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TOXIC CYANOBACTERIA STRAINS IN LOWLAND DAM RESERVOIR (SULEJÓW RES., CENTRAL POLAND): AMPLIFICATION OF *MCY* GENES FOR DETECTION AND IDENTIFICATION

ABSTRACT: Excessive eutrophication causes the growth of microcystin-producing *Cyanobacteria* and leads to an increased human health risk. This paper reports the analyses of *Cyanobacteria* toxigenicity (the degree of potential toxicity) in lowland dam reservoir (Sulejów Reservoir, Central Poland) by the use of PCR-based methods. The reservoir (area 22 km^2, mean depth 3.3 m) is very eutrophic water body permanently blooming with blue-green algae (mainly *Microcystis aeruginosa*). For identification of cyanobacterial genus the *16S rRNA* region was used. Cyanobacterial genus was detected during the whole monitoring period in summer 2003. The potential toxicity of cyanobacteria was determined by amplification of selected *mcyA,B,E* genes in the microcystin biosynthesis pathway. All of the analyzed genes were detected at the beginning of the growing season during low cyanobacterial biomass (0.67 mg l^{-1}). 89% of the samples were found to be positive for *mcyA* detection. Early detection of *mcy* genes at the beginning of summer preceded a period of the highest microcystins concentration (2.91 µg l^{-1} in maximum) and toxicity established by ELISA (enzyme-linked immunosorbent assay – enables determination of microcystins concentration) and PPIA (protein phosphatase inhibition assay – enables estimation of microcystins toxicity). We show that toxigenic (potentially toxic) strains of cyanobacteria occurred in Sulejów Reservoir throughout the summer and genetic markers were effective in early identification of microcystin-producing genera. Application of molecular methods in parallel with toxicity testes can provide complete information to prevent any human health risk.

KEY WORDS: Cyanobacteria, microcystins, *mcy* gene cluster, toxicity

1. INTRODUCTION

An enhancement of eutrophication process leads usually to higher frequency of cyanobacterial blooms occurrence (Foulds *et al.* 2002). The expansion of *Cyanobacteria* (blue-green algae) and production of hepatotoxic microcystins, can cause serious health problems and influence water resources management (Chorus and Bartram 1999, Dittman *et al.* 2001, Briand *et al.* 2003, de Figueiredo *et al.* 2004, Codd *et al.* 2005). Recently in many countries including Poland the problem of toxicity caused by cyanobacterial blooms has important consequences (Fleming *et. al.* 2002, de Figueiredo *et. al.* 2004, Pawlik-Skowrońska *et al.* 2004, Mankiewicz *et al.* 2003, 2005). The increase distribution of hepatotoxins during the last decade in Central Poland emphasizes the need for early detection of toxic *Cyanobacteria* to prevention of water quality and human health (Tarczyńska *et al.* 2001a,b, Jurczak *et al.* 2004, Izydorczyk *et al.* 2005).

In this study we reported the results of analysis of water samples containing toxic cyanobacterial strains, collected from Sulejów Reservoir (Central Poland), between May and October 2003. This reservoir is a popular place for sport activities such as swimming, sailing, or canoeing. Moreover, it is an alternative source of drinking water for the city of Łódź, a town with approximately 1 million inhabitants. The health risk in this reservoir is caused by microcystin-producing genera with *Microcystis aeruginosa* dominating (Zalewski *et al.* 2000, Tarczyńska *et al.* 2001a,b). Microcystins are harmful hepatotoxins, they inhibit the eukaryotic enzyme protein phosphatase 1 and 2A (Falconer 1994, Mankiewicz *et al.* 2003, de Figueiredo *et al.* 2004, Codd *et al.* 2005). At least over 70 microcystin variants are known (Spoof 2004, Codd *et al.* 2005). Contact with microcystin-producing *Cyanobacteria* in bathing water can cause skin irritations, allergic reactions and gastrointestinal symptoms (Chorus and Bartram 1999, Mankiewicz *et al.* 2003). Swimmers involuntarily can swallow some water while swimming, and bathing suits, particularly wet suits, could accumulate cyanobacterial material and enhance disruption of cells and liberation of cell content (WHO, 2003). Moreover, chronic exposure to low microcystins concentration in drinking water can led to cancer promotion (Carmichael 2001, Briand *et al.* 2003).

Due to the health hazard character of cyanobacterial blooms there exists a need to identify and monitor toxigenic (i.e. potentially toxic) strains in water bodies (Baker *et al.* 2002). The amplification of the *16S rRNA* gene, specific for *Cyanobacteria,* can identify the cyanobacterial genus, but does not indicate the toxin production potential. Also the inability to differentiate between toxic and nontoxic strains of cyanobacteria by morphological analysis lead to applying sensitive molecular methods, which enable identification of microcystin-producing strains (Kurmayer *et al.* 2002, Hisbergues *et al.* 2003, Vaitomaa *et al.* 2003). To distinguish between toxic and nontoxic strains *mcy* genes in the microcystin biosynthesis pathway have been applied. Microcystins are synthesized nonribosomally by *mcyABCDEFGHIJ* gene cluster including peptide synthetase, polyketide synthase and modifying enzymes. The total size of this region is 55 kb of DNA. The *mcy* genes in *Microcystis* genera are transcribed bidirectionally as two putative operons: *mcyABC* and *mcyDEFGHIJ*. The transcription start sites are situated between *mcyA* and *mcyD* genes. *McyA*, *mcyB* and *mcyC* genes encode peptide synthetase. Genes from the second operon (*mcyDEFGHIJ*) code polyketide synthase and modifying enzymes. The most significant is *mcyE* gene which encodes the glutamate-activating adenylation domain in ADDA chain (Kaebernick *et al.* 2002, Christiansen *et al.* 2003, Rouhiainen *et al.* 2004). This unique hydrophobic amino acid, ADDA (2S,3S,8S,9S-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-deca-4,6-dienoic acid) (Eriksson *et al.,* 1987) is indispensable for the biological activity of microcystin. The progressive knowledge about the microcystin synthesis genes has contribute to the development of PCR primers used for discrimination between toxic and nontoxic strains.

We have shown that it is possible to obtain routinely PCR products from water samples and then determine occurrence of toxic strains in reservoir. The toxic potential of *Cyanobacteria* can be detected at the beginning of bloom expansion, and monitored throughout the vegetation season, to observe development of the toxic strains. Analysis of toxigenicity (i.e. microcystins production) by application of *mcy* genes was never done before in Sulejów Reservoir. Moreover, presented molecular data are the first published application of *mcy* genes to study of eutrophic, blooming water bodies in Poland. Early detection of toxic cyanobacterial strains in water is indispensable for health hazard prevention because they allow the early application of corrective actions.

The aim of this study was: 1) application of molecular methods for identification of microcystin synthetase genes in water samples from Sulejów Reservoir 2) prediction of toxigenic strains occurrence and 3) determination of their toxicity.

2. STUDY AREA, MATERIAL AND METHODS

The Sulejów Reservoir is a shallow polymictic and eutrophic lowland reservoir with dominant *Microcystis aeruginosa* species, situated in Central Poland, in the middle course of the Pilica River (Tarczyńska *et al.* 2001a,b). At its maximum capacity ($75\times10^6 m^3$), the reservoir covers 22 km^2, with a mean depth of 3.3 m and a mean retention time of about 30 days (Ambrożewski 1980). This reservoir is emergency source of drinking water for the city of Łódź (1 million inhabitants) and a popular place for sport activities such as swimming, sailing or canoeing.

2.1. Sampling, phytoplankton analysis and *Cyanobacteria* culture.

Water samples of the surface layer (0–0.5 m) were collected every two weeks from Sulejów Reservoir, from 21st May to 1st October 2003. Surface samples were stored at 4°C for a maximum of 24 h. Physico-chemical parameters of monitored Sulejów Reservoir were determined according to standard methods (Golterman *et al.* 1988). After that, 1 ml of the filtrated surface water without concentrating the samples for determination of microcystins concentration by ELISA (enzyme-linked immunosorbent assay) and their toxicity by PPIA (protein phosphatase inhibition assay) was used. For DNA analysis 100–200 ml of water or cell culture samples were filtered on a Gelman Supor filter 0.45 μm (Pall Life Science, USA). Then, the filter was put into the lysis buffer containing 40 mM EDTA, 400 mM NaCl, 0.75 M sucrose and 50 mM Tris-HCl (pH 8.3) and frozen as soon as possible.

Water samples for phytoplankton estimation were preserved in Lugol's solution and sedimented in the laboratory. Phytoplankton was counted using a Fusch-Rosenthal counting cell. The phytoplankton biomass (fresh weight) was determined based on a volumetric analysis of cells using geometric approximation. Biomass computed in volume units was transposed to freshmass (FM) assuming the specific mass of phytoplankton as a unit (=1) (Komárkova *et al.* 1995).

The toxic strain of *Microcystis aeruginosa* PCC 7820 was obtained from Pasteur Culture Collection, Paris, France and cultured in Department of Ecopysiology and Plant Development, University of Łódź. The strains were grown in Z8 medium under continuous light at 22°C (Sivonen *et al.* 1990).

2.2. PCR – sample preparation and amplification

Nucleic acid extraction from the filters was performed according to Giovannoni *et al.* 1990, with some modifications. For the centrifugation, a speed of 13,000 × g instead of 10,000 × g was used. For the enzymatic lysis step, a final concentration of proteinase K (Fermentas, Lithuania) of 275 μg ml^{-1} instead of 160 μg ml^{-1} was used. During the phenol/chloroforme step, a volume of chloroforme/isoamyl alcohol (24:1) equal to the volume of supernatant was used.

The first step of the molecular analysis was the identification of cyanobacterial DNA by amplification of the *16S rRNA* gene. For PCR amplification part of *16S rRNA* was used with the forward primer 16S27F (5'-AGAGTTTGATCCTGGCTCAG-3', Wilmotte 1994) and the cyanospecific reverse primer 23S30R (5'-CTTCGCCTCTGTGTGCCTAGGT-3', Taton *et al.* 2003).

In Sulejów Reservoir occur several genera of *Cyanobacteria*, with *Microcystis aeruginosa* dominating. For this reason were used primers which enable detection of *mcy* genes from different *Cyanobacteria* i.e. *Microcystis, Anabaena* or *Planktothrix*.

For *mcyA* (291–297 bp region), PCR was performed with the pair primers Cd1F (5'AAAATTAAAAGCCGTATCAAA3') and Cd1R (5'AAAAGTGTTTTATTAGCGGCTCAT-3'). The thermal cycling conditions were performed according to Hisbergues *et al.* 2003.

For *mcyB* (758 bp region), PCR was performed with FAA (5'-CTATGTTATTTATACATCAGG-3') and RAA (5'-CTCAGCTTAACTTGATTATC-3') pair primers, according to Bittencourt-Oliveira 2003.

For *mcyE* (809–812 bp region), PCR was performed with the pair primers mcyE-F2 (5'-GAAAATTTGTGTAGAAGGTGC-3') and MicmcyE-R8 (5'-CAATGGGAGCATA-

ACAG-3'). The thermal cycling conditions were performed according to Vaitomaa *et al.* 2003.

We decided to employ some modifications, because environmental factors can influence water sample quality and inhibit PCR. The first modification was addition of BSA (0.1 mg ml^{-1} per sample) to the PCR mixture as a PCR amplifier. We also recommend to use more stable DyNaZyme II DNA polymerase then Taq polymerase, which was proposed in previous publications (Bittencourt-Oliveira 2003, Hisbergues *et al.* 2003).

After that, PCR products were separated on 1.5% agarose gels with ethidium bromide.

2.3. Determination of microcystins concentration and their toxicity

Microcystins concentration in sampled water was estimated by ELISA (enzyme-linked immunosorbent assay). The concentration of microcystins was determined using the commercial EnviroLogix microcystin plate kit (No EP 022, Portland, USA). ELISA enabled detection of microcystins at the 0.1 μg l^{-1} level, without concentrating the sample.

Microcystins toxicity, i.e. their biological activity, was described by PPIA (protein phosphatase inhibition assay). The inhibition of protein phosphatase type 1 (PP1) from rabbit skeletal muscle (BioLabs, New England) by microcystins was estimated according to An and Carmichael (1994) with minor modification. The samples were incubated with 2.5 U of PP1 and 15 mM p-nitrophenol phosphate (Fluka, UK). PPIA enabled detection of microcystins at the 0.125 μg l^{-1} level, without concentrating the sample

3. RESULTS

3.1. Physico-chemical parameters

In Sulejów Reservoir the physico-chemical conditions such as water temperature above 18°C, pH between 6–8 and low N:P ratio supported cyanobacterial blooms occurrence (Table 1). In summer 2003 the average water temperature was 20.5°C and did not decrease below 17°C. The average water pH was 7.5 with the tendency to decrease from 8 (May) to 7 (September) (Table 1). The main nutrients such as phosphorus and nitrogen are transported by the inflowing rivers, groundwater and surface flow from the direct catchment (Tarczyńska *et al.* 2001a,b). Total phosphorus (TP) and total nitrogen (TN) in Sulejów Reservoir exceeded the lim-

Table 1. Physico-chemical parameters, cyanobacteria biomass, microcystins concentration (estimated by ELISA) and toxicity (estimated by PPIA) in Sulejów Reservoir in summer 2003.

Date of collection	TP [mg l^{-1}]	TN [mg l^{-1}]	pH	Temp. [°C]	Biomass[1] [mg l^{-1}]	PPIA		ELISA	
						extr[2] MCs	intr[3] MCs	extr[2] MCs	intr[3] MCs
21st May	0.15	1.70	8.43	17.13	0.67	0.00	0.08	0.00	0.13
4th Jun.	0.17	2.30	8.42	21.34	18.34	0.00	0.62	0.11	0.17
18th Jun.	0.19	2.60	8.51	21.90	23.90	0.06	1.96	0.26	2.65
2nd Jul.	0.20	2.50	8.52	20.84	2.62	0.00	0.84	0.16	0.28
16th Jul.	0.22	2.00	8.23	20.56	2.18	0.00	0.26	0.24	0.53
6th Aug.	0.20	2.00	8.08	23.91	14.24	0.18	1.46	0.18	2.50
20th Aug.	0.11	0.70	7.78	21.86	29.30	0.00	1.76	0.17	2.50
10th Sept.	0.30	2.10	7.00	19.24	10.50	0.00	0.27	0.00	0.13
1st Oct.	0.17	1.60	7.10	17.63	5.40	0.15	0.73	0.22	0.20

[1] hepatotoxic cyanobacteria biomass; MCs – microcystins; [2] extracellular microcystins; [3] intracellular microcystins

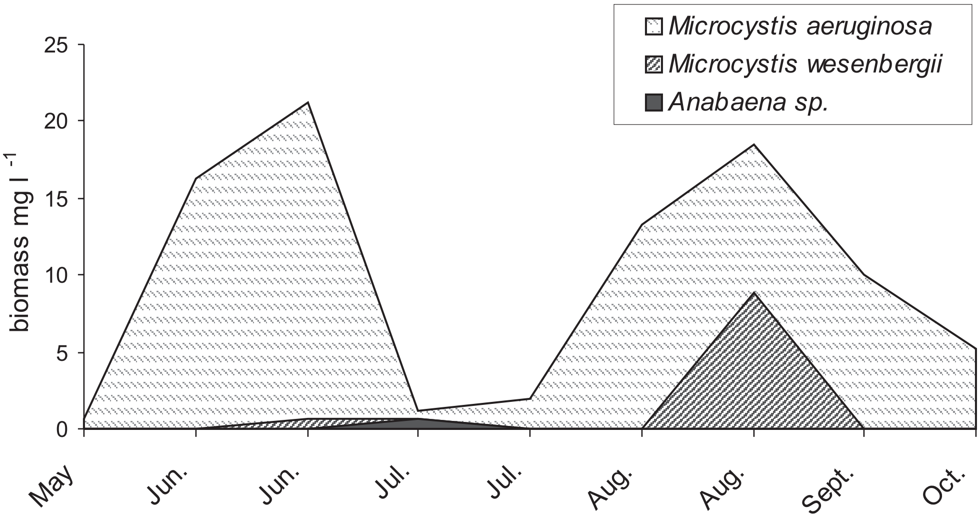

Fig. 1. Changes in cyanobacteria composition in Sulejów Reservoir in summer 2003.

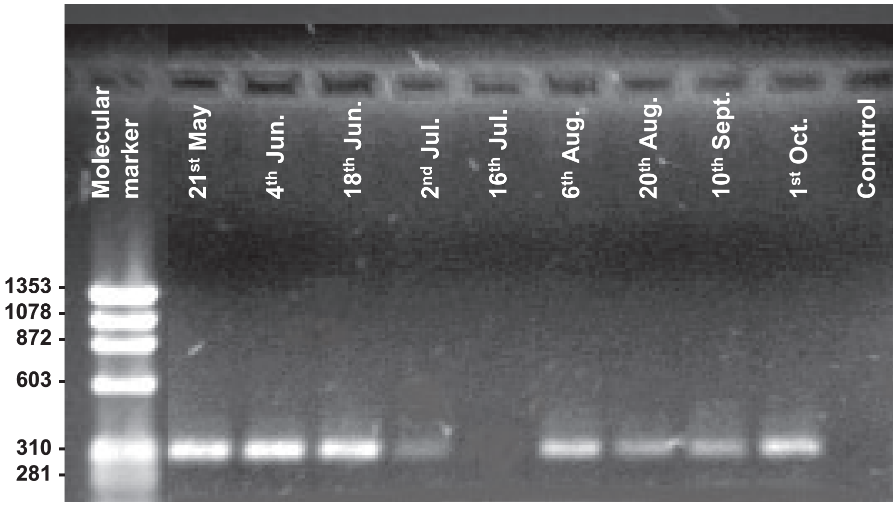

Fig 2. PCR amplification of the *mcyA* with DNA extracted from water samples of Sulejów Reservoir. ΦX174 DNA – HaeIII digest (72–1353 bp) – molecular marker; DNA samples collected on: (21SU) 21st May; (23SU) 4th Jun.; (25SU) 18th Jun.; (27SU) 2nd Jul.; (29SU) 16th Jul.; (32SU) 6th Aug. 03; (34SU) 20th Aug.; (37SU) 10th Sept. 03 and (40SU) 1st Oct. 03; each sample contains 3.5 ng DNA; Control – control without DNA.

its for eutrophication of reservoirs (0.1 mg P l^{-1} and 1.5 mg N l^{-1}) (OECD, 1983). Average TP about 0.19 mg l^{-1} and TN of 1.94 mg l^{-1} were maintained throughout summer period (Table 1). The ratio of N:P decrease from 14 (June) to 6 (August), with average of 10 (Table 1).

3.2. Diversity of Cyanobacteria

Amplification of the *16S rRNA* gene was used to determine the cyanobacterial genus in water samples collected from 21st May to 1st October 2003. Cyanobacterial genus was identified throughout summer (Table 2).

Table 2. Presence (+) and absence (–) of the molecular marker of *16SrRNA* gene in cyanobacterial genus and *mcy* genes involved in the biosynthesis of microcystins in culture of *Microcystis aeruginosa* PCC 7820 and in water samples collected from Sulejów Reservoir in summer 2003.

Genes	Date of collection									
	M. aeruginosa culture	21st May	4th Jun.	18th Jun.	2nd Jul.	16th Jul.	6th Aug.	20th Aug.	10th Sept.	1st Oct.
16S rRNA	+	+	+	+	+	+	+	+	+	+
mcyA	+	+	+	+	+	–	+	+	+	+
mcyB	+	+	+	+	+	–	+	–	+	–
mcyE	+	+	+	+	+	+	–	–	–	+

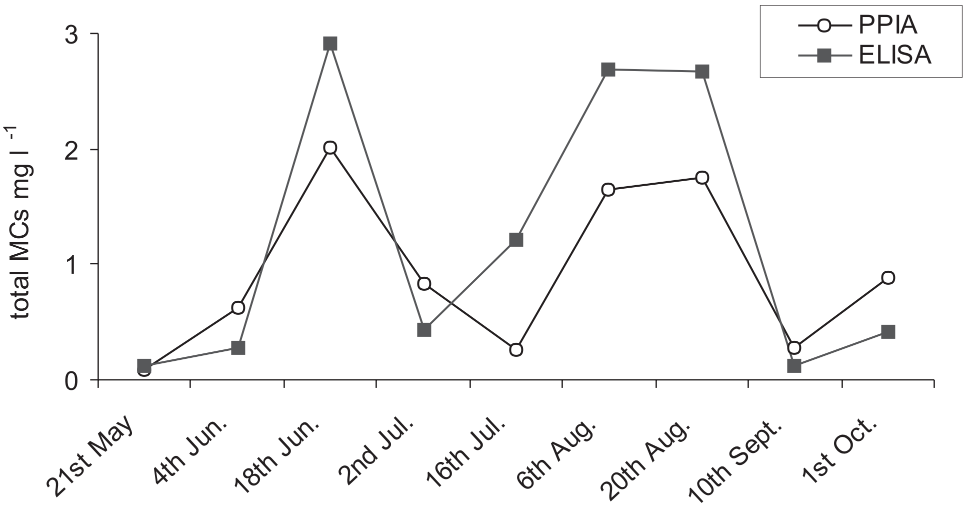

Fig. 3. Seasonal microcystins concentration (estimated by ELISA) and toxicity (estimated by PPIA) in water samples collected from Sulejów Reservoir in summer 2003. Total MCs – extracellular and intracellular concentration of all variants of microcystin contained in water sample.

The cyanobacterial composition of each sample was determined by microscopic analysis. The cyanobacterial sample collected at the end of May, was dominated by *Microcystis aeruginosa*. The biomass of this species amounted to 0.67 mg l^{-1} (Table 1). The first maximum of *Microcystis aeruginosa* biomass occurred at 18th June (24.19 mg l^{-1}) and the second one at 20th August 2003 (18.49 mg l^{-1}) (Fig. 1). *Microcystis wesenbergii* was observed three times during the vegetation season at 18th June (0.63 mg l^{-1}), 2nd July (0.66 mg l^{-1}) and 20th August 2003 (8.89 mg l^{-1}) (Fig. 1). *Anabaena* sp. (0.68 mg l^{-1}) was observed only at 2nd July (Fig. 1). In the summer of 2003, the Sulejów Reservoir was dominated by *Microcystis aeruginosa* in 89% of the samples.

3.3. Toxigenic strains of *Cyanobacteria*

To assess the toxigenic potential to produce microcystins selected *mcy* genes i.e. *mcyA*, *mcyB* and *mcyE* from two different operons were used.

Firstly, the DNA from toxigenic strain of *Microcystis aeruginosa* PCC 7820 was used to test PCR conditions and suitability of selected primer pairs for *mcy* genes amplification. The positive result was obtain for all applied primers of *mcy* genes (Table 2).

After this step, the utility of the selected primers to detect hepatotoxic strains in the water samples collected from Sulejów Reservoir was tested. The results of *mcy* genes analysis showed that toxigenic strains of *Cyanobacteria* occurred throughout summer period of 2003 (Table 2). The Cd1F and Cd1R primers were used successfully to amplify *mcyA* gene in 89% water samples (Fig. 2 and Table 2). The *mcyB* and *mcyE* genes were positively amplified in 67% of samples (Table 2).

3.4. Toxicity of water samples

Both the ELISA and PPIA analyses of water samples showed the presence of microcystins in Sulejów Reservoir. In analysed water samples cell-bind (intracellular) microcystins dominated. The concentration of microcystins (determined by ELISA) and their biological activity (determined by PPIA) ranged from 0.13 to 2.91 μg l^{-1} and from 0.08 to 2.02 μg l^{-1}, respectively (Table 1 and Fig. 3). The lowest microcystins concentration (0.13 μg l^{-1}) and toxicity (< 0.1 μg l^{-1}) was observed at the beginning of summer period on 21st May and it corresponded with low cyanobacterial biomass 2.62 mg l^{-1} (Table 1). The highest microcystins concentration 2.91 μg l^{-1} and 2.67 μg l^{-1} with high toxicity 2.02 μg l^{-1} and 1.76 μg l^{-1} was observed at 18th June and 20th August, respectively. These results corresponded with higher cyanobacterial biomass 23.90 mg l^{-1} on 18th June and 29.30 mg l^{-1} on 20th August (Table 1 and Fig. 1). At the end of summer season in September the microcystins concentration and toxicity decreased to 0.13 μg l^{-1} and 0.27μg l^{-1}, respectively (Table 1, Fig. 3).

4. DISCUSSION

The Sulejów Reservoir, situated in Central Poland is a eutrophic water body receiving nutrients mainly from Pilica river and its catchment area. The favorable physicochemical conditions support the development of cyanobacterial blooms (Table. 1). Health risk in this reservoir is caused by microcystin-producing genera with *Microcystis aeruginosa* dominating (Fig. 1). Samples collected from Sulejów Reservoir during summer period from 21st May till 1st October 2003 contained toxigenic *Cyanobacteria*. The presence of selected genes from *mcy* cluster in water samples indicated the genetic potential to produce hepatotoxins. The most intensively amplified was the *mcyA* gene (89%) (Table 2 and Fig. 2). PCR amplification of the *mcyB* and *mcyE* genes were obtained in 67% of samples. The higher number of positive amplification results of *mcyA* (291–297 bp region) compared to *mcyB* (758 bp region) was probably caused by the higher amplification efficiency for smaller PCR products (Via-Ordorika et *al.* 2004). Additionally, Kurmayer and Kutzenberger (2003) reported significant variations between PCR products for *mcyB* gene, but mechanism of this result is unknown. The low amplification of *mcyE* gene fragment, obtained only during first cyanobacterial maximum, could be attributed to the environmental factors changes, such as: nutrient distribution, light and temperature and influence on microcystins synthetase (Oh *et al.* 2001). Moreover, water samples may contain (micro)organisms, humic acid or other materials in sediments, which might remain associated with cyanobacterial cells, inhibiting DNA polymerases and thus preventing PCR (Pan *et al.* 2002, Hisbergues *et al.* 2003). Therefore, in the present study, bovine serum albumin was added to the PCR mixture of water samples to alleviate this negative influence caused by the environment.

The results shown that the occurrence of *mcy* genes in water samples collected from Sulejów Reservoir in May, at the beginning of growing season, corresponded with further microcystins production in June, July and August (Tables 1 and 2, Figs 2 and 3). The highest microcystin concentration over 2 μg l^{-1} and toxicity about 2 μg l^{-1} were observed at 18th June and 20th August. These data corresponded with the highest cyanobacterial biomass of 23.90 mg l^{-1} and 29.30 mg l^{-1} at 18th June and 20th August, respectively (Table 1 and Fig. 1).

In conclusion, genetic analysis of water samples collected from Sulejów Reservoir indicated that the potential for toxins production was present throughout the summer in 2003. Positive detection signals of *mcyA*, *mcyB* and *mcyE* were obtained, as early as, at the beginning of study season (21st May) when a low cyanobacterial biomass (0.67 mg l^{-1}) was determined. Application of DNA primer sets for PCR assays of *mcy* genes enabled toxigenic and nontoxigenic strains present in water body to be distinguished simply and rapidly and can provide information about alternation of the proportion of toxic genotypes within the population of cyanobacteria in studied water. This confirmed the usefulness of the molecular method as the early warning system for water resources management. For routine environmental monitoring in water body with dominant *Microcystis aeruginosa* we suggest to apply two *mcy* genes from both different operons: *mcyABC* (encodes peptide synthetase) and *mcyGDJEFI* (contains genes for peptide synthetase, polyketide synthase and modyfing enzymes). We recommend *mcyA* gene which encode nonribosomal peptide synthetase and *mcyE* gene which is responsible for activation of ADDA chain in microcystin. In addition, combination of molecular methods with toxicity tests provide complete information used to protect the public from health risks.

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