Mycrocystin-LR degradation by indigenous bacterial community of Rybinsk Reservoir

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Abstract. The fate of microcystin-LR (MC-LR) in water samples of Rybinsk Reservoir taken before and during water bloom was assessed. The decrease in the MC-LR concentration in the both samples was due to its biodegradation by indigenous bacterial community. The highest biodegradation rate and shortest half-life of MC-LR in the water sample taken during water bloom were observed. A microcystin degrading bacterium was isolated and shown be 98.92% similar to Sphingopyxis sp. based on a fragment of 16S rRNA gene sequence. The rate of MC-LR biodegradation by Sphingopyxis sp. S7 was strongly dependent on initial concentration of toxin and temperature.

1. Introduction
The Rybinsk Reservoir is located on the Volga River and is part of the Volga-Kama Cascade of reservoirs. The surface area of the Rybinsk Reservoir is 4580 sq. km, the maximum depth is 28 m (on average 5.6 m) and the trophicity level is from mesotrophic to eutrophic [1].

The reservoir is widely used for domestic, drinking and industrial water supply; it is of great recreational importance. The observed regular water blooms reduce the biological productivity of the reservoir, create problems at water intake stations, and reduce the recreational attractiveness. In the plankton of the Rybinsk Reservoir during the "bloom" period, cyanobacteria are numerically leading with the dominance of Aphanizomenon flos-aquae (L.) Rafs and Microcystis aeroginosa Kutz [1, 2].

Cyanobacterial blooms have become a serious global environmental problem due to unpleasant odor and cyanobacterial toxins produced. Cyanobacteria Microcystis aeroginosa, which dominate during the "blooming" of the Rybinsk Reservoir, are producers of algotoxins-microcystins (MCs) classified as hepatotoxic and carcinogenic [3]. In the Rybinsk Reservoir, the presence of microcystins, including the highly toxic MC-LR was recorded. So, in 2010 year, the content of MCs in water was 0.079 μg/l [2], in 2016 year 0.01 μg/l and 0.3 μg/l in algal biomass [1]. During the growth of cyanobacteria, MC, remain in the cells and are found to be released during senescence and breakdown process, which poses a serious environmental risk [4].

Microcystins are cyclic heptapeptides with general structure: cyclo-(D-Ala-L-X-D-MeAsp-L-Z-Adda-D-Glu-Mdha) (figure 1); X and Z – variable amino acids, D-MeAsp – D-erythro-b-methylaspartic acid, Adda – (2S, 3S, 8S, 9S) 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4, 6-dienoic acid and Mdha – N-methyldehydroalanine.
By now approximately 246 variants of MCs have been characterized. MCs variants differ in toxicity [5]. According to WHO recommendations, the concentration of the most toxic MC-LR in drinking water should not exceed 1 μg/l [3].

The cyclic structure of MCs is associated with their resistance to temperature and pH. MCs are able to survive at temperatures above 300 °C in the absence of sunlight, as well as at extremely high and low pH [6, 7].

Advanced oxidation water treatment processes (ozonation, chlorination, solar or UV/chlorine process, photocatalytic degradation, permanganate oxidation, et al.) are generally expensive, ineffective at removing or destroying MCs [5]. In connection with this, biological treatment approach to degrade and remove MCs, from water bodies has received great attention in recent years [8, 9].

Despite their stability and resistance to peptidases of pro- and eukaryotic microorganisms, MCs can be degraded by bacteria and fungi. The process of microbial degradation is the main one in the destruction of MCs in water bodies. Most bacteria capable of degrading microcystins belong to Proteobacteria (α, β, γ), Actinobacteria and Bacilli. A number of MCs bacteria-destructors have cluster genes Mlr – MlrA, MlrB, MlrC, MlrD, which are responsible for the biodegradation of these toxins. However, in some bacteria-destructors, for example, Artrobacter and Brevibacterium Mlr-genes were not found, which suggests the possible participation of other genes in the destruction of MCs [5].

Natural microbial communities from lakes [10], drinking water reservoirs [11], sea water and soil [12] have demonstrated the ability of indigenous microbiota to degrade algotoxins, including MCs.

In Russia, despite the urgency of the problem of toxic “blooms” of water bodies, studies related to the biodegradation of microcystins by aquatic microbiota have not been carried out to date.

This study aimed to assess MC-LR biodegradation by indigenous bacterial community of Rybinsk Reservoir and isolation and identification of biodegrading responsible bacterium.

2. Materials and methods

2.1. Environmental water sampling
Volumes of 1 l surface water were collected from the Rybinsk Reservoir in area (58.065545, 38.255244) with frequent CyanoHABs, in 20.05.2018 (before water bloom) and 01.08.2018 (during water bloom). Water samples were kept in plastic bottles until arriving at the laboratory.

2.2. Bath degradation experiments
To determine the rate of microcystin biodegradation by the natural community of microorganisms, MC-LR was added to the samples of natural waters at a dose of 100 μg/l. The samples were incubated...
at a temperature of 20 ± 1°C, illumination 1000 lx, with a light/dark regime - 12 hours/12 hours. Samples of natural water sterilized at 1 atm within 30 minutes were used as a control. For the determination of content of MC-LR in water, water samples (5 ml) were lyophilized and dissolved in 400 μl of 25 % (vol/vol) aqueous methanol. The supernatant was separated by centrifugation at 10,000 rpm for 5 min. The MC-LR concentration was determined by HPLC on a Hewlett-Packard HP1090 chromatograph with a DAD detector (at 238 nm with 1.2 nm resolution) according to the previously described method [13]. The microcystin standard was purchased from Sigma-Aldrich (St. Louis, MO).

2.3. Isolation of MC-LR degraders
After incubation of water sample with MC-LR for 20 days, aliquots were streaked on BG11 agar plate with 0.2 mg/l MC-LR and incubated at 28°C for 2-3 days. The bacterial colonies with different colony colour, shape, elevation were picked and re-suspended in liquid BG11 medium with MC-LR. The liquid cultures were incubated at 28°C for 3 days and they were used to prepare pure bacterial cultures. Single colonies were re-streaked for at least five times to obtain pure isolates.

The ability of the isolated cultures to destroy MC-LR was determined under static conditions in 20 ml tubes with 10 ml of BG11 medium (pH 7.0) containing 1 mg/l MC-LR, at 20°C for 7 days. Negative control consisted of 10 ml BG11 medium containing 1 mg/l MC-LR, without bacterial inoculation.

2.4. Identification of isolated bacterium
Isolated bacterial strain was identified by its morphological, physiological and biochemical characteristics [14], and the analysis of 16S rRNA. Genomic DNA was isolated using a reagent kit AxyPrep Multisource Genomic DNA Miniprep Kit (Corning, USA) in accordance with the manufacturer’s recommendations. The following PCR primers were used for sequencing of the 16S rRNA: fD1 5’ AGAGTTTGATCCTGGCTCAG-3’ and rD1 5’ CTTAAGGAGGTGATCCAGCC-3’[15]. PCR was performed in 50-μl reaction mixtures containing 5 μM dNTPs (Helicon, Russia), 50 pmol of each primer (Eurogen, Russia), 0.5 μl (5 U/μl) of Taq polymerase (Helicon, Russia) and 100 - 200 ng of purified template DNA. For amplification the C1000™ Thermal Cycler was used (BioRad, USA). PCR conditions were following: initial denaturation at 95°C for 3 min 30 sec; 35 cycles of denaturation at 94°C for 1 min, annealing at 54°C for 1 min and extension at 72°C for 2 min; final extension at 72°C for 6 min 10 sec. Electrophoresis was carried out with 1% agarose gel (Invitrogen, USA) in TAE. A 100-bp GeneRuler™ and Lambda DNA/HindIII markers (Fermentas, USA) were used for sizing and approximate quantification of DNA fragments. Purification of the PCR products was usually performed by using PureLink™ Quick kit (Invitrogen, USA) according to the manufacturer’s guidance. The direct sequencing of PCR products was performed by an ABI PRISM 3500xl genetic analyser (Applied Biosystems, USA). The sequences were compared with related sequences available in the GenBank databases using BLAST analysis (http://www.ncbi.nlm.nih.gov). The strain was identified in the Russian Collection of Agricultural Microorganisms (RCAM, WDCM 966).

2.5. MC-LR biodegradation by strain S7
Autoclaved BG11 medium (20 ml) containing the strain S7 was incubated in sterilized 50 ml flask. The starting OD 540 for strain S7 was approximately 0.1. Each flask was spiked with filter sterilized MC-LR to initial concentrations – 0.3 μg/ml, 1.0 μg/ml, 5.0 μg/ml for MC-LR. The controls were prepared in autoclaved BG11 medium with same levels of MC-LR. Liquid cultures were incubated under static conditions at 24°C. In addition, BG11 medium containing 0.3 μg/ml MC-LR was prepared to examine the MC-LR biodegradation by strain S7 at different temperatures: 9°C, 24°C. An aliquot (1ml) of samples was withdrawn at intervals and centrifuged, and residual MC–LR was measured according to the protocol described previously in Section 2.2.
2.6. **Statistical analysis**

Results were expressed as mean values ± standard deviation (mean ± SD). All statistical analyses were performed by the Mann–Whitney U tests (STATISTICA version 8 (Statsoft, Inc.)). Statistically significant differences were reported with p values < 0.05.

3. **Results and discussion**

The concentration of microcystin LR decreased during the incubation of water samples taken in the Rybinsk Reservoir before and during water bloom (figure 2). The observed concentration decreases were considered as biodegradation of MC-LR since the control experiments remained constant during the experimental period.

![Figure 2](image_url)  
**Figure 2.** Microcystin-LR degradation in natural water samples of the Rybinsk Reservoir: 1 – sterile samples; 2 – samples taken before CyanoHABs; 3 – samples taken during CyanoHABs.

The absence of lag phase in both experimental samples may indicate adaptation of the indigenous bacterial community of the Rybinsk Reservoir to microcystin due to regular "blooms". These findings are in agreement with the data of other authors who showed that bacterial community isolated from natural waters with previous cyanobacterial contamination was noted to entirely removal MC-LR without lag phase [16]. Exposure to MCs drives change in structure and physiology of indigenous microbial community and may also enhance biodegradation of MCs [17].

The highest biodegradation rate (18.0 µg l⁻¹ day⁻¹) and shortest half-life (2.2 days) were observed in the water samples taken during CyanoHABs (table 1).

| Water samples     | MC–LR biodegradation rate (µg l⁻¹ day⁻¹) | Degradation rate constant, k* (day⁻¹) | Determination coefficient R² | Half life, t₁/₂ (day) |
|-------------------|-----------------------------------------|--------------------------------------|-----------------------------|----------------------|
| before CyanoHABs  | 6.3±0.4ᵃ                              | 0.319±0.021ᵃ                         | 0.9599                      | 6.1±0.5ᵃ             |
| during CyanoHABs  | 18.0±1.1ᵇ                             | 0.114±0.012ᵇ                         | 0.9854                      | 2.2±0.2ᵇ             |

*calculated from first-order rate law.

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Three bacteria showed microcystin-degradative activity were isolated from a water sample taken during the "blooming" of the Rybinsk Reservoir and inoculated with 100 µg/l MC–LR for 20 days. One isolated single strain (S7) which showed the highest degradative activity was selected for subsequent studies.

Strain S7 was aerobic, Gram-negative, motile bacterium. Cell size 0.8×0.2 µm. After 72 hours of incubation on the SPA medium (1:1) the strain formed shiny beige colonies with a diameter of 1.2-2.0 mm. Catalase- and oxidase-positive. Indole production and nitrate reduction are negative. Acid is produced from trehalose, D-glucose and D-ribose. Assimilates D-glucose, D-mannose, D-ribose, fructose, xylose and trehalose. The 16s rRNA sequence of the strain was determined and compared with the sequence available from GenBank databases to obtain more definitive information on the taxonomic and phylogenetic position of strain S7. Based on morphological, physiological and biochemical analysis and a fragment of 16S rRNA gene sequence, the isolate S7 was identified as Sphingopyxis sp.

The rate of MC–LR biodegradation by S7 strain was strongly dependent on initial concentration of toxin and temperature (figure 3, 4, table 2, 3).

![Figure 3.](image)

**Figure 3.** MC–LR biodegradation by MC-degrading bacterium Sphingopyxis sp. S7 at different concentration: 1 – 0.3 µg/ml; 2 – 1.0 µg/ml; 3 – 5.0 µg/ml.

| MC-LR concentration, µg/ml | MC-LR degradation rate (µg ml⁻¹ h⁻¹) | Degradation rate constant, k* (h⁻¹) | Determination coefficient R² | Half-life, t₁/₂ (hour) |
|---------------------------|--------------------------------------|-----------------------------------|-----------------------------|---------------------|
| 0.3                       | 0.143±0.01ᵃ                        | 1.55±0.12ᵃ                       | 0.9727                      | 0.45±0.05ᵃ          |
| 1.0                       | 0.25±0.02ᵇ                        | 0.345±0.019ᵇ                     | 0.9581                      | 2.0±0.3ᵇ           |
| 5.0                       | 0.60±0.05ᶜ                        | 0.164±0.01ᶜ                      | 0.9624                      | 4.2±0.4ᶜ           |

The duration of the process of 100% destruction of MC–LR, depends on its concentration, ranged from 2 to 6 hours. The rate of biodegradation increased with increasing toxin concentration and reached its maximum value (0.6 µg ml⁻¹ h⁻¹) at 5.0 µg/ml.

The lowest rate of MC-LR biodegradation was noted at +9°C. The highest MC-LR biodegradation rate (0.143 µg ml⁻¹ h⁻¹) was at +24°C (figure 4, table 3).
Figure 4. Effect of temperature on MC–LR degradation by MC-degrading bacterium *Sphingopyxis* sp. S7: 1 - +9°C; 2 - +24°C.

Table 3. Kinetic parameters of MC-LR degradation by *Sphingopyxis* sp. S7 at different temperature.

| Temperature | MC–LR biodegradation rate (µg ml⁻¹ h⁻¹) | Degradation rate constant, k* (h⁻¹) | Determination coefficient R² | Half life, t₁/₂ (hour) |
|-------------|--------------------------------------|-----------------------------------|-----------------------------|---------------------|
| +9°C        | 0.047±0.004ᵃ                       | 0.215±0.021ᵃ                      | 0.9651                      | 3.2±0.3ᵃ            |
| +24°C       | 0.143±0.015ᶜ                      | 1.55±0.15ᶜ                       | 0.9727                      | 0.45±0.03ᶜ          |

Our results were consistent with findings of previous studies. Some authors stating that the MC–LR degradation rate by bacteria strongly depends on the temperature. Biodegradation rates have been shown the tendency to be higher as higher the temperature. At elevated temperatures, bacteria become metabolically active, enhancing enzymatic activity and bacterial metabolic on MC–LR [18].

4. Conclusion
Microcystin-LR can be biodegraded by aquatic indigenous bacterial community of Rybinsk Reservoir. A bacterial strain capable of degrading MC-LR was isolated. This strain was identified as *Sphingopyxis* sp. The rate of MC-LR biodegradation by *Sphingopyxis* sp. S7 was strongly dependent on initial concentration of toxin and temperature. The strain *Sphingopyxis* sp. S7 theoretically could be directly added to HABs to degrade MC or could be used in biofilters to remove MC-LR from drinking water.

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References
[1] Chernova E, Sidelev S, Russkikh I, Korneva L, Solovyova V, Mineeva N, Stepanova I and Zhakovskaya Z 2020 Spatial distribution of cyanotoxins and ratios of microcystin to biomass
indicators in the reservoirs of the Volga, Kama and Don Rivers, the European part of Russia Limnologica 84 125819 doi:10.1016/j.limno.2020.125819.

[2] Korneva L G, Soloveva V V, Jackowskaya Z A, Russkikh Y V, Chernova E N 2014 Fitoplankton i soderzhanie cianotoksinov v Rybinskom i Gor’kovskom i Cheboksarskom vodohranilishchah v period anomal’no zharkogo leta 2010 g [Phytoplankton and content of cyanotoxins in Rybinsk, Gorky and Cheboksary reservoirs during the anomalously hot summer of 2010] Voda: himiya i ekologiya pp 24–29

[3] Cyanobacterial toxins: microcystins. Background document for development of WHO Guidelines for drinking-water quality and Guidelines for safe recreational water environments. Geneva: World Health Organization 2020 (WHO/HEP/ECH/WSH/2020.6). Licence: CC BY-NCSA 3.0

[4] Carmichael W W 1992 Cyanobacteria secondary metabolites—the cyanotoxins J. Appl. Bacteriol. 72 pp 445–459

[5] Massey I Y and Yang F 2020 A mini review on microcystins and bacterial degradation Toxins 12(4) 268 pp1-21 doi.org/10.3390/toxins12040268

[6] Tsuji K, Naito S, Kondo F, Ishikawa N, Watanabe M F, Suzuki M, Harada K 2004 Stability of microcystins from cyanobacteria: Effect of light on decomposition and isomerization Environ. Sci. Technol 28 pp173–177

[7] Harada K, Tsuji K, Watanabe M F and Kondo F 1996 Stability of microcystins from cyanobacteria—III. Effect of pH and temperature Phycologia 35 pp 83–88

[8] Huang F Y, Feng H, Li X Y, Yi X P, Guo J, Clara T and Yang F 2019 Anaerobic degradation of microcystin-LR by an indigenous bacterial Enterobacter sp. YF3 J. Toxicol. Environ. Health Part A 82(21) pp 1120–1128 doi: 10.1080/15287394.2019.1699345

[9] Zhang J, Lu Q, Ding Q, Yin L and Pu Y A 2017 Novel and native microcystin-degrading bacterium of Sphingopyxis sp. isolated from lake Taihu Int. J. Environ. Res. Public Health 14 1187

[10] Lezcano M Á, Quesada A and El-Shehawy R 2018 Seasonal dynamics of microcystin-degrading bacteria and toxic cyanobacterial blooms: Interaction and influence of abiotic factors Harmful algae 71 pp19–28

[11] Ho L, Dreyfus J, Boyer J, Lowe T, Bustamante H, Duker P, Meli T and Newcombe G 2012. Fate of cyanobacteria and their metabolites during water treatment sludge management processes Sci Total Environ 424 pp 232–238

[12] Redouane E M, El S A Z, El F K, Oufdou K, Oudra B, Lahrouni M, et al 2019 Mode of action and fate of microcystins in the complex soil-plant ecosystems Chemosphere 225 pp 270–281

[13] Medvedeva N, Zaytseva T and Kuzikova I 2017 Cellular responses and bioremoval of nonylphenol by the bloom-forming cyanobacterium Planktothrix agardhii 1113 Journal of Marine Systems 171 pp 120–128 doi.org/10.1016/j.jmarsys.2017.01.009

[14] Holt J G 1994 Bergey's manual of determinative bacteriology. Baltimore: Williams & Wilkins 787 pp

[15] Weisburg W G, Barns S M, Pelletier D A and Lane D J 1991 16S ribosomal DNA amplification for phylogenetic study Journal of Bacteriology 173 pp 697–703

[16] Christoffersen K, Lyck S and Winding A 2002 Microbial activity and bacterial community structure during degradation of microcystins Aquat. Microb. Ecol. 27 pp 125–136

[17] Giaramida L, Manage P M, Edwards C, Singh B K and Lawton L A 2013 Bacterial communities' response to microcystins exposure and nutrient availability: Linking degradation capacity to community structure International Biodeterioration and Biodegradation 84 pp 111–117 doi:10.1016/j.ibiod.2012.05.036

[18] Krishnan A, Zhang Y-Q and Mou X 2018 Isolation and characterization of microcystin-degrading bacteria from Lake Erie Bulletin of Environmental Contamination and Toxicology 101 pp 617–623 doi:10.1007/s00128-018-2468-4