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Antti Rissanen

Nitrogen Removal by Microbial Processes in Aquatic Systems

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ABSTRACT

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Denitrification and anaerobic ammonium oxidation (anammox) are microbial processes that produce N_2 gas. They mitigate the effects of excess nitrogen (N) loads by permanently removing reactive N from the ecosystems. N_2 gas producing processes are, therefore, important components of the N cycling in lakes and are also widely applied in wastewater treatment plants (WWTP). Therefore, lakes and WWTPs significantly reduce N loads from inland to coastal areas. However, the factors controlling N_2 gas production in lakes are poorly understood. In addition, the biological controls of these processes in WWTPs are not known. In this study, variations in the N_2 gas production in lake sediments were studied across local and continental scales. A new method to measure N_2 gas production, based on concentration and natural stable isotopic ratio ($\delta^{15}N$) of the N_2 gas in the water column, was also tested in oxygen-stratified humic lakes. In addition, the function and the bacterial community of a methanol-utilizing denitrification system of a large WWTP was temporally followed and compared with those of methanol-utilizing denitrification systems of a smaller WWTP and a saline water aquarium. Denitrification was the primary N_2 gas producing process in the lakes. It varied considerably seasonally and spatially, and was mainly driven by variations in the nitrate availability. Boreal lakes were less efficient in removing N as N_2 gas than temperate lakes. The structure of the lake denitrifier community did not affect denitrification rates. The new measurement method indicated denitrification in the water columns of oxygen-stratified lakes, but did not provide quantitative assessment of the N_2 production. Both the function and the bacterial community were stable in the denitrification system of the WWTP, but the communities varied among the systems. This study increases the knowledge on the natural capacity of lakes to remove excess N loads. It also gives important information on the relationship between the structure and the function of denitrifier communities both in natural and engineered ecosystems.

Keywords: Anammox; denitrification; isotope pairing technique (IPT); lake; microbial community; wastewater treatment.

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CONTENTS

LIST OF ORIGINAL PUBLICATIONS

1 INTRODUCTION 7
1.1 Nitrogen cycle 7
1.2 N_2 gas producing processes mitigating the effects of anthropogenic N loads to coastal areas 11
1.2.1 Lakes 11
1.2.2 Wastewater treatment 12
1.3 Methods to study N_2 gas producing processes in aquatic systems 14
1.3.1 N_2 gas production rate measurement techniques 14
1.3.2 Characterization of microbial communities 19
1.4 Aims 21

2 MATERIALS AND METHODS 22
2.1 Study areas 22
2.1.1 Boreal study lakes (II, III, IV) 22
2.1.2 Methanol-utilizing denitrification systems (V) 23
2.2 Sampling and data collection 24
2.2.1 Storage of the sediment samples (I) 24
2.2.2 Within-lake and inter-lake variation in the sediment N_2 gas producing processes (II, III) 24
2.2.3 Vertical N_2 gas profiles and denitrification in the lake water columns (IV) 25
2.2.4 Methanol-utilizing denitrification systems (V) 26
2.3 N_2 gas analyses and calculations 26
2.4 Microbial analyses 27
2.5 Statistical analyses 29
2.6 Background data analyses 29

3 RESULTS AND DISCUSSION 31
3.1 Storage of sediment samples (I) 31
3.2 Within-lake and inter-lake variation in the sediment N_2 gas producing processes (II, III) 31
3.3 N_2 gas producing processes in the lake water columns (IV) 36
3.4 Microbial communities of the methanol-utilizing denitrification systems (V) 38

4 CONCLUSIONS 41

Acknowledgements 44

YHTEENVETO (RÉSUMÉ IN FINNISH) 45

REFERENCES 47

LIST OF ORIGINAL PUBLICATIONS

The thesis is based on the following original papers, which will be referred to in the text by their Roman numerals I-V.

I planned the experiment in I together with MT. I planned the samplings in II and III together with MT and AO and in V together with MT. Sampling in IV was planned by MT, MS and HN, except for experiments with ^{15}N tracers that were planned by MS and me. I conducted the sampling and laboratory work in II and III, and in I with MT and EK. In IV, the sampling and analyses were conducted jointly by MT, MS, HN and me. I conducted the sampling jointly with TF, MD and JT in V. TF, MD and JT also provided background data for V. I conducted the laboratory analyses in V. I did all the statistical analyses in II, III and V by myself and in I together with TO and TA. I wrote the papers I, II, III and V. The paper IV was written jointly with MT. All papers were finished together with all co-authors.

I Rissanen A.J., Kurhela E., Aho T., Oittinen T. & Tiirola M. 2010. Storage of environmental samples for guaranteeing nucleic acid yields for molecular microbiological studies. *Applied Microbiology and Biotechnology* 88: 977–984.

II Rissanen A.J., Tiirola M. & Ojala A. 2011. Spatial and temporal variation in denitrification and in the denitrifier community in a boreal lake. *Aquatic Microbial Ecology* 64: 27–40.

III Rissanen A.J., Tiirola M., Hietanen S. & Ojala A. 2012. Interlake variations in microbial N_2 gas producing processes. Submitted manuscript.

IV Tiirola M.A., Rissanen A.J., Sarpakunnas M., Arvola L. & Nykänen H. 2011. Stable isotope profiles of nitrogen gas indicate denitrification in oxygen-stratified humic lakes. *Rapid Communications in Mass Spectrometry* 25: 1497–1502.

V Rissanen A.J., Ojala A., Fred T., Dernjatin M., Toivonen J. & Tiirola M. 2012. Bacterial communities in methanol-utilizing denitrification systems. Manuscript.

1 INTRODUCTION

1.1 Nitrogen cycle

Nitrogen (N) is one of the key elements sustaining life. N occurs in dissolved inorganic (DIN: NH_4^+, NO_2^-, NO_3^-), dissolved organic (DON) as well as in gaseous inorganic (e.g. NH_3, NO, N_2O, N_2) and particulate organic forms (PON), representing a wide range of oxidation states from –III (NH_4^+ and organic N) to +V (NO_3^-) (e.g. Hulth et al. 2005). N is constantly transformed and recycled in microbially mediated processes both within and between ecosystems and the atmosphere. It can be retained within ecosystems and is also physically transported between different ecosystems through water and air (Fig. 1 & 2).

The largest pool of N on earth is the atmospheric molecular N_2 gas. N_2 gas is available to the majority of the biota only through N fixation. This can take place biologically by diazotrophic (N-fixing) bacteria, such as cyanobacteria, many heterotrophs and some methanotrophs, that can reduce N_2 to fixed N forms. Non-biological N fixation can also occur both naturally, through lightning, and industrially, through the Haber-Bosch process and during combustion at high temperatures (Fig. 1). Fixed N is then released into the environment in the form of DIN. Besides these immediate atmospheric sources, fixed N, stored in bedrock over longer geological time scales, can also be released as DIN into environment (Holloway et al. 1998). DIN can then be assimilated into living biomass to form PON. When organisms die, PON breaks down into DON. In addition, DON is secreted by the living biota into their surroundings. DON can then be transformed back to DIN (NH_4^+) in ammonification. NH_4^+ can be assimilated again to form PON or it can be used in oxidative reactions.

In oxic conditions, NH_4^+ can be oxidized into NO_2^- and NO_3^- in a chemolithoautotrophic reaction, nitrification (Fig. 2). Some N_2O gas can also be formed during the process especially at very low O_2 availability. Nitrification takes place in two phases. In the first phase, NH_4^+ is oxidized into NO_2^- by ammonia-oxidizing bacteria or by ammonia-oxidizing archaea. In the second phase, NO_2^- is oxidized into NO_3^- by nitrite-oxidizing bacteria, and this process is

usually very tightly coupled to NH_4^+ oxidation. In aquatic systems, most nitrification activity is usually located in oxic layers close to the oxic–anoxic interfaces in sediments or in water columns (Fig. 2). However, NH_4^+ oxidation to NO_3^- can also take place in anoxic conditions by other oxidants, such as manganese oxides (e.g. Hulth et al. 2005, Pearson et al. 2012).

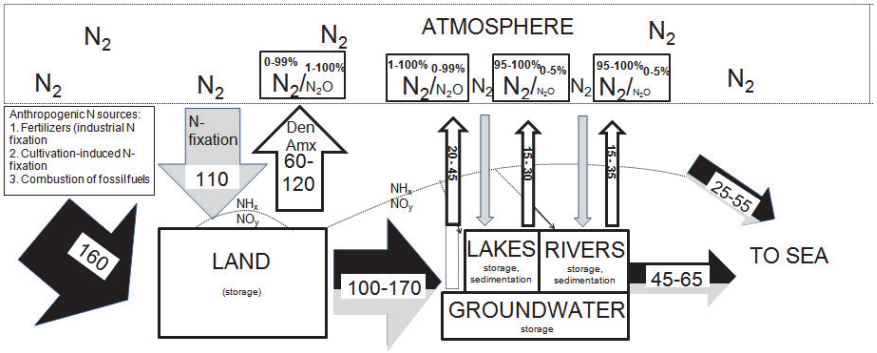

FIGURE 1 Modern global nitrogen (N) cycle on land (Tg N year^{-1}). The proportions of naturally and anthropogenically fixed N in the flow and transport of N is depicted with solid grey and black arrows, respectively. Microbial reactions removing N in gaseous forms (N_2 and N_2O), denitrification (Den) and anaerobic ammonium oxidation (anammox, Amx) are depicted with black outlined arrows. Relative percentages of N_2 and N_2O in gaseous emissions are given as a range reported in previous studies. Data are compiled from various sources (Seitzinger 1988, Groffman et al. 2000, Seitzinger et al. 2000, Well et al. 2001, Galloway et al. 2004, Seitzinger et al. 2006, Gruber & Galloway 2008, Schlesinger 2009).

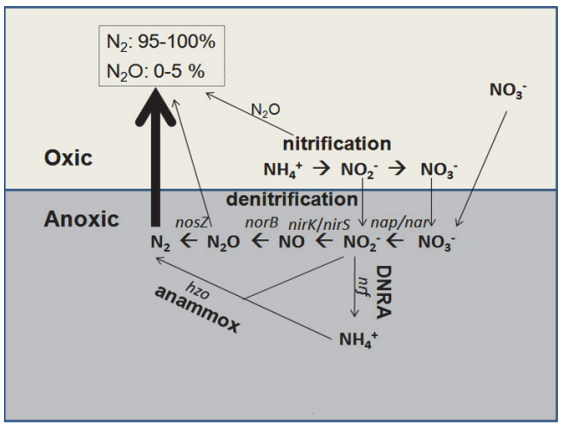

FIGURE 2 Nitrogen transforming reactions and associated functional genes coding for enzymes utilized in different steps of the processes at the oxic–anoxic interface. Relative percentages of N_2 and N_2O in gaseous emissions are given as a range reported in previous studies (see Fig. 1).

NO_3^- (and NO_2^-) can either be taken up by primary producers or diffused into anoxic site of the oxic–anoxic interface and reduced. There are several NO_3^-/NO_2^--reducing reactions that can take place in anoxic conditions. Some of them, like denitrification and anaerobic ammonium oxidation (anammox), produce N gases and, thus, remove N from the ecosystem, while some, such as dissimilatory nitrate reduction to ammonium (DNRA), retain N in the ecosystem. In denitrification, facultatively anaerobic bacteria use nitrogen oxides as alternative terminal electron acceptors during the oxidation of organic matter (heterotrophic denitrification) or inorganic matter, for example reduced sulphur or iron compounds (autotrophic denitrification) (Zumft 1997, Burgin & Hamilton 2007). During the process, inorganic N is reduced from dissolved forms (NO_3^-, NO_2^-) into gaseous forms (NO, N_2O, N_2). In aquatic systems, the majority (> 95 %) of the end product of denitrification usually consists of N_2 gas, while N_2O might be very significant in soils (Fig. 1). The ability to denitrify is widely dispersed among Bacteria and Archaea (e.g. Philippot 2002) and has also been found in some eukaryotes (Piña-Ochoa et al. 2010). Analyses on the complete genomic sequences of cultivated denitrifying bacteria indicate that 2/3 of bacteria can perform the whole chain of reactions from NO_3^- to N_2, whereas 1/3 of bacteria have a truncated pathway, which halts at N_2O (Jones et al. 2008).

Anammox is a chemoautotrophic reaction, where NH_4^+ is oxidized with NO_2^- resulting in the formation of N_2 gas, and it is being carried out only by a specific group of anaerobic bacteria within Planctomycete (genera: *Brocadia*, *Kuenenia*, *Scalindua*, *Anammoxoglobus*, *Jettenia*) (review by Hu et al. 2011). Denitrification was regarded as the major N_2 gas forming process before the discovery of anammox in mid-90s. Since then, anammox bacteria and activity have been detected in various ecosystems (reviewed by Hu et al. 2011). Global estimates suggest that anammox is especially significant in marine environments, accounting for half of the N_2 gas produced (Dalsgaard et al. 2005). In natural freshwater ecosystems, anammox activity *in situ* has so far been detected only in sediments of non-saline parts of estuaries (Dale et al. 2009, Koop-Jakobsen & Giblin 2009) and in the anoxic water column of lakes Tanganyika (Schubert et al. 2006) and Rassnitzer (Hamersley et al. 2009), and its wider importance in freshwater N cycling remains to be studied. In addition to denitrification and anammox, oxides of N can be reduced to N_2 during other less well known reactions, such as microbially mediated anaerobic oxidation of methane (CH_4) by NO_2^- (Ettwig et al. 2010), and abiotic oxidation of Fe^{2+} or Mn^{2+} by NO_3^- (e.g. Hulth et al. 2005, Burgin & Hamilton 2007). NH_4^+ can also be oxidized to N_2 by other oxidants besides NO_2^-, for example by manganese oxides (e.g. Pearson et al. 2012).

DNRA is a process where bacteria use NO_3^-/NO_2^- during fermentation of organic matter or in oxidation of inorganic matter (reduced sulphur compounds) resulting in the formation of NH_4^+ (Burgin & Hamilton 2007). DNRA is one of the least understood N processes, and most DNRA reporting studies so far have been conducted in coastal and estuarine ecosystems (e.g. Christensen et al. 2000, Jäntti et al. 2011). DNRA prevails when O_2 is in short supply (Jäntti & Hietanen

2012) and in highly reducing conditions, as in sediments with high organic matter content and low NO_3^- availability (Christensen et al. 2000).

Anthropogenic activities have more than doubled the input of fixed N to the terrestrial biosphere and this anthropogenic N input is continuously increasing (e.g. Gruber & Galloway 2008) (Fig. 1). This N-fertilization is a significant part of the human-induced global change and has led to severe health and environmental problems including nitrate-pollution, photochemical smog, eutrophication and acidification. Fertilization also contributes to climate change and stratospheric ozone depletion due to increases in N_2O emissions. Coastal and estuarine ecosystems, where primary production is mostly N-limited, are especially vulnerable to N-induced eutrophication (Howarth & Marino 2006). For instance, in the Baltic Sea, where N-limitation prevails (Kivi et al. 1993, Tamminen & Andersen 2007), increased N loading has caused severe algal blooms, anoxia in the bottom layers and loss of animals (Anon. 2009). Anthropogenic sources dominate the N load to the Baltic Sea and most of the N entering the Baltic Sea comes via rivers from inland sources (> 70 %) (Anon. 2009) (Fig. 3). While the importance of point sources such as wastewaters in N loads to the Northern Baltic Sea have decreased during recent decades due to more efficient N removal, the river-borne N loads have increased (Räike et al. 2009). Consequently, detailed investigations of the fate of N in the whole catchment area of the Baltic Sea have become increasingly important.

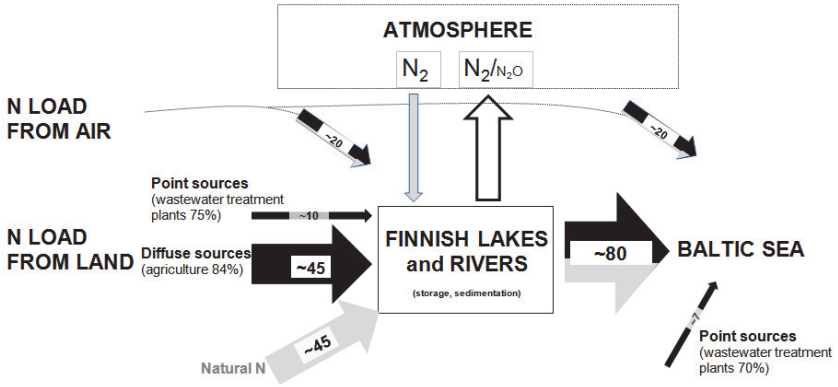

FIGURE 3 The sources and the flow of nitrogen (N) in the surface waters of Finland (Gg N $year^{-1}$). The proportions of naturally and anthropogenically fixed N in the flow and transport of N is depicted with solid grey and black arrows, respectively. Figure compiled from Pietiläinen (2008) and references therein & Räike et al. (2009).

1.2 N_2 gas producing processes mitigating the effects of anthropogenic N loads to coastal areas

1.2.1 Lakes

Global models suggest that lakes are ´hot spots' of N removal and significantly reduce the N load from land to the coastal ecosystems (Seitzinger et al. 2006, Harrison et al. 2009). Although lakes comprise only 2 – 3 % of global land surface, they may account for 13 % of the total terrestrial gaseous N emissions and remove 23 – 46 % of the estimated inputs (65 Tg N $year^{-1}$) to surface waters (Seitzinger et al. 2006, Wollheim et al. 2008, Harrison et al. 2009) (Fig. 1). This takes place through sedimentation, and, most importantly, through the microbially mediated gas emissions via denitrification (e.g. Saunders & Kalff 2001a), and possibly through anammox (e.g. Schubert et al. 2006). Models are crucial for predicting the fate of anthropogenic N inputs in the landscape and for predicting the response of ecosystems to changes in the N loads. There are, however, considerable uncertainties in the current biogeochemical models, which is partly due to inadequate background data of the different components of N removal and recycling (e.g. Harrison et al. 2009). More data are needed especially regarding the magnitude, variations and factors affecting the N_2 gas production in lakes.

N_2 gas producing processes, with the main focus on denitrification, have been studied during recent decades in a variety of lake ecosystems (reviewed by Seitzinger 1988, Saunders & Kalff 2001b). Most studies have focused on temperate lakes, leaving the large number of lakes in the boreal zone, including the catchment area of the Northern Baltic Sea, almost totally unstudied. Models, however, suggest that small lakes (surface area < 50 km^2), typical of the boreal zone, have larger area-specific N retention rates than large lakes, and are globally very important sinks of N (Harrison et al. 2009). For instance, in Finland there are almost 190 000 lakes, yet no previous studies on the N_2 formation in these lakes. However, N mass balances indicate that the N retention in Finnish lakes, although quite variable among lakes, may be as high as 82 % of the N input (Pietiläinen 2008). Boreal lakes often form chains and networks and are shallow, which provide opportunities for sediment–water contact, and may promote N removal by N_2 gas producing processes. Indeed, the modeled N retention by river basins in Finland, 0 – 68 % of the N input, was highest in basins with a high abundance of lakes (Lepistö et al. 2006), highlighting the role of lake networks in the N removal. In addition, the previous denitrification studies have concentrated almost solely on sediments (e.g. Seitzinger 1988, Piña-Ochoa & Álvarez-Cobelas 2006). In many small, stratified forest lakes of the boreal zone, hypolimnetic anoxia with high NH_4^+ concentrations may give rise to the N_2 producing processes also in the water column.

Previous studies of lake sediments indicate that temperature (e.g. Saunders & Kalff 2001b), NO_3^- concentration in the water above the sediment (e.g.

Risgaard-Petersen et al. 1999) and the organic matter content of sediment (Saunders & Kalff 2001b) can affect the denitrification rates. In addition, O_2 concentration of the water overlying the sediment can indirectly affect the processes (e.g. Rysgaard et al. 1994). However, previous studies have been mostly case studies on single lakes and the range in the measured process rates and environmental factors has, therefore, been quite limited, hampering generalizations. The few multiple lake studies indicate that NO_3^- concentration, in general, affects the sediment denitrification rates (McCrackin & Elser 2010, 2012). These studies, however, were conducted using techniques (sediment slurries + acetylene inhibition) which are known to produce erroneous estimates of the N_2 gas production rates (e.g. Groffman et al. 2006) (chapter 1.3).

Besides environmental factors, the structure of microbial communities can affect the N_2 producing processes (e.g. Magalhaes et al. 2008, Enwall et al. 2010). However, only recently has attention been paid to the denitrifier and anammox communities in lake sediments (e.g. Kim et al. 2011, Yoshinaga et al. 2011) and water columns (Schubert et al. 2006, Hamersley et al. 2009), with most of the attention previously on soil (e.g. Rich et al. 2003) or marine (e.g. Braker et al. 2001), wetland (e.g. Kjellin et al. 2007) and river sediments (e.g. Perryman et al. 2008). Simultaneous analyses of *in situ* N_2 production rates and microbial communities are especially scarce in lakes and limited to studies on water column anammox (Schubert et al. 2006, Hamersley et al. 2009).

1.2.2 Wastewater treatment

Microbial N_2 gas producing reactions are increasingly being applied in the treatment of municipal and industrial wastewaters. In addition, the processes are also utilized in controlling DIN levels in aquaria, especially in those with closed water circuit (e.g. Labbé et al. 2003). Despite the recent technological advances in N removal technology, such as anammox, CANON (completely autotrophic nitrogen removal over nitrite) and OLAND (oxygen limited autotrophic nitrification and denitrification) processes, the most often used process in wastewater N removal is nitrification–denitrification. Denitrification can be integrated as a part of activated sludge process (organic matter oxidation), either as pre- or post-denitrification. In these cases, the activated sludge tank is divided into oxic and anoxic parts, for nitrification and denitrification to take place, respectively. Post-denitrification can also be applied as a separate anoxic unit after the oxic (e.g. activated sludge) unit. Separate anoxic denitrification is also in use in aquaria (e.g. Labbé et al. 2003). Both types, denitrification integrated with the activated sludge process and separate post-denitrification unit, can be applied in some wastewater treatment plants in Finland, as it is in the Viikinmäki wastewater treatment plant in Helsinki. Separate post-denitrification usually takes place in filters, where the water flows through granular filter bed and NO_3^- is reduced to N_2 gas by the bacteria living attached to granules (e.g. Koch & Siegrist 1997). An external carbon source, usually acetate, ethanol, glucose or methanol, has to be applied to post-denitrification filters due to low C:N ratio in

the water. Methanol is the most often used carbon source because of its relatively cheap price and the small amounts of sludge produced. Methanol-utilizing denitrification filters have been used in municipal wastewater treatment in the USA since the 1980s and in Europe since the 1990s (Koch & Siegrist 1997). In Finland, the first full-scale plant was launched in 1999, and since then the technique has been successfully applied in six wastewater treatment plants located in coastal areas (Niemelä 2009). This has raised the total N removal in the plants to very high levels (70 – 90 % N removal) (Niemelä 2009). Methanol-utilizing denitrification systems are also applied in some sea water aquaria, for example Sealife in Helsinki (Dernjatin 2008) and at Biodome in Montreal (e.g. Labbé et al. 2007).

The study of microbiology of wastewater treatment systems has gained interest in recent years because understanding the identity and ecology of bacteria involved in the processes may help the design, successful application and optimization of the processes. It is crucial to understand the relationships between function and community structure/diversity, such as between functional stability and community dynamics (e.g. Wang et al. 2011). The physico–chemical and technical aspects of full-scale methanol-fed denitrification systems have been regularly studied (e.g. Koch & Siegrist 1997, Lemmer et al. 1997, Bosander & Westlund 2000, Jonsson 2004); however, there are only a few studies on the microbial communities inhabiting the systems (e.g. Neef et al. 1996, Labbé et al. 2003). Most of the previous studies on methanol-utilizing denitrifier communities have focused on laboratory-scale systems (e.g. Ginige et al. 2004, Osaka et al. 2006). Thus, information about the variability in communities within and between different full-scale filters and how these variations relate to function is scarce. Methanol is a C-1 compound and only specific methylotrophic bacteria can utilize it (e.g. Madigan et al. 2007). This can limit biodiversity in the filters making them good candidates for studies of community variations. Indeed, previous studies that have utilized methods linking substrate use with bacterial identity [e.g. stable isotope probing (SIP) and MAR-FISH (fluorescent in situ hybridization combined with microautoradiography)] indicate low diversity communities mostly dominated by Betaproteobacteria [Methylophilaceae (genera: *Methylophilus*, *Methylobacillus*); Rhodocyclaceae (genus: *Methyloversatilis*)] and less by Alphaproteobacteria [Hyphomicrobiaceae (genus: *Hyphomicrobium*)] (e.g. Ginige et al. 2004, Osaka et al. 2006, Baytshtok et al. 2009) in non-saline lab-scale systems. In contrast, a different type of community consisting mostly of Gammaproteobacteria [Piscirickettsiaceae (genus: *Methylophaga*)], and less of Alphaproteobacteria [Hyphomicrobiaceae (genus: *Hyphomicrobium*)] dominated the denitrification reactor of a saline water aquarium (e.g. Labbé et al. 2003, 2007). However, the structure and the diversity of microbial communities might be different in full-scale denitrification filters of municipal wastewater treatment plants. In addition, most previous studies of wastewater systems have been based on characterization of the bacterial phylogenetic marker gene, 16S rRNA gene. Identifying denitrifiers based on 16S rRNA sequences alone might be difficult and, therefore, community characterizations should also focus on the functional

genes of denitrification (Fig. 2), but this has seldom been attempted in methanol-utilizing denitrification systems (Hallin et al. 2006, Osaka et al. 2006, Auclair et al. 2011).

1.3 Methods to study N_2 gas producing processes in aquatic systems

1.3.1 N_2 gas production rate measurement techniques

A variety of methods have been developed to study N_2 gas production in aquatic systems. These methods include NO_3^- disappearance, acetylene inhibition (AI), ^{15}N tracers, N_2 gas flux quantification, mass-balance (MB), stoichiometric approaches, sediment pore-water profiles and methods based on natural stable isotope ratios (see reviews by Cornwell et al. 1999, Groffmann et al. 2006, Piña-Ochoa & Álvarez-Cobelas 2006, Song & Tobias 2011). In aquatic studies, the most often used methods have been AI, N_2 gas flux, ^{15}N tracers and MB (e.g. Saunders & Kalff 2001b, Piña-Ochoa & Álvarez-Cobelas 2006). The first three mentioned have been applied mainly *in vitro* (e.g. Saunders & Kalff 2001b), but in some cases also *in situ* mesocosms, specifically in flux chambers (e.g. Mengis et al. 1997) or in enclosures (Risgaard-Petersen et al. 1999) (Fig. 4). Laboratory incubations have been conducted both in batch-mode assays, either as end-point or time series measurements, or in flow-through systems (e.g. Cornwell et al. 1999, Steingruber et al. 2001).

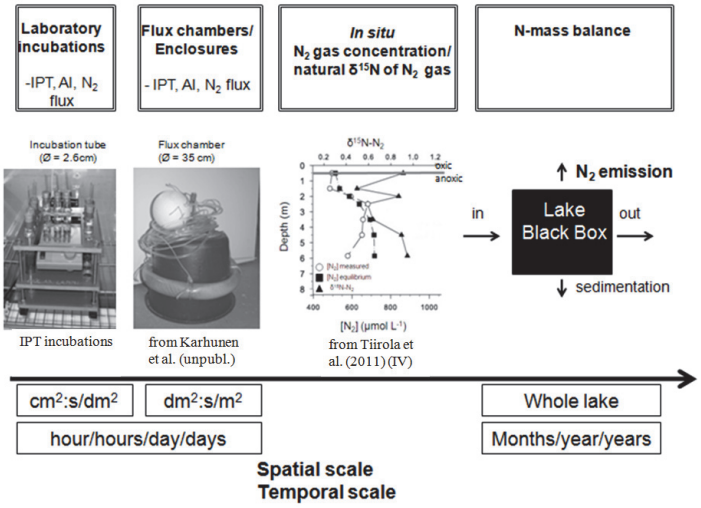

FIGURE 4 Methods of N_2 gas production rate measurements used in lakes and divided by the temporal and spatial measurement scale. IPT = isotope pairing technique, AI = acetylene inhibition.

AI is based on the inhibition of the N_2O reduction to N_2 in denitrification, and so the accumulated N_2O can be measured using gas chromatograph (GC) (Sørensen 1978). This method has been used in numerous studies, especially in the 1970s and early 1980s (Groffman et al. 2006). AI is easily applied and very cost-effective, but has several limitations leading to biased estimates of N_2 production. These limitations include the inhibition of nitrification by acetylene, preventing the measurement of coupled nitrification-denitrification; the incomplete blockage of N_2O reduction at low NO_3^- concentrations, particularly in the presence of H_2S (reviewed by Cornwell et al. 1999) and the inability to measure anammox. Thus, the applicability of the method is limited to ecosystems with no anammox, low levels of H_2S and denitrification supported solely by NO_3^- in water column. Despite these limitations, AI is still used in aquatic research (e.g. McCrackin & Elser 2010, 2012) and as a valuable method in assays of denitrification potential (Groffman et al. 2006). However, ^{15}N tracer methods (e.g. isotope pairing technique, IPT) and N_2 flux quantification are currently considered more reliable when estimating N_2 gas production rates (e.g. Groffman et al. 2006).

^{15}N tracer methods are based on incubating water or sediment samples with additions of $^{15}NH_4^+$, $^{15}NO_3^-$ or $^{15}NO_2^-$ and by measuring the production of ^{15}N-labeled N_2 gas (D15) using either isotope ratio mass spectrometer (IRMS) or quadrupole mass spectrometer (QPMS). Tracers are usually added in such amounts that isotopic fractionation and the natural occurrence of ^{15}N (3.66 ‰ of the total N pool) can be neglected facilitating simple separation between the added (^{15}N) and the natural nitrogen (^{14}N). In incubations with added $^{15}NO_3^-$ and denitrification as the only process producing N_2 gas, the ^{15}N production rate (D15) can be translated into estimates of natural N_2 production (D14, denitrification of natural $^{14}NO_3^-$) if the ratio of ^{15}N to ^{14}N in the NO_3^- being denitrified (in the NO_3^- reduction zone) is known. This ratio can be derived in anoxic water or sediment slurry incubations, particularly in conditions where nitrification does not produce NO_3^-, either by measuring the isotopic composition of NO_3^- or by estimating it using known concentrations of natural $^{14}NO_3^-$ and added $^{15}NO_3^-$. If nitrification takes place, such as in intact sediment core incubations with oxic sediment surface, the isotopic ratio in NO_3^- reduction zone cannot be estimated straightforwardly. In this case, using the $^{15}NO_3^-/^{14}NO_3^-$ ratio in the oxic surface water will only yield estimates of the denitrification supported by the NO_3^- in the overlying water. One solution to this problem is parallel incubations with $^{15}NH_4^+$ to measure N_2 gas production via coupled nitrification–denitrification (Nishio et al. 1983); however, this approach has assumptions that are difficult to fulfill and test (Groffmann et al. 2006). It was not until Nielsen (1992) adopted the idea of random isotope pairing originally suggested by Hauck et al. (1958) for soil studies (according to Groffmann et al. 2006) that it has been possible to get reliable estimates of D14 in intact sediments using $^{15}NO_3^-$ additions to the water overlying the sediment. In IPT, it is assumed that the added $^{15}NO_3^-$ and natural $^{14}NO_3^-$ pair randomly and the production of N_2 gas molecules ($^{14}N^{14}N$, $^{15}N^{14}N$ and $^{15}N^{15}N$) follows binomial distribution. Therefore, the ratio of ^{15}N-labeled gas molecules produced ($^{15}N^{14}N$ and $^{15}N^{15}N$) reflects the isotope ratio of NO_3^- in the NO_3^- reduction zone (Nielsen 1992). N-fixation in

sediments has little effect on IPT and, therefore, this method determines D14, which is very close to actual gross N_2 gas production (Eyre et al. 2002). In addition, IPT can differentiate between different NO_3^- sources in one incubation, in other words between D_n (denitrification of the $^{14}NO_3^-$ produced in oxic sediment surface zone in nitrification) and D_w (denitrification of the $^{14}NO_3^-$ diffusing into the sediment from the water overlying the sediment) (e.g. Steingruber et al. 2001).

Since the beginning of 1990s, IPT has been used in various aquatic ecosystems (e.g. Steingruber et al. 2001). There are, however, several assumptions underlying IPT, for example homogenous mixing of $^{14}NO_3^-$ and $^{15}NO_3^-$ in the water column and sediment, independence of natural denitrification activity on the amount of added $^{15}NO_3^-$ and a positive linear dependency of D15 on the $^{15}NO_3^-$ concentration, that should be tested before analysis. This can be done by parallel incubations with differing $^{15}NO_3^-$ concentrations. In these tests, D14 should be independent and D15 linearly and positively dependent on the added $^{15}NO_3^-$ concentration (Fig. 5).

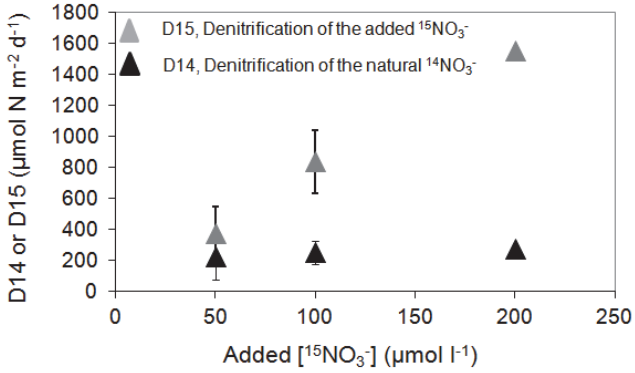

FIGURE 5 An example of a successful $^{15}NO_3^-$-concentration test of the assumptions underlying the isotope pairing technique. Data (mean ± SD) are from the profundal sediment of Ormajärvi in October 2006 (II).

Discovery of anammox has complicated the application of ^{15}N tracer methods (e.g. Risgaard-Petersen et al. 2003, 2004). In anoxic conditions when nitrification does not take place, anammox activity can be measured in incubations of water and sediment slurries by different combinations of $^{15}NO_3^-$, $^{15}NO_2^-$, $^{15}NH_4^+$ and their natural or added ^{14}N analogues (Thamdrup & Dalsgaard 2002, Trimmer et al. 2003). In anoxic incubations with $^{15}NH_4^+$ (+natural or added $^{14}NO_3^-$ or $^{14}NO_2^-$), the anammox activity is estimated from the production $^{15}N^{14}N$. In anoxic incubations with $^{15}NO_3^-$ or $^{15}NO_2^-$ (+ natural or added $^{14}NH_4^+$), anammox and denitrification can be measured and separated using the production rates of $^{14}N^{15}N$ and $^{15}N^{15}N$ and the isotopic ratio of NO_3^-/NO_2^- (e.g. review by Song & Tobias 2011). In measurements of intact sediment cores, anammox activity can cause severe overestimation of N_2 gas production if the original IPT is applied (Risgaard-Petersen et al. 2003, Hietanen 2007). This is because the presence of

anammox skews the binomial distribution of produced N_2 gas molecules (see above); more $^{14}N^{15}N$ is produced (via pairing of $^{15}NO_3^-$ and $^{14}NH_4^+$) than would be through denitrification only. Therefore, the isotopic ratio of NO_3^- in the NO_3^- reduction zone can no longer be estimated using the ratios of N_2 gas molecules produced. This has led to a revision of the original IPT (r-IPT) (Risgaard-Petersen et al. 2003, 2004, Trimmer et al. 2006). In r-IPT, the $^{15}NO_3^-/^{14}NO_3^-$ ratio can be estimated in three ways requiring either 1) parallel slurry incubations to quantify the relative importance of anammox, 2) different sets of intact sediment core incubations with different concentrations of $^{15}NO_3^-$ (Risgaard-Petersen et al. 2003, 2004) or 3) the isotopic analysis of N_2O produced in incubations of intact sediment cores (Trimmer et al. 2006). Thus, IPT incubations with differing $^{15}NO_3^-$-concentrations to test the assumptions underlying IPT will also aid in quantifying the possible anammox activity (e.g. Risgaard-Petersen at al. 2003, Jäntti et al. 2011).

Besides measuring denitrification and anammox, $^{15}NO_3^-$-incubations can potentially be extended for measurement of other N cycling reactions, such as DNRA (analysis of $^{15}NH_4^+$ formation) and nitrification (analysis of $^{15}NO_3^-$ dilution) (e.g. Christensen et al. 2000, Jäntti et al. 2011). In addition, production of N_2O can also be measured using the IPT (analysis of isotopic composition of N_2O) (e.g. Master et al. 2005, Dong et al. 2006).

N_2 gas flux technique, which quantifies the production of N_2 by measurement of N_2 gas concentration change, has the advantage of providing reliable data on net N_2 gas production (= gross N_2 gas production – N fixation) with minimum disturbance of the system since no inhibitors or tracers are added. This technique, however, requires extremely gas-tight incubations and sampling systems and a very sensitive analysis due to high concentrations of the N_2 gas in the atmosphere and in natural waters. Contrary to IPT (and r-IPT), the technique is also unable to separate between anammox and denitrification and between D_n and D_w. The N_2 gas concentration is measured with gas chromatograph (GC) (e.g. Saunders & Kalff 2001b) or from N_2:Ar ratio using headspace or membrane inlet mass spectrometry (MIMS), which provides superior precision over GC (e.g. Kana et al. 1994, Groffmann et al. 2006). Currently, IPT and the N_2 flux measurements using N_2:Ar on MIMS are the most frequently used methods to measure N_2 production in undisturbed sediments (e.g. Ferguson & Eyre 2007). Several methodological comparisons between N_2 flux and IPT have been performed showing either a good agreement (Risgaard-Petersen et al. 1998, Eyre et al. 2002) or some differences between the methods (van Luijn et al. 1996, Ferguson & Eyre 2007). These techniques can also be combined allowing simultaneous measurements of N_2 production and N fixation (e.g. Scott et al. 2007).

Laboratory incubations are important when the variations and the abiotic and biotic controls of N_2 gas production are studied. However, caution must be used when N_2 production rates based on laboratory incubations are extrapolated to the ecosystem scale (e.g. whole lake scale) (e.g. Groffman et al. 2006). For instance, laboratory studies usually underestimate N_2 gas production in lakes when compared to estimates with MB, where N_2 gas production is indirectly

calculated from N budgets (e.g. Seitzinger et al. 1988, Ahlgren et al. 1994, Mengis et al. 1997, Risgaard-Petersen et al. 1999) (Fig. 4). There are several possible reasons for this. Conditions in laboratory incubations may not correctly simulate the *in situ* conditions ("enclosure effect" in Groffmann et al. 2006) (e.g. Risgaard-Petersen et al. 1999). Disturbances during sample collection and handling may affect the chemical and physical properties (e.g. redox profiles) of the sediment cores. The temporal and spatial heterogeneity of the ecosystems might not be adequately covered, with incubations lasting only for some hours and with samples covering the sediment at cm^2/dm^2-scale (Fig. 4). The larger scale spatial (e.g. different depth zones and bottom types) and temporal (e.g. monthly) variations might be inadequately covered due to logistical reasons and/or laborious field and laboratory work, and in some cases due to expensive analyses of N_2 gases. In addition, sediments with macrophyte roots and bioturbation by fish and invertebrates, which affect the *in situ* redox profiles, are a challenge in laboratory measurements (e.g. Groffmann et al. 2006). Measurements *in situ* in mesocosms exclude some of the disadvantages and should be, therefore, more widely used. Conditions in mesocosms reflect the *in situ* conditions, and only minimal disturbance of sediments is involved. In addition, small scale spatial variation is adequately covered (Fig. 4). Mesocosm measurements also generally last longer than laboratory incubations (Fig. 4). When applying IPT in mesocosms, the $^{15}NO_3^-$-concentration tests can still be performed in laboratory reducing the number of required field replicates (e.g. Nielsen & Glud 1996, Risgaard-Petersen et al. 1999).

Although mesocosm measurements provide clear advantages over laboratory incubations, they may still fail to cover the larger scale spatial and temporal variation, which may then lead to underestimation of ecosystem scale N_2 production when compared to MB estimates (e.g. Mengis et al. 1997). On the other hand, MB method may also yield inaccurate estimates of the N_2 gas production due to difficulties in measuring or estimating all the components of the N budget (Groffman et al. 2006) (Fig. 4). A rare alternative approach, but with potential to integrate large scale spatial and temporal variability, is direct measurements of *in situ* N_2 gas concentrations (e.g. Deemer et al. 2011) (Fig. 4). This method is based on detection of oversaturation and accumulation of N_2 from N_2 gas producing processes (Groffmann et al. 2006). Additional information on the processes may be acquired by concurrent analyses of natural isotopic ratios of N_2 gas and DIN (e.g. NO_3^-) (e.g. Fuchsman et al. 2008, Pearson et al. 2012).

Natural isotopic ratios are expressed in terms of δ values (expressed as ‰), which are parts per thousand differences from a standard (atmospheric N_2 gas):

$$\delta^{15}N = [(R_{sample}/R_{standard})-1]*10^3,$$

where R is the $^{15}N/^{14}N$ ratio. During each N cycling process (e.g. denitrification), isotopic fractionation takes place, in other words ^{14}N reacts faster than ^{15}N. As denitrification proceeds, this leads to a lower $\delta^{15}N$ value of the product (N_2) compared to the substrate (NO_3^-). When the substrate is consumed

completely, the $\delta^{15}N$ of the product will represent that of the substrate initially. $\delta^{15}N$ of the total N_2 gas pool may decrease or increase during the N_2 producing processes as a result of the mixing of new N_2 with other components of the N_2 in the system. The $\delta^{15}N$ of produced, excess gas can then be calculated using mass and isotopic balance equations. Fractionation ranging from –5 to –40 ‰ has been shown to take place during denitrification (reviewed by Robinson 2001, Kendall et al. 2007), but the fractionation associated with anammox is currently unknown. Together, the excess concentration and lower $\delta^{15}N$ of excess N_2 than $\delta^{15}N$ of NO_3^- confirmed denitrification in the Black Sea (Fuchsman et al. 2008). Fractionation of N_2 in the Black Sea was exceptionally high since the $\delta^{15}N$ of the excess N_2 was very low (–30 – –40 ‰) in the lower part of the suboxic zone (Fuchsman et al. 2008), indicating the potential of the natural abundance isotope analysis in the aquatic denitrification studies. In contrast, in a stratified, eutrophic lake $\delta^{15}N$ of N_2 gas increased during denitrification (Pearson et al. 2012) because no isotopic fractionation took place during denitrification; NO_3^- produced in nitrification was rapidly denitrified, and, therefore, the changes in $\delta^{15}N$ of N_2 gas were controlled by $\delta^{15}N$ of PON and fractionation during preceding N cycling processes (ammonification, nitrification) (Pearson et al. 2012). Due to scarcity of data, it is not known how the N_2 producing processes affect the stable isotope and concentration profiles of N_2 gas in other aquatic systems, such as boreal lakes.

1.3.2 Characterization of microbial communities

Culture-independent, molecular biology methods dominate the current research in microbial ecology. The most often used techniques are based on polymerase chain reaction (PCR)-amplification of the gene target from nucleic acid extract of an environmental sample, and subsequent resolution of the sequence differences either using cloning/sequencing or molecular microbiological fingerprinting, for example denaturing gradient gel electrophoresis (DGGE) (Muyzer et al. 1993) and length heterogeneity analysis of PCR-amplified 16S rRNA gene (LH-PCR) (Suzuki et al. 1998) (Spiegelman et al. 2005) (Fig. 6). The recent breakthrough of cost-effective, next generation sequencing technologies (e.g. 454-pyrosequencing) has added much to these analyses, now enabling comprehensive insights into the variation and diversity of communities (e.g. Mao et al. 2011) (Fig. 6).

The microbial ecological studies are usually based on the bacterial phylogenetic marker genes, especially on the 16S rRNA gene. In the case of denitrification, however, this approach is unsuitable since the ability to denitrify is so widely spread among bacteria. Therefore, denitrifier communities are studied by characterizing the functional genes coding for different reductase enzymes acting in the nitrate reduction chain (Fig. 2) (e.g. Wallenstein et al. 2006). The studies have mostly focused on genes coding for nitrite (*nirK* and *nirS*) and nitrous oxide reductase (*nosZ*). There are two structurally different, but functionally equal nitrite reductases, copper nitrite reductase (coded by *nirK*) and cytochrome cd1 nitrite reductase (coded by *nirS*) (Fig. 2 & 6), which have been shown to be mutually exclusive in cultivated strains (e.g. Jones et al. 2008). Thus,

denitrifying bacteria can be grossly divided into two groups based on the nitrite reductase system. In contrast to denitrifiers, anammox bacteria form a phylogenetically distinct group within the Planctomycete and they can be studied by targeting the 16S rRNA gene (e.g. Dale et al. 2009). An alternative approach is through focusing on *hzo* gene encoding hydrazine oxidation, the key step for gaining energy in the anammox pathway (review by Song & Tobias 2011).

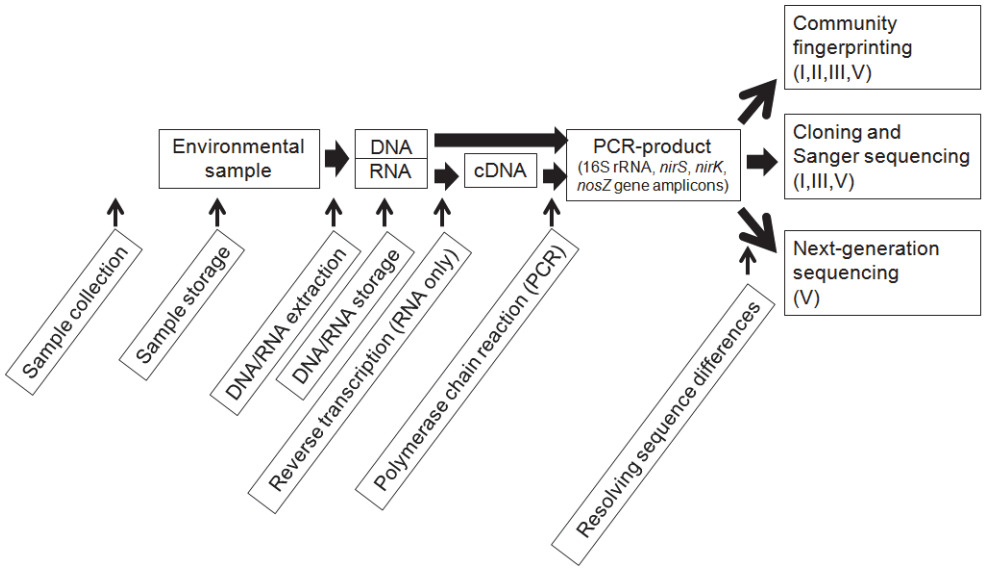

FIGURE 6 Typical workflow of polymerase chain reaction (PCR)-based analyses in a molecular microbiological study of environmental samples.

Several factors may affect and bias the outcome of molecular microbiological analyses. Methods utilized in the collection, handling and storage of the environmental samples (e.g. Rochelle et al. 1994), as well as during nucleic acid extraction (e.g. Luna et al. 2006), can all affect the quantity and the quality of nucleic acid extractions. In addition, all PCR-based methods share the same weaknesses, for example, primer bias, inhibition by humic and fulvic acids and chimera formation, which might bias the representation of the studied community (review by Spiegelman et al. 2005). These potential biases received much attention previously and suggestions for conducting reliable analyses have also been made (e.g. review by Spiegelman et al. 2005). However, studies focusing on the effects of different sample storage methods are scarce. Only a few studies have addressed the effects of storing soil samples (e.g Sessitsch et al. 2002) and only one study on sediment samples (Rochelle et al. 1994). Storage by freezing is usually considered the most reliable method for preservation (e.g. Wallenius et al. 2010). Freezing, however, is not always possible, for example during extended field trips. Consequently, different storage solutions (e.g.

RNAlater®) have been developed, but their effect on the outcome of molecular microbiological studies of sediments has never been assessed.

1.4 Aims

Lakes and denitrification systems of wastewater treatment plants significantly reduce the N load from land to vulnerable coastal areas. Thus, it is necessary to learn to understand the magnitude, variations and the factors controlling N removal by N_2 gas production in these 'hot spot' systems. Therefore, the aims of this thesis are to

1) clarify the abiotic and biotic factors that control N_2 gas production in lakes,

2) test and develop a novel, spatially and temporally integrative technique based on *in situ* concentration and $\delta^{15}N$ of N_2 gas to measure N_2 gas production in lakes and

3) study the relationship between functional stability and community dynamics within denitrification systems, as well as variations in community structure between different systems.

These aims were achieved by studying within lake spatial and temporal variations in sediment N_2 gas production rates and in denitrifying communities with samples collected from 1 boreal lake in Finland (II). In addition, interlake variations of these processes and microbial communities were studied with samples collected from 4 boreal lakes also located in Finland. A wider analysis on environmental controls of N_2 production in lakes was conducted with data on N_2 production rates of boreal lakes combined with data from 9 previously studied lakes from northern, central and southern Europe (III). A specific experiment to test the effects of different sediment sample storage methods on the quantity and quality of nucleic acid extracts as well as on the outcome of molecular microbiological studies was also conducted (I). The potential of *in situ* measurements of N_2 gas concentration and $\delta^{15}N$ of N_2 gas to detect and measure N_2 gas production was tested by analyzing vertical N_2 gas profiles from 9 oxygen-stratified humic lakes (IV). The relationship between functional stability and microbial community dynamics of denitrification systems was studied by a 10-week monitoring of one methanol-utilizing denitrification filter of a municipal wastewater treatment plant. In addition, differences in the community structures were studied between methanol-utilizing denitrification systems of 2 municipal wastewater treatment plants and a seawater fish aquarium (V).

2 MATERIALS AND METHODS

2.1 Study areas

2.1.1 Boreal study lakes (II, III, IV)

Lakes in II and III, Pääjärvi, Ormajärvi, Suolijärvi and Lehee, are located in southern Finland (61.07 – 61.17 °N, 24.79 – 25.13 °E), and represent typical, small to medium-sized boreal lakes with differences in morphometry and trophic status (Fig. 7, Table 1). Pääjärvi and the chain of Ormajärvi, Suolijärvi and Lehee form two separate headwater lake systems of the Kokemäenjoki River Basin draining into the Bothnian Sea in the northern part of the Baltic Sea (Fig. 7).

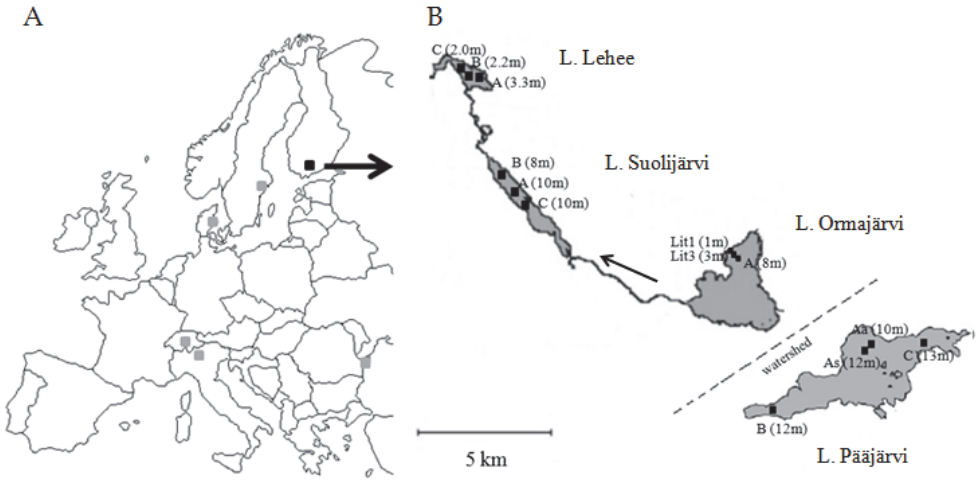

FIGURE 7 A) A map of Europe showing the locations of the boreal study lakes (black square) and the other lakes of the continental scale analysis (grey squares). B) A map of the boreal study lakes showing the locations of the sampling sites (black squares, depths shown) (II, III).

Pääjärvi is mesotrophic, whereas the lake chain is classified as eutrophic. Except for Lake Lehee, which is polymictic, all lakes are dimictic and stratify thermally in summer. All the lakes have an oxic hypolimnion throughout the open-water period. Ormajärvi has a lower total organic carbon (TOC) concentration and colour value in the epilimnion (TOC 9.0 g C m^{-3}; colour 34 g Pt m^{-3}) than Pääjärvi, Suolijärvi and Lehee (TOC 11 – 12.3 g C m^{-3}; colour 87 – 100 g Pt m^{-3}). The lakes have forested catchments; forests cover approximately 60 % of the catchments around Pääjärvi and Ormajärvi and approximately 70 % around Suolijärvi and Lehee. Agricultural land use is highest in the catchment area of Ormajärvi (26 %) followed by Pääjärvi and Suolijärvi (18 %), whereas the agricultural land use is lowest in the catchment area of Lehee (13 %).

TABLE 1 Characteristics of the study lakes (II and III).

		Pääjärvi	Ormajärvi	Suolijärvi	Lehee
Area	km^2	13.4	6.5	2.1	1.1
Catchment area	km^2	199.0	116.0	214.0	243.0
Max. depth	m	87.0	31.0	10.0	4.0
Mean depth	m	15.0	9.0	5.0	1.0
Mean residence time	y	3.3	2.9	0.4	0.1

The 9 study lakes in IV are located in southern Finland, within 40 km of the study lakes in II and III (61.32 – 61.38 °N, 25.18 – 25.40 °E), and they represent typical, small (A = 0.3 – 25 ha), humic, forest lakes with low pH values (4.7 – 5.9 in epilimnion) and high colour content (100 – 460 g Pt m^{-3} in epilimnion). The high colour content combined with the small lake surface area contribute to the strong temperature stratification and hypolimnetic anoxia during summer and often incomplete mixing during turnover periods, especially in spring. NO_3^- concentrations in the water column are low (NO_3^- + NO_2^-: 2 – 7 µmol l^{-1}). NH_4^+ concentrations are low in the epilimnion (0.7 – 7 µmol l^{-1}), but can be very high in the hypolimnion (up to 320 µmol l^{-1}). In addition, CH_4 concentrations are very high in hypolimnion (160 – 1000 µmol l^{-1}) during stratification.

2.1.2 Methanol-utilizing denitrification systems (V)

The studied denitrification systems were located in two municipal wastewater treatment plants (WTPA and WTPB) and in one public, sea-water fish aquarium (AQUAR). The study systems differ considerably in their size and filter bed characteristics. WTPA is a large plant with one of the largest denitrification filter systems in the world whereas WTPB is a medium-sized plant (Niemelä 2009) (Table 2). The aquaria in AQUAR are divided into 2 water recirculation systems (warm, tropical and cold North Atlantic recirculation systems) and have a total water volume of 420 m^3. The denitrification reactors of AQUAR are very small

compared to WTPA and WTPB (Table 2). In addition, WTPA and WTPB are non-saline systems and AQUAR is a saline water system (salinity 27 – 34 ppt). The studied denitrification systems utilized mainly methanol as their carbon source (Table 2).

TABLE 2 Characteristics of denitrification systems in the wastewater treatment plants and the seawater fish aquarium. * = Reactor connected to warm, tropical recirculation system (T = 24 °C) utilized methanol (AQUAR_M). Reactor connected to cold North Atlantic recirculation system (T = 14 °C) utilized a mixture of methanol and saccharose (AQUAR_MS).

Site	Population equivalent	Average flow $m^3\ d^{-1}$	No. of denitr. cells	Bed volume $m^3\ cell^{-1}$	Carrier
WTPA	740000	280000	10	432.000	polystyrene
WTPB	31000	14000	6	56.000	polystyrene
AQUAR		5	2*	0.125	oolitic sand

2.2 Sampling and data collection

2.2.1 Storage of the sediment samples (I)

Storage of microbial samples was studied using three sediment types of varying contents of dry matter and carbon. Sediments were collected from the eutrophic Lake Jyväsjärvi (sediment dry matter = 12 %, loss on ignition (LOI) = 13 %), the polyhumic Lake Mekkojärvi (dry matter = 4 %, LOI = 57 %) and from a coastal station of the Baltic Sea (dry matter = 20 %, LOI = 10 %). Replicate sediment aliquots were stored in different storage preservative conditions [no solution, ethanol, RNAlater®, phenol-chloroform-isoamylalcohol (PCIAA) (25:24:1)] and at different temperatures (+4 °C, –20 °C, –80 °C) for 24 h or 1 month before analyses. In addition, replicate aliquots of control samples were subjected to analyses immediately after application of the storage treatments.

2.2.2 Within-lake and inter-lake variation in the sediment N_2 gas producing processes (II, III)

Sediment cores were collected from a boat using plexiglass tubes (ø = 7 cm) connected to a gravity corer. The profundal and littoral samples of Lake Ormajärvi were collected three times during the open water period 2006 (June, August, October) and once in winter 2007 (February-March) (II) (sites Lit1, Lit3, A in Fig. 7). N_2 production rates, microbial communities and background data [surface sediment characteristics (LOI, porosity), inorganic nutrients 2 – 3 cm

above sediment, T and O_2 concentration 10 cm above sediment] were analyzed each time. The profundal sediments of the lakes Pääjärvi, Suolijärvi and Lehee were sampled in June and October 2007, from three sites at differing depths (A, B, C in Fig. 7) (III). N_2 gas production rates were measured from each site A and from site C of Pääjärvi in October, whereas microbial communities and background data were analyzed from each site. In Pääjärvi, Suolijärvi and Lehee, inorganic nutrients were also determined from the sediment pore-water (top-most 0 – 2 cm) of each site A and site C in Pääjärvi in October.

N_2 gas production rates were measured in laboratory using IPT (Nielsen 1992, Risgaard-Petersen et al. 2003). Sampling cores were subsampled into smaller plexiglass tubes (ø = 2.6 cm, length = 16 cm) so that about half of the tube was filled with sediment and the rest with overlying water. Water overlying the sediment was amended with $^{15}NO_3^-$ to concentration of 100 µmol N l^{-1} in samples of Ormajärvi (n = 3), except in a separate $^{15}NO_3^-$-concentration test conducted in October 2006, when the samples were amended to concentrations of 50, 100 and 200 µmol N l^{-1} (n = 2 – 3 in each concentration). The samples of other lakes were amended with $^{15}NO_3^-$ to concentrations of 40, 80, 120 and 160 µmol N l^{-1} (n = 2 – 4). After incubations (3 – 6 h, in darkness, at *in situ* temperature), microbial activity was terminated by adding 1 ml $ZnCl_2$ (1 g ml^{-1}), the samples were mixed, and subsamples of the sediment–water slurry were transferred to gas-tight glass vials (12 ml; Exetainer®; Labco).

In addition, data on denitrification rates and environmental factors, as well as N loads, were obtained for 9 previously studied lakes, located in northern, central and southern Europe (III) (Fig. 7). This data were combined with the data collected as a part of this thesis.

2.2.3 Vertical N_2 gas profiles and denitrification in the lake water columns (IV)

Water samples were collected vertically from different depths of the study lakes in July 2009. The samples were collected into gas tight vials (12 ml; Exetainer®; Labco) using a Limnos tube sampler (2.8 l) (Limnos, Turku, Finland). Vials were flushed by twice their volume before closing them carefully with screw-capped septa avoiding entrapment of gas bubbles. Subsequently, biological activity was stopped by injecting 0.1 ml of $ZnCl_2$ (1 g ml^{-1}) into vials. Vials were stored upside down at room temperature for 1 – 3 months before analyses.

In addition, ^{15}N tracer experiments were conducted with water samples collected from anoxic zones (meta- and hypolimnion) of two study lakes, as explained above. Two types of incubation experiments (both at +10 °C for 0 h, 12 h, 36 h and 72 h, n = 4 during each time point) were carried out: one with additions of $^{15}NO_3^-$ (8 – 11 µmol N l^{-1}) and one with additions of $^{15}NH_4^+$ + $^{14}NO_2^-$ (both 250 µmol N l^{-1}), to test denitrification and anammox potentials, respectively. After incubations, samples were processed as explained above.

2.2.4 Methanol-utilizing denitrification systems (V)

Since direct sampling from inside the denitrification filters of WTPA and WTPB was impossible, samples were taken indirectly from the backwash water. Backwashing consists of repeating sequences of air-sparging and washing, which efficiently detached biomass from the carrier material. The biomass samples of WTPA were collected at 5 to 9 day time intervals during a 10 week follow-up period (27 August 2008 – 28 October 2008) from the same denitrification cell each time. The samples of WTPB (WTPB: biomass; WTPB_Car: escaped carrier material) were collected once from one denitrification cell (2 October 2008). The samples of AQUAR were collected directly from inside the reactors. The carrier material samples were collected from a reactor utilizing methanol (AQUAR_M) and from a reactor utilizing a mixture of methanol and saccharose (AQUAR_MS) as a carbon source (10 November 2008) (Table 2).

2.3 N_2 gas analyses and calculations

The samples of studies II and III were commercially analyzed for their mass ratios and concentrations of N_2 using a mass spectrometer (Europa Scientific, Roboprep-G-Plus and Tracermass) at the National Environmental Research Institute in Silkeborg, Denmark. D15 (denitrification rate of added $^{15}NO_3^-$), D14 (denitrification rate of natural $^{14}NO_3^-$), D_w (denitrification of the $^{14}NO_3^-$ from the water overlying the sediment) and D_n (denitrification of the $^{14}NO_3^-$ produced in the sediment via nitrification) were calculated using IPT equations according to Nielsen (1992).

Before analyzing the samples of IV, a He-headspace (6 ml) was injected into each sample vial. Analyses of concentration and $\delta^{15}N$ of N_2 were carried out with a Gasbench II (Thermo Fisher Scientific, Bremen, Germany), an on-line gas preparation and introduction system for isotope ratio mass spectrometry, coupled to a Delta Plus Advantage isotope ratio mass spectrometer (Thermo Fisher Scientific) at the University of Jyväskylä. Standard samples, with added air, were used for the calibration of the N_2 concentrations and standardization of the $\delta^{15}N$ values. The original N_2 concentration in water was calculated from the headspace concentration using the Bunsen coefficient. The equilibrium concentrations were calculated according to Weiss (1970) (101.325 kPa pressure, 0 % salinity) using data of *in situ* temperatures. The excess concentration of N_2 was calculated from the difference between the observed N_2 concentration and the calculated equilibrium concentration. $\delta^{15}N$ of excess N_2 gas was calculated using mass and isotope balance equations. Denitrification potential was determined by calculating the accumulation of $^{29}N_2$ and $^{30}N_2$ in incubations with $^{15}NO_3^-$. Anammox potential was studied as the accumulation of $^{29}N_2$ in incubations with $^{15}NH_4^+ + {}^{14}NO_2^-$.

2.4 Microbial analyses

Nucleic acids of sediment samples (DNA in I, II, III; RNA in I) were extracted from samples (200 µl aliquots) using a modified version of the bead-beating and phenol-chloroform extraction protocol of Griffiths et al. (2000) (full description in I). Samples of denitrification systems (V) were processed using standard molecular biological methods (bead-beating, phenol-chloroform extraction and subsequent precipitation of DNA by isopropanol).

In the sample storage comparison study, quantities of DNA and RNA were measured using fluorometric methods (I). The quality of DNA and RNA was assessed by LH-PCR analysis conducted as in Tiirola et al. (2003) with minor modifications. RNA was subjected to DNAase treatment and reverse-transcription before PCR amplification. The phylogenetic affiliations of major LH-PCR peaks were predicted based on the 16S rRNA clone library data (91 sequences) from control samples. Primers used in LH-PCR and clone library analysis were F8 and Prun518R (Table 3). Lengths and phylogenetic affiliations of sequences in clone library were determined with BioEdit and SeqMatch tool of the Ribosomal Database Project (RDP) (Anon. 2012a), respectively.

Spatial, i.e. intra-lake and inter-lake, and temporal variations in sediment denitrifier communities (II, III) were assessed using DGGE. DNA was subjected to PCR amplification with primer pairs F1aCu/R3Cu and cd3aF/R3cd (Table 3), for examination of *nirK*- and *nirS*-carrying, nitrite-reducing communities, respectively. A GC-clamp was attached to 5´-end of R3Cu and R3cd. PCR worked fine for both genes. DGGE worked well for *nirK* gene, but, unfortunately, it could not resolve the sequence differences of *nirS* gene. Therefore, *nirS*-carrying community was left out of further analyses. The presence of anammox bacteria in the sediments of the study lakes was analyzed using anammox-specific primers and a nested PCR-protocol, which is highly specific for sequences related to known anammox genera (Dale et al. 2009, Song & Tobias 2011). The primer pairs used in the analyses were Pla46F/1037R and Amx368/Amx820 for the first- and second-round PCR, respectively. The specificity of the protocol was verified by cloning and sequencing of three samples showing positive PCR result (20 to 34 sequences per library). Phylogenetic affiliations of sequences were determined by comparisons with databases using BLASTN (Altschul et al. 1997).

The temporal and spatial variations in microbial communities between different denitrification systems (V) were studied by microbial fingerprinting of 16S rRNA gene using LH-PCR and DGGE. In addition, denitrifier communities were specifically addressed by DGGE analysis of *nirS* and *nosZ* genes. Primer pairs used in PCR amplifications for these analyses were F8/Prun518R and 63F/518R for LH-PCR and DGGE analyses of 16S rRNA, respectively, and cd3aF/R3cd and nosZ-F/nosZ1622R for DGGE analyses of *nirS* and *nosZ*, respectively (Table 3). A GC-clamp was attached to 5´-end of 63F, R3cd and nosZ1622R. DGGE analysis of *nirK*-carrying community was not conducted due

to unspecific PCR amplification and an inadequate amount of gene fragment of the right size from WTPA samples. In addition, the structure, phylogeny and diversity of communities were studied by clone library and 454-pyrosequencing analyses. Separate 16S rRNA gene clone libraries (60 to 79 sequences per library) were generated from each sample type (WTPA, WTPB, WTPB_Car, AQUAR_M, AQUAR_MS). WTPA and AQUAR_M samples were chosen to 454-pyrosequencing analysis of 16S rRNA (WTPA: 3646 sequences; AQUAR_M: 1855 sequences), *nirS* (WTPA: 6947 sequences; AQUAR_M: 7561 sequences) and *nirK* (WTPA: 3480 sequences; AQUAR_M: 4374 sequences) genes. Primer pairs used in PCR reactions were 27F/907R and 341F/805R for 16S rRNA clone library and 454-pyrosequencing analyses, respectively, and for other genes as explained above for DGGE analyses, but without a GC-clamp (Table 3). Primers used in PCR reactions for 454-pyrosequencing carried 454FLX-adaptors and one of the primers carried a 5 basepair (bp) molecular barcode specific for each sample. 454-pyrosequencing was conducted with FLX chemistry using a 454 GS-FLX system (454 Life Sciences, Branford, CT) at the Institute of Biotechnology, University of Helsinki. Subsequent phylogenetic and diversity analyses were conducted using various programs: BioEdit, ClustalX, HMMER3-aligner of Functional Gene Pipeline & Repository (Anon. 2012b), Mothur and Mega 5.05.

TABLE 3 Primers used in the PCR – reactions.

Gene: primer	Sequence (5´ - 3´)	Reference
16S rRNA: 341F	CCTACGGGNGGCWGCAG	Herlemann et al. (2011)
16S rRNA: 805R	GACTACHVGGGTATCTAATCC	Herlemann et al. (2011)
16S rRNA: F8	AGAGTTTGATCMTGGCTCAG	Weisburg et al. (1991)
16S rRNA: Prun518R	ATTACCGCGGCTGCTGG	Muyzer et al. (1993)
16S rRNA: 63F	CAGGCCTAACACATGCAAGTC	El Fantroussi et al. (1999)
16S rRNA: 518R	ATTACCGCGGCTGCTGG	El Fantroussi et al. (1999)
16S rRNA: 27F	AGAGTTTGATCMTGGCTCAG	Lane (1991)
16S rRNA: 907R	CCGTCAATTCMTTTGAGTTT	Johnson (1994)
16S rRNA: Pla46F	GGATTAGGCATGCAAGTC	Neef et al. (1998)
23S rRNA: 1037R	CGACAAGGAATTTCGCTAC	Ludwig et al. (1992)
16S rRNA: Amx368	TTCGCAATGCCCGAAAGG	Schmid et al. (2003)
16S rRNA: Amx820	AAAACCCCTCTACTTAGTGCCC	Schmid et al. (2000)
nirK: F1aCu	ATCATGGTSCTGCCGCG	Hallin & Lindgren (1999)
nirK: R3Cu	GCCTCGATCAGRTTGTGGTT	Hallin & Lindgren (1999)
nirS: cd3aF	GTSAACGTSAAGGARACSGG	Michotey et al. (2000)
nirS: R3cd	GASTTCGGRTGSGTCTTGA	Throbäck et al. (2004)
nosZ: nosZ-F	CGYTGTTCMTCGACAGCCAG	Kloos et al. (2001)
nosZ: nosZ1622R	CGSACCTTSTTGCCSTYGCG	Throbäck et al. (2004)

2.5 Statistical analyses

Spatial and temporal variations in sediment N_2 gas production rates were analyzed using t-test and ANOVA. Environmental factors affecting N_2 gas production were studied using correlation and regression analyses. Variations in the microbial communities studied by DGGE and LH-PCR were analyzed using permutational multivariate analysis of variance (PERMANOVA) and visualized using principal coordinate analysis (PCoA), non-metric multidimensional scaling (NMS) and moving window analysis (MWA). Factors affecting the microbial communities were studied using distance based linear model (DISTLM) and Mantel´s test. Dependencies between the community structure and process rates were analyzed using Mantel´s test. (Table 4)

TABLE 4 Statistical analyses used in the study.

Analysis	Description	Reference
Analysis of variance (ANOVA)	I,II,III	
Principal coordinate analysis (PCoA)	I	
Mantel´s test	I,II,III	
Spearman correlation analysis	II,III	
Non-metric multidimensional scaling (NMS)	II,III	
PERMANOVA	II,III	Anderson (2001), McArdle & Anderson (2001)
distance based linear model (DISTLM)	II	Anderson (2001), McArdle & Anderson (2001)
t-test	III	
Regression analysis	III	
Hierarchical cluster analysis	V	
Moving window analysis (MWA)	V	Marzorati et al. (2008)

2.6 Background data analyses

The vertical profiles of temperature, O_2 and pH (only in II) of the boreal study lakes were measured during each sampling using a portable device (YSI model 58 or YSI 556 MPS, Yellow Springs Instruments, Yellow Springs, OH, USA) (II, III, IV). Inorganic nutrients, NH_4^+, NO_3^- + NO_2^- and PO_4^{3-} of the water overlying the sediment (II, III) as well as of sediment pore-waters (III) were analyzed using standard methods (QuikChem®8000, Zellweger Analytics Inc., Lachat Instruments Division, Milwaukee, 160 Wisconsin, USA). In addition, sediments were characterized for their content of organic matter (loss on ignition, LOI) and

porosity (I, II, III). Process parameters of the denitrification systems (e.g. NO_3^-, O_2, methanol addition) were measured by the staff at the wastewater treatment plants and the aquarium.

3 RESULTS AND DISCUSSION

3.1 Storage of sediment samples (I)

The highest nucleic acid yields and microbiological fingerprints most similar to those of non-preserved control profiles were obtained from samples which were stored without storage solutions or in PCIAA. In contrast, ethanol and RNAlater® preservation decreased nucleic acid yields drastically at all temperatures and caused a slight bias towards certain microbial types in the community analyses, and, therefore, should not be used as a preservative of humic acid containing, environmental samples. Thus, freezing (at –20 – –80 °C) is the recommended method to store environmental samples, e.g from sediment (I) and soil (e.g. Wallenius et al. 2010). Since sediment samples in this thesis were stored frozen (II, III), these results confirm that storage did not affect the outcome of the community characterizations. Preservation in phenol–chloroform solution can be recommended as an alternative storage method when freezing is not possible, such as during extended field sampling. However, PCIAA has a disgusting odour and evaporates, which might limit its use only to short periods (< 30 days). Preliminary trials with Lifeguard™ (MoBio), a recently introduced, odourless, non-evaporative, commercial preservative, showed that it effectively preserved DNA and RNA of sediment samples during the 30 day storage period at +4 °C (data not shown).

3.2 Within-lake and inter-lake variation in the sediment N_2 gas producing processes (II, III)

No anammox activity was detected. Therefore, denitrification was the primary N_2 gas producing process in the four boreal study lakes (III). This study (II,III), together with some previous findings, show that denitrification rates in lake sediments vary considerably seasonally (e.g. Ahlgren et al. 1994) and spatially, in

other words, there are intra-lake (e.g. Saunders & Kalff 2001b) and inter-lake differences (e.g. McCrackin & Elser 2010). The study of Ormajärvi (II) is one of the most thorough ever carried out that reveals spatial and temporal variation in lacustrine denitrification, and interesting similarities as well as contrasts were observed in comparison with previous studies. Denitrification in the profundal of Ormajärvi was almost three times higher than in the littoral zone in mid-summer (560 vs. about 190 µmol N m^{-2} d^{-1}), which contrasts with the few previous studies on spatiality that showed higher rates in the littoral zone with higher temperatures (e.g. Saunders & Kalff 2001b). Thus, spatial variation may be different in different types of lakes. The seasonal variation in the denitrification in the profundal zone of Ormajärvi, with highest rates in mid-summer, is consistent with previous studies (e.g. Piña-Ochoa & Álvarez-Cobelas 2006), but contrasting seasonalities, with highest rates in winter, as measured from the shallow littoral zone (Lit1, Fig. 7), have also been recorded (Risgaard-Petersen et al. 1999, Hasegawa & Okino 2004). Thus, temporal variation differs between the lakes, but it can also differ between sites within lakes.

Inter-lake comparison of profundal denitrification rates revealed that denitrification was especially low in the polymictic Lake Lehee (about 50 – 70 µmol N m^{-2} d^{-1}), but quite stable among other lakes (about 200 – 300 µmol N m^{-2} d^{-1}) except for the peak rates in Ormajärvi in mid-summer (560 µmol N m^{-2} d^{-1}) (III). As in previous studies in Norway (McCrackin & Elser 2010) and Colorado, USA (McCrackin & Elser 2012), the variation among the study lakes close to each other was small compared to the substantial variations at the global scale (0 – 15000 µmol m^{-2} d^{-1}) (e.g. Saunders & Kalff 2001b and references therein). In contrast, very large variations among closely located lakes (40 – 12900 µmol m^{-2} d^{-1}) were observed in Danube Delta area, Romania (Friedrich et al. 2003). So far, the N_2 production has been studied in 6 boreal lakes, 4 Finnish lakes (II, III) and 2 Swedish lakes (Ahlgren et al. 1994), using a reliable measurement method (IPT, see Chapter 1.3), and these studies indicate that the denitrification rates of boreal lakes (0 – 600 µmol N m^{-2} d^{-1}) are very low compared to the lakes in temperate areas.

TABLE 5 Simple and multiple regression models ($p < 0.001$) on variation in denitrification (D14 and D_w) at local and continental scale.

Model	Scale	Equation	N	r^2
A	Local	$\log_{10}D14 = 0.354*\log_{10}[NO_3^-] + 1.86$	18	0.55
B	Contin.	$\log_{10}D14 = 0.666*\log_{10}([NO_3^-]+1) + 1.556$	67	0.43
C	Contin.	$\log_{10}(D_w+1) = 1.353*\log_{10}([NO_3^-]+1) + 0.209$	53	0.75
D	Contin.	$\log_{10}D14 = 1.052*\log_{10}([NO_3^-]+1) +0.067*T + 0.388$	57	0.70
E	Contin.	$\log_{10}D14 = 0.764*\log_{10}([NO_3^-]+1) – 0.002*[O_2] + 2.151$	56	0.53

The environmental (e.g. NO_3^-, O_2, T in the water overlying the sediment, sediment LOI) and the biological (structure of the *nirK*-carrying community) factors affecting denitrification rates were studied within a single lake, Ormajärvi (II), and then on a broader local scale using the four study lakes (III). In addition, the effects of environmental factors were specifically studied on a larger continental scale (Fig. 7). The correlation and regression analyses (Table 5), in conjunction with previous studies, indicate that NO_3^- concentration in the water above the sediment is the most important factor explaining the denitrification rates within individual lakes (e.g. Risgaard-Petersen et al. 1999, Hasegawa & Okino 2004, II) and among the lakes, both at the local scale (e.g. McCrackin & Elser 2010, III) and at the wider geographical scale (III). This is in accordance with studies on wetlands (Kjellin et al. 2007), streams (Mulholland et al. 2008) and estuaries (Nielsen et al. 1995), as well as with a meta-analysis on various aquatic systems (oceans, coastal ecosystems, estuaries, lakes and rivers) (Piña-Ochoa & Álvarez-Cobelas 2006). There were higher NO_3^- concentrations and denitrification rates in temperate areas than in boreal areas and this stems at least partly from higher anthropogenic N loads (Seitzinger et al. 2002).

A large proportion of the variability in denitrification rates (D14) was still left unexplained (Table 5), which is probably due to different environmental controls for the coupled nitrification/denitrification (D_n) and for denitrification supported by the nitrate in the overlying water (D_w) (e.g. Cornwell et al. 1999). NO_3^- concentration in the water controls the diffusive flux of nitrate from water to anoxic sediment. Increasing NO_3^- concentration in the water column increases denitrification directly through the increase in D_w (e.g. Nielsen et al. 1995), as was shown at both study scales as positive correlation between NO_3^- concentration and D_w (III, Table 5). However, at the local scale the direct causality between NO_3^- concentration and denitrification rates was not as evident, because besides D_w, D_n was also positively correlated with the NO_3^- concentration. O_2 concentration of the water overlying the sediment controls the relative importance of D_n and D_w through controlling the thickness of the oxic sediment surface in which nitrification is possible (e.g. Rysgaard et al. 1994). Low O_2 can favour D_w over D_n by decreasing nitrification and reducing the diffusional distance of NO_3^- from the water column to the denitrification zone (e.g. Rysgaard et al. 1994). In contrast, high O_2 can favour D_n over D_w by increasing nitrification and increasing the diffusional distance of water column NO_3^- to the anoxic sediment. Local scale correlation analyses indicated that NO_3^- concentrations increased when O_2 concentration was high and sediment organic matter content low. In these conditions, nitrification was probably increased by the extension of the oxic nitrification zone (Rysgaard et al. 1994) and by lowered competition for inorganic N with heterotrophic bacteria (Strauss & Lamberti 2000). This led to increased D_n, but also to increased flux of NO_3^- from sediment to the water above the sediment, explaining the positive correlation between D_n and NO_3^- concentration. The effect of O_2 concentration on denitrification was also observed in the variability of D_n and D_w. For instance, the peak rates of denitrification in the profundal zone of Ormajärvi in mid-summer were caused by concurrent high concentration of NO_3^- and low levels of O_2 in the water overlying the sediment

affecting through increased D_w. However, D_w decreased considerably in autumn due to replenishment of O_2.

The multiple regression models (D and E in Table 5) at the continental scale provided further insights into the factors affecting denitrification. The models also reflected the differing controls of D_w and D_n. In both models, NO_3^- explained most of the variation in denitrification and stimulated denitrification mainly through D_w component. In model E, the low O_2 concentration further stimulated D_w, as explained above. In model D, the increasing temperature stimulated denitrification through the effects on D_n. Increasing temperature has actually been suggested to have a strong positive effect on nitrification (Berounsky & Nixon 1990, Bruesewitz et al. 2009), which may explain this relationship. Sediment organic matter content has also been shown to affect denitrification rates (Saunders & Kalff 2001b), however, that was not observed in this study (II, III). Variations in the quality of organic matter can also result in variation in denitrification rates (Hietanen & Kuparinen 2008), but these variations were not taken into account in this study. Especially denitrification in the littoral zone might be affected by easily degradable periphyton and aquatic plant litter.

Denitrification activity may, in some cases, be affected by denitrifier community composition, but often environmental factors are the dominant determinants (Wallenstein et al. 2006). In the boreal study lakes, the variation in denitrification rates was not dependent on the variation in the *nirK*-carrying community (II, III). This suggests that denitrification is controlled by environmental factors rather than by the structure of the *nirK*-community. In addition, the *nirK*-community was seasonally very stable despite large fluctuations in environmental conditions. The community differed only spatially both within lake, for instance between different depths in Ormajärvi, and between lakes (II, III). These variations were best explained by differences in sediment characteristics (LOI, porosity) and NO_3^- concentration in the water above the sediment and in the sediment porewaters (II, III). This is consistent with studies of other aquatic sediments (e.g. Wallenstein et al. 2006, Magalhães et al. 2008). Unfortunately, the *nirS*-carrying community could not be addressed in this study due to technical problems with the existing DGGE primers. It is possible that the response to environmental factors may vary between *nirK*- and *nirS*-communities (e.g. Enwall et al. 2010, Kim et al. 2011), and in some cases the structure of *nirS*-community may be related to activity, even when that of *nirK* is not (Enwall et al. 2010). Evidence from river sediments also show that *nirS* gene may be more abundant than the *nirK* gene (Huang et al. 2011), which further suggest the importance of *nirS*-community in sediment denitrification. Besides community structure, the total abundance of denitrifiers may also vary spatially and temporally, and have also been shown to correlate with process activities (e.g. O´Connor et al. 2006). The denitrifier communities of this study are now being reanalyzed using 454-pyrosequencing and Q-PCR of *nirS*, *nirK* and also *nosZ* genes.

The annual removal of N by denitrification was roughly estimated for each lake and compared to the N input data from two years of contrasting hydrological conditions representing low (year 2003) and high (year 2004) N

inputs to lakes (Tulonen et al. unpublished). This was 0.4 – 1.2 %, 3.8 – 10.6 %, 10.2 – 22.5 % and 13.3 – 26.6 % of the N input, in Lehee, Suolijärvi, Ormajärvi and Pääjärvi, respectively. The estimates from two Swedish lakes measured using IPT, 5 – 12 % of the N input (Ahlgren et al. 1994), agree quite well with our estimates. A proportion of N denitrified was positively dependent on the average hydraulic residence time, as has also been more generally shown for aquatic systems (Seitzinger et al. 2006). With longer residence times, there are more opportunities for sediment–water contact and, hence, promotion of N cycling processes (Saunders & Kalff 2001a). Nitrification was not measured in this study, but D_n was much higher in Pääjärvi and Ormajärvi with long residence times, reflecting higher nitrification promoted denitrification than in Lehee and Suolijärvi. In addition, sediment porewater NH_4^+ concentrations were higher in Lehee and Suolijärvi than in Pääjärvi, and the NH_4^+ concentrations in the water above the sediment were higher in Suolijärvi than in other lakes. This suggests that DNRA is more active in Lehee and Suolijärvi and further lowers the N removal in these lakes. Differences in the quality of N load that the lakes receive might also partially explain the differences in N removal. The ratio of easily recyclable inorganic N to organic N in the N loads, and hence N removal, might be larger in Pääjärvi and Ormajärvi due to smaller forest coverage and higher agricultural land use in the catchment areas than in Suolijärvi and Lehee. The estimated N removal by the 6 boreal lakes (II, III, Ahlgren et al. 1994), however, is low compared to other aquatic systems. It is only 1 – 50 % of that calculated by the generalized equation between hydraulic residence time and denitrification in aquatic systems (Seitzinger et al. 2006). In addition, the similarly calculated estimates of 60 – 90 % from two temperate lakes (measured using IPT), in Denmark (Risgaard-Petersen et al. 1999) and in Switzerland (Mengis et al. 1997) are considerably higher. These results indicate that besides having generally low area-specific denitrification rates, boreal lakes might also be less efficient in removing their N loads by denitrification. Similarly to boreal lakes, this might stem from latitudinal differences in the quality of N load, in other words, the proportion of easily recyclable inorganic N might be larger in more populated and agriculturally dominated temperate areas with higher use of inorganic fertilizers (Seitzinger et al. 2002) than in the boreal areas. Likewise, it is also possible that rates of other N cycling reactions vary between lakes and might explain the differences. The generally lower temperatures in the boreal areas may limit the cycling of N and production of NO_3^-. In addition, DNRA might be more significant in boreal lakes than in temperate lakes. In future studies, the different processes of the N cycle (nitrification, denitrification, and DNRA) should be simultaneously addressed. However, it is possible that the laboratory incubation measurements utilized in this study underestimate the removal of N due to inadequate spatial and temporal coverage or unsuccessful simulation of the *in situ* conditions (see Chapter 1.3.1). Mesocosm measurements (e.g. Mengis et al. 1997) and measurements of *in situ* concentration (Deemer et al. 2011) and isotopic composition (Pearson et al. 2012) of N_2 gas might provide more reliable methods to estimate the ecosystem scale N removal.

3.3 N_2 gas producing processes in the lake water columns (IV)

Denitrification should result in oversaturation of N_2 gas and also potentially in the decreased $\delta^{15}N$ values of the produced N_2 gas (e.g. Fuchsman et al. 2008). Indeed, there was a negative dependency between concentration and $\delta^{15}N$ of N_2 gas in 5 of the 9 study lakes indicating active denitrification. In 4 of these lakes, the site of maximum denitrification was in the anoxic zone 2 – 5 m below the lake surface, as deduced from the N_2 maximum and $\delta^{15}N$ minimum. The maximum N_2 oversaturation and the $\delta^{15}N$ of excess N_2 was 7, 5, 20 and 38 % and –5.3, –3.5, 0.7 and –1.3 ‰, respectively, in these lakes. The N_2 gas saturation was high compared to values reported previously from suboxic zones of the Black Sea (2 – 4 %) (Fuchsman et al. 2008) and an eutrophic reservoir (about 4 %) (Deemer et al. 2011), indicating very high denitrification. The $\delta^{15}N$ values of excess N_2, however, were very close to that of atmospheric N_2 gas (about 0 ‰) and much higher than in the Black Sea (–30 – –40 ‰) (Fuchsman et al. 2008) suggesting very low fractionation during denitrification.

The low fractionation can be explained by considering nitrification as a rate limiting step for denitrification, resulting in a complete turnover of NO_3^- during denitrification. Nitrification might be limited by the low pH and competition for O_2 with CH_4 oxidizers in these lakes. In the case of slow nitrification, all the NO_3^- produced should be denitrified completely and, therefore, further isotopic fractionation does not occur. The $\delta^{15}N$ of excess N_2 gas is, thus, most likely controlled by $\delta^{15}N$ of the NH_4^+ pool and the isotopic fractionation during nitrification. Measurement of $\delta^{15}N$ of NH_4^+ and NO_3^- are needed to confirm this.

There are, however, also alternative explanations for the high N_2 oversaturation and the high $\delta^{15}N$ of excess N_2 in some of the lakes. In these lakes, the water column mixes either completely or partially in the spring. At that time, N_2 gas in the water is in equilibrium with the atmosphere and the N_2 gas solubility is high due to low water temperatures (around +4 °C). This leads to high concentration of N_2 in the whole mixing layer. During onset of temperature stratification in early summer, the solubility of N_2 gas decreases due to warming. It is then possible that some of the released excess N_2 gas in the metalimnion and upper parts of the hypolimnion is trapped and cannot enter the epilimnion and escape into the atmosphere. It has been shown that the thermocline serves as an effective trap, slowing down the exchange of N_2 gas (e.g. Deemer et al. 2011). The excess N_2 gas can, thus, originate both from spring mixing and denitrification, and the contributions of these two sources cannot be separated. If most of the excess gas is derived from spring mixing, with $\delta^{15}N$ near that of atmospheric N_2 gas (about 0 ‰), then this would explain the moderately high $\delta^{15}N$ of excess N_2 gas, even though denitrification should produce N_2 gas with very low $\delta^{15}N$ value (–30 – –40 ‰). In this case, denitrification would have produced N_2 oversaturation of only 0 – 3 % in the study lakes. This is slightly lower than previously reported from aquatic systems (Fuchsmann et al. 2008, Deemer et al. 2011) and only 1 – 20 % of the excess N_2 gas concentration. In 4 other lakes, there

was no oversaturation of N_2 gas, and the N_2 concentrations followed the equilibrium concentrations more closely, although they were also oxygen and temperature stratified. Thus, these inter-lake differences might stem from differences in either spring mixing or denitrification, or their combination.

Stable isotope tracer experiments in two lakes detected slow starting denitrification (within 36 h from $^{15}NO_3^-$ addition), but no signs of anammox. This suggests that *in situ* denitrification was actually stopped or severely slowed down, supporting the idea that the turnover of NH_4^+ to NO_3^- was very low in these lakes. On the other hand, it is always possible that O_2 contamination during sampling might have caused the delay in the onset of denitrification, and that *in situ* denitrification was actually taking place. In the future, more care should be taken to assure the anoxic conditions during incubations.

The previous studies of *in situ* N_2 gas concentrations were conducted by measuring N_2:Ar ratio (Fuchsmann et al. 2008, Deemer et al. 2011) with the assumptions that Ar changes only through physical factors (e.g. temperature), while N_2 changes are due to both physical and biological (e.g. denitrification and N_2 fixation) factors. If N_2 had been proportioned to Ar, it may have aided in the detection of N_2 gas produced in the denitrification. On the other hand, there was undersaturation of N_2 gas in the hypolimnion of some lakes indicating either N_2 fixation or ebullition of N_2 gas with CH_4. Also, Ar concentrations are known to be affected by ebullition. The accuracy of N_2:Ar measurement might therefore, decrease in systems with high N_2 fixation and high concentrations of CH_4. Method comparison between measuring *in situ* N_2 concentration either directly or as N_2:Ar is currently being conducted.

Time will show the applicability of *in situ* N_2 gas measurements as a spatially and temporally integrative tool to quantify N_2 gas production in different types of aquatic ecosystems. Deemer et al. (2011) pointed out that this approach in lakes and reservoirs is limited only to the hypolimnion of stratified, stable systems. But also in these cases, there is slow transport of N_2 gas through the thermocline that must be taken into account, which may be difficult to model (Deemer et al. 2011). In addition, there is still need for the development of integrative *in situ* approaches that work under nonstratified conditions (e.g. in littoral zones and polymictic lakes). This study indicated that the vertical variation in $\delta^{15}N$ of N_2 gas may be used in detection of denitrification. Combining the measurement of *in situ* concentration and $\delta^{15}N$ of different components of N cycle, i.e. NH_4^+, NO_3^- and N_2, in water column and sediment porewaters, would provide a more complete view of the different co-occurring N cycling processes. This may then aid in the estimation of N_2 gas production in nonstratified systems.

3.4 Microbial communities of the methanol-utilizing denitrification systems (V)

The relationship between functional stability and microbial community dynamics in wastewater treatment processes is currently unclear (e.g. Wang et al. 2011). Studies in lab-scale wastewater treatment systems have shown highly variable microbial communities despite stable function (e.g. nitrification system in Wittebolle et al. 2008). An equilibrium model based on island biogeography predicts more stable microbial communities in large-scale systems (Briones & Raskin 2003; Curtis et al. 2003). Indeed, in a series of full-scale, aerobic reactors treating pharmaceutical wastewater, LaPara et al. (2002) found a relatively stable community structure during an 87-day period of constant influent characteristics. In contrast, Wang et al. (2011) detected a dynamic bacterial community in a functionally stable pilot-scale BOD removal system. The large, denitrification cell of WTPA was stable in function during the 10-week follow-up period as judged from stable $[NO_3^-]_{outflow}$ (70 – 100 µmol N l^{-1}) and NO_3^- reduction (84 – 93 %). The results of concurrent variations in the bacterial community were, however, controversial and dependent on the study technique. According to guidelines in Marzorati et al. (2008), the overall bacterial community dynamics were either low or high depending on whether they were studied by LH-PCR or DGGE, respectively. Denitrifier community was specifically monitored by DGGE analysis of *nirS* and *nosZ* genes and appeared temporally stable. The differences between LH-PCR and DGGE analyses of the 16S rRNA genes are probably due to higher resolution of the DGGE, and they emphasize the variable effects of different methods on the outcome of microbial analyses. However, together these results of microbial fingerprinting analyses indicate that bacterial community can be stable in large scale systems. Besides size differences between the operational units, the discrepancy between this and the previous studies (Wittebolle et al. 2008, Wang et al. 2011) can be due to differences in the treatment processes.

In general, the bacterial and the denitrifier communities varied among the systems, with overlap between the two wastewater treatment plants (WTPA and WTPB), but were different between the wastewater treatment plants and the saline water aquarium (AQUAR). Betaproteobacteria dominated in the municipal wastewater treatment plants (30 – 70 % of 16S rRNA gene sequences), but were absent in the aquarium. In contrast, Alphaproteobacteria were much less abundant (7 – 11 %) in the municipal plants, but dominated the reactors of the aquarium (35 – 69 %). Gammaproteobacteria were fairly abundant in AQUAR_M (20 – 29 %), but not in AQUAR_MS (6 %) or in the municipal plants (2 – 6 %). Of the phylogenetic groups previously shown to be dominant in methanol-utilizing denitrification (e.g. Osaka et al. 2006, Labbé et al. 2007), Methylophilaceae (Betaproteobacteria) were dominant in WTPA (25 – 28 %) and WTPB (WTPB_Car: 16 %) followed by Hyphomicrobiaceae (Alphaproteobacteria) in much lower abundance (2 – 8%). In contrast, Hyphomicrobiaceae dominated AQUAR_M (26 – 56 %) and AQUAR_MS (21 %) followed by *Methylophaga*

(Gammaproteobacteria; Piscirickettsiaceae) in AQUAR_M (13 – 16 %), while they were marginal in AQUAR_MS. The proportion of these bacteria was higher in WTPA than in WTPB and higher in AQUAR_M than in AQUAR_MS. *Methyloversatilis* (Rhodocyclaceae), abundant in a previously studied lab-scale system (Baytshtok et al. 2009), were found in very low abundance and only from WTPB. Hyphomicrobiaceae were the only methanol-utilizing group common in both non-saline and saline systems. BlastP-searches indicated that denitrifiers in WTPA, divided to *nirK*- and *nirS*-carrying communities, were dominated by Alphaproteobacteria (70 % of *nirK* sequences) and Betaproteobacteria (75 % of *nirS* sequences), respectively, which agrees with the results of the 16S rRNA gene based studies. In contrast, Gammaproteobacteria dominated both communities in AQUAR_M (*nirK* 96 %; *nirS* 54 %), whereas Alphaproteobacteria that dominated the overall bacterial community, accounted only for 10 % of the *nirS*-community and were absent in the *nirK*-community.

The results of this study conflict with findings from the previously studied saline water, methanol-fed denitrification system at Montreal Biodome (Labbé et al. 2007, Auclair et al. 2011), which suggest that the dominant Gammaproteobacteria, *Methylophaga*, is only capable in reducing NO_3^- to NO_2^-, while Hyphomicrobiaceae is responsible for further reduction of NO_2^-. The phylogenies of *nirS*/*nirK* genes are not coherent with the 16S rRNA phylogeny due to differences in evolutionary events and horizontal gene transfer (Jones et al. 2008), and this could be – at least partly – behind the results. In addition, the protein-coding genes were only studied at the level of potential denitrifiers (DNA level). Whether Gammaproteobacteria really acted as active denitrifiers (mRNA level) is still unknown, and this might also explain the differences between this and the previous studies. However, nitrite reductase genes have been recently found from the cultivated *Methylophaga* strains of Montreal Biodome (*Methylophaga* sp. JAM1, NCBI accession: YP_006294968; *Methylophaga* sp. JAM7, NCBI accession: YP_006291956), which together with the results of this study show that Gammaproteobacteria are also responsible for methanol-utilizing denitrification.

Variations in system size (Van Der Gast et al. 2006) or in chemical, physical and microbiological properties of the inflowing water or in physico–chemical conditions of reactors/filters might all contribute to differences among systems. Since salinity is known to be the dominant factor controlling the structure of overall bacterial communities (e.g. Silveira et al. 2011) and the denitrifier communities (Jones & Hallin 2010), the differences between the wastewater treatment plants and the aquarium most likely stem from that. The lower proportion of methanol-utilizing denitrifiers and higher bacterial diversity in AQUAR_MS than in AQUAR_M further suggest that the sugar addition to AQUAR_MS was the reason for the differences between the reactors in the aquarium, however, the possible effect of temperature variations cannot be ruled out either. The differences between WTPA and WTPB can also be explained by significant variations in the physico-chemical (e.g. water flow: WTPB < WTPA; O_2: WTPB > WTPA; NO_3^-: WTPB > WTPA) properties of the inflow water. In addition, the microbiological quality of the inflow water probably differs

between the systems, due to differences in the microbial processes in the preceding stages, which might also affect the communities within the denitrification systems.

One interesting genus-level difference was observed between clone library and 454-pyrosequencing analyses in the taxonomic classification of overall bacterial community of WTPA; Methylophilaceae was dominated either by genus *Methylophilus* or *Methylotenera,* depending on whether the classification was based on short 454-pyrosequences or long clone library sequences, respectively. *Methylophilus* has previously been shown to be important in methanol-fed denitrification systems (e.g. Osaka et al. 2006), but *Methylotenera*, a methanol-utilizing, denitrifying genus, was quite recently discovered from lake sediments (Kalyuhznaya et al. 2009), and has never before been discovered from wastewater systems. The variable taxonomic results probably stem from differences in the length of the sequences subjected to classification. The longer sequence in the clone library analyses leads to a more reliable classification. Therefore, it is likely that *Methylotenera* is important in WTPA. It was also abundant in WTPB indicating that it is generally important in methanol-fed denitrification filters of wastewater treatment plants. A denitrification pathway, recently reconstructed from composite genome of *Methylotenera*, and experiments with one cultivated strain of *Methylotenera* suggest that it reduces NO_3^- into N_2O, but not into N_2 (Kalyuzhnaya et al. 2009, Chistoserdova 2011). Thus, the abundance of *Methylotenera* may play a role in the N_2O production of methanol-utilizing denitrification systems of wastewater treatment plants. Altogether, the results of this study imply that more studies should be carried out on denitrification systems to gain better understanding of the relationship between community structure and function and how these affect the N_2/N_2O production of the systems. This could be done using single-cell genomics as well as by temporal monitoring of the structure and function of bacterial communities at systems of varying sizes by utilizing deep-sequencing and Q-PCR of functional genes combined with measurements of the N_2/N_2O production.

4 CONCLUSIONS

This thesis provides information on the magnitude, variations and controlling factors of N removing and N_2 gas producing processes in natural lake ecosystems. In addition, it reveals the structure and variations of bacterial communities maintaining the biotechnological methanol-utilizing denitrification systems in the wastewater treatment works. By combining process measurements with molecular analysis of the bacterial communities, it was possible to characterize the microbial "black box" and reveal the biotic factors controlling the processes in these ecosystems. The results of this thesis can be used in environmental protection and policy making, for example through the modelling of the N flow from the lake ecosystems to the Baltic Sea. Furthermore, the molecular methods used for connecting the bacterial community structure to the functional performance can be useful for designing and operating engineered denitrification processes in the future.

Denitrification rates of lake sediments varied seasonally and spatially, in other words, there was both intra-lake and inter-lake variation. The inter-lake variation was evident not only at the regional scale, but also at larger geographical, continental and global scales. The denitrification rates of northern boreal lakes were among the lowest ever reported from lake sediments. This study, in conjunction with previous studies, indicate that NO_3^- concentration in the water above the sediment is the most important factor explaining the denitrification rate within individual lakes and among lakes, both on the local scale and at wider geographical scales. Varying NO_3^- concentrations reflect differences in the N loads to lakes from catchment area as well as differences in the capacity of lakes to cycle N into NO_3^-. The lower denitrification rates in northern boreal lakes compared to temperate lakes are at least partially explained by the lower N loads to lakes in the boreal zone. Also, the O_2 concentration and temperature of the water above the sediment affect denitrification rates through effects on D_w (denitrification of the NO_3^- of the water above the sediment) and D_n (denitrification coupled to nitrification). The structure of the denitrifier community of boreal lake sediments was seasonally very stable and independent of the denitrification rates, but varied spatially within lakes and among lakes

being mainly controlled by NO_3^- availability and sediment characteristics (organic content and porosity of sediments). In addition, the results of this study indicated that anammox was not an important process in the lake sediments, although bacteria assigned to anammox are generally found in the sediments, including two boreal lakes of this study.

Besides variations in the denitrification rates *per se*, the lakes differed considerably in their efficiency to remove their annual N loads through N_2 gas emissions. This variation was best explained by the hydraulic residence time of the lake. In lakes with longer residence times, there are more opportunities for sediment–water contact and, hence, promotion of the N cycling processes. However, taking into account the variable residence times, it still seems that, besides having low area-specific denitrification rates, boreal lakes are also less efficient in removing their annual N load by means of denitrification than lakes in temperate zones. Whether this is due to differences in the quality of N loads, for instance in the proportions of DIN and D/PON the lakes receive, or due to variations in other N cycling processes, such as in nitrification and dissimilatory NO_3^- reduction to NH_4^+, requires further studies. Since measurements based on laboratory incubations may lead to biased estimates of the annual N removal, future studies should utilize techniques that measure the processes *in situ*, for example mesocosms and *in situ* concentration and isotopic composition ($\delta^{15}N$) of the N_2 gas.

The vertical *in situ* profiles of concentration and $\delta^{15}N$ of N_2 gas measured using IRMS can be used to detect denitrification in the hypoxic zones of lake water columns. However, oversaturation of N_2 accompanied by only slightly depleted $\delta^{15}N$ of excess N_2 could arise from low isotopic fractionation indicating that slow nitrification limits denitrification. Another explanation might be the inability of the method to separate between the N_2 gas produced in the denitrification and that originating from spring mixing, which could be trapped under the thermocline. Measurements of N_2:Ar ratio might provide a more accurate way to separate between the two sources of the excess N_2. However, the concentrations of both N_2 and Ar can be affected by ebullition, which further complicates the quantitative analysis of denitrification using this method. Simultaneous assessment of the concentrations and $\delta^{15}N$ of N_2 and components of DIN would provide better insight in the different co-occurring N cycling processes and may aid in the estimation of the N_2 gas production. Further studies in lakes of different types will show the applicability of these methods.

This study also showed that functional stability was accompanied by the bacterial community stability in a large methanol-fed denitrification filter of a wastewater treatment plant. Methanol-utilizing denitrifier communities differed among treatment processes with overlap between filters of wastewater treatment plants, but non-saline wastewater treatment plant systems and saline aquarium systems had different bacterial compositions. However, Hyphomicrobiaceae were common to all systems. The variations among the systems are explained by variations in the properties of inflowing water. A recently described, methanol-utilizing denitrifying bacterial genus, *Methylotenera*, was abundant in the wastewater treatment plants, and its abundance may affect the N_2O production

of these systems. Single-cell genomics, quantitative PCR and high throughput sequencing would offer further tools to elucidate the role of this microbial group in the N_2/N_2O production of the wastewater treatment processes.

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YHTEENVETO (RÉSUMÉ IN FINNISH)

Typpeä poistavat mikrobiprosessit vesiekosysteemeissä

Typen määrä maapallon ravinnekierrossa on kasvanut voimakkaasti ihmistoiminnan seurauksena esiteolliseen aikaan verrattuna. Tämä on aiheuttanut useita ympäristöongelmia, kuten ekosysteemien rehevöitymistä ja happamoitumista. Matalat meret ja rannikkoalueet ovat erityisen herkkiä typen aiheuttamalle rehevöitymiselle, ja esimerkiksi Itämeressä typpikuormitus on aiheuttanut runsaita leväkukintoja ja pohja-alueiden hapettomuutta. Bakteerit voivat tuottaa epäorgaanisista typpiyhdisteistä typpikaasua denitrifikaation ja anaerobisen ammoniumin hapettumisen (anammox) kautta. Nämä prosessit toimivat vesistöjen sedimenteissä ja vesipatsaissa sekä maaperässä luontaisesti. Tämän lisäksi varsinkin denitrifikaatiota sovelletaan jätevedenpuhdistamoiden typenpoistossa.

Maailmanlaajuisten mallinnusten tulokset viittaavat siihen, että järvissä tapahtuva typpikaasun tuotanto vähentää merkittävästi sisämaasta meriin joutuvaa typpikuormaa. Järvien typpikaasun tuotantoa säätelevistä fysikaalisista, kemiallisista ja biologisista tekijöistä on kuitenkin ollut vain vähän tietoa saatavilla. Erityisesti boreaalisen vyöhykkeen järvien typpikaasutuotannon määrä, ajallinen ja paikallinen vaihtelu sekä vaihteluun vaikuttavat tekijät on tunnettu huonosti. Tällainen tieto on kuitenkin avainasemassa vesistöjen hoidossa ja ekosysteemien toiminnan mallintamisessa. Käytetyimmät mittausmenetelmät ovat perustuneet laboratoriokokeisiin, jotka kuitenkin saattavat arvioida väärin luonnossa tapahtuvien prosessien nopeuksia, minkä vuoksi on herännyt tarve kehittää uusia mittausmenetelmiä. Myös jätevedenpuhdistuksen denitrifikaatioprosesseja säätelevät biologiset tekijät tunnetaan huonosti. Tietoa näistä tekijöistä voitaisiin mahdollisesti käyttää hyödyksi jätevedenpuhdistamoiden suunnittelussa sekä typenpoiston ohjaamisessa ja optimoinnissa.

Tässä tutkimuksessa selvitettiin boreaalisissa järvisedimenteissä tapahtuvan typpikaasutuotannon vuodenaikaista ja alueellista vaihtelua. Järvisedimenttien typpikaasutuotanto mitattiin laboratoriossa käyttämällä typen vakaisiin isotooppeihin (^{14}N ja ^{15}N) perustuvaa leimaustekniikkaa. Järvisedimenttien denitrifikaatiobakteeriyhteisöjen rakennetta tutkittiin samanaikaisesti molekyylibiologisin menetelmin. Tulosaineistoa verrattiin myös aiemmin tutkittujen eurooppalaisten järvien aineistoihin laajemman kuvan saavuttamiseksi järvisedimenttien typpikaasutuotantoa säätelevistä tekijöistä. Denitrifikaatio havaittiin ainoaksi typpikaasua tuottavaksi prosessiksi järvissä. Denitrifikaationopeus vaihteli merkittävästi vuodenaikojen, eri syvyysvyöhykkeiden ja järvien välillä. Boreaalisten järvisedimenttien denitrifikaationopeudet (0 – 600 µmol N m^{-2} d^{-1}) olivat matalia verrattuna muiden alueiden järvistä mitattuihin arvoihin (0 – 15000 µmol N m^{-2} d^{-1}). Merkittävin vaihtelua selittävä tekijä, niin boreaalisten järvien mittakaavassa kuin laajemmassa eurooppalaisessa mittakaavassa, oli sedimentin yläpuolisen veden nitraattipitoisuus. Typpikaasuna poistuvan typen määrä vaihteli merkittävästi boreaalisten järvien välillä ollen 0,4 – 26,6 %

vuotuisesta typpikuormasta, ja tämä vaihtelu selittyi parhaiten järvien erilaisilla viipymäajoilla. Boreaalisten järvien havaittiin olevan tehottomampia poistamaan typpikuormaansa typpikaasuna kuin eteläisempien alueiden järvien. Tämä saattaa johtua alueellisista eroista joko typpikuorman epäorgaanisten ja orgaanisten jakeiden osuuksissa tai muiden typpeä muokkaavien prosessien, kuten nitrifikaation ja DNRA:n (dissimilatorinen nitraatin pelkistyminen ammoniumiksi), nopeuksissa. Tuloksen taustalla olevien tekijöiden selvittäminen vaatii lisätutkimuksia.

Tutkimuksessa testattiin myös vaihtoehtoista typpikaasutuotannon mittausmenetelmää, joka perustuu vesipatsaan typpikaasun pitoisuuteen ja vakaiden isotooppien luonnolliseen suhteeseen ($^{15}N/^{14}N$). Typpikaasun tuotannon pitäisi johtaa typpikaasun ylikyllästyneisyyteen ja prosessin aikana tapahtuvan isotooppien runsaussuhteiden muuttumisen (fraktioitumisen) myötä typpikaasun alhaiseen $^{15}N/^{14}N$-suhteeseen vesipatsaassa. Erillisissä laboratoriossa tehtävissä isotooppileimauskokeissa varmistettiin denitrifikaation olevan ainoa typpikaasua tuottava prosessi happikerrostuneissa tutkimusjärvissä. Typpikaasun määrään ja isotooppisuhteeseen perustuva menetelmä osoittautui herkäksi havaitsemaan syvyysvyöhykkeet, joissa denitrifikaatiota oli tapahtunut. Denitrifikaation nopeuden ja määrän selvittämiseen menetelmä ei kuitenkaan suoraan soveltunut, mikä johtui alhaisesta typen isotooppien fraktioitumisesta ja typpikaasun mahdollisesta karkaamisesta kuplinnan myötä. On myös mahdollista, että fysikaalisten tekijöiden (veden kevätkierron ja lämpötilavaihtelujen) vaikutuksesta ilmakehästä veteen liuenneen typpikaasun taustapitoisuudet voivat vaihdella merkittävästi.

Lopuksi tutkimuksessa verrattiin metanolia hiilenlähteenään hyödyntävien denitrifikaatiosysteemien bakteeriyhteisörakenteita käyttämällä molekyylibiologisia menetelmiä. Suuren kunnallisen jätevedenpuhdistamon denitrifikaatiosuodattimen toiminnan ja bakteeriyhteisön rakenteen ajallista vaihtelua seurattiin 10 viikon seurantajakson ajan. Näitä tuloksia verrattiin pienemmän jätevedenpuhdistamon denitrifikaatiosuodattimen ja merivesiakvaarion denitrifikaatioreaktorin bakteeriyhteisöjen rakenteeseen. Suuren jätevedenpuhdistamon denitrifikaatiosuodattimen toiminta ja bakteeriyhteisön rakenne olivat molemmat ajallisesti vakaita. Bakteeriyhteisöjen rakenne erosi tutkimuskohteiden välillä. Jätevedenpuhdistamojen denitrifikaatiosuodattimien bakteeriyhteisöissä oli samankaltaisuutta, mutta yhteisöt olivat hyvin erilaisia jätevedenpuhdistamoissa verrattuna merivesiakvaarion denitrifikaatioreaktoriin. Systeemien väliset erot selittyivät denitrifikaatioon tulevan veden ominaisuuksien eroilla. Metanolia hiilenlähteenään käyttävän denitrifioivan *Methylotenera*–suvun bakteerit olivat runsaita jätevedenpuhdistamojen denitrifikaatiosuodattimilla. Viljeltyjen *Methylotenera*–bakteerien on havaittu denitrifioidessaan tuottavan typpioksiduulia (N_2O), joka on kasvihuonekaasu. *Methylotenera*–bakteerien runsaus ja runsauden vaihtelut voivat siis vaikuttaa jätevedenpuhdistamojen typenpoistosuodattimien kasvihuonekaasupäästöihin.

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