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EXPERIMENTAL ARTICLES

Analysis of Phytoplankton in Tsimlyansk Reservoir (Russia) for the Presence of Cyanobacterial Hepato- and Neurotoxins

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Abstract—Although the water bodies of southern Russia experience the most extreme effects of cyanobacterial blooms, molecular genetic data on the composition of toxigenic cyanobacteria in this region have been absent. Screening for the genes responsible for the synthesis of hepatotoxins (microcystins and cylindrospermopsin) and neurotoxins (anatoxin-a and saxitoxins) in cyanobacteria from the Tsimlyansk Reservoir on the Don River was carried out. The presence of microcystin-producing *Microcystis* and *Planktothrix* populations, as well as of cyanobacteria capable of synthesis of a neurotoxin anatoxin-a, was revealed by polymerase chain reaction (PCR). A hypothesis of the presence of anatoxin-a-producing *Planktothrix rubescens* population in the phytoplankton of the Tsimlyansk Reservoir is proposed. The obtained PCR data were confirmed by the results of enzyme-linked immunosorbent assay (ELISA) and liquid chromatography/mass-spectrometry (LC/MS). Anatoxin-a and five microcystin variants were identified in the phytoplankton biomass.

Keywords: cyanobacterial toxins, microcystins, anatoxin-a, cylindrospermopsin, saxitoxins, PCR, enzyme-linked immunosorbent assay, liquid chromatography/mass-spectrometry, Tsimlyansk Reservoir

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Cyanobacteria are among the major components of phytoplankton in aquatic ecosystems worldwide. In the periods of prolonged high temperatures and elevated content of biogenic elements in the water, massive development of these microorganisms often occurs, which results in water blooms. The consequences of this event in the basins of the southern Russian regions may be extreme. The emergency occurring in the Tsimlyansk Reservoir in October 2009, when accumulation of cyanobacteria resulted in clogging of the water inlets, terminating water supply to Volgodonsk, is a recent example (Matishov and Kovaleva, 2010). Secretion of cyanobacterial toxins, such as hepatotoxins (microcystins and cylindrospermopsin) and neurotoxins (saxitoxins and anatoxin-a), is another negative effect on human health (Apeldoorn et al., 2007). Among these toxins, the first group causes necrotic liver damage, while the second one causes paralysis of respiratory muscles, which may result in death of respiratory arrest (Apeldoorn et al., 2007).

In spite of the practical importance of this issue, no information was available on the composition of toxigenic cyanobacteria and cyanotoxins in the Tsimlyansk Reservoir. Due to their ubiquitous occurrence in aquatic ecosystems, microcystins attract special attention of researchers. To our knowledge, molecular genetic identification of neurotoxin-producing cyanobacteria, particularly anatoxin-a producers, in Russian reservoirs has never been carried out.

The goal of the present work was to identify the hepato- and neurotoxigenic cyanobacteria in the Tsimlyansk Reservoir phytoplankton using molecular genetic and chemical analytic techniques.

MATERIALS AND METHODS

Characterization of the reservoir and sampling. The Tsimlyansk Reservoir is among the largest lake-type water body in southern Russia, which was formed at the conjunction of the mid- and lower reach of the Don River. It is characterized by weak water exchange (the yearly water exchange coefficient 1.05), relatively shallow depth (8–9 m on average), extended vegetation period (205–215 days), and high productivity of biocenoses (Lapitskii, 1970). The catchment area is 255000 km^2, the water surface area is 2702 km^2, full volume of the reservoir at the normal headwater level 36.0 meters according to the Baltic System is 23.86 km^3. The reservoir has four reaches: Verkhnii, Chirskoi, Potemkinskii, and Priplotinnyi (Fig. 1), which are separated by shoreline constrictions and have different morphometric, hydrological, and commercial-biological characteristics. The water belongs to the hydrocarbonate class of type II calcium group

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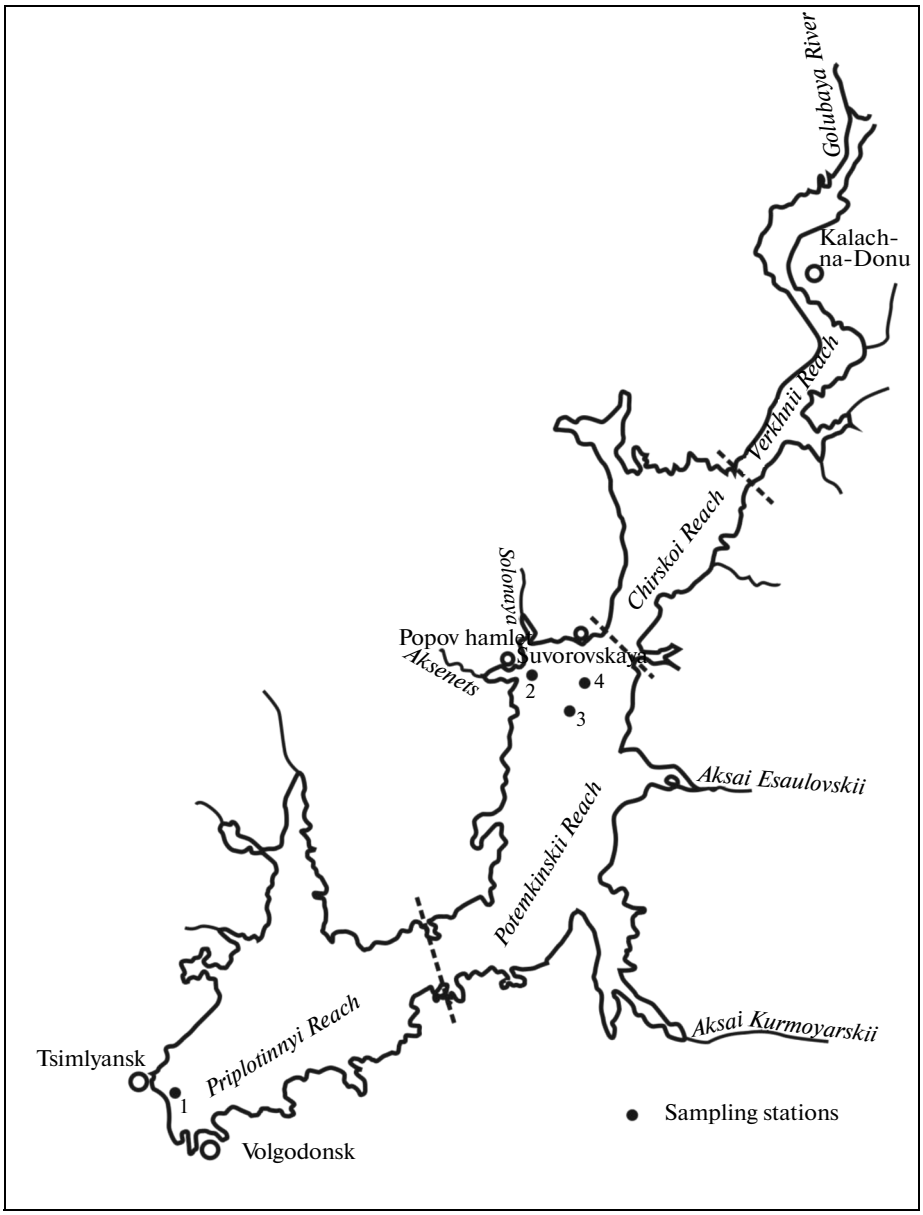

Fig. 1. Schematic map of the Tsimlyansk Reservoir with location of the sampling stations.

with total mineralization from 200 to 500 mg/L. At the present stage of its existence, the ratio of biogenic elements in the water changed: the concentration of ammonium increased, and the ratio of nitrogen and phosphorus concentrations decreased (Kuchishkina, 2002).

The present work was carried out using phytoplankton samples collected on August 5–15, 2013 in the Priplotinnyi (station no. 1) and Potemkinskii reaches (station no. 2 opposite Popov hamlet, station no. 3 between the Chaus section and Suvorovskii hamlet, and station no. 4 in the area closer to Suvorovskii) (Fig. 1). Phytoplankton samples were collected from the surface, concentrated by filtration through Vladis-art membranes (5 μm pore diameter), and fixed with a Lugol's iodine-based solution (Kuz'min, 1975). Species identification of cyanobacteria was carried out using the relevant identification manuals (Komárek and Anagnostidis, 1998, 2005; Komárek, 2013). The algae were enumerated in a Nageotte chamber (0.01 mL), and the biomass was determined using the counting-volumetric method (Methodical recommendations..., 1984). Determination of the taxonomic composition and calculation of the biomass were carried out for the samples of two stations, nos. 1 and 2.

Amplification of the cyanotoxin synthesis gene fragments. DNA was isolated from filtration-concentrated

phytoplankton using the Diatom DNA Prep 200 reagent kit (Isogene Lab Ltd., Russia) according to the manufacturer's recommendations. For detection of toxigenic cyanobacteria, a search for the *mcyE, aoaA, anaC*, and *stxA* genes, which are responsible for synthesis of microcystins, cylindrospermopsin, anatoxin-a, and saxitoxins, respectively, was carried out. Polymerase chain reaction (PCR) was carried out using specific primers HEPF/HEPR (amplicon size 472 bp), CatF1/CatR1 (881 bp), anaC–genF/anaC–genR (366 bp), and sxtaf/sxtar (600 bp) under amplification conditions described in the literature (Jungblut et al., 2006; Ballot et al., 2010b; Rantala-Ylinen et al., 2011; Baron–Sola et al., 2012). For direct genus-level identification of microcystin and anatoxin-a producers in the samples with mixed cyanobacterial composition, PCR analysis with genus-specific primers was used: mcyE–F2/MicmcyE–R8 (for *Microcystis*, 250 bp), mcyE–F2/AnamcyE–12R (for *Anabaena*, 250 bp), and mcyE–F2/mcyE–plaR3 (for *Planktothrix*, 250 bp) and anaC–anab F/R (for *Anabaena*, 263 bp) and anaC–osc F/R (for *Oscillatoria*, 216 bp), respectively (Vaitomaa et al., 2003; Rantala et al., 2006; Rantala-Ylinen et al., 2011). PCR was carried out in 20 μL of the GenPak PCR Core reagent mixture (Isogene Lab., Russia). DNA of axenic cultures of microcystin-producing cyanobacteria *Microcystis aeruginosa* strain PCC 7806 and *Planktothrix agardhii* strain NIVA–CYA 126 (provided by Elke Dittmann, Potsdam University), as well as of the cylindrospermopsin-producing strain *Aphanizomenon* sp. 10E9 and of the saxitoxin-producing strain *Aphanizomenon* sp. AB59 (provided by Jutta Fastner, Federal Environmental Agency, Berlin) were used as positive controls. No control strains of microcystin-producing *Anabaena* species and of anatoxin-a-synthesizing cyanobacteria were used. In these cases, the known sizes of specific PCR amplicons were used for orientation. For the negative control reaction, cyanobacterial strains producing none of the studied cyanotoxins were used (the non-axenic culture of *Gloeocapsa decorticans* (A. Braun) Richter in Wille, provided by the Department of Botany and Microbiology, Yaroslavl State University). PCR products were separated by electrophoresis in 1.5% agarose gel, stained with ethidium bromide, and analyzed under UV illumination. The size of the amplified DNA fragments was determined using the FastRuler Low Range DNA Ladder molecular mass marker (Thermo Scientific, United States).

Liquid chromatography/mass-spectrometry and enzyme-linked immunosorbent assay of cyanotoxins. Preliminary search for cyanotoxins (microcystins, cylindrospermopsin, saxitoxins, and anatoxin-a) was carried out in water extracts from filtration-concentrated phytoplankton (stations nos. 1–4) by enzyme-linked immunosorbent assay using the relevant ELISA test systems (Abraxis LLS, United States) according to the manufacturer's recommendations. For cyanotoxin extraction from the cells, distilled water was added to the filters, followed by a freezing–thawing cycle and ultrasonic treatment.

Precise identification of individual microcystins and anatoxin-a was carried out using high-performance liquid chromatography/tandem mass-spectrometry, which is widely used for such studies (Spoof, 2005; Mazur-Marzec et al., 2008; Meriluoto and Spoof, 2008).

Water and 75% methanol extracts from the phytoplankton-carrying filter from station no. 1 were analyzed for microcystins and anatoxin-a using LTQ Orbitrap Hybrid Ion Trap-Orbitrap Mass Spectrometer (Thermo Finnigan, United States). The standard solutions of anatoxin-a fumarate (A.G. Scientific, United States), a mixture of LR, RR, and YR microcystins (Sigma Aldrich, United States), and a mixture of LR, RR, YR, LY, LW, and LF microcystins, demethyl-MC-RR, and demethyl-MC-LR isolated by preparative liquid chromatography were used for identification.

The mixture was separated by reversed-phase chromatography on a Thermo Hypersil Gold column (100 × 3 mm, 3 μm) with a gradient elution mode (from 0 to 75% B) at 0.2 mL/min flow rate. Eluent A was 0.05% solution of formic acid in water. Eluent B was 0.05% solution of formic acid in acetonitrile. The volume of injected sample was 25 μL. Mass-spectrometry was carried out using electrospray ionization in the positive ions detection mode. The scanned mass ranges (m/z) were 500–530 for detection of doubly-charged molecular ions of microcystin-RR derivatives (Edwards et al., 1993; Yuan et al., 1999) and 850–1200 for single-charged molecular ions of microcystins. Analysis was carried out under unit resolution conditions (detection in an ITMS linear quadrupole trap). The temperature of the ion source was 275°C, the flow rate of carrier gas (nitrogen) was 25 arb, and ISpray voltage was 3.2 kV. To obtain MS/MS spectra, the CID mode (collision-induced dissociation) was used at 35% collision energy. Retention time in the chromatographic column and the masses of detected ions confirmed by the data of MS/MS spectra (coincidence of at least three diagnostic ion products) were used for identification. Thus, the presence of m/z 135 (Adda side-chain fragment $[C_9H_{10}O + H]^+$) and m/z 375 [Adda–Glu–Mhda + H-134]$^+$ ion products in the tandem spectra, indicating a compound as a microcystin (Table 1), was used for microcystin identification. Arginine-containing microcystins were identified using the m/z 599 ion product corresponding to the structure [Arg–Adda–Glu + H]$^+$ or [MeAsp–Arg–Adda + H]$^+$ (Table 1). Individual variants of microcystins were confirmed by the presence of [M + H-134]$^+$ fragments on the tandem spectra (Table 1), using also a number of other diagnostic ion products.

Table 1. Characteristic ion products used for microcystin identification

Microcystin	Protonated molecule $[M + H]^+$	Characteristic ion products for microcystins, *m/z*		
		all variants	arginine-containing microcystins	individual variants $[M + H\text{-}134]^+$
dmMC-LR	981	135 375	599	847
MC-LR	995			861
dmMC-RR	1024			890
MC-RR	1038			904
MC-YR	1045			911

Special attention was paid to identification of anatoxin-a (*m/z* 166 for the protonated molecule) in order to avoid false positive results due to the possible presence of an isobaric compound, phenylalanine, in the water and biomass (the masses of these compounds differ by 0.05 Da). It was confirmed by the data of MS/MS (tandem) spectra. The ion products at *m/z* 149 ($[M + H\text{-}NH_3]^+$) and *m/z* 131 ($[M + H\text{-}NH_3\text{-}H_2O]^+$) are diagnostic for anatoxin-a, which, unlike phenylalanine, has ion products at *m/z* 120 and *m/z* 131 in MS/MS spectra (Furey et al., 2005). The ratio of intensities of the listed ion products and the absence of an intense ion at *m/z* 120 typical of phenylalanine were also considered in the course of analysis.

RESULTS AND DISCUSSION

During the period of investigation, phytoplankton of the Priplotinnyi (station no. 1) and Potemkinskii reaches (station no. 2) contained cyanobacteria, diatoms, cryptophytes, chlorophytes and chrysophytes. In 2013, the average phytoplankton abundance and biomass for the reservoir were 319×10^6 cells/L, 12 mg/L, respectively, with cyanobacteria responsible for 98 and 72% of these values, respectively. At the sampling sites, quantitative phytoplankton abundance was below average, although still relatively high, with total numbers and biomass 286.7×10^6 cells/L and 6.1 mg/L at station no. 1 and 77.6×10^6 cells/L and 6.8 mg/L at station no. 2. Cyanobacteria were predominant in both reaches both numerically (96–99% of the total cell number) and by biomass (70–85% of the total biomass). Microscopic analysis of the phytoplankton samples revealed potentially toxigenic cyanobacteria in both reaches (Table 2). At stations nos. 1 and 2, *Microcystis* and *Planktothrix* species were predominant, while *Aphanizomenon* and *Anabaena* species were present in small numbers (Table 2). *Aphanizomenon* and *Microcystis* species visually predominated at stations nos. 3 and 4, respectively. This ratio of cyanobacteria with and without heterocyts is generally typical of the Tsimlyansk Reservoir in high-water years, such as 2013. Thus, it was previously shown that *Microcystis, Anabaena, Aphanizomenon*, and *Planktothrix* species constituted most of the summer cyanobacterial complex in the reservoir, with nitrogen-fixing *Anabaena* and *Aphanizomenon* predominant in low-water years, while heterocyt-free *Microcystis* and *Planktothrix* prevailed in high-water years (Lysak, 2002).

The presence of cylindrospermopsin and saxitoxin-producing cyanobacteria was not revealed by PCR with specific primers or by ELISA. Fragments of the *mcyE* and *anaC* genes were, however, amplified in the total phytoplankton DNA, indicating ability of cyanobacteria from this reservoir to synthesize microcystins and anatoxin-a, respectively. ELISA analysis revealed intracellular microcystins at all sampling stations. At stations nos. 1–4, *Microcystis* populations containing the *mcyE* gene were identified (Fig. 2a). Since *M. aeruginosa* was the only *Microcystis* species revealed in the Priplotinnyi Reach (Table 2), and PCR analysis for the presence of *Microcystis* populations containing the microcystin synthetase gene *mcyE* was positive, *M. aeruginosa* from the Tsimlyansk Reservoir was able to produce microcystins. This species is known as a microcystin producer worldwide (Sivonen and Jones, 1999). The *mcyE* gene of *Planktothrix* species was detected only in the total phytoplanktonic DNA sample from the Priplotinnyi Reach (Fig. 2b). PCR results were in perfect agreement with those obtained by microscopic analysis of phytoplankton. *Microcystis* colonies were present at the stations of both Potemkinskii and Priplotinnyi reaches (Table 2), while *Planktothrix* were predominant only in the Priplotinnyi Reach (Table 2). Since the early 2000s, the share of *P. agardhii* and *P. rubescens* among the dominant species increased, so that in summer in the Priplotinnyi Reach it may reach 40% of the weighted average biomass (Golokolenova, 2007). The *mcyE* gene fragment specific for microcystin-producing *Anabaena* species was not revealed. The negative PCR result was probably caused by low abundance of *Anabaena flos-aquae* during the sampling period (Table 2).

Apart from the results of ELISA and PCR analysis, the presence of microcystin-producing cyanobacteria in the Tsimlyansk Reservoir was also confirmed by LC/MS. In the analyzed samples of water and 75% methanol extracts of phytoplankton biomass from sta-

Table 2. Species composition and biomass of potentially toxigenic cyanobacteria

Species	Biomass, mg/L	
	Station no. 1	Station no. 2
Microcystis flos-aquae (Wittr.) Kirch.	–	0.05
Microcystis viridis (A. Braun in Rabenh.) Lemm.	–	0.20
Microcystis wesenbergii (Kom.) Kom.	–	0.08
Microcystis aeruginosa (Kütz.) Kütz.	0.03	3.74
Anabaena flos-aquae Breb. ex Born. et Flah.	0.04	0.01
Planktothrix agardhii (Gom.) Anag. et Kom.	3.20	–
Planktothrix rubescens (D. C. ex Gom.) Anagn. et Kom.	0.80	–
Oscillatoria limosa Ag. ex Gom.	0.10	–
Aphanizomenon gracile Lemm.	0.03	–
Aphanizomenon flos-aquae Ralfs ex Born. et Flah.	–	0.72

The species not found in the samples are indicated by dashes

tion no. 1, the following arginine-containing microcystins (MC) and their demethylated variants (dmMC) were detected: MC-LR (m/z 995), dmMC-LR (m/z 981), MC-RR (m/z 1038), dmMC-RR (m/z 1024), and MC-YR (m/z 1045). Extracted LC/MS chromatograms for above mentioned m/z of the water extract showing the presence of microcystins are presented on Fig. 3. Retention times for detected microcystins and for the standards differed by not more than 0.3 min. Individual microcystin variants were also confirmed by the presence of $[M + H\text{-}134]^+$ ion fragments in the tandem spectra (Table 1). Demethylated microcystin-RR was quantitatively predominant among the observed variants. In order to determine its isomeric structure, ion products corresponding to characteristic fragments of the structure were analyzed. Of the three possible isomeric variants of demethylated microcystin-RR, the presence of [D-Asp^3]MC-RR was confirmed by analysis of the fragment spectra (Fig. 4). The most intensive characteristic ion product at m/z 499 [Ala–Arg–D–Asp–Arg + H]$^+$, as well as the product ions at m/z 582 [Mdha(Dhb)–Ala–Arg–D–Asp–Arg + H]$^+$, m/z 714 [D–Asp–Arg–Adda–Glu + H]$^+$, and m/z 343 [Ala–Arg–D–Asp+H]$^+$ registered in the MS/MS spectrum indicated the loss of a methyl group on Asp in position 3 (Fig. 4). Other ion fragments at m/z 213 [Glu–Mdha(Dhb) + H]$^+$, m/z 311[Mdha(Dhb)–Ala–Arg + H]$^+$, and m/z 446 [$C_{11}H_{15}O$–Glu–Mdha(Dhb)–Ala]$^+$ indicated the presence of the structural fragment either of dehydrobutyrine (Dhb) or *N*-methyldehydroalanine (Mdha) in position 7 (Mazur-Marzec et al., 2008). Since the masses of these fragments are identic, it was impossible to determine the structural fragment in position 7 of the isomer based on the data from unit resolution MS/MS spectra.

In environmental samples with predominance of *P. agardhii*, the isomeric structure [D–Asp^3,

Mdha[7]]MC-RR was mainly present (Fastner, 1999). In the case of *P. rubescens*, [D–Asp[3], Dhb[7]]MC-RR was the predominant variant. Since both *Planktothrix* species were simultaneously dominant in the Priplotinnyi Reach (Table 2), the presence of both [D–Asp[3]]MC-RR is possible.

The isomeric structure [D-Asp[3]] MC-LR was most probable for demethylated microcystin-LR, since the ion products obtained indicated the absence of a methyl group in position 3 on Asp: *m/z* 866 $[M + H–D–Asp]^+$, *m/z* 714 $[D–Asp–Arg–Adda–Glu + H]^+$, *m/z* 556 $[Mdha–Ala–Leu–D–Asp–Arg + NH_4]^+$, *m/z* 539 $[Mdha–Ala–Leu–D–Asp–Arg + H]^+$, and *m/z* 456 $[Ala–Leu–D–Asp–Arg + H]^+$.

Our results are in agreement with the literature data on predominance of demethylated forms of microcystins in environmental samples with predominance of *Planktothrix* species (Fastner, 1999; Sivonen and Jones, 1999; Kurmayer et al., 2004; Mazur-Marzec et al., 2008).

Both the *anaC* gene (Fig. 2d) and intracellular anatoxin-a were found in the Priplotinnyi Reach, which was confirmed by ELISA and by LC/MS. Data comparison of mass chromatograms and single-resolution tandem mass spectra for the water extract from phytoplankton biomass of station no. 1 and of anatoxin-a standard solution (Fig. 5) confirmed the presence of this cyanotoxin in the Priplotinnyi Reach sample. The water extract contained the ion products at *m/z* 149 ($[M + H\text{-}NH_3]^+$) and *m/z* 131 ($[M + H\text{-}NH_3\text{-}H_2O]^+$), which are characteristic of anatoxin-a (Fig. 5).

In this region, anatoxin-a has been detected in the lower Don area (Matishov et al., 2006), which indicates the possibility of considerable occurrence of anatoxin-a-synthesizing cyanobacteria in southern Russian water bodies. Several cyanobacterial species producing anatoxin-a have been reliably determined: various *Oscillatoria* sp. strains, *O. formosa* Bory ex Gom., *Planktothrix rubescens, Cylindrospermum* sp., *Anabaena flos-aquae, A. planktonica* Brunnth., *Phormidium favosum* (Bory) Gom., *P. autumnale* (Ag.) Trevisan ex Gom., *Raphidiopsis mediterranea* Skuja, *Aphanizomenon flos-aquae, A. issatchenkoi* (Ussaczev) Pr.-Lavr., *Arthrospira fusiformis* (Voronikhin) Kom. et Lund (Sivonen and Jones, 1999; Viaggiu et al., 2004; Gugger et al., 2005; Ballot et al., 2010a; Rantala-Ylinen et al., 2011). Thus, *Anabaena flos-aquae, Oscillatoria limosa,* and/or *P. rubescens* were the possible anatoxin-a producers in the Priplotinnyi Reach (Table 2). However, PCR analysis with genus-specific primers did not reveal *Anabaena* or *Oscillatoria* populations containing the *anaC* responsible for anatoxin-a synthesis (Fig. 2a). This may point to the possible presence of anatoxin-a producing *P. rubescens* populations in the Tsimlyansk reservoir. A single report exists presently concerning ability of *P. rubescens* populations from Lake Spino (Italy) to produce anatoxin-a (Viaggiu et al., 2004). Thus, methodical

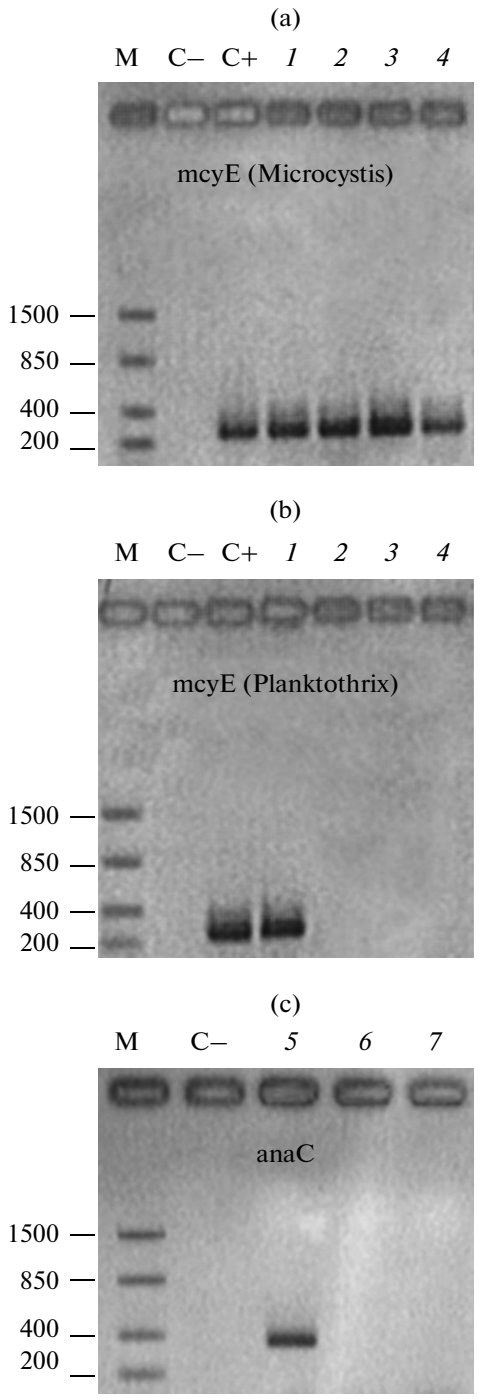

Fig. 2. PCR analysis of total DNA of the Tsimlyansk Reservoir phytoplankton: for the *mcyE* microcystin synthetase genes in *Microcystis* species (a); for the *mcyE* microcystin synthetase genes in *Planktothrix* species (b); and for the *anaC* gene of anatoxin synthesis (c). Lanes: M, molecular mass marker (bp); C–, negative control; C+, positive control; *1–4*, total DNA of phytoplankton (stations nos. 1–4, respectively); *5–7*, result of amplification of the total phytoplankton DNA (station no. 1) with the primers anaCgen (all anatoxin-a producers), anaC-anab (*Anabaena* producers), and anaC-osc (*Oscillatoria* producers), respectively.

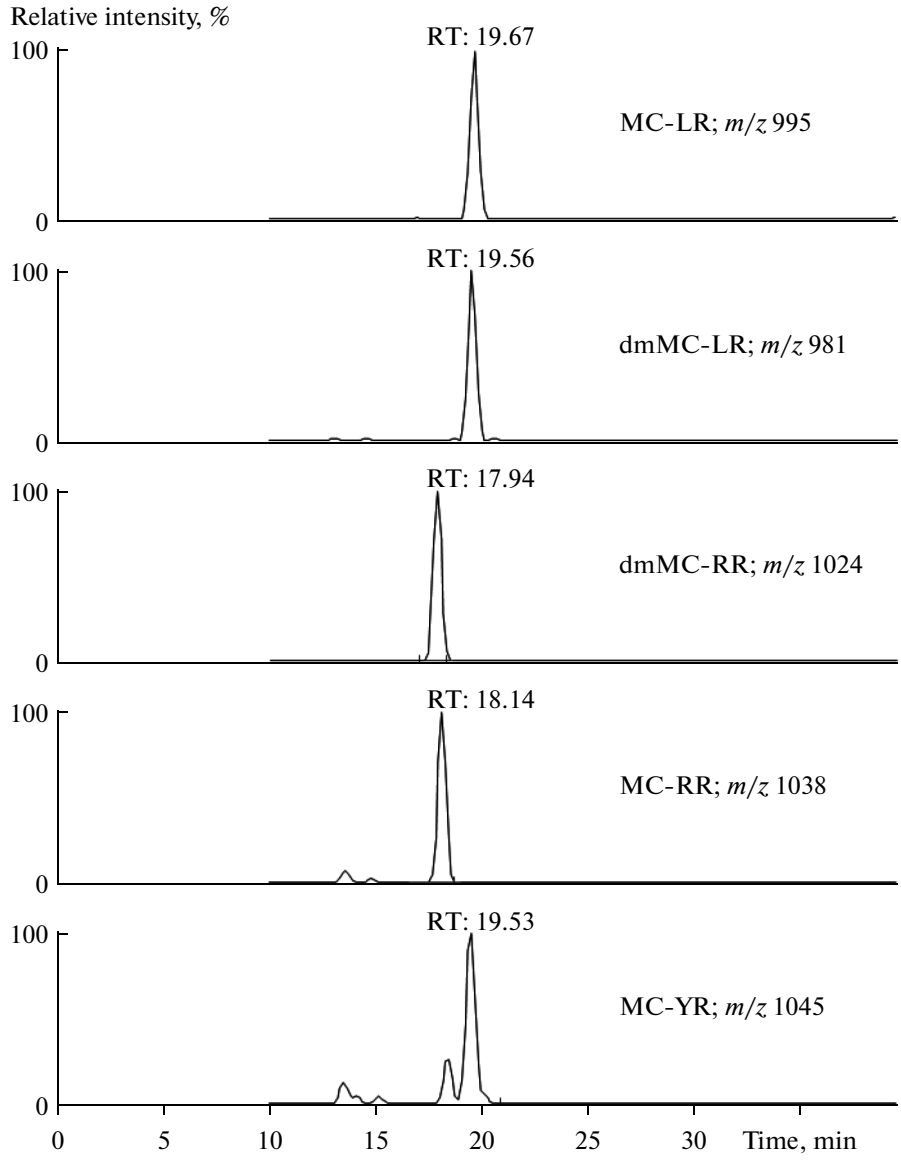

Fig. 3. Mass chromatogram of separation of microcystins from the water extract of phytoplankton biomass (station no. 1) obtained in the tandem analysis mode (ITMS linear quadrupole trap, electrospray ionization for registration of positive ions ESI(+), fragmentation energy 35% CID).

approaches used in the present work (light microscopy, PCR, ELISA, and chromatography/mass-spectrometry) yielded identical results.

Microcystin-producing *Microcystis* and *Planktothrix* have not been previously identified in the Tsimlyansk Reservoir. PCR and ELISA revealed no cyanobacteria producing cylindrospermopsin and saxitoxins. Five microcystin variants were found: MC-LR, MC-RR, MC-YR, and demethylated forms [D–Asp^3]MC-LR and [D–Asp^3]MC-RR. Quantitative predominance of demethylated microcystin variants at station no. 1 was an additional confirmation of the ability of *Planktothrix* from the Tsimlyansk reservoir to synthesize these toxins. Molecular genetic identification of anatoxin-a-producing cyanobacteria has not been previously carried out in Russia. The presence of intracellular anatoxin-a in the phytoplankton biomass of the Priplotinnyi Reach was confirmed by ELISA and LC/MS. The presence of *P. rubescens* populations capable of anatoxin-a synthesis in the reservoir was hypothesized.

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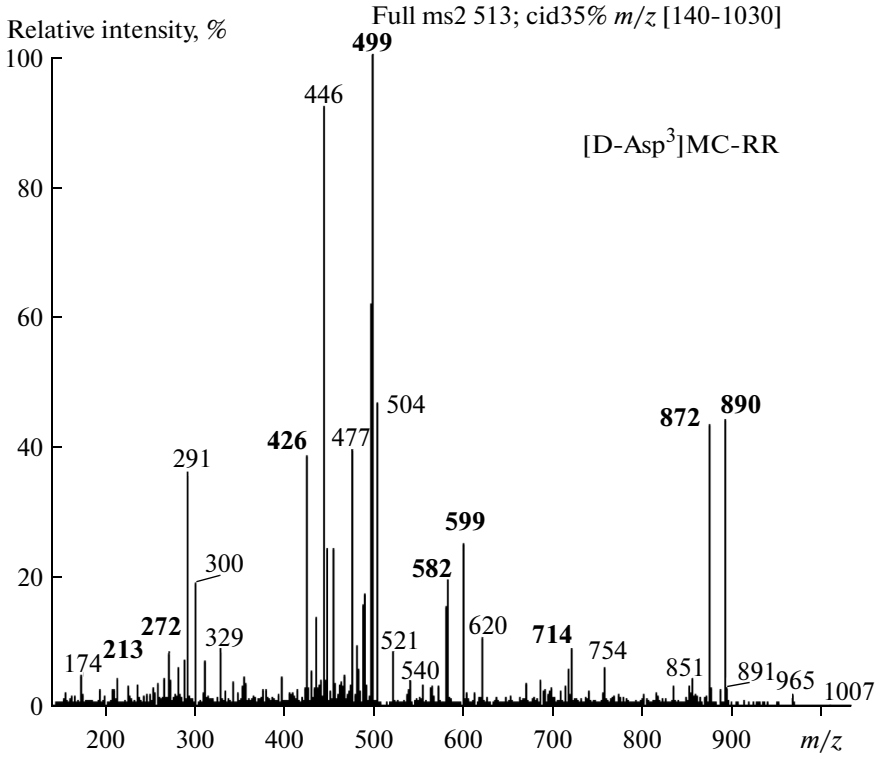

Fig. 4. Tandem mass spectrum for the precursor ion *m/z* 513 (doubly protonated molecule of demethylated MC-RR). The variant of isomeric structure [D-Asp^3]MC-RR.

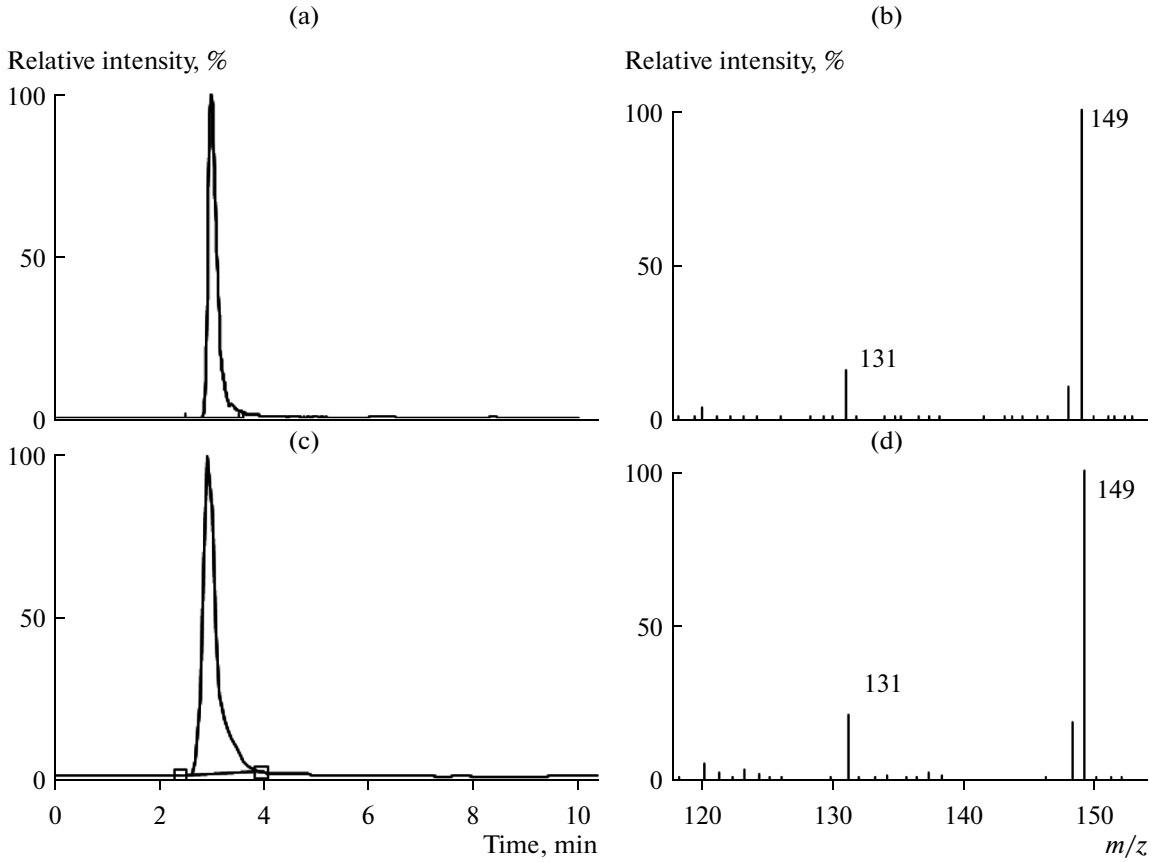

Fig. 5. Mass chromatograms (a, c) and single-resolution tandem mass spectra (b, d) (linear quadrupole trap, electrospray ionization for registration of positive ions, fragmentation energy 35% CID) confirming the presence of anatoxin-a in the sample: water extract from phytoplankton biomass, station no.1 (a, b) and the standard anatoxin-a solution, 100 ng/mL (c, d).

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