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Microcystin-Producing Cyanobacteria in Water Reservoirs of Russia, Belarus and Ukraine

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Abstract

A review of hepatotoxic microcystins produced by cyanobacteria of different genera is presented. The chemical structure and properties of microcystins, the mechanism of their action and toxicokinetics are considered. Methods of microcystin analysis and the genetic basis of their production are described. The results of the studies of cyanobacteria and their toxins (microcystins) in different water reservoirs of Russia, Belarus and Ukraine are presented.

Key words: toxic cyanobacteria, *Microcystis*, *Anabaena*, Lake Baikal, Lake Kotokelskoye, Baltic Sea, Beresh water reservoir, Svisloch River, Dnepr River, Kanev water storage reservoir, microcystin, genetic markers

Contents

INTRODUCTION

Cyanobacteria (cyanoprocaryotes) are one of the oldest groups of organisms on the Earth. They originated about 3.5 billion years ago and played important part in the formation of the Earth's atmosphere having enriched it with oxygen [1]. At present, cyanobacteria are still inalienable component of aqueous and ground ecosystems and are widespread almost every-

where including extremal ecological niches: deserts, thermal springs, lakes of Arctic and Antarctic [2].

Cyanobacteria synthesize large amounts of secondary metabolites. The most rapt attention of researchers is attracted to toxins because they bring danger to human and animal life and health. Mass development ("blossom") of cyanobacteria in water reservoirs leads to the release of toxins and to the appearance of technical and aesthetic problems for the use of water for domestic water consumption and for recreation purposes, and in some cases it becomes the reason of damage and degradation of the whole ecosystem. During water reservoir "blossoming" the concentrations of toxins in water increases multiply at the stage of cyanobacteria population death, cell lysis and escape of toxins; the intracellular concentration of dissolved toxins in young cells is usually not high (0.1–10 μg/L). In fresh and salty water reservoirs, two major groups of cyanotoxins are distinguished according to their chemical structures: cyclic peptides (microcystins and nodularine) and alkaloids (cylindrospermopsin, anatoxins, and saxitoxins) [3, 4]. In addition to secondary metabolites that do not participate in the general metabolism, toxicity is also exhibited by the structural components of cyanobacterial cell walls – lipopolysaccharides. Cyanotoxins cause both acute and chronic poisoning; according to their effect on target organs they are divided into hepato-, neuro- and dermatotoxins.

Microcystins (MC) are among the most widely known and widespread cyanotoxins in fresh water; their major producers are cyanobacteria of *Anabaena, Microcystis, Planktothrix* genera that often cause toxic "blossom" of water in productive water reservoirs all over the world [5].

CHEMICAL STRUCTURE AND PROPERTIES OF MC

MC were isolated for the first time from the *Microcystis aeruginosa* strain and named in agreement with the genus name of these cyanobacteria. MC include seven amino acids: *cyclo*-($_{D}$-Ala-**X**-$_{D}$-MeAsp-**Z**-Adda-$_{D}$-Glu-Mdha), where $_{D}$-Ala is $_{D}$-alanine, $_{D}$-MeAsp is $_{D}$-erythro-β-methylaspartic acid, Adda is 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-deca-4,6-dienoic acid, $_{D}$-Glu is $_{D}$-glutamate, Mdha is *N*-methyldehydroalanine, **X** and **Z** are variable *L*-amino acids [6, 5]. The molecular mass of MC varies from 500 to 4000, being equal to 900–1100 for the majority of versions. The most unusual structure in this group of cyclopeptides is amino acid Adda. It provides absorption at 238 nm (which is characteristic of MC and nodularine) due to the presence of conjugated diene group, and this feature is used for HPLC separation [7]. By present, more than 90 versions of MC have been identified [8]. Structural versions were detected in all the seven amino acids but most frequently in the position of variable *L*-amino acid: **X** (**X** is most often leucine, arginine or tyrosine but also homotyrosine, alanine, phenylalanine, homophenylalanine, methionine-*S*-oxide or tryptophane are possible) and **Z** (**Z** is arginine or alanine; aminoisobutylic acid, homoarginine or methionine-S-oxide are also possible). Most frequent is MC-LR in which variable *L*-amino acids are represented by leucine and arginine. Isoforms RR (arginine-arginine) and YR (tyrosine-arginine) are less widespread. Many versions are formed by methylation/demethylation of $_{D}$-MeAsp and/or Mdha. The majority of MC contains β-methylaspartic acid, glutamic acid and alanine with methylamine attached to glutamic acid. In some versions Mdha is replaced by *L*-serine and $_{D}$-Ala-$_{D}$-serine. Some nontoxic versions were identified as the components containing 6Z-stereoisomer of Adda. In general, some structural modifications of Adda in the region or acylation of glutamate cause a decrease in the toxicity of MC and even provide its non-toxicity. Esterification of the free carboxylic group of glutamic acid leads to the formation of inactive structures (versions).

Microcystins are soluble in water, methanol and ethanol but are insoluble in acetone, ether, chloroform and benzene. They remain stable in water reservoirs for 7 days but are stable in filtered or deionised water for a long time. Microcystins are resistive to chemical hydrolysis or oxidation at pH close to neutral. Microcystins are not destroyed during boiling for several hours. At high temperature (40 °C) and extremally high or low pH values (pH > 12 or pH ~ 1, respectively) more than 90 % of MC degrade within 10 weeks as a consequence of slow hy-

drolysis. Rapid chemical hydrolysis of MC is possible under laboratory conditions, for example at high temperature and with the addition of 6 M HCl solution. Microcystins are oxidized with ozone and other strong oxidizers [3], they are very stable under sunlight, while get rapidly destroyed under the action of UV within the range of absorption maximum [9].

MECHANISM OF MC ACTION

Microcystins are mainly hydrophilic peptides (due to the presence of free carboxylic groups and arginine) and are unable to penetrate through the membranes of animal cells, so that ATP-dependent carrier is necessary for their transport. Multispecific carrier of organic anions (or carrier of bile acids) was described as the carrier of cyclic peptides including MC [3]. As a consequence of this, the toxicity of MC is directed toward the organs transporting organic ions through cell membranes, first of all toward liver. After peroral introduction, MC get into blood through the ileum (small intestines) and then are concentrated in the liver as a result of active capture by hepatocytes. Some MC, more hydrophobic than MC-LR, may penetrate through cell membranes in other ways including diffusion [3]. Microcystins serve as inhibitors of eukaryotic serine/threonine phosphatase 1 and 2A (PP1 and PP2A). Inhibition of PP1 and PP2A leads to hyperphosphorylation of the proteins of cytoskeleton, which causes their destruction and leads to the deformation of hepatocytes, the loss of cell contacts and promotes the formation of vast hemorrhages; as a result, the liver expands [10, 11]. It was shown that inhibition of phosphatases and hyperphosphorylation of cytokeratin leads to the development of cancer in hepatocyte culture [12, 13]. The action of MC-LR in experiments with mice caused immune response and the production of eight kinds of cytokines stimulating inflammation and cancer [14]. Adda plays the key part in MC toxicity; it is this region of heptapeptide that interacts with phosphatases. Antagonists of MC are cyclosporine A, riphampicine and silymarin preventing the toxicity of MC in experiment [15].

TOXICITY OF MC

MC versions differ from each other in toxicity: LD_{50} (semilethal dose) after intraperitoneal introduction in mice varies from 50 to 1200 μg/kg of body mass [5, 16]. The most toxic compound turned out to be MC-LR: its peroral dose for mice is 500–10 900 μg/kg of body mass. For intraperitoneal introduction of MC-LR in mice, LD_{50} varies from 25 to 150 μg/kg of body mass (50–60 μg/kg of body mass as average) [3, 17]. Humans are affected by MC through drinking water, tabletted preparations of cyanobacteria or through skin when bathing in lakes and rivers [15]. According to the recommendations of WHO, the concentration of MC-LR in drinking water should not exceed 1 μg/L for single use and 0.1 μg/L for multiple use [18]. For recreational use of water reservoirs, the dangerous level of cyanobacteria is $20 \cdot 10^6$ cl/L and the concentration of MC 2–4 μg/L [19]. The consumption of biologically active additive s containing algae (usually *Aphanizo-menon flosaquae* and *Spirulina*) is dangerous because in addition to these species the tablets often contain toxic *M. aeruginosa* [3, 20]. The first report on cyanotoxins appeared in 1878 when horses, small cattle, dogs and pigs were poisoned by *Nodularia spumi-gena* which formed thick films on the bottom of the Murray River in Australia [21]. After 100 years, it was demonstrated that this species produces nodularine – a cyclic pentapeptide similar in clinical symptoms and pathohystological signs with MC. The most widely known example of MC toxicity was acute poisoning of hundreds of patients of hemodialysis centre in Brazil; among them, 54 patients died [22].

TOXICOKINETICS

Investigations with laboratory animals showed that 50–70 % of MC get accumulated in liver, 7–10 % in small intestines, 1–5 % in kidneys, and the rest get spread over the whole organism. Microcystins are resistant to digestion in the digestive tract because peptides are bound in a chain by d-amino acids and are insensitive to hydrolytic enzymes; MC are stable to degradation in tissues [7]. Bile is necessary

for their excretion, so liver plays the major part in MC detoxication [3].

At the cell level, the action of MC is exhibited as increased permeability of hepatocyte membranes, their vacuolization, cell contraction and redistribution of organelles in fibroblasts, in endothelial and epithelial cells, lymphocytes [23–25]. Cardiotoxic action of MC was also observed [26]. In addition, phosphatase inhibition may cause cell proliferation, transformation and therefore to the formation of tumour as the studied of the National Cancer Centre in Japan showed [27] and other works confirmed [12, 13, 28–30]. Long-term action of the low doses of MC explains the formation of hepatocellular carcinoma in the inhabitants of Southern China where «blossoming' of water reservoirs is abundant; after the adoption of drinking water quality control the level of cancer morbidity decreased [3].

Clinical signs for MC intoxication include diarrhea, nausea, rigor, asthenia, bleach [28]. Death caused by acute MC poisoning is a result of hypovolemic shock caused by rapid and severe obstruction of liver vessels and vast liver hemorrhages. Dermatological signs of contact with MC include lip blisters, allergic reaction (contact dermatitis, asthma, hay fever, and conjunctivitis) [15]. A syndrome named bather dermatitis is known: it includes diarrhea, emesis, fever signs, skin eruption, mouth ulceration, eye and ear irritation within 7 days after contact with water containing toxic cyanobacteria. These symptoms are directly connected with the amount of cyanobacteria cells [3, 7].

METHODS OF MC ANALYSIS

A number of methods have been developed for the analysis of cyanotoxins in water and cyanobacteria cells. The most important criteria for the choice of the methods are their specificity and sensitivity. Three major approaches exist: biological, physicochemical and biochemical. Biological methods (bioanalysis) include the use of small animals (mice, rabbits, crustacea *etc.*) and microorganisms as test objects. The advantage of this method is simplicity and high speed of analysis, but its application is restricted due to ethical considerations and due to the fact that this method does not give information about the nature of the toxin. Physicochemical methods include chromatographic techniques: thin layer chromatography (TLC), high performance liquid chromatography (HPLC), mass spectrometry with fast atom bombardment (FAB), ionization by electrospraying (ESI), using registration relying on selected ion record (SIM) or selected reactions (SRM), nuclear magnetic resonance (NMR). Physicochemical methods are sensitive and highly efficient for qualitative and quantitative evaluation of toxins. Biochemical methods are represented by immunoanalysis (ELISA) and enzyme analysis (inhibition of protein phosphatases, acetylcholinesterases *etc.*). These methods became a good replacement of bioanalysis but they are less sensitive than physicochemical procedures and are best suitable for rapid screening of samples during monitoring.

GENETIC FOUNDATIONS OF MC PRODUCTION

Biosynthesis of MC proceeds in a multienzymatic complex composed of different units of non-ribosomal peptide synthetase (NRPS), polyketide synthase (PKS) and additional modifying enzymes [31–34]. The NRPS units contain ATP-dependent adenylation domain which activates specific or preferable amino acids, peptidyl carrier domain (PCP) to bind the substrate during assembling, and condensing domain which catalyses the formation of amide bridges between PCP bound substrates. Assembled peptide molecule is liberated from the enzymatic complex, usually with the help of thioesterase domain which can also participate in the direct cyclization of the final product. Non-ribosomal peptide synthetases perform the synthesis of linear, cyclic and branched cyclic peptides including antibiotics, such as penicillin, vancomycin, and cyclosporin [35]. Polyketide synthase – megasynthase formed by repeated functional modules – plays the role of ketoacyl synthase, ketoacyl reductase, acyl- and methyltransferase, dehydratase and acyl-carrying protein.

A cluster of genes coding the complex of MC synthetase was discovered and described for the first time for *Microcystic aeruginosa* K-139 [31], PCC 7806 strains [32]. Its length is

55 thousand kb, it is composed of six open reading frames (ORFs) of mixed NRPS/PKS (*mcy*ABCDE and *mcy*G) and 4 ORFs coding domains with assumed function of modification of precursor molecules and MC (*mcy*ABCDE and *mcy*G). Recently, genes responsible for MC synthesis were detected in *Planktothrix agardhii* 126/8 [33] and *Anabaena* sp. 90 [34].

There are some differences in major MC-producing genera *Microcystis*, *anabaena* and *Plaktothrix*, both in *mcy* gene arrangement and in nucleotide sequence [32–34]. Discovery of the (*mcy*ABCDEFGHIJ) gene cluster allowed developing markers that were later used to reveal potentially toxic species containing the genes of MC synthesis [31, 32, 36, 37]. It is known that the same species in different lakes may produce toxins or not, depending on ecological conditions [38]. Moreover, different strains of the same species may be toxic or non-toxic; microscopic investigation is unable to distinguish between them. Molecular biological detection of the genes of toxin synthesis allows determining potential danger even before the toxins appear in water, when they may be detected using analytical methods [39]. The most frequently used gene targets are *mcy*ABDCE genes with the help of which the presence of toxicogenic cyanobacteria in many water reservoirs of the world was revealed [40–44].

OUR STUDIES

We were the first in Russia in 2005 to start molecular biological investigation of toxic cyanobacteria. With the help of genetic markers, search for potentially toxic cyanobacteria was carried out in Lake Baikal and in water reservoirs of the Angara system (Irkutsk, Ust'-Ilimsk and Bratsk water reservoirs) [45, 46]. Before our studies, there were no data on the toxic species of cyanobacteria in Lake Baikal; the problem connected with toxic blossoming had not been studied yet. Lake Baikal is the largest and the deepest lake in the world; it is oligotrophic water reservoir and contains enormous resources of the purest fresh water. At the same time, the presence of sors and shallow-water regions well warmed in summer (to 17–22 °C), increasing anthropogenic load as a consequence of intense tourism and recreation, and implied mass development of phytoplankton including cyanobacteria in the regions near the lakeside determined our attention to the investigation of this object. Previously, for the Alpine lakes of Switzerland where the death of cattle after drinking water from the lakes was observed for several years, the risk of the development of toxic cyanobacterial also in oligotrophic water reservoirs was demonstrated [3].

During the entire period of investigations (2005–2012), samples were collected from water reservoirs in Russia (Irkutsk Region, Republic of Buryatia, Krasnoyarsk Territory, and Kaliningrad Region), Ukraine and Belarus. In the Irkutsk Region, sampling was carried out in pelagic environment and in the regions near the banks of Lake Baikal, in water reservoirs of the Angara system: Irkutsk (Yelovy Bay near Patrony settlement), Ust'-Ilimsk (Vikhorev Bay, Sedanovo settlement, Nizhneilimsk area, Karapchan Bay near Zheleznodorozhny settlement) and Bratsk (Ust′-Uda settlement, Tanguy Bay, Sukhoy Log Bay within Bratsk city, Dolonovo broadening), in the Krasnoyarsk Territory – in Beresh water reservoir, in Buryatia – Lake Kotokelskoye, in Kaliningrad – in the Russian part of the Kursh Bay of the Baltic Sea.

Plankton was samples with Upstain's net and Ruttner batometer from the depth of 0–25 m at the intervals of 5 m. Temperature and water transparency according to Secchi disk were determined at the same time. Batometric samples were fixed with Lugol solution and concentrated with the help of precipitation method. Net samples were fixed with formalin for microscopic observations and with ethanol for molecular biological analysis (final concentration: 2 and 80 %, respectively). Chemical analysis of water samples including the concentrations of total and phosphate phosphorus, nitrate, nitrite and ammonium nitrogen, permanganate and dichromate oxidability was carried out using the methods that are generally accepted in fresh water hydrochemistry as described in [47]. To determine the concentrations of chlorophyll (*a*), the samples were filtered through the Millipore polycarbonate filters (USA) with pore diameter 0.45 μm. Extraction was carried out with hot methanol [48], parameter *a* was determined with the help of SmartSpec^{TM} Plus spectrophotometer (Bio-Rad, USA).

To study the species composition and to estimate the number of phytoplankton organisms, light microscope Axio Imager (Zeiss, Germany) was used. Algae and cyanobacteria were examined with the magnification of 400 and 1000 times with triple repeatability. The biological volume of each species was established from the average cell size measured in microphotographs that were obtained with the help of AxioScan MRm camera (Pixera Corp., USA) and Axio Vision software (version 4.7.2.0) included in the microscope kit.

A set of primers to the genes of MC synthetase *mcy*E and *mcy*A were used in the first works [45, 46] for the molecular genetic detection of potentially toxic species [40, 49, 50]. In subsequent works, the domain of aminotransferase (AMT) was chosen as the marker [51–53]. Aminotransferase is incorporated into all the known MC-synthetases and, as shown recently, nodularin synthetase (*nda*) coding the synthesis of another known hepatotoxin – nodularin produced by the species of *Nodularia* genus [54]. Aminotransferase is localized between NRPS and PKS modules of *mcy*E and *nda*F genes and plays a key part in the biosynthesis of MC and nodularins transferring amino group to the Adda motive [32, 54]. The AMT domain was found in *Microcystis*, *Anabaena*, *Nodularia*, *Nostoc*, *Planktothrix*, *Phormidium* that are present in the samples from different water reservoirs; the presence of AMT domain had a positive correlation with the production of MC and nodularins [50].

Two strains of toxic cyanobacteria *Microcystis aeruginosa* CALU 972 and 973 submitted by L. N. Voloshko (Biological Institute, St. Petersburg) were used as a positive reference. The toxicity of the strains was tested preliminarily with crustacea *Daphnia magna* and endemic *Epishura baicalensis* [45].

The presence of MC in samples was established by means of immune-enzyme detection (IED) with the help of Microcystins kit (ADDA), ELISA kit (Abraxis, USA) which is intended for the quantitative, sensitive and highly specific determination of MC in water. Analysis is based on the antigen-antibody interaction and represents a sandwich-IED version. Analysis procedure was carried out according to the manufacturer's instruction; MC concentration was calculated by measuring the optical density with an EL300 immune-enzyme analyzer (BioTek, USA) at the wavelength of 450 nm. The optical density in the wells, measured using the spectrophotometric method, is inversely proportional to MC concentration in the samples under investigation. Results were processed with the help of RIDA SOFT Win software (R-Biofarm, Germany). Calibration curve was plotted by means of linear regression relying on the measurements of the optical density in reactions with standard solutions containing known concentrations of MC. The statistical treatment of the data (calculation of standard deviation) was carried out with the help of Microsoft Excel 2010 for Windows (Microsoft, USA).

The versions of toxins in the samples were determined by means of liquid chromatography-mass spectrometry. To detect, MC, the concentrated phytoplankton sample was subjected to freezing-melting procedure for five times, and dried at a temperature of 60 °C to the constant weight. The dried sample was extracted with methanol, evaporated to the dry state with a rotary evaporator IKA RV 05 Basic (IKA-WERKE, Germany). The dry residue was re-dissolved in 0.5 mL of methanol before determination by means of liquid chromatography-mass spectrometry (LC-MS). The LC-MS analysis was carried out with an Agilent HP 1200 chromatograph (Hewlett Packard, USA) in combination with the time-of-flight mass spectrometer Agilent 6210 (Agilent Technologies, USA) with ionization by means of electrostatic spraying (ESI) in the mode of positive ion registration.

Siberia

Water reservoirs of the Angara system. In the Ust′-Ilimsk water reservoir, mass development of cyanobacteria *Aphanizomenon flos-aquae*, *M. aeru-ginosa, M. pulverea* was observed in August 2005. The number of *A. flos-aquae* was 100 million cl/L, *M. aeruginosa* – 500 thousand cl/L, *M. pulverea* – 300 thousand cl/L. Water "blossoming" characteristic of this water reservoir, with the domination of *A. flos-aquae* (up to 1.4 billion cl/L), is observed since the time of water reservoir filling (1975–1977) [55]. It should be noted that during preceding

years the number of *M. aeruginosa* during summer and autumn was lower, 200–400 thousand cl/L as average.

In 2010, the number of biomass of cyanobacteria in the Ust'-Ilimsk water reservoir were 4.0 million cl/L and 16.2 mg/m^3, respectively. *Aphanizomenon flos-aquae* and *Anabaena lemmermennii* dominated in biomass. In the samples taken in 2005 and 2010, the genes of MC synthesis belonging to *Microcystis* and *Anabaena* genera were detected (Figs. 1, 2). The concentration of MC in water, measured by means of IEA in 2010, was (0.25±02) μg/L. The major species causing water blossoming in the Ust'-Ilimsk water reservoir is *Aphanizomenon flos-aquae*. According to the data obtained, it does not synthesize MC but may be potential producer of other cyanotoxins.

In the Bratsk water reservoir, the mass development of *Anabaena flosaquae* cyanobacteria containing the genes of MC synthesis was observed in 2005 in the Sukhoy Log Bay near the city water supply of Bratsk, in 2012 – in the Tanguy Bay (see Fig. 2). MC concentrations in water were not measured [56].

In the Irkutsk water reservoir, monitoring of toxin-producing cyanobacteria was carried out in 2005, 2006, and 2010 in the Yelovy Bay near Patrony settlement. Potentially dangerous species were detected in phytoplankton in 2005 and 2006; the PCR analysis for the genes of MC synthesis was negative. According to the data of the author of [55], potentially toxic species *Anabaena lemmermannii*, *A. flos-aquae* and *Aphanizomenon flos-aquae* occur frequently in the Irkutsk water reservoir, but they only rarely dominate in the community. So, the risk of toxic blossoming in the Irkutsk water reservoir is not high.

According to the results of investigations, potentially toxic cyanobacteria of *Microcystis* and *Anabaena* general containing the genes of MC synthesis are present in the Ust'-Ilimsk and Bratsk water reservoirs; these water reservoirs are characterized from the viewpoint of algae biomass and chlorophyll concentration as mesotrophic with eutrophic regions [56]. These water reservoirs are prone to strong anthropogenic impact, the concentrations of organic compounds and biogenic elements in them exceed the MPC levels. At present, the Vikhoreva River, with blossoming of toxin-producing species observed near its mouth, is included in the list of most heavily polluted water objects. Waste water from the Bratsk PCM and sewage from the housing and communal services of Bratsk and Vikhorevka towns enter this river; the concentrations of organic substances and biogenic elements (ammonium and nitrite nitrogen, mineral phosphorus) in the river exceed MPC by a factor of several times [57].

Lake Baikal. Microscopic observation of phytoplankton in Lake Baikal showed that during winter and spring five species of nanoplankton cyanobacterial are present in the pelagic environment: *Microcystis pulverea*, *Anabaena* sp., *Chroococcus limneticus*, *Gomphosphaeria lacustris*, *Planktolyngbia* sp. The number of these cyanobacteria did not exceed 10 thousand cl/L. In summer, *Microcystis aeruginosa*, *Coelosphaerium kuetzingianum*, *Planktolyngbya* sp. were observed in plankton in concentrations up to 10 thousand cl/L. No genes coding the synthesis of MC were detected in the samples of phytoplankton from pelagic environment of Lake Baikal in 2005–2009.

In 2010–2011, samples for monitoring of the genes of cyanotoxin synthesis were taken not only in the pelagic region of Lake Baikal but also in bays and the regions near lakeside in July–August, while water was the warmest. It was established that there are no potentially dangerous species of cyanobacteria in the pelagic regions; seven species were observed in the bays. Among them, dominant species were those of *Anabaena* genus: *A. lemmermannii*, *A. spiroides*, *A. scheremetievi*, *A. zinserlingii*. Also *Gloeotrichia echinulata* and *Trichodesmium lacustre* also occurred.

The positive PCR result was obtained for the samples collected in the Barguzin and Chivyrkuy bays, Kurkut and Mukhor bays (Maloye More channel), in the Turka Bay. The genes of MC synthesis were revealed for the first time since 2005 during plankton monitoring in Lake Baikal. Comparative and phylogenetic analysis of the obtained sequences of AMT domain of *mcy*E gene showed that the plankton from the bays of Lake Baikal contained cyanobacteria of *Anabaena* and *Microcystis* genera (see Figs. 1, 2). The concentrations of MC in water were determined by means of IEA. In Lake Baikal, MC were detected in the zone near lakeside near

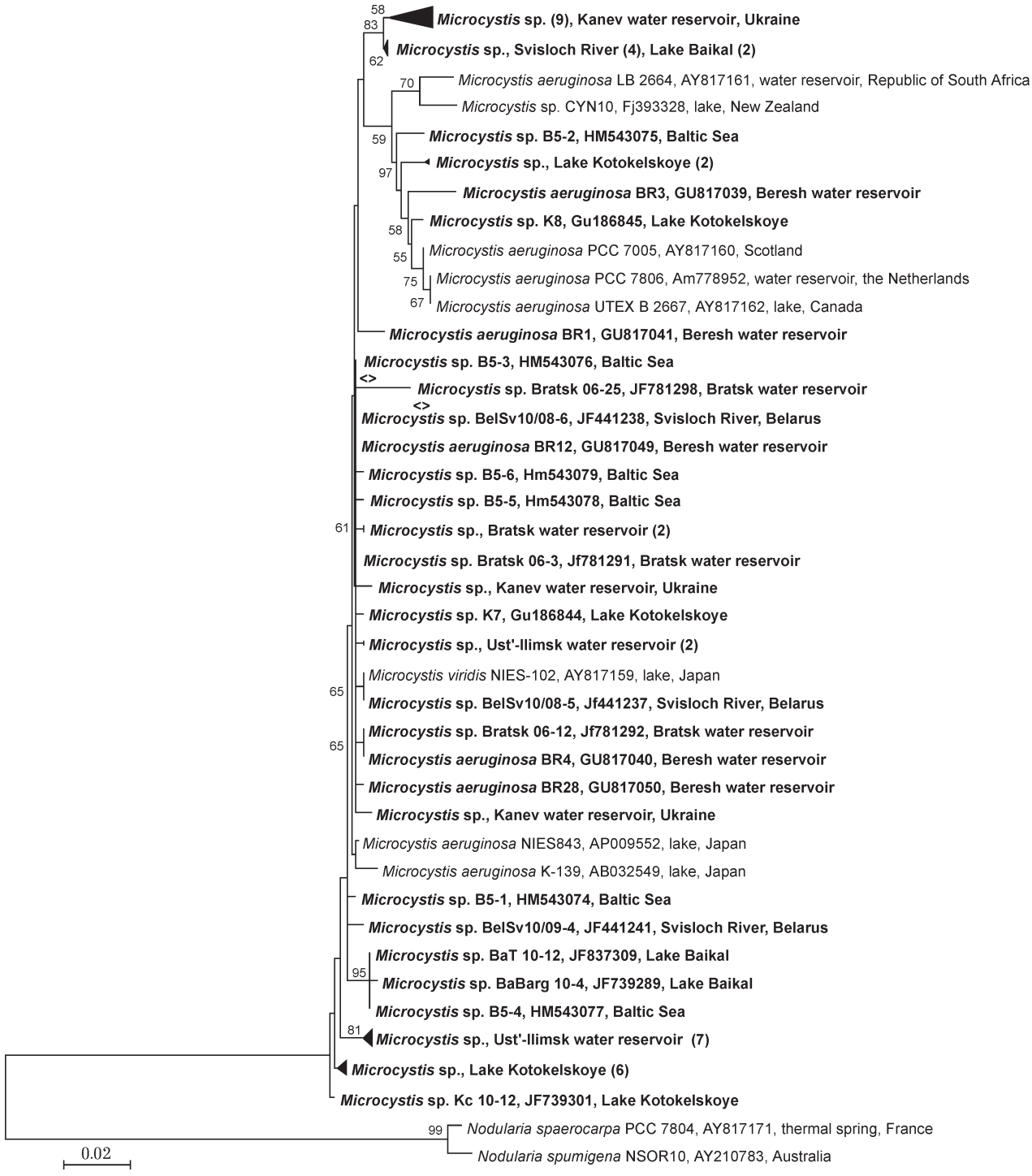

Fig. 1. Phylogenetic tree built on the basis of the sequences of AMT domain of *mcy*E gene of cyanobacteria of *Microcystis* genus. The sequences of *nda*F gene of *Nodularia* genus were used as outgroup. Nucleotide sequences obtained by the authors are typed in bold. In branching centres, the results of boot strap analysis are shown. The scale corresponds to two nucleotide replacements per each 100 nucleotides.

Turka settlement, their concentration was (0.17±0.02) μg/L, which does not exceed the MPC adopted by the World Health Organization [18]. The bank near Turka settlement is included into the Special Economic zone of tourist and recreation type Baikalskaya Gavan. In 2010, construction of tourist objects was launched near Turka settlement. An increase in the number of persons on leave and water pollution with biogenic elements may worsen the situation with summer

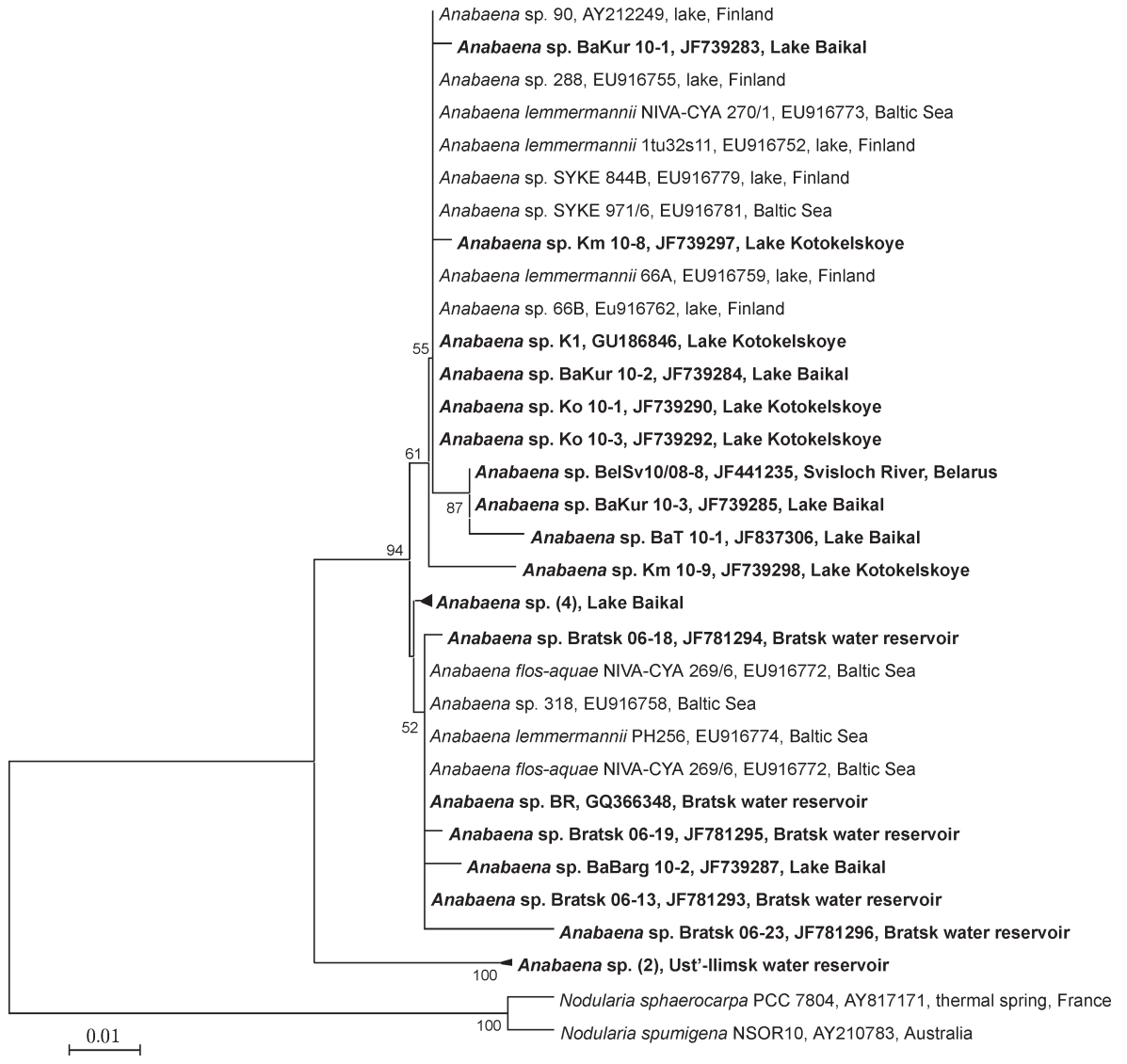

Fig. 2. Phylogenetic tree built on the basis of the sequences of AMT domain of *mcy*E gene of cyanobacteria of *Anabaena* genus. The sequences of *nda*F gene of *Nodularia* genus were used as outgroup. Nucleotide sequences obtained by the authors are typed in bold. In branching centres, the results of boot strap analysis are shown. The scale corresponds to two nucleotide replacements per each 100 nucleotides.

cyanobacterial water "blossoming" near lakeside, while the presence of toxigenic strains in phytoplankton brings potential danger for population.

Lake Kotokelskoye. The integrated approach with the use of microscopic, molecular genetic and analytical methods tested by us previously when studying the water reservoirs of the Irkutsk Region was used to study the samples from Lake Kotokelskoye (Buryatia) [51, 53]. This lake is a large commercial water reservoir situated at a distance of 2 km from the eastern side of Lake Baikal and connected with it through a system of small rivers. Lake Kotokelskoye is an object of the tourist and recreation zone Baikalskaya Gavan. Many tourist centres and boarding houses are situated there. Mass death of fish, waterfowls and cats was detected here in summer 2008; 16 cases of poisoning were detected in humans after they ate bream fished out from the lake. The signs of Haff disease (alimentary toxic paroxysmal myoglobinuria) were observed in all of them. Aetiology of Haff disease has not been studied thoroughly yet; one of the possible reasons may be

the action of biologically active substances of cyanobacteria. Since June 2009, it is prohibited to eat fish caught in the lake, to bath and to use water for domestic and economic purposes.

Our studies showed that Lake Kotokelskoye is related to eutrophic water reservoirs, for total phosphorus content, chlorophyll *a* concentration and transparence. Cyanobacteria of 25 species were detected in the phytoplankton of the lake. Dominating species was *Aphanocapsa* (53–98.5 % of total number). Among cyanobacteria detected in the plankton of Lake Kotokelskoye, the most well known potential producers of are the species of *Microcystis* (*M. aeruginosa*, *M. viridis*, *M. wesenbergii*) and *Anabaena* (*A. lemmermannii*, *A. flos-aquae*, *A. circinalis*) genera. The number of biomass of cyanobacteria in batometric samples was 6.17 million cl/L and 3.87 g/m^3, respectively. Search for toxic cyanobacteria in the plankton of Lake Kotokelskoye in August 2008–2009 was carried out with the help of markers to the domain of aminotransferase of *mcy*E and *nda*F genes [51, 53]. PCR analysis gave a positive result. The data obtained in phylogenetic studies revealed a high similarity (97–100 %) of amplicons obtained with the *mcy*E genes of toxic cyanobacteria *Microcystis* and *Anabaena* (see Figs. 1 and 2). The sequences of *mcy*E gene of *Microcystic* spp. had a 98–99 % similarity with the sequences of *M. viridis*, *M. aeruginosa* and *M. wesenbergii* strains isolated from toxic "blossoming" of lakes Kasumigaura and Kawaguchi (Japan) and water reservoirs of Europe and North America. The sequences of *mcy*E gene of *Anabaena* sp. were distinguished by a 100 % homology with the sequences of the strains of *Anabaena* genus from the lakes of Scandinavia. The number of clones of *Microcystis* spp. exceeded that of *Anabaena* spp. Our data are in agreement with the results obtained in the analysis of phytoplankton from the lakes of Finland [38]. The authors showed that the *Microcystis* genus includes a larger number of toxic genotypes in comparison with *Anabaena*. So, in spite of the higher concentration of *Anabaena* than *Microcystis*, the latter was the major producer of MC.

According to the data of chromatographic analysis, three peaks with retention time 14.5, 16.55 and 16.86 min are observed for the extract of phytoplankton. The UV spectra of these peaks are characteristic of microcystins (Fig. 3, *a*). The data of LC-MS confirmed the presence of three MC with characteristic m/z ratios: MC-RR (m/z 519.8089 $[M + 2H]^{2+}$) MC-LR (m/z 995.5874 $[M + H]^{+}$), MC-YR (m/z 1045.5661 $[M + H]^{+}$) (see Fig. 3, *b*–*d*). The concentrations of MC were calculated using the molar extinction coefficient for MC-LR [58]. The MC ratio was the following: MC-RR 49 %, MC-LR 42.5 %, MC-YR 8.5 %, total MC content calculated for the dry mass was 53 μg/g. For comparison: the total concentration of MC in phytoplankton of fresh water reservoirs of China reached 7300, Portugal – 1600–7100 μg/g of the dry mass [5]. In the lakes of Scandinavia, during the mass development of *Microcystis*, the average MC concentration reached 770 μg/g of the dry mass, while for *Anabaena* it was 107 μg/g of the dry mass.

The strains of *Microcystis* and *Anabaena* usually synthesize more than two MC versions at a time; the representatives of these genera are able to produce MC of the same version [5, 59]. For example, both *Microcystis* and *Anabaena* can synthesize MC versions detected by us in Lake Kotokelskoye. In the samples containing usually several strains of one or more toxin-producing species, diversity of MC versions was revealed. During *Microcystis* spp. "blossoming" in Lake Homer (USA), 19 different MC were identified; in Australia during the mass development of *M. aeruginosa*, 23 versions of MC were determined; MC-LR version was not detected among them. A smaller amount of isoforms was observed in the samples from toxic "blossoming" of *Anabaena* spp.; quite contrary, *Anabaena* strains isolated from fresh lakes in Finland produced 17 MC versions in which, in addition to MC-LR, MC-RR and demethylated forms were also determined [5].

In Lake Kotokelskoye, the least toxic MC-RR dominated insignificantly, while the fraction of highly toxic MC-LR in total MC content was smaller, and the contribution from MC-YR characterized by medium toxicity turned out to be the lowest. The composition and ratios of MC in Lake Kotokelskoye are comparable with those for lakes in Japan where mainly MC-LR, MC-RR and MC-YR dominate. However, MC content in the lakes of Japan is

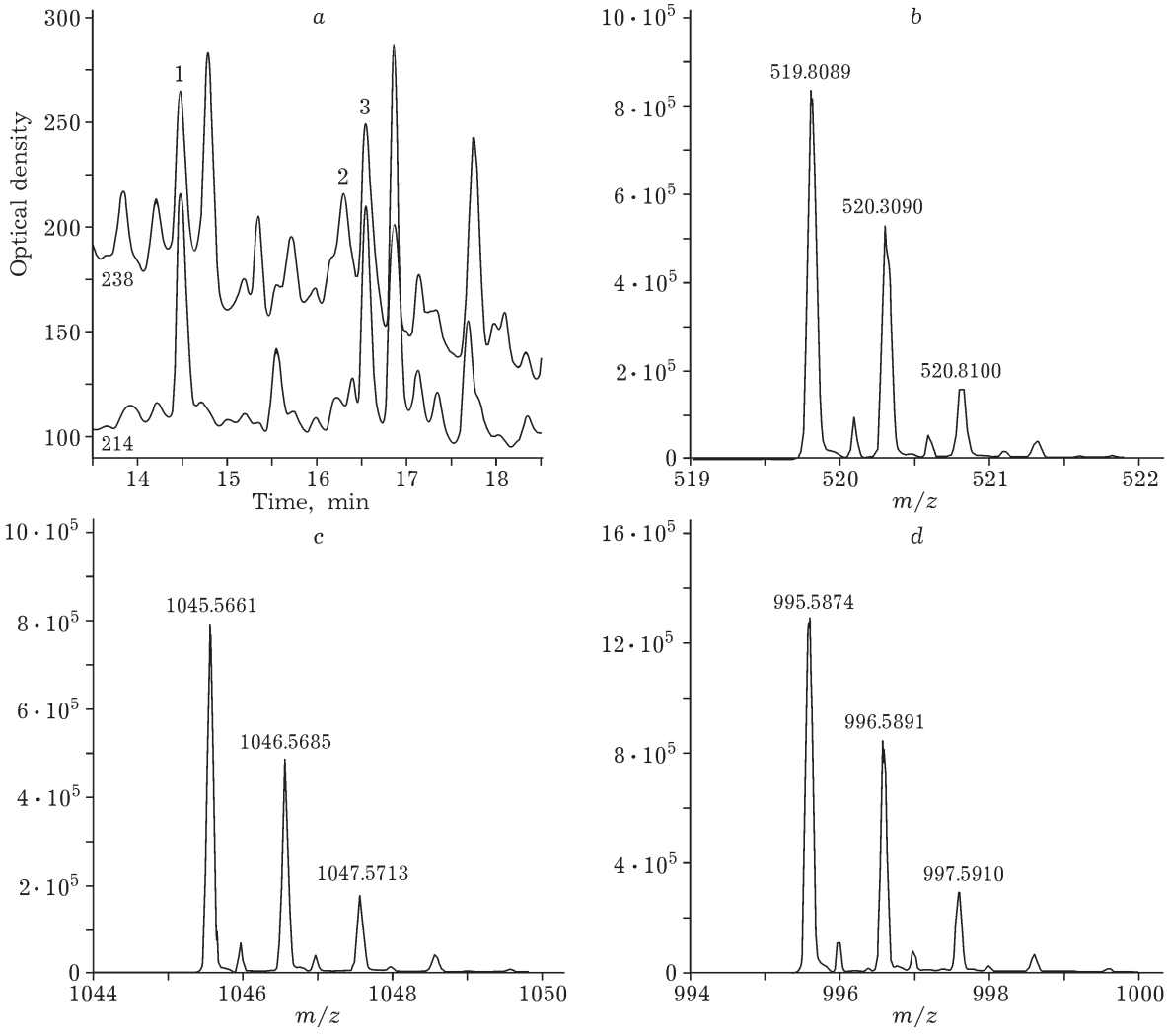

Fig. 3. HPLC-MS determination of microcystins in the extract from phytoplankton of Lake Kotokelskoye: *a* – chromatogram of the extract of microcystins: *1* – microcystin-RR (T_R = 14.50 min), *2* – microcystin-YR (T_R = 16.55 min), *3* – microcystin-LR (T_R = 16.86 min); *b*–*d* – mass spectra for peaks 1–3, respectively.

higher: the sum of three MC reached 2100 μg/g of the dry mass [5]. It was demonstrated that for the majority of strains from the lakes of Japan MC-RR is prevailing [59, 60]. In water reservoirs of Thailand, among three major MC versions 9LR, YR and RR), MC-RR dominates.

In general, MC-LR is the dominating toxin in water reservoirs of Europe, Canada and North America [3]. In Lake Ontario with toxicogenic *Microcystis, Anabaena* and *Planktothrix*, five MC versions were determined, including RR, YR, and LR [44]. Similarly to the western part of lakes Erie and Huron, MC-LR dominates there (50–100 %), as well as MC-RR or MC-LA, depending on station [61]. Subsequent analysis of MC composition in another part of Lake Erie showed that here mostly demethylated isoforms dRR (63 %) and dLR (12 %) occur, in the regions near the banks MC-LR prevailed (78 %), while the fraction of MC-RR reached 22 % [62]. In Great Lakes, MC-YR turned out to be practically minor component; its fraction was 9–23 % in the total MC content [44, 61, 62].

The similarity between Lake Kotokelskoye and small lakes in Japan is observed not only in the versions and relations of MC. We also revealed genetic relationship between toxigenic *Mictrocystis* species for these lakes. In general, geographical confinement is observed for MC versions. For example, substantial diversity of versions in the position of *L*-amino acids was

detected in South Africa, demethylated isoforms occur rather frequently in the strains from the lakes of Finland [5]. This distribution in part depicts regional differences in the species composition of cyanobacteria. In the case of Lake Kotokelskoye, some similarity of MC versions with the lakes of East Asian regions is observed, but at the same time some MC-producing species from the lake exhibit high similarity of *mcy*E gene with isolates from the lakes of Europe and North America.

For the strains of *A. Lemmermanii* from the lakes of Finland, which are phylogenetically similar to *Anabaena* sp. sequences from Lake Kotokelskoye, major MC are MC-LR, MC-RR and demethylated versions of Mdha. Demethylated versions of MeAsp were also detected in low concentrations [63]. For the *A. lemmermanii* 66A strain, derivatives of homotyrosine (Hty) and homophenylalanine (Hph) dominated.

Cyanobacteria containing the genes of MC synthetase and synthesizing MC in water reservoir situated near Lake Baikal and having a direct waterway connection with the latter may be potentially dangerous for the deepest lake in the world. The presence of toxic cyanobacteria and MC in bays and shallow regions of Lake Baikal, especially near Turka settlement, where the water of Lake Kotokelskoye outfalls through a system of rivers, provides evidence of possible inwash of MC-producing cyanobacteria from that water reservoir. It is known that propagation of cyanobacterial "blossoming" promotes migration of MC-producing genotypes between water reservoirs. Toxic species get into the lake from neighbouring small lakes and pools as a result of the transfer of water masses during storms or with fishing boats, equipment and so on. A similar fact was also described for the Great Lakes (Lake Ontario) [44].

Beresh water reservoir. In addition to water reservoirs of the Baikal region, in Siberia we also studied the Beresh water reservoir, which is a hypereutrophic cooling reservoir of the Berezovo Power Plant (Krasnoyarsk Territory). Mass development of *M. aeruginosa* and *Anabaena flos-aquae* was observed there. According to the data of genetic analysis, the samples from the cooling water reservoir contain large amounts of the sequences of AMT domain which are similar by 97–99 % to the isolates of *M. aeruginosa*, causative agents of toxic "blossoming" in different water reservoirs over the world [64].

Baltic Sea

Cyanobacteria of *Microcystis* genus containing the genes of MC synthesis were detected with the help of molecular genetic detection method in the Kursh Bay, a hypereutrophic freshwater lagoon of the Baltic Sea [52]. During the period of monitoring since April to November 2002–2008, 91 species of cyanobacteria were revealed. Among them, six dominating a potentially toxic species were found: *Aphanizomenon flos-aquae*, *Anabaena* sp., *Microcystis aeruginosa*, *M. viridis*, *M. we-senbergii*, *Planktothrix agardhii*. The biomass of cyanobacteria was substantial varying from 10 to 113 g/m^3 as average, which was up to 82 % of the total biomass of phytoplankton. The sequences of AMT domain of *mcy*E genes from the Kurshsky Bay had the best similarity with those in *M. aeruginosa* and *M. viridis*. The water area of the bay is included into the tourist and recreation zone Kurshskaya Kosa and is actively used by the population. In view of intense and long-term water "blossoming" caused by potentially toxic and toxicogenic cyanobacteria, the ecological situation in the Kursh Bay is considered unfavourable.

Ukraine

Unlike for the cold water reservoirs of East Siberia, the presence of toxic MC-producing cyanobacteria in the productive water reservoirs of Ukraine was predictable [65]. Samples were taken from the Kiev and Kanev water reservoirs, in the pools and lakes of Kiev and Kiev Region, in the Dnepr River and its bays in 2009–2010. In 2009, PCR analysis for the presence of genes coding the synthesis of microcystins was positive for 68 % of the examined water reservoirs of Ukraine, in 2010 – for 90 % of water reservoirs. It was demonstrated that "blossoming" of toxic cyanobacteria is characteristic of the communities with the domination of several species, while it was not revealed in monodominant communities. We

analyzed 34 cultures from the collection of the Institute of Hydrobiology, National Academy of Sciences of Ukraine. The PCR analysis of strains revealed the presence of *mcy*E gene in one culture which, according to the data on determined sequence of 16S rRNA gene, belongs to *Fischerella* genus. So, we established for the first time the presence of *mcy*E gene in this genus. With the help of chromatography-mass spectrometry, microcystins LR, RR, YR *etc*., as well as aeruginosins (linear peptides possessing antithrombotic activity) were determined in the phytoplankton of the Kanevskoye water storage reservoir.

So, toxicogenic cyanobacteria occur with a high rate in water reservoirs of Ukraine, for example in the majority of samples collected within the territory of Kiev and its suburbs. Analysis showed that potentially toxic are representatives of *Microcystis* genus. It is evident that nature conservation organs and enterprises engaged in water supply and water preparation should pay attention to the obtained results.

Belarus

The species composition of cyanobacteria, their quantitative development (number and biomass) was studied in economically important and recreational water reservoirs of Minsk (the Republic of Belarus): the Drozdy water storage reservoir, Komsomolskoye lake and running-water regions of the Svisloch River within the boundaries of the city [66]. The Svisloch with man-made water reservoirs survives various anthropogenic actions. At present, in spite of the measures aimed at improvement of the quality of its water, this is the most heavily polluted river in Belarus. In August 2006–2010, the species that dominated in biomass were *Aphanothece clathrata* (10–66 %), *Microcystis aeruginosa* (8–57 %), *M. we-senbergii* – (5–18 %), *M. viridis* (8–13 %), *Syne-chococcus aeruginosus* (25–50 %), *Aphani-zomenon flos-aquae* (10–20 %), among nine species of *Anabaena* genus, most essential were *A. flos-aquae* and *A. lemmermannii,* among other genera – *Planktothrix agardhii*. The biomass of cyanobacteria at the stations on the Svisloch in recreation sites reached 30–40 mg/L in some years, and the number of cyanobacteria was $2 \cdot 10^9$ cl/L, which is many times higher than the parameters recommended by WHO for recreation water reservoirs – $20 \cdot 10^6$ cl/L [19]. From the samples of phytoplankton of the Svisloch River taken at the station in Vaneev str. in August and September 2010, eight sequences of *mcy*E gene were obtained; seven of them belonged to cyanobacteria of *Microcystis* genus and one to *Anabaena* (see Figs. 1, 2). The sequences of *Microcystis* sp. had a high degree of similarity (98–99 %) with the sequences of *M. aeruginosa*, *M. viridis* and *M. wesenbergii* strains isolated during toxinc "blossoming" from water reservoirs in Japan, the Netherlands, the USA and South African Republic.

So, the species of *Microcystis* and *Anabaena* genera containing *mcy*E genes coding the synthesis of MC were detected in all the studied water reservoirs. Dominating species were the representatives of *Microcystis* genus, similarly to the majority of fresh water reservoirs of the world.

It is known that more than 80–90 % of samples from water reservoirs of Denmark, Germany, Czechia and Korea where *Microcystis* dominate contain MC [5]. The maximal amount of toxic strains was found for *M. aeruginosa*, approximately half of them have *mcy* genes and produce MC; the concentrations of MC for *M. aeruginosa* strains are higher in comparison with the toxic strains of other *Microcustis* species [59, 60, 67, 68]. Analysis of *Microcystis* spp. from Lake Wannsee (Germany) revealed that 73 % of *M. aeruginosa* colonies contained *mcy* genes [69]. The abundance of *Microcystis* spp. during toxic "blossoming" often exceeds limiting values not only in tropical regions but also in the temperate zone. For example, in the Great Lakes Huron and Erie the number of *Microcystis* spp. was 13–50 and 5–34 million cl/L, respectively [61], while in Lake Wannsee it reached 610 million cl/mL [70], which is many times higher than the concentrations of *Microcystis* spp. in water reservoirs studied by us.

MC-producing strains of *Anabaena* were detected in many water reservoirs of the world; blossoming of *Anabaena* is abundant in salty waters of the Baltic Sea [5]. However, in fresh water reservoirs of different trophic levels *Anabaena* is rarely the only dominating species. In 15 % of mesotrophic and 25 %

eutrophic lakes, toxicogenic *Microcystis* and *Anabaena* occur at the same time [49]. *Anabaena*, due to its ability to fix nitrogen, may develop under the conditions of nitrogen limitation.

CONCLUSION

According to the data of international hydrological programme of UNESCO "CYANONET", blossoming of cyanobacteria and related appearance of toxins was revealed in water reservoirs and water flows of 65 countries of the world [71]. In one of the recent reviews, Russia and FSU countries were not marked at the map of toxic blossoming [4]. We established that toxic cyanobacteria able to synthesize MC are present in the majority of the studied water reservoirs of East Siberia, Ukraine, Belarus. The data obtained agree with the results of Finnish researchers who demonstrated that MC-producing species inhabit 91 % of lakes in the countries with cold climate [49].

To evaluate the possibility of economic and recreation use of water reservoirs in which the mass development of cyanobacteria is observed, ensuring the absence of any health risk, it is necessary to carry out monitoring of the concentration of cyanotoxins in water with the help of analytical methods.

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