Master’s Thesis

Seasonality of bacterioplankton in subarctic ponds above and below treeline

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TIIVISTELMÄ

Bakteerien vuodenaikaisuutta tarkasteltiin kuudessatoista tunturilammikossa, jotka sijaitsivat puurajan ylä- ja alapuolella Kilpisjärvellä, Pohjois-Suomessa. Tuloksista havaittiin että bakteerien aktiivisuus kasvaa kevään ajan saavuttaen huipun kesällä tämän jälkeen jälleen vähentyen syksyä kohden. Puurajan alapuolisissa lammissa kesän aktiivisuuspiikki on kuitenkin selvästi terävämpi kuin puurajan yläpuolisissa ja molemmissa tapauksissa aktiivisuus seuraa liuenneen orgaanisen hiilen määrän muutoksia. Aktiivisuutta kuvattiin bakteerimäärällä, -tuotannolla ja -biomassalla. Alapuolisten lammikoiden bakteerit muodoistuivat suurimmaksi osaksi actinobakteereista (32.1%) sekä betaproteobakteereista (34.4%). Lisäksi alphaproteobakteerit muodostivat pienen erillisen ryhmän (7.9%) Yläpuolisten lampien bakteerit olivat vielä voimakkaammin jakautuneet kahteen ryhmään actinobakteereihin (46.2%) sekä betaproteobakteereihin (27.0%). Monimuotoisuus kuitenkin vaihteli voimakkaasti läpi näytteenottokauden viitaten vahvasti bakteerien kykyyn reagoida nopeasti resurssien tai ympäristömuuttujien vaihteluun. Koko aineistoa käsiteltäessä liuennut orgaaninen hiili ja lämpötila nousivat regressiomallissa tärkeimmiksi bakteerien aktiivisuutta selittäviksi tekijöiksi. Lammikoiden bakteerien aktiivisuuden ollessa vahvasti yhteydessä muuttuviin resursseihin ja ympäristömuuttujiin, ilmaston muutoksen myötä mahdollisesti lisääntyvillä sateilla ja tällä tavoin lisääntyneillä resursseilla saattaisi olla voimakas vaikutus pieniin subarktisiin lammikoihin.

UNIVERSITY OF JYVÄSKYLÄ, Faculty of Science

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ROIHA TONI, M.: Seasonality of bacterioplankton in subarctic ponds below and above treeline

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ABSTRACT

Activity of bacteria was studied seasonally in 16 shallow ponds situated below and above the treeline in Northern Finland, Kilpisjärvi. Seasonal results showed that bacteria activity increased during the spring time and reached the maximum values in the summer period then declining during the autumn. Highest values in bacteria abundance, biomass and production were all measured in the summer sampling. Comparison between the ponds situated above and below treeline revealed that activity peak is much sharper at lower altitudes but in both cases activity followed the trend of dissolved organic carbon concentration. In low altitude ponds main bacteria phyla were actinobacteria (32.1%) and betaproteobacteria (34.4%). Also small separated group of alphaproteobacteria (7.9%) was found. Similar main phyla, actinobacteria (46.2%) and betaproteobacteria (27.0%) were found in high altitude ponds with increased relative proportion. Within these groups bacteria diversity differed strongly during the sampling season indicating bacteria population ability to react rapidly to changes in environment. When the whole data were considered DOC (dissolved organic carbon) and temperature explained best the changes in bacteria activity in regression model. Bacteria activity is strongly connected to resources and environmental variables suggesting that increased precipitation induced by global warming and consequent higher DOC runoff from the catchment could have strong impact on small subarctic ponds.

Contents

1. INTRODUCTION **5**
2. RESEARCH BACKGROUND **6**
2.1. Developments in bacteria research 6
2.1. Influence of allochthonous dissolved organic carbon in aquatic ecosystems 7
3. MATERIALS AND METHODS **9**
3.1. Study Site 9
3.2. Environmental data 11
3.3. Bacteria Biomass 12
3.4. Bacteria Production 13
3.5. Bacteria Diversity 15
4. RESULTS **17**
4.1 Seasonality in characteristics 17
4.2 Seasonal changes in bacteria abundance 17
4.3 Seasonal changes in bacteria productivity 18
4.4 Bacteria biomass with three methods 18
4.5 Bacteria diversity 20
4.6 Environmental variables explaining bacteria dynamics in Kilpisjärvi ponds. 21
5. DISCUSSION **25**
5.1 Environmental control of bacterioplankton in subarctic ponds 25
5.2 Bacteria activity in high-latitude freshwaters 27
5.3 The still unknown diversity of aquatic bacteria 29
5.4 Choice of methods 30
5.5 Future of arctic research and climate change 31
ACKNOWLEDGEMENTS **33**
References **34**

1. INTRODUCTION

Until the 1980s aquatic bacterioplankton were thought to be simple and inactive. Only recently their role as a basic element in water ecosystem has been understood (Cole et al. 1988). Bacteria play fundamental role in the microbial loop (Azam et al. 1983) but they are also necessary in decomposing processes. Their ability to produce particles from dissolved organic carbon (DOC) makes them essential for other organisms in the water ecosystem. Biomass of bacteria and their biological efficiency are dependent on nutrients and grazing, but are also determined by temperature and allocthonous DOC inputs. This makes bacterioplankton's role important when estimating the effects of global warming (Karlsson et al. 2001). Climate warming and increasing precipitation have significant impact on the interaction between the lake and the catchment area by increasing the organic material inputs (ACIA 2005). From these inputs DOC has the biggest impact on the ecosystem. It works as an energy source for bacteria production, attenuates light and decreases the primary production in lakes and it leads to new relationship between primary producers and heterotrophic organisms that is favorable to bacterioplankton (Hessen et al. 1990). A part of the organic load from drainage area is released to the atmosphere by bacteria respiration and with this mechanism it is possible that carbon accumulated to soils contribute to adding green house gasses to the atmosphere.

The first high latitude aquatic microorganism studies were primarily targeted to demonstrate the presence of microorganism in water supply. Therefore bacteria were first microorganism to be examined. These first studies described the presence of coliforms and thermophilic bacteria (McBee & McBee 1956). First non-pathogenic descriptions from bacteria were done by Boyd & Boyd (1963) when isolating strains belonging to species of *Chromobacteriun.* First molecular description from arctic freshwater system was not until Bahr et al. (1996). Their study reported a diversity of bacteria in Lake Toolik (Alaska, USA) in which they isolated cultures grouping into eleven phenotypes. One of the latest descriptions of arctic freshwater bacteria diversity is by Crump et al. (2003) which revealed globally distributed freshwater phylogenetic clusters from Lake Toolik. However the biodiversity of bacteria in arctic lakes is still very little known (Pearce & Galand 2008).

My master's thesis work is part of Milla Rautio's and her group's research where we study food web structure and cycling of carbon in subarctic lakes and ponds. Purpose of my study was to find out what kind of effects different inputs of organic material from the catchment area have on bacteria biomass, production and diversity in different seasons in small ponds in subarctic region. Study was carried out in a group of shallow ponds with differing altitudes situated in Northern Finland, Kilpisjärvi.

2. RESEARCH BACKGROUND

2.1. Developments in bacteria research

After the invention of microscope, bacteria were discovered in water with other microorganisms. Antonie Van Leeuwenhoek was the first person to provide illustrations of different types of bacteria in 1683. However presence of bacterial communities in all types of environments and their extraordinary variety was not understood until the 19th century. One factor which increases variety of bacterial communities in aquatic systems is that besides truly aquatic bacteria a number of other bacteria from different habitats can also be found (Rheinheimer 1992).

Method where nucleopore filters were used to numerate bacteria by using fluorescence microscopy was proved to be efficient by Hobbie et al. (1977) who demonstrated how bacteria were not trapped inside nucleopore filters like they were trapped when using cellulose filters. This was seen in results which showed that twice as many bacteria were retained on nucleopore filter than in cellulose filter. Study which compared fluorescence dyes DAPI (4’6-diamidino-2-phenylindole) and acridine orange was published by Porter & Feig (1980). They concluded that use of DAPI improved visualization of bacteria and extended sample storage period at least to 24 weeks. Bacteria biomass determination by image analysis was first studied by Bjornsen (1986). Epifluorescense microscope was mounted with a video camera and still pictures and video footage were taken from acridine orange stained bacteria. From these still pictures the length and the width of individual bacteria were measured for calculation of biovolume. Bacterial biomass was also estimated from bacterial abundance using coefficient of 20 fg C cell^{-1} which is mentioned to be mean average for bacteria cell carbon content (Lee & Fuhrman 1987). Recently flow cytometric application which is rapid, automated and accurate method to evaluate for example cell viability, biomasses and productions has been introduced to replace the laborious microscopic analysis (Troussellier et al. 1993). Also the fluorescence dyes has been under development. Suitability of the dyes for cytometric measurements were tested by Lebaron et al. (1998) and they came to conclusion that Sybr Green II is the most appropriate for measurements. Sybr Green dyes has also been tested in traditional microscopic analysis. Comparison between Sybr Green I and acridine orange reported very strong correlations when enumerating bacteria and also Sybr Green I stained samples did not need desalting or heating thus resulting total preparation time under 25 minutes (Noble & Fuhrman 1998). Recent comparison between Sybr Green I and Sybr Gold suggested that use of Sybr Gold enhances the persistent of fluorescence signal (Shibata et al. 2006).

There are different approaches to measure bacterial carbon production with radioisotopes. First approach is to measure production based on DNA synthesis by the tritiated thymidine corporation (Fuhrman & Azam 1980, 1982). This method measures cell multiplication and therefore results needs to be transformed into bacterial production by using knowledge of carbon content of the growing bacteria. Second approach to measure bacterial production is to use ^{35}S-sulfate (Cuhel et al. 1982) and ^{3}H-leucine (Kirchman et al. 1985) radiotracer incorporation. ^{35}S-sulfate incorporation method is mainly limited to marine environments because there plankton sulfate incorporation is not bacteria-specific and ^{35}S-sulfate isotope is excessively diluted. ^{3}H-leucine method is based on protein synthesis and bacterial production can be calculated in basis of leucine incorporation. Parameters which have to be taken account are ^{3}H-leucine isotope dilution, per-cell protein

content of natural planktonic bacteria, the protein:cell volume relationship and mol% leucine in protein. ^{3}H-leucine method is however simple and economical method of measuring bacterial protein synthesis (Simon & Azam 1989).

16S rRNA based techniques lead to molecular identification of variety of micro-organism and novel microbial groups in different environments in mid 1980s. From a normal sample, sequences can be obtained by polymeraine chain reaction (PCR) using primers corresponding to conserved bacterial priming sites. There has been development of PCR-based community fingerprinting methods because of limitation of slow and laborious "clone and sequence" method. Denaturing gradient gel electrophoresis (DGGR) (Muyzer et al. 1993), length heterogeneity of PCR-amplified 16S rDNA (LH-PCR) (Suzuki et al. 1998), single-strand conformation polymorphis (SSCP) (Lee et al. 1996), restriction fragment length polymorphism (RFLP) and ribosomal intergenic spacer region analysis (RISA) are all methods used to structural and serial analyses of microbial communities. The most recent technique pyrosequencing dispenses the need for labelled primers, labelled nucleotides and gel-electrophoresis where all the earlier methods relied on. The problems so far has been the throughputs and costs of the single run which has made the method unavailable for many studies (Ronaghi 2001).

2.1. Influence of allochthonous dissolved organic carbon in aquatic ecosystems

Bacterioplankton have an important role in aquatic food chains and their place in aquatic ecosystems is illustrated in Fig. 1. They act as decomposers of carbon and nitrogen in biochemical processes and they are grazed by heterotrophic and mixotrophic nanoflagellates (Vincent and Laybourn-Parry 2008). They transform larger molecular structures to smaller particles but still they can be primary producers in food webs. Studies in the Northern American tree line have shown that plankton < 2 µm in diameter can provide 10 to 80 % of a lake's primary production (Milot-Roy and Vincent 1994). Heterotrophic bacteria, on the contrary, use mainly allochthonous humic carbon as an energy source.

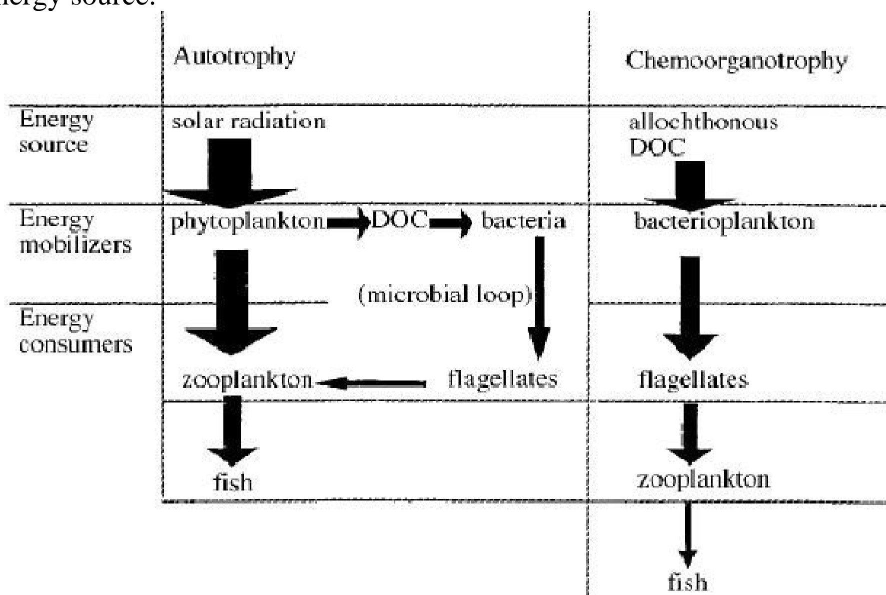


Figure 1. Schematic illustration to demonstrate the place of bacterioplankton in autotrophic and chemotrophic aquatic ecosystems (Jansson 2000).

Humic substances can alter the physical environment by attenuating light especially by reducing amount of PAR (photosynthetically active radiation) and therefore having

negative effect on primary production. Humic substances can also have an effect on chemical environment and they can also act as energy source for organisms. Humic substances acting as energy source was discovered when there was no explanation found for many humic lakes having annual CO2 production from respiration exceeding the phytoplankton primary production (Jones 1992).

Another reason for the importance of allocthonous humic carbon is that although < 15 % of it is poorly readily available for growth, the supply can be many times higher than that of autocthonous carbon (Tranvik 1988). For example in lake Toolik, Alaska, the measured annual loadings of DOC was 98 gCm^{-2} which is seven higher than the carbon from annual primary production which measured 14 g $Cm^{-2}year^{-1}$ (Hobbie & Laybourn-Parry 2008).

Majority of bacterioplankton needs organic carbon as a food source. In small arctic ponds the importance of DOC inputs from the catchment is great but it varies spatially. Location of the lake, soil type of the catchment and annual variation in precipitation and runoff have also a direct impact on biomass and production of bacteria. Normally the amount of DOC in arctic lakes is very low (<5 mg l^{-1}) therefore even a small increase may change the quality of runoff water and have strong and rapid impacts on lake condition (ACIA 2005).

Threshold for lakes to turn from truly autotrophy to chemoorganotrophy has been studied in Scandinavia. Amount of DOC, when lake turns to be more chemoorganotrophy, has considered to be around 10 mg l^{-1}. This concentration of DOC is only valid through summer months. When the whole year is taken into account the concentration will be lower around 4 mg/l. This is because heterotrophic bacteria can be productive also during the winter in absence of light. Simple ratio between net primary production and net bacterial production was made by Jansson et al. (2000) to estimate this balance between autotrophy and heterotrophy. If ratio between these two is < 1 it means that bacterial production produces more carbon to higher trophic levels than phytoplankton suggesting to lakes heterotrophy. If ratio is >1 phytoplankton is dominant source of carbon to higher trophic levels indicating to autotrophy of the lake. Generally this means that very oligotrophic lakes are the closest from being truly autotrophic and with increasing DOC gradient lakes turn to heterotrophy and eventually in very humic lakes energy is based on heterotrophic sources (Jansson et. al 2000).

Energy transferred to next trophic level when shifting from lower trophic level to higher varies from 5 % to 15 % (Wetzel 2001). Heterotrophy effects on ecological efficiency of the foodweb because it has one extra trophic level when compared to autotrophic foodweb like shown in Fig. 1. In practice this means that less energy is transferred to top of the food web in heterotrophic system (Jansson et al. 2000).

In addition to energy transfer, DOC has also other effects on higher trophic levels in the lake. Increased concentrations of DOC increase the absorption of the damaging UV radiation but still organisms are often exposed to detrimental amounts of radiation (Laurion 1997). UV radiation is more lethal to planktonic species than terrestrial organisms because of their very thin outer layer with little protection against the radiation. On the other hand increasing DOC concentration with decreased transparency can make the photosynthetically active layer thinner for phytoplankton. Acidification might lead to decreasing concentrations of DOC because of the removal of UV-absorbing particles from water column by coagulation. This leads to increased transparency of the water body and increased doses of lethal UV radiation (Molot & Dillon 1997 & Gjessing et al. 1998).

3. MATERIALS AND METHODS

3.1. Study Site

Study site contains sixteen shallow ponds in Kilpisjärvi region (Fig. 2). These particular ponds were chosen because they had already been studied before (Rautio 1996). Eight of the ponds were situated below the treeline (approximately 600 m), two were at the treeline and six ponds were situated above the treeline.

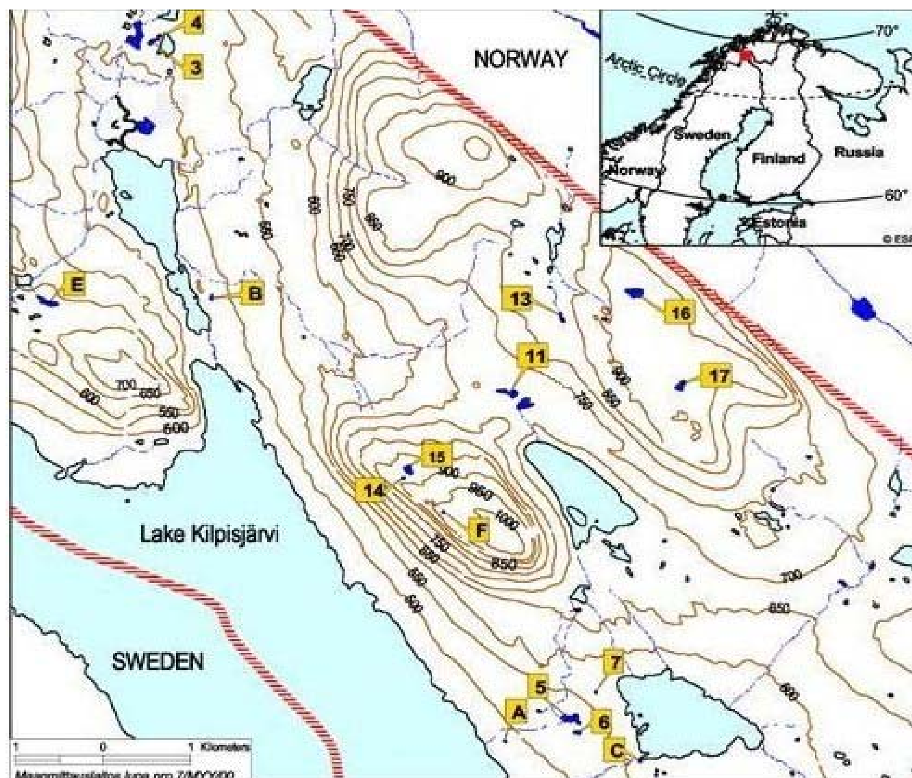


Figure 2. Sampling sites in Kilpisjärvi region. Treeline follows the 600 m contour.

The ponds were originally divided to three different groups according to their location in relation to the treeline, but the factor analysis in SPSS 16.0 (Statistical Package for the Social Sciences) showed that separation to two groups would be also possible based on physico-chemical measurements from water (temperature, pH, DOC and specific conductance). In the new grouping ponds were separated to low altitude ponds (< 600 m) and high altitude ponds (>600 m). Table 1 presents physiological characteristics of the ponds and from there it is seen that larger and deeper ponds were situated at higher altitudes and they also had larger variance in their size.

Table 1. Physical characteristics from ponds below and above treeline. Numbers outside parenthesis are averages from ponds in group. Numbers in parenthesis are minimum and maximum values.

	< 600m	> 600m
Lake Area (ha)	0.3 [0.1-0.8]	0.8 [0.1-1.4]
Drainage Area (ha)	3.6 [0.6-17.2]	17.3 [0.6-41.6]
Depth (m)	1.1 [0.5-2.0]	3.0 [0.5-7.5]
Altitude (m)	527 [485-570]	825 [600-950]

In Fig. 3 important physico-chemical properties were inserted by using the extraction method to PCA (principal component analysis). PCA axis 1 separates the ponds by using changes in temperature and specific conductance. PCA axis 2 divides the ponds by using DOC and pH. Principal component analysis gives even better separation power between the groups, when seasonality is also included. Seasonal factor PCA-figures are shown in supplementary materials given in the end.

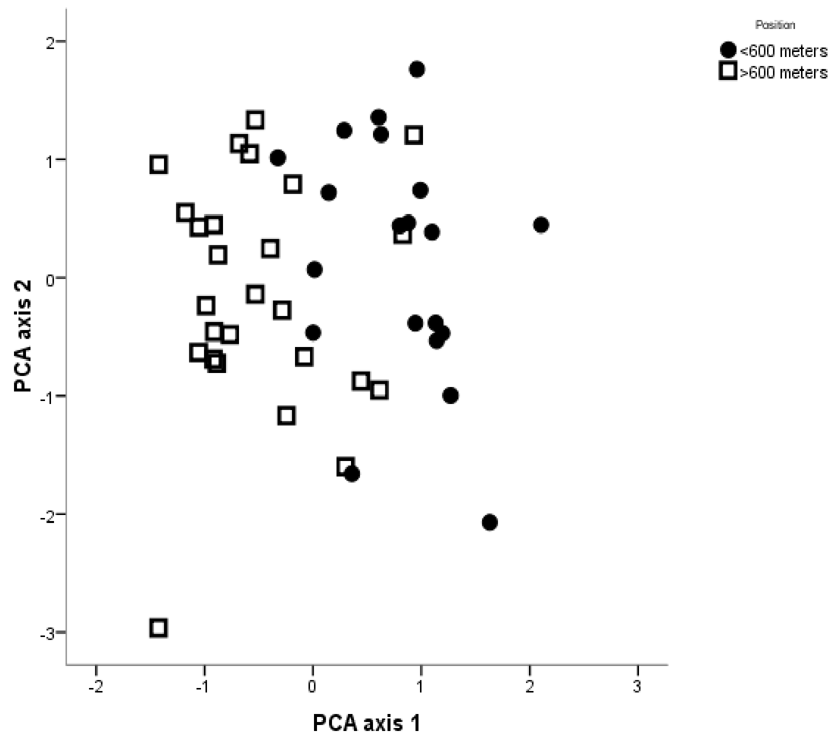


Figure 3. PCA-plot based on physico-chemical (Temperature, DOC, Specific Conductance and pH) properties change during whole sampling season.

Study ponds are frozen solid in winter and they are active only in approximately four summer mounts from late May to early June to late September. Generally ponds are rarely stratified because their water circulation is frequent and nutrient loadings, including carbon, are often proportionally higher than in larger lakes. It is normal in these kinds of ponds that organic matter sources are mainly from allocthonous origin. In this study part of the ponds were temporary and dried out during summer. Drying that is caused by evaporation has strong effects on ionic composition and nutrient availability. Because of these reasons organisms living in these ponds have to be strongly adapted to rapid changes in environment. Variation among the ponds is presented in Fig. 4.

Figure 4. Low altitude pond (Tsâhkal 5) and high altitude ponds (Saana 14 & 15) in autumn sampling.

3.2. Environmental data

All 16 ponds were sampled three times; in May-June right after ice-out (26.5-12.6. & 27-28.6.08), in end of June and mid-July (15-18.7.08) and in September (7-10.9.08) 2-3 weeks before the freezing of the ponds. Additional knowledge from surface- and drainage basin area of each pond was estimated by using Geographical information systems (GIS) in Lapland's Regional Environment Centre. Measurements for environmental data were taken from each pond in spring, summer and autumn. This way time series analysis was possible to do. Procedure for sampling was that the water was scooped to three different buckets from three different locations around the pond and then mixed together. Measurements for temperature, conductivity, specific conductance at 25℃ and pH were done from this mixed water with YSI 63 or YSI professional

Sample water for DOC was filtered through a cellulose acetate filter with a pore size 0.2 µm to exclude particles. After filtration, 200 ml of filtered water was poured to an acid-washed plastic bottle and was then stored in fridge. The samples were later transported in to Lapland's Regional Environment Centre in Rovaniemi for DOC analyses.

For Chl-*a* samples, 200 ml sample water was filtered through a fine carbon fibre filter (CF/F) pore size 0.7 µm. The Filter was then placed inside an aluminium foil and stored in a freezer. Chl-*a* samples were analysed with a fluorometer in Lammi field station owned by university of Helsinki. Extraction of chl-*a* from the filters started by placing the capped tubes filled with ethanol 95% to hot bath (70℃). When ethanol had reached wanted temperature filters were placed inside the tube for 5 minutes. Samples were then placed to fridge in darkness to cool down for one hour. After homogenization (vortex) samples were left to stay in dark place in room temperature until measurements in fluorometer.

Standard curve with a known ethanol 95 % concentration was made in the beginning and in the end of the measuring to make sure that the fluorometer was working properly. These curves are shown in Fig. 5

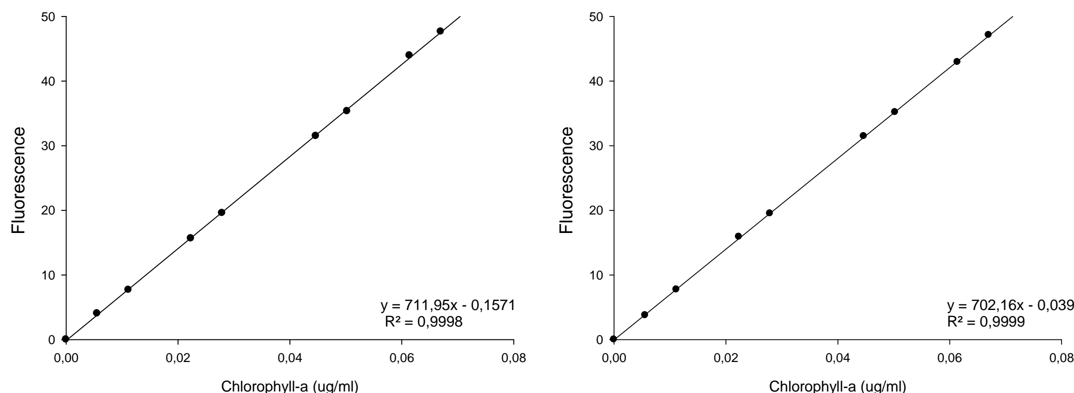


Figure 5. Standard curves before and after Chl-*a* measurements.

All the samples were injected to fluorometer first without acid and then after acidification. In fluorometric method acidification is used to correct natural degradation products (phaeopigments) (Yentsch & Mentzel 1963). All results from fluorometer were also automatically corrected by the blank ethanol 95 % sample before calculation (Nusch 1980). The formula [1] was used to calculate final chl-*a* concentration in a sample (Jeffrey & Walschmeyer 1997).

$$Chl - a(\mu g / L) = \frac{1/K * Fm * v(F0 - Fa)}{Vf(Fm - 1)} \qquad [1]$$

Where K is slope of the standard curve. F0 and Fm are fluorescences measured before and after the acidification. Fm is fluorescence before acidification divided by after acidification fluorescence. V stands for volume of EtOH used in extraction and Vf is the volume that was filtered in the sample.

3.3. Bacteria Biomass

Staining samples with fluorescent stains, in this case DAPI (4-,6-diamido-2-phenylindole), is used often to verify bacteria in the natural sample. DAPI is bound to DNA and shows light blue when examined with an epifluorescence microscope and UV (365 mm) excitation (Porter et al. 1980).

Process started by fixing 2 ml 0.2 µm filtered formaldehyde to 10 ml of water sample. Formaldehyde kills all the bacteria in the water sample. After fixation the sample was stored in cold and dark from 1 to 6 hours. The sample was then filtered with a low pressure through a 0.2 µm 25mm black polycarbonate filter that was placed on top of a glass fiber filter 25 mm (Hobbie et al. 1977). When there was only 1 ml left to filter 60 µm DAPI with a concentration 80 µm/ml was added to the sample. The sample was left to stain for some minutes and was then filtered to the end. Slide for microscopic examination was prepared by dropping immersion oil to the cover slip and the object glass. The black carbonate filter was placed on the top of the cover slip which was then placed on the object glass. The sample was then sealed with a nail polish and stored in -20℃ and dark (Turley et al. 1992). From each sample three replicates were made with same methods. Counting of the samples was done with an epifluorecense microscope (Leica Leitz DMRB) with 1000x magnification number of bacteria and length of random individuals from known area was counted from each sample and replica (Kirchman et al. 1982). Bacterial abundance was calculated by using formula [2] (JGOFS 1994):

$$\text{Bacterial Abundance (cells/litre)} = (C_f \times R) + F_s \quad [2]$$

Where C_f is mean number of cells per field, R is active area of filter divided by area of field counted and Fs is volume of water filters in liters

After calculating the bacteria abundance, three still images were taken through UV-microscope and width and length of 11 individual bacteria were measured from each image with screen ruler software program (Perfect Screen Ruler 2.01) and bacteria volumes were calculated using equation [3](Sieracki et al. 1985).

$$V = \pi/4 \times w^2(l - w/3) \quad [3]$$

Where w is width of individual bacteria and length of individual bacteria.

Calibration for the software was done from previously measured nanoflagellates from where the coefficient for converting pixels to µm was done. All together volume of 99 individuals was measured per sample site. Then bacteria abundances were multiplied with bacterial volume to get biovolume for each individual sampling site.

Biovolumes were then transformed to biomass with three different biomass coefficients. These were obtained from 1) Fry (1988) where the coefficient 308 fg C μm^{-3} is used to convert bacterial biovolumes to bacterial biomass, 2) Combination of Fry (1988) coefficient 308 fg C μm^{-3} and estimates of biovolumes that are calculated from bacteria abundances and from average bacteria size that are obtained from Swedish lakes (Karlsson et al. 2001). 3) Lee & Fuhrman (1987) where bacterial biomasses were estimated with cell carbon content coefficient. Coefficient used in this study was 20 fg C cell which is mentioned to be mean average for individual cell carbon content.

3.4. Bacteria Production

Productivity of heterotrophic bacteria was in my work measured with ^{3}H-leucine method. ^{3}H-leucine measures incorporation of leucine to bacterial protein during a certain incubation time. Physiologically this method is based on protein synthesis. Calculation of biomass is based on rates on protein synthesis and this is possible because proteins create fairly constant 60 % fraction of bacteria biomass (JGOFS 1994).

Work started with working solution preparation. ^{3}H-leucine with specific activity 73 Ci/mmol was mixed with MQ-water (Milli-Q) to obtain a concentration 59 µCi/ml. The correct incubation time was determined first (Moriary and Polard 1981). Determination was done with a time series where the total incorporated radioactivity was measured during various uptake times. Correct incubation time was determined by radioisotope uptake stabilization measured as CPM or DPM. The target is to maximize incorporation of leucine. One representative from low (Tsâhkal A) and high (Saana 11) altitude were chosen to determine the final incubation time and leucine concentration. Suggested default time for incubation is two hours but because lower productivity in arctic lakes incubation time can be between three and four hours. Three hours incubation time was chosen based on place where curve stabilizes in Fig. 6. After this the final concentration of ^{3}H-leucine in the sample was defined by saturation curve. In saturation curve total incorporation of leucine is plotted against leucine concentration in the sample. Leucine concentrations between 10 nM and 20nM were recommended for oligotrophic lakes (Simon and Azam 1989). In the beginning of the study the leucine concentration of 20 nM was used but saturation curves presented in Fig. 7 seemed to stabilize in 30nM and because of this leucine concentration was changed. Difficulty is that if the amount of radioactive leucine

concentration is too low in the sample it does not induce enough difference to natural non-radioactive leucine incorporation. On the other hand too high concentration of radioactive leucine may inflict artificial uptake by bacteria, prevention of uptake or uptake by other organisms (Kirchman et al. 1986).

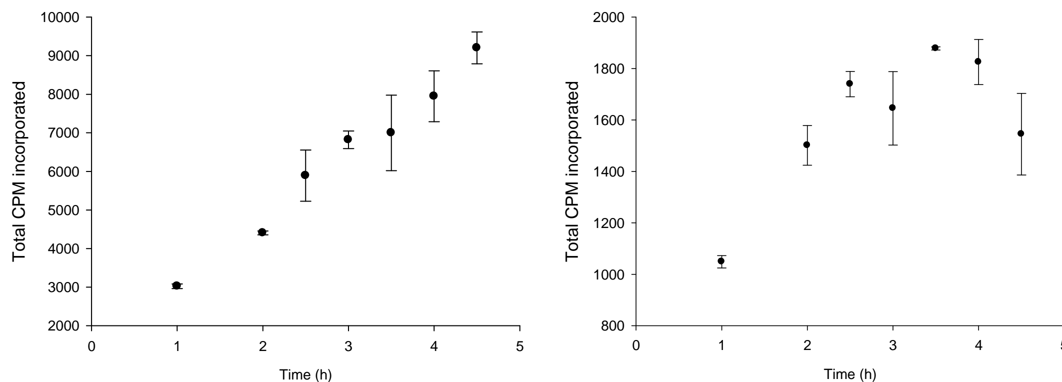


Figure 6. Determination of incubation time in low (Tsâhkal A) and high (Saana 11) altitude pond with standard error bars.

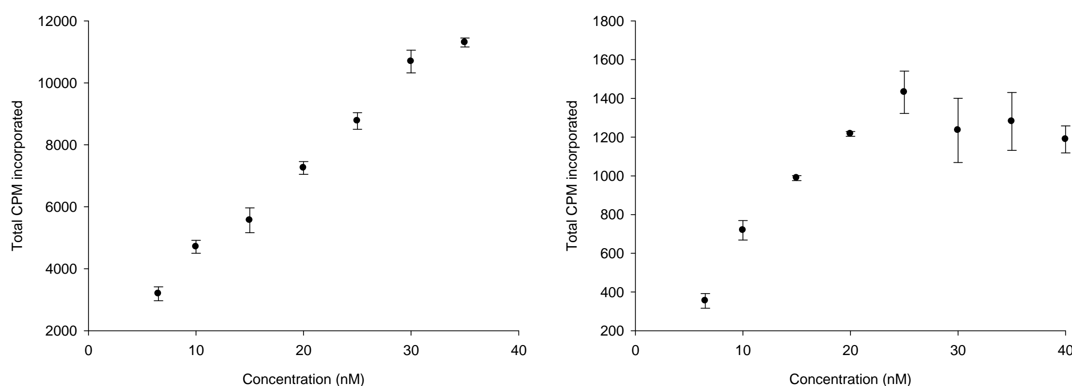


Figure 7. Saturation curves of leucine incorporation in a low (Tsâhkal A) and a high (Saana 11) altitude pond with standard error bars.

All productivity measures were conducted in 1.5 ml microvials at 4°C. The microvials were numbered to identify the samples and 1.25 ml of sample water was added to each vial in three replicates. Two control samples were also prepared in this phase by adding TCA 50 % (Trichloroacetic acid) to kill all the bacteria in the sample (Chin-Leo and Kirchman 1988). Subsequently ^{3}H-leucine was added to the samples to start the incubation. After the incubation time the bacteria in the three replicates were killed with TCA to prevent further bacteria production in the vial. Samples were now ready to be frozen and stored.

Frozen samples were melted before centrifugation in room temperature for 30 minutes. Centrifugation was done at speed of 12 000 rounds per minute for ten minutes (Kirschner & Velimirov 1999). During centrifugation a bacteria pellet was developed in the exterior side of the vial. All liquid was sucked out from the vial with a Pasteur pipette which was connected to a vacuum pump. If this phase is performed with care bacteria pellet stays in the vial. Subsequently TCA 5 % was added to the vial to remove last unbound leucine. The sample was centrifuged and the supernatant was sucked out from the

vial as earlier. Scintillation cocktail (1 ml) was added to the vial and the samples were stored in dark for 24 hour. The ^{3}H-leucine activity of the samples was measured with a scintillation counter (RackBeta). Leucine incorporation was then calculated with formula [4] (JGOFS 1994).

$$\frac{L}{h} = (4{,}5 \times 10^{-13}) \times (DPM_{sample} - DPM_{blank}) \times SA^{-1} \times T^{-1} \times V^{-1} \quad [4]$$

Where L/h is incorporation of leucine in millimoles per time. SA is the specific activity of the leucine solution (Ci/mmol). T is the time used in incubation and C is incubation volume used. Coefficient 4.5 x 10^{-12} (Ci/dpm) is the constant factor. Leucine incorporation then used to calculate bacterial production with equation [5] (JGOFS 1994).

$$mgC\,L-1\,h-1 = Leu\,(mmols\,Leu\,L-1\,h-1) * 131.2 * (\%Leu)-1 * (C:Protein) * ID \quad [5]$$

Where 131.2 g/mol is the molecular weight of leucine. Proportion of total leucine in protein. This assumed to be 7.3 %. Carbon/protein ratio is assumed to 86 % and 2 is assumed to be isotope dilution (ID) factor.

3.5. Bacteria Diversity

One important part of my final thesis is a rough definition of bacterial diversity and seasonal changes of different bacteria types in the ponds. This work started by freeze drying 250 ml of frozen sample water. Four milligrams of freeze dried sample material was then weighted and put into PowerBead tubes provided in PowerSoilTM DNA Isolation Kit. In some cases water was so oligotrophic and poor in organic carbon that the 4 mg sample was not enough. In that case all the available material was weighted and then diluted to liquid in PowerBead tube. DNA isolation followed MOBIO Laboratories Inc. PowerSoilTM DNA Isolation Kit user protocol and as a final result 100 µl of isolated DNA was ready for PCR.

After preparing the Master Mix (Biotools buffer, 5’-primer, 3’-primer, dNTPs mix, sterile water and biotools polymerase) for PCR it was mixed in relation of 24 µl Master Mix to 1 µl template. Samples were then placed to Perkin-Elmer GeneAMP 9600 and 35 cycles of PCR was performed to amplify bacteria 16S rRNA-genes from the sample. Fragments were then separated by using agarose gel electrophoresis which is a conventional method to get good resolution from 100 bp-5000 bp DNA-fragments. Medium sized agarose gel was prepared mixing 0.8 g of 0.8% agarose to TAE-buffer. EtBr solution was then added to about 60℃ mixture and the gel was poured to electrophoresis tray and at the same time comb was laid on the tray. Owl horizontal electrophoresis system was then filled with TAE-buffer and the tray was put into device. Lambda DNA digestion was then added to first sample slot and after it 6x loading buffer is added to template so that to final volume consisted 1/6 of this buffer. Then gel was run at 100 V for one hour. Finally the agarose gel was photographed to illustrate separation of DNA-fragments. Results from PCR showed only one strong band if the primers were specific for 16S rRNA like showed in Fig. 8.

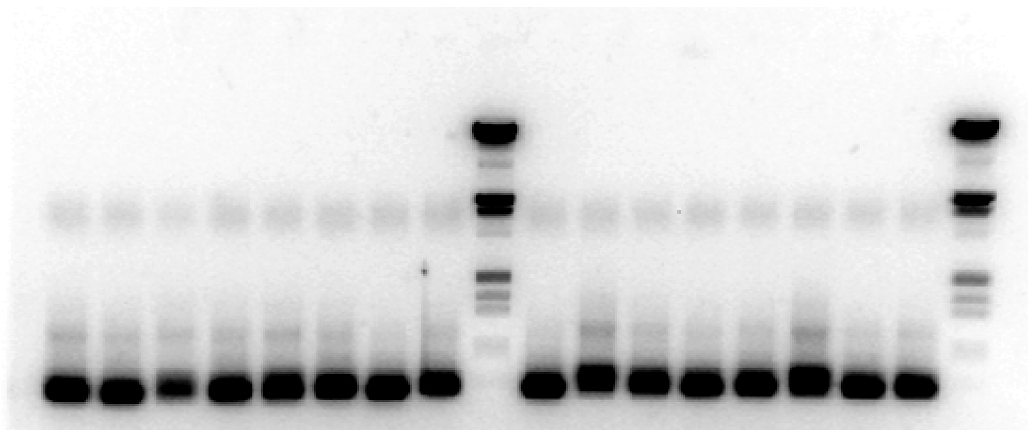


Figure 8. Successful PCR illustrated with digital image from electrophoresis gel.

LH-PCR was the method that was used to compare distributions of 16S rRNA fragments among sampling sites. It is based on the natural length variation within 16S rRNA genes. In LH-PCR the fluorescence emission from labelled PCR primers were used to estimate proportions of PCR amplicons originating from genes from the original sample. Loading mixture (buffer, Licor loading dye and sterile water) and PCR product were mixed in relation 19 µl of loading mixture and 1 µl of PCR product and then injected to Licor sequencer. Automated LI-COR 4200 sequencer (LI-COR BioTech, Lincoln, NE) was used to run the electrophoresis gel for six hours or overnight using 6% Long Ranger denaturing polyacrylamide gel (FMC Bioproducts, Rocland, ME). Gel preparing started with assembling the glass plates, spacers and rails for casting the gel. Then 250 µl ammoniumpersulfate and 25 µl TEMED was added to 50 ml of Long Ranger 6 % acrylamide solution. Avoiding generating bubbles between the glass plates the gel was casted and let to dry for one hour or more. The gel was then pre-run in 1x TBE buffer for half an hour. Size standards of 470, 527 and 553 base pairs were used in the run. Data was then analyzed by using Quantity One software (Bio-Rad Laboratories, Hercules, CA) Example of the Successful LH-PCR gel is illustrated in Fig 9.

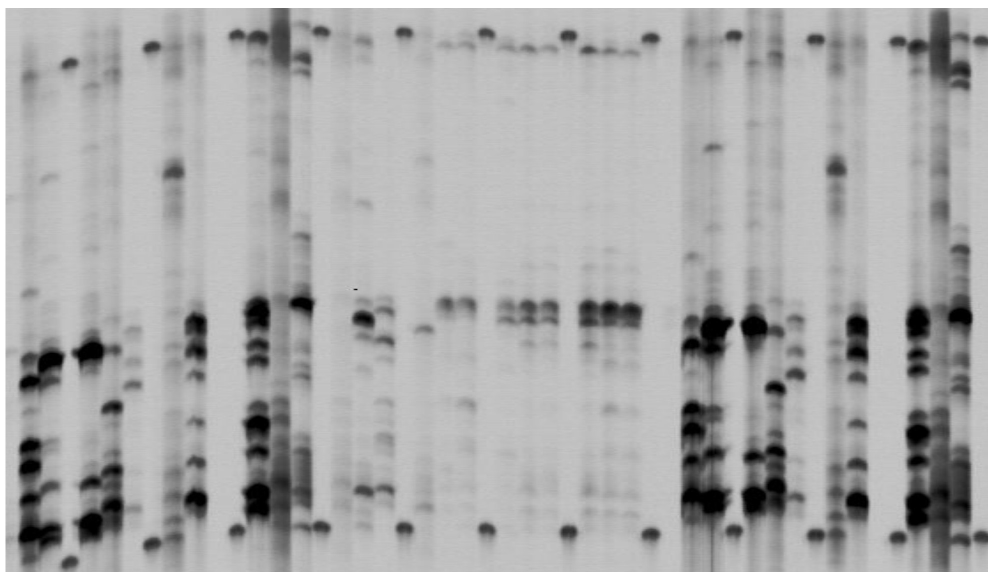


Figure 9. Successful LH-PCR illustrated with digital image from electrophoresis gel.

LH-PCR clone library is created from cultured bacteria and it is used to identify the possible phylum that generally exists has 16S rRNA-gene of certain length. Same methods to study bacteria diversity was applied in study by Taipale et al. (2009). They studied vertical bacteria diversity in humic lakes and they made phylogenetic affiliations between simulated fragment lenghts and clone libraries. With the clone library these definitions are used for identify the main bacterial phylums in the ponds (Taipale et al. 2009).

4. RESULTS

4.1 Seasonality in characteristics

The groups had different characteristics; ponds below the treeline tend to have more DOC runoff from the catchment and therefore are more humic than ponds at higher altitudes. Environmental characteristics were sampled during spring, summer and autumn. They differed clearly between ponds below and above the treeline. Especially fluctuation of DOC values is very interesting. DOC concentration in ponds below the treeline tends to rise during the sampling season but this kind of fluctuation cannot be found in ponds above the treeline. Reason for this DOC increase is likely the drying of lower altitude ponds in summertime. Other clearly visible trends were decreasing temperature and conductance with increasing altitude and higher chl-*a* concentrations at lower altitudes.

Table 2. Seasonality of environmental characteristics of ponds below and above treeline. Numbers outside of the parenthesis are seasonal averages. Numbers inside parenthesis are seasonal minimum and maximum values.

	Spring		Summer		Autumn	
	< 600m	>600m	< 600m	> 600m	< 600m	> 600m
Temperature (°C)	6.0 [1.5-11.6]	2.0 [0.6-4.5]	16.5 [15.9-17.5]	12.0 [9.7-15.7]	7.4 [5.9-8.3]	6.4 [5.0-7.8]
Conductance (µS/cm)	19.8 [8.6-28.8]	7.3 [5.0-10.0]	37.1 [16.2-71.1]	15.1 [5.3-24.0]	39.1 [12.2-70.5]	21.7 [4.4-62.6]
pH	6.1 [5.3-6.6]	6.5 [5.8-8.0]	6.8 [5.6-7.4]	7.1 [6.7-8.1]	6.7 [5.2-7.7]	7.1 [6.5-7.7]
DOC (mg/l)	4.3 [2.6-6.9]	2.1 [1.3-4.1]	7.6 [3.9-13]	2.4 [1.4-3.5]	5.25 [2.4-8.8]	2.6 [1.9-4.0]
Chl-a (µg/l)	-	-	1.3 [0.6-2.2]	0.4 [0.2-0.8]	1.0 [0.4-2.6]	0.5 [0.5-0.7]

4.2 Seasonal changes in bacteria abundance

First of the studied bacteria community variables was bacteria abundance which expresses the number of bacteria individuals in known volume of sample. In ponds that are situated below treeline bacteria abundance showed similarity between spring and autumn sampling. Two exceptionally high values in spring sampling Malla B and Tsâhkal C were absent in autumn because Malla B dried out during summer and Tsâhkal C value is barely in the limits of error bars. Peak in abundance was seen clearly in the summer sampling in low altitude ponds with values almost two times higher than in other occasions. In ponds above treeline the seasonal trend showed similarity with low altitude ponds but the actual values were approximately two times lower during the whole season. Also the peak in summer sampling was as sharp as in ponds below treeline. Independent t-test analysis was run to verify the significant difference between the groups ($T_{28.890}$=4.560 $p<0.000$). Bacterial abundance seasonality is shown in Fig. 10

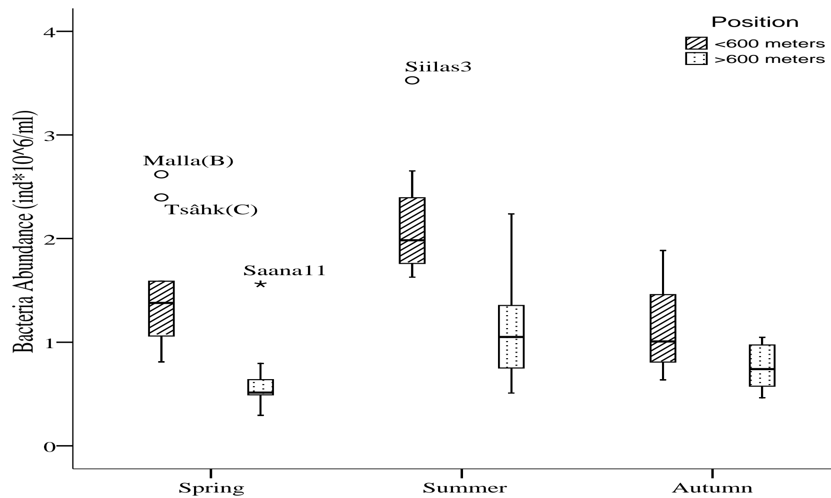


Figure 10. Seasonality of bacteria abundance in ponds below and above treeline.

4.3 Seasonal changes in bacteria productivity

Bacteria production was carried out in the same time than bacteria abundance measurements. In ponds below treeline seasonal trend is clear, low values in spring and autumn sampling and then high peak in summer sampling. It is noticeable that the error bars are rather large even after removing the clearly contaminated samples. The high spring time value of the pond Tsâhkal A is absent in other sampling occasions because the pond dried out during summer. Interestingly the trend was not similar in the ponds above treeline. There was no summer peak and the variation among the sampling occasions was very low. Only the pond Jeähkkas 17 above treeline showed exceptionally high production values. The seasonal results for bacteria production are shown in Fig. 11. Independent t-test did not found significant difference between ponds below and above treeline ($T_{25.330}$=1.823 p=0.080) but when the t-test was run seasonally significant difference was found in summer sampling ($T_{7.573}$=2.531 p=0.037).

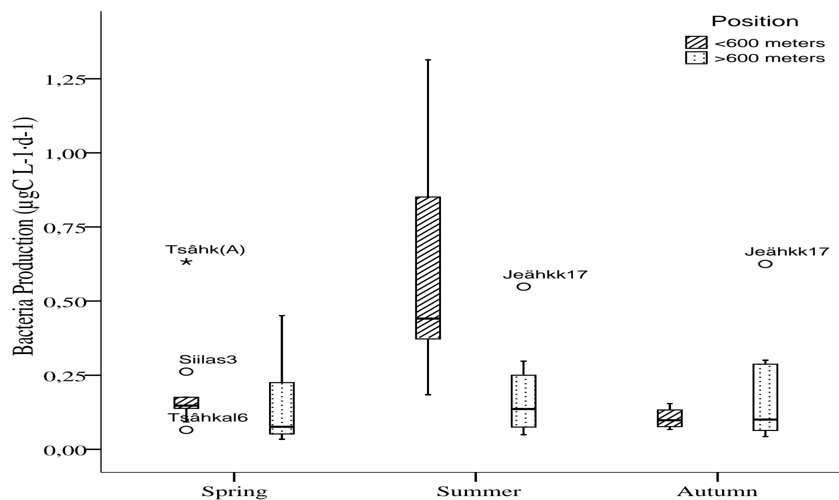


Figure 11. Seasonality in bacteria production seasonality in ponds below and above treeline.

4.4 Bacteria biomass with three methods

Bacteria abundance and average biovolume per individual were the components that were needed to calculate bacteria biomass. In first method the bacteria abundance and my

own bacteria size measurements were multiplied together to get bacteria biovolume. This biovolume is then multiplied with coefficient 308 fg C μm^{-3}. Seasonality of biomass received from first method is illustrated in Fig. 12. Second method also applies the same calculation method only difference is that average bacteria sizes from Swedish lakes (Karlsson 2001) were used to calculate the biovolume. Seasonal biomasses achieved are shown in Fig. 13. Third method is only based on rather constant carbon value per cell. Coefficients from 10 to 25 fg C cell were found from literature. These coefficients are then multiplied by bacteria abundance. Bacteria carbon content average coefficient 20 fg C cell was used in the calculations. Results from this method are shown in Fig. 14.

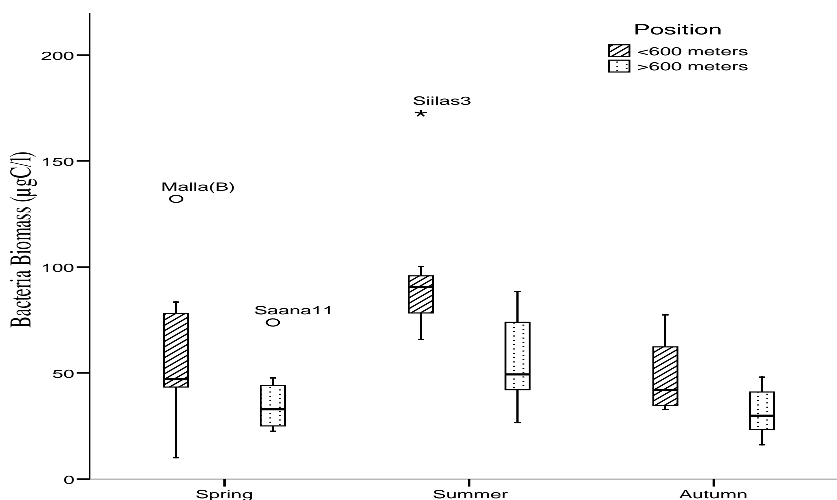


Figure 12. Bacteria Biomass seasonality below and above treeline. Bacteria biomass is determined based on (Fry 1988).

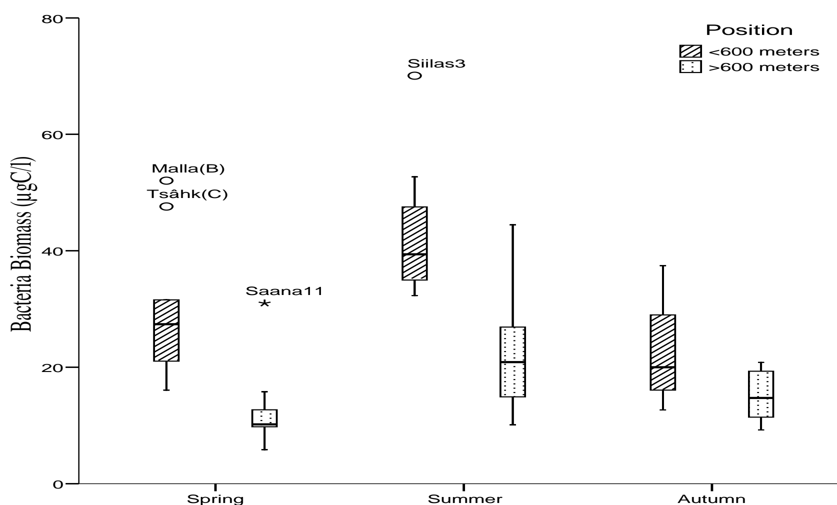


Figure 13. Bacteria Biomass seasonality below and above treeline. Bacteria biomass is determined based on (Fry 1988) and bacteria size averages from (Karlsson 2001) are used.

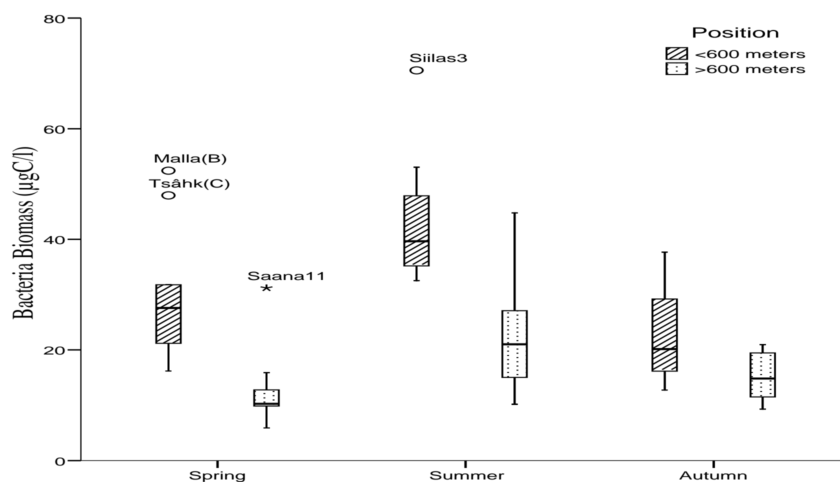


Figure 14. Bacteria Biomass seasonality below and above treeline. Bacteria biomass is determined based on (Lee & Fuhrman 1987).

4.5 Bacteria diversity

Fig. 15 decribes intesity peaks between sequences 465 bp and 565 bp in LH-PCR. The upperchart illustates the situation in ponds below 600 meter contour and lower chart is explaining the situation in high altitude ponds. First traces of bacteria in the samples below the treeline were found between fragments 470 bp and 475 bp. There is also one single peak in trace intensity at point 488 bp. Second intensity peak group occurs between 500 bp and 508 bp. Then there are several peaks between fragments 513 bp and 525 bp. Ponds above treeline follow the same general trend but there was only a single peak of trace intensity in the beginning at point 473 bp. After that first intensity peaks occur between bands 502 bp and 507 bp and the latter group of peaks starts at 517 bp and continue to 527 bp. Generally there seems to be large bacteria diversity between the ponds in both below and above treeline because of the number of different peaks. Even bacteria diversity inside one single pond seems differ seasonally. Seasonal diversity of every pond are presented in appendices. These intensity peaks are illustrated in Fig. 15.

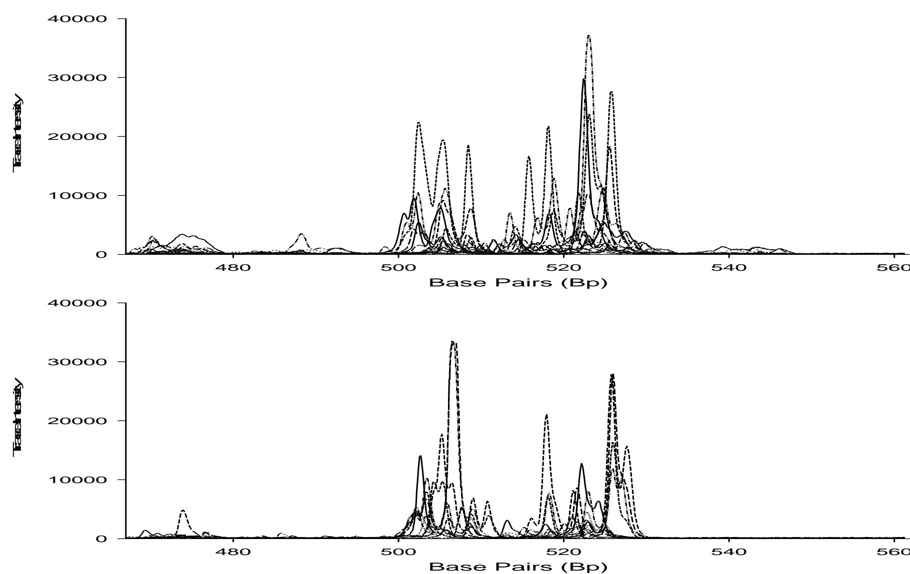


Figure 15. Trace intensities in LH-PCR analysis of ponds below and above 600 meter contour.

Relative proportions of main fragments are presented in Fig 16. Proportions of three different bacteria phyla were estimated based on their theoretical length variation and artificial grouping was made based on clone library and by grouping suggested by Taipale (2009). These groups were alphaproteobacteria which are assumed to present under 475 base pair length. Second group is actinobacteria occuring lengths between 498 bp and 508 bp and last betaproteobacteria which are usually dominant between sequences 521 bp and 527 bp. Average proportions of different phylum groups for low altitude ponds were alphaproteobacterio 7.9 %, actinobacteria 32.1 % and betaproteobacteria 34.4 %. Proportions among these three bacteria groups were distributed differently in high altitude ponds. Alphaproteobacteria phylum was relatively less abundant with 4.4 % proportion. Actinobacteria groups on the other hands had 46.2 % proportion and betaproteobacteria group 27.0 % proportion of total base pair length variation.

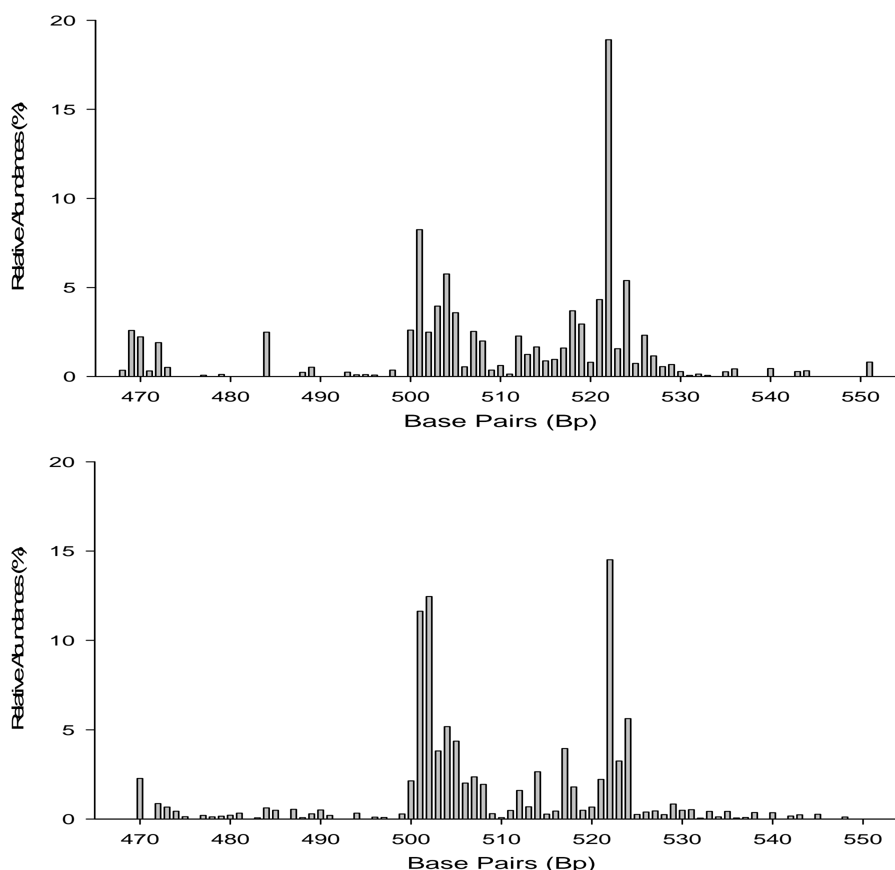


Figure 16. Proportions of the main fragment size classes in LH-PCR analysis in ponds below and above treeline. The bars represent relative band densities in each size class.

4.6 Environmental variables explaining bacteria dynamics in Kilpisjärvi ponds.

Multiple regression analysis with stepwise method with all the environmental and physico-chemical variables (DOC, Temperature, Specific conductance, pH, chl-*a*, lake area, drainage area, depth, altitude) was entered to SPSS 16.0 (Statistical package for the social sciences) to find out the variables responsible for bacteria abundance change. This was done with three different data sets; 1) all the data included, 2) seasonally and 3) divided in below and above treeline groups. In situation where all data is included to run regression analysis it gives two explaining variables for bacteria abundance; DOC and temperature ($r=0.636$, $p<0.000$, $n=46$) with Durbin-Watson value (1.616) for detecting

autocorrelation between residuals. Results showed no similarity when seasonality was included in to the model. In spring sampling session specific conductance (r=0.416, p=0.004, n=18) was found to be explaining variable for bacteria abundance with Durbin-Watson value (2.081). Chl- *a* (r=0.502, p=0.003, n=15) was calculated to be the explaining variable in summer sampling period with Durbin-Watson value (1.382). In autumn sampling session DOC was the explaining variable (r=0.610, p=0.003, n=12) and with Durbin-Watson (2.316). In multiple regression model with all pond data below 600 meter contour specific conductance and temperature were variables significantly (r=0.765, p=0.003, n=20) shaping the bacteria abundance values with Durbin-Watson value (2.213). Interestingly regression model calculation for ponds above 600 meter contour came out without any result although clear correlation (PEARSON) with temperature (r=0.424, p=0.031, n=26) and depth (r=-0.456, p=0.019, n=26) was found. Bacteria abundance Pearson's correlation charts of significantly correlating environmental variables are presented in Fig. 17.

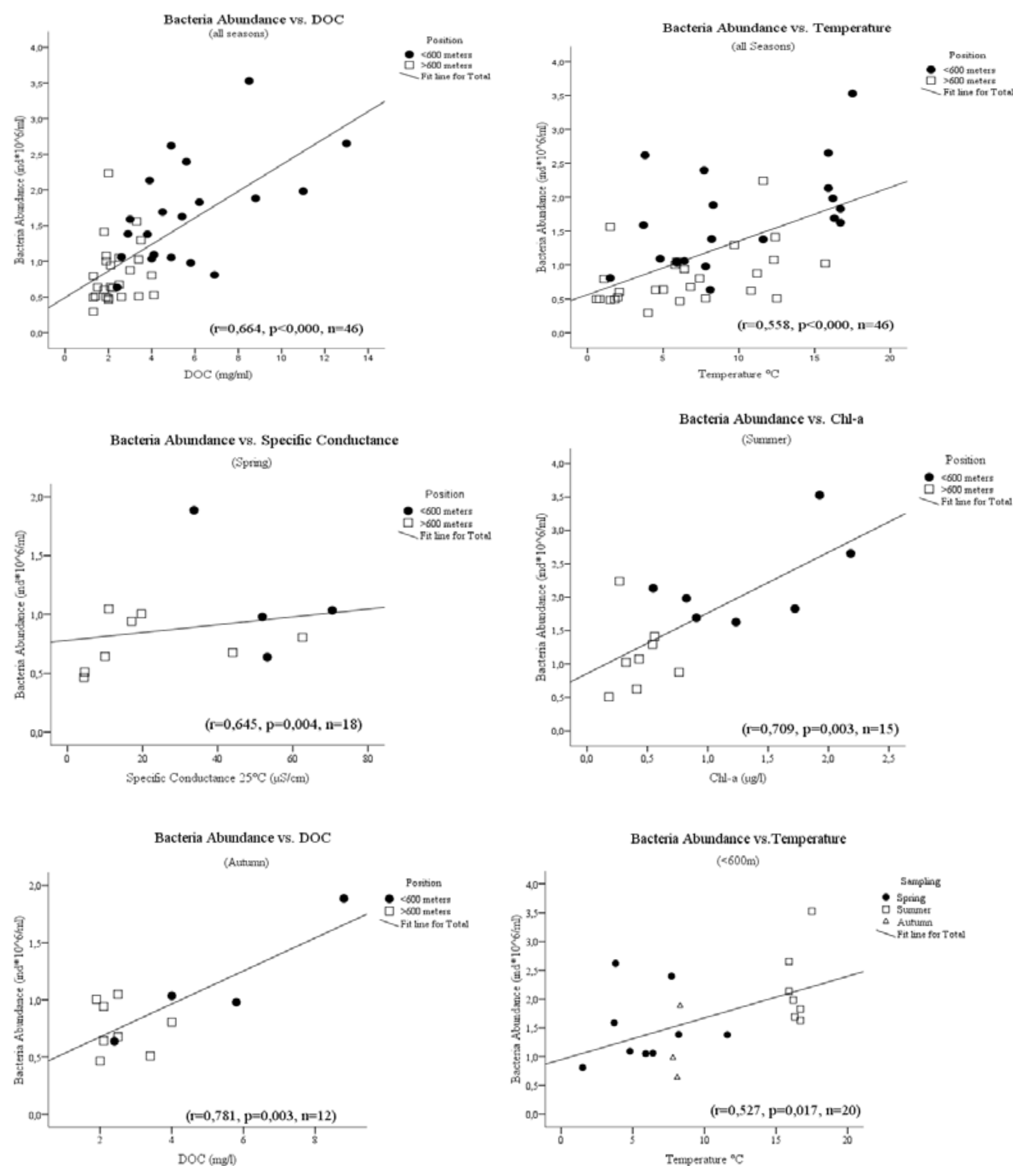


Figure 17. Pearsson's correlations explaining variables that are in regression model for bacteria abundance.

Secondly variables effecting bacteria production rate were tested with multiple regression analysis with same methods than earlier. DOC was found to be the most important variable to explain variation in bacteria production rates. DOC (r=0.277, p=0.005, n=46) was calculated to explain 27.7 % of variation in bacteria production rates with Durbin-Watson (1.519). After dividing the data to seasonal groups multiple regression model was harder to calculate. Only in summer sampling period model with chl-*a* (r=0.481, p=0.004, n=15) was produced. In spring sampling variable, which correlated the most with bacteria production was depth (r=-0.369, p=0.120, n=19) and in the autumn sampling slight correlation with temperature (r=-0.427, p=0.166, n=12) was found. When multiple regression model was applied to data considering only ponds in lower altitude pH (r=0.469, p=0.020, n=20) was found to be the explaining variable for bacteria production changes with extremely low Durbin-Watson value (0.593). Also significant correlation with DOC (r=0.521, p=0.018, n=20) was found. At higher altitude no significantly explaining variable for bacteria production was found with regression analysis. Only correlation was found with depth (r=-0.391, p=0.048, n=26). Pearson's correlations between bacteria productivity and significantly correlating variables are presented in charts in Fig. 18.

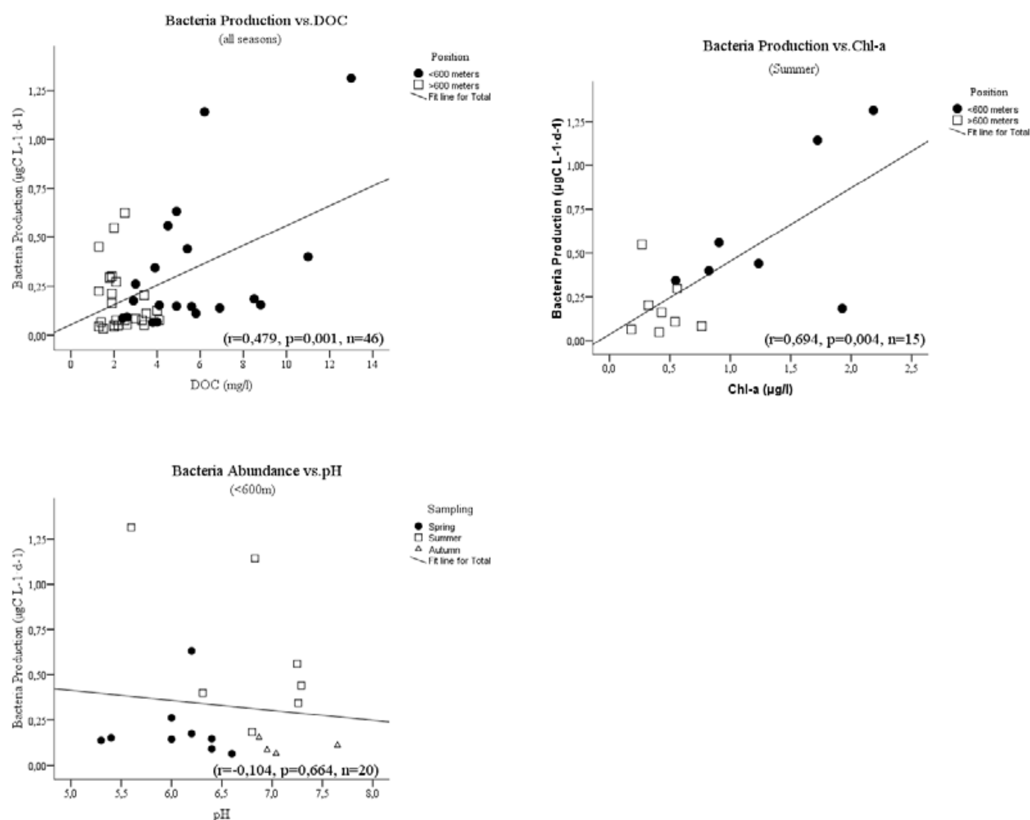
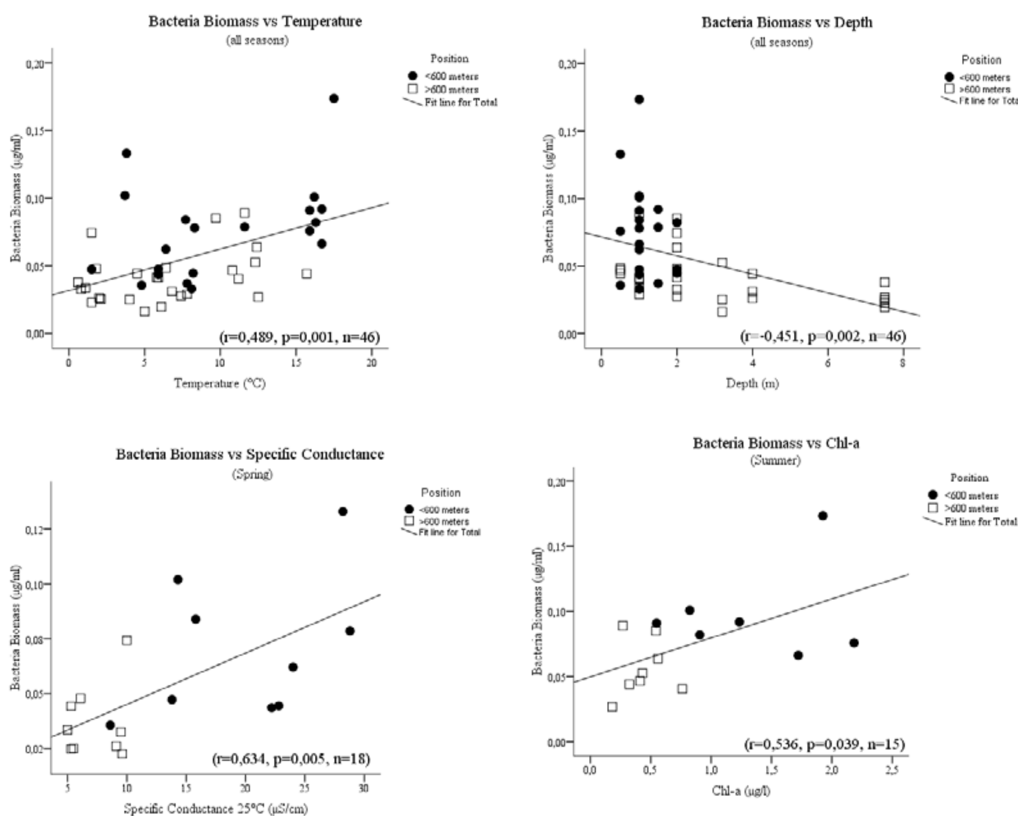


Figure 18.Pearson's correlations explaining variables in regression model for bacteria production.

The methods used to find out the variables responsible for change in bacteria biomass values were the same as described earlier with bacteria abundance. Regression model was run in method that was using only data collected in this study. When whole data was entered to regression analysis, temperature and depth (r=0.576, p<0.000, n=46) were evaluated to be responsible for bacteria biomass change with Durbin-Watson value (1.076). Then data was divided seasonally and entered to SPSS 16.0. Samping data from spring pointed that specific conductance (r=0.402, p=0.005, n=19) was the one that had

most impact on bacteria biomass. Durbin-Watson values detecting autocorrelation was (1.797). In the same way than with bacteria abundance and bacteria production also bacteria biomass data from summer sampling pointed out that the variable chl-*a* (r=0.481, p=0.004, n=15) had the biggest impact on bacteria biomass with Durbin-Watson value (1.488). Autumn data showed that bacteria biomass was dependent on chl-*a* and depth (r=0.735, p=0.003, n=12). Durbin-watson value for autumn sampling was (2.555). Then data was entered in two groups below and above treeline ponds. In ponds below 600 meter altitude bacteria biomass was found to be dependent on temperature changes (r=0.469, p=0.020, n=20) and autocorrelation was tested with Durbin-Watson test (1.107). In ponds above 600 meter treeline multiple regression analysis was unable to build model. Pearson's test showed signifant correlation for bacteria biomass only with depth (r=-0.464, p=0.017, n=26). Seasonal results calculated in two different ways are shown in fig. 18 and 19 (20). Significant Pearson's correlations between bacteria biomass and other variables are shown in Fig. 19.



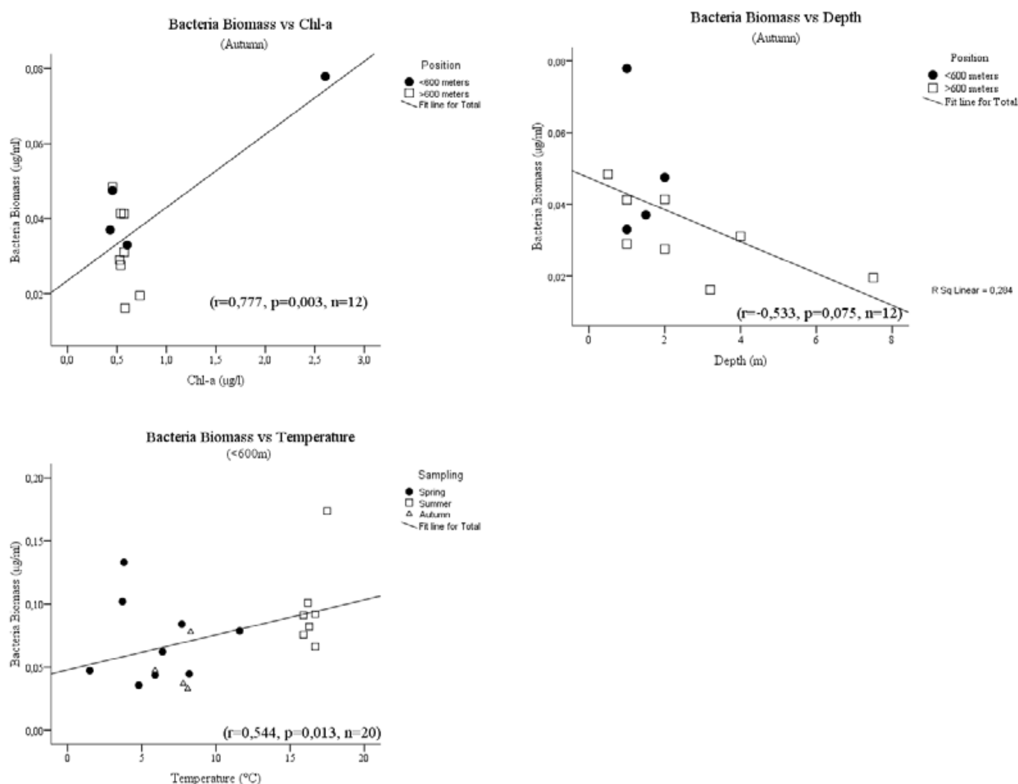


Figure 19. Explaining variables in regression model for bacteria biomass based on (Fry 1988).

5. DISCUSSION

5.1 Environmental control of bacterioplankton in subarctic ponds

Rapid fluctuation of physical and chemical conditions is typical for subarctic ponds as a habitat. Growing season in Finnish Lapland lasts approximately four months from end of May till late September. This season is characterized with large fluctuations in temperature and concentration of dissolved organic carbon and nutrients, which all play important role in shaping bacteria communities.

In subarctic environments temperature has been observed to be one of the key factors effecting bacteria activity (Ochs et al. 1995 & Rae & Vincent 1998) slowing down their metabolic activity. Temperature is negatively dependent on altitude and this study was no exception. This is one of the reasons why lower bacteria activity was seen in higher altitude ponds. During growing season temperature in shallow ponds in Alaska varied as much 10 ℃ within a day (Hobbie 1980). In this study the biggest temperature difference between two ponds (Saana 11 and Siilasvuoma 3) in summer was 7.8℃. Temperature has been shown to be one of the limiting factors for bacterioplankton growth particularly in relatively low temperatures (< 12-15℃) although many of bacteria are adapted to colder environment. Generally in higher temperatures correlation with bacteria growth is weaker (Wetzel 2001).

It is known that photosynthetic plankton in arctic and subarctic lakes has strong response to nutrient addition, especially phosphorus (Lizotte 2008). Chl-*a* results in this

study were used to reflect the nutrient availability in the ponds and reveal the possible nutrient limitation of bacteria. In general, chl-*a* concentrations in all ponds in this study were low (< 1.5 µg/l) suggesting low amount of nutrients and possible nutrient limitation. Earlier phosphorus measurements from the same ponds have shown that total phosphorus range from 4 to 18 µg/l, the highest values found in low latitude humic ponds (Rautio 2001). Seasonal studies often suggest that the number of bacteria is greater in productive periods and increasing number of studies is indicating that nutrients could be the main limiting factor effecting bacteria activity (Watanabe 1996 & Simon et al. 1998). Bacterioplankton has high-affinity uptake system for especially inorganic forms of phosphorus and this way bacterioplankton dominates the competition for phosphorus against phytoplankton. Nutrient limitation can shift rapidly seasonally and even in diurnal bases and with changes in composition of microbiota (Wetzel 2001).

The problem with the chlorophyll-*a* results is that there were only two samplings, in summer and in autumn. In small subarctic ponds chl-a peak is not usually in spring time like normally in boreal dimictic lakes. This is mainly due to electrolyte poor melting water which does not provide enough nutrients for algae growth. Therefore it is possible that observed summer peak is the actual season maximum. When chl-*a* concentrations were compared seasonally with altitude change two times higher values were found at lower altitudes compared to higher altitudes and no significant change was found between seasons at neither low or high altitudes. Multiple regression models suggest that in summer sampling that we can predict changes in bacteria variables with chl-*a* values. This suggests that nutrients could be the factor controlling bacteria abundance, production and biomass in summer period. However, chl-*a* had significant correlation ($r=0.802$, $p<0.000$, $n=15$) with DOC indicating that DOC could be also the explaining factor controlling bacteria. Bacterioplankton benefits from high primary production also by the input of autochthonous DOC produced by algae. Similarity between DOC and chl-*a* was also found when the results were observed from point of altitude change. Another factor correlating significantly with chl-*a* concentration in summer sampling was temperature ($r=0.639$, $p=0.010$, $n=15$). In this case, however, all these variables were connected to altitude change among the observed ponds.

There are two different sources of DOC. Autochthonous DOC can be the dominant source of carbon when inputs from the drainage area are very low (Baron et al. 1991 & Forsström et al. unpublished). This might be the case with the low nutrient ponds situated above the treeline. Allochthonous carbon on the other hand is organic carbon derived from the drainage area. Allochthonous carbon inputs relieve the heterotrophic bacteria from dependency on phytoplankton carbon. Autotrophic production is often nutrient limited in the oligotrophic lakes. This is the reason why allochthonous carbon increases the importance of heterotrophic bacteria as an energy mobilizer. Amount and quality of DOC is known to be one of the most important factors controlling the bacteria communities in the lakes (Crump et al 2003). In Lake Toolik bacteria community shift was measured during the spring. The shift was associated with the flux of allochthonous carbon from melting water. Also peaks in bacteria production was encountered in spring (Crump et al. 2003). Effects of DOC addition on bacteria has also been tested on enclosure experiments both in oligotrophic lake in Northern-Finland (Saanajärvi) and in high-arctic lake in Svallbard. In both cases DOC addition had positive impact on bacteria production (Hessen et al. 2004 & Forsström et al. unpublished)

Specific conductance is a measure of electrolyte content of water and it is closely related to discharge from the drainage area. (Kalf 2002) This was also the case in studied ponds because in spring time inputs from drainage areas are increased by melting snow

water that increases erosion from soil. According to multiple regression model in spring time bacteria abundance and biomass changes could be explained by specific conductance. Specific conductance itself does not explain the variation but it indicates to other factor. In this study specific conductance change is mainly explained by the altitude change ($r=-0.804$, $p<0.000$, $n=18$) and therefore change in vegetation in the drainage area. Lower altitude drainage areas were covered with *Sphagnum moss* and vegetation was lacking in higher altitudes were the soil were mostly covered with rocks and boulders. Also the pH values that were constantly higher in higher altitudes indicated the fact that the soil is more acidified in the lower areas. Interestingly no trends concerning bacteria production was found from spring data. Reason for this might be that there were no obvious changes found in bacteria production when moving from lower to higher altitude and many variables react strongly to altitude change.

Abiotic regulation of communities is more common in low productive systems, for example in polar lakes, while in more productive systems increased biotic regulation controls food-web structure more (Menge & Sutherland 1987). I therefore assume that in this study the bacteria communities were mostly controlled by the abiotic variables measured and discussed above. However, some grazing may have occurred, especially in the low altitude ponds. Grazing pressure by heterotrophic nanoflagellates is considered to be main biotic factor causing mortality to bacterial communities. Studies in two oligotrophic lakes in Svallbard suggested that heterotrophic nanoflagellates removed up to 28 % bacterial biomass per day (Laybourn-Parry & Marshall 2003) and even situations when heterotrophic nanoflagellates are able to consume all bacteria production per day has been encountered in lower latitude lakes (Bloem & Bär-Gilissen 1989). On the other hand in ultraoligotrophic Antarctic lake heterotrophic nanoflagellates removed only between 0.1 and 9.7 % of daily bacterial production (Laybourn-Parry et al. 1995). In our study heterotrophic nanoflagellates were 1.5 times more abundant in low altitude than in high altitude ponds density averages varying from above treeline 308 ind/ml to below treeline 448 ind/ml (Mariash & Cazzanelli unpublished) therefore causing more grazing pressure to lower altitude ponds. Measured heterotrophic nanoflagellate densities are well comparable to those measured in Svallbard 50-320 ind/ml (Laybour-Parry & Marshall 2003) and Lake Toolik 100-600 ind/ml (O'Brien et al. 1997). Larger zooplankton like cladoceras are also known to consume bacteria (Kankaala 1988 & Rautio & Vincent 2006). Species like *Daphia longispina*, *Ceriodaphnia quadrangula* and *Bosmina obtusirostris* have been collected from many of the sampled ponds below treeline (Rautio 1998, Mariash & Cazzanelli unpublished) therefore presenting a possible top-down pressure to bacterioplankton. However, in some cases cladocerans grazing on bacterioplankton, did not have any major effect on bacterioplankton biomass (Güde 1988). Viral infections are also known to be one important factor causing rapid changes to bacteria communities, mortality often exceeding 50 % (Wetzel 2001).

5.2 Bacteria activity in high-latitude freshwaters

The harsh abiotic conditions and their great annual fluctuation have contributed to the lower biomass and biodiversity in high latitude regions among all taxonomic groups, including the bacteria. The biomass and productivity of aquatic bacteria in high latitude water bodies is generally low but may still vary considerably in different polar regions and types of waters (Pearce & Galand 2008). The lowest known bacteria abundances, 0.01-0.02 x 10^9/l, have been measured in Char Lake in High Arctic Canada (Morgan & Kalf 1972), and are lower than abundances measured for Antarctic lakes, while tundra ponds in Barrow, Alaska are more bacteria rich with abundances reaching 2-6 x 10^9/l (Hobbie et al.

1980). Research on smaller ponds was done in Canadian high arctic where bacterial abundance results varied between 2.1-6.5 x 10^9/l (Graneli et al. 2004). Results from Kilpisjärvi ponds are therefore in same range with other ponds and lakes in the subarctic-arctic region with bacterial abundances ranging from 0.30 to 3.53 x 10^9/l.

Karlsson et al. (2001) studied bacteria abundances along an altitude gradient in northern Sweden and showed that lower altitude lakes had more bacteria. My results in Kilpisjärvi show similar increasing bacteria abundance with lower altitude. A closer comparison of bacteria abundances in Kilpisjärvi ponds below treeline with sub-alpine Swedish lakes (375m-510m) reveal that mean bacteria abundance values were 50% lower in larger Swedish lakes. Calculated two year summer mean for sub-alpine lakes was 1.13 x 10^9/l while summer mean for ponds below the treeline in Kilpisjärvi was 2.21 x 10^9/l. High altitude ponds in Kilpisjärvi are comparable with Karlsson's low, middle and high alpine lakes (710m–1050m) because there is no vegetation and the soil is covered only by rock and boulders. Two year mean value for low, middle and high alpine lakes was 1.21 x 10^9/l but mean value only for high-alpine lakes with no vegetation was 0.52 x 10^9/l. Results from Kilpisjärvi go nicely in the middle of this range with mean bacteria abundance 1.13 x 10^9/l in high altitude ponds.

Bacterial biomasses in observed Kilpisjärvi ponds varied between ponds and seasons but also depending on the method. Fry's method gave the greatest variability that was 10.0-172.5 µg C/l while combined Fry & Karlsson's method gave an average biomass 5.9-70.1 µg C/l and Lee & Fuhrman's method nearly the identical 5.9-70.6 µg C/l. Mean biomass results from Swedish lakes varied between 9.2–66.1µg C/l (Karlsson et al. 2001), which is similar to my results when they are calculated with Fry & Karlsson and Lee & Fuhrman's methods. Even Fry's method still gives values that are clearly below the concentrations found at lower latitudes; i.e. bacterial biomass varied between 251-684µg C/l (Bjornsen 1986) in two temperate Danish lakes.

All the methods show that there is almost two times higher bacteria biomass in the lower altitude ponds. Depending on the method the average biomass in the low altitude ponds ranged from 33.4 (Lee & Fuhrman's method) to 43.8 (combined Fry & Karlsson's method) and to 96.7 µg C/l (Fry's method). The corresponding values for high altitude ponds were 16.6, 22.5 and 55.7 µg C/l.

Bacterial production in our ponds ranged from 0.03 to 1.31 C/l/d. Similar low productivity has been found in both arctic lakes and Antarctica. Among the lowest measured bacterial production is Beaver Lake in MacRoberson Land were production varied between 0.05-0.29 C/l/d (Laybourn-Parry et al. 2006). In arctic lakes the measured bacterial production has generally been higher than in Antarctica. In Toolik lake the production values were between 1.6 and 22.4 C/l/d (O'Brien et al. 1997) and in Franz Joseph Land lake between 1.2 and 3.9 C/l/d (Panzenböck et al. 2000). On the other hand productivity results from Lake Saanajärvi that is situated in same Kilpijärvi region represent similar bacterial productivity, values between 0.02-0.25 C/l/d ,than high altitude ponds (Forsström et al. unpublished) but still it seems that our bacteria productivity values from the observed ponds were very low compared to other studies.

Comparison of separated low and high altitude pond results show that bacteria production values in Kilpisjärvi ponds were >50% lower than the values in bigger lakes in Northern Sweden (Karlsson et al. 2001). Mean bacteria production in summer sampling in Karlsson's sub-alpine lakes was 2.2 µg C L/l/d and mean production in summer for low, middle and high-alpine lakes was 1.5 µg C/l/d. Mean summer bacteria production in low altitude ponds in Kilpisjärvi was 0.63 µg C/l/d and in high altitude ponds 0.19 µg C/l/d.

Studies on bacteria seasonality are rare in the Arctic. In 1980 Hobbie et al. characterized seasonal patterns concerning bacteria in arctic tundra ponds in Alaska. They found out that there was three separate phases during the growing season; 1) runoff phase, 2) growth phase and 3) winter phase. Highest bacterial abundances and biomasses were in that case measured during the runoff phase (early June). Higher bacteria activity in runoff phase was strongly connected to allocthonous nutrient inputs from melting water. As runoff flow decreases also the number of bacteria decreases (late June). Growth season lasts majority of the summer season and in late August another peak in bacterial activity was observed. In winter period the bacterial activity was measured to be low and stable because bacterioplankton is limited by temperature and reduced inputs of organic substances (Hobbie et al. 1980). More recently bacteria community shifts were studied in oligotrophic Toolik Lake in Alaska. Results support the earlier view by Hobbie et al. (1980) and also community shifts were divided in three different categories; winter, inlet-influenced and summer communities. Although there were persistent bacteria groups identified during the whole sampling season the highest bacterial production values were measured during the inlet-influenced season when lake temperature was only 4 ℃ (Crump et al. 2003). Seasonality in our ponds differed from this pattern. Highest values from abundance, production and biomass were all measured during the summer sampling period in mid-July approximately a month after ice-out period and both spring and autumn measurement showed lower bacteria activity. This differece is likely due to DOC concentration peak in our sampling that occurred during the summer. Chl-*a* concentrations were also higher in summer sampling indicating better availability of nutrients. Bacteria community seasonality was also observed in sampled pond and the results pointed out that in many cases only minor similarity between seasons was found but persistent groups of bacteria occurred.

5.3 The still unknown diversity of aquatic bacteria

Results from bacteria diversity in species level indicated 1) very little similarity between low and high altitude ponds, 2) only occasional similarity between ponds within low or high altitudes and 3) only occasional seasonal similarity in single ponds. Clearest similarity was found in ponds situated below treeline in autumn sampling. When comparing single ponds that were connected in some part of season, for example Saana 14 and 15, occasional similarity was found. This indicates that strong variation in bacteria diversity might be due to the change in environmental conditions supporting the assumption that bacteria respond quickly to environmental changes (Wetzel 2001).

There was a small group of intensities in the beginning of the fragment scale between 470 bp and 475 bp. According to Taipale et al. (2009) alphaproteobacteria exist between fragment 470 bp and 472 bp. Below treeline there was many trace intensity peaks between sequenses 500 bp and 508 bp and 73.7 % of those were were assigned to actinobacteria according to LH-PCR clone library. Other peaks between lengths 513 bp and 525 bp. Between this variation there are many possible phylums but in Taipale et al. (2009) for example 514 bp represented bacteroidetes, fragment between 518 bp and 519 bp delta- and betaproteobacteria and fragments between 522 bp and 535 bp betaproteobacteria. The single peak in 485 bp might represent trace from epsilonproteobacteria (Taipale et al. 2009).

When comparison was made among phyla, results showed that two groups dominated the diversity; betaproteo- and actinobacteria. Alphaproteobacteria was also found but its relative abundance was much lower than betaproteobacteria and actinobacteria. Similar main groups were also found to be dominant in small humic lake

Mekkojärvi where proteobacteria, especially betaproteobacteria with 33 % proportion was found to be to very abundant phylum and actinobacteria were the second largest group with 10 % proportion. The greatest difference in comparison between Mekkojärvi and the sampled ponds was the small number of chlorobi which was the most abundant group in Mekkojärvi. In Mekkojärvi relative number of chlorobi increased in anoxic layers. Sampled ponds were mixing so oxygen was always available this way probably causing the absence of chlorobi (Taipale et al. 2009).

Comparison between Kilpisjärvi bacterioplankton communities and communities found in oligotrophic tundra lake, Lake Toolik was also done (Crump et al. 2003). Similarity between main phyla were found. Dominated groups were identified as alpha- and betaproteobacteria, cytophaga-flavobacteria-bacteroides and actinobacteria groups. Only cytophaga-flavobacteria-bacteroides group was missing from pond communities in Kilpisjärvi.

Water sampling method for determining the bacteria diversity can be questioned when interpreting the results. There were no exact sampling places when three different water samples from same pond were integrated together. This way we cannot be sure that the integrated water sample from different seasons represents the same communities. There might be changes in bacteria communities even inside the small pond. Also there might a problem with interpreting the LH-PCR results and for example overloading the LI-COR-sequencer with template might lead to problems with the result scale. This way the intensity peaks can go out from the scale. Also there is a problem with too strong template traces which does not fit to their own narrow line this way causing artifacts to samples placed on surrounding lines. Despite these error sources this study is among the first ones to describe freshwater bacteria aquatic communities in the North, and perhaps the first one done for subarctic ponds.

5.4 Choice of methods

Bacteria biomass calculation that is based on individual bacteria numbers and volume is a really robust method. Still comparison of the bacteria biomasses revealed almost three time differences between the lowest and highest estimates. Lee & Fuhrman's method gives coherently lower values for bacteria biomass than Fry's method when using Karlsson's bacteria size averages. Fry's methods applied with my own averages for bacteria biovolume gives clearly largest values. There are several reasons for the observed differences. Lee & Fuhrman's method only estimates the carbon content in single cell and it does not take an account the differentiation in bacteria size. It is known that smaller bacteria contain more carbon according to size than bigger ones (Lee & Fuhrman 1987). In Fry's method volumes of individual bacteria are calculated from bacteria diameters and then used with bacteria abundance to calculate bacteria biomass. Fry's method was applied twice in this study. In first case the measured bacteria sizes from this study were used and secondly average volumes from lakes studied by Karlsson (2001).

Lee & Fuhrman's method provided always the lowest values when comparison among these three methods was done. This is probably due the coefficient which was estimate of single cell carbon content. Coefficients provided in literature varied between 10 and 25 fg. Lowest value was used with bacterioplankton in Arctic Ocean and the highest value represented eutrophic freshwater lake. Average value for carbon cell content was suggested to be 20 fg C cell. Fry's method produced coherently higher average biomasses than Lee & Fuhrman's method. When results from biomass averages from Fry's method were compared the reason for almost two times higher biomass average with my own data was difference in average bacteria diameter and volume.

Very likely the reason behind this is the inaccurate method for measuring the dimensions for the individual bacteria in my study. Most of the bacteria was only sized around 2-4 pixels and therefore measuring errors might have been done. It is also possible that some other organism (nanoflagellates) than bacteria has been measured from images therefore increasing the average bacteria biovolume. In Karlsson's article automated Neural network-based computer software was used to calculate the average biovolumes. Automated image analysis gave good results for bacterial biovolume when they compared results to earlier studies. Only problem was that they could not compare the results seasonally, because there was no data available. This would have been important, because faint objects caused problems when they estimated bacterial concentration in winter period (Blackburn et al.1998). For further research automated bacterial dimension measurement would be much more reliable, even with its problems, than laborious manual dimension measuring. On the other hand even easier way to estimate bacterial biomass results was Lee & Fuhrman's methods where only calculated coefficient was used. In our study results from these two separate methods were surprisingly similar.

As a method bacteria production measurement is much more complex than abundance and biomass measurements. One of the biggest assumptions is the fact that the method rely on incorporation of taken up leucine into protein. In some environments uptaken leucine can degrade to amino acids. This is suggested to happen in oligotrophic environments with little organic carbon inputs (Kirchman et al. 1985). Also the procedures in laboratory needed special caution so possibility to human mistake is larger than in bacteria abundance and biomass measurements although the variation among the three replicates was quite small (average c.v. 8.2 %). Due the small water volume few of the lower altitude ponds dried out during the sampling. In three occasions, one in summer and two in autumn, the depth of water body were so low that the prepared samples were contaminated.

Like said earlier there were many assumptions made to calculate production. Incubation temperature might be one reason behind lower production values. Especially in summer sampling the water temperature was much higher (ca. 15 °C) that the incubation temperature (4°C)**.** This will cause lower activity on bacteria. Also one major difference in our method was the use of the unit count per minute (CPM) instead of disintegration per minute (DPM) in equation 4. This makes the bacterial production seem higher than it should but because the colour of the samples did not vary in eppendorf tubes when samples where run in the scintillation counter the unit error does not affect relative values from bacteria production. Complexity of bacteria production method can also cause difference in results when comparing them to Swedish ones. In our study saturation curves were used to estimate the right leucine concentration and concentrations from 20 nM to 30 nM were chosen. Comparison with Karlsson (2002) revealed that they used concentrations between 32nM and 73 nM. This might cause artificial uptake of leucine in bacteria and cause too high bacteria productivity.

5.5 Future of arctic research and climate change

In future arctic limnology will benefit greatly on development of technology because much of polar latitudes is inaccessible for normal study. Remote sampling provides solution to this problem and it has been tested for example on Lake Fryxell over winter (McKnight et al. 2000) and in remote sensing platform in Crooked Lake in the Westfold hill (Palethorpe et al. 2004). This kind of remote data collecting and transmitting is called wireless networking (Porter et al. 2005) and it provides possibility for extensive datasets. Developments in surface imaginary allow the defining on the catchment characteristics for

example vegetation coverage (Markon & Derksen 1994) which is very important factor when estimating organic material loads to waterbody. When only bacterioplankton is considered the most important development has been done in area of genomic tools where DNA- and RNA-based technology allow the description of phylogenetic diversity for viruses, bacteria, archaea and eukarya. Now the challenge is to find out how these organisms function in the aquatic systems.

The results from observed ponds illustrated that DOC, nutrients and temperature are responsible for changes in bacteria activity. After long public debate on occurrence of climate change it has now been widely acknowledged. These results support the hypothesis where increased precipitation caused by global warming could cause increased inputs of DOC and this way shape the bacteria communities (Hobbie et al. 1996 & Karlsson et al. 2001). It is also predicted that air temperature rises especially in the subarctic and arctic regions (ACIA 2005).

High Arctic or Antarctic lakes and ponds are very sensitive to changes and therefore can work as good barometer for climate change. In smaller scale the observed higher altitude ponds are more sensitive to global warming than ponds that were situated lower altitudes because of their very low DOC concentration. This means that the amount of DOC increases relatively most in high altitude lakes and ponds (Vinebrook et al. 1998). Global warming should also increase the amount of nutrients available that way having almost identical consequences than DOC in subarctic aquatic environment. This way global warming would create also more beneficial environment for the microorganism at subarctic ponds and therefore have great impact on the lowest trophic level and leading to changes also in the higher trophic levels. Arctic water research has developed considerably during last decades but there is still need for further research to better estimation for change in bacterioplankton communities in subarctic-arctic lakes and ponds because their crucial part in aquatic food webs and also because of their role in methane release from arctic thermocarst ponds (Walter et al. 2006).

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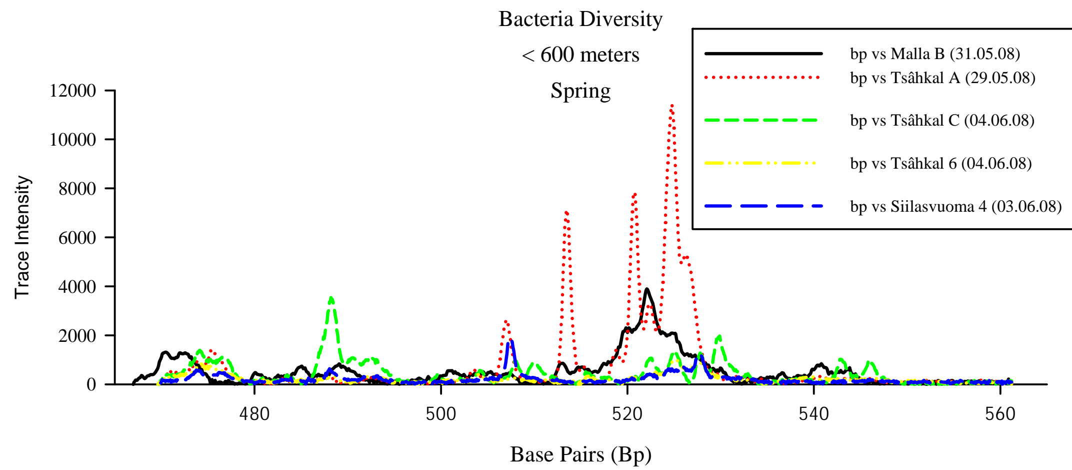
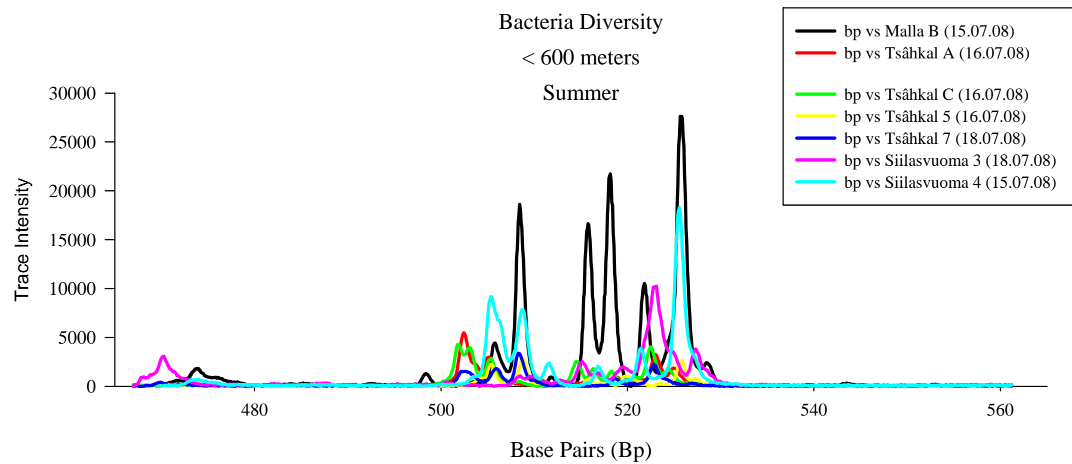
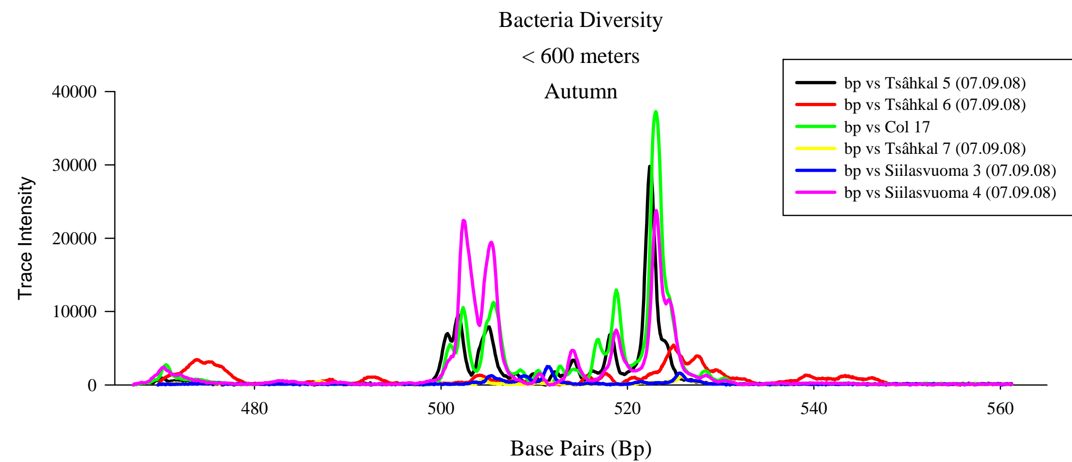
APPENDICES:

Page 1. Bacteria Diversity Seasonality in low altitude ponds







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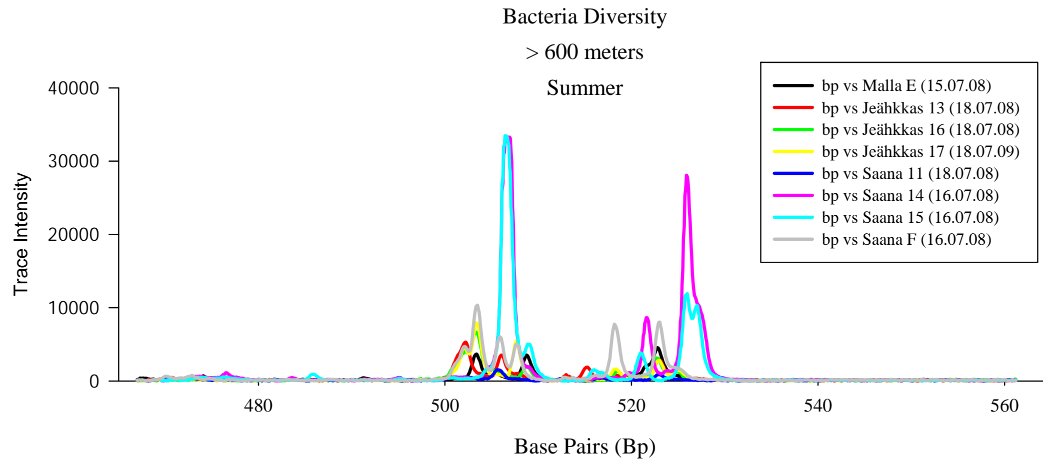
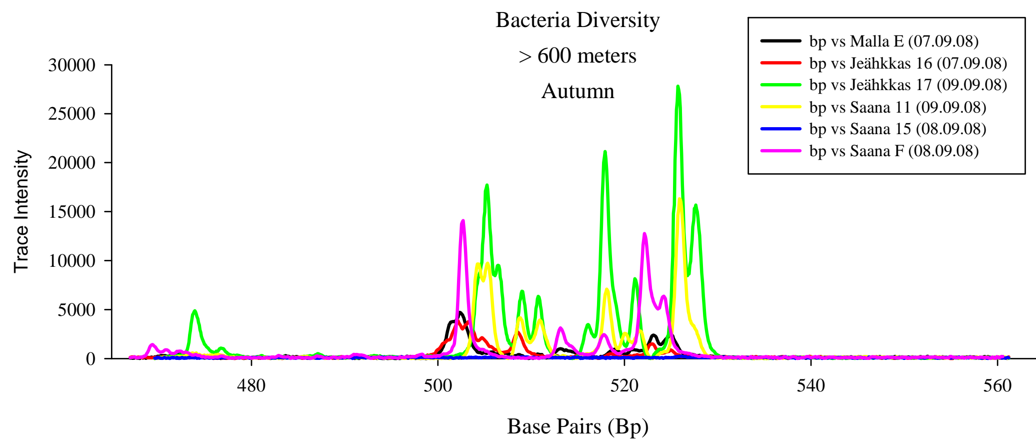
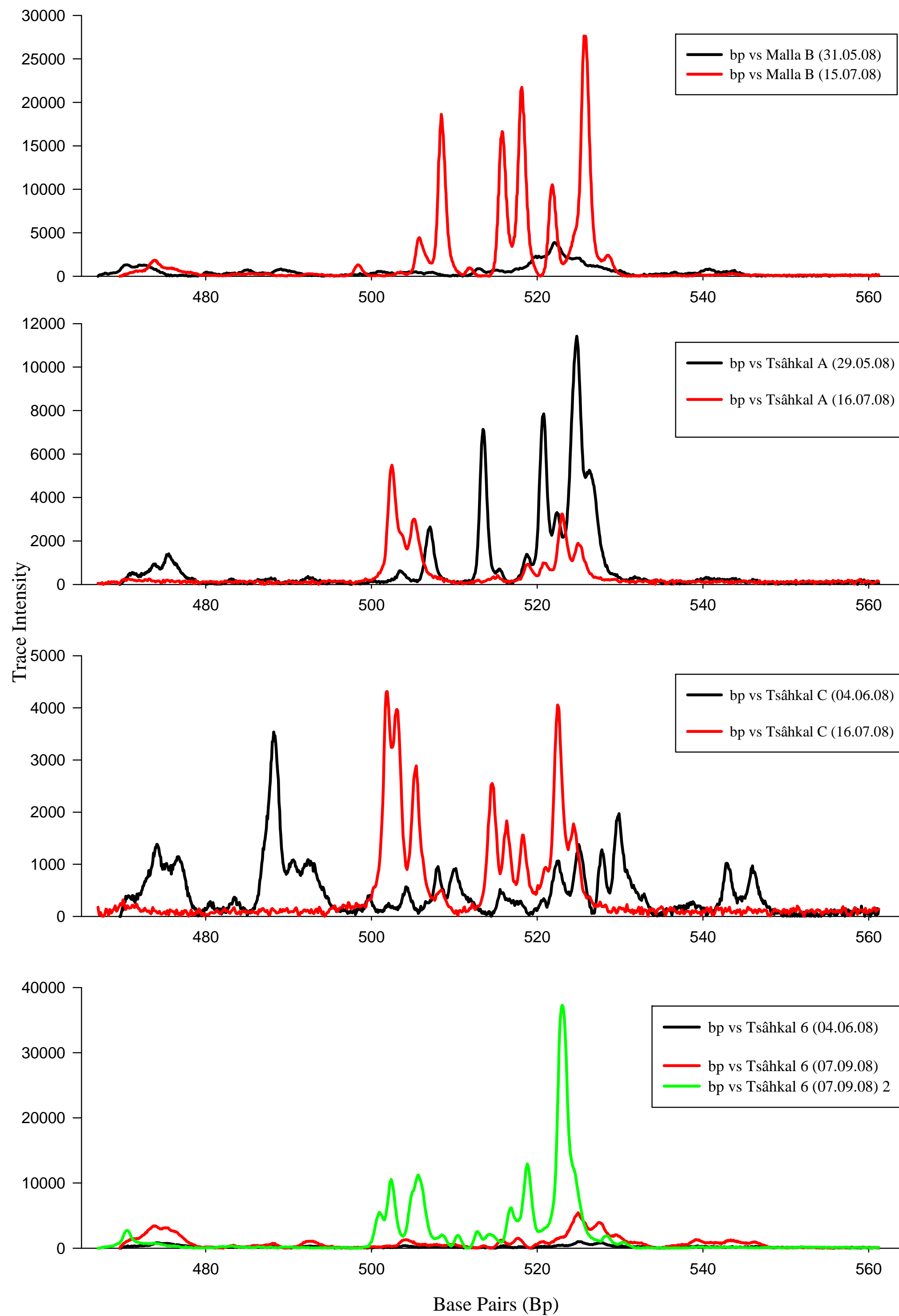




Page 3. Bacteria Diversity Seasonality in single low altitude ponds



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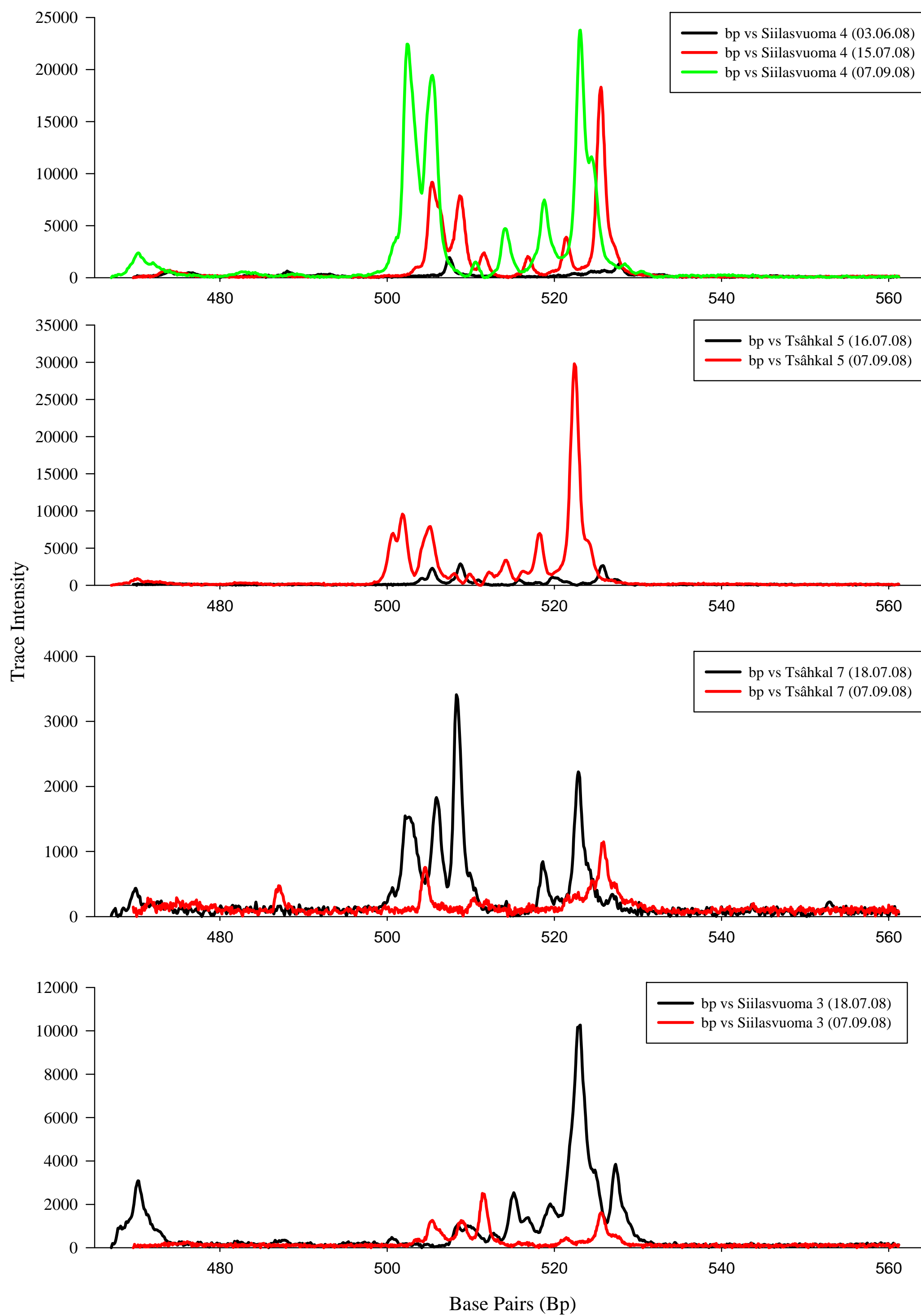

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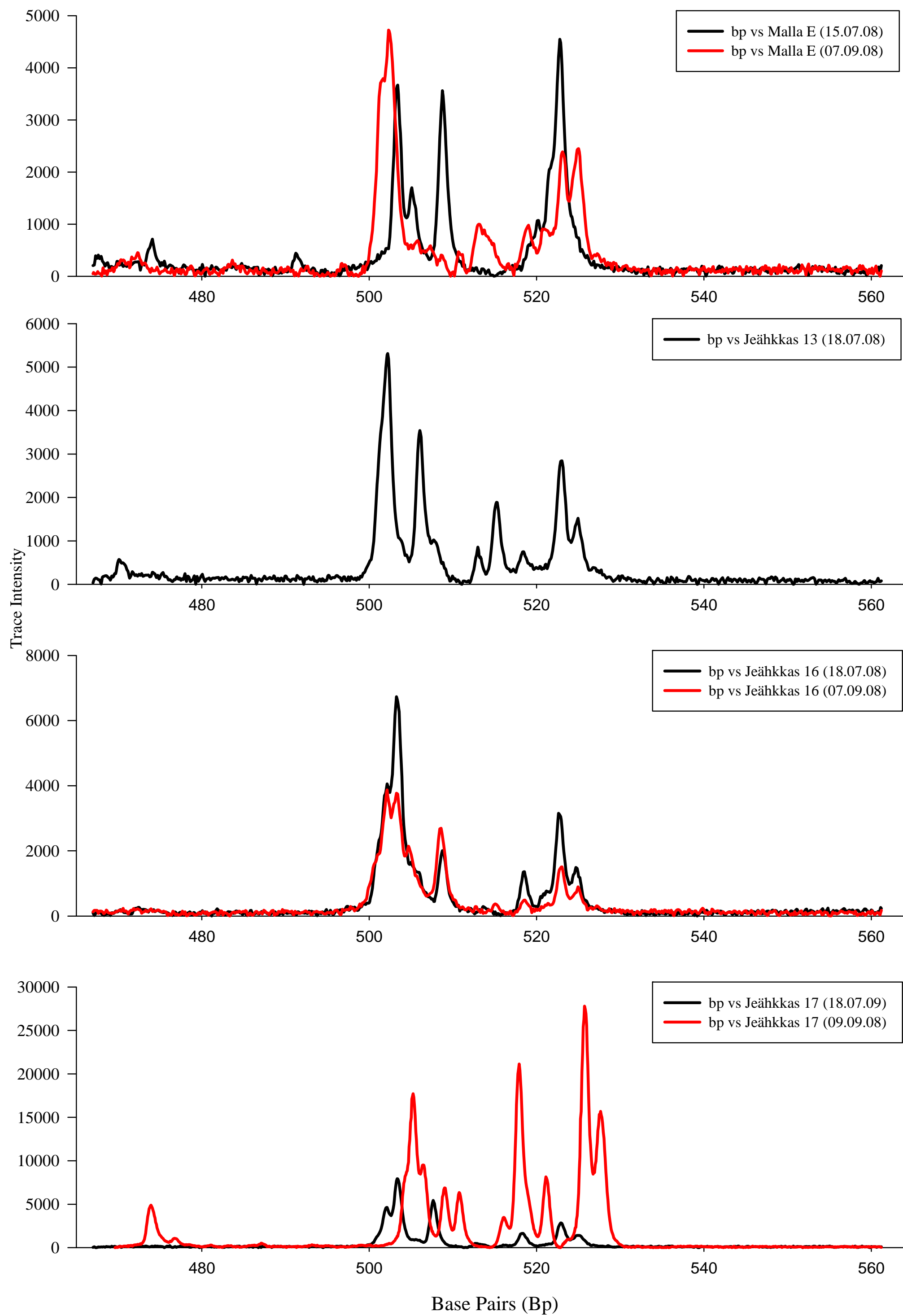

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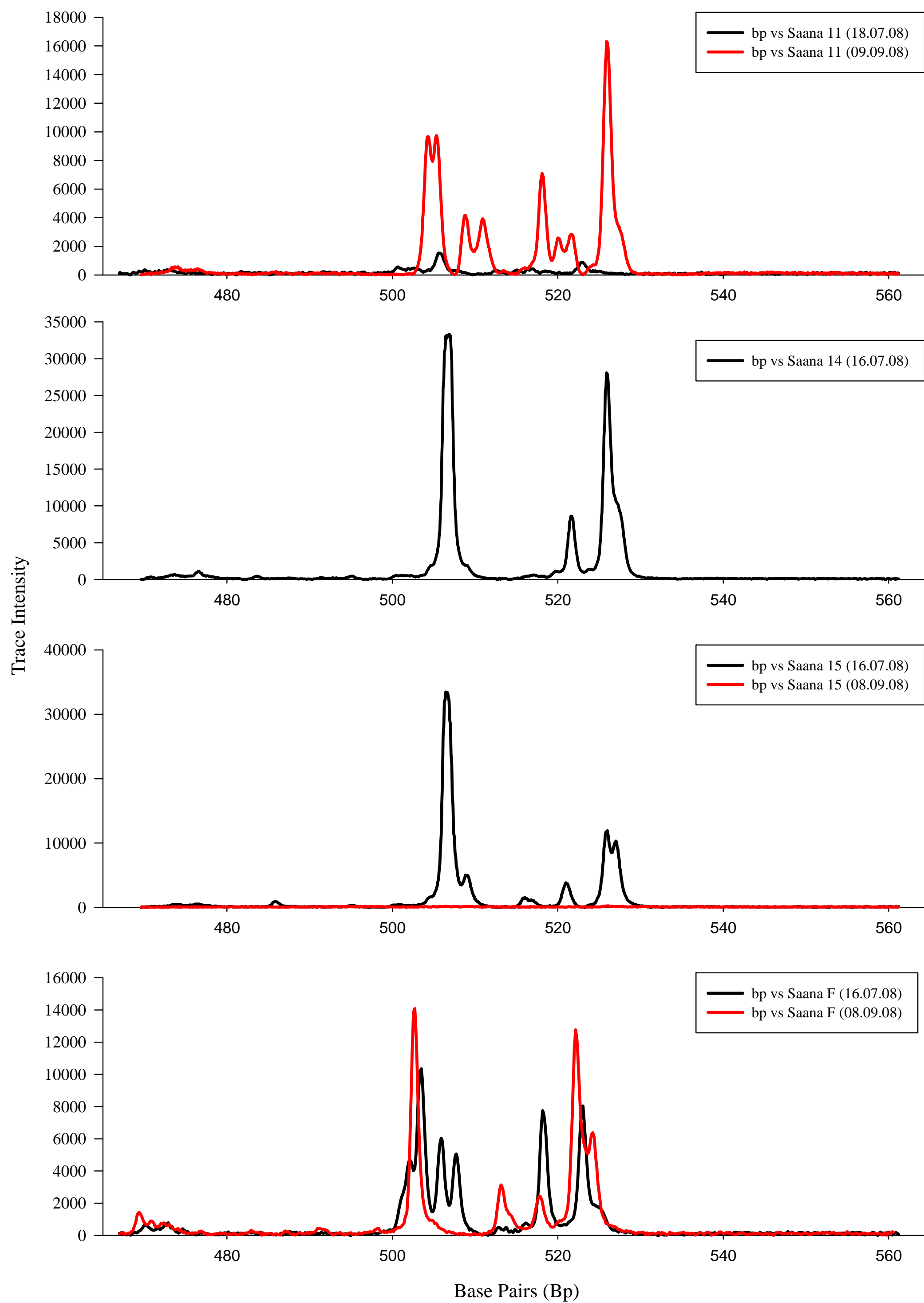
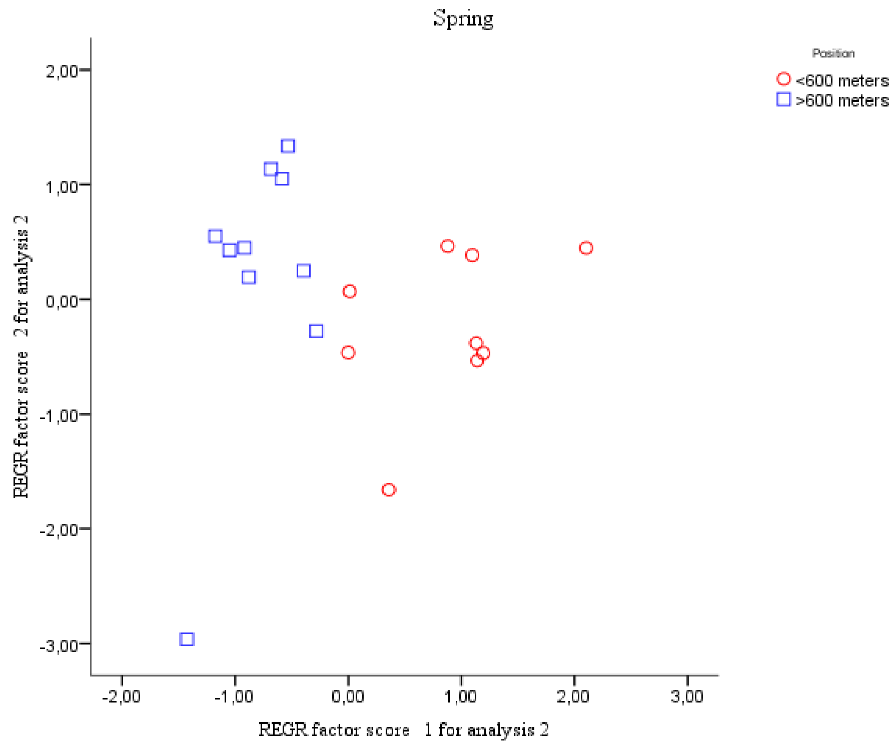
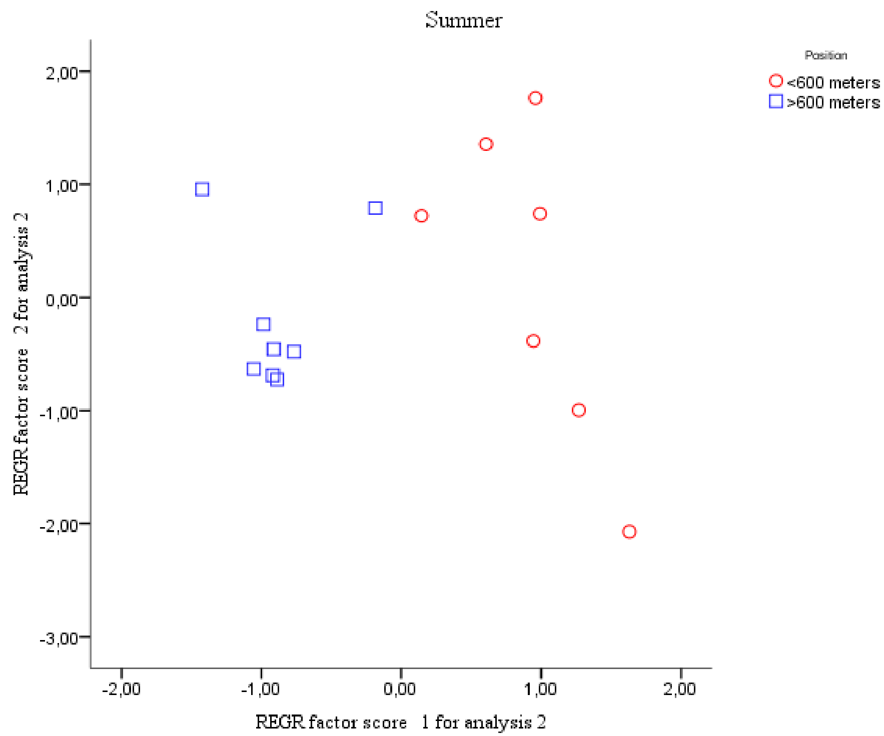


Page 7. Seasonal factor-analysis results





Page 8. Seasonal factor-analysis results