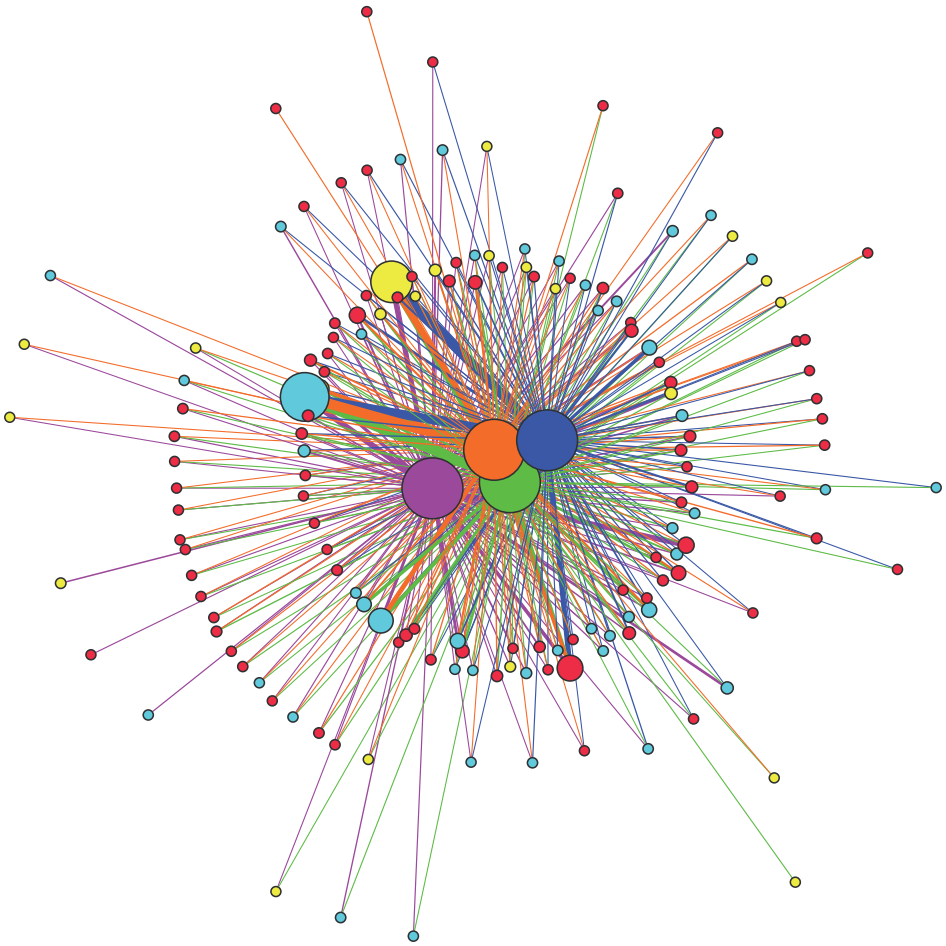
JYVÄSKYLÄ STUDIES IN BIOLOGICAL AND ENVIRONMENTAL SCIENCE
299

Jatta Saarenheimo

Microbial Controls of Greenhouse Gas Emissions from Boreal Lakes



JYVÄSKYLÄN YLIOPISTO

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Esitetään Jyväskylän yliopiston matemaattis-luonnontieteellisen tiedekunnan suostumuksella
julkisesti tarkastettavaksi yliopiston Ambiotica-rakennuksen salissa YAA303,
maaliskuun 28. päivänä 2015 kello 12.

Academic dissertation to be publicly discussed, by permission of
the Faculty of Mathematics and Science of the University of Jyväskylä,
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UNIVERSITY OF JYVÄSKYLÄ

JYVÄSKYLÄ 2015

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Editors
Timo Marjomäki
Department of Biological and Environmental Science, University of Jyväskylä
Pekka Olsbo, Ville Korkiakangas
Publishing Unit, University Library of Jyväskylä

Jyväskylä Studies in Biological and Environmental Science

URN:ISBN:978-951-39-6127-5
ISBN 978-951-39-6127-5 (PDF)

ISBN 978-951-39-6126-8 (nid.)
ISSN 1456-9701

Jyväskylä University Printing House, Jyväskylä 2015

ABSTRACT

Saarenheimo, Jatta
Microbial controls of greenhouse gas emissions from boreal lakes
Jyväskylä: University of Jyväskylä, 2015, 56 p.
(Jyväskylä Studies in Biological and Environmental Science
ISSN 1456-9701; 299)
ISBN 978-951-39-6126-8 (nid.)
ISBN 978-951-39-6127-5 (PDF)
Yhteenveto: Metsäjärvien kasvihuonekaasujen mikrobitasoinen säätely
Diss.

Biogeochemical processes in stratified boreal lakes promote or reduce greenhouse gas (GHG; CO_2, CH_4 and N_2O) emissions in distinct compartments. In this thesis environmental and biotic controls of microbially-mediated GHG processes were studied at a multi-lake scale as well as in a whole-lake experiment. Microbial communities, specific microbial groups and functional genes were studied using molecular methods, and data were combined with environmental and gas concentration measurements. Aerobic, microaerobic and anaerobic zones of a small stratified lake were shown to support distinct microbial communities, which were mostly controlled by seasonal succession and oxygen concentration. High biomasses of anaerobic photosynthetic green sulphur bacteria (GSB) of the genus *Chlorobium* were found where light intensities were sufficient to support their growth in the anoxic zone. During a three year whole-lake experiment, the effect of trophic cascades on the microbial communities and processes was studied by adding fish (European perch, *Perca fluviatilis*) to one basin of an experimentally divided fishless lake. This removed nearly all zooplankton and released methanotrophic bacteria from grazing by *Daphnia* sp., allowing more methane oxidation. These cascading impacts reduced CH_4 efflux from the basin to which fish were added to one quarter the efflux from the fishless basin. The genetic potential for denitrification (studied with functional genes *nirS*, *nirK*, *nosZ*$_{I}$ and *nosZ*$_{II}$), whereby N_2O is produced as an intermediate product, was wide spread in all studied lakes and was driven by only a few core proteobacterial species. Inter-lake variation in N_2O accumulation was connected to the relative abundance of nitrite versus N_2O reductase genes, which co-varied with the nitrate concentration. Thus the abundance of GSB, methanotrophs, and denitrifiers were all found to impact on GHG emissions from boreal lakes, with a strong control by dissolved oxygen concentration.

Keywords: *Chlorobium*; denitrification; humic lakes; methanotrophs; next generation sequencing; nitrous oxide; stratification.

Jatta Saarenheimo, University of Jyväskylä, Department of Biological and Environmental Science, P.O. Box 35, FI-40014 University of Jyväskylä, Finland

Author's address Jatta Saarenheimo (née Karhunen)
Department of Biological and Environmental Science
P.O. Box 35
FI-40014 University of Jyväskylä
Finland
jatta.saarenheimo@jyu.fi

Supervisors Professor Marja Tiirola
Department of Biological and Environmental Science
P.O. Box 35
FI-40014 University of Jyväskylä
Finland

Docent Jari Syväranta
Department of Biological and Environmental Science
P.O. Box 35
FI-40014 University of Jyväskylä
Finland

Reviewers Docent Paula Kankaala
University of Eastern Finland
Department of Biology
P.O. Box 111
FI-80101 Joensuu
Finland

Professor David Lipson
Department of Biology
San Diego State University
San Diego, CA 92182-4614
USA

Opponent Professor Stefan Bertilsson
Uppsala University
Limnology Department
Evolutionary Biology Centre, EBC
Norbyvägen 18 D
752 36 Uppsala
Sweden

CONTENTS

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 was responsible for the molecular laboratory work for all papers. I planned papers I and II together with Marja Tiirola, and conducted the experiments and sampling. Paper III was planned together with Marja Tiirola and Antti Rissanen and the samples were collected by Antti Rissanen. Experiments for IV and V were planned together with Roger Jones and Jari Syväranta, and the sampling was conducted jointly with Jari Syväranta and Shawn Devlin mainly responsible. I was responsible for writing I, II, III and V. Paper IV was written jointly with Shawn Devlin. All papers were finalised together with all co-authors.

I Karhunen J., Arvola L., Peura S. & Tiirola M. 2013. Green sulphur bacteria as a component of the photosynthetic plankton community in small dimictic humic lakes with an anoxic hypolimnion. *Aquatic Microbial Ecology* 68: 267–272.

II Saarenheimo J., Rissanen A., Nykänen H., Arvola L., Lehmann M.F. & Tiirola M. 2015. Genetic and environmental factors controlling nitrous oxide accumulation in lakes. *PLoS ONE*. In press.

III Saarenheimo J., Tiirola M. & Rissanen A. 2015. Functional gene pyrosequencing indicates that denitrification is driven by a few core proteobacterial populations in boreal lakes. Submitted manuscript.

IV Devlin S., Saarenheimo J., Syväranta J. & Jones R.I. 2015. Top consumer abundance influences lake methane efflux. Submitted manuscript.

V Saarenheimo J., Syväranta J., Devlin S., Aalto S.L., Tiirola M. & Jones R.I. 2015. Influence of a trophic cascade on the bacterial community in a whole-lake manipulation. Manuscript.

1 INTRODUCTION

1.1 Stratification patterns in boreal lakes

The Finnish landscape is rich in lakes that cover approximately 10 % of the total surface area (Raatikainen and Kuusisto 1990). Of the 180 000 lakes with area > 500 m^2, 93 % are considered humic (Kortelainen 1993), having high dissolved organic carbon (DOC) concentrations and dark water colour. The dark water warms quickly after spring icemelt leading to characteristic steep lake temperature stratification. The epilimnion (surface layer) and hypolimnion (lowest layer) are separated by a metalimnion (transition zone), which can be particularly narrow in boreal lakes due to the steep stratification. The metalimnion isolates the hypolimnion from the mixed surface waters, restricting vertical mixing to the hypolimnion and reducing gas exchange with the upper water layers and the atmosphere. The depth of the epilimnion is strongly influenced by the size of the lake and by the strength of wind mixing. Small boreal lakes are often very well sheltered from wind and during stratification the height of their hypolimnion typically exceeds that of the epilimnion (Salonen *et al.* 1984, Korhonen 2002). Hence small humic lakes typically exhibit particularly steep and stable vertical stratification. When the physical stratification is well established, many chemical and biological changes follow in the water column that further impact on biochemical processes.

Thermal stratification is often accompanied by steep oxygen (O_2) stratification in boreal lakes. The strong physical stratification that separates the different water layers acts as a barrier for O_2 transport to the hypolimnion. This, combined with high O_2 consumption in humic waters containing high DOC concentrations, leads to typical anoxic conditions in the hypolimnion (Eloranta 1999). The epilimnion is well oxygenated due to photosynthesis and diffusion from the atmosphere, whereas the dark water colour of humic lakes prevents light absorption to deeper layers resulting in characteristically shallow photic layers with sufficient light intensities for photosynthesis (Lindholm 1992, Salonen and Lehtovaara 1992). The hypolimnion is thus a heterotrophic zone where free O_2 is consumed and anoxic conditions can develop quickly after the thermal stratification. The humic substances not only restrict light penetration

to deeper layers but also affect the spectral composition of the light. With increasing humic substances red light (wave length ca. 620–740 nm) predominate in the deeper water column (Eloranta 1978), and this light climate favours some specific groups, such as anaerobic green sulphur bacteria (GSB; Vila and Abella 2001).

Stratification also strongly impacts on the nutrient and gas movements in the water column. High phosphorus (P) concentrations, mainly in the form of phosphate (PO_4^{3-}), are found from the hypolimnion. P accumulation can result from several processes. In anoxic conditions PO_4^{3-} becomes more soluble and is released from the sediment (Wetzel 1983, Boström *et al.* 1988). The decomposition of organic material in the hypolimnion further increases the PO_4^{3-} concentrations. A particular feature in highly humic lakes is also the binding of humic particles and iron with free PO_4^{3-}, decreasing the amount of truly available P (De Haan 1992). Ammonium (NH_4^+) concentrations follow the same pattern as the phosphorus with higher concentrations in the anoxic hypolimnion. In the epilimnion NH_4^+ is nitrified to nitrate (NO_3^-) and used by different prokaryotes and eukaryotes, whereas in the anoxic environment nitrification is inhibited by the lack of O_2. In the epilimnion, where light and O_2 are supporting primary production, nutrients are used for algal and autotrophic and heterotrophic bacterial growth. This, combined with the physical barrier that prevents the export of nutrients from hypolimnion to epilimnion, results in low nutrient concentrations in the epilimnion. When the hypolimnetic waters are protected from wind driven mixing, and no external forces are mixing the water, the nutrient movements and gas diffusion are very slow (diffusion at the rate of 10^{-4} to 10^{-6} cm^2 s^{-1}, Ferrell and Himmelblau 1967). Therefore, the gases produced in the hypolimnion during stratification do not readily enter the epilimnion. This means that the hypolimnion is usually supersaturated with produced gases, such as carbon dioxide (CO_2) and methane (CH_4).

A distinct biological profile typically follows the thermal and chemical stratification. As outlined above, the O_2 concentration not only affects biogeochemical processes in the lakes, but also restricts the suitable habitat for many animals, such as zooplankton and fish. The outcome is a situation where fish, zooplankton and primary producers all mainly occupy the shallow oxic epilimnion. However, some zooplankton species, like the cladoceran *Daphnia longispina,* are known to enhance their fitness by vertically migrating to the anoxic parts of the lake, partly to feed on available prey such as vertically migrating algae and bacteria, but also to escape predation (Salonen and Lehtovaara 1992). Bacterial communities also show zonation patterns leading to distinct communities in the oxic epilimnion and anoxic hypolimnion (Peura *et al.* 2012). One good example is methanotrophs that are most abundant at the oxic/anoxic interface where they can exploit both the CH_4 slowly diffusing upwards from the hypolimnion and O_2 from the epilimnion (Kiene 1991, Bastviken *et al.* 2002).

Lakes are typically dimictic at the Finnish latitudes of the boreal zone (Korhonen 2002), meaning that their water column mixes twice each year; in

spring after ice melt and in autumn when the surface water becomes cooler. At these times less energy is needed for the water column circulation due to the more similar densities of different water layers. The stratification that develops in spring lasts the entire summer efficiently separating the different water layers from each other. However, the spring or autumn mixing events can be incomplete in small lakes, making the lakes partly meromictic and resulting in prolonged periods of anoxia in the hypolimnion (Saura *et al.* 1995).

1.1.1 Redox conditions

O_2 is typically used as an electron acceptor in microbial respiration processes. When the environment becomes anoxic various other substances can be used as electron acceptors in the order of their thermodynamic energy yield (O_2 > NO_3^- > Mn^{4+} > Fe^{3+} > SO_4^{2-} > CO_2; Capone and Kiene 1988). The electron acceptors are used either in the water column or in the sediment, resulting in a vertical zonation of mineralization processes, where the occurrence and the ratio between oxidized and reduced compounds regulates the redox potential (Conrad 1996). Thus the redox potential is an indication of which types of electron acceptors are available. However, it is important to understand that the disappearance of O_2 itself does not give the water a reducing power; for a reduction to occur electrons are necessary. At the lowest redox potential phase CO_2 can be reduced in methanogenesis to CH_4 (Fig. 1), which based on its warming potential is 30 times more harmful as a greenhouse gas (GHG) than CO_2 (Anon. 2013). SO_4^{2-}, Fe^{3+}, Mn^{4+} and NO_3^- are reduced in more energy yielding processes, which can outcompet methanogenesis (Fig. 1). However, these inorganic electron acceptors have been coupled to anaerobic CH_4 oxidation (Boetius *et al.* 2000, Beal *et al.* 2009, Ettwig *et al.* 2010). In highly humic boreal lakes complex humic substances may have an important role as electron acceptors in anaerobic microbial respiration (Lovley *et al.* 1996), especially when the redox cycling of humic substances suppresses CH_4 production (Klüpfel *et al.* 2014). In addition, nitrous oxide (N_2O), the third most important GHG, can be produced in anaerobic NO_3^- reducing processes, such as denitrification or dissimilatory NO_3^- reduction to NH_4^+ (DNRA) (Fig. 1). Redox conditions change drastically within the vertical water profile of small boreal lakes. O_2 concentrations and redox conditions are thus the major factors determining which biogeochemical processes are active and the redox potential is a good indicator of the relative dominance of microbial processes in anaerobic systems.

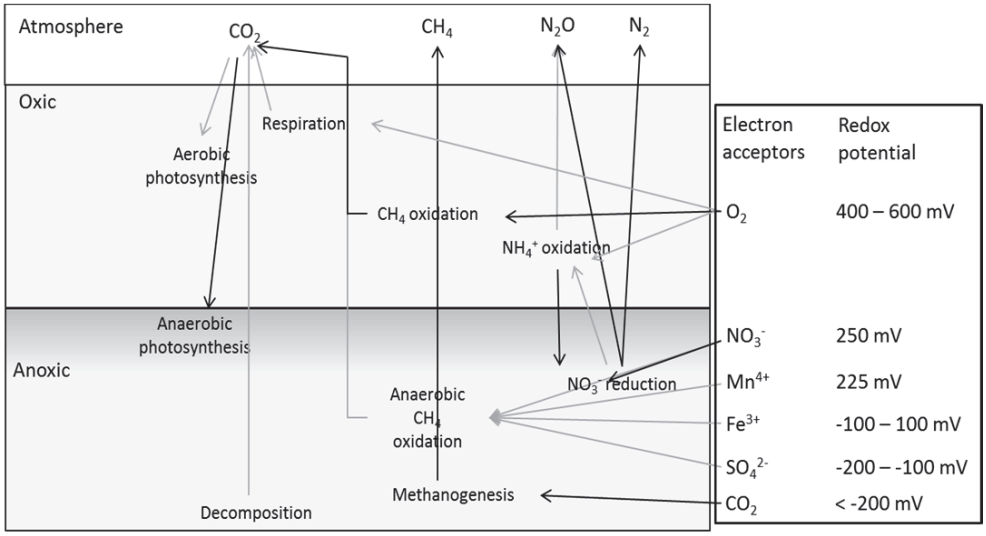

FIGURE 1 Biogeochemical processes in oxic and anoxic environments in lake sediment or in the stratified lake water column. The processes studied here are indicated with black arrows. Redox potentials are from Kirchman (2012).

1.2 Microbes in boreal lakes

Microbes are key players in biogeochemical processes. Their role in carbon and nutrient cycling in boreal lake ecosystems with high allochthonous (terrestrial) dissolved organic carbon load from the catchment area impacts on the balance between system heterotrophy vs. autotrophy. Microbes are spread throughout the lake water column, but are found in the highest densities from the sediment, both the oxic and anoxic layers. Clear water lakes and humic lakes differ by their water colour and DOC concentrations. Euphotic layers of humic lakes are often nutrient poor, and allochthonous DOC is an important carbon source for heterotrophic microbes (Jones 1992, Tranvik 1992) that compete with algae for inorganic nutrients. The restricted depth of the photosynthetic layer, combined with low nutrient concentrations in the epilimnion, strongly limits the primary production. In addition, the heterotrophic bacteria that more efficiently utilize allochthonous DOC in both oxic and anoxic compartments can outcompete autotrophs, resulting in dominance of decomposition and respiration processes in boreal lakes, and making them net heterotrophic (Salonen *et al.* 1983).

Redox conditions, light and nutrients are factors that determine the microbial community composition and functioning. In stratified boreal lakes these controlling factors are highly variable across the lake depth profiles. The most common microbes and their ecology in freshwater ecosystems were recently reviewed by Newton *et al.* (2011). However, this and other studies that reveal the bacterial taxa composition in freshwater ecosystems (e.g. Zwart *et al.* 2002, Burkert *et al.* 2003, Chróst *et al.* 2009) mostly cover only the oxic epilimnion,

while it is now known that the microbial communities in the anoxic hypolimnion are very distinct from those in the epilimnion (Taipale *et al.* 2009a, Peura *et al.* 2012). These recent studies of hypolimnetic communities have revealed the importance of previously known methanotrophs and anoxic phototrophs (green sulphur bacteria; GSB) in small humic lakes (Taipale *et al.* 2011), but have also identified a new group of candidate division OD1 and shown it to be extremely abundant in these boreal lakes (Peura *et al.* 2012).

In addition to controlling biogeochemical processes in lakes, microbes play an important role in lacustrine food webs through the "the microbial link" (Jones 1992). The energy from DOC is mobilized (*sensu* Jones 1992) by heterotrophic microbes and thus made available for transport to the higher trophic levels within the ecosystem. The role of this microbial carbon subsidy is considered more important in humic lakes than in clear water systems (Jones 1992, Jansson *et al.* 2000). The consumption of bacteria by phagotrophic micro-organisms (flagellates and ciliates; Muylaert *et al.* 2002) and zooplankton (cladocera and copepods; Ojala and Salonen 2001, Zöllner *et al.* 2003) has been shown in various studies and suggested as a potential top-down control of the microbial community compositions (e.g. Muylaert *et al.* 2002, Grossart *et al.* 2008, Berdjeb *et al.* 2011,). Especially in humic lakes GSB (Salonen and Lehtovaara 1992) and methanotrophs (Jones and Grey 2011) are grazed by zooplankton, and the carbon acquired from methanotrophic bacteria could comprise as much as 80 % of the total diet of *Daphnia* in autumn (Taipale *et al.* 2009b). Carbon availability has also been shown to affect the bacterial community composition, as *Betaproteobacteria* and *Bacteroidetes* are known to favour higher DOC concentrations (Burkert *et al.* 2003, Eiler *et al.* 2003, Hutalle-Schmelzer *et al.* 2010). In addition, *Actinobacteria* and *Verrucomicrobia* were reported to be the most abundant microbial groups of the epilimnetic layers in some boreal lakes (Lindström and Leskinen 2002, Burkert *et al.* 2003, Haukka *et al.* 2005, Arnds *et al.* 2010).

1.3 Controls of microbially-mediated greenhouse gas emissions

CO_2, CH_4 and N_2O are the three most important greenhouses gases, which naturally occur in the atmosphere and are produced in soils, sediments and waters in various microbiological processes (Fig. 1). Over recent decades human activities, such as combustion of fossil fuels, agriculture and clearing of forests, have substantially increased the GHG concentrations in the atmosphere, leading to accelerated climate warming (Anon. 2013). Lakes can be either sources or sinks of GHG, depending on the microbial processes that produce and consume these gases. In most lakes worldwide the community respiration exceeds primary production making these lakes net heterotrophic systems and conduits for returning terrestrially fixed carbon to the atmosphere (Cole *et al.* 1994, Algesten *et al.* 2003, Raymond *et al.* 2013). This is especially pronounced in the boreal zone, where lakes with dark water colour and high allochthonous DOC concentrations originating from the catchment areas (Kortelainen 1993) are continuously

reported as supersaturated with GHGs and thus emitters to the atmosphere (Huttunen *et al.* 2003, Liikanen *et al.* 2003, Kankaala *et al.* 2013, Kortelainen *et al.* 2013). While many microbiological, other biotic and environmental factors are known to control GHG emissions, it is crucial to understand these processes in boreal lakes better, to improve predictions of the possible effects of global warming and increasing eutrophication (increasing nitrogen and phosphorus concentrations in the aquatic ecosystems) on GHG emissions from lakes in the future.

1.3.1 Microbial controls

CO_2 is produced in both aerobic and anaerobic oxidation of organic material. The organic material can be produced either in the water column (autochthonous) or it can originate from the catchment area (allochthonous). CO_2 is also produced in fermentation processes. CO_2 is consumed in autotrophic processes where it is fixed to organic compounds and biomass by various bacteria and in particular algae. Photoautotrophic processes in boreal lakes mainly take place in the epilimnion where the bacteria and algae derive their energy from the sun and further produce O_2 as a by-product. Chemoautotrophs derive their energy from inorganic oxidation and use CO_2 as a C source. In the anoxic systems CO_2 can also be reduced to CH_4 in methanogenesis. However, high abundances of anaerobic photosynthetic GSB have been found from the anoxic water of small boreal lakes (Taipale *et al.* 2009a, 2011). These GSB are well adapted to extremely low light intensities where they may comprise up to 47 % of the microbial biomass (Taipale *et al.* 2011). Abundant GSB deriving energy through photosynthesis might shift the whole lake towards net autotrophy during the stratification period.

CH_4 is produced in anoxic environments by methanogens that reduce CO_2 to CH_4 together with H_2, formate, alcohols or CO as an electron donor (Boone 1991). In freshwater sediments, most of the CH_4 is produced by acetoclastic methanogenesis, where the methyl group of acetate is used (Woltemate *et al.* 1984, Jones 1991). All known methanogens belong to the domain of Archaea. The CH_4 produced is mainly oxidised by methanotrophs at the oxic/anoxic interface where both CH_4 and O_2 are available (Kiene 1991, Bastviken *et al.* 2002, Kankaala *et al.* 2006). These aerobic methanotrophs use CH_4 as their sole C and energy source. Three main types of aerobic methane-oxidizing bacteria (MOB; type Ia, Ib and II) all belong to the group of Proteobacteria (Hanson and Hanson 1996). Type Ia and Ib MOB are *Gammaprotepbacteria* and belong to the family of *Methylococcaceae*. Type II MOB belong to subdivision of *Alphaproteobacteria* and family *Methylocystaceae* (Bodrossy *et al.* 2003). In addition to the aerobic Proteobacteria, a group of MOB has been found from *Verrucomicrobia* phyla. These *Verrucomicrobia* methanotrophs have been found from extremely acidic environments (Op den Camp *et al.* 2009) and they use CO_2 as a C source and are thus autotrophic microbes. CH_4 oxidation can, however, also occur already in the anoxic environment either by bacteria oxidizing CH_4 with NO_2^- as electron acceptor (*Candidatus Methylomirabilis oxyfera*, Ettwig *et al.* 2010), by anaerobic

methanotrophic archaea (ANME archaea), or by a consortium of ANME and bacteria where SO_4^{2-}, Fe^{3+}, Mn^{4+}, NO_3^- and NO_2^- are used as electron acceptors (Eller *et al.* 2005, Schubert *et al.* 2010). Anaerobic CH_4 oxidation is mainly observed in marine environments (Boetius *et al.* 2000, Valentine 2002, Beal *et al.* 2009) but recently also in freshwater lakes (Eller *et al.* 2005, Schubert *et al.* 2010). However, the importance of anaerobic CH4 oxidation or the microaerophilic glycolysis-based CH_4 assimilation (Kalyuzhnaya *et al.* 2013) in boreal lakes is not currently known. Nonetheless, the presence of either aerobic or anaerobic CH_4 oxidizers is crucial in reducing its emissions from lakes.

N_2O can be produced as an intermediate product in denitrification or as a by-product in nitrification or in the dissimilatory NO_3^- reduction to NH_4^+ (DNRA) process (Stevens *et al.* 1998, Rütting *et al.* 2011). Of these, denitrification is thought to be the main source of N_2O in freshwater ecosystems (McCrackin and Elser 2010, Freymond *et al.* 2013). Ability to denitrify has been found from a wide variety of different bacteria (Zumft 1997), archaea (Philippot 2002) and fungi (Shoun *et al.* 1992, Tanimoto *et al.* 1992). The denitrification pathway consists of four reduction steps where facultative anaerobic microbes reduce NO_3^- to N_2 gas, by oxidizing organic material, through intermediates of NO_2^-, nitric oxide (NO) and N_2O. The different reductive steps are catalysed by distinct enzymes and the accumulation of N_2O is finally determined from the ratio of N_2O production and reduction. The microbial community composition is suggested to have a role in controlling the N_2O production, when even up to 1/3 of the denitrifying microbes are found with a truncated pathway, meaning that they are missing the enzyme of *nosZ* that catalyses the last reductive step where N_2O is reduced to N_2 (Jones *et al.* 2008). In addition, microbes harbouring *nirK* gene, one of the two possible NO_2^- reducers, are more likely to have the truncated pathway than microbes containing *nirS* (Jones *et al.* 2008). The fact that these two functionally equivalent reductases (*nirS* and *nirK*) have never been found from the same organism would suggest that communities dominated by *nirK* are likely to produce more N_2O. The only known sink of N_2O is catalysed by *nosZ* gene, which has two different clades (Sandorf *et al.* 2012, Jones *et al.* 2013). The familiar *nosZ* cladeI has only been identified from denitrifying microbes and for a long time full denitrification was thought to be the only process able to reduce N_2O. However, the newly reported *nosZ* cladeII has also been found from organisms that do not possess the up-stream denitrification steps, suggesting that N_2O accumulation can be partly controlled by non-denitrifying organisms (Sandorf *et al.* 2012).

1.3.2 Environmental controls

Environmental parameters control GHG emissions either directly through the physical characteristics (such as gas solubility in water) or by controlling the microbial communities. O_2 has a major role in determining which processes are present and thus it determines whether the gases are produced or reduced. Photoautotrophic processes are the sink for CO_2, and mainly occur in the aerobic layers where O_2 is produced during primary production. The exception are the

anaerobic phototrophs, such as GSB, that are strictly anaerobic and do not produce O_2 in their photosynthesis. CH_4 production is strictly anaerobic process whereas the CH_4 oxidation mainly occurs in the presence of O_2 at the oxic/anoxic interface (Bastviken *et al.* 2002, Kankaala *et al.* 2006). N_2O reduction to N_2 is an O_2 sensitive process and thus N_2O can be reduced only in anoxic environments (Betlach and Tiedje 1981), even though it can be produced both in aerobic (nitrification) and anaerobic processes (denitrification and DNRA).

Whether lakes are sinks or sources of GHG depends on the surface water gas concentration and the atmospheric equilibrium concentration. Based on Henry´s (1803) law, more gas can dissolve in colder water and thus the equilibrium concentrations are dependent on the water temperature. This means that at colder temperatures less gas will be released to the atmosphere. However, highest CH_4 and CO_2 fluxes are repeatedly observed during and immediately after water column mixing events when the gas concentrations accumulated in the hypolimnion are released to the surface waters and further to the atmosphere (Kankaala *et al.* 2006, López Bellido *et al.* 2009). In addition, CH_4 is poorly soluble in water and ebullition, whereby the CH_4 is released as bubbles from the bottom straight to the surface bypassing the oxidation, is a common phenomenon in highly methanogenic environments (Chanton and Whiting 1995, Casper *et al.* 2000, Bastviken *et al.* 2011). Temperature has also an important role on the microbial activity. In short, the rates of all chemical reactions increase with temperature following the Arrhenius' (1889) equation. Although microbes can have different optimal activity temperatures, many processes like denitrification are more effective at higher temperatures (Ahlgren *et al.* 1994, Saunders and Kalff 2001), and the N_2O production itself has been shown to be most active at 16°C in a boreal lake (Liikanen *et al.* 2002). Increased temperatures also increase the mineralization of organic materials, thus leading to higher CO_2 and CH_4 production (Liikanen *et al.* 2002). Liikanen *et al.* (2002) predicted that a 1–3 °C increment in temperature would increase CO_2 production by 13–66 % and CH_4 release from anoxic sediments by 13–39 %.

Carbon and nutrient concentrations affect the GHG fluxes both by shaping the microbial community composition and controlling the process rates. As stated above the O_2 concentration and redox conditions have a major role on the vertical distribution and concentrations of NO_3^-, SO_4^{2-}, Fe^{3+} and Mn^{4+}, which control the possible reactions. NO_3^- concentration plays an important role in controlling N_2O production by increasing the denitrification rates (Pina-Ochoa and Alvares-Cobelas 2006, McCrackin and Elser 2010), but also by inhibiting N_2O reduction under high NO_3^- concentrations (Betlach and Tiedje 1981). Microbes prefer NO_3^- as an electron acceptor over N_2O, because it is less energy efficient to reduce N_2O than NO_3^- (Blackmer and Bremmer 1978, Baggs *et al.* 2003), and in high NO_3^- concentrations relatively more N_2O is produced in the denitrification pathway (e.g. Weier *et al.* 1993). The most important factor controlling the competition between denitrification and the DNRA process, which also reduces NO_2^-, appears to be the C/N ratio (Kelso *et al.* 1997, Burgin and Hamilton 2007), where a high ratio favours DNRA over denitrification. In addition, the NO_3^-

/NO_2^- ratio and microbial generation time are identified as key environmental factors controlling whether NO_3^- is reduced to N_2 in denitrification, or retained in the system as NH_4^+ in DNRA (Kraft *et al.* 2014). However, whether the environmentally controlled changes in community composition affect biogeochemical cycles is not clear, because the same metabolic pathways can be controlled by a wide variety of different bacteria.

1.3.3 Top-down controls

Predator size, predator abundance and food web structure have all been shown to impact on CO_2 emissions from lakes (Schindler *et al.* 1997, Atwood *et al.* 2013). When planktivorous fish were added to lake ecosystems their grazing reduced zooplankton abundance, allowing greater phytoplankton biomass and primary production, which led to decreased CO_2 concentrations and emissions (Schindler *et al.* 1997). Food web structure substantially affected lake/atmosphere C exchange turning the ecosystem from a CO_2 source to a CO_2 sink (Atwood *et al.* 2013). Trophic cascades are also shown to affect N_2O emissions from soil, where predatory mites regulated fungivores and further led to increased N_2O emissions (Thakur *et al.* 2014). In addition, the top-down control of bacterivorous protists was shown to regulate and modify microbial communities (Kent *et al.* 2004, Zöllner *et al.* 2003). The cladoceran *Daphnia longispina* is known to feed on methanotrophs (Jones *et al.* 1999, Jones and Grey 2011), which have a key role in controlling CH_4 emissions from lakes (Bastviken *et al.* 2003, Kankaala *et al.* 2006). A recent study in a small boreal lake indicated that methanotrophs contributed up to 80 % of *Daphnia* diet in autumn during lake mixing (Taipale *et al.* 2009b). This supports the results from a laboratory experiment, where CH_4 oxidation was lower when *Daphnia* were present than in a control treatment (Kankaala *et al.* 2007, Jones and Lennon 2009). However, how the top-down control on microbes relates to biogeochemical cycling and GHG dynamics *in situ* is not known.

1.4 Aims

Boreal lakes are hot spots for biogeochemical processes that produce and reduce GHGs (CO_2, CH_4 and N_2O). Even though GHG reducing biochemical pathways are well known, there is still a lack of information on the abundance and ecological importance of microbes involved in these processes in boreal lakes, in which special characteristics create certain well-established environments for the processes to occur in different water or sediment layers. In addition, stratified water columns can create a suitable environment for some specific anaerobic microbial groups (for example *Chlorobium,* OD1), whose abundance and role in lake ecosystems are very poorly understood. Therefore, the main aims of this thesis were to study the diversity and abundance of the special microbial groups in boreal lakes and to determine their role in controlling GHG emissions from the lakes. Under these general aims the more specific study hypotheses were:

1. Stratified humic lakes harbour high abundances of anaerobic photosynthetic green sulphur bacteria (GSB) *Chlorobium* depending on the light climate in the anaerobic hypolimnion, creating a previously unacknowledged C sink in these lakes (I, V).
2. Denitrification gene abundances and gene ratios (*nir*/*nos*) control the reduction and production of N_2O and thus explain the N_2O accumulation in lakes (II). The denitrifying communities are well adapted to different environmental conditions resulting in unique communities on a spatial scale. The community composition with gene abundances can be used to predict the prevailing process rates (III).
3. Trophic interactions influence microbial communities (IV, V) and further alter methane fluxes by controlling the abundance of methanotrophs (IV).

2 MATERIALS AND METHODS

The aims and hypotheses were addressed by a combination of multi-lake studies and a whole-lake experiment. Microbial communities were analysed using molecular tools, and accumulation of GHG using gas chromatographic concentration measurements. The abundance and annual occurrence patterns of GSB were studied in a set of 13 stratified boreal lakes with varying water transparency, and in a whole-lake study during a three year experiment. The role of denitrification genes and their ratios in controlling N_2O accumulation in lakes together with environmental factors were studied in a set of 12 boreal lakes, which were divided into two groups based on their NO_3^- concentrations. The denitrifying community adaptation between lakes and the potential for community composition and gene abundances to predict the denitrification rates were studied at the inter-lake scale, covering four boreal lakes, as well as at the intra-lake scale, covering a depth transect from littoral to profundal sediments. The whole-lake experiment, where the trophic structure and interactions were altered by manipulating the grazing pressure from planktivorous fish, was used to study the impacts of trophic cascades on microbial community composition and especially on CH_4 emissions through cascading impacts via zooplankton (*Daphnia*) biomass on methanotroph abundance.

2.1 Study areas, sampling and experimental design

2.1.1 Green sulphur bacteria (GSB) in small humic boreal lakes (I)

A set of 13 small steeply stratified lakes from the Southern Finland Evo forest region (Fig. 2, lakes 1–13) with varying humic matter content were chosen for the analyses of the abundance and spatial distribution of GSB in the water column. The study lakes are shallow with a maximum depth from 3 to 12 m. The pH in the lakes is low (5.2–6.3) while the variation in the water colour is high (65–459 mg Pt l^{-1}). The lakes were stratified with respect to temperature,

O_2, light and nutrients. Annual spring mixing can be missing or incomplete in the study lakes, but the autumn mixing occurs more regularly.

The lakes were sampled 1–4 times in 2009. All the study lakes were sampled at mid-summer, seasonal changes in the abundance of GSB were followed in lakes Alinen Mustajärvi, Halsjärvi and Mekkojärvi, and GSB communities under ice were studied in Alinen Mustajärvi, Halsjärvi, Nimetön and Horkkajärvi. Water samples were taken with a Limnos water sampler (height 30 cm, volume 2.1 l) from the entire water column of each lake at 1 m intervals and at 0.5 m interval from the oxic-anoxic boundary layer (O_2 saturation between 0–10 %). Abundance of GSB and selected variables for background information were analysed from each sample. *In situ* profiles of water temperature, O_2 concentration, redox and pH were measured from every sampling point using a portable field meter (YSI 556 MPS, Yellow Spring Instruments). Other physico-chemical, biological, and molecular background data analyses are presented in Table 1.

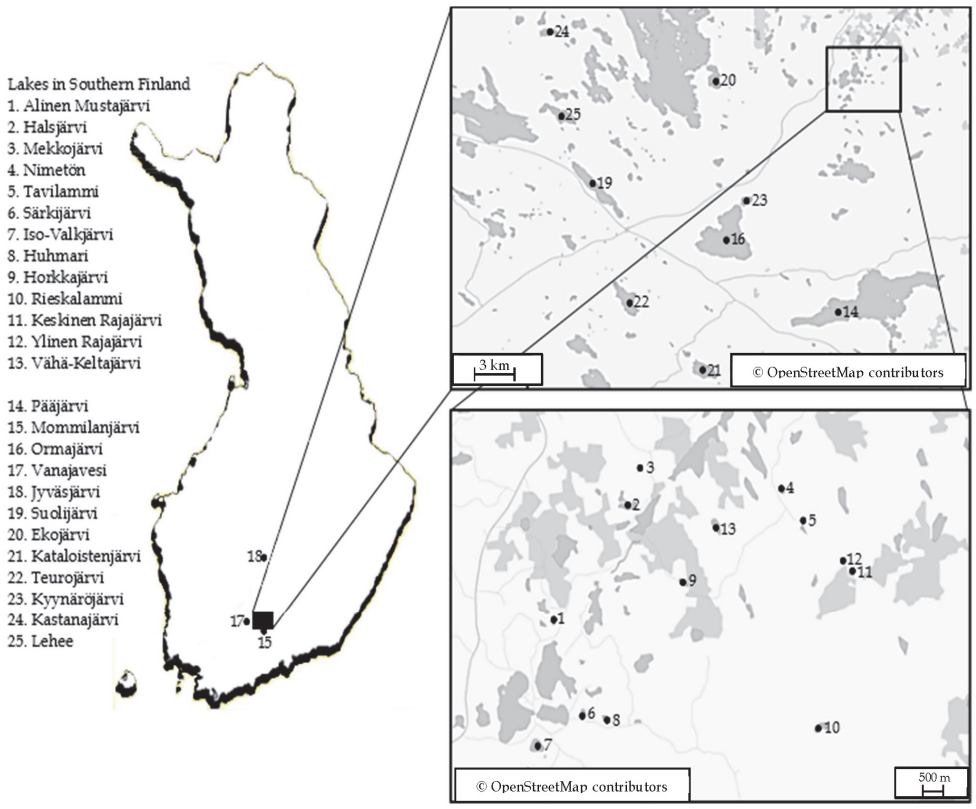

FIGURE 2 Locations of the study lakes in Southern Finland.

2.1.2 N_2O accumulation in boreal lakes (II)

To study the genetic and environmental controls of N_2O accumulation in boreal lakes, 12 lakes were sampled in mid-summer 2011. All the lakes are located in the boreal forest region in southern Finland (Fig. 2, lakes 14–25) apart from Lake Jyväsjärvi (lake 18) which is located in central Finland. The lakes represent a cross-section of Finnish boreal lakes with varying size (25–12 000 ha), morphometry and trophic status from mesotrophic to hypereutrophic (Supplementary material in II). All lakes typically have ice-cover from November to April and normally undergo spring and autumn overturns. Some of the lakes develop water column stratification with respect to temperature and O_2 during summer (Supplementary material in II). The lakes were divided into two groups based on their hypolimnetic NO_3^- concentrations. High-NO_3^--lakes (n=6) have NO_3^- concentrations between 15.7 and 79.4 µmol l^{-1} while low-NO_3^--lakes (n=6) between 0.6 and 1.5 µmol l^{-1}.

Water samples for gas analyses were collected from ca. 0.5 m, 1 m, 3 m and 5 m above the lake bottom (if the lake was deep enough) and at the surface (0.5 m water depth). Water for nutrient analyses was collected in 1 l bottles from the near-bottom waters of the lakes and transported to the laboratory on ice. Sediment cores for analyses of the sediment denitrifier communities were collected from all the lakes using a mini gravity corer with plexiglass tubes (ø = 3.5 cm). Water column profiles of temperature and O_2 concentrations were measured *in situ* using a portable field meter (YSI model 58, Yellow Springs Instruments). Sediment samples from the surface layer (0–2 cm) of the sediment cores were collected in 15 ml plastic tubes and freeze-dried (Alpha 1–4 LD plus, Christ). For the other physico-chemical, biological and molecular background data analyses are presented in Table 1.

2.1.3 Denitrifying communities in lakes (III)

Lakes Pääjärvi, Ormajärvi, Suolijärvi and Lehee (Fig. 2, lakes 14, 16, 19 and 25) were selected to study whether the denitrifier community size or composition could be linked to denitrification rates measured earlier (Rissanen *et al.* 2011, 2013). The study covered both spatial variation at the inter-lake scale and temporal variation within Lake Ormajärvi. Ormajärvi, Suolijärvi and Lehee form a connected lake chain which is classified as eutrophic, whereas Pääjärvi is mesotrophic. All the lakes have an oxic hypolimnion throughout the open water period.

Sediment samples (0–1 cm layer) from Ormajärvi were collected from shallow littoral (1 m), deep littoral (3 m) and profundal (8 m) sites during four seasons (spring, summer, autumn and winter) in 2006–2007. For studies at the inter-lake scale, samples (0–2 cm layer) were collected from profundal sites of Pääjärvi (10 and 12 m), Suolijärvi (10 m) and Lehee (3.3 m) during early-summer and autumn in 2007. Sediment denitrification rate (N_2 production rate) variables, measured using the isotope pairing technique (IPT) (Nielsen *et al.* 1992), included D14 (denitrification of the natural NO_3^-), Dn (natural coupled

nitrification-denitrification), and Dw (denitrification of the natural NO_3^- in the water above the sediment). More detailed sampling and IPT methods, and the IPT results are presented in Rissanen *et al.* (2011, 2013). The other physico-chemical, biological, and previous molecular analyses are presented in Table 1.

2.1.4 Whole-lake experiment in Lake Mekkojärvi (IV, V)

Mekkojärvi is a small (area 0.35 ha), shallow (mean depth 3 m), and highly humic lake located in the Evo forest area in Southern Finland (Fig. 2, lake 3). The lake water is dark brown due to high DOC concentrations (20–45 mg C l^{-1}; Taipale *et al.* 2009a) leaching from the catchment area. During summer the dark coloured lake becomes steeply stratified with respect to temperature, O_2 and nutrients, and the euphotic zone is limited to only 0.5 m. The lake has ice-cover from November to April and the whole water column becomes anoxic during winter. The lake can be considered practically fishless (with some rare exceptions of pike, *Esox lucius*) and hence high abundances of cladoceran (*Daphnia*) zooplankton dominate the water column.

The study was conducted during 2011–2013 to investigate whether trophic interactions can control methanotrophy via regulation of bacterial community dynamics. Mekkojärvi was divided into two treatment basins with a plastic curtain reaching from the surface down to the sediment. The curtain was lowered in spring after any spring mixing was complete. The lake was divided from a small inlet to a small outlet to obtain as similar treatment basins as possible (Fig. 3). During the course of 3 years, early in each July adult European perch (*Perca fluviatilis*) were added to one basin and an equivalent biomass of juvenile perch was introduced to the other basin. However, due to a brief pulse of hypoxic conditions (repeated in every study year) in one basin the established juvenile populations died shortly after introduction leaving the basin fishless. The experiment thus created two contrasting fish treatments: fish present (high planktivory) and fish absent (no planktivory).

The lake was sampled 1–2 times per month every year during the open water season, from epi- (~ 0–0.5 m), meta- (~ 0.5–1 m) and hypolimnion (~ 1–3 m). Gas samples for CH_4, zooplankton samples and samples for DNA analysis were always collected from both basins of the lake. On each sampling occasion temperature and O_2 profiles were measured *in situ* using a portable field meter. The other physico-chemical, biological and molecular background data analyses are presented in Table 3.

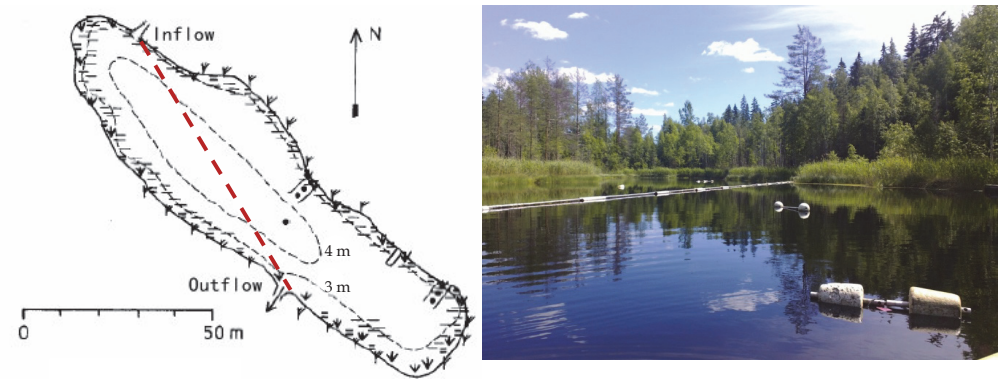

FIGURE 3 Lake Mekkojärvi divided into two treatment basins with a plastic curtain from inflow to outflow (Map: Kairesalo et al. 1992, Photo: Jari Syväranta).

2.2 Microbial analysis

2.2.1 LH-PCR (I)

The fingerprinting method by length heterogeneity analysis of PCR-amplified 16S rRNA gene (LH-PCR) (Suzuki *et al.* 1998) was used to identify GSB *Chlorobium* from the stratified water columns of small humic boreal lakes. Earlier studies have shown that the genus *Chlorobium* has a distinct fragment length at 512 (±1) bp, which was therefore used as a specific biomarker for *Chlorobium*, marked as LH-PCR$_{512}$. LH-PCR analysis was done according to Taipale *et al.* (2009a) with minor modifications, using primers 27f and 518r (Table 2) for 16S rRNA amplification. The data were analyzed using Quantity One software (Bio-Rad Laboratoires, CA).

2.2.2 Cloning and Sanger sequencing (I)

To confirm the LH-PCR results and to study the diversity of *Chlorobium* sp. genotypes and anaerobic microbial communities, clone libraries were constructed from lakes Alinen Mustajärvi, Halsjärvi, Mekkojärvi, Nimetön, Tavilammi, Särkijärvi, Iso-Valkjärvi and Huhmari from the depth of the maximum bacteriochlorophyll (BChl) concentration. The primers 27f and 907R primers were used to amplify ~ 880 bp long 16S rRNA fragments (Table 2). Cloning and sequencing of the PCR products were performed as described by Taipale *et al.* (2009a) by using TOPO TA Cloning Kit for Sequencing (Invitrogen). A total of 192 sequences was analyzed (24 clones were sequenced from each sample). DNA sequences were edited with ContiqExpress (Invitrogen) and the sequences (~ 880 bp and shorter part of the sequence ~ 450 bp corresponding to LH-PCR fragment) were compared to the GENBANK database using BLAST software (Altschul *et al.* 1997) and the Ribosomal Database Project II programs Seqmatch and Classifier (Anon. 2010). MEGA4

(Tamura *et al.* 2007) was used for sequence alignment and to construct a neighbour-joining tree for selected *Chlorobium* sp. clones. The sequences were divided into operational taxonomic units (OTUs) by using CD-hit program (Huang *et al.* 2010) with 97 % sequence identity cut-off level.

2.2.3 Next generation sequencing (III, V)

Next generation sequencing techniques, 454-pyrosequencing (III) and Ion Torrent sequencing (V), were used to study the role of functional denitrifying communities in controlling denitrification process rates (III) and changes in the microbial community composition based on 16S rRNA during the whole-lake experiment in Mekkojärvi (V).

The community structure, richness and diversity of organisms harboring *nirS*, *nirK* and *nosZ* genes were studied with 454-pyrosequencing (III). PCR was conducted with primer pairs nirScd3aF/nirSR3cd for *nirS*, F1aCu/R3Cu for *nirK* and nosZF/nosZ1622R for *nosZ* (Table 2). Sequencing was done with Titanium chemistry using a 454 GS-FLX system (454 Life Sciences, Branford, CT, USA) at the Institute of Biotechnology hosted by Helsinki University. The primers used carried 454FLX adaptors at their 5´ ends, and 5-basepairs long barcodes were incorporated between the 454FLX adapter and the forward primer to distinguish each sample in the mixed reaction.

Amplification of bacterial 16S rRNA genes to observe changes in community composition during the whole-lake experiment was conducted using general bacterial primers 27f and 338r (V, Table 2) with Ion Torrent sequencing. Primer 27f carried a specific 11-12 bp long barcode for each sample followed by a KEY tag and an Ion Torrent adaptor A at the 5′ end, and primer 338R carried a P1 adaptor at the 5′ end. The sequencing of the pooled library was conducted from A adaptor by using the Ion Torrent Personal Genome Machine (PGM; Life Technologies) with Ion Sequencing 400 kit and Ion 314 Chip.

Barcodes and primer sequences, as well as low-quality sequences (containing ambiguous nucleotides and homopolymers longer than eight nucleotides) were removed from the sequence libraries using Mothur (Schloss *et al.* 2009). All nucleic acid sequences were checked for chimeras in Mothur and any sequences denoted chimeric were removed from the alignment. The functional denitrifying sequences were translated into amino acid sequences (III), and sequences with ambiguous amino acid residues and stop codons were removed. Amino acid sequences were aligned using HMMER3-aligner tool at FunGene (Functional gene pipeline & repository; Fish *et al.* 2013) and the 16S rRNA sequences were aligned against the SILVA database in Mothur (Quast *et al.* 2013). The amino acid sequences were classified into OTUs at the 90 % similarity level (III) and the 16S rRNA sequences at 97 % similarity level (V) in Mothur. From the 16S rRNA sequences, OTUs containing at least 50 reads were included in further analysis (covering 92 % of all sequences) (V). A representative sequence from each of the 597 OTUs obtained was classified using the Least Common Ancestor tool of the SINA ALIGNER v1.2.11 (Pruesse

et al. 2012). Classification was based on the SILVA taxonomy for the 10 nearest neighbors retrieved from the curated SILVA SSU Ref database Release 106 (Pruesse *et al.* 2007) with minimal identity of 0.80. In addition, Mothur was used to calculate diversity (inverted Simpson´s diversity index), richness (Chao 1 richness estimate) and coverage (Good´s coverage), an estimate of the proportion of amplified gene amplicons represented by sequence libraries for each sample. The sequence data were analysed further with several statistical methods (III, V, Table 3).

2.2.4 Quantitative PCR (qPCR) (II, III, IV)

qPCR was used to measure the abundance of various functional genes and 16S rRNA as a reference gene in the studies. The abundance of the two functionally equivalent but structurally divergent NO_2^- reducing enzymes *nirS* and *nirK* (II, III) were the focus of interest in the denitrification pathway. In addition, the last step in denitrification is controlled by *nosZ* reducer that converts N_2O to N_2 and thus has a key role in controlling N_2O production in the denitrification pathway (II, III). Recent studies have shown that there are two different *nosZ* clades that need to be studied with separate primers (Jones *et al.* 2013, Sandorf *et al.* 2012, II). In II and III the primers for *nirS* were nirSCd3aF and nirSR3cd, for *nirK* nirK876 and nirK1040 and for *nosZ* cladeI: nosZ2F and nosZ2R, and in addition in II *nosZ* cladeII was included with primers nosZII-F and nosZII-R. All primer sequences are presented in Table 2. The annealing temperatures for *nirS* was 55 °C, *nirK* 60 °C, *nosZ* cladeI 60 °C and *nosZ* clade II 54 °C.

Methanotrophs were studied with two separate primer pairs to obtain as complete estimate of the methanotrophic communities as possible (IV). After testing different primers (from Kolb *et al.* 2003, Rahman *et al.* 2010 and Sharp *et al.* 2013) *pmoA* (specific membrane protein in methanotrophs), the primer pairs MCOC: A189F and MC 468R, and MBAC: A189F and Mb601R (Table 2) were chosen to cover as many as possible of the MOBs present. The annealing temperatures for MCOC was 60 °C and for MBAC 54 °C.

Gene abundances were calculated as relative abundances compared to the abundance of the reference gene (16S rRNA; primers 27f and 338r) and as a gene copy number per ng of DNA. Amplification of qPCR and fluorescent data collection was carried out with a Bio-Rad CFX96 thermal cycler (Bio-Rad Laboratorios) using SYBR Green supermix (BioRad). Standard curves were constructed from PCR amplicons extracted from agarose gel with a BioRad Gel Extraction Kit (BioRad). Amplicons were re-amplified using PCR, and the PCR products were purified with Agencourt AMPure XP (Beckman Coulter). A dilution series of 10^7–10^2 gene copies was used as a standard in each qPCR run. A set of random samples with dilutions of 1, 1^{-10} and 1^{-100} was used to check possible inhibition, but no significant inhibition was observed in our studies. Finally, an increase of 0.5 °C s^{-1} from 65 °C to 95 °C was performed to obtain the melting curve analysis of PCR products.

2.3 Natural N_2, N_2O and CH_4 gas concentrations from the water column

Natural N_2, N_2O (II) and CH_4 (IV) gas concentrations were measured from the water columns of the study lakes. Nitrogen gas (N_2) samples for membrane inlet mass spectrometry (MIMS) measurements were taken in 12 ml borosilicate glass vials (6 replicates) with screw-capped butyl rubber septa (Labco Ltd.). Water was allowed to overflow for at least 3 vial volumes to avoid atmospheric contamination, and samples with air bubbles were discarded. Microbial processes in borosilicate glass tubes were stopped by adding 100 µl ZnCl through the septum with a needle under water.

Samples for N_2O and CH_4 were taken in 60 ml polypropylene syringes, which were closed with three-way stopcocks after removing any gas bubbles, and transported to the laboratory on ice. The N_2O and CH_4 samples were processed according to López Bellido *et al.* (2009), using a headspace equilibration technique. N_2O samples were analysed according to Maljanen *et al.* (2009) with a gas chromatograph (Agilent 6890N, Agilent Technologies) equipped with an auto sampler (Gilson) and an electron capture detector (ECD) and the CH_4 samples according to Ojala *et al.* (2011) with a gas chromatograph equipped with a flame ionization detector (temperature 210 °C) and thermal conductivity detector (temperature 120 °C, oven 40 °C, PlotQ capillary column, flow rate 12 ml min^{-1}, He as a carrier gas).

N_2O equilibrium concentrations were calculated based on Henry's law (modified from Lide and Frederikse 1995 and Anon. 2007). Concentration of N_2O accumulated due to microbial reactions (N_2O_{excess}) was calculated from the difference between observed N_2O concentration and the calculated equilibrium concentration (II). The overall amount of accumulated N_2O m^{-2} was estimated from integration of the N_2O_{excess} concentration profiles, and the depth-integrated N_2O_{excess} m^{-3} was obtained by division through the water depth at the sampling site. Assuming cumulative N_2O production in the hypolimnion, with low atmospheric exchange after the mixing period, net N_2O production rates were calculated according to Mengis *et al.* (1997) (with slight modifications) by dividing the amount of accumulated N_2O m^{-2} by the number of days from ice-off (i.e., the onset of water column stratification in early May) to the sampling date (end of July) (II).

CH_4 effluxes in summer (IV) were estimated using the boundary layer diffusion equation (Kling *et al.* 1992, Phelps *et al.* 1998),

$$CH_4 \text{ efflux} = Db/zb\ (Csur\text{-}Ceq),$$

where the Csur is the concentration of CH_4 measured in the epilimnion (0–50 cm depth), Ceq is the concentration of CH_4 in equilibrium with air, zb is the thickness of the boundary layer and the Db is the diffusion coefficient. Db (cm^2 s^{-1}) and zb (µm) were calculated with the following equations,

$Db = (1.33 + (0.055\ T))\ 10^{-5}$, and

$zb = 10^{2.56\ -\ 0.133\ ws}$,

where T is the water temperature (°C) at the surface and ws is wind speed at 10 m height (m s^{-1}). The wind speed is an average wind speed measured and reported (Huotari *et al.* 2011, Peura *et al.* 2013) between 2002–2009 from the lake Valkea Kotinen climate station located within 10 km of lake Mekkojärvi. The Ceq was calculated with Henry's law constants for surface temperatures (Lide & Frederikse 1995) assuming a stable atmospheric CH_4 concentration of 1.745 ppm (Houghton *et al.* 2001).

N_2/Ar gas concentration ratios were determined using membrane inlet mass spectrometry (MIMS) as described in Kana *et al.* (1994). Equilibrium concentrations were calculated according to Weiss (1970). $N_{2excess}$ was then calculated from N_2/Ar in the sample divided by the N_2/Ar at equilibrium for a given temperature.

2.4 Background data analyses, primer sequences and statistical analyses

TABLE 1 Physico-chemical, biological, and molecular analyses conducted in the thesis.

Analysis	Description	Reference
Chl *a*	I	
BChl	I	
pH	I, III, IV, V	
Water colour	I	
DOC	I, IV, V	
Inorganic nutrients	I, II, III, IV, V	
Zooplankton abundance & diversity	IV, V	
Light intensities	I	Wetzel 1983
DNA extraction	II, III	Griffiths *et al.* 2000
DNA extraction with lysate technique	I	
DNA extraction with MO-BIO kit	I, IV, V	

TABLE 2 Primer sequences used in the PCR-reactions.

Gene:primer	Sequence (5′ - 3′)	Reference	Paper
16S rRNA: 27f	AGAGTTTGATCMTGGCTCAG	Lane 1991	I, II, III, IV
16S rRNA: 338r	TGCTGCCTCCCGTAGGAGT	Universal primer	II, III, IV
16S rRNA: 518r	ATTACCGCGGCTGCTGG	Muyzer *et al.* 1993	I
16S rRNA: 907r	CCGTCAATTCMTTTRAGTTT	Amann *et al.* 1992	I
nirS: Cd3aF	AACGYSAAGGARACSGG	Kandeler *et al.* 2006	II, III
nirS: R3cd	GASTTCGGRTGSGTCTTSAYGAA	Kandeler *et al.* 2006	II, III
nirK: 876	ATYGGCGGVAYGGCGA	Henry *et al.* 2004	II, III
nirK: 1040	GCCTCGATCAGRTTRTGGTT	Henry *et al.* 2004	II, III
nirK: F1aCu	ATCATGGTSCTGCCGCG	Hallin and Lindgren 1999	III
nirK: R3Cu	GCCTCGATCAGRTTGTGGTT	Hallin and Lindgren 1999	III
nosZ: 2F cladeI	CGGRACGGCAASAAGGTSMSSGT	Henry *et al.* 2006	II, III
nosZ: 2R cladeI	CAKRTGCAKSGCRTGGCAGAA	Henry *et al.* 2006	II, III
nosZ: II-F cladeII	CTIGGICCIYTKCAYAC	Jones *et al.* 2013	II
nosZ: II-R cladeII	GCIGARCARAAITCBGTRC	Jones *et al.* 2013	II
nosZ: nosZF	CGYTGTTCMTCGACAGCCAG	Kloos *et al.* 2001	III
nosZ: nosZ1622R	CGSACCTTSTTGCCSTYGCG	Throbäck *et al.* 2004	III
pmoA: A189F	GGNGACTGGGACTTCTGG	Kolb *et al.* 2003	IV
pmoA: Mc468R	GGNGACTGGGACTTCTGG	Kolb *et al.* 2003	IV
pmoA: Mb601R	ACRTAGTGGTAACCTTGYAA	Kolb *et al.* 2003	IV

TABLE 3 Statistical analyses used in the study.

Analysis	Description
t-test	II
Mann-Whitney U-test	II, V
Spearman's rank correlation	I, II, III
Pearson correlation analysis	II, III
Non-Metric Multidimentional scaling (NMDS)	III, V
distance based linear model (DISTLM)[1]	III
PERMANOVA[1]	III, V
Mantel's test	III
2-way ANOVA	V

[1]Anderson (2001), McArdle & Anderson (2001)

3 RESULTS AND DISCUSSION

3.1 Anaerobic phototrophic GSB in boreal stratified lakes (I, V)

GSB are strictly anaerobic and phototrophic microbes that require stable anoxic conditions (Van Gemerden and Mas 1995). All the study lakes were steeply stratified with an anoxic hypolimnion and thus provided a suitable environment for GSB (I, V). In 8 of the 13 lakes significant amounts of GSB were found from LH-PCR_{512} and sequencing techniques, and in 2 of the study lakes they occurred in low densities (I). This finding was supported by a strong correlation between the LH-PCR_{512} results and the BChl concentrations (Sperman's correlation; ρ = 0.827, $p < 0.001$). In addition to anoxic conditions, GSB requires the presence of reduced sulphur compounds and low amounts of light for growth (Van Gemerden and Mas 1995). Based on BChl concentration profiles the GSB communities were located in the anoxic hypolimnion, with a maximum abundance usually close to the oxic-anoxic boundary layer. The PAR intensity at the depth of the oxic-anoxic interface was higher in those lakes where GSB were abundant (1.1–24.2 μE m^{-2} s^{-1}, corresponding to 0.08–1.7 % of the surface PAR) than in the lakes without GSB (< 0.1 μE m^{-2} s^{-1}, corresponding to < 0.007 % of the surface PAR). The clear relationship between PAR intensity at the oxic-anoxic interface and BChl concentration (ρ = 0.813, $p < 0.001$) further supported this finding. Thus even though the light conditions in boreal humic lakes are poor, many of these lakes provide a suitable niche for GSB.

Based on clone sequence libraries constructed from anoxic water columns (I), 13 bacterial phyla were identified, the main phyla being *Proteobacteria*, *Chlorobi* and *Bacteroidetes*, which have been classified as freshwater species (Pfennig and Overmann 2001). The proportion of GSB varied between 14–38 % of the bacterial community in the lakes with GSB and a close phylogenetic relationship in GSB communities was observed among lakes. The clustering analysis at $OTU_{0.97}$ level (sequence length ~ 880 bp) divided the clones into 4 clusters, of which the most common OTU1 had the closest match with type strain *Chlorobium clathratiforme* (I). *C. clathratiforme* is known to belong to the green subset of the GSB, with a

pigment composition of BChl *c*, *d* or both (Gich *et al.* 2001). In Lago di Cadagno *Chlorobium clathratiforme* covered 95 % of the anoxygenic phototrophic community (Habicht *et al.* 2009).

The seasonal sampling indicated a substantial increase in BChl concentrations from spring to the end of summer within the 3 study lakes, suggesting that the abundance of GSB could be even higher by the end of the summer in the samples (I). In addition, the BChl concentrations and LH-PCR$_{512}$ profiles at the end of the winter under ice suggest that GSB can survive under ice during winter. However, the amount of GSB was substantially lower and indicates that the growth mainly occurs during summer when sufficient light can penetrate to the depth of GSB communities. GSB have also been found under ice from Lake Burton, Antarctica, where the communities are isolated under ice for 3 months of the year (Burke and Burton 1988). The survival of GSB during winter time can be explained by their efficient maintenance metabolism and by their great efficiency in utilizing low-intensity light (Burke and Burton 1988).

In a whole-lake experiment in Lake Mekkojärvi (V), *Chlorobium* was the most abundant bacterial group found from the lake water column. On average they comprised 31 % of the bacterial 16S rRNA genes from the meta- and hypolimnetic communities. Taipale *et al.* (2011) previously reported that during the summer stratification on average 47 % of the bacterial rRNA genes in Mekkojärvi were related to the GSB, which is slightly more than in our study. However, as much as 74 % of the bacterial community was observed in the hypolimnion during our experimental study in Mekkojärvi (V). Based on OTU$_{0.97}$ classification of the 16S rRNA sequences, the most abundant OTU was classified as *Chlorobium* and together 64 OTUs (from the OTUs that contained at least 50 reads) belonged to this group. The abundance patterns followed the assumptions as highest abundances were observed in summer and the community then collapsed during the autumn overturn (Fig. 4).

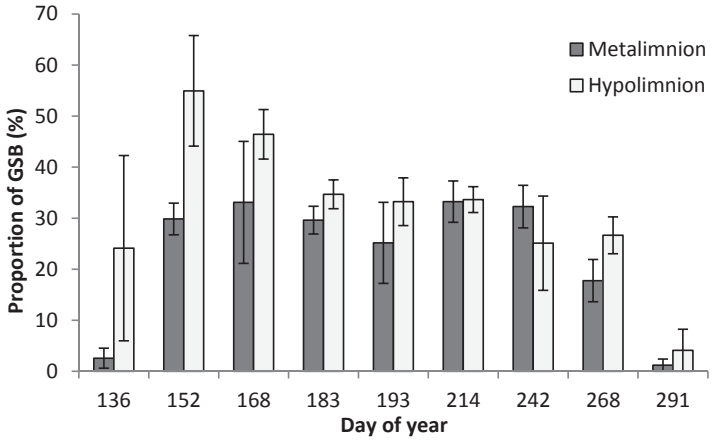

FIGURE 4 Proportion of green sulphur bacteria (GSB) *Chlorobium* in the total bacteria in the meta- and hypolimnion during 2011–2013 in Lake Mekkojärvi (mean ± SE), based on the 16S rRNA gene sequences (data from V).

Thus the results confirm that GSB are widespread in small boreal lakes despite the very low PAR in the anaerobic zone. The measured BChl concentrations and the high proportion of the characteristic LH-PCR_{512} biomarker further proved that GSB were abundant in more than half of the study lakes. According to the results of Arvola *et al.* (1992), hypolimnetic GSB comprised from 61 to 78 % of the autochthonous carbon in Mekkojärvi in summer 1986. Even though more attention should be paid to the actual photosynthetic activity and primary production rates of *Chlorobium* sp. before their ecological significance in the energy mobilization of boreal lakes can be determined, the results suggest that these lakes are more autotrophic than previously thought and the GSB may constitute a significant, but largely neglected, part of the microbiology of these boreal lakes.

3.2 Genetic and environmental factors controlling denitrification and N_2O production (II, III)

Denitrification rates measured from the 4 study lakes (III) ranged between 44.8 and 560.8 µmol N m^{-2} d^{-1} (Rissanen *et al.* 2011, 2013) and were at the levels previously reported from Swedish lakes (Ahlgren *et al.* 1994). The rates are low on the global scale, but the prevailing NO_3^- concentrations are lower in the boreal zone than in temperate regions, from where most denitrification results have been reported (see Rissanen *et al.* 2011). Boreal lakes are shown to be possible sources of N_2O (Kortelainen *et al.* 2000), which can be produced as an intermediate in the denitrification pathway or as a by-product in nitrification and in DNRA. Most of the 12 Finnish lakes in our dataset were oversaturated with N_2O (11– 337 % oversaturation) and the highest concentrations were observed either near the sediment surface or, in stratified lakes, from the oxic-anoxic interface within the water column (II). NO_3^- concentration was the most important factor controlling both the denitrification rates (III) and the N_2O accumulation (Pearson's correlation, $r = 0.66$, $p = 0.01$) (II). A meta-analysis from various aquatic environments reported the importance of NO_3^- in controlling denitrification rates (Pina-Ochoa and Alvares-Cobelas 2006) and the relationship between higher NO_3^- concentrations and higher N_2O concentrations has been shown in various studies (Weier *et al.* 1993, Kortelainen *et al.* 2000, McCrackin *et al.* 2011). In addition, coupled nitrification-denitrification (Dn) is suggested to be important in the denitrification pathway whereby nitrification produces NO_3^- through ammonium reduction (Seitzinger *et al.* 2006, Vila-Costa *et al.* 2014). The measured Dn was connected to certain gene abundances (relationship of *nirS* and *nosZ*) and community compositions (*nirS, nirK* and *nosZ*) (II), and thus confirms the role of nitrification in boreal lakes where the natural NO_3^- concentrations are low (Rissanen *et al.* 2013).

The denitrifying genes (*nirS*, *nirK* and *nosZ*) were present in all studied lakes and sites on every sampling occasion (II, III). The relative abundances were

at the same level as previous results (Henry *et al.* 2006, Cuhel *et al.* 2010, Martins *et al.* 2011), varying between 0.9–8.2 % *nirS*, 1.3–9.5 % *nirK*, 0.9–6.1 % *nosZ*$_{I}$ and 0.6–12.9 % *nosZ*$_{II}$, and indicating the important biogeochemical role of denitrification in boreal lake sediments. Previously *nirS* has been suggested to be more dominant in freshwater sediments (Martins *et al.* 2011). However, average ratios were found between *nirS* and *nirK* genes of 1.0 and 1.3 (II, III) indicating that these two NO_2^- reducers were equally important in boreal lakes. Unlike the subtropical lakes studied by Martins *et al.* (2011), boreal lakes experience substantial seasonal variations in O_2, redox and other physicochemical conditions, which may increase the diversity of ecological niches and prevent out-competition of certain microbial ecotypes. The recently identified *nosZ* cladeII has been found to be nearly as frequent as the typical *nosZ* cladeI in freshwater sediments (ratio *nosZ*$_{I}$/*nosZ*$_{II}$ on average 1.9) (II). This highlights the importance of taking the new clade into account, and the need for further study of the ecology of *nosZ*$_{II}$ encoding organisms, when studying N_2O reduction processes in different ecosystems.

Gene abundances or ratios could not be linked to the observed denitrification rates (III), but the ratio *nir/nos* was important in controlling N_2O accumulation in boreal lakes (II). The gene ratio *nir/nos* in the denitrification pathway determine whether N_2O is produced by *nir* genes and further reduced to N_2 by *nosZ* genes (Zumft 1997). NO_3^- is generally preferred over N_2O as an electron acceptor among the denitrifying bacteria, and high availabilities of NO_3^- and NO_2^- are shown to increase N_2O accumulation, elevating the $N_2O/(N_2O+N_2)$ in the gaseous denitrification products (e.g. Weier *et al.* 1993). At the inter-lake scale, lakes in the high-NO_3^--group (NO_3^- > 15.7 µmol l^{-1}) had significantly higher (*nirS*+*nirK*)/*nosZ*$_{I}$ than lakes in the low-NO_3^--group (NO_3^- < 1.5 µmol l^{-1}) ($p = 0.006$), and the (*nirS*+*nirK*)/*nosZ*$_{I}$ gene ratio correlated positively with the estimated net N_2O production ($r = 0.62$, $p = 0.03$) (II). The same trend was seen in the higher ratios of (*nirS*+*nirK*)/*nosZ*$_{II}$ and (*nirS*+*nirK*)/(*nosZ*$_{I}$ + *nosZ*$_{II}$) in the high NO_3^- lakes group, although the differences were not statistically significant ($p > 0.05$ and $p = 0.062$, respectively). At the depth transect of Lake Vanajavesi, NO_3^- concentrations were consistently high (24.0 - 44.9 µmol l^{-1}) at all sampling sites. Contrary to the inter-lake scale, the NO_3^- concentration did not show correlation with the (*nirS*+*nirK*)/*nosZ*$_{I}$ gene ratio within the Vanajavesi transect, but with the (*nirS*+*nirK*)/*nosZ*$_{I+II}$ ratio and NO_3^- concentrations ($r = 0.98$, $p = 0.001$) (II). The observed gene ratios and their connection to NO_3^- concentrations and observed N_2O accumulation indicate that ambient NO_3^- concentrations control N_2O production, either directly by increasing the production rates or indirectly by controlling the balance between NO_2^- versus N_2O reductase carrying organisms (II).

The balance between N_2O production and reduction is sensitive to redox conditions (e.g. Codispoti *et al.* 2001), as N_2O can be produced both in oxic and anoxic environments, whereas the reduction is strongly inhibited by the presence of O_2 (Betlach and Tiedje 1981), thus occurring only in anaerobic processes. N_2O accumulation patterns were found to be clearly linked to O_2 concentrations (II),

where concentrations peaked at the oxic-anoxic interface and even undersaturated concentrations were observed from the anoxic bottom layers (II). This could be due to O_2 availability just above the oxic-anoxic interface, that would increase N_2O production via nitrification or nitrifier-denitrification (Goreau *et al.* 1980, Kampschreur *et al.* 2009), further inhibiting N_2O reduction (Kampschreur *et al.* 2009). The reduced, or absent, N_2O accumulation in the anoxic water column of the lakes, where a redox cline developed, further supports the notion that stable anoxic conditions are conducive to full denitrification to N_2, while more unstable redox conditions within the redox cline would rather support truncated denitrification and/or delayed nitrous oxide reduction. In Alpine lakes, highest N_2O production was related to hypolimnetic oxygen deficiency (but not anoxia) (Mengis *et al.* 1997).

To study further the community composition and the stability of denitrifying communities, *nirS*, *nirK,* and nosZ genes were pyrosequenced (III). The sequencing of the genes resulted in 388, 435 and 147 OTUs (at 90 % sequence similarity level) in *nirS*, *nirK* and *nosZ* libraries, respectively. The community compositions differed between the lakes, indicating unique communities on a spatial scale (III), as shown previously in aquatic ecosystems (Junier *et al.* 2008, Kim *et al.* 2011). NO_3^-, both directly as NO_3^- concentration and through nitrification-denitrification (Dn), was the most important environmental factor shaping the communities (Mantel's test: r = 0.71, p = 0.001; r = 0.57, p = 0.001; r = 0.83, p = 0.001, respectively) (III). The other environmental factors that affected the community structure were porosity and loss on ignition (LOI) for all genes. Even though the communities were spatially different, the denitrifying communities shared most of the operational taxonomic units (OTUs) found. The core OTUs comprised 7, 6 and 4 OTUs for *nirS, nirK* and *nosZ*, respectively, and were found from each lake at different sampling times. Up to 53 % of the *nosZ* sequences belonged to OTU1 (III). The extremely low number of core OTUs suggests that only a small number of microbial dominants are responsible for the denitrification process. Jayakumar *et al.* (2013) hypothesized that under low NO_3^- concentrations higher diversity assemblages should dominate. However, a fertilization study in a salt marsh showed contradictory results where higher nutrient concentrations increased the number of endemic OTUs (Bowen *et al.* 2013). The results showed that not only a low number of core OTUs was dominating, but also that most of the OTUs were shared between the lakes, even though NO_3^- concentrations varied. However, the unique *nirS*, *nirK* and *nosZ* communities between the study lakes would suggest that, even though the core dominant OTUs were shared between the lakes, less abundant OTUs which were "endemic" to certain lakes were adaptations to specific lake environments.

The most abundant *nirS*, *nirK* and *nosZ* sequences all belonged to *Proteobacteria* phyla (III), where they were clearly divided in different classes (Fig. 5). The observed phylogenetic divisions followed the same division patterns as those reported previously (Jones *et al.* 2008, Vila-Costa *et al.* 2014), where the most abundant *nirK* sequences belonged to *Alphaproteobacteria*, the *nirS* sequences to *Betaproteobacteria*, and the *nosZ* sequences to *Beta-* and *Alphaproteobacteria*

subclasses. All the *nirK* sequences were further related to sequences of diazotrophs, which suggests that these bacteria are also capable of nitrogen fixation (III). Reoccurring denitrification and nitrogen fixation has been previously reported from aquatic systems (Halm *et al.* 2009, Fernandez *et al.* 2011). The classification of the most abundant *nosZ* OTU to *Burkholderiales* was only at the 65 % confidence level and could thus show unique *nosZ* communities from boreal lake sediments (III). The stability of the community was studied at the profundal site of Lake Ormajärvi (III). The ratio between *nirS* and *nirK* genes decreased towards autumn and was more linked to changes in the *nirK* abundance (III). However, most OTUs in the communities of *nirS, nirK* and *nosZ* did not show seasonal variations in their relative frequencies. Thus the community compositions remained stable, whereas the ratios and total abundances of the functional genes varied among the seasons. A succession study in a salt marsh soil showed that the community responded significantly to different successional stages (Bowen *et al.* 2013), thus indicating that the sediment communities in boreal lakes are already well adapted to the redox fluctuations.

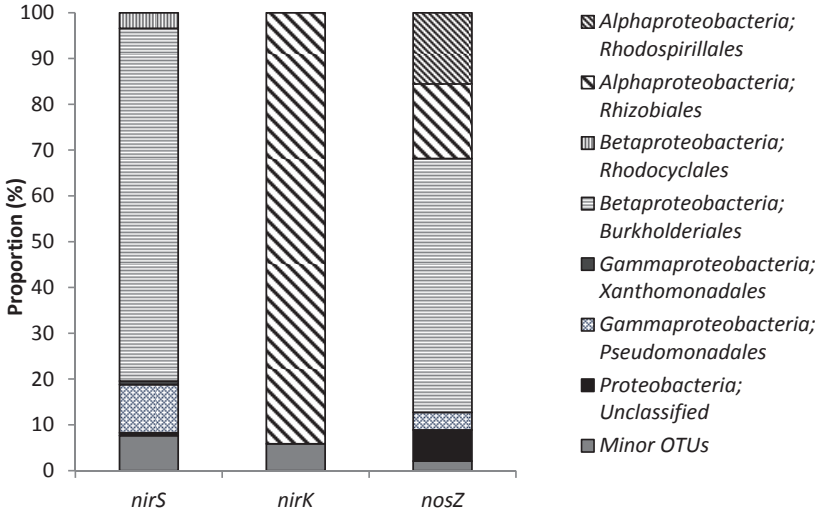

FIGURE 5 Assignment of *nirS*, *nirK* and *nosZ* sequences to orders of cultivated species, based on the pyrosequencing of 8 sediment samples from boreal lakes (data from III).

3.3 Trophic interactions controlling methanotrophs and microbial community composition in a whole-lake experiment (IV, V)

Mekkojärvi was divided with a plastic curtain during the open water periods of 2011–2013, when the trophic structure was experimentally altered by adding fish to the treatment basin of the lake. The fish addition had a clear impact on the zooplankton population, which mainly consisted of *D. longispina*. In the fish-

present treatment basin the biomass of zooplankton dropped to less than 0.1 mg C m^{-3} after fish were introduced, whereas in the fish-absent basin the zooplankton densities increased towards autumn until water column mixing. High grazing pressure and altered trophic cascades following addition of fish were earlier found to control the zooplankton biomass in this lake (Järvinen and Salonen 1998).

The typical boreal humic lake stratification patterns were found from Mekkojärvi every study year, with a shallow oxic epilimnion (0.5 m), steep temperature and O_2 profiles, and higher nutrient concentrations in the hypolimnion than in the epilimnion (IV, V). The steep stratification was also seen in the distinct bacterial communities in different depth layers (epilimnion, metalimnion and hypolimnion) (V). The observed bacterial groups agreed well with previous results from the same lake (Taipale *et al.* 2009a, Peura *et al.* 2012) with high abundances of green sulphur bacteria, *Chlorobium,* and of the previously unknown group of Candidate division OD1 in the anoxic hypolimnion and metalimnion (V). Boreal humic lakes are considered to be net heterotrophic and large amounts of CH_4 can be produced in the anoxic compartments (Huttunen *et al.* 2002, Ojala *et al.* 2011). As a consequence, high densities of CH_4-oxidizing bacteria were found at the oxic/anoxic interface, mainly from the group *Methylococcales* (IV, V). These observed microbial groups have been shown to be an important part of the food web in humic boreal lakes, transforming energy from CH_4 to biomass and making it available to higher trophic levels (Taipale *et al.* 2009b).

The microbial communities were dominated by a few core OTUs, which were present every study year (V). The low diversity and the consistent community succession among years show that these extreme humic and anoxic environments have only a few microbial groups that occupy a specialised niche in the ecosystem. The spring and autumn overturn are important in shaping the communities, as the strictly anoxic groups are destroyed during the mixing while other minor groups become highly abundant (Garcia *et al.* 2013). After spring mixing, O_2 concentration declined quickly from the hypolimnion and metalimnion. The changing O_2 concentrations impacted on both epilimentic and metalimnetic communities, while the hypolimnetic communities were dominated by anaerobic groups. Such seasonal succession occurred every year and largely explained the changes in community composition (V). Seasonal succession and consistent community patterns have been reported previously (Shade *et al.* 2007, Crump *et al.* 2009, Eiler *et al.* 2012), but also contrasting results have also been reported (Kent *et al.* 2004), where little similarity in bacterial community composition was observed between 3 study years. The environmental factors that determine the microbial community composition in Mekkojärvi are similar every year enabling the same few microbial groups develop and dominate in the water column.

The altered trophic cascades were seen to extend all the way to the microbial level, where the bacterial cell numbers were significantly lower in the basin where *Daphnia* biomass was not removed by fish (V). *Daphnia* are known to

control the distribution and biomass of algae and bacteria throughout the water column due to their ability to migrate vertically (Arvola *et al.* 1992), and this was evident also in Mekkojärvi as *Daphnia* impacted on bacterial cell numbers in all depth layers (V). Visualizing the distribution of the 200 most abundant OTUs at the different treatment phases (before fish addition, transition phase and after fish addition) indicates the difference in the bacterial cell numbers between the lake basins (Fig. 6) This is particularly evident in the epilimnion and metalimnion. It has been widely shown that *Daphnia* use bacteria, such as GSB and MOB as an important food source (Ojala and Salonen 2001, Kankaala *et al.* 2006, Taipale *et al.* 2009b, Jones and Grey 2011). The main phylogenetic groups in V consisted of these microbial groups and thus would indicate that the *Daphnia* grazing is likely impacting on these major microbial groups within the ecosystem (V).

Reduction in *Daphnia* biomass released MOB from grazing pressure by zooplankton and allowed MOB abundance to increase significantly after the fish addition, whereas MOB abundance did not increase when fish were not present (IV). Further, the reduced MOB number was linked to observed differences in the epilimnetic CH_4 concentrations, which were higher in the fish-absent basin where lower MOB number was detected with qPCR and thus represented lower potential for oxidation of CH_4 (IV). The hypolimnetic CH_4 concentrations were at the same level in the two basins, indicating that the production (methanogenesis) was not altered; only the CH_4 oxidation was different in the different lake basins. Moreover, when fish were present, the calculated CH_4 effluxes to the atmosphere were only about 25 % of the release from the fish-absent basin (IV). These findings thus present the first evidence that CH_4 efflux from lakes can be regulated by planktivorous fish via trophic cascades from fish down to microbes.

The proportional microbial community compositions was not affected only by the altered trophic cascades and reduced top-down pressure, but also bottom-up factors and physicochemical factors, which were linked to the season and to lake stratification, were found to control the communities (V). Actually, it seems that environmental factors were more significant in controlling the microbial community than biotic factors (e.g. *Daphnia*) in this boreal lake. One reason for this could be that *Daphnia* removal opened a feeding niche for other bacterivores (Jürgens *et al.* 1999, Zöllner *et al.* 2003). Previous studies have shown that when *Daphnia* are sparse the density of other bacterivores (rotifers and ciliates) increases (Järvinen and Salonen 1998), which may have happened in the experiment. In fact, even though the numbers of rotifers and ciliates are not available, the preliminary results indicate that rotifers did not become more abundant during the experiment in contrast to the study of Järvinen and Salonen (1998), but the ciliate abundance may have increased (Dr Jari Syväranta, Jyväskylän yliopisto, pers. comm.). However, the grazing effectivity of ciliates towards large MOB cells is presumably low. Thus top-down pressure was probably not altogether removed, but the bacterivore composition changed. In addition, as *Daphnia* are non-selective filter feeders they are expected to prey equally on all microbes within their reach and thus not favour some specific

groups and change the microbial community composition, unless these are only found from specific depths that the *Daphnia* target. The results discussed previously nonetheless indicate that *Daphnia* rely on the bacterial diet and are effective grazers as seen in the decreased bacterial cell numbers (IV, V).

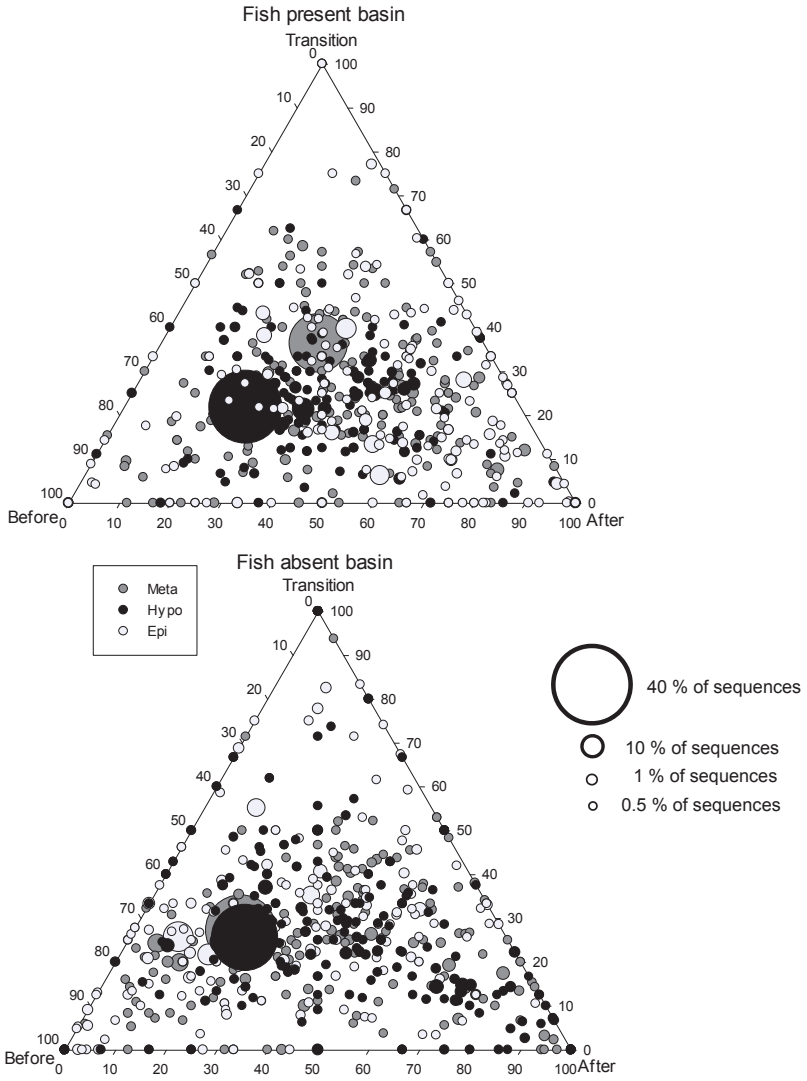

FIGURE 6 Ternary plots showing the distribution of the 200 most abundant 16S rRNA gene OTUs ($OTU_{0.90}$) at different lake basins. Axes present the three treatment phases (before fish addition, transition phase and after fish addition) and the percentage of reads associated with different treatment phases for each OTU. The size of the symbol indicates relative abundances of each OTU in different depth layers, which are marked with different colours (data from V).

4 CONCLUSIONS

Microbial processes are fundamental in controlling the circulation of greenhouse gases, particularly CO_2, CH_4 and N_2O. Stratified boreal lakes are hot spots for greenhouse gas production, as shown by the high CH_4 concentrations and N_2O oversaturation in the study lakes. Since lakes have also been recognized as early indicators of global environmental changes (Adrian *et al.* 2009), it is important to understand how microbiological processes in lakes are controlled. Climate warming is expected to increase inland water temperatures, which would result in steeper, more stagnant, and longer stratification periods and shortened ice cover periods (Elo *et al.* 1998). In addition, the predicted higher precipitation rates may increase nutrient and allochthonous C runoff from the catchments, resulting in increased eutrophication and/or brownification, particularly in the boreal region where the impacts of global warming will be amplified (Hongve *et al.* 2004, Lepistö *et al.* 2014). This thesis provides more information on the microbial controls of greenhouse gas emissions from boreal lakes.

The lake survey revealed that GSB (*Chlorobium* sp.) formed high abundances in the anoxic layers of small humic lakes in the boreal zone. The actual process rates of these phototrophic microbes are still not known, but during the three experiments in Lake Mekkojärvi GSB comprised almost half of the bacteria in the anoxic lake layers, which indicates that GSB are a neglected C sink in humic lakes, which are generally considered net heterotrophic. However, as GSB are obligate anaerobic microbes, overturn leads to breakdown of their biomass, facilitating the heterotrophic food chain. During the summer stratification, bacterial communities were distinct at different depth layers from the oxic epilimnion to the anoxic hypolimnion. Environmental conditions were drastically different following the steep water column stratification. In addition to GSB, high numbers of bacteria of the Candidate division OD1 were found throughout the open water season confirming previous findings of the presence of these presumably anaerobic microbes in boreal humic lakes. As a consequence of the steep stratification and anoxic hypolimnion providing suitable environment for methanogenesis, high concentrations of CH_4 were

measured in Lake Mekkojärvi. Therefore CH_4 oxidizing bacteria, mainly *Methylococcales*, were found especially from the metalimnetic layers, where their abundance was further connected to the epilimnetic CH_4 concentrations.

Denitrifying genes were found with high abundance when compared to housekeeping genes (16S rRNA genes) from all study lakes showing that the ability to denitrify is widespread in boreal lake sediments. The core denitrifiers comprised only a few Proteobacterial variants, as the communities were dominated just by 7, 6 and 4 core OTUs of *nirS, nirK* and *nosZ*, respectively, and these OTU sequences best matched sequences of Proteobacterial isolates. The results further suggested that the $nosZ_I$ in boreal lakes belongs to a distinct environmental cluster. The ratio between NO_2^- reducers and N_2O reducers (*nir*/*nos*) was > 1 in almost all lakes, indicating that the microbial community had a higher potential to produce N_2O than to reduce it. This implies that genetic factors can be used for prediction of N_2O accumulation and emissions.

In addition to direct microbial controls, several different environmental factors were found to control the abundance of microbial groups and their functions. Environmental factors linked to lake stratification were fundamentally important. O_2 concentrations and the depth of the oxic/anoxic interface determined the lake microbial community composition. The depth of this interface determined whether the light intensities were sufficient at the anoxic layers to support the growth of GSB. O_2 concentration and the oxic/anoxic interface were also linked to N_2O accumulation. Highest N_2O concentrations were measured at the oxic/anoxic interface while only negligible N_2O concentrations were found from the anoxic water columns. This suggests that O_2 inhibited N_2O reduction at oxic/anoxic interfaces and supported a truncated denitrification pathway, while in anoxic conditions the N_2O was further reduced to N_2. In addition, the NO_3^-concentrations were linked to higher denitrification rates and to the N_2O accumulation and $(nirS+nirK)/nosZ_I$. Even though the genetic potential and substantial denitrification indicated that boreal lakes can reduce excess NO_3^- through denitrification, the results also indicated that more N_2O was accumulating in the water column under higher NO_3^- concentrations. The high NO_3^- concentrations were presumably both inhibiting N_2O reduction and adapting the genetic communities to have more potential for reducing NO_3^-/ NO_2^- than N_2O in lake sediments.

The addition of fish to a naturally fishless lake showed, that top-down control by fish can lead to trophic cascades down to GHG processes. Reduced zooplankton biomass led to decreased grazing pressure, which further reduced epilimnetic CH_4 concentrations and GHG fluxes from the lake. The study showed that CH_4 fluxes to atmosphere were fourlfold lower when higher abundance of methanotrophs was detected than when methanotrophs were less abundant, which comparison was possible in the divided and experimentally manipulated lake. This is a clear indication that microbial abundance controlled the biogeochemical process. The effect of *Daphnia* presence or absence was seen throughout the whole water column highlighting that *Daphnia* were able to vertically migrate to the hypolimnion, despite the anoxic conditions.

As the stratification patterns and O_2 concentrations were seen to control microbial communities, expected climate warming is likely to impact on GHG processes. If lakes become more steeply stratified or the stratification patterns change from dimictic towards monomictic or meromictic, prolonged anoxic conditions in hypolimnetic layers may further increase the importance of anaerobic microbial communities and processes in boreal lakes. The anoxic hypolimnion favours heterotrophic processes which can produce fermentation products and CH_4. However, the stagnant stratification conditions also favour the occurrence of GSB, which is a neglected sink for CO_2. The higher precipitation predicted by climate change models may, however, increase the colour of the lakes, especially in areas where the catchment is rich in peatland and forest. This may further reduce light intensities for GSB growth and production. Predicted higher precipitation is also expected to increase nutrient concentrations. As the presence of fish drastically decreased CH_4 emission during the open water stratification period, it is important whether the number of naturally fishless lakes and bonds increases in the future, which is dependent on the wintertime thickness of aerobic water layers.

Acknowledgements

This study was carried out at the Department of Biological and Environmental Science, University of Jyväskylä and in the Lammi biological station, University of Helsinki. The first year of the study was financed by the grant from Maj and Tor Nessling foundation (to Prof. Marja Tiirola). The next three years were funded by the Finnish Doctoral Programme in Environmental Science and Technology (EnSTE). Travel grants were provided by EnSTE and Nordic Stable Isotope Research network (NordSIR). I am grateful for all the financial support that I have received over the years.

First I would like to express my gratitude to my supervisors Marja Tiirola and Jari Syväranta. I want to thank Marja for guiding me through the microbiological world and for encouraging me to follow and strengthen my own interests and for counting on me. I want to thank Jari for helping me when I needed it most and for widening your own research area to help me in addition to limnological parts. Especially I want to thank you both for giving me extremely good support at the end of the thesis.

I would also like to thank Roger Jones for letting me participate in his Mekkojärvi project and all the wise advice and support. I owe a lot to Antti Rissanen in many ways, for helping with the samples, data analysis, writing papers and for the never-ending willingness to help. Hannu Nykänen has guided me through the gas world and is thus acknowledged for that. I thank Lauri Arvola for introducing me with green sulphur bacteria *Chlorobium* and for guidance around the many Evo lakes. I would also like to thank all my collaborators and co-authors for their help and involvement in my thesis, especially Moritz Lehmann and the Tanzanian group. I thank my support group Antti R., Heikki Hämäläinen and Timo Huttula for keeping my timetable on track and the staff at the Lammi Biological Station are acknowledged for their valuable help in the field and lab. In addition thanks to Miika Sarpakunnas and Saara-Maria Haapala for your help and company at the sampling trips.

I have had a privilege to work with many people and within two research groups. I want to thank all the previous molecular microbiology-group members Sari, Sanna, Emilia, Anna, and Antti for all the work and non-work related help and for the guidance you have given me, and the present microbiology-group members Sanni, Anu, Anita, Kristiina, Sukhitar, Ville and Eveliina for sharing the everyday work and for supporting me till the end. Mekkojärvi group, Jussi, Jari and Shawn, has provided me with the best samples and the best company around this project, thank you for that. I also want to thank all the fellow students and the other personnel at the WET section and especially the coffee-break company for the refreshing conversations.

I am really grateful on having best friends around me, who support me on my work, but also give me other things to listen and to do. We have shared the best trips and weekends together. I want to thank you all, and your families.

In addition, I want to thank my parents for believing in me and for supporting me to follow my own dreams. I also thank my dearest sisters Jonna and Lotta for being in the same mental level with me that no one else can understand, and for Jonna bringing Aada and Aleksi to my life. I am privileged to have the best grandmothers and grandfather in my life, who shake their heads when listening to my newest ideas and whims, but still fill me with love. I also want to thank my parents-in-law and sister-in-law (with family) on all the support and interest they have had on my work.

Finally, I want to thank my Sampo who has given me the never ending support and shoulder to lean on. In addition, thank you for giving me all the energy, love, and happiness in my everyday life.

YHTEENVETO (RÉSUMÉ IN FINNISH)

Metsäjärvien kasvihuonekaasujen mikrobitasoinen säätely

Hiilidioksidin (CO_2), metaanin (CH_4) sekä dityppioksidin (N_2O) määrä ilmakehässä on noussut viime vuosisatojen aikana ihmistoiminnan seurauksena. Näitä kasvihuonekaasuja muodostuu ja myös käytetään luonnon omissa mikrobiologisissa prosesseissa niin maaperässä, vesistöissä kuin pohjasedimenteissä. Järvien on todettu olevan maailmanlaajuisesti kasvihuonekaasujen nettotuottajia, minkä vuoksi kasvihuonekaasuja kontrolloivien tekijöiden ymmärtäminen on erittäin tärkeää. Suomen tuhansista järvistä suurin osa luokitellaan humuspitoisiksi, mikä tarkoittaa, että ne sisältävät runsaasti liuennutta orgaanista ainetta ja ovat tummavetisiä. Tyypillistä on, että näihin pieniin tummavetisiin järviin muodostuu lämpötilakerrostuneisuus kesän aikana. Lämpötilakerrostuneisuudesta seuraa monia biologisia, kemiallisia sekä fysikaalisia ilmiöitä vesipatsaassa, kuten alusveden hapettomuus, ravinnepitoisuuksien suuret erot pinta- ja alusvedessä sekä kaasujen kerääntyminen alusveteen. Hapettomat olosuhteet sekä muut kerrostuneisuuden vaikutukset edesauttavat kasvihuonekaasujen tuotantoa.

Tässä tutkimuksessa selvitettiin metsäjärvien mikrobiyhteisöjen rakennetta, yhteisöjä sääteleviä tekijöitä sekä mikrobien säätelyä ja osuutta kasvihuonekaasujen tuotannossa. Tutkimukseen sisältyi niin järvien välistä vertailua kuin yhden järven kokeellista ravintoverkon muuttamista. Vesipatsaan ja pohjasedimentin mikrobiyhteisöjä sekä toiminnallisia mikrobiryhmiä kartoitettiin molekyylibiologisin menetelmin. Näiden lisäksi vesipatsaan kaasupitoisuudet mitattiin, jotta voitiin laskennallisesti arvioida kasvihuonekaasujen vapautumista tutkimusjärvistä.

Humuspitoisten järvien perustuotannon on oletettu olevan vähäistä alhaisten valo- ja ravinnepitoisuusolosuhteiden vuoksi, minkä seurauksena järvien yhteisöhengitys olisi suurempaa kuin perustuotanto ja järvet toimisivat hiilidioksidin lähteenä ilmakehään. Hapettomista olosuhteista löytyneiden yhteyttävien *Chlorobium*-sukuun kuuluvien vihreiden rikkibakteerien määrä kuitenkin osoittaa selvästi, että ne voivat muodostaa hapettomissakin olosuhteissa merkittävän hiilinielun. Valon määrä hapellisen ja hapettoman vesikerroksen rajalla vaikutti eniten järven soveltuvuuteen näiden mikrobien tehokkaaseen lisääntymiseen.

Pienen humuspitoisen Mekkojärve mikrobiyhteisön säätelijöitä sekä ravintoverkon kokeellisen muuttamisen vaikutuksia mikrobiyhteisön rakenteeseen ja bakteerisolujen määrään tutkittiin järvikokein. Eri syvyysvyöhykkeiden mikrobiyhteisöt erosivat selvästi toisistaan. Yleisesti yhteisöt koostuivat hyvin harvoista bakteerilajeista, joista merkittävimmät olivat e.m. *Chlorobium* sekä fysiologialtaan vielä tuntematon OD1 hapettomassa vesikerroksessa. Hapellisen ja hapettoman vyöhykkeen rajapinnasta löytyi myös suuria määriä metaania hapettavia bakteereita. Pieni lajimäärä sekä samojen yleisimpien bakteeriryhmien dominointi eri vuosina osoittaa, että pienen humusjärven hyvin rajoitettu

elinympäristö suosii vain tiettyjä lajeja. Planktonsyöjäkalojen lisääminen kalattomaan järveen vaikutti selvästi yleisimmän eläinplanktonryhmän, vesikirpun (*Daphnia* sp.), määrään. Vesikirput katosivat vesipatsaasta lähes kokonaan kalojen lisäämisen jälkeen. Vesikirpun on osoitettu käyttävän ravinnokseen mikrobeja, joten saalistuspaine mikrobiyhteisöön ennakko-oletuksen mukaisesti aleni kalojen lisäyksen ja vesikirpun häviämisen myötä. Tutkittaessa tarkemmin yksittäisen bakteeriryhmän, metaaninhapettajien, solumääriä havaittiin, että näiden bakteerien tiheys oli korkeampi sillä puolella järveä, johon kalat oli lisätty. Tämän lisäksi metaaninhapettajien määrä voitiin liittää havaittuihin pintakerroksen metaanipitoisuuksiin, jotka olivat huomattavasti alemmat sillä puolella järveä, jolla oli enemmän metaaninhapetukseen osallistuvia bakteereita. Laskennallisesti järvestä vapautuvan metaanin määrä oli kalattomalla alueella nelinkertainen kalalliseen verrattuna. Tässä työssä pystyttiin osoittamaan ensimmäistä kertaa kokeellisesti muutetun ravintoverkon vaikutusten ulottuvan planktonia syövistä kaloista aina mikrobitasolle asti, jossa mikrobit vaikuttavat koko järven biogeokemiallisiin prosesseihin ja järvestä vapautuvan haitallisen kasvihuonekaasun metaanin määriin. Vaikka mikrobien määrä lisääntyi, mikrobiyhteisön suhteellinen rakenne ei kuitenkaan muuttunut kalojen lisäämisen seurauksena.

Dityppioksidia (N_2O) muodostuu sivutuotteena denitrifikaatio-prosessissa, joka vähentää typpikuormaa vesistöissä pelkistämällä nitraattia typpikaasuksi. Funktionaalisia reduktaasigeenejä voidaan käyttää tunnistamaan denitrifikaatioon osallistuvien mikrobien osuus. Kolmentoista järven tutkimuksessa havaittiin typpiyhdisteiden pelkistämiseen liittyvien reduktaasi-geenien olevan yleisiä ja havaittavissa kaikissa tutkimusjärvissä. Tutkimuksessa havaittiin, että suurimassa osassa järvistä dityppioksidia muodostavien geenien määrä oli suurempi kuin sitä pelkistävien geenien, mikä osoittaa että havaittu dityppioksidin kertyminen vesipatsaaseen voidaan kytkeä geenien koodaamien prosessien epätasapainoon. Ilmaston lämpenemisen myötä sademäärän on arvioitu kasvavan, mikä saattaa lisätä valuma-alueilta tulevien ravinteiden määrää vesistöissä ja näin ollen myös typen määrää. Tulokset osoittivat, että denitrifioivat mikrobit pystyvät vastaamaan korkeampiin nitraattipitoisuuksiin kiihdyttämällä denitrifikaatioprosessia, mutta tulosten perusteella voidaan myös olettaa, että mikrobiyhteisö sopeutuu korkeampiin nitraattipitoisuuksiin tuottamalla suhteellisesti enemmän dityppioksidia. Denitrifioiva yhteisö koostui funktionaalisten geenien perusteella vain muutamista keskeisistä bakteeriryhmistä, jotka kuuluivat proteobakteereiden pääjaksoon.

Tutkimuksessa havaittiin, että sekä kalojen määrä, metaania hapettavien bakteerien ja vihreiden rikkibakteereiden runsaus että myös denitrifioivien mikrobiyhteistöjen toiminta säätelee kasvihuonekaasupäästöjä. Tärkein näitä kaikkia kontrolloiva ympäristötekijä on vesistön happiprofiili. Tulevaisuuden ilmastomuutoksen aiheuttamat muutokset tuhansien metsäjärvien ja metsälammikoiden happiprofiileissa vaikuttavat siis hyvin merkittävästi siihen, kuinka kasvihuonekaasujen nettotuotanto näissä järvissä muuttuu.

SAMMANDRAG (RÉSUMÉ IN SWEDISH)

Mikrobiologisk kontrollering av skogssjöars växthusgasproduktion

Växthusgaser koldioxid (CO_2), metan (CH_4) och dikväveoksid (N_2O) koncentrationerna har ökat i atmosfären på grund av mänskliga åtgärder. De här växthusgaserna produceras också och används i naturens egna mikrobiologiska processer både i jord, i sediment och i vattendrag. Internationellt är sjöarna märkta att vara nettoproducent av växthusgaser. Därför är det mycket viktigt att förstå de kontrollerade faktorerna bakom processerna, som producerar växthusgaser. Den största delen av Finlands tusen sjöar är klassificerade som humushaltiga, vilket betyder att de innehåller stora mängder av upplöst kol och sjövattnet är mörkfärgat. Det är typiskt att de här små mörkfärgade sjöarna temperaturstratifierades under sommaren. Temperaturstratifiering är en följd av många biologiska, kemiska och fysikaliska förändringar i vattenpelare, såsom oxygenbristning i hypolimnion, stor skillnad i näringsämne koncentrationer mellan epilimnion och hypolimnion, och ansamling av gaser i hypolimnion. Oxygenfattigt tillstånd och andra faktorer som följer stratifiering bidrar till producerande av växthusgaser.

I denna undersökning klargjordes boreala skogssjöars mikrobiologiska gruppers struktur och reglering samt andel av växthusgasproduktionen. Forskningen innehöll både jämförelse mellan många sjöar och experimental förändring av näringsväv i en sjö. Molekylärbiologiska metoder användes för att studera mikrobiologiska grupper från vatten och sediment tillsammans med forskning av funktionella mikrobiologiska grupper. Härutöver mättes gaskoncentrationer från vattenpelare, för att kunna räkna mängden av växthusgaser som befrias från sjön.

Primärproduktion i humushaltiga sjöar är tänkt att vara lågt på grund av dåliga ljus- och näringsämne omständigheter, vilket betyder att samfund respiration är större än assimilering och sjöarna är källan till koldioxid (CO_2). Från syrefria förhållanden hittades stora mängder assimilerande gröna svavelbakterier, som hör till *Chlorobium*-släkten, vilket bevisar att de kan utforma kolsänkan i syrefria förhållanden, vilket inte har beaktats tidigare. Ljuskvantitet fastställdes vara den viktigaste faktor som definierade om sjön var lämplig livsmiljön för de här bakterierna eller inte.

Den lilla humushaltiga sjöns (Mekkojärvi) mikrober undersöktes under tre år. Experimentalt förändrade näringsvävseffekter i bakteriernas antal och struktur examinerades. Mikrobiologiska grupper var klart avskilda mellan olika djuplager. Generellt består det hela mikrobiologiska samfundet av bara få olika bakteriearter, av vilka den viktigaste var tidigare nämnd *Chlorobium* och inte så väl kända OD1 i den syrefattiga hypolimnion. Från gränsytan mellan syrerikt och syrefattigt vatten fanns det stora mängder av metanotrofer som oxiderar metan. Låga mängder av olika bakteriegrupper och dominerande av samma mikrobiologiska grupper varje år, bevisar att den strikt levande miljön i humushaltig sjö kan stöda bara några viktiga mikrobiologiska grupper. Tillförning

av planktonätande fiskar i sjön, var det naturligt inte fanns fiskar, hade klar påverkning på antalet av djurplankton *Daphnia*. Antalet av *Daphnia* föll till ner nära noll efter fisktillförningen. Tidigare har det påvisats att *Daphnia* använder mikrober som sin näring och därför antogs att jakttryckt mot mikrobier var förminskad efter fisktillförning och förminskning av *Daphnia*. I närmare forskning av den enskilda gruppen, metanotrofer, kunde man se att mängden av de här bakterierna var högre på den sida av sjön vart fisken hade tillförts. Mängden av metanotrofer kunde därefter kopplas till metan koncentrationer i epilimnion, vilket var betydligt lägre på den sida varifrån mer metanoxiderande bakterier hade hittats. Kalkylerad mängd av metan, som befriats från sjön var en fjärdedel på den sida vart fisken hade tillförts jämfört med den fisklösa sidan. För första gången kunde i denna forskning påvisas att experimental förändring i näringsväv hade påverkning från planktonätande fiskar ända till mikrobiologisk nivå, var effekten i biogeokemiska processer kunde påvisas och i mängden av befriade växthusgasmetan från sjön.

Dikväveoksid (N_2O) är producerats som biprodukt i denitrifikation process, vilket är en ekologist viktigt process som förminskar kvävelast i vattendrag genom renodling av nitrat till kvävgas. I en forskning som omfattat över 13 sjöar kunde man se att funktionella gener, som kan användas att känna till mikrober som deltar i denitrifikation, är allmänna och de hittades i alla undersökta sjöar. Mängden av dikväveoksid producerat i denitrifikation beror på relation mellan processer som produceras och använder den. I största delen av sjöarna var mängden av mikrober som producerar dikväveoksid större än vad mängden av de som använder och reducerar den, vilket påvisar att observerad dikväveoksid samling i vattenpelare kunde kopplas till obalans mellan kodande gener. På grund av uppvärmning av klimatet är det också beräknat att regnmängden kommer att öka, vilket kan öka mängden av näringsämnen från avrinningsområdet till vattendrag och följaktligen öka koncentrationen av kväve. Resultat påvisar att denitrifierande mikrober kan bemöta högre nitrathalt genom att utföra snabbare denitrifikationsprocess, men det var också sett att mikrobiologiskt samfund var anpassat i den högre nitrathalt med anledning av proportionellt högre dikväveoksid produktion.

Som slutsats kunde man se att fiskarna antal, metanoxiderande bakterier, gröna svavelbakterier och denitrifierande bakterier alla kontrollerade växthusgas befriande från sjöarna. Den viktigaste faktorn var den syreprofil som hittats från vattenpelare. Hur framtidens klimatförändringar påverkar syreprofilerna i de tusen skogssjöarna och träsken är mycket viktig för skogssjöarnas växthusgasproduktion.

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ORIGINAL PAPERS

I

GREEN SULPHUR BACTERIA AS A COMPONENT OF THE PHOTOSYNTHETIC PLANKTON COMMUNITY IN SMALL DIMICTIC HUMIC LAKES WITH AN ANOXIC HYPOLIMNION.

by

Jatta Karhunen, Lauri Arvola, Sari Peura & Marja Tiirola 2013

Aquatic Microbial Ecology 68: 267–272.

II

GENETIC AND ENVIRONMENTAL FACTORS CONTROLLING NITROUS OXIDE ACCUMULATION IN LAKES

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III

FUNCTIONAL GENE PYROSEQUENCING INDICATES THAT DENITRIFICATION IS DRIVEN BY A FEW CORE PROTEOBACTERIAL POPULATIONS IN BOREAL LAKES

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IV

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V

INFLUENCE OF A TROPHIC CASCADE ON THE BACTERIAL COMMUNITY IN A WHOLE-LAKE MANIPULATION

by

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