

Sari Peura

Bacterial Communities  
in Stratified Humic Lakes



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*Dedicated to Venla, Oskari, Eemeli, Senni and Fanni.*

## ABSTRACT

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Yhteenveto: Humuspitoisten metsäjärvien mikrobiyhteisöt

Diss.

Humic lakes are a prominent feature of the boreal landscape and have a substantial role in influencing the future climate, as many of them are net sources of greenhouse gases (GHG) to the atmosphere. Carbon concentrations in humic lakes and other surface waters have been observed to increase during the past two decades. Even though the bacteria play a crucial role in nutrient cycles and redistribution of this organic material, microbial communities in humic lakes largely remain to be explored. For this thesis bacterial communities in humic boreal lakes were studied in their natural state and further, community responses to increased organic carbon were addressed in a whole-lake study and to increased nutrients in a microcosm experiment. Seasonal dynamics and responses of bacterial community to carbon and nutrient amendments were explored using 454 pyrosequencing. Compared to older methods, this new fast and cost effective sequencing technique enables much more detailed analysis of bacterial community composition. The results revealed that bacterial communities in humic lakes differ markedly from their clearwater counterparts and that these lakes harbour poorly known bacterial phyla. Typical community members in the epilimnion were *Actinobacteria* and *Betaproteobacteria*, while the hypolimnetic communities were dominated by Candidate division OD1, *Chlorobia* and *Bacteroidetes*. Network analysis suggested distinct ecological properties for suboxic hypolimnetic communities. Furthermore, the carbon and nutrient amendments showed that changes in community composition cannot be explained or predicted based on current knowledge of bacterial ecology. Thus, to reveal the functions and significance of bacterial communities in the ecological processes and metabolism of humic lakes, more information must be gathered taking advantage of recent advances in molecular microbiology. The data presented in this thesis increase our knowledge of these communities and represent a first step along a road to disentangle the bacterial community functions in these unique environments.

Keywords: 454 pyrosequencing; bacterial community; carbon cycle; greenhouse gas; humic lake; nutrients.

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## 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-IV.

I did the molecular laboratory work for all papers. I planned and conducted the experiments in II together with Hannu Nykänen. The experiments in III and IV were planned by Roger Jones and Paula Kankaala; of these the former was conducted by Paula Kankaala and I conducted the latter together with Hannu Nykänen. 454 pyrosequencing data were analysed with Alexander Eiler. I was responsible for writing I, II and IV. Paper III was written jointly with Paula Kankaala. All papers were finished together with all co-authors.

- I Peura S., Eiler A., Bertilsson S., Nykänen H., Tiirola M. & Jones R.I. 2012. Distinct and diverse anaerobic bacterial communities in boreal lakes dominated by candidate division OD1. *ISME Journal*. doi: 10.1038/ismej.2012.21
- II Peura S., Eiler A., Hiltunen M., Nykänen H., Tiirola M. & Jones R.I. 2012. Bacterial and phytoplankton responses to nutrient amendments in a boreal lake at different taxonomic resolutions. Submitted manuscript.
- III Kankaala P., Peura S., Nykänen H., Sonninen E., Taipale S., Tiirola M., Jones R.I. 2010. Impacts of added dissolved organic carbon on boreal freshwater pelagic metabolism and food webs in mesocosm experiments. *Fundamental and Applied Limnology* 177: 161–176.
- IV Peura S., Kankaala P., Nykänen H., Eiler A., Tiirola M., Jones R.I. 2012. Effects of a sustained increase in DOC loading to a small boreal lake. Manuscript.

## ABBREVIATIONS

bp	base pair
DIC	dissolved inorganic carbon
DOC	dissolved organic carbon
DOM	dissolved organic matter
GHG	greenhouse gas
LH-PCR	length heterogeneity analysis of PCR amplified 16S rRNA genes
LSA	local similarity analysis
Mb	megabases
NMDS	non-metric multidimensional scaling
OTU	operational taxonomic unit
POC	particulate organic carbon
POM	particulate organic matter
rRNA	ribosomal RNA

# 1 INTRODUCTION

## 1.1 Humic lakes

Small lakes located in forests and peatlands are typical of the boreal landscape. In Finland alone there are over 130 000 lakes smaller than 1 ha, which represents approximately 70 % of all Finnish lakes (Suomen ympäristökeskus 2011). Many of these small boreal lakes are characterized by heavy loadings of detrital organic matter (humus) originating from production by terrestrial plants within the surrounding catchment area (allochthonous production). Indeed, as many as 93 % of Finnish lake are considered to be humic (Kortelainen 1993), that is to have a high concentration of dissolved organic carbon (DOC) in the water column. Most of the humic material entering the lake is high-molecular weight, recalcitrant carbon compounds but approximately 15 % of the carbon has been estimated to be in labile compounds (Tranvik 1998). This allochthonous carbon, and especially the labile fraction, can be used as an energy subsidy for heterotrophic bacteria (Jones 1992) and bacterial respiration in humic lakes is often very high (Hessen 1992). In addition, the high DOC concentration results in distinctive, very dark watercolour in these lakes, which restricts the primary production in the lakes to a relatively thin surface layer. The combination of enhanced bacterial respiration and constrained primary production means that in very many of these lakes bacterial respiration exceeds primary production making them net heterotrophic (e.g. Cole et al. 1994, del Giorgio & Peters 1994, Kankaala et al. 1996, Jones et al. 2001). Ultimately this means that these lakes are net emitters of greenhouse gases (GHG), especially carbon dioxide (CO<sub>2</sub>) and methane (CH<sub>4</sub>) to the atmosphere (Kankaala et al. 2006). Thus, even though the area of these lakes might not be substantial, they may have a significant role in the global carbon cycle (Eloranta 1999). In fact, the global annual emissions of carbon dioxide from inland waters to the atmosphere are similar in magnitude to the carbon dioxide uptake by the oceans while the global burial of organic carbon in inland water sediments exceeds organic carbon sequestration on the ocean floor (Tranvik et al. 2009).

Besides restricting primary production, the dark watercolour of humic lakes has other consequences. In the spring, after ice break, dark water warms rapidly from the surface and consequently a humic lake more quickly stratifies to layers with differing environmental conditions. The upper layer (epilimnion) remains oxic and is characterized by low nutrient concentrations, while the bottom layer (hypolimnion) turns suboxic or even anoxic and nutrient concentrations are much higher. Between these layers is a transition layer (metalimnion). The most typical stratification type in northern latitudes is considered to be dimictic lakes. In these lakes the stratification is disturbed by spring and autumn overturns, when the whole water mass is mixed and even the bottom layer becomes oxic, while in the upper layer the nutrient concentrations increase. Some shallow and exposed humic lakes are holomictic, with no stratification developing during the summer season. However, in some lakes full mixing at either spring or autumn overturn (or both) may be absent with the temperature pattern rapidly transformed between summer and winter stratification. In these partly or totally meromictic lakes the lower hypolimnion may remain anoxic even for several years.

In stratified lakes the division to oxic and anoxic compartments has a profound impact on the fate of carbon. DOC “mobilization” for the food web via a “microbial loop” or “microbial link” (Jones 1992) recycles carbon that otherwise would be flushed from the lake or perhaps stored in the lake sediment. However, following its integration into the food web, the carbon re-enters the active carbon cycle and might end up in the atmosphere as CO<sub>2</sub> or CH<sub>4</sub> emissions from the lake. When carbon is incorporated into biomass in an oxic environment, the main by-product is CO<sub>2</sub>, while in anoxic environments CH<sub>4</sub> is produced along with CO<sub>2</sub>. This difference between oxic and anoxic metabolisms is also fundamental for climate, as CH<sub>4</sub> is 20 times more efficient as a greenhouse gas than CO<sub>2</sub> (IPCC 2007), and accounts for over 20 % of the greenhouse effect in the atmosphere (Gauci et al. 2004). Energy mobilization from allochthonous DOC and virtually all processes producing or consuming greenhouse gases are microbially-mediated (Cole et al. 1994). Thus, microbial communities have a key role in determining whether these lakes are carbon sinks or sources in the global carbon cycle.

## 1.2 Bacteria and food webs in humic lakes

The particular importance of the microbial link in humic lakes for the transfer of energy stored in allochthonous DOC was recognized only some two to three decades ago when it became apparent that dissolved organic matter (DOM) including humic substances provides an important carbon source for pelagic bacteria (Jones 1992, Tranvik 1992). It was also suggested that the relative importance of the microbial loop is greater in humic lakes than in clearwater systems (Jones 1992), due to diminished photosynthetic capacity of humic lake phytoplankton (Ask et al. 2009). Since then it has become clear that aquatic bacteria drive transformations and the cycling of most biologically active

elements in freshwater ecosystems (Newton et al. 2011). Heterotrophic bacteria contribute to the cycles of nutrients and carbon in 2 major ways: by producing new bacterial biomass (secondary production) and by re-mineralizing organic carbon and nutrients (del Giorgio & Cole 1998). Bacterial growth efficiency may vary significantly and more than 90 % of energy derived from organic substrates may be lost in respiration (Smith & Prairie 2004). In fact, bacterial respiration is the major component of total respiration in most aquatic systems, so that changes in bacterial respiration have profound effects on the overall carbon and gas balance in aquatic ecosystems (del Giorgio & Cole 1998). It has been suggested that the main factor differentiating clearwater and humic lake ecosystems is the carbon itself, rather than other effects of humic substances, such as reduced light penetration (Blomqvist et al. 2001). While in clearwater lakes bacterial production is thought to be mainly limited by primary production (Blomqvist et al. 2001), in humic lakes the bacteria can be limited by phosphorus (Jansson et al. 1996), DOC or both (Vrede 2005). Unproductive lakes are especially sensitive to input of allochthonous DOM through its negative effects on the light climate and the concomitant repression of benthic primary production (Ask et al. 2009).

Along with energy mobilization from allochthonous DOC, virtually all processes producing or consuming greenhouse gases are microbially-mediated (Cole et al. 1994). In the epilimnion bacterial metabolism, while incorporating carbon compounds into biomass, is producing  $\text{CO}_2$  and consuming  $\text{O}_2$ , while the phytoplankton is consuming  $\text{CO}_2$  and producing  $\text{O}_2$ . Thus, part of the  $\text{CO}_2$  produced by bacteria can be incorporated into the phytoplankton biomass and emissions from the lake are then diminished. Upon cell death this carbon incorporated to the phytoplankton biomass may then be buried to the lake sediment, even though some of it is again degraded and adds to the potential  $\text{CO}_2$  emissions. The lake type has an evident impact on the amount of emitted  $\text{CO}_2$ , as the light extinction depth and thus the depth of the layer where phytoplankton can photosynthesize is highly dependent on the watercolour and shading. In the anoxic conditions of the hypolimnion, the end products of microbial metabolism include  $\text{CH}_4$  along with  $\text{CO}_2$ . The produced  $\text{CH}_4$  may then be either emitted to the atmosphere or oxidized by methane-oxidizing bacteria (methanotrophs) and hence incorporated to the microbial biomass. Thus, methanotrophs can be an important food source for zooplankton in humic lakes (Bastviken et al. 2003, Jones & Grey 2011, Taipale et al. 2011) and a gateway of DOC to higher trophic levels (Bastviken et al. 2003, Taipale et al. 2008), even though some opposing reports on the importance of methanotrophs also exist (Jones & Lennon 2009). Bacterial methane oxidation in freshwater lakes has been mainly assumed to occur in the presence of  $\text{O}_2$  (Rudd et al. 1976, Fallon et al. 1980, Kuivila et al. 1988, Frenzel et al. 1990, Bastviken et al. 2002, Kankaala et al. 2006), often in the oxic/anoxic interface. Until recently anaerobic methane oxidation was thought to occur only in saline environments (Joye et al. 1999, Valentine 2002) by archaea in a consortium with sulphate-reducing bacteria (Boetius et al. 2000). However, anaerobic methane oxidation has now been reported in freshwater lakes (Liikanen et al. 2002, Eller et al. 2005,

Bastviken et al. 2008, Biderre-Petit et al. 2011), although its overall importance to the freshwater methane cycle remains uncertain.

While in clearwater lakes autotrophic energy mobilization in the benthic habitat has been suggested to dominate, in humic lakes energy mobilization is usually governed by heterotrophic metabolism in pelagic habitats (Ask et al. 2009). An exception to this may be humic lakes with an exceptionally shallow oxic layer. In these lakes an appreciable proportion of energy may be derived from autotrophic green sulphur bacteria (Taipale et al. 2009, 2011), thus shifting the whole lake towards net autotrophy. Still in most humic lakes the energy is essentially derived from allochthonous DOC. It should be noticed that the DOC concentration in inland waters in the boreal region has been reported to have increased during the recent decades (Hongve et al. 2004, Vuorenmaa et al. 2006, De Wit et al. 2007, Hruska et al. 2009, Weyhenmeyer & Karlson 2009). Considering the crucial role of allochthonous organic carbon in determining the net metabolism and also magnitude of gas emissions from lakes (Hanson et al. 2003), the impact of any increase in lake carbon concentration may have global consequences. However, experimental tests of the possible effects of increased loading of organic carbon on lake ecosystems are still scarce, particularly at the realistic whole-lake scale.

The impact of increased organic carbon on the foodweb of a humic lake is rather challenging to study, as it requires the separation of the direct carbon effect from the impact of light availability (Carpenter et al. 1998, Jones 1992). Addition of coloured humic substances to a lake changes the light climate and even though the direct impact of decreased light on the bacterial community is minimal, the photosynthetic capacity of the phytoplankton community decreases with increasing watercolour (Jones 1992, Drakare et al. 2003). As the bacterial community is linked to the phytoplankton community (Weisse et al. 1990, Sundh & Bell 1992, Jansson 1998, Kent et al. 2006), any decrease in the algal photosynthetic capacity will affect the bacterial community as well. To decouple these impacts, it is necessary to increase the carbon availability without changing the watercolour. Previously this has been done by the use of colourless carbon sources, such as sugar compounds (e.g. Blomqvist et al. 2001, Drakare et al. 2002, Kamjunke et al. 2008, Stets and Cotner 2008, Hitchcock et al. 2010, Faithfull et al. 2011). Even though these compounds are not comparable with the complex carbon substances in natural aquatic ecosystems, their use may be justified to eliminate the change in light climate. Thus, the use of a colourless source of organic carbon allows the assessment of carbon impact *per se*.

## 1.3 Bacterial communities

### 1.3.1 Community composition

The composition of bacterial communities from oxic freshwater habitats has been studied extensively during the past decade (Zwart et al. 2002, Newton et

al. 2011). The first rigorous comparison of 16S rRNA gene sequences retrieved from freshwater, marine and soil environments by Zwart et al. (2002) showed that the 689 sequences from freshwater habitats from different continents were most closely related to each other while relatively few were closest to sequences recovered from soils or marine habitats. They therefore concluded that these freshwater clusters represent species or groups of species that are indigenous to freshwater and that rivers and lakes therefore have a specific bacterial community distinct from bacteria in neighbouring environments such as soil and sediments. According to this freshwater grouping, the putative freshwater clusters were found among the *Alpha*-, *Beta*- and *Gammaproteobacteria*, the *Cytophaga-Flavobacterium-Bacteroides* group (currently known as *Bacteroidetes*), the *Cyanobacteria*, the *Actinobacteria*, the *Verrucomicrobia*, the green non-sulphur bacteria (currently known as *Chloroflexi*) and candidate division OP10 (Zwart et al. 2002). Of those, *Gammaproteobacteria* is currently associated with saline environments (Zwart et al. 2002, Wu et al. 2006) rather than with freshwater lakes and they are considered to be only transient members of bacterial communities in lakes (Newton et al. 2011). Another classification of typical freshwater bacteria was presented recently by Newton et al. (2011) based on 12 000 16S rRNA gene sequences. They summarised the main phyla in epilimnetic freshwater microbial communities as being *Proteobacteria* (especially *Betaproteobacteria*), *Actinobacteria*, *Bacteroidetes*, *Cyanobacteria* and *Verrucomicrobia*. The last of these has recently been suggested to be generally underrepresented in studies as the phylum is biased against some commonly used primers (Bergmann et al. 2011).

However, as stated above, humic lakes are distinct environments among freshwater lakes and they can be expected to harbour microbial populations different from their clearwater counterparts. To date, bacterial community structure in humic lakes has received much less attention than clearwater communities, and the studies have often been done with methods that do not give specific phylogenetic data (Lindström 2000, Yannarell et al. 2003, Kent et al. 2004, Shade et al. 2008). Furthermore, the few studies including phylogenetic data only comprise the oxic layer of the lake (Burkert et al. 2003, Newton et al. 2006, Grossart et al. 2008, Chróst et al. 2009) or the analysis has been done from samples pooled from the whole water column (Haukka et al. 2005). In the oxic layer of humic lakes the main bacterial groups have been shown to be *Actinobacteria* (Lindström & Leskinen 2002, Burkert et al. 2003, Haukka et al. 2005, Newton et al. 2006, Taipale et al. 2009) and *Betaproteobacteria* (Burkert et al. 2003, Grossart et al. 2008, Chróst et al. 2009, Taipale et al. 2009), but there are some reports also of *Verrucomicrobia* as one of the most abundant bacterial groups (Haukka et al. 2005, Arnds et al. 2010). However, to my knowledge only one study has so far addressed the bacterial community composition in the anoxic hypolimnion using phylogenetic methods (Taipale et al. 2009), according to which in the suboxic hypolimnion of a small humic lake, the main phylum was *Chlorobia*. Thus, data concerning the suboxic or anoxic bacterial communities in humic lakes are extremely scarce.



### 1.3.2 Impact of DOC and nutrients

Bacterial communities in clearwater as well as humic lakes have been reported to change with season (Crump et al. 2003, Yannarell et al. 2003, Kent et al. 2004, Newton et al. 2006, Newton & McMahon 2011, Eiler et al. 2012). This seasonal succession has been suggested to be driven by bacteria-phytoplankton interactions (van Hannen et al. 1999, Crump et al. 2003, Kent et al. 2007) as well as other environmental factors, such as pulses of allochthonous DOM (Crump et al. 2003, Kent et al. 2004). Experimental evidence suggests that in bacterial communities derived from a clearwater lake the proportion of *Betaproteobacteria* increases with increasing carbon availability (Hutalle-Schmelzer & Grossart 2009) and the same has been reported for communities from a humic lake (Burkert et al. 2003, Hutalle-Schmelzer et al. 2010). Furthermore, in a lake divided to compartments with differing DOC concentration, *Betaproteobacteria* were found to be more abundant in the compartment with higher DOC concentration (Burkert et al. 2003). Thus, it seems that *Betaproteobacteria* prefer environments with high DOC concentration. Similarly, members of *Bacteroidetes* have been found to prefer high DOC concentration (Eiler et al. 2003, Hutalle-Schmelzer et al. 2010). In contrast, the *Actinobacteria* do not appear to gain a similar benefit when the DOC concentration increases; in the same divided lake mentioned earlier the *Actinobacteria* were evenly distributed between compartments (Burkert et al. 2003), while in enrichment experiments the relative abundance of *Actinobacteria* has been found to decline or remain stable with increasing DOC concentration (Burkert et al. 2003, Hutalle-Schmelzer et al. 2010). Experimental results for *Alphaproteobacteria* are conflicting, as it has been related to low DOC concentration (Eiler et al. 2003) but also reported to benefit from increase in DOM concentration (Hutalle-Schmelzer et al. 2010).

Nutrients and resource limitation may also significantly shape the bacterial community. In lake ecosystems the elements most frequently considered to limit production are carbon or phosphorus for bacteria and phosphorus or nitrogen for phytoplankton (Hessen et al. 1994, Jansson et al. 1996, Brett et al. 1999, Vrede 2005, Bergström et al. 2008). Still there is a wide variation in nutrient requirements between organisms and, although functional differentiation within bacterial taxa has been recognized (Schauer et al. 2005, Allgaier et al. 2007, Jezbera et al. 2011), available techniques have not previously enabled detailed resolution of bacterial populations. Hence conclusions assessing impacts of carbon and nutrients have been drawn for rather broad and diverse groups; for example, as stated above, claims have been made that *Actinobacteria* decrease or are non-responsive following carbon amendment (Burkert et al. 2003, Hutalle-Schmelzer et al. 2010) and that *Betaproteobacteria* respond to ammonium but not to nitrate (Barlett & Leff 2010). However, taxa within phylum level groups are not necessarily functionally or metabolically coherent and individual communities might be limited by different resources than the total community (Danger et al. 2008). When even bacteria closely related to each other may differ markedly in their elemental composition and nutrient limitation, the response of any bacterial community will be highly dependent on the individual populations present within the community.

Therefore, as the studies regarding alphaproteobacterial carbon responses hint, conclusions drawn for high taxonomic levels (e.g. phylum-level) may be conflicting or even misleading.

### 1.3.3 Identification of bacterial communities

While macro-organisms may be identified relatively easily by visual inspection, microorganisms are not identifiable by their appearance (Amann et al. 1995). Consequently the microbial communities in various environments were for a long time “black boxes” as there were no means to identify the species present in the communities. Indeed, it is apparent that “species” as they are defined for macro-organisms do not even exist for bacteria, which are asexual organisms exchanging genes in unique and unusual ways, such as through bacteriophages (Cole et al. 2010). This makes the species concept blurred, and thus in microbial ecology those organisms sharing a certain proportion of phylogenetic similarity are today often referred to as Operational Taxonomic Units or OTUs, rather than as species. OTUs are constructed from sequence libraries based on the similarity in the DNA sequence. If similarity between 2 sequences is over 97 %, OTU comprised of these sequences may be regarded as bacterial “species”, while 95 % similarity may be regarded as equivalent to genus level and 80 % similarity as equivalent to phylum level. Here OTUs are constructed based on their 16S rRNA genes, but elsewhere OTUs have also been constructed based on various functional genes, such as genes related to nitrification (Fortunato et al. 2009).

The identification and classification of microbial species and later OTUs has gone through enormous changes during the past 30 years (Fig. 1). Modern DNA sequencing was originally developed already in the 1970s (Sanger et al. 1977) and the marker molecules in molecular microbiology were chosen to be 16S rRNA genes (Woese & Fox 1977). Nevertheless, until the 1990s the identification of bacteria was still mainly based on the isolation of pure cultures (or defined co-cultures) followed by testing for multiple physiological and biochemical traits. Even though it was early on acknowledged that only a minor fraction of the whole community was growing in laboratory conditions (Jannash & Jones 1959) and viable but non-culturable cells were first recognized as early as the late 1800s (Roszak & Colwell 1987), culture-based surveys from various environments still lead researchers to believe that the organisms inhabiting soil and aquatic environments were quite similar (Newton et al. 2011). The first entire microbial genome (*Haemophilus influenzae*) was published in the 1990s beginning the genomic era (Fleischmann et al. 1995). Cloning of bacterial sequences from environmental samples then gave access to enormous bacterial diversity, of which 95–99 % has been estimated to be non-culturable with known methods (Amann et al. 1995). The first collection of 16S rRNA genes (708 sequences) was released in 1992 and it formed the backbone for the Ribosomal Database Project (Maidak et al. 1994; <http://rdp.cme.msu.edu/>), which aims to help researchers classify 16S rRNA sequences. Besides cloning, another fast method to identify bacteria from environmental samples was fluorescence *in situ* hybridization (FISH) (DeLong et al. 1989). This also

facilitated the estimation of abundance of individual taxa in the environment. Further, the development of PCR (Mullis & Faloona 1987) enabled the use of various fingerprinting methods (e.g. DGGE, LH-PCR, t-RFLP, reviewed by Grant & Ogilvie 2004) to overcome the quantitative limitation related to sequencing of clone libraries. Even though only some of these methods give phylogenetic information (e.g. LH-PCR, Taipale et al. 2008; t-RFLP, Morales et al. 2006), they offer a fast and cost effective comparison of bacterial communities between different environments and the possibility to track changes in community composition following perturbations (Amann et al. 1995). However, studies of freshwater bacterial communities remained largely based on culturing methods even in the late 1990s. Since then the sequencing of clone libraries has become more accessible and the first classification of freshwater bacteria was published in 2002, including 689 sequences derived from various freshwater habitats (Zwart et al. 2002).

The development of next generation sequencing methods was initiated already in the mid 1990s (Nelson et al. 2010) and in 2005 Margulies et al. (2005) published the first paper using 454 pyrosequencing. Pyrosequencing, as well as other next generation sequencing methods (e.g. Illumina, Gunderson et al. 2004; SOLiD, Barski et al. 2007; BacBio, Korlach et al. 2010; Ion Torrent, Rothberg et al. 2011) frees the sequencing from the laborious and time consuming cloning step and costs are also considerably lower (typically around 1500 €/Mb for Sanger vs. 12.4 €/Mb for 454 pyrosequencing with Titanium chemistry, Glenn 2011). In 454 pyrosequencing of 16S rRNA genes the samples are first amplified with PCR using primers carrying an adaptor sequence and in multiplexing additionally also sample specific barcodes. Then amplicons are bound to beads under conditions that favour one fragment per bead and the beads are captured in the droplets of a PCR-reaction-mixture-in-oil emulsion. PCR amplification takes place within each droplet, resulting in beads carrying 10 million copies of a unique DNA template. Beads carrying DNA clones are deposited into wells of a fibre-optic slide, each of the 1 000 000 wells including only a single bead. Thus, sequencing of the slide produces 1 million sequences in a single run. The first published 454 pyrosequencing run yielded almost 500 000 sequences with an average length of 108 bases. As well as number of sequences, also the length of the sequences has steadily increased, the maximum length with pyrosequencing currently being 1 000 bases/sequence. Thus since I began analysis for this thesis the number of DNA sequences has increased from approximately 100 to 4000 sequences per sample and from 1 000 to 1 000 000 sequences per library!

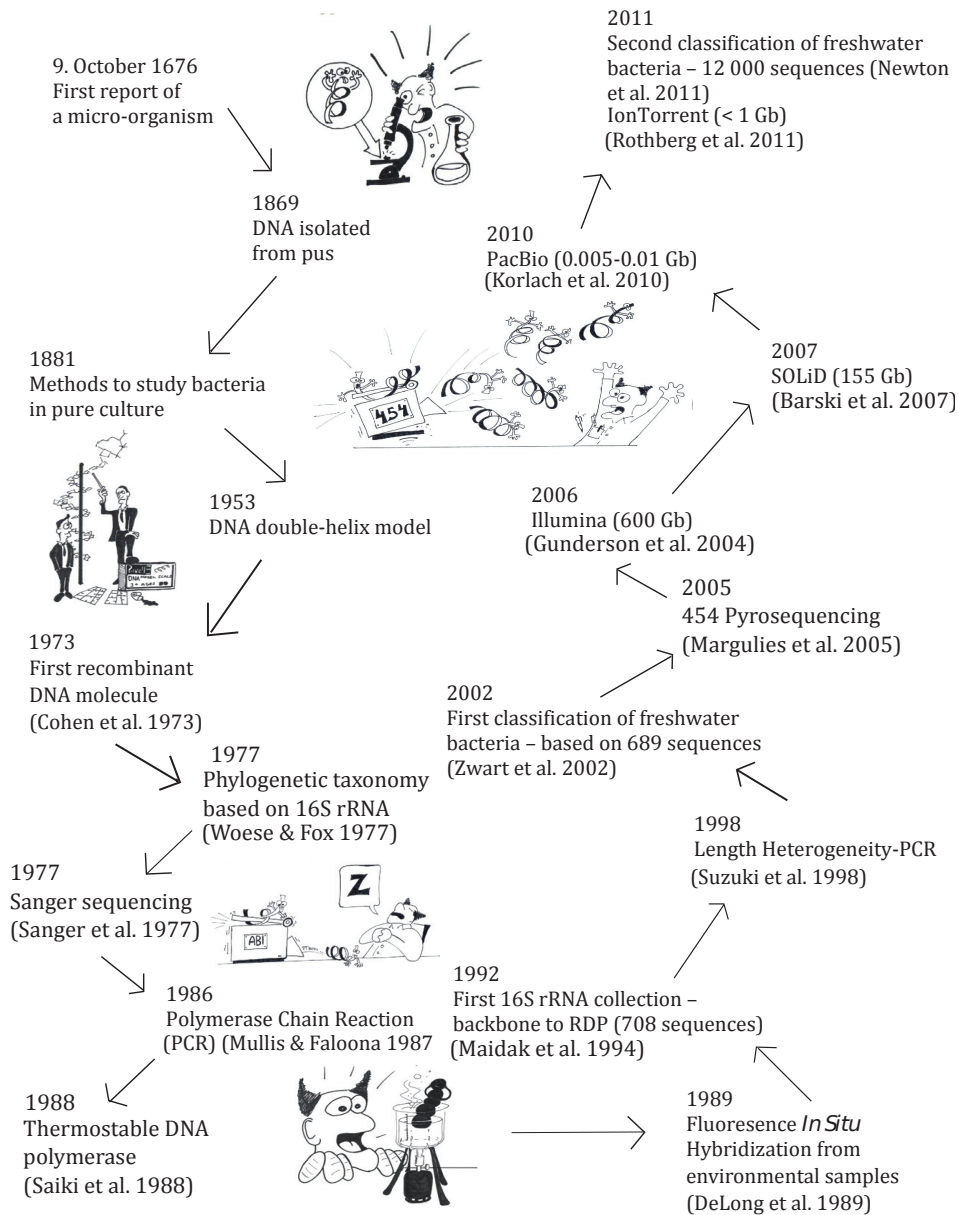


FIGURE 1 Short history of bacterial identification (© 2012 Jussi Peura).

## 1.4 Aims

The aim of this thesis was to characterize the microbial community composition in the different depth layers of a humic lake, and to elucidate the impact of carbon and nutrients on bacterial communities and hence on lake metabolism within seasonal and annual timescale. The hypotheses for the study were:

- 1) composition and functions of microbial communities in a stratified lake are layer-specific,
- 2) bacteria and phytoplankton communities are quantitatively and structurally affected by changes in the C:N:P stoichiometry, and
- 3) external increase of labile DOC has a specific impact on the whole-lake metabolism whereby it
  - i) enhances GHG emissions to the atmosphere (IV) and
  - ii) increases bacterial production while primary production remains unaffected or even decreases (III, IV).

These hypotheses were addressed mainly in a small humic lake, Alinen Mustajärvi, but data from 4 other lakes representing a DOC gradient were also included to extend the findings to related lakes. The bacterial community composition in these lakes was studied at a high taxonomic resolution to reveal the temporal and spatial variability between lakes and depth layers (I). The nutrient-mediated changes to bacteria and phytoplankton were elucidated with 3 experimental setups with water from lake Alinen Mustajärvi (II). Carbon flux to the atmosphere, impact of increased DOC on bacteria and phytoplankton together with usability of cane sugar as a tracer in boreal environment were addressed with a whole lake experiment (IV). The stable isotope tracing of carbon flow through bacterioplankton to phyto- and zooplankton was studied in 2 experiments in mesocosms and at a whole lake scale (III, IV).

Sequencing and environmental data (including isotopic data) were combined to explore the potential functions of poorly known bacterial groups in Alinen Mustajärvi using local similarity analysis (LSA) (I). The 454 pyrosequencing also allowed the estimation of the impact of different levels of OTU-resolution on the nutrient-mediated changes in bacterial community (II). Similarly, 454 pyrosequencing enabled elucidation of the fine scale changes and ecologically coherent groups related to the experimental increase in organic carbon concentration in Alinen Mustajärvi (IV).

## 2 MATERIALS AND METHODS

### 2.1 Description of Lake Alinen Mustajärvi and sampling

Lake Alinen Mustajärvi (61° 12' N, 25° 06' E, area 0.007 km<sup>2</sup>, volume 31 × 10<sup>3</sup> m<sup>3</sup>, mean depth 4.2 m, maximum depth 6.5 m) is a head water lake located in the Evo forest area in southern Finland (Fig. 2). The catchment area (< 0.5 km<sup>2</sup>) consists of > 90 % coniferous forest and < 10 % peatland and the natural DOC concentration of the lake is around 10 mg l<sup>-1</sup> in the epilimnion and 20 mg l<sup>-1</sup> in the hypolimnion (Table 1). The lake has ice cover from late November to late April and it is spring meromictic; thus winter stratification is usually replaced by summer stratification without an intervening period of full water column mixing. The autumn overturn is often also partial and hence the bacterial communities in the bottom may experience several years without exposure to oxygen. The watercolour is dark throughout the water column while nutrient concentrations are low in the epilimnion and high in the hypolimnion. The lake has low pH, and CH<sub>4</sub> concentration is high especially in the bottom layer.

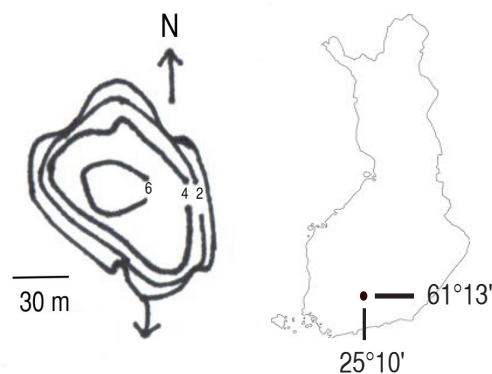


FIGURE 2 Bathymetry and location of Lake Alinen Mustajärvi (bathymetry according to Rask & Arvola 1985).

The lake was sampled intensively through 2007 to 2009 over the deepest point. Additional samples were taken twice in 2006 and 4 times in 2010. Water samples were taken with a 30-cm-long acrylic tube sampler (Limnos vol 2 l) in the open water season, every other week in 2007–2008 and once a month in 2009. Oxygen concentration in the water column was measured every 0.5 m with a YSI 55 probe (Yellow Springs Instruments) and samples for epi-, meta- and hypolimnion, respectively, were taken according to this vertical profile. Epilimnion samples in 2006–2008 were pooled from 0, 1 and 2 m depths and in 2009–2010 from 0 and 1 m depths. Metalimnion samples in 2006–2008 were pooled from 3 and 4 m depths, and in 2009–2010 from 2 and 3 m depths. Hypolimnion samples in 2006 were from 5 m depth, in 2007–2008 pooled from 5 and 6 m depths and in 2009–2010 pooled from 4, 5 and 6 m depths. Water was passed through a 50  $\mu\text{m}$  sieve and larger zooplankton was collected for the determination of community composition. Pooled water samples were transported to the laboratory and kept at +4 °C until they were concentrated by tangential flow filtration (Durapore cassette, pore size 0.22  $\mu\text{m}$ , Millipore) within 6 h of sampling. Concentrated water samples for bacterial community and DOM and POM stable isotope analyses were frozen and freeze-dried with an Alpha 1–4 LD plus (Christ, Osterode, Germany).

The other 4 nearby lakes were sampled in May and October 2006 and the sampling and environmental characteristics of lakes Nimetön, Mekkojärvi, Valkea Kotinen, and Valkea Mustajärvi have been described in Kankaala et al. (2010).

TABLE 1 Lake characteristics in epi-, meta- and hypolimnion of Alinen Mustajärvi during summer stratification 2007. Values represent means  $\pm$  standard deviation.

Layer	Epilimnion	Metalimnion	Hypolimnion
DOC (mg l <sup>-1</sup> )	10.1 $\pm$ 0.7	11.2 $\pm$ 0.7	20.1 $\pm$ 1.5
POC (mg l <sup>-1</sup> )	0.5 $\pm$ 0.2	0.8 $\pm$ 0.2	1.6 $\pm$ 0.3
O <sub>2</sub> (mg l <sup>-1</sup> )	7.1 $\pm$ 0.9	0.8 $\pm$ 1.0	0.2 $\pm$ 0.0
Colour (mg Pt l <sup>-1</sup> )	101.0 $\pm$ 11	134.0 $\pm$ 8	293.0 $\pm$ 18
pH	5.1 $\pm$ 0.1	5.0 $\pm$ 0.3	5.9 $\pm$ 0.2
CH <sub>4</sub> (nmol l <sup>-1</sup> )	551.0 $\pm$ 203	1.0*10 <sup>4</sup> $\pm$ 7*10 <sup>3</sup>	4.5*10 <sup>5</sup> $\pm$ 8.6*10 <sup>4</sup>
Total N ( $\mu\text{g l}^{-1}$ )	396.0 $\pm$ 39	669.0 $\pm$ 83	2576.0 $\pm$ 195
NO <sub>2</sub> +NO <sub>3</sub> <sup>-</sup> ( $\mu\text{g l}^{-1}$ )	13.4 $\pm$ 5.7	11.8 $\pm$ 4.2	11.8 $\pm$ 2.5
NH <sub>4</sub> <sup>+</sup> ( $\mu\text{g l}^{-1}$ )	21.2 $\pm$ 8.1	212.0 $\pm$ 63	1521.0 $\pm$ 186
Total P ( $\mu\text{g l}^{-1}$ )	11.0 $\pm$ 3.5	19.9 $\pm$ 2.5	129.0 $\pm$ 15
PO <sub>4</sub> <sup>-</sup> ( $\mu\text{g l}^{-1}$ )	1.3 $\pm$ 0.6	1.3 $\pm$ 0.5	78.0 $\pm$ 13

## 2.2 Experimental design

### 2.2.1 Whole lake experiment (I, IV)

The experiment comprised 3 phases: in 2007 the lake was monitored in its natural state; in 2008–2009 the DOC concentration was increased by monthly additions of cane sugar during the open water season; and the recovery of the lake was observed in 2010. Prior to the addition to the lake, the sugar was dissolved into a large container of lake water and then spread evenly to the lake surface while rowing a boat around the lake. On each occasion 66 kg of cane sugar was added, which corresponds to 2 mg C l<sup>-1</sup> in the epilimnetic water. Sugar addition was conducted a day after lake sampling and the subsequent sampling was conducted 2 weeks after each addition to measure long-term, rather than immediate responses to increased carbon concentration.

### 2.2.2 Nutrient amendment experiment (II)

Three nutrient addition experiments (at the beginning of May, July and September) were conducted during the open water season of 2009 when regular sugar addition to the lake was in progress. Water for the experiments was taken from the upper 1.5 m of the water column. Each experiment had 4 treatments (control, +N, +P and +NP) in triplicates. Nutrient additions were made only at the beginning of each experiment with the target rise in concentrations being 0.35 mg l<sup>-1</sup> for N and 0.05 mg l<sup>-1</sup> for P. The nutrient sources used were NH<sub>4</sub>NO<sub>3</sub> for nitrogen and Na<sub>3</sub>PO<sub>4</sub> for phosphorus. The water for each experiment was taken with a 30-cm-long acrylic tube sampler (Limnos vol 2 l). Water was sieved with a 50 µm mesh to remove larger zooplankton and mixed thoroughly prior to and after nutrient amendments. Replicates (2 l) were measured into polypropylene bags, which were then sealed and incubated *in situ* at 0.5 m depth, approximating the effective light climate of the mixed layer of the water column. Each experiment lasted for 7 d and sampling was conducted at the start and on days 4 and 7.

### 2.2.3 Pre-experiment for whole lake experiment (III)

Two mesocosm experiments were carried out during the open water season of 2007, each with water from 1 of 2 contrasting boreal mesohumic lakes: 1) a relatively large lake, Pääjärvi (61° 04' N, 25° 08' E, area 13.4 km<sup>2</sup>, volume 206 × 10<sup>6</sup> m<sup>3</sup>), the catchment area (119 km<sup>2</sup>) of which is 15 % agricultural land, 59 % coniferous forests, 11 % peatlands and the rest mainly smaller lakes, and 2) Alinen Mustajärvi. The concentrations of total organic carbon and total phosphorus are similar in both lakes, but the concentration of nitrogen, and especially that of nitrate, was ca. 80-fold higher in Pääjärvi water. The water was from 1 m depth and experiments included 3 treatments (control, cane sugar and humic matter) in triplicates. Mesocosms were 70 l open top polypropylene containers.



Cane sugar was used as a carbon source to keep the light climate unaffected and to trace the carbon transfer from bacteria to zooplankton. The stable isotope composition of carbon in cane sugar (from a C<sub>4</sub> plant;  $\delta^{13}\text{C}$  value -12 ‰) is distinct from that of “local” DOC (derived from C<sub>3</sub> plants;  $\delta^{13}\text{C}$  value around -28 ‰) in the boreal region; therefore it can serve as an isotope tracer, allowing the use of stable isotope analysis to track the fate of the added DOC through the food web. Thus, the transfer of bacterial carbon up the food web to higher trophic levels, a task that previously has been notoriously difficult to measure directly, can be determined.

## 2.3 Analysis of bacterial community composition

### 2.3.1 454 pyrosequencing (I, II, IV)

Bacterial community composition was analysed from the DNA extracted from freeze-dried water samples. The DNA was extracted from 0.4 mg of freeze-dried material, and the procedure was modified (Taipale et al. 2009) from the protocol described by Griffiths et al. (2000). Amplification of bacterial 16S rRNA genes (*E. coli* positions 341 to 805) was conducted using general bacteria primers 341F (5'-CCTACGGGNGGCWGCAG-3') and 805R (5'-GACTACHVGGGTATCTAATCC-3') (Herlemann et al. 2011). Primer 341F carried a 454FLX adaptor B at the 5' end and primer 805R carried a 5 basepair (bp) molecular barcode specific for each sample followed by a 454FLX adaptor A at the 5' end. Amplicons were sequenced with Titanium chemistry from adaptor A, using a 454 GS-FLX system (454 Life Sciences, Branford, CT) at the Institute of Biotechnology hosted by the University of Helsinki, Finland. The resulting reads carried the sample-specific molecular barcode and covered the entire V4 region of the 16S rRNA gene as well as flanking regions. Samples from Alinen Mustajärvi included 380 512 sequences (I, IV), nutrient experiment samples 97 610 sequences (II) and samples from the other 4 lakes 77 146 sequences (Nimetön, Mekkojärvi, Valkea Kotinen and Valkea Mustajärvi, I). Further, 4 samples from the mesocosm experiment conducted with water from Alinen Mustajärvi were sequenced (from day 0 and from day 18 from each of the 3 replicate mesocosms with added cane sugar, the latter 3 thereafter called Sugar1-3) which resulted 13 367 sequences. Ambiguous sequences were removed from the dataset, including reads with low quality as inferred from their flowcharts and those that did not carry the exact primer sequence. After implementation of these quality control criteria, remaining sequences were denoised using AmpliconNoise Version 1.22 (Quince et al. 2011). AmpliconNoise implements algorithms that remove PCR and 454 pyrosequencing noise as well as the novel chimera removal tool Perseus. Before statistical analysis all samples were resampled to same size using perl-script DaisyChopper (Gilbert et al. 2009).

Resampled sequence data were analysed with several statistical methods (I, II and IV, Table 2). Samples from the mesocosm experiment were

transformed to Z-scores to estimate divergence of OTUs between triplicate samples. To estimate the responses of OTUs to DOC amendment, OTUs were divided into 10 ecologically coherent clusters according to their Z-scores using k-means clustering (Hartigan & Wong 1979) for each depth layer respectively. Z-scores inside clusters were tested for differences between years with Kruskal-Wallis test with post hoc test. OTUs in each cluster were considered to respond to treatment if  $p < 0.001$  and trend between years was uniformly increasing or decreasing from 2007 to 2009.

TABLE 2 Statistical analyses used in the study.

Analysis	Description	Reference
K-means clustering	I	Hartigan & Wong 1979
Kruskal-Wallis test with post hoc testing	I, II, IV	
Linear models	IV	
Local Similarity Analysis (LSA)	I	Ruan et al. 2006
Non-Metric Multidimensional scaling (NMDS)	I, II, III, IV	
Paired Wilcoxon test	I, IV	
PERMANOVA	I, IV	McArdle & Anderson 2001
Procrustes test	II	Perez-Neto & Jackson 2001
Spearman rank correlation	I	
Standardized variance	I	Burt 1995

### 2.3.2 LH-PCR (II, III)

Bacterial community composition was also analysed by length heterogeneity analysis of PCR-amplified 16S rRNA gene (LH-PCR) (Suzuki et al. 1998). LH-PCR was executed and analysed according to Taipale et al. (2009) with minor modifications. The phylogenetic affiliations of *Actinobacteria* and  $\alpha$ - and  $\beta$ -*Proteobacteria* were predicted based on the 16S rRNA gene clone library data from Alinen Mustajärvi (313 clones). For that purpose, a vertical profile of the lake was sampled in summer 2008 and the bacterial community was analysed with LH-PCR and Sanger sequencing. Primers used in LH-PCR were 27f (Edwards et al. 1989) and 518r (Neefs et al. 1990) and in sequencing 27f and 907r (Johnson 1994). From the sequencing results an LH-PCR simulation was conducted according to Taipale et al. (2009), which gave an interpretation of various LH-PCR marker lengths. LH-PCR fragments with lengths between 466–473 bp were assigned to *Alphaproteobacteria*, lengths between 500–508 bp to *Actinobacteria* and lengths between 520–524 bp to *Betaproteobacteria*.

## 2.4 Stable isotope analysis (I, III, IV)

Stable isotopes of carbon were analysed from natural as well as added sources. Natural variation in  $\delta^{13}\text{C}$  values was measured from methane. Methane concentration in the lake water column may diminish due to methane oxidation

by methanotrophic bacteria, or because of diffusion to the atmosphere. Natural variation of  $\delta^{13}\text{C}\text{H}_4$  reflects microbial methane oxidation, which preferentially uses the light isotope ( $^{12}\text{C}$ ) leading to enrichment of the residual methane with the heavier isotope ( $^{13}\text{C}$ ) (Whiticar 1999). Determining the fraction of  $^{13}\text{C}$  in methane allowed evaluation of whether the decrease in methane concentration in Alinen Mustajärvi was due to oxidation or diffusion and in which depths methane oxidation was occurring (I).

A system may also be deliberately enriched with some compound that may be used as an isotopic tracer. Here cane sugar was used for this purpose. The isotopic value of cane sugar is -12 ‰, while the natural value of DOC in boreal zone lakes is around -28‰. This difference enabled the tracing of carbon added as sugar through the lake food chain from bacteria via zooplankton up to the fish by analysing the isotopic composition of DOM, POM, zooplankton and fish muscle (III, IV).

## 2.5 Other analyses

The specific analyses made in the study and in which of the papers the methods are fully described are presented in Table 3.

TABLE 3 Analyses made in the study.

Analysis	Description	Reference
Bacterial abundance	II, IV	
Bacterial production	III, IV	Tulonen 1993
CH <sub>4</sub>	I, IV	Kankaala et al. 2010
Chl a	I, II, III, IV	
DNA extraction	I, II, III, IV	Griffiths et al. 2000
DOC	I, II, III, IV	
Nutrients	I, II, III, IV	
pH	IV	
Phytoplankton biovolume & community	II, IV	
POC	II, III, IV	
Primary production	III, IV	Keskitalo & Salonen 1994
Zooplankton abundance & community	I, III, IV	
$\delta^{13}\text{C}$ from DOM, POM and zooplankton	III, IV	
$\delta^{13}\text{C}\text{-CH}_4$	I	Kankaala et al. 2007

### 3 RESULTS AND DISCUSSION

#### 3.1 Bacterial community dynamics and composition between lakes and layers (I)

The study included 5 lakes representing a DOC gradient of 5.8–26.3 mg l<sup>-1</sup> in the epilimnion and 5.4–43.8 mg l<sup>-1</sup> in the hypolimnion. Detailed sequencing of the study lakes confirmed that bacterial communities are clearly different between lakes as well as between layers, as has been suggested earlier based on fingerprinting methods and small clone libraries (e.g. Kent et al. 2004, Shade et al. 2008, Taipale et al. 2009, Barberan & Casamayor 2011). However, at least some of the disparities in community composition seemed to be consistent with changes in DOC concentration (Fig. 3). For example, *Actinobacteria* would seem to be more abundant in the hypolimnion of lakes with low DOC concentration, while the proportion of *Betaproteobacteria* was rather stable among the epilimnia. In some lakes the proportion of *Gammaproteobacteria* was relatively high in hypolimnion, which contradicts previous suggestions that they are only transient members of the bacterial community (Newton et al. 2011). The abundance of *Alphaproteobacteria* seemed to increase with decreasing DOC concentration, which is in line with previous studies (Eiler et al. 2003). A key finding was that the representation of tribes previously described as typical for freshwaters (Newton et al. 2011) declined with increasing DOC concentration among and within lakes. Accordingly, the proportion of candidate divisions and other poorly described phyla was high especially in the hypolimnia. Furthermore, the number of OTUs increased with increasing DOC concentration, again both between and within lakes. Community composition also changed with season, but this temporal variation was higher in the upper layers than at the bottom of the lake. Possible reasons for high variation in the epilimnion include more pronounced seasonal shifts in temperature, light and nutrient concentrations compared to the more stable hypolimnion. Also selective feeding by heteronano-flagellates (Fu et al. 2003; Massana et al. 2009) or irregular inputs of organic compounds from allochthonous production may have in part resulted in higher community fluctuation in the epilimnia.

(Bergström & Jansson 2000). Conversely, the low variance in hypolimnetic OTUs may be due to anaerobic conditions, where metabolic pathways have lower energy yield than in the presence of oxygen, thus limiting the growth of fast-growing copiotrophic bacteria.

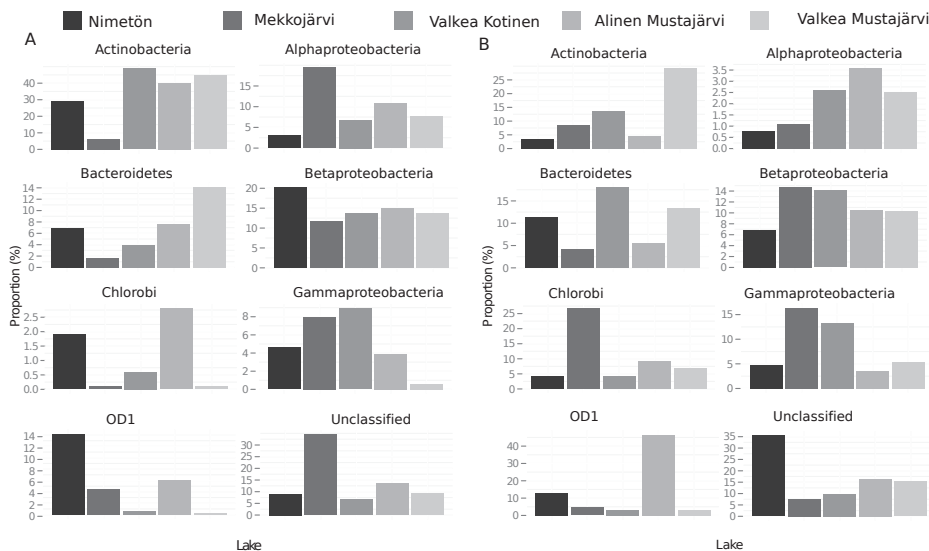


FIGURE 3 Bacterial community composition in A) epilimnion and B) hypolimnion of study lakes. Order of the lakes follows the DOC gradient from highest (Nimetön) to lowest (Valkea Mustajärvi).

The time series data from Alinen Mustajärvi clearly indicated that the bacterial community composition is strikingly different between the depth layers of a humic lake. After the phyla typical for surface and bottom layers were resolved, by far the most abundant phylum in the surface was *Actinobacteria*, followed by *Betaproteobacteria*. *Actinobacteria* has been found to contribute over 50 % of the bacteria in the epilimnion in previous lake studies (Glöckner et al. 2000, Sekar et al. 2003, Warnecke et al. 2004, Allgaier & Grossart 2006, Newton et al. 2007,) and it was also the major phylum in 3 of the 4 other lakes studied here. Even though *Actinobacteria* are present also in the hypolimnion (Allgaier & Grossart 2006, Taipale et al. 2009), their abundance has been said to decrease with decreasing oxygen concentration (Taipale et al. 2009). This pattern was partly supported by the present findings, as at the bottom the *Actinobacteria* were a minor group in most study lakes, but it would seem that they might be more common in the bottom layers of lakes with low DOC concentration. Still, the lake with the highest proportion of *Actinobacteria* in the hypolimnion also has the lowest proportion of anoxic volume compared to other lakes. Thus, the high proportion of *Actinobacteria* in lake Valkea Mustajärvi might be related to oxygen environment as well as to DOC concentration. The major group in the hypolimnion of Alinen Mustajärvi was candidate division OD1, with *Chlorobi* and *Bacteroidetes* also well represented. In previous studies *Chlorobi* (Taipale et al. 2009, Barberan & Casamayor 2011) and *Bacteroidetes* (Barberan & Casamayor,

2011) have also been found to be the major groups in the anoxic hypolimnion, but the dominance of OD1-related sequences has only been reported once (Bri e et al. 2007), from a small suboxic pond. Members of this phylum were also recovered from the other 4 lakes and were positively correlated with DOC and methane concentrations. That is, the lakes with higher concentrations of methane and DOC also harboured a higher proportion of OD1-related reads. The wider diversity of the hypolimnion compared to the epilimnion could be seen in the number of layer-specific phyla of Alinen Mustaj arvi, as in the hypolimnion there were representatives from 20 phyla and in the epilimnion from only 7 phyla.

The most abundant phylum in the hypolimnion of Alinen Mustaj arvi, candidate division OD1, constituted an abundant and persistent population in the lake. This still uncultured phylum has previously been detected mainly in sulphur-rich, anoxic environments (e.g. Elshahed et al. 2005, Bri e et al. 2007, Barberan & Casamayor 2011), and a partial genome has been recovered, revealing genes known only from anaerobic or facultatively anaerobic microorganisms. In Alinen Mustaj arvi the abundance of OD1 increased during the summer, but collapsed right after the beginning of autumn overturn, supporting the supposition that these microbes might be strict anaerobes. The genomic organization of the previously recovered partial genome was similar to methanotrophic *Archaea* (Elshahed et al. 2005) found in anoxic marine sediments (Hallam et al. 2004). Stable isotope data indicated methane oxidation at the same depth where OD1 OTUs were highly abundant, and in the LSA several OTUs annotated as OD1 were positively related to CH<sub>4</sub> concentrations and also to ammonium. Further LSA revealed that OTUs annotated as OD1s were often significantly positively correlated to each other, suggesting that these OTUs share many ecological properties with considerable niche overlap. In turn OTUs affiliated with *Chlorobi* were negatively related to the OD1 OTUs, suggesting competition or differences in growth constraints among these OTUs. Overall, OTUs affiliated with OD1 often had a high number of connections to other OTUs. OTUs with a high number of associations can be regarded as a "hub" in the complex network (see Montoya et al. 2006) that anaerobic lake bacterioplankton communities form in the hypolimnion of boreal lakes. Together these findings suggest that members of the candidate division OD1 are very likely anaerobic, possibly play a role in anaerobic methane oxidation and probably have an important ecological role in the environment.

### 3.2 Impact of nutrients on bacterial community composition (II)

Changes in bacterial community composition were more pronounced across seasons than in response to nutrient amendments; the main phyla in the May experiment were *Betaproteobacteria* and *Actinobacteria* whereas by September the community was dominated by *Alphaproteobacteria*. The seasonal succession in the phytoplankton community followed a parallel trajectory to the bacterial community. This kind of synchrony of bacteria and phytoplankton has been

suggested earlier (Kent et al. 2007). However, the communities showed opposing trends in abundance/biovolume, as the bacterial abundance in the lake declined towards autumn whereas phytoplankton biovolume increased. Interactions between these groups may have shaped both communities through a number of means. Phytoplankton exudates are readily available substrates for bacterioplankton (Cole 1982) and may induce bacterial growth, but phytoplankton may also shape bacterial communities by the release of antimicrobial agents (Cole 1982). Some phytoplankton taxa are also capable of mixotrophic growth by selective feeding on bacteria and thus affecting bacterial community composition (Massana et al. 2009). Conversely, bacteria may affect the composition of the phytoplankton community as the algal growth may be affected by certain members of the bacterial community (Grossart & Simon 2007).

Physicochemical conditions, such as temperature and nutrient concentrations, might also be the reason for contrasting seasonality of the groups (bacterial responses most pronounced in spring and phytoplankton responses in autumn). The affinity for nutrients has been suggested to vary between bacteria and phytoplankton with bacteria having generally stronger affinity for nutrients due to their higher surface-to-volume ratios (Currie & Kalff 1984). In contrast, phytoplankton is assumed to have higher nutrient uptake rates compared to bacteria and thus to benefit from rapid and pronounced changes in nutrient concentrations (Suttle et al. 1990). Further, for bacteria an increase in nutrient concentration has been reported to reduce the influence of low temperature (Felipe et al. 1996). Thus, the outcome of competition for nutrients is determined by complex interactions between physical and biological factors (Lewitus et al. 1998, Newton & McMahon 2011) including the occurrence and distribution of populations present in these communities. The growth dynamics of the communities might also have been linked via zooplankton as there are signs that the bacterial growth in the autumn might have been under top-down control. Thus a decline in the competition for nutrients with bacteria might have enhanced the phytoplankton growth towards autumn.

Responses of the bacterial community to the treatments were different between experiments and the strength of the treatment effects was dependent on the level of resolution used for sequence clustering (75 > 99 % sequence similarity). Hence, at the low sequence similarities there were less pronounced treatment effects than at 99 % similarity. The only uniform treatment response at phylum level was that the proportion of *Actinobacteria* declined in the N+P treatment in all experiments. Overall the dissimilarities in community composition between treatments were in most cases greatest between the Ctrl and N+P treatments and the distance was increasing with increasing OTU resolution. Nevertheless, the treatment effects on the community were generally smaller than the seasonal changes between experiments. The bacterial community in the September experiment was more coherent than in May or July while the diversity was highest in May. One reason for the drop in richness might have been increases in flagellate grazers, as a drop in bacterial community richness has been shown to coincide with an increase in the

flagellate abundance (Kent et al. 2004). This may have also in part induced the more coherent autumnal bacterial community structure.

The 97 % sequence similarity OTUs showed highly diverse treatment responses inside tribes. Most OTUs were found only from 1 experiment and there were very few patterns in OTU behaviour as a response to treatments; rather OTUs annotated to a single tribe could appear in any experiment and treatment. Thus, while at phylum level the treatment responses were minimal, highly variable responses were seen at high taxonomic levels. This kind of niche partitioning among highly similar organisms has recently been observed for strains of *Polynucleobacter necessarius asymbioticus* in respect to pH, conductivity, DOC and oxygen concentration (Jezbera et al. 2011) and for actinobacterial phylotypes from contrasting layers of a lake that were indistinguishable based on 16S rRNA genes (Allgaier et al. 2007). Thus it seems that ecologically coherent populations responding to changes in nutrient stoichiometry are difficult to resolve even with current methods.

### 3.3 DOC- induced changes in bacterial community (III, IV)

#### 3.3.1 Bacterial abundance

The increase in the DOC concentration of Alinen Mustajärvi resulted in a decline in bacterial abundance, but only in the lower layers of the lake (Fig. 4). In the metalimnion the decrease was subtle ( $\chi^2 = 8.43$ ,  $p < 0.05$ ), but in hypolimnion the abundance was clearly declining from 2007 to 2009 ( $\chi^2 = 14.51$ ,  $p < 0.005$ ). Bacterial abundance has previously been observed to increase with increasing DOC concentration (Blomqvist et al. 2001, Eiler et al. 2003, Anesio et al. 2004), indicating that bacterial production has been carbon limited. That would suggest that bacterial production in Alinen Mustajärvi was not carbon limited, and the limiting elements might instead have been nitrogen together with phosphorus as suggested for the epilimnion by the nutrient amendment experiments. Towards autumn it is also possible that the bacterial growth was top-down rather than bottom up controlled as suggested by the nutrient amendment experiments. However, as the nutrient concentrations in the lower layers of the lake are higher, bacterial production in the hypolimnion might also have been limited by some other factor. Generally, bacterial metabolism in anoxic environments is slow and typically the available substrates in the hypolimnion are recalcitrant. Thus, it is possible that many of the hypolimnetic bacteria cannot metabolize glucose, which could explain lack of growth despite the increased carbon availability.

Competition for resources with other organisms might also have resulted in a lack of bacterial response. Phytoplankton biovolumes were recorded for the surface layer and the total volume did not change between 2007–2010 (data not shown). Also changes within phytoplankton phyla were minor; only the biovolume of *Cryptophyceae* increased from 2007 to 2009 ( $\chi^2 = 13.23$ ,  $p < 0.005$ ) and an increase in the biovolume of *Raphidophyceae* was almost significant ( $\chi^2 =$



7.68,  $p = 0.053$ ). The biovolume of heterotrophic flagellates also increased from 2007 to 2009 ( $\chi^2 = 8.08$ ,  $p < 0.05$ ), which could have had an impact on the bacterioplankton abundance. Unfortunately the abundances or biomasses of other planktonic organisms were not studied. Possible other planktonic organisms include *Archaea*, which are shown together with bacteria when stained with DAPI, but the method does not distinguish between these organisms. Thus it cannot be known whether the abundance of both decreased or one increased while the other decreased drastically. Another often disregarded group are the fungi, which are seldom included in lake studies, but may have a profound role in plankton communities (Wurzbacher et al. 2010). However, currently no fungi are considered to inhabit the suboxic hypolimnion (Wurzbacher et al. 2010), so this was not likely to affect the bacterial growth in the hypolimnion.

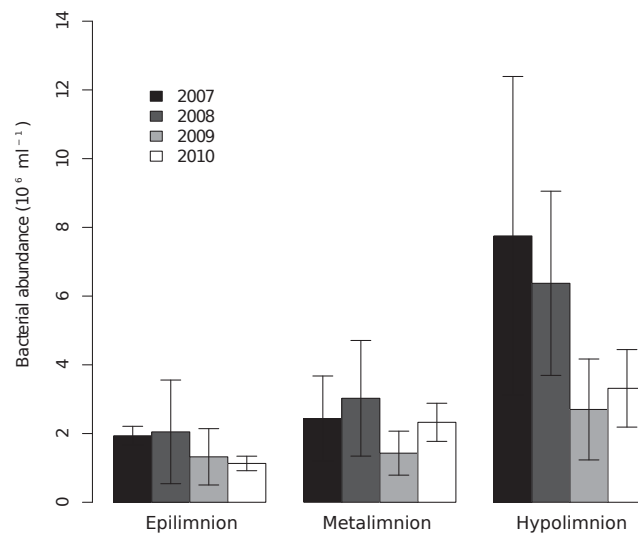


FIGURE 4 Average bacterial abundance in epi-, meta- and hypolimnion of Alinen Mustajärvi during summer stratifications from 2007 to 2010. Error bars represent standard deviation.

### 3.3.2 Bacterial community composition

The preliminary experiment in mesocosms suggested relatively few changes in bacterial community composition following the cane sugar amendments. At phylum level LH-PCR results were supported by 454 pyrosequencing, in which the proportion of *Actinobacteria* increased (from 30.4 % in the beginning to 37.6 % (s.d.  $\pm 2.3$ ) on day 18) while the proportion of *Betaproteobacteria* decreased (from 16.3 % to 8.8 % ( $\pm 1.9$ )). Pyrosequencing also showed that the proportion of *Gammaproteobacteria* had increased from 1.8 % to 11.3 % ( $\pm 3.4$ ) and the proportion of *Verrucomicrobia* and unclassified bacterial sequences decreased (from 7.4 % to 3.6 % ( $\pm 0.3$ ) and from 13.6 % to 6.4 % ( $\pm 2.6$ ), respectively). Changes in *Gammaproteobacteria*, *Verrucomicrobia* and unclassified bacteria were not visible in the LH-PCR due to lack of known marker gene lengths.

Regardless of these seemingly uniform changes at phylum level, there was a great variation between individual samples at OTU level. Of 1055 OTUs derived from mesocosm sequences, more than half were singletons and over 90 % included less than 20 reads. Regardless of the above-mentioned uniform treatment responses, mainly different OTUs responded to added carbon in each of the triplicates (Fig. 5). For example in Sugar1 sample, actinobacterial OTUs 128 and 229 seemed to benefit the most from added carbon, while in Sugar2 actinobacterial OTUs 28, 59, 83 and 777 were flourishing and in Sugar3, it was OTUs 24, 122, 245 and 769. Thus, the same high diversity and diverse treatment responses as in nutrient amendment experiment were observed.

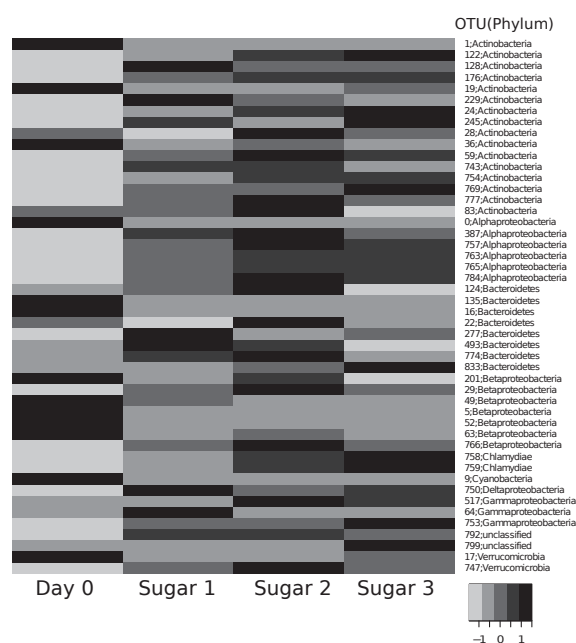


FIGURE 5 Heatmap visualizing the frequencies of OTUs in triplicate mesocosms with added cane sugar. Frequencies expressed as z-scores. Only OTUs with over 50 reads included.

At phylum level the long-term effects of DOC on the bacterial community seemed to be opposite to short-term effects, as the proportion of *Actinobacteria* decreased ( $\chi^2 = 11.43$ ,  $p < 0.001$ ) and the proportion of *Betaproteobacteria* and *Verrucomicrobia* increased during study period ( $\chi^2 = 9.45$ ,  $p < 0.05$  and  $\chi^2 = 17.56$ ,  $p < 0.001$ , respectively) (Fig. 6). These results are supported by previous studies by Burkert et al. (2003) where *Betaproteobacteria* were enriched in media based on water from a humic lake, while the proportion of *Actinobacteria* declined relative to the natural state of the community. Further, the proportion of Candidate division OD1 declined ( $\chi^2 = 12.26$ ,  $p < 0.01$ ) and the proportion of *Bacteroidetes* increased ( $\chi^2 = 23.46$ ,  $p < 0.001$ ). Change in DOC concentration did not seem to affect *Alphaproteobacteria* even though it has been earlier said to favour low DOC environments (Eiler et al. 2003) and was found to be more

abundant in low DOC lakes among the study lakes here. The NMDS analysis suggested that the composition of the bacterial community in Alinen Mustajärvi was clearly different between the study years.

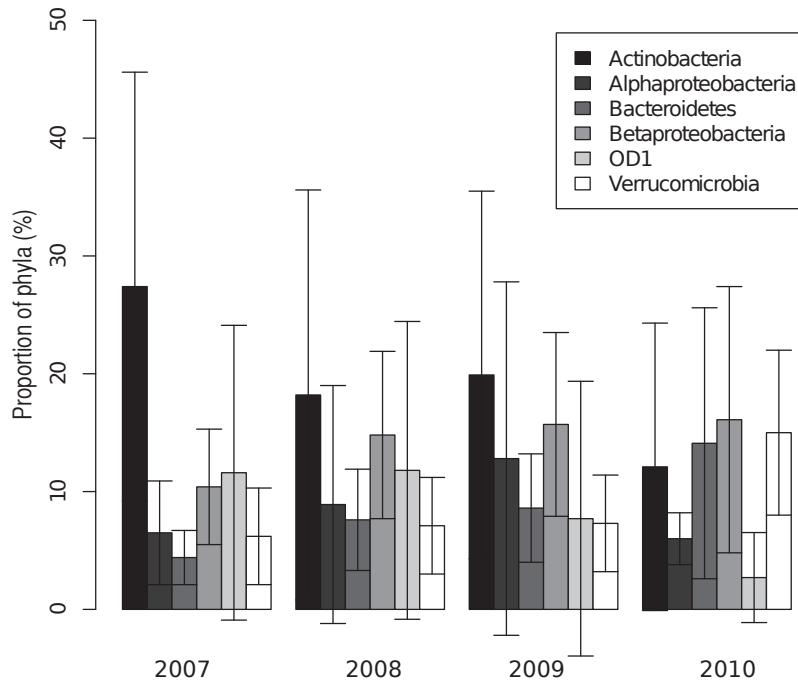


FIGURE 6 Proportion of main bacterial phyla in Alinen Mustajärvi during summer stratifications from 2007 to 2010. Error bars represent standard deviation.

The finer level changes in bacterial community were diverse. After all the OTUs were divided to ecologically coherent clusters using k-means clustering, certain trends could be seen among those groups (Fig. 7). Distinctively, clustering suggested functionally coherent groups crossing phylum borders as all clusters included OTUs from multiple phyla. This again emphasizes that taxa inside phyla are not necessarily ecologically coherent. In the epilimnion, OTUs in cluster 1 seemed to benefit from carbon amendment, as their abundance increased during the treatment ( $\chi^2 = 31.09$ ,  $p < 0.001$ ). Correspondingly, in the hypolimnion OTUs in clusters 1, 5, 6 and 7 seemed to benefit ( $\chi^2 = 82.57$ , 36.60, 40.77 and 29.67, respectively,  $p < 0.001$  for all). The losers seemed to be OTUs in clusters 2 and 7 ( $\chi^2 = 33.59$  and 52.49, respectively;  $p < 0.001$  for both) in the epilimnion and OTUs in clusters 4 and 9 ( $\chi^2 = 49.02$  and 82.0, respectively;  $p < 0.001$ ) in the hypolimnion. Phylogenetic connections between OTUs were produced using freshwater taxonomy (Newton et al. 2011). This could be used to show similarities and disparities between and within taxa. For example, among OTUs belonging to cluster 1 in the epilimnion, there were 2 OTUs from tribe Novo-A1 and also 2 from clade Alf-VI, both alphaproteobacterial taxa. All of those were distinctively present in the community in 2008 and 2009, especially towards the end of summer, but missing in 2007 and 2010. Still, the

most abundant OTU in epilimnetic cluster 7, that clearly suffered from DOC amendment, was also affiliated to *Alphaproteobacteria*, more specifically to tribe LD12. While this OTU was highly abundant in 2007, in 2008 it was only found during midseason and then disappeared totally. Also in the epilimnion among the OTUs in clusters appearing to gain from DOC, was OTU2989 affiliated with *Methylomonas*. This taxon belongs to the methanotrophic bacteria using methane as a sole carbon and energy source (Hanson & Hanson 1996). This OTU was especially abundant in autumn 2009, coinciding with the highest measured methane concentrations at the lake. Similarly in the hypolimnion, while 2 clusters (7 and 8) increasing towards the end of the treatment period included mainly OTUs affiliated with *Chlorobia* and candidate division OD1, OTUs in another cluster (9), mainly affiliated with OD1, were disappearing.

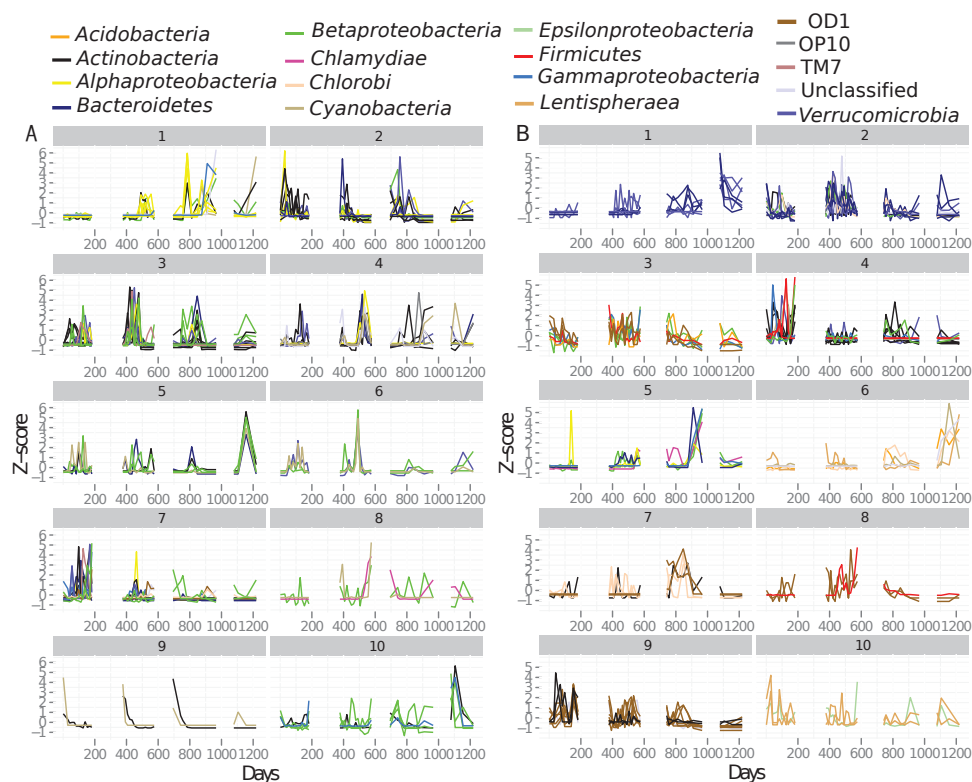


FIGURE 7 Dynamic behaviour of bacterial clusters in A) epi- and B) hypolimnion expressed with z-scores. Numbering of days starts from the first sampling in 2007 and is continuous through the study period. Breaks in the curves indicate winter seasons when no samples were collected.

### 3.4 Carbon flow in the foodweb (III, IV)

Previously, demonstrating the transfer of bacterial carbon in the food web has been difficult. Here this was done using stable isotope analysis and addition of cane sugar. The transfer of carbon was first studied in preliminary experiments in mesocosms where it was confirmed that some sugar-derived carbon was indeed transferred through the food chain as the  $\delta^{13}\text{C}$  values in crustacean zooplankton increased. The same phenomenon has also been shown in mesocosms by Karlsson et al. (2007) and Faithfull et al. (2012). Further the mesocosm experiments proved cane sugar to be a more available carbon source for bacteria than natural humic matter as the bacterial production, community respiration and bacterial growth efficiency were all higher in the sugar treatment than in the humic treatment. Also the  $\delta^{13}\text{C}$  DIC values were higher in sugar treatment further indicating use of the added cane sugar in bacterial production and respiration.

In the whole-lake experiment the carbon transfer was clearly seen through the food web, and the change in  $\delta^{13}\text{C}$  values was confirmed even in the fish (data not shown), corroborating the previous observations from a mesocosm study (Faithfull et al. 2012). However, as the bacterial production or respiration in the epilimnion did not change during the experiment, it seems that bacteria were mainly using the added DOC in preference to the indigenous DOC and the overall DOC usage did not increase. However, as the process measurements did not include the deeper layers, it cannot be verified whether there were changes in their metabolism following the amendments, although the decrease in POC and nutrient concentrations in the meta- and hypolimnion do suggest increased carbon usage in these layers. Possible mechanisms there include a priming effect, whereby the arrival of fresh carbon substrate triggers the use of stored carbon compounds (Kuzyakov et al. 2000), although for the lower layers of the lake the changes in metabolism and possible reasons remain speculative. Nevertheless, in the epilimnion the combined results from nutrient amendment, mesocosm and whole lake experiments suggest that even though the added carbon was incorporated to the biomass, it did not increase the carbon usage and thus, in Alinen Mustajärvi the epilimnetic bacterial community was under bottom-up nutrient control especially during spring, while by autumn, top-down control may also have had some effect.

### 3.5 GHG emissions

The daily  $\text{CH}_4$  and  $\text{CO}_2$  fluxes from Alinen Mustajärvi were similar to previous measurements (Huttunen et al. 2003, Huotari et al. 2011), as were the methane concentrations in the surface layer (Bastviken et al. 2004, Juutinen et al. 2009). Following the DOC amendments the daily  $\text{CO}_2$  emissions at Alinen Mustajärvi increased, being higher in 2008 than in 2007 (Fig. 8, pairwise Wilcox test  $p < 0.005$ ) while  $\text{CH}_4$  emissions in both 2008 and 2009 were higher than in 2007 ( $p <$

0.05 for both). As the process measurements in the epilimnion suggested no changes for bacteria or phytoplankton, increased emissions must be related to processes in the deeper layers of the lake. For CO<sub>2</sub> emissions the most likely explanation would seem to be increased degradation of organic substrates in the lower layers of the lake. This could have also increased the competition for nutrients and/or electron acceptors in these layers, which in turn might have affected other processes, for instance CH<sub>4</sub> oxidation. Further, at the bottom of the lake the bacterial abundance declined during the study. Even though the proportion of methane oxidizing bacteria did not change during the study years, as the overall number of bacteria in the meta- and hypolimnion decreased, the net abundance of methanotrophs did decrease. This may also at least partly explain the increase in CH<sub>4</sub> emissions. Another reason for the increased emissions could be increased methanogen activity, but unfortunately neither abundance of methanogens nor methane production rate were measured from the lake. Nevertheless, it would seem that even in a lake that is not carbon limited, an increase in DOC concentration may enhance GHG emissions, but further studies are needed to resolve the mechanisms leading to this.

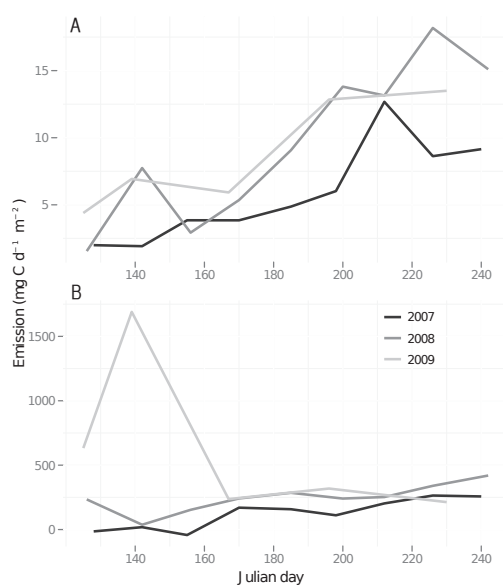


FIGURE 8 Daily emissions of A) CH<sub>4</sub> and B) CO<sub>2</sub> in Alinen Mustajärvi in 2007–2009.

## 4 CONCLUSIONS

Thorough examination of bacterial communities in stratified humic lakes showed that, especially in high-DOC lakes, bacterial communities in the suboxic hypolimnion are very different from previously defined typical freshwater bacteria. Analysis indicated that bacterial communities in different depth layers of humic lakes are clearly distinct from each other, and while in the epilimnion the typical bacterial phyla are *Actinobacteria* and *Betaproteobacteria*, in the hypolimnion they are candidate division OD1, *Chlorobi* and *Bacteroidetes*. Highly divergent communities between layers as well as disparities in community diversities indicate that the organisms in the different layers were ecologically distinct. LSA together with stable isotope analysis suggested that poorly known candidate division OD1 might play a key role in the hypolimnetic community and may be involved in carbon and methane cycles. Still, the functions and metabolic characteristics of this phylum need to be verified with more function-oriented methods. Compared to other environments where candidate divisions have been found (e.g. hydrothermal vents, sulphur springs), humic lakes offer a wide spread and accessible environment to study these organisms.

Changes in bacterial communities in response to carbon and nutrient amendments were similar in that they were subtle at phylum level, but considerable at tribe and especially at 97 % sequence similarity level. There seemed to be ecologically coherent bacterial clusters beyond phylum borders that benefited or suffered from enhanced DOC concentration. In turn nutrient amendment experiments indicated that seasonal succession of the bacterial community has a profound impact on the community and that nutrient responses were highly dependent on season. Even though some uniform treatment responses could be found, a key conclusion was that changes in community composition cannot be explained or predicted based on current knowledge of bacterial ecology. Thus as the methodology evolves, new techniques like metagenomics should be used to define the functions of bacterial groups at tribe and finer level.

Increase in DOC concentration apparently resulted in elevated GHG emissions from the lake to the atmosphere. There were no significant changes in

bacterial or primary production in the epilimnion, but due to lack of process data from the deeper layers of the lake, it is not possible to say what kind of changes there might have been in the bottom layers. However, for the surface layer the results suggest that the bacteria in Alinen Mustajärvi were not carbon limited and in turn, the phytoplankton did not suffer from increased DOC level when there was no shading effect from the allochthonous matter. If the daily GHG emissions from the lake did indeed increase, the likely reasons for this include increased degradation of organic substrates, increased competition for nutrients and electron acceptors and the decline in bacterial abundance in the bottom layers of the lake and hence in the numbers of methanotrophs. Furthermore, it seems that even in high-DOC lakes further increase in DOC concentration may lead to increased GHG emissions and thus, if the increase in DOC concentrations in surface waters continues, this may affect the future climate. Moreover, based on stable isotope values part of the added carbon could be traced from DOM up to the fish confirming that DOC can indeed contribute to carbon flow through food webs to support top consumers.

Overall the results suggest that bacterial communities in stratified humic lakes are still poorly characterized and may harbour poorly known bacterial phyla. Even though knowing the exact composition of bacterial community does not in itself currently tell us much, it is clear that the community composition does matter as metabolic functions and the ability to adapt to a changing environment are drastically different between different species. For example, certain bacteria are capable of survival in oxic as well as anoxic environments, whereas other species are strict aerobes or anaerobes. Thus, the impact of changes in the environmental parameters is determined by the make-up of the community. Although the current focus in microbial ecology is still largely in community compositions, there is more and more interest in the processes in microbial communities and the ultimate goal is to determine the actual functions of the individual community members. However, this is not possible unless we know what the community members are. Thus, to discover the functions and significance of bacterial communities to processes and metabolism of a humic lake, we must first unravel the composition of bacterial communities in stratified lakes, as the results acquired from other freshwater habitats cannot be extrapolated to them. The data presented in this thesis increase our knowledge of these communities and represent a first step along a road to disentangle the bacterial community functions in these unique environments.



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## YHTEENVETO (RÉSUMÉ IN FINNISH)

### Humuspitoisten metsäjärvien mikrobiyhteisöt

Pienet, metsissä ja soilla sijaitsevat järvet ovat kiinteä osa suomalaista maisemaa sekä koko boreaalista vyöhykettä. Näihin järviin huuhtoutuu huomattava määrä orgaanista ainesta (humusta) ympäröivältä valuma-alueelta, ja liunneen orgaanisen hiilen pitoisuus järvissä on korkea. Lisäksi viime vuosikymmenten aikana pintavesien hiilipitoisuuksien on havaittu yleisesti nousseen. Tämän on arveltu johtuvan mm. lisääntyneistä sademääristä, jotka puolestaan liitetään globaaliin ilmastomuutokseen. Erityisen merkitykselliseksi humusjärvet tekevät niiden happiolosuhteet, sillä useimmat pienet humusjärvet ovat alusvedeltään hapettomia lämpötilakerrostuneisuuden aikana. Siinä missä hapellisissa järvissä hiilimetabolian lopputuotteena muodostuu hiilidioksidia, humusjärvissä orgaanisen aineen hajotuksen lopputuotteena muodostuu lisäksi metaania, joka on hiilidioksidia huomattavasti voimakkaampi kasvihuoneilmiön edistäjä. Tämän vuoksi humusjärvillä saattaa olla huomattava vaikutus tulevaisuuden ilmasto-olosuhteisiin. Runsaslukuisuudestaan ja merkityksellisyydestään huolimatta humusjärvien mikrobiyhteisöt tunnetaan huonosti. Erityisesti hapettoman alusveden mikrobiologinen tutkimus sekä yhteisöjen rakenteeseen että toimintaan liittyen on suhteellisen vähäistä. Silti juuri mikrobit ovat vastuussa sekä orgaanisten aineiden hajotuksesta että kasvihuonekaasujen tuottamisesta, ja siten ne pohjimmiltaan määrittävät koko järven metabolian ja mahdolliset ilmastovaikutukset.

Tässä tutkimuksessa humusjärvien mikrobiyhteisöjen rakennetta selvitettiin DNA-menetelmin uusinta sekvensointitekniikkaa (454 pyrosekvensointi) ja bioinformatiikan menetelmiä käyttäen. Humusjärvien mikrobiyhteisöjen rakenne eri kerroksissa (päälyysvesi, harppauskerros ja alusvesi) määritettiin luonnontilassa, ja lisäksi ravinne- ja hiilenlisäyskokeiden avulla määritettiin, kuinka typpi, fosfori ja hiili vaikuttavat yhteisön rakenteeseen. Viiden humusjärven mikrobiyhteisöjen kartoitus osoitti, että mikrobiyhteisöt järvien hapettomassa alusvedessä poikkeavat huomattavasti hapellisen päälyysveden yhteisöistä. Lisäksi osoittautui, että etenkin järvissä, joissa hiili- ja metaanipitoisuudet ovat korkeita, merkittävässä osassa ovat mikrobit, joiden ekologiasta tiedetään hyvin vähän. Tutkimuksessa havaittiin myös, että makeavetisille järville tyypillisinä pidettyjen mikrobien osuus oli hapettomassa alusvedessä erittäin alhainen. Tarkimmin tutkitun järven alusvedessä yleisin mikrobipääjakso oli OD1, jonka yhtäkään jäsentä ei ole onnistuttu kasvattamaan laboratorio-olosuhteissa. Käyttäen sosiaalitieteistä tunnettua verkostanalyysiä ja kerättyjä taustatietoja muun muassa metaani- ja ravinnepitoisuuksista pystyttiin päättämään, että järven OD1 pääjaksoon kuuluvilla bakteereilla näyttäisi olevan huomattava merkitys alusveden yhteisön toiminnalle. Lisäksi tulokset viittaavat siihen, että OD1-ryhmän runsaus saattaa liittyä joko metaani- tai ammonium-metaboliaan.

Hiilen vaikutusta mikrobiyhteisöihin ja koko järven metaboliaan tutkittiin nelivuotisessa koko järven mittakaavan kokeessa, jossa pienen humusjärven (Alinen Mustajärvi) hiilipitoisuutta nostettiin  $2 \text{ mg l}^{-1}$  kahden vuoden ajaksi. Tämä toteutettiin lisäämällä järveen toukokuun ja lokakuun välisenä aikana kuukausittain ruokosokeria. Käyttämällä hiilenlähteenä ruokosokeria hiilen siirtyminen ravintoketjussa ylemmille tasoille voitiin todeta hiilen eri fraktioiden sekä eläinplanktonin stabiili-isotooppiarvojen avulla. Lisäksi tämän kokeen aikana järvellä toteutettiin kolme ravinnelisäyskoetta, joissa selvitettiin typen ja fosforin vaikutusta (yhdessä ja erikseen) järven päällysveden yhteisöihin. Sekä hiili- että ravinnekoheet osoittivat, että jopa samaan heimoon kuuluvien mikrobien välillä ravinnevasteissa oli erittäin suurta vaihtelua. Huolimatta kattavasta yhteisörakenteen kartoituksesta tämä toiminnallinen hajanaisuus vaikeuttaa ekologisesti yhtenäisten mikrobiryhmien määrittämistä. Lisäksi hiilenlisäyskoe antoi viitteitä siitä, että hiilipitoisuuden kohoaminen lisää järven metaani- ja hiilidioksidipäästöjä jopa niissäkin järvissä, joissa hiilipitoisuus on jo alun perin korkea. Ottaen huomioon humusjärvien yleisyyden tällä saattaa olla huomattava merkitys ilmastolle.

Kaiken kaikkiaan humusjärvien mikrobiyhteisöjen kartoitus osoitti, että näiden järvien anaerobisen alusveden mikrobiologia tunnetaan erittäin huonosti, sillä suuri osa hapettomissa kerroksissa vallitsevista bakteereista kuului viljelemättömiin ja siten hyvin heikosti tunnettuihin pääjaksoihin. Hiili- ja ravinnekoeteiden perusteella näyttää siltä, että nykyinen laajojen mikrobipääryhmien ekologian tuntemus ei riitä selittämään ravinteiden vaikutuksia yksittäisille populaatioille. Tämä korostaa tarvetta lisätä humusjärvien mikrobiyhteisöjen rakenteen ja toiminnan tutkimusta käyttäen uusimpia, biotekniikkaan ja bioinformatiikkaan perustuvia menetelmiä. Humusjärvien tuntemattomat mikrobiryhmät saattavat sisältää sekä ekologisesti että bioteknisesti merkittäviä geenivarantoja, ja tämä väitöskirja lisää huomattavasti tietämystä näiden mikrobiyhteisöjen rakenteesta ja merkityksestä.

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