and the end of the incubations were measured using Winkler titration (Parsons et al., 1984). Oxygen consumption was calculated from the difference in concentration between start and end values, assuming constant O_2 uptake and compensating for

the dilution by refill water. Oxygen fluxes based on MIMS data were consistent with those based on Winkler titration. During summer, additional samples for DIC concentrations were taken in all cores at the start and the end of the incubations. The samples for DIC were analyzed within 24 h by the flow injection technique of to Hall and Aller (1992).

4.2.3 Porewater extraction and sediment characteristics

Separate cores were collected to determine pore water profiles of NH_4^+ and NO_3^-, grain size characteristics of the upper 5 cm and porosity. The cores were sliced at 1 cm depth intervals and porewater extracted by centrifugation (30 min., 3000G) and subsequent filtration, with pre-combusted Whatman GF/F filters, of the supernatant. The extracted porewater was analyzed for nutrients as described above. Grain size was determined in duplicate via laser-diffraction with a Malvern laser particle sizer 3600 EC. Porosity was calculated from water loss of a known sediment volume after drying at 50 °C for 48 h.

4.3 Results

4.3.1 Sediment characteristics and porewater profiles

The porewater concentration profiles of NH_4^+ from winter and summer were similar at larger depths (> 8 cm) where concentrations of up to 200 μM were observed (Figure 4.4). During summer the top 5 cm of the sediment contained lower concentrations of NH_4^+ than during winter. The porewater profiles of NO_3^- were different between the two seasons. The summer profile followed the expected trend with low NO_3^- concentrations decreasing with depth (Figure 4.4 D) while the winter showed relatively high NO_3^- concentrations increasing with sediment depth (Figure 4.4 B). Moreover, during winter the standard deviation among replicates for NO_3^- was large compared to the other porewater profiles. During winter the median grain size was 106 μm (sand) for the upper 2 cm of the sediment and 28 μm (silt) for sediment at 2-5 cm depth. During summer the upper sediment fraction had a median grain size of 120 μm and the subsurface layer was similar (128 μm). The average organic carbon content in the

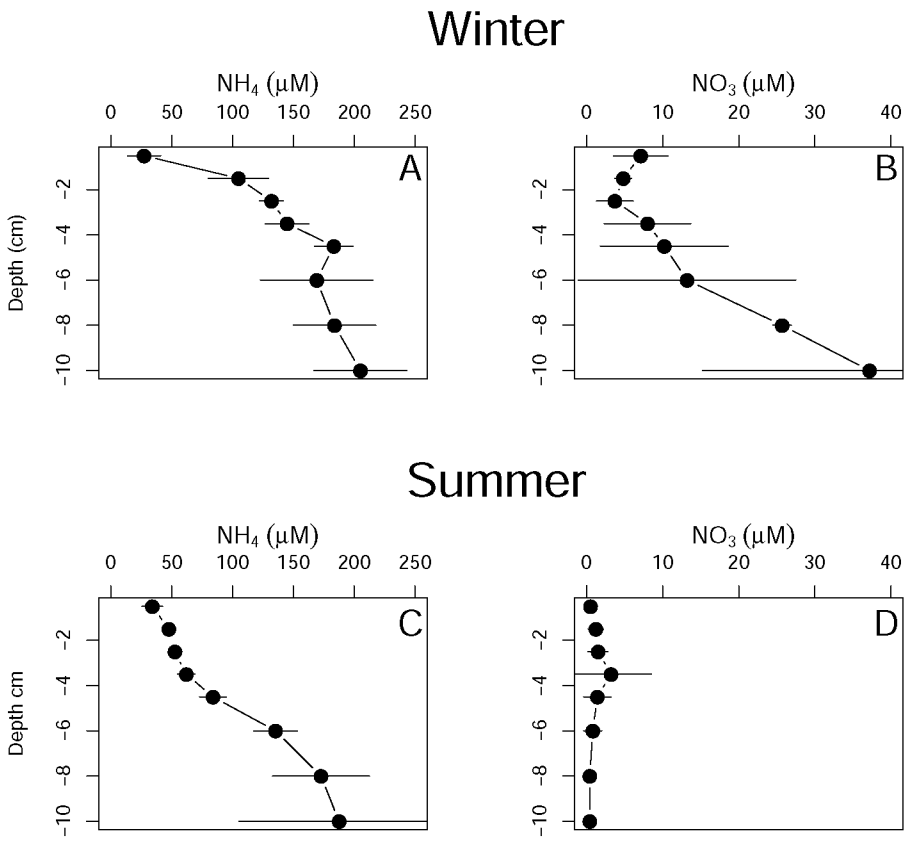

Figure 4.4: Porewater profiles of NH_4^+ and NO_3^- down to 10 cm depth during winter (n=2) and summer (n=3).

top two cm of the sediment were 0.75 % during winter and 0.26 % during summer. Average nitrogen content in the top 2 cm were 0.05 % and 0.04 % during winter and summer respectively. The molar C:N ratios were 16 during winter and 7.4 during summer. Macrofauna diversity was low and dominated by small specimens of *Arenicola marina*. During winter the upper 1-2 cm and during summer the upper 2-3 cm of the sediment were oxidized, based on visual observations.

4.3.2 Response to elevated temperatures and NO_3^- loading

Ambient NO_3^- concentration and *in situ* water temperature during the winter experiment were 40 μM and 10 °C. Increasing the temperature had no significant effect (Anova, p=0.09) on the N_2 production rate (Figure 4.5 A) which averaged 1.9±0.6 mmol N m^{-2} d^{-1} for all temperatures. After addition of 75 μM NO_3^- however, temperature had a significant effect on N_2 production (Anova, p=0.02, Figure 4.5 B). At 10 °C there was no increase but at 15 and 20 °C N_2 production rate increased to 3.7±1.2 mmol N m^{-2} d^{-1} averaged over both temperatures compared to 1.6±0.7 mmol N m^{-2} d^{-1} at 10 °C. During the summer experiments the *in situ* water temperature was 15 °C and the bottom water concentration of NO_3^- was 10 μM. The N_2 production rate in unamended cores at *in situ* temperature was 63 % higher in summer, 3.1±0.2 mmol N m^{-2} d^{-1}, compared to winter, 1.9±0.9 mmol N m^{-2} d^{-1} (Tables 4.1, 4.2). Increasing the temperature in summer had a significant effect on N_2 production rates (Anova, p=0.005, Figure 4.5 C). An increase of 10 °C at *in situ* nitrate levels resulted in a N_2 production rate of 6.1±0.2 mmol N m^{-2} d^{-1} compared to 3.1±0.2 mmol N m^{-2} d^{-1}. There was also an effect of nitrate addition; this was however not statistically significant (Anova, p=0.06, Figure 4.5 D).

Response of N_2 production to intermediate additions of NO_3^- (25 and 50 μM) followed the trend discussed above (Figure 4.6). During winter increased NO_3^- concentrations corresponded with increased N_2 production at elevated temperatures while during summer the response of N_2 production to additional NO_3^- could only be seen at the highest temperature. The Q_{10} value for unamended cores were 1.0 during winter and 1.3 during

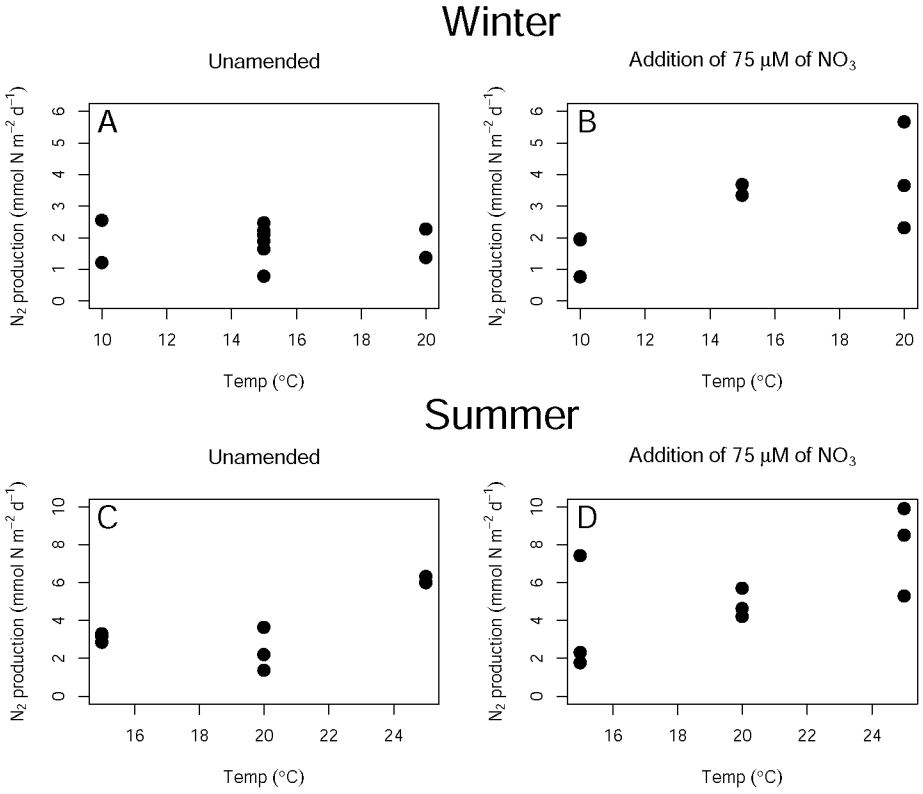

Figure 4.5: Response of increased temperature in unamended cores and with addition of 75 μM NO_3^- in winter and in summer (note differences in scale).

summer ($Q_{10} = e^{(10s)}$; s being the slope of the regression: temperature vs. logarithm of the rate (Berounsky and Nixon, 1990)). The Q_{10} values for the 75 μM NO_3^- addition were 1.5 and 1.4 during winter and summer respectively.

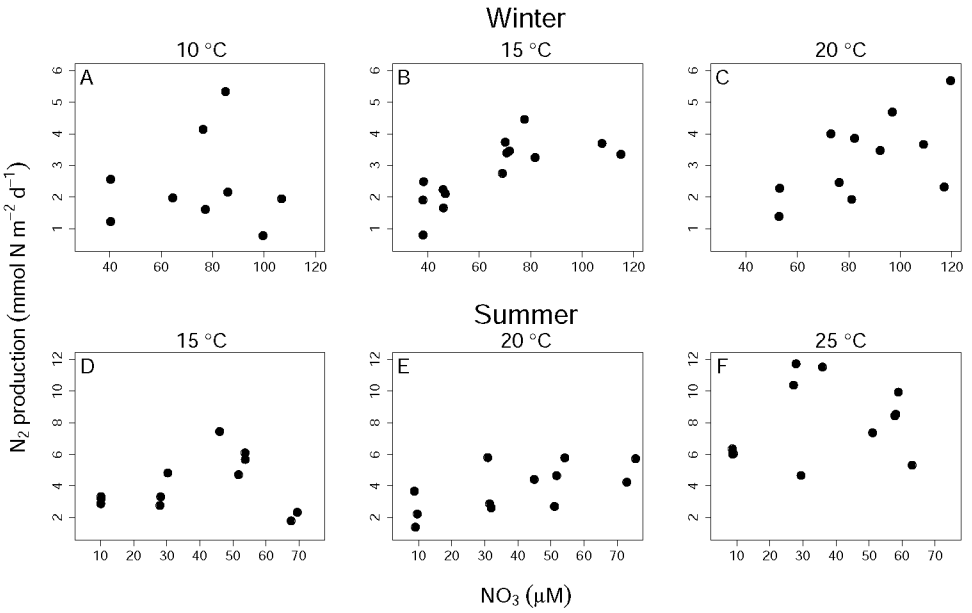

Figure 4.6: Response to different pulse addition of NO_3^- to the overlying water in the cores taken in winter and in summer.

4.3.3 Benthic metabolism and nutrient flux

During winter the average flux of NH_4^+ increased in unamended cores from 1.7 to 3.2 mmol N m^{-2} d^{-1} when the temperature was raised 10 °C (Table 4.1, Figure 4.7 A). During summer the average increase in NH_4^+ flux was larger, from 1.3 to 17 mmol N m^{-2} d^{-1}, with the same raise in temperature (Figure 4.7 C). At ambient temperatures NH_4^+ efflux rates in winter and summer were similar. During winter there was no effect of added NO_3^- on NH_4^+ efflux at the two lower temperatures, but at 20 °C the efflux of NH_4^+ increased (with up to 70 %) with additional NO_3^- (Figure 4.7 B).

During summer NH_4^+ efflux rates increased with increasing NO_3^- concentration at the lowest temperature, but there was additional release at 20 and 25 °C. A similar pattern was found for oxygen consumption rates with no or little effect of added NO_3^-. During the winter experiment elevated temperatures (at *in situ* NO_3^- levels) had no effect on oxygen consumption, while we found and increase of 16 and 63 % of the oxygen consumption at 20 and 25 °C respectively, compared to at *in situ* temperature, during summer (Tables 4.1, 4.2). The production of dissolved inorganic carbon (summer only) followed the same pattern as oxygen consumption with a small increase (15 %) at 20 °C and a large increased (170 %) at 25 °C. There was no effect of NO_3^- loading on the production of DIC. The respiration quotient (RQ; DIC:O_2 ratio) was 1.8 ± 0.3 at 15 and 20 °C combined and increased to 2.5 ± 0.4 at 25 °C. Fluxes of NO_3^- were generally very low and in several cases there was no significant change of NO_3^- concentration over time in the overlying water.

4.4 Discussion

Sediment in tidal systems may be subject to rapid changes in nitrate concentrations and temperature because they are covered by different water masses that move with the tides. These rapid and transient changes in overlying water conditions may induce a sedimentary response. While the addition of NO_3^- was nearly instantaneously, increases in water and sediment temperature were slower and we therefore decided to pre-incubate the cores at elevated temperature for 48 hours prior to the incubations. We speculate that this may not be sufficient time for the bacterial community to adapt to the higher temperature and that a shorter or longer pre-incubation time could have given different results. In this study we showed that elevated temperatures alone had little to no effect on the N_2 production rate at *in situ* concentrations of NO_3^- during winter and only an effect at the highest temperature (+ 10 °C) during summer (Figure 4.5 A, C). Sedimentary respiration did however respond to imposed changes in temperature (Table 4.2). Increased loadings of NO_3^- to the overlying water did not affect N_2 production rates at ambient temperatures, irrespectively of season (Figure 4.6 A, D). However, as the temperatures were increased above ambient levels the N_2 production rate increased with additional

Table 4.1: Average N_2 production, fluxes of NH_4^+ and NO_3^- and O_2 consumption rates at three different temperatures during winter on unamended samples and with additional NO_3^- added. Ambient concentration of NO_3^- was 40 μM. Numbers in superscript (^{x}) indicate number of replicates when different from 3, due to problems during incubation or measurements. ND - no significant flux could be detected in any of the replicate cores. Positive values refer to effluxes; negative values to influxes.

Temp °C	added NO_3^- μM	N_2 prod mmol N m^{-2} d^{-1}	NH_4^+ flux mmol N m^{-2} d^{-1}	NO_3^- flux mmol N m^{-2} d^{-1}	O_2 consump mmol m^{-2} d^{-1}
Winter					
10	0	1.9±0.9^2	1.7±0.5	ND	47±5.6
10	25	3.7±2.2^2	1.9±1.4	-1.1^1	49±12
10	50	2.9±1.8^2	1.2±0.9	-2.2±0.8	39±2.9
10	75	1.6±0.7	ND	-3.8±1.1	44±10
15	0	1.9±0.6^6	2.4±0.8^5	-0.5^1	35±7.8^7
15	25	3.3±0.5	4.3±1.2	-2.4^1	34±14
15	50	3.7±0.6	2.5±1.5^4	-2.3^1	36±9.4^4
15	75	3.5±0.2^2	1.8^1	-7.3±2.7^2	52±18
20	0	1.8±0.6^2	3.2±0.9	-1.4±0.8	50±12
20	25	2.8±1.1	3.2±1.1	-2.5±0.7	63±11
20	50	4.0±0.6	4.5±1.3	-7.6±3.8^2	67±5.8
20	75	3.9±1.7	5.4±2.0	-6.9±3.7^2	62±19

Table 4.2: Average N_2 production, fluxes of NH_4^+ and NO_3^-, O_2 consumption, DIC production and RQ at three different temperatures during summer on unamended samples and with additional NO_3^- added. Ambient concentration of NO_3^- was 10 μM. Numbers in superscript (^{x}) indicate number of replicates when different from 3, due to problems during incubation or measurements. ND - no significant flux could be detected in any of the replicate cores. Positive values refer to effluxes; negative values to influxes.

Temp °C	added NO_3^- μM	N_2 prod mmol N m^{-2} d^{-1}	NH_4^+ flux mmol N m^{-2} d^{-1}	NO_3^- flux mmol N m^{-2} d^{-1}	O_2 consump mmol m^{-2} d^{-1}	DIC prod mmol m^{-2} d^{-1}	RQ
15	0	3.1±0.2	1.3±1.5	-1.1±0.4^2	57±4.4^2	102±26	1.8
15	25	3.6±1.1	3.3±1.5	4.2^1	63±6.0	96±25	1.5
15	50	5.5±0.7	3.9±1.8	8.1±2.2	72±15	108±7^2	1.5
15	75	3.8±3.1	4.1±2.3	29±6.5	64±30	137±48	2.1
20	0	2.4±1.1	3.2±2.8	-1.1±0.1^2	66±8.2	117±12	1.8
20	25	3.7±1.8	6.5±5.5^2	0.7±4.2^2	72±13	124±49	1.7
20	50	4.3±1.5	5.8±4.6	ND	71±16	173±12	2.4
20	75	4.9±0.8	4.9±1.4	37^1	55±6.9	97±2	1.8
25	0	6.1±0.2	17±6.2	-1.3±0.6	93±11	276±67	3.0
25	25	8.9±3.7	16±5.0	-8.4^1	111±8.6	220±9	2.0
25	50	9.1±2.2	15±1.9	-7.4±13	95±21	251±10	2.6
25	75	7.9±2.4	16±4.9	17^1	92±20	229±45	2.5

NO_3^-, both during winter and summer (Figure 4.5 B, D). Some studies have shown a strong correlation between N_2 production and both NO_3^- concentrations in the overlying water (Rysgaard et al., 1994; Nielsen et al., 1995; Kana et al., 1998; Meyer et al., 2001) and temperature (Seitzinger, 1988). In other studies however, no effect of temperature (Fear et al., 2005) or no correlation between N_2 production and overlying water NO_3^- concentrations (Seitzinger, 1994; An and Gardner, 2002) were observed. Our study shows that the interaction between two factors, temperature and NO_3^- concentrations in the overlying water, governs N_2 production, whereas these two factors separately have a minor effect.

When the temperature was increased above ambient levels, both during winter and summer, the flux of NH_4^+ out of the sediment increased (Figure 4.7, A, C). Production of N_2 was not affected by the increased temperature (except at 25 °C during summer, Figure 4.7 C). This indicates that production of NH_4^+ has a faster response to increased temperature than nitrification or denitrification and Anammox (Figure 4.1 b, c). Our experimental manipulations allowed us to assess whether nitrification or denitrification had a slower response to increased temperature. When NO_3^- was added to the overlying water and therefore not limiting the system, there was an increase in N_2 production with enhanced temperature (Figure 4.5 B, D). This suggests that the nitrifying population was likely not responding to the same extent as the ammonifying, denitrifying and Anammox populations to increased temperatures. Previous studies of the benthic processes have shown the Q_{10} value for NH_4^+ production to be higher than those of nitrification and denitrification (Berounsky and Nixon, 1990). In our study we found Q_{10} values for benthic N_2 production between 1 and 1.5, similar to those reported by Berounsky and Nixon (1990).

In this study we have shown that with increasing temperature the efflux of NH_4^+ from the sediment increased (Figure 4.7, A, C). Sediment efflux of NH_4^+ results from the net balance between NH_4^+ production during ammonification and DNRA on the one hand and NH_4^+ consumption during nitrification, Anammox (and assimilation) on the other hand. Increased ammonification at higher temperatures appears to be the most likely cause for the increased efflux of NH_4^+, because O_2 uptake and DIC release increased with temperature (Table 4.1, 4.2). One would then expect a corresponding increase in NH_4^+ efflux and oxygen consumption.

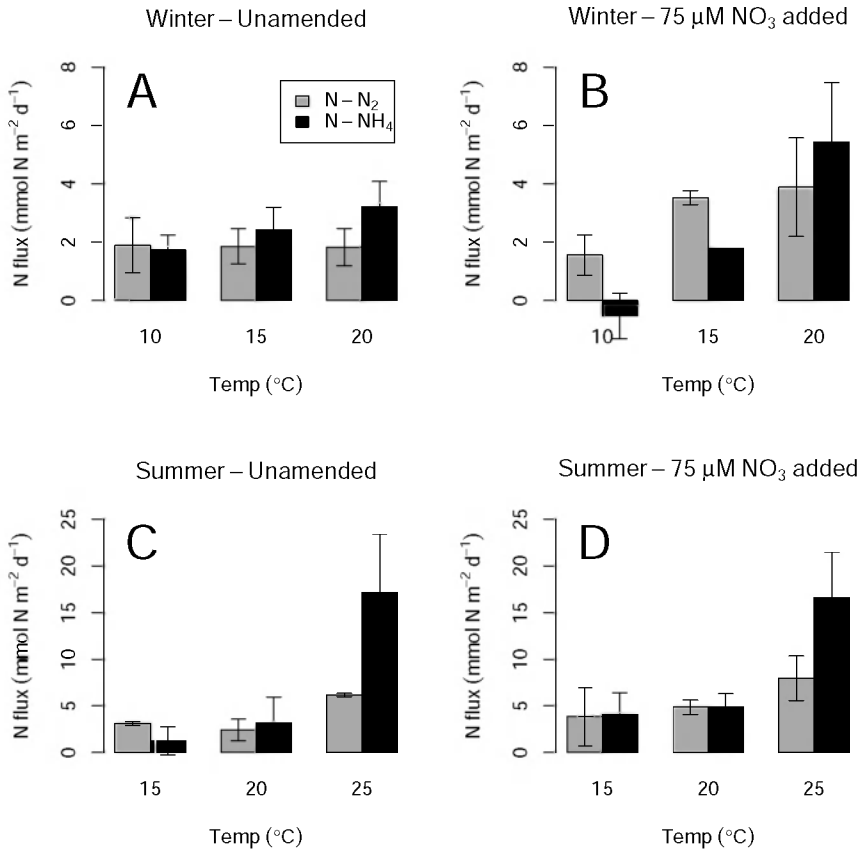

Figure 4.7: Production of NH_4^+ and N_2 at the three different temperatures during winter and summer for unamended samples. Note difference in scale.

This was indeed observed during summer (Figure 4.8) but not during winter. NH_4^+ effluxes during winter were far lower than expected based on ammonification, indicating that nitrification was stimulated by elevated temperature.

Dissimilatory nitrate reduction to ammonium (DNRA, Figure 4.1 d) is resulting in the formation of NH_4^+. This process competes with denitrification (Figure 4.1 c) for NO_3^- as substrate. Compared to denitrification, DNRA has received relatively little attention and the ecological significance of this process is not completely clear (Cornwell et al., 1999). Since it affects both NO_3^- and NH_4^+ concentrations it may have a large impact on several other processes of the nitrogen cycle. Sørensen (1978) was, to our knowledge, the first to quantify the importance of DNRA versus denitrification. His data suggested similar importance of the two processes in marine sediments. Addition of NO_3^- caused an increase in efflux of NH_4^+ at the highest temperature during winter and the two lower temperatures during summer (Figure 4.7), suggesting that DNRA may have been significant in these experiments.

Degradation of organic matter (OM) can occur aerobically, with oxygen as electron acceptor, or anaerobically through denitrification, manganese, iron or sulfate reduction (SR) using NO_3^-, manganese oxides (MnO_2), iron oxides (Fe_2O_3) and sulfate (SO_4^{2-}) as electron acceptors respectively. If the degradation of OM only occurs via aerobic respiration or if all the reduced compounds from anaerobic degradation are re-oxidized, a 1:1 relationship between DIC production and O_2 consumption is expected. In our study the DIC to O_2 ratios were much higher than 1:1 (Figure 4.9) indicating that reduced products of anaerobic mineralization, escape the sediments or accumulate in the sediment, most likely as iron sulfides (FeS_x). This discrepancy can only partly be explained by the escape of N_2 (Figure 4.9). Therefore manganese oxide, iron oxide or sulfate reduction (SR) must contribute significantly to mineralization and their products should not be fully oxidized. Ferro et al. (2003) showed that in sediment from a nearby site, SR was the dominant anaerobic mineralization process and manganese oxide and iron oxide reduction were of limited importance. If we neglect metal oxide reduction and attribute the remaining discrepancy between O_2 and DIC to SR and assuming that none of the reduced products of SR are re-oxidized, SR accounted for 40-62 % of the mineralization rate. This is a minimum estimate since some of the reduced

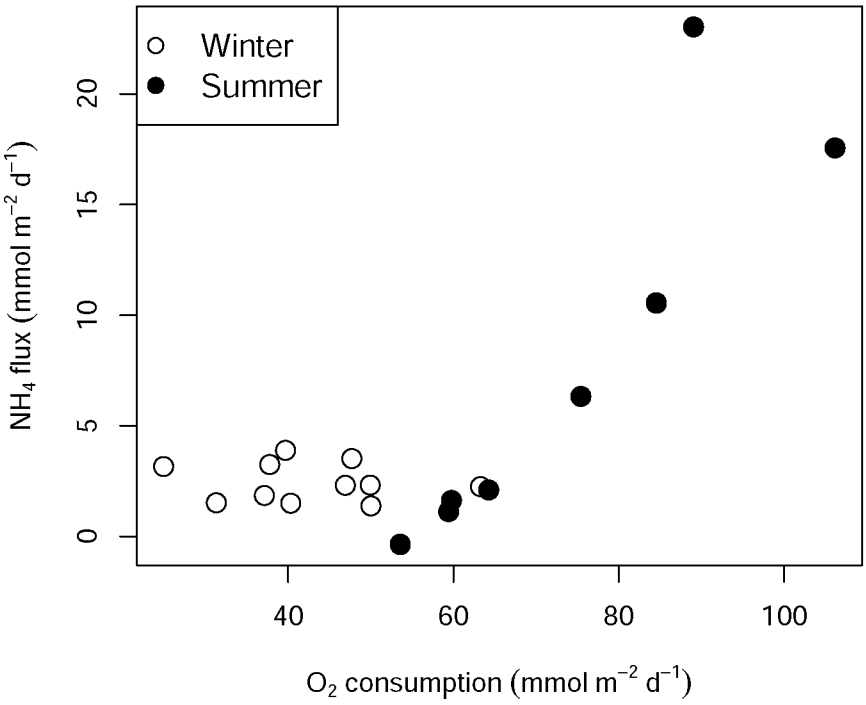

Figure 4.8: NH_4^+ flux plotted versus oxygen consumption in unamended samples during winter: ○ and summer: ●

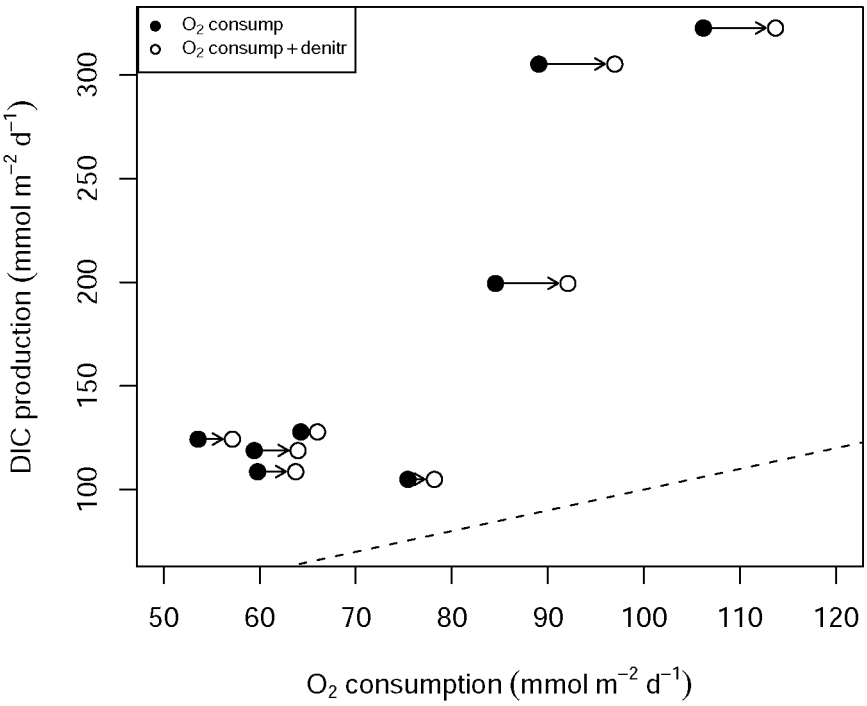

Figure 4.9: Relationship between DIC production and oxygen consumption at unamended incubations during summer experiments. The dashed line shows 1:1 ratio. The tie-lines between open and solid symbols reflect the contribution of N_2 production

compounds may have been re-oxidized with O_2. Using oxygen consumption as a measure for total mineralization in this type of systems may give significant errors due to the storage of reduced sulfur in the sediment. This contribution of SR and accumulation of reduced S is similar to results reported for other temperate intertidal mudflats in particular (Gribsholt and Kristensen, 2003) and marine systems in general (Thamdrup and Canfield, 2000). There was no significant change in the average fraction of mineralization that was due to SR with increased temperature in unamended cores. Estimated average fraction of mineralization due to SR were, however, more than 3 times higher at 25 °C compared to lower temperatures which falls within the range of Q_{10} values for SR of 2.6-3.6 reported previous in the literature (Jørgensen, 1977; Urban et al., 1994) and for intertidal sediment (Middelburg et al., 1996a). It has been suggested that high sulfide concentrations can inhibit nitrification and denitrification while stimulating DNRA (An and Gardner, 2002). Our results with high production of NH_4^+ together with high sulfide storage at 25 °C during summer supports these findings. However, we saw an increase in N_2 production with additional NO_3^- both during winter and summer, indicating that a slow response in growth of the nitrifying community is more likely limiting N_2 production at *in situ* NO_3^- concentrations rather than inhibition of both nitrification and denitrification due to high sulfide concentrations.

4.5 Conclusions

N_2 production was found to be governed by the interaction between temperature and NO_3^- concentration in the overlying water. Increasing temperature resulted in increased rates of oxygen uptake and DIC release and stimulated effluxes of NH_4^+ from the sediment but had a minor effect on N_2 production rates. DNRA is possibly occurring in the Oosterschelde with increasing importance at elevated temperatures. DNRA was however, not of the same order of magnitude as N_2 production. Sulfate reduction accounted for a large part of the mineralization rate and 40-62 % of the reduced products escaped re-oxidation or accumulated, at least temporarily, in the sediment.

5 Recovery of biogeochemical processes following an anoxic event: Results from in situ defaunation study

Maria G. I. Andersson, Britta Gribsholt, Francesca Rossi, Jack J. Middelburg

5.1 Introduction

Hypoxia, low oxygen concentrations in bottom waters, influences biogeochemical cycling and leads to significant structural changes in benthic communities (Diaz and Rosenberg, 1995; Gray et al., 2002). The intensity, duration and frequency of coastal hypoxia are changing due to human-induced alteration of coastal ecosystems (e.g. enhanced delivery of nutrients and/or organic matter) and climate change induced modification of ocean circulation patterns, water column stratification and water temperature (Rabalais et al., 2001; Kemp et al., 2005). More sluggish circulation and strengthened stratification result in less oxygen supply. Increased water temperatures lower the solubility of oxygen in water and enhance respiration. Many of the factors causing hypoxia are natural processes and have occurred for many years but their extent has increased in time and space due to eutrophication (Gray et al., 2002).

Whether natural, human enhanced or human induced, hypoxia causes major changes in coastal, in particular benthic, communities because of differential abilities of species to deal with low oxygen conditions (Levin, 2003). Hypoxia may result in large-scale mortality, changes in biodiversity and behaviour, physiological stress, and other sub-lethal effects, such as

reduced growth and reproduction (Rabalais et al., 2001). It is important to distinguish between the response of communities to permanent hypoxia such as those found in ocean oxygen minimum zones and the deep Black Sea, and to seasonal or intermittent hypoxia in coastal systems because there is less adaptation and more mortality in the latter.

Experimental and modelling research has generated a first-order understanding of the effects of low-oxygen bottom water concentrations on sediment biogeochemistry and sediment-water exchange fluxes. Lower bottom-water oxygen concentrations are expected to result in lower oxygen consumption rates, less re-oxidation of reduced metabolites and higher effluxes of ammonium and phosphate (Soetaert et al., 1996; Kemp et al., 2005). However, the response can be non-linear as reported for iron recycling (Wijsman et al., 2001) and can be partly compensated by co-varying concentrations of other oxidants in the bottom water, e.g. nitrate (Middelburg et al., 1996b; Soetaert and Middelburg, 2007).

While we have some understanding of the role of hypoxia on sediment biogeochemistry and on benthic fauna distribution patterns, these consequences are often studied in isolation and we have little knowledge on synergistic effects (Kemp et al., 2005; Herbert et al., 2007; Morse and Eldrigde, 2007): has the effect of hypoxia on fauna consequences for sediment biogeochemistry and sediment-water exchange fluxes? Such indirect effects are expected because of the major role of animals on sediment biogeochemistry (Meysman et al., 2006b). Benthic animals have some direct effects on sediment biogeochemistry through their respiration and excretion activity, but they have major effects on sediment biogeochemistry through modification of sediment texture, in particular through increasing heterogeneity and via their bio-irrigation and bioturbation activities (Herman et al., 1999; Meysman et al., 2006a).

Coastal hypoxia has many consequences that are detrimental to coastal ecosystems and it is therefore essential to study the combined ecological and biogeochemical response to decreasing and increasing oxygen concentrations. While decreasing oxygen concentrations can lead to rapid and dramatic responses such as die-off of benthic animals with consequences for bioturbation and sediment biogeochemistry, the response towards increasing oxygen concentrations are often much slower because recovery of benthic communities may take years to decades. This asymmetric response to decreasing and increasing oxygen (i.e. hysteresis) has consequences

for the functioning of coastal ecosystems with intimate interactions between pelagic and benthic compartments (Kemp et al., 2005; Soetaert and Middelburg, 2007). As stated in Gray et al. (2002) 'Eutrophication is a problem of global significance which, once it has occurred, is difficult to reverse'. After hypoxia and die-off of fauna, recolonization begins once the system returned to oxic conditions. Adult fauna migrate from surrounding oxic areas and larvae and juvenile fauna settle from the pelagic zone. The process of recolonization can take months to years before the system has recovered and returned to the condition prior to an anoxic/hypoxic event.

In this study we deliberately created hypoxic conditions to defaunate intertidal sediments and studied several biogeochemical processes during the recovery of fauna communities. We hypothesize: (1) That defaunation would lower variance of biogeochemical process measurements because of reduced heterogeneity related to animals presence and activity. (2) That denitrification rates in defaunated sediments would be lower than in control sediments because of lower bio-irrigation (3) That sediment community respiration ratios (O_2 to DIC effluxes) would be lower in defaunated sediments because of less particle mixing by the animals. Finally, we expect that the dynamics of reassembling faunal communities governs the recovery of fauna regulated biogeochemical processes.

5.2 Materials and Methods

5.2.1 Study area and sampling

The experiments were conducted on an intertidal flat, Paulinapolder, in the Scheldt estuary (Figure 5.1). The Scheldt estuary is located in the Southwest of the Netherlands and Belgium and is among the most nutrient-rich systems in the world (Soetaert et al., 2006). The tidal flat covers an area of approximately 1.0 km^2 and has a mean tidal range of 3.9 m with a semidiurnal regime. The macrofauna community of this muddy (50 % $<$ 63 μm) sediment is dominated by polychaetes (*Heteromastus filiformis*, *Arenicola marina*, *Pygospio elegans*) and molluscs (*Macoma balthica*, *Cerastoderma edule*, *Hydrobia ulvae*). The study site was characterized by homogeneous sediment properties with respect to median

particle size, total organic matter, porosity and chlorophyll content.

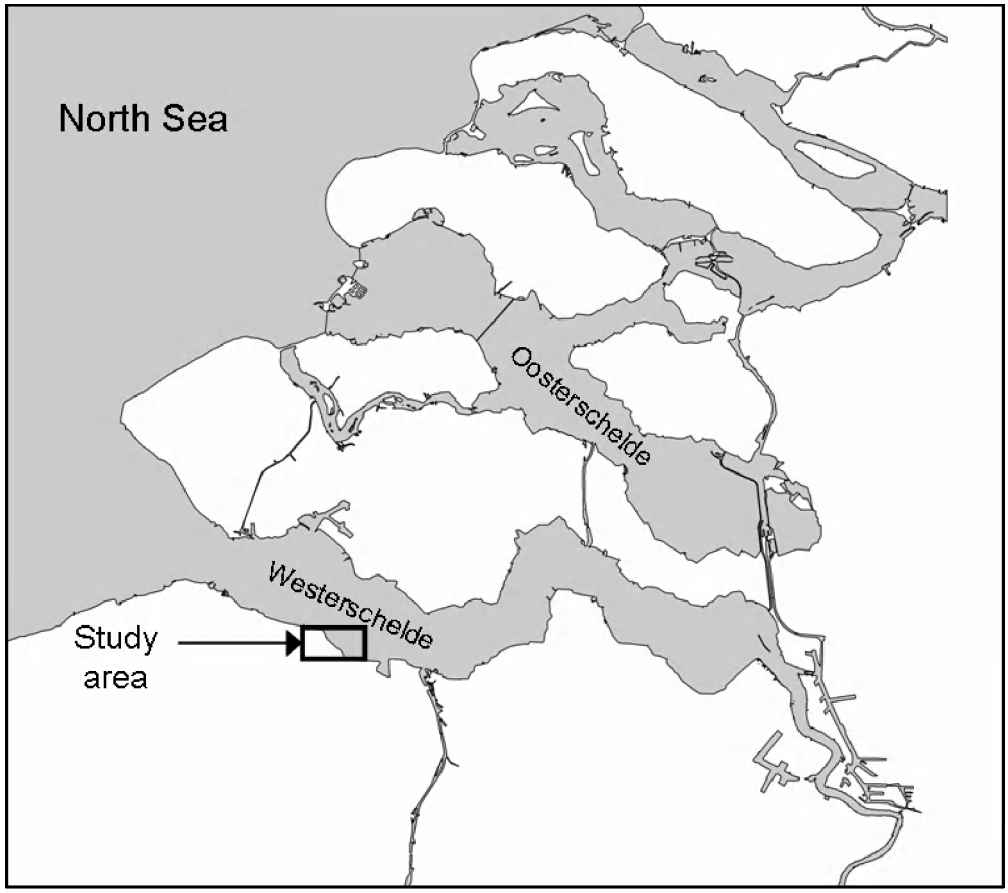

Figure 5.1: Location of the study site, Paulinapolder, in the Scheldt estuary.

At a 50 x 50 m location (51° 21' 23" N, 3° 42' 49" E), four relatively large patches (4x 4 m), 5 to 10 m apart were covered with black plastic sheets for two months to create severe hypoxia in the sediment. Two patches were covered from January 30 until March 30 and the other two from May 9 until July 6, 2005. Two undisturbed (e.g. no hypoxia) patches were chosen as controls. These treatments will be referred to as Defaunated: defaunated until March and allowed to recolonise for 5 months; Defaunated-J: defaunated until July and allowed to recolonise for 2 months and Fauna:

unmanipulated controls. Measurements were done in April (a few days after uncovering the plots), June and September to follow the effect of recolonization of the defaunated plots on sediment biogeochemistry and sediment-water exchange fluxes.

Undisturbed sediment cores were sampled by hand at low tide. The Plexiglas cores (10 cm i.d.) were gently inserted into the sediment and withdrawn without disturbing the sediment surface in the core. Within one hour the cores were brought to a climate room held at constant *in situ* temperature: 10, 16 and 20 °C in April, June and September respectively. Water was carefully added to the cores, avoiding resuspension to be created (water constituted 2/3 and sediment 1/3 of the core). The cores were placed uncapped, submerged in an open tank containing aerated water from the Scheldt estuary, for 48 hour in darkness. Teflon coated magnets, rotated by a central magnet, were placed approximately 5 cm above the sediment surface in each core to ensure movement of the water and to prevent anoxia at the sediment-water interface. All equipment used for the incubations (sediment cores, lids, Teflon tubings, Luer stopcocks) was pre-incubated in Scheldt water to avoid introduction of new surfaces for O_2, N_2 and argon (Ar) adsorption and desorption.

5.2.2 Flux measurements

Incubations

After 48 hours of pre-incubation each core was closed with a gas tight lid. Incubations were made at *in situ* temperature in triplicate cores. The cores were incubated under dark conditions and incubations lasted between 6-10 hours. The incubation time depended on the decrease of oxygen in the overlying water, average oxygen decrease during incubation was 17 % (maximum decrease was 51 %).

Sampling

Before the start of the incubations samples were taken for initial concentrations. During incubation samples for N_2, O_2, dissolved inorganic carbon (DIC) and nutrients were withdrawn from the overlying water via Luer stopcocks sample ports with a gas tight glass syringe. Refill water was

allowed to enter via Luer stopcocks from the tank water surrounding the cores. For N_2 and Ar triplicate samples were taken at the start and end of the incubation and duplicate samples at time intervals of 1.5-2 hours during incubation. The samples were taken from the sample ports with a glass syringe and transfered to 10 mL Pyrex glass test tubes with glass stoppers avoiding introduction of air bubbles. The samples were preserved with 20 μL $HgCl_2$ (saturated solution), stored submerged in water from the Scheldt estuary at the same temperature as used for the incubations.

Analyzes and calculations

The samples for N_2 were analyzed within one week using membrane inlet mass spectrometry (MIMS). N_2 and Ar were detected using an Omnistar QMS 200 quadropole mass spectrometer (Baltzers Instruments, Liechtenstein). The inlet tube consists of a 60cm stainless steel tube (1/16 inch O.D., 1mm I.D.) with 14 holes of 0.7 mm close to the silver soldered closed end, and was kept at 150 °C. Medical silicon tubing (Raumedic, 1 mm O.D., 0.5 mm I.D.) covering the 14 holes forms the gas permeable inlet. Due to the high background concentration of N_2 and relatively small changes due to N_2 effluxes, N_2 was normalized versus Ar to eliminate the influence of processes other than N_2 production. Ar concentrations were assumed to be at equilibrium with the water at the measured temperature and salinity since it is not affected by biological processes (Kana et al., 1994). N_2 fluxes across the sediment-water interface were calculated by linear regression of the concentration data, corrected for the refill water (Figure 5.2). The rates are presented as average values ± standard error of replicate cores.

Water was filtered through pre-weighed, pre-combusted Whatman GF/F filters (47 mm), stored frozen and analyzed for NH_4^+, NO_3^- and NO_2^- using automated colorimetric techniques. Oxygen concentrations in all cores at the start and the end of the incubations were measured using Winkler titration (Parsons et al., 1984). In addition to oxygen, samples for DIC concentrations were also taken in all cores at the start and the end of the incubations. The samples for DIC were analyzed within 24 h by flow injection technique according to Hall and Aller (1992). Oxygen consumption, DIC release and nutrient exchange were calculated from the difference in concentration between start and end values, assuming constant

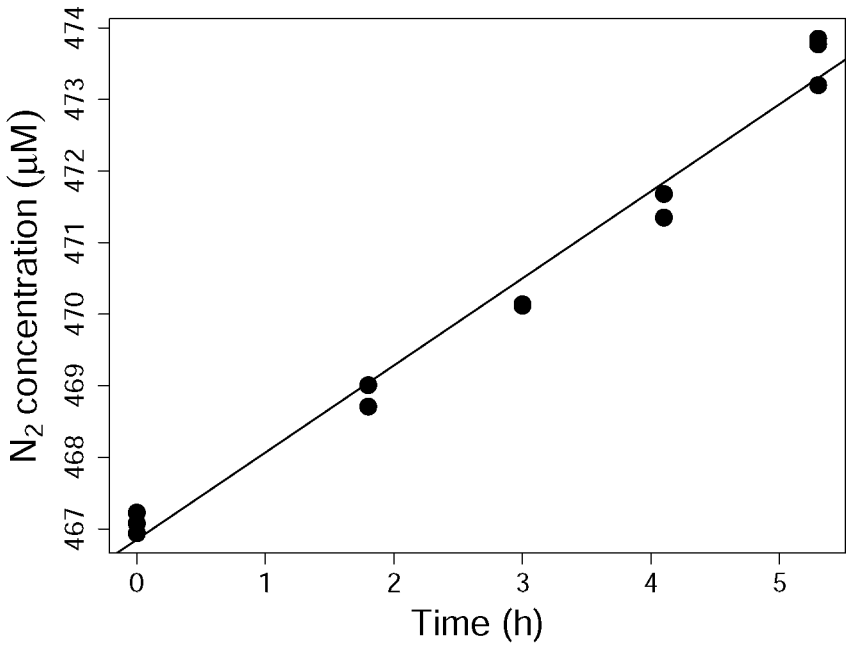

Figure 5.2: Linear increase of N_2, after normalizing versus Ar, during an incubation.

uptake/release and compensating for the dilution by refill water.

5.2.3 Macrofauna

After incubation the cores were sieved over 1 mm to determine benthic macrofaunal density (hereafter referred to as fauna) and species composition. All visible fauna was collected and preserved in 4 % buffered formalin and stored until analysis. Animals were identified to species level under a dissecting microscope, enumerated and the total dry weight of each species determined after drying at 60 °C for 48 h. Biomass of the fauna (ash free dry weight, AFDW) of each macrofaunal species was determined following combustion (520 °C for 6 h).

5.3 Results

5.3.1 Defaunation and recolonization

Defaunation of the sediment was successful and there was basically no biomass present at the first sampling after reopening the plots (Figure 5.3 B). However, there were 5 small specimens of *Heteromastus filiformis* in one of the three cores in April (Figure 5.3 A). In terms of species richness, average numbers of species in the three replicates, there were no differences between the defaunated plots and the control plots in June and September (Figure 5.3 A). However, in terms of biomass large discrepancies were observed between defaunated and control plots in September (Figure 5.3 B). It should be noted that in a more extensive faunal study and using a mesh-size of 0.5 mm Rossi et al. (2007) found differences in speices richness between defaunated and control sediment.

The facultative surface deposit feeding bivalve *Macoma balthica* and polychaete *Hediste diversicolor* were the first to recolonize the sediment. The *Macoma balthica* found in defaunated sediment however, were small, < 10 mm. The abundances of the suspension feeder *Cerastoderma edule* and the deep deposit feeder *Heteromastus filiformis* were still low in September compared to control plots (Figure 5.4). Abundance of *Hediste diversicolor* was even higher in recolonised plots than in control plot in September (Figure 5.4).

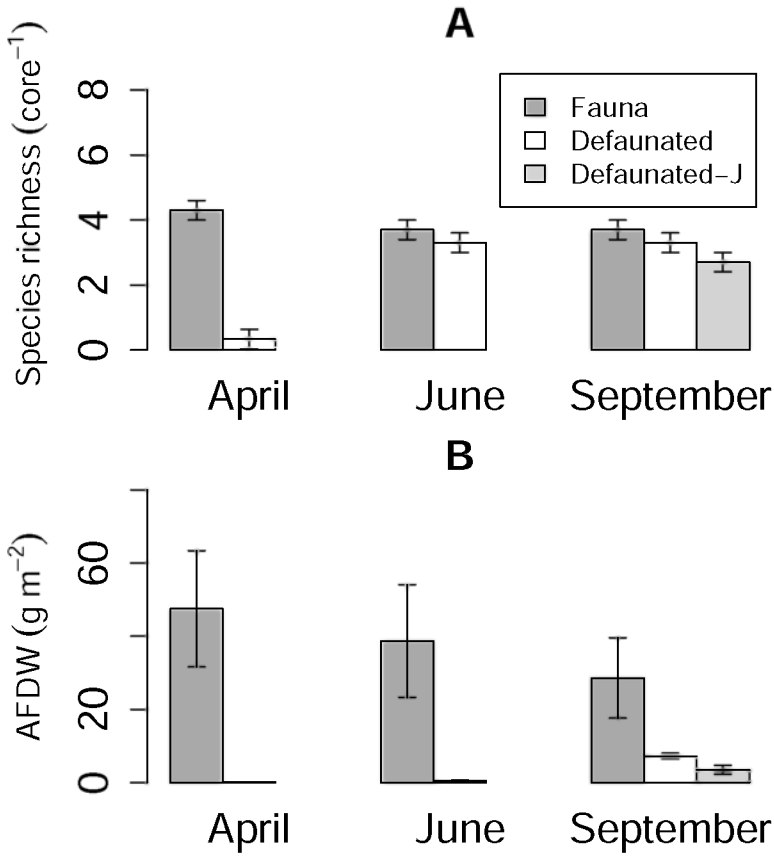

Figure 5.3: Upper graph: average number of species found per core, lower graph: average biomass found per core (g m^{-2}) during April, June and September in control plots (Fauna) and plots defaunated in April (Defaunated) and June (Defaunated-J).

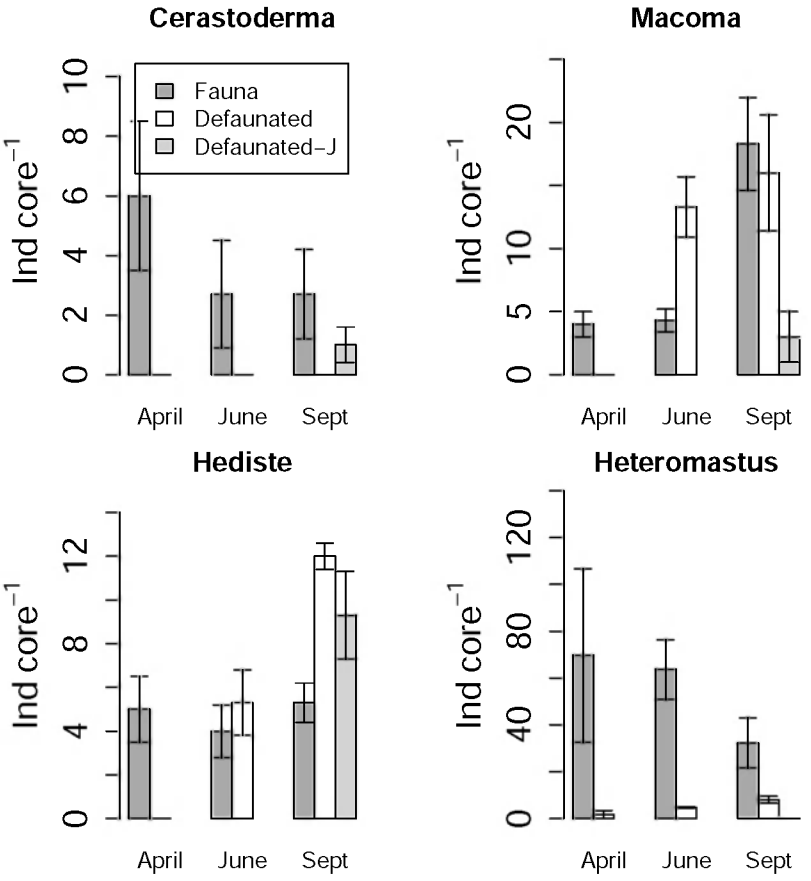

Figure 5.4: Average numbers of individuals of the bivalves *Cerastoderma edule* and *Macoma balthica* and the polychaetes *Hediste diversicolor* and *Heteromastus filiformis* per core during April, June and September in control plots (Fauna) and plots defaunated in April (Defaunated) and June (Defaunated-J).

5.3.2 Sediment water exchange and sediment biogeochemistry

The N_2 production rate in the control plots were in the same order of magnitude during the three sampling occasions with rates from 13 to 18 mmol m^{-2} d^{-1} (Table 5.1, Figure 5.5), higher rates in April and June and lower rates in September. The N_2 production rates in the defaunated plots varied between 5 and 8 mmol m^{-2} d^{-1} which were significantly lower (p-value=0.004) and had lower variance (i.e. standard errors; p-value=0.005) than the rates in the control plots. The ratio between N_2 production in control and defaunated plots increased with recolonisation from 0.30 in April via 0.43 in June to 0.52 in September. In Defaunated-J plots recolonised for two months, N_2 production rates were 38 % those of controls in September with an average N_2 production rate of 5.1 mmol m^{-2} d^{-1}.

Consumption of oxygen increased during the sampling period in control plots with average rates from 83 to 123 mmol $m^{-2}d^{-1}$ from April to September (Table 5.1, Figure 5.5). The variance among replicates in the control plots were larger (p-value=0.002) than those in defaunated and recolonizing plots. Oxygen consumption rates in the defaunated plots were 42-55 % of the controls with rates between 35 and 65 mmol m^{-2} d^{-1} (Table 5.1). In September the cores from Defaunated-J plots showed higher oxygen consumption rates, 69 mmol m^{-2} d^{-1} on average, compared to samples from plots defaunated in April, 57 mmol m^{-2} d^{-1}.

No clear difference in DIC release between control and defaunated plots was found during any of the measurements. In September however, there was a strong indication of lower production rates in the defaunated plots with rates of 100 mmol C m^{-2} d^{-1} (average of all measurements from defaunated plots defaunated) compared to 175 mmol C m^{-2} d^{-1} in the control plots. In April the average production rate of DIC for all cores was around 110 mmol C m^{-2} d^{-1} and in June the average was 210 mmol C m^{-2} d^{-1}.

Effluxes of NH_4^+ from control and defaunated plots were similar in April and June with values around 10 mmol $m^{-2}d^{-1}$ (Figure 5.6). In September however, there was a clear difference (p-value=0.004) between NH_4^+ efflux in the control plots, on average 20 mmol $m^{-2}d^{-1}$ and the plots defaunated in April, average efflux 7 mmol m^{-2} d^{-1}, and defaunated in

Table 5.1: Rates of denitrification, oxygen consumption, total DIC production, and fluxes of NH_4^+, NO_3^- and NO_2^- from natural and defaunated sediment in April, June and September. The unit for all rates are mmol m^{-2} d^{-1} and expressed in single N units. ND = no significant fluxes could be detected.

Month	Fauna	N_2 prod	O_2 consumed	DIC prod	NH_4^+ efflux	NO_3^- influx	NO_2^- efflux
Apr	Fauna	14	59	118	5.6	10	1.0
		21	122	126	14	13	1.4
		18	69	134	11	15	1.6
	Defaunated	4.1	34	90	9.5	ND	1.6
		5.2	35	79	11	6.5	0.7
		6.0	37	132	13	ND	0.7
Jun	Fauna	17	77	197	10	11.4	1.4
		18	148	230	16	34	1.3
		17	126	226	19	13	2.2
	Defaunated	7.2	62	189	13	11	1.1
		7.2	61	221	13	12	1.2
		8.4	72	192	11	25	1.0
Sep	Fauna	9.7	54	142	12	7.2	1.2
		20	147	243	28	9.5	0.8
		11	168	138	20	8.0	0.7
	Defaunated	6.4	62	36	6.4	5.2	1.0
		7.6	66	82	7.5	ND	0.9
		6.9	44	142	6.5	3.4	1.1
	Defaunated-J	6.7	68	70	7.9	6.5	1.0
		4.3	82	139	13	6.7	1.5
		4.3	57	116	7.6	8.1	0.6

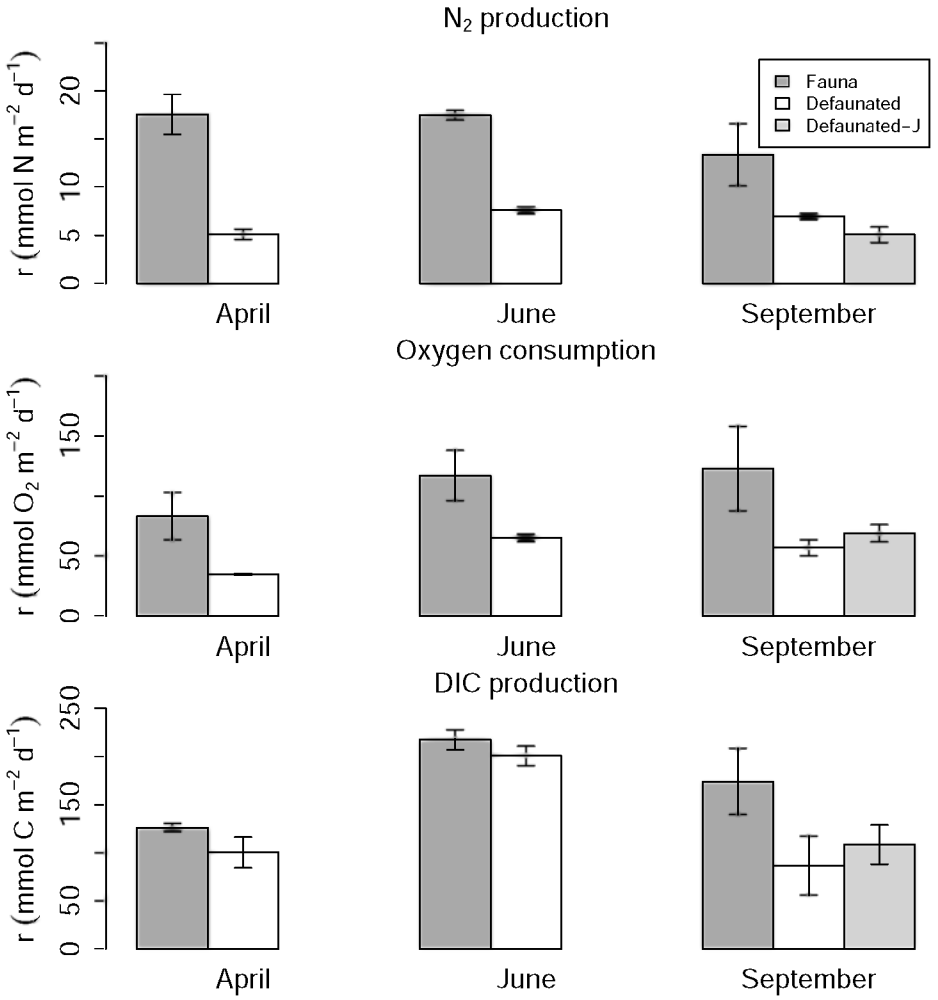

Figure 5.5: Rates of N_2 production, oxygen consumption and DIC production during April, June and September in control plots and plots defaunated in April and July (in July rates from September only).

July, average 10 mmol m^{-2} d^{-1}. Similar results were found for influx of NO_3^- with significant differences between control plots and defaunated plots only in September, between control plots and plots defaunated in April, (p-value=0.03) with average rates of 8, 4 and 7 mmol m^{-2} d^{-1} in control, Defaunated and Defaunated-J plots respectively. In April we could only measure a significant influx of NO_3^- in one of three cores from the defaunated plots. The average rate in control plots during April was 13 mmol m^{-2} d^{-1}. In June the average influx rates were 20 and 16 from control and defaunated plots respectively. Effluxes of NO_2^- were lower than efflux of NH_4^+ and influx of NO_3^-. A significant difference between control and defaunated plots was found in April (p-value=0.04) with average rates of 1.3 and 0.6 mmol m^{-2} d^{-1} respectively. During June and September no clear differences were found. The results however indicate that the efflux of NO_2^- were higher in the control plots in June with average rates of 1.6 mmol m^{-2} d^{-1} compared to 1.1 mmol m^{-2} d^{-1} in the defaunated plots. During September the efflux of NO_2^- was around 1.0 mmol m^{-2} d^{-1} in all treatments.

5.3.3 Species-flux relationships

Table 5.2: Correlation, expressed as r-value, between the biomass (AFDW) of each species and N_2 production, O_2 consumption and DIC production. Bold numbers show significant correlations.

	Cerastoderma	Heteromastus	Macoma	Hediste	Total
N_2	**0.82**	**0.72**	0.46	**0.61**	**0.84**
O_2	**0.65**	0.25	**0.73**	0.34	**0.77**
DIC	0.42	-0.09	0.24	-0.09	0.22

The effect of individual species on sediment-water exchange fluxes and biogeochemical processes can be investigated using a correlative approach. Tables 5.2 and 5.3 present the correlations of individual species biomass and abundance respectively with biogeochemical processes. The biomass and abundance of all species, but *Macoma balthica*, and those of the total community significantly correlated with N_2 production, suggesting

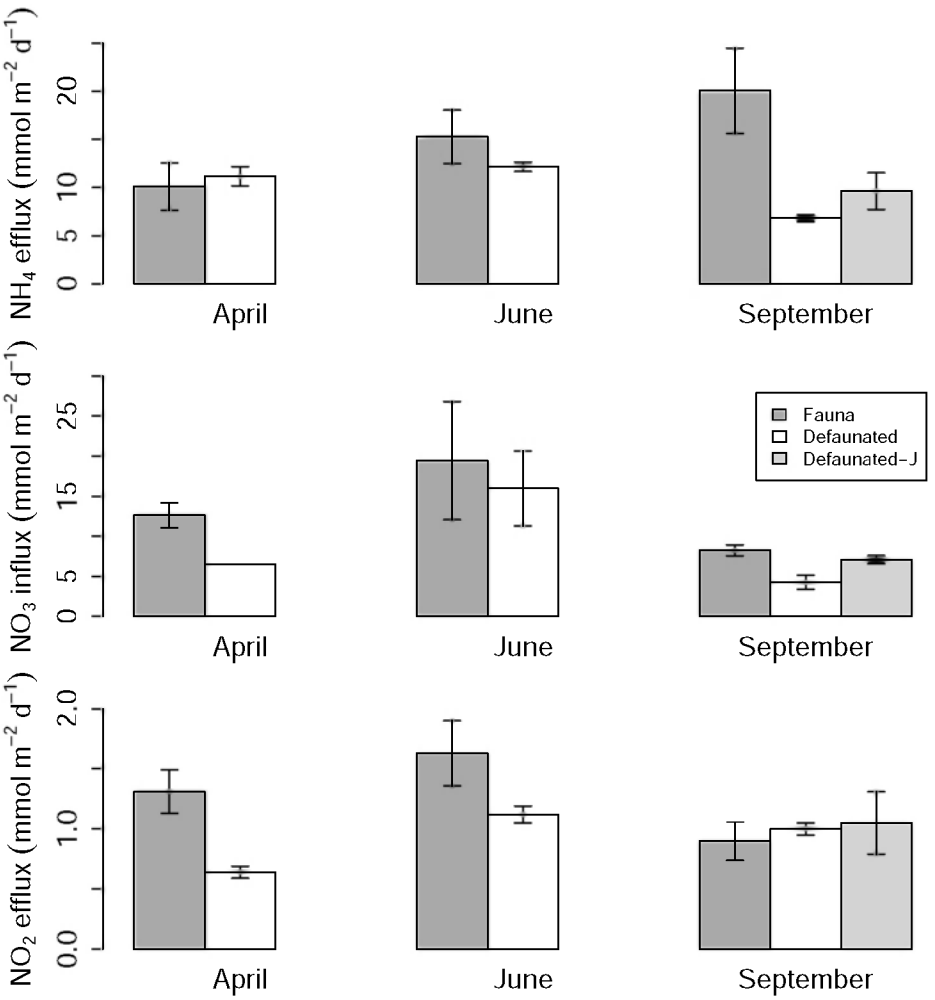

Figure 5.6: Rates of NH_4^+ efflux, NO_3^- influx and NO_2^- efflux during April, June and September in control plots and plots defaunated in April and July (in July rates from September only).

Table 5.3: Correlation, expressed as r-value, between the abundance of each species and N_2 production, O_2 consumption and DIC production. Bold numbers show significant correlations.

	Cerastoderma	Heteromastus	Macoma	Hediste	Total
N_2	**0.68**	**0.66**	-0.22	**0.62**	**0.7**
O_2	0.36	0.13	0.35	-0.41	0.31
DIC	0.06	0.04	-0.05	**-0.51**	0.23

that they stimulate microbial N_2 production. The biomass of the bivalves *Macoma balthica* and *Cerastoderma edule* has a positive effect on oxygen consumption. While fauna biomass may influence oxygen consumption directly via animal respiration, faunal abundance influences biogeochemical processes mainly via bio-irrigation (e.g. the number of burrows).

5.4 Discussion

Benthic macrofauna has been recognized to govern sediment biogeochemical processes and to play a key role in benthic-pelagic exchanges (e.g. Heip, 1995; Meysman et al., 2006b; Karlson et al., 2007)). Through their presence and feeding and moving activities, they create three-dimensional sediment structure and thus high spatial and temporal heterogeneity (Meysman et al., 2006a). Their movement and feeding activities also result in extensive particle mixing (Meysman et al., 2003). Bioturbation, biologically induced particle transport, has multiple consequences for sediment biogeochemistry, in particular for transport of labile organic matter into the sediment (Herman et al., 1999) and reduced components such as iron sulfide from deeper, more reduced zones to shallower, more oxygenated zones. Bioturbational transport of solid phase sulfides is a key process needed for closure of the sedimentary sulfide and redox balance (Berner and Westrich, 1985; Thamdrup et al., 1994). Macrofauna creates burrows and burrow networks in which they live and which they oxygenate in order to respire in an otherwise anoxic environment. This oxygenation occurs by passive or active ventilation of their burrows and causes not only additional oxygen transport into the sediments, but also enhanced transport in and out of sediments of nitrate, ammonium and other nutrients and metabolites,

a process called bio-irrigation (Aller, 2001).

This key role of macrobenthos in sediment biogeochemistry and sediment-water exchange has resulted in many studies and an extensive literature. On the one hand there is a large body of knowledge from the geochemical literature that focuses on quantification of bio-irrigation and bioturbation and the consequences for biogeochemical transformation rates (Aller, 2001; Meysman et al., 2006b). On the other hand, ecologists have studied extensively the effects of single species, or artificial, randomly assembled communities on sediment-water exchange rates of nutrients (Hansen and Kristensen, 1997; Emmerson et al., 2001; Raffaelli et al., 2003; Lohrer et al., 2004; Karlson et al., 2005, 2007). Many of these experiments involved the use of defaunated sediments to which one, or combinations of two or three species, were added. Such type of experiments have the advantage that changes in biogeochemical processes can be attributed in a simple way to a certain species or combinations there of (Ieno et al., 2006), but have limited relevance to natural communities.

Our aim was to study the recovery of biogeochemical processes following defaunation. Rather than adding selected organisms to manipulate macrobenthos communities, we allowed communities to re-assemble naturally. To our knowledge there have been few previous attempts to perform similar studies in situ (Bolam et al., 2002). In our study we successfully defaunated the sediment, there was basically no biomass present in the defaunated plots after the opening in April (Figure 5.3 B). The experiment did not last long enough for the sediment to return to natural conditions since the biomass in the defaunated plots was still substantially lower than in the control plots at the last sampling campaign in September. Despite long-lasting low biomass values in defaunated sediments, species richness was close to control levels after two months of colonization (Figure 5.3 A) and densities of early recolonizers such as *Macoma balthica* and *Hediste diversicolor* were even higher in recovering than in control communities (Figure 5.4). These surface deposit feeders apparently took advantage of the low abundance of other species. In general all animals in the defaunated sediment were smaller in size compared to the control sediment. For more information regarding the recolonization of the fauna and consequences for foodweb functioning see Van Colen et al. (2007) and Rossi et al. (2007).

Our first hypothesis was that defaunation would lower the variance

in biogeochemical process rates because of lower sediment heterogeneity. Macrobenthos creates a three-dimensional, dynamic pattern of biogeochemical environments (Aller, 2001; Burdige, 2006; Meysman et al., 2006a,b) and higher variability. We would therefore expect lower standard errors in the defaunated compared to control plots. The data shown in Figure 5.5 and 5.6 and listed in Table 5.1 do indeed show that the presence of fauna results in higher standard errors of process rates.

Nitrogen gas production rates, whether due to traditional denitrification (respiration with nitrate) or to anaerobic oxidation of ammonium (Anammox), were indeed lower in defaunated plots compared to controls plots (Figure 5.5), consistent with our second hypothesis and with a large body of knowledge (Pelegri and Blackburn, 1995; Aller, 2001). Bio-irrigation activities of fauna lead to enhanced aerobic zones and larger redox interfaces, leading to more nitrification and subsequently denitrification (coupled nitrification-denitrification) or to more Anammox. N_2 gas production correlated strongly with total faunal biomass (0.84) and biomasses of *C. edule* (0.82), *H. filiformis* (0.72) and *H. diversicolor* (0.61) (Table 5.2). Similar correlations were found between N_2 gas production and total faunal density (0.70) and densities of *C. edule* (0.68), *H. filiformis* (0.66) and *N. diversicolor* (0.62) (Table 5.3).

Presence of fauna seemed to have no or a limited effect on fluxes of NH_4^+ and NO_3^- (Figure 5.6), although many studies reported statistical significant effects (Hansen and Kristensen, 1997; Emmerson et al., 2001; Raffaelli et al., 2003; Ieno et al., 2006). In April and June there were higher effluxes of NO_3^- in defaunated versus control plots, supporting that the presence of fauna stimulates nitrification by bio-irrigation. However, higher nitrification would have had a negative influence on the NH_4^+ effluxes and this was not observed. Both NH_4^+ and NO_3^- fluxes are effected in multiple, indirect ways by benthic animals with the result that non-linear relationship are expected (Aller, 2001) and observed (Emmerson et al., 2001; Ieno et al., 2006).

Respiration of sediment communities was quantified using oxygen uptake and DIC release measurements. DIC release provides a direct, simple measure for depth integrated respiration provided that carbonate dissolution/precipitation reactions can be ignored (Anderson et al., 1986). Oxygen consumption is not only due to aerobic respiration of micro-organisms and fauna, but also related to re-oxidation of reduced products such as

ammonium and dissolved and particulate reduced forms of iron and sulfur (Burdige, 2006). If there is no carbonate dissolution/precipitation and if all reduced metabolites are efficiently oxidized using oxygen as electron acceptor, then the O_2 consumption to DIC release ratio should approach 1. However, actual ratios may be lower depending on composition of organic matter, the contribution of denitrification and accumulation of reduced sulfur at depth. The accumulation of reduced iron sulfide in sediments depends primarily on bioturbational mixing of these solid-phase reduced compounds to the oxygenated surface layers (Berner and Westrich, 1985; Thamdrup et al., 1994). Accordingly, one would expect lower O_2:DIC ratios in defaunated sediments with lower bioturbation activities than in control sediments. This expectation was indeed observed in April and July (Figure 5.7).

Our study has clearly documented that the presence of fauna results in enhanced variance of biogeochemical process rates, enhanced rates of N_2 gas formation and enhanced re-oxidation of reduced substances and thus oxygen consumption (Figure 5.4). Although our deliberately created anoxic events are artificial and unlike that occurring naturally, we believe that the recovery from defaunation following an anoxic event represents a close to natural processes. The re-assembling communities entered the treatment plots by natural dispersion mechanism (by movement as adults or via settlement of larvae and juveniles) and survived depending on their performance and competitive abilities. The recovery of N_2 gas formation and sediment oxygen consumption rates to level before the disturbance did not occur within the period of investigation. Moreover, dynamics of recovery of N_2 gas formation, a microbial process, appears to be dominated by the dynamics of macrofauna recovery. This delayed recovery of microbial processes because of slow faunal recovery dynamics has consequences for functioning of coastal ecosystems experiencing seasonal or short-term anoxic events.

Anoxic events have a direct influence on oxygen-dependent biogeochemical processes and sediment-water exchange processes and indirectly via macrofauna (Rabalais et al., 2001; Kemp et al., 2005; Karlson et al., 2007). These direct and indirect responses operate on different timescales. Severe events cause relatively rapid mortality of macrobenthos and subsequent slow recovery of macrofauna upon re-oxidation will cause a period of months, perhaps years, with lower oxygen uptake rates and lower N_2

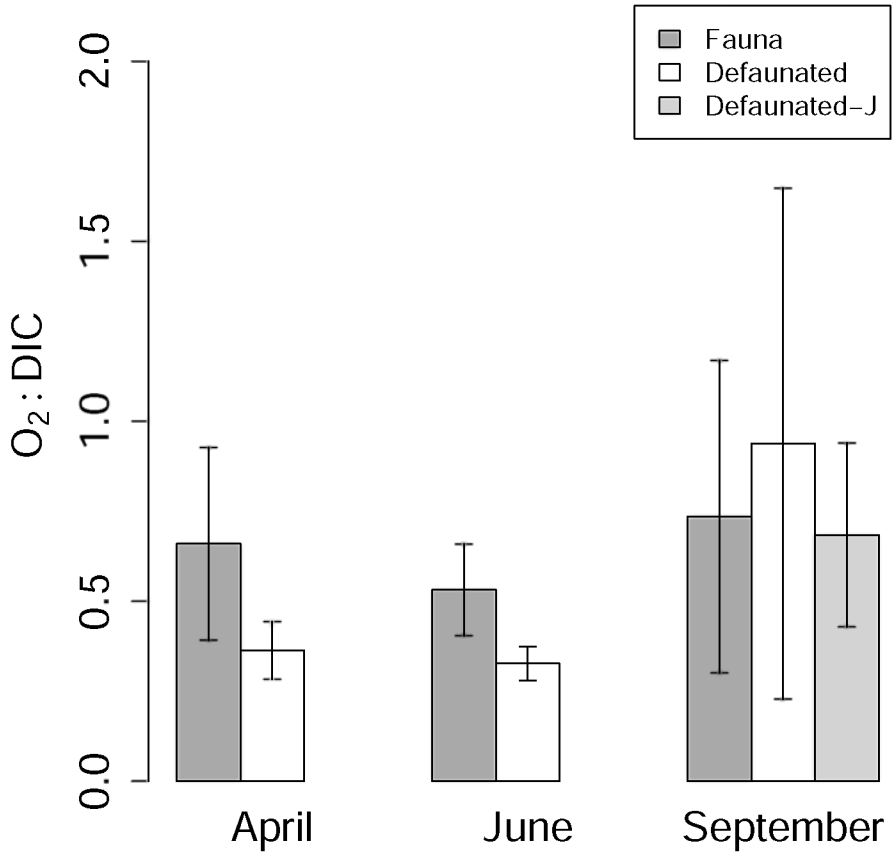

Figure 5.7: O_2:DIC ratios during April, June and September in control plots and plots defaunated in April and July).

gas formation because the facilitating essential macrofauna functions bio-irrigation and bioturbation have not been fully restored. Reduced fixed N removal for more than 6 months following a severe anoxic events as observed here, represents a positive feedback towards eutrophication. Eutrophication induced enhanced primary production results in enhanced respiration in bottom water and sediments with the consequence that anoxic conditions establish if physical condition do not support enough supply of oxygen. These anoxic conditions then result in faunal mortality and reduced fixed nitrogen removal and enhanced phosphate release (Herbert et al., 2007; Morse and Eldrigde, 2007; Kemp et al., 2005) with the consequence that more nutrients become available for primary producers. To conclude this study, fauna inhabiting the sediment is very important for biogeochemical processes taking place in the sediment in general and for processes in the nitrogen cycle in particular. Presence of fauna has a strong positive effect on production of N_2. The direct and indirect (via fauna) effects of hypoxia on sediment biogeochemistry and sediment-water exchange should be incorporated in coastal ecosystem studies if we are to further our understanding and predictive capabilities of the response of coastal ecosystems to climate change, eutrophication and oligotrophication.

6 Nitrogen processing in a tidal freshwater marsh: A whole-ecosystem ^{15}N labeling study

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6.1 Introduction

Estuaries are well known to modify and attenuate nitrogen (N) transfer from rivers to the coastal sea (Billen et al., 1991), and tidal marshes have been reported to play a major role in nitrogen retention (Bowden, 1987; Howarth et al., 1996; Cai et al., 2000). Marshes have high surface areas of sediment and biofilms in contact with the water, promoting processes that may change nitrogen speciation and may remove dissolved inorganic and organic nitrogen and enhance deposition of particulate nitrogen (Dame et al., 1996; Merrill and Cornwell, 2000). There is considerable interest in exploiting the nitrogen 'filtration' capacity of wetland ecosystems in order to improve estuarine and coastal marine water quality. However, despite the widely held belief that wetland systems are important nutrient sinks, the literature regarding the influence of tidal freshwater wetlands on water quality is remarkably small (Merrill and Cornwell, 2000). There is still much uncertainty about the underlying mechanisms and controls of nitrogen retention in marsh ecosystems, thus hindering their potential

use and effective management for improvement of water quality.

The importance of freshwater marshes to the estuarine nitrogen budget is most often estimated with a mass-balance approach. This technique, however, is subject to considerable error and yields little information regarding the role and spatial and temporal distribution of the underlying processes. Use of stable isotope tracers to track N through aquatic ecosystems *in situ* is a relatively recent technique and has provided increased understanding of N processing in groundwater plumes (Tobias et al., 2001), lakes (Kling, 1994), streams (Peterson et al., 1997; Tank et al., 2000; Hamilton et al., 2001), and small estuaries (Holmes et al., 2000; Hughes et al., 2000; Tobias et al., 2003b). Here we present the results of a whole-ecosystem $^{15}NH_4^+$ tracer addition experiment to quantify the fate and transport of watershed-derived ammonium through a tidal freshwater marsh system. Combined with a mass-balance study, this approach allows a simultaneous examination of transport and processing of nitrogen in an intact ecosystem, something that is impossible to achieve in traditional bottle, enclosure, or mesocosm experiments (Schindler, 1998; Holmes et al., 2000). Furthermore, this technique allows us to estimate whole-ecosystem nitrogen uptake and transformation without the confounding effects of altering floodwater N concentrations significantly.

The whole-ecosystem ^{15}N labeling experiment was conducted in a tidal freshwater marsh fringing the nutrient-rich Scheldt River. Although many tidal marshes of the Scheldt basin have been reduced to very small size today (mainly by embankment for polder reclamation) (Desender and Maelfait, 1999), they are still a prominent feature of the Scheldt, and the surfaces of the $\sim$4.5 km^2 of tidal freshwater marshes could represent important potential N sinks. Yet the importance of these marshes as nutrient sinks within the estuary remains largely unassessed. In this article we present results of the ^{15}N tracing through the water-phase components of the marsh system. ^{15}N tracer incorporation into sediments, macrophytes, and higher trophic levels (pelagic and benthic) will be described in detail in a separate article. Our results show that the marsh is an intense site for nitrification and that nutrient-rich tidal freshwater marshes function not only as nutrient sinks but also as nutrient transformers.

6.2 Materials and methods

6.2.1 Study area

The study was conducted in a tidal freshwater marsh fringing the Scheldt and Durme Rivers at Tielrode (51°06"N, 4°10"E), Belgium, where the small tributary Durme flows into the Scheldt approximately 100 km from the mouth of the Scheldt estuary. Because of the funnelshaped morphology of the Scheldt estuary, the tidal range (5.3 m) and turbidity are here at their maxima. A detailed description of the hydrodynamic features and basin morphology of the Scheldt estuary is given by Soetaert and Herman (1995). The Scheldt estuary is a heterotrophic, low oxygen, nutrient-rich system with riverine dissolved inorganic nitrogen concentrations of >400 μmol L^{-1} (Struyf et al., 2004; Soetaert et al., 2006). Except for the period ranging from July to August, dissolved ammonium concentrations at Tielrode are generally high (>60 μmol L^{-1}), and concentrations decrease toward the more seaward part of the estuary. Consequently, nitrification has been identified as an important process in the water column of this estuary, accounting not only for the oxidation of ammonium but also for a significant fraction of the carbon fixation and oxygen demand (Soetaert and Herman, 1995).

The study area is located in the northern end of the ~100,000 m^2 Tielrode Marsh (Figure 6.1a). This triangular 3,477 m^2 section of the marsh is isolated on two sides by dikes, and water is fed into the area by only one high-order tidal creek (Figure 6.1b). Groundwater input is negligible, as the marsh is higher than the neighboring embanked polder. Prior to arriving at the study area, the tidal water has travelled a considerable distance (~1 km) from the main river through tidal marsh creeks. During the entire experimental period, the southern boundary of the study area was screened off by 1-m-high wooden screens embedded 10-20 cm into the sediment (Figure 6.1b). Only the 4.5-m-wide span of the tidal creek and creek banks were not screened off. Instead, a flume channel was constructed by placing 3-m-long boards along the creek. Tidal water could thus only enter and exit the study area via the creek, and surface water covering the study area during high tide was partitioned from that overflowing the remaining marsh. A 4.5-m-long sampling and tracer release platform was placed across the creek at the entrance to the study area.

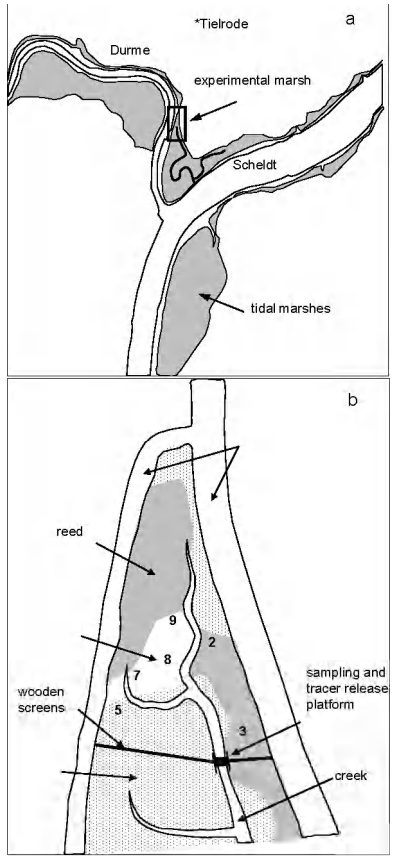

Figure 6.1: (a) Tielrode tidal freshwater marsh fringing the Scheldt and Durme Rivers, with location of experimental marsh marked and (b) experimental marsh with vegetation distribution. Numbers represent sampling stations.

The vegetation in the study marsh is typical for freshwater marshes, with large patches of common reed (monospecific stands of *Phragmites australis*) in the lower elevations, and willows (2-6-m-high specimens of *Salix* sp.) and ruderal vegetation (dominated by *Impatiens grandulifera, Epilobium hirsutum* and *Urtica dioica*) at the higher elevations (Figure 6.1b). The vegetation cover is very dense, with *P. australis* and *I. grandulifera* growing to exceptional heights of up to 4 m, as is commonly observed in Scheldt marshes. Three sampling stations within each of the vegetation types and on unvegetated creek banks were made accessible by walking boards. Marsh sampling stations within each vegetation type were chosen to represent the variation in topography as well as distance from the sampling bridge. Benthic microalgal mats dominated by the filamentous yellow-green alga *Vaucheria* sp. were abundant throughout the marsh, particularly in the creek bank and willow sites. A digital terrain model of the study area was constructed using a geographic information system and a detailed mapping of the topography.

The entire study area is flooded only at the highest tides (26 % of the tides), and the experiment was initiated just before a spring tide on 24 May 2002, when a maximum of consecutive high tides followed (12 tides), and sampling continued until the following spring tide cycle 31 tides after labeling (10 June 2002), when the entire study area was once again flooded. Measurements were carried out prior to labeling (T_{-2}) to establish natural abundance levels of ^{15}N; at the tide when tracer was released (T_0), and at five subsequent tides (T_1, T_2, T_5, T_9, and T_{31}). The subscript denotes the tide number relative to tracer release.

6.2.2 Tracer release

On 25 May 2002 (T_0), 1.98 moles of ^{15}N-ammonium was added to the floodwater entering the study area. The tracer solution consisted of 1,247 g of 10.7 % ^{15}N labeled ammonium sulfate $[(NH_4)_2SO_4]$ and 50 kg of the conservative tracer NaBr dissolved in 250 L of deionized H_2O. The added tracer increased the ^{15}N content of the ammonium pool to 1.3 % and increased the total NH_4^+ concentration by approximately 14 % (but see following). An even distribution of ^{15}N tracer throughout the marsh was accomplished by adding the tracer solution to the incoming tidal creek water proportionally to the water volume entering the study area. For

this purpose, a simple tracer addition device was constructed. A 4.5-m-long, 24-mm-diameter polyvinyl chloride (PVC) pipe was placed on the railing of the sampling platform 2.67 m above the creek bed, fitted with 30 outlets and 150-cm-long 3.2-mm (inner diameter [ID]) Tygon tubing attached to each outlet. Each of the 30 outlet tubes was fitted with a clamp and placed into open PVC pipes (15-mm ID) attached vertically to the bridge and ending at various heights above the creek bed (release points, Figure 6.2). Thus, three, four, five, five, six, and seven open pipes

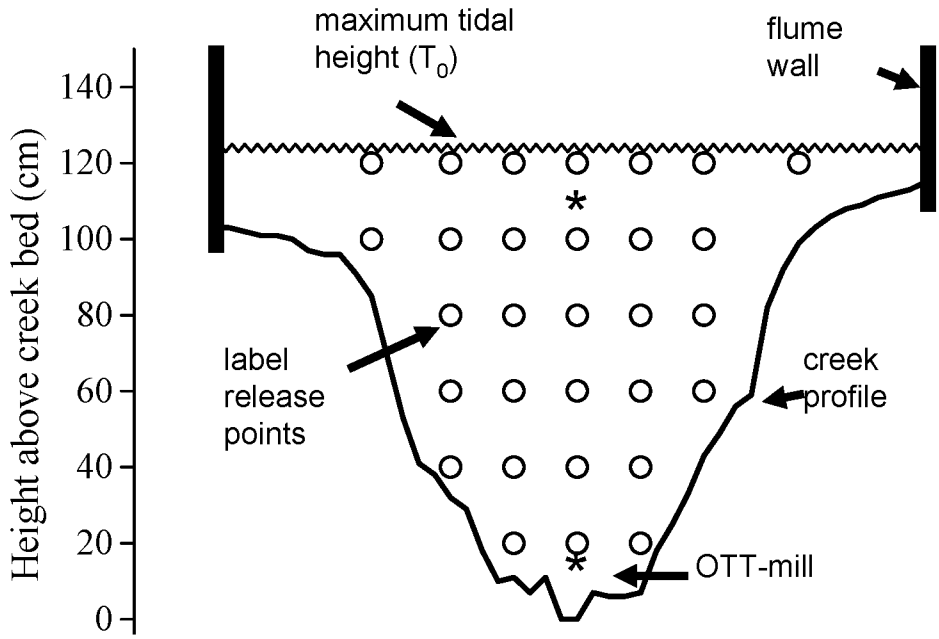

Figure 6.2: Cross section of creek below sampling platform indicating labeling release points (o) and OTT-mill flow meters (*). Span between flume walls was 4.5 m. Label was released on the landward side of the 1.5-m-wide labeling platform, while flow measurements were conducted on the river (downstream) side.

ended 20, 40, 60, 80, 100, and 120 cm above the creek bed, respectively. During floodwater labeling (T_0), tracer flowed passively from an elevated small reservoir (25 L) into the main pipe, where pressure, and thus the

tracer flow rate through open valves, were kept constant (2.5 mL s^{-1}) by maintaining a constant solution level in the small reservoir. The tracer level in the small reservoir was kept constant by continuously pumping tracer solution from a larger tank into the small reservoir, where a closed overflow returned excess tracer solution to the large tank. Tests prior to deployment confirmed that the flow out of each individual outlet was the same regardless of how many outlets were open. Enrichment solution then dripped via the Tygon tubes into the vertical pipes. The number of outlets open was continuously regulated to maintain a constant degree of labeling. Thus, initially, only outlets to the lowest situated release points were open, but as the water level and flow velocity in the creek rose, an increasing number of outlets releasing tracer higher above the creek bed were manually opened. Labeling was initiated when the first floodwater arrived at the sampling and tracer release platform.

6.2.3 Creek-water sampling and analysis

Creek water was sampled from the sampling bridge using an 80-cm-long (6-cm ID) water sampler fitted with a bottom valve. Care was taken to get an integrated water sample over the entire water column. At each time point, three samples were pooled and a 1-L subsample was immediately brought to the field laboratory. 12 creek-water samples were collected during the main tide, defined here as the duration from floodwater arrival below sampling platform until there was no change in water level for 30 min (flood + ebb), and three were collected from the seepage water during low tide. Additional 1-liter floodwater samples were collected from the 12 marsh sampling stations at high water during T_0 (maximum tide) and from the main river (the Scheldt).

Water samples (300-500 mL) were immediately filtered under vacuum through precombusted, preweighed GF/F Whatman filters. The filters were stored frozen for particulate ^{15}N nitrogen analysis (see following). Filtered water samples (30 mL) for isotopic composition analysis of ammonium and nitrate were immediately transferred to 100-mL Schott bottles, capped tightly, and stored frozen. Replicate samples for determination of $^{15}N_2O$ and $^{15}N_2$ were preserved with 500 or 50 μL NaOH (2 mol L^{-1}) in tightly capped (gas-tight crimpseal septum) 60-mL and 20-mL headspace vials, respectively, stored at room temperature with no headspace, and

were analyzed within 1 week. Preserving the samples with NaOH also trapped the carbon dioxide from the headspace later introduced for analysis (see following). Filtered subsamples for determination of NH_4^+, NO_3^-, and NO_2^- concentration were stored cold (4°C) and analyzed within 3 d, while samples for Br^- determination were stored frozen.

Nitrogen isotopic compositions of NH_4^+ and NO_3^- + NO_2^- were determined in two steps using a modification of the ammonia diffusion procedure (Sigman et al., 1997; Holmes et al., 1998), followed by mass spectrometry. Briefly, 30-mL filtered water was transferred to a 100-mL Scott incubation bottle and 0.1 g NaCl was added. $^{15}NH_4^+$ was determined by adding MgO, converting NH_4^+ to NH_3, which was trapped on an acidified (H_2SO_4) 10-mm GF/D filter sandwiched between two Teflon filters floating on the sample surface. After 8 d of shaking at room temperature, filters were removed, dried for 2 d in an exicator, and analyzed using a Fisons elemental analyzer (EA-1500) coupled online via a Finnigan CONFLO II interface, with a Finnigan Delta S isotope ratio mass spectrometer (EA-IRMS). Tests confirmed complete ammonium removal from samples after 8 d of incubation. Devarda's Alloy was subsequently added to the sample to convert NO_3^- + NO_2^- to NH_3, which was collected onto a new acidified filter as described above. Bulk suspended particulate nitrogen (SPN) on GF/F filters was determined after drying (6 h, 60°C) using a Carlo-Erba CN analyzer following Nieuwenhuize et al. (1994) and the isotopic composition determined by EA-IRMS (described previously).

Dissolved nitrous oxide (N_2O) concentrations and isotopic composition in headspace gas (10 mL He headspace in 60-mL vials, then shaken for 30 min) were determined by gas chromatography coupled to isotope ratio mass spectrometry (GC-IRMS, Hewlett Packard G1530 GC, with Thermo Finnigan Delta-plus IRMS). The GC was fitted with a Porabound Q column (dimensions: length, 50 m; 0.32-mm ID; film, 5 μm, Chrompack) operated at 30°C with a 2 mL min^{-1} column flow of helium. The oxidation and reduction reactors of the GC-IRMS interface were bypassed. A sample of the headspace (500 μL) was injected in split mode (split ratio: 1:10) at 110°C. Concentrations were calibrated against a certified gas mixture (5 ppmv N_2O) and recalculated to water concentrations using the temperature- and salinity-dependent partitioning coefficient (Weiss and Price, 1980). Stable isotope ratios of N_2O were measured on masses 44 and 45 and calibrated against carbon dioxide reference gas. Reported

^{15}N:^{14}N-ratios are based on the average of multiple gas injections from the same sample. This method of N_2O analysis is very sensitive for N_2O concentrations, and good results were obtained for the complete tidal cycle. Although our method of stable isotope analysis is not optimal for natural abundance ^{15}N studies, precision was sufficient (standard deviation [SD] above 40 nmol L^{-1} N_2O, approximately 0.020 atom % ^{15}N) to detect significant labeling of the N_2O pool during the main tide (see Results). Isotopic composition of dissolved dinitrogen ($^{29}N_2$, $^{30}N_2$) in headspace gas (2 mL He headspace in 20-mL vials) was determined by EA-IRMS equipped with a Haysep Q column. Samples were injected directly in front of the EA chromatography column. Results are based on the average of three gas injections from the same sample. For calculations of enrichment of the total N_2 pool, an equilibrium concentration of 550 μmol L^{-1} (20°C) was assumed.

Nutrient concentrations were measured colorimetrically on an automated Segmented Flow Analyzer. Bromide concentrations were determined by high-pressure ion chromatography with ultraviolet detection.

6.2.4 Ex situ rate measurements

Water-column nitrification rates (WNR) (T_{-2}, six times over the tidal cycle) were determined in bottle incubations by measuring the transfer of ^{15}N in ammonium to nitrate according to a modification of Horrigan et al. (1990). Briefly, creek-water samples were spiked with $^{15}NH_4Cl$ (98 atom %) to approximately 2 % of the ambient NH_4^+ concentration and incubated in the dark at in 14 situ temperature in 100-mL Scott bottles for 3 h. Incubations were ended by filtration through precombusted Whatman GF/F filters (21 mm), the ^{15}N content of NH_4^+ and NO_3^- + NO_2^- in the filtrate measured by the diffusion method described above and nitrification rate calculated from the (linear) increase of ^{15}N in NO_3^- + NO_2^-. Absolute uptake rate of ammonium (μmol N L^{-1} h^{-1}) into the particulate nitrogen pool (PN) was determined according to Middelburg and Nieuwenhuize (2000b).

6.2.5 Discharge characteristics and additional measurements

Two OTT-mill flow meters continuously (at 1-min intervals) measured current velocity at the bottom and surface of the creek center. The bottom flow meter was fixed ~20 cm above the creek bed, the top one maintained at ~30 cm below the water surface, according to the tidal level (monitored every minute). In addition, lateral surface flow rates were measured every 5-10 min at fixed points (0.5-m interval) between the flume walls with an electromagnetic flow meter (EMF, Valeport Model 801). During the seepage phase, here defined as the period after the main tide during which no decrease in water level was observed for 30 min, EMF measurements were conducted every hour. The creek profile was subdivided into lateral subsegments and discharge calculated per minute in each segment by multiplying the cross-sectional area of the channel by water velocity. These data were used to calculate advective water fluxes in and out of the study area.

Dissolved oxygen, specific conductivity, temperature, pH, and turbidity were recorded continuously (at 2-min intervals) during all tides using a Hydrolab Datasonde 3.

6.2.6 Water traps in the marsh

To evaluate if the $^{15}NH_4^+$ tracer successfully mixed with the floodwater and was distributed evenly over the entire study area, water traps were placed at each marsh sampling station (Figure 6.1 b) during T_0. Each water trap consisted of eight 30-cm-long PVC pipes (15-mm ID) attached at 10-cm height intervals to a bamboo pole. As the water rose over the marsh surface, pipes captured water at 10-cm depth intervals, with the lowest trap collecting initial floodwater and highest trap capturing water at high tide. Rhodamine addition tests revealed negligible mixing with overlying water once traps were filled. Traps were sampled with a 30-mL syringe by withdrawing 10 mL via a polyethylene tube gently inserted to the bottom of the trap. Water traps were sampled immediately following T_0 and analyzed for Br^- (conservative tracer) as described previously.

6.2.7 Calculations

Water budgets for each tide were calculated from the measured water flows (Table 6.1). Furthermore, the total volume of water covering the study area in a given tide and duration of water cover at each sampling station were calculated from the digital terrain model using the maximum water levels measured at the sampling platform. Stock size for all components dissolved or suspended in the water column over the tidal cycle were calculated from concentration and discharge measurements for each sampling point. Nitrogen isotopic ratios are expressed in the delta notation (δ^{15}N, ‰) relative to atmospheric nitrogen and mostly given as $\Delta\delta^{15}$N (isotopic enrichment), corrected for natural abundance levels of ^{15}N by subtracting the δ^{15}N value of similar samples collected at the same time relative to maximum tide at $T_{(-2)}$. The tracer content (excess ^{15}N) in each nitrogen pool was determined from the isotopic enrichment and stock size, and a total ^{15}N inventory and ^{15}N-flow balance was constructed for each tide. Similar inventories were made for the conservative tracer (Br^-) and unlabeled nitrogen pools. Net ecosystem nitrification rate was calculated from the net transfer of ^{15}N from the added ammonium tracer pool to the nitrate pool over the first tide (T_0) (^{15}N mass balance) and from the linear increase in isotopic enrichment of NO_3^- + NO_2^- during T_0 ebb.

Airwater exchange fluxes of $^{15}N_2O$ and $^{15}N_2$ were calculated from the product of gas transfer or piston velocity (k) and the partial pressure of the gas. Gas transfer velocities in estuaries depend on various factors, including current velocity, wind velocity, and fetch, and we have therefore calculated a lower and upper limit based on k values of 5 (in the absence of wind) and 50 cm h^{-1}, the range of observations in the Scheldt estuary (Borges et al., 2004). Water surface area available for exchange was based on water height and digital terrain model.

6.3 Results

6.3.1 Hydrodynamics and creek characterization

The average tidal cycle in the creek showed a normal diurnal sinusoidal tide. The flood lasted ~80 min (Table 6.1), and after high tide, the water dropped according to a normal tidal curve until the bottom of the creek

Table 6.1: Duration of main tide (flood and ebb), maximum water height above creek bed (2.47 m above mean sea level) below measuring platform, water volume estimated from the digital terrain model and calculated water budget computed by mass balance model during the 7 tides. Values in parenthesis are balances in percentage of flood.

	Duration				Water budget					
Tide	Flood (min)	Ebb (min)	Water height (cm)	Water volume[1] m^3	Flood m^3	Ebb m^3	Seepage m^3	Balance m^3	Import in % of total volume[2] (%)	Export in % of total volume[3] (%)
T_{-2}	78	107	105	1,336	1,234	1,206	66	-38 (-3.1)	92	95
T_0	80	131	125	2,099	1,823	2,103	65	-345 (-18.9)	87	103
T_1	77	119	117	1,800	1,700	1,642	61	-3 (-0.2)	94	95
T_2	82	144	136	2,511	2,364	2,580	64	-277 (-11.7)	94	105
T_5	78	139	129	2,249	1,912	2,218	65	-372 (-19.5)	85	102
T_9	79	120	109	1,500	1,376	1,305	65	7 (0.5)	92	91
T_{31}	76	106	100	1,173	900	955	68	-123 (-13.7)	77	87

[1]Calculated from the digital terrain model and maximum water height.
[2]Calculated floodwater volume from mass balance model in percentage of water volume estimated from [1].
[3]Calculated ebb water volume + seepage from mass balance model in percentage of water volume estimated from [1].

was nearly reached (Figure 6.3 a). The main ebb lasted for 106 to 144 min depending of the tidal height. Then water seeping out of the sediment and between vegetation kept flowing out (average speed ~6 cm s^{-1}) until the next flood (after 8.5-9.5 h). Current speeds in the creek strongly depended on the level of the high tide, but the same spatial pattern was observed during all seven tides. Flood currents were relatively constant (~20 cm s^{-1}), while ebb currents showed more variation (20-50 cm s^{-1}) with time and depth. Maximum ebb current were up to 66 % faster than maximum flood current and current speeds were always higher near the surface than at the bottom (up to 60 % higher). The amount of water covering the study area estimated by the digital terrain model varied by a factor of 2.1 among the seven tides studied, while the calculated amount of water entering and leaving the marsh varied by factors of 2.6 and 2.5, respectively (Table 6.1). Except for T_9, the exported water volume exceeded the incoming volume. The calculated export generally deviated less from the volume estimated by the digital terrain model than the estimated import volume, indicating the import to be underestimated. However, Br^- mass balance during T_0 indicates the ebb water budget to be slightly overestimated (see following). Between tides, 65 $\pm$2 m^3 water (seepage) was exported, corresponding to 2.5-7.1 % of the total water volume depending on maximum tidal height.

Water temperature and pH varied from 17°C to 19°C and from 7.4 to 7.7 across all tides, respectively. Except at T_0 maximum tide, when the conservative tracer (Br^-) increased the conductivity by 30 % (see following), conductivity was relatively constant (~1 mS cm^{-1}) over the tidal cycle and between tides. Initial turbidity in the floodwater was high (80-200 nephelometric turbidity units [NTU]), but turbidity decreased rapidly to < 50 NTU in most tides. In the highest tides, however, turbidity remained high during flood and the first part of the ebb, showing erratic temporal patterns. The initial floodwater was relatively oxic (40-90 %), but dissolved oxygen concentrations rapidly decreased to ~10 % saturation, and the water was hypoxic (<50 μmol L^{-1}) during most of the main tide (Figure 6.3b). During ebb [O_2] increased linearly ($r^2 = 0.92$, $p < 0.01$) as a function of decreasing water height to ~50 % saturation at the end of all tides. Generally, T_{31} water was slightly higher in dissolved oxygen compared to the other tides.

Ammonium concentrations in the floodwater varied greatly and re-

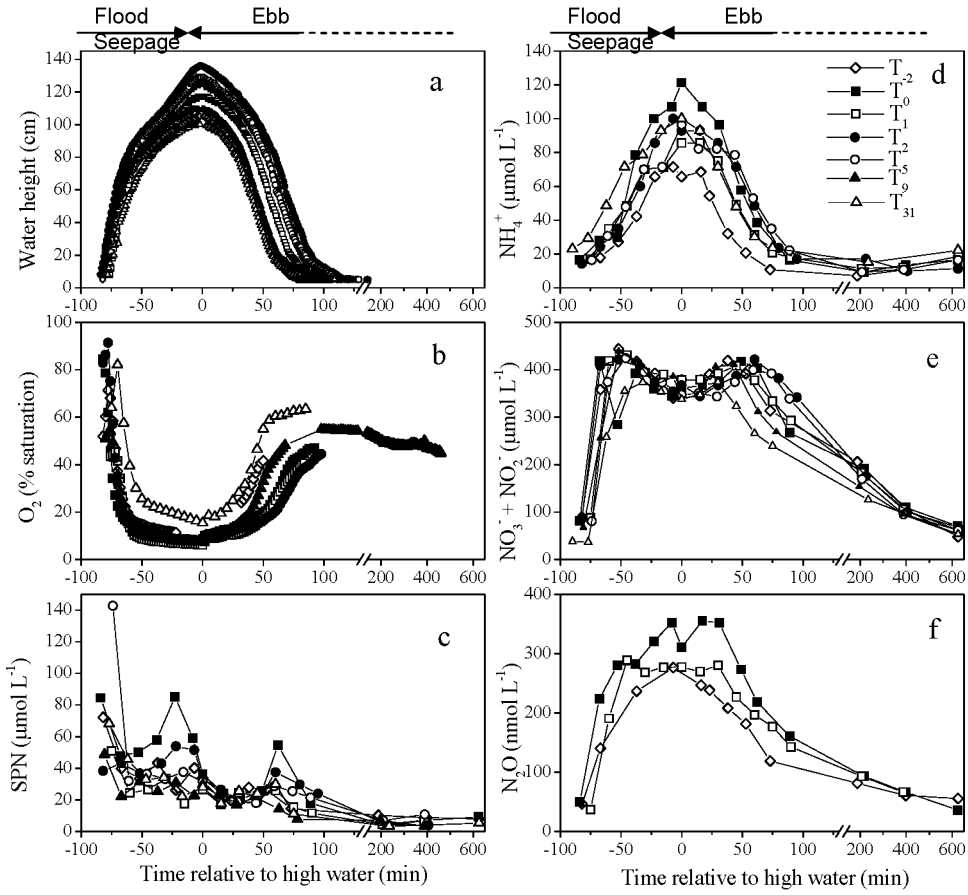

Figure 6.3: Water level, O_2 , and nitrogen pools in the creek below the sampling platform as a function of time relative to maximum tidal height in all seven tides. (a) Water height , (b) O_2 saturation, (c) suspended particulate nitrogen concentration (SPN), (d) dissolved ammonium concentration (NH_4^+), (e) dissolved nitrate and nitrite concentration (NO_3^- + NO_2^-), and (f) dissolved nitrous oxide concentration (N_2O). The three phases of the tidal cycle (flood, ebb and seepage) are indicated above figures, with arrow indicating the direction of the water flow.

vealed a bell-shaped distribution pattern over the tidal cycle (Figure 6.3d). Initially below 20 μmol L^{-1}, concentrations gradually increased to 60-120 μmol L^{-1} at maximum tide, followed by a gradual decrease to 10-20 μmol L^{-1} at the end of the main ebb tide. During seepage, a slight increase in $[NH_4^+]$ was observed. Similar patterns were observed in all tides, but the maximum concentration varied by a factor of two between tides. Only at maximum tidal height did $[NH_4^+]$ match that of the main river (105-120 μmol L^{-1}. $[NH_4^+]$ was inversely correlated with O_2 saturation ($O_{2\%}$) ($[NH_4^+] = 463.5 \cdot O_{2\%}^{0.83}$; $r^2 = 0.79$, $p < 0.001$). The $NO_3^- + NO_2^-$ concentrations showed a bimodal distribution curve over the tidal cycle (Figure 6.3e). From relatively low (40-90 μmol L^{-1}) concentrations in the initial floodwater, $[NO_3^-] + [NO_2^-]$ rapidly increased to 400-450 μmol L^{-1} halfway into the flood period, but subsequently decreased slightly to ~350-400 μmol L^{-1} at maximum tide. Following a second $[NO_3^-] + [NO_2^-]$ peak midway through the ebb tide, concentrations gradually decreased to ~250-350 μmol L^{-1} at the end of the main tide and continued to decrease in the seepage. During most of the main tide, $[NO_3^-] + [NO_2^-]$ was significantly higher than in the main river (300-320 μmol L^{-1}). Nitrite (NO_2^-) accounted for 2-17 % of the $NO_3^- + NO_2^-$, with maximum contributions at maximum tide. Overall, the dissolved inorganic N pool ($DIN = NH_4^+ + NO_3^- + NO_2^-$) was relatively constant (450-480 μmol L^{-1}) for the main duration of all tides. From the end of ebb, however, [DIN] gradually decreased to relatively low (60-100 μmol L^{-1}) concentrations in the seepage water. Similar low concentrations were observed in all initial floodwater samples. Onward $NO_3^- + NO_2^-$ will collectively be referred to as nitrate (NO_3^-).

Nitrous oxide (N_2O) concentrations were only determined in the first three tides, in which the temporal patterns roughly mimicked that of nitrate, but concentrations were three orders of magnitude lower (Figure 6.3f). Concentrations of SPN were generally highest (40-148 μmol L^{-1}) in the initial floodwater, but decreased with time (Figure 6.3c). In the three highest tides (T_0, T_2, and T_5), additional peaks in SPN coincided with maxima in flow rates and turbidity during flood as well as ebb. Low [SPN] (3-11 μmol L^{-1}) was always observed in the seepage, likely reflecting particle settling, consistent with low NTU values.

6.3.2 Conservative tracer distribution

Addition of a conservative tracer (NaBr) to the tracer solution enabled us to monitor the distribution of the label within the study site and indeed verified that a rather even distribution was obtained (Table 6.2). Bromide concentrations were, however, slightly higher (0.35 ± 0.05 mmol L^{-1}) in lower-placed water traps than in higher ones (0.27 ± 0.06 mmol L^{-1}), indicating that tracer was initially released at a slightly higher relative rate than during the bulk of the tide. The peak in bromide concentration (0.6 mmol L^{-1}) in the first sample of the outgoing tide shows that tracer addition was carried out for too long and/or at too high a rate at the turn of the tide (Figure 6.4). It appears that the tracer addition valves were not closed sufficiently fast at the rather abrupt halt in tidal inflow. However, this last labeled body of water quickly left the creek again and therefore did not mix with the main body of water covering the marsh. The very uniform concentration (0.26 ± 0.02 mmol L^{-1}) in the remaining water leaving at T_0 together with the water trap (Table 6.2) and T_0-maximum tide water samples (0.24 ± 0.11 mmol L^{-1}) substantiate that the tracer was relatively evenly distributed over the entire study area. Recovery of the added Br^- in the mass balance (T_0) was 115 %, indicating that the ebb water volume during T_0 was overestimated. In addition, bromide was slightly elevated in the last part of the outflow of T_1 - T_5, with minor influence on the Br^- mass balance due to low water volumes.

6.3.3 Stable isotopes

The ammonium pool in the T_0 ebb water (directly after the ~80-min period of tracer addition to the floodwater) was enriched 1,975-4,735 ‰ ($\Delta\delta^{15}NH_4^+$) (Figure 6.5a). Enrichment of samples collected from the 12 marsh sampling stations at T_0-maximum tide was well within this range (2,878 ± 844 ‰). The initial peak in ebb water $\Delta\delta^{15}NH_4^+$ is attributed to overlabeling around the turn of the tide (see Conservative tracer distribution). In the seepage water, the enrichment decreased over time from 545 ‰ to 119 ‰, indicating dilution with unlabeled NH_4^+ from mineralization or exchange with particulate pools. In following tides, only the initial flood sample of T_1 showed significant enrichment (69 ‰) of the ammonium pool (Figure 6.5 a, insert).

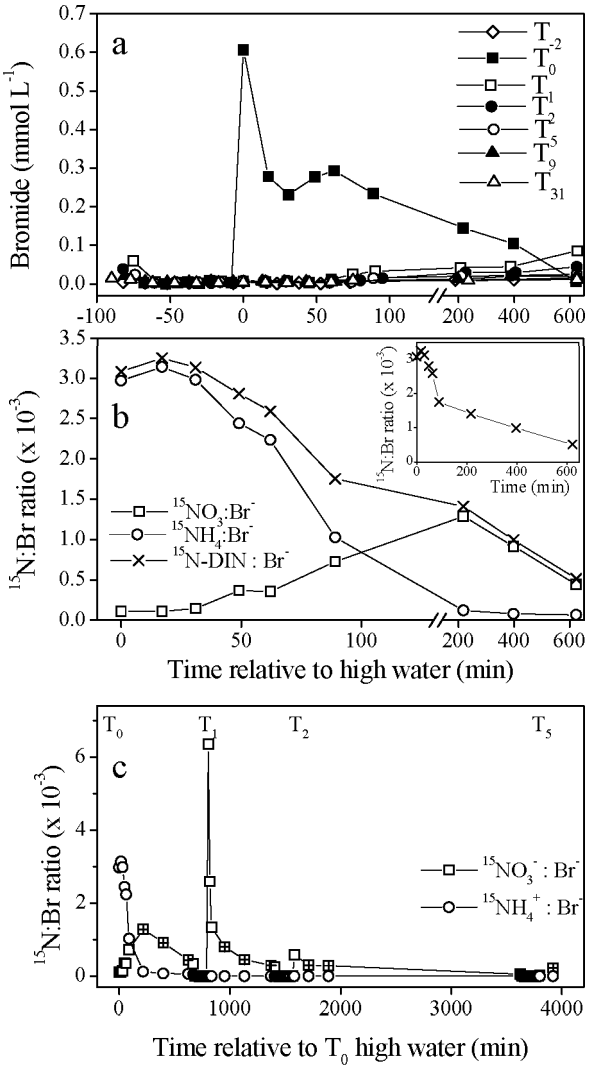

Figure 6.4: (a) Bromide concentration in the flood water during the seven tides in relation to time relative to maximum tidal height, and (b) changes in ratio between excess ^{15}N in NH_4^+, NO_3^- and DIN and conservative tracer Br^- concentrations during T_0-ebb and -seepage (insert shows $DI^{15}N : Br^-$ on an unbroken timescale), and (c) changes in ratio between excess ^{15}N in NH_4^+, NO_3^- and DIN and conservative tracer Br^- concentrations from end of label addition (T_0-maximum) to T_5. Symbols with (+) indicate seepage phase. Note the different scales on the x-axis.

Table 6.2: Vegetation type, topographic level (relative to mean sea level), duration of flooding at T_0, and water trap bromide concentrations (T_0) at the 12 sampling stations. Water traps are divided into traps placed above and below 3.4 m above mean sea level. Locations of sampling stations are shown in Figure 6.1 b.

				Bromide	
Vegetation type	Station no.	Surface height (m)	Flooding duration (T_0) (min)	<3.4 m (μmol L^{-1})	>3.4 m (μmol L^{-1}
Reed	1	3.23	117	339±54	189±23
	2	2.97	139	352±80	239±53
	3	3.06	133	323±11	250±12
Willow	4	3.15	124	381±63	331±32
	5	3.34	105	—	228 ±26
	6	3.40	92	—	292
Ruderal	7	3.31	109	—	230±22
	8	3.25	116	358	262±31
	9	3.31	109	262	235±6
Creek	10	3.11	129	366	283±23
	11	2.87	148	360±16	371±5
	12	2.67	163	385±50	264±51

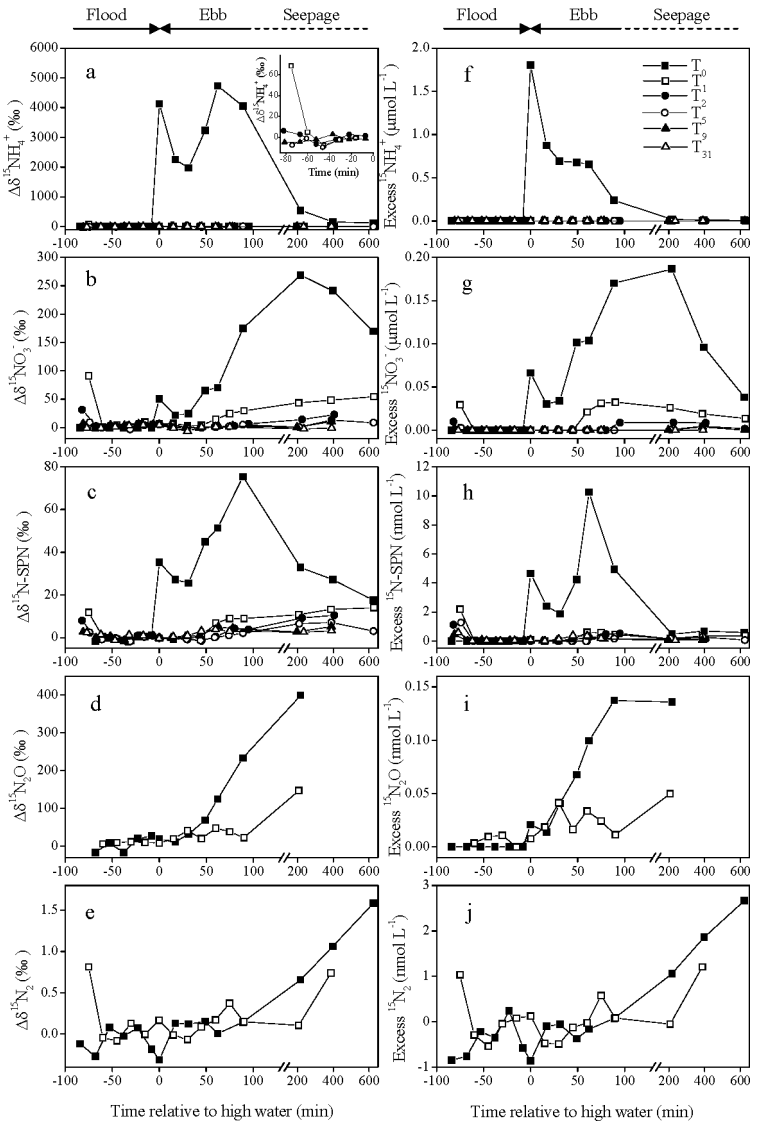

Figure 6.5: Isotopic enrichment ($\Delta\delta$) (above natural abundance level) in (a) NH_4^+, (b) NO_3^-, (c) SPN, (d) N_2O and (e) N_2, and excess ^{15}N concentration of (f) $^{15}NH_4^+$, (g) $^{15}NO_3^-$, (h) ^{15}N-SPN, (i) N_2O, and (j) N_2 in the floodwater of the six tides. Insert in panel a shows an enlargement of the lower left part of panel a. The three phases of the tidal cycle (flood, ebb and seepage) are indicated above figures, with arrow indicating the direction of the water flow.

^{15}N enrichment to the nitrate pool was observed immediately around the turn of the T_0 tide, and enrichment increased linearly over time to 175 ‰ at the end of the main tide (Figure 6.5 b). Enrichment was even higher (269 ‰) in the first seepage sample (217 min after T_0-maximum), but subsequently decreased to 170 ‰ over the following 7 h. In the following two tides (T_1 and T_2), the initial floodwater was enriched in ^{15}N-nitrate (91 ‰ and 31 ‰, respectively), then decreased to below < 10 ‰ enrichment, followed by a gradual increase in the ebb and seepage water (to 54 ‰ and 23 ‰, respectively). The temporal ^{15}N enrichment pattern of SPN was similar to that of the nitrate pool, except the maximum enrichment was much lower (75 ‰) and occurred at the end of the ebb tide and not during seepage (Figure 6.5 c). The enrichment was predominantly in the smallest, most abundant SPM fractions (< 20 μm) (data not shown).

Coinciding with the ^{15}N enrichment to the nitrate pool, the nitrous oxide pool was also enriched during T_0 and T_1 (Figure 6.5d). The enrichment increased linearly (T_0) and was ~50 % higher (up to 399 ‰) than in the nitrate pool. The isotopic composition of N_2O could only be determined during the main tide and in the initial seepage sample. A slight but significant increase (2 ‰) in ^{15}N was observed in the N_2 pool during T_0 seepage (Figure 6.5e), indicating the occurrence of denitrification.

The initial excess concentration of ^{15}N-NH_4^+ (calculated as the measured ^{15}N-NH_4^+ concentration minus the natural abundance concentration) in the T_0 ebb water was high (1.8 μmol L^{-1}; Figure 6.5f), since too much label was added at the end of the label addition period (see Conservative tracer distribution). Thereafter, the concentration of excess ^{15}N-NH_4^+ was relatively constant (0.7 μmol L^{-1}) for the main duration of the ebb tide but decreased to 0.3 μmol L^{-1} toward the end of the main tide. Removal of ^{15}N from the ammonium pool during T_0 was, however, clearly evident from the decrease in $^{15}NH_4^+$:Br^- concentration ratio from $3.1 \cdot 10^{-3}$ initially to $1.0 \cdot 10^{-3}$ at the end of the ebb tide (Figure 6.4b). In the seepage water following T_0 and initial T_1 samples, low concentrations of ^{15}N-NH_4^+ were detected. Thereafter, $^{15}NH_4^+$ concentrations were not significantly different from natural abundance levels.

The temporal pattern of excess $^{15}NO_3^-$ (Figure 6.5g) roughly mimicked that of the $\Delta\delta^{15}NO_3^-$, except that the maximum at T_0 was observed in the seepage and at the end of ebb in the following tides. During T_0 ebb, the concentration of excess $^{15}NH_4^+$ decreased 4.15 times faster than the cor-

responding excess $^{15}NO_3^-$ concentration increased (inferred from slopes of curves in Figure 6.5f,g), indicating that 24 % of the transformed ^{15}N ammonium was nitrified. An increase in the $^{15}NO_3^-$:Br^- concentration ratio over time further demonstrates incorporation of ^{15}N into the nitrate (NO_3^-) pool during T_0 (Figure 6.4b). In subsequent tides, high ^{15}NO:Br^- concentration ratios were also observed, especially halfway through T_1 ebb, at which time $^{15}NO_3^-$:Br^- was twice as high as ^{15}N:Br^- in the tracer solution (Figure 6.4c). Overall, the excess dissolved inorganic $^{15}N(DI^{15}N)$:Br^- concentration ratio decreased linearly during both T_0 ebb and seepage, but the decrease was one order of magnitude faster during ebb (Figure 6.4b, insert). This decreasing ratio further demonstrates that NO_3^- was not the only sink for $^{15}NH_4^+$. Excess ^{15}N concentration in the particulate pool showed a temporal pattern similar to nitrate (Figure 6.5h), but concentrations were more than one order of magnitude lower.

Excess ^{15}N-N_2O concentrations (Figure 6.5i) mimicked those of nitrate over the tidal cycle, but concentrations were three orders of magnitude lower. Estimated water-air gas exchange from the entire water surface could account for a loss of 6-60 μmol ^{15}N-N_2O to the atmosphere during the main T_0 tide. During T_0 seepage, the concentration of excess $^{15}N_2$ increased linearly to 2.7 nmol L^{-1} (Figure 6.5j). No ^{15}N was recovered in the N_2 pool during the main tide, and estimated water-air exchange of $^{15}N_2$ was zero. Sediment-air gas exchange during emersion was not determined.

6.3.4 Nitrogen transformation rates

Bottle incubations with ^{15}N revealed WNR rates of 0.40-0.67 μmol L^{-1} h^{-1} over the T_{-2} tidal cycle (Figure 6.6). WNR were linearly correlated with $[NH_4^+]$ ($r^2 = 0.80$, $p < 0.05$), except during seepage, when relatively high rates were observed. WNR were seven to ten times higher than uptake rates of ammonium into the particulate pool, except in one ebb sample, in which exceptionally high (0.27 μmol L^{-1} h^{-1}) uptake rates were observed.

In situ transfer of ^{15}N from the added ammonium to the nitrate pool as a result of nitrification is directly evident from the increase in $\Delta\delta^{15}NO_3^-$ during T_0 ebb (Figure 6.5b). A simple estimate of whole-ecosystem nitrification rate during marsh flooding can be obtained from the linear increase ($r^2 = 0.93$, $p < 0.05$) in the excess ^{15}N-NO_3^- concentration (Figure 6.5g)

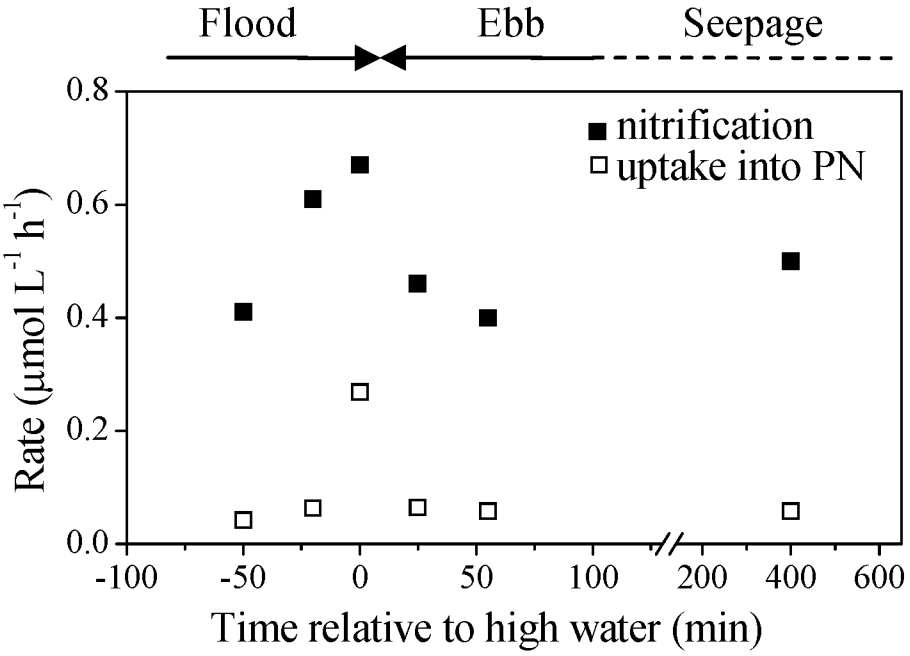

Figure 6.6: Nitrification rate and rate of ammonium uptake into the suspended particulate nitrogen (PN) pool in closed bottle water incubations. The three phases of the tidal cycle (flood, ebb and seepage) are indicated above figures, with arrow indicating the direction of the water flow.

during T_0 ebb tide. Assuming complete symmetry of the flow patterns of flood and ebb and no or limited mixing of the water (i.e. the first body of water entering the study area is the last to leave), the net ecosystem nitrification rate (ENR) can be expressed according to:

$$\mathrm{ENR}_{\mathrm{regression}} = \frac{\alpha}{\%^{15}N_{NH_4^+}} \tag{6.1}$$

where α is the slope of excess $^{15}NO_3^-$ during T_0 ebb as a function of $2 \cdot$ sampling time after T_0-maximum (accounting for residence time effectively being twice the duration between T_0-maximum and sampling time), and $\%\ ^{15}N_{NH_4^+}$ is the average percentage of the ammonium pool labeled with ^{15}N. Another estimate of ENR during T_0 can be determined from mass balance calculations according to:

$$\mathrm{ENR}_{\mathrm{mass-balance}} = \frac{\sum^{15} NO_3^-}{V \cdot T \cdot \%^{15}N_{NH_4^+}} \tag{6.2}$$

where $\sum NO_3^-$ is the total amount of $^{15}NO_3^-$ exported during T_0 ebb, V is the total water volume in the study area, T is the duration of the main tide (flood + ebb), and $\%\ ^{15}N_{NH_4^+}$ is the average percentage of the ammonium pool labeled with ^{15}N. Both estimates of ENR are significantly (four to nine times) higher than WNR (Table 6.3), revealing that the marsh system as a whole is an intense site for nitrification and that most nitrification was associated with sediment and biofilms rather than the water column. Similarly, a mass-balance estimate of the uptake rate of $^{15}NH_4^+$ into SPN is almost twice as high (0.11 μmol h^{-1}) as those measured in most bottle incubations (0.06 ± 0.1; exceptionally high value at maximum tide excluded; Figure 6.6).

The N_2O production rate based on the linear increase in $^{15}N_2O$ concentration (Figure 6.5i) and calculated using the same approach as $\mathrm{ENR}_{regression}$ was 6.96 nmol L^{-1} h^{-1}, while a rate of 1.41 nmol L^{-1} h^{-1} was obtained from the $^{15}N_2O$ mass balance. The NO_3^- to N_2O ratio was 663 to 1,441, based on the regression and mass-balance approaches, respectively. Including seepage water in the mass-balance estimates rendered a similar NO_3^-:N_2O ratio (1,446:1).

Table 6.3: Nitrification rate estimates for the water phase (WNR) and whole marsh ecosystem (ENR). Water phase incubations were done during T_{-2}, while whole ecosystem rates were determined from T_0.

Compartment	Method	Nitrification rate (μmol L^{-1} h^{-1}
Water phase	^{15}N bottle incubations	0.5±0.1
Whole ecosystem	Regression (Eq. 6.1)	4.6
	^{15}N mass balance (Eq. 6.2)	2.0
	Nutrient mass balance[4]	-7.3-21.2

[4]All seven tides, derived from NO_3^- mass balance.

6.3.5 The fate of N

^{15}N mass-balance budgets revealed that 31 % of the added tracer was either transformed or taken up by the marsh biota (Table 6.4). Of the 1.98 moles of ^{15}N added, 8.7 % was nitrified, while 69 % was exported as ammonium (unprocessed) during the first tide. An additional 0.5 % was exported as SPN, and only 4 % (T_0) was retained in the marsh (sinks = sediment, plants, roots, plant litter) (Gribsholt et al. unpubl. data). Collectively, less than 0.01 % of the ^{15}N was recovered in dissolved gas (N_2O and N_2). The cumulative transfer of ^{15}N to the different pools changed only slightly in subsequent tides. Thus, the only difference was the amount of label recovered in marsh sinks other than nitrate that accounted for 6.9 % and 5.0 % after T_5 and T_{31}, respectively, while the importance of nitrification increased from 8.7 % to 9.1 % (Table 6.4). We were able to account for 83-86 % of added ^{15}N. The remaining 14-17 % was probably transferred to the dissolved organic nitrogen (DON) pool and exported during ebb water or was denitrified and lost to the atmosphere as N_2 during low tide (sediment-air flux) (see Discussion). Nitrification was quantitatively the most important transformation process for ^{15}N.

Whether the tidal freshwater marsh net imported or exported nitrate and/or ammonium could not be determined from the mass balance based solely on nutrient concentrations. Although almost closed, proper error accumulation estimates showed that a balanced nitrogen budget based on

Table 6.4: ^{15}N mass balance budget. Recovery in the various N-pools after T_0, T_5 and T_{31} (cumulative). Numbers in parenthesis are percentage of the total ^{15}N added.

Compartment	T_0 (mmol)	T_0-T_5 (mmol)	T_0-T_{31} (mmol)
Tracer input	1,976 (100)		
^{15}N exported unchanged (as $^{15}NH_4^+$)	1,370 (69)	1,369[5] (69)	1,369 (69)
^{15}N transformed	607 (31)		
$^{15}NO_3^- + {}^{15}NO_2^-$	172 (8.7)	181 (9.1)	181 (9.1)
$^{15}N_2O$	0.13 (0.0)	0.13 (0.0)	0.13 (0.0)
$^{15}N_2$	0.11 (0.0)	0.11 (0.0)	0.11 (0.0)
$SP^{15}N$	9.6 (0.5)	10.0 (0.5)	10.0 (0.5)
SINKS[6]—stored	79 (4.0)	135 (6.9)	98.1 (5.0)
Balance not accounted for	345 (17)	280 (14)	318 (16)

[5]Decrease in export of $^{15}NH_4^+$ due to small amount pushed back into the marsh during T_1 flood.
[6]SINKS collectively refer to uptake into plants, roots, sediment, and plant litter.

concentration and water fluxes could not be derived. Thus, nitrate export relative to nitrate import varied between -4 and +26 %, while ammonium export relative to import varied from -11 to +20 % among the seven tides. Ammonium transformation rates (main tides) based on the mass balance varied between -1.8 and 3.2 μmol L^{-1} h^{-1}, while overall changes in nitrate ranged from -7.3 to 21.2 μmol L^{-1} h−1 (Table 6.3). Generally, the highest tides (including T_0) showed a net export of both NH_4^+ and NO_3^-, because ebb water estimates during these tides greatly exceeded flood (Table 6.1).

6.4 Discussion

6.4.1 Whole-ecosystem labeling

The relatively new technique of *in situ* stable isotope enrichment has increased our understanding of N processing under natural conditions. It allows for the examination of N flow through multiple pools simultaneously while maintaining natural hydrologic and biogeochemical gradients and ecosystem processes. Unconfined, whole-ecosystem ^{15}N enrichments have been carried out in lakes (Kling, 1994), streams (Peterson et al., 1997; Hamilton et al., 2001; Wollheim et al., 2001), and estuaries (Holmes et al., 2000; Hughes et al., 2000; Tobias et al., 2003b). The present study is, to our knowledge, the first of its kind in tidal marshes, and it yielded

several new insights about the functioning of these ecosystems. First, our results unequivocally show that nitrification is one of the most important transformation processes associated with eutrophic freshwater marshes. Moreover, we demonstrated that the large reactive surface area of marsh sediment and biofilms is a key site for nitrification. Finally, we found that freshwater marshes function as nutrient transformers as well as nutrient sinks.

Numerous studies have attempted to draw inferences about marsh-estuarine interactions by measuring nutrient concentrations of flooding and draining water over tidal cycles (mass balance) (Merrill and Cornwell, 2000). However, possible net changes are often obscured by large uncertainties in the hydrologic budget, which is also the case in this study. The mass-balance deficit in floodwater at most tides compared to mass-balance ebb water and estimates of maximum standing water volume (digital terrain model) indicate that some water enters the marsh through pathways other than the main creek. Though care was taken, screens transecting the downstream end of the marsh may not have been completely watertight, and water may have flown into the study area, where screens joined the dikes at the edge of the experimental marsh. Alternatively, model calculations may underestimate the water volume entering the marsh, possibly because of turbulence and heterogeneity in flow velocity profiles. However, the high bromide recovery relative to known amount of tracer release (115 %) indicates instead a 15 % overestimation of the T_0 ebb water volume. A reduction of the T_0 ebb by 15 % would indeed result in an almost closed T_0 water budget, the balance between import and export being only 1.6 % rather than the 18.9 % reported in Table 6.1. Whatever the reason, the above-mentioned factors illustrate the shortcomings of traditional mass-balance studies.

Large errors in our conventional mass-balance budget did not allow for reliable whole-system N-transformation estimates. Although the water budget is also used in the calculations of the ^{15}N mass-balance budget, experimental errors in water estimates are of less consequence, because a known amount of tracer is added (import), and only the export relies on the water volume estimates. Thus, the 15 % error (overestimation) in the water budget (T_0) indicated by the bromide data and discussed above results in a 15 % error in the ^{15}N budget estimates. The clear observations that $^{15}NH_4^+$ is nitrified and that marsh surfaces are key sites

for nitrification as well as nitrogen accumulation highlight the strengths of the whole-ecosystem ^{15}N labeling approach.

The ^{15}N mass-balance calculations revealed that, surprisingly, only a relatively small fraction of the added label was retained within the marsh. Uptake into the various marsh compartments is beyond the scope of this article and is discussed elsewhere (Gribsholt et al. unpubl. data), but collectively, sediment, roots, plants, and plant litter retained only ~4 %, corresponding to 13 % of the ^{15}N transformed (i.e. not exported as $^{15}NH_4^+$). Uptake by suspended algae and bacteria was limited (< 2 % of transformed ^{15}N), while nitrate was the largest single pool, accounting for 9 % of the added tracer or 30 % of the transformed ^{15}N. Nitrification has also been demonstrated to rival assimilative uptake of NH_4^+ in forested streams (e.g. (Mulholland et al., 2000; Hamilton et al., 2001; Peterson et al., 2001). Overall, our study shows that the marsh is quite important for N transformation, with almost one-third of the $^{15}NH_4^+$ added to floodwater transformed within the first tide. Assuming we overestimated the export of untransformed tracer by 15 %, as indicated by the Br^- budget, the fraction of transformed ^{15}N was as high as 40 %.

We were able to account for about 86‘% of the added ^{15}N and ascribe the missing 14 % to either export as DON or denitrification to N_2. Detailed process studies summarized by Berman and Bronk (2003) have shown that ^{15}N uptake by microbes can result in rapid formation of ^{15}N-enriched DON. Moreover, uptake of ^{15}N by macrophytes and subsequent leaching of leaves may also result in ^{15}N enrichment of DON. However, methodological restrictions prevent us from evaluating the significance of DON. Although water-air fluxes were insignificant, gas emission during emersion (sediment-air flux) of $^{15}N_2O$ and $^{15}N_2$ produced by sedimentary nitrification and denitrification may also account for some loss. Furthermore, we recognize that several omitted compartments may represent minor losses as well. For instance, benthic and pelagic faunal components were omitted as a result of very low abundances.

To successfully trace the ^{15}N and construct a reliable budget for the entire marsh ecosystem, a prerequisite is an even distribution of tracer through the entire study area. For this purpose, we constructed a simple tracer addition device that allowed us to add tracer to the floodwater at a rate approximately proportional to the water volume. Simultaneous addition of the conservative tracer Br^- allowed us to confirm that a

relatively even distribution was indeed obtained. Too much tracer solution was, however, added directly around the turn of the tide, resulting in Br^- concentrations twice as high as those observed in the main tide, and this was also reflected in a peak in excess concentrations of $^{15}NH_4^+$, $^{15}NO_3^-$, and ^{15}N-SPM. Since the last body of water entering the study site is also the first to exit, we believe this overlabeling to be of little consequence for the overall labeling and N processing within the study site and therefore for the interpretation of the results. This perception is corroborated by the relatively even Br^- concentrations recovered in the marsh water traps, indicating that we successfully obtained an even tracer distribution. On average, the added tracer increased the ^{15}N content to 1.3 % of the ammonium pool and increased the total NH_4^+ concentration by approximately 14 %. Nutrient concentration variability during flood tide (Figure 6.3), however, resulted in a higher initial degree of labeling compared to the main tide. This initial period accounted for only a minor part of the total water volume, and therefore, we consider this of little significance. The added tracer probably did not accelerate *in situ* rates but merely substituted for in situ N species.

In this study the ^{15}N tracer was added in a short pulse (1.5 h) as opposed to the long-term (up to 6 weeks) continuous tracer addition approach adopted in previous ^{15}N whole-ecosystem tracer studies (e.g. (Mulholland et al., 2000; Tobias et al., 2003b; Ashkenas et al., 2004)). The constraint of periodic, two-directional water flows in the tidal marsh and the complete drainage between tides renders a longer addition unfeasible. The short pulse labeling is, however, adequate for tracing the short-time scale processes such as nitrification. We added ^{15}N in the form of NH_4^+ primarily because ammonium is preferred over nitrate by most macrophytes as well as microphytes. Thus, a higher turnover of ammonium rather than nitrate would be expected. Also, nitrate concentrations in the Scheldt are much higher than those of ammonium, and ^{15}N enrichment of the NO_3^- pool would require a large addition of ^{15}N-NO_3^- , with high related costs.

6.4.2 Nitrification and the importance of the marsh surface area

Nitrate was quantitatively the most important fate for ^{15}N in our study, with nitrification accounting for 30 % of the ^{15}N transformation. The validity of this mass-balance estimate is strongly supported by the estimate of the relative importance of nitrification (24 %) based on the straightforward relation during T_0 ebb between the observed decrease and increase in excess $^{15}NH_4^+$ and $^{15}NO_3^-$ concentrations, respectively. Nitrification has also been reported to account for a substantial fraction (up to 50 %) of the total $^{15}NH_4^+$ removal in *in situ* ^{15}N tracer studies of forested streams (Hamilton et al., 2001; Merriam et al., 2002; Ashkenas et al., 2004).

The occurrence of nitrification in the marsh is evident in several ways and is most clearly evident in the direct transfer of ^{15}N from the added ammonium pool to the nitrate pool, as seen in the increase in $\Delta\delta^{15}NO_3^-$ (Figure 6.6b). Although the pelagic ammonium pool was only isotopically enriched during T_0, enriched nitrate was also exported in the following tides. This indicates that the marsh acts as at least a temporary sink for N, either through pore-water DIN exchange, sorption, or rapid assimilation by benthic microbes and subsequent mineralization. The very high $^{15}NO_3^-$:Br^- ratios observed, especially during T_1, indicate a biological mechanism (microbial uptake and subsequently remineralization by nitrifiers) rather than physicochemical sorption as the main sink.

The significantly higher (four to nine times) nitrification rates observed on a whole-ecosystem scale (ENR) compared to the water column alone (WNR) clearly demonstrate that even in this nutrient-rich system, the large reactive surface area of sediments and biofilms in the marsh are the key sites for nitrification. Three factors may potentially be important controls on nitrification in the marsh ecosystem: nitrifier biomass, oxygen dynamics, and ammonium availability for nitrifiers. The distribution, abundance, and activity of nitrifiers is known to be influenced by their attachment to particles (Prosser, 1989), and the surfaces of the dense vegetation and plant litter in the marsh may provide an excellent substratum for microbial colonization, just as epiphytic communities on submersed macrophytes have been demonstrated to be a major source of nitrification in nutrient-rich aquatic environments (Eriksson and Weisner, 1999). Several authors have argued that establishment of macrophyte beds, a con-

spicuous, defining feature of the tidal marsh, should shift the location of greatest algal and bacterial growth from the water column and sediment to benthic plant surfaces because of the multiple advantages associated with an epiphytic biofilm habitat (e.g. Wetzel and Søndergaard, 1998). Benthic nitrification may, however, also be enhanced as oxygen-releasing roots of marsh plants provide oxic subsurface microniches in otherwise anoxic sediments in which ammonium-oxidizing bacteria may thrive (Bodelier et al., 1996; Gribsholt and Kristensen, 2002). Nitrifying bacteria can survive as viable inactive cells during periods of low substrate concentrations or poor growing conditions such as low [O_2] (e.g. Verhagen et al., 1992; Bodelier et al., 1996) and can react instantaneously to favourable conditions. Although hypoxic conditions prevailed, O_2 exchange is rapid in the shallow, turbulent water, and O_2 was never fully depleted. The rapid response of the nitrifying population to inundation indicates adaptation to the specific environmental conditions in the tidal freshwater marsh. Because of the relatively high N availability in the floodwater, plant and heterotrophic bacteria competition for NH_4^+ becomes low enough to allow nitrifiers to flourish. Some ammonium limitation may occur, though, as at least WNR was correlated with ammonium concentrations. The importance of the marsh surface in N transformation is even evident from the intriguing temporal patterns in nutrient loadings observed in all tides. Only around the turn of the tides (maximum tide) did the [NH_4^+] equal that of the main river, while [NO_3^-] even exceeded it approximately halfway through flood as well as ebb tides. These large differences between floodwater and Scheldt water nutrient loading can be explained by the long distance over marsh creeks and surfaces through which the water travels before arriving at the study site and by the influence (uptake as well as transformation) this estuarine environment has already exerted on the water. The body of water initially entering the study site appears to experience the most contact with the sediments and biofilms, and thus the nitrifying (and denitrifying) microorganisms associated with it. Correspondingly, the pattern of decreasing [NH_4^+] and increasing [NO_3^-] in the first part of the ebb clearly shows that ammonium is being oxidized to nitrate within our study site while inundated. During the seepage phase, however, low and decreasing [DIN] could indicate N removal through temporary assimilation and denitrification.

Between tides, water continued to trickle out of our study site. The

long distance to the main river, however, meant that although the water had left the study site, not all water left the marsh before the next flood. Some water remained in the marsh creek system and was pushed back into the study site with the next tide, as is evident from the elevated bromide concentration in the initial T_1-T_5 samples. While residing within the marsh, this water had been in intimate contact with the reactive marsh surface sediment, and N removal was very evident, as all initial floodwater samples were extremely depleted in DIN relative to the river water.

6.4.3 Denitrification

Denitrification is often considered the most important ultimate nitrogen removal pathway from marshes and other aquatic systems and has thus attracted much attention. On ecosystem scales, the importance of denitrification has most often been inferred from mass-balance studies, as the 'missing part' and is generally reported responsible for most nitrogen retention in freshwater ecosystems. Unfortunately, denitrification was not completely covered by our measurements. A small increase (2 ‰) in the isotopic ratio of N_2, however, as well as the temporal DIN concentration dynamics, indicate that denitrification occurs, especially during the seepage phase. During low tide, topographic and hydraulic gradients along creek banks can lead to drainage of marsh pore water into the creek; thus, the seepage water consists of a mixture of surface water trapped in depressions and between vegetation and pore water. Thus, this water has been in intimate contact with sediments and biofilms, where oxic and anoxic microniches may support high rates of coupled nitrification-denitrification.

No significant labeling of the N_2 pool was observed during the main tides. The lack of $^{15}N_2$ accumulation, however, does not exclude the possibility that denitrification took place. First, ^{15}N was added as ammonium, and the relatively short duration of the tide (~3 h) may not be sufficient for the transfer of ^{15}N via nitrification and denitrification to N_2 to occur at a sufficient rate for detection, even with a tight coupling between nitrification-denitrification. Second, the NO_3^- formed (~0.15 μmol L^{-1}) was diluted in the very high background [NO_3^-] (~350 μmol L^{-1}), and $^{15}N_2$ derived from the labeled NO_3^- pool ($\Delta\delta^{15}N$, Figure 6.5) is therefore rather low. Third, since denitrification occurs mainly, perhaps even only, in the sediment, and the $\delta^{15}NO_3^-$ of pore water may not have been enriched

significantly (because it is derived mainly from sedimentary ammonification and nitrification), it is likely that N_2 produced was not isotopically enriched. Fourth, part of the N_2 produced may have escaped to the atmosphere.

Besides being an end product of denitrification, the $^{15}N_2$ recovered during seepage could theoretically also be produced by anaerobic ammonium oxidation coupled to nitrate reduction (anammox)
($^{15}NH_4^+ + NO_2^- \rightarrow {}^{15}N_2 + 2\ H_2O$) (Van de Graaf et al., 1995). However, sediment incubations (performed according to Thamdrup and Dalsgaard, 2002) in 2003 revealed that anammox is not an important process in this tidal freshwater marsh. Whole-core incubation of marsh sediment, on the other hand, revealed high denitrification rates (~0.5 mmol m^{-2} h^{-1}) in May 2003 (Gribsholt unpubl. data), similar to those reported for a nearby tidal freshwater marsh by Verhoeven et al. (2001) and in the Scheldt estuary by Middelburg et al. (1995), and much higher than those reported for more oligohaline North American freshwater marshes. More work is needed to determine the spatial and temporal patterns of denitrification and its importance for N-transformation in tidal freshwater marshes.

6.4.4 N_2O formation

Coinciding with the increase in $^{15}NO_3^-$ during T_0, we observed a clear increase in ^{15}N-labeled dissolved N_2O. Nitrous oxide is produced by three microbial processes: nitrification, denitrification, and dissimilatory reduction of nitrate to ammonia (Seitzinger, 1988). While the importance and mechanism for N_2O production by denitrification are relatively well understood, our appreciation of the importance of nitrification for the global N_2O budget is still limited. The concurrent increase in $^{15}N_2O$ and $^{15}NO_3^-$ after addition of labeled ammonium observed in our study strongly indicates that the observed $^{15}N_2O$ production is largely due to nitrification. Thus, during T_0 excess $^{15}N_2O$ is directly correlated with excess $^{15}NO_3^-$ concentrations ($r^2 = 0.80$, $p < 0.05$). Moreover, the maximum $\Delta\delta^{15}N$ of N_2O is higher than that of NO_3^-, indicating that it comes directly from ammonium. This conclusion is further corroborated by the observed hypoxic conditions during large parts of all tides, as concentrations of O_2 have been clearly recognized as the key control on N_2O production by both nitrification and denitrification, especially during high nutrient availability

(e.g. (Goreau et al., 1980; Jørgensen et al., 1984; de Bie et al., 2002a).

During nitrification, oxidation of NH_4^+ has been shown to produce a NO_3^-:N_2O ratio between 10:1 and 300:1 (Caraco and Cole, 2001). Although largely produced by nitrification, the observed marsh N_2O production rate was quite low, and the NO_3^-:N_2O ratio (663:1 to 1,441:1) is high compared to previous estimates based on studies with isolates. Middelburg et al. (1995) reported sediment-air N_2O fluxes and nitrification rates in tidal sediments along the Scheldt estuary corresponding to NO_3^-:N_2O ratios of 278 to 3,333. Our whole-system estimates include not only the production but also the consumption of N_2O and NO_3^-. Since most nitrification occurs in the sediment, a large portion of the produced N_2O is most likely immediately consumed by denitrifying bacteria in the sediment, and thus never reaches the water phase.

Globally, human alterations of the nitrogen cycle have increased concentrations of the potent greenhouse gas N_2O (Vitousek et al., 1997), and while the increasing concentration of N_2O is clearly documented, the source of that increase remains a matter of some discussion. Most likely, many anthropogenic sources contribute (Vitousek et al., 1997), and although not quantified in this study, our results indicate that nitrification in wetlands receiving hypoxic, nutrient-rich water may contribute as well. While the importance of nitrification as a source of nitrous oxide triggered by low oxygen concentrations (in conjunction with high ammonium availability) has previously been shown on the estuarine scale (Barnes and Owens, 1998; de Bie et al., 2002a; Punshon and Moore, 2004), this is, to our knowledge, the first study specific to tidal marsh ecosystems.

6.4.5 Marshes and the Scheldt estuary

To determine the role of tidal-freshwater-marsh nitrification to the nitrogen budget of the Scheldt estuary, we scaled our estimates of nitrification rates in our study site during water cover to the total area of fringing tidal freshwater marshes in the estuary. Estimates are based on the T_0 nutrient and water balances and T_0 nitrification rates (1.0-2.4 mmol m^{-2} h^{-1}) and a yearly inundation period of our study site of 691 h yr^{-1} (based on topography and mean tidal height at Tielrode [2002]). Annually, 36-81 kg N is nitrified in our study site, corresponding to 1,030-2,340 kg in the ~100,000 m^2 Tielrode marsh. Consequently, ~46,000-105,000 kg of

river borne ammonium will be nitrified yearly in the 4.5 km^2 of freshwater marshes fringing the Scheldt, equalling ~1.5-3.5 % of the yearly ammonium load to the Scheldt (~3,000,000 kg yr^{-1}, Struyf et al. (2004). Variation in duration of water cover and reactive surface area (sediment, plant stems, litter) due to differences in topography and variations in heterotrophic activity due to nutrient availability and season will determine the processing of N. Therefore, extrapolation from our spring/early summer experiment with relatively high temperatures may overestimate annual nitrification. Water residence time and inundation frequency, however, are longer in large parts of the marsh compared to our study site; thus, our extrapolation to the entire Tielrode marsh may underestimate nitrification. In spite of these limitations, our extrapolations are reasonable because the relative large study area, covering all relevant vegetation types, is a good representative of freshwater marshes along the river. Overall, our results, in combination with those of other studies throughout the estuary, reveal that tidal freshwater marshes contribute little to the overall N-cycling of the Scheldt estuary, which is mainly governed by main-stream water column processes. This is likely due to a combination of the small surface of marshes relative to the overall surface of the estuary, very high nutrient loading, and short water residence time. However, if the large areas of brackish marshes in the Scheldt estuary (~20 km^2) transform NH_4^+ at comparable rates to our freshwater marsh site, as much as 252,000-573,000 kg, or 8.4-19.1 % of the yearly ammonium load to the Scheldt, is potentially nitrified in tidal marshes.

Summary

In this thesis I investigated nitrification, dissolved inorganic and organic nitrogen uptake, and the relative importance of nitrification and ammonium assimilation. All of these processes occur in the pelagic zone and they refer to internal cycles. I have also investigated exchange with (freshwater) marshes and with sediments which refer to external nitrogen cycling. In addition to this, nitrogen cycling, within the marsh and sediment compartments, has been studied.

Nitrification, oxidation of NH_4^+ to NO_3^- is a central process in the nitrogen cycle. It is the first step for removal of nitrogen from a system, counteracting eutrophication. Evaluating what could be a suitable method to quantify nitrification in estuarine systems was the subject of **Chapter 2**. One method quantifying nitrification as nitrifier activity was compared to another method using growth of nitrifying bacteria in the Scheldt estuary over a salinity gradient. Measurements were made during 4 seasons using ^{15}N enriched ammonium and ^{14}C labeled carbon incorporation. Established conversion ratios are often used to convert the growth of nitrifiers (measured as the incorporation of carbon) to nitrifying activity (i.e. oxidation of ammonium to nitrate). Our study demonstrated that the conversion of growth rates to nitrifying activity induces uncertainty because activity and growth of nitrifiers may be uncoupled. The N:C conversion ratio appeared to be oxygen and temperature dependent. We advocate the use of ^{15}N stable isotope techniques to study nitrification: this technique measured the actual activity of the nitrifiers without the disadvantages involved in using inhibitors, and also allowed light inhibition to be measured.

Simultaneously to nitrification measurements, uptake of NH_4^+ was quantified, in combination with other inorganic and organic nitrogen compounds in **Chapter 3**. Hereby we could assess the fate of NH_4^+ and the relative importance of uptake of inorganic versus organic nitrogen compounds. Uptake of dissolved ammonium, nitrate, nitrite, urea and

amino acids was studied in the Scheldt estuary during the same cruises mentioned above. The importance of inorganic nitrogen sources was compared to that of urea and amino acids and the relative uptake of urea and amino acid nitrogen and carbon was studied. Urea and amino acids constituted up to 43 and 29 % of total nitrogen uptake, respectively, and were of similar importance as inorganic substrates. Ammonium oxidation by nitrifiers and ammonium uptake by algae and bacteria were of similar magnitude. In January, April and November amino acids constituted a source of both nitrogen and carbon while urea mainly constituted a source of carbon. During the summer months amino acids were used mainly as a source for nitrogen, while urea was a source for both carbon and nitrogen; urea was rarely used as nitrogen substrate alone. Due to this seasonal uncoupling of nitrogen and carbon assimilation, dually labeled substrates are necessary to assess the importance of urea and amino acids as substrate for organic nitrogen and carbon.

After exploring a number of pelagic processes I took another step in studying the nitrogen cycle in **Chapter 4** and focused on production of N_2. This is of considerable interest since bioavailable nitrogen is permanently removed from the estuarine system. Sediment nitrogen removal via production of N_2, depends on a number of factors. This study showed that the interaction between two factors, temperature and NO_3^- concentration in the overlying water, governs benthic production of N_2, whereas these factors separately have a minor effect. Increasing temperatures above ambient levels, both during winter and summer, caused the flux of NH_4^+ out of the sediment to increase. Since no response in N_2 production without additions of NO_3^- was found, the additional produced NH_4^+ was not efficiently nitrified and subsequently denitrified. Addition of nitrate did not increase sediment respiration in terms of oxygen consumption and dissolved inorganic carbon release, while enhancing temperature did. Sulfate reduction could account for a large part of the mineralization rate and 40-62 % of the reduced products were not oxidized, implying escape to the water column or accumulation in the sediment.

After investigating the abiotic factors, temperature and NO_3^- concentration on N_2 production, attention turned to biotic factors, such as macrofauna. In **Chapter 5** we successfully defaunated the sediment of a tidal flat *in situ* and were able to follow the effect of recolonization on biogeochemical processes. The experiment did not last long enough for the

sediment to return to natural conditions. Nevertheless, a strong effect of faunal community on biogeochemical processes were found. N_2 production rates were up to 70 % lower in defaunated sediment compared to control sediment. Fauna inhabiting the sediment is very important for biogeochemical processes taking place in the sediment in general and for processes in the nitrogen cycle in particular. Presence of fauna had a strong positive effect on production of N_2 and greatly stimulated coupled nitrification/denitrification.

In an effort to put all our knowledge regarding the nitrogen cycle, into a whole-ecosystem context, we quantified the fate and transport of watershed-derived NH_4^+ in a tidal freshwater marsh in a whole-ecosystem labelling experiment in **Chapter 6**. ^{15}N-NH_4^+ was added to the floodwater entering a tidal marsh area, and marsh NH_4^+ processing and retention were traced in six subsequent tide cycles. Data were presented for the water phase components of the marsh system, in which changes in concentration and isotopic enrichment of NO_3^-, NO_2^-, N_2O, N_2, NH_4^+ and suspended particulate nitrogen were used in a mass balance study. All analyzed dissolved and suspended N pools were labeled. 31 % of added ^{15}N-NH_4^+ was retained or transformed. NO_3^- was the most important pool for ^{15}N, with nitrification accounting for 30 % of ^{15}N-transformation. Whole-ecosystem nitrification rates were four to nine times higher than those in the water column alone, implying a crucial role for the large reactive marsh surface area in N-transformation. Under conditions of low oxygen concentrations and high ammonium availability, nitrifiers produced N_2O. Our results show that tidal freshwater marshes function not only as nutrient sinks but also as nutrient transformers.

With the work presented in this thesis we have elucidated some of the remaining mysteries of the nitrogen cycle. The use of stable isotopes has proven invaluable to the quantification of many processes, e.g. nitrification. Recent findings have shown that microorganisms belonging to Archaea, such as the group Crenarchaeota, can play an important role in marine nitrification (Wuchter et al., 2006). In order to take our study one step further one could investigate whether nitrification by Archaea could explain the variable N:C ratios we found in Chapter 2. We have studied the effect of fauna, temperature and nitrate concentration on production of N_2 (Chapter 4, 5). There are two known processes in which N_2 is produced, denitrification and anaerobic ammonium oxidation (Anam-

mox). One interesting continuation of our study would be to quantify the relative importance of the denitrification vs. Anammox and if they are affected differently by the factors studied.

Samenvatting

Dit proefschrift gaat over mijn onderzoek naar nitrificatie, de opname van organisch en anorganisch stikstof en het relatieve belang van nitrificatie en ammonium assimilatie. Al deze processen vinden plaats in de pelagische zone en hebben betrekking op interne kringlopen. Daarnaast heb ik ook onderzoek gedaan naar de uitwisseling van stikstof met (zoetwater) schorren en sedimenten, hierbij gaat het om externe stikstofkringlopen. Daarnaast is ook de stikstofcyclus binnen een schor en in het sediment onderzocht.

Nitrificatie, de oxidatie van NH_4^+ naar NO_3^-, is een proces met centrale rol in de stikstofcyclus. Het is de eerste stap in de verwijdering van stikstof uit een systeem, en geeft zodoende tegenwicht aan eutrofiëring. In **Hoofdstuk 2** worden een potentiële methode voor het kwantificeren van nitrificatie in estuariene systemen geëvalueerd. Met een methode wordt nitrificatie gemeten als de activiteit van nitrificeerders. Met een andere methode wordt nitrificatie gekwantificeerd als de groei van nitrificerende bacterin. Deze twee methodes zijn vergeleken in een studie langs een saliniteitsgradient in het Schelde estuarium gedurende vier seizoenen waarin gebruikt is gemaakt van ^{15}N verrijkt ammonium en incorporatie van ^{14}C-gelabelde koolstof. Vaak worden vaste waardes gebruikt om groeisnelheden van nitrificerende bacteriën (gemeten als koolstof incorporatie) om te rekenen naar nitrificatie activiteit (de oxidatie van ammonium naar nitraat). Uit onze studie blijkt dat de groei van nitrificerende bacteriën en nitrificatie activiteit niet altijd direct gekoppeld zijn wat onzekerheid geeft met betrekking tot de conversie van groeisnelheden naar nitrificatie. De betreffende N:C conversiefactor lijkt afhankelijk te zijn van zuurstof en temperatuur. We bevelen de ^{15}N methode aan voor het kwantificeren van nitrificatie aangezien met deze methode direct de nitrificatie activiteit wordt gemeten zonder de nadelen van het gebruik van inhibitors. Bovendien kan met deze methode ook lichtinhibitie worden gemeten.

Tijdens de cruises in het Schelde estuarium voor de nitrificatiemetingen

uit **Hoofdstuk 2** is ook de opname van ammonium en een aantal andere anorganische (nitraat en nitriet) en organische (urea en aminozuren) substraten gemeten. De resultaten hiervan worden gepresenteerd in **Hoofdstuk 3**. Het doel van deze metingen was het bepalen van het relatieve belang van microbiële ammoniumopname als verliesproces voor ammonium (in vergelijking met verlies door nitrificatie) en het onderzoeken van het belang van de verschillende anorganische en organische substraten als stikstofbronnen voor de microbile gemeenschap. Ook wordt voor de organische substraten de opname van koolstof en stikstof uit deze substraten vergeleken. Urea en aminozuren droegen respectievelijk tot 43 en 29 % bij aan de totale stikstofopname en waren als stikstofbronnen even belangrijk als de anorganische substraten. De opname van ammonium door algen en bacteriën was van dezelfde orde van grootte als de consumptie van ammonium door ammonium oxidatie door nitrificeerders (nitrificatie). In januari, april en november werden de aminozuren gebruikt als bron voor zowel koolstof als stikstof terwijl urea alleen als koolstofbron fungeerde. In de zomermaanden werden de aminozuren voornamelijk gebruikt als stikstofbron terwijl van urea zowel de koolstof als stikstof werd opgenomen. Urea werd zelden alleen als stikstofbron gebruikt. Deze seizoensgebonden koppeling tussen de assimilatie van stikstof en koolstof geeft aan dat het gebruikt van dubbel gelabelde substraten nodig is voor het bestuderen van het belang van urea en aminozuren als bronnen van organisch koolstof en stikstof.

Na de pelagische processen zoals die in **Hoofdstuk 2** en **3** aan de orde zijn gekomen richt ik me in **Hoofdstuk 4** op een ander proces binnen de stikstofcyclus: de productie van N_2. Dit is een belangrijk proces aangezien langs deze weg biobeschikbare stikstof permanent uit een systeem wordt verwijderd. De verwijdering van stikstof uit het sediment door productie van N_2 is afhankelijk van een aantal factoren. Uit de resultaten van deze studie blijkt dat de combinatie van twee van deze factoren, te weten de temperatuur en de NO_3^- concentratie in het overstaande water, bepalend was voor de productie van N_2 in het sediment terwijl het individuele effect van deze factoren veel kleiner was. In zowel de zomer als de winter resulteerde een stijging van de temperatuur boven normale waardes in een toename van de NH_4^+ flux vanuit het sediment. Zonder toevoeging van NO_3^- resulteerde dit echter niet in productie van N_2 wat er op duidt dat de gevormde NH_4^+ niet efficiënt kon worden genitrificeerd en vervolgens

gedenitrificeerd. De toevoeging van nitraat resulteerde niet in een verhoogde sedimentrespiratie in de vorm van zuurstofopname en productie van opgelost anorganisch koolstof, terwijl dit bij verhoging van de temperatuur wel het geval was. Een groot deel van de mineralisatiesnelheid kon worden toegeschreven aan sulfaatreductie en 40-62 % van de gereduceerde producten werd niet geoxideerd, dit duidt op verlies naar de waterkolom of accumulatie in het sediment.

Na het effect van de abiotische factoren temperatuur en nitraat concentratie op N_2 productie te hebben onderzocht wordt in **Hoofdstuk 5** de aandacht gericht op biotische factoren zoals macrofauna. *In situ* sediment werd succesvol gedefauneerd waarna het effect van rekoloniserende fauna op verschillende biogeochemische processen werd onderzocht. Ondanks dat het experiment niet lang genoeg duurde om het sediment terug te laten keren naar zijn natuurlijke toestand was er sprake van een duidelijk effect van de faunagemeenschap op biogeochemische processen. Productiesnelheden van N_2 waren tot 70 % lager in gedefauneerd sediment als in onverstoord sediment. De aanwezigheid van fauna in het sediment is van groot belang voor de biogeochemische processen die zich in het sediment afspelen, in het bijzonder processen die deel uit maken van de stikstofcyclus. De aanwezigheid van fauna had een duidelijk positief effect op de productie van N_2 en stimuleerde gekoppelde nitrificatie/denitrificatie sterk.

In **Hoofdstuk 6** wordt al onze kennis met betrekking tot de stikstofcyclus gecombineerd en toegepast op ecosysteem niveau. Om het lot van het ammonium dat met opkomend tij een zoetwaterschor binnenstroomt te onderzoeken werd ^{15}N-gelabeld ammonium toegevoegd aan het binnenstromende water waarna de ^{15}N gedurende zes op elkaar volgende getijdecycli werd getraceerd in verschillende stikstofhoudende componenten van de waterkolom. Concentraties en ^{15}N verrijking werden gemeten voor NO_3^-, NO_2^-, N_2O, N_2, NH_4^+ en gesuspendeerd particulair stikstof en er is een massabalansanalyse uitgevoerd. Alle gemeten stikstof pools waren ^{15}N gelabeld en 31 % van het toegevoegde ^{15}N-NH_4^+ werd vastgehouden of getransformeerd. Het meeste ^{15}N kwam terecht in NO_3^- en nitrificatie was verantwoordelijk voor 30 % van de ^{15}N transformatie. Nitrificatiesnelheden voor het complete schor waren 4 tot 9 keer hoger als die voor alleen de waterkolom. Dit duidt er op dat het reactieve schoroppervlak een cruciale rol speelde in de stikstoftransformaties binnen het schor. In situaties met

lage zuurstofconcentraties en hoge beschikbaarheid van ammonium werd er N_2O geproduceerd door nitrificeerders. Onze resultaten laten zien dat zoetwaterschorren niet alleen een rol spelen in de retentie van nutrienten maar ook in de transformatie van deze nutrienten.

Met het in dit proefschrift gepresenteerde werk hebben we een aantal van de mysteries van de stikstofcyclus weten te ontrafelen. Het gebruik van stabiele isotopen is onmisbaar gebleken voor het kwantificeren van vele processen, bijvoorbeeld nitrificatie. Uit recent onderzoek is gebleken dat microorganismen behorende tot de Archaea, zoals bijvoorbeeld de Crenarchaeota, een belangrijke rol kunnen spelen in nitrificatie in mariene systemen (Wuchter et al., 2006). In het verlengde van het werk in dit proefschrift zou het interessant zijn om te onderzoeken of nitrificatie door Archaea een verklaring zou kunnen vormen voor de variabele N:C ratios in Hoofdstuk 2. We hebben onderzoek gedaan naar het effect van fauna, temperatuur en nitraat concentraties op de productie van N_2 (Hoofdstukken 4 en 5). N_2 wordt niet alleen geproduceerd door denitrificatie maar kan ook worden geproduceerd door anaërobe ammonium oxidatie (Anammox). Als vervolg op het onderzoek in dit proefschrift zou het interessant zijn om het belang van denitrificatie versus Anammox te kwantificeren en te onderzoeken of deze twee processen verschillend worden benvloed door de bestudeerde factoren.

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Curriculum Vitae

Maria Andersson was born on the 8^{th} of July 1977 in Stenstorp Sweden. She studied chemistry at Göteborg University, Sweden specializing in marine chemistry. She received her MSc degree (in swedish *Fil. Mag.*) in 2002, the title of her thesis was "Recycling of nitrogen and carbon in sediments of the Han Bay, the Baltic Sea". The research was conducted at the department of Analytical and Marine Chemistry at Göteborg University under the supervision of Prof. Per Hall.

Between April 2002 and May 2006 she worked as a PhD student at the department of ecosystem studies at the Netherlands Institute of Ecology (NIOO-CEME). The topic of her research was "Nitrogen processes in the Westerschelde estuary" using stable isotopes as tracers and flux measurements. The work resulted in this thesis. After leaving the NIOO Maria went on to teaching science to high school students.

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