Master's thesis

Phototrophic green sulphur bacteria in small stratified lakes with varying humic matter concentrations

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ABSTRACT

Chlorobium is a bacterial genus belonging to the family of green sulphur bacteria (*Chlorobiaceae*). These microbes are obligately anaerobic phototrophic organisms, which are often found in the hypolimnia of stratified lakes. The development of green sulphur bacteria requires stable anoxic conditions with the presence of reduced sulphur compounds and availability of light. Phototrophic growth with CO_2 as the carbon source is typical for green sulphur bacteria, and the carbon dioxide is fixed through the reductive TCA cycle. Small humic lakes with dark water colour warm up rapidly after ice break, and stratification with respect to temperature and oxygen develops providing an optimal growing habitat to *Chlorobium.* In this study the abundance of *Chlorobium* was studied in 13 humic lakes in Southern Finland with two different methods, length heterogeneity analysis of PCR-amplified 16S rRNA gene (LH-PCR) and bacteriochlorophyll concentration with spectrophotometer (BChl). *Chlorobium* was found from the hypolimnion of eight (62 %) of the study lakes. Water colour and light intensity on the oxic/anoxic boundary was found to best correlate with the depth of maximum abundance. Samples taken on April showed that *Chlorobium* bacteria can survive even in the heavily light limited conditions under ice, but the *Chlorobium* population was found closer to the bottom during winter than during the summer time. The calculated *Chlorobium* growth rates based on BChl concentrations showed variation between lakes implying that different physical and chemical conditions had prevailed in the lakes affecting the growth. The biomass generation times integrated and calculated over the whole water column indicates that although the whole *Chlorobium* biomass was growing relatively slowly in the lake the maximum observed generation times calculated for the depth of maximum abundance varied between 13-30 days.

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TIIVISTELMÄ

Chlorobiaceae-heimoon kuuluvat *Chlorobium*-suvun bakteerit ovat yhteyttäviä vihreitä rikkibakteereita, joita löydetään kerrostuneiden humuspitoisten järvien alusvesistä. Tämä mikrobiryhmä vaatii kasvaakseen hapettoman elinympäristön, jossa on saatavilla pelkistettyjä rikkiyhdisteitä sekä riittävä määrä valoa. Tyypillisesti vihreät rikkibakteerit kasvavat fototrofisesti hiilidioksidin avulla käyttäen hiilidioksidia pelkistävän TCA-syklin kautta. Tumman vetensä ansiosta pienet humuspitoiset järvet lämpiävät nopeasti jäiden lähdön jälkeen, ja muodostuva kerrostuminen lämpötilan sekä hapen suhteen luo optimaalisen kasvuympäristön *Chlorobium*-bakteereille. Tässä tutkimuksessa selvitettiin *Chlorobium*-suvun esiintyvyyttä 13 eteläsuomalaisessa humusjärvessä. Perustuen sekä DNA-pohjaiseen määritykseen (LH-PCR, length heterogeneity polymerase chain reaction) että bakteeriklorofyllin (BChl) määrään, *Chlorobium* löydettiin merkittävissä määrin kahdeksan (62 %) tutkimusjärven alusvedestä. Valon intensiteetti hapellisen ja hapettoman vesikerroksen rajapinnalla huomattiin olevan tärkeimpiä esiintymistä ja maksimikasvun syvyyttä selittäviä tekijöitä. *Chlorobium*-populaation todettiin selviytyvän talven yli rajoittuneissakin valo-olosuhteissa jään alla, mutta *Chlorobium*-bakteerit paikannettiin talvella lähempänä pohjaa kuin kesällä. Bakteeriklorofylli-konsentraatiosta lasketut *Chlorobium*-suvun kasvunopeudet osoittivat eroja eri järvien välillä, mikä saattaa johtua järvien erilaisista fysikaalisista ja kemiallisista olosuhteista. Vaikka järvien koko vesipatsaan *Chlorobium*-biomassat kasvoivat melko hitaasti huomattavasti nopeampia kasvunopeuksia (12-30 vrk) havaittiin laskennallisesti maksimiesiintymän syvyydellä.

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1. INTRODUCTION

Phototrophic green sulphur bacteria (GSB) are anaerobic phototrophic organisms that are found in the anoxic hypolimnia of stratified lakes (Van Gemerden and Mas 1995). Besides anoxic conditions they require reduced sulphur compounds and light for photosynthesis. CO_2 as a carbon source is typical for GSB, and carbon dioxide is fixed through the reductive TCA cycle. GSB cells contain special antenna complexes called chlorosomes that absorb the light energy with bacteriochlorophyll photopigments (Van Gemerden and Mas 1995). The abundance of green sulphur bacteria has been studied in different environments around the world, which has shown that the GSB can dominate in the anaerobic water layers of stratified water bodies (Van Gemerden and Mas 1995). Light play a critical role in photosynthetic reactions and it has been suggested that light is the main factor controlling primary production of GSB (Overmann 2006).

Stable physical and chemical conditions support the growth of GSB. In holomictic lakes such conditions can be provided only during steep thermal stratification in summer, while in meromictic lakes the physical and chemical gradients in the water column are more stable, and thus more suitable for the GSB (Van Gemerden and Mas 1995). Small lakes with dark water colour warm up rapidly in spring and as a result deep thermal stratification may develop very quickly (Salonen et al. 1984). Also spring and autumn overturns can both be incomplete in such lakes (Salonen et al. 1984) and these enable GSB survival in small sheltered lakes. In addition red light penetrates deeper than other wavelenghts in lakes with high humic matter concentrations (Eloranta 1978) and may favour the growth of GSB.

In small forest lake ecosystem *Chlorobium,* belonging to GSB family *Chlorobiaceae,* has been recognised as the most abundant phototrophic sulphur bacteria (Arvola et al. 1994, Taipale et al. 2009). The importance of GSB and especially *Chlorobium* in lake ecosystem is not fully understood. However, it seems that the abundace of *Chlorobium* in anoxic lakes may form an important carbon source after spring and autumn overturns (Taipale et al. 2009) as illustrated in Fig. 1. This dissolved organic carbon (DOC) can then be further used for heterotrophic bacterial growth. Another important influence for freshwater ecosystems is the ability of *Chlorobium* for mercury methylation in anoxic hypolimnia with the presence of sulphate (Verta et al. 2010).

This Master's thesis work was made in collaboration with the University of Helsinki and Lammi Biological Station. My purpose was to determine the abundance of *Chlorobium* in 13 steeply stratified small lakes with varying humic matter concentrations and light conditions. The hypothesis for the work was that *Chlorobium* sp. is abundant in stratified anaerobic water columns where sufficient amount of light is available. If this is not the situation, to find out what could be the other factors limiting the growth of *Chlorobium*. One aim was to study what happens to the *Chlorobium* community during the open water season and under ice. I also studied how well the length heterogeneity analysis of PCR-amplified 16S rRNA gene (LH-PCR) and bacteriochlorophyll concentration with spectrophotometer (BChl) methods correlate with each other, and if the simplified DNA extraction method correlates with the more laborious DNA extraction method with Mo-BIO DNA extraction kit.

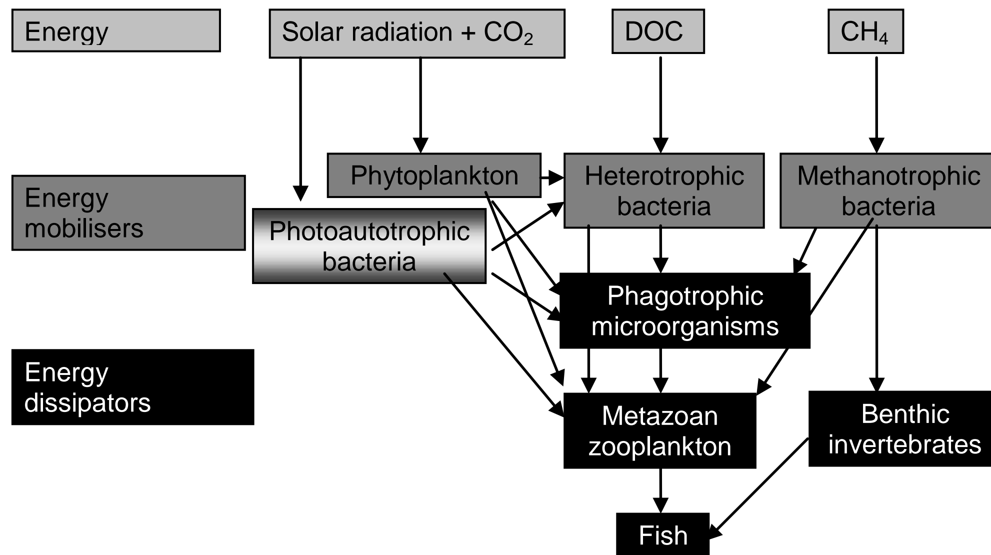


Figure 1. Energy mobilisation concept of lake ecosystem trophic structure (Jones & Grey 2010), modified with the addition of photoautotrophic bacteria as a source of autochthonous fixed carbon.

2. RESEARCH BACKGROUND

2.1. Ecology of green sulphur bacteria

The green sulphur bacteria (GSB) are organisms that have the ability to perform photosynthesis in anoxygenic conditions. Unlike cyanobacteria and eukaryotic algae, GSB are unable to use water as an electron donor and thus oxygen is not produced in the photosynthesis (Van Gemerden and Mas 1995). The anoxygenic photosynthesis is carried out on the basis of bacteriochlorophyll mediated processes. As stated before, instead of water as a photosynthetic electron donor, sulphide and other reduced sulphur compounds, but also hydrogen and a number of small organic molecules can be used, because of the required lower redox potential. Green sulphur bacteria are metabolic specialists, strictly anaerobic and obligately phototrophic (Van Gemerden and Mas 1995). However, GSB can also be potentially mixotrophs in the presence of inorganic reductants and CO_2, by using simple organic compounds for biomass formation (Overmann 2006).

Chlorobiaceae (GSB) are non-motile, spherical, and ovoid or vibrio shaped cells. All species lack flagella and phototrophic growth with CO_2 as carbon source is typical for green sulphur bacteria. GSB contain special structures called chlorosomes that are light-harvesting pigments attached to the internal face of the cytoplasmic membrane. Chlorosomes contain the major photosynthetic pigments (bacteriochlorophylls) of green sulphur bacteria (Imhoff 1995).

In the GSB, carbon dioxide is fixed through the reductive TCA cycle (Ormerod and Sirevåg, 1983). This metabolic route of CO_2 fixation is less energy-demanding, which may, at least in part, explain why GSB are able to photosynthesise at low light intensities. A green sulphur bacterium has been found to be growing in the Black Sea at a depth of 80m, where the light intensity is really low (<4 µE m^{-2} s^{-1}) (Overmann et al. 1992). Additionally, a green sulphur bacterium has recently been recovered from 2200 m below the surface of the Pacific Ocean, where it is believed to survive phototrophically on the black body radiation emitted by a black smoker (Beatty et al. 2005). Their abundance in

small boreal lakes, where the light penetration is heavily modified due to high humic concentrations (Eloranta 1978), has also been reported (Arvola et al. 1992; Taipale et al. 2009).

Two different species of green sulphur bacteria are known, green and brown. Green contains bacteriochlorophyll *c* or *d* and the carotenoid chlorobactene and OH-chlorobactene as light-harvesting pigments. The brown species has BChl *e* and the carotenoids isorenieratene and B-isorenieratere as light-harvesting pigments (Van Gemerden and Mas 1995). Green sulphur bacteria can be considered as a family of genetically related species. The similarity of the 16S rRNA gene of all GSB taxa (except on) one is >90.1% (Overmann 2006). Genus *Chlorobium* belonging to family *Chlorobiaceae* is the target organism in this study.

Chlorobium was prevailing in the hypolimnion of the small boreal lake Mekkojärvi (Taipale et al. 2009), and the high biomass of GSB was suggested to form an important carbon source for the whole lake after overturns. However, there is no clear evidence that the carbon they fix would have any impact overall for the trophic structure of their environments, because the anoxygenic hydrogen sulfide rich environment they occur is toxic for the most aerobic grazers (Van Gemerden and Mas 1995).

2.1.1. Growth rates and limitations

In addition to anoxygenic conditions other environmental factors required for the growth of GSB are the availability of light and the presence of reduced sulphur compounds. Light plays a critical role in photosynthesis and a good correlation between light and GSB has been observed suggesting that light is the main factor controlling primary production of phototrophic sulphur bacteria. Solar radiation is absorbed and scattered in the water column and the irradiance is usually very low, on the order of a few $\mu E\ m^{-2}\ s^{-1}$. Not only the irradiance is low, but also the spectral composition of light is modified due to specific absorption of certain wavelengths by water itself, and very often by populations of algae, other phototrophic bacteria and/or DOM and POM. This makes it possible for GSB to be abundant both in shallow lakes and in deeper clean water lakes, where the algae concentration is lower. This relationship, however, does not necessarily indicate that phototrophic bacteria are limited by light in all cases, because all the other required conditions also have to be present. (Van Gemerden and Mas 1995)

The GSB are oxygen sensitive and can survive only in the anoxic water layers of lakes. In stratified holomictic environments, the exposure to oxygen occurs only during the mixing periods. Another cause of viability loss is insufficient supply of energy (Van Gemerden and Mas 1995). Phototrophic bacteria are unable to gather enough energy to fuel vital processes when the irradiance falls below a certain threshold. As hydrogen sulfide is a toxic compound for most of the potential predators, it is assumed that predation does not play a very important role in controlling GSB populations (Van Gemerden and Mas 1995). Yet, the presence of crustaceans and rotifers on top of the oxic/anoxic interface feeding on the phototrophic bacteria underneath has been repeatedly reported (Takahashi and Ichimura 1968; Caumette et al. 1983; Salonen & Lehtovaara 1992). However, any actual impact of this feeding on dynamics of the microbial community hasn't been indicated. Since sedimentation is one of the main mechanisms through which planktonic organisms are removed from the water column (Van Gemerden and Mas 1995), it is presumably so also for *Chlorobium*.

2.1.2. Chlorosomes and bacteriochlorophylls

GSB contain antenna complexes known as chlorosomes. These membrane associated antenna complexes are found only in green sulphur bacteria. Cohen-Bazire et al. (1964) were the first ones who reported chlorosomes. Chlorosomes are elliptical structures, which are arranged in a single layer underlying the cytoplasmic membrane (CM). Their function is to absorb light and transfer the energy to the photochemical reaction center, where photochemical energy storage takes place. Green sulphur bacteria contain also another unique antenna complex: a bacteriochlorophyll a (BChl *a*) containing protein known as the Fenna-matthews-Olson (FMO) protein (Blankenship et al. 1995). Most of this BChl *a* is localized in the CM while most of the BChls *c, d* or *e* in chlorosomes (Oelze and Golecki 1995).

Chlorosomes constitutes of bacteriochlorophylls which are the central pigments of the photosynthetic bacteria. Bacteriochlorophylls function as antenna pigments in the light harvesting complexes and as accessory and special pair chlorophylls in the chlorosome reaction center (Senge and Smith 1995). All GSB contain BChl *c, d* or *e* which is found inside the chlorosome and is thought to be associated with rod-element ca. 5-10 nm in diameter.

In addition to chlorophylls the chlorosomes contain also lipids, proteins and carotenoids. The protein concentration in chlorosome is surprisingly low, relative to the large amount of pigment (Oelze and Golecki 1995). The GSB contain considerable amounts of glycolipids, the major part of which (more than 80 %) is associated with the chlorosomes (Imhoff and Bias-Imhoff 1995).

2.2. Quantifying methods of green sulphur bacteria

The green sulphur bacteria *Chlorobium* have been studied since it was found in 1906 (Nadson). Different methods are used and with rising knowledge more accurate techniques are developed. The most often used method is to study the presence and concentration of bacteriochlorophyll (BChl). The BChl absorption maximum can be detected with wavelengths 654 nm and 656 and the amount of bacteria can be calculated from equation given by Takahashi and Ichimura (1970). 16S rRNA gene sequencing technique has opened many different DNA based methods to study the bacterial composition and existing 16S rRNA gene libraries are useful tools for bacteria indication. Other methods to quantify the microbial community structure are phospholipid fatty acid analysis and cell cultures. Microscope techniques are also used in identification and characterization.

2.2.1. 16S rRNA LH-PCR

The most used phylogenetic chronometer with bacteria is 16S rRNA (small subunit ribosomal RNA), which has proven to be a stable and specific phylogenetic marker (Brock et al. 1994). The degree of similarity in ribosomal sequences between two organisms indicates their relative evolutionary relatedness (Fox et al. 1980, Woese 1987). Wayne et al. (1987) suggests that organisms sharing more than 97 % identity in their full-length 16S rRNA sequences might belong to one and same species.

With the availability of the 16S rRNA based techniques new methods for molecular identification of variety micro-organism products have been developed. Denaturing gradient gel electrophoresis (DGGE) (Muyzer et al. 1993), length heterogeneity of PCR-amplified 16S rDNA gene (LH-PCR) (Suzuki et al. 1998), single-strand conformation polymorphis (SSCP) (Lee et al. 1996), restriction fragment length polymorphism (RFLP) (Avaniss-Aghajani et al. 1994), terminal restriction fragment length polymorphism (T-

RFLP) (Liu et al. 1997) and ribosomal intergenic spacer region analysis (RISA) (Bonerman & Triplet 1997) are all fingerprinting methods used to analyse microbial communities.

Saiki et al. (1988) introduced polymerase chain reaction (PCR) in 1988, which is an in vitro technique to exponentially amplify specific DNA sequences using primers corresponding to conserved microbial priming sites. Suzuki et al. (1998) developed LH-PCR, which determines the relative proportions of amplicons originating from different organisms by measuring the fluorescence emission of a labelled primer used in the amplification region. The identification of different organisms in LH-PCR is based on natural variation in the length of small-subunit rRNA genes (Suzuki et al. 1998). LH-PCR is an effective tool for assessing microbial community structure. The band sizes (Fig. 2) can be compared against 16S rRNA sequence libraries to specify bacterial groups that may correspond in size to the size of the band (Tiirola 2002).

Taipale et al. (2009) studied the vertical diversity of bacteria in an oxygen-stratified humic lake, Mekkojärvi, by using DNA techniques and phospholipid fatty acid (PLFA) analysis. They noticed that sequences assigned to the photoautotrophic green sulphur bacterium *Chlorobium sp.* dominated the anoxic water column. The sequence analysis allowed Taipale et al. (2009) to connect sequence data with distinct lengths in the LH-PCR profiles. The sequence size 512 pb was a specific biomarker for Chlorobi, especially for the genus *Chlorobium*.

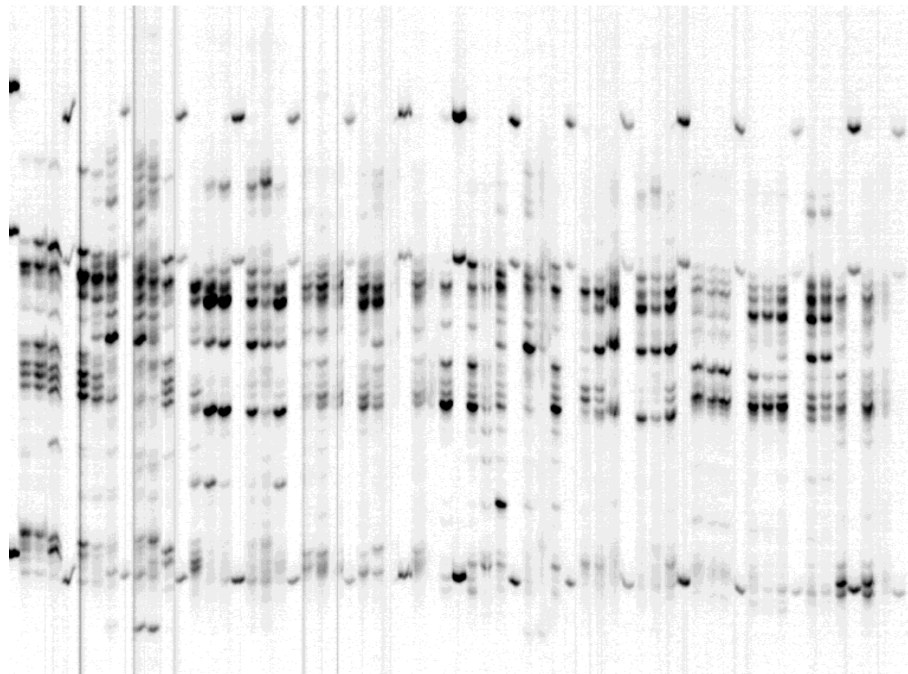


Figure 2: LH-PCR illustrated with digital image from electrophoresis gel.

2.2.2. Spectrophotometry

The chlorophyll a (Chl *a*) and bacteriochlorophylls *c, d* and *e* (BChl) have characteristic absorption spectra, which can be determined with spectrophotometer. The absorption properties of Chl *a* and BChls are different and can be separated according to this variations from each other. The samples can be diluted in different solvents, such as methanol, acetone, ether, ethanol, or chloroform. The Chl *a* absorption can be detected with wavelength 665 nm and BChls with wavelength 654 nm and 656 nm, while the wavelength 750 nm is used as a background noise (Arvola et al. 1992). The Table 1 below

shows the normalized absorption maximums of Chl *a* and b and BChl *a, b, c, d* and *e* in methanol or ethanol.

Table 1. Absorption maximums of Chl and BChl in methanol or ethanol

Pigment	Chl *a*	Chl *b*	BChl *a*	BChl *b*	BChl *c*	BChl *d*	BChl *c*
Absoprtion maxima (nm)	430, 664	463, 648	364, 770	373, 795	434, 666	427, 655	469, 654

3. MATERIALS AND METHODS

3.1. Study site

The study lakes are located in the Evo forest region in southern Finland (Fig. 3). All together 13 different lakes were sampled according to the sampling program shown in Table 3. The lakes were chosen to the study because of their small size, water colour, and steeply stratified water column regarding to oxygen, temperature, and hydrogen sulphite concentration. All the lakes have ice cover approximately from November to May. After ice-break the dark water in humic lakes warms rapidly resulting steeply stratified water column. Spring and autumn overturn mixing is often incomplete.

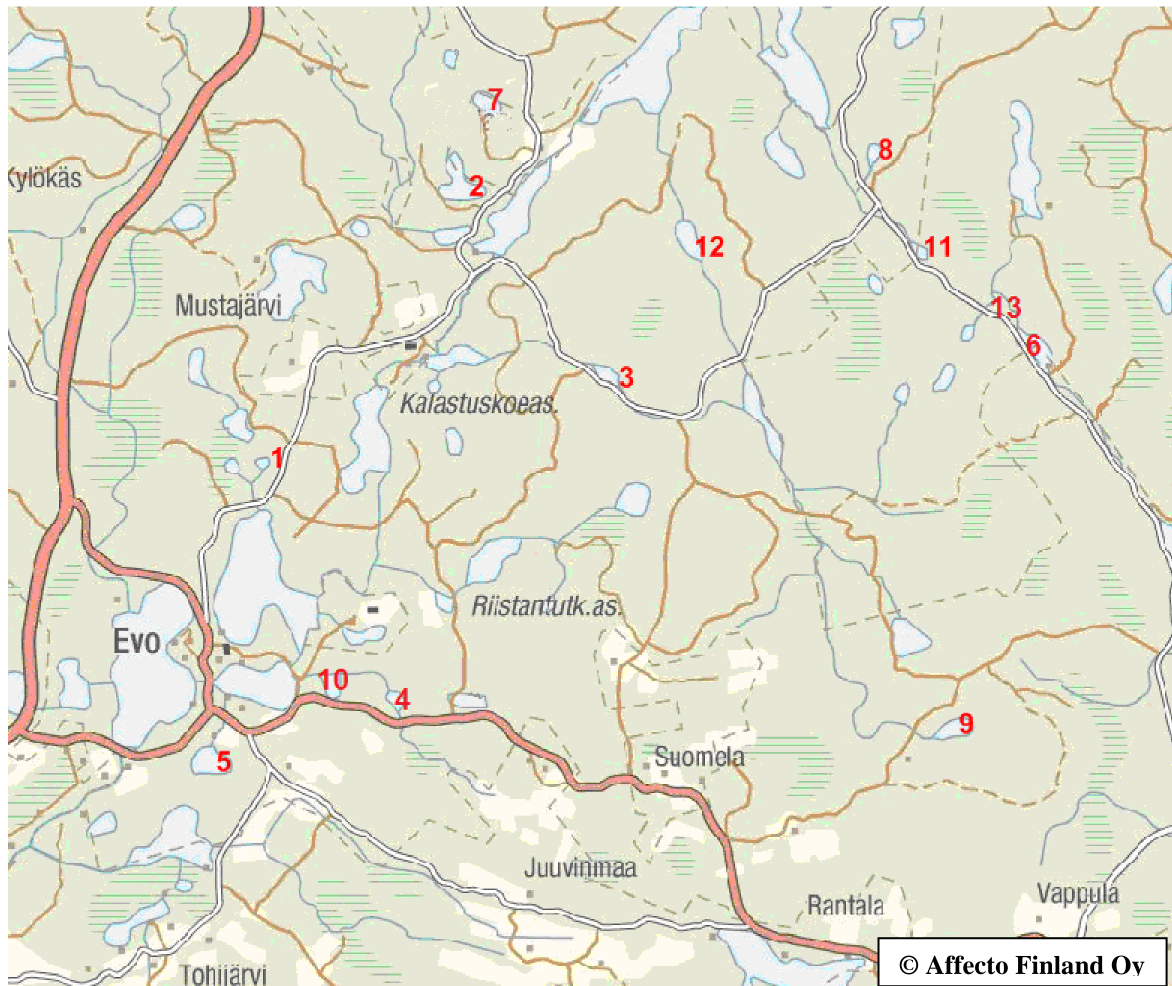


Figure 4: Sampling sites in Evo region Southern Finland: 1 Alinen Mustajärvi, 2 Halsjärvi, 3 Horkkajärvi, 4 Huhmari, 5 Iso-Valkjärvi, 6 Keskinen Rajajärvi, 7 Mekkojärvi, 8 Nimetön, 9 Rieskalampi, 10 Särkijärvi, 11 Tavilampi, 12 Vähä-Keltajärvi, 13 Ylinen Rajajärvi

Table 2 gives some background information of the lakes. All the lakes except Ylinen Rajajärvi and Keskinen Rajajärvi belong to long-term monitoring program of the Lammi Biological Station, University of Helsinki, and the Finnish Game and Fisheries Research Institute, where water chemistry of the lakes is monitored regularly twice a year.

Table 2. Background information of the lakes. SA=surface area, CA= catchment area, GW= groundwater lake, SW= surface runoff lake, S= sand, T= till, * Colour values from year 2008 (Karhu 2010)

Lake	SA	CA:SA	Max. depth (m)	Hydrology	Soil type	pH	Colour (mg Pt/L)
Alinen Mustajärvi*	0.7	6.0	7	GW	S	5.37	101
Halsjärvi	4.7	2.9	6	SW	T/S	6.29	130
Horkkajärvi*	1.1	56.4	10	SW	T	5.68	304
Huhmari*	1.6	63.8	9	SW	S	6.20	226
Iso-Valkjärvi*	4.2	6.1	7	GW	S	5.64	71
Keskinen Rajajärvi	1.2	151.2	12	SW	T	5.00	271
Mekkojärvi*	0.3	82.5	4	SW	T	5.94	432
Nimetön*	0.4	80.8	10	SW	T	5.51	240
Rieskalammi*	2.5	9.0	5	SW	T	6.24	459
Särkijärvi*	1.8	26.6	3	GW	S	6.39	236
Tavilampi*	0.8	22.6	7	SW/GW	T/S	5.90	149
Vähä-Keltajärvi	2.5	52.9	4	SW	T	5.82	341
Ylinen Rajajärvi	0.1	1098.5	6	SW	T		243

From the background information it is possible to calculate different parameters. Formula [1] shows the extinction coefficient (Jones & Arvola 1984), formula [2] shows the depth of euphotic layer which threshold is normally considered 1 % (Jones & Arvola 1984), formula [3] shows the maximum amount of light in different depths on a bright sunny day (Wetzel 1983) and formula [4] shows the approximate amount of dissolved organic carbon (DOC) (Karhu 2010).

$$y= 0.011x + 0.60 \quad [1]$$

Where x is the water colour mg Pt/L

$$y = 26.7*x^{-0.53} \quad [2]$$

Where x is the water colour mg Pt/L

$$I_z = I_o e^{-\eta z} \quad [3]$$

Where I_o is the maximum light intensity at the surface during a bright day (approximately 1400 $\mu E\ m^{-2}\ s^{-1}$), e is neper value (2.718), η is extinction coefficient and z is the depth distance in meters.

$$y=0.050x+3.534 \quad [4]$$

Where x is the water colour mg Pt/L

3.2. Sampling

The lakes were sampled in four different sampling times (Table 3), four lakes in April under ice cover (6-9.4.2009), three lakes in May just after ice cover was melted (14-15.5.2009), 13 lakes in July when the lakes are steeply stratified (13-14.7.2009 and 20-23.7.2009) and three in August before the onset of autumn mixing (11.8.2009). Deepest part of the lake was located with echo-sounder and the sampling was performed on that site. Water samples were taken from the whole water column of the lake on 1 m intervals with Limnos-type water sampler (height 30 cm, volume 2.1 L) and with 0.5 m interval from the oxic/anoxic layer. Water temperature, oxygen concentration, redox and pH were measured *in situ* with YSI-meter (YSI 556 MPS, Yellow Springs Instruments) on the lake.

Table 3. Showing the sampling times of each lake

Lake	6-9 April	14-15 May	13-27 July	11 August
Alinen Mustajärvi	x	x	x	x
Halsjärvi	x	x	x	x
Mekkojärvi		x	x	x
Horkkajärvi	x		x	
Nimetön	x		x	
Särkijärvi			x	
Huhmari			x	
Iso-Valkjärvi			x	
Keskinen Rajajärvi			x	
Ylinen Rajajärvi			x	
Vähä-Keltajärvi			x	
Tavilampi			x	
Rieskalampi			x	

Two bottles were filled from each depth, 1 L in dark bottle for chlorophyll analyses and 1 L in plastic bottle for DNA experiments. The bottles were placed in cold and protected from the light. The samples were further processed during the same day.

3.3. Chlorophyll

For Chl and BChl samples, 120-500 mL sample water was filtered through 45 mm Ø Whatman glass fiber filters (GF/C) in the dark. The filters were then placed in 10 ml plastic tubes enfold with aluminium foil and stored in a freezer. Rest of the filtered water was used to measure nutrient fractions of inorganic nitrogen (N/NO_2+NO_3) and ammonium (N/NH_4).

Chlorophyll *a* and bacteriochlorophyll *d* were measured with spectrophotometer with different wavelengths. 10 mL of 96 % ethanol was added to plastic tubes with the filters. Tubes were incubated in 75 ˚C water bath for 5 minutes and then placed in dark to cool down. After that the samples were filtered through Whatman glass fiber filters 25 mm Ø and the extract was placed in plastic scintillation bottles and measured with Shimadzu UV-

visible recording spectrophotometer UV-2100 (Shimadzu Corporation, Japan). Measured wavelengths were 665 and 750 nm for Chl *a* and 654, 656 and 750 nm for BChl *d*. The samples were protected from the light whole time.

The spectrophotometer was autozeroed and baselined with the same 96 % ethanol that was used to extract the samples and every 20th sample was sole 96 % ethanol to control that the measuring level was stable. From the spectrophotometer results Chl *a* concentrations were calculated with formula [5] (Lorenzon 1967) and BChl *d* with formula [6] (Takahashi & Ichimura 1970).

$$\text{Chl } a = \frac{(D_{665} - D_{750}) \text{ x } 10000}{83.4 \text{ x V}} \qquad [5]$$

Where the background noise D_{750} is taken in to consideration and V is the amount of filtered water in litres.

$$\text{BChl } d = 10.2 \text{ x } (D_{654} - D_{750}) \text{ x v/V} \qquad [6]$$

Where the background noise is taken in consideration and v is the volume of ethanol in ml and V is the amount of filtered water in litres.

Arvola et al. (1992) suggests that the 665/656 ratio can be used to indicate the shift from Chl *a* to BChl *d* in the hypolimnion. When the ratio is >1 Chl *a* is a dominant pigment and when the ratio declines clearly under 1 BChl *d* is the dominant pigment. The concentration of other pigments (Chl *b, c*) also influence the result, but the ratio gives a rough picture of the situation. Normally the ratio changes steeply in the metalimnion or in the upper part of the hypolimnion, where the amount of photosynthetic bacteria rises (Arvola et al. 1992).

3.4. 16S rRNA LH-PCR

From all of the summer samples (May, July and August) bacterial lysates were made instead of normal DNA extraction to save time and to test this method. Water samples in 2 ml eppendorf tubes were melted down and centrifuged 20 000 g for 5 minutes. Supernatant was removed (carefully; because the pellet gets easily loose) by pipetting, and 50 µl of 0.05 M NaOH/ 0.25 % SDS-solution was added to the tube. The samples were incubated in 95 ˚C water bath for 15 minutes and finally the samples were diluted with 950 µl sterile water.

DNA extraction with MO BIO PowerSoil DNA isolation kit (MO BIO Laboratories, CA) was made from under ice samples (April) following the instructions. The samples were first freeze dried (Dryer Christ ALPHA 1-4 LD Plus, Germany) and then further processed. To test that the same results are achieved with DNA extraction and bacterial lysates methods four samples were prepared with both methods. 16S rRNA LH-PCR was performed of each sample and the results were compared.

From bacterial lysates and DNA extractions 16S rRNA gene was amplified with PCR. The PCR was performed using IRD labeled forward primer 27 F (Lane 1991) and PRUN518r (Muyzer et al. 1993) reverse primer. Amplified fragments are 465 to 565 bp long (Tiirola 2003). PCR Master Mix was prepared (Final concentrations: 1 x DreamTaq Green buffer, 0.3µM 5'-primer 27F/$^{1/5}$-ird F8, 0.3µM 3'-primer PRUN 518r, 0.2mM dNTPs mix, 0.5mg/ml BSA, sterile water and 0.05U/µl Dream Taq polymerase) and mixed with templates in relation of 24 µl Master Mix to 1 µl template. PCR was performed with Bio-Rad S1000 Thermal Cycler (Bio-Rad Laboratorios, CA). Initial denaturation and

enzyme activation step of 5 minutes at 95 ºC was followed by amplification for 35 cycles at the following conditions: 30 seconds at 94 ºC, one minute at 52 ºC and three minutes at 72ºC. A final 20 minute extension at 72 ºC completed the protocol. For a positive control a known *Pseudomonas* sample was used. Agarose gel electrophoresis was used to control PCR outcome. Large agarose gel (0.8%) was prepared by mixing 1.6 g of agarose with 200 ml 1xTAE and 2.5 µl SybrSafe DNA-binding dye. The agarose/buffer solution was heated up in a microwave oven until all the agarose was completely dissolved. After cooling down to about 60 ˚C the SybrSafe dye was added and the gel was poured on the electrophoresis tray. The gel was placed in electrophoresis system and filled with TAE-buffer. No loading buffer was needed since to the loading buffer was already included in the DreamTaq Green buffer. The gel was run at 160 V for 60 minutes. To illustrate separation of DNA-fragments the agarose gel was photographed with blue light table.

The gel electrophoresis for LH-PCR was performed with an automated LI-COR 4200 sequencer (LI-COR BioTech, NE). The data was analyzed using Quantity One software (Bio-Rad Laboratoires, CA). Size standards of 470, 527 and 553 bp were prepared earlier by amplifying *Sphingomonas* sp., *Yersinia* sp. and *Lactobacillus* sp. by the same method and primers as used for the samples. 6 % acrylamide gel was prepared by adding 250 µl ammoniumpersulfate and 25 µl TEMED to 50 ml of Long Ranger 6 % acrylamide solution (FMC Bioproducts, ME). The gel was casted between glass plates that were assembled with spacers and rails. The gel was casted avoiding bubbles and let to dry for 1 hour or more. A loading mixture was prepared (10 % buffer, 33% of Licor Loading Dye, 57% water) and 1 µl of the PCR product was added to 19 µl of the loading mixture. Before loading the samples in the gel they were heated at 94 ˚C for 5 minutes and cooled down with ice. Finally the electrophoresis was performed for six hours. LH-PCR biomarker 512 bp was selected based on Taipale et al. (2009), since in the library of 453 clones all the clones related to this size was assigned to the phylum Chlorobi (from all the clones 43% was assigned to this), so the data was analyzed and samples that contained bands with length of 512 ± 1 bp were recorded to allow some inaccuracy in the length estimation.

3.5. *Chlorobium* diagnostics

For *Chlorobium* diagnostics in lakes three different parameters were used. Set limits for significant *Chlorobium* biomasses were: 665/656 ratio <0.7 in hypolimnion indicating the shift from Chl *a* to BChl, maximum BChl concentration >4 µg/l in the anoxic hypolimnion and the maximum relative abundance of 511-513 bp long fragments in LH-PCR >10%.

3.6. Data analysis

For analysing the data Microsoft office Excel (Microsoft®) and SigmaPlot 11.0 (Systat Software Inc., California) were used. Statistical analysis was made with PASW Statistics 18 (IBM Corporation, NY).

4. RESULTS

4.1. Lake characteristics

All the sampled lakes were steeply stratified with respect to temperature and oxygen and in all the lakes the anoxic hypolimnion was thicker than the oxic epilimnion, however, water volume was smaller. The anoxic water from the bottom of the lakes smelled like rotten egg, indicating the presence of H_2S. Measured ammonium (N/NH_4) concentrations varied between 5-100 µg/L in the epilimnion and metalimnion, but were much higher (600-4600 µg/L) in the hypolimnion. Nitrate/nitrite concentrations (NO_3/NO_2) were low in all lakes varying between 30-100 µg/L throughout the water column. Other measured or calculated variables of lakes are shown in Table 4 and nitrate/nitrite, ammonium, temperature, oxygen and light intensity profiles in Appendix 1.

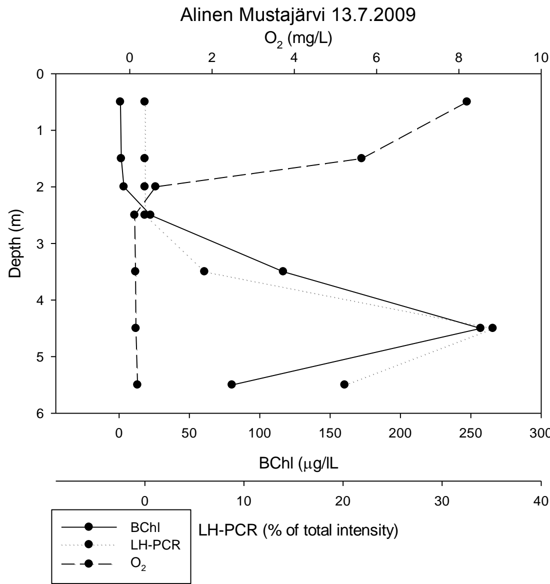
Table 4. Showing measured and calculated variables of study lakes on July.

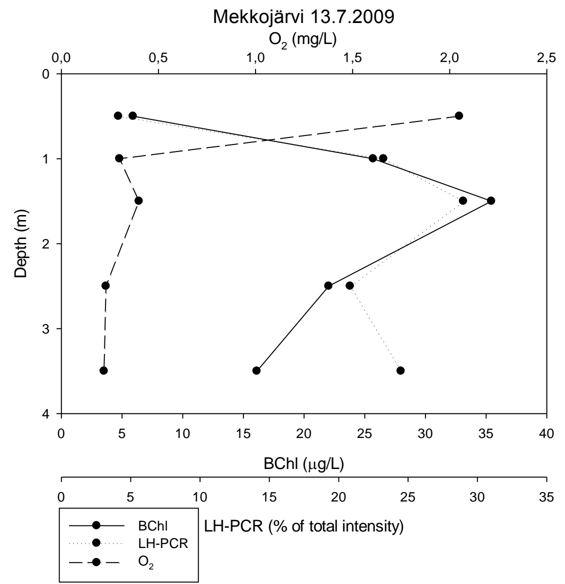
Lake	Extinction coefficient[1]	Euphotic layer[1] (m)	DOC[1] (mg C/L)	Depth of oxic epilimnion[2] (m)
Alinen Mustajärvi	1.7	2.3	8.6	2
Halsjärvi	2.5	1.8	12.0	2
Horkkajärvi	3.9	1.3	18.7	1.5
Huhmari	3.1	1.5	14.8	1.5
Iso-Valkjärvi	1.4	2.8	7.1	4
Keskinen Rajajärvi	2.0	1.4	17.1	5.5
Mekkojärvi	5.4	1.1	25.1	1
Nimetön	3.2	1.5	15.5	2
Rieskalammi	5.6	1.0	26.5	1
Särkijärvi	3.2	1.5	15.3	1.5
Tavilampi	2.2	1.9	11.0	2
Vähä-Keltajärvi	4.3	1.2	20.6	2
Ylinen Rajajärvi	3.3	1.5	15.7	4.5

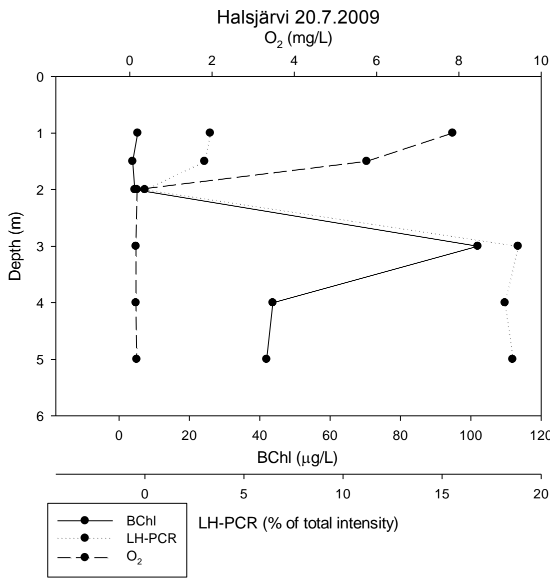
1) Calculated from color values, formulas given in chapter 3.1.
2) Measured in this study

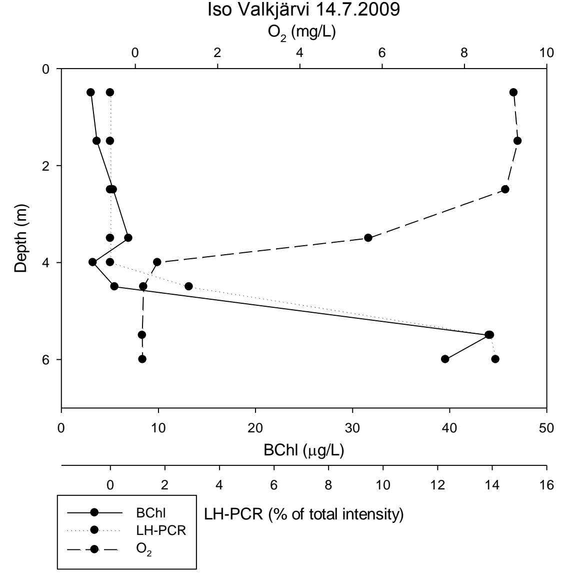
4.3. Abundace of *Chlorobium*

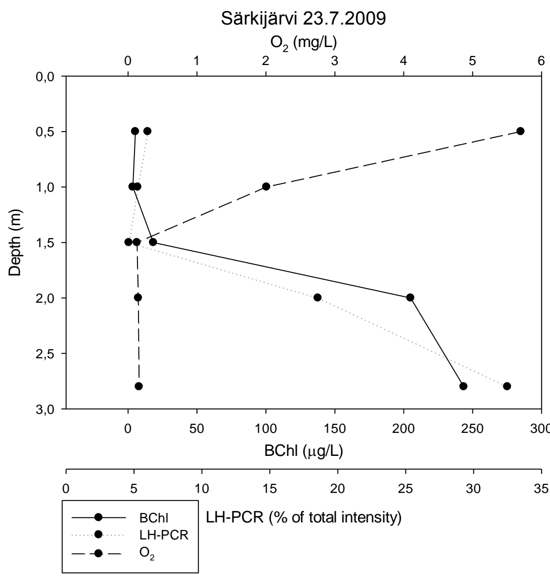
In eight lakes out of 13 there was a clear evidence of significant *Chlorobium* community (Fig. 4). Only in Ylinen Rajajärvi, Keskinen Rajajärvi and Vähä-Keltajärvi no *Chlorobium* was detected. In lakes Horkkajärvi and Rieskalammi some BChl concentrations were observed, but all set limits were not fulfilled (chapter 3.5.). Based on the BChl concentrations the *Chlorobium* biomass communities were located in the anoxic hypolimnion and the maximum abundance was usually detected in the upper part of the hypolimnion

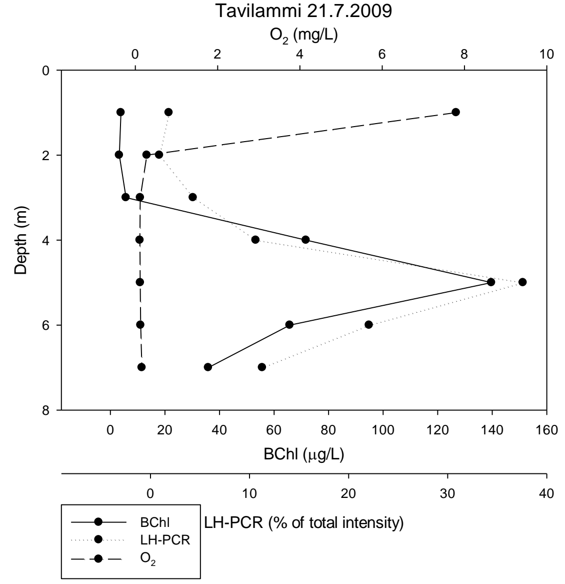

Alinen Mustajärvi 13.7.2009
O2 (mg/L)
Depth (m)
BChl (μg/L
LH-PCR (% of total intensity)
BChl
LH-PCR
O2


Mekkojärvi 13.7.2009
O2 (mg/L)
Depth (m)
BChl (μg/L)
LH-PCR (% of total intensity)
BChl
LH-PCR
O2


Halsjärvi 20.7.2009
O2 (mg/L)
Depth (m)
BChl (μg/L)
LH-PCR (% of total intensity)
BChl
LH-PCR
O2


Iso Valkjärvi 14.7.2009
O2 (mg/L)
Depth (m)
BChl (μg/L)
LH-PCR (% of total intensity)
BChl
LH-PCR
O2


Särkijärvi 23.7.2009
O2 (mg/L)
Depth (m)
BChl (μg/L)
LH-PCR (% of total intensity)
BChl
LH-PCR
O2


Tavilammi 21.7.2009
O2 (mg/L)
Depth (m)
BChl (μg/L)
LH-PCR (% of total intensity)
BChl
LH-PCR
O2

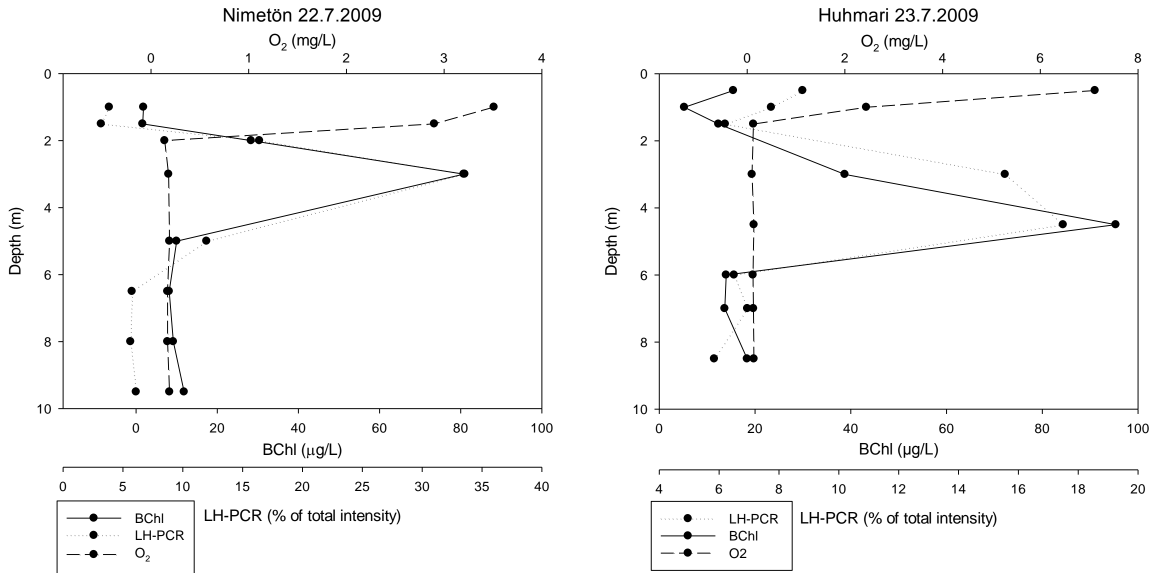


Figure 4. Vertical distribution of oxygen, BChl and LH-PCR in the eight lakes where significant *Chlorobium* biomass and abundance were detected.

In those lakes where *Chlorobium* was abundant, the results from two different methods (LH-PCR and BChl) gave very consistent results (Fig. 5). The results from other study lakes are presented in Appendix 1 and Appendix 2. To study the correlation between LH-PCR and BChl results the values were transformed to % of the lake maximum value so that in every lake the highest measured value was set to 100%. Regression model calculation between these two methods showed clear correlation r^2=0.786 and p<0.001.

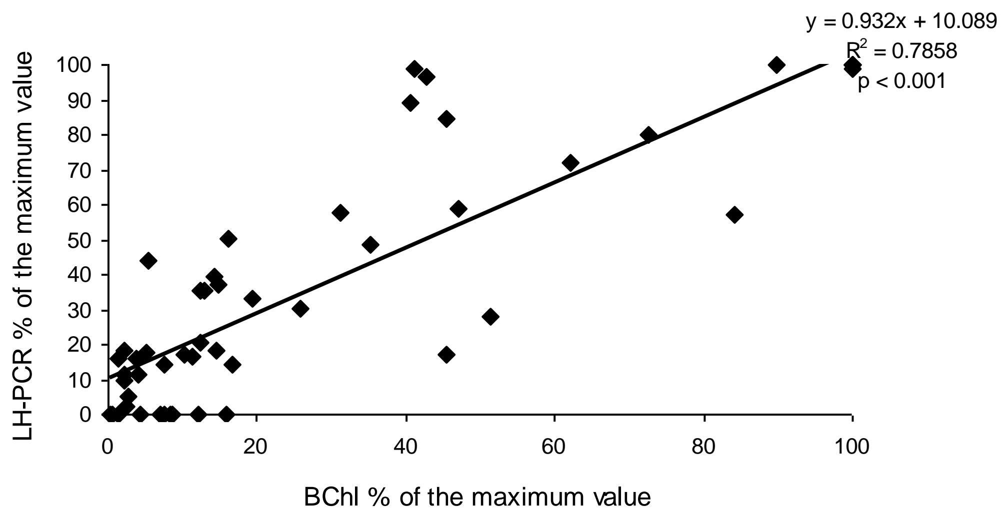


Figure 5. The relationship between LH-PCR and BChl methods to detect GSB.

The high correlation between BChl and LH-PCR methods shows that the connection between these parameters is reliable and makes it possible to investigate the relationship between vertical distribution of bacteria and water chemistry. Both BChl and LH-PCR methods showed the maximum values for the same depths in different lakes r^2=0.98 (data not shown).

4.2. The role of light intensity

The light intensities from different depths were calculated and the intensity on the oxic/anoxic boundary was determined (Fig. 6). Both, lowest irradiance on the oxic/anoxic boundary and lowest BChl concentrations (Fig. 7) were found from lakes Keskinen Rajajärvi, Ylinen Rajajärvi and Vähä-Keltajärvi.

The lakes were divided in two groups: 1) lakes where *Chlorobium* is abundant (n=8) and 2) lakes without significant *Chlorobium* biomass (n=5) based on criteria described in chapter 3.5. Differences between the light intensities at the oxic/anoxic boundary were compared between the two lake groups (Fig. 8). When the average light intensities were calculated, group one had 13.9 µE m^{-2} s^{-1} and group two 1.8 µE m^{-2} s^{-1}. An independent samples T-test was made to observe the statistical importance of the difference in the light intensity at the oxic/anoxic boundary, and a statistical difference was observed (p=0.052). Of the lakes in group two Horkkajärvi and Rieskalammi are lakes in which some BChl and LH-PCR fragments were observed, but in those lakes the *Chlorobium* community was seemingly very sparse. These lakes increased the average light intensity value of group two.

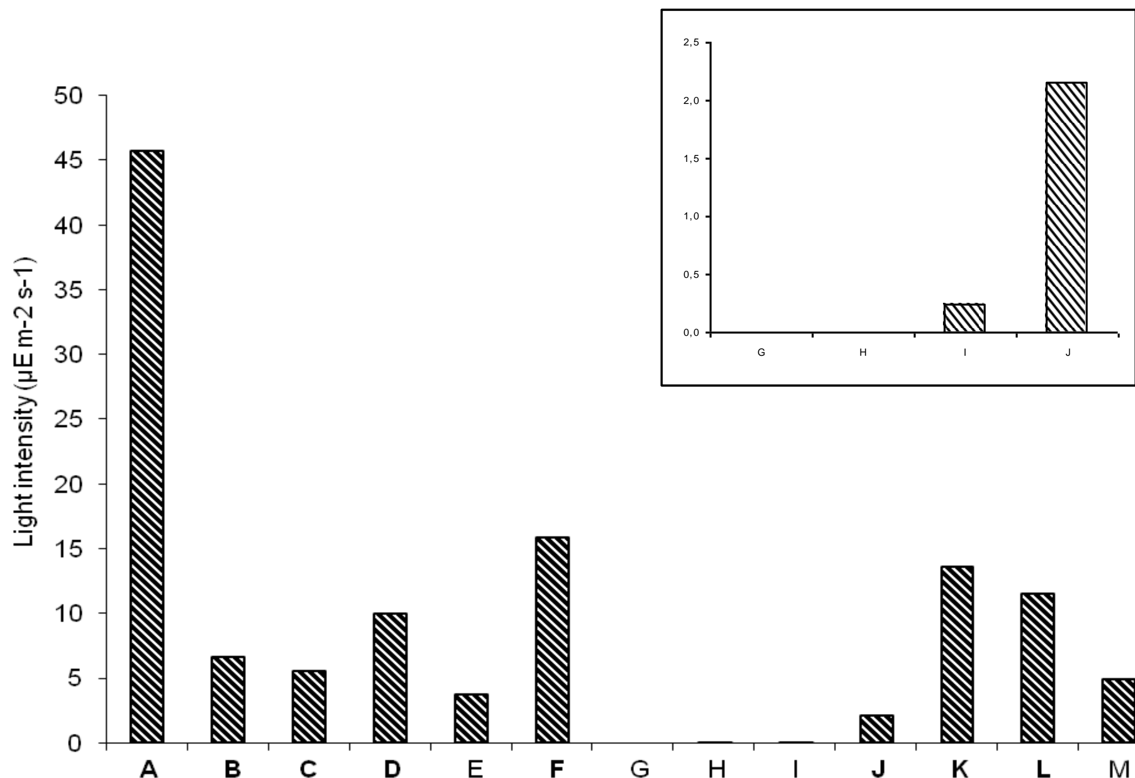


Figure 6. Light intensities (µE m^{-2} s^{-1}) at the boundary between the oxic and anoxic water layers. A= Alinen Mustajärvi, B= Mekkojärvi, C= Iso Valkjärvi, D=Halsjärvi, E=Horkkajärvi, F=Tavilammi, G=Keskinen Rajajärvi, H=Ylinen Rajajärvi, I=Vähä-Keltajärvi, J=Nimetön, K=Huhmari, L=Särkijärvi and M=Rieskalammi. Lake characters A-D, F, J-L are bolded to indicate lakes with significant *Chlorobium* growth. Inner picture: upscaled data of lakes with lowest light intensities.

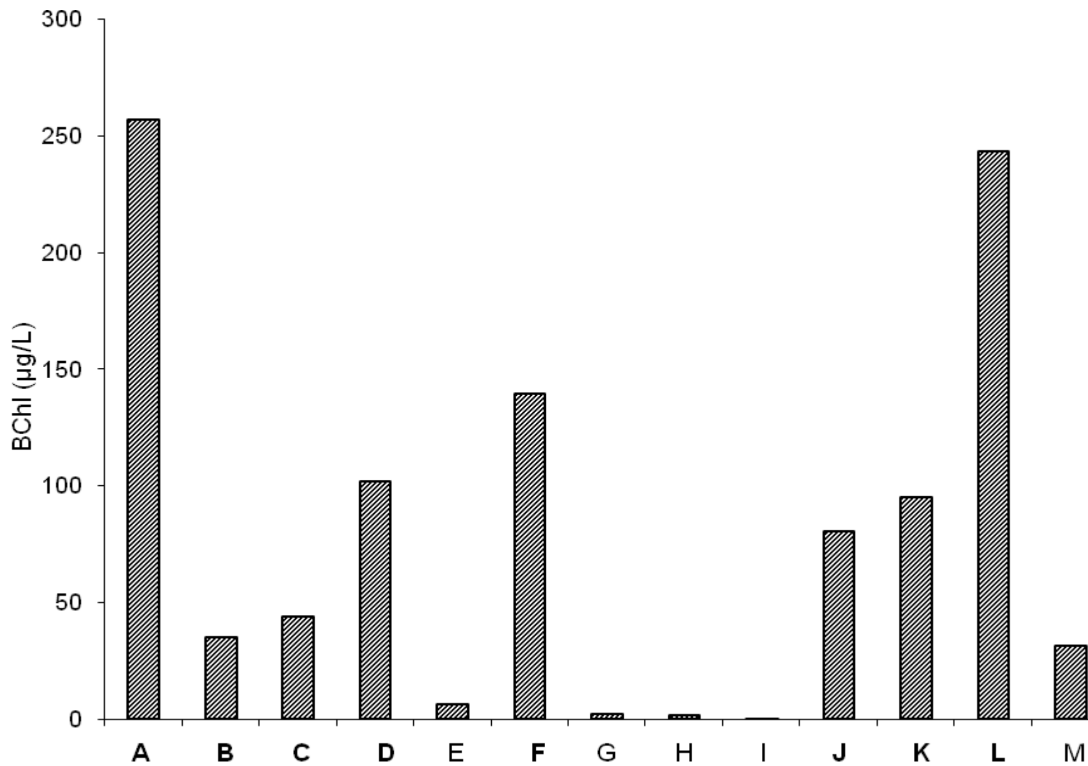


Figure 7. Maximum observed BChl (µg/L) concentrations in the study lakes. A= Alinen Mustajärvi, B= Mekkojärvi, C= Iso Valkjärvi, D=Halsjärvi, E=Horkkajärvi, F=Tavilammi, G=Keskinen Rajajärvi, H=Ylinen Rajajärvi, I=Vähä-Keltajärvi, J=Nimetön, K=Huhmari, L=Särkijärvi and M=Rieskalammi. Lake characters A-D, F, J-L are bolded to indicate lakes with significant *Chlorobium* growth.

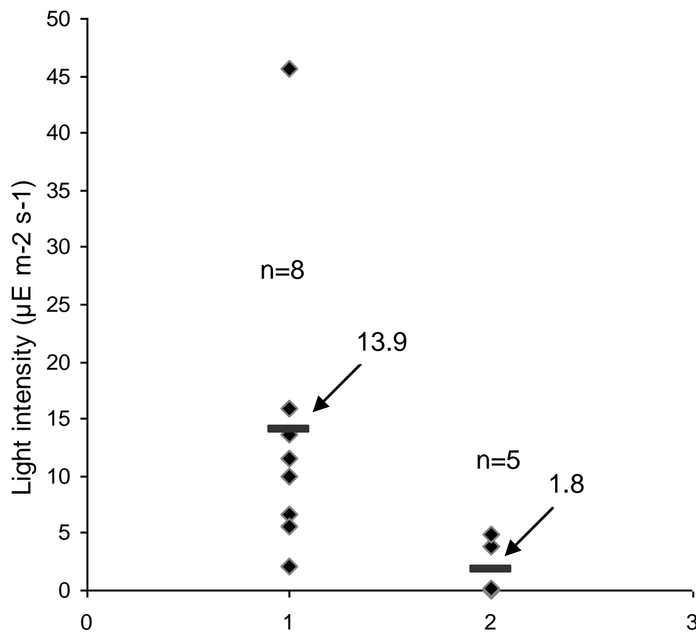


Figure 8. Light intensities on the oxic/anoxic boundary in lakes belonging to group 1 and 2. Group 1 with *Chlorobium*, group 2 without significant *Chlorobium* biomass.

Calculated and measured parameters were compared to see which ones have an effect on the microbial abundance and which parameters could be used to predict the abundance. Lakes where *Chlorobium* was found were used (n=8). A clear correlation was found between water colour and the depth of maximum BChl abundace (p=0.006 and r^2=0.743) (Fig. 9) and between light intensity on the oxic/anoxic boundary and maximum BChl concentration (p=0.038 and r^2=0.540) (Fig. 10). No correlation could be seen between the maximum BChl concentration and light intensity on this depth (p=0.861 and r^2=0.006) (Fig. 11). The results are showing that the maximum BChl correlated better with the light intensity on the oxic/anoxic boundary than the light intensity on the depth of maximum abundance.

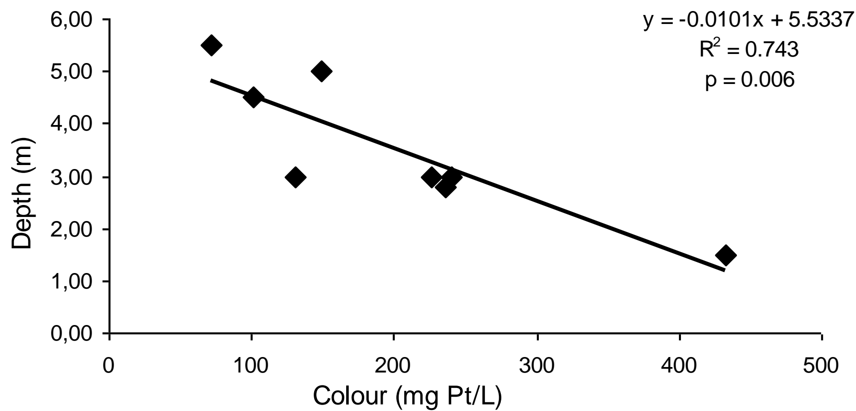


Figure 9. Relationship between the depth of maximum BChl abundance and lake colour.

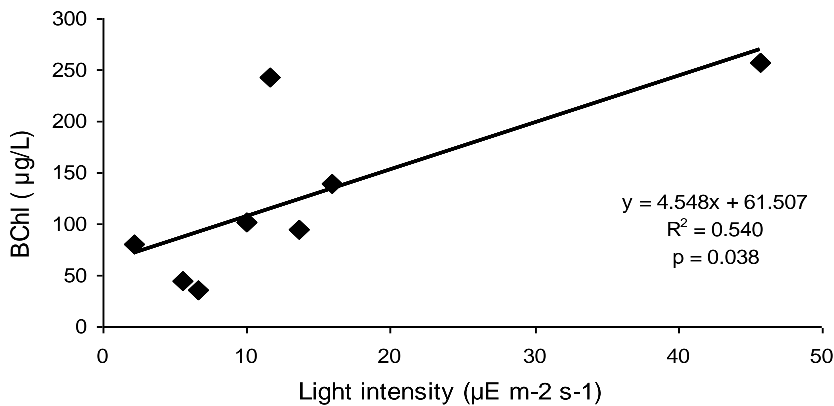


Figure 10. Relationship between light intensity on the oxic/anoxic boundary and maximum BChl.

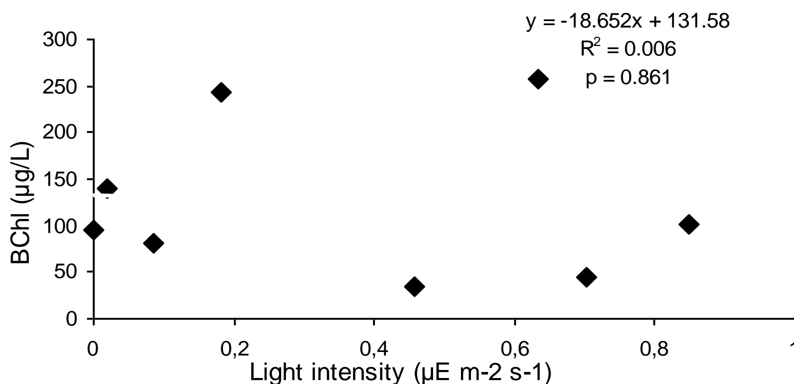


Figure 11. Relationship between light intensity and BChl in the depth of maximum abundance.

4.4. Seasonal changes

Seasonal changes in *Chlorobium* concentrations were followed in Alinen Mustajärvi, Mekkojärvi and Halsjärvi (Fig. 12). The amount of BChl was more than doubled in all the lakes during the open water season (Table 5). In lake Alinen Mustajärvi the maximum abundance of *Chlorobium* was reached already in July, but in lakes Mekkojärvi and Halsjärvi the amount of BChl increased in the course of the summer season. The generation time of integrated bacteriochlorophyll concentration varied between 69-118 days. The development of *Chlorobium* communities in the lakes (based on integration of the BChl concentrations) are showed in Fig. 13. In Halsjärvi the over whole lake integrated generation (doubling) time (from May to August) was 84 days and in Mekkojärvi 97 days. In Alinen Mustajärvi the generation time during growth season (form May to July) was 100 days but the growth stopped after July. In the depth of maximum summer abundance much greater doubling times were observed: Halsjärvi 13 days, Alinen Mustajärvi 30 days and Mekkojärvi 38 days (between May and July). In Mekkojärvi the growth rate in the depth of maximum abundance between July and August was even higher (23 days).

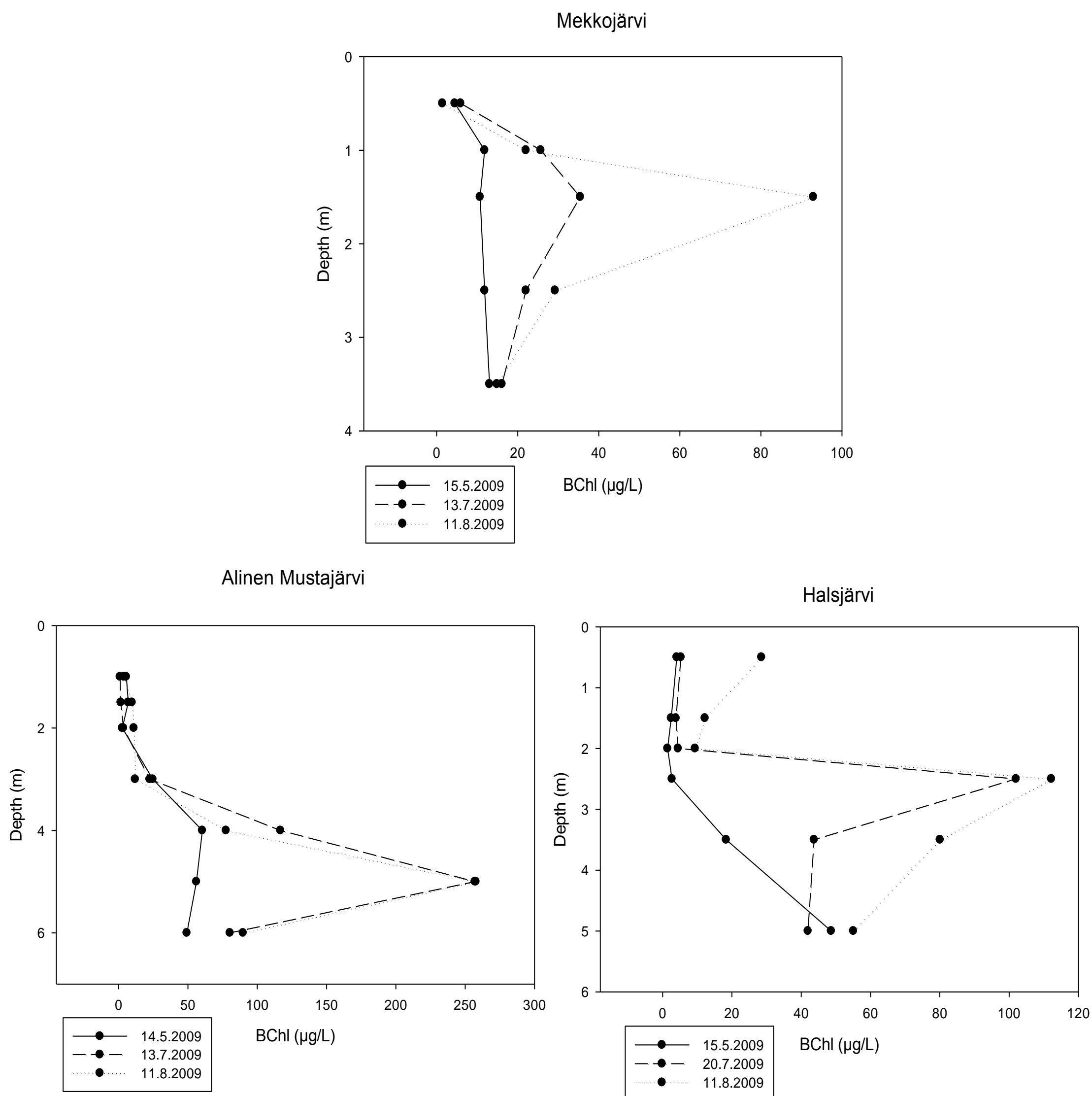


Figure 12. Vertical profiles of BChl in different sampling times in lakes Mekkojärvi, Halsjärvi and Alinen Mustajärvi showing seasonal changes in lake *Chlorobium* communities.

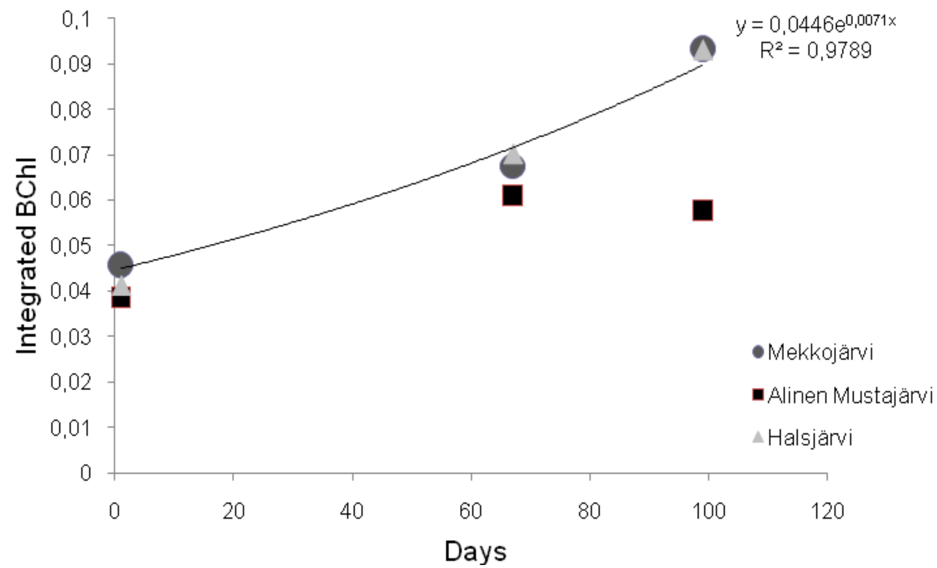


Figure 13. Integrated BChl concentrations from May (14-15.5) to August (11.8) in Alinen Mustajärvi, Mekkojärvi and Halsjärvi.

Table 5. Measured parameters of seasonal observation

Lake	Date	Thermocline (m)	Max. BChl (μg/L)
Mekkojärvi	15.5.2009	1	13.1
Mekkojärvi	13.7.2009	1	35.5
Mekkojärvi	11.8.2009	1	93.0
Halsjärvi	9.4.2009	2	161.7
Halsjärvi	15.5.2009	2.5	48.7
Halsjärvi	20.7.2009	2	102.0
Halsjärvi	11.8.2009	2	112.2
Alinen Mustajärvi	6.4.2009	2	54.2
Alinen Mustajärvi	14.5.2009	2	60.4
Alinen Mustajärvi	13.7.2009	2	257.0
Alinen Mustajärvi	11.8.2009	2.5	257.7

4.5. *Chlorobium* under ice

In three of the four lakes *Chlorobium* community was found in winter under ice, namely in Alinen Mustajärvi, Halsjärvi, and Nimetön while not in Horkkajärvi. BChl concentrations were measured from each lake (Fig. 14) and the 665/656 ratio declined under 0.7 in the anoxic parts. In lakes Alinen Mustajärvi, Nimetön and Halsjärvi the set limits (chapter 3.5.) for *Chlorobium* identification were filled.

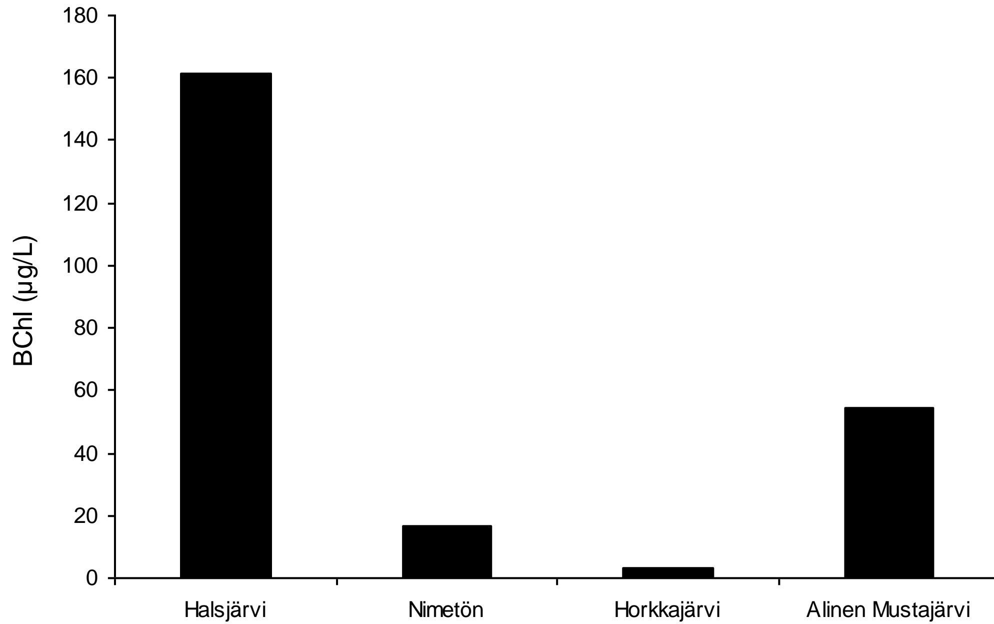
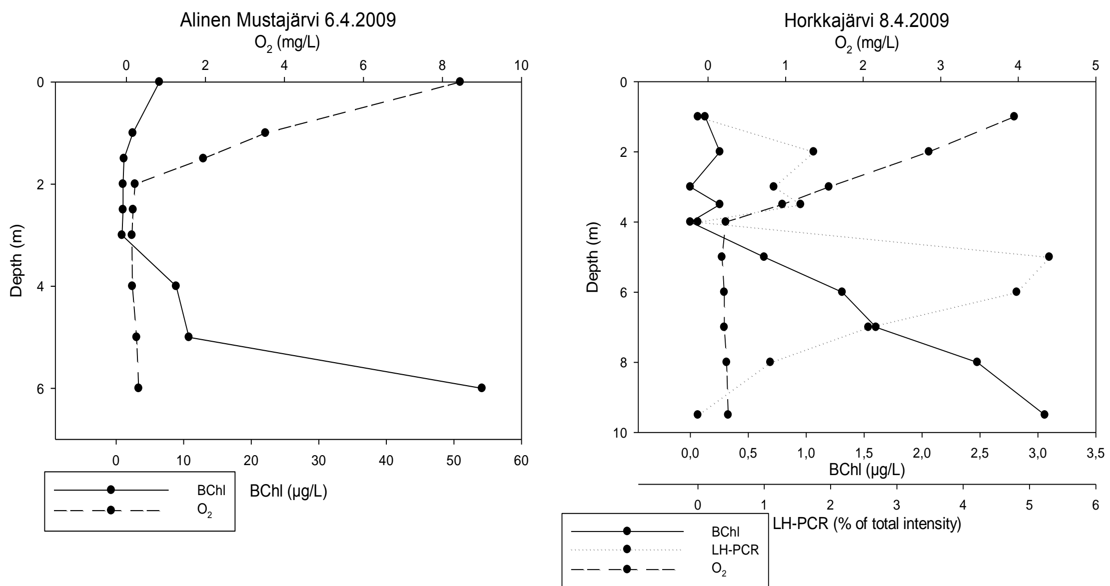


Figure 14. Maximum observed BChl concentrations on winter sampling on April.

In lake profile pictures (Fig. 15) it can be seen that the maximum BChl concentrations in winter were observed near the bottom in all lakes. Steep oxygen stratification was found in all lakes and the oxic/anoxic boundary layer was located at the same depth as in July (Table 5). The LH-PCR relative intensities are found from higher depth, but still the *Chlorobium* communities are found deeper compared to summer vertical profiles (Fig. 4).



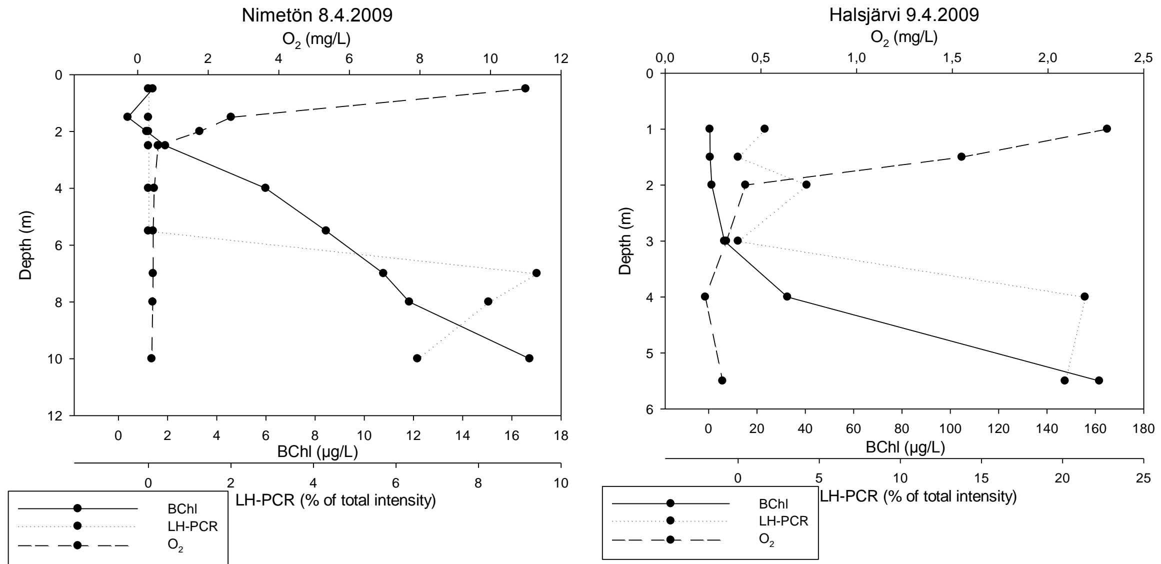


Figure 15. Lake profiles from winter sampling

Results from lake oxygen profiles and BChl concentrations on different sampling time gives a rough estimation of lakes spring and autumn overturns (Table 5). It seems that the largest study lake Halsjärvi had mixed on spring and lake Alinen Mustajärvi on autumn. This could explain the higher BChl concentration in Halsjärvi in winter than in summer and an opposite in lake Alinen Mustajärvi (Fig. 16). According to Salonen et al. (1984) lakes Nimetön and Horkkajärvi are meromictic lakes, without spring and autumn overturns.

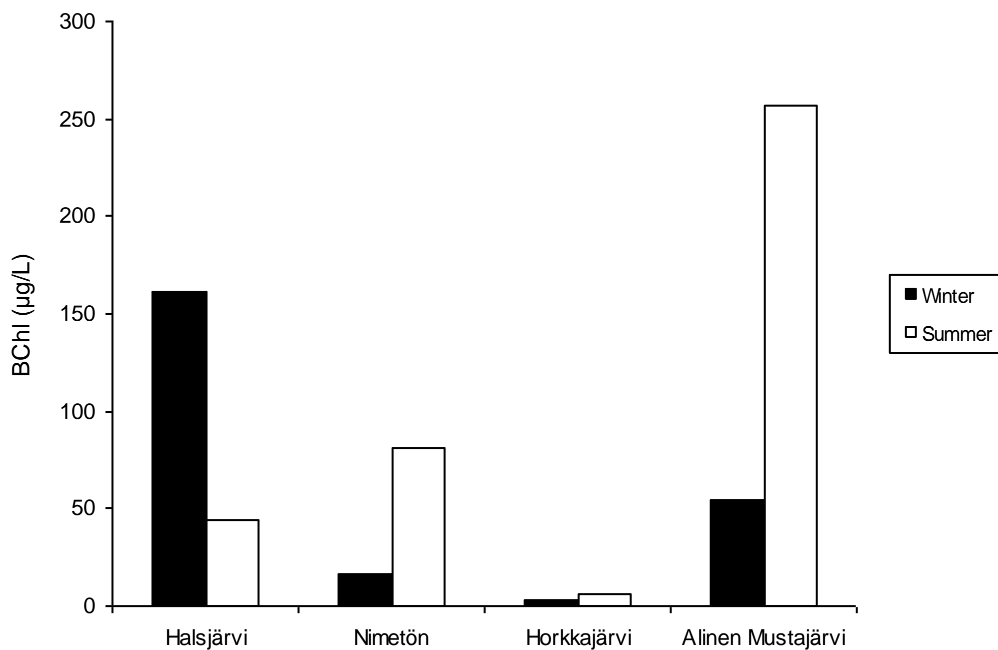
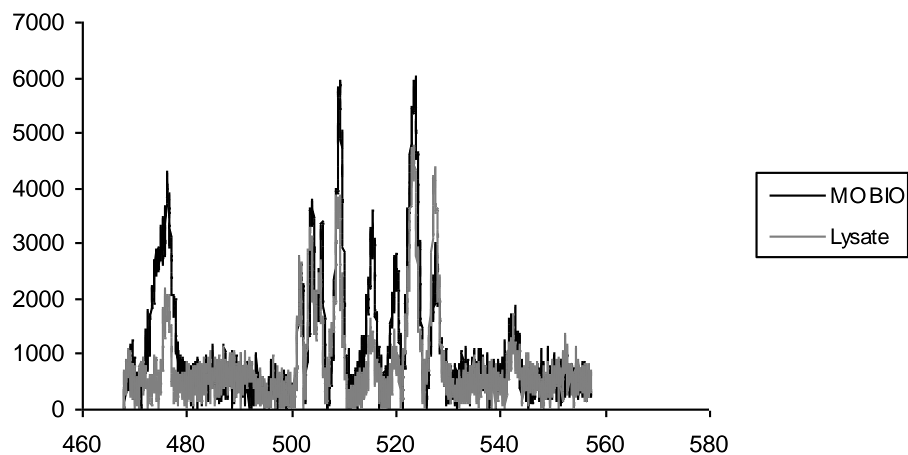
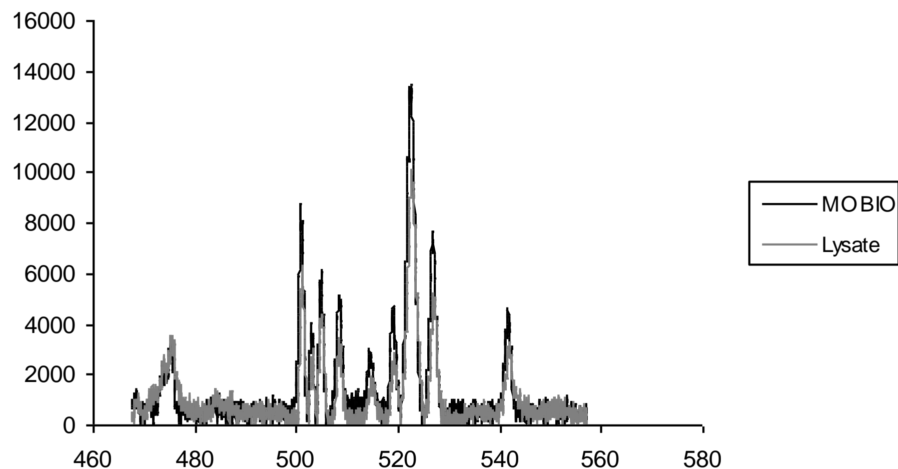


Figure 16. Maximum BChl concentrations (µg/L) in the study lakes before the ice break-up on April in winter and on July in summer.

4.6. DNA extraction

Two DNA extraction methods were used and results compared. Methods were i)bacterial lysates made using NaOH and heating directly from the sample water and ii)DNA extraction with MO BIO kit from freeze dried samples. Intensity peaks between 465 and 565 bp on LH-PCR measurements are showed in Figure 17. Peaks between two methods are located on same places, meaning that both methods generated the same 16S rRNA fragments. A regression model calculation was made between intensity peaks from all samples relative % values of the two methods and a clear correlation was seen (r^2=0.824 and $p<0.001$). The results are indicating that the bacterial lysate technique is consistent with the more commonly used MO BIO kit, and the use of the lysate method in this study was justified.





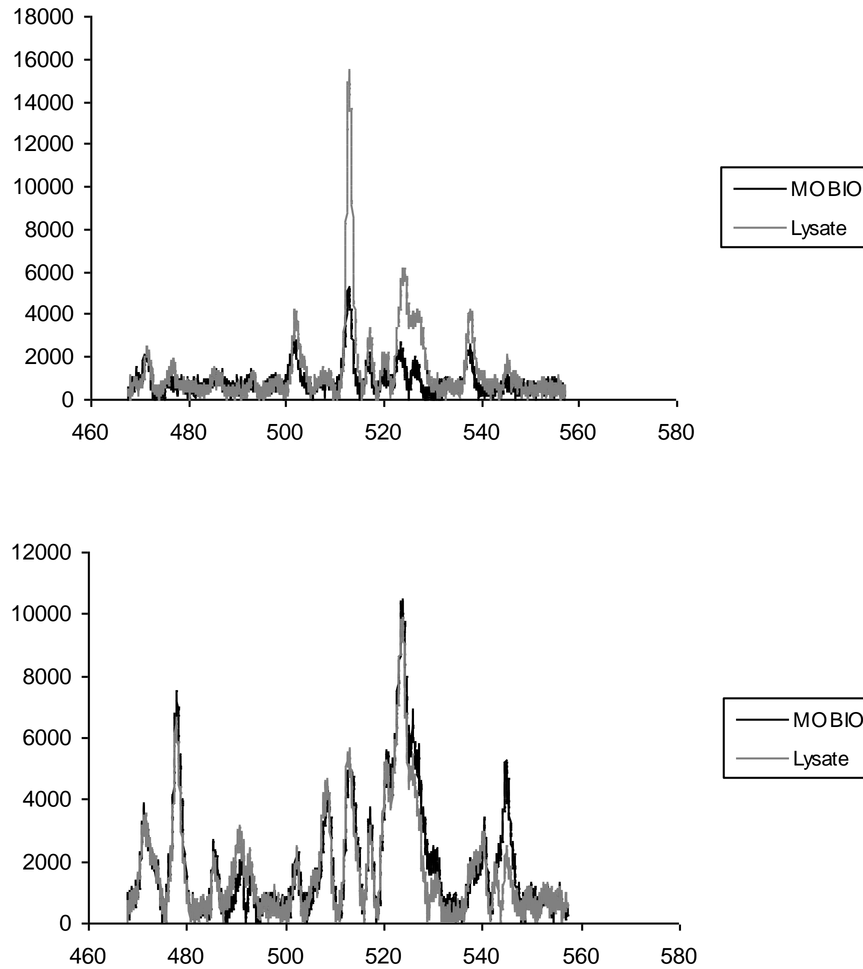


Figure 17. DNA extraction method comparison. Samples from lake Alinen Mustajärvi 14.5.2009 from depths 1m, 2m, 4m and 6m.

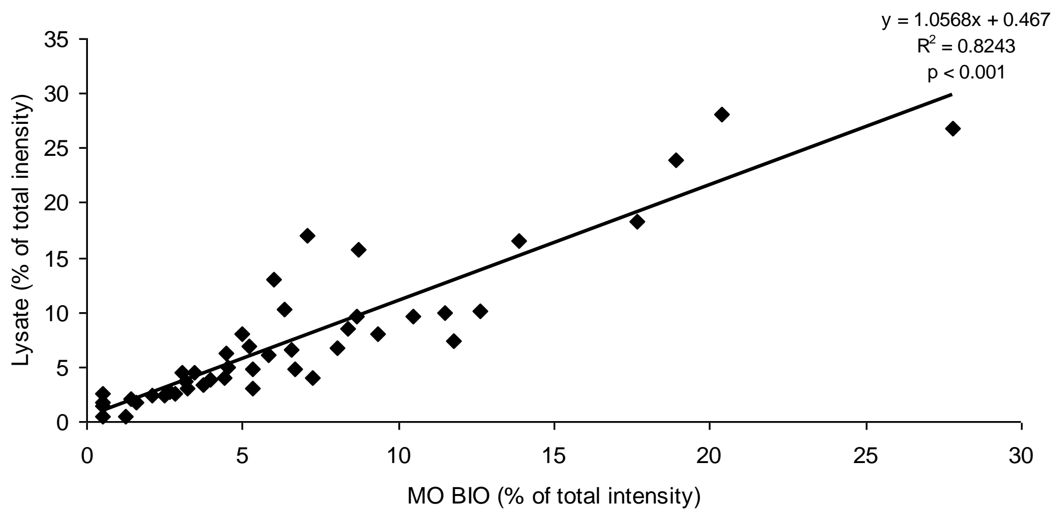


Figure 18. Relationship between % of total intensity peaks of MO BIO and Lysate methods.

5. DISCUSSION

The *Chlorobium* genus was described first time at the beginning of 20th century (Nadson 1906). After this the presence of the genus has been repeatedly reported, and the ecology of phototrophic green sulphur bacteria has been studied in detail (Van Gemerden and Mas 1995, Overmann 2006). However, their abundance and actual impact on the lake ecosystem are still far from fully understood. This study gives some estimation how common *Chlorobium* communities are in the small boreal humic lakes in Southern Finland and what factors are affecting the growth of bacterium.

Requirements for *Chlorobium* growth seem to be at least anoxic conditions with sufficient amount of light. All the study lakes were steeply stratified with respect to temperature and oxygen, and the study by Salonen et al. (1984) indicates that most of the Evo region small boreal lakes are meromictic or spring-meromictic providing stable or rather stable anoxic conditions. In the same study Salonen et al. (1984) pointed out that even if the hypolimnetic water with reduced substances is mixed with oxygenated water from the epilimnion, the resulting concentration of oxygen may still remain zero or close to that. This may explain why the *Chlorobium* communities can be so abundant in these lakes, although only three of them were listed by Hakala (2004) as meromictic lakes. In fact, Hakala (2004) estimated that in Finland there are only a few dozen meromictic lakes, which is such a low number that it may be an underestimation even based on the results of this study.

The abundance of *Chlorobium* was detected and reported for the first time in most of the study lakes. In eight lakes of 13 the diagnostic set limits for significant *Chlorobium* growth were filled (see chapter 3.5.). The growth was found only in strictly anoxic conditions, in agreement with the literature (Imhoff 1995; Van Gemerden and Mas 1995; Overmann 2006). Earlier Arvola et al. (1984) noticed that the growth of *Chlorobium* is restricted in small humic lakes to a narrow zone in the upper part of the anoxic water layer where still sufficient amount of light is available. The same phenomenon was noticed in all the study lakes with narrow density peaks in the hypolimnion.

In three lakes out of 13 no significant *Chlorobium* growth was observed, although some small quantities would be possible in all of them. The light intensity on the oxic/anoxic boundary layer was so low that it could not support intense phototrophic growth. This result supports the hypotheses that in small boreal lakes with steep stratification light has a key role in controlling the *Chlorobium* community and that light is, indeed, necessary. The maximum *Chlorobium* biomass correlated better with the light intensity on the oxic/anoxic boundary than the light intensity on the depth of maximum abundance, a result which was consistent with the observation of Parkin and Brock (1980). They concluded that light intensity at the thermocline is the major factor controlling photosynthetic bacterial production in lakes. Schanz et al. (1998) found that the *Chlorobium* cells are also sensitive to light intensities higher than the optimum (~0.036 E m^{-2} h^{-1}, is equal to 0.01 µE m^{-2} s^{-1}). In our study the light intensity on the depth of the maximum BChl concentration varied between 0.8 - 0.001 µE m^{-2} s^{-1}. In Alinen Mustajärvi where the light intensity at the oxic/anoxic boundary was highest (45.7 µE m^{-2} s^{-1}) among the study lakes the maximum BChl peak was deeper than in other lakes relative to the depth of oxic/anoxic boundary. This may indicate that *Chlorobium* cells could not tolerate such high light intensities. Another explanation might be too low H_2S concentration in upper part of the anoxic hypolimnia. Schanz et al. (1998) found that light intensity at the density peak of bacterial population was on average 0.4% of the subsurface radiation. In this study the light intensity at the density peak varied between 0.06-0.00009 % (with an

average 0.026 %) of the maximum surface PAR (1400 µE m^{-2} s^{-1}), and in the oxic/anoxic boundary layer the respective values were 3.25-0.15 % (with a mean 0.99 %). This may suggest that different *Chlorobium* populations can be adapted to slightly different light conditions or that in the study lakes the other required parameters were not fulfilled on the depth where the light intensity would have been optimal.

In lakes Rieskalammi and Horkkajärvi the results were not as clear and obvious as in the other study lakes, although in general the conditions seemed to be suitable for *Chlorobium*. Horkkajärvi has been listed as a meromictic lake by Hakala (2004) and it has been expected that the amount of H_2S is high enough in the hypolimnion of all stratified lakes in the area to provide GSB growth because of strong sulphide smell of the hypolimnetic water. Anyway, too low H_2S concentration (Vila et al. 1998; Van Gemerden and Mass 1995) inhibits the growth of GSB, and therefore it is possible, at least in theory, that in Horkkajävi, which is quite deep (12 m) lake and with a very shallow thermocline, the amount of H_2S was not high enough for *Chlorobium* to grow in the boundary layer. In Lake Rieskalammi, which is a beaver lake, the water level fluctuations due to the animals, which may have affected the lake chemistry and as a consequence the whole ecosystem may have changed (Karhu 2010).

The high bacterial biomass of the anaerobic phototrophic microbes in the hypolimnia can play an important role in the lake carbon cycle as has been shown by Taipale et al. (2011). The measured BChl concentrations from the study lakes and the high proportion of characteristic LH-PCR peak profiles clearly proved that the GSB can be abundant in many of the small forest lakes in the study area, and perhaps also in other similar boreal lakes worldwide. Taipale et al. (2011) have noticed earlier, that *Chlorobium* made up nearly 80 % of the total bacterial biomass in one of the study lakes with only moderate BChl concentration in this study.

This was the first time *Chlorobium* was measured and detected under ice. High BChl concentrations were found near the bottom. The results show that the *Chlorobium* bacteria can survive through the winter in the water column. Beneath the ice light conditions are extremely poor in mid-winter, and phototrophical growth must be even more light limited than during the summer. The high BChl concentrations closer to the bottom relative to the LH-PCR biomarker peaks indicate that the *Chlorobium* bacteria may have lost some amount of their pigmentation during the winter. Schmidt et al. (1980) studied the BChl c-synthesis in *Chloroflexus aurantiacus* and noticed that decrease in light illumination caused an increase in the BChl c content of chlorosome. The LH-PCR peaks were also closer to the bottom than during the summer time, maybe a result of the sedimentation and lack of growth of the community during prolonged winter conditions. Another explanation could be that the cells needed substances which were released from the bottom sediments. In any case the phototrophic bacteria were capable to support their energy demands during the winter, and to be ready for the photosynthesis in spring when the light conditions rapidly improved. It is known that GSB are potentially mixotrophic in the presence of inorganic reductants and CO_2, and can use simple organic compounds for biomass formation (Overmann 2006). The strong relationship between light intensity and *Chlorobium* abundance in summer indicates, however, that light has a key role in controlling the growth of GSB in the study lakes.

The profile measurements gave a rough estimation of the efficiency of overturns in the lakes as well as their effects on the GSB communities in Alinen Mustajärvi and Halsjärvi. For example, in Halsjärvi which is the largest study lake the results indicated that the lake had a spring overturn, and as a consequence of the spring mixing the

concentration of BChl declined from April to May. However, at the same time it is obvious that the mixing period was a short one and perhaps incomplete, and at least part of the deeper water layers may have provided anoxic conditions for GSB. Otherwise it would be difficult to understand how such high BChl concentrations could be possible to find later in the summer. This is in accordance with Arvola et al. (unpubl.) who have observed earlier that due to the artificial mixing of water column the GSB community in Halsjärvi collapsed almost completely but recovery of the community took place very quickly after that. Thus there must be somewhere living cells for the new inoculum, because otherwise the recovery presumably would take much longer time. In Alinen Mustajärvi no spring mixing was observed, however, but the autumnal overturn was evident. This autumnal mixing may explain why the GSB community was suppressed under ice in comparison to the last summer.

The *in situ* growth rates of phototrophic green sulphur bacteria can be determined by two different approaches, either measuring the carbon dioxide fixation rate or measuring the growth rates *in situ* (Van Gemerden and Mas 1995). In this study the generation times of GSB were calculated based on the BChl concentrations rather than the real growth rates of the cells. The generation times of GSB varied to some extent between the lakes which implies that the different physical and chemical conditions prevailed in the lakes affected the growth rates and/or that net growth rates were also influenced by other factors such as grazing by metazooplankton, ciliates and other protists. It is also possible that the species composition of the communities varies between the lakes and it affects the growth rates. By comparing the integrated BChl concentrations during the open water season from May to August in Mekkojärvi, Halsjärvi and Alinen Mustajärvi the development of the *Chlorobium* community between May and July was almost identical in all lakes. Since July untill August Halsjärvi and Mekkojärvi *Chlorobium* community development followed each other, but Alinen Mustajärvi not. In that lake something special happened because the net growth rate was negative. The simplest explanation might be that the maximum abundance of GSB was achieved already in July and the alternative explanation might be that grazing pressure has reduced the community.

This study clearly proved that the two independent methods gave very consistent results of the *Chlorobium* abundance. The best understanding was achieved when the result of both methods (i.e. Taipale et al. (2009) suggesting that 512 pb long fragments indicated *Chlorobium* in boreal lakes and Takahashi & Ichimura (1970) introducing BChl method) were compared with each other. The wavelength ratio of 665/656 (Arvola et al. 1992) was a good indicator of the shift in the dominance from chlorophyll a to bacteriochlorophyll d in the study lakes.

A strong relationship between water colour of the lake and the depth of the maximum GSB community was found. The lakes with darker water colour and higher humic matter concentration have maximum peak of GSB higher in depth than the lakes with more transparent water and low humic matter concentration. If the light intensity on the oxic/anoxic boundary and the lake colour are known, it can be predicted if *Chlorobium* is abundant or not and in which depth the maximum density is likely to be located.

5.2. Conclusions

In this study *Chlorobium* was commonly found in small stratified lakes where sufficient amount of light was available in the anoxic hypolimnia. The results showed that in the study lakes the most important factor for GSB growth was light intensity. Light intensity at the oxic/anoxic boundary layer was better correlated with the maximum BChl concentration than with the light intensity in the depth of the maximum BChl abundance.

Besides light, the mixing depth is a critical factor because it may destroy the oxic/anoxic boundary layer and elevate oxygen concentration in the hypolimnion and delince H_2S concentration therein. LH-PCR and BChl methods gave very consistent and supplementing results. The overall results implied that water colour of a lake can be used to predict the depth of the maximum GSB community when other prerequisites for their appearance are fulfilled. *Chlorobium* communities were also found under the ice cover meaning that the bacteria can survive over the winter season in the water column when light conditions in the lake are extremely poor.

Many aspects related to *Chlorobium* and more widely to the whole family of phototrophic green sulphur bacteria are still unknown and the research continues to reveal the ecology of these organisms. The importance of *Chlorobium* in lake ecosystems is an interesting and challenging study object with high potential importance. One such issue is the abundance of *Chlorobium* in winter under ice, and its role in the nutrition of zooplankton including protists. The methods used were sensitive enough to detect the vertical distribution patterns of the *Chlorobium* communities, but new methods are needed to determine the quantitative role of GSB in the food webs of humic lakes.

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