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Bacteria homologus to *Aeromonas* capable of microcystin degradation

Abstract: Water blooms dominated by cyanobacteria are capable of producing hepatotoxins known as microcystins. These toxins are dangerous to people and to the environment. Therefore, for a better understanding of the biological termination of this increasingly common phenomenon, bacteria with the potential to degrade cyanobacteria-derived hepatotoxins and the degradative activity of culturable bacteria were studied. Based on the presence of the *mlrA* gene, bacteria with a homology to the *Sphingopyxis* and *Stenotrophomonas* genera were identified as those presenting potential for microcystins degradation directly in the water samples from the Sulejów Reservoir (SU, Central Poland). However, this biodegrading potential has not been confirmed in *in vitro* experiments. The degrading activity of the culturable isolates from the water studied was determined in more than 30 bacterial mixes. An analysis of the biodegradation of the microcystin-LR (MC-LR) together with an analysis of the phylogenetic affiliation of bacteria demonstrated for the first time that bacteria homologous to the *Aeromonas* genus were able to degrade the mentioned hepatotoxin, although the *mlrA* gene was not amplified. The maximal removal efficiency of MC-LR was 48%. This study demonstrates a new aspect of interactions between the microcystin-containing cyanobacteria and bacteria from the *Aeromonas* genus.

Keywords: microcystins, biodegradation, *mlrA* gene, *Aeromonas*, *Stenotrophomonas*, *Sphingopyxis*

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DOI 10.1515/biol-2015-0012
Received January 13, 2014; accepted August 20, 2014

1 Introduction

Cyanobacteria that are able to produce hepatotoxins known as microcystins are the key indicators of increasing eutrophication caused by the excessive inflow of nutrients into freshwater aquatic environments [1]. Thus, a limitation in nutrient inflow from the catchment must be the first step in reducing cyanobacterial blooms [2-5]. However, the investigation and selection of methods for removing nutrients requires time and specific physicochemical and biological data for a particular body of water. Therefore, it is important to develop methods to treat areas where toxic cyanobacteria already exist and affect the quality of drinking and recreational water resources. For this task, implementation of biological methods with the use of controlling agents such as bacteria capable of microcystins removal seems to be promising.

In the study of Ho *et al.* [6] the rapid biological sand filtration with natural indigenous bacteria (with domination of *Sphingopyxis* sp. LH21) aggregated in the biofilm was reported as an effective treatment process for the complete removal of microcystins. Also, Bourne *et al.* [7] reported the usefulness of applying selected cultured bacteria *Sphingomonas* sp. MJ-PV strain for removing of microcystin-LR (MC-LR) in sand filtration columns.

An example of possible microcystins removal from surface water was described in the pilot study of Ji *et al.* [8]. In a meso-scale experiment performed in Lake Taihu (China), artificial media were submerged in the flowing water from the lake. The biofilm containing indigenous bacteria (with domination of *Pseudomonas* spp. and *Bacillus* spp.), which was created on artificial media, was able to degrade microcystins.

As indicated by cited studies, the removal of microcystins by a diverse community of bacteria is considered to be the dominant proces responsible for the disappearance of cyanobacterial-derived hepatotoxins

in water. Therefore this biological termination of microcystins by bacteria is currently being intensively studied. Bacteria capable of microcystins degradation belong to the genus: *Pseudomonas* (Australia, Japan, China), *Sphingomonas* – including *Sphingosinicella* (Japan, Argentina, New Zealand), *Sphingopyxis* (Australia, China), *Novosphingobium* (China), *Stenotrophomonas* (China), *Ochrobactrum* (China), *Methylobacillus* (China), *Methylosinus* (China), *Ralstonia* (China), *Bacillus* (Saudi Arabia), *Morganella* (USA), *Rhizobium* (USA), *Microbacterium* (USA), *Burkholderia* (Brazil), *Methylotenera* (USA) *and* various *Burkholderiales*, including *Bordetella* (USA, China) [9-12].

In Europe, there is limited data on the specific bacteria capable of degrading cyanobacterial hepatotoxins in fresh water. The first strain of bacteria was isolated from sediment of Lake Vihnusjärvi in 2005 and classified as a novel bacterium: *Paucibacter toxinivorans* [13]. In Scotland, three new strains of bacteria were discovered: *Arthrobacter* sp., *Brevibacterium* sp. and *Rhodococcus* sp. These species were isolated from Lake Rescobie, Lake Forfar, and the River Carron [14-15].

The process of microcystins degradation, as was already mentioned, can be performed by different groups of bacteria, but the only described and continuously studied route of degradation of microcystin molecule was presented by Bourne *et al.* [16]. This 3-step sequential enzymatic process was based on proteolytic hydrolysis of peptide bonds, in which a crucial role is played by the *mlr* gene cluster, consisting of the genes: *mlrA*, *mlrB*, *mlrC* and *mlrD*, coding intracellular enzymes. The first step of this process (activation of *mlrA* gene) involves the linearization of the microcystin molecule. The product of the first enzymatic step was reported to be 160-fold less reactive than the cyclic microcystin. Both the second and third steps involved the gradual cutting of the linearized microcystin chain, which resulted in degradation into its individual components.

The objectives of the present study were: 1) to assess the co-occurrence of bacteria with the potential for microcystins degradation (based on *mlrA* genes presence) and microcystin-producing cyanobacteria (based of *mcyE* gene presence), together with determination of the concentration of cyanobacteria-derived hepatotoxins in Sulejów Reservoir (SU), the lowland dam reservoir in Central Poland; and 2) to identify culturable bacteria isolated from the reservoir actively degrading microcystin molecules, and determine their respective removal efficiencies. The phylogenetic affiliation of culturable bacteria based on sequencing of the 16S rRNA gene fragment was also performed.

2 Experimental Procedures

2.1 The study site

In the present study, water samples were collected from the Sulejów Reservoir at Tresta Station located near the dam in the lacustrine zone of the reservoir (+51°27′42.53″, +19°58′40.88″). The reservoir located in Central Poland was formed by damming at 138.9 km of the Pilica River (Fig. 1). This reservoir is used for flood control, recreation, fishing and power generation. The Sulejów Reservoir is also used as an alternative source of drinking water for the city of Lodz. It is an example of a dam reservoir with progressive anthropogenic eutrophication, in which cyanobacterial blooms dominated by toxic *M. aeruginosa* appear regularly every year [17-23]. During the bloom accumulation, the total microcystins concentration (intra- and extracellular) in the water could increase to 30 μg L^{-1} [19].

2.2 Preparation and molecular analysis of environmental samples

Integrated water samples were collected every 2 weeks during the summer season from May to October 2010. To obtain material for DNA analysis, each water sample (100 mL) was filtered using a sterile filter with a pore size of 0.45 μm for the analysis of cyanobacteria or a pore size of 0.22 μm for the analysis of other bacteria (Millipore, USA). The filters were placed in 2 mL of lysis buffer (40 mM EDTA, 400 mM NaCl, 0.75 M sucrose and 50 mM Tris-HCl, pH 8.3) and stored at -20°C until DNA extraction. The DNA was isolated by hot phenol extraction from the filters based on the protocol by Giovannoni *et al.* [24] with the modifications described in Mankiewicz-Boczek *et al.* [20].

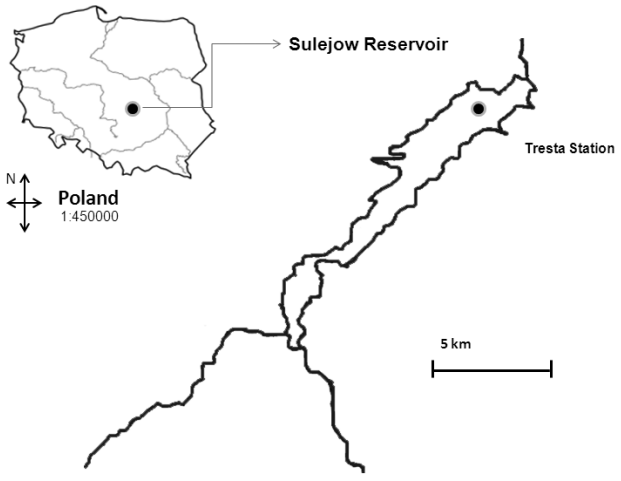

Figure 1: Study site. Sampling point located in Tresta Station, Sulejów Reservoir, between Tresta Gulf and Borki Gulf.

2.2.1 Amplification of mcyE gene

Molecular analysis using polymerase chain reaction (PCR) was performed to determine the presence of potential microcystin-producers via the amplification of the *mcyE* gene with *mcyE-R1*/*mcyE-S1* primers (Table 1). In the present study, the primers were designed, using Vector NTI Advance™ 9 software (Invitrogen), to hybridize to the *mcyE* consensus sequence - a sequence of DNA having similar structure and function in microcystin-producing cyanobacteria: *Microcystis aeruginosa*, *Planktothrix agardhii* and *Anabaena* sp. (currently *Dolichospermum*). The cyanobacterial *mcyE* gene takes part in the synthesis and integration of the Adda moiety (3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-4(E),6(E)-decadienoic acid) into the microcystin molecule. The Adda moiety is required for microcystin toxicity and binding the hepatotoxin to protein phosphatases [25]. The amplification of the *mcyE* gene fragments was performed for 11 isolated DNA samples.

The PCR was performed in a 20 µL volume reaction containing 1x PCR buffer, 0.25 µM each primer, 3 mM $MgCl_2$, 0.25 mM dNTP, 0.1 mg mL^{-1} BSA and 1 U of *Taq* polymerase (Qiagen). For one reaction, 1 µL of cyanobacteria DNA was used (DNA concentration range from 25 – 1,116 ng μL^{-1}). The PCR consisted of an initial denaturation step at 95°C for 5 min, followed by 30 cycles of DNA denaturation at 94°C for 30 s, primer annealing at 59°C for 30 s, and strand extension at 72°C for 1 min, and a final extension step at 72°C for 10 min.

The PCR products were separated on a 1.5% agarose gel by electrophoresis using a constant voltage (70 V), and the DNA was visualized using ethidium bromide (2 µg mL^{-1}).

2.2.2 Amplification of mlrA gene

For amplification of the *mlrA* gene fragments specific to the microcystin-degrading bacteria, primers designed by Saito *et al.* [26] were used. The *mlr*A gene encoding methylopeptidase (MlrA enzyme) catalyzes the first step of bacterial degradation of cyanobacterial hepatotoxin associated with hydrolysis and ring opening of microcystin molecule at the Adda-Arg peptide-bond formation site [16]. Both *mlrA* gene fragments were amplified in 5 of 11 isolated DNA samples. To amplify the longer fragment of the *mlrA* gene (807 bp), the first set of primers MF/MR were used (Table 1). The PCR reaction was performed according to Saito *et al*. [26] with minor modifications. The PCR reaction was performed in a final volume of 20 µL containing 1x PCR buffer, 5 µM each MF/MR primer, 2.5 mM $MgCl_2$ (Qiagen), 0.2 mM dNTP, 0.1 mg mL^{-1} BSA (Fermentas), and 0.5 U of *Taq* polymerase (Qiagen). For each reaction, 1 µL of bacterial DNA was diluted 20 times (DNA concentration range from 3 – 113 ng μL^{-1}). The PCR protocol consisted of an initial denaturation step at 94°C for 1 min, followed by 35 cycles of DNA denaturation at 94°C for 20 s, primer annealing at 60°C for 10 s, and strand extension at 72°C for 30 s, and a final extension step at 72°C for 10 min.

In the second stage, a nested PCR was performed with the products of the *mlrA* gene amplification containing fragments 807 bp in length (11 samples in total). Amplification of the shorter fragment of the *mlrA* gene, with a length of 453 bp, was performed using the primer pairs MF2/MR (Table 1). The PCR reaction was performed in a final volume of 20 µL containing 1x PCR buffer, 5 µM each primer MF2/MR, 2.5 mM $MgCl_2$, 0.2 mM dNTP, 0.1 mg mL^{-1} BSA (Fermentas), and 0.5 U of *Taq* polymerase

Table 1: Molecular markers and primer sequences used in the present study.

Genes & Primers	Sequence (5' to 3')	Size [bp]	Source
mcyE		405	Present study
mcyE-R1	ATAGGATGTTTAGAGAGAATTTTTTCCC		
mcyE-S1	GGGACGAAAAGATAATCAAGTTAAGG		
16S rRNA		1300-1400	[28]
B27F	AGAGTTTGATCCTGGCTCAG		
U1492R	GGTTACCTTGTTACGACTT		
mlrA		453 and 807	[26]
MF	GACCCGATGTTCAAGATACT		
MF2	TCGCCATTTATGTGATGGCTG		
MR	CTCCTCCCACAAATCAGGAC		

(Qiagen). Instead of the DNA, for each reaction, 1 µL of the *mlrA* PCR product (807 bp) from the previous reaction was used. The initial denaturation step was performed at 94°C for 1 min followed by 35 cycles of DNA denaturation at 94°C for 20 s, primer annealing at 58°C for 10 s, and strand extension at 72°C for 20 s, and a final extension step at 72°C for 5 min. Visualization of the results was performed as described above.

For the sequence analysis of the *mlrA* gene, the shorter PCR product (453 bp) obtained with specific MF2/MR primers (Table 1) was used. The PCR product was initially purified using a QIAEX® II Gel Extraction Kit (Qiagen) and then cloned into a pJET1.2/blunt vector (MBI Fermentas), followed by sequencing. Homology searches were performed using the National Center for Biotechnology Information microbial and nucleotide BLAST network service (http://blast.ncbi.nlm.nih.gov/Blast.cgi) [27] and Vector NTI Advance™ 9 software (Invitrogen).

2.3 In vitro experiments with environmental culturable bacteria

2.3.1 Preparation of bacterial cultures

Immediately after water sample collection, 100 µL of the unfiltered water taken on July 13th 2010 from Sulejów Reservoir, was placed on nutrient broth medium (8 g L^{-1} NB medium, 10 g L^{-1} glucose, 2 mL L^{-1} Tween 80, 1.5% agar) at dilutions made with distilled water: 0, 10^{-1}, and 10^{-2}. One sample dilution was used for one plate. The plates were incubated at 25°C in the dark. The initial plating of the water samples resulted in bacterial colonies with different morphologies. After 3 days of incubation, the bacterial colonies were washed from the plate, suspended in liquid NB medium, and mixed with sterile glycerol (final concentration 25%). The bacterial stocks prepared from the 0, 10^{-1}, and 10^{-2} dilutions containing the total pool of culturable bacteria were stored at -70°C. In further analysis with the total pool (experiment *no.* 1) or selected bacteria (experiment *no.* 2), only bacterial stocks prepared from the undiluted water sample was used. This plate contained the highest variability of bacterial colonies based on morphological characteristics.

2.3.2 Experiment with total pool of culturable environmental bacteria – no. 1

Before starting the *in vitro* experiment with MC-LR standard (Alexis®, USA), the previously prepared bacterial stocks were thawed and plated on solid NB medium in a volume of 50 µL. The plate was incubated at 25°C for 3 days. After passaging the bacteria from the thawed glycerol stocks (stored at -70°C), only morphologically homogenous colonies were obtained.

In the first experiment, the distilled water aliquots were spiked with MC-LR standard (Alexis®, USA) at a final concentration of 10 µg mL^{-1}. A high concentration of MC-LR was used to determine hepatotoxin levels with an analytical method (HPLC-DAD, High Performance Liquid Chromatography with Diode Array Detection). The bacteria isolated from the plate were added to the prepared MC-LR water solutions. As an experimental control, sterile distilled water without added bacteria was spiked with MC-LR standard. The prepared samples and controls were incubated with continuous shaking (50 rpm) in the dark at 25°C for 2 weeks. To determine the remaining MC-LR concentration, 400 µL subsamples were taken after 7 and 14 days.

2.3.3 *Experiment with selected culturable environmental bacteria – no. 2*

Bacteria from the stocks were prepared with undiluted water samples and plated on agar plates. The plates were incubated in the dark at 25°C for 3 days. Serial dilutions of the bacteria (dilutions in distilled water from 0 to 10^{-5}) were plated to obtain single bacterial colonies. The material originating from 192 individually grown bacterial colonies was randomly pooled into mix containing 6 colonies (cultivated bacteria were scratched from plate). Each bacterial mix was suspended in 100 µL of distilled water, and the suspensions were used in experiment *no.* 2. This process created 32 bacterial mixes. The control without bacteria was spiked with MC-LR and incubated according to the description in experiment *no.* 1. Subsamples from each individual bacterial colony from experiment *no.* 2 were stored in glycerol stocks (final concentration 25%) for further cultivation. Other subsamples from experiment *no.* 2 were taken for further phylogenetic analysis using molecular methods (see next subsection).

Similar *in vitro* experiments with individual bacterial colonies were also performed. However, passaging the bacteria from thawed glycerol stocks reduced the growth of individual colonies. As a result, no MC-LR degradation was observed in the experiments with individual bacterial colonies. Therefore, this part of the study was not included in the Results section.

2.4 Preparation and molecular analysis of culturable bacteria

The bacterial colonies from mixes 2, 3, 8, 10 and 12 (chosen due to their high degrading potential >40% in experiment *no.* 2) were subjected to chromosomal DNA isolation and further phylogenetic analysis to identify bacteria capable of MC-LR degradation. Additionally, the bacteria from mixes 22 and 23 were selected as samples with low potential (<10%) for MC-LR degradation. The bacteria were suspended in 200 µL of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8) containing 0.1 mm diameter zirconia/silica beads (BioSpec Products, Bartlesville, OK). The cells were lysed using a Mini-BeadBeater-8 cell disruptor (BioSpec Products). An equal volume of DNAzol ® reagent (Invitrogen) was added, and the DNA was then extracted from the lysate using chloroform:isoamyl alcohol (24:1). After centrifugation (15 minutes at 4°C, 12,000×*g*), the upper aqueous phase was collected and ethanol precipitated by adding 3 volumes of 96% ethanol in the presence of 0.1 volumes of 5 M CH_3COOK. The DNA was incubated at -70°C for 30 minutes. After drying, the precipitate was dissolved in 200 µL of sterile deionized water.

2.4.1 Amplification of 16S rRNA gene specific for bacteria

The amplification of the 16S rRNA gene fragment (approximately 1300 to 1400 bp) was performed in 40 bacterial isolates using the specific primer pairs B27F/U1492R, as described by Orphan *et al.* [28] (Table 1). The PCR reaction was performed in a final volume of 25 µL per reaction. The PCR mix contained 1x PCR buffer with dNTP (Buffer A, *no.* 11), 7.5 µM each primer, and 0.5 U of Accu Prime™ *Taq* Polymerase High Fidelity (Invitrogen). Each reaction contained approximately 25 ng of DNA isolated from bacterial samples selected based on *in vitro* experiments with MC-LR. The initial denaturation step was at 94°C for 1 min. This step was followed by 35 cycles of DNA denaturation at 94°C for 30 s, primer annealing at 58°C for 30 s and strand extension at 68°C for 1.5 min. Visualization of the DNA was performed as previously described.

The amplification products were purified using Wizard ® SV Gel and PCR Clean-Up System (Promega) according to the manufacturer's instructions. The purified products were subjected to sequencing, and the homology searches were performed using BLAST and Vector NTI Advance™ 9 software (Invitrogen), as described for *mlrA* sequence analysis.

Rectangular phylogram representing the phylogenetic distance between the 16S rDNA sequence of *Aeromonas* and other microcystin-degrading bacteria was generated using ClustalW2 with Neighbour-joining clustering method and visualized by Dendroscope V3.2.9 software [29].

2.5 Determination of microcystins concentration

2.5.1 Environmental samples

One liter water samples from the Sulejów Reservoir (11 samples in total) were filtered through GF/C filters (Whatman) immediately after sampling. The microcystins concentration in both forms (cell-bound and dissolved in water) after extraction were identified using the HPLC-DAD (model 1100, Hewlett Packard) according to Jurczak *et al.* [18]. Microcystins in the suspended material were extracted in 75% aqueous methanol [18]. To analyze the dissolved microcystins, the filtered water samples were concentrated using solid phase extraction (SPE) [18]. The identification of microcystins were based on the comparison of retention times of MC-LR, -RR and -YR standards and UV spectra. In the present study focus was put on the above-mentioned variants because, as described in previous studies [18], they are main variants of microcystin found in the Sulejow Reservoir. The microcystins concentrations were calculated automatically by calibration curves prepared for standards of MC-RR and MC-LR (Calbiochem). The limit of detection (LOD) was 4 ng of microcystin per injection (20 µL). The limit of quantification (LOQ) was 10 ng of microcystin per injection (20 µL).

2.5.2 Samples from bacterial experiments

Subsamples (400 µL) were collected after the 1st and 2nd weeks of the bacterial experiments from the total pool of bacteria (experiment *no.* 1) and selected culturable environmental bacteria in 32 mixes (experiment *no.* 2). The samples were stored at -20°C until further analysis. Prior to analysis, the subsamples were prepared similar to the environmental samples with some modifications. The subsamples were evaporated to dryness at 40°C using the vacuum centrifuge SC 110A SpeedVac Plus1 (Thermo-Savant). The dried subsamples were reconstituted in the same volume of 400 µL of 75% methanol and then filtrated through a Gelman GHP Acrodisc 13 mm syringe filter (with 0.45 mm GHP membrane and minispike outlet; East Hills, NY, USA). The samples were analyzed as described with

the MC-LR standard. The LOD and LOQ were the same as those for the environmental samples.

2.6 Nucleotide sequence accession numbers

In the present study, sequencing results showed high homology with sequences deposited in GeneBank with accession numbers: AB468058, AB468058 and JF490063.

3 Results and Discussion

To assess the co-occurrence of bacteria with potential for microcystins degradation and microcystin-producing cyanobacteria, the identification of the *mlrA* and the *mcyE* genes respectively was performed in summer season of 2010. Bacteria with the potential to degradation of microcystin molecule were identified directly in the water collected from the lowland Sulejów Reservoir (Fig. 2). The molecular analysis of *mlrA* in the water samples from the reservoir confirmed the presence of bacteria from late June to the end of August 2010 (Fig. 2). The *mcyE* gene, which indicates the presence of microcystin-producers, was amplified in all 11 samples in the summer season from May until October 2010 (Fig. 2). In turn, the microcystins were present from June until the end of the monitoring period on October 2010, with maximum concentration of 3.45 μg L^{-1} on August 4 (Fig. 2). It was observed that bacteria with the potential to degrade microcystins were found in water samples in which cyanobacteria-derived hepatotoxins were also detected (Fig. 2), and physico-chemical conditions favored the development of phytoplankton [30]. According to Orr and Jones [31], products of microcystin molecule degradation can be utilized as the source of carbon and nitrogen. In consequence, this process provides energy necessary for growth of planktonic bacteria associated with cyanobacterial blooms.

To determine the bacteria with the potential to degrade microcystin molecule, an analysis of the *mlrA* gene sequence was performed. The nucleotide sequence of the PCR products was blasted with a DNA database. The results showed 95% homology with the *mlrA* gene of the *Sphingopyxis* strain C-1 (GeneBank AB468058.1) and the *Stenotrophomonas* sp. strain EMS (GeneBank GU224277.1) (Fig. 3). These bacteria genera had been previously isolated from Chines lakes [32-33] (Fig. 4). Collectively, our genetic study of water samples obtained directly from the Sulejów Reservoir showed that bacteria comparable to the *Sphingopyxis* sp. C-1 strain and/or *Stenotrophomonas* sp. EMS may be responsible for microcystins degradation.

To assess the actual ability to degrade microcystins, we analyzed the cultures of pelagic bacteria collected from the Sulejów Reservoir in July 2010. First, the *in vitro* experiment *no.* 1 was performed with the total pool of bacteria and standard MC-LR. After one week, the MC-LR level was reduced by 19% compared to the control sample. After two weeks, the level of MC-LR degradation by the total pool of culturable bacteria reached 34% (Fig. 5A). Next, in experiment *no.* 2, the active degradation of MC-LR

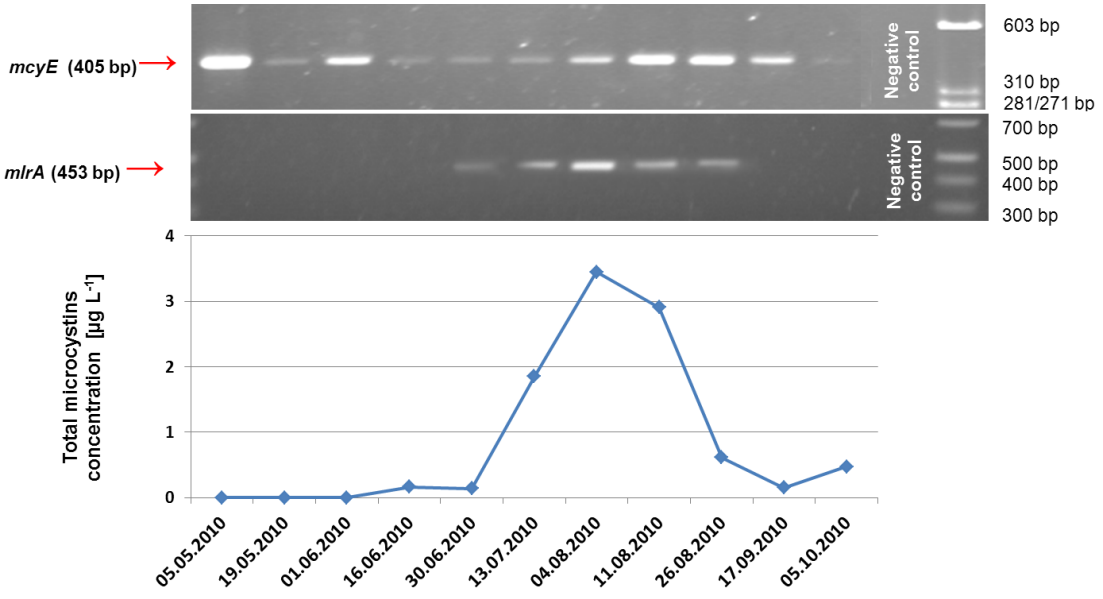

Figure 2: The results of: 1) determination of microcystins concentration, 2) molecular monitoring of microcystin-producing cyanobacteria – presence of *mcyE* gene, and 3) molecular monitoring of microcystin-degrading bacteria – presence of *mlrA* gene, in Tresta Station, in Sulejów Reservoir.

Figure 3: Homology analysis of *mlrA* gene fragment (453 bp) amplified in sample from Tresta Station, Sulejów Reservoir. (Query – obtained sequence; *Sphingopyxis* – strain C1 AB468058.1; *Stenotrophomonas* - strain EMS GU224277.1).

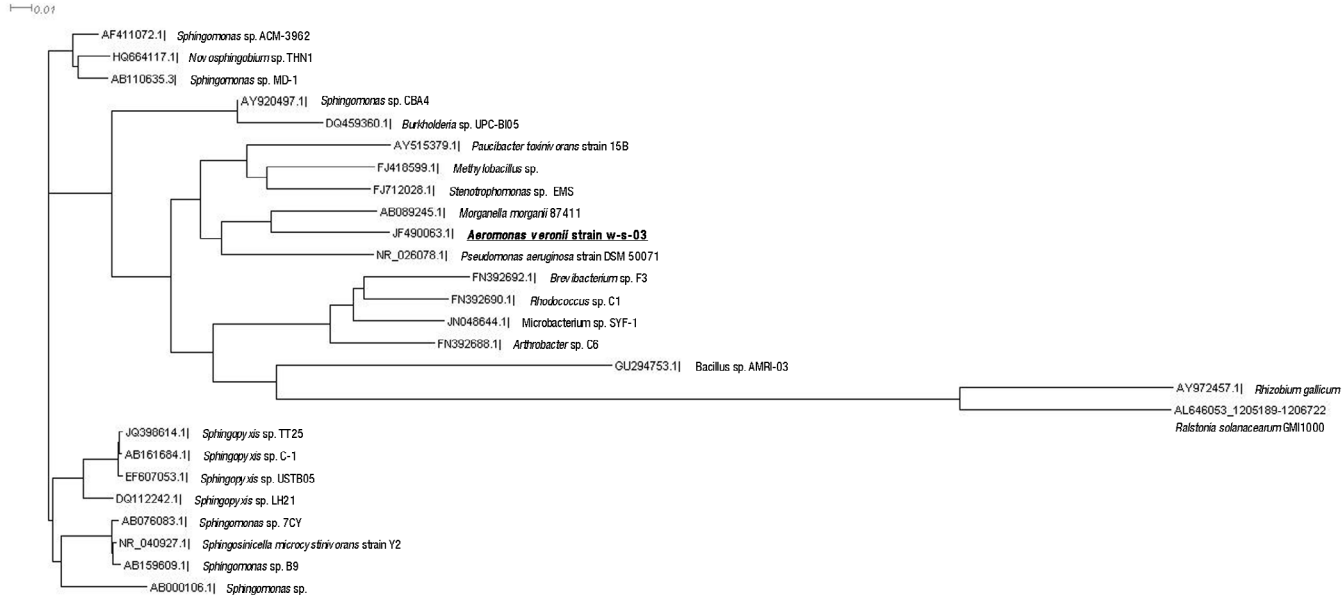

Figure 4: The approximate phylogenetic distance between the 16S rDNA sequence of *Aeromonas* sp. and other microcystin-degrading bacteria.

was determined in 32 bacterial mixes (6 colonies per mix). The level of MC-LR degradation was dependent on the bacterial mix used. After one week, the bacterial mixes 1-5, 8, 10-13, 20 and 24 reduced MC-LR levels by more than 20% (Fig. 5B). After two weeks, degradation was also observed in mixes 27 and 28. The highest degradation after two weeks was identified in mixes 8 and 12, in which the loss of MC-LR reached 48% (Fig. 5B). In the control mix without bacteria, there was a 2% degradation of MC-LR after both the first and second week of the experiment (Fig. 5B).

Taking into account the maximal 48% loss of MC-LR (from 10 μg mL^{-1} to 5.2 μg mL^{-1}) in relation to the duration of the experiment (14 days) it could be established that the degradation rate reached up 0.4 μg mL^{-1} per day. Previous studies on the identification of bacteria capable of degrading of mentioned cyanobacterial hepatotoxin and assessment of its activity demonstrated

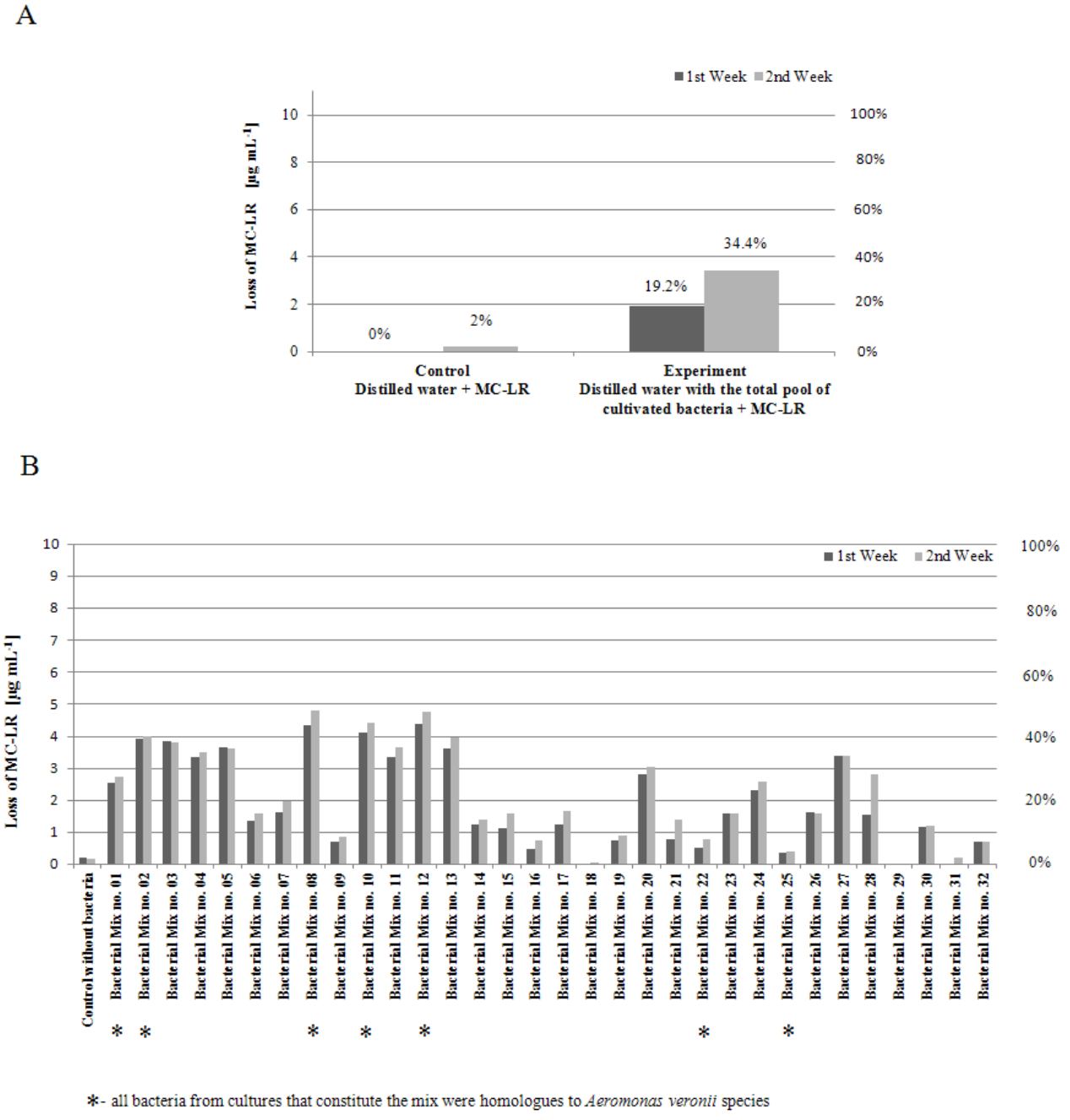

Figure 5: The results of the analysis of MC-LR degradation in *in vitro* experiments with: A) total pool of culturable bacteria – experiment no. 1, and B) mixes of selected culturable environmental bacteria – experiment no. 2.

even 100 % degradation of MC-LR for bacteria mainly of the family *Sphingomonadaceae* [6, 33-39]. The rate of MC-LR degradation was determined from 0.0015 μg mL^{-1} up to 101.5 μg mL^{-1} per day (depending on the initial amount of bacteria and the concentration of MC-LR) [6; 36-38]. The reason that the degradation of MC-LR did not exceed 50 % could have been influenced by high initial concentration of MC-LR (10 μg mL^{-1}). The application of high initial concentration was dictated by the sensitivity of the available HPLC–DAD method to ensure accurate and reliable measurement.

To determine the phylogenetic affiliation of culturable bacteria from the mixes, the 16S rRNA gene fragment was amplified and sequenced. The results indicated that regardless of the ability to cause MC-LR degradation, the 40 bacterial isolates belonged to the *Aeromonas* genus (100% homology) (Figs 4 and 5B). This phenomenon could partly result from the activity of various pathogenic factors associated with *Aeromonas*, such as exotoxins, extracellular lytic enzymes, iron-binding and secretion systems, or an ability to survive low temperatures [40-42]. These factors might facilitate the total domination of *Aeromonas* in laboratory cultures. An interesting conclusion was formulated in the study of Gaoshan *et al.* [43], which demonstrated that the crude microcystin may be an important factor stimulating the transition of *Aeromonas sobria* from the VBNC state (viable but non-culturable) to the active growth stage. Therefore, it was presumed that in the present experiments (*no.* 1 and 2), entering the VBNC state could contribute to the great variability in MC-LR degradation.

The analysis of the sequences showed that isolates represented the strain of *Aeromonas veronii* w-s-03 (GenBank record number JF490063.1) (Fig. 4). According to our knowledge, no one has yet demonstrated directly that bacteria of the genus *Aeromonas* (family *Aeromonadaceae*) are capable of MC-LR degradation.

Aeromonas belongs to the class of Gammaproteobacteria, which contains three types of bacteria capable of degrading microcystins: *Pseudomonas*, *Stenotrophomonas* and *Morganella* (see Introduction). Previous studies indicated that the bacteria originating from the *Aeromonas* genus might coexist with cyanobacterial blooms [44-45]. Østensvik *et al.* [46] and Bomo *et al.* [47] reported antibacterial activity of *Microcystis aeruginosa* extracts on *Aeromonas hydrophila*. On the other hand, Liu *et al.* [48] observed a strong algicidal effect of bacterium *Aeromonas* sp. strain FM against cyanobacterium *M. aeruginosa*.

When it comes to research directly associated with the relationship between cyanobacteria-derived hepatotoxins and *Aeromonas*, Lee *et al.* [49] identified *Aeromonas* among the pool of different bacteria potentially capable of degrading microcystins. These bacteria were absorbed on a GAC (granular active carbon) filter from a water treatment facility, creating a biofilm. When the biofilm was used as an inoculum in the experiment, bacteria were found capable of microcystin molecule degradation. However, *Aeromonas* itself was not isolated nor tested for the potential to remove microcystins from water.

To verify whether *Aeromonas*, isolated in the present study, contained the *mlrA* gene, a genetic analysis was performed. The *mlrA* gene amplification product was not detected in either of the cultivated bacteria belonging to the *Aeromonas* genus. It is likely that these bacteria might be able to degrade MC-LR differently than described by Bourne *et al.* [7, 16]. In general, the fate of the degradation products and enzymatic character of the decomposition process in different types of microcystin-degrading species are still relatively unknown [50].

The *mlr* genes were also found to be absent in other microcystin-degrading bacteria, including *Burkholderia* sp. [51], *Paucibacter toxinivorans* [13], *Methylobacillus* sp. [52], *Pseudomonas aeruginosa* [53], *Morganella morganii* [54], *Arthrobacter* sp. [14,15], *Brevibacterium* sp. [14,15], *Rhodococcus* sp. [14,15] and *Stenotrophomonas acidiminiphila* strain MC-LTH2 [55].

4 Conclusion

Based on the presence of the *mlrA* gene, bacteria with the potential for microcystins degradation were identified in the water samples from the Sulejów Reservoir in Central Poland. The genetic analysis allowed classification of the bacteria with a high homology to the *Sphingopyxis* and *Stenotrophomonas* genera (95%). In the study cultures, the above-mentioned bacteria were not detected. The *in vitro* MC-LR degradation tests on culturable bacteria demonstrated, for the first time, that bacteria homologous to *Aeromonas* genus (100%) could degrade cyanobacterial hepatotoxins – microcystins, although the *mlrA* gene was not amplified. In further studies, we plan to determine the degradation activity of bacteria by modifying the cultivation conditions and controlling bacterial growth in relation to the removal of microcystins at different phases of the experiment.

The data obtained in the present study suggest that microcystins can be degraded and used by *Aeromonas* genus as a necessary energy source. Thus, the *Aeromonas* genus not only accompanies cyanobacterial blooms but also interacts with them. The nature of this complex interaction requires further clarification.

Acknowledgements: The authors would like to acknowledge the European Cooperation in Science and Technology, COST Action ES 1105 "CYANOCOST - Cyanobacterial blooms and toxins in water resources: Occurrence, impacts and management" for adding value to this study through networking and knowledge sharing with European experts and researchers in the field. The Sulejów Reservoir is a part of the Polish National Long-Term Ecosystem Research Network and the European LTER site.

Conflict of interest: Authors declare that this research was funded by the National Science Centre, project *no.* NN305 096439 - "Explanation of cause-effect relationships between the occurrence of toxigenic cyanobacterial blooms and abiotic and biotic factors with particular focus on the role of viruses and bacteria".

References

[1] Carvalho L., Miller C.A., Scott E.M., Codd G.A., Davies P.S., Tyler A.N., Cyanobacterial blooms: Statistical models describing risk factors for national-scale lake assessment and lake management, Sci. Total. Environ., 2011, 409, 5353–5358

[2] Bednarek A., Stolarska M., Ubraniak M., Zalewski M., Application of permeable reactive barrier for reduction of nitrogen load in the agricultural areas - preliminary results, Ecohydrology and Hydrobiology, 2010, 10, 355–362

[3] Kelly J.M., Kovar J.L., Modelling phosphorus capture by plants growing in a multispecies riparian buffer, Appl. Environ. Soil Sci., 2012, 2012, 1–7

[4] Kiedrzyńska E., Kiedrzyński M., Zalewski M., Flood sediment deposition and phosphorus retention in a lowland river floodplain: impact on water quality of a reservoir Sulejów, Poland, Ecohydrology and Hydrobiology, 2008, 8, 281–289

[5] Schmidt C.A., Clark. M.W., Evaluation of a denitrification wall to reduce surface water nitrogen loads, J. Environ. Quality., 2012, 41, 724–731

[6] Ho L., Hoefel D., Saint C.P., Newcombe G., Isolation and identification of a novel microcystin-degrading bacterium from a biological sand filter, Water Res., 2007, 41, 4685–4695

[7] Bourne D.G., Blakeley R.L., Riddles P., Jones G.J., Biodegradation of the cyanobacterial toxin microcystin-LR in natural water and biologically active slow sand filters, Water Res., 2006, 40, 1294–302

[8] Ji R.P., Lu X.W., Li X.N., Pu Y.P., Biological degradation of algae and microcystins by microbial enrichment on artificial media, Ecol. Eng., 2009, 35, 1584–1588

[9] Gągała I., Mankiewicz-Boczek J., Natural degradation of microcystins (cyanobacterial hepatotoxins) in fresh water – the future of modern treatment systems and water quality improvement. Pol. J. Environ. Stud., 2012, 21, 1125–1139

[10] Mou X., Lu X., Jacob J., Sun S., Heath R., Metagenomic identification of bacterioplankton taxa and pathways involved in microcystin degradation in Lake Erie, PLoS One., 2013, 8, e61890

[11] Jing W., Sui G., Liu S., Characteristics of a microcystin-LR biodegrading bacterial isolate: *Ochrobactrum* sp. FDT5. Bull. Environ. Contam. Toxicol., 2014, 92(1), 119-122

[12] Ma G., Pei H., Hu W., Xu X., Ma C., Li X., The removal of cyanobacteria and their metabolites through anoxic biodegradation in drinking water sludge, Bioresour. Technol. 2014, 165C, 191-198

[13] Rapala J., Berg K.A., Lyra C., Niemi R.M., Manz W., Suomalainen S., *et al.*, *Paucibacter toxinivorans* gen. nov. sp. nov. a bacterium that degrades cyclic cyanobacterial hepatotoxins microcystins and nodularin, Int. J. Syst. Evol. Microbiol., 2005, 55, 1563–1568

[14] Lawton L.A., Welgamage A., Manage P.M., Edwards C., Novel bacterial strains for the removal of microcystins from drinking water, Water Sci. Technol., 2011, 63, 1137–1142

[15] Manage P.M., Edwards C., Singh B.K., Lawton L.A., Isolation and identification of novel microcystin-degrading bacteria, Appl. Environ. Microbiol., 2009, 75, 6924–6928

[16] Bourne D.G., Riddles P., Jones G.J., Smith W., Blakeley R.L. Characterisation of a gene cluster involved in bacterial degradation of the cyanobacterial toxin Microcystin-LR, Cultures, 2001, 16, 523–534

[17] Izydorczyk K., Jurczak T., Wojtal-Frankiewicz A., Skowron A., Mankiewicz-Boczek J., Tarczyńska M., Influence of abiotic and biotic factors on microcystin content in *Microcystis aeruginosa* cells in a eutrophic temperate reservoir, J. Plankton Res., 2008, 30, 393–400

[18] Jurczak T., Tarczyńska M., Izydorczyk K., Mankiewicz J., Zalewski M., Meriluoto J., Elimination of microcystins by water treatment processes — examples from Sulejów Reservoir, Poland, Water Res., 2005, 39, 2394–2406

[19] Jurczak T., Zastosowanie monitoringu toksyn sinicowych w celu optymalizacji technologii uzdatniania wody oraz strategii rekultywacji zbiorników zaporowych, PhD dissertation, University of Lodz, Poland, 2006, (in Polish)

[20] Mankiewicz-Boczek J., Izydorczyk K., Romanowska-Duda Z., Jurczak T., Stefaniak K., Kokociński M., Detection and monitoring toxigenicity of cyanobacteria by application of molecular methods, Environ. Toxicol., 2006, 21, 380–387

[21] Mankiewicz-Boczek J., Urbaniak M., Romanowska-Duda Z., Izydorczyk K., Toxic Cyanobacteria strains in lowland dam reservoir (Sulejów Res. Central Poland): Amplification of *mcy* genes for detection and identification, Pol. J. Ecol., 2006, 54, 171–180

[22] Tarczyńska M., Romanowska-Duda Z., Jurczak T., Zalewski M., Toxic cyanobacterial blooms in a drinking water reservoir - causes consequences and management strategy, Water Sci. Technol.: Water Supply., 2001, 1, 237–246

[23] Zalewski M., Ecohydrology - The scientific background to use ecosystem properties as management tools toward sustainability of water resources, Guest Editorial Ecol. Eng., 2000, 16, 41647

[24] Giovannoni S.J., DeLong E.F., Schmidt T.M., Pace N.R., Tangential flow filtration and preliminary phylogenetic analysis of marine picoplankton, Appl. Environ. Microbiol., 1990, 56, 2572-2575

[25] Rantala A., Rajaniemi-Wacklin P., Lyra C., Lepistö L., Rintala J., Mankiewicz-Boczek J., *et al*., Detection of microcystin-producing cyanobacteria in Finnish lakes with genus-specific microcystin synthetase gene E (*mcy*E) PCR and associations

with environmental factors, Appl. Environ. Microbiol., 2006, 72, 6101–6110
[26] Saito T., Okano K., Park H., Itayama T., Inamori Y., Neilan B.A., *et al*., Detection and sequencing of the microcystin LR-degrading gene, *mlrA*, from new bacteria isolated from Japanese lakes, FEMS Microbiol. Lett., 2003, 229(2), 271-276
[27] Zhang Z., Schwartz S., Wagner L., Miller W., A greedy algorithm for aligning DNA sequences, J. Comput. Biol., 2000, 7, 203-221
[28] Orphan V.J., Hinrichs K., Iii W.U., Paull C.K., Taylor L.T., Sylva S.P., *et al.*, Comparative analysis of methane-oxidizing archaea and sulfate-reducing bacteria in anoxic marine sediments, J. Appl. Microbiol., 2001, 67, 1922–1934
[29] Huson D.H., Scornavacca. C., Dendroscope 3: An Interactive Tool for Rooted Phylogenetic Trees and Networks, Syst. Biol., 2012, 61, 1061-1067
[30] Gągała I., Izydorczyk K., Jurczak T., Pawełczyk J., Dziadek J., Wojtal-Frankiewicz A., *et al*., Role of environmental factors and toxic genotypes in the regulation of microcystins-producing cyanobacterial blooms, Microbial Ecol., 2014, 67(2), 465-479
[31] Orr P.T., Jones. G.J., Relationship between microcystin production and cell division rates in nitrogen-limited *Microcystis aeruginosa* cultures, Limnol. Oceanogr., 1998, 43, 1604
[32] Okano K., Shimizu K., Kawauchi Y., Maseda H., Utsumi M., Zhang Z., *et al*., Characteristics of a microcystin-degrading bacterium under alkaline environmental conditions, J. Toxicol., 2009, 2009, 41648
[33] Chen J., Hu L., Bin Zhou W., Yan S.H., Yang J.D., Xue Y.F., *et al.*, Degradation of microcystin-LR and RR by a *Stenotrophomonas* sp. strain EMS isolated from Lake Taihu, China, Int. J. Mol. Sci., 2010, 11, 896–911
[34] Park H.D., Sasaki Y., Maruyama T., Yanagisawa E., Hiraishi A., Kato K., Degradation of the cyanobacterial hepatotoxin microcystin by a new bacterium isolated from a hypertrophic lake, Environ. Toxicol., 2001, 16, 337–343
[35] Ishii H., Nishijima M., Abe T., Characterization of degradation process of cyanobacterial hepatotoxins by a gram-negative aerobic bacterium, Water Res., 2004, 38, 2667–2676
[36] Wang J., Wu P., Chen J., Yan H., Biodegradation of microcystin-RR by a new isolated *Sphingopyxis* sp. USTB-05, Chin. J. Chem. Eng., 2010, 18, 1-5
[37] Zhang, M., Pan G., Yan H., Microbial biodegradation of microcystin-RR by bacterium *Sphingopyxis* sp. USTB-05, 2010, J. Environ. Sci., 22, 168–175
[38] Yan H., Wang J., Chen J., Wei W., Wang H., Wang H., Characterization of the first step involved in enzymatic pathway for microcystin-RR biodegraded by *Sphingopyxis* sp. USTB-05, Chemosphere, 2012, 87, 12-18
[39] Ho L., Tang T., Monis P.T., Hoefel D., Biodegradation of multiple cyanobacterial metabolites in drinking water supplies, Chemosphere, 2012, 87, 1149-1154
[40] Mateos D., Anguita J., Naharro G., Paniagua C., Influence of growth temperature on the production of extracellular virulence factors and pathogenicity of environmental and human strains of *Aeromonas hydrophila*, J. Appl. Bacteriol., 1993, 74, 111–118
[41] Mano S., Growth/survival of natural flora and *Aeromonas hydrophila* on refrigerated uncooked pork and turkey packaged in modified atmospheres, Food Microbiol., 2000, 17, 657–669
[42] Tomás J.M., The main *Aeromonas* pathogenic factors, ISRN Microbiol., 2012, 2012, 1–22
[43] Gaoshan P., Zhangli H., Anping L., Shuangfei L., Effect of crude microcystin on the viable but non-culturable state of *Aeromonas sobria* in aquatic environment, J. Lake Sci., 2008, 20, 105-109 (in chinese)
[44] Berg K.A., Lyra C., Niemi R.M., Heens B., Hoppu K., Erkomaa K., *et al.*, Virulence genes of *Aeromonas* isolates bacterial endotoxins and cyanobacterial toxins from recreational water samples associated with human health symptoms, J. Water Health., 2011, 9, 670-679
[45] Berg K.A., Lyra C., Sivonen K., Paulin L., Suomalainen S., Tuomi P., *et al.*, High diversity of cultivable heterotrophic bacteria in association with cyanobacterial water blooms, ISME J., 2009, 3, 314–325
[46] Østensvik O., Skulberg O.M., Underdal B., Hormazabal V., Antibacterial properties of extracts from selected planktonic freshwater cyanobacteria - a comparative study of bacterial bioassays, J. Appl. Microbiol., 1998, 84, 1117–1124
[47] Bomo A-M., Tryland I., Haande S., Hagman C.H.C., Utkilen H., The impact of cyanobacteria on growth and death of opportunistic pathogenic bacteria, Water Sci. Technol., 2011, 64, 384–390
[48] Liu Y-M., Chen M-J., Wang M-H., Jia R-B., Li L. Inhibition of *Microcystis aeruginosa* by the extracellular substances from an *Aeromonas* sp., J. Microbiol. Biotechnol., 2013, 23, 1304-1307
[49] Lee Y-J., Jung J-M., Jang M-H., Ha K., Joo G-J. Degradation of microcystins by adsorbed bacteria on a granular active carbon (GAC) filter during the water treatment process, J. Environ Biology/Academy of Environmental Biology, India, 2006, 27, 317–322
[50] Dziga D., Wasylewski M., Wladyka B., Nybom S., Meriluoto J. Microbial degradation of microcystins, Chem. Res. Toxicol., 2013, 26, 841-852
[51] Lemes G.A.F., Kersanach R., Pinto L.D.S., Dellagostin O.A., Yunes J.S., Matthiensen A., Biodegradation of microcystins by aquatic *Burkholderia* sp. from a South Brazilian coastal lagoon, Ecotoxicol. Environ Saf., 2008, 69, 358–365
[52] Hu L., Bin Yang J.D., Zhou W., Yin Y.F., Chen J., Shi Z.Q., Isolation of a *Methylobacillus* sp. that degrades microcystin toxins associated with cyanobacteria, New Biotechnol., 2009, 26, 205–211
[53] Takenaka S., Watanabe. M.F., Microcystin-LR degradation by *Pseudomonas aeruginosa* alkaline protease, Chemosphere, 1997, 34, 749–757
[54] Eleuterio L., Batista. J.R., Biodegradation studies and sequencing of microcystin-LR degrading bacteria isolated from a drinking water biofilter and a fresh water lake, Toxicon, 2010, 55, 1434–1442
[55] Yang F., Zhou Y., Yin L., Zhu G., Liang G., Pu Y., Microcystin-degrading activity of an indigenous bacterial strain *Stenotrophomonas acidaminiphila* MC-LTH2 isolated from Lake Taihu. PLoS ONE, 2014, 9(1): e86216