Denitrification in Ditches, Streams and Shallow Lakes

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CHAPTER 1

General Introduction

1.1 Causes and consequences of nitrogen pollution in freshwater ecosystems

Causes

Food and energy production have greatly increased the availability of reactive nitrogen in the environment, which has considerably altered the nitrogen cycle locally, regionally and globally (Galloway et al., 2004; Rockström et al., 2009). Reactive nitrogen includes organic nitrogen forms such as proteins, amines and nucleic acids; as well as reduced and oxidized inorganic nitrogen forms like ammonium, nitrate and nitrous oxide. Creating reactive nitrogen from highly non-reactive dinitrogen gas (N_2) is difficult because of the extreme stability of the triple nitrogen-nitrogen bond. As a result, there are only two natural processes that transform N_2 to reactive nitrogen: lightning and microbial nitrogen fixation. In the past century, however, due to the growing human population, nitrogen for food production was in short supply, urging scientists to find a way of artificially fixing N_2 for fertilizer production (Galloway et al., 2004). This led to the development of the Haber-Bosch process in 1913, which produces NH_3 from N_2 and H_2; arguably the most important technological development of the 20th century (Smil, 2004). In the years that followed, artificial nitrogen fixation rapidly became a necessity as the human population continued to grow. Furthermore, increased industrialization led to greatly increased fossil-fuel combustion, which is a major source of nitrogen pollution through emission of reactive nitrogen (NO) into the atmosphere as a waste product. Atmospheric nitrogen pollution (NH_3 and NO_x) from food and energy production cascades through the environment, increasing atmospheric ozone, fine particle matter and N-deposition to terrestrial and aquatic ecosystems (Galloway et al., 2003). Intense agriculture causes large nitrogen surpluses in many parts of the world (see Fig. 1.1 for a European example). This leads to leaching of nitrogen to surface waters, through seepage of nitrogen polluted groundwater, subsurface runoff and surface runoff (Verhoeven et al., 2006). Additionally, domestic and industrial waste disposal form point sources of nitrogen pollution. As a result, riverine N-fluxes have increased 2-20 fold in the past century (Howarth et al., 1996).

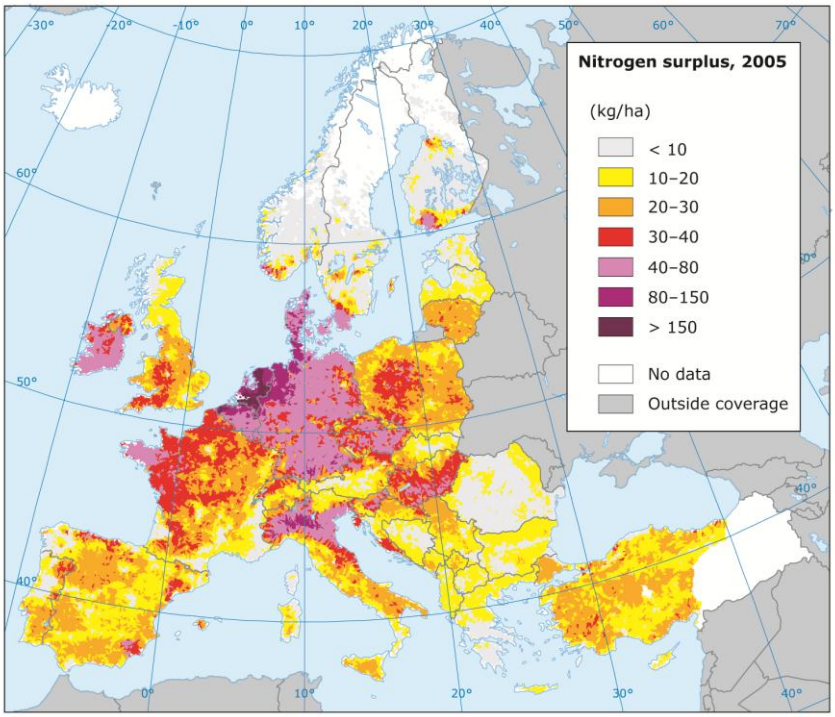

Figure 1.1. Estimated nitrogen surplus (the difference between inorganic and organic fertilizer application, atmospheric deposition, fixation and uptake by crops) for the year 2005 across Europe. Source: JRC European Commission, 2011. http://ies.jrc.ec.europa.eu.

Consequences

In surface waters, increased nitrogen concentrations contribute to acidification and eutrophication effects, such as altered plant productivity, harmful phytoplankton blooms, floating plant cover, temporal anoxia, and consequently fish-kills, biodiversity loss and losses of ecosystem services (Smith et al., 1999; Rabalais, 2002). Drainage ditches and low-order streams in agricultural areas are often the first in line to receive nitrogen loads from (sub)surface runoff and groundwater seepage. Many ditches and streams in these areas are therefore highly eutrophic, and contribute to the eutrophication of receiving waters, namely rivers, lakes and reservoirs and finally also coastal zones and oceans (Seitzinger et al., 2006).

Increased nitrogen-loads alone may lead to eutrophication effects in surface waters, but often these effects occur from a combination of phosphorus and nitrogen enrichment, as many freshwater ecosystems are co-limited by N and P (Elser et al., 1990; Smith et al., 1999; Elser et al., 2007).

The most evident effects of eutrophication in shallow freshwater ecosystems are shifts in the dominant vegetation, altered biogeochemistry and loss of biodiversity. Pristine systems are usually dominated by a diverse community of rooted macrophytes that mainly take up nutrients trough their roots. With increasing nutrient loads, plant biomass increases, and plants that can absorb nutrients from the water-column gain a competitive advantage and start to dominate. Plant growth-strategies then shift from vertical, optimizing nutrient uptake, to horizontal, optimizing exposure to light (Janse & Van Puijenbroek, 1998). In small waters, e.g. ditches and ponds, floating plants dominate in the hypertrophic state, whereas phytoplankton dominates in shallow lakes (Scheffer et al., 1993; Scheffer et al., 2003).

As plant biomass increases, diurnal fluctuations in oxygen become larger, eventually leading to oversaturation during the day, and hypoxia at night. This can result in fish-kills and loss of sensitive invertebrates, thus further altering food webs and biogeochemistry (Scheffer, 1998). Importantly, hypoxia can lead to internal eutrophication, through the release of reactive phosphorus from the sediment (Smolders et al., 2006).

Additionally, eutrophication can stimulate decomposition and alter coupling of biogeochemical cycles, with cascading effects on water quality (Scheffer, 1998; Howarth et al., 2011). For example, nitrate addition can couple sulphide oxidation to denitrification (Burgin & Hamilton, 2008), resulting in a release of sulphate from the sediment, and consequently iron reduction and release of iron bound phosphorous into the water column (Smolders et al., 2006).

1.2 Nitrogen removal by denitrification

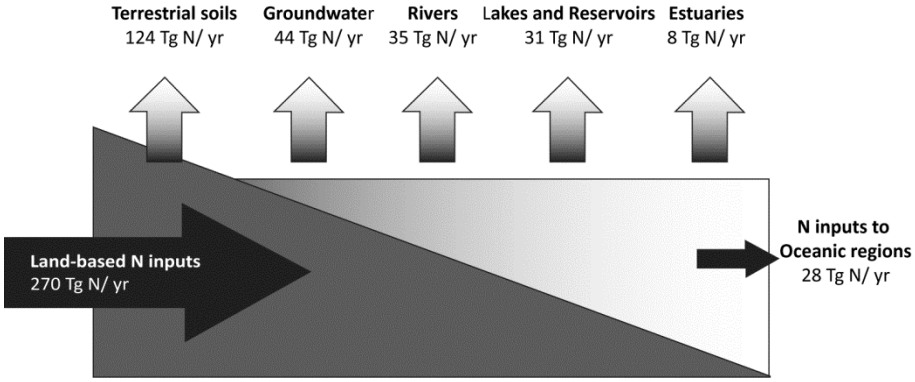

Figure 1.2. Nitrogen removal by denitrification from terrestrial to marine habitats. Estimates of global nitrogen inputs and losses through denitrification are based on Seitzinger et al. (2006).

Denitrification is the stepwise reduction of nitrate to gaseous nitrogen. It removes a large proportion of reactive nitrogen from the aquatic environment before it reaches the oceans (Seitzinger et al., 2006, Fig. 1.2). However, the extent of this loss is still one of the largest uncertainties in regional and global N-budgets (Galloway et al., 2004), as denitrification rates vary widely in space and time, and the most important factors controlling denitrification rates may differ among systems. For example, denitrification rates in lakes have been found to range from 0.10 to 3.54 mol N m^{-2} y^{-1}, whereas those in coastal areas range from 0.09 to 1.10 mol N m^{-2} y^{-1} (Piña-Ochoa & Álvarez-Cobelas, 2006), with considerable seasonal variability (Christensen & Sørensen, 1986; Christensen et al., 1990; Rissanen et al., 2011).

Heterotrophic denitrification (also known as respiratory denitrification) is carried out by a wide array of facultative anaerobic microorganisms, including bacteria, archaea and eukaryotes. It requires easily degradable organic carbon as electron donor, and nitrate as electron acceptor. The overall reaction-equation can be described as follows:

$$5CH_2O+4NO_3^-+4H^+ \rightarrow 5CO_2+2N_2+7H_2O \quad (1)$$

Some denitrifiers are chemolithoautotrophs and use other electron donors, such as sulphide and iron, at the expense of nitrate reduction (Burgin & Hamilton, 2007; Burgin & Hamilton, 2008) e.g.:

$$5HS^- + 8NO_3^- + 3H^+ \rightarrow 5SO_4^{2-} + 4N_2 + 4H_2O \quad (2)$$

Abiotic or chemodenitrification can also occur, when nitrite reacts with reductors present in the environment. It is difficult to distinguish this process from other forms of denitrification because it largely depends on intermediates formed during nitrification and denitrification (van Cleemput, 1998). Heterotrophic denitrification is considered the dominant form of denitrification in non-sulfidic systems with high carbon loads, such as many ditches, streams and shallow lakes, (Burgin & Hamilton, 2007), while non-heterotrophic denitrification, through sulphide oxidation coupled to nitrate reduction likely occurs in ditches found in pyrite areas. In this thesis, we use the term denitrification as "heterotrophic denitrification" unless otherwise stated.

Most microorganisms preferentially denitrify under anoxic or suboxic conditions (Knowles, 1982), because aerobic oxidation of organic carbon yields more energy. However, aerobic denitrification has been found to occur as well (Robertson et al., 1995; Gao et al., 2010).

The denitrification pathway

Total denitrification from nitrate to dinitrogen gas is performed in 4 different reactions (Fig. 1.3), each catalysed by different enzymes: nitrate reductase (Nar, Nap) which reduces nitrate to nitrite, nitrite reductase (Nir), nitric oxide reductase (Nor) and nitrous oxide reductase (Nos). Nir reduces nitrite to nitric oxide. There are 2 different types of nitrite reductases: a cytochrome cd1 enzyme encoded by the *nirS* gene and a Cu-containing enzyme encoded by *nirK* (Throbäck et al., 2004).

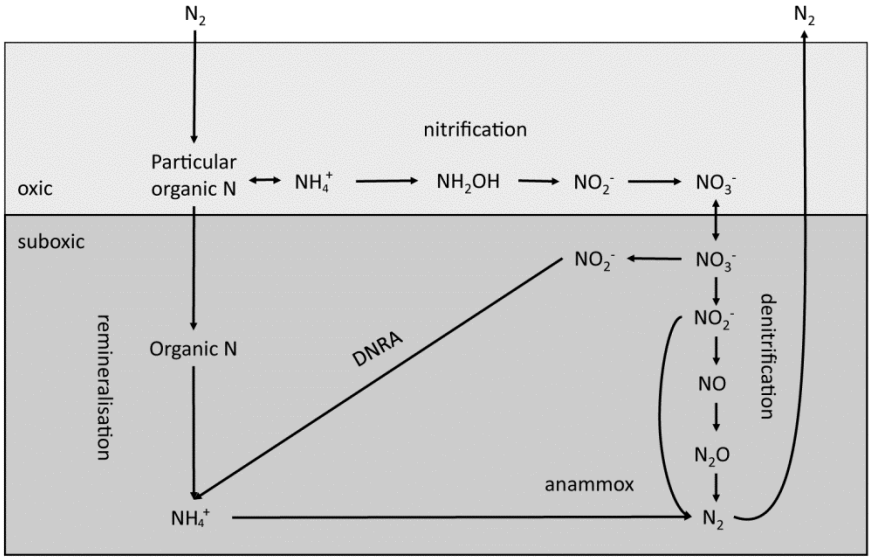

Figure 1.3. The microbial Nitrogen cycle. Modified from Francis et al. (2007).

These genes are nowadays the most commonly used biomarkers in denitrifier community studies (Braker et al., 1998; Philippot & Hallin, 2005; Wallenstein et al., 2006; Graham et al., 2010). The Nor enzyme catalyses the reduction of nitric oxide to nitrous oxide. The last step in the denitrification pathway is the reduction of nitrous oxide, which is catalysed by nitrous oxide reductase (Nos), encoded by the *nosZ* gene. Under certain conditions the last denitrification reaction is not performed, either because the denitrifiers do not possess the necessary Nos-enzyme, or because it is blocked by environmental factors. In this case, nitrous oxide is the end-product of denitrification. This is undesirable because it is a strong greenhouse gas - with a 310 times stronger warming potential than CO_2 - and involved in stratospheric ozone depletion (Seitzinger et al., 2000).

Regulating factors

Denitrification rates are regulated by the availability of substrates: nitrate and organic carbon (Knowles, 1982). Furthermore, denitrification rates are strongly regulated by oxygen availability, the process usually takes place under anoxic conditions (Nõmmik, 1956). Denitrifiers benefit from spatially and temporally heterogeneous oxygen conditions, as oxic conditions stimulate nitrification of ammonium to nitrate, which feeds the denitrification process in nitrate-limited systems. Furthermore, denitrification rates, in the same way as other enzymatic processes, increase with temperature, though literature values on the temperature-dependence of denitrification vary widely (Dawson & Murphy, 1972; Bachand & Horne, 1999; Holtan-Hartwig et al., 2002; Barnard et al., 2005). Optimal pH for denitrification is usually between 6-8, with a higher proportion of N_2O compared to N_2 as end-product at lower pH (Nõmmik, 1956; Focht, 1974; Knowles, 1982; Šimek et al., 2002).

Macrophyte presence may indirectly affect denitrification by changing concentrations of oxygen and nitrate in the sediment and water column, and by changing the quality and quantity of available organic matter (Christensen & Sørensen, 1986). Furthermore, macrophytes provide habitats for both nitrifiers and denitrifiers in the root zone and periphyton. These habitats can present heterogeneous oxygen conditions that stimulate denitrification rates through coupled nitrification-denitrification (Christensen & Sørensen, 1986; Eriksson & Weisner, 1997; Körner, 1999).

Besides instantaneous effects of oxygen, carbon, nitrate, pH and temperature on the activity of denitrifying microorganisms, these factors also regulate abundance and structure of denitrifying communities in the long term (Wallenstein et al., 2006). Abundance of denitrifiers reflects denitrification potential, although the denitrifiers present in a system are not always active (Graham et al., 2010). Predation and disturbances, like removal of organic matter, may affect both abundance and diversity of denitrifiers, resulting in altered denitrification potential

1.3 Objectives and outline of the thesis

This thesis focusses on denitrification in shallow freshwater ecosystems, such as ditches, lakes and streams. The primary objective is to identify factors controlling nitrogen removal through denitrification in these ecosystems, using experiments, field studies and modelling. A second objective is to quantify denitrification rates in ditches, streams and lakes.

First, I elucidate direct and indirect effects of temperature and oxygen on denitrification rates in laboratory experiments. I quantify effects of temperature using a microcosm set-up, and explore synergistic effects of temperature and temperature dependent oxygen concentrations (Chapter 2). In a second microcosm experiment, I explore the effect of macrophyte presence on denitrification rates. Denitrification was measured in systems with submerged macrophytes, floating macrophytes and no macrophytes, under dark as well as light conditions. In this way capturing the different effects on denitrification of plant-specific modifications of oxygen concentration through photosynthesis (Chapter 3).

Next, I study relevant controlling factors in a field experiment, combining warming and plant presence in a factorial design, in temperate and subtropical lakes. The aim of this experiment is to examine if effects of temperature and macrophyte presence are climate-dependent, and if macrophytes and warming can have synergistic effects (Chapter 4).

I quantify denitrification rates in ditches and streams in two field studies (Chapters 5 & 6). In addition to quantifying denitrification rates, I explore factors affecting denitrification, including direct factors like nitrate availability and temperature, as well as more indirect factors such as stream restoration. Furthermore, I here aim to elucidate relations between denitrification and the denitrifier community-structure and abundance, examined through the richness and abundance of the *nirK* gene.

Last, a simple energy-based model of the nitrogen cycle is described, which was created to study basic principles behind the driving factors of denitrification and other processes of the nitrogen cycle (Chapter 7).

Chapter 2

Effects of temperature and oxygen on denitrification rates

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Abstract

Background: Global warming and the alteration of the global nitrogen cycle are major anthropogenic threats to the environment. Denitrification, the biological conversion of nitrate to gaseous nitrogen, removes a substantial fraction of the nitrogen from aquatic ecosystems, and can therefore help to reduce eutrophication effects. However, potential responses of denitrification to warming are poorly understood. Although several studies have reported increased denitrification rates with rising temperature, the impact of temperature on denitrification seems to vary widely between systems.

Methodology/ Principal Findings: We explored the effects of warming on denitrification rates using microcosm experiments, field measurements and a simple model approach. Our results suggest that a three degree temperature rise will double denitrification rates. By performing experiments at fixed oxygen concentrations as well as with oxygen concentrations varying freely with temperature, we demonstrate that this strong temperature dependence of denitrification can be explained by a systematic decrease of oxygen concentrations with rising temperature. Warming decreases oxygen concentrations due to reduced solubility, and more importantly, because respiration rates rise more steeply with temperature than photosynthesis.

Conclusions/ significance: Our results show that denitrification rates in aquatic ecosystems are strongly temperature dependent, and that this is amplified by the temperature dependencies of photosynthesis and respiration. Our results illustrate the broader phenomenon that coupling of temperature dependent reactions may in some situations strongly alter overall effects of temperature on ecological processes.

Introduction

Anthropogenic activities have greatly increased reactive nitrogen inputs to aquatic ecosystems, which has led to numerous eutrophication problems such as harmful phytoplankton blooms, temporal hypoxia and fish-kills (Vitousek et al., 1997). Denitrification is the main nitrogen removing process in freshwater ecosystems, it reduces nitrate to gaseous nitrogen under anoxic conditions (Seitzinger, 1988). Effects of climate change on denitrification have been difficult to predict because of the complex of biogeochemical interactions involved (Barnard et al., 2005). Predicting these effects for aquatic ecosystems is even more difficult as data on the effects of temperature on denitrification in aquatic ecosystems are sparse (Smith, 1997). As most biochemical reactions occur at higher rates when temperature increases (Thomann & Mueller, 1987) we expect increased denitrification rates at elevated temperatures. However, the intensity of the impact of temperature on denitrification rates appears to vary widely between systems (Seitzinger, 1988; Barnard et al., 2005). In anaerobic soil slurries and batch reactors under controlled conditions (Dawson & Murphy, 1972; Holtan-Hartwig et al., 2002) denitrification shows only a moderate effect of temperature, whereas a study in constructed wetlands shows much stronger temperature effects (Bachand & Horne, 1999). This suggests that the strong temperature dependence of denitrification might arise from coupled temperature dependent processes that are excluded in the bioreactors but captured in more natural environmental settings. As denitrification is strongly affected by oxygen levels, we explored whether the direct effect of temperature on denitrification could be amplified by a temperature dependence of dissolved oxygen concentrations. Temperature affects dissolved oxygen concentration (DO) in aquatic ecosystems in different ways. Solubility of oxygen in water decreases with temperature, and high temperatures also tend to promote respiration more than photosynthesis (Allen et al., 2005), potentially implying a decrease of DO beyond the solubility effect. The resulting drop in oxygen could boost denitrification rates (Fig. 2.1). We tested this idea by analyzing field data on denitrification, temperature and DO, in combination with lab experiments assessing the effect of temperature on DO and the effect of DO on denitrification separately. In addition, we used a simple model to further explore if the temperature effect on denitrification can be realistically explained by the coupled temperature dependencies of respiration, primary production and oxygen solubility.

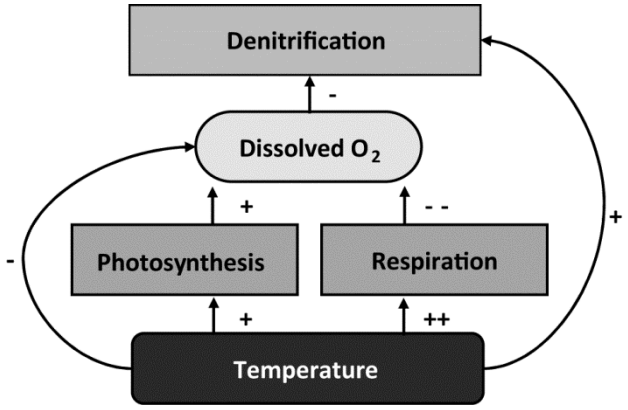

Figure 2.1 Schematic overview of the major direct and indirect effects of temperature on denitrification.

Results and Discussion

We found an exponential increase of denitrification with temperature in both the microcosms and in the field (Fig. 2.2 A, B). The overall temperature effect on denitrification could be quantified by a modified Arrhenius expression (Kadlec & Reddy, 2001):

$$D_T = D_{20}\theta_s^{(T-20)} \quad (1)$$

where D_T is the denitrification rate in µmol N m^{-2} h^{-1}, at temperature T (°C), D_{20} is the denitrification in µmol N m^{-2} h^{-1} at 20°C, and θ_s is the overall system temperature coefficient (dimensionless) (Thomann & Mueller, 1987; Kadlec & Reddy, 2001). For most biochemical reactions in this temperature range reaction rates double with a ten degree temperature increase, which corresponds to a θ_s of around 1.07 (Q_{10}=θ^{10}) (Kadlec & Reddy, 2001). We observed a stronger temperature response of denitrification, with temperature coefficients θ_s with a value of 1.24 in the microcosms (*n*=12), and 1.28 in the ditch enclosures (*n*=29). This means that a one degree temperature rise led to 24 to 28 percent higher denitrification rates. These temperature effects resemble those found in constructed wetlands (Bachand & Horne, 1999). However, they are nearly three times stronger, in terms of percent increase, than those found in controlled batch reactors and anoxic soil slurries, where temperature coefficients ranged from 1.06 to 1.13 (Dawson & Murphy, 1972; Stanford et al., 1975; Keeney et al., 1979; Holtan-Hartwig et al., 2002).

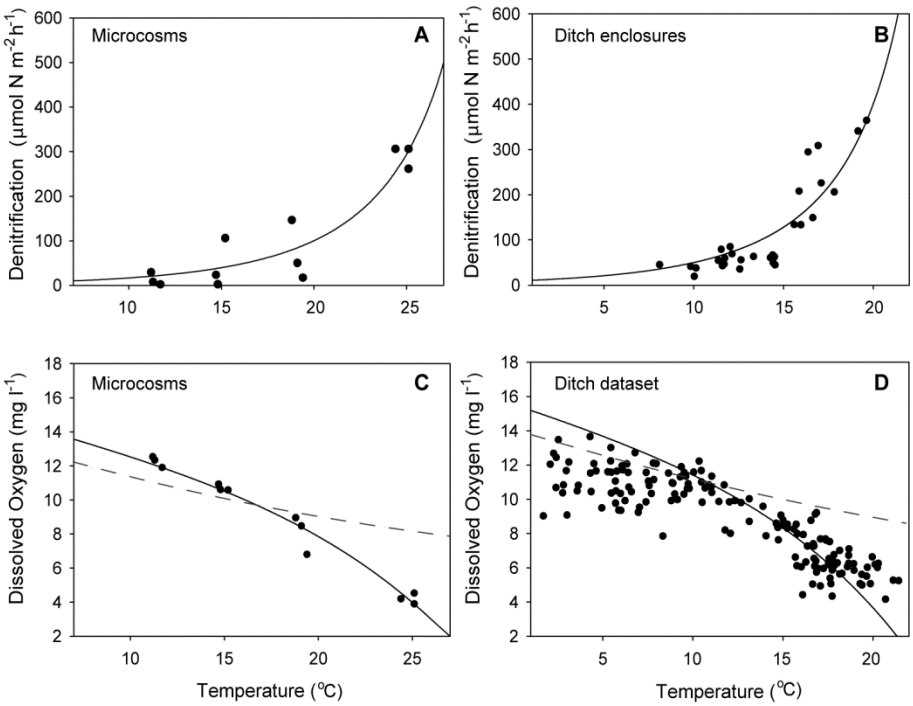

Figure 2.2. Temperature dependence of denitrification and dissolved oxygen concentrations. Panels show: Denitrification rates at different temperatures in vegetated microcosms (A) and vegetated drainage ditches (B). Dissolved oxygen concentrations at different temperatures in vegetated microcosms (C) and in drainage ditches based on monthly average values (April-July) at 3100 sites for the years 1980-2005 (D). Solid lines show the model predictions based on equation (2) (panels A and B), and equation (3) (panels C and D). Dashed lines represent the temperature dependence of the saturation concentration of oxygen (Equation 4).

Besides the effect of temperature on denitrification, this experiment also clearly showed a temperature dependence of DO (Fig. 2.2C). As predicted, oxygen levels dropped faster with rising temperatures than can be expected from reduced solubility alone. A similar temperature dependence of DO was observed in a dataset containing 100 monthly averages of DO and temperature (April-July) for all years in the period 1980-2005, based on measurements in 3100 ditches throughout the Netherlands (Fig. 2.2D). These results indicate that indeed respiration is more strongly affected by temperature than photosynthesis, as has also been found in other studies (Allen et al., 2005). The fundamentally different temperature dependencies of photosynthesis (in primary producers) and respiration (on all

trophic levels) are determined by their specific enzymatic temperature dependencies. Photosynthesis is constrained by the temperature dependence of Rubisco carboxylation in the chloroplasts (Allen et al., 2005), whereas respiration is constrained by the temperature dependence of ATP synthesis in respiratory complexes (Gillooly et al., 2001; Allen et al., 2005).

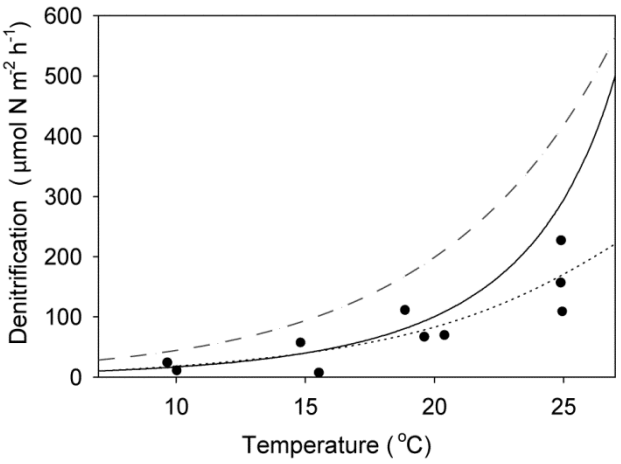

Figure 2.3. Temperature dependence of denitrification rates at constant low dissolved oxygen levels. Denitrification rates measured in vegetated freshwater microcosms, as compared to the fitted Arrhenius equation (Dotted line; Equation 1), the modelled denitrification rates (Solid line; Equations 2-4 with parameters from Table 2), and modelled denitrification with O_2 fixed at 1 mg l^{-1} (Dashed line; Equation 2).

To test if this temperature dependence of DO can explain the strong temperature dependence of denitrification we performed a second microcosm experiment in which we excluded the oxygen effect. Keeping DO constant and low (0.6-1 mg l^{-1}) we found a temperature coefficient θ_s for denitrification of only 1.15 (n=12, Fig. 2.3, Table 2.1), which was substantially lower than the temperature coefficient observed in the previous experiment in which DO was left free to vary with temperature. These results indicate that the temperature dependence of DO may indeed boost the effect of temperature on denitrification. Our simple model approach supported this finding, using parameters from the literature and our experiments (see methods), the model could reproduce the experimental data well (Fig. 2.2 A, B, Equation 2, R^2=0.86). Non-linear regression using equation (1) on the

modelled data yielded an estimated temperature coefficient θ_s of 1.30 (R^2 =0.995). By contrast, the model predicted a much lower temperature coefficient for denitrification if we mimicked a situation in which oxygen was unaffected by temperature by keeping DO at 1mg l^{-1}. The resulting temperature dependence of denitrification (θ_s = 1.16, Table 2.1) is in good agreement with the temperature coefficient of 1.15 for denitrification at fixed oxygen concentrations that we found in the corresponding experiment. Thus the model confirms that the observed effects of temperature on denitrification may reasonably be explained by correlated temperature effects on DO.

Obviously, several other biochemical reactions preceding denitrification may be directly affected by temperature. For instance, higher temperatures may promote the production of ammonium by mineralization and the conversion of ammonium to nitrate by nitrification. On the other hand, increased temperature may indirectly affect denitrification through its effect on other factors, for example by decreasing redox potential and organic carbon availability (Jenkins & Kemp, 1984; Tscherko et al., 2001; Barnard et al., 2005). In nitrate limited systems direct and indirect effects of temperature on mineralization and nitrification may play a larger role than in this study, as they provide nitrate for the denitrification process. This may work out in different ways as nitrification rates generally increase with temperature (Barnard et al., 2005), while at the same time lowered dissolved oxygen concentrations caused by increased temperature may reduce nitrification rates and thereby reduce denitrification in nitrate limited systems (Jenkins & Kemp, 1984).

Table 2.1. Overall temperature effects on denitrification (equation 1) with either temperature dependent or fixed dissolved oxygen levels. Q_{10} Estimates indicate the reaction rate increase at a 10 degree temperature rise.

Set-up	*DO*	D_{20}	*Overall* θ_s	R^2	$\approx Q_{10}$
Microcosm	T dependent	100.6	1.24	0.87	9
Field	T dependent	430	1.28	0.79	12
Microcosm	Fixed (0.6-1mg l^{-1})	81.2	1.15	0.74	4
Model	T dependent (exp.)	85.4	1.30	1.00	14
Model	T dependent (field)	370.5	1.23	1.00	8
Model	Fixed (1 mg l^{-1})	198.5	1.16	1.00	4

Table 2.2. Model parameter values.

Symbol	*Description*	*Unit*	*Value*	*Source*
D_{20max}	Denitrification at 20 degrees under anoxic conditions	µmol N m^{-2} h^{-1}	232; 645	Exp 1; Ditch enclosures
θ_D	Temperature activity coefficient denitrification	Dimensionless	1.16	Exp 1
K_s	Half saturation constant of denitrification for oxygen	mg l^{-1}	6.0	Exp 1
P	DO Production rate	g m^{-3} d^{-1}	1.94	Exp 1
R	Temperature activity coefficient photosynthesis	g m^{-3} d^{-1}	2.28; 3.26	Exp 1; Ditch dataset
θ_P	Temperature activity coefficient photosynthesis	Dimensionless	1.04	Gillooly et al., 2001; Allen et al., 2005
θ_R	Temperature activity coefficient respiration	Dimensionless	1.10	Gillooly et al., 2001; Allen et al., 2005
K_R	Re-aeration constant	d^{-1}	0.30	Spellman, 1996

The dissimilatory reduction of nitrate to ammonium (DNRA), a process that competes with denitrification, is affected by warming as well. Similar to denitrification, DNRA occurs under anoxic conditions (Burgin & Hamilton, 2007), and increases with warming (King & Nedwell, 1984; Ogilvie et al., 1997; Gruca-Rokosz et al., 2009). Thus, effects of warming on DNRA are likely also amplified by temperature effects on dissolved oxygen. Availability of organic carbon and nitrate determines whether denitrification or DNRA dominates in absolute nitrate reduction (Tiedje et al., 1982).

This illustrates the complexity of predicting the effect of warming on environmental processes. Counteracting effects may buffer overall temperature effects, while in other situations synergy between positive effects can lead to greatly amplified temperature sensitivity. Our results strongly indicate that the latter is the case for denitrification in freshwater ecosystems such as ditches and shallow lakes. The fact that such synergistic temperature effects can build up to a very steep overall temperature dependence has recently also been demonstrated in a study of newly developing ecosystems (Anderson-Teixeira et al., 2008). While freshwater ecosystems may be particularly sensitive to the effect we describe, a similar synergistic effect of temperature on denitrification has been hypothesized for terrestrial soils (Smith, 1997; Castaldi, 2000).

The overall consequences of an alteration of aquatic denitrification with warming are difficult to oversee. Increased denitrification may help to reduce eutrophication effects in shallow lakes and coastal waters. On the other hand, warming may also alter nitrogen loading through changes in mineralization, nitrogen deposition, precipitation and land-use (Jeppesen et al., 2011). Thus, although absolute denitrification rates may increase, warming does not necessarily lead to higher nitrogen removal efficiency. Importantly, greenhouse gas emissions could rise with denitrification rates. Lowered oxygen levels can affect the fraction of N_2O produced in denitrification and nitrification in various ways (Goreau et al. 1980, Focht et al. 1974, Maag & Vinther 1996) making the overall effect of temperature difficult to predict. Nonetheless, as denitrification inevitably produces the greenhouse gases N_2O and CO_2 (Seitzinger, 1988), a doubling of denitrification with a 3 degree temperature rise implies a potentially significant positive feedback on global warming (Smith, 1997).

Clearly, we are still far from understanding many aspects of the human alteration of the world's nitrogen cycle. Nonetheless, our results indicate that

denitrification in freshwater ecosystems may be particularly sensitive to warming due to the strong synergistic oxygen effects in these systems.

Materials and Methods

Microcosm setup

Two similar microcosm experiments were performed, in the first experiment oxygen concentrations were not controlled, in the second one they were kept below 1 mg l^{-1} O_2. For each experiment we set up 12 microcosms. Each microcosm contained a litre of organic sediment originating from a nearby eutrophic pond, 7 litre of Smart and Barko growth medium containing 1.3 mg N l^{-1} (as NH_4NO_3) and 0.19 mg P l^{-1}(as K_2HPO_4), and 60 gram wet weight of *Elodea nuttallii* (Planch.) St. John, which originated from an experimental drainage ditch (Sinderhoeve experimental station, Renkum, the Netherlands 51°59'55.08"N, 5°45'21.40"E). The microcosms were kept in water baths at 17.5°C at a 12/12 D/L cycle for 5 weeks before the start of the denitrification measurements to allow biofilm development. Twenty hours before the denitrification measurements we applied 4 different temperature treatments (in triplicate) to the microcosms: 10, 15, 20 and 25 °C.

In the experiment with controlled oxygen, water column dissolved oxygen levels in the microcosms were set to < 1.2 mg l^{-1} by gently bubbling the water column with helium, as previous tests showed that DO would further drop to 1.0mg l^{-1} in the 4 hours acclimatization period before the denitrification measurements. When a concentration of 1.2 mg l^{-1} was reached the microcosms were closed by an airtight disc. Denitrification measurements in these microcosms started 4 hours after setting the low oxygen levels.

Denitrification measurements.

Denitrification measurements were performed in the dark after 8 hours of darkness to prevent the production of gas bubbles due to photosynthesis which may disturb the measurements. For the denitrification measurements the microcosms were closed with airtight lids. Each lid had a screw opening for a stirrer, which gently stirred the water continuously to provide mixing of substrates, and a screw cap-opening with a septum. The lids where positioned 4 cm under the water surface.

The growth medium under the lids of the microcosms was enriched with 1.16 mg l^{-1} ^{15}N and 0.56 mg l^{-1} ^{14}N both in the form of $NaNO_3$, which was injected through the septum. We added 0.5 mg l^{-1} glucose as a source of easily oxidizable carbon to prevent carbon limitation of the denitrifying bacteria during the denitrification measurements. Water was sampled 0.25, 1, 2 and 3 hours after injection of the $^{15}N[Na\text{-}NO_3]$ solution. Samples (5ml, in triplicate) were taken through the septum using a 10 ml airtight glass syringe, after which they were injected into 12ml Exetainers (Labco, high Wycombe, UK). These exetainers contained 100 µl 50% (w:v) $ZnCl_2$ solution to stop biological processes in the samples, and were pre-flushed with helium to prevent air contamination of the samples, after which 5ml of helium was removed to create space for the water sample (Dalsgaard et al., 2000). Samples were stored at room temperature and before analysis they were vigorously shaken to transfer the dissolved N_2 into the helium headspace. Denitrification rates were calculated from accumulation of $^{14,15}N_2$ and $^{15,15}N_2$ in the headspace (Nielsen, 1992), measured at a SerCon Cryoprep trace gas concentration system interfaced to a PDZ Europa 20-20 isotope ratio mass spectrometer (Sercon Ltd., Cheshire, UK) at the UC Davis stable isotope facility (Davis, CA, USA).

Field study

A field study was performed in 3 vegetated experimental ditches (de Nieuwlanden experimental station, Wageningen, the Netherlands, 51°58'26.05"N, 5°38'35.02"E). Measurements were performed weekly between July and August 2001 and 2002, and daily for 20 days in September and October 2002. A split-box measuring device which contained three separate compartments was placed under water over the sediment in order to trap the produced dinitrogen gas. Each chamber was spiked with $^{15}N[Na\text{-}NO_3]$ to reach a concentration of 0.5 to 0.9 mg N l^{-1}. The water in the chambers was gently stirred continuously. Water was sampled (5 ml in triplicate) 0.5, 1.5 and 2.5 hours after spiking. Denitrification rates where further determined as described above.

Model approach

To further explore if the strong effect of temperature on denitrification can be realistically explained by the coupled temperature dependencies of respiration, primary production and oxygen solubility, we formulated a simple model for the

dependence of denitrification on temperature and oxygen. We assumed that the temperature effect, for the temperature range between 5 and 25 °C, can be described by a modified Arrhenius expression (Thomann & Mueller, 1987; Kadlec & Reddy, 2001) , and the effect of oxygen follows inverse Michaelis-Menten kinetics (Thomann & Mueller, 1987):

$$D_T = D_{20max}\theta_D{}^{(T-20)}\left(\frac{K_S}{K_S+DO_T}\right) \quad (2)$$

where D_T is the denitrification rate in µmol N m^{-2} h^{-1}, at temperature T(°C), D_{20max} is the denitrification at 20°C in µmol N m^{-2} h^{-1} in the absence of oxygen, θ_D is the temperature coefficient for denitrification under fixed oxygen conditions (dimensionless), K_S is the half saturation constant of denitrification for oxygen in mg l^{-1}, and DO_T is the ambient dissolved oxygen concentration in mg l^{-1} at temperature T(°C). In steady state conditions DO can be described by:

$$DO_T = C_T + \frac{\left(P\theta_p{}^{(T-20)}\right)-\left(R\theta_R{}^{(T-20)}\right)}{K_R} \quad (3)$$

where P is an overall DO-production rate (g m^{-3} d^{-1}), θ_P is the temperature coefficient of DO-production (dimensionless), R is an overall DO-consumption rate (g m^{-3} d^{-1}), θ_R is the temperature coefficient of DO-consumption (dimensionless), K_R is the re-aeration constant (d^{-1}), and C_T is the saturation concentration of DO (mg l^{-1}) at a certain temperature T(°C) (Thomann & Mueller, 1987) which is quantified as:

$$C_T = 0.003T^2 - 0.32T + 14.13 \quad (4)$$

Parameter values were taken from the literature (θ_P, θ_R and K_R) (Allen et al., 2005; Gillooly et al., 2001; Spellman 1996) and previous experiments in vegetated microcosms (K_s) (Veraart et al., 2011a). The remaining parameters (P, R, D_{20max}, θ_D) were estimated by fitting the model to the experimental and field data, as they are system specific (Table 2.2). Production (P) should be proportional to plant biomass and vary with plant productivity. The calibrated values for P correspond well to values for oxygen production found in the literature (Sorrell & Dromgoole, 1987). Respiration (R) varies with organic matter availability, which in turn depends on

long term production and respiration. For R we calibrated different values for the experimental data and for the ditch data (2.3 g m^{-3} d^{-1} and 3.3 g m^{-3} d^{-1}), which is likely due to the fact that the microcosm sediments were less organic than the ditch sediments. Still, both calibrated values are in agreement with commonly observed rates of DO-consumption (macrophyte respiration and sediment oxygen demand) in aquatic ecosystems, which range from 0.4-2 g m^{-2} d^{-1} (Graneli, 1978; van Luijn et al., 1999). The calibrated value for D_{20max} is in the range of what might be expected for vegetated drainage ditches (Piña-Ochoa & Álvarez-Cobelas, 2006).

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“Zoo heeft zich de geweldige veroveraar tenslotte nog doen kennen als een lief en nuttig waterplantje. Maar de leelijke naam ,,waterpest” heeft ’t behouden.”

Smeets & Zwegers in: “Uit de Levende Natuur – In en bij het water”, Malmberg, ’s-Hertogenbosch (ongedateerd).

Chapter 3

Effects of aquatic vegetation type on denitrification

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Abstract

In a microcosm ^{15}N enrichment experiment we tested the effect of floating vegetation (*Lemna* sp.) and submerged vegetation (*Elodea nuttallii*) on denitrification rates, and compared it to systems without macrophytes. Oxygen concentration, and thus photosynthesis, plays an important role in regulating denitrification rates and therefore the experiments were performed under dark as well as under light conditions. Denitrification rates differed widely between treatments, ranging from 2.8 to 20.9 µmol N m^{-2} h^{-1}, and were strongly affected by the type of macrophytes present. These differences may be explained by the effects of macrophytes on oxygen conditions. Highest denitrification rates were observed under a closed mat of floating macrophytes where oxygen concentrations were low. In the light, denitrification was inhibited by oxygen from photosynthesis by submerged macrophytes, and by benthic algae in the systems without macrophytes. However, in microcosms with floating vegetation there was no effect of light, as the closed mat of floating plants caused permanently dark conditions in the water column. Nitrate removal was dominated by plant uptake rather than denitrification, and did not differ between systems with submerged or floating plants.

Introduction

Nitrogen inputs to aquatic ecosystems have dramatically increased in the past decades. Excess nutrient loading has caused numerous problems in aquatic ecosystems worldwide, such as harmful phytoplankton blooms, closed mats of floating plants, hypoxia and loss of biodiversity (Smith et al., 1999; Scheffer et al., 2003). Denitrification, the reduction of nitrate to gaseous nitrogen, is an important process for permanent nitrate removal from aquatic systems (Seitzinger et al., 2006). It occurs under anoxic conditions and requires the presence of sufficient nitrate and organic carbon (Knowles, 1982). In aquatic ecosystems, denitrification mainly takes place in the sediment (Eriksson & Weisner, 1999), but it also occurs in biofilms on macrophyte surfaces (Eriksson & Weisner, 1999; Körner, 1999; Eriksson, 2001).

Macrophytes may influence denitrification rates directly and indirectly. Directly, they provide surface area for attached biofilms, where the heterogeneous oxygen conditions may favor both nitrification and denitrification (Eriksson &

Weisner, 1996; Eriksson & Weisner, 1999; Körner, 1999), although most surface area is available in the sediment itself where conditions are more suitable for denitrification. Indirectly, they affect denitrification rates by changing the nutrient concentrations by uptake and release during growth and senescence, and by influencing oxygen levels, pH and organic carbon availability in the sediment and the water column (Carpenter & Lodge, 1986; Weisner et al., 1994; Körner, 1999).

In this way, rooted submerged macrophytes may create favorable conditions for coupled nitrification-denitrification in the sediment by creating heterogeneous oxygen conditions in the rootzone, and by excreting organic carbon from their roots (Christensen & Sørensen, 1986; Reddy et al., 1989; Caffrey & Kemp, 1992; Weisner et al., 1994). On the other hand, submerged macrophytes may inhibit denitrification when photosynthesis generated oxygen levels become too high, and by competing for nitrate with denitrifying bacteria (Weisner et al., 1994; Toet et al., 2003).

There are thus both negative and positive effects of macrophytes on denitrification. A meta- analysis of 136 data-sets showed no significant difference in denitrification rates in absence or presence of macrophytes (Piña-Ochoa & Álvarez-Cobelas, 2006). However, this study was done on datasets gathered in systems with different types of vegetation, which may have different effects on denitrification, and in sites with various environmental conditions. Several direct comparisons of denitrification rates in vegetated and non-vegetated sediment patches showed positive effects of macrophytes on denitrification (Iizumi et al., 1980; Christensen & Sørensen, 1986; Caffrey & Kemp, 1992). However, the effect of the type of vegetation, either floating or submerged, on denitrification is still unclear. Studying the effects of these vegetation types on denitrification is particularly interesting as many shallow waterbodies such as ditches and ponds are dominated by either submerged or floating vegetation, depending on nutrient loading (Janse & Van Puijenbroek, 1998; Scheffer et al., 2003).

In this study we compared the effect of floating macrophytes (*Lemna* sp.) and submerged macrophytes (*Elodea nuttallii* (Planch.) H. St John) on total denitrification rates in a microcosm experiment. We included microcosms without macrophytes to study effects of macrophyte presence. To explore the effect of photosynthesis-driven variation in oxygen levels, we performed the experiments both under light and dark conditions.

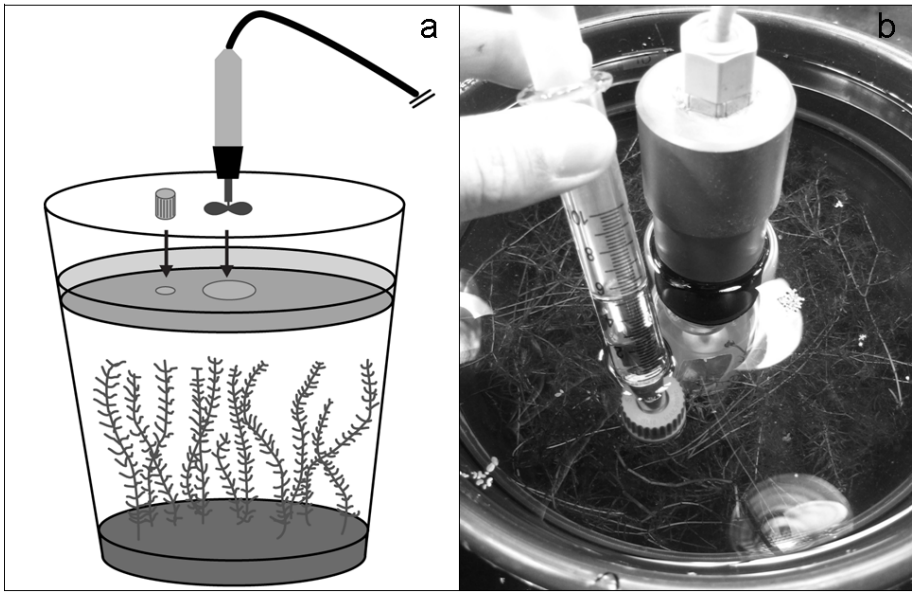

Fig. 3.1. Schematic overview (a) and picture (b) of a microcosm containing *Elodea nuttallii*.

Methods

Experimental setup

Eighteen microcosms (8l v., 20cm Ø, 30cm h) were used, in which we introduced a 2 cm thick layer of sediment (330ml) and 7 liter of Smart and Barko macrophyte growth medium with 1mg N l^{-1} and 0.19 mg P l^{-1} (Smart & Barko, 1985; van Liere et al., 2007). The sediment contained 7% organic matter and originated from a nearby eutrophic pond. We applied 3 treatments to the microcosms with 6 replicates each: they were either covered completely by the floating macrophyte duckweed (*Lemna sp.*) (corresponding to ca. 5.3 gram dry weight), filled with 70 gram wet weight of the submerged macrophyte western waterweed (*Elodea nuttallii* (Planch.) St. John) (corresponding to 4.5 gram dry weight), or kept without vegetation. We chose these species as they are representative types of floating and submerged vegetation in mesotrophic and eutrophic ditches and lakes, and dominate many waterbodies in the Netherlands. Waterweed was collected in a nearby mesotrophic

artificial drainage ditch (Sinderhoeve Experimental Station, Renkum, the Netherlands 51°59'55.08"N, 5°45'21.40"E). Duckweed was collected in a nearby eutrophic drainage ditch (Wageningen, the Netherlands 51°59'14.29"N, 5°45'21.40"E). We kept all macrophytes on Smart and Barko growth medium at 20 °C for 4 weeks, and removed all visible snails before placing them into the microcosms.

The microcosms were incubated for 4 weeks in a water bath at 20.5 ± 0.9 sd °C at a 12h/12h light/ dark cycle to ensure biofilm development. After the incubation period denitrification was measured. We measured denitrification in 3 microcosms of each treatment during light conditions after a light period of 4 h, and in the remaining 3 under dark conditions after a dark period of 8 h. The experiment was run twice leading to a total of 36 measurements.

Water quality analysis

Dissolved O_2 (DO), pH, temperature and electric conductivity were measured with a HQ multiprobe with a luminescent dissolved oxygen sensor (Hach Company, Loveland, Colorado, USA) directly before the denitrification measurements. We took 3 water samples from each microcosm directly before and after the denitrification measurements. Two samples were filtered immediately with a Whatman 0.45µm cellulose membrane filter (Whatman International Ltd, Maidstone, England) and then frozen until analysis. The other one was left unfiltered. Filtrated samples were analysed colorimetrically for NO_3^-+ NO_2^-, NH_4^+ and ortho-PO_4^{3-}, using a SAN^{plus} autoanalyzer (Skalar Analytical, Breda, the Netherlands). Nitrate and nitrite (hereafter: nitrate) were determined by the sulfanilamide/ naphthylethylene-diamine dihydrochloride method with cadmium reduction (Green et al., 1982), ammonium by the indophenolblue method (Bietz, 1974), and ortho-phosphate using the ammonium-molybdate method (Murphy & Riley, 1962). Chlorophyll-*a* was determined by Pulse Amplitude Modulation fluorometry (Phyto-PAM) in the unfiltered samples immediately after collection, as described by Lürling & Verschoor (2003).

Light irradiance at the sediment surface in the microcosms was measured before the denitrification measurements with a subsurface light intensity meter (LI-COR, Lincoln, NE, USA). Water losses due to evaporation and sampling were compensated for by adding deionized water.

Denitrification measurements

For the denitrification measurements the microcosms were closed with airtight lids. Each lid had a screw opening for a stirrer and a screwcap-opening with a septum. The lids were positioned 4 cm under the water surface, gently pushing down the macrophytes (Fig. 3.1). The growth medium under the lids of the microcosms was enriched with 1.07 mg l^{-1} ^{15}N by injecting 0.5 mmol $^{15}N[Na\text{-}NO_3]$ (98 atom %) through the septum. We added 0.5 mg l^{-1} glucose as a source of easily oxidisable carbon to prevent carbon limitation of the denitrifying bacteria during the denitrification measurements. Water was sampled 0.25, 1, 2 and 3 hours after injection of the $^{15}N[Na\text{-}NO_3]$ solution. Water samples (5ml, in triplicate) were taken through the septum using a 5ml airtight glass syringe, and were injected into 12ml Exetainers (Labco, High Wycombe, UK). Exetainers contained 100µl 50% (w:v) $ZnCl_2$ solution to stop biological processes in the samples, and were pre-flushed with helium to prevent air contamination, after which 5ml of helium was removed to create space for the water sample (Dalsgaard et al., 2000). Samples were stored at room temperature and before analysis they were vigorously shaken to transfer the dissolved N_2 into the helium headspace. Dinitrogen concentrations and ratios of $^{14,15}N_2$ and $^{15,15}N_2$ over $^{14,14}N_2$ were measured using a SerCon Cryoprep trace gas concentration system interfaced to a PDZ Europa 20-20 isotope ratio mass spectrometer (Sercon Ltd., Cheshire, UK) at the UC Davis stable isotope facility (Davis, CA, USA). We calculated denitrification rates from the change in ratios of $^{14,15}N_2$/ $^{14,14}N_2$ and $^{15,15}N_2$/$^{14,14}N_2$ in time, following Nielsen (1992).

Nitrate removal

Nitrate removal rates R_T (mg N l^{-1} h^{-1}) were calculated from the difference between the nitrate concentration in the microcosm before the denitrification measurements ($N_{t=0}$) and after the denitrification measurements ($N_{t=t}$) using equation 1:

$$R_T = \frac{N_{t=0} - N_{t=t}}{t} \quad (1)$$

Where t is the duration of the denitrification measurements (h). Nitrate removed from the microcosm by denitrification (R_D, mg N l^{-1} h^{-1}) was calculated as:

$$R_D = \frac{D \cdot M \cdot A \cdot t}{V} \quad (2)$$

Where D is the denitrification rate (mmol N m^{-2} h^{-1}), M is the molar mass of nitrogen (g), A is the microcosm area (m^2), t is the duration of the experiment (h) and V is the microcosm volume (l). Percentage of N removed by denitrification ($\%D$) was calculated as:

$$\%D = \frac{R_D}{R_T} \cdot 100 \quad (3)$$

Data analysis

Data of the two experimental runs were combined because their results were not significantly different (independent samples t-test: $t_{21.405}$=-0.457 P=0.652). If necessary, data were ln(x+1) transformed to achieve homogeneity of variances. Hierarchical nested ANOVA was used to test for effects of vegetation treatment (fixed factor) and light nested within vegetation treatment (random factor) on denitrification rates, nitrate removal, and DO. Tukey HSD post-hoc tests were used to test for differences among the individual vegetation treatments. One-way ANOVA was used to test for differences in nutrient concentrations between the three vegetation treatments before the denitrification measurements. Stepwise multiple linear regression was used to test which factors were most important in influencing denitrification rates.

Table 3.1. Physical and chemical variables in microcosms with 3 different vegetation types during dark and light conditions measured before (t0) and after (t3) 3 hour denitrification measurements. Values given for NO_3^-+NO_2^- t0 are after addition of 1.07 mg N l^{-1} $^{15}N[NaNO_3]$. Values are given as: mean (standard error) *n*=6.

		Vegetation type					
		No Macrophytes		*Floating*		*Submerged*	
		dark	*light*	*dark*	*light*	*dark*	*light*
NO_3^- t0	(mg N l^{-1})	1.08 (0.00)	1.08 (0.00)	1.08 (0.00)	1.08 (0.00)	1.16 (0.08)	1.08 (0.00)
NO_3^- t3	(mg N l^{-1})	1.04 (0.02)	0.91 (0.08)	0.82 (0.09)	0.74 (0.02)	0.70 (0.16)	0.77 (0.08)
NH_4^+ t0	(mg N l^{-1})	0.00 (0.00)	0.00 (0.00)	0.01 (0.00)	0.00 (0.00)	0.01 (0.00)	0.02 (0.02)
NH_4^+ t3	(mg N l^{-1})	0.02 (0.01)	0.01 (0.01)	0.02 (0.01)	0.01 (0.00)	0.02 (0.00)	0.01 (0.00)
pH		9.22(0.15)	9.12(0.13)	6.80(0.15)	6.84(0.08)	9.22(0.15)	9.42(0.15)
chl-a green algae	(µg l^{-1})	5.13 (1.13)	5.54 (3.38)	6.89 (1.04)	7.98 (3.03)	8.33 (5.29)	4.88 (1.40)
Light at sediment*	(µmol phot. m^{-2} s^{-1})		80		0.5		25

* *n*=1

Results

Conditions in the microcosms

Floating macrophyte cover greatly reduced light irradiance in the microcosms, leading to near dark conditions at the sediment surface (Table 3.1). Presence of submerged macrophytes also reduced light irradiance in the microcosms, though some light still reached the sediment surface. We observed some periphyton and planktonic algal growth in all microcosms (Table 3.1).

Nitrate and ammonium were depleted after the 4 week incubation period. After addition of $^{15}N[NaNO_3]$ at the start of the denitrification measurements nitrate levels in all treatments were around 1.1 mg N l^{-1}. Ammonium concentrations were below the detection limit before and after denitrification measurements (Table 3.1).

The pH was significantly lower in microcosms covered by floating vegetation than in microcosms without macrophytes (Tables 3.1 & 3.2, Tukey post-hoc test $P<0.001$) or with submerged macrophytes (Tukey post-hoc test $P<0.001$).

Denitrification rates

Denitrification rates differed significantly between the treatments (Fig 3.2A, Table 3.2). Denitrification rates in microcosms covered by floating plants were 3.7 times higher than in microcosms without macrophytes (Tukey post-hoc test $P<0.001$) and 3.2 times higher than in microcosms with submerged macrophytes (Tukey post-hoc test $P<0.001$).

Light tended to have an effect on the denitrification rates within the different vegetation treatments, although this was only significant at the $P<0.1$ level, probably due to the different effects of light for the different treatments. There was no difference in denitrification rates under dark or light conditions in systems covered by floating plants. However, we found 1.8 times more denitrification under dark conditions than under light conditions in systems without macrophytes and 3.5 times more denitrification under dark conditions than under light conditions in systems with submerged macrophytes.

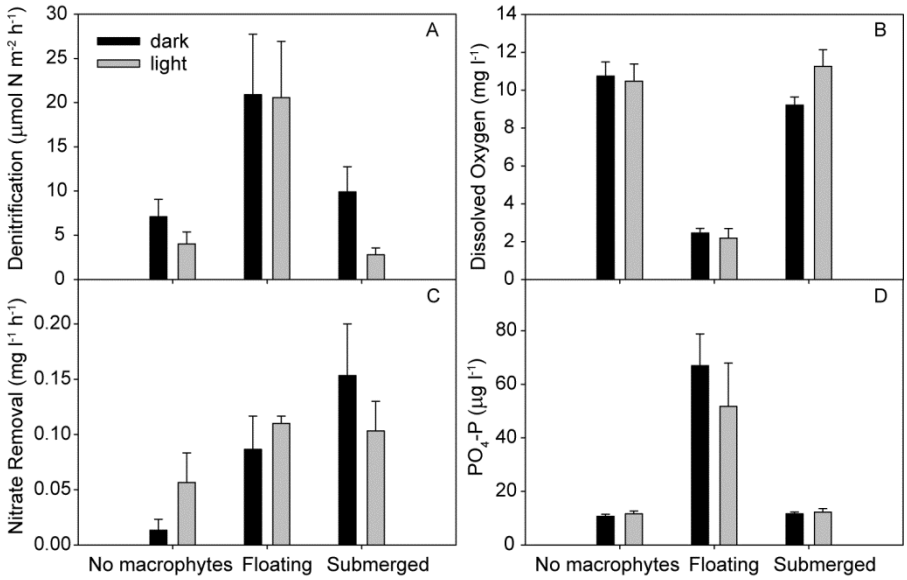

Fig. 3.2. Total denitrification rates (A), water column dissolved oxygen levels (DO) (B), NO_3-removed from the water column during the 3h denitrification measurement (C) and PO_4-P concentrations at the start of denitrification measurements (D) (mean ± se, *n*=6) in microcosms without macrophytes, covered by floating macrophytes (*Lemna minor*) or filled with submerged macrophytes (*Elodea nuttallii*). Black bars show measurements performed under dark conditions, starting after 8 hours of darkness. Grey bars show measurements performed under light conditions, starting after 4 hours of light.

Table 3.2. Hierarchical nested ANOVA results comparing different vegetation treatment effects and effects of light nested within the vegetation treatment on denitrification rate, dissolved oxygen, pH and NO_3-N removal.

	Vegetation				*Light in vegetation*			
	df	MS	*F*	*P*	*df*	MS	*F*	*P*
Denitrification rate	3	55.48	46.29	0.005	3	1.20	2.48	0.08
Dissolved Oxygen	3	52.35	1085.97	<0.001	3	0.05	0.94	0.719
pH	3	869.50	16827.20	<0.001	3	0.23	1.01	0.433
NO_3-N removal	3	0.67	28.34	0.011	3	0.02	1.01	0.401

Dissolved oxygen

DO in the water column differed significantly between the treatments (Fig 3.2B, Table 3.2). Multiple regression analysis including DO, pH, temperature, plant dry weight, plant surface area and chlorophyll-*a* as explanatory variables showed that DO was the most important factor explaining denitrification rates (adjusted R^2 = 0.356). DO in microcosms covered by floating vegetation was significantly lower than in microcosms without macrophytes (Tukey post-hoc test $P<0.001$) or with submerged macrophytes (Tukey post-hoc test $P<0.001$), whereas DO in microcosms without macrophytes and in microcosms with submerged macrophytes was similar. Effect of light on DO within the treatments was not significant (Table 3.2).

Nitrate removal and phosphate release

The NO_3-N removed from the water column during the 3-hour denitrification measurements differed significantly between the treatments (Fig 3.2C, Table 3.2). Nitrate removal in microcosms without macrophytes was lower than nitrate removal in microcosms with floating macrophytes (Tukey post-hoc test $P=0.035$) or submerged macrophytes (Tukey post-hoc test $P=0.004$). There were no significant differences between the nitrate removal in microcosms with floating vegetation and microcosms with submerged macrophytes. Effects of light on NO_3-N removal within the treatments were not significant (Table 3.2). Only about 6% of the total nitrate removal could be attributed to the measured denitrification.

After the 4 week incubation period PO_4-P concentrations in the water column differed significantly between the treatments (One-way ANOVA: $F_2=49.958$ $P<0.001$). Microcosms covered by floating vegetation had highest PO_4-P concentrations, whereas those without macrophytes had the lowest PO_4-P concentrations (Fig. 3.2D).

Discussion

Denitrification rates were affected by the presence of macrophytes as well as the type of macrophytes. These effects differed depending on the light conditions. In the dark differences between microcosms were small. However, in the light denitrification in our systems with submerged macrophytes and in those without macrophytes was lower than in the duckweed covered systems. This was likely due to the permanently dark conditions under the duckweed, which inhibited oxygen production by photosynthesis. The floating plant cover also provides a barrier to re-aeration (Morris & Barker, 1977).

Our results thus suggest that oxygen production by photosynthesis of microalgae and plants inhibited denitrification in the top layer of the sediment and in biofilms on the macrophyte surface. Such oxygen mediated inhibition of denitrification rates under light conditions was also found in other studies (Christensen & Sørensen, 1986; Nielsen et al., 1990b; Sündback & Miles, 2002).

By contrast, several studies in nitrate-limited systems have found positive effects of illumination on denitrification due to coupled nitrification-denitrification (Laursen & Seitzinger, 2004). If nitrate availability is limited, denitrification rates may be largely dependent on the production of nitrate during nitrification. As nitrification requires oxygen, it is enhanced by photosynthesis (Risgaard-Petersen et al., 1994; Eriksson & Weisner, 1999). In our study, coupled nitrification-denitrification is less important because sufficient nitrate was available and ammonium concentrations were low.

Despite the different denitrification rates, overall nitrate removal rates observed during the experiment were similar for all treatments in the light. Denitrification only accounted for ca. 6% of the nitrate removal. Most of it was probably removed by plant uptake. Both *E. nuttallii* and *L. minor* are capable of rapid nitrate uptake from the water column (Ozimek et al., 1993; Cedergreen & Madsen, 2002). An uptake rate of 0.1 mg N g^{-1} plant dry weight h^{-1} (Cedergreen & Madsen, 2002) would imply that 77% of nitrate removal in our experiments could be attributed to uptake by plants. In the microcosms without macrophytes, nitrate assimilation by algae may explain part of the nutrient removal during the light period.

It is possible that dissimilatory nitrate reduction to ammonium (DNRA) may have removed some of the nitrate from our systems too. However, DNRA uses

more carbon per nitrate than denitrification and will therefore only be favored over denitrification when nitrate becomes limiting (Burgin & Hamilton, 2007), which did not occur in our systems. It is therefore likely that denitrification was the dominant pathway for N-reduction in our systems.

Denitrification rates compared to natural systems

Our microcosm setup allowed us to isolate the effects that different functional groups of macrophytes may have on denitrification rates. Rather than distinguishing between denitrification in the water column, biofilm and sediment, we considered effects of macrophytes on the whole system, as effects of macrophytes in the water column may also influence sediment biogeochemical processes. Nonetheless, there are of course profound differences between our microcosms and many natural systems. For example, natural systems have deeper sediments. Therefore, sediment denitrification may play a larger role than in our microcosm experiment. Importantly, if sufficient nitrate is available, denitrification may continue in deeper layers of the sediment even if the upper sediment layers are oxygenated. Also, the degree to which oxygen produced in photosynthesis affects denitrification rates varies with factors such as plant density, respiration rates, and bioturbation (Mermillod-Blondin et al., 2008).

Although denitrification rates in our microcosms were similar to those found in littoral plant covered sediments and mesotrophic lakes (Christensen & Sørensen, 1986; Seitzinger, 1988), rates observed in agricultural ditches and streams are an order of magnitude higher (de Klein, 2008; Smith et al., 2009). The high denitrification in such systems could be due to the fact that they receive very high nutrient loads (Janse & Van Puijenbroek, 1998) and also tend to contain large quantities of organic matter (Smolders et al., 2006; Needelman et al., 2007).

Phosphorus release

Obviously, from a practical nutrient management perspective it is important to consider effects of macrophytes not only on nitrogen but also on phosphorus. Although the low dissolved oxygen concentration under the floating vegetation stimulated denitrification rates, it also reduced the P-binding capacity of the sediment, which led to increased water column phosphorus concentrations. In natural systems this may lead to eutrophication of connected waterbodies.

Furthermore, the combined nitrogen removal and phosphorus release may alter the systems chemical stoichiometry, which may affect ecosystem functioning in various ways (Sterner & Elsner, 2002).

Our findings illustrate the strong interaction between biota and chemistry in aquatic systems. While increased nutrient loads are a major driver of aquatic vegetation presence and type (Scheffer, 1998), our results show that such an alteration of vegetation in turn has profound effects on nutrient dynamics.

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Chapter 4

Influence of warming and macrophyte presence on denitrification in temperate and subtropical shallow lakes

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Abstract

Denitrification is a major nitrogen removing process in freshwater ecosystems. Both warming and macrophyte presence can considerably affect denitrification rates in temperate lakes. However, it is not known if the same relationships hold in subtropical lakes, where process rates may be resource rather than temperature limited. In this study, we tested the response of denitrification to warming and submerged macrophyte presence in six temperate and four subtropical lakes. Denitrification was measured *in situ* using the ^{15}N isotope pairing technique in chambers under ambient and warmed conditions.

Denitrification rates ranged from <0.1 to 114 µmol N m^{-2} h^{-1} in the temperate lakes and from <0.1 to 4 µmol N m^{-2} h^{-1} in the subtropical lakes. Warming did not affect denitrification in either of the climatic regions. Denitrification decreased with water column dissolved oxygen and sediment oxygen demand in the temperate lakes, but was not related to macrophyte presence. However, in the subtropical lakes, denitrification increased with macrophyte biomass and organic matter content of the sediment. Patches with macrophytes had twice as high denitrification rates as patches without macrophytes, likely due to the higher organic matter content of the macrophyte covered sediments in these lakes. Our results indicate the importance of macrophytes for the biogeochemistry of subtropical shallow lakes, and furthermore show that resource limitation may mask effects of temperature on denitrification,

Introduction

Global warming and the alteration of the global nitrogen cycle are two major anthropogenic threats to the stability of ecosystems worldwide. Yearly, human activities convert around 120 million tons of atmospheric nitrogen into more reactive forms of nitrogen, a large proportion of which ends up in groundwater, rivers and lakes (Rockström et al., 2009). This has contributed to eutrophication effects, such as harmful phytoplankton blooms, hypoxia and fish-kills (Galloway, 1998; Bergström & Jansson, 2006). Global change scenarios predict a substantial increase in nitrogen inputs to surface waters in the next decades, both directly from anthropogenic sources and indirectly due to the increased deposition of NH_x and NO_x (Dentener et al., 2006), which may lead to further deterioration of water quality (Bergström & Jansson, 2006). In addition, the expected increase in global temperature is expected to amplify eutrophication effects, as warming increases nutrient recycling (Genkai-Kato, 2005; Jensen et al., 1992; Moss et al., 2011)

Denitrification, the microbial reduction of nitrate to nitrogen gas, is a major nitrogen removing process in freshwater ecosystems (Seitzinger et al., 2006). Rates of denitrification are determined by availability of nitrate and organic carbon, oxygen, temperature and presence of macrophytes (Knowles, 1982), which are all affected by global change (IPCC, 2007; Mooij et al., 2007; Kosten et al., 2010). Denitrification, in the same way as other enzymatic processes, exponentially increases with temperature, but most studies on the effects of global change on denitrification in aquatic environments have been performed in areas with a temperate climate (Bachand & Horne, 1999; Kadlec & Reddy, 2001; Veraart et al., 2011b). Extrapolating these findings to (sub)tropical lakes could lead to year-round higher denitrification rates in these lakes, supporting the classic hypothesis that tropical and subtropical lakes are nitrogen rather than phosphorus limited (Lewis, 1996; Downing et al., 1999). However, several recent studies found no evidence for higher N-limitation (Huszar et al., 2006; Elser et al., 2007; Kosten et al., 2009a), or higher denitrification rates (Kosten *et al.*, 2009a, Piña-Ochoa & Álvarez-Cobelas, 2006) in the tropics. Furthermore, denitrification studies in subtropical and tropical lakes are scarce (Piña-Ochoa & Álvarez-Cobelas, 2006).

Besides the hypothesized difference in denitrification rates between temperate and subtropical lakes, there may also be a difference in the mechanisms involved in how denitrification in these lakes responds to a rise in temperature.

Warming can affect denitrification rates by several different mechanisms, some having instant effects, others having effects in the long term. A short term effect of warming is the stimulation of denitrifier enzyme activity, both as a direct effect of warming and as an indirect effect of the lowered oxygen concentrations with warming (Knowles, 1982; Veraart et al., 2011b). Effects in the longer term may include changes in the denitrifier community composition (Wallenstein et al., 2006), and availability of organic carbon and nitrate due to increased mineralization (Kosten et al., 2010). Denitrification rates in subtropical lakes may show only a moderate response to warming, as process rates in these lakes are likely restrained by resource availability rather than temperature (Lewis, 1996).

Another factor that can be affected by global change is the presence of submerged macrophytes in shallow lakes. A recent study shows that the critical P and N load at which lakes shift from a clear, macrophyte dominated, system to a turbid, phytoplankton dominated, system is lower for warm than for cold lakes (Kosten et al., 2009b), which suggests that likeliness of macrophyte dominance decreases with warming. The predicted change in macrophyte presence may have an impact on denitrification rates, as macrophytes modify the biogeochemistry of aquatic systems (Carpenter & Lodge, 1986). Macrophytes may reduce local denitrification rates by increasing oxygen concentrations in the water column and oxygen penetration into the sediment (Christensen & Sørensen, 1986; Nielsen et al., 1990a), but may also stimulate denitrification due to increased nitrate concentrations produced in the aerobic nitrification process (Eriksson & Weisner, 1999), or by increasing organic carbon availability (Golterman, 2004). Several studies found positive effects of macrophyte presence on denitrification (Christensen & Sørensen, 1986; Caffrey & Kemp, 1992), but these effects appear to be site-specific; local conditions as well as the type of macrophytes determine whether macrophytes have stimulating or suppressing effects on denitrification (Piña-Ochoa & Álvarez-Cobelas, 2006; Veraart et al., 2011a).

In this study, the effect of warming and macrophyte presence on denitrification rates was tested using short-term warming experiments, in six temperate and four subtropical lakes. Specifically, we tested if denitrification responds differently to warming, macrophyte presence and their interaction in the different climatic regions. We hypothesize that denitrification is favored by warming as well as macrophyte presence, but responds more strongly to warming in temperate than subtropical systems, as denitrifying communities in subtropical systems are likely limited by resources rather than temperature. Furthermore we

expect synergistic effects of macrophytes and warming in both systems, as macrophytes create favorable conditions for denitrification.

Methods

Setup and study sites

We measured the effect of warming and macrophyte presence on denitrification rates in six temperate and four subtropical lakes. The temperate lakes were situated in the Netherlands, the subtropical lakes in Uruguay. In the Netherlands, monthly average air temperatures range from 2°C in January to 17°C in July. In Uruguay, monthly average air temperatures range from 11°C in July to 23°C in January. All lakes were relatively small and shallow (Table 4.1). All Uruguayan lakes had sandy sediments, whereas in the Netherlands two lakes had sandy sediments and four lakes had organic sediments. In each lake we performed denitrification measurements under ambient and warmed circumstances (3°C above ambient temperature), both with and without macrophytes, resulting in four different conditions. We performed these measurements at two different sites in the littoral zone, leading to two replicates of each condition in each lake. Each lake was sampled once, subtropical lakes December 2008, temperate lakes August 2009, these months were chosen to minimize the temperature difference between the climatic zones in order to compare effects of warming at similar temperatures.

Warmed chamber method

Denitrification was measured using the ^{15}N isotope pairing technique in *in situ* measurement chambers. Benthic chamber measurements allow to study the systems response to environmental changes under field conditions, and were found to give similar results as laboratory batch-mode assays (Nielsen & Glud, 1996; Mengis et al., 1997). To compare denitrification rates at ambient temperatures and at 3°C above ambient, we designed cylindrical denitrification chambers that could be warmed. These chambers were 25 cm wide and 30 cm high. They were open at the bottom and had a removable airtight lid. The ambient chambers had a single stainless steel wall, whereas the warmed chambers had an extra outer wall made of insulating PVC.

Table 4.1. Location, climatic region and area of the studied lakes.

Lake nr	*Name*	*Climate*	*Latitude*	*Longitude*	*Area(ha)*	*Sampling date*
T1	Forumvijver	Temperate	51°59'03.86"N	5°39'53.09"E	0.4	20-08-'09
T2	Naardermeer-zuid	Temperate	52°16'59.46"N	5°07'43.22"E	28.7	21-08-'09
T3	Zwanewater	Temperate	51°56'14.00"N	5°57'02.96"E	24.9	24-08-'09
T4	Groene Heuvels	Temperate	51°50'48.73"N	5°41'19.95"E	21.0	25-08-'09
T5	Immerlooplas	Temperate	51°57'20.57"N	5°55'17.95"E	20.3	26-08-'09
T6	Donderven	Temperate	51°46'30.34"N	5°48'38.50"E	0.2	27-08-'09
S1	Blanca	Subtropical	34°54'01.98"S	54°50'12.71"W	54.0	03-12-'08
S2	del Diario	Subtropical	34°54'13.17"S	55°00'27.26"W	49.9	04-12-'08
S3	Clotilde	Subtropical	34°17'40.64"S	53°48'12.02"W	16.3	05-12-'08
S4	Javier	Subtropical	34°52'00.62"S	56°02'41.34"W	5.0	08-12-'08

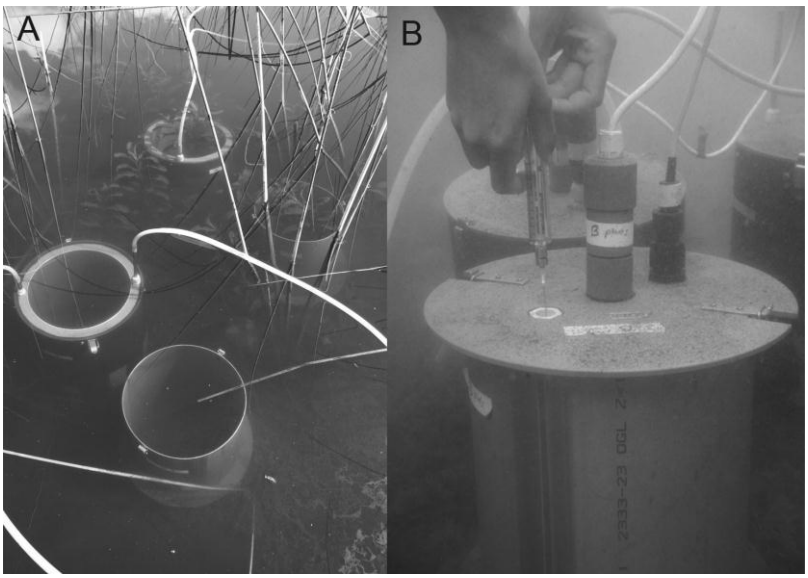

Figure 4.1. Denitrification chamber setup. A) Warmed double walled chambers and 'ambient' single walled chambers before placing the airtight lids. The figure also shows the plastic tubes through which hot water was added to maintain the 3 °C above ambient temperature. B) 'Ambient' chamber during the denitrification experiment, showing the stirrer and DO temperature probe attached to the lid, as well as the septum through which the denitrification sample was taken.

The reservoir between the PVC and steel wall could be filled with hot water. They were placed under water without the lid and inserted 5cm into the sediment, either on bare sediment or over patches of macrophytes, to create the two different vegetation conditions (Fig. 4.1A). Subsequently, the chambers were closed with the airtight lids to trap N_2 produced during denitrification (Fig. 4.1B). After closing them, the chambers were continuously stirred by a mechanical stirrer in the lid. Temperature was continuously monitored during the denitrification measurements using temperature probes inside the chambers. Hot water was added to the reservoir of the warmed chambers whenever necessary to maintain a constant 3°C difference between the ambient and warmed chambers (Fig. 4.1A). In a pilot experiment we found that a 3°C temperature rise in the water of the chamber corresponded with a 2.0°C temperature rise in the sediment at 1 cm depth (t_{10}=-6.723, P<0.001) and a 1.2°C temperature rise at 5 cm depth (t_{10}=-6.690, P<0.001).

Denitrification measurements

Denitrification measurements were started about 1.5 h after chamber deployment, when the ambient and warmed chambers had reached a stable 3 degree temperature difference for at least 30min (average difference was 2.9 ± 0.6 sd °C). The water in the chambers was enriched with 1 mg l^{-1} ^{15}N by injecting $^{15}N[Na\text{-}NO_3]$ (99 atom %) through a septum in the lid. Background $^{14}NO_3$ concentrations ranged from 0.00 to 0.08mg N l^{-1}, resulting in 91-99% ^{15}N in the chambers. Water was sampled (5ml, in triplicate) through the septum 0.25, 1, 2 and 3 hours after injection of the ^{15}N solution using a 10 ml airtight glass syringe. Because sampling removed less than 1% of the chamber volume, we did not replace the volume of the water sample to the chamber. Water samples were injected into helium flushed 12ml Exetainers (Labco, high Wycombe, UK) resulting in 5ml water samples and a 7ml helium headspace. The exetainers contained 100µl 50% (w:v) $ZnCl_2$ solution to stop biological processes in the samples (Dalsgaard et al., 2000). All samples were stored at room temperature. In the laboratory, samples were vigorously shaken to transfer the dissolved N_2 into the helium headspace.

Then, dinitrogen concentrations and ratios of $^{14,15}N_2$ and $^{15,15}N_2$ over $^{14,14}N_2$ were measured using a SerCon Cryoprep trace gas concentration system, which was interfaced to a PDZ Europa 20-20 isotope ratio mass spectrometer (Sercon Ltd., Cheshire, UK) at the UC Davis stable isotope facility (Davis, CA, USA). We calculated denitrification rates per chamber from the change in ratios of $^{14,15}N_2/^{14,14}N_2$ and $^{15,15}N_2/^{14,14}N_2$ in time, using ^{15}N natural abundance in air as standard. We averaged the triplicate samples taken at every time step (Nielsen, 1992; Steingruber et al., 2001).

Water analysis

We measured profiles at 10cm depth intervals of dissolved oxygen, temperature, electric conductivity and pH in the water column of the lakes using an HQ multiprobe with a luminescent dissolved oxygen sensor (Hach Company, Loveland, Colorado, USA). Measurements were performed twice in each lake; once in the morning and once in the afternoon. Measurements were performed within 3 meters from the chambers, water samples were taken at the same location but only in the morning.

We measured NO_3^-+ NO_2^-, NH_4^+, ortho-PO_4^{3-} and Dissolved Organic Carbon (DOC) in filtered (Cellulose nitrate, 0.45 µm, Whatmann) lake water samples and total nitrogen (TN) and total phosphorus (TP) in unfiltered samples. Nutrient concentrations (NO_3^-+ NO_2^-, NH_4^+, ortho-PO_4^{3-}, TN and TP) were measured colorimetrically using a SAN^{plus} autoanalyzer (Skalar Analytical, Breda, the Netherlands) as described by Veraart et al. (2011a) and references therein. DOC was measured using a Total Organic Carbon Analyzer (Model 700, O.I. International, College Station, TX, USA). Acid binding capacity was determined by titration with 0.05N HCl of unfiltered samples directly after sampling.

Phytoplankton, periphyton and macrophyte analysis

Chlorophyll-*a* concentrations in the water column were determined by hot ethanol extraction from GF/C filters (Nusch, 1980). After all analyses macrophytes were harvested from each chamber. They were identified, hand-centrifuged for 1 minute and the total macrophyte wet weight was determined for each chamber. Periphyton biomass on macrophytes in the denitrification chambers was determined as described by Eriksson & Weisner (1996).

Sediment analysis

After the denitrification measurements, we opened the chambers and took triplicate samples of the top 3cm of sediment from each denitrification chamber using a Kajak corer. We determined organic matter percentage from the loss on ignition at 550°C for 3h, as described by Kosten *et al*. (2009a). Water soluble organic carbon from the sediment (WSOC) was measured as described by Burford & Bremner (1975), but with the modification that after the shaking and filtration steps, the concentration of dissolved organic carbon in the water was measured on a total organic carbon analyzer (Model 700, O.I. International, College Station, TX, USA). This variable was used as a proxy of organic carbon available to denitrifiers. Sediment Oxygen Demand (SOD) was measured in the field by inserting a closed dark chamber (10 cm high, 10 cm wide) in bare sediment and monitoring oxygen decrease in the static water at 5 min. intervals for at least three hours using a luminescent dissolved oxygen sensor (Hach Company, Loveland, Colorado, USA).

Data analysis

We used t-tests to determine if denitrification rates, average lake temperature and nutrient concentrations differed between the sets of temperate and subtropical lakes. Because differences in denitrification rates among the lakes were large, which masked the effects of the treatments within each lake, we normalized the denitrification rate data. The normalization was expressed as the percentage of difference in denitrification rate compared to the treatment without macrophytes at ambient temperature, for each treatment in each lake.

Factorial ANOVA models were used to determine differences in normalized denitrification rates and oxygen levels between the different temperature and macrophyte presence conditions, and the interaction between temperature and macrophyte presence. Multiple linear regression was used to determine the most important factors influencing denitrification rates, in each climatic region. Only variables significantly correlating (Pearson correlation, Supplementary Tables 4.1 and 4.2) to denitrification were entered in the regression equation. In the case of collinear variables, only the variable that correlated strongest to denitrification was entered in the regression equation. If necessary, variables were ln(x+1) transformed to obtain a normal distribution. Normalized denitrification rates were ln(x/100)+1) transformed. Results were considered statistically significant when $P<0.05$. Analyses were performed using SPSS statistics 19.

Table 4.2. Physical and chemical characteristics of water and sediment of the studied lakes. Values are given as: mean (sd). DR= Denitrification rate (mean of all 4 chambers per warming treatment per lake, Amb.= ambient temperature, '+3'= 3°C above ambient), SD=Secchi disc depth (cm), DO=dissolved O_2, ABC=Acid Binding Capacity, EC=electric conductivity, OM= organic matter content in the sediment, TN=total nitrogen, TP=total phosphorus, DOC=dissolved organic carbon, Chl-a= Chlorophyll-a, WSOC= water soluble organic carbon from dry sediment, SOD= sediment oxygen demand.

Lake		*T1*	*T2*	*T3*	*T4*	*T5*	*T6*	*S1*	*S2*	*S3*	*S4*
DR Amb.	(µmol N m^{-2} h^{-1})	8.1 (4.1)	23.1 (3.8)	3.3 (2.2)	18.8 (32.7)	36.4 (20.4)	3.3 (3.4)	1.2 (0.8)	1.4 (1.6)	2.4 (1.0)	1.1 (0.6)
DR +3	(µmol N m^{-2} h^{-1})	4.9 (2.9)	33.2 (6.2)	5.9 (1.5)	7.0 (5.1)	77.4 (31.8)	6.6 (1.0)	1.4 (0.9)	2.0 (1.5)	1.1 (1.0)	0.3 (0.1)
SD	*(cm)*	30	29	>90	>90	>90	45	50	89	102	70
pH	**	9.24 (0.04)	7.70 (0.07)	8.54 (0.07)	8.40 (0.09)	8.33 (0.13)	7.08 (0.25)	7.67 (0.15)	8.24 (0.02)	7.74 (0.40)	8.27 (0.22)
DO	*(mg l^{-1}) ** *	12.14 (2.09)	5.16 (1.68)	10.37 (0.55)	9.61 (1.60)	9.11 (0.70)	9.00 (0.81)	8.34 (0.84)	8.63 (1.33)	8.74 (0.68)	9.96 (4.86)
T	*(°C) ** *	23.34 (1.39)	21.81 (0.30)	22.95 (0.39)	22.39 (0.16)	21.40 (0.63)	20.09 (0.98)	21.44 (0.81)	19.79 (1.19)	24.07 (1.16)	27.12 (0.33)
ABC	*(Meq l^{-1})*	3.52	2.31	2.09	1.75	1.83	0.57	3.34	4.6	0.84	7.58
EC	*(µS cm^{-1}) ** *	408	647	438	286	337	91	333	594	200	493
NO_{3^-} $+NO_2^-$	*(mg N l^{-1}) *** *	<0.01 (0.01)	<0.01 (0.01)	0.02 (0.01)	<0.01 (0.00)	0.07 (0.01)	0.08 (0.00)	0.01 (0.01)	0.01 (0.00)	0.01 (0.01)	0.04 (0.01)
NH_4^+	*(mg N l^{-1})*	0.01 (0.00)	0.01 (0.00)	0.01 (0.00)	0.01 (0.00)	0.01 (0.00)	0.01 (0.00)	0.04 (0.01)	0.02 (0.00)	0.01 (0.01)	0.02 (0.01)
PO_4^{3-}	*(µg P l^{-1})*	42 (0)	5 (1)	4 (1)	2 (2)	3 (0)	35 (2)	12 (4)	11 (1)	<0.1 (0)	5 (3)
OM	*%*	2.8 (1.8)	0.9 (0.1)	0.5 (0.1)	0.7 (0.3)	5.1 (0.4)	32.1 (14.7)	1.08 (0.8)	0.8 (0.8)	0.8 (0.1)	0.3 (0.2)
TN	*(mg l^{-1}) *** *	1.15 (0.30)	1.05 (0.09)	0.35 (0.02)	0.36 (0.18)	0.27 (0.20)	1.47 (0.74)	0.51 (0.12)	0.47 (0.07)	0.34 (0.00)	0.65 (0.04)
TP	*(mg l^{-1})*	0.16 (0.03)	0.10 (0.02)	<0.01 (0.02)	<0.01 (0.00)	<0.01 (0.00)	0.06 (0.04)	0.07 (0.01)	0.05 (0.01)	0.02 (0.01)	0.02 (0.00)
DOC	*(mg C l^{-1})*	19.44 (0.08)	20.75 (0.52)	8.14 (0.09)	5.78 (0.14)	10.67 (0.02)	40.05 (0.06)	13.97 (1.28)	13.80 (0.52)	12.37 (0.42)	16.08 (1.06)
Chl-a	*(µg l^{-1})*	47.36*	45.28 (15.20)	3.24 (0.20)	2.01*	5.17 (1.11)	156.21 (39.73)	27.38 (1.05)	3.77 (0.10)	3.70 (1.05)	11.51 (1.40)
WSOC	*(µg g^{-1})*	132	104	72	63	119	189	52	59	55	53
SOD	*(g m^{-2} d^{-1})*	2.43 (1.14)	0.58 *	1.13 (0.35)	0.43 (0.21)	0.76 (0.15)	5.40 (2.95)	1.54 (0.98)	0.68*	0.96 (0.57)	2.57 (1.24)

* *n*=1, ** mean of all measurements in the depth intervals, *** before addition of 1 mg N l^{-1} $^{15}N[NaNO_3]$

Table 4.3. Macrophyte species, average macrophyte wet biomass (MB) and periphyton biomass per gram macrophyte (PB) in the vegetated chambers in the lakes. Values are given as: mean (sd)

Lake	*MB (kg m^{-2})*	*PB (mg g^{-1})*	*Dominant Species*
T1	0.58 (0.13)	12.87 *	*Elodea nuttallii*
T2	0.06 (0.03)	4.85 *	*Elodea nuttallii*
T3	1.2 (0.42)	10.79 (3.76)	*Elodea nuttallii*
T4	0.21 (0.15)	6.04 (5.41)	*Chara* sp., *Potamogeton perfoliatus*
T5	0.86 (0.33)	6.47 (5.89)	*Elodea nuttallii*
T6	0.29 (0.08)	9.22 (8.90)	*Potamogeton natans*
S1	0.61 (0.12)	2.64 (1.07)	*Egeria densa*
S2	0.62 (0.39)	1.66 (0.53)	*Egeria densa*, *Myriophyllum* sp.
S3	0.38 (0.21)	17.59 (7.07)	*Potamogeton illinoensis*
S4	0.75 (0.51)	5.45 (3.17)	*Chara* sp.

* n=1

Table 4.4. Linear regression models explaining denitrification rates (as ln(DR + 1)) in the temperate and subtropical lakes. All entered variables significantly correlated to ln(DR + 1) and are shown in the table. DO = dissolved O_2 in the denitrification chamber (mg l^{-1}), SOD= sediment oxygen demand (g m^{-2} d^{-1}), T= temperature in the denitrification chamber (°C), MB = macrophyte wet biomass in the denitrification chamber (kg m^{-2}), OM = Fraction of organic matter in the sediment (%).

Model	R^2_{adj}	*P*
Temperate		
4.38-1.05ln(DO+1)	0.102	<0.05
3.15-0.89Ln(SOD+1)	0.158	<0.01
5.52-1.21Ln(DO+1)-0.92 ln(SOD+1)	0.294	<0.001
Subtropical		
0.622+0.1ln(MB+1)	0.159	<0.05
4.58-1.19ln(T+1)	0.109	<0.05
0.48+0.62ln(OM+1)	0.138	<0.05
2.310-0.560ln(T+1)+0.531ln(OM+1)	0.132	<0.10

Results

Conditions in the lakes

During the experiments, subtropical lakes were on average 1°C warmer than temperate lakes (T-test, $P<0.05$), with an average water temperature of 22.0 ± 0.4 sd °C in the temperate lakes and of 23.1± 1.0 (sd) °C in the subtropical lakes.

Nutrient concentrations in the water column were low in all lakes (Table 4.2), with NO_3-N below 0.08 mg l^{-1}, NH_4-N below 0.04 mg l^{-1} and PO_4-P below 42 µg l^{-1}. In most of the lakes, the percentage of organic matter in the sediment was lower than 2%. Exceptions were temperate lakes T1, and T5, which had organic rather than sandy sediments (OM 2-6%), and T6, which is a peat fen and therefore has a naturally high (32%) fraction of organic matter in the sediment. In the temperate lakes we found no overall difference in OM% between chambers with and without macrophytes (without macrophytes OM% = 9.08 ± 9.74 sd, with macrophytes OM% = 5.08 ± 4.14 sd Mann-Whitney U, $Z_{23}=70$, $P=0.932$), whereas sediments of subtropical sediments with macrophytes tended to be more organic than those without macrophytes (without macrophytes OM% = 0.43 ± 0.40 sd, with macrophytes OM% = 1.14 ± 0.52 sd $n=14$, Mann-Whitney U, $Z_{13}=38$, $P<0.1$). However, the temperate lakes had a larger variability in OM%, and relations between OM% and macrophyte presence differed from lake to lake in this region, whereas subtropical lakes were more similar and relations between macrophytes and OM% were more straightforward (Supplementary Figures 4.1 and 4.2). Macrophyte biomass significantly correlated to OM% in the subtropical lakes (R=0.505, $P<0.01$, Supplementary Table 4.2) but not in the temperate lakes (R=-0.043, $P=0.771$, Supplementary Table 4.1).

Temperate lakes were mostly dominated by *Elodea nuttallii* (Planch.) st. John and *Potamogeton* sp., whereas *Egeria densa* Planch., *Potamogeton illinoensis* Morong and *Chara* spp. were the most common species found in the subtropical lakes (Table 4.3).

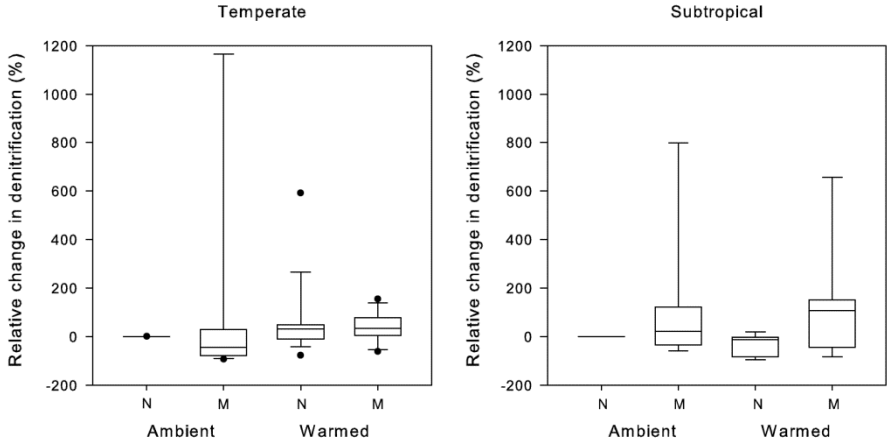

Figure 4.2. Relative change in denitrification rates (%) compared to the chambers without macrophytes at ambient temperature. Boxes indicate the 25th-75th percentiles, lines indicate the mean, whiskers indicate 10th-90th percentiles, points indicate the 5th and 95th percentiles. N = No macrophytes, M = Macrophytes

Denitrification rates

Denitrification rates in the ambient chambers ranged from <0.1 to 67.9 µmol N m^{-2} h^{-1}, whereas denitrification rates in the warmed chambers ranged from <0.1 to 114.1 µmol N m^{-2} h^{-1}. Highest denitrification rates were observed in lakes T2 and T5 (Table 4.2). Sediment oxygen demand and dissolved oxygen in the water column of the denitrification chamber explained 29% of the variation in absolute denitrification rates in the temperate lakes (linear regression, Table 4.4). Furthermore denitrification significantly negatively correlated to PO_4^{3-} (R=-0.329, *P*<0.05, Supplementary Table 4.1). In the subtropical lakes macrophyte biomass was the best predictor of denitrification rates, explaining 16% of the variation (linear regression, Table 4.4). In these lakes denitrification also significantly correlated to sediment organic matter (R=0.416, P<0.05) and negatively to temperature (R=-0.375, *P*<0.05, Supplementary Table 4.2).

When comparing denitrification rates at ambient temperatures in the different climatic regions, denitrification rates were 10 times higher in the temperate lakes than in the subtropical lakes (Temperate 15.5 ± 18.7 sd µmol N m^{-2} h^{-1}, subtropical 1.6 ± 1.1 sd µmol N m^{-2} h^{-1}, Mann-Whitney U, *P*<0.001).

We found no overall effect of warming on denitrification rates, combining the data of the temperate and subtropical lakes (Mann-Whitney U, P=0.618), and also found no significant effects of warming when testing the climatic regions separately (factorial ANOVA on normalized data, macrophyte presence and heating, heating NL: P=0.122, UY: P=0.209, Fig. 4.2).

When we combined data of temperate and subtropical lakes, macrophytes had no overall significant effects on denitrification rates (Mann-Whitney U, P=0.972). However, denitrification rates related differently to macrophyte presence in the different climatic regions (factorial ANOVA on normalized data, interaction macrophyte presence and climatic region: P<0.05). In the subtropical lakes, denitrification rates in chambers with macrophytes were on average 2 times higher than those without macrophytes (factorial ANOVA, normalized data, P<0.1), whereas in temperate lakes macrophyte presence did not seem to be related to denitrification (factorial ANOVA, normalized data, P=0.407) (Fig. 4.2). The interaction between warming and macrophyte presence was significant in neither of the climatic regions (factorial ANOVA, normalized data, temperate P=0.347, subtropical P=0.347).

Discussion

Denitrification rates differed between the climatic regions, with highest rates in the temperate lakes. Denitrification rates in the sampled temperate lakes were in the normal range for lakes, but low compared to those in rivers and constructed wetlands (Piña-Ochoa & Álvarez-Cobelas, 2006; Seitzinger et al., 2006), which may be due to the low availability of nitrate in the lakes during the sampling period. Denitrification rates in the subtropical lakes were on average about an order of magnitude lower, which besides nitrate limitation can be explained by the low fraction of organic matter in the sediments of these lakes.

Contrary to our expectations, in neither of the climatic regions denitrification was affected by the three degree warming, and no synergistic effects of warming and macrophyte presence were found. In the temperate lakes denitrification was also unrelated to macrophyte presence, whereas in the subtropical lakes denitrification was highest in patches with macrophytes. Macrophytes might have alleviated carbon limitation of the denitrifiers in the subtropical lakes, as patches with macrophytes had higher fractions of organic matter in the sediment than those without macrophytes. Furthermore, submerged macrophytes can provide ample oxic–anoxic interfaces and surface area for denitrifiers (Reddy et al., 1989; Weisner et al., 1994; Eriksson & Weisner, 1997), further enhancing local denitrification rates.

The absence of an effect of warming on denitrification suggests that while temperature stimulates denitrification at local scales when organic carbon and nitrate are sufficiently available (Bachand & Horne, 1999; Veraart et al., 2011b), across ecosystems other factors than temperature are more important drivers of denitrification. This notion was substantiated by a regression analysis of the overall denitrification rates in this study, which revealed no effect of temperature in the temperate lakes. Our finding is in agreement with other multi-system studies where site-specific conditions were found to overrule climatic conditions (Piña-Ochoa & Álvarez-Cobelas, 2006) However, we found a weak negative effect of temperature in the subtropical lakes. This could indicate that denitrifiers in the subtropical lakes were already at their temperature optimum, but the low amount of lakes studied (4) prevents us from a detailed analysis of this finding. Additionally, it is important to note that we studied only short-term effects of warming, influencing enzyme activity rather than microbial abundance or community structure (Wallenstein et al., 2006). Long-term effects of warming may change

resource availability, macrophyte presence and oxygen dynamics, complicating the prediction of changes in denitrification in the long term.

Although we found no clear temperature effect, other factors did influence denitrification rates, and those varied between the temperate and subtropical lakes. In the temperate lakes, denitrification was highest at low oxygen concentrations in the water column, reflecting suitable denitrification conditions in the sediment. Interestingly, denitrification in these lakes negatively correlated to sediment oxygen demand (SOD) and phosphate concentration. This is different from what one would expect, as both SOD and phosphate release are signs of reducing conditions in the sediment supposedly favoring denitrification. Other studies indeed found a positive correlation between soluble reactive phosphorus and denitrification rates (Inwood et al., 2005; Graham et al., 2010). Furthermore some denitrifying clades have been found to couple nitrate reduction to anoxic P-uptake in activated sludge (Zeng et al., 2003; Flowers et al., 2009). However, contrary to these studies, but similar to our findings, low phosphorus concentrations related to high seasonal denitrification rates in a meta-analysis (Piña-Ochoa & Álvarez-Cobelas, 2006). The authors suggested that this might be explained by an imbalance of N and P supply to denitrifiers as well as higher competition for N with microalgae at low N:P ratios. These contradicting findings point out that the mechanism through which phosphorus affects environmental denitrification rates, is not yet clearly understood.

It is unclear what causes the negative correlation between SOD and denitrification in this study. Such an effect may be expected at high organic C/ nitrate ratios, but this was not the case in our study. Potentially, SOD in some of the lakes may have related to electron donors that are unsuitable for denitrifiers, or occurred through respiration by macrofauna or phytobenthos rather than microorganisms, though this was not examined in this study.

In the subtropical lakes macrophytes and the related higher organic matter content in the sediment, were the most important variables explaining denitrification rates. This underlines the importance of macrophytes for lake biogeochemistry. The influence of macrophytes on nitrogen concentrations may work in two ways. By stimulating denitrification they contribute to permanent nitrogen removal, and additionally they can remove nitrate temporally through N-uptake (Veraart et al., 2011a). Both mechanisms contribute to stabilizing the macrophyte-dominated state in lakes.

In conclusion, we found no effect of warming on denitrification in the temperate and subtropical lakes, which we argue to be masked by resource limitation. In the subtropical lakes, denitrification was enhanced by macrophyte presence, associated with higher organic carbon content of macrophyte-covered sediments. These results indicate that denitrification rates may be influenced by different factors in different climatic regions, which should be taken into account when scaling up regional N-budgets. In the context of global warming scenarios clearly it is important to not only consider short term effects of warming on biogeochemical cycles, but to take into account climatic effects on resource availability and ecology as well. Climate change may, for instance, alter sediment organic matter content and macrophyte coverage and, as our analysis confirms, these may be more important drivers of biogeochemical functioning than the sole effect of temperature.

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Supplementary Table 4.1. Correlation matrix for the temperate lakes (abbreviations as in table 4.2 of the main text, ch = in the denitrification chamber).

		ln_DR	*ln_PB_ch.*	*ln_DO_ch.*	*ln_T_ch.*	*ln_MB*	*ln_SOD*	*ln_OM%*	*ln_DOC*	*ln_NH4*	*ln_PO4*	*ln_NO3*	*ln_TN*	*ln_TP*	*pH*
ln_DR	Pearson Correlation	1	-.116	-.350*	-.021	-.072	-.419**	-.055	-.050	.285*	-.329*	.064	-.214	-.059	-.057
	Sig. (2-tailed)		.432	.017	.887	.626	.003	.712	.737	.050	.022	.665	.145	.688	.701
	N	48	48	46	48	48	48	48	48	48	48	48	48	48	48
ln_PB_ch.	Pearson Correlation	-.116	1	.231	.133	.983**	.069	-.053	-.042	.092	.049	.058	-.065	-.035	.120
	Sig. (2-tailed)	.432		.122	.366	.000	.642	.721	.776	.532	.740	.697	.659	.811	.418
	N	48	48	46	48	48	48	48	48	48	48	48	48	48	48
ln_DO_ch.	Pearson Correlation	-.350*	.231	1	.394**	.273	-.121	-.362*	-.600**	-.072	-.128	-.289	-.476**	-.238	.683**
	Sig. (2-tailed)	.017	.122		.007	.067	.422	.013	.000	.635	.396	.051	.001	.111	.000
	N	46	46	46	46	46	46	46	46	46	46	46	46	46	46
ln_T_ch.	Pearson Correlation	-.021	.133	.394**	1	.133	-.124	-.333*	-.219	-.167	.026	-.383**	-.093	.168	.469**
	Sig. (2-tailed)	.887	.366	.007		.367	.403	.021	.135	.257	.859	.007	.531	.254	.001
	N	48	48	46	48	48	48	48	48	48	48	48	48	48	48
ln_MB	Pearson Correlation	-.072	.983**	.273	.133	1	.053	-.043	-.074	.133	.020	.087	-.114	-.080	.133
	Sig. (2-tailed)	.626	.000	.067	.367		.721	.771	.616	.366	.892	.554	.442	.587	.368
	N	48	48	46	48	48	48	48	48	48	48	48	48	48	48
ln_SOD	Pearson Correlation	-.419**	.069	-.121	-.124	.053	1	.794**	.788**	-.217	.894**	.565**	.732**	.403**	-.331*
	Sig. (2-tailed)	.003	.642	.422	.403	.721		.000	.000	.139	.000	.000	.000	.005	.021
	N	48	48	46	48	48	48	48	48	48	48	48	48	48	48
ln_OM%	Pearson Correlation	-.055	-.053	-.362*	-.333*	-.043	.794**	1	.720**	-.016	.600**	.837**	.520**	.112	-.570**
	Sig. (2-tailed)	.712	.721	.013	.021	.771	.000		.000	.914	.000	.000	.000	.448	.000
	N	48	48	46	48	48	48	48	48	48	48	48	48	48	48

Table S4.1 continued

		ln_DR	*ln_PB_ch.*	*ln_DO_ch.*	*ln_T_ch.*	*ln_MB*	*ln_SOD*	*ln_OM%*	*ln_DOC*	*ln_NH4*	*ln_PO4*	*ln_NO3*	*ln_TN*	*ln_TP*	*pH*
ln_DOC	Pearson Correlation	-.050	-.042	-.600**	-.219	-.074	.788**	.720**	1	-.262	.822**	.431**	.924**	.646**	-.552**
	Sig. (2-tailed)	.737	.776	.000	.135	.616	.000	.000		.072	.000	.002	.000	.000	.000
	N	48	48	46	48	48	48	48	48	48	48	48	48	48	48
ln_NH4	Pearson Correlation	.285*	.092	-.072	-.167	.133	-.217	-.016	-.262	1	-.466**	.474**	-.566**	-.660**	-.151
	Sig. (2-tailed)	.050	.532	.635	.257	.366	.139	.914	.072		.001	.001	.000	.000	.304
	N	48	48	46	48	48	48	48	48	48	48	48	48	48	48
ln_PO4	Pearson Correlation	-.329*	.049	-.128	.026	.020	.894**	.600**	.822**	-.466**	1	.234	.857**	.760**	-.071
	Sig. (2-tailed)	.022	.740	.396	.859	.892	.000	.000	.000	.001		.110	.000	.000	.630
	N	48	48	46	48	48	48	48	48	48	48	48	48	48	48
ln_NO3	Pearson Correlation	.064	.058	-.289	-.383**	.087	.565**	.837**	.431**	.474**	.234	1	.106	-.327*	-.587**
	Sig. (2-tailed)	.665	.697	.051	.007	.554	.000	.000	.002	.001	.110		.472	.023	.000
	N	48	48	46	48	48	48	48	48	48	48	48	48	48	48
ln_TN	Pearson Correlation	-.214	-.065	-.476**	-.093	-.114	.732**	.520**	.924**	-.566**	.857**	.106	1	.801**	-.401**
	Sig. (2-tailed)	.145	.659	.001	.531	.442	.000	.000	.000	.000	.000	.472		.000	.005
	N	48	48	46	48	48	48	48	48	48	48	48	48	48	48
ln_TP	Pearson Correlation	-.059	-.035	-.238	.168	-.080	.403**	.112	.646**	-.660**	.760**	-.327*	.801**	1	.159
	Sig. (2-tailed)	.688	.811	.111	.254	.587	.005	.448	.000	.000	.000	.023	.000		.279
	N	48	48	46	48	48	48	48	48	48	48	48	48	48	48
pH	Pearson Correlation	-.057	.120	.683**	.469**	.133	-.331*	-.570**	-.552**	-.151	-.071	-.587**	-.401**	.159	1
	Sig. (2-tailed)	.701	.418	.000	.001	.368	.021	.000	.000	.304	.630	.000	.005	.279	
	N	48	48	46	48	48	48	48	48	48	48	48	48	48	48

* Correlation is significant at the 0.05 level (2-tailed).

** Correlation is significant at the 0.01 level (2-tailed).

Supplementary Table 4.2. Correlation matrix for the subtropical lakes (abbreviations as in table 4.2 of the main text, ch = in the denitrification chamber).

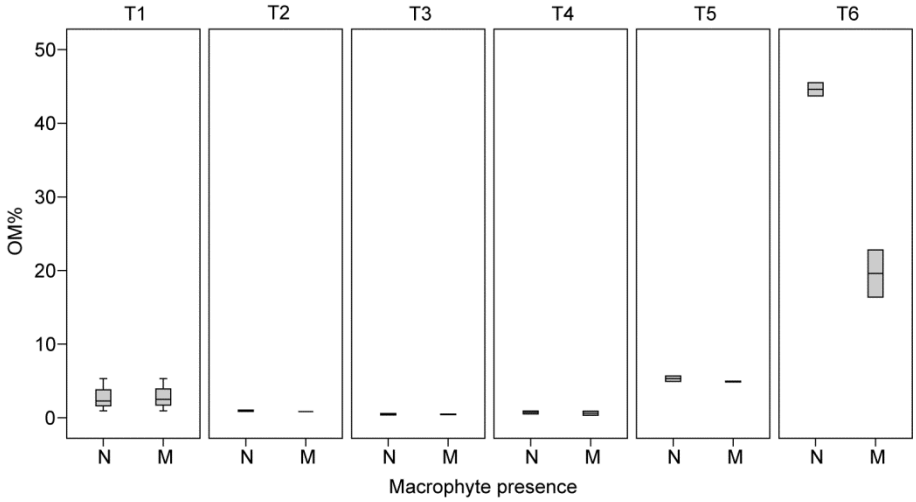
		ln_DR	*ln_PB_ch*	*ln_DO_ch*	*ln_T_ch*	*ln_MB*	*ln_SOD*	*ln_OM%*	*ln_DOC*	*ln_NH4*	*ln_PO4*	*ln_NO3*	*ln_TN*	*ln_TP*	*pH*
ln_DR	Pearson Correlation	1	.325	.070	-.375*	.382*	-.323	.416*	-.331	-.084	-.089	-.353	-.318	.096	-.187
	Sig. (2-tailed)		.091	.769	.045	.041	.088	.035	.079	.664	.645	.060	.093	.620	.332
	N	29	28	20	29	29	29	26	29	29	29	29	29	29	29
ln_PB_ch	Pearson Correlation	.325	1	.003	.036	.960**	.041	.457*	.011	-.011	-.048	.040	.007	-.053	-.006
	Sig. (2-tailed)	.091		.990	.851	.000	.828	.017	.952	.954	.803	.834	.973	.781	.975
	N	28	30	22	30	30	30	27	30	30	30	30	30	30	30
ln_DO_ch	Pearson Correlation	.070	.003	1	.026	-.034	-.092	-.085	.068	-.634**	-.413	.212	-.049	-.632**	.504*
	Sig. (2-tailed)	.769	.990		.907	.882	.683	.708	.763	.002	.056	.344	.829	.002	.017
	N	20	22	22	22	22	22	22	22	22	22	22	22	22	22
ln_T_ch	Pearson Correlation	-.375*	.036	.026	1	-.075	.746**	-.358	.491**	-.059	-.364*	.778**	.427*	-.670**	.206
	Sig. (2-tailed)	.045	.851	.907		.688	.000	.061	.004	.750	.041	.000	.015	.000	.258
	N	29	30	22	32	31	32	28	32	32	32	32	32	32	32
ln_MB	Pearson Correlation	.382*	.960**	-.034	-.075	1	.018	.505**	.038	.038	.054	.016	.042	.039	.017
	Sig. (2-tailed)	.041	.000	.882	.688		.923	.006	.841	.839	.772	.933	.823	.834	.928
	N	29	30	22	31	31	31	28	31	31	31	31	31	31	31
ln_SOD	Pearson Correlation	-.323	.041	-.092	.746**	.018	1	-.310	.803**	.469**	.126	.918**	.798**	-.237	.169
	Sig. (2-tailed)	.088	.828	.683	.000	.923		.108	.000	.007	.493	.000	.000	.191	.354
	N	29	30	22	32	31	32	28	32	32	32	32	32	32	32
ln_OM%	Pearson Correlation	.416*	.457*	-.085	-.358	.505**	-.310	1	-.401*	.168	.040	-.461*	-.349	.352	-.400*
	Sig. (2-tailed)	.035	.017	.708	.061	.006	.108		.035	.393	.840	.014	.069	.066	.035
	N	26	27	22	28	28	28	28	28	28	28	28	28	28	28

* Correlation is significant at the 0.05 level (2-tailed). **Correlation is significant at the 0.01 level (2-tailed).

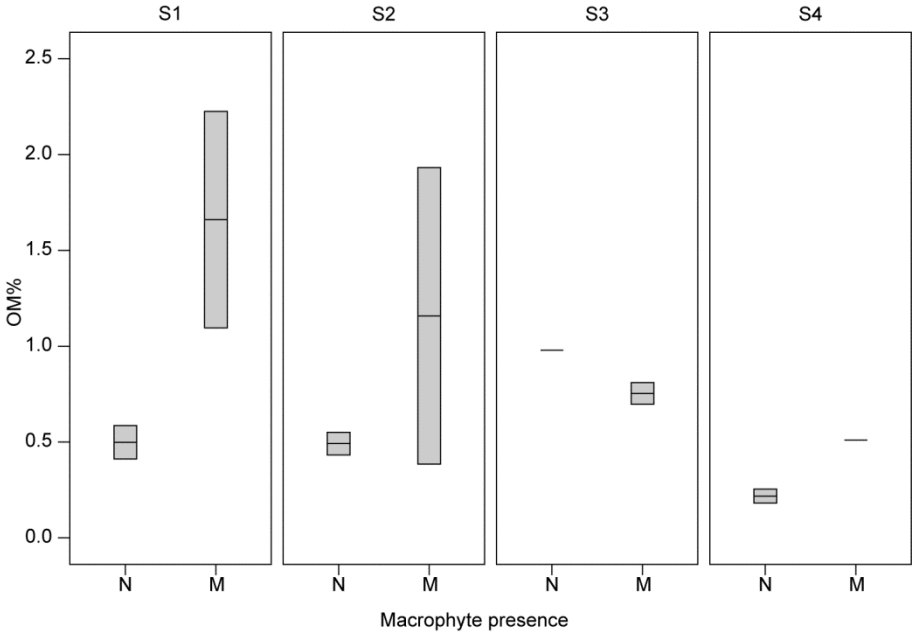
Supplementary Table 4.2. Continued

		ln_DR	*ln_PB_ch.*	*ln_DO_ch.*	*ln_T_ch.*	*ln_MB*	*ln_SOD*	*ln_OM%*	*ln_DOC*	*ln_NH4*	*ln_PO4*	*ln_NO3*	*ln_TN*	*ln_TP*	*pH*
ln_DOC	Pearson Correlation	-.331	.011	.068	.491**	.038	.803**	-.401*	1	.333	.531**	.895**	.992**	-.068	.646**
	Sig. (2-tailed)	.079	.952	.763	.004	.841	.000	.035		.063	.002	.000	.000	.712	.000
	N	29	30	22	32	31	32	28	32	32	32	32	32	32	32
ln_NH4	Pearson Correlation	-.084	-.011	-.634**	-.059	.038	.469**	.168	.333	1	.604**	.151	.445*	.709**	-.415*
	Sig. (2-tailed)	.664	.954	.002	.750	.839	.007	.393	.063		.000	.409	.011	.000	.018
	N	29	30	22	32	31	32	28	32	32	32	32	32	32	32
ln_PO4	Pearson Correlation	-.089	-.048	-.413	-.364*	.054	.126	.040	.531**	.604**	1	.107	.613**	.765**	.306
	Sig. (2-tailed)	.645	.803	.056	.041	.772	.493	.840	.002	.000		.561	.000	.000	.088
	N	29	30	22	32	31	32	28	32	32	32	32	32	32	32
ln_NO3	Pearson Correlation	-.353	.040	.212	.778**	.016	.918**	-.461*	.895**	.151	.107	1	.851**	-.443*	.528**
	Sig. (2-tailed)	.060	.834	.344	.000	.933	.000	.014	.000	.409	.561		.000	.011	.002
	N	29	30	22	32	31	32	28	32	32	32	32	32	32	32
ln_TN	Pearson Correlation	-.318	.007	-.049	.427*	.042	.798**	-.349	.992**	.445*	.613**	.851**	1	.054	.575**
	Sig. (2-tailed)	.093	.973	.829	.015	.823	.000	.069	.000	.011	.000	.000		.767	.001
	N	29	30	22	32	31	32	28	32	32	32	32	32	32	32
ln_TP	Pearson Correlation	.096	-.053	-.632**	-.670**	.039	-.237	.352	-.068	.709**	.765**	-.443*	.054	1	-.329
	Sig. (2-tailed)	.620	.781	.002	.000	.834	.191	.066	.712	.000	.000	.011	.767		.066
	N	29	30	22	32	31	32	28	32	32	32	32	32	32	32
pH	Pearson Correlation	-.187	-.006	.504*	.206	.017	.169	-.400*	.646**	-.415*	.306	.528**	.575**	-.329	1
	Sig. (2-tailed)	.332	.975	.017	.258	.928	.354	.035	.000	.018	.088	.002	.001	.066	
	N	29	30	22	32	31	32	28	32	32	32	32	32	32	32

* Correlation is significant at the 0.05 level (2-tailed). **. Correlation is significant at the 0.01 level (2-tailed).



Supplementary Figure 4.1. Sediment organic matter fraction (OM%) in patches with (M) and without (N) macrophytes in the temperate lakes (T1-T6). Boxes indicate the 25^{th}-75^{th} percentiles, lines indicate the median.



Supplementary Figure 4.2. Sediment organic matter fraction (OM%) in patches with (M) and without (N) macrophytes in the subtropical lakes (S1-S4). Boxes indicate the 25^{th}-75^{th} percentiles, lines indicate the median.

Chapter 5

Abundance, richness and activity of denitrifiers in drainage ditches - in relation to sediment characteristics, vegetation and land-use.

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Abstract

Background. Agricultural drainage ditches are often polluted by nitrogen, which contributes to eutrophication of the lakes, canals and reservoirs in which they drain. Nitrogen can be removed from ditches by microbial denitrification, but factors affecting denitrification in these systems are still poorly understood.

Methodology/ Principal findings. We measured *in-situ* denitrification rates and environmental conditions in 13 Dutch drainage ditches, to explore which factors are most important in influencing denitrification rates. Furthermore, we investigated which factors regulate abundance, richness and community structure of denitrifying microorganisms, and how these metrics relate to denitrification, using denaturing gradient gel electrophoresis and quantitative-PCR of the *nirK* gene. Denitrification rates varied widely between the ditches, ranging from 6 to 24344 µmol N m^{-2} h^{-1}. Ditches with fine, sandy sediments, that were covered by duckweed and contained high nitrate concentrations, were denitrification hotspots. Denitrification rates differed between different land-use types. Highest rates were found in ditches next to arable land, followed by those in grasslands; lowest rates were observed in bogs and nature reserves. Denitrification correlated to nitrate concentrations, but not to *nirK* abundance or richness, whereas *nirK* abundance correlated to organic matter content of the sediment but not to nitrate concentrations. Despite high organic matter content in the sediment, denitrification in peat ditches was rather low.

Conclusions/ significance. Denitrification in ditches is affected by sediment, vegetation and land-use types, and can contribute considerably to reducing nitrogen loads to adjacent waters. Our results indicate that organic material in the sediment influences denitrifier abundance, whereas nitrate concentrations determine instantaneous denitrification rates, creating denitrification hotspots in nitrate rich drainage ditches in agricultural areas.

Introduction

Drainage ditches are essential for the hydrology and biodiversity of reclaimed wetlands and low lying agricultural areas of western Europe and the USA (Painter, 1999; Herzon & Helenius, 2008). In the Netherlands, they comprise a total length of 300.000 km, creating a unique landscape (Nijboer, 2000). Ditches often receive run-off and nitrogen rich groundwater from adjacent fields, leading to excessive production of macrophytes and macroalgae, floating plant dominance, and consequently hypoxia and biodiversity loss (Janse & Van Puijenbroek, 1998; Scheffer et al., 2003). Nitrogen loads from ditches contribute to eutrophication in connected waters, such as canals, lakes and reservoirs (Needelman et al., 2007). This can be partly counteracted by denitrification (de Klein, 2008), a major N removing process that reduces nitrate to gaseous nitrogen. Denitrification is performed by microorganisms and requires an electron donor like easily degradable organic carbon and nitrate as terminal electron acceptor (Knowles, 1982). In freshwater sediments, denitrification usually takes place in anoxic-oxic boundary layers, where nitrate is supplied from the oxic zone (Seitzinger et al., 2006). Similar to wetlands, agricultural ditches are potential denitrification hotspots, due to their tight terrestrial-aquatic coupling; high nitrate inputs, ample anoxic-oxic interface and organic sediments (McClain et al., 2003; Veraart et al., 2011a). However, denitrification rates in ditches have been found to vary widely (de Klein, 2008), and it remains unclear which factors are most important in regulating denitrification in these systems.

Factors affecting denitrification act on two different levels: they drive the abundance and diversity of the denitrifying microorganisms present, but also the amount of nitrate converted by the resultant denitrifying community (Wallenstein et al., 2006). Although most denitrifiers present in the environment remain uncharacterized (Philippot & Hallin, 2005), abundance of genes coding for denitrification enzymes can be used to probe denitrification potential in these systems (O'Connor et al., 2006; Graham et al., 2010), whereas variation within functional genes may be used as indicator of denitrifier diversity (Hallin & Lindgren, 1999; Throbäck, 2006).

In this study, we quantify denitrification rates in drainage ditches of different sediment, vegetation and land-use types, and explore which environmental factors are most important in explaining denitrification rates. Furthermore, we examine which are the most important environmental factors

regulating denitrifier abundance, and how abundance, community structure and richness of the *nirK* gene, coding for nitrite reductase, relate to denitrification in drainage ditches.

Methods

Study sites and sampling design

We sampled 13 drainage ditches (Table 5.1), seven of these ditches were located in peat areas, and were also used to quantify greenhouse gas emission from peat ditches (P1-P4 & SP1-SP3, Schrier-Uijl et al., 2011). The other six ditches were selected to include ditches with clay (C1-C4) and fine, sandy sediments (FS1, FS2), containing some well decomposed organic sludge. The ditches were situated in agricultural areas (crops or meadows) and nature reserves or protected areas (mostly peat bogs), and therefore differed in yearly N-loads (table 5.1). Each ditch was sampled once. We measured denitrification rates, dissolved oxygen, temperature, dissolved organic carbon (DOC), NO_3^-, NH_4^+, electric conductivity (EC) and pH in the water column, and the organic matter content (OM%) and oxygen demand of the sediment (SOD). Additionally, the composition and abundance of the *nirK* gene pool in the top layer of the sediment were analysed. Furthermore, we determined dominant macrophyte structures by estimating the coverage of floating and submerged vegetation in each ditch.

Denitrification measurements

Denitrification rates were measured *in situ* using the ^{15}N isotope paring technique in split-box benthic measuring chambers. Use of benthic chambers allows to study denitrification rates under field conditions, and was found to give similar results as more labour intensive laboratory batch-mode assays (Nielsen & Glud, 1996; Mengis et al., 1997). The perspex split-box chambers consisted of a frame and 3 parallel chambers (12 L each). Frames were placed in the sediment at least 1 h before starting the measurements. After settling of the disturbed sediment the 3 parallel chambers were placed on the frame, fully submersed in the ditch water, capturing submerged vegetation, when present. The top of the chamber contained a screw cap opening with a septum, through which ^{15}N could be injected and water samples could be taken. We placed a stirrer next to the septum opening in the chamber, to

gently mix the ^{15}N through the chamber water, and to optimise diffusion of ^{15}N into the sediment. Measurements were performed in the morning, and each day one ditch was sampled. We injected 5ml 0.16 M $^{15}N[NaNO_3]$ through the septum of each chamber, to enrich the water in each chamber with 1 mg l^{-1} ^{15}N. Water samples for N_2 analysis were taken with an airtight glass syringe, whereupon 5 ml of sample was transferred into a helium flushed pre-vacuated 12 ml exetainer (Labco Wycombe), which contained 100 µl (50% w:v) $ZnCl_2$, to stop microbial activity. Water samples (triplicates) were taken 0.25, 1, 2 and 3 hours after injecting the ^{15}N solution. Samples were stored at room temperature until analysis. Before analysis, samples were vigorously shaken to transfer the dissolved N_2 into the helium headspace. Denitrification rates were calculated from the increase of $^{29}N_2$ and $^{30}N_2$ in the headspace (Nielsen, 1992; Steingruber et al., 2001), measured at a SerCon Cryoprep trace gas concentration system interfaced to a PDZ Europa 20-20 isotope ratio mass spectrometer (Sercon Ltd., Cheshire, UK).

Conditions in the water column

Dissolved oxygen, temperature (T), electric conductivity (EC) and pH in the ditches were measured using an HQ multiprobe with a luminescent dissolved oxygen sensor (Hach Company, Loveland, Colorado, USA), at a location undisturbed by placing the denitrification chambers. We measured depth profiles at 10 cm intervals for O_2 and T; EC and pH were measured at 20 cm depth in the water column.

For nutrient analysis, mixed water column samples were collected within 2 m from the denitrification chambers. Samples were filtered in the field using 0.45 µm cellulose nitrate filters (Whatman ltd., Kent, UK), kept cool on ice during transportation and stored at -20 °C upon arrival in the laboratory. Nutrient concentrations (NO_3^-+ NO_2^-, NH_4^+, ortho-PO_4^{3-}) were measured colorimetrically using a SAN^{plus} autoanalyzer (Skalar Analytical, Breda, the Netherlands) as described by Veraart et al. (2011a) and references therein. DOC was measured using a Total Organic Carbon Analyzer (Model 700, O.I. International, College Station, TX, USA). Estimates of groundwater seepage in the ditch area were obtained from hydrological maps (van der Gaast et al., 2006).

Table 5.1. Locations, sediment types and sampling dates of the 13 Ditches. Vegetation= the dominant type of macrophytes in the ditch, characterised as either floating (water surface fully covered by *Lemna* sp.), submerged (sediment surface covered by more than 25% submerged macrophytes, mainly *Elodea* sp.) or no/ little vegetation (less than 25% sediment surface covered by submerged macrophytes).

Ditch	*Location*	*Latitude*	*Longitude*	*Soil type* **	*Sediment* ***	*Vegetation*	*Land-use* †	*N-load* ††	*Sampling date*
C1	Randwijk	51° 57'15.35"	5° 43'51.02"E	R7	Clay	None/ little	Grassland	95.5	July 9, 2009
C2	Randwijk	51° 57'15.55"N	5° 43'51.02"E	R7	Clay	None/ little	Grassland	95.5	July 9, 2009
C3	Lienden	51° 55'29.10"N	5° 31'47.55"E	R8	Clay	Submerged	Grassland	65.4	July 10, 2009
C4	Lienden	51° 55'29.22"N	5° 31'49.09"E	R8	Clay	Submerged	Crops	65.4	July 10, 2009
FS1	Wageningen	51° 59'16.01"N	5° 39'02.54"E	Z20	Fine Sand	Floating	Crops	174.6	July 7, 2009
FS2	Wageningen	51° 59'16.47"N	5° 39'00.28"E	Z20	Fine Sand	Floating	Crops	174.6	July 7, 2009
P1	Nieuwkoop *	52° 08'32"N	4° 47'28"E	V1	Peat	None/ little	Bog /NR.	22.3	June 24, 2009
P2	Nieuwkoop *	52° 08'22"N	4° 47'50"E	V1	Peat	None/ little	Bog /NR.	22.3	July 2, 2009
P3	Oukoop	52° 02'10.76"N	4° 46'48.42"E	V1	Peat	Floating	Grassland	23.3	June 23, 2009
P4	Stein	52° 01' 08.13"	4° 46' 43.67"E	V1	Peat	None/ little	Grassland	27.5	July 1, 2009
SP1	Doosje	52° 41' 10.81"N	6° 07' 57.88"E	V13	Sand/Peat	Submerged	Bog /NR.	23.6	June 25, 2009
SP2	st Jansklooster	52° 40' 48.53"N	6° 00' 28.66"E	Z18x/A1 (V)	Sand/Peat	None/ little	Grassland	28	June 29, 2009
SP3	Horstermeer *	52° 14' 25"N	5° 04' 17"	V6	Sand/Peat	None/ little	Bog /NR.	27	June 26, 2009

* Approximate coordinates

** Soil classification of the ditch area according to the Dutch classification system (Steur et al., 1985), classes are explained in Supplementary Information Table S5.1.

*** Sediment type classified by the main soil type in the ditch area. In sand/peat ditches both sand and peat occur in the first 120cm of the soil.

† Dominant land-use type surrounding the ditch; Bog/ Nr. are peat ditches in nature reserves or protected areas with mostly swamp vegetation in its surroundings.

†† Average annual N-load to the surface water (g m^{-2} y^{-1}) of the polder area surrounding the ditch (1985-2005), estimated by the STONE model (Wolf et al., 2003).

Sediment characteristics

The top 3 cm of the sediment was sampled using a Kajak corer at 3 locations within 1 m from to the denitrification chambers. Samples were mixed to create one mixed sediment sample per ditch, kept on ice during transportation and frozen at -20 °C until analysis. Organic matter content was determined from the loss on ignition at 550 °C for 3 h. Sediment oxygen demand was measured in the field by inserting a closed dark chamber (10 cm high, 10 cm wide) in bare sediment and monitoring oxygen decrease in the static water at 5 min intervals for at least three hours using a luminescent dissolved oxygen sensor (Hach Company, Loveland, Colorado, USA).

nirK composition and richness

The diversity of the *nirK* gene encoding for the copper containing nitrite-reductase, a key enzyme in denitrification which transforms nitrite to nitric oxide, was used as a proxy of the denitrifier community structure. Sediment samples from the top 3 cm of sediment were collected and stored as described above. Total DNA was extracted from each sediment sample using a FastDNA® Kit for Soil (MP Biomedicals, Irvine, CA, USA), according to the manufacturers protocol. After extraction, DNA templates were purified using a OneStep™ PCR Inhibitor Removal Kit (Zymo Research, Orange, CA, USA). DNA quality and quantity were checked using a Nanodrop ND-100 spectrophotometer (Thermo Scientific, San Jose, CA, USA) and 1% (w/v) agarose gel electrophoresis, after which the DNA templates were diluted to 20 ng DNA μl^{-1}.

Fragments of the *NirK* gene were amplified with the primers F1ACu (5'-ATC ATG GT (C/G) CTG CCG CG-3') (Hallin & Lindgren, 1999) and R3Cu with a 33-bp GC-clamp attached to the 5'end (5'- GGC GGC GCG CCG CCC GCC CCG CCC CCG TCG CCC GCC TCG ATC AG(A/G) TTG TGG TT-3') (Hallin & Lindgren, 1999; Throbäck, 2006). PCR amplification was performed in a total reaction volume of 50 µl, containing 10 µl of 5× Green GoTaq Reaction Buffer (Promega), 200 µM of each dNTP, 0.4 µM of each primer, 1.25 U of GoTaq DNA polymerase (Promega) and 2 µl (40 ng) of DNA. Bovine Serum Albumin (BSA) was added to a final concentration of 200 ng μl^{-1} to improve PCR performance.

A touchdown PCR was performed in order to increase specificity. Initial DNA denaturation was performed at 94 °C for 2min, followed by 10 cycles of 30 s at 94 °C, 40 s of annealing at 64 °C (decreasing 1 °C per cycle) and 1 min of elongation

at 72 °C, followed by 25 cycles of 30 s at 94 °C, 40 s at 53 °C and 1 min at 72 °C. The reaction was completed with a final elongation for 5 min at 72 °C. BSA added to the PCR reaction was digested by adding 1 µl of proteinase K (20 mg ml^{-1}) in order to avoid interference with DGGE (Denaturing Gradient Gel Electrophoresis) reagents. PCR reactions were analyzed by 1% (w/v) agarose gel electrophoresis visualized under UV light after SYBRSafe (Invitrogen) staining.

Denaturing Gradient Gel Electrophoresis analysis of amplicons was performed as described by Muyzer & Smalla (1998), using a Dcode Universal Mutation Detection System (Bio-Rad, Hercules, CA, USA). DGGE was performed on 8% polyacrylamide gels with a denaturant gradient from 40% to 70% (100% denaturing acrylamide was defined as 7 M urea and 40% (v/v) formamide). Aliquots of PCR products were loaded on the gel and electrophoresis was carried out with 0.5% Tris acetic acid EDTA buffer at 60 °C and at 85 V for 16 h, initiated by a pre-run of 10 min at 120 V. After electrophoresis, gels were silver-stained (Sanguinetti et al., 1994) and scanned using a GS-800 Calibrated Densitometer (Bio-Rad Hercules, CA, USA).

Quantitative PCR

Abundance of the *nirK* gene was quantified using real-time PCR. DNA was isolated and purified as described above. Fragments of the *nirK* gene were amplified using primers nirK876 (5'-ATY GGC GGV CAY GGC GA-3') and nirK1040 (5'-GCC TCG ATC AGR TTR TGG TT-3') (Henry et al., 2004). The 25 µl final volume reaction contained 12.5 µl iQ™ SYBR® Green supermix (BioRad Hercules, CA, USA), 1.4 µM of each primer, 0.25 µl BSA (final concentration 200ng BSA μl^{-1}), and 5 µl (corresponding to 10 ng) of sample DNA. Thermal cycling was performed using a BioRad CFX96 real-time thermal cycler (Bio-Rad, Hercules, CA, USA), according to the following protocol: 3 min enzyme activation at 90 °C, followed by 45 cycles of 15 s of denaturation at 90 °C, 30 s annealing at 60 °C, 30 s elongation and data acquisition at 72 °C, after which a melting curve was constructed in a last step from 60 to 95 °C incrementing 0.5 °C s^{-1}. Specificity of *nirK* fragment amplification was checked by observing a single band of expected size in a 1.5% agarose gel, and the presence of a single melting peak on the melting curve.

Data analysis and Bionumerics

DGGE band detection was performed using Bionumerics software (version 4.61 Applied Maths, Belgium), with an optimum of 0.5% and a 0.5% position tolerance, but with manual adjustment to avoid misplacing of bands. A reference marker, included on the gel in three different positions, was used as standard for normalization, ensuring sample-to-sample comparability. Similarity between DGGE profiles was determined by calculating similarity indices using the Dice similarity coefficient that takes into account the presence or absence of specific bands. The unweighted pair group method with arithmetic means (UPGMA) algorithm was used for dendrogram construction. *NirK* richness was obtained from the number of visible bands (representing Operational Taxonomic Units, OTUs).

We tested for differences in denitrification rates between sediment types and vegetation types using one-way ANOVA. Relations between denitrification and potential explaining variables were tested by linear regression analysis. If necessary, data were ln(x+1) transformed to achieve a normal distribution. We used redundancy analysis (RDA) to test how environmental variables explained variation in *nirK* OTUs. Absence/ presence for each of the observed OTUs was entered as species data in the ordination. Nitrate, NH_4-N, PO_4-P, DOC, O_2, T, EC and sediment OM% were ln(x+1) transformed and entered as environmental data. Scaling was focused on inter-sample distances. Significance of the canonical axes was evaluated by Monte-Carlo permutation tests (499 permutations). Statistical analyses were performed in SPSS 19 (IBM SPSS statistics, Armonk, NY, USA) and CANOCO 4.55 (Ter Braak and Smilauer, Biometris, Wageningen, the Netherlands).

Results

Conditions in the ditches

The sampled ditches varied considerably in water column conditions (Table 5.2). Dissolved oxygen in the water column ranged from 1.0 to 7.3 mg l^{-1} (average 4.3 ± 2.2 sd mg l^{-1}). Nitrate could only be detected in FS1, FS2 and C3. FS2 had a 5 fold higher nitrate concentration than FS1 and a 15 fold higher nitrate concentration than C3. Sediment organic matter averaged 30.0 ± 27.5 sd%. Ditch temperatures were on average 19.7 ± 2.8 sd °C. SP3 had highest EC, due to high amounts of chloride-rich groundwater seepage.

Denitrification

Denitrification rates varied widely between the ditches, averaging 2261 ± 6718 sd µmol N m^{-2} h^{-1} (Table 5.2), with considerable within ditch variability (*n*=3, Table 5.2). Denitrification rates differed among sediment types (One-way ANOVA, F_3=33.683, *P*<0.001, Fig. 5.1A), vegetation types (One-way ANOVA, F_2=19.307, *P*<0.001, Fig. 1B) and land-use types (One-way ANOVA, F_2=35.129, *P*<0.001, Fig. 5.1C). Denitrification rates were highest in the ditches with sediments consisting of fine sand (FS1 & FS2, Tukey post hoc, *P*<0.01, Fig. 5.1A), followed by clay and peat ditches, with significantly higher rates in clay ditches than in ditches containing mixtures of sand and peat. Furthermore rates were highest in ditches covered by floating vegetation (FS1, FS2, P3; Tukey post hoc, *P*<0.01), and in ditches in agricultural areas (Crops > Grasslands > Bogs/ Nature reserves, Tukey post hoc, *P*<0.01). Denitrification rates significantly correlated to nitrate (R=0.816, *P*=0.001, Fig. 5.2A) and ammonium (R=0.581, *P*<0.05), but not to any of the other measured variables.

NirK richness, composition and abundance

A total number of 94 *nirK* OTUs were observed in the ditches. On average, ditches had 39 ± 6 sd OTUs (Table 5.3).

About 50% of the *nirK* bands were similar in all samples, however, there were several distinct clusters of ditches with similar denitrifier communities, based on the presence/absence of the different *nirK* OTUs (Fig. 5.3). Band analysis clustered the ditches mostly in groups that related to their sediment characteristics or vegetation type; with a cluster of clay ditches (C1-C4), a cluster of all ditches with floating vegetation (P3, FS1 and FS2), and a cluster of peat ditches in a nature reserve (P1 and P2). Ditches SP2 and P4, both ditches without vegetation, clustered together but were not as similar as samples within the other clusters. Ditch SP3 formed the overall outgroup, with only 50% similarity with the other ditches.

Redundancy analysis clustered the ditches by sediment type, vegetation and trophic status. The first RDA axis was mainly explained by conditions in the sediment and explained 19% of the variance in OTUs. The second RDA axis explained 15% of the variation and was mostly defined by concentrations of

solutes, and primary production (Fig. 5.4), with vegetated ditches on the negative side of the axis, and most unvegetated ditches on the positive side of the axis.

Copy numbers of *nirK* per gram dry sediment were on average $9*10^4 \pm 1.7*10^5$ sd. *NirK* copies per ng sample DNA were on average $1*10^2 \pm 2*10^2$ sd (Table 5.3). Copy numbers of *nirK* were significantly correlated to organic matter percentage of the sediment (ln(*nirK* g dry sed^{-1})+1) vs OM%, R=0.649, *P*<0.05, Fig. 5.2B), but negatively correlated to seepage (R=-0.598, P<0.05). Ditches with high copy numbers of *nirK* also had a richer *nirK* community structure (#OTUs R=0.564, *P*<0.05, Fig. 5.2C).

Discussion

Denitrification rates related to environmental factors.

Denitrification rates of the studied ditches varied widely, and differed among sediment, vegetation and land-use types. Denitrification rates in the agricultural ditches in our study are high, similar to those previously found in agricultural streams and rivers (Garcia-Ruiz et al., 1998; Pattinson et al., 1998; Laursen & Seitzinger, 2004; Schaller et al., 2004). These are among the highest denitrification rates observed in aquatic systems (Piña-Ochoa & Álvarez-Cobelas, 2006). Ditches containing fine sand (FS) had higher denitrification rates than those with clay or peat sediments. Fine-textured sediments may support higher denitrification rates, because they have a larger proportion of anoxic microsites compared to coarser sediments (Valett et al., 1996; Garcia-Ruiz et al., 1998; Martin et al., 2001; Findlay et al., 2011). Clay sediments have even smaller particle size, but may have had lower denitrification rates in this study due to their lower porosity and therefore hampered diffusion of nitrate to the denitrification zone, but possibly also because these ditches were situated in areas with lower nitrate loads and different vegetation types. In this study, FS ditches had significantly higher nitrate concentrations than ditches of other sediment types, probably causing the extreme differences in denitrification rates. Additionally - and potentially as a consequence of high nutrient loads - these ditches were covered by duckweed, which likely further improved the conditions for denitrification, due to the resultant decrease in dissolved oxygen concentration (Veraart et al., 2011a).

Table 5.2. Water column conditions and sediment characteristics, presented as single measurement or mean (sd). DOC = dissolved organic carbon (*n*=2), DO = average of depth profile of dissolved oxygen in the water column, T= average of depth profile of temperature in the water column, EC= electric conductivity (*n*=1), SOD= sediment oxygen demand (*n*=1), OM= organic matter percentage of the sediment (*n*=1). Seepage= the estimated groundwater seepage for the ditch area (van der Gaast et al., 2006).

Ditch	*Denitrification (µmol N m^{-2} h^{-1})**	*NO_3+NO_2^- (mg N l^{-1}) ***	*NH_4^+ (mg N l^{-1}) ****	*PO_4^{3-} (µg P l^{-1}) ****	*DOC (mg C l^{-1})*	*DO (mg l^{-1})*	*T (°C)*	*pH ****	*EC (µS cm^{-1})*	*SOD (g m^{-2} day^{-1})*	*OM (%)*	*Seepage (mm d^{-1})*
C1	497 (93)	<0.01	0.11	18	10.0 (0.2)	3.7 (0.6)	17.7 (0.1)	7.2	470	1.10	14.8	0.23
C2	31 (31)	<0.01	0.04	32	9.7 (0.1)	6.4 (0.0)	18.2 (0.0)	7.5	389	n.a.	9.9	0.23
C3	50 (4)	0.32	0.03	42	6.7 (0.4)	7.2 (0.0)	16.3 (0.0)	7.3	491	2.86	12.6	0.06
C4	143 (22)	<0.01	0.03	42	7.8 (2.2)	7.3 (0.1)	16.3 (0.1)	7.4	491	n.a.	12.8	0.06
FS1	3888 (983)	0.88	0.14	159	10.2 (0.1)	4.6 (0.3)	19.3 (0.2)	7.0	294	2.76	2.9	0.70
FS2	24344 (9955)	4.86	0.07	14	12.3 (0.4)	3.0 (1.5)	18.4 (0.3)	7.1	430	n.a.	20.3	0.66
P1	13 (6)	<0.01	<0.01	21	14.6 (0.2)	5.9 (3.2)	21.0 (0.3)	n.a.	n.a	1.10	74.5	-0.71
P2	48 (7)	<0.01	<0.01	11	15.6 (0.1)	3.3 (1.6)	25.8 (0.9)	7.2	408	0.21	74.8	-0.75
P3	137 (45)	<0.01	0.02	91	37.9 (0.2)	2.2 (1.7)	18.7 (1.6)	7.3	541	0.25	57.6	-0.37
P4	57 (15)	<0.01	<0.01	30	39.2 (1.7)	3.2 (2.0)	23.5 (0.3)	6.7	231	1.73	62.9	-0.38
SP1	6 (8)	<0.01	<0.01	18	17.1 (0.0)	6.9 (2.4)	22.1 (1.2)	7.8	420	1.52	1.0	0.40
SP2	167 (6)	<0.01	0.20	255	16.4 (0.8)	1.0 (0.5)	19.7 (2.1)	7.1	325	0.74	11.7	0.00
SP3	14 (5)	<0.01	<0.01	12	10.8 (1.7)	1.7 (1.3)	18.6 (1.7)	6.8	832	n.a.	35.2	2.00

***** *n*=3**, **** Before adding 1 mg l^{-1} ^{15}N-NO_3^- to the denitrification chambers (*n*=1), ******* *n*=1

Table 5.3. Richness and abundance of the *nirK* gene in 13 drainage ditches.

Ditch	*# OTUs*	*copy number nirK/ g dr sed.*	*copy number nirK/ng DNA*
C1	34	2.30E+03	4
C2	39	8.20E+03	21
C3	34	1.60E+04	24
C4	45	1.90E+04	26
FS1	45	5.70E+03	22
FS2	45	2.50E+04	28
P1	36	2.50E+05	217
P2	40	3.70E+05	199
P3	49	5.10E+05	791
P4	31	4.50E+03	8
SP1	39	1.80E+03	11
SP2	32	2.10E+02	1
SP3	32	8.30E+02	1

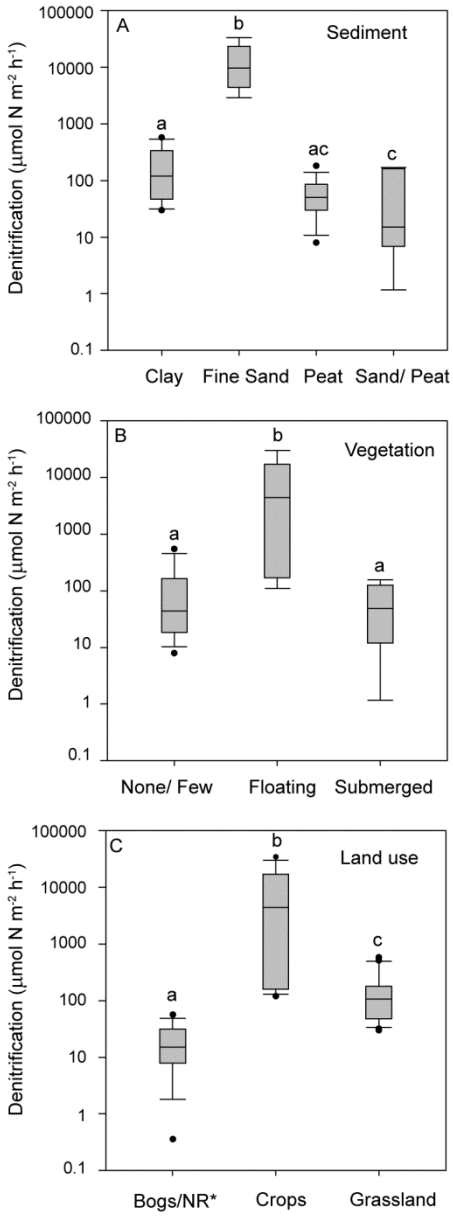

Figure 5.1. Denitrification rates for each of the sediment (A) vegetation (B) and land-use (C) types studied. Data of all denitrification chambers sampled are plotted. Boxes indicate the 25th-75th percentiles, lines indicate the mean, whiskers indicate 10th-90th percentiles, points indicate the 5th and 95th percentiles. Letters indicate homogeneous subsets (Tukey post-hoc, α = 0.05). * NR = Nature reserves and protected areas.

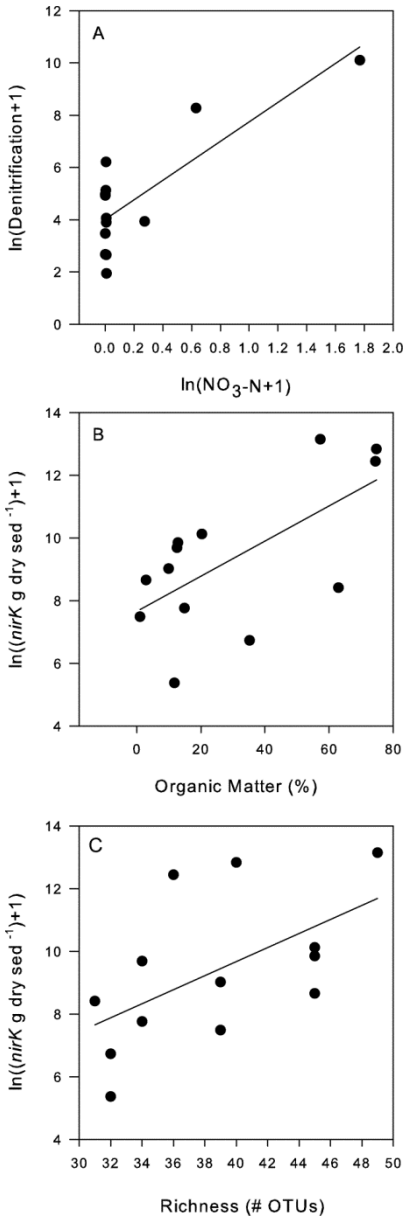

Figure 5.2. Relations between (A) denitrification and nitrate, (B) *nirK* gene copies per g. sediment and organic matter % of the sediment, (C) richness of the *nirK* gene measured by PCR-DGGE and *nirK* copies per g. sediment. Points indicate ditches, lines indicate linear regressions (A: R^2=0.67, P<0.01, B: R^2=0.42, P<0.05, C: R^2=0.32, P<0.05).

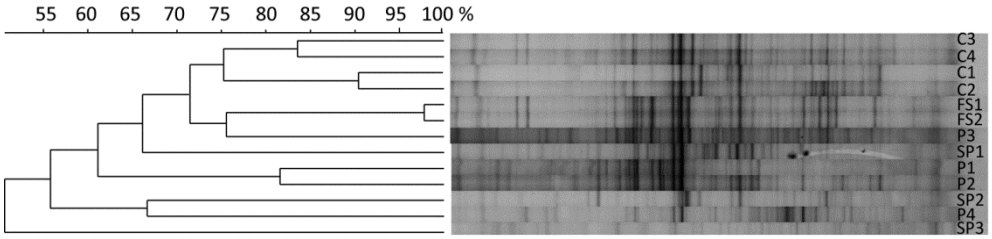

Figure 5.3. Clustering of the ditches based on DGGE analysis of PCR-amplified *nirK* gene pools. Each band is one operational taxonomic unit.

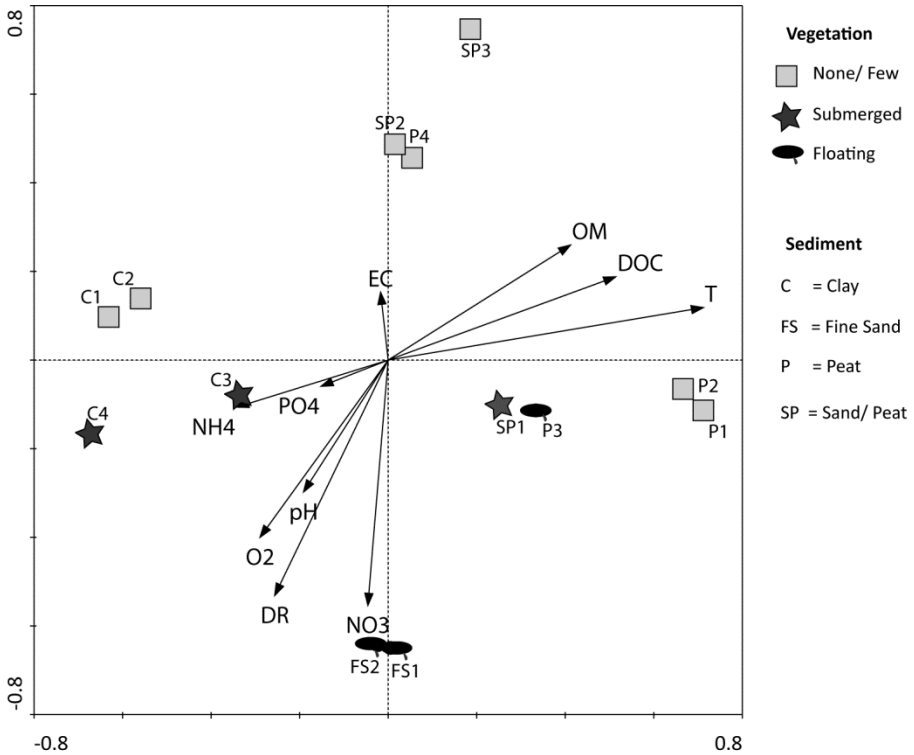

Figure 5.4. Redundancy analysis of *nirK* OTUs and environmental variables. DOC= dissolved organic carbon, DR= denitrification rate, EC= electric conductivity, OM= organic matter content of the sediment, T= temperature. Units are as in Table 5.2. Larger distance on the plot indicates greater dissimilarity between ditches based on *nirK* OTUs. Arrows indicate the direction of the largest gradient in each environmental variable. Eigenvalue of axis 1 (x) = 0.188, *P*=0.280; eigenvalue of axis 2 (y) = 0.149. Significance of all canonical axes: *P*<0.05.

Denitrification rates in peat ditches were relatively low. Due to the high C/N ratios in the peat sediments dissimilatory nitrate reduction to ammonium (DNRA) may have been the dominant nitrogen reducing process in these ditches (Tiedje et al., 1982; Burgin & Hamilton, 2007). However, the organic carbon may not have been available for nitrate reducing bacteria (those performing either DNRA or denitrification) due to the presence of phenolic compounds in peat. Phenolic substances are potent enzyme inhibitors that may inhibit nitrate reducing enzymes directly, but also slow down microbial decomposition under anaerobic conditions (Freeman et al., 2001; Freeman et al., 2004), resulting in lower nitrate reduction rates due to carbon limitation.

Ditches with floating plants had higher denitrification rates than those without plants or with submerged vegetation. However, distinguishing net vegetation effects on denitrification is complex, because the type of dominant vegetation present in aquatic ecosystems largely depends on nutrient loads, sediment conditions, and maintenance strategies (Janse & Van Puijenbroek, 1998; Scheffer et al., 2003; Kosten et al., 2009b) which all influence denitrification as well.

Overall, denitrification rates were most explained by nitrate and ammonium concentrations, although in the case of nitrate this was mainly caused by the presence of nitrate in 3 ditches. The relation between nitrate and aquatic denitrification rates is well known (Inwood et al., 2005; Piña-Ochoa & Álvarez-Cobelas, 2006; Mulholland et al., 2008). Ammonium availability in the ditches potentially related to denitrification through coupled nitrification-denitrification (Eriksson & Weisner, 1999), but may also reflect anoxia in the sediment; at which both ammonification and denitrification are favoured.

Similar to the meta-analysis of Piña-Ochoa & Álvarez-Cobelas (2006), temperature was no significant factor explaining denitrification rates in the ditches. This opposed to studies of Veraart et al. (2011b) and Bachand & Horne (1999) which both found a strong temperature effect on denitrification in experimental and single wetland setups covering a similar temperature range. This indicates that temperature plays a role within ecosystems, but when comparing rates in different ecosystems other factors limiting denitrification, such as nitrate availability, are most probably more important.

Thus, our data show that certain ditches are indeed denitrification hotspots. These hotspots are mostly related to high nitrate availability, but given the within ditch variability local sediment conditions play a role as well.

Denitrification and nitrogen removal in Dutch drainage ditches.

When recalculating the measured denitrification rates to yearly values, the studied ditches would remove 0.7 to 2986 g m^{-2} y^{-1}, corresponding to 3.1 to 1710% of their yearly nitrogen loads. The median nitrogen removal efficiency by denitrification in agricultural areas (grasslands and crops) would be 45%, suggesting that denitrification in ditches can indeed significantly contribute to purification of nitrogen polluted surface waters. However, in practise there will be a seasonal mismatch between N-loads and denitrification potential, including the observed hotspots or 'hot moments' of this study. Highest loads occur in winter, when denitrification rates are low due to the low temperatures. In summer denitrification potential can be high, but nitrogen loads are lower than in winter, and incoming nitrogen is rapidly assimilated by the ditch vegetation (de Klein, 2008). Nonetheless, the differences in denitrification rates found for different sediment types and land-use types could contribute to calculating regional nitrogen budgets.

Denitrifier (nirK) richness and abundance

In line with previous studies (Hallin et al., 2009; Attard et al., 2011), denitrification rates in the ditches were not significantly related to *nirK* richness. The absence of a richness-functioning relationship in denitrifying communities may be explained by a high functional redundancy of denitrifiers (Wertz et al., 2006). However, it should be taken into account that only one denitrifying enzyme was considered in this study, and little is known about environmentally important denitrifiers.

Abundances of the *nirK* gene observed in this study were in the same range as those reported for soils (Henry et al., 2004). Abundance of *nirK* was not significantly related to denitrification rates, which is in agreement with results of Graham et al. (2010), but contrasts to the findings of O'Connor et al. (2006). The absence of such a relation may be partly caused by the fact that presence of a denitrification gene does not mean that it is expressed – and thus functional – in the environment. Interestingly, denitrifier abundance was significantly related to organic matter in the sediment, as was also found by Kandeler et al. (2006), but organic matter in the sediment was not related to denitrification. Furthermore, denitrifier abundance in the ditches was not related to nitrate concentration. These results confirm that denitrifier abundance is mainly controlled by organic matter

availability, whereas instantaneous denitrification rates are largely determined by nitrate availability (Graham 2010, Wallenstein 2006). In the studied ditches, nitrate limitation may explain the absence of a strong *nirK* abundance-denitrification relationship. Additionally, ditches with a thick layer of well decomposed sludge and low oxygen concentrations, in which we found high denitrification rates (e.g. FS2), may have had a larger proportion of denitrifiers containing the cytochrome *cd1*-nitrate reductase enzyme NirS (Knapp et al., 2009), resulting in a weaker relation between *nirK* abundance and denitrification.

Denitrifier (nirK) community structure related to environmental conditions.

Community structures based on the *nirK* gene were similar for similar sediment and vegetation types. Part of the variation in *nirK* community structure was explained by environmental variables, indicating that specific conditions favour some denitrifiers more than others. About 2/3 of the total variation in OTUs remained unexplained and may be due to specific conditions in the sediment or geographical factors. Interestingly, not all ditches that were geographically close to each other clustered together in the RDA (eg. P3-P4, SP1-SP2), indicating that local environmental conditions, and in relation to this the dominant vegetation present, may play a more important role in structuring denitrifying communities. Another example of specific conditions creating different communities is ditch SP3, which was the overall outgroup in the DGGE clustering analysis and RDA. This ditch is situated in a polder with high rates of groundwater seepage, rich in chloride and arsenic; very different hydrological conditions than the other ditches. These different conditions likely led to the different *nirK* community structure in ditch SP3.

Conclusions

Drainage ditches can sustain high denitrification rates, which can contribute to reducing N-loads to adjacent surface waters. Denitrification was mainly explained by availability of nitrogen, but not significantly related to *nirK* abundance or richness, indicators of denitrifier presence. Agricultural ditches with fine, sandy sediments, a closed cover of floating plants, and high nitrate concentrations were denitrification hotspots. Despite high organic matter content in the sediment, denitrification in peat ditches was low. Furthermore, our results show that *nirK*

abundance is mostly governed by presence of organic material in the sediment, whereas nitrate concentrations determine instantaneous denitrification rates.

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Supplementary Information 5

Table S5.1. Soil classification of the ditch locations (Steur et al., 1985).

Soil type class	*Definition*
A1	Peat excavations
R	Holocene riverine clay deposits
R7	Silt and clay
R8	Heavy clay
V	Histosols
V1	Thick layer of peat with wood debris
V13	Sphagnum peat, usually with a humic podzol within 120 cm and a sandy topsoil
V6	Alder carr/ reed sedge peat, usually with sand within 120 cm
Z	Sandy soils
Z18x	Fine sand with boulder clay or other Pleistocene clay starting between 40 and 120 cm
Z20	Fine sand / Loamy fine sand

Chapter 6

Denitrification in restored and unrestored Danish streams

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Abstract

Restoring channelized streams is an effective tool to reduce nitrogen loads to downstream waterbodies. However, although overall restoration effects at the catchment level are well established, it is still unclear how in-stream denitrification is affected by restoration. In this study, denitrification rates as well as factors controlling denitrification in unrestored and restored sections of two Danish streams (S1 and S2) were compared. The ^{15}N isotope pairing technique was used to measure denitrification *in situ*. Denitrifier presence was analyzed by denaturing gradient gel electrophoresis (DGGE) and quantitative PCR of the nitrite reductase gene (*nirK).* Denitrification rates were highly variable, with denitrification rates of 3106 µmol N m^{-2} h^{-1} in the unrestored section of S1, but no detectable denitrification in the restored section of S1, whereas in S2 restored and unrestored sections had similar denitrification rates of around 250 µmol N m^{-2} h^{-1}. These large differences in denitrification rates were mainly due to differences in hydrologic conditions and sediment characteristics. High nitrate fluxes from upwelling groundwater created denitrification hotspots in the unrestored section of S1. Moreover, a lack of organic matter in the restored section of S1 may have resulted in a low abundance of denitrifiers and consequently no detectable denitrification. Our results indicate the importance of hydrology for stream nitrogen dynamics, which should be considered in restoration design. Additionally, our results show that removal of organic sediment as a restoration measure may reduce the potential for denitrification.

Introduction

Increased anthropogenic nitrogen loads have led to a strong deterioration of water quality worldwide, causing hypoxia, excessive growth of plants and algae, biodiversity loss and fish-kills (Smith et al., 1999). Ditches and small streams are often the first waterbodies to receive nitrogen rich water from agricultural and urban areas. They can transport high nutrient loads to downstream areas, but can also be important nitrogen sinks due to plant uptake and denitrification (de Klein, 2008; Klocker et al., 2009), which is the microbial reduction of nitrate to nitrogen gas.

An important disturbance to a stream's natural capacity to retain nitrogen is channelization. In the past century, many streams in Western Europe and the US have been channelized to improve drainage of adjacent fields (Erickson et al., 1979; Iversen et al., 1993). Often, this resulted in an uncoupling of the stream from its floodplain wetland, a more homogeneous stream with uniform flow and morphology without riffles and pools, likely leading to reduced nitrogen retention capacity and consequently higher nitrogen loads on downstream areas.

Restoring channelized streams has proved to be an effective tool to reduce nitrogen loads to downstream waterbodies (Craig et al., 2008), especially when the stream is hydrologically reconnected to its floodplain, enabling nitrogen retention in the groundwater of connected stream banks and wetlands (Hoffmann & Baattrup-Pedersen, 2007; Kaushal et al., 2008). Stream restoration may also affect in-stream nitrogen biogeochemistry. However, there are only a few studies investigating restoration effects on nitrogen biogeochemistry in streams (Klocker et al., 2009).

There may be different short term (up to one year after restoration), and long term effects of stream restoration on nitrogen dynamics. Short term effects may be due to sediment allocation and rewetting of old meanders, whereas long term effects may be due to the altered stream hydrology, morphology, sediment characteristics and nutrient loading. The overall effect of restoration on denitrification is difficult to predict. For instance, in newly created pools fine-textured sediments, and accumulations of fine benthic material may support higher denitrification rates, as these substrate types can have a large proportion of anoxic microsites (Garcia-Ruiz et al., 1998; Martin et al., 2001; Findlay et al., 2011). However, in the newly created riffles denitrification rates may be low, due to the high flow velocity and coarser sediment with a lower proportion of organic matter.

Klocker et al. (2009) found a strong correlation between flow velocity and potential denitrification in streams, with highest denitrification rates in the restored streams which had lower flow velocities. Related to this, nitrogen removal increases with hydrologic residence time, favoring denitrification in restored streams, provided that restoration reduces flow velocity (Kaushal et al., 2008; Klocker et al., 2009; de Klein & Koelmans, 2011). However, in restored streams less nitrate may be available for denitrification because compared to unrestored streams a higher proportion of the nitrate is denitrified in the groundwater before it reaches the stream. Therefore, denitrification in the restored stream sediment may be more dependent on coupled nitrification-denitrification.

Furthermore, restoration may potentially affect the abundance and community composition of denitrifiers, by changing the conditions in the sediment. Although it is not yet possible to accurately determine the number of denitrifiers present in a system, the abundance of genes involved in denitrification can be used as a surrogate (Graham et al., 2010). In-stream denitrification rates, as well as potential denitrification rates have been found to correlate with abundance of the *nir*-gene, encoding for the nitrite reductase enzyme which converts nitrite to nitric oxide (O'Connor et al., 2006; Graham et al., 2010).

In this study we compare denitrification rates, stream hydrology, stream water, pore water and sediment characteristics, denitrifier community structure, richness and abundance in restored and unrestored sections of two Danish streams, to study effects of restoration on the in-stream nitrogen biogeochemistry. To better understand potential variability in denitrification rates we also estimated nitrogen transport to the denitrification zone for each stream section.

Table 6.1. Stream characteristics

Stream	*S1*	*S2*
Name	Bæksgård bæk	Hjarup å
Year of restoration	2005	2004
Catchment area (km^2)	14.1	16.2
Land-use		
Agriculture (%)	54	85
Forest/ Wetland (%)	26	11
Urban (%)	20	4
Soil type of catchment		
Loam (%)	0	32
Sandy loam (%)	16	68
Sand (%)	84	0
Strahler order	2	2
Latitude	55°26'06.48"N	55°52'09.92"N
Longitude	9°20'29.08"E	9°09'18.62"E

Methods

Study sites

The study was conducted in two Danish agricultural streams that both have been partly restored more than 5 years ago. The first stream, Bæksgård bæk (hereafter referred to as S1) is located in Jutland near Give whereas the second stream, Hjarup å (hereafter referred to as S2) is located 10 km south-west of Kolding (Table 6.1).

Restoration in both streams consisted of remeandering the channel and disconnecting tile drainage from the adjacent fields. Additionally, the streambeds were raised to restore the streams natural dimensions and reconnect them to the riparian area. In the restored sections of both streams the morphology of the stream bed is heterogeneous with alternating riffles and pools. The unrestored parts of the two streams are channelized and deeply incised, which prevents flooding. The morphology of the unrestored section of S2 is very homogenous, it has a deeply entrenched channel and low flow velocity. The unrestored section of S1, however, has alternating riffles and pools. The sediment in S1 is sandy, with numerous pebbles, cobbles and wood debris. The S2 sediment has a clayish top layer without pebbles. The restored sections of both streams were located upstream of the unrestored sections; sampling distances between restored and unrestored sections were 200 m for S1 and 75 m for S2.

During the field experiments, the banks of S1 were vegetated, and in both the restored and unrestored section the dominant in-stream vegetation consisted of *Sparganium emersum* Rehmann and *Ranunculus fluitans* Lam. with *Iris pseudacorus L.* dominating on the banks. In S2 the dominant stream vegetation consisted of *Phragmites australis* (Cav.) Trin. ex Steud. and *Sparganium erectum* L. growing from the banks into the stream, with some *Elodea nuttallii* (Planch.) H. St. John growing on the stream bed.

Denitrification measurements

Denitrification rates were measured *in situ,* using the ^{15}N isotope pairing technique in split-box measuring chambers (Nielsen, 1992; Dalsgaard et al., 2000). Chambers were placed in the pools, because we expected highest denitrification rates there. The chambers consisted of a Perspex frame that could be inserted into the

sediment, and a closed top that could be attached after the sediment had settled. Each chamber consisted of three 12 L sub-chambers. Each sub-chamber had a screw-cap opening with a septum, a stirrer, and a combined oxygen and temperature probe. To start the denitrification measurements 5 ml of 0.16 M $^{15}N[Na\text{-}NO_3]$ was added through the septum. Water was sampled (5 ml, in triplicate) 0.25, 1, 2 and 3 hours after injection of the $^{15}N[Na\text{-}NO_3]$ solution, as described by Veraart et al. (2011a). Dinitrogen concentrations and ratios of $^{29}N_2$ and $^{30}N_2$ over $^{28}N_2$ were measured using a Robo-Prep-G+ in line with a TracerMass mass spectrometer (Europa Scientific, Crewe, UK). Denitrification rates were calculated from the change in N_2 isotope ratios in time, following Nielsen (1992).

Sediment characteristics and biogeochemical profiles

Profiles of oxygen in the sediment were made in sediment cores directly after sampling, using an O_2 micro-profiler (Unisense, Aarhus, Denmark). Profiles of NH_4^+ and NO_3^- in the porewater where made using Diffusive Equilibrium in Thin films (DET) probes (DGT research Ltd., Lancaster, UK, Krom et al., 1994, see Supplementary Information 6 for detailed procedures).

We determined the fraction of organic matter in the sediment (OM%) from the loss on ignition at 550 °C for 3 h. Textural composition of the sediment was determined after removal of C and $CaCO_3$ by dispersing the samples in a sodium pyrophosphate solution. Clay and silt content were determined using a hydrometer, sand fractions were separated using the wet-sieving technique. C/N content in the sediment was measured with an automated C/N analyser (Roboprep-G+) in line with a Tracermass mass spectrometer (Europa Scientific, Crewe, UK).

Water column conditions & hydrological measurements

Dissolved oxygen, pH, temperature and electric conductivity were measured at four locations and three depths in each of the four stream sections using an HQ multiprobe with a luminescent dissolved oxygen sensor (Hach Company, Loveland, CO, USA). Discharge was calculated based on measurements of flow velocity at four locations and three depths in each of the four stream sections using an electromagnetic flow velocity meter (Sensa-RC2, Aqua data services, Wiltshire, England).

Seepage was measured using a low-profile seepage meter following design and recommendations specified in Rosenberry (2008). The seepage meter was placed into the stream bed and water seeping into the cylinder was collected in a prefilled (1 L) collection bag which was placed in a bag shelter. Seepage was calculated from the volume change of the bag after thirty minutes.

Dissolved nutrients (NO_3^-, NO_2^-, NH_4^+, PO_4^{3-}) were measured in filtrated water samples (0.45 µm cellulose membrane filters, Whatman International Ltd, Maidstone, England). Nitrate and nitrite were measured on a ICS-1500 ion chromatograph (Dionex, Sunnyvale, CA, USA) and ammonium and orthophosphate were analyzed colorimetrically. Chlorophyll-*a* and turbidity were measured in the field using a fluorometer (AquaFluor Turner designs, Sunnyvale, CA, USA).

Calculation of Nitrogen fluxes to the denitrification zone

The nitrate seepage flux (g m^{-2} h^{-1}) was calculated by multiplying nitrogen concentrations (mg N l^{-1} measured by the DET probes) in the anoxic zone of the sediment with seepage velocities (m h^{-1}). Diffusion of nitrate from the water column to the denitrification zone in the sediment was estimated by multiplying the sediment nitrate concentration gradient (g m^{-4}), with the diffusion rate constant (m^2 h^{-1}), calculated according to Portielje & Lijklema (1999). The nitrate concentration gradient is the gradient between the nitrate concentration of the water column and the nitrate concentration of the pore water in the anoxic zone of the sediment where most denitrification takes place (Christensen et al., 1989).

Composition and abundance of the nirK gene pool

The diversity of the gene encoding for the Cu containing nitrite-reductase enzyme (*nirK*), which transforms nitrite to nitric oxide, was used as a proxy for structure and richness of the denitrifier community. Sediment samples from the top 2 cm of sediment and the underlying sediment at 3 to 5 cm depth were collected in the field using a Kajak corer, and frozen at -20 °C upon arrival in the lab. Total DNA was extracted from each sample using a FastDNA® Kit for Soil (MP Biomedicals, Irvine, CA, USA). After extraction, DNA templates were purified using a OneStep™ PCR Inhibitor Removal Kit (Zymo Research, Orange, CA, USA). DNA quality and quantity were checked using a Nanodrop ND-100 spectrophotometer (Thermo Scientific,

San Jose, CA, USA) and 1% (w/v) agarose gel electrophoresis, after which the DNA templates were diluted to 20 ng DNA μl^{-1}.
PCR amplification was performed using primers F1ACu and R3Cu with a 33-bp GC-clamp attached to the 5'end (Hallin 1999), using a touchdown PCR protocol (See Chapter 5). Denaturing Gradient Gel Electrophoresis (DGGE) analysis of amplicons was performed as described by Muyzer & Smalla (1998) (Chapter 5). *NirK* richness was obtained from the number of visible bands on the gel (which represent Operational Taxonomic Units, OTUs).

Abundance of the *nirK* gene was quantified using quantitative real-time PCR. DNA was isolated and purified as described above. Fragments of the *nirK* gene were amplified using primers nirK876 and nirK1040 (Henry et al., 2004, see Chapter 5 for details).

Data analysis

We tested for differences in in-stream conditions between both streams, or restored and unrestored sections within each stream, using t-tests if the data followed a normal distribution. If necessary, data were ln(x+1) transformed. If the data did not follow a normal distribution after transformation we used Mann-Whitney U tests on untransformed data. In the case of denitrification, in S1 the results from one denitrification chamber from each stream section were excluded from statistical analysis because the chambers had not been properly closed during sampling. Zero values in denitrification measurements were considered to be below a 0.01 µmol N m^{-2} h^{-1} detection limit, therefore we assigned each zero value a value randomly drawn from a uniform dataset containing values between 0 and 0.01. Statistical analysis was performed in PASW statistics 17 (IBM SPSS statistics, Armonk, NY, USA). Analysis of the composition of the *nirK* gene pool was performed using Bionumerics (version 4.61, Applied Maths, Belgium, See Chapter 5 for details).

Table 6.2. Conditions in the unrestored and restored sections of both streams. Values are given as mean (sd). 0-2 = top 0-2 cm of the sediment; 2-5 = 2 to 5cm depth in the sediment.

	Stream 1		*Stream 2*	
	Unrestored	*Restored*	*Unrestored*	*Restored*
Morphology & Hydrology				
Depth (cm) (*n*=12)	23 (12)	34 (10)	42 (27)	22 (21)
Width (cm)*	196	370	205	212
Flow Velocity (m s^{-1}) (*n*=12)	0.21 (0.04)	0.22 (0.23)	0.06 (0.01)	0.07 (0.04)
Discharge (l s^{-1})*	114.7	153.7	42.4	33
Seepage (l m^{-2} h^{-1})*	8.6	1.1	0.2	0.2
Water column conditions				
Temperature (^{o}C) (*n*=12)	16.3 (0.1)	16.1 (0.2)	18.6 (0.5)	19.2 (0.8)
pH (*n*=12)	6.9 (0.1)	6.7 (0.2)	7.7 (0.2)	7.8 (0.0)
Dissolved Oxygen (mg l^{-1}) (*n*=12)	9.7 (0.0)	9.4 (0.0)	8.0 (0.1)	8.4 (0.2)
Electric Conductivity (µS cm^{-1}) (*n*=4)	286 (1)	277 (1)	553 (2)	550 (12)
NO_3^- (mg N l^{-1})	3.18	3.72	3.52	3.83
NH_4^+ (mg N l^{-1})	0.06	0.03	0.08	0.11
PO_4^{3-} (µg P l^{-1})	14	24	24	16
Dissolved Inorganic Carbon (mg l^{-1}) (*n*=2)	12.5 (0.0)	13.8 (0.1)	31.0 (0.5)	29.8 (0.1)
Dissolved Organic Carbon (mg l^{-1}) (*n*=2)	7.6 (0.3)	8.2 (0.1)	6.6 (0.4)	10.6 (0.6)
Chlorophyll-*a* (µg l^{-1}) (*n*=4)	0.6 (0.0)	0.6 (0.0)	0.6 (0.0)	0.6 (0.0)
Turbidity (ntu) (*n*=4)	1.7 (0.2)	1.1 (0.2)	5.2 (0.6)	7.7 (0.1)
Sediment conditions				
Organic Matter 0-2cm (%)	0.5 (0.2)	0.1 (0.0)	1.9 (0.2)	1.3 (0.4)
Organic Matter 2-5cm (%)	0.2 (0.1)	0.1 (0.0)	1.2 (0.7)	0.9 (0.6)
Sediment Oxygen Demand (g m^{-2} d^{-1})*	0.0	0.3	1.6	0.2
Anoxic Depth (mm) (*n*=2)	-38*	-19*	-12 (4)	-23 (6)
Clay <2µm (%)*	3	2.5	14.6	13.4
Silt 2-20µm (%)*	0	0	10.4	10.1
Coarse Silt 20-63µm (%)*	1.9	2.7	10.6	16.5
Very Fine Sand 63-125µm (%)*	4.0	1.0	6.4	14.5
Fine Sand 125-200µm (%)*	16.2	4.8	9.5	15.8
Coarse Sand 200-500µm (%)*	53.5	69.1	21.2	22.4
Very Coarse Sand 500-2000µm (%)*	20.7	19.6	21.0	5.6
Humus (%)*	0.7	0.4	3.6	1.7
Total Carbon (%)*	0.39	0.22	2.43	0.98
$CaCO_3$ (%)*	<0.1	<0.1	2.58	<0.1
C/N	11.5 (7.7)	2.9 (8.6)	13.7 (0.7)	12.9 (1.7)

* *n*=1

Results

Conditions in the water column and stream hydrology

Both streams had low ammonium and ortho-phosphate concentrations but high nitrate concentrations (3.6 ± 0.3 sd mg N l^{-1}). S1 had three times higher flow velocities, and significantly lower pH and DO than S2 (Table 6.2, Table 6.3). S2 had twice as high EC and DIC and 2.7 °C higher water temperatures than S1 (Table 6.2, Table 6.3).

In both streams we found some small but significant differences in water column conditions between the restored and unrestored sections (Table 6.3). The restored section of S1 had 0.3 mg l^{-1} lower DO, and slightly lower pH and EC than the unrestored section. In S2 we found the opposite: the restored section had 0.3 mg l^{-1} higher DO and a slightly higher pH than the unrestored section (Table 6.2). Flow velocities were similar among the restored and unrestored section of each stream, but overall restored sections tended to have a larger spatial heterogeneity in flow rates (Table 6.2). S1 had about 3x higher discharge than S2. Seepage was also highest in S1, with 8 times higher seepage in the unrestored than in the restored section (Table 6.2).

Conditions in the sediment

The sediments of S2 were more organic and had a higher C/N ratio than those of S1, although both streams had mineral sediments with a relatively low amount of organic matter (Table 6.2, Table 6.3). In S1 the sediment of the unrestored section had a significantly higher proportion of organic matter and C/N ratio than the restored section, which hardly had any organic material. In S2, which was overall more organic, we found no significant difference in organic matter content or C/N ratio between the restored and unrestored section (Table 6.3). The top 2-cm of the sediment tended to be more organic than the underlying layer. S2 had finer sediments than S1, with higher amounts of clay, silt and fine sand, and lower amounts of coarse sand (Table 6.2).

Table 6.3. Statistics for comparisons of physical-chemical conditions between the 2 streams (S1 and S2), Unrestored (U) and Restored (R) sections of both streams and the top 2 and underlying 2-5 cm of sediment of both streams. Flow= flow velocity, T= temperature, DO= dissolved oxygen, DR= denitrification rate, EC= electric conductivity, OM= Organic matter fraction of the sediment, C/N= carbon to nitrogen ratio. Comparisons were performed using either t-tests (for normally distributed datasets or Mann-Whitney U tests (for datasets that did not follow a normal distribution), *M* and *t* values indicate test-statistics for Mann-Whitney U and t-tests, respectively. *= Ln(x+1) transformed.

		S1	*S2*	*S1 and S2*
	S1 vs S2	*U vs. R*	*U vs. R*	*0-2 vs. 2-5 cm depth*
Flow (m s-1)*	$t_{23.838}$=4.455, ***P*<0.001**	$t_{10.605}$=0.186, *P*=0.856	$t_{9.556}$=-1.316, *P*=0.219	
T (ºC)	*M*=0.0, ***P*<0.001**	*M*=9.5, *P*=0.062	*M*=41, *P*=0.071	
pH	*M*=0.0, ***P*<0.001**	*M*=17, *P*=0.001	*M*=26, ***P*<0.01**	
DO (mg l^{-1})	*M*=0.0, ***P*<0.001**	*M*=0.0, ***P*<0.001**	*M*=12, ***P*<0.01**	
DR (µmol N m^{-2} h^{-1})*	$t_{9.157}$=1.495, *P*=0.168	$t_{4.221}$=2.796, ***P*<0.05**	$t_{9.983}$=-0.237, *P*=0.818	
EC (µS cm^{-1})*	$t_{12.250}$=-80.589, ***P*<0.001**	t_{6}=14.085, ***P*<0.001**	$t_{3.117}$=0.466, *P*=0.672	
OM (%)	$t_{32.653}$=8.927, ***P*<0.001**	$t_{11.174}$=5.098, ***P*<0.01**	t_{22}=1.642, *P*=0.115	t_{52}=1.662, *P*=0.103
C/N	$t_{21.984}$=-3.267, ***P*<0.01**	t_{20}=2.465, ***P*<0.05**	t_{18}=1.453, *P*=0.163	t_{34}=2.041, ***P*<0.05**

Profiles of O_2, NH_4^+, and NO_3^- in the sediment

Oxygen penetrated deeper into the sediment in S1 than in S2 (Table 6.2, Fig. 6.1). Unrestored sediments of S2 had the smallest oxic layer, of on average 12mm, whereas unrestored sediments of S1 had 3x deeper sediment oxygen penetration. S1 sediments showed a peak in oxygen concentration a few millimetres below the sediment-water interface, indicating primary production by phytobenthos (Fig. 6.1).

S2 had higher porewater NH_4^+ concentrations than S1. In general, NH_4^+ concentrations increased with depth in the top 4cm of the sediment, after which it decreased again; another NH_4^+ peak was observed deeper in the sediment of S2 at around 11-14 cm depth. S1 had porewater NO_3^- concentrations ranging from 0.5 to 3 mg N l^{-1} whereas those in S2 were lower, ranging from 0 to 0.5 mg N l^{-1}. All nitrate profiles showed a decrease with depth in the top 6 cm of the sediment in S1 and the top 3 cm in S2 (Fig. 6.2).

Denitrification rates and nitrogen fluxes to the denitrification zone

Denitrification rates were highly variable, both within and among streams (Fig. 6.3A). In S1 the unrestored section had significantly higher denitrification rates than the restored section, with 3106 ± 3302 sd µmol N m^{-2} h^{-1} denitrification in the unrestored section and no detectable denitrification in the restored section. In S2 both sections had similar denitrification rates, averaging 230 ± 300 sd µmol N $m^{-2}h^{-1}$ in the unrestored and 270 ± 288 sd µmol N m^{-2} h^{-1} in the restored section.

To better understand variability in denitrification rates between both streams and within the 2 sections of each stream we estimated nitrate and ammonium transport to the denitrification zone (Table 6.4). In the unrestored section of S1 (S1U) seepage provided the largest source of nitrogen needed to sustain its high denitrification rates, whereas nitrogen diffusing from the water column was of minor importance. In the other stream sections, the major source of nitrogen to the denitrifiers was from the water column (Table 6.4). This result is supported by the ratios of $^{28}N_2$, $^{29}N_2$ and $^{30}N_2$ produced by denitrification (Fig. 6.3B). In S2 the fraction of N_2 species were in line with the theoretical ratio (based on the $^{14}NO_3/^{15}NO_3$ ratio in the water column), indicating that in this stream NO_3^- for denitrification was mainly derived from the water column. However, in S1U the

fraction $^{28}N_2$ produced was much higher, indicating significant transport of $^{14}NO_3^-$ from groundwater and/or nitrification.

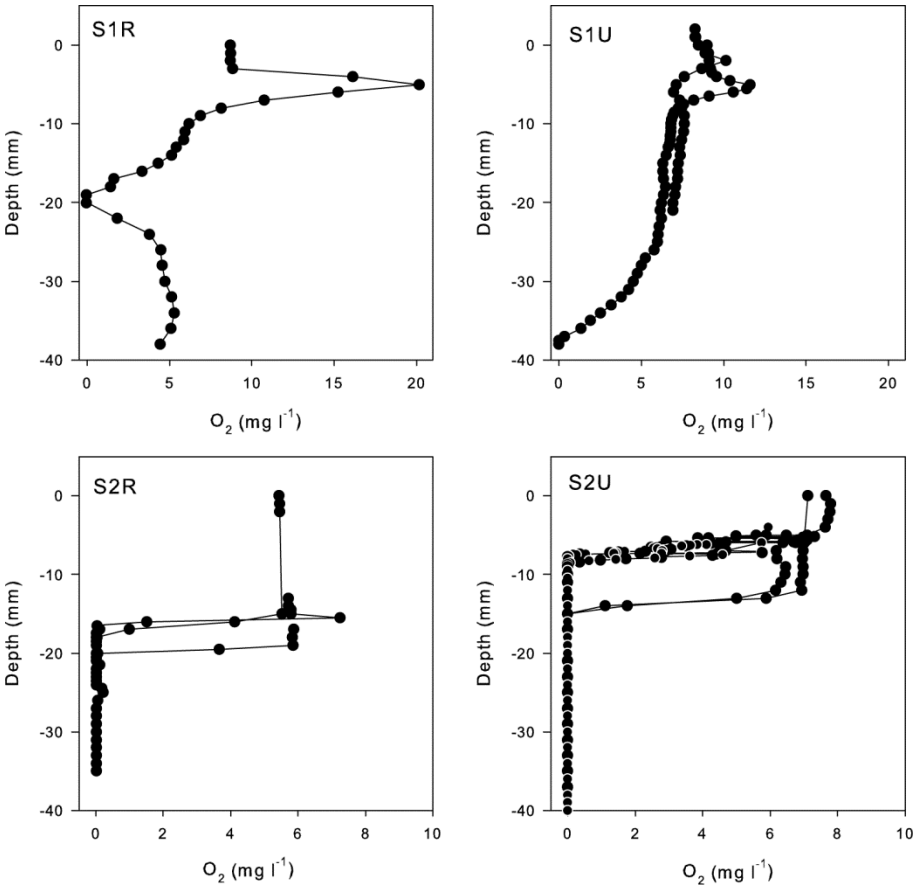

Figure 6.1. Dissolved oxygen profiles in the top 4cm of the sediment in unrestored (U) and restored (R) sections of both streams.

Table 6.4. Mean denitrification rates and nitrogen transport to the denitrification zone by groundwater seepage and diffusion from the water column (mg N m^{-2} h^{-1}).

	Stream 1		*Stream 2*	
	Unrestored	*Restored*	*Unrestored*	*Restored*
Denitrification rate	36.3	0.0	3.2	3.8
Seepage flux (NO_3+NH_4)	15.2	1.8	2.6	2.3
Estimated Diffusion flux NO_3	0.6	3.5	10.5	3.5

Composition and abundance of the nirK gene pool

Each stream had a distinct *nirK* community composition (Fig. 6.4). Only about 40% of the *nirK* OTUs were similar for both streams (Fig. 6.4). In S1 the restored section had a different *nirK* composition than the unrestored section (similarity of only 57%). By contrast, in S2 the unrestored and restored sections had a similar community composition, with higher similarity among both samples from the top layer of the sediment of the restored and unrestored section than among the samples within each section.

S2 had about twice as high *nirK* richness as S1 ($t_{3.386}$=-3.775, P<0.05, Fig. 6.5A), but we found no overall significant effect of restoration on *nirK* richness (t_6=-0.839, P=0.433; Figs. 6.4 & 6.5). Differences in *nirK* richness at the two different depths in the sediment were not significant (t_6=0.627, P=0.554).

NirK abundance ranged from 69 to $3.14*10^5$ copies gr. dry $sediment^{-1}$, and were on average around 70 times higher in S2 than in S1 (Fig. 6.5B). Lowest *nirK* gene copy numbers were found in stream S1R, which also had lowest denitrification rates. The top 2 cm of the sediment contained most denitrifiers: 4 times more than the underlying layer in S1, 33 times more than the underlying layer in S2.

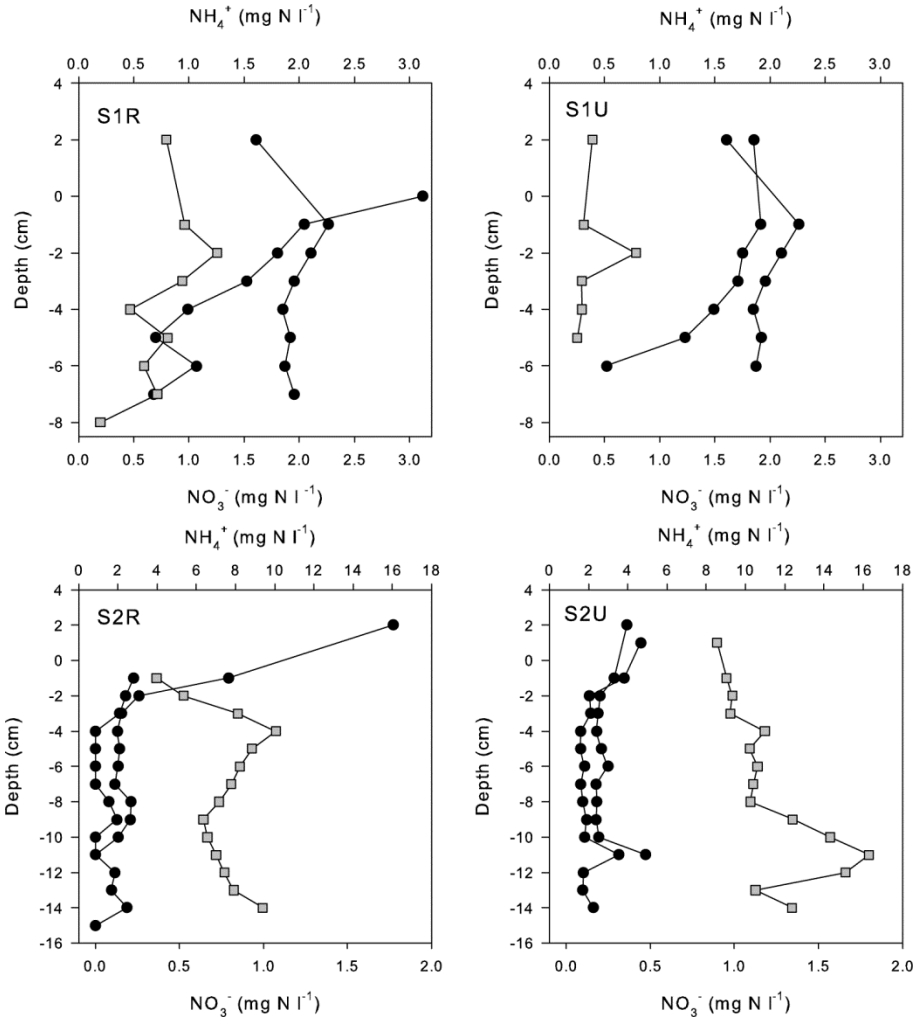

Figure 6.2. Profiles of NO_3^- and NH_4^+ in unrestored (U) and restored (R) sections of both streams. Black circles indicate NO_3^- profiles (2 profiles). Grey squares indicate NH_4^+ profiles (1 profile).

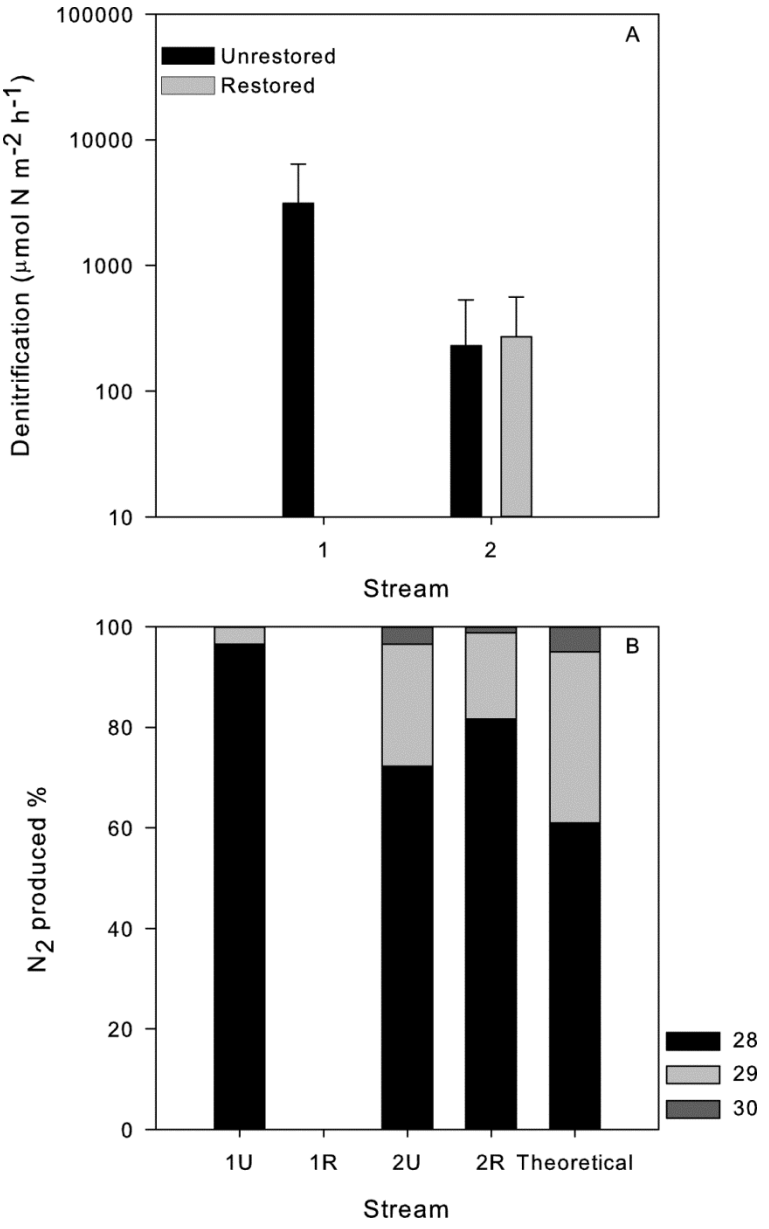

Figure 6.3. Denitrification (A) and fractions of $^{28}N_2$, $^{29}N_2$ and $^{30}N_2$ produced by denitrification (B) in unrestored (U) and restored (R) sections of both streams. Error bars denote standard deviations. Theoretical fractions are based on the $^{14}NO_3^-/^{15}NO_3^-$ ratio in the water column.

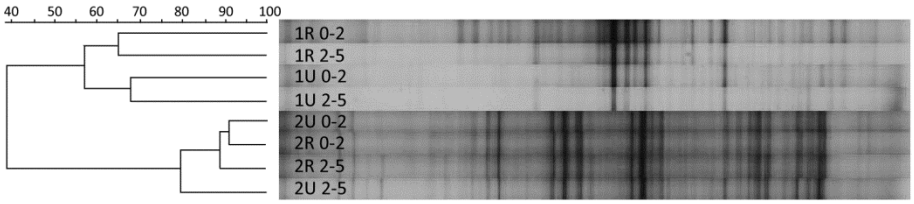

Figure 6.4. Clustering of unrestored (U) and restored (R) sections of both streams, based on DGGE analysis of PCR-amplified *nirK* gene pools. Each band is one operational taxonomic unit. Every stream section was sampled at two depths: 0-2 indicates the top 2 cm of the sediment, 2-5 indicates the underlying 3 cm of the sediment.

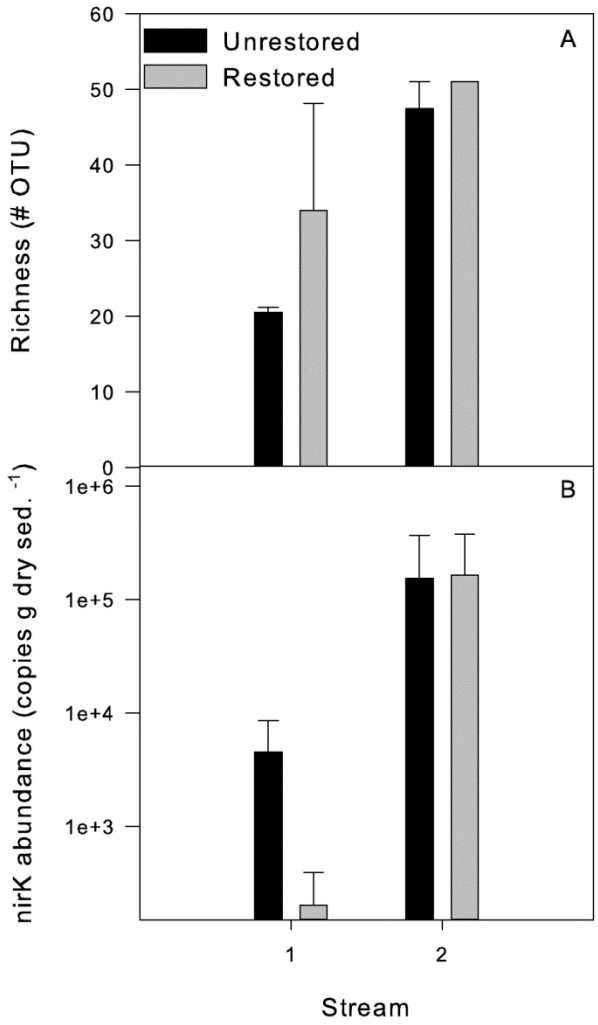

Figure 6.5. Denitrifying community characteristcs. A) Richness, based on nr. of bands representing operational taxonomic units (OTUs) and B) Abundance of the *nirK* gene obtained from quantitative PCR. Error bars indicate standard deviations.

Discussion

Denitrification rates in relation to hydrology, sediment conditions, and denitrifier (nirK) abundance, richness and community structure.

In this study we compared denitrification rates and factors affecting denitrification in restored and unrestored sections of two Danish streams. For stream S1 denitrification rates were significantly higher in the unrestored as compared to the restored section. In the other stream (S2) denitrification rates in the restored and unrestored section were similar. The difference in denitrification between the restored and unrestored section in S1 can be explained by differences in sediment characteristics and hydrology. The restored section, in which no denitrification was detected, had inorganic sediment, with a low C/N ratio and low amounts of fine sand. The absence of organic material as an electron donor probably explains why we did not detect any denitrification in this section. This is in line with the findings of Smith et al. (2009) and Lefebvre et al. (2004) who found highest denitrification rates in the finest sediments with most organic matter, in which the probability of anaerobic conditions is higher (Garcia-Ruiz et al., 1998; Martin et al., 2001). The low quantity of *nirK* gene copies in S1R, which is an indication of the streams intrinsic capacity of enzymatic denitrification, supports this finding. Likely, the inorganic sediment did not create favorable growth conditions for denitrifiers. Such a relation between in-stream denitrification and *nirK* abundance has recently also been found by Graham (2010).

The unrestored stream section of S1 had more organic matter, more denitrifiers (estimated from *nirK* copies) and higher proportions of fine sediment than the restored section. Furthermore, this stream section had 8 times higher seepage rates than the restored section, supplying the denitrification zone with nitrogen, which likely created denitrification hotspots in this stream section. Seepage has been found to considerably influence nitrogen biogeochemistry of streams and stream-riparian interfaces, and can create denitrification hotspots (Duval & Hill, 2007). In both sections of S2, downwards diffusion of nitrate from the water column into the sediment has likely provided nitrate to the denitrifiers, as also showed from the isotopic ratios of N_2 produced in denitrification.

The streams appeared to have different *nirK* community composition, with only about 40% similarity between the two. In S1, where differences in denitrification rates were most distinct, the restored and unrestored sections had

different denitrifying communities. However, in S2, where denitrification rates in the two sections were more similar, the community compositions of the restored and unrestored sections were similar. We did not find a strong richness-functioning relationship between denitrification rates and *nirK* richness. Other factors, like availability of organic matter, were more important in determining denitrification rates than diversity or community composition of the denitrifiers, which is in line with previous studies (Hallin et al., 2009; Attard et al., 2011) and may be explained by a high functional redundancy within denitrifying communities (Wertz et al., 2006), and the fact that genes are not always expressed, and thus functional, in the environment.

Conditions in the water columns of both streams

Restoration only moderately altered conditions in the water column; "natural" differences between both streams were larger than differences between restored and unrestored stream sections. Restored sections had more heterogeneous flow than the unrestored sections, resulting from more heterogeneous stream beds with alternating riffles and pools. Interestingly, the restored section of S1 had significantly lower DO and pH, indicating lower primary production, as compared to the unrestored section, whereas in S2 we found the opposite. These findings indicate that stream restoration may work out in different ways in terms of water quality. The overall effect of restoration will depend on both initial conditions and the nature and extent of the restoration measures.

Profiles of O_2, NH_4^+ and NO_3^- in the sediment

The concentrations of O_2, NH_4^+ and NO_3^- in the porewater differed between the streams, but were similar between restored and unrestored sections of each stream. Although concentrations were different, the profiles of both streams followed similar patterns. The observed profiles indicate the importance of oxygen in the sediment for nitrogen transformations in streams. Ammonium levels increased with depth in the sediment, whereas nitrate decreased. This indicates nitrification in the more oxygenated top layer of the sediment, but may in theory also indicate increased dissimilatory nitrate reduction to ammonium (DNRA) in the anoxic deeper layers of the sediment. However, occurrence of DNRA in these streams is unlikely, as this usually occurs when plenty of organic C is available,

because DNRA requires higher organic C to nitrate ratios than denitrification (Kelso et al., 1997; van de Leemput et al., 2011). The observed profiles are thus likely due to increased nitrification in the sediment top layer, and increased denitrification in the deeper layers.

Concluding remarks

Our findings indicate that stream restoration may work out in different ways for in-stream denitrification. Other studies found increased denitrification rates in restored streams, but with considerable variability in restoration designs (Kaushal et al., 2008). It is important to take into account that restoration has site-specific as well as wider-scale effects on nitrogen removal. For example, while denitrification per square meter may decrease due the removal of organic sediment, in-stream nitrogen removal may still increase if the remeandering increases the residence time. Effects of restoration will thus largely depend on the restoration design.

Our results highlight the importance of hydrologic conditions for in-stream denitrification. Stream restoration may not have the expected effect if stream hydrology is not considered. In addition, restoration may change the hydrologic characteristics of the stream, including upwelling and hydraulic conductivity of the sediment, which should be considered to reach the desired restoration effect.

Furthermore our results show that regeneration of the streambed, including organic matter accumulation and development of denitrifying communities, may take more than 5 years. The time needed to regain pre-restoration denitrification rates after sediment removal depends on organic matter deposition rates and vegetation development (Craft, 1996), which again depend on restoration design. In many situations, organic matter will be deposited in the floodplains during flood events, whereas little organic material accumulates in the streambed due to faster flow velocities, increasing the time needed to restore denitrification potential.

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on statistics. We thank Liz Palmer-Felgate for advice on the use of DET probes. This project was funded by the Danish Council for Strategic Research and is part of the MONITECH project.

Supplementary Information 6

Diffusive Equilibrium in Thin Films – Procedure

Profiles of NH_4^+ and NO_3^- in the porewater where made using Diffusive Equilibrium in Thin films (DET) probes (DGT research Ltd., Lancaster, UK, Krom et al. (1994)). Before deploying the probes they were placed in de-ionized water which was bubbled with N_2 for 12-24 h to minimize oxygen levels in the probe. The day before the denitrification measurements were performed, in each stream section (restored and unrestored) three probes were placed into the sediment, within one meter from the denitrification chambers. They were left in the sediment for at least 20 hours to equilibrate with the porewater. After equilibrating they were carefully removed from the sediment, and quickly sliced into 1 cm segments starting from the sediment water interface. Segments were placed in pre-weighed sterile vials, and stored cool (2 ^oC) and dark until further processing. After weighing the vials to determine segment mass, 2 ml of ultra-pure water was added and the samples were shaken at 160 rpm at 10 ^oC for 24 h to re-equilibrate the solutes in the gel with the added water. Ammonium in the equilibrated solution was measured on a spectrophotometer (UV-1700, Shimadzu, Kyoto, Japan) following Grasshoff (1983). Nitrate was measured on an ion-chromatograph (ICS-1500, Dionex, Sunnyvale, CA, USA).

Chapter 7

Predicting microbial nitrogen pathways from basic principles

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Abstract

Nitrogen compounds are transformed by a complicated network of competing geochemical processes or microbial pathways, each performed by a different ecological guild of microorganisms. Complete experimental unraveling of this network requires a prohibitive experimental effort. Here we present a simple model that predicts relative rates of hypothetical nitrogen pathways, based only on the stoichiometry and energy yield of the performed redox reaction, assuming competition for resources between alternative pathways. Simulating competing pathways in hypothetical freshwater and marine sediment situations, we surprisingly found that much of the variation observed in nature can simply be predicted from these basic principles. Investigating discrepancies between observations and predictions led to two important biochemical factors that may create barriers for the viability of pathways: enzymatic costs for long pathways and high ammonium activation energy. We hypothesize that some discrepancies can be explained by non-equilibrium dynamics. The model predicted a pathway that has not been discovered in nature yet: the dismutation of nitrite to the level of nitrate and dinitrogen gas.

Introduction

In nature, several competing microbial pathways are responsible for the transformation of nitrogen compounds. Each pathway is performed by a different guild of microorganisms under different environmental conditions. This network of microbial nitrogen pathways is known as the microbial nitrogen cycle, and includes pathways such as nitrification, denitrification, dissimilatory nitrate reduction to ammonium (DNRA, also called nitrate ammonification), anaerobic ammonium oxidation (anammox), nitrate reduction to nitrite, and nitrogen fixation (Fig. 7.1). The biogeochemical nitrogen cycle is affected intensely by human activity (Rockström et al., 2009), and a good understanding of the outcome of competition between nitrogen pathways as a function of the environmental conditions is essential if we wish to project the consequences of the human induced changes.

Previous studies have modeled the competitive strength of nitrogen pathways using a semi-empirical approach: activity rates of competing pathways were measured in experiments or in the field and used as input for models, to estimate nitrogen conversion rates under different environmental conditions (e.g. Spérandio & Queinnec, 2004; Liu et al., 2005; Canavan et al., 2007). However, because of the immense experimental effort required, no study so far has addressed all nitrogen pathways simultaneously. Besides, it could well be that our current inventory of nitrogen transforming pathways is still incomplete; for example, only very recently a pathway was discovered that dismutates nitric oxide into oxygen and nitrogen (Ettwig et al., 2010).

Here, we circumvent these problems by taking a completely different approach that may complement the semi-empirical results obtained so-far. We assume no prior knowledge on the microbial nitrogen cycle as we know it. Instead, following Broda's proposition that microbes may realize all energetically profitable pathways (Broda, 1977), we use the chemical properties of the nitrogen compounds in a simple energy-based model to predict the relative success of catabolic, thermodynamically feasible, pathways as a function of the environmental conditions. This may sound strange from an ecological perspective, as in nature organisms compete, not reactions. However, a functional approach that puts reactions rather than species or populations central is increasingly used in microbial ecology. For instance, locally collected samples of enzymes (metaproteomics) or genetic material (metagenomics) can give a clue of the reactions occurring on a site.

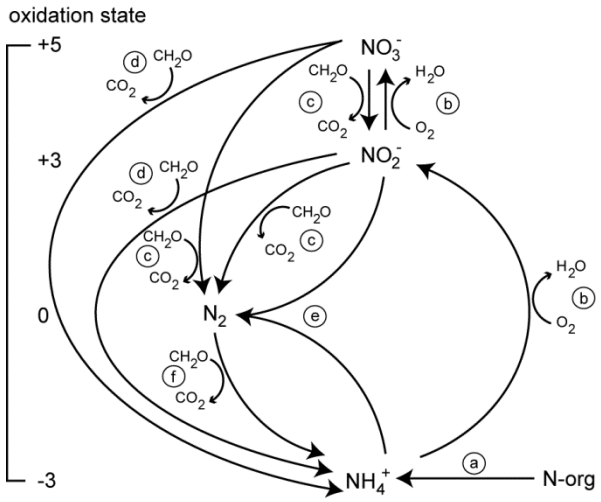

Figure 7.1. The microbial nitrogen cycle, involving known pathways between nitrogen compounds, oxygen and organic material: (a) mineralization, (b) nitrification in two steps: ammonium-oxidation and nitrite-oxidation, (c) denitrification from nitrite and nitrate, (d) dissimilatory nitrite and nitrate reduction to ammonium (DNRA), (e) anaerobic ammonium oxidation (anammox) and (f) nitrogen fixation.

We define differential equations for the dynamics of the substrates nitrate, nitrite, nitric oxide, nitrous oxide, ammonium, dinitrogen gas, organic material and oxygen. For simplicity the only non-nitrogen substrates considered are oxygen (as an alternative electron acceptor) and organic compounds (as an alternative electron donor). Furthermore, we define a differential equation for each thermodynamically feasible redox reaction, in other words a 'theoretical pathway'. The equations describe the growth of the 'volume' of that pathway as a function of its substrate affinity, determined by the stoichiometry of the reaction, and its energy yield. To make it less abstract one may think of the volume of a pathway as the biomass of a guild of microbes performing that particular pathway even though we do not model mass balances explicitly. Pathway volume is in this sense directly related to pathway activity. The mathematical formulation of our model is given in the Methods section and in Table 7.1.

Note that it is not our ambition to produce a model for accurate quantitative prediction of nitrogen dynamics in nature. Rather we seek to explore what is a minimum set of assumptions needed to explain which pathways are found in practice. More specifically we ask how much of the observed reactions can

be simply explained from resource competition, stoichiometry and energy yield. The model is based on three main assumptions.

Firstly, we assume that the rates of all transformations are controlled by the delivery of fresh substrates (by mass transport), rather than by microbial processing capacity (enzyme turnover). This is a reasonable assumption because most nitrogen conversion takes place in the chemocline where the delivery of fresh substrates is limiting (Halm et al., 2009; Jensen et al., 2009).

Secondly, we assume that the volume yield for each microbial guild depends linearly on the energy yield (the Gibbs free energy change) of the associated pathway. This linear relationship was previously shown to approximate reality (Liu et al., 2007). Some pathways are obviously less efficient than others in this sense (Tijhuis et al., 1993). However, for simplicity we neglect these differences here.

Thirdly, we assume for all microbial guilds that the substrate affinity is proportional to the stoichiometric factor, that is, the relative consumption of that substrate in that pathway. In reality, substrate affinity is mainly determined by the flux of substrate towards the single cells and is therefore proportional to the stoichiometric factor as well as the cell size (Schulz & Jørgensen, 2001). However, assuming cell size to be roughly equal for each microbial guild, we neglect this source of variation.

In addition to these three assumptions, we impose a thermodynamic restriction on pathways consuming ammonium, because of its high activation energy (Strous et al., 1999; Dosta et al., 2008). Both anammox and nitrification need additional electrons for the ammonium reaction, which are transferred from reactions later in the pathway (Fig. 7.2). Anammox bacteria use a NO radical to activate ammonium to the level of hydrazine (N_2H_4) (Fig. 7.2A) (Strous et al., 2006), while nitrifying bacteria oxidize ammonium directly with activated oxygen yielding hydroxylamine (NH_2OH) (Fig. 7.2B). It is therefore assumed that ammonium can only be activated directly by a reactive species (Hooper et al., 2004). In our model, a theoretical pathway is considered thermodynamically feasible when each ammonium molecule can react at a one-to-one ratio with a reactive chemical species (e.g. NO or O_2) as the primary substrate or intermediate.

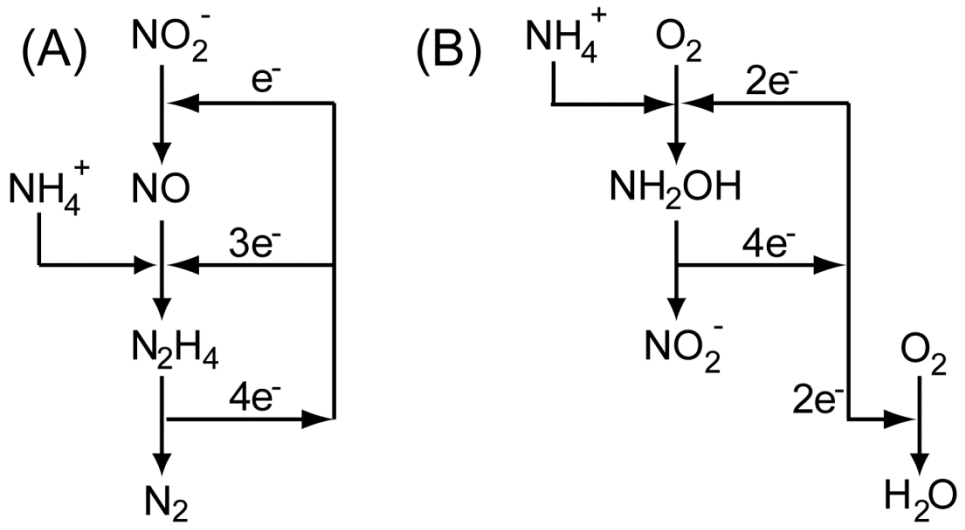

Figure 7.2. Microbial ammonium oxidation pathways of: (A) anammox (Strous *et al.* 2006) and (B) nitrification (Ferguson, 2007). Anammox bacteria make use of the nitric oxide radical to activate ammonium directly to the level of hydrazine while nitrifying bacteria use activated oxygen to react with ammonium directly to hydroxylamine. It is hypothesized that ammonium has to react with a reactive species at a one-to-one ratio, to overcome its high activation energy. This could explain why electrons are transported from other oxidation reactions in the pathways to the ammonium oxidation step.

Table 7.1. Equations and parameters of the model.

Description	*Model Equations*			
Nutrient concentration (in 'molS')	$\frac{dN_k}{dt} = i_k - \sum_{j=1} s_{kj}\ M_j\ P_j + \sum_{j=1} p_{kj}\ M_j\ P_j - eN$		*k*=1,..8, *j*=1,...45	(1)
Electron transfer rate (in 'molP'$^{-1}$ t^{-1})	$M_j = r\left(\frac{[N_1]}{[N_1]+s_{1,j}}\right)\left(\frac{[N_2]}{[N_2]+s_{2,j}}\right)$		*j*=1,..45	(2)
Pathway volume (in 'mol P')	$\frac{dP_j}{dt} = b\Delta G_j M_j P_j - dP_j$		*j*=1,..45	(3)
Parameters	*Description*	*Value*		*Units*
ΔG_j	Gibbs free energy yield for the transfer of one electron, for pathway *j*	Table S1		kJ
s_{kj} and p_{kj}	Stoichiometric factor for nutrient *k,* for pathway *j*	Table S1		'molS'
i_k	Inflow of nutrient *k*	0-10		'molS' t^{-1}
e	Outflow rate	1		t^{-1}
r	Maximum electron transfer rate	1		'molP'$^{-1}$ t^{-1}
b	Energy-to-volume-conversion rate	-0.1		'molP' kJ^{-1}
d	Maintenance costs	1		t^{-1}

These assumptions led to a model that allows us to simulate the competition of thermodynamically feasible pathways simultaneously. The only inputs to this model are the continuous inflow of nitrogen compounds, oxygen and organic material into the system. For simplicity, organic material is assumed to be completely labile. We did not consider the inflow of nitrous oxide or nitric oxide, as the concentrations of these compounds are typically too low to contribute significantly to mass balances of nitrogen. Thus, nitric oxide and nitrous oxide in the model are only available if produced.

A dynamic equilibrium arises when pathway growth equalizes decay and continuous inflow and outflow equalize both consumption and production. The model outputs are the volume of each theoretical pathway and the final concentration of each nutrient, after stabilization of the system.

Table 7.2. The 11 predicted viable nitrogen pathways out of the 45 thermodynamically feasible pathways. We considered a pathway viable with an equilibrium activity higher than 0.001 in at least one of the 10.000 simulations of the model with random inflow levels of nitrate, nitrite, ammonium, dinitrogen gas, oxygen and organic material.

	Pathway	*Reaction*
1	Anammox	$NO_2^- + NH_4^+ \rightarrow N_2 + 2\ H_2O$
2	Denitrification NO_2^-	$4\ NO_2^- + 3\ CH_2O + 4\ H^+ \rightarrow 2\ N_2 + 3\ CO_2 + 5\ H_2O$
3	Denitrification NO_3^-	$4\ NO_3^- + 5\ CH_2O + 4\ H^+ \rightarrow 2\ N_2 + 5\ CO_2 + 7\ H_2O$
4	DNRA NO_2^-	$2\ NO_2^- + 3\ CH_2O + 4\ H^+ \rightarrow 2\ NH_4^+ + 3\ CO_2 + H_2O$
5	DNRA NO_3^-	$NO_3^- + 2\ CH_2O + 2\ H^+ \rightarrow NH_4^+ + 2\ CO_2 + H_2O$
6	Heterotrophic N_2 fix.	$2\ N_2 + 3\ CH_2O + 4\ H^+ + 3\ H_2O \rightarrow 4\ NH_4^+ + 3\ CO_2$
7	Nitrification (NH_4^+-ox.)	$2\ NH_4^+ + 3\ O_2 \rightarrow 2\ NO_2^- + 4\ H^+ + 2\ H_2O$
8	Nitrification (NO_2^--ox.)	$2\ NO_2^- + O_2 \rightarrow 2\ NO_3^-$
9	Respiration	$CH_2O + O_2 \rightarrow CO_2 + H_2O$
10	'Total nitrification'	$NH_4^+ + 2\ O_2 \rightarrow NO_3^- + 2\ H^+ + H_2O$
11	'Nitrite dismutation'	$5\ NO_2^- + 2\ H^+ \rightarrow 3\ NO_3^- + N_2 + H_2O$

Results

Even if a pathway could take place in isolation (i.e. the pathway is 'feasible'), this does not mean that it is 'viable' in the sense that it would yield a positive volume in a situation where it is competing with other pathways. In order to screen the overall viability of the theoretical pathways, the model was run for 10.000 random sets of substrate inflow rates. From all 45 thermodynamically feasible pathways, 11 were found to be viable in this analysis (Table 7.2). Most pathways known to occur in nature are covered by this theoretically predicted list (Table 7.2: Eqns. 1-9). This suggests that the thermodynamic principles implemented may indeed explain much of the patterns observed in reality. The model predicted two pathways that have not been discovered in nature: complete nitrification from ammonium to nitrate by a single organism, which we call 'total nitrification' (Table 7.2: Eqn.10), and simultaneous oxidation and reduction of nitrite, producing nitrate and dinitrogen gas, which we call 'nitrite dismutation' (Table 7.2: Eqn. 11).

In order to compare the model predictions with patterns in natural systems, we defined a number of hypothetical environments in terms of their relative inflow of ammonium, organic material, nitrate and nitrite. To have a concrete situation in mind we think of these environments as freshwater and marine sediments. We chose the conditions in such a way that each viable theoretical pathway in the model can be discussed. Then, we simply assume a sediment to have a linear gradient of oxygen inflow. For pristine freshwater and marine sediments (i.e. without any anthropogenic inflow), we assumed a constant inflow of organic material. The only nitrogen source was ammonium, assumed to be produced by mineralization (Fig. 7.3A and 7.4A). For freshwater sediments the inflow of organic material was assumed to be five times higher than ammonium, while in marine sediments it was assumed to be five times lower, due to the activity of for example sulphate reducers (Fig. 7.4A). We considered both nitrate and nitrite inflow for eutrophic freshwater sediments (Fig. 7.3B, C and D), and only nitrate inflow for the eutrophic (e.g. coastal) marine sediment (Fig. 7.4B).

Along the oxygen inflow gradient in each hypothetical sediment, the model produced stable estimates of: the relative volume of each pathway (Fig. 7.3 and 7.4: Pathway volume), the relative concentration of each substrate and product (Fig. 7.3 and 7.4: Nutrient concentration),

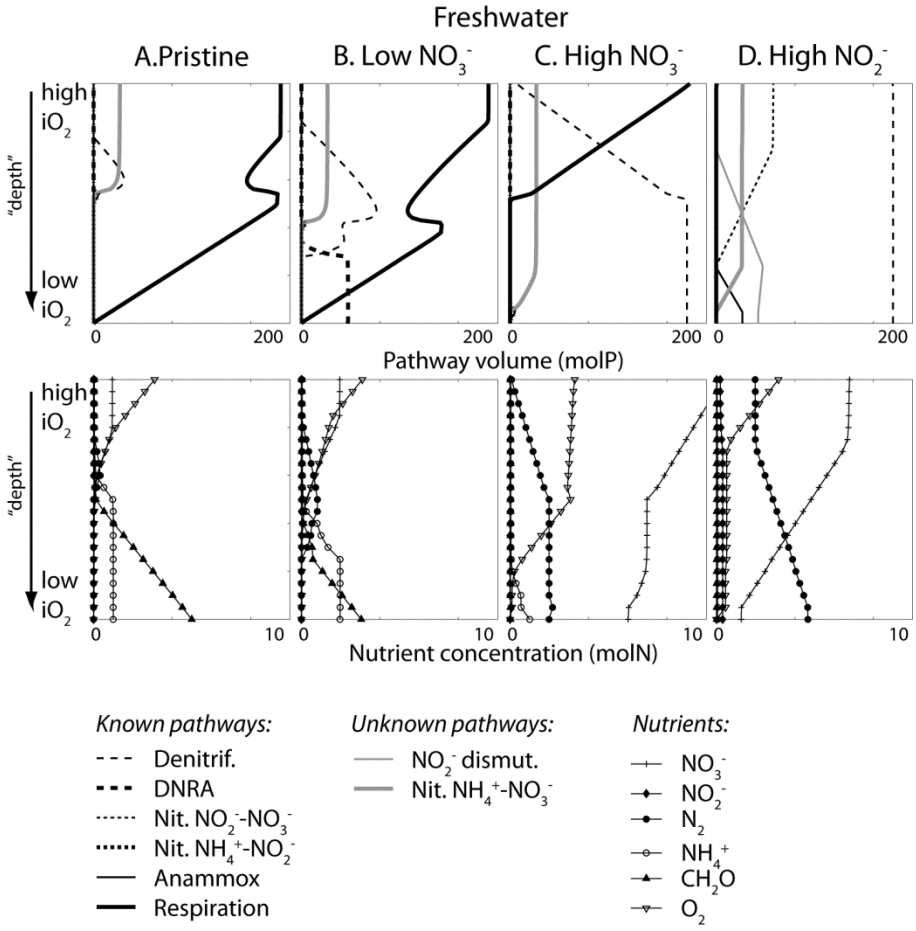

Figure 7.3. Competition simulations of the model representing four hypothetical freshwater sediments. Steady state conditions, for each simulation, of pathway volume (activity), nutrient concentration, and percentage of nutrient inflow consumed per pathway for different combinations of nutrient inflow levels along a gradient of oxygen inflow. A: Pristine sediment (iNO_3^-=0, iNO_2^-=0, iNH_4^+=1, iCH_2O=5), B: Eutrophic (iNO_3^- low) sediment (iNO_3^-=1, iNO_2^-=0, iNH_4^+=1, iCH_2O=5), C: Eutrophic (iNO_3^- high) sediment (iNO_3^-=10, iNO_2^-=0, iNH_4^+=1, iCH_2O=5), D: Eutrophic (iNO_2^- high) sediment (iNO_3^-=0, iNO_2^-=10, iNH_4^+=1, iCH_2O=5)

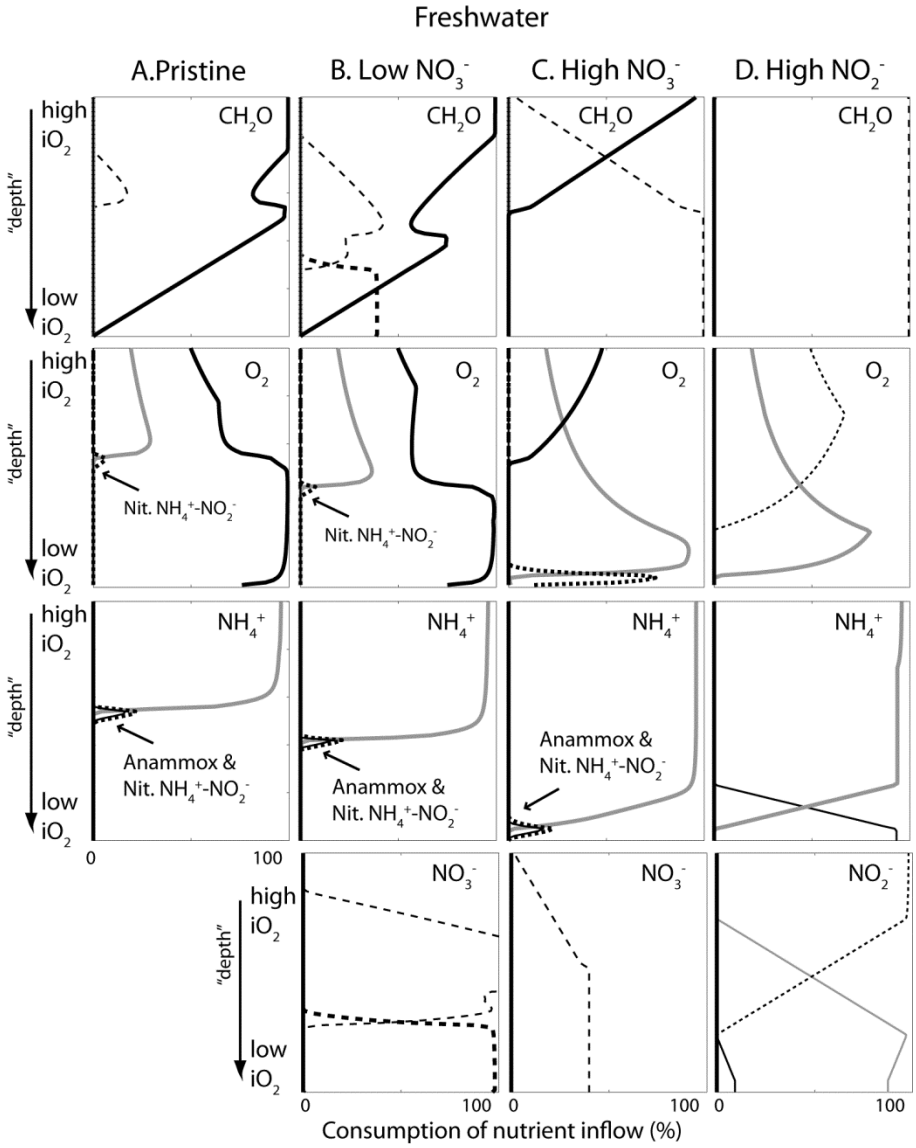

Figure 7.3 (Continued)

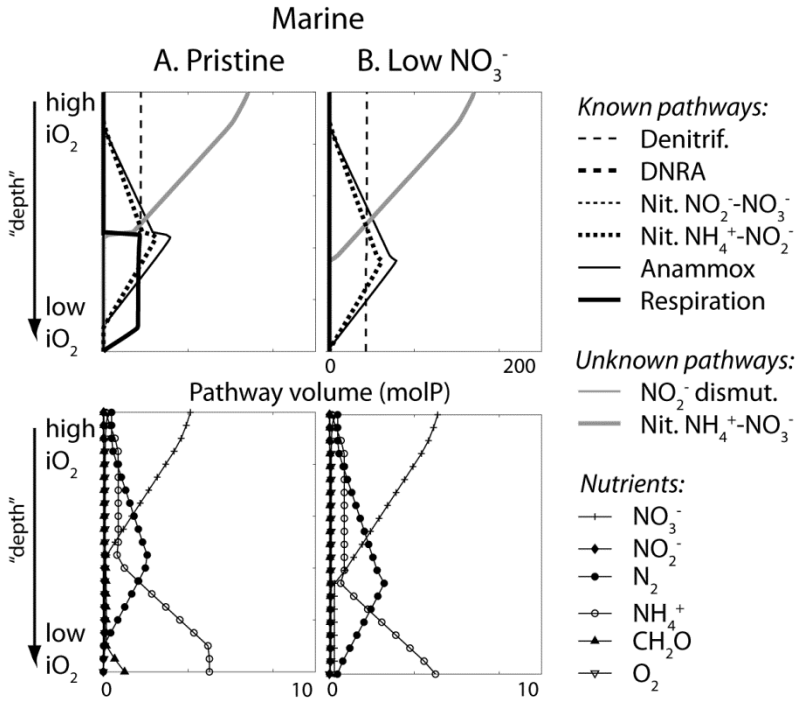

Figure 7.4. Competition simulations of the model representing two hypothetical marine sediments (as in Fig. 7.3). A: Pristine sediment (iNO_3^-=0, iNO_2^-=0, iNH_4^+=5, iCH_2O=1), B: Eutrophic (iNO_3^- low) sediment (iNO_3^-=1, iNO_2^-=0, iNH_4^+=5, iCH_2O=1).

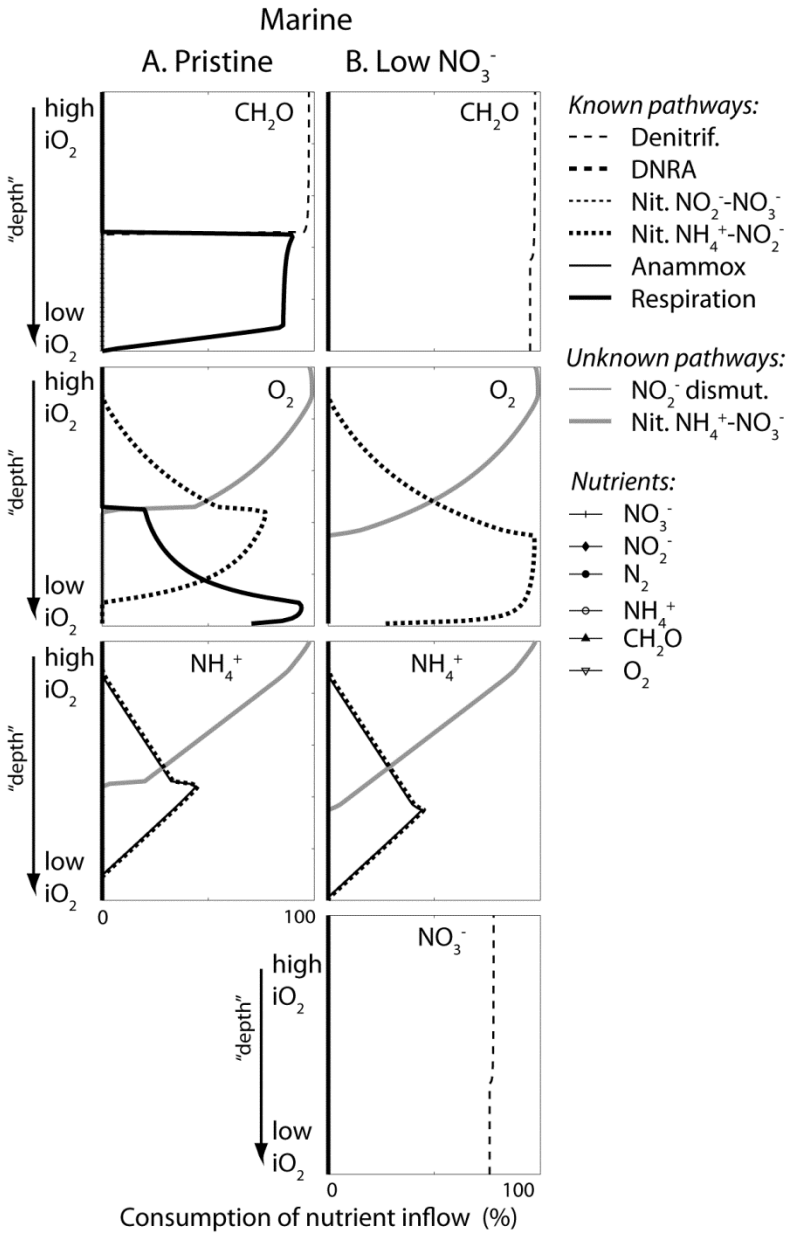

Figure 7.4. (Continued)

and the percentage of substrate uptake relative to the inflow per pathway (Fig. 7.3 and 7.4: Consumption of nutrient inflow; CH_2O, O_2, NH_4^+, NO_2^- and NO_3^-).

In a pristine freshwater sediment, the model predicted dominance of aerobic respiration, total nitrification and denitrification (Fig. 7.3A: Pathway volume). At high oxygen inflow, the equilibrium oxygen concentration was high, while at low oxygen inflow, anoxic conditions were established (Fig. 7.3A: Nutrient concentration). This was mainly due to the activity of the aerobic respiration pathway (Fig. 7.3A: O_2). At high oxygen inflow, organic material (Fig. 7.3A: CH_2O) and ammonium (Fig. 7.3A: NH_4^+) were consumed by aerobic respiration and nitrification pathways, while both substrates accumulated under low oxygen levels (Fig. 7.3A: Nutrient concentration). At intermediate oxygen inflow levels, where the equilibrium oxygen concentration was low (Fig. 7.3A: Nutrient concentration), nitrification was predicted to be coupled to denitrification (Fig. 7.3A: Pathway volume). At the oxic-anoxic boundary, some partial nitrification to the level of nitrite coupled to anammox activity was predicted (Fig. 7.3A: O_2 and NH_4^+).

Freshwater sediments with an inflow of nitrate (Fig. 7.3B and C) were predicted to be dominated by nitrification and aerobic respiration at high oxygen levels, and by denitrification and DNRA at low oxygen levels. At relatively low nitrate inflow levels, DNRA outcompeted denitrification at low oxygen inflow levels (Fig. 7.3B), while at high nitrate inflow levels, DNRA was outcompeted by denitrification along the whole oxygen inflow gradient (Fig. 7.3C). The amount of nitrate inflow influenced the location of the oxic-anoxic boundary. This can be understood from the competition for substrates. At high nitrate and low oxygen inflow, denitrification wins the competition from respiration and completely consumes the available organic material. The available oxygen is then consumed by nitrification. However, ammonium is more limited than organic material, so the available oxygen is not completely consumed by the nitrification pathway. Therefore, the depth at which positive oxygen levels may be found (the thickness of the oxic layer) increased with nitrate inflow levels.

A freshwater sediment with a high inflow level of nitrite (Fig. 7.3D) and high inflow levels of oxygen, was predicted to be dominated both by nitrification from nitrite to nitrate and by total nitrification. In the layers with lower oxygen inflow levels, the unknown 'nitrite dismutation' pathway consumed the available nitrite. At the lowest levels, anammox was predicted to coexist with this pathway.

A pristine marine sediment (Fig. 7.4A) was predicted to be dominated by total nitrification coupled to denitrification at high oxygen inflow levels, shifting to

ammonium-to-nitrite nitrification coupled to anammox with decreasing oxygen inflow. Due to high ammonium levels, oxygen was the limiting substrate over the whole inflow gradient, so anoxic conditions were created even at high oxygen inflow levels.

A marine sediment with an inflow of nitrate (Fig. 7.4B) showed similar pathway activity rates as a pristine marine sediment, only denitrification rates were higher at low oxygen levels. Even higher nitrate inflow levels in the simulated marine sediments showed the same pathway activity rates, because nitrate was not a limiting substrate (results not shown).

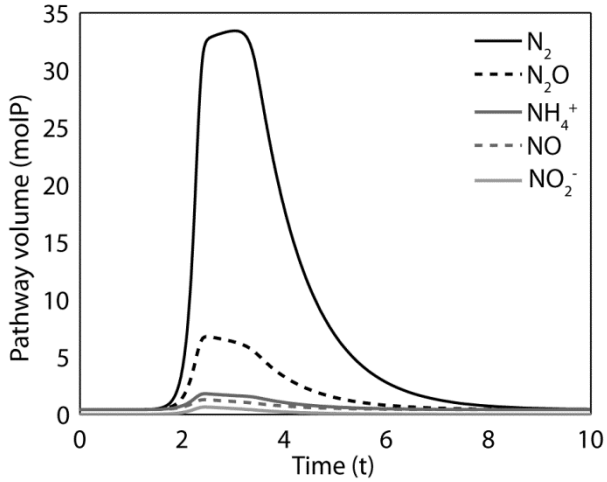

Figure 7.5. Activity of nitrate reduction pathways to the level of N_2, N_2O, NO, NH_4^+ and NO_2^- in time after a pulse of nitrate (iNO_3^-=10) at t=1 (iNO_2^-=0, iNH_4^+=0, iO_2=0, iCH_2O=10).

Because truncated denitrification to the level of nitrite, nitric oxide or nitrous oxide, were not predicted to be viable pathways (Table 7.2), we followed the non-equilibrium dynamics of the truncated denitrification pathways. We simulated a pulse of nitrate influx in an anoxic, organic environment and followed the pathway activity in time (Fig. 7.5). As long as both substrates were not limited, denitrification to the level of nitrite, nitric oxide and nitrous oxide were predicted. However, when pathways started to compete (at t≈2.4), the activity rate of denitrification to the level of dinitrogen gas continued increasing, while the activity of other pathways decreased.

To test if our results are sensitive to the implemented limitation on ammonium activation, we explored the outcome of the model without this restriction. Two additional strong ammonium-consuming pathways were predicted: direct ammonium oxidation with oxygen to the level of dinitrogen gas (as a single redox reaction) and anammox using nitrate (Fig. S7.1 and S7.2). None of these pathways have been found in nature, suggesting that, indeed, ammonium oxidation requires a one-to-one reactive species.

Discussion

The model predicted the viability of almost all nitrogen pathways known to exist in nature (Table 7.2). Interestingly, this suggests that the combination of basic thermodynamic principles (stoichiometry, energy yield), and competition for substrates can already explain why most pathways are found in practice. In other words, these principles may largely determine the competitive strength of a pathway.

Strikingly, our basic principles model also predicted the viability of two unknown pathways, namely 'nitrite dismutation' and 'total nitrification' (Table 7.2). One possibility is that these pathways do exist in nature but are not discovered. A likely alternative explanation for their absence in observations is that other biochemical restrictions may impact the viability of these pathways. Some pathways were not predicted by the model, but observed in nature, such as the truncated denitrification pathways. Here, we first compare the activity of known pathways for model simulations and for measurements in natural systems, then we discuss the possible existence of the two unknown pathways predicted by the model.

In the hypothetical sediments oxygen inflow was modeled as a gradient. This gradient can be thought of as sediment depth, with high oxygen inflow close to the sediment water interface. However, it is important to realize that oxygen inflow layers were modeled separately, so we did not consider diffusion of substrates through the modeled layers or porosity of the sediment. The model predictions show many similarities, but also show some deviations from what is found in nature (Fig. 7.3). These deviations may give us a hint about additional biochemical limitations or factors influencing the activity of a certain pathway. Here, we discuss for each predicted viable pathway how the model results relate to observations in nature.

DNRA is only predicted by the model in eutrophic organic sediments, when nitrate inflow is not too high (Fig.7.3B). In nature, DNRA is indeed encountered at high C:NO_3^- ratios, which is explained by the fact that DNRA, despite having a lower energy yield, consumes less nitrate per C than denitrification (Table 7.2) (Tiedje, 1988; Kelso et al., 1997). Also, DNRA is only predicted at strictly anoxic conditions, whereas denitrification is predicted at both low and high equilibrium oxygen concentrations (Fig. 7.3B,C and 7.4B). In natural environments, DNRA is indeed measured under anoxic conditions and in deeper sediment layers than denitrification (Buresh & Patrick, 1981; Jørgensen, 1989; Kelso et al., 1997). Most denitrification activity is reported in anoxic or suboxic conditions (Seitzinger et al., 2006).The model suggests that denitrification could thrive in oxic conditions, when nitrate co-occurs with oxygen (Fig.7.3C), as recently found by Gao *et al.* (2010). The lack of predicted DNRA activity in marine sediments, could be due to the fact that we did not consider sulfur compounds, while in natural systems, DNRA activity can be coupled to sulfur cycling (Brunet & Garcia-Gil, 1996; An & Gardner, 2002).

Anammox is mainly predicted to occur coupled to partial nitrification, due to the necessity of nitrite. In the modeled freshwater sediments, both pathways are only found at the oxic-anoxic boundary at very low rates (Fig.7.3A, B, and C), while in the marine sediments, they are predicted over a larger range of depth under anoxic conditions. This follows from the competition for oxygen between nitrification and respiration: when ammonium levels are high compared to organic material levels (i.e. marine sediment), partial nitrification can flourish along a large range of depth by consuming the incoming oxygen and ammonium, creating anoxia, and supplying anammox with nitrite (Fig.7.4A and B). Anammox coupled to partial nitrification is indeed a process used in ammonium-rich wastewater treatment under oxygen limitation (Sliekers et al., 2002; Jetten et al., 2003). Not much evidence has been found for anammox in natural freshwater systems. Schubert *et al*. (2006), however, showed anammox activity at the oxic-anoxic boundary of the water column of a large lake. In marine environments, anammox is indeed found at high rates (Dalsgaard et al., 2005; Brandes et al., 2007) under strictly anoxic conditions (Kuypers et al., 2003; Dalsgaard et al., 2005; Schmid et al., 2007). While anammox is expected to have a high global contribution to dinitrogen production (Strous & Jetten, 2004), under laboratory conditions, anammox has a very low growth rate (Strous et al., 1999). This cannot be explained by the model.

This suggests that the low growth rate may be due to other factors than thermodynamic feasibility or resource competition.

The model predicts that partial nitrification may win the competition over total nitrification in marine sediments when oxygen is more limited than ammonium (Fig.7.4A and B), because partial nitrification consumes less oxygen per unit of N. Experiments indeed show that partial nitrification is supported by high ammonium inflow and relatively low oxygen conditions (Ciudad et al., 2005). At high ammonium and oxygen inflow levels, total nitrification is predicted to feed denitrification with nitrate (Fig. 7.4A and B). Nitrification-denitrification coupling is indeed a common process found in oxic sediments and close to macrophytes (Risgaard-Petersen et al., 1994; Eriksson & Weisner, 1999). However, as discussed before, total nitrification is in nature performed in two steps by two different pathways.

One of the unknown pathways predicted by our model, the 'nitrite dismutation' pathway, has already been suggested as a potential microbial pathway by Strohm *et al.* (2007), based on its energy yield. Generally, nitrite input in nature is much lower than nitrate input, therefore the chance to encounter this pathway is expected to be low. However, our model suggests that 'nitrite dismutation' could be directly coupled to denitrification, through nitrate (in the presence of organic material). Therefore, we suggest that it might have been overlooked in natural environments. If this pathway has evolved, we speculate that, based on our model results, 'nitrite dismutation' could be found in deeper, anoxic, layers of freshwater systems with a high inflow of nitrite.

From the viability tests and the above comparison of predicted conditions with observed conditions, we can deduce that resource competition proves to be a crucial model ingredient to understand why pathways are active in certain conditions. In our model pathways and substrates are interacting dynamically, so each pathway can both limit substrate availability (i.e. anoxia), and create conditions in which other pathways can flourish (i.e. coupled nitrification-denitrification). As a result, the ecological fitness of each pathway is determined by its efficiency of consuming substrates.

There is, however, a conspicuous deviation between the model predictions and the patterns observed in nature. Our model predicted the highest fitness for the longest pathways, whereas this is not always the case in nature. For instance, complete denitrification in the model always outcompeted the truncated denitrification found in nature. In addition, the second unknown pathway predicted

by our model, total nitrification, existed side by side in the simulations with ammonium oxidation to nitrite, while nitrification from nitrite was not predicted at all. What might explain such differences between our theoretical prediction and reality?

Interestingly, after a pulse of nitrate, activity of denitrification pathways to the level of nitrous oxide, nitric oxide and nitrite is temporarily high (Fig. 7.5). This result nicely shows that during an ecological feast, when resources are abundant, the selective forces are weak, as there is hardly any competition. Experiments indeed show higher denitrification activity to the level of nitrous oxide with high nitrate concentrations (Firestone et al., 1980). In our former analyses, we considered only equilibrium conditions (arising when at least one of the substrates becomes limited). Thus, the pulse simulation illustrates that the lack of dynamic conditions (feast and famine) may explain why these analyses do not predict truncated denitrification pathways.

Still, such non-equilibrium dynamics do not appear to explain the fact that total nitrification is not observed in nature. Costa et al. (2006) suggested that the more steps are included in a pathway, the more enzymes and (possibly toxic) intermediates are involved, which makes long pathways less beneficial than short pathways. We explored whether this could explain the lack of total nitrification in nature, by adapting individual maintenance costs relative to an estimated number of steps in a pathway (Supplementary Information 7). Indeed, we found a parameter range of pathway length costs where total nitrification was always outcompeted by the two partial nitrification pathways and where nitrate to nitrite reduction was additionally predicted at high nitrate inflow levels. However, for that parameter range, anammox showed relatively high activity, also in oxic conditions, while denitrification and DNRA were relatively weak competitors (Fig. S7.3 and S7.4). This suggests that costs may indeed be an important factor influencing the fitness of long pathways, and thus the existence of pathways like total nitrification. However, assuming a linear relation between the number of enzymes and costs is probably too simplistic. Obviously, differences in regulatory mechanisms, protein sizes, and the level of toxicity of intermediates may also play a role.

Apart from the two exceptions described, our analysis suggests that the current inventory of nitrogen cycle pathways may be complete. However, many of the other pathways of table S7.1 that were predicted to have low fitness today may still have been important in the past, before the evolution of the currently observed pathways. For example, it has been suggested that the oxidation and

reduction of toxic nitrogen cycle intermediates such as nitric oxide was a prerequisite for the evolution of the current nitrogen cycle processes (Klotz et al., 2008; Klotz & Stein, 2008). In our model systems such toxic compounds never accumulated, only occurred as intermediates or were consumed by other processes. Therefore, there was no need to consider toxicity or inhibition in our model.

Obviously, many relevant aspects were excluded or highly simplified in our model. For instance, we do not account for the inflow of alternative electron donors or acceptors, such as S-, and Fe- compounds (Brunet & Garcia-Gil, 1996; Weber et al., 2001; Weber et al., 2006), or the effect of temperature on pathway activity (Tijhuis et al., 1993). Also, we did not consider environmental variation in inflow of substrates, such as the availability and quality of organic material which could affect the activity of the nitrogen pathways (Burford & Bremner, 1975). Inclusions of such aspects in future models may allow a more complete prediction of theoretical pathways. However, our minimal model analysis suggests that much of the seemingly complex repertoire of nitrogen pathways in nature may be understood from a few simple basic principles.

Conclusions

The realism of the results we obtained suggests that our minimal model may capture much of the essence of what drives microbial nitrogen processes in the real world. Our findings imply that the fitness of a catabolic nitrogen pathway may be determined largely by stoichiometry and energy yield of the performed redox reaction, and that the activity of each pathway at certain environmental conditions can simply be explained from competition for limited resources.

The few discrepancies between predictions and observations hint at the importance of non-equilibrium dynamics and of biochemical barriers that may exclude certain nitrogen pathways, such as high ammonium activation energy and costs relative to pathway length. An interesting remaining discrepancy is the prediction of the dismutation of nitrite, to the level of nitrate and dinitrogen gas. We suggest that this could well be a viable, yet undiscovered, pathway that might well play a role in systems with high nitrite and low oxygen levels.

Methods

Theoretical pathways

We created a list of theoretical pathways (Table S7.1) in several steps. First, we determined all possible half reactions involving organic material (CH_2O), oxygen (O_2) and the nitrogen compounds nitrate (NO_3^-), nitrite (NO_2^-), nitric oxide (NO), nitrous oxide (N_2O), dinitrogen gas (N_2) and ammonium (NH_4^+). Then, we combined the electron accepting and electron donating half reactions resulting in a complete list of possible redox reactions. We then removed redox reactions with overlapping intermediates in both half reactions, because if a reductant of a half reaction is the oxidant in the complementary half reaction and vice versa, the reaction cannot proceed. Finally, we calculated the Gibbs free energy change per electron transfer for each reaction, by subtracting the total standard formation energy of substrates from the total standard formation energy of products, for standard conditions: 1 atm pressure, 1 M concentration, pH =7 (as in Madigan, 2003). For organic material, we used the standard formation energy of glucose. Reactions with a negative Gibbs free energy change, and thus energy yield, were included in the final pathway list. This resulted in 62 thermodynamically possible pathways (Table S7.1).

The list of theoretical pathways was modified to include the ammonium activation restriction. Pathways in which NH_4^+ is not oxidized by O_2 as the primary substrate, or NO as an intermediate or primary substrate, were removed from the list. NO was considered an intermediate or primary substrate when the oxidation state of the substrate was lower or the oxidation state of the product was higher or equal than that of NO (+2). Pathways that could be activated by O_2 or NO, were removed from the list when the [oxidizer: NH_4^+] ratio was smaller than 1. The final model consisted of 45 pathways (Table S7.1).

Model description

Our model is an ordinary differential equation (ODE) model with 53 differential equations, namely 45 competing theoretical pathways and 8 nutrients (NO_3^-, NO_2^-, NO, N_2O, N_2, NH_4^+, O_2 and CH_2O). The model equations and parameters are given in table 2.

In this study, we are interested in relative rather than absolute differences between pathway volume and between nutrient concentrations, therefore the units of these variables are undefined, and called 'molP' and 'molS'. For the same reason, most general parameters applying to all pathways were simply set to one. The individual pathway parameters are the nutrient concentrations involved in the transfer of one electron (*s*), and the energy yield for the transfer of one electron (ΔG). Both parameters were derived from the theoretical pathways list (Table S7.1).

Nutrients (*N*) are assumed to flow through the system with a constant inflow (*i*) and a concentration dependent outflow rate (*e*), while being consumed and produced by the theoretical pathways, like a chemostat (Table 7.1: Eqn. 1). Nutrient consumption and production of each pathway are calculated by multiplying the nutrient concentration involved in the transfer of one electron (*s* and *p*), the electron transfer rate per molP (*M*) and the pathway volume (*P*).

The electron transfer rate per molP (*M*) for each pathway is assumed to be limited by mass transfer. We captured this in the model by implementing double Monod kinetics, with the nutrient concentration of the substrates involved in the transfer of one electron (*s*) as the half saturation factor (Table 7.1: Eqn. 2). In this way, the maximum electron transfer rate (*r*) is reached at lower nutrient concentrations when nutrient consumption per electron is low.

The growth of each pathway per molP is calculated by multiplying the energy yield for the transfer of one electron (ΔG), the electron transfer rate per

molP (*M*), and the pathway volume (*P*). Maintenance costs (*d*) are considered constant per molP (Table 7.1: Eqn. 3). Because energy yield is negative, ΔG is converted to volume by an energy-to-volume-conversion factor (*b*), in this way the maximal growth rate ranges from approximately 1 to 17 molP/t.

An equilibrium is established when nutrient concentrations and pathway activity do not change in time. The final nutrient concentration is the residual of inflow, consumption and production. Total consumption stabilizes when each pathway has depleted one of its nutrients. Thus the final pathway volume in the model is determined by the relative differences in nutrient inflow rates. Therefore, pathway volume, or activity is a relative measure as well, so it can only be judged qualitatively, not quantitatively. We studied different combinations of substrate inflow levels, for both random and competition simulations. All simulations were carried out in GRIND for MATLAB (http://www.aew.wur.nl/uk/grind).

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Supplementary Information 7

Theoretical pathway list

Table S7.1. Theoretical pathways involving the nitrogen compounds nitrate (NO_3^-), nitrite (NO_2^-), nitric oxide (NO), nitrous oxide (N_2O), nitrogen gas (N_2), ammonium (NH_4^+), organic material (CH_2O) and oxygen (O_2). The reaction coefficients and the energy yield are calculated per electron transfer. The pathways indicated with a star were not included in the model, due to the ammonium activation restriction.

Nr	*Reactants*		*Products*	*ΔG*
1	1/2 NO_3^- + NO + 1/2 H_2O	→	1 1/2 NO_2^+ + H^+	-7.8645
2 *	1/2 NO_3^- + 1/6 NH_4^+	→	2/3 NO_2^- + 1/3 H^+ + 1/6 H_2O	-8.7375
3 *	1/2 NO_3^- + 1/5 NH_4^+	→	1/2 NO_2^- + 1/5 NO + 1/5 H^+ + 3/10H_2O	-8.9121
4 *	1/2 NO_3^- + 1/4 NH_4^+	→	1/2 NO_2^- + 1/8 N_2O + 1/4 H^+ + 3/8 H_2O	-29.027
5 *	1/2 NO_3^- + 1/3 NH_4^+	→	1/2 NO_2^- + 1/6 N_2 + 1/3 H^+ + 1/2 H_2O	-8.421
6	1/2 NO_3^- + 1/4 CH_2O	→	1/2 NO_2^- + 1/4 CO_2 + 1/4 H_2O	-82.65
7 *	1/3 NO_3^- + 1/5 NH_4^+ + 2/15 H^+	→	8/15 NO + 7/15 H_2O	-6.2906
8 *	1/3 NO_3^- + 1/4 NH_4^+ + 1/12 H^+	→	1/3 NO + 1/8 N_2O + 13/24 H_2O	-26.405
9 *	1/3 NO_3^- + 1/3 NH_4^+	→	1/3 NO + 1/6 N_2 + 2/3 H_2O	-65.8
10	1/3 NO_3^- + 1/4 C H_2O + 1/3 H^+	→	1/3 NO + 1/4 CO_2 + 5/12 H_2O	-80.028
11*	1/4 NO_3^- + 1/4 NH_4^+	→	1/4 N_2O + 1/2 H_2O	-44.947
12*	1/4 NO_3^- + 1/3 NH_4^+	→	1/8 N_2O + 1/6 N_2 + 1/12 H^+ + 5/8 H_2O	-84.342
13	1/4 NO_3^- + 1/4 CH_2O + 1/4 H^+	→	1/8 N_2O + 1/4 CO_2 + 3/8 H_2O	-98.57
14*	1/5 NO_3^- + 1/3 NH_4^+	→	4/15 N_2 + 2/15 H^+ + 3/5 H_2O	-98.989
15	1/5 NO_3^- +1/4 CH_2O + 1/5 H^+	→	1/10 N_2 + 1/4 CO_2 + 7/20 H_2O	-113.22
16	1/8 NO_3^- + 1/4 CH_2O + 1/4 H^+	→	1/8 NH_4^+ + 1/4 CO_2 + 1/8 H_2O	-76.097
17	NO_2^- + 1/2 H^+	→	1/2 NO_3^- + 1/4 N_2O + 1/4 H_2O	-31.841
18	5/6 NO_2^- + 1/3 H^+	→	1/2 NO_3^- + 1/6 N_2 + 1/6 H_2O	-50.946
19	1/2 NO_2^- + NO	→	1/2 NO_3^- + 1/2 N_2O	-71.547
20	1/2 NO_2^- +1/2 NO	→	1/2 NO_3^- + 1/4 N_2	-80.352
21	1/2 NO_2^- + 1/2 N_2O	→	1/2 NO_3^- + 1/2 N_2	-89.157
22	1/2 NO_2^- + 1/4 O_2	→	1/2 NO_3^-	-37.067
23*	NO_2^- + 1/5 NH_4^+ + 4/5 H^+	→	6/5 NO + 4/5 H_2O	-1.0476
24*	NO_2^- + 1/4 NH_4^+ + 3/4 H^+	→	NO + 1/8 N_2O + 7/8 H_2O	-21.162
25*	NO_2^- + 1/3 NH_4^+ + 2/3 H^+	→	NO + 1/6 N_2 + H_2O	-60.557
26	NO_2^- + 1/4 CH_2O + H^+	→	NO + 1/4 CO_2 + 3/4 H_2O	-74.785
27	1/2 NO_2^- + 1/4 NH_4^+ + 1/4 H^+	→	3/8 N_2O + 5/8 H_2O	-60.868
28	1/2 NO_2^- + 1/3 NH_4^+ +1/6 H^+	→	1/4 N_2O + 1/6 N_2 + 3/4 H_2O	-100.26
29	1/2 NO_2^- + 1/4 CH_2O + 1/2H^+	→	1/4 N_2O + 1/4 CO_2 + 1/2 H_2O	-114.49
30	1/3 NO_2^- + 1/3 NH_4^+	→	1/3 N_2 + 2/3 H_2O	-119.37

Table S7.1 continued.

Nr	*Reactants*		*Products*	*ΔG*
31	1/3 NO_2^- + 1/4 CH_2O + 1/3 H^+	→	1/6 N_2 + 1/4 CO_2 + 5/12 H_2O	-133.6
32	1/6 NO_2^- +1/4 CH_2O + 1/3 H^+	→	1/6 NH_4^+ + 1/4 CO_2 + 1/12 H_2O	-73.912
33	4/3 NO + 1/6 H_2O	→	1/3 NO_3^- + 1/2 N_2O + 1/3 H^+	-74.168
34	5/6 NO + 1/6 H_2O	→	1/3 NO_3^- + 1/4 N_2 + 1/3 H^+	-82.974
35	1/3 NO + 1/2 N_2O + 1/6 H_2O	→	1/3 NO_3^- + 1/2 N_2 + 1/3 H^+	-91.779
36	1/3 NO + 1/4 O_2 + 1/6 H_2O	→	1/3 NO_3^- + 1/3 H^+	-39.689
37	2 NO + 1/2 H_2O	→	NO_2^- + 1/2 N_2O + H^+	-79.411
38	3/2 NO + 1/2 H_2O	→	NO_2^- + 1/4 N_2 + H^+	-88.216
39	NO + 1/2 N_2O +1/2 H_2O	→	NO_2^- + 1/2 N_2 + H^+	-97.022
40	NO + 1/4 O_2 + 1/2 H_2O	→	NO_2^- + H^+	-44.931
41	NO + 1/4 NH_4^+	→	5/8 N_2O +1/4 H^+ + 3/8 H_2O	-100.57
42	NO + 1/3 NH_4^+	→	1/2 N_2O + 1/6 N_2 + 1/3 H^+ + 1/2 H_2O	-139.97
43	NO + 1/4 CH_2O	→	1/2 N_2O + 1/4 CO_2 + 1/4 H_2O	-154.2
44	1/2 NO + 1/3 NH_4^+	→	5/12 N_2 + 1/3 H^+ + 1/2 H_2O	-148.77
45	1/2 NO + 1/4 CH_2O	→	1/4 N_2 + 1/4 CO_2 + 1/4 H_2O	-163
46	1/5 NO +1/4 CH_2O + 1/5 H^+ +1/20 H_2O	→	1/5 NH_4^+ + 1/4 CO_2	-73.738
47	5/8 N_2O + 1/8 H_2O	→	1/4 NO_3^- +1/2 N_2 + 1/4 H^+	-73.236
48	1/8 N_2O +1/4 O_2 + 1/8 H_2O	→	1/4 NO_3^- + 1/4 H^+	-21.146
49	3/4 N_2O + 1/4 H_2O	→	1/2 NO_2^- + 1/2 N_2 + 1/2 H^+	-57.316
50	1/4 N_2O + 1/4 O_2 + 1/4 H_2O	→	1/2 NO_2^- + 1/2 H^+	-5.2258
51	N_2O	→	NO + 1/2 N_2	-17.61
52*	1/2 N_2O + 1/3 NH_4^+	→	2/3 N_2 + 1/3 H^+ + 1/2 H_2O	-157.58
53	1/2 N_2O + 1/4 CH_2O	→	1/2 N_2 + 1/4 CO_2 +1/4 H_2O	-171.81
54	1/8 N_2O + 1/4 CH_2O + 1/4 H^+ + 1/8H_2O	→	1/4 NH_4^+ + 1/4 CO_2	-53.623
55	1/10 N_2 + 1/4 O_2 + 1/10 H_2O	→	1/5 NO_3^- + 1/5 H+	-6.4991
56	1/6 N_2 + 1/4 CH_2O + 1/3 H^+ + 1/4 H_2O	→	1/3 NH_4^+ + 1/4 CO_2	-14.228
57	1/8 NH_4^+ + 1/4 O_2	→	1/8 NO_3^- + 1/4 H^+ + 1/8 H_2O	-43.62
58	1/6 NH_4^+ + 1/4 O_2	→	1/6 NO_2^- + 1/3 H^+ + 1/6 H_2O	-45.804
59*	1/5 NH_4^+ + 1/4 O_2	→	1/5 NO + 1/5 H^+ + 3/10 H_2O	-45.979
60*	1/4 NH_4^+ + 1/4 O_2	→	1/8 N_2O + 1/4 H^+ + 3/8 H_2O	-66.094
61*	1/3 NH_4^+ + 1/4 O_2	→	1/6 N_2 + 1/3 H^+ + 1/2 H_2O	-105.49
62	1/4 CH_2O +1/4 O_2	→	1/4 CO_2 +1/4 H_2O	-119.72

Model without ammonium activation restriction

To investigate the effect of the ammonium activation restriction, we repeated the simulations as in Figure 7.3 and 7.4 in the main text, including pathways in which ammonium cannot be activated one-to-one with a reactive species (Table S7.1: starred pathway numbers). Two unknown pathways showed strong activity (Fig. S7.1 and S7.2): ammonium oxidation to the level of dinitrogen gas (Table S7.1, Eqn. 61) and anammox using nitrate (Table S7.1, Eqn. 14).

Maintenance costs relative to pathway length

A theoretical explanation considering enzymatic and intermediate costs has been proposed by Costa et al (2006). The more steps are included in a pathway, the more enzymes and intermediates are involved. This implies that a long pathway is associated with relatively high costs, since enzyme synthesis requires ATP, carbon and other substrates while intermediates can have toxic effects (Pfeiffer & Bonhoeffer, 2004; Costa et al., 2006).

To implement this constraint in our model, we made a general estimation for the number of enzymes involved in a theoretical pathway. We assumed that every step involving the making and breaking of N-O, N-N, C-O and O-O bonds needs to be catalyzed by one enzyme. For example for denitrification from NO_3^- to N_2 three oxygen atoms have to be removed and one nitrogen atom has to be added, therefore the estimated number of enzymes for this half reaction is four. This estimation is correct for the majority of the known pathways, for example the denitrification pathway includes four enzymes catalyzing the following steps: NO_3^- → NO_2^- → NO → N_2O → N_2 (Van Spanning, 2007). We included the pathway length costs as a constant decay rate in the pathway biomass equation:

$$\frac{dP_j}{dt} = b\Delta G_j M_j P_j - dP_j - cLP_j$$

where L is the estimated pathway length, and *c* a pathway-length-to costs factor.
We performed several simulations with different costs factors. At *c* = 0.4, 'total nitrification' could not win the competition in any of the simulations with random inflow levels (Fig. S7.3 and S7.4). For this parameter setting, two other known 'short pathways' appeared: nitrate reduction to nitrite (active at high nitrate levels), and denitrification from the level of nitrite to dinitrogen (active at high

nitrite levels under anoxic conditions). Anammox showed relatively high activity, also at oxic conditions, while denitrification and DNRA were weak competitors.

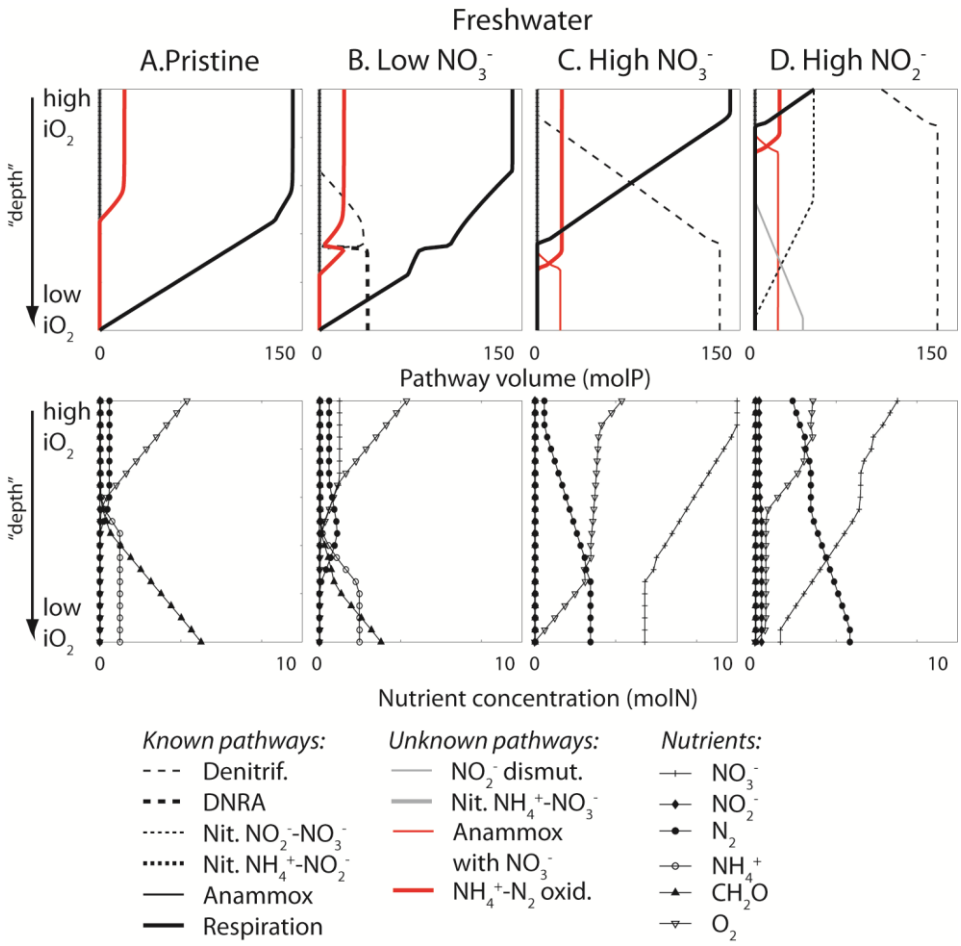

Figure S7.1. Competition simulations of the model without ammonium activation restriction representing four hypothetical freshwater sediments. Steady state conditions, for each simulation, of pathway volume (activity), nutrient concentration, and percentage of nutrient inflow consumed per pathway for different combinations of nutrient inflow levels along a gradient of oxygen inflow. A: Pristine sediment (iNO_3^-=0, iNO_2^-=0, iNH_4^+=1, iCH_2O=5), B: Eutrophic (iNO_3^- low) sediment (iNO_3^-=1, iNO_2^-=0, iNH_4^+=1, iCH_2O=5), C: Eutrophic (iNO_3^- high) sediment (iNO_3^-=10, iNO_2^-=0, iNH_4^+=1, iCH_2O=5), D: Eutrophic (iNO_2^- high) sediment (iNO_3^-=0, iNO_2^-=10, iNH_4^+=1, iCH_2O=5).

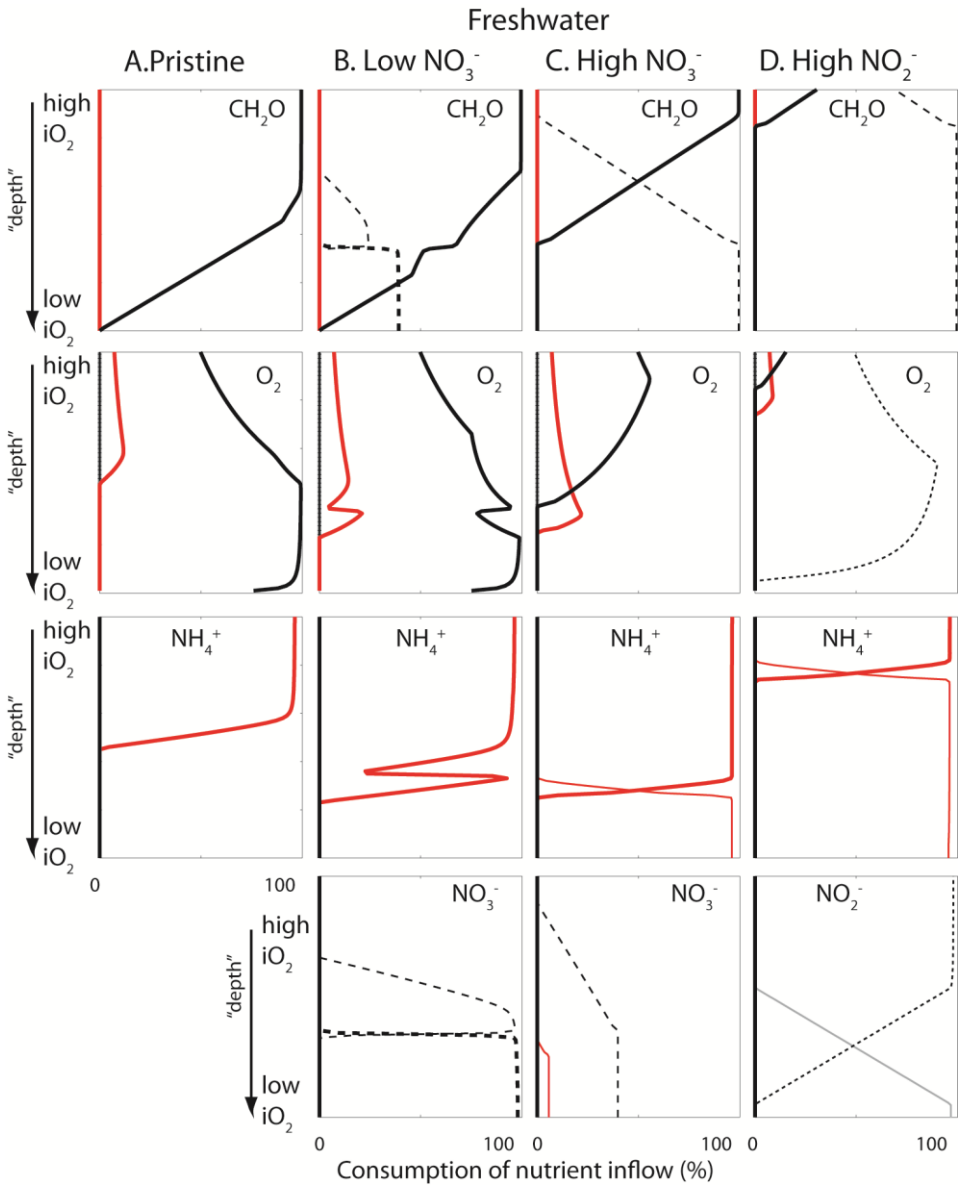

Figure S7.1. (Continued)

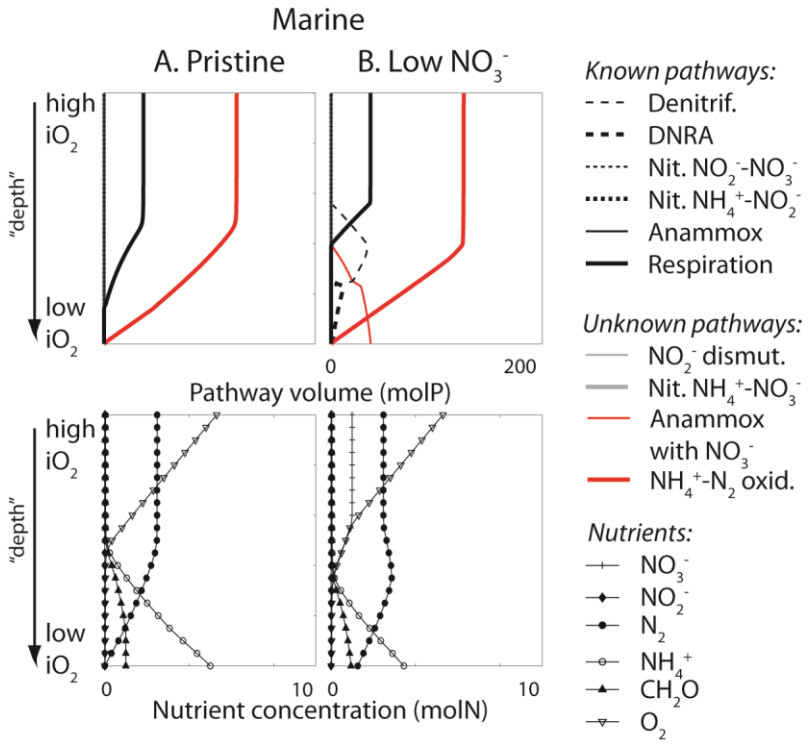

Figure S7.2. Competition simulations of the model without ammonium activation restriction representing two hypothetical marine sediments (as in Fig. S7.1). A: Pristine sediment (iNO_3^-=0, iNO_2^-=0, iNH_4^+=5, iCH_2O=1), B: Eutrophic (iNO_3^- low) sediment (iNO_3^-=1, iNO_2^-=0, iNH_4^+=5, iCH_2O=1).

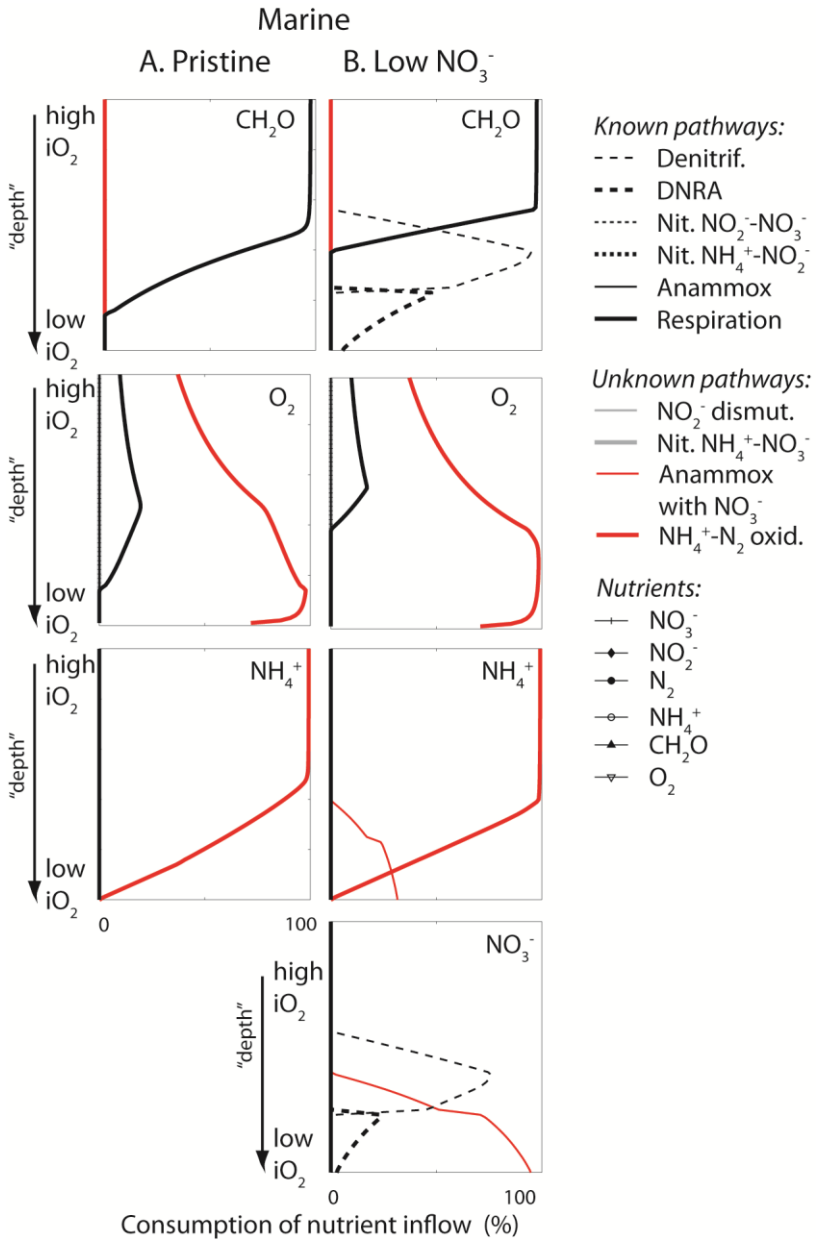

Figure S7.2 Continued

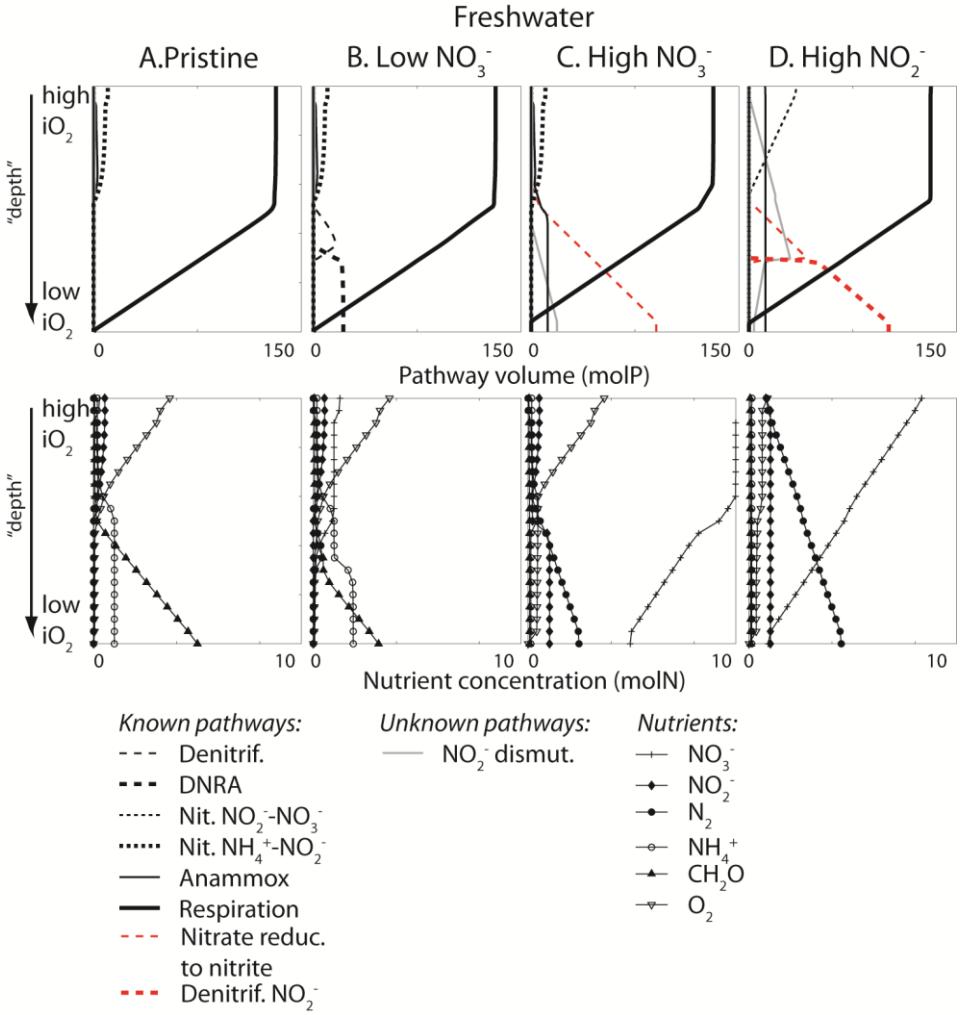

Figure S7.3. Competition simulations of the model including the costs for pathway length assumption, representing four hypothetical freshwater sediments. Steady state conditions, for each simulation, of pathway volume (activity), nutrient concentration, and percentage of nutrient inflow consumed per pathway for different combinations of nutrient inflow levels along a gradient of oxygen inflow. A: Pristine sediment (iNO_3^-=0, iNO_2^-=0, iNH_4^+=1, iCH_2O=5), B: Eutrophic (iNO_3^- low) sediment (iNO_3^-=1, iNO_2^-=0, iNH_4^+=1, iCH_2O=5), C: Eutrophic (iNO_3^- high) sediment (iNO_3^-=10, iNO_2^-=0, iNH_4^+=1, iCH_2O=5), D: Eutrophic (iNO_2^- high) sediment (iNO_3^-=0, iNO_2^-=10, iNH_4^+=1, iCH_2O=5).

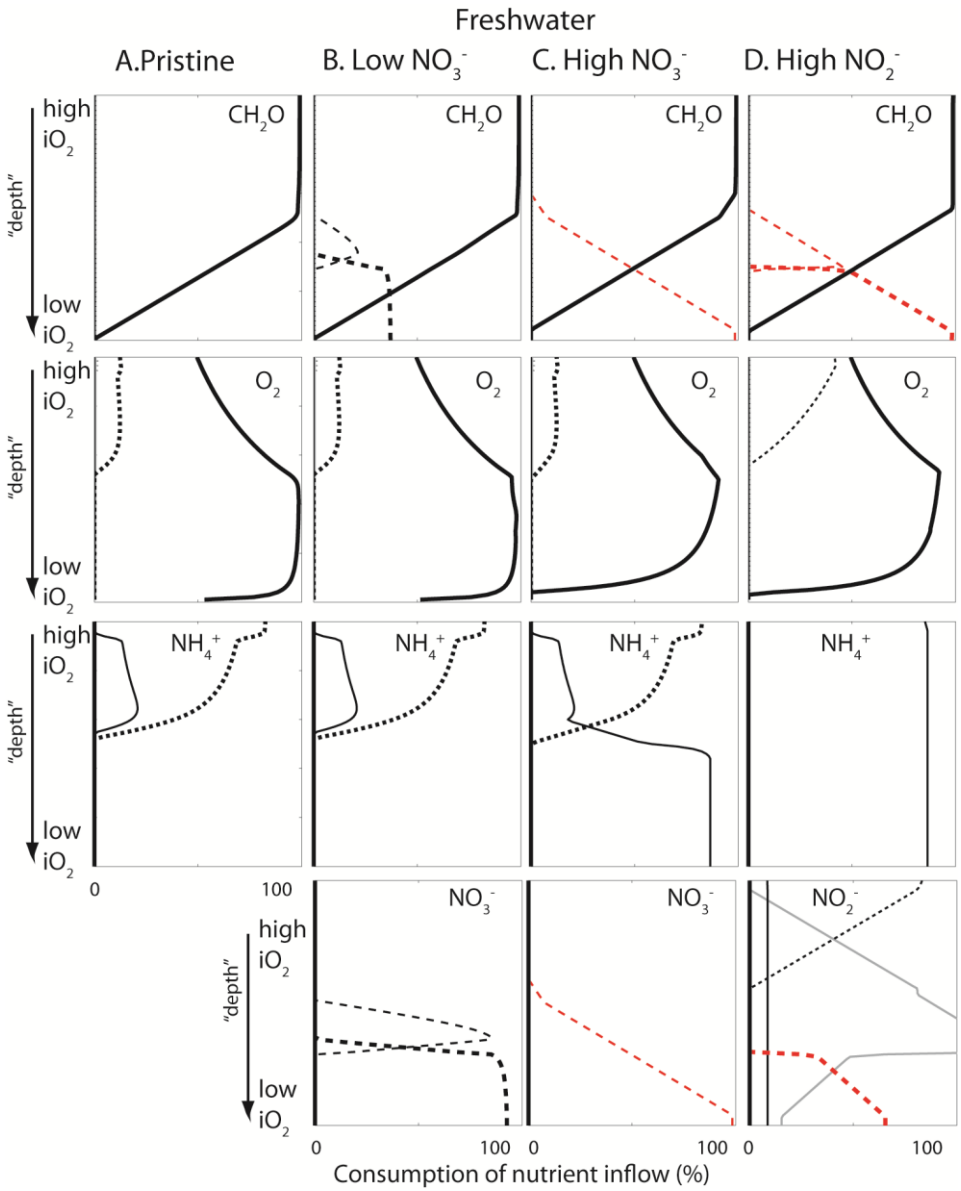

Figure S7.3. Continued

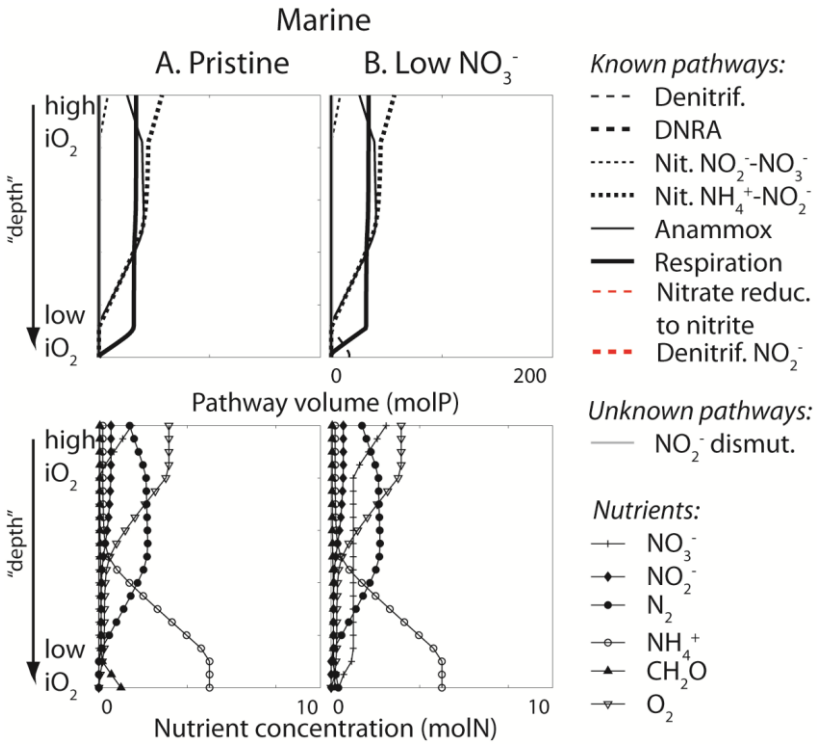

Figure S7.4. Competition simulations of the model including the costs for pathway length assumption, representing two hypothetical marine sediments (as in Fig. S 7.3). A: Pristine sediment (iNO_3^-=0, iNO_2^-=0, iNH_4^+=5, iCH_2O=1), B: Eutrophic (iNO_3^- low) sediment (iNO_3^-=1, iNO_2^-=0, iNH_4^+=5, iCH_2O=1).

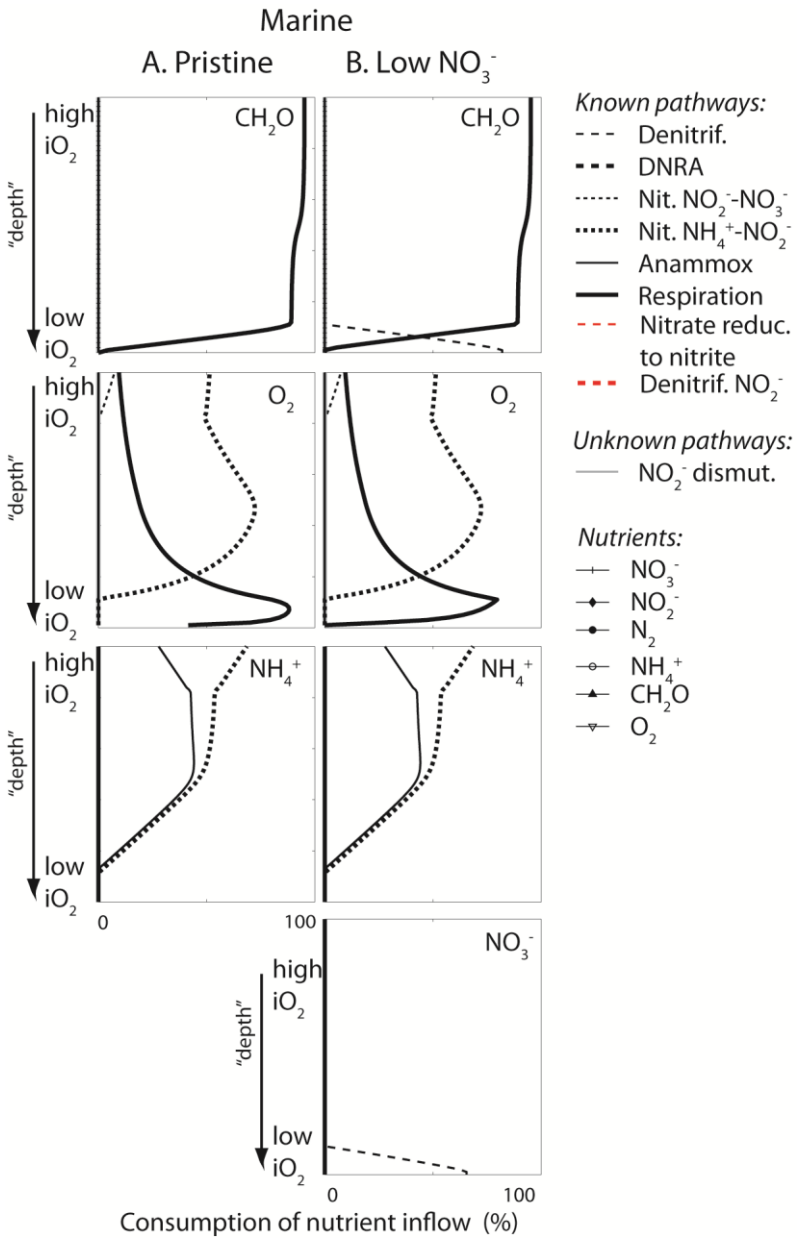

Figure S7.4. (Continued)

Chapter 8

Synthesis

"It is also worth noting that unlike many of humanity's other effects on the planet, the remaking of the nitrogen cycle was deliberate."

(pg. 74 "The Anthropocene", The Economist, May 28th 2011)

"To be sure, advances have been made (...) on understanding the factors that control classical biological denitrification, although the basic understanding of electron donors and acceptors provided by Nõmmik (1956) still stands" – Davidson & Seitzinger, 2006)

In the 2006 review "The enigma of progress in denitrification research", Davidson & Seitzinger reflect on over a century of denitrification research. As they point out, despite a considerable research effort, and even though significant progress has been made in the past decades, the main difficulties in denitrification research are still the same as roughly 60 years ago. Denitrification remains under-sampled relative to its spatial and temporal heterogeneity and its ecological and societal importance, and furthermore we are still optimising nitrogen budgets and pointing out controlling factors beyond 'electron donors and acceptors' across ecosystem types.

In this thesis I aim to add some small pieces to this puzzle by quantifying denitrification in ditches, streams and shallow lakes, and elucidating factors controlling denitrification within and across these ecosystems. Below I will briefly discuss the results presented in the previous chapters and give recommendations for research and (water-) management.

8.1 Denitrification in ditches, streams and shallow lakes – rates and efficiency

Denitrification rates varied considerably within and among systems, covering the whole range of previously reported rates in freshwater ecosystems (Figure 8.1). Rates ranged from undetectable in streams and lakes that contained little organic matter in their sediments (Chapters 4 & 5), to up to 20 mmol N m^{-2} h^{-1} in ditches and streams in agricultural areas that were rich in nitrate and organic matter (Chapters 5 & 6). However, in all measured systems, mean rates were lower than those averagely reported in the literature. This may partly arise from differences in methodology. All field measurements for this thesis were performed using *in-situ* benthic measurement chambers using the ^{15}N isotope pairing technique. This makes them comparable among each other, but comparisons with data obtained from other methodologies (e.g. batch mode assays, acetylene inhibition experiments, mass balances, N_2/Ar and N_2 flux methods) should be treated with caution (Risgaard-Petersen et al., 1998; Eyre et al., 2002). Besides variability arising from methodological differences, low mean denitrification rates in the lakes

studied in this thesis likely also arose from the inclusion of a relatively large proportion of lakes with very little organic matter in the sediments.

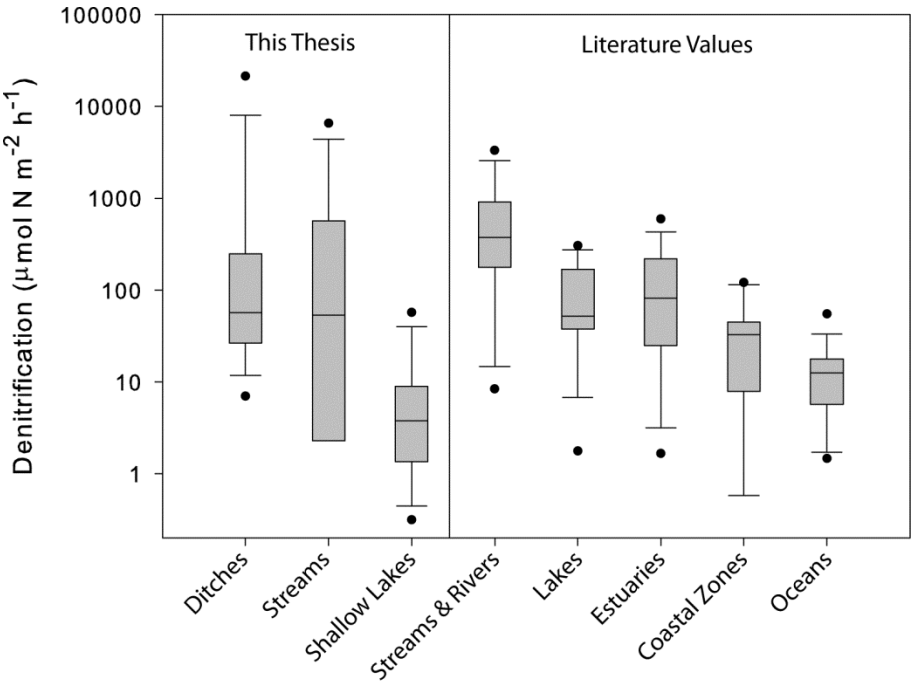

Fig. 8.1. Denitrification rates in ditches, streams and shallow lakes studied in this thesis compared to those reported in the literature between 1971-2005, obtained from a compilation by Piña-Ochoa & Álvarez-Cobelas (2006). Boxes display means and 25^{th}-75^{th} percentiles, whiskers show 10^{th}-90^{th} percentiles, dots show 5^{th} and 95^{th} percentiles.

Importantly, denitrification rates in systems receiving large N-loads were high, indicating the importance of denitrification for nitrogen removal from these systems (Chapters 5 & 6). Agricultural drainage ditches, for example, can potentially remove about half of the incoming nitrate (Chapter 5, de Klein, 2008), and thereby protect downstream water quality. However, nitrogen removal efficiency depends not only on absolute denitrification rates, but also on quantity and timing of N-loads. A recent multi-ecosystem study showed that nitrogen removal efficiency significantly declines with nitrogen load (Mulholland et al., 2008). Additionally, in practise there will be a seasonal mismatch between N-loads and denitrification potential (de Klein, 2008), which may result in lower annual nitrogen removal efficiency. Furthermore, occurrence of small areas (hotspots) and

brief periods (hot moments) of high denitrification activity, arising from spatial and temporal variability of nitrate, carbon and oxygen availability, complicate scaling-up denitrification rates and N-removal efficiencies (Chapters 5 & 6; McClain et al., 2003; Groffman et al., 2009). Quantifying denitrification rates and nitrogen loads over a seasonal range and across land-use types could therefore provide better insights in nitrogen removal efficiency of such systems.

To conclude, although progress has been made in quantifying denitrification in shallow freshwater ecosystems, there is still a need to quantify denitrification rates - and potential explanatory variables - at multiple spatial and temporal resolutions, to better understand variability in rates and efficiency of denitrification, including the occurrence of hotspots and hot moments.

8.2 Factors regulating denitrification rates in ditches, streams and shallow lakes

Classic experiments in the early 20th century already pointed out the most important factors regulating denitrification: the involved electron donors (organic C) and electron acceptors (nitrogen oxides and oxygen) (Nõmmik, 1956). As confirmed in chapter 7, the influence of these factors largely arises from resource competition, stoichiometry and energy yield of denitrification, as well as from respiration and other processes involved in the nitrogen cycle. However, there may be variations in secondary factors regulating denitrification in different ecosystem types. Below, I discuss which factors related to denitrification in the experiments and field studies presented in this thesis. I also consider relations that hold across ecosystems as well as site-specific effects tested in microcosm experiments and field studies.

Factors affecting denitrification across ecosystems

When considering all field data collected in this thesis, the most important factors explaining denitrification rates were ammonium, nitrate and sediment organic matter content, together explaining 57% of the variation[1]. Effects of nitrate and organic matter content arise from denitrification stoichiometry (Chapter 7), whereas the effect of ammonium may point at the importance of coupled nitrification-denitrification as a source of nitrate in many freshwater ecosystems. On the other hand, the relation to ammonium may be correlative, as ammonium is released under the anoxic conditions that favour denitrification.

However, multi-ecosystem determinants reported in this thesis differed across ecosystem types. In ditches and streams nitrate was the most important driver of denitrification (Chapters 5 & 6). Additionally, in the studied ditches denitrification related to land-use, sediment type and the type of macrophyte vegetation present. All of these factors were interrelated and also varied in nitrate concentrations (Chapter 6). Interestingly, we found no overall relation between denitrifier (*nirK*) abundance or richness and denitrification activity in the ditches and streams, a result for which there are several possible reasons. On the one hand, our methodology only enabled us to study part of the *nirK* denitrifiers. First of all, optimizing primers that target denitrification genes, and including *nirS*-denitrifiers will give a better picture of actual relations between denitrifier abundance, composition and functioning. Secondly, present denitrifiers may not always be active; nitrate availability (Chapter 5) and oxygen concentration largely determine their activity (Knowles, 1982; Wallenstein et al., 2006). On the other hand, a high functional redundancy in denitrifying communities may explain the absence of a diversity-functioning relationship (Wertz et al., 2006). We therefore need to study this relation in more detail.

Factors regulating denitrification in the studied shallow lakes differed between climatic regions. Denitrification in temperate lakes related negatively to both water column dissolved oxygen as well as to sediment oxygen demand (SOD), whereas denitrification in subtropical lakes related positively to organic matter

[1] *Stepwise multiple linear regression. Denitrification= 25.34 NH_4-N + 0.67 OM + 1.751 NO_3-N + 0.92; $R^2_{adj.=}$ 0.57, n=38 (ditches, streams, shallow lakes), P<0.001, variables tested: NH_4-N, NO_3-N, PO_4-P, sediment organic matter % (OM), mean water column temperature, mean water column dissolved oxygen, electric conductivity, chlorophyll-a. All variables were ln(x+1) transformed.*

and, in association to this, macrophyte biomass, but related negatively to phosphate concentration (Chapter 4).

Because both SOD and phosphate-release are signs of reducing conditions in the sediment, supposedly favoring denitrification, these findings are quite unexpected. The negative correlation between SOD and denitrification in the temperate lakes is still not clearly understood. This may be associated to SOD arising from respiration by macrofauna or phytobenthos rather than from microbial activity, or from oxidation of electron donors that are unsuitable for denitrification, though these hypotheses still need to be studied in more detail. The occurrence of low denitrification rates at high phosphate concentrations possibly arises from an imbalance of N and P supply to denitrifiers as well as a higher competition for N with microalgae at low N:P ratios (Piña-Ochoa & Álvarez-Cobelas, 2006). However, other studies have reported positive relations between phosphorus and denitrification (Inwood et al., 2005; Graham et al., 2010), thus there is more to the mechanism through which phosphorus affects environmental denitrification rates than we currently understand.

Interestingly, although temperature strongly affected denitrification in microcosm experiments (Chapter 2), temperature was neither significantly related to denitrification within sets of ditches and lakes, nor across all sampled ecosystems in this thesis. Our results indicate that temperature is only important when denitrification is not limited by its primary resources, as has been previously reported (Smith et al., 1985; Herbert, 1999; Piña-Ochoa & Álvarez-Cobelas, 2006). In addition, temperature effects on denitrification will depend on the level of coupling between nitrification and denitrification (Groffman et al., 2009). In cases where denitrification is strongly dependent on nitrate supply from nitrification, decreased oxygen at higher water temperatures may result in reduced nitrification, and consequently lower denitrification, which in turn might for example lead to higher denitrification rates in spring than in summer (Jenkins & Kemp, 1984).

Site-specific determinants of DR in this study

The field studies presented in this thesis indeed point to sediment organic matter, nitrate availability and dissolved oxygen as major regulating factors of denitrification. Besides these direct regulators, temperature and macrophyte presence are site-specific determinants of denitrification (Chapters 2-4). Because these factors not only directly or indirectly interact with denitrifier activity, but also interact among each other, it is difficult to tease apart their separate effects in the environment. The most valuable data in this respect were therefore obtained from microcosm experiments (Chapters 2 & 3). The experiments described in chapter 2 show that when nitrate and organic carbon are sufficiently available, denitrification can increase exponentially with temperature. The strong temperature dependence was found to arise from the different temperature dependences of respiration and photosynthesis, which lead to strongly reduced dissolved oxygen at increasing temperatures. These findings illustrate the complexity in predicting effects of warming on freshwater ecosystems. Some effects, such as those of respiration and denitrification, may be synergistic, whereas others, like respiration and coupled nitrification-denitrification, may buffer overall temperature effects.

Besides such site-specific temperature effects, the experiments presented in chapters 3 and 4 showed site-specific and structure-specific effects of macrophytes. Denitrification rates in duckweed-covered microcosms were higher than those without macrophytes or those containing submerged macrophytes, because of lower oxygen concentrations under duckweed. Furthermore, in subtropical lakes patches with submerged macrophytes had higher denitrification rates than patches without macrophytes. In carbon-limited cases (such as the subtropical lakes studied in this thesis) macrophytes can considerably improve availability of easily degradable organic carbon to denitrifiers, stimulating denitrification (Bastviken et al., 2007). Absence of a clear macrophyte effect in the temperate lakes indicates that these effects are site- and condition-specific.

8.3 Optimizing denitrification to increase nutrient removal.

Importantly, the most effective strategy to reduce nitrogen concentrations in surface waters is to reduce emission of N to the aquatic environment, by optimizing agricultural practises, installing buffer zones and improving waste water treatment (Carpenter et al., 1998; Hefting & de Klein, 1998; Ju et al., 2008). Therefore, optimizing nutrient removal by denitrification within freshwater ecosystems should only be considered as a management strategy when directly reducing N loads is impossible or will only be effective after a long period (de Klein, 2008).

Surface waters in agricultural areas receive highest nitrogen loads (Chapter 1, Chapter 5). In ditches that drain agricultural fields about half of the incoming nitrogen can be removed by denitrification (Chapter 5; de Klein, 2008). Denitrification rates in these systems are associated to several different factors, including organic matter availability, nitrate concentration, sediment type, temperature, and macrophyte presence and type (Chapters 2-5, 7). Though easy to manipulate in the laboratory, most of these factors are difficult to change within ecosystems. However, macrophyte presence may be optimized quite easily, since it is already frequently managed in agricultural ditches and streams. Additionally, hydraulic residence time and organic carbon availability can be changed during stream restoration, which will be discussed in more detail below.

Macrophytes stimulate temporal nitrogen removal through uptake and permanent removal by enhancing denitrification rates. Effects of macrophytes on denitrification are biochemical, through changes in oxygen dynamics and organic carbon availability (Chapters 3, 4, 6; Christensen & Sørensen, 1986; Caffrey & Kemp, 1992); physical, by providing colonisable surface area for nitrifiers and denitrifiers (Körner, 1999); and hydrologic, by increasing the hydraulic residence time (de Klein & Koelmans, 2011). A closed cover of floating plants such as duckweed (*Lemna* sp.) is most effective in stimulating denitrification rates because it hampers re-aeration and photosynthesis in the water column, ultimately resulting in anoxia (Chapter 3). However, duckweed cover is not always desirable in practise because the associated anoxia has detrimental effects on fish and causes release of phosphate and ammonium toxicity (Chapter 3; Scheffer et al., 2003; Netten, 2011). Generally, submerged macrophytes are less likely to cause such detrimental effects on the ditch ecosystem, and at the same time still enhance (nightly) denitrification rates and nitrate removal (Chapters 3 & 5). These findings

can help to improve maintenance strategies. For example, denitrification potential could be protected without compromising drainage capacity by not removing all macrophyte vegetation at once. In addition, management practices aiming to keep shallow lakes in a clear, macrophyte dominated state will contribute to maintaining denitrification capacity (Chapter 4), especially in lakes containing little organic matter in the sediment.

Restoring streams by reconnecting them to their floodplain wetlands is a good strategy to reduce nitrogen leaching to streams, as a large proportion of the nitrate will be denitrified in the wetland (Hoffmann & Baattrup-Pedersen, 2007; Craig et al., 2008). Also, re-meandering can result in a longer hydraulic residence time, which can optimise nitrogen retention. However, if organic sediments are removed during restoration, in-stream denitrification may be greatly reduced (Chapter 6; Craig et al., 2008) and regaining biogeochemical functioning can take decades (Antheunisse et al., 2008; Moreno-Mateos et al., 2012). Ideally, reducing internal nutrient loading by removing sediments should be carefully balanced against losing denitrification potential. There is clearly still a great need for monitoring studies on effects of restoration and maintenance on denitrification rates, preferably designed in a before-after / control-impacted set-up, and carried out on a long enough time-scale (multi-decade) to draw conclusions about effects and efficiency on the long term (Feld et al., 2011).

8.4 Implications of global environmental change for denitrification and N-cycling.

Climate change scenarios predict a global temperature rise of one to several degrees in the next century (IPCC 2007). At the same time, changes in nitrogen loading will follow altered atmospheric deposition, precipitation regimes, mineralisation rates and land-use. Denitrification is affected by these environmental changes but in turn also affects the climate, through N_2O emission, stratospheric ozone depletion and links with the global carbon cycle (Wallenstein et al., 2006). The overall effect of climate change on denitrification rates and nitrogen removal efficiency is difficult to predict. At local scales warming can enhance denitrification rates (Chapter 2). On regional scales however, effects of nitrogen and carbon are more important (Chapter 4 & 5), although these are in turn also affected by climate change through a complex of effects on terrestrial and aquatic biogeochemistry. In northern temperate regions overall denitrification efficiencies (the proportion of incoming nitrate removed by denitrification) may be reduced by

increased nitrogen loads to surface waters because of increases in precipitation and mineralization as well as of changes in cropping patterns (Mulholland et al., 2008; Jeppesen et al., 2011). By contrast, in warmer climates, the effects of warming on denitrification rates might be less pronounced (Chapter 4), and climate change effects on nitrogen concentrations are more difficult to predict. Even though N-loading in these regions is thought to be decreasing, enhanced evaporation and changes in biogeochemistry can still lead to overall increased nitrogen concentrations (Jeppesen et al., 2011). These issues make the outcome of global environmental change on nitrogen removal efficiency difficult to predict. Moreover, although short-term warming experiments have contributed to our understanding of effects of temperature on denitrification, studies on long-term effects of warming have yet to be performed.

Understanding effects of environmental change on nitrogen cycling is important as human activities continue to change regional and global nitrogen budgets. Although this thesis mainly considers detrimental effects of excess nitrogen in the environment, it is important to note that many areas in the developing world are still facing a shortage of nitrogen for food production, and that the world population is still highly dependent on artificially fixed nitrogen. Additionally, as a consequence of human population growth, there will be a rising fuel demand, resulting in increased release of NO_x and NH_x from fossil-fuel burning. Although use of biofuels is intended to reduce greenhouse gas emissions, CO_2 savings may be cancelled out by increased N_2O emission from biofuel production sites (Crutzen et al., 2008; Crutzen et al., 2009). A recent study indicated biofuel production as a new and important factor in global nitrogen budgets (Galloway et al., 2008), especially in (sub)tropical areas where the production of biofuel crops such as corn and sugar cane is growing rapidly.

8.5 Concluding Remarks

Denitrification rates vary widely among ditches, streams and shallow lakes. Most of this variation arises from availability of nitrate, oxygen and organic carbon, which can be largely explained by energy yield and stoichiometry of denitrification and competing pathways. Ammonium also strongly relates to denitrification in these systems, which may be due to coupled nitrification-denitrification processes. Furthermore, temperature and macrophytes can both strongly affect denitrification rates, although the extent of their effects depends on local biogeochemical conditions.

Our understanding of denitrification in freshwater ecosystems is still far from complete. The occurrence of denitrification hotspots, as shown in this thesis, underlines the need for measuring denitrification at increased spatial and temporal resolution. Furthermore, long-term studies on effects of warming, land-use change and restoration on nitrogen cycling (including N_2O as well as N_2 production rates) are much needed to optimize future nitrogen budgets and management strategies.

Summary

Food and energy production have greatly increased the availability of nitrogen in terrestrial and aquatic ecosystems, which is now recognized as one of the major threats to the environment. Increased nitrogen loads to freshwater ecosystems have caused dramatic eutrophication effects, including harmful phytoplankton blooms, hypoxia and fish-kills. Denitrification, the microbial conversion of nitrate to dinitrogen gas, permanently removes reactive nitrogen from ecosystems and can thereby help counteract eutrophication effects. However, besides availability of organic carbon, nitrate and oxygen, it is still unclear which environmental factors are most important in influencing denitrification rates. Certainly, denitrification remains under-studied relative to its environmental significance. The aims of this thesis were therefore to quantify denitrification in ditches, streams and shallow lakes and to identify the most important factors affecting this process in these ecosystems.

Effects of Temperature

Temperature affects all enzymatic processes, including denitrification. However, reported temperature effects on denitrification rates are highly variable. In addition, climate change scenarios predict considerable changes in N-deposition to freshwater ecosystems, making unravelling the effects of warming on denitrification even more important. In Chapter 2 I explored temperature effects on denitrification. I found a strong temperature dependence of environmental denitrification, indicating a doubling of denitrification rates upon a three degree temperature rise. Microcosm experiments and modelling revealed that this strong temperature dependence largely arises from a systematic decrease of oxygen concentrations with rising temperature. Warming not only decreases oxygen concentrations due to reduced solubility, but also because respiration rates rise more steeply with temperature than photosynthesis. Although temperature has clear potential to affect denitrification rates in the environment, it is important to note that availability of nitrate and easily oxidizable organic carbon are the principal prerequisites of denitrification, and temperature effects will be absent when these requirements are not met (Chapters 4 & 5).

Effects of Macrophytes

The presence of macrophytes in freshwater ecosystems has often been found to affect denitrification, although effects appear to vary among systems. In Dutch drainage ditches studied in this thesis, denitrification rates differed among macrophyte vegetation types. Highest rates were found in ditches covered by floating macrophytes, followed by those with submerged macrophytes and lowest rates were found in ditches without macrophytes (Chapter 5). These results clearly hint towards relations between the dominant type of macrophytes present and denitrification rates. However, because macrophyte cover in ditches strongly relates to nutrient availability and sediment characteristics, which both affect denitrification as well, it was not possible to tease apart macrophyte effects in this field study. In the studied shallow lakes effects of macrophyte presence were variable (Chapter 4). Macrophytes were not significantly related to denitrification in temperate lakes, whereas I observed a positive correlation between denitrification and macrophyte biomass in subtropical lakes. This difference may be due to the lower availability of easily degradable organic carbon in subtropical lakes, which was alleviated by macrophyte presence. These results indicate that effects of macrophytes depend on local resource availability. To study macrophyte effects in more detail, I tested the effect of floating and submerged macrophytes on denitrification in a microcosm experiment (Chapter 3). I found significantly enhanced denitrification rates under a closed duckweed cover, likely because the floating cover resulted in low oxygen concentrations in the water column and consequently in the sediment top layer

Denitrification and nitrogen removal in drainage ditches

The field study in drainage ditches showed that denitrification rates differ among land-use types, sediment types, and vegetation types (Chapter 5). Highest rates were found in agricultural areas, which also received highest nitrogen loads. In these areas denitrification can remove a substantial part of the incoming nitrogen (median 45%), and thereby contribute to reducing nitrogen loads to connected waters. Overall, denitrification rates positively related to nitrate and ammonium concentration, but not to any of the other measured variables. Denitrifier (*nirK*) abundance related to organic matter content of the sediment, but not to denitrification rates. These results indicate that organic material in the sediment

influences denitrifier abundance, which reflect denitrification potential, whereas nitrogen availability determines instantaneous denitrification rates.

Restoration Effects

Restoring channelized streams and their floodplain wetlands can reduce nitrogen loads to downstream waterbodies. Although overall effects of stream and wetland restoration on the catchment level are well established, effects on in-stream nitrogen cycling are still poorly understood. I measured denitrification rates in restored and unrestored sections of two streams longer than 5 years after restoration, to explore its potential consequences (Chapter 6). Effects of restoration on denitrification are difficult to predict because of the complexity of biogeochemical, hydrological and biological factors involved. Restoration effects were found to be variable, and likely depend on initial sediment composition and hydrology as well as the restoration design. Importantly, I found low numbers of denitrifiers, and likely as a consequence, no detectable denitrification, in a restored stream section in which most of the organic sludge was removed. This may indicate that removal of organic sludge can result in decreased denitrification potential in the years after restoration.

Modelling the Nitrogen Cycle

Understanding the competition between different nitrogen pathways is essential to predict effects of global environmental change on denitrification. Modelling is an effective tool to achieve this. Most modelling efforts so-far approached the nitrogen cycle from a semi-empirical perspective. However, no empirical study addressed all pathways simultaneously, and furthermore our current inventory of the N-cycle may be incomplete. Therefore nitrogen transforming pathways were modelled using a completely different approach, based only on resource competition, energy yield and stoichiometry of the involved redox reactions (Chapter 7). Surprisingly, these few basic principles were found to already resemble the N-cycle as we know it. With the additional assumption of enzymatic costs for long pathways and a high activation energy for ammonium, the model predicted much of the variation in nitrogen transformations observed in nature. These results indicate that the outcome of competition of nitrogen transforming pathways can be largely predicted from energy yield and reaction stoichiometry. Additionally, the

model predicted a pathway that has not yet been discovered in nature: the dismutation of nitrite to nitrate and dinitrogen gas. The process could play a role in systems with high nitrite and low oxygen levels. Time will tell if this is indeed a viable, yet undiscovered, pathway.

Conclusions

Denitrification is an important nitrogen removing process in shallow freshwater ecosystems. However, denitrification rates are highly variable within and among ditches, streams and shallow lakes. Most of this variation arises from availability of nitrate, oxygen and organic carbon, which can be largely explained by the energy yield and stoichiometry of denitrification and competing pathways. When sufficient nitrate and organic carbon are available, temperature can exponentially increase denitrification, which is enhanced by the temperature dependency of dissolved oxygen. Macrophyte presence can stimulate denitrification rates. Their effects are related to the type of macrophyte structure and composition. Restoration effects on in-stream denitrification will depend on initial conditions and restoration design. When too much of the organic material is removed during restoration denitrification rates may be hampered for at least several years. These findings contribute to understanding denitrification variability, and help to unravel the complex nitrogen cycle in the environment.

Samenvatting

Door een steeds groeiende productie van voedsel en energie is de hoeveelheid stikstof in het milieu in de afgelopen eeuw sterk toegenomen. De hoge stikstofbelasting wordt op dit moment gezien als een van de belangrijkste bedreigingen voor het milieu, naast de klimaatverandering, de fosfaatproblematiek en het verlies van biodiversiteit. In zoetwater ecosystemen draagt een teveel aan stikstof bij aan eutrofiëring, dat zich uit in een verslechterde waterkwaliteit met overmatige groei van waterplanten of algen, met zuurstofloosheid en vissterfte tot gevolg. Denitrificatie, de microbiële omzetting van nitraat naar stikstofgas, verwijdert stikstof uit ecosystemen en is daarom een natuurlijk proces dat eutrofiëring kan tegengaan. Randvoorwaarden voor het optreden van denitrificatie zijn de aanwezigheid van organisch koolstof en nitraat en zuurstofarme omstandigheden. Afgezien van deze randvoorwaarden is het tot nog toe onduidelijk welke andere factoren belangrijk zijn voor het optreden van denitrificatie. Ondanks de grote ecologische relevantie van denitrificatie is dit proces nog te weinig onderzocht in open water. De belangrijkste doelen van dit proefschrift zijn dan ook: 1) het kwantificeren van denitrificatie in sloten, beken en meren en 2) het bepalen van de belangrijkste factoren die de denitrificatie in deze ecosystemen beïnvloeden.

Effecten van de omgevingstemperatuur op denitrificatie

Alle enzymatische processen, en dus ook denitrificatie, worden beïnvloed door de omgevingstemperatuur. De meeste processen zullen bij tien graden stijging in temperatuur twee keer sneller verlopen. De in de literatuur beschreven effecten van temperatuur op denitrificatie zijn echter sterk variabel. In het kader van de voorspelde verandering van het klimaat en de daarmee gepaard gaande verandering van zowel temperatuur als stikstof-depositie in zoetwater-ecosystemen is het belangrijk om de effecten van temperatuur op denitrificatie te onderzoeken. In Hoofdstuk 2 beschrijf ik het onderzoek naar temperatuurseffecten op denitrificatie in zowel een veld- als een laboratorium studie. Ik vond een sterke temperatuursafhankelijkheid van denitrificatie in zoetwater-ecosystemen, die een verdubbeling van denitrificatie met drie graden temperatuurstijging lieten zien. Uit experimenten met microcosmen en een simpele modelanalyse bleek dat dit sterke effect van temperatuur veroorzaakt wordt door de temperatuursafhankelijkheid van opgelost zuurstof. In warm water kan minder zuurstof oplossen dan in koud water en daarnaast neemt de zuurstofproductie door fotosynthese minder sterk

toe met stijgende temperatuur dan de zuurstofafname door respiratie. Hierdoor ontstaat met stijgende temperatuur een systematische afname van opgelost zuurstof in de waterkolom en het sediment. Omdat denitrificatie over het algemeen sneller zal verlopen onder zuurstofarme omstandigheden zal dit effect denitrificatie dus versnellen bij hogere temperaturen. Het is daarbij wel van belang dat er voldoende nitraat en organisch koolstof aanwezig zijn, als aan deze voorwaarde niet wordt voldaan zullen temperatuurseffecten op denitrificatie uitblijven.

Effecten van waterplanten

Uit eerder onderzoek is gebleken dat waterplanten van invloed kunnen zijn op denitrificatie, maar effecten van waterplanten kunnen per situatie verschillen. In de Nederlandse sloten die in dit proefschrift onderzocht zijn verschilden denitrificatie-snelheden tussen vegetatietypen. De hoogste denitrificatiesnelheden werden gevonden in sloten die volledig bedekt werden met drijvende waterplanten (zoals kroos), gevold door sloten met ondergedoken waterplanten (zoals waterpest), terwijl de laagste denitrificatie gemeten werd in sloten zonder waterplanten (Hoofdstuk 5). Dit geeft een sterke aanwijzing voor een effect van vegetatietype op denitrificatie. Echter, omdat het type waterplanten sterk gerelateerd is aan nutriëntengehalten in de waterkolom en het sediment, en deze ook van invloed zijn op denitrificatie, is het lastig om de netto effecten van waterplanten in een veldstudie te onderzoeken. In de onderzochte ondiepe meren waren effecten van waterplanten op denitrificatie variabel (Hoofdstuk 4). In de Nederlandse meren correleerde biomassa van waterplanten niet significant met denitrificatie, terwijl dit in subtropische meren wel het geval was. Dit kan veroorzaakt zijn door de lage hoeveelheid makkelijk afbreekbaar organisch koolstof dat beschikbaar was in het sediment van subtropische meren; waterplanten verhoogden in die meren de hoeveelheid beschikbaar organisch materiaal, vermoedelijk ten gunste van de denitrificatie. Effecten van waterplanten kunnen dus afhankelijk zijn van lokale beschikbaarheid van nitraat en koolstof. Om de effecten van ondergedoken en drijvende waterplanten in meer detail te onderzoeken heb ik deze onderzocht in een laboratorium experiment in microcosmen (Hoofdstuk 3). Ik vond significant verhoogde denitrificatiesnelheden onder een gesloten kroosdek, waarschijnlijk door de lagere zuurstofgehalten onder het kroos en in de toplaag van het sediment. Door zo'n kroosdek wordt de aanvoer van zuurstof uit de atmosfeer naar

de waterkolom bemoeilijkt. Bovendien neemt het kroos het licht weg, waardoor geen zuurstofproductie door fotosynthese meer plaatsvindt.

Denitrificatie en stikstofverwijdering uit sloten

Denitrificatiesnelheden verschilden tussen sloten met een verschillend landgebruik, sediment type en vegetatietype (Hoofdstuk 5). De hoogste snelheden werden gevonden in landbouwgebieden, die ook het sterkst belast werden met stikstof. In deze gebieden kan denitrificatie een aanzienlijk deel van het inkomende stikstof verwijderen (mediaan 45%) en daarmee bijdragen aan het verlagen van stikstofbelasting op aangrenzende wateren. Denitrificatie correleerde met nitraat en ammoniumgehalten, maar niet met andere gemeten variabelen. De aanwezigheid van denitrificerende micro-organismen, gemeten als het aantal kopieën van het *nirK* gen, een indicator voor aanwezigheid van denitrificeerders, was juist gerelateerd aan de fractie organisch materiaal in het sediment, maar niet gerelateerd aan denitrificatiesnelheden. Deze resultaten laten zien dat organisch materiaal van invloed is op de hoeveelheid denitrificeerders, en hiermee de mogelijkheid voor denitrificatie, terwijl beschikbaarheid van stikstof de directe denitrificatiesnelheid bepaald.

Effecten van ecologische herstelmaatregelen

Het herstel van gekanaliseerde beken en de aangrenzende wetlands kan een effectieve maatregel zijn om stikstofbelasting naar benedenstroomse wateren te reduceren. Hoewel de effecten op het stroomgebiedsniveau goed bekend zijn, zijn de effecten op de stikstofdynamiek binnen de beken zelf nog onduidelijk. Ik heb denitrificatiesnelheden gemeten in herstelde en niet herstelde segmenten van twee Deense beken, die elk langer dan vijf jaar geleden hersteld waren, om zo mogelijke effecten van beekherstel op denitrificatie te onderzoeken (Hoofdstuk 6). Effecten van beekherstel zijn lastig te voorspellen door de complexiteit van de betrokken biogeochemische, hydrologische en biologische factoren. De gevonden effecten van herstel verschilden tussen beide beken. De effecten van herstel zijn waarschijnlijk sterk afhankelijk van de uitgangssituatie, de samenstelling van het sediment en de aard van de herstelmaatregelen. Een belangrijke bevinding van dit onderzoek is dat in één van de twee herstelde beken, waar een groot deel van de organische bagger verwijderd was, zeer lage hoeveelheden denitrificeerders

aanwezig waren en waarschijnlijk als een gevolg hiervan geen denitrificatie werd waargenomen. Dit kan betekenen dat het verwijderen van het organisch sediment nog jarenlang het vermogen tot denitrificatie zal verminderen.

Het modeleren van de stikstofcyclus

Om de effecten van de sturende factoren op denitrificatie goed te kunnen voorspellen is een gedegen begrip van de stikstofcyclus noodzakelijk, met name van de competitie tussen verschillende stikstofomzettingen. Wiskundig modeleren is een effectieve manier om dit te bereiken. Tot nu toe hebben de meeste modelstudies de stikstofcyclus benaderd vanuit een semi-empirisch perspectief. Dit heeft echter praktische beperkingen, omdat het tot nu toe niet mogelijk geweest is om in de praktijk alle stikstofomzettingen tegelijkertijd te meten en daarnaast onze huidige beschrijving van de stikstofcyclus mogelijk nog incompleet is. Daarom hebben we stikstofomzettingen vanuit een andere invalshoek gemodelleerd (Hoofdstuk 7). Het model werd alleen gebaseerd op competitie voor koolstof (C) en stikstof (N), en energiewinst en stoichiometrie van de betrokken redoxreacties. Verassend genoeg waren deze basisprincipes al voldoende om een theoretische stikstofcyclus te beschrijven die sterk lijkt op de stikstofcyclus zoals we die in de natuur vinden. Een volgende stap was naast de hierboven genoemde basisprincipes ook aan te nemen dat er kosten zijn voor enzymen (zodat lange reactieketens minder voordelig worden) en er daarnaast een hoge activeringsenergie nodig is voor het gebruik van ammonium. Hiermee voorspelde het model veel van de variatie in stikstofomzettingen zoals die in de natuur gevonden worden. Deze resultaten wijzen erop dat de uitkomst van competitie tussen stikstofomzettende reacties vrijwel volledig voorspeld kan worden uit energiewinst en stoichiometrie van de reacties. Daarnaast voorspelde het model een mogelijk proces dat nog niet ontdekt is in de natuur: de dismutatie van nitriet naar nitraat en stikstofgas. Dit proces zou een rol kunnen spelen in systemen met veel nitriet en lage zuurstofconcentraties. De tijd zal leren of dit inderdaad een levensvatbaar doch onontdekt proces is.

Conclusies

Denitrificatie is een belangrijk stikstofverwijderend proces in ondiepe zoetwaterecosystemen. Denitrificatiesnelheden zijn echter sterk variabel binnen en

tussen sloten, beken en ondiepe meren. Veel van deze variatie ontstaat door variatie in de beschikbaarheid van nitraat, zuurstof en organisch koolstof, wat verklaard kan worden door de stoichiometrie van denitrificatie en de processen waarmee denitrificatie concurreert. Als voldoende nitraat en organisch koolstof aanwezig is kan denitrificatie exponentieel toenemen met stijgende temperatuur, dit effect wordt versterkt door de temperatuursafhankelijkheid van opgelost zuurstof. De aanwezigheid van waterplanten kan denitrificatie stimuleren, afhankelijk van de lokale omstandigheden en het type waterplanten dat aanwezig is. Effecten van beekherstelmaatregelen op denitrificatie zullen afhangen van de begincondities en de aard van de herstelmaatregelen. Als te veel organische bagger verwijderd wordt kan de denitrificatie voor meerdere jaren geremd worden. Deze bevindingen zullen helpen variatie in denitrificatiesnelheden beter te begrijpen en onze kennis over de complexe stikstofcyclus in het milieu vergroten.

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Publications

Annelies J. Veraart (Zaanstad, 1981) obtained her BSc in Biology at the University of Amsterdam, after which she specialised in Limnology and Oceanography at the same university. During three MSc thesis projects she studied animal-plant interactions, competition for light between different phytoplankton species, and effects of nutrient enrichment on epiphytic algae. Annelies obtained her MSc degree in Biological Sciences in 2006 (*cum laude*). She worked as a research assistant at the Aquatic Ecology and Ecotoxicology department of the University of Amsterdam until she started her PhD at the department of Aquatic Ecology and Water Quality Management of Wageningen University in 2007.

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The Netherlands Research School for the
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- Phosphorus in Wetlands - Hydrology, biogeochemistry and management
- Theoretical Ecology Seminar Series
- iGIS - A practical Postgraduate GIS course
- Writing Grant Proposals
- Supervising Master Thesis students
- Techniques for Writing and Presenting Scientific Papers

Didactic and Management Activities

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