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ECOLOGY

Microcystins in Cyanobacterial Biofilms from the Littoral Zone of Lake Baikal

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Abstract—Some species of cyanobacteria synthesize toxins whose concentration during water bloom can reach values dangerous for human and animal health. Planktonic cyanobacteria are the most common and well-studied microcystins producers, hepatotoxic cyclic heptapeptides, whereas microcystin-producing benthic cyanobacteria are less known. In recent years, the mass development of benthic cyanobacteria forming extensive fouling on different substrates has been detected in the littoral zone of Lake Baikal. We found microcystins produced by benthic cyanobacteria in the biofouling on different natural and artificial substrates, including diseased and dead endemic sponges *Lubomirskia baicalensis* and *Baikalospongia* spp. collected from the littoral area of Lake Baikal. Microscopic analysis of the biofouling revealed prevalence of representatives of *Nostocales* and *Oscillatoriales* with predominance of *Tolypothrix distorta* that is likely the main microcystin producer in Lake Baikal. According to enzyme-linked immunosorbent assay (ELISA), microcystin concentrations in biofouling were 29.8–3050 μg/kg dry weight. We identified eight microcystin variants using MALDI-TOF/TOF; [Dha7]MC-YR was detected in most samples. The presence of microcystins in biofilms formed on the surface of the artificial substrate by *Phormidium autumnale* was also recorded. The data obtained demonstrated the necessity to monitor potentially toxic species and concentrations of cyanotoxins in plankton and benthos in the littoral zone of Lake Baikal, especially in the regions with intense tourist and recreational activities.

Keywords: Lake Baikal, cyanobacteria, *Tolypothrix distorta*, microcystins, sponges, MALDI-TOF/TOF, ELISA.

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INTRODUCTION

Cyanobacteria (blue-green algae (obs.)) inhabiting the water column, the bottom of water bodies, the surface of aquatic plants, and other objects are permanent component of freshwater and marine ecosystems. Elevated temperature and light, excess of biogenic elements, and absence of intensive mixing in water bodies cause rapid growth of cyanobacteria resulting in water bloom [1–4]. Approximately 60% of the cases of the bloom are caused by toxic species of cyanobacteria, which represent a serious threat for human and animal health [1].

Microcystins (MC), cyclic peptides consisting of seven amino acid residues, are the most common cyanotoxins in freshwater. To date, over 90 variants of MC have been characterized, and the most common are MC-LR, MC-RR, and MC-YR [1, 2]. MC are chemically stable compounds, retain their toxic properties in water for several weeks, and are resistant to the heating and cleavage by digestive enzymes. MC possess high hepatotoxicity and cause destruction of the hepatocyte cytoskeleton inhibiting activity of liver serine-threonine phosphatases [1, 2]. Poisoning with high MC concentrations causes clinical symptoms of acute intoxication and even extensive hemorrhage in the liver in severe cases. Long-term exposure to MC in low doses leads to tumor formations. According to the recommendation of the World Health Organization (WHO), MC-LR concentration should not exceed 1 μg/L in drinking water and 2–4 μg/L in bathing and recreational water [1].

Planktonic cyanobacteria have been considered as the main MC producers for a long time; however, benthic species often have been MC producers causing animal death in recent years [3]. The first report on poisoning in animals caused by MC produced by cyanobacterial mats in Alpine lakes that resulted in the death of more than a hundred head of cattle was registered in 1994–1995 in Switzerland. Numerous poisonings in wild and domestic birds were registered over the years in Africa, North America, Europe, Australia, and New Zealand during the mass development of benthic cyanobacteria [3].

MC were shown to be toxic for many water animals [2]. It is known that associations of filamentous MC-producing cyanobacteria and heterotrophic bacteria can cause disease and death of corals due to the necrosis [6]. Large number of filamentous cyanobacteria was found in the bodies of mortally deceased marine sponges, but data on the presence of cyanotoxins in these cyanobacteria are absent [7].

Since 2011, essential changes in the littoral zone of Lake Baikal, among which mass death of sponges associated with intensive development of filamentous cyanobacteria on their surface occupies a special place, have been observed [8]. In some parts of the lake, the proportion of sick endemic sponges *Lubomirskia baicalensis* (Pallas, 1773), which covered approximately 47% of the bottom surface of the lake before the described events, varies from 30 to 100%. The "ecological crisis" in Lake Baikal—one of the largest lakes in the world and an object of UNESCO World Heritage Site containing more than 80% of Russian and 20% of the Earth's freshwater reserves—is of high interest for researchers; however, the reasons for the changes are still unknown [8, 9].

The goals of the present work were to study species composition of cyanobacteria in fouling on different substrates in the littoral zone of Lake Baikal, determine MC concentration by enzyme immunoassay (EIA), and identify their structure by mass spectrometry.

MATERIALS AND METHODS

Samples were collected in May, July, and September 2015 in the coastal zone of South Baikal near settlements Listvyanka and Bol'shiye Koty by divers at a depth of 3 to 15 m. In total, 14 samples from the surface of the different substrates were collected: samples 11, 16–19, and 23 were collected from the surface of *Lubomirskia baicalensis*; samples 13 and 22 were collected from the surface of *Baikalospongia* sp.; samples 12, 14, 15, and 24 were collected from stones; sample 21 was collected from the rock surface; and sample 25 was collected from underwater part of a wooden pier (Table). One of the water samples was collected using a syringe in close proximity to the surface of sponge 11. Fouling from deceased and dead sponges was collected along with parts of their bodies. The samples were fixed with 4% formaldehyde for microscopy and were frozen for genetic studies, EIA, and mass spectrometry.

Species identification of cyanobacteria was performed using an Axio Imager light microscope (Zeiss, Germany) equipped with an HBO 100-W mercury lamp and an AxioCam camera according to the manuals for cyanobacteria identification [10–12]. Frequency of occurrence of species was determined using the Starmach's scale.

EIA was performed using an Abraxis Microcystins-ADDA ELISA kit (Abraxis LLC, United States) according to the manufacturer's recommendation. The results were processed using RIDA®SOFT Win software. The analysis was performed in two stages. In the first stage, screening for MC presence was carried out directly on the research ship; in the second stage, MC concentrations were determined in the laboratory. The samples were preliminary dried at 60°C and weighed. To perform mass spectrometric determination of MC, dry biomass of the fouling was twice subjected to the extraction with 75% ethanol for 60 min under ultrasonic treatment. The extracts obtained were combined and evaporated to dryness using a rotary evaporator at 45 ± 2°C and then dissolved in methanol [13]. MC identification was performed on a matrix-assisted laser desorption ionization-time of flight mass spectrometer (MALDI-TOF/TOF) (Bruker Daltonics, Germany); α-Cyano-4-hydroxycinnamic acid was used as a matrix. Detection was performed in the positive-ion detection mode (laser working wavelength was 355 nm) in a mass range of 500 to 3500 Da.

RESULTS AND DISCUSSION

Visual Observations

Visual observations of the fragments of three branchy sponges *L. baicalensis* collected at the depths of 10–15 m demonstrated destructed branches devoid of characteristic green coloring and covered with blue–green or brown–violet bushy fouling and brown films. Red–brown lesion focuses were detected on the surfaces of two sponges (samples 11 and 16), whereas extensive discolored areas were detected on the surface of one sponge (sample 23). Destructed brown-green branches and brown and dark green fouling were observed in three specimens of *L. baicalensis* (17–19) collected from the depths of 3–7 m. Two essentially destructed cortical sponges *Baikalospongia* sp. were obviously dead. Sample 13 was characterized by brown color of body as well as by red–brown foci of necrosis and fouling. Sample 22 was completely discolored and its surface was covered with pink sediment. Samples from stones and rock were represented by brown–green and red–brown bushy fouling. The sample collected from the stone (sample 24) consisted of hemispherical blue–green colonies. Scraping from the pier surface was similar to the dark green leathery film.

Species Composition of Cyanobacteria

Microscopy revealed that biomass of the fouling was mainly represented by cyanobacteria. In total, 23 cyanobacterial species belong to the orders *Synechococcales*, *Chroococcales*, *Oscillatoriales*, and *Nostocales* (Table). Five species of cyanobacteria of the eight most frequent were represented by the filamentous forms. Only discolored *Baikalospongia* sp. did not

Species composition of benthic cyanobacteria in Lake Baikal in 2015 and relative abundance of the species according to the Starmach's scale ("–" is absence; "+" is extremely rare; 1 is 1–6 cells in the specimen; 2 is 7–16 cells in the specimen; 3 is 17–30 cells in the specimen; 4 is 31–50 cells in the specimen; 5 is absolute predominance, more than 50 specimens).

Sampling site Order. Species	Listvyanka (51°51.58′ N, 104°51.84′ E)									Bol'shiye Koty (51°54.29′ N, 105°4.47′ E)				
	11	12	13	14	15	16	17	18	19	21	22	23	24	25
Synechococcales														
Chamaesiphon fuscus (Rostafinski) Hansgirg	2	2	–	–	–	–	–	–	2	–	–	5	–	–
Heteroleibleinia kuetzingii (Schmidle) Compère	2	1	–	–	–	–	–	–	2	–	–	4	–	–
Leibleinia epiphytica (Hieronymus) Compère	2	2	1	3	3	1	1	–	–	2	–	2	–	–
Leptolyngbya sp.	–	–	–	–	–	–	–	–	–	–	–	3	–	+
L. margaritata (Kufferath) Anagnostidis	5	–	–	–	–	–	–	–	–	–	–	–	–	–
*Merismopedia glauca (*Ehrenberg) Kützing	–	–	–	–	–	–	–	–	–	1	–	–	–	–
Pseudanabaena galeata Böcher	3	–	4	4	3	2	–	–	–	3	–	–	–	–
P. mucicola (Naumann et Huber-Pestalozzi) Schwabe	–	–	–	–	+	–	–	–	–	–	–	–	–	–
Synechococcus/Cyanobium spp.	4	4	4	4	4	5	–	–	–	–	5	5	–	–
Chroococcales														
Aphanocapsa parasitica (Kützing) Komárek et Anagnostidis	1	1	2	3	3	2	1	–	5	+	–	+	–	–
Chroococcus minutus (Kützing) Nägeli	+	–	–	–	–	–	–	+	–	–	–	–	–	–
C. minor (Kützing) Nägeli	–	–	+	–	–	–	–	–	–	+	–	–	–	–
Microcystis aeruginosa (Kützing) Kützing	–	–	–	–	+	–	–	–	–	–	–	+	–	–
M. wesenbergii (Komárek) Komárek ex Komárek	–	–	–	–	–	–	–	–	–	–	–	+	–	–
Oscillatoriales														
Kamptonema formosum (Bory ex Gomont) Strunecký, Komárek et J.Smarda	5	2	3	2	–	1	–	–	–	–	–	–	–	–
Microcoleus paludosus Gomont	+	–	–	–	–	–	–	–	–	–	–	–	–	–
Oscillatoria curviceps C.Agardh ex Gomont	1	1	–	1	1	5	–	–	–	1	–	–	–	–
Phormidium autumnale Gomont	–	–	–	–	–	–	–	–	–	–	–	–	–	5
Symplocastrum sp.	–	–	–	5	5	1	–	–	–	5	–	2	–	–
Tychonema sp.	3	3	2	2	–	1	–	–	–	–	–	–	–	–
Nostocales														
Rivularia borealis P. Richter	–	+	–	–	–	–	–	–	–	–	–	–	–	–
Tolypothrix distorta Kützing ex Bornet et Flahault	5	5	5	+	2	5	5	5	5	5	–	5	5	–
Calothrix sp. Ag. ex Born. et Flah. sp.	–	–	–	–	–	–	–	–	+	–	–	–	–	–
Total	**13**	**10**	**8**	**9**	**9**	**9**	**3**	**2**	**5**	**8**	**1**	**10**	**1**	**2**

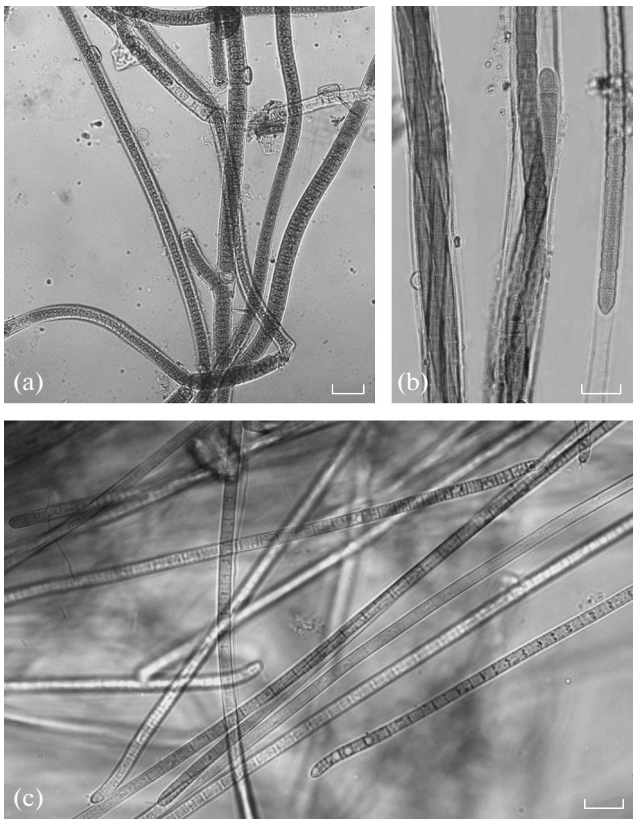

Fig. 1. Cyanobacteria from fouling of different substrates. (a) *Tolypothrix distorta*, (b) *Symplocastrum* sp., (c) *Tychonema* sp. Scale bar: 20 μm.

contain filamentous cyanobacteria. Cells corresponding to the morphotypes of the genera *Synechococcus* C. Nägeli and *Cyanobium* R. Rippka et G. Cohen-Bazire were detected on the surface of its body and pink sediment. These genera are morphologically similar and, therefore, combined in the cluster of picoplanktonic cyanobacteria. Numerous picocyanobacteria developing in the plankton of Lake Baikal (up to 3×10^6 cells/mL) penetrate into the sponge body along with water flow during filtration and comprise an essential fraction of microbiome of the healthy sponges [14]. Probably, some representatives of the genera *Synechococcus* and *Cyanobium* are also symbionts of Baikal sponges. Intracellular *Synechococcus spongiarum* was found in marine sponges [7]; however, related phylotypes were not found in Baikal sponges [14]. To date, reliable information on the production of toxins by cyanobacteria of the genera *Synechococcus* and *Cyanobium* is absent.

Among representatives of the order Synechococcales species *Chamaesiphon fuscus* and *Leptolyngbya margaritata* are also numerous in biofilms. *Ch. fuscus* are usually detected on the spicules in necrotic areas of the sponge bodies, whereas *L. margaritata* formed brown films on the surface of *L. baicalensis*.

Aphanocapsa parasitica, representative of the order *Chroococcales*, was detected in most biofilms. A low number of planktonic representatives of the order *Chroococcales*, *Microcystis aeruginosa* and *M. wesenbergii*, producing MC were found in two samples.

Tolypothrix distorta, representative of the order *Nostocales*, was predominant in 72% of the samples (Fig. 1a). Its blue–green and red–brown trichomes (partially dead) were found in all fouling, with the exception of discolored sponge and biofilm from the pier. The fouling covering the stone (sample 24) were numerous colonies of *T. distorta*. According to long-term observations, *T. distorta* was previously detected along the entire coast of Lake Baikal on stones and macrophytes mainly at the depths of 1.5–6 m. Its biomass comprised approximately 0.25 g/m^2 [15]. Since 2015, *T. distorta* has been detected at the depths of 0–1.5 m, in the first plant level, and its biomass increased hundreds of times [8]. In this period, *T. distorta* was detected for the first time in sponges, but reasons of the colonization and its role in the disease and further death of these endemic animals were not understood [9]. Based on the results of genetic analysis and mass spectrometry, it has been proposed that *T. distorta* is capable of producing paralytic shellfish toxins, including the most toxic saxitoxin [9]. Other species of the order *Nostocales* were rarely found on the stones and in the sponges. It should be noted that filamentous cyanobacteria was not previously detected even in metagenomic studies [14].

Symplocastrum sp., representative of the order *Oscillatoriales*, was numerous in the studied species (Fig. 1b). A large number of its bushy tufts with brown trichomes were detected on solid substrates and rarely on the branchy sponges. Other widespread representatives of the order *Oscillatoriales*, *Kamptonema formosum* and *Tychonema* sp., formed red–brown films in foci of necrosis on the surface of the sponges (Fig. 1c). The presence of *Oscillatoria curviceps* was registered in six samples. *Phormidium autumnale* was predominant in the biofilms formed on the underwater part of a wooden pier.

Study of species composition of cyanobacterial fouling from Lake Baikal demonstrated the presence of known MC producers, first of all, species *Pseudanabaena mucicola*, *P. galeata*, and *K. formosum*. Their toxicity was revealed by chemical analysis and animal biotesting; in one case, it was confirmed by the presence of the genes encoding enzymes involved in MC synthesis [2, 16, 17]. MC production in *T. distorta* colonies from benthic communities of the Alharabe River (Spain) was revealed [18]. It should be noted that, in other water bodies, MC-producers were found among representatives of the genera *Leptolyngbya* Anagnostidis et Komárek and *Rivularia* C. Agardh ex Bornet et Flahault, which were also detected among benthic cyanobacteria of Lake Baikal [3, 16].

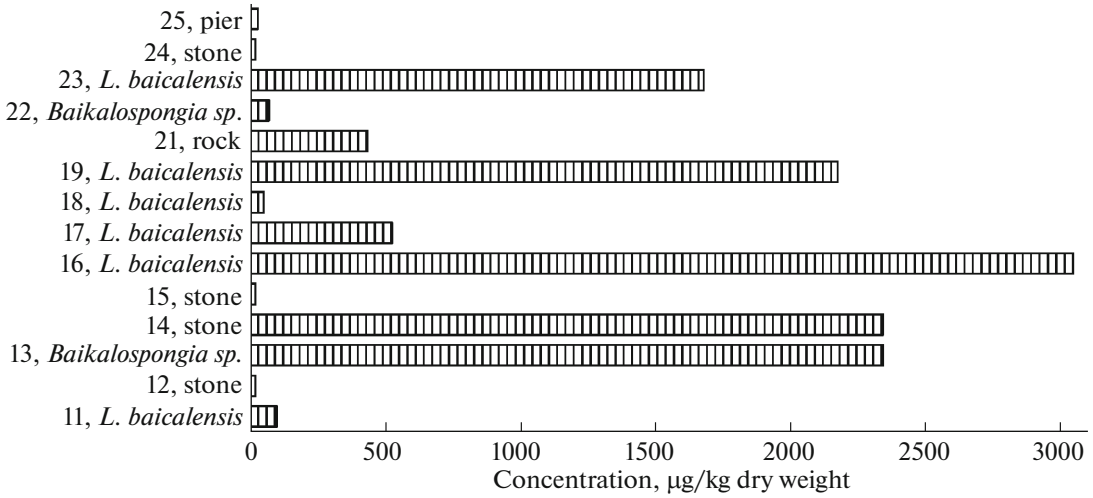

Fig. 2. MC concentration of cyanobacterial fouling of different substrates from Lake Baikal. MC variants in the samples: 11 is MC-YR, [Dha^7]MC-YR; 12 is [Dha^7]MC-YR; 13 is MC-LA, [Dha^7]MC-YR; 14 is [Dha^7]MC-YR; 17 is MC-LF; 18 is [L-MeSer^7]MC-LR; 19 is MC-YR; 24 is [Dha^7]MC-YR; 25 is [D-Asp^3,Dha^7]MC-HtyR, MC-RR, MC-HtyR, [Dha^7]MC-YR; 15, 16, 21–23 are not identified.

Enzyme Immunoassay and Mass Spectrometry

EIA detected MC in all studied samples, including 14 fouling samples (Fig. 2) and the water sample collected in the close proximity to the surface of a branchy sponge (sample 11). MC concentration in water was 0.1 μg/L, which was ten times lower than WHO standards for drinking water. In biofilms, MC concentration was 29.8–3050 μg/L. Highest MC content was detected in sponge fouling and in *T. distorta* colonies. Minimal MC concentration was detected in the biofilms formed by *P. autumnale*. This sample contained four MC variants, three of which were not detected in other samples. MALDI-TOF identified eight MC variants in the biofilms from Lake Baikal (Fig. 2). Profiles of toxins were mainly similar; demethylated MC-YR–[Dha^7] MC-YR (N-methyldehydroalanine replaced with dehydroalanine) revealed in sponge (samples 11 and 13) and stone (samples 12 and 14) fouling as well as in *T. distorta* colonies (sample 24) and *P. autumnale* biofilms (sample 25) was the most common.

Thus, MC-producing cyanobacteria were found in all foulings developing on different substrates in Lake Baikal. As *T. distorta* capable of MC synthesis was predominant in almost all samples, this species is the most likely producer of these toxins in Lake Baikal.

Cyanobacterial species producing other groups of toxins were also found in Lake Baikal. For example, *K. formosum*, *Tychonema bourellyi*, and *P. autumnale* were shown to be able to synthesize anatoxins, neurotoxic alkaloids [2, 3, 19, 20]. *P. autumnale* is known as the most widespread producer of antitoxins. In New Zealand, this species caused numerous poisonings and deaths of dogs [20]. At the same time, MC synthesis was not revealed in *P. autumnale*.

Possible discovery of MC-producing *P. autumnale* and *T. distorta* in Lake Baikal requires further studies mainly to evaluate the potential risk for humans and animals.

MC concentration in biofilms of Lake Baikal was considerably lower than in plankton of blooming water bodies [1, 2] but was comparable to that in cyanobacterial mats from Antarctic and Arctic water bodies [21–23]. In benthic samples, the highest MC content was detected in the Nile River (up to 4.1×10^6 μg/kg dry weight) [3]. In the present work, the real risk of development of toxic cyanobacteria in Lake Baikal was difficult to evaluate due to the absence of universal recommendations approved by WHO for benthic MC-producing cyanobacteria. Regional standards for MC content are usually used in countries where monitoring of potentially toxic species and cyanotoxins are performed [3]. At the same time, despite low MC content in foulings and water of Lake Baikal, the potential risk of drinking water poisoning exists.

Cyanobacterial communities of Lake Baikal similar to mats from Polar regions in species composition and MC content are characterized by a unique MC profile. In algobacterial mats from Antarctic and Arctic water bodies, MC-RR and MC-LR variants produced by *Nostoc* sp. were predominant, although *T. distorta* was also widespread in arctic samples [21–23]. MC variants in Baikal synthezied mainly by predominant species *T. distorta* differed from those found in *T. distorta* from a river in Spain producing five MC variants with prevalence of MC-YR [18]. The demethylated variant [Dha^7]MC-YR was predominant in

Baikal samples, whereas MC-YR was detected in only two samples, in which its concentration reached 2178 μg/kg dry weight, which was comparable to MC content in *T. distorta* from subtropical latitudes [18]. MC content and composition significantly differed not only between species but also between strains of the same species and depended primarily on the environmental abiotic factors [1, 2]. A large number of MC variants (approximately 20) is typical for *Microcystis aeruginosa* strains, whereas benthic cyanobacteria do not possess this wide of MC diversity [1–3].

Numerous studies demonstrated that mass development of benthic cyanobacteria is a consequence of global climate change and eutrophication of water bodies [1–4]. Nevertheless, the reasons for the increase in the frequency and abundance of cyanobacterial water bloom, as well as factors affecting cyanotoxins synthesis, are poorly understood. Benthic cyanobacteria often can produce high biomass in unproductive water bodies, for example, in lakes and river in Switzerland and New Zealand [3, 5, 20]. In recent years, a similar phenomenon is observed in Baikal. Numerous cyanobacteria develop in the lake and reach biomass values typical for eutrophic water bodies, despite the fact that the current trophic status of the lake corresponds to oligotrophic according to hydrochemical parametrs [24]. On the other hand, the first signs of eutrophication manifested by changes in the species composition of algae and increase in phytoplankton biomass were detected in shallow areas of the lake in the 1970s [25]. Water blooms in the bays caused by *Anabaena lemmermannii* P.G. Richter were registered for the first time in the 1980s. In 2011–2016, filamentous algae *Spirogyra* Link—unusual for the lake—and high concentration of thermotolerant coliform bacteria demonstrating anthropogenic pollution were detected in many sites of the coastal zone [8]. We previously reported on the high number of cyanobacteria and presence of toxic species in littoral plankton and noted increased dangerous toxic blooms [9, 13]. Lake biota (mainly primary producers) is the most sensitive indicator of the eutrophication that usually begins in the coastal zone.

Researchers of Antarctic and Arctic algobacterial mats consider temperature as the main factor affecting species cyanobacterial composition and MC production. It was shown that increase in temperature leads to increase in MC concentration [22, 23]. According to many authors, ongoing climate change can lead to increase in toxin production and the appearance of toxic strains [1, 22, 23]. Since 1896, average annual air temperature in Lake Baikal increased by 1.2°C per century. In last 60 years, the temperature of upper water layers in the warm season has gradually increased [24].

MC presence in foulings of different underwater substrates, including endemic sponges and artificial objects, demonstrates the widespread range of toxic cyanobacteria in the littoral zone of Lake Baikal and the potential risk for human and animals. Climate change and increase in anthropogenic load on the lake ecosystem, especially in the recreational zone, makes it necessary to perform regular monitoring of toxic bloom in Lake Baikal. Since MC are toxic for many hydrobionts, the role of MC in the appearance and development of disease of endemic Baikal sponges requires further studies.

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