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Master thesis

Bacterial diversity of sediment in large eutrophic and shallow lake

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Contents

Abbreviations

DGGE – Denaturing Gradient Gel Electrophoresis

DWS – Dry weight of sediment

GPS – global positioning system

GuSCN – guanidinium thiocyanate

ITS – internal transcribed spacer of rDNA

LSU rRNA – large subunit of ribosomal RNA

MMD – multiple melting domains

OTU – operational taxonomic unit

PCR – Polymerase Chain Reaction

rDNA – ribosomal DNA

rRNA – ribosomal RNA

S – Svedberg unit

SIP – stable isotope probing

srRNA – small ribosomal RNA

SSU rRNA – small subunit of ribosomal RNA

tRNA – transfer RNA

V – hypervariable region of 16S rRNA gene

Introduction

The diversity of lake sediment bacterial communities has been investigated for many years using methods based on isolating and culturing the microorganisms. The proportion of cells which can be cultured is estimated to be 0.1% or at most 10% of the total population and few data are available concerning how closely they reflect the actual composition of these communities. Recent advances in the field of molecular biology (extraction of nucleic acids, polymerase chain reaction (PCR) amplification, fingerprinting methods) have made it possible to develop techniques which no longer require the isolation and cultivation of bacteria.

Denaturing gradient gel electrophoresis (DGGE) analysis of 16S rRNA gene fragments has been used to profile complex microbial communities and to infer the phylogenetic affiliation of the community members. Few cultivation-independent studies of microbial diversity in sediments have been conducted, but the importance and role of microorganisms in the lake ecosystem requires further investigation, especially with respect to their potentially important role in the significant lake sediment processes.

For present study PCR-DGGE was employed as it provides the resolution needed to assess and compare the microbial diversity in the natural sediment samples. Because the bacterial communities in sediments of shallow lakes are poorly described, this study was undertaken to investigate some fundamental characteristics of bacterial community composition. Shallow lakes are specific because the frequent resuspension on the upper layer of sediment makes conditions in these lakes different for other water bodies.

1. Review of the literature

Microscopy and cultivation have limited usefulness in case of natural bacterial samples, since phylogenetically and metabolically diverse microorganisms lack conspicuous external features for a reliable and robust grouping. Cultivation on rich media is known for its selectivity and is not considered representative of the extent and diversity of the bacterial community (Ferris *et al*., 1996; Amann *et al*., 1995; Muyzer, 1998). Simple phenotypic traits such as morphology and more complex properties such as spore formation and the presence of a photosynthetic apparatus are grossly inadequate to define higher taxonomic ranks. The only exceptions, in which morphological traits are indicative of phylogenetic coherence, were found among the spirochetes and the fruiting forms of myxobacteria (Stackebrandt and Pukall, 1999).

Approximately 1 – 10% of environmental bacteria are culturable by using standard techniques (Hugenholtz *et al*., 1998). This observation is now often named `the great plate count anomaly' (Spring *et al*., 2000). The culturable fraction of a bacterial population is neither quantitatively nor qualitatively representative of the total microbial community in the respective environment. These results exclude the possibility that the fraction of non-cultivable cells is composed of bacteria which cannot be grown in the laboratory because they are dead or temporarily in a metabolically inactive state (Spring *et al*, 2000). Moreover, the directed cultivation of distinct microorganisms demands time-consuming and laborious trial-and-error experiments with an unpredictable outcome, which in consequence prevents a rapid accumulation of data in this field (Spring *et al*., 2000). However, the application of molecular techniques offers new opportunities for the analysis of microbial communities (Muyzer, 1998).

Limitations and difficulties with discriminating morphologically similar bacteria have compelled microbial ecologists to use molecular techniques to investigate the species composition of bacterial communities (Zwart *et al*., 1998). The application of several molecular biological techniques to field samples has widened the knowledge about microbial diversity largely. Molecular techniques avoid the need for isolation of pure cultures by focusing directly on the nucleic acids present in the environment. From

the diversity of nucleic acids isolated directly from environment, the biological (phylogenetic) diversity can be investigated (Acinas *et al*., 1997). The development of techniques for the analysis of small-subunit ribosomal RNA (SSU rRNA) genes sequences in natural samples has greatly enhanced our ability to detect and identify bacteria or any other microbes in nature (Ferris *et al*., 1996). Molecular techniques generate valuable information on microbial diversity and community structure in any environment, taking into account both the culturable and unculturable fractions of microorganisms (van Elsas *et al*., 1998). These techniques allow the direct sampling and more complete analysis of the genetic diversity of microbial communities, without the need for cultivation (Spring *et al*., 2000). These molecular techniques have successfully been employed to survey the diversity of bacteria of various habitats: in the oceans, seas, rivers and lakes (Zwart *et al*., 1998; Ferris and Ward, 1997). By using molecular approach we now know that microbial diversity is much greater than previously anticipated, and that culture techniques are insufficient for exploring this enormous reservoir of hidden diversity (Muyzer, 1999).

1.1. Tools to investigate bacterial community structure and function

Two main molecular approaches are available to study bacterial communities (Figure 1):

• molecular approaches which try to investigate all the genetic information in the extracted DNA and that are called ‘whole community DNA analysis', nowadays meaning mostly metagenomics

• molecular approaches which usually investigate parts of this information by focusing on genome sequences which are targeted and amplified by PCR that are called ‘partial community DNA analysis' (Ranjard *et al*., 2000).

Metagenomics so-called whole community DNA analysis' refers to the study of the collective genomes in an environmental community. Metagenomics is employed as a means of systematically investigating, classifying, and manipulating the entire genetic material isolated from environmental samples. This is a multi-step process that relies on the efficiency of four main steps. The procedure consists of (i) the isolation of genetic material, (ii) manipulation of the genetic material, (iii) library construction, and the (iv)

the analysis of genetic material in the metagenomic library (sequencing, total genomic cross-DNA hybridization, etc) (Xu, 2006).

The ‘partial community DNA analysis' approaches are based on limited number of marker genes, the most commonly on the ribosomal operon, and particularly 16S rDNA gene. These methods include:

• PCR fragment cloning followed by restriction and / or sequence analysis;

• “Genetic fingerprinting”, which provides a global picture of the genetic structure of the bacterial community (Ranjard *et al*., 2000).

The general strategy for genetic fingerprinting of microbial communities consists of first, the extraction of nucleic acids, second, the amplification of genes encoding the 16S rRNA, and, third, the analysis of PCR products by a genetic fingerprinting techniques (Ranjard *et al*., 2000). These techniques are used to distinguish between species using only samples of their DNA based on the physical/electrophoretic separation and to provide a pattern or profile of the genetic diversity in a microbial community (Muyzer and Smalla, 1998). Although PCR products can be analysed by cloning or genetic fingerprint, the clone/sequencing approach is time and labor intensive, which has no doubt increased the popularity of molecular profiling techniques that give a ‘snapshot’ of the entire community (Burr *et al.*, 2006). Denaturing gradient gel electrophoresis (DGGE) is probably the most popular because of its relatively high resolution and because DNA sequence information can be recovered from gel bands (Burr *et al*., 2006). The banding pattern of the DGGE gel is being used for community analysis by correlating the number of bands with environmental factors or by calculating different indices to trace changes in community structure with changes in environmental conditions (Dahllöf *et al*., 2000).

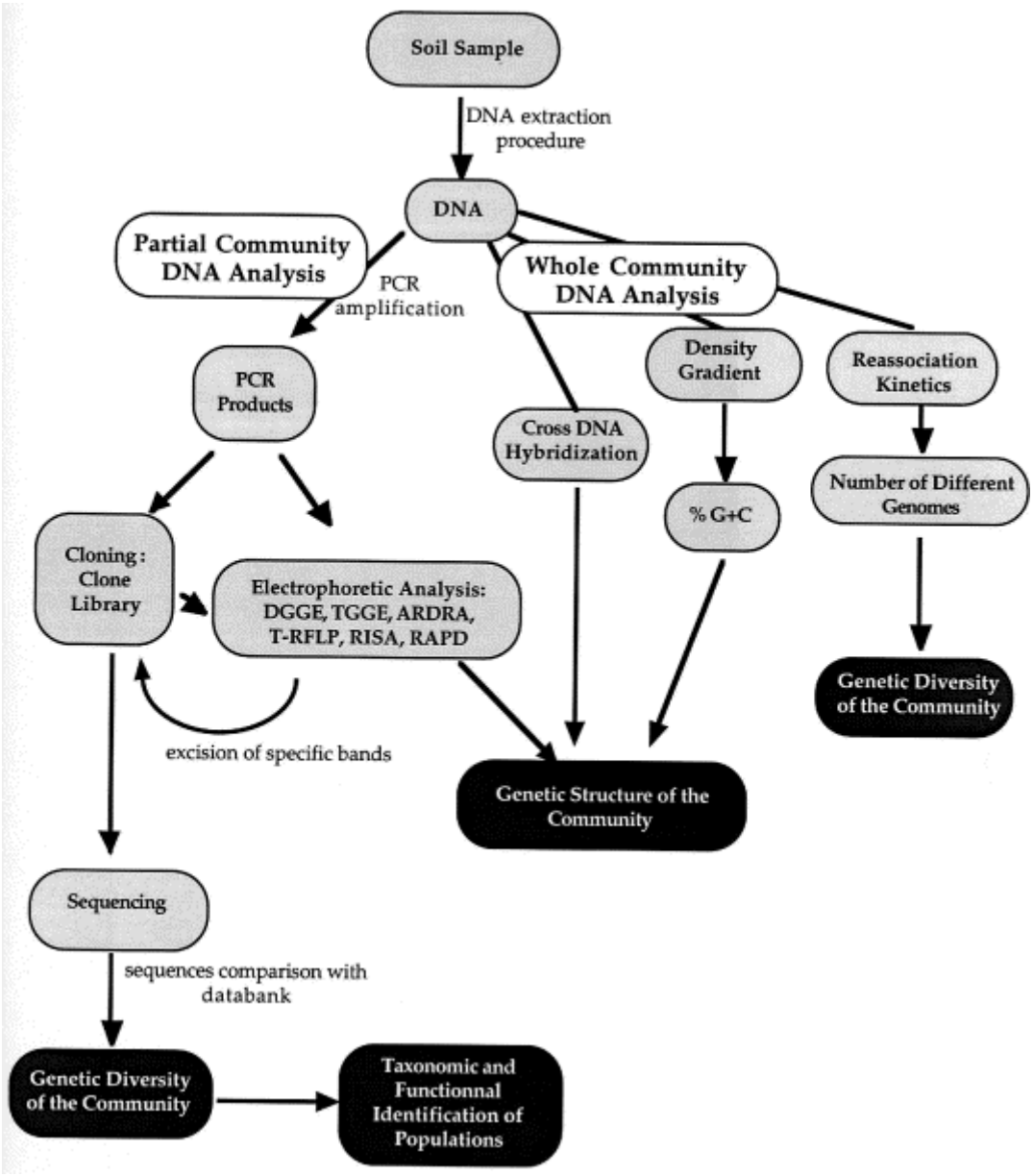

Figure 1. Schematic representation of the different molecular approaches for assessing the genetic diversity and structure of soil bacterial communities. DNA is directly extracted from soil samples and subsequently analysed either by characterizing particular sequences targeted and amplified by PCR (approaches called 'partial community DNA analysis') or by characterizing all the genetic information (approaches called 'whole community DNA analysis') (Ranjard *et al*., 2000).

1.1.1. Theoretical and practical aspects of PCR-DGGE finger printing method

Denaturing gradient gel electrophoresis (DGGE) is a gel system that separates DNA fragments according to their melting properties (Gonzalez and Saiz-Jimenez, 2004). DGGE of PCR-amplified ribosomal DNA fragments has been introduced into microbial ecology in 1993 (Muyzer *et al*., 1993). It was originally developed to detect specific mutations within the human genome, and has been adapted to analyse bacterial communities in space and time (Sekiguchi, 2001). Within a short period of time this method has attracted the attention of many environmental microbiologists and today PCR-DGGE is a well-established molecular tool in environmental microbiology that allows rapidly screen multiple samples and study complexity, the phylogenetic affiliation of the community members and behavior of microbial communities based on temporal and geographical differences (Muyzer and Smalla, 1998; Muyzer *et al*., 1993). Furthermore, this technique is reliable, reproducible, rapid and inexpensive. This approach has now been variously and easily combined with different other method like group-specific amplification, band excision and sequence analysis (Brüggemann *et al*., 2000).

DGGE is used to separate DNA fragments recovered from environment of the same length but with different nucleotide sequences. Separation is based on the decreased electrophoretic mobility of DNA fragment through a linearly increasing gradient of denaturants (a mixture of urea and formamide). The fragment remains double stranded until it reaches the concentration of denaturants equivalent to a melting temperature (Tm) that causes the lower-temperature melting domains of the fragment to melt (Figure 2). At this point, a DNA molecule is (partially) melted and the fragment undergoes a change in conformation and, as a consequence, its electrophoretic mobility is reduced. The lower-temperature melting domains of DNA fragments differing by as little as a single-base substitution will melt at slightly different denaturant concentrations because of differences in stacking interactions between adjacent bases in each DNA strand. These differences in melting cause two DNA fragments to begin slowing down at different levels in the gel, resulting in their separations from each other. In short, DGGE separates DNA fragments according to their highly sequence-dependent melting behaviour

(Muyzer *et al*., 1993; Muyzer and Smalla, 1998; Sheffield *et al*., 1989; Gonzalez and Saiz-Jimenez, 2004).

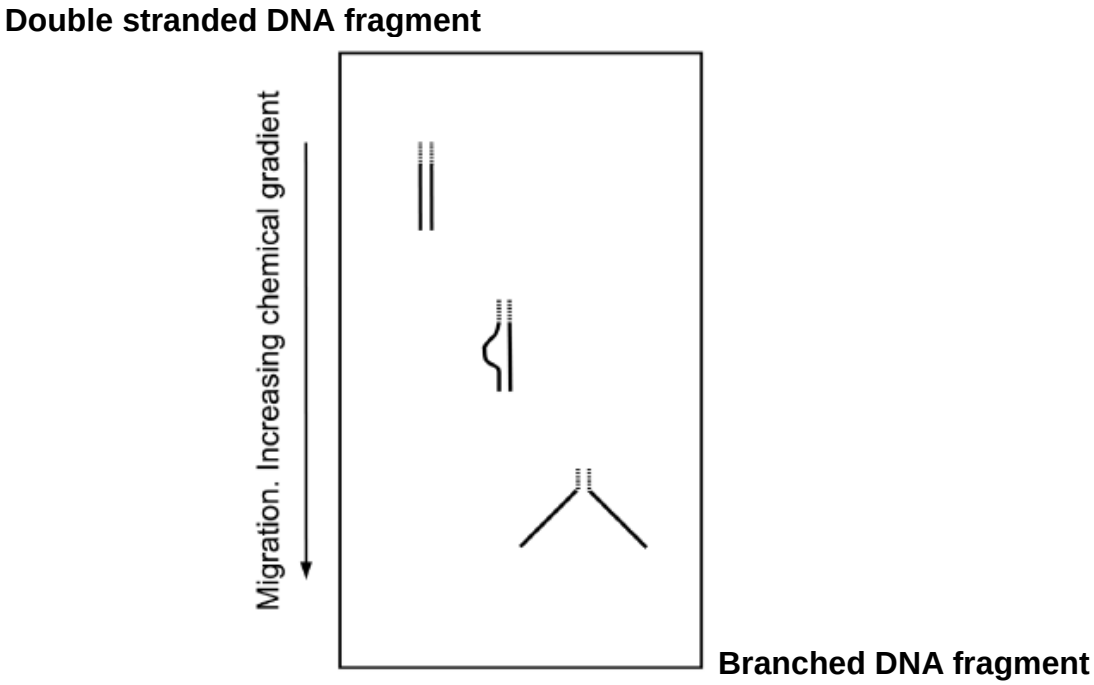

Figure 2. The scheme of melting behaviour of DNA in denaturing gradient gel. The progressive denaturation of the double-stranded DNA molecules through the increasing chemical gradient. The elevated resistance to denaturation of the GC-rich tail (dotted bar) stabilizes the migration of the amplified DNA fragments (continuous bar) avoiding that higher gradient levels are reached. The double-stranded DNA fragment melts resulting in the branched molecule shown in the bottom of the scheme (Gonzalez and Saiz-Jimenez, 2004).

Successful application of this methodology needs optimized technical experimental conditions for each DNA fragment. It is important to determine when it becomes partially melted and when it becomes single-stranded while running in a DGGE gel (Muyzer *et al*., 1998). In other words, it is important to predict the melting behavior of DNA fragments using special computer programs designed for this purpose, because this melting profile can be used to select the positions of PCR primers that will generate fragments suitable for DGGE and for determining the range of denaturant concentrations to be used. Moreover, sometimes it is difficult to predict melting behaviour due to the presence of two or more melting domains (Wu *et al*., 1998).

DGGE techniques requires the improvement of genomic DNA fragments with the insertion of a 35-45 bp GC-clamp by PCR primers, because GC-rich sequence acts as a high melting domain preventing the two DNA strands from complete dissociation into single strands. So, it will increase the range of DNA fragments detection in DGGE from 50% to 100% (Muyzer *et al*., 1998; Sheffield *et al*., 1989).

The DGGE successful application also depends on the calibration of the linear gradient of DNA denaturants and duration of electrophoresis (Ranjard *et al*, 2000). Perpendicular gels should help in choosing the optimal denaturant concentrations for nature genomic DNA fragments and *time travel* experiment on parallel gels should determine the duration of electrophoresis (Figure 3A, 3B) (Muyzer and Smalla, 1998). Although it is well recognized that the quality of information produced by PCR-DGGE is dependent on both the number and resolution of the amplicons in denaturing gradient gels, few authors have explained their justification of primer choice and DGGE conditions (Yu and Morrison, 2004). In this study a combination of three hypervariable (V) regions were studied. The technical experimental conditions of this study were optimized according to Yu and Morrison (2004). For the V3 and V5 regions of *rrs* genes were used.

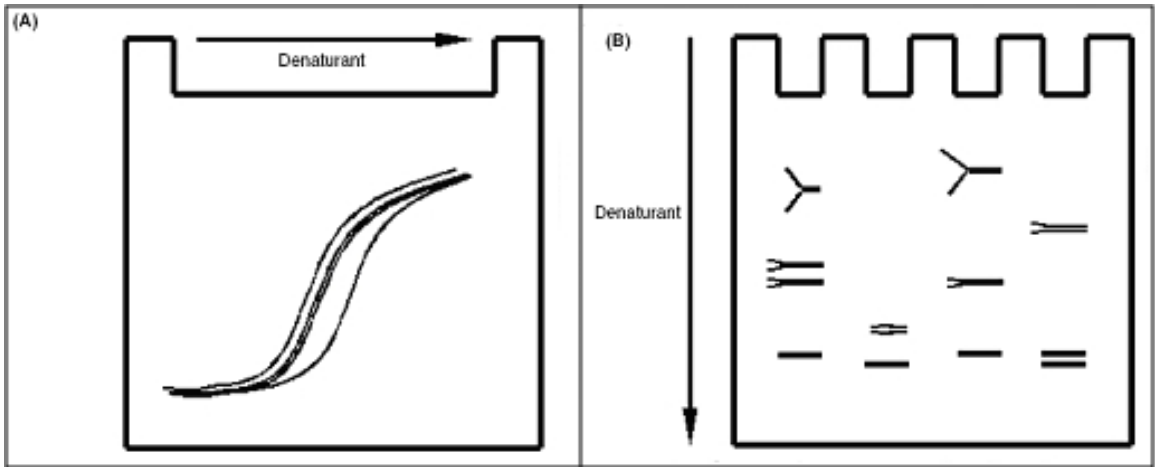

Figure 3. (A) The optimal denaturing gradient for nucleotide sequence separation is determined by using a perpendicular DGGE. Each line corresponds to different DNA fragments.
(**B)** Multiple nucleotide sequences, from various species, are separated on the appropriate parallel denaturing gradient. DNA sequences that denature rapidly will move through the gel at a slower rate than alleles that denature more slowly (Knapp, 2005).

1.1.2. Limitations and problems of PCR-DGGE microbial ecological studies

It must be emphasized that as with every method, also the molecular techniques are not free from errors and biases, especially when applied to environmental samples. Biases may be introduced at different steps of molecular methods; for instance, already by sample handling. When a sediment sample are taken under anaerobic conditions, but stored aerobically for 24 hours before freezing it was noticed, that after sequencing the samples were dominated by sequences belonging to *beta-* and *gamma-Proteobacteria,* while a duplicate sample which was kept anaerobically and frozen within 2 hours after sampling showed a greater diversity with sequences from *alpha-*, *gamma-*, and *delta-Proteobacteria* and gram-positive bacteria (Muzer and Smalla, 1998). Common results are shown by Suomalainen *et al*. (2006). They argue, that freezing and thawing of samples can negatively affect the molecular detection of a specific bacterial species, as well as diversity analysis in general (Suomalainen *et al*., 2006).

The next step in the molecular characterization of microbial communities is the extraction of nucleic acids from bacterial cells present in the samples; also this step is not free from biases (Menking *et al*., 1999). It is estimated, that DNA extraction methods can affect both phylotype abundance and composition of the indigenous bacterial community. The methods for extraction and purification of environmental samples especially soil and sediment DNA suffer from low efficiency, mainly due to incomplete cell lysis and DNA sorption to soil and sediment particles (Martin-Laurent *et al*., 2001). Problems are encountered with the reliable and reproducible lysis of all bacterial cells as well as with the extraction of intact nucleic acid, and the removal of substances, such as humic acids and bacterial exopolysaccharides, which may inhibit DNA digestion with PCR amplification (Wintzigerode *et al*., 1997). For instance, soluble humic material in quantities as small as 1 µg has been shown to inhibit PCR (Menking *et al*., 1999).

However, the results of molecular analysis of microbial communities rely not only on the extraction of DNA representative of the indigenous bacterial community composition but also on factors related to PCR, such as the choice of primers, the concentration of amplified DNA, errors in the PCR, or even the method chosen for analysis (Martin-Laurent *et al,* 2001). Although PCR is a routine method for pure cultures, several problems arise when the method is applied to environmental

communities (Wintzigerode *et al*., 1997). For instance, amplification efficiency of genes using whole bacterial cells as template instead of extracted DNA can be affected by the physiological state of the cells. The source of the DNA that is originating not only from active bacterial cells but also from dead or dormant cells and persistent DNA adsorbed onto soil particles, can affect the interpretation of the analysis of the results, leading to an over- or underestimation of detected genomes/species (Silva and Batt, 1995). Farrelly *et al*. (1995) have showed that the genome size and the copy number of 16S rRNA genes effect on the quantities of PCR products. Another problem in the use of PCR to amplify mixed target DNA is the formation of artifacts – and first of all formation of so-called chimeric molecules. Chimera formation is thought to occur when a prematurely terminated amplicon reanneals to a foreign DNA strand and is copied to completion in the following PCR cycles. This results in a sequence composed of two or more phylogenetically distinct parent sequences and, when comparatively analysed with other 16S rDNA sequences, suggests the presence of a non-existent organism (Hugenholtz and Huber, 2003). For preventing such kind of biases computer algorithms, such as the CHECK CHIMERA, have been developed to detect chimeric sequences (Wang and Wang, 1997).

In addition, the formation of heteroduplex molecules that have strands from two different PCR products, during the amplification process might contribute to difficulties in the interpretation of community complexity from DGGE patterns (Acinas *et al*., 2005). The heteroduplex formation would be expected to increase in the later cycles of mixed PCR amplifications, when the amplified DNA species reach concentrations high enough to compete with primers for binding sites (Speksnijder *et al*., 2001).

There is also established, that some errors can be introduced by polymerase. As a result a formation of point mutations can be observed (Wintzigerode *et al*., 1997). Some other biases as due to primer selectivity or erroneous product ratios are caused by product saturation in the later cycles of amplification (Ravenschlag *et al*., 1999) or primers dimerization could be observed during PCR. Detection of sequence hybrids between closely related 16S rDNA molecules is problematic, making it difficult to estimate the amount of real sequence microvariation in extant databases (Speksnijder *et al*., 2001).

In addition, for successful application the appropriate selection of the PCR fragments and PCR primers is crucial. The sequence of interest should always be within the domain with the lowest melting temperature (Wu *et al*., 1998). And of course, contaminating DNA can lead to both amplification in negative controls without external DNA being added and co-amplification in experimental reaction (Wintzigerode *et al*., 1997). Such kind of sources of sequence error may be high enough to affect genetic diversity estimates (Speksnijder *et al*., 2001).

Although we cannot exclude any of these errors, the number of errors of the first two categories (chimeric and heteroduplex molecules) can be limited by employing a low number of amplification cycles and by obtaining sequence data from both strands. Several factors, such as number of 16S rRNA gene copies per cell, the DNA isolation efficiency and the amplification efficiency in PCR, may influence the intensity of the signal in an uncertain manner. Caution should however be taken with sequences extracted and amplified directly from the environment (Zwart *et al*., 1998).

1.1.3. DGGE problems

DGGE technique has also some disadvantages. Informative gels with well-resolved bands are difficult to produce, especially from samples that contain low biomass and contaminated with humics or other compounds that inhibit PCR (Burr *et al*., 2006). Furthermore, one of the limitations of DGGE is that only relatively small fragments, up to 500-1000 basepairs can be separated. But it is not always possible to separate DNA fragments despite sequences variation, because DGGE images from sediments are very likely capture a mix of real diversity and artifact of natural microheterogeneity introduced by PCR in the DNA sequence of 16S rRNA gene (Burr *et al*., 2006; Sekiguchi, 2001). Theoretically, if the heterogeneous 16S rRNA genes are present within a single strain (the existence of naturally occurring heterogeneous 16S rRNA genes within a single microorganism is not rare), multiple bands should appear instead of a single band (Ueda *et al*, 1999). The intraspecies heterogeneity observed in a DGGE banding pattern is the result of the presence of multiple copies of the ribosomal genes and the fact that the gene copies have evolved differently (Fogel *et al*., 1999). However, little information has been published on the frequency of 16S rDNA heterogeneity in species isolated from the

environment (Dahllöf *et al*., 2000). Although the band appears as a single band, it may include a small amount of heterogeneous DNA. It means that, a single DGGE band does not always represent a single bacterial strain and that several bands may be also generated from a single species and also the band which migrated to the same position in different lanes may be consist of different bacteria. The heterogeneity may also been ascribed to the presence of faint bands, which are located very close to or overlapped the targeted band. The implications of such heterogeneity for community analysis have been discussed but nor resolved (Dahllöf *et al*., 2000).

PCR-DGGE analysis of microbial communities may often demonstrate poor resolution at the species level (Wintzigerode *et al*., 1997). One reason of such low resolution due to the sequences of nucleotides is the occurrence of 16S rDNA with multiple melting domains (MMD) (Kisand and Wikner, 2002). When a DNA fragment with two or more different melting domains is separated by electrophoresis in a DGGE gel, the fragment will be arrested at the position in the gel where the denaturant concentration dissociates the fragment at its lowest melting domain. As mobility decreases, the fragment may not reach the position in the gel where the second melting domain will melt. Most of these partially melted fragments appear as sharp and focused bands. When fragments contain two melting domains (not taking into account the GC-clamp) MMD can be observed (Wu *et al.*, 1998). These MMDs typically result in an extended (fuzzy) band in the migration direction, hampering band resolution (Kisand and Wikner, 2002). These phenomena only complicate the interpretation of DGGE patterns derived from microbial communities.

Individual bands of DGGE pattern can be assigned to taxa by the sequencing and can be determined and phylogenetically analyzed. However, the ecological role and metabolic activity of an organism often cannot be inferred from a comparison of its 16S rRNA sequence to those of known bacteria. This is another limitation of DGGE (Teske *et al*., 1996; Spring *et al*., 2000).

Another one actual problem is tied together with previous one and depends on the limited sequence database. The limited sequence database may lack a well-studied closely related reference strain, or the strain may differ in the trait of interest even if the sequence of the 16S rDNA regions used for DGGE is identical (Heuer *et al*., 1999).

Although sequencing of bands for analysis of DGGE fingerprints provides insight into the community structure through the phylogenetic affiliations of community members, the information about their physiological and ecological traits derived from the partial sequences is often rather limited (Heuer *et al*., 1999).

1.1.4. Applications of DGGE in microbial ecology

DGGE allows the simultaneous analysis of multiple samples and the comparison of microbial communities based on temporal and geographical differences (Sekiguchi, 2001). Denaturing gradient gel electrophoresis is nowadays used for a variety of purposes. DGGE of PCR-amplified DNA fragments is used to:

1. Study community structure and complexity and measure phylotype diversity (Ferris *et al*., 1996)
2. Analysis enrichment cultures and access culture purity (Ward *et al*., 1997; Muyzer, 1999)
3. Detect sequence heterogeneities of 16S rRNA genes in single genomes (Nübel *et al*., 1996)
4. Monitor community changes (Muyzer and Smalla, 1998)
5. Comparison of different DNA extraction methods/protocols (Yang *et al*., 2007)
6. Screen clone libraries (Burr *et al*., 2006)
7. Determine PCR and cloning biases (Muyzer *et al*., 1996)
8. Obtain sequence data of dominant species from individual DGGE bands (Sekiguchi, 2001).

1.1.4.1. Analyzing community diversity and dynamics

PCR-DGGE as well as cloning and sequencing has been used to determine the genetic diversity of total bacterial communities or particular populations without further characterization of the individual inhabitants and to obtain more information about identity of the microbial community members (Ferris *et al*., 1996).

One of the strongest points of the application of DGGE in microbial ecology is the simultaneous analysis of multiple samples, which allows monitoring of the complex dynamics that microbial communities may undergo by diel and seasonal fluctuations or

after environmental perturbations (Muyzer, 1999). This makes the DGGE techniques a powerful tool for monitoring community behavior after environmental changes (Muyzer and Smalla, 1998).

1.1.4.2. Monitoring the enrichment and isolation of bacteria

Although DGGE analysis was originally used to study community complexity, the techniques are also suited to monitor mixtures of microorganisms. For instance, DGGE was used to monitor enrichment cultures of aerobic chemoorganotrophic bacteria from hot spring cyanobacterial mats and in many other studies (Ward *et al*., 1997). Furthermore, DGGE is used to ensure isolates purity and correct migration of excised bands (Freitag and Prosser, 2003).

1.1.4.3. Detection of microheterogeneity in rRNA encoding genes

One of the observations in analysing PCR products from pure bacterial cultures is the presence of more than one band in the DGGE pattern. By using DGGE Nübel *et al.* (1996) found a pattern of ten different bands obtained after PCR amplification of the 16S rRNA genes from a pure culture of *Paenibacillus polymyxa.* A more detailed analysis of this observation revealed microheterogeneity in the different rRNA operons present in this species. As indicated by the authors this finding has important consequences for the use of 16S rRNA sequence data for biodiversity estimates and phylogenetic reconstruction (Nübel *et al*., 1996).

1.1.5. Perspectives of DGGE in microbial ecology

Future innovations might include the use of double-gradient DGGE (i.e. the combined application of a gradient of acrylamide and a gradient of denaturants to obtain a better resolution), and the use of terminally labeled fluorescent PCR products and the addition of fluorescent intra-lane standards for detection of rare community members and an accurate sample-to-sample comparison. The routine use of functional genes as molecular markers also could be used to discriminate between closely related but ecologically different populations (Muyzer, 1999). In addition, stable isotope probing (SIP) is a molecular technique that allows investigators to follow the flow of atoms in isotopically

enriched molecules through complex microbial communities into metabolically active microorganisms. This allows specific separation of active bacteria using SIP-DGGE (Madsen, 2006).

1.2. Bacterial community diversity based on rDNA

The small subunit of the bacterial ribosome (SSU rRNA gene or 16S rDNA) has been proven extensively to be an important and useful molecular marker for quantitating evolutionary relationships between organisms and for estimating the diversity of bacterial communities in modern prokaryotic taxonomy. Numerous studies have applied 16S rDNA as a molecular target to characterize soil and sediment bacterial communities (Amann and Ludwig, 2000).

Although any gene may be used as a genetic marker, rRNA genes offer distinct advantages:

- The 16S molecule is ancient and at the same time stable over long evolutionary times, i.e. during 40-50x10^6 years the primary structure will change by 1% (Stackebrandt and Pukall, 1999)
- All bacteria (and all living organisms) harbour these genes, which are essential for ribosome functioning in protein synthesis (Amann, 1995; Gonzalez and Saiz-Jimenez, 2004)
- 16S rDNA has both conserved and highly variable regions that permit the discrimination of taxa at multiple taxonomic levels (Amann, 1995)
- Informative length of about 1500 and 3000 nucleotides for 16S and 23S, respectively (Bautista-Zapanta *et al*., 2002)
- Unlike phenotypic, morphological and certain chemotaxonomic properties, the primary structure of such molecular sequences is independent of culture conditions (Stackebrandt and Pukall, 1999)
- Evolutionary relationships between organisms can be compared in same extent (molecular clock) (Britschgi and Giovannoni, 1991; Stackebrandt and Pukall, 1999)
- Availability of huge 16S rDNA databases with a large number of 16S sequences of different organisms for comparative sequence analysis and for identifying unknown

microbes by database comparisons (for instance, RNA Data Base Project)(MacGregor, 1999).

In prokaryotes 16S, 23S, and 5S rRNA genes are typically organized as a co-transcribed *rrn* operon (Figure 5). There may be one or more copies of the operon dispersed in the genome. According to Fogel *et al*. (1999), there is only a little correlation apparent between the genome size and *rrn* operon copy number (Figure 4). This copy number varies greatly. There are more exceptions than positive correlation between genome size and *rrn* operon copy number between different bacterial inter- and intraspecies. For instance, the organisms with 10 to 12 operons have genome size less then 5 Mb, whereas organisms with the larger genomes (10-12 Mb) have only 4-6 operons (Fogel *et al*., 1999). Such varieties influence on the PCR amplification efficiency in case of estimating nature microbial population diversity. PCR may over- or under-represent species with multiple operon copies (MacGregor, 1999).

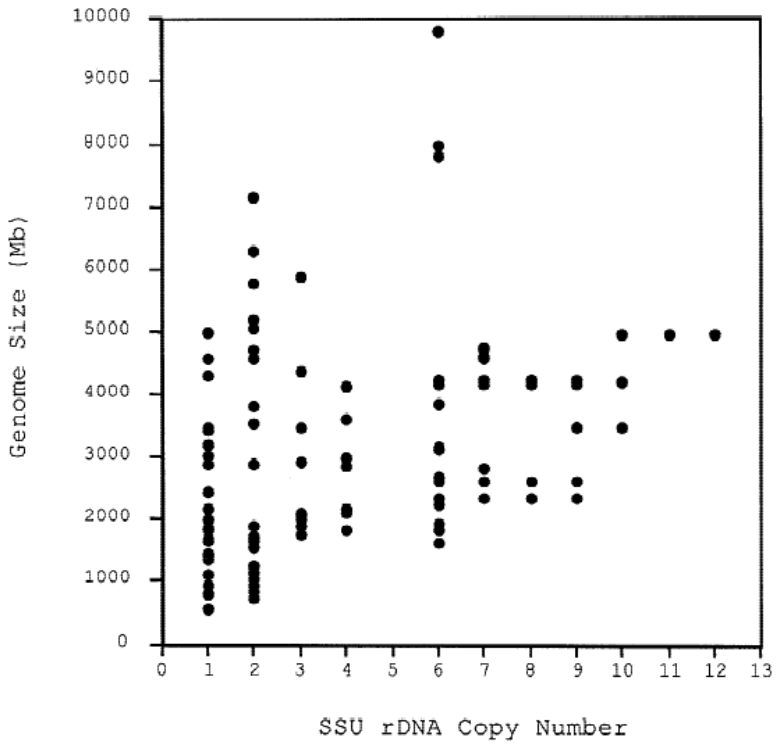

Figure 4. Distribution of prokaryotic genome size versus SSU rRNA copy number (N = 101). Increased genome size shows only a slight correlation with increased copy number (Fogel *et al*., 1999).

Primer extension analyses revealed that the primary transcription products for 16S and 23S rRNAs are about 1500 and about 2900 bases long, respectively and whole *rrn* operon is ~6 kbp in length. The general organization of each *rrn* operon of prokaryotes is shown in the Figure 5 (Bautista-Zapanta *et al*., 2002). As usual, the 16S-23S internal spacer (ITS) of *rrn* operon contains genes for tRNA-De and tRNA-Ala and tRNA-Met downstream of 5S rDNA gene, while the intergenic spacer between 23S rDNA and 5S rDNA lacked tRNA genes (Yasuda and Shiaris, 2005).

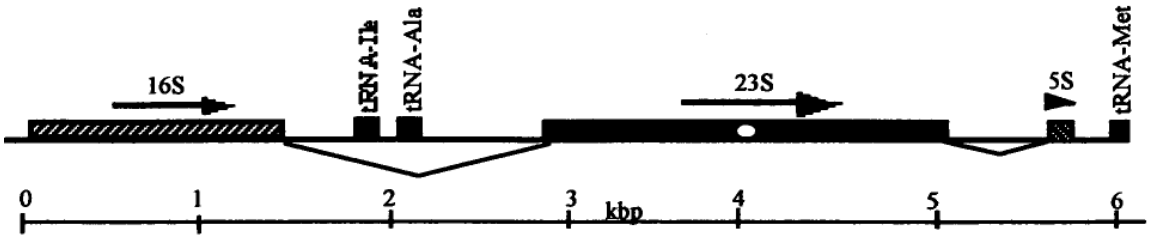

Figure 5. Diagram of the ~6 kbp rRNA operon in prokaryotes (on *A. tumefaciens* MAFF301001 example). Ribosomal RNA genes and tRNA genes are illustrated as shaded boxes drawn to scale in kbp. Internal transcribed spacers (ITS) are shown with brackets. An arrow above each rRNA gene reveals the direction of transcription (Bautista-Zapanta *et al*., 2002).

The 16S rRNA gene sequence displays an alternating pattern of conserved and hypervariable regions reflecting the functional importance of the conserved regions in the gene product's secondary and tertiary structure (Figure 6) (Case *et al*., 2007). The differences among the 16S rRNA genes are localized in nine hypervariable regions of the 16S molecule (Figure 7) (Neefs *et al*., 1990).

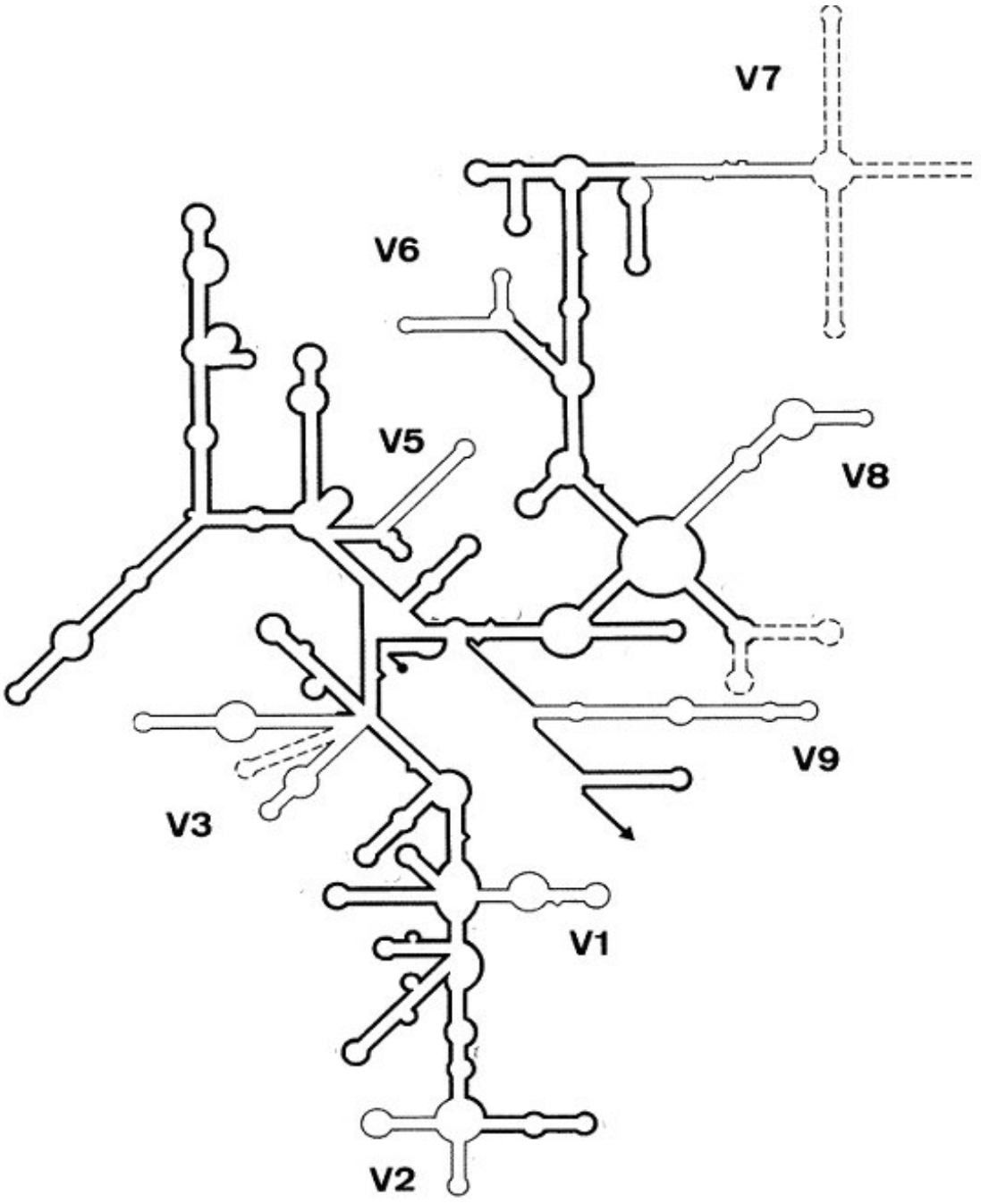

Figure 6. The secondary structure model of prokaryotic 16S rRNA molecule.
The 5'-terminus is symbolized by a filled circle and the 3'-terminus by an arrowhead. Helices are numbered in the order of occurrence from 5'- to 3'-terminus. Helices bearing a single number are common to the prokaryotic model. Relatively conserved areas are drawn in bold lines, areas of sequence – and length variability in thin lines. Eight variable areas, numbered V1 to V9, are distinguished, V4 being absent in prokaryotic srRNAs. Helices drawn in broken lines are present in a small number of known structures only (Neefs *et al*., 1990).

Microheterogeneities in 16S rDNA gene families may account for some of the variability, particularly among very closely related genes (Britschgi and Giovannoni, 1991). The heterogeneity sometimes found among the rRNA operons of a single organism may also be a problem, because significant 16S rDNA interoperon differences have been found in different bacterial species (Amann and Ludwig, 2000). The intragenomic heterogeneity can influence 16S rRNA gene tree topology, phylogenetic resolution and operational taxonomic unit (OTU) estimates at the species level or below (Case *et al*., 2007). In virtually all species, the sequences of multicopy rRNA genes are identical or nearly identical and the homogeneity is thought to be governed by concerted evolution, which may originate from stringent selective pressure on the primary sequences of rRNA molecules to maintain their precise interactions with components of the complex protein-synthesizing machinery (Ueda *et al*., 1999). Several recent studies using rDNA sequencing have reported the existence of divergent 16S rRNA sequences within a single organism. These studies clearly showed the presence of sequence heterogeneity between *rrn* operons on single genomes, but it has not been elucidated whether such intra-rRNA heterogeneity is peculiar or general. To clarify this, the extent of such sequence heterogeneity should be systematically studied (Ueda *et al*., 1999). It is generally considered that mutations, such as random base anomalies, depend on a number of factors, including mis-incorporation and misrepair by DNA polymerase and the number of PCR cycles used during DNA replication or sequencing (Speksnijder *et al*., 2001). In case of estimating the natural microbial diversity, it is used to know, that the 16S rRNA may be too well-conserved to discriminate between closely related populations. Different species may have almost identical 16S rRNA sequences. In such cases, the 23S rRNA may be useful (Case *et al*., 2007).

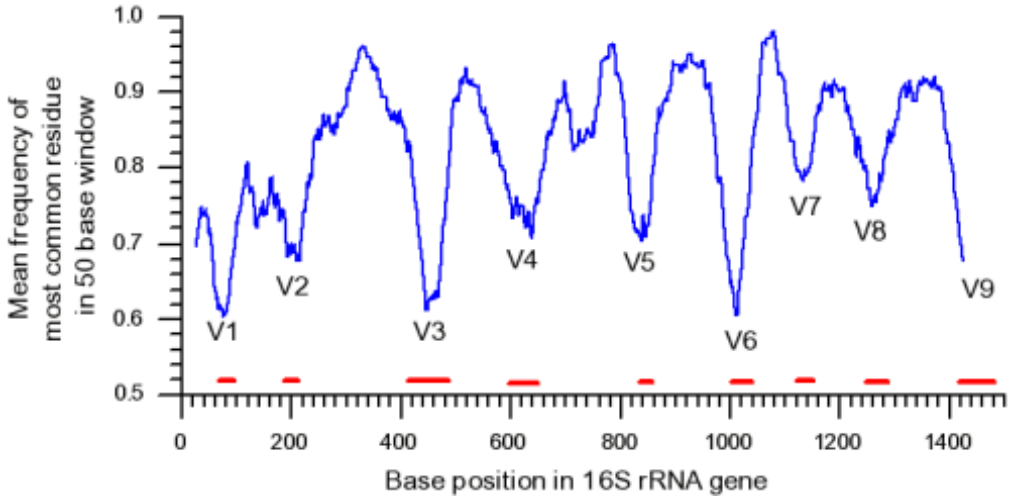

Figure 7. A representation of hypervariable regions within the 16S rRNA gene. The plotted line reflects fluctuations in variability amongst aligned 16S rRNA gene sequences; peaks reflect greater conservation, whilst troughs correspond to the known hypervariable regions V1 to V9 indicated by the red bars (Neefs *et al*., 1990).

1.3. Microbes in lake sediments

Bacteria are an important part of the sediment microflora because of their abundance, their species diversity and the multiplicity of their metabolic activities (Miskin *et al*., 1998). They play a key role in the decomposition of organic matter and in the biogeochemical cycles of the main elements (carbon, nitrogen, phosphorus, etc) and of trace elements (iron, nickel, mercury, etc) and are therefore heavily implicated in energy and nutrient exchanges within the sediment (Ranjard *et al*., 2000; Urakawa *et al*., 2000; Miskin *et al*., 1998). Besides the geographical location and morphology of a lake, the chemical composition of its water and sediments is important in the determination of the biotic complex inhabiting the lake. Abiotic factors such as sediment types, plant species, nutrient status, contamination with pollutants, predation, temperature, light, oxygen gradients and other environmental variables influence bacterial communities which, in turn, adapt and modify the environment (Edwards *et al*., 2001; Horner-Devine *et al*., 2004; Haberman *et al*., 2004; Bühring *et al*., 2005). So this is therefore essential to understand the interrelationships between bacteria and their environment by studying the structural and functional diversity of sediment bacterial communities and how they respond to various natural conditions (Ranjard *et al*., 2000). However, defining or

quantitatively describing the microbial community structures often difficult and incomplete, because sediments probably represent some of the most complex microbial habitants on earth: a single gram of sediment may contain thousands of bacterial species (Urakawa *et al*., 2000).

Bacterial activity is generally highest near the sediment-water interface, although there is indirect evidence that microbial activity continues to considerable depths within the sediment (Miskin *et al*., 1998). Complexity in environmental conditions can vary at multiple spatial scales and could potentially influence bacterial diversity. Most freshwater sediments are quite heterogeneous ecosystems which give rise to many different environmental niches even on a millimeter scale (Spring *et al*., 2000). Microscale heterogeneity (e.g. at the scale of a sediment particle) can be very high and potentially allows for high microbial diversity in a relatively small area. For example, within a single sediment particle, oxygen concentrations can range strongly only with a few millimeters (Horner-Devine *et al*., 2004). A consequence of this heterogeneity is that growth conditions are not uniform for different groups of bacteria, and biotic and abiotic factors may promote successive changes in the community composition. For instance, phytoplankton blooms fuel the water with new particulate and dissolved organic matter providing new niches. In response, bacterial production and ectoenzyme activity increase (Riemann *et al*., 2000). As a result, the sediments were found to be sites of metabolic processes not found in the water column (Hollibaugh *et al*., 2000). In general, the depth-related gradient of physical and chemical properties provides niches for a wide variety of metabolically diverse microorganisms in sediments. In such environments, different interactions occur between different physiological types of microorganisms (Spring *et al*., 2000).

1.4. Sediments

Lake sediment can be regarded as a mirror that provides long-term records of past changes in climate–catchment processes as well as changes in biological communities in lakes. Every particle that has reached into the lake and accumulated into the sediment can be regarded as carrier of information about its origin and pathways (Håkanson, 2004).

Sedimentation is a process, when the interplay between the deposition of organic matter from the overlying water column and decomposition processes leads to the formation of sediment layers which differ in carbon content (Ambrosetti *et al*., 2003). Moreover, the complexity of this environment is often increased by mixing of the sediment by currents or the activity of invertebrates (Spring *et al*., 2000).

Sedimentation process is affected by the vegetation in the lake, when the bulk of the (organic) material in the sediment has formed in the lake in the course of plant life (Hannon and Gaillard, 1997) and also rivers are playing an important role in the input of organic and carbonate matter to the lake (Haberman *et al*., 2004).

1.4.1. Classification and distribution of bottom sediments

The Lake Võrtsjärv sediments are divided into organic (lake mud or sapropel), carbonaceous (lake marl) and terrigenous (gravel, sand, silt, clay) deposits. The mud is divided into highly plastic detrital mud, organic-rich detrital mud (one part of detrital mud and three parts of organic matter), detrital mud with moderate organic matter content and peat mud or dry mud (Haberman *et al*., 2004).

Sapropel is the most widespread sediment in Lake Võrtsjärv. It forms about two-thirds of the topmost part of sediments. The upper layers of sapropel consist of liquid (water content up to 95%) material, which in deeper layers is replaced by greenish black slightly plastic material. The content of organic matter reaches 87-92%. In the northern part of the depression organic rich sediments are absent or form only a thin layer, some dozen centimeters in thickness. Till occurs only in the northern part of the lake depression and in the coastal zone (Haberman *et al*., 2004). The map (Figure 10B) gives the overview of sediments distribution of Lake Võrtsjärv bottom sediments.

1.4.2. Sediments resuspension

The sediment resuspension in the Lake Võrtsjärv is a common phenomenon (Haberman *et al*., 2004). As Lake Võrtsjärv is shallow, it lacks the temperature stratification. During the ice-free period the wind is the dominant factor causing waves and currents and thus acting as the principal force in sediment and nutrient resuspension

from bottom (Bogucki *et al*., 1997; Ambrosetti *et al*., 2003). The model of bottom resuspension is illustrated in Figure 8.

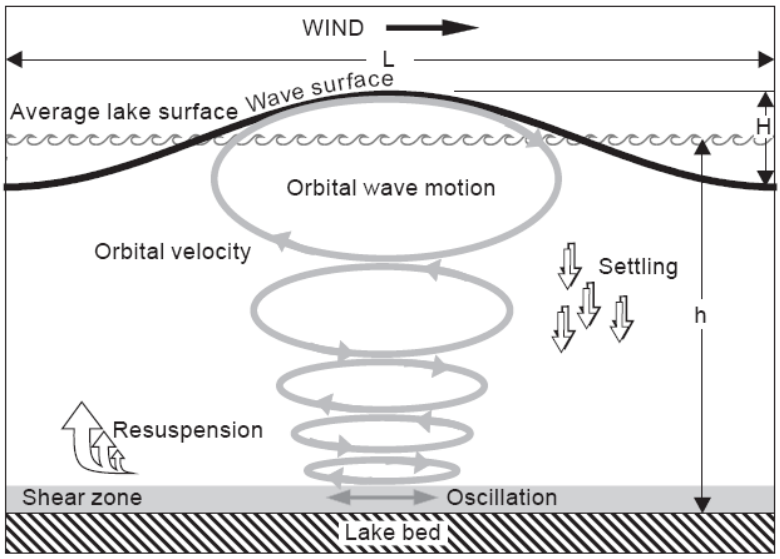

Figure 8. Forces induced by wind that cause resuspension of bed sediments. H - wave height ; h - water depth ; L - wave length (Laenen and LeTourneau, 1996).

Lake Võrtsjärv is characterized by peculiar wind currents. Changes in the direction of water movement caused by the changed wind direction occur very fast – already in three hours (Kivimaa *et al*., 1998). Even with the southerly wind the water in the northern part of the lake seem to circle anticlockwise, while in the southern part it moves anticlockwise towards the south. This together with the ongoing land uplift may be the reason why the light and fine-grained fractions such as sapropel and sapropelic silty clay are eroded from the shallow northern areas of the lake and transported to deeper and sheltered places in the central and southern parts of the lake. During floods an intensive south-north orientated water movement takes place along the eastern coast towards the outflow of the Emajõgi River. This is an important factor of accumulating of sediments in this area (Haberman *et al*., 2004).

Wind-induced sediment resuspension of bed sediments in Lake Võrtsjärv occurs frequently and affects the water quality at least in two ways. First, sediments suspended in the water column decrease light penetration. Second, the sediments are capable of acting as an internal source of nutrients. This is mostly expressed as an increase in the

amount of nitrogen and phosphorus in the lake water column (Luettich *et al*., 1990; Webster and Lemckert, 2002).

Resuspension of sediment appears to be an important process and may play a significant role in the dynamics, the functionality and health of whole lake system, including structuring the physical characteristics of the bottom, providing a mechanism for the recycling of biogeochemically important materials to the water column (such as contaminants and metals), and driving horizontal and cross margin transport of suspended particulates (Webster and Lemckert, 2002). An understanding of such dynamic behavior of suspended particles is particularly important in shallow lakes since there they may repeatedly settle to the bottom and can be resuspended throughout the water column over and over again (Luettich *et al*., 1990).

1.5. The nature of Lake Võrtsjärv

Shallow lakes, typically, fall into the eutrophic category. The Lake Võrtsjärv is strongly eutrophic and is characterized by water turbidity and much oxygen depletion from the lower water of the plankton and bacteria respiration (Haberman and Laugaste, 2003; Dent *et al*., 2002).

Eutrophication, a lake aging process, is the enrichment of water body by increased nutrient input to a lake over the natural supply. This process is considered a natural part of the lake succession (Carpenter**,** 2005). At this stage the lake is rich in plant nutrients and thus its productivity is high. Phosphorus and nitrogen are the two main and excessive macronutrients in lake, which (especially phosphorus) often lead to algal bloom and excessive production of organic matter by primary producers - algae and/or aquatic plants (Pickhardt *et al*., 2002; Clarke *et al*., 2006). After the growing season, most nutrients are withdraw from plant tissues and accumulated in sediments within detritus, and only a small fraction is released into the water column (Haberman *et al*., 2004). Another feature of lake eutrophication is the production of high numbers of zooplankton and minnows and other small fish that feed on the zooplankton. These small fish in turn provide food for the growth of larger fish (Haberman *et al*., 2004). As a result, in such productive conditions much of organic matter drifts to the bottom and forms organic sediments. This sediment in turn provides the food for high numbers of bacteria (Ambrosetti *et al*., 2003).

1.6. Objectives

The major goal of this study was to characterize season and depth related changes in whole microbial population of upper sediments of large, shallow and eutrophic lake using PCR-DGGE.

The specific objectives of the present study were:

(i) To use 16S rDNA for studying community structure of lake sediment bacteria

(ii) To compare bacterial diversity of lake sediment in depth profile (depth intervals from 0-0.5 cm to 25-30 cm)

(iii) To investigate the seasonal dynamics of the sediment bacterial over one year period

(iv) To compare spatial variation of the bacterial diversity in sediment.

2. Material and methods

2.1. Study area and sediment sampling

Studied strongly eutrophic large and shallow Lake Võrtsjärv is the second largest lake in the Baltic countries and the largest domestic water body in Estonia (Haberman and Laugaste, 2003). The main limnological features are given in Table 1. Lake Võrtsjärv is located in the southern part of Estonia at 57^{o} 50'- 58^{o} 30' N and 25^{o} 35' – 26^{o} 40' E. The average water level in L. Võrtsjärv is 33.7 m above sea level. The official register of Estonian running waters includes 154 streams belonging to the drainage basin of Lake Võrtsjärv. Among 18 inflows to the lake are the rivers Väike Emajõgi, Õhne and Tänassilma; the single outflow - the Emajõgi River (Haberman *et al*., 2004).

	Lake Võrtsjärv
Surface area	270 km^2
Catchment area	3,374 km^2
Average depth **Maximal depth**	2.8 m 6 m
Average water turnover rate	once a year
Average ice cover - days	135
Trophic State	eutrophic

Table 1. The main limnological features of Lake Võrtsjärv (Haberman *et al*., 2004).

Samples were collected during one year from June 2005 to June 2006. Seasonal sampling included monthly samples from stationary sampling station indicated as marked hatched area (Figure 10A). Additionally, 7 samplings with distance 3-400 m intervals were carried out the transect study in June 2006 from the inflowing River Õhne mouth to stationary station. Sediment samples were collected at locations, which are numbered and represented on the map (Figure 10A). On-board GPS (global positioning system) was used for accurate positioning.

Sediment samples were collected using Willner sediment corer (Figure 9). The cores were brought up from the bottom of the lake and capped at both ends and transported in the dark to the laboratory for further processing usually within about 0.5 - 3 h. Sediment cores were sliced into the following layers: 0–0.5, 1, 2, 5, 10, 15, 20 and 25 cm (top-down). The samples (0.2 – 0.4 mg of sediment) were frozen and stored at -20 ^{o}C until DNA was extracted. Environmental and weather conditions at each sampling site were also recorded.

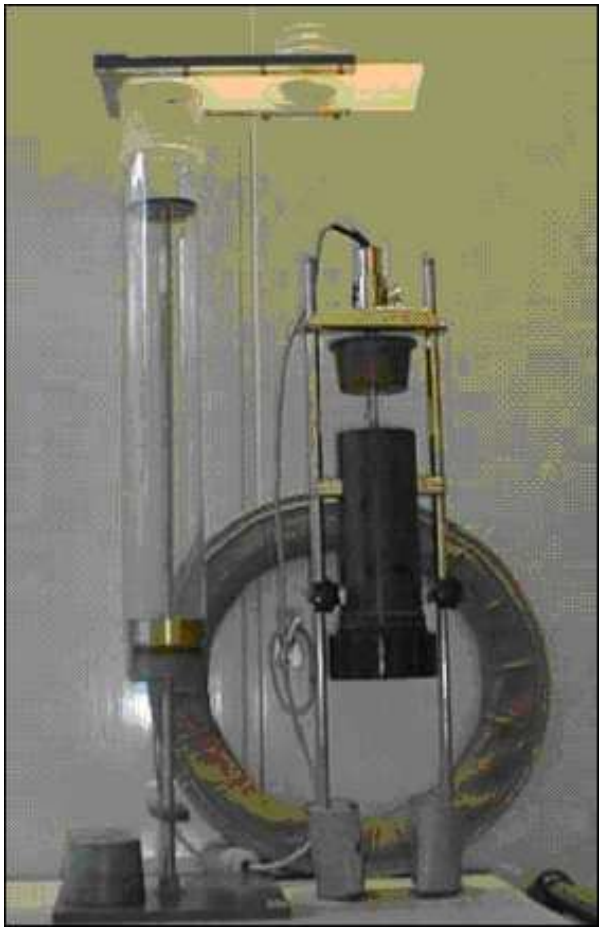

Figure 9. The photo of the sediment corer.

Figure 10A **Figure 10B**

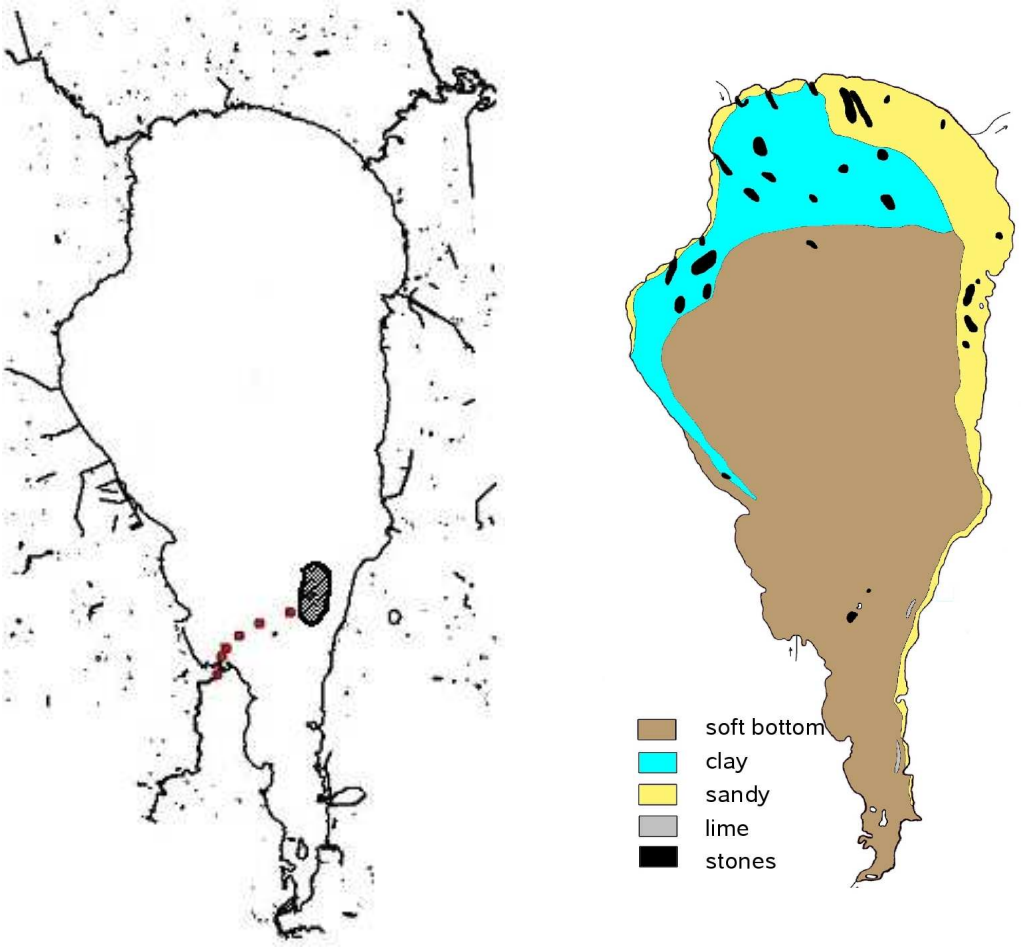

Figure 10A. Sampling sites for sediments in Lake Võrtsjärv. Seasonal study (monthly sampling in 2005-2006) area is indicated by marked hatched area and transect study (June 21, 2006) by red dots (numbers 14-20 from the left).
Figure 10B. The bottom composition of Lake Võrtsjärv (Haberman *et al*., 2004).

2.2. DNA extraction

Genomic DNA was extracted from sediment samples using Zirconium beads (0.1 mm-diameter; burned at 500 ^{o}C), phenol-chloroform-isoamylalcohol (PCI, 25:24:1, vol/vol/vol, pH 8), 100 mM phosphate buffer (PP) (Na_2HPO_4, pH 8.3) and sodium dodecyl sulfate (SDS). Nucleic acids were precipitated with NaOAc and isopropanol for overnight at -20 °C. After the centrifugation at 13,200 x g at 4 °C during 30 min, the pellet was washed with 80% ethanol, dried under a vacuum, and redissolved in 100 µl of MQ water or TE buffer.

2.3. Additional DNA purification

DNA was purified by GuSCN-Silica bead extraction method of Boom *et al*. (1998). Method was used for nucleic acid purification and removal of PCR inhibitors. Briefly, 900 µl of guanidinium thiocyanate lysis buffer (GuSCN) was added to a mixture of 20 µl of silica particle and earlier extracted probes at room temperature for 5 minutes. The mixture was then centrifuged, supernatant discarded, and pellet washed. The pellet was dried briefly and nucleic acid was eluted using 50 µl of TE buffer (pH 7.4) (Boom, *et al*, 1998.). The DNA was eluted and stored at -20 ^{o}C.

2.4. 16S rDNA amplification

Nearly full 16S rRNA genes from natural mixed bacterial DNA were amplified by PCR using universal primers PCRI and PCRII (Table 2) (Lane, 1991). The nested bacterial primers GM5-Forward with 40 bp GC clamp at positions ~341 (*Escherichia coli* numbering) and DS907-Reverse at ~928 position have amplified 580 bp fragment of the 16S rDNA suitable for subsequent DGGE analysis including the highly variable V3, V5 regions (Teske *et al*, 1996). Numbering refers to the *E. coli* 16S rRNA gene position corresponding to the 3´ end of the primers.

PCR reactions were performed using termocycler (Eppendorf, Germany) in 25 µl reaction mixture containing approximately 200 µM of each deoxynucleotide, 2.5 mM $MgCl_2$, 1 U of Red *Taq* polymerase (Sigma) and 0.5 µM of each primer per reaction.

The temperature cycling conditions were as follows. A preincubation at 94 ^{o}C for 5 min; then 30 cycles of 94 ^{o}C for 1 min, the annealing temperature of 45 ^{o}C (for primers PCRI and PCRII) and 55 ^{o}C (for primers GM5-F and DS907-R) for 1 minute, the extension temperature was 72 ^{o}C (2 min) and final extension for 7 min at 72 ^{o}C.
The PCR products were run through 0.8% agarose gel and were purified with PCR Kleen spin columns (Bio-Rad Inc.) according to the manufacturer's instructions.

Table 2. Primers used in this study.

Primer	Positions	Sequence	Description
PCRI	6-26	5´-AGAGTTTGATCATGGCTCAG-3´	Universal bacterial primer for 16S rRNA gene DGGE fragment amplification
PCRII	1513-1492	5´-TACGGYTACCTTGTTACGACTT-3´	Universal bacterial primer for 16S rRNA gene DGGE fragment amplification
GM5F-GC-clamp[a]	341–357	5´-GC-clamp-CCTACGGGAGGCAGCAG-3´	General bacterial primer for 16S rRNA gene DGGE fragment amplification
DS907-Reverse	907–928	5´-CCCCGTCAATTCCTTTGAGTTT-3´	General bacterial primer for 16S rRNA gene DGGE fragment amplification

[a] GC-clamp: 5´-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG-3´.

2.5. DGGE

DGGE was performed essentially as described by Muyzer *et al* (1993). The PCR amplicons were run on a 6.5% (wt/vol) polyacrylamide gel (acrylamide:bisacrylamide, 37,5:1) (1 mm thick, polymerized at least 2 h) at 100 V for a maximum of 20 h at 60 ^{o}C in 1 x TAE using CBS Scientific DGGE-2001 system. Different denaturing gradients between 35% and 70% (7 M urea and 40% formamide (vol/vol) as 100% denaturants), increasing in the direction of electrophoresis (from the top to the bottom), were used. The gel was stained with 1000 x SybrGold (Molecular Probes) for 30 min in 1x TAE buffer and photographed on a UV transillumination table (Spectroline, USA) or variable mode imager (Typhoon Trio, Amersham Biosciences).

A set of four DGGE standards was generated by PCR amplification of 16S rDNA extracted from known isolates to allow comparison of different DGGE runs.

2.6. DGGE analysis

In order to compare DGGE patterns, the gels were analyzed with soft program (ImageQuant TL, AmershamBiosciences) that was used to create a dendrogram describing pattern similarities.

3. Results

3.1. Characterization of the lake and lake sediment environment

At each sampling site environmental conditions and physical parameters of Lake Võrtsjärv were recorded. As it is shown in Tables 3 and 4 temperature, concentration of dissolved oxygen and Chl *a* were measured.

Date	T (^{o}C)	Chl *a* (mg m^{-3})
30.06.2005	17.3	40.19
13.07.2005	23.6	36.24
17.08.2005	17.8	48.16
22.09.2005	12.8	64.87
11.01.2006	2.8	11.49
13.02.2006	2.6	11.15
20.03.2006	2.1	3.39
03.04.2006	3.2	3.20
25.05.2006	12.4	48.83
30.06.2006	19.1	37.31

Table 3. Temperature was measured in the water layer above the sediment. Chl *a* concentration was determined from integrated water sample.

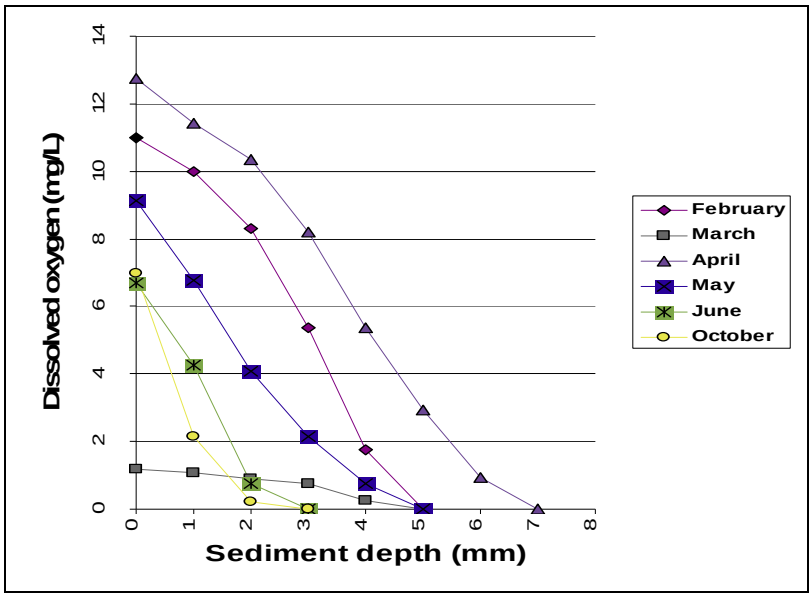

Table 4. Dissolved oxygen concentrations within upper sediment layer of Lake Võrtsjärv measured from October 2005 to June 2006.

Site of sampling	DWS %
14	1.28600
15	1.75000
16	4.26900
17	0.51900
18	0.55700
19	0.51500
20	0.45700

Table 5. Dry weight of sediment (DWS) concentrations measured during transect study from upper sediment layers in June 21, 2006. See M&M section for sampling points numbers.

3.2. Development and optimization of the PCR-DGGE method for profiling bacterial diversity in lake sediment

The bacterial diversity was investigated by analysis of 16S rRNA gene (16S rDNA) recovered from sediment samples through PCR amplification. The DNA extracted from samples was amplified in two ways. First of all, nearly full-length 16S rRNA (~1500 bp) gene (Figure 11) was amplified with universal bacterial PCRI and PCR II primers. For DGGE analysis, however, the entire 16S rRNA gene is too large. So specific bacterial primers GM5-Forward and DS907-Reverse primers were used to amplify hypervariable V3-V5 regions of 16S rDNA and the product of 580 bp (540 bp insert + 40 bp GC-clamp) (Figure 12) was directly analyzed using denaturing gradient gel electrophoresis (DGGE).The separation of 16S rDNA fragment by DGGE is shown in Figure 13. There was observed sufficient separation of environmental bacteria. The chemical linear gradient was increased from primary 20% and 60% to 30% and 70%. The 0% was added on the gel top. These optimizations have provided a marked increase in resolution, resulting in precise sharp banding and reproducible separation. After 20h at 100V 60 ^{o}C bands were clearly defined and showed reduced mobility. High density of bands was obtained in the bottom of the gel.

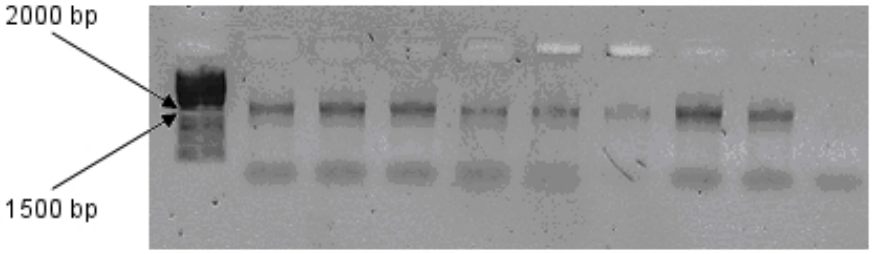

Figure 11. The ~1500 bp 16S rDNA fragment amplified with universal bacterial PCRI and PCRII primers (Table 2).

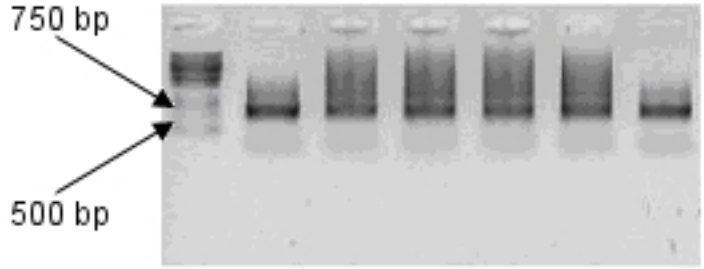

Figure 12. The ~580 bp 16S rDNA fragment amplified with 'nested' general bacterial primers GM5-Forward with 40 bp GC-clamp and DS907-Reverse (Table 2).

3.3. Diversity of bacterial community

PCR-DGGE of 16S rDNA fragments as molecular marker, obtained after enzymatic amplification of nucleic acids from the upper sediment layer of the Lake Võrtsjärv sediments, has been used to examine seasonal, vertical and temporal variations in bacterial sediment communities and to compare bacterial community compositions.

This DGGE study was focused on the fingerprint of bands as estimation of community diversity (amount of bands and their position). All DGGE patterns were analyzed as follows. First, the total number of different bands was determined for the samples being compared. Then each sample was scored based on the presence or absence of each band in its profile when compared to the profile of each of the other samples.

Supposing a constant PCR amplification bias for (or against) a specific bacteria rDNA sequence, the appearance or disappearance of a DGGE band reflect the increase and decrease of the corresponding bacterial populations. Intensities of different DGGE bands, derived from different bacterial species, do not allow quantitative conclusions

about the abundance of different bacteria, because of a possible unknown PCR bias in the amplification of different templates.

Some bands within the sample lanes appeared to align with standards, permitting a tentative identification based on their locations in the gradient.

3.4. Variation of the community structure in depth

In recent study 10 samples were taken from the area that is indicated by marked hatched area (Figure 10A) and 10 DGGE gels were analysed to compare bacterial depth-related changes. DGGE patterns of sediment bacterial communities were composed of between 33 and 39 bands when profiles were sorted by depths. These numbers include bands of 16S rRNA gene PCR products amplified from the sediment cores found within the whole denaturing gel. Each sample displayed a unique banding pattern within the DGGE gels, although many samples contained bands at similar locations within the gels. The DGGE fingerprints obtained for the surface samples were similar and the fingerprints obtained for the deep samples were similar, and there were clear differences when surface and deep fingerprints were compared within 10 samples.

As example of depth related bacterial community changes was analyzed January 2006 gel pattern, which DGGE profile of the bacterial diversity at different lake sediment depths is shown in Figure 13. This sample produced a complex fingerprint composed of a large number of bands. The January sample DGGE banding profile resulted in total 34 different bands. The maximum number of DGGE bands in one track was 24, and the minimum was 17. The DGGE pattern of sediment sample can be interpreted as specific bands emerge and disappear at different sediment depths. Some bands were unique to surface samples, whereas other bands were obtained only with deep samples. Twelve bands in the DGGE profile occurred in all sediment layers; 6 bands are found in the sediment surface only (0-0.5 cm) and 5 bands in the depth of 20-25 cm only (the deepest depth that was taken).

Cluster dendrogram analysis revealed that DGGE fingerprinting effectively differentiated the environmental bacterial communities according at different sediment depths. Figure 14 shows that there is a high degree of microbial diversity based on 16S rRNA gene within the surface sediments (0-5 cm) of the Lake Võrtsjärv than at deeper

layers (20-25 cm). At first glance the DGGE patterns from the different depths look similar because the most intensely stained bands appear in all lanes. This indicates that there is probably a relatively large, stable population of microorganisms detectable by molecular techniques. Similarities between DGGE banding patterns of sediment samples taken at different depths are indicative of the existence of a stable, dominant community. Nevertheless, a more thorough analysis showed quite a number of low-intensity bands which differed in the various depths. These low-intensity bands are responsible for the differences between the depth samples. These results show a marked community structure variation with depth in the sediments of the Lake Võrtsjärv.

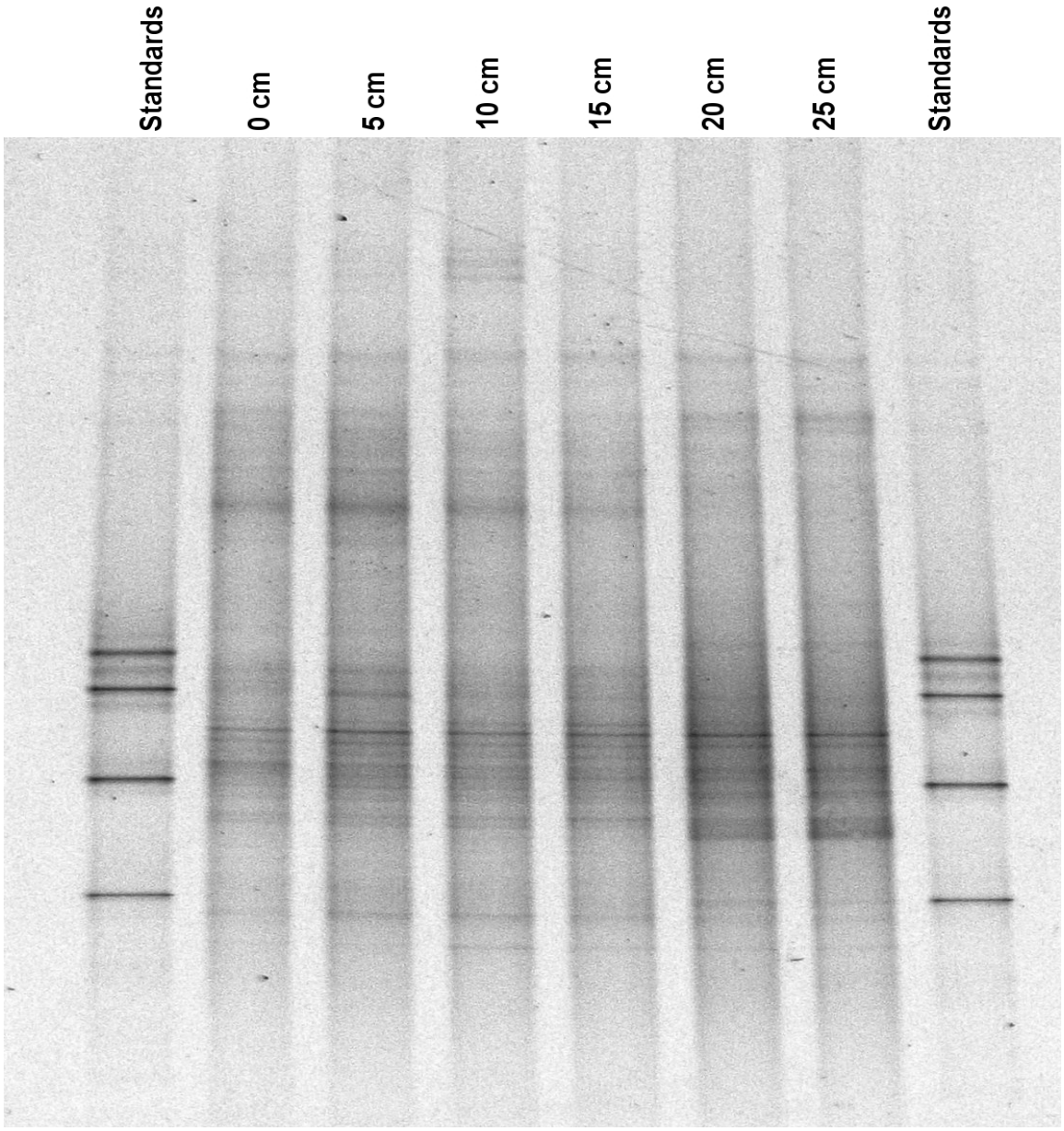

Figure 13. Direct DGGE fingerprinting pattern of depth-related bacterial community composition from upper sediment layer with depth intervals from 0 to 25 cm.

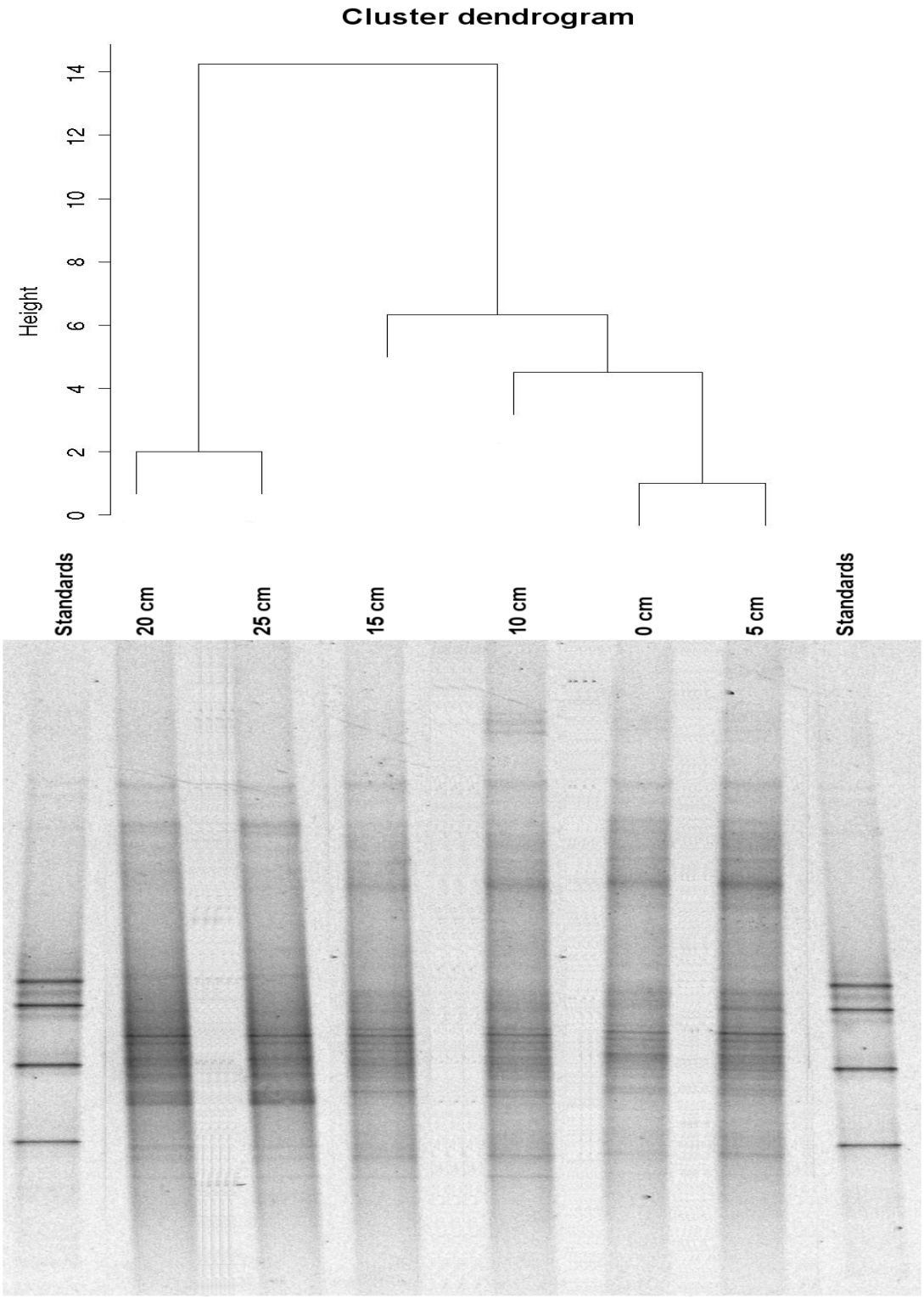

Figure 14. Cluster dendrogram describes DGGE profile of 0, 5, 10, 15, 20, 25 cm sediment depths.

3.5. Seasonal variation of microbial community

The changes in the composition of the bacterial community in Lake Võrtsjärv sediment upper layer were followed during one year, from June 2005 to June 2006. 10 samples were collected at the area that is indicated on the scheme as hatched area. The total 10 DGGE gel patterns of sediment bacterial communities were composed of between 32 and 40 bands when profiles were sorted by months.

As an example DGGE profiles of 0 cm (the sediment surface) depth of Lake Võrtsjärv sediments month-by-month were compared to look for seasonal changes of microbial community structure (Figure 15). Each sample displayed a unique banding pattern within the DGGE gels. The seasonal DGGE banding profile resulted in 33 different bands, which were assumed to represent separate populations. The maximum number of DGGE bands in one track was 16, and the minimum was 4 (February 2005). There was not identifying any of bands present in all samples during the year. Twelve bands on DGGE pattern were rare (present only in 2-4 samples). The highest bands numbers were observed in August (14 bands), January (16 bands) and March (14 bands). In late spring and summer (Aprill - July) the diversity (bands number) was the lowest. An increasing number of bands were observed in the DGGE profiles, indicating an increase in bacterial species. The pronounced seasonal succession of the pattern of bands occurred, with the small differences, in the summer, while winter and spring communities clustered separately (Figure 15). The high abundance of bacteria was associated with summer and early autumn as well as a characteristic community of low productivity occurring in spring.

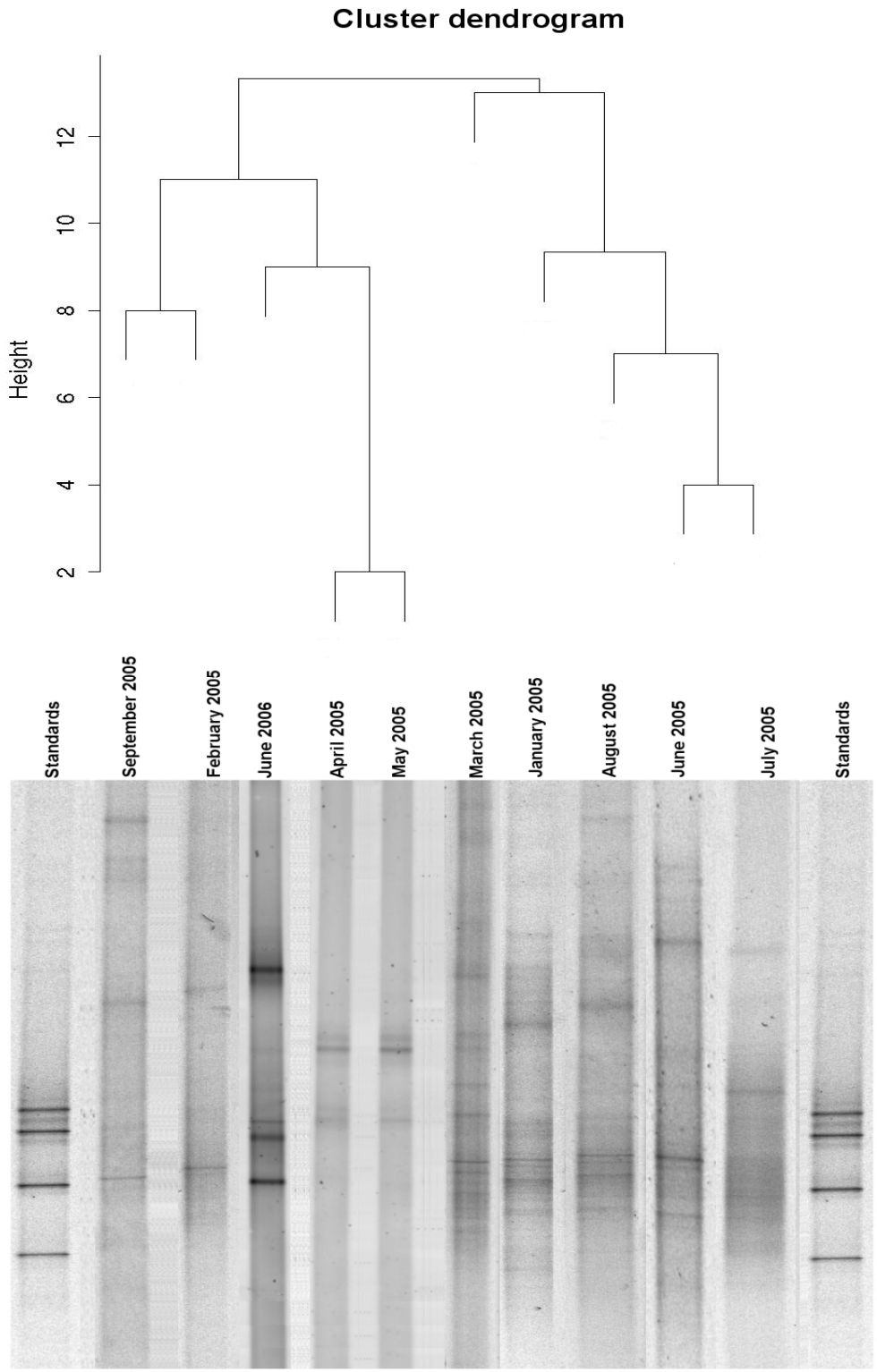

Figure 15. Cluster dendrogram of DGGE fingerprinting of microbial community season changes in upper sediment layer of Lake Võrtsjärv. Samples were taken during one year 2005/2006 month-by-month (October, November, December 2006 not included because of thick ice cover).

3.6. The transect and spatial studies

A transect study and 2 one-day sampling campaigns were carried out covering 7 points of Lake Võrtsjärv sediments to analyze the bacterial community composition, diversity and changes over spatial scale. The transect study samples were taken with distance 3-400 m from the inflowing river mouth to stationary station in June 2006. Sediment samples were collected at locations, which are numbered and represented on the map (Figure 10A).

As an example DGGE profiles of 0 cm (the sediment surface) depth of Lake Võrtsjärv sediments from the transect study were compared to look for changes of microbial community structure. Each of 7 samples displayed a unique DGGE banding pattern within the DGGE gels. A total of 33 band positions were observed after DGGE (Figure 16). The maximum number of DGGE bands in one track was 17, and the minimum was 15. All bands showed different patterns in their appearance. No identical DGGE patterns were found between any sampling sites, because it was not identify any of bands present in all samples. In total 13 unique bands were identified for different sampling site on the DGGE pattern of the sediment surface.

Cluster analysis of the profiles revealed distinct differences between sediment samples of River Õhne and Lake Võrtsjärv sediment sites (Figure 16). According to cluster dendrogram analysis, the number 14 river site profiles were very different from those from lake samples (site numbers 18, 19, 20), and were clustered separately.

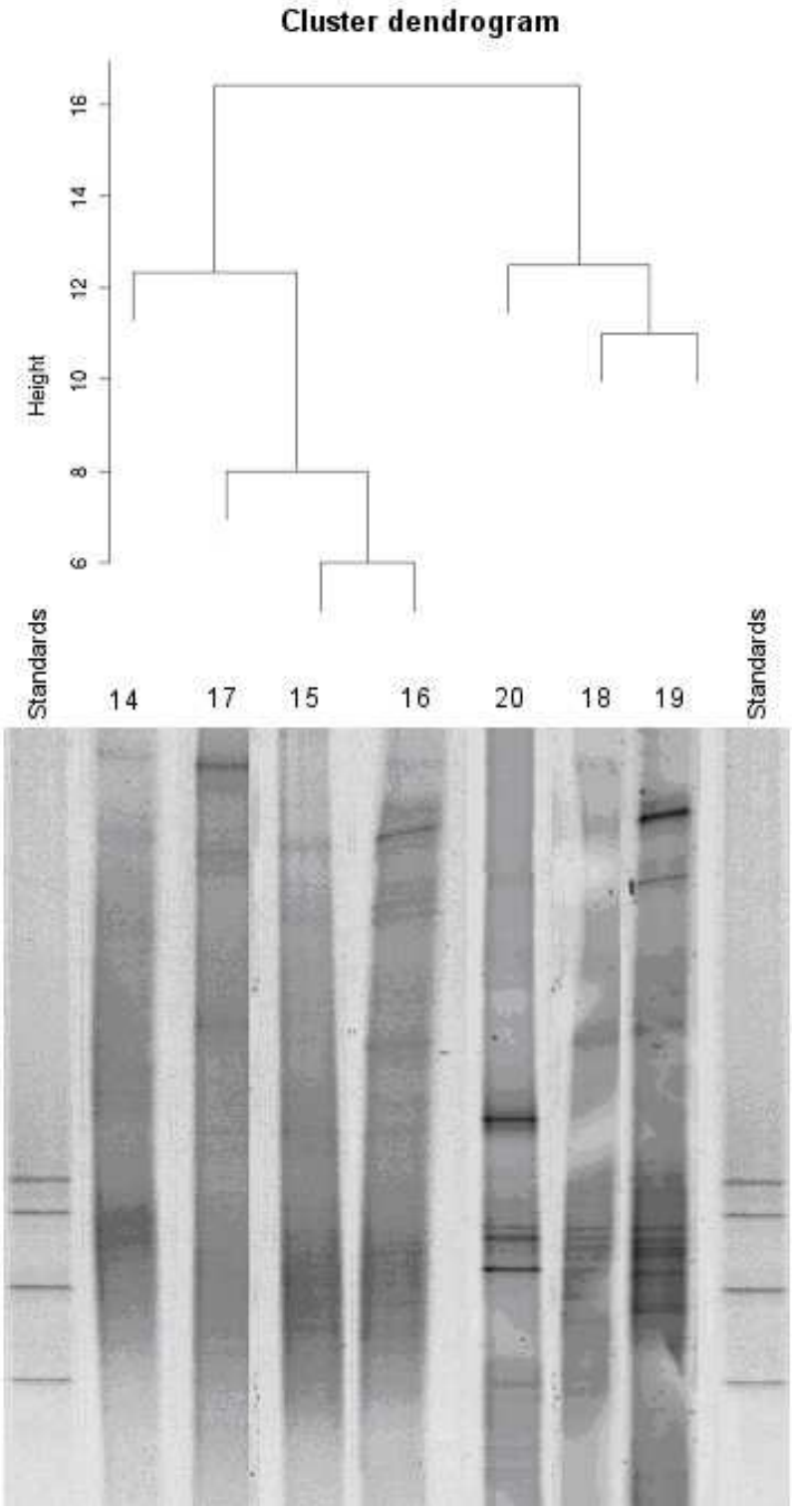

Figure 16. Dendrogram of DGGE fingerprinting of the first transect study with distance 3-400 m from the inflowing river mouth to stationary station. See M&M section for sampling points numbers.

In addition, spatial differences between seasonal (Figure 15) and transect (Figure 16) DGGE profiles were observed. Differences of the sample variance between seasonal and transect datasets was tested by Levene's Test for Homogeneity of Variance. Variance in seasonal dataset did not differ statistically from the dataset variation in transect ($F=1.85$, $p=0.19$). This shows that bacterial diversity of the sediment has similar degree of variation in the seasonal scale compared to the variation in a changing space.

4. Discussion

The aim of this study was to analyze the bacterial community composition, diversity and changes in Lake Võrtsjärv upper sediment layers over temporal, spatial and vertical scales. The primary reason that the heterogeneity of bacterial populations of the Võrtsjärv sediment layers has not been thoroughly investigated is the labor-intensiveness and time-consuming of cloning and sequencing.

We know little about the distributions of bacterial populations within the sediment in space and time, because bacterial communities associated with sediments are not well studied. In the present study, DGGE of PCR amplified 16S rRNA fragments was exploited for analyzing the community structure, diversity and changes within temporal, spatial and vertical scales in environmental samples from Lake Võrtsjärv, because several characteristics of the 16S rRNA gene, such as its essential function, ubiquity and evolutionary properties, have allowed it to become the most commonly used molecular marker in microbial ecology (Case *et al*., 2006).

4.1. Depth-related changes of sediment bacterial communities

Results show a marked community structure variation with depth in the sediments of the Lake Võrtsjärv, suggesting that the depth into sediment has a consistent effect on the bacterial communities. This can be explained in part by the great influence of oxic conditions on bacterial communities. Oxygen is rapidly depleted with depth into the sediment, typically within millimeters (Tabel 4). However, James *et al*. (2006) showed that macrophytes exude oxygen into the sediment creating an oxygen gradient into the rhizosphere extending to 50 mm from the root surface outward into the sediment. In the case of Lake Võrtsjärv macrophytes grow only 18.8% of lake area, most of all in the central part of the lake, around the Tondisaar island (Haberman *et al*., 2004). So this is not significant factor influencing sediment bacterial community diversity in case of whole lake. Cluster dendrogram revealed clear differences within bacteria communities of surface and deeper sediment layers. Upper sediment layer bacteria are in divided two communities suggesting the transition of aerobic bacteria to anaerobic bacteria

communities. The results of our study correlated positively with similar studies described bacterial community changes within sediment depths.

Another noticeable influence on bacterial community variations within sediment depth is provided by the sediments itself, because sediments contain different organic matter concentrations within the depth (Table 5). As it is revealed by cluster dendrogram, the microbial communities of 20-25 and 0-5 cm different significantly and that the diversity is higher within the 0-5 cm than at deeper layers. It can be assumed that organic matter mineralization process at depth of 20-25 cm can reduce sediment carbon and nutrient concentrations. On the sediment surface there is uninterrupted supply of organic matter, because the eutrophic state of Lake Võrtsjärv is characterized as an increase in the rate of organic matter production in the lake ecosystem. As a result high sedimentation rates can therefore contribute to higher concentrations of carbon and nutrients in sediment (Harnett *et al*., 1998) providing enough nutrients for successful and diverse bacterial community. As a result, it was found that the two assemblages were different in species composition, probably as a result of the availability of different kinds of organic substrates.

4.2. Microbial community variations influenced by seasons

It is obvious, that a large number of environmental factors influence bacterial communities in the sediments. It is not surprise that bacterial community varies greatly during the year. Every season has its specific complex of natural conditions for water bodies and its biota. The influence of weather conditions, first of all the temperature, on the seasonal development of the lake's ecosystems is the leading criterion of bacterial community composition changes. As we can see from Table 3, the temperature of lake sediment surface varies greatly during the year from 2.1 to 23.6 ^{o}C. As cluster dendrogram revealed, during year 2005/2006 bacterial community in upper sediment layer of Lake Võrtsjärv varied significantly. According to Haberman *et al*. (2004) Thought the season bacterial abundance and biomass are typically low during the cold winter months. It is confirmed by poor DGGE banding profile of February 2005. Bacterial abundance is increased in March and reaches the highest values from June to August (Figure 15) with regard to temperature increase.

However, the direct effect of temperature is often eclipsed by other factors, mainly by substrate availability. The bacterial communities for a spring month were likely responding to the seasonal status of the macrophytes and phytoplankton algae. Microbial activities in sediments showed strong seasonality and are very low when the plants are actively growing and during algae bloom (April and May 2005). The highest bacterial diversity is revealed in June and lasted until the end of summer. A common feature was that Chl *a* peaks always corresponded to the low bacterial abundance and diversity and vice versa (Tabel 3). Nutrients become available to bacteria only after the decay of algae cells. Because of low temperatures during autumn, algae dyes, sediments on the lake bottom and gets decomposed by bacteria and repackaged into a new wave of nutrients. On the seasonal scale, bacterial growth in Lake Võrtsjärv seems to be related positively to the total nutrient concentrations and negatively to the temperature.

The another efficient nutrient utilization could be due to increased mean nutrient supply to individual cells in a population dispersed by resuspension or to reduced transport limitation of nutrient supply to suspended cells. Increased light attenuation after resuspension may decrease microbes primary production in natural systems (Garstecki and Wickham, 2001).

It is expected that during different seasons different bacteria species dominate. The highest extracellular enzymatic activities are usually found during period after phytoplankton bloom when algae are dying and being lysed. It is expected to observe some additional bands on DGGE or stronger intensivities of dominant bands. Some ectoenzymes (leucine aminopeptidase for example) specific activity increases and correlates more strongly with the presence of specific populations than with the total number of bacteria (Haberman *et al*., 2004).

The ice cover also plays an essential role in the formation of the lake's ecological state. The ice-cover of Lake Võrtsjärv is characterized by variability in ice thickness, as well as in the time and extent of the disappearance of the ice cover. Freezing of the lake usually begins in the middle of November, and the ice melts by the end of April (Haberman *et al*., 2004). DGGE profile confirms the data of strong bacterial diversity differences between winter months and other seasons (Figure 15). Under the ice primary production is controlled mainly by availability of light. Winter conditions in a lake,

especially the amount and concentration of oxygen in sediment (Table 4), depend on the duration of the ice cover and on the thickness of ice and snow. The poorest ecological conditions in Lake Võrtsjärv occurred in the winter period with a low water level (monthly means below 33.00 m above sea level) accompanied by a thick ice cover (>50 cm) of long duration (>130 days). When the ice cover lasted over 130 days, the O_2 concentration in the bottom layer is lower than 3 mg l^{-1}. Thick ice cover in winter may cause a reduction in the oxygen content below the critical point (<2 mg l^{-1}) (Haberman *et al*., 2004). It was showed on the cluster dendrogram that it may be significant differences in bacterial population diversity between summer and winter seasons, especially in the upper sediment layers.

However, many other factors can influence bacterial seasonal activity, e.g. grazing and excretion of nutrients by protozoa or metazooplankton. Short-term (during one month, for instance) changes may be less affected by physical parameters but may depend on the biological features of the community.

Because seasonal variability of lake ecosystems strictly follows annual cyclicity of climate changes, microbial community changes can be predicted according to each of four seasons.

4.3. The transect study

An important characteristic of freshwater lake sediments is that they are not spatially separated from adjacent habitats, but are part of complex ecosystems. The composition of microbial communities in freshwater sediments may therefore be largely influenced by interactions with surrounding aquatic and terrestrial habitats. Some bacteria species may be transported by the rivers from terrestrial sites, that provide the possibility of a continuous input of terrestrial microorganisms by transport and sedimentation of particles (Urakawa *et al*., 2000). The Lake Võrtsjärv has 18 river inflows that can play significant role in forming microbial population diversity in lake sediments. However, the transport of terrestrial microorganisms into lake sediments have not been well studied. The comparison of river and lake microbial might provide the basis for a better understanding of the ecological relationships between lake sediment and terrestrial microorganisms in lake sediments.

As cluster dendrogram revealed there is no much similarity between bacterial communities between River Õhne and Lake Võrtsjärv. Bacterial diversity in the lake appears not to be influenced by the rapid movement of water through the system. Therefore, may be more site numbers and later analyses reveal some general characteristic of bacterial communities, because according to literature such factors as river inflow and water resuspension can significantly influence on sediment bacterial composition. Water masses entering the shallow lake from the river are mixed by wind action and are then washed out into the central part of the lake (Crump *et al*., 1999). However, the resuspension effect on sediment bacteria composition is also influenced by decreased light attenutation due to reduced selfshading, and nutrient and chlorophyll *a* fluxes from the sediment. It remains to be seen whether enhanced growth due to better nutrient access in suspension is a general phenomenon of resuspended sediment bacteria, and how different light regimens affect the response of these bacteria and further effects on the microbial food web (Garstecki and Wickham, 2001).

Bacterial populations can undergo rapid turnover in both time and space, but the composition of bacterial communities and the dynamics of their changes over long time scale are not well understood. Spatial comparisons within Lake Võrtsjärv revealed no similarity from different lake sediment sites, suggesting noticeable influence on bacterial community variations within the spatial scale is provided by the sediments itself. In Lake Võrtsjärv at least 4 sediment types are present (clay, sandy, lime, sapropel or soft bottom) (Figure 10B) that consist of different water and organic matter content, and mineral composition of sediments differ clearly within the lake bottom. Another factor explains such spatial variations may be that macrophyte bed sediments support higher numbers of bacteria and greater bacterial activities than nonvegetated sediments because of enrichment with organic carbon. Bacterial communities in unvegetated sediment do not experience these inputs and would be expected to differ at some level from the vegetated bed sediment communities (James *et al*., 2006). The microbial community in each type of lake sediment is likely to have its own distinct characteristics.

4.4. PCR-DGGE optimization and limitations

In this study powerful, easy and quick DGGE has provided precise picture of microbial community diversity and changes of lake upper sediment layer of Lake Võrtsjärv. As with all methods used to study microorganisms in nature, DGGE is not without limitations. Although biases associated with the molecular methods applied to microbial ecology are not yet well understood, they appear to be less limiting than those associated with culture-based methods. Moreover, no methodological biases have yet been described that would invalidate the conclusions reached by studies such as this (Rappe *et al*, 1997). Obviously, there are many possible reasons why all populations might not be detected. It can be argued that the number of bands on the DGGE gels can over/underestimate the actual diversity of the sample due to the formation of heteroduplex (Dahllöf *et al*., 2000) or chimeric molecules during PCR amplification (Lindström, 1998), and also because of sequence heterogeneities of different copies and copy numbers of the 16S rRNA gene (Rappe *et al*, 1997). Such biases serve to increase, rather than decrease, the diversity of genes in PCR products derived from natural samples, because of high amplicons concentrations during late rounds of amplification (Suzuki and Giovannoni, 1996). For eliminating such possible biases Brügemann *et al*. (2000) suggest the minimizing of amplification cycles number and/or template concentration. As one step to prevent inhibitory effects of some (like humic acids) substances present in sediments BSA was added to the amplification reaction, because BSA may be able to scavenge a variety of substances and thereby prevent their binding and inactivation of *Taq* DNA polymerase. BSA has also been added to PCR to relieve inhibition from samples containing endogenous protease activity (Kreader, 1996).

In addition, positive PCR amplification is not surprising, because it is well-known that PCR is sufficiently sensitive to detect even a single copy of a gene (Saiki *et al*., 1988). All of these potential biases can change the relative concentrations of PCR products so that the resulting profile of phylotypes no longer reflects the composition of the native community (Burr *et al*., 2006). In this case all bands on DGGE should be analyzed, because, in addition to PCR amplification problems, sometimes DNA from species gives rise to two bands instead of one or DNA fragments from different species may have the same melting temperature and give rise to one band instead of two

(Lindström, 1998), although such strategy is time-consuming. Although population analyses based on the retrieval and amplification of rRNA genes enable a more comprehensive view of bacterial diversity, the results should be interpreted with caution due to the limitations of the used methods.

Our working definition was that each band in a DGGE gel represents a separate microbial species and two bands are common if they migrated the same distance on a gel. In present study it was possible to estimate general similarities and differences between communities based on the gel-patterns obtained by DGGE due to high resolution of gel. It can be assumed that our results may simply be a true reflection of the populations (individual bands on DGGE) present or absence at these sites when the samples were collected. We suggest that PCR-DGGE has provided a precise overview of the community profiles, because visual observation of DGGE profiles revealed strong differences between the fingerprints of the microbial communities within sediments (Figure 13). Results of our experiments demonstrate that the DGGE pattern can be assumed to give a reasonable view of the composition of a bacterial community, assuming that each band represents a population in the lake sample. In present study no attempt to quantify the abundance of the members of each population was made since band intensity may not entirely reflect the abundance of an organism in the sample.

Earlier investigations of a lake sediments phylogeny have included the complete 16S rRNA molecule for strains identifications and monitoring community changes. We therefore used only the V3-V5 regions to obtain bacterial communities. Therefore we regard the V3-V5 regions as suitable to readily assess the microbial community from natural samples, because they contain highly variable regions. These regions of bacteria from natural sediment samples were PCR amplified with primers previously determined to be specific for all *Bacteria*. Findings according to literature revealed these primers high efficiency (Kisand and Wikner, 2003; Teske *et al*, 1996). The V3-V5 regions of 16S rDNA also suggested Yu and Morrison (2004) for being routinely used in PCR-DGGE analyses.

In addition, although sequencing of bands for analysis of DGGE fingerprints provide insight into the community structure through the phylogenetic affiliations of community members, the information about their physiological and ecological traits

derived from the partial sequences is often rather limited. Only when the retrieved sequence can be clearly affiliated to a monophyletic lineage characterized by a common phenotypic trait can some conclusions be drawn about the function of the corresponding microorganism. In most cases, however, the simple knowledge of phylogenetic diversity in an environment helps very little in understanding the interacting metabolic processes and the factors which control them (Spring *et al*., 2000; Heuer *et al,* 1999).

4.4.1. Standards

For better gel-to-gel comparison it is possible to load not only external (as presented in present study) but also internal standard markers in each gel lane mixing them with PCR products of environmental samples. It is possible to predict from earlier DGGE gels analyses that 16S rDNA fragments (home-made ‘standards’) from known organisms would denature at unique positions in a denaturing gradient, allowing clear separation of these products from environmentally-derived 16S rDNA fragments. We suggest, this simple modification enables additional DGGE versatility (Neufeld and Mohn, 2005).

Summary

Genetic fingerprinting method, PCR-denaturing gradient gel electrophoresis (PCR-DGGE) has been used to assess the composition of microbial communities of upper sediment layer (0 - 25 cm) of Lake Võrtsjärv. Fragment of 16S rDNA was used as phylogenetic marker gene for comparison of bacterial diversity in lake sediment depth profiles (depth intervals from 0-0.5 cm to 20-25 cm), studying seasonal dynamics of the sediment bacterial over one year period and comparison of spatial variation.

Environmental changes (environmental gradients), such as spatial, seasonal or depth-related fluctuations, influence composition and dynamics of bacterial community within the sediments. Patchiness of the similar sediment type was not higher that differences in environmental gradients. Marked community structure variation with depth in the sediments of the Lake Võrtsjärv was observed, suggesting that such factors as oxygen depth profiles and different kinds and concentrations of organic substrates can sufficiently influence on bacterial communities composition and changes. In addition, all sediment samples analyzed showed a higher degree of microbial diversity within upper that deeper sediment layers.

The seasonal studies revealed that sediment microbial community undergone significant changes. Seasonal variations in microbial abundance and diversity are strongly affected by physical parameters suggesting a leading role to temperature fluctuations, ice-cover parameters and phytoplankton bloom during the spring.

The transect study revealed clear differences between bacterial communities of river bed sediment (River Õhne), littoral zone of the lake and typical soft bottom sediment in the middle of Lake Võrtsjärv.

In conclusion, present study showed that general characteristics of the sediment type and seasonal changes in environment are the major factors influencing the upper 5 cm of sediment.

Kokkuvõte

Töös uuriti madala järve (Võrtsjärv) sette ülakihi mikroobse koosluse mitmekesisust. Mitmekesisuse uurimiseks kasutati geneetilise sõrmejälje meetodit, PCR-denatureerivat gradient geeli elektroforeesi (PCR-DGGE). Markerina kasutati 16S rDNA fragment Bakterite mitmekesisuset võrreldi järve settes sügavuti (sügavuse intervallid 0-0.5 cm kuni 20-25 cm), sesoonset dünaamikat ühe aasta jooksul ja ruumilist muutlikust.

Keskkonna muutused (keskkonna gradiendid), nagu ruumiline, sesoonne või sügavusega seotud kõikumised, mõjutavad bakterite kooslusi ja dünaamikat. Sarnast tüüpi settes ei olnud ruumiline koosluste varieeruvus suurem kui eri tüüpi setete vaheline erinevus. Bakterite kooslus muutus oluliselt sette sügavuse kasvades. See oli tingitud keskkonna tingimuste muutustest nagu hapniku kadumine ning orgaaniliste ainete koostise ning omaduste tõttu. Sügavama sette (alla 5 cm) kooslused olid võrreldes ülakihiga homgeensema mitmekesisusega.

Sesoonsed uuringud näitasid, et bakterite kooslus varieerub oluliselt. Bakterite mitmekesisuse sesoonne dünaamika oli mõjutatud tugevasti füüsilist faktorist. Kõige olulisemad olid temperatuuri kõikumised, jää olemasolu (resuspensiooni puudumine ning hapniku defitsiit) ja fütoplanktoni õitsengud.

Ruumilise muutlikuse uuring näitas, et bakterite kooslused erinevad sõltuvalt asukohast, jõe (Õhne) ning järve litoraali kooslused olid erinevad järve keskkosa pehmesetteliseste regiooni kooslusest.

Kokkuvõtteks, käesolev uuring näitas et üldised järve sette omadused ja sesoonsed kliima muutused on põhilised abiootilised faktorid, mis mõjutavad järve sette ülemise kihi (0-5 cm) bakterite kooslust.

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