MOLECULAR GENETIC IDENTIFICATION OF PLANKTONIC BACTERIA IN THE YENISEI RIVER BASIN AND EXPERIMENTAL STUDY OF THEIR BIOGEOCHEMICAL FUNCTIONS

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Abstract

Each species has its unique and intrinsic geochemical function, i.e. it consumes certain types of energy and matter, and it synthesizes other specific substances including its own biomass. In aquatic ecosystems, research of species composition and functions of certain types of producers and consumers such as algae, invertebrates and fish, has been successfully conducted for a long time. However, the studies of decomposers have until recently been limited by a lack of adequate research methods.

In pelagic ecosystems, decomposers are primarily represented by planktonic bacteria. Light microscopy and cultivation on solid selective media have been principal methods of bacterial species identification for a long time. However, the species with characteristic morphology could only be identified microscopically. The culture method also has significant limitations since no more than 1% of free-living planktonic bacteria grow on the media. Thus, until recently, the species composition of the vast majority of aquatic bacteria has remained virtually unknown. Obviously, if it is impossible to identify an organism and distinguish between species, one will not be able to determine species-specific functions of an individual species in an ecosystem. In the last two decades, these limitations have been overcome with the advent of molecular genetic methods for bacterial species identification.

Since then the bacterial composition of oceans, seas and freshwater reservoirs has been extensively studied. However, watercourses, including major rivers, attracted much less attention. The most effective method of describing taxonomic composition of natural bacterial communities is to determine the nucleotide sequence of 16S rRNA genes by next-generation sequencing. There are only a few studies of composition and diversity of bacteria in rivers based on next-generation sequencing in literature.

Research on biogeochemical functions of natural microbial communities has been conducted by many authors, but most studies did not aim to determine what species of bacteria consumed a particular substance, and they defined the functions of a bacterial community as a whole.

The purpose of this work was to determine the species composition of planktonic bacteria in the Yenisei River basin by molecular genetic techniques including next-generation sequencing, and to estimate their biogeochemical functions using the experimental study of bacterial

consumption of certain types of specific organic substances. To achieve this goal the following objectives were set:

1) studying the biodiversity of bacteria in the Yenisei River by next-generation sequencing.

2) identifying planktonic bacteria consuming a certain range of amino acids: the Bugach reservoir case study.

3) studying the seasonal dynamics of bacterial response to the addition of various amino acids using experimental mesocosms based on water samples from the Bugach reservoir.

For the first time, the bacterial biodiversity in the Yenisei River over the length of about 1800 km was studied. Three thousand and twenty two operational taxonomic units (OTUs) of planktonic bacteria were found in the Yenisei River. The alpha-diversity of bacterial communities and the relative proportion of Cyanobacteria reached maximum values in the middle of the studied section.

It was found that there were three bacterial assemblages differing significantly in the species composition and inhabiting different parts of the Yenisei River located in the mountain taiga (the upper part of the river), the lowland taiga (middle portion) and the tundra (lower portion). Presumably these assemblages were formed as a result of biogeochemical influence of the surrounding landscape. The dominant taxa of each assemblage specialized in the consumption of various organic substances. The obtained results can be used for integrated environmental monitoring of the Yenisei River and to determine the contribution of the ecosystem of the largest Arctic river to the global sink of carbon in the biosphere.

The bacteria consuming lysine and glycine in the Bugach reservoir were identified with the use of non-sterile cultivation in experimental mesocosms. The obtained data indicated that different species of planktonic bacteria specialized in the consumption of individual amino acids. The response of summer bacterial community to added lysine was stable and repeated in different years. In contrast to summer experiments, the addition of lysine in spring and fall did not cause significant changes in quantitative and qualitative composition of pelagic bacterial community. This should be considered when assessing the ability of water ecosystem in biological self-purification from organic pollution.

Introduction

Each biological species has its own set of unique biogeochemical functions, i.e. it consumes certain types of energy and matter and it synthesizes other specific substances including its own biomass (Vernadsky, 1978). In aquatic ecosystems, the studies of species composition and functional role of certain types of producers and consumers such as algae, invertebrates and fish, have been successfully carried out since the inception of Limnology. At the same time, the study of decomposers has until recently been limited due to the lack of adequate research methods.

In pelagic ecosystems decomposers are represented by bacterioplankton. For a long time light microscopy and culturing on solid selective media were the main identification methods of bacterial species. However, only a few species with peculiar morphology, for example, large filamentous bacteria and cyanobacteria, were identifiable microscopically (Zavarzin, 2003). The culture method also has significant limitations since no more than 1% of water bacteria determined by direct counting grow on media (Cole, 1982; Amann et al., 1995). The reservoirs with low trophic level have the greatest gap between the numbers of aquatic bacteria determined by microscopy and culture methods. This gap can reach tens of thousandfolds for oligotrophic reservoirs (Zavarzin, 2003). Many bacterial species cannot be cultured under laboratory conditions (Cases, de Lorenzo, 2002). Thus, until recently, a vast majority of aquatic bacterial species have remained virtually unknown.

It is not possible to cultivate many species of bacteria for several reasons: 1) a cell can lose its culturability (Nyström, 2001); or 2) it is an unknown species for which culture conditions have not been found yet (Amann et al., 1995; Suzuki et al., 1997). Thus, uncultured bacterial species could more correctly be called "not-yet-cultured" (Yokokawa, Nagata, 2010). According to some reports, certain species of bacteria cannot be isolated in a pure culture, as they only exist in a close cooperation with other organisms in the environment (Connon, Giovannoni, 2002). In any case, the separation of "living matter" in the pure form inevitably simplifies real phenomena and is contrary to the natural state of things, while so-called "combined species" is more important. It is a symbiosis of species and a geochemical organic mixture (Vernadsky, 1978). Even if we can figure out physiological characteristics of isolated and identified bacteria, this

does not mean that this species has the natural characteristics of the reservoir from which it was isolated. A species may respond differently to a change in environmental factors, for example, to an increase in nutrient concentration, depending on whether there are other species around, and what kind of species they are (Lawrence et al., 2012). Tested species may utilize an added substance due to the unavailability of other nutrients which it normally consumes under natural conditions (figuratively speaking, if you feed vegetables to a hungry caged lion, the animal will probably swallow them, which does not mean that wild lions are vegetarians). Moreover, it may happen that competitors consume the substance more successfully than the species of interest, and the latter has to settle for other resources. Thus, if natural factors, such as heterogeneity of the environment, competition for resources, predation and other interactions are not simulated in the laboratory conditions, cultivation of the isolated species does not enable determining physiological and biotic functions of these microorganisms in natural ecosystems (Maron et al., 2007).

Obviously, impossibility to identify species prevents the exploration of species-specific functions of individual species in the ecosystem. Because of this obstacle researchers determined some groups of bacteria united by a common biogeochemical function (Ouverney, Fuhrman, 1999). Detailed studies were devoted to the role of aggregated groups of aquatic microorganisms in photosynthesis, chemosynthesis, degradation of organic substances (carbohydrates, proteins, amino acids, amino sugars, alginic acids, hydrocarbons, and other humic substances), nitrogen cycle and so on (Weiss, Simon, 1999; Grover, Chrzanowski, 2000; Rosenstock, Simon, 2001; Perez et al., 2003; Zubkov et al., 2008). However, the role of individual species in the degradation of organic matter has remained unknown for a long time because of the difficulty of bacterial species identification. At the same time, many authors showed the need to determine phylogenetic affiliations of bacteria involved in various biogeochemical processes (Grover, Chrzanowski, 2000; Perez et al., 2003). These limitations have been overcome in the last two decades with the advent of molecular genetic methods of bacterial identification.

Nowadays, there is a number of molecular genetic methods available for identification of uncultivated bacteria, such as sequence analysis of 16S ribosomal RNA gene, multilocus sequence typing (MLST) and analysis of multilocus variable number of tandem repeats (MLVA) (Hofle et al., 2008). The molecular systematics of bacteria is based on the nucleotide sequences of 16S ribosomal RNA gene (16S rDNA), the differences in which serve as a measure of the

evolutionary distance between organisms. Various sequencing technologies are used to determine the nucleotide sequence in genes. A classical method of sequencing is chain termination method, also known as Sanger sequencing. It is based on the selective inclusion of dideoxynucleotides by DNA polymerase, which terminates chain synthesis during DNA replication in vitro. Then, the DNA fragments are separated by electrophoresis on a gel plate or in a capillary and dideoxynucleotide inclusion site is detected. Sanger sequencing is intended to separate pure samples of DNA fragments of up to 1,000 base pairs long, and therefore requires a preliminary separation of the amplified gene fragments of various natural community members by molecular fingerprinting and cloning. However, the number of organisms in natural samples exceeds the potential of Sanger sequencing manyfold (Shokralla et al., 2012).

The most high-throughput sequencing method is massive parallel, also known as next-generation sequencing (NGS) (Barriuso et al., 2011). NGS is characterized by its ability to simultaneously read DNA sequences from different matrices, which eliminates the need for continuous and laborious separation of genetic material of various organisms contained in the sample. The obtained sequences are compared with the ever-growing database of 16S rRNA genes (Engel et al., 2013). Modern computer technologies allow drawing conclusions on biodiversity measures in time and space by clustering DNA sequences with phylogenetic methods. Avalanche-like increase in the number of publications on next-generation sequencing applications shows a paradigm shift in environmental research towards the use of large amounts of sequencing data (Shokralla et al., 2012).

There are several NGS technologies used in sequencing platforms from different manufacturers: 454 pyrosequencing (Roche), sequencing-by-synthesis approach (Illumina), ionic semiconductor (Ion Torrent), sequencing-by-ligation (SOLiD) and others (Mardis, 2008; Shokralla et al., 2012). The emulsion PCR is used when applying pyrosequencing which is the most well-known technique. Single DNA molecules are attached to beads covered with primers and then amplified in a water droplet dispersed in the oil solution. Sequencing occurs on plates with multiple cells and each cell contains one bead. When a nucleotide is attached to a DNA chain, a flash of light is detected by a laser (Shokralla et al., 2012).

In most cases DNA fragments are amplified with a primer comprising a specific adapter and a unique barcode before sequencing. The barcode allows splitting the sequences relating to different samples in the subsequent bioinformatic analysis. In addition, low-quality, short and

chimeric sequences are sorted out during bioinformatic analysis. Similar sequences are merged into clusters of operational taxonomic units (OTUs) and they are identified by comparison with the reference sequences in databases, for example, Greengenes database (DeSantis et al., 2006; McDonald et al., 2012). Further, the indexes of alpha and beta diversity of communities can be calculated based on data on occurrence and abundance of OTUs. The number of information units (base pairs) obtained in one cycle of NGS is several orders of magnitude greater than the capability of devices based on Sanger sequencing.

Currently, microbiological studies are conducted based on two groups of methods. Firstly, classic culture-based methods are used. Historically, a prerequisite for the identification of bacteria was isolation of pure culture strains on the basis of physiological and biochemical characteristics (Holt et al, 1994). Secondly, molecular genetic methods are increasingly used leading to exponentially growing amount of data on bacterial gene sequences and diversity of natural bacterial communities (Hofle et al., 2008). There is a paradox: original taxonomic identification of bacteria was based on their biogeochemical functions which were determined by cultivating on selective media, and those which failed to be cultivated and identified were discarded. Nowadays it is easy to identify bacteria using molecular genetics but it is difficult to determine their function. Knowledge about the function of bacterial populations and communities is required to predict their response to changing environmental conditions and understanding of biogeochemical processes taking place in the ecosystem (Zehr, 2010). Thus, there is an urgent need for adequate methods to identify species-specific environmental (biogeochemical) functions of uncultured bacteria identified by means of molecular genetics.

Methods for determination of species-specific biogeochemical features of planktonic bacteria

1 Analysis of the functional genes, transcripts and proteins

This group of methods is aimed at the functional genes and gene expression products of bacterial community; thereby it identifies the potential ability of the studied bacteria to perform certain biogeochemical functions.

1.1 Omics methods

A number of methods – metagenomics, metatranscriptomics, metaproteomics and metabolomics – is united by the neologism "omics" based on the common part of their names. An important feature of omics which distinguishes it from all other methods for determining bacterial biogeochemical functions is that they give an idea of all the metabolic processes that can potentially take place in the community at the same time (Maron et al., 2007).

1.1.1 Metagenomics

Metagenomic analysis is a molecular method potentially able to identify all biogeochemical functions of bacterial communities. It acquired great popularity by the second decade of the XXI century. Metagenomics studies the genetic material of all members of a community (Logue et al., 2008). The technology of metagenomic analysis has recently been upgraded: previously DNA was extracted from natural water samples, and a library of vectors was created followed by a physiological or genetic screening and determination of the nucleotide sequence (Nardini et al., 2010). Next-generation sequencing methods have eliminated the time-consuming process of creating a library of clones (Schuster, 2008). Since a result of metagenome sequencing is a set of separated nucleotide sequences belonging to different members of the community, the final stage is the assembly of these sequences into gene sequences. However, none of the omics methods is able to determine what particular species consume a given substance. Omics only determine some potential properties and functions of the whole community.

Metagenomics is widely used to identify potential functions of uncultivated bacterial communities. For example, genes responsible for the consumption of reduced sulphur and carbon monoxide were found in the bacterial samples from the ocean surface (Hofle et al., 2008). It was subsequently found that relevant biogeochemical functions were really characteristic of the investigated bacterial community. However, in some cases, the observed composition of the metagenome contradicts the basic metabolic specialization of bacterial communities (Mou et al., 2008). For example, the presence of proteorodopsin genes in a marine bacterial metagenome should indicate that cells are able to use proteorodopsin for energy production, i.e., they will grow faster in the light than in the dark. However, different taxa containing proteorodopsin gene responded differently to the light or the lack thereof, and most of them did not increase the rate of growth in the light (Fuhrman and Steele, 2008). The presence of a gene in a bacterial community does not mean that it is expressed in a particular environment (Maron et al., 2007). Thus, no final conclusions on functions of a microbial community can be based on the detection of functional genes in the community metagenome.

For metagenomic analysis sampling representativeness is particularly important. Typically, a water sample is filtered through a plankton net to remove large particles and organisms, particularly eukaryotes, which can interfere with the process of sample handling using metagenomic techniques developed for prokaryotes (Grossart, 2010). Thus, seston macroaggregates and organisms carrying bacteria on their surface are removed. Many bacteria, even free-living ones, spend most of their time concentrating in the nutritional areas surrounding seston particles and living organisms. That is why the traditional sampling methods used for metagenomic analysis of bacteria ignore the differences in the lifestyle of bacteria and often overlook the "hot spots" of concentration of microbial activity and interactions between microorganisms (Grossart, 2010). There are other methodological complications and "pitfalls" of metagenomic analysis: 1) the difficulty of assembling the gene sequences from short fragments, resulting in stocking gene databases with non-existent chimeric and artificial sequences (Hofle et al., 2008, Kunin et al., 2008); 2) it is not always possible to achieve a sufficient overlap of sequences for gene assembly for all organisms, especially those whose share in the community is small; 3) inability to functionally characterize genes which encode proteins with unknown functions (Heidelberg et al., 2010).

Overall metagenomics is a reliable tool for finding new genes. However, data on the functions of bacterial communities received by means of metagenomic analysis does not reveal information about species-specific functions of individual populations.

1.1.2 Metatranscriptomics

The main disadvantage of metagenomics is that the presence of a gene is not indicative of its expression. Another omics method is metatranscriptomics, which explores the composition of gene transcripts of the whole community, namely messenger RNA. The presence of gene transcripts in bacterial cells indicates the expression of genes coding for functional proteins (Logue et al., 2008). Thus, transcriptomic study can potentially provide information about specific functions of the bacterial community.

However, a number of metatranscriptomics features limit its applicability to the study of biogeochemical functions of aquatic bacteria. These limitations include a rapid degradation of mRNA, difficulties of prokaryotic mRNA isolation and humic acids removal during the extraction, different transcription kinetics of similar gene types in different populations, low correlation between the level of mRNA and protein synthesis (Maron et al., 2007; Poretsky et al., 2005). Due to the rapid degradation of RNA the extraction should be carried out as quickly as possible. After RNA isolation and rRNA subtractive hybridization, the reverse transcription of mRNA into cDNA is performed, and the latter is analyzed (Poretsky et al., 2005). Some metatranscriptomics protocols use DNA microarrays, but their sensitivity to the presence of transcripts is limited by number of probes on the chip (Nardini et al., 2010).

The same effect occurs as in the case of metagenomics: the results of transcriptome analysis of natural communities have the potential to generate new hypotheses about bacterial functions but there is no proof of implementation of these functions in natural ecosystems.

1.1.3 Metaproteomics

Metaproteomics is the study of all proteins of a natural microbial community. Since proteins, namely enzymes, are involved in metabolic processes, metaproteomics is a tool for measuring functions of bacterial community (Maron et al., 2007). If a detection of a gene by metagenomics does not mean that the gene is expressed into a protein, the presence of the protein indicates an implementation of a particular biogeochemical function with more confidence. After extraction and separation of the proteins they are identified by mass spectrometry (Maron et al., 2007).

Some authors believe that metaproteomics of natural samples will be more effective if it is supplemented with the information on the species composition of the community (Wilmes, Bond, 2006). However, like all omics methods, metaproteomics cannot answer the question of what species of bacteria has the revealed function. Metaproteomics can only serve as a complement to other methods of studying biogeochemical functions providing information on the enzyme set possessed by the community.

It has been noted that, compared with an estimated potential of metaproteomics, the results derived from the use of it are still very scarce (Schneider, Riedel, 2010). Current research is mainly focused on simple microbial communities, such as the community of activated sludge or acid mine drainage (Wilmes, Bond, 2006). Because of the complexity of bacterial communities, it is difficult to distinguish all the different proteins. The main difficulties of metaproteomics are a wide range of expression level of proteins in bacterial cells and an enormous genetic heterogeneity of bacterial populations. Constantly improving methods of protein isolation, together with advances in mass spectrometry and the steadily growing fund of bioinformatics data, may help to overcome the complications and constraints of metaproteome research in the future. However, even in such a case metaproteomics will not become an independent method of determining the species-specific biogeochemical functions of aquatic bacteria.

1.1.4 Metabolomics

Metabolome is the complete set of metabolites produced by an organism. Metabolome reflects the enzymatic pathways and networks that are encoded in the genome (Tang, 2011). Metabolomics complements functional genomics data because metabolic intermediates play an important role in bringing together various biochemical reactions taking place in the cell. An analysis of metabolites is performed using the method of nuclear magnetic resonance and mass spectrometry (Tang, 2011). However, the study of community metabolites in itself cannot give sufficient information on the functional role of bacterioplankton. Metabolomics can be taken up by the researchers of biogeochemical functions of bacteria only as an additional method.

1.2 Single amplified genomes

As noted above, direct community DNA sequencing (metagenomics) is not suitable to assemble the genomes of individual members of complex natural communities because they do not match particular species and functions. However, DNA of single bacterial cells can be amplified

individually (Nardini et al., 2010). Bacterial cells are separated by dilution, micromanipulation, or fluorescence sorting of activated cells. Next, genomic DNA of a cell is amplified in a special way and its nucleotide sequence is determined. It is necessary to take into account that it is not always possible to extract all the genomic DNA of a bacterial cell and that rearrangement of nucleotide sequences may occur during PCR.

A single genome amplification can detect functional genes of particular species of uncultured bacteria, and can be used as an addition to other methods of determining biogeochemical functions of aquatic bacteria.

2 Methods with nutrient additions

The most logical way to ascertain the biogeochemical functions of heterotrophic water bacteria is to add an organic substrate to the water they inhabit and to find out which species of bacteria are consuming this substance. Many authors believe that methods with additions of organic matter can provide important information on species-specific biogeochemical functions of bacteria if these methods are used in a combination with the method of experimental mesocosms (Carlson et al., 2002; Giovannoni, Stingl, 2005; Schafer et al., 2001; Zubkov et al., 2008; Trusova, Gladyshev, 2006). The basic idea of the experimental mesocosm method is that bacteria function as a part of the natural plankton community there while the biogeochemical process of interest is enhanced by the addition of tested nutrients. Natural bacterial community in mesocosms should remain intact because the interactions among bacterial populations, as well as the impact of consumers, for example protozoans, will ultimately affect the implementation of various biogeochemical functions of bacterial species in the ecosystem. Only with *in situ* conditions bacteria can be expected to perform their "natural" functions (Hofle et al., 2008).

The problem of determining the role of individual taxa of bacteria in the ecosystem is complicated by the fact that in a stable bacterial community only a small part of the metabolic capacity and variability of this community is actively expressed (White, 1995). The bacteria are ready to benefit from the nutrients that are available after the intervention of the experimenter. Some bacteria start consuming nutrients only when the substance is added in a large excess, therefore it cannot be concluded that the bacterium feeds on the substance under normal conditions.

It is assumed that the structure of a microbial community changes in mesocosms just as it would change in a natural pond with a similar intervention (Caron, Countway, 2009). The experimental approach can give a relatively accurate assessment of the community functioning, and there is now almost no alternatives.

All methods described in this section are used in conjunction with the cultivation of bacterial community in the experimental mesocosms with addition of nutrients. The methods described below are aimed to determine which bacterial species (or another taxon, depending on phylogenetic resolution) consumed an added substrate.

2.1 Stable isotope probing

An effective way to study the consumption of various substrates by uncultivated bacteria is their probing with stable isotopes (Hofle et al., 2008; Radajewski et al., 2000). Typically, stable but relatively rare isotopes ^{13}C and ^{15}N are used as labels. ^{2}H deuterium atoms are not applicable since hydrogen can enter bacterial cells not only with nutrients but also with water from the culture medium (Kreuzer-Martin, 2007).

After exposure to a medium containing the labelled substance bacteria that absorbed it have an increased content of heavy isotopes in their composition. DNA, RNA, or cell membrane lipids and fatty acids such as polar lipids and (less often) hopanoids are usually probed (Adamczyk et al., 2003; Kreuzer-Martin, 2007). The labelled DNA or RNA is separated out of the unlabelled one by density gradient centrifugation, and then species composition is analyzed, revealing bacteria that absorbed a stable isotope (Kreuzer-Martin, 2007). Phospholipids composition is analyzed by isotope ratio mass spectrometry or gas chromatography-mass spectrometry (Gray, Head, 2001).

2.1.1 Stable isotope probing of lipids

Stable isotope probing (SIP) of lipids is more sensitive than the analysis of nucleic acids (Kreuzer-Martin, 2007). All labelled and unlabelled lipids of a sample are extracted together and separated by gas chromatography. This method reveals even small differences in the content of ^{13}C in a molecule. Sensitivity is especially significant when the experimental conditions do not allow achieving a high level of labelling. Such conditions include the limited duration of the experiment and the availability of alternative sources of the studied atom to the

community. These factors may hamper DNA-SIP or RNA-SIP if the number of absorbed labels is not enough for detection. In this case, isotope probing of lipids is preferable.

SIP of lipids impedes phylogenetic affiliation of bacteria that consumed the labelled substrate. The same fatty acids may be synthesized by various species, and fatty acid profiles of individual organisms may vary depending on environmental conditions. Lipids of the entire community are extracted as a whole and lipid profiles of uncultured bacteria are generally unknown. Determination of label incorporation into fatty acids of bacterial communities is informative only when a limited number of organisms are metabolically active and these organisms synthesize species-specific fatty acids.

2.1.2 Stable isotope probing of DNA

DNA probing is preferable when the duration of an experiment is not a limitation. A significant advantage of DNA probing is that the DNA enriched with heavy isotopes contains the entire genome of each functionally active member of the community (Gray, Head, 2001). Cloning of large fragments of labelled DNA molecules using vectors provides an opportunity for a more thorough genomic analysis of uncultured bacteria which perform specific biogeochemical functions. The data obtained can be used for designing probes for fluorescence *in situ* hybridization (FISH) (Kreuzer-Martin, 2007). Some researchers have noted that sometimes labelled and unlabelled DNA fractions are not separated enough in the density gradient and both fractions are mixed (Kreuzer-Martin, 2007). However, special approaches are developed to achieve the complete separation of fractions (Chauhan, Ogram, 2006). A disadvantage of DNA-SIP is the need to carry out the experiment long enough to ensure that a replication has occurred (Kreuzer-Martin, 2007). Replication time varies for different types of bacteria, depending on environmental conditions. The longer lasts the experiment, the more likely it is that the added nutrients are metabolized by bacteria into chemical compounds that are available for consumption by other bacterial species. DNA of bacteria that consumed the metabolites of the labelled substances will also be labelled and extracted along with the DNA of bacteria that feed on the initially added substance.

2.1.3 Stable isotope probing of RNA

RNA-SIP combines the possibility to identify the bacteria that consumed the labelled substrate and the benefits of short incubation time. RNA incorporates isotopic labels faster than DNA (Adamczyk et al., 2003). The rate of label incorporation into RNA varies depending on various

factors: the type of the substrate, RNA stability and metabolism of the consumer. These factors must be considered when choosing the duration of the experiment. A disadvantage of the analysis of the labelled RNA is its instability.

The common shortcoming of all methods based on labelled nutrient supplements is that bacteria equally absorb labelled and unlabelled natural substrates thereby reducing the relative quantity of labels incorporated into DNA (Radajewski et al., 2000). In order to obtain a detectable amount of isotopic labels, the labelled substrate needs to be added in an excess compared with the natural substrate concentration, and incubation time should be long enough to increase the level of label incorporation. The addition of high concentrations of nutrient can trigger the growth of copiotrophic organisms which are not able to function actively under natural conditions (Gray, Head, 2001).

The increased incubation time can also lead to the formation of labelled metabolites which are assimilated by secondary consumers. However, the label can be traced in the food chain of community decomposers if an experimenter repeatedly takes samples at intervals after the addition of the labelled substrate. After sampling, the DNA is extracted and electrophoresis profiles of 16S rDNA fragments from the unlabelled and labelled fractions are prepared. Then, nucleotide sequences of the 16S rDNA fragments of bacteria that absorbed the label are determined. By comparing the gene sequences to databases, phylogenetic analysis is carried out and organisms that consecutively incorporated the label are identified. A separate experiment with the addition of the labelled degradation intermediates of the initially tested substrate will also help to identify the consumers of these metabolites (Gray, Head, 2001).

Despite some limitations, SIP is a reliable method to determine bacterial species-specific biogeochemical functions in natural communities.

2.2 Application of bromodeoxyuridine and iodonitrotetrazolium violet

Sometimes bromodeoxyuridine and iodonitrotetrazolium violet, the indicators of metabolic activity of bacterial cells, are used as a methodically simpler alternative to isotope probing.

Thymidine analogue bromodeoxyuridine is added to experimental mesocosms as a tracer simultaneously with the tested nutrient. Metabolically active cells consume bromodeoxyuridine, and the more metabolically active the cell is, the more bromodeoxyuridine it incorporates in its DNA. DNA containing bromodeoxyuridine may be separated from DNA that

does not contain this thymidine analogue using immunocapture techniques (Gray, Head, 2001). Bromodeoxyuridine-enriched DNA of metabolically active members of a microbial community is visualized by immunofluorescence method. For this purpose, anti-bromodeoxyuridine monoclonal antibodies or fluorescently labelled secondary antibodies are used. Bromodeoxyuridine-labelled DNA can be also isolated using immunochemical capture with paramagnetic beads coated with antibodies.

Iodonitrotetrazolium violet can also be used for the detection and subsequent identification of active members of microbial communities responding to experimental substance additives (Gray, Head, 2001). Iodonitrotetrazolium method is used to study the bacterial response to the addition of substrates oxidized by bacteria. Respiratory activity of bacteria reduces tetrazolium salts into insoluble formazan crystals inside the cells. Intracellular formazan deposition changes the density of active cells allowing them to be sorted by density gradient centrifugation for subsequent community analysis, the same occurs for SIP, too.

A disadvantage of the methods using bromodeoxyuridine and iodonitrotetrazolium violet is that a selective stimulation of bacteria that were inactive prior to the addition of the excess amounts of a substrate is not excluded. Application of iodonitrotetrazolium violet is limited because its reduction is associated with specific sites of the electron transport chain. Some bacteria cannot recover iodonitrotetrazolium violet or do so insufficiently for cells to be separated by centrifugation. A significant limitation of the method using bromodeoxyuridine is that not all heterotrophic microbial populations consume nucleotides, even when they are metabolically active. In this case, the cell that actively consumes the added substance does not accumulate bromodeoxyuridine and is not visualized as metabolically active. Thus, the use of bromodeoxyuridine and iodonitrotetrazolium violet to determine the biogeochemical functions of bacteria is less informative than the stable isotope probing.

2.3 Methods based on fluorescence *in situ* hybridization

Fluorescence *in situ* hybridization (FISH) is a good method to identify, locate and count individual microbial cells and clusters (Schramm, 2003). Cells are hybridized with fluorescently labelled oligonucleotide probes complementary to specific regions of the 16S rRNA, and then examined under a microscope. Probes specific for certain taxa are selected from databases (Pernthaler, Amann, 2005).

Use of FISH to study bacteria can be complicated by some technical difficulties, such as a weak signal, high background fluorescence and cell counting errors (Schramm, 2003). In addition, it is not easy to pick up specific probes for the uncultured microorganisms. Sometimes a limitation of FISH is the focus on the ribosomal RNA. RRNA genes are highly conserved and their phylogenetic resolution is less than that of the functional genes, or variable coding regions of the bacterial genome, such as intergenic spacer regions (Gray, Head, 2001). It is important to carefully select FISH probes, allowing detection of the target groups with minimal inclusion of false-positive results (Yokokawa, Nagata, 2010). In addition, hybridization efficiency varies in different taxa, which may lead to a systematic error in the estimate of the community structure. With FISH, bacteria are not identified down to species level, but successfully linked to higher ranks of phylogenetic affiliation, such as families.

To increase the accuracy and sensitivity of the method up to the quantitative assessment of a single cell activity, CARD-FISH is used instead of the standard fluorescent hybridization technology. The CARD-FISH method is based on the use of labelled tyramine molecules. Tyramine is a phenolic compound used for the amplification of the fluorescent signal (Pernthaler et al., 2002). The fluorochrome-labeled tyramine is deposited in the site of hybridization. Accumulation of fluorescent molecules in the hybridization site leads to a strong increase of FISH sensitivity compared to conventional probes. Another kind of methodological upgrade is RING-FISH, based on the use of multiple labelled polynucleotide probes, which are organized in large networks around the target sequence (Logue et al., 2008). FISH is widely used to identify different groups of bacteria in conjunction with the methods that enable to trace the consumption of certain substrates.

2.3.1 Combined microautoradiography and FISH

Microautoradiography gives an opportunity to track the bacterial uptake of certain substances labelled with radioactive isotopes ^{3}H, ^{14}C, ^{33}P and ^{35}S (Gray, Head, 2001; Hofle et al., 2008). Initially, microautoradiography was used to study the consumption of various substrates by cultured bacteria (Andreasen, Nielsen, 1997). The simultaneous use of FISH allows identification of non-culturable bacteria that consumed the labelled substrate (Ouverney, Fuhrman, 1999). Slightly differing methods are used: microautoradiography combination with FISH (MAR-FISH and Micro-FISH varieties) and the combination of substrate tracking autoradiography with FISH (STAR-FISH) (Hofle et al., 2008).

The most important advantage of microautoradiographic method is the possibility to track the inclusion of radionuclide-labelled components by a cell (Hofle et al., 2008). A disadvantage of the microautoradiography in combination with FISH is its laboriousness, which limits the number of parallel experiments (Adamczyk et al., 2003). Labelled substrate is metabolized by primary consumers and may be used by the other members of the microbial food chain that do not consume the initially added substrate. Bacteria consuming metabolites would also be labelled with radioactive isotopes, which may lead researchers to false conclusions. Short incubation time reduces but does not eliminate the problem of metabolite consumption by bacteria that do not consume the tested nutrient.

In some cases, the use of microautoradiography led to ambiguous conclusions. For example, the combination of autoradiographic determination of the substrate inclusion and FISH (STAR-FISH) was used to determine biogeochemical functions of marine planktonic archaea (Ouverney, Fuhrman, 1999). After addition of a mixture of ^{3}H-labelled L-amino acids to a sample of water, about 60% of archaea included the radioactive label, and for this reason they were allocated to heterotrophs. However, subsequent studies have shown that archaea are principally chemoautotrophs (Fuhrman, Steele, 2008). Incorporation of a labelled substrate indicates the activity of the transport system of the body, rather than the ability of archaea or bacteria to breathe and grow under given conditions, or to store the substrate as a reserve matter (Andreasen, Nielsen, 1997). However, microautoradiographical studies greatly expand our knowledge of the biogeochemical functions of microbial communities (Cottrell, Kirchman, 2000).

2.3.2 Combined Raman microscopy and FISH

The uptake of labelled substrates by individual cells of uncultured bacteria can be studied with high sensitivity through a joint application of FISH, stable isotopes and Raman microspectroscopy (Raman-FISH by the name of one of the discoverers of the phenomenon of Raman scattering). In Raman spectroscopy, an excitation laser is used to measure the vibration energy of chemical bonds in a sample. Scattered laser light is detected by a camera, and the Raman spectrum with distinct peaks is obtained (Neufeld et al., 2007).

Raman spectroscopy determines the structure of chemical bonds in biological molecules of individual microbial cells (Huang et al., 2007). Uptake of ^{13}C stable isotope by a cell causes a significant change in the observed resonance spectrum as compared with the conventional

spectrum of molecules with ^{12}C atoms. Since the oscillation frequency is inversely proportional to the square root of the atomic mass, after inclusion of the heavy isotopes the increase in molecular weight causes a modification of the vibration state of the bond. This modification of bond fluctuation is called redshift due to the formation of longer wavelengths in the spectrum of Raman scattering. Studies have also shown that the spectral shifts are independent of the type of studied bacteria, and correlate with the content of the tracer in the cell, thus, Raman spectroscopy is a quantitative method (Neufeld et al., 2007).

Huang et al. (2007) demonstrated the possibility of using Raman-FISH for studying biogeochemical functions of microorganisms at a single cell level in complex samples using ^{13}C-labeled substrate. Compared with the MAR-FISH, Raman-FISH has similar or higher resolution and does not need the use of radioactive isotopes as labels. Raman-FISH allows the quantitative analysis of stable isotope incorporation at a single cell level.

Key benefits of the combination of Raman spectroscopy, isotopic labels and fluorescence *in situ* hybridization are: 1) higher spatial resolution than MAR-FISH (approximately 1 μm), 2) possibility to use stable isotopes as a label, 3) tracing what particular cell compartments incorporated the label. However, some improvements of Raman-FISH are still needed. For example, it is still unclear whether the redshift is also caused by substrates labelled with other stable isotopes (for example, ^{15}N). The calibration curves for each pair of a particular organism and labelled substrate are also needed. If redshift has a similar magnitude for a large number of microorganisms and substrates, then the method is applicable to uncultured bacteria from natural environmental samples (Huang et al., 2007). A common limitation of all methods based on FISH is applicable to Raman-FISH: hybridized cells should be relatively numerous for microscopic detection (Neufeld, Murrell, 2007).

2.3.3 Multi-isotope imaging mass spectrometry

The most sensitive method to register the incorporation of isotope labels into a cell is multi-isotope imaging mass spectrometry (MIMS) (Hofle et al., 2008). This is a new generation of secondary ion mass spectrometry (SIMS), united with FISH, with a complex ion optics and software for quantitative analysis of the image (Lechene et al., 2006). After incubation with substrates containing stable isotopes, the number of isotopes in cells that absorbed the label is analyzed with a very high spatial and mass resolution, high sensitivity and reproducibility. MIMS technology analyzes the composition of stable and radioactive isotopes of single cells with a

lateral resolution of 50 nm, exceeding the Raman spectroscopy (Neufeld et al., 2007). MIMS is 1000 times more sensitive than microautoradiography, and has an accuracy of counting stable isotopes of ± 1%. As with Raman-FISH, FISH combination with MIMS enables phylogenetic and isotope analysis of natural samples in a single scan. Behrens et al. (2008) combined their own modification of the CARD-FISH with MIMS and demonstrated its applicability to establish biogeochemical functions of uncultured bacteria.

Multi-isotope imaging mass spectrometry would be an ideal method for studying biogeochemical functions of bacteria if it enabled identification of bacteria feeding on an added substrate down to species level.

2.3.4 Combination of beta microimaging and FISH

Beta microimaging determines the number of isotopes absorbed by a bacterial cell and measures the two-dimensional distribution of radioactive isotopes with high sensitivity (Gieseke et al., 2005). For beta microimaging, autoradiographic film and emulsion are replaced by a more sensitive β-particles imaging system (Laniece et al., 1998). The disadvantage is a relatively low spatial resolution of about 10 microns, which does not enable to distinguish individual cells. Usually beta microimaging is used in conjunction with microautoradiography or an equivalent, which allows to study the distribution of isotopes with higher resolution.

Gieseke et al. (2005) used a combination of microautoradiography and beta microimaging with FISH to study the consumption of substrates by bacteria inhabiting a biofilm. Benefits of microautoradiography allowed to track the consumption of the substrate by individual cells, and beta microimaging was used to determine the amount of consumed labelled carbon. Treude et al. (2007) studied the consumption of methane and carbon dioxide labelled with ^{14}C by methanotrophic microbial mats from the Black Sea. The combination of different methods, including beta microimaging to study the amount of incorporated ^{14}C, secondary ion mass spectrometry to study the distribution of ^{14}C, and CARD-FISH, has identified methanotrophs in the microbial consortium. As an addition to microautoradiography, beta microimaging was applied to test the activity of methane-producing organisms (Collins et al., 2007).

Currently because of the low spatial resolution beta microimaging is only suitable for use in well-structured microbial communities, such as biofilms, microbial mats and anaerobic granules of treatment facilities (Neufeld et al., 2007).

2.4 Microarrays

Using microarrays to study the biogeochemical functions of bacteria is one of the most high-performance techniques tested for various bacterial communities (Ward, 2005). The microarrays are glass plates, which bear probes (oligonucleotides or PCR products) in numerous small dots (Logue et al., 2008). Modern type of microarrays looks like a polyacrylamide "pad" with a side of 100 mm and a depth of 20 microns (Stahl, 2004). For the identification of specific bacteria present in the sample, microarray is coated with different versions of the gene corresponding to the various organisms. When a complimentary DNA or RNA from a bacterial sample, which was incubated in medium containing a substrate with an isotopic label, hybridizes to a suitable sequence on the microarray and associates with the corresponding dot, the isotope pattern indicates the presence of the desired genes in a sample according to their position on the microarray. Microarrays as a method of direct detection of 16S rRNA enable a simultaneous study of the community structure and specific consumption of the substrate by its members (Adamczyk et al., 2003).

The use of microarrays for biogeochemical functions study has a number of drawbacks. Just as in the case of SIP and MAR-FISH, it is difficult to distinguish between primary consumers of the substrate from microorganisms that feed on the lysis products or exometabolites of the primary consumers. In some studies, there was a problem of low resolution of phylogenetic microarrays, since they allow to distinguish sequences that differ by more than 12% (Wilmes, Bond, 2006). For comparison, other methods, such as 16S rDNA sequencing, enables distinguishing between the nucleotide sequences that differ by less than a percent. Analysis using microarrays prevents collection and analysis of genomic DNA of metabolically active organisms. Humic acids and other organic substances contained in natural samples may inhibit hybridisation of DNA with microarrays. Some authors question the applicability of isotopic microarrays for the study of complex natural communities (Sharkey et al., 2004).

On the other hand, microarrays have several unique advantages. Unlike DNA-SIP and RNA-SIP, isotope microarrays directly measure the substrate inclusion in target nucleic acids, thereby virtually eliminating false positive results. Compared with the MAR-FISH, microarrays enable application of many probes in parallel, which makes it faster compared with other methods to study the biogeochemical functions of microbial communities.

2.5 Community fingerprinting

Molecular fingerprinting is a quick way to see changes in the composition of bacterial communities, it is widely used to study a variety of habitats, including aquatic environments (Yan, Yu, 2011). The method does not allow tracing the intake of nutrients by individual bacterial cells. Molecular fingerprints are community profiles, which separate the DNA band corresponding to a bacterial population. If a population increases its share in a community and becomes dominant after nutrient addition, it is assumed that it feeds on the added substance (Trusova, Gladyshev, 2006).

Currently, the most commonly used fingerprinting techniques for aquatic ecosystem studies are denatured / temperature gradient gel electrophoresis (DGGE / TGGE), single-stranded conformation polymorphism (SSCP), random amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (T-RFLP), restriction analysis of amplified ribosomal DNA (ARDRA), analysis of ribosomal intergenic spacers (RISA) and automatic analysis of ribosomal intergenic spacers (ARISA) (Yan, Yu, 2011). All of these methods provide a fast and reproducible way to assess changes in natural microbial communities (Logue et al., 2008).

Molecular fingerprinting creates a profile of the genetic diversity of microbial communities and provides an opportunity to track genotypic changes in communities over time. The best methods to determine biogeochemical functions of individual species are DGGE, TGGE and SSCP (Nocker et al., 2007), since they give an opportunity to cut gel bands containing the DNA of certain species of bacteria for subsequent determination of its nucleotide sequence. Fingerprinting is a semi-quantitative method as obtained community profiles are subject to potential bias caused by PCR, such as uneven amplification of genes of different bacteria and the formation of chimeric sequences. Bands at the community profiles correspond to the genes of different species of bacteria, but are not directly translated into taxonomic information, and provide data only on the most numerous populations (Logue et al., 2008; Zajec et al., 2012). It is now believed that DNA-based fingerprints reflect the presence of species in the community while RNA-based fingerprints match metabolically active members of the community (Brettar et al., 2012).

Fingerprinting of amplified fragments of 16S rDNA using DGGE is an effective approach for monitoring changes in the composition of the dominant species in bacterial communities in experimental mesocosms, for example, (Ovreas et al., 2003; Schafer et al., 2001; Trusova,

Gladyshev, 2006). Moreover, fingerprinting allows studying the effect of the addition of organic substances on bacterial communities and, importantly, the community reaction to changes in other physical, chemical, and biotic factors (Brettar et al., 2006; Brown et al., 2012).

Fingerprints allow monitoring changes in the structure of bacterial plankton community during the uptake of nutrients. Usually, a substance is added directly to the water sample containing the whole plankton community. The dynamics of bacterial community is monitored by increase of the brightness (density) of bands at the profile corresponding to the individual species consuming the substance in the course of mesocosm experiment. The populations that do not consume the added substance remain stable, and the density of corresponding bands at the profile is unchanged. Some researchers use DGGE in conjunction with other methods of distinguishing a functionally active part of the community, for example by adding bromodeoxyuridine to the original sample (Hamasaki et al., 2007).

Sequencing of DNA extracted from the bright bands facilitates the design of highly specific primers used to obtain the entire gene sequence of 16S rRNA. Then, using real-time PCR the number and activity of individual species can be determined *in situ* and in experimental conditions. In addition, the sequence data is used for the design of oligonucleotide probes for Micro-FISH. Applying Micro-FISH together with fingerprinting would confirm whether the bacterial population increased its share in the community as a result of added substrate uptake.

3. Selection of an optimal method

Development of molecular genetic methods for studying biogeochemical functions of bacteria has reached a new level. Bacterial ecology, thanks to the improvement of tools and the constantly growing productivity of methods, is running out of white spots (Logue et al., 2008).

The methods to determine bacterial species-specific biogeochemical functions can be evaluated with the following criteria: 1) phylogenetic resolution of the method; 2) the opportunity to study the process as close as possible to *in situ* conditions; 3) the ability to monitor the process in time; 4) the ability to monitor the bacterial community as a whole. Analysis of nucleic acids labelled with stable isotopes and molecular fingerprinting have the best phylogenetic resolution. Methods combined with FISH (microautoradiography, RAMAN-FISH, multi-isotope imaging mass spectrometry) allow creating conditions closest to *in situ*,

which do not require the addition of large amounts of substrate for the study of its uptake by bacterioplankton.

The best method for studying the process in time is fingerprinting. Isotopic techniques allow keeping track of the entire community. The most adequate way to study biogeochemical functions of water bacteria is to use a combination of several methods chosen depending on the situation and the objectives of the study. Ideally, in order to study what species of bacteria consume the substance, experimental incubation in mesocosms supplemented with a radiolabelled nutrient should be combined with molecular fingerprinting, MIMS and FISH. Fingerprinting followed by a phylogenetic analysis will help to track the population dynamics in the community. MIMS results will confirm if communities which increase their share of the population are actually consuming the added substance. FISH will confirm the increase in the number of populations whose share in the community profile increased during the experiment. If phylogenetic resolution of FISH is not good enough, the isolated community DNA can be divided in a density gradient in order to separate the heavy fraction, and species that absorbed the added substrate will be identified.

Many studies of the biogeochemical role of bacteria are focused on revealing the relations between the structure and function of the whole bacterial community but some researchers suggest it is better to focus on identifiable bacterial populations whose biogeochemical functions in aquatic ecosystems can be calculated and determined (Pernthaler, Amann, 2005). In many ways, a "bacterial community" is more a theoretical concept rather than a real biological object with its own structure, genome and evolutionary history. A bacterial community can be regarded as a free collection of individual populations whose characteristics may change over time.

Ecosystem stability depends on the resistance (the ability of ecosystem to maintain its structure and functional properties when exposed to external factors), resilience (the ability of ecosystem to return to its original or close to original condition after cessation of treatment) and functional redundancy (the same function is performed by different populations) (Maron et al., 2007). These characteristics are well known for animals and plants, but they have not been sufficiently studied in microbial ecology.

Insufficient knowledge of ecological characteristics of water bacteria is associated with the enormous taxonomic and functional diversity of microbial communities, which until recently

was impossible to study. Modern methods of determining the functional role of different bacterial taxa will help to solve the most important questions in microbial ecology, such as the relationship between genetic and functional diversity and the relative contribution of the taxonomic and functional diversity to ecosystem stability.

Thus, one of the best solutions to determine biogeochemical functions of bacterial communities is cultivating water bacteria in experimental mesocosms with nutrient additives followed by molecular genetic identification of species that consume this nutrient. It is obvious that the methods used should give the information about the abundance dynamics of the studied populations. As mentioned before, the dynamics of populations of individual species can be tracked by community fingerprinting. We used mesocosm cultivation with additives of tested substances (amino acids) and subsequent analysis of fingerprints based on DGGE as a method of experimental study of reservoir planktonic bacteria.

Aims of the Thesis

1) To study the bacterial biodiversity of the Yenisei River by next-generation sequencing.

2) To identify bacterioplankton consuming a certain spectrum of amino acids by molecular genetic methods: Bugach reservoir case study.

3) To study the seasonal dynamics of species composition of bacterial response to the addition of various amino acids.

Bacterial biodiversity of the Yenisei River

1. Brief limnological description of the Yenisei River

Water samples were collected from the Yenisei River (Fig. 1), which is the longest watercourse (4803 km) and has the greatest discharge (636 km^3 yr^{-1}) of all the rivers flowing into the Arctic Ocean (Amon et al., 2012).

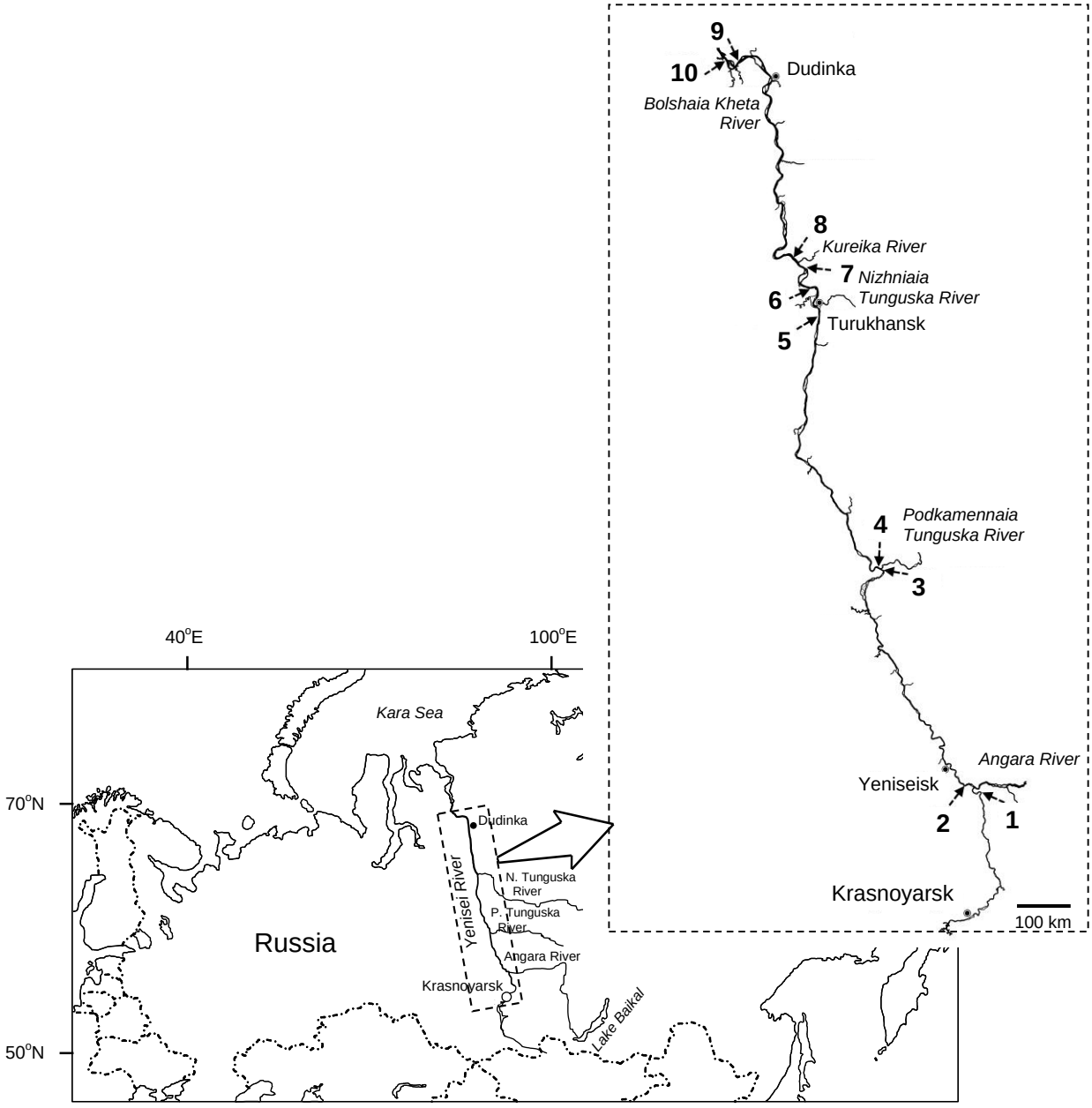

Figure 1. Map of the Yenisei River. Sampling transect locations are indicated by arrows and labelled with transect numbers.

The Yenisei is also the largest Arctic river in terms of discharge of dissolved and particulate organic carbon – $4.5 \cdot 10^{12}$ g yr^{-1} and $0.2 \cdot 10^{12}$ g yr^{-1}, respectively (Dittmar, Kattner, 2003). Its main hydrochemical features are low turbidity (<100 mg L^{-1}), high oxygen content (c. 100% saturation), and hardness of 21 – 23 mg L^{-1} Ca^{2+}. The concentrations of inorganic nutrients, PO_4-P and NH_4-N, are 0 – 0.1 and 0.3 – 1.6 mg L^{-1}, respectively (Gladyshev *et al.*, 1993). The water catchment area of the Yenisei River is asymmetric: its eastern part is 5.5 times bigger than its western part (Dryukker, Petrova, 1988).

Geographically, the riverbed of the Yenisei is divided into 3 sections: 1) its upper part (from the source of the Bolshoy Yenisei River to the mouth of the Angara River), 2) medium (from the mouth of the Angara River to the mouth of the Lower Tunguska River), 3) lower (from the mouth of the Lower Tunguska to the inflow in the Yenisei Gulf) (Galaziy et al., 1993). In the lower part of the upper section, the Yenisei passes the Yeniseiskii Kryage Mountains. The banks in this region are rocky and covered with taiga. The character of the river here is mountain-like, that is it has a high flow speed 1-3 m s^{-1}, and considerable depths alternate with rapids. Sample transects 1 and 2 are situated here (Fig. 1). The middle and lower stream of the river passes the West-Siberian Plain and the region of permafrost. The Yenisei has the features of a plain river and taiga is gradually replaced by forest-tundra on the banks. Sample transects 3-10 are situated here.

The first studies on bacteria of the Yenisei River were conducted by the research team headed by V. Dryukker in the 1970s (Dryukker, Petrova, 1988). The spatial distribution and dynamics of the total number of aquatic bacteria as well as proportion of mesophilic and heterotrophic microorganisms were studied along the entire length of the river from its source to its estuary. Various physiological groups of cultured bacteria were characterized, bacterial biomass and generation time was determined. In the area downstream of the Krasnoyarsk Reservoir the authors observed significant fluctuations in the total number of bacteria (1-4 million cells ml^{-1}) at different times and sampling stations. Also, downstream of the village of Korkino the bacterial population was not evenly distributed across the transect. The authors attributed the spatial distribution of bacterial numbers in the lower reach of the upper Yenisei to the influence of anthropogenic factors. In the middle section of the Yenisei River, the total number of bacteria was low in all seasons. In the lower section, the number of bacteria increased downstream from the mouth of the Nizhnyaya Tunguska to the Yenisei Gulf. In 1974, the highest rate of bacterial reproduction – from the Krasnoyarsk Reservoir to Korkino – was in

March, the lowest – in August. At the section from the mouth of the Angara River to the mouth of the Nizhnyaya Tunguska, the maximum multiplication rate was in July, and the minimum was observed in September. The authors concluded that the doubling time of bacterial numbers could give an idea on the intensity of the processes of self-purification in the river and on the impact of wastewater on the ecosystem of the watercourse. The number of bacteria in the Yenisei River was later repeatedly studied by different authors (Aponasenko et al., 2010; Meon, Amon, 2004; Kopylov et al., 2012), and varied from 1 to 10 million cells ml^{-1}.

The first study of water bacteria of the Yenisei River using molecular genetic methods have been carried out in the mid-2000s by Crump et al. (2009). The water samples were collected in the area of Dudinka, which is close to the river estuary. DGGE profiles of bacterial communities were obtained in different periods of two vegetation seasons. Nucleotide sequences of 80 clones of bacterial 16S rRNA gene were identified by Sanger sequencing (Crump et al., 2009).

2. Materials and methods

2.1 Sampling

Samples were taken during a research cruise at the section from 58°N to 69°N in June 2012, that is the period of the highest monthly water discharge (Gebhardt et al., 2004) (Fig. 1). Ten sampling transects were located downstream and upstream of inflows of major tributaries (Table 1). At each transect, three sample sites were established: near the left bank, right bank and in the mainstream. Samples were taken from surface to bottom by a large-volume low-pressure pump, just like in a similar study of Fortunato et al. (2012). Depth-integrated sampling in the Yenisei River is conventional practice (Crump et al. 2009; Holmes et al., 2012), because there are no significant depth-dependent differences of measured ecological parameters (e.g. Gebhardt et al., 2004; Hessen et al., 2010). At the first two transects, the temperature was measured with Long-Stem Thermometer, F/C, 8, (Cole-Parmer, USA), at all other stations by YSI 6600v2 probe (YSI, USA).

Immediately after sampling, 1 L samples, pooled from surface to bottom, were filtered through a 0.22 μm acetate cellulose membrane (Vladipor, Russia) and kept at -20°C until further laboratory analysis. To calculate the number of bacterial cells, 4.8 mL of water was fixed with 0.2 ml 50% glutaraldehyde and stored at 4°C.

Table 1. Sampling stations at the Yenisei River in June 2012

Date	Sampling transect	Samling station	Coordinates	
			Latitude	Longitude
12.06.12	**Transect 1** Above the Angara River	Centre	58°00.801'	93°12.667'
		Right bank	58°00.894'	93°12.767'
		Left bank	58°00.727'	93°12.338'
13.06.12	**Transect 2** Below the Angara River	Mainstream	58°07.586'	92°42.270'
		Right bank	58°07.604'	92°43.190'
		Left bank	58°07.115'	92°42.488'
17.06.12	**Transect 3** Above the Podkamennaya Tunguska River	Mainstream	61°34.349'	90°09.467'
		Right bank	61°34.267'	90°11.310'
		Left bank	61°33.956'	90°10.254'
	Transect 4 Below the Podkamennaya Tunguska River	Mainstream	61°36'46.62"	90° 0'3.06"
		Right bank	61°37.194'	90°01.350'
		Left bank	61°36.266'	90°00.575'
20.06.12	**Transect 5** Above the Nizhnyaya Tunguska River	Mainstream	65°35.662'	88°02.997'
		Right bank	65°35.079'	88°03.665'
		Left bank	65°34.461'	88°00.991'
22.06.12	**Transect 6** Below the Nizhnyaya Tunguska River	Mainstream	66°05.124'	87°47.341'
		Right bank	66°05.561'	87°45.412'
		Left bank	66°04.563'	87°49.140'
23.06.12	**Transect 7** Above the Kurejka River	Mainstream	66°25.392'	87°18.337'
		Right bank	66°25.770'	87°19.772'
		Left bank	66°25.118'	87°17.322'
24.06.12	**Transect 8** Below the Kurejka River	Mainstream	66°32.750'	87°01.940'
		Right bank	66°33.368'	87°03.163'
		Left bank	66°32.451'	86°59.461'
28.06.12	**Transect 9** Below the Bolshaya Kheta River and the Malaya Kheta River	Mainstream	69°38.644'	84°32.760'
		Right bank	69°39.448'	84°32.544'
		Left bank	69°38.412'	84°34.207'
27.06.12	**Transect 10** Above the Bolshaya Kheta River and the Malaya Kheta River	Mainstream	69°43.368'	84°00.114'
		Right bank	69°43.510'	84°00.358'
		Left bank	69°42.923'	84°04.111'

2.2 Sequencing

Frozen filter membranes with collected bacterial cells were used for DNA extraction and purification with the GeneJET Genomic DNA Purification Kit (Thermo Scientific, USA) according to the Gram-Positive Bacteria Genomic DNA Purification Protocol. Although reading short sequences allows comparison and identification of known species, longer sequences are more desirable for a more precise taxonomic assignment of unknown bacteria. A variable region surrounded by conservative fragments should be chosen for sequencing (Baker et al., 2003; Wang, Qian, 2009). V3 and V4 hypervariable regions of the 16S rDNA were amplified with the universal primers 343F (5' – CTCCTACGGRRSGCAGCAG) and 806R (5' - GGACTACNVGGGTWTCTAAT). PCR reactions of 30 µl contained 0.67 µM of each primer, 0.7 µL of Herculase II Fusion DNA Polymerase (Agilent Technologies, USA), 6 µL of 5× Herculase II Reaction Buffer containing $MgCl_2$ in a final concentration of 2 mM, 0.33 µM of dNTPs and 2 µL of DNA template. The reaction mixture for two samples also contained 10 µg of bovine serum albumin to eliminate the effect of inhibitors. PCR was conducted with a MyCycler Thermal Cycler (Bio-RAD, USA) and included the following steps: initial denaturation at 95^{o}C, 2 min, followed by six cycles each at 95^{o}C for 20 s, 48^{o}C at 30 s and 68^{o}C for 2 min; and then 26 cycles each at 95^{o}C for 15 s; 52^{o}C for 25 s; 68^{o}C for 2 min; and final polymerisation at 72^{o}C for 5 min. The PCR product was confirmed by electrophoresis.

A 1-µL aliquot of PCR product was used as a template for amplicon library construction using barcoded primers 343F and 806R (Caporaso et al., 2012):

343F – 5' – AATGATACGGCGACCACCGAGATCTACAC xxxxxxxx AGTCAGTCAG GT CTCCTACGGRRSGCAGCA

806R – 5' – CAAGCAGAAGACGGCATACGAGAT xxxxxxx ACGTACGTACG CC cGGACTACNVGGGTWTCTAAT

Thus, DNA was amplified twice as recommended in Berry et al. (2011). Environmental DNA often is not amplified sufficiently, which is why complementary primers were used without any barcodes for the first round of amplification. The annealing temperature was very low for the designated pair of primers so that the fragments that did not completely match the primers would not be lost. In such conditions, extra overhangs lead to nonspecific products, and in some cases the reaction does not occur because the annealing temperature of the primers with

barcodes and adaptors is high. The second PCR is aimed to amplify DNA fragments with barcodes and adaptors.

For each sample a unique pair of barcoded primers with Illumina adaptors was used. A 25-µL PCR reaction mixture contained 0.2 µM of each primer, 7% of dimethylsulfoxide, 0.2 µL of Herculase II Fusion DNA Polymerase (Agilent Technologies), 5 µL of 5× Herculase II Reaction Buffer containing $MgCl_2$ in a final concentration 2 mM and 0.8 µM of dNTPs. The samples were amplified using an initial denaturing step of 2 min at 94°C, followed by five cycles of 10 s denaturation at 94°C, 10 s annealing at 50°C, 30 s elongation at 72°C, then 25 cycles under the same conditions except that the annealing temperature increased to 53°C, and a final elongation for 3 min at 72°C. The PCR products were run on agarose gel and purified using the GeneJET Gel Extraction Kit (Thermo Scientific).

Thirty samples were sequenced using Illumina MiSeq platform with MiSeq reagent kit v.2 in the Laboratory of Evolutionary Genomics at Moscow State University. Sequencing and preliminary quality filtering was done according to Caporaso et al. (2011).

2.3 Processing of sequence data

Paired-end reads were joined together with PANDAseq (Masella et al., 2012). A total of 487228 16S rRNA sequence reads were filtered, denoised and processed using QIIME v. 1.7.0 (Caporaso et al., 2010). The reads were quality-checked using the built-in USEARCH v5.2.236 analysis tool (Edgar, 2010). The reads were first sorted from longest to shortest. USEARCH was then used to cluster the reads together into the groups in which each sequence was an exact match to a portion of the seed sequence for each cluster. The sequences were again sorted from longest to shortest, and USEARCH clustered the sequences at a 97% similarity. Chimera checking and filtering was done *de novo* with UCHIME and against a reference Gold database (http://drive5.com/uchime/gold.fa). Chimera-free consensus sequences were sorted by abundance, and any cluster containing fewer than four member sequences was removed from consideration, a conventional default setting in QIIME. After quality filtering, denoising and chimera checking, the remaining 257714 reads were clustered into 3226 OTUs. The representative sequence for each OTU was queried against the Greengenes database version 13_5 of high-quality sequences (DeSantis *et al.*, 2006; McDonald *et al.*, 2012) by UCLUST v1.2.22q. OTUs classified as chloroplasts (n = 204) were discarded.

Alpha-diversity calculations were performed at the best taxonomic level available. Individual samples (n = 30) were rarefied to the lowest number of reads in a sample (4460), with 10 iterations per sample using QIIME. Shannon's diversity index was calculated conventionally (Schultz et al., 2013).

Beta-diversity was calculated using the Bray-Curtis similarity coefficient (Schultz et al., 2013). A distance matrix for all samples was made using QIIME and 3D and 2D plots were visualized by EMPeror (Vázquez-Baeza et al., 2013) and the STATISTICA software package, version 9 (StatSoft Inc., USA). STATISTICA was also used to carry out multidimensional scaling (MDS) and calculate Fisher's least significant difference (LSD) *post hoc* test after ANOVA. In addition, the software Community Analysis Package, version 5.2.1.448 (Pisces Conservation Ltd, UK) was used to calculate ANOSIM and SIMPER.

2.4 Bacterial cell counting

In the laboratory, 0.5-mL samples were stained with the fluorescent dye DAPI (4 ', 6-diamidino-2-phenylindole dihydrochloride). After staining, the sample was filtered through nuclear membrane filters pre-painted with Sudan black B, with a pore size of 0.22 μm (JINR, Russia). The same method of staining and bacterial counting was used in other studies of the Yenisey (Meon, Amon, 2004; Kopylov et al., 2012). Cells were photographed using the photographic documentation system of epifluorescene microscope 40 Axioscope (Zeiss, Germany). To calculate the number of cells in the frame, each image was processed by ImageJ v. 1.44 p software (Wayne Rasband National Institutes of Health, USA, http://imagej.nih.gov/ij). To determine the number of cells on the entire filter surface, the average number of cells per frame was multiplied by a coefficient linking the frame area with the total area of the filter.

3. Results and discussion

3.1 Cell counts

The number of planktonic bacteria in the lower transects 9 and 10 was 9.7 and 5,6 $\cdot 10^6$ cells mL^{-1} respectively and corresponded to the results of Kopylov et al. (2012), although other authors give smaller values of $\approx 1.5 \cdot 10^6$ cells mL^{-1} (Meon, Amon, 2004), or about 2-4 $\cdot 10^6$ cells mL^{-1} (Aponasenko et al., 2010). Overall, the bacterial counts did not vary considerably

along the studied section of the Yenisei (Fig. 2) and were close to those of other rivers (Ellis et al., 2012; Vargas et al., 2013).

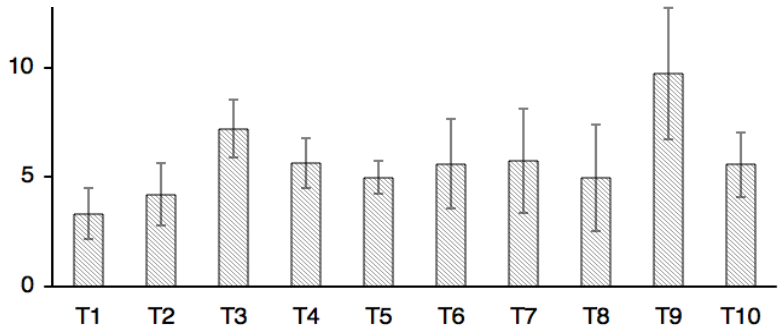

Figure 2. Bacterial counts in the Yenisei River, June 2012

3.2 Alpha-diversity

In all samples, 240500 reads were obtained and 3022 OTUs (genetic "species") of prokaryotes were identified (Table 2). The pair of oligonucleotide primers used for the library of amplicons was aimed at amplification of 16S rRNA genes of bacteria allowing their identification down to genera rank (Klindworth et al., 2013), so archaeal sequences were accidental. Since there were only two OTUs of archaea and they had comparatively negligible abundance (Table 1), for simplicity it was decided to use the term "bacteria" for the studied prokaryotes. The identified OTUs belonged to 17 known phyla, and also to 4 genera *incertae sedis* (Table 1). In addition, 552 sequences were not identified; those were not found in conventional databases. Among the identified phyla, Proteobacteria, Actinobacteria, Bacteroidetes and Verrucomicrobia had comparatively high numbers of unique OTUs, and Proteobacteria and Actinobacteria comprised the highest numbers of sequences (Table 1). The DNA sequence information is deposited to the Sequence Read Archive, accession number SRP036054.

At all the sampling sites, Actinobacteria and Proteobacteria were the dominant phyla in terms of their relative abundance (Fig. 3). At transects 1 and 2, Firmicutes had comparatively high abundance, and at transects 3-5 Bacteroidetes and Cyanobacteria were abundant phyla (Fig. 3). The average percentages of Firmicutes at transects 1-2 (6.3±0.9%) were significantly higher than at transects 3-5 (1.2±0.2%, p = 0.0000) and 6-10 (0.8±0.1%, p = 0.0000), according to Fisher's LSD *post hoc* test. In turn, the average percentages of Cyanobacteria at transects 3-5 (5.1±0.9%) were significantly higher (LSD test) than at transects 1-2 (1.3±0.4%, p = 0.0001) and

6-10 (1.1±0.2%, p = 0.0000). Average percentages of Verrucomicrobia decreased from 6.9±1.2% at transects 1-2 to 4.6±0.6% at transects 3-5, and to 2.7±0.3% at transects 6-10 (the averages all differed significantly according to LSD test, p = 0.0180, p = 0.0000 and p = 0.0195, respectively).

Table 2. List of phyla of prokaryotes, bacteria and archaea (Euryarchaeota), found in studied samples from the Yenisei River in June 2012, their number of operational taxonomic units (OTU) and number of sequences (N)

No.	Phyla	OTU	N
1	Proteobacteria	914	84152
2	Actinobacteria	837	109953
3	Unknown bacteria	552	9579
4	Bacteroidetes	200	9313
5	Verrucomicrobia	162	10029
6	Firmicutes	60	4869
7	Planctomycetes	58	4835
8	TM7 genera *incertae sedis*	58	553
9	Cyanobacteria	55	5520
10	Acidobacteria	37	373
11	Chlamydiae	32	286
12	OD1 genera *incertae sedis*	13	456
13	Armatimonadetes	9	171
14	Chloroflexi	7	32
15	Gemmatimonadetes	7	173
16	SR1 genera *incertae sedis*	5	42
17	Deinococcus-Thermus	4	64
18	Fusobacteria	4	48
19	OP11 genera *incertae sedis*	4	27
20	Euryarchaeota	2	10
21	Chlorobi	1	6
22	Nitrospirae	1	9
	Total	**3022**	**240500**

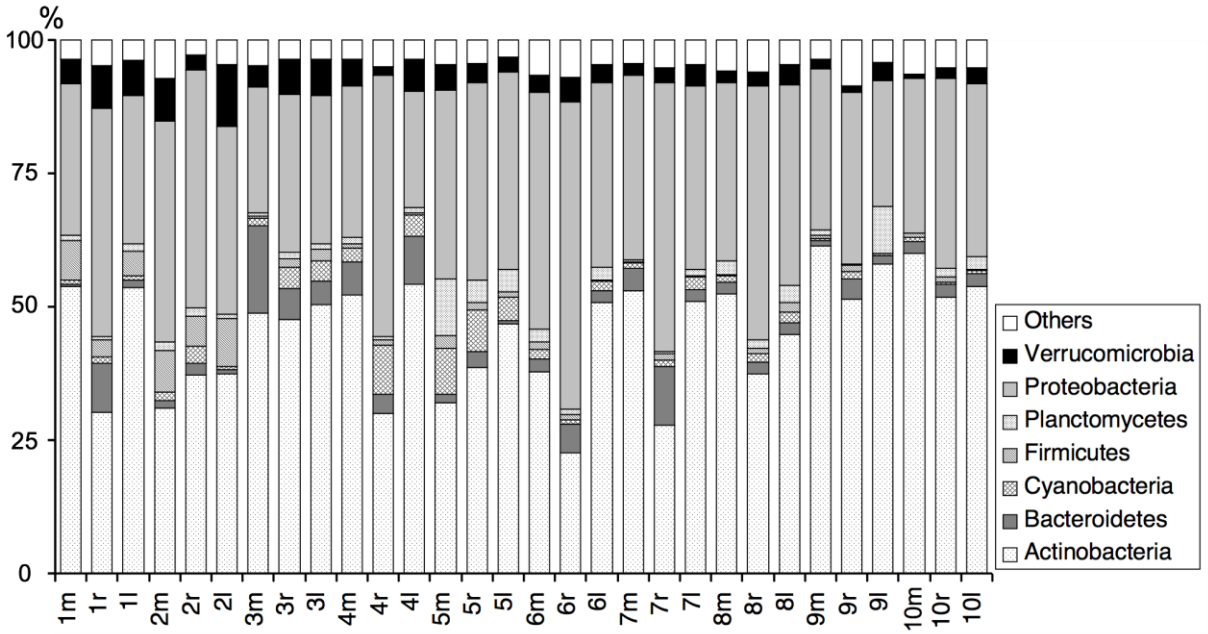

Figure 3. Percent abundances of the quantitatively prominent bacterial phyla in the Yenisei River, June, 2012. See Fig. 1 for sample site numbers; m – mainstream, r – right bank, l – left bank

Regarding OTU richness, sampling sites 2l and 4r had rarefaction curves which rose more slowly than the others (Fig. 4), that is, sites 2l and 4r had the lowest OTU abundance in the assemblages and their OTU abundance distributions were highly uneven. In turn, rarefaction curves of sampling sites 6m and 6r rose more rapidly, that is sites 6m and 6r had more OTUs in the assemblages and a more even OTU abundance distribution compared with other sites (Fig. 4). Indeed, sites 2l and 4r had the lowest values of Shannon index of α-diversity, and the sites 6m and 6r had the highest values of this index (Table 3).

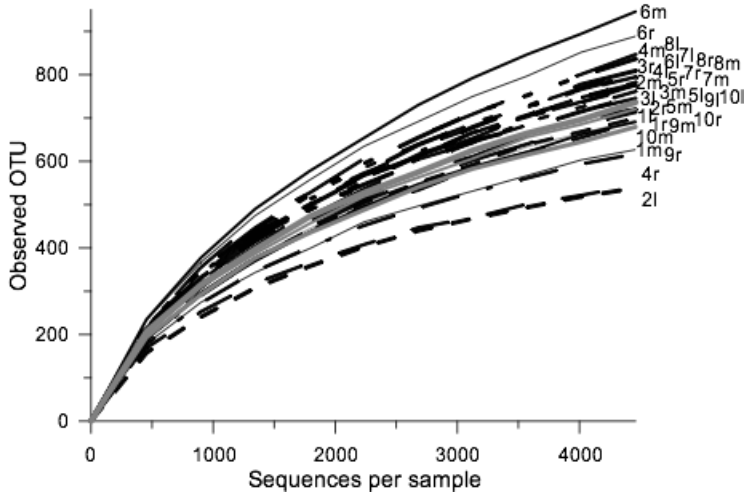

Figure 4. Rarefaction curves for bacterial richness from the Yenisei River. See Fig. 1 for sample site numbers; m – mainstream, r – right bank, l – left bank; OTUs – operational taxonomic units

Table 3. Number of operational taxonomic units (OTUs) and values of diversity by Shannon index *(H*) of bacterial communities from the Yenisei River, June 2012. See Fig. 1 for sample site numbers; m – mainstream, r – right bank, l – left bank

Sample	Reads	OTUs	*H*
1m	7780	776	7.18
1r	5698	767	7.55
1l	9708	923	7.43
2m	8052	968	7.82
2r	8967	908	7.51
2l	8673	692	6.89
3m	9494	1000	7.91
3r	8166	1016	7.92
3l	8161	930	7.92
4m	9859	1146	8.04
4r	5690	600	7.07
4l	8819	1009	7.93
5m	8579	939	7.48
5r	6824	892	7.66
5l	8409	927	7.66
6m	8159	1192	8.25
6r	8455	1115	8.17
6l	10410	1101	7.89
7m	9766	1052	7.65
7r	7991	964	7.85
7l	7954	1020	7.87
8m	7914	963	7.65
8r	4466	795	7.86
8l	5066	878	7.91
9m	8213	877	7.44
9r	8256	778	7.16
9l	7631	913	7.66
10m	7321	822	7.46
10r	8141	909	7.57
10l	7578	896	7.68

Rivers are one-way flowing systems which accumulate more and more nutrients washed in from surrounding soils. Intuitively, the diversity of bacteria should also increase downstream (Besemer et al., 2013). However, in the Yenisei River we did not find such a monotonous increase of the alpha-diversity of bacteria. In contrast, we found the highest alpha-diversity values approximately in the middle of the studied river section, at transect 6 (sampling sites near the right bank and in the mainstream). Indeed, rarefaction curves 6m and 6r rose more rapidly than others, and the bacterial assemblages therefore had more OTUs and more even distribution of OTU abundance than those in the other sites (Gotelli, Colwell, 2011). Water temperature also reached maximum values of about 20°C in the middle of the studied section.

It is worth noting that at many transects in the Yenisei River there were conspicuous differences in ecological parameters between its left and right banks, including those of bacterial assemblages. These differences were caused by large right-side tributaries, such as the Angara River and both Tunguska Rivers. After inflows, river waters flow several kilometres practically without mixing, and this phenomenon is conspicuous even to the naked eye due to different water colors and/or transparency. The Yenisei River is situated at the interface of ecoregions: West Siberian taiga and Yamal-Gydan tundra are on the left bank, East Siberian taiga and Taimyr Central Siberian tundra are on the right bank (Olson et al., 2001). The highest percentage of Cyanobacteria was observed in the middle section of the Yenisei. In the upper section, the photosynthesis of phytoplankton, that is Cyanobacteria, was probably inhibited by too high flow speed and concomitant turbulence. In the lower section, the phytoplankton might be inhibited by a low transparency of water because of its brown colour.

3.3 Beta-diversity

Bray-Curtis similarity coefficients for beta-diversity of the bacterial communities are given in Table 4. In general, the similarity decreased down the river from transect 1 to transect 10 (Table 4). The results of MDS of the similarity coefficients matrix (Table 4) are given in Fig. 5. Three groups can be distinguished in the first two meaningful dimensions: sampling sites of transects 1-2, of transects 3-5 and of transects 6-10 (Fig. 5). The ANOSIM test confirmed the significance of the differences between the groups: sample statistic (global R) = 0.583, P = 0.001. Thus, there were three distinct sections of the river with different OTU composition: section I (transects 1-2), section II (transects 3-5) and section III (transects 6-10).

Ten OTUs from each section with the highest average percentage (relative abundance) are listed in Table 5. As the three lists of top-ten OTUs partly overlapped, there are only 20 OTUs in Table 5. OTU 1297 *Ilumatobacter* were the dominant bacteria in sections I and II, and subdominant bacteria in section III, and contributed the most to the dissimilarity between the sections after SIMPER test (Table 5). Section I had significantly higher average percentage of OTU 8 Microbacteriaceae, OTU 13 *Rhizobium*, OTU 15 *Rhodobacter*, OTU 25 *Arthrobacter*, OTU 4 Actinomycetales, OTU 47 Microbacteriaceae, OTU 12 Actinobacteria and OTU 16 Sphingomonadaceae, but significantly lower percentage of OTU 671 *Ilumatobacter* than in sections II and III (Table 5). In section II, the highest percentages of OTU 18 Chitinophagaceae and OTU 21 *GpIIa* compared to those in sections I and III occurred (Table 5). Section III had the highest percentages of OTU 2179 Actinomycetales (the dominant bacteria of this section), OTU 2 Rhizobiales, OTU 2588 Actinomycetales, OTU 5 *Polynucleobacter* and OTU 17 *Acinetobacter* (Table 5).

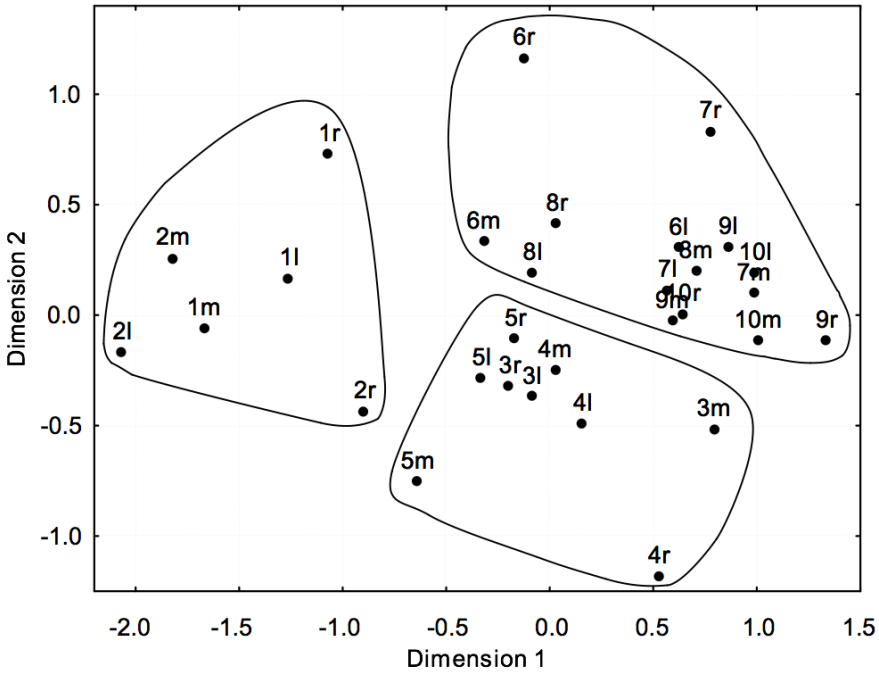

Figure 5. Results of MDS of the degree of similarity among samples between bacterial communities of the Yenisei River, based on Bray-Curtis community similarity, calculated as relative abundance of operational taxonomic units (OTUs). Sampling site abbreviations: numerals means transect number (see Fig. 1); m – mainstream, r – right bank, l – left bank.

Table 4. Bray-Curtis similarity coefficients for bacterial β-diversity in the Yenisei River in June, 2012. Identical communities have a value of 1.00. Sampling site abbreviations: numeral means transect number (see Fig. 1); m – mainstream, r – right bank, l – left bank

	1m	1r	1l	2m	2r	2l	3m	3r	3l	4m	4r	4l	5m	5r	5l	6m	6r	6l	7m	7r	7l	8m	8r	8l	9m	9r	9l	10m	10r
1r	0.46	1.00																											
1l	0.70	0.56	1.00																										
2m	0.47	0.40	0.44	1.00																									
2r	0.42	0.40	0.45	0.53	1.00																								
2l	0.51	0.42	0.49	0.58	0.47	1.00																							
3m	0.24	0.40	0.33	0.21	0.34	0.20	1.00																						
3r	0.39	0.44	0.47	0.41	0.55	0.38	0.53	1.00																					
3l	0.40	0.44	0.47	0.36	0.51	0.35	0.55	0.69	1.00																				
4m	0.38	0.43	0.47	0.35	0.48	0.33	0.59	0.71	0.69	1.00																			
4r	0.23	0.33	0.28	0.24	0.36	0.18	0.42	0.44	0.47	0.44	1.00																		
4l	0.33	0.40	0.42	0.32	0.43	0.30	0.63	0.65	0.67	0.70	0.45	1.00																	
5m	0.36	0.32	0.37	0.41	0.47	0.34	0.31	0.53	0.46	0.50	0.30	0.44	1.00																
5r	0.36	0.38	0.41	0.36	0.49	0.31	0.44	0.60	0.56	0.60	0.44	0.54	0.64	1.00															
5l	0.33	0.31	0.37	0.39	0.55	0.35	0.40	0.57	0.54	0.56	0.36	0.51	0.55	0.59	1.00														
6m	0.41	0.39	0.41	0.44	0.49	0.34	0.38	0.53	0.52	0.52	0.38	0.46	0.54	0.59	0.55	1.00													
6r	0.31	0.34	0.32	0.40	0.40	0.27	0.32	0.38	0.40	0.36	0.46	0.32	0.33	0.42	0.36	0.55	1.00												
6l	0.27	0.33	0.32	0.22	0.35	0.19	0.50	0.49	0.50	0.54	0.42	0.50	0.39	0.50	0.51	0.48	0.39	1.00											
7m	0.23	0.33	0.29	0.21	0.31	0.17	0.55	0.45	0.47	0.48	0.43	0.49	0.30	0.43	0.39	0.43	0.40	0.60	1.00										
7r	0.22	0.34	0.26	0.23	0.31	0.18	0.51	0.40	0.42	0.42	0.49	0.41	0.29	0.41	0.35	0.45	0.52	0.51	0.55	1.00									
7l	0.28	0.35	0.33	0.25	0.36	0.22	0.54	0.51	0.51	0.54	0.39	0.53	0.41	0.53	0.52	0.50	0.39	0.65	0.65	0.52	1.00								
8m	0.25	0.32	0.31	0.22	0.35	0.18	0.53	0.48	0.50	0.52	0.47	0.50	0.37	0.50	0.45	0.51	0.44	0.67	0.69	0.55	0.66	1.00							
8r	0.36	0.36	0.39	0.36	0.45	0.29	0.41	0.52	0.52	0.51	0.45	0.47	0.47	0.56	0.53	0.61	0.56	0.55	0.49	0.51	0.55	0.58	1.00						
8l	0.36	0.36	0.41	0.38	0.44	0.31	0.41	0.54	0.51	0.56	0.33	0.50	0.55	0.59	0.59	0.59	0.42	0.53	0.45	0.43	0.59	0.50	0.62	1.00					
9m	0.33	0.35	0.40	0.25	0.37	0.24	0.53	0.49	0.49	0.54	0.37	0.51	0.41	0.52	0.49	0.47	0.36	0.58	0.61	0.46	0.65	0.62	0.53	0.56	1.00				
9r	0.21	0.31	0.28	0.21	0.29	0.21	0.50	0.38	0.38	0.42	0.33	0.44	0.28	0.39	0.34	0.35	0.32	0.43	0.61	0.45	0.53	0.52	0.39	0.40	0.59	1.00			
9l	0.27	0.27	0.33	0.18	0.31	0.17	0.48	0.44	0.45	0.50	0.32	0.48	0.38	0.46	0.48	0.44	0.31	0.65	0.58	0.43	0.63	0.63	0.49	0.52	0.62	0.44	1.00		
10m	0.25	0.34	0.32	0.23	0.32	0.21	0.53	0.43	0.44	0.47	0.36	0.48	0.33	0.44	0.40	0.40	0.34	0.50	0.61	0.47	0.58	0.58	0.46	0.46	0.68	0.70	0.52	1.00	
10r	0.28	0.34	0.35	0.27	0.38	0.25	0.52	0.50	0.48	0.53	0.33	0.50	0.43	0.53	0.50	0.47	0.36	0.56	0.58	0.49	0.64	0.60	0.54	0.58	0.71	0.60	0.61	0.67	1.00
10l	0.23	0.29	0.28	0.17	0.31	0.14	0.51	0.43	0.46	0.48	0.42	0.48	0.30	0.42	0.42	0.41	0.36	0.64	0.66	0.51	0.64	0.67	0.48	0.45	0.62	0.52	0.65	0.60	0.61

Table 5. Average (± SE) of percent abundances of the quantitatively prominent (top 10 in each section) OTUs in the three sections of the Yenisei River, June 2012: section I (transects 1-2), section II (transects 3-5) and section III (transects 6-10). Dis – contribution (%) to average dissimilarity between the sections after SIMPER. Means labeled with the same letter are not significantly different at $P < 0.05$ after Fisher's LSD *post hoc* test for one-way ANOVA. When ANOVA is insignificant, letter labels are absent

OTU	Class	Order	Family	Genus	Section I	Section II	Section III	Dis
1297	Actinobacteria	Acidimicrobiales	Acidimicrobiaceae	*Ilumatobacter*	5,80 ± 1,49	3,99 ± 0,59	4,57 ± 0,48	2,66
2	α-Proteobacteria	Rhizobiales			4,25 ± 1,18^{A}	3,87 ± 0,76^{A}	1,62 ± 0,32^{B}	2,51
2179	Actinobacteria	Actinomycetales			0,34 ± 0,16^{A}	2,69 ± 0,46^{A}	5,42 ± 0,73^{B}	1,98
18	Sphingobacteria	Sphingobacteriales	Chitinophagaceae		0,31 ± 0,18^{A}	1,95 ± 0,53^{B}	1,26 ± 0,27^{A}	1,88
13	α-Proteobacteria	Rhizobiales	Rhizobiaceae	*Rhizobium*	2,79 ± 1,09^{A}	0,67 ± 0,15^{B}	0,73 ± 0,20^{B}	1,64
8	Actinobacteria	Actinomycetales	Microbacteriaceae		2,89 ± 0,68^{A}	0,65 ± 0,13^{B}	0,29 ± 0,08^{B}	1,47
10	β-Proteobacteria	Burkholderiales	Comamonadaceae	*Limnohabitans*	1,01 ± 0,60	1,94 ± 0,58	1,96 ± 0,29	1,40
15	α-Proteobacteria	Rhodobacterales	Rhodobacteraceae	*Rhodobacter*	2,73 ± 0,21^{A}	0,90 ± 0,14^{B}	0,17 ± 0,04^{C}	1,20
25	Actinobacteria	Actinomycetales	Micrococcaceae	*Arthrobacter*	1,91 ± 0,57^{A}	0,25 ± 0,04^{B}	0,34 ± 0,09^{B}	1,13
21	Cyanobacteria		Family II	*GpIIa*	0,41 ± 0,13^{A}	1,93 ± 0,46^{B}	0,32 ± 0,07^{A}	1,13
2588	Actinobacteria	Actinomycetales			0,69 ± 0,33^{A}	1,60 ± 0,37^{A}	2,23 ± 0,27^{B}	1,12
671	Actinobacteria	Acidimicrobiales	Acidimicrobiaceae	*Ilumatobacter*	0,18 ± 0,09^{A}	1,59 ± 0,32^{B}	2,32 ± 0,25^{B}	1,05
4	Actinobacteria	Actinomycetales			2,88 ± 0,52^{A}	1,83 ± 0,19^{B}	0,80 ± 0,12^{C}	1,02
6	α-Proteobacteria	Rhizobiales	Methylocystaceae	*Methylocystis*	1,71 ± 0,46	2,44 ± 0,31	1,61 ± 0,21	1,01
47	Actinobacteria	Actinomycetales	Microbacteriaceae		2,08 ± 0,41^{A}	0,62 ± 0,08^{B}	0,36 ± 0,05^{B}	0,96
12	Actinobacteria				1,76 ± 0,52^{A}	0,90 ± 0,19^{B}	0,88 ± 0,11^{B}	0,94
11	β-Proteobacteria	Burkholderiales	Alcaligenaceae		1,29 ± 0,09	2,27 ± 0,51	1,85 ± 0,24	0,94
16	α-Proteobacteria	Sphingomonadales	Sphingomonadaceae		1,79 ± 0,73^{A}	0,68 ± 0,13^{B}	0,33 ± 0,05^{B}	0,91
5	β-Proteobacteria	Burkholderiales	Burkholderiaceae	*Polynucleobacter*	1,76 ± 0,23^{A}	2,01 ± 0,51^{A}	3,53 ± 0,52^{B}	0,87
17	γ-Proteobacteria	Pseudomonadales	Moraxellaceae	*Acinetobacter*	0,10 ± 0,04^{A}	0,65 ± 0,36^{A}	2,19 ± 0,50^{B}	0,59

According to our data on beta-diversity of bacterial assemblages analysed by MDS and ANOSIM, the Yenisei River ecosystem can be subdivided into three sections: the upper (section I), middle (section II) and lower (section III). The upper section of the river (transects 1-2) was situated in the Yeniseiskii Kryage Mountains around the inflow of the Angara River. Bacterial assemblages of this section had significantly higher percentages of Firmicutes and Verrucomicrobia compared to other sections. However, the dominant specific OTUs in this section belonged to Actinobacteria and Proteobacteria. The dominant indicator taxa of section I, the percentage of which was significantly higher than those in sections II and III, were OTU 13 *Rhizobium*, OTU 15 *Rhodobacter* and OTU 25 *Arthrobacter*. Genus *Rhizobium* is a plant root nodule bacteria (Castagno et al., 2011), and the high percentage of this genus in the river water of the "mountain" section I was surprising. Species of *Rhodobacter* were found to increase their abundance in river biofilms after an addition of pesticides (Tien et al., 2013). The finding might explain the dominance of OTU 15 of this genus in section I which has a stony bottom, is covered with biofilms, and has undergone a weak anthropogenic pollution (Gladyshev et al., 2012), which nevertheless seems to be higher than that in significantly less populated sections II and III. The river *Arthrobacter* was known to degrade aromatic pollutants (Narancic et al. 2012), and we reported elsewhere the highest activity of phenol-degrading bacteria just in this 'mountain' section of the Yenisei River (Gladyshev et al., 1993). The abundant *Arthrobacter* may be responsible for the highest rates of degradation of phenolic compounds in section I. It is interesting to note, that different OTUs from the same genus, *Ilumatobacter*, had a different distribution between the river sections: OTU 1297 was the dominant bacteria in section I and had the highest percentage among all OTUs, while OTU 671 had significantly lower percentage in section I compared to sections II and III. Evidently, the two different species (strains) of this genus had different ecological features.

The highest percentage of Cyanobacteria was the main peculiarity of the middle section of the Yenisei River (transects 3-5). An intermediate percentage of Verrucomicrobia was characteristic of this section. The indicator taxa of section II were Cyanobacteria, namely OTU 21 *GpIIa*.

The lower section III (transects 6-10) was separated from the middle section by the inflow of the Nizhnyaya Tunguska River. Just after the inflow of this river, the water color changes dramatically from transparent to brown which might be explained by a high content of humic substances from the drained lands (Gladyshev et al., 1993). Thus, downstream the inflow of Nizhnyaya Tunguska (Fig. 1), the total organic carbon content in the Yenisei increased from c.

7 mg L^{-1} to c. 12 mg L^{-1} (Gladyshev et al., 1993). The dominant indicator taxa of section III was OTU 5 *Polynucleobacter*. This is a well-known cosmopolitan genus, which has been isolated from both lentic and lotic habitats all over the world (Ghai et al., 2011). Some of *Polynucleobacter* subclusters were especially abundant in waters with a high contribution of allochthonously derived dissolved organic matter, including soil humic substances (Watanabe et al., 2012). As mentioned above, a high concentration of dissolved organic matter, including allochthonous humic substances, was the characteristic of the lower section of the Yenisei River (Gladyshev et al., 1993), and this might cause the high percentage of *Polynucleobacter* in section III. The other indicator taxa of section III was OTU 17 *Acinetobacter*. Species of *Acinetobacter* were reported among copiotrophic river bacteria (Bhadra et al., 2007), and this might also correspond to the high content of organic matter in section III mentioned above.

Hence, beta-diversity of bacterial assemblages in the river is shaped by the surrounding landscape (biome). In the three sections of the Yenisei River, situated in the Yeniseiskii Kryage Mountains, West-Siberian Plain taiga and in forest-tundra and tundra, in a region of permafrost, respectively, three distinctly different bacterial assemblages occurred. Section II was evidently separated from section III by the input of a large tributary, Nizhnyaya Tunguska. Thus, one of the mechanisms by which the landscape might influence the bacterial diversity in the Yenisei River is via dispersal of different bacterial communities in tributaries arising from the different types of landscape.

Summary

It was found that the bacterial composition of the Yenisei includes more than 3000 OTE. Alpha diversity of bacterial communities was the highest in the middle of the studied area. Downstream, while mountain taiga turns into plain taiga and tundra into the permafrost region, the species composition of bacterial communities in the river water changed in accordance with the change of the biome, which is probably due to the influence of tributaries, carrying their own bacterial communities shaped by these biomes. Characteristics of the species composition of bacteria in the upper (mountain) section of the river were probably due to the high flow rates and a pebbly bottom which contributed to the development of biofilms and dominance of *Rhodobacter* associated with biofilms.

In addition, the upper section is potentially exposed to an anthropogenic load which may determine the high number of *Rhodobacter* and *Arthrobacter*, capable of degrading phenolic pollutants. In the middle portion, the conditions are favorable for growth (photosynthesis) of cyanobacteria: this section displays a relatively slow flow velocity compared to the upper section and high transparency of water compared to the lower section. High concentrations of the dissolved organic carbon (humic substances) in the water of the lower section is probably causing the dominance of *Polynucleobacter* and *Acinetobacter* which usually prevail in the river water with the high concentrations of allochthonous organic substances.

Thus, downstream the Yenisei River, from mountain taiga to plain taiga and forest-tundra in permafrost, the diversity of bacterial assemblages in the river water changed according to the landscape (biome) succession driven by tributary inputs. Different bacterial assemblages may vary in their impact on the carbon cycles in the river. Revealing a relation between the biodiversity (species composition) of river bacteria and their biogeochemical function will be the next important step to study the role of rivers in global carbon cycles.

Experimental study of amino acid consumption by bacteria of a eutrophic reservoir

Experimental mesocosms containing samples of natural bacterial communities and enriched with tested nutrients are a promising method to study consumption of various substances by individual species of water bacteria. It has been found that mesocosms maintained a natural plankton community, including phytoplankton, zooplankton and bacterioplankton, for at least a week (Gladyshev, 1992, Gladyshev, 1999).

In order to monitor changes in the composition of dominant species in the bacterial communities in mesocosms, the method for separating the amplified fragments of 16S ribosomal RNA by DGGE is often used (Martin et al., 2012).

Amino acids were selected as test additives for heterotrophic bacteria because these substances are excreted from growing and dying out phytoplankton and zooplankton in large quantities, and are one of the most preferred by bacteria organic components.

1 Materials and methods

1.1 Sampling for inoculation

Bacterial samples were taken in the coastal zone from the surface of the Bugach Reservoir. This small pond used for hatchery and recreational purposes is located in the northwestern part of the city of Krasnoyarsk (56° 03' N, 92° 43' E) (Gladyshev et al., 2007). The reservoir is formed by the Bugach River, a secondary tributary of the Yenisei River. The area of the reservoir is about 32 hectares. The average depth of the reservoir is about 2-3 m; the maximum depth near the dam is 7 m (Gladyshev et al., 2007). The reservoir is characterized by a weak thermal stratification, relatively low transparency of water (0.25 m to 0.8 m by Secchi disk), high pH (from 8.0 to 9.7) and a relatively high concentration of organic nitrogen and inorganic phosphorus (Kravchuk et al., 2011). The Bugach Reservoir is eutrophic with cyanobacterial blooms taking place in summer, and a relatively high content of dissolved organic substances including amino acids (Kalachova et al., 2004; Gladyshev et al., 2007; Kravchuk et al., 2011). Some of the amino acids detected in the Bugach Reservoir are lysine at concentration of 0.05 – 0.8 mg L^{1}, glycine 0.1 to 0.8 mg L^{-1}, arginine from 0.02 to 0.7 mg L^{-1} (Kalachova et al., 2004).

1.2 Experimental design

Experiments were conducted in laboratory mesocosms. Water samples from the reservoir containing natural plankton were inoculated in three mesocosms. Mesocosm had a form of glass cylinder 26 cm in diameter and 25 cm in height with a working volume of 10 liters, placed in the external circuit of cooling and thermal insulation. The temperature in the mesocosms was maintained by circulation cryothermostate Cryo KRIO-VT-11 (IPC SB RAS, Tomsk, Russia). The temperature in each mesocom corresponded to that of the reservoir measured at the time of sampling. Mesocosms were illuminated by fluorescent light with intensity 7.0 W m^{-2}. During the experiments, natural periodic lighting mode was maintained: photoperiod in the Experiments I, III, IV and V was 16 h light: 8 hours dark; in Experiment II, conducted in the second half of August, 14 hours light: 10 hours dark. Water in the mesocosms was mixed with a glass spatula once or twice a day.

Overall at different times five experiments with additives of amino acids were conducted (see Table 6). The first three experiments were carried out without the participation of the author but the molecular genetic analysis of the samples collected in the course of these experiments was partially carried out by the author.

Glycine, lysine and arginine selected for the experiments are characteristic substances in the total amino acid pool of the reservoir (Kalachova et al., 2004). However, the seasonal dynamics of concentrations of these amino acids in the reservoir was different; moreover, they came from different sources (Kalachova et al., 2004). To ensure the response of the bacterial community in the first three experiments, initially the amino acids were added in a 100-fold concentration compared to the natural level, and later the concentration was reduced and brought closer to natural level.

Table 6. List of mesocosm experiments with the additions of amino acids

No.	Date	Additions		
		Mesocosm 1	Mesocosm 2	Mesocosm 3
I	30 June – 6 July 2004	lysine 100 mg L^{-1}	control	glycine 100 mg L^{-1}
II	17 – 23 August 2005	lysine 100 mg L^{-1}	control	arginine 100 mg L^{-1}
III	17 – 23 May 2006	lysine 100 mg L^{-1}	control	glycine 100 mg L^{-1}
IV	31 July – 5 August 2009	lysine 100 mg L^{-1}	lysine 100 mg L^{-1}	lysine 10 mg L^{-1}
V	14 – 20 May 2013	lysine 100 mg L^{-1}	control	lysine 5 mg L^{-1}

1.3 Sampling during the experiments

The initial sample was taken just before filling the mesocosms. During the experiments, 100 mL of water were sampled daily from each mesocosm to analyze the bacterial composition by PCR-DGGE.

In Experiments III and V, 10-50 mL of water were taken daily from each experimental mesocosm to determine the dynamics of the added amino acids. Also at the beginning and at the end of Experiment V, 500 mL of water were selected to determine the pool of suspended and dissolved amino acids in water, and also amino acids in seston. In order to determine the concentration of suspended and dissolved amino acids, water sample was concentrated by evaporation in a water bath. To determine the total amino acids, 500 mL of seston sample volume was filtered through a membrane filter MFAS-OS-2 (Vladipor, pore size 0.45 µm) coated with hardened $BaSO_4$ to facilitate the separation of the precipitate. The filters were dried and the precipitate was separated from the filter. Dr A. Kolmakova determined the concentration of amino acids in water and seston of the mesocosms. For this, a dry precipitate was transferred to a thick-walled 50 mL glass vial, and 20 mL of 6N HCl were added. The vial was sealed, dry sediment hydrolysis was performed in an oven for 22 hours at 110°C. Then the sample was cooled and filtered and the acid was evaporated in a boiling water bath with the addition of a small amount of water to remove the traces of acid. The residue was dissolved in citrate buffer with pH 2.2. The concentration of amino acids was determined by HPLC chromatograph Knauer A0326V2 (Germany) equipped with Knauer A0992-13v1 column (Germany) 125 × 3 mm, ninhydrin reaction unit and UV detector 2500. The detection limit was 0.1 nmole.

1.4 DNA extraction

Bacterial biomass was concentrated from a volume of 100 mL on sterile 0.22-µm bacterial filters. Genomic DNA was extracted using the method of Boström et al. (2004). For this, membranes were cut into pieces, placed in 2-mL plastic sterile tubes, and incubated with 525 µL of lysis buffer (400 mM NaCl, 750 mM sucrose, 20 mM EDTA, 50 mM Tris-HCl pH 9.0) and 11 µL of lysozyme solution (1 mg mL^{-1} final concentration) for 30 min at 37°C. After that, 60 µL of sodium dodecyl sulfate (SDS, 1% final concentration) and 3 µL of proteinase K (final concentration 100 µg mL^{-1}) were added, and the samples were incubated at 55 °C for 12 hours. The lysis products were transferred to clean test tubes, filters were washed with 500 mL of TE

buffer (10 mM Tris-HCl pH 8.0, 2 mM EDTA), which was then added to the lysates. The DNA-containing solutions were mixed with 1/10 volume 3M sodium acetate, and the DNA was precipitated with 2.5 volumes 96% ethanol at -20°C for 1 h. The tubes were centrifuged at 20000 g and 4°C for 20 minutes. The precipitates were washed with 70% ethanol. After removal of ethanol, the precipitates were air-dried and dissolved in 100 µL of TE buffer. The quality and quantity of the isolated high molecular weight DNA was analyzed by electrophoresis in 1% agarose gel.

1.5 PCR-DGGE

16S rDNA fragments were amplified with the universal primers GC-341F (5'-CCT ACG GGA GGC AGC AG-3', with "GC-clamp" 5'-CGC CCG CCG CGC CCC GCG CCC GGC CCG CCG CCC CCG CCC C-3' at the 5'-end) and 926R (5'-CCG TCA ATT CA/CT TTG AGT TT-3') (Schauer et al., 2000). The PCR mixture was optimized for a given primer pair and contained 75 mM Tris-HCl (pH 8.8 at 25°C), 0.01% Tween 20, 20 mM $(NH_4)_2SO_4$, 2.5 mM $MgCl_2$, 2% dimethyl sulfoxide (DMSO), 10 mg BSA, 0.25 mM dNTP, 1 µM primers, 0.25 units of Taq DNA polymerase, and the template. PCR was conducted in the following conditions: 95°C initial denaturation, one cycle of denaturation at 95°C for 30 s; annealing at 63°C for 40 s; elongation at 72°C for 50 s; followed by 10 cycles with the annealing temperature decreasing by 1°C per cycle; then 20 cycles – at 95°C for 30 s; 53°C for 40 s; 72°C for 50 s; and the final polymerization at 72°C for 7 minutes. PCR products were analyzed by electrophoresis in a 1.2% agarose gel. PCR products were concentrated by alcohol precipitation, and 800 ng of DNA was used for DGGE analysis.

Changes in the structure of the bacterial community in the experimental and control mesocosms were monitored using DGGE and sequencing of the 16S rDNA, as in the studies of other authors (Lebaron et al., 2001; Lindh et al., 2013; Øvreås et al., 2003; Schäfer et al., 2001). DGGE was performed using a DCode Universal Mutation Detection System (BioRad, USA) in a 6% polyacrylamide gel with a denaturing gradient factor from 25% to 70% (the factor of 100% denaturant is a mixture of 7M urea solution and 40% deionized formamide). Electrophoresis was run in 1X TAE buffer at 60°C first at 50V for 30 min, followed by 100 V for 16 h 45 min. Upon completion of electrophoresis, the gel was stained with ethidium bromide dye or Gel Green (Biotium, USA) and photographed in the UV light (302 nm) using an AlphaImager (Alpha Innotech Corp., USA). The bands with DNA corresponding to the subdominant bacterial species that presumably consumed the added amino acid were excised from the gel with a sterile

scalpel. Digitized photos of the gel were subjected to densitometry analysis using 1D-Multi tab of AlphaEase v5.5 software (Alpha Innotech Corp., USA).

The Shannon index was calculated to estimate of the diversity of bacterial communities (Dunbar et al., 1999). One-link cluster analysis was carried out according to Jeffers (1978) using the Euclidean distances. All calculations were performed using STATISTICA, version 9 (StatSoft Inc., USA).

1.6 16S rRNA cloning

1.6.1 Experiment I

The excised fragments of the 16S rDNA were eluted in a PCR buffer as described in (Frost, Guggenheim, 1999) and reamplified with the same primers without GC-clamp.
For cloning of PCR product, a T-vector was prepared: pBluescript II plasmid (Stratagene, USA) was digested with EcoR V (SibEnzyme, Russia), and the sticky 3'-T-ends were extended by two-hour incubation with Taq DNA polymerase and dTTP at 72°C. The T-vector was purified by preparative electrophoresis in a 0.8% agarose gel and extracted from the gel using a MinElute Gel Extraction Kit (QIAGen, Germany).

The 16S rDNA fragments and the T-vector were ligated by highly active T4 DNA ligase (SibEnzyme, Russia) at 16°C. The ligation mixture was used to transform *E. coli* XL-1 Blue cells (Stratagene, USA). The competent *E. coli* cells were prepared by the standard method using TFB buffer as described by Sambrook et al. (1989). The cell suspension was seeded on a 1.5% LB-agar containing 100 μg mL^{-1} ampicillin, 80 μg mL^{-1} X-Gal (5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside) and 80 μg mL^{-1} IPTG (isopropyl-beta-D-thiogalactopyranoside). Clones containing the inserts were selected by blue-white screening (Sambrook et al., 1989). White colonies were transferred to microtubes containing 20 μL of 10 mM Tris-HCl, pH 8.0. The insert size was analyzed by PCR with 2 μL of the cell suspension as a template, using primers complementary to plasmid sites in the insert region (M13-Up 5'-CCT TTG TCG ATA CTG GTA-3', M13-Down 5'-GTT GTA AAA CGA CGG CCA GTG A-3'). The reaction conditions were as follows: the first cycle at 94°C for 2 min; 60°C for 1 min; and 72°C for 1 min; then 29 cycles at 94°C for 30 s; 60°C for 40 s; 72°C for 40 s, and the final polymerization at 72°C for 5 min. The obtained reaction products were analyzed by electrophoresis in a 1.2% agarose gel. After staining of the gel with ethidium bromide, the bands of the desired size (1124 bp) was excised from the gel under long wavelength UV, and DNA was isolated as described above.

1.6.2 Experiment IV

DNA bands were cut from the denaturing polyacrylamide gel, eluted with 2x PCR buffer and reamplified with the primer pair 341F-907R (without the GC-clamp). Reamplification products were run in a 1.5% agarose gel and then extracted using an illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare, UK) according to the manufacturer's protocol. The purified DNA fragments were cloned into a vector using a pJET1.2 CloneJET PCR Cloning Kit (Thermo Fisher Scientific Inc., USA). *E. coli* XL-1 Blue cells (Stratagene, USA) were transformed using a TransformAid Bacterial Transformation Kit (Thermo Fisher Scientific Inc., USA). Transformed bacteria were inoculated on 1.5% LB-agar containing 100 μg mL^1 ampicillin. According to the manufacturer, only colonies containing the cloned fragment grow in the medium after transformation, since the recircularized pJET1.2 plasmid without an insert expresses a protein that is lethal to cells. The selection of colonies containing the insert of a correct size (586 bp) was carried out by PCR of colonies. To do this, PCR mixture was prepared with primers complementary to the plasmid sequence near the region of insert (pJET1.2 forward sequencing primer 5'-CGA CTC ACT ATA GGG AGA GCG GC-3', pJET1.2 reverse sequencing primer 5'-AAG AAC ATC GAT TTT CCA TGG CAG-3'). Colony cells were touched with a sterile microbiological loop and resuspended in 20 μL of PCR mix. Reaction conditions were the following: initial denaturation at 95°C for 3 min; 24 cycles at 94°C for 30 s; 60°C for 30 s; 72°C for 45 s. The reaction products were analyzed by electrophoresis in a 1.2% agarose gel. Colonies containing the plasmid with the desired insert size were incubated overnight in a liquid LB-medium with ampicillin at 37°C with stirring. Plasmids were isolated from the cell biomass with a GeneJET Plasmid Miniprep Kit (Thermo Fisher Scientific Inc., USA).

1.7 Sequencing

1.7.1 Experiment I

The inserts were sequenced on an ALFexpress II sequencer (Amersham Pharmacia Biotech, USA) at the Center of Collective Use of the Yenisei REC (Siberian Federal University, Krasnoyarsk) using a Thermo Sequenase Cy5 Dye Terminator Kit (Amersham Pharmacia Biotech, USA) according to manufacturer's recommendations. Universal M13 plasmid primers were used for sequencing. The obtained nucleotide sequences of 16S rRNA genes were deposited in the GenBank database under accession numbers HM072086 and HM072087, and in the EMBL database under numbers FN773496-FN773499.

1.7.2 Experiment IV

The nucleotide sequences of the inserts in the plasmids were determined by sequencing with a fluorescently labeled primer on a NEN Model 4300L DNA Analyzer (LI-COR, USA). Sequencing reactions were performed with 2 pmol of IRDye800 labeled T7 Promoter primer (5'-TAA TAC GAC TCA CTA TAG GG-3'), complementary to plasmid sequence in the region of insert, and 500 ng of plasmid DNA using a USB Thermo Sequenase Cycle Sequencing Kit (USB, USA) according to the protocol recommended by LICOR. Separation of the reaction products was performed in a polyacrylamide gel (5.5% concentration, ratio of acrylamide: bis-acrylamide was 29 : 1, 7M urea) 41 cm long and 0.2 mm thick in the conditions recommended by the manufacturer.

1.8 Phylogenetic analysis

The obtained 16S rDNA sequences were compared to the sequences in the GenBank and EMBL databases using the BLAST online service (URL: http://www.ncbi.nlm.nih.gov/blast). Then, a phylogenetic analysis was performed with the Mega software (URL: http://www.megasoftware.net/). We used the neighbor-joining and maximum likelihood methods with 1000 bootstrap replicates. The archea *Halobacterium sp.* was used as the root species in the phylogenetic tree.

2. Results

2.1 Experiment I (July 2004)

DGGE gel analysis revealed 25 bands of 16S rDNA corresponding to different genotypes of bacteria in the initial sample (Fig. 6). No significant qualitative and quantitative changes in the composition of bacteria were detected throughout the experiment in the control mesocosm compared to the initial sample. This implies that the natural bacterial community of the reservoir functioned in the control mesocosm throughout the entire experiment.

On the day 2 of the experiment the bacterial composition already differed greatly from the initial sample in the mesocosm with addition of glycine (Fig. 6). A band labeled *Gly1*, which was present in the initial sample, became dominant judging by the relative density on the day 2 and continued to dominate until the day 4 of the experiment. On the day 3, a subdominant band

Gly2 appeared in the mesocosm with the addition of glycine. On the day 4 of the experiment, the profile did not change significantly except that the fraction of *Gly3* band began to increase. On the day 5, *Gly1* band disappeared from the bacterial community of the mesocosm, but a new dominant band *Gly4* emerged. On the last day, it was replaced by another new band *Gly5*. In days 3-5, two more bands had a positive dynamics in the community of the mesocosm with glycine addition, but they did not become dominant by the end of the experiment. Thus, with the addition of glycine to the bacterial community, there was an increase in relative densities of two initially prominent bands, one of which disappeared by the end of the experiment, and three bacterial species appeared that were not detected in the initial sample.

In the mesocosm with lysine addition, *Lys1* band occupied a dominant position on the day 2 of the experiment, but by the end of the experiment its fraction decreased (Fig. 6). By the day 4, *Lys2* band became dominant, but later it also gave way to two new bands.

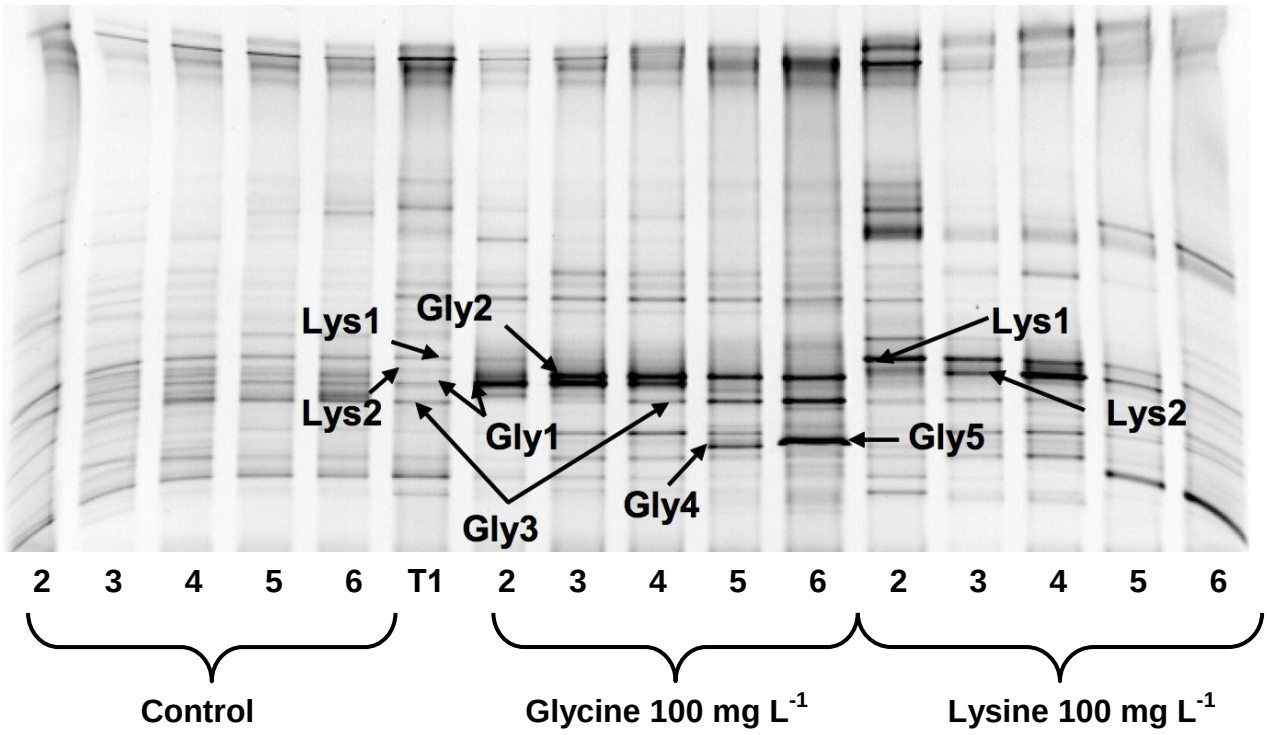

Figure 6. 16S rDNA PCR-DGGE of bacterial samples from the mesocosm with addition of glycine, the mesocosm with addition of lysine, the control mesocosm, and the initial sample (lane T1) of the Experiment I. Lane number corresponds to the day of the experiment. Arrows indicate the sequenced DNA bands

Cluster analysis, based on the presence of bands and their relative intensity, gave four main clusters (Fig. 7). The first cluster joined all samples from the control mesocosm, the initial sample (T1) and the sample of the mesocosm with the addition of lysine on day 2 of the

experiment (L2). Clustering of the initial sample with the samples of the control mesocosm confirms that bacterial community of the control mesocosm remained invariable throughout the experiment. Sample L2 clustered with T1, which means that the addition of lysine to the mesocosm caused no significant changes immediately after the start of the experiment. The next two clusters united samples taken on days 3-6 from the mesocosm with the addition of lysine (L3-L6). The most remote cluster contained samples taken at days 2 - 6 of the mesocosm with the addition of glycine (G2 - G6). The Shannon diversity index, based on the number of bands and their relative density, ranged from 3.7 to 4.54 in all mesocosms, indicating no significant trends during the experiment.

According to the results of phylogenetic analysis (Fig. 8), bands *Lys1* and *Lys2*, which dominated in the mesocosm with the addition of lysine for the first three days, belonged to the family Burkholderiaceae of Betaproteobacteria. Betaproteobacteria are now the most explored and often the most numerous group of bacteria which inhabit the upper layer of lake water (Newton et al., 2011).

The nucleotide sequence of the studied 16S rDNA region of band *Lys1* was 100% identical to the immobile hemoorganogeterotrophic aerobic bacteria *Polynucleobacter difficilis* (Hahn et al., 2012). This genotype was also found in the waters of Armenian Lake Sevan (Hahn et al., 2012), Tibetan Kelike lake, Lake Michigan (Mueller-Spitz et al., 2009), and the Chesapeake Bay (Shaw et al., 2008). *Lys2* was 99% identical to the strain of *Limnobacter thioxidans* found in sedimentary samples a freshwater spring. Similar uncultivated bacteria were detected in an aerosol sample from the Dumont d'Urvill coastal Antarctic station, on the surface of the snow blocks at an altitude of 6350 m in the Andes, in a fjord of the Arctic Ocean above the place of methane emission along the edge of the North American continent (http: //blast.ncbi .nlm.nih.gov/Blast.cgi). *Limnobacter thiooxidans* consumes aspartate and glutamate in laboratory cultures, but the uptake of other amino acids was not studied (Spring et al., 2001). Thus, bacterial populations whose increase can be attributed to the addition of lysine belonged to the same family.

The closest relatives of bacteria corresponding to bands *Gly2*, *Gly4* and *Gly5* of the mesocosm with the addition of glycine, belonged to the Betaproteobacteria family Comamonadaceae. *Gly2* was 99% similar to an uncultured bacterium found in wastewater. *Gly4* had 98% similarity with the strain of *Comamonas testosteroni*, isolated from wastewater of a tetracycline production plant. According to the literature data, *Comamonas testosteroni* consumes glycine and some other amino acids (lysine uptake was not tested) in laboratory cultures (Tamaoka et

al., 1987). The band *Gly5* formed a separate branch on the phylogenetic tree, remote from all the other members of the family Comamonadaceae (Fig. 8). The nucleotide sequence of the analyzed 16S rDNA region of *Gly5* band had only 96% similarity with the closest relatives of the family Comamonadaceae. Thus, according to the generally accepted "97% rule" (Giovannoni, Stingl, 2005), *Gly5* is a new species which has not been described previously. The band *Gly1* was 100% identical to the 16S rDNA region from uncultured bacteria most closely related to the freshwater bacterial phylum Chloroflexi (Fig. 8) (Newton et al., 2011). Uncultivated representatives of Chloroflexi, including *Gly1*, clustered separately, far from all the known cultivated species of this group (see Fig. 8). This bacterium is found in the water of lakes in China, Greece, North, Central and South America [http://blast.ncbi.nlm.nih.gov/Blast.cgi]. Sequencing of the studied 16S rDNA region of band *Gly3* was not successful.

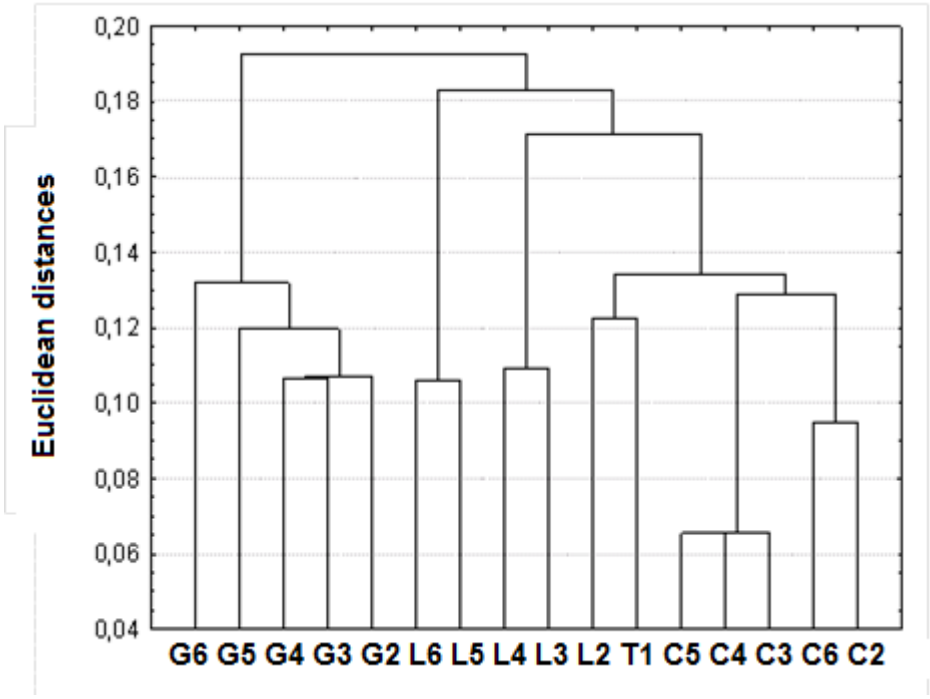

Figure 7. Clustering of bacterial samples from mesocosms of the Experiment I according to PCR-DGGE. T1 – initial sample; C2–C6 – control mesocosm, G2–G6 – mesocosm with the addition of glycine, L2–L6 – mesocosm with the addition of lysine. The numerical value in the designation of the samples corresponds to a day of the experiment

Additions of amino acids glycine and lysine to a natural bacterial community in the mesocosms of the Experiment I led to the dominance of different species of bacteria. This observation means that various amino acids are consumed by different bacterial taxa. Currently, little is known about whether certain types of bacteria specialize on consumption of specific types of dissolved organic carbon (Mou et al., 2008). It is assumed that some bacterial taxa prefer to utilize specific carbon sources available at high concentrations (Buck et al., 2009). Our data

supports this assumption. Selective consumption of individual amino acids leucine, methionine and tyrosine was found in the ocean pelagic microbial community (Zubkov et al., 2008).

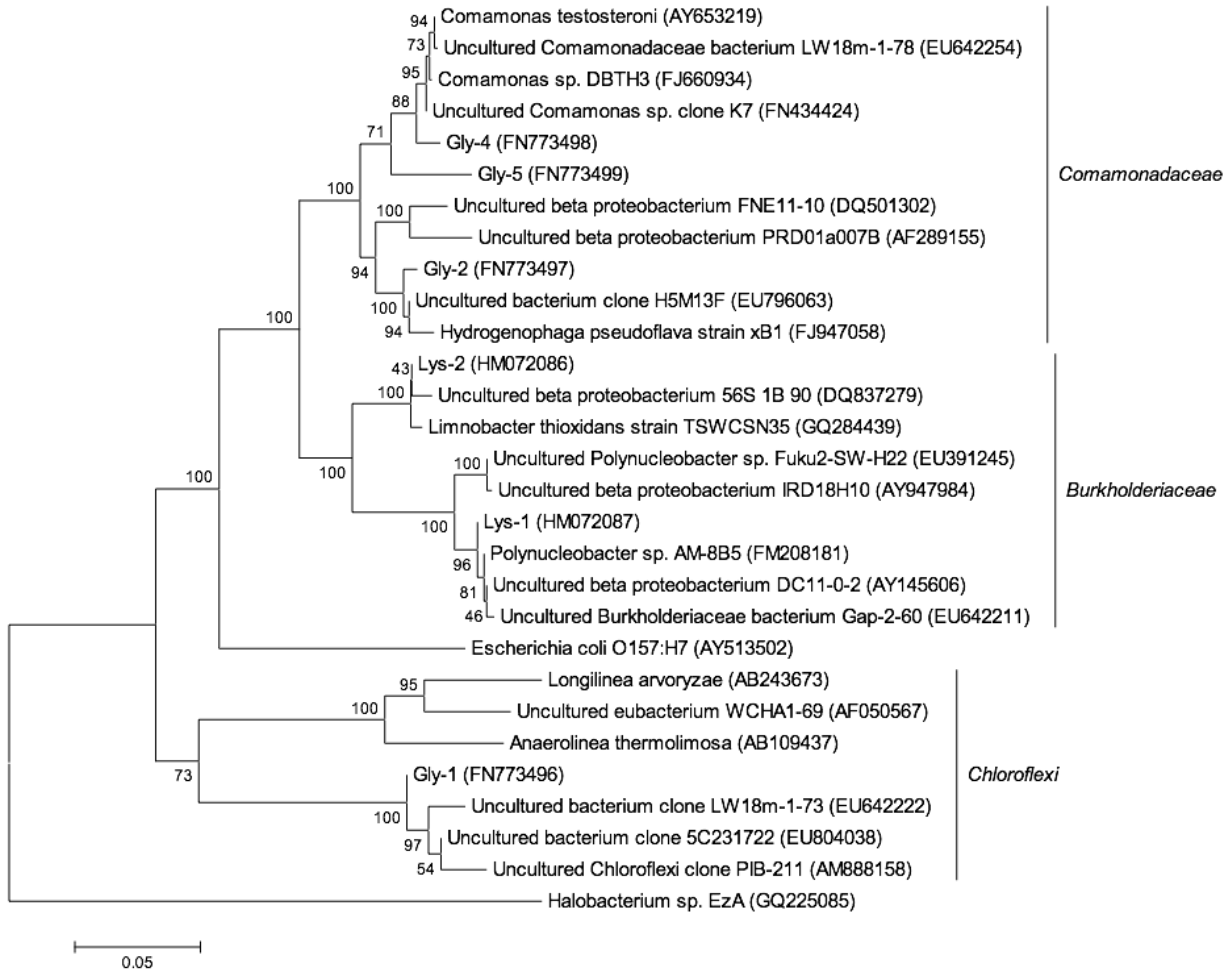

Figure 8. Phylogenetic tree of the 16S rDNA sequences of clones isolated from the DGGE bands of the Experiment I. Random sequences from GenBank database and the reference strains are included for comparison. Bootstrap values are listed on the branches of a tree constructed by neighbour-joining method. The scale bar represents 5% divergence

It has previously been suggested (Trusova, Gladyshev, 2006) that, in contrast to traditional concept, according to which groups of aquatic bacteria are consumers of the whole class of certain substances (e.g., lipids, sugars, amino acids), dominant bacterial species may be highly specialized in consumption of certain simple substances. It is well known that the same bacterial species is found in totally different ecosystems with various compositions of producers and consumers (Glockner et al., 2000; Pommier et al., 2007; Trusova, Gladyshev, 2002). It is possible that such cosmopolitanism (habitat versatility) is the evidence, that these bacteria consume simple substances, universal for almost all aquatic ecosystems, such as amino acids. If only these free-living planktonic bacteria consumed complex organic metabolites and degradation products of other organisms, their presence would depend on the species

composition of other plankton groups, as it happens in the case of attached bacteria (Cole, 1982). Simple food substrates require specialization of heterotrophs, otherwise it is impossible to achieve such an important factor in the stability of communities as biodiversity.

Taking into consideration the above-mentioned cosmopolitanism (habitat versatility), the question arises whether all identified bacteria were originally present in natural water of the reservoir, or the latter was contaminated from the air or equipment during the deliberately non-sterile cultivation? First of all, the bands *Gly1*, *Gly3*, *Lys1* and *Lys2* were present in the initial sample from the reservoir and also in the control, but in much smaller amounts than in the experimental mesocosm with the corrsponding additives (see Fig. 6). Probably, *Gly2*, *Gly4* and *Gly5* were also contained in the initial sample from the reservoir, but were not revealed by PCR-DGGE. It should be noted that the DGGE detects genotypes, that amount more then 1-2% of the community population (Muyzer et al., 1993), therefore, the appearance of a new DNA band at the community profile is usually due not to the occurrence of a new species in the ecosystem, but to an increase of its abundance up to a measurable value. It is important to emphasize that throughout the experiment the control mesocosm (Fig. 6) did not reveal any appreciable qualitative or quantitative changes in the bacterial composition compared to the initial sample. This is in a good agreement with earlier evidence for sustainable functioning of natural plankton communities in similar mesocosms (Gladyshev, 1992). Thus, in the mesocosms with added amino acids, the occurrence and increase of all *Gly* and *Lys* genotypes is due to the addition of certain amino acids, and not to artifacts related to non-sterile cultivation. In fact, it is known that changes in the concentration and quality of the organic material can influence the success of a variety of bacterial species in aquatic ecosystems (Buck et al., 2009). The added amino acids were also conventional components of the Bugach reservoir water. In previous years, glycine has been noted among the amino acids with the highest concentration; the content was on average 7.72% of their total amount. The content of lysine was somewhat lower (4.46%), but this amino acid was also present in samples throughout the growing season (Kalachova et al., 2004).

The structure of the bacterial community in the control mesocosm remained relatively stable throughout the experiment in comparison with the abrupt shift in the community profile of the mesocosms with the addition of amino acids. Thus, data on the control mesocosm allows to suggest that the changes in the experimental mesocosms were caused by additives. It is interesting to note that the Shannon index did not change significantly over time. The Shannon

index values in our experiments were similar to those in studies of other authors (Øvreås et al., 2003).

Although we associate the expression and the domination of the *Gly* bands with the addition of glycine, it is possible that only bacterial genotypes *Gly1* and *Gly2* consumed this amino acid directly. The abundance of these bacteria increased sharply immediately after the addition of glycine, and on the fifth day sharply dropped (Fig. 6). It is unlikely that the dropping of *Gly1* genotype was influenced by the decline of glycine concentration, as was the case in the experiments of some other authors (Øvreås et al., 2003). The concentration of this amino acid many times exceeded the maximum concentration characteristic of the reservoir. Perhaps an unknown limiting factor played a role in decline of these bacterial populations. Firstly, their decline could be caused by protozoan predation. Indeed, grazing plays a key role in the formation of the morphological, phenotypic and genotypic composition of bacterial communities, and less active cells are more likely to survive than growing and propagating cells which do not elaborate defense mechanisms against predation (Lebaron et al., 2001). Secondly, reduction of the growing populations could be due to species-specific lysis by viruses (Øvreås et al., 2003).

After the decrease of *Gly1* abundance, the bands *Gly3*, *Gly4* and later *Gly5* became dominant in the bacterial community of the glycine-enriched mesocosm according to DGGE profile analysis (Fig. 6). Emerging secondary bacterial populations with genotypes *Gly3*, *Gly4* and *Gly5*, as well as unidentified clones from the mesocosm with the addition of lysine, may have increased their numbers for two reasons. Firstly, they could have specialized in the consumption of the added amino acids, but had lower growth rate than the bacterial populations that dominated at the beginning of the experiment. If secondarily emerged dominants were highly resistant to predation and viral lysis, they could have displaced the primary dominant rapidly growing populations (Lebaron et al., 2001). Secondly, bacteria that increased their abundance at the end of the experiment could have consumed metabolites excreted by primarily dominating populations, as happened in other studies (Mou et al., 2008). In general, the dynamics of bacterial communities in mesocosms, namely a very fast response of populations that had only faint bands in the lane of the original sample, and the preservation of populations initially present during the experiment in the control mesocosm, are in a good agreement with the results of similar experiments in nutrient-enriched marine mesocosms (Schäfer et al., 2001).

Thus, certain species of free uncultivated bacteria were highly specialized in their ability to consume certain amino acids. Not the whole bacterial community, but only a small number of

populations that were present in relatively low abundances in the original samples, responded to the addition of an individual amino acid. These results are fully consistent with the previously conducted experiments (Trusova, Gladyshev, 2006), but the highly specialized bacteria were identified for the first time.

2.2 Experiment II (August 2005)

DGGE profile of the bacterial community of the Experiment II is shown in Fig. 9.

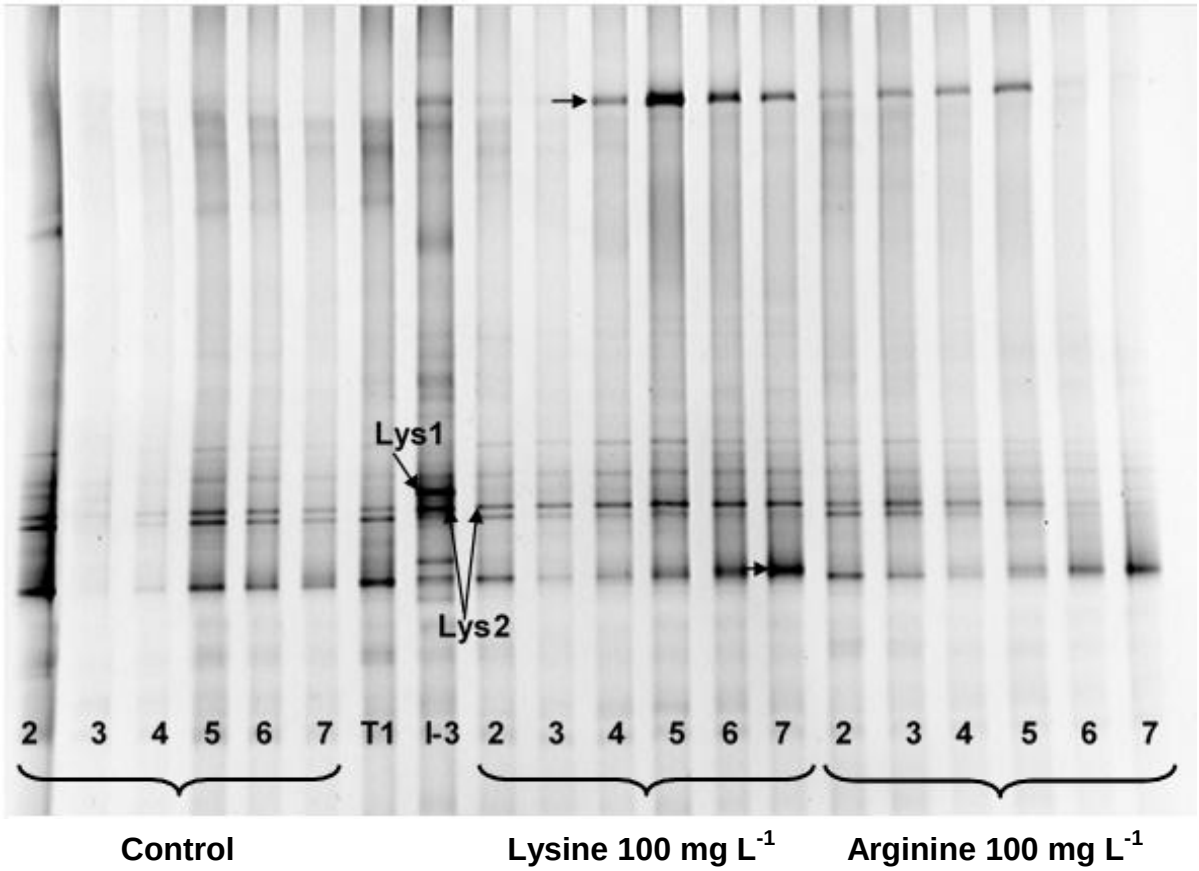

Figure 9. 16S rDNA PCR-DGGE of bacterial samples from the mesocosm with the addition of lysine, the mesocosm with the addition of arginine, the control mesocosm, and the initial sample (lane T1) of the Experiment II and the reference sample (I-3). Lane number corresponds to the day of the experiment. For comparison, a sample from the mesocosm with lysine addition on the day 3 of the Experiment I (lane I-3) is included as a reference sample

No significant qualitative and quantitative changes in the bacterial composition of the control mesocosm compared to the initial sample were detected throughout the entire experiment. This result means that the natural bacterial community of the reservoir functioned in the control mesocosm. At the same time no significant changes in the composition of bacterial communities in comparison with the initial sample were also revealed in the experimental

mesocosms within the first days after the addition of lysine and arginine. Only at the end of the experiment the density of several bands increased (Fig. 9, indicated by unsigned arrows) in both mesocosms with the addition of amino acids. Perhaps this reaction was not directly caused by the addition of amino acids, but by other factors discussed above in Section 2.1. Bands corresponding to genotypes *Lys1* and *Lys2* that consumed lysine in the Experiment I were present in all samples of the Experiment II. This was revealed by applying a sample from the Experiment I, taken on the third day of cultivation of the bacterial community in the mesocosm with the addition of lysine, to the gel. Thus, these species were present in the mesocosms of the Experiment II, however, they did not consume the added amino acids, and their abundance did not increase.

2.3 Experiment III (May 2006)

Picture of the DGGE gel with samples of the bacterial community from the Experiment III is shown in Fig. 10.

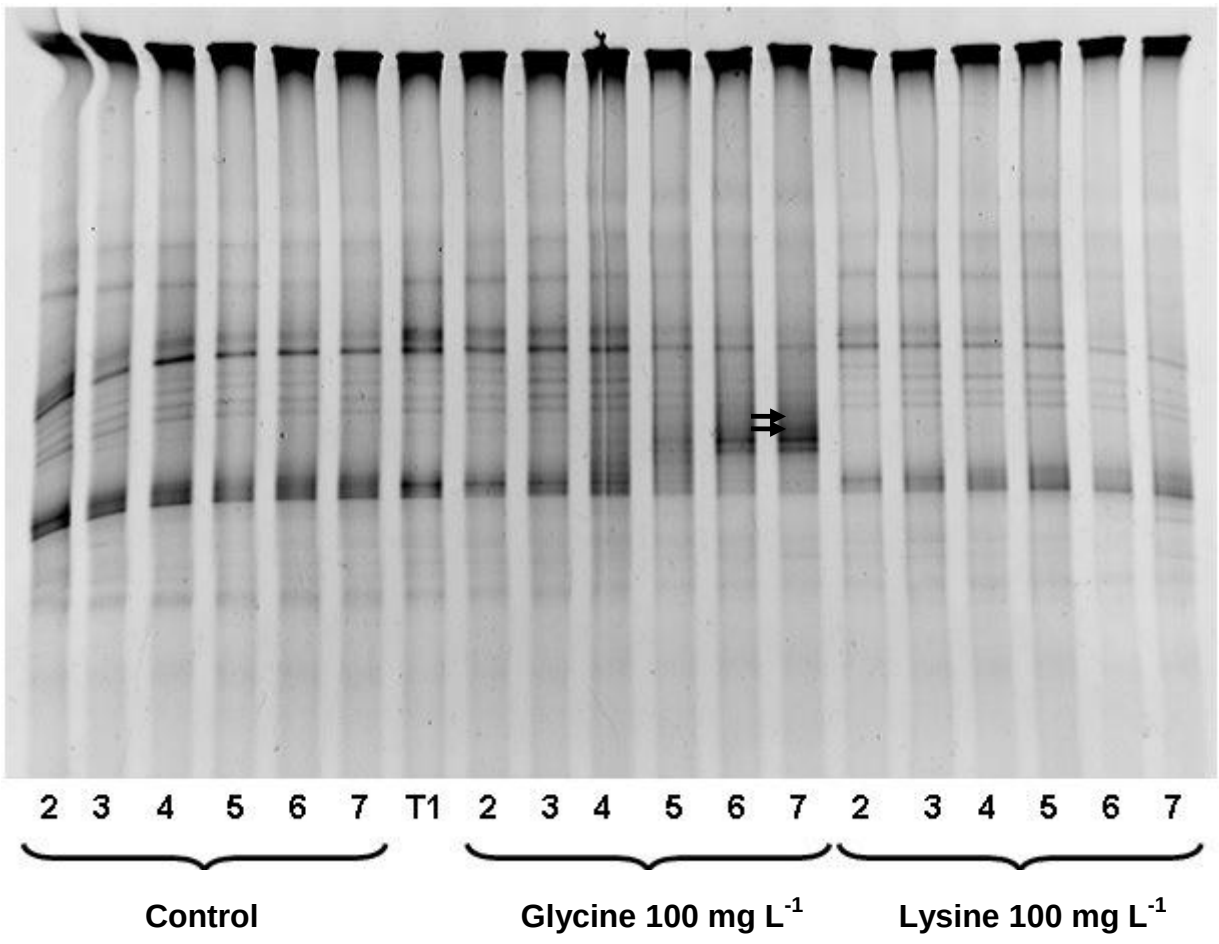

Figure 10. 16S rDNA PCR-DGGE of bacterial samples from the mesocosm with the addition of glycine, the mesocosm with the addition of lysine, the control mesocosm, and the initial sample (lane T1) of the Experiment II. Lane number corresponds to the day of the experiment

As in the Experiment II, no significant changes in qualitative and quantitative composition of bacterial community were revealed immediately after the addition of amino acids to the experimental mesocosms. Only in the mesocosm with the addition of lysine two new bands became dominant in the last two days of the experiment. The concentration of dissolved lysine in water gradually decreased during the experiment (Table 7). Presumably, lysine was used for constructive metabolism by the entire bacterial community.

Table 7. Concentration (mg L^{-1}) of the added amino acids dissolved in water of experimental mesocosms in the Experiment III

Date	**18 May 2006**	**19 May 2006**	**20 May 2006**	**21 May 2006**	**22 May 2006**	**23 May 2006**
Mesocosm with the addition of 100 mg L^{-1} of lysine	93.62	90.46	87.75	87.67	88.16	87.60
Mesocosm with the addition of 100 mg L^{-1} of glycine	99.14	98.17	96.68	96.25	92.12	94.37

2.4 Experiment IV (August 2009)

DGGE gel with samples of the bacterial community from the Experiment IV is shown in Fig. 11. Two subdominant bands, labeled IVa and IVb, were detected in the initial sample. Significant changes in the qualitative and quantitative composition of the bacterial communities were detected in all mesocosms of this experiment after the addition of lysine. Bands *Lys1* and *Lys2*, which were the same genotypes that consumed lysine in the experiment I, already became dominant on the second day of the experiment. This was revealed by applying the sample from the Experiment I that was taken on the third day of cultivation of the bacterial community in the mesocosm with the addition of lysine to the gel. Band *Lys1* dominated during the first day of the experiment IV in all mesocosms, and *Lys2* remained the dominant band throughout the experiment in the mesocosm with the addition of 10 mg L^{-1} of lysine (MES10) and in the mesocosm with the addition of 100 mg L^{-1} of lysine (MES100) (Fig. 11). Since the same genotypes Lys1 and Lys2 became dominant after adding lysine in the Experiment IV, as in the Experiment I, this means that the response of the summer communities of bacteria to amino acid amendments is stable and repeated over different years.

Band *IVa* was dominant for the first four days only in the mesocosm supplemented with 1 mg L^{-1} lysine (MES1). Also *IVb* band remained dominant in MES1 up to the end of the experiment. In MES10 these genotypes were dominant only on the first 2-3 days of the

experiment, and in MES100 their abundance declined on the day 3. Band *IVc*, which was present but not dominant in the initial sample, disappeared from all mesocosms on the days 3 and 4 of the experiment. Band *IVd*, which presence in the initial sample was not obvious because of the closely located bands in the compression zone, became dominant in all mesocosms on the day 2, and remained abundant in MES1 and MES10 until the end of the experiment. Band *IVe* became dominant in MES 10 and MES1 on the days 3 and 4, respectively, and remained so until the end of the experiment. Bands *IVf* and *IVg* appeared only in MES100 during the experiment. Bands *IVh* and *IVj*, which were absent in the original sample, appeared only in MES10 and MES100. Band *IVj* dominated only in MES1.

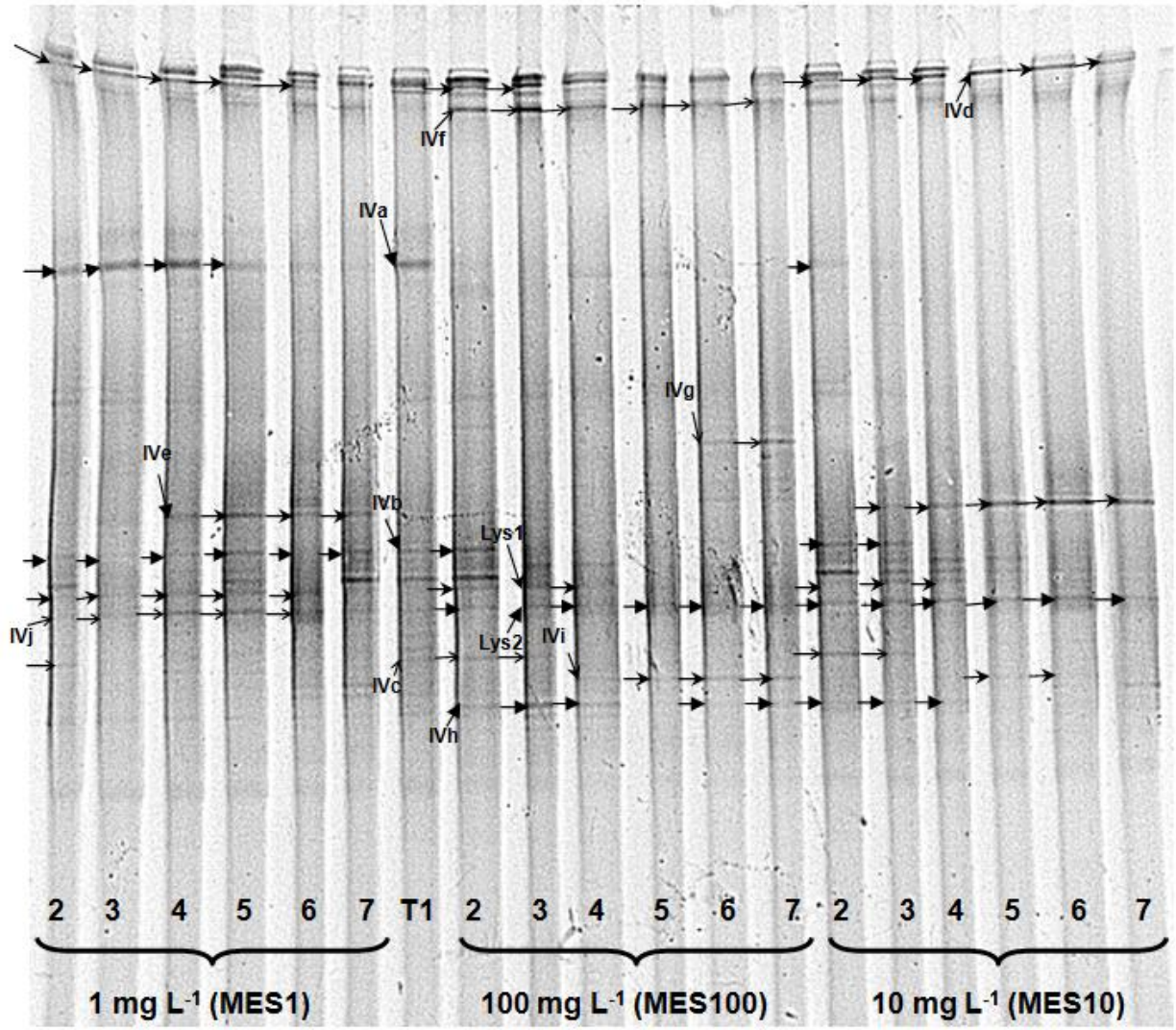

Figure 11. 16S rDNA PCR-DGGE of bacterial samples from the mesocosms with the addition of lysine in the concentrations of 1 mg L^{-1}, 10 mg L^{-1} and 100 mg L^{-1}, and the initial sample (lane T1) of the Experiment IV. A lane number corresponds to the day of the experiment. Arrows indicate the sequenced DNA bands

Certain patterns are observed in the dynamics of bacterial communities in the Experiment IV (Fig. 11). Genotype *Lys1* dominated in all mesocosms regardless of the concentration of the

added lysine. Throughout the experiment the proportion of species that dominated in the initial sample (*IVa*, *IVb*) declined, and the higher the concentration of amino acids was in the mesocosm, the sooner it happened. Densities of other bands (*IVf*, *IVh*) increased during the experiment, and the higher was the concentration of lysine, the faster it happened. Certain genotypes dominated only in mesocosms with the highest (*IVf*, *IVh*, *IVi*, *IVg*) or lowest (*IVe*, *IVd*, *IVj*) concentrations of lysine. Thus, the bacterial species responded differently to different concentrations of the same added amino acid.

Cluster analysis of similarity of samples in Experiment IV, which is based on presence and density of the bands on the gel, gave three main clusters (Fig. 12).

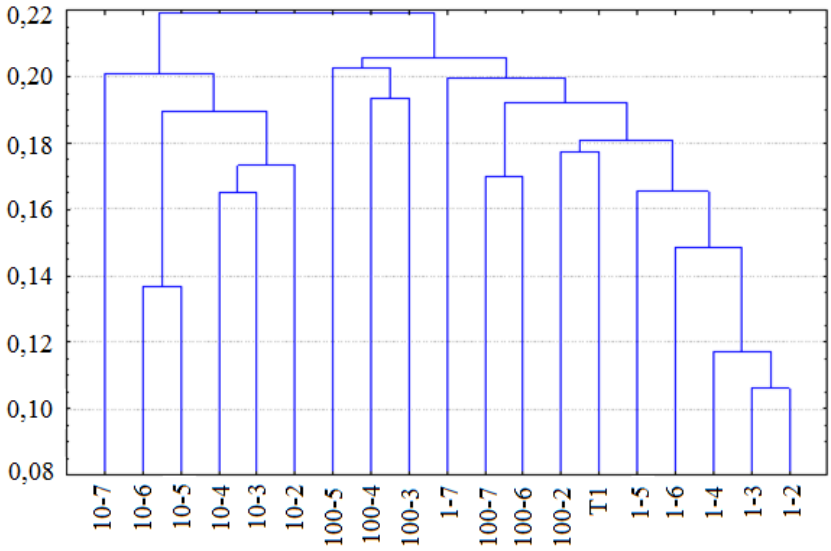

Figure 12. Clustering of bacterial samples from the mesocosms of experiment IV based on DGGE data. T1 - initial sample; 1-2 - 1-7 - mesocosm with the addition of 1 mg L^{-1} of lysine, 10-2 - 10-7 - mesocosm with the addition of 10 mg L^{-1} of lysine, 100-2 - 100-7 - mesocosm with the addition of 100 mg L^{-1} of lysine. A numerical value in the designation of the samples corresponds to the day of the experiment

All samples of mesocosm supplemented with 10 mg L^{-1} of lysine clustered together. Samples of MES1 and MES100 distributed between two adjacent clusters: one included samples from days 3-5 of the experiment of MES100, another cluster joined the initial sample, all samples of MES1 and samples taken on the days 2, 6 and 7 of MES100.

Fig. 13 is a phylogenetic tree containing all bands of the Experiment IV, whose nucleotide sequence of the 16S rDNA was determined, as well as bands *Lys1* and *Lys2* of Experiment I. Bands *IVh* and *IVi* were clustered with uncultured water bacteria belonging to order Acidimicrobiales of Actinobacteria (97% and 99% similarity with the closest relatives, respectively). Proteobacteria and Fibrobacteres-Bacteriodetes-Chlorobi (FCB) group clustered in the sister clades. Inside the FCB group two sister clades formed, Bacteroidetes and

Fibrobacteres. The latter included band *IVa*, whose similarity of the nucleotide sequence of 16S rDNA with the most related uncultivated representatives was 99%. Bacteriodetes consisted of two clades, corresponding to families Flavobacteriaceae (class Flavobacteriia, order Flavobacteriales) and Sphingobacteriaceae (class Sphingobacteriia, order Sphingobacteriales).

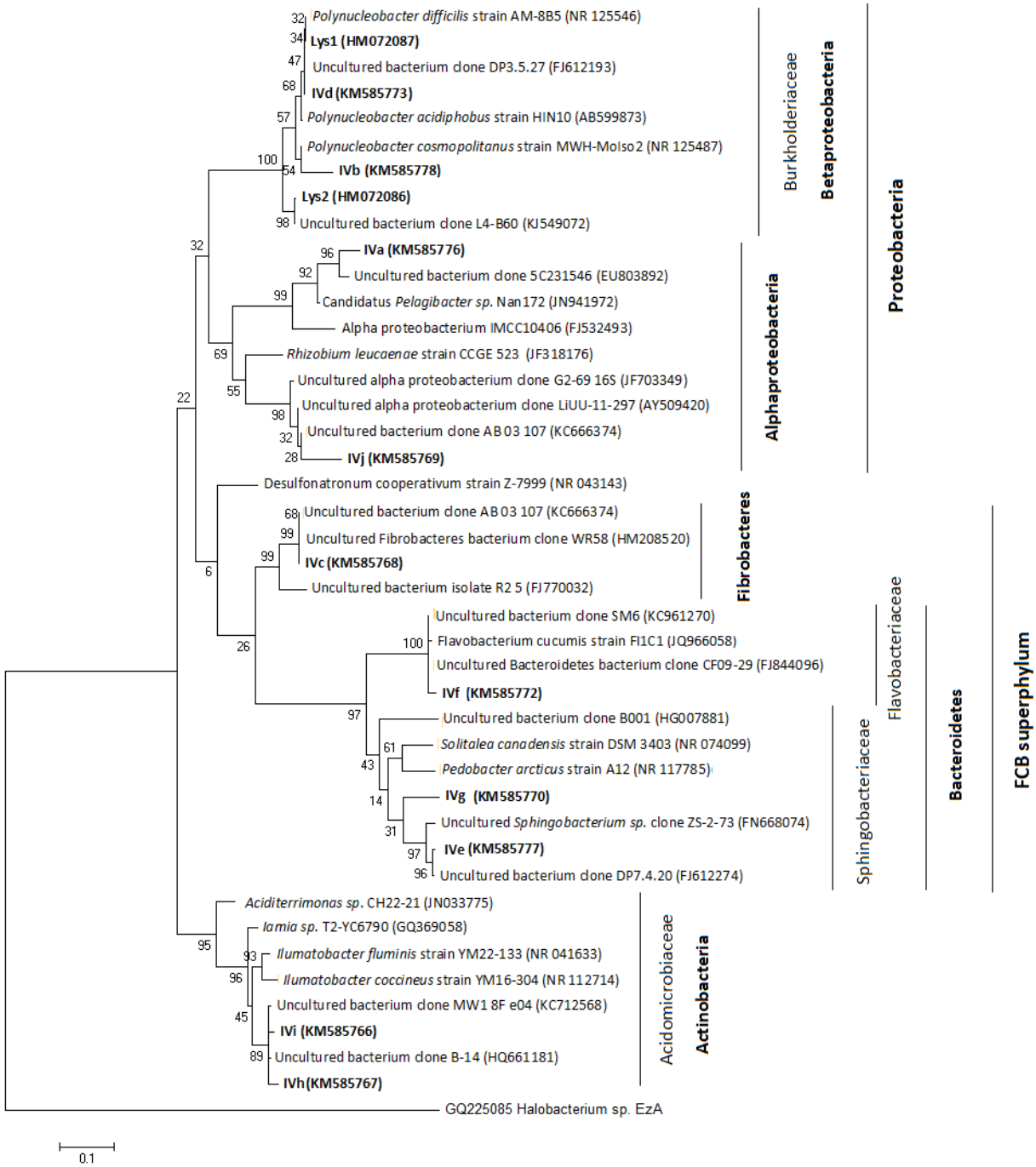

Figure 13. Phylogenetic tree of the 16S rDNA sequences of clones isolated from the DGGE bands of the lysine-enriched mesocosms in Experiments I and IV. Random sequences from GenBank database and the reference strains are included for comparison. The scale bar represents 10% divergence

The Flavobacteriaceae clade included band *IVf*, which was 98% similar to uncultured bacteria of this family isolated from a variety of natural habitats. Bands *IVe* and *IVg* clustered together with

representatives of the family Sphingobacteriaceae, and while band *IVe* was 99% identical to uncultivated clones of this family, *IVg* had a very distant 89% similarity with the closest relatives.

Most of the bacteria identified in the mesocosms, belonged to Proteobacteria, namely Alpha- and Betaproteobacteria, which formed the sister groups on the phylogenetic tree. The band *IVa* clustered together with a group of representatives of uncultivated Alphaproteobacteria, however, had only 94% similarity to the closest relative - a bacterium from Lake Gatun (Panama). The band *IVj* belonged to another branch of uncultivated Alphaprteobacteria and had a 99% similarity with the closest relatives isolated from different soil and water sources. Bands *IVb* and *IVd* belonged to Burkholderiacea, the same family of Betaproteobacteria, which *Lys1* and *Lys2* belonged to. *IVb* had a distant 95% similarity with *Polynucleobacter cosmopolitanus* – a heterotrophic aerobic attached bacteria isolated from freshwater habitats (Hahn et al., 2010). Lane *IVd* was 99% similar to *Polynucleobacter difficilis* (Hahn et al., 2012) and to the band *Lys1*. However, it cannot be stated on the basis of high similarity that *IVd* belongs to this species, as only a fragment of the 16S rRNA gene has been studied.

Thus, different response of bacterial communities in the mesocosms was observed to the addition of lysine at various concentrations. However, the same species that consumed lysine in the Experiment I also became dominant in the Experiment IV after the addition of this amino acid.

2.5 Experiment V (May 2013)

DGGE gel with the samples of bacterial community of the Experiment V is shown in Fig. 14. As in the Experiments II and III, no qualitative or quantitative changes in the composition of bacterial community of the mesocosms were revealed in the Experiment V. However, bands *Lys1* and *Lys2*, which consumed lysine in the Experiments I and IV, were present in the community profile of the mesocosms. The concentration of dissolved lysine in water gradually decreased throughout the experiment (Table 8). Presumably, lysine was used for constructive metabolism by the entire bacterial community.

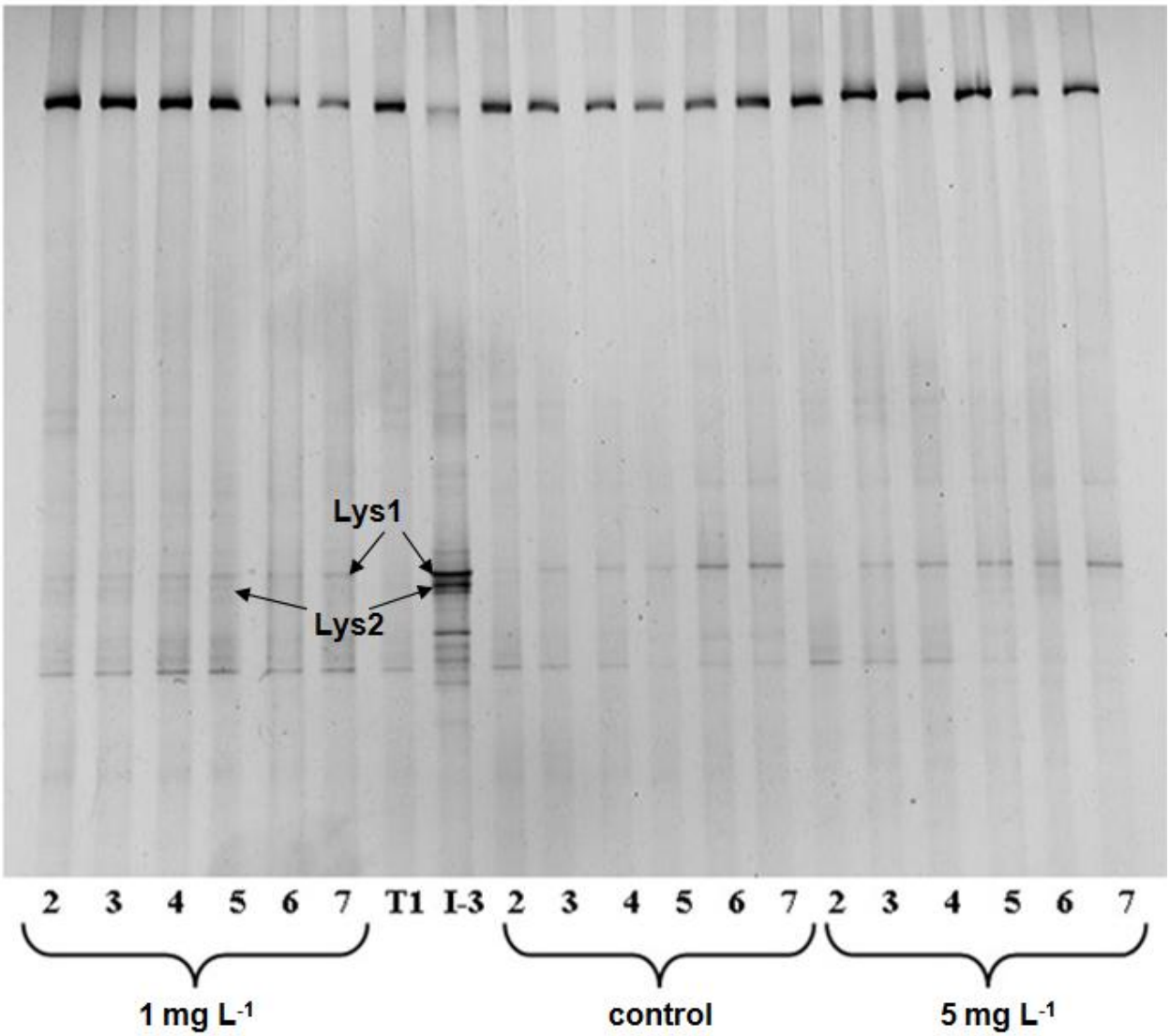

Figure 14. 16S rDNA PCR-DGGE of bacterial samples from the mesocosms with the addition of lysine in the concentrations of 1 mg L^{-1} and 5 mg L^{-1}, the control mesocosm, and the initial sample (lane T1) of the Experiment V. A lane number corresponds to the day of the experiment. For comparison, a sample from the mesocosm with lysine addition on the day 3 of the Experiment I (lane I-3) is included as a reference sample

Table 8. Concentration (mg L^{-1}) of the dissolved lysine in water of experimental mesocosms in the Experiment V

Date	**13 May 2013**	**14 May 2013**	**15 May 2013**	**16 May 2013**	**17 May 2013**	**18 May 2013**
Mesocosm with the addition of 1 mg L^{-1} of lysine	0.62	0.45	0.46	0.45	0.35	0.1
Mesocosm with the addition of 10 mg L^{-1} of lysine	4.06	3.94	3.96	3.59	3.49	3.57

3. Discussion

Despite some limitations, experimental mesocosms are a good tool for environmental studies of aquatic bacterial communities (Schäfer et al., 2001). Non-sterile cultivation of the whole communities in mesocosms is the only experimental method for the investigation of uncultivated bacterial species, many of which cannot exist beyond a holistic plankton community (Amann et al., 1995). Many authors cultured whole bacterial communities to study the effect of added substrates on the communities (Carlson et al., 2002; Volova et al., 2007). A combination of experiments in mesocosms and molecular identification methods of bacteria is the most appropriate method to determine the relationship between the structure (genotype) and function (phenotype) in a bacterial community (Øvreås et al., 2003).

Identifying bacterial species is important for phylogeny and it is even more important for ecology. Knowledge of particular species and the substrates they utilize can now be formalized in mathematical models of aquatic ecosystems designed to forecast and manage the quality of natural waters (Gladyshev, 1999). Forecast accuracy and success of management will undoubtedly be increased by replacing aggregated bacteria and aggregated organic matter in such models with particular bacterial species and particular substances they utilize (Adamowicz 1992; Gladyshev, 1999; Cottrell, Kirchman, 2000; Degermendzhi, 2010).

Notably, significant changes in bacterial communities of experimental mesocosms in Experiments I (held in July) and IV (early August) happened after the addition of amino acids, whereas in Experiments II (late August), III and V (May) these changes were not observed. A possible reason for the observed differences may be the functional characteristics of the reservoir bacterial community in spring and autumn (late summer). In spring and in late summer the bacterial species specializing in the consumption of lysine were functionally inactive in the community. Perhaps their activity was suppressed by low water temperature or other unfavorable environmental factors (see table 9).

Variability of the Shannon diversity indices in Experiments II and III was much lower than in Experiments I and IV. That is, the diversity index confirms that in July and early August the bacterial community responded to the added lysine with a significant change in species composition, and in May and in the end of August, such a response was not observed.

Table 9. Ecological characteristics of the experimental mesocosms with the addition of lysine at a concentration of 100 mg L^{-1} in the Experiments I-IV

Sampling date	Sample No.	Water temperature, °C	Number of DGGE bands	Shannon's index (H)	Simpson's index (D)
Experiment I					
30.06.2004	0	20.1	28	4.66	0.043
01.07.2004	1	21.6	25	4.44	0.052
02.07.2004	2	21.6	22	4.29	0.057
03.07.2004	3	21.6	19	4.08	0.064
04.07.2004	4	21.6	20	4.14	0.064
05.07.2004	5	21.5	23	4.22	0.069
SD	–	–	–	0.214	0.010
Experiment II					
17.08.2005	0	18.8	21	4.29	0.054
18.08.2005	1	18.5	22	4.26	0.059
19.08.2005	2	18.7	21	4.11	0.071
20.08.2005	3	20.6	20	4.15	0.065
21.08.2005	4	21.6	26	4.55	0.049
22.08.2005	5	20.9	23	4.29	0.060
23.08.2005	6	20.5	21	4.21	0.061
SD	–	–	–	0.143	0.007
Experiment III					
17.05.2006	0	7.2	23	4.35	0.057
18.05.2006	1	7.2	23	4.3	0.062
19.05.2006	2	7.7	22	4.23	0.064
20.05.2006	3	7.4	24	4.37	0.059
21.05.2006	4	7.8	24	4.38	0.056
22.05.2006	5	7.5	21	4.11	0.068
23.05.2006	6	7.4	21	4.07	0.070
SD	–	–	–	0.126	0.005
Experiment IV					
31.07.2009	0	22	20	4.08	0.069
01.08.2009	1	22.8	18	3.87	0.082
02.08.2009	2	22.8	14	3.53	0.101
03.08.2009	3	22.7	19	3.89	0.072
04.08.2009	4	22.7	14	3.61	0.093
05.08.2009	5	22.7	19	4.08	0.065
SD	–	–	–	0.231	0.014

The presumed narrow specialization of bacterial plankton species in the consumption of certain organic substances, as well as the low activity of specialized species in some seasons, could be important for understanding the processes of self-purification of the reservoir ecosystem from organic pollutants. Indeed, if for some periods of time there are no species capable of utilizing the contaminant in the reservoir (or they are inactive), the ecosystem in such periods becomes particularly vulnerable to anthropogenic pollution. For instance, in the study of dynamics of reservoir self-purification from phenol conducted in the same mesocosms (Gladyshev et al., 1998), it was found that during summer months phenol added to the mesocosm completely dissapeared within 2 -5 days, whereas in May it is not used for 10 days.

Summary

In mid-summer the addition of lysine and glycine to mesocosms caused changes in the structure of bacterial community, namely the abundance of species that consumed the added amino acids increased, compared to the initial sample and the control mesocosm. The reaction of bacterial communities to added lysine depended on the concentration of added substances. In spring and late summer plankton communities of bacteria almost did not react to additions of lysine, glycine and arginine. These data suggest that the ability of the reservoir ecosystem to self-purify to remove various organic compounds can greatly depend on the seasonal characteristics of the structure and function of the bacterial community.

Conclusions

3022 operational taxonomic units of bacteria were found in the Yenisei River.

Alpha diversity of bacterial communities and the relative proportion of Cyanobacteria in the Yenisei River reached maximum values in the middle of the river.

Three bacterial assemblages were detected in the Yenisei River, significantly differing in species composition and structure. These assemblages were probably formed as a result of the biogeochemical influence of the surrounding biome: mountain taiga (the upper part of the river), the plain taiga (middle section) and forest-tundra and tundra (the lower section).

Dominant taxa of each assemblage specialized in the consumption of various groups of organic substances.

Bacteria consuming lysine and glycine were identified by means of non-sterile cultivation in experimental mesocosms of bacterial community from the Bugach Reservoir. These bacteria specialized in consumption of a narrow spectrum of amino acids.

In summer, the response of bacterial community from Bugach Reservoir to lysine additions was stable and repeated in different years.

In contrast to the midsummer experiment, the addition of lysine to pelagic community of the reservoir in spring and late summer did not cause significant changes in the quantitative and qualitative composition of bacterial community. The capability of aquatic ecosystems for self-purification to remove certain organic substances may vary considerably during the growing season.

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