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Diversity of Cyanobacterial Species and Phylotypes in Biofilms from the Littoral Zone of Lake Baikal

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The majority of naturally occurring biofilms contain numerous microorganisms that have not yet been cultured. Additionally, there is little information available regarding the genetic structure and species diversity of these communities. Therefore, we characterised the species diversity, structure and metagenome of biofilms grown on stones and steel plates in the littoral zone of Lake Baikal (East Siberia, Russia) by applying three different approaches. First, light microscopy enabled identification of the species diversity of biofilm-forming cyanobacteria on different substrates with the dominance of *Rivularia rufescens*, *Tolypothrix limbata*, *Chamaesiphon fuscus*, *Ch. subglobosus*, and *Heteroleibleinia pusilla*. Additionally, scanning electron microscopy was used to show the spatial structure of biofilms. Finally, sequence analysis of 30,660 16S rRNA clones indicated a high diversity within the biofilm communities, with the majority of the microbes being closely related to Cyanobacteria (8–46% sequences), Proteobacteria (14–43%), and Bacteroidetes (10–41%). *Rivularia* sp., *Pseudanabaena* sp., and *Chamaesiphon* spp. were the dominant cyanobacterial phylotypes.

Keywords: cyanobacteria, biofilm, epilithon, Lake Baikal, *Rivularia*, pyrosequencing

Introduction

Because of the unique characteristics of cyanobacteria, such as involvement in oxygenic photosynthesis, fixation of atmospheric molecular nitrogen, and considerable resistance to unfavourable factors, they are able to colonise different ecological niches, including extreme environments (Rozanov, 2002). Numerous studies and reviews have been devoted to the fouling of monuments and historical buildings by epi- and endolithic microorganisms, as well as to the development of methods to prevent biodeterioration of stone (Tomaselli *et al*., 2000; Crispim *et al*., 2003; Scheerer *et al*., 2009). Stone colonisation by autotrophic microorganisms (cyanobacteria and algae) starts with the formation of biofilms, which then facilitate and promote the growth of heterotrophic bacteria and fungi, resulting in the formation of complex and well-organized biofilms. In the tropics, marine ecosystems are dominated by cyanobacteria on the stony littoral substrates, whereas in temperate zones, the majority of diatoms are recorded among epilithic microorganisms because of the better resistance of cyanobacteria to high temperatures (Narváez-Zapata, 2005). In the large oligotrophic freshwater lakes of North America, diatoms prevail in the upper littoral zone, while in the deep littoral zone they are replaced by cyanobacteria of the genera *Hapalosiphon*, *Calothrix*, *Tolypothrix*, *Nostoc*, *Lyngbya*, and *Gloeocapsa* (Stevenson *et al*., 1996).

Cyanobacteria in biofilms from Lake Baikal have not been studied using a wide variety of methods to date. Investigation of the systematics and ecology of meio- and macrophytes (>0.1 mm) provide more data on over 60 species and forms of benthic cyanobacteria (Meyer, 1930; Izhboldina, 2007). In studies conducted from 1997–2000, more than 25 species of cyanobacteria were registered in the microphytobenthos of Lake Baikal; however, the species list was not published (Rodionova and Pomazkina, 2003). Hence, simultaneous taxonomic studies of all size groups of cyanobacteria in epilithic biofilm communities growing in Lake Baikal have not yet been conducted.

Use of the 16S rRNA gene as a marker enables a wide variety of bacteria in different ecological niches to be recorded. Currently, pyrosequencing, which is a new technology of sequencing by synthesis, is used worldwide. This method can identify a much higher number of sequences to reveal the diversity of the microbial community at a lower cost than the Sanger method (Sogin *et al*., 2006). Pyrosequencing of PCR amplicons from variable regions of the 16S rRNA gene has recently enabled deep bacterial diversity surveys (> 1,000 phylotypes) in oceans (Sogin *et al*., 2006; Rusch *et al*., 2007), freshwater lakes (Pope and Patel, 2008; Debroas *et al*., 2009; Oh *et al*., 2011), and thermal springs (Gumerov *et al*., 2011). Studies of the Baikal biota by means of pyrosequencing have recently been started (Gladkikh *et al*., 2011; Kadnikov *et al*., 2012). Comparative studies of phylotype diversity in plankton and fouling on steel plate have shown that the communities have similar primary bacterial phyla, but differing structures (Gladkikh *et al*., 2011; Parfenova *et al*., 2013). An extremely high percentage of the planktonic cyanobacteria, *Synechococcus* spp., was recorded in deep methane hydrate-bearing sediments dominated by methanotrophic bacteria (Kadnikov *et al*., 2012). Analysis of microbial biofilms using pyrosequencing is of significance to both the water suppliers and consumers; however, ecological and microbiological inves-

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tigations are rarely performed, and focused on surveys of the bacterial species diversity of biofilms that form in marine (Narváez-Zapata, 2005; Edwards *et al.*, 2010; Mobberley *et al.*, 2012) and extreme ecosystems (Thibault *et al.*, 2010; Klatt *et al.*, 2011).

Therefore, this study was conducted to investigate cyanobacterial communities inhabiting different types of substrates at the bottom of Lake Baikal in the littoral zone using microscopic methods and pyrosequencing of a 16S rRNA gene fragment.

Materials and Methods

Sampling

The plates for the analysis of biofilm communities were made of the three most common rock types in the shore area of Lake Baikal (marble, granite, and amphibolite). The plates had an area of 16–24 cm^2 and a stainless steel plate of 34 cm^2. In March of 2005, these plates were placed at a depth of 7 m in the southern basin of Lake Baikal near Cape Berezovy (51°50′50.4″ N, 104°54′43.3″ E). An experimental polygon is located near this cape where regular sampling takes place (Timoshkin *et al.*, 2003). The stone plates were exposed for 6 years (up to April of 2011) and the steel plate was exposed for 5 years (May of 2010). After the exposure periods, the experimental plates were lifted by scuba divers, placed into the sterile containers, and transported to the laboratory. A nearby stone that served as a control was also lifted with the experimental plates. The control stone was plagiogranite. The biofilm (1 cm^2) was then removed with a sterile scalpel and put into sterile Eppendorf tubes, after which the samples were fixed in formalin (final concentration 2%). DNA was extracted immediately after removal of biofilms from the substrates as described below.

Microscopic analysis

Scanning electron microscopy (SEM) was used for the detailed analysis of the biofilm structures. The biofilms were dehydrated in ethyl alcohol solution by gradually increasing the alcohol concentration. Subsequently, the samples were dried at 40°C, coated in gold using a Balzers SCD 004 sputter-coater (Bal-Tec AG, Liechtenstein), and examined using a SEM Quanta 200 (FEI CO., USA).

Qualitative analysis of the cyanobacteria was performed on an Axio Imager light microscope (Carl Zeiss, Germany) equipped with a HBO 100W mercury lamp and AxioCam MRm and MRc5 cameras. A green filter was used for fluorescence microscopy. Cyanobacterial species were identified based on their morphology according to available guides (Gollerbakh *et al.*, 1953; Komárek and Anagnostidis, 1999, 2005). The Sørensen index was used to define the similarity of the cyanobacterial species composition.

Pyrosequencing and data analysis

Total DNA was isolated from the samples using a DNASorb kit (Interlabservice, Russia). Briefly, 60 µl of lysozyme (1 mg/ml, Sigma-Aldrich, USA) was added to a pellet of biofilm sample followed by incubation for 1 h at 37°C. The remainder of the DNA extraction was continued according to the manufacturer's instructions. Genomic DNA was amplified using primers targeting the V1–V3 hypervariable regions of the bacterial 16S rRNA gene. DNA pyrosequencing was performed by ChunLab Incorporation (Seoul, Korea) using the 454 GS Junior Sequencing System (Roche, Switzerland).

The Mothur Programme v.1.22.0 (http://www.mothur.org) was used for primary analysis of pyrosequencing data, removal of short and chimeric sequences, and calculation of the ACE and Chao 1 estimators. The maximum length of the sequences obtained was 558 nucleotides. Sequences that were shorter than 300 nucleotides were excluded from the analysis. Available tools from the RDP pyrosequencing pipeline (Cole *et al.*, 2009) were used to determine the taxonomic composition at the 75% confidence threshold and build rarefaction curves. For determination of operational taxonomic units (OTUs), we defined the species (phylotypes), genus, family, order, and phylum level at 3, 5, 10, 15, and 25% sequence divergence, respectively, according to Schloss and Handelsman (2005).

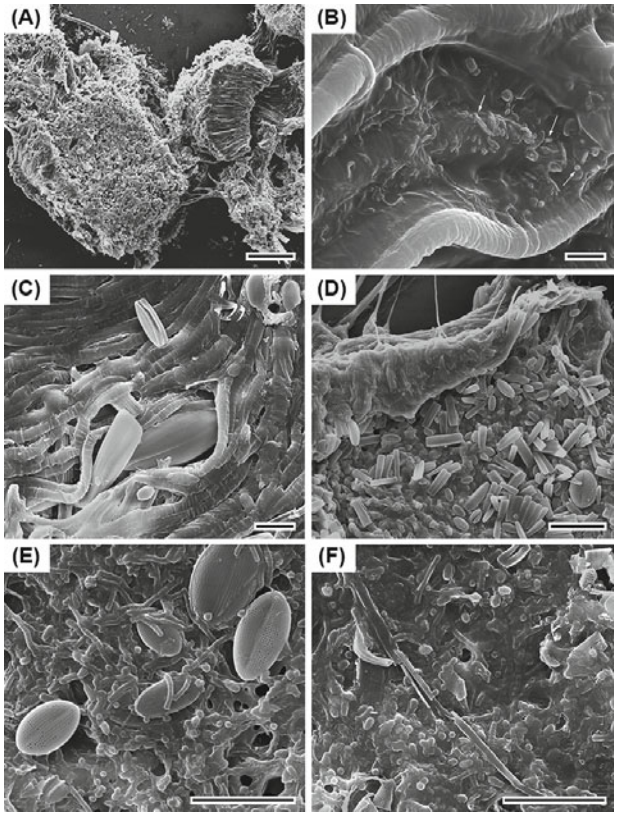

Fig. 1. Scanning electron microscopy of biofilms from different substrates. (A–D) colony structure from fouling on stony substrates: (A) general view of a colony from above and in longitudinal section; (B) rod-shaped and coccoid bacterial cells in the mucous matrix of a colony (arrows); (C) diatom cells between trichomes; (D) colony surface covered with diatoms. Biofilms from the steel plate with the dominance of filamentous (E) and coccoid (F) cyanobacteria. Scale bars: A=200 µm; B=5 µm; C, E, F= 20 µm; D=50 µm.

Table 1. Cyanobacterial species composition on different substrates

Orders/Species	Marble	Granite	Amphibolite	Control stone	Steel plate
Chroococcales					
Aphanocapsa holsatica [a] (Lemm.) Cronb. et Kom.	+				
A. parasitica [a] (Kütz.) Kom. et An.	+	+	+	+	
Chamaesiphon fuscus [a] (Rost.) Hansg.	+	+	+	+	+
C. subglobosus [a] (Rost.) Lemm.	+	+	+	+	+
Chroococcus limneticus [a] Lemm.	+			+	
Nostocales					
Anabaena sp.					+
Calothrix epiphytica [a] W. et G.S. West					+
Rivularia rufescens [a] Näg. in Kütz. ex Born. et Flah.	+	+	+	+	
Tolypothrix limbata Thur. ex Born. et Flah.	+			+	
Oscillatoriales					
Heteroleibleinia pusilla [a] (Hansg.) Compere					+
Leibleinia epiphytica [a] (Hier.) Compere	+			+	
Leptolyngbya rivulariarum [a] (Gom.) An. et Kom.	+	+		+	
Phormidium inundatum Kütz. ex Gom.				+	
Phormidium sp.				+	
Pseudanabaena sp. [a]	+			+	+
Schizothrix rivulariarum [a] Voronich.	+	+	+	+	
Total	11	6	5	12	5

[a] Species described for Lake Baikal for the first time.

Results

Biofilm structure by SEM

Visual examination of the samples showed that the stony substrates were fouled with dense brown hemispherical colonies 1–5 mm in diameter located close to each other. The densest fouling by colonies was observed on the control stone, while experimental plates were fouled with thin green and brown biofilms between colonies. Unlike the stony substrates, the steel plate was completely covered with thin green and brown biofilms. Scanning electron microscopy showed that colonies contained long vertically oriented trichomes enclosed in mucilaginous sheaths. Filaments were adjoined close to each other and interspersed with coccoid and rod-shaped bacterial cells submerged in mucilage (Figs. 1A–1B), as well as diatom frustules (Fig. 1C) that sometimes formed a dense layer on the top surface of the colony (Fig. 1D). The biofilm from the steel plate showed minimal vertical development and contained short filaments and coccoid cells surrounded by mucilage and diatom frustules embedded in the biofilm matrix (Figs. 1E–1F).

Cyanobacterial species diversity according to light microscopy

Sixteen species of cyanobacteria belonging to the orders Chroococcales, Oscillatoriales, and Nostocales (Table 1) were detected on five different substrates using light and epifluorescence microscopy. Most of the species were representatives of benthos, mainly epilithic and epiphytic cyanobacteria, with the exception of the typical planktonic species *Aphanocapsa holsatica* and *Chroococcus limneticus*. Two dominant species associations were distinguished in the fouling on the stony substrates. The *Rivularia* association included *Rivularia rufescens* with species-satellites inhabiting its colonies, *Leptolyngbya rivulariarum* and *Schizothrix rivulariarum*. This association inhabited the marble, granite, amphibolite, and the control stones (Fig. 2A and 2B). The *Tolypothrix* association consisting of *Tolypothrix limbata* colonies and *Leibleinia epiphytica* on its filaments inhabited the marble and control stone (Fig. 2C). Colonies of the epiphyte *Aphanocapsa parasitica* were not associated with a specific host and were recorded in the biofilm communities on all types of stone. Few periphytic cyanobacteria of the genera *Phormidium* and *Pseudanabaena* were observed on the marble and the control stone. The epiphytic and epilithic chroococcoid cyanobacteria *Chamaesiphon fuscus* and *C. subglobosus* inhabited all types of and were dominant on the steel plate forming a biofilm with the filamentous cyanobacteria *Heteroleibleinia pusilla* (Fig. 2D). The singular heterocystous species *Calothrix epiphytica* and *Anabaena* sp. were detected in the cyanobacterial community on the steel plate.

The dominant *Rivularia rufescens* involved in stone fouling possessed a number of interesting morphophysiological attributes. Specifically, it formed hemispherical gelatinous colonies, tapered trichomes, a basal heterocyst, false-branching, a thick sheath, was often coloured brown with scytonemin, and deposited calcite crystals inside the mucilage (Figs. 2A–2B). The crystals occurred in concentric zones, and colonies could contain a series of calcite laminations. The scytonemin pigment distribution also varied continuously throughout the colony, appearing as a series of dark brown concentric zones. The *Rivularia* colonies are perennial, and alteration of the pigmented zones shows changes in light intensity during a year. It should be noted that the largest *R. rufescens* colonies were found on the control stone with 3–4 zones coloured with scytonemin and calcite crystal deposits. The colonies on the experimental plates contained

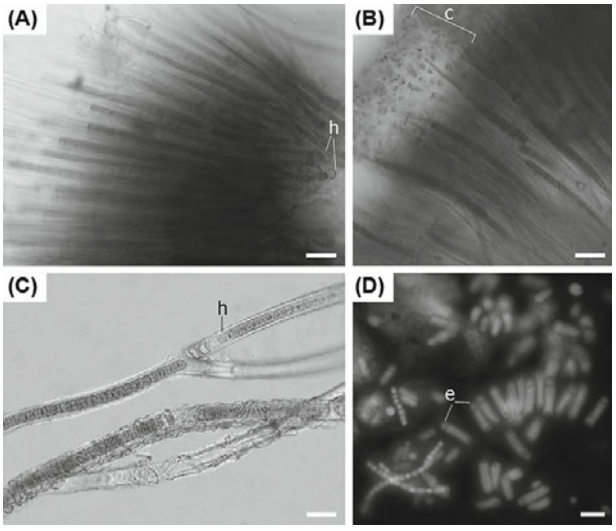

Fig. 2. Dominant cyanobacterial species in biofilms. (A–C) light microscopy, (D) fluorescence microscopy, autofluorescence of cyanobacteria, green filter. (A–B) *Rivularia rufescens*, (C) *Tolypothrix limbata*, (D) *Chamaesiphon fuscus* (cylindrical cells), and *Heteroleibleinia pusilla* (thin trichomes). Designations: c, calcite crystals zone; e, exocyte; h, heterocyst. Scale bars: A–C=30 μm; D=5 μm.

no visible calcite crystals, but did have 1–2 dark brown concentric zones. Morphological differences were likely due to the younger age of colonies on the experimental plates than on the control stone.

The highest diversity of cyanobacteria (11–12 species) was recorded on the marble and control stones (Table 1). According to the Sørensen index, the species composition of the communities from the stony substrata was 59–91%. The maximum similarity of the species composition was detected on the granite and amphibolite (K_s=91%), and marble and control stones (K_s=87%). The species cyanobacterial composition on the steel plate was least similar to the communities that developed on the natural substrates (K_s=33–36%).

Green algae of filamentous and laminar morphotypes were observed among autotrophic microorganisms in the biofilms. There were recorded numerous benthic diatoms of the genera *Cocconeis* and *Gomphonema*, and, additionally, planktonic diatoms *Synedra* sp. settled to the biofilm surface from the water column.

Taxonomic structure of biofilm communities identified by pyrosequencing

A total of 30,660 reads with an average length 430 bp was retrieved from pyrosequencing of PCR amplicons (12,844; 3,670; 3,854; 4,393; and 5,899 reads from the marble, granite, amphibolite, control stone, and stainless steel, respectively). Overall, 1.6 to 3.1% reads obtained from lithophytic communities (mean 2.2%, STD=0.6) and 10.1% from the steel plate communities belonged to the Eukarya domain, and these primarily included diatoms. Genotypes of cryptophytic and green algae were also recorded. The Bacteria domain prevailed on the substrates. The taxonomic structure of the biofilm communities was diverse on different substrates consisting of 30 bacterial phyla, and over 85% of the sequences belonged to the phyla Cyanobacteria, Proteobacteria, and Bacteroidetes (Fig. 3A).

The phylum Cyanobacteria was the most abundant in phy-

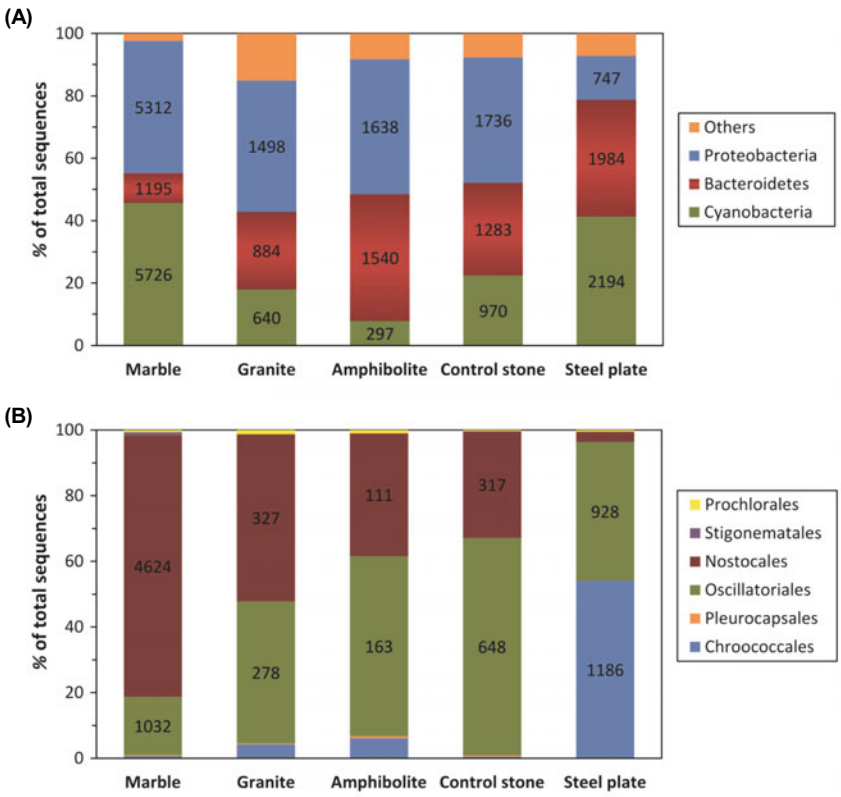

Fig. 3. Taxonomic structure of biofilm communities on different substrates. (A) the ratio of main microbial phyla; (B) the ratio of Cyanobacteria orders (according to NCBI Taxonomy Browser).

Table 2. The most numerous phylotypes in cyanobacterial biofilm communities from a 16S rRNA gene (according to GenBank taxonomy)

Substrate	Phylotype	No. of sequences	Percentage	Accession no. of closest relative	Identity (%)
Marble	*Rivularia* sp.	4 367	76.23	AM230702	95–96
	Pseudanabaena sp.	835	14.57	AJ580007	98–100
	Nostocaceae_uc	176	3.07	AM230702	94
Granite	*Rivularia* sp.	284	44.38	AM230702	95–96
	Pseudanabaenaceae_uc	235	36.72	AB179527 EF032660	93–94 93–94
	Nostocaceae uc	41	6.41	AF091150	93–94
	Microcystaceae_uc	23	3.59	CP001701	92–94
	Geitlerinema carotinosum	14	2.19	AY423710	98–99
Amphibolite	*Rivularia* sp.	103	34.68	AM230702	96
	EF580958_s	42	14.14	EF580958	99–100
	Pseudanabaenaceae_uc	26	8.75	AB179527	93–94
	AF448080_s	23	7.74	AF448080	98
	Leptolyngbya sp.	15	5.05	EF122600	96–97
	Limnothrix sp.	11	3.70	AJ580007	95
	AF448080_g_uc	10	3.37	AF448080	95–97
Control stone	*Pseudanabaena* sp.	381	39.32	AJ580007	99–100
	Rivularia sp.	201	20.72	AM230702	96
	Pseudanabaena limnetica	144	14.86	AJ007908	99–100
	Rivulariaceae_uc	106	10.93	AM230683	93
	AF448080_g_uc	33	3.41	AF448080	97
	Pseudanabaenaceae_uc	29	2.99	AB179527	93–94
	AF448080_s	20	2.06	AF448080	98
	EU340161_s	14	1.44	EU340161	99–100
	Geitlerinema carotinosum	13	1.34	AY423710	98–99
Steel plate	EF580937_s	386	17.59	EF580937	99–100
	Chamaesiphonaceae_uc	330	15.04	AY170472	93–94
	Chamaesiphon subglobosus	288	13.13	AY170472	98–100
	EF580958_s	276	12.58	EF580958	100
	EU340161_s	252	11.49	EU340161	99–100
	Chamaesiphon sp.	248	11.30	AY170472	96–97
	EF580937_g_uc	161	7.34	EF580937	95–96

lotypes on the marble and steel plate (45.7% and 41.4%, accordingly), whereas it was the third most abundant phylum on the control stone, granite and amphibolite (22.4, 18.0, and 7.8%, respectively) (Fig. 3A). Cyanobacterial taxonomic diversity was high in the biofilms. Specifically, phylotypes of six orders were heterogeneously spread on different substrates, with the maximum being observed on the marble and the minimum on the steel plate (Fig. 3B). Sequences of the orders Nostocales and Oscillatoriales dominated the communities of epilithic cyanobacteria, accounting for 80.8% and 18.0% on the marble, 51.3% and 43.6% on the granite, 37.2% and 54.7% on the amphibolite, and 32.6% and 66.6% on the control stone, respectively. Members of the orders Chroococcales (54.1%) and Oscillatoriales (42.3%) were dominant on the steel plate.

The composition of dominant phylotypes of cyanobacterial communities was similar on all stony substrates, but was quite different from the communities on the steel plate. The metagenome analysis revealed that the phylotype *Rivularia* sp. dominated on the marble, granite, amphibolite, and control stones, possessing an average of 93% of the OTUs of the order Nostocales (Fig. 3B and Table 2). Phylotypes *Pseudanabaena* sp. and *P. limnetica* dominated among Oscillatoriales on the marble, granite, and control stone, accounting for 85% and 19% of the OTUs on the amphibolite. Pyrosequencing and microscopy revealed the phylotype *Chamaesiphon* among Chroococcales on the granite, amphibolite, and control stone. All stony substrates were inhabited by *Limnothrix* sp., *Geitlerinema carotinosum*, phylotypes of the order Pleurocapsales, and the family Prochlorococcaceae. Differences in the community structures recorded on the stony substrates were attributed to minor phylotypes represented by several sequences in the communities. Phylotypes EF580987, *Chamaesiphon* sp., and C. *subglobosus* prevailed on the steel plate (Table 2). A unique phylotype EF580987 was likely referred to *Heteroleibleinia pusilla*, which is commonly found in large amounts in the biofilm during microscopic studies. Minor phylotypes with 1–2 sequences were homologous (99–100%) to *Pseudanabaena* sp., *P. limnetica*, *Pseudoformidium* sp., and uncultivated clones from the microbial mats in fresh water and thermal springs.

The results of metagenome analysis and light microscopy were consistent with each other and revealed a dominant phylotype, *Rivularia* sp. and *R. rufescens*, on the stony substrates and *Chamaesiphon* spp. on the steel plate. However, the taxonomic diversity of cyanobacterial biofilms revealed

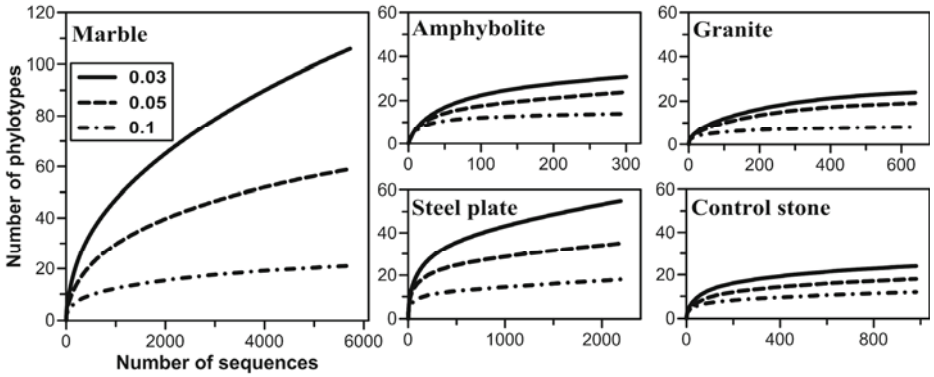

Fig. 4. Estimation of cyanobacterial diversity in biofilm communities from different substrates. Dependence of phylotype number of different level on the number of analysed sequences of a 16S rRNA gene fragment. Phylotypes are defined at different distances, as shown in the legend on the plot for the marble. Designations of x- and y-axes are as on the plot for the marble.

by pyrosequencing was one order of magnitude richer than that found in samples identified by microscopy, with 122 phylotypes versus 16 species.

To determine the cyanobacterial richness on different substrates, the non-parametric estimators ACE and Chao 1 were calculated for every library (genetic distance 0.03) and compared with the OTUs observed. For marble, the numbers of Chao 1 and ACE observed were 124.58 and 257.59, respectively, while only 35 OTUs were observed. For granite, 21.50 and 21.71 instances of Chao 1 and ACE were observed, respectively, which was close to the number of OTUs observed (21). The values of Chao 1 and ACE in the amphibolite cyanobacterial community were 41.00 and 34.38, respectively, whereas 28 OTUs were observed. For the control stone, the values of Chao 1 and ACE were 19.50 and 26.81, respectively, while the observed OTUs were calculated to be 20. For the steel plate, the values of Chao 1 and ACE were 111.00 and 153.04, respectively, while only 30 OTUs were observed. The number of unseen phylotypes was presented by the gap between the observed and estimated (ACE and Chao 1) OTUs. Thus, the cyanobacterial communities were analysed in detail in the biofilms from the granite, amphibolite, and control stone.

The species richness of communities was estimated from rarefaction curves that showed the dependence of the revealed phylotype number on the analysed sequence number (Fig. 4). The shape of rarefaction curves showed that the pyrosequencing depth obtained in this study was sufficient for complete characterisation of the cyanobacterial taxonomic composition in the biofilms on the granite, amphibolite, and control stone. By the end of the analysis, the curves reached a plateau at a cluster distance of 0.03. Distances of 0.03, 0.05, and 0.1 corresponded to species (phylotypes), genus, and family at the taxonomic level. The rarefaction curves for marble and steel plates suggested that the pyrosequencing depth was insufficient (Fig. 4); however, analysis of taxonomic composition indicated that the most numerous taxa have been already identified, and further sequencing will only help identify rare species of cyanobacteria represented by singular sequences.

Discussion

SEM studies of the biofilm structure on different substrates in Lake Baikal showed that cyanobacteria were basic components of biofilms. Filamentous and coccoid cyanobacterial species covered with mucilaginous sheaths and pseudovaginae served as a matrix for heterotrophic bacteria and diatoms. Phototrophic organisms are considered to initiate colonisation of stony substrates at sufficient light intensity, whereas heterotrophic organisms that need organic matter colonise the biofilm later. Mucilage extracted by cyanobacteria possesses adhesive properties and serves as a nutrient substrate for heterotrophic bacteria (Ljaljević-Grbić *et al.*, 2010).

The species composition of cyanobacterial biofilms in Lake Baikal consisted of the genera *Tolypothrix* and *Calothrix*, which are common cyanobacteria of the stony littoral area in large freshwater lakes (Stevenson *et al.*, 1996). According to long-term observations by Izhboldina (1990), species such as *Stratonostoc verrucosum*, *Tolypothrix distorta*, *Calothrix* sp., *C. parietina*, *Schizothrix* sp., *Oscillatoria* sp., and *Phormidium* sp. dominated annually in meio- and microphytobenthos near Bolshie Koty on the western shore of Southern Baikal (the nearest area to our observation site). In 1997–2000, *Oscillatoria amoena* and unidentified representatives of the genera *Oscillatoria* and *Lyngbya* were recorded in microphytobenthos in the same area (Rodionova and Pomazkina, 2003). The dominance of *Rivularia rufescens* during fouling of stony substrates has not been described before in Lake Baikal. The nearest known habitat of this species is freshwater oligotrophic Lake Hovsgol in Mongolia (Elenkin, 1949). However, cyanobacteria of the genus *Rivularia* are recorded more often in calcareous streams, and their ability to calcify and form stromatolites was described by Pentecost and Edwards (2002). Visible areas of deposition of calcite crystals were only recorded by us in *Rivularia* colonies on the control stone, which was likely due to their older age when compared to the colonies on the experimental plates. The water in Lake Baikal is ultra-fresh, containing bicarbonate and calcium with low mineralisation. The total content of dissolved salts in the Baikal water does not exceed 100 mg/L and Ca^{2+} – 16 mg/L, respectively (Grachev *et al.*, 2004), and it is likely that the formation of calcified zones in the colonies occurs very slowly under such conditions.

The difference in the species composition of epilithic cyanobacteria on experimental stony plates exposed to similar conditions (habitation depth, temperature, pH, light, concentration of nutrients in water, and exposition time) is probably

associated with the physical and chemical characteristics of the substrates. The rock structures have differing porosities, which influence the absorption of microorganisms and colonisation of the substrate (Guillitte and Dreesen, 1995). In the series marble-granite-amphibolite, which was positioned in this study according to a decrease in cyanobacterial species diversity, the marble had the highest porosity and amphibolite the lowest, confirming the significance of this factor. Different chemical compositions of substrates can also affect epiliths. Microorganisms in biofilms play a role in biodestruction of rocks, as well as metal leaching (Gorbushina and Krumbein, 2005). The chemical composition of stony plates used in this study was identified earlier (Parfenova *et al.*, 2008). For example, the marble contained minimal concentrations of heavy metals able to inhibit the growth of cyanobacteria, whereas the experimental granite and amphibolite plates had high and very high concentrations of heavy metals. The inhibitory effect of zinc, lead, cobalt, nickel, and chrome on physiological processes and growth of cyanobacteria has been demonstrated in numerous studies (Azeez and Banerjee, 1991; Prasad *et al.*, 1991; Ybarra and Webb, 1999; Sekar *et al.*, 2004). Hence, the low species diversity of epilithic cyanobacteria on the granite and amphibolite and high diversity on the marble were consistent with the presence of high concentrations of certain heavy metals. In addition, the preference of cyanobacteria for the marble may be attributed to its high calcium content. It is known that many species of epilithic cyanobacteria are calciphils characterised by selective growth on different substrates. For example, the analysis of fouling in 45 European monuments showed that the species diversity of cyanobacteria on marble was 5.5 times higher than that on granite (Tomaselli *et al.*, 2000).

The low species diversity in the biofilm from the stainless steel plate with the dominance of *Chamaesiphon fuscus*, *Ch. subglobosus*, and *Heteroleibleinia pusilla* (which were minor species on the stony substrates) was likely due to the smooth steel surface and its chemical inertness. The cyanobacteria on the steel plate had small cell sizes, sheaths, and pseudovaginae, which likely allowed them to attach to the smooth substrate and colonise the steel plate while consuming nutrients from the aquatic environment. Experiments with different types of substrates demonstrated that cyanobacteria, green algae and diatoms attached more easily to titanic and stainless steel surfaces than to glass, copper, and its alloys (Sekar *et al.*, 2004).

Pyrosequencing of the 16S rRNA gene revealed that the taxonomic structure of biofilm communities was diverse on different substrates. However, 85% of sequences belonged to the phyla Cyanobacteria, Proteobacteria, and Bacteroidetes. The same phyla (DGGE analysis) dominated the epilithon structure in European and Japanese rivers (O'Sullivan *et al.*, 2002; Honma *et al.*, 2009). The planktonic bacteria of Lake Baikal were dominated by Bacteroidetes, Actinobacteria and Proteobacteria (Parfenova *et al.*, 2013). Metagenome analysis and light microscopy revealed that the dominant phyla were *Rivularia* sp. and *R. rufescens* on the stony substrates and *Chamaesiphon* spp. on the steel plate. The phylotype *Pseudanabaena limnetica* (=*Oscillatoria limnetica*), a freshwater cosmopolitan species capable of anaerobic photosynthesis (Oren and Padan, 1978), was detected on all substrates except amphibolite during metagenome analysis, but not upon microscopic analysis.

Overall, cyanobacterial species diversity in the biofilms was one order of magnitude higher when pyrosequencing was used than when microscopic analysis was conducted. Discrepancies in estimates of species diversity based on microscopic and genetic approaches are often observed during comprehensive studies of different microbial communities (Fuhrman and Campbell, 1998; Brambilla *et al.*, 2001). One of the reasons for this may be microdiversity, which is commonly found in molecular ecology studies (Fuhrman and Campbell, 1998). However, technical problems should also be taken into account. For example, a recent communication by Gomez-Alvarez *et al.* (2009) reported a systematic error in metagenomes generated by 454 pyrosequencing and introduced by artificial replicates that may cause overestimation of the gene and taxon abundance by up to 35%. A number of publications dedicated to this problem (Quince *et al.*, 2009; Reeder and Knight, 2009; Kunin *et al.*, 2010) revealed obvious overestimation of microbial diversity by studies using pyrosequencing. The 3% threshold of intraspecific differences is the average used for all bacterial groups; however, this value is currently the subject of debate. Conversely, the divergence of bacterial strains per 1% of nucleotide substitutes in a marker 16S rRNA gene allows their identification as separate species (Stackebrandt and Ebers, 2006). Nevertheless, intraspecific differences among certain species of filamentous cyanobacteria can reach 7% (Ezhilarasi and Anand, 2009), which also adds to errors in estimation of phylotype number during the analysis of pyrosequencing data.

In conclusion, this paper demonstrated the presence of distinct dominant cyanobacterial species on stony substrates and a steel plate. The biofilm from the steel plate was thinner, with minimal vertical development, and consisted of *Chamaesiphon* spp. and *Heteroleibleinia pusilla*. Macroscopic colonies were observed on stony plates and were dominated by *Rivularia* and *Tolypothrix* associations. Differences in the cyanobacterial species richness are more likely to be attributed to physical and chemical characteristics of the substrates. Twelve of 16 species identified in our study have not been described for Lake Baikal before. Metagenome analysis of microbial communities showed significantly higher diversity of phylotypes when compared with the species richness revealed by microscopy. However, the dominant phylotypes were similar to the species identified by microscopy.

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