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PRIMARY RESEARCH PAPER

Presence and genetic diversity of microcystin-producing cyanobacteria (*Anabaena* and *Microcystis*) in Lake Kotokel (Russia, Lake Baikal Region)

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Abstract A survey was conducted for the presence of cyanobacteria toxins in Lake Kotokel due to a few cases of Haff disease registered in 2008–2009 caused by consumption of fish from Lake Kotokel, and wildlife mortality including large fish kill. The aims of this study were to determine what cyanotoxins (if any) were present in the lake, to describe phytoplankton composition including morphology, density, and species diversity of cyanobacteria, as well as to evaluate the trophic state of the lake. Samples were collected from both nearshore and central sites in August of 2009. *Aphanocapsa holsatica* dominated the phytoplankton. The presence of toxigenic genotypes of *Microcystis* spp. and *Anabaena lemmermannii* was detected by sequencing of PCR-amplified aminotransferase domain of microcystin synthetase gene. LR, RR, and YR microcystin (MC) variants were detected with liquid chromatography-UV mass spectrometry. The data do not shed light on the etiology of Haff disease in Lake Kotokel region, nevertheless taking into account the recreational importance of the lake and its direct connection to Lake Baikal, a necessity to monitor cyanobacteria in these water bodies is evident. This is the first report on simultaneous detection of MC-producing genotypes and MCs in the Lake Baikal region.

Keywords Toxic cyanobacteria · Microcystin · *mcy*E gene · HPLC · LC/MS · Lake Kotokel · Lake Baikal

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Introduction

Cyanobacteria produce a range of secondary bioactive metabolites, including toxins. One of the best studied cyanotoxins in freshwater ecosystems is the group of microcystins (MCs). MCs are cyclic heptapeptides produced nonribosomally by peptide synthetases, polyketide synthases, and tailoring enzymes. Over 90 variants of MCs are known (Welker & Von Döhren, 2006), the most common of them differ in L-amino acids and in modification by methyl groups. MCs specifically inhibit the eukaryotic serine/threonine phosphatases 1 and 2A rendering the hyperphosphorylation of proteins in the hepatocyte cytoskeleton, which leads to massive hepatocyte necrosis and pooling of blood into the liver (MacKintosh et al., 1990). Chronic exposure to sublethal doses of MCs resulted in endemic cancer of the liver in one of the provinces of China (Chorus & Bartram, 1999).

Predominant MC-producing cyanobacteria belong to the *Anabaena*, *Microcystis*, *Planktothrix*, *Anabaenopsis*, *Hapalosiphon*, and *Nostoc* genera (Chorus & Bartram, 1999). Planktonic cyanobacteria, including

unicellular colony forming *Microcystis* and the filamentous, heterocystous *Anabaena*, commonly form toxic blooms in highly productive freshwater ecosystems worldwide (Codd et al., 2005). Therefore, many countries monitor the presence and safe concentrations of highly toxic MC-LR, which contains leucine and arginine as variable L-amino acids. The LD50 (lethal dose, 50%) of MC-LR (given intraperitoneally to mice) is 50 μg kg^{-1}, MC-RR- (arginine–arginine) and MC-YR- (tyrosine–arginine) variants are less toxic. The World Health Organization (1998) has set an advisory level for MCs in drinking water of 1.0 μg l^{-1} MC-LR.

Several detection methods for MCs are commonly used, including high performance liquid chromatography (HPLC), immunoassays, and protein phosphatase inhibition assays (reviewed in Carmichael & An, 1999). However, liquid chromatography–mass spectrometry (LC/MS) provides a greater specificity and sensitivity (Chorus & Bartram, 1999).

Identification of a gene locus of microcystin synthetase provided a powerful tool to detect potential toxigenic species (Nishizawa et al., 1999; Rouhiainen et al., 2004). MCs producing multienzyme complex is encoded by a 55 kb-long gene cluster containing 10 genes designated mcyA–mcyJ (Rouhiainen et al., 2004). One of the enzymes, the aminotransferase (AMT) plays a crucial role in cyanotoxin producing by transferring amino groups to the side chain of the Adda moiety (Moffitt & Neilan, 2004). The gene for AMT domain locating between microcystin synthetase gene E and nodularin synthetase gene F has been found suitable for the detection of MC or nodularin producing *Microcystis*, *Planktothrix, Anabaena, Nostoc* and *Nodularia* strains (Jungblut & Neilan, 2006). In phylogenetic analysis, *mcy*E sequences from different toxin producer genera form their own groups and are not affected by lateral gene transfer (Rantala et al., 2004). Thus, the primers for AMT region are suitable for reliable detection and identification of main cyanotoxin producers in environmental samples.

Lake Kotokel is situated close to the eastern coast of Lake Baikal (Eastern Siberia, Fig. 1). In 2008, mass mortality of fish, birds, and domestic animals was recorded in Lake Kotokel and its neighborhoods. Sixteen persons were hospitalized with symptoms of Haff disease: severe muscular pains, rhabdomyolysis (rapid breakdown of skeletal muscle), and myoglobinuria. The etiology is not yet known, but there is little doubt that the disease is due to an unknown, natural toxin. In June of 2009, the Ministry of Health and Social Development of Russia forbade eating fish caught in the lake, as well as bathing in and drinking the water. Analysis of phytoplankton samples obtained in Lake Kotokel in August of 2008 revealed the presence of potentially toxic *Microcystis* bearing microcystin synthetase gene E, however, the analysis for the appearance of MCs in the water samples was not performed (Belykh et al., 2010). Therefore, Lake Kotokel became a subject of the interdisciplinary investigation.

Lake Kotokel was thoroughly studied in the 1950–1980s due to its recreational and mainly commercial value for the region—the annual fish catch exceeded 1,200 tons (Kuzmich, 1988). It was a eutrophic water body, cyanobacteria were the most abundant phytoplankton group, and *Gloeotrichia echinulata* was the dominant species (Korde, 1968; Kuzmich, 1988). *Microcystis aeruginosa*, *M. pulverea*, and *Anabaena lemmermannii* proliferated less intensively. In summer, the water transparency dropped to 0.6–0.8 m and the water temperature sometimes reached 25.8°C. Mass development of phytoplankton in July–August resulted in a decrease of phosphate and mineral nitrogen concentration; in other seasons, the content of these nutrients was higher (Kuzmich, 1988).

In Siberia, the first report on finding of microcystin synthetase genes for potentially toxic *M. aeruginosa* and *Anabaena* sp. was concerned to Ust-Ilim and Bratsk reservoirs fall within of Lake Baikal watershed (Tikhonova et al., 2006, 2007). However, *mcy* genes have not been detected in any samples either from the pelagic zone or the shallow areas of Lake Baikal.

The aim of this work was to comprehensively investigate the phytoplankton of Lake Kotokel including morphology, density, and species diversity of cyanobacteria, to evaluate the trophic state of the lake, to measure the concentration of different variants of the MCs (if they were), as well to find potential toxin producers by the PCR-amplified *mcy*E genes.

Materials and methods

Site description

Lake Kotokel is located in the south-central region of Siberia (Russia), 2 km off the eastern coast of Lake

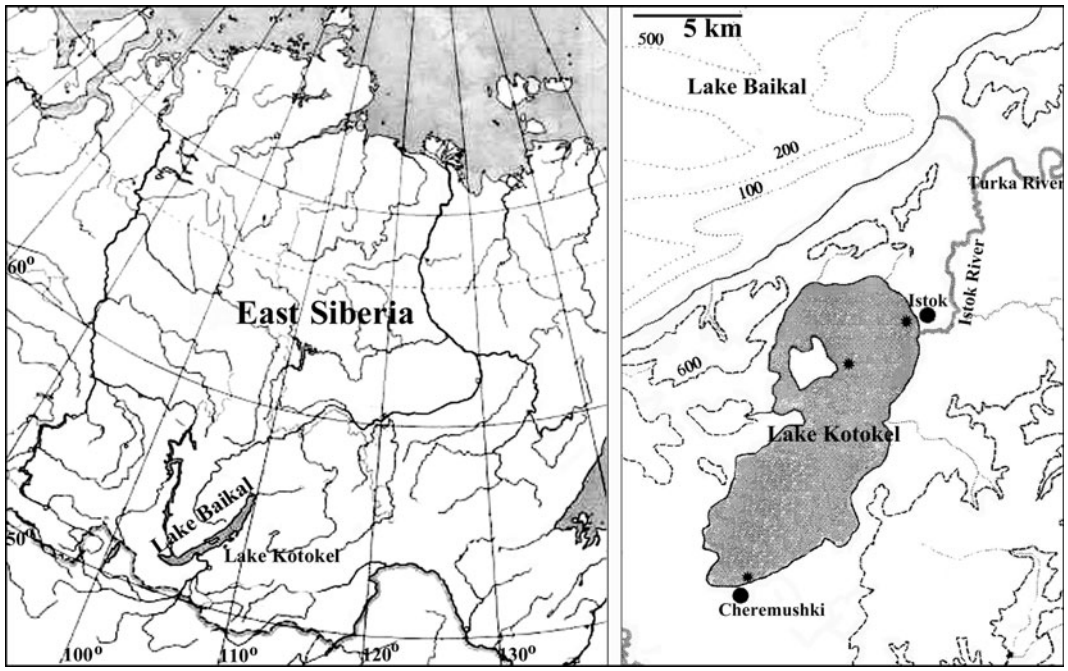

Fig. 1 Sampling sites in Lake Kotokel, 2009

Baikal (Fig. 1). It is separated from Baikal by a low mountain chain. The elevation of Lake Kotokel is only 5 m higher than that of Lake Baikal. The Istok River flows from Lake Kotokel and joins the Turka River, which flows into Lake Baikal. The area of Lake Kotokel is 68.9 km^2 and the catchment area is 150 km^2. The water balance of the lake provides continuous discharge into Lake Baikal at a rate of 1.3 $m^3\ s^{-1}$, and the time of conventional water retention is close to 7 years. The lake is 15 km long and 6 km wide. It has an average depth 3.5 m, and its maximum depth is 14 m (Kuzmich, 1988).

The lake has low-lying and swampy shores often covered with an aquatic plant community consisting of *Carex*, *Equisetum*, and *Typha*. Aquatic macrophytes *Phragmithes*, *Potamogeton*, *Polygonum,* and species *Elodea canadensis* grow up to 1.5 m, and *Ceratophyllum* occurs up to a depth of 3 m (Korde, 1968; Kuzmich, 1988).

Sample collection and processing

In August of 2009, plankton samples were collected with a Ruttner water sampler and Apstein net (mesh size 16 μm) at a pelagic site in the central part (52°49′543″, 108°07′357″, maximal depth 14 m) and at a littoral site near village Cheremushki (52°45′533″, 108°07′557″, depth 1 m) in the southern part of Lake Kotokel (Fig. 1). Sampling depths were 0, 6.5, and 13 m. Water temperature and Secchi depth were also measured. Bottle phytoplankton samples (0.5 l) were fixed with Lugol's solution and concentrated by sedimentation to a volume 0.1 l. Net phytoplankton samples were fixed with 2% formalin for microscopic observations and with 80% ethanol for molecular analysis. A 0.1-ml aliquot of the settled sample was pipetted in a Nageotte chamber, and cyanobacteria were counted in triplicate, under a light microscope at a magnification of 400× and 1000× (Axiovert 200, Zeiss). The biovolume of cyanobacteria cells was calculated from microphotographs obtained using a Penguin 600CL camera (Pixera Corp., USA) and the VideoTest-Razmer 5.0 software package (www.videotest.ru).

Nutrients were measured by colorimetric methods: phosphates with phosphorous-molybdate, ammonium with Nessler's reagent, and nitrite with the Griess reagent (Semenov, 1977). Nitrate was analyzed by HPLC. The total phosphorus was measured in water samples after oxidation with persulphate. The total phosphate was then detected by the methods

described above for inorganic phosphate. Determination of permanganate and dichromate oxidizability was carried out by titration in an acid medium.

The concentration of chlorophyll *a* was measured by spectrophotometry. Samples (0.5 l) were filtered onto 0.45 μm Millipore polycarbonate filters, extracted using hot methanol, and then analysed with a spectrophotometer (Bio-Rad SmartSpec Plus, USA).

Microcystin analyses

Net samples of phytoplankton were frozen and thawed (5 times) and then dried (60°C) prior to toxin extraction. The dried phytoplankton biomass (178.2 mg) was extracted twice (10 ml each time) with methanol by sonication in an ultrasonic bath (1 h). The combined raw extracts were centrifuged (at 5,263 rpm for 15 min), and the supernatant was dried in a rotary vacuum evaporator IKA RV 05-ST (Werke GmbH & Co. KG, Germany). The extract obtained was redissolved in 0.5 ml of methanol prior to Liquid chromatography–mass spectrometry analysis. LC/MS analyses were performed on an Agilent HP1200 chromatographic system that was coupled to an Agilent time-of-flight mass spectrometer (ESI-TOF) equipped with an electrospray ionization source operating in the positive ionization mode. Chromatographic separation of MCs was carried out using a Zorbax 300 SB C18 column (2.1 × 150 mm, 5 μm) maintained at 35°C, at a flow rate of 0.3 ml min^{-1}, with a gradient of 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B). The solvent program was as follows: 10% B at time zero, 10-80% B for 30 min, 100% B for 5 min, and 100% B for 40 min. The injected sample volume was 10 μl, and the detection was performed with a diode array UV detector at 214, 222, 238, and 330 nm. Quantification was achieved by measuring the chromatographic signals of each MC against calibration curve produced from "Microcystins-LR standard" (Biosense Laboratories, Norway), as they have the same extinction coefficients as MC-LR.

Genetic analyses

Amplification, cloning, and sequencing

DNA from the phytoplankton samples was extracted using "DNA-sorb" extraction kit (InterLabService, Russia). *Mcy* gene fragments were PCR amplified with the HepF and HepR gene specific primers (Jungblut & Neilan, 2006) in the Peltier Thermal Cycler (MJ Research, USA). PCR was performed in 50 μl reaction mixtures containing 0.1–0.4 μM of each primer, 0.25 mM of dNTPs, 2.5 mM of $MgCl_2$, buffer (20 mM TrisCl, pH 8.4, KCl 40 mM), Taq DNA polymerase (1.2 units) (Fermentas, MD, USA), and 10–15 ng of DNA template. Cycling conditions were as follows: initial denaturation at 95°C for 5 min, then 35 cycles of 95°C for 0.5 min, 55°C for 0.5 min, and 72°C for 1 min followed by 10 min at 72°C to complete the extension. PCR products of expected size (~470 bp) were gel-purified using the "PCR Clean-Up Gel Extraction NucleoSpin Extract II kit" (Macherey-Nagel, Germany) and subcloned into *E. coli* XL1BL cells using "InsTAclone" (Fermentas, MD, USA). 100 cloned amplicons were processed for sequencing. Plasmid inserts were sequenced bidirectionally by using vector-specific primers on the CEQ 8800 Genetic Analysis System and using DTSC Quick Start Kit (Beckman Coulter, USA). All unique sequences from this study have been deposited in GenBank under accession numbers GU186843–GU186846.

Phylogenetic analyses

Newly decoded *mcyE* gene sequences were used in a tBLASTn search against the NCBI "nr" database (http://www.ncbi.nlm.nih.gov/BLAST/) to find close relatives. The most similar reference sequences, preferably of cultivated strains, were downloaded and aligned with Kotokel isolate sequences using the Clustal W program (MEGA software; Tamura et al., 2007). The data set was analyzed by neighbor-joining (NJ), maximum likelihood (ML), and Bayesian (BI) methods to infer trees and estimate branch support. Evolutionary distances for the NJ tree (MEGA software; Tamura et al., 2007) were calculated by the Kimura-2 parameter model; the tree topology was evaluated by bootstrap support in 1,000 replicons. ML calculations were performed using PhyML software (Guindon & Gascuel, 2003), and the most appropriate evolutionary model (GTR + G) for the nucleotide sequences was set. The model was generated by the Model Generator program (http://distributed.cs.nuim.ie/multiphyl.php). BI tree topology was calculated using a Bayesian Markov chain

Monte Carlo method implemented by the program Mr. Bayes v3.1.2. (Ronquist & Huelsenbeck, 2003). One million generations were run, and the likelihood function stabilized after 2,000 generations; these trees were discarded, and every hundredth of the remaining trees was used to drive the final consensus tree according to the 50% majority rule.

Results

Environmental parameters

Temperature, Secchi disk transparency, concentrations of total phosphorus, nutrients, permanganate and dichromate oxidation, and chlorophyll *a* in the Lake Kotokel pelagic area are presented in Table 1. Based on these data, the trophic state of Lake Kotokel was assigned as eutrophic according to the Vollenweider & Kerekes classification (1982).

Intracellular microcystin concentration

The extracts from phytoplankton samples analyzed by HPLC exhibited three major peaks with the following retention times (T_R): 14.50, 16.55, and 16.86 min (Fig. 2); the UV spectra were typical of MCs. LC/MS analysis revealed the presence of three MCs with characteristic m/z values of MC-RR (m/z = 519.8089 $[M + 2H]^{2+}$), MC-LR (m/z = 995.5874 $[M + H]^{+}$), and MC-YR (m/z = 1045.5661 $[M + H]^{+}$) (Fig. 2). The total content of MC was 53 μg g^{-1} of dry weight and the ratio of MCs was the following: MC-RR—49%; MC-LR—42.5%; and MC-YR—8.5%.

Species composition and abundance

Twenty-five species of planktonic cyanobacteria were identified in the pelagic samples taken in August, 2009. Half of them were considered dominant species (the number of each exceeded 50×10^3 cell l^{-1}), namely: *Aphanocapsa holsatica*, *Aph. delicatissima*, *Aph. conferta*, *Aph. incerta*, *Microcystis aeruginosa*, *M. viridis*, *Chroococcus limneticus*, *Coelosphaerium kuetzingianum*, *Snowella rosea*, *Romeria* sp., *Pseudanabaena voronichinii*, and *Planktolyngbya contorta*. *Microcystis wesenbergii*, *A. lemmermannii*, *A. scheremetievi*, *A. circinalis*, and *A. spiroides* only occurred in the net samples. Diatom *Aulacoseira granulata* and green algae *Coelastrum cambricum*, *Scenedesmus communis*, *Dimorphococcus lunatus,* and *Pediastrum* spp. dominated in the eukaryotic phytoplankton.

The mean abundance and biomass of cyanobacteria in the 0–13 m layer were 7.13×10^9 cell l^{-1} and 3.87×10^3 mg m^{-3} that consisted of 99.97 and 83.07% of total phytoplankton values, accordingly. *Aph. holsatica* had the highest cell density (7.11×10^9 cell l^{-1}) and the maximum biomass (3.77×10^3 mg m^{-3}); its contribution to the total cyanobacterial biomass reached 97.28%. The quantitative indices of *Microcystis* species were low and their contribution to cyanobacteria total biomass did not exceed 0.55%. The concentration of *Microcystis viridis*, which was the most abundant among *Microcystis* spp., was 2.14×10^5 cell l^{-1} (biomass 14.03 mg m^{-3}), whereas that of *M. aeruginosa* was 1.12×10^5 cell l^{-1} (biomass 7.15 mg m^{-3}). The cell number and biomass of *Anabaena* spp. were low, 3.30×10^4 cell l^{-1}, 2 mg m^{-3}, respectively.

Genetic analysis of *mcy*E

Decoded sequences of one hundred of the cloned amplicons can be divided into four groups, designated as K1-09, K6-09, K7-09, and K8-09 (index "09" underlines clones obtained in the study), and each of them contained 12, 30, 30, and 28 identical sequences, respectively.

A sequence of each type served as a query in a tBLASTn search against the NCBI "nr" database. The search revealed that close homologes of K6-09, K7-09,

Table 1 Mean limnological variables at the central pelagic site (0 m) of Lake Kotokel, August 2009

	T (°C)	Secchi depth (m)	TP (mg l^{-1})	PO_4^{3-} (mg l^{-1})	NO_3^- (mg l^{-1})	NH_4^+ (mg l^{-1})	NO_2^- (mg l^{-1})	PO (mgO l^{-1})	DO (mgO l^{-1})	Chl *a* (μg l^{-1})
Mean	22.6	0.7	0.037	0.006	0.08	0.34	0.016	6.3	21.6	11.8
SD	0.3	0.1	0.005	0.002	0.01	0.04	0.005	0.6	3.1	1.2

T water temperature, *TP* total phosphorus, *PO* permanganate oxidation, *DO* dichromate oxidation, *Chl a* chlorophyll *a*, *SD* standard deviation

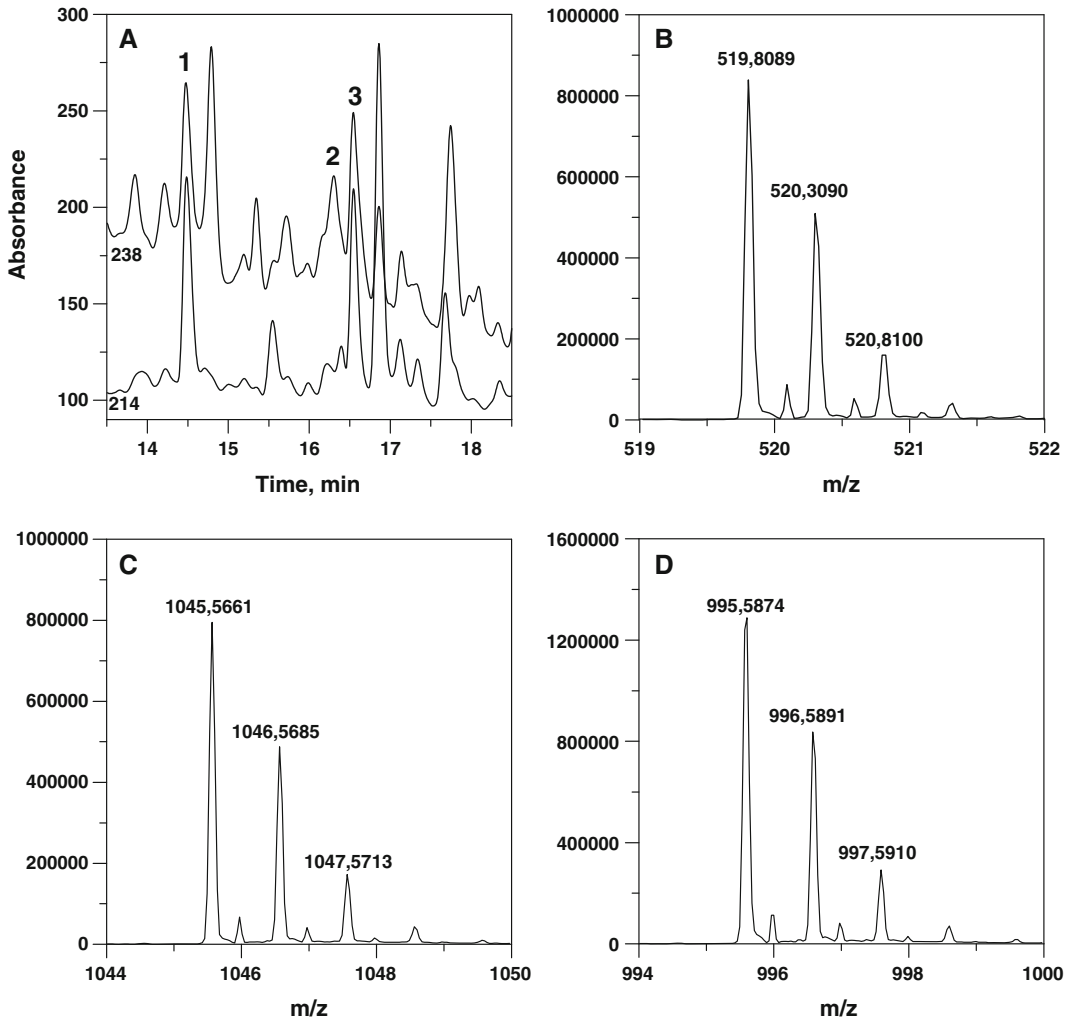

Fig. 2 HPLC/MS determination of microcystins extracted from the Lake Kotokel phytoplankton (for experimental details see text). **A** HPLC/UV chromatogram of raw extract of microcystins: *1* microcystin-RR ($T_R = 14.50$ min), *2* microcystin-YR ($T_R = 16.55$ min), *3* microcystin-LR ($T_R = 16.86$ min). **B–D** ESI-TOF mass spectra for the peaks *1* (**B**), 2 (**C**), *3* (**D**)

and K8-09 sequences belong to *Microcystis* spp., whereas *Anabaena* spp. are the nearest relatives of the K1-09 sequence. Pair-wise comparisons of *mcy*E gene fragments of cyanobacteria isolated from geographically distinct freshwater bodies, including Lake Kotokel, show that *Microcystis* spp. differ by no more than 4% of nucleotide changes, whereas the dissimilarities in sequences of *Anabaena* and *Microcystis* genera reach up to 21% of the substitutions. K6-09 and K7-09 *mcy*E gene sequences share up to 98–99% identity with the strains of *M. viridis* NIES-102, *M. aeruginosa* NIES-843, *M. aeruginosa* K-139, and *M. wesenbergii* NIES-107 isolated during toxic bloom from Lakes Kasumigaura and Kawaguchi in Japan. K8-09 fragment shows 99% similarity with sequences of some strains of *M. aeruginosa* (PCC 7806, PCC 7005, UTEX B 2667) isolated from different locations in Europe and North America. K1-09 shows 100% homology with many of the sequences of *Anabaena*-species found in 9 Scandinavian lakes, for example *Anabaena* sp. SYKE 971/6 (Lake Kotojärvi, Finland) or *A. lemmermannii* NIVA-CYA 270/1 (Arefjordsvatnet, Norway).

As all tree-constructing methods resulted in similar topologies of the *mcy*E gene phylogeny, the unrooted neighbor-joining tree only is shown in Fig. 3. The branching of *mcy*E genes corresponds to highly supported diversification according to

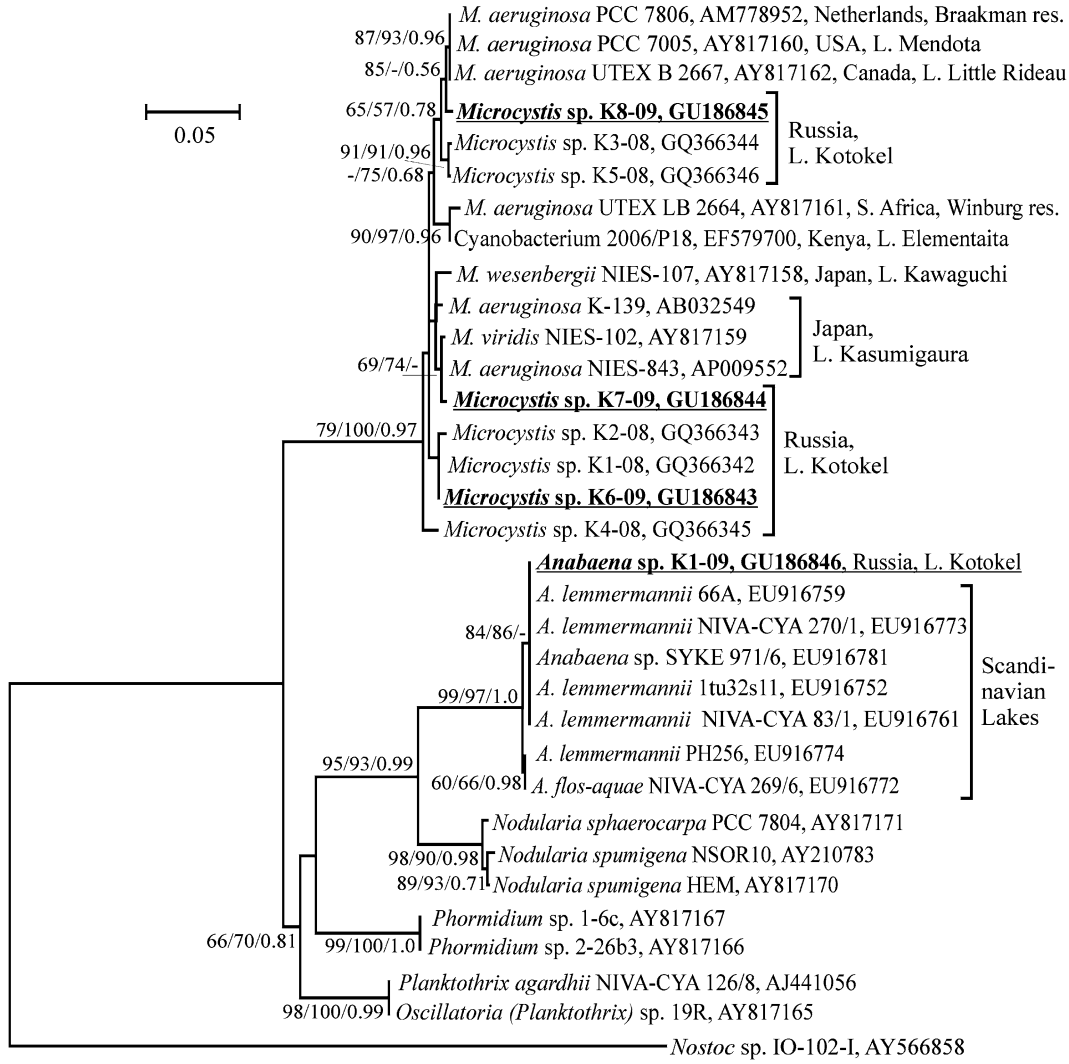

Fig. 3 Phylogenetic analysis of 470 bp *mcy*E and *nda*F gene sequences. The neighbor-joining phylogenetic tree is shown. Neighbor-joining, maximum likelihood bootstrap values (BP > 50%), and Bayesian posterior probabilities (PP > 0.5) are given at common branch points (NJ/ML/BI). Sequences of the *mcy*E gene obtained by authors are underlined. The strain *Nostoc* sp. IO-102-I has been chosen as an out-group

Microcystis, *Anabaena*, *Nodularia*, *Planktothrix*, and *Phormidium* genera. Three new *mcy*E sequences from Lake Kotokel (K6-09, K7-09, and K8-09 sequences) are reliable examples of the *Microcystis* genus. The internal relationships of the *Microcystis* clade are uncertain and represented by threefold polytomies. K6-09 and two more sequences from Lake Kotokel (K1-08 and K2-08), isolated in 2008 (Belykh et al., 2010), form a monophyletic group. The monophyly of K8-09 and two more Lake Kotokel sister sequences K3-08 and K5-08, as well as the relatedness of K8-09 to sequences from geographically distant water bodies has weak support (37%). K7-09 is the closest relative to the gene fragments of cyanobacteria from mesotrophic lakes of Japan. Finally, the K1-09 sequence belongs to the *Anabaena* genus clade and is identical to some of *A. lemmermannii* strains sequences from Scandinavian lakes. In general, among potentially MC-producing genotypes identified in Lake Kotokel in 2009, the single K1-09 is attributed to *A. lemmermannii*, whereas K6-09, K7-09, and K8-09 sequences belong to different species within the *Microcystis* genus.

Discussion

Lake Kotokel is a eutrophic water body, as stated earlier (Kuzmich, 1988), and confirmed by our data on the concentration of total phosphorus, content of chlorophyll *a* and water transparency. In the summer of 2009, the concentrations of phosphate and mineral nitrogen were lower 1.7 and 3.6 times, accordingly, in comparison with the 1990s (N. M. Pronin, personal communication). It was probably caused by some reduced anthropogenic impact on the lake due to the depletion of agricultural activity in this area. Moreover, in the summer of 2009, the low concentrations of nutrients in Lake Kotokel might be attributed not only to the phytoplankton bloom but also to the high development of aquatic plants, which are diverse in the lake.

An invasive macrophyte *Elodea canadensis*, unlike in most water bodies of Siberia, proliferates in Lake Kotokel, especially in the coastal zone near camp sites and residential buildings. This species facilitates the utilization of the nutrients in the lake, preventing the transition of the lake to the hypereutrophic state (Kuzmich, 1988). It has been shown that there are competitive relationships between cyanobacteria and aquatic macrophytes—the major primary producers in small eutrophic lakes (Li et al., 2009). Aquatic plants such as *E. canadensis*, *Ceratophyllum*, and *Phragmithes* spp. overgrown the lake are known to accumulate and transform MCs (Pflugmacher et al., 1999, 2001). Considering that the littoral zone covered by aquatic plants extends to a depth of 2 m and occupies 23% of the Lake Kotokel surface (Kuzmich, 1988), it is possible that macrophytes can decrease the content of toxins in the water.

Aphanocapsa holsatica (previously known as *Microcystis holsatica*) was a dominant species of planktonic cyanobacteria in the lake in August of 2009 (97.28% of total abundance), as well as in the summer of the previous year (53% of total abundance, Belykh et al., 2010). This species is nontoxic (Yasuno et al., 1998) and differs in 16S rDNA sequence from the *Microcystis* genus, despite minor morphometric differences (Neilan et al., 1997). Previously, the morphological similarity of *Aph. holsatica* and *Microcystis* spp. probably often led to their misidentification in Lake Kotokel samples. In August of 2008, besides *Aph. holsatica*, the potentially toxic *M. viridis* also dominated in the phytoplankton of Lake Kotokel (36% of total abundance). Other cyanobacteria species, including *M. aeruginosa*, made up less than 5% of the total number (Belykh et al., 2010). As there were no data on water chemistry in August of 2008, we can speculate only about the decreasing of the development of *Microcystis* compared to August of 2009. Heavy organic input from nearby camp sites and residential buildings might result in the dominance of *M. viridis* as a more resistant beta-mesosaprob to pollution (Barinova et al., 2006). Its abundance reduced in 2009 possibly due to the ban of the recreational use of the lake.

In 2009, the samples were collected in August, after the bloom. The development of cyanobacteria was low (3.87 g m^{-3}), and the biomass was 4 times lower when compared to the maximal biomass recorded in July 1986 by other authors (Kuzmich, 1988). The chlorophyll *a* concentration was high due to abundant development of cyanobacteria ($\sim$80% of total biomass) and also to the impact of green and diatom algae. Previous results (Korde, 1968; Kuzmich, 1988) and our survey show that a complex of species typical of shallow well-warmed productive water bodies has been developed in Lake Kotokel over the last 50 years. It is characterized by a high abundance of diatoms, green algae and cyanobacteria.

The plankton cyanobacteria of Lake Kotokel like *Microcystis aeruginosa*, *M. viridis*, *M. wesenbergii*, *A. lemmermannii*, *A. flos-aquae*, and *A. circinalis* are known as potential MC producers. We amplified 10 diverse cyanobacterial sequences of microcystin synthetase genes from Lake Kotokel samples collected during 2 years of our observations (Belykh et al., 2010): nine of them belonged to *Microcystis* genus, the tenth was identical to *A. lemmermannii*. The total number of clones containing the *Microcystis* gene insert was 9 times higher than those with the *Anabaena* gene. Similarly, in lakes of Finland where *Anabaena* and *Microcystis* often form hepatotoxic mass occurrences, *Microcystis mcy*E gene copy numbers were more abundant than those of *Anabaena*; both toxic and nontoxic strains of *Anabaena* existed, whereas all strains of *Microcystis* spp. were toxic (Vaitomaa et al., 2003). The data correlate with records that *Microcystis*-species are the most common and significant MC producers in freshwater environments. Eighty to ninety percent of the samples

from the water bodies of Denmark, Germany, Czech Republic, and Korea, where *Microcystis* dominated, contained MCs (Chorus & Bartram, 1999). Coexistence of toxigenic *Microcystis* and *Anabaena* species, like in Lake Kotokel, was detected in 15% of mesotrophic and 25% of eutrophic lakes in Finland (Rantala et al., 2006). In four other examined Siberian water bodies, the genera occurred as well (Tikhonova et al., 2006, 2007). In oligotrophic Lake Baikal and Irkutsk reservoir, cyanobacteria were nontoxic. In a mesotrophic Bratsk reservoir, the *mcyA* genes were found in *Anabaena* species. In Ust-Ilim reservoir, which is a mesotrophic also, both *mcyA* and *mcyE* genes of *Microcystis* genus were detected (Tikhonova et al., 2006, 2007). Thus, simultaneous development of toxic cyanobacteria of the both genera was observed only in the eutrophic Lake Kotokel.

The total value of potentially toxic *Microcystis* and *Anabaena*-species in Lake Kotokel in August 2009 was low (3.59×10^5 cell l^{-1} and 23.2 mg m^{-3}) and similar to that detected in the oligotrophic Lake Baikal during the warmest season (Belykh et al., 2007). Nevertheless, the total abundance of cyanobacteria in Lake Kotokel was higher than that recommended by WHO guidelines for recreational water reservoirs averaging 7.13×10^9 cell l^{-1}. The recreational use of water is not recommended in many countries when the concentrations of cyanobacteria exceed 20×10^6 cell l^{-1} (Chorus & Bartram, 1999).

However, the intra-cellular concentrations of MCs (MC-RR, MC-LR, and MC-YR) were not high in the Lake Kotokel phytoplankton (53 μg g^{-1} DW). For comparison, the highest total concentration of MCs detected by HPLC in phytoplankton of Kenya water bodies was 19,822 μg g^{-1} DW, which is similar to that found in the cyanobacterial bloom samples from Germany that contained 14,700 μg g^{-1} DW (Jungmann et al., 1996; Ballot et al., 2003). In general, the average MC concentration in water during the *Microcystis* bloom was seven times higher than that for mass development of *Anabaena* (Fastner et al., 1999).

Microcystis and *Anabaena* strains commonly produce two or more MC variants simultaneously, which may be the same for these genera (Sivonen et al., 1992; Sivonen & Jones, 1999). In the cyanobacterial blooms caused by more than one toxin-producing species, several MC types can be detected, but the maximum contribution to the total is provided by a few main types of MCs. Of the three MC variants recorded in Lake Kotokel, the most toxic MC-LR concentration was slightly lower than that of the least toxic MC-RR. MC-YR, being eight times more toxic than MC-RR, was in the minor portion of the microcystin pool.

The composition and ratio of MCs in Lake Kotokel were similar to those in field samples and *Microcystis* strains from Japan where variants of MC-LR, -RR, and -YR mainly dominated; however, the total concentration of MCs during cyanobacterial bloom reached 2,100 μg g^{-1} DW (Yasuno et al., 1998; Sivonen & Jones, 1999). MC-RR was also more abundant in the majority of *Microcystis* strains from Japanese lakes (Yasuno et al., 1998). The same, three MC types were also detected in Lake Wannsee, which was dominated by *Microcystis* (Kurmayer et al., 2002). In general, MC-LR was proposed to be the major toxin in bloom samples and strains from temperate waters of Europe and Canada (Chorus & Bartram, 1999). Based on this assumption, and on the data on blooming of Indian ponds and lakes, it was suggested that the production of MC-RR was more common under tropical conditions (Ghosh et al., 2008). The prevalence of MC-RR in temperate Lake Kotokel conflicts with this conclusion. MC-YR is generally a minor component of the MC pool, for example, in the Great Lakes its contribution ranged between 9–23% of total MCs (Hotto et al., 2007; Dyble et al., 2008; Allender et al., 2009). The low content of the dominating, the least toxic MC-RR in Lake Kotokel was not likely to lead to animal or human disease, nevertheless deaths were reported in 2008–2009. Apparently, our finding of MCs does not shed light on the etiology of Haff disease because the clinical symptoms associated with the hepatotoxic MCs and those typical for Haff disease are different (Buchholz et al., 2000). However, although the etiology of Haff disease is not clear, outbreaks of the disease usually correlate to cyanobacterial blooms (Smith, 2000).

It should be noted that Lake Kotokel is similar to small Japanese lakes in the composition of MCs, ratios of MC variants, and genetic affinity of toxigenic *Microcystis* strains. The Japanese strains *M. wesenbergii* NIES-107 and *M. viridis* NIES-102 contained the same three MC variants and were

grouped into the same clade as the K7-09 sequence from Lake Kotokel. MC-RR dominated in these two strains, and MC-YR was a minor component (Yasuno et al., 1998).

The K1-09 sequence from Lake Kotokel is identical to *A. lemmermannii* strains from Finnish lakes, where MC-LR, MC-RR, and their demethylated forms are produced as dominant MCs (Sivonen et al., 1992).

Toxic cyanobacterial blooms have been well documented from over sixty-five countries, including the European part of Russia (Codd et al., 2005). However, the data from Asian regions of Russia were absent. This work and previous studies (Tikhonova et al., 2006, 2007; Belykh et al., 2010) demonstrate that toxigenic cyanobacterial *mcy*A and *mcy*E genes present in two water bodies of East Siberia, and both *mcy*E gene and toxins have been found in Lake Kotokel.

One of the factors facilitating the introduction of toxic cyanobacteria is the spread of MC-producing genotypes into the nearby shallow lakes and ponds due to storm-induced water currents or by external carriers like fishing boats and animals (Hotto et al., 2007). The direct water connection between Lake Kotokel, in which toxic cyanobacteria were found, and Lake Baikal may be a threat to the latter lake, which holds $\sim$20% of the world's fresh water reserve. The water temperature at the coastal zone of Lake Baikal rises to 20°C in the summer, i.e., there are conditions favorable for dense growth of cyanobacteria. The species composition of cyanobacteria in Lake Kotokel is similar to that described for bays and sors that are located on the eastern shore of Lake Baikal (Korde, 1968; Belykh et al., 2007). The abundance of cyanobacterial plankton is high in these areas of Lake Baikal. Therefore, the emergence of toxic genotypes in Lake Baikal is not improbable.

Conclusions

Light microscopy analysis showed the mass development of *Aphanocapsa holsatica* in Lake Kotokel. Potentially toxic *Microcystis* and *Anabaena* species (*M. aeruginosa*, *M. viridis*, *M. wesenbergii*, *A. lemmermannii*, *A. circinalis*, and *A. spiroides*) were recorded in small quantities (less than 24 mg m^{-3}). Toxigenic cyanobacteria of *Microcystis* and *Anabaena* containing the *mcy*E gene were also present in the lake. The ratio of MC-RR:-LR:-YR found in Lake Kotokel was 49:42.5:8.5. Intra-cellular MC concentration in the phytoplankton was 53 μg g^{-1} DW. The presence of toxic cyanobacteria in the lake can pose a serious threat to the humans living in the Lake Baikal region. Taking into account the recreational importance of Lake Kotokel and its direct connection with Lake Baikal, it is necessary to perform regular assessment of the toxic cyanobacteria in this lake to prevent cases of mass poisoning of people and animals in this area.

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